ANTISPASMODIC EFFECTS OF VOLATILE OIL FROM GINGER RHIZOMES (*ZINGIBER OFFICINALE* ROSCOE) ON ISOLATED RAT TRACHEAL SMOOTH MUSCLE

Thitiya Mangprayool

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ฤทธิ์ต้านการหดตัวของน้ำมันขิง (*ZINGIBER OFFICINALE* ROSCOE) ต่อกล้ามเนื้อเรียบของหลอดลมที่แยกจากหนูขาว

นางสาวฐิติยา มั่งประยูร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ขิงหรือเหง้าของ *Zingiber officinale* Roscoe (วงศ์ Zingiberaceae) ใช้เป็นส่วนประกอบในอาหารทั่วโลกมีสรรพคุณทางเภสัชวิทยาหลายอย่าง ขิงถูกนำมาใช้เป็นยาสมุนไพรตั้งแต่ครั้งโบราณเพื่อบำบัดอาการเจ็บป่วยของมนุษย์หลายประการโดยเฉพาะอย่างยิ่ง ใช้เพื่อช่วยย่อยอาหาร บรรเทาอาการปวดท้อง ท้องเสีย และคลื่นไส้อาเจียน พบว่าสารที่มีกลิ่นฉุนซึ่งเป็นส่วนประกอบในขิงและพืชวงศ์ Zingiberaceae อื่นๆ มีคุณสมบัติต้านการตอบสนองที่มากเกินไปต่อสิ่งกระตุ้นและมีฤทธิ์ต้านการอักเสบของทางเดินหายใจ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ขยายหลอดลมของน้ำมันขิงรวมทั้งระบุสารออกฤทธิ์ น้ำมันขิงที่ใช้ศึกษาในครั้งนี้ทำการสกัดแยกด้วยวิธีกลั่นด้วยน้ำ จากนั้นนำมาวิเคราะห์หาองค์ประกอบทางเคมีด้วยวิธีแก๊สโครมาโทกราฟี-แมสสเปกโตรมิทรี (GC-MS) พบว่าสารประกอบส่วนใหญ่คือ ซิทรัล (62.4%) ยูคาลิปตอล (6.9%) และแคมฟีน (4.6%) การทดสอบทางเภสัชวิทยาแบบ *in vitro* ทำในหลอดลมที่แยกจากกายของหนูขาว นำไปแขวนในอ่างจำลองสภาวะเสมือนร่างกาย เพื่อวัดแรงตึงของกล้ามเนื้อเรียบของหลอดลมด้วยเครื่อง Power Lab electronic recorder พบว่าน้ำมันขิงและซิทรัล ยกเว้นแคมฟีน สามารถยับยั้งการหดตัวของหลอดลมที่กระตุ้นโดย carbachol (CCh) ความเข้มข้น 1 ไมโครโมลาร์ ส่วนสารยูคาลิปตอลนั้นพบว่า มีฤทธิ์ในการคลายตัวกล้ามเนื้อเรียบของหลอดลมสอดคล้องกับการทดลองที่เคยมีรายงานมาก่อนแล้ว อย่างไรก็ตามเนื่องจากในน้ำมันขิงมีปริมาณสารยูคาลิปตอลค่อนข้างต่ำ ดังนั้นยูคาลิปตอลจึงน่าจะมีส่วนร่วมต่อฤทธิ์คลายกล้ามเนื้อเรียบของหลอดลมเพียงเล็กน้อย สำหรับการศึกษาหากลไกการออกฤทธิ์ที่รับผิดชอบต่อฤทธิ์คลายกล้ามเนื้อเรียบของน้ำมันขิงและซิทรัล ทำโดยใช้ verapamil (สารปิดกั้นแคลเซียม) propranolol (สารต้านบีต้ารีเซพเตอร์) L-NAME (สารต้านไนตริกออกไซด์ซินเทรส) และ indomethacin (สารต้านเอนไซม์ COX) จากผลการทดลองพบว่าทั้งน้ำมันขิงและซิทรัลมีฤทธิ์ทำให้เส้นโค้งความสัมพันธ์ระหว่างความเข้มข้นของแคลเซียมและการหดตัวของกล้ามเนื้อเรียบของหลอดลมเคลื่อนไปทางขวาเช่นเดียวกับ verapamil ยิ่งไปกว่านั้นยังพบว่า propranolol สามารถยับยั้งฤทธิ์คลายกล้ามเนื้อของน้ำมันขิงและซิทรัลได้ แต่ฤทธิ์ยับยั้งดังกล่าวนี้ไม่เกิดขึ้นเมื่อใช้ L-NAME และ indomethacin จากผลการทดลองที่กล่าวมาทั้งหมดชี้แนะว่าฤทธิ์คลายกล้ามเนื้อเรียบของน้ำมันขิงและซิทรัลมีกลไกการออกฤทธิ์ผ่าน Ca^{2+} channel และ β_2-adrenergic receptor

สรุป การศึกษานี้ได้แสดงให้เห็นถึงฤทธิ์ขยายหลอดลมของน้ำมันขิงและสารออกฤทธิ์ซิทรัล โดยพบว่าน่าจะมีกลไกการออกฤทธิ์คือไปปิดกั้น Ca^{2+} channel และกระตุ้น β_2-adrenergic receptor งานวิจัยชิ้นนี้ทำให้เกิดหลักฐานด้านเภสัชวิทยาเพื่อใช้สนับสนุนการใช้ขิงเป็นยาช่วยขยายหลอดลมเพื่อประโยชน์ในการรักษาโรค

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

THITIYA MANGPRAYOOL : ANTISPASMODIC EFFECTS OF VOLATILE OIL FROM GINGER RHIZOMES (*ZINGIBER OFFICINALE* ROSCOE) ON ISOLATED RAT TRACHEAL SMOOTH MUSCLE. THESIS ADVISOR : ASSOC. PROF. NUANNOI CHUDAPONGSE, Ph.D. 80 PP.

ZINGBIBER OFFICINALE/GINGER OIL/CITRAL/BRONCHODILATION/ ANTI-SPASMODIC/TRACHEAL SMOOTH MUSCLE/ASTHMA

Ginger, the rhizome of *Zingiber officinale* Roscoe (Zingiberaceae) which is a common constituent of diets around the world, exhibits several pharmacological activities. Ginger has been traditionally used from time immemorial for various human aliments worldwide, especially to relieve digestion and treat stomach upset, diarrhea, and nausea. Some pungent constituents present in ginger and other zingiberaceous plants have been shown to produce potent anti-hyperactivity and anti-inflammatory activities on airway. The present study was aimed to describe bronchodilatory activity of ginger oil and identify its active compounds. Ginger oil was extracted by hydro-distillation. The compositions of ginger oil were analyzed by gas chromatography and mass spectrometer (GC-MS). Citral (62.4%), eucalyptol (6.9%), and camphene (4.6%) were found to be the major components. The *in vitro* pharmacological study was conducted in isolated tracheal preparation of Wistar rat. The tissue was suspended in organ bath to measure the isometric response, using Power Lab electronic recorder. Ginger oil and citral, but not camphene, suppressed rat tracheal contraction induced by 1 µM carbachol (CCh). Consistent with previous report, eucalyptol also showed a relaxing effect on rat airway. However, the content of eucalyptol in ginger oil was relatively low, therefore the contribution of eucalyptol to the bronchodilatory effect of

ginger oil was small. To elucidate the mechanisms responsible for the myorelaxing effect, verapamil (a Ca^{2+} channel blocker), propranolol (a β-adrenergic receptor antagonist), L-NAME (a NOS inhibitor) and indomethacin (a COX inhibitor) were used to test the inhibitory effects of ginger oil and citral. The results showed that both ginger oil and citral shifted concentration-response curve of Ca^{2+} in CCh-induced tracheal smooth muscle contraction towards right similar to verapamil (positive control). Moreover, propranolol, but not L-NAME and indomethacin, inhibited bronchodilatory effects of both ginger oil and citral. Taken together, the results suggested that ginger oil and citral relax airway smooth muscle through Ca^{2+} channel and β_2-adrenergic receptor.

In conclusion, herein the brochodilatory effects of ginger oil and citral, its major active ingredient have been demonstrated. The possible mechanisms underlying this relaxing effect appeared to involve β_2-adrenergic receptor stimulation and Ca^{2+} channel blockade. This study provides the pharmacological basis supporting therapeutic potential of *Z. officinale* rhizomes as a bronchodilator.

School of Pharmacology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

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LIST OF ABBREVIATIONS

α	=	alpha, adrenoceptor
β	=	beta, adrenoceptor
µg	=	microgram
µl	=	microliter
µM	=	micromolar
µm	=	micrometer
ACh	=	acetylcholine
amu	=	atomic mass unit
ATPase	=	adenosine triphosphatase
^{o}C	=	degree Celsius
Ca^{2+}	=	calcium ion
$[Ca^{2+}]$	=	calcium ion concentration
$[Ca^{2+}]_i$	=	cytosolic calcium ion concentration
$CaCl_2$	=	calcium chloride
cAMP	=	adenosine 3', 5'-cyclicmonophosphate
CCh	=	carbachol
cGMP	=	guanine 3', 5'-cyclicmonophosphate
Cl	=	chloride
COPD	=	chronic obstructive pulmonary disease
COX	=	cyclooxygenase

LIST OF ABBREVIATIONS (Continued)

CRCs	=	concentration-response curves
EC_{50}	=	half maximal effective concentration
EI	=	electron ionization
EGTA	=	ethylene glycol tetraacetic acid
eNOS	=	endothelial nitric oxide synthase
eV	=	electron volt
g	=	gram
GC/MS	=	gas chromatography/mass spectrometry
GMP	=	guanosine 5'-monophosphate
hr	=	hour
IC_{50}	=	half maximal inhibitory concentration
IFN-γ	=	interferon gamma
IgE	=	immunoglobulin E
IL	=	interleukin
iNOS	=	inducible nitric oxide synthase
IP_3	=	inositol (1,4,5)-tris-phosphate
IP_3R	=	inositol (1,4,5)-tris-phosphate receptor
l	=	liter
L-NAME	=	N_ω -nitro-L-arginine methyl ester hydrochloride
LTs	=	leukotrienes
M	=	molar

LIST OF ABBREVIATIONS (Continued)

Mg^{2+}	=	magnesium ion
mg	=	milligram
min	=	minute
ml	=	milliliter
MLCK	=	myosin light chain kinase
mm	=	millimeter
mM	=	millimolar
min	=	minute
mol	=	mole
mRNA	=	messenger ribonucleic acid
mV	=	millivolt
MS	=	mass spectrometry
nNOS	=	neuronal nitric oxide synthase
NA	=	noradrenaline
Na	=	sodium
NO	=	nitric oxide
NOS	=	nitric oxide synthase
%	=	percent
p	=	probability
PDE	=	phosphodiesterase enzyme
pH	=	-log of hydrogen concentration

LIST OF ABBREVIATIONS (Continued)

PG	=	prostaglandin
PK	=	protein kinase
ROCC	=	receptor-operated cation channels
S.E.M	=	standard error of the mean
SOC	=	store-operated calcium
SR	=	sarcoplasmic reticulum
TNF-α	=	tumor necrosis factor-alpha
TLR4/NF-κB	=	toll-like receptor 4/ nuclear factor kappa-light-chain-enhancer of activated B cells
VOCCs	=	voltage-operated calcium channels
v/v	=	volume/volume
w/w	=	weight/weight
wt	=	weight

CHAPTER I

INTRODUCTION

1.1 Problem background

Asthma, an inflammatory disease of the airway, is a serious global health problem. Its common symptoms and signs including wheezing, coughing and tightness of the chest, occur after exposure to allergens. The breathing difficulty and shortness of breath are induced by bronchial hyperresponsiveness and airway remodeling. People who suffer from this chronic condition (long-lasting or recurrent) are said to be asthmatic. The inside walls of an asthmatic's airways are swollen or inflamed. This swelling or inflammation makes the airways extremely sensitive to irritations and increases susceptibility to an allergic reaction. An estimated 300 million people worldwide are affected by this chronic airway disorder which can be severe and sometimes fatal (Peters, Ferguson, Deniz, and Reisner, 2006). Moreover, the prevalence of asthma is increasing everywhere, especially among children. Recently, medical researchers have verified that asthmatic people living in highly urbanized and sparsely vegetated areas are at a greater risk of suffering severe asthma attacks that lead to hospital admissions (Ayres-Sampaio *et al*., 2014). It causes a significant burden, not only in terms of health care costs but also lost productivity and reduced participation in family life.

The pharmacological treatments for asthma can be divided into bronchodilators, which give rapid relief of symptoms mainly through relaxation of airway smooth

muscle and (2) controllers, which inhibit the underlying inflammatory process (Boushey, 2009). There are three classes of bronchodilators in current use: (1) β_2-adrenergic agonists, (2) anticholinergics, and (3) theophylline. For asthma controller, inhaled corticosteroids are the most effective drugs. Corticosteroids act primarily by reducing the number of inflammatory cells and their activation on the airways. Although the standard therapy is accepted to be effective and safe, many patients cannot access to these drugs because most of them are expensive. Thus, the quest for anti-asthmatic therapy from medicinal plants still remains an area of active pharmacological research.

Ginger rhizome of the *Zingiber officinale* Roscoe (family Zingiberaceae) has been used as traditional medicine in Asian countries, including Thailand. For example, it has been widely used to aid digestion and treat stomach upset, diarrhea, motion sickness and nausea related to pregnancy or chemotherapy (Apariman, Ratchanon, and Wiriyasirivej, 2006; Willetts, Ekangaki, and Eden, 2003; Yamahara, Rong, Naitoh, Kitani, and Fujimura, 1989). It also has beneficial effects in common cold, headaches and several other diseases. It has been shown effectiveness in aroma-massage therapy as an alternative method for short-term knee pain relief (Yip and Tam, 2008). Osteoarthritis patients who received ginger extract twice daily experienced less pain and required fewer pain-killing medications compared to those who received placebo (Altmann, Gilman, Hayoz, Willis, and Himpsel, 2001). It has been proposed that ginger has potential to cause smooth muscle relaxation and relieve pain in dysmenorrheal. Buddhakala and co-workers (Buddhakala, Talubmook, Sriyotha, Wray, and Kupittayanant, 2008) have discovered that ginger oil inhibits spontaneous and prostaglandin (PG)-induced myometrial contraction. In this study, citral, but not camphene, was identified as bioactive compound of ginger oil for its smooth muscle

relaxation. Moreover, a group of researchers from Iran found that ginger reduced the symptoms, such as nocturnal cough and dyspnea in asthmatic patients (Rouhi, Ganji, and Nasri, 2006); however the mechanisms underlie this effect have not been clearly elucidated.

