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Effects of the root of *Platycodon grandiflorum* on airway mucin hypersecretion in vivo and platycodin D_3 and deapi-platycodin on production and secretion of airway mucin *in vitro*

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ABSTRACT

We investigated whether aqueous extract of the root of *Platycodon grandiflorum* A. de Candolle (APG), platycodinD_3 and deapi-platycodin significantly affect the production and secretion of airway mucin using *in vivo* and *in vitro* experimental models. Effect of APG was checked on hypersecretion of pulmonary mucin in sulfur dioxide-induced bronchitis in rats. Confluent NCI-H292 cells were pretreated with platycodinD_3 or deapi-platycodin for 30 min and then stimulated with PMA (phorbol 12-myristate 13-acetate) for 24 h. The MUC5AC mucin production and secretion were measured by ELISA. The results were as follows: (1) APG stimulated the secretion of airway mucin in sulfur dioxide-induced bronchitis rat model; (2) platycodinD_3 and deapi-platycodin inhibited the production of MUC5AC mucin induced by PMA from NCI-H292 cells, respectively; (3) however, platycodinD_3 and deapi-platycodin did not inhibit but stimulated the secretion of MUC5AC mucin induced by PMA from NCI-H292 cells, respectively. This result suggests that aqueous extract of *P. grandiflorum* A. de Candolle and the two natural products derived from it, platycodinD_3 and deapi-platycodin, can regulate the production and secretion of airway mucin and, at least in part, explains the traditional use of aqueous extract of *P. grandiflorum* A. de Candolle as expectorants in diverse inflammatory pulmonary diseases.

Introduction

Mucus in the pulmonary system is very important in defensive action against various particles, noxious chemicals and invading pathogenic microbes. This defensive action of pulmonary mucus is attributed to the physicochemical property of mucins, i.e. viscoelasticity. Mucins are high molecular weight glycoproteins present in the airway mucus and produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, hypersecretion of airway mucus is one of the major symptoms associated with severe pulmonary diseases including chronic bronchitis, cystic fibrosis, bronchiectasis and asthma (Lee et al., 2002; Heo et al., 2007, 2009; Voynow and Rubin, 2009; Kim et al., 2012). According to traditional oriental medicine, the root of *Platycodon grandiflorum* A. de Candolle has been used for controlling pulmonary inflammatory diseases (Jang, 2003). Platycodin D, a compound derived from the root of *P. grandiflorum* A. de Candolle, was reported to have diverse biological effects including anticancer effect (Kim et al., 2001, 2008; Shin et al., 2002; Ahn et al., 2006; Chung et al., 2008; Yu and Kim, 2010). PlatycodinD_3 was reported to modulate nitric oxide production and TNF-α secretion from RAW 264.7 cells (Wang et al., 2004). Deapi-platycodin showed antiproliferative effect on cancer cells (Choi et al., 2010). However, to the best of our knowledge, there is no report about the potential effect of aqueous extract of *P. grandiflorum* A. de Candolle and, platycodinD_3 and deapi-platycodin, the two natural products derived from *P. grandiflorum* A. de Candolle, on production and secretion of airway mucin. Therefore, in this study, we checked whether aqueous extract of *P. grandiflorum* A. de Candolle, platycodinD_3 and deapi-platycodin significantly affect the production and secretion of airway mucin using in vivo and *in vitro* experimental models reflecting the hypersecretion and/or hyperproduction of mucus observed in inflammatory

Abbreviations: EGF, epidermal growth factor; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate.

