Crude ethanolic extracts of *Zingiber cassumunar* ROXB. inhibit PMA-induced MUC2 and MUC5AC expression via ERK inhibition in human airway epithelial cells

Thidarat Limvuttegrijerat,[1] Orapan Poachanukoon,[1,2] Sittichai Koontongkaew[2,3] and Thaweephol Dechatiwongse Na Ayudhya[2,3]

Summary

Background: **Over expression of mucin often leads to serious airway pathologies. The rhizome of *Zingiber cassumunar* Roxb. ("Phlai" in Thai) has been used as an anti-asthmatic drug in Thai traditional medicine. However, the effect of this plant on mucin production has not been reported.**

Objective: **The aim of the present study was to investigate whether crude ethanolic extracts of *Zingiber cassumunar* (CEZE) suppress phorbol12-myristate 13-acetate (PMA)-induced mucin production and gene expression in human airway epithelial cells and if so, to examine whether the suppression of mucin gene expression is mediated via the mitogen-activated protein kinase (MAPK) signal transduction pathways.**

Methods: **Confluent NCI-H292 cells were pretreated with CEZE for 2 hours and then stimulated with 100 or 200 nmol/l PMA for 8 h. The levels of *MUC2* and *MUC5AC* mRNA were determined by RT-PCR and real-time PCR. Levels of total mucin; MUC2 and MUC5AC in culture supernatants were measured using ELLA and ELISA assays, respectively. Extracellular signal-regulated kinase (ERK), JNK, p38 MAPK protein levels were analyzed by Western blotting.**

Results: **CEZE (5-100µg/ml) significantly inhibited total mucin production, including MUC2 and MUC5AC mRNA and proteins induced by PMA in NCI-H292 cells. The extracts obviously inhibited the phosphorylation of ERK, but not JNK and p38 in PMA-stimulated NCI-H292 cells. Our results suggest that *Z. cassumunar*-mediated suppression of PMA-induced MUC2 and MUC5AC mRNA operates via ERK inhibition.**

Conclusion: ***Z. cassumunar*** **suppresses PMA-induced MUC2 and MUC5AC gene expression in human airway epithelial cells via inhibition of ERK MAPK-dependent pathway.** ***(Asian Pac J Allergy Immunol 2014;32:328-36)***

Keywords: *Zingiber cassumunarRoxb.,airway epithelial cells, mucin, MUC2, MUC5AC*

Introduction

The respiratory tract has a surface area of approximately 70 m^2 that is in direct contact with the external environment. Approximately 12,000 L of air are inhaled daily, exposing the airway epithelium to up to 25 million particles an hour.[1] As a result, the airway epithelium has developed mucus to combat the attack of dust, microbes, and allergens. However, airway mucus hypersecretion and accumulation in the airway lumen are pathological processes associated with various chronic airway diseases.[2] Mucus obstruction of the airway is the major cause of morbidity and mortality in patients with asthma.[3]

Twenty human MUC genes have so far been identified. Of these, only nine, namely MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7,

From 1. Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.
2. Medicinal Herb Research Unit for Asthma, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.
3. Oral Biology Laboratory, Faculty of Dentistry, Thammasat University, Pathum Thani 12121, Thailand
Corresponding author: Orapan Poachanukoon
E-mail: orapanpoachanukoon@yahoo.com
Submitted date: 12/5/2014
Accepted date: 27/5/2014

MUC8, MUC11, and MUC13, are expressed in the human respiratory tract. Three of the nine, MUC2, MUC5AC, and MUC5B (the classic gel-forming mucins), are found in airway secretions.[4] Overexpression of these mucins correlates strongly with secretory cell hyperplasia and metaplasia in human airway.[5,6] MUC2 and MUC5AC expression are altered in inflamed airways[7,8] and, therefore, may contribute to the pathogenesis of several respiratory diseases.

A small number of key molecules, including mitogen-activated protein kinases are (MAPKs), may be involved in translating the actions of the different inflammatory mediators into airway mucus hypersecretion.[9,10]

According to traditional medicine, the rhizome of *Zingiber* (*Z.*) *cassumunar* Roxb. (Phlai in Thai) has been use for controlling diverse inflammatory diseases including asthma. [11] After sixteen weeks of treatment of asthmatic patients with *Z. cassumunar* at a dosage of 500-750 mg per day for body weight, the plant was found to provide a significant protection against asthmatic attacks in 19 out of 22 cases (86.4%).[12] However, to the best of our knowledge, there is no report about the effect of *Z. cassumunar* on mucin.

NCI-H292 is a human pulmonary mucoepidermoid cell line, which has been frequently used for the purpose of studying airway mucin production and gene expression.[9] Phorbol 12-myristate 13-acetate (PMA) is a well-known stimulant for secretion and gene expression of airway mucin, especially MUC2 and MUC5AC.[13] Therefore, the aim of this study was to investigate whether *Z. cassumunar* suppresses PMA-induced mucin synthesis in human airway epithelial cells and, if so, to examine whether the suppression operates via the MAPK signal transduction pathway.

Methods

Reagents

All chemicals and reagents used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Cell culture

The NCI-H292 (human mucoepidermoid pulmonary carcinoma ATCC No. CRL-1848) cell line was cultured in RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco-Invitrogen), 10 µg/ml of amphotericin B (PAA, Austria), 100 U/ml of penicillin-G and 100 µg/ml of streptomycin (Gibco-Invitrogen). For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and re-cultured in serum-free RPMI 1640 medium for 24 h.

