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To cite this article: Wafda Rohhimi, Ji Wei Tan, Kong Yen Liew, Alain Jacquet, Hanis Hazeera Harith, Daud Ahmad Israf & Chau Ling Tham (2021): Zerumbone attenuates house dust mite extract-induced epithelial barrier dysfunction in 16HBE14o- cells, Immunopharmacology and Immunotoxicology, DOI: [10.1080/08923973.2021.1992633](https://doi.org/10.1080/08923973.2021.1992633)

To link to this article: <https://doi.org/10.1080/08923973.2021.1992633>



Published online: 25 Oct 2021.



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ORIGINAL ARTICLE



Zerumbone attenuates house dust mite extract-induced epithelial barrier dysfunction in 16HBE14o- cells

Wafda Rohhimi^{a*}, Ji Wei Tan^{a,b*}, Kong Yen Liew^{a*}, Alain Jacquet^c, Hanis Hazeera Harith^a, Daud Ahmad Israf^a and Chau Ling Tham^a 

^aDepartment of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia; ^bSchool of Science, Monash University Malaysia, Subang Jaya, Malaysia; ^cCenter of Excellence in Vaccine Research and Development, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

ABSTRACT

Context: The airway epithelial barrier can be disrupted by house dust mite (HDM) allergens leading to allergic airway inflammation. Zerumbone, a natural monocyclic sesquiterpene, was previously found to possess anti-asthmatic effect by modulating Th1/Th2 cytokines. However, the protective role of zerumbone on epithelial barrier function remains to be fully explored.

Objective: To investigate the effect of zerumbone on HDM extract-induced airway epithelial barrier dysfunction.

Materials and methods: Human bronchial epithelial cells 16HBE14o- were incubated with 100 µg/mL HDM extract and treated with non-cytotoxic concentrations of zerumbone (6.25 µM, 12.5 µM, and 25 µM) for 24 h. The epithelial junctional integrity and permeability were evaluated through transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC)-Dextran permeability assays, respectively. The localization of junctional proteins, occludin and zona occludens (ZO)-1, was studied using immunofluorescence (IF) while the protein expression was measured by western blot.

Results: Zerumbone inhibited changes in junctional integrity (6.25 µM, $p \leq .05$; 12.5 µM, $p \leq .001$; 25 µM, $p \leq .001$) and permeability (6.25 µM, $p \leq .05$; 12.5 µM, $p \leq .01$; 25 µM, $p \leq .001$) triggered by HDM extract in a concentration-dependent manner. This protective effect could be explained by the preservation of occludin (12.5 µM, $p \leq .01$ and 25 µM, $p \leq .001$) and ZO-1 (12.5 µM, $p \leq .05$ and 25 µM, $p \leq .001$) localization, rather than the prevention of their cleavage.

Discussion and conclusion: Zerumbone attenuates HDM extract-induced epithelial barrier dysfunction which supports its potential application for the treatment of inflammation-driven airway diseases such as asthma.

ARTICLE HISTORY

Received 11 February 2021
Accepted 7 October 2021

KEYWORDS

House dust mite; epithelial barrier dysfunction; asthma; tight junction; zerumbone

Introduction

Human airways contain three barriers of protection to prevent the development of diseases when exposed to airborne pollutants, pathogens, or allergens. The three barriers, namely chemical, physical, and immunological barriers, have different protective roles to maintain the airway integrity. Chemical barrier is characterized by the mucociliary clearance in which the mucus traps dust particles from inhaled air while the cilia sweep dust particles upwards to remove it from the airway passage [1]. If this first line of defense is compromised, the airway epithelial cells are the first cells that will get in contact with and insulted by the environmental stimuli. These stimuli will then cause the airway epithelium to release a variety of cytokines and chemokines, resulting in an acute inflammatory response. Other than causing direct damage, allergens and pathogens could also penetrate the epithelial barrier and interact with the dendritic cells beneath the airway epithelium consequently drive

the adaptive immune response via antigen presentation to T cells [2].

The physical barrier of the airways consists of pseudostratified columnar epithelial cells attached to each other by junctional proteins. This semi-permeable epithelial barrier creates a protective layer toward inhaled foreign material besides separating the immunological cells compartment and the airway lumen passage [3,4]. The junctional system of the airway epithelial lining is made up of junctional protein groups namely adherens junctions (AJs), gap junctions, and most importantly, tight junctions (TJs). Examples of tight junctional proteins include occludin, zona occludens (ZO), claudins, and tricellulin [5–7]. The integral transmembrane (occludin, claudins, and tricellulin) and scaffolding proteins (ZO-1, -2, and -3) constitute the core components of epithelial TJ architecture [8,9]. TJs play an essential role in the barrier protection of the airway tract as they provide the lining with the ability to control the passage of substances through the paracellular route of the epithelial lining and

CONTACT Chau Ling Tham  chauling@upm.edu.my  Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia

*These authors contributed equally to this work.

maintain the apical-basal plasma membrane polarity [10,11]. However, tight junctional proteins can be disrupted by allergens, respiratory viruses, as well as cigarette smoke, resulting in changes in the epithelial barrier permeability leading to inappropriate or exaggerated immune reactions such as asthma [12–15].

Among more than 35 different house dust mite (HDM) allergens identified, the HDM species *Dermatophagoides pteronyssinus* and *farinae* represent two of the most important sources of airborne allergens which can be found in households. HDM is associated with microbial compounds such as lipopolysaccharides (LPS), chitin, and β -glucans, all of which could contribute to the development of the allergic airway inflammation through innate immune activation [16,17]. The cysteine proteinase Der p 1, the most abundant HDM allergen present in the mite fecal pellet, as well as other serine proteases such as Der p 3, Der p 6, and Der p 9 are able to initiate the cleavage of junctional proteins (occludin, claudin, and E-cadherin) in bronchial epithelial cells *in vitro*. In particular, the proteolytic activity of the major allergen Der p 1 has been shown to enhance allergenicity in a mouse inhalation model [18]. Other than directly cleaving epithelial junctions proteolytically, the activation of pattern recognition receptors (PRRs) on airway epithelial cells, such as protease-activated receptors (PAR), C-type lectins (CLR), and purinergic receptors, by HDM components could also lead to the degradation and/or delocalization of the junctional proteins. Thus, this will facilitate the passage of allergens through the epithelial barrier for subsequent presentation to the host immune system [19–22].

Zerumbone, a natural monocyclic sesquiterpene compound extracted from the rhizome of *Zingiber zerumbet* (L) Smith, was found to possess therapeutic properties. For example, *in vitro* studies showed that zerumbone possesses anti-cancer effects such as its ability to prevent Epstein-Barr virus activation [23] and its inhibitory effect on the proliferation of human colonic adenocarcinoma or breast cancer cells [24,25]. Zerumbone was also found to inhibit the colon and lung carcinogenesis due to its ability to induce apoptosis in colon/lung cancer cells and suppress nuclear factor- κ B (NF- κ B)/heme-oxygenase-1 (HO-1)-mediated inflammatory response [26]. Antinociceptive effect of zerumbone has been documented as well [27]. An *in vivo* study showed that zerumbone also possesses anti-inflammatory and anti-proliferative effects in mouse models of paw edema and granuloma, respectively [28].