From the study in mouse lung slices, the hydromethanolic crude extract of *Z. officinale* has been shown to inhibit acetylcholine-induced contraction of airway smooth muscle cells, possibly via blockade of plasma membrane Ca^{2+} channels (Ghayur, Gilani, and Janssen, 2008). In addition to parasympathetic nervous system, there are other mechanisms that involve in controlling airway diameter, such as β_2-adrenoceptor and mast cells. Mast cells sensitized with immunoglobulin E (IgE) for specific antigen will release stored substances on subsequent exposure to that antigen. These substances include several enzymes, chemotactic factors, leukotrienes, histamine, bradykinin and prostaglandin D_2 (PGD_2) (Pelaia *et al*., 2008). The inhibition of leukotrienes is considered as a new asthmatic drug discovery because the first leukotriene receptor antagonists has been available to the market since 1996 and the first lipoxygenase inhibitor has been released in 2006. It has been found that the rhizomes of ginger contain potent inhibitors against prostaglandin biosynthesizing enzyme. Moreover, the active compounds of *Z. officinale*, gingerols and diarylhepatanoids have been found to inhibit 5-lipoxygenase, an enzyme of leukotriene biosynthesis in rat basophilic leukemia cells (Kiuchi, Iwakami, Shibuya, Hanaoka, and Sankawa, 1992). A recent study has reported that zingerone, one of the active components of ginger, has a protective effect on LPS-induced acute lung injury in mice (Xie *et al*., 2014).

To support the beneficial activity of *Z. officinalae* rhizomes on respiratory system, the present study was designed to determine the bronchodilatory effect of

ginger oil using isolated trachea of rats. The possible mechanism(s) underlying the airway relaxation of ginger oil including (1) β_2-adrenergic receptor (2) voltage-gated Ca^{2+} channel and (3) eicosanoid pathways were examined. The postulated bioactive substances responsible of the relaxation effect of ginger oil were identified as well.

1.2 Research objectives

1.2.1 To examine the effects of ginger oil on the contraction of rat tracheal smooth muscle.

1.2.2 To identify a major component that is responsible for the effects of ginger oil on rat tracheal smooth muscle contraction.

1.2.3 To find the pathways that involve in the effect of ginger oil on airway contraction.

1.3 Research hypotheses

1.3.1 The volatile oil from *Z. officinale* has bronchodilatory effects on isolated rat tracheal smooth muscle.

1.3.2 The effect on airway relaxation of ginger oil involves β_2-adrenergic receptor and voltage-gated Ca^{2+} channel.

1.3.3 Citral contributes to the inhibitory effect of ginger oil on the smooth muscle contraction as a major active ingredient.

1.4 Significance of the study

This study provided more definitive evidence to establish the activity and mechanisms of the Thai medicinal plant *Z. officinale* in the inhibition of airway

contraction. A detailed understanding of the mechanisms of action of ginger and its active ingredients on the function of airway smooth muscle justifys the rationale use of Thai medicinal herb as an alternative or additive asthmatic therapy.

CHAPTER II

LITERATURE REVIEW

2.1 Ginger (*Zingiber officinale* Roscoe)

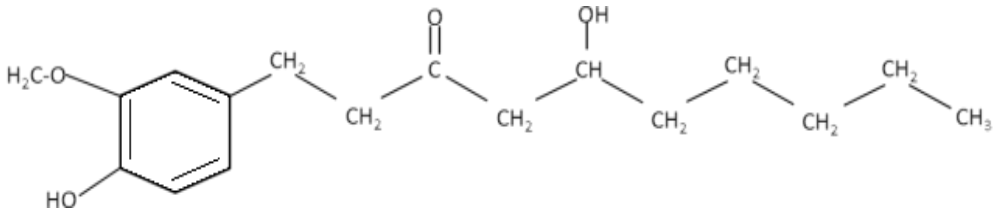
Ginger is the rhizome of *Zingiber officinale* (family: Zinggiberaceae), which is widely used as a culinary agent and traditional medicines throughout the world, especially in Asia. Ginger is commonly used as a spice in cuisines. It is also employed in the beverage and fragrance industries. The part used for medicine is roots or rhizomes.

Ginger is a rhizomatous perennial herb with height of up to 90 cm above cultivation. Rhizomes are aromatic, thick lobed, pale yellowish, bearing simple alternate distichous narrow oblong lanceolate leaves. The herb develops abundant lateral shoots in clumps, which begin to dry when the plant matures. Leaves are long and 2-3 cm broad with sheathing bases, the blade gradually tapering to a point. In florescence solitary, lateral radical pedunculate oblong-cylindrical spikes. Flowers are rare, rather small, calyx superior, gamosepalous, three toothed, open splitting on one side, corolla of three subequal oblong to lanceolate connate greenish segments. It is widely cultivated all over and perennially grows in warm climates such as Thailand, India, Bangladesh, Taiwan, Jamaica, and Nigeria (Kemper, 1999).

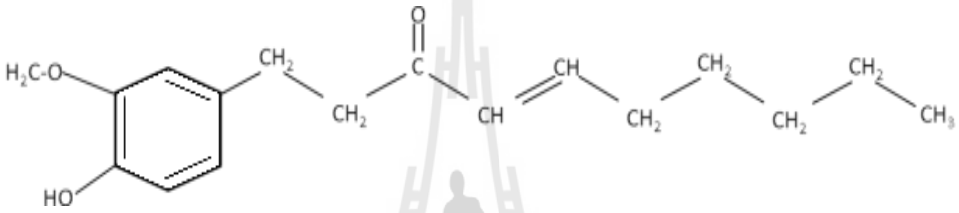
For centuries, ginger is useful for medicinal purposes in Asia. Because of its strong aromatic properties, it is characterized in traditional medicine as spicy and hot. It is recommended by the traditional healers for use in dyspepsia, flatulence, colic,

diarrhea, headaches, nausea, rheumatism, and colds (Borrelli, Capasso, Pinto, and Izzo, 2004; Ghayur, Gilani, Afridi, and Houghton, 2005). The aromatic, spasmolytic carminative and absorbent properties of ginger suggest that it has direct effects on the gastrointestinal tract (Ernst and Pittler, 2000). Thomson and co-worker (Thomson *et al*., 2002) have discovered that ginger could be used as an cholesterol-lowering, antithrombotic and anti-inflammatory agent. Ginger is now the most important herb for international trade.

Phytochemical reports have shown that the main constituents and active ingredients of ginger are gingerols, shogaols, zingerone, and paradol (Langner, Greifenberg, and Gruenwald, 1998). Previous quantitative analyses have shown that gingerols can be changed to shogaols, zingerone, and paradol, which are a family of homologous constituents differentiated by the number of carbon atoms in their side chain. [6]-Gingerols and [6]-shogaol are the major components in the rhizome of ginger (Figure 2.1) and [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol are known as pungent constituents (Connell and McLachlan, 1972; Ghayur *et al*., 2005). The compound [6]-gingerol appears to be responsible for a variety of interesting pharmacological effects and has been used as a marker property of ginger. Some studies have shown that [6]-gingerol possessed analgesic and anti-inflammatory activites (Young *et al*., 2005). The pungency of ginger is also from the shogaol family, resulting from dehydration of gingerols. They can appear naturally and are formed chemically from the analogous gingerols in optimal condition (Connell and Sutherland, 1969; Jolad *et al*., 2004). This may be the reason why shogaols are found in small amounts in fresh ginger and in larger amounts in dried or extracted products.



[6]-Gingerol



[6]-Shogaol

Figure 2.1 Active principles of ginger (Shukla and Singh, 2007).

The property of aroma is involved with a volatile oil. Ginger has been reported to contain usually 1-3% of volatile oil. Its pungency is attributed to ginger oleoresin. The oleoresin is a mixture of homologue gingerols that have differences of number of carbon atom in the side chain (Shadmani *et al*., 2004). Ginger oleoresin contains mainly the pungent principles gingerols and shogaols as well as zingiberone. Shogaols have recently been found to be twice as pungent as gingerols. Ginger oleoresin is used widely as a flavoring agent in the food and beverage industries. Another component of ginger oil contains zingiberene and bisaboline as major constituents along with other sesqui and monoterpenes (Wohlmuth, Smith, Brooks, Myers, and Leach, 2006). At present, ginger oil is well-known in aroma therapy and perfume industry.

Recently, medical researchers have verified that ginger contains many active substances. Ginger is listed in modern pharmacopoeias and repertories, and has extensive range of confirmed pharmacological properties and its active ingredients. For example, a meta-analysis study of clinical properties of ginger showed that it is more effective for treatment of nausea and vomiting postoperative symptoms than placebo (Chaiyakunapruk, Kitikannakorn, Nathisuwan, Leeprakobboon, and Leelasettagool, 2006). Likewise, it has been shown in women with less than 16 weeks of pregnancy that ginger is more effective than acupressure to relieve mild to moderate nausea and vomiting (Saberi, Sadat, Abedzadeh-Kalahroudi, and Taebi, 2013). From previous report, a study was conducted by a group of researchers in atherosclerotic, apolipoprotien E-deficient mice to demonstrate an anti-atherogenic activity of ginger. It was found that prevention of atherosclerosis by ginger extract was associated with reduced oxidation of low density lipoprotein produced by macrophage (Fuhrman, Rosenblat, Hayek, Coleman, and Aviram, 2000). In addition, hepatoprotective effect of ginger has been shown in streptozotocin-induced diabetic rats. The result showed that free polyphenols extracted from *Z. officinale* could alleviate liver damage caused by streptozotocin (Kazeem, Akanji, Yakubu, and Ashafa, 2013). Similarly, ethanolic extract, but not n-hexane extract, from *Z. officinale* has been shown to possess protective effects in pancreatic β cells against hydrogen peroxide (Rackova *et al*., 2013). Moreover, Rodrigues and co-worker also reported the nephroprotective effect of gingerol against gentamicin-mediated nephropathy. Gentamicin is an antibiotics that is widely known to cause renal dysfunction by generation of oxidative stress and induction of inflammatory processes. It was found that gingerol significantly reduced mRNA transcription for TNF-α, IL-2, and IFN-γ in dose-dependent fashion (Rodrigues *et al*., 2014). In addition, analgesic and anti-inflammatory effects were investigated in

mice (Young *et al*., 2005). The data suggested that ginger could be used as analgesic and anti-inflammatory agent since an intraperitoneal administration of [6]-gingerol, a majaor component, inhibited paw edema induced by carrageenin. Furthermore, [6]-shogaol, another major component of ginger, prevents the growth of human pancreatic tumors and potenitate the anti-tumorigenesis effect of gemcitabine by blocking of TLR4/NF-κB-mediated inflammatory pathways (Zhou *et al*., 2014). There was an attempt to test anticancer potential and mechanism of action of gingerol. Modulation of testosterone induced antiapoptotic proteins by [6]-gingerol has been studied in *in vitro*, using androgen sensitive LNCaP cells. It has been found that [6]-gingerol induces cell apoptosis, downregulates Bcl-2 and Survivin and upregulates Bax expression (Shukla and Singh, 2007). From the same study, similar results have been reported *in vivo*, ventral prostate of Swiss albino mice.

Ginger has also shown beneficial properties involved in relaxation of smooth muscle cells. Pancho and co-workers (Pancho, Kimura, Unno, Kurono, and Kimura, 1989) reported the effects of crude ginger extracts, S-(+)-[6]-gingerol and [6]-shogaol on noradrenaline(NA)-induced contraction by using mouse mesenteric veins. The result showed that both S-(+)-[6]-gingerol and [6]-shogaol inhibited the contractile responses to NA-induced contraction. Moreover, ginger, [6]-gingerol, [8]-gingerol, and [6]-shogaol, relax airway smooth muscle and [8]-gingerol meliorate airway hyperresponsiveness, partly by modulating intracellular Ca^{2+} regulation. It was suggested that these active compounds could be useful in the treatment of airway diseases, such as asthma in the therapeutic option as sole drug or in the combination with accepted therapeutic drugs, including β_2-agonists (Townsend *et al*., 2013). Very recently, intracellular signal transduction mediated by the constituents of ginger extracts has been studied in human airway smooth muscle cells. It has been

demonstrated that [6]-gingerol, [8]-gingerol and [6]-shogaol potentiate brochodilatory effect of β-adrenergic receptor agonists, by inhibition of the functions of phosphodiesterase type 4 and cytoskeletal regulatory proteins (Townsend, Zhang, Xu, Wakita, and Emala, 2014). On smooth muscle of reproductive system, it has been reported that ginger oil inhibits spontaneous and $PGF_{2\alpha}$-induced myometrial contraction (Buddhakala *et al*., 2008). In addition, the aqueous extracts of ginger blocked contraction of isolated rat aorta induced by phenylephrine and atropine, suggesting that its relaxing effects mediated via both stimulation of muscarinic receptor and Ca^{2+} channel blockade (Ghayur *et al*., 2005). Finally, similar result has been demonstrated in airway smooth muscle using mouse lung slices. Inhibitory effect of the aqueous methanolic crude extract of ginger on airway contraction has been found to be associated with blockade of plasma membrane Ca^{2+} channels (Ghayur *et al*., 2008). This data suggests its beneficial uses in the treatment of cough as well as asthma. However, the effect of ginger oil on isolated tracheal smooth muscle and its signaling mechanisms have not yet been reported.

2.2 Anatomy and physiology of trachea

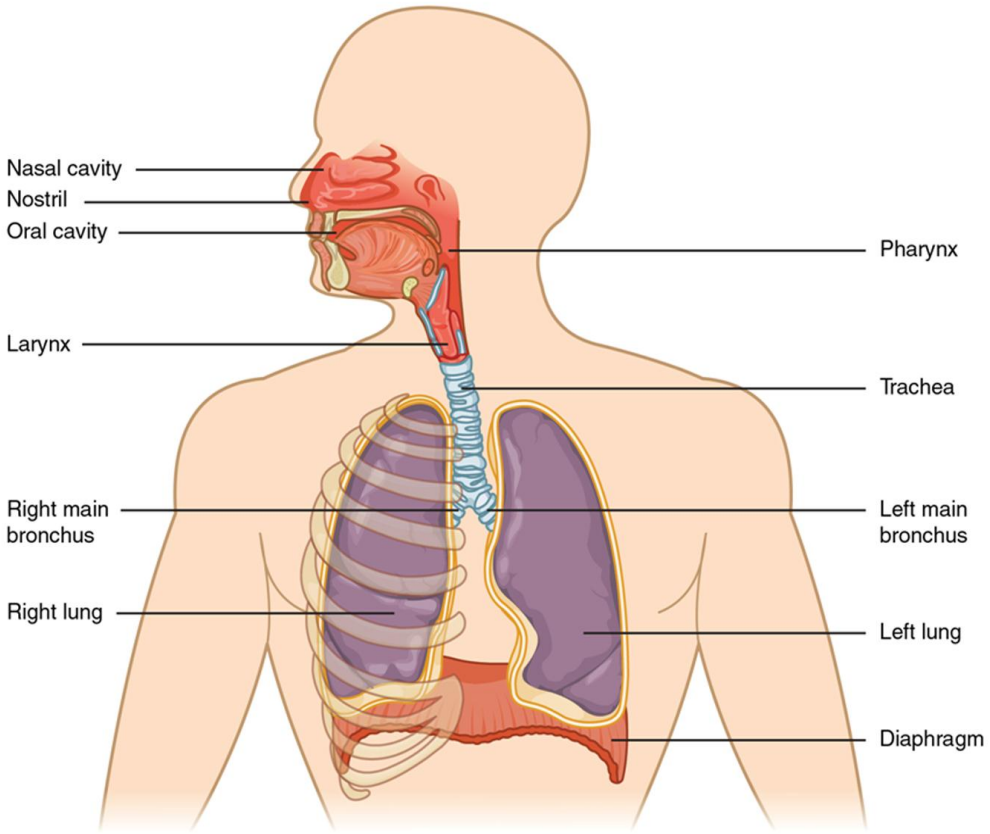


Figure 2.2 The major respiratory structures span the nasal cavity to the diaphragm (College, 2013).

