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Inhibition of Proinflammatory Cytokine Generation in Lung Inflammation by the Leaves of *Perilla frutescens* and Its Constituents

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Abstract

This study was designed to find some potential natural products and/or constituents inhibiting proinflammatory cytokine generation in lung inflammation, since cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are pivotal for provoking airway inflammation. In our preliminary screening procedure, the 70% ethanol extract of the leaves of *Perilla frutescens* (PFE) was found to clearly inhibit TNF-α production in the lung at 100 mg/kg, after intranasal lipopolysaccharide treatment of mice. Based on this result, ten constituents including phenylpropanoids (allyltetramethoxybenzene, caffeic acid, dillapiole, elemicin, myristicin, nothoapiole, rosmarinic acid methyl ester, rosmarinic acid) and monoterpenes (perilla aldehyde and perilla ketone) were successfully isolated from the extract. Among them, elemicin and myristicin were found for the first time to concentration-dependently inhibit IL-1β-treated IL-6 production from lung alveolar epithelial cells (A549) at concentrations of 10-100 μM. These findings suggest that the phenylpropanoids including elemicin and myristicin have the potential to be new inhibitory agents against lung inflammation and they may contribute, at least in part, to the inhibitory activity of PFE on the lung inflammatory response.

Key Words: *Perilla frutescens*, Lung inflammation, Bronchitis, Phenylpropanoid, Elemicin

INTRODUCTION

The chronic obstructive pulmonary diseases (COPD) are complex disorders that have complex origins and pathology (Al-Kassini and Alhamad, 2013). They include chronic asthma, chronic bronchitis and emphysema. With the aid of several classes of drugs such as anti-inflammatory steroids, antitussives, mucolytics and/or bronchodilators, the symptoms of COPD can be relieved and controlled in clinics. Recently, some new classes of drugs such as leukotriene receptor antagonist and phosphodiesterase IV inhibitor have been introduced in clinical trials (Jeffery, 2001; Reid and Pham, 2012). But they are not expected to completely cure these diseases. Thus, there is a need for new agents to treat COPD. In this respect, herbal drugs and compounds from plant origins have been continuously explored.

Perilla frutescens (L.) Britton var. *acuta* Kudo (Labiatae) is distributed in China, Japan and Korea, and the leaves of this plant have been frequently prescribed in traditional medicine for treating lung inflammatory conditions especially for treating cough, asthma and chronic bronchitis (Bae, 2000). Previously, some pharmacological activities of this plant material were demonstrated. The ethyl acetate fraction of the plant and the constituents such as luteolin, chrysoeriol and rosmarinic acid, and its methyl ester inhibited 5- and 12- lipoxygenase from rat platelets and leukocytes (Yamamoto *et al*., 1998). In particular, anti-allergic activity against the immediate type was demonstrated (Shin *et al*., 2000; Makino *et al*., 2001). As its constituents, luteolin was found to possess anti-inflammatory activity (Ueda *et al*., 2002). And rosmarinic acid was demonstrated to inhibit *in vivo* allergic inflammation induced by diesel exhaust particles and mite allergens (Sanbongi *et al*., 2003, 2004).

Despite these previous investigations, no attempt has been made to find the effect of the leaves of *P. frutescens* (L.) Britton var. *acuta* Kudo on lung inflammation and to identify their active principle constituents. In our screening procedure, the

