A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Isolation of secondary metabolites derived from marine bacteria in Jeju island, Korea and their biological activities

Chao Zhang

Department of Marine Life Science

GRADUATE SCHOOL

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(Supervised by Professor You-Jin Jeon)

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The marine environment is a proven rich source of natural products that have a wide variety of biological activities. During the last three decades, more than 15,000 natural products have been isolated from marine organisms [1]. In many cases, it is not the marine invertebrates themselves, but their associated microbes that are the true producers of the pharmaceutically interesting compounds [2-6]. Again, sponges often harbor significant amounts of bacteria in their tissues. In some cases bacteria make up more than 40% of sponge biomass [7,8]. Symbiotic functions that have been attributed to marine sponge microbial associates include nutrient acquisition and secondary metabolite production [9].The various secondary metabolites synthesized by microbial associates inhabiting marine sponges also possess good bioactivities in many studies [10-14]. In this respect, the micro-organisms, assuming they can be cultured, would represent a more attractive source of marine natural products.

Inflammation is an essential aspect of the host response to infection and injury and is required for the maintenance of good health in response to bacterial and viral infections. However, excessive or aberrant inflammation contributes to many acute and chronic human diseases [15,16]. Macrophages play an important role in inflammatory diseases related to overproduction of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and inflammatory mediators, including reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin E_2 (PGE_2), which are generated by activated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [17,18].

Apoptosis is a selective process of physiological cell deletion that regulates the balance between cell proliferation and cell death. The failure of apoptosis is considered to contribute generally to the development of human malignancies [19]. Because it was recently suggested that cancer chemotherapeutics exert part of their pharmacological effects by triggering apoptotic cell death, the induction of apoptosis in cancer cells has become a target of cancer treatment [20,21]. Reactive oxygen species (ROS) are unwanted metabolic byproducts of normal aerobic metabolism. High ROS levels lead to apoptosis and necrosis. Therefore, recently, an increasing number of studies have implicated ROS in anti-cancer drug-mediated apoptosis [22,23]. Cell cycle control mechanisms serve major regulatory functions for cell growth. Many cytotoxic agents and/ or DNA-damaging agents induce apoptosis by arresting the cell cycle [24,25]. In fact, the anti-cancer properties of many anti-cancer agents act through the induction of cell cycle arrest and/ or apoptotic cell death.

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Supernatants were collected, and the IL-1β concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments.

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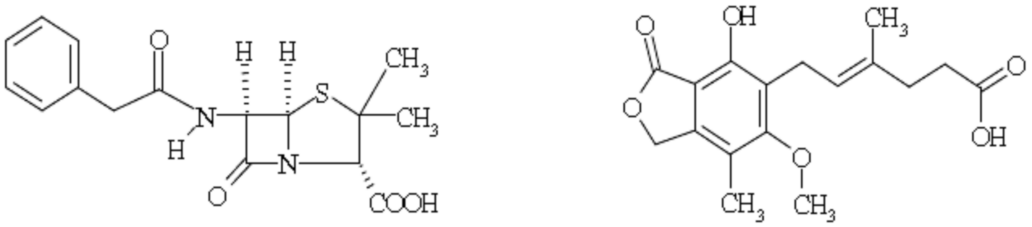
Introduction

1.1. Natural products

The story of bioactive natural products started more than 100 years ago. Their usual definition in the widest sense is chemical compounds isolated/derived from the nature i.e. living organisms such as plants, animals and microorganisms. These compounds may be derived from primary or rather secondary metabolism of these organisms [26]. Chemistry of natural products is related to the isolation, biosynthesis and structure elucidation of new products that led to new medical and crop protection agents. Due to their chemical diversity and various activities against diseases, they have been playing an important role in pharmaceutical and agricultural research [27].

The World Health Organization (WHO) estimated that 80% of the earth inhabitants mainly depend on traditional medicines for their health care [28]. Plants have been the roots of the traditional medicine that has existed for thousands of years starting from the first records about 2600 $_{BC}$. Some of these plants are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation [29].

Since the discovery of penicillin (penicillin G, **1**) in 1928 [30], intensive studies, mainly on soil derived bacteria and fungi, have shown that microorganisms are a rich source of structurally unique bioactive substances [31]. Penicillin represents the first antibiotic in the history of natural products from microorganisms despite that mycophenolic acid (**2**) was identified in the end of the nineteenth century by Bartolomeo Gosio [32].



1 **2**

Fig. I. Penicillin G (**1**) and mycophenolic acid (**2**), two natural products from *Penicillium* species.

1.2. Marine environment as a new source for bioactive metabolites

Marine organisms represent a promising source for natural products of the future due to the incredible diversity of chemical compounds that were isolated. The oceans, which cover almost 70% of the earth's surface and over 90% of volume of its crust [31,33], contain a variety of species, many of which have no terrestrial counterparts. 34 of the 36 phyla of life are represented in oceans in contrast to 17 phyla representing the terrestrial environment [34]. The pioneers of marine microbiology, such as Claude Zobell, became active in delineating the vast numbers and diversity of true marine bacteria. One of the early isolations of secondary metabolites from marine sources was the isolation of cephalosporin in 1948 by Giuseppe Brotzu. Cephalosporin (cephalosporin C) was isolated from the fungus *Cephalosporium acremonium*. In the early 1950s, Bergmann and his colleagues isolated two compounds from a marine sponge, spongouridine and spongothymidine [35,36]. They were the first naturally occurring nucleosides with a sugar moiety other than ribose or deoxyribose. Later on

Burkholder and his co-workers had isolated the first marine metabolite from the bacterium *Pseudomonas bromoutilis*, the highly brominated pyrrole antibiotic pentabromopseudiline [37].

The systematic investigations of marine environment as sources of novel biologically active agents began intensively in the mid 1970s. Among the many phyla found in the oceans, bacteria (including cyanobacteria), fungi, certain group of algae, sponges, coelenterates, sea hares, bryozoans, tunicates and nudibranchs were the most studied organisms. During over 60 years of an increase in the number of natural products from marine resources, the number of publications in 2000 has declined slightly when compared with 1999 (**Fig.** II**.** a). Sponges followed by coelenterates were the most studied marine organisms. The share of microorganisms in marine studies since that time has not lost its significance and stayed more or less in a constant level in contrast to sponges that have lost the interest of the natural products' scientists (**Fig.** II**.** b, c and d). The bioactivity profiles of marine metabolites include neurotoxic, antiviral, antitumor, antimicrobial or cytotoxic properties and are of considerable biotechnological interest.

1.3. Marine secondary metabolites with interesting activities

Many of the marine derived anti-inflammatory, neurotoxic and antitumor compounds had been included in clinical research in clinical trials. *Conus* venoms are small, highly constrained peptides, 10-30 amino acids in length. They target nicotinic acetylcholine receptors, voltage-sensitive calcium channels and sodium channels [38]. A synthetic version of ω-contoxin MVIIA (ziconotide), the first isolated peptide from the venom of *Conus magus*, is

used as a potential treatment for patients suffering from chronic pain. It is in the registration stage under the name Prialt in both US and Europe [39].

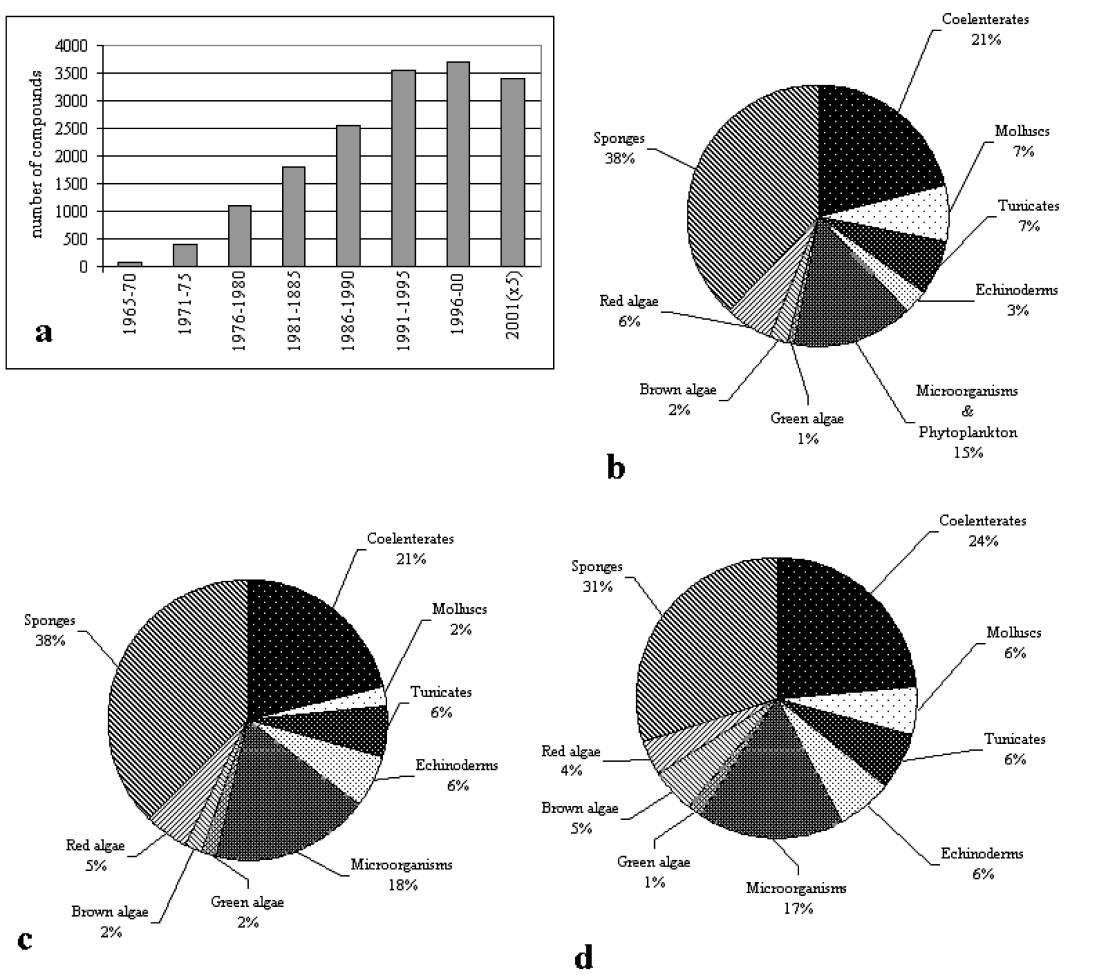


Fig. II. Statistics of marine natural products. a) Since 1965 onwards, b) percentage of isolated compounds from different phyla till 2001, c) till 2002, d) till 2003 (modified from Blunt *et al.,* 2003, 2005).

Marine compounds that interfere with protein kinase C (bryostatin 1) or inhibit the synthesis of macromolecules (didemnin B) in cancer cell lines were considered as promising antitumor drugs. Bryostatins, macrocyclic metabolites isolated from the bryozoans *Bugula neritina* [40] and *Amathia convulata* [41], were used as a partial agonist of protein kinase C (PKC). Bryostatin 1 is currently in phase II clinical trials.

Didemnin B, a depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum* [42], inhibits the synthesis of RNA, DNA and proteins in various cancer cell lines. It shows anti-viral and immunosuppressive activities as well as being an effective agent in treatment of leukaemia and melanoma. Due to its toxicity, it was withdrawn from phase II clinical trials [43,44].

In 1997 a group from Spain found a novel bioactive depsipeptide, thiocoraline. It was isolated from the mycelial extract of the bacterium *Micromonospora marina* associated with a marine soft coral in the Indian Ocean. Thiocoraline showed potent cytotoxic activity at a nanomolar concentration against several tumor cell lines. It inhibits DNA polymerase-α [45,46]. Thiocoraline is currently in preclinical phase by PharmaMar.

Manoalide, a sesterterpenoid isolated from the sponge *Luffariella variabilis* [47] inhibits irreversibly the release of arachidonic acid from membrane phospholipids and subsequently inhibits the inflammatory reactions [48,49]. The work on this compound was discontinued in phase II clinical trial due to formulation problems [46].

Pseudopterosins, tricyclic diterpene glycosides isolated from the Caribbean sea whip *Pseudopterogorgia elisabethae*, possess anti-inflammatory and analgesic activities as they inhibit PLA_2 and degranulation and leukotriene formation in human neutrophils, but do not affect eicosanoid biosynthesis in stimulated murine macrophages *in vivo* [50,51]. Recently, it was reported that the real origin of this metabolite is the dinoflagellate symbiont *Sympoidinium* sp. localized within the tissues of the sea whip [52]. Clinically it has not found its way yet as an anti-inflammatory drug, but it is used as an additive to prevent irritation caused by exposure to sun or chemicals under the name of the cosmetic care product,

Resiliene®. Finally, scytonemin isolated from the sheath of many cyanobacteria as a yellow-green pigment [53], has recently been patented as anti-inflammatory agent.

The low amounts produced from the above mentioned compounds as well as the striking structural similarities between some pharmaceutically active agents and known microbial metabolites addressed a question about their biosynthetic origin. Inspection of structural features of ecteinascidin-743 (ET-743) from tunicate reveals similarities to saframycin B isolated from *Streptomyces lavendulae* [54] and safracin isolated from *Pseudomonas fluorescens* [55]. Such observation represents one of several clues on the microbial origin of these chemicals (for reviews see [56-58]).

Bacteria are regularly observed in unique microhabitats on surfaces and internal spaces of marine invertebrates. The cytotoxic macrolide swinholide 1, isolated from the sponge *Theonella swinhoei*, was found to be produced by the symbiotic unicellular bacteria inhabiting the endosome of this sponge [59]. Symbioses can range from relatively loose coexistence to highly interdependent interacellular associations. Sponge- bacterial association are probably the most thoroughly described. Several studies showed that the associated bacteria could be distinct from those in the surrounding seawater (specific association) [60]. Association does not include just eubacterial groups; archaea, cyanobacteria and fungi are also sponge microsymbionts [61].

1.4. Marine bacteria as a source for natural products

The oceans are massively complex and consist of diverse assemblages of life forms. The water column of the oceans contains approximately 10^6 bacterial cells per ml [62]. Marine bacteria and other marine microorganisms develop unique metabolic and physiological capabilities. These capabilities enable them to survive in extreme habitats and to produce compounds that might not be produced by their terrestrial counterparts. Since 1990, the number of bioactive metabolites from marine bacteria has exponentially increased (**Fig.** III) [31,63-75].

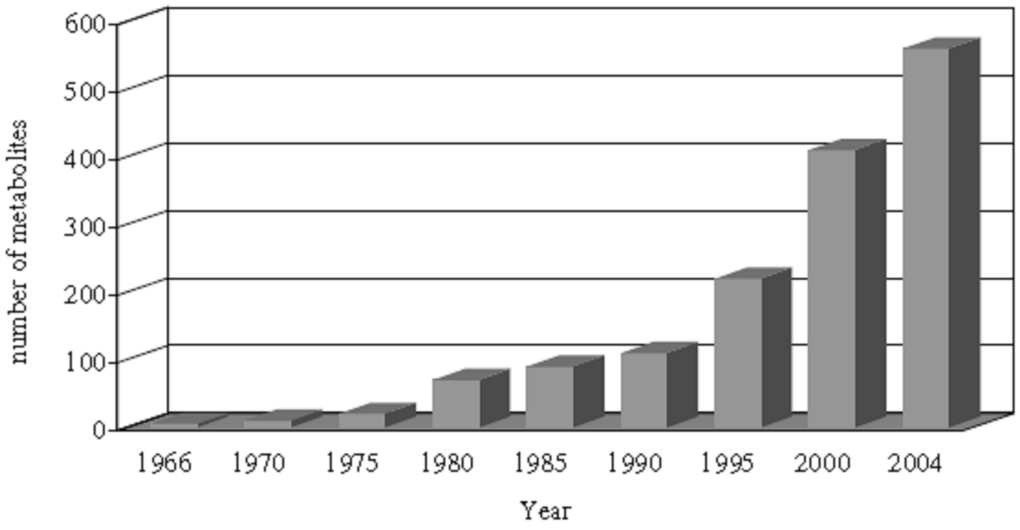


Fig. III**.** Annual increase in the number of marine bacterial metabolites, according to AntiBase (Laatsch, 2005).

The search for new bioactive chemicals from marine organisms resulted in the isolation of about 10000 metabolites [76], many of which are potential biomedicals. These agents show a broad spectrum of biological activities.

Up to now, bioactive agents were isolated extensively from *Streptomyces*, *Altermonas*/ *Pseudoalteromonas*, *Bacillus*, *Vibrio*, *Pseudomonas*, and *Cytophaga* (**Fig.** IV). These

microorganisms were isolated from seawater, sediments, algae and marine invertebrates. They are able to produce quinones, polyenes, macrolides, alkaloids, peptides and to a lesser extent terpenoids.

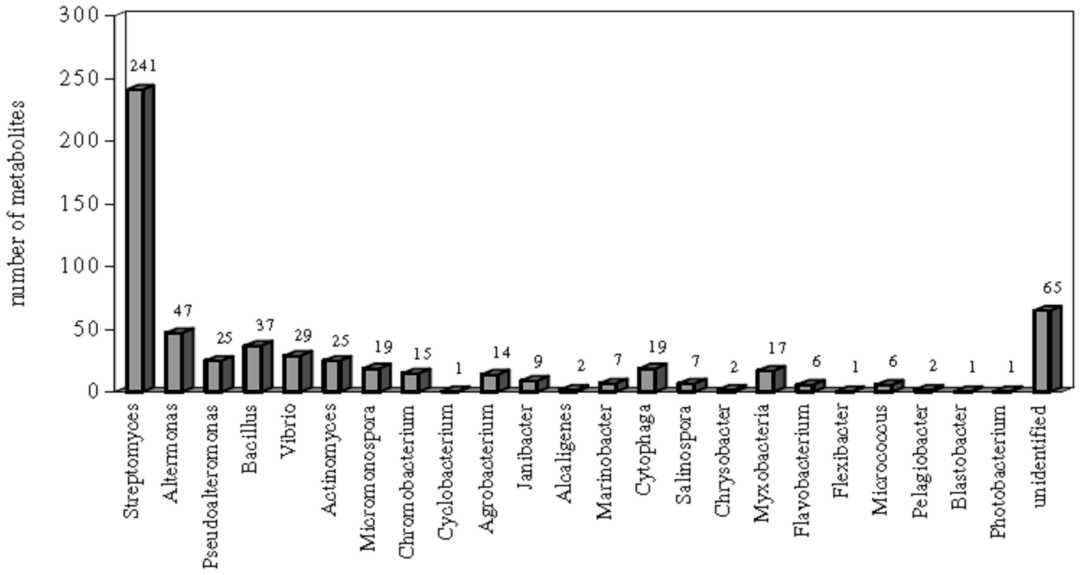


Fig. IV**.** Number of secondary metabolites isolated from some marine bacteria according to their taxonomic origin since 1966 till 2004 (modified from Laatsch, in preparation).

Some of the first marine metabolites were isolated from seawater bacteria. The highly brominated pyrrole antibiotic (pentabromopseudiline) was active against Gram-positive bacteria. Its biosynthesis was not apparent from its structure, which led to studies by Laatsch and co-workers with *Alteromonas luteoviolaceus* on the biosynthesis [77] as well as on the structure-activity relationships [78].

The pioneering work of Okami and co-workers represents the first building unit in the knowledge of the chemistry of marine derived bacteria, in particular of Actinomycetes. They have reported the isolation of a benzanthraquinone antibiotic from the actinomycete *Chainia purpurogena* [79] and istmycins A and B antibiotics from the *Streptomyces tenjimariensis*.

One of the early marine metabolites is the 3,2-indolinedione (isatin). This compound is produced by a bacterium colonizing the surface of the embryos of the shrimp *Palaemon macrodactylus* and protects the eggs against the pathogen fungus *Lagenidium callinectes* [80]. A new macrolide with antibacterial, antiviral and cytotoxic activities was isolated from a deep sea unidentified unicellular bacterium, macrolactin A [81]. Two bicyclic depsipeptides, salinamide A and B, were isolated from a *Streptomyces* sp. from the surface of the jellyfish *Cassiopeia xamachana*. They exhibited moderate antibiotic activity, but were potent topical anti-inflammatory in chemically induced mouse ear edema assays [82,83].

1.5. Newly described metabolites from marine bacteria

Since the beginning of this century nearly 250-300 marine compounds have been described. Interestingly, within the same period the number of described metabolites produced by terrestrial bacteria does not exceed 150 compounds. Nearly 100 marine compounds from bacterial origin were isolated within the year 2004. Actinomycetes were as usual the most studied group. A novel bioactive macrolide was isolated from the culture of the *Micromonospora* sp. IB-96212 [84]. The group of Laatsch isolated novel anticancer compounds, chandrananimycins A, B and C, from *Actinomadura* sp. [85]. These compounds exhibited antibacterial and cytotoxic activities.

A bactericidal antibiotic, MC21-A (3,3′, 5,5′-tetrabromo-2, 2′-biphenyldiol) was isolated from the new species *Pseudoalteromonas phenolica*. It is a brominated anti-MRSA substance that rapidly permeabilizes the cell membranes of MRSA, but it has no lytic activity against

bacterial cells or human erythrocytes [86]. Two years later, mechercharmycin A and mechercharmycin B were isolated the bacterium *Thermoactinomyces* sp. X-ray crystallographic analysis of these compounds showed that mechercharmycin A is a cyclic-peptide and mechercharmycin B is its linear congener. Mechercharmycin A showed cytotoxic activity against human lung carcinoma and human leukaemia [87].

The isolation of highly cytotoxic proteasome inhibitor, salinosporamide A, from *Salinospora* sp. represents one of the remarkable studies in this century. This new genus was proposed by Fenical and his colleagues in 2002 to a group of rare obligate marine actinomycetes isolated from the ocean sediments [88]. This compound displayed a potent *in vitro* cytotoxic activity against human colon carcinoma. This effect was due to the inhibition of the 20S proteasome [89]. Recently halogenated macrolides, sporolides A and B, were isolated from *Salinospora tropica*. Sporolides A and B were neither antibacterial nor cytotoxic [90].

Despite this interest on metabolites from marine derived bacteria, studies in this field encounter some problems. Firstly, not more than 5% of the marine bacteria observed in marine samples are amenable to be cultured with the normal microbiological techniques [91]. Secondly, the taxonomy of marine bacteria is very poorly defined and many publications describe compounds isolated from numbered strains of otherwise partially or totally unidentified bacteria. Thirdly, very low fermentation yields that may be in some cases in the range of milligrams per litre from dense culture are common.

In order to overcome such obstacles, microbiologists developed PCR-based screening assays that may increase the screening efficiency for bioactive compounds. Progression in the

knowledge of the genes involved in the biosynthesis of secondary metabolites and the knowledge with different biosynthetic systems, e.g. polyketide synthetases (PKS), nonribosomal polypeptide synthetases (NRPSs), halogenases, allow completely new approaches, such as combinatorial biosynthesis, to the discovery of novel antibiotics and add a another source of data for the elucidation of metabolites structure [92,93]. Such knowledge has led to the discovery of the bacterial origin of bryostatins [94].

1.5. Separation of natural products by centrifugal partition chromatography (CPC)

In liquid-liquid chromatography, also called centrifugal partition chromatography or more common counter-current chromatography, the mobile and the stationary phase are liquid. The two phases of a biphasic liquid system, obtained by mixing two or more solvents, are used as mobile and stationary phase. One of the phases is kept stationary by means of centrifugal forces [95,96]. One advantage of this technology is the free choice of the stationary phase. Namely, the upper or the lower phase of the biphasic liquid system can be used as a stationary phase. Furthermore, the role of the stationary phase can be switched during the separation run [97]. With the expansion and operational strategy of CPC technology [98,99], research on the separation of natural products [100,102] has been streamlined due to the advantages of the technology such as the elimination of irreversible absorption, the high recovery of target compounds and the high throughput compared with other traditional separation methods such as thin-layer chromatography and column chromatography. Despite

the advancement of CPC technology, the choice of a suitable solvent system is fundamental and can require a significant time investment, which can occupy up to 90% of the time devoted to CPC experimental design [103]. The mixture of heptanes, ethyl acetate, methanol and water (termed the HEMW at family) works well for many classes of natural compounds, but there is no widely accepted convention on how the proportions were listed. The results of shake-flask experiments are expressed in terms of the partition coefficient for each analyte of interest in each solvent system, which become more laborious when isolating a group of compounds.

However, there is no report about screening anticancer, anti-inflammatory and antioxidant activities of EtOAc extracts isolated from marine bacteria in Jeju island. Hence, the goal of my study was to identify the marine bacteria with interesting biological activities from the sources in Jeju island.

Part Ⅰ.

Isolation, culture of marine-derived bacteria and screening the biological activities of their extracts

Part Ⅰ.

Isolation, culture of marine-derived bacteria and screening the biological activities of their extracts

1. Abstract

In order to explore marine microorganisms with medical potential, we tried to isolate and identify the marine-derived bacteria from four types of marine samples including float, marine algae, animal, and sponge collected from Jeju Island, Korea. Finally, 21 different strains have been successfully isolated and identified from these samples by 16S rRNA analysis. Then, all strains were cultured in the Marine Broth and were extracted with ethyl acetate, were assessed in a series of bioassays. The results of these assays showed that four extracts inhibited DPPH by more than 50%, four extracts inhibited hydrogen peroxide by greater than 50%, one extract inhibited the growth of HL-60 cells by more than 95%, 14 extracts inhibited nitric oxide production more than 50% and 12 of them without cytotoxicity in LPS-induced RAW 264.7 cells. These data revealed the tested samples to have many and varied activities, Moreover, the large number of samples demonstrating activity in only one or sometimes two assays accentuates the potential of the bacterial strains isolated from marine-derived samples could be the good sources for biological natural products.

2. Materials and methods

2.1 General reagents

All solvents used for preparation of crude sample were in analytical grade (Daejung Chemicals &Metals Co., Seoul, Korea). 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-picrylhydeazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Other all reagents and solvents were purchased from Sigma (St. Louis, MO, USA).

2.2 Bacterial strains isolation from collected samples

Four types of samples including float, marine algae, animal, and sponge were collected around the coast of Jeju Island, Korea, in August, 2011. Thereafter, all the samples were rinsed three times with sterile seawater for 12hr at room temperature in order to remove loosely attached microorganisms. The samples were aseptically cut into small pieces using sterile surgical scissors and carefully placed onto the prepared petri-dish plates containing Marine Agar 2216 media. 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 °C with humidity.

2.3 Isolation and Identification of bacterial strains in pure culture

The plates were monitored for bacterial growth. After 3-5 days, when several bacterial colonies observed growing on the surface of the above culture media plates. New colonies were streaked on new agar plates for purification. The plates were incubated again in the incubator at 29°C with humidity. This process was repeated several times till finally pure culture plates were obtained. These bacteria were identified to the species level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database. Total twenty-one different bacterial strains have been isolated. The voucher specimens are deposited at Marine Bio-Resource Technology Laboratory of Jeju National University.

2.4 Liquid Culture and Extraction

A single colony from a well grown agar plate was used as an inoculum and was transfer to the 300 mL Erlenmeyer flasks containing 200 mL of Marine Broth 2216 medium containing peptone (0.5%), yeast extract (0.1%) and seawater (100%) for the production of secondary metabolites. The liquid culture flasks were incubated on a rotatory shaker at 121 rpm at 29°C with humidity for 10 days. Thereafter, the broth was centrifuged (10000 rpm, 15

min) to remove the cells. Then the supernatants were extracted with equal volumes of ethyl acetate (200 mL). After separation, the organic phases were concentrated *in vacuo* at 35 °C. The extracts were subjected in the further biological activity screening experiments.