The respiratory tract is functionally divided into the conducting and the gas exchanging part. As shown in Figure 2.2, the conducting airways can be divided into 2 parts, the upper airways including nose (nostrils), mouth, nasopharynx, oropharynx, hypopharynx, and larynx and the lower airways from trachea to alveolar ducts (Kimoff, 2005). The upper and lower respiratory airways form a continuous tract with anatomical and histological properties. The one important function is passage of air in and out of the lungs.

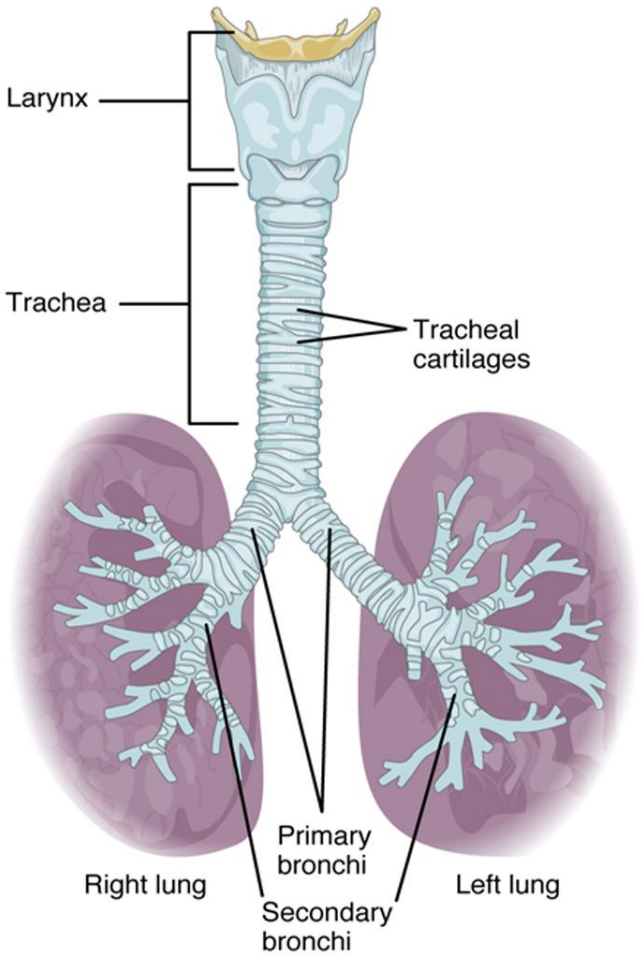


Figure 2.3 The tracheal tube is formed by stacked, C-shaped pieces of hyaline cartilage (College, 2013).

The trachea, or windpipe, is a 10-12 cm long tube with 2 cm in diameter which begins below the larynx corresponding to the sixth cervical vertebra. It is supported by irregular ring cartilages. The C-shaped cartilages (Figure 2.3) are connected by ligaments and banded together posteriorly, by a smooth muscle. The esophagus which lies posteriorly shares its anterior wall with the posterior wall of the trachea. The proximity of these two structures is a reason why some asthmatic patients can experience a bronchoconstriction when ingesting very cold drinks or food. At T5 to T7

vertebral level, the trachea bifurcates into two lobar bronchi for the left lung and into three lobar bronchi for the right lung. As seen in Figure 2.4, distal to the bifurcates, respiratory bronchioles and alveoli are present and constitute the gas exchanging part of the lung (Gaga, Vignola, and Chanez, 2001; Ritz *et al*., 2002).

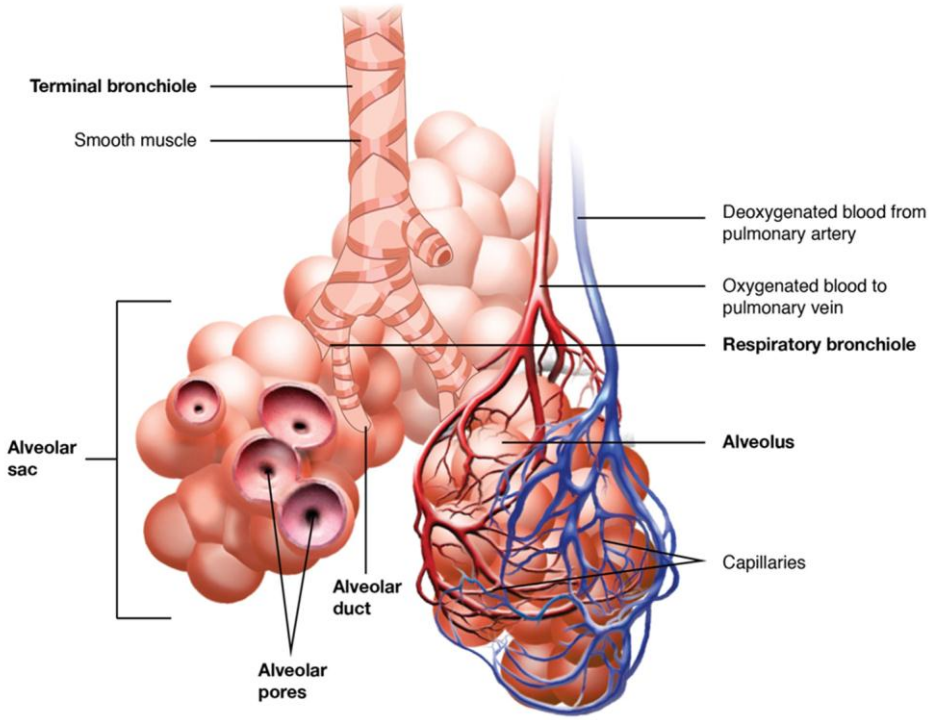


Figure 2.4 Bronchioles lead to alveolar sacs in the respiratory zone, where gas exchange occurs (College, 2013).

2.3 Asthma

Asthma is an airway disorder that causes contraction of the smooth muscles in the airway, breathlessness, cough, wheezing, and dyspnea. It results from respiratory hypersensitivity, inflammation, and spasmodic obstruction. Several million people worldwide are affected by this chronic airway disorder which sometimes can be fatal in severe cases (Peters *et al*., 2006). The prevalence of asthma is increasing everywhere,

especially among children. Most patients with asthma may have a significant trouble, because of the cost of drugs and lost of participation with their family.

Inflammation, airway hyperresponsiveness, and changeabled airway blockage are the primary characteristics of asthma. Previous studies regarding the pathogenesis of asthma have concentrated on the advancement of an inflammatory response orchestrated by a subset of T-helper cells (Figure 2.5). These inflammatory mediators initiate airway smooth muscle contraction, vasodilation, plasma extravasation, mucus secretion, and inflammatory cell influx. Allergens also can interact directly with IgE bound to the high affinity IgE receptor on occupant mast cells to precipitate these effects. The large airway enlargement in airway smooth muscle is a structure of fatal asthma, as is the luminal obstruction because mixtures of inflammatory exudates and mucus (Jeffery, 2001a). The result in inflammation and airway obstruction generates the clinical diagnosis of asthma. With chronic allergic inflammation, the airways may become remodeled, with thickening of the mucosal basement membrane airway smooth muscle and goblet cell hyperplasia. Airway obstruction, and the continuously inflammation and subepithelial fibrosis are related with the existence of exacerbations and nonspecific bronchial hyperresponsiveness (Bousquet, Jeffery, Busse, Johnson, and Vignola, 2000).

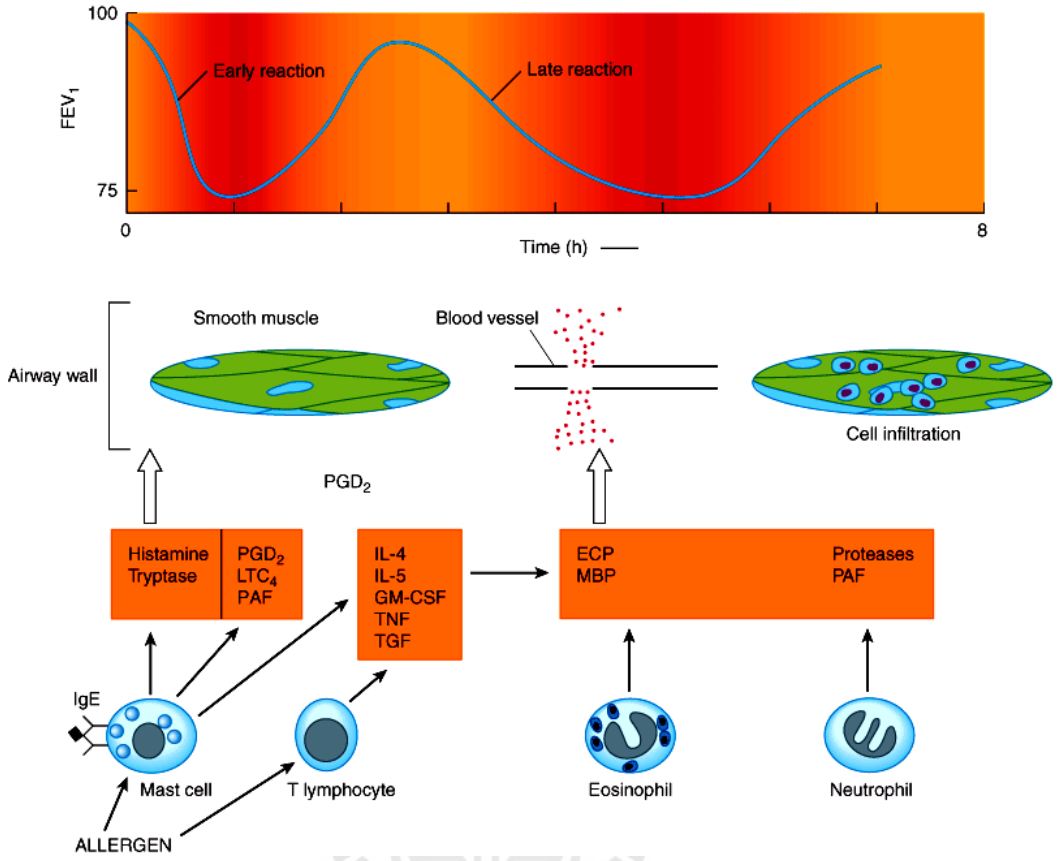


Figure 2.5 Conceptual model for the immunopathogenesis of asthma (Boushey *et al.*, 2009).

There is increasing evidence suggesting that changes in airway structure by collection of eosinophils, lymphocytes, mast cells, macrophages, dendritic cells, and myofibroblasts (Chetta, Marangio, and Olivieri, 2003). The airway remodeling can be observed by hypertrophy and hyperplasia of airway smooth muscle, augmentation in mucous glands, thickening of the reticular basement membrane (Figure 2.6). Thus, this has in turn been connected to the enhancement of physiological dysfunction, providing a probable mechanism for the development of fixed air flow limitation seen in many asthmatic patients (Ward *et al.*, 2002).

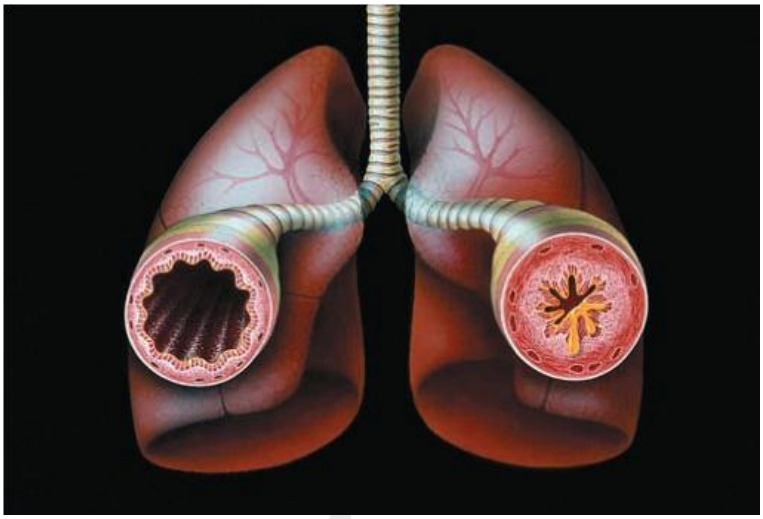


Figure 2.6 The open airways of the lung (left) and narrowed bronchioles (right) of patient who suffering from asthma. Repeated cycles of inflammation result in airway reformation, fibrosis and the accumulation of many materials including collagen (Jeffery, 2001b).

Bronchial hyperresponsiveness is a well-established hallmark of airway function in asthma and is determined by an exaggerated response of the airways to non-specific stimuli. The two reasons of bronchial hyperresponsiveness are an increase in reactivity as well as sensitivity of the airways. The airway remodeling is considered to provide significantly to both components of bronchial hyperresponsiveness. First, the adventitial thickening can attenuate the load on airway smooth muscle which is provided by lung elastic contract. Secondly, the thickening of the inner wall can increase the effects of airway smooth muscle moderation (King, Pare, and Seow, 1999). For the second reason, airway remodeling in bronchial asthma is reversibility of airflow obstruction. The reversibility of airflow obstruction can be reverse both

spontaneously and after treatment. Furthermore, bronchial asthma is associated with an enlargement in the rate of descent in respiratory factors.

