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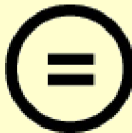
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A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Secondary Metabolites and their Biological Activity from Marine Bio-resource: Marco-alga and Microorganism

Xiudong Yang

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIOANL UNIVERSITY

August, 2012

Secondary Metabolites and their Biological Activity from Marine Bio-resource: Marco-alga and Microorganism

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(Supervised by Professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

2012.08

This thesis has been examined and approved by

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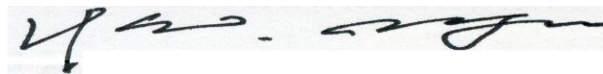

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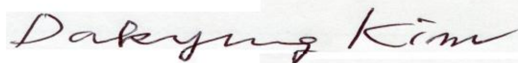

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SUMMARY

Natural products have long been used as foods, fragrances, pigment, insecticides, medicines, and so on. From 1970s, marine natural products have attracted the attention of biologists and chemists in the world. Marine organisms produce secondary metabolites that are structurally distinct from those produced by terrestrial organisms, possibly due to factors unique to marine environments such as high salinity, pressure and a relatively constant temperature. Unusual functional groups, such as isocyanate, isonitrile, dichloroimine, and halogenated functionalities, occur predominantly in marine metabolites. So far, approximately 16,000 have been isolated from marine organisms. Among the marine organisms, marine macro-algae, known as seaweeds are normally used as foods and crude drug in the Asian countries. In the recent years, many secondary metabolites have been isolated from marine macro-algae with interesting biological activities and potential beneficial effects. Furthermore, marine-derived fungi have proved to be a rich and promising source of novel bioactive natural products. Most of these microorganisms grow in a unique and extreme habitat and therefore have the capability to produce unique and unusual secondary metabolites.

Therefore, in this study, the chemical composition of the brown alga, *Padina arborescens* Holmes has been investigated. Three new glyceroglycolipid compounds (SQMG, OGG and NGG) and one known compound (POGG) were isolated from the $CHCl_3$ and EtOAc fractions. The new compounds, SQMG, OGG and NGG showed higher cell growth inhibitory effects than POGG in HL-60 cancer cells. The

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treatments with SQMG, OGG and NGG could induce the apoptosis in HL-60 cells through up-regulation of BAX and caspase-3, down-regulation of Bcl-xL.

In order to develop the new marine bio-resource, we tried to isolate marine-derived fungi. Thirty six fungal strains were isolated from four types of marine samples. Most broth and mycelium extracts of marine fungi showed strong anti-oxidant activities against DPPH radical, especially the strains from *Penicillium* sp. and *Eurotium* sp. Several extracts exhibited inhibitory effects on NO production in LPS-stimulated RAW 264.7 cells without cytotoxicity. The extracts of an unidentified strain 013-1 exhibited highest cell growth inhibitory activity in HL-60 cells among all the isolated marine fungi.

The isolated marine-fungus, *Eurotium amstelodami* was selected as the target strain for research of natural products and their biological activities. Four compounds were isolated from broth extracts. Among them, asperflavin and questinol showed inhibitory effects of NO and PGE2 production in LPS-stimulated RAW 264.7 cells without cytotoxicity. They were also found to decrease the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). The mechanism of the potential anti-inflammatory effects of asperflavin and questinol may attribute to the suppression of iNOS. Therefore, our study suggests a potential use of these compounds might be selected as a promising agent for the prevention and therapy of inflammatory disease.

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1. INTRODUCTION

1.1 Marine Organisms

It is well known that more than 70% of our planet's surface is covered by oceans and the life on Earth has its origin in the sea [1]. Oceans provide a home for over 30 phyla and 500,000 species of marine organisms. Their resources are varied and vast and partly comprise of fish, shellfish, other animals, vegetation, algae, bacteria and fungi [2, 3]. In recent years, many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, bryozoans, sea slugs and marine organisms. Due to the extreme environment, such as high salinity, pressure and a relatively constant temperature or darkness, the marine organisms have been found to produce a diverse of marine natural products, including terpenes, steroids, polyketides, peptides, alkaloids, phlorotannins, and porphyrins, which have novel structures and are often biologically active. Unusual functional groups, such as isocyanate, isonitrile, dicholoroimine, and halogenated functionalities, occur predominantly in marine metabolites. In recent years, natural products from marine bio-resources and their biological activity have been drawn considerable attention as many of them are structurally unique and possess interesting biological activities.

1.2 Marine Macro-algae

Among marine organisms, marine algae are rich sources of bioactive compounds with various biological activities. About 30,000 species of algae are found the world over

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which occur at all places where there is light and moisture and are found in abundance in sea. Marine macro-algae or seaweeds have been used as foods especially in China and Japan and crude drugs for treatment of many diseases such as iodine deficiency (goiter, Basedow's disease and hyperthyroidism). Some seaweeds have also been used as the source of additional vitamins, treatment of various intestinal disorders, as vermifuges, as hypocholesterolaemic and hypoglycemic agents. Seaweeds have been employed as dressings, ointments and in gynecology [4]. Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities. Marine macro-algae, known as seaweed, are classified into three divisions such as Chorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae) according to their composition of pigments. For example, the presence of xanthophyll pigment, fucoxanthin, is responsible for the color of brown seaweeds [5, 6]. Over the last several decades, a lot of researches have reported the biological active compounds with unusual and exciting carbon skeletons from marine algae. These compounds exhibited various biological activities, such as anti-oxidant, anti-inflammation, anti-hypertension, anti-bacteria, and anti-cancer activities.

1.2.1 Natural Products and Biological Activity of Marine Macro-algae

There are several reviews focusing on the natural products and bioactivity of marine macro-algae. The biological importance of marine algae has been reviewed in 2010 [7]. Phlorotannins from marine brown algae with their health beneficial biological

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activities were reviewed. Phlorotannins, consisting of phloroglucinol subunits, contain four types as fucols, fucophlorethols, phlorethols, and eckols according the type of connection between the subunits (Fig. I) [3, 8]. The phlorotannins isolated from marine brown algae have been reported to show several pharmacological activities, including antioxidant, enzyme inhibition, anti-bacteria, anti-HIV, anti-cancer, radioprotection, anti-allergy, and other beneficial effects.

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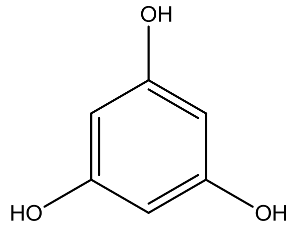
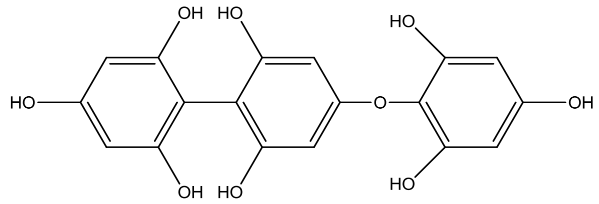
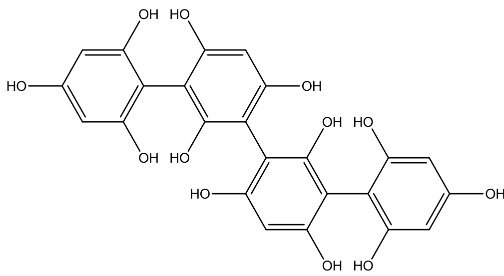


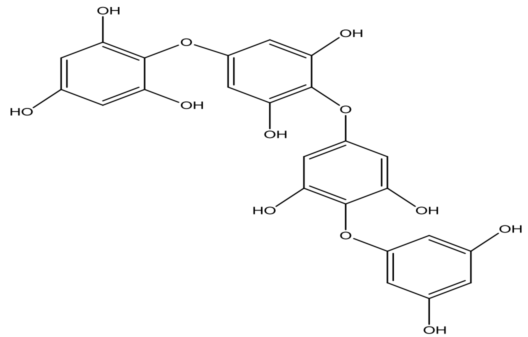
Phloroglucinol



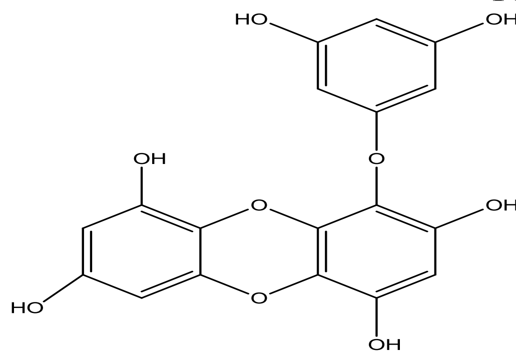
Fucophlorethol A



Tetrafucol A



Tetraphlorethol C



Eckol

Figure I. Four types of phlorotannins isolated from marine algae

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The natural pigments derived from marine algae including chlorophyll a, pheophytin a, pheophorbide a, pyrophenophytin a, phycoerythrobilin, lutein, β-carotene, fucothxanthin, and siphonaxanthin have been reviewed. These natural pigments exhibit various beneficial biological activities (Fig. II) [9]. Fucoxanthin was found to have strong radical scavenging activities against DPPH, hydroxyl and singlet oxygen. In addition, cytoprotective effect of fucoxanthin against ROS formation induced by H_2O_2 has been observed *in vitro* [10]. Fucoxanthin and its deacetylated metabolite fucoxanthinol also showed stronger anti-adult T-cell leukemia effects than those of β-carotene and astaxanthin. The anti-inflammatory effect of fucoxanthin was reported by Heo et al. Fucoxanthin treatment could reduce production of NO and PGE_2 expressions and levels of inflammatory cytokines such as TNF-α, IL-6, and IL-1β [11]. Other biological activities of fucoxanthin have been reported, including anti-obsesity [12, 13], neuroprotection [14], anti-angiogenicactivity [15] and photo-protective-activity [16, 17]. Those activities of fucoxanthin and other natural pigments were reviewed by Pangestuti & Kim [9].

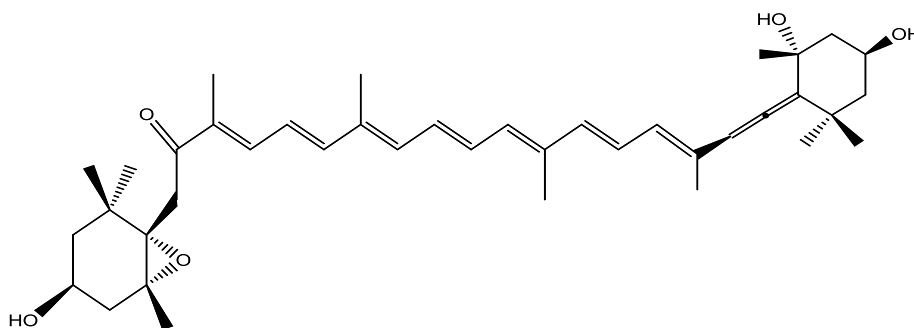

Figure II. Chemical structure of fucoxanthin isolated from marine algae.

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Glycolipids are widely distributed in the marine algae. Certain species of red algae contains glycolipids from 50.3% to 75.1% total lipids, from 47.2% to 83.1% in brown algae, and from 68.0% to 75.1% in green algae [18]. Several reports have shown that the glycolipids isolated or identified from marine organism exhibited various biological activities. Two natural glucosyldiacylglycerols isolated from a brown alga, *Sargassum fulevellum* could enhance reciprocal activation of pro-u-PA and plasminogen *in vitro* [19]. Avrainvilloside has been isolated from a green alga, *Avrainvillea nigricans*, and it has cytotoxicity on WEHI164cells (murine frbrosarcoma) [20]. A sulfoquinovosyldiacylglycerol compound was isolated from the *n*-buthanol extract of the green alga, *Caulerpa racemosa*. This compound exhibited an excellent antiviral effect against HSV-2 with an IC_{50} of 15.6 μg/ml [21]. Other literatures showed that the glycolipids also showed inhibition of HIV-1 RT enzyme, anti-inflammatory, mammalian DNA polymerase α inhibitory, Myt1 kinase inhibitory activities (Fig.III) [22-24].

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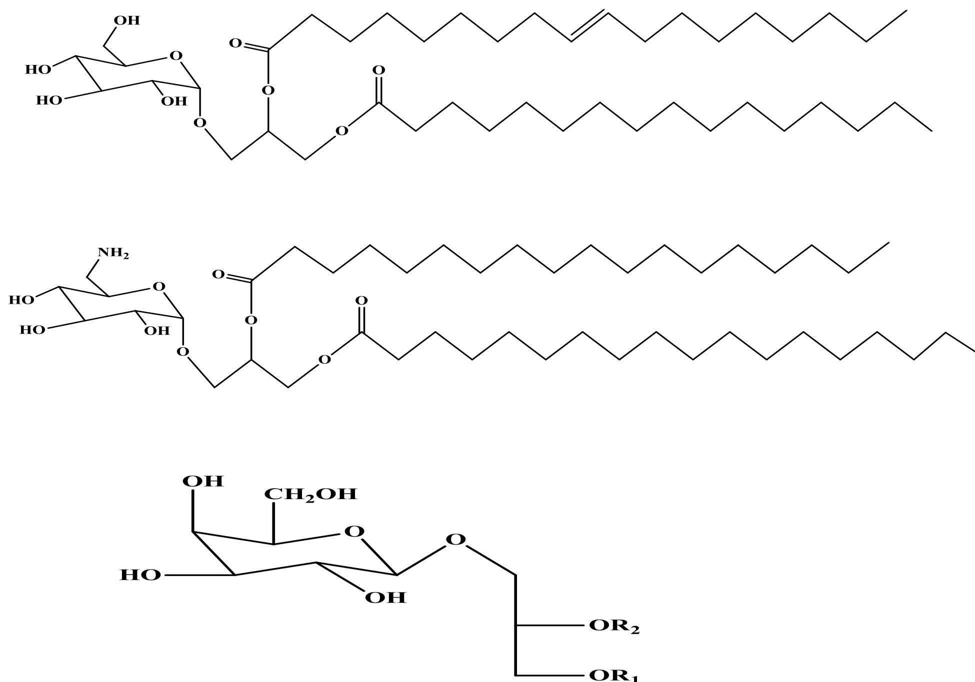


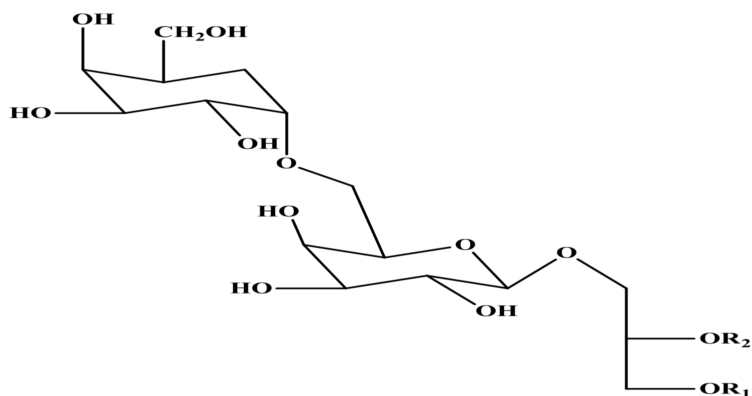
R1= (7Z,10Z,13Z)-hexadecatrienoyl, R2 = (7Z,10Z)-hexadecadienoyl

R1, R2 = (7Z,10Z)-hexadecadienoyl

R1 = linolenoyl, R2= (7Z,10Z,13Z)-hexadecatrienoyl

R1 = linolenoyl , R2 = (7Z,10Z)-hexadecadienoyl

R1, R2 = linolenoyl,



R1 = linolenoyl, R2= (7Z,10Z,13Z)-hexadecatrienoyl

R1 = linolenoyl , R2 = (7Z,10Z)-hexadecadienoyl

Figure III. The glycolipids isolated from marine algae.

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Other bioactive metabolites from marine marco-algae, such as sesquinterpenes, diterpenes, meroterpenoids, diterpenes, C15-acetogenins, and steroids, have been frequently reported from various species of seaweeds. Most of these skeletons have been reported to exhibit antitumor, cytotoxic, and antibacterial activities. For example, two new sesquinterpenes from the red alga *Laurencia microcladia* showed cytotoxicity against five human tumor cell lines (Fig. IV-1, IV-2) [25]. A halogenated C15 acetogenin, (12E)-lembyne-A (Fig. IV-3), was isolated from *Laurencia mariannensis*. It showed inhibitory activity against *Alcaligenes auqa-marinus*, *Azomonas agilis*, *Erwinia amylovora*, and *Escherichia coli* with MIC in the range of 20-30 μg/disk [26]. Steroids, a class of functionally important triterpeniod lipid are typical metabolites occurring in marine algae (Fig. IV-4) [27, 28].

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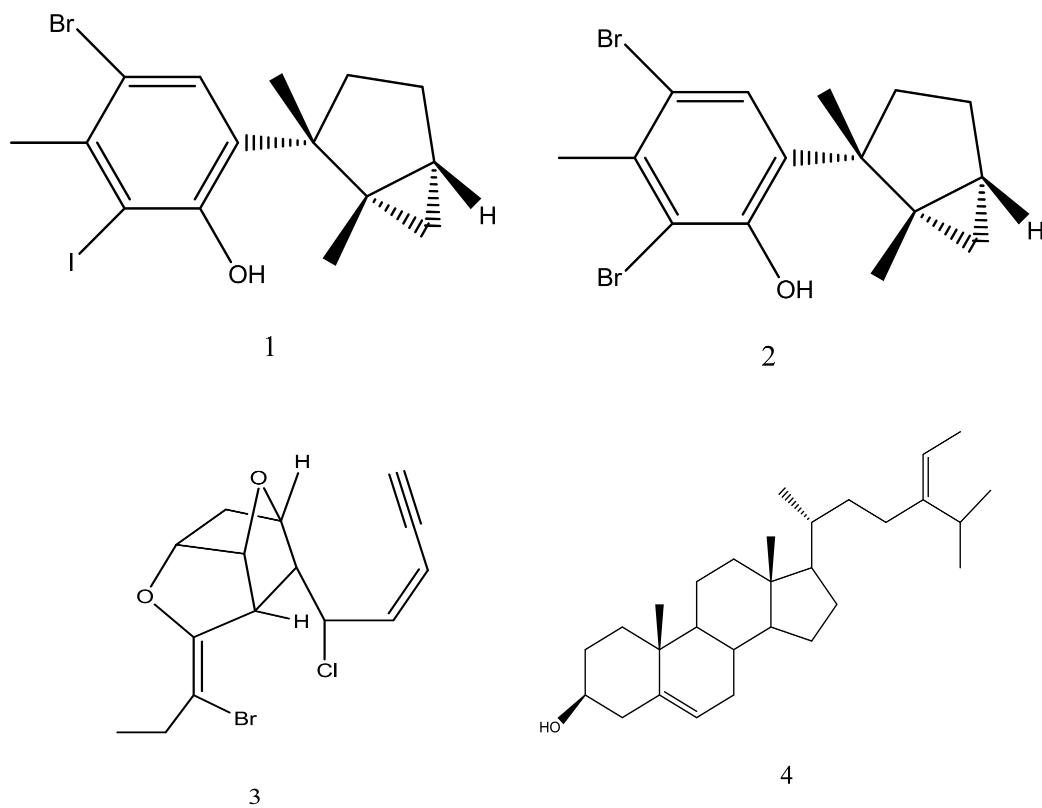

Figure IV. Other bioactive metabolites isolated from marine algae.

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1.3 Marine-Derived Fungi

Marine-derived fungi have recently been proved to be a rich source of structurally unique and biologically active secondary metabolites [29]. The generally accepted estimate of the number of the fungal species on Earth is a conservative 1.5 million [30]. However, most of the fungi inhabiting the world have not yet been described. So far, less than 5% of all species, about 72,000 to 100,000 species have been known already. Therefore, fungi represent a large source for natural products with diverse chemical structural and activities [31]. According to the "classic" definition that appears to be universally accepted in the scientific community, marine fungi are divided into two groups, obligate and facultative marine fungi-obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, while facultative marine fungi are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment [32, 33]. The research showed that over 60% of marine fungi genera have been obtained from sponge, wood and alga. Sponges have yielded the greatest taxonomic diversity [34].

1.3.1 Natural Products from Marine-Derived Fungi

Historically, the first secondary metabolite isolated from a marine-derived fungal strain was cephalosporin C, produced by a culture of a *Cephalosporium* sp., isolated in 1949 close to a sewage outlet off the Sardinian coast [35]. However, that was a more or less incidental discovery, and it took another 30 years until marine-derived fungi were analyzed more systematically. It was only in the late 1980s that sizable

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quantities of new secondary metabolites were discovered from this long neglected source [36]. Before 1990s, the reports on natural products chemistry from marine derived fungi were rare. A few studies can be found between 1970 and 1990. The researches of secondary metabolites from marine derived fungi blossomed in the 1990s and notably since 1998. To date, the total number of new natural products from marine-derived fungi has exceeded 1000. 21% and 19% of these new compounds have been reported from marine fungi derived from algae and sponges, respectively. The majority of the isolated compounds (almost 50%) belonged to polyketides and their isoprene hybrids, followed by alkaloids, terpenes, and peptides, which contributed 14–20%. These new compounds were produced mostly from members of the fungal genera *Penicillium* and *Aspergillus*. Representatives of other genera that were less common were *Acremonium*, *Emericella*, *Epicoccum*, *Exophiala*, *Paraphaeospaeria*, *Phomopsis*, and *Halarosellinia* [37].

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Part I

Isolation and Biological Activities of Secondary Metabolites from the Marine Brown Alga, *Padina arborescens* Holmes

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Abstract:

In this study, three new glyceroglycolipid compounds, 3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-2-*O*-palmitoyl-glycerol (SQMG), 1-*O*-oleoyl-3-*O*-(*β*-D-gluco-pyranosyl)-glycerol (OGG), and 1-*O*-nonadecyl-3-*O*-(*β*-D-glucopyranosyl)-glycerol (NGG) along with a known compound 1–*O*–palmitoyl–2–*O*–oleoyl–3–*O*–(*β*–D–glucopyranosyl)–glycerol (POGG) were isolated from a marine brown alga, *Padina arborescens* Holmes using different column chromatography. The structures of the isolated compounds were determined based on the analysis of NMR and MS spectral data. Furthermore, the anti-cancer effects of the glyceroglycolipid compounds were examined in human leukemia (HL-60) cells via MTT assay. The results showed that SQMG, OGG and NGG showed higher cell growth inhibitory effects than POGG in the HL-60 tumor cells. In order to detail elucidate the anti-cancer effects of SQMG, OGG and NGG, the morphology changes using nuclear staining and DNA population distribution via cell cycle analysis were also investigated. These results revealed that treatment with SQMG, OGG and NGG could induce the apoptosis in HL-60 cells. Western blot analysis showed that the treatments of SQMG, OGG and NGG could up-regulate the expression level of caspase-3 and Bax, inhibit the expression of Bcl-xL in HL-60 cells. Collectively, these findings suggest that the new glyceroglycolipids, SQMG, OGG and NGG isolated from *P. arborescens* Holmes induce apoptosis in HL-60 cells through regulation of Bcl-2 family and caspase-3.

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1. Materials and methods

1.1 General materials

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. Column chromatography was carried out by Silica Gel 60 (230–400 mesh, Merck, Germany), ODS (12 nm, YMC, Japan), Sephadex LH-20 (Sigma, St. Louis, MO). Thin-layer chromatography (TLC) was run on pre-coated Merck Kieselgel 60 F254 plates (0.25 mm). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

1.2 Plant Materials

The marine brown seaweed, *Padina arborescens* Holmes (PA) was collected from the coast of Jeju Island, Korea, in July, 2008. The fresh seaweed were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and maintained in a medical refrigerator at -20 ℃.The lyophilized PA was ground into powder before extraction.

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Figure 1-1. The photograph of the brown alga, *Padina arborescens* Holmes

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1.3 Extraction and isolation

The dried PA powder (300 g) was extracted by stirring with 80% methanol (3 × 3 L) at room temperature for 12 h and filtered. The solvent was removed under vacuum at 40 ℃. This methanol extract was suspended in distilled water (800 mL). The extract was partitioned with *n*-hexane, $CHCl_3$, EtOAc in sequence. The $CHCl_3$ extract (PAC), which exhibited a potent activity, was subject to a silica gel flash chromatography eluted with $CHCl_3$/MeOH (gradient) to yield twelve sub-fractions (PAC1-PAC12). The active fraction F12 was further purified by Sephadex LH-20 column with MeOH to give nine fractions (PAC12.1-PAC12.9).The active fraction PAC12.5 was further purified on ODS column chromatography, eluted with a gradient solvent system of H_2O-MeOH (50:50) to H_2O-MeOH (0:100), afford 60 fractions 15 mL in volume to yield the compound 1 (35.7 mg). The active fraction F12.6 was purified by ODS column chromatography, eluted with 50% to 100% MeOH in distilled water, and yielded compound 2 (5.5 mg) (Fig. 1).