2.5 Screening antioxidant effects of the extracts from Marine-Derived Bacteria

2.5.1 DPPH radical scavenging capacity using ESR spectrometer

The radical tested here was generated according to the previously described procedures [104], and the spin adducts were recorded using JES-FA electron spin resonance (ESR) spectrometer (JES-FA ESR, JEOL, Tokyo, Japan).

DPPH has been widely used to evaluate free radical generation. The free radical scavenging activity of the test samples was determined using an electron spin resonance (ESR) spectrometer in accordance with the method described by Nanjo et al. [105]. A methanol solution of 60 μL of each sample (or methanol as a control) was added to 60 μL DPPH (60 μmol/L) in methanol solvent, and the sample was mixed vigorously. After 2 min, the solution was transferred to a capillary tube and fitted into the cavity of the ESR spectrometer. The spectrum was recorded with an ESR spectrometer (JES-FA machine, JEOL, Tokyo, Japan). The experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 1 mW, modulation frequency, 100 kHz; amplitude,10 × 100; modulation width, 0.8 mT; sweep width, 10 mT; sweep time, 30 s; and time constant, 0.03 s. The extent of scavenging activity was calculated as follows.

Scavenging activity % = (HC - HS) / HC × 100

where HC is the relative peak heights of the radical signals without sample, and HS is the relative peak heights of the radical signals with sample.

2.5.2 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of [106]. A hundred μl of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96 microwell plate. A 20 μl of hydrogen peroxide was added to the mixture, and then incubated at 37 °C for 5 min. After the incubation, 30 μl of 1.25 mM ABTS and 30 μl of peroxidase (1 unit/ml) were added to the mixture, and then incubated at 37 °C for 10 min. The absorbance was read with an ELISA reader at 405 nm.

2.6 Screening anti-inflammatory effects of the extracts from Marine-Derived Bacteria

2.6.1 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°C and were sub-cultured every 2 days.

2.6.2 Assessment of cytotoxicity in Raw 264.7 cells

The cells were seeded in 24-well plate at a concentration of 1×10^5 cells/ml (450 μL). After 24 h incubation at 37°C under an atmosphere of 5% CO_2, the cells were treated with LPS (1 μg/mL) and 25 μL of the samples, to a total reaction volume of 500 μL and further incubated for 24 h. The 100 μL of MTT stock solution (2 mg/ml) was then applied to the wells. After 4 h of incubation, the supernatants were aspirated. The formazan crystals in each well were dissolved in 200 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.

2.6.3 Determination of Nitric Oxide (NO) Production

The anti-inflammatory activity of the EtOAc extracts were determined on the basis of nitric oxide (NO) production in macrophage culture supernatants, and the nitrite concentrations were measured using the Griess reaction, as described by Green et al. [107]. Raw 264.7 cells (1×10^5) were plated and incubated with samples in the absence or presence of LPS (1 μg/mL) for 24 h at 37°C under an atmosphere of 5% CO_2. The cultured cell supernatant (100 μL) was mixed with an equal volume of Griess reagent (1% sulfanilamide in

2.5% phosphoric acid and 0.1% naphthylenediamine dihydrochloride in water) and incubated at room temperature for 10 min [108]. The optical density at 540 nm was measured using an ELISA microplate reader (Amersham Pharmacia Biotech, UK, USA). The nitrite concentration was calculated by comparison with the absorbance at 540 nm of standard solutions of sodium nitrite prepared in culture medium.

2.7 Screening anti-cancer effects of the EtOAc extracts from Marine-Derived Bacteria

2.7.1 Cell culture

HL-60 (Human promyelocytic leukemia cell line) was maintained at 37°C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured at a concentration of 5 × 10^4 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.

2.7.2 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 5 × 10^4 cells/ml (190 μL). After 24 h incubation at 37°C under a humidified atmosphere, the cells were treated with 10 μL of the EtOAc extracts from marine bacteria, 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 4 h of incubation, the plates were centrifuged for 5 min at 1500 rpm, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 100 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.

2.8. Statistical analysis

The Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data are expressed as means ± standard errors (SE) and the results are taken from at least three independent experiments performed in triplicate.

3. Results

3.1 Culture and isolation of marine-derived bacteria

Total twenty-one different bacterial strains have been isolated and identified from the marine samples which were collected around the coast of Jeju island. After taxonomic study it was concluded that these bacterial strains belong to seventeen genera. Most of the isolated strains belong to the genus of *Bacillus* (5 strains). The others belong to the genus of *Paracoccus*, *Exiguobacterium*, *Halomonas*, *Halobacillus*, *Burkholderia*, *Kytococcus*, *Staphylococcus*, *Cobetia*, *Streptomyces*, *Dietzia*, *Lysinibacillus*, *Maribacter*, *Planococcus*, *Intrasporangium*, *Micrococcus* and *Klebsiella sp*, respectively (**Table 1-1**).

For evaluation of the biological activities of the extract from the bacterial strains, all the strains were cultured in the flasks with 200 mL of liquid culture medium. After 10-15 days, the bacterial strains were further extracted to give extracts. All the extracts were prepared with ethyl acetate for further experiments.

No.	Host	Name	Similarity (%)
001-2	Alga	Paracoccus denitrificans	98
002-3	Sponge	Cobetia marina	99
003-2	Float	Halomonas marina	100
004-4	Alga	Streptomyces griseus	99
012-1	Aniaml	Dietzia cinnamea	99
015-1	Aniaml	Burkholderia cepacia	99
024-1	Alga	Halobacillus halophilus	95
030-1	Alga	Lysinibacillus sphaericus	99
032-5	Alga	Staphylococcus saprophyticus	98
033-2	Alga	Maribacter goseongensis	99
036-2	Float	Planococcus donghaensis	99
036-5	Float	Bacillus licheniformis	98
041-1	Alga	Kytococcus sedentarius	100
041-2	Alga	Intrasporangium calvum	97
063-1	Aniaml	Bacillus coagulans	98
063-3	Aniaml	Micrococcus luteus	100
072-1	Alga	Klebsiella oxytoca	99
074-2	Alga	Exiguobacterium sibiricum	99
075-1	Float	Bacillus badius	99
075-3	Float	Bacillus pseudofirmus	94
076-2	Alga	Bacillus megaterium	100

Table 1-1. Scientific names of the 21 species of marine bacteria isolated from marine samples

3.2 Screening antioxidant effects of the extracts from Marine-Derived Bacteria

3.2.1 DPPH radical scavenging capacity using ESR spectrometer

ESR spin trapping provides a sensitive, direct, and accurate means of monitoring reactive species. DPPH is a stable free radical donor that is widely used to test the free radical scavenging effect of natural antioxidants. Therefore, this study used ESR to compare the DPPH radical scavenging abilities of the EtOAc extracts isolated from marine samples. The percentage scavenging activities of the EtOAc extracts towards DPPH free radicals is shown in **Fig. 1-1**. Significant differences in the activities among different bacterial species were observed. Among all the marine bacteria, the extracts from *Streptomyces griseus*, *Bacillus coagulans*, *Bacillus badius and Bacillus pseudofirmus.* (004-4, 063-1, 075-1 and 075-3, respectively) showed higher activities than other species with activities of more than 50% at the concentration of 1 mg/mL. Especially, *Bacillus coagulans* (063-1) scavenged 64.7% of the DPPH free radicals at 1 mg/mL.

3.2.2 Hydrogen peroxide scavenging activity

Hydrogen peroxide together with reactive oxygen species (ROS) can damage several cellular components. Hydrogen peroxide is relatively an unstable metabolic product being responsible for the generation of hydroxyl radical and singlet oxygen, which is formed by fenton reaction and initiate lipid peroxidation or be toxic to cells [109,110]. Hydrogen peroxide

scavenging activity of the marine-derived bacterial EtOAc extracts were showed in **Fig. 1-2**. As shown in the results, remarkable scavenging effects of the marine bacterial EtOAc extracts were observed in hydrogen peroxide scavenging assay, compared to the DPPH scavenging assay at the concentration of 0.36 mg/mL and 0.71 mg/mL. The EtOAc extracts from *Maribacter goseongensis* (033-2) and *Bacillus badius* (075-1) at 0.36 mg/mL and *Maribacter goseongensis* (033-2)，*Halobacillus halophilus* (024-1), *Micrococcus luteus* (063-3) and *Bacillus badius* (075-1) at 0.71 mg/mL yielded approximately 50% scavenging activities. In particular *Bacillus badius* (075-1) EtOAc extract recorded the highest activity (approximately 73%) and *Maribacter goseongensis* (033-2) EtOAc extract exhibited relatively higher scavenging activities (approximately 67%) at the concentration of 0.71 mg/mL.

3.3 Screening anti-inflammatory effects of the extracts from Marine-Derived Bacteria

3.3.1 Assessment of cytotoxicity in Raw 264.7 cells

In order to evaluate cytotoxicity of the extracts from marine-derived bacteria 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 [111]. As shown in **Fig. 1-3**, the cells treated with LPS showed 80% cell viability. After incubation with LPS

and extracts from marine bacteria, three were from *Streptomyces griseus, Burkholderia cepacia* and *Bacillus pseudofirmus* (004-4, 015-1 and 075-3 respectively) of the twenty-one EtOAc extracts from marine bacteria showed cytotoxicity in RAW 264.7 cells. Especially, the EtOAc extracts of *Streptomyces griseus* (004-4) and *Burkholderia cepacia* (015-1) showed lowest cell viabilities (approximately 14.0% and 47.3% respectively). However, eighteen of the twenty-one EtOAc extracts without cytotoxicity in RAW 264.7 cells at the concentration of 200 μg/mL.

3.3.2 Effects of the extracts from marine-derived bacteria 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 [112,113]. 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 [114]. In this study, the anti-inflammatory effect of the EtOAc extracts from marine bacteria at the concentration of 200 μg/mL were observed using Raw 264.7 macrophages and investigating the released amount of NO. To evaluate the effect of EtOAc extracts from marine bacteria on NO production, RAW 264.7 macrophages were treated with LPS (1 μg/mL) for 1 h and then treated with EtOAc extracts of marine bacteria for 24h. NO production was measured in the

culture supernatants by the Griess reaction and ELISA assay. LPS treatment significantly increased the production of NO.

As shown in **Fig. 1-4**, Among the twenty-one tested EtOAc extracts from marine bacteria, fourteen EtOAc extracts showed more than 50% inhibitory effects of NO production at the concentration of 200 μg/mL. six were from *Kytococcus sedentarius*, *Halomonas marina*, *Halobacillus halophilus*, *Klebsiella oxytoca*, *Bacillus badius* and *Bacillus megaterium* (041-1, 003-2, 024-1, 072-1, 075-1 and 076-2 respectively) of the twenty-one tested EtOAc extracts inhibited nitric oxide production more than 80%. Especially, the EtOAc extracts of *Kytococcus sedentarius* (041-1) and *Klebsiella oxytoca* (072-1) showed significant inhibitory activities of NO production as 89.5% and 89.2%, respectively.

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

To determine the cytotoxic effects of EtOAc extracts of various bacteria on HL-60 cells, the cells were exposed to various bacterial EtOAc extract at a concentration of 200 μg/mL for 24 h. Cells treated with 0.1% DMSO were used as controls. As shown in **Fig. 1-5**, most EtOAc extracts from the marine bacteria, except the extract of *Streptomyces griseus* (004-4), showed poor cell growth inhibitory effects. The ethyl acetate extract of *Streptomyces griseus* (004-4) showed the highest level of inhibition of the proliferation of HL-60 cells (approximately 90%), as compared to the other bacterial EtOAc extracts. The EtOAc extracts

of *Bacillus coagulans* (063-1), *Bacillus badius* (075-1), *Halomonas marina* (076-1) showed inhibitory effects more than 30% in HL-60 cells.

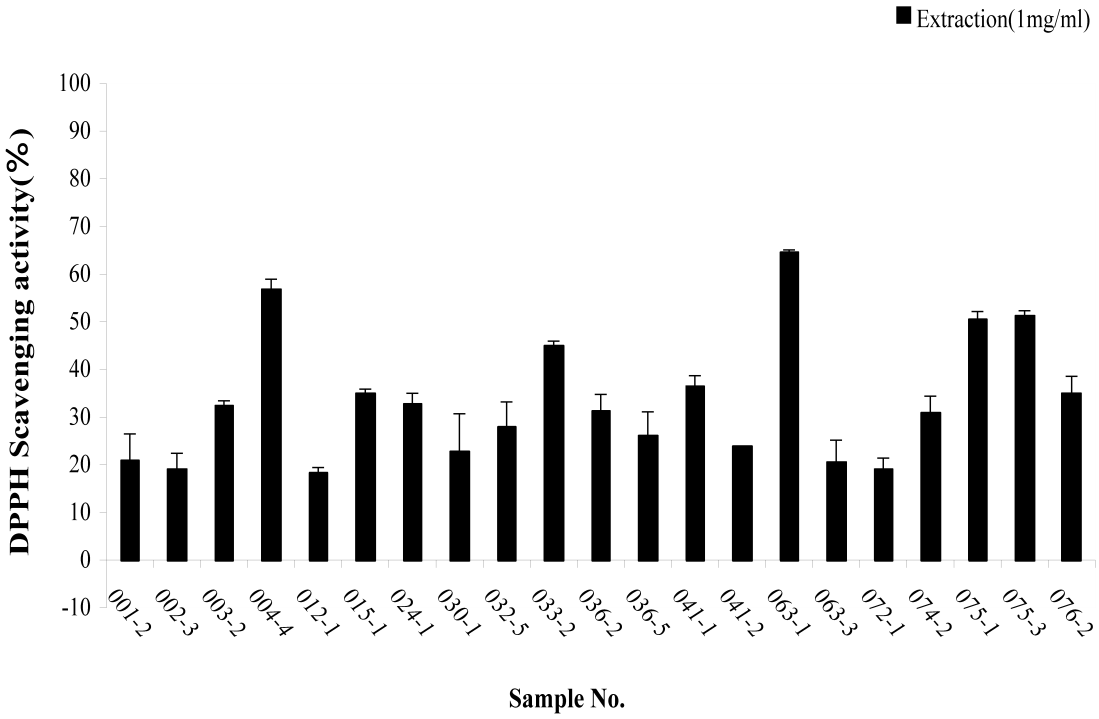


Fig. 1-1. DPPH radical scavenging activity of EtOAc extracts from marine-derived bacteria. Each value indicates that the mean ± standard error from three independent experiments.

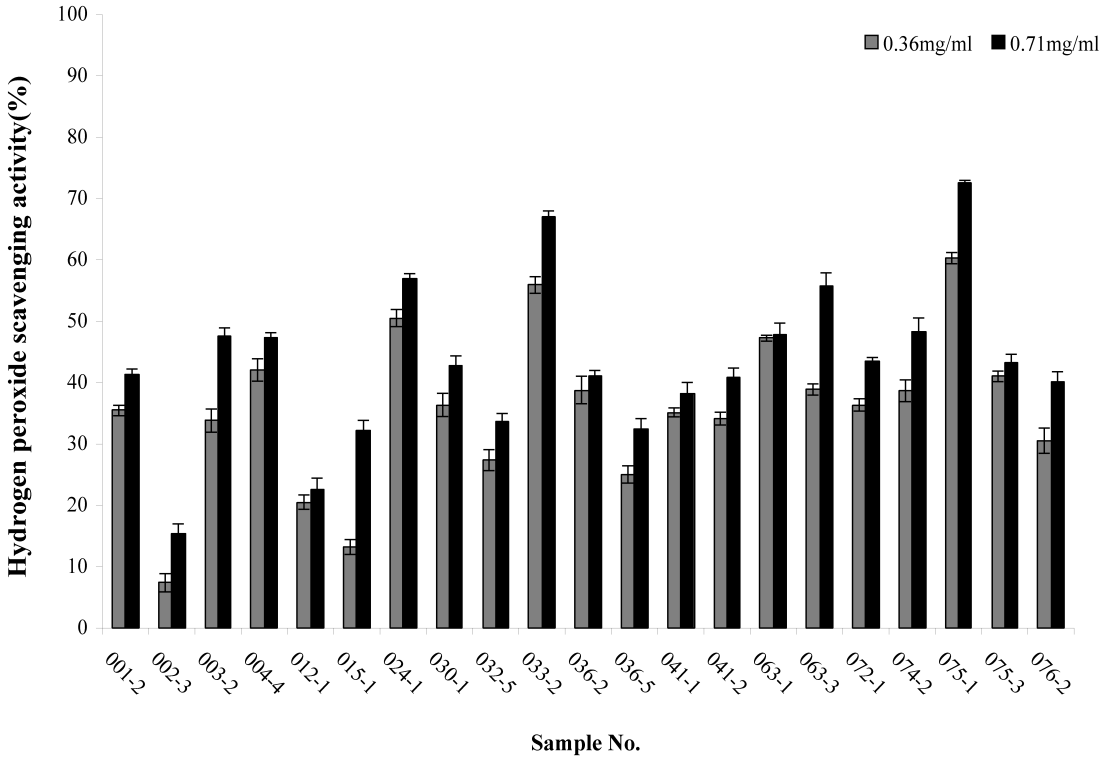


Fig. 1-2. Hydrogen peroxide scavenging activity of EtOAc extracts from marine-derived bacteria. Each value indicates that the mean ± standard error from three independent experiments.

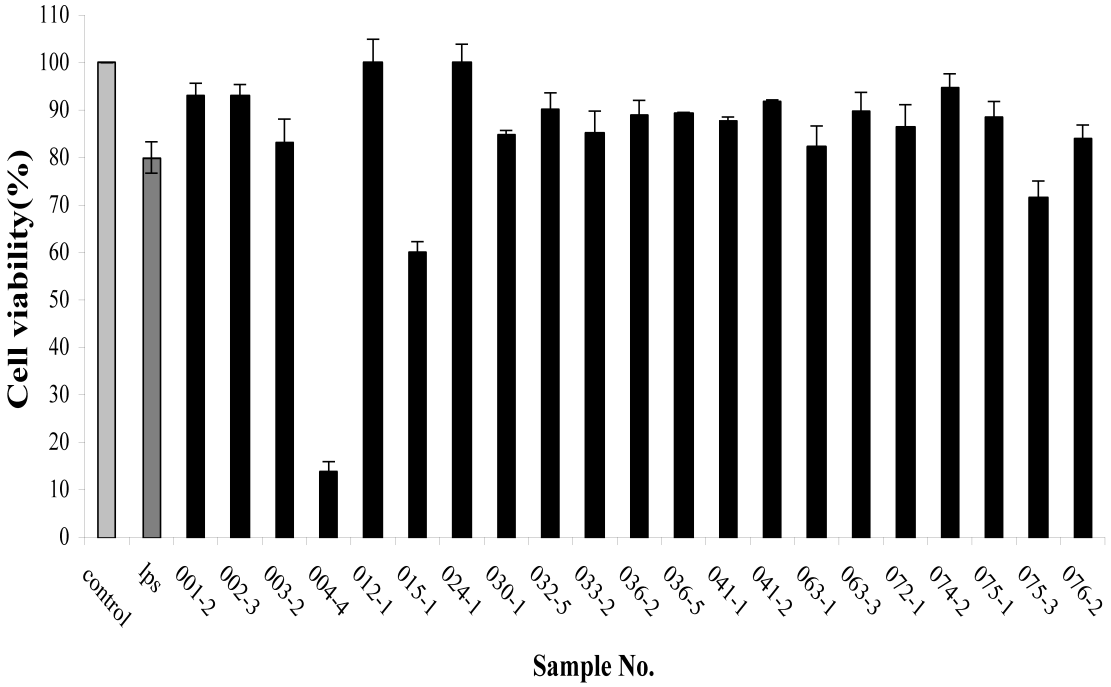


Fig. 1-3. Cell viability of the EtOAc extracts from marine-derived bacteria in RAW 264.7 macrophages. Each value indicates that the mean ± standard error from three independent experiments.

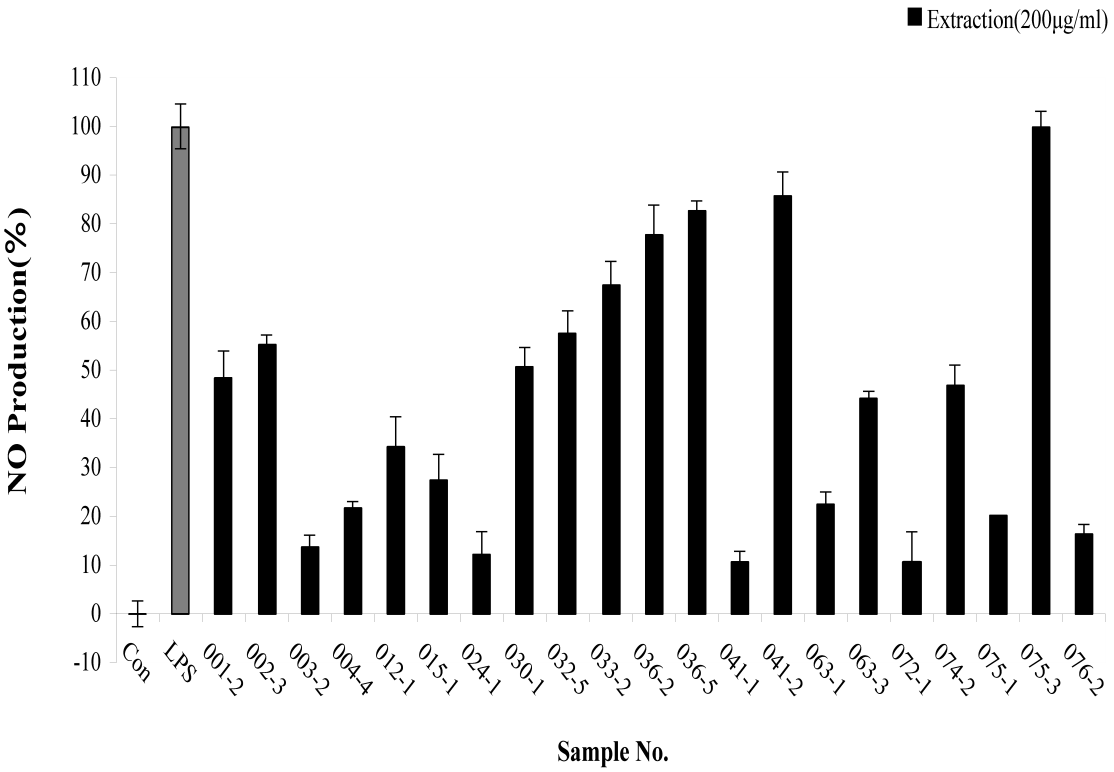


Fig. 1-4. NO production inhibitory effects of the EtOAc extracts from marine-derived bacteria. Each value indicates that the mean ± standard error from three independent experiments.

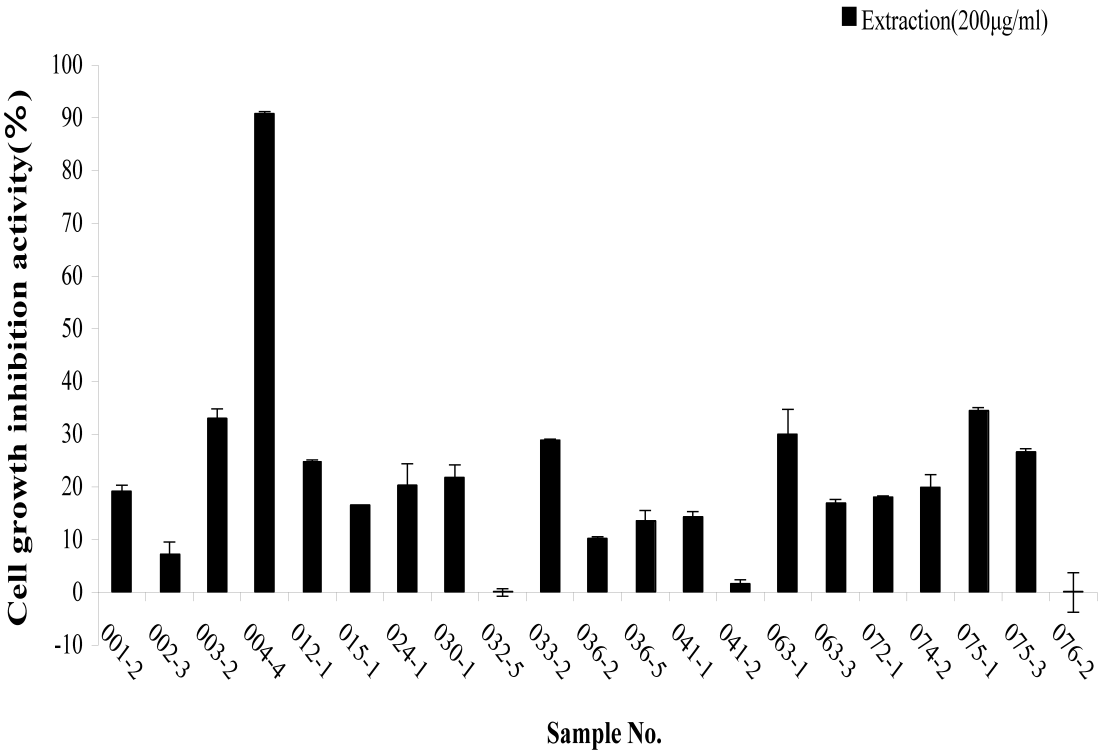


Fig. 1-5. Cell growth inhibitory effects of the EtOAc extracts from marine-derived bacteria in HL-60 cells. HL-60 cells were incubated with 21 EtOAc extracts for 48 h and the cell viability was examined by an MTT assay. Each value indicates that the mean ± standard error from three independent experiments.

4. Discussion

Jeju Island is located in the southwest sea of the Korean peninsula and is highlighted for its uniqueness. Especially, in the coastal area of this Island the seawater level fluctuates rapidly [115]. Therefore, the bacterial species present along the shoes of Jeju Island may require specific biological protection as an adaptative response to this especial environment. However, yet there are few or less systematically studied reports regarding the potential biological activities of secondary metabolites isolated from Jeju Island bacteria. We are confident we have been able to identify numerous extracts with interesting biological activity from bacterial strains isolated from Jeju Island. The present study reports for the first time the antioxidant, anti-inflammatory, anti-cancer activities of the ethyl acetate extracts of marine bacteria isolated from Jeju Island.

Twenty-one different bacterial crude extracts derived from the marine samples collected off Jeju Island were assessed in a series of bioassays. These assays included: Inhibition of nitric oxide (NO) production, inhibition 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), inhibition of HL-60 cell proliferation and inhibition hydrogen peroxide radical scavenging.

Firstly, the antioxidant activities of the crude EtOAc extracts had been investigated by assessing their roles on DPPH radical scavenging activity and hydrogen peroxide scavenging activity. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases.

They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms [116].

The free radical scavenging activity of the EtOAc extracts was investigated in relation to 1,1-diphenyl-2-picrylhydrazyl (DPPH) using an electron spin resonance (ESR) system. As shown in the results, moderate scavenging effects of the extracts from marine bacteria were observed in DPPH free radical scavenging assay. Four extracts (*Streptomyces griseus*, *Bacillus coagulans*, *Bacillus badius and Bacillus pseudofirmus*) yielded approximately 50% scavenging activities at the concentration of 1 mg/mL. In particular the extract of *Bacillus coagulans* (063-1) showed significantly higher inhibition percentage (64.7 %) at 1 mg/mL. This finding is line with Kodali et al. (2011) [117] who reported *Bacillus coagulans* RK-02, produces an EPS, which is a heteropolymer composed of four monosaccharides has significant antioxidant and free radical scavenging activities. The result of this assay indicates that this bacterial extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage and *B. coagulans* would appear to be a good potential DPPH free radical scavenger. Based on this, further chemical and pharmacological investigations to isolate and identify minor chemical constituents in *B. coagulans* and to screen other potential bioactivities may be recommended.