According to the current international guidelines, the therapeutic control of asthma is based on bronchodilators, which give rapid relief of symptoms mainly through relaxation of airway smooth muscle and controllers, which inhibit the underlying inflammatory process (Rodrigo, 2006). There are three classes of bronchodilators in current use:

1) β_2-Adrenergic agonists: β_2-adrenoceptor agonist bronchodilators are the most effective and extensively used to produce rapid reversal of bronchoconstriction. Even though usually classified by their duration of action, as short-acting and long-acting β_2-agonists (Nelson, 2009). β_2-agonists are based on adrenaline and early forms, such as isoprenaline, lacked bronchial selectivity and had unpleasant side effects. Recent β_2-agonists are more specific selective for the β_2-adrenoceptors located in bronchial smooth muscle and have less cardiotoxicity. Traditional β_2-agonists, such as salbutamol, terbutaline and fenoterol, were characterized by a rapid onset but relatively short duration of action (Sears and Lotvall, 2005).

2) Anticholinergics: Anticholinergic agents have considerably been used as bronchodilators for the treatment of obstructive airway disorder. While their effectiveness in chronic asthma proposes no preference over β_2-agonists, evidence continues to accumulate suggesting substantial important benefit in acute asthma attacks. This increasing reaction to anticholinergics suggests that cholinergic bronchoconstriction is increased in acute asthma. Several mechanisms correlate with changes in expression and function of inhibitory M_2 muscarinic receptors on the

airway parasympathetic nerves may be involved (Gross, 2006; Jacoby and Fryer, 2001).

3) Theophylline: It has been shown profit in the control of asthma in patients. Theophylline is extensively used as a bronchodilator in patients with airflow restriction diseases such as bronchial asthma and chronic obstructive pulmonary disease (COPD). It is an inexpensive drug suggested by international guidelines as the central treatment for the COPD management (Iiboshi *et al*., 2007; Ram *et al*., 2005).

For asthma controller, inhaled corticosteroids are the most effective drugs. Corticosteroids act primarily by reducing the number of inflammatory cells and their activation on the airways. Inhaled steroids are able to down-regulate several airway inflammatory cytokines (Wang, Trigg, Devalia, Jordan, and Davies, 1994), to reduce cell infiltration of bronchial wall (Booth *et al*., 1995), and may have some in vitro anti-angiogenic properties, because they directly inhibit the presentment of the vascular endothelial growth factor gene (Nauck, Karakiulakis, Perruchoud, Papakonstantinou, and Roth, 1998). Inhaled steroids are quite efficient on inflammatory changes of airway wall in asthma (Chetta *et al*., 2003).

2.4 Smooth muscle contraction and relaxation

Smooth muscle cells are a type of tissue which enables them to constrict or dilate. They consist of the cardiovascular, gastrointestinal, genitourinary, and respiratory smooth muscle cells. They response for contraction from the innervation from the autonomic nervous system and signals from hormones (autocrine/ paracrine agents) and other local chemicals (Webb, 2003). Smooth muscle cells have two different mechanism of activation, which are tonic and phasic contractions to changes

in load or length (Petkov and Boev, 1999). Nevertheless the stimulus, smooth muscle cells use cross-bridge cycling between actin and myosin to originate force, and calcium ions (Ca^{2+}), which activates the Ca^{2+}-calmodulin-dependent enzyme myosin light-chain kinase (MLCK), serve to commence contraction (Figure 2.7) (Gerthoffer and Larsen, 2000).

The role of smooth muscle contraction is controlled by receptor and mechanical stimulation of proteins myosin and actin (Gerthoffer and Larsen, 2000). Contraction is triggered by influx of calcium through transmembrane calcium channels (Floyd and Wray, 2007). Calcium ions entry from the extracellular into intracellular smooth muscle cells by the voltage-dependent calcium channel. After that, the calcium combines with calmodulin to form a complex that converts MLCK to its active form. MLCK must phosphorylate the 20 kDa light chain of myosin cooperated with actin. Therefore, the phosphorylation state of the light chain of myosin a highly regulated the contractile activity (Webb, 2003). Sarcoplasmic reticular (SR) play an important role in intracellular Ca^{2+} rise as well. Ca^{2+} release from the SR store proceeds via two main receptor controlled channels, the inositol-1,4,5-trisphosphate (IP_3) receptor (IP_3R) and the ryanodine receptor (RyR). Thus, the change in concentration of calcium channels can occur from the activity of channels in the plasma membrane or receptor on SR membrane (Floyd and Wray, 2007).

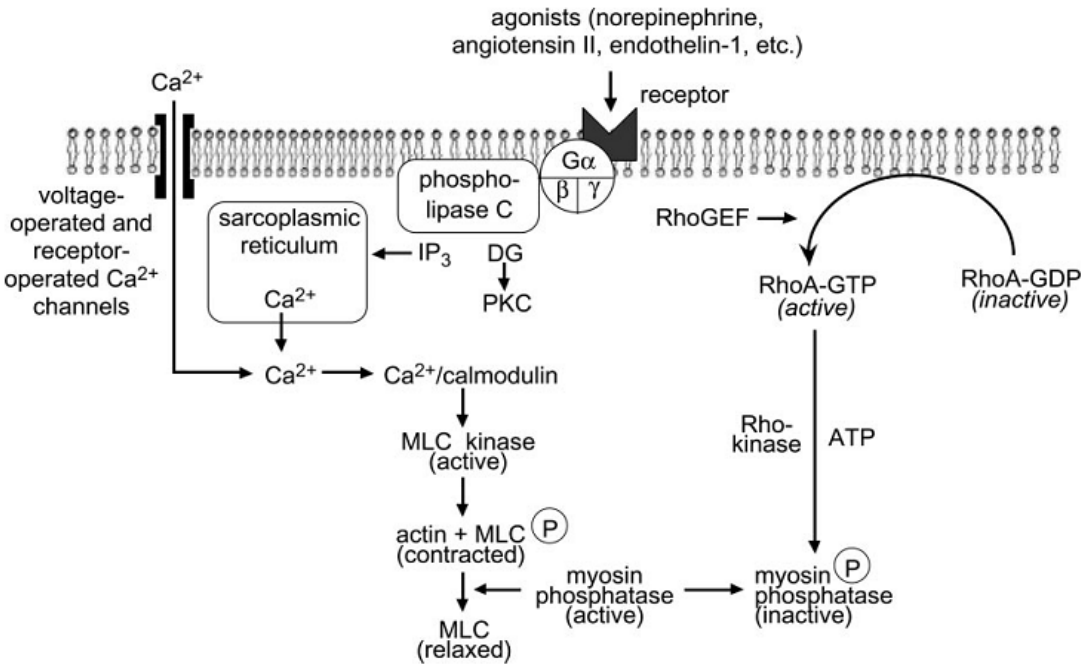


Figure 2.7 Regulation of smooth muscle contraction (Webb, 2003).

The relaxation of smooth muscle occurs via a decrease in the concentration of intracellular Ca^{2+} and an increase MLC phosphatase activity (Webb, 2003). Various mechanisms are associated in the removal of cytosolic Ca^{2+} (Chalmers, Olson, Macmillan, Rainbow, and McCarron, 2007). Mg^{2+}-ATPase is one of two mechanisms that responsible for reducing the concentration of activator Ca^{2+} in the cell by pumping Ca^{2+} outside (Floyd and Wray, 2007). The other mechanism which assisting in reducing intracellular Ca^{2+} is Na^{+}/Ca^{2+} exchangers, located on the plasma membrane (Figure 2.8). Meanwhile, receptor-operated and voltage-operated Ca^{2+} channels located in the plasma membrane also close, resulting in a reduced Ca^{2+} entry in to the cell.

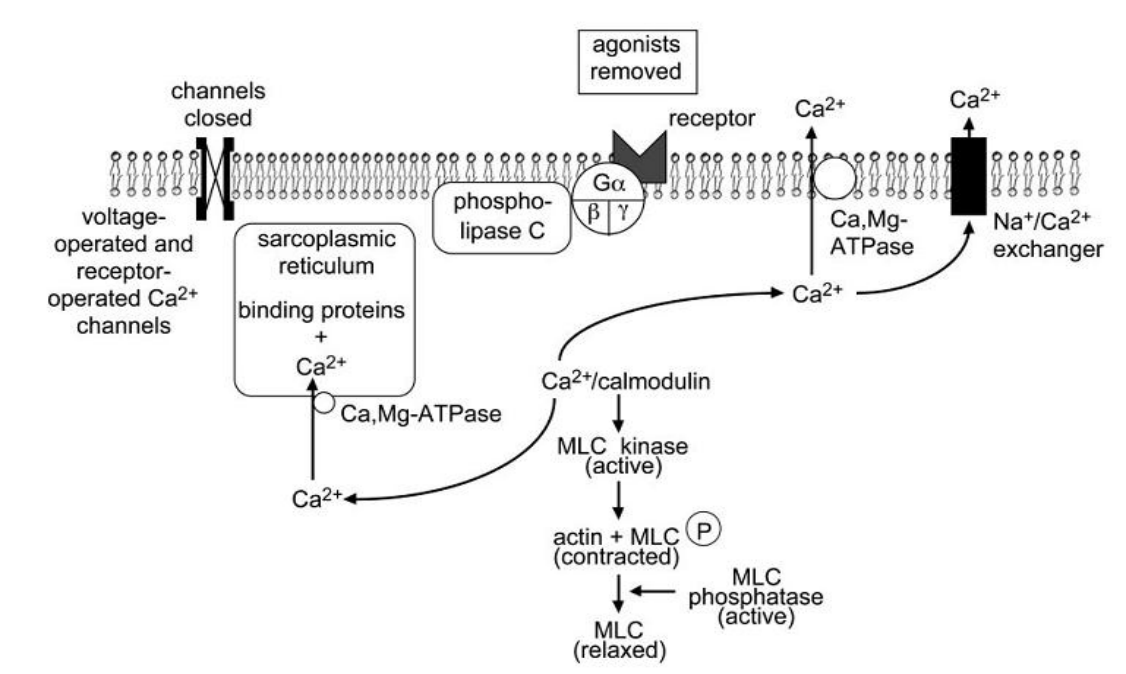


Figure 2.8 Relaxation of smooth muscle (Webb, 2003).

2.5 Regulation of the bronchial tone

Airway or bronchial tone is controlled by various neurotransmitters, inflammatory mediators, and drugs which relate both direct or indirectly with specific surface receptors on smooth muscle cells. As shown in Figure 2.9, the bronchial tone is the functional expression of a dynamic equilibrium between different excitatory and inhibitory mechanisms (Robuschi, 1988).

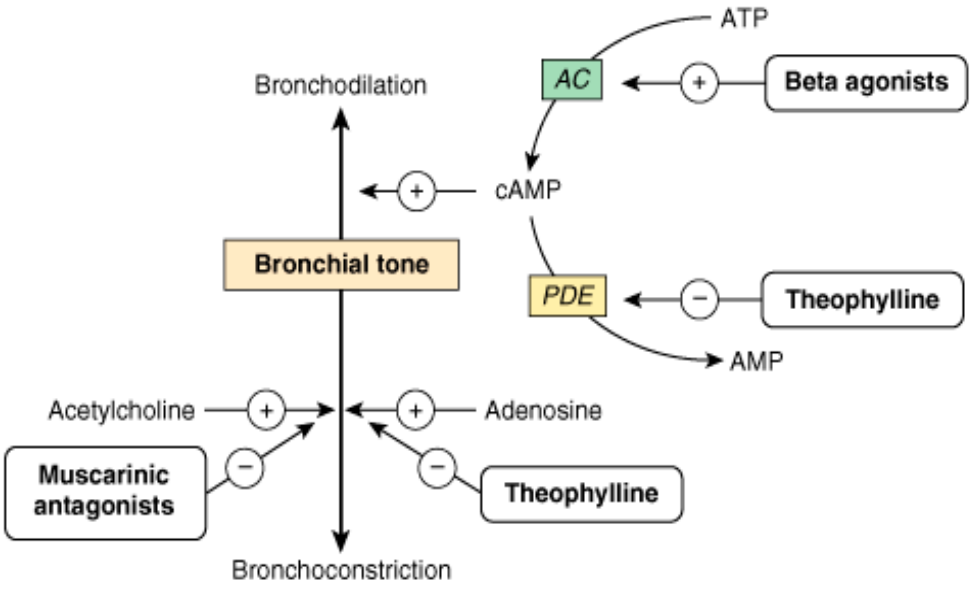


Figure 2.9 Regulation of the bronchial tone (Boushey *et al*., 2009).

On the bronchoconstriction, the mainly mechanism is about the vagal reflex mechanism which is activated by central airway irritant receptors. Bronchial epithelium acts a pivotal mechanism in the regulation of the responsiveness of these receptors. The response of cholinergic nervous system on the bronchial muscle are mediated by muscarinic receptors (Canning, 2006). Airway smooth muscle expresses M_2 and M_3 muscarinic receptors were found in binding studies and northern blot analysis in previous study (Mitchelson, 1988). M_3 muscarinic receptors in airway smooth muscle are coupled to phospholipase C via GTP-binding regulatory protein. Stimulation of phospholipase C catalyzes the production of inositol triphosphate and diacylglycerol from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, resulting in phosphorylation of several proteins by activation of protein kinases (Grandordy, Cuss, Sampson, Palmer, and Barnes, 1986; Meurs *et al*., 1989).

On the bronchodilation, the mainly mechanism is about the β_2-adrenergic receptor. β_2-adrenoceptor is located on smooth muscle cells and on epithelial cells (Morrison, Gao, and Vanhoutte, 1993). The mainly relaxant pathway in airway smooth muscle cells (ASM) is β_2-adrenoceptor mediated stimulation to adenylyl cyclase activity, appearing in the structure of the intracellular second messenger cyclic AMP (cAMP). cAMP alternately activates the cAMP dependent protein kinase (PKA) (Langner *et al*., 1998). PKA phosphorylates various intracellular targets resulting in inhibition of IP_3 formation, increased Ca^{2+} reuptake, and downregulation of myosin light chain kinase. All of these promote ASM cell relaxation (Billington and Penn, 2003). β_2-adrenoceptor agonists may act on smooth muscle tone indirectly by controlling mucociliary clearance and the paracellular exchange of inflammatory mediators (Morrison *et al*., 1993).

Oxidative metabolism of arachidonic acid is increased in inflamed tissues. There are two principal pathways of arachidonic acid oxygenation related in inflammatory mechanism, the cyclooxygenase enzyme (COX) which produces PGs and the 5-lipoxygenase which produces leukotrienes (LTs) (Salmon and Higgs, 1987). Generally made up of phospholipids, the enzyme phospholipase A2, which is occurred in leukocytes and platelets, is stimulated by proinflammatory cytokines. After that, this enzyme takes to the degradation of phospholipids, resulting in production of arachidonic acid. This metabolite froms leukotrienes through the activity of the enzyme lipoxygenase, and prostaglandins, prostacyclins and thromboxances, through the COX (Figure 2.10) (Brune and Hinz, 2004).

