

Water extract of *Osmanthus fragrans* attenuates TGF- β 1-induced lung cellular fibrosis in human lung fibroblasts cells

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Abstract

Introduction: Idiopathic interstitial pneumonia, also known as idiopathic pulmonary fibrosis, is a type of diffuse parenchymal lung disease of unknown etiology. *Osmanthus Fragrans*, referred to as sweet olives, possesses various functions, including neuro-protection, the scavenging of free radicals, and anti-oxidation. Transforming growth factor beta (TGF- β) is a vital growth factor that is essential for regulating cell growth, differentiation, immune-regulation, and extracellular matrices. This study investigates the mechanism underlying water extract of *Osmanthus fragrans* on the regulation of pulmonary cellular fibrosis.

Materials and Methods: The HFL-1 cells, human lung fibroblasts, were cultured with 5 ng/mL of TGF- β 1 for 24 h. After the induction of cellular fibrosis, 200 μ g/mL water extract of *Osmanthus fragrans* was added and cultured for another 24 h. Subsequently, the enzyme-linked immunosorbent assay was performed to quantify the extracellular fibronectin, and Western blot analysis was conducted to examine the expression of TGF- β 1 and its downstream SMAD proteins.

Results: The findings indicated that 200 μ g/mL of water extract of *Osmanthus fragrans* significantly attenuates TGF- β 1-induced increase in fibronectin. In addition, *Osmanthus fragrans* dramatically suppresses TGF- β 1-induced increases in the expression of pSmad2/3.

Conclusion: We propose that water extract of *Osmanthus fragrans* is a potential fibrosis antagonist for lung fibroblasts. Thus, water extract of *Osmanthus fragrans* may exert its beneficial effects through suppressing post-receptor signaling of TGF- β 1 and restoring lung fibroblasts cells character by blocking the expression of pSmad2/3.

Keywords: *Osmanthus Fragrans*, lung fibrosis

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial lung disease (ILD) of poor prognosis, for which there is no effective therapy available.[1-3]. IPF is a chronic, progressive fibrotic lung disease, characterized by deposition of interstitial matrix within the alveolar walls and fibroblast foci in the lung regions, which has a poor prognosis with a median survival of between 2.5 and 3.5 years after diagnosis. [3-7] There is no effective therapy to prevent or reverse pulmonary fibrosis, emphasizing the need to explore novel targets. Transforming growth factor-beta1 (TGF- β 1) is considered to be one of the critical mediators in the pathological processes of IPF, linking inflammation to fibrogenesis.[3,7,8]. Thus, TGF- β 1-induced pulmonary fibrosis is widely accepted as model of IPF.

Osmanthus fragrans flower, recently certificated as a new natural antioxidant, has been used in various foods. It has been found to inhibit lipid peroxidation through ferrous chloride in the mitochondria in rat brain, liver, heart, and kidney.[9] It may also exert neuroprotective actions through its upregulation of survival pathway, attenuating neurotoxicity. In addition, extract of *Osmanthus fragrans* flower have been demonstrated to have anti-depressive and anti-allergic effects. [10] All of these studies showed a strong enhanced antioxidant status after the consumption of the extract from the *Osmanthus fragrans* flower. However, the possible role of *Osmanthus fragrans* in the treatment of pathogenesis of pulmonary fibrosis remains unclear.

There is compelling evidence that the cytokine TGF- β (transforming growth factor- β) plays a central role in lung fibrosis.[3,11-13]. TGF- β has profound effects on fibroblasts, which are central to the pathogenesis of pulmonary fibrosis.[5,12]. TGF- β assembles TGF- β type I and type II receptors and, then, transmits signals by the Smad family of transcriptional activators. Activated type I receptors phosphorylate receptor-regulated Smad (R-Smad), Smad 2 and 3 that, in turn, binds to the common mediator Smad, Smad4, then translocate into the nucleus and bind to specific Smad-binding elements in the regulatory region of TGF- β target genes and direct the trans activation.[11,12,14]. Such complexes subsequently translocate into the nucleus to regulate the expression of target genes (e.g., fibronectin). These suggest that TGF- β /Smad signaling is essential for ECM formation in the pathogenesis of tissue fibrosis.[1,12,15-17]. Thus, the effects of *Osmanthus fragrans* in cultured human fetal lung fibroblasts (HFL-1 cell line) were examined.

