

Anti-Inflammatory and Anti-Fibrotic Activities of *Nocardiosis* sp. 13G027 in Lipopolysaccharides-Induced RAW 264.7 Macrophages and Transforming Growth Factor Beta-1-Stimulated Nasal Polyp-Derived Fibroblasts

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Nocardiosis species produce bioactive compounds, such as antimicrobial and anti-cancer agents and toxins. However, no reports have described their anti-inflammatory and anti-fibrotic effects during nasal polyp (NP) formation. In this study, we investigated whether marine-derived bacterial *Nocardiosis* sp. 13G027 exerts anti-inflammatory and anti-fibrotic effects on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages and transforming growth factor (TGF)- β 1-induced NP-derived fibroblasts (NPDFs). Nitric oxide (NO) and prostaglandin E₂ (PGE₂) levels were analyzed. Extract from *Nocardiosis* sp. 13G027 significantly inhibited the upregulation of NO and PGE₂ in LPS-activated RAW 264.7 macrophages. The expression of mitogen-activated protein kinases (MAPKs) and protein kinase B (Akt/PKB) in LPS-induced RAW 264.7 macrophages was evaluated; smooth muscle alpha-actin (α -SMA), collagen type I (Col-1), and fibronectin also phosphorylated small mothers against decapentaplegic (SMAD) 2 and 3 in TGF- β 1-stimulated NPDFs. The *Nocardiosis* sp. 13G027 extract suppressed the phosphorylation of MAPKs and Akt and the DNA-binding activity of activator protein 1 (AP-1). The expression of pro-fibrotic components such as α -SMA, Col-1, fibronectin, and SMAD2/3 was inhibited in TGF- β 1-exposed NPDFs. These findings suggest that *Nocardiosis* sp. 13G027 has the potential to treat inflammatory disorders, such as NP formation.

Keywords: Nasal polyp, *Nocardiosis* sp. 13G027, anti-inflammatory, anti-fibrotic

Introduction

Nasal polyps (NPs) are exogenous in the shape of tear-drops in beingn, pink, and nasal sinus. NPs typically include tissue remodeling, such as substrate and fibrosis, proliferation of epithelial cell, permeation of

inflammatory cell, basal membrane thickening, formation of pseudocyst, and have high recurrence risks [1, 2]. Most patients diagnosed with NPs show common symptoms, such as anosmia, headaches, nasal obstruction, and reduced taste, leading to reduced quality of life [3]. NPs generally occur through the chronic infiltration of inflammatory cells. Because NPs commonly recur after surgery, additional treatments are necessary in many cases. The cause and pathophysiology perspective of dis-

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ease in NP formation needs to be studied in more detail. However, other research groups proposed involvement in the remodeling process, and the mucosal epithelial damage is associated with inflammatory cell penetration and accumulation of extracellular matrix (ECM) protein [4].

Nocardiosis is known as an ecologically multi-functional and biotechnologically meaningful genus. Species belonging to the genus *Nocardiosis* are known to produce a variety of bioactive substances, such as anti-cancer agents and antimicrobial compounds (pendolmycin, apoptolidins, griseusins, and naphthospiroones), immunomodulators, and toxins [5–11].

We examined the anti-inflammatory and anti-fibrotic activities of *Nocardiosis* sp. 13G027 in lipopolysaccharide-induced RAW 264.7 macrophage cells and transforming growth factor- β 1-treated nasal polyp-derived fibroblasts. We measured the proteins expression related to MAPK, Akt, and AP-1 pathways following *Nocardiosis* sp. 13G027 treatment in LPS-induced RAW 264.7 macrophages. We also examined that the *Nocardiosis* sp. 13G027 extract inhibited the expression of fibrosis-related proteins such as α -SMA, Col-1, fibronectin, and activation of Smad2 and Smad3 pathways in TGF- β 1-activated NPDPs. We studied that the intracellular signaling pathways in the macrophages and NPDPs for the regulatory activities of *Nocardiosis* sp. 13G027.