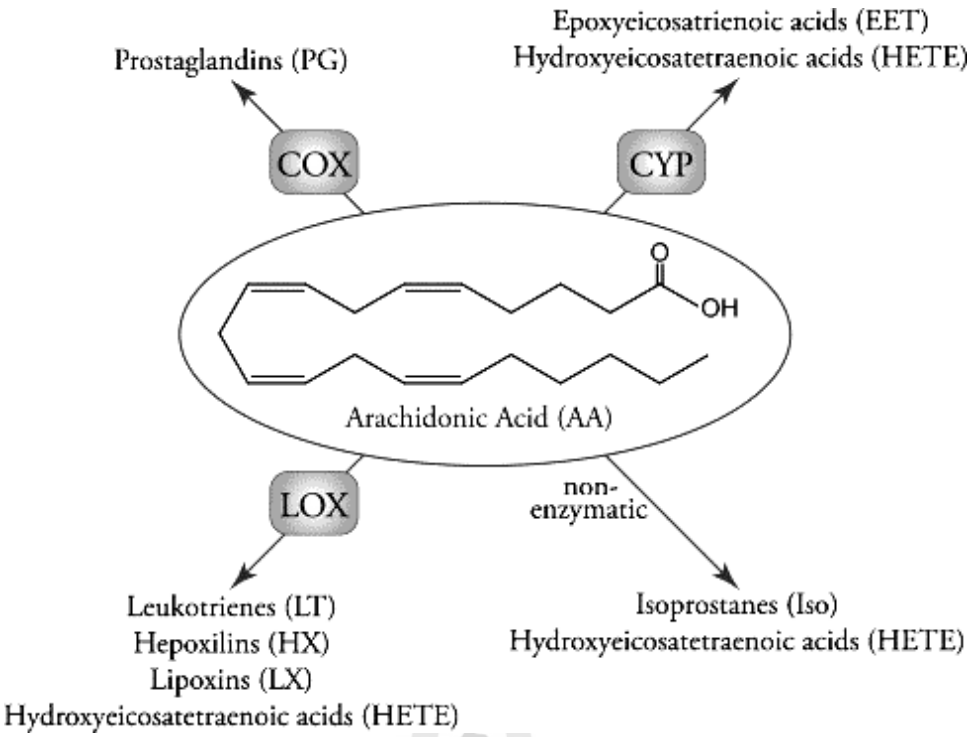


Figure 2.10 Mechanism of synthesis of prostaglandins and leukotriene (Buczynski, Dumlao, and Dennis, 2009).

COX is the initial enzyme related in production of protaglandins from arachidonic acid. It initially converts the arachidonic acid by oxygenation into two unstable component namely PGG_2 and PGH_2 (Cronstein, 2002). COX-1 and COX-2 are two important isofroms for anti-inflammatory therapy. COX-1 is exhibited in almost all tissues, for example blood vessel, platelets, stomach, intestine, and kidney. COX-1 is connected with PG production and a collection of physiological effects (FitzGerald and Patrono, 2001; Cronstein, 2002). COX-2 appears in the site of inflammation. It is originally expressed by cells that involved in the inflammatory step, such as macrophages, monocytes. COX-2 is induced by cytokines such as interleukin-1 (IL-1), IL-2 and tumor necrosis factor and other mediators at the site of inflammation.

It is probably also expressed in the central nervous system and regulates mechanisms in central mediation of pain and fever (Chandrasekharan *et al*., 2002).

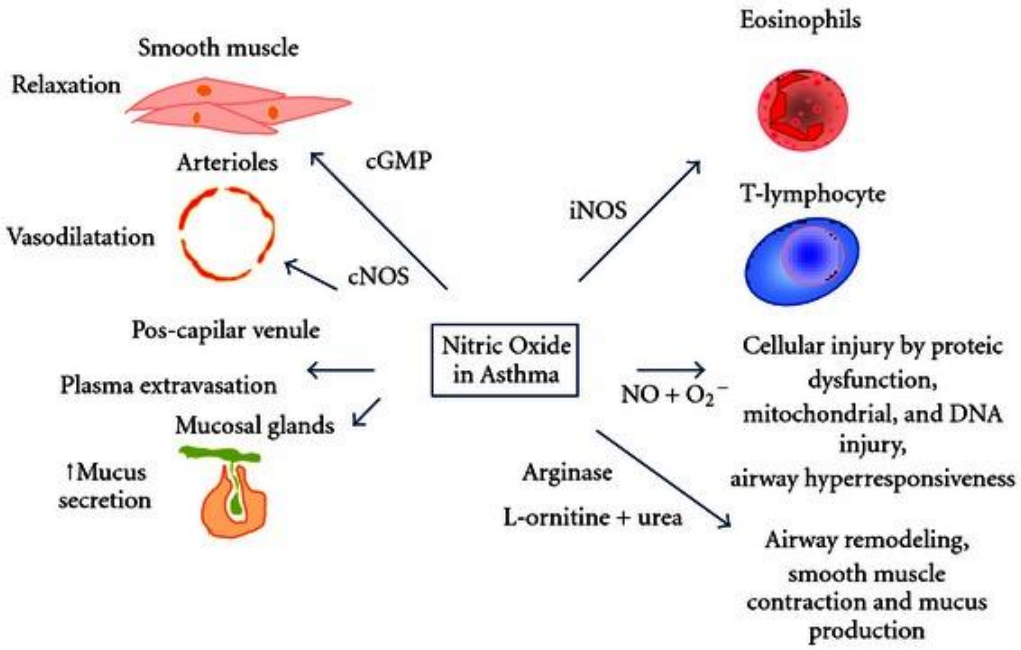


Figure 2.11 The dual effects of nitric oxide in asthma pathology (Prado *et al*., 2011).

In addition to prostanoids and leucotrienes, it is widely accepted that nitric oxide involves in inflammation. Nitric oxide can be found in abundance in both the central and peripheral nervous systems. Nitric oxide is generated from L-arginine amino acid by oxidative enzymatic activity of nitric oxide synthase (NOS) (Prado, Martins, and Tiberio, 2011). It consists of three distinct isoforms, encoded by three distinct genes, including neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3) (Dellamea, Leitao, Friedman, and Canani, 2014). Nitric oxide biosynthesis in excitable tissues is regulated by increases in intracellular calcium, which activate NOS through the enzyme's dependence upon calmodulin

(Christopherson and Bredt, 1997). However, some authors have found increases of these isoforms in inflammatory situations such as asthma (De Sanctis *et al*., 1997; Kobayashi *et al*., 2006). The nitric oxide production can be inhibited by the blockage of nitric oxide synthase enzyme, such as L-NAME (Prado *et al*., 2011).

In asthma, nitric oxide can have both beneficial and adverse effects. The endogenous nitric oxide stimulates soluble guanylyl cyclase, which in turn generates intracellular cyclic GMP and causes airway smooth muscle relaxation and regulation inducing bronchodilation and vasodilation (Tamaoki, Nakata, Kawatani, Tagaya, and Nagai, 2000). However, nitric oxide can also regulate the mucosal glands, increasing the mucus secretion. By substrate competition, nitric oxide can control the arginase pathway and induces airway remodeling, smooth muscle contraction and mucus production (Figure 2.11).

However, the role of nitric oxide in asthma is not so simple as it has been initially described because various *in vitro* and *in vivo* studies with animal experimental models and humans showed different results. The nitric oxide effects seem to be dependent on the animal mode and type of nitric oxide inhibitor as well as the parameters evaluated (Prado *et al*., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemical sources

Citral (cis/trans), eucalyptol, camphene, carbachol, propranolol, L-NAME, hexane, isoproterenol, and indomethacin being used in this study were purchased from Sigma-Aldrich (USA). NaCl, $NaHCO_3$, $CaCl_2$, KCl, NaH_2PO_4, $MgSO_4$, and glucose were purchased from Carlo Erba (Italy).

3.2 Materials and methods

3.2.1 Plant material

Fresh rhizomes of *Z. officinale* (at 10 months of age) were obtained from local markets in Muang district, Nakhon Ratchasima province, Thailand. The plant sample was identified at the Forest Herbarium of Thailand and confirmed by Dr. Surapon Saensouk, a plant taxonomist from Mahasarakham University. The specimen has been kept at School of Phamacology, Institute of Science, Suranaree University of Technology (SUT). The voucher specimen number is Pharm-Chu-004.

3.2.2 Preparation of oil extract

Prior to distillation procedure, unpeeled rhizomes were washed and chopped into small pieces. The sample was put into extract apparatus and subjected to hydro-distillation for 8 hours at 80°C. The obtained ginger oil was dried over anhydrous sodium sulfate and then kept in tightly sealed containers at 4°C for subsequent

experiments. A stock solution was obtained by dissolving small aliquots of ginger oil in hexane (1:1 v/v).

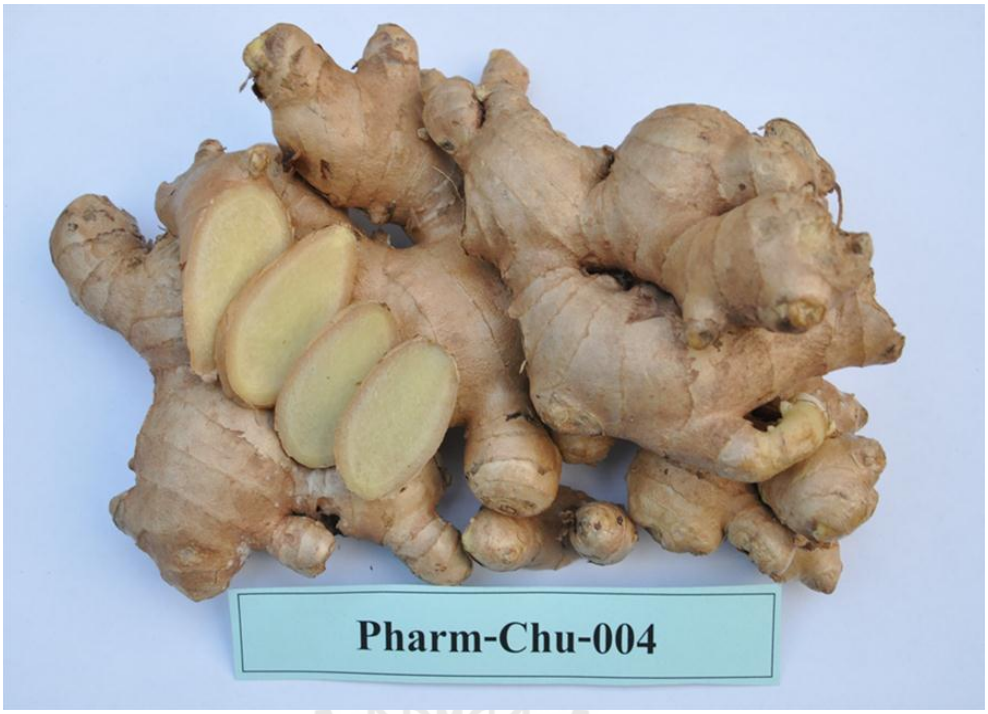


Figure 3.1 Rhizomes (underground stem) of *Zingiber officinale*.

3.2.3 Determination of oil yield

The yield of ginger oil was expressed as gram of distillated ginger oil per gram of fresh ginger weight, calculated as follows;

$$\text{Yield} = \frac{\text{Weight of distillation oil (g)}}{\text{Fresh sample (g)}} \times 100\%$$

3.2.4 Determination of constituents of ginger oil by GC/MS

Ginger oil was analyzed chemically by gas chromatography and mass spectrometry (GC/MS; Model CP-3800-1200 L Quadrupole MS/MS). Briefly, compounds were separated on a FactorFour capillary column VF-5ms (30 m x 2.25 mm; 0.25 µm) using the carrier gas helium (0.7 ml/min). The injector temperature was

250°C and the column temperature was maintained at 40°C for 5 min and then programmed at 4°C/min to 250°C. The spectrometers were operated in electronionization (EI) mode at 70 eV ionization energy, the scan range was 35-400 amu. The detector was set as fixed voltage at 1200 V and the scan rate was 0.5 s per scan. The ionization source temperature was 250°C. The identification of the major compounds was performed by comparing their mass spectra with the NIST library available in the instrument and confirmed by comparing with standards.

3.2.5 Pharmacological experiment

3.2.5.1 Amimals preparation

Male Wistar rats, weighing 230-380 g, were used in this study. The Wistar rats were obtained from Laboratory Animal, SUT. Animals were acclimatized for 7 days prior to the experiments. The rats were housed in polypropylene cage, with free access to normal diet and water *ad libitum*. The rats were maintained at room temperature (25 ± 0.5°C), relative humidity 45-50% and a 12 h light/dark cycle. All procedures in this study were approved and conducted according to guidelines of the Institutional Animal Care and Use Committee, SUT. All efforts were made to minimize the number of rats used and their suffering.

3.2.5.2 Rat tracheal rings preparation

Rat trachea was prepared according to method previously described (Evangelista *et al*., 2007). Rats were sacrificed by cervical dislocation. The trachea was carefully dissected and cleaned of external connective tissue and then cut into trachea several two-cartilage segments (2-3 mm). Each tracheal segment was mounted vertically in an organ bath (10 ml capacity) containing Kreb's solution (37°C, pH 7.4) and aerated with carbogen (O_2 95% and CO_2 5%). The composition of Kreb's solution (mM) were: NaCl (120), $NaHCO_3$ (22), $CaCl_2$ (2.5), KCl (4.6), NaH_2PO_4 (1.2),

$MgSO_4$ (1.2), and glucose (11.5). One end of tracheal ring was tied by fine cotton thread to a hook at the bottom of the bath and the other to a force transducer (Force Transducer Model MLT 1030/D, ADInstruments).

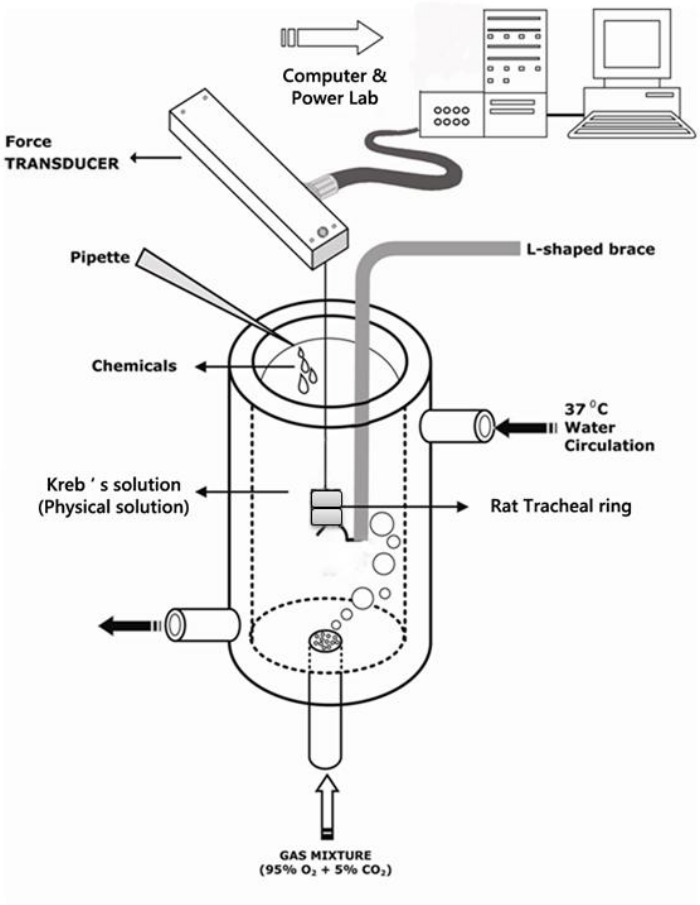


Figure 3.2 A schematic diagram of rat trachea ring preparation in an organ bath, set up used for tension measurement (Modified from Yildiz, Seyrek, and Gul, 2003).

