Combinatorial prophylactic effect of phlorotannins with photobiomodulation against tracheal stenosis

Highlights

Phlorotannins (PTs) with photobiomodulation (PBM) ameliorates fibrotic responses

PT-combined PBM downregulates SMAD and MAPK pathways

The combined treatment preserved the opening in fenestrated rodent tracheostoma

PT-combined PBM has a potential to be a preventive treatment for tracheal stenosis

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Combinatorial prophylactic effect of phlorotannins with photobiomodulation against tracheal stenosis

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SUMMARY
Several conventional treatments are used to manage tracheal stenosis after intubation and surgical procedures; however, patients are at risk of restenosis because of the absence of effective preventative therapy. In this study, we evaluate the biomodulatory effect of PT-combined blue light (BL) PBM in tracheostomal stenosis-induced animal models. The PT-combined BL group showed a significant decrease in the fibrotic protein synthesis by downregulating the release of stenosis-triggering fibrotic signals, without cytotoxicity or thermal damage. Moreover, the combined treatment ameliorated excessive granulation and collagen formation, and consequently preserved the opening of the trachecotoma ten days after fenestration. The current study demonstrated the biomodulatory effect of PT-combined BL on human tracheal fibroblasts and tracheal fenestration rodent models. Hence, PT-combined BL has the potential to be an effective preventative treatment for tracheal stenosis but also as an alternative option for fibrotic disorders.

INTRODUCTION
Tracheostomal stenosis is not an infrequent complication after tracheal fenestration such as tracheostomy and laryngectomy, and continues to be a serious clinical problem.1,2 Tracheal injury during and after intubation or surgery causes inflammation, edema, ischemia, and eventually leads to fibrosis, resulting in severe tracheal stenosis in up to 21% of patients.3,4 Recently, the number of patients who have undergone tracheostomy or intubation has increased because of the spread of coronavirus disease (COVID-19), and a large number of studies have been conducted on tracheal stenosis for efficient management after the procedure.5-9 Several surgical options (e.g., balloon dilation, bronchoscopy, and tracheal resection) are currently in use to improve airway patency depending on the degree of stenosis.1,10 Nonetheless, patients still suffer from breathing interference because of restenosis after these treatment methods. Restenosis is accompanied by excessive granulation or fibrosis, and attenuated stenosis can be life-threatening.3,4 Furthermore, the treatment of established fibrosis remains an unmet clinical challenge.11,12 Hence, preventative approaches are urgently needed to treat tracheal stenosis, which is pivotal not only for the recovery of patients but also for reducing complications and side effects after tracheostomy or tracheal intubation.

Although pirfenidone and mitomycin have been used clinically to prevent fibrosis, synthetic drugs often cause side effects including heartburn, stomach pain, nausea, and headache.4,13-15 As an alternative anti-fibrotic agent, phlorotannins (PT), a group of functional polyphenols, have been screened from natural materials to develop a potential preventative approach. Previous studies have reported that PT could ameliorate excessive collagen synthesis by suppressing the release of transforming growth factor (TGF)-β1-related fibrotic protein.16,17 Photobiomodulation therapy (PBM) has also been introduced as a non-invasive anti-fibrotic modality to modulate biological activities, including inflammation, proliferation, and migration.18,19 A number of clinical studies have reported that red (600-700 nm) and far-red (700-800 nm) light promotes recovery from injury and ameliorates infection and inflammation.20-22 Although most wavelengths at high power and energy density can downregulate biological activities, blue light (BL; 400-500 nm) is particularly effective in inhibiting fibroblast activity in vitro even at a low level of laser irradiation.23 However, these studies are based at the cellular level and merely reveal the limited therapeutic effect of a single agent or modality on fibrosis as a result of multifactorial disease processes.11,12 To the best of our knowledge, no previous studies have reported the biomodulative effect of PT-combined BL in the prevention of tracheal stenosis.
The current study examined whether the combination of PT and BL could cause a synergistic therapeutic effect for the prevention of tracheal stenosis. The present study developed an animal model of tracheostomal stenosis using surgical techniques that induce mechanical stress on the trachea and skin from the suture knot. Both PT and BL treatments were applied directly to the fenestrated wound site. In this study, we evaluated the potential anti-fibrotic effects and signal release of the combined treatment by evaluating TGF-induced human tracheal fibroblasts and developed tracheal fenestrated rodent models (Figure S1).

