UNIVERSITI PUTRA MALAYSIA

IN VITRO PROTECTIVE EFFECT OF ZERUMBONE ON HOUSE DUST MITE-INDUCED AIRWAY EPITHELIAL BARRIER DISRUPTION

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By

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October 2018

Chairman : Tham Chau Ling, PhD
Faculty : Medicine and Health Science

Human airway system is lined by epithelial cells attached to each other by junctional system. This epithelial barrier can be disrupted by common allergen such as house dust mites (HDM) and consequently resulting in airway diseases such as asthma. Zerumbone was found to possess anti-asthmatic effect by modulating Th1/Th2 cytokines. However, there is yet study done to assess whether zerumbone protects the epithelial barrier from junctional disruption before the allergen invades into the immunological barrier of the airway system. This study aims to investigate the effect of zerumbone on HDM-induced airway epithelial barrier disruption. A transformed epithelial cell line derived from human bronchial epithelium (16HBE14o-), which possesses characteristics of *in vivo* human airway lining, was treated with a range of concentrations of zerumbone (6.25 µM-200 µM) for 24 hours to investigate the non-cytotoxic concentrations of the compound on 16HBE14o- cells. As a result of the cytotoxicity assay, the cells were then co-treated with 100 µg/mL HDM and three selected concentrations of zerumbone (6.25 µM, 12.5 µM and 25 µM) for 24 hours in subsequent experiments. Transepithelial electrical resistance (TEER) assay and FITC-Dextran permeability assay were carried out to study the effect of zerumbone on HDM-induced junctional integrity and permeability of the epithelial monolayer respectively. Zerumbone has shown protective effect on the junctional integrity as all three concentrations (6.25 µM, 12.5 µM and 25 µM) possess significantly lower TEER change and supressed the change by 27%, 48.8% and 49.7% respectively, compared to the HDM group. The permeability assay result complement the result of the TEER assay as 6.25 µM, 12.5 µM and 25 µM of zerumbone shows inhibition in the flux of FITC-Dextran through the epithelial monolayer by 37.8%, 58.6% and 62.2% respectively compared to the HDM group. The localization of junctional proteins, occludin and ZO-1, was studied by using immunofluorescence (IF) while the protein and gene expression were studied by immunoblotting and Real Time-Polymerase Chain Reaction (qPCR) respectively. However, no significant changes can be seen in both protein and gene expression of occludin and ZO-1 as compared to the HDM group. This

study has proven that zerumbone preserves both HDM-induced junctional integrity and permeability by maintaining the localization of occludin and ZO-1 without affecting both proteins and gene expression. As a conclusion, zerumbone possesses protective effect on HDM-induced airway epithelial barrier disruption by preserving the junctional permeability and localization without affecting the junctional protein and mRNA expression. This study has proven that zerumbone possess protective effect on the allergen-induced airway barrier disruption therefore enabling it to be further studied as a potential drug for epithelial barrier-related airway inflammatory diseases.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN PERLINDUNGAN *IN VITRO* ZERUMBONE TERHADAP GANGGUAN PENGHALANG EPITELIUM SALURAN PERNAFASAN OLEH HAMA RUMAH TERARUH

Oleh

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Saluran pernafasan manusia dilapisi oleh sel epitelium yang bercantum antara satu sama lain menerusi sistem persimpangan *(junctional system*). Penghalang epithelium ini terdedah kepada gangguan alergen biasa seperti hama rumah yang seterusnya boleh membawa kepada penyakit saluran pernafasan seperti asma. Zerumbone telah terbukti mempunyai kesan anti-asma melalui modulasi sitokin Th1/Th2. Namun begitu, masih belum ada kajian yang membuktikan sama ada zerumbone melindungi penghalang epitilium daripada gangguan persimpangan sebelum alergen menyerang halangan imunolgi sistem saluran pernafasan. Oleh itu, kajian ini bertujuan untuk mengkaji kesan zerumbone terhadap gangguan penghalang epitelium saluran pernafasan yang diakibatkan oleh hama rumah. Barisan sel epitelium diubah yang diperoleh daripada epitelium bronkial manusia (16HBE14o-) mempunyai sifat-sifat *in vivo* sel saluran pernafasan manusia telah dirawat dengan julat kepekatan zerumbone (6.25 µM-200 µM) selama 24 jam untuk menyiasat bukan sitotoksik terhadap kepekatan sel 16HBE14o- . Berdasarkan hasil ujian kesitotoksikan asai, sel kemudian dirawat dengan 100 µg/mL hama rumah dan tiga kepekatan zerumbone (6.25 µM, 12.5 µM dan 25 µM) dalam eksperimen seterusnya selama 24 jam. Sel 16HBE14o- didedahkan secara serentak dengan hama rumah (100 µg/mL) dan zerumbone selama 24 jam dalam eksperimen berikutnya. Pengujian rintangan transepitelium (TEER) dan kebolehtelapan asai FITC-Dextran masing-masing dijalankan untuk mengkaji kesan zerumbone terhadap integriti simpang dan kebolehtelapan ekalapisan sel-sel epitelium. Zerumbone telah menunjukkan kesan perlindungan terhadap integriti persimpangan kerana ketiga-tiga kepekatan (6.25 µM, 12.5 µM dan 25 µM) mempunyai perubahan TEER yang jauh lebih rendah dan menahan perubahan masing-masing sebanyak 27%, 48.8% dan 49.7% berbanding kumpulan hama rumah. Keputusan ujian kebolehtelapan asai melengkapkan keputusan ujian TEER kerana 6.25 µM, 12.5 µM dan 25 µM zerumbon menunjukkan perencatan dalam fluks FITC-Dextran melalui ekalapisan epitelium masing-masing sebanyak 37.8%, 58.6% dan 62.2% berbanding kumpulan hama rumah. Penyetempatan penyimpangan protein, occludin dan ZO-1 dikaji melalui

pengujian imunopendarfluor manakala protein dan ekpresi gen masing-masing dikaji melalui pengujian immunoblot dan Tindak Balas Berantai Polimerase Masa Nyata (qPCR). Walau bagaimanapun, tiada perubahan ketara dapat dilihat dalam kedua-dua protein dan ekspresi gen occludin dan ZO-1 berbanding kumpulan hama rumah. Kajian ini telah membuktikan bahawa zerumbone mengekalkan kedua-dua integriti persimpangan dan kebolehtelapan dengan mengekalkan penyetempatan occludin dan ZO-1 tanpa menjejaskan protein dan ekpresi gen. Kesimpulannya, zerumbone mempunyai kesan perlindungan terhadap gangguan penghalang epitelium yang disebabkan oleh hama rumah melalui pemuliharaan kebolehtelapan dan penyetempatan simpang tanpa menjejaskan protein dan ekpresi mRNA. Kajian ini telah membuktikan bahawa zerumbone mempunyai kesan perlindungan terhadap gangguan penghalang saluran pernafasan yang diakibatkan oleh hama rumah. Oleh itu, hasil dapatan ini membolehkan zerumbone untuk dikaji dengan lebih lanjut sebagai bahan yang berpotensi dibangunkan sebagai rawatan terhadap masalah keradangan saluran pernafasan yang berkaitan dengan penghalang epitelium.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 19 December 2019

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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Name of Member of Supervisory Committee: _____

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LIST OF ABBREVIATIONS

AJs	Adheren junctions
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CaCo-2	Human colon colorectal cell line
CCD	Charged-coupled device
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Egtazic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Relative centrifugal force
HBSS	Hepes buffered saline
HCL	Hydrochloric acid
HDM	House dust mite
HO-1	Heme oxygenase-1
HPLC	High Performance Liquid Chromatography
HRP-conjugated	Horseradish peroxidase-conjugated

HT29	Human colon cancer cell line
IFN-ɣ	Interferon-ɣ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
MCF-7	Breast cancer cell line
MHC II	Major histocompatibility complex II
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
OVA	Ovalbumin
PAR-2	Proteinases activated receptor-2
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris buffered saline with Tween
TEER	Transepithelial electrical resistance
Th1	Type 1 T helper cells
Th2	Type 2 T helper 2 cells
TJs	Tight junctions
ZO-1	Zona occluden-1

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Human airway system is exposed to air contaminants through the process of respiration. Contaminants or allergen from the inhaled air may interact with the immunological cells of the protective mechanism of the human body and thus initiating various immunological reactions such as inflammation. However, excessive inflammations may result to changes in the morphology of the human respiratory lining system and thus may lead to more chronic condition such as asthma. Human airway system has been equipped with great protective mechanism as it possesses three barriers of protective mechanisms namely the chemical, physical and immunological barriers. The physical barrier of the airway system refers to the epithelial lining made up of multiple pseudostratified columnar epithelium which are attached to each other by the junctional system which also contributes to the semi-permeable properties of the physical barrier of the airway system. The junctional system is made up of different types of junctional protein groups namely adherens junctions, gap junctions and most importantly tight junctions. Two most significant tight junction proteins are occludin and zona occluden (ZO)-1.

However, these junctional proteins of the protective barrier was found to be prone to disruption after being exposed to the most common source of allergen, house dust mite (HDM). HDM or *Dermatophagus pteronyssinus* was found to possess the ability to cleave junctional proteins and thus causing the passage of allergen to the cellular compartment of the airway system with the presence of its cysteine proteinase Der p 1. Thus, zerumbone come into context as the monocyclic sesquiterpene active compound extracted from the rhizome of *Zingiber zerumbet* has been proven to possess anti-inflammatory activity on asthmatic model. Zerumbone may be a potential solution to this problem as the failure of the epithelial barrier may results in worsening the inflammatory conditions and thus resulting in conditions such as asthma. Therefore, the study has been carried out to investigate the effect of the compound on HDM-induced epithelial barrier disruption.

1.2 Problem Statement

The physical barrier of the airway system is very crucial in the protective mechanism of the system as it is the first line of barrier which will interact with the inhaled allergens. HDM is the most common source of allergens found in households and the presence of it will results in allergic reactions such as itchiness and runny nose. Unfortunately, the effect of continuous exposure to the allergens will be worse than just having runny nose as it will lead to more chronic

condition such as asthma. Among the crucial components of HDM which may lead to such conditions is Der p 1, the cysteine proteinase which has been proven to be able to directly cleave the junctional proteins and thus lead to the passage of the allergen to the cellular compartment of the airway system. The interaction between the allergen with the immunological cells of the airway system such as the dendritic cells will then lead to inflammatory events which will consequently resulting to more chronic condition such as asthma. The presence of dexamethasone (DEXA) as the current intervention does help in reducing the effect caused by the allergens (Abaya, Jones, & Zorc, 2018). However, dexamethasone has also been proven to possess many side effects such as stomach upset, dizziness and weight gain. Therefore, a search for a potential compound which able to be developed as an asthma treatment may help in providing an alternative to DEXA.

Zerumbone has been proven to possess many therapeutic activities including anti-inflammatory activity. Research done by Shieh et al. has proven that zerumbone was found to possess anti-asthmatic activity as it modulates the Th1/Th2 cytokines. However, there is yet a study done to prove the ability of the compound to protect the junctional protein of the epithelial barrier from the disruption caused by HDM.

1.3 Objectives

1.3.1 General objective

To investigate the effect of zerumbone on allergen-induced airway epithelial barrier disruption.

1.3.2 Specific objectives

1) To establish the relationship between the effect of zerumbone and junctional integrity in HDM-induced airway epithelial disruption.

2) To determine the effect of zerumbone on tight junctions in HDM-induced airway epithelial disruption.

3) To identify the mechanisms used by zerumbone in the regulation of tight junctions in HDM-induced airway epithelial disruption.

1.4 Hypothesis

Zerumbone is able to preserve HDM-induced airway epithelial disruption in 16HBE14o- cells through the preservation of junctional integrity, permeability, localization, protein and gene expression.

CHAPTER 2

LITERATURE REVIEW

2.1 Human Airway System

Respiration is a crucial process to any living things. Human beings are equipped with a well-structured respiratory system to ensure that the whole body can be delivered with oxygen and for the carbon dioxide to be exhaled. Human respiratory system consists of three sections namely upper respiratory tract, lower respiratory tract and the lungs. The upper respiratory tract is made up of the nose, pharynx and larynx whereas the lower respiratory tract consists of the trachea and bronchi which will then branch into the lungs as shown in Figure 1.

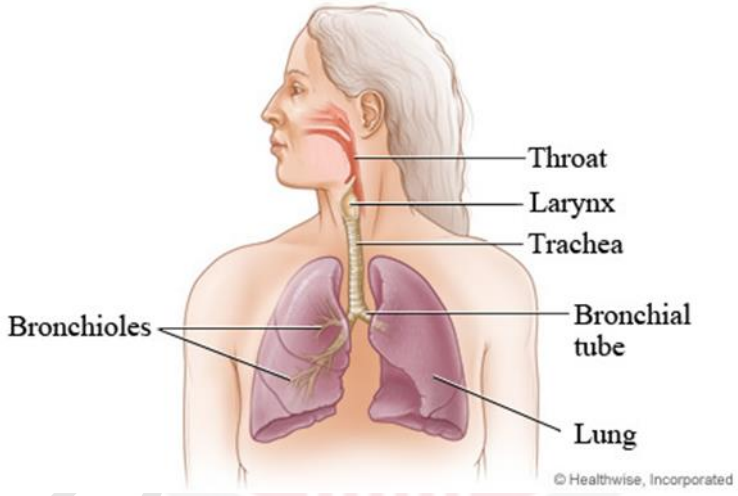

Figure 1: Human Respiratory System. The upper and lower respiratory tract of a human. (Adapted from Thompson & Poinier, 2017).