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms and food [118]. H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage [119]. In our assay, four isolated strains (*Maribacter goseongensis*, *Halobacillus halophilus*, *Micrococcus luteus* and *Bacillus badius*) showed anti-oxidant

activity against hydrogen peroxide, above 50% at the 0.71 mg/mL level. Especially the ethyl acetate extract of *Bacillus badius* (075-1) efficiently scavenged hydrogen peroxide (approximately 73%) which may be attributed to the presence of bioactive secondary metabolites that could donate electrons to hydrogen peroxide, thereby neutralizing it into water. Previous study carried out by other researcher, reported that Nitrite-oxidizing enzyme I (NiOx I) was purified from *Bacillus badius* I-73. NiOx I is a catalase and the purified enzyme also catalyzed H_2O_2 degradation [120]. The scavenged DPPH effect of *B. badius* was also higher (50.6 % at 1 mg/mL) implying that this bacterium can be utilized as a useful natural radical scavenger and a potential supplement for the food, pharmaceutical, and cosmetic industries because of its antioxidant capacities.

The anti-inflammatory activity of the extracts was investigated by lipopolysaccharide (LPS) activated NO production in a murine macrophage-like cell line, RAW 264.7. Combination of MTT and NO production inhibitory results, twelve extracts of marine bacteria inhibited production by 50 % or more without cytotoxicity in RAW 264.7 cells. Furthermore, the ethyl acetate extracts from the strains belonged to the *Kytococcus sedentarius* (041-1) and *Klebsiella oxytoca* (072-1) were observed to show higher NO production inhibition (89.5% and 89.2%, respectively) than those of other bacterial strains and didn't show any cytotoxic effect. This is the first report of NO production inhibitory activities of *Kytococcus sedentarius* and *Klebsiella oxytoca*. Moreover, no report on the isolation and characterization of anti-inflammatory constituents from these bacteria has been made. On the basis of above-mentioned evidence, the active marine bacteria, *Kytococcus sedentarius* and *Klebsiella oxytoca* may be useful for the treatment of various inflammatory diseases.

It has been established that reactive oxygen species (ROS) are implicated in inflammation [121]. There exists a link of antioxidants with respect to scavenging ROS and antiinflammatory effects and therefore play an important role in the treatment of inflammatory diseases [122]. The demonstration of both antioxidant and anti-inflammatory activities by *Bacillus coagulans* may confirm this relationship.

Finally, the cytotoxic potential of the extracts was determined employing HL-60 cells in culture. Inhibition of proliferation or induction of apoptosis in cancer cells is one characteristic of many anti-cancer drugs. Only one extract was active in the HL-60 cell assay with inhibition level of 90% at a concentration of 200 μg/mL. Our report is well in agreement with Wendt-Pienkowski et al. (2005) [123]who reported Fredericamycin (FDM) A biosynthetic gene cluster has been previously cloned from *Streptomyces griseus* ATCC 49344, the FDM exhibits potent cytotoxicity and has been studied as a new anticancer drug lead. The potent cytotoxic activity of *S. griseus* against the HL-60 cells suggested that *S. griseus* may be a potential cancer chemopreventive agent for use in the treatment of leukemia. Moreover, *S. griseus*, it can be considered for compound isolation in order to detect future anti-tumour compounds.

In sum, of the 21 extracts, 15 showed a positive result in at least one of the applied tests. Of these, four were active in two of the assay systems, and only one in three assays. Of the extracts showing positive results in two assays, two of them (*Halobacillus halophilus*, *Micrococcus luteus*) were positive in both the H_20_2 (57.3 and 55.8% inhibition, respectively, at 0.71 mg/mL) and NO (87.9% and 55.9% inhibition, respectively, with 100% cell survival at 200 μg/mL) test systems, the remaining two being active in DPPH (56.9% inhibition at 1

mg/mL) and HL-60 (90.1% cell mortality at 200 µg/mL) (*Streptomyces griseus*), and DPPH (64.7% inhibition at 1 mg/mL) and NO (77.7% inhibition at 200 µg/mL, with 100% cell survival) (*Bacillus coagulans*). Extract *Bacillus badius* was active in DPPH (50.6% inhibition at 1 mg/mL), H_20_2 (72.5% inhibition at 0.71 mg/mL) and NO (79.8% inhibition with 100% cell survival) assays.

Aside from these, all other extracts were active in either only one assay system or apparently devoid of any activity in the applied assays. Eight extracts were active in NO (greater than 50% inhibition and 100% survival) assay. In both the DPPH (greater than 50% inhibition) and H_20_2 (greater than 50% inhibition) assays, one extract was found to have activity, respectively.

On the basis of these findings, fractionation was initiated with one of the active extracts, a sample of the bacterium *Bacillus badius* that demonstrated significant activity in three assays; DPPH, NO and H_20_2. This process is ongoing.

5. Conclusion

In this study, we have isolated and identified twenty-one different bacterial strains from the marine samples which were collected around the coast of Jeju island and studied on their biological activities of cytotoxicity, anti-inflammation, and anti-oxidation. These data revealed the tested samples to have many and varied activities, making them, useful starting points for further fractionation and purification. Moreover, the large number of samples demonstrating activity in only one or sometimes two assays accentuates the potential of Jeju Island, as a rich source, for the discovery of selectively bioactive compounds. However, further studies are necessary to elucidate the mechanism lying with these effects. This study may serve as a foot step regarding the biological and pharmacological activities of the crude EtOAc extracts of marine bacteria isolated from Jeju Island. Based on these screening results we are now in the process of fractionation and purification of novel, pharmacologically active compounds from the most promising lead extracts.

Part II.

Isolation and Anti-cancer Activity of Secondary Metabolites from the Marine Bacterium, *Streptomyces griseus* by centrifugal partition chromatography

Part II.

Isolation and Anti-cancer Activity of Secondary Metabolites from the Marine Bacterium, *Streptomyces griseus* by centrifugal partition chromatography

1. Abstract

Based on the result of screening for the anti-cancer activity of the extracts from marine bacteria, the bacterial strain, *Streptomyces griseus* (004-4) has been selected as our target strain for the researches of bioactive natural products and their bioactivities. Bioactivity-guided fraction was rechromatographed by preparative centrifugal partition chromatography (CPC) to obtain three compounds, namely cyclo[L-(4-hydroxyprolinyl)-L-leucine] (1), cyclo(L- Phe-trans-4-hydroxy-L-Pro) (4) and phenethyl acetate (9) from the EtOAc extract of *S. griseus.* Then, we adopted active track method to select phenethyl acetate as our target compound for further experiments, because phenethyl acetate showed the highest level of inhibition of the proliferation of HL-60 cells. Treatment of HL-60 cells with various concentrations of phenethyl acetate (0.15-0.6 μM) resulted in the effects included the formation of apoptotic bodies and fragmented DNA, as well as the accumulation of DNA in the G_0/G_1 phase of the cell cycle and reactive oxygen species (ROS). Analysis of the mechanism of these events indicated that phenethyl acetate treated cells had an increased ratio

of Bax/Bcl-xL, activated caspase-3, and cleaved poly-ADP-ribose polymerase (PARP); these observations are hallmarks of apoptotic events. In simultaneous *in vitro* and *in vivo* toxicity tests, toxicity was not detected in the Vero cells and in the zebrafish treated with phenethyl acetate. In this study, it was demonstrated for the first time that phenethyl acetate generated ROS and that the accumulation of ROS performed a crucial role in the phenethyl acetate-induced Bcl-xL signaling pathway.

2. Materials and methods

2.1.1 General materials

RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco/BRL (Burlington, ON, Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, Dihydroethidium (DE), 2' 7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), propidium iodide (PI), dimethyl sulfoxide (DMSO), and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Bax, Bcl-xL, cleaved caspase-3, poly-ADP-ribose polymerase (PARP) and GAPDH were purchased from Cell Signaling Technology (Bedford, MA, USA). All solvents used for preparation of crude sample and CPC separation were of analytical grade (Daejung Chemicals &Metals Co., Seoul, Korea). HPLC grade solvents were purchased from Burdick & Jackson (MI, USA).

2.1.2 Apparatus

LLB-M high performance CPC 240 (system instrumets co., LTD., Japan) was used in preparative CPC. The total cell volume is 240 mL. A four-way switching valve incorporated in the CPC apparatus allows operating in either the descending or the ascending mode. This CPC system was equipped with a FLO 214 dual pump (FLO, Japan), an S-3702 UV/Vis detector (Soma optics., LTD, Japan), and a Advantec CHF 122SC fraction collector (Toyo

seisakusho kaisha LTD., Japan). The samples were manually injected through a ES injector (ES, Japan) with a 2 mL sample loop.

^{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 Methanol-d_4 solvent peak (3.31 ppm in ^{1}H and 49.15 ppm in ^{13}C NMR) as an internal reference standard. Mass spectra (FAB-MS and EIMS) were recorded on a JEOL JMS 700 spectrometer. The HPLC system in this experiment consisted of two 515 HPLC pump, In-Line Degasser AF, Pump control module II, 2998 Photodiode array detector and 2707 Autosampler (Water, USA).

2.2. Culture and Extraction procedure of *S. griseus*

Based on the result of screening for the anti-cancer activity of the extracts from marine bacteria, the bacterial strain, *Streptomyces griseus* (004-4) has been selected as our target strain for the researches of bioactive natural products and their bioactivities.

The phylogenetic position of the isolated strain (004-4) was assessed by performing a nucleotide sequence database search using the BLAST program from NCBI GenBank. 2L Erlenmeyer flasks containing 2L of medium containing of peptone (0.7%), yeast extract (0.2%), D-(+)-Glucose (0.2%) and seawater (100%) were inoculated with a single colony from a well grown agar plate. The bacterial strain was cultured (18 L) for 20 days (static) at 29 °C with humidity. The cultured broth of the strain *S. griseus* was centrifuged (10000 rpm, 15 min) to remove the cells. Then the supernatants were extracted with equal volumes of ethyl acetate. After separation, the organic extracts were combined and concentrated in vacuo to

dryness at 35°C yielding 0.6 g of a dark red-colored solid extract was stored in a refrigerator for CPC separation.

2.3. Centrifugal partition chromatography (CPC) separation procedure

The EtOAc extract was suspended in H_2O and fractionated with *n*-hexane to give water fraction and *n*-hexane fraction. The CPC experiments were performed using a two-phase solvent system composed of *n*-hexane:EtOAc:MeOH:water (0:10:1:9, v/v). The two phases were separated after thoroughly equilibrating the mixture in a separating funnel at room temperature. The upper organic phase was used as the mobile phase, whereas the lower aqueous phase was employed as the stationary phase. The CPC column was initially filled in ascending mode with the aqueous stationary phase and then rotated at 1000 rpm while the mobile phase was pumped into the column at the flow rate used for the separation (2 mL/min). When the mobile phase emerged from the column, indicating that hydrostatic equilibrium had been reached (back pressure : 2.0 MPa), The bioactivity-guided fraction of ethyl acetate extract from *S. griseus* was dissolved in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system phases was injected through the Rheodyne injection valve. The effluent from the CPC was monitored in the UV at 254 nm and fractions were collected with 6ml in 8ml tube by a Advantec CHF 122SC fraction collector (Toyo seisakusho kaisha LTD., Japan).

2.4. HPLC-DAD analyses

The HPLC system in this experiment consisted of a binary FLEXAR UHPLC pump, a FLEXAR PDA detector, a FLEXAR PDA auto sampler (PERKIN ELMER, USA). A 10μl of 5mg/ml sample solution was directly injected on Atlantis T3 3μm 3.0 × 150mm column (Waters, Ireland) using stepwise gradient mixtures of acetonitrile (solvent A) and distilled water (solvent B) as eluents at a flow rate of 0.2 ml/minute. The gradient was from 5% to 50% for solvent A in 50 minutes, from 50% to 100% for solvent A in 10 minutes with a 10-minute hold at 100% for solvent A. Multiple wavelength monitoring was performed at 210, 254, 280 and 365 nm and photodiode array detector measured from 200 to 400 nm.

2.5. HPLC-DAD-ESI/MS analysis of purified compounds

HPLC-DAD-ESI/MS analyses were carried out using a Hewlett-Packard 1100 series HPLC system equipped with an autosampler, a column oven, a binary pump, a DAD detector, and a degasser (Hewlett-Packard, Waldbronn, Germany) coupled to a Finnigan MAT LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a Finnigan electrospray source and capable of analyzing ions up to m/z 2000. Xcalibur software (Finnigan MAT) was used for the operation. The chromatographic conditions are identical to those described in Section 2.4 and the outlet of the flow cell was connected to a splitting valve, from which a flow of 0.2 mL/min was diverted to the electrospray ion source via a short length of fused silica tubing. Negative ion mass spectra of the column eluate were

recorded in the range m/z 100-2000. The source voltage was set to 4.5 kV and the capillary temperature to 250°C. The other conditions were as follows: capillary voltage, -36.5 V; inter-octapole lens voltage, 10 V; sheath gas, 80 psi (551.6 kPa); auxiliary gas, 20 psi (137.9 kPa).

2.6. ^{1}H-NMR and ^{13}C -NMR analysis of purified compounds

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 Methanol-d_4 solvent peak (3.31 ppm in ^{1}H and 49.15 ppm in ^{13}C NMR) as an internal reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer.

2.7. Cell culture

HL-60 (Human promyelocytic leukemia cell line) was maintained at 37°C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured at a concentration of 5 × 10^4 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.

2.8. 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 5 × 10^4 cells/ml (190 μL). After 24 h

incubation at 37 °C under a humidified atmosphere, the cells were treated with 10 μL of the samples, and further incubated for 36 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 4 h of incubation, the plates were centrifuged for 5 min at 1500 rpm, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 100 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. According to the result of cell growth inhibitory assay of the three isolated compounds, we selected the compound showed the highest level of inhibition of the proliferation of HL-60 cells as our target compound for further experiments.

2.9. Nuclear staining with Hoechst 33342

The nuclear morphology of cultured HL-60 cells was studied using the cell-permeable DNA dye Hoechst 33342 and PI. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis [124,125]. HL-60 cells were seeded into 24-well plates at a density of 1×10^5 cells/mL. The cells were then treated with various concentrations of the target compound and incubated for an additional 36 h. Then, Hoechst 33342 and PI, a DNA specific fluorescent dye was added to the culture medium at a final concentration of 10 and 5 μg/mL, respectively, and

the plates were incubated for an additional 15 min at 37 °C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to determine the degree of nuclear condensation.

2.10. Cell cycle analysis

The HL-60 cells were seeded into 5 cm dishes at a density of 1×10^5 cells/mL. The cells were then treated with the target compound and incubated for 36 h. The cells were harvested at the indicated times and fixed in 1 mL of 70% ethanol and kept at -20 °C until analysis. The cells were washed twice with phosphate buffered saline (PBS) and incubated in darkness with 1 ml of PBS containing 100 μg PI and 100 μg RNase A for 30 min at 37 °C. Flow cytometric analysis was conducted with an FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect of the target compound on 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 [126].

2.11. Measurement of ROS

The accumulation of intracellular H_2O_2 was measured using the probe $DCFH_2$-DA as previously described [127,128]. In brief, the HL-60 cells were placed in 6-well plates at a concentration of 5×10^4 cells/mL. The cells were treated with various concentrations of the target compound. After 48 h, the cells were labeled with 10 μM $DCFH_2$-DA for 30 min at

37 °C. The labeled cells were then washed in phosphate-buffer saline (PBS), and the fluorescence was analyzed using a flow cytometer.

2.12. Western blot analysis

Cells (1×10^5 cells/mL) were treated with various concentrations of the target compound and 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% sodium dodecyl sulfate (SDS), and 1 mmol/L EDTA). Cell lysates were washed and collected via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). 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, PARP, and GAPDH in TTBS (25 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20, pH 7.4) 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.

2.13. Origin and maintenance of zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Korea) and 10 fish were kept in a 3-L acrylic tank under the following conditions: 28.5 °C, with a 14/10 h light/dark cycle. Fish were fed three times a day, 6 days a week, with Tetramin flake food supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Collection of embryos was completed within 30 min.

2.14. Waterborne exposure of embryos to phenethyl acetate

From approximately 3 hour post-fertilisation (3 hpf), embryos (group = 32 embryos) were transferred to individual wells of a 24-well plate and maintained in embryo medium containing 50 µl of vehicle (0.1% DMSO) or 0.15, 0.3 and 0.6 µM phenethyl acetate for up to 120 hours post-fertilisation (120 hpf). The embryo medium was changed everyday.

2.15. Measurement of heartbeat rate

The heartbeat rate of both atrium and ventricle were measured at 35 hpf to determine the sample toxicity [129]. Counting and recording of atrial and ventricular contractions were performed for 3 min under a microscope, and results were presented as the average heartbeat rate per min.

2.16. Measurement of phenethyl acetate-induced cell death in zebrafish embryo

Cell death was detected in live embryos using acridine orange staining, a nucleic acid selective metachromatic dye that interacts with DNA and RNA by intercalation or electrostatic attractions. Acridine orange stains cells with disturbed plasma membrane permeability so it preferentially stains necrotic or very late apoptotic cells. At 72 hpf, The embryos were transferred into 96-well plates and treated with acridine orange (AO) solution (7 μg/ml), and the plates were incubated for 30 min in the dark at 28.5 °C. After incubation, the embryos were rinsed in embryo medium and anaesthetised before visualisation. Individual embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B). The images of stained embryos were observed using a fluorescence microscope, which was equipped with a CoolSNAP-Pro colour digital camera (Olympus).

2.17. Statistical analysis

The Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data are expressed as means ± standard errors (SE) and the results are taken from at least three independent experiments performed in triplicate.

3. Results

3.1. Cytotoxic effects of solvent fractions of ethyl acetate extract of *S. griseus* on HL-60 cell lines

The EtOAc extract of *S. griseus* was suspended in H_2O and fractionated with *n*-hexane to give water fraction and *n*-hexane fraction, and their growth inhibitory effects on HL-60 cells were evaluated (**Fig. 2-1**), the cells were exposed to *n*-hexane and water fractions at three concentrations of 200, 100 and 50 μg/mL for 48 h. Cells treated with 0.1% DMSO were used as controls. The water fraction showed the higher level of inhibition of the proliferation of HL-60 cells (91%, 23% and 9% at 200, 100 and 50 μg/mL, respectively). Thus, we selected water fraction for next experiments.

3.2. HPLC analysis of EtOAc extract of *S. griseus*

The water fraction of EtOAc extract was analyzed by described HPLC condition and chromatogram depicted in **Fig. 2-2**. In the result, HPLC peak 1, 2, 4 and 9 are the main peaks showed on HPLC analysis. Therefore, we separated and collected the focused compounds (1, 2, 4 and 9) using preparative CPC.

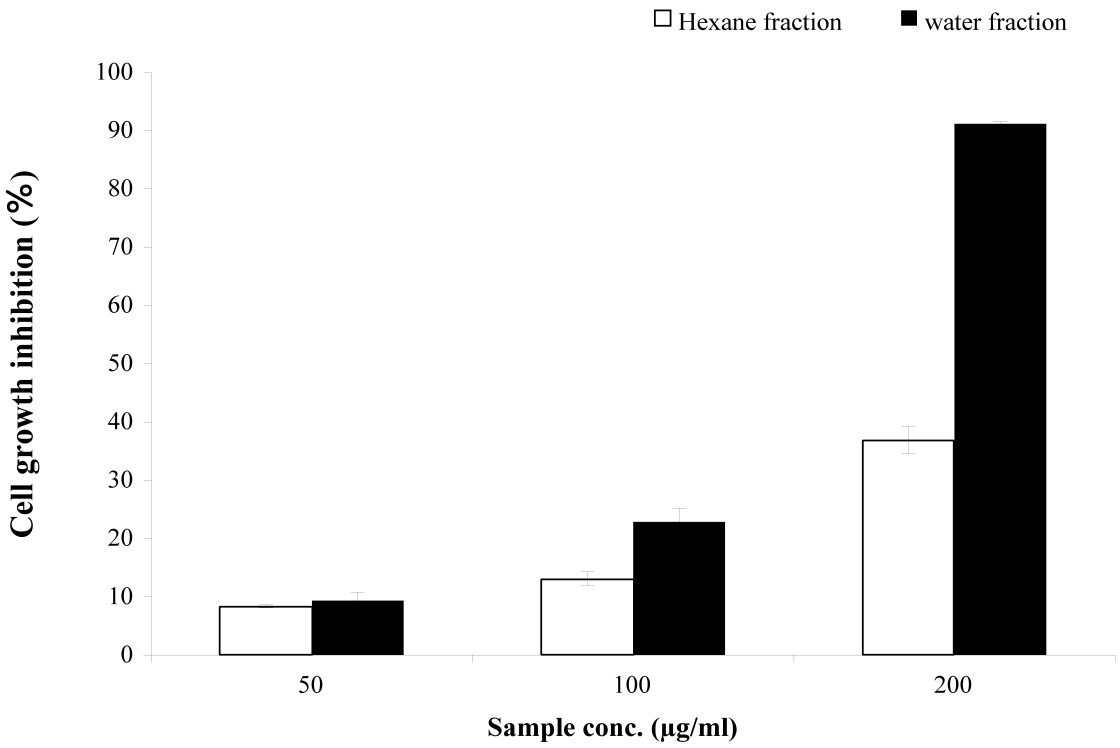
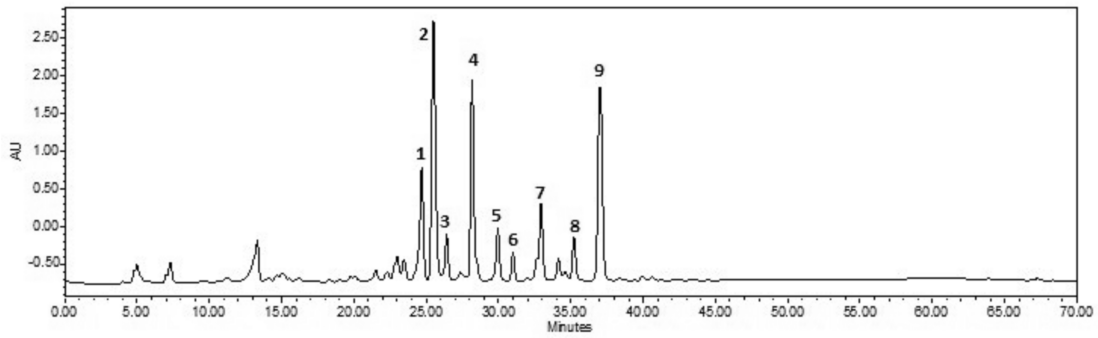


Fig. 2-1. Inhibitory effects of two fractions from *S. griseus* against the growth in the HL-60 cells. HL-60 cells were incubated with various concentrations of two fractions for 48 h and the cell viability was examined by an MTT assay. Each value indicates that the mean ± standard error from three independent experiments.



HPLC condition：0-50min 5-50% ACN, 50-60min 50-100% ACN, 60-70min 100% ACN

Fig. 2-2. HPLC analysis of water fraction from culture filtrate extract of *S. griseus.*

3.3. Optimization of two-phase solvent system

Partition coefficient (*K*) for selection of a suitable two phase solvent systems were the most important for successful separation of target samples by preparative CPC. In order to choice efficient separation, several two-phase solvent system was performed through different compositions and volume ratios of two immiscible solvents such as *n*-hexane:EtOAc:MeOH: water (v/v), and then their *K* values was calculated and showed in **Table 2-1**. Two-phase solvent system composed of 0:10:1:9 (*n*-hexane:EtOAc:MeOH:water, v/v) exhibited good *K* values to separate compounds confirmed by HPLC. The most efficient separation of each compounds was performed under the solvent condition, *n*-hexane:EtOAc:MeOH:water (0:10:1:9, v/v) by preparative CPC.

3.4. Centrifugal partition chromatography (CPC) separation procedure of anticancer compounds

The *K*-values of most of target compounds exhibited upper numerical values than 1. Therefore, preparative CPC was operated on ascending mode selected upper phase as mobile phase and lower phase as stationary phase, respectively. The retention of the stationary phase in the coil retained 140 mL and pressure exhibited 2.0 MPa during the operation. Preparative CPC chromatogram was described in **Fig. 2-3**. We confirmed that the compound 9 is included in fraction 1, compound 4 is included in fraction 4 and compound 1 is included in fraction 5 according to analysis by HPLC peak area. The yields of compound 9, compound 4 and

compound 1 isolated from 500 mg of water fraction of EtOAc extract of *S. griseus* by one-step of CPC system was 29.7 mg, 24.9 mg and 15.7 mg, respectively. The separated fraction of the HPLC peak 2, which showed on HPLC analysis, was not pure enough and didn't show any anti-cancer activity. Hence, we selected compound 1, 4 and 9 for further experiments. The entire flow chart showing extraction and purification of bioactive metabolites from the culture filtrate of *Streptomyces griseus* is presented in **Fig. 2-4**.

3.5. Identification and Structural elucidation of bioactive metabolites produced by *Streptomyces griseus*

The structures of the isolated compounds were determined by analysis of the MS (**Fig. 2-5**) and NMR spectral data, and also comparing with previously published data.

Compound 1 (cyclo[L-(4-hydroxyprolinyl)-L-leucine]): pale yellow liquid, ^{1}H NMR (Methanol-d_3, 400 MHz) δ 4.51 (1H, m, H-8), 4.46 (1H, m, H-6), 4.17 (1H, m, H-3), 3.65 (1H, dd, J = 12.3 and 3.5 Hz, H-9a), 3.43, (1H, m, H-9b), 2.10 (1H, m, H-7a), 2.08 (1H, m, H-7b), 1.92 (1H, m, H-10a), 1.89 (1H, m, H-11), 1.52 (1H, m, H-10b), 0.97 (3H, d, J = 2.0 Hz, H-12), 0.96 (3H, d, J = 2.0 Hz, H-13) (**Fig. 2-6**); ^{13}C NMR (Methanol-d_3, 100 MHz) δ 173.2 (C-5), 169.2 (C-2), 69.3 (C-6), 58.9 (C-8), 55.3 (C-9), 54.7 (C-3), 39.5 (C-10), 38.3 (C-7), 25.9 (C-11), 23.5 (C-12), 22.3 (C-13) (**Fig. 2-7**); ESI/MS *m/z* 227.09 $[M+1]^{+}$.

Compound 4 (cyclo(L-Phe-trans-4-hydroxy-L-Pro)): pale yellow liquid, ^{1}H NMR (Methanol-d_3, 400 MHz) δ 7.26 (5H, m, H-12-16), 4.49 (1H, m, H-3), 4.28 (1H, m, H-6), 4.20 (1H, m, H-8), 3.68 (1H, m, H-9a), 3.20 (1H, m, H-9b), 3.16 (2H, m, H-10), 2.08 (1H, m,

H-7a), 1.29 (1H, m, H-7b) (**Fig. 2-8**); ^{13}C NMR (Methanol-d_3, 100 MHz) δ 171.4 (C-5), 167.2 (C-2), 137.5 (C-11), 131,2 (C-13, 15), 129.6 (C-12, 16), 128.2 (C-14), 68.7 (C-11), 58.5 (C-12), 57.7 (C-13), 55.4 (C-11), 39.0 (C-11), 38.2 (C-11) (**Fig. 2-9**); ESI/MS *m/z* 261.20 $[M+1]^+$.