In this study, we investigated the mechanism underlying the attenuation of lung fibrosis exerted by *Osmanthus fragrans*. The crude extract of *Osmanthus fragrans* may contain some natural compounds with potential therapeutic effects for pulmonary fibrosis that are associated with antagonization of TGF- β -induced fibrogenic signals (i.e., Smad pathways) in interstitial fibroblast cells. Collectively, we proposed that *Osmanthus fragrans* might act as a novel fibrosis antagonist for treating pulmonary fibrosis

2. Materials and methods

2.1 Preparation of water extracts of *Osmanthus fragrans*

Dried flowers of water extract of *Osmanthus fragrans* were purchased from Hung Chao Co., Ltd., Taipei, Taiwan, in 2009. The *Osmanthus fragrans* (10 kg) were milled, suspended in 150 L of distilled water, and boiled for 60 min at 100°C. After filtration with filter paper, the combined extracts were concentrated under reduced pressure and freeze-dried to provide dark syrup which was stored at -20 °C for further use.

2.2 HPLC analysis

Constituents of water extract of *Osmanthus fragrans* were analyzed using a Hitachi instrument with L-7100 series quaternary gradient pump (Hitachi, Japan), and a diode array detector (L-7455) linked to a Hitachi lachrom software data handling system (D-7000 Multi-HSM-Manager). Reverse-phase separations were carried out using a C₁₈ SUPELCO Discovery column (4.6 × 250 mm i. d., 5 μ m; Sigma-Aldrich Co. LLC., USA). Reverse phase HPLC was performed by using 0.1% acetic acid and methanol(65:35) as a mobile phase, and the column temperature was maintained at 25°C. The flow rate was 1 mL/min and the injection volume was 10 μ L. The eluted components were identified based on the retention time in comparison with the used reference standards. The identity of constituents was also confirmed with a photodiode array detector by comparison with UV spectra of standards over the wavelength range 190-400 nm.

2.3 Cell Culture:

HFL-1 (CCL-153, ATCC), a human lung, normal fibroblast, was cultured in Dulbecco's modified Eagle's medium (Hyclone Labs, UT) medium supplemented with F12K and 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Hyclone Labs) at 37°C in 5% CO₂. The cells were trypsinized using 0.5% trypsin-EDTA (GIBCO, Canada).

2.4 Western Blotting assay

Western blot assay was utilized to evaluate the protein expression for the TGF- β receptors downstream signal (i.e. Smads) and fibronectin. In brief, cells were lysed using lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mM dithiothreitol and 50 mM PMSF). The crude protein lysate was resolved by 7.5% SDS-PAGE. After protein transfer to a polyvinylidene difluoride (PVDF) membrane using an electrotransfer unit, the PVDF membrane was blocked with 10% (w/v) de-fat milk in Tris-buffered saline (TBS-T) for 2 h at 37°C. The blots were probed with a 1:2,000 (v/v) dilution of polyclonal antibodies fibronectin, all purchased from Santa Cruz Biotechnology, Inc. (SantaCruz, CA). After hybridization at 37°C, the blots were washed and hybridized with 1:4,000 (v/v) dilutions of goat anti-rabbit IgG or horseradish peroxidaseconjugated secondary antibody (Calbiochem, Germany) or donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.). TBS-T buffer with 10% defat milk was used for blocking. The signal for respective proteins was generated by adding enhanced chemiluminescent reagent, with b-actin used as an internal control.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to evaluate the expression of secreted fibronectin. Conditioned culture media from HFL-1 cells were collected and centrifuged at 1,200 rpm for 5 min to remove particulate. The clear supernatant was then collected and concentrated and finally stored at -80°C for further use. A commercial sandwich enzyme-linked immunosorbent assay kit was utilized for detection of extracellular fibronectin (Tarkara Bio, Inc., Shiga, Japan). The procedure was performed according to the manufacturer's instruction manual. The absorbance (450 nm) for each sample was analyzed using an ELISA reader, and the concentration of each sample determined by interpolation with the standard curve, which was generated using an exogenous fibronectin (0, 1.56, 6.25, and 25 ng/ml) as the standard.

2.6 Immunofluorescent analysis

HFL-1 cells were grown as monolayers on glass cover slips in 24 well culture plates. When 80% confluent, Cells were treated with 5 ng/ml TGF- β 1 in 0.5% FBS for 24 h, followed by treatment with water extract of *Osmanthus fragrans* (200 μ g/ml) for another 24 h incubated at 37 °C with 5% CO₂, Fix the samples in 4% paraformaldehyde for 30 min at room temperature. Wash the samples twice with ice cold PBS, Triton X-100 for 10 min at room temperature, Wash the samples twice with ice cold PBS, and then blocked 1h, Wash the samples twice with ice cold PBS, The fixed samples were incubated overnight at 4 °C with fibronectin antibody (1:200) (ab23751; Abcam, Cambridge, UK), and then incubated with Goat Polyclonal Anti-rabbit FITC, ab6717; (Abcam, Cambridge, UK) (1:400) for 1 hrs. At room temperature in dark and washed three times with PBS, 4,6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnology, USA) was used to stain nuclei, and observed by using an Olympus IX-71 fluorescence microscope, using image J software.

