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Evaluation of the Biological Activities of Marine Bacteria Collected from Jeju Island, Korea, and Isolation of Active Compounds from their Secondary Metabolites

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Abstract

To explore marine microorganisms with medical potential, we isolated and identified marine bacteria from floats, marine algae, animals, and sponges collected from Jeju Island, Korea. We isolated and identified 21 different strains from the marine samples by 16S rRNA analysis, cultured them in marine broth, and extracted them with ethyl acetate (EtOAc) to collect secondary metabolite fractions. Next, we evaluated their anti-oxidative and anti-inflammatory effects. Among the 21 strains, the secondary metabolite fraction of *Bacillus badius* had both strong antioxidant and anti-inflammatory activity, and thus was selected for further experiments. An antioxidant compound detected from the secondary metabolite fraction of *B. badius* was purified by preparative centrifugal partition chromatography (*n*-hexane:EtOAc:methanol:water, 4:6:4:6, v/v), and identified as diolmycin A2. Additionally, diolmycin A2 strongly inhibited nitric oxide production. Thus, we successfully identified a significant bioactive compound from *B. badius* among the bacterial strains collected from Jeju Island.

Key words: Marine microorganism, Secondary metabolite, Jeju Island, *Bacillus badius*, Diolmycin A2, Biological activities

Introduction

Marine organisms represent a promising future source of natural products due to the incredible diversity of chemical compounds they contain. Since the mid-1970s, marine environments have been systematically investigated as a source of novel biologically active agents, and marine bacteria, fungi, algae, sponges, coelenterates, seahares, bryozoans, tunicates, and nudibranchs have been especially studied (Mearns-Spragg et al., 1998; Schmitt et al., 2012).

Marine organisms have recently been shown to be a rich source of structurally unique and biologically active secondary metabolites. Among these, marine bacteria represent a major source of natural products with diverse chemical structures and biological activities (Kim et al., 2008), and are regularly found both on and within marine invertebrates. Marine and terrestrial bacteria differ due to the influence of their respective environmental conditions (ZoBell and Johnson, 1949). Microorganisms living in the sea must survive and grow in a marine environment with low nutrition, high salinity, and high pressure. To survive under such conditions, marine bacteria often produce compounds with unique biological properties, which are attractive to researchers. Marine *Streptomyces*, *Pseudomonas*, *Bacillus*, *Pseudoalteromonas*, *Vibrio*, and *Cytophaga* isolated from seawater, sediments, algae, and marine invertebrates have all been found to produce bioactive agents. They can produce indole derivatives (quinones and violacein: Collins and Jones, 1981; Faulkner, 2000), alkaloids (prodiginines and tambjamines: Kochanowska-Karamyan and Hamann, 2010), polyenes (Bae et al., 2013), macrolides

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(Hirata and Uemura, 1986), peptides (Suarez-Jimenez et al., 2012), and terpenoids (Soria-Mercado et al., 2005). Recently, marine microbes have become important agents in the study of novel microbial products exhibiting antibacterial, antiviral, antitumor, anticoagulant, and cardioactive properties (Holmstrom and Kjelleberg, 1999). Therefore, the goal of this study was to identify suitable marine bacteria, and evaluate the antioxidative and anti-inflammatory activities of their secondary metabolites. To that end, we identified and evaluated the bioactive compounds of several marine bacteria.

Materials and Methods

General reagents

All solvents used for crude sample preparation were of analytical grade (Daejung Chemicals & Metals Co., Seoul, Korea). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin streptomycin, and trypsin-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), and all other reagents and solvents were also purchased from Sigma Aldrich.