Compound 9 (phenethyl acetate): pale yellow liquid, ^{1}H NMR (Methanol-d_3, 400 MHz) δ 7.23 (5H, m, H-1-5), 3.34 (2H, t, J = 1.6 and 1.8 Hz, H-7), 2.78 (2H, t, J = 7.6 and 7.3 Hz, H-8), 1.90 (3H, s, H-11) (**Fig. 2-10**); ^{13}C NMR (Methanol-d_3, 100 MHz) δ 171.8 (C-10), 140.3 (C-6), 129.8 (C-2, 4), 129.7 (C-1, 5), 127.8 (C-3), 42.2 (C-8), 36.4 (C-7), 22.3 (C-11) (**Fig. 2-11**); ESI/MS *m/z* 164.08 $[M]^+$.

The chemical structures of the three isolated compounds are presented in **Fig. 2-22**.

H:E:M:W	1	2	3	4	5	6	7	8	9
3737	0.00	1.11	0.71	0.14	0.52	0.21	0.03	0.49	1.71
2828	0.00	1.97	0.32	0.23	0.76	0.39	0.80	1.48	2.76
1919	0.00	2.11	0.72	0.38	1.18	0.60	1.28	2.31	3.88
01019	0.00	5.36	0.94	0.66	1.90	1.17	2.34	3.58	5.90

Table 2-1. Partition coefficient (K values) as solvent condition of water fraction from culture filtrate extract of *S. griseus.*

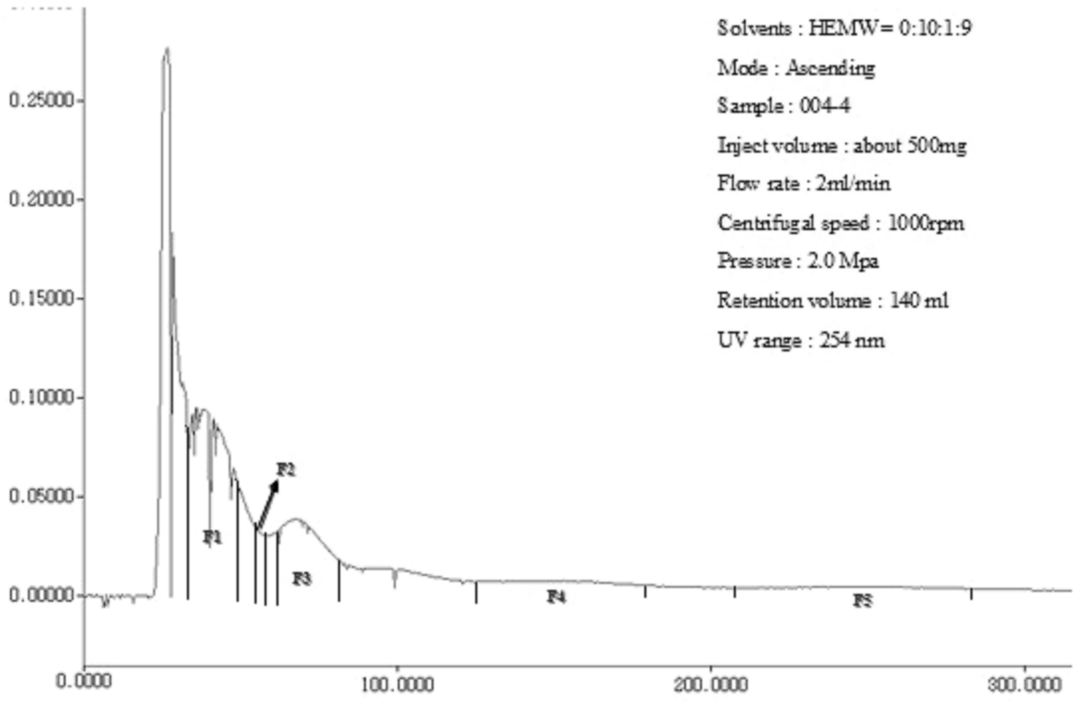


Fig. 2-3. Preparative CPC chromatogram of water fraction from culture filtrate extract of *S. griseus.*

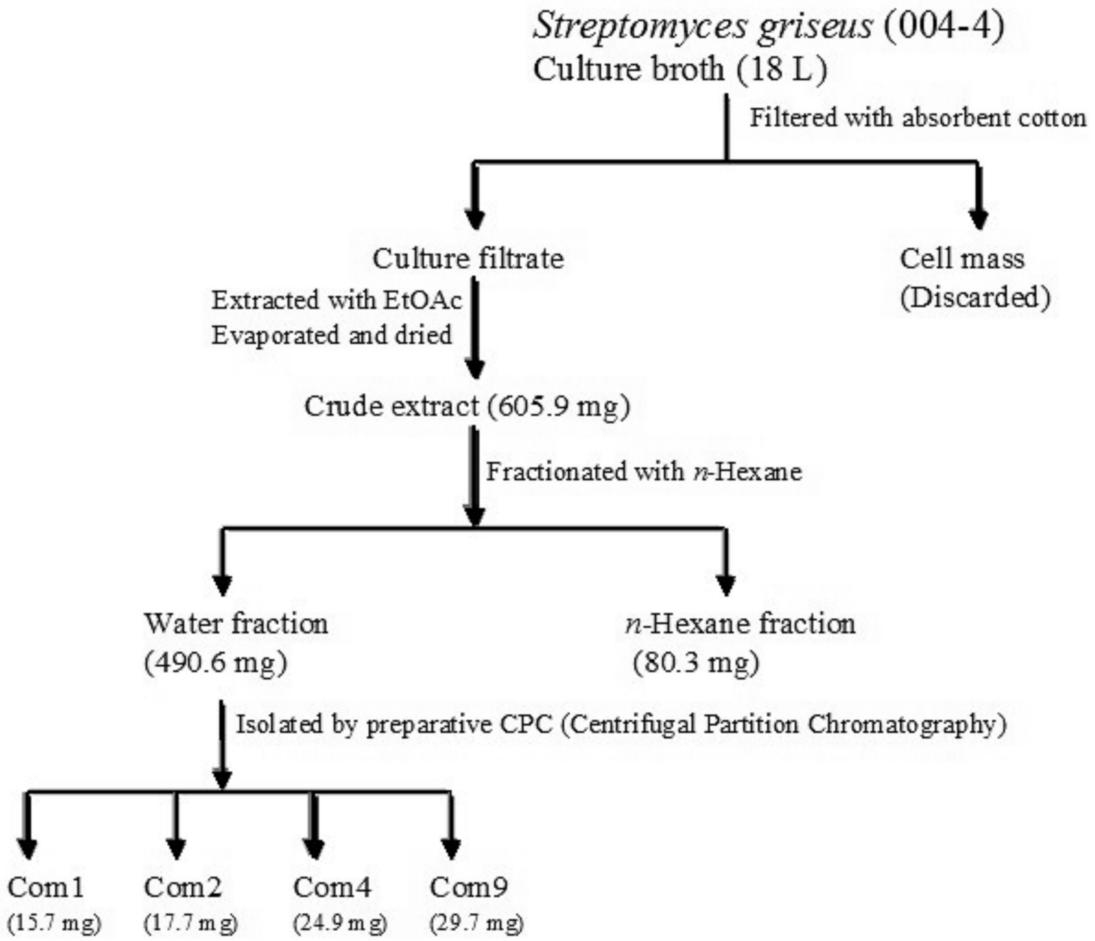
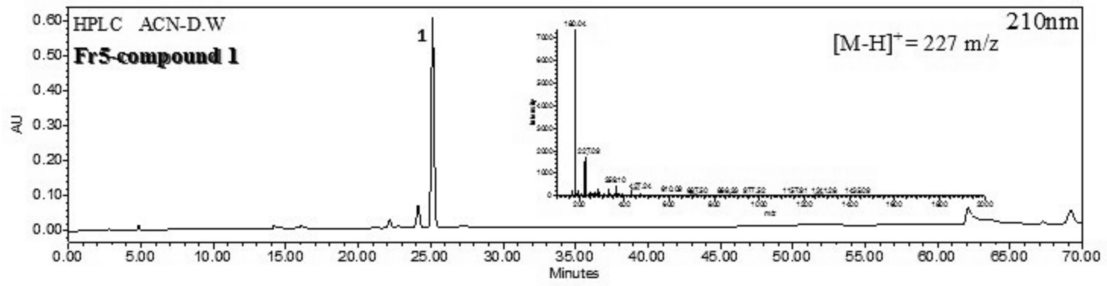
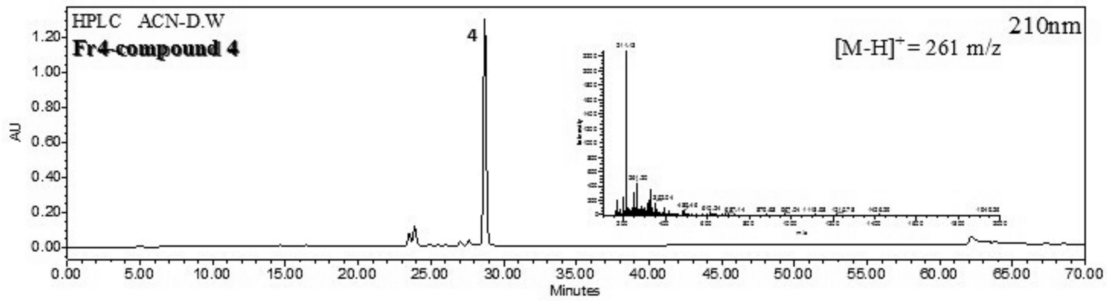
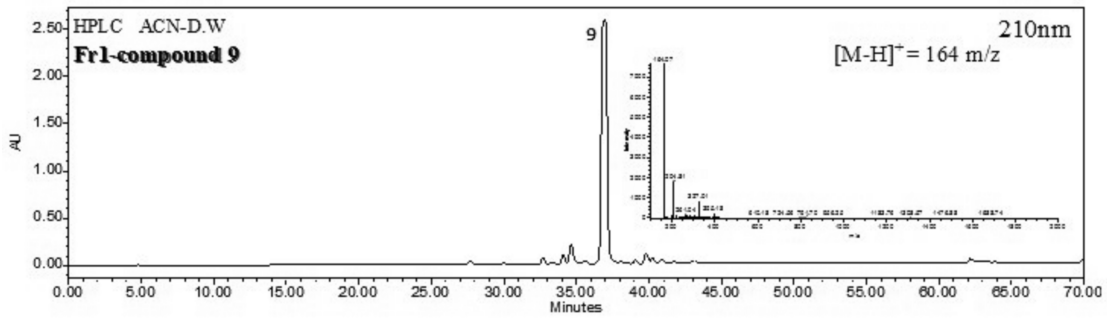


Fig. 2-4. Flow chart illustrating the extraction and purification of bioactive metabolites from *Streptomyces griseus*.







HPLC condition：0-50min 5-50% ACN, 50-60min 50-100% ACN, 60-70min 100% ACN

Fig. 2-5. HPLC and MS analysis of compound1, 4 and 9 from the EtOAc extract of *S. griseus.*

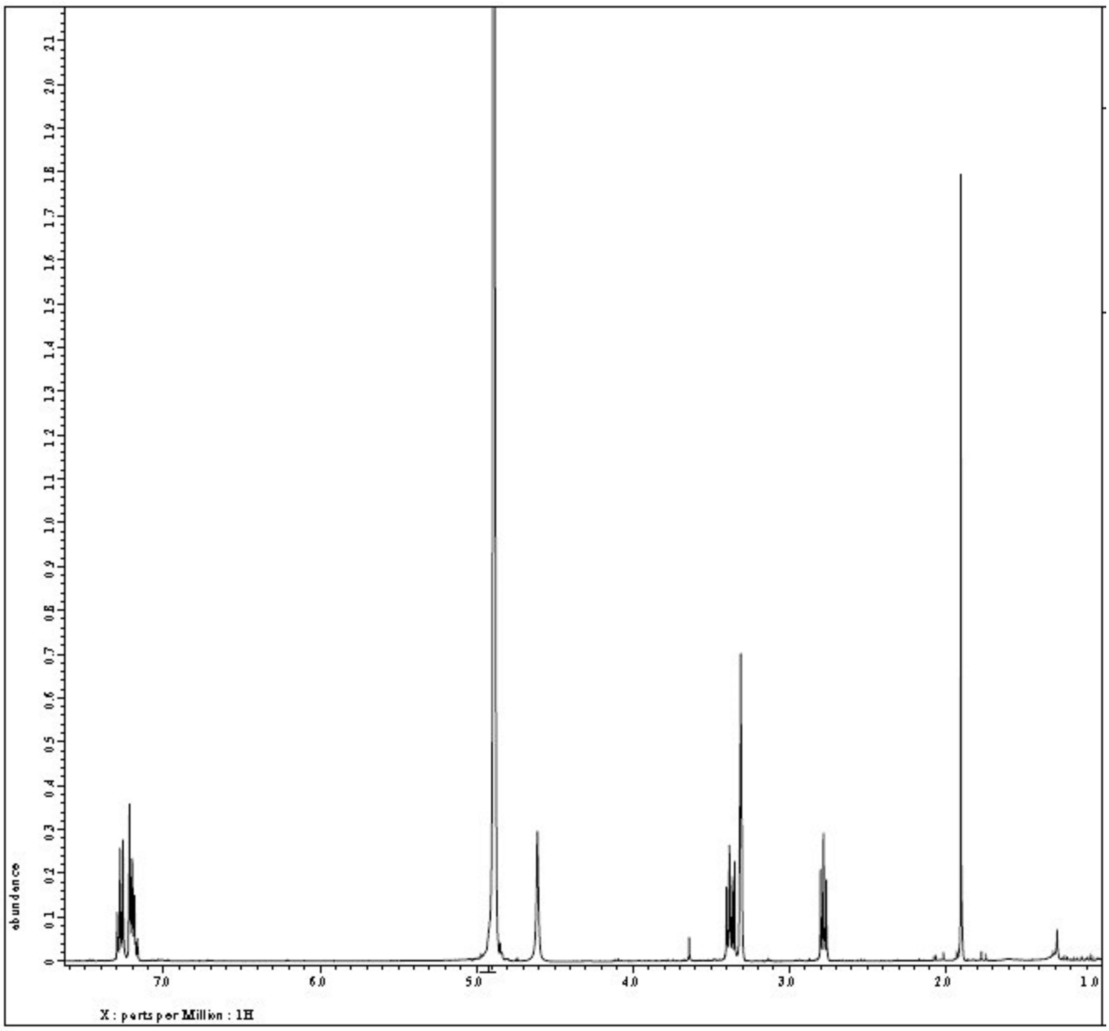


Fig. 2-6. ^{1}H-NMR spectrum (MeOH-d_3, 400 MHz) of compound 9 from the EtOAc extract of *S. griseus.*

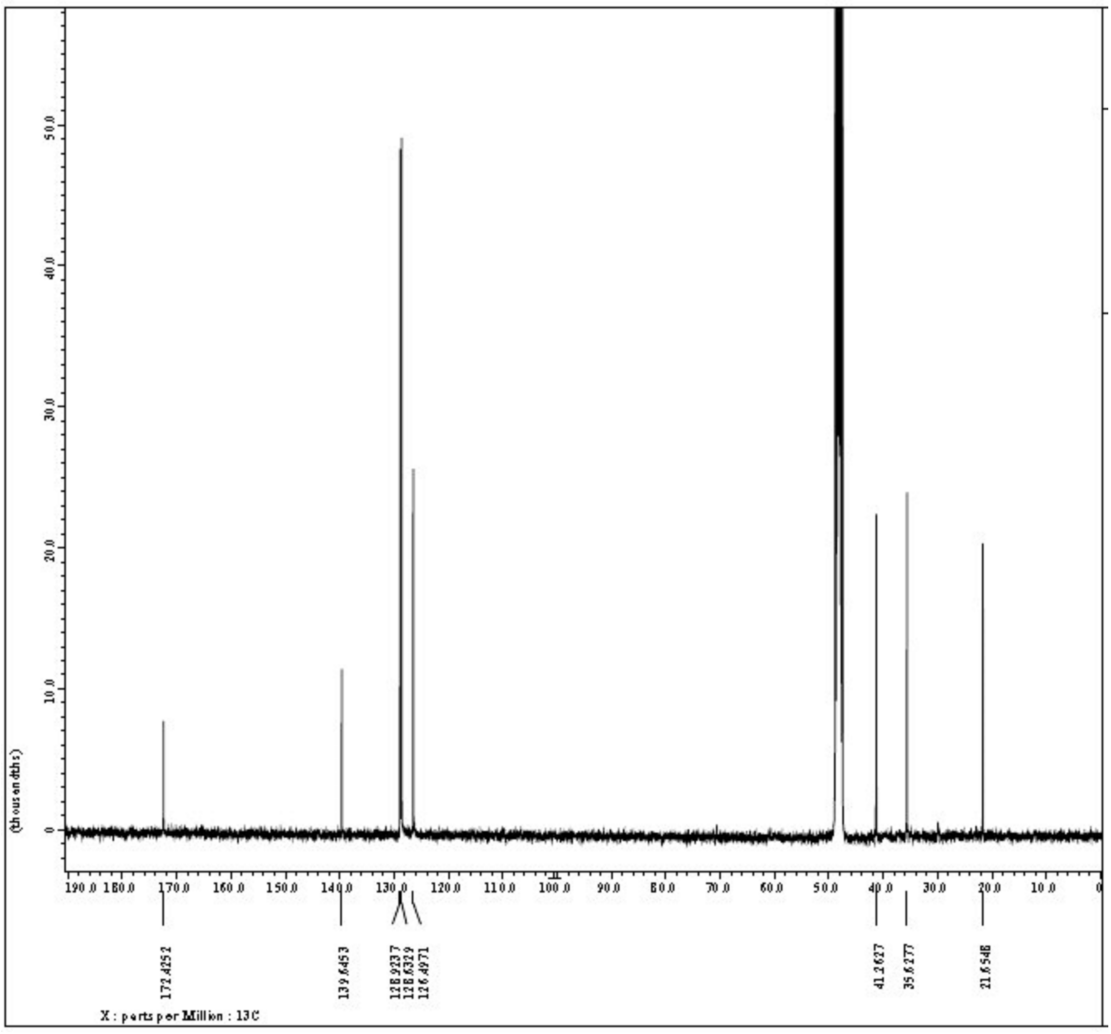


Fig. 2-7. ^{13}C-NMR spectrum (MeOH-d_3, 100 MHz) of compound 9 from the EtOAc extract of *S. griseus.*

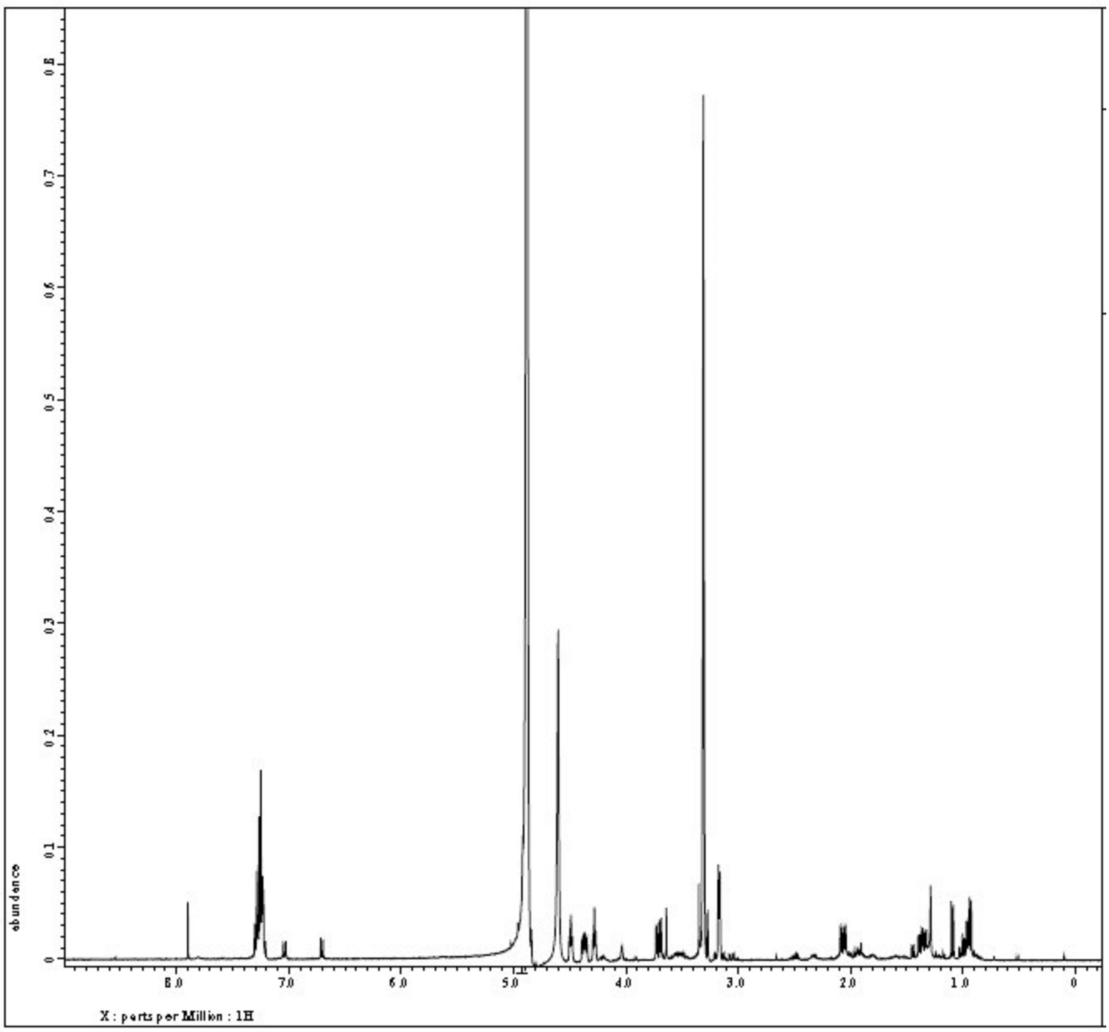


Fig. 2-8. ^{1}H-NMR spectrum (MeOH-d_3, 400 MHz) of compound 4 from the EtOAc extract of *S. griseus.*

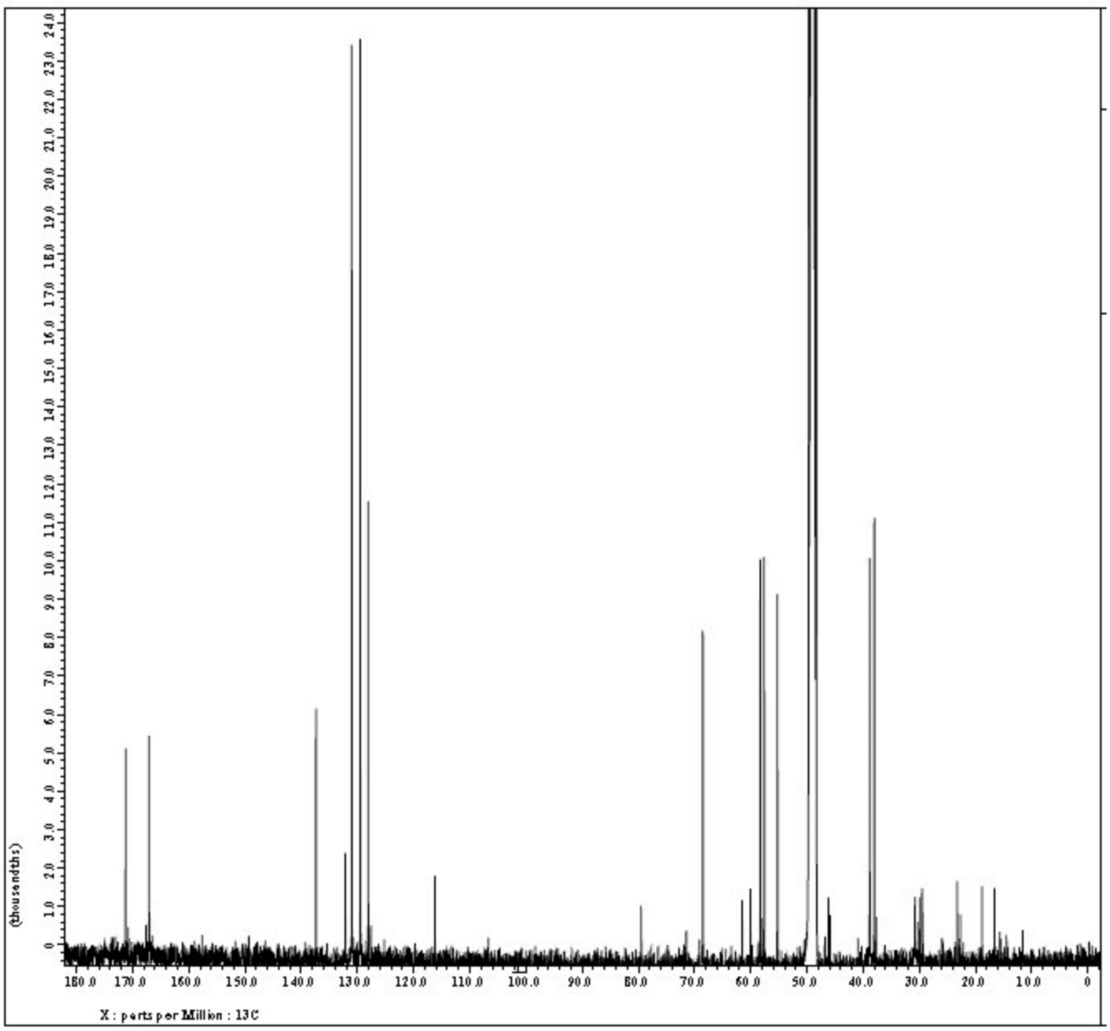


Fig. 2-9. ^{13}C-NMR spectrum (MeOH-d_3, 100 MHz) of compound 4 from the EtOAc extract of *S. griseus.*