Materials and Methods

Reagents

LPS (from *Escherichia coli* O111:B4) and TGF- β 1 were purchased from Merck (USA) and R&D Systems, Inc. (USA) respectively. We used Cell Counting Kit-8 (CCK-8) manufactured by DOJINDO Laboratories (USA). We purchased antibodies against α -SMA and Col-1 from Abcam (USA, cat. no. ab5694 and ab88147, respectively). Antibodies against actin and fibronectin were purchased from BD Biosciences (USA, cat. no. 612656 and 610077, respectively). Antibodies against GAPDH and goat anti-mouse IgG (HRP) conjugate were purchased from Young In Frontier (Korea, cat. no. LF-PA0018 and LF-SA8001, respectively). Antibodies against p-Smad2, p-Smad3, c-Jun N-terminal kinase (JNK), p-JNK, p-extracellular signal-related kinase (ERK), Akt, p-Akt, and p-p38 MAPK were purchased

from Cell Signaling Technology (USA, cat. no. 3101, 9520, 9252, 9251, 9106, 9272, 4058, and 9211, respectively). Antibodies against p38 MAPK and ERK were purchased from Santa Cruz Biotechnology (USA, cat. no. sc-535 and sc-94, respectively).

Isolation and identification of strain 13G027

Strain 13G027 was associated from an unidentified marine sponge 13G003 collected at Jeju Island, South Korea in 2013. The sponge 13G003 was washed by sterile seawater, and diluted with sterile seawater (1:10). One hundred microliters of the sponge suspension were spread onto SYP agar (soluble starch 10 g, yeast extract 4 g, peptone 2 g, agar 18 g, and 1 L sterile seawater). The spreading plate was incubated under aerobic conditions at 27°C for 2 weeks. Strain 13G027 was observed as a white colony that was transferred to a SYP agar before culture in liquid medium. The colony was inoculated in 25 ml SYP liquid medium with shaking (170 rpm) at 27°C for 7 days. The 13G027 bacterial strain was stored in liquid medium containing 15% (*v/v*) glycerol at -80°C.

The 16S rRNA gene sequence was analyzed for identification of 13G027. The resulting sequence was compared with other sequences from the NCBI GenBank nucleotide database by the BLAST search [12].

Preparation of 13G027 extract

Strain 13G027 was cultured in 2.5-L flasks each containing 1 L SYP liquid medium (total, 5 L). The culture flasks were incubated with shaking (150 rpm) at 25°C for 7 days. The 13G027 culture broth was extracted with the same volume of ethyl acetate (EtOAc) after 7 days. After combining from individual extraction processes, we dried the EtOAc-soluble components of 13G027 culture using a vacuum evaporator to give 150 mg of crude extract.

Free radical scavenging ABTS assay

The 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ABTS assay was tested as previously described for antioxidant activity [13]. 7 mM ABTS was combined with 2.45 mM potassium persulfate and the mixture was incubated at 25°C for 16 h. This mixture 100 μ l and 100 μ l of 13G027 extract at distinct concentrations (25 to 400 μ g/ml) were divided in 96-well plates and the absorbance was measured at 734 nm. Ascorbic

acid (1.25 to 10 µg/ml) was used as a positive control.

Culture and viability of RAW 264.7 macrophage cells

We maintained RAW 264.7 macrophages (ATCC TIB-71™, USA) using Dulbecco's modified Eagle's (DMEM) (FUJIFILM Wako, Japan) with 10% fetal bovine serum; FBS (Gibco BRL, USA) and 1% penicillin-streptomycin; PS (FUJIFILM Wako) in 5% CO₂ incubator at 37°C. Cell viability was measured using CCK-8, which measured the activity of dehydrogenase in cells. In short, cells were treated with 13G027 extract (0 to 1000 µg/ml) for 24 h at a density (2 × 10⁴ cells/well) followed by incubation with or without 1 µg/ml LPS for 18 h. Then, the absorbance at 450 nm was measured via an SH-1200 microplate reader (CORONA Electric, Japan). Whole-cell was extracted via cell lysis by 2% (*v/v*) Triton X-100.

Production of NO and PGE₂

First, we treated the 13G027 extract of various concentrations in RAW 264.7 macrophages for 24 h. Then we removed the media and washed the cells using phosphate-buffered saline (PBS). Next, using LPS in DMEM (1 µg/ml) the pretreated cells were stimulated for 18 h. For quantification of NO content, we removed 0.1 ml of the conditioned medium and mixed the cells with a same volume of Griess reagent dissolved in H₂O at room temperature for 10 min. The absorbance at 540 nm was checked with a microplate reader, and the NO contents were calculated with a standard curve using NaNO₂ [14]. PGE₂ expression was measured via a ELISA kit following the manufacturer's instructions. Briefly, we plated RAW 264.7 macrophages (2 × 10⁵ cells/ml) in 24-well plate wells and pretreated the cells with the 13G027 extract 1 h prior to stimulation using 1 mg/ml LPS. After 24 h of incubation, 100 µl of supernatant were collected to assess PGE₂ levels via ELISA.