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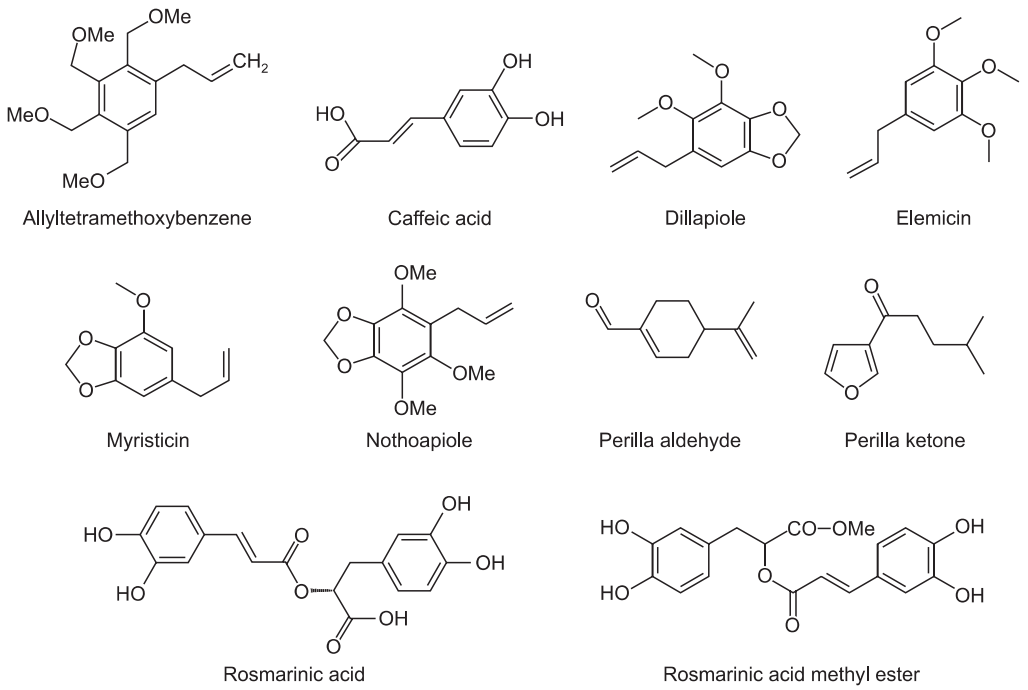

Fig. 1. The chemical structures of the isolated compounds from the leaves of *P. frutescens.*

70% ethanol extract of the leaves of *P. frutescens* (L.) Britton var. *acuta* Kudo (PFE) was found to inhibit TNF-α production in the lung after intranasal lipopolysaccharide (LPS) treatment of mice. Thus, in the present investigation, the active compounds from the extract of *P. frutescens* (L.) Britton var. *acuta* Kudo were investigated and their pharmacological activities were evaluated against proinflammatory cytokine generation in lung inflammation for the purpose of finding new potential inhibitory agents to treat lung inflammatory disorders.

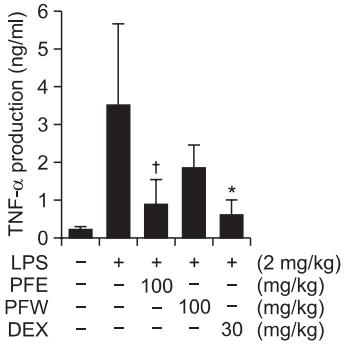

Fig. 2. Inhibition of the 70% ethanol extract of the leaves of *P. frutescens* (PFE) on TNF-α production in LPS-induced airway inflammation in mice. LPS was administered to mice intranasally. TNF-α concentration in the BALF was measured. PFW: water extract of the leaves of *P. frutescens*; DEX: dexamethasone; †p<0.1, *p<0.05, Significantly different from the LPS-treated control group (n=5).

MATERIALS AND METHODS

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dexamethasone, interleukin-1β (IL-1β) and LPS (*Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO, USA). Minimum Essential Media (MEM) and other cell culture reagents including fetal bovine serum (FBS) were products of Gibco BRL (Grand Island, NY, USA). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA, USA).

Plant materials

The aerial parts of *P. frutescens* (L.) Britton var. *acuta* Kudo (25 kg) were collected on Nam Won, Korea in January 2012, and authenticated by Prof. J. H. Lee (Dongguk University, Gyoungju, Korea). A voucher specimen (SKKU NPL 1207) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Preparation of the water and ethanol extracts, and isolation of the compounds

The dried leaves of *P. frutescens* (L.) Britton var. *acuta* Kudo (500.0 g) were extracted with water and 70% EtOH under reflux (2×3 h) and then filtered. The filtrates were evaporated under reduced pressure to give water extract (68.0 g, 13%) and EtOH extract (57.0 g, 11%). These are used for the pharmacological assays.