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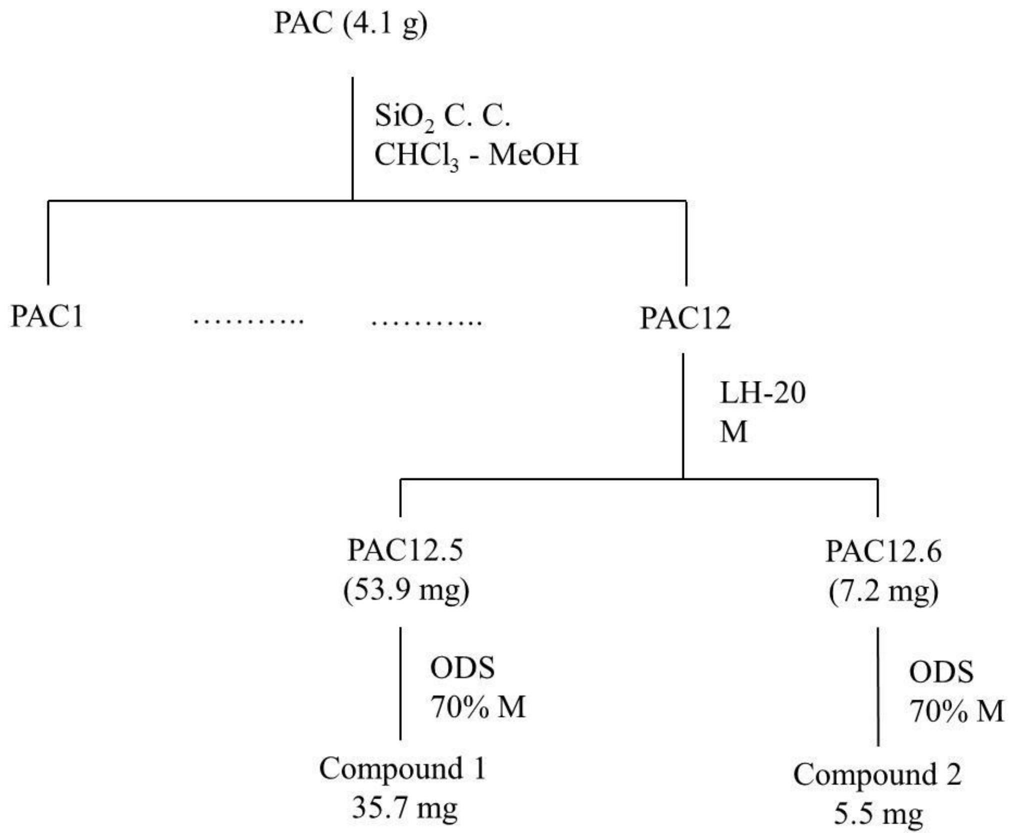

Figure 1-2. Isolation scheme of the active compounds from $CHCl_3$ fraction of *P. arborescens* Holmes.

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The EtOAc extract (PAE), which exhibited a potent activity, was subject to a silica gel flash chromatography eluted with $CHCl_3$/MeOH (gradient) to yield eleven sub-fractions (PAE1-PAE11). The fraction PAE7 was further purified by Sephadex LH-20 column with MeOH to give nine fractions (PAE7.1-PAE7.9). The fraction PAE7.2 was purified by ODS column chromatography, eluted with 50% to 100% MeOH in distilled water, yield compound 3 (14.6 mg) and compound 4 (3.5 mg).

The ^{1}H NMR, ^{13}C NMR and 2D NMR spectra of the isolated compounds were recorded on a JEOL JNM-ECP 400 MHz NMR spectrometer, using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. The MS spectra were obtained on a JEOL JMS-700 spectrometer.

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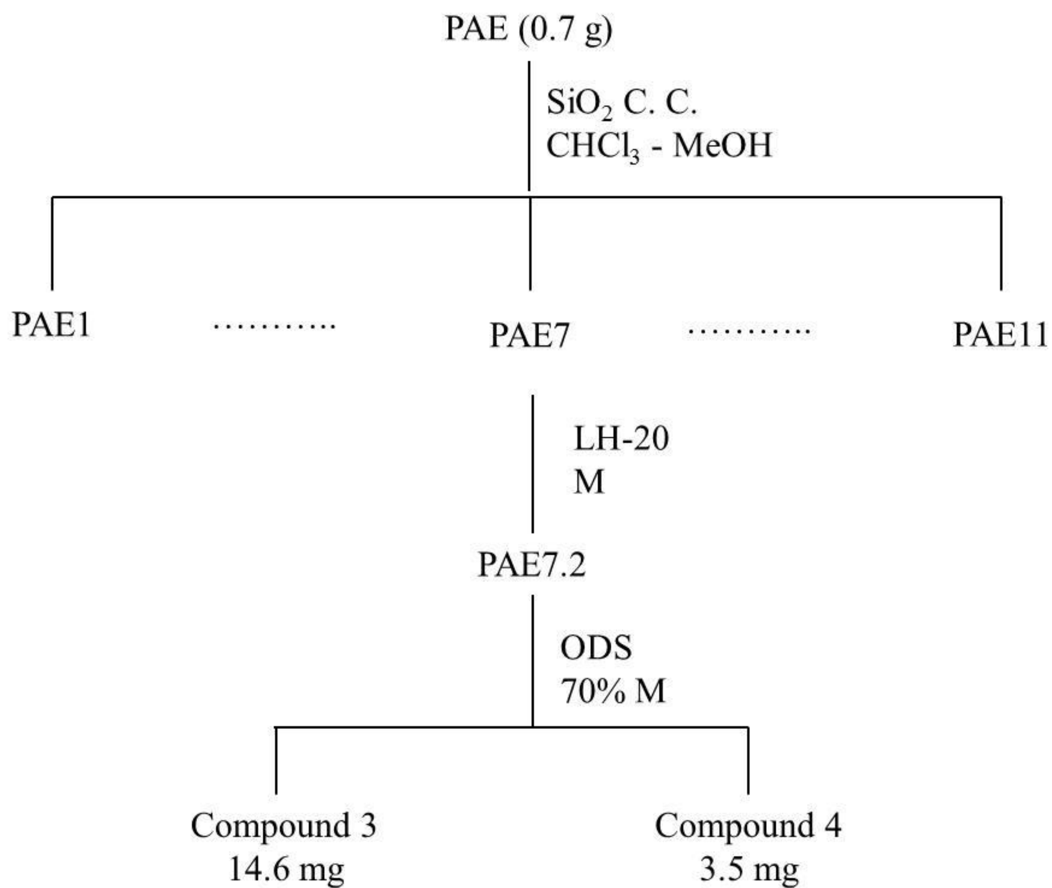

Figure 1-3. Isolation scheme of the active compounds from EtOAc fraction of *P. arborescens* Holmes.

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1.4 Cell culture

HL-60 (Human promyelocytic leukemia cell line) was maintained at 37 ℃ in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured at a concentration of 1×10^5 cells/ml in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) for further experiments.

1.5 Cell growth inhibitory assay

The cell growth inhibitory assays of isolated compounds were examined by MTT assay. The cells were seeded in 96-well plate at a concentration of 1×10^5 cells/ml (180 μl). After 16 h incubation at 37 ℃ under a humidified atmosphere, the cells were treated with 10 μl of various concentrations of the compounds (25, 50, 100 and 200 μM), and further incubated for 24 h. The 50 μl of MTT stock solution (2 mg/ml) was then applied to the wells, to a total reaction volume of 250 μl. After 3 h of incubation, the plates were centrifuged for 5 min at 800 $\times g$, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. The percentage inhibitory effect was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

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1.6 Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis [38, 39]. The cells were placed in 24-well plates at a concentration of 1 $\times 10^5$ cells/ml (950 μl). Sixteen hours after plating, the cells were treated with various concentrations of the compounds (50 μl). Twelve hours later, 3 μl of Hoechst 33342 (stock 10 mg/ml), a DNA-specific fluorescent dye, were added to each well, followed by 10 min of incubation at 37 ℃. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.

1.7 Cell cycle analysis

Cell cycle analysis was conducted to determine the proportion of apoptotic sub-G1 hypodiploid cells [40]. The cells (HL-60) were plated in 6-well plates at a concentration of 4.0 $\times 10^5$ cells/mL and then incubated for 24 h at 37 ℃ under a humidified atmosphere. The cells were then treated with the different concentrations of the isolated compounds. After incubation for 24 h at 37 ℃, the cells were harvested and fixed in 1 ml of 70% ethanol for 30 min at 4 ℃. The cells were washed twice with PBS and incubated in darkness in 500 μl of PBS containing 50μg PI and 50μg RNase A for 30 min at 37 ℃. The flow cytometric analysis was conducted with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect on

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the cell cycle was determined by changes in the percentage of cell distribution at each cell cycle phase, and assessed by histograms generated by the Quest and Mod-Fit computer programs [41].

1.8 Western blot analysis

Cells (2×10^5 cells/mL) were treated with different concentration of the isolated compounds for 24 h, and then harvested. The cell lysates were prepared with lysis buffer (50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/L EDTA). Cell lysates were washed via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit. The lysates containing 30 μg of protein were subjected to electrophoresis on 10% or 12% sodium dodecyl sulfate-polyacrylamide gels, and the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, and β-actin in TTBS (25 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20, pH7.4) containing 0.5% nonfat dry milk at 1 h. The membranes were then washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL Western blotting detection kit and exposed to X-ray films.

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2. Results

2.1 Isolation of the active compounds from marine brown alga, *Padina arborescens* Holmes

In our continuous study on the bioactive compounds from marine algae, MeOH extracts of several marine algae have been investigated for their anti-cancer activities in different cell lines. As a result, the MeOH extract of marine brown alga, *P. arborescens* Holmes exhibited most potential anticancer effects in HL-60 cells. Therefore, the MeOH extract of *P. arborescens* Holmes was further partitioned between different solvents, including *n*-hexane, chloroform, and ethyl acetate. The $CHCl_3$ and EtOAc fractions with the highest activity were further purified by different column chromatography. Finally, four glyceroglycolipid compounds were successively isolated and the structures of the isolated compounds were determined on basis of the MS and NMR (^{1}H and ^{13}C) spectral data, and also comparing with previously published data.

The ^{1}H and ^{13}C NMR spectral data of the isolated compounds suggested three common structural features including a sugar moiety, a glycerol system, and long aliphatic chains.

1–*O*–palmitoyl–2–*O*–oleoyl–3–*O*–(*β*–D–glucopyranosyl)–glycerol (POGG, 1): White, amorphous powder, LR-EIMS: *m/z* 757 $[M + H]^+$. ^{1}HNMR (DMSO-d_6, 400 MHz): δ 5.31 (2H, t, J = 4.8), 5.13 (1H, m), 4.56 (1H, d, J = 3.68), 4.35 (1H, dd, J = 2.3, 9.8), 4.14 (1H, dd, J = 4.6, 7.32), 3.87 (1H, dd, J = 5.9, 11.1), 3.77 (1H, ddd, J = 5.2, 5.9, 9.4), 3.41 (1H, dd, J = 5.7, 10.7), 3.19 (1H, dd, J = 3.7, 9.6), 2.91 (1H, t, J =

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8.7), 2.53 (1H, dd, *J* = 6.6, 14.0), 2.26 (4H, m), 1.98 (4H, m), 1.49 (4H, m), 1.23 (36H, br. s), 0.85 (6H, t, *J* = 6.4); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 173.9 (C-1′), 172.4 (C-1″), 129.6 (C-9′), 129.5 (C-10′), 104.1 (C-1‴), 74.3 (C-5‴), 72.9(C-3‴), 71.6 (C-2‴) , 69.8 (C-2) , 68.5 (C-3), 64.6 (C-3), 62.6 (C-1) , 54.6 (C-6‴), 33.5 (C-2′), 33.4 (C-2″) , 31.2 (C-16′, C-14″), 28.5-29.0 (C-4′-7′, C-12′- C-15′, C-4″-C-13″) , 26.6 (C-8′), 26.5 (C-11′), 24.4 (C-3′), 24.2 (C-3′), 22.0 (C-15″, C-17′), 13.9 (C-18′, C16″).

Compound 1 was isolated as a white, amorphous powder. The LR-EIMS data give a molecular ion peak at *m/z* 757 $[M + H]^+$. Compared with the published data, the structure of the compound 1 was determined as 1–*O*–palmitoyl–2–*O*–oleoly –3–(*α*–D–glucopyranosyl)–glycerol. It was first isolated from a brown alga, *Sargassum fulvellum* and showed fibrinolytic activity (Fig.1-4-1) [19]. In this study, this compound was first isolated from the brown alga, *P. arborescens* Holmes.

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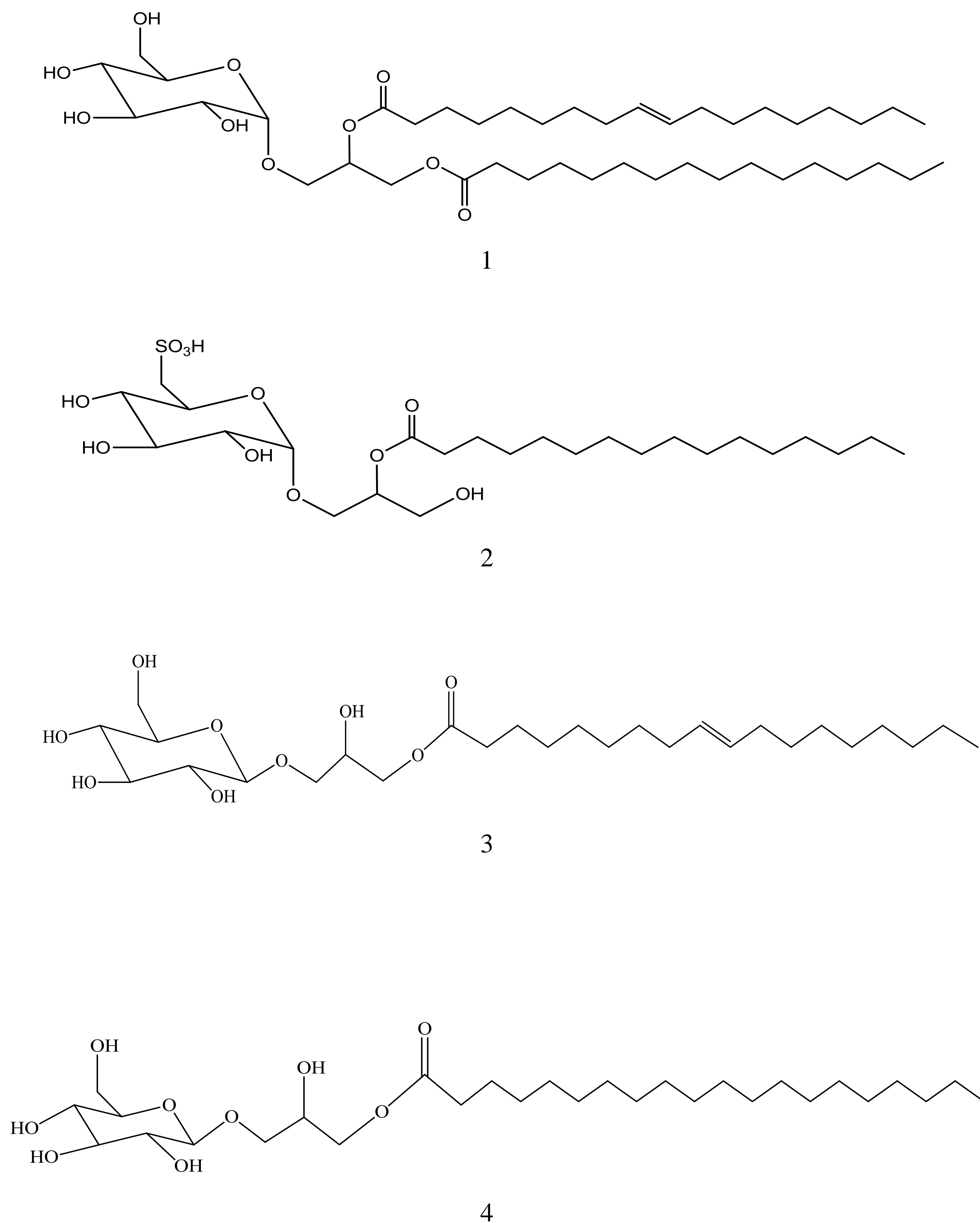

Figure 1-4. The structures of four compounds isolated from *P. arborescens* Holmes.

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3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-2-*O*-palmitoyl-glycerol (SQMG, 2). White, amorphous powder, HR-EIMS: *m/z* 555.2868 $[M-H]^-$. ^1H NMR (DMSO-d_6, 400 MHz): δ 5.11 (1H, m, H-2), 4.57 (1H, J = 3.9, H-1′), 4.10 (1H, dd, J = 5.2, 10.0, H-1a), 3.91 (1H, dd, J = 10.8, 4.2, H-1b), 3.85 (1H, m, H-3a), 3.85 (1H, m, H-5′), 3.69 (1H, dd, J = 4.28, 9.7, H-3b), 3.17 (1H, dd, J = 5.6, 10.4, H-2′), 2.93 (1H, t, J = 4.6, H-4′), 2.89 (1H, m, H-6′a), 2.56 (1H, dd, J = 6.7, 14.0, H-6′b), 2.29 (2H, t, J = 7.6, H-2″), 1.50 (2H, m, H-3″), 1.23 (H, m, H-4″-15″), 0.85 (3H, t, J =7.1, H-16″); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 172.9 (C-1‴), 98.8 (C-1′), 74.4 (C-4′), 73.1 (C-3′), 71.9 (C-2′), 69.2 (C-2), 68.4 (C-5′), 65.3 (C-3), 67.1 (C-1), 32.4 (C-2″), 31.2 (C-14″), 28.5 – 29.0 (C-4″- C-13″), 24.4 (C-3″), 22.0 (C-15″), 13.9 (C-16″).

Compound 2 was isolated as a white amorphous solid and gave a quasimolecular ion peak at m/z 555.2868 $[M-H]^-$ in the negative HR-EIMS, corresponding to a molecular formula $C_{25}H_{48}O_{11}S$, and this was supported from its NMR data. The first spin system was assigned to a glycerol system (δ_H 4.10 and 3.91, δ_c 67.1); (δ_H 5.11, δ_c 69.2); (δ_H 3.94 and 3.69, δ_c 65.3); the second group of signals were attributed to a long-chain saturated fatty acid, where the terminal methyl signals appeared at δ_H 0.85 (3H, t, J = 7.0 Hz) and δ_c 13.9, while the third spin system indicated that the presence of a glycosyl moiety. The anomeric signals at δ_C 98.8 and δ_H 4.57 (d, 3.9) indicated the α configuration of this sugar. The ester group signals (δ_C 172.9) and methyl carbon signals (δ_C 13.9) in the ^{13}C NMR spectrum, as well as methyl proton signals (δ_H 0.85, 3H) in the ^1H NMR spectrum, suggested that one saturated fatty acid chain was linked to the glycerol moiety. The presence of one acyl fatty acid was confirmed by the

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MS/MS fragment m/z 255, which suggested loss of palmitic acid. In the HMBC spectrum, the glycerol, the glycerol methylene proton at δ_H5.11 was correlated to the ester carbonyl at δ_C 172.9, indicating the linkage of the palmitoyl acid to the glycerol portionat C-2. Consequently, the structure of 2 was determined as 3-*O*-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-2-*O*-palmitoyl-glycerol (SQMG). Although, the sulfoquinovosyldiacylglycerol (SQDG) compounds have been isolated from marine algae, especially from green and brown algae [42, 43], this compound was obtained from the marine brown alga, *P. arborescens* Holmes as a new compound, sulfoquinovosylmonoacylglycerol (SQMG). (Fig. 1-4-2)

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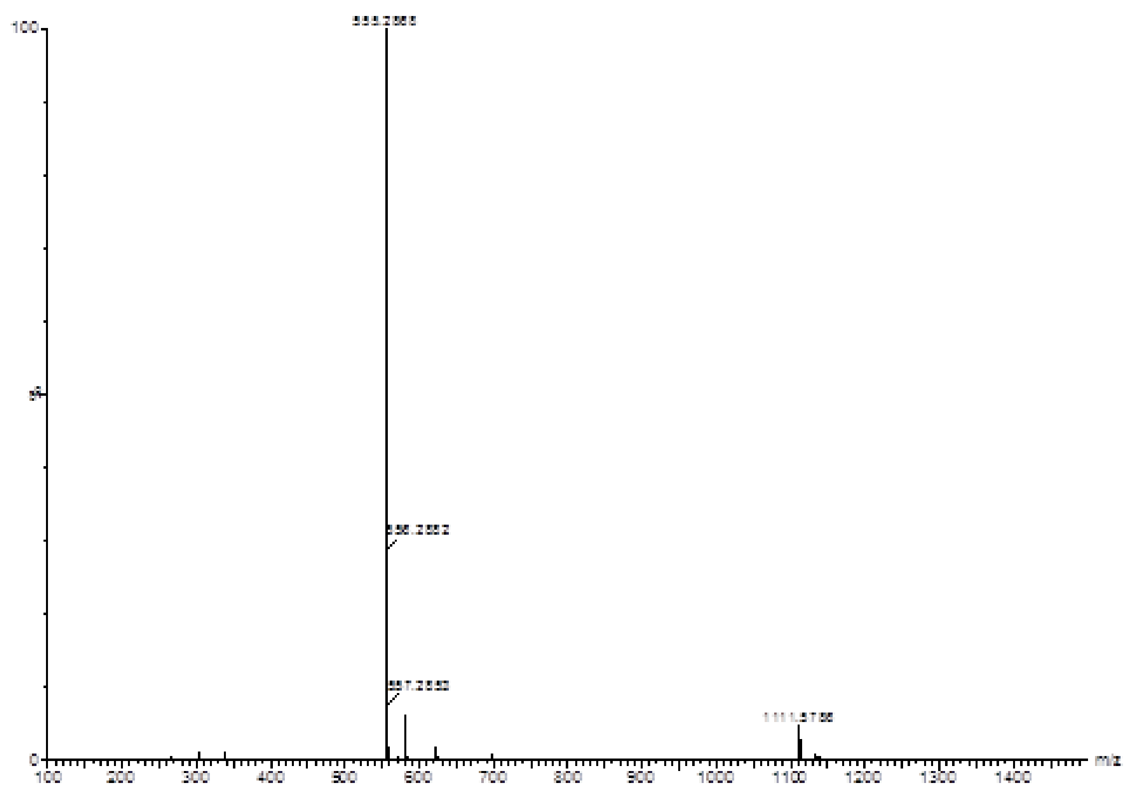

Figure 1-5. The mass spectrum of SQMG (2) isolated from *P. arborescens* Holmes.

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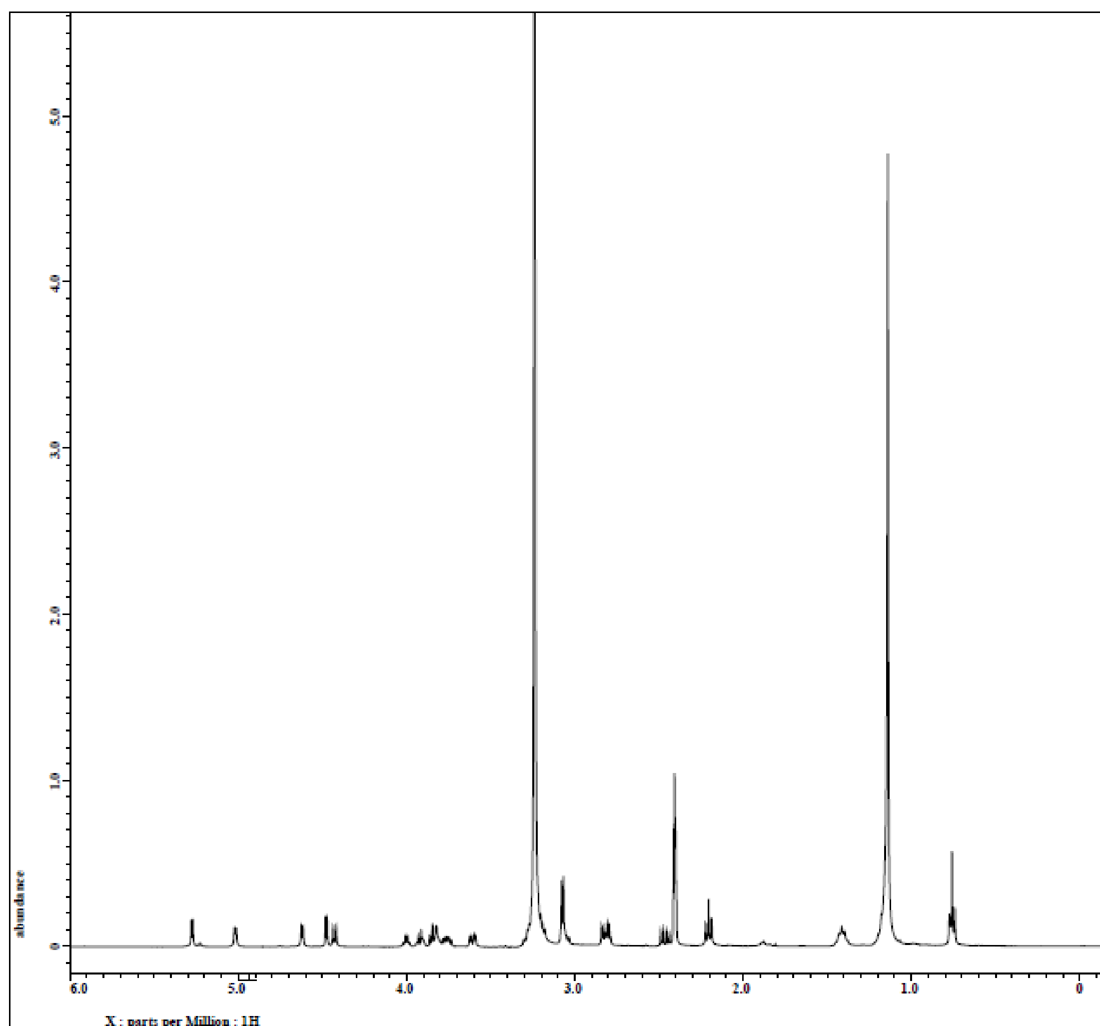

Figure 1-6. The ^{1}H-NMR spectrum of SQMG (2) isolated from *P. arborescens* Holmes.