NP-derived fibroblast culture

We cultured NPDFs as previously reported [15]. NPDF cells were cultured in DMEM containing 10% FBS and 1% PS in 5% CO₂ incubator at 37°C. NPDF cells were used in the third to fifth cell passage.

Viability of 13G027-treated NPDFs

The effects of 13G027 on NPDF viability was evaluated by the CCK-8 test. First, the 13G027 extract (30 to

100 µg/ml) was divided to the 96-well plate wells containing NPDFs (1 × 10⁵ cells/well) and incubated in 5% CO₂ incubator at 37°C for 24 h. Then, the cells were cultured with CCK-8 for 1 h after washing twice with PBS. The absorbance was checked at 450 nm through a microplate reader. All experiments were repeated three times.

Western blotting

Cell lysis using Mammalian Cell-PE lysis buffer (G-Biosciences, USA), the protein content was quantified via the Bradford assay (Bio-Rad Laboratories, USA). The protein (20 mg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, UK). Membrane was blocked using 5% non-fat dry milk (NFDM) suspended in 0.1% Tween-20 (TBST) at room temperature for 1 h. The membrane was incubated with the primary antibody and phospho-specific antibodies (α -SMA, Col-1, fibronectin, pSmad2, pSmad3, β -actin, p38, p-p38, JNK, ERK, p-ERK, AKT, p-AKT, p-JNK, and GAPDH) of 1:1000 dilution at 4°C for overnight. The goat anti-mouse IgG was used to incubated the membranes with secondary antibody conjugated to HRP of 1:1000 dilution for 1 h at room temperature. After washing three times with TBST, the immunoreactivity band was visualized by improved chemical light emitting detection system (Thermo Fisher Scientific, USA), and the strength of protein expression was quantitatively evaluated using Multi gauge software (FUJIFILM).

Electrophoretic mobility shift assay (EMSA)

Nuclear extract was obtained by NE-PER nuclear extraction reagent (Thermo Fisher Scientific). For DNA-protein binding assay, an oligonucleotide used as a probe was designed as follow: We inserted the AP-1 DNA-binding site sequence (5'-CGCTTGATGACTCAGCCGGAA-3') to the oligonucleotide, and labeled the 3' end of the probe by the biotin 3'-end DNA labeling kit (Thermo Fisher Scientific). The binding reaction was composed of 5 µg nuclear extract protein, 50 ng poly (dI-dC), and 20 fM biotin-labeled DNA. Following the mixture incubated at room temperature for 20 min. The competition reaction was carried out through adding AP-1, which has no labels more than 100 times the mixture. The reaction mixture was then separated via electrophoresis

on a 5% PAGE in $0.5 \times$ Tris-borate buffer and transferred to nylon membranes. The LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) was used for biotin-labeled DNA detection.

Statistical analysis

The mean \pm standard error of the mean was used to describe all data in this study. All statistical analysis was conducted using GraphPad Prism (GraphPad Software, USA). The comparison analysis between tests was conducted using Dunnett's multiple range tests, and $p < 0.05$ showed statistical significance.

Results

Species identification and solvent extraction of 13G027

Homogenous bacterial strain 13G027 was associated from marine sponge and identified via 16S rRNA gene analysis. The bacterial strain 13G027 displayed 99.6% similarity with *Nocardiopsis* sp. TZQ47 (accession no. HQ143638) according to a BLAST search of the GenBank database [12]. The NCBI GenBank accession number for the 16S rRNA gene of strain 13G027 was registered as MT823009. Crude extracts (150 mg) were prepared via EtOAc extraction of the culture broth (5 L) of *Nocardiopsis* sp. 13G027 and used for subsequent analyses.

