PHYTOCHEMICAL PROFILES, ANTI-ASTHMATIC AND IMMUNOMODULATORY EFFECTS OF *Lignosus rhinocerus* IN AIRWAY INFLAMMATION MODEL

by

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Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

August 2016

DECLARATION

I declare that all the contents presented in this thesis are my original research work. The research was conducted at Craniofacial Science Laboratory, School of Dental Sciences, School of Health Sciences and School of Medical Sciences, Universiti Sains Malaysia, Health Campus, Kubang Kerian, Kelantan. The thesis has not been previously submitted for any other degree elsewhere either in part or in full.

JOHNATHAN A/L MALAGOBADAN

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor, Dr. Nurul Asma Abdullah for the opportunity to work on this research. I am very thankful for her patience, motivation, enthusiasm and immense knowledge that guided me. Also I am truly honoured to my Co-supervisors. My sincere appreciation to Prof. Gan Siew Hua for her care and advices throughout the research. Thanks to Dr Wan Ezumi Mohd Fuad and Dr Wan Majdiah for their insightful comments, guidance and encouragement. Their supervisions have strengthened my research from various perspectives and helped me throughout my PhD candidature in Universiti Sains Malaysia.

I would also like to thank Siti Nurshazwani, for being very supportive and helpful. Thanks to Siti Aminah, Alphy, Zati for their co-operation and support. I am obliged to my friends Nurul Hidayat, Shifat A Nowrin, Fazal Shahid khan for their moral support and guidance over the years. I am also thankful for the members of Craniofacial Lab (PPSG), for helping me during difficulties in performing laboratory work, Ms. Asiah Abu Bakar, Ms. Siti Fadilah Abdullah, Mr. Marzuki Yusof, Mr. Mohd Yusof Soon Abdullah, Ms. Eda Sharip, Ms. Khadijah Mohd Ali, Mr. Mohamad Hairie Sahabudin and Mr Mohamad Ezany Yusoff in the Craniofacial Laboratory. My appreciation and thanks to PPSK laboratory and research facilities, Dr Faezatul Arbaeyah Hussein from Department of Pathology, Mr Jamaruddin from Department of Immunology, as well as Animal Research and Service Centre (ARASC) who have offered their unconditional support at some point during my research.

I extend my most heartfelt gratitude to my Mother for her prayers, trust and unconditional love, my Father for being a life mentor, my wife for the inspiration for my success, my brother and sister for their faith throughout my years. Finally, thanks to Shaminea for being a great strength during the hard times, Prakash, Shalini, Moses and Nanthini for always encouraging me and being my strongest supporters. I would not have been able to complete this journey without them. My deepest appreciation credited to the Ministry of High Education, Malaysia for awarding me MyBrain 15 (MyPhD). This research project was financially supported By Research University Grant (RU) from Universiti Sains Malaysia (1001/PPSG/813065).

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

x	Multiply
%	Percentage
°C	Degree celsius
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
ml	Millilitre
mM	Millimolar
µl	Microliter
µm	Micrometre
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APC	Allophycocyanin
BALF	Bronchoalveolar lavage fluid
B.p	Bordetella pertussis
CO_2	Carbon dioxide
CRD	Crude
CWE	Cold water extract
DC	Dendritic cells
DEPC	Diethyl pyrocarbonate treated water
dH_2O	Distilled water
DPX	Distrene, Plasticiser, Xylene mounting medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbant assay
et al.	et alii- and others
FACS	Fluorescent-activated cell sorter
FITC	Fluorescein isothiocyanate
GC-MS	Gas Chromatography Mass Spectrometry
HWE	Hot water extract
HRP	Horseradish peroxide
HCl	Hydrochloric acid
IFNγ	Interferon gamma
IL	Interleukin
i.p.	Intraperitoneal
MHC	major histocompatibility complex
mRNA	Messenger ribonucleic acid
ng	Nano gram
nm	Nanometre
OD	Optical density
OVA	Ovalbumin
PAS	Periodic Acid Schiffs

PBS	Phosphate buffered saline
PE	Phycoerythrin
PERCP	Peridinin chlorophyll
PHA	Phytohemagglutinin
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
T cell	Thymus-derived lymphocyte
TGF	Transforming growth factor
TGF-B1	Transforming growth factor beta 1
Th 2	T helper 2
Th 17	T helper 17
TIC	Total Ion Chromatogram
TNF	Tumor necrosis factor
Treg	Regulatory T cell

PROFIL FITOKIMIA, KESAN ANTI-ASMA DAN IMMUNOMODULASI DARIPADA *Lignosus rhinocerus* DALAM MODEL KERADANGAN SALUR PERNAFASAN

ABSTRAK

Lignosus rhinocerus (*L. rhinocerus*) atau lebih dikenali sebagai cendawan susu harimau, digunakan secara tradisional di kalangan masyarakat tempatan di Malaysia bagi merawat pelbagai penyakit termasuk asma. Walau bagaimanapun, keberkesanannya dalam rawatan asma dan potensi immunomodulasinya masih belum disahkan secara saintifik. Selain itu, maklumat tentang juzuk aktif cendawan ini juga jarang dilaporkan. Kajian ini mengkaji juzuk mudah meruap daripada ekstrak air panas (HWE), ekstrak air sejuk (CWE) dan *L. rhinocerus* mentah (CRD) dengan menggunakan kromatografi gas-jisim spektrometri (GC-MS). Kesan potensi ekstrak-ekstrak *L. rhinocerus* terhadap aktiviti anti-asma telah disiasat pada tikus jantan Sprague Dawley yang telah dicetuskan keradangan pada saluran udaranya dengan menggunakan ovalbumin (OVA). Kesan immunomodulasi pula telah dikaji pada mencit BALB/c. Pengekstrakan berjujukan ke atas *L. rhinocerus* menggunakan lima pelarut (eter petroleum, eter, heksana, etil asetat dan metanol) telah dijalankan sebelum analisis GC-MS. Secara keseluruhan, 44 juzuk telah dikenal pasti daripada analisis CRD *L. rhinocerus*. Sebatian daripada kumpulan asid lemak adalah komponen yang paling utama (68.58%) manakala kompaun yang paling utama adalah asid linoleik (49.39%). Analisis GC-MS HWE pula mengenalpasti 18 juzuk dengan kompaun yang paling utama juga adalah asid linoleik (21.35%). Sebaliknya, GC-MS CWE hanya menunjukkan kehadiran metilsiklopentana (97.74%) dan sikloheksana (2.26%) sahaja. Untuk kajian asma, tikus jantan Sprague Dawley

