EVALUATION OF *Lignosus rhinocerus* AQUEOUS EXTRACT ON MUSCARINIC RECEPTOR IN ISOLATED TRACHEA OF SPRAGUE-DAWLEY RATS

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by

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JANUARY 2019

CERTIFICATE

This is to certify that the dissertation entitled "Evaluation of *Lignosus rhinocerus* aqueous extract on muscarinic receptor in isolated trachea of Sprague-Dawley rats" is the bona fide record of research done by Mr. Syed Muhamad Asyraf Bin Syed Taha during the period of February 2018 until December 2018 under my supervision.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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"In the name of Allah, most Gracious, most Compassionate"

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LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

%	Percentage
±	plus minus
µg	micro gram
µM	micro molar
AHR	Airway hyperresponsiveness
BALF	bronchoalveolar lavage fluid
°C	Degree Celsius
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
$CaCl_2$	Calcium chloride
Carbachol	Carbamylcholine chloride
cm	Centimeter
CO_2	Carbon dioxide
DRC	Dose response curve
EC_{50}	Concentration of agonist that produce 50% maximal response
GC-MS	Gas chromatography mass spectrometry
gm	Gram
IC_{50}	Concentration of antagonist that produce 50% inhibition of the response of an agonist
IP	intraperitoneal
KCl	Potassium chloride
kg	Kilogram
KH_2PO_4	Potassium hydrogen phosphate
LRE	*Lignosus rhinocerus* aqueous extract

M	Molar
M_2	Muscarinic acetylcholine receptor subtype 2
M_3	Muscarinic acetylcholine receptor subtype 3
mg	milligram
$MgSO_4$	Magnesium sulfate
ml	milliliter
mm	millimeter
mM	millimolar
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
ng	nanogram
nM	nanomolar
O_2	Oxygen
pEC_{50}	The negative logarithm to base 10 of the EC_{50} of an agonist
pIC_{50}	The negative logarithm to base of the IC_{50} of an antagonist
r^2	regression correlation coefficient
SEM	Standard error of mean
V	volume
WHO	World Health Organization
β	beta

ABSTRAK

Asma adalah masalah kesihatan awam yang serius. Terdapat peningkatan kes asma di seluruh dunia dan ia berada di kalangan sepuluh penyakit kronik yang paling biasa di dunia. *Lignosus rhinocerus* (cendawan susu rimau) telah digunakan secara tradisional dalam rawatan asma. Oleh itu, adalah penting untuk menentukan reseptor yang terlibat untuk ekstrak ini di dalam tindak balas terlampau saluran pernafasan dan menilai peranannya dalam mekanisme relaksasi otot lembut saluran pernafasan. Objektif utama kajian ini adalah untuk menilai kesan ekstrak akues *L. rhinocerus* (LRE) terhadap fungsi reseptor muskarin dalam trakea terasing tikus Sprague Dawley. LRE telah disediakan menggunakan proses pengekstrakan soxhlet panas dan ia dibeku-keringkan. Cincin trakea tikus terasing telah digunakan dalam kajian miograf untuk menilai kesan penguncupan LRE dengan dibandingkan kepada antagonis tidak selektif muskarin, atropin pada tona penguncupan-karbakol (agonis tidak selektif muskarin). LRE menghasilkan >50% tindak balas penguncupan pada 30 mg/ml pengecutan 3 µM aruh-karbakol dengan pIC_{50} 1.62 ± 0.01. Atropin mengecutkan secara penuh pada 9 nM dengan pIC_{50} 5.71 ± 0.05. Kesimpulannya, kajian ini menunjukkan bahawa kesan pengenduran LRE adalah disebabkan oleh otot lembut trakea dikawal dengan kesan antagonis pada reseptor muskarin sistem kolinergik.

Keywords: *Lignosus rhinocerus*, asma, ekstrak akues, reseptor muskarin, trakea, miograf.

ABSTRACT

Asthma is a serious public health concern. There is an increase of asthma cases worldwide and it ranks among the top ten most common chronic disease in the world. *Lignosus rhinocerus* (Tiger milk mushroom) has been traditionally used in treatment of asthma. Therefore, it is important to elucidate the receptor involved for this extract on airway hyperresponsiveness and evaluate its roles in the mechanism of airway smooth muscle relaxation. The main objective of the study was to evaluate the effect of *L. rhinocerus* aqueous extract (LRE) on the muscarinic receptor functions in isolated trachea of Sprague Dawley rats. LRE was prepared using hot soxhlet extraction process and it was freeze-dried. Isolated rat trachea ring was used in functional myograph study to evaluate the relaxation effect of LRE in comparison to non-selective muscarinic antagonist, atropine on carbachol-induced contractile (non-selective muscarinic agonist) tone. The LRE produced more than >50% relaxation effect at 30 mg/ml in 3 µM carbachol-induced contraction with pIC_{50} 1.62 ± 0.01. Atropine produced complete relaxation at 9 nM with pIC_{50} of 5.71 ± 0.05. In conclusion, this study suggests that the LRE induced relaxation in trachea smooth muscle was mediated by antagonistic effect on muscarinic receptor of cholinergic system.

Keywords: *Lignosus rhinocerus*, asthma, aqueous extract, muscarinic receptor, trachea, myograph.