Plant materials and extraction processes

Rhizomes of *Z. cassumunar* were purchased from a local herbal store in Bangkok, Thailand. Identification of the rhizomes was performed by a taxonomist at the Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Thailand. A voucher specimen has been deposited at the Oral Biology Research Unit, Faculty of Dentistry, Thammasat University (Rangsit campus), Prathum Thani, Thailand.

The plant materials were washed and cleaned thoroughly with tap water and then with distilled water. They were then finely ground to powder and the powdered tissues (60 g) were extracted with 500 ml of 95% ethanol in a Soxhlet apparatus (Buchi Laboratory, Flawil, Switzerland) for approximately 8 hours. The extracts were then filtered through Whatman No. 1 filter paper. All of these extracts were evaporated under reduced pressure at 50°C using a Buchi Rotavapor R-200 (BuchiLabortechnik AG, Flawil, Switzerland). The final yield was approximately 4-5 g of each respective extract. The crude ethanolic extracts of *Z. cassumunar* (CEZE) were stored at -20°C until use.

For sample preparation, a 1000 µg/mL stock solution of CEZE was prepared in distilled water. The extract was filtered and further diluted with the culture medium to the indicated concentration before use. The final concentration of ethanol in the culture medium was less than 0.01%.

Cytotoxicity Assay

To determine the nontoxic doses of CEZE, PMA and budesonide, cell viability was measured using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay.[14] Briefly, NCI-H292 cells ($1x10^4$ cells/well) were cultured in a 96-well plate (NUNC, Thermo, USA) in the presence of the indicated concentrations of CEZE, PMA or budesonide. Cells treated with medium only served as the negative control. After incubation for 72 h, each well was washed with PBS and then 50 µl of MTT solution (5 mg/ml) was added. The plates were incubated at 37 ° C for 4 h with protection from light. The resultant formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured by a microplate reader (Sunrise by Tecan, Salzburg, Austria) at 570 nm with a reference of 620 nm. Each experiment was performed with triplicate

samples. The percentage of growth was expressed as a percentage relative to the untreated cells.

Treatment of cells with CEZE

After 24 hours of serum deprivation, NCI-H292 cells were pretreated with the indicated concentrations of CEZE for 2 hours and then stimulated with PMA (100 or 200 nmol/l) for 8 hours in serum-free RPMI 1640 media. For controls, the cells were incubated with the medium alone. Supernatants were collected to measure the production of total mucin, MUC2 and MUC5AC proteins. The cells were then harvested. The total RNA from the cells was extracted for measurement of the expression of *MUC5AC and MUC2* genes using semi-quantitative and real-time quantitative PCR. The total protein from the cells was extracted for measurement of the phosphorylation of ERK, JNK and p38 using Western blotting. Budesonide[15] and genistein, which is a tyrosine kinase inhibitor,[16] were used as positive controls for mucin and MAPK inhibitions, respectively in the present study.

Semi-Quantitative RT-PCR for MUC genes

Total RNA was isolated from NCI-H292 cells using TRIzol® Reagent (Gibco-Invitrogen) according to the manufacturer's instructions. RNA samples (500 ng) were reverse-transcribed and amplified in a typical PCR using Super ScriptTM III Platinum® one-step Quantitative RT-PCR Kit (Invitrogen) 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to standardize the quantification of RNA samples. Primer sequences and annealing temperatures were as follows: MUC2 forward primer: 5'-TGCCTGG CCCTGTCTTTG-3', reverse primer: 5'-CAGCT CCAGCATGAGTGC-3', size: 440 bp; MUC5AC forward primer: 5'- ATCACCGAAGGCTGCTTCT GTC-3', reverse primer: 5'-GTTGATGCTGCA CACTGTCCAG-3' size: 410 bp and GAPDH forward primer: 5'-ACGCATTTG GTCGTATTGG G-3', reverse primer: 5'-TGATTTTGGAGGGAT CTCGC-3', size: 239 bp. Each cycle lasted 15 sec at 95°C for denaturation, 30 sec at 60°C for annealing, and 1 min at 72°C for extension. The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The signal intensity was analyzed using Quantity One® 1-D analysis software. (Gel Doc; BioRad, Hercules, CA, USA).

Quantitative real-time PCR for MUC genes

Total RNA was isolated from NCI-H292 cells using TRIzol® Reagent according to the manufacturer's instructions. Five hundred nanograms of total RNA was reverse transcribed to cDNA using RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas, USA). Quantitative real-time PCR reactions were set up in triplicate with the TaqMan® Fast Universal PCR master mix (Roche Diagnostic Gmbh, Mannheim, Germany) and run on the iQ5 Cycler Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA., USA). PCR primers and TaqMan probes were designed and used as reported previously.[17] The primers and TaqMan probes for human mucin genes were as follows: *MUC2*, 5′-GCTGG CTGGATTCTGGAAAA-3′(sense), 5′-TGGCTCTGCAAGAGATGTTAGC-3′ (antisense), 5′-ACCAGGTGGAGACACAGAATT GATTGGAGA-3′ (probe); *MUC5AC*, 5′-CAGCC ACGTCCCCTTCAATA-3′ (sense), 5′-ACCGCA TTTGG GCATCC-3′ (antisense), 5 ′-CCACCT CCGAGCCC GTCACTGAG-3 ′ (probe). The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold. To normalize *MUC* gene expression, real-time quantitative PCR for *GAPDH* was performed using the PCR primers and Taqman probe [5′-GGAAGGACTCATGACC ACAGT-3′(sense), 5′GCCATCACGCCACAGTT TC-3′ (antisense), 5′-TGCCATCACTGCCACCC AGAAGAC-3′ (probe)] as reported previously.[18] Relative quantification of target gene expression was performed using iCycler iQ5 TM software using the comparative cycle threshold method.

