

# Effects of Lupenone, Lupeol, and Taraxerol Derived from *Adenophora triphylla* on the Gene Expression and Production of Airway MUC5AC Mucin

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**Background:** *Adenophora triphylla* var. *japonica* is empirically used for controlling airway inflammatory diseases in folk medicine. We evaluated the gene expression and production of mucin from airway epithelial cells in response to lupenone, lupeol and taraxerol derived from *Adenophora triphylla* var. *japonica*.

**Methods:** Confluent NCI-H292 cells were pretreated with lupenone, lupeol or taraxerol for 30 minutes and then stimulated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) for 24 hours. The MUC5AC mucin gene expression and production were measured by reverse transcription–polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Additionally, we examined whether lupenone, lupeol or taraxerol affects MUC5AC mucin production induced by epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA), the other 2 stimulators of airway mucin production.

**Results:** Lupenone, lupeol, and taraxerol inhibited the gene expression and production of MUC5AC mucin induced by TNF- $\alpha$  from NCI-H292 cells, respectively. The 3 compounds inhibited the EGF or PMA-induced production of MUC5AC mucin in NCI-H292 cells.

**Conclusion:** These results indicated that lupenone, lupeol and taraxerol derived from *Adenophora triphylla* var. *japonica* regulates the production and gene expression of mucin, by directly acting on airway epithelial cells. In addition, the results partly explain the mechanism of *Adenophora triphylla* var. *japonica* as a traditional remedy for diverse inflammatory pulmonary diseases.

**Keywords:** Mucin; Lupenone; Lupeol; Taraxerol

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## Introduction

Mucus in respiratory system plays a pivotal role in defense against invading pathogenic microorganisms, noxious chemicals and particles. The protective function of airway mucus is attributed to the viscoelasticity of mucins. However, any abnormality in the quality or quantity of mucins not only cause altered airway physiology but may also impair host defenses often leading to severe airway pathology as exemplified in chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis<sup>1</sup>. Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excessive mucin secretion (production) by various medicinal plants. We have tried to investigate the possible activities of some natural products on mucin secretion from airway epithelial cells. As a result of

our trial, we previously reported that several natural products affected mucin secretion and/or production from airway epithelial cells<sup>2-4</sup>. According to traditional oriental medicine, *Adenophora triphylla* var. *japonica* has been utilised for controlling airway inflammatory diseases<sup>5</sup>. Also, luponone, lupeol and taraxerol—its components—were reported to have diverse biological effects including antioxidative and anti-inflammatory effects<sup>6-9</sup>. However, to the best of our knowledge, there are no reports about the potential effects of luponone, lupeol, and taraxerol on the gene expression and production of mucin from airway epithelial cells. Among the twenty one or more MUC genes coding human mucins reported up to now, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium<sup>1,10</sup>. Therefore, we examined the effect of luponone, lupeol, or taraxerol on tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-induced MUC5AC mucin gene expression and production from NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of elucidating intracellular signaling pathways involved in airway mucin production and gene expression<sup>11-13</sup>.

## Materials and Methods

### 1. Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. Luponone (purity, 98.0%), lupeol (purity, 98.0%), and taraxerol (purity, 98.0%) (Figure 1) were isolated, purified and identified by analytical chemists in the Laboratory of Natural Product Science, Division of Bioscience, Dongguk University (Gyeongju, Korea). Briefly, roots of *Adenophora triphylla* var. *japonica* (cultivated in Jeongsun-gun, Gangwon-do, Korea) were provided and authenticated by Professor Je-Hyun Lee (College of Oriental Medicine, Dongguk University, Korea). Air-dried and chopped roots (300 g) were extracted with each of distilled water and 70% ethanol twice at 95°C for 3 hours. The combined extracts were filtered and concentrated under reduced pressure to give 31.1 g (10.7%) and 38.4

g (12.8%) of crude extracts, respectively. In order to isolate its active constituents the ethanol extract was suspended in distilled water, then consecutively partitioned with organic solvents to give the corresponding hexane (3.6 g), dichloromethane (0.23 g), ethylacetate (0.09 g), and *n*-butanol (1.4 g) fractions. The main hexane fraction was subjected to column chromatographic separation on a silica gel column using a stepwise elution with dichloromethane, dichloromethane-MeOH 50:1, and dichloromethane-MeOH 1:1 to yield fractions F1-F12. Fraction F3 was recrystallized with dichloromethane-EtOH (two-phase system) 1:1 to give taraxerol (10.8 mg, 0.3%). Fraction F7 was further chromatographed with dichloromethane to afford five subfractions SF1-SF5. Among the subfractions, SF5 was separated by silica gel column chromatography using dichloromethane and MeOH (10:1 to 1:1 gradient) to furnish luponone (136.8 mg, 3.8%) and lupeol (28.8 mg, 0.8%). The isolated compounds were identified by comparison of their spectral data with literature values<sup>14-16</sup>.