3.7 Statistics

Results were expressed as mean \pm SEM. The unpaired Student's t test was used for comparison between two groups. $P < 0.05$ was considered to be statistically significant. All values were calculated using GraphPad Prism version 3.00 for Macintosh (GraphPad Software, San Diego, CA).

3. Results

During HPLC analysis, *Osmanthus fragrans* displayed peaks at 8.48 min for verbascoside and 13.4 min for rutin (Fig. 1). The present study identified that the major antioxidant compound in *Osmanthus fragrans* is verbascoside, which can be easily separated [10]. Rutin is another compound which contains in *Osmanthus fragrans*. Verbascoide and rutin in *Osmanthus fragrans* appeared to be significant antioxidants. [10] Hung et al. (2012) reported that the fractions 5 and 6 exhibited high antioxidant activity than other fraction. Rutin (8.48 min) and verbascoside (13.4 min) revealed strong DPPH radical scavenging activity, with IC₅₀ values of 10.3, and 6.2 μ M, were isolated from fraction 5 and 6. There appears to be a prominent compounds occurring at approx. 65 minutes. However these were a mixture of compound without strong antioxidant capability. Water extract of *Osmanthus fragrans* flower, contained rutin, and verbascoside compounds were, therefore, selected as the test materials for *in vitro* experiments.

We further investigated the fibrosis-regulatory effects of water of extract *Osmanthus fragrans* on human fetal lung fibroblasts cells. TGF- β -induced over-production of ECM provided an *in vitro* model for pulmonary fibrosis. As shown in Fig. 2, water of extract *Osmanthus fragrans* does not inhibit cell growth or induce cell death. To evaluate the regulatory effects of *Osmanthus fragrans* in TGF- β -induced ECM proteins accumulation, ELISA analysis and immunofluorescent staining were performed for detection of extracellular fibronectin. According to Fig. 3 and 4, *Osmanthus fragrans* treatment (200 μ g/ml for 24-h) significantly decreased the production of fibronectin in a dose-dependent manner in HFL1 cells compared with TGF- β 1 (5 ng/ml) treating group ($P > 0.01$). In addition, the parallel results were observed from an ELISA and immunofluorescence assay. TGF- β 1 treatment showed an increase in the extracellular deposition of fibronectin by immunofluorescence staining. More importantly, *Osmanthus fragrans* treatment (200 μ g/ml for 24-h) significantly restored TGF- β 1-induced increase in fibronectin (Fig. 3 and 4). So far, this study is the first demonstration discussing the lung fibrosis regulatory effects of *Osmanthus fragrans*.

To elucidate the underlying mechanism by which *Osmanthus fragrans* regulates lung cellular fibrosis, the expressions of two TGF- β receptor types (type II TGF- β 1 receptors and type I TGF- β 1 receptors) were investigated. As shown in Fig. 5, TGF- β 1-induced a significant increase in the level of type II TGF- β 1 receptors. Most importantly, *Osmanthus fragrans* statistically significant reduced protein expression of type II TGF- β 1 receptors instead of type I receptor according to Western blot. Smads-related signal molecules were examined under the treatment of *Osmanthus fragrans* because the Smad family is the most important mediator for post-receptor signaling of TGF- β 1. The TGF- β 1 (5 ng/ml) significantly increased pSmad2/3 and Smad2/3 (Fig. 5). Intriguingly, water extract of *Osmanthus fragrans* dose-dependently (25, 50, 100, and 200 μ g/ml) and dramatically suppressed TGF- β 1-induced increases in pSmad2/3 and Smad2/3. These results (see also Fig. 5) suggest that *Osmanthus fragrans* may have the potential to reverse TGF- β 1-induced cellular fibrosis through down-regulation of TGF- β 1 down-stream signals.

Previous studies showed that TGF- β signals within the cell through the pSmad2/3 of transcriptional activators. Thus, pSmad2/3 is the most important mediator for TGF- β signaling. We demonstrated that 5 ng/ml of TGF- β 1 time course-dependently (0, 1, 4, 8, 12 and 24 hrs) induced a significant increase in pSmad2/3. Again, these effects have been dramatically attenuated by *Osmanthus fragrans* (200 μ g/ml) especially at the time point of 24 h (Fig. 6). The results demonstrated *Osmanthus fragrans* has the potential to retard TGF- β 1-induced pulmonary cellular fibrosis in HFL-1 cells.

