



## Effects of Nodakenin, Columbianadin, and Umbelliferone Isolated from the Roots of *Angelica decursiva* on the Gene Expression and Production of MUC5AC Mucin from Human Airway Epithelial NCI-H292 Cells

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**Abstract** – *Angelica decursiva* has been utilised as remedy for controlling the airway inflammatory diseases in folk medicine. We investigated whether nodakenin, columbianadin, and umbelliferone isolated from the roots of *Angelica decursiva* inhibit the gene expression and production of MUC5AC mucin from human airway epithelial cells. Confluent NCI-H292 cells were pretreated with nodakenin, columbianadin or umbelliferone for 30 min and then stimulated with epidermal growth factor (EGF), phorbol 12-myristate 13-acetate (PMA) or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) for 24 h. The MUC5AC mucin gene expression was measured by reverse transcription - polymerase chain reaction (RT-PCR). Production of MUC5AC mucin protein was measured by enzyme-linked immunosorbent assay (ELISA). The results were as follows: (1) Nodakenin did not affect the expression of MUC5AC mucin gene induced by EGF, PMA or TNF- $\alpha$ . Columbianadin inhibited the expression of MUC5AC mucin gene induced by EGF or PMA. However, umbelliferone inhibited the expression of MUC5AC mucin gene induced by EGF, PMA or TNF- $\alpha$ ; (2) Nodakenin also did not affect the production of MUC5AC mucin protein induced by EGF, PMA or TNF- $\alpha$ . Columbianadin inhibited the production of MUC5AC mucin protein induced by PMA. However, umbelliferone inhibited the production of MUC5AC mucin protein induced by EGF, PMA or TNF- $\alpha$ . These results suggest that, among the three compounds investigated, umbelliferone only inhibits the gene expression and production of MUC5AC mucin stimulated by various inducers, by directly acting on airway epithelial cells, and the results might explain the traditional use of *Angelica decursiva* as remedy for diverse inflammatory pulmonary diseases.

**Keywords** – Mucin, Nodakenin, Columbianadin, Umbelliferone

### Introduction

Mucus in the airway is very important for defensive action against pathogenic microorganisms, invading particles and noxious chemicals. The protective function of airway mucus is due to the physicochemical properties e.g. viscoelasticity of mucins.<sup>1</sup> Mucins are multimillion-dalton glycoproteins present in respiratory mucus that are produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, any abnormality (increase or decrease) 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 asthma, chronic

bronchitis, cystic fibrosis, 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 investigated the activities of some natural products derived from several medicinal plants on mucin production and/or secretion from airway epithelial cells.<sup>2-6</sup> According to traditional oriental medicine, the roots of *Angelica decursiva* has been utilised as remedies for controlling the airway inflammatory diseases.<sup>7</sup> Also, nodakenin, columbianadin, and umbelliferone, the compounds isolated and purified from the roots of *Angelica decursiva*, were reported to have diverse biological effects including antioxidant and anti-inflammatory effects.<sup>8-11</sup>

However, to the best of our knowledge, there is no report about the potential effects of nodakenin, columbianadin, and umbelliferone, on the gene expression and production of mucin from airway epithelial cells. Among the twenty one or more MUC genes coding human

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mucins reported up to now, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium.<sup>1,12</sup>

Therefore, we examined the effects of nodakenin, columbianadin, and umbelliferone on EGF-, PMA- or TNF- $\alpha$  induced MUC5AC mucin gene expression and production in NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of elucidating mechanisms involved in airway mucin production and gene expression.<sup>13-15</sup>

## Experimental

**General experimental procedures** – All the chemicals and reagents used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise specified.