Collection and identification of marine bacteria

We collected four types of samples, from floats, marine algae, animals, and sponges from around the coast of Jeju Island, Korea in August 2011. Thereafter, all samples were rinsed three times with sterile seawater for 12 h at room temperature to remove loosely attached microorganisms. Samples were aseptically cut into small pieces using sterile surgical scissors and carefully placed onto prepared Petri-dishes containing Marine Agar 2216 (BD, Sparks, MD, USA) media. These plates were sealed and labeled by sample code and initial culturing date. Next, they were incubated at 29°C with humidity. After 3-5 days, cultivated 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. In this manner, we isolated 21 bacterial strains, and deposited voucher specimens in the Marine Bio-Resource Technology Laboratory of Jeju National University.

Preparation of secondary metabolite extracts

A single colony from a well grown agar plate was used as an inoculum and transferred to 300 mL Erlenmeyer flasks with 200 mL of Marine Broth 2216 (BD) medium containing peptone (0.5%), yeast extract (0.1%), and seawater (100%) for the production of secondary metabolites. These liquid culture flasks were incubated on a rotary shaker at 121 rpm and 29°C with humidity for 10 days. Thereafter, the broth was centrifuged (10,000 rpm, 15 min) to remove cells. Next, the supernatants were extracted with an equal volume of ethyl acetate (EtOAc; 200 mL). After separation, the organic phases were concentrated *in vacuo* at 35°C and used as secondary metabolite extracts in screening for further biological activity.

Hydrogen peroxide (H_2O_2) scavenging activity

H_2O_2 scavenging activity was determined according to the method of Müller (1985), wherein 100 μL of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96 microwell plate. Next, 20 μL of H_2O_2 was added, and the mixture, was incubated at 37°C for 5 min. Finally, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added, and the mixture was again incubated at 37°C for 10 min, after which we measured the absorbance with an enzyme-linked immunosorbent assay (ELISA) reader at 405 nm.

Cell culture

We purchased the murine macrophage cell line RAW 264.7 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. Cells were incubated in an atmosphere of 5% CO_2 at 37°C and subcultured every 2 days.

Determination of nitric oxide (NO) Production

Anti-inflammatory effects were evaluated following the protocol of Lee et al. (2013), with some modifications. 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, and the supernatant of the cultured cells was used for NO quantification. A mixture of 100 μL of the supernatant and 100 μL of Griess reagent was incubated at room temperature for 10 min and the absorbance at 540 nm was measured on a microplate reader. We performed MTT and lactate dehydrogenase (LDH) assays to measure cytotoxicity: RAW 264.7 cells (1×10^5 cells/mL) plated in 24-well plates were preincubated and subsequently treated with LPS (1 μg/mL) coupled with samples at 37°C for 24 h. Next, 100 μL of MTT stock solution (2 mg/mL) was applied to the wells, and after 4 h of incubation, the supernatants were aspirated. The formazan crystal in each well was dissolved in 200 μL of DMSO and the absorbance measured via ELISA at a wavelength of 540 nm. The medium was carefully removed from each well, and LDH activity in the medium was determined using an LDH cytotoxicity detection kit.