### 2. NCI-H292 cell culture

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100 µg/mL), and HEPES (25 mM) at 37°C in a humidified, 5% CO<sub>2</sub>/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640 with 0.2% FBS for 24 hours.

### 3. Treatment of cells with luponone, lupeol, or taraxerol

After 24 hours of serum deprivation, cells were pretreated with varying concentrations of luponone, lupeol, or taraxerol for 30 minutes and treated with TNF- $\alpha$  (0.2 nM), epidermal growth factor (EGF; 25 ng/mL), or phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) for 24 hours in serum-free RPMI 1640. Luponone, lupeol, and taraxerol were dissolved in di-

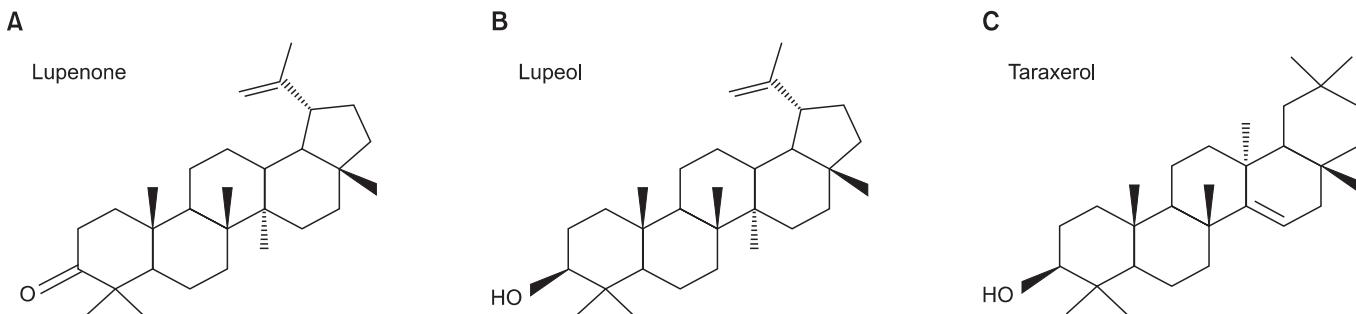


Figure 1. Chemical structure of luponone (A), lupeol (B), and taraxerol (C).

methylsulfoxide and treated in culture medium (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulfoxide did not affect mucin gene expression and production from NCI-H292 cells. After 24 hours, cells were lysed with buffer solution containing 20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and collected to measure the production of MUC5AC protein (in 24-well culture plate). The total RNA was extracted for measuring the expression of *MUC5AC* gene (in 6-well culture plate) by using reverse transcription–polymerase chain reaction (RT-PCR).

#### 4. Total RNA isolation and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (Intron Biotechnology, Inc., Seongnam, Korea) and reverse transcribed by using AccuPower RT Premix (Bioneer Co., Daejeon, Korea) according to the manufacturer's instructions. Two micrograms of total RNA was primed with 1 µg of oligo(dT) in a final volume of 50 µL (RT reaction). Two microliters of RT reaction product was polymerase chain reaction (PCR) amplified in a 25 µL by using Thermorprime Plus DNA Polymerase (ABgene, Rochester, NY, USA). Primers for *MUC5AC* were (forward) 5'-TGA TCA TCC AGC AGG GCT-3' and (reverse) 5'-CCG AGC TCA GAG GAC ATA TGG G-3'. As quantitative controls, primers for R18/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used. Primers for R18/S15 were (forward) 5'-TTC CGC AAG TTC ACC TAC C-3' and (reverse) 5'-CGG GCC GGC CAT GCT TTA CG-3'. The PCR mixture was denatured at 94°C for 2 minutes followed by 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. After PCR, 5 µL of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

#### 5. MUC5AC mucin analysis

*MUC5AC* airway mucin production was measured by enzyme-linked immunosorbent assay. Cell lysates were prepared with PBS at 1:10 dilution, and 100 µL of each sample was incubated at 42°C in a 96-well plate, until dry. Plates were washed three times with PBS and blocked with 2% bovine serum albumin (fraction V) for 1 hour at room temperature. Plates were again washed three times with PBS and then incubated with 100 µL of 45M1, a mouse monoclonal *MUC5AC* antibody (1:200, NeoMarkers, Fremont, CA, USA), which was diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 hour, the wells were washed three times with PBS, and 100 µL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3,000) was dispensed into each well.

After 1 hour, plates were washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine peroxide solution and stopped with 1 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm.

#### 6. Statistics

Means of individual group were converted to percent control and expressed as mean±SEM. The difference between groups was assessed using one-way ANOVA and Holm-Sidak test as a *post-hoc* test. p<0.05 was considered as significantly different.