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Peak	Rt (min.)	Compound
1	6.1	Platycoside E
2	11.2	Platycodin D_3
3	26.5	Deapiplatycodin D
4	30.5	Platycodin D
5	35.3	Polygalacin D
6	37.6	Platycodin J
7	40.1	2''-O-aectyl Platycodin D
8	42.9	Platycodin K

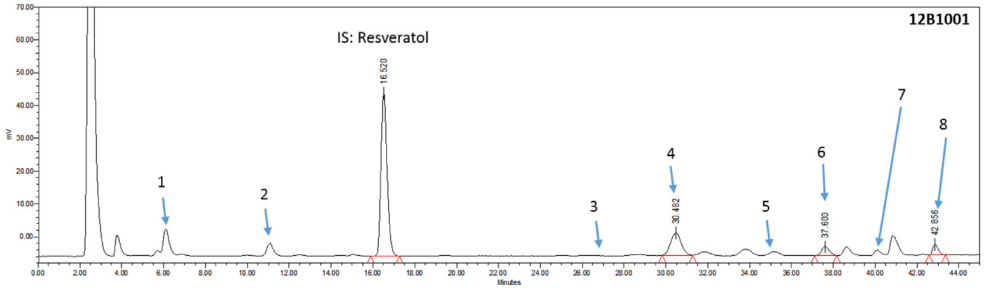

Fig. 1. HPLC-fingerprint analysis of the aqueous root extract of *Platycodon grandiflorum* A. de Candolle.

pulmonary diseases (Li et al., 1997; Takeyama et al., 1999; Shao et al., 2003).

Materials and methods

Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified. PlatycodinD_3 (purity: 98.0%), deapi-platycodin (purity: 98.0%) were isolated, purified and identified by analytical chemists in the Laboratory of Pharmacognosy, Department of Pharmacy, Seoul National University (Seoul, Korea). Briefly, small pieces of the roots of *P. grandiflorum* A. de Candolle 100 g were extracted twice with 1 L of distilled water for 3 h in water bath. Aqueous extract (41.0 g) were obtained after removing solvent by lyophilization. The HPLC-fingerprint analysis of the aqueous root extract of *P. grandiflorum* A. de Candolle was performed (Fig. 1). To isolate several compounds including platycodinD_3 and deapi-platycodin, the roots of *P. grandiflorum* A. de Candolle were extracted with 80% MeOH 3 l in ultrasonic bath and evaporated under reduced pressure. The extract was subjected to HP-20 column chromatography with water, 30%, 70% and 100% EtOH. 70% EtOH fraction was applied to RP-MPLC (20–40% MeOH) to give three fractions (Fr. 1–3). Fraction 3 was separated to 5 fractions (Fr. 3–1–3–5) by high speed counter current chromatography (HPCCC). A two-phase solvent system composed of BuOH–water–*n*-hexane [10:10:1 (v/v)] was used for the separation. A column was filled with upper stationary phase and the HSCCC instrument was revolved at 1.400 rpm while lower mobile phase was pumped in at a flow rate of 10.0 ml/min. Each fraction was purified by C_{18} RP HPLC [CH_3CN–H_2O (25:75–35:65)] to give platycodin D_3 and deapi-platycodin D (Fr. 3–2). Chemical structures of natural products derived from *P. grandiflorum* A. de Candolle including platycodinD_3 and deapi-platycodin can be seen in Fig. 2.

Animals

Pathogen-free male Sprague-Dawley rats (Daehan Biolink, Seoul, Korea), 5 weeks of age weighing 200–220 g, were used. The animals were housed five per cage and were provided with the distilled water and food ad libitum. They were kept under a 12 h light/dark cycle (light on 08:00–20:00) at constant temperature (22.5 °C) and humidity (55%). Animals were cared through all of the experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals regulated by Chungnam National University, Daejeon, Korea.

Experimental design

Twenty five rats were randomly divided into the following five groups: normal control; sulfur dioxide (SO_2)-only exposure; SO_2 exposure – aqueous extract of *P. grandiflorum* A. de Candolle (APG) 100 mg/kg; SO_2 exposure – APG 300 mg/kg; SO_2 exposure – dexamethasone 0.5 mg/kg. SO_2 was exposed to rats by inhalation and APG was administered per oral. A positive control, dexamethasone, was administered to rats via intraperitoneal injection. A 15% solution of sodium metabisulfite was aerosolized into a Plexiglas exposure chamber, using an ultrasonic humidifier (Samsung Electronics Inc., Seoul, Korea). The concentration of sulfur dioxide (SO_2) gas generated by this apparatus was measured to be 150 ppm. Rats were exposed to SO_2 for 3 h per day, 5 days per week, 3 weeks and APG was administered during the last 2 weeks out of 3 weeks in total. Normal control group were exposed to fresh air in a similar environment without SO_2 exposure.