The respiratory system can also be classed into two zones namely the conducting zone and the respiratory zone (Ganesan, Cormstock, & Sajjan, 2013). The conducting zone or passages as can be seen in Figure 2 refers to nasal cavities, pharynx, larynx, trachea, bronchi and bronchioles where it helps keeping the inhaled air to be moist, warm and clean while the respiratory zone plays a role in gas exchanges and the oxygenation of blood.

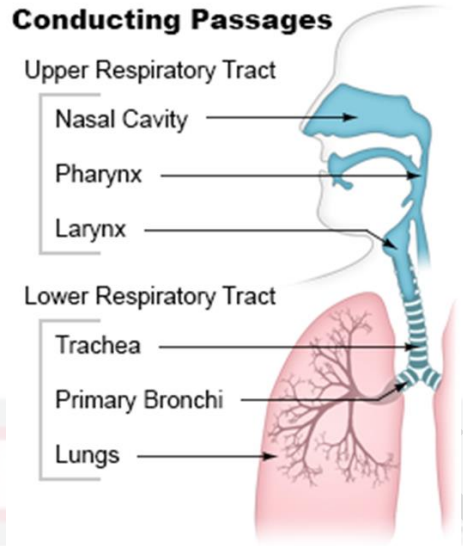

Figure 2: Conducting Passages of Human Respiratory System. Division of the conducting passages in the human respiratory system. (Adapted from National Cancer Institute).

This process has, thus, allowed lung to be the second organ after skin to be exposed to the external environment which may consist of allergens and pollutants. Therefore, it is crucial for the respiratory system to be equipped with a defence mechanism.

2.1.1 Defence Mechanism of Airway System

The human respiratory system is equipped with the defence mechanism which can be grouped into three barriers namely the chemical, physical and immunological barrier as described in Figure 3 (Swindle, Colllins, & Davies, 2009). The chemical barriers refers to the mucus which can be found at the lining of the respiratory system. Mucus combined action with cilia, a hair-like projection of the epithelial lining, helps to remove the foreign particles resulted from the inhaled air by sweeping them upwards allowing the foreign materials to be removed to the external environment through coughing. Mucus also helps to moisten the dry air inhaled through breathing process (Fahy & Dickey, 2010).

The immunological barrier refers to the immunological cells such as localized dendritic cells and other antigen presenting cells which plays a role in recognizing foreign materials and will then emit signals for further immunologic reactions (Wills-Karp, 2010).

The physical barrier refers to the epithelial lining of the respiratory system. The conducting zone except bronchioles were lined with ciliated pseudostratified columnar epithelium. As has been mentioned previously, the cilia of the epithelial lining works with mucus to propel out foreign materials from the respiratory system. The epithelial lining provides a barrier separating the airway lumen and the cellular compartment.

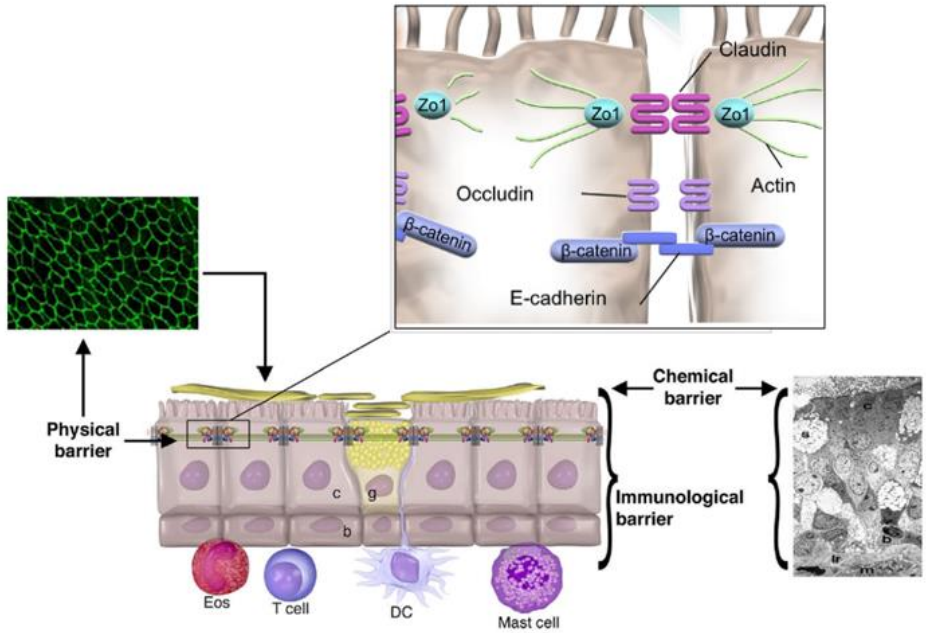

Figure 3: The Three Layers of Protection of the Epithelial System. The airway epithelial lining is protected by three layers of protection namely the physical, chemical and immunological barrier. (Adapted from Swindle, Colllins, & Davies, 2009; Gon & Hashimoto, 2017).

2.1.2 Epithelial lining as first line of defence

Epithelial lining is an important structure in the protective mechanism of the respiratory system. Its hair-like projection as shown in Figure 4 helps in maintaining the cleanliness of the inhaled air as it works with mucin to sweep out foreign materials. Individual columnar epithelium binds to each other closely and forms a protective layer preventing the entrance of allergens into the cellular compartments of the respiratory systems. Interaction of allergen with the antigen-presenting cells will consequently result in immunologic reactions such as inflammation. The barrier properties of the epithelial lining come from intercellular junctions which helps to regulate the epithelial permeability. The epithelial lining also possess the ability to secrete the antimicrobial products to kill pathogens.

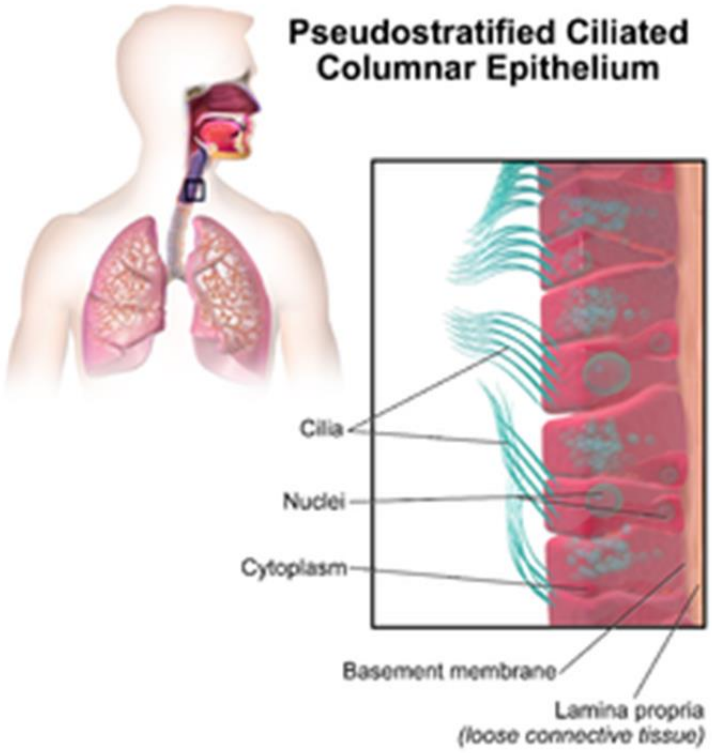

Figure 4: Airway Epithelial Lining. Lining of the respiratory epithelium equipped with cilia which helps in removing dust particles from the system. (Adapted from Blausen.com staff, 2014)

2.1.3 Tight junctions

There are few types of intercellular junction such as tight junctions (TJs), adherens junctions (AJs) and gap junctions. However, TJs and AJs are the types of junctions which play a role in maintaining the barrier properties of the airway epithelial lining. AJs and TJs both possess transmembrane protein which is anchored intracellularly to actin cytoskeleton and provide links extracellularly for adjacent cells to be bound tightly to each other as has been illustrated in Figure 5. AJs have a role in maintaining and regulating the assembly of TJs. AJs protein, E-Cadherin binds to the actin cytoskeleton by the cytoplasmic catenin. TJs is a highly dynamic molecular structure comprises of the transmembrane proteins and cytoplasmic plaques which contributes to TJs ability in the maintenance and regulation of cell permeability and polarity (Aijaz, Balda, & K, 2006; Balda & Matter, 2008). Various pulmonary diseases such as asthma and lung cancer has been the result of the dysregulation in TJs permeability and thus leading to the extensive study on the possible molecular targets which causes such condition to reside (Sawada, 2013).

TJs is a complex structure made up of different proteins such as ZO-1 which is localized in the cytoplasm of the cells and occludin which interacts with TJs protein from neighbouring cells to seal the gap to allow limited passage of the substances through the spaces (Wan, et al., 2000). However, despite the in depth information that has been gained on the importance of the membrane components of TJs which plays great role in the adhesion of neighbouring cells, the cytoplasmic components of TJs has also gained recognition as it regulates the biogenesis of TJs in addition to the cellular signals transmission between TJs plaques various components (Balda & Matter, 2008). The maintenance of TJs barrier function was also believed to be associated with the activity of protein kinases and phosphatases on residues which consequently affects the TJs-associated proteins expression, subcellular localization and phosphorylation (Dorfel & Huber, 2012). This complex system of junctional proteins is as graphically illustrated in Figure 6.

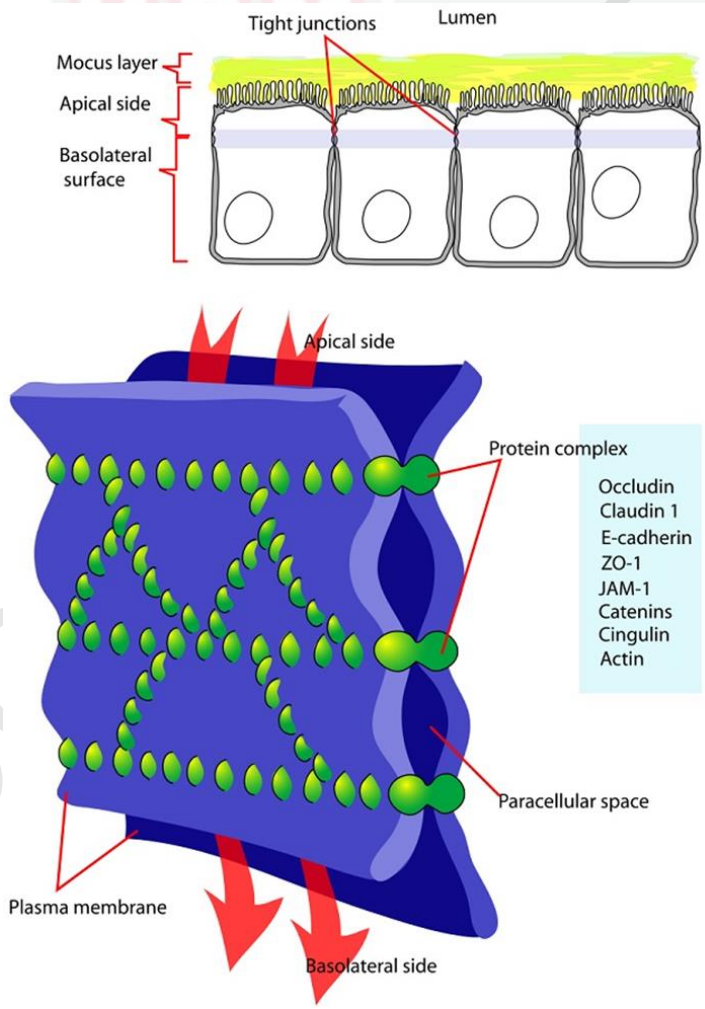

Figure 5: Tight Junctions. The junctional system positioned in between two neighbouring cells with some proteins reside in the cytoplasmic are and the others reside in the intercellular space. (Adapted from Ruiz, 2006)

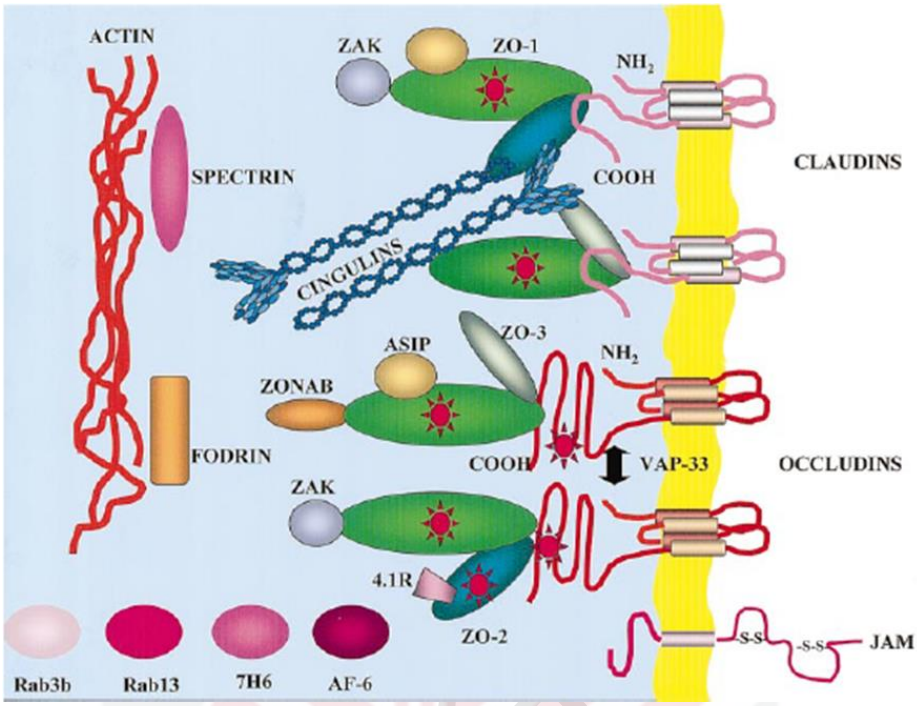

Figure 6: The Junctional System. The junctional system consists of multiple protein complex working together in the maintenance of epithelial barrier properties. (Adapted from Cereijido, Shoshani, & Contreras, 2000).