Antioxidant activity of the 13G027 EtOAc extract

In order to check the antioxidant activity of the 13G027 EtOAc extract, radical-scavenging activity was indirectly evaluated using the ABTS assay. This experiment showed that the free radical-scavenging activity of the extract increased linearly as the concentration increased (Fig. 1). In the ABTS assay, the concentration of the 13G027 extract to scavenge 50% (SC_{50}) of the free radicals was 30.1 μ g/ml (equivalent to 24.7 μ g/ml vitamin C, Fig. 1). This result suggests that the 13G027 EtOAc extract effectively scavenges free radicals.

Effect of the strain 13G027 for cell viability in RAW 264.7 macrophages

We tested the cell viability of the 13G027 extract using RAW 264.7 cells to identify the optimal concentration of the extract for anti-inflammatory effects with minimal toxicity. At a concentration of 300 μ g/ml, the extract

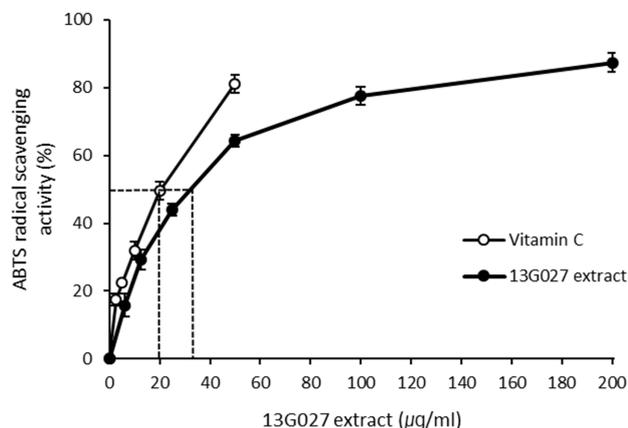


Fig. 1. Radical-scavenging activity of the *Nocardiopsis* sp. 13G027 extract in the ABTS assay. Vitamin C was used as a positive control. After incubation, the absorbance at 734 nm was checked, and radical-scavenging activity was analyzed. The dotted line showed the concentration of 13G027 and positive control required to scavenge 50% of radicals (SC_{50}). The error bar represents the mean standard deviation of the three repeated experiments. Significant differences between the 13G027 extract and vitamin C control groups ($n = 3$) were evaluated using the t -test.

reduced cell survival by approximately 10% (Fig. 2A). No cytotoxicity was observed at lower concentrations.

13G027 extract inhibiting NO production on LPS-induced RAW 264.7 macrophages

NO is known as signaling molecule, generated by inductive nitric oxide synthases that are critical for inflammatory reaction, and is increased by inflammation or damage [16, 17]. We investigated the anti-inflammatory effect of the 13G027 extract on LPS-induced RAW 264.7 macrophages. NO production was significantly increased by LPS stimulation. However, pretreatment with the extract significantly inhibited the level of NO production (91.6%, 78.2%, and 14.4%) at 50, 100, and 300 μ g/ml, respectively (Fig. 2B).

13G027 extract inhibiting the production of PGE₂ in LPS-induced RAW 264.7 macrophages

PGE₂ production in macrophage culture was tested by the method of enzyme immunoassay kits (Cayman Chemical, USA). 13G027 extract considerably inhibited the level of PGE₂ production (39.3%, 26.9%, and 5.8%) at 50, 100, and 300 μ g/ml, respectively (Fig. 2C).