CHAPTER 1

INTRODUCTION

1.1 Asthma in general

Asthma is one of the highly complex airway-obstruction diseases and is also considered to be one of the most common respiratory diseases worldwide that affected about 300 million peoples globally (Masoli *et al.*, 2004). Asthma kills around 1000 people every day and affects as many as 339 million people and the prevalence is rising continuously. According to the Global Asthma Report (2018), the death caused by asthma in Malaysia has reached 1,642 or 1.29% of total deaths with the age adjusted death rate at 8.22 per 100,000 of population. Over the years, the global prevalence, morbidity, mortality, and economic burden of asthma have increased particular in children. As a result, it causes alarming concern on both individuals and healthcare system.

Asthma is characterized by persistent attacks of breathlessness and wheezing which may varied in severity and frequency. In the event of an acute asthma attack, the lining of the bronchial tubes would be inflamed and constricted causing the airways to narrow and reducing the flow of air into and out of the lungs (Figure 1.1). Therefore, chronic airways inflammation and hyperresponsiveness are recognized as the major underlying causes of asthma (Global Asthma Network, 2018). Although, both of these symptom are considered as the characteristics of asthma, their relationship is still poorly understood (Sheffer, 1991). Inflammation in asthmatic airways does not only occurs at the trachea and bronchi, it also extends to the terminal bronchioles and parenchyma (Tulic *et al.*, 2001). On the other hand, airway hyperresponsiveness can be sustained by airway wall remodeling (Wiggs *et al.*, 1992) and inability to reduce the trachea and bronchial smooth muscle contraction (Skloot *et al.*, 1995). The pathophysiology of asthma is complex involving several different inflammatory cells and multiple mediators resulting in acute and chronic inflammatory effects on the airways. The search for treatment of asthma should be based on the understanding of the its underlying pathophysiologic mechanisms and how this this knowledge be used appropriately. Fortunately, there have been significant technology advancement in understanding asthma pathogenesis with the application new techniques that help with the development of plausible treatment on the complex mechanism of action that is activated in asthma.

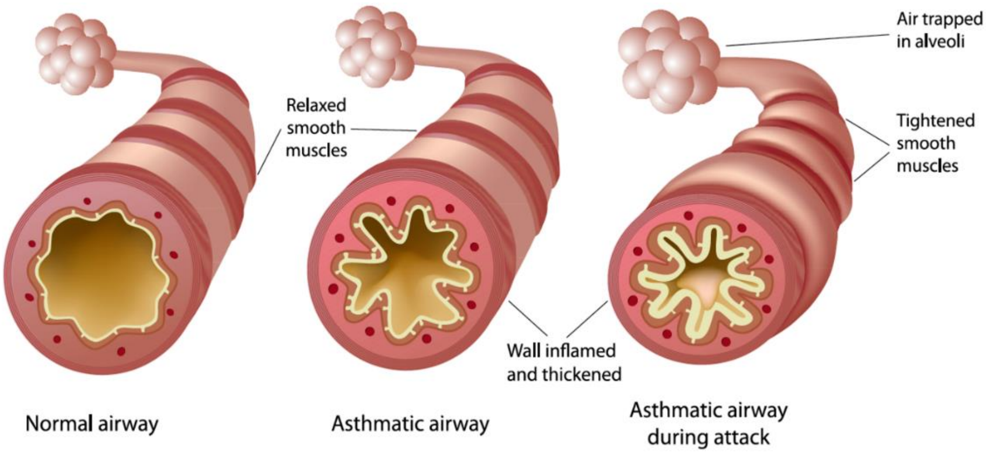


Figure 1.1 Illustration of asthma airway. Adapted from Gandy (2017)