Taken together with above results, we identified an antifibrogenic role of *Osmanthus fragrans* and elucidated its interactions with TGF- β 1 and its downstream signal transducers as shown in Fig. 7. In this study, we found that *Osmanthus fragrans* antagonizes TGF- β 1-induced pulmonary fibrosis by enhancing the inhibitory pSmad2/3. Thus, we propose for the

first time that *Osmanthus fragrans* has the potential to regulate the pathogenesis of pulmonary fibrosis. Our findings demonstrate the beneficial role of water extract of *Osmanthus fragrans* in treating pulmonary fibrosis. We suggested that water extract of *Osmanthus fragrans* is implicated in TGF- β -induced pulmonary fibrosis. *Osmanthus fragrans* may act as a novel therapeutic agent for treating pulmonary fibrosis.

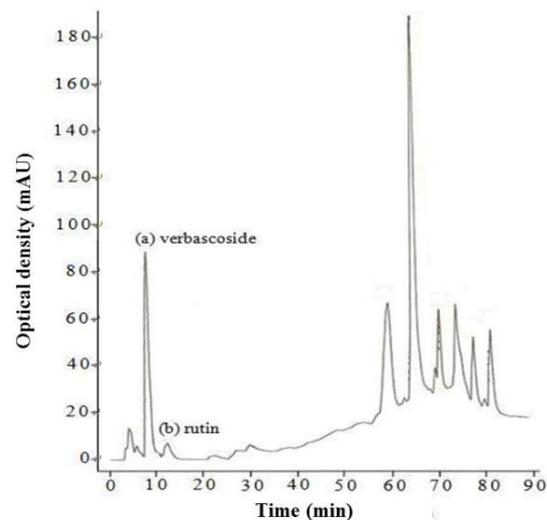


Figure 1: High performance liquid chromatographic spectrum of Osmanthus Fragrans. (a):Verbascoside, (b):rutin. The total phenolic, flavonoid contents and dpph scavenging effects in Osmanthus Fragrans.

The total phenolic contents were 340.68 ± 19.47 mg GAE/g extract, while the total flavonoid contents were 49.97 ± 4.40 mg QE/g extract. The water extract DPPH IC₅₀ was $15 \mu\text{g/mL}$, less than methanol extract ($12.8 \mu\text{g/mL}$), and trolox ($4.9 \mu\text{g/mL}$). As previous report, the *Osmanthus fragrans* was a rich phenolic- and flavonoid-content, also exhibited greater antioxidative activity.