For the isolation of the constituents, the dried leaves of *P. frutescens* (L.) Britton var. *acuta* Kudo (25 kg) were extracted with petroleum ether, and methanol, successively and evaporated under reduced pressure to give residues (264 g and 2 kg, respectively). The methanol extract (1 kg) was dissolved in water (800 ml) and partitioned with solvent to give hexane (190.0 g), $CHCl_3$ (134.0 g), EtOAc (60.0 g), and BuOH (87.0 g) soluble portions.

The petroleum ether extract (130.0 g) was chromatogra-

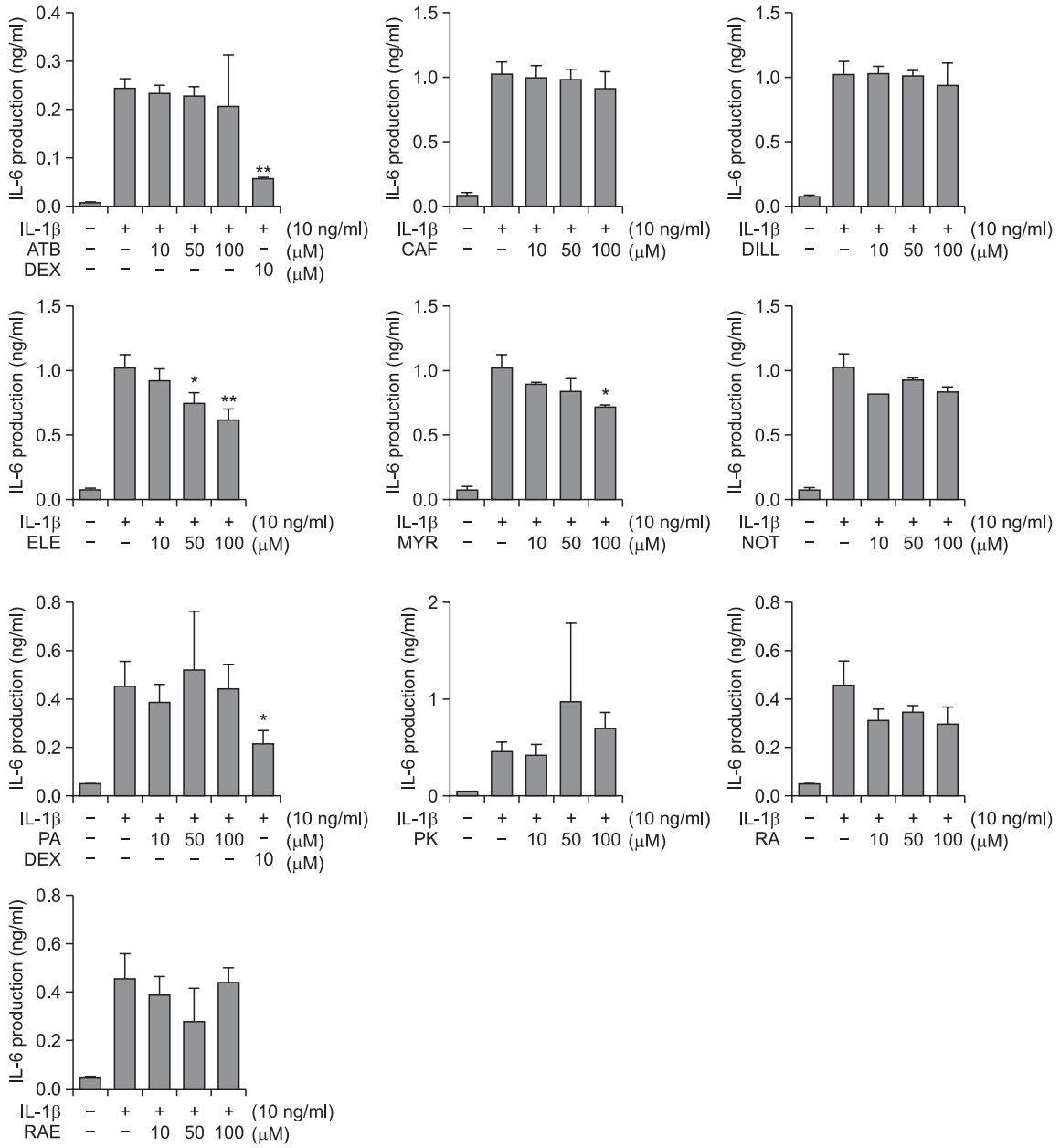

Fig. 3. Inhibition of the constituents isolated on IL-6 production in A549 cells. IL-1β and compounds were simultaneously treated. ATB (allyltetramethoxybenzene), CAF (caffeic acid), DILL (dillapiole), ELE (elemicin), MYR (myristicin), NOT (nothoapiole), PA (perilla aldehyde), PK (perilla ketone), RA (rosmarinic acid), RAE (rosmarinic acid methyl ester), DEX (dexamethasone), **p*<0.05, ***p*<0.01, Significantly different from the IL-1β-treated control group (n=3).