### Results

#### 1. Effect of luponone, lupeol, or taraxerol on TNF-α-induced *MUC5AC* gene expression from NCI-H292 cells

As can be seen in Figure 2, *MUC5AC* gene expression induced by TNF-α from NCI-H292 cells was inhibited by pretreatment with luponone, lupeol, and taraxerol, respectively (Figure 2). Cytotoxicity was checked by lactate dehydrogenase assay and there was no remarkable cytotoxic effect of luponone, lupeol, or taraxerol, at the treatment concentrations (data not shown).

#### 2. Effect of luponone on TNF-α-induced *MUC5AC* mucin production from NCI-H292 cells

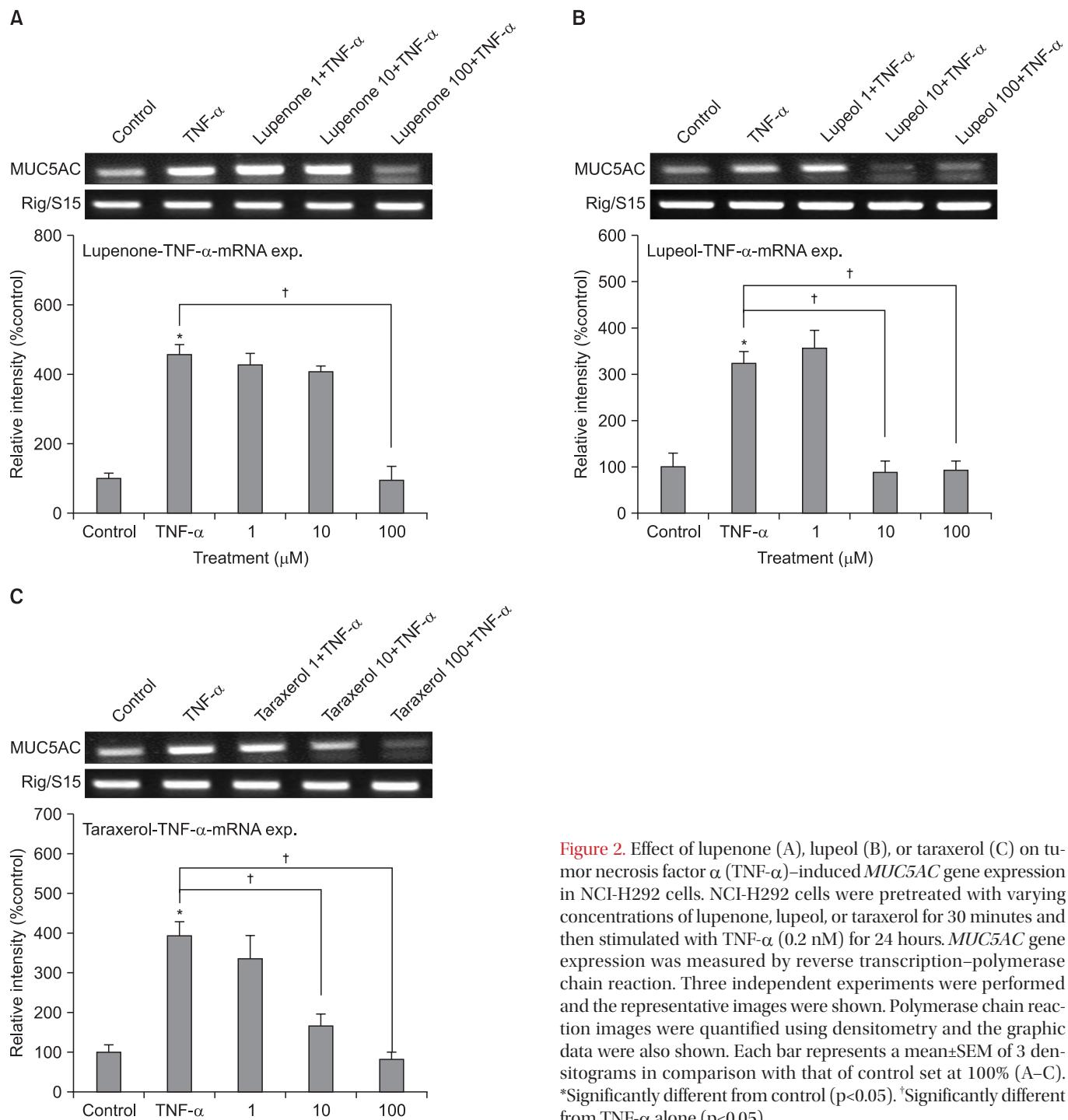
Luponone inhibited TNF-α-induced *MUC5AC* mucin production. The amounts of *MUC5AC* mucin in the cells of luponone-treated cultures were 100±6%, 543±29%, 362±14%, 324±7%, and 114±7% for control, 0.2 nM of TNF-α alone, TNF-α plus luponone 10<sup>-6</sup> M, TNF-α plus luponone 10<sup>-5</sup> M, and TNF-α plus luponone 10<sup>-4</sup> M, respectively (Figure 3A).