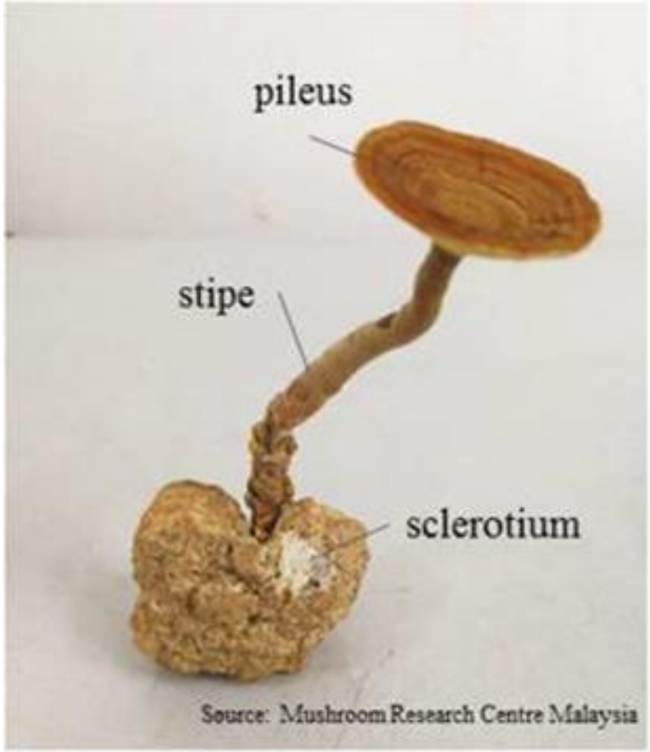
1.2 Natural products and asthma

Conventional asthma treatment is still currently effective in managing asthma. However, there are increased concerns regarding the risk of adverse effects and low satisfactory curative efficacy of current treatment (Abramson *et al.*, 2003; Slader *et al.*, 2006). Therefore, it is relevant for the current asthma research to find for effective low-risk, natural product medicine that provides a valuable alternative treatment for asthma management. In traditional medicine, many natural products found to be a good replacement in treating asthma (Busse *et al.*, 2004). Due to high of demand for the treatment, the interest in complementary and alternative medicine and its use in treating asthma is growing exponentially (Slader *et al.*, 2006). Herbal medicine is still believed as the mainstream medication in 75-80% of the world population for primary health care (Kamboj, 2000). The main reasons of this trend of belief are that herbal medicines is cheaper, locally available and possess no side effects (Verma and Singh, 2008). According to World Health Organization (WHO), traditional medicine including herbal drugs have existed hundreds of years prior to any development and spread of modern medicine and still currently practiced and used. Studies have suggested that a medical plant that could be used asthma treatment should have anti-inflammatory, immunomodulatory, antihistaminic, smooth-muscle relaxants and allergic activity (Kasirajan *et al.*, 2007; Taur and Patil, 2011).

1.3 *Lignosus rhinocerus* mushroom

Lignosus rhinocerus (Cooke) Ryvarden is a mushroom that is known as "cendawan susu rimau" in Malay language or tiger's milk mushroom in English. *L. rhinocerus* is a unique "national treasure" that can typically be found in Southeast Asia and Southern China. Mushroom has been consumed by many communities around the world due to its high nutritional value, culinary flavors and medicinal properties. *L. rhinocerus* is also one of the common mushrooms that has been widely used by the indigenous community in Malaysia for treatment in variety of ailments. It has more than 15 medicinal uses including antipruritic, antipyretic, general tonic, starve off hunger, anti-cancer, treat food poisoning, swollen breasts, fever, cough, anti-asthma, as wound healing, and others (Nallathamby *et al.*, 2018).

The taxonomy of *L. rhinocerus* based on its morphological characteristics, such as the shapes, forms and size of pileus (cap), stipe (stem) and sclerotia (tuber). (Figure 1.2). The sclerotium is white and gives a milk-like solution; and it even tastes like milk (Tan *et al.*, 2010). The sclerotium of *L. rhinocerus* is the part of this mushroom with medicinal value. This rare mushroom has been successfully cultivated and optimized which making it possible to be fully explored for its medicinal and functional benefits (Abdullah *et al.*, 2013). Previous animal study has showed that *L. rhinocerus* sclerotia water extract significantly reduced the asthmatic parameters which involved in inflammatory response (Johnathan *et al.*, 2016). However, there is very limited scientific evidence that has reported any direct effect of airway relaxation mediated by bioactive fractions or compounds of *L. rhinocerus*.



Kingdom	**:**	**Fungi**
Phylum	**:**	**Basidiomycota**
Class	**:**	**Agaricomycetes**
Order	**:**	**Polyporales**
Family	**:**	**Polyporaceae**
Genus	**:**	***Lignosus***
Species	**:**	***Lignosus rhinocerotis*** **(Cooke) Ryvarden**

Figure 1.2 The morphology of *L. rhicocerus* (*L. rhinocerotis*) and its taxonomic classification (Nallathamby *et al.*, 2018).

1.4 Justification of the study

Asthma is a serious public health concern. There is an increase of asthma cases worldwide and it ranks among the top ten most common chronic disease in the world. Asthma is most prevalent among young children at the age 5 – 17 years old (Akinbami *et al.*, 2012). This chronic disease costs our healthcare system large amount of money each year, with the majority of this money going towards direct medical costs rather than prevention and research. As a result, there is a growing interest in the usage of dietary herb and medicinal plants as an alternative management of asthma.

Previous study had showed that of the treatment of *L. rhinocerus* extracts on ovalbumin induced allergic in Sprague Dawley rats significantly reduced the asthmatic parameters, such as Th2 cytokines (IL4, IL5 and IL13) levels in bronchoalveolar lavage fluid (BALF), and immunoglobulin E (IgE) level in serum (Johnathan *et al.*, 2016). It also suppressed eosinophil number in BALF and attenuated eosinophil infiltration in the lungs. Therefore, *L. rhinocerus* could be one of the potential alternative treatment that is cost-effective in helping lung function by controlling smooth muscle contraction.

However, there is very scarce scientific evidence on the efficacy of *L. rhinocerus* and its bioactive components on regulation of systemic pharmacology. Therefore, this study is designed to elucidate the receptor involved for this extract on airway hyperresponsiveness and evaluate its roles in the mechanism of airway smooth muscle relaxation.

1.5 Objectives of the study

The general objective of the study was to evaluate the effect of *L. rhinocerus* aqueous extract (LRE) on the muscarinic receptor functions in isolated trachea of Sprague Dawley rats. The specific objectives that is included are:

i. To evaluate the effect of muscarinic receptor agonist (carbachol) on the isolated tracheal ring

ii. To evaluate the effect of muscarinic receptor antagonist (atropine) against carbachol on the isolated tracheal ring

iii. To evaluate the effect of *L. rhinocerus* aqueous extract against carbachol on the isolated tracheal ring

1.6 Focus of the study

The focus of this study was to explore and evaluate the effect of LRE on the isolated tracheal ring of Sprague Dawley. Therefore, evaluation of the LRE dose response curve and its potential mechanism of action on muscarinic receptor in trachea smooth muscle would be the consideration of this study. This study hypothesized that the LRE would able to behave as an antagonist of the muscarinic receptor and induce airway smooth muscle relaxation.