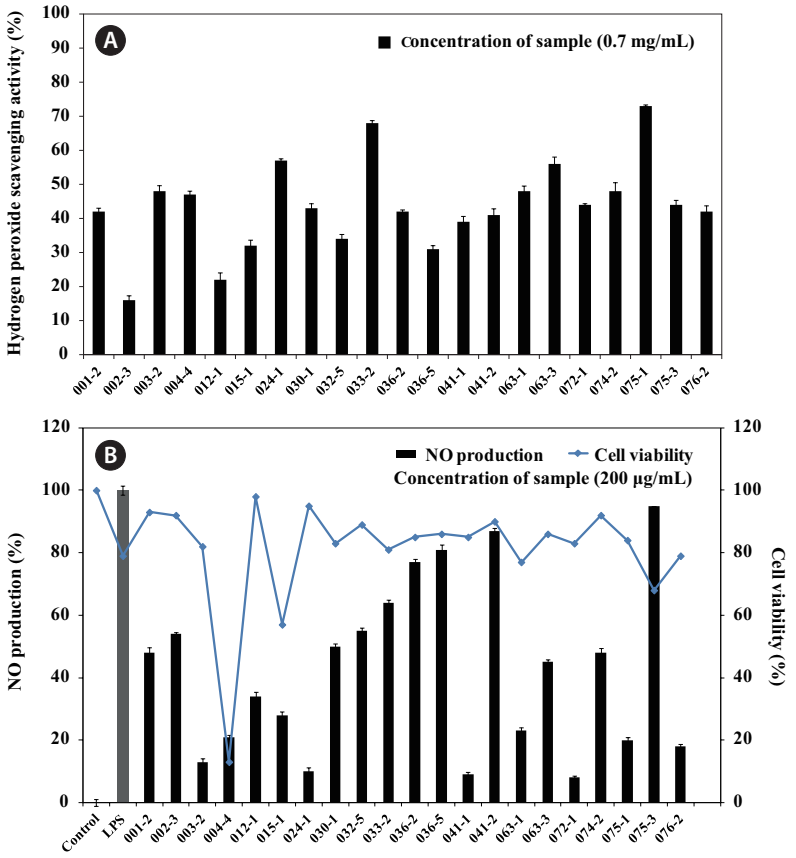

Fig. 1. Hydrogen peroxide scavenging (A) anti-inflammatory activities and cytotoxicity (B) of the secondary metabolites fractions from marine bacteria. Hydrogen peroxide effect was measured by the above described protocol. To evaluation of anti-inflammation, Raw 264.7 cells were pretreated with the secondary metabolites fractions for 2 h and then the produced nitric oxide (NO) was measured after incubation with lipopolysaccharide (LPS) for 24 h. Names of all bacteria species were expressed using the number indicated in Table 1. Each value indicates the mean ± standard error from three independent experiments.

Online high-performance liquid chromatography (HPLC)-ABTS$^+$ assay

HPLC and ABTS assays were performed according to the method of Lee et al. (2013), with some modifications. For HPLC processing, the mobile phase comprised several ratios of acetonitrile to water in gradient mode as follows: acetonitrile: water (0-50 min: 20:80→60: 40, v/v; 50-60 min: 60:40, v/v; and 60-70 min: 100:0, v/v). The flow rate was 0.2 mL/min and the ultraviolet (UV) absorbance was detected at 280 nm.

Preparative centrifugal partition chromatography (CPC) separation procedure

The CPC experiment was performed using the assay of Lee et al. (2014), and the two-phase solvent system was composed of *n*-hexane:EtOAc:MeOH:water (4:6:4:6, v/v). When the mobile phase emerged from the column, hydrostatic equilibrium had been reached at 2.0 MPa. We dissolved 490 mg of *B. badius* secondary metabolite extract in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system phases and injected them through the Rheodyne injection valve. The effluent from the CPC separation procedure was monitored in UV light at 254 nm.

Statistical analysis

All measurements were made in triplicate and all values are represented as means ± standard error (SE). The results were subjected to an analysis of variance (ANOVA) using Turkey's HSD post hoc test to analyze the differences between groups, with a significance level of 0.05.

Results

Culture and isolation of marine bacteria

The marine bacteria collected from the floats, marine algae, animals and sponges were cultured in marine agar media, and we successfully isolated 21 bacterial strains exhibiting morphological differences. We identified these bacterial strains

to 17 genera: five belonged to the genus *Bacillus*, while one each belonged to *Paracoccus*, *Exiguobacterium*, *Halomonas*, *Halobacillus*, *Burkholderia*, *Kytococcus*, *Staphylococcus*, *Cobetia*, *Streptomyces*, *Dietzia*, *Lysinibacillus*, *Maribacter*, *Planococcus*, *Intrasporangium*, *Micrococcus* and *Klebsiella* (Table 1).

To evaluate the biological activity of the secondary metabolites extracted from the bacterial strains, all strains were cultured in flasks with 200 mL of liquid culture medium. After 10–15 days, the media and bacterial strains were extracted by adding EtOAc in preparation for further experiments.