Bronchoalveolar lavage fluid (BALF) collection and quantitation of in vivo mucins in BALF

Rats were euthanized on the last day of experiment and the trachea was cannulated by using sterile polyethylene tube. BAL was performed four times with 5.0 ml of ice-cold PBS (pH 7.4) with 80% of recovery rate. Floating cells and cell debris were removed by centrifugation of BALF at 12,000 × *g* for 5 min. The BALF samples were stored at −70 °C until assayed for their mucin contents. The amount of mucins in each BALF sample was measured by using enzyme-linked immunosorbent assay (ELISA). The BALF samples 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% BSA (fraction V) for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μl of 45M1 (NeoMarkers, CA, U.S.A.), a mouse monoclonal

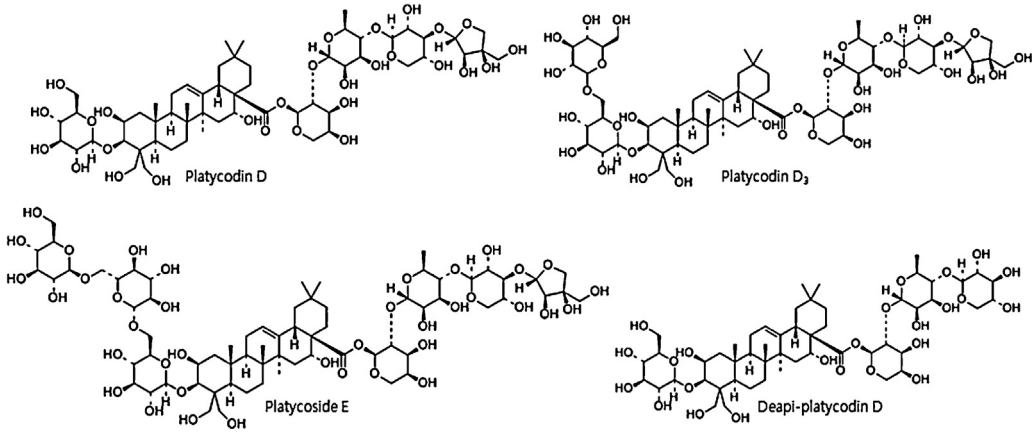

Fig. 2. Chemical structures of natural products derived from *Platycodon grandiflorum* A. de Candolle including platycodinD_3 and deapi-platycodin.

MUC5AC antibody (1:200), 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 μl of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3000) 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_2SO_4. Absorbance was read at 450 nm.

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 (seeding density: 1×10^4 cells/well in 24 well plate) 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_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells (5×10^5 cells/well in 24 well plate) 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 platycodinD_3 and deapi-platycodin

After 24 h of serum deprivation, cells were pretreated with platycodinD_3 (1, 10 and 100 μM) and deapi-platycodin (1, 10 and 100 μM) for 30 min and treated with PMA (10 ng/ml) for 24 h in serum-free RPMI 1640, respectively. PlatycodinD_3 and deapi-platycodin were dissolved in dimethylsulfoxide, diluted in PBS 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 in medium did not affect mucin production and secretion from NCI-H292 cells. After 24 h, the spent media were collected to measure the secretion of MUC5AC mucin and 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 mucin (in 24-well culture plate).

MUC5AC mucin analysis using ELISA

MUC5AC mucin was measured by using ELISA. 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% BSA for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μl of 45M1, a mouse monoclonal MUC5AC antibody (NeoMarkers, CA, U.S.A.) (1:200), 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 μl of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3000) 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_2SO_4. Absorbance was read at 450 nm.

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 Duncan's Multiple Range test as a post hoc test. $P < 0.05$ was considered as significantly different.