In a mouse model of allergic asthma, oral treatment of zerumbone has been proven to attenuate asthmatic condition by promoting Th1 cytokine (interferon (IFN)- γ) production while reducing Th2 cytokines (eotaxin, keratinocyte-derived chemokine (KC), interleukin (IL)-4, IL-5, IL-10, and IL-13) production [29]. As the airway epithelium serves as the first barrier against external environment and the detrimental effects are primarily triggered by the exposure of immune cells to the allergens as a result of the disruption of airway epithelial barrier, it would be interesting to determine whether zerumbone is able to suppress HDM extract-induced airway epithelial barrier disruption, which is a crucial event

that increases the vulnerability of asthmatic patients to different noxious agents. Therefore, the goal of the present study was to investigate the *in vitro* effect of zerumbone on airway epithelial barrier dysfunction induced by HDM extract by using 16HBE14o- cell line as an *in vitro* model of the human airway system as this cell line has been shown to express tight junctional proteins such as occludin and ZO-1. This is the first study that investigates the effect of zerumbone on epithelial barrier function in HDM-induced airway epithelial cells.

Materials and methods

Materials

Cell culture

16HBE14o- cell line was a kind gift from Dr Dieter C. Gruenert (University of California, San Francisco, CA). The cell line was developed by the transformation and immortalization of normal bronchial epithelial cells obtained from a 1-year-old male heart and lung transplant patient. The cells were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA). For each passage, cells were washed with HEPES-buffered saline solution (HBSS) and trypsinized with PET solution (10% polyvinylpyrrolidone (Sigma, USA), 0.2% ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma, USA), and 0.25% trypsin with 0.02% ethylenediaminetetraacetic acid (EDTA) (Gibco, USA)). LHC basal medium containing 100 μ g/mL bovine serum albumin (Nacalai Tesque, Japan), 30 μ g/mL bovine collagen I (Corning, USA), and 10 μ g/mL human fibronectin (Corning, USA) was used to prepare fibronectin coating solution. The tissue culture flasks, plates, inserts, and chamber slides for 16HBE14o- cultures were all coated with the fibronectin coating solution before use.

Antibodies

Anti-occludin rabbit polyclonal antibody (71-1500) was purchased from Gibco, USA, whereas anti-ZO-1 (D6L1E) rabbit monoclonal antibody (#13663) was purchased from Cell Signaling Technology, USA. HRP-conjugated goat anti-rabbit IgG (sc-2030) and HRP-conjugated mouse monoclonal anti- β -actin IgG (sc-47778) were obtained from Santa Cruz Biotechnology, USA. Alexa Fluor 488-conjugated goat anti-rabbit IgG (ab150077) was purchased from Abcam, United Kingdom.

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4 kDa Fluorescein isothiocyanate (FITC)-Dextran, and paraformaldehyde were purchased from Sigma-Aldrich, USA. Phosphate buffered saline (PBS), glycine, and Triton X-100 were purchased from Amresco, USA. Protease and phosphatase inhibitors cocktails were purchased from Nacalai Tesque, Japan. Prolong antifade reagent was purchased from Thermo Fisher Scientific, USA. Western Bright Sirius enhanced

chemiluminescence (ECL) was purchased from Advanta, USA. Polyvinylidene difluoride (PVDF) membrane was purchased from Merck Millipore, USA. Zerumbone $\geq 98\%$ (HPLC) was purchased from Sigma-Aldrich, USA. The stock solution of zerumbone (200 mM) was prepared by dissolving the powder in dimethyl sulfoxide (DMSO). To ensure the compound integrity and activity, the stock solution was stored in aliquots at -20°C to avoid repeated freeze/thaw cycles. Lyophilized HDM extract (dry weight: 12.6 mg/vial; Der p 1: 114.57 μg /vial; endotoxin: 805 EU/vial) was purchased from Greer Laboratories, USA and was stored at 4°C . The HDM extract was resuspended in ultrapure water at a concentration of 20 mg/mL and was stored in aliquots at -20°C to avoid repeated freeze/thaw cycles. DTT (1,4-Dithiothreitol), the reducing agent purchased from Sigma, USA was dissolved in ultrapure water to prepare the stock solution (10 mM).

Methods

Cell culture, cell treatment and HDM induction

16HBE14o- cells were incubated in a 5% CO_2 humidified incubator at 37°C . Passage 68–80 of 16HBE14o- cells were used throughout this study. All vessels used for culturing cells were pre-coated with fibronectin coating solution (refer to *Materials* section for the composition of the coating solution) for 2 h at 37°C .

To achieve the desired concentrations of zerumbone (Figure 1) used for cell treatment, the stock solution (200 mM) was diluted with serum-free EMEM. The final concentration of DMSO in the culture medium was $\leq 0.1\%$. On the other hand, the HDM stock solution (20 mg/mL) was first incubated with 10 mM of DTT at 37°C for 30 min to activate the enzymatic activity of the Der p 1 allergen [30]. The DTT-activated HDM extract was then diluted with serum-free EMEM to obtain the concentration of 200 μg /mL (which contained 100 μM DTT) for subsequent cell treatment. Upon reaching desired cell confluency, the 16HBE14o- cells were simultaneously induced with DTT-activated HDM extract and treated with different concentrations of zerumbone for 24 h. The final concentration of the DTT-activated HDM extract used in the treatment was 100 μg /mL HDM (which contained 50 μM DTT). For cells cultured in ALI condition, HDM and zerumbone were only added into the apical compartment of the cell culture inserts [31,32].

Cytotoxicity assay

16HBE14o- cells (2×10^4 cells/well) were seeded in 96-well plate overnight at 37°C . The cells were then exposed to 100 μg /mL DTT-activated HDM extract and a range of different concentrations of zerumbone (6.25 μM –200 μM) for 24 h using serum free medium. The supernatant was then removed and the cells were incubated with 100 μL of fresh serum-free EMEM containing 10 μL of 5 mg/mL MTT solution for 4 h. After 4 h incubation, the supernatants were carefully removed and 100 μL of DMSO was added to each well. The absorbance reading at 570 nm wavelength for each well was

measured by using a microplate reader (Molecular Devices Versa Max, Sunnyvale, CA).

Transepithelial electrical resistance (TEER) assay

For TEER assay, the 16HBE14o- cells were cultured in ALI condition. Briefly, the cells (1×10^5 cells/well) were seeded onto 0.4 μm pore size fibronectin-coated inserts in 24-well plate. They were then left to attach overnight and the medium was removed from the apical compartment of the cell monolayer the next day. On the other hand, the basal compartment of the cell monolayer was fed with 600 μL of fresh complete EMEM and the medium was replaced once every two days. When the cells reached 100% confluency, they were exposed to 100 μg /mL of DTT-activated HDM extract simultaneously with either zerumbone (6.25, 12.5, and 25 μM) or dexamethasone (Dexa) (10 μM). The TEER readings were measured at 0 h and 24 h after treatment. Coated inserts without cell monolayer were used as the blank and the resistance readings obtained from each well were corrected by subtracting with the resistance reading from the blank. The resistance reading of the monolayer was calculated as previously described [33] with modifications shown below:

$$\text{TEERChange}(\%) = \frac{(a)-(b)}{a} \times 100\%$$

Where, a = average 0 h reading

b = average 24 h reading

FITC-Dextran permeability assay

16HBE14o- cells were cultured in ALI condition as described earlier for the TEER assay. Briefly, 1×10^5 cells/well were seeded onto 1.0 μm pore size fibronectin-coated inserts, which allowed the paracellular flux of 4 kDa FITC-Dextran, and placed in a 24-well plate. The cells were then exposed to 100 μg /mL of DTT-activated HDM extract simultaneously with either zerumbone (6.25, 12.5, and 25 μM) or Dexa (10 μM). For Normal group controls, the cells were treated with complete EMEM alone while for the vehicle group, the cells were treated with complete EMEM with 0.1% DMSO. FITC-Dextran solution (final concentration of 1 μg /mL) was added to the apical compartment of the cell monolayer and incubated in CO_2 incubator for 1 h. The solution from the basal compartment was then collected into a 96-well black microplate and readings were measured using a fluorescence microplate reader (Tecan Infinite 200, Switzerland) with excitation and emission wavelength of 490 nm and 520 nm, respectively. The ratio of flux was calculated as previously described with modifications. Briefly, the permeability values of each treatment group (P_A) were normalized to the Normal group (P_N) (Ratio of flux = P_A/P_N) [34].