#### 3. Effect of lupeol on TNF-α-induced *MUC5AC* mucin production from NCI-H292 cells

Lupeol also inhibited TNF-α-induced *MUC5AC* mucin production. The amounts of *MUC5AC* mucin in the cells of lupeol-treated cultures were 100±10%, 405±13%, 337±21%, 307±24%, and 110±11% for control, 0.2 nM of TNF-α alone, TNF-α plus lupeol 10<sup>-6</sup> M, TNF-α plus lupeol 10<sup>-5</sup> M, and TNF-α plus lupeol 10<sup>-4</sup> M, respectively (Figure 3B).

#### 4. Effect of taraxerol on TNF-α-induced *MUC5AC* mucin production from NCI-H292 cells

Taraxerol also inhibited TNF-α-induced *MUC5AC* mucin production. The amounts of *MUC5AC* mucin in the cells of

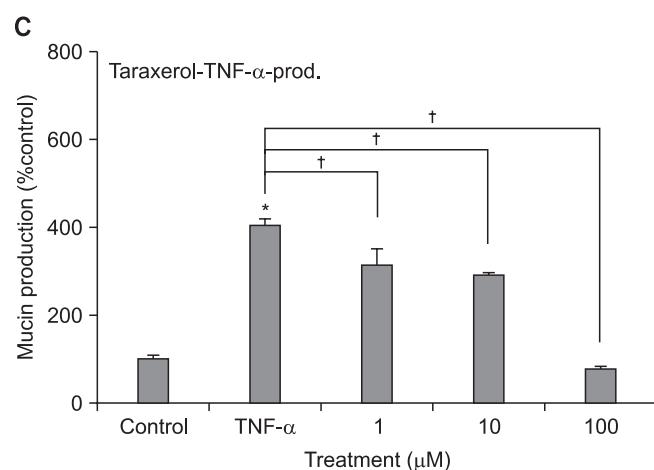
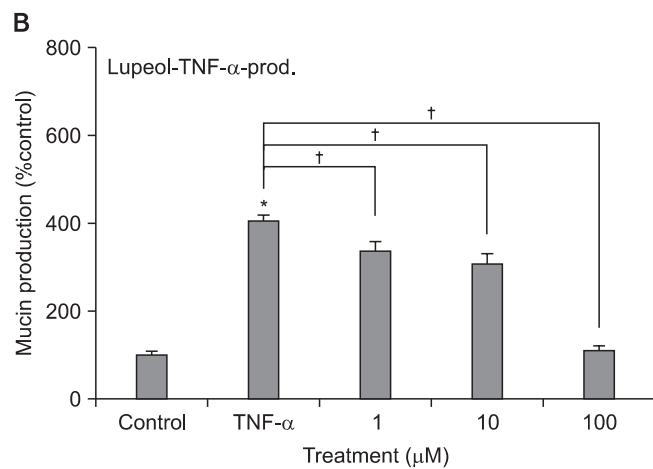
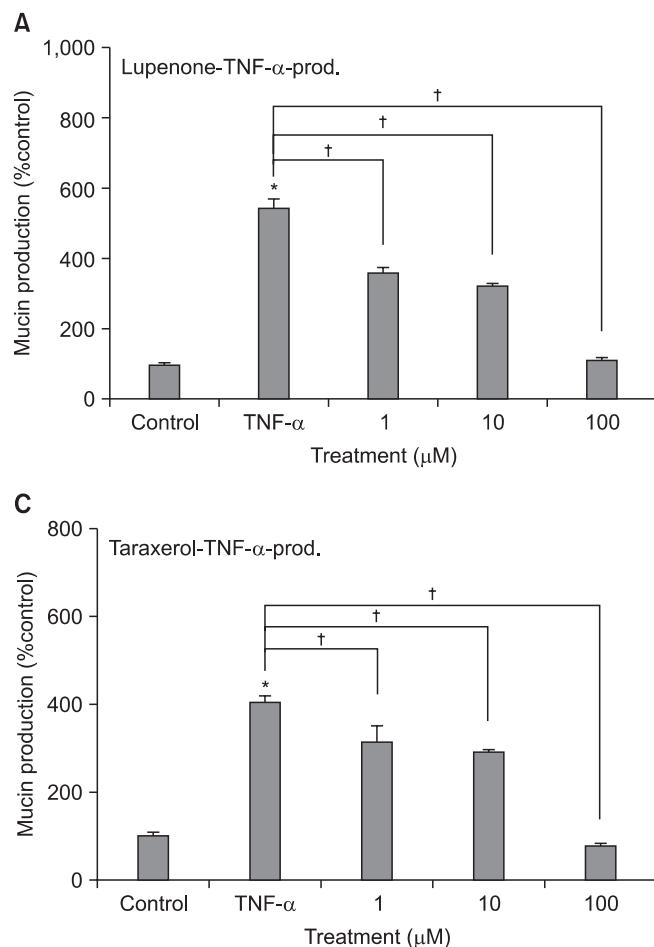