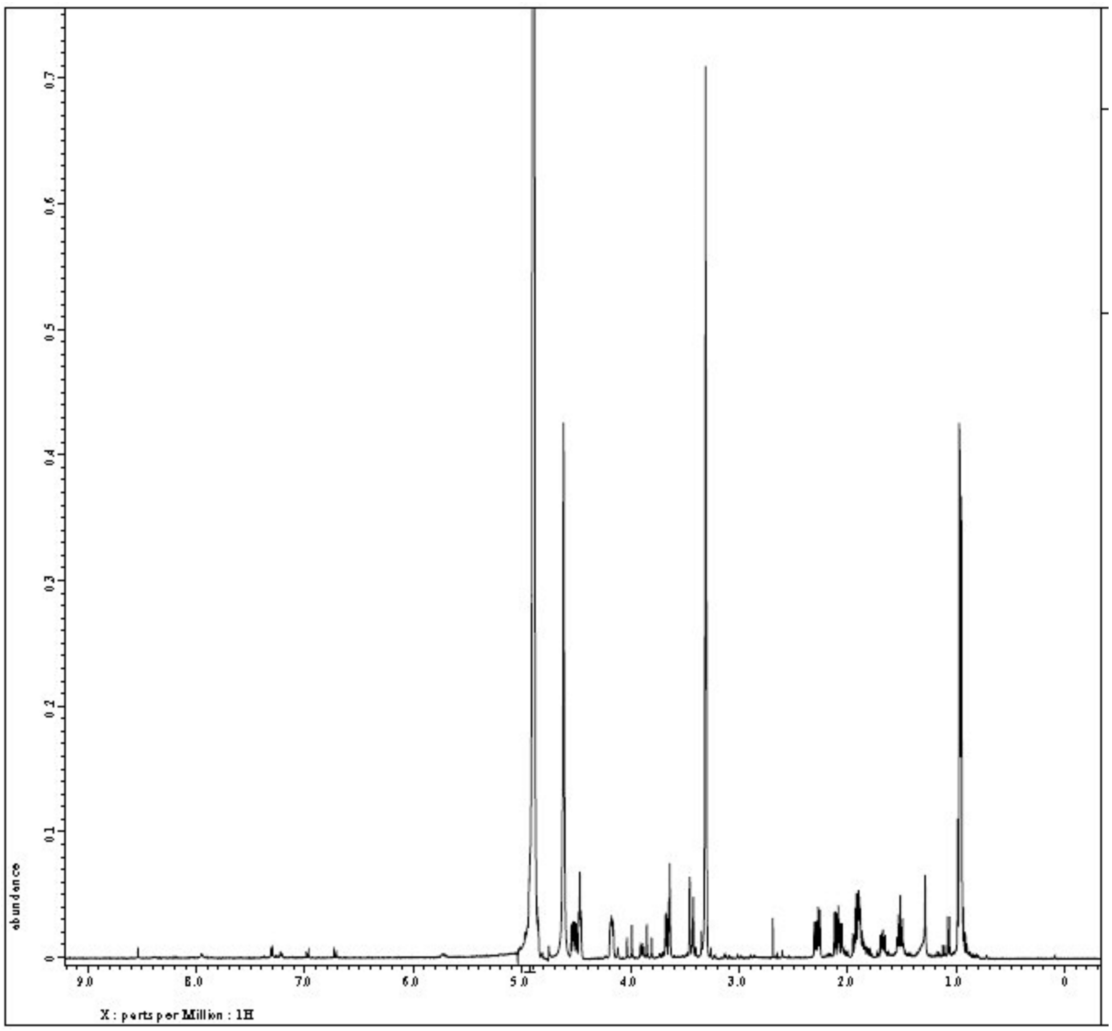


Fig. 2-10. ^{1}H-NMR spectrum (MeOH-d_3, 400 MHz) of compound 1 from the EtOAc extract of *S. griseus.*

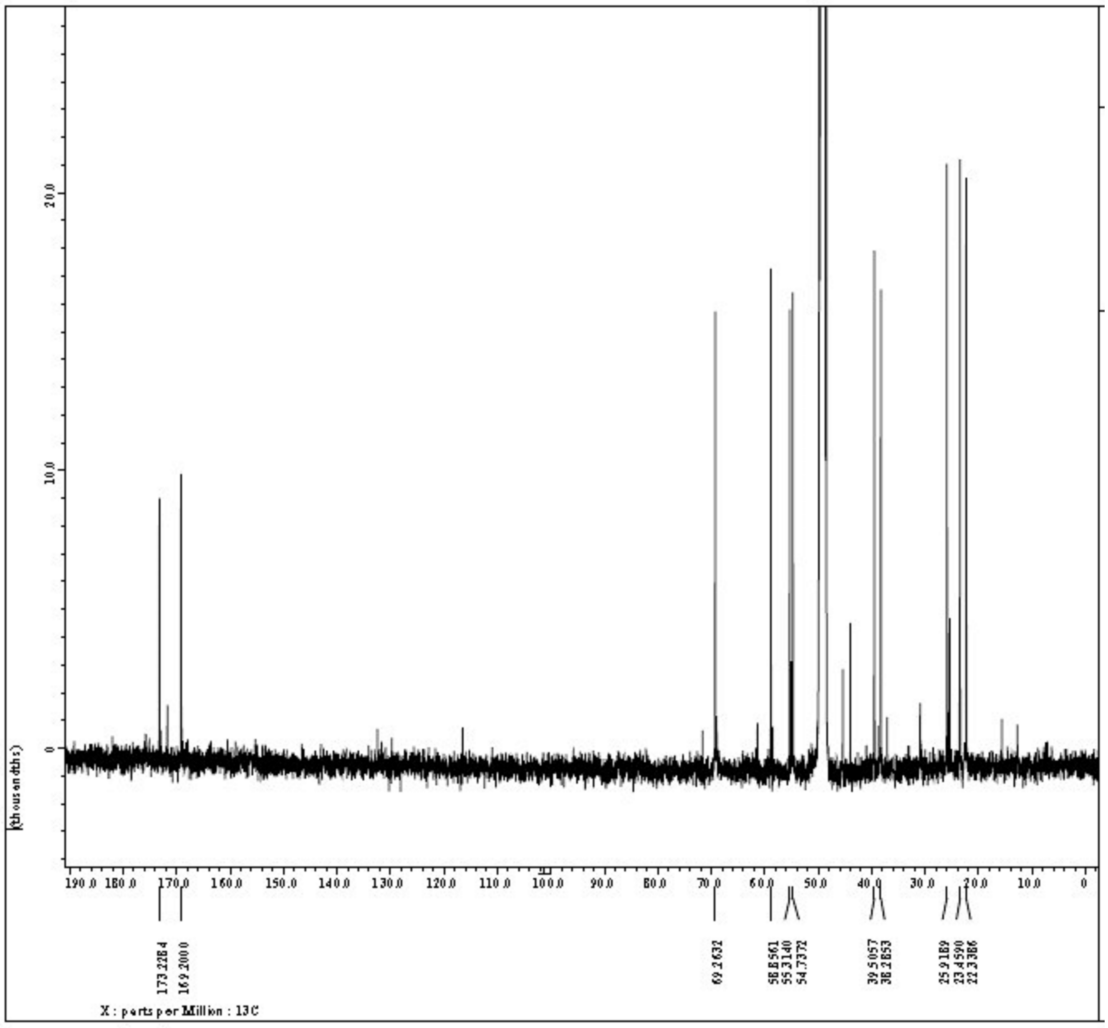


Fig. 2-11. ^{13}C-NMR spectrum (MeOH-d_3, 100 MHz) of compound 1 from the EtOAc extract of *S. griseus.*

3.6. Inhibitory effects of three pure compounds on the growth of HL-60 cell lines

As shown in **Fig. 2-12**, compound 9 (phenethyl acetate) evidenced the highest level of inhibition on the proliferation of HL-60 cells as compared to the other compounds in a dose-dependent increment, with an inhibition rate of 93%, 66.6% and 35.4% at 200, 100 and 50 μg/mL, respectively. In addition, there was no cytotoxicity effect of phenethyl acetate on normal cell lines (Vero cells) at the tested concentration (**Fig. 2-13**). Therefore, phenethyl acetate was selected as our target compound for use in further experiments.

3.7. Induction of apoptosis by phenethyl acetate in HL-60 cells

Because the HL-60 cell line was highly sensitive to the antiproliferation effect of phenethyl acetate, we examined whether phenethyl acetate could interfere with cell cycles using flow cytometry. In a given cell, the amount of bound dye correlates with the DNA content; thus DNA fragmentation in apoptotic cells translates into fluorescence intensity. Accordingly, HL-60 cells were incubated with phenethyl acetate (0.15, 0.3 and 0.6 μM) for 48 h. Taken together, the increased proportions of cells in the G_0/G_1 phase confirmed that phenethyl acetate induced apoptosis in HL-60 cells, resulting in DNA degradation. The percentage of cells at the G_2/M phase decreased, which corresponded to an increase in the percentage of cells at the G_0/G_1 phase, indicating an arrest of the cell cycle at the G_0/G_1 phase by phenethyl acetate (**Fig. 2-14**). Induction of cell death and apoptosis by phenethyl acetate was further studied by Hoechst-PI double staining assay. Apoptotic cell death was confirmed

by apoptotic body and nuclear condensation as detected by Hoechst 33342 staining assay. Co-staining of the cells with PI allows the discrimination of dead cells from apoptotic cells. The control, without phenethyl acetate exposure, exhibited no DNA damage. However, obvious cell damage was noted in the phenethyl acetate-treated cells. Cells treated with phenethyl acetate at different concentrations showed dramatically increased numbers of apoptotic bodies (**Fig. 2-15**). Thus, there is a good correlation between the extent of apoptosis and the inhibition of cell growth, suggesting that HL-60 cells may undergo apoptosis after phenethyl acetate pretreatment.

3.8. ROS generation triggers phenethyl acetate-induced apoptosis

It has been previously noted that ROS generation performs an important function in the pro-apoptotic activities of a variety of anti-cancer agents [130,131]. Therefore, we tested the possibility that phenethyl acetate induces apoptosis, allowing for ROS accumulation in HL-60 cells. DCFH-DA (for H_2O_2) was used to measure ROS production in the cells. As is shown in **Fig. 2-16**, phenethyl acetate treatment increased H_2O_2 production, as we observed an increase in fluorescence intensity (FI) from 85 in the vehicle-treated HL-60 cells to 1100 in the phenethyl acetate (0.6 μM)-treated HL-60 cells, respectively. Phenethyl acetate treatment induced a dose-dependent increase in DCF fluorescence as compared to what was observed in control cells. This result showed that ROS generation performs an important function in the phenethyl acetate-induced growth inhibition of HL-60 cells.

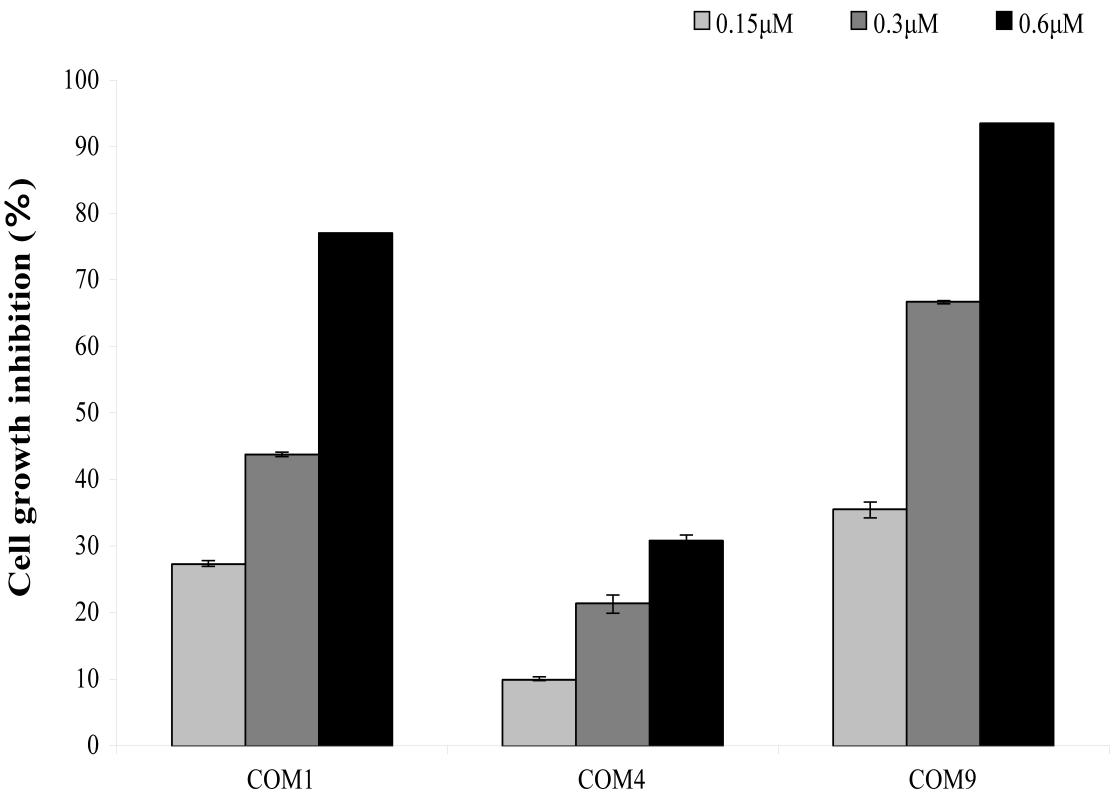


Fig. 2-12. Inhibitory effects of three compounds from *S. griseus* against growth of HL-60 cells. HL-60 cells were incubated with various concentrations of three compounds for 48 h and the cell viability was examined by an MTT assay. Each value indicates that the mean ± standard error from three independent experiments.

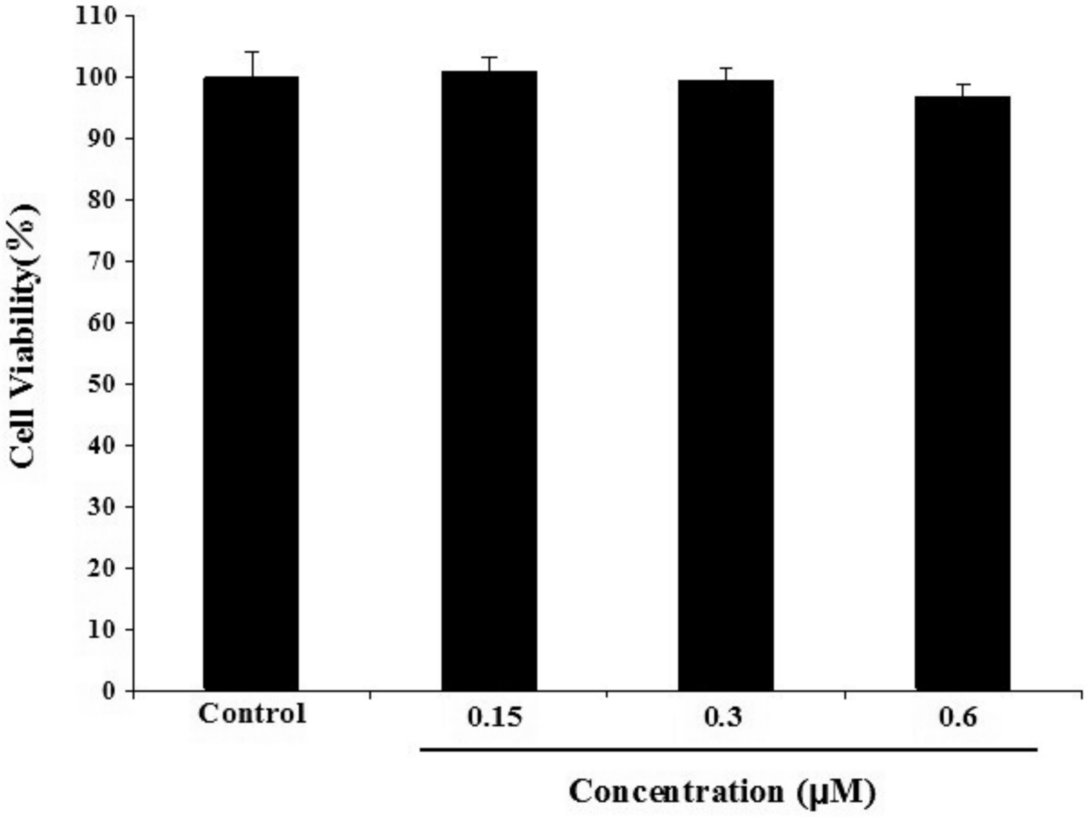


Fig. 2-13. Cell viability of phenethyl acetate with various concentrations in normal (Vero cells) cells was examined by an MTT assay. Each value indicates that the mean ± standard error from three independent experiments.

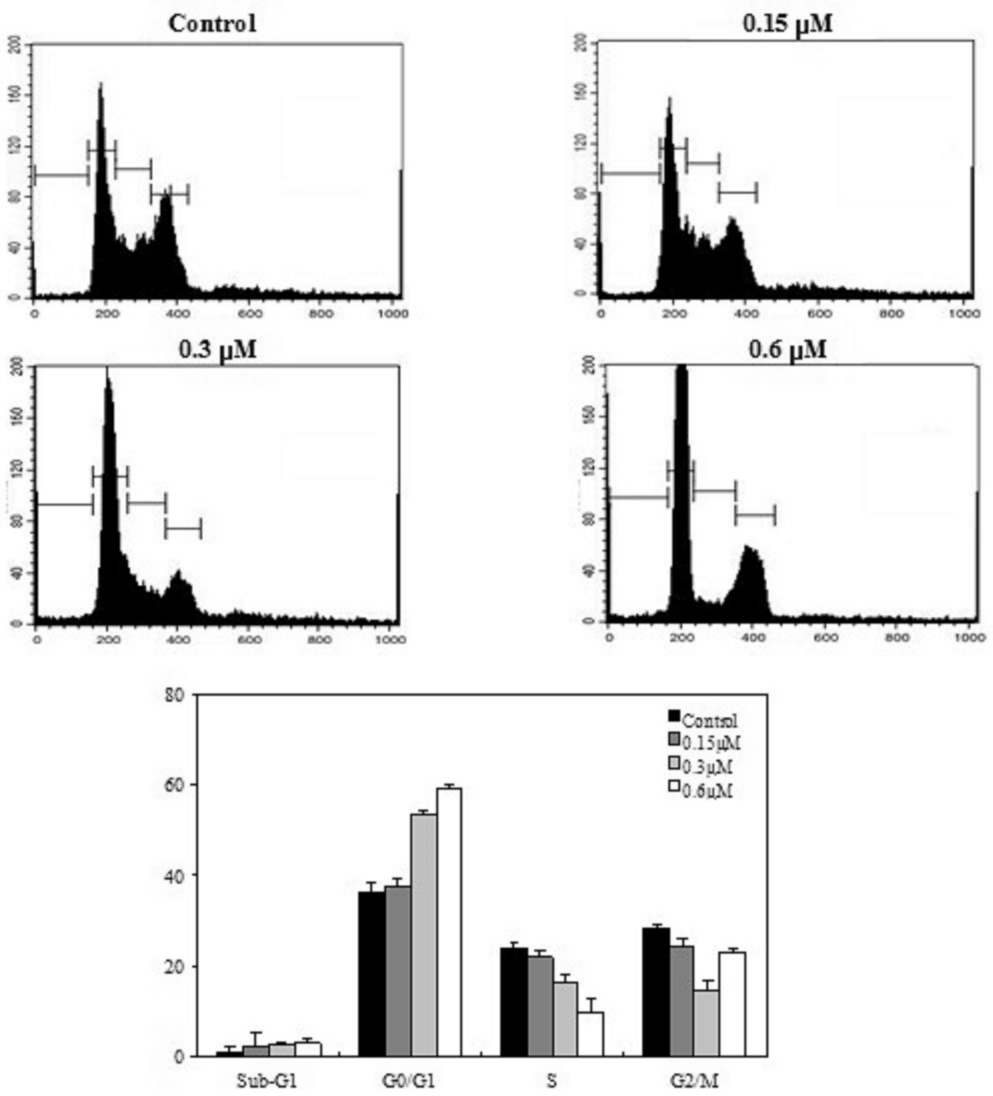


Fig. 2-14. Effects of phenethyl acetate on the cell cycle progression of HL-60 cells. HL-60 cells were exposed to various concentrations of phenethyl acetate (0.15-0.6 μM) for 48 h followed by cell-cycle distribution assay. Phenethyl acetate caused a significant increase in the level of cells in G_0/G_1 phase compared with control. All the data are presented as means ± SD and are the results of at least three individual experiments.

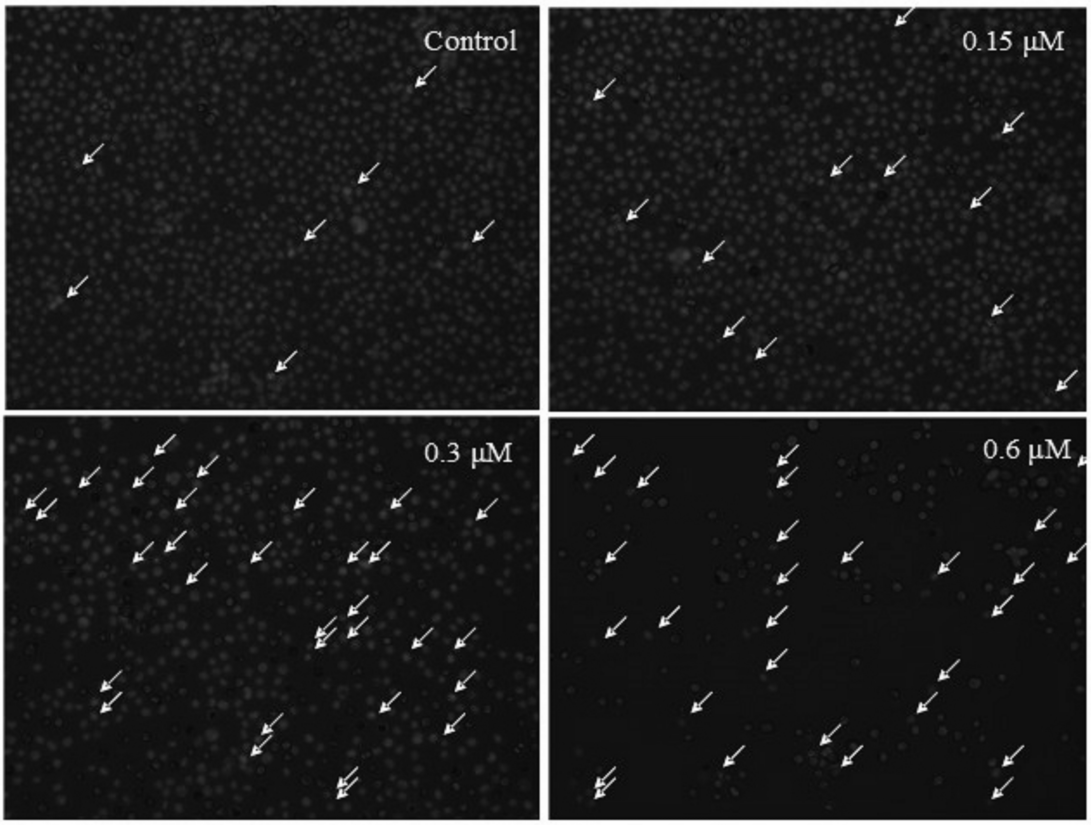


Fig. 2-15. Induction of apoptosis by phenethyl acetate treatment of HL-60 cells. HL-60 cells were seeded at 5×10^4 cells/mL and treated with different phenethyl acetate concentrations for 48 h. Apoptotic bodies were double stained with Hoechst 33342 and PI solution and then observed under a fluorescent microscope using a blue filter and red filter.

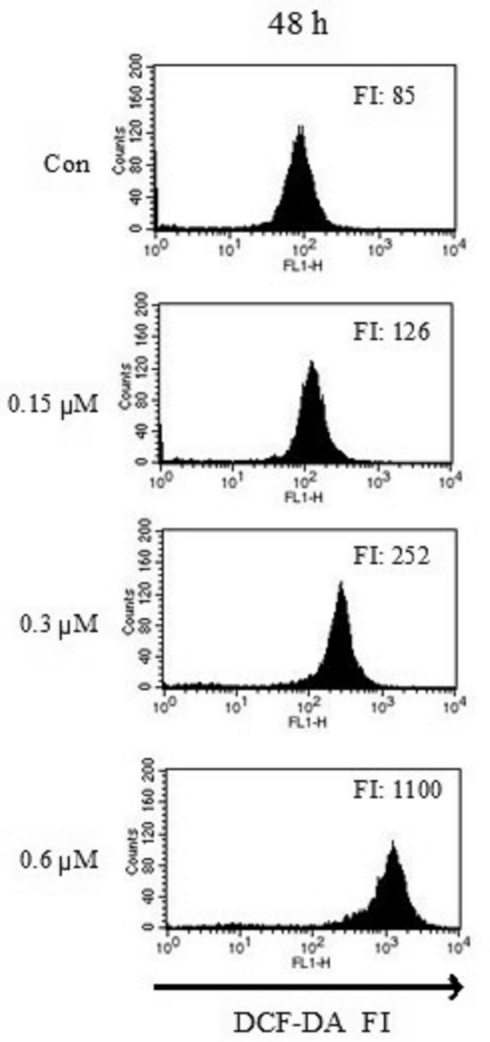


Fig. 2-16. Phenethyl acetate-induced ROS generation in HL-60 cells. HL-60 cells were seeded at 5×10^4 cells/mL and treated with different phenethyl acetate concentrations. After 48 h, the cells were labeled with 10 μM $DCFH_2$-DA (for H_20_2) for 30 min at 37 °C, and subjected to subsequent FACS analyses for intracellular ROS accumulation.

3.9. Phenethyl acetate-induced effects on the apoptosis-related protein levels in HL-60 cells

To study the mechanism of phenethyl acetate-induced apoptosis, the expression of Bax, Bcl-xL, caspase-3 and PARP were evaluated by Western blot analysis (**Fig. 2-17**). The expression level of Bcl-xL, an anti-apoptotic protein, decreased in a dose-dependent manner, following phenethyl acetate exposure. Conversely, the expression of Bax, a pro-apoptotic protein, was similarly increased following phenethyl acetate exposure. Furthermore, the expression of the active form of caspase-3 was increased at a concentration of 0.6 μM; PARP was cleaved at the same concentration.

3.10. Toxicity of phenethyl acetate in zebrafish embryo

In order to determine the toxicity of phenethyl acetate, we monitored the survival rate and growth patterns of zebrafish embryos. The adopted endpoints experiment used to assess the toxicity of the compound included embryo mortality and heartbeat disturbances. The survival rates of zebrafish embryos treated with phenethyl acetate at different concentrations are shown in **Fig. 2-18**. The survival rates were observed are 100, 100 and 93.8% in embryos treated with phenethyl acetate at 0.15, 0.3 and 0.6 μM, respectively. Hence, phenethyl acetate was not associated with mortality in this experiments. When evaluating the morphological malformations, phenethyl acetate did not show conspicuous adverse effects, whereas the total edema rate of zebrafish embryos exposed to phenethyl acetate at the concentration of 0.6 μM

was 6.7%, which showed a little typical morphological defects, a curled tail and a dilated pericardial sac were observed, suggesting pericardial oedema (**Fig. 2-19**). In the heartbeat test, on the other hand, phenethyl acetate at concentrations of 0.3 and 0.6 μM generated a slight increase, whereas at the concentration of 0.15 μM did not generate any heartbeat rate disturbances as compared with the control (untreated phenethyl acetate, **Fig. 2-20**). To evaluate whether phenethyl acetate-induced cell death in live zebrafish, cell death induced by phenethyl acetate treatment was measured via acridine orange as fluorescence intensity in the body of the zebrafish (**Fig. 2-21A**). The microscopic pictures in **Fig. 2-21B** show that the control zebrafish had intact cells, while phenethyl acetate at the highest dosage tested (0.6 μM) treatment caused a slight increase in the intensity of acridine orange. In simultaneous *in vivo* toxicity tests, toxicity was not detected in the fish treated with phenethyl acetate at 0.15 and 0.3 μM, whereas a slight toxicity was observed in the fish treated with phenethyl acetate at 0.6 μM.