Evaluation of biological effects of the extracts

The results for H_2O_2 scavenging activity of the extracts containing secondary metabolites are shown in Fig. 1A. The secondary metabolite extracts from *Halobacillus halophilus* (024-1), *Maribacter goseongensis* (033-2), *Micrococcus luteus* (063-3), and *B. badius* (075-1) exhibited high radical scavenging activities. In particular *B. badius* (075-1) extract recorded the highest activity (approximately 73%) and *M. goseongensis* (033-2) extract also exhibited relatively high scavenging activity (approximately 67%) at a concentration of 0.7 mg/mL. NO production of LPS-induced RAW 264.7 cells was measured by the Griess reaction, and cytotoxicity of the secondary metabolite extract in LPS-induced RAW 264.7 cells was estimated by the MTT assay (Fig. 1B). Among the 21 extracts, 14 showed ≥50% inhibition of NO production at 200 μg/mL. Six extracts in particular, including *Kytococcus sedentarius*, *Halomonas marina*, *H. halophilus*, *Klebsiella oxytoca*, *B. badius* and *Bacillus megaterium* (041-1, 003-2, 024-1, 072-1, 075-1 and 076-2, respectively) exhibited ≥80% NO inhibition without any cytotoxicity, with *K. sedentarius* (041-1) and *K. oxytoca* (072-1) showeing the highest inhibitory activities (89.5% and 89.2%, respectively). The extracts from *Streptomyces griseus, Burkholderia cepacia* and *Bacillus pseudofirmus* (004-4, 015-1 and 075-3 respectively) showed high cytotoxicity in LPS-induced RAW 264.7 cells, and therefore were excluded from further experiments. Thus, the *B. badius* extract showed both the strongest anti-oxidative and anti-inflammatory effects, and we proceeded with the identification and bioactive evaluation of compounds from the secondary metabolites in this extract.

Online HPLC-ABTS$^+$ analysis of the of *B. badius* extract

We analyzed chromatography peaks and their radical scavenging activities in the *B. badius* extract according to the online HPLC-ABTS$^+$ conditions described previously (Fig. 3). The HPLC peak 1 had stronger antioxidant activity than the other peaks. Therefore, we performed the isolation and purification of the active peak using preparative CPC.

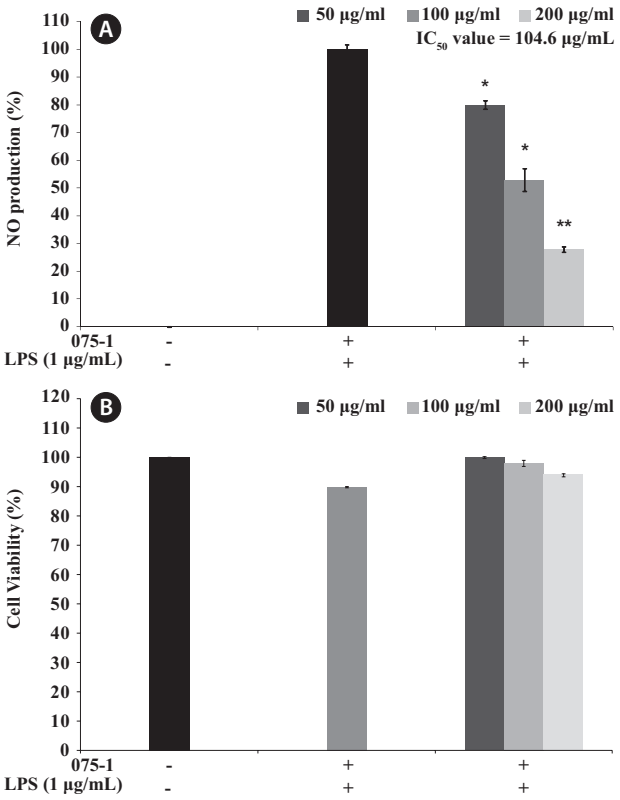

Fig. 2. Anti-inflammatory activities and cytotoxicity of *Bacillus badius* (075-1) secondary metabolites fraction against lipopolysaccharide (LPS)-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with the secondary metabolites fraction for 2 h, and then incubated with LPS for 24 h. Each value indicates the mean ± standard error (SE) from three independent experiments. Experiments were performed in triplicate and the data are expressed as mean ± SE. $^*P < 0.1$, $^{**}P < 0.05$.

