

# *Zingiber cassumunar* ROXB. and its active constituent inhibit MMP-9 direct activation by house dust mite allergens and MMP-9 expression in PMA-stimulated human airway epithelial cells

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## Summary

**Background:** House dust mite (HDM) induced-matrix metalloproteinase (MMP)-9 plays a role in asthma. *Zingiber cassumunar* Roxb. (Phlai in Thai) has been used in folk medicine for asthma treatment.

**Objective:** We investigated effects of Phlai and its constituent (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D) on the cleavage of pro-MMP-9 by HDM. The effects of these compounds on phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 gene and protein expression in airway epithelial cells (NCI-H292) were also investigated.

**Methods:** Pro-MMP-9 was directly activated *in vitro* with HDM in the presence or absence of the ethanolic extracts of Phlai or compound D for 1 hour. The amount of activated MMP-9 was determined using gelatin zymography. To study the cellular response of Phlai, NCI-H292 cells were pretreated with crude Phlai extracts or compound D for 2 hours, and then the cells were stimulated with PMA for 48 hours. The mRNA

and protein levels of MMP-9 were measured by RT-PCR and Western blotting, respectively. MMP-9 activity was determined by gelatin zymography.

**Results:** Crude Phlai extracts (0.25 - 2.0 mg/ml) and compound D (0.5 - 4.0 mg/ml) inhibited pro-MMP-9 cleavage by HDM. Furthermore, crude Phlai extracts (100 µg/ml) and compound D, at concentrations of 50 and 100 µg/ml, attenuated the PMA-induced MMP-9 gene and expression in NCI-H292 cells. These compound also suppressed MMP-9 release from PMA-induced NCI-H292 cells.

**Conclusion:** The crude ethanolic extract of *Z. cassumunar* and its active constituent compound D inhibited the cleavage of pro-MMP-9 by HDM. They also inhibited PMA-induced MMP-9 gene and protein synthesis in human airway epithelial cells. (*Asian Pac J Allergy Immunol* 2015;33:42-51)

**Keywords:** *Zingiber cassumunar*, Phlai, house dust mite, MMP-9, NCI-H292

## Introduction

Proteins excreted in the fecal pellets of house dust mites (HDM) belonging to the genus *Dermatophagoides* (e.g. *D. pteronyssinus*, *D. farinae*) are major causes of allergic asthma.<sup>1</sup> It has been shown that allergens from HDM, particularly those possessing protease activity, are significant modulators of epithelial functions.<sup>2</sup>

The matrix metalloproteinase (MMP) family is a group of structurally related proteins that degrade extracellular matrix (ECM) and basement membrane in a zinc-dependent manner at physiological pH. It comprises at least 26 secreted and membrane-tethered endopeptidases, classified according to their structures and substrate specificities.<sup>3</sup> By degrading components of the ECM, MMPs are thought to play a role in tissue remodeling and the accumulation of

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inflammatory cells associated with asthma.<sup>4</sup> Among the MMP family, MMP-9 is believed to be the major MMP in asthma, because its level and activity are increased in the exhaled breath condensate (EBC),<sup>5</sup> sputum,<sup>6</sup> serum,<sup>7</sup> and bronchial epithelium<sup>8</sup> of asthmatic patients. In addition, HDM extracts have been shown to directly activate proMMP-9 *in vitro* suggesting that HDM may enhance airway inflammation in asthma by increasing MMP-9 activity.<sup>9</sup>

*Zingiber cassumunar* Roxb. (Zingiberaceae) commonly known, as Phlai in Thai has been used as folk medicine in Thailand for treatment of various diseases, including inflammation and asthma. Several phenylbutanoids and cyclohexene derivatives have been identified in the rhizomes of *Z. cassumunar*. (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D) and (E)-1-(3,4-dimethoxyphenyl)but-1,3-diene (DMPBD) isolated from the rhizomes of *Z. cassumunar* possess a potential anti-inflammatory activity.<sup>10,11</sup> Additionally, compound D has an antispasmodic effect in the guinea pig ileum and tracheal smooth muscle.<sup>12</sup> Pulverized rhizome of Phlai is effective in relieving asthmatic symptoms in children.<sup>13</sup>

Phorbol myristate acetate (PMA), a phorbol ester, is known to substitute for diacylglycerol (DAG) as a high affinity ligand for protein kinase C (PKC).<sup>14</sup> It has been shown to strongly stimulate MMP-9 expression in various human airway epithelial cell lines including NCI-H292 cells.<sup>15</sup> NCI-H292 cells can secrete MUC5 AC and MUC 2, which are involved in asthma pathogenesis.<sup>16,17</sup> In addition, MMP-9 could mediate ligand-dependent activation of EGFR and leads to an increase in MUC5 AC production in NCI-H292 cells.<sup>18</sup>

To date, however, the precise molecular mechanisms by which Phlai modulates allergic airway diseases have not been completely elucidated. We hypothesized that Phlai might attenuate HDM induced asthma either by (1) inhibition of HDM-activated proMMP-9 or (2) inhibition of MMP-9 production. Therefore, in this study, we determined whether the Phlai extract and compound D could directly inhibit the cleavage of pro MMP-9 by HDM. The inhibitory effect of those compounds on PMA-induced MMP-9 in human airway epithelial cells was also determined in the present study.