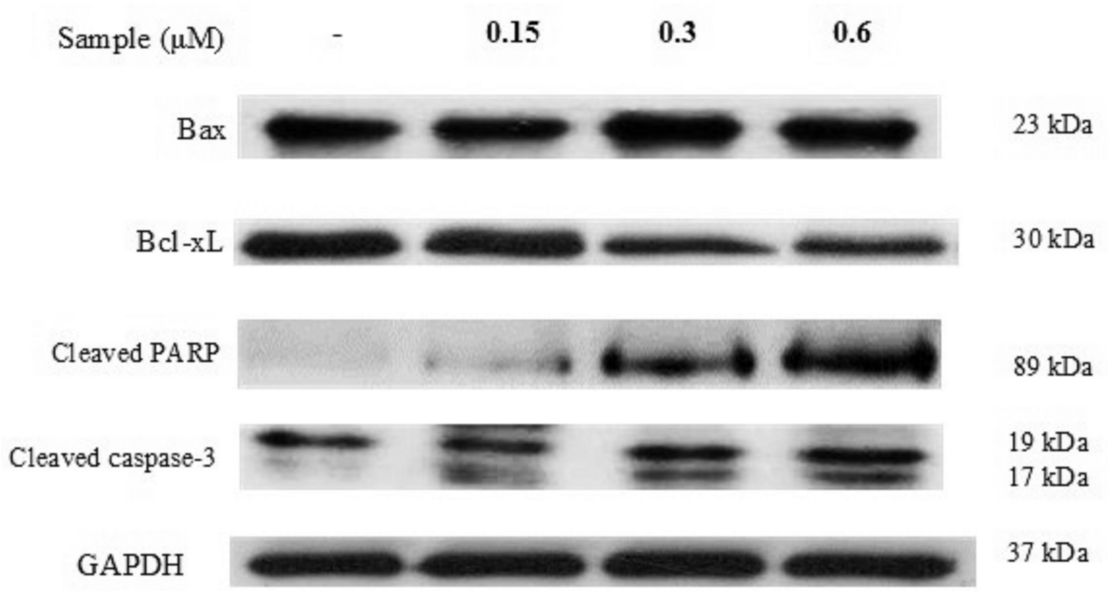


Fig. 2-17. Effect of phenethyl acetate on apoptosis-related proteins in HL-60 cells. Cells were treated with phenethyl acetate at the indicated concentration for 24 h. Whole cell lysates were subjected to Western blot analysis of anti-Bax, -Bcl-xL, -cleaved-caspase-3 and -PARP monoclonal antibodies. GAPDH was used as internal control.

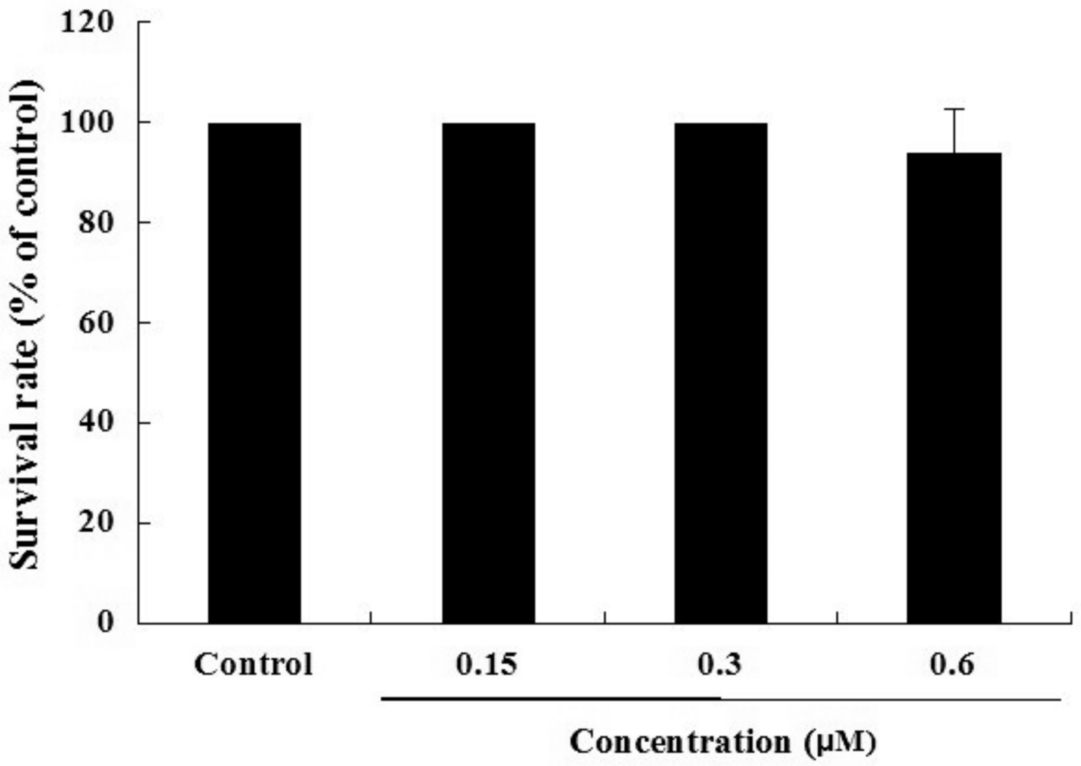


Fig. 2-18. Survival rate after treated with various concentrations of phenethyl acetate. Experiments were performed in triplicate.

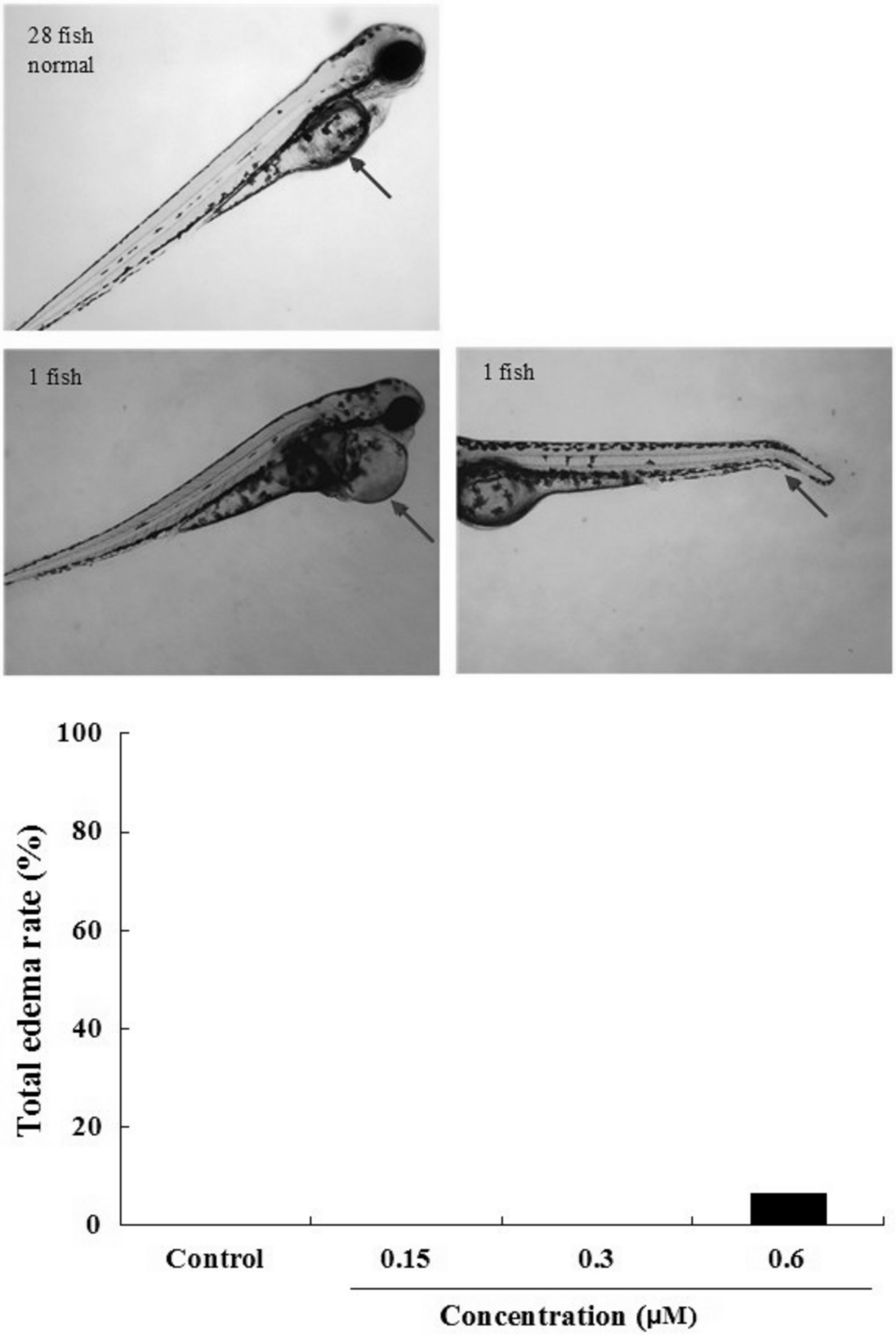


Fig. 2-19. The edema rate of zebrafish by phenethyl acetate. The embryos were treated with various concentrations of phenethyl acetate in zebrafish. Experiments were performed in triplicate.

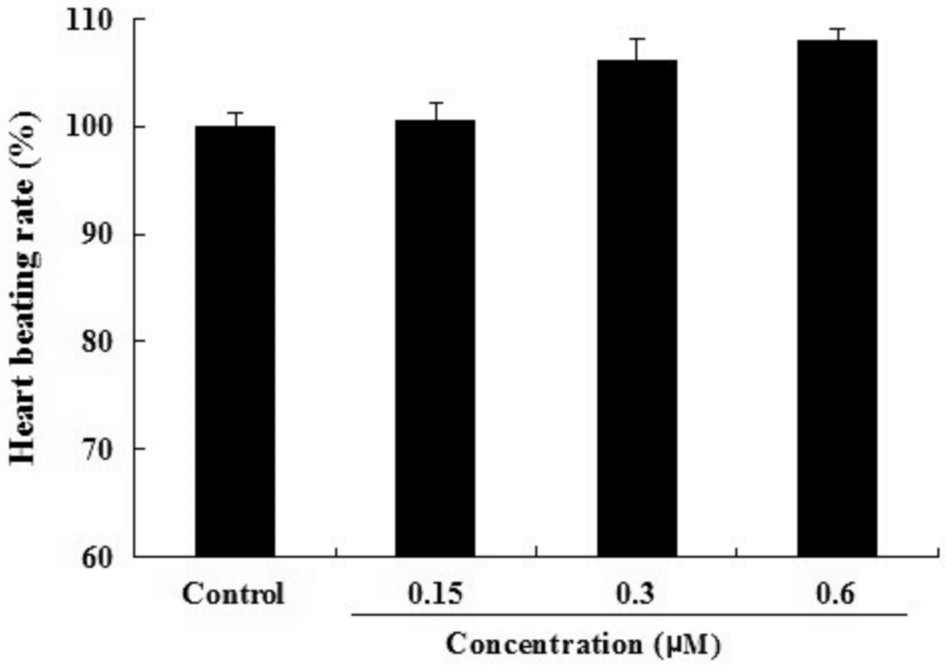


Fig. 2-20. Change of the heart-beat rate of zebrafish by phenethyl acetate. The embryos were treated with various concentrations of phenethyl acetate in zebrafish. Experiments were performed in triplicate.

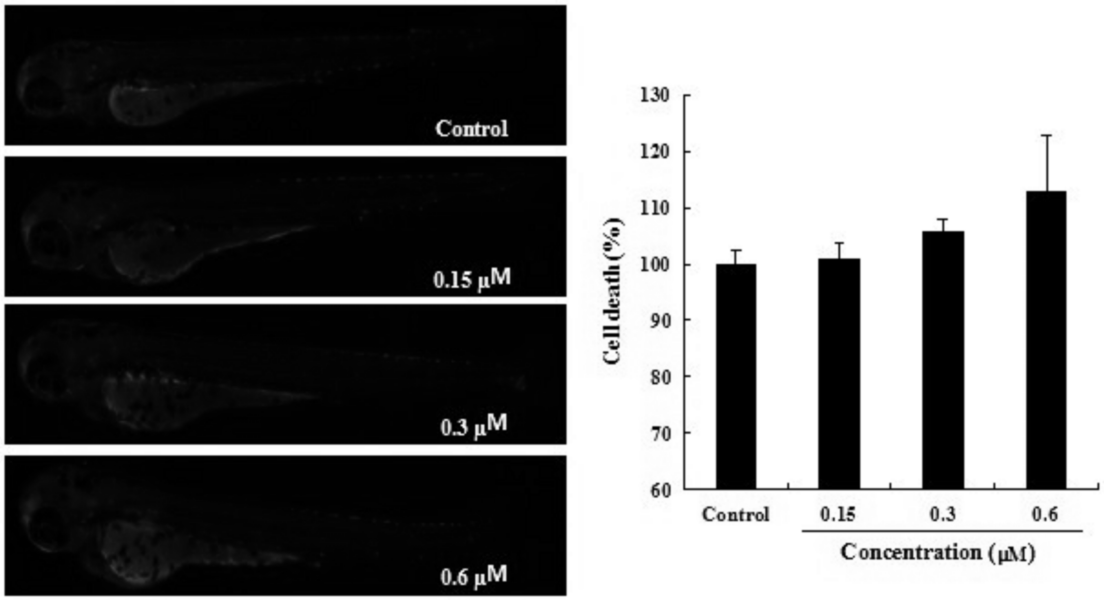
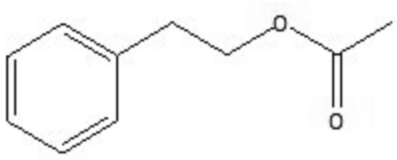
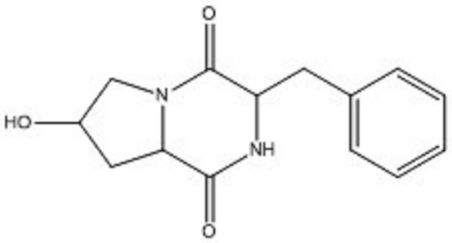


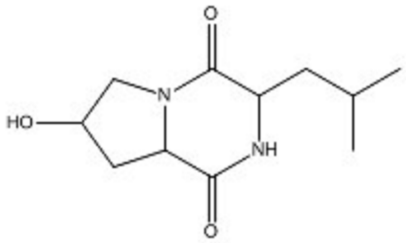
Fig. 2-21. Phenethyl acetate-induced cell death in live zebrafish embryos (**A**). The embryos were treated with various concentrations of phenethyl acetate. After incubation, the cell death was detected by fluorescence spectrophotometry after acridine orange staining. Photographs of phenethyl acetate-induced cell death in live zebrafish embryos (**B**). The cell death levels were measured by image analysis and fluorescence microscopy. Experiments were performed in triplicate and the data are expressed as mean ± SE.



Name: Phenethyl acetate
Molecular formula: $C_{10}H_{12}O_2$
Molecular weight: 164



Chemical Formula: $C_{14}H_{16}N_2O_3$
Molecular Weight: 260.29
Compound 4
Cyclo(L-Phe-trans-4-hydroxy-L-Pro)



Chemical Formula: $C_{11}H_{18}N_2O_3$
Molecular Weight: 226.27
Compound 1
Cyclo[L-(4-hydroxyprolinyl)-L-leucine]

Fig. 2-22. Chemical structures of the three isolated compounds.

4. Discussion and conclusion

Apoptosis plays an important role in the maintenance of cellular homeostasis by regulating cell division and cell death [132]. Abnormally growing cancer cells are characterized by uncontrolled proliferation and reduced apoptosis. As a result, activation of apoptotic pathways is a key mechanism by which cytotoxic drugs kill cancer cells [133]. To the best of our knowledge, this paper is the first to report that phenethyl acetate-induced apoptosis in HL-60 cells.

Previous studies have demonstrated that phenethyl acetate is a clear, colorless to pale yellow liquid with a floral odor of considerable tenacity [134]. Phenethyl acetate has been reported to occur in nature, with highest quantities observed in evergreen trees (cinnamomum species) and cloves (eugenia caryophyllata thunberg) [135]. In this study, phenethyl acetate isolated from marine bacteria is being reported for the first time. Rumy-antsev et al., 1987 reported that the oral LD_{50} of phenethyl acetate in rats was 5.2 g/kg, but in our study, we investigation of phenethyl acetate that inhibits proliferation and mediates apoptosis of HL-60 cells in low concentration (50-200 μg/mL). The results of this study demonstrated that phenethyl acetate exerts cytotoxic effects on HL-60 cells, and then demonstrated that phenethyl acetate could induce apoptosis in HL-60 cells in a dose-dependent fashion.

Cell-cycle arrest in cancer cells has become a major indicator of anticancer effects. Accordingly, cell-cycle analysis was carried out on the HL-60 cells. Anticancer agents may alter the regulation of the cell-cycle machinery, resulting in an arrest of cells in various phases of the cell cycle, thereby reducing the growth and proliferation of cancerous cells. In this

study, we found that phenethyl acetate exerted a strong antitumor activity via the G_0/G_1 arrest of the cell cycle. This result indicates that phenethyl acetate suppresses the growth of HL-60 cells in association with cell-cycle arrest in the G_0/G_1 phase.

One major biochemical change in cancer cells after treatments with anti-cancer agents is the increase in ROS generation, which is frequently considered a cancer-promoting factor [136-138]. Previous studies have demonstrated that high ROS levels can induce cellular damage [139-142] and may play an important role in mediating apoptosis [143,144]. In this study, we determined that ROS are generated during phenethyl acetate-induced apoptosis in HL-60 cells. We focused on intracellular H_2O_2 generation using FACS, as well as for further clarification of the relationship between ROS generation and apoptotic body formation, when HL-60 cells were treated with phenethyl acetate. our results showed that phenethyl acetate-mediated apoptosis is associated with ROS generation.

The Bcl-2 protein family is a critical regulator of apoptotic pathways. Many types of cancer, including leukemia, are associated with the overproduction of the Bcl-2 protein [145]. The anti-apoptotic Bcl-2 and Bcl-xL proteins reside on the outer membranes of the mitochondria and can inhibit apoptosis in the presence of various apoptotic stimuli, thereby promoting cell survival [146]. Bax is a pro-apoptotic member of the Bcl-2 family that resides in the cytosol and translocates to the mitochondria upon the induction of apoptosis [147]. The present data indicate that phenethyl acetate-induced apoptosis is associated with augmented levels of the Bax protein and downregulation of Bcl-2 expression. Other reports have also suggested that the Bax/Bcl-2 ratio is a key factor in regulating the apoptotic process [148,149]. An analysis of the present data indicates that phenethyl acetate may increase the Bax/Bcl-2

ratio, and this may lead to the induction of mitochondrial dysfunction and subsequent apoptosis of HL-60 cells. In addition, the Western blot experiments indicated that cleavage of caspase-3 and PARP appears to be correlated with phenethyl acetate-induced apoptosis in HL-60 cells. Caspase-3 is one of the key executioners of apoptosis, because it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as PARP [150]. PARP is important for cell viability, but its cleavage facilitates cellular disassembly and serves as a marker of cellular apoptosis [151,152]. Hence, these data suggest that phenethyl acetate-induced apoptosis likely occurs through the caspase-dependent pathway.

In conclusion, this study is, to the best of our knowledge, the first to demonstrate the biological mechanisms underlying the anti-cancer effects of the phenethyl acetate in HL-60 cells. our data reveal that HL-60 cells are highly sensitive to growth inhibition and apoptosis induction by phenethyl acetate. We further showed that phenethyl acetate-induced apoptosis of HL-60 cells was associated with the upregulation of Bax, downregulation of Bcl-xL, activation of caspase-3 and ROS generation. Hence, the results of this study indicate that the phenethyl acetate isolated from *S. griseus* might be a potential candidate for the development of anti-cancer drugs for use in the treatment of leukemia.

Part III.

Natural Products from the Marine-Derived Bacterium *Bacillus badius* and their Anti-inflammatory Activity

Part III.

Natural Products from the Marine-Derived Bacterium *Bacillus badius* and their Anti-inflammatory Activity

1. Abstract

In this study, potential anti-inflammatory effect of two compounds isolated from the marine-derived bacterium *Bacillus badius* was assessed via inhibitory effect of nitric oxide (NO) production in lipopolysaccharide (LPS) induced RAW 264.7 macrophage cells. The *Bacillus badius* was selected for further experiments due to its profound NO inhibitory effect, and was purified by preparative centrifugal partition chromatography to isolate two pure compounds. Then, we adopted active track method to select the compound showed higher level of inhibition of NO production and without cytotoxicity in LPS-induced RAW cells as our target compound. The target compound induced dose-dependent reductions in the levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins and concomitant reductions in the production of NO and PGE2. Additionally, the target compound was shown to suppress the production of inflammatory cytokine, IL-6. Hence, these results suggest that the use of the target compound may be a useful therapeutic approach for the various inflammatory diseases.

2. Materials and methods

2.1.1 General materials

All solvents used for preparation of crude sample and CPC separation were in analytical grade (Daejung Chemicals &Metals Co., Seoul, Korea). HPLC grade solvents were purchased from Burdick & Jackson (MI, USA). Lipopolysaccharide (LPS) was purchased from sigma chemical Co., Ltd (ST. Louis, MO). LDH cytotoxicity detection kit (Promega, Madison, WI, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin-strptomycin and trypsine-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, TNF-α and Prostaglandin E_2 (PGE_2) were purchased from R & D Systems Inc. (Minneapolis, MN, USA). Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amercham Biosciences (Piscataway, NJ, USA), respectively. All solvents used for preparation of crude sample and CPC separation were of analytical grade (Daejung Chemicals &Metals Co., Seoul, Korea). HPLC grade solvents were purchased from Burdick & Jackson (MI, USA).

2.1.2 Apparatus

LLB-M high performance CPC 240 (system instrumets co., LTD., Japan) was used in preparative CPC. The total cell volume is 240 mL. A four-way switching valve incorporated in the CPC apparatus allows operating in either the descending or the ascending mode. This CPC system was equipped with a FLO 214 dual pump (FLO, Japan), an S-3702 UV/Vis detector (Soma optics., LTD, Japan), and a Advantec CHF 122SC fraction collector (Toyo seisakusho kaisha LTD., Japan). The samples were manually injected through a ES injector (ES, Japan) with a 2 mL sample loop.

^{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 Methanol-d_4 solvent peak (3.31 ppm in ^{1}H and 49.15 ppm in ^{13}C NMR) as an internal reference standard. Mass spectra (FAB-MS and EIMS) were recorded on a JEOL JMS 700 spectrometer. The HPLC system in this experiment consisted of two 515 HPLC pump, In-Line Degasser AF, Pump control module II, 2998 Photodiode array detector and 2707 Autosampler (Water, USA).

2.2. Culture and Extraction procedure of *B. badius*

Based on the result of screening for the anti-inflammatory and antioxidant activities of the extracts from marine bacteria, the bacterial strain, *Bacillus badius* (075-1) has been selected as our target strain for the researches of bioactive natural products and their bioactivities.

The phylogenetic position of the isolated strain (075-1) was assessed by performing a nucleotide sequence database search using the BLAST program from NCBI GenBank. 2L Erlenmeyer flasks containing 2L of medium containing of peptone (0.7%), yeast extract (0.2%), D-(+)-Glucose (0.2%) and seawater (100%) were inoculated with a single colony from a well grown agar plate. The bacterial strain was cultured (18 L) for 20 days (static) at 29°C with humidity. The cultured broth of the strain *B. badius* was centrifuged (10000 rpm, 15 min) to remove the cells. Then the supernatants were extracted with equal volumes of ethyl acetate. After separation, the organic extracts were combined and concentrated in vacuo to dryness at 35°C yielding 0.49 g of a brown-colored solid extract was stored in a refrigerator for CPC separation.

2.3. Centrifugal partition chromatography (CPC) separation procedure

The CPC experiments were performed using a two-phase solvent system composed of n-hexane:EtOAc:MeOH:water (4:6:4:6, v/v). The two phases were separated after thoroughly equilibrating the mixture in a separating funnel at room temperature. The lower aqueous phase was used as the mobile phase, whereas the upper organic phase was employed as the stationary phase. The CPC column was initially filled in descending mode with the organic stationary phase and then rotated at 1000 rpm while the mobile phase was pumped into the column at the flow rate used for the separation (2 mL/min). When the mobile phase emerged from the column, indicating that hydrostatic equilibrium had been reached (back pressure : 2.0 MPa), The concentrated water fraction (490mg) of ethyl acetate extract from B. badius

was dissolved in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system phases was injected through the Rheodyne injection valve. The effluent from the CPC was monitored in the UV at 254 nm and fractions were collected with 6ml in 8ml tube by a Advantec CHF 122SC fraction collector (Toyo seisakusho kaisha LTD., Japan).

2.4. HPLC-DAD analyses

The HPLC system in this experiment consisted of a binary FLEXAR UHPLC pump, a FLEXAR PDA detector, a FLEXAR PDA auto sampler (PERKIN ELMER, USA). A 10μl of 5mg/ml sample solution was directly injected on Atlantis T3 3μm 3.0 × 150mm column (Waters, Ireland) using stepwise gradient mixtures of acetonitrile (solvent A) and distilled water (solvent B) as eluents at a flow rate of 0.2 ml/minute. The gradient was from 20% to 60% for solvent A in 50 minutes, from 60% to 100% for solvent A in 10 minutes with a 10-minute hold at 100% for solvent A. Multiple wavelength monitoring was performed at 210, 254, 280 and 365 nm and photodiode array detector measured from 200 to 400 nm.

2.5. HPLC-DAD-ESI/MS analysis of purified compounds

HPLC-DAD-ESI/MS analyses were carried out using a Hewlett-Packard 1100 series HPLC system equipped with an autosampler, a column oven, a binary pump, a DAD detector, and a degasser (Hewlett-Packard, Waldbronn, Germany) coupled to a Finnigan MAT LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a Finnigan

electrospray source and capable of analyzing ions up to m/z 2000. Xcalibur software (Finnigan MAT) was used for the operation. The chromatographic conditions are identical to those described in Section 2.4 and the outlet of the flow cell was connected to a splitting valve, from which a flow of 0.2 mL/min was diverted to the electrospray ion source via a short length of fused silica tubing. Negative ion mass spectra of the column eluate were recorded in the range m/z 100-2000. The source voltage was set to 4.5 kV and the capillary temperature to 250°C. The other conditions were as follows: capillary voltage, -36.5 V; inter-octapole lens voltage, 10 V; sheath gas, 80 psi (551.6 kPa); auxiliary gas, 20 psi (137.9 kPa).

2.6. ^{1}H-NMR and ^{13}C-NMR analysis of purified compounds

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 Methanol-d_4 solvent peak (3.31 ppm in ^{1}H and 49.15 ppm in ^{13}C NMR) as an internal reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer.

2.7. On-line HPLC-ABTS^{+} assay

HPLC coupled with ABTS assay was performed by using the method developed by Koleva *et al*. (2001) [153] with some modifications. A stock solution containing 3.5 mM potassium persulphate and 2 mM ABTS was prepared and kept at room temperature in darkness for 12 h in order to stabilize the radical. The radical reagent was prepared by diluting

the stock solution with pure water to an absorbance of 0.70 ± 0.02 at 680 nm. The extracts (10 μL) were injected into an Waters HPLC system. HPLC separation was carried out as described in the previous section. HPLC eluates from the column then arrived at a T-junction, where the ABTS reagent was added. The ABTS reagent flow rate was 0.7 mL/min delivered by a Waters Reagent Pump (Waters Corporation, USA). After the eluates mixed with ABTS reagent in a reaction coil (15 m x 0.25 mm i.d. PEEK tubing), the negative peaks were measured by UV spectrometer at 680 nm. Water was used as the control by replacing $ABTS^{+}$ in terms of above procedure. Data were analysed using Empower Software.