**Figure 2.** Effect of luponone (A), lupeol (B), or taraxerol (C) on tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )–induced *MUC5AC* gene expression in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of luponone, lupeol, or taraxerol for 30 minutes and then stimulated with TNF- $\alpha$  (0.2 nM) for 24 hours. *MUC5AC* gene expression was measured by reverse transcription–polymerase chain reaction. Three independent experiments were performed and the representative images were shown. Polymerase chain reaction images were quantified using densitometry and the graphic data were also shown. Each bar represents a mean $\pm$ SEM of 3 densitograms in comparison with that of control set at 100% (A–C). \*Significantly different from control ( $p<0.05$ ). †Significantly different from TNF- $\alpha$  alone ( $p<0.05$ ).

taraxerol-treated cultures were 100 $\pm$ 10%, 405 $\pm$ 13%, 315 $\pm$ 36%, 290 $\pm$ 7%, and 77 $\pm$ 7% for control, 0.2 nM of TNF- $\alpha$  alone, TNF- $\alpha$  plus taraxerol 10 $^{-6}$  M, TNF- $\alpha$  plus taraxerol 10 $^{-5}$  M, and TNF- $\alpha$  plus taraxerol 10 $^{-4}$  M, respectively (Figure 3C).

##### 5. Effect of luponone on EGF-induced MUC5AC mucin production from NCI-H292 cells

Luponone significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of luponone-treated cultures were 100 $\pm$ 13%, 330 $\pm$ 14%,



**Figure 3.** Effect of lupenone (A), lupeol (B), or taraxerol (C) on tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-induced MUC5AC mucin production in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of lupenone, lupeol or taraxerol for 30 minutes and then stimulated with TNF- $\alpha$  (0.2 nM, 10 ng/mL) for 24 hours. Cell lysates were collected for measurement of MUC5AC mucin production by enzyme-linked immunosorbent assay. Each bar represents a mean $\pm$ SEM of three culture wells in comparison with that of the control set at 100% (A–C). Three independent experiments were performed and the representative data were shown. \*Significantly different from control ( $p<0.05$ ). †Significantly different from TNF- $\alpha$  alone ( $p<0.05$ ).

232 $\pm$ 2%, 116 $\pm$ 8%, and 75 $\pm$ 4% for control, 25 ng/mL of EGF alone, EGF plus lupenone 10 $^{-6}$  M, EGF plus lupenone 10 $^{-5}$  M, and EGF plus lupenone 10 $^{-4}$  M, respectively (Figure 4A).

## 6. Effect of lupeol on EGF-induced MUC5AC mucin production from NCI-H292 cells

Lupeol significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of lupeol-treated cultures were 100 $\pm$ 31%, 369 $\pm$ 15%, 340 $\pm$ 38%, 336 $\pm$ 13%, and 79 $\pm$ 18% for control, 25 ng/mL of EGF alone, EGF plus lupeol 10 $^{-6}$  M, EGF plus lupeol 10 $^{-5}$  M, and EGF plus lupeol 10 $^{-4}$  M, respectively (Figure 4B).

## 7. Effect of taraxerol on EGF-induced MUC5AC mucin production from NCI-H292 cells

Taraxerol significantly inhibited EGF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of taraxerol-treated cultures were 100 $\pm$ 31%, 369 $\pm$ 15%, 344 $\pm$ 29%, 327 $\pm$ 36%, and 100 $\pm$ 11% for control, 25 ng/mL of EGF alone, EGF plus taraxerol 10 $^{-6}$  M, EGF plus taraxerol 10 $^{-5}$