## Methods

### Reagents

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

### House dust mite and commercial Der p 1 extracts

House dust mites (HDM) were purchased from the Siriraj Dust Mite Center for Service and Research, Mahidol University, Bangkok, Thailand. HDM extracts were obtained from both mite bodies and fecal pellet. Mite bodies and their fecal remnants (100g) were incubated in 1 ml of phosphate buffered saline (PBS) at 4°C for 2 hours with continual rocking. After centrifugation at 10,000xg for 5 minutes, the supernatant was collected. For each extract, total protein was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction. In this study, effects of HDM extracts on pro-MMP-9 cleavage were compared with that of the commercial Der p 1 extract obtained from ALK-Abello' (Horsholm, Denmark).

In our preliminary experiment, we found that HDM extract itself possessed endogenous protease activity as assessed by gelatin zymography, however, heat treatment (60°C for 3 h)<sup>19</sup> could abolish protease activities of HDM extracts (data not shown). Therefore, we have used heat-inactivated HDM extracts for some subsequent experiments.

### Measurement of total protease activity of HDM and commercial Der p1 extracts

The protease activity of HDM and Der p 1 extracts was determined by monitoring the hydrolysis of casein as described by Kumari et al.<sup>20</sup> with minor modification. The reaction mixture contained 0.25 ml of the test substance and 0.5 ml of 1% w/v casein in 0.02 M Tris-HCl buffer (pH 8.0), and was incubated at 37°C for 24 hours. The reaction was terminated by adding 1ml of 10% trichloroacetic acid (TCA) and kept at 4°C for 10 minutes. The resultant precipitate was removed by centrifugation at 13,000xg for 10 minutes. The TCA-soluble peptides were measured by Lowry's method using tyrosine as a standard. PBS and the protease of *Streptomyces griseus* were used as negative and positive controls, respectively. The specific protease activity was expressed as tyrosine released in  $\mu\text{mol}/\text{mg}$  protein of HDM extracts.

### Identification of proteolytic activation of pro-MMP-9 by HDM extracts and Der p1 antigens

SDS-PAGE of pro-MMP-9 treated with the HDM or Der p 1 extract was performed to examine the change in molecular size of ProMMP-9 during activation. Aminophenylmercuric acetate (APMA, 1mM) was used for activation of proMMP-9 as a positive control. Pro-MMP-9 (0.5 - 5 ng, CalBiochem, San Diego, CA, USA) was incubated with 1mM APMA, HDM extracts (300 and 600 ng), or Der p 1 extract (200 ng) in a buffer solution (20  $\mu$ l) containing 100 mM TrisHCl, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, 0.01% Brij 35, pH7.5 at 37°C for 1-3 hours. Aliquots of the reaction mixture were subjected to gelatin zymography.

To identify the nature of the protease, in some experiments pro-MMP-9 was activated with HDM or Der p1 in the presence of PMSF (serine protease inhibitor, 25-1250  $\mu$ M) or E-64 (cysteine protease inhibitor, 10-500  $\mu$ M). Proteolytic activation of proMMP-9 was performed in the same manner as described above.

### Cell cultures

The human mucoepidermoid pulmonary carcinoma cell line (NCI-H292, ATCC#CRL-1848) was cultured in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) amphotericin B (2.5  $\mu$ g/ml) and 100  $\mu$ g/ml penicillin-streptomycin (Gibco-Invitrogen). The cells were grown at 37°C in 5% carbon dioxide at 95% humidity. To prevent the presence of serum affecting the response of the cells to HDM extracts, the cells were cultured in serum-free medium with 0.1% bovine serum albumin (BSA) before doing the experiments.

### Effects of HDM extracts on MMP-9 activation in airway epithelial cells

NCI-H292 cells were seeded in a 6-well plate at  $1 \times 10^5$  cells/ well. When the cultured cells were in a confluent state, they were incubated in serum-free RPMI 1640 medium containing 0.1% BSA. After 24 hours of incubation with HDM extracts, culture supernatants were collected and stored at -20°C. MMP-9 activity in the culture supernatants was determined. Corresponding cell monolayers were trypsinized and counted.