2.8. 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°C and were sub-cultured every 2 days.

2.9. Latic dehydrogenase (LDH) cytotoxicity assay

RAW 264.7 cells (1×10^5 cells/ml) plated in 24-well plates were pre-incubated and subsequently treated with LPS (1 μg/ml) coupled with the two isolated compounds at 37 °C for 24 h. The medium was carefully removed from each well, and the LDH activity in the medium was determined using an LDH cytotoxicity detection kit. Briefly, 100 μl of reaction

mixture were added to each well, and the reaction was incubated for 30 min at room temperature in darkness. The absorbance of each well was measured at 490 nm using a microplate reader (ThermoMax).

2.10. Determination of nitric oxide (NO) production

After pre-incubation of RAW 264.7 cells (1×10^5 cells/ml) with LPS (1 μg/ml) plus the two isolated compounds at 37 °C for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production [154]. Briefly, a 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader (ThermoMax, CA, USA). Fresh culture medium was used as a blank in every experiment.

2.11. Determination of PGE_2 production

The target compound was diluted with DMEM prior to treatment. Cells were treated for 24 h with LPS (1 μg/ml) to permit cytokine production. The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immunoassay kit (R&D Systems Inc., MN, USA) in accordance with the manufacturer's instructions. The production of PGE_2 was measured relative to that observed after control treatment.

2.12. Measurement of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) production

The target compound solubilized with DMSO was diluted with DMEM before treatment. The inhibitory effect of fucoxanthin on the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) production from LPS (1 μg/ml) treated RAW 264.7 cells was determined as described by Cho et al. (2000) [155]. Supernatants were used for pro-inflammatory cytokines assay using mouse ELISA kit.

2.13. Western blot analysis

Murine macrophage RAW 264.7 cells were pre-incubated for 24 h, then stimulated with LPS (1 μg/ml) in the presence of the target compound for the indicated times. After incubation, the cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer [50 mMTris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 25 μg/ml leupeptin] and maintained on ice for 30 min. The cell lysates were washed via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit. Aliquots of the lysates (30-50 μg of protein) were separated on 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% methanol (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin

(BSA), the membrane was incubated overnight with specific primary antibody at 4 °C. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

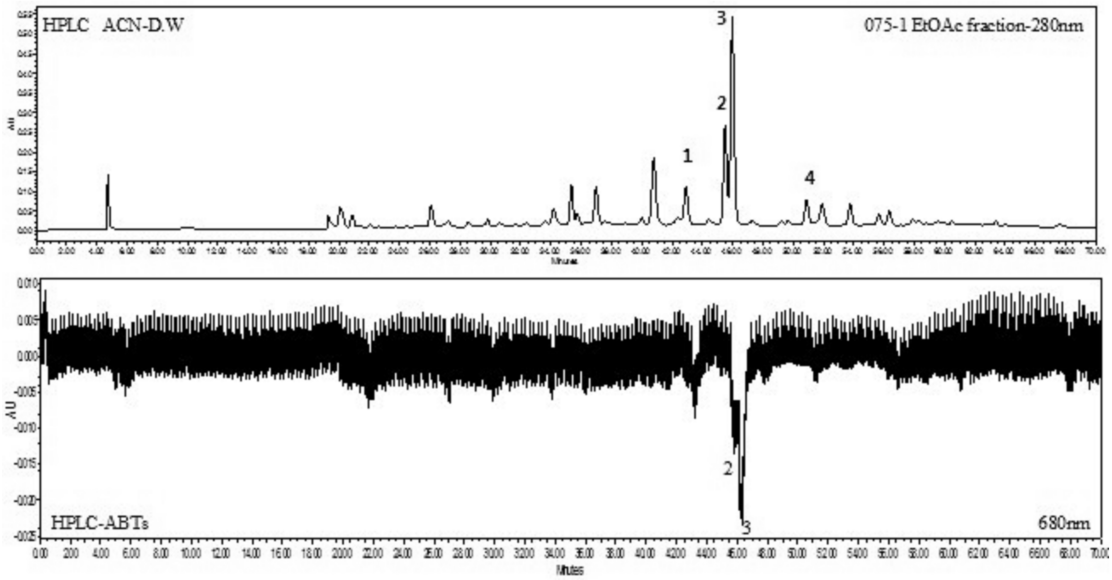
2.14. Statistical analysis

The Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data are expressed as means ± standard errors (SE) and the results are taken from at least three independent experiments performed in triplicate.

3. Results

3.1. HPLC and on-line HPLC-ABTS$^+$ analysis of EtOAc extract of *B. badius*

The EtOAc extract of *B. badius* was analyzed by the described on-line HPLC-ABTS$^+$ condition and chromatogram (**Fig. 3-1**). The determination of the antioxidant activity on the on-line HPLC was based on a decrease in absorbance of 680 nm after the postcolumn reaction of HPLC separated the antioxidants with ABTS$^+$. In the results, the HPLC peak 2 and 3 had the strongest antioxidant activities than the other peaks. Therefore, we separated and collected the focused active compound 2 and compound 3 using the preparative CPC.



HPLC condition：0-50min 0-100% ACN, 50-60min 100% ACN

Fig. 3-1. Online-HPLC analysis of EtOAc extract of *Bacillus badius.*

3.2. Optimization of the two-phase solvent system

Partition coefficient (*K*) for the selection of a suitable two-phase solvent system was the most important for the successful separation of the target samples by the preparative CPC. In order to choose the most efficient separation, several two-phase solvent ratios were applied with the different compositions and volume ratios of the two immiscible solvents such as *n*-hexane:EtOAc:MeOH:water (v/v). The *K* values were then calculated. The two-phase solvent system composed of 4:6:4:6 (*n*-hexane:EtOAc:MeOH:water, v/v) exhibited good *K* values to separate the antioxidative compounds confirmed by the on-line HPLC. The most efficient separation of each compound was performed under the solvent condition, *n*-hexane:EtOAc:MeOH:water (4:6:4:6, v/v) by preparative CPC.

3.3. Separation of antioxidative compounds by preparative CPC

The *K*-values of most target compounds exhibited lower numerical values than 1. Therefore, the preparative CPC was operated in the descending mode (upper phase: stationary phase and lower phase: mobile phase, respectively). The retention of the stationary phase in the coil retained 140 mL and the pressure exhibited 4.1 MPa during the operation. The preparative CPC chromatogram was described in **Fig. 3-2**. We confirmed that the compound 2 is included in fraction A, and fraction B contained the compound 3 according to the analysis by the HPLC peak area (Fig. 2(b)). The HPLC-DAD-ESI/MS and NMR data of the two isolated compounds were shown from **Fig. 3-3** to **Fig. 3-7**. The yields of compound 2 and

compound 3 isolated from 490 mg of the EtOAc extract by the one-step of CPC system were 6.8 mg and 16.7 mg, respectively. The entire flow chart showing extraction and purification of bioactive metabolites from the culture filtrate of *Bacillus badius*. is presented in **Fig. 3-8**.

3.4. Effects of the two isolated compounds on LPS-induced NO production and cytotoxicity

To evaluate the effect of the two isolated compounds on NO production, RAW 264.7 macrophages were stimulated with LPS (1 μg/ml) for 24 h to evoke NO, and the accumulation of its metabolite, nitrite, in the culture medium was measured. NO was produced by the treatment of LPS. Among these two isolated compounds, compound 2 showed higher level of inhibitory effect on NO production in a concentration-dependent manner: 27%, 42% and 66% at 12.5, 25, and 50 μg/ml (**Fig. 3-9**). The 50% inhibitory concentration (IC_{50}) of compound 2 and compound 3 were calculated to be 34.61 μg/ml and 60.03 μg/ml, respectively. To exclude the possibility that the inhibition of NO production was due to cytotoxicity caused by the isolated compounds treatment, LDH assays were investigated in RAW 264.7 macrophages treated with the two isolated compounds. As shown in **Fig. 3-9**, the two isolated compounds did not affect cell viability in macrophages at the employed concentrations (12.5, 25, and 50 μg/ml). Thus, the inhibitory effects of the two isolated compounds on LPS-induced NO production was not due to any cytotoxic action on RAW 264.7 macrophages. Hence, the compound 2 was selected as our target compound for further experiments.

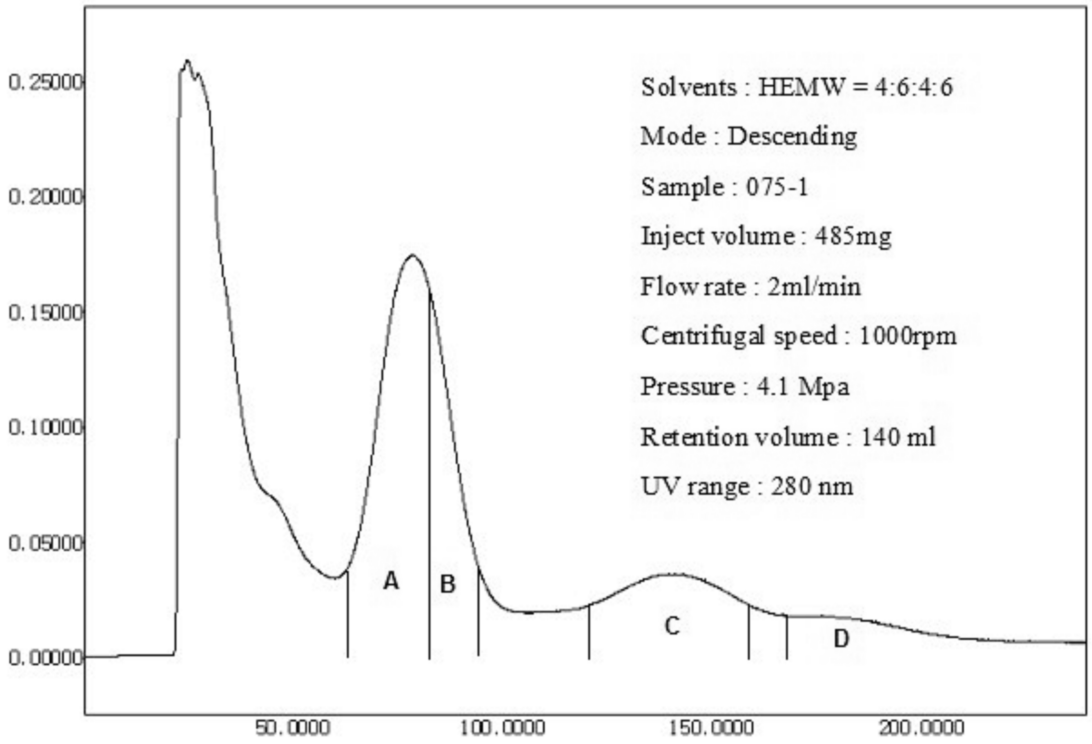
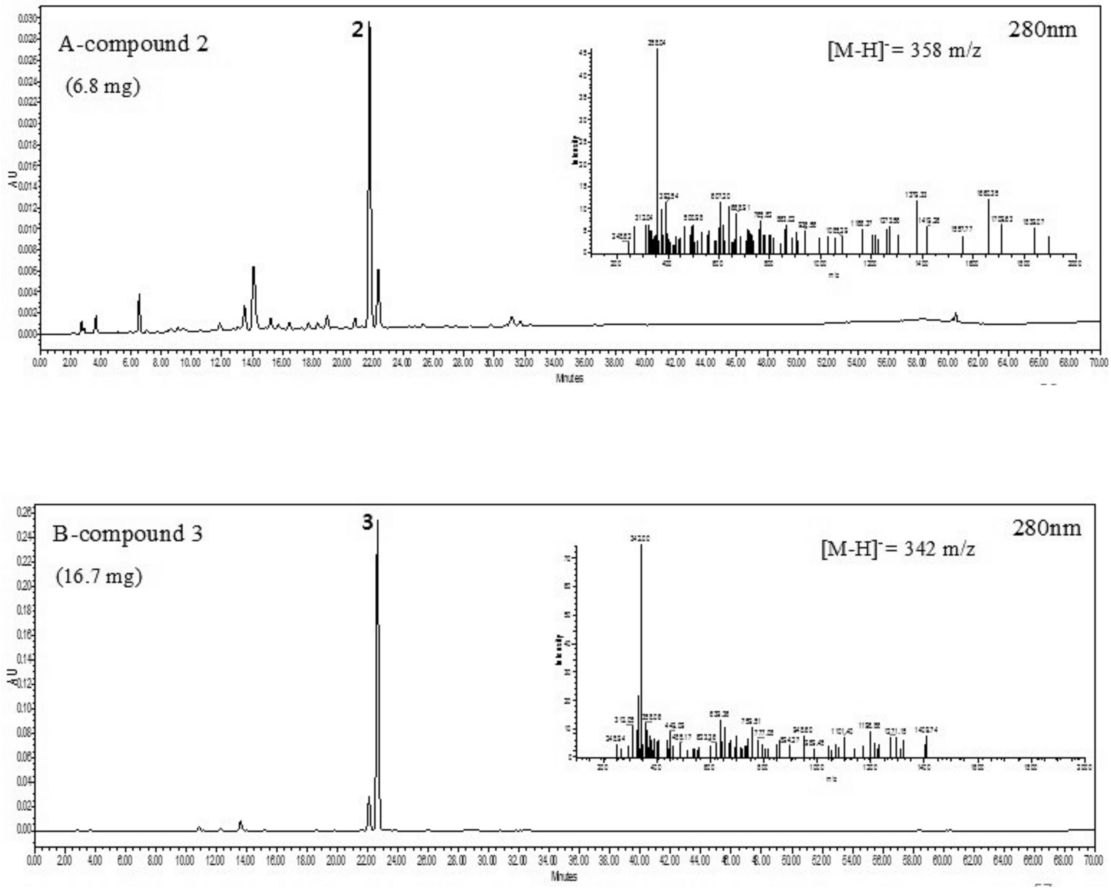


Fig. 3-2. Preparative CPC chromatogram of water fraction from culture filtrate extract of *B. badius.*



HPLC condition: 0-50min 20-60% ACN, 50-60min 60-100% ACN, 60-70min 100% ACN

Fig. 3-3. HPLC and MS analysis of compound 2 and 3 from the EtOAc extract of *B. badius.*

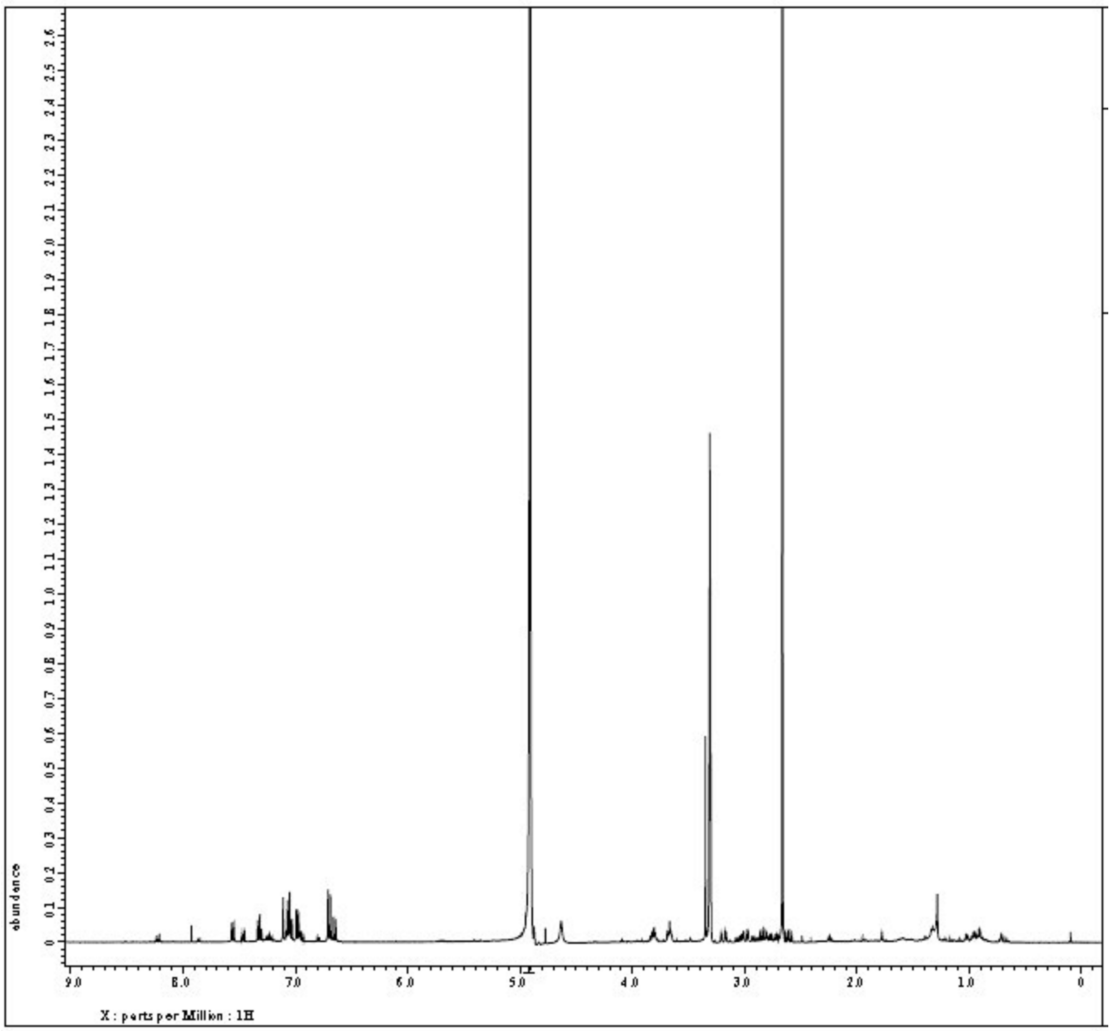


Fig. 3-4. ^{1}H-NMR spectrum (MeOH-d_3, 400 MHz) of compound 2 from the EtOAc extract of *B. badius.*

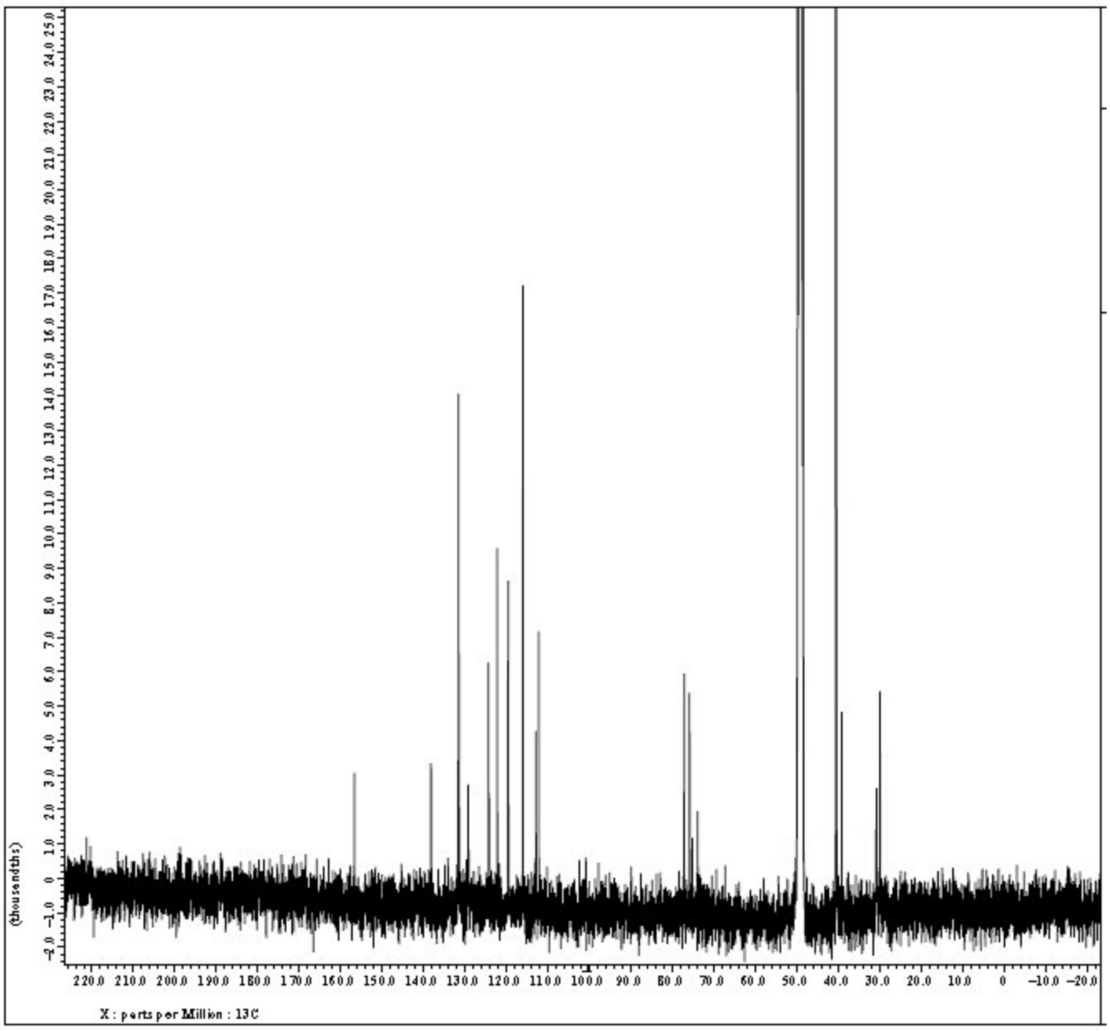


Fig. 3-5. ^{13}C-NMR spectrum (MeOH-d_3, 100 MHz) of compound 2 from the EtOAc extract of *B. badius.*

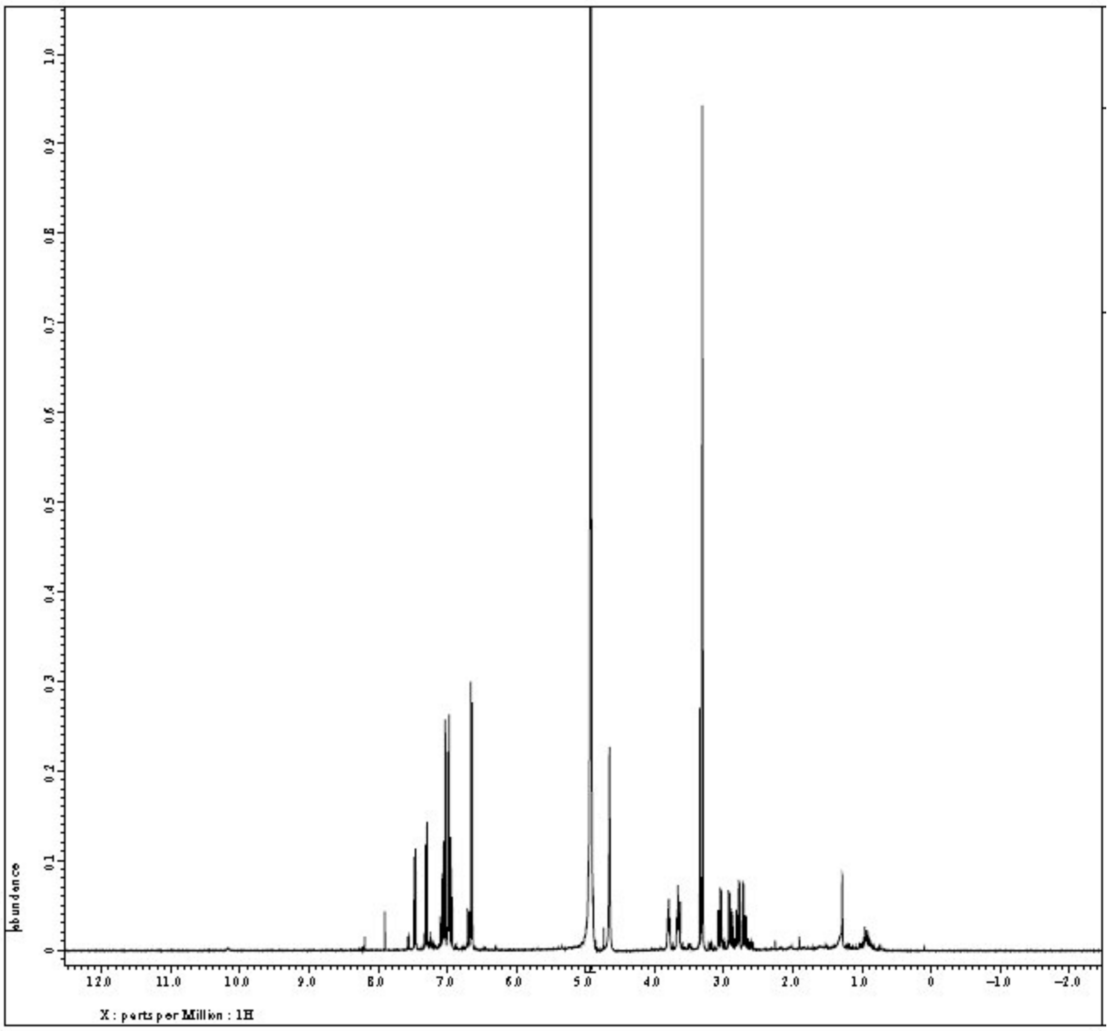


Fig. 3-6. ^{1}H-NMR spectrum (MeOH-d_3, 400 MHz) of compound 3 from the EtOAc extract of *B. badius.*

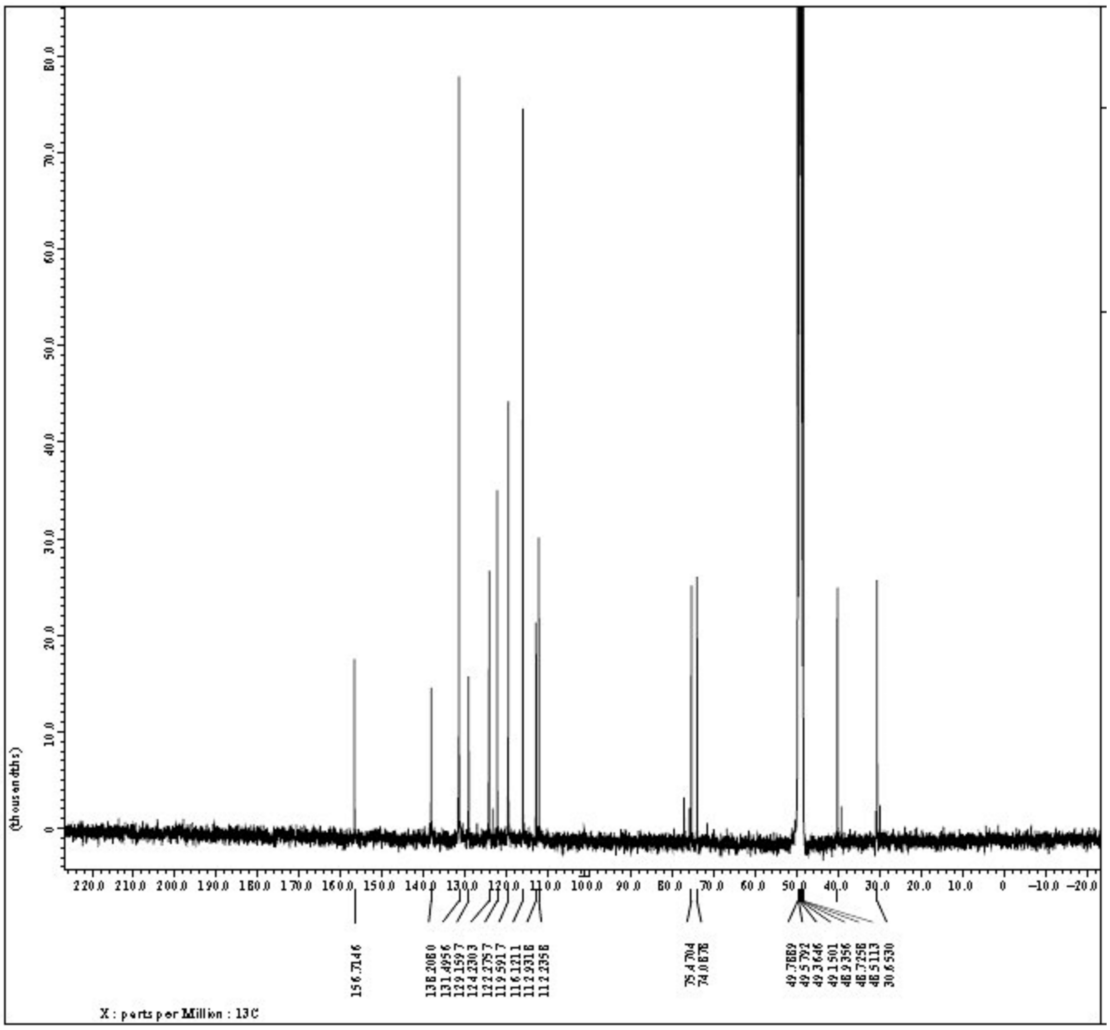


Fig. 3-7. ^{13}C-NMR spectrum (MeOH-d_3, 100 MHz) of compound 3 from the EtOAc extract of *B. badius.*