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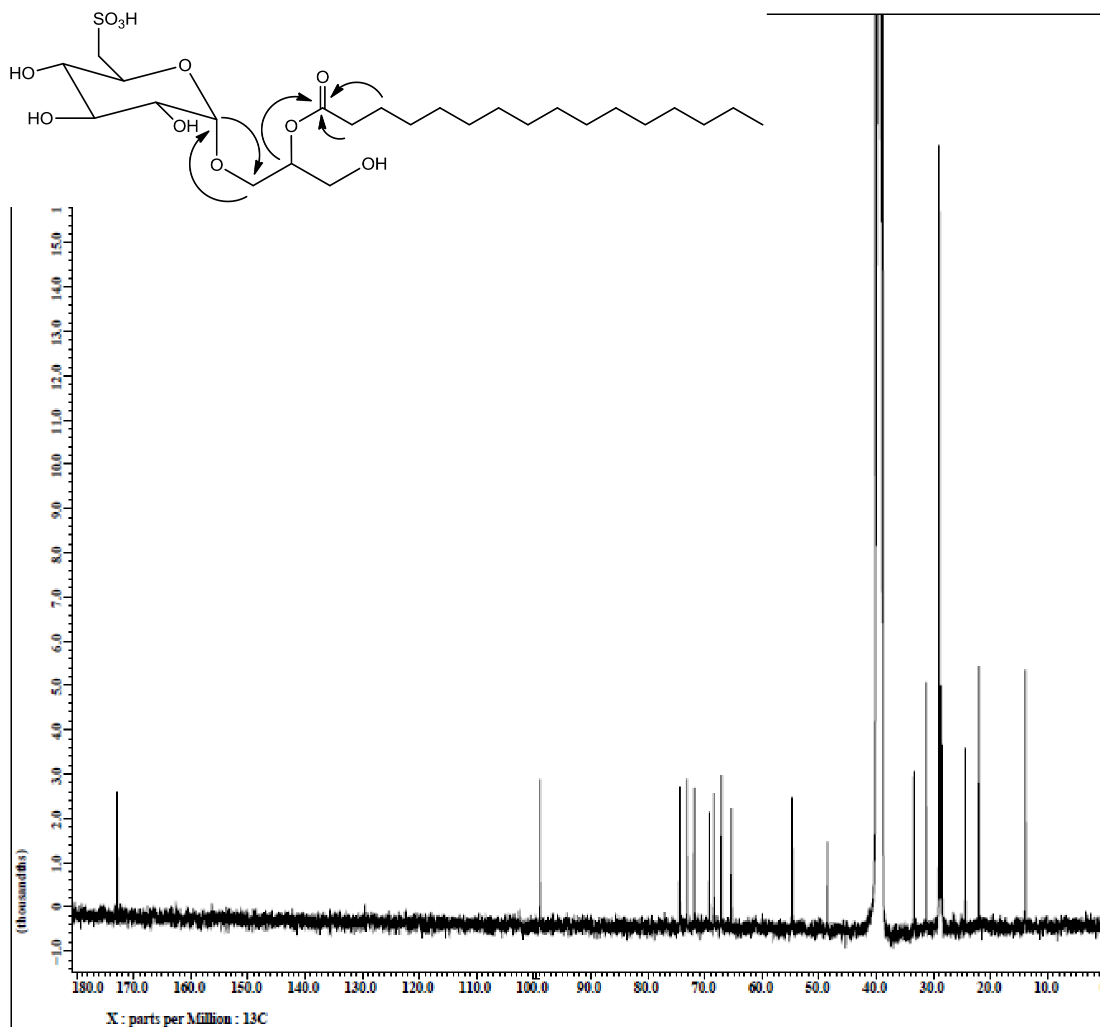

Figure 1-7. The ^{13}C-NMR spectrum of SQMG (2) isolated from *P. arborescens* Holmes.

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1-*O*-oleoyl-3-*O*-(β-D-glucopyranosyl)-glycerol (OGG, 3), White, amorphous powder, HR-EIMS: *m/z* 517.3401 [M-H]$^-$. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 4.95 (1H, m, H-2), 4.06 (1H, d, J = 7.1, H-1′), 4.05 (1H, dd, J = 4.1, 11.2, H-1a), 3.97 (1H, dd, J = 6.4, 11.2, H-1b), 3.79 (1H, dd, J = 5.7, 10.3, H-3a), 3.68 (1H, dd, J = 5.2, 10.3, H-3b), 3.62 (1H, br. d, H-5′), 3.51 (1H, dd, J = 5.9, 10.4, H-6′a), 3.44 (1H, dd, J = 5.7, 10.4, H-6′b), 3.42 (1H, dd, J = 5.6, 10.4, H-4′), 3.27 (1H, m, H-3′), 3.17 (1H, m, H-2′), 2.29 (2H, t, J = 7.6, H-2″), 1.51 (2H, m, H-3″), 1.23 (H, m, H-4″-7″, H-12″-17″), 0.85 (3H, t, J =7.1, H_3-18″); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 172.9 (C-1″), 129.6 (C-9″), 129.6 (C-10″), 104.0 (C-1′), 75.2 (C-4′), 73.2 (C-3′), 70.5 (C-2′), 70.4 (C-2), 68.0 (C-5′), 67.3 (C-1), 65.4 (C-3), 33.4 (C-2″), 31.2 (C-16″), 28.4-29.0 (C-4″-C-7″, C-12″-C-15″), 24.4 (C-3″), 22.1 (C-17″), 13.9 (C-18″).

Compound 3 was isolated as a white amorphous solid, displayed a quasimolecular ion peak in the negative HR-EIMS at m/z 517.3401 [M-H]$^-$, which corresponded to a molecular formula $C_{27}H_{50}O_9$, and this was supported from its NMR data. It showed an almost identical NMR parttern to 2, except for the presence of a signal at δ_C 129.6 (C-9″, C-10″) and the absence of a signal at δ_C 54.7 in the ^{13}C NMR spectrum. The anomeric carbon signal appeared at δ_C 104.0 (C-1′), whereas anomeric protons resonated at δ_H 4.06 (d, J = 7.1), indicated the β configuration of this sugar. The ester group signals (δ_C 172.9), methyl signals (δ_C 13.9, δ_H 0.85) and olefinic resonances at δ_C 129.6 (C-9″, C-10″) and δ_H 5.32 (2H, m), suggested that one unsaturated fatty acid chain was linked to the glycerol moiety. The presence of one

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acyl fatty acid was confirmed by the MS/MS fragment m/z 253, which suggested loss of oleic acid. In the HMBC spectrum, the glycerol methylene proton at δ_H 4.05 was correlated to the ester carbonyl at δ_C 172.9, indicating the linkage of the oleic acid to the glycerol portion at C-1. Consequently, the structure of 3 was determined as a new compound, 1-*O*-oleoyl-3-*O*-(β-D-glucopyranosyl)-glycerol (Fig. 1-4-3).

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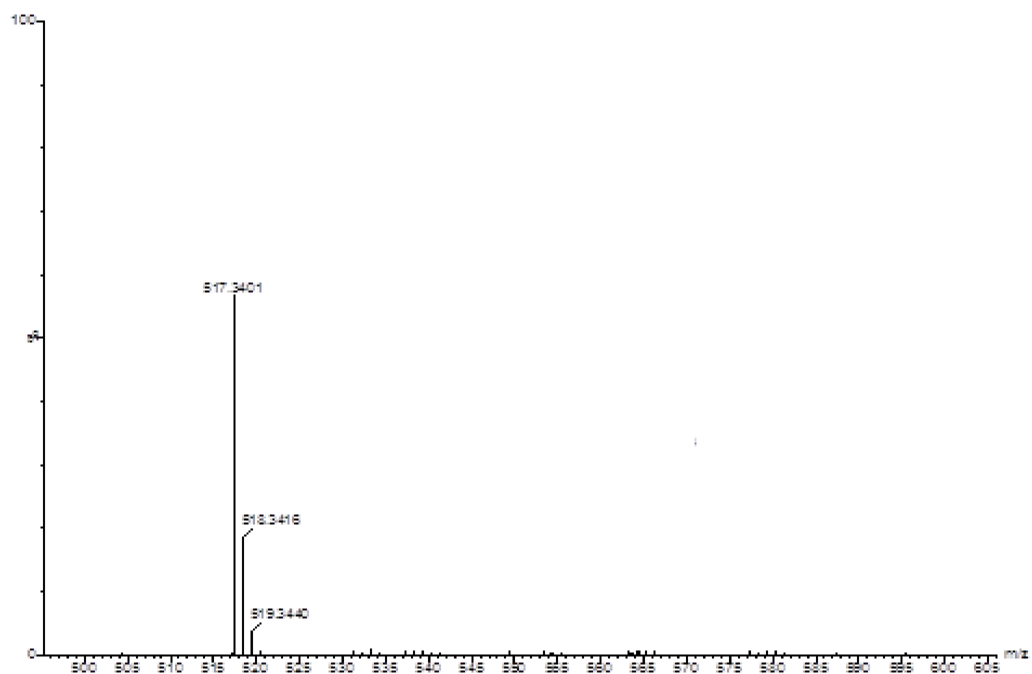

Figure 1-8. The mass spectrum of OGG (3) isolated from *P. arborescens* Holmes.

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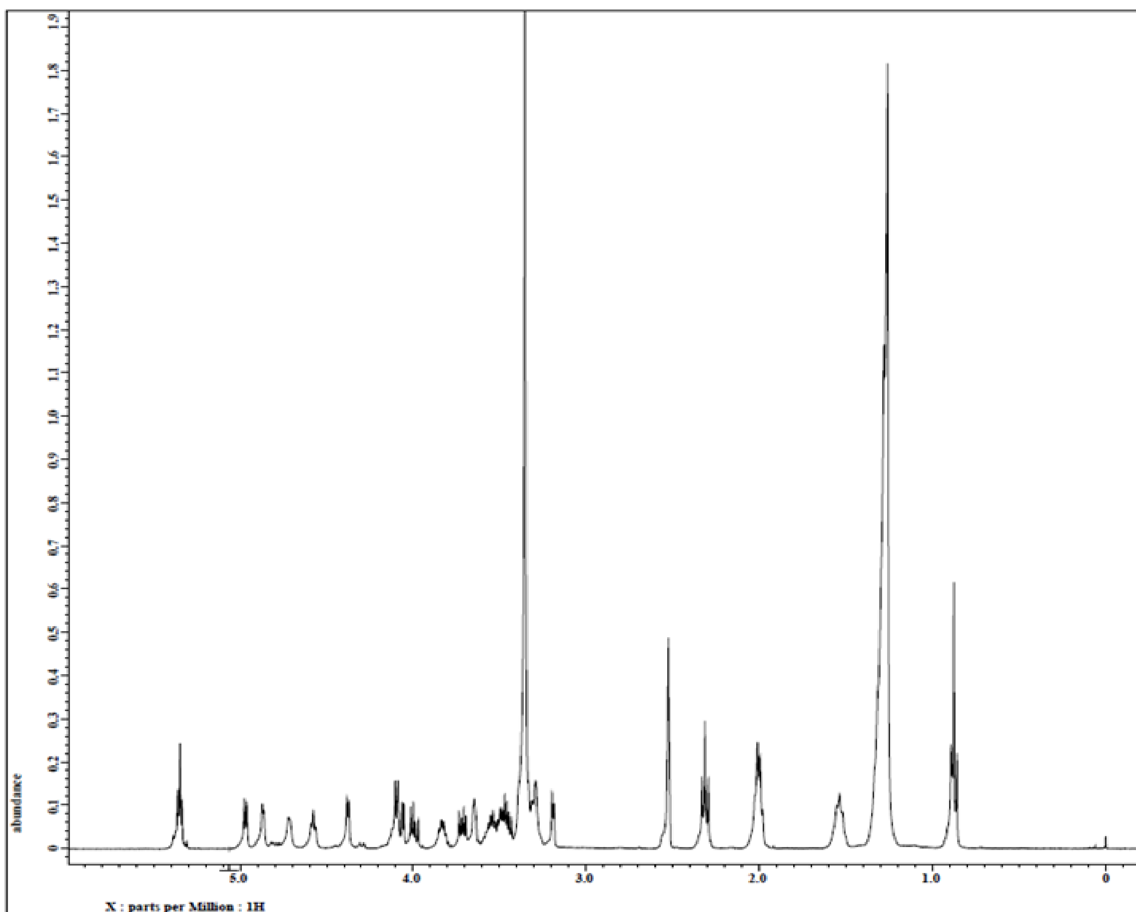

Figure 1-9. The ^{1}H-NMR spectrum of OGG (3) isolated from *P. arborescens* Holmes.

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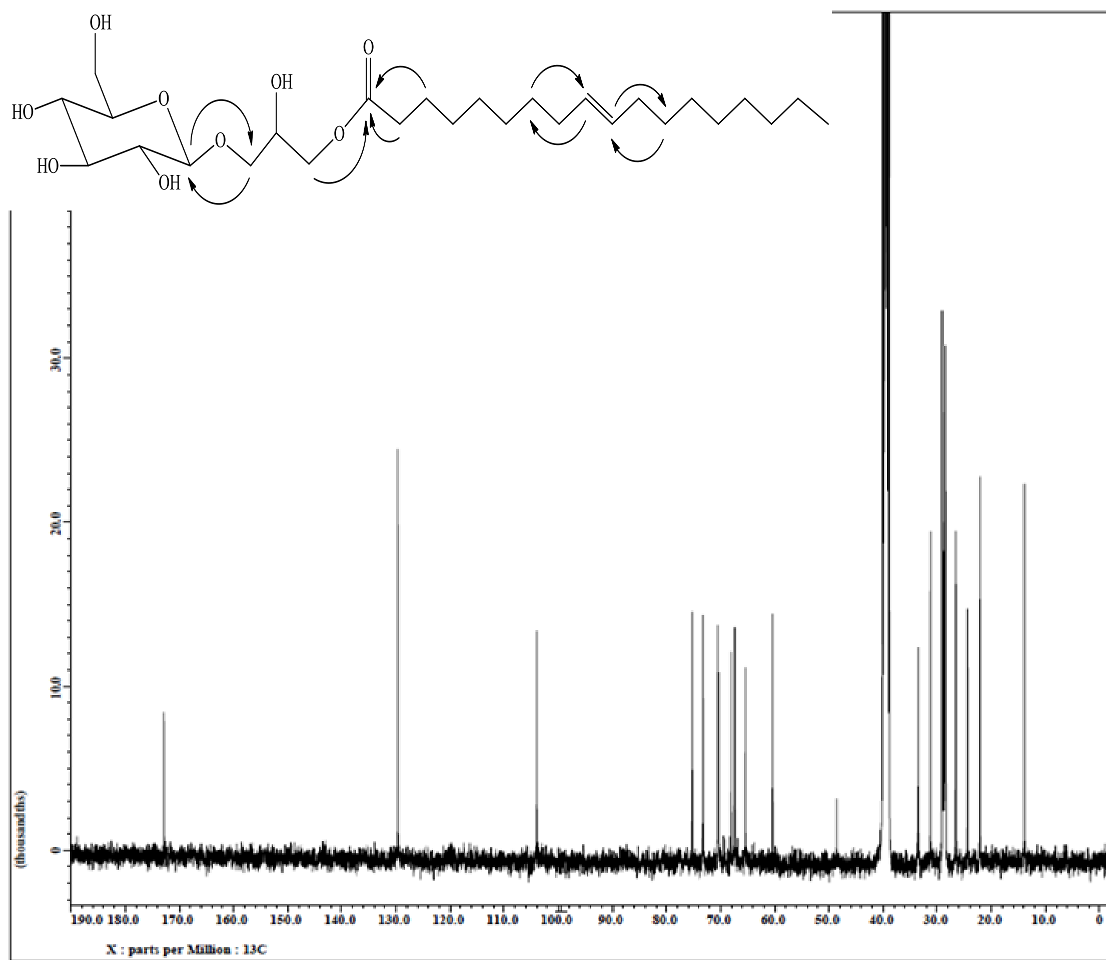

Figure 1-10. The ^{13}C-NMR spectrum of OGG (3) isolated from *P. arborescens* Holmes.

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1-*O*-nonadecyl-3-*O*-(β-D-gluco-pyranosyl)-glycerol (NGG, 4). White, amorphous powder. HR-EIMS: *m/z* 533.3331 $[M-H]^-$. ^1H NMR (DMSO-d_6, 400 MHz): δ 4.96 (1H, m, H-2), 4.08 (1H, J = 7.1, H-1′), 4.04 (1H, dd, J = 4.1, 11.2, H-1a), 3.97 (1H, dd, J = 6.4, 11.2, H-1b), 3.81 (1H, dd, J = 5.7, 10.3, H-3a), 3.68 (1H, dd, J =5.2, 10.3, H-3b), 3.62 (1H, br. d), 3.51 (1H, dd, J = 5.9, 10.4, H-6′a), 3.46 (1H, dd, J = 5.7, 10.4, H-6′b), 3.43 (1H, dd, J = 5.6, 10.4, H-4′), 3.27 (1H, m, H-3′), 3.17 (1H, m, H-1′), 2.29 (2H, t, J = 7.3, H_2-2″), 1.51 (2H, m, H_2-3″), 1.23 (H, m, H-4″-18″), 0.85 (3H, t, J =7.1, H_3-19″); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 172.9 (C-1″), 104.0 (C-1′), 75.2 (C-4′), 73.2 (C-3′), 70.5 (C-2′), 70.4 (C-2), 68.0 (C-5′), 65.4 (C-3), 67.4 (C-1), 33.4 (C-2″), 31.2 (C-17″), 28.5 – 29.0 (C-4″-16″), 24.4 (C-3″), 22.1 (C-18″), 13.9 (C-19″).

Compound 4, a white amorphous solid, displayed a quasimolecular ion peak in the negative HREIMS at m/z 533.3331 $[M-H]^-$, which corresponded to a molecular formula $C_{28}H_{54}O_9$, and this was supported from its NMR data. It also showed an almost identical NMR parttern to compound 3, except for absence of a signal at δ_C 129.6 (C-9″, C-10″) in the ^{13}C NMR spectrum. The anomeric carbon signal appeared at δ_C 104.0 (C-1′), whereas anomeric protons resonated at δ_H 4.06 (d, J = 7.1), indicated the β configuration of this sugar. The ester group signals (δ_C 172.9) and methyl signals (δ_C 13.9, δ_H 0.85), suggested that one saturated fatty acid chain was linked to the glycerol moiety. The presence of one acyl fatty acid was confirmed by the MS/MS fragment *m/z* 297, which suggested loss of nonadecanoic acid. In the HMBC spectrum, the glycerol methylene proton at δ_H 4.05 was correlated to the ester

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carbonyl at δ_C 172.9, indicating the linkage of the oleic acid to the glycerol portion at C-1. Therefore, the structure of 1 was determined as a new compound, 1-*O*-nonadecyl-3-*O*-(β-D-glucopyranosyl)-glycerol (Fig. 1-4-4).

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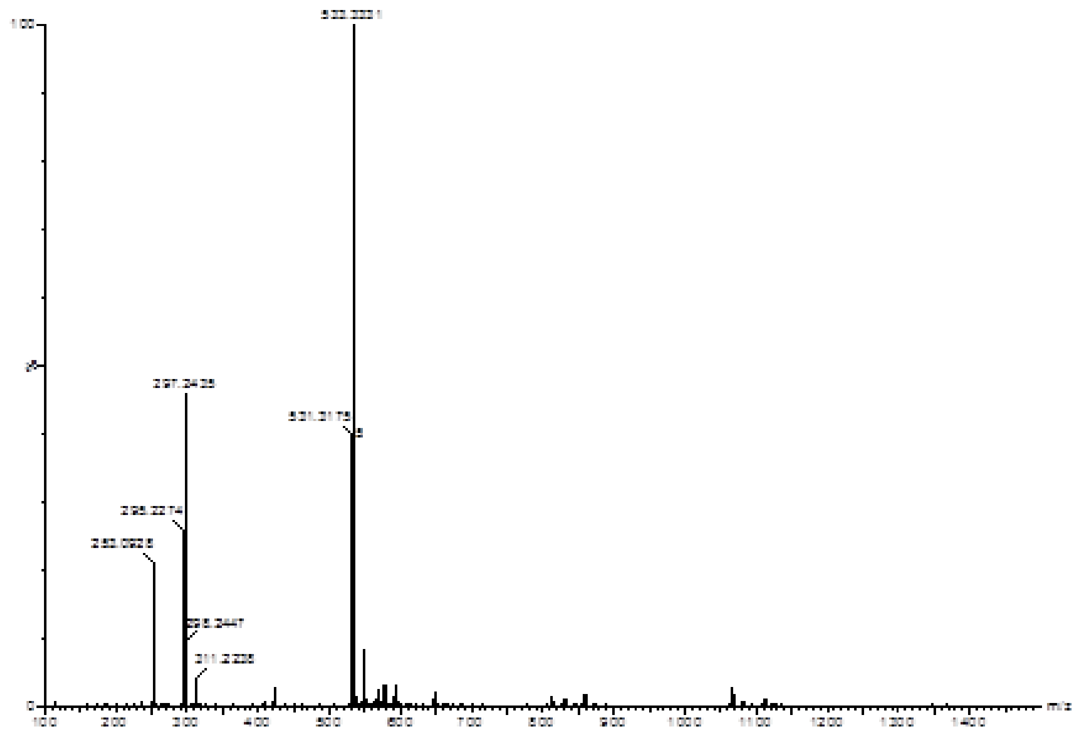

Figure 1-11. The mass spectrum of NGG (4) isolated from *P. arborescens* Holmes.

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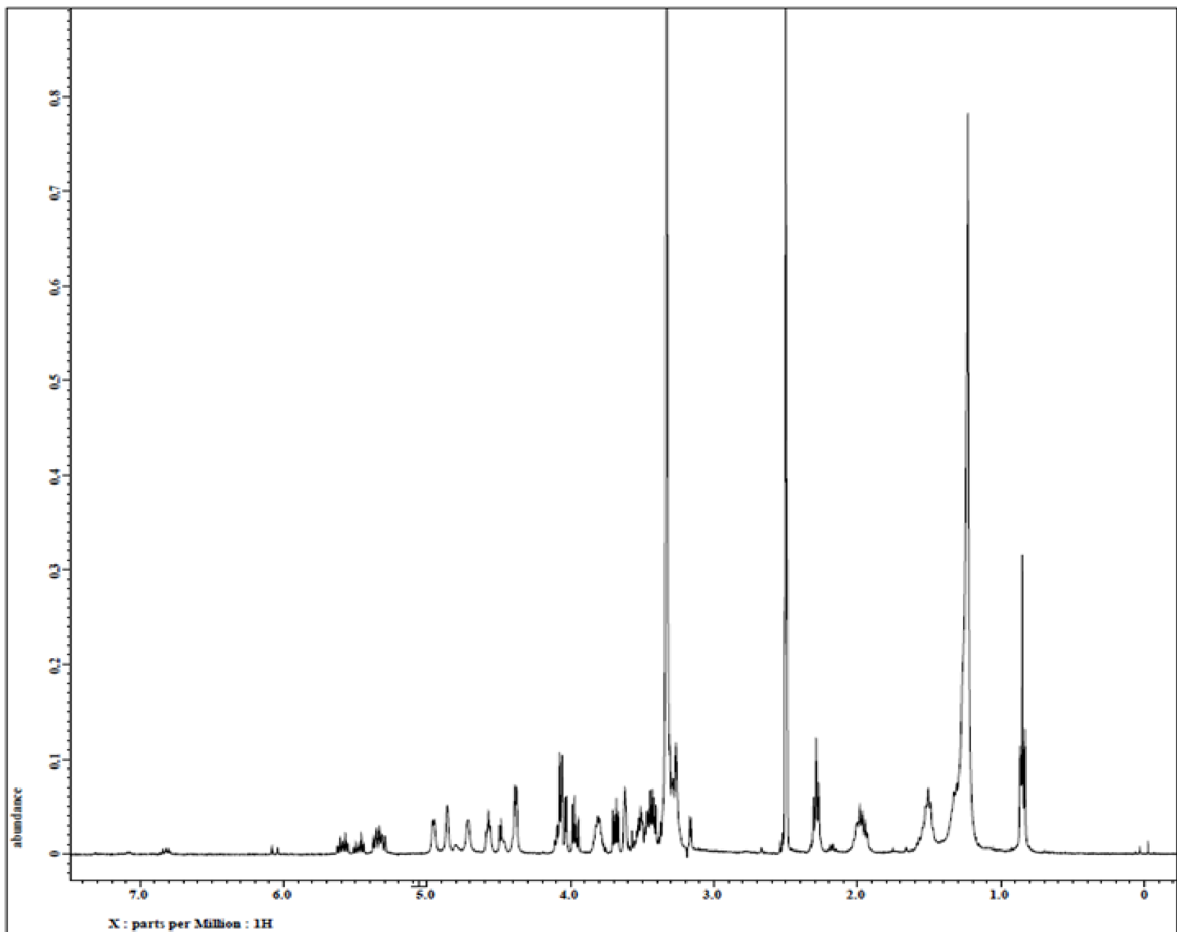

Figure 1-12. The ^{1}H-NMR spectrum of NGG (4) isolated from *P. arborescens* Holmes.