2.2 Epithelial Barrier Disruption

Even though, the airway system has been equipped with such well-structured barrier, there are instances in which the barriers can be broken or passed through by the allergens such as pollens and house dust mite. Some of the allergens possess the ability to pass through the epithelial barrier by directly cleaving the junctional proteins of the epithelial lining. The loose gap between the epithelial cells will then allow the passage of the allergen through the epithelial barrier and allows them to interact with the immunological cells. An example of the source of allergen which has the ability to cleave junctional proteins is house dust mite (HDM). HDM can be found in any indoor environment (Wan H. , et al., 2000). Introduction of HDM to the airway causes disruption of the epithelial tight junction and thus affecting the permeability of the lining (Asokananthan, et al., 2002).

2.2.1 House Dust Mite

Dermatophagus pteronyssinus or house dust mite (HDM) as illustrated in Figure 7 is the most known household source of allergen. Thirteen out of 47 species of

the main family of mites, *Pyroglyphidae*, can be found in house dust. *D. pteronyssinus* is the most encountered species among the four most common species of mite family (Millian & Diaz, 2004). Most of the time, it also carries lipopolysaccharides, cysteine and serine peptidase and many more as illustrated in Figure 8. Der p 1, a cysteine peptidase, from HDM possesses the ability to break through the airway epithelial barrier by causing the breakage of tight junction proteins such as occludin and ZO-1 (Jacquet, 2010). Proteinases originates from HDM such as Der p 1 acts on the proteinases activated receptor (PAR)-2 to cause subsequent allergic responses which may result in asthmatic condition. These proteinases have the ability to directly cleave the junctional proteins and thus jeopardize the ability of the airway lining to maintain its barrier properties (Gandhi, Davidson, Asaduzzaman, Nahirney, & Vliagoftis, 2013). Der p 1 has even been studied to be able to cause the activation of other proteases found in the house dust mites (Herman, et al., 2014).

Figure 7: House Dust Mite. House dust mite is the most common source of allergen found in households. (Adapted from Vyszenski-Moher, Arlian, & Neal, 2002).

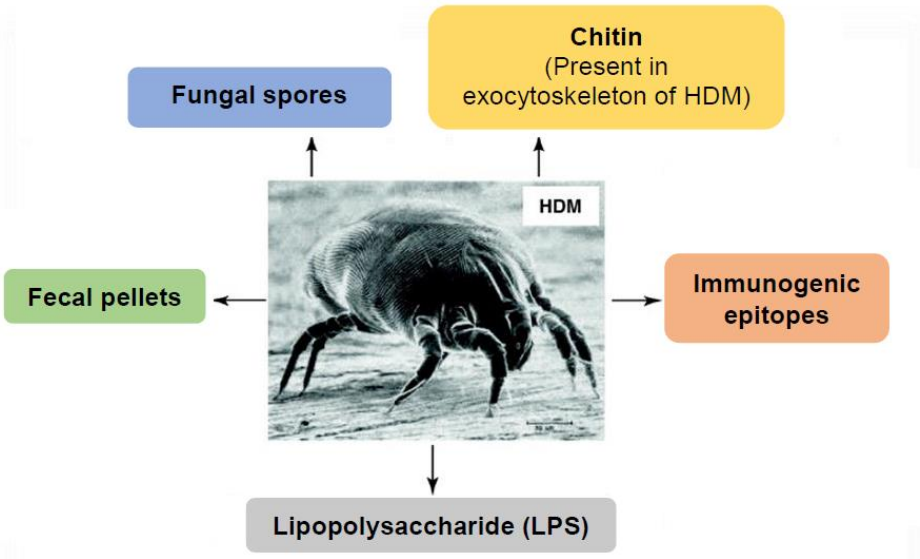

Figure 8: Allergens from House Dust Mites. HDM carries with it multiple allergens with it and Der p 1 the cysteine protease of HDM possess an effect on the disruption of the junctional integrity. (Adapted from Gregory & Lloyd, 2011).

2.2.2 Immunologic response of the airway system

As a result of the impairment of the epithelial barrier through the disruption of the junctional proteins, the allergens pass through the protective layer and interact with the next level of protective mechanism which is the immunological barrier. The allergen will then interact with the dendritic cells. Allergen uptake will occur and dendritic cells as the antigen-presenting cells will present it to the naïve T cell through major histocompatibility complex (MHC) II. As has been mentioned in the previous section, Der p 1 possesses proteolytic effect, thus allowing it to enhance the interaction between dendritic cells and T cells as it cleaves the protease activated receptor which can be found on the surface of dendritic cells. This will eventually results in the recruitment of Th2 cells and the release of pro-inflammatory cytokines by the mast cells (Holgate, 2007). This may lead to allergen-induced airway hyperresponsiveness, inflammation and airway remodelling which are the characteristics of a chronic condition, allergic asthma (Heijink, Postma, Noordhoek, Broekema, & Kapus, 2010).

2.3 Zerumbone

Zerumbone can be found predominantly in the essential oil of the rhizome of the plant. Zerumbone (2,6,10-cy-cloundecatrien-1-one, 2,6,9,9-tetramethyl-,(E,E,E)-) as illustrated in Figure 9 is a monocyclic sesquiterpene which consists of three double bonds: an isolated one at C2 and two at C6 and C9 which are a part of cross-conjugated dienone system (Kitayama, et al., 1999). However, zerumbone can also be bought in powder form as it is available commercially. There have been many studies done on the therapeutic effects of this compound and its plant origin. Seventeen zerumbone derivatives has been studied to investigate the cytotoxicity of these derivatives on the cholangiocarcinoma cell lines. Based on the study, it was proven that one of the derivatives of zerumbone, (±)-[6E,10E]-3-amino-2,6,9,9-tetramethylcycloundeca-6,10-dienone, shows the most potent anti-proliferative activity (Songsiang, Pitchuanchom, Boonyarat, Hahnvajanawong, & Yenjai, 2010). Zerumbone which is extracted from another member of genus *Zingiber*, *Zingiber aromaticum*, exhibits anticancer effect as it causes growth inhibition of three human cell lines namely HT29 colon, CaCo-2 and MCF-7 breast cancer cells. Zerumbone inhibits DNA synthesis and blocking cells at the G0/G1 and G2/M leading to the anti-proliferation effect (Kirana, Mcintosh, Record, & Jones, 2009). There are *in vivo* studies that have been carried out to identify the anticancer properties of zerumbone. Zerumbone was found to inhibit the colon and lung carcinogenesis due to its anti-proliferative, apoptosis inducing, and anti-inflammatory properties as well as the suppression of NF-κB and HO-1 expression (Kim, Miyamoto, Yasui, & Oyama, 2009).

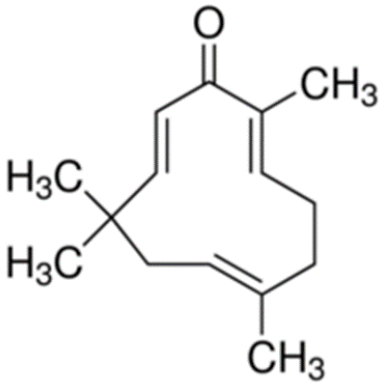

Figure 9: Structural Formula of Zerumbone.

Antinociceptive effect of zerumbone has been proven by the study carried out by (Sulaiman, et al., 2009) where zerumbone administration exhibit 19.3% of inhibition on acetic acid-induced visceral nociceptive response in mice. Two novel analogues of zerumbone analogues namely azazerumbone 1 and azazerumbone 2 was synthesized and tested for their potential antibacterial activity. The results obtained through the study showed that azazerumbone 2 possesses better antibacterial compared to azazerumbone 1 and zerumbone (Kumar, Srinivas, Negi, & Bettadaiah, 2013). In addition, this compound has also been proven to be able to contribute in lipid metabolism (Tzeng, Liou, Chang, & Liu, 2013). From the investigation on the immunomodulation of the antigen-presenting dendritic cells (DCs) and therapeutic effects against ovalbumin (OVA)-induced T helper 2 (Th2)-mediated asthma, zerumbone has shown to enhanced the T-cell proliferation and increased interferon-ɣ (IFN-ɣ) secretion by T-cells besides efficiently inhibit the OVA-specific IgE production in OVA-induced mice models (Shieh, et al., 2015).

2.4 16HBE14o- Cell Line

16HBE14o- cell line is a human bronchial surface epithelium which was first isolated from 1 year old male heart-lung transplant patient by Dr Dieter C. Gruenert. The cells were first primary cells before they were transformed by calcium phosphate transfection with pSVori- plasmid (Cozens, et al., 1994). 16HBE14o- cell line has been widely used in the study of barrier function of the airway epithelium to mimic *in vivo* airway bronchial epithelium. Among the characteristics which allow it to be a suitable model for airway epithelial barrier studies are, they form polarized cell layer and express major intercellular junctional proteins such as occludin and ZO-1 (Forbes, 2000).

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Cell Culture

16HBE14o- cell line (refer Appendix A: 1 and 2) was a gift from Dr Dieter C. Gruenert from University of California, San Francisco. Eagle's Minimum Essential Medium (EMEM) which was purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). Fetal Bovine Serum (FBS), 200 mM L-Glutamine, 100X Penicillin/Streptomyocin, Trypsin-EDTA 10X and LHC basal medium were purchased from Gibco (Waltham, Massachusetts, USA). HEPES sodium salt, sodium chloride, glucose, sodium phosphate dibasic heptahydrate, phenol red solution, polyvinylpyrrolidone, 0.2% EGTA and dimethyl sulfoxide were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Tissue culture flasks, multi-well plates and inserts were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA) and multi-well chamber slide was purchased from Merck Millipore (Berlington, Massachusetts, USA). Bovine serum albumin was purchased from Nacalai Tesque (Nakagyo-ku, Kyoto, Japan). 3 mg/mL bovine collagen type I and 1 mg/mL human fibronectin were purchased from Corning (Corning, New York, USA).

3.1.2 Antibodies

Occludin rabbit polyclonal antibody (71-1500) was purchased from Gibco (Waltham, Massachusetts, USA) while ZO-1 (D6L1E) rabbit monoclonal antibody (#13663) was purchased from Cell Signalling Technology (Danvers, Massachusetts, USA). HRP-conjugated goat anti-rabbit IgG (sc-2030) and HRP-conjugated mouse monoclonal beta actin IgG (sc-47778) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Alexa Fluor 488-conjugated goat anti-rabbit IgG (ab150077) was purchased from Abcam (Cambridge, UK).

3.1.3 Zerumbone, HDM and Dexamethasone

Zerumbone ≤98% (HPLC) was purchased from Sigma-Aldrich (St Louis, Missouri, USA). Lyophilized house dust mite was purchased from Greer Laboratories, Inc. (Lenoir, North Carolina, USA) while 1,4-Dithiothreitol (DTT) and dexamethasone were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

3.1.4 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 (St Louis, Missouri, USA). Phosphate buffered saline (PBS), glycine and Triton-X100 were purchased from AMRESCO, Inc. (Solon, Ohio, USA). Prolong antifade reagent was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). WesternBright Sirius enhanced chemiluminescence (ECL) was purchased from Advansta Inc. (Menlo Park, California, USA). Polyvinylidene difluoride (PVDF) membrane and (Bicinchoninic Acid) BCA protein assay kit was purchased from Merck Millipore (Berlington, Massachusetts, USA). Rneasy Plus Mini Kit, QuantiNova Reverse Transcription Kit and QuantiNova SYBR Green Kit were purchased from Qiagen (Hilden, Germany).