Immunofluorescence assay

16HBE14o- cells (5×10^5 cells/well) were seeded in fibronectin-coated 8-well chamber slide until the cells reached 90% confluency. The cells were exposed to 100 μg /mL DTT-activated HDM extract and zerumbone (6.25, 12.5, and 25 μM) for 24 h. The cells were then washed with cold PBS and fixed

with freshly prepared 4% paraformaldehyde for 20 min. The cells were then washed with 100 mM glycine in PBS thrice. Triton X-100 in PBS (0.2%) was used to permeabilize the cells for 15 min and the cells were washed thrice with 0.1% Triton X-100 in PBS before incubation with the blocking solution (1% BSA in 0.1% Triton X-100 in PBS) for 30 min. Primary antibody prepared in blocking solution was used to stain the cells at 1:100 dilution overnight at 4 °C. After that, the cells were rinsed with 0.1% Triton X-100 in PBS, followed by staining with Alexa Fluor 488-conjugated secondary antibody prepared in blocking solution at 1:200 ratio for 2 h in the dark at room temperature. The cells were then washed with PBS thrice and mounted with Prolong Antifade Reagent. The fluorescent images were viewed using a fluorescent microscope (Leica, Germany). Gap area was then quantified using ImageJ software. In brief, the fluorescent images were converted to black and white images followed by quantitation of the tight junctional proteins gap area in the images by measuring their respective pixilation. The result was expressed as the percentage of gap area compared to HDM-induced control group.

Immunoblotting assay

To determine the effect of zerumbone on the junctional protein expression, 16HBE14o- cells (1×10^6 cells/well) were seeded in a 6-well plate until the cells reached 100% confluency. The cells were then co-treated with 100 µg/mL DTT-activated HDM extract and zerumbone for 24 h. At the end of the incubation period, treated or untreated 16HBE14o- cells were washed with cold PBS twice and lysed with RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% protease and phosphatase inhibitors cocktail). The soluble cell lysate fraction was obtained by centrifugation and the protein quantification was later carried out using bicinchoninic acid (BCA) assay (Merck Millipore, USA). The protein samples were separated using 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to the PVDF membrane using a wet transfer device (Biorad, USA). The membrane was blocked using 5% BSA in tris-buffered saline with Tween-20 (TBST) for 1 h and subsequently incubated with primary antibody specific for occludin or ZO-1 in blocking buffer solution (1:1000) at room temperature overnight. The membrane was rinsed with TBST thrice before incubation with goat anti-rabbit IgG secondary antibody in 5% BSA (1:5000) for 1 h at room temperature. The membrane was then washed with TBST three times and incubated with chemiluminescent substrate solution followed by visualization using the Fusion FX gel documentation system (Vilber Lourmat, Eberhardzell, Germany). β -actin was used as the loading control in this study.

Statistical analysis

Data from three individual experiments was presented in means $\pm$ standard error of mean (SEM) by using GraphPad Prism 5. One-Way Analysis of Variance (ANOVA) followed by

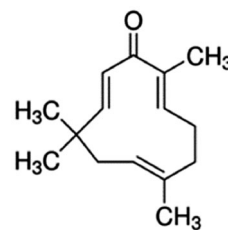


Figure 1. The structural formula of zerumbone. Adapted from Sulaiman et al. [28].

post hoc comparison using Dunnett test was used to determine statistical significance ($p \leq .05$).

Results

Zerumbone is non-cytotoxic to 16HBE14o- cells at 25 µM and lower concentrations

16HBE14o- cells were treated with zerumbone at a concentration ranging between 6.25 µM to 200 µM for 24 h to determine the highest non-cytotoxic concentration of zerumbone on 16HBE14o- cells using MTT assay. As shown in Figure 2, zerumbone exerted cytotoxic effect on HDM extract-exposed 16HBE14o- cells only at the concentration of 50 µM and above. The results also showed that the amount of HDM used in this study did not have cytotoxic effect on the cells in comparison to the Normal (N) group. In order to determine which concentration of zerumbone poses cytotoxic effect on HDM-induced 16HBE14o- cells, the treatment groups were all compared to the HDM only group. Based on the data, zerumbone at the concentrations of 50, 100, and 200 µM caused 39%, 75%, and 88% reduction in cell viability, respectively, compared to the HDM only group. On the other hand, zerumbone at 25 µM and lower concentrations caused less than 6% reduction in cell viability compared to the HDM only group. Therefore, zerumbone at 25 µM and lower concentrations were selected for subsequent assays in this study.

Zerumbone inhibits the disruption of 16HBE14o- junctional integrity induced by HDM extract

TEER assay was carried out to investigate the effect of zerumbone on the junctional integrity of the HDM extract-induced epithelial monolayer. The cells were exposed to 100 µg/mL DTT-activated HDM extract for 24 h to induce epithelial disruption while co-treated with zerumbone or Dexa at the same time. Based on Figure 3, the N group showed 5.2% TEER change between the readings at 0 and 24 h, which was the lowest TEER change among the other groups. On the other hand, the increase of TEER change of the HDM extract-exposed group was 7.2-fold in comparison to the N group. When compared to the HDM extract-exposed group, Dexa which was the positive control in this experiment showed a significant decrease in TEER change by 81.76%, whereas 6.25, 12.5, and 25 µM of zerumbone had shown a decrease in TEER change by 27.1%, 48.6%, and 49.6%, respectively. Our results suggest that zerumbone was able to

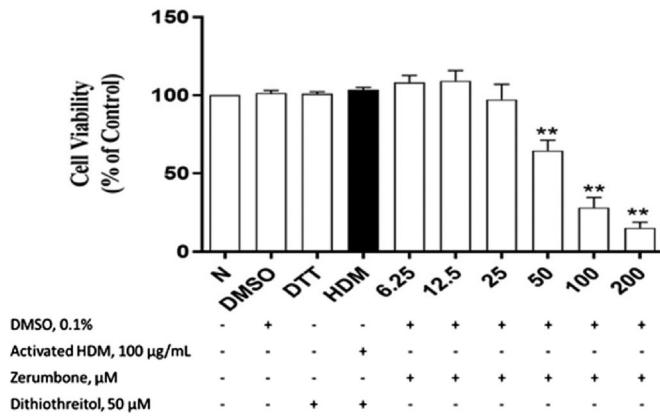


Figure 2. The cytotoxicity of zerumbone on 16HBE140- cells. 16HBE140- cells were seeded in a 96-well plate for 24 h, followed by treatment with zerumbone at indicated concentrations. The HDM group represents the HDM extract-exposed control group. The N group represents cells under normal condition which were only fed with complete EMEM. The DMSO vehicle group represents cells which were only fed with 0.1% of DMSO in EMEM. The DTT group represents cells which were only fed with 50 µM of DTT solution in EMEM. Results were expressed as mean $\pm$ SEM of three independent experiments performed in triplicates. $**p \leq .01$ indicates significant difference from the HDM group.

maintain the junctional integrity in HDM extract-exposed 16HBE140- monolayer.