disensitasi dengan 2 suntikan intraperitoneal 10 mg/ml OVA yang diemulsifikasi dalam 100 mg/ml aluminium hidroksida bersama-sama 50 ng/ml *Bordetella pertussis* pada hari 0 dan 14 diikuti rawatan ektrak *L. rhinocerus* pada hari ke-21 selama 7 hari. Dos yang optimum bagi *L. rhinocerus* HWE ialah 500 mg/kg, CWE ialah 250 mg/kg dan CRD pula 250 mg/kg. Rawatan dengan ekstrak *L. rhinocerus* telah berjaya menambahbaik beberapa parameter yang berkaitan asma di dalam tikus yang diuji. Rawatan dengan HWE telah berjaya mengurangkan jumlah eosinofil dalam BALF, IgE dalam serum, sitokin Th2 (IL-4, IL-5 dan IL-13) dalam BALF dan infiltrasi eosinofil dalam paru-paru. Analisis sitometri aliran mendedahkan bahawa rawatan dengan HWE telah mengurangkan eosinofil dan meningkatkan sel T-regulatori berbanding CRD dan CWE. Sebanyak 21 gen yang berkaitan penyakit asma telah berjaya di kawal atur selepas rawatan HWE. Kesan immunomodulasi ekstrak *L. rhinocerus* HWE telah dijalankan di dalam model *in vitro* dan *in vivo.* Mencit yang dirawat dengan ekstrak HWE *L. rhinocerus* menunjukkan penambahan populasi splenosit terutamanya populasi sel-sel $CD3^+$ $CD4^+$ dan $CD3^+$ $CD8^+$ berbanding kumpulan mencit yang tidak dirawat. Kajian ini juga menunjukkan proliferasi splenosit dan aktiviti fagositosis yang bertambah baik dan pengeluaran NO yang terkawal. Kajian ini menyimpulkan bahawa *L. rhinocerus* mempunyai potensi untuk digunakan sebagai alternatif bagi rawatan alergik asma.

PHYTOCHEMICAL PROFILES, ANTI-ASTHMATIC AND IMMUNOMODULATORY EFFECTS OF *Lignosus rhinocerus* IN AIRWAY INFLAMMATION MODEL

ABSTRACT

Lignosus rhinocerus (*L. rhinocerus*) or locally known as Tiger's Milk mushroom, is traditionally used in the treatment of various diseases including asthma by indigenous communities in Malaysia. However, to date, its efficacy on asthma and its immunomodulating potential have not been confirmed by scientific studies. Also, there is sparse information available on its active constituents. This study investigated volatile constituents of hot water extract (HWE), cold water extract (CWE) and crude (CRD) *L. rhinocerus* using gas chromatography mass spectrometry (GC-MS). The anti-asthmatic potential of *L. rhinocerus* extracts was investigated on ovalbumin (OVA)-induced airway inflammation Sprague Dawley rats. Immunomodulatory effects of *L. rhinocerus* were carried out in BALB/c mice. Sequential extractions of *L. rhinocerus* using five solvents (petroleum ether, diethyl ether, hexane, ethyl acetate and methanol) were conducted prior to GC-MS analysis. Overall, 44 constituents were identified from CRD *L. rhinocerus*. Compounds from the fatty acid group were the most predominant (68.58%) and the main constituent was linoleic acid (49.39%). GC-MS analysis of HWE *L. rhinocerus* identified 18 constituents with the main compound also linoleic acid (21.35%). In contrast, CWE only demonstrated the presence of methylcyclopentane (97.74%) and cyclohexane (2.26%). Male Sprague Dawley rats were sensitized with two intraperitoneal injections of 10 mg/ml OVA emulsified in 100 mg/ml aluminium hydroxide with the presence of 50 ng/ml of *Bordetella pertussis*, on days 0 and 14 followed by treatment

with *L. rhinocerus* extracts on day 21 for 7 days. Optimization study indicated optimized dosage for *L. rhinocerus* HWE was 500 mg/kg, CWE (250 mg/kg) and CRD (250 mg/kg). Treatments with *L. rhinocerus* extracts significantly ameliorated related asthmatic parameters in the induced rats. Specifically, HWE comparatively reduced eosinophils numbers in BALF, IgE in serum, Th2 cytokines (IL-4, IL-5 and IL-13) levels in BALF, and eosinophil infiltrations in the lungs than the CWE and CRD. Flow cytometry analysis revealed HWE to reduce eosinophils and improved T regulatory cells compared to CRD and CWE. A total of 21 asthma related genes were successfully down-regulated with HWE treatment. Immunomodulatory effects of HWE were studied in *in vitro* and *in vivo* model. Treatment with HWE *L. rhinocerus* extract demonstrated improved splenocyte population mainly of $CD3^+CD4^+$ and $CD3^+CD8^+$ when compared with untreated mice groups. This study also demonstrated improved splenocytes proliferation and phagocytosis activity and controlled NO production. In conclusion, *L. rhinocerus* has the potential to be used as an alternative for the treatment of allergic asthma.

CHAPTER ONE

INTRODUCTION

1.1 Asthma

The term ‘allergy’ was perceived by Von Pirquet in 1905 (Mekori, 1996). Allergic disorder is the consequence of an exaggerated reaction against innocuous environmental proteins or antigens. Some of the common allergic disorders include asthma, eczema, allergic rhinitis, anaphylaxis and autoimmune disorders (Porter *et al.*, 2011). Despite continuous exposure to harmless antigens, some individuals experience adverse immunological responses to these antigens. This could be supported with the fact that normal immune response to allergens is linked with the initiation of tolerance. Thus, retraction of tolerance leading to further tolerance may cause induction and prolongation of active immune responses (Romagnani, 2000). There are four types of allergic responses based on the mechanism of immunological involvement which are known as Types I to IV allergic reactions. Immediate hypersensitivity reaction is the Type I allergic reaction where major components involve allergen, immunoglobulin (Ig) E antibody, mast cell and their mediators in which asthma is a known example (Tortora and Derrickson, 2008).