Results

Effect of aqueous extract of P. grandiflorum A. de Candolle (APG) on secretion of in vivo airway mucin from rats exposed to sulfur dioxide

As can be seen in Fig. 3, SO_2 exposure to rats for 3 weeks resulted in significant increase in mucin secretion, compared with the

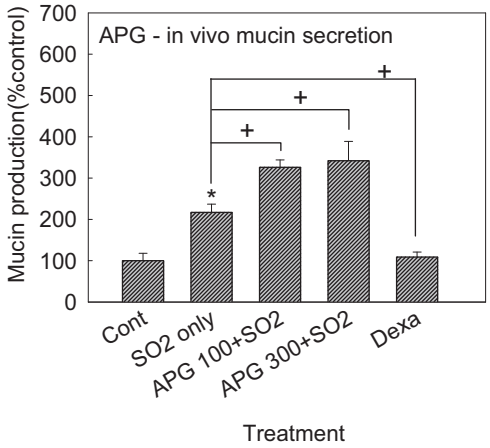

Fig. 3. Effect of aqueous extract of *Platycodon grandiflorum* A. de Candolle (APG) on secretion of in vivo airway mucin from rats exposed to sulfur dioxide. Rats were exposed to sulfur dioxide and effect of orally-administered APG on secretion of in vivo airway mucin was investigated as described in Materials and methods. Each bar represents a mean ± SEM from 5 rats (cont, control; Dexa, dexamethasone; SO_2, sulfur dioxide; concentration unit is mg/kg body weight).

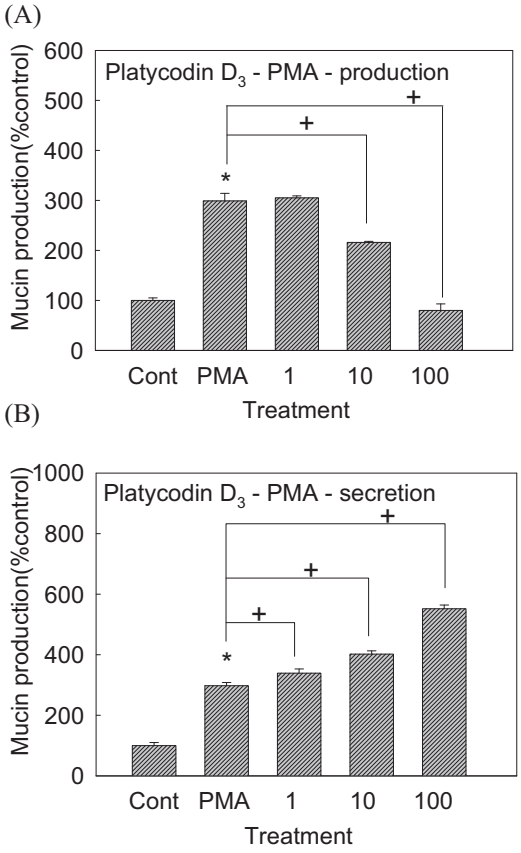

Fig. 4. Effect of on platycodinD_3 on PMA-induced MUC5AC mucin production and secretion from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of platycodinD_3 for 30 min and then stimulated with PMA (10 ng/ml) for 24 h. Spent media (for secretion) and cell lysates (for production) were collected for measurement of MUC5AC mucin secretion and production by ELISA, respectively. Three independent experiments were performed and the representative data were shown. Each bar represents a mean ± SEM of 3 culture wells in comparison with that of control set at 100%. *Significantly different from control ($p < 0.05$). +Significantly different from PMA alone ($p < 0.05$). (cont, control; concentration unit is μM).