RESULTS
Cytotoxicity suggests a permissible dose of combined treatment in preclinical studies

The cytotoxicity of PT and BL in HTF/Fs was assessed using the MTT assay, and the maximal permissible dose of both treatments was quantitatively determined based on the cell viability results.
A cell viability percentage above 80% (dashed line) was considered to be non-cytotoxic pursuant to ISO 10993-5. As shown in Figure 1A, the cell viability did not decrease up to 50 μg/mL of PT. The viability began to decrease at 100 μg/mL and continued to decrease at higher concentrations. Although a significant difference in the cell viability was observed at concentrations of 200 μg/mL and above, 100 μg/mL was selected for the PT treatment without cytotoxicity (*p< 0.05, **p< 0.005). Likewise, 200 mW/cm² and 12 J/cm² BL were considered for the combined treatment with 100 μg/mL PT (Figures 1B and 1C). RL, PT + RL, and BL hardly showed a remarkable reduction in the cell viability, whereas PT + BL significantly decreased the cell viability compared to the other treatments. Among all the power and energy densities, 200 mW/cm² and 12 J/cm² of BL were the maximal doses that preserved the viability above 80%. Therefore, 100 μg/mL of PT and 12 J/cm² (200 mW/cm² for 60s) of BL were determined as the maximal permissible dose of the combined treatment and were applied for further experiments.

### Combined treatment inhibits TGF-β1-stimulated cell migration and proliferation

The inhibitory effects of the proposed treatment doses on the cell migration and proliferation were compared and verified in TGF-β1-stimulated HTrFs. The cell migration at two different time points (0 and 24 h) was observed as representative images, with yellow lines indicating each cell boundary (Figure 1D). The migration rate was quantified and expressed as a percentage of wound closure (Figure 1E). The wound closure rate was increased by TGF (89% ± 6%) compared to that in the control (77% ± 5%). In contrast, both PT and BL decreased to 68 ± 5% and 75 ± 8%, respectively. Furthermore, PT + BL showed a significant difference (p< 0.005) from the other groups and decreased the wound closure by up to 55% when compared with the TGF group.

As shown in Figure 1F, a similar tendency of reduction was observed in the cell proliferation at 24 and 48 h after the PT and BL treatments. Cellular proliferation was also demonstrated by the microscopic images of cells at 48 h (Figure S3). All groups showed an increased rate over time, with the greatest increase observed in the TGF group (119% of the control). The PT and BL treatment reduced proliferation by 90 and 95% in the TGF group, respectively. Among all the treated groups, PT + BL showed a significant reduction in proliferation, which was less than that of the control (78% of TGF; p< 0.05). These results revealed that the PT-combined BL inhibited cell proliferation and migration in TGF-β1-stimulated fibroblasts.

### Combined treatment modulates TGF-β1-stimulated fibrotic protein release

To determine the modulatory effect of the combined treatment, PT and BL were tested under the induction of TGF-β1-stimulated fibrotic phenotypic markers, type-1 collagen, and α-SMA. Figures 1G and 1H show the highly induced protein release in the TGF group. The relative expression of type-1 collagen and α-SMA increased by up to 122 and 159%, respectively, compared to the control (p< 0.05). Nevertheless, all the PT and BL treatments markedly decreased the TGF-β1-induced protein release. Following PT treatment, type-1 collagen and α-SMA were downregulated by up to 66 and 87%, respectively, in the TGF group. BL treatment decreased the expression by up to 60% (Type-1 collagen) and 82% (α-SMA), respectively, compared with the TGF group (p< 0.05). The PT + BL treatment markedly decreased the relative protein levels (Type-1 collagen = 50% and α-SMA = 64% of TGF group), with a substantial difference between the other treated groups (p< 0.05). Thus, the PT-combined BL modulated TGF-β1-stimulated fibrotic protein release.

### TGF-β/SMAD and non-SMAD pathways elucidate the dominant treatment mechanism

To elucidate the mechanism underlying the inhibition of type-1 collagen and α-SMA, we determined whether PT and BL could modulate TGF-β1-stimulated SMAD and non-SMAD (microtubule-associated protein kinases; MAPKs) release via western blot analysis (Figure 2). As shown in Figures 2C–2F, all the relative expressions were highly induced by TGF from 1.3 to 2.2 times of the control. In contrast, both PT and BL suppressed the TGF-stimulated phosphorylation of SMAD 2/3 and MAPK subunits, including JNK, ERK, and p38, respectively. Phosphorylation was the lowest in the PT + BL group. Moreover, PT + BL significantly diminished the phosphorylated of SMAD 2/3, ERK, and p38 pathways, compared with the other treated groups. Hence, it was evident that PT-combined BL modulated the SMAD and non-SMAD signaling pathways in TGF-stimulated human tracheal fibroblasts.
Combined treatment preserves the opening on fenestrated tracheostoma