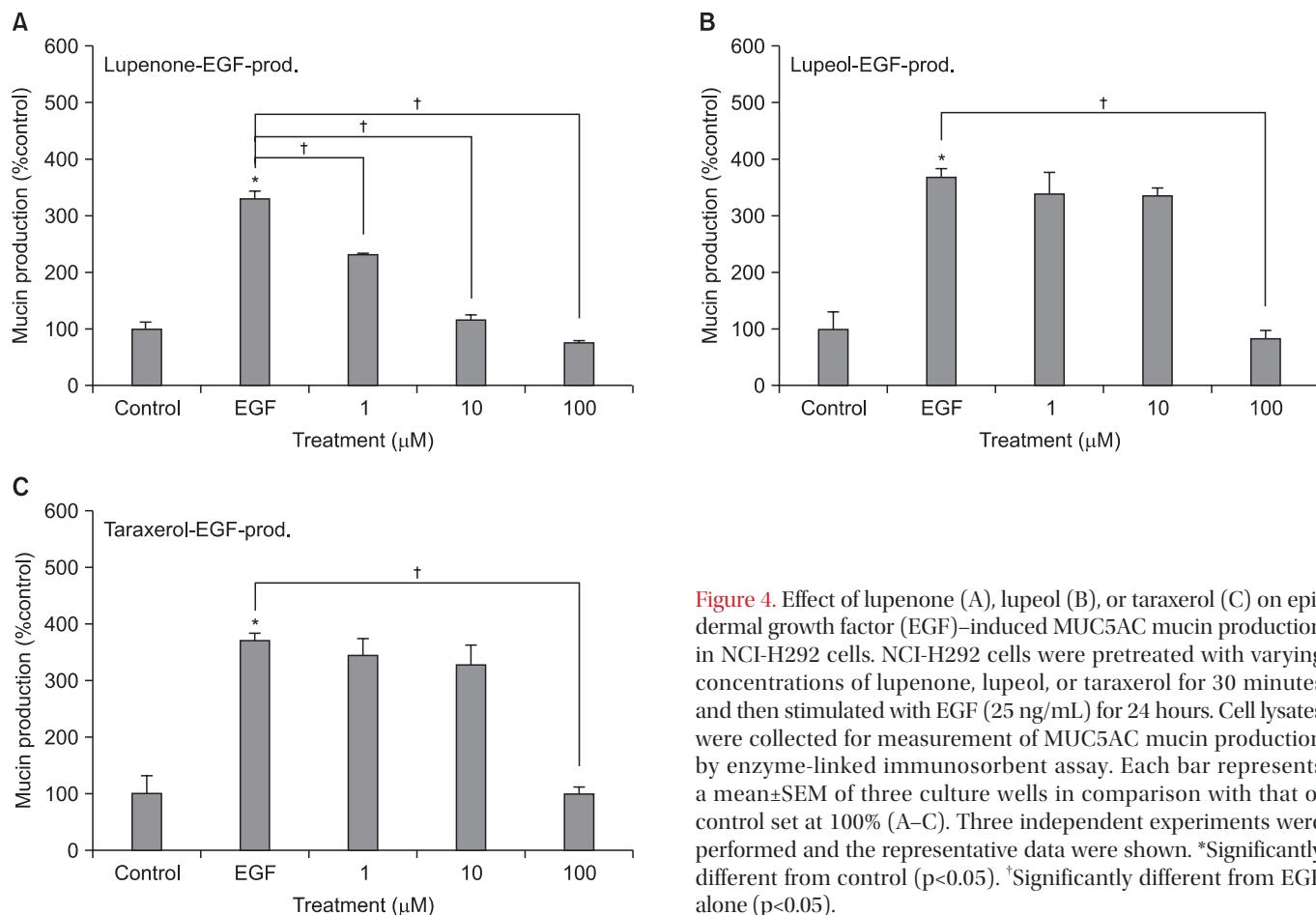
M, and EGF plus taraxerol 10 $^{-4}$  M, respectively (Figure 4C).

## 8. Effect of lupenone on PMA-induced MUC5AC mucin production from NCI-H292 cells

Lupenone significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of lupenone-treated cultures were 100 $\pm$ 14%, 247 $\pm$ 2%, 261 $\pm$ 1%, 147 $\pm$ 8%, and 55 $\pm$ 3% for control, 10 ng/mL of PMA alone, PMA plus lupenone 10 $^{-6}$  M, PMA plus lupenone 10 $^{-5}$  M, and PMA plus lupenone 10 $^{-4}$  M, respectively (Figure 5A).

## 9. Effect of lupeol on PMA-induced MUC5AC mucin production from NCI-H292 cells

Lupeol significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of lupeol-treated cultures were 100 $\pm$ 6%, 273 $\pm$ 6%, 262 $\pm$ 14%, 205 $\pm$ 7%, and 123 $\pm$ 15% for control, 10 ng/mL of PMA alone, PMA plus lupeol 10 $^{-6}$  M, PMA plus lupeol 10 $^{-5}$  M, and PMA plus lupeol 10 $^{-4}$  M, respectively (Figure 5B).



**Figure 4.** Effect of lumenone (A), lupeol (B), or taraxerol (C) on epidermal growth factor (EGF)-induced MUC5AC mucin production in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of lumenone, lupeol, or taraxerol for 30 minutes and then stimulated with EGF (25 ng/mL) for 24 hours. Cell lysates were collected for measurement of MUC5AC mucin production by enzyme-linked immunosorbent assay. Each bar represents a mean $\pm$ SEM of three culture wells in comparison with that of control set at 100% (A–C). Three independent experiments were performed and the representative data were shown. \*Significantly different from control ( $p<0.05$ ). †Significantly different from EGF alone ( $p<0.05$ ).