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

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	Animal	*Dietzia cinnamea*	99
015-1	Animal	*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	Animal	*Bacillus coagulans*	98
063-3	Animal	*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

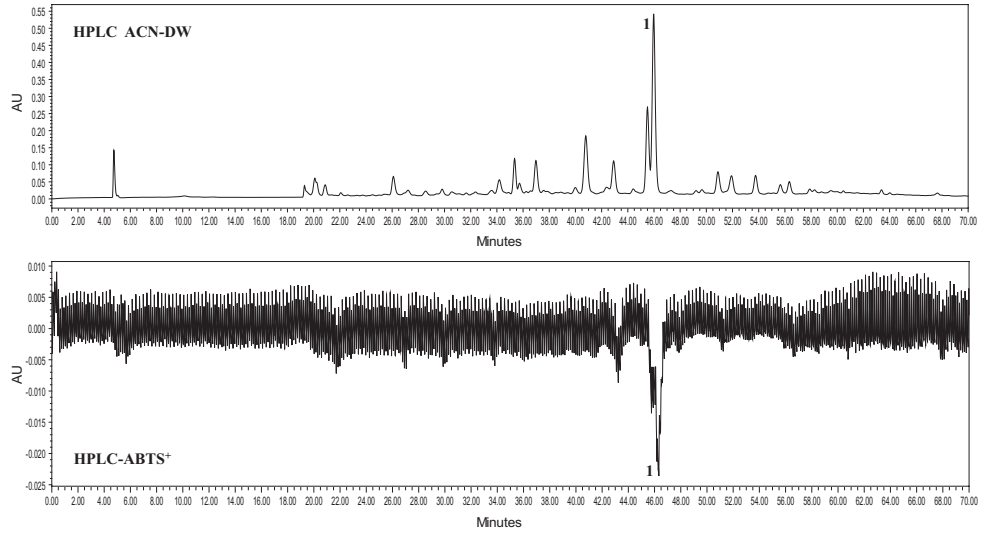

Fig. 3. $ABTS^{+}$ on-line HPLC chromatogram of secondary metabolites fraction from *Bacillus badius*. The mobile phase comprised acetonitrile– distilled water (ACN-DW) in gradient mode as follows: ACN-DW (0-50 min: 20:80→60:40, v/v; 50-60 min: 60:40, v/v; and 60-70 min: 100:0, v/v). The flow rate was 0.2 mL/min, and the UV absorbance was detected at 280 nm. Peak 1 represents compound 1.