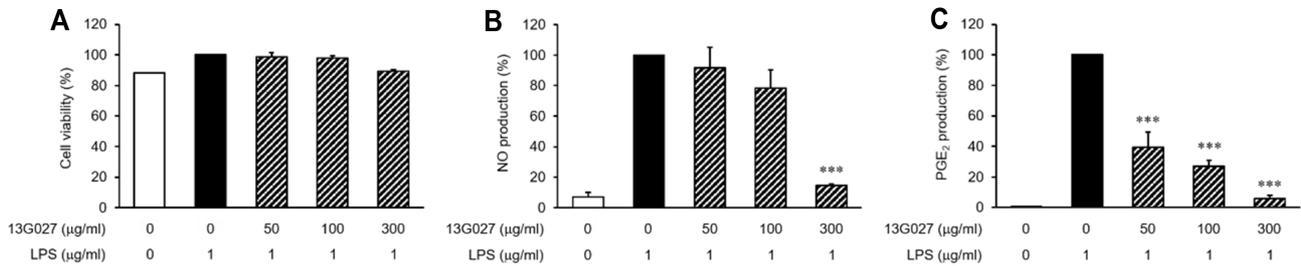


Fig. 2. Effect of the *Nocardiosis* sp. 13G027 extract on NO and PGE₂ production on LPS-stimulated RAW 264.7 macrophages. The pretreated cells with the 13G027 extract were washed using PBS, and stimulated with 1 µg/ml LPS. RAW 264.7 cells were treated with the 13G027 extract for 24 h, and cell viability was examined (A). NO and PGE₂ contents into media were quantified by Griess reagent (B), and the PGE₂ concentration (C) was checked by ELISA. Each bar shows the average \pm standard error of the mean in three repeated experiments. *** $p < 0.001$ vs LPS stimulation.

Effect of 13G027 extract on the MAPKs and Akt phosphorylation in LPS-induced RAW 264.7 macrophages

MAPK and phosphoinositide-3-kinase (PI3K)/Akt signaling pathways function upstream of the nuclear factor-kappa B signaling pathways and are important for LPS-stimulated inflammatory reactions in RAW 264.7 macrophages [18, 19]. Western blot tests showed that the 13G027 extract effectively inhibited the MAPKs

(p38, JNK, and ERK) and Akt phosphorylation (Fig. 3). Thus, the 13G027 extract inhibited inflammatory response through blocking the MAPK and Akt signaling pathways in the RAW 264.7 macrophages.

13G027 extract inhibiting AP-1 activity on LPS-induced RAW 264.7 macrophages

The production of inflammatory mediators is regu-

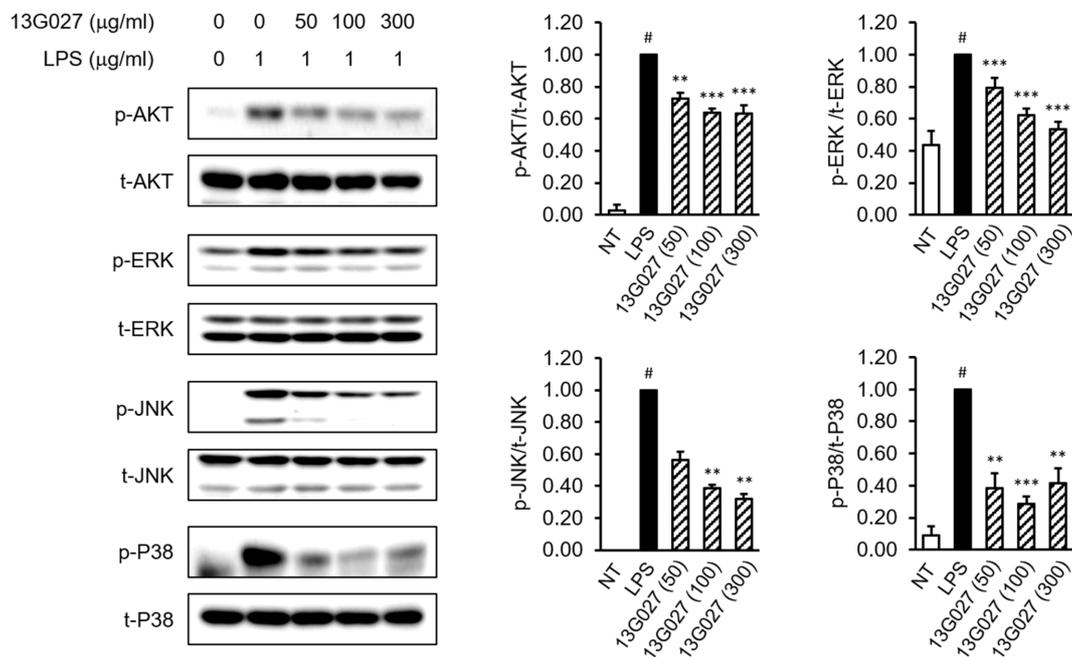


Fig. 3. Effects of the *Nocardiosis* sp. 13G027 extract on the phosphorylation of MAPKs and Akt in LPS-activated RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with vehicle or 13G027 extract for 30 minutes before LPS (1 µg/ml) stimulation. (Left) Cell extract was prepared and Western blotting was performed by specific antibodies to the total and phosphorylated forms of MAPKs (p38, JNK, and ERK; for 15 min) and AKT (for 4 h). (Right) t-AKT, t-ERK, t-JNK, and t-P38 were measured as control of p-AKT, p-ERK, p-JNK, and p-P38 for relative intensity. These results are expressed in the mean \pm SD of triple experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