Measurement of total mucin secretion

An enzyme-linked lectin assay was used to measure mucin-like glycoprotein secretion as previously described.[9] Briefly, wells of a Maxisorb 96-well plate (NUNC™, Denmark) were coated with samples or standard bovine submaxillary gland mucin diluted in sodium carbonate buffer (0.05 mol/l, pH 9.6) and incubated overnight at 4°C. The plate was then washed with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked with a blocking buffer (1% gelatin in PBS-Tween) for 2 hours at 37 ° C.

Following washing with PBS-Tween, horseradish peroxidase-conjugated *Helix pomatia* lectin (5 μg/ml) in a blocking buffer was added and the plate was incubated for 2 hours at 37°C. After washing, the plate was incubated with the tetramethylbenzidine (TMB) solution at room temperature. The reaction was stopped after 10 min by the addition of 0.5 mol/l of hydrochloric acid. Color development was read at 450 nm using a

micro plate reader. The concentration of total mucin, measured in ng/ml, was calculated by comparison with the standard.

Immunoassay of MUC2 and MUC5AC proteins

MUC2 protein levels in conditioned media were determined by sandwich ELISA.[19] Briefly, an anti-MUC2 monoclonal antibody (SC-59956, Santa Cruz Biotechnology, Santa Cruz, CA., USA) at 1: 500 in a blocking buffer (1% gelatin in PBS-Tween) was applied to each well of a Maxisorb 96-well plate. After incubation at 4°C overnight, the plate was washed with PBS-Tween and blocked with the blocking buffer at 37°C for 1 hour. Following washing with PBS-Tween, samples were added to each well and the plate was incubated at 37° C for 2 hours. After washing with PBS-Tween, the plate was incubated with HRP-conjugated *H. pomatia* lectin (5 µg/ml in the blocking buffer) for 1 h at 37°C. The reaction in each well was visualized by addition of the TMB solution. After stopping the reaction with 0.5 mol/l of hydrochloric acid, color development was read at 450 nm using a microplate reader.

MUC5AC protein in culture supernatants was measured using a modified version of a previously described ELISA.[9] Each sample was incubated at 37°C in a Maxisorb 96-well plate until dry. The plate was washed with PBS-Tween, blocked with a blocking buffer (1% gelatin in PBS-Tween) at 37°C for 1 hour and then incubated with 100 µl of mouse monoclonal MUC5AC (MS-145-P1, clone 45M1; Neomarkers, Fremont, CA., USA; 1: 1,000) at 37° C for 2 hours. The plate was washed, and incubated with 100 µl of horseradish peroxidase-conjugated goat anti-mouse antibody (product No. 31430; Pierce, Rockford, IL, USA; 1: 5,000) at 37°C for 2 h. Color was developed using the TMB solution and stopped with 0.5 mol/l of hydrochloric acid. The absorbance was read at 450 nm. Because there are no commercial standard available for human MUC2 and MUC5AC, protein expressions are presented as absorbance values (arbitrary units) in the present study.

Western blotting

NCI-H292 cells were lysed in RIPA buffer for 10 min. Then, cell lysates were centrifuged at 10,000 g at 4°C. The protein concentrations were determined using Bradford reagents with bovine serum albumin as a standard (Pierce). Sample proteins of 100 µg/ml were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk for 2 hours, washed with Tris Buffered Saline Tween-20 (TBST), incubated in p-ERK, p-JNK, p-p38 mouse monoclonal antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA); ERK, JNK, p38 rabbit polyclonal antibody (1:1000, Santa Cruz Biotechnology), or actin rabbit polyclonal antibody (1:5000, Sigma-Aldrich) at 4°C overnight. Then the membranes were washed with TBST and incubated with goat anti-mouse or goat anti-rabbit antibody (1:40,000, Pierce) for 1 hour at room temperature. After the final washes, enhanced chemiluninescence was performed for 5 minutes with Supersignal substrate (Pierce). Semi-quantitative determination of protein expression was performed using a densitometer (Gel Documentation Systems, Bio-Rad, CA, USA).

Statistical analysis

Data are expressed as mean ± SEM. The difference between groups was assessed using one-way ANOVA and the Dunnett's multiple comparison test as a *post hoc* test. Statistical significance was considered for p values ≤ 0.05. Graphs and statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

Effects of PMA, CEZE and budesonide on cell viability of NCI-H292 cells

PMA concentrations as high as 200 nmol/l had no toxic effect on NCI-H292 cells (Figure 1 A). Prior to investigating the pharmacological potential of CEZE on PMA-induced mucin release, we determined the dose-dependent cytotoxicity of the extracts at concentrations of 25–1000 µg/ml on NCI-H292 cells. We found that concentrations of CEZE as high as 100 µg/ml had no cytotoxicity on NCI-H292 (Figure 1 B). Budesonide at the concentrations of 0 - 400 nmol/l did not have an affect on cell viability of NCI-H292 cells (Figure 1 C). Thus, PMA at 100 or 200 nmol/l, budesonide at 50 nmol/l and CEZE up to 100 µg/ml were selected for use in all subsequent experiments.