To clarify whether the effect observed resulted from the protease activity of HDM extracts, we performed similar experiments using heat-inactivated HDM extracts.

### Plant materials

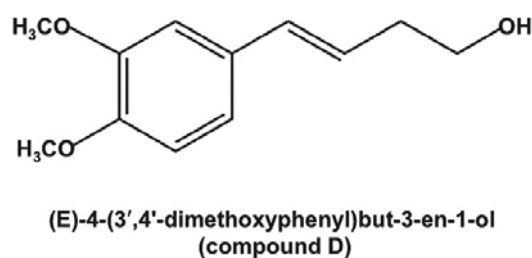
Dried rhizomes of Phlai were obtained from a traditional medicine shop in Bangkok, Thailand. The species was identified by a botanist from Department of Medical Science, Ministry of Public Health, Thailand. The voucher specimen was deposited in the Faculty of Dentistry, Thammasat University, Thailand.

The rhizomes of Phlai were ground into a powdered form using a pulverizer. Each 40 g of Phlai powder was extracted with 400 ml of absolute ethanol for 6 hours using a Soxhlet apparatus. The extracts were collected and evaporated to dryness by rotatory evaporation at 40°C and 175 mbar. The ethanol soluble portion was subjected to silica gel column chromatography with hexane-ethyl acetate (Merck, Whitehouse station, NJ, USA) (90:10 $\rightarrow$ 70:30). Fractionation of the ethanol extract on silica gel (gradient) gave 13 major fractions. Fraction 11 was further purified to give compound D. Compound D was prepared from the ethanol extracts of Phlai, as described in a previous paper.<sup>21</sup> In our preparation Phlai contained a proportion of compound D approximately of 1 %, which was in the same range as in a previous report.<sup>22</sup>

The structure of compound D is shown in Figure 1. Its structure was unambiguously established by its comparing spectroscopic properties, including UV, IR, MS and NMR spectroscopy with those reported in literature.<sup>23</sup>

### Effect of Phlai and compound D on HDM-mediated pro-MMP-9 activation

To determine the inhibitory effects of Phlai and compound D on HDM-mediated pro-MMP-9 activation, pro-MMP-9 (250 ng/ $\mu$ l) was pretreated with APMA (1mM) or HDM extracts (200  $\mu$ g/ $\mu$ l) in the presence or absence of various concentrations of test compounds at 37°C for 1 hour. Then aliquots of the reaction mixture were subjected to gelatin zymography.



**Figure 1.** The chemical structure of (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (compound D).

**MTT assay**

Prior to investigating the pharmacological potential of Phlai and its constituents on PMA-induced MMP-9 expression in cultured NCI-H292 cells, we first determined the dose dependence of the cytotoxic effects of PMA, crude Phlai extracts and compound D. PMA was initially dissolved in DMSO and then diluted with culture media to indicated concentrations. Crude Phlai extracts and compound D were diluted with cultured media.

Cytotoxicity was determined by the thiazolyl blue tetrazolium bromide (MTT) assay. The methodology described here is a modification of the original MTT colorimetric assay developed by Mosmann.<sup>24</sup> Briefly, the cell suspensions were dispensed (200 µl) in triplicate into 96-well culture plates at 5 x 10<sup>4</sup> cells/ml. After a 24-hour recovery, the medium was changed, and PMA, crude Phlai extracts or compound D were added at various concentrations. After an additional 72 hours of culture, the medium in each well was aspirated and replaced with 200 µl of MTT working solution to obtain a final concentration of 0.5 mg/ml. After incubation at 37°C for 4 hours, the medium was aspirated, and DMSO was added to dissolve the formazan crystals formed in the cells. The culture plates were shaken for 5 minutes and the absorbance at 570 nm was measured by using a microplate reader. The relative viability of the treated cells as compared to the control cells was expressed as the % viability, using the following formula:

$$\% \text{ viability} = \frac{[\text{A570 of treated cells}]}{[\text{A570 of control cells}]} \times 100\%$$

Conditions were considered toxic if the % viability was < 80% compared to the control.

**Effect of crude Phlai extract and compound D on PMA-activated MMP-9 in NCI-H292 cells**

After 16 hour-incubation in serum-free DMEM with 0.1% BSA, NCI-H292 cells were pretreated with crude Phlai extracts or compound D for 2 hours at various concentrations. Then cells were stimulated with PMA at the indicated concentration for 48 hours. Culture supernatants were then collected and stored at -70°C until the MMP-9 activity was measured. Cells were harvested and lysed. Lysates were subjected to RT-PCR and Western blotting.