3.2 Methods

3.2.1 Preparation of Culture Media

16HBE14o- cells were cultured in complete EMEM which was prepared by supplementing the EMEM with 10% FBS, 1% 200 mM L-Glutamine and 1% 100X Penicillin/Streptomyocin. However, the FBS concentration in the complete EMEM was reduced to 5% in assays. Preparation of the complete EMEM was carried out in the biosafety chamber. (Refer Appendix B1(a))

3.2.2 Preparation of Fibronectin Coating Solution

The coating solution used to coat the vessels in which 16HBE14o- cells were cultured, was prepared by supplementing LHC basal medium with 10% of 1 mg/mL BSA, 1% 3 mg/mL bovine collagen type I and 1% of 1 mg/mL human fibronectin. 1 mg/mL BSA was sterile-filtered by using syringe filter before being added to the LHC basal medium. Human fibronectin powder was prepared as stock solution at concentration of 1 mg/mL by dissolving the powder in sterilized ultrapure water. Preparation of the coating solution was carried out in the biosafety chamber. LHC basal medium, BSA, bovine collagen type I and human fibronectin powder were stored in 4°C. The coating solution was freshly prepared prior usage. (Refer Appendix B1(b))

3.2.3 Preparation of Coated Vessels

Vessels in this study refers to the culture flasks and plates used to culture 16HBE14o- cells. The vessels used in this study were coated by adding 1 mL of the fibronectin coating solution in T25 flasks or 2 mL in T75 flasks. The coating

solution will then was distributed across the surface of the flasks and the flasks was left with the coating solution for 2 hours at 37°C. After the incubation, the solution was completely removed from the flasks and the flasks are ready for usage. The coated vessels was kept at room temperature.

3.2.4 Preparation of Trypsinizing Solution (PET)

The trypsinizing solution (PET) used for the detachment of 16HBE14o- cells was prepared by adding 10% of 10% polyvinylpyrrolidone, 10% of 0.2% EGTA and 8% of Trypsin 5X to HBSS. 10% polyvinylpyrrolidone and 0.2% EGTA were prepared by dissolving each powder in HBSS and sterile-filtered the solutions by using sterile syringe filter. Preparation of PET was carried out in the biosafety chamber and the solution was stored as aliquots of 5 mL at -20°C and was used over two weeks period to ensure best detachment performance. (Refer Appendix B1(e))

3.2.5 Preparation of Hepes-buffered Saline (HBSS)

HBSS was used in the study as the washing solution for 16HBE14o- cells and also as solvent for some reagents in the study. 1 L of HBSS was prepared by dissolving 4.76 g hepes, 7.13 g sodium chloride, 0.2 g glucose and 1.7 g sodium phosphate dibasic heptahydrate in ultrapure water. The solution was then sterile-filtered in the biosafety chamber by using 0.22 µm bottle top filter unit. HBSS solution was kept at 4°C until further use. (Refer Appendix B1(d))

3.2.6 Cell Culture

16HBE14o- cells were incubated in a 5% CO2 humidified incubator at 37°C. Passage 68-80 of 16HBE14o- was used throughout this study. The cells arrived in a T25 flask with 100% confluency at passage 66 (Refer Appendix D(1)). The cells were trypsinized and subcultured at 1:4 subcultivation ratio. 16HBE14o- cells were cultured in vessels which have been pre-coated with fibronectin coating solution. 16HBE14o- cells were stored as stock in freezing solution containing 50% FBS, 40% complete EMEM and 10% DMSO (Refer Appendix B1(e)) at -80°C overnight before being transferred to liquid nitrogen for longer term storage.

The methodologies depicted in section 3.2.1 until 3.2.6 was adapted from the protocol given by Dr Dieter C. Gruenert along with 16HBE14o- cells.

3.2.7 Preparation of Zerumbone and House Dust Mite Stock Solution

Zerumbone (molecular weight: 218.33) was purchased from the manufacturer in powder form. Stock solution with concentration 20 mM was prepared by dissolving zerumbone powder in DMSO. HDM with Der p 1 concentration at 114.57 µg/vial purchased was stored at 4°C. Stock solution of HDM with concentration 1 mg/mL was prepared by dissolving HDM in sterilized ultrapure water. This process was carried out in the biosafety chamber to preserve the sterility of HDM. Dexamethasone (molecular weight: 392.46) was prepared as stock solution at concentration 20 mM by dissolving dexamethasone powder in DMSO. The stock solutions of zerumbone, HDM and dexamethasone was kept as aliquots at -20°C. (Refer Appendix B2)

3.2.8 Cell Counting

16HBE14o- cells were harvested by removing the used media from the culture flask and the cells were then washed twice with sterile HBSS. 16HBE14o- were incubated with PET for 3-5 minutes at 37°C to allow the cells to be detached from the surface of the flask. Fresh complete EMEM was added to the cells to stop trypsinization and the cell suspension were transferred from the culture flask to a sterile centrifuge tube. The cells suspension were then centrifuged at 1200 rpm for 4 minutes. The supernatant was removed and 1 mL of fresh complete EMEM was added to the cell pellet. Viability test was carried out by using trypan blue exclusion test. 10 µL of the cell suspension was mixed with 10 µL of 0.4% trypan blue and the solution was then loaded to a haematocytometer. The cells loaded on the haematocytometer were then viewed under microscope. The number of cells which were viable (unstained) and non-viable (stained) was counted separately. Calculation of the cell concentration was carried out by using the formula as follows (Refer Appendix C):

$$\left(\frac{Number\ of\ viable\ cells}{4}\right) \times 10^4 \times Dilution\ factor$$

Where,

Dilution factor = 2

3.2.9 Cell Treatment

16HBE14o- cells were seeded on the multi-well plate until it reach 100% confluency. 16HBE14o- cells were divided into 7 different groups with different treatments. The different groups of treatment used in this study are briefly illustrated in table below:

Table 1: Treatment Groups for 16HBE14o- Cells

Group	N	HDM	6.25	12.5	25	Dexa
HDM (100 µg/mL)	-	+	+	+	+	+
Zerumbone (µM)	-	-	6.25	12.5	25	-
Dexamethasone (10 µM)	-	-	-	-	-	+

Group N: Normal control, HDM: HDM control, 6.25, 12.5 and 25: Zerumbone and HDM co-treatment, Dexa: Positive control.

3.2.10 Cytotoxicity Assay

16HBE14o- cells were harvested when the cells confluency reached ≥ 90%. The cells were trypsinized and centrifuged at 1200 rpm for 4 minutes. 16HBE14o- cells concentration were then adjusted to 2×10^5 cells/mL (Refer Appendix D2). 100 µL of cell suspension were seeded into each well of 96-well plate and cultured overnight in 37°C incubator. The cells were then treated with a range of concentrations of zerumbone (6.25 µM-200 µM) for 24 hours. The supernatant was then removed and replaced with 100 µL of fresh complete EMEM and 10 µL of 5 mg/mL MTT solution in each well and the cells were then incubated for 4 hours. After 4 hours incubation, 100 µL of DMSO was added to each well and the plate was read at 570 nm wavelength. Absorbance reading for each well was measured by using ELISA microplate reader (ASYS Hitech UVM 340, Austria).

3.2.11 Transepithelial Electrical Resistance (TEER) Assay

16HBE14o- cells (1×10^5 cells/well) were seeded onto 24 well plate which contained 0.4 µm pore size coated inserts. The cells were let to attach overnight and the cells were introduced to air-liquid interface condition at day 2 of seeding by removing the media from the apical compartment of the cell monolayer. The basal compartment of the cell monolayer of the cells were fed with 600 µL of fresh complete EMEM and the media was replaced once every two days. The cells were treated with 100 µg/mL HDM and respective concentration of zerumbone (6.25, 12.5 and 25 µM) after the cells reached 100% confluency. The Transepithelial Electrical Resistance was measured before and after a 24 hrs treatment. Coated inserts without the presence of cells were used as the blank and resistance readings obtained of each wells was corrected by substracting with the resistance reading obtained of the blank. The resistance reading of the monolayer was calculated as follows as modified from (Kuhlmann, et al., 2007):

$$TEER\ Change\ (100\%) = \frac{(a) - (b)}{a} \times 100\%$$

Where, a = average 0 hours reading
b = average 24 hours reading

3.2.12 FITC-Dextran Permeability Assay

16HBE14o- cells (1 × 10^5 cells/well) were seeded onto 24 well plate which contained 1.0 µm pore size inserts. The cells were let to attach overnight and introduced to air-liquid interface condition at day 2 of seeding by removing the media from the apical compartment of the cell monolayer. The basal compartment of the cell monolayer of the cells were fed with 600 µL of fresh complete EMEM and the media was replaced once every two days. The cells were treated with 100 µg/mL HDM for 24 hours and respective concentration of zerumbone (6.25, 12.5 and 25 µM) after they reached 100% confluency. As Normal control, the cells were treated with complete EMEM only while for the Vehicle group, the cells were treated with complete EMEM with 0.1% DMSO. Trypsin EDTA 10X which has been diluted to 2X was used as the negative control where the 16HBE14o- cells was treated with trypsin for 15 minutes. FITC-Dextran solution (1 µg/mL) was added to the apical compartment of the cell monolayer. The cells were then incubated in CO2 incubator for 1 hour. The solution from the basal compartment was then collected into a 96 well black microplate before reading the plate with a fluorescence microplate reader (Tecan Infinite 200, Switzerland) with excitation and emission wavelength, 490 nm and 520 nm respectively. The ratio of flux was calculated by normalizing the permeability values of each treatment group (PA) to the Normal group (PN). (Ratio of flux = PA/PN) as modified from (Dong, et al., 2017).

3.2.13 Immunofluorescence Assay

16HBE14o- cells (5 × 10^5 cells/well) were seeded onto fibronectin coating solution-coated 8 well chamber slide until reached ~90% confluency. The cells were then treated accordingly (Section 3.2.3) for 24 hours. The cells were washed with cold PBS once. The cells were then fixed with freshly prepared 4% paraformaldehyde for 20 minutes. The cells were then washed with 100 mM glycine in PBS three times. Triton X-100 in PBS (0.2%) was used to permeabilize the cells for 15 minutes. The cells were then washed thrice with 0.1% Triton X-100 in PBS before incubating the cells with the blocking solution (1% BSA in 0.1% Triton X-100 in PBS) for 30 minutes. The cells were stained with primary antibody prepared in blocking solution at 1:100 ratio overnight at 4°C. The cells were then rinsed with 0.1% Triton X-100 in PBS before being stained with Alexa Fluor 488-conjugated secondary antibody prepared in blocking solution at 1:200 ratio for 2 hours in the dark at room temperature. The cells were washed with thrice with PBS before being mounted with Prolong Antifade Reagent. The fluorescent image was viewed by using a fluorescent microscope (Leica, Germany).

3.2.14 Immunoblotting Assay

3.2.14.1 Sample Preparation

16HBE14o- cells (2×10^5 cells/well) was seeded on pre-coated 6 well-plate. The cells were incubated in 37°C, 5% CO2 incubator until the cells reached ≥90% confluency. The EMEM was replaced every 2 days. After reaching the intended confluency, the cells were then treated and incubated with the treatment for 24 hours excluding the trypsin group which incubation time was 15 minutes. The treatment was carried out as follows:

Table 2: 16HBE14o- Cells Treatment Groups for Immunoblotting Assay

Group	N	HDM	6.25	12.5	25	Dexa	Trypsin
HDM (100 µg/mL)	-	+	+	+	+	+	-
Zerumbone (µM)	-	-	6.25	12.5	25	-	-
DEXA (10 µM)	-	-	-	-	-	+	-
Trypsin	-	-	-	-	-	-	+

Group N: Normal control, HDM: HDM control, 6.25, 12.5 and 25: Zerumbone and HDM co-treatment, Dexa: Positive control, Trypsin: Negative control

3.2.14.2 Whole Cell Protein Extraction

Treated 16HBE14o- cells were then rinsed twice with 4°C 1X PBS (pH 7.0). Complete removal of the PBS was ensured before adding 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) to lyzed the cells. The cells were incubated with the lysis buffer for 5 minutes at 4°C before being scraped gently by using the rubber scraper. The cell lysates were collected and transferred to a microcentrifuge tube before being vortexed for around 1-2 minutes. The cell lysates were then centrifuged at 13000 × g, 4°C for 15 minutes. The supernatants were collected into new microcentrifuge tubes and were stored at -80°C for further use.

3.2.14.3 Protein Quantification

Protein in the cell lysates were quantified by using BCA protein assay kit. The quantification was carried out according to the manufacturer's protocol. The standard graph used in the protein assay was generated by using BSA standard (2 mg/mL) which was prepared by using serial dilution. The BCA working solution used in the experiment was prepared by adding BCA solution and 4% cupric sulphate at ratio 50:1 respectively. Standard solution (25 µL) or 5× diluted

sample (25 µL) was added to the wells of 96 well plate. BCA working solution (200 µL) was then added to the wells and the plate was incubated at 37°C for 30 minutes. The absorbance reading of the mixtures were then read at 562 nm by using the ELISA microplate reader. Protein concentration was calculated by using the obtained absorbance reading and the standard graph (Refer Appendix D3).