phed over a silica gel column with hexane-EtOAc (1:0-1:1) as the eluent to give three fractions (P1-P3). The P2 fraction (37 g) was subfractionated with a silica gel column with hexane-EtOAc (1:0-1:1) as the eluent to give seven fractions (P21-P27). The P25 fraction (8.7 g) was separated by silica gel column chromatography using a solvent system of hexane-EtOAc (30:1-1:1) as the eluent to yield five fractions (P251-255). The P253 fraction (1.0 g) was also subjected to a RP-C_{18} silica gel column with 100% MeCN and purified by preparative normal-phase high performance liquid chromatography (HPLC) with solvent system of hexane-EtOAc (20:1) to yield myristicin (40 mg), dillapiole (200 mg), perilla ketone (24 mg), nothoapiole (75 mg), and perilla aldehyde (40 mg). The P3 fraction (32.0 g) was subfractionated with a silica gel column with hexane-EtOAc (40:0-1:1) as the eluent to give five fractions (P31-P35). The P33 fraction (4.0 g) was also subjected to a RP-C_{18} silica gel column with 50% MeOH as the eluent to afforded eight fractions (P331-P338). The subfraction P331 (1.9 g) was purified by preparative normal-phase HPLC with solvent system of hexane-EtOAc (8:1) to yield elemicin (1.6 g). The subfraction P334 (466 mg) was purified by preparative normal-phase HPLC with solvent system of hexane-EtOAc