CEZE inhibits PMA-Induced MUC2 and MUC5ACgene expression from NCI-H292 cells

Figures 2A and B show that PMA-stimulated *MUC2* gene expression was significantly inhibited when NCI-H292 cells were pretreated with CEZE (5-50 µg/l) ($p < 0.05$). Budesonide, the positive

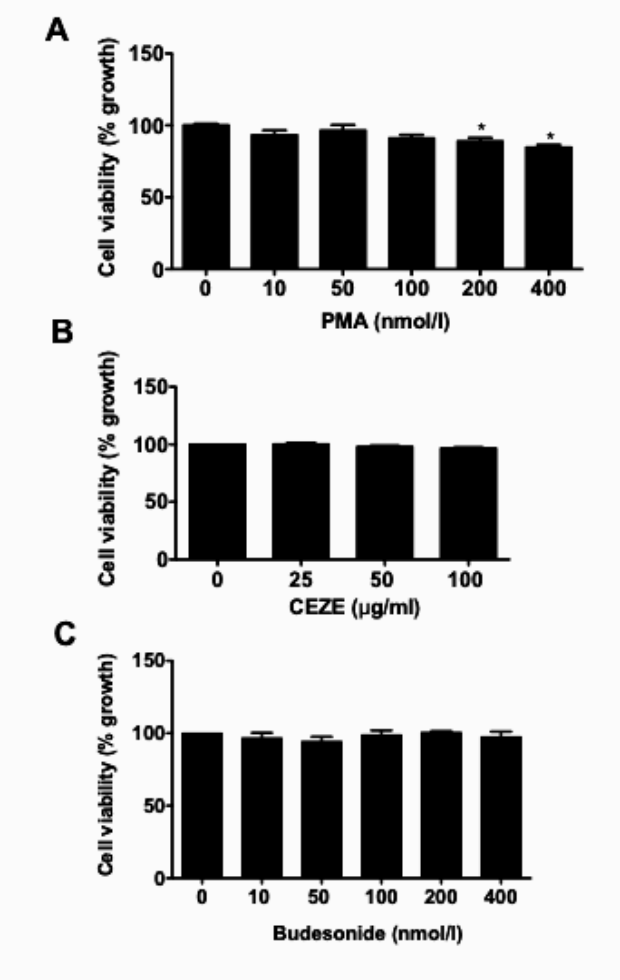

Figure 1. Effects of PMA (**A**), CEZE (**B**) and budesonide (**C**) on the viability of NCI-H292 cells. Cells were incubated for 72 h with PMA, CEZE or budesonide at the indicated concentrations. Cell viability testing was performed using an MTT assay. Viable cell numbers were denoted as a percentage of untreated cells at the concurrent concentration point. Data were means ± SEM (n = 3). * $p< 0.05$ versus the control. (PMA: phorbol 12-myristate 13-acetate; CEZE: crude ethanolic extract of *Zingiber cassumunar*).

control, at 50 nmol/l markedly inhibited PMA-stimulated *MUC2* gene expression.

As can be seen in Figures 2C and D, PMA-induced *MUC5AC* gene expression from NCI-H292 cells was inhibited by pretreatment with CEZE at concentrations of 5-50 μg/ml, compared to that of PMA alone ($p < 0.05$).

Quantitative real-time PCR confirmed that CEZE at concentrations of 5-100 μg/ml significantly reduced the expression of *MUC2* and *MUC5AC* genes of PMA-stimulated NCI-H292 cells when compared with PMA alone ($p < 0.05$) (Figures 3A and B).

Suppression of total mucin, MUC2 and MUC5AC protein production by CEZE

As expected, pretreatment with CEZE at concentrations of 5-100 μg/l significantly suppressed total mucin secretion from PMA-stimulated NCI-H292 cells ($p < 0.05$) as shown in Figure 4A. The inhibition was dose-dependent. CEZE also significantly inhibited the secretion of MUC2 (Figure 4B) and MUC5AC (Figure 4C) proteins, $p < 0.05$.

ERK mediates suppression of PMA-induced MUC-2 and MUC5AC mRNA expression by CEZE

To investigate inhibitory effect of CEZE on up-stream regulation of mucin synthesis in NCI-H292 cells, Western blotting was performed to evaluate the phosphorylation of ERK, JNK and p38. The expression of non-phosphorylated ERK, JNK and p38 was observed in NCI-H292 cells under basal conditions.

PMA markedly activated the phosphorylation of ERK (Figures 5A and B), but it did not activate the phosphorylation of JNK (Figures 5C and D) and p38 (Figures 5E and F). CEZE at 100 μg/ ml significantly inhibited the phosphorylation of ERK while the phosphorylation suppression was not observed in JNK and p38 ($p < 0.05$).

Discussion

In chronic inflammatory airway diseases, an increase in mucus production is one of the major pathologic manifestations. The development of agents that can control mucus secretion through the regulation of mucin genes is important for suppression of inflammatory airway diseases and improvement of symptoms.[3] A previous study demonstrated that patients treated with *Z. cassumunar* had reduced asthmatic symptoms.[12] In addition, animal studies have demonstrated that one of *Z. cassumunar* constituents, namely (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) possesses a potent anti-inflammatory activity through the inhibition of cyclooxygenases (COX) and lipoxygenases (LOX).[20] Therefore, based on the fact that *Z. cassumunar* has efficacy in asthmatic symptoms and anti-inflammatory effects in traditional medicine, we attempted to determine whether the expression of MUC5AC and MUC2 genes and proteins that are induced by PMA in airway epithelial cells, could be suppressed by CEZE.