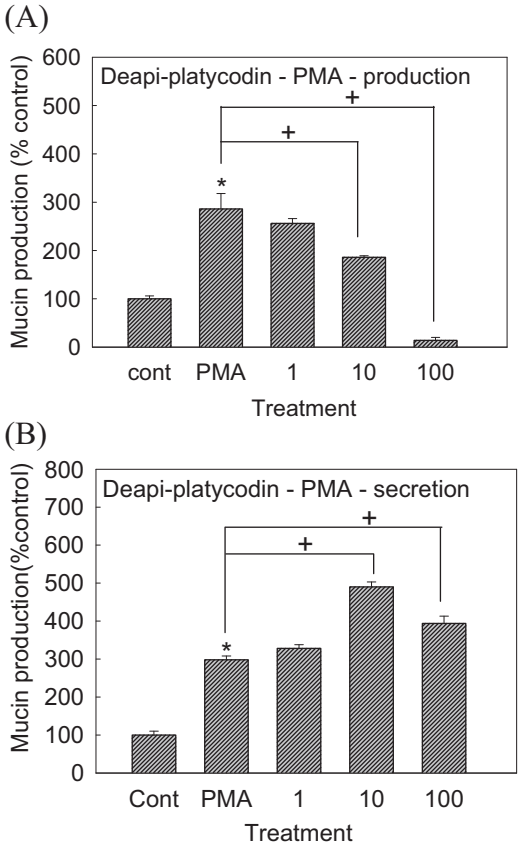

Fig. 5. Effect of on deapi-platycodin on PMA-induced MUC5AC mucin production and secretion from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of deapi-platycodin for 30 min and then stimulated with PMA (10 ng/ml) for 24 h. Spent media (for secretion) and cell lysates (for production) were collected for measurement of MUC5AC mucin secretion and production by ELISA, respectively. Three independent experiments were performed and the representative data were shown. Each bar represents a mean ± SEM of 3 culture wells in comparison with that of control set at 100%. *Significantly different from control ($p < 0.05$). +Significantly different from PMA alone ($p < 0.05$). (cont, control; concentration unit is μM).

normal control group. Dexamethasone, a positive control, significantly inhibited mucin secretion, due to its prominent antiinflammatory effect. However, APG stimulated the secretion of mucin in this acute bronchitis rat model. The amounts of mucin in the BALF samples were 100 ± 18%, 217 ± 20%, 326 ± 18%, 342 ± 47% and 109 ± 12% for control, SO_2 alone, SO_2 plus APG 100 mg/kg, SO_2 plus APG 300 mg/kg and SO_2 plus dexamethasone 0.5 mg/kg, respectively (Fig. 3).

Effect of platycodinD_3 on PMA-induced MUC5AC production and secretion from NCI-H292 cells

As can be seen in Fig. 4(A), platycodinD_3 significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of platycodinD_3-treated cultures were 100 ± 5%, 299 ± 15%, 305 ± 4%, 216 ± 2% and 80 ± 13% for control, 10 ng/ml of PMA alone, PMA plus platycodinD_3 10^{-6} M, PMA plus platycodinD_3 10^{-5} M and PMA plus platycodinD_3 10^{-4} M, respectively (Fig. 4(A)). However, platycodinD_3 did not inhibit but stimulated PMA-induced MUC5AC secretion from NCI-H292 cells. The amounts of mucin in the spent media of platycodinD_3-treated cultures were 100 ± 10%, 298 ± 10%, 339 ± 14%, 402 ± 11% and 552 ± 12% for control, 10 ng/ml of PMA alone, PMA plus platycodinD_3 10^{-6} M, PMA plus platycodinD_3 10^{-5} M and PMA plus platycodinD_3 10^{-4} M, respectively (Fig. 4(B)).