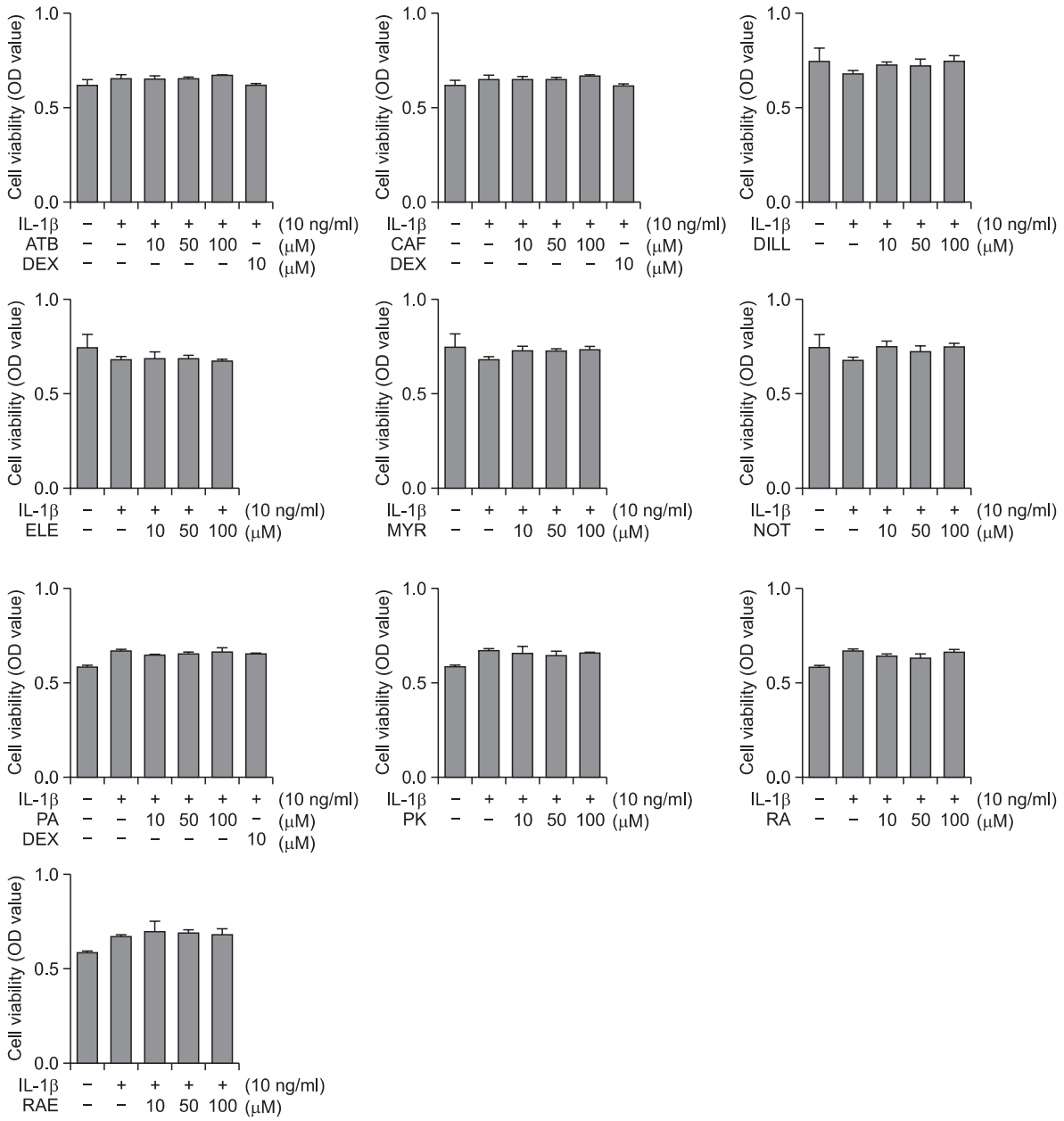

Fig. 4. MTT assay for the isolated compounds in A549 cells. IL-1β and compounds were simultaneously treated. ATB (allyltetramethoxybenzene), CAF (caffeic acid), DILL (dillapiole), ELE (elemicin), MYR (myristicin), NOT (nothoapiole), PA (perilla aldehyde), PK (perilla ketone), RA (rosmarinic acid), RAE (rosmarinic acid methyl ester), DEX (dexamethasone), Note: No cytotoxicity was observed in all tested compounds (n=3).

(20:1) to yield allyltetramethoxybenzene (100 mg).

The EtOAc fraction (33.0 g) was chromatographed over a silica gel column with $CHCl_3$-MeOH (20:1-1:1) as the eluent to give seven fractions (E1-E7). The E5 fraction (14 g) was also subjected to a RP-C_{18} silica gel column with 50% MeOH as the eluent to afford eight fractions (E51-E58). The E51 fraction (7.9 g) was subfractionated with a silica gel column with $CHCl_3$-MeOH (20:1-1:1) as the eluent to give eight fractions (E511-E518). Subfraction E515 (750 mg) was subjected to a Sephadex LH-20 (80% MeOH) and purified by RP-C_{18} preparative HPLC (30% MeOH) to give caffeic acid (80 mg). Subfraction E518 (4.2 g) was subjected to a Sephadex LH-20 (80% MeOH) and purified by RP-C_{18} preparative HPLC (30% MeCN, 60% MeOH) to give rosmarinic acid (1.9 g) and rosmarinic acid methyl ester (245 mg).