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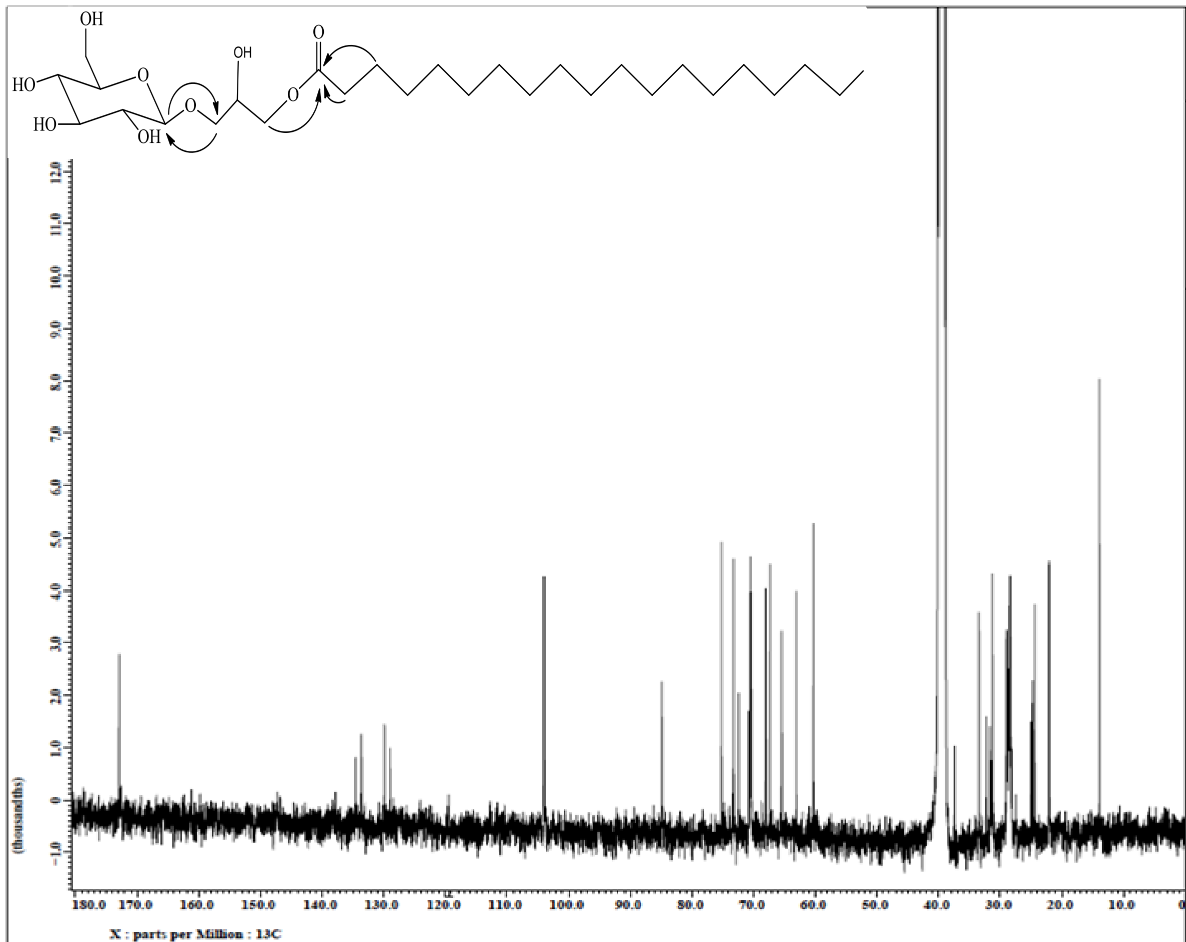

Figure 1-13. The ^{13}C-NMR spectrum of NGG (4) isolated from *P. arborescens* Holmes.

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2.2 Anticancer effects of the glyceroglycolipids from *P. arborescens* Holmes

In order to determine the inhibitory effects of the four isolated compounds on tumor cell growth, human promyelocytic leukemia cell line (HL-60) has been adopted. As shown in the Fig. 1-14, the compounds, OGG and NGG showed higher cell growth inhibitory effects than POGG and SQMG. At the concentrations of 200 μM, OGG and NGG showed the inhibition rate were 89.1% and 79.3%, respectively. SQMG, OGG and NGG also showed dose-dependent inhibitory effects of cancer cell growth in HL-60 cells. At the highest concentration of 200 μM, the growth inhibitory effect of SQMG on cancer cell is 60.4%. On the other hand, POGG didn't show a dose-dependent inhibitory effect and the activities at all the concentrations were less than 20%.

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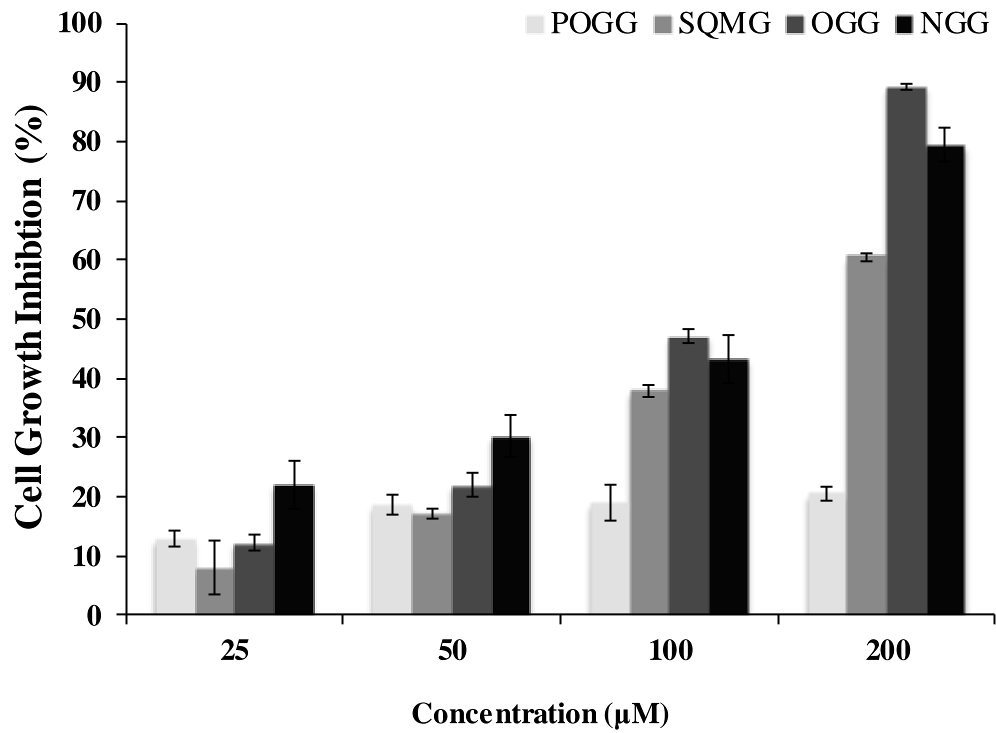

Figure 1-14. Inhibitory effects of the four compounds isolated from the marine brown alga, *P. arborescens* Holmes against the growth in the cancer cell (HL-60).

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2.3 Induction effects of apoptosis in HL–60 cells by the isolated compounds

Since three new compounds have shown the growth inhibitory effects in HL–60 cancer cells, the induction effects of apoptosis by treatments of these compounds have been investigated via nuclear staining with Hoechst 33342. Hoechst 33342 is used for specifically staining DNA, and has been used extensively for the detection of nuclear shrinkage, including chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, all of which are indicative of apoptosis [44]. As shown in the Figure1-15, in the control, which was non-treated, uninjured nuclei were clearly observed. However, the additions of SQMG, OGG and NGG at the different concentrations, especially at 100 and 200 μM, indicated significant levels of nuclear fragmentation, which is also indicative of apoptosis. This result suggests that the treatments of SQMG, OGG and NGG could induce the apoptosis in the HL–60 cells.

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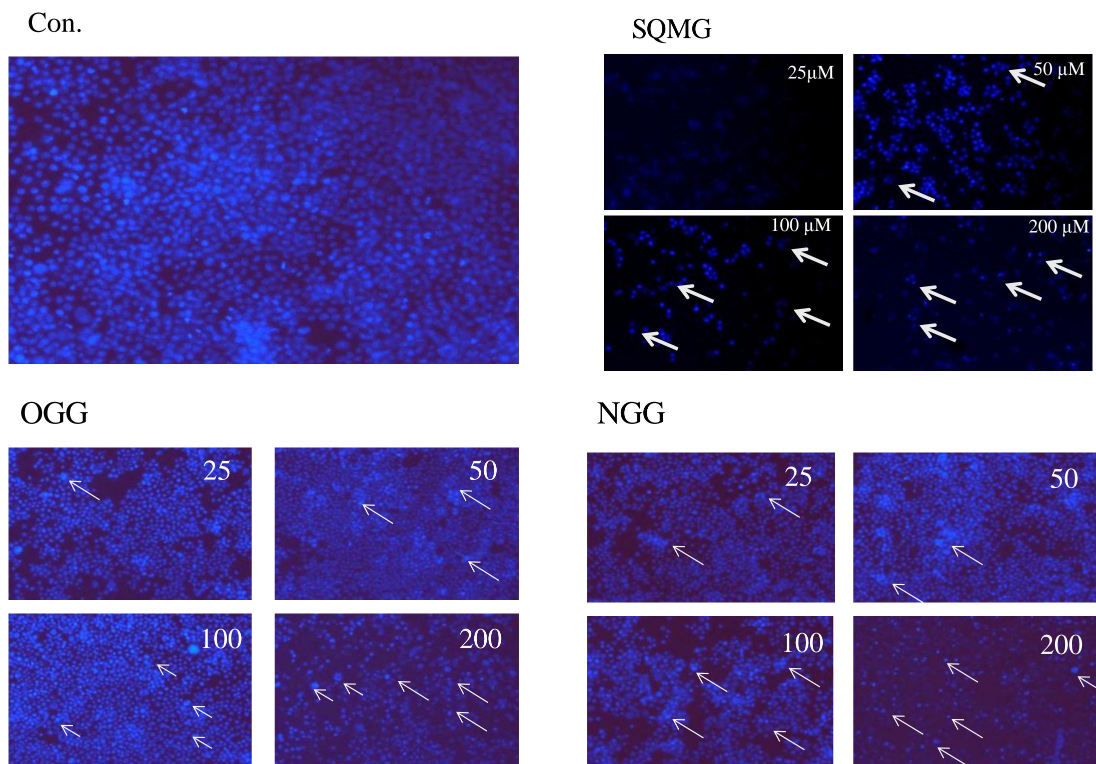

Figure 1-15. Induction of apoptosis by the treatment of SQMG, OGG and NGG at the different concentrations. HL-60 cells were seeded at 1 $\times 10^5$ cells/ml and treated with the different concentrations of the compounds. After an incubation of 24 h, cellular morphological changes were observed under a fluorescence microscope after Hoechst 33342 staining.

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2.4 Sub-G1 DNA content

Using flow cytometric analysis of PI-stained fixed cells, the anti-cancer effects of three new compounds associated with changes in the cell cycle distribution have been examined. Compared with the non-treated cells (Fig. 1-16), the sub-G1 DNA content was significantly increased when the cells were treated with the different concentrations of SQMG. Especially, the sub-G1 content increased to 67.2%at the concentration of 200 μM, (Fig. 1-16A). Furthermore, the treatments of OGG and NGG were also found to increase the sub-G1 contents. However, at the concentration of 200 μM, the sub-G1 contents of OGG and NGG treated groups were 37.1% and 22.1%, respectively (Fig. 1-16B). This result indicated that the treatment with three new compounds, especially SQMG could induce a sub-G1 phase arrest of the HL-60 cancer cell cycle.

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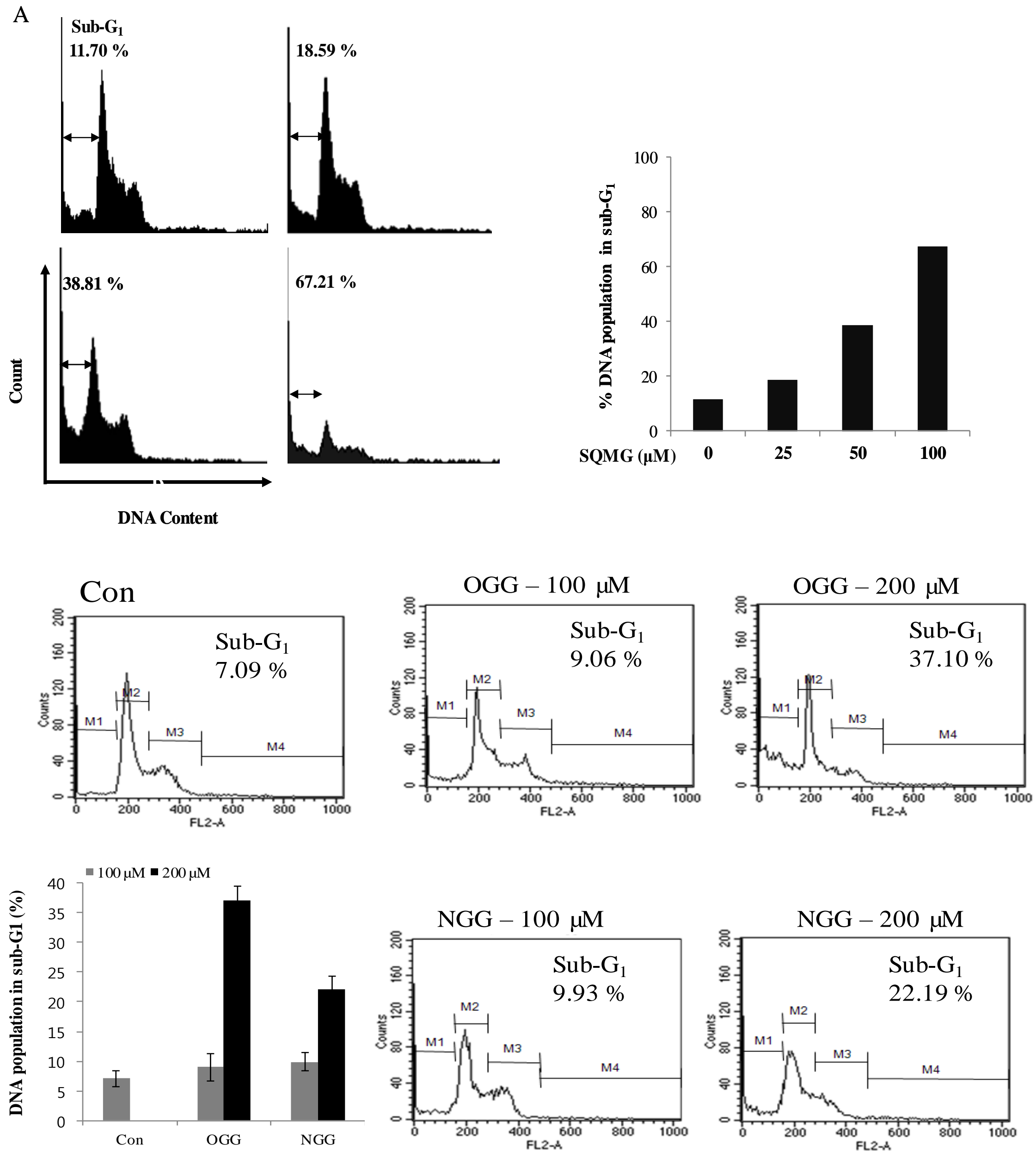

Figure 1-16. Effects of SQMG, OGG and NGG on the cell cycle distribution of HL-60 cells. HL-60 cells were treated with the different concentrations of the compounds for 24 h. Cells were fixed in 70% ethanol, stained with PI and the percentage of cells in G1 phase of the cell cycle was determined by flow cytometry.

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2.5 Regulations of caspase-3, Bax and Bcl-xL by the isolated compounds.

The activation of caspase cascade is a key element in the apoptotic process [45]. Caspase-3 is one of the key executioners in apoptosis, as it is cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP)[46].The Bcl-2 family, including anti-apoptotic (such as Bcl-xL and Bcl-2) and pro-apoptotic (such as Bax and Bad) members, also play important roles in the regulation of mitochondrial-mediated apoptosis [47]. In order to further analyze the possible mechanism of the cell apoptosis induced by SQMG, OGG and NGG, we examined the expression of caspase-3, Bax, and Bcl-xL in HL-60 cells after the treatment with 100 and 200 μM of three new compounds. The expression levels of Bax, a pro-apoptotic protein were increased by the compounds treatments at the concentrations of 100 and 200 μM, while the Bcl-xL protein levels were decreased in a dose-dependent manner. Furthermore, the treatments with SQMG, OGG and NGG were found to activate the caspase-3 in HL-60 cells (Fig.1-17). These findings suggest that three news compound isolated from *P. arboresens* induce apoptosis in HL-60 cancer cells via down-regulation of Bcl-xL and up-regulation of Bax and caspase-3.

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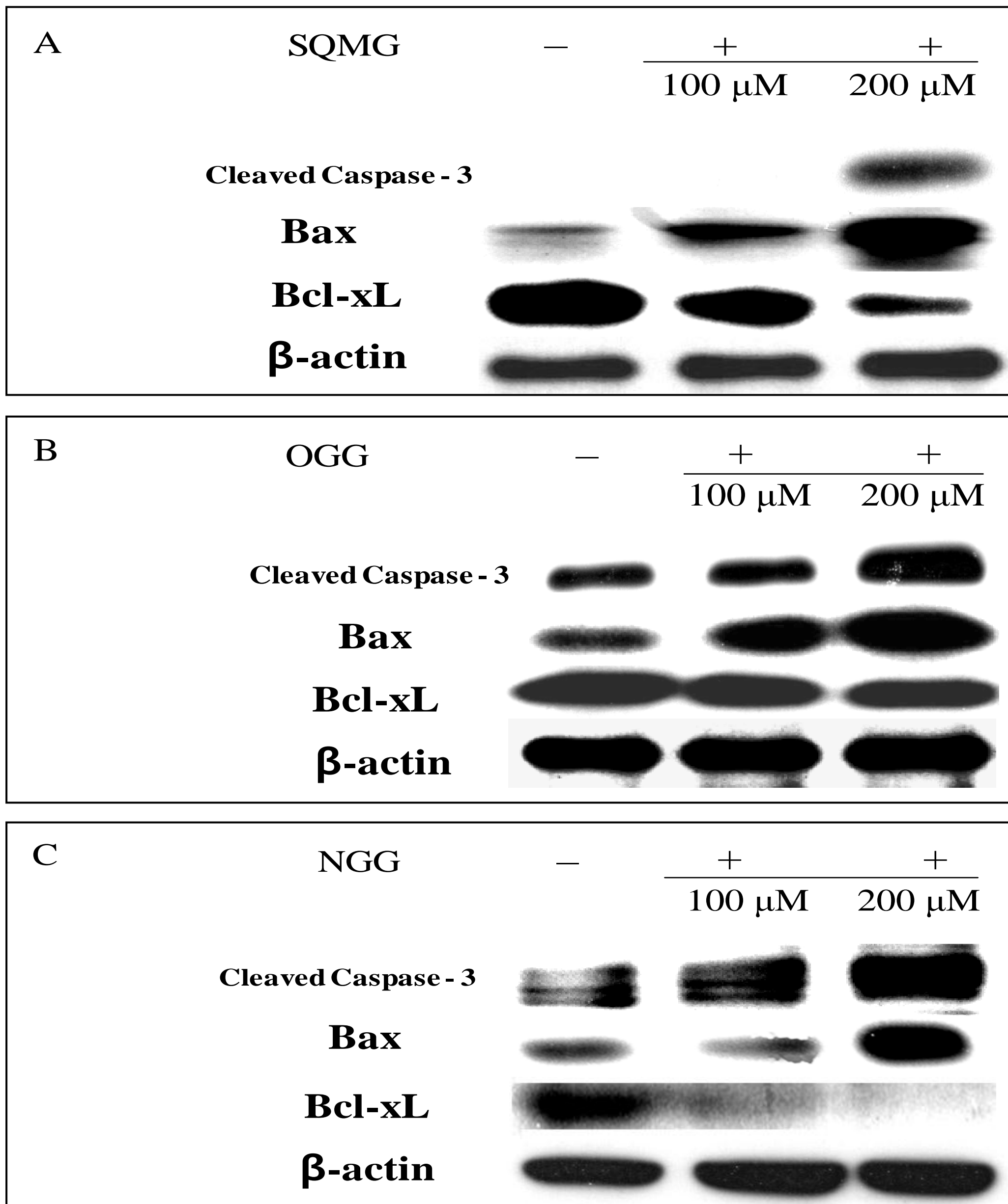

Figure 1-17. Effects of SQMG, OGG and NGG on up-regulation of Bax and caspase-3, down-regulation of Bcl-xl. The HL-60 cells were treated with the different concentrations of the compounds for 24 h. Equal amounts of cell lysate (30μg) were resolved via SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bax, anti-BCL-xl, and anti-caspase-3).

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3. Discussion

This is the first study on chemical composition of the marine brown alga, *Padina arborescens* Holmes. In this study, three new glyceroglycolipids, SQMG, OGG and NGG along with a known compound, 1–*O*–palmitoyl–2–*O*–oleoyl–3–*O*–(*β*–D–glucopyranosyl)–glycerol were isolated from marine brown alga, *P. arborescens* Holmes. The anti-cancer effects of the four glycerolglycolipids have been investigated on HL-60 cell. The results showed that OGG and NGG showed higher growth inhibitory effects than SQMG. The morphology changes of the HL-60cells treated with SQMG, OGG and NGG were also observed. When the cell treated with SQMG, OGG and NGG at the different concentrations, the obvious apoptosis were observed. Furthermore, the results of sub-G1 DNA contents indicated that the SQMG, OGG and NGG could significantly induced accumulation of sub-G1 DNA contents.

Apoptosis is an evolutionarily conserved and genetically regulated process that plays an essential role in the development and maintenance of cell homeostasis and is morphologically and biochemically different from necrosis [48, 49]. Many anticancer agents exert their effect by apoptosis. The Bcl-2 family proteins are key regulators of apoptosis. The anti-apoptotic Bcl-2 family of proteins (especially Bcl-2, Bcl-xL, Mcl-1) are frequently over expressed in cancer cells, including solid tumors. Therefore, they prevent death in corresponding cells and increase resistance to traditional treatments [50]. On the other hand, activation of the pro-apoptotic protein, Bax promotes cytochrome c release from the mitochondria. Released cytochrome c will activate initiator caspase-9 to activate sequential cascade of caspases. The

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proteolytic activation of executioner, such as caspase-3, results in the proteolysis of death substrates and subsequent DNA degradation and apoptotic death [51]. Our results clearly demonstrated that SQMG, OGG and NGG treatments to HL-60 cells resulted in a dose-dependent increase in the levels of Bax with a concomitant decrease in Bcl-xL levels. Moreover, the SQMG, OGG and NGG also found to up-regulate the level of caspase-3 in the cancer cells.

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4. Conclusion

In conclusion, this is the first study on natural product chemistry and biological activity of the isolated compounds from *P. arborescens* Holmes. Three new glyceroglycolipid compounds, SQMG, OGG and NGG isolated from *P. arborescens* Holmes were shown to inhibit the cell growth in HL-60 cells, and arrest cell cycle at sub-G1 phase. It is capable of inducing apoptosis in HL-60 cells through up-regulation of Bax and caspase-3, down-regulation of Bcl-xL level.

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Part Ⅱ

Isolation, Culture of Marine-Derived Fungi and Screening the Biological Activities of their Extracts

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Abstracts

In order to find new bio-resource, we tried to isolate and identify the marine-derived fungi from four types of marine samples including float, marine algae, animal, and wood collected from Jeju Island, Korea. Finally, 36 strains have been successfully isolated and identified from these samples. Then, all trains were cultured in the SWS medium. After extraction, the bio-activities including DPPH scavenging, NO production inhibitory effects and anti-cancer effects of the broth and mycelium extracts of each strain were determined. Most of isolated strains showed strong anti-oxidant activity against DPPH, especially the strains from *Penicillium* sp. and *Eurotium* sp. Combination of MTT and NO production inhibitory results, several extracts (B extracts of 021-2, 042-1, 047-2, 063-1, 074-2, 080-1, 82-2-2 and M extracts of 021-2, 042-1, 069-1) of marine fungi showed higher inhibitory effects on LPS-induced NO production without cytotoxicity in RAW 264.7 cells than thoes of other fungi. Furthermore, the extracts from the strains belonged to the *Penicillium* species showed higher cell growth inhibitory activity than other strains. And an unidentified strain 013-1 showed highest inhibitory activity among all fungal strains. Therefore, the fungal strains isolated from marine-derived samples could be the good sources for biological natural products.

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1. Materials and methods

1.1. General reagents

Lipopolysaccharide (LPS) was purchased from sigma Chemical Co (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin–streptomycin and trypsine-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).The other chemicals and reagents used were of analytical grade.

1.2. Samples and fungal strain isolation from collected samples.

Four types of samples including float, marine algae, animal, and wood were collect around the coast of Jeju Island, Korea, in August, 2011. Thereafter, all the samples were immersed by saline for 12hr at room temperature. The samples were aseptically cut into small piece using sterile surgical scissors and carefully placed onto the prepared petri-dish plates containing selected solid YPG culture media with antibiotics. These plates were sealed and labeled by sample code and initial culturing date. Then, they were put into the incubator at an incubation temperature of 29 ℃ with humidity.