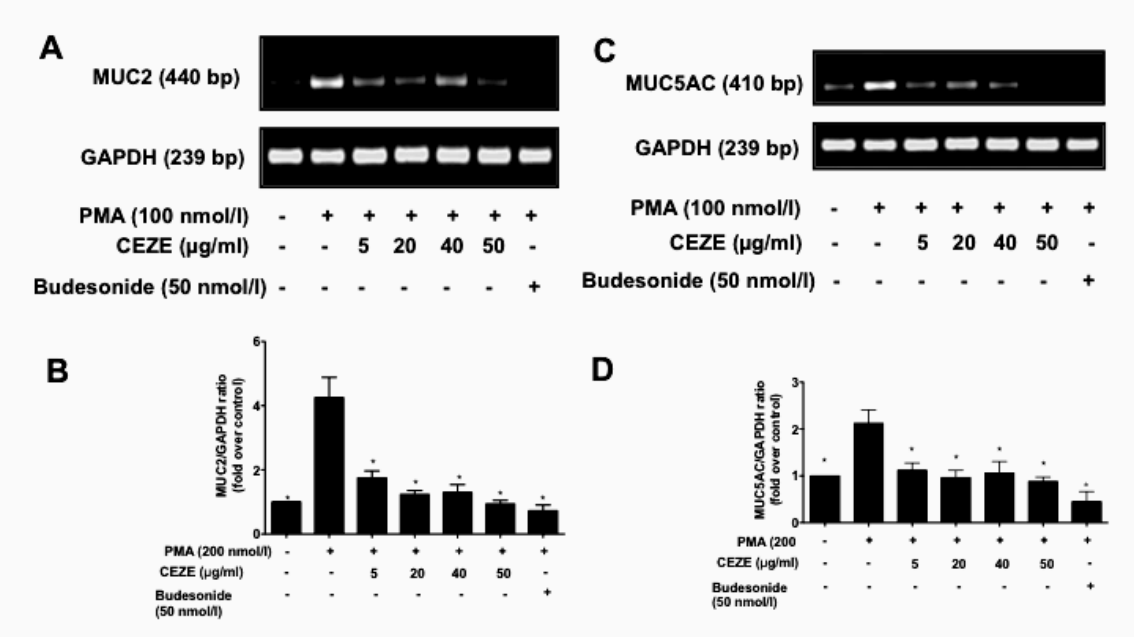

Figure 2. Effects of CEZE pre-treatment on PMA-induced *MUC2* and *MUC5AC* mRNA levels in NCI-H292 cells detected by conventional RT-PCR. Cells were pretreated with CEZE or budesonide at the indicated concentrations for 2 hours and then stimulated with 100 nmol/l of PMA for 8 hours. Steady state levels of *MUC2* (**A**) and *MUC5AC* (**C**) mRNA were measured by RT-PCR and quantified by densitometry of the reactions run on agarose gels (**B** & **D**). GAPDH was used as a quantitative control. Results are expressed as fold change over the control (NCI-H292 cells alone). Values are expressed as mean ± SEM (n = 3). * $p < 0.05$ compared with the PMA alone-treated cells. (PMA: phorbol 12-myristate 13-acetate; CEZE: crude ethanolic extract of *Zingiber cassumunar*).

The NCI-H292 cell line, which is known to express a high levels of MUC2 and MUC5AC, has been commonly used to understand the regulation of mucin genes and protein expression.[9,21, 22] As shown in our results, PMA enhanced the expression of MUC2 and MUC5AC genes and proteins in NCI-H292 cells. The concentrations of PMA (100 and 200 nmol/l) used in our study are relatively high, compared with the range of concentrations commonly used in previous studies.[9,23] However, we found that those concentrations are not toxic to NCI-H292 cells. PMA was reported to stimulate the endogenenous activator of protein kinase C (PKC), diacylglycerol,[24] and it can induce MUC2 and MUC5AC production from NCI-H292 cells.[13,25]

Our findings have demonstrated that CEZE obviously inhibits PMA-induced mucin gene and protein expression. It is known that the downstream effectors of the PKC pathway are MAPKs. MAPKs are serine/threonine kinases and tyrosine residues in response to a wide array of extracellular stimuli. Three distinct groups of MAPKs have been identified, including ERK, JNK and p38.[26,27] MAPKs are important enzymes in the production of mucin.[28]

To clarify the mechanism by which PMA-induced MUC2 and MUC5AC genes expression was suppressed by CEZE, we examined which MAPKs were activated within NCI-H292 cells stimulated by PMA. Western blotting revealed the expression of non-phosphorylated ERK, JNK and p38 in NCI-H292 cells under basal conditions. This indicated that ERK, JNK and p38 were constitutively expressed in this cell line. However, the level of phosphorylated ERK was increased 15 min after the stimulation of PMA, while that of JNK and p38 did not change. Our findings are in line with those of previous studies.[9,29,30]

We found that pretreatment with CEZE suppressed PMA-induced ERK activation in NCI-H292 cells. The results indicate that, in this cell line, ERK, but not JNK and p38, is activated with PMA treatment and the activation is suppressed by CEZE. Therefore, we can infer that ERK is involved in the inhibition of MUC2 and MUC5AC genes