**Preparation of nodakenin, columbianadin, and umbelliferone** – The root of *A. decursiva* was authenticated by Prof. Dr. Je-Hyun Lee in Department of Herbology, College of Oriental Medicine, Dongguk University (Kyoung-ju, Korea), after purchase from a local retailer of medicinal herbs. The voucher specimen (No. 20120220) was deposited in the laboratory of Professor Jae Sue Choi, Department of Food Science and Nutrition, Pukyong National University, Busan, Korea. For an isolation of active constituents, the powder of roots of *A. decursiva* (2.7 kg) was refluxed with methanol (MeOH) for 3 h ( $3 \times 10$  L). The total filtrate was then concentrated to dryness *in vacuo* at 40 °C in order to render the MeOH extract (864.32 g). This extract was suspended in distilled water and then successively partitioned with dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield CH<sub>2</sub>Cl<sub>2</sub> (212.47 g), EtOAc (19.06 g), and *n*-BuOH (151.07 g) fractions, respectively, as well as water residue (434.32 g). The part (80.0 g) of CH<sub>2</sub>Cl<sub>2</sub> fraction was first chromatographed over a Si gel column (8 × 80 cm) using a mixed solvent of *n*-hexane and EtOAc (*n*-hexane : EtOAc = 20 : 1 → 1 : 1, gradient) to afford 10 subfractions (F01 ~ F10). Compounds columbianadin (10.5 mg) and umbelliferone (68 mg) were separately purified from F03 (4.8 g), with a solvent mixture of *n*-hexane and EtOAc (*n*-hexane : EtOAc = 6 : 1). Compound nodakenin (180 mg) was purified from F08 (8.2 g) (Fig. 1). The isolated compounds were identified and characterized by different spectroscopic methods, including <sup>1</sup>H- and <sup>13</sup>C-NMR as well as by comparisons with published spectral data and TLC analyses.

**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, U.S.A.) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100  $\mu$ g/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% fetal bovine serum for 24 h.

**Treatment of cells with nodakenin, columbianadin, and umbelliferone** – After 24 h of serum deprivation, cells were pretreated with varying concentrations of columbianadin, umbelliferone or nodakenin for 30 min and then treated with EGF (25 ng/mL), PMA (10 ng/mL) or TNF- $\alpha$  (0.2 nM) for 24 h in serum-free RPMI 1640. Nodakenin, columbianadin, and umbelliferone were dissolved in dimethylsulfoxide 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 in NCI-H292 cells. After 24 h, 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, IN, U.S.A.) 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 RT-PCR.

**MUC5AC mucin analysis** – MUC5AC airway mucin production was measured using ELISA. Cell lysates were prepared with PBS at 1:10 dilution, and 100  $\mu$ L 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% BSA (fraction V) for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100  $\mu$ L of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, U.S.A.), which was diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 h, the wells were washed three times with PBS, and 100  $\mu$ L of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3,000) was dispensed into each well. After 1 h, plates were washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm.

**Total RNA isolation and RT-PCR** – Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc. Kyung-gi-do, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER

Corporation, Daejeon, Korea) according to the manufacturer's instructions. 2  $\mu$ g of total RNA was primed with 1  $\mu$ g of oligo (dT) in a final volume of 50  $\mu$ L (RT reaction). 2  $\mu$ L of RT reaction product was PCR amplified in a 25  $\mu$ L by using Thermoprime Plus DNA Polymerase (ABgene, Rochester, NY, U.S.A.). 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 a quantitative control, primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used. Primers for Rig/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 min followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s. After PCR, 5  $\mu$ L of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

**Statistics** – Means of individual group were converted to percent control and expressed as mean  $\pm$  S.E.M. 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 and Discussion

Of the twenty one or more mucin genes which are identified until now, MUC5AC has been known as a major type of airway gel-forming mucin because it is highly expressed in the goblet cells<sup>1,12,16</sup> and is regulated by proinflammatory cytokines.<sup>17</sup>