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YPG solid media:

Glucose	1.0%
Nutrient Agar	2.0%
Peptone	2.0%
Yeast	1.0%

Dissolve in the filtered seawater, stirred and autoclaved at 121 ℃ for 20 minutes. Then, these media were laid the media at room temperature under UV light. The antibiotic was added when the temperature was decreased to less than 55 ℃. The medium were transferred to petri-dish.

1.3 Isolation and Identification of fungal strains in pure culture

After several days, when several fungal colonies observed growing on the surface of the above culture media plates, colonies that differ in time of appearances, size, color, and morphological shapes were further cultured. A loop full of each pure colony was picked up and inoculated onto solid culture media plates. The plates were incubated again in the incubator at 29 ℃ with humidity. The culture and transfer was repeated several times until finally pure isolates were obtained. Total thirty-six fungal strains have been isolated. The fungal strains were identified base on the sequencing of the ITS region. (Table 1) The voucher specimens are deposited at Marine Bio-Resource Technology Labortary of Jeju National Unversity.

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1.4 Liquid Culture and Extraction

The isolated marine-derived fungi were transfer to the 300 mL Erlenmeyer flasks containing 100 mL of SWS medium containing soytone (0.1%), solube starch (1.0%) and seawater (100%). The liquid culture flasks were put in the incubator at 29 ℃ with humidity for several days. Thereafter, the culture broth and mycelium were separated by filtration. Then broth was extracted with EtOAC (100 mL) three times to give broth extract (B). The mycelium was extracted with $CHCl_3$-MeOH (1:1) to provide mycelium extract (M). The broth and mycelium extracts were checked TLC and subjected in the further biological activity screening experiments.

1.5 DPPH radical scavenging assay

The free radical scavenging activity of the test samples was determined using the ESR spin-trapping technique. DPPH has been widely used to evaluate free radical generation. The DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine, JEOL, Tokyo, Japan) according to the previous described by Nanjo et al. [52]. 60 µL of each sample was added to 60 µL of DPPH (60 µmol/L) in ethanol. After 10 seconds of vigorous mixing, the solutions were transferred to Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adduct was determined by the ESR spectrometer exactly 2 min later under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, and temperature 298 K. All the radical scavenging activities (%) in the present study were

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calculated using the following equation, in which A and A_0 were the relative peak areas of the radical signal with and without a sample, respectively.

$$\text{Radical scavenging activity} = [1-(A/A_0)] \times 100$$

1.6 Screening nitric oxide (NO) production inhibitory effects of the extracts from marine-derived fungi

1.6.1 Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% FBS. The cells were incubated in an atmosphere of 5% CO_2 at 37 ℃ and were sub-cultured every 3 days.

1.6.2 MTT assay

The cells were seeded in 96-well plate at a concentration of 1×10^5 cells/ml (180 μL). After 24 h incubation at 37 ℃ under a humidified atmosphere, the cells were treated with LPS (1 μg/mL) and 10 μL of the samples, and further incubated for 24 h. The 50 μl of MTT stock solution (2 mg/ml) was then applied to the wells, to a total reaction volume of 250 μl. After 24 h of incubation, the plates were centrifuged for 5 min at $800 \times g$, and the supernatants were aspirated. The formazan crystals in each well were

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dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. The percentage inhibitory effect was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

1.6.3 Determination of Nitric Oxide (NO) Production

RAW 264.7 cells (2×10^5) were plated and incubated with samples in the absence or presence of LPS (1 μg/mL) for 24 h. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [53]. One hundred microliters of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the mixture was measured with a microplate reader (Ultraspec 2100 pro) at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

1.7 Screening anti-cancer effects of the extracts from Marine-Derived Fungi

1.7.1 Cell culture

HL-60 (Human promyelocytic leukemia cell line) was maintained at 37 ℃ in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured at a concentration of 1×10^5 cells/ml in RPMI-1640 medium supplemented with 10% (v/v)

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heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) for further experiments.

1.7.2 Cell growth inhibitory assay

The cell growth inhibitory assays of the test samples were examined by MTT assay. The cells were seeded in 96-well plate at a concentration of 1×10^5 cells/ml (180 μl). After 16 h incubation at 37 ℃ under a humidified atmosphere, the cells were treated with 10 μl of the samples, and further incubated for 30 min. The 50 μl of MTT stock solution (2 mg/ml) was then applied to the wells, to a total reaction volume of 250 μl. After 5 h of incubation, the plates were centrifuged for 5 min at 800 × *g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. The percentage inhibitory effect was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

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2. Results and Discussion

2.1 Culture and isolation of marine-derived fungi

Total thirty-six fungal strains have been isolated and identified from four types of samples, including alga, animal, float and some unknown samples. These fungal strains belong to five genera and several unidentified strains. Most of the isolated strains belong to the genus of *Penicillium* (19 strains). Four species of them belong to the genus of *Aspergillus*. Two species belong to the genus of *Eurotium*, two were from *Fusarium*, and one is *Auricularia sp.* Although some of the isolated strains were same, they were isolated from different hosts.

For evaluation of the biological activities of the extract from the fungal strains, all the strains were culture in the flask with 100 mL of liquid culture medium. After several days, the fungal strains were further extracted to give B and M extracts. The B and M extracts from each strain were checked by TLC to observe the profile of chemical composition (date do not show). All the extracts were prepared with different solvent for further experiments.

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Table 2-1. Total fungal strains isolated from marine resources.

No.	Host	Name	Similarity (%)
002-2	Float	*Aspergillus tamarii*	98
003-2	Float	-	
006-1	Unknown	*Aspergillus tamarii*	98
007-1	Float	-	
010-1	Alga (Br.)	*Penicillium chrysogenum*	100
010-2	Alga (Br.)	*Penicillium* sp.	96
013-1	Float	-	
015-1	Animal	*Eurotium repens*	100
015-2	Animal	*Eurotium amstelodami*	100
015-3	Animal	*Penicillium crustosum*	100
021-2	Unknown	*Penicillium* sp.	99
042-1	Alga (Br.)	-	
043-1	Alga (Br.)	*Auricularia polytricha*	99
045-1	Float	*Eurotium amstelodami*	100
045-2	Float	*Penicillium aff. sclerotiorum*	100
045-3	Float	*Aspergillus clavatus*	100
047-2	Float	*Penicillium chrysogenum*	100
050-1	Alga (Red)	*Fusarium* sp.	100
053-1	Animal	*Penicillium janthinellum*	96
063-1	Alga	*Aspergillus* sp.	98
063-2	Alga (Br.)	*Penicillium citreonigrum*	100
063-3	Alga (Br.)	*Aspergillus* sp.	98
064-1	Alga (Br.)	*Penicillium herquei*	95
065-2	Alga (Br.)	*Penicillium crustosum*	100
066-2	Alga (Br.)	*Penicillium expansum*	100
068-1	Alga (Br.)	*Penicillium* sp.	100
069-1	Alga (Red)	*Fusarium oxysporum*	100
074-2	Alga (Gre.)	*Penicillium* sp.	100
075-1	Alga	*Penicillium oxalicum*	100
076-1	Float	*Eurotium amstelodami*	98
077-1	Float	*Penicillium* sp.	100
079-1	Alga (Red)	*Penicillium* sp.	99
079-2	Float	*Penicillium chrysogenum*	99
079-3	Alga (Red)	*Penicillium* sp.	100
080-1	Alga (Red)	*Penicillium* sp.	100
082-2-2	Alga (Red)	-	

-Indicate unidentified fungi.

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2.2 DPPH scavenging activities

DPPH is a free-radical generating compound and has been widely used to evaluate the free-radical scavenging ability of various antioxidant compounds [54, 55]. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [56]. The DPPH scavenging activities of extracts from marine fungi are shown in Fig 2-1. Most broth extracts of marine fungi showed higher DPPH scavenging activity than the mycelium extracts. That may be due to the containing of salt in the mycelium extract. Over 20 species of marine fungi show scavenging activity more than 60% against DPPH at concentration of 1 mg/mL. Two strains including *Eurotium repens* (015-1) and *Penicillium crustosum* (015-3) didn't grow in the SWS medium. The DPPH activities of the extracts from them were not measured. Among all the marine fungi, the broth extracts from *Penicillium chrysogenum, Eurotium amstelodami, Aspergillus clavatus, Penicillium herquei, Penicillium sp.* (010-2, 074-2, 079-1, 077-1, 079-1, 079-3, 080-1) showed higher activities than other species with activities of more than 70% at the concentration of 1 mg/mL. The mycelium extracts of *Penicillium* sp. (010-2), *Eurotium amstelodami, unidentified* (013-1), *Penicillium chrysogenum,* and *Penicillium sp.* (068-1, 079-3, and 080-1) showed higher DPPH activities than those of other mycelium extracts at concentration of 1 mg/mL (Figure 2-1). The results indicated that both B and M extract from these fungi could be the resources to provide antioxidant natural products.

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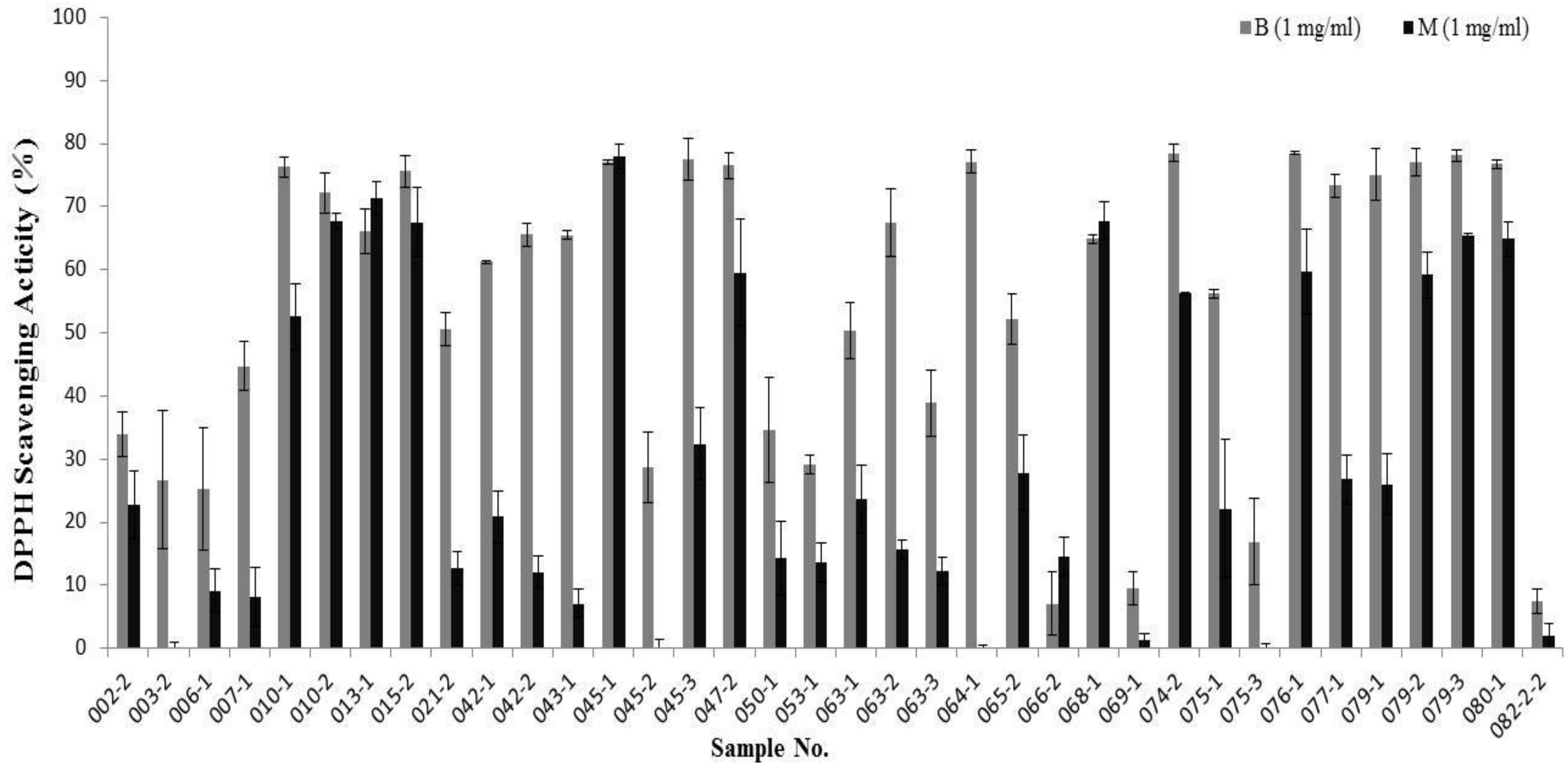

Figure 2-1 DPPH radical scavenging activity of broth and mycelium extract from marine-derived fungi. Experiments were performed in triplicated and the data are expressed as mean ±SE.

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2.3 Assessment of cell viability

In order to evaluate cytotoxicity of the extracts from marine-derived fungi in RAW 264.7 cells, cell viability was estimated via an MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells [57]. The results were shown in the figure 2-3. The cell treated with LPS showed 87% cell viability. After incubation with LPS and extracts from marine fungi, some extracts of marine fungi showed cytotoxicity in RAW 264.7 cell. Especially, the broth extracts of unidentified strain 013-1, *Aspergillus clavatus* (045-3), and *Penicillium janthinellum* (053-1), the mycelium extracts of *Penicillium janthinellum* (053-1) and 82-2-2 showed lowest cell viabilities. Some extracts of marine fungi showed slight cytotoxicity. However, the B extracts of 021-2, 042-1, 047-2, 050-1, 063-1, 074-2, 077-1, 080-1, 82-2-2 and M extracts of 015-2, 021-2, 042-1, 045-1, 063-3, 069-1 did not showed cytotoxicity in RAW 264.7 cells.

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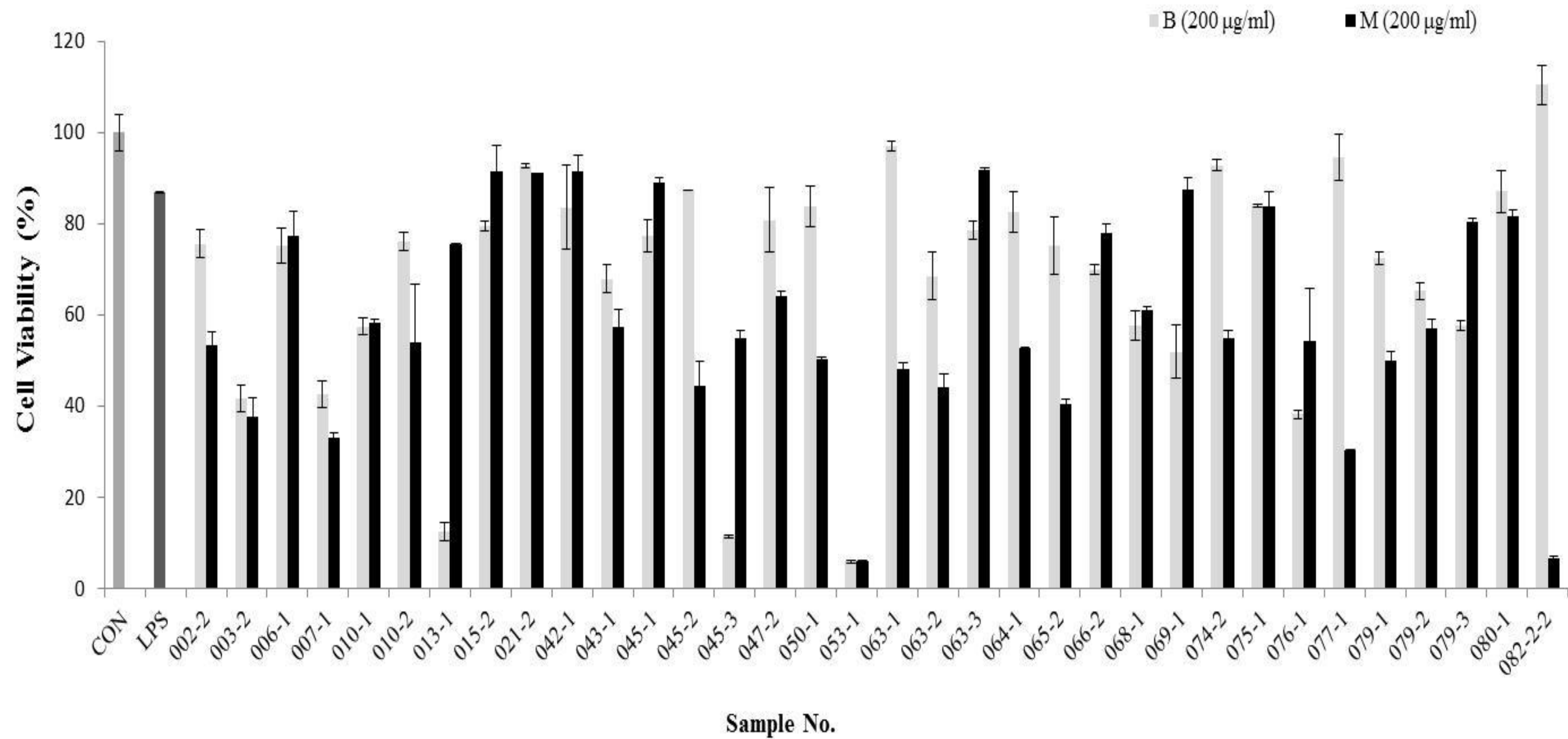

Figure 2-2. Cell viability of the extracts from marine-derived fungi in RAW 264.7 macrophages. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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2.4 Effects of the extracts from marine-derived fungi on LPS-induced NO production.

Nitric oxide (NO) plays an important role in the regulation of many physiological functions such as vasodilation, neurotransmission, and inflammation [58, 59]. NO is produced from L-arginine by a chemical reaction catalyzed by NO synthase (NOS) in living systems. However, excessive NO has been implicated in various pathological processes including septic shock, tissue damage following inflammation, and rheumatoid arthritis [60]. To evaluate the potential anti-inflammatory effects of the extracts from marine fungi on NO production in RAW 264.7 cells, cell were treated with extracts of marine fungi for 1 h and then treated with LPS (1 μg/ml) for 24 h. NO concentrations were measured in the culture supernatants by the Griess reaction and ELISA assay. LPS treatment significantly increased the production of NO. According to the results of cell viability of extracts in RAW 264.7 cells, the NO inhibitory effects of some extracts from marine fungi maybe were caused by cell damage or death. However, the B extracts of 021-2, 042-1, 047-2, 063-1, 074-2, 080-1, 82-2-2 and M extracts of 021-2, 042-1, 069-1 showed significant inhibitory effects of NO production without cytotoxicity at concentration of 200 μg/ml. Especially, the B extracts 021-2, 047-2, 074-2 and 82-2-2 showed highest inhibitory activities of NO production as 17.1%, 0.9%, 1.0%, and 0%, respectively. Therefore, these marine-derived fungi may produce anti-inflammatory nature products.

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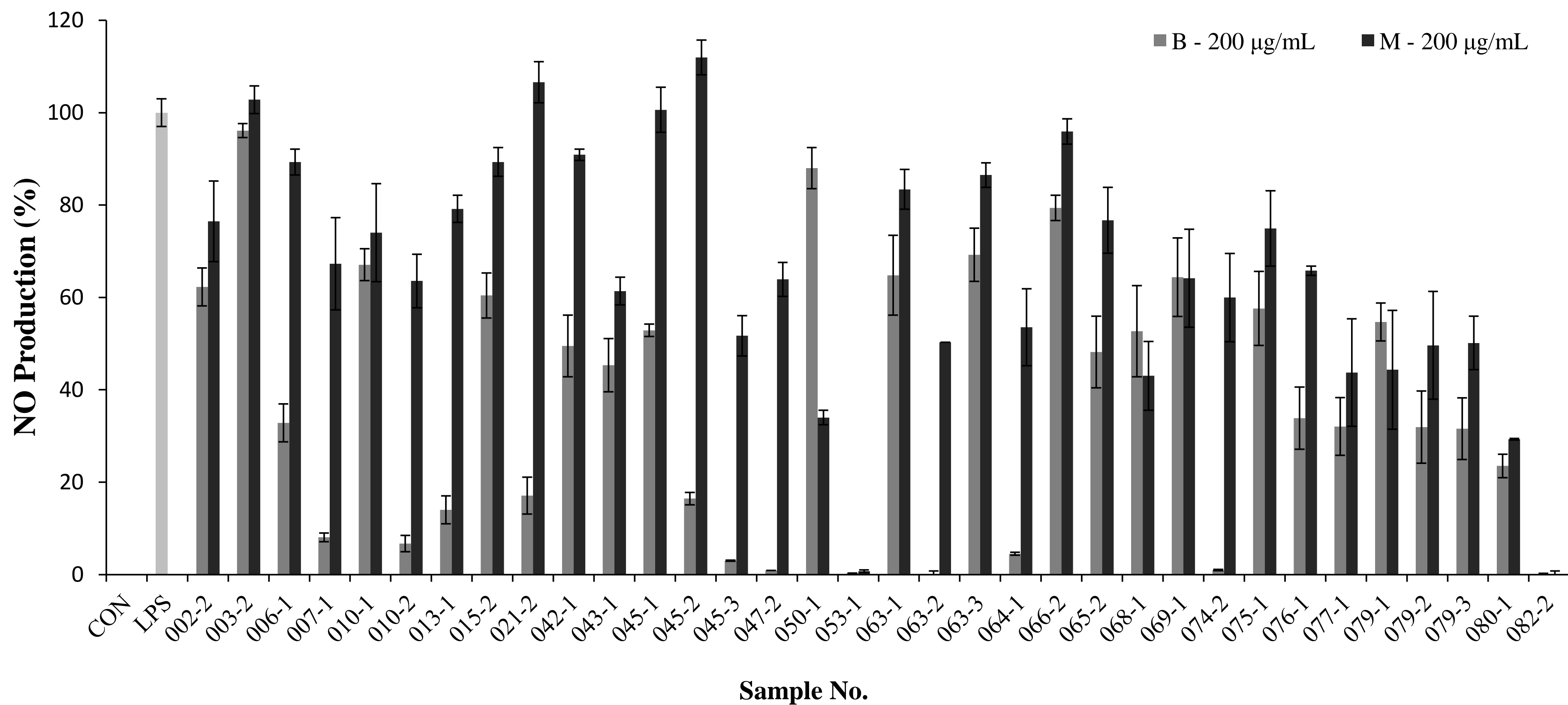

Figure 2-3. No production inhibitory effects of the extracts from marine-derived fungi. Experiments were performed in triplicate and the data are expressed as mean ±SE.

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2.5. Cell growth inhibitory effects of the extracts from marine-derived fungi in HL-60 cells.

In this study, we investigated the cell growth inhibitory effects of the extracts from marine fungi in HL-60 (Figure 2-4). The B extracts of unidentified fungi 013-1 and *Aspergillus clavatus* (045-3) showed highest growth inhibitory activity in HL-60 cells. At the concentration of 200 μg/mL, the activities of them were 94.7% and 92.0%, respectively. The B extracts of 007-1, *Penicillium* sp. (010-2), *Eurotium amstelodami* (015-2), *Auricularia polytricha* (043-1), *Penicillium chrysogenum* (047-2), *Penicillium janthinellum* (053-1), *Penicillium citreonigrum* (063-2), *Penicillium herquei* (064-1) and 82-2-2 showed inhibitory effects more than 60% in HL-60 cells. In case of M extracts from marine fungi, the extracts of *Fusarium* sp. (050-1), *Penicillium janthinellum* (053-1), and 82-2-2 exhibited the activities more than 60% on cell growth inhibition at concentration of 200 μg/mL. Many reports have been showed that the polyketide and alkaloids isolated from marine fungi exhibited anti-cancer activities in various cancer cell lines. Therefore, the extracts of marine-fungi used in this study could be the potential sources of polyketides and alkaloids with anti-cancer effects.