The histological changes in the tracheal tissue were analyzed by HE staining on day 10 after fenestration (Figure 3). The bottom images (40X) exhibit the magnified images of the black squares in each top image (10X; Figure 3A). The insets in the top-right corner of the bottom images magnify a representative section (400X) of the wound sites. The cyan dashed lines represent the tracheal cartilage around the fenestrated tracheal tissue with the neo-dermis (N) and granulation tissue (GT). The black arrows indicate the inflammatory infiltrate with neutrophils, and the double-headed arrow represents the width of the stoma (WS). The histological features of fibrosis and stenosis were evaluated in four stages based on a semi-quantitative scoring system (Figure 3B). Generally, re-epithelization was completed or nearly completed in all groups on day 10. Marked acute inflammation and granulation tissue formation were observed in the control group. Although PT or BL reduced the fibrotic features, only acute inflammation treated with PT showed a significant difference compared with the control (p< 0.05). However, the PT + BL group showed moderate inflammation and mild granulation tissue formation around the fenestrated trachea. Particularly, PT + BL treatment significantly reduced the granulation tissue formation compared with the other groups (p< 0.05). Owing to the granulation tissue formation at the fenestration site, the width of the stoma shown in Figure 3C was narrow on day 10. The stoma was completely obstructed in the control, whereas the width was increased by PT (91 ± 85 μm) and BL (113 ± 112 μm) treatments. Furthermore, a substantial difference in the stomal width was observed in the PT + BL group (455 ± 100 μm) compared to the other groups (p< 0.005). Hence, it was concluded that PT + BL ameliorated fibrotic changes with reduced acute inflammation and granulation tissue formation and preserved the stoma opening in fenestrated rodent tracheostoma.

Combined treatment ameliorates excessive collagen accumulation and fibrogenesis

MT-stained tissues were analyzed to compare the degree of collagen accumulation in fenestrated tracheostomas on day 10 (Figure 4). The bottom images (40X) exhibit the magnified images of the black squares in each top image (10X; Figure 4A). The insets in the top-right corner of the bottom images magnify a representative section (400X) of the wound sites. The stained tissues are presented with collagen fibers (blue), nuclei (dark red/purple), and cytoplasm/muscle fibers (red/pink). Tangled...
collagen fibers (TCs), aligned collagen fibers (ACs), and tracheal cartilage (yellow dashed line) are indicated in the images. Tangled collagen fibers were observed on both sides of the wounds, and aligned collagen fibers, which are characteristic of fibrotic tissues, were present at the fenestrated sites. The control group had an obstructed stoma consisting of massive collagen accumulation, whereas the PT + BL group exhibited significantly reduced collagen deposition with a preserved opening of the stoma. As shown in the quantitative graphs (Figure 4B), tracheal fenestration resulted in an immense collagen area fraction in the control group (63.0%). The PT and BL groups showed decreased collagen levels of up to 53.6 and 56.6%, respectively. Furthermore, the collagen area fraction significantly diminished to two-thirds of the control in the PT + BL group (43.7%), and a substantial difference was observed between the PT + BL group and the other treated groups (p < 0.05). These results demonstrate that PT-combined BL effectively ameliorated excessive collagen accumulation and fibrogenesis induced by tracheal fenestration.

Combined treatment alleviates release of proinflammatory and profibrotic cytokines
IHC-stained tissues were analyzed to validate the anti-inflammatory and anti-fibrotic effects of the fenestrated tracheostoma on day 10 (Figure 5). The brown areas represent the release of proinflammatory and profibrotic cytokines in the representative IHC staining images (400X, bar = 30 μm). To compare the
release of proinflammatory cytokines, TNF-α and IL-6 were detected on days 3 and 10, respectively. The fibrotic responses to TGF-β1 and fibronectin were assessed. As shown in Figures 5A and 5B, the expressions of TNF-α and IL-6 were not significantly different between days 3 and 10. Similarly, no substantial difference was observed in the TGF-β1 and fibronectin expression levels on day 3. However, the PT + BL group showed decreased expression of fibrotic markers (TGF-β1, 24.9%; fibronectin, 10.3% of the control) on day 10 (Figures 5C and 5D). Moreover, the PT + BL group showed significantly reduced levels of fibronectin on day 10 compared to the other treated groups. (p< 0.005). The current findings prove that PT-combined BL alleviates the release of inflammatory and fibrotic cytokines, especially fibronectin, in fenestrated tracheostoma.