**MMP-9 activity by gelatin zymography**

The MMP-9 activity was determined using gelatin zymography as previously described.<sup>25</sup> Briefly, a total of 10 µl of sample underwent

electrophoresis in 10% polyacrylamide containing 1mg/ml gelatin, in the presence of SDS under non-reducing conditions. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 minutes to remove SDS, washed three times in water, and then incubated in a renaturing buffer (50 mM Tris, pH8, 5 mM CaCl<sub>2</sub> and 1 µM ZnCl<sub>2</sub>, 3 mM NaN<sub>3</sub>, pH7.4) at 37°C overnight. Following incubation, the gels were stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA, USA) for 1 hour at room temperature and de-stained in a solution of 10% glacial acetic with 50% methanol. The gelatinolytic activity was estimated using densitometry with GeneTools software program (Syngene, Frederick, MD, USA). Calculated values were normalized with the numbers of corresponding cells in each culture. A parallel culture of HT1080, a human fibrosarcoma cell line that produces both MMP-2 and MMP-9 was used as a positive control.<sup>26</sup>

**MMP-9 detection by Western blot analysis**

Treated NCI- H292 cells were washed in cold PBS, lysed with RIPA buffer [15 NP-40, 0.5% deoxycholic acid sodium salt, 0.1% SDS and PBS (pH7.4)] supplemented with 10mM sodium orthovanadate, 1 mM PMSF and 1X proteinase inhibitor (Complete mini EDTA-free, Roche Applied Science, Indianapolis, IN, USA). Cell debris was spun down at 10,000X g for 10 minutes, and the protein content of the supernatants was then determined using the BCA Protein Assay Kit (Pierce Biotechnology). Equal amounts of protein (50 µg) were separated on 10% SDS-PAGE, and transferred on to nitrocellulose membranes. The membranes were blocked in 5% (w/v) non-fat powder before probing with appropriated antibodies. The following primary antibodies were used: rabbit anti-actin (1:4,000, Sigma-Aldrich) and mouse anti-MMP-9 (1:500, Sigma-Aldrich). Membranes were then incubated with HRP (horseradish peroxidase) conjugated secondary antibodies (1:40,000, Pierce Biotechnology) before undergoing a chemiluminescence HRP substrate reaction (Super Signal West Pico Chemiluminescent substrate, Pierce Biotechnology). Signals were detected using Gene Gnome system (Syngene).

**RNA isolation and RT-PCR analysis**

Total RNA was isolated from cells with Trizol (Quiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed with the following primer for MMP-9 (sense: 5'-GGTCCCCCACTGCTGGCCCTTCTACGGCC-3';

antisense: 5'-GTCCTCAGGGCACTGCAGGATGT CATAGGT-3') and GAPDH (sense: 5'-ACGC ATTTGGTCGTATTGGG-3'; antisense 5'- TGA TTTTGGAGGGATCTCGC-3'). GAPDH was used to verify that equal amount of RNA were used for reverse transcription and PCR amplification from different experiment conditions. The RT-PCR was carried out using One-step RT-PCR kit (Quiagen). Each PCR reaction was performed in 50  $\mu$ l of a reaction mix, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.4 mM dNTP, 600  $\mu$ M primers, 300 ng RNA templates and three units of Hotstar TagDNA polymerase. Thermocycling was performed at 50  $\mu$ C for 30 minutes, 95°C for 15 minutes and then 30 cycle of 95°C (denature) for 1 minute, 60°C (annealing) for 1 minute and 72°C (extension) for 1 minute.

RT-PCR products were run on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. Amplified fragment sizes for MMP-9 and GAPDH were 640 bp and 231 bp, respectively. A computer densitometer (Syngene) was used to determine the density of PCR product bands. The signals of MMP-9 mRNA were normalized by comparison with GAPDH mRNA signal.

#### Data analysis

All experiments were performed at least three times. Unless otherwise stated, values are presented as mean  $\pm$  standard error (SEM). When applicable, all comparisons were made with One-way analysis variance (ANOVA) followed by Bonferroni's posttest analysis for significance between groups. Significance was defined as a *p* value  $\leq$  0.05. All statistical analyses were performed using Prism 5 (GraphPad software, San Diego, CA).

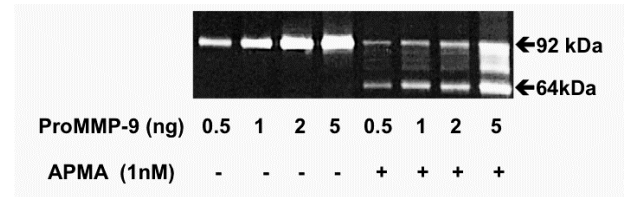
## Results

### Effects of APMA on the cleavage of pro-MMP-9

In preliminary experiments, we confirmed that proMMP-9 (0.5-5.0 ng) was cleaved by APMA from the 92-kDa form to the 64-82 kDa active forms (Figure 2). Therefore, we used proMMP-9 at 5 ng as a substrate in subsequent experiments.