Asthma is a disease affecting 300 million lives globally and is a result of complex interplay between genetic and environmental factors (WHO, 2013). It is a disease characterized by persistent wheezing, shortness of breath, chest tightness and coughing (Konno *et al.*, 2002). The airway disorder is commonly characterized by airway eosinophil infiltration, airway hyperresponsiveness (AHR), excessive airway mucus production and increased serum IgE and T-helper 2 cytokine levels including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13)

(Wardlaw *et al.*, 1988; Lukacs, 2001; Elias *et al.*, 2003). Generally asthma is mediated by an over exuberant response of Th2 inflammatory responses, leading to exaggerated airway eosinophilia infiltration and lung mucus production (Issekutz and Sapru, 2008).

1.1.1 History

Ancient ways of treatment and drugs used against asthma have been documented for as long as 5000 years ago (Huntley and Ernst, 2000). Various countries have contributed to this knowledge including Ancient China, Egypt, Greece, Rome and India. It is apparent that their medicinal approach, specifically for asthma, was closely interwoven with religious and cultural practices. One of the earliest document called 'Su Wen' from China, reported on how asthma is interpreted by seasonal aspect of the respiratory disease (Cserhati, 2004). This writing is dated by scholars between 475 and 221 BC (Veith, 2002). It explains that the disease when located on lung region is improved during winter season and it will escalate and worsened by summer. It has also been reported that if the disease withstand and continued without any mortalities, the conditions can be warded off during summer evening although the disease can exarcebate again during autumn. Such common environmental factors of asthma is also reported in other regions in the world (Cserhati, 2004).

In Greece, Hippocrates the Great, also known as 'father of medicine' (460-377 BC) was one of the first to establish the relationship between the environment and respiratory ailments (Chadwick, 1950). The original term of asthma came from the verb "azein" which in Greek means exhalation with an open mouth and to pant (Marketos and Ballas, 1982). The term was continued among Greek physicians in

describing the degree of respiratory defects. On another account, asthma also means for ‘wind’ or ‘to blow’ in Greek (Cserhati, 2004).

In Chinese culture, the ancient healers named asthma as ‘xiao-chiran’ which means wheezy breathing as a result of imbalance in the life force, “Qi”. Therefore, the imbalance life “Qi” has to be restored by means of herbs, acupuncture, massage, diet and exercises (Lao *et al.*, 2012). Meanwhile, Indian medicinal practitioners related breathing difficulties to human’s soul in connection to the mind, body and spirit in which yoga was often the most applied method of meditation along with breathing techniques (Singh, 2006).

1.1.2 Prevalence of asthma

It is estimated that 250,000 people die every year due to asthma (Bateman *et al.*, 2008; WHO, 2013). According to the World Health Organization (WHO), the number of asthma patients are increasing and is expected to reach to 400 million by 2025 (Pawankar, 2014). The disability-adjusted life years (DALYs) lost rate due to asthma worldwide is estimated to be 15 million per year which is comparable with diabetes, liver cirrhosis and schizophrenia (Bousquet *et al.*, 2005). In addition, asthma has been estimated to account for at least one death for every 250 mortality worldwide (Masoli *et al.*, 2004). Poor management and uncontrolled asthmatic condition often affected the quality of life and daily activities of the sufferers (Haselkorn *et al.*, 2010). In some cases, it causes depression and subsequently leads to increased hospital visits (Sullivan *et al.*, 2007).

In Asia, asthma mortality rates in more affluent areas such as Hong Kong and Japan, are similar to those reported in Western countries (Lai *et al.*, 1996). The severity of

asthma and control measures are important in evaluating patients and their response towards treatment not only in public health registries but also in research (clinical trials, epidemiologic, genetic and mechanistic studies) (Bousquet *et al.*, 2010). Therefore, the Global Initiative for Asthma (GINA) was developed by WHO in collaboration with the US National Heart, Lung, and Blood Institute to establish a more generally accepted diagnostic and management strategies in its proper diagnosis.

1.1.3 Asthma in Malaysia

In Malaysia, approximately 80% to 90% of asthmatic children and young adults were found to have allergy asthma (Gendeh *et al.*, 2004). The International Study of Asthma and Allergies in Childhood (ISAAC III) has estimated that the prevalence of asthma in Malaysia has increased from 6.4% to 9.4% in children from between 6 and 7 years of age to 9% to 13% among children between 13 and 14 years (Pearce *et al.*, 2007; Yadav *et al.*, 2014). Allergic asthma is common among children and often related to genetic inheritance. On the other hand, non-allergic asthma (20%) is commonly found in middle-aged and the elderly. Statistical studies conducted among Malaysians reported that children with asthma showed various responses to specific allergens. Approximately 90% of patients are allergic to house-dust mites, 67% to cockroach, 23% to cat dander or dog epithelium and 10% to 22% to foods including cow's milk, soya bean, egg, peanut, fish, shrimp, crab, banana, and wheat (Gendeh *et al.*, 2004).

1.1.4 Aetiology

The development of asthma involves both exogenous and endogenous factors as evidenced by its onset at the beginning of infancy or childhood (Dietert, 2011). Combination of genetic predisposition with environmental factors affect its severity and responses to treatment (Choudhry *et al.*, 2007). Factors that further provoke the allergic reactions or irritate the airways includes indoor allergens including cockroaches, mold, house dust mites in bedding, carpets and pet dander (Arshad, 2010; Kelly and Fussell, 2011). Surprisingly, indoor volatile organic compound such as formaldehyde is also a known trigger (McGwin Jr *et al.*, 2010). Phthalates in PVC has been also associated with asthma in children as well as adults (Bornehag and Nanberg, 2010). Meanwhile, perfumes are common cause of acute attacks in women and children (Lessenger, 2001). Chemical irritants in the workplace, air pollution, other environmental chemicals and low air quality caused by traffic pollution or high ozone levels has been widely reported as causes of asthma (Kroegel, 2009). Smoking during and after pregnancy delivery is highly associated with a greater risk of asthma (Bousquet *et al.*, 2007). Moreover, psychological stress is also believed to worsen the symptoms, where it alters immune system thus increases the airway inflammatory response to allergens and irritants (Gold and Wright, 2005).

Some viral respiratory infections, such as respiratory syncytial virus and rhinovirus, cause high risk of asthma when acquired as young children (Murray John and Nadal Jan, 2000) and infections of upper respiratory tract can further worsen the asthmatic condition (Baxi and Phipatanakul, 2010). Nutritional aspect also plays a major role in the development of allergic disease. Deficiency of vitamins, fatty acids, antioxidants

sources as well as imbalance of the gut micro flora causes interference of this homeostasis and leads to development of allergy (Holgate and Polosa, 2008). Family history is also one of the contributing factor for asthma development (Burke *et al.*, 2003).