**DISCUSSION**

To date, the lack of scientific evidence for the treatment of tracheal stenosis, a multifactorial disease, has created the need to evaluate novel therapeutic interventions. The complex pathophysiology of tracheal stenosis has prompted the exploration of an effective treatment to prevent stenosis before occurrence. Although surgical options are common for tracheal stenosis, surgery increases the risk of recurrence and may worsen stenosis. In addition, adjuvant medications, such as mitomycin C, are hardly effective in preventing stenosis, and the improper use of the drug may provoke adverse clinical outcomes. Hence, the present study attempted to validate the synergistic biomodulatory effect of
PT combines the anti-fibrotic effects of nintedanib and pirfenidone, whereas various side effects such as diarrhea, fatigue, skin-related events, and decreased appetite have been reported.4,14,15 Unlike synthetic drugs, PT from natural products has the advantage of minimal or no side effects.26–28 Thus, we hypothesized that PT can be used to induce synergistic effects to improve the anti-fibrotic activity with PBM. PBM was previously known as low-level laser light (LLLT) and can activate mitochondria through preferential absorption.31 However, BL, the highest energy wavelength in the visible light spectrum, has not been well described owing to its high absorption and shallow light penetration in tissue.32 BL has been relatively unexplored in terms of how it is absorbed into cells and how its effects are exerted. Because the characterization of the biological effects of BL PBM on cells is still lacking in comprehensive studies, the signaling process and fundamental differences between RL and BL PBM remain elusive. However, recent studies have shown that BL has the potential to inhibit fibroblast activity, providing a valuable perspective for the treatment of fibrotic disorders.23 Similarly, BL was suggested to reduce the expression of fibrotic phenotypic markers. Furthermore, in terms of infection during tracheal intubation, BL irradiation can be expected to yield an antimicrobial effect to inactivate bacteria such as resistant Staphylococcus aureus.33,34 Using PT and BL together, the combined approach leads to a synergistic ameliorative effect on tracheal stenosis by downstream signal release, which forms the basis for stenosis-triggering fibrotic responses. These findings suggest that PT-combined BL is an effective treatment for the prevention of tracheal stenosis.

Although PT is derived from edible seaweed and is generally considered safe in the absence of BL, the relative toxicity of BL-irradiated PT has not been reported and may raise concerns about its safety. This study evaluated the safety profile of PT-combined BL, including its toxicity and thermal effects (Figures 1 and S6). The results showed the synergistic modulatory effect of the combined treatment on fenestrated tracheostomy with no or minimal cytotoxicity and heat generation. For preclinical applications, the maximal permissible dose of the combined treatment was determined in a dose-and time-dependent manner. Sufficient photons are necessary for cellular photoactivation, but metabolic effects are not observed during excessive heat generation.35 Inappropriate use of laser light may yield unexpected or negative outcomes, such as irreversible changes in cells and tissues.35,36 As shown in Figures S5 and S6, 100 μg/mL of PT used in this study hardly absorbed 405 nm of BL and resulted in no significant temperature increase (ΔT = 0.8°C). Consequently, the current study confirmed the appropriate parameters of the combined method for tracheal stenosis, which can regulate bioactivity without cellular damage by toxic and thermal effects.

In terms of the efficacy profile, the therapeutic effects of PT and BL were validated in tracheal fenestration rodent models developed by a surgical technique of tracheostomy, which induces stenosis-triggering granulation tissue and fibrosis (Figures 4C, S2, and S4). According to the histological results, tracheostoma obstruction was gradually induced after tracheostomy. Granulation tissue and fibrosis, the main causes of tracheal stenosis, may occur because of mechanical stretching, irritation, and compressive stress caused by suturing the skin and trachea around the fenestrated wounds.37,38 TGF-β1 released by stress plays a crucial role in the differentiation of fibroblasts and the extracellular matrix (ECM) deposition, which are related to fibrosis and granulation tissue formation.3,12 Fibroblasts maintain tissue homeostasis through the regulation of ECM. However, the fibroblasts that are constantly activated by growth
factors, such as TGF-β, produce excessive ECM and eventually lead to fibrosis.\textsuperscript{39} TGF-β1 also contributes to cell proliferation and migration involved in tissue remodeling by activating the SMAD and MAPK signaling pathways.\textsuperscript{40} Therefore, the modulation of TGF-β1-induced differentiation, ECM deposition, and signaling pathways in fibroblasts is a competitive strategy for preventing the progression of tracheal stenosis. Our results showed that PT-combined BL significantly alleviated the TGF-β1-induced fibrotic expression of collagen and α-SMA (biomarker of ECM and myofibroblasts) by modulating the SMAD and MAPK signaling pathways. Moreover, the opening on the fenestrated tracheostoma was preserved by PT-combined BL treatment, reducing acute inflammation, granulation, and collagen accumulation. In summary, the current study demonstrated the efficacy profile of PT-combined BL treatment in an animal model of tracheostomal stenosis and emphasized the synergistic advantage of the combination approach for multifactorial disease therapy.