These compounds were identified to be perilla aldehyde (Zou *et al*., 2008), perilla ketone (Bassoli *et al*., 2009), allyltetramethoxybenzene (Lopes *et al*., 1986), dillapiole, myristicin (Benevides *et al*., 1999), elemicin (Mohammad and Waterman, 1985), nothoapiole (Laouer *et al*., 2009), rosmarinic acid (Ha *et al*., 2012), rosmarinic acid methyl ester (Woo and Piao, 2004), caffeic acid (Lee *et al*., 2012) by comparison of their spectroscopic and physical data with previously reported values (Fig. 1).

Measurement of tumor necrosis factor-α (TNF-α) concentration in LPS-induced acute lung injury in mice

Since TNF-α is important to provoke and maintain lung inflammatory disorders including COPD (Mueller *et al*., 1996), TNF-α concentration was measured as a biomarker of airway inflammation. For inducing acute lung injury, LPS (800 μg/ml, saline, 10 μl/mouse, 5 times) was administered intranasally to ICR mice (male, 4 weeks, specific pathogen-free, Nara Biotech. Ltd., Seoul) according to the previously published procedures (Lim *et al*., 2013). Four hours later, mice were sacrificed and bronchoalveolar lavage fluid (BALF) was collected via intratracheal cannulation after 700 μl of saline was administered 3 times. From the BALF, TNF-α concentration was measured with an enzyme-linked immunoabsorbant assay (ELISA) kit (eBioscience) according to the manufacturer's recommendation. Test compounds dissolved in 0.3% carboxymethylcellulose were orally administered to mice 1 h prior to LPS treatment (n=5). The experimental design using the animals was approved by the local committee for animal experimentation, Kangwon National University (KNU, KIACUC-13-0003). In addition, the ethical guideline described in the Korean Food and Drug Administration guide for the care and use of laboratory animals was followed throughout the experiments.

A549 cell culture and measurement of IL-6 concentration

A549 cells, a human lung epithelial cell line, obtained from American type culture collection (Rockville, VA) were cultured with RPMI 1640 supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37°C. After pre-incubation for 24 h, interleukin-1β (IL-1β, 10 ng/ml) was added simultaneously with/without test compounds. Four hours later, media was collected and the concentration of IL-6 was determined from the media with an ELISA kit (eBioscience) according to the manufacturer's recommendation. The cell viability was checked using an MTT bioassay as previously described (Mossman, 1983).

The test compounds including the reference drug, dexamethasone, were dissolved in dimethyl sulfoxide (DMSO) and diluted with complete RPMI media. The final concentration of DMSO in the cell culture was adjusted to 0.1% (v/v), and this concentration of DMSO did not affect the cell viability and the levels of IL-6 (data not shown).

Statistical analysis

Experimental values were represented as arithmetic mean ± SD. One way ANOVA followed by Dunnett's test was used to determine the statistical significance.

RESULTS

LPS-treated lung injury in mice is a frequently used animal model for establishing the effects on lung inflammation (Chapman *et al*., 2007; Lim *et al*., 2013). LPS treatment of mice provokes proinflammatory cytokine generation in the lung by an activation of the inflammatory response. In our experiment, LPS treatment by intranasal administration increased TNF-α concentration in the BALF to 3.5 ± 2.2 ng/ml, while the control group showed a level of only 0.2 ± 0.1 ng/ml (n=5). Under these conditions, PFE (100 mg/kg) significantly reduced TNF-α production (79.1% reduction) whereas the water extract showed 50.7% inhibition (Fig. 2). Dexamethasone used as a reference compound strongly inhibited TNF-α production at 30 mg/kg (88.2%). These results indicate that PFE possesses higher inhibitory activity against lung inflammation, and the active constituent(s) may be contained in this extract.