CHAPTER 2

LITERATURE REVIEW

2.1 Asthma and its medication

Asthma is characterized by variable airflow obstruction, airway hyper-responsiveness, and chronic airway inflammation. The treatment for asthma should follow the approved asthma management guideline recommended by the authority. Currently available anti-inflammatory and bronchodilator drugs are very effective and good asthma control can be achieved for most patients. Inhaled short acting β_2-andrenoceptor agonists such as salbutamol and terbutaline are effective bronchodilators and should be prescribed to all patients with symptomatic asthma (Walters *et al.*, 2003). Their mechanism of action is thought to occur primarily by the relaxation of airway smooth muscle cells, but they also increase mucosal clearance. Corticosteroids are currently considered to be the most effective anti-inflammatory agents for the treatment of asthma (BTS/SIGN, 2003). The anti-inflammatory effects of corticosteroid are exerted through a diverse range of mechanisms including the activation of the glucocorticoid receptor and the direct inhibition of a range of inflammatory cells, particularly eosinophils (Jatakanon *et al.,* 1999). The study showed that treatment with regular inhaled corticosteroids results in significant improvements in airway inflammation in asthma. However, there are concerns about the possibility of ineffective effects of inhaled corticosteroids. Regular inhaled corticosteroids over period of time has been showed to affect the lung functions in adult and children (Agertoft and Pedersen, 1994; Childhood Asthma Management Program, 2000). Long acting β_2-agonists (salmeterol and formoterol) are currently generally recommended as additional treatment for patients who have symptoms that persist despite regular inhaled corticosteroids (Van Noord *et al.*, 1996). At higher dose, these drugs may cause side effects such as tachycardia, tremor, and muscle cramps. Moreover, tolerance to the effects of β_2-agonists has been reported in which the bronchodilator activity was attenuated (Newnham *et al.*, 1995).

There is increasing range of treatments available for asthma which could be rationalized the future management of asthma by identifying individual treatment goals and carefully assessing the likely underlying pathophysiology. Novel herbal products which act on specific mechanistic pathways in asthma are emerging. The future management of patients with asthma may well involve the use of these newer herbal products in combination with more established therapies.

2.2 Airway pathophysiology of asthma

In asthma, the airways are highly responsive to variety of internal and external stimuli causing airway narrowing and reduce airflow which may affect the airway inflammatory response (Figure 2.1). Asthmatic airway inflammation is characterized by excessive production of airway mucosa and thickening of the airway epithelium. The composition of airway mucosa includes cellular debris from necrotic airway epithelial cells, and inflammatory cell including lymphocytes, eosinophils, and neutrophils, plasma protein exudate, and mucin that is produced by goblet cells. Repeated airway inflammation influences the remodeling of the airway walls which causes the thickening of epithelium due to formation of goblet cells, subepithelial fibrosis, smooth muscle hypertrophy, and proliferation of submucosal glands (Dunnill, 1960; Bousquet *et al.*, 1990). The asthmatic airway showed a thickness with inflammatory cell infiltration consisting of mixture of T lymphocytes and eosinophils, mast cells (Hamid *et al.*, 1997).

Acetylcholine is the primary parasympathetic neurotransmitter in the airways, and is traditionally associated with inducing airway smooth muscle contraction and mucus secretion. A study has showed that acetylcholine production in the airways is not restricted to the parasympathetic nervous system in which the acetylcholine is also can be released from non-neuronal origins such as the bronchial epithelium and inflammatory cells (Wessler and Kirkpatrick, 2001). Moreover, acetylcholine has been showed to play an essential regulatory role in the mechanisms that drive the structural changes in the airways, called airway remodeling, that are associated with chronic airway inflammation (Gosens *et al.*, 2004; Gosens *et al.*, 2005). These evidences could suggest that acetylcholine acting on muscarinic receptors may contribute to the pathophysiology and pathogenesis of asthma to a much larger extent than is currently expected.