Despite the potential of PT and BL combination therapy in the prevention of tracheal stenosis, the current study has several limitations. The current method can be considered a primary photodynamic response because of the dose and manner of use of laser, and exogenous chromophores. However, the current study applied the immediate laser treatment without waiting for cellular uptake unlike the conventional photodynamic therapy. Further studies will be investigated to clarify whether the reaction is photobiomodulation or photodynamic response by evaluating the formation of free radicals and other oxidizing agents. In the wound healing process consisting of three phases (inflammatory, proliferative, and remodeling), the selection of the optimal timing and adequate frequency of the combined treatment is unclear. The current results were obtained in a short period of 10 days owing to the rapid healing rate of rodent models. In addition, many factors can interfere with one or more pathways in the fibrotic process.\textsuperscript{3,12} In addition to TGF-β, myeloid cells, regulatory T cells, and profibrotic type 2 immune responses have been described as key fibrotic pathways for therapeutic targeting.\textsuperscript{12,41} Therefore, the use of small molecule inhibitors against TGF-β will be considered in further studies to convince the biological responses of the proposed treatment. For clinical application, further in vivo tests are necessary to determine the optimal timing and frequency of treatment and to verify the therapeutic and sideeffects in the long-term application of the porcine model, which is similar to the human skin anatomically and physiologically.\textsuperscript{30,42} Further studies will be conducted to elucidate several unproven factors that have not been validated in this study to improve the efficiency of the proposed treatment.

Based on cellular and histological analyses, the current study demonstrated the safety and efficacy profile of PT-combined 405 nm BL treatment for tracheal fenestration in an in vivo model. The combination of PT and BL ameliorated the formation of granulation tissue by modulating both the TGF/SMAD and MAPK signaling pathways, and consequently prevented the obstruction of the fenestrated tracheostoma. The current findings not only revealed the potential of combined methods as a prophylactic approach for tracheal stenosis but may also pave the way for new anti-fibrotic therapies for the effective treatment of multifactorial fibrotic disorders.