1.1.5 Types of asthma

Asthma can be divided into several types, namely allergic, non-allergic, exercise induced, nocturnal, occupational and steroid resistant asthma (Paliwal, 2012). Allergic asthma is highly related to family history of allergic diseases such as eczema and allergic rhinitis (Bel, 2004; Moore *et al.*, 2010; Wenzel, 2012). Non-allergic asthma (intrinsic) on the other hand, occurs when there is no allergy involved (Paliwal, 2012). Many non-allergic asthma populations experience exercise-induced asthma. In nocturnal asthma, patients experience asthma when they are sleeping (Yawn, 2008). Long periods of direct exposure to chemical fumes, wood dust or other irritants leads to occupational asthma. Lastly, steroid-resistant asthma is caused by steroid overdose which leads to severe asthmatic attack that may not respond to medication and may require ventilation (Paliwal, 2012). Other common phenotypes identified include late-onset asthma which occurs particularly among women for the first time in adult life and asthma with fixed airflow limitation which is caused by airway wall remodelling. Asthma also occurs in an obese where such patient may have noticeable respiratory symptoms with slight eosinophilic airway inflammation (Bel, 2004; Moore *et al.*, 2010; Wenzel, 2012). Previously the WHO also proposed a uniform definition of severe asthma in 2009. Finally, following a review by the Global Alliance against Chronic Respiratory Disease, severe asthma is classified into

three groups; (1) untreated severe asthma, (2) difficult-to-treat severe asthma and (3) treatment-resistant severe asthma (Bousquet *et al.*, 2010).

1.1.6 Immunopathogenesis

Asthma and other allergic related mechanisms are known to be a T helper 2 (Th2) dominated response. The response is initially triggered when an airway allergen is taken by antigen presenting cells and presented to specific Th cells by means of major histocompatibility complex (MHC) class II followed by initiation of the immunological synapse (Valenta *et al.*, 2003). This activated allergen-specific T helper cells then polarize into Th1 or Th2 effector populations, followed by differentiation into a Th2 subpopulation. These activated Th2 cells then engage in recruiting and activating cytokines such as interleukin (IL)-4, IL-5 and IL-13 followed by stimulation of eosinophil. At the same time, B lymphocytes produce antigen-specific IgE that binds to its high-affinity receptor on the mast cells to enable mast cell degranulation by crosslinking (Amin, 2012) leading to IgE release and binding to IgE receptors on mast cells, basophils, and eosinophils (Maes *et al.*, 2014). Upon allergen crosslinking with mast cell-bound IgE, a large number of preformed and newly synthesized mediators are released, including histamine, cysteinyl leukotrienes and proteases (Figure 1.1) (Holgate and Polosa, 2008). Generally, the immediate release of histamine and leukotrienes occurs before the prostaglandins release, followed by the production of cytokines after a few hours (Maes *et al.*, 2014). In an allergic individual, the IgE-sensitized mast cells degranulates in a very short period of making contact with the allergen as an early allergic reaction (Holgate and Polosa, 2008). The mediators released basically causes vascular permeability, bronchoconstriction and excessive mucus production which

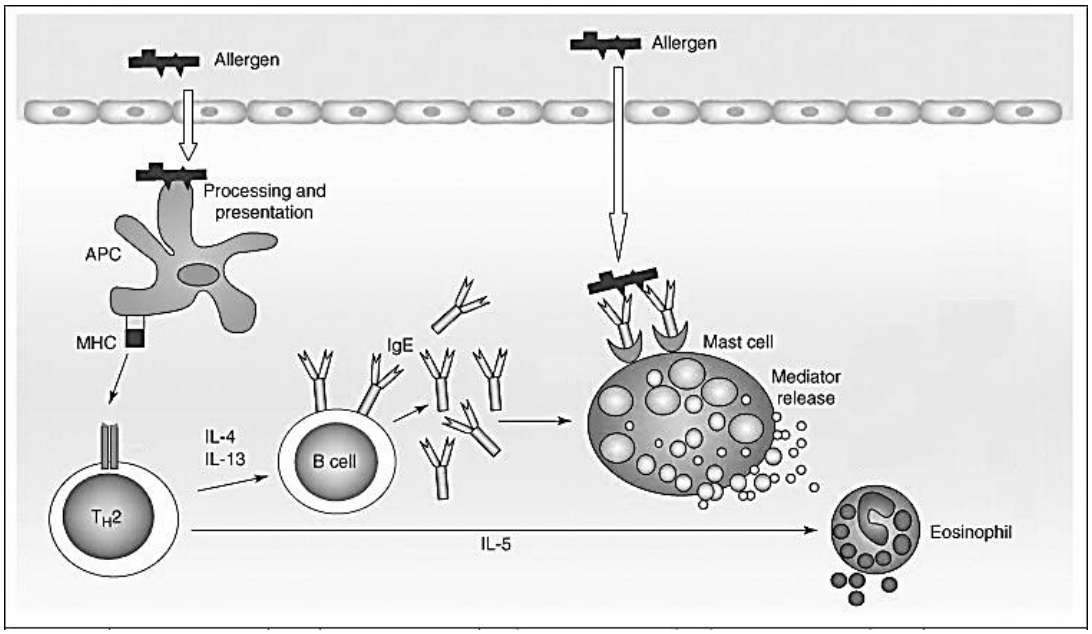

Figure 1.1: Initiation of immunological cascade after allergen exposure. Allergen is taken by APC and presented to specific Th2 cells by means of major histocompatibility complex (MHC) class II. The activated IL-4 and IL-13 stimulates B-cell for IgE, while IL-15 regulates eosinophils recruitments. Upon repeated exposure, allergen crosslinking with mast cell-bound IgE and leads to mediators release by degranulation thereby further exacerbating the allergic inflammatory responses. Adapted from (Singh and Bhalla, 2008). Abbreviations: APC, antigen presenting cells; Th2, T-helper 2; IL-, interleukin-, IgE, immunoglobulin E.

subsequently intensify the allergic manifestations (Holgate and Polosa, 2008; Amin, 2012). The roles of prostaglandin E_2 (PGE_2) and nitric oxide (NO) have been highlighted as their upregulation has been identified to allow enabling histamine to modulate the cytokine networks upon allergic reactions (Packard and Khan, 2003).