3.2.5.3 Measurement of contractility

Tracheal rings were mounted into the organ bath under an initial resting tension of 1 g and left to equilibrate for a period of 30 min before processing with the experimental protocol. Tension changes were recorded using isometric force transducers connected to a Power Lab Chart recorder (Model ML866, ADInstruments) and the preparation were washed with Kreb's solution every 15 min during the

equilibrating period. After the equilibration period, control contractions were induced by adding 1 µM carbachol (CCh), a non-selective muscarinic receptor agonist, to the organ bath. When two successive control contractions showed similar amplitude, preparations were considered to be equilibrated. The CCh-induced contraction was used as reference for maximal percentage response. The contractile amplitude was measured at the peak deflection and tension were recorded continuously using Chart 5 software (PowerLab; ADInstruments).

3.2.6 Study on the effect of ginger oil on CCh-induced contraction

A tracheal segment was incubated in Kreb's solution and equilibrated for 30 min. Tracheal contractions were induced by 1 µM CCh. Once sustained contractions elicited by 1 µM CCh were established for 5 min, ginger oil (0-1125 µg/ml final concentration) was added to obtain the dose response curve. The responses were expressed as percent reduction of the CCh-induced tracheal contraction. Control experiments were performed with the vehicle (0.125% hexane) which showed no effect on tracheal contraction. Protocol timeline of this experiment is shown in Figure 3.3.

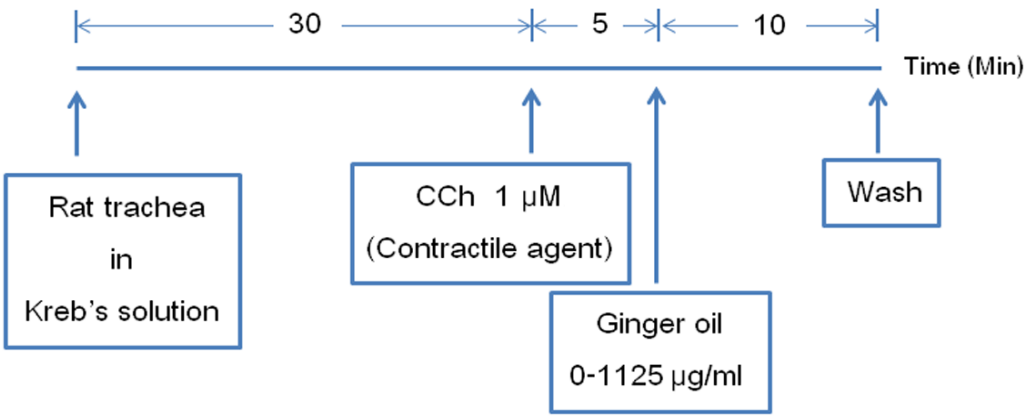


Figure 3.3 The experimental design for studying the bronchodilatory effect of ginger oil.

3.2.7 Determination of active ingredients of ginger oil on the relaxing effect

To examine the myorelaxant effect of ginger oil and its active ingradients, a tracheal segment was firstly incubated in Kreb's solution and equilibrated for 30 min. Tracheal contractions were then induced by 1 µM CCh. As shown in Figure 3.4A, once sustained contractions elicited for 5 min, tracheal preparations were treated with half-maximal inhibition concentration (IC_{50}) of ginger oil (225 µg/ml) and its major constituents (citral, eucalyptol, and camphene). The final concentrations of citral, eucalyptol and camphene used (corresponding to the contents in 225 µg/ml ginger oil) were 140, 15, and 10 µg/ml, respectively. As it was found that citral produced strongest inhibitory effect on bronchial contraction, dose response curve of citral (0-562 µg/ml) was further conducted to evaluate its IC_{50}. The timeline of the later experiment is presented in Figure 3.4B

(A)

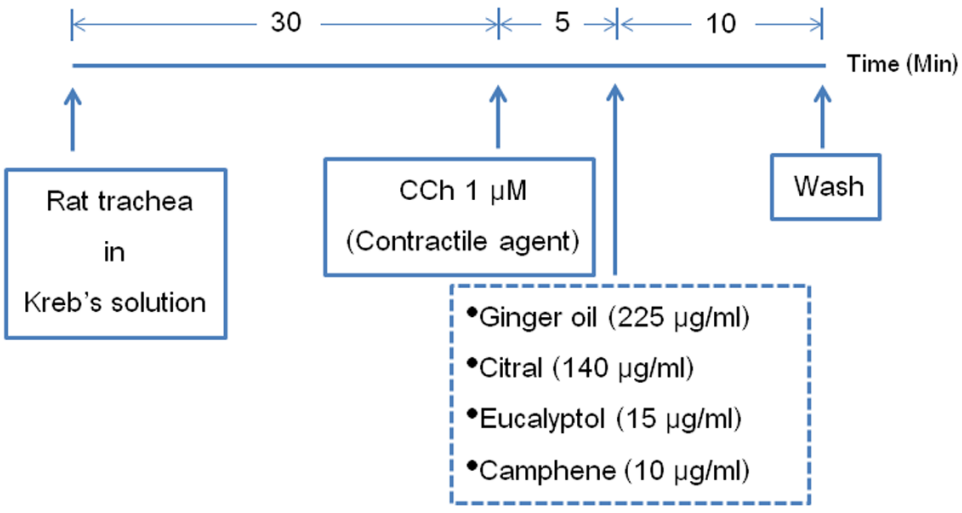


Figure 3.4 Experimental design for investigation of active compound of ginger oil. (A) Study on the effect of ginger oil and its major constituents on CCh-induced contraction and (B) Timeline for studying on dose response curve of citral.

(B)

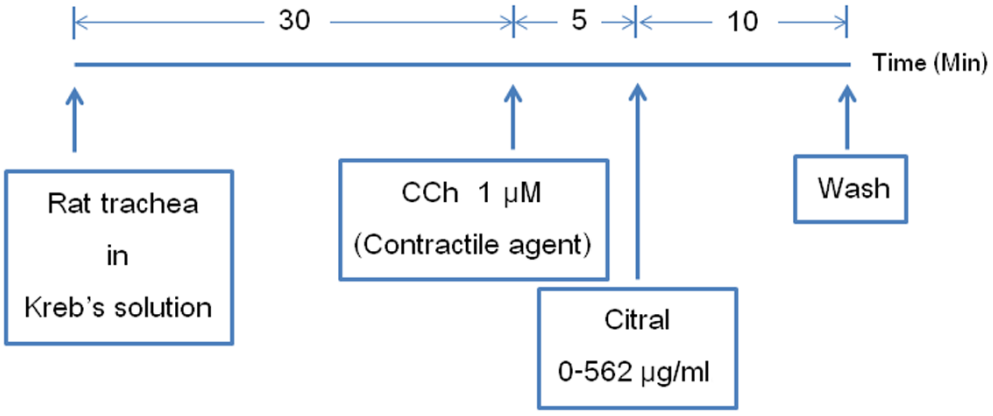


Figure 3.4 Experimental design for investigation of active compound of ginger oil. (A) Study on the effect of ginger oil and its major constituents on CCh-induced contraction and (B) Timeline for studying on dose response curve of citral (Continued).

3.2.8 Study on the effect of ginger oil and citral on the external calcium

A concentration-response relationship of the external calcium was studied in the calcium-free Kreb's solution on the rat trachea in the presence of ginger oil or citral. The tracheal rings were allowed to equilibrate for 30 min in normal Kreb's solution and washed every 15 min. After equilibration periods, normal Kreb's solution was removed and exchanged with calcium free Kreb's solution (80 mM KCl instead Ca^{2+} plus 1 mM EGTA) for 15 min. The tissues were pre-incubated with 112.5 µg/ml of ginger oil or 70 µg/ml of citral prior to the addition of 1 µM CCh, which produced a small contraction. When a stable plateau (almost baseline level) responses to 1 µM CCh was established, series of measurement for a cumulative contraction-response to Ca^{2+} from 0.5 to 5 mM were performed. A positive control experiment was also carried out using the same protocol as described above. Verapamil hydrochloride

(10 µM), a standard calcium channel blocker, was used as a positive control. Experimental design is shown in Figure 3.5.

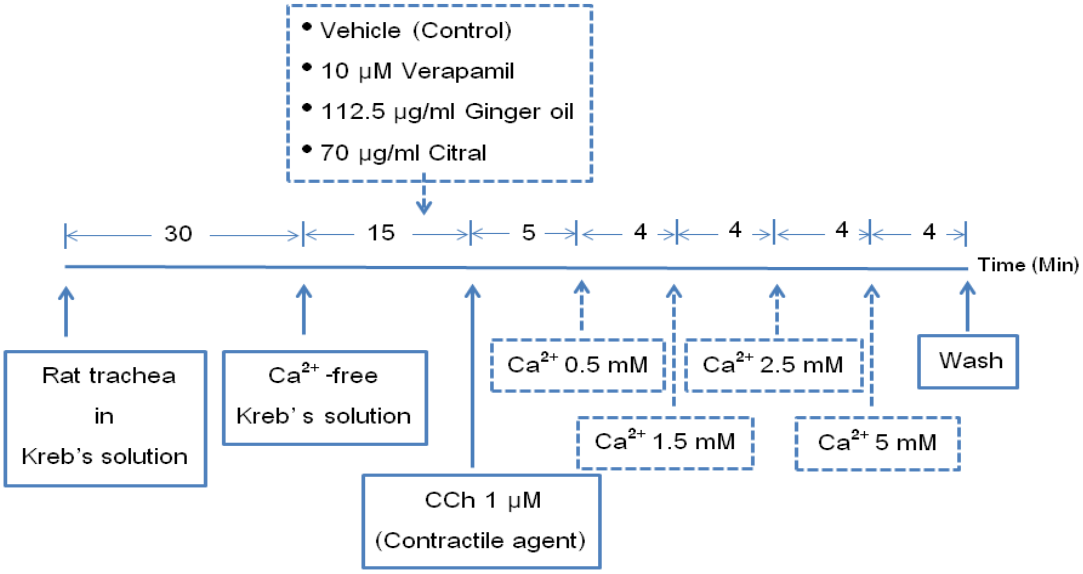


Figure 3.5 Experiment protocol for studying the effect of external calcium on bronchodilatory effect of ginger oil and citral.

3.2.9 Study on the effect of β-adrenergic antagonist, NOS and COX inhibitors on the relaxation produced by ginger oil and citral

To examine the possible mechanism of tracheal relaxant effect of ginger oil and citral, the tracheal rings were pretreated with 3 µM propranolol (a β-adrenergic blocker) for 30 min (Fehri, Ahmed, and Aiache, 2011), and then contracted with 1 µM CCh. After sustained CCh-induced contraction, ginger oil (112.5 µg/ml) or citral (70 µg/ml) was added into the organ bath. Changes of contraction force were recorded. Protocol timeline of this experiment is shown in Figure 3.6A.

For interaction with NOS and COX inhibitors, the tracheal rings were pretreated with 100 µM L-NAME (a nitric oxide synthase (NOS) inhibitor) for 15 min (Estrada-Soto *et al*., 2012) or 10 µM indomethacin (a cyclooxygenase (COX) inhibitor) for 30 min (Sydney de Sousa *et al*., 2010), recpectively. After that the contraction were induced by CCh and then ginger oil or citral was added into the bath similar to the method described above for studying β-adrenergic pathway. Experimental designs for NOS and COX pathways are shown in Figure 3.6B and Figure 3.6C, recpectively.

3.3 Data analysis

Data are expressed as mean ± standard error of mean (S.E.M). Maximal contraction after each concentration of ginger oil or citral was used to construct a concentration-response curve. It was expressed as percentage of control contraction evoked by CCh.

(A)

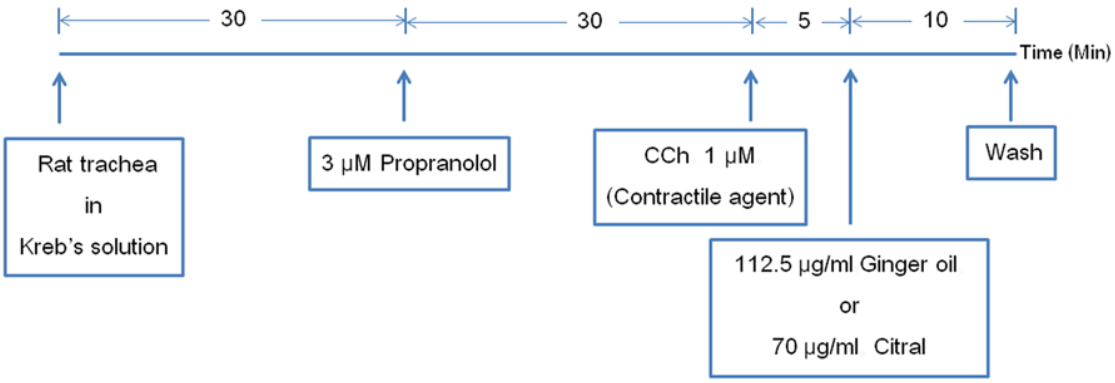
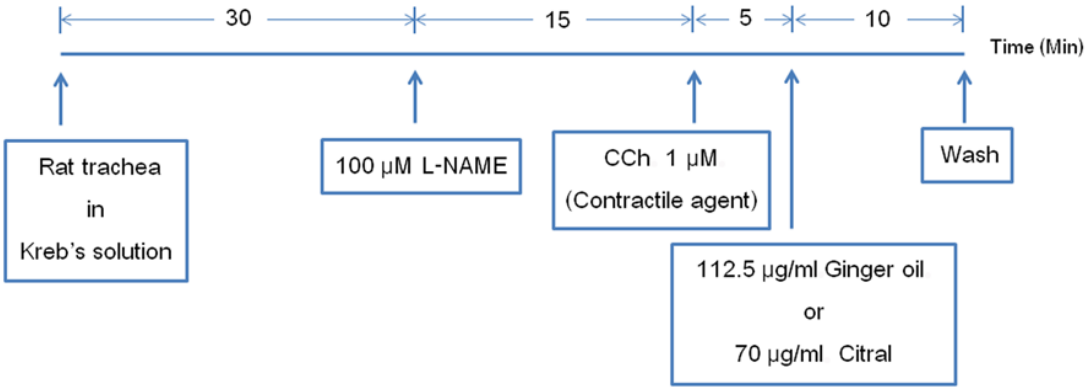


Figure 3.6 Experiment timelines of the studies on (A) β-adrenergic, (B) NOS and (C) COX pathways.