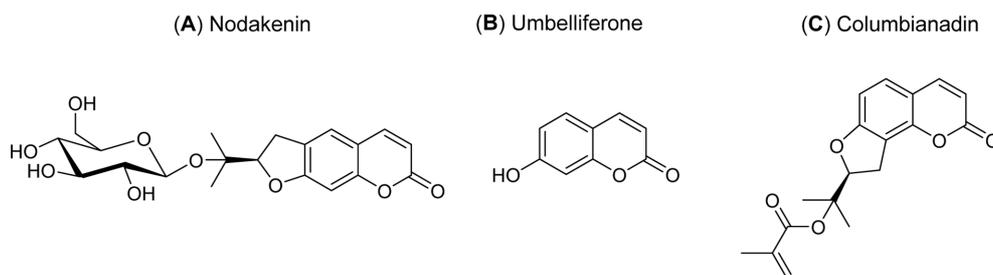
EGF was reported to regulate MUC5AC gene expression in the lung. MUC5AC mRNA expression was increased after binding to the EGF receptor and activation of the MAPK (mitogen-activated protein kinase) cascade.<sup>14,17</sup>

PMA stimulates the protein kinase C (PKC), diacylglycerol (DAG)<sup>18</sup> and is an inflammatory stimulant that can control a gene transcription<sup>19</sup> and cell growth and differentiation.<sup>20</sup> PMA also induces MUC5AC gene expression in

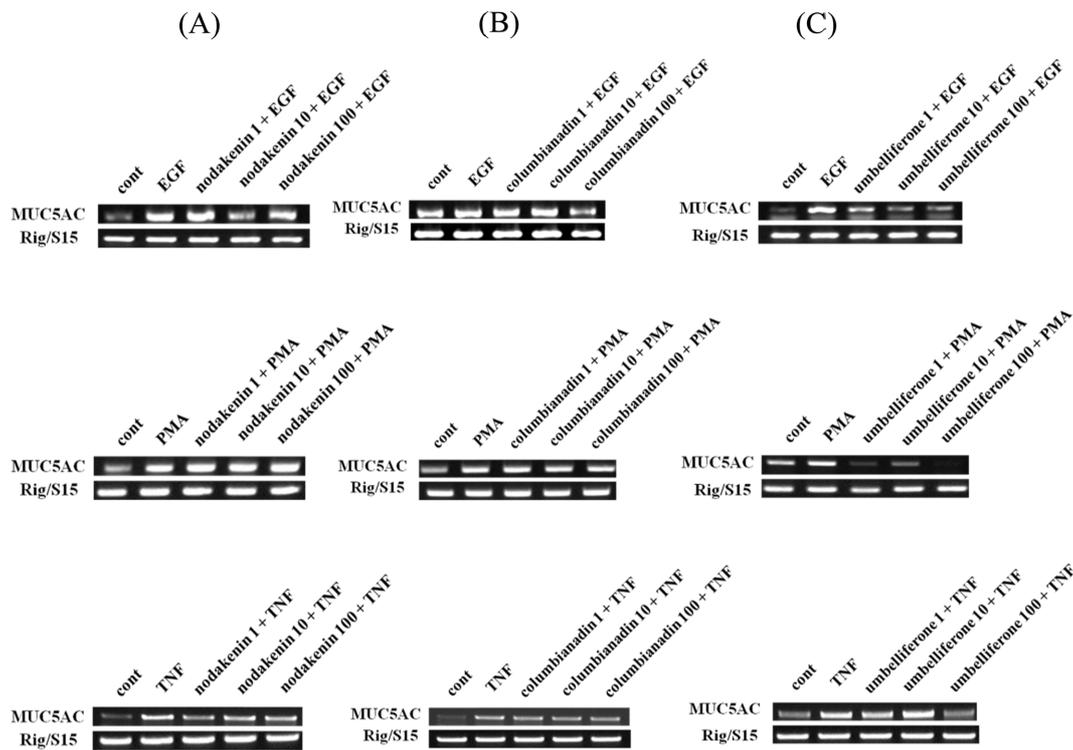
NCI-H292 cells.<sup>21</sup> PMA activates a type of PKC isoforms. This activates matrix metalloproteinases (MMPs), which cleave pro-EGFR ligands from the cell surface to become mature EGFR ligands.<sup>19</sup> These ligands bind to the EGF receptor, provoking the phosphorylation of its intracellular tyrosine kinase. This leads to activation of MEK leading to ERK activation.<sup>19,20</sup> 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>19</sup>