Recently, the involvement of regulatory T (Treg) cells which is another important subset of CD4+ T cells have been highlighted for its protective role in suppressing Th2 cell responses in allergic inflammations (McAlees *et al.*, 2015). Their mechanisms are specifically associated with the neutrophilic inflammatory events that follows disease exacerbation and tissue remodelling (Holgate and Polosa, 2008). In addition, they also directly inhibit the proliferation of effector T cells or through antigen-presenting cells (Workman *et al.*, 2009). Th2-mediated airway inflammation in asthma may also result from the suppression of Tregs (Boulet *et al.*, 2012), which is being studied for potential therapeutic focus in treating asthma (Jin *et al.*, 2013). Development of asthma is unclear and may lead to worse condition if left untreated. An exacerbated hyper reaction and symptoms of allergy may affect the respiratory, gastrointestinal, nervous and even cardiovascular systems. In addition, severe complex condition of allergy, namely anaphylaxis, can also occur resulting in an anaphylactic shock with hypotension and finally leading to death (Abril-Gil *et al.*, 2012).

Currently, many studies mainly focus on various parameters such as neutrophils, eosinophils, immuglobulin and interleukins on the levels of which are variable in asthmatic patients. Although various studies reported that neutrophil is associated with acute bronchial hyperresponsiveness and is claimed to be active participant in epithelial damage, the presence of several neutrophils in epithelium is noted to be a normal phenomenon, since they are also reported to be present in the bronchial lavage fluid (BALF) of normal subjects (Kaliner *et al.*, 1990). In addition, eosinophils serve as an important marker during the development and expression of allergic inflammation. These multifunctional granulocytes are produced and derived in the bone marrow from myeloid progenitors (Stevens *et al.*, 2007). Eosinophils secrete various proteins including eosinophil cationic protein, peroxidase and histamines (MacKenzie *et al.*, 2001; Humbles *et al.*, 2004). During an exaggerated asthma reaction, eosinophils accumulate in the respiratory tracts and infiltrate the lung regions (Stevens *et al.*, 2007) which allows their quantitation as a standard measurement in inflammation on murine allergic airway sensitization models, including those exposed to chicken egg albumin administration for sensitization (Varga *et al.*, 2000; Varga *et al.*, 2001).

Since IgE is involved in allergy development it is also associated with asthma. Generally, IgE has a very short life and is present in low amount compared to other immunoglobulin (Oettgen and Geha, 1999). However, the low concentration of IgE in the circulation does not reveal its true allergic activity and therefore, they are highly sensitive to allergens despite being present in low concentration in the circulation (Platts-Mills, 2001).

Cytokines such as interleukin plays important roles as mediators amid leukocytes and mediates inflammation in asthmatic patients. Increased expression of Th2 cytokines (IL-4, IL-5 and IL-13) has been reported in the BALF of asthmatic patients. It has been highlighted that the imbalance between Th1 [interferon gamma (IFN-γ) and IL-2] and Th2 (IL-4, IL-5,and IL-13) cytokines is an important mechanism that leads to asthma (Busse and Lemanske, 2001). IL-4 has been hypothesized for its key role in the pathogenesis of asthma. It has a crucial role in causing class switching of Ig isotype B cells, resulting in IgE synthesis (together with IL-13) which is involved in mast cell degranulation by crosslinking with IgE receptors (Bateman *et al.*, 2008; Lee *et al.*, 2010). Generally, asthmatic individuals have elevated IL-4 protein levels in BALF and serum as well as increased IL-4 mRNA and protein in bronchial biopsies (Humbert *et al.*, 1996; Kotsimbos *et al.*, 1996). It has been reported that aerosol nebulization of IL-4 in individuals who are mildly asthmatic prompts airway hyper responsiveness (AHR) and eosinophilia (Shi *et al.*, 1998). Various strategies therefore target IL-4 as potential therapies. For instance, a study on IL-4–deficient mice indicated a reduced eosinophilic inflammation and peribronchial inflammation. It was also reported that the IL-4–deficient mice neither produced total and allergen-specific IgE nor expressed the development of AHR (Brusselle *et al.*, 1994). Molecular signalling of IL-4 via IL-4Ra activates the transcription factor Stat6, causing up-regulation of Th2 lineage–specific transcription factor GATA-binding protein 3(GATA-3) expression. Nonetheless, IL-4 dominates by directing Th2 cell polarization (Maes *et al.*, 2014).

IL-5 enhances eosinophil recruitment, activation and survival at the inflammatory sites (Fujisawa *et al.*, 2000; Farahi *et al.*, 2007). It also modulates the eosinophil

progenitors in asthmatic individuals (Menzies-Gow *et al.*, 2007) and influences the growth and differentiation of bone marrow eosinophils (Sanderson, 1987; Yamaguchi *et al.*, 1988). Studies on allergen-induced inflammatory responses and AHR using anti-IL-5 revealed that the IL-5 knockout mice are protected from AHR (Foster *et al.*, 2002), acute allergic inflammation and chronic airway remodeling (Cho *et al.*, 2004). However, focusing on single interleukin alone as therapeutic target has not been very successful. For instance, IL-5 antagonists has been ineffective in clinical studies (Kips *et al.*, 2003; Nials and Uddin, 2008).

On the other hand, IL-13 known as ‘‘central mediator of allergic asthma’’ plays an important role in allergic response in asthmatic patients (Bateman *et al.*, 2008). IL-13 is involved in class switching of B cells to produce IgE, that regulates eosinophilic inflammation, airway smooth-muscle hyperplasia and the recruitment of monocytes, as well as macrophages and T cells in airway (Wynn, 2003; Holgate and Polosa, 2008). In fact, it has been shown that IL-13 alone is sufficient to induce responses in murine models (Ingram and Kraft, 2012). In a study using allergen sensitized mice, the selective blockade of IL-13 was achieved by using a soluble form of IL-13Rα2 that competes with IL-13 binding leading to airway hyper responsiveness and reversal of mucus production (Grünig *et al.*, 1998). Nevertheless, IL-13 is mainly involved in inducing goblet cell hyperplasia, airway remodeling mucus hypersecretion and AHR (Wills-Karp *et al.*, 1998).