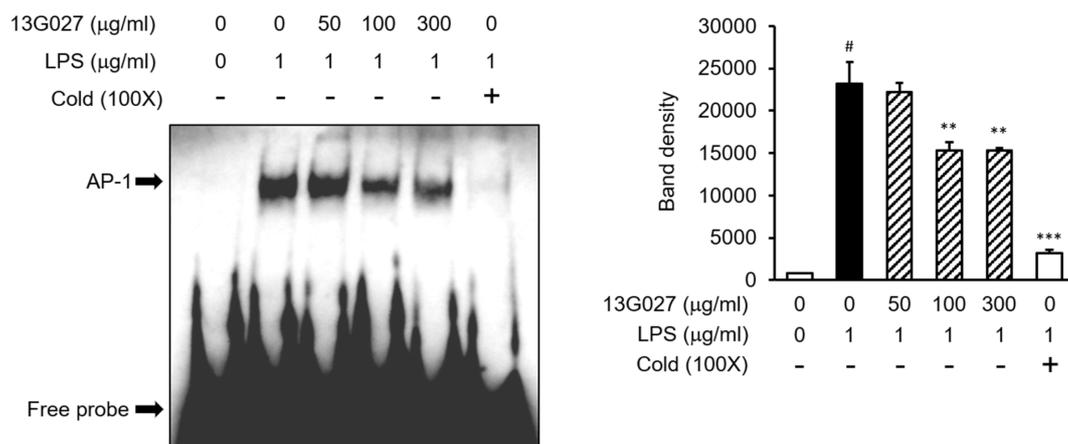


Fig. 4. Effect of the *Nocardiosis* sp. 13G027 extract on AP-1 activation in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were preprocessed with 13G027 extract (30, 50, and 100 $\mu\text{g/ml}$) for 1 h and induced for 2 h using LPS. Nuclear extracts were prepared and evaluated for AP-1 expression by EMSA. Cold: cold (unlabeled) probe. These results are expressed in the mean \pm SD of triple experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

lated through AP-1 in the inflammatory process [20–22]. Thus, to clarify the mechanism of 13G027 as inflammatory mediators, we examined the effects of its extract on the DNA-binding activity of AP-1 by EMSA. LPS stimulation was increased the DNA-binding activity of AP-1, whereas this activity was significantly inhibited by pretreatment with the 13G027 extract (Fig. 4).

Effect of the 13G027 extract on the NPDFs viability

The viability of NPDFs was investigated by the DMEM assay after the 13G027 extract treatment. The extract was not cytotoxic to NPDFs at concentrations up to 100 $\mu\text{g/ml}$ (Fig. 5A). For further study, 13G027 extract ranging from 30 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ was selected.

Effect of 13G027 extract on the expression of α -SMA, Col-1, and fibronectin on TGF- β 1-activated NPDFs

We questioned whether 13G027 weakened the effects of TGF- β 1 on NPDFs. To answer to this question, the cells were treated with the 13G027 extract (30–100 $\mu\text{g/ml}$) for 30 min before TGF- β 1 (1 ng/ml) stimulation for 24 h. In the result, expression of α -SMA, Col-1, and fibronectin appeared significantly reduced by treatment of a 13G027 extract (Fig. 5B).

Effect of 13G027 extract on Smad2 and Smad3-dependent signaling pathways in TGF- β 1-stimulated NPDFs

TGF- β 1 induction in the nucleus significantly

increased Smad2 and Smad3 phosphorylation in NPDFs (Fig. 5C). However, when the cells were pretreated with 13G027 extract for 30 min, particularly at 30–100 $\mu\text{g/ml}$, prior to 24 h of TGF- β 1 activation, significantly reduced the accumulation of phosphorylated Smad2 and Smad3 in the nucleus. Therefore, the anti-fibrotic effects of 13G027 can be mediated by inhibition of the Smad2 and Smad3 signaling pathways induced by TGF- β 1.