On the other hand, TNF- $\alpha$  is a well-known inducer for secretion and gene expression of airway mucin.<sup>15,16,22</sup> TNF- $\alpha$  level in sputum was reported to be increased, with further increases during exacerbation of diseases.<sup>14,23</sup> TNF- $\alpha$  converting enzyme (TACE) mediated MUC5AC mucin expression in cultured human airway epithelial cells<sup>15</sup> and TNF- $\alpha$  induced MUC5AC gene expression in normal human airway epithelial cells.<sup>16</sup>

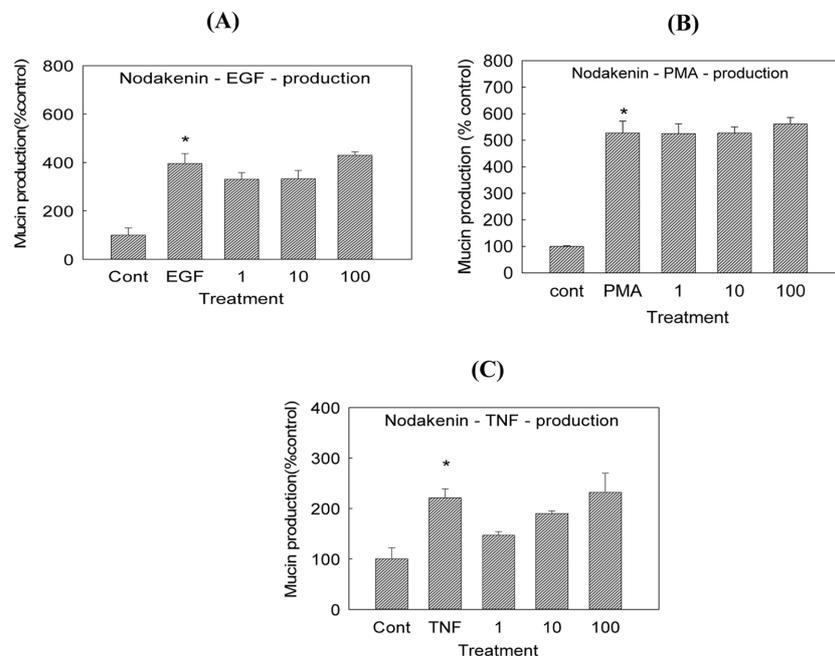
Based on the above reports, we examined whether nodakenin, columbianadin, and umbelliferone isolated from the roots of *Angelica decursiva* suppress the gene expression and production of airway MUC5AC mucin induced by EGF, PMA or TNF- $\alpha$ . As can be seen in Fig. 2, MUC5AC gene expression induced by EGF-, PMA- or TNF- $\alpha$  in NCI-H292 cells was inhibited by pretreatment with umbelliferone (Fig. 2C). However, nodakenin did not affect MUC5AC gene expression induced by EGF-, PMA- or TNF- $\alpha$  and columbianadin inhibited the expression of MUC5AC mucin gene induced by EGF or PMA (Fig. 2A, B). Cytotoxicity was checked by lactate dehydrogenase (LDH) assay and there was no significant cytotoxic effect of nodakenin, columbianadin or umbelliferone, at 1, 10, and 100  $\mu$ M (data were not shown). Also, nodakenin did not affect the production of MUC5AC mucin protein induced by EGF, PMA or TNF- $\alpha$ . Each datum represents a mean  $\pm$  S.E.M. of 3 culture wells in comparison with that of control set at 100%. The amounts of mucin in the nodakenin-treated NCI-H292 cells were 100  $\pm$  30%, 396  $\pm$  41%, 331  $\pm$  28%, 333  $\pm$  34% and 430  $\pm$



**Fig. 1.** Chemical structures of nodakenin, columbianadin, and umbelliferone.



**Fig. 2.** Effect of nodakenin, columbianadin or umbelliferone on EGF-, PMA- or TNF- $\alpha$ -induced MUC5AC gene expression in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations (1, 10, and 100  $\mu$ M) of nodakenin, columbianadin or umbelliferone for 30 min and then stimulated with EGF (25 ng/mL), PMA (10 ng/mL) or TNF- $\alpha$  (0.2 nM) for 24 h. MUC5AC gene expression was measured by RT-PCR. Three independent experiments were performed and the representative data were shown (cont: control, concentration unit is  $\mu$ M).