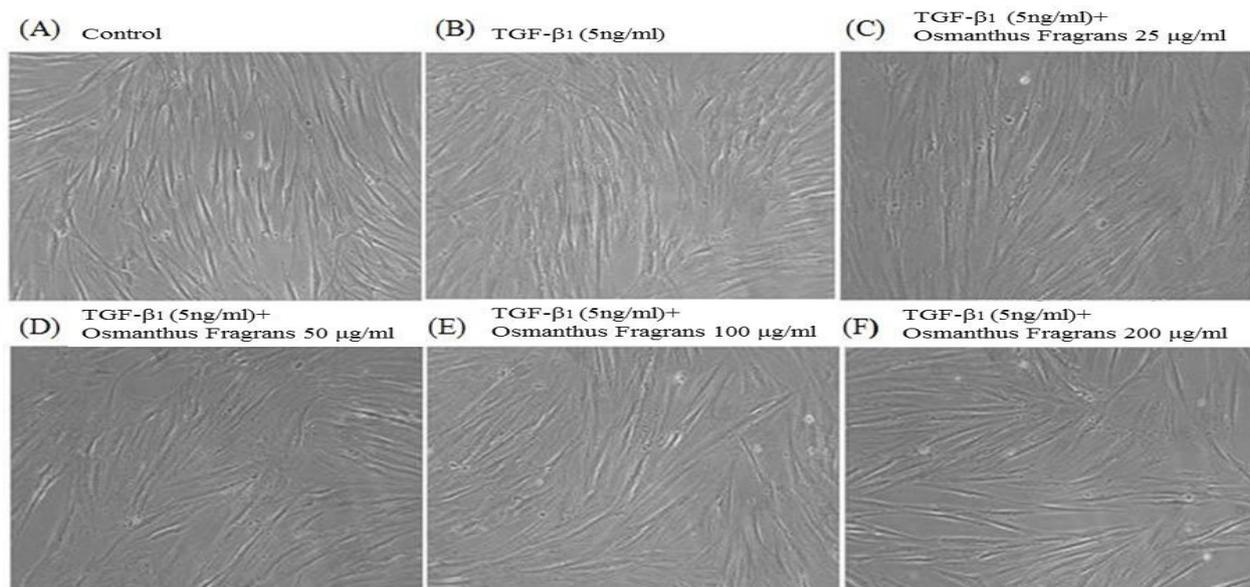


Figure 2: Effect of Osmanthus fragrans on phenotypic in Human fetal lung fibroblasts induced by TGF- β 1. Phase contrast microscopy 200 \times : Cells were treated with 5 ng/ml TGF- β 1 in 0.5% FBS for 48hrs. Treatments with different water extract of *Osmanthus fragrans* for 24hrs. Water extract of *Osmanthus fragrans* (0-200 $\mu\text{g/ml}$) for another 24 hrs. (A)Control (B)TGF- β 1 (C)Water extraction of *Osmanthus fragrans* (25 $\mu\text{g/ml}$) (D) Water extraction of *Osmanthus fragrans* (50 $\mu\text{g/ml}$) (E) Water extraction of *Osmanthus fragrans* (100 $\mu\text{g/ml}$) (F) Water extraction of *Osmanthus fragrans* (200 $\mu\text{g/ml}$)

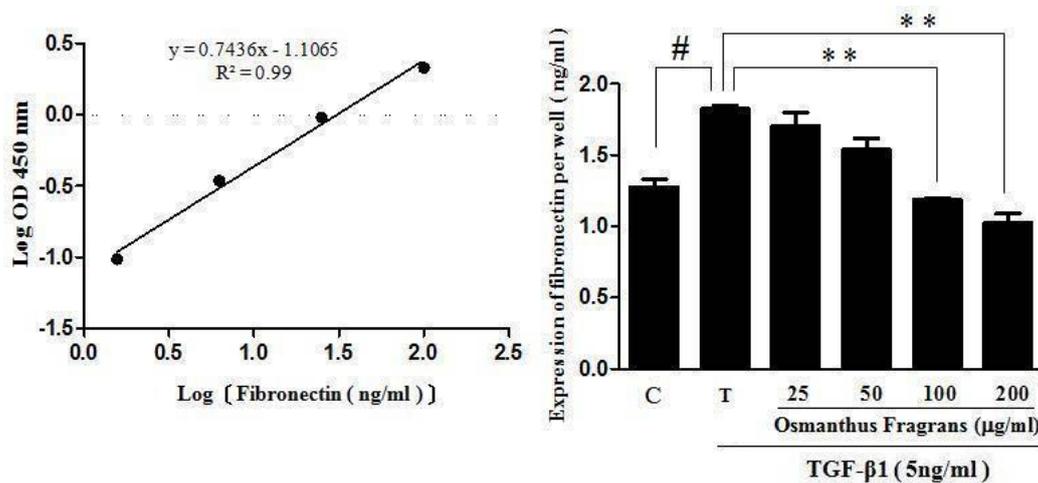


Figure 3: Antagonizing effects of Chinese herb *Osmanthus fragrans* extracts on TGF-β1-induced fibronectin expression in Human fetal lung fibroblasts (HFL-1) cells. **A:** Standard curves were generated using known concentrations of fibronectin (0, 1.56, 6.25, and 25 ng/ml). ELISA was performed as the manufacturer's instructions. The absorbance (450 nm) of each sample was then analyzed by an ELISA reader. **B:** Cells were cultured in F12K within 10% fetal bovine serum and 2% penicillin/streptomycin in 25cm² flask for 24 hrs. The following, Cells were treated with 5 ng/ml TGF-β1 in 0.5% FBS for 24 h, followed by treatment with water extract of *Osmanthus fragrans* (200 μg/ml) for another 24 h. Supernatant was collected and subjected to fibronectin ELISA analysis. Fibronectin level was determined by interpolation with the standard curve. The fibronectin level of each condition was normalized to the cell number of each well. It is evident that TGF-β1-induced a significant increase in fibronectin levels. In addition, water extracts of *Osmanthus fragrans* dose-dependently attenuated TGF-β1-induced increase in fibronectin level. 0 vs. T, P* < 0.05; T vs. 100 and T vs. 200, P** < 0.05.