\textbf{STAR METHODS}

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  - Combined treatment on stimulated fibroblasts
  - Combined treatment on fenestrated wounds
  - Cell viability and proliferation
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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Alpha smooth muscle actin antibody | Abcam | ab7817; RRID: AB_262054 |
| Anti-collagen 1 antibody | Abcam | ab34710; RRID: AB_731684 |
| Smad2/3 antibody | Cell Signaling Technology | #5678S; RRID: AB_10693547 |
| Phospho-Smad2(Ser465/467)/Smad3(Ser423/425) antibody | Cell Signaling Technology | #8828S; RRID: AB_2631089 |
| Anti-JNK antibody | Santa Cruz Biotechnology | sc-7345; RRID: AB_675864 |
| Phospho-SAPK/JNK(Thr183/Tyr185) antibody | Cell Signaling Technology | #92515; RRID: AB_331659 |
| Anti-ERK 1/2 Antibody | Santa Cruz Biotechnology | sc-514302; RRID: AB_2571739 |
| Phospho-ERK antibody | Santa Cruz Biotechnology | sc-7383; RRID: AB_627545 |
| Anti-p38 antibody | Santa Cruz Biotechnology | sc-7149 |
| Phospho-p38 MAPK(Thr180/Tyr182) antibody | Cell Signaling Technology | 9211S; RRID: AB_331641 |
| Goat anti-mouse IgG Secondary antibody, HRP | Invitrogen | Cat#31430; RRID: AB_228307 |
| Goat anti-rabbit IgG-HRP | AbFrontier | LF-SA8002; RRID: AB_2916036 |
| Anti-TNF alpha antibody | Abcam | ab6671; RRID: AB_305641 |
| Anti-interleukin-6 antibody | Abcam | ab9324; RRID: AB_307175 |
| TGF beta-1 monoclonal antibody (TB21) | Invitrogen | MAS-16949; RRID: AB_2538424 |
| Anti-fibronectin antibody | Abcam | ab2413; RRID: AB_2262874 |
| Chemicals, peptides, and recombinant proteins | | |
| Recombinant human TGF-β1 | PeproTech | Cat#100-21 |
| Isofluurane (Terrell) | Piramal Critical Care | CAS: 26675-46-7 |
| Povidone-Iodine solution | Green pharmaceutical | ATC code: D08AG02 |
| Thiazolyl Blue Tetrazolium Bromide | Sigma-Aldrich | P/N: M5655 |
| Dimethyl sulfoxide | Duksan | CAS: 67-68-5 |
| Phosphatase inhibitor cocktail 2 | Sigma-Aldrich | Cat#P8340 |
| Protease inhibitor cocktail | Sigma-Aldrich | Cat#P5726 |
| Critical commercial assays | | |
| BrdU cell proliferation assay kit | Cell signaling Technology | Cat#68135 |
| Pierce BCA protein assay kit | Thermo Fisher Scientific | Cat#23225 |
| Experimental models: Cell lines | | |
| Human tracheal fibroblast cells (HTF) | ScienCell Research Laboratories | Cat#3430 |
| Experimental models: Organisms/strains | | |
| Sprague-Dawley rat | Hana Biotech | Male, 12 weeks |
| Software and algorithms | | |
| ImageJ | Schneider et al., 2012⁴⁵ | https://imagej.nih.gov/ij/ |
| Motic DSAssistant | Motic | https://www.motic.com/As_Support_Download/ds3.html |
| IBM SPSS Statistics V22.0 | IBM, SPSS | https://www.ibm.com/support/pages/spss-statistics-220-available-download |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hyun Wook Kang (wkang@pukyong.ac.kr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All data reported in this article will be shared by the lead contact on request.
- This article does not report original code.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and in vitro model
Human tracheal fibroblast cells (HTrF, ScienCell Research Laboratories, CA, USA) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Corning) and 1% antibiotic-antimycotic solution (Gibco, NY, USA) in an incubator (MCO-18AC, Sanyo, Osaka, Japan) at 37°C and 5% CO2. The cells were counted and seeded at three different densities (8 × 103 cells/well in 96-well plates for MTT and BrdU assay, 4 × 104 cells/well in 24-well plates for scratch assay, and 2 × 105 cells/well in 6-well plates for western blot analysis) with 2-well intervals to avoid the overlap of the scattered light. Fibroblast cells were stimulated to differentiate into myofibroblasts by adding 2 ng/mL of recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ, USA). All treatments with PT and BL were applied consecutively after the addition of TGF-β1.

In vivo tracheal fenestration model
Forty-four male Sprague-Dawley rats (age = 12 weeks, weight = 200–250 g) were anesthetized in a chamber with 3% isoflurane (Terrell™ isoflurane, Piramal Critical Care, PA, USA) and 1 L/min oxygen using a respiratory system (Classic T3, SurgiVet, USA). During the experiments, 1.5% isoflurane was added to sustain anesthesia via a nosecone. After hair removal, povidone-iodine solution (Green Pharmaceutical Co., Korea) was administered to prevent infection at the surgical site. The skin and strap muscles were dissected to expose

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Other               |        |            |
| CO2 incubator       | Sanyo  | MCO-18AC   |
| Classic T3 Vaporizer| Smiths Medical | VCT302 |
| Nylon suture        | AILIEE | NBS17      |
| Microplate spectrophotometer (Multiskan Go) | Therm Fisher Scientific | Cat#S1119300 |
| 405 nm diode laser system | CNI | FC-W-405-SW |
| 635 nm diode laser system | CNI | SD-635-HS-1W |
| Power meter (Nova II) | Ophir | P/N: 7201550 |
| Low Power sensor    | Ophir  | P/N: 7202696 |
| Thermal camera      | FLIR System | FLIR A300   |
| SPLScar Scratcher   | SPL Life Science | Cat: 201924 |
| Cell culture microscope | Olympus | CKX53  |
| Nitrocellulose/Filter Paper Sandwich | Invitrogen | LC2001 |
| IBright imaging system | Invitrogen | FL1000 |
| Motic Digital Slide Assistant System | Motic | EasyScan Infinity 60 |
the trachea, and fenestration was created using a 5-mm vertical midline incision from the second tracheal ring. The upper and lower parts of each side (total four points) of the incised tracheal wall were sutured to the skin using 6-0 Blue Nylon (NB617, AILEE Co., Korea) to complete tracheal fenestration (Figure S2). After the surgery, all the animals were separated into dry cages, and food and water intake were monitored every day. Suction was performed around the fenestration site to prevent the obstruction of the stoma caused by sputum and foreign substances. The study protocol was approved by the Institutional Animal Care and Use Committee of the Pukyong National University (Number PKNUIACUC-2019-31).