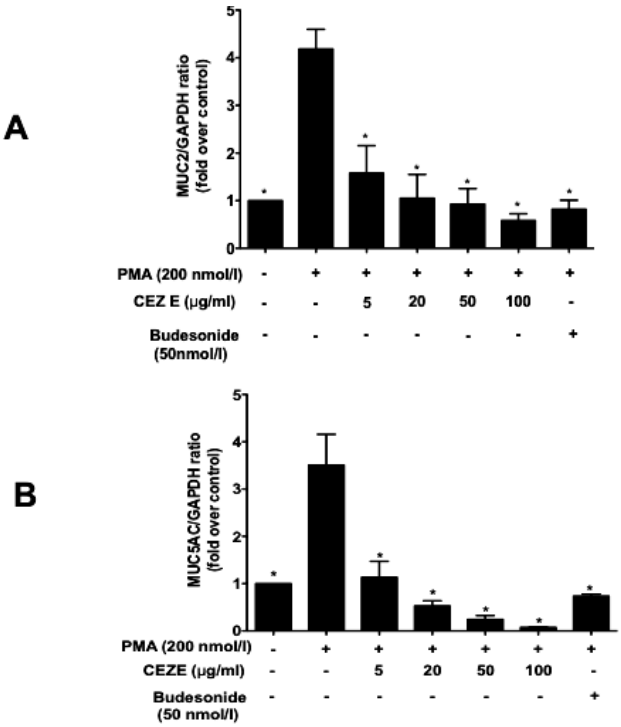

Figure 3. Effects of CEZE on PMA-induced *MUC2 and MUC5AC* gene expression in NCI-H292 cells detected by quantitative real-time PCR. Cells were pre-treated with CEZE or budesonide at the indicated concentrations for 2 hours and then stimulated with PMA (200 nmol/l) for 8 hours. *MUC2* (**A**) and *MUC5AC* (**B**) mRNA levels were analyzed by quantitative real-time PCR. GAPDH was used as a quantitative control. The results are expressed as fold change over the control (NCI-H292 cells alone). Data are means ± SEM of 3 independent samples. * $p < 0.05$ versus cells treated with PMA alone. (PMA: phorbol 12-myristate 13-acetate; CEZE: crude ethanolic extract of *Zingiber cassumunar*).

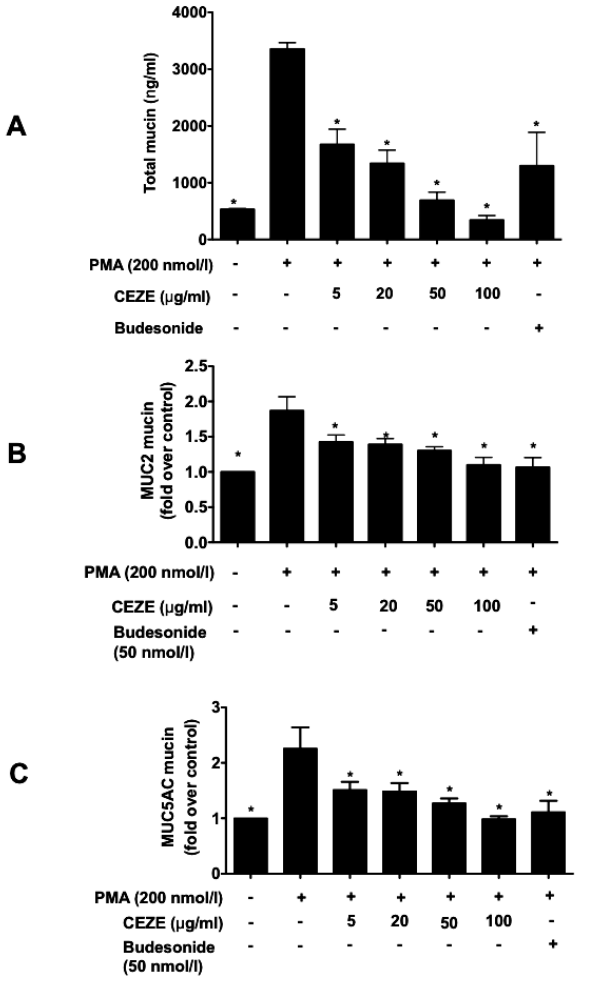

Figure 4. Effects of CEZE pre-treament on PMA induced mucin production. NCI-H292 cells were pre-treated with CEZE or budesonide at the indicated concentrations for 2 hours and then stimulated with PMA (200 nmol/l) for 8 h. Conditioned media were collected for measurement of total mucin (**A**); MUC2 (**B**) and MUC5AC (**C**) proteins by ELLA and ELISA, respectively. Data are means ± SEM of 3 independent samples. * $p < 0.05$ versus cell treated with PMA alone. (PMA: phorbol 12-myristate 13-acetate; CEZE: crude ethanolic extract of *Zingiber cassumunar*).

expression by CEZE in PMA-stimulated NCI-H292 cells.

Our findings are in accordance with reports that various constituents of plants can inhibit PMA-induced MUC5AC gene and protein expression in NCI-H292 cells.[23,31] It should be noted that there are few reports on natural inhibitors of MUC2. However, in the present study we found that CEZE inhibited not only *MUC5AC* but also *MUC2* gene and protein expression in NCI-H292 cells.

There may be other mechanisms involved in the inhibitory effect of CEZE on PMA-induced mucin synthesis. COX and prostaglandin E2 are also known to be involved in the signaling pathway of *MUC2* and *MUC5AC* gene expression.[32] Previous studies have showed that *Z. cassumunar* contains anti-COX constituents and the crude extract of this plant could inhibit COX expression.[20,33,34] Therefore, it is possible that CEZE may inhibit PMA-stimulated mucin production via COX inhibition. In addition, CEZE attenuates PMA-induced mucin protein synthesis in airway epithelial cells, possibly by inhibiting oxidative stress and inflammation via a mechanism involving NF-kappaB pathway activation and EGFR phosphorylation.[22,35]