3.2.14.4 SDS-polyacrylamide Gel Electrophoresis (PAGE)

Preparation of the polyacrylamide gel used in this experiment is described in the appendix. Samples with known concentration of proteins (20-40 µg) were added with loading buffer which contained β-mercaptoethanol. The mixture was then loaded into each well of the polyacrylamide gel (Refer Appendix B4). The protein ladder loaded in a well which was located at the left end of the polyacrylamide gel was used as protein molecular weight reference. The chamber of the Mini-PROTEAN® vertical electrophoresis system (Bio-rad Laboratories Inc., Hercules, CA, USA) was filled with cold 1× electrophoresis buffer. The preparation of the electrophoresis buffer is further described in the Appendix B3. The electrophoresis was carried out at 50V until the blue dye reached the resolving gel. The voltage was then increased to 100V until the dye reached near the bottom of the gel.

3.2.14.5 Wet Transfer

The gel was carefully cut separating the empty wells of the polyacrylamide gel and the section with separated proteins. The gel was then lifted from the glass plate gently. The gel and the methanol-activated PVDF membrane were sandwiched between two blotting papers and foam pads. The blotting papers, foam pads, membrane and gel was were equilibrated in the transfer buffer before being sandwiched together. The preparation of the sandwich was carried out while being soaked in the transfer buffer. Bubbles trapped in between each layers of the sandwich are eliminated by using the mini roller. The sandwich was then placed in between the cassette holder before being transferred to the wet electrophoretic transfer system (Bio-rad Laboratories Inc., Hercules, CA, USA) which have been filled with the transfer buffer. Preparation of the transfer buffer is further described in Appendix B3. Ice packs was added to the system as cooling agents. The transfer process was carried out at 0.35 A for 1 hour and 30 minutes.

3.2.14.6 Immunoblotting

After the wet electrophoretic transfer process, the PVDF membrane was blocked with 5% BSA in Tris Buffered saline with Tween (TBST) for 1 hour. The membrane was then rinsed three times with TBST for 10 minutes each wash before being incubated with primary antibody at room temperature (1:1000) specific for occludin or ZO-1 in TBST containing 5% BSA overnight. The membrane was then rinsed with TBST three times before being incubated with goat anti-rabbit IgG secondary antibody (1:5000) diluted in 5% BSA for 1 hour at room temperature. The membrane was then rinsed again with TBST three times before visualization.

3.2.14.7 Visualization

After being rinsed with TBST, the membrane was then incubated with chemiluminescent substrate solution before being visualized using the Fusion FX gel documentation system (Vilber Lourmat, Eberhardzell, Germany). Charge-coupled device (CCD) camera was used to capture the protein bands. The bands intensity were later being quantified by using the ImageJ software and the bands are normalized to the loading control used in this study, β-actin.

3.2.15 Real Time Polymerase Chain Reaction (qPCR)

3.2.15.1 Cell Treatment

To determine the effect of zerumbone on the gene expression of occludin and ZO-1, 16HBE14o- cells were harvested when the cells at ≥90% confluency. The cells were trypsinized and centrifuged at 4°C, 1200 rpm for 4 minutes. 16HBE14o- cells were seeded at concentration of 2×105 cells/well onto 6 well plate in 37°C, 5% CO2 incubator until reached confluency. The cells were then co-treated with 100 µg/mL HDM and zerumbone for 6 hours.

3.2.15.2 RNA Extraction

RNA extraction was carried out according to the manufacturer's protocol. Cell culture medium was completely aspirated from each wells of the 6 well plate. The cells were disrupted by the addition of 350 µL of RLT Plus (cell lysis) buffer to each well. B-mercaptoethanol was added to the RLT Plus buffer prior usage (10 µL of β-mercaptoethanol to 1 mL of RLT Plus buffer). A rubber scraper was used to gently scrape the cells from the surface of the wells. The samples were

then collected into a microcentrifuge tube. The cell lysate was vortexed for 1 minute to mix. The homogenized lysate was then transferred to a genomic deoxyribonucleic acid (gDNA) eliminator spin column which was placed in a 2 mL collection tube. Lysate were centrifuged at 8000 g for 30 seconds. The column was discarded and the flow-through was kept for subsequent steps. 350 µL of 70% ethanol was added to the flow –through and was mixed well by pipetting. 700 µL of the sample was then transferred to the RNeasy spin column which was placed in a 2 mL collection tube. The lid was closed gently and centrifuged at 8000 g for 15 seconds. The flow-through was discarded after centrifugation and 700 µL of RW1 (washing) buffer was added to the RNeasy spin column. The lid was closed gently and the lysate were centrifuged at 8000 g for 15 seconds. The flow-through was then discarded. 500 µL of RPE (mild washing) buffer was added to the Rneasy spin column. The lid was closed gently and the lysate was centrifuged at 8000 g for 15 seconds. The flow-through was then discarded. Another 500 µL of RPE buffer was added to the lysate and centrifugation was carried out at 8000 g for 2 minutes. Prior usage, RPE buffer was prepared by adding 4 parts of absolute ethanol to 1 part of RPE buffer. The Rneasy spin column was then placed in a new 1.5 mL microcentrifuge tube and 40 µL of Rnase-free water was added directly to the spin column membrane. The lid was closed gently and centrifuged for 1 minute at 8000 g. The step was repeated again to gain as much RNA as possible.

3.2.15.3 RNA Quantification

RNA sample obtained from the step in 3.2.15.3 was examined for its purity and concentration by using a nanophotometer (IMPLEN Nanophotometer® P300). The procedure was carried out according to the manufacturer's protocol. The NanoPhotometer® P-Class submicroliter Cell was inserted into the cell holder of the nanophotometer prior to the addition of 1 µL of the RNA sample onto the measuring window. Lid 50 which have the pathlength of 0.2 mm was used to cover the measuring window. The automated software provided by the manufacturer was used to measure the purity and concentration of the RNA samples. RNA samples purity was estimated by using the ratio A260/A280 in which 1.98-2.05 readings shows high purity of the RNA samples obtained.

3.2.15.4 RNA Reverse Transcription

After the RNA quantification was carried out as in 3.2.15.3, the reverse transcription was carried out for the RNA samples according to the manufacturer's protocol. The genomic DNA removal reaction was carried out on ice. RNA sample (1 µg) was added to 2 µL of gDNA removal buffer and Rnase-free water with final volume of 15 µL. This mixture was then incubated for 2 minutes at 45°C. Reverse transcription master mix was prepared by adding 1 µL

of reverse transcription enzyme and 4 µL of reverse transcription mix. The reverse transcription master mix was then added to the gDNA elimination reaction mixture prepared previously making up the final volume of 20 µL. This process was carried out on ice. The mixture was incubated in 25°C for 3 minutes for annealing step, followed by incubation at 45°C for 10 minutes and finally at 85°C for 5 minutes to inactivate reverse transcriptase. Analysis of the samples by qPCR was carried out after this step.

3.2.15.5 SYBR® Green Real Time qPCR Analysis

Real time qPCR was carried out accordingly as stated in the manufacturer's protocol. 10 µL of SYBR® green PCR master mix, 2 µL of each forward and reverse primers (occludin/ZO-1/GAPDH) and RNase-free water were added to the cDNA template which makes up the final volume of the mixture as 20 µL. The prepared mixtures were then placed in the 96-well block Rotor gene and were proceeded to the cycling protocol described in Table 3:

Table 3: qPCR Cycling Condition

Step \ Condition	Time	Temperature	Number of cycles
PCR initial heat activation	2 minutes	95°C	-
2-step cycling			
Denaturation	5 seconds	95°C	40
Combined annealing/extension	10 seconds	60°C	40
Melting curve analysis*	-	-	-

*Refer Appendix D4

Table 4: qPCR Primer Sequence

Gene name	Oligonucleotide sequences (5'-3')
GAPDH*	F: 5'-GGCACAGTCAAGGCTGAGAATG-3
(internal control)	R: 5'-ATGGTGGTGAAGACGCCAGTA-3'
Occludin	F: 5'-TACAGCAATGGAAAACCACACT-3'
	R: 5'-CAAAGGAATGGGAAACGACTAA-3'
ZO-1	F: 5'-TCCGTGTTGTGGATACCTTGTA-3'
	R: 5'-GCCTCGTTCTACCTCCTTATGA-3'

*glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

The fold-changes in the transcription levels were calculated using the 2−ΔΔCT method where $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{GAPDH})_{treated\ groups} - (Cttarget\ gene - Ct_{GAPDH})_{Normal}$.

3.2.16 Statistical Analysis

Data of three individual experiments was presented in means ±SEM by using GraphPad Prism 5. One-Way Analysis of Variance (ANOVA) followed by post hoc comparison by using Dunnet test was used to determine statistical significance ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 Cytotoxic Effect of Zerumbone on 16HBE14o- Cells

MTT assay or cytotoxicity assay is a colorimetric assay which involves the reduction of tetrazolium dye (MTT) to insoluble formazan crystal by an action of the metabolic enzymes of the cells. The purple formazan crystal will then be solubilized by DMSO and the absorbance reading was measured to investigate the activity of the metabolic enzyme which will then reflect the presence of viable cells.

MTT assay has been carried out by incubating 16HBE14o- cells with zerumbone at concentrations ranging from 6.25 µM to 200 µM for 24 hours as to determine the highest non-cytotoxic concentration of zerumbone on the epithelial cells 16HBE14o-.

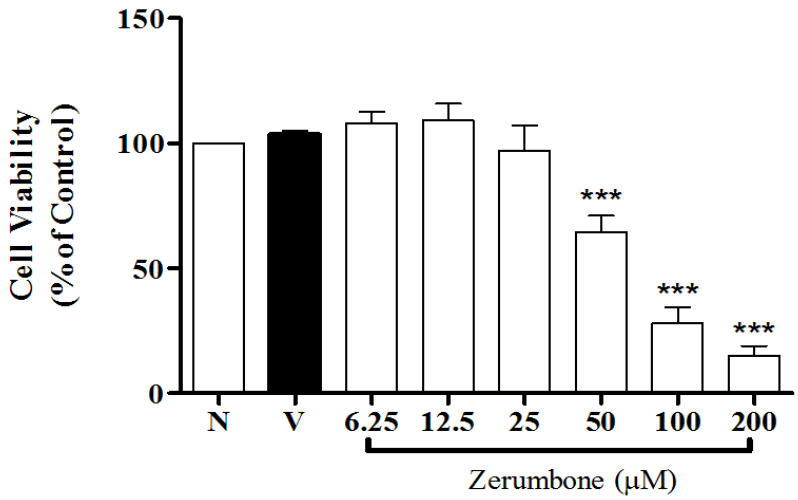

Figure 10: Zerumbone is Non-cytotoxic on 16HBE14o- Cells at 25 µM and Below. Zerumbone were seeded in 96-well plate overnight before being treated with zerumbone at concentration ranging from 6.25 µM to 200 µM. The vehicle (V) group represents as the control group. The Normal (N) group represents the cells at its normal condition where the cells were only fed with complete EMEM. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. ***p ≤ 0.001 significantly different from V group.
From Figure 10, it shows that zerumbone poses cytotoxic effect on 16HBE14o- cells at concentration 50 µM and above. Normal (N) group represents the cells

at its normal condition and the vehicle (V) group represents the cells which have been exposed to 0.1% of DMSO which is a solvent used to dissolve zerumbone in the preparation of the compound stock solution. This group was included in the experimental design to prove that the cells will not encounter significant effect when it was exposed to the amount of DMSO found in the zerumbone solution used in the treatment of the cells. From this experiment, it was proven to be true as the cell viability results show that there is no significant difference between the N group and the V group. Therefore, to determine which concentration of zerumbone poses an effect on the cell viability of 16HBE14o-, the group was compared to the control group (V). From the experiment, zerumbone at concentration 50, 100 and 200 µM shows 39%, 75% and 88% reduction in cell viability respectively compared to the control group. On the other hand, concentrations of zerumbone from 25 µM and below shows less than 6% of reduction in cell viability compared to the control group and thus proving that the concentrations are suitable to be used in the subsequent assays.

4.2 Effect of Zerumbone on HDM-induced Junctional Integrity of 16HBE14o- Monolayer

TEER assay was carried out to investigate the effect of zerumbone on the junctional integrity of the epithelial cells monolayer which was concurrently induced with 100 µg/mL HDM. The cells were co-treated with HDM and zerumbone for 24 hours. The results of the experiment is shown in Figure 11.