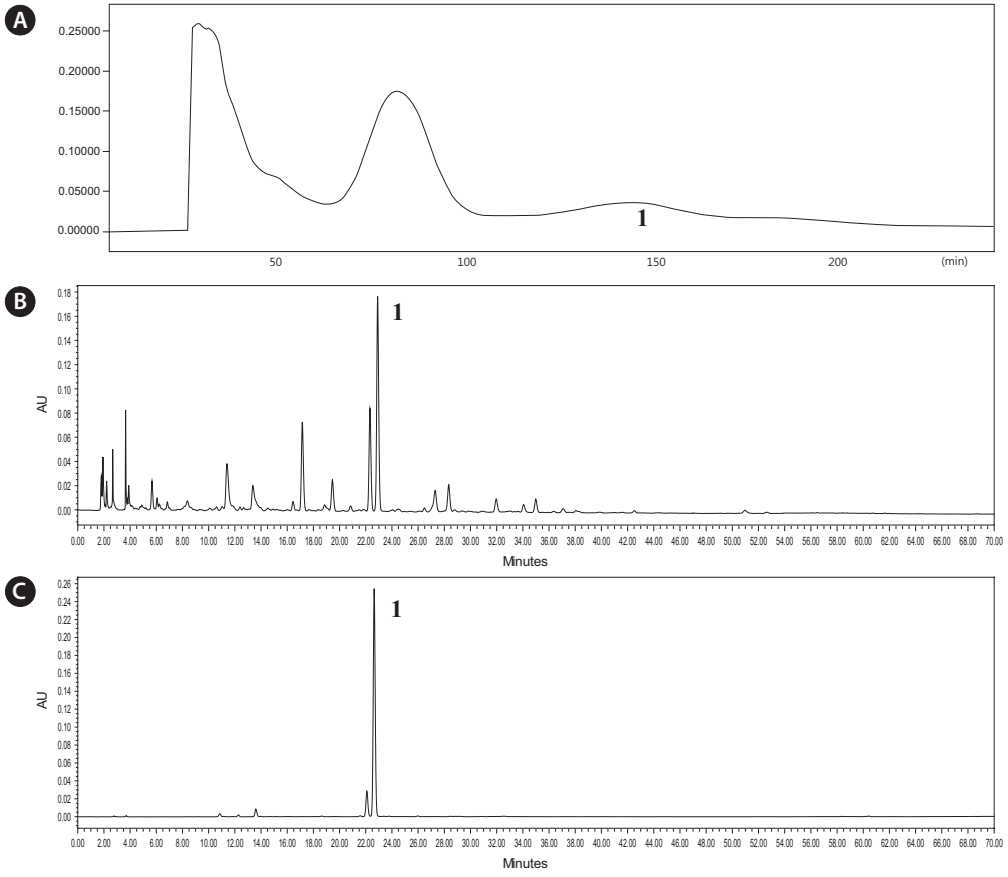

Fig. 4. Chromatograms of the secondary metabolites fraction. The CPC chromatogram of secondary metabolites fraction (A), HPLC chromatograms of the secondary metabolites fraction (B), and the compound 1 (C) from *Bacillus badius*. For operation of CPC, stationary phase: upper organic phase, mobile phase: lower aqueous phase, flow rate: 2 mL/min, rotation speed: 1000 rpm, and sample: 500 mg dissolved in 6 mL mixture of lower phase and upper phase (1:1, v/v) of the solvent system. For operation of HPLC, column: SUNFIRE™ C18 5μm ODS column (250 × 4.6 mm i.d.; Waters, Milford, MA, USA); mobile phase: acetronitrile (20:80 v/v to 60:40 v/v at 0-50 min, 60:40 v/v to 100:0 v/v at 50-60 min, 100:0 v/v to 100:0 v/v at 60-70 min); flow rate: 1 mL/min, monitored at 280 nm. Peak 1 represents compound 1.