(B)



(C)

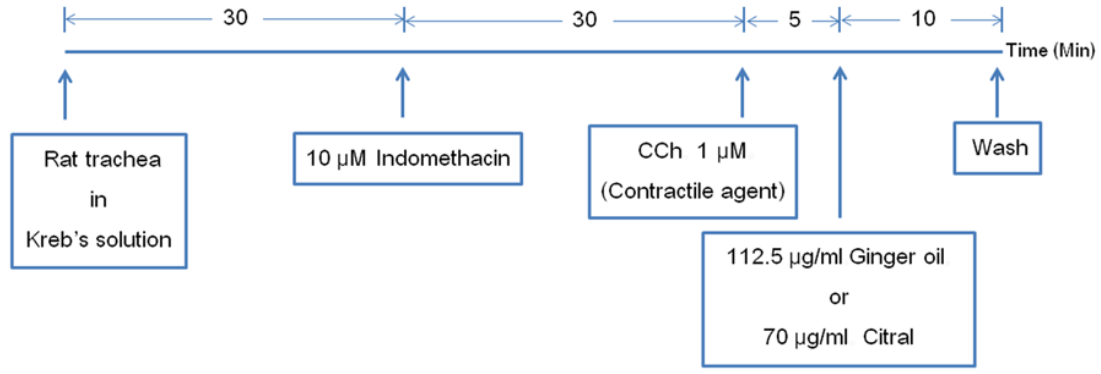


Figure 3.6 Experiment timelines of the studies on (A) β-adrenergic, (B) NOS and (C) COX pathways (Continued).

The IC_{50} and EC_{50}, defined as the concentration (in µg/ml) of each compound at which the maximal CCh-induced contraction was inhibited by 50% and the concentration of antagonist required to inhibit the agonist's response by 50% relative to the control response, respectively. The IC_{50} or EC_{50} were calculated by interpolation from semi-logarithmic plots, and expressed as means ± S.E.M. The significance

(p<0.05) of the results was assessed by Student's paired t-tests and one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls comparison test.

CHAPTER IV

RESULTS

4.1 Constituents of ginger oil

Ginger oil extracted by water distillation obtained in this study was yellow pungent essential oil. The yield was about 0.13% (w/w). After comparison with mass spectra from NIST library, several compounds were predicted. However, due to a limitation to obtain standards, only three major compounds with high possibility of being the active ingredients were identified and quantified.

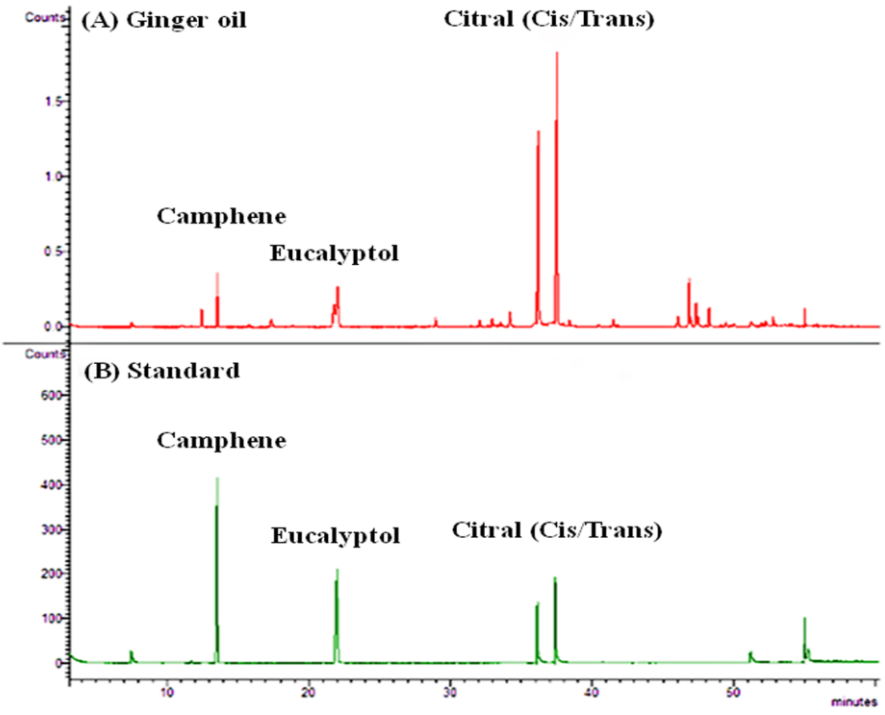


Figure 4.1 Chemical compositions obtained by GC-MS analysis of ginger oil.

The results from GC/MS analysis (Figure 4.1) revealed that citral was the major component in the ginger oil prepared by hydrodistillation procedure. It was found that the ginger oil was composed of (values in % of oil weight): citral (62.4), eucalyptol (6.9), and camphene (4.6). The remaining (26.1%) were unidentified chemicals.

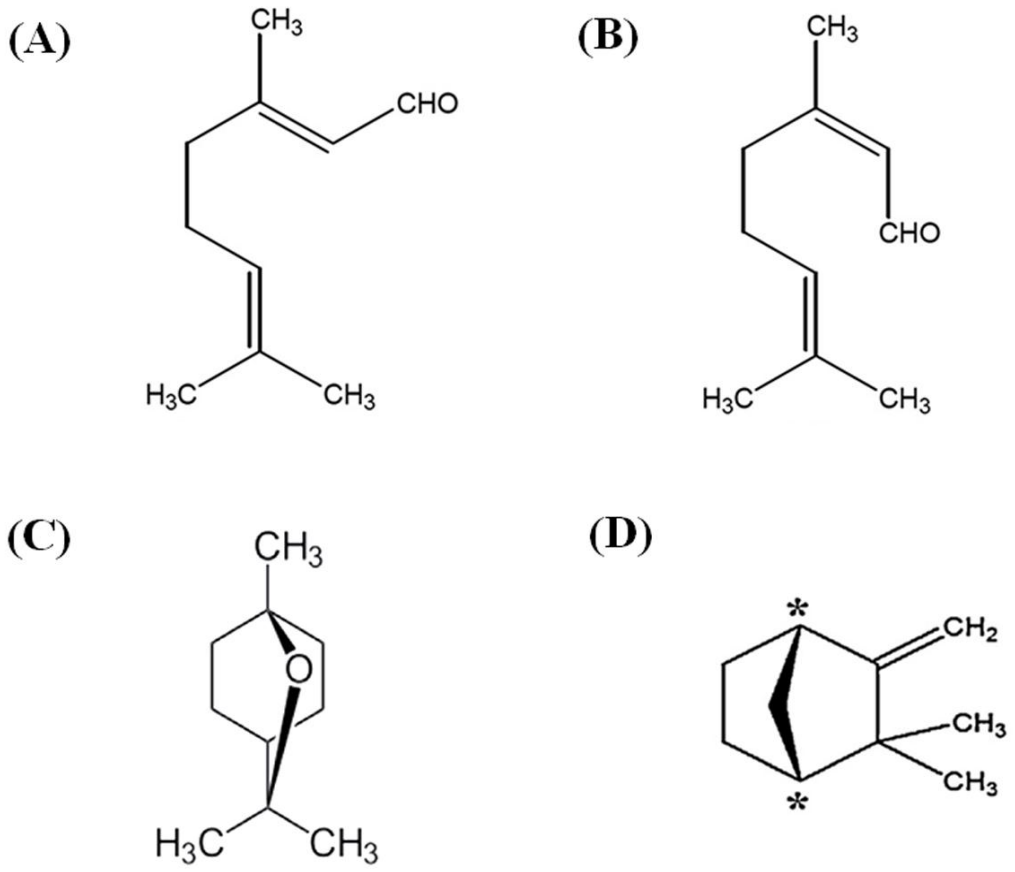


Figure 4.2 Chemical structures of major compounds found in ginger oil. (A) Geranial (*trans*-citral or citral A), (B) Neral (*cis*-citral or citral B) (Dudai *et al*., 2000), (C) 1,8-cineole or eucalyptol (Rodenak Kladniew *et al*., 2014), and (D) DL-camphene (*denotes the chiral centers) (Lee, Chiu, Lee, and Lee, 2014).

As shown in Figures 4.2A and 4.2B, citral (3,7-dimethyl-2,6-octadienal) is a linear monoterpene with two geometric isomers-geranial (*trans*-citral or citral A) and neral (*cis*-citral or citral B). It is also present in the essential oil of several other aromatic plants (Dudai *et al*., 2000; Dudai, Weinstein, Krup, Rabinski, and Ofir, 2005). The structures of eucalyptol and camphene which were found in ginger oil as well are shown in Figures 4.2C and Figure 4.2D, respectively.

4.2 Effect of ginger oil on CCh-induced contraction

In the present study, *in vitro* myorelaxant properties of ginger oil in rat isolated trachea contracted with the muscarinic agonist carbachol was studied. Figure 4.3 showed that ginger oil (22.5-1125 µg/ml) inhibited the contractile responses induced by 1 µM CCh in a concentration-dependent manner ($p<0.05$). The IC_{50} value for ginger oil-induced relaxation was 120 ± 17 µg/ml. Vehicle (the same concentrations used to dissolve ginger oil) produced no significant change in CCh-induced contraction.

4.3 Active ingredients of ginger oil responsible for the relaxing effect

Ginger oil at 225 µg/ml practically abolished the CCh-evoked tracheal contraction; and 140 µg/ml citral (the concentration corresponding to its content in ginger oil used) reduced the contraction to 23.7 ± 9.9% as compared with the control ($p<0.05$). In the case of eucalyptol (1,8-cineole), the concentration of ginger oil (225 µg/ml) required for 100% airway relaxation contained only 16 µg/ml of eucalyptol. At this concentration, eucalyptol reduced the amplitude of tracheal contraction to 79.8 ± 6.7%, but not significantly as shown in Figure 4.4.

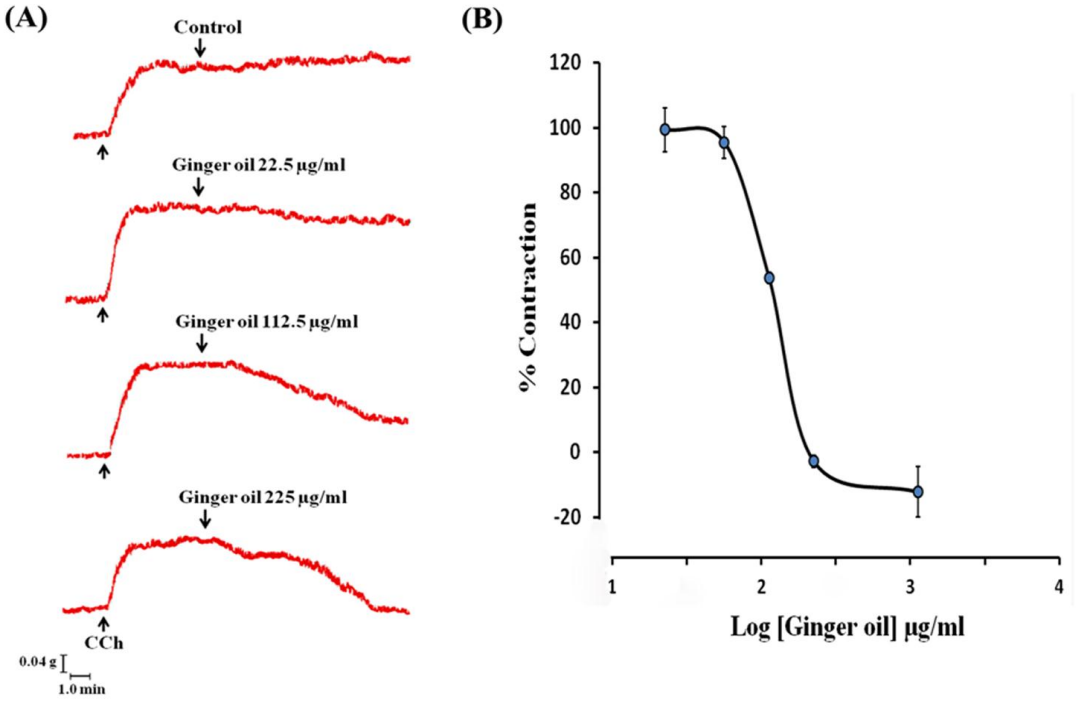


Figure 4.3 Tracings demonstrating effect (A) and concentration-response curve (B) of ginger oil on rat tracheal contraction induced by 1 µM CCh. Values in panel B were expressed as % of contractions produced by CCh (1 µM) in the absence of ginger oil and vertical bars indicate S.E.M. The ginger oil at 225 µg/ml produced 100% relaxation and the calculated IC_{50} was 120 ± 17µg/ml.

4.4 Inhibitory effect of citral on CCh-induced contraction

In vitro myorelaxant properties of citral in rat isolated trachea contracted with carbachol was examined. In Figure 4.5, it was shown that citral (0-562 µg/ml) inhibited the contractile responses induced by CCh (1 µM) in a concentration-dependent fashion (p<0.05). The IC_{50} value for citral-induced relaxation was 110 ± 9 µg/ml (n = 6).