**METHOD DETAILS**

**Phlorotannins (PT)**

PT was isolated from Ecklonia cava, as described previously by Heo et al. (2020). Briefly, E. cava powder was extracted thrice with 70% ethanol and filtered. The filtrate was evaporated and lyophilized to obtain ethanol extracts. The ethanol extracts were sequentially partitioned using n-hexane, chloroform, ethyl acetate, and distilled water. The ethyl acetate fraction was evaporated and dissolved in phosphate-buffered saline (PBS, Corning, NY, USA) for further experiments (50 mg/mL stock solution). The absorbance spectra of the PT solutions were measured between 400 and 700 nm using a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, MA, USA).

**Laser light source**

A diode laser system was used for blue light (BL, \(\lambda = 405\) nm; FC-W-405-5W, CNI, Changchun, China) photobiomodulation in the continuous-wave mode. Red light (RL, \(\lambda = 635\) nm; SD-635-HS-1W, CNI, Changchun, China) was used to compare the cell viability between BL and RL. A multimode flat optical fiber (core diameter = 600 \(\mu\)m, TeCure, Inc.) was used to deliver laser light to the target surface of a cell culture plate and fenestrated wound site. The laser output from the optical fiber was monitored using a power meter (Nova II, Ophir) and power sensor (50(150)A-BB-26, Ophir, Jerusalem, Israel) to ensure consistent experimental conditions. The laser output was calculated by considering the changes in a beam spot size to maintain the equivalent irradiance (See below table). The temperature of the media was measured using an infrared camera (FLIRA300, FLIR System, Stockholm, Sweden) during the PT and BL treatments.

| Treatment parameter of blue light photobiomodulation | In vitro | In vivo |
|-----------------------------------------------------|---------|-------|
| Parameter [unit] | 96-well plate | 24-well plate | 6-well plate | Fenestrated wound |
| Wavelength [nm] | 405 | | | |
| Operating mode | Continuous wave (CW) | | | |
| Power [mW] | 66 | 380 | 1,920 | 400 |
| Beam size [cm\(^2\)] | 0.33 | 1.9 | 9.6 | 2 |
| Power density [mW/cm\(^2\)] | 200 | | | |
| Irradiation time [sec] | 60 | | | |
| Energy [J] | 3.96 | 22.8 | 115.2 | 24 |
| Energy density [J/cm\(^2\)] | 12 | | | |

**Combined treatment on stimulated fibroblasts**

Twelve hours after the addition of TGF-\(\beta_1\), PT and BL were applied to the stimulated fibroblasts consecutively. To verify the maximum permissible dosage of the treatments, various irradiation conditions were tested: 0, 25, 50, 100, 200, 400, and 800 \(\mu\)g/mL for PT, and 0, 50, 100, 200, 400, and 800 mW/cm\(^2\) for BL. Time (energy density)-dependent cytotoxicity was confirmed using 200 mW/cm\(^2\) for various irradiation times: 30 s (6 J/cm\(^2\)), 60 s (12 J/cm\(^2\)), 120 s (24 J/cm\(^2\)), 240 s (48 J/cm\(^2\)), and 480 s (96 J/cm\(^2\)). Based on the cell viability results, 100 \(\mu\)g/mL of PT and 12 J/cm\(^2\) (200 mW/cm\(^2\) for 60 s) of BL were selected as the optimal dosage of the treatment and used for the rest of the experiments.

**Combined treatment on fenestrated wounds**

The animals were randomly assigned to four groups to compare the therapeutic effect of each treatment (CTRL, no treatment; PT, phlorotannins only; BL, blue light only; PT + BL, phlorotannins with blue light).
Based on the in vitro results, 100 μg/mL PT and 12 J/cm² BL (200 mW/cm² for 60 s) were used for the treatment of the fenestrated wounds. PT was applied to the fenestrated wounds using gauze dressing. For BL irradiation, an optical fiber was placed 7 cm above the wound surface to completely cover the wound (spot size = 2 cm²; see above table). All treatments were applied once daily for three days, beginning on the day of tracheal fenestration. All wounds were monitored for ten days after fenestration when the fenestration sites were almost closed in the CTRL group.