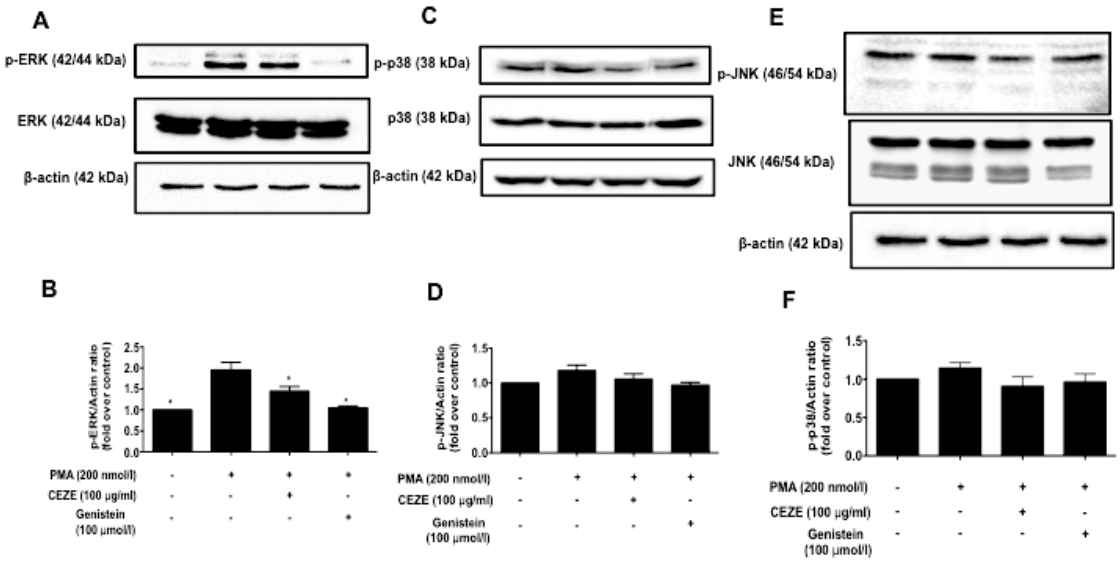

Figure 5. Effect of CEZE pre-treatment on PMA-induced MAPK phosphorylation in NCI-H292 cells. Cells were incubated with CEZE at 100 µg/ml for 2 hours and then treated with PMA (200 nmol/l). The cell culture was continued for 15 minutes and then harvested, lysed and subjected to Western blotting using (**A**) p-ERK, ERK; (**B**) p-JNK, JNK; (**C**) p-p38 MAPK, p38 MAPK, and β-actin antibodies. The expression of MAPKs was quantified on the basis of densitometry relative to β -actin (**B**, **D** and **F**). The results are expressed as fold change over the control (NCI-H292 cells alone). Data are means ± SEM of 3 independent samples. * $p < 0.05$ versus cell treated with PMA alone. (PMA: phorbol 12-myristate 13-acetate; CEZE: crude ethanolic extract of *Zingiber cassumunar*).

Taken together, our results can explain, at least in part, the traditional use of *Z. cassumunar* as an anti-inflammatory agents for reducing asthmatic symptoms that are accompanied by hypersecretion of mucus. The results from this study suggests that there is a possibility of developing *Z. cassumunar* as a candidate for a new anti-asthmatic agents, although further studies are required.

Acknowledgments

This study was supported in part by research funding from Faculty of Medicine, Thammasat University, Thailand in 2013. The authors thank Dr. Visakha Aupaphong for providing the samples of *Zingiber cassumunar* for the present study. We also thank Mr. Thanakorn Inthachart for his technical support.

References

1. Osorio F, Lambrecht B, Janssens S. The UPR and lung disease. Semin Immunopathol. 2013;35:293-306.
2. Thai P, Loukoianov A, Wachi S, Wu R. Regulation of airway mucin gene expression. Annu Rev Physiol. 2008;70:405-29.
3. Evans CM, Kim K, Tuvim MJ, Dickey BF. Mucus hypersecretion in asthma: causes and effects. Curr Opin Pulm Med. 2009;15:4-11.
4. Rogers DF. Airway mucus hypersecretion in asthma: an undervalued pathology? Curr Opin Pharmacol. 2004;4:241-50.
5. Voynow JA. What does mucin have to do with lung disease? Paediatr Respir Rev. 2002;3:98-103.
6. Rose MC, Nickola TJ, Voynow JA. Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. Am J Respir Cell Mol Biol. 2001;25:533-7.
7. Voynow JA, Selby DM, Rose MC. Mucin gene expression (MUC1, MUC2, and MUC5/5AC) in nasal epithelial cells of cystic fibrosis, allergic rhinitis, and normal individuals. Lung. 1998;176:345-54.
8. Vinall LE, Fowler JC, Jones AL, Kirkbride HJ, de Bolos C, Laine A, et al. Polymorphism of human mucin genes in chest disease: possible significance of MUC2. Am J Respir Cell Mol Biol. 2000;23:678-86.
9. Hewson CA, Edbrooke MR, Johnston SL. PMA induces the MUC5AC respiratory mucin in human bronchial epithelial cells, via PKC, EGF/TGF-alpha, Ras/Raf, MEK, ERK and Sp1-dependent mechanisms. J Mol Biol. 2004;344:683-95.
10. Kim YD, Jeon JY, Woo HJ, Lee JC, Chung JH, Song SY, et al. Interleukin-1beta induces MUC2 gene expression and mucin secretion via activation of PKC-MEK/ERK, and PI3K in human airway epithelial cells. J Korean Med Sci. 2002;17:765-71.