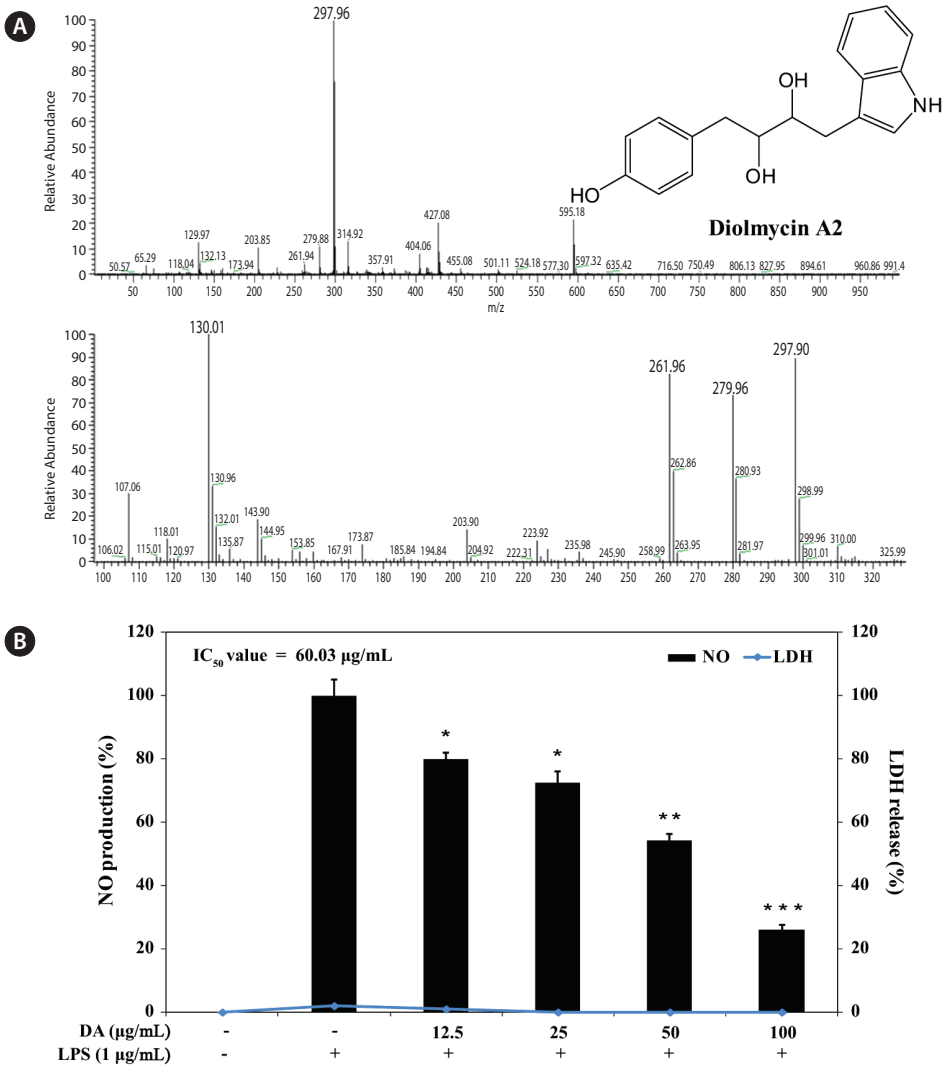

Fig. 5. Mass spectrometry (MS) and MS/MS analysis data (A) and anti-oxidative effect (B) of diolmycin A2 from the secondary metabolite fraction of *B. badius*. The production of nitric oxide (NO) was assayed in the culture medium of cells stimulated with lipopolysaccharide (LPS, 1 μg/mL) for 24 h in the presence of diolmycin A2. Cytotoxicity was determined using the lactate dehydrogenase (LDH) method. Experiments were performed in triplicate and the data are expressed as mean ± standard errors. $^{*}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.01$.

Purification and identification of anti-oxidative compounds from *B. badius*

The *K*-value of the target compound 1 was <1. Therefore, we operated the preparative CPC in descending mode (upper phase = stationary phase, and lower phase = mobile phase, respectively). Approximately 140 mL of the stationary phase was retained in the coil, and the pressure reached 4.1 MPa during operation. The preparative CPC chromatogram is described in Fig. 4A; in all, 16.7 mg of compound 1 was isolated from 490 mg of the EtOAc extract. We identified compound 1 as diolmycin A2 by liquid chromatography/mass spectrometry (LC/MS) and one-dimensional nuclear magnetic resonance (1D-NMR) spectroscopy, as well as by comparison with a previous report (Tabata et al., 1993). We obtained diolmycin A2 of up to 90% purity.