Zerumbone down-regulates permeability of 16HBE140- monolayer induced by HDM extract

In order to determine whether zerumbone can modulate epithelial permeability, FITC-Dextran permeability assay was carried out using HDM extract-exposed 16HBE140- monolayer. As shown in Figure 4, the increase in the permeability of HDM extract-exposed group was 1.65-fold in comparison to the N group. Three concentrations of zerumbone used in the study, 6.25, 12.5, and 25 µM showed a decrease in permeability by 41.9%, 48.4%, and 52.8%, respectively, while the Dexa group showed a decrease in permeability by 82.5% in comparison to the HDM extract-exposed group. A higher fold change in permeability indicates an increased permeability of the monolayer thus allowing more solutes to pass through the epithelial monolayer. The highest fold change in permeability for the HDM extract-exposed group reflects that HDM extract increased the permeability of the epithelial monolayer. Zerumbone treatment groups, on the other hand, exhibited lower fold changes in permeability in comparison to the HDM extract-exposed group. This indicates that zerumbone possesses the ability to reduce HDM extract-induced increase in epithelial permeability.

Zerumbone inhibits the redistribution/delocalization of tight junctional proteins in HDM extract-exposed 16HBE140- cells

We had demonstrated that zerumbone exhibits protective effect toward the disruption of junctional integrity and permeability of epithelial monolayer when exposed to HDM extract. It remains questionable whether the protective effect of zerumbone is attributed to its ability to inhibit the

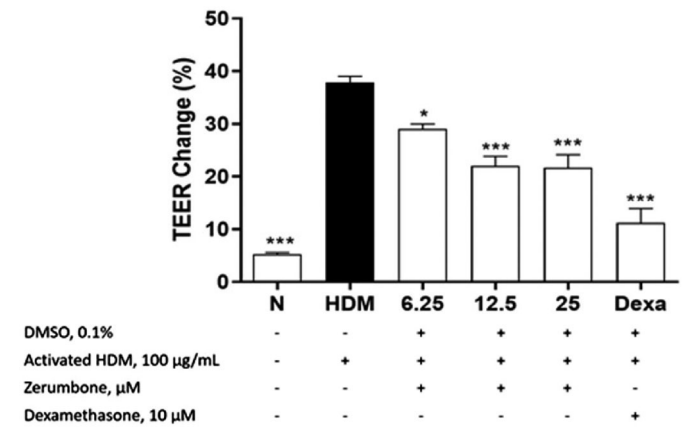


Figure 3. Zerumbone preserves the junctional integrity of 16HBE140- monolayer exposed with HDM extract. 16HBE140- cells were seeded on pre-coated inserts in a 24 well plate. The cells were then treated simultaneously with zerumbone and HDM extract for 24 h. The TEER reading was recorded before and after the 24 h incubation. The graph represents the TEER change of different groups of treatment on 16HBE140- monolayer. N: Normal; HDM: HDM extract-exposed group; 6.25, 12.5, and 25: Co-treated with HDM extract and zerumbone, thus, each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM extract and 10 µM dexamethasone. Results were expressed as mean $\pm$ SEM of three independent experiments performed in triplicates. $*p \leq .05$ and $***p \leq .001$ indicate significant difference from the HDM group.

redistribution/delocalization of tight junctional proteins, or its ability to prevent the cleavage of occludin and ZO-1. In order to elucidate the mechanism of action of zerumbone, we examined the effect of zerumbone on the localization and distribution of the tight junctional proteins, occludin and ZO-1, by immunofluorescence staining. Under normal condition, occludin and ZO-1 were expressed at the periphery of the cells in a continuous manner and in 'honey-comb'-like shape (Figures 5(A) and 6(A)). On the other hand, when the cells were exposed to HDM extract, both junctional proteins underwent redistribution or delocalization where occludin and ZO-1 expressions were found scattered in a discontinuous manner (Figures 5(B) and 6(B)). All three concentrations of zerumbone (Figures 5(C-E) and 6(C-E)) as well as Dexa (Figures 5(F) and 6(F)) inhibited the redistribution or delocalization of both junctional proteins by preserving the expression of occludin and ZO-1 at the periphery of the cells in a continuous manner. The qualitative immunofluorescence data was also analyzed quantitatively by comparing the gap areas found in different experimental groups. Figures 5(G) and 6(G) show that treatment with 12.5 µM zerumbone was able to suppress the formation of gap areas of occludin and ZO-1 by 25.1% and 41.6%, respectively, in comparison to the HDM extract-exposed group. Notably, the suppressive effect of 25 µM zerumbone on the formation of gap areas was as high as 55.3% for occludin and 67.7% for ZO-1, which was almost comparable to Dexa (64.9% for occludin and 78.4% for ZO-1).

Zerumbone does not prevent the degradation of tight junctional proteins in HDM extract-exposed 16HBE140- cells

Since zerumbone inhibited the redistribution or delocalization of occludin and ZO-1 in HDM extract-exposed epithelial monolayer (Figures 5 and 6), the effect of zerumbone on the

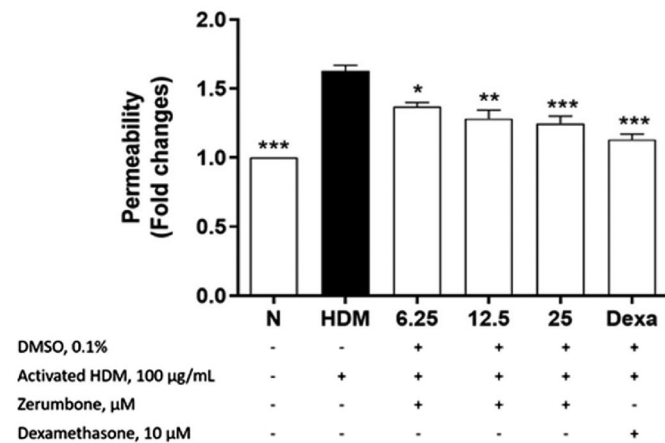


Figure 4. Zerumbone reduces the increase in permeability of 16HBE14o- monolayer exposed with HDM extract. 16HBE14o- cells were seeded on pre-coated inserts in a 24 well plate. The cells were treated simultaneously with zerumbone and HDM extract for 24 h. One mg/mL FITC-Dextran was applied at the apical compartment of inserts. After 1 h incubation at 37 °C, the solution in the basal compartment was collected and measured using fluorescence microplate reader. The graph represents the ratio of flux of solutes through 16HBE14o- monolayer normalized to the N group. N: Normal; HDM: HDM extract-exposed group; 6.25, 12.5, and 25: Co-treated with HDM extract and zerumbone, thus, each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM extract and 10 µM dexamethasone. Results were expressed as mean $\pm$ SEM of three independent experiments performed in triplicates. * $p \leq .05$, ** $p \leq .01$ and *** $p \leq .001$ indicate significant difference from the HDM group.

protein degradation of occludin and ZO-1 was examined by Western Blotting. As shown in Figure 7(A,B), both occludin and ZO-1 were highly expressed under normal condition. However, occludin was cleaved into 45 kDa and 25 kDa products following exposure to 100 µg/mL HDM extract for 24 h. Similarly, ZO-1 was also cleaved into 192 kDa and 164 kDa products upon exposure to HDM extract. The cleavage was further confirmed through inclusion of a trypsin control group to demonstrate enzymatic cleavage. Consistent with its protective effect on HDM extract-induced epithelial barrier dysfunction as reported earlier, the positive control Dexa preserved high levels of intact occludin and ZO-1 in HDM extract-exposed 16HBE14o- cells, similar to that of the N group. Conversely, all three concentrations of zerumbone failed to inhibit HDM extract-induced degradation of occludin or ZO-1. These results indicate that the protective effect of zerumbone on the HDM extract-induced epithelial barrier disruption is not attributed to the preservation of intact tight junctional proteins expression particularly occludin and ZO-1.