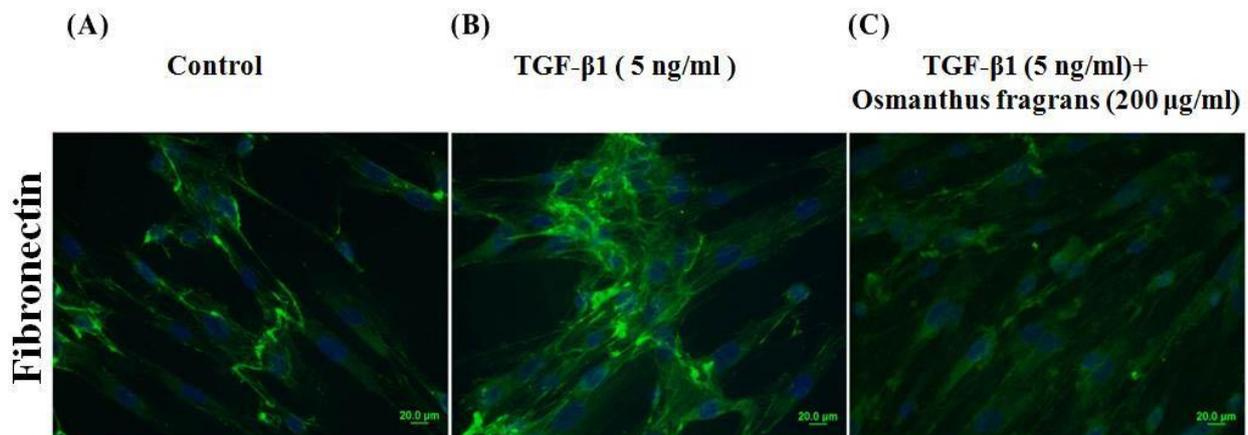


Figure 4: Water extracts of *Osmanthus fragrans* revers TGF-β-induced fibronectin. Analysis used immunofluorescence stain.

Cells were cultured in F12K within 10% fetal bovine serum and 2% penicillin/streptomycin in 24 well for 24 hrs. The following, Cells were treated with 5 ng/ml TGF-β1 in 0.5% FBS for 24 h, followed by treatment with extract of O.F. extracts (200 μg/ml) for another 24h. (A) control group; (B) TGF-β1 group; (C) O.F. water extract group (200 μg/ml) ; using the fluorescence microscope to observe the performance of the fiber protein.