11. Thai pharmacopoeia committee. Thai herbal pharmacopoeia.Nonthaburi, Thailand: Department of medical sciences, ministry of public health; 1995.
12. Youngchaiyud P, Suthamsmai T, Kanchanapee P, Dechatiwongse T. Clinical Assessment of the Efficacy and Tolerance of *Zingiber cassumunar* Roxb in Bronchial Asthma. Siriraj Hosp Gaz. 1985;37:435-40.
13. Poachanukoon O, Koontongkaew S, Monthanapisut P, Pattanacharoenchai N. Macrolides attenuate phorbol ester-induced tumor necrosis factor-alpha and mucin production from human airway epithelial cells. Pharmacology. 2014;93:92-9.
14. Freshney RI. Culture of animal cells: A manual of basic technique.New york: Wiley-liss; 2000.
15. Kim YD, Kwon EJ, Kwon TK, Baek SH, Song SY, Suh JS. Regulation of IL-1beta-mediated MUC2 gene in NCI-H292 human airway epithelial cells. Biochem Biophys Res Commun. 2000;274:112-6.
16. Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, et al. The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. Am J Respir Cell Mol Biol. 2002;27:329-35.
17. Inoue D, Yamaya M, Kubo H, Sasaki T, Hosoda M, Numasaki M, et al. Mechanisms of mucin production by rhinovirus infection in cultured human airway epithelial cells. Respir Physiol Neurobiol. 2006;154:484-99.
18. Li R, Meng Z. Effects of SO2 derivatives on expressions of MUC5AC and IL-13 in human bronchial epithelial cells. Arch Toxicol. 2007;81:867-74.
19. Bamrungphon W, Prempracha N, Bunchu N, Rangdaeng S, Sandhu T, Srisukho S, et al. A new mucin antibody/enzyme-linked lectin-sandwich assay of serum MUC5AC mucin for the diagnosis of cholangiocarcinoma. Cancer Lett. 2007;247:301-8.
20. Jeenapongsa R, Yoovathaworn K, Sriwatanakul KM, Pongprayoon U, Sriwatanakul K. Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl) butadiene from *Zingiber cassumunar* Roxb. J Ethnopharmacol. 2003;87:143-8.
21. Berger JT, Voynow JA, Peters KW, Rose MC. Respiratory carcinoma cell lines. MUC genes and glycoconjugates. Am J Respir Cell Mol Biol. 1999;20:500-10.
22. Ishinaga H, Takeuchi K, Kishioka C, Suzuki S, Basbaum C, Majima Y. Pranlukast inhibits NF-kappaB activation and MUC2 gene expression in cultured human epithelial cells. Pharmacology. 2005;73:89-96.
23. Lee HJ, Lee SY, Lee MN, Kim JH, Chang GT, Seok JH, et al. Inhibition of secretion, production and gene expression of mucin from cultured airway epithelial cells by prunetin. Phytother Res. 2011;25:1196-200.
24. Hong DH, Petrovics G, Anderson WB, Forstner J, Forstner G. Induction of mucin gene expression in human colonic cell lines by PMA is dependent on PKC-epsilon. Am J Physiol. 1999;277: G1041-7.
25. Kim JO, Sikder MA, Lee HJ, Rahman M, Kim JH, Chang GT, et al. Phorbol ester or epidermal growth-factor-induced MUC5AC mucin gene expression and production from airway epithelial cells are inhibited by apigenin and wogonin. Phytother Res. 2012;26:1784-8.
26. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem. 1995;270:16483-6.
27. Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med (Berl). 1996;74:589-607.
28. Andrianifahanana M, Moniaux N, Batra SK. Regulation of mucin expression: mechanistic aspects and implications for cancer and inflammatory diseases. Biochim Biophys Acta. 2006;1765:189-222.
29. Lee HW, Ahn DH, Crawley SC, Li JD, Gum JR, Jr., Basbaum CB, et al. Phorbol 12-myristate 13-acetate up-regulates the transcription of MUC2 intestinal mucin via Ras, ERK, and NF-kappa B. J Biol Chem. 2002;277:32624-31.
30. Yuan-Chen Wu D, Wu R, Reddy SP, Lee YC, Chang MM. Distinctive epidermal growth factor receptor/extracellular regulated kinase-independent and -dependent signaling pathways in the induction of airway mucin 5B and mucin 5AC expression by phorbol 12-myristate 13-acetate. Am J Pathol. 2007;170:20-32.
31. Ryu J, Lee HJ, Park SH, Kim J, Lee D, Lee SK, et al. Effects of the root of *Platycodon grandiflorum* on airway mucin hypersecretion in vivo and platycodin D3 and deapi-platycodin on production and secretion of airway mucin in vitro. Phytomedicine. 2014;21:529-33.
32. Kim YD, Kwon EJ, Park DW, Song SY, Yoon SK, Baek SH. Interleukin-1beta induces MUC2 and MUC5AC synthesis through cyclooxygenase-2 in NCI-H292 cells. Mol Pharmacol. 2002;62:1112-8.
33. Han AR, Kim MS, Jeong YH, Lee SK, Seo EK. Cyclooxygenase-2 inhibitory phenylbutenoids from the rhizomes of *Zingiber cassumunar*. Chem Pharm Bull (Tokyo). 2005;53:1466-8.
34. Koontongkaew S, Meesuk L, Aupaphong V, Ayudhaya TD, Poachanukoon O. Inhibitory effect of *Zingiber cassumunar* extracts on lipopolysaccharide-induced cyclooxygenase-2 and matrix metalloproteinase expression in human gingival fibroblasts. J Periodontal Res. 2013;48:507-16.
35. Yang T, Luo F, Shen Y, An J, Li X, Liu X, et al. Quercetin attenuates airway inflammation and mucus production induced by cigarette smoke in rats. Int Immunopharmacol. 2012;13:73-81.