**Cell viability and proliferation**

The dose- and time-dependent cytotoxicities of PT and BL were assessed based on the cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The treated cells were incubated with 1 mg/mL MTT tetrazolium bromide solution (Sigma-Aldrich, St. Louis, MO, USA) for 4 h and replaced with DMSO for 20 min. The absorbance was measured using a microplate spectrophotometer at a wavelength of 540 nm. The cell proliferation was analyzed using a bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit (#68135; Cell Signaling Technology, MA, USA) according to the manufacturer’s instructions. Each experiment was conducted in duplicate and repeated thrice independently.

**Cell migration**

To evaluate the cell migration, a scratch wound assay was performed using a scratcher with a 0.5 mm tip size (201,924, SPL Life Science, Korea). After scratching, the cells were washed with PBS and treated under the experimental conditions. The scratched cell monolayers were imaged using a cell culture microscope (CKX53, Olympus, Japan) at 0 and 24 h after scratching. The scratched area was measured using ImageJ (National Institute of Health, MD, USA) and calculated as the percentage wound closure using the following equation:

\[
\text{Percentage wound closure} = \frac{\text{Initial area (0 h)} - \text{Measured area (24 h)}}{\text{Initial scratched area (0 h)}} \times 100
\]

**(Equation 1)**

**Protein expressions and cell signaling pathways**

Western blot analysis was performed to validate the release of fibrosis-related proteins and to elucidate the cell signaling pathways using α-SMA (ab7817, 1:1000), type-I collagen (ab34710, 1:1000), total and phospho-SMAD (5678S, sc7345, 9251S, 1:2000), total and phospho-JNK (sc7345, 9251S, 1:2000), total and phospho-ERK (sc7149, 9251S, 1:2000), and total and phospho-p38 (sc7149, 9251S, 1:2000) antibodies. The treated cells were washed twice with PBS and lysed with lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl2, 0.5% Triton X-100, 1 mmol/L EDTA, 1% phosphatase inhibitor, 1% protease inhibitor (Sigma, St. Louis, MO)] at 30 min (SMAD, JNK, ERK, and p38) and 48 h (α-SMA and collagen) after treatments. The supernatant was collected after centrifugation at 12,000 rpm for 15 min and quantified using a BCA protein assay kit (Thermo Fisher Scientific). Protein (30 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (LC2001, Invitrogen, MA, USA). After blocking with 5% bovine serum albumin (BSA), the membrane was incubated with primary and secondary antibodies (HRP-anti-mouse; Thermo Fisher Scientific, HRP-anti-rabbit; AbFrontier, Korea, 1:5000). The signals were detected using an imaging system (iBright FL1000, Thermo Fisher Scientific) and quantified using the gel analysis plugin in ImageJ.

**Histology and immunohistochemistry**

Biopsied tissues were fixed in 4% paraformaldehyde, and 4 μm serial sections were stained with hematoxylin and eosin (H and E), Masson’s trichrome (MT), and immunohistochemistry (IHC) to evaluate the morphological changes, collagen distribution, and fibrosis-related cytokine release. For IHC, TNF-α (ab6671), IL-6 (ab9324), TGF-β1 (MA5-16949), and fibronectin (ab2413) were used as the primary antibodies. The stained histology images were observed using the Motic Digital Slide Assistant System (Motic, British Columbia, Canada) and quantified by an expert pathologist (S.O.) using a semi-quantitative scoring system (See below table).
QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using the SPSS software 22 (SPSS Inc., Chicago, IL, USA), and the mean ± standard deviation was calculated for at least three independent experiments. The Mann–Whitney U-test was used for nonparametric statistical analysis between two groups, and the statistical significance was set at p < 0.05.

| Scale | Acute inflammation (predominantly neutrophil infiltrate) | Granulation tissue | Re-epithelization | Fibrosis |
|-------|---------------------------------------------------------|--------------------|-------------------|---------|
| 0     | Absent to minimal                                       | Absent to minimal  | Absent to minimal | Absent to minimal |
| 1     | Mild                                                    | Mild               | Epithelial proliferation in the margin | Mild |
| 2     | Moderate                                                | Moderate           | Continuation of epithelialization     | Moderate |
| 3     | Marked                                                  | Marked             | Complete           | Marked  |