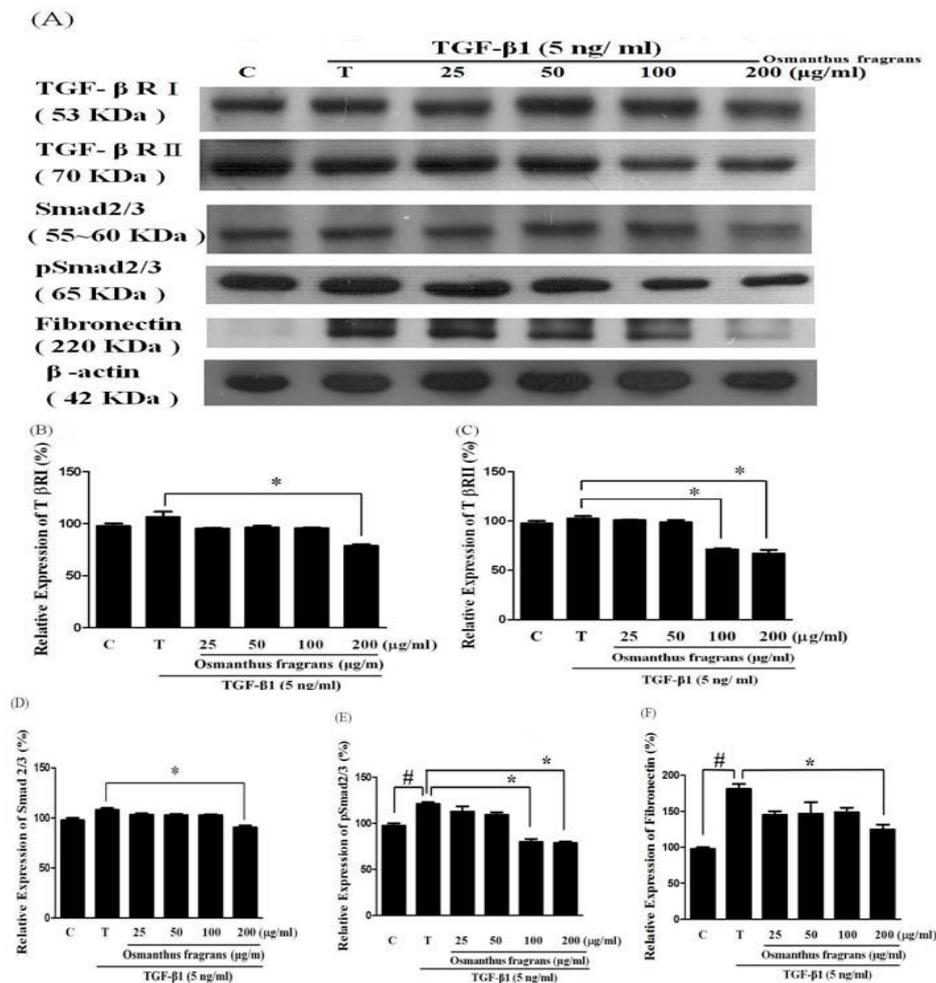


Figure 5: Effects of *Osmanthus fragrans* revers TGF-β-induced Signal pathway (TbetaR1 TbetaR2 Smad2/3 pSmad2/3, Smad4 Smad7) in HFL-1 cells: Western blot analysis *Osmanthus fragrans* in the TbetaR1 TbetaR2 Smad2/3 pSmad2/3, Smad4 Smad7 level. HFL-1 cells were cultured in 25 cm² flask. Cells were treated with 5 ng/ml TGF-β1 in 0.5% FBS for 24 h and incubated in different *Osmanthus fragrans* concentration (0-200 μg/ml) condition for 24 hrs.

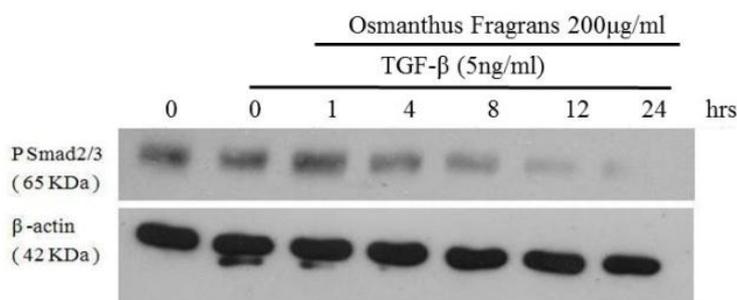


Figure 6: TGF-β1-induced a significant increase in pSmad2/3 levels. Water extract of (200 μg/ml) attenuated TGF-β1-induced increase in different time point pSmad2/3 level. Cells were cultured in F12K within 10% fetal bovine serum and 2% penicillin/streptomycin in 25cm² flask 1 for 24 hrs. The following, Cells were treated with 5 ng/ml TGF-β1 in 0.5% FBS for 24 h, followed by treatment with extract of *Osmanthus fragrans* extracts (200 μg/ml) for another 24h. It is evident that TGF-β1-induced a significant increase in pSmad2/3 levels. In addition, *Osmanthus fragrans* extracts attenuated TGF-β1-induced increase in different time points Psmad 2 / 3 level.

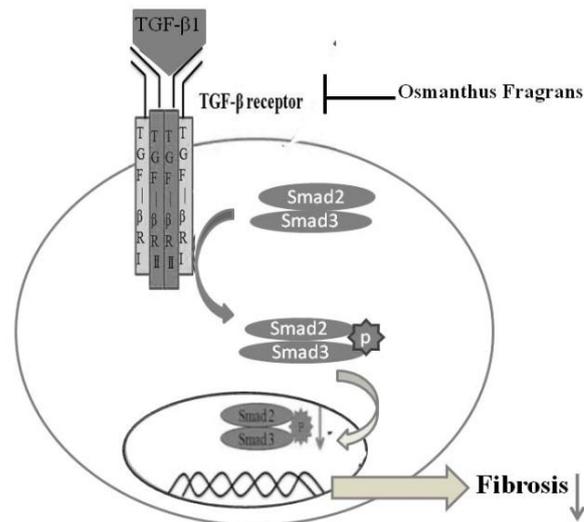


Figure 7: We suggested that *Osmanthus fragrans* antagonizes TGF-β1-induced pulmonary fibrosis by inhibit of pSmad2/3.