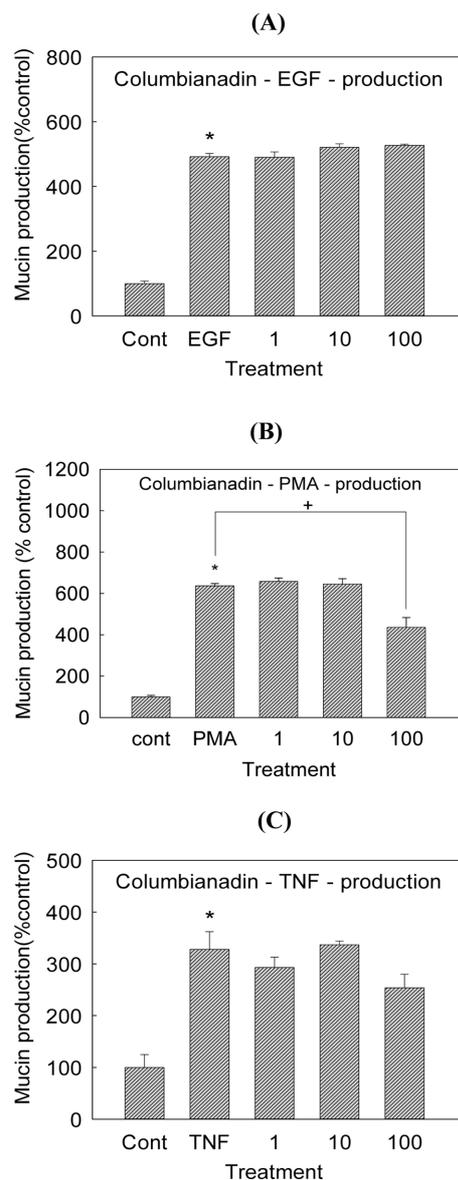


**Fig. 3.** Effect of nodakenin on EGF-, PMA- or TNF- $\alpha$ -induced MUC5AC mucin production in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations (1, 10, and 100  $\mu$ M) of nodakenin for 30 min and then stimulated with EGF (25 ng/mL), PMA (10 ng/mL) or TNF- $\alpha$  (0.2 nM) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean  $\pm$  S.E.M. of 3 culture wells in comparison with that of control set at 100%. Three independent experiments were performed and the representative data were shown \* significantly different from control ( $p < 0.05$ ) (cont: control, concentration unit is  $\mu$ M).

15% for control, 25 ng/mL of EGF alone, EGF plus nodakenin  $10^{-6}$  M, EGF plus nodakenin  $10^{-5}$  M and EGF plus nodakenin  $10^{-4}$  M, respectively (Fig. 3A). The amounts of mucin in the nodakenin-treated NCI-H292 cells were  $100 \pm 2\%$ ,  $527 \pm 45\%$ ,  $525 \pm 37\%$ ,  $527 \pm 23\%$  and  $561 \pm 25\%$  for control, 10 ng/mL of PMA alone, PMA plus nodakenin  $10^{-6}$  M, PMA plus nodakenin  $10^{-5}$  M and PMA plus nodakenin  $10^{-4}$  M, respectively (Fig. 3B). The amounts of mucin in the nodakenin-treated NCI-H292 cells were  $100 \pm 22\%$ ,  $221 \pm 18\%$ ,  $147 \pm 7\%$ ,  $190 \pm 5\%$  and  $232 \pm 38\%$  for control, 0.2 nM of TNF- $\alpha$  alone, TNF- $\alpha$  plus nodakenin  $10^{-6}$  M, TNF- $\alpha$  plus nodakenin  $10^{-5}$  M and TNF- $\alpha$  plus nodakenin  $10^{-4}$  M, respectively (Fig. 3C).