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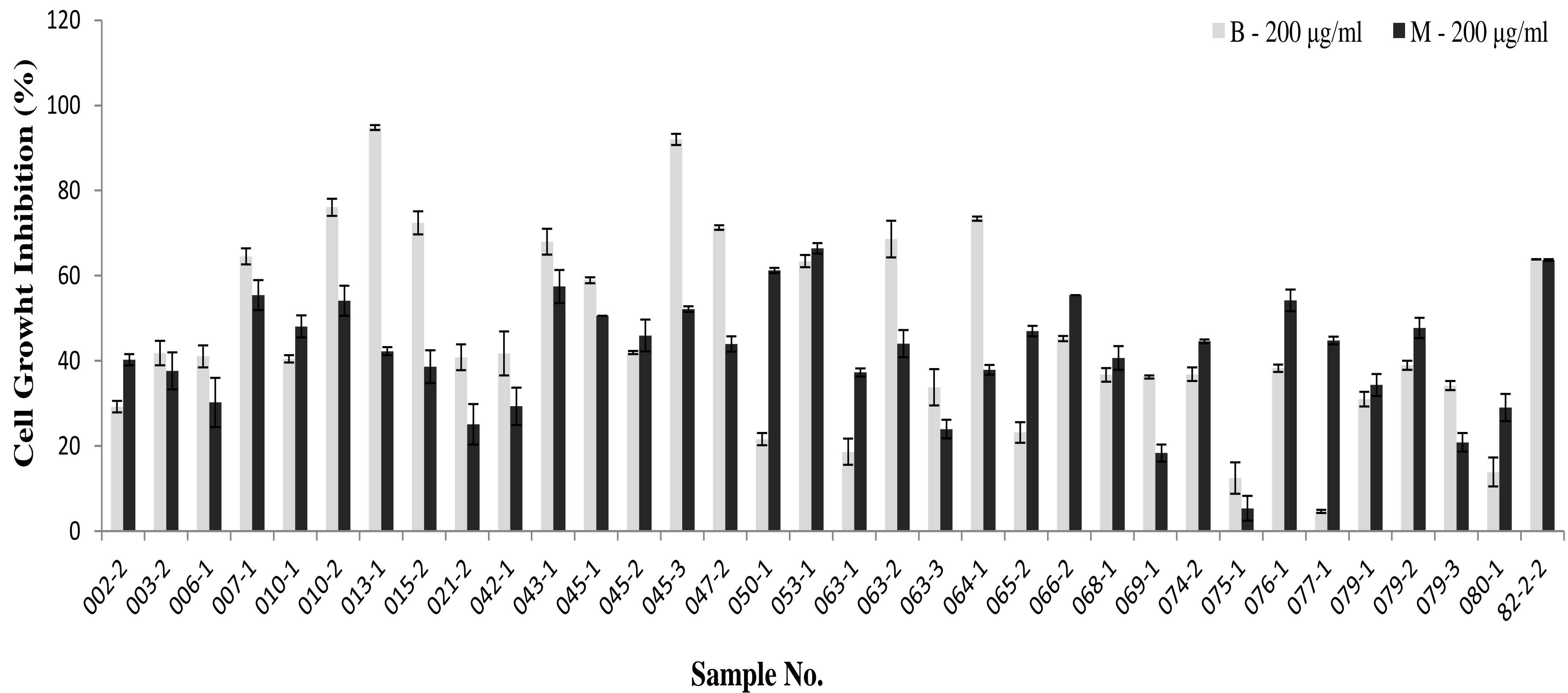

Figure 2-4. Cell growth inhibitory effects of the extracts from marine-derived fungi in HL-60 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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3. Conclusion

In this study, 36 strains have been isolated and identified from marine-derived samples. Most of them showed strong anti-oxidant activity against DPPH. Several extracts of marine fungi showed inhibitory effect on LPS-induced NO production without cytotoxicity in RAW 264.7. The extracts from the strains belonged to the *Penicillium* species showed higher cell growth inhibitory activity than other strains. And an identified strain 013-1 showed highest inhibitory activity among all fungal strains. Therefore, the fungal strains isolated from marine-derived samples could be the good sources for biological natural products.

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Part III

Natural Products from the Marine-Derived Fungus, *Eurotium amstelodami* and their Biological Activities

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Abstract

Due to the extracts from *Eurotium amstelodami* (015-2) have shown strong bioactivities (DPPH scavenging, inhibition of NO production and anti-cancer effects) among all marine fungi, we selected *E. amstelodami* as our target strain for the researches of natural products and their biological activity. Four compounds were successfully isolated from the B extract of *E. amstelodami*. The structures of the compounds were determined as asperflavin (1), questinol (2), neoechinulin A (3) and preechinulin (4) base on the analysis of the MS and NMR spectral data as well as comparison of those data with the published data. Among them, questinol was isolated from *E. amstelodami* for the first time. Moreover, the anti-inflammatory effects of asperflavin and questinol in LPS-stimulated RAW264.7 cells were investigated. The results showed that treatment with asperflavin and questinol at indicated concentrations could significantly inhibit NO and PGE_2 production. Asperflavin and questinol were also found to inhibit the production of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6. Furthermore, asperflavin and questinol suppressed the expression level of iNOS in a dose-dependent manner, respectively. However, they could not affect the expression of COX-2. Therefore, our study suggests a potential use of these compounds might be selected as a promising agent for the prevention and therapy of inflammatory disease.

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1. Materials and Methods

1.1 General materials

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. Column chromatography was carried out by Silica Gel 60 (230–400 mesh, Merck, Germany), ODS (12 nm, YMC, Japan), Sephadex LH-20 (Sigma, St. Louis, MO, USA). Thin-layer chromatography (TLC) was run on pre-coated Merck Kieselgel 60 F254 plates (0.25 mm). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylte-trazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

1.2 Fungal Material

Base on the results of screening for the biological activities of the extracts from marine fungi, the fungal strain, *Eurotium amstelodami* (015-2) has been selected as our target strain for research of bioactive natural products and their bioactivities. The fugal strain was cultured (8 L) for 30 d (static) at 29 ℃ in SWS medium containing of soytone (0.1%), soluble starch (1.0%) and seawater (100%) (Fig. 3-1).

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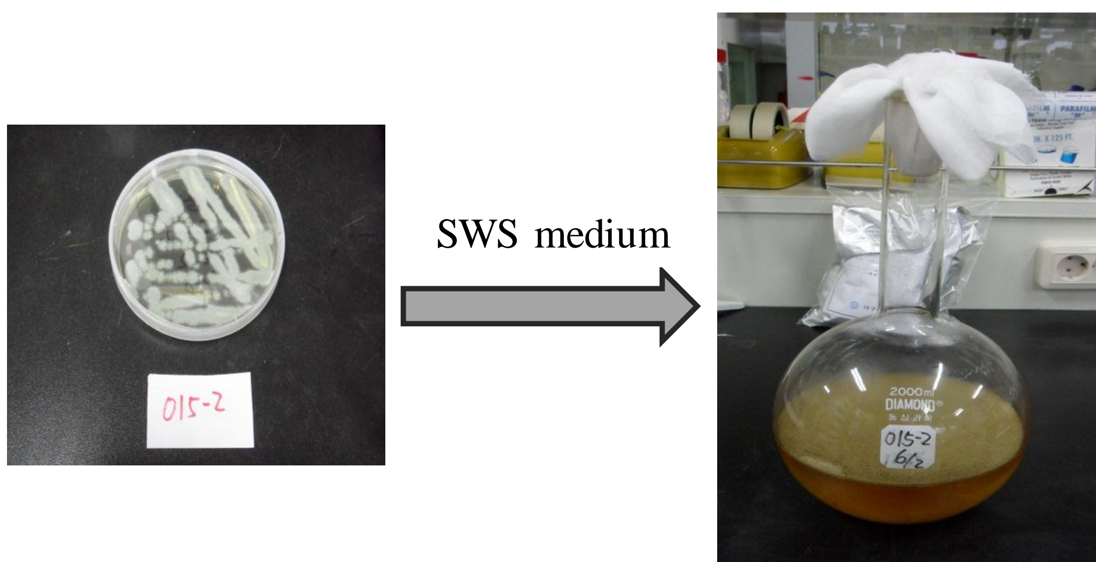

Figure 3-1 Morphology of *E. amstelodami* and culture in SWS medium.

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1.3 Extraction and Isolation of Natural Products

The cultured broth of the strain *E. amstelodami* was filtered through cheese cloth to separate into broth and mycelium. The broth was extracted with EtOAc, and the EtOAc solution was concentrated under reduced pressure to give a broth extract (015-2B) (0.7 g). The mycelium was freeze-dried and extracted three times with $CHCl_3$-MeOH (1:1) for 2 h using sonication and gave a mycelium extract (015-2 M) (2.2 g) after evaporation and dry. The broth extract (0.7 g) was subjected to silica gel flash chromatography (*n*-hexane/EtOAc, EtOAc/MeOH) to furnish twelve fractions (B1-B12) on the basis of TLC analysis. Fr. B7 (161.2 mg) was further purified by Sephadex LH-20 column eluting with MeOH to give compound 1 (7.6 mg) and crude compound 2 and 3. Final purification of the each fraction by HPLC (Sunfire, Waters, 50% MeOH) yielded the compounds 2 (25.2 mg) and 3 (35.6 mg). Fr. B10 (130.5 mg) was further purified by Sephadex LH-20 column eluting with MeOH to give crude compound 4. Then the fraction was finally purified by HPLC to afford compound 4.

The ^{1}H NMR and ^{13}C NMR spectra of the isolated compounds were recorded on a JEOL JNM-ECP 400 MHz NMR spectrometer, using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer.

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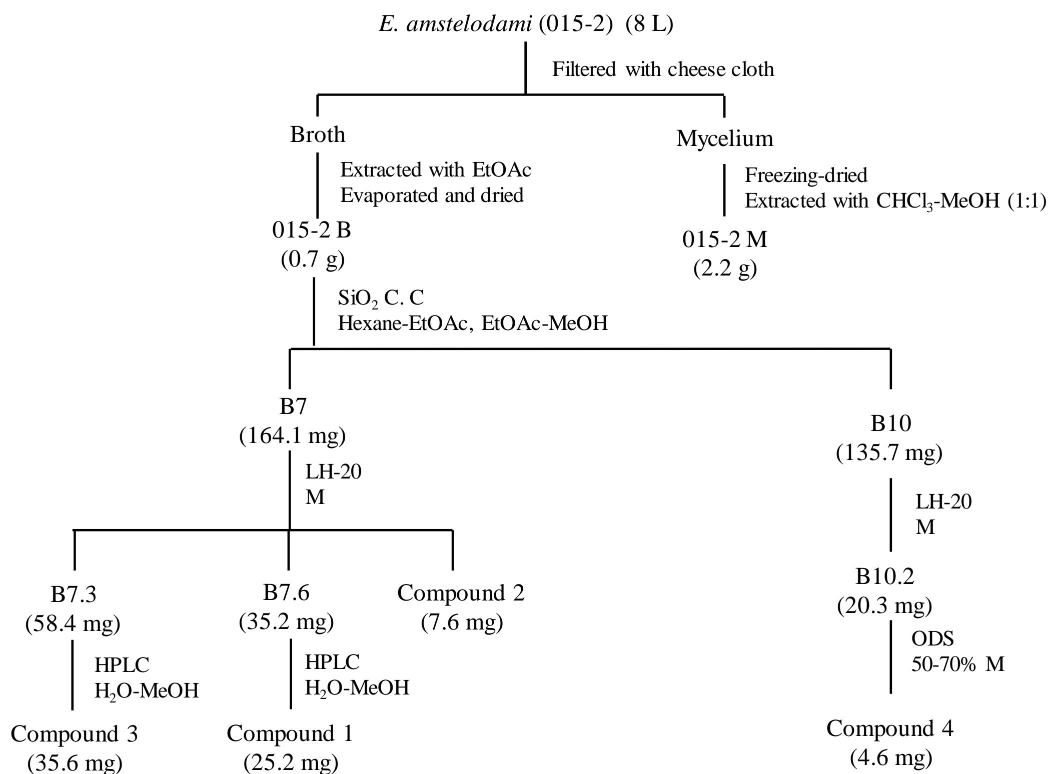

Figure 3-2. Isolation scheme of the natural products from *E. amstelodami*.

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Compound 1 (asperflavin): greenish amorphous powder, EI-MS *m/z*: 287 $[M-H]^-$. ^1H NMR (DMSO-d_6, 400 MHz): δ 6.78 (1H, s, H-10), 6.54 (1H, d, *J* = 2.0, H-5), 6.42 (1H, d, *J* = 2.0, H-7), 3.84 (3H, s, CH_3O-8), 2.95 (2H, s, H-4), 2.85 (1H, d, *J* = 16.9, H-2a), 2.79 (1H, d, *J* = 16.9, H-2b), 1.26 (3H, s, CH_3-3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 203.2 (C-1), 165.0 (C-9), 161.2 (C-8), 160.6 (C-6), 141.6 (C-10a), 137.8 (C-4a), 115.7 (C-10), 108.8 (C-8a), 108.1 (C-9a), 101.9 (C-5), 97.8 (C-7), 69.4 (C-3), 55.7 (CH_3O-8), 51.5 (C-2), 42.7 (C-4), 28.9 (CH_3-3).

Compound 2 (questinol): yellow powder, EI-MS *m/z*: 287 $[M-H]^-$. ^1H NMR (DMSO-d_6, 400 MHz): δ 13.31 (1H, s, OH-1), 7.57 (1H, d, *J* = 1.6, H-4), 7.20 (1H, d, *J* = 1.6, H-2), 7.20 (1H, d, *J* = 2.3, H-5), 6.83 (1H, d, *J*= 2.3, H-7), 4.58 (2H, s, $C\underline{H}_2OH$-3), 3.90 (3H, s, 6-OCH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 186.3 (C-9), 182.3 (C-10), 164.8 (C-1), 163.5 (C-6), 161.7 (C-8), 151.2 (C-3), 136.8 (C-10a), 132.1 (C-4a), 120.9 (C-2), 115.8 (C-4), 115.1 (C-8a), 112.5 (C-9a), 107.2 (C-5), 105.0 (C-7), 62.0 (CH_2OH-3), 56.3 (OCH_3-8).

Compound 3 (neoechinulin A): white powder. EI-MS *m/z*: 324 $[M+H]^+$, ^1H NMR (DMSO-d_6, 400 MHz): δ 1.38 (3H, d, *J* = 6.8, H-12), 1.47 (6H, s, CH_3-18/19), 4.16 (1H, qd, *J* = 7.2, 1.6, H-12), 5.01 (1H, d, *J* = 13.1, H_a-17), 5.05 (1H, d, *J* = 6.2, H_b-17), 6.07 (1H, dd, *J* = 13.1, 6.2, H-16), 6.89 (1H, s, H-14), 7.01 (1H, dd, *J* = 7.6, 7.3, H-6), 7.08 (1H, dd, *J* = 7.8, 7.6, H-5), 7.19 (1H, d, *J* = 7.8, H-4), 7.42 (1H, d, *J* = 7.3, H-7), 8.32 (1H, s, H-8), ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.4 (C-13), 159.9 (C-10), 145.1 (C-16), 143.9 (C-2), 135.1 (C-7a), 125.9 (C-3a), 124.9 (C-9), 120.7 (C-6), 119.3 (C-5), 118.8 (C-4), 111.6 (C-8), 111.5 (C-7), 110.1 (C-17), 103.4 (C-3), 50.5

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(C-12), 38.9 (C-15), 27.5 (C-18/19), 19.6 (C-20).

Compound 4 (preechinulin): white powder. EI-MS *m/z*: 326 $[M+H]^+$, ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.23 (3H, d, J = 7.1, H-20), 1.49 (6H, s, CH_3-18/19), 3.07 (1H, dd, J = 9.0, 14.4, H_a-8), 3.36 (1H, d , J = 4.6, H_b-8), 3.79 (1H, qd, J = 2.5, 7.0, H-12), 3.96 (1H, m, H- 9), 5.04 (2H, d, J = 17.6, H-17), 6.18 (1H, dd, J = 10.5, 17.4, H-16), 6.93 (1H, dd, J = 7.3, 7.5, H-5), 7.02 (1H, dd, J = 7.7, 7.3, H-6), 7.31 (1H, d, J =7.5, H-4), 7.42 (1H, d, J = 7.7, H-7), 7.49 (1H, d, J = 2.9, NH-14), 8.16 (1H, d, J = 2.5, NH-11), 10.5 (1H, s, NH-1). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 167.8 (C-13), 167.3 (C-10), 146.5 (C-16), 141.3 (C-2), 134.9 (C-7a), 128.9 (C-3a), 120.5 (C-6), 118.3 (C-5), 117.9 (C-4), 111.0 (C-17), 110.1 (C-7), 104.6 (C-3), 55.7 (C-9), 50.3 (C-12), 31.0 (C-8), 28.0 (C-19), 27.9 (C-18), 20.6 (C-20).

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1.4 Anti-inflammatory effects of the compounds isolated from *E. amstelodami*.

1.4.1 Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% FBS. The cells were incubated in an atmosphere of 5% CO_2 at 37 ℃ and were sub-cultured every 3 days.

1.4.2 MTT assay

The cells were seeded in 96-well plate at a concentration of 1×10^5 cells/ml (180 μl). After 24 h incubation at 37 ℃ under a humidified atmosphere, the cells were treated with 10 μl of the samples, and further incubated for 30 min. The 50 μl of MTT stock solution (2 mg/ml) was then applied to the wells, to a total reaction volume of 250 μl. After 24 h of incubation, the plates were centrifuged for 5 min at 800 $\times g$, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. The percentage inhibitory effect was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

1.4.3 Determination of Nitric Oxide (NO) Production

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RAW 264.7 cells (5×10^5) were plated and incubated with samples in the absence or presence of LPS (1 μg/mL) for 24 h. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. One hundred microliters of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the mixture was measured with a microplate reader (Ultraspec 2100 pro) at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

1.4.4 Determination of PGE_2 Production

RAW 264.7 macrophages were plated on 24well plates at a density of 5×10^5cells/ml. The cells were pre-treated with 1 h with the compounds at the concentrations of 50, 100, 200 μM prior to 24h of stimulation with LPS (1 μg/ml). The culture supernatants were immediately utilized for PGE_2 determination. The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immunoassay kit according to the manufacturer's instructions. The production of PGE_2 was measured relative to that following control treatment.

1.4.5 Measurement of Pro-inflammatory Cytokines (TNF-α, IL-1β and IL-6) Production.

To determine the effects of the compounds on production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, the RAW 264.7 macrophages were incubated with the compounds (50, 100 and 200μM) in the presence or absence of LPS (1μg/ml) for 24 h. The inhibitory effects of compound 1 and 2 on the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) production from LPS-treated

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RAW 264.7 cells was determined as described in the cho et al. [61] protocols. Supernatants were used for pro-inflammatory cytokines assay using mouse ELISA kit.

1.4.6 Western Blot Analysis

Murine macrophage cell line RAW 264.7 were pre-incubated for 24 h, and then stimulated with LPS (1 μg/ml) in the presence of the test compounds for the indicated time. After incubation, the cells were collected and washed twice with cold-PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin] and kept on ice for 30 min. Cell lysate were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay kit.. Aliquots of the lysates (30-50 μg of protein) were separated on a 12%SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane(BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin (BSA), the membrane was then incubated with specific primary antibody at 4 ℃forovernight.Themembranewas further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The immune-active proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

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1.5 Statistical Analysis

All the data were presented as mean ± S.D. from three independent experiments unless stated otherwise. Statistical comparisons between different treatments were done by one way ANOVA with Student Newman Keul's post hoc tests using SPSS program (version 19.0).*$p<0.05$,and** $p<0.01$,vs. LPS-stimulated group.

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2. Results and Discussion:

2.1 Isolation and identification of bioactive natural products from *E. amstelodami*

Base on the results of the bioactivities of marine-derived fungi, the strain *Eurotium amstelodami* has been selected for the isolation of the bioactive compounds, especially the broth extract. Therefore, 8 L of the strain, *E. amstelodami* was cultured for in SWS medium for 30 d (static) at 29 ℃ in SWS medium. The broth was extracted with EtOAC to obtain the B extract. Then, the B extract was purified using silica column chromatography followed by Sephadex LH-20 and ODS or HPLC chromatography. Four compounds have been successfully isolated. The structures of the isolated compounds were determined by analysis of the MS and NMR spectral data as well as comparison of those data with the previous reports.

Asperflavin (1) was isolated as greenish amorphous powder. The LR-EIMS data give a molecular ion peak at *m/z* 287 $[M - H]^-$. The molecular formula of 1 was determined as $C_{16}H_{16}O_5$ base on the MS and NMR data. The ^1H and ^{13}C NMR data showed the presence of a carbonyl, a methoxyl, two aliphatic methylenes, three aromatic protons among which two were meta-coupled with each other, an aliphatic quaternary carbon bearing oxygen, seven aromatic quaternary carbons among which three bore oxygen and a ketone carbonyl in 1. It was determined as asperflavin (**Fig. 3-3-1**) as comparison with the previous report [62, 63].

Questinol (2) was isolated as yellow powder, and its molecular weight was determined as m/z 299 $[M-H]^-$, and that corresponded with $C_{16}H_{12}O_6$. The ^1H and ^{13}C

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NMR signals indicated it might be an anthraquinone compound. In comparison of the NMR data with those of questinol in the literature [64, 65], it was determined as quetinol, as shown in Figure 3-3-2. This compound was first isolated from *E. amstelodami* according our best knowledge.

Neoechinulin A (3), a white powder, has a molecular ion peak at m/z 322 $[M-H]^-$. The molecular formula of 3 was determined as $C_{19}H_{21}N_3O_2$ base on the MS and NMR data. It was identified as neoechinulin A (**Fig. 3-3-3**), which was previously isolated from fungal genera *Eurotium* and *Apsergillus* [66].

Preechinulin (4), a white powder, showed a molecular ion peak at m/z 326 $[M+H]^+$. The molecular formula of 4 was determined $C_{19}H_{23}N_3O_2$ base on the MS and NMR data. The ^1H and ^{13}C NMR were identical to those of compound 3 except for the absence of olenfinic protons and carbons signals. Therefore, compound 4 was determined as preechinulin (**Fig. 3-3-4**) by comparison of the NMR data with published data [67].

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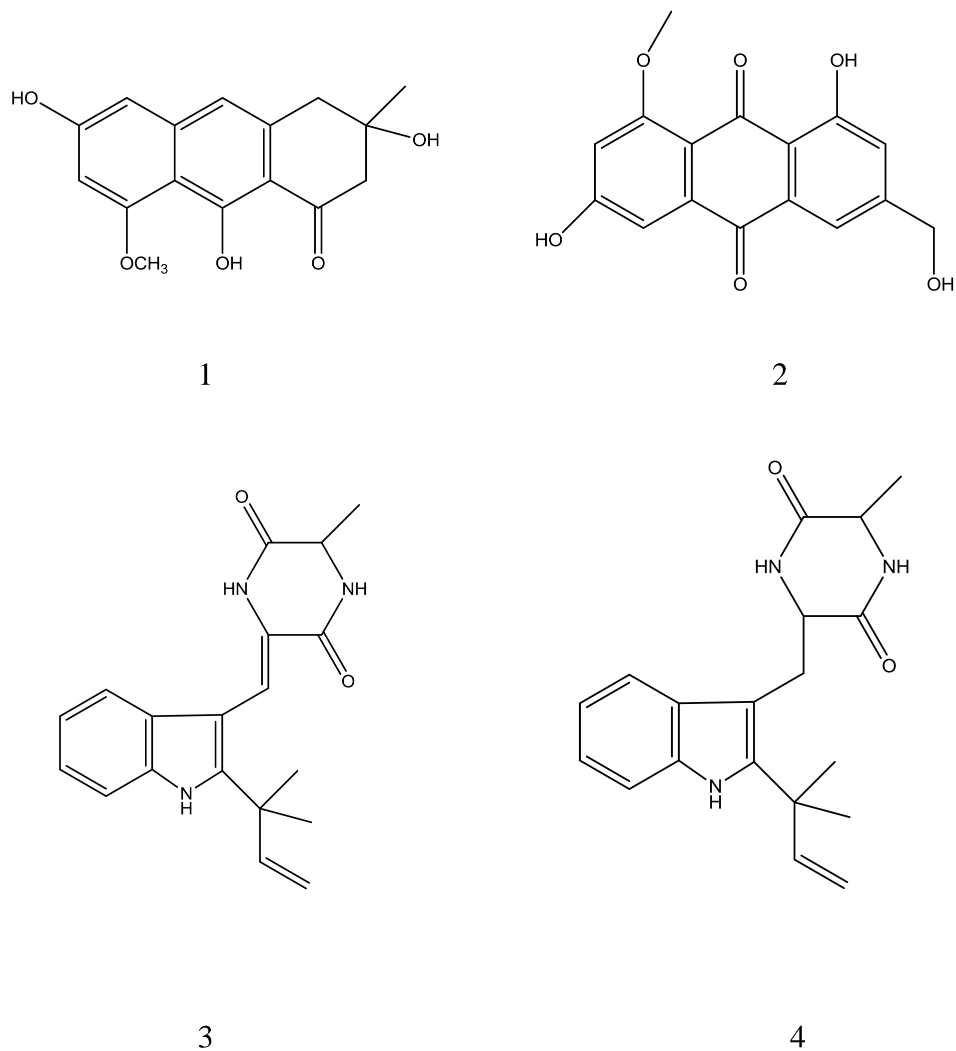

Figure 3-3. The chemical structures of the compounds isolated from *E. amstelodami*.