### 10. Effect of taraxerol on PMA-induced MUC5AC mucin production from NCI-H292 cells

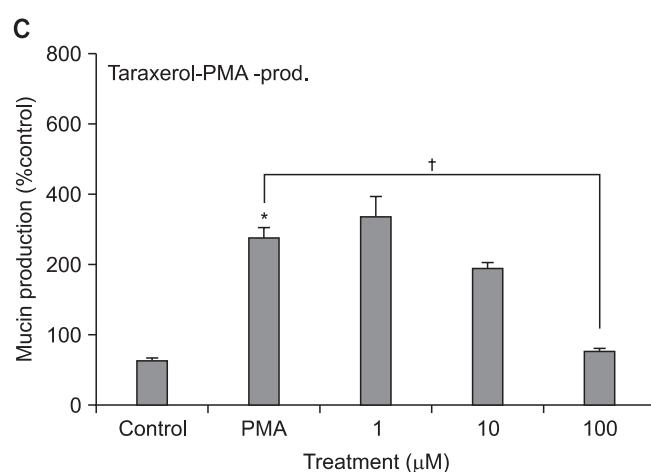
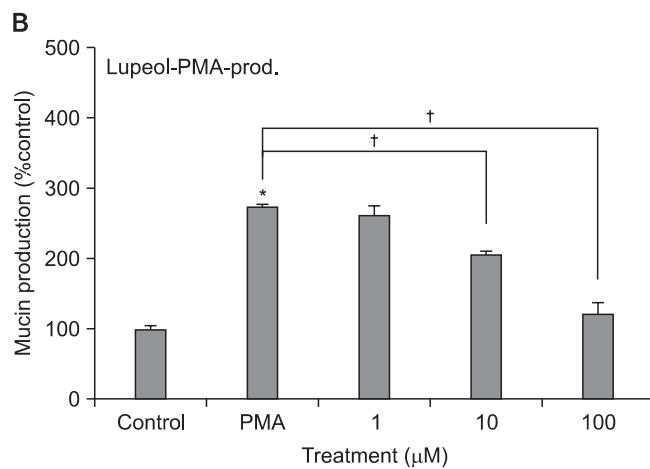
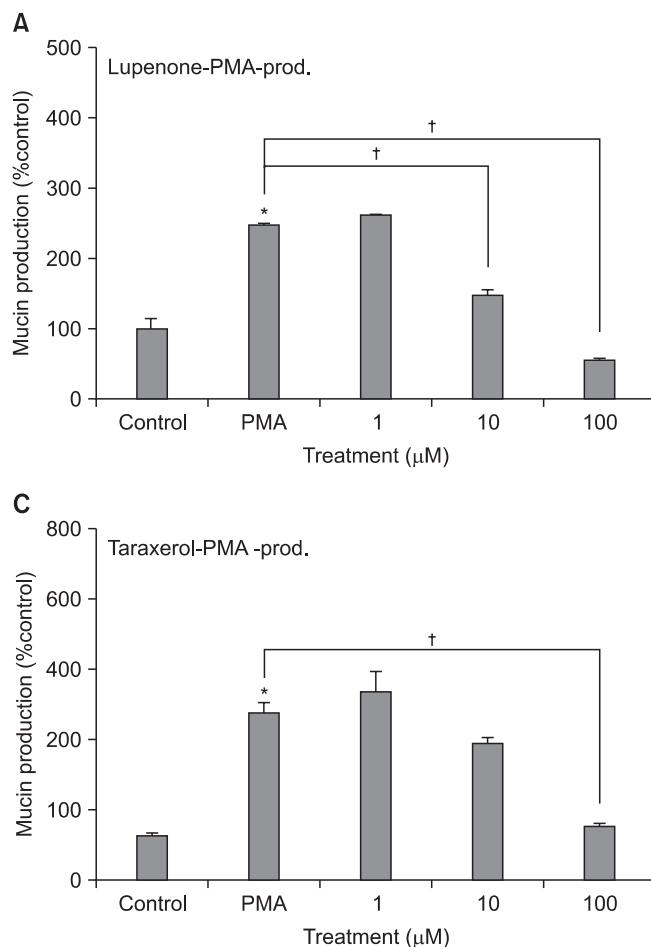
Taraxerol significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of taraxerol-treated cultures were  $100\pm 5\%$ ,  $378\pm 24\%$ ,  $426\pm 48\%$ ,  $308\pm 14\%$ , and  $121\pm 7\%$  for control, 10 ng/mL of PMA alone, PMA plus taraxerol  $10^{-6}$  M, PMA plus taraxerol  $10^{-5}$  M, and PMA plus taraxerol  $10^{-4}$  M, respectively (Figure 5C).