Since asthma pathogenesis is extremely complex, efforts to exclusively target on Th2-type cells alone had little effect and failed to progress to clinical trials (Leckie *et al.*, 2000). Moreover, the balance between Th1 and Th2 cells are claimed to be

inadequate to elicit many experimental observations (Wills-Karp *et al.*, 2001; Herrick and Bottomly, 2003). Often, Th1 cells itself contribute to exacerbations and disease manifestations of asthma in mouse models (Hessel *et al.*, 1997; Hansen *et al.*, 1999; Ford *et al.*, 2001). The roles of Treg cells in asthma are gaining great research interest (van Oosterhout and Bloksma, 2005; Li *et al.*, 2012). The immunoregulatory effects of Tregs is becoming more important as it is purported to be involved in suppressing conventional CD4 T cells-mediated immune responses.

Recent candidates of Tregs being widely investigated are Th3 (T helper 3), TR1 (Type 1 regulatory T cells), CD4+, CD25+ and natural killer (NK) T cells. TR1 cells can prevent experimental colitis and Th2 responses (Tsitoura *et al.*, 2000). TR cells have also been shown to inhibit AHR. Naturally occurring regulatory CD25+ cells are involved in preventing autoimmune disease (Umetsu *et al.*2003). For example, a study conducted on asthmatic children reported significantly decreased levels of CD4+CD25hi Tregs and Foxp3 mRNA expression, highlighting a decrease of these Treg cells influences Th2 balance (Hartl *et al.*, 2007). Nonetheless, exact roles of Tregs cells in controlling the development of asthma are still unclear. Nevertheless, TR1 cells plays roles in inhibiting Th2 and Th1 responses (Cottrez *et al.*, 2000) and regulate the growth of Th2 responses and AHR (Akbari *et al.*, 2002). CD25+ cells is speculated to inhibit the development of airway eosinophilia (Suto *et al.*, 2001) but not AHR (Hadeiba and Locksley, 2003).

1.1.7 Pathophysiology and clinical features

The presence of respiratory epithelial cells in the sputum was one of the first pathological abnormalities as described by Curschman in 1885. Thus, many

approaches focused on sputum for studies on patients suspected of suffering from asthma or airway inflammation. Many indirect approaches on examination of airway structure, such as airway inflammation and airway hyperresponsiveness have been performed although difficulties lies in obtaining the representative specimens of humans airways (Melder, 2005). The Beta Adrenergic Theory of Asthma in 1968 led to one of the most important watershed moment in research related to allergic. The theory brought an understanding of asthma pathogenicity where a study has reported that the blockage of beta-2 receptors of pulmonary smooth muscle cells lead to asthma (Szentivanyi, 1968). Later, it was demonstrated that over production of IgE causes blocking of beta-2 receptors, thereby making clear the central role of IgE in allergic diseases (Szentivanyi *et al.*, 1993).

The hallmark of asthma is the functional changes that is associated with airways obstruction (Dunnill, 1960) In asthma, the "hypersensitive" inflamed airways respond to the presence of environmental allergens or triggers, causing airways narrowing and increase production of mucus, leading to difficulty in breathing. In an asthmatic patient, the walls of the airways in the lungs are irritated and swollen as the muscles surrounding airways becomes more sensitive and they form a more spontaneously tightened and compressed muscles (Cockcroft, 1983; Fahy and Dickey, 2010). Hence, contraction of smooth muscle of bronchi (large airways) leads to spasm, resulting in an asthmatic attack. Other airway remodelling and structural changes include subepithelial fibrosis, enlarged smooth muscle mass, glands and thickening of airway walls as well as bronchial hyper responsiveness. This is also followed by airway oedema and excessive mucous hypersecretion (Bergeron *et al.*, 2009). Other known airway remodelling characteristics are angiogenesis, loss of

cartilage integrity and inflammation (Bergeron *et al.*, 2010). Further contraction follows after inflammation of the airways and mucus production leading to coughing and breathing difficulties (Figure 1.2). On the other hand, AHR is an occurrence in the presence of excessive airway narrowing upon sensitive chemical or physical stimuli that exert little or no harm to a normal individual (Brusasco and Pellegrino, 2013). A study on the relationship between inflammation and AHR reported that alteration on inflammatory signalling pathways on mouse and its effects of sensitization which revealed that inflammation and AHR do not directly correlate. Thus, it was proposed that a more mechanistic investigation may examine clearly the potential causal impact of inflammation on AHR (Janssen-Heininger *et al.*, 2012).

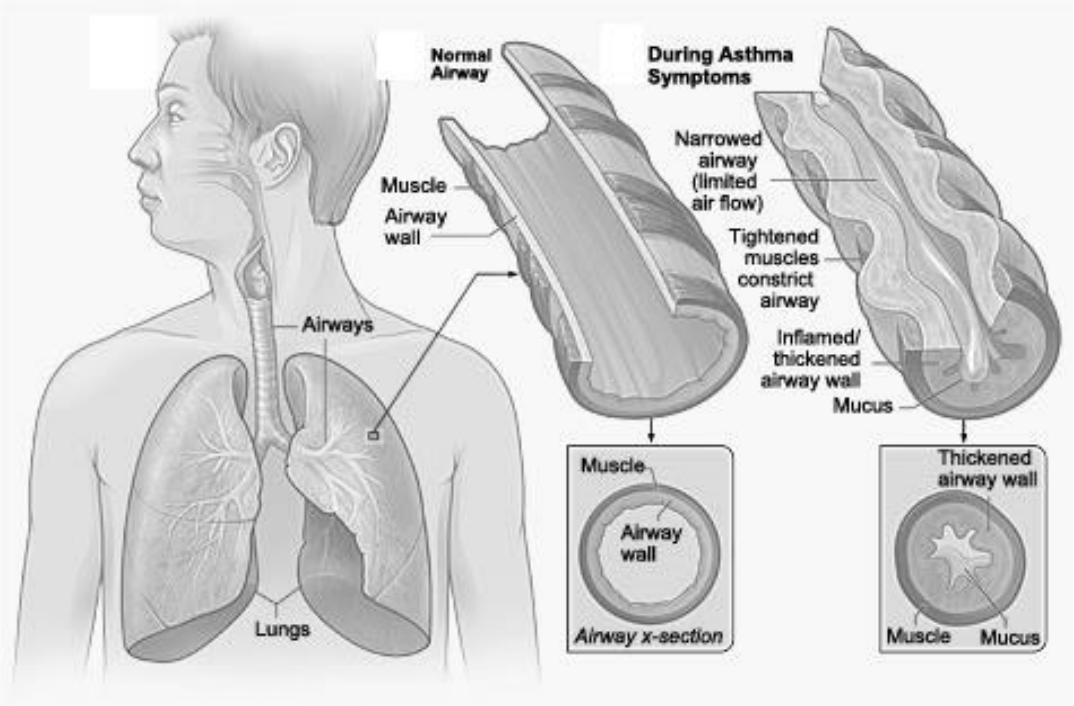

Figure 1.2: Airways narrowing and the release of excess mucus, causing breathing difficulties . Adapted from Doeing and Solway (2013).