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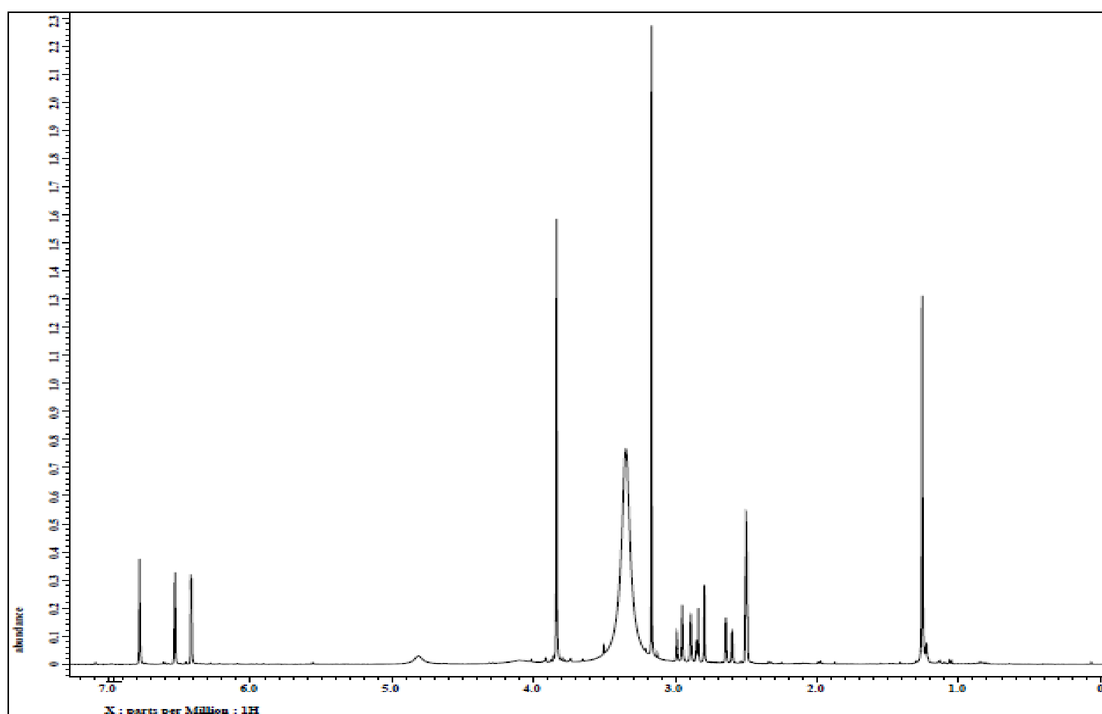

Figure 3-4. The ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of asperflavin

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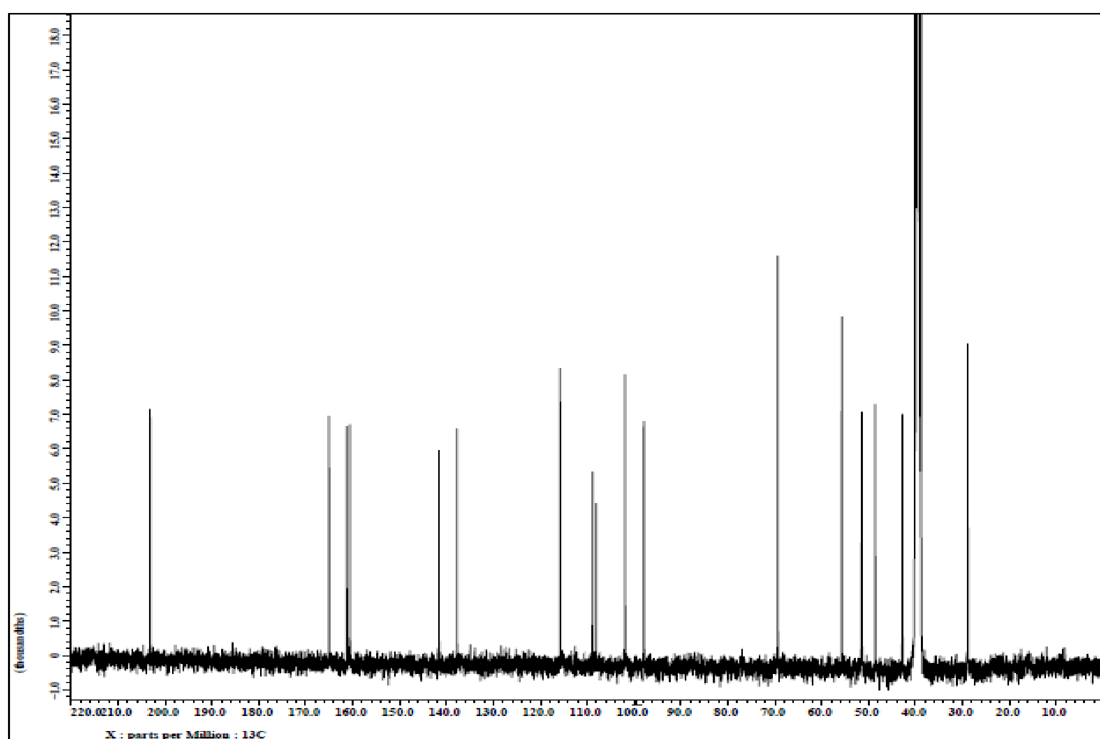

Figure 3-5. The ^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of asperflavin

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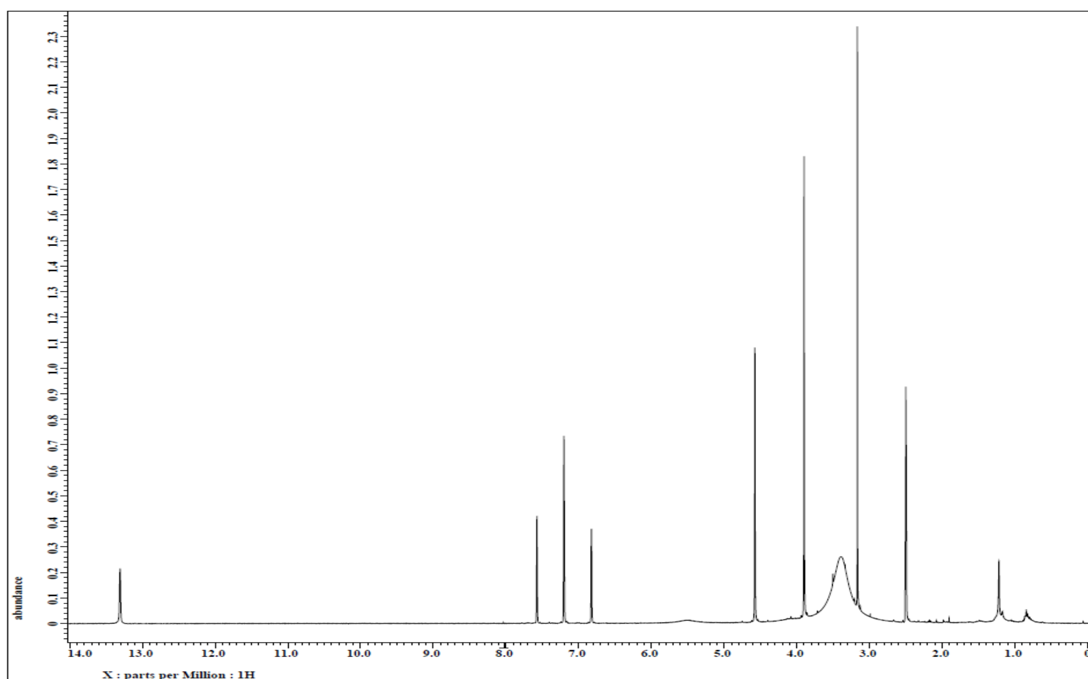

Figure 3-6. The ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of questinol

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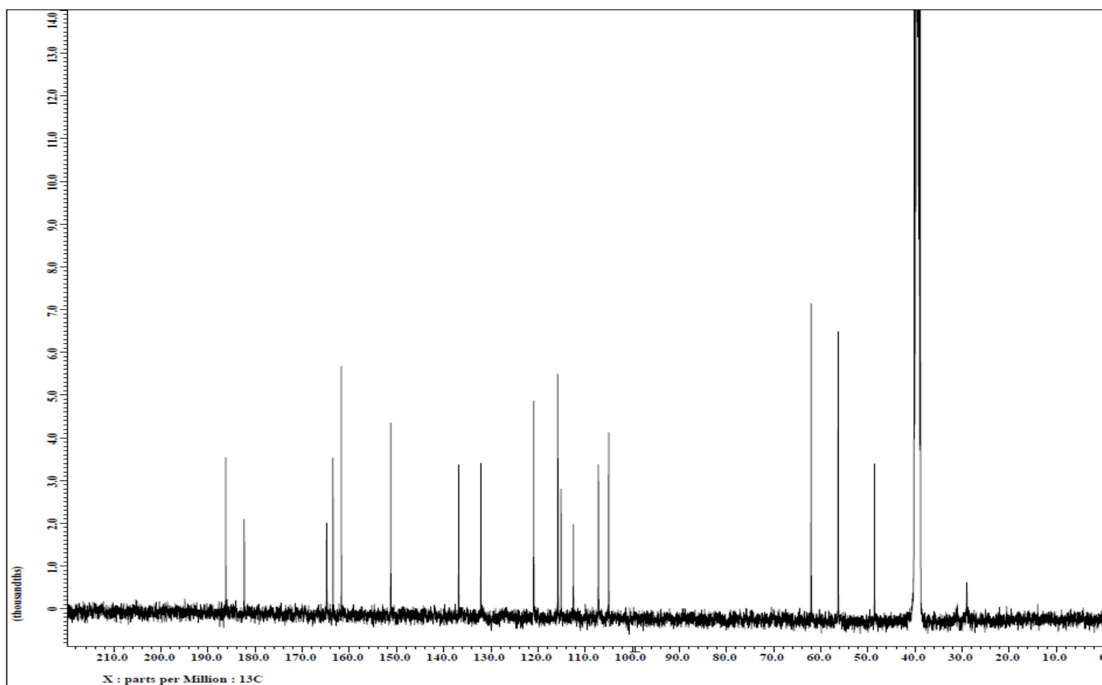

Figure 3-7. The ^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of questinol

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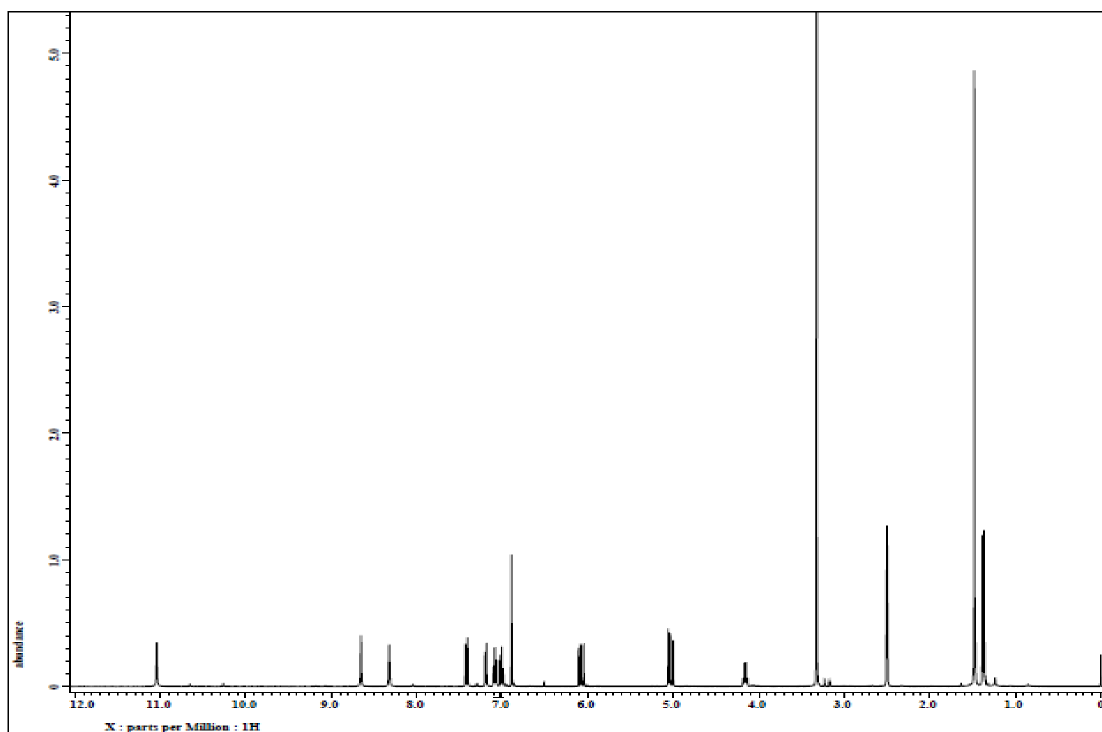

Figure 3-8. The ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of neoechinulin A

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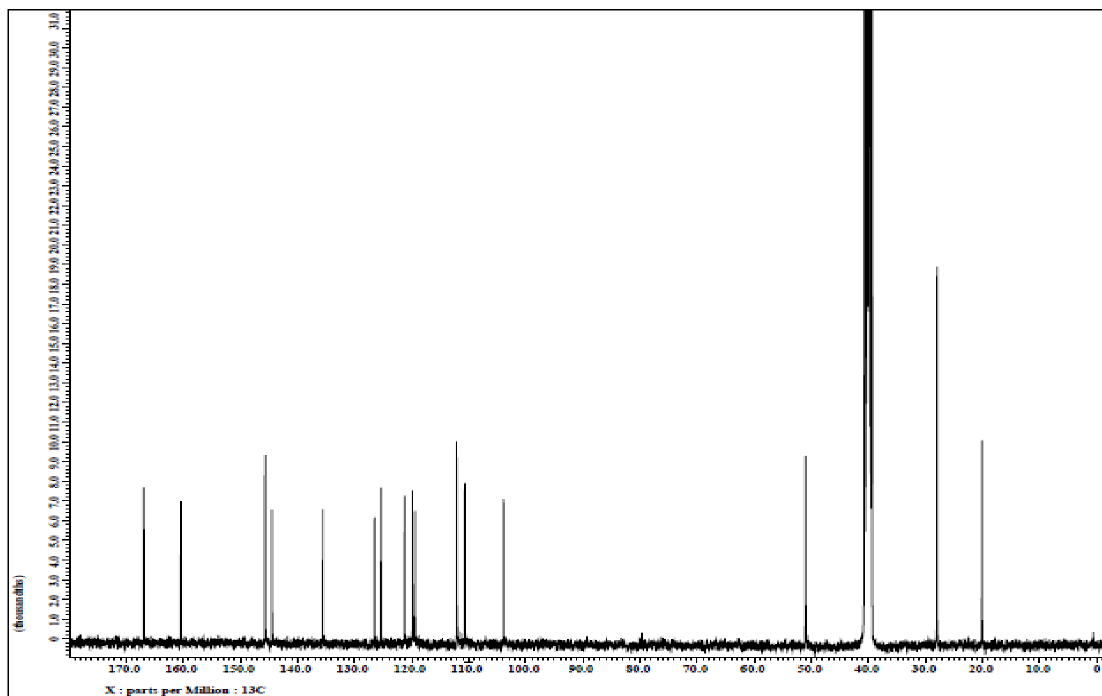

Figure 3-9. The ^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of neoechinulin A

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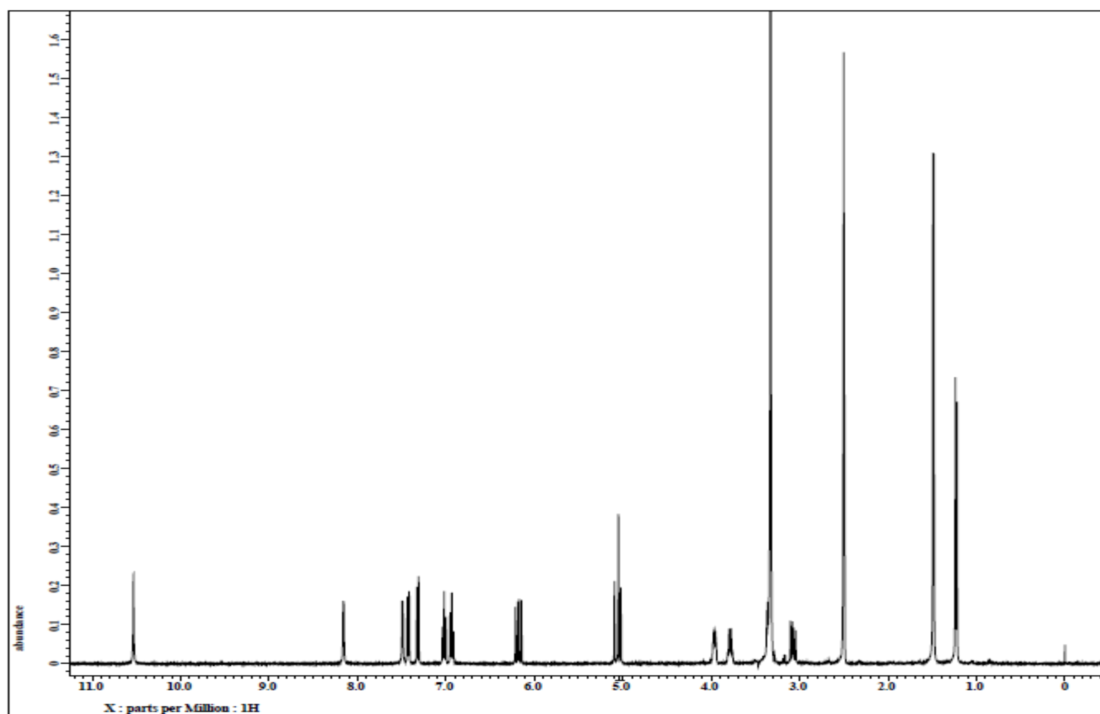

Figure 3-10. The ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of preechinulin

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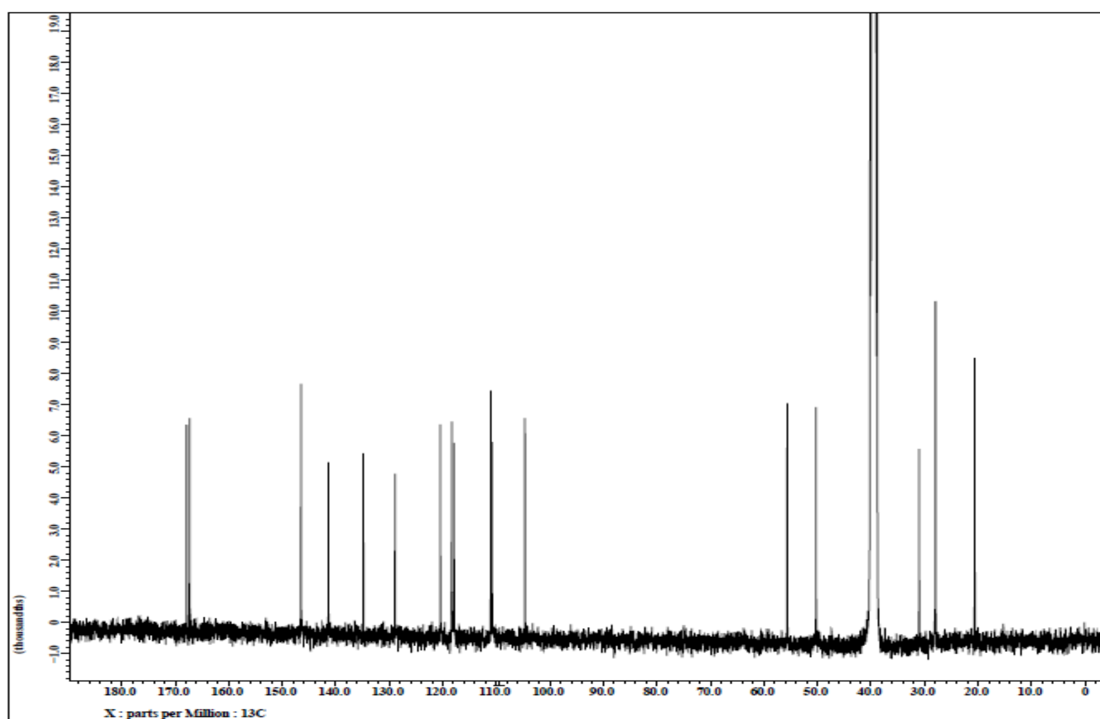

Figure 3-11. The ^{13}C-NMR (100 MHz, DMSO-d_6) spectrum of preechinulin

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2.2 Anti-inflammatory effects of asperflavin and questinol in LPS-stimulated Raw 264.7 cells

2.2.1 Cell viability in Raw 264.7 cells

To investigate the cytotoxic effects of asperflavin and questinol in RAW 264.7 cells in the presence of LPS (1 μg/ml), MTT assays were employed. As shown in the **Fig. 3-12**, treatment of LPS (1 μg/ml) alone showed cytotoxic effect to RAW264.7 macrophages, while no significant differences between the LPS-treated group and control group. When the cells treated with 50, 100, and 200 μM of asperflavin and questinol in the presence of LPS, no significant differences of cell viability were found between the these groups and control group, respectively. The results indicated that up to the concentration of 200 μM, asperflavin and questinol did not affect the cell viability in RAW 264.7 cells. Thus, the concentrations of 50, 100, and 200 μM of asperflavin and questinol will be used for further experiments.

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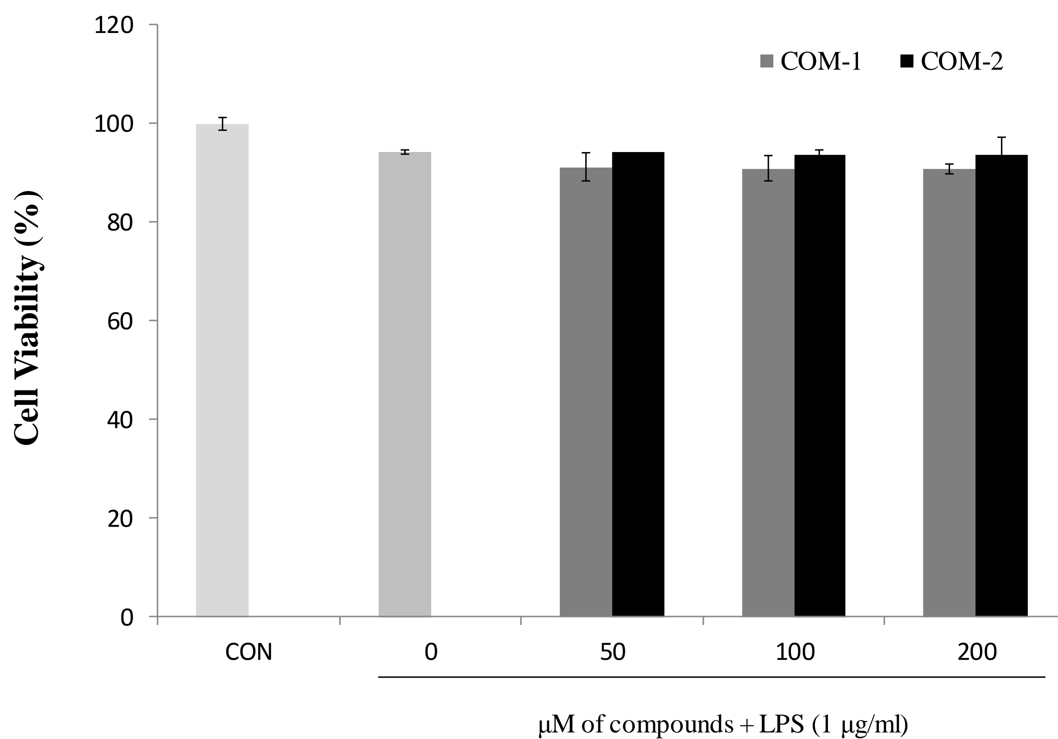

Figure 3-12. Effects of asperflavin (COM-1) and questinol (COM-2) on cell viability in RAW 264.7 cells. RAW cells were cultured with the different concentrations (50, 100, and 200 μM) of asperflavin and questinol, respectively, and LPS (1 μg/ml) for 24 h, respectively. Values are the mean ± SD of triplicate experiments.

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2.2.2 Effects of asperflavin and questinol on NO production in LPS-treated RAW 264.7 cells

The inhibitory effects of asperflavin and questinol on NO production, RAW 264.7 cells were incubated with LPS (1 μg/ml) for 24 h. The production of NO in the culture medium was evaluated by measurement the release of nitrite, which is stable metabolite of NO. As shown in the **Fig. 3-13**, LPS-treatment could significantly elevate the production of NO. The concentration of NO in the LPS-treatment group was defined as 100% of NO production. Pre-treatment with asperflavin and questinol could significantly inhibit LPS-induced NO production in a concentration-dependent manner, respectively. Asperflavin showed NO production of 58.5%, 41.4%, and 4.6% at the concentrations of 50, 100 and 200 μM, respectively. Questinol showed a little weaker inhibitory effects of NO production. The NO production of questinol treated groups were 80.9%, 42.1% and 23.0% at the concentrations of 50, 100 and 200 μM, respectively. Combination of the results of cell viability and NO production inhibitory effects, the inhibitory effects of asperflavin and questinol on NO production was not caused by cytotoxic effects in RAW 264.7 cells.