Taken together with previous results, we identified a mechanism for the antifibrogenic role of *Osmanthus fragrans* and elucidated its interactions with TGF-β1 and its downstream signal transducers as shown in Fig. 7. In this study, we found that *Osmanthus fragrans* antagonizes TGF-β1-induced pulmonary fibrosis by inhibit of pSmad2/3. Thus, we propose for the first time that *Osmanthus fragrans* has the potential to regulate the pathogenesis of pulmonary fibrosis. Our findings demonstrate the beneficial role of *Osmanthus fragrans* in pulmonary fibrosis. We suggested that *Osmanthus fragrans* is implicated in TGF-beta-induced pulmonary fibrosis. *Osmanthus fragrans* may act as a novel therapeutic agent for treating pulmonary fibrosis.

4. Discussion

The *Osmanthus fragrans* flower contains several antioxidant compounds. Ben Cao Gang Mu, traditional Chinese medical literature, describes the usefulness of these flowers for phlegm and stasis reduction, arrest of dysentery with blood in the bowel, and stomachache and diarrhea treatment.[22] Few studies have examined the medical applications of Osmanthus Fragrans. However, little is known about the role of *Osmanthus fragrans* in regulating fibrosis in pulmonary fibrosis.

A key finding of our study is the demonstration of the beneficial effects of water extract of *Osmanthus fragrans* on human fetal lung fibroblasts cellular fibrosis. In this study, 2-day exposure of TGF-β1 in lung cells induced significant cellular fibrosis in human fetal lung fibroblasts cell. Intriguingly, water extract of *Osmanthus fragrans* dramatically reduced TGF-β- induced increase in fibronectin of both intercellular and extracellular origin (Fig. 3, 4). We show that water extract of *Osmanthus fragrans* is a novel and powerful lung fibrosis regulator. Water extract of *Osmanthus fragrans* dramatically attenuates TGF-β1-induced increase in fibronectin in human fetal lung fibroblasts cells. Moreover, water extract of *Osmanthus fragrans* significantly inhibited TGF-β1-induced increases in type I TGF-β receptors and it down stream Smads signaling.

The mechanism underlying the fibrosis-regulatory effects of water extract of *Osmanthus fragrans* might be closely correlated with type I TGF-β receptors. Water extract of *Osmanthus fragrans* has the potential to counteract the effects of fibrogenic growth factor TGF-β1 by regulating type I TGF-β receptors and their down-stream signal transducers, like Smad2/3s and Smad7 (Fig. 5). So far, this study is the first demonstration discussing the fibrosis regulatory effects of water extract of Osmanthus Fragrans. In addition, we demonstrate that water extract of *Osmanthus fragrans* plays a pivotal role in cellular fibrosis in human lung fibroblast cells, possibly by regulating post-receptor signal transducers (e.g., pSmad2/3) of fibrogenic growth factor, TGF-β. (Fig. 6)

Although modern evidence for the in vivo therapeutic activity of water extract of *Osmanthus fragrans* is yet to be provided, a few studies have examined the in vitro bioactivity of water extract of Osmanthus Fragrans. Lin *et al.*, demonstrated no evidence of adverse effects. Ajith *et al.*, demonstrated *Osmanthus fragrans* inhibited lipid peroxidation occurring through ferrous chloride in the mitochondria in rat brain, liver, heart and kidney.[10,18-20] Jeong *et al* showed that *Osmanthus fragrans* exert neuroprotective actions through the upregulation of the AKT survival pathway, which

attenuates neurotoxicity.[9] Lee *et al* showed *Osmanthus fragrans* can suppress NO production in LPS-induced macrophage.[20] In addition, *Osmanthus fragrans* may prove beneficial in the development of natural agents for prevention and treatment of inflammatory diseases.

In this study, we propose that water extract of *Osmanthus fragrans* might have potential applications in treating lung fibrosis. Although this inference requires further intensive investigations, the notion is compatible with some previous studies. Hung *et al.*, demonstrated that water extract of Osmanthus Fragrans, which contains many antioxidants, promotes a positive antioxidative state in an animal model of allergic airway inflammation.[10] It also has protective effects including decreasing the OVA-specific IgE production and inflammatory cell infiltration in lung.[1-5,21]

We propose that water extract of *Osmanthus fragrans* is a potential fibrosis antagonist for lung fibroblasts. *Osmanthus fragrans* might act through suppressing post-receptor signaling of TGF- β 1 and restoring tubule epithelial character by blocking the expression of pSmad2/3. Taken together, our studies indicate that water extract of *Osmanthus fragrans* is an effective fibrosis regulator. Future clinical and basic research studies are needed to be investigated in lung cells. Moreover, the present data inspire further study to explore the effects and mechanisms of anti-fibrotic herbal compounds in preventing lung fibrosis and urges us to screen more plant materials to discover novel anti-fibrotic drugs.

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