Based on the above results, isolation of the active compounds was carried out. And the standard extraction and isolation procedures successfully yielded ten compounds including phenylpropanoids (allyltetramethoxybenzene, caffeic acid, dillapiole, elemicin, myristicin, nothoapiole, rosmarinic acid methyl ester, rosmarinic acid) and monoterpenes (perilla aldehyde and perilla ketone). These compounds were evaluated for their inhibitory action on proinflammatory cytokine generation in the activated lung cells. When they were incubated in IL-1β-treated A549 lung epithelial cells, elemicin and myristicin significantly inhibited IL-6 production (Fig. 3). Rosmarinic acid showed a tendency of inhibition at 10-100 μM, although it was not statistically significant. In particular, elemicin showed the strongest inhibitory activity at 10-100 μM (42.7% inhibition at 100 μM). Dexamethasone used as a reference compound showed a potent inhibitory action as expected (86.0% inhibition at 10 μM).

When cytotoxicity was examined using an MTT assay, none of the compounds showed a meaningful cytotoxic effect on A549 cells at the tested concentrations (Fig. 4).

DISCUSSION

The present study demonstrates for the first time that the leaves of *P. frutescens* (L.) Britton var. *acuta* Kudo possess inhibitory activity against lung inflammation. And the constituents such as elemicin, myristicin and rosmarinic acid showed similar activity, suggesting their contribution to the *in vivo* activity of *P. frutescens*.

Since TNF-α is one of the most prominent inflammatory cytokines to produce an inflammatory response in the lung (Mueller *et al*., 1996), the inhibitory effect on TNF-α generation in the BALF strongly supports the scientific rationale of the clinical use of the leaves of *P. frutescens* in lung inflammatory disorders in traditional medicine.

The search for the active compounds yielded three phenylpropanoids including elemicin, myristicin and rosmarinic acid. These compounds clearly inhibited IL-6 production in lung epithelial cells. Since IL-6 is another important biomarker in inflammatory disorders including lung inflammation, it is thought that they may possess inhibitory action on lung inflammatory disorders. Although *P. frutescens* (L.) Britton var. *acuta* Kudo is known to contain various components, such as phenylpropanoids and terpenes (Fujita *et al*., 1994; Liu *et al*., 2000) and to possess various pharmacological actions including antidepressant-like effects (Nakazawa *et al*., 2003) and β-secretase inhibiting activity (Choi *et al*., 2008), the present study has clearly shown the importance of phenylpropanoids in expressing inhibitory activity against lung inflammation.

To date, some herbal extracts including the extracts of *Hedera helix* (Guo *et al*., 2006), *Echinacea purpurea* (Sharma *et al*., 2006; Agbabiaka *et al*., 2008) and *Pelargonium sidoides* (Matthys and Funk, 2008) have been used for treating lung inflammatory conditions such as bronchitis. Some other plant extracts from *Morus alba* (Lim *et al*., 2013) and *Broussonetia papyrifera* (Ko *et al*., 2011) have also been shown to

possess similar activity by our laboratory. The present study was carried out to find the active principle constituents which had the potential to be used as new and alternative medicines. And the results show that PFE and the constituents possess meaningful activity. To the best of our knowledge, this is the first report demonstrating the potential therapeutic effects of PFE and its constituents on lung inflammation.

In conclusion, all results of the present study demonstrated that the leaves of *P. frutescens* (L.) Britton var. *acuta* Kudo inhibited lung inflammation in vivo. And several phenylpropanoid derivatives such as elemicin, myristicin and rosmarinic acid were found to inhibit the production of inflammatory biomarker in lung epithelial cells (A549), and they possibly contribute to the inhibitory action of *P. frutescens* (L.) Britton var. *acuta* Kudo. Taken together, *P. frutescens* (L.) Britton var. *acuta* Kudo and the phenylpropanoid constituents have potential as new therapeutic agents for lung inflammation.

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