## Discussion

TNF- $\alpha$  has been reported to stimulate the secretion and gene expression of airway mucin<sup>13,17,18</sup>. TNF- $\alpha$  converting enzyme provoked MUC5AC mucin expression in cultured human airway epithelial cells<sup>13</sup> and TNF- $\alpha$ -induced MUC5AC gene expression in normal human airway epithelial cells<sup>18</sup>. TNF- $\alpha$  level in sputum was reported to be increased, with further increases during exacerbation of pulmonary diseases<sup>19,20</sup>. It also induced mucin secretion from guinea pig tracheal epithelial cells<sup>17</sup>. Based upon these reports, we investigated

whether lumenone, lupeol, or taraxerol affects TNF- $\alpha$ -induced MUC5AC mucin gene expression and production from NCI-H292 cells. As shown in results, MUC5AC mucin gene expression induced by TNF- $\alpha$  from NCI-H292 cells was inhibited by pretreatment with lumenone, lupeol, and taraxerol, respectively (Figure 2). At the same time, lumenone, lupeol or taraxerol suppressed TNF- $\alpha$ -induced production of MUC5AC mucin protein (Figure 3). These results suggest that lumenone, lupeol, or taraxerol can regulate the gene expression and production of MUC5AC mucin induced by TNF- $\alpha$ , by directly acting on airway epithelial cells.

Next, we tried to investigate whether lumenone, lupeol, or taraxerol affects MUC5AC production induced by EGF or PMA, the other well-known stimulator of mucin production from airway epithelial cells. EGF has been reported to regulate MUC5AC gene expression in the lung. MUC5AC mRNA expression was reported to increase after ligand binding to the EGF receptor and activation of the mitogen-activated protein kinase (MAPK) cascade<sup>12,19</sup>. On the other hand, PMA was reported to stimulate the endogenous activator of protein kinase C (PKC), diacylglycerol<sup>21</sup> and to be an inflammatory stimulant that can control a gene transcription<sup>22</sup>, cell growth and differ-



entiation<sup>23</sup>. PMA also can induce *MUC5AC* gene expression in NCI-H292 cells<sup>24</sup>. PMA activates a type of PKC isoforms. This activates matrix metalloproteinases, which cleave pro-EGF receptor ligands from the cell surface to become mature EGF receptor ligands. These ligands bind to the EGF receptor, provoking the phosphorylation of its intracellular tyrosine kinase. This leads to activation of MEK leading to ERK activation. Following is the activation of the transcription factor, Sp1, and binding of the factor to specific sites with the *MUC5AC* gene promoter. Finally, the promoter is activated and produced the gene transcription and translation to *MUC5AC* mucin protein<sup>22</sup>. As shown in results, EGF-induced production of *MUC5AC* mucin protein was suppressed by pretreatment of lumenone, lupeol, and taraxerol, respectively (Figure 4). Also, PMA-induced production of *MUC5AC* mucin protein was inhibited by pretreatment of lumenone, lupeol, and taraxerol, respectively (Figure 5).

The underlying mechanisms of action of lumenone, lupeol and taraxerol on *MUC5AC* mucin gene expression and production are not clear at present, although we are investigating whether lumenone, lupeol and taraxerol act as potential regulators of nuclear factor kB signaling pathway and/or the

**Figure 5.** Effect of lumenone (A), lupeol (B), or taraxerol (C) on phorbol 12-myristate 13-acetate (PMA)-induced *MUC5AC* mucin production in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of lumenone, lupeol, or taraxerol for 30 minutes and then stimulated with PMA (10 ng/mL) for 24 hours. Cell lysates were collected for measurement of *MUC5AC* mucin production by enzyme-linked immunosorbent assay. Each bar represents a mean $\pm$ SEM of three culture wells in comparison with that of control set at 100% (A-C). Three independent experiments were performed and the representative data were shown. \*Significantly different from control ( $p<0.05$ ). †Significantly different from PMA alone ( $p<0.05$ ).

MAPK cascade after ligand binding to the TNF or EGF receptor, in mucin-producing NCI-H292 cells.

Taken together, the inhibitory action of lumenone, lupeol or taraxerol on airway mucin production and gene expression might explain, at least in part, the traditional use of *Adenophora triphylla* var. *japonica* as an anti-inflammatory mucoregulator for pulmonary inflammatory diseases, in folk medicine. We suggest it is valuable to find the natural products that have specific inhibitory effects on mucin production and gene expression—in view of both basic and clinical sciences—and the result from this study suggests a possibility of using lumenone, lupeol or taraxerol as a new efficacious mucoregulator for pulmonary diseases, although further studies are essentially required.

## Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

## Acknowledgements

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