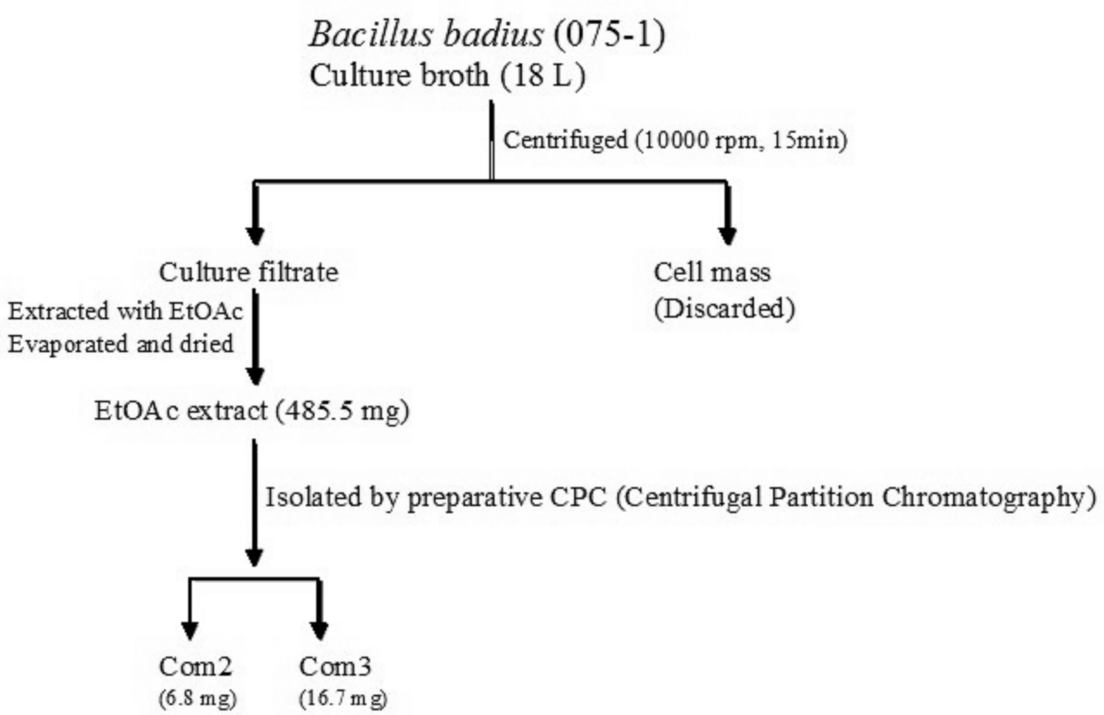


Fig. 3-8. Flow chart illustrating the extraction and purification of bioactive metabolites from *B. badius*.

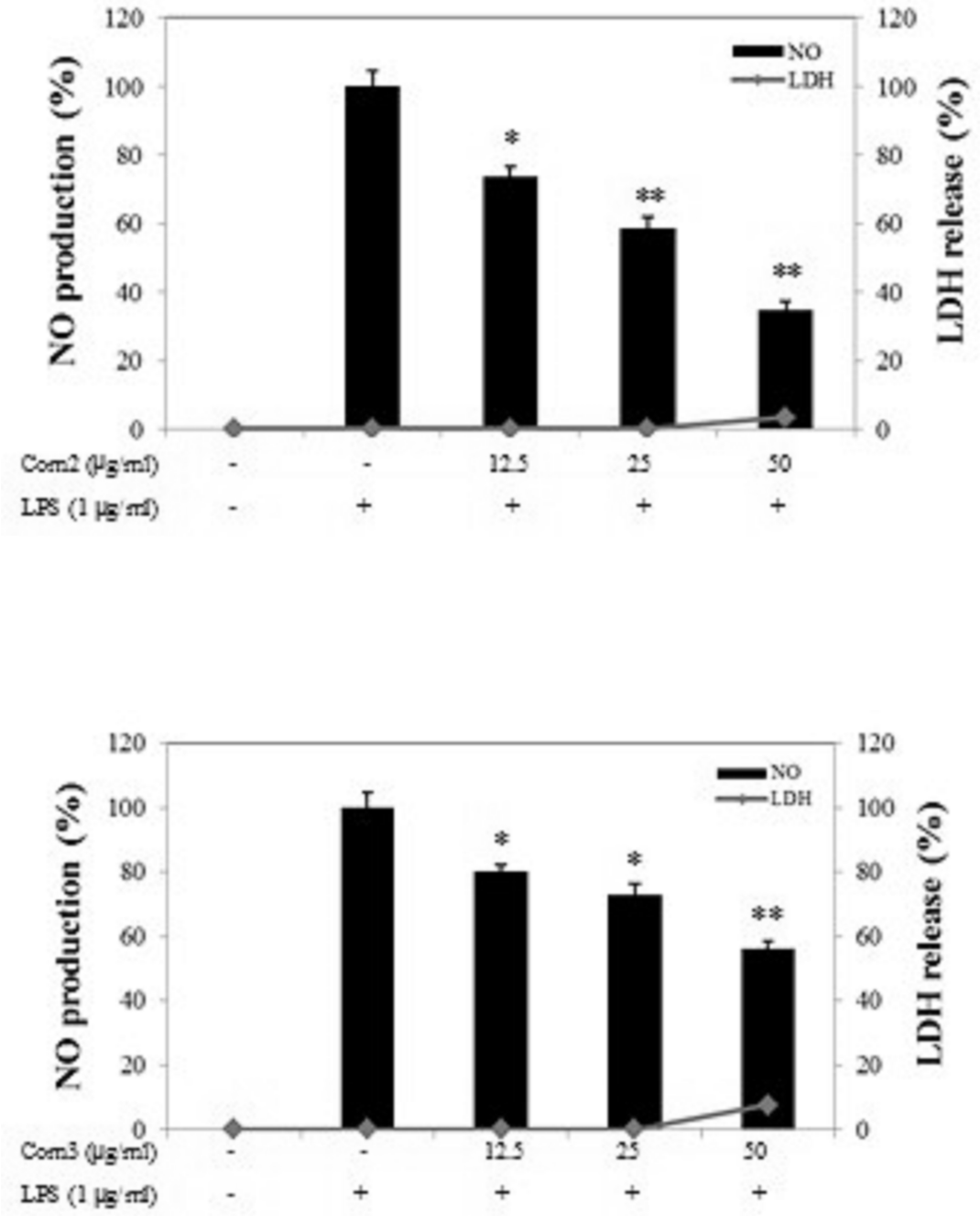


Fig. 3-9. Inhibitory effects of compound 2 and compound 3 on the NO production in RAW 264.7 cells. The production of nitric oxide was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of compound 2 and 3 (12.5, 25 and 50 μg/ml). Cytotoxicity was determined using the LDH method. Values are the mean ± SD of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$.

3.5. Effects of compound 2 on LPS-induced PGE_2 production

The inhibitory effect of compound 2 on PGE_2 production in LPS-induced RAW 264.7 macrophages were similar pattern to its effect on NO inhibition, in that compound 2 inhibited LPS-induced PGE_2 production by 98% at 50 μg/ml (**Fig. 3-10**). Thus, the inhibitory effect on PGE_2 production was stronger than that exhibited in the inhibition of NO production.

3.6. Effects of compound 2 on LPS-induced TNF-α, IL-1β, and IL-6

To determine the effects of compound 2 on the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, the RAW 264.7 macrophages were incubated with compound 2 (0, 12.5, 25, and 50 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h, and the cytokine levels were measured by ELISA. The treatment of RAW 264.7 cells with LPS alone resulted in significant increases in cytokine production relative to the control group. However, the levels of TNF-α, IL-1β, and IL-6 in the supernatants from the compound 2-treated cells were reduced relative to the LPS group, in a dose-dependent manner (from **Fig. 3-11** to **Fig. 3-13**). Especially, compound 2 was significantly inhibited the IL-6 production in LPS-induced macrophages, and the production rate was recorded as 83.8%, 61.7%, 48.5% at 12.5, 25, and 50 μg/ml, respectively.

3.7. Effects of compound 2 on increases in iNOS and COX-2 protein levels in LPS-stimulated RAW 264.7 cells

Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent of these include the production of NO by iNOS and the formation of prostaglandins by COX-2 [156]. In an effort to characterize the antiinflammatory activities of compound 2, we assessed the effects of compound 2 on LPS-induced iNOS and COX-2 protein upregulation in RAW 264.7 cells, via Western blotting. The levels of iNOS and COX-2 expression were profoundly induced by LPS treatment. However, the inhibitory effects of compound 2 on iNOS and COX-2 protein expression were significantly suppressed in a concentration-dependent manner with the addition of compound 2 to the macrophages mixed with LPS (**Fig. 3-14**).

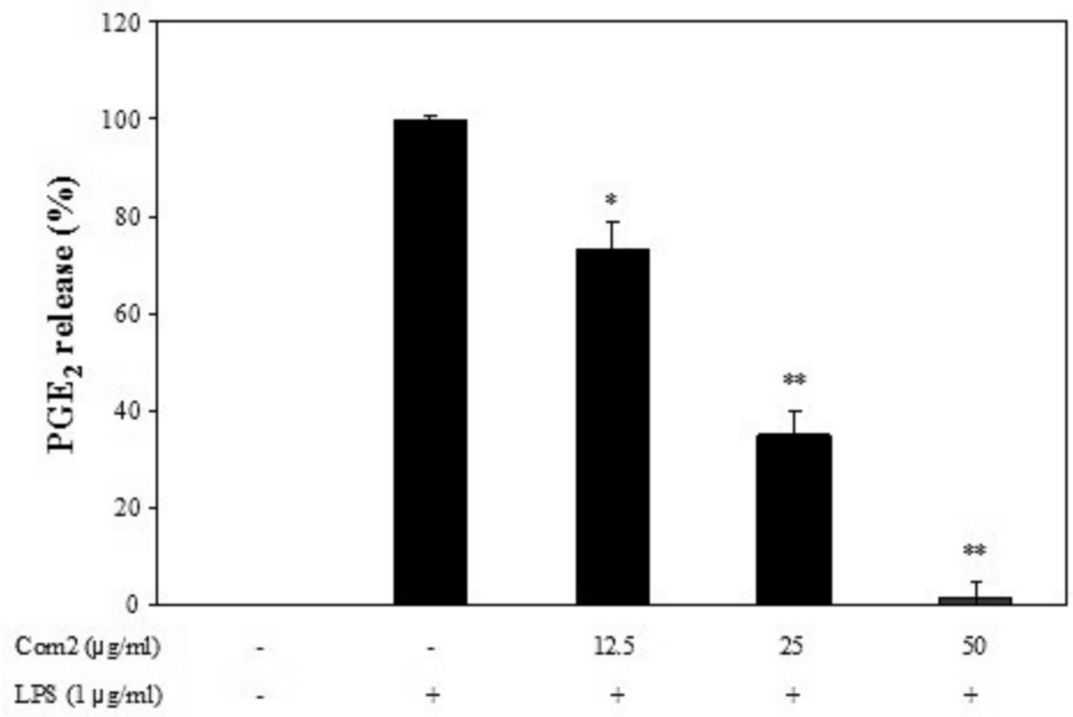


Fig. 3-10. Inhibitory effect of compound 2 on PGE_2 production in RAW 264.7 cells. Cells were stimulated by LPS (1 μg/ml) for 24 h in the presence of compound 2 (12.5, 25 and 50 μg/ml). Supernatants were collected, and the PGE_2 production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$.

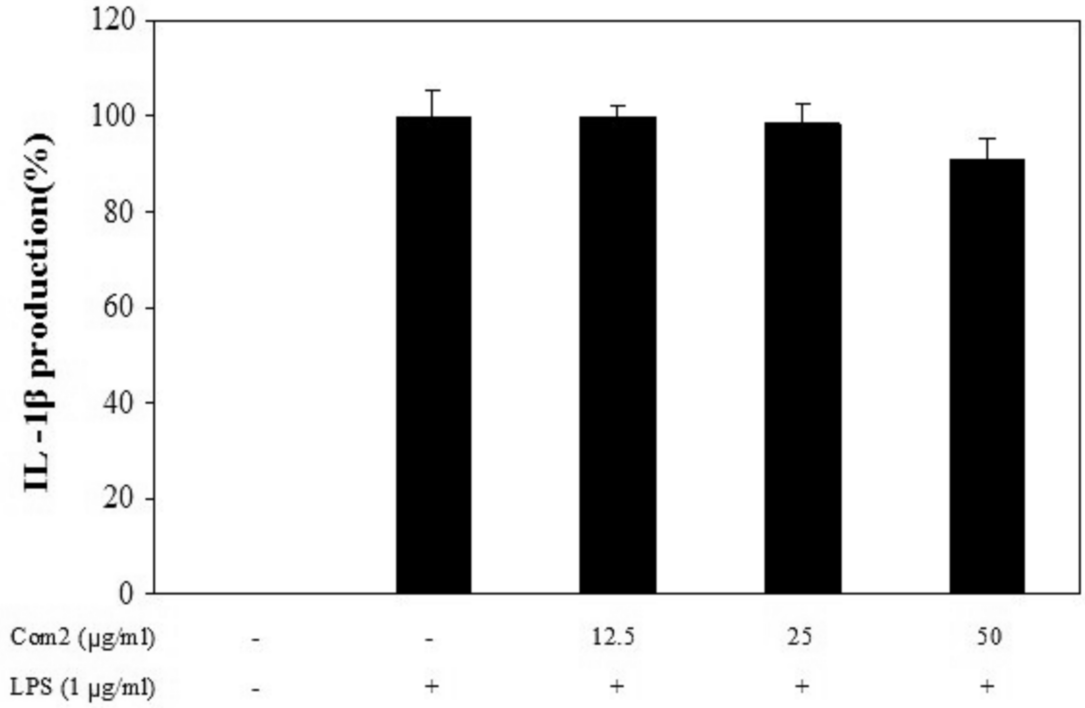


Fig. 3-11. Inhibitory effect of compound 2 on the IL-1β production in RAW 264.7 cells. The production of IL-1β was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of compound 2 (12.5, 25 and 50 μg/ml). Supernatants were collected, and the IL-1β concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments.

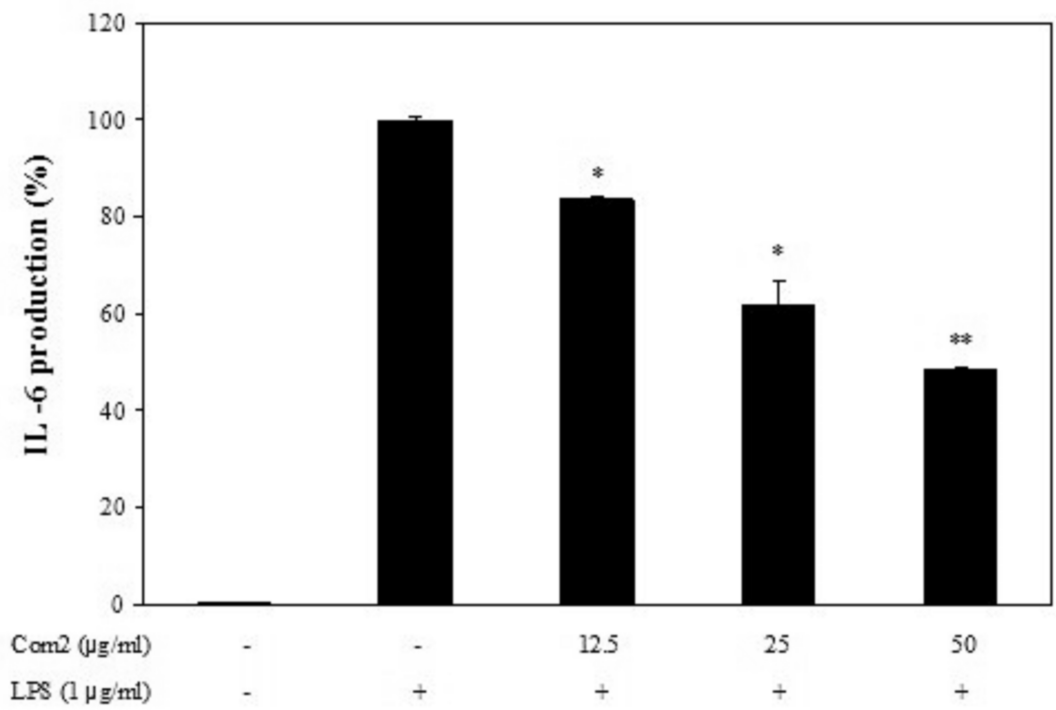


Fig. 3-12. Inhibitory effect of compound 2 on the IL-6 production in RAW 264.7 cells. The production of IL-6 was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of compound 2 (12.5, 25 and 50 μg/ml). Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$.

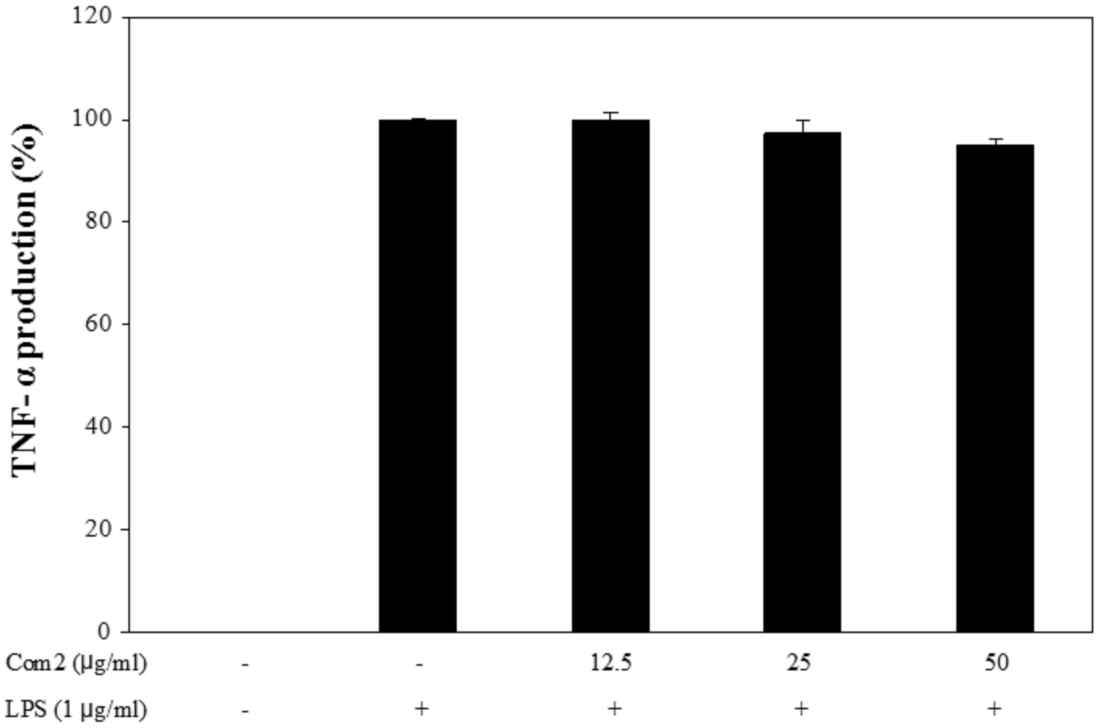


Fig. 3-13. Inhibitory effect of compound 2 on the TNF-α production in RAW 264.7 cells. The production of TNF-α was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of compound 2 (12.5, 25 and 50 μg/ml). Supernatants were collected, and the TNF-α concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments.

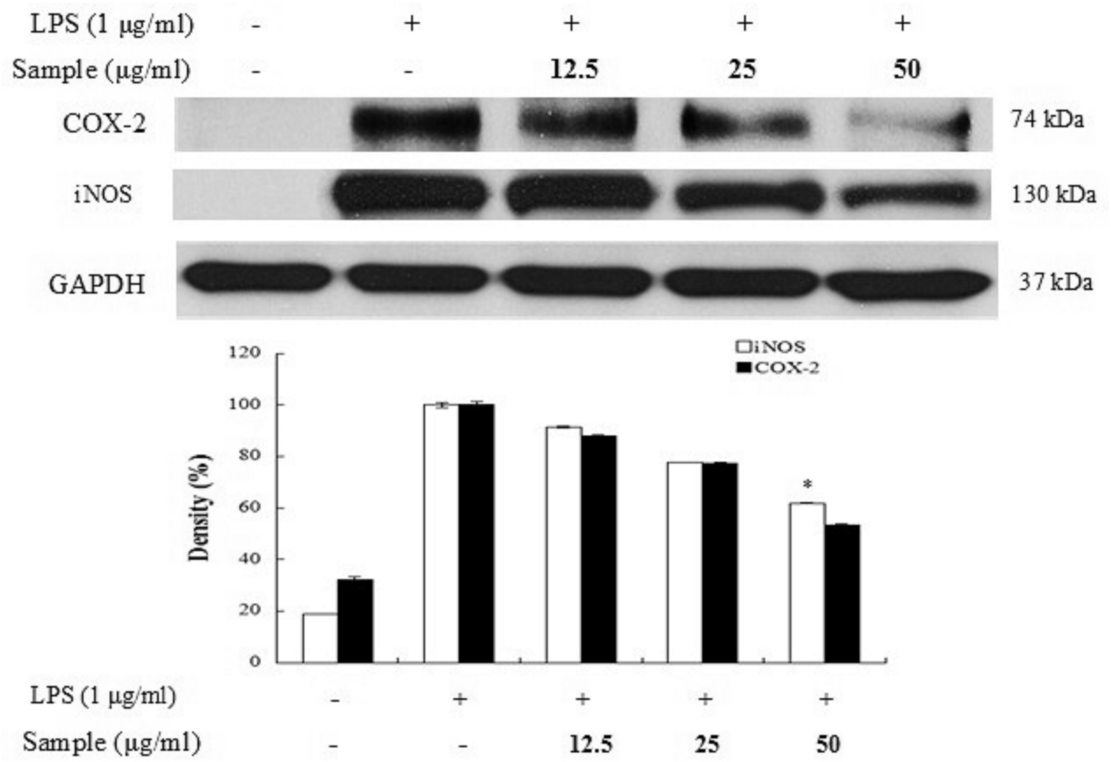


Fig. 3-14. Inhibitory effect of compound 2 on the protein level of iNOS and COX-2 in RAW 264.7 cells. RAW 264.7 cells were pre-incubated for 24 h, and the cells were stimulated with LPS (1 μg/ml) in the presence of compound 2 (12.5, 25 and 50 μg/ml) for 24 h. iNOS and COX-2 protein level were determined using immunoblotting method. Values are the mean ± SD of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$.

4. Discussion and conclusion

NO is an important inflammatory mediator which is synthesized from arginine by nitric oxide synthase (NOS). Generally, NO plays an important role as a vasodilator, neurotransmitter and in the immunological system as a defense against tumor cells, parasites, and bacteria [157]. However, under pathological condition, NO production is increased by the inducible NOS (iNOS), subsequently, brings about cytotoxicity, and tissue damage [158]. Therefore, NO inhibitors are essential for the prevention of inflammatory diseases. PGE_2 is produced at inflammatory site by COX-2 and it also has been implicated as important mediator in the processes of inflammation [159]. Chang et al. (2006) reported that the induction of COX-2 activity and subsequent generation of PGE_2 are closely related to the NO production [160]. Thus, reducing the levels of PGE_2 and COX-2 may be an effective strategy for inhibiting the inflammation. In the present study, we isolated two anti-oxidant compounds by preparative CPC with anti-inflammatory activity. According to the inhibitory effect on NO production and cytotoxicity assay, we selected compound 2 as our target compound for further experiments. Our results demonstrated that the target compound (compound 2) isolated from *B. badius* inhibited LPS-induced NO and PGE_2 production in a concentration-dependent manner in RAW 264.7 macrophages which were attributed to its ability to down regulate the protein expression of iNOS and COX-2. Especially, 50 μg/ml of compound 2 completely suppressed PGE_2 production and COX-2 expression. Moreover, the inhibitory effects of compound 2 on the LPS-induced expressions of iNOS and COX-2 in

RAW 264.7 macrophages were not due to the cytotoxicity of compound 2, as assessed by LDH assay. Thus, compound 2 can be considered as an effective therapeutic agent for preventing inflammatory diseases.

It has been reported that an abnormality in the production or function of cytokines, such as TNF-α, IL-6, and IL-1β, play roles in many inflammatory lesions [161]. TNF-α is a potent activator of macrophages and can stimulate the production or expression of IL-6, IL-1β, PGE_2, collagenase, and adhesion molecules. It elicits a number of physiological effects, including septic shock, inflammation, and cytotoxicity [162]. Interleukin 6 is well known pro-inflammatory cytokine and regarded as an endogenous mediator of LPS-induced fever [163]. Interleukin 1β is also considered to be a pivotal pro-inflammatory cytokine, primarily released by macrophages, and it is believed to play an important role in the pathophysiology of rheumatoid arthritis [164]. Inflammatory stimuli, such as LPS, induce cytokines in the process of macrophage activation, which mediates tissue response in different phases of inflammation [165,166]. Thus, the inhibition of cytokine production or function is a key mechanism in the control of inflammation. Our results exhibited that the compound 2 isolated from *B. badius* significantly inhibited the production of pro-inflammatory cytokine IL-6 in RAW 264.7 macrophages stimulated by LPS, but showed less effect on TNF-α and IL-1β formation, suggesting that the inhibition of COX-2 / PGE_2 pathway by compound 2 may be associated with the attenuation of IL-6 formation and less mediated by enhancing TNF-α and IL-1β release.

In conclusion, we provided a mechanism to explain the antiinflammatory activity of the compound 2 isolated from *B. badius* by suppressing NO and PGE_2 production and COX-2

expression, which may be associated with the attenuation of IL-6 formation in LPS-stimulated RAW 264.7 macrophages. Hence, these results suggest that the compound 2 isolated from *B. badius* possesses potential anti-inflammatory activity and might have a beneficial effect on the treatment for inflammatory diseases.

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