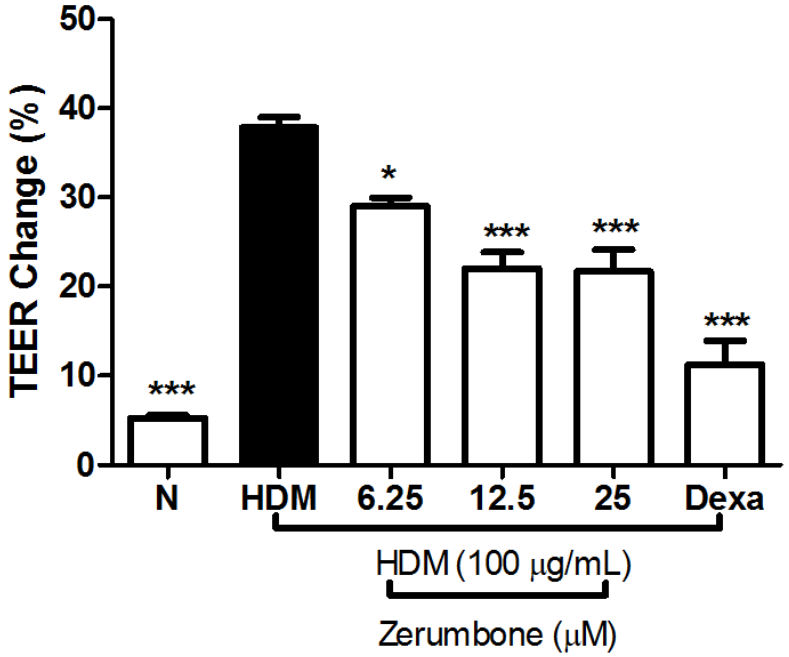

Figure 11: Zerumbone Preserves the Junctional Integrity of 16HBE14o- Monolayer Treated with HDM Allergen Extracts. 16HBE14o- cells were seeded on the pre-coated inserts in 24 well plate. The cells were then treated simultaneously with zerumbone and HDM for 24 hours. The TEER reading was recorded before and after the cells incubation with zerumbone and HDM. The graph represents the TEER change of different groups of treatment on 16HBE14o- monolayer. N: Normal; HDM: HDM-induced group; 6.25, 12.5 and 25: Co-treated with HDM and zerumbone, thus, each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM and 10 μM dexamethasone. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. *p ≤ 0.05 and ***p ≤ 0.001 significantly different from HDM group.

Measurement of the transepithelial electrical resistance in TEER assay reflects the strength of junctional integrity of the monolayer. The junctional integrity may refers to the strength of the junctional proteins in between cells and the amount of electric charges allowed to pass through the monolayer. The smaller amount of the electrical charges pass through reflects the junctional integrity in cell-cell contact and thus can be measured as higher resistance reading. The 0 hours resistance reading was measured before the cells were exposed to its respective treatments while the 24 hours reading reflects the resistance reading 24 hours after the cells were exposed to its respective treatment. In Figure 2, the Y-axis bar represents the TEER change in percentage form as the reading of resistance of the 0 hours and 24 hours was subtracted to investigate the difference between the two readings. The difference was then expressed in percentage form. The normal (N) group represents the cells in its normal condition where the cells were

supplemented with complete media while the HDM group represents the cells which were exposed to HDM only and this group was used as the control group. From the graph, it shows that the N group possess 5% TEER change between the reading of 0 hours and 24 hours, which is the lowest TEER change among all groups of treatment. On the other hand, the TEER change of the HDM group was the highest which is 37.8% of TEER change. Dexamethasone which was used as the positive control drug in the experiment also shows 11.2% of TEER change. 6.25, 12.5 and 25 μM of zerumbone used in this experiment has shown to possess 29%, 22% and 21% in TEER change respectively. Therefore, this shows that there is slight dose-response effect of zerumbone on the HDM-induced junctional integrity.

4.3 Effect of Zerumbone on HDM-induced Junctional Permeability of 16HBE14o- Monolayer

The barrier properties of the epithelial monolayer of the airway system was also contributed by the semi-permeable properties of the epithelial monolayer. TEER assay results has proven that zerumbone possess protective effect on HDM-induced junctional integrity disruption. However, it is still insufficient as the assay cannot directly prove the effect of the compound on the permeability properties of the epithelial monolayer. Therefore, to investigate the effect of zerumbone on this property of the epithelial monolayer, FITC-Dextran permeability assay was carried out. The results of the experiment is as shown in Figure 12.

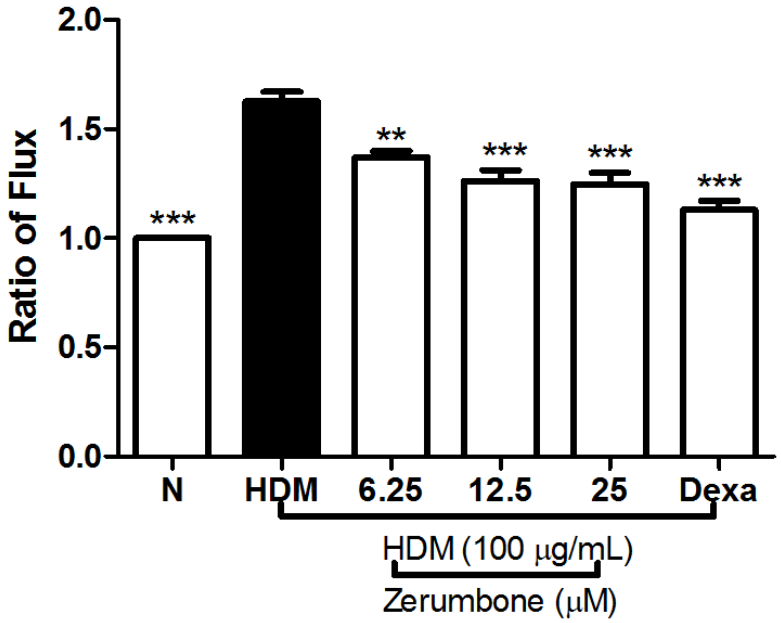

Figure 12: Zerumbone Reduces HDM-induced Permeability of 16HBE14o- Monolayer. 16HBE14o- cells were seeded on pre-coated inserts in 24 well plate. The cells were treated simultaneously with zerumbone and HDM for 24 hours. 1 mg/mL FITC-Dextran was applied at the apical compartment of inserts. After 1 hour incubation in 37°C, the solution in the basal compartment was collected and measured by using fluorescence microplate reader. The graph represents the ratio of flux of solutes normalized to N group through 16HBE14o- monolayer. N: Normal; HDM: HDM-induced group; 6.25, 12.5 and 25: Co-treated with HDM and zerumbone. Each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM and 10 μM dexamethasone. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. **p ≤ 0.01 and ***p ≤ 0.001 significantly different from HDM group.

The FITC-Dextran assay was carried out by using dextran as the solutes which will pass through the epithelial monolayer through the paracellular route. The cells were exposed to their respective treatment for 24 hours. After 24 hours, the solutes was introduced to the apical compartment of the cell monolayer .The cell monolayer was then allowed to equilibrate in 37°C incubator to allow the passage of the solutes from the apical compartment towards the basal compartment. The amount of solutes found in the basal compartment of the cell monolayer indicates the permeability of the monolayer. The amount of solutes in the basal compartment was measured by the intensity of the fluorescence signal emitted by the fluorescein isothiocyanate (FITC) conjugated to the Dextran. HDM group was be used as the control group in the experiment. Ratio of flux in the Y-axis group of the graph reflects the number of solutes found in the basal compartment of the epithelial monolayer in proportion to the solutes found in the basal

compartment of the normal group. The HDM group shows 0.6 fold increase in the ratio of flux compared to the N group while Dexa group shows 0.13 fold increase in ratio of flux. Three concentrations of zerumbone used in the study, 6.25, 12.5 and 25 μM shows 0.37, 0.28 and 0.25 fold increase in ratio of flux compared to the N group respectively. From the result, 6.25, 12.5 and 25 μM of zerumbone reduced epithelial hyperpermeability as compared to the HDM group as much as 38.3%, 53.3% and 58.3% respectively.

4.4 Effect of Zerumbone on the Localization of Occludin and ZO-1 in HDM-induced 16HBE14o- Monolayer

Even though previous experiments has proven that zerumbone poses protective effect on the junctional integrity and permeability of epithelial monolayer exposed to HDM, it is still remain unknown whether this compound affects directly on the junctional protein thus causing the protective effect. Therefore, immunofluorescence was carried out to study the effect of zerumbone on HDM-induced junctional proteins localization namely occludin and ZO-1. The results of the experiment is as demonstrated in Figure 13 and 14.

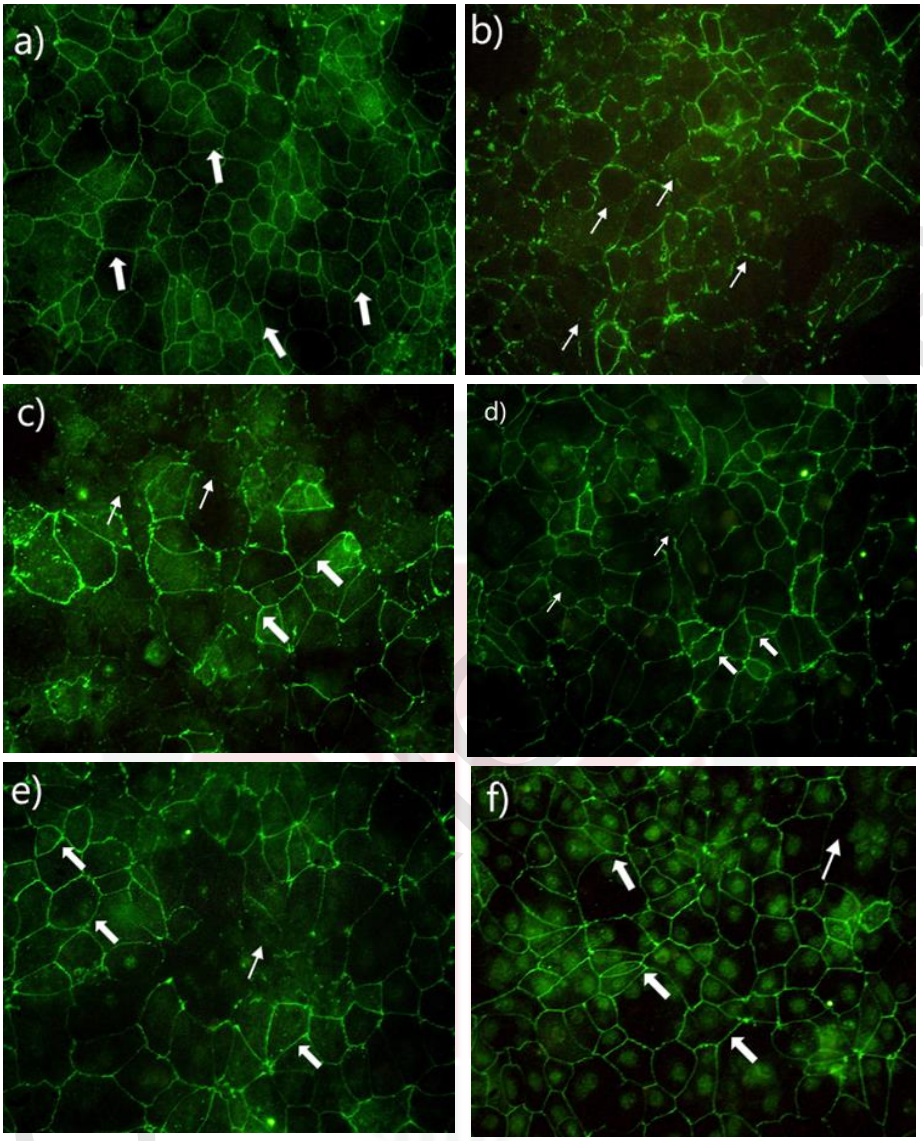

Figure 13: Zerumbone Inhibits Redistribution of Occludin in HDM-induced 16HBE14o- Cells. 18HBE14o- cells were seeded on the well the chamber slides. The cells were treated with zerumbone and HDM for 24 hours after the cells reached >90% confluency. The cells were incubated with occludin antibody and subsequently with Alexa Fluor 488-conjugated IgG. Each figures represent each different treatment groups. (a) Normal group; (b) HDM-induced group; (c) 6.25 μM zerumbone group; (d) 12.5 μM zerumbone group; (e) 25 μM zerumbone group; (f) Dexamethasone group. Thick arrows represent the “honey-comb”-like structure of the junctional protein while the thin arrows represent the disrupted area of the junctional protein. Magnification: 40X.