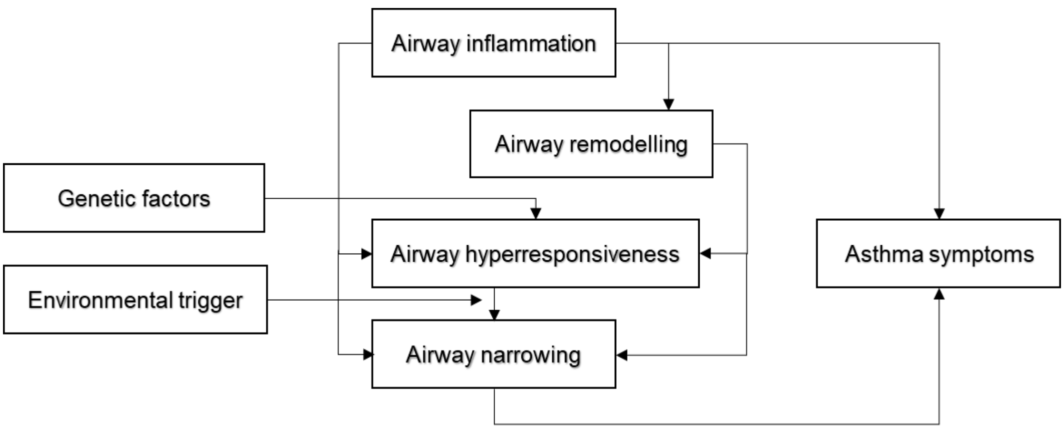


Figure 2.1 Mechanism of asthma

2.3 Cholinergic receptors in airways

The airway smooth muscle is primarily mediated by the innervation of parasympathetic fibers that are carried in the vagus nerves. The vagus nerves relay the transmission of the preganglionic nerve fibers from the vagal nuclei in the medulla to ganglia in the airways (Richardson, 1979). These parasympathetic ganglia are found to be distributed along the posterior aspect of the wall of the trachea and major bronchi (Honjin, 1956). Major bronchi are the site that has the densest cholinergic innervation which is also indicated as the site of bronchoconstriction in patients with asthma (van Koppen *et al.*, 1988).

Acetylcholine acts on both muscarinic and nicotinic receptors. There are five muscarinic receptors subtypes have been recognized by International Union of Pharmacology which are M_1, M_2, M_3, M_4 and M_5 (Caulfield and Birdsall, 1998). These M_1 – M_5 muscarinic receptor subtypes can also be differentiated based on differing binding affinities between different antagonists. All five muscarinic receptor subtypes are expressed in the lung. Study on the primary cultures of postganglionic cholinergic neurons from the trachea showed that only the messenger RNA for M_2 receptors was observed (Fryer *et al.*, 1996). Furthermore, study on airway smooth muscle cells showed the expression of M_2 and M_3 muscarinic receptors (Maeda *et al.*, 1988).

2.4 Muscarinic receptor regulation of airway smooth muscle

In the airway smooth muscle, the vagal parasympathetic nervous system (vagus nerve) via muscarinic receptors acts as the dominant autonomic control of airway smooth muscle tone (Figure 2.2). Acetylcholine that is released at neuromuscular junctions binds to M_3 muscarinic receptors in the smooth muscle and promotes airway contraction (Roffel *et al.*, 1990). Stimulation of this parasympathetic transmission also promotes the glandular tissue to secrete mucus (Gallagher *et al.*, 1975) and the bronchial circulation to dilate (Widdicombe, 1963). Evidences from human and animal studies have showed that the vagus nerve also maintain baseline tonic contraction of the airway smooth muscle (Colebatch and Halmagyi, 1963; Cabezas *et al.*, 1971). Acetylcholine also feeds back onto neuronal M_2 muscarinic receptors located on the postganglionic cholinergic nerves. This is an important negative feedback mechanism that helps inhibit acetylcholine release, so these M_2 muscarinic receptors would be known as autoreceptors. Therefore, the regulation of airway smooth muscle is induced mainly by activation of muscle M_3 receptor subtype and inhibited by activation of vagal M_2 receptor subtype.

Functional study finding showed that contraction induced by muscarinic ligands in isolated trachea and bronchi is mediated by M_3 receptors in all species including humans (Haddad *et al.*, 1991; Struckmann *et al.*, 2003). Moreover, an *in vivo* study in muscarinic receptor gene-deficient mice found that only M_3 receptors contribute to bronchoconstriction induced by electrical stimulation of the vagus nerves or intravenous methacholine (Fisher *et al.*, 2004). Activation of smooth muscle M_3 receptor has been demonstrated in the absence of acetylcholine by membrane depolarization induced chemically with KCl (Liu *et al.*, 2009).

Although the number of M_2 receptors presence is more than M_3 receptor in the airway, it also has an indirect role in airway smooth muscle contraction. M_2 receptors on airway smooth muscle inhibit relaxation induced both by β-adrenoreceptor agonists and adenylyl cyclase activation with forskolin (Fernandes *et al.*, 1992). Therefore, M_2 receptors contribute to smooth muscle contraction by functionally antagonizing Gαs-induced relaxation. A study using isolated trachea from mice deficient for M_2 receptors showed that the muscarinic agonist potency is reduced, but the maximum contraction is still achieved (Stengel *et al.*, 2000). This finding further proved M_3 receptors alone are sufficient for smooth muscle contraction but M_2 receptors still have influence in acetylcholine-induced smooth muscle contraction.