Columbianadin inhibited PMA-induced MUC5AC production from NCI-H292 cells, at the highest concentration, 100  $\mu$ M. The amounts of mucin in the columbianadin-treated NCI-H292 cells were  $100 \pm 7\%$ ,  $637 \pm 11\%$ ,  $658 \pm 17\%$ ,  $645 \pm 26\%$  and  $436 \pm 47\%$  for control, 10 ng/mL of PMA alone, PMA plus columbianadin  $10^{-6}$  M, PMA plus columbianadin  $10^{-5}$  M and PMA plus columbianadin  $10^{-4}$  M, respectively (Fig. 4B). Columbianadin did not affect EGF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the columbianadin-treated NCI-H292 cells were  $100 \pm 8\%$ ,  $492 \pm 10\%$ ,  $490 \pm 17\%$ ,  $521 \pm 11\%$  and  $527 \pm 4\%$  for control, 25 ng/mL of EGF alone, EGF plus columbianadin  $10^{-6}$  M, EGF plus columbianadin  $10^{-5}$  M and EGF plus columbianadin  $10^{-4}$  M, respectively (Fig. 4A). Columbianadin did not affect TNF-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the columbianadin-treated NCI-H292 cells were  $100 \pm 25\%$ ,  $328 \pm 34\%$ ,  $293 \pm 20\%$ ,  $337 \pm 7\%$  and  $254 \pm 26\%$  for control, 0.2nM of TNF- $\alpha$  alone, TNF- $\alpha$  plus columbianadin  $10^{-6}$  M, TNF- $\alpha$  plus columbianadin  $10^{-5}$  M and TNF- $\alpha$  plus columbianadin  $10^{-4}$  M, respectively (Fig. 4C).

Umbelliferone inhibited the production of MUC5AC mucin protein induced by EGF, PMA or TNF- $\alpha$ . The amounts of mucin in the umbelliferone-treated NCI-H292 cells were  $100 \pm 8\%$ ,  $492 \pm 10\%$ ,  $445 \pm 37\%$ ,  $310 \pm 19\%$  and  $265 \pm 7\%$  for control, 25 ng/mL of EGF alone, EGF plus umbelliferone  $10^{-6}$  M, EGF plus umbelliferone  $10^{-5}$  M and EGF plus umbelliferone  $10^{-4}$  M, respectively (Fig. 5A). The amounts of mucin in the umbelliferone-treated NCI-H292 cells were  $100 \pm 9\%$ ,  $722 \pm 45\%$ ,  $542 \pm 18\%$ ,  $510 \pm 39\%$  and  $234 \pm 17\%$  for control, 10 ng/mL of PMA alone, PMA plus umbelliferone  $10^{-6}$  M, PMA plus umbelliferone  $10^{-5}$  M and PMA plus umbelliferone  $10^{-4}$  M, respectively (Fig. 5B). Umbelliferone inhibited TNF-induced MUC5AC production from NCI-H292 cells, at the highest concentration, 100  $\mu$ M. The amounts of mucin



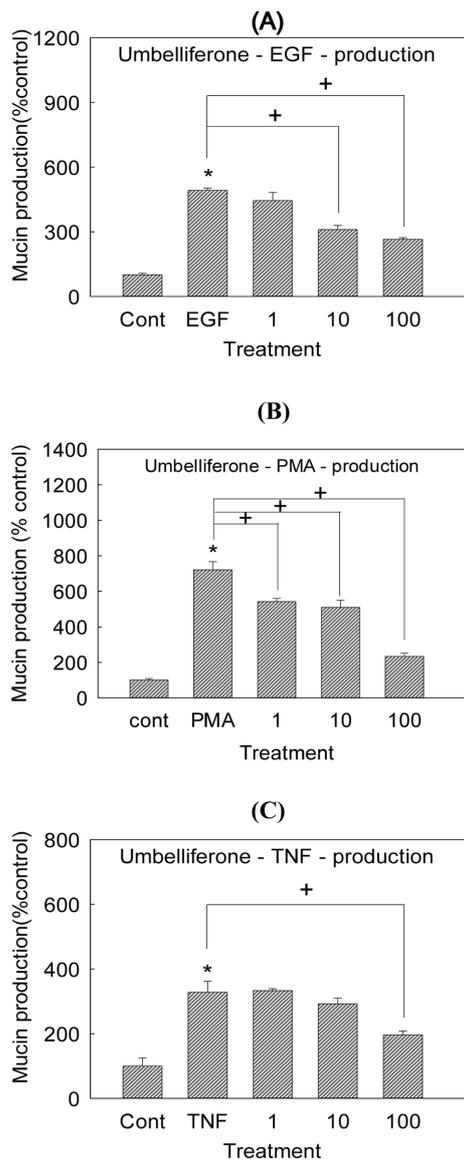
**Fig. 4.** Effect of columbianadin on EGF-, PMA- or TNF- $\alpha$ -induced MUC5AC mucin production in NCI-H292 cells.