Effect of deapi-platycodin on PMA-induced MUC5AC production and secretion from NCI-H292 cells

As can be seen in Fig. 5(A), deapi-platycodin also significantly inhibited PMA-induced MUC5AC production from NCI-H292 cells. The amounts of mucin in the cells of deapi-platycodin-treated cultures were 100 ± 6%, 286 ± 32%, 256 ± 10%, 186 ± 3% and 14 ± 6% for control, 10 ng/ml of PMA alone, PMA plus deapi-platycodin 10^{-6} M, PMA plus deapi-platycodin 10^{-5} M and PMA plus deapi-platycodin 10^{-4} M, respectively (Fig. 5(A)). However, deapi-platycodin did not inhibit but stimulated PMA-induced MUC5AC secretion from NCI-H292 cells. The amounts of mucin in the spent media of deapi-platycodin-treated cultures were 100 ± 10%, 298 ± 10%, 328 ± 10%, 490 ± 13% and 394 ± 19% for control, 10 ng/ml of PMA alone, PMA plus deapi-platycodin 10^{-6} M, PMA plus deapi-platycodin 10^{-5} M and PMA plus deapi-platycodin 10^{-4} M, respectively (Fig. 5(B)).

Discussion

As shown in results, SO_2 exposure through aerosolized sodium metabisulfite to rats for 3 weeks resulted in significant increase in

mucin secretion, compared with the normal control group. Dexamethasone, a positive control, showing prominent antiinflammatory effect, significantly inhibited mucin secretion. However, aqueous extract of *P. grandiflorum* A. de Candolle (APG) stimulated the secretion of airway mucin in this acute bronchitis rat model. This result can explain, at least in part, the traditional use of APG as expectorants for controlling diverse pulmonary inflammatory diseases that are accompanied by hypersecretion of mucus. APG might stimulate the secretion of airway mucus and then expel the mucus from airway via inducing cough reflex. That is to say, APG can provoke the removal of sputum by inducing cough reflex via stimulation of secretion of mucus in airway luminal surface under inflammatory status. Dexamethasone, a corticosteroidal compound, might suppress the overproduction of in vivo airway mucin and resultantly might decrease the amount of secretion of mucin, under inflammatory condition. Next, since APG showed the stimulatory action on airway mucin secretion in in vivo model, we tried to investigate which component of APG can contribute to its pharmacologic activity. Of the twenty one MUC genes coding human mucins reported up to now, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium (Rogers and Barnes, 2006; Voynow and Rubin, 2009). Phorbol 12-myristate 13-acetate (PMA) was reported to stimulate the endogenous activator of protein kinase C (PKC), diacylglycerol (DAG) (Hong et al., 1999) and to be an inflammatory stimulant that can control a gene transcription (Hewson et al., 2004), cell growth and differentiation (Park et al., 2002). PMA also can induce MUC5AC gene expression in NCI-H292 cells (Kim et al., 2012). Based upon these reports, we investigated the effects of platycodinD_3 and deapi-platycodin, the two natural products derived from *P. grandiflorum* A. de Candolle, on PMA-induced MUC5AC mucin production and secretion from NCI-H292 cells, a human pulmonary mucoepidermoid cell line. As can be seen in results, platycodinD_3 and deapi-platycodin inhibited the production of MUC5AC mucin induced by PMA. However, the two compounds did not inhibit but stimulated the secretion of MUC5AC mucin induced by PMA. These results suggest that platycodinD_3 and deapi-platycodin can inhibit de novo production of airway mucin under inflammatory or stimulatory conditions, although they can stimulate the secretion of airway mucin already produced by inflammatory condition. This interpretation of the result is based on the record that aqueous extract of *P. grandiflorum* A. de Candolle (APG) was utilized as folk remedy which shows an antiinflmmatory activity and expectorating activity (Jang, 2003). Also, the stimulatory effect of platycodinD_3 on airway mucin secretion (release) was reported by Shin and his colleagues, although they examined the effect of platycodinD_3 through direct intratracheal nebulization to normal rat trachea. They reported that the effect of platycodinD_3 on mucin release was more potent than that of adenosine triphosphate (ATP), a well-known mucin secretagogue (Shin et al., 2002). Taken together, these results can explain, at least in part, the traditional use of APG as expectorants and anti-inflammatory agents for controlling diverse pulmonary inflammatory diseases that are accompanied by hypersecretion of mucus. The result from this study suggests a possibility of developing platycodinD_3 and deapi-platycodin as a candidate for the new efficacious expectorants for inflammatory pulmonary diseases, although further studies are required.

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