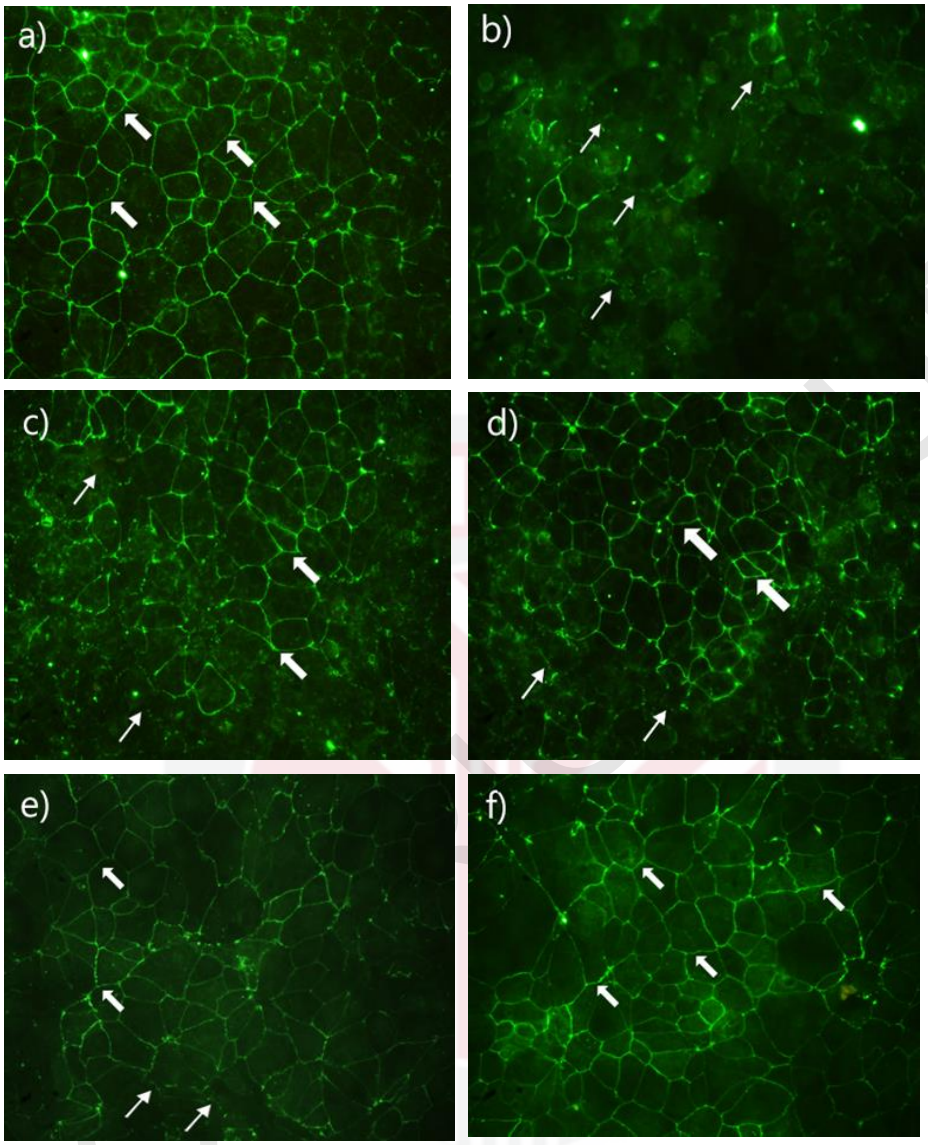

Figure 14: Zerumbone Inhibits Redistribution of ZO-1 in HDM-induced 16HBE14o- Cells. 16HBE14o- cells were seeded on the well of the chamber slides. The cells were treated with zerumbone and HDM for 24 hours after the cells reached >90% confluency. The cells were incubated with ZO-1 antibody and subsequently with Alexa Fluor 488-conjugated IgG. Each figures represent each different treatment groups. (a) Normal group; (b) HDM-induced group; (c) 6.25 μM zerumbone group; (d) 12.5 μM zerumbone group; (e) 25 μM zerumbone group; (f) Dexamethasone group. Thick arrows represent the “honey-comb”-like structure of the junctional protein while the thin arrows represent the disrupted area of the junctional protein. Magnification: 40X.

Normal groups in both Figure 13(a) and 14(a) represent the localization of both junctional proteins, occludin and ZO-1, in normal condition whereas the HDM group (Figure 13(b) and 14(b)) represents the localization of both junctional proteins which have been exposed to HDM for 24 hours. The localization of occludin and ZO-1 in normal group was displayed as the proteins were positioned at the periphery of the cells in continuous manner in “honey-comb”-like shape whereas junctional proteins in HDM group was scattered out and the expression of the junctional proteins were in discontinuous manner. Based on the fluorescence images obtained from the experiment, Dexamethasone which was used as the positive control of this experiment shows protective effect on the localization of both junctional proteins as they remained at the periphery of the cells in continuous manner despite being exposed to HDM at the same time. All three concentrations of zerumbone were also displayed to be able to preserve the localization of the localization of both junctional proteins as both remained in “honey-comb”-like shape even after being exposed to the allergen.

4.5 Effect of Zerumbone on HDM-induced Protein Expression Level of Occludin and ZO-1

Immunofluorescence assay results have demonstrated that zerumbone preserve the localization of both junctional proteins, occludin and ZO-1, in HDM-induced epithelial monolayer. However, despite preserving the localization of these proteins which thus lead to the protective effect of zerumbone on the barrier properties, it is still unknown whether this compound also preserves the expression of the junctional proteins which are disrupted by HDM. Therefore, to study the effect of zerumbone on the junctional proteins expression, immunoblotting assay was carried out. The results of the experiment is as shown in Figure 15 and 16.

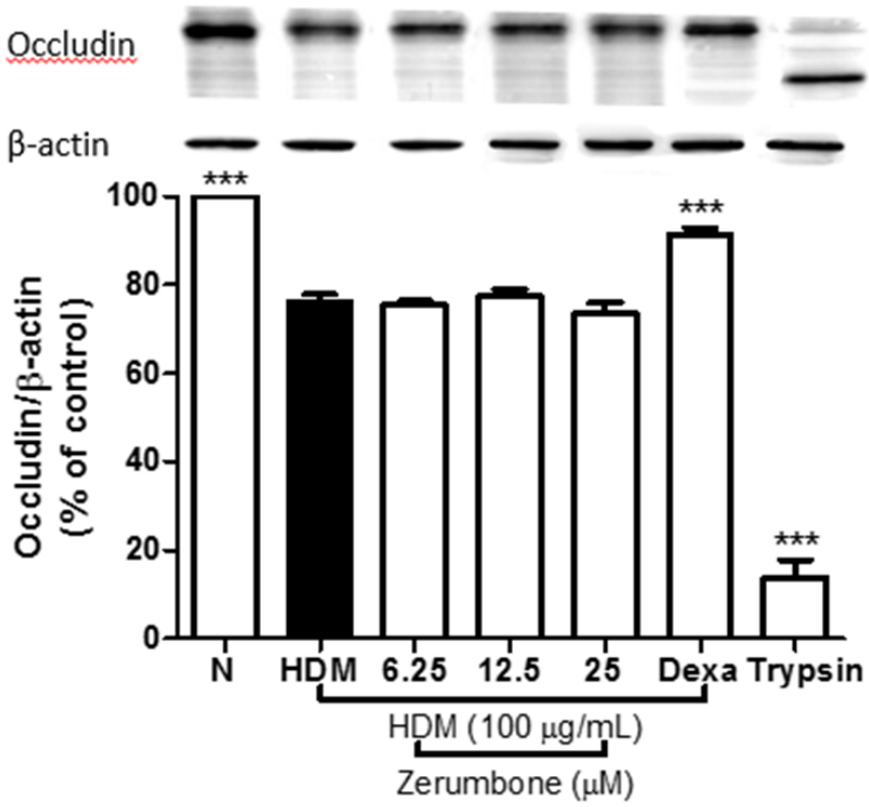

Figure 15: Zerumbone Does Not Protect the Degradation of Occludin Protein in HDM-induced 16HBE14o- Cells. 16HBE14o- cells were seeded on 6-well plate until 100% confluent. The cells were treated with zerumbone and HDM for 24 hours. The cell lysate was collected and SDS-PAGE was conducted. The treatment groups were labelled as follows: N: Normal group; HDM: HDM-induced group; 6.25, 12.5 and 25: Co-treated with HDM and three different concentrations of zerumbone (µM). Each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM and 10 µM dexamethasone; Trypsin: Trypsin-treated group. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. ***$p \leq 0.001$ significantly different from HDM group.

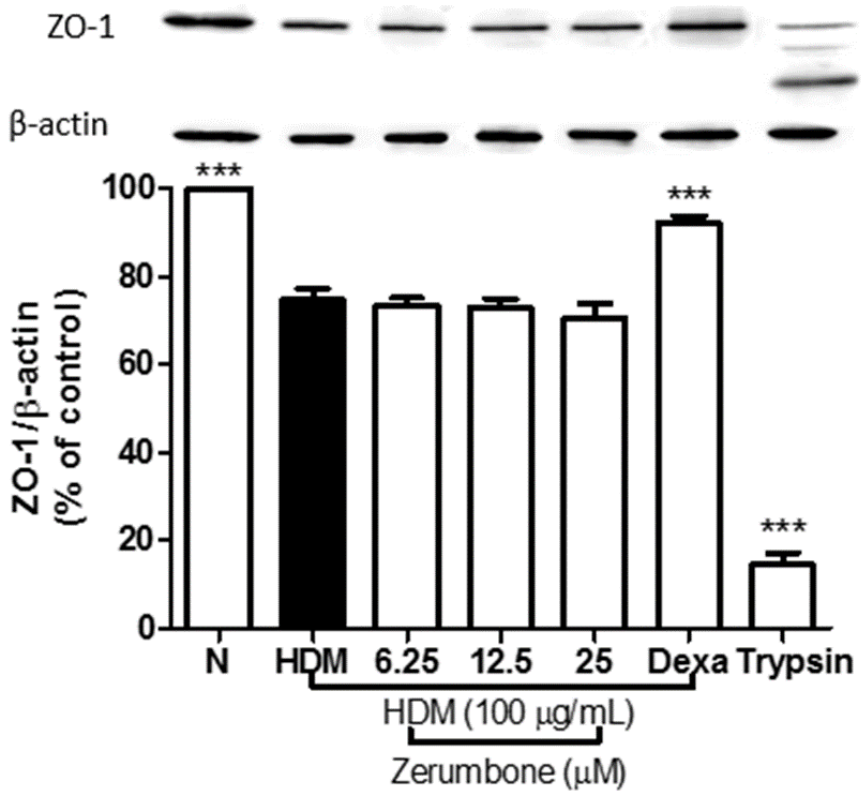

Figure 16: Zerumbone Does Not Protect the Degradation of ZO-1 Protein in HDM-induced 16HBE14o- Cells. 16HBE14o- cells were seeded on 6-well plate until 100% confluent. The cells were treated with zerumbone and HDM for 24 hours. The cell lysate was collected and SDS-PAGE was conducted. The treatment groups were labelled as follows: N: Normal group; HDM: HDM-induced group; 6.25, 12.5 and 25: Co-treated with HDM and three different concentrations of zerumbone (μM). Each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM and 10 μM dexamethasone; Trypsin: Trypsin-treated group. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. ***$p \leq 0.001$ significantly different from HDM group.

The protein expression of both occludin and ZO-1 in normal condition was as demonstrated in normal group (N) whereas the HDM group represents the protein expression of both occludin and ZO-1 which have been exposed to 100 μg/mL HDM only for 24 hours. The protein expression of both occludin and ZO-1 was reduced in HDM group as compared to the N group. Trypsin group which is the negative control group used in this experiment shows significant reduction of both occludin and ZO-1 while the positive control group, Dexamethasone was shown to possess protective effect on the junctional protein expression as it shows significant difference in the junctional protein expression as compared to

the HDM group. All three concentrations of zerumbone, however, shows no significant difference in the expression of both junctional protein as compared to the HDM group. Thus, this shows that the compound does not possess protective effect on the disruptive effect imposes by HDM on the protein expression of the junctional proteins, occludin and ZO-1.

4.6 Effect of Zerumbone on HDM-induced Gene Expression Level of Occludin and ZO-1

Protein is encoded by gene before it can actually being produced and plays a role in the dictation of the cell function. Reduced gene expression may results in reduced production of the junctional proteins. Therefore, the study of the effect of zerumbone on gene expression of occludin and ZO-1 is necessary in investigating the protective effect of zerumbone on HDM-induced epithelial barrier.

Results from immunoblotting assay has proven that zerumbone does not possess protective effect on the protein expressions of both occludin and ZO-1. Therefore, Real Time PCR was carried out for further confirmation of this results. The results obtained from the experiment are as shown in Figure 17 and 18.

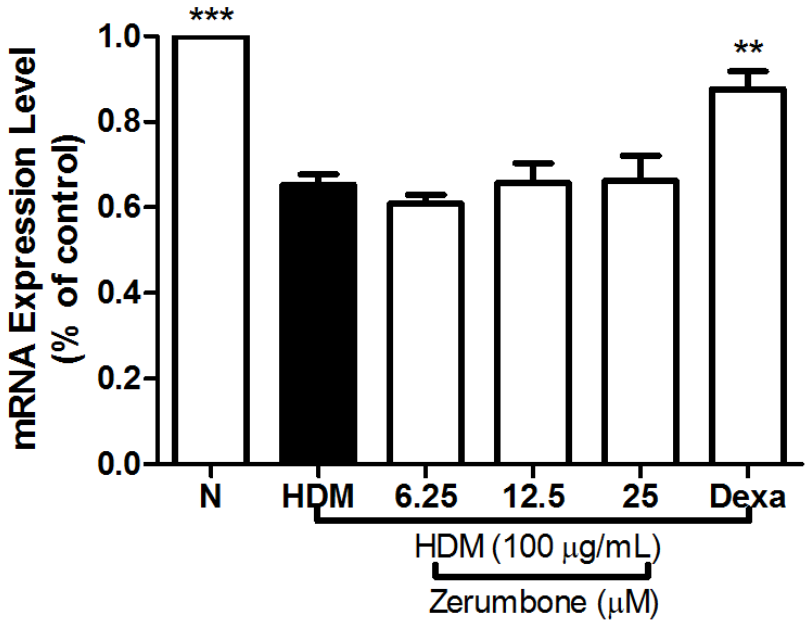

Figure 17: Zerumbone Does Not Regulate the mRNA Expression of Occludin in HDM-induced 16HBE14o- Cells. The cells were seeded on a 6-well plate until 100% confluent. The cells were treated with HDM and zerumbone for 6 hours. RNA was extracted and transcribed and followed by qPCR. The treatment groups were labelled as follows: N: Normal group; HDM: HDM-induced group; 6.25, 12.5 and 25: Co-treated with HDM and three different concentrations of zerumbone. Each bar represents three different concentrations of zerumbone used in the study; Dexa: Co-treated with HDM and 10 μM dexamethasone. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. **p ≤ 0.01 and ***p ≤ 0.001 significantly different from HDM group.