Inhibitory effect of diolmycin A2 on NO production in LPS-stimulated RAW 264.7 cells

To evaluate the inhibitory effect of diolmycin A2 on anti-inflammatory action, RAW 264.7 macrophages were stimulated with LPS (1 μg/mL) for 24 h to generate NO, and the accumulated NO in the culture medium was measured by the Griess reaction. The 50% inhibitory concentration (IC_{50}) of diolmycin A2 was 60.03 μg/mL with no cytotoxicity on LPS-stimulated RAW 264.7 cells (Fig.5). Thus, diolmycin A2 strongly but dose-dependently inhibited NO production in LPS-induced RAW 264.7 cells.

Discussion

Various secondary metabolites biosynthesized by bacteria inhabiting marine creatures have been shown to possess good bioactivity (Bringmann et al., 2003, 2005; Liu et al., 2005; Zheng et al., 2005; Pimentel-Elardo et al., 2010). Previous studies have shown that *Bacillus* spp. and *Halobacillus* spp. have antifungal activity, and *Streptomyces* spp. may exhibit anticancer, anti-inflammation, antioxidant, antifungal and other biological activities (Hayashi et al., 1996; Nakae et al., 2000; Sohn et al., 2008). Therefore, we collected 21 species of bacteria from various sources near Jeju Island, and assessed their extracted secondary metabolites for anti-oxidative, anti-inflammatory, and other biological activities. Among these, the secondary metabolite fraction of *B. badius* efficiently scavenged H_2O_2 (approximately 73%). Kodali et al. (2011) reported that *Bacillus coagulans* RK-02, which is a heteropolymer composed of four monosaccharides, also shows significant anti-oxidative activity, and that this bacterial extract contains phytochemical constituents capable of donating hydrogen to a free radical. Sakai et al. (2000) purified nitrite-oxidizing enzyme I (NiOx I), which catalyzes H_2O_2 degradation, from *B. badius* I-73.

Here, we investigated the anti-inflammatory activity of secondary metabolite extracts from various bacteria species based on NO produced in LPS-stimulated RAW 264.7 cells without cytotoxicity. In the combined results of both cell viability and NO inhibitory activity, the secondary metabolite extracts from *H. halophilus*, *B. badius*, *K. sedentarius* and *K. oxytoca* inhibited NO production. This is the first report of NO production inhibition in these species. Moreover, no previous reports have described the isolation and characterization of anti-inflammatory constituents from these bacteria. Our results suggest that the secondary metabolites of *B. badius* may be useful not only as an antioxidant but also as an anti-inflammatory agent. Aruoma (1998) reported that reactive oxygen species (ROS) are implicated in inflammation, and consequently, antioxidants play an important role in inflammatory diseases (Conner and Grisham, 1996). The demonstration of both anti-oxidative and anti-inflammatory activity in *B. badius* may confirm this relationship.

Overall, we confirmed the presence of biologically active compounds in *B. badius* secondary metabolites. Diolmycin A2 is the main bioactive compound and showed a strong antioxidant effect in $ABTS^+$ online HPLC significantly reducing the inflammatory response in LPS induced macrophages. Diomycin A2 was previously isolated from *Streptomyces* spp., but has not previously been reported from other natural organisms (Tabata et al., 1993). Diolmycins comprise four active compounds, including diolmycin A1, diolmycin A2, diolmycin B1, and diolmycin B2. The order of anticoccidial activity *in vitro* using monensin-resistant *Eimeria tenella* was diolmycin A1 > A2 >> B1 = B2, indicating that the indole unit is important to the anticoccidial effect (Sunazuka et al., 1993). Furthermore, diolmycins are produced from *Streptomyces* spp. as part of the scytonemin biosynthetic pathway (Tabata et al., 1993). However, diolmycin A2 has not previously been isolated from *B. badius* and neither antioxidant nor anti-inflammatory effects have been reported. Our results, show that *B. badius* extract and diolmycin A2 are potential marine sources of biologically active natural products, highlighting the potential value of marine bacteria from Jeju Island for the development of drugs or nutraceuticals.

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