### Protease activities of HDM and Der p1 and their effects on pro-MMP-9 activation

We found that the specific protease activity of commercial Der p1 extract was 0.0027 units. It is relatively low compared to that of the HDM extract (0.75 units). To determine if active proteases in HDM extracts play a role in cleavage of pro-MMP-9, we



**Figure 2.** Effect of APMA on proMMP-9 activation *in vitro*. ProMMP-9 (0.5 - 5 ng) was incubated with or without APMA (1mM) in 20  $\mu$ l of reaction solution at 37°C for 3 hours. The MMP activity was determined using gelatin zymography.

incubated pro-MMP-9 with HDM extracts that had either been pretreated with PMSF (serine protease inhibitor) (Figure 3A) or E-64 (cysteine protease inhibitor) (Figure 3B). We found that the inhibition of serine protease or cysteine protease attenuated the conversion of pro-MMP-9 to active forms. These data demonstrate the importance of cysteine and serine proteases of HDM in mediating pro-MMP-9 cleavage.

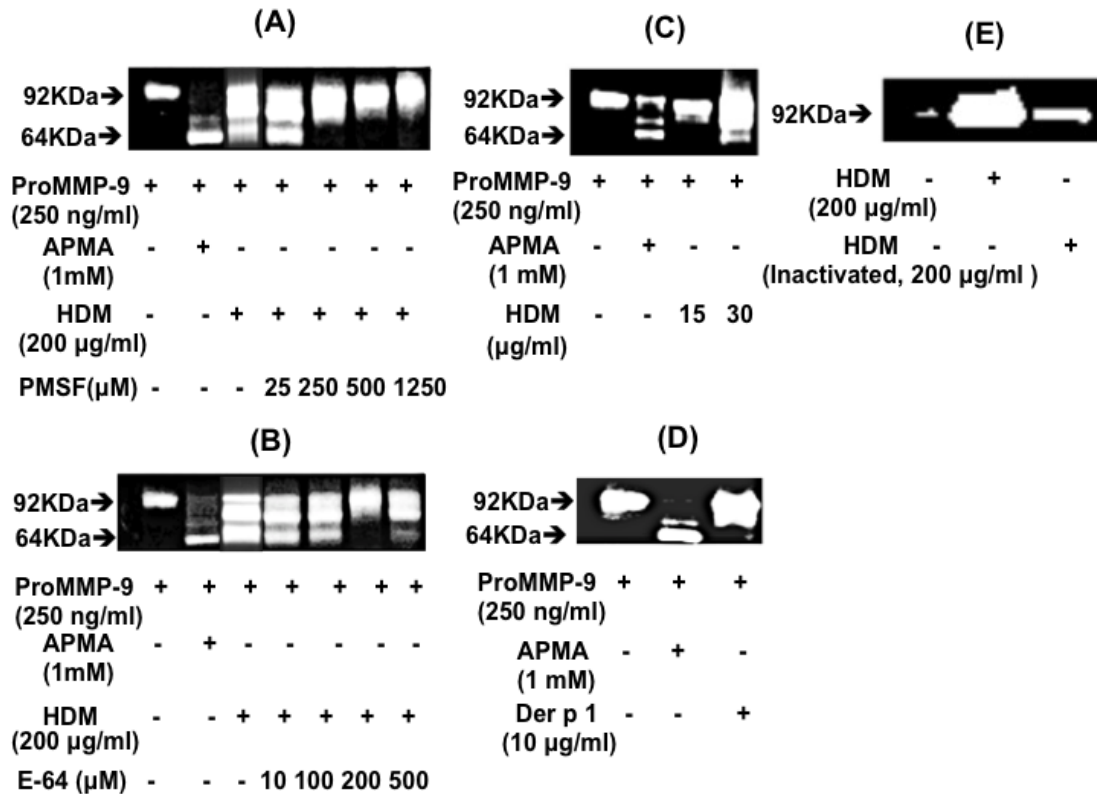
Next, we compared pro-MMP-9 cleavage between HDM and Der p1 extracts. Figure 3C shows that HDM extracts markedly activated pro-MMP-9 at the concentration employed compared to the control. In contrast, the Der p1 extract did not obviously activate pro-MMP-9 (Figure 3D). We also determined the effects of HDM extracts on the activation of MMP-9 in NCI-H292 cells. HDM extracts stimulated MMP-9 production from NCI-H292 cells (Figure 3E). Heat treatment caused a decrease in HDM-induced MMP-9 synthesis. These data suggest that proteases of HDM are involved in MMP-9 production in airway epithelial cells.