Discussion

Airway epithelium forms a protective barrier against environmental stimuli such as aeroallergens, airborne particles, and respiratory viral infections. Disruption of the airway epithelial barrier may lead to increased risk of allergen sensitization and consequently chronic inflammation in allergic asthma [35]. Thus, preservation of the airway barrier function has been suggested to be a novel therapeutic approach for asthma [36]. As zerumbone has been shown to alleviate asthmatic symptoms in an ovalbumin-induced mouse model of allergic asthma by modulating Th1/Th2 cytokines [29], we

further investigated its potential effect on airway barrier dysfunction using an *in vitro* model of human airway epithelium induced by HDM extract. Commonly used airway epithelial cell lines include A549, BEAS-2B, Calu-3, and 16HBE14o-. A previous study suggested that A549 and BEAS-2B cell lines are not suitable for airway epithelial barrier studies as these cells do not form functional TJs. In contrast, 16HBE14o- (immortalized human bronchial epithelial cell line) and Calu-3 (human lung adenocarcinoma epithelial cell line) express AJs, TJs, and desmosomes, enabling them to develop functional barrier properties required for the study of barrier function of airway epithelium [37,38].

HDM allergen is a ubiquitous aeroallergen that has been shown to disrupt the epithelial intercellular junctions *in vitro* which may consequently lead to airway barrier dysfunction. An *ex vivo* study by Steelant et al. [39] has demonstrated that the nasal biopsies from patients with HDM-induced allergic rhinitis showed lower trans-tissue resistance with a higher transmucosal passage of FITC-Dextran compared to healthy subjects, suggesting an impaired barrier function in patients with HDM-induced allergic rhinitis. Compromised epithelial barrier could facilitate the access of allergens to submucosal antigen presenting cells (APCs), resulting in the activation of innate immunity and a cascade of inflammatory responses including airway inflammation [40]. Therefore, in this study, we aimed to investigate whether zerumbone has the ability to prevent HDM-induced airway barrier dysfunction. We have demonstrated here that zerumbone inhibited the increase in epithelial permeability and maintained the transepithelial electrical resistance of ALI cultures of 16HBE14o- cells induced by HDM extract. These findings suggest that zerumbone may have potential therapeutic effect on allergic asthma by attenuating airway epithelial barrier dysfunction and subsequently prevent the transepithelial delivery of allergens.

As the integrity of the airway epithelium is mainly regulated by the intercellular junctions, particularly TJs, we investigated the effect of zerumbone on the tight junctional proteins, specifically occludin and ZO-1. Occludin is a trans-membrane protein of the TJ complex that is anchored to the actin cytoskeleton by the cytoplasmic protein ZO-1 [41]. Previous study by Wan et al. [20] found that the major HDM allergen Der p 1 induced cleavage of both occludin and ZO-1. It was hypothesized that the cysteine protease Der p 1 directly cleaves the extracellular region of occludin whereas ZO-1 is degraded by an intracellular proteolysis process which likely results from the dissociation of the TJ complex [20]. Cleavage of occludin and ZO-1 has also been reported for serine proteases of *D. pteronysinnus*, which are Der p 3, Der p 6, and Der p 9 [42]. Additionally, HDM extract was also shown to induce the cleavage of occludin and ZO-1 which might be due to the presence of heat-insensitive and yet to be identified proteases in the HDM extract [43]. Consistent with these previous reports, we observed that HDM extract induced the cleavage of occludin and ZO-1 in 16HBE14o- cells. However, this process was not influenced by the treatment with zerumbone. Apart from degradation of the intercellular junctions, numerous studies have reported