NCI-H292 cells were pretreated with varying concentrations (1, 10, and 100  $\mu$ M) of columbianadin for 30 min and then stimulated with EGF (25 ng/mL), PMA (10 ng/mL) or TNF- $\alpha$  (0.2 nM) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean  $\pm$  S.E.M. of 3 culture wells in comparison with that of control set at 100%. Three independent experiments were performed and the representative data were shown.

\* significantly different from control ( $p < 0.05$ ).

+ significantly different from EGF, PMA or TNF- $\alpha$  alone ( $p < 0.05$ ) (cont: control, concentration unit is  $\mu$ M).

in the umbelliferone-treated NCI-H292 cells were  $100 \pm 25\%$ ,  $328 \pm 34\%$ ,  $333 \pm 6\%$ ,  $292 \pm 18\%$  and  $196 \pm 12\%$  for control, 0.2 nM of TNF- $\alpha$  alone, TNF- $\alpha$  plus umbelliferone  $10^{-6}$  M, TNF- $\alpha$  plus umbelliferone  $10^{-5}$  M and



**Fig. 5.** Effect of umbelliferone on EGF-, PMA- or TNF- $\alpha$ -induced MUC5AC mucin production in NCI-H292 cells.

NCI-H292 cells were pretreated with varying concentrations (1, 10, and 100  $\mu$ M) of umbelliferone for 30 min and then stimulated with EGF (25 ng/mL), PMA (10 ng/mL) or TNF- $\alpha$  (0.2 nM) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean  $\pm$  S.E.M. of 3 culture wells in comparison with that of control set at 100%. Three independent experiments were performed and the representative data were shown.

\* significantly different from control ( $p < 0.05$ ).

+ significantly different from EGF, PMA or TNF- $\alpha$  alone ( $p < 0.05$ ) (cont: control, concentration unit is  $\mu$ M).

TNF- $\alpha$  plus umbelliferone 10<sup>-4</sup> M, respectively (Fig. 5C).

These results suggest that umbelliferone only inhibits the gene expression and production of MUC5AC mucin stimulated by the three inducers, EGF, PMA, and TNF- $\alpha$ , by directly acting on airway epithelial cells. This means

that umbelliferone can inhibit MUC5AC mucin gene expression and production provoked by *both* growth factor signaling (EGF and PMA) *and* pro-inflammatory signaling (TNF- $\alpha$ ), whereas columbianadin can inhibit MUC5AC mucin gene expression and production provoked by *only* growth factor signaling (EGF and PMA).

Taken together, findings in this study might explain the traditional use of the roots of *Angelica decursiva* as a folk remedy for treating several pulmonary inflammatory diseases that are accompanied by hypersecretion of sticky mucus. The underlying mechanism of action of umbelliferone on MUC5AC gene expression and production is not clear at present, although we are investigating whether umbelliferone acts as potential regulator of the MAPK (mitogen-activated protein kinase) cascade after binding to the EGF receptor and/or potential regulator of NF- $\kappa$ B signaling pathway, in mucin-producing NCI-H292 cells.

We suggest it is valuable to find the natural products that have specific regulatory 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 developing umbelliferone as a new efficacious mucoregulator for pulmonary diseases, although further studies are essentially required.

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