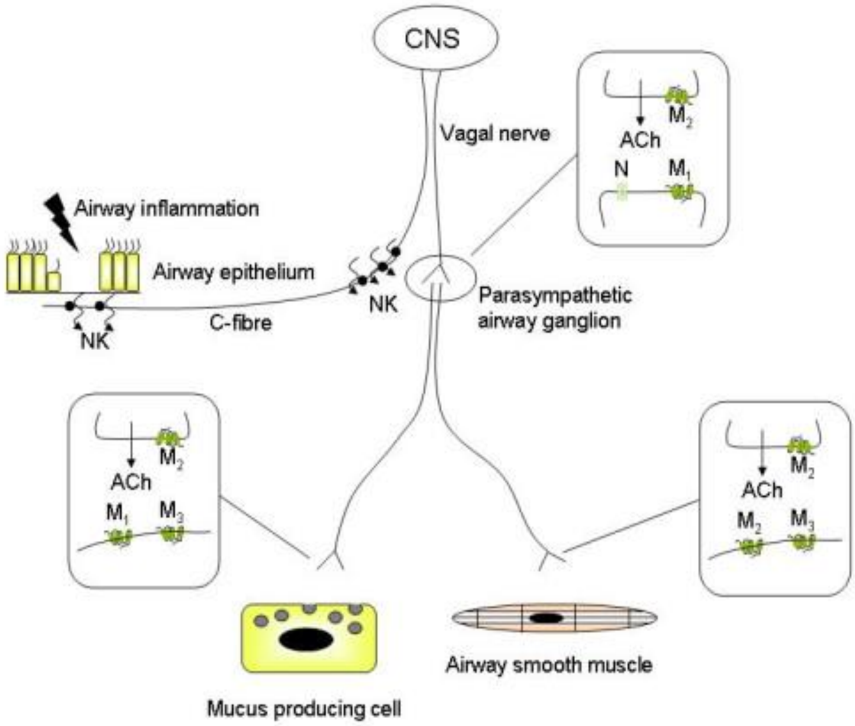


Figure 2.2 Cholinergic receptors involved in neuronal acetylcholine release and function (Gosens *et al.*, 2006).

2.5 Muscarinic receptor function in asthma

2.5.1 Mucus hypersecretion

The airway narrowing in asthma is frequently associated with the production of airway mucus in the central airways which is under cholinergic regulation (Rogers, 2000). Airway mucus is a protective film that serves to prevent inhaled particles from damaging the airway epithelium. It is composed of electrolytes, water and contains high amounts of mucins (Rogers, 2004). Mucins are glycoproteins that are responsible for the high viscosity of mucus. Acetylcholine is the dominant neurotransmitter involved in mucus secretion in the central airways (Figure 2.2). Mucus hypersecretion is a pathological feature of asthma that contributes significantly to airflow narrowing by obstructing the airways (Jeffery, 2004). Furthermore, cholinergic receptor stimulation interacts synergistically with epidermal growth factor (EGF) on mucus cell activation in airway submucosal glands which regulate goblet cell hyperplasia and mucus gland hypertrophy in asthma (Iwase *et al.*, 2002). This is supported by an animal study which has showed that repeated administration of the muscarinic agonists such as pilocarpine and methacholine can promote goblet cell hyperplasia and mucus gland hypertrophy (Rogers, 2001).

2.5.2 Airway inflammation

Recent evidences have showed that muscarinic receptors play a pro-inflammatory role in the structural cells of airway wall (Gosens *et al.*, 2006). An increased expression of M_3 muscarinic receptors on airway structural cells of asthma patients has been reported (Profita *et al.*, 2009). In addition, reduced expression of the auto-inhibitory M_2 receptor on airway neurons in asthma has been reported (Belmonte, 2005). These findings suggest that acetylcholine can regulate inflammatory processes by directly via M_3 receptor and/or indirectly via M_2 auto-receptor mechanisms (Kirkpatrick *et al.*, 2003).

There are considerable evidences that cholinergic system plays a role in lymphocytes regulation. The expression of muscarinic receptor profile and acetylcholine has been reported in mononuclear leukocytes consisting mainly lymphocytes (Fujii, 2004). Another study also showed the presence of muscarinic receptor subtype in macrophages, neutrophils and eosinophils of asthmatic patients (Profita *et al.,* 2009). These findings suggest that regulated expression of muscarinic receptor subtypes is a feature of inflammatory cells that migrate to the airways.

2.5.3 Airway remodeling

The pathophysiology of asthma is always associated with the development of structural changes of the airways which commonly known as airway remodeling. It is progressive structural changes in the airway that correlate with asthma severity. These structural changes include increased in airway muscle mass and mucus gland hypertrophy which progressively contribute to the disease severity over time and irreversible decline in lung function in patient with chronic symptoms. Previous studies suggested that cholinergic system could plays prominent regulatory role for endogenous acetylcholine in promoting allergen-induced airway remodeling (Gosens *et al.,* 2004; Halayko *et al.,* 2006). Muscarinic receptor stimulation induces profound proliferation of primary cultured human lung fibroblasts (Matthiesen *et al.*, 2005). The effect of muscarinic receptor agonists could also potentiate the responses to epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) in both human and bovine airway smooth muscle (Krymskaya *et al.,* 2000).

2.6 *Lignosus rhinocerus* sclerotium

The sclerotium of *L. rhinocerus* are the part with medicinal value. It is a compact mass of hardened fungal mycelium and represents one of the stages in the fungal life cycle. This structure is morphologically difference, nutrient-rich, multi-hyphal aggregate that serves as a food reserve and it can remain dormant until favorable growth conditions arise (Willetts and Bullock, 1992). They are long-lived compared to mycelia due to the ability to survive environmental extremes. The sclerotium of *L. rhinocerus* appears in different shapes and sizes from being spherical to oval or irregular with a diameter of 4–5 cm. The pale to the grayish- brown outer skin (rind) appears rough and wrinkly to keep the internal compacted hyphal mass from drying out.