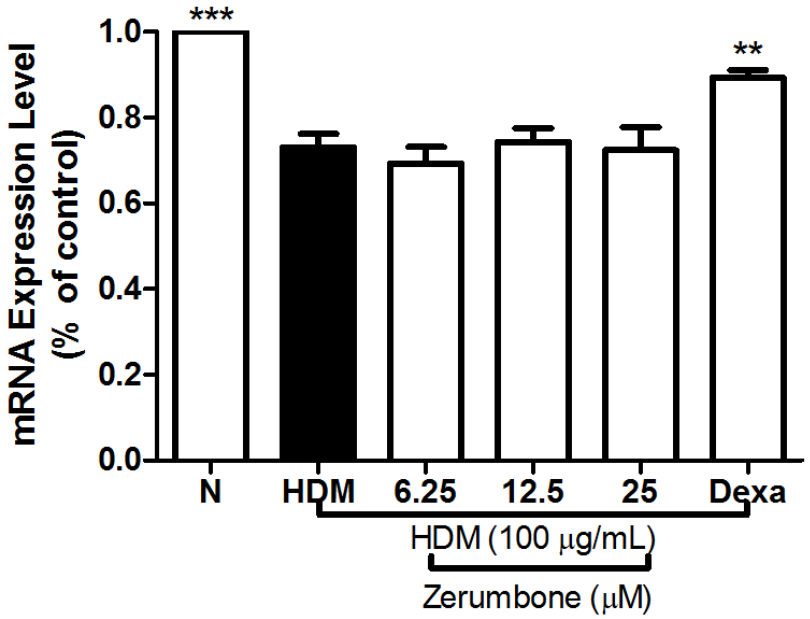

Figure 18: Zerumbone Does Not Regulate the mRNA Expression of ZO-1 in HDM-induced 16HBE14o- Cells. The cells were seeded on a 6-well plate until 100% confluent. The cells were treated with HDM and zerumbone for 6 hours. RNA was extracted and transcribed and followed by qPCR. The treatment groups were labelled as follows: N: Normal; HDM: HDM-induced group; 6.25, 12.5 and 25: Co-treated with HDM and zerumbone. Each bar represents three different doses of zerumbone used in the study; Dexa: Co-treated with HDM and 10 μM Dexamethasone. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 significantly different from HDM group.

From the experiment, it was shown that zerumbone treated group does not possess significant difference compared to the HDM-induced group. Thus, concludes that zerumbone does not possess protective effect on mRNA expression level of both occludin and ZO-1 in HDM-induced airway epithelial 16HBE14o- monolayer.

CHAPTER 5

DISCUSSION

5.1 Introduction

Asthma has been one of the most prevalent airway diseases in the world. The exposure to household common allergens has causes such diseases to occur. The interaction of the immunological cells and allergen such as HDM causes cascade of immunological reactions such as inflammation. The continuous inflammatory condition will then results to chronic condition such as asthma. Thus, to prevent the allergen from interacting with the immunological cells was by studying the barrier protective effect of a compound such as zerumbone which may help in developing a treatment to such condition and may then provide a solution to more chronic condition such as asthma. Therefore, the important conditions that were studied related to the maintenance of the epithelial barrier after being exposed to allergen. Thus, the study design involved the exposure of the 16HBE14o- monolayer to HDM and zerumbone simultaneously to study the effect of zerumbone on allergen-exposed epithelial monolayer. The epithelial barrier properties possessed by the epithelial lining of the airway system is contributed majorly by the presence of junctional proteins. Thus, it is important to assess the effects of zerumbone on HDM-exposed junctional proteins of the epithelial monolayer. Epithelial cell lines was used to mimic the airway epithelial barrier. There are few cell lines which are available as a tool in studying the airway system. Among the most well-known cell lines are Calu-3, A549, BEAS-2B and 16HBE14o- (Winton, et al., 1998). A549 and BEAS-2B has low TEER reading in addition of BEAS-2B having no functional tight junctions making these two cell lines unsuited for this study (Bhowmick & Gappa-Fahlenkamp, 2016). Calu-3 cell line has initially being used in this study, however, due to its slow growth rate it has impedes the progress of the study. Therefore, 16HBE14o- was used as *in vitro* model of the human airway system as it possesses characteristics similar to the epithelial cells exist *in vivo* such as the formation of junctional systems (Vllasaliu, Fowler, Gamett, Eaton, & Stolnik, 2011; Winton, et al., 1998)

5.2 Zerumbone Protects the Junctional Integrity of the HDM-induced Airway Epithelial Barrier Disruption

A way to assess the junctional proteins properties is by using the TEER assay which is an assay used to assess the integrity of a cell monolayer by using the measure of resistance across the monolayer. Higher resistance reading possessed by an epithelial monolayer indicates greater junctional integrity possessed by the monolayer. HDM was predicted to possess low resistance reading in TEER as the allergen cause the epithelial monolayer to have reduced junctional integrity in the epithelial monolayer. Similar result has been shown from a study done by (Post, et al., 2011) in which the research group carried out

an experiment to compare the effect of different HDM extracts on the barrier properties of 16HBE14o- cells monolayer. It has been proven from the study, Greer HDM extract, which was used in this study, able to reduce the resistance reading of the 16HBE14o- monolayer more significantly compared to other types of HDM extract. As for the zerumbone-treated group, the resistance reading has shown significant increase as compared to the HDM group and thus indicating the ability of the compound in maintaining the integrity of HDM-induced 16HBE14o- monolayer. However, this only proves that zerumbone possess the ability to maintain the structural integrity of the monolayer. Zerumbone should also have protective effect on the function of the HDM-induced junctional protein of the monolayer for it to serves the purpose in searching an alternative treatment to the allergen-induced inflammation. Therefore, the effect of zerumbone on the permeability of HDM-induced 16HBE14o- monolayer was studied by using the FITC-Dextran permeability assay.

5.3 Zerumbone Protects the Junctional Permeability of HDM-induced Epithelial Barrier Disruption

HDM-induced 16HBE14o- monolayer was expected and found to be the most permeable as the junctional protein has been disrupted and thus disable the junctional proteins to reduce the passage of dextran from the apical to the basal compartment of the epithelial monolayer. From this study, it was shown that zerumbone-treated 16HBE14o- monolayer was significantly less permeable compared to the HDM-induced 16HBE14o- monolayer. Thus, this has proven that zerumbone does protect the structural and functional aspect of the HDM-induced 16HBE14o- monolayer by inhibiting the increase passage of dextran to the basal compartment of the epithelial monolayer. Similar results has also been demonstrated in a study done by (Dong, et al., 2017) in which the epithelial monolayer was less permeable in epithelial monolayer with high resistance reading. However, it is not sufficient to only figure out the effect zerumbone possess on the integrity and permeability of the monolayer but it is also important to ultimately search for exactly which part and how does zerumbone actually affects the HDM-induced epithelial monolayer.

5.4 The effects of zerumbone on the Junctional Proteins Localization and Expression in HDM-induced 16HBE14o-

Thus, the localization of the junctional proteins was studied by using immunofluorescence and zerumbone was shown to be able to maintain the localization of both junctional proteins studied, occludin and ZO-1, in HDM-induced epithelial monolayer. Significant difference can be seen as the occludin and ZO-1 which had been induced with HDM for 24 hours shows jagged and discontinuous localization while zerumbone-treated group showed localization of both junctional proteins in “honey-comb”-like shape and continuous in expression. As in (Wan H. , et al., 1999) HDM was proven to possess the ability to cause junctional permeability by directly cleaving the junctional proteins, there are expectations in which zerumbone protective effect on the barrier properties

of HDM-induced epithelial monolayer maybe due to its ability in protecting the expression of the junctional proteins. However, both immunoblotting and qPCR assay results carried out in this study show that zerumbone protective effect does not came from its ability to protect the expression of junctional proteins, occludin and ZO-1 as the compound possess no significant effect on the expression of both proteins in HDM-induced 16HBE14o- monolayer. Similar findings has also been reported by (Dong, et al., 2017) in which HDM causes hyperpermeability in 16HBE14o- monolayer by affecting the localization of the junctional protein E-cadherin. However, HDM does not affect the E-cadherin expression of the 16HBE14o- monolayer in the experiment carried out by using immunoblotting. Thus, the researcher emphasized that HDM may have impact on the E-cadherin/β-catenin complex. On the other hands, it has been reported by Grainge & Davies (2013) that the defect in the formation of the tight junction in asthmatic patient was not resulted purely due to the localization but it can be caused by decreasing level of common tight junction proteins despite the normal level of mRNA. This is just to show that the permeability of the epithelial monolayer can be affected either through the localization or the expression level of the junctional proteins. It is not necessarily true that the junctional proteins have to be affected in both aspects for the compound to be proven to possess an effect on the epithelial monolayer.

5.5 The Effect on the Hyperpermeability of the Epithelial Barrier Also Affected By the Action of Actin Cytoskeleton

As has been mentioned previously, occludin is a transmembrane protein which is anchored to ZO-1 which is directly linked to the actin cytoskeleton. Previous studies has shown that alteration on the actin cytoskeleton possess an effect on the epithelial permeability (Rodgers & Fanning, 2011). It has also been reported that the permeability properties of the epithelial monolayer and reorganization of the junctional proteins also can be affected by the interaction of actin cytoskeleton and the ZO proteins which functions as the junctional adaptor at the cytoplasm of the epithelial cells (Matter, Aijaz, Tsapara, & Balda, 2005). It was also reported that the inhibition of the RhoA/MLC signalling worsen the effect of HDM on the bronchial epithelial barrier dysfunction (Dong, et al., 2017). This has shown that actin cytoskeleton possess significant role in the HDM-induced hyperpermeability. Therefore, a study should be carried out to investigate whether the protective mechanism of zerumbone on the HDM-induced barrier disruption originates from its effect on actin cytoskeleton. As the junctional systems are comprises of multiple types of proteins which may thus leads to the barrier properties of the epithelial lining, it is necessary to study the effect of zerumbone on these proteins to further understand its protective mechanism on epithelial cells exposed to allergen.

This study was carried out with the purpose of investigating whether zerumbone possess effect on HDM-induced barrier dysfunction and it has served the purpose as zerumbone has been proven to possess protective effect on the HDM-induced hyperpermeability. However, the exact mechanism of the protective effect has to be further studied in depth to obtain deeper

understanding on the protective mechanism possessed by this compound on HDM-induced 16HBE14o- cell monolayer.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

6.1 Summary

Epithelial barrier plays pivotal role in maintaining the health of the airway system. Junctional system which binds these epithelial cells together to form the lining are susceptible to the disruption caused by allergen inhaled during respiration. House dust mite which is the common source of allergens carries Der p 1 which possess proteolytic properties which contributes to the degradation of the junctional proteins. Zerumbone, the active compound extracted from the plant, was known to possess many therapeutic effects including anti-inflammatory properties. Thus, this study was carried out to investigate the effect possessed by zerumbone on the HDM-induced junctional proteins in 16HBE14o- cells. 6.25, 12.5 and 25 μM of zerumbone were selected as the three concentrations used throughout the study as it was proven that these concentrations of zerumbone do not possess cytotoxic effect on 16HBE14o- cells. It has also been proven in this study that zerumbone possess a protective effect on the junctional integrity and permeability of HDM-induced 16HBE14o- cells. Zerumbone also showed protective effect on HDM-induced localization of junctional proteins in 16HBE14o- cells. On contrary, the protein expression and gene expression studies showed that zerumbone does not possess effect on the HDM-induced junctional proteins expression in 16HBE14o- cells.

6.2 Conclusion

This study has shown that zerumbone preserve the junctional integrity and permeability of epithelial barrier by inhibiting the redistribution of junctional proteins such as occludin and ZO-1 in HDM-induced 16HBE14o-. Interestingly, zerumbone does not regulates the expression of occludin and ZO-1, either the transcriptional.

6.3 Recommendation

Previous study has shown that actin cytoskeleton do possess effect on permeability as the ZO proteins are directly anchored to the cytoskeleton. Therefore, further study on the effect of zerumbone on the actin cytoskeleton of HDM-induced 16HBE14o- cells should be carried out for further understanding of the protective mechanism of the compound on HDM-induced epithelial barrier disruption. As this study can only cover the superficial surface of the problem, deeper understanding on the protective effect of zerumbone on HDM-induced

epithelial barrier dysfunction can be further studied by investigating the signalling pathways involved in the process.

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