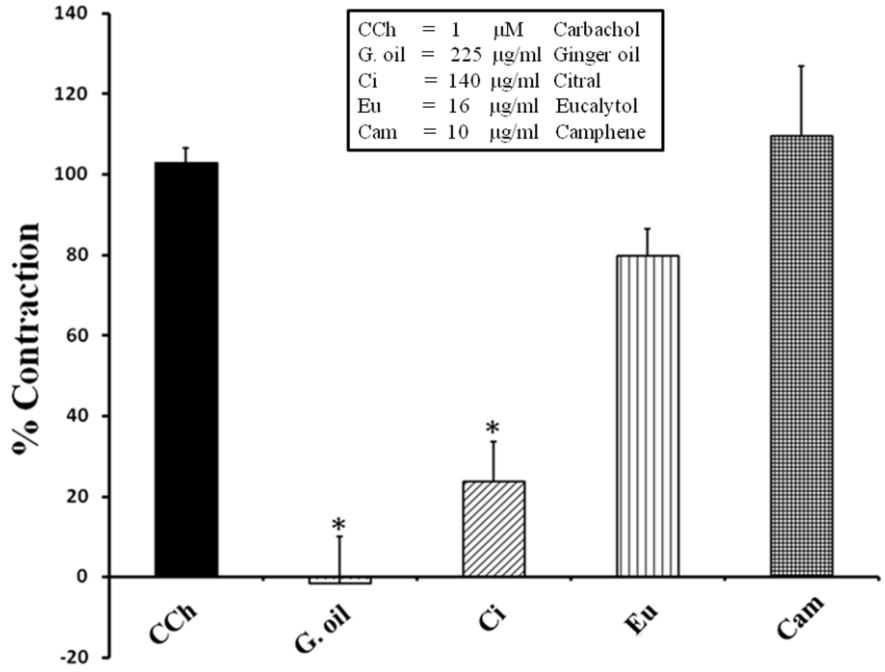


Figure 4.4 Myorelaxing effects of ginger oil and its major compositions. Ginger oil (225 µg/ml), citral (140 µg/ml), eucalyptol (16 µg/ml), and camphene (10 µg/ml) were added on the plateau of a steady sustained contraction elicited by CCh (1 µM) in isolated rat trachea. Ginger oil, citral, and eucalyptol reduced the amplitude of tracheal contraction to -1.5 ± 11.5, 23.7 ± 9.9, and 79.8 ± 6.7%, respectively compared to control (102.9 ± 3.7%). In contrast, camphene did not affect rat tracheal smooth muscle contraction as the amplitude was 109.5 ± 17.5%. Results are reported as mean ± S.E.M. *p<0.05 (n = 5-8).

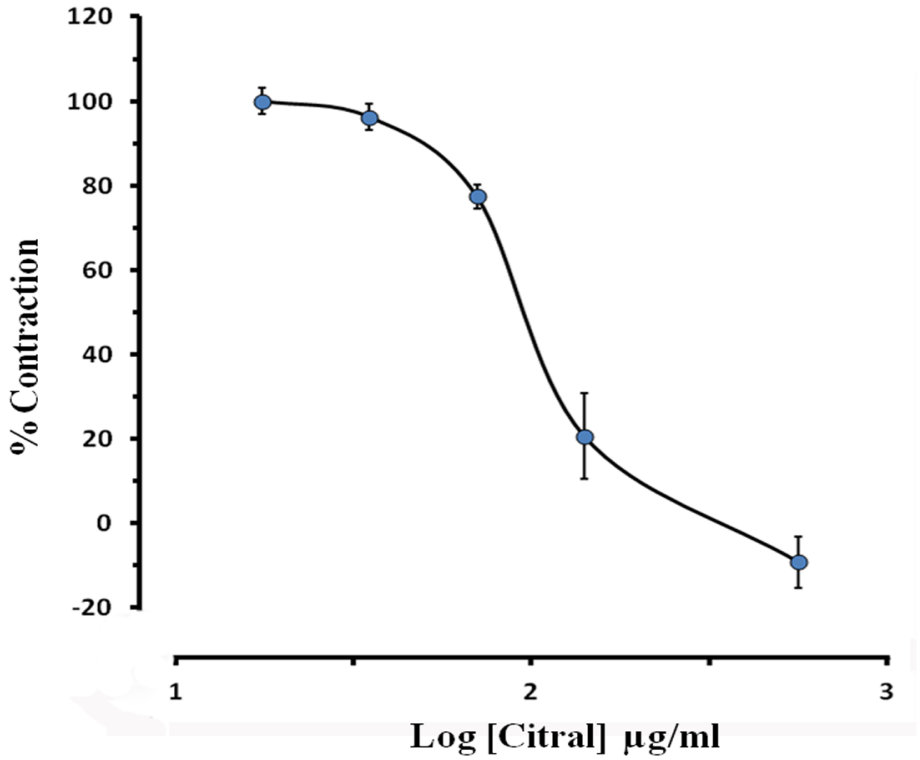


Figure 4.5 Effect of citral (0-562 µg/ml) on rat tracheal contraction induced by CCh (1 µM). Values were expressed as % of contractions produced by CCh (1 µM) in the absence of citral and vertical bars indicated S.E.M.

4.5 Effect of extracellular calcium on the tracheal relaxation induced by ginger oil and citral

Concentration-response curve of trachea to external $CaCl_2$ (0.05-5 mM) against CCh-induced contraction in the presence of ginger oil or citral compared with verapamil (positive control) are presented in Figure 4.6. The EC_{50} of $CaCl_2$ alone was 2.4 ± 0.2 (n = 7). Meanwhile, in the presence of ginger oil (112.5 µg/ml), citral (70 µg/ml), as well as verapamil (10 µM), the EC_{50} of $CaCl_2$ were 4.3 ± 0.2, 6.6 ± 1.1, and

6.5 ± 1.4 (n = 7), respectively. As observed on Ca^{2+} plots, the ginger oil or citral shifted Ca^{2+} concentration-response curves (CRCs) towards right similar to verapamil.

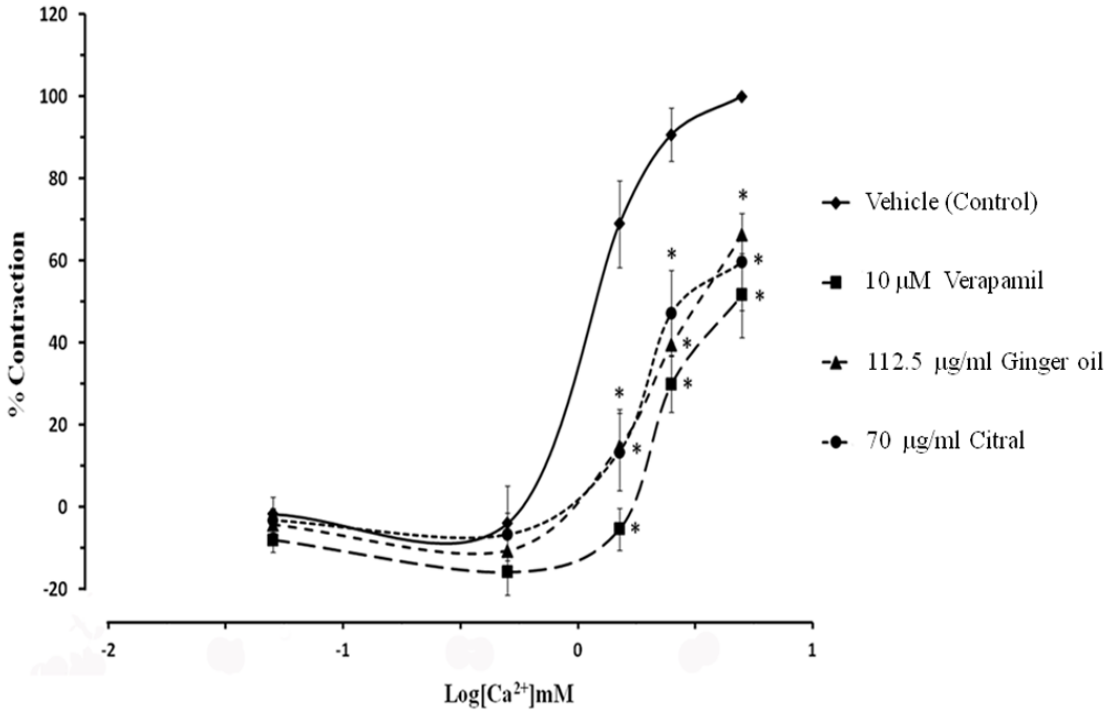


Figure 4.6 Effect of ginger oil (112.5 µg/ml), citral (70 µg/ml), and verapamil (10 µM) on concentration-response curve for Ca^{2+} in rat isolated trachea contracted by 1µM CCh in Ca^{2+}-free medium. Data are reported as mean ± S.E.M. *p<0.05 (n = 5-8) compared to control group.

4.6 Effect of β-adrenergic antagonist on the relaxation produced by ginger oil and citral

Figure 4.7 shows the effect of an absence and a presence of propranolol on a trachea response during the pre-incubation. The relaxing effects of ginger oil (112.5 µg/ml) and citral (70 µg/ml, concentration corresponding to its content in ginger oil

used) were reduced significantly (p<0.05). Propranolol reversed the bronchodilatory effects of ginger oil and citral on CCh-induced tracheal contraction from 66.1 ± 4.0 to 83.5 ± 4.9% and 75.3 ± 3.7% to 104.7 ± 6.0%, respectively.

4.7 Effect of NOS inhibitor on the tracheal relaxation produced by ginger oil and citral

The relaxing effect of ginger oil in this study was not related to nitric oxide because L-NAME did not significantly alter the inhibitory effects of ginger oil and citral (p>0.05). In Figure 4.8, the contraction responses to CCh in the presence of ginger oil with and without pre-incubated with L-NAME were 73.9 ± 9.6% and 68.9 ± 9.6%, respectively. Whereas those of citral were 64.6 ± 11.4% and 70.7 ± 7.5% respectively.

4.8 Effect of COX inhibitor on the relaxation produced by ginger oil and citral

Similar to nitric oxide, the relaxing effect of ginger oil in the study was not related to prostaglandins because indomethacin did not significantly alter the inhibitory effects of ginger oil and citral (p>0.05). In Figure 4.9, the contraction responses to CCh in the presence of ginger oil with and without pre-incubated with indomethacin were 68.2 ± 15.0% and 56.1 ± 4.5%, respectively. Whereas those of citral were 66.4 ± 8.7% and 72.0 ± 6.2% respectively.

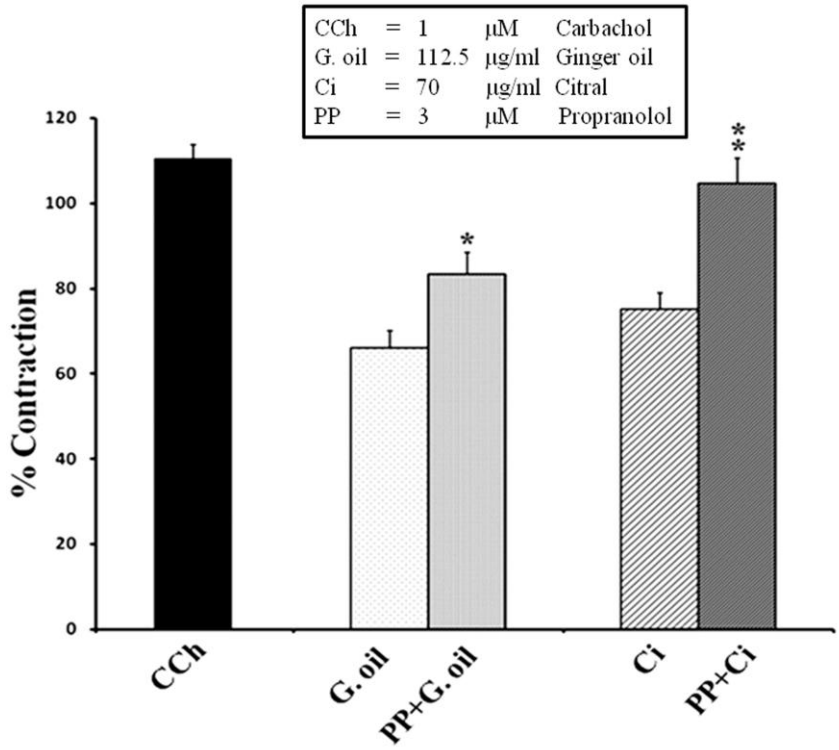


Figure 4.7 Effects of β-adrenergic receptor antagonist on bronchodilatory activity of ginger oil and citral. The effects of ginger oil (112.5 µg/ml) and citral (70 µg/ml) were evaluated in the presence of propranolol (3 µM; for 30 min) on sustained contraction elicited by CCh (1 µM) in isolated rat trachea. Propranolol significantly reversed the inhibitory effects of ginger oil and citral. Data are reported as mean ± S.E.M. *p<0.05 (n = 5-8) compared to ginger oil group; and **p<0.05 compared to citral group (n = 5-8).

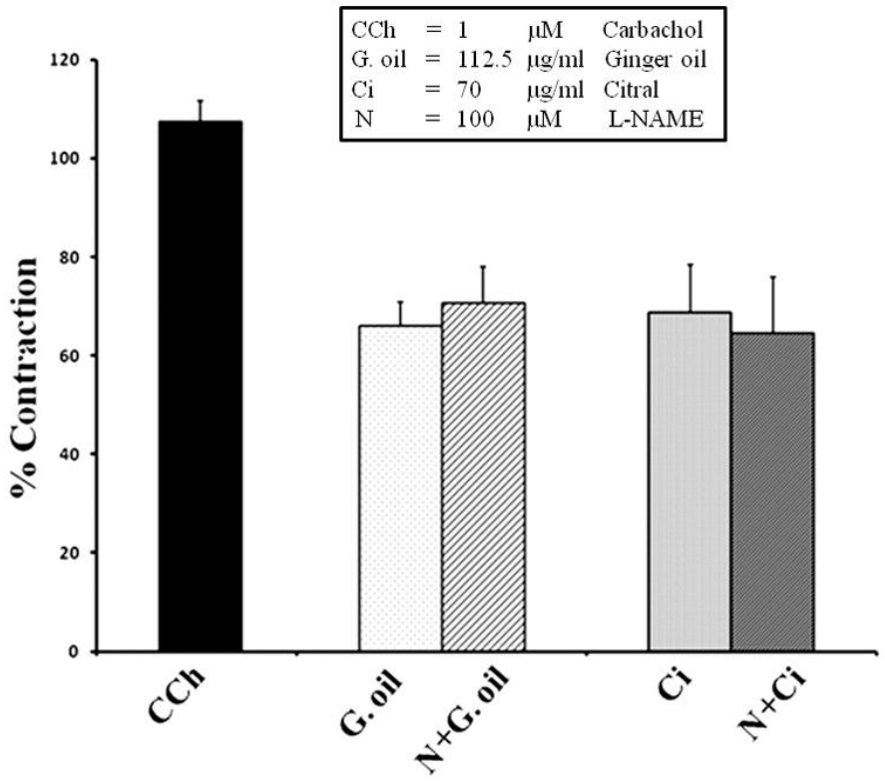


Figure 4.8 Effects of NOS inhibitor on bronchodilatory activity of ginger oil and citral. The effects of ginger oil (112.5 µg/ml) and citral (70 µg/ml) were evaluated in the presence of L-NAME (100 µM; for 15 min) on sustained contraction elicited by CCh (1 µM) in isolated rat trachea. L-NAME did not significantly alter the relaxation of trachea smooth muscle rings induced by ginger oil and citral. Data are reported as mean ± S.E.M. $p>0.05$ (n = 5-8).