There are quite number of previous studies used sclerotium of this mushroom to examine its bioactivities including anti-proliferative (Lai *et al.*, 2008; Suziana Zaila *et al.*, 2013), anti-inflammatory (Lee *et al.*, 2014), immunomodulatory (Wong *et al.*, 2011), anti-oxidative (Lau *et al.*, 2014) and neuritogenesis (Eik *et al.*, 2012). However, the chemical nature of many bioactive, low-molecular-weight compounds in the extracts remains unidentified (Eik *et al.*, 2012)

2.6.1 Nutritional composition

Mushrooms are a good source of proteins and contain amino acid compositions that are comparable to plant proteins. *L. rhinocerus* sclerotia is a good source of non-starch polysaccharides as it is composed mostly of insoluble fiber with very low amount of soluble fiber. The major constituents of *L. rhinocerus* sclerotia are carbohydrates (monosaccharides and disaccharides) while the fat content was <1%. Phytochemical study showed that the beta (β)-glucans represented the dominant glucans in the aqueous extracts of *L. rhinocerus*, which was 82–93% of total glucan (w/w) (Lau *et al.*, 2013). This dietary fiber substances such as β-glucans and polysaccharide-proteins have been reported to be able to stimulate immune systems and exert anti-tumor, immunomodulatory and anti-cancer activities (Wong *et al.*, 2009; Guo *et al.*, 2011).

Aqueous extraction of *L. rhinocerus* has been showed to produce the highest yield of extracted material compare to other solvent (Kong *et al.*, 2016). Higher yield of aqueous extraction suggested that *L. rhinocerus* sclerotia powder contain mainly water-soluble substances with high polarity. Study using the sequential extraction of five solvents, i.e., petroleum ether, diethyl ether, hexane, ethyl acetate, and methanol, of *L. rhinocerus* extract was conducted prior to gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS finding has revealed five major groups (alkane, fatty acids, benzene, phenol, and dicarboxylic acid) with a total of 18 constituents in *L. rhinocerus*. Linoleic acid, octadecane, and 2,3-dihydroxypropyl elaidate were present in abundance in the *L. rhinocerus* extract (Johnathan *et al.*, 2016). The content of the phenolic and terpenoid compounds in the *L. rhinocerus* sclerotia extracts are affected by the temperatures of extraction process in which cold preparation process has higher content of phenolics and terpenoids as compared to the hot preparation (Lau *et al.*, 2013). However, *in vivo* study showed that cold aqueous extract of *L. rhinocerus* exhibited significantly stronger cytotoxicity effect on both cancer and normal cell line when compare to hot aqueous extract (Lau *et al.*, 2013). This finding suggests that hot aqueous extraction process would destroy the thermolabile water soluble protein/peptide which justify the use of this extraction process for explorative pharmacology study.

2.6.2 Ethnomedicinal uses

The use of *L. rhinocerus* in the Malay states date back to the early 1900s when the mushrooms were valued by the Malays for treating tuberculosis, asthma, coughs and chest complaints (Ridley, 1890; Heyne, 1987). *L. rhinocerus* is considered to be part of Malay traditional medicine and is usually consumed with other medicinal herbs. The Malays feed this mushroom to cool the body. It is also used to nurse women after childbirth (during postpartum period) and also to stave off hunger (Laderman, 1987). Various tribes of the Orang Asli in Malaysia (the Semai, Temuan, and Jakun) are still widely using *L. rhinocerus* to relief asthma, cough, food poisoning, swollen breasts, joint pain, liver illness, swollen body parts, and as a general tonic (Lee *et al.*, 2009; Ismail, 2010). The *L. rhinocerus* is not only popular among the indigenous people but also the urban population in Malaysia (Hattori *et al.*, 2007). The sclerotia of *L. rhinocerus* are occasionally sold in Traditional Chinese Medicine (TCM) store in Malaysia. They are used by the TCM practitioner to revitalize the body of the patients. Infusions of *L. rhinocerus* is believed to improve the overall wellness of the individual by enhancing the vitality, energy, and alertness (Sabaratnam *et al.*, 2013).

There are many ways this mushroom is prepared and consumed to treat illness. In earlier years, the sclerotium was pounded and the juice was infused with water and drunk as a tonic. The mushrooms are grounded or sliced, then boiled with water for drinking or soaked into Chinese wine for external applications (Chang and Lee, 2004). The sclerotium is also eaten raw and with betel leaves to relieve a cough and sore throat. The preparation methods of decoction and/or topical medicine vary among tribes.

2.6.3 Therapeutic value in anti-asthmatic activity

There is quite a number published traditional claim that *L. rhinocerus* have therapeutic value in asthma. However, very limited validated scientific evidence is available. With the advancement of new technology, the efficacy of *L. rhinocerus* in treating asthmatic symptoms has been demonstrated in several *in vitro* and *in vivo* studies. The treatment with *L. rhinocerus* extracts on ovalbumin-induced Sprague Dawley rats showed significantly reduction of asthmatic parameters, for instance, the total immunoglobulin E (IgE) in serum, and T-helper type 2 (Th2) cytokine levels (IL-4, IL-5, and IL-13) in bronchoalveolar lavage fluid (BALF) (Johnathan *et al.*, 2016). The finding also showed the extract reduced of the number of eosinophil in BAFL and diminished the infiltration of eosinophil in the lungs (Johnathan *et al.*, 2016). The hot aqueous extract of *L. rhinocerus* (500 mg/kg) was effective in reducing asthma related parameters. Another ex vivo study showed that *L. rhinocerus* water extracts were able to relax both trachea and bronchus at 3.75 mg/ml and 2.5 mg/ml respectively (Lee *et al.*, 2018a).