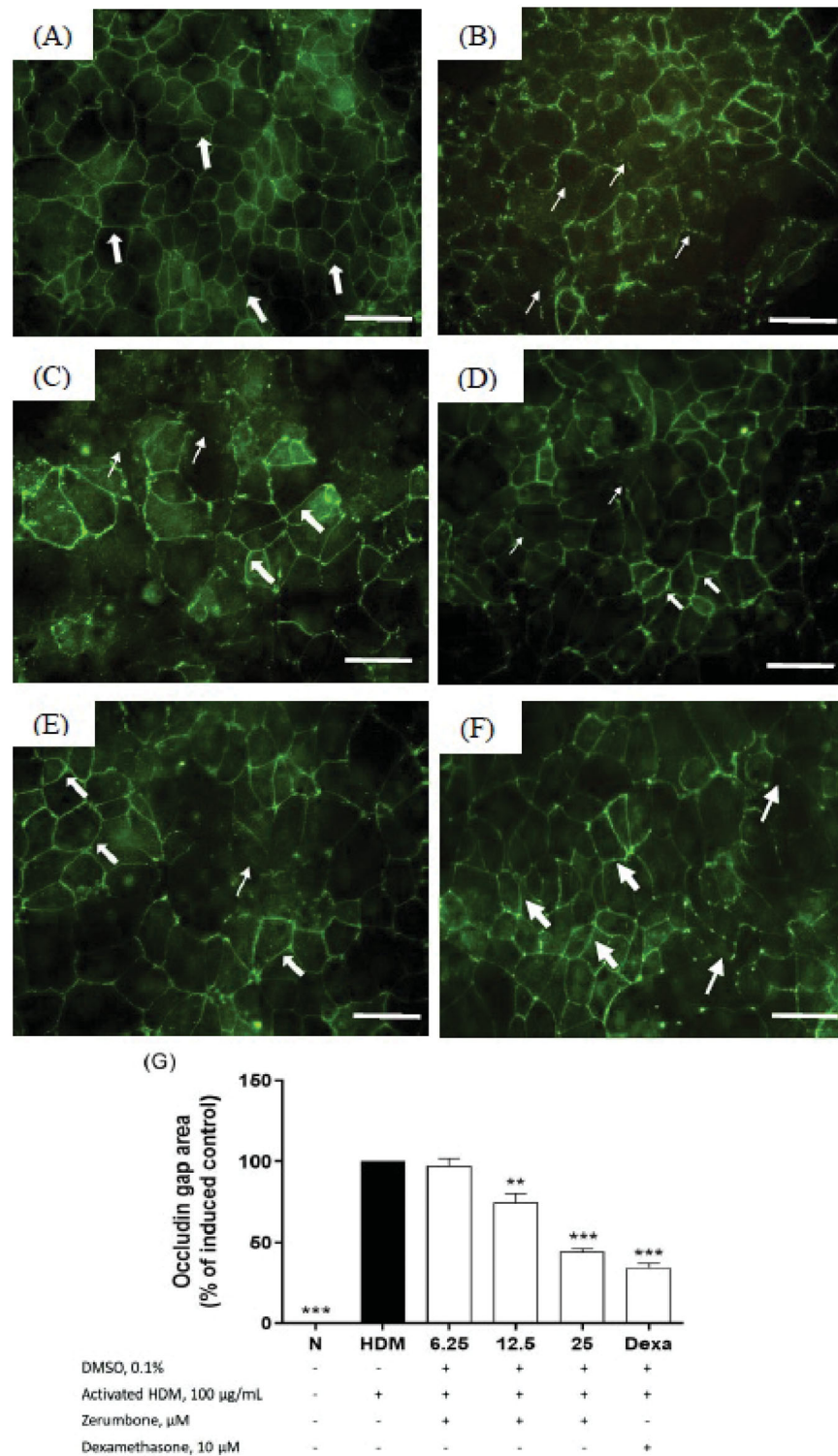


Figure 5. Zerumbone inhibits the delocalization of occludin in HDM extract-exposed 16HBE14o- cells. 16HBE14o- cells were seeded on chamber slides. The cells were treated with zerumbone and HDM extract for 24 h after the cells reached 90% confluency. The cells were incubated with occludin antibody and subsequently with Alexa Fluor 488-conjugated IgG. Each figure represents each different treatment groups. (a) Normal group; (b) HDM extract-exposed group; (c) 6.25 μM zerumbone group; (d) 12.5 μM zerumbone group; (e) 25 μM zerumbone group; (f) Dexa group. (g) Quantitative analysis of the disrupted area (gap) of the junctional protein which was quantified using ImageJ software. Thick arrows represent the 'honey-comb'-like structure of the junctional protein, while the thin arrows represent the gaps of the junctional proteins. $**p \leq .01$ and $***p \leq .001$ indicate significant difference from the HDM group. Magnification: 40 $\times$. Scale bar = 50 μm.

the ability of HDM to disrupt the distribution of TJs as well as AJs. As such, we next examined the distribution of occludin and ZO-1 in response to HDM extract exposure in the presence or absence of zerumbone. This study demonstrated that HDM extract-exposed 16HBE14o- cells displayed a

discontinuous distribution of occludin and ZO-1 at adjacent cell borders. This is in line with other studies which demonstrated delocalization of occludin, ZO-1, and E-cadherin in cells exposed to HDM extract in serum-free culture medium [43,44]. Wan et al. [42] have also described the redistribution

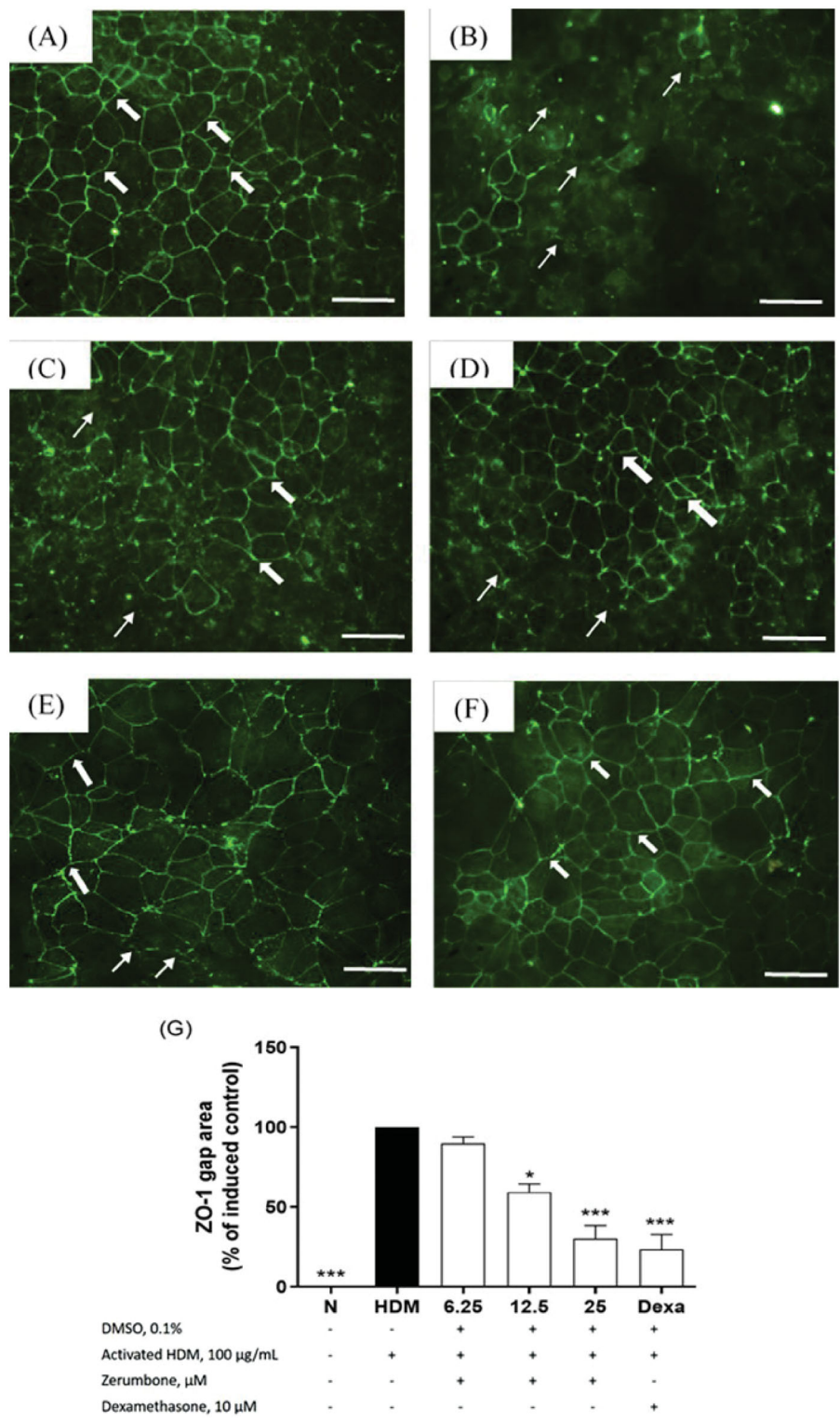


Figure 6. Zerumbone inhibits the delocalization of ZO-1 in HDM extract-exposed 16HBE14o- cells. 16HBE14o- cells were seeded on chamber slides. The cells were treated with zerumbone and HDM extract for 24 h after the cells reached 90% confluency. The cells were incubated with ZO-1 antibody and subsequently with Alexa Fluor 488-conjugated IgG. Each figure represents each different treatment groups. (a) Normal group; (b) HDM extract-exposed group; (c) 6.25 μ M zerumbone group; (d) 12.5 μ M zerumbone group; (e) 25 μ M zerumbone group; (f) Dexa group. (g) Quantitative analysis of the disrupted area (gap) of the junctional protein which was quantified using ImageJ software. Thick arrows represent the 'honey-comb'-like structure of the junctional protein, while the thin arrows represent the gaps of the junctional proteins. ** $p \leq .01$ and *** $p \leq .001$ indicate significant difference from the HDM group. Magnification: 40 $\times$. Scale bar = 50 μ m.

of TJ proteins (occludin and ZO-1) from the cell-cell junctions into a cytoplasmic compartment in Calu-3 cells induced by HDM fecal pellet serine peptidases. Besides that, the aberrant distribution of junctional proteins, particularly AJs, was also frequently observed in *in vivo* models of asthma.