Discussion

Antioxidants are critical for redox process through protecting cells from inflammation and apoptosis [23]. Oxidative stress induced by LPS or other factors might cause macrophage activation, resulting in an excessive inflammatory response [24]. In this study, we demonstrated that the 13G027 extract can alleviate several inflammatory symptoms owing to its anti-inflammatory characterizations.

We investigated the phosphorylation of MAPKs, Akt, and AP-1. The findings suggest that 13G027 has an inhibitory activity against the regulation mechanisms associated with anti-inflammatory reactions and that it is a potential candidate for treating LPS-activated inflammatory diseases. MAPKs (p38, JNK, and ERK) regulate pro-inflammatory signal transduction pathways critical for the inflammatory reaction of LPS-stimulated macrophage cells [25]. The PI3K/Akt pathway is

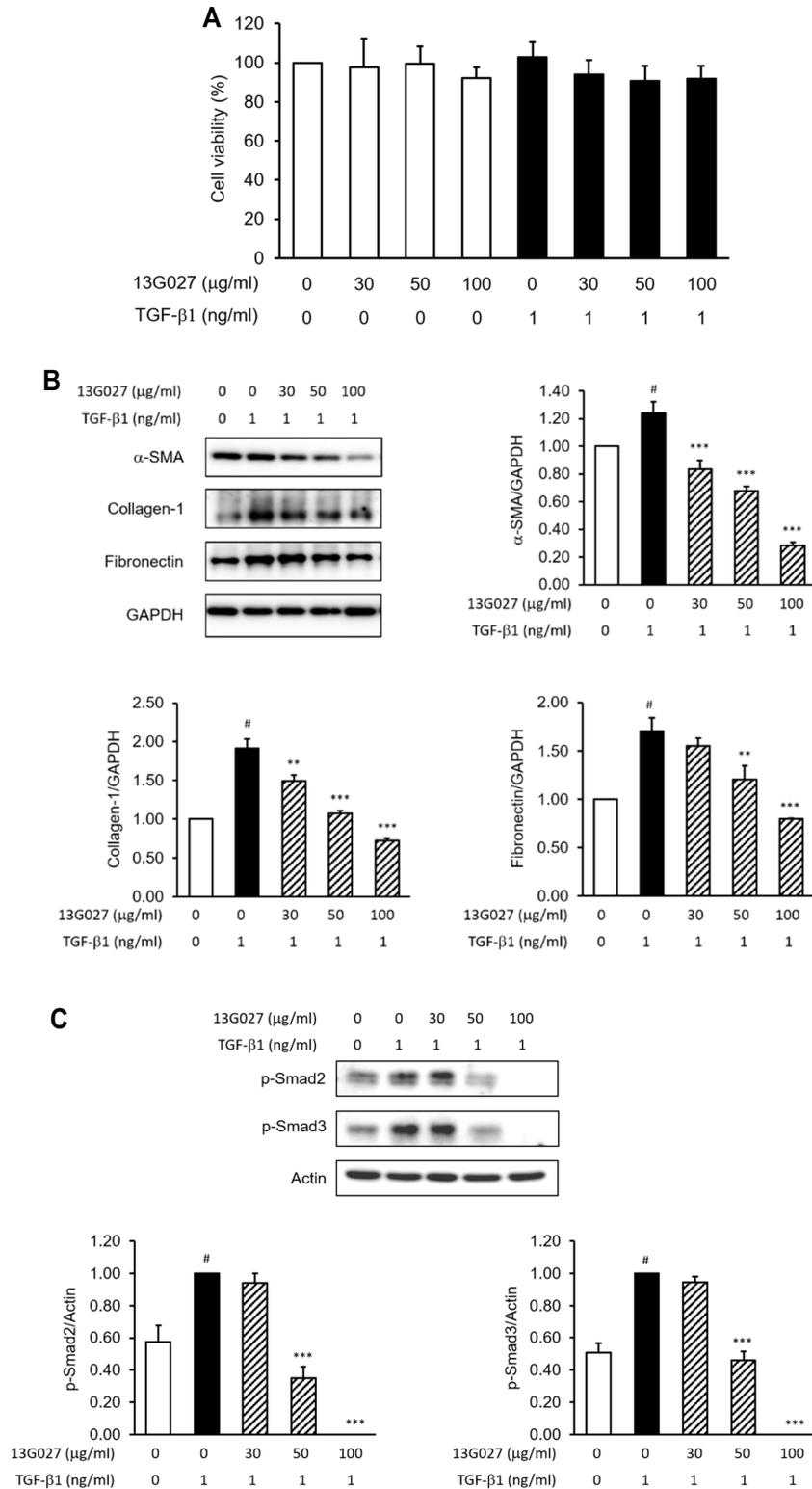


Fig. 5. Effect of the *Nocardiosis* sp. 13G027 extract on the protein expression of α -SMA, Col-1, fibronectin, p-Smad2, and p-Smad3 in TGF- β 1-stimulated NPFDs. Cell viability (A). Expression of α -SMA, collagen-1 (Col-1), and fibronectin (B). p-Smad2 and p-Smad3 expression (C). All experiments were conducted in triplicate, and representative data are presented ([#] $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$).

also associated with the induction of pro-inflammatory factors [26]. Thus, the use of anti-inflammatory agents, effectively modulating MAPK and Akt pathways, are potential modalities for treating inflammatory diseases. To elucidate the signaling process related with the 13G027-mediated regulation of pro-inflammatory mediator expression, the activity of 13G027 on the phosphorylation of MAPKs and Akt was confirmed. Our results suggested that 13G027 can suppress LPS-activated inflammatory process mediated by MAPK and Akt signaling pathways.

Previous studies demonstrated that AP-1 regulates the production of several inflammatory mediators [20, 21]. Thus, we checked the activities of 13G027 on the AP-1 activation in LPS-stimulated RAW 264.7 cells. The activated transcription factors enter the nucleus and induce inflammatory mediator production. thus, we evaluated the DNA-binding activity of AP-1, confirming that the 13G027 extract inhibited the LPS-induced activity of AP-1 in a concentration-dependent manner.

After observing the anti-inflammatory effects of the 13G027 extract in LPS-stimulated RAW264.7 macrophages, we examined the relationship between NP formation and inflammation. NPs may arise from the mucosa of the nose or paranasal sinuses, and they are believed to result from inflammation of the mucosa [27]. α -SMA, Col-1, and fibronectin are expressed in myofibroblasts, which proliferate and display contractile properties [28]. ECM accumulation can play an important role for NP formation in the pathogenesis [29]. α -SMA expression is known to be associated with increased fibroblast contraction and decreased fibroblast motility, which are characteristics of mature myofibroblasts. Collagen is known to play an important role in extracellular space of various connective tissues in animals [30]. In addition, Col-1 deposition is higher in NPs than in normal nasal tissue. Thus, we investigated the inhibitory activities of *Nocardiosis* sp. 13G027 on ECM accumulation on NPfDs stimulated by TGF- β 1 at non-cytotoxic concentrations.

It is known that damage of the mucosal epithelium activities TGF- β 1 expression [31]. TGF- β 1 of high levels expressed in NP tissues, is related with the structural modifications that characterize NP formation [32]. The TGF- β 1 upregulated proteins expression of α -SMA, Col-1, and fibronectin in NPfDs in the previous reports [1,

33]. The current study results suggested that 13G027 reduces the TGF- β 1-activated production of α -SMA, Col-1, and fibronectin through inhibiting TGF- β 1-stimulated signal pathways. TGF- β 1 activates fibrous reactions primarily via the Smad signaling pathways, where Smad is the standard component of the signaling pathway of TGF- β family. Thus, regulation of Smad pathway supports a useful therapeutic strategy for NP, in which, upon TGF- β 1 activation, Smad is phosphorylated through a specific cell surface receptor (receptors, type I and type II) interacting with the TGF- β receptors, where Smad2 and Smad3 are oligomerized with Smad 4 and then transferred to the nucleus, which activates the transcription of the TGF- β -response gene [33]. From this point of view, we studied whether 13G027 inhibits the Smad2/3 phosphorylation and nuclear accumulation in NPfDs. The finding indicated that 13G027 can induce α -SMA, Col-1, and fibronectin production in NPs by modulating Smad2/3 signal activation.

This study suggested that *Nocardiosis* sp. 13G027 extract is a useful therapeutic agent for treating NP formation. Moreover, this possibility provides important implications for the development of new therapeutic approaches for fibrotic disease management in the future.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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