2.7 Pharmacological evaluation

2.7.1 Myograph

Wire myograph is a laboratory technique used to study the mechanical activity of smooth muscle in *ex vivo* mounted experiment (del Campo and Ferrer, 2015). Airways smooth muscle such as trachea is segmented and maintained in organ bath setting which then mounted to between two wires that go through the lumen. The trachealis muscle on the segmented airways consists the cartilage ring that is connected to it. One wire is connected to a force transducer that records the isometric tension developed by the trachealis muscle, and the other is connected to a micrometric screw that allows setting up the initial tension. The mounting placement of the trachea smooth muscle procedures are tricky and complicated which require adequate training and technical competency during the conduct of study. However, wire myography technique is a very useful and reliable technique in evaluation of the contractility and relaxation properties of the smooth muscle.

The bronchorelaxant effect of Chinese herbal medicines on isolated rat tracheal ring has been showed to use the myograph system and this study has also established agonist-induced concentration response curve (Yue *et al.*, 2012). Another study also used the myograph to measure the response of constriction and dilation of the trachea, main bronchi and lobar bronchi of ovalbumin-induced rat model of asthma (Long *et al.*, 2009). Therefore, current study has adapted to use wire myograph technique as pharmacological procedure.

2.7.2 Pharmacology parameters

It is important to adequately describe the dose response relationship in explorative pharmacology study in which acts as indicator of successful treatment development. Therefore, the evaluation of pharmacodynamics relationship in current study involves in the activation and regulation of cholinergic parasympathetic system. The dose response curve (DRC) is constructed in order to describe the process of understanding pharmacodynamics *L. rhinocerus* extract on the contractility of airway smooth muscle. In the contractility study, the sigmoidal E_{max} DRC model is constructed which include parameters of efficacy (E_{max}) and agonist potency (EC_{50}). However, the relaxation study would produce different parameters such as the percentage of relaxation response and the antagonist potency (IC_{50}). These parameters would be used in current study to determine the magnitude of response in activation of cholinergic system and evaluate the effect of *L. rhinocerus* extract as muscarinic receptor antagonist.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

The overall design of this study includes both the extraction process using hot Soxhlet extraction and the *ex vivo* myograph study (Figure 3.1). This experimental protocol was aimed to evaluate the effect of *L. rhinocerus* extract (LRE) on the contractility of isolated rat's trachea via the muscarinic receptor function.

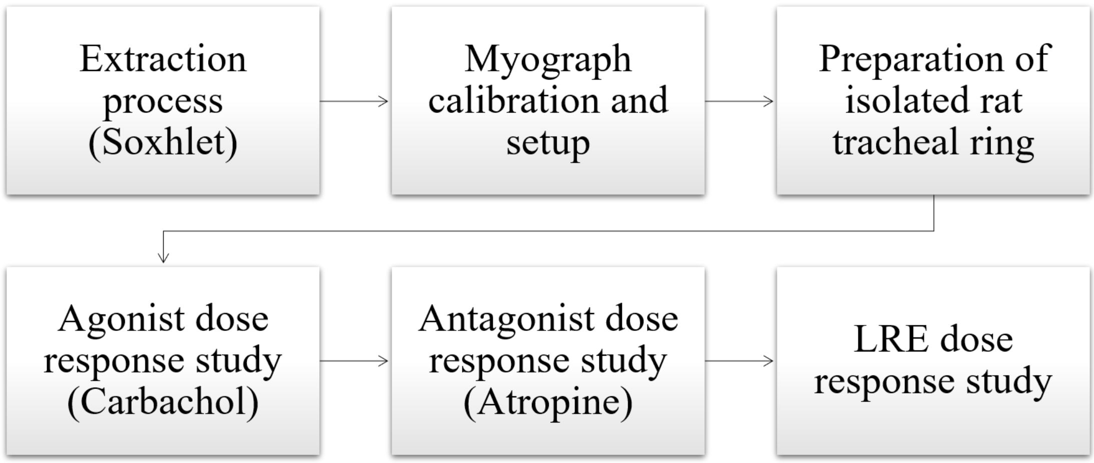


Figure 3.1 The overall experimental design of the study

3.2 *Lignosus rhinocerus* aqueous extract extraction

Dried powdered form of *L. rhinocerus* sclerotia (TM02 cultivar) was provided from Ligno Biotech Sdn. Bhd. (Selangor, Malaysia). This powdered *L. rhinocerus* was subjected to hot aqueous extraction (100℃) using soxhlet extraction method for 24 hours and then subsequently went through freeze-drying process using Freeze-drier (Ilshin BioBase, Gyeonggi-do, Korea) before milled into LRE powder. The extraction preparation was adopted from previous study (Johnathan *et al.*, 2016). The LRE powder was kept in refrigerator prior to use.

3.2.1 Soxhlet extraction

Hot aqueous extraction using soxhlet preparation was performed in the study. The justification for this method is that the previous study has shown that this process removes any undesirable toxic compounds that is presence in the *L. rhinocerus* sclerotia (Lau *et al.*, 2013). Fifty grams of the dried powdered of *L. rhinocerus* sclerotia was placed inside a thimble and blocked with cotton gauze to prevent spillage during extraction. The thimble was then loaded into the main chamber of the soxhlet extractor. Six hundred milliliters of distilled water was filled into the round-bottom flask and placed on the soxhlet heating mantle. The round-bottom flask was connected to the soxhlet chamber that was attached to a condenser (Figure 1).

Figure 3.2 Hot aqueous extraction process using Soxhlet preparation.