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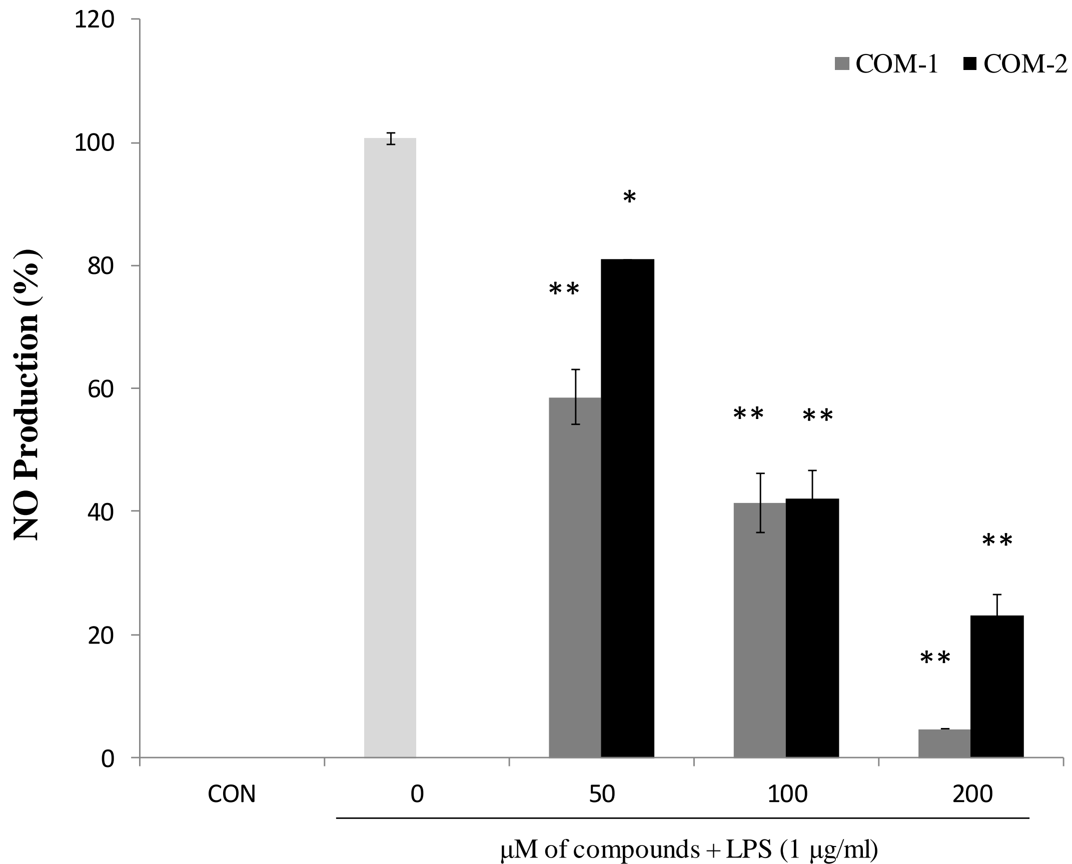

Figure 3-13. Inhibitory effects of asperflavin (COM-1) and questinol (COM-2) on NO production in LPS-stimulated RAW 264.7 cells. Cells (1 × 10^5 cells/ml) were stimulated by LPS (1 µg/ml) for 24 h in the presence of asperflavin and questinol (50, 100, and 200 µM), respectively. The NO production was assayed in the culture medium. Values are the mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ indicate significant differences from the LPS-stimulated group.

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2.2.3 Effects of asperflavin and questinol on PGE_2 production in LPS-treated RAW 264.7 cells

To determine the potential effects of asperflavin and questinol on the inhibition of PEG_2 production, the amount of released PGE_2 was measured using anti-PGE2-coated ELISA plates. Conditioned media was prepared by treating the RAW264.7 cells with asperflavin and questinol (50, 100 and 200 μM) for 1 h followed by 24 h of stimulated with LPS (1 μg/ml). As shown in the Fig. 3-14, the PGE_2 production was significantly increased by the treatment of LPS. However, pre-treatment with asperflavin and questinol could significantly inhibit LPS-induced PGE_2 production in a concentration-dependent manner, respectively, except for the group treated by 50μM of asperflavin. Asperflavin showed PGE_2 production of 99.7%, 87.6%, and 55.9% at the concentrations of 50, 100 and 200 μM, respectively. Questinol showed higher inhibitory effects of PGE_2 production. The PGE_2 production of questinol treated groups were 93.8%, 78.9% and 56.5% at the concentrations of 50, 100 and 200 μM, respectively.

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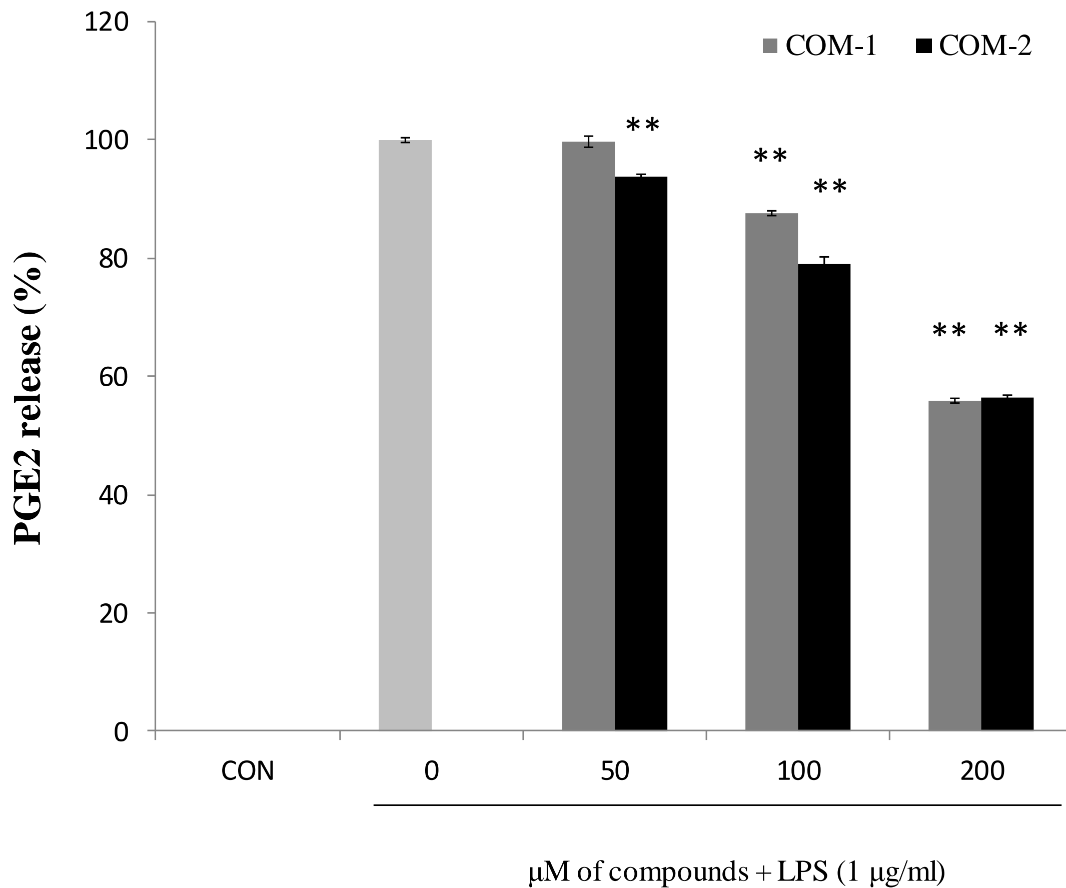

Figure 3-14. Inhibitory effects of asperflavin (COM-1) and questinol (COM-2) on PGE_2 production in LPS-stimulated RAW 264.7 cells. Cells (1×10^5 cells/ml) were stimulated by LPS (1 μg/ml) for 24 h in the presence of asperflavin and questinol (50, 100, and 200 μM), respectively. Supernatants were collected, and the PGE_2 production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. **, $P < 0.01$ indicates significant differences from the LPS-stimulated group.

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2.2.4 Effects of asperflavin and questinol on production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells

The release of pro-inflammatory cytokines is an important mechanism by which the immune cells regulate the inflammatory responses and contribute to various inflammatory and autoimmune disorders. Therefore, we examined the effects of asperflavin and questinol on LPS-induced TNF-α, IL-1β, and IL-6 production using ELISA kit. LPS could induce a significant increase of cytokines including TNF-α (Fig. 3-15), IL-1β (Fig. 3-16) and IL-6 (Fig. 3-17) compared to the control group. However, pre-treatment with asperflavin and questinol was found to reduce the TNF-α level. At the concentrations of 100 and 200 μM, the levels of TNF-α were significantly reduced. Pretreatment with questinol at the concentration of 50 μM, the level of TNF-α was also significantly reduced. But, no significant difference has been found between the LPS-treated group and 50 μM of asperflavin-treated group.

The results of IL-1β production inhibitory effects of asperflavin and questinol were shown in Figure 3-16. At the concentration of 200 μM, pre-treatment of the asperflavin were found to significantly inhibit the production of IL-1β. However, at the concentrations of 50 and 100 μM, no significant differences were found compared to LPS-treated group. The questinol could significantly reduce the production of IL-1β at the concentrations of 100 and 200 μM.

Similar to the results of IL-1β production inhibitory effects of asperflavin and questinol, pre-treated with questinol at the concentrations of 100 and 200 μM could significantly inhibit the production of IL-6. Pre-treatment of asperflavin at the concentration of 200 μM also significantly reduced the levels of IL-6. While, at other concentrations, asperflavin and questinol could not significantly inhibit the production of IL-6.

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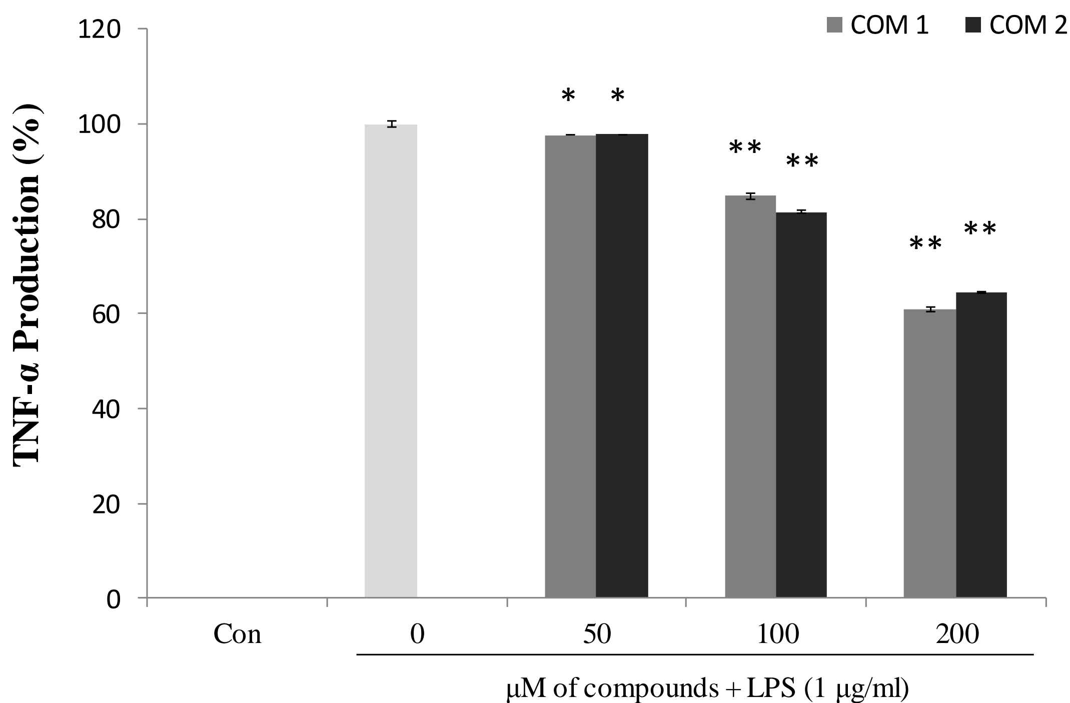

Figure 3-15.Inhibitory effects of asperflavin (COM-1) and questinol (COM-2) on TNF-α production in LPS-stimulated RAW 264.7 cells. The production of TNF-α was assayed in the culture medium of cells stimulated by LPS (1 μg/ml) for 24 h in the presence of asperflavin and questinol (50, 100, and 200 μM), respectively. Supernatants were collected, and the TNF-α production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *, $P < 0.05$ and **, $P < 0.01$ indicate significant differences from the LPS-stimulated group.

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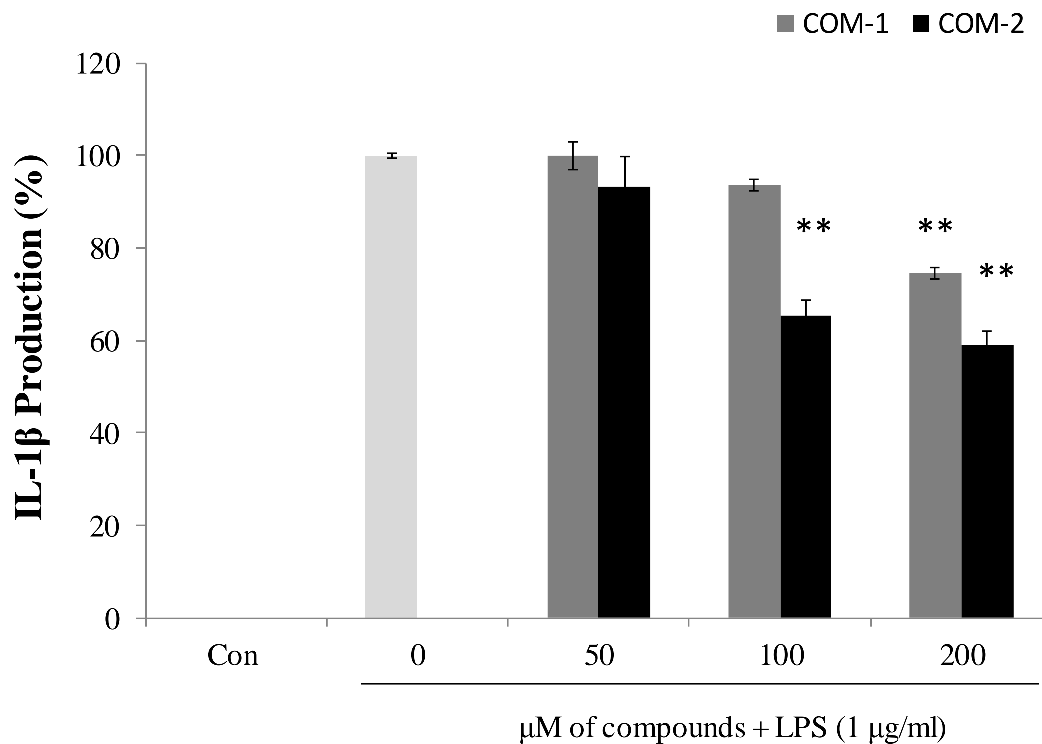

Figure 3-16. Inhibitory effects of asperflavin (COM-1) and questinol (COM-2) on IL-1β production in LPS-stimulated RAW 264.7 cells. The production of IL-1β was assayed in the culture medium of cells stimulated by LPS (1 µg/ml) for 24 h in the presence of asperflavin and questinol (50, 100, and 200 µM), respectively. Supernatants were collected, and the IL-1β production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. **, $P < 0.01$ indicates significant differences from the LPS-stimulated group.

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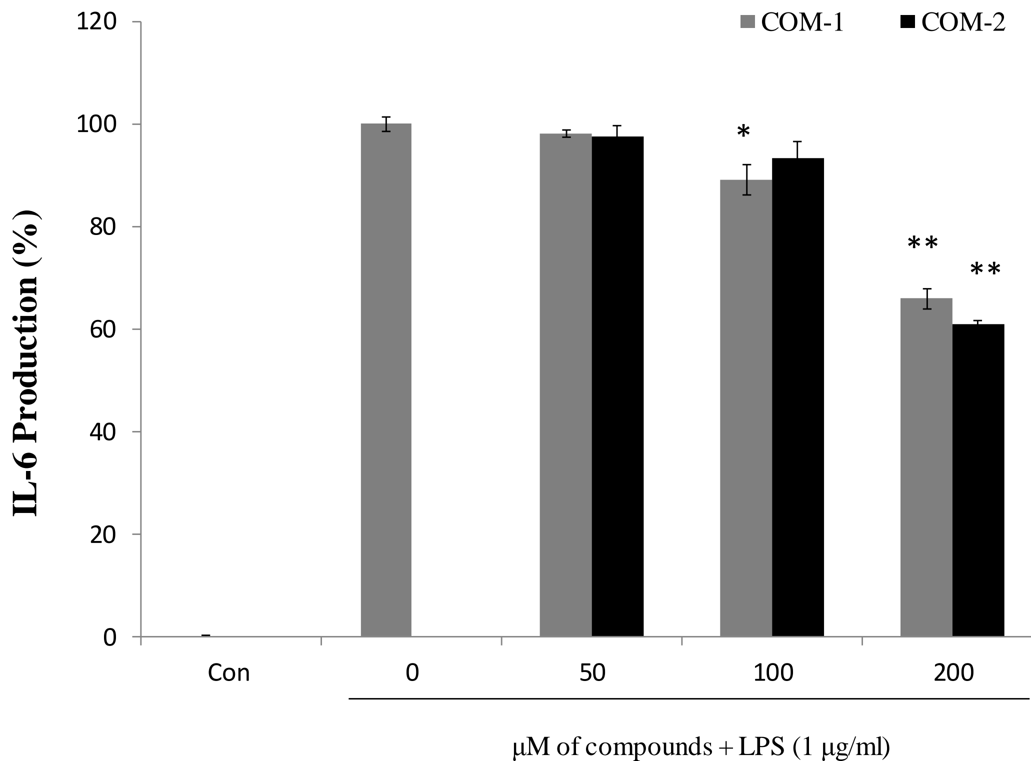

Figure 3-17. Inhibitory effects of asperflavin (COM-1) and questinol (COM-2) on IL-6 production in LPS-stimulated RAW 264.7 cells. The production of IL-6 was assayed in the culture medium of cells stimulated by LPS (1 µg/ml) for 24 h in the presence of asperflavin and questinol (50, 100, and 200 µM), respectively. Supernatants were collected, and the IL-6 production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *, P < 0.05 and **, *P* < 0.01 indicate significant differences from the LPS-stimulated group.

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2.2.5 Effects of asperflavin and questinol on expression of iNOS and COX-2 protein in LPS-stimulated RAW 264.7 cells.

To determine the mechanism by which the compounds reduce LPS-induced NO and PGE_2 production, we investigated the ability of asperflavin and questinol on expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. As shown in the figure 3-18, treatment of LPS (1μg/ml) could significantly increase the expression levels of iNOS and COX-2 compared to the control without LPS and asperflavin/questinol in RAW 264.7 cells. However, pre-treated with asperflavin could inhibit the expression of iNOS in a dose-dependent manner. On the other hand, the expression of COX-2 was not decreased by treatment of asperflavin at all concentrations (Fig. 3-18).

Similarly, questinol was also found to inhibit the expression of iNOS at the concentrations of 50, 100, and 200 μM. And the expression of COX-2 was not affected by treatments of questinol at all concentrations (Fig. 3-19).

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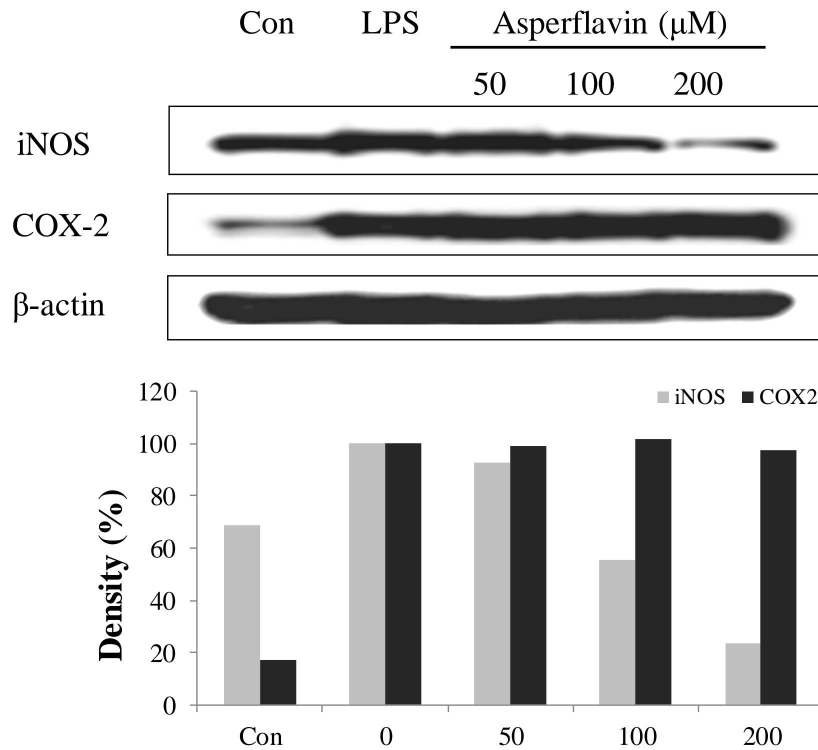

Figure 3-18. Inhibitory effects of asperflavin on the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The RAW 264.7 cells were pre-incubated for 18 h, and stimulated with LPS (1 μg/ml) for 24 h in the presence of asperflavin (50, 100, and 200 μM). The expression levels of iNOS and COX-2 were determined using immunoblotting method.

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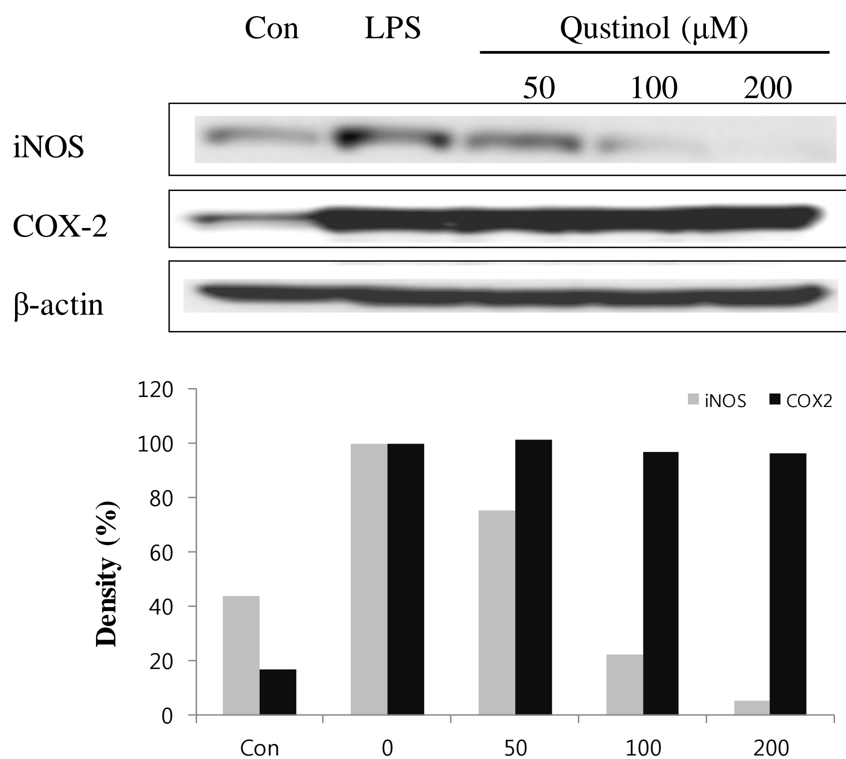

Figure 3-19. Inhibitory effects of questinol on the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The RAW 264.7 cells were pre-incubated for 18 h, and stimulated with LPS (1 μg/ml) for 24 h in the presence of questinol (50, 100, and 200 μM). The expression levels of iNOS and COX-2 were determined using immunoblotting method.

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3. Discussion

NO plays a vital role in the regulation of the vascular and immune system. It has been proven to be an important signaling molecule involved in regulating a wide range of biological activities in vascular, neural, and immune systems [68]. However, over production of NO from inflammatory cells is found to cause the pathophysiology in a variety of diseases, carcinogenesis and inflammation. No is formed by L-arginine by No synthase (NOS), which is generated by man cell types [69]. Three distinct isoforms of NOS have been identified including constitutive endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [70]. In the macrophages, iNOS is significantly induced by LPS-stimulation. PGE_2 was considered one of the strongest inflammatory mediators in inflammatory response. It was transformed from arachidonic acid via the COX-2 catalytic reaction. COX-2 also could be affected directly at its enzymatic activity by NO and iNOS [71]. In our study, the compounds, asperflavin (1) andquestinol (2) were found to significantly inhibited the LPS-induced NO and PGE_2 production in a concentration-dependent manner in RAW 264.7 cells. Especially, asperflavin showed strong inhibitory effects of NO production at concentration of 200 μM. Accordingly, the levels of iNOS were also significantly suppressed by the asperflavin and questinol at the concentration of 100 and 200 μM. However, asperflavine and qustinol could not decrease the expression levels of COX-2. Furthermore, the inhibitory effects on the LPS-induced NO and PGE2 production, iNOS and COX-2 expression were not due to the cytotoxicity according to the results of cell viability in RAW 264.7 macrophages.

Activated macrophages secrete a number of different inflammatory mediators, including TNF-α, IL-1β and IL-6. The overproduction of these mediators has been implicated in several inflammatory diseases and cancer [72]. TNF-α is an important

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pro-inflammatory cytokine and, like NO, is involved in normal physiological immune and inflammatory processes. However, when inappropriately expressed, TNF-α also plays a role in the development of chronic inflammation and associated diseases [73, 74]. IL-1β and IL-6 are also considered to be the pivotal pro-inflammatory cytokine, for example, IL-1β is believed to play an important role in the pathophysiology of rheumatoid arthritis [75, 76], and IL-6 is regarded an endogenous mediator of LPS-induced fever [77]. Our finding indicated that treatment of asperflavin and questinol significantly inhibited the production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in the LPS-stimulated RAW 264.7 macrophages.

4. Conclusion

In conclusion, we have demonstrated that the compounds, asperflavin and questinol isolated from the marine-derived fungus, *E. amstelodami* inhibited the production of NO and PGE_2 production through suppression of iNOS expresion in LPS-stimulated RAW 264.7 macrophages. The compounds, asperflavin and questinol were found to inhibit the pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 as well. Therefore, our study suggests a potential use of these compounds might be selected as a promising agent for the prevention and therapy of inflammatory disease.

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