### Effects of crude Phlai and compound D on pro-MMP-9 activation

Pretreatment of HDM extracts (200 $\mu$ g/ml) with crude Phlai extracts (0.25-2.0 mg/ml) (Figure 4A & B) or compound D (0.5-4.0 mg/ml) (Figure 4C & D) obviously inhibited the conversion of pro-MMP-9 to active forms in a dose-dependent manner.

### Cell viability with crude Phlai extract treatment

The effects of crude Phlai extracts compound D and PMA on viability of NCI-H292 cells were determined by MTT assay. Crude Phlai extracts showed a cytotoxic action toward NCI-H292 cells at concentrations over 250  $\mu$ g/ml (Figure 5A). Compound D did not show any cytotoxicity at concentrations



**Figure 3.** Protease activities of HDM and commercial Der p 1 extracts and their effects on proMMP-9 direct activation. ProMMP-9 (250 ng/ml) was treated with HDM (200 µg/ml) in the presence or absence of (A) serine protease inhibitor (PMSF) or (B) cysteine protease inhibitor (E-64) at the indicated concentrations at 37°C for 1-3 h, aliquots of the reaction mixture were subjected to gelatin zymography. APMA (1mM) was used as a positive control. To compare effects of HDM and Der p 1 on proMMP-9 activation, proMMP-9 (250 ng/ml) was incubated with and without (C) HDM extracts (15 and 30µg/ml) or (D) commercial Der p 1 extract (10 µg/ml) at 37°C for 3 h, gelatin zymography of the reaction mixture was determined. To determine the role of protease activity in MMP-9 production in cells, NCI-H292 cells treated with or without HDM (200µg/ml) and heat-inactivated HDM (200 µg/ml) for 48h. The MMP-9 activity in conditioned media was determined (E).

lower 200 µg/ml (Figure 5B). Therefore, all experiments were performed with test substances at non-cytotoxic concentrations. MTT assay shows that PMA at concentrations lower than 800 nM had no cytotoxic effect on NCI-H292 cells (Figure 5C).

#### **Effects of crude Phlai and compound D on PMA stimulated MMP-9 in cells**

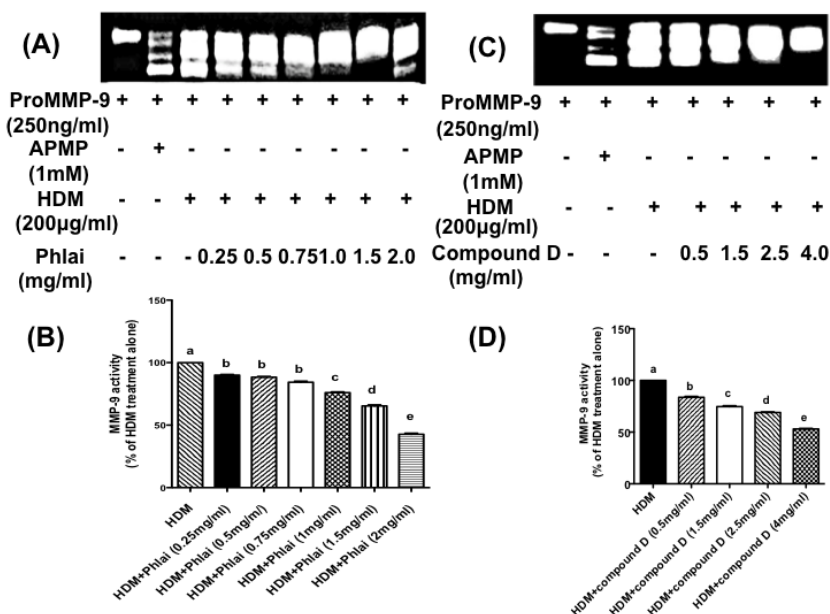
Preliminary data demonstrated that PMA (20-100 nM) enhanced MMP-9 expression in NCI-H292 cells in a dose dependent manner (data not shown). Therefore in this study, we used PMA at the concentration of 100 nM to stimulate MMP-9 expression in NCI-H292 cells.

RT-PCR revealed that crude Phlai at 100 µg/ml and compound D at 50 µg/ml could inhibit PMA-induced MMP-9 gene expression in NCI-H292 cells. (Figure 6A). We next used a Western blot assay and

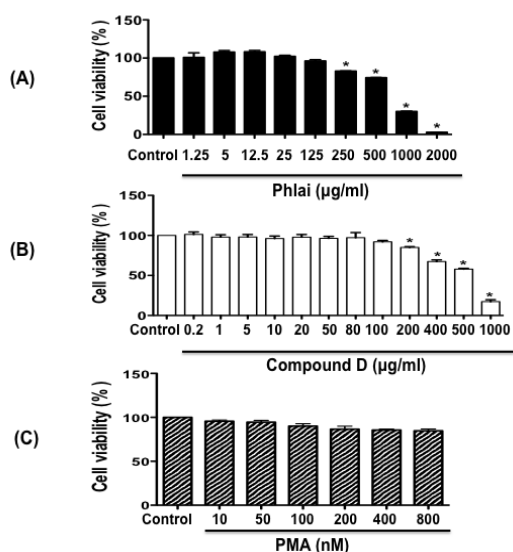
gelatin zymography to investigate the inhibitory effect of Phlai and compound D on PMA-induced MMP-9 protein expression and enzyme activity. Western blotting (Figure 6B) and gelatin zymography (Figure 6C & D) also demonstrated the inhibitory effects of crude Phlai and compound D on PMA-mediated MMP-9 release from NCI-H292 cells.

#### **Discussion**

In this study, we confirmed that HDM extracts contain both cysteine and serine activities.<sup>27</sup> Using this *in vitro* model, our findings are in agreement with observations that allergens including mite extracts directly cleave pro-MMP-9.<sup>9, 28</sup> This and the fact that inhibition of cysteine and serine proteases resulted in a reduction of enzyme activation,



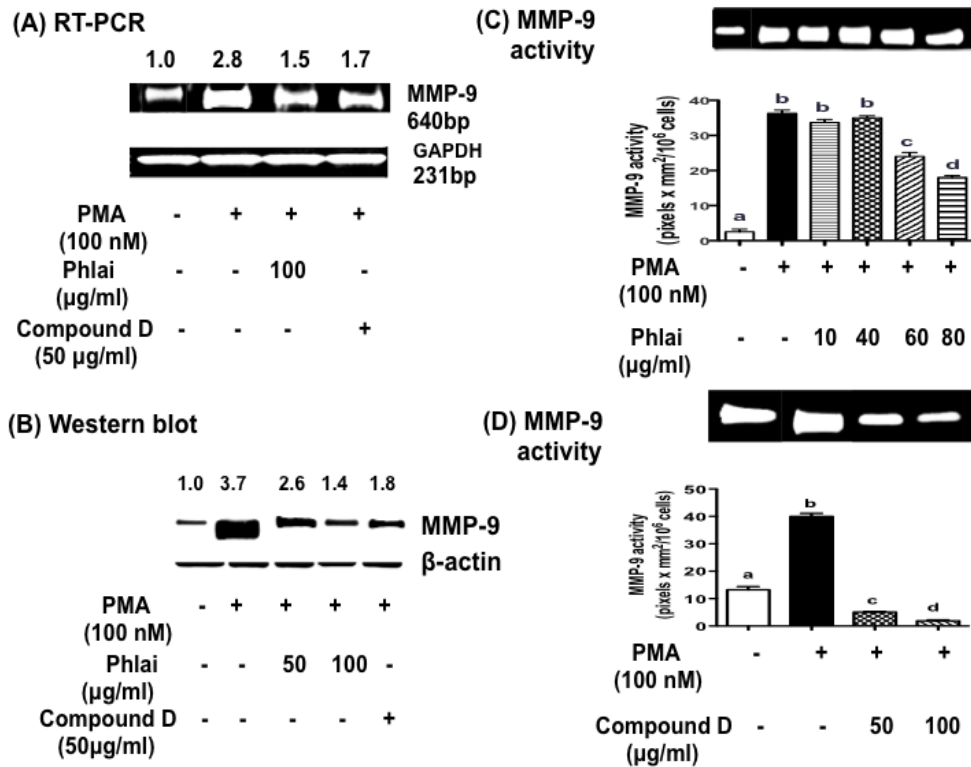
**Figure 4.** Inhibitory effects of crude Phlai extracts on HDM-activated proMMP-9. ProMMP-9 (250ng/ml) was pretreated with 1mM APMA (a positive control) or HDM extracts (200µg/ml) in the presence or absence of crude Phlai extracts (0.25-2.0 mg/ml) (A) or compound D in the range of 0.5-4.0 mg/ml (C) at 37°C for 1-3h. Aliquots of the reaction mixture were subjected to gelatin zymography. Densitometric values of proMMP-9 and its active forms were calculated from their gelatinolytic activities by using a densitometer (B & D). Data are expressed as a percentage of the response that is obtained from cells treated with HDM alone. Values represent mean ± SE of three independent experiments. Means followed by different alphabetical letters are significantly different ( $p \leq 0.05$ ).



**Figure 5.** Effects of crude Phlai extracts (1.25-2,000 µg/ml) (A), compound D (0.2-1,000 µg/ml) (B) and PMA (10-800 nM) (C) on the cell viability of NCI-H292 cells. NCI-H292 cells were treated without (control) and with the test compounds at indicated concentrations for 72 hours. The cell cytotoxicity was measured by the MTT assay. Values (mean ± SE) represent the percentage of controls containing no test compounds ( $n=3$ ). \*Significantly different from untreated cells ( $p \leq 0.05$ ).

suggests the importance of protease in MMP-9 activation.<sup>3</sup> However, the commercial Der p1 extract used in this study could not induce cleavage of pro-MMP-9. It is possible that the amounts of proteases in the commercial Der p1 extract are insufficient to elicit this response. Lack of serine protease in commercially available HDM was also reported.<sup>9</sup> This discrepancy between HDM extract and commercial Der p1 with respect to pro-MMP-9 cleavage may be due to the difference in their compositions. HDM is a mixture of various compounds; it can also contain some compound that has an activating effect *in vitro* and has been removed from HDM during Der p1 preparation. Although the concentrations of Phlai and compound D used in our study of Pro-MMP-9 cleavage were relatively high and probably toxic to cells, the concentration of crude phlai 2 g (equivalent to 15 mg of compound D) had no toxic effect in animals.<sup>22</sup>

We were able to provide evidence that protease inactivation by heat treatment abrogate HDM-induced MMP-9 release from NCI-H292 cells. This indicates that the activation of MMP-9 in cells depends on the proteolytic activities of HDM allergens. It has been known that asthma is



**Figure 6.** Inhibitory effects of crude Phlai and compound D on PMA-mediated MMP-9 expression. Serum-starved NCI-H292 cells were pretreated with the crude Phlai extract or compound D at indicated concentrations for 2 hours. Then cells were stimulated with PMA (100nM) for 48 hours. **(A)** RT-PCR was performed to determine the MMP-9 mRNA expression level. GAPDH mRNA levels were used as an internal control. **(B)** The MMP-9 protein expression was analyzed by Western blot assay. The  $\beta$ -actin expression was included as an internal control. The number shown on the top indicates the fold-change of MMP-9 level of treated cells versus control cells (taken as 1.0). **(C&D)** The MMP-9 activity in conditioned media was analyzed by zymogram assay. Densitometric values of gelatinolytic activities of zymogram were analyzed. Data represent mean  $\pm$  SE of three independent experiments. Means followed by different alphabetical letters are significantly different ( $p \leq 0.05$ ).

associated with increased protease-activated receptor (PAR) -2 expression in the bronchial epithelium,<sup>29</sup> suggesting a potential role for PAR-2 as a mediator involved in airway inflammation. PARs are present on airway epithelial cells, including NCI-H292.<sup>30</sup> Proteases bind and cleave the PAR-2 receptors, which trigger their own intracellular signaling in activation of G-coupled protein activation including ERK. The end result is an increase in MMP-9 secretion from airway epithelial cells.<sup>31</sup> It has been reported that Der p 1 activates the release of IL-6 and IL-8 from air way epithelial cells, A549, in a PAR-independent manner.<sup>32</sup> HDM proteases activate ERK in airway epithelial cells resulting in an increase of IL-8 production.<sup>33</sup> Therefore it is possible that the stimulating effect of HDM on MMP-9 production may be mediated through cleavage of PARs, which leads to ERK activation.

Our findings demonstrate that Phlai at 10 - 80  $\mu$ g/ml (equivalent to 0.1 - 0.8  $\mu$ g/ml of compound D) markedly inhibit MMP-9 activity in PMA-induced NCI-H292 cells, compared to that of compound D at 50 and 100  $\mu$ g/ml. This suggests that Phlai contains other anti-inflammatory constituents, which might inhibit MMP-9 activities.<sup>34</sup> Although the underlying mechanism of the action of Phlai and compound D on PMA-mediated MMP-9 activation in airway epithelial cells is not clear at present, our previous work has shown that crude Phlai extract suppresses MMP-2 via the inhibition of ERK activation in LPS-stimulated human gingival fibroblasts.<sup>35</sup> Therefore, it is conceivable that Phlai and compound D may suppress PMA-induced MMP-9 production in NCI-H292 cells through the inhibition of PAR-2 and ERK activation.



In conclusion, our results show that crude phlai extract and its constituent compound D inhibit the cleavage of pro-MMP-9 and MMP-9 production in PMA-induced airway epithelial cells. Accordingly, it is suggest that Phlai and its constituent should be benefit to ameliorate inflammation and hypersensitiveness of airway epithelium in response of HDM.

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### Conflict of interests

The authors declare that they have no conflict of interests related to the present study.

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