1.2 Drugs and treatment

1.2.1 Current drugs and therapies

Various drugs were used decades ago, such as adrenaline and isoproterenol in treating asthma. However, over the years, side effects of these adrenergic agonists lead to the production of newer drugs with major advantages over the former. For instance, the newer drugs such as metaproterenol, terbutaline and salbutamol expressed better and longer duration of action while acting selectively as pure beta2 stimulators, thus alleviating undesirable cardiovascular stimulation (Goldberg, 1976). Currently, pharmacological agents such as inhaled corticosteroids, anti-histamines and β2-adrenoceptor agonists such as short- and long-acting β2-adrenoceptor agonists (SABAs and LABAs) are the mainstay of asthma treatment as supported by asthma management guidelines. These drugs are aimed to treat different allergic symptoms (Holgate and Polosa, 2008; Abril-Gil *et al.*, 2012). To date, inhaled corticosteroids and leukotriene inhibitors such as beclomethasone, budesonide and dexamethasone have long been used for the management of asthma for more than 50 years. They have been efficiently reducing the severity of symptoms and improving the lung functions while β-adrenergic agonists (salbutamol, terbutaline and fenoterol) and anticholinergics (Brown *et al.*, 1992; Mali and Dhake, 2011) have been used for maintenance therapy. These drugs help to relieve asthmatic symptoms and attacks by relaxing the airway of smooth muscle (Kim and Yang, 2011). In most cases, the combination of drug that relieves and control the symptoms present as the foundation of disease management (Holgate and Polosa, 2008).

Meanwhile, new approach as anti-IgE drug known as omalizumab, has been used to control asthma severity. Omalizumab was the first biological agent used for allergy

treatment and paved the way for more studies on such potential biological agent to target cells and receptors. It is a humanized IgE-specific and non-anaphylactic IgG1 drug which has been developed and confirmed in clinical trials to control the disease symptom in patients when combined with lower doses of inhaled corticosteroids (Holgate *et al.*, 2005). Reducing circulating IgE levels has been the target for asthma treatment, as also seen with lumiliximab, an antibody specific to the low-affinity IgE receptor (Poole *et al.*, 2005). Otherwise, methods such as vaccine and allergen-based therapies are also some of the important strategies in allergy prevention and treatment, specifically with the involvement of recombinant allergens and peptide fragments. The coupling of allergens with oligonucleotide stimulatory sequences also transformed the whole conceptual prospect of asthma treatment (Holgate and Polosa, 2008).

1.2.2 Side effects and limitation of drugs

Despite their efficiencies in treating asthma, current medications for asthma which are mainly steroid-based have some side-effects and limitations. For example, synthetic drugs such as salbutamol, terbutaline and fenoterol only prevent early stages of asthma attacks. They cannot be used for chronic persistent asthma attacks, which need steroids inhalation. Steroids are anti-inflammatory drugs that affect and act directly on the lungs to provide a long-term relief. The side effects of corticosteroid is however apparent. Inhaled corticosteroid is expected to be more effective in reducing symptom compared to the orally consumed corticosteroid. However, drugs such as beclomethasone dipropionate (BDP) and budesonide are claimed to have weak active metabolites following liver biotransformation in the liver. In contrast to BDP and budesonide, dexamethasone is more effective when

taken orally rather than via the inhalation mode (Mali and Dhake, 2011). Inhaled corticosteroid also has negative side effects as they are absorbed into the systemic circulation and may lead to undesired effects such as impaired growth, decreased bone mineral density and cataracts and many more (Dahl, 2006; Kaliner *et al.*, 1990). Since anticholinergic, nevertheless are reported to be less effective when compared to β-adrenergic agonists, they may be used together for additional results or effects (Mali and Dhake, 2011). However, physicians are still uncertain in long-acting beta2 agonist drugs role for asthma which cannot be used alone due to severe side effects. Therefore, Food and Drugs Association (FDA) recommends using these products together with steroids (Chowdhury and Dal Pan, 2010).

In fact, many complications from synthetic drug have been reported such as detrimental effects on calcium metabolism leading to osteoporosis following long term medications. Other local and systemic side effects are oral candidiasis, dysphonia, growth failure, elevated intraocular pressure and mild tachyphylaxis (Fanta, 2009). In addition, resistances towards drugs and treatments were also increasing despite more efficient response in treating and relieving symptoms (Kaliner *et al.*, 1990). Basically, with the application of current drugs, the occurrence of recurrent attacks and asthma exacerbations still occur. A new drug called Altrakincept which involves targeting of IL-4 for the treatment of asthma has failed phase III trial due to some bioavailability issues (Holgate and Polosa, 2008).

Currently, the majority of available drugs are only effective in preventing early stages of asthma and are unable to provide immediate symptomatic relief especially for acute attacks. Due to the inadequacy of modern medicine, natural products and

nutritional interventions are frequently utilized to aid in the prevention, diagnosis and management of asthma (Lee *et al.*, 2007; Lee *et al.*, 2010; Abril-Gil *et al.*, 2012). The Th2 cells driven inflammatory responses has been known to lead to enhancement of airway eosinophilia and lung mucus production. Various researches are therefore primarily focused on products which have the potential to reduce IgE, IL-4, IL-5, IL-13 and eosinophils as therapeutic targets in the context of identifying anti-asthmatic properties (Lee *et al.*, 2007; Lee *et al.*, 2010; Zhang *et al.*, 2011; Abril-Gil *et al.*, 2012).