Delocalization of E-cadherin and β -catenin has been shown in mice receiving intranasal exposure of HDM extract dissolved in deionized water or PBS compared to mice receiving deionized water or PBS alone [34,45–49]. The inhibitory effects of zerumbone on TEER change and FITC-Dextran

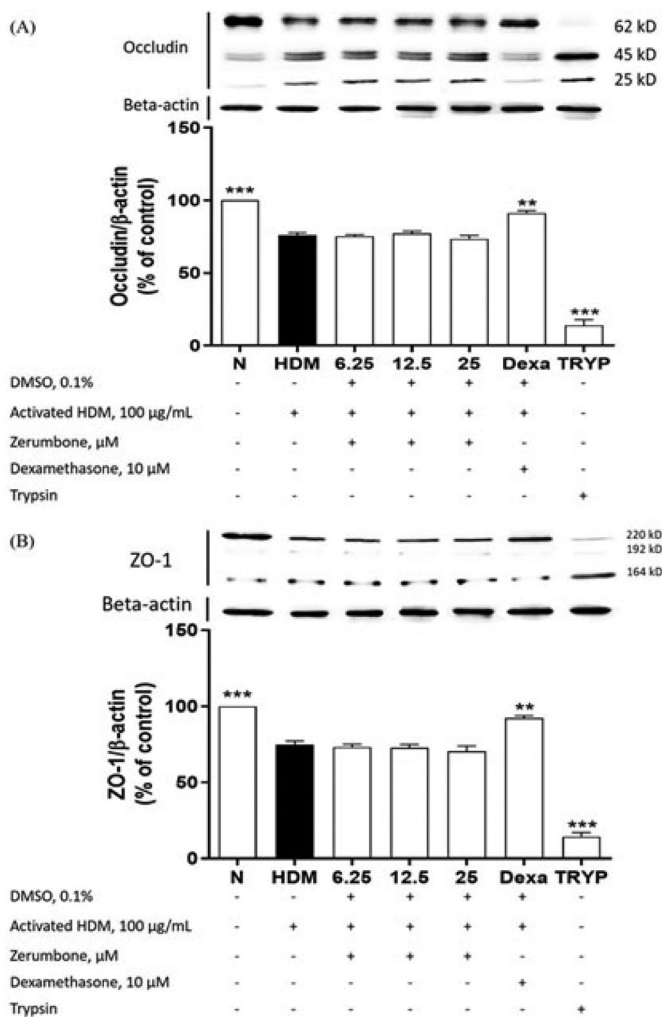


Figure 7. Zerumbone does not prevent the degradation of (A) occludin and (B) ZO-1 in HDM extract-exposed 16HBE14o- cells. 16HBE14o- cells were seeded on a 6-well plate until 100% confluent. The cells were treated with zerumbone and HDM extract for 24 h. The cell lysate was collected and SDS-PAGE was conducted. The treatment groups were labeled as follows: N: Normal group; HDM: HDM extract-exposed group; 6.25, 12.5, and 25: Co-treated with HDM extract and three different concentrations of zerumbone (µM). Each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM extract and 10 µM dexamethasone; Trypsin: Trypsin-treated group. Results were expressed as mean $\pm$ SEM of three independent experiments performed in triplicates. $**p \leq .01$, and $***p \leq .001$ indicate significant difference from the HDM group.

paracellular flux in HDM extract-induced 16HBE14o- cells were similar but zerumbone did not seem to act in a concentration-dependent manner in these two assays. This was probably due to the role of various intercellular junctional proteins in maintaining the epithelial integrity and permeability. Nevertheless, our data demonstrates that co-treatment with zerumbone was able to maintain the 'honeycomb' structure of both occludin and ZO-1 at the periphery of 16HBE14o- cells in a concentration-dependent manner, suggesting that the inhibitory effect of zerumbone against HDM-induced loss of junctional integrity and increased permeability might be primarily due to the preserved TJ localization in the 16HBE14o- cell monolayer.

Although a previous study by Jyothilakshmi et al. [50] showed that zerumbone has trypsin-inhibiting activity, it is

very unlikely that zerumbone is capable of inhibiting the enzymatic activity of the proteases within the HDM extract as we demonstrated here that zerumbone did not prevent the degradation of occludin and ZO-1 in HDM extract-induced 16HBE14o- cells. However, our finding that zerumbone was capable of inhibiting the delocalization of occludin and ZO-1 induced by HDM extract suggests that zerumbone might inhibit a particular signaling pathway that regulates the distribution of occludin and ZO-1. It has been previously demonstrated that epidermal growth factor receptor (EGFR) signaling regulates the distribution of ZO-1 in HDM-induced 16HBE14o- cells [43]. Heijink et al. [43] reported that the inhibition of EGFR signaling pathway by the inhibitor AG1478 did not prevent the cleavage of ZO-1 but resulted in increased membrane localization of ZO-1. Most importantly, the EGFR inhibitor AG1478 also suppressed HDM-induced fall in epithelial resistance of 16HBE14o- cell monolayer [43]. Thus, targeting a particular signaling pathway like EGFR offers an alternative approach in preventing HDM-induced airway barrier dysfunction and it may be a possible mechanism that mediated the protective effect of zerumbone demonstrated in this study. Besides that, several studies have shown that the modulation of other signaling pathways could also attenuate HDM-induced airway epithelial barrier dysfunction. Zhang et al. [46] demonstrated that blocking the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway in HDM extract-exposed airway epithelial cells (16HBE14o- and BEAS-2B) using the PI3K inhibitor LY294002 increased TEER, decreased FITC-Dextran permeability, as well as reduced the expression of VEGF. Besides that, the inhibition of PI3K/Akt pathway also alleviated VEGF-induced epithelial barrier dysfunction [46]. A similar increase in TEER and a decrease in FITC-Dextran permeability in HDM-induced 16HBE14o- cells due to the inhibition of PI3K/Akt pathway have been reported by Ye et al. [48]. Moreover, the inhibition of PI3K/Akt pathway has also been shown to attenuate E-cadherin delocalization in HDM-induced 16HBE14o- cells [47]. Other than EGFR and PI3K/Akt pathways, HDM has been shown to stimulate Ca^{2+} influx in bronchial epithelial cells via adenosine triphosphate (ATP)/purinergic receptor signaling which could result in epithelial barrier dysfunction due to the accumulation of intracellular Ca^{2+} [Ca^{2+}]_i [51]. The effects of zerumbone on these signaling pathways need further investigation to elucidate the protective mechanism of zerumbone against HDM extract-induced epithelial barrier dysfunction. Stabilizing the epithelial barrier function by targeting a particular signaling pathway may offer an alternative therapeutic strategy in attenuating allergic responses triggered by HDM allergens, aside from inhibiting the proteolytic activity of group 1 HDM allergens using allergen delivery inhibitors (ADIs) [22].

Nevertheless, there are some limitations to this study. First, the exact composition of the HDM extract used in this study is not known, except for the levels of Der p 1 and endotoxin. We did not perform further experiment to determine the component(s) which were responsible for HDM extract-induced epithelial barrier dysfunction observed in our study, for example, using cysteine protease inhibitor (E-64) or

serine protease inhibitor (aprotinin, benzamidine, or phenylmethylsulfonyl fluoride (PMSF)). Thus, we could not rule out the possible involvement of proteases other than Der p 1 that might be present in our HDM extract. Although Der p 1 is the major allergen in HDM extract that primarily induces airway epithelial barrier dysfunction due to its proteolytic activity [20], this study selected a crude HDM extract as the inducer as allergic individuals are naturally exposed to the different HDM constituents from the environment in real life. Post et al. [44] reported that the Greer HDM extract caused the most pronounced epithelial barrier dysfunction *in vitro* as well as allergic sensitization and asthma phenotypes *in vivo* compared to the other two commercial sources. In this study, the Greer HDM extract was used but characterization of the constituents including their levels and activities was not performed as it is beyond the scope of this study. In addition, this study only focused on selected intercellular junctional proteins, namely occludin and ZO-1. Besides these two proteins, there are other structures that regulate the epithelial barrier properties and function including AJ proteins such as E-cadherin and catenins, TJ proteins such as claudins and junctional adhesion molecule (JAM)-A, and actin microfilaments, particularly F-actin cytoskeleton that connects to the apical junctional complex and regulates the distribution of AJ and TJ proteins [52]. It is also important to note that HDM disrupts the epithelial barrier function *via* two different mechanisms which are degradation and delocalization of junctional proteins [53], with the former had only been demonstrated in *in vitro* studies [20,42,43]. Although degradation

of junctional proteins has not been shown in *in vivo* studies, an *ex vivo* study by Steelant et al. [39] provided evidence that the nasal biopsies from patients with HDM-induced allergic rhinitis had an impaired barrier function, suggesting that HDM exposure may possibly disrupt airway epithelial barrier function in humans. While this study demonstrates that the barrier protective effects of zerumbone against HDM extract-induced epithelial barrier dysfunction could be partly explained by its ability to inhibit the delocalization of occludin and ZO-1, investigating the effect of zerumbone on other intercellular junctional complexes may increase our understanding on the effect of zerumbone on the airway barrier properties. Lastly, although we did not repeat the experiments on primary bronchial epithelial cells isolated from asthmatic patients in this study to provide additional evidence of the barrier protective effects of zerumbone against HDM extract challenge, they should be performed in the future study to further support the development of zerumbone as a potential treatment for airway epithelial barrier dysfunction.

This study has shown that zerumbone exerts protective effects against HDM extract-induced epithelial barrier dysfunction by inhibiting the delocalization of tight junctional proteins, occludin and ZO-1, instead of preventing their degradation. These findings highlight the potential therapeutic benefits of zerumbone in the treatment of allergic asthma by maintaining an optimal airway barrier function, which is crucial to prevent the insults by environmental stimuli and allergens. Further study on the effect of zerumbone on the

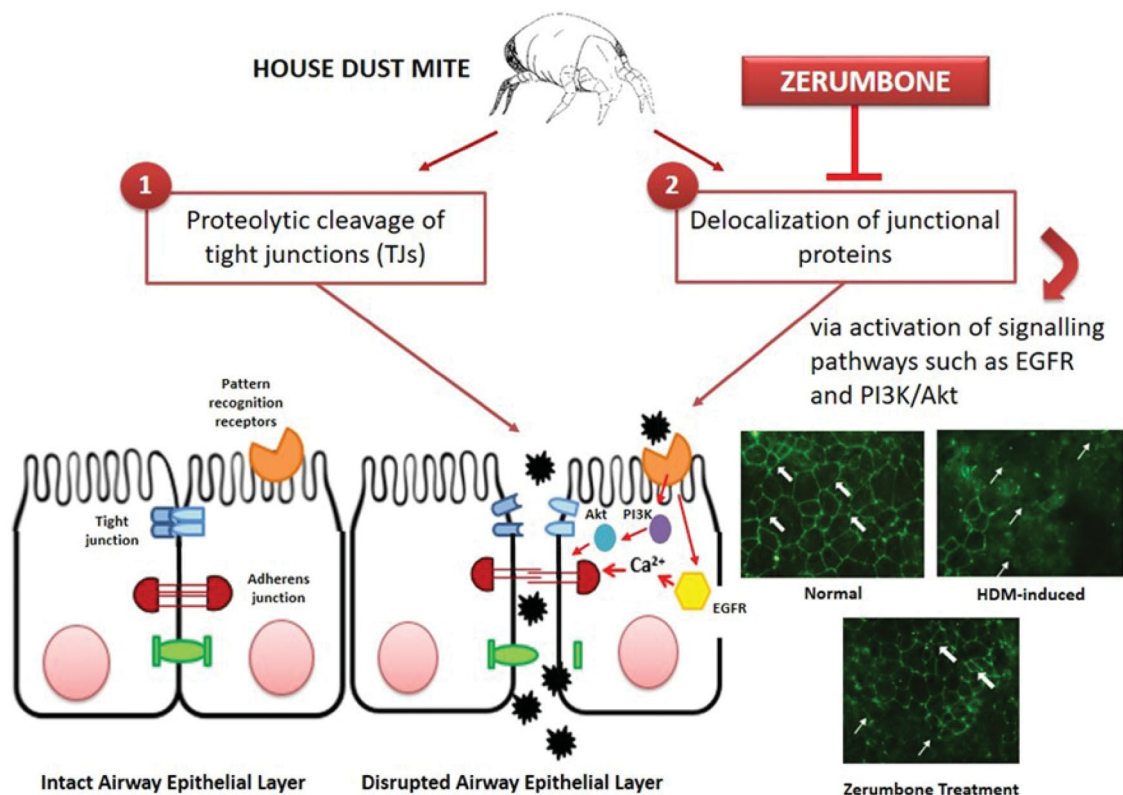


Figure 8. Diagram illustrating the mechanism of zerumbone in HDM extract-induced airway epithelial barrier dysfunction. HDM allergens can directly disrupt the epithelial barrier by proteolytically cleaving the tight junctional proteins such as occludin and claudin. HDM allergens can also activate various pattern recognition receptors (PRRs) which can induce the degradation and/or delocalization of junctional proteins *via* downstream signaling pathways such as EGFR and PI3K/Akt pathways. Zerumbone inhibits HDM extract-induced delocalization of occludin and ZO-1 but does not prevent their cleavage. Modified from Heijink et al. [53].

signaling pathway(s) regulating the epithelial barrier function, particularly the distribution of tight junctional proteins, such as EGFR and PI3K/Akt pathway should be carried out to uncover the underlying mechanism of its barrier protective effects (Figure 8). In addition, the barrier protective effect of zerumbone should also be further evaluated in *in vivo* model of airway epithelial barrier disruption using HDM-induced asthmatic mice. Based on previous studies that had tested zerumbone in both *in vitro* and *in vivo* models, the concentrations of zerumbone used in our study were within the range of effective concentrations (5–37 μ M) commonly reported in *in vitro* models [54,55]. In these studies, zerumbone had been shown to exhibit analgesic and anti-inflammatory effects at a dose as low as 1 mg/kg [54], and exert modulatory effects on receptors in chronic constriction injury (CCI)-induced neuropathic pain mice at a dose of 10 mg/kg [55]. Thus, these findings indicate that the concentrations of zerumbone we used are likely to be within a range that could be achieved *in vivo* and should be taken into considerations to design *in vivo* experiments in future studies. Nevertheless, it should also be noted that there is yet any clinical trial which evaluates the safety and efficacy of zerumbone [56] and thus more findings, especially *in vivo* studies, are required in the future to support the testing of zerumbone in the clinical phase for potential therapeutic purpose in humans.

Acknowledgements

The researchers thanked the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for the support given throughout this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Universiti Putra Malaysia under Grant Putra Young Initiative-Universiti Putra Malaysia (IPM-UPM) [GP-IPM/2015/9455600] and Malaysia Toray Science Foundation [Grant No: 6378700]. WR was funded by Universiti Putra Malaysia Graduate Research Fellowship (GRF). JWT was a recipient of MyPhD scholarship under MyBrain15 programme. KYL was funded by Universiti Putra Malaysia Graduate Research Assistantship (GRA).

ORCID

Chau Ling Tham  <http://orcid.org/0000-0002-9734-0372>

Data availability statement

The data used to support the findings in the study are available on reasonable request to the corresponding authors.

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