Thesis for the Degree of Master of Engineering

Inhibitory Effect of *Ecklonia cava* Phlorotannin on Vocal Fold Fibrosis: *In vivo* and *In vitro* Study

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Interdisciplinary Program of Biomedical Mechanical & Electrical Engineering

College of Engineering

Pukyong National University

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성대 섬유화에 대한 감태 플로로타닌의 억제 효과: *In vivo* 와 *In vitro* 연구

Advisor: Prof. Won-Kyo Jung

by

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Abstract

성대 섬유화(Vocal fold fibrosis)는 체내의 재생 및 반응 과정에서 섬유아세포(Fibroblast)의 비정상적인 증식(Proliferation) 및 분화(Differentiation)로 인해 섬유성 결합조직이 과도하게 형성되는 상태를 의미한다. 특히, 재생 과정에서는 손상 부위에서의 섬유모세포의 이주(Migration)와 증식, 섬유모세포(Myofibroblasts)에 의한 세포외 기질(Extracellular matrix, ECM)의 과도한 축적에 따라 진행되며, 선천적 및 후천적 요인에 의해 다양하게 발병하는 것으로 알려져 있다.

이러한 성대 섬유화를 예방 및 치료하기 위해 여러 연구가 진행되었으나 현재까지는 항암제로 쓰이며, 다양한 부작용을 지닌 마이토마이신 C (Mitomycin C)와 부데소나이드(Budesonide)와 베타메타손(Betamethasone)과 같은 당질 코르티코이드(Glucocorticoid) 계열의 치료제 이외에는 마땅한 성대 섬유화 치료제는 없는 실정이다. 이에 본 연구에서는 성대 섬유화를 예방 및 치료를 위한 생리활성물질을 해양으로부터 찾고자 본 연구를 진행하였다.

본 연구에서는, 해양 생물 중 항염증, 항산화, 항암, 항당뇨 등 다양한 생리활성 효과를 지닌 것으로 알려진 감태 플로로탄닌(*Ecklonia cava* phlorotannin)을 이용하여 성대 섬유화 예방 및 치료 효과를 확인하였다. 그 결과, 1470 nm 레이저 조사를 통해 성대 섬유화가 유도된 토끼 모델에서의 성대 섬유화와 극심한 염증 반응을 감소시켰으며, 인간 유래 성대 섬유아세포(Human vocal fold fibroblast,

hVFFs)에 대하여 전환성장인자-베타1(Transforming growth factor-β1, TGF-β1)의 자극에 의해 증가된 1형 아교질(Type Ⅰ collagen)의 발현을 억제하였다. 이러한 결과들은 감태 플로로탄닌이 성대 섬유화 예방 및 치료 효과를 지닌 것을 시사한다. 또한, 감태 플로로탄닌의 항섬유화 효과에 대한 작용 기전(Action mechanism)을 밝히기 위해 섬유화와 관련된 신호전달경로(Signaling pathway)에 대하여 western blot 분석을 실시한 결과, 감태 플로로탄닌이 p38 미토겐 활성화 단백질 인산화효소(p38 mitogen-activated protein kinases, p38 MAPK)와 세포외 신호조절 인산화 효소(Extracellular signal-regulated kinase, ERK)의 인산화(Phosphorylation)를 억제함으로써 항섬유화 효과를 나타내는 것을 확인하였다. 결론적으로, 감태 유래 플로로탄닌의 p38 미토겐 활성화 단백질 인산화 효소와 세포외 신호조절 인산화 효소의 활성화 억제를 통한 항섬유화 효과를 통해 추후 성대 섬유화의 예방 및 치료가 가능할 것으로 사료된다.

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Abbreviations

Abbreviations	Full name
VFs	Vocal folds
LP	Lamina propria
ECM	Extracellular matrix
hVFFs	Human vocal fold fibroblasts
GAGs	Glycosaminoglycans
α-SMA	α-smooth muscle actin
bFGF	Basic fibroblast growth factor
COX-2	Cyclooxygenase-2
IL1 β	Interleukin-1β
TGF-β1	Transforming growth factor-β1
E. cava	*Ecklonia cava*
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
Trypsin-EDTA	Tyrpsin-ethylenediminetetraacetic acid
MTT	3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
DW	Distilled water
NEAA	Non-essential amino acid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
TBS	Tris buffered saline
TBS-T	Tris buffered saline containing Tween 20
Smad 2/3	Mothers against decapentaplegic homolog 2/3
p38 MAPK	p38 mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
ERK	Extracellular signal–regulated kinase
H&E	Hematoxylin and eosin

1. Introduction

1.1. Molecular and cellular structure of vocal fold

The human vocal folds (hVFs) are two strips of tissue located in the larynx and play important roles in the phonation for critical effective oral communication (Gaston and Thibeault 2013, Kim, Yi et al. 2013). It is composed of multiple layers including the epithelium, the lamina propria (LP), and the vocal muscle (Allen 2010). Its sophisticated layered structure contributes to the unique vibratory property of the hVFs (Friedrich, Dikkers et al. 2013).

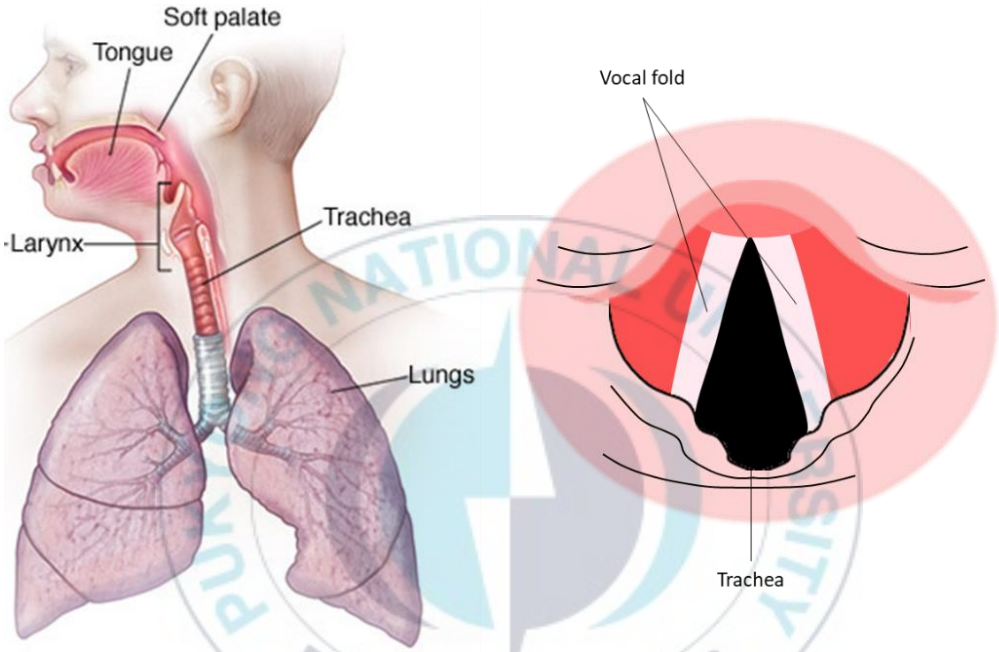
The epithelium layer, the outermost layer of the VFs, is stratified by five to ten squamous epithelium layers and plays a role as a protective barrier for deeper tissues (Tse, Zhang et al. 2015, Valerie, Vassiliki et al. 2016). The LP is subdivided into three layers: the superficial layer, the intermediate layer, and the deep layer (Valerie, Vassiliki et al. 2016). The superficial layer of the LP is contributed biomechanical properties of vocal cover (viscosity and stiffness) because of its cellular and molecular composition and organization. It is composed of myofibroblasts and macrophages, which surrounded by the loose extracellular matrix (ECM), composed of fibrous elements including collagen, elastin, fibronectin, hyaluronic acid, and proteoglycans (Chhetri and Mendelsohn 2010, Finck, Harmegnies et al. 2010). At the boundary between the epithelium layer and the superficial layer of the LP, there is the basal lamina (basement membrane), a thin supportive structure for epithelium. It consists of collagen and laminin and its cell is arranged columnar or polyhedral (Lee, Lee et al. 2015, Valerie, Vassiliki et al. 2016, Sato 2018). The intermediate layer of LP consists

of cellular components similar to the superficial layer, but the major element of ECM is the hyaluronic acid and the elastic fibers (Valerie, Vassiliki et al. 2016). The deep layer, the densest layer consisted of collagenous fibers among LP, is mostly composed of macrophages and fibroblasts (Catten, Gray et al. 1998). The vocal cover included the epithelium and superficial layer of the LP and can be vibrated for sound in speech due to their biomechanical property which is relatively more pliable than the deeper body (intermediated and deep layers of the LP and thyroarytenoid muscle) (Friedrich, Dikkers et al. 2013). The vocal muscle, the main body of the vocal fold, is an upper portion of thyroarytenoid muscle and provides stability and mass on the VF (Yang, Wang et al. 2015).

1.2. Injury of vocal folds and fibrosis

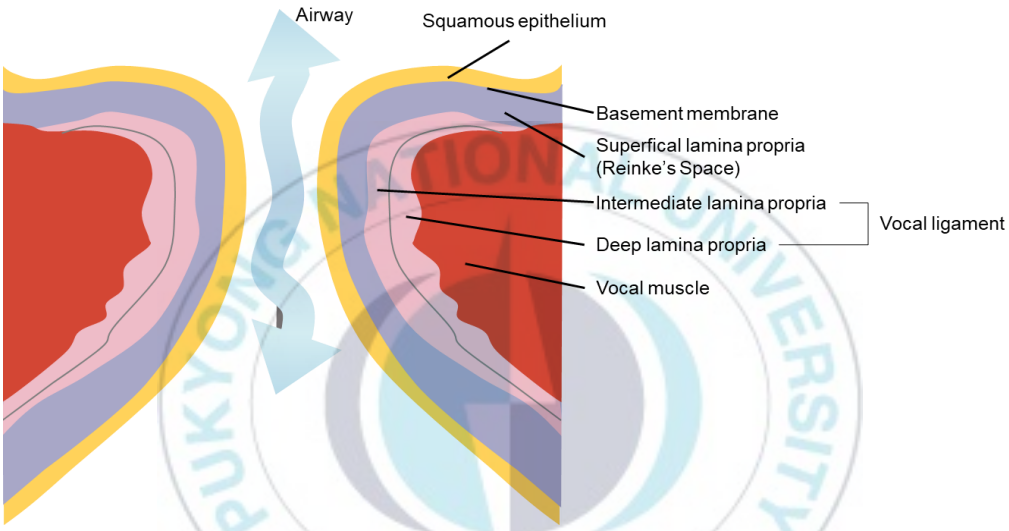
When the injury to the VFs occurs, patients can experience various structural and functional VF abnormalities such as atrophy, nodules, polyps, scar through vocal fold fibrosis, dysphonia, vocal fold paralysis, sulcus vocalis, irregular vibration, and incomplete glottal closure (Welham, Choi et al. 2011, Dankbaar and Pameijer 2014, Sinclair, Bumpous et al. 2016, Stephenson and Wyatt 2016, Cho, Choi et al. 2017). Especially, vocal fold fibrosis are most commonly caused by trauma related to prolonged intubation, tracheostomy, inhalational burns, irradiation, infection, and surgical defect (George, Jaquet et al. 2010, Mortensen 2010, Svensson, Nagubothu et al. 2010),(Campagnolo, Tsuji et al. 2010, Vorasubin, Vira et al. 2014, Stephenson and Wyatt 2016). It occurs from abnormal wound healing, which progresses proliferation of fibroblasts and increases fibrous protein such as collagen, elastin, hyaluronic acid, fibronectin, and other constituents of the ECM around the wound site through specific molecular pathways within several cells in the normal process (Lim, Tateya et al. 2006).

There are diverse treatments for vocal fold fibrosis such as invasive procedures, laser treatment, stent placement, cryosurgery, treatment of corticosteroids, and treatment of mitomycin C (Mortensen 2010, Woo, Jeong et al. 2014, Suzuki, Kawai et al. 2017). In particular, mitomycin C is used primarily for the treatment of vocal fold fibrosis, but it has several side effects including lung fibrosis, bone marrow depression, and edema (Whited and Dailey 2015). Therefore, it is not appropriate as an effective and safe treatment (George, Lang et al. 2005).



[Figure adapted from the site of Mayo Foundation for Medical Education and Research (MFMER) and Anatomylibrary]

Figure 1. Anatomical position of vocal folds



[Figure adapted from Ref. (Rosen and Simpson 2008)]

Figure 2. The layered microanatomical structures of vocal folds

1.3. Vocal fold fibroblasts

Human vocal fold fibroblasts (hVFFs) play an important role in the healing processes and production of ECM, mainly constituted by various fibrous proteins and glycosaminoglycans (GAGs) (Thibeault, Klemuk et al. 2011). In addition, the hVFF's role in wound healing is regulated by different cells including inflammatory cells, epithelial cells, and other fibroblasts (Vyas, Ishikawa et al. 2010, Berg, Kolachala et al. 2011). The components of ECM give LP unique structure and pliability of vocal fold (Allen 2010). During wound repair proceeds, hVFFs are activated and then differentiated into α-smooth muscle actin (α-SMA)-expressing human vocal fold myofibroblasts (Branco, Bartley et al. 2016). Vocal fold myofibroblasts play an important role in the earlier stages of wound healing and can secrete excessive amounts of type I collagen, but their number normally decreases when the wound is closed (Mann, Oakley et al. 2007). If hVFFs aberrantly proliferate or over-activate during wound healing processes, it can result in fibrosis due to over-production of ECM (Dolivo, Larson et al. 2017). Moreover, several pro-fibrotic or inflammatory mediators such as basic fibroblast growth factor (bFGF), cyclooxygenase-2 (COX-2), interleukin-1β (IL1β), and transforming growth factor-β1 (TGF-β1) modulate ECM production through control of fibroblast response and activation (Dolivo, Larson et al. 2017, Erndt-Marino, Jimenez-Vergara et al. 2017). Among them, TGF-β1 is a major promoter of fibrosis through indirect mechanisms and induced the transition of fibroblasts into α-SMA-expressing myofibroblasts (Akdogan, Selcuk et al. 2009, Mitchell, Kojima et al. 2014, Hiwatashi, Bing et al. 2017, Hiwatashi, Hirano et al. 2017). Based on these facts, it is important for the prevention and/or treatment of vocal fold fibrosis to modulate fibroblast response to injury during wound healing processes.

1.4. *Ecklonia cava* phlorotannin

Seaweeds are classified into three broad groups into brown, green, and red algae according to their color (Nguyen, Ko et al. 2016). Particularly, brown algae are a large group of marine seaweeds and contain fucoxanthin, a valuable pigment that determines their color (Wijesinghe and Jeon 2011, Wijesinghe and Jeon 2012). They inhabit all over the world and some are investigated their commercial values in the pharmacological application (Davis, Volesky et al. 2003). In previous studies, they contain a variety of biological compounds including pigments, alginate, different polysaccharides, fucoidan, vitamins, and unique secondary metabolites (Cho, Jung et al. 2009, Heo, Ko et al. 2009, Heo, Yoon et al. 2010). Additionally, their content varies depending on the season, age, species, geographical area, and environmental factors (Balboa, Conde et al. 2013).

Ecklonia cava (Figure 4) is a brown alga found usually in Jeju Island in Korea and Japan and has been reported to be utilized in biotechnology and industrial fields such as food, animal feed, fertilizer, and medicine (Lee, Kang et al. 2015, Kim, Youn et al. 2018). It possesses diverse bioactive compounds and shows biological activities (Choi, Lee et al. 2015, Kong, Kim et al. 2015). Among bioactive compounds extracted from *E. cava*, phlorotannin is one of the most diverse and widespread groups of natural compounds and included organic polymers of phloroglucinol (1,3,5-trihydroxybenzene) (Heo, Yoon et al. 2012, Rengarajan, Rajendran et al. 2013, Zhang, Fang et al. 2014). It is composed various phloroglucinol derivatives such as 6,6’-bieckol (a hexamer), dieckol (a hexamer), eckol (a closed-chain trimer of phloroglucinol), phlorofucofuroeckol (a pentamer), and triphlorethol-A (a opened-chain trimer of phloroglucinol) (Chung, Choi et al. 2013) (Figure 5). Furthermore, they exhibits

various biological effects including anti-matrix metalloproteinase activity (Urikura, Sugawara et al. 2011), anti-oxidative (Peña, Campos et al. 1997), anti-allergic (Fuks, Filipiuk et al. 2006), anti-obesity (Thu, Zulfakar et al. 2012), anti-hypertensive (Yu, Du et al. 2010), anticoagulant (Hunt, Smith et al. 2010), anti-inflammatory effects(Sharma, Sanpui et al. 2012). The biological activities of *E. cava* summarized in Table 1 and 2. Although the diverse biological activities of *E. cava* phlorotannin are investigated widely, there is no study reporting their antifibrotic activity on vocal fold.

Figure 3. Morphology of human vocal fold fibroblasts (hVFFs)

1.5. Goal of this study

The aim of this study was to study that inhalation of *E. cava* phlorotannin suppresses vocal fold fibrosis in the animal model which was induced fibrosis of vocal fold by a cylindrical diffuser and *E. cava* phlorotannin has the antifibrotic effect and its underlying mechanisms on hVFFs, which can be stimulated by TGF-β1 to mimic the conditions of vocal fold fibrosis.

[Figure adopted from Site of Jeju innovation resources integrated management system]

Figure 4. *Ecklonia cava* (*E. cava*)

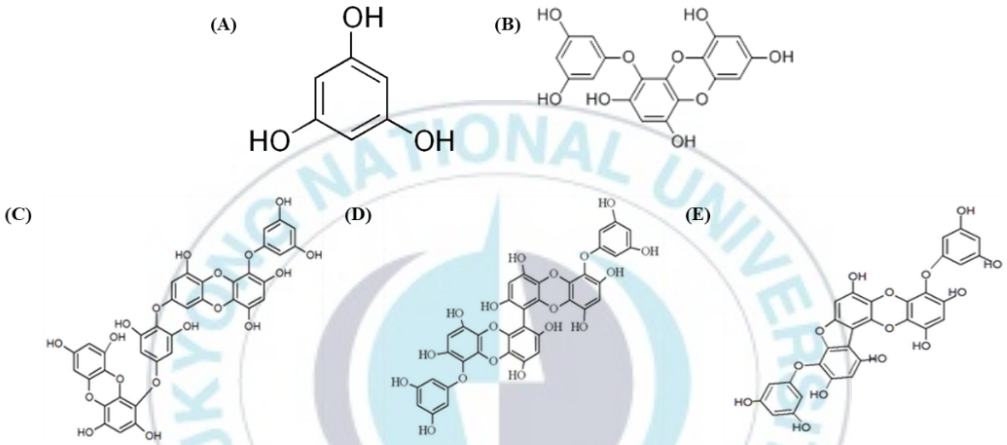


Figure 5. Chemical structure of single compounds contained in *Ecklonia cava* phlorotannin. **(A)** Phloroglucinol, **(B)** Eckol, **(C)** Dieckol, **(D)** 6,6'-bieckol, **(E)** Phlorofucofuroeckol A

Table 1. Biological activity of *Ecklonia cava* extracts

Extract solvent	Health effect	Reference
Enzymatic extracts	Antioxidant, Anti-inflammatory, Anti-allergy, Anti-proliferative, Anti-cancer, Anti-tumor, Anti-hypertensive, and etc	(Athukorala and Jeon 2005, Heo, Park et al. 2005, Athukorala, Kim et al. 2006, Kim, Heo et al. 2006, Ahn, Hwang et al. 2008, Ahn, Park et al. 2008)
Ethanol	Anti-inflammatory, Ant-iallergy, Cytoprotective, and etc.	(Kim, Lee et al. 2008, Jung, Ahn et al. 2009, Choi 2016)
Methanol	Antioxidant, Anti-inflammatory, Anti-allergy, Anti-diabetic, Anti-HIV, and etc.	(Ahn, Yoon et al. 2002, Shim, Quang-To et al. 2009, Kang, Jin et al. 2010, Lee, Ko et al. 2010)
Organic solvent	Anti-inflammatory, Anti-allergy, and etc.	(Kim and Bae 2010)
Water	Antimicrobial, Antioxidant, and etc.	(Kim, Moon et al. 2008)

Table 2. Biological activity of single compounds in *Ecklonia cava* phlorotannin

Singe compounds	Health effect	Reference
6,6'-bieckol	Anti-HIV, Antihypertensive, Antioxidant, Anti-inflammatory, Anti-obesity, etc.	(Artan, Li et al. 2008, Kwon, Wu et al. 2015, Park, Heo et al. 2015, Kim, Lee et al. 2016, Ko, Kang et al. 2017)
Dioxinodehydroeckol	Antiproliferative, Anti-adipogenic, Skin protection, Hair growth–promotion, Osteogenesis activity, etc	(Kong, Kim et al. 2009, Choi, Jeon et al. 2015, Ryu, Ahn et al. 2015, Ahn, Karadeniz et al. 2016, Shin, Cho et al. 2016)
Eckol	Radioprotective, Antioxidant, activation of hemo oxygenase-1 expression, cytoprotective effect against oxidative stress, tyrosinase inhibition, etc.	(Kang, Lee et al. 2005, Zhang, Kang et al. 2008)
Dieckol	Antidiabetic, Radioprotective, Aantiinflammatory, Anticancer, Anti-diabetic, etc.	(Heo, Ko et al. 2009, Eom, Lee et al. 2012, Ahn, Yang et al. 2015, Lee, Kang et al. 2015, Kim, Lee et al. 2016)
7-Phloroeckol	Skin protection, Anti-diabetic, Anti-inflammatory, anti-glycation, anti-adipocyte differentiation	(Yoon, Eom et al. 2009, Lee, Park et al. 2012, Jung, Jin et al. 2013, Lee and Jeon 2013, Bak, Sung et al. 2014)
Phlorofucofuroeckol A	Antiallergic, Memory-enhancing, Anti-diabetic, Anti-oxidant, anti-lipid peroxidation, etc.	(Myung, Shin et al. 2005, Lee, Kwon et al. 2012, Ahn, Yang et al. 2015, Lee, Ko et al. 2015)

2. Materials and methods

2.1. Materials

Human vocal fold fibroblasts kindly provided by Professor Byung-Joo Lee from the Medical Research Institute, Pusan National University, Busan, Korea. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/ streptomycin/amphotericin (10,000 U/ml, 10,000 μg/ml, and 2,500 μg/ml, respectively), phosphate buffered saline (PBS), and Tyrpsin-ethylenediminetetraacetic acid (trypsin n-EDTA) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), recombinant human transforming growth factor-β1 (TGF-β1), Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The specific antibodies used for western blot analysis were: Collagen I (ab34710, abcam), p-Smad2/3 (8828S, Cell signaling), Smad2/3 (5678, Cell signaling), p-Akt (9271, Cell signaling), Akt (sc-1618, Santa Cruz), ERK 1/2 (sc-292838, Santa Cruz), p-ERK 1/2 (sc-7383, Santa Cruz), JNK 1/2 (sc-734, Santa Cruz), p-JNK 1/2 (sc6254, Santa Cruz), p38 MAPK (sc-7149, Santa Cruz), p-p38 MAPK (sc-7973, Santa Cruz), GAPDH (sc25778, Santa Cruz), β-actin (sc-130656, Santa Cruz), donkey anti-goat IgG-HRP (sc-2020, Santa Cruz), goat anti-mouse IgG-HRP (sc-2031, Santa Cruz), goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody. The other chemicals and reagents used were of analytical grade and were commercially available.

2.2. Extraction of *Ecklonia cava* phlorotannin

The *E. cava* phlorotannin was isolated as previously described by Heo et al (Heo, Ko et al. 2009) with slight modifications. The marine alga *E. cava* was collected at the coast of Jeju Island, Korea. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface. Then carefully rinsed with fresh water, and maintained in a medical refrigerator at 20°C. Thereafter, the frozen samples were lyophilized and homogenized with a grinder prior to extraction. The dried *E. cava* powder was extracted three times with 70% ethanol (1:10 w/w) and then was filtered. The *E. cava* phlorotannin was freshly dissolved in saline or distilled water (DW) before using *in vivo* and *in vitro* experiments, respectively.

2.3. Animal model of vocal fold fibrosis

The fibrosis-induced animal model was made following the previous study with slight modifications (Lee, Kim et al. 2017). Briefly, twelve New Zealand white rabbits (Taesung Laboratory Animal Science, Busan, Korea) weighing 2.0-2.5 kg (male) were enrolled in this study. The experiment protocol was approved by the Committee on Animal Research of the College of Medicine at Kosin University (Rosen and Simpson 2008). Each of the rabbits was intramuscularly anesthetized with 5 mg/kg of Zoletil and 5 mg/kg of Rompun. Each rabbit was placed in the supine position on a heated operating table. As a light source, a 1,470 nm diode laser system (FCW-1470, CNI, Changchun, China) was employed to entail tissue coagulation (defined as discoloration in tissue) in the vocal folds. The cylindrical diffuser was inserted trans-orally under bronchoscopy view and laser irradiation was conducted at the intended location within the vocal fold. Under the endoscopic view, the ‘active segment’ of the diffusing device

was positioned in the center of the vocal folds. Laser irradiation was performed at the power of 10 W for 5 seconds (applied energy = 50 J). In our previous study, we demonstrated that the conditions of 10W, 7 seconds irradiation led to severe grade (≥ 75%) of vocal fold fibrosis, which led to death in most rabbits.

2.4. *In vivo* evaluation of vocal fold fibrosis

The degree of vocal fold fibrosis was observed weekly up to 1 weeks after laser irradiation with endoscopic examination (Panoview Plus sinuscopeTM, 2.7mm, 58, Richard Wolf, Knittlingen, Germany). The bronchoscope was inserted transorally and the distal end of bronchoscope was located vocal cord in every documentation procedure. The rabbits were euthanized at 1 weeks or when costal retraction, due to airway narrowing was identified. After sacrifice or death of the rabbits, the segment of vocal fold fibrosis was removed at the site of fibrosis and was fixed with 10% neutral buffered formalin. Removed vocal fold was embedded in paraffin and serial sections of 4 mm thickness were stained with hematoxylin and eosin (H&E) and examined by microscopy. All sections were examined under light microscopy and scored semiquantitatively. Seven sections were obtained from each fourteen rabbits; seven from the site of vocal fold in saline inhalation groups and seven from the site of vocal fold in *E. cava* phlorotannin inhalation groups. Histological evaluations were performed separately in saline inhalation groups and *E. cava* phlorotannin inhalation groups. Each section was evaluated for reepithelialization, inflammatory reaction, amount of granulation tissue, and then scored using the following scale; 1 = continuation of epithelialization, 2 = incomplete epithelialization, 3 = necrotic epithelium/epithelial proliferation in the margin of ulcer in reepithelialization, 1 = minimal/mild, 2 = moderate, 3 = marked in inflammatory reaction and amount of

granulation tissue. Six sections of the specimens were used in assessment of the mean score of the inflammatory parameters. Sections were evaluated by the same histologist blinded to the groups. The scoring method of reepithelialzation, inflammatory reaction, and the amount of granulation tissue is summarized in Table 3.

2.5. Isolation and culture of human vocal fold fibroblasts

Two primaries, normal hVFF lines-p59 and p21-were obtained from specimens excised from non-cancerous donors in accordance with our IRB-approved protocol; their derivation has been previously described (Hanson, Kim et al. 2010). Additionally, two immortalized hVFF lines-T59 and T21-whose transduction are reported elsewhere were used in this investigation. Primary and immortalized hVFF were grown in DMEM with 10% FBS and 1× Non-essential amino acid (NEAA). All experiments performed on primary and immortalized cells ranged between passages 4 through 6.

2.6. Cell viability

The hVFFs were seeded in a 96-well plate at a density of 10^4 cells/well. After 24 h, cells were exposed to sample. Cells treated for 24 h with various concentrations of sample and with or without TGF-β1 (2 ng/ml). MTT stock solution (1 mg/ml in PBS) was then added to each well. After 2 h of incubation, the supernatants were aspirated. The formazan crystals in each well were dissolved in 100 μl of DMSO, and the absorbance was measured at 540 nm by using with the microplate reader (Powerwave XS2, BioTek Instruments, Inc., Winooski, USA). Relative cell viability was evaluated in accordance with the quantity of MTT converted to the formazan generated in the

untreated cells was considered to represent 100% viability. The data are expressed as the mean percentage of the viable cells versus the respective untreated cells.

2.7. *In vitro* model on human vocal fold fibroblasts through TGF-β1 treatment

The hVFFs were grown in 100 cm^2 dish at a density of approximately 3×10^5 cells. The *E. cava* phlorotannin was dissolved in DW and filtered through 0.20 μm cellulose membranes. Cells were pre-treated with the human recombinant protein TGF-β1 (R&D systems) followed by stimulation with various concentrations of phlorotannin (31.25, 62.5, 125, and 250 μg/ml) for 24 h.

2.8. Cell migration assay

The hVFFs were seeded in 6 well. The cells were incubated for 24 h in complete medium to adhere to have a density of about 90%. After cells attached to plates, cells made injury line with a 2-mm width tip on the cells. The cells were then rinsed with PBS and allowed to migrate in various concentrations of the sample in the presence or absence of TGF-β1 (2 ng/ml) and a photograph was captured using a Zeiss Axio Observer A1 microscope (Zeiss, Jena, Germany) at time points.

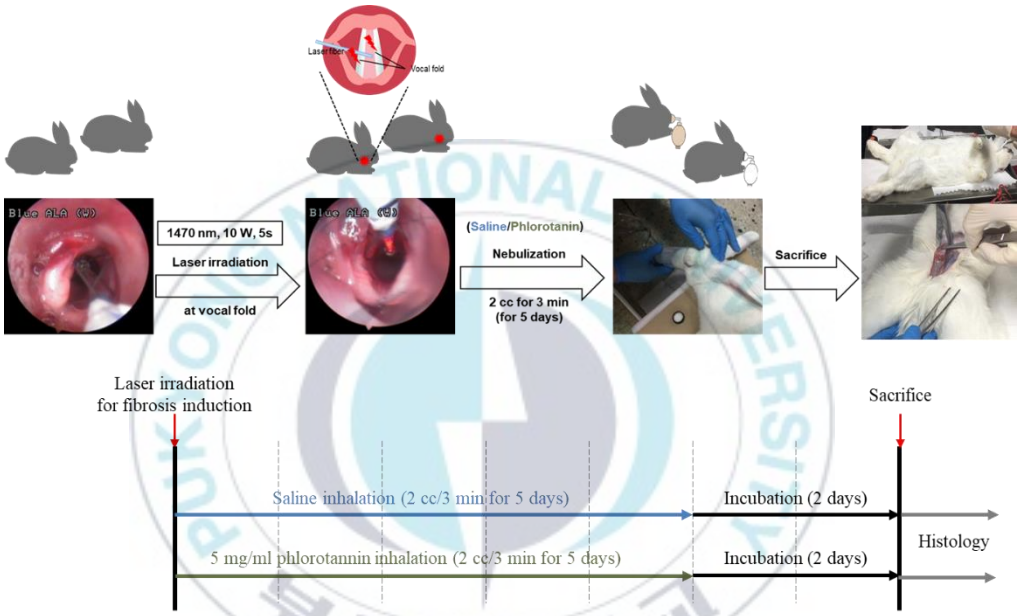


Figure 6. Scheme and experiment time schedule of *in vivo* experiment

2.9. Protein expression analysis by western blot

The cells were lysed in lysis buffer (20 mM Tris, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM PMSF) for 30 min and then centrifuged at 14,000 rpm for 15 min at 4°C. The concentrations of protein that were extracted from the cells were determined using a Bicinchoninic acid (BCA) protein assay with bovine serum albumin (BSA) as a standard. Cell lysates were electrophoresed in Sodium dodecyl sulfate (SDS)–polyacrylamide gels (10%), and the separated proteins were transferred to nitrocellulose blotting membrane (Amersham Protran Premium 0.45 μm NC, GE Healthcare Life Sciences) for 1 h. The membranes were pre-incubated with blocking in 5% nonfat dry milk in tris-buffered saline (TBS) (25 mM Tris-HCl, 137 mM NaCl, 2.65 mM KCl, pH 7.4) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature and then incubated with primary antibodies (1:1,000 dilution) at 4°C for 24 h. After washing with TBS-T, the membranes were incubated with secondary antibodies (1:5,000 dilution) at room temperature for 2 h. The bands were visualized using Davinch Western Imaging System (Davinch-K, Younghwa Science, Korea).

2.10. Statistical analysis

All of the data are presented as the mean ± standard deviation (SD) of three determinations. Statistical comparisons of the mean values were performed by analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS software. The statistical significance of the differences was defined at the $*p < 0.05$ level.

3. Results

3.1. Vocal fold fibrosis and inflammatory reaction suppressed by *Ecklonia cava* phlorotannin inhalation on the fibrosis-induced rabbit model

To assess the degree of fibrosis and inflammation reaction on the vocal fold, we conducted an endoscopic examination immediately after laser irradiation and after 1-week incubation. It observed mucosal damage at vocal fold after the fibrosis inducements by laser irradiation compared with the normal vocal fold (Figure 7 (A), (B), and (D)). After 1-week incubation, fibrosis and ulceration of mucosa progressed in the saline inhalation group (Figure 7 (C)), *E. cava* phlorotannin inhalation group showed slightly vocal fold swelling without significant fibrosis or ulceration (Figure 7 (D)).

In addition, histological analysis of hematoxylin and eosin (H&E)-stained sections after 1-week incubation showed that epithelialization with the thin layer of squamous epithelium continually progressed in the *E. cava* phlorotannin inhalation group whereas ulceration and inflamed granulation tissue persisted without reepithelialization in the saline inhalation group. Moreover, the *E. cava* phlorotannin inhalation group showed mild inflammatory response with a small number of neutrophils and progression of chronic inflammation with an increasing number of lymphocytes (Figure 8 and Figure 9). In the saline inhalation group, marked acute inflammatory reaction was observed and a large number of neutrophils infiltrated not only lamina propria but also muscle.

In based on results of endoscopy and histology, *E. cava* phlorotannin inhalation suppressed the progression of vocal fold fibrosis and marked inflammatory reaction.

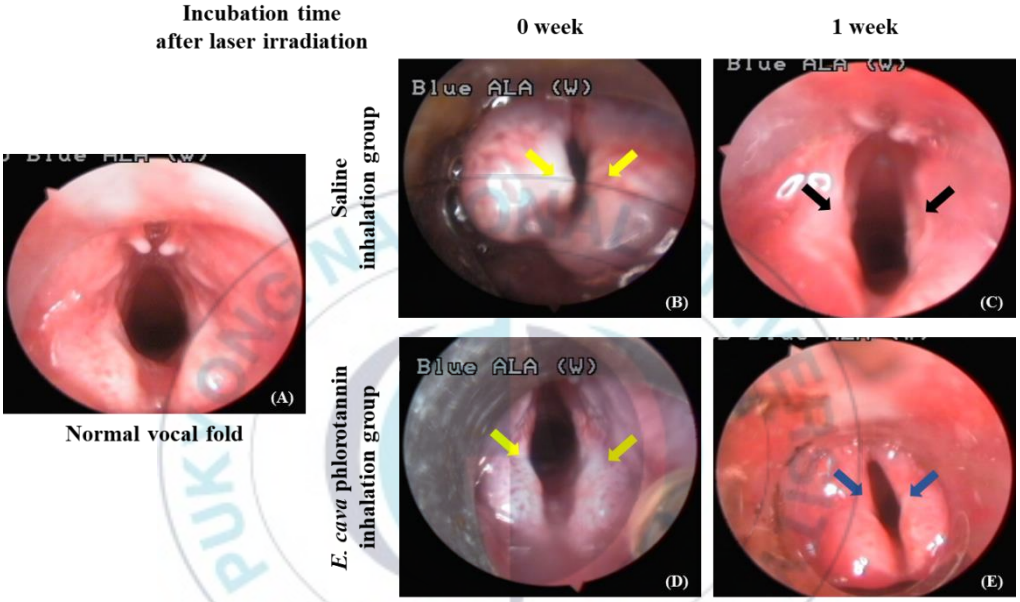


Figure 7. The endoscopic image of vocal fold on fibrosis-induced rabbit model. The endoscopic images were acquired normal vocal fold before injury **(A)**, after injury using laser **(B, D)**, and 1-week incubation **(C, E)**. The yellow arrows indicate injury by laser irradiation on vocal fold, the black arrows indicate fibrosis and ulceration of mucosa, and the blue arrows indicate mild mucosal swelling without significant fibrosis or ulceration.

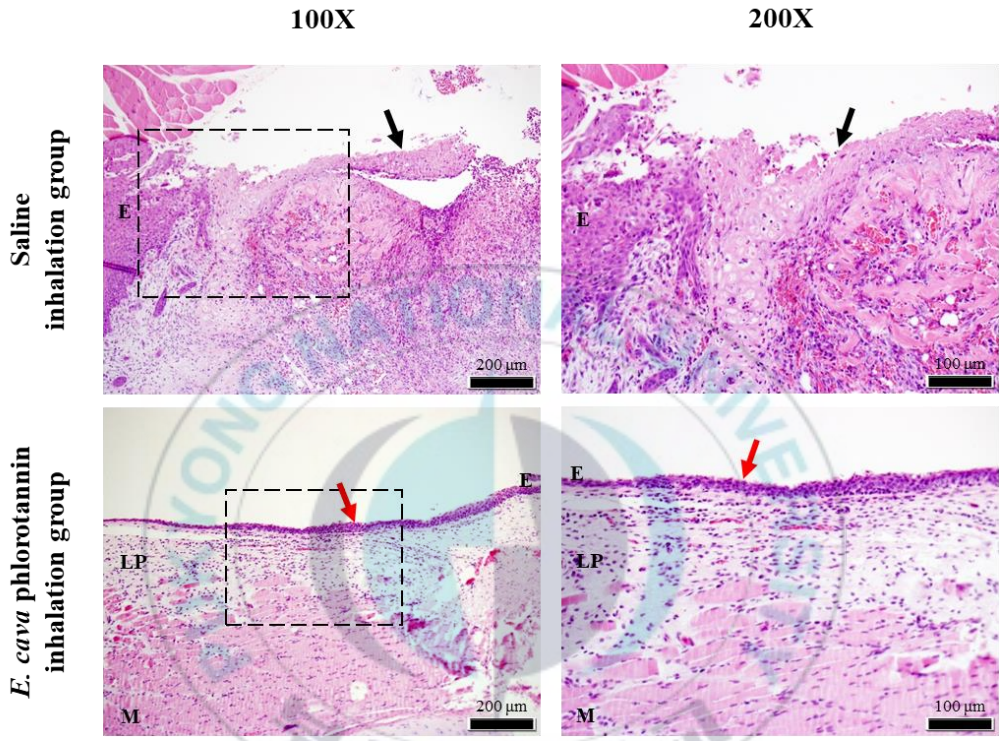


Figure 8. H&E staining of vocal fold on animal model after 1-week incubation (magnification: 100X and 200X). Black arrows indicated ulcerated area with formation of granulation tissue and necrosis and red arrows means regenerated epithelium. E: Epithelium, LP: Lamina propria, M: Muscle

Table 3. Scoring method (based on the calculation of 1 vision field on the object magnification 100 times)

Score	Histopathological Observations		
	Reepithelialization	Inflammatory reaction	Amount of granulation tissue
1	Continuation of epithelialization	Minimal/Mild	Minimal/Mild
2	Incomplete epithelialization	Moderate	Moderate
3	Necrotic epithelium/epithelial proliferation in the margin of ulcer	Marked	Marked

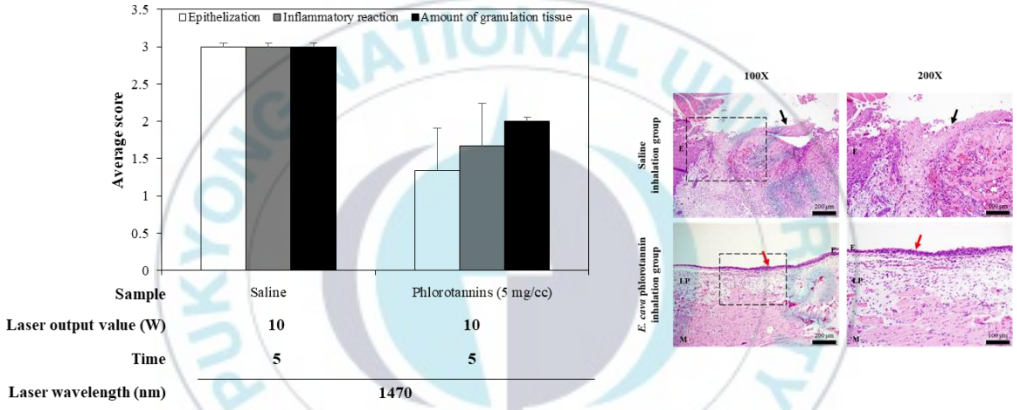


Figure 9. The average score of reepithelialization the structure of fibrosis-induced rabbit vocal fold, inflammatory reaction degree, amount of granulation tissue. Each section was evaluated for reepithelialization, inflammatory reaction, amount of granulation tissue, and then scored using the following scale; 1 = continuation of epithelialization, 2 = incomplete epithelialization, 3 = necrotic epithelium/epithelial proliferation in the margin of ulcer in reepithelialization, 1 = minimal/mild, 2 = moderate, 3 = marked in inflammatory reaction and amount of granulation tissue

3.2. *Ecklonia cava* phlorotannin inhalation inhibited collagen synthesis of the vocal fold on the fibrosis-induced rabbit model

After histological evaluation, the collagen synthesis inhibitory effect by inhalation of *E. cava* phlorotannin on fibrosis-induced rabbit was investigated by western blot analysis. The result indicated that the type I collagen protein expression level was decreased against to saline inhalation group (Figure 10).

3.3. TGF-β1 concentration to induce fibrosis in human vocal fold fibroblasts was selected by MTT assay and western blot

Before checking the antifibrotic effect of *E. cava* phlorotannin on hVFFs *in vitro*, we selected the proper TGF-β1 concentration for inducing *in vitro* fibrosis model to mimic fibrosis of vocal fold on hVFFs through MTT assay and western blot. Different concentrations of TGF-β1 (0.5, 1, and 2 ng/ml) were treated on hVFFs for 1 days. As depicted Fig 11, the results indicated that TGF-β1 has no cytotoxicity at tested concentrations (0.5, 1, and 2 ng/ml) and treatment of TGF-β1 at 2 ng/ml is proper to induce type I collagen expression level for *in vitro* fibrosis model without cytotoxicity on hVFFs (Figure 11).

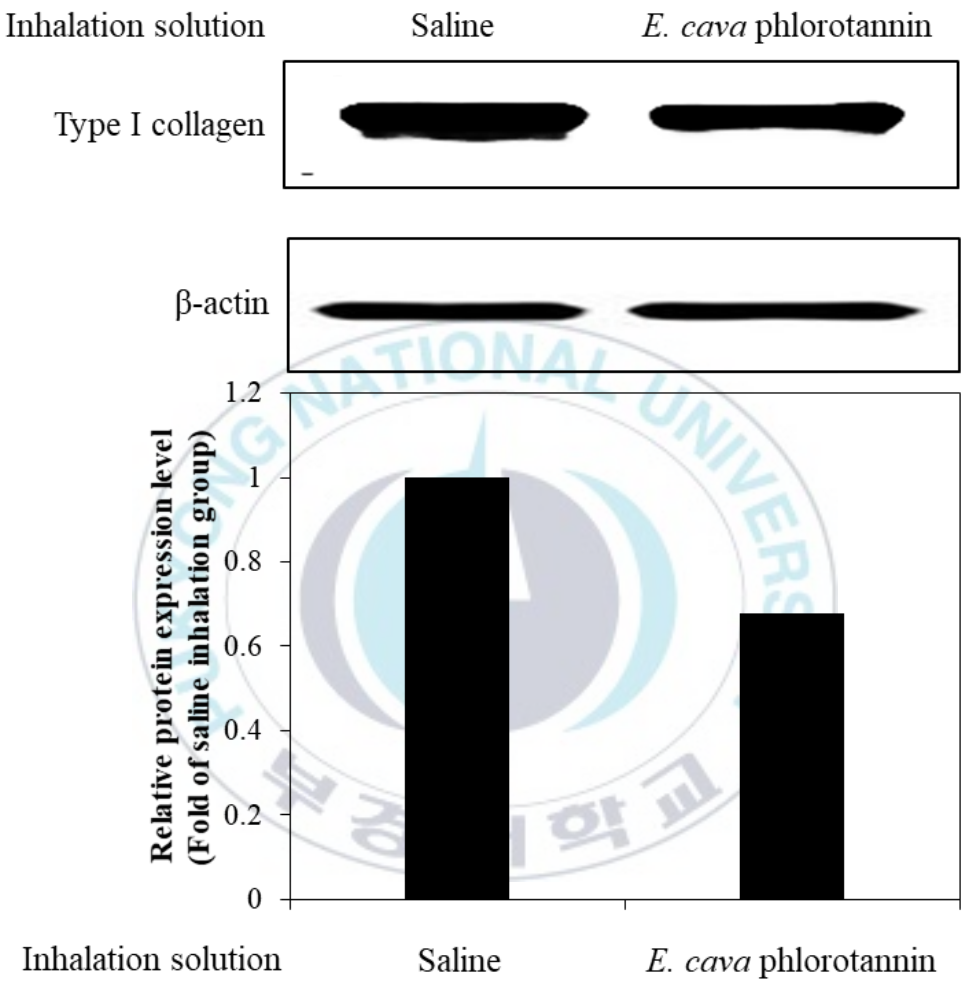


Figure 10. The antifibrotic effect of inhalation of *Ecklonia cava* phlorotannin (25 mg/ml) on fibrosis-induced rabbit model. After 1 week, the vocal fold tissue harvest from fibrosis-induced rabbit. The protein expression level of type I collagen in vocal fold tissue was determined by western blot analysis

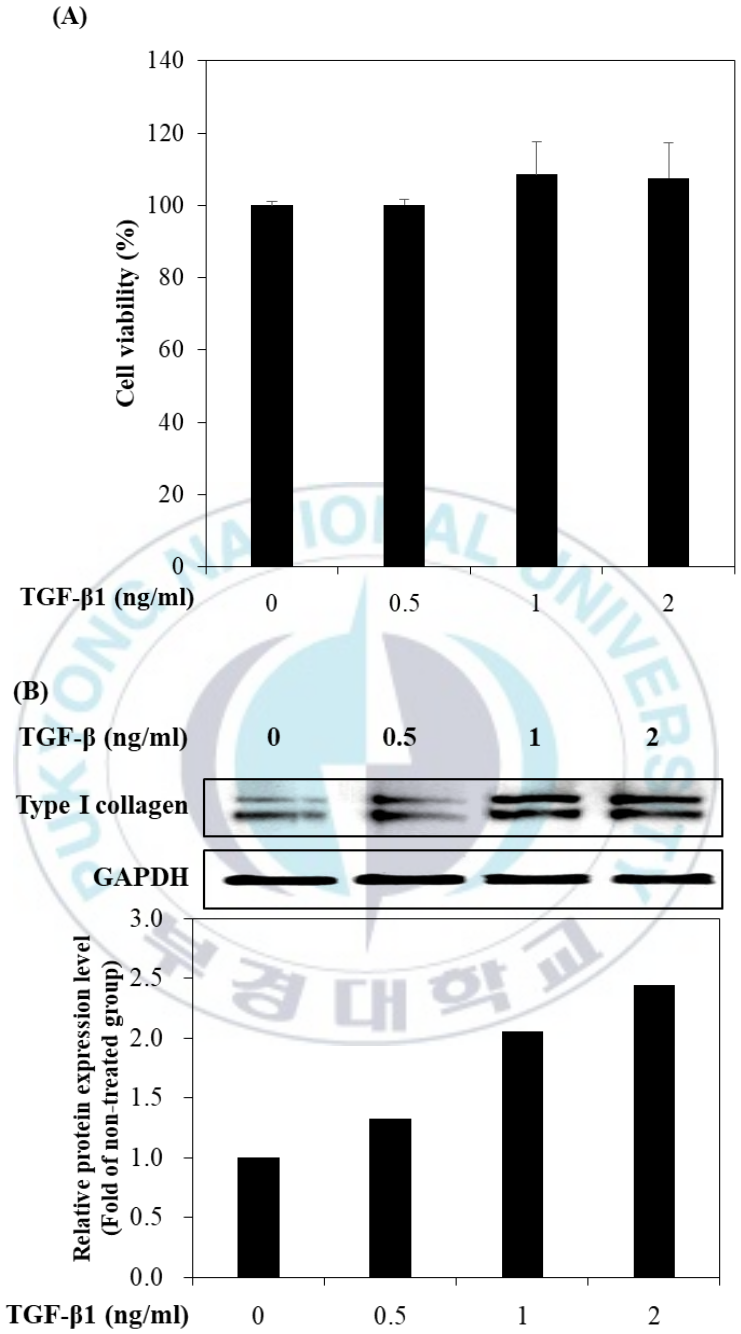


Figure 11. The effect of TGF-β1 on human vocal fold fibroblasts. **(A)** The cytotoxic and **(B)** fibrotic effect of TGF-β1 (0.5, 1, and 2 ng/ml) on human vocal fold fibroblasts. The cytotoxic effect and fibrosis effect were measured by MTT assay and western blot, respectively.

3.4. Cell viability effect of *Ecklonia cava* phlorotannin on human vocal fold fibroblasts

To determine the cytotoxic effect of *E. cava* phlorotannin on hVFFs, various concentrations of *E. cava* phlorotannin (31.25, 62.5, 125, and 250 μg/ml) were treated on hVFFs for 1 day and mitomycin C (MMC) was used as positive control. As described in Figure 12 (A), *E. cava* phlorotannin have no significant cytotoxic effect for any concentration. Then, we treated TGF-β1 (2 ng/ml) with *E. cava* phlorotannin on hVFFs to investigate whether *E. cava* phlorotannin exhibits no significant effect on cell viability in fibrosis condition (Figure 12 (B)). The results indicated that *E. cava* phlorotannin has no cytotoxic effect in presence or absence TGF-β1 on hVFFs.

3.5. *Ecklonia cava* phlorotannin inhibited cell migration of human vocal fold fibroblasts

We performed wound scratch assay in the presence or absence of TGF-β1 to determine whether *E. cava* phlorotannin inhibited the cell migration of hVFFs. As shown in Figure 13 (A), the wound area of the non-treated group was highly covered. Whereas, the *E. cava* phlorotannin inhibited cell migration in a dose-dependent manner. Additionally, the wound areas of the non-treated group and TGF-β1-treated group were also completely covered the injury line, in contrast, covered wound area of the *E. cava* phlorotannin-treated groups was decreased (Figure 13 (B)). Thus, *E. cava* phlorotannin inhibited cell migration of hVFFs in the presence or absence of TGF-β1.

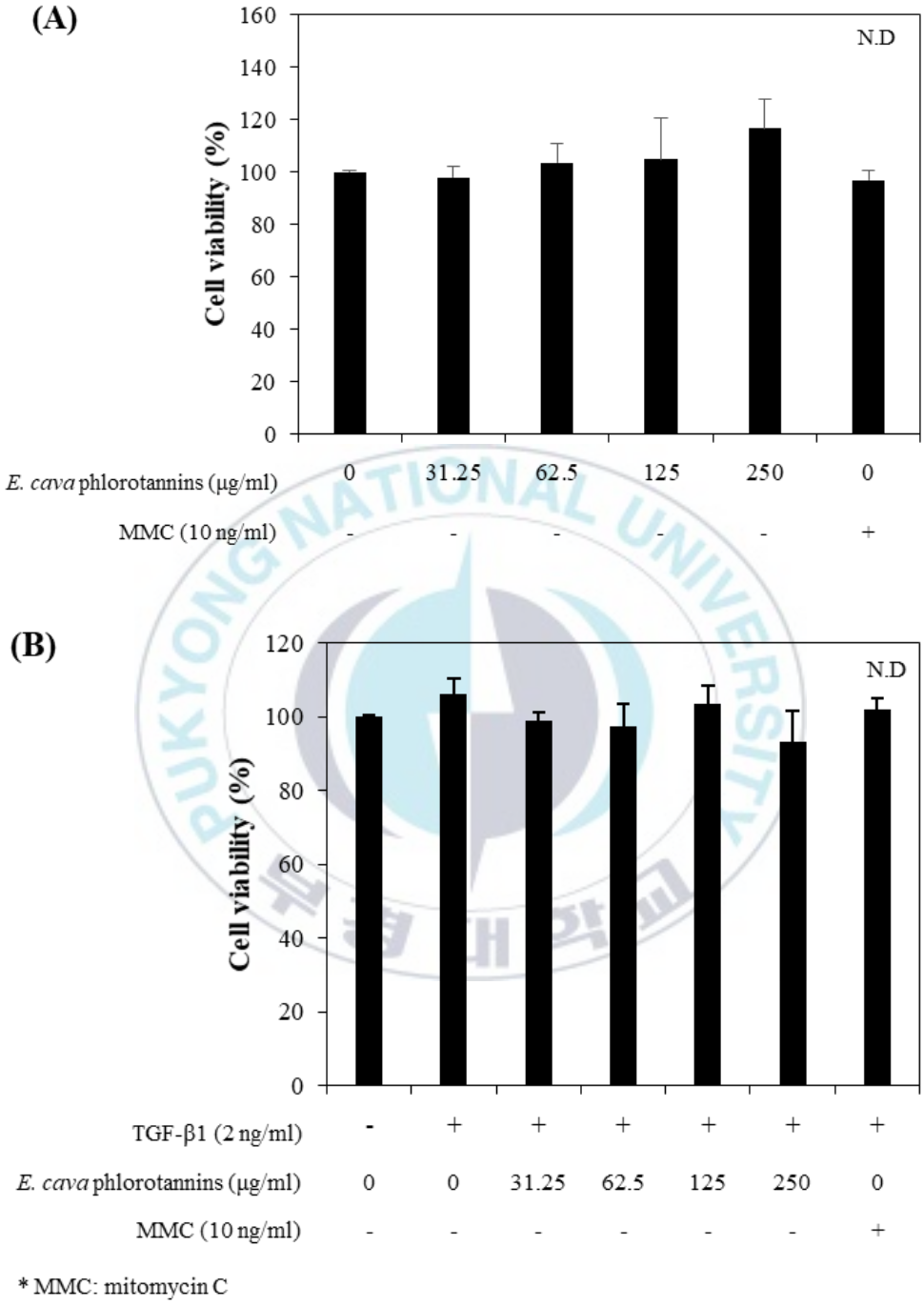
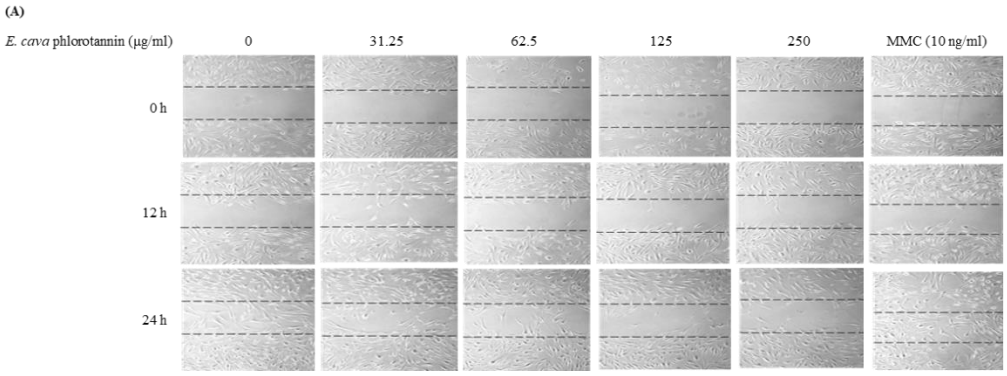
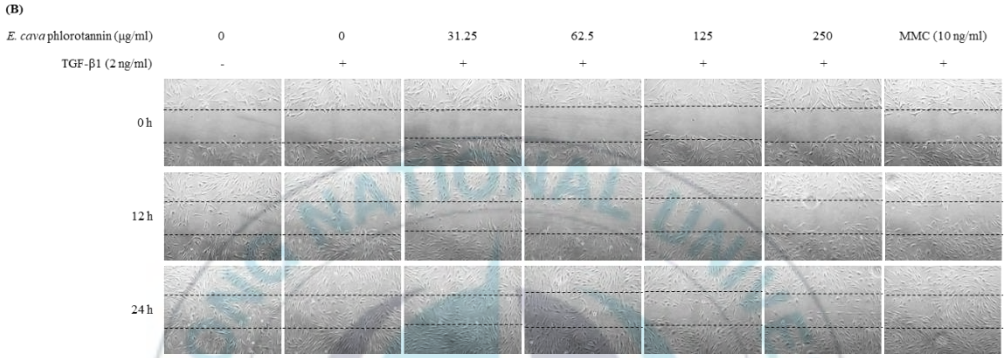
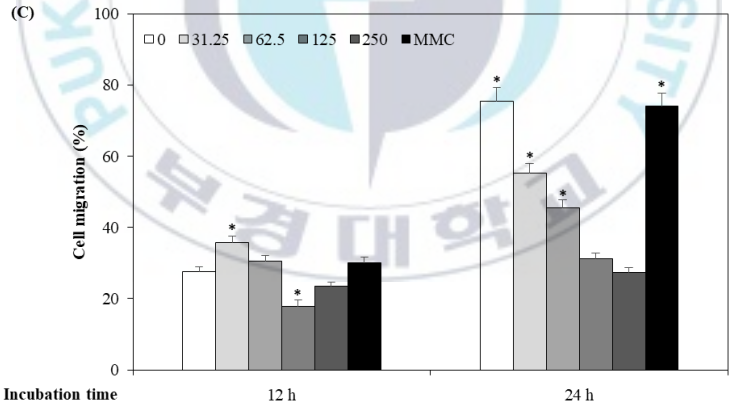


Figure 12. The cytotoxic effect of *Ecklonia cava* phlorotannin in the **(A)** presence or **(B)** absence TGF-β1 (2 ng/ml) on human vocal fold fibroblasts.


(A)
E. cava phlorotannin (μg/ml)
0
31.25
62.5
125
250
MMC (10 ng/ml)
0 h
12 h
24 h


(B)
E. cava phlorotannin (μg/ml)
0
0
31.25
62.5
125
250
MMC (10 ng/ml)
TGF-β1 (2 ng/ml)
-
+
+
+
+
+
+
0 h
12 h
24 h


(C)
0
31.25
62.5
125
250
MMC
Cell migration (%)
100
80
60
40
20
0
Incubation time
12 h
24 h

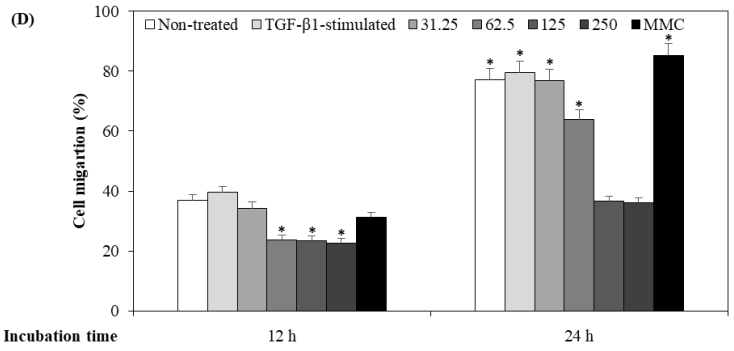

(D)
Non-treated
TGF-β1-stimulated
31.25
62.5
125
250
MMC
Cell migartion (%)
100
80
60
40
20
0
Incubation time
12 h
24 h

Figure 13. Cell migration inhibitory effect of *Ecklonia cava* phlorotannin in the (A) absence or (B) presence of TGF-β1 (2 ng/ml) on human vocal fold fibroblasts using a wound scratch assay. The injury line was made on the confluent monolayer of cells. The human vocal fold fibroblasts were treated with various concentrations of *Ecklonia cava* phlorotannin (31.25, 62.5, 125, and 250 μg/ml) with or without of TGF-β1 (2 ng/ml) for 24 h. Cell motility was examined with a light microscope at indicated time points. The cell migration percentage indicated as graph in (C) and (D), respectively. The values are expressed as the means ± S.D. of triplicate experiments. $*p < 0.05$ indicates significant differences compared with the non-treated groups

3.6. Type I collagen expression inhibitory effect of *Ecklonia cava* phlorotannin on TGF-β1-stimulated vocal fold fibroblasts

In this study, the type I collagen protein expression level was investigated using western blot after hVFFs were treated with MMC or various concentrations of *E. cava* phlorotannin and stimulated TGF-β1. As shown in Figure 14, the type I collagen protein expression level increased on the TGF-β1-stimulated group as compared to other groups. However, the protein expression level of *E. cava* phlorotannin-treated group decreased in a dose-dependent manner. Furthermore, the level of *E. cava* phlorotannin-treated group at 250 μg/ml similarly to the untreated group.

3.7. TGF-β1 activated phosphorylation of Smad 2/3, Akt, and p38 MAPK

Before confirming molecular explanation for antifibrotic effect of *E. cava* phlorotannin on hVFFs, we examined whether TGF-β1 induces phosphorylation of Mothers against decapentaplegic homolog 2/3 (Smad 2/3), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun N-terminal kinase (JNK) and investigated proper activation time on Smad 2/3, p38 MAPK, and JNK. The Smad 2/3 and JNK were strongly activated at 30 min by TGF-β1 the thereafter the phosphorylation of Smad 2/3 and JNK were decreased. In the case of p38 MAPK, the phosphorylation of p38 MAPK was gradually increased until 2 h and was highest increased at 2 h compared to 0 h (Figure 15).

3.8. The antifibrotic mechanism of *Ecklonia cava* phlorotannin on TGF-β1-stimulated human vocal fold fibroblasts

Western blot was conducted to estimate the inhibitory effect of *E. cava* phlorotannin on the phosphorylation levels of Smad 2/3 and MAPKs. In Figure 16, *E. cava* phlorotannin suppressed phosphorylation of Smad 2/3, p38 MAPK, and extracellular signal-regulated kinase (ERK) but not that JNK on TGF-β1-stimulated hVFFs.

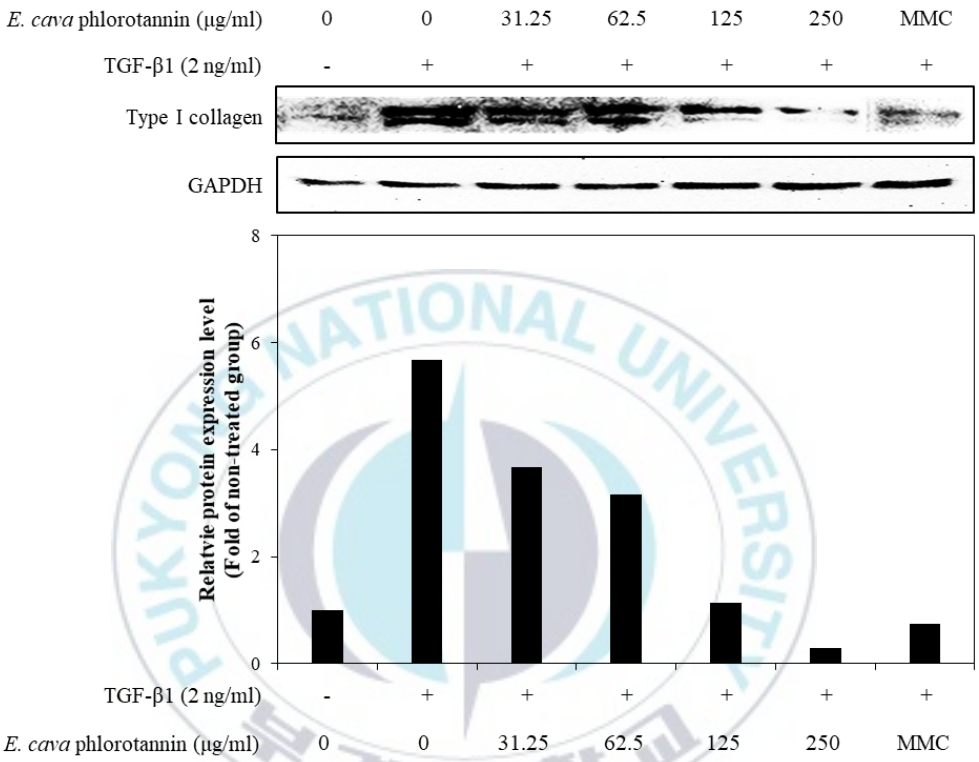


Figure 14. TGF-β1-induced type I collagen protein expression on TGF-β1-stimulated human vocal fold fibroblasts. Human vocal fold fibroblasts were treated with various concentrations of *Ecklonia cava* phlorotannin, then treated with or without TGF-β1 (2 ng/ml) for 24 h. The protein levels of type I collagen were determined by western blot.

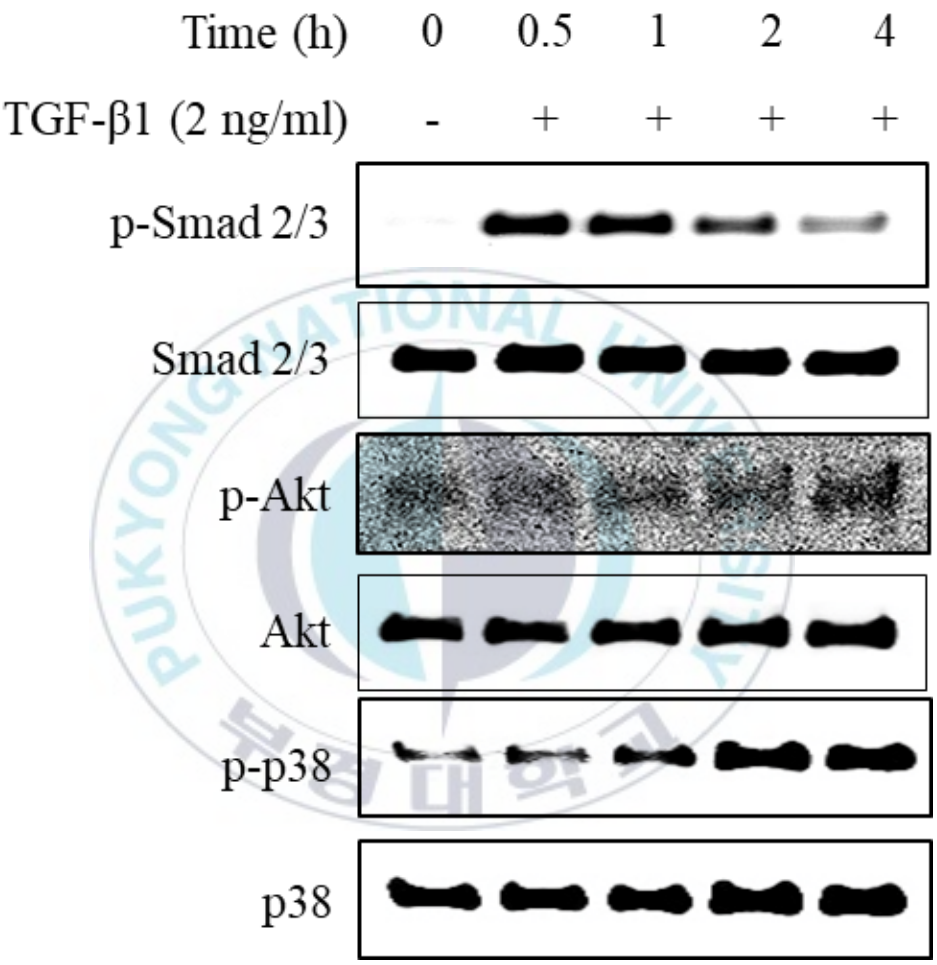


Figure 15. Effect of TGF-β1 on phosphorylation of Smad 2/3, p38 MAPK, and JNK according to time. The phosphorylation of Smad 2/3, p38 MAPK, and JNK were measured by western blot analysis.

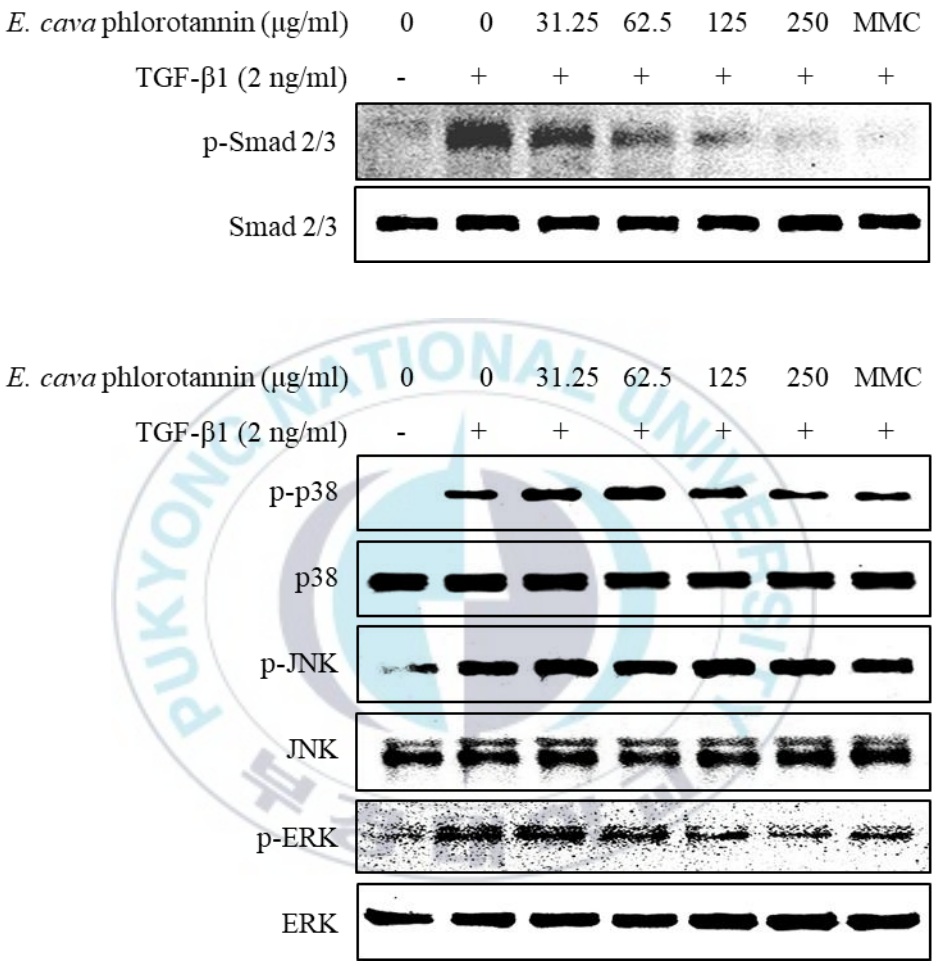


Figure 16. Effect of *Ecklonia cava* phlorotannin on TGF-β1-stimulated phosphorylation of MAPKs pathway in human vocal fold fibroblasts. Cells were stimulated with TGF-β1 (2 ng/ml) in the presence of *Ecklonia cava* phlorotannin (31.25, 62.5, 125, and 250 μg/ml) for 30 min. The phosphorylation levels of MAPKs pathway were measured by western blot analysis.

4. Discussion

Vocal fold fibrosis occurs by the exuberant inflammatory environment which was progressed after injury at the vocal fold and usually caused by trauma. The exuberant inflammatory environment lead to abnormal wound healing process resulted from excessive fibroblast proliferation and migration or type I collagen and α-SMA deposition through activation of several signaling pathways such as Smad 2/3, Akt, and MAPKs signaling. The vocal fold fibrosis ultimately yielded scar formation caused intractable loss of tissue viscoelasticity (Mortensen 2010, Shi, Giraldez-Rodriguez et al. 2016, Hiwatashi, Bing et al. 2017). Although various treatment such as invasive procedures, laser treatment, stent placement, and MMC applied to treat vocal fold fibrosis, it has several side effects such as lung fibrosis, bone marrow depression, and congestive heart failure, candidiasis, skin thinning (Mortensen 2010, Woo, Jeong et al. 2014, Matera, Cardaci et al. 2015, SFAR 2016). Therefore, we investigated the compounds from marine to prevent and/or treat vocal fold fibrosis without any side effect.

Ecklonia cava, a brown alga, has various nutrients and shows biological activities such as anti-oxidative, anti-inflammatory, and anti-allergic activities (Peña, Campos et al. 1997, Fuks, Filipiuk et al. 2006, Sharma, Sanpui et al. 2012). Among *E. cava*-extracted natural compounds, phlorotannin is polyphenolic compounds containing phloroglucinol derivatives such as phloroglucinol, eckol, bieckol, dieckol, and phlorofucofuroeckol A and was reported their biological activities (Table 2). In addition, the previous study investigated the antistenosis effect on the trachea through *in vivo* stenosis-induced animal model and *in vitro* experiments on human tracheal fibroblasts. However, it is not reported their antifibrotic effect on vocal fold and mechanism of

action. Thus, the aim of the present study was to prevent and/or treat vocal fold fibrosis through *E. cava* phlorotannin containing biological polyphenolic compounds.

TGF-β1, a central pro-fibrotic factor involved in chemotaxis and proliferation of fibroblasts, promotes vocal fold fibroblasts differentiation into myofibroblasts and is a ubiquitous mediator of fibrosis in both uninjured and injured tissue (Branski, Bing et al. 2016, Gonzalez, Contreras et al. 2017). It is involved various fibroblast activities such as cell proliferation, survival, migration, production of ECM components, and ECM accumulation (Conte, Gili et al. 2014, Rockey, Bell et al. 2015, Castellone and Laukkanen 2017). In relation to the fibrosis which finally leads to hypertrophic scar formation, TGF-β1 promotes producing and deposition plenty of ECM that was progressed by fibroblasts across numerous fibrotic condition and activates several signaling pathways related to fibrosis. Therefore, TGF-β1 used for inducing *in vitro* fibrosis model to mimic vocal fold fibrosis.

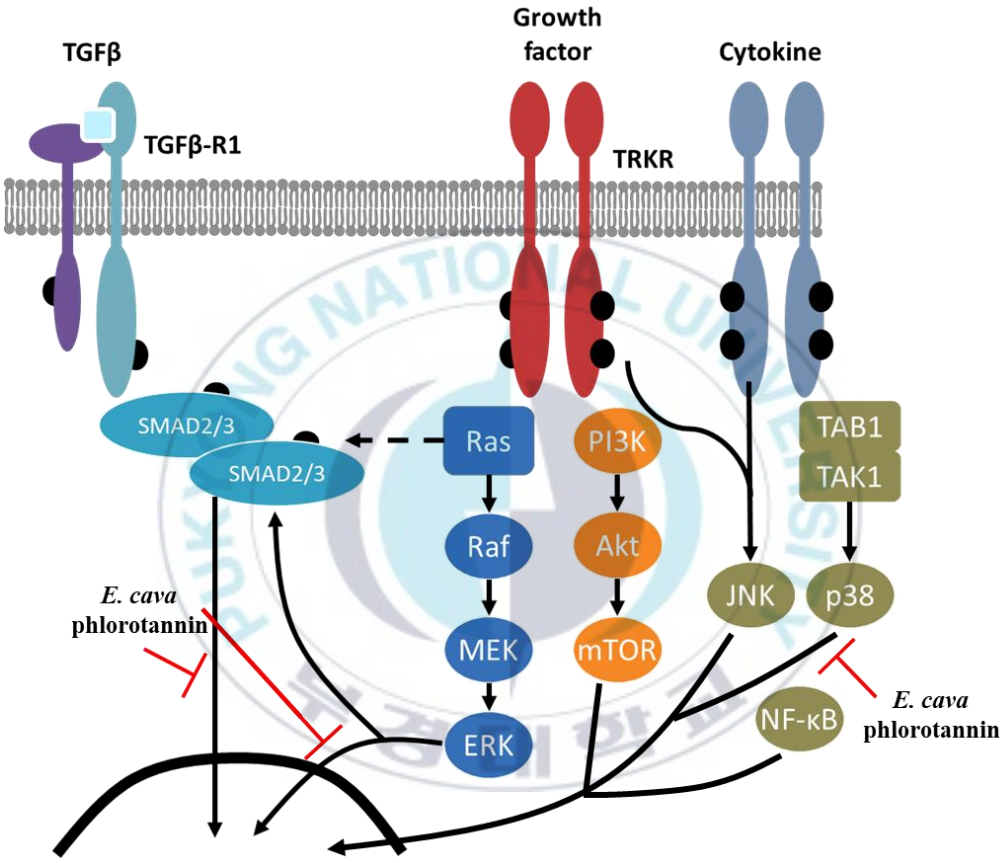
It is commonly known that TGF-β1 activates several pathways including Smad 2/3, Akt, and MAPKs pathways (Heldin and Moustakas 2012, Khalil, Kanisicak et al. 2017). The Smad2/3 pathway is activated by p-Smad2/3 complex nucleus translocation to induce the expression of profibrotic target genes including plasminogen activator inhibitor-1 (PAI-1), connective tissue growth factor (CTGF), and extracellular matrix proteins(Samarakoon, Rehfuss et al. 2016). In addition, it is involved in epithelial cell apoptosis, fibroblasts activation, regulating virus infection and innate immune response (Xiao, Zhang et al. 2014, Yoshimoto, Fujita et al. 2015, Pokharel, Shil et al. 2016). Akt pathway activated through phosphorylation of Akt is the excellent marker of cell proliferation and regulates cell cycle, angiogenesis, cellular differentiation, and biological characteristics of malignant cells (Xu, Yang et al. 2015, Liu, Cao et al. 2016,

Rahmaniah, Yuyuntia et al. 2018). MAPK pathway regulates various physiological processes such as cell proliferation, differentiation, apoptosis and ECM synthesis, (Wang, Ma et al. 2002, Ha and Lee 2003). This pathway activated by phosphorylation of MAPK family members, including JNK, ERK, and p38 MAPK. Based on these facts, Smad, Akt, and MAPKs pathways which are activated by TGF-β1 are normally regarded as the mechanism to understand the type I collagen synthesis and cell proliferation.

In vivo experiments using the vocal fold fibrosis-induced rabbit model, we examined that *E. cava* phlorotannin inhalation suppressed fibrosis and ulceration of vocal fold mucosa, unlike saline inhalation that developed fibrosis and ulceration together with the marked inflammatory reaction. Additionally, we confirmed that the *E. cava* phlorotannin inhalation resulted in the inhibiting type I collagen expression which was increased by laser irradiation.

In vitro experiments on the hVFFs, our study found that *E. cava* phlorotannin has no effect on cell viability up to 250 μg/ml and inhibited type I collagen protein expression level through suppressing phosphorylation of p38 MAPK and ERK on TGF-β1-stimulated hVFFs (Figure 17).

Based on these results, we investigated that *E. cava* phlorotannin has the antifibrotic effect both *in vivo* and *in vitro*. Therefore, it is considered that *E. cava* phlorotannin may be used as a potential antifibrotic drug candidate for prevention or treatment.



[Figure adapted from the paper (Neuzillet, Cindy, et al. Targeting the TGFβ pathway for cancer therapy, Pharmacology & therapeutics, 147 (2015): 22-31)]

Figure 17. The antifirotic mechanism of *Echlonia cava* phlorotannin

5. Conclusion

In the present study, we demonstrated that *E. cava* phlorotannin inhibited vocal fold fibrosis in the rabbit model and showed inhibitory effects on cell migration and TGF-β1-induced type I collagen in hVFFs. Additionally, *E. cava* phlorotannin suppressed phosphorylation of p38 MAPK and ERK. Based on these finding, we suggest that that *E. cava* phlorotannins may be used in the prevention or treatment of vocal fold fibrosis.

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