1.2.3 Complementary and alternative medicine in asthma

The complementary and alternative medicine (CAM) is becoming more popular now among the public due to the enormous increase in access to worldwide information through the World Wide Web and widespread news and media coverage. Generally, CAM can be categorized into whole medical systems, natural products, manipulative and body-based practices, movement therapies, traditional and energy field healing (George and Topaz, 2013). Natural products have been widely benefited since centuries ago, although the necessity of its scientific evident on its efficacy was overlooked. The preference towards such herbal alternative is surprisingly high in the modern era. Interestingly, patients are also aware that such interventions are not wholly curative. Instead they function more towards symptom relieving and improving the quality of life. A study in the United States reported that 55% of adults prefer to use herbal remedies along with conventional medicines, while 28% of adults solely rely on natural products due to the assumption that synthetic drugs are more toxic while others feel that synthetic drugs are also expensive (Mahmoudi, 2009). Nevertheless, there is limited evidence supporting the value of CAM

treatments from many clinical trials. Many studies have described its use among patients (Blanc *et al.*, 2001; Berg *et al.*, 2015; Ward and Baptist, 2016) and reported their effectiveness for allergic-related diseases (Lakshmana *et al.*, 2001; Zhang *et al.*, 2011; Abril-Gil *et al.*, 2012).

Promising Anti-asthma Herbal Medicine Intervention (ASHMI) is run along with plant-based medicines in some clinical studies of asthma. ASHMI is a combination of three herbs known as *Ganoderma lucidum* mushroom, *Radix Sophora flavescentis* and Radix *Glycyrrhiza uralensis* (Gan Cao) (Wen *et al.*, 2005). ASHMI confirms therapeutic effects on major pathogenic mechanisms of asthma including AHR, airway inflammation and remodelling as well as alleviation of Th2 immune reaction. The direct inhibitory role on airway smooth muscle was also reported (Srivastava *et al.*, 2005; Li and Brown, 2009). In addition, a preclinical trial of Gan Cao confirmed to its anti-inflammatory properties and potential control over 'neutrophilic asthma'. The intervention decreased the levels of mediators such as IL-8, eotaxin 1 and Signal transducer and activator of transcription6 (STAT6) in human lung fibroblast cell line (Matsui *et al.*, 2006; Nair *et al.*, 2015). The clinical trial was conducted after the novel herbal formula found to show effective therapy in a murine model of allergic asthma. The clinical success was observed with apparently a harmless and effective alternative medicine for treating asthma. In contrast with prednisone, of oral ASHMI capsules had no adverse effects on the adrenal function (Wen *et al.*, 2005).

1.2.4 Development of natural-based medication for asthma

Many studies reported on the effectiveness of various natural products on asthmatic animal models. *Viola mandshurica* (VM), a Korean traditional herbal medicine has

been reported to show anti-asthmatic effects in an animal model (Lee *et al.*, 2010). Another study also showed the efficacy of *Elaeagnus pungens* in anti-asthmatic formulations (Ge *et al.*, 2009). Similarly, *Rhus succedanea* (Japanese wax tree), *Solanum xanthocarpum* (yellow-fruit nightshade), *Tylophora indica* (vine plant), *Albizzia lebbeck* (flea tree), *Glycyrrhiza glabra* (licorice) and *Achyranthes aspera* (prickly chaff flower) have been reported to cause mast cell stabilization, have anti-histamine, anti-cholinergic, anti-anaphylactic actions and is effective against allergic asthma disorders (Lakshmana *et al.*, 2001; Gopumadhavan *et al.*, 2005). Other therapeutic relieve of bronchoalveolar inflammation in airway of the sensitized murine model have also been shown by *Ganoderma tsugae* mushroom extract (Lin *et al.*, 2006). Similarly, the extract from the medicinal *Basidiomycetes* mushroom also demonstrated anti-allergic and anti-inflammatory effects (Hetland *et al.*, 2015). Another herb, *Cordyceps sphecocephala,* known for its traditional use in Korea, China and Japan was widely used to treat cancer and hypocholesterolaemia has also been reported to alleviate the expression of IL-4, IL-13 and IL-25 in allergic murine model (Heo *et al.*, 2010) highlighting their potentials.

1.3 Mushroom and its potential immune response

Mushroom is one of the most consumed medicinal plants due to of their nutritive values and low calories (Agrahar-Murugkar and Subbulakshmi, 2005), essential fatty acids (Manzi *et al.*, 2001) vitamin and minerals (Manzi *et al.*, 2001). In addition, it has anti-bacterial and anti-viral properties (Breene, 1990). Various types of mushrooms are also traditionally used for their effects against cancer, asthma and inflammatory responses (Rowan *et al.*, 2003). Mushrooms are also well known for their abundant polysaccharides chitin and beta-glucan compositions that have

prominent roles in modulating airway inflammation. Previous studies have shown that chitin can inhibit the development of the quintessential features of asthmatic disease including chronic airway inflammation, AHR and pathological remodelling changes in mouse models of allergy (Catalli and Kulka, 2010). Notably, the boiled extract of *Lignosus rhinocerus* sclerotium has been widely used to treat cough and asthma traditionally (Chang and Lee, 2004). The active compounds, β-glucan is confirmed in many studies to be the key essence in asthma-healing (Catalli and Kulka, 2010), yet *L. rhinocerus*'s potential in asthma still remains unclear.

The immunomodulating effect of mushroom has gained some interest (Mau *et al.*, 2001; Rowan *et al.*, 2003) where it is explored in many studies (Rowan *et al.*, 2003; Lindequist *et al.*, 2005; Nurul *et al.*, 2014). *Pleurotus sajor caju* (PSC) an edible mushroom commonly found in the tropical region has shown immunomodulatory effects in BALB/c mice (Nurul *et al.*, 2014). Similarly, the immunomodulatory effects of *Polyporus rhinocerus* mushroom from China has also been reported (Wong *et al.*, 2011) where it improves the general immune status such as balancing T helper cells and T cytotoxic cells, increase B cell as well as macrophages populations (Nurul *et al.*, 2014).

1.4 *Lignosus rhinocerus* "Tiger's milk mushroom"

Lignosus rhinocerus of Polyporaceae family is one of the exclusive wild mushrooms found in Malaysia. It is locally known as "cendawan susu rimau" or "tiger's milk mushroom". This unique mushroom is one of the total 38 types of edible mushrooms in Malaysia which is used for its medicinal purposes by the rural and indigenous communities (Lee *et al.*, 2009). The name "tiger milk mushroom" was originated

from the belief that it vegetates from the ground where the tigress leaked its milk while feeding her cubs. The structures of *L. rhinocerus* comprise of pileus, stipe and sclerotium (Figure 1.3). It is believed that the tuber or sclerotium of *L. rhinocerus* can remain in the ground for periods ranging from several months to decades, and the mushroom will only grow solitarily when the nature calls. The underground tuber is the most treasured portion of *L. rhinocerus* due to its medicinal value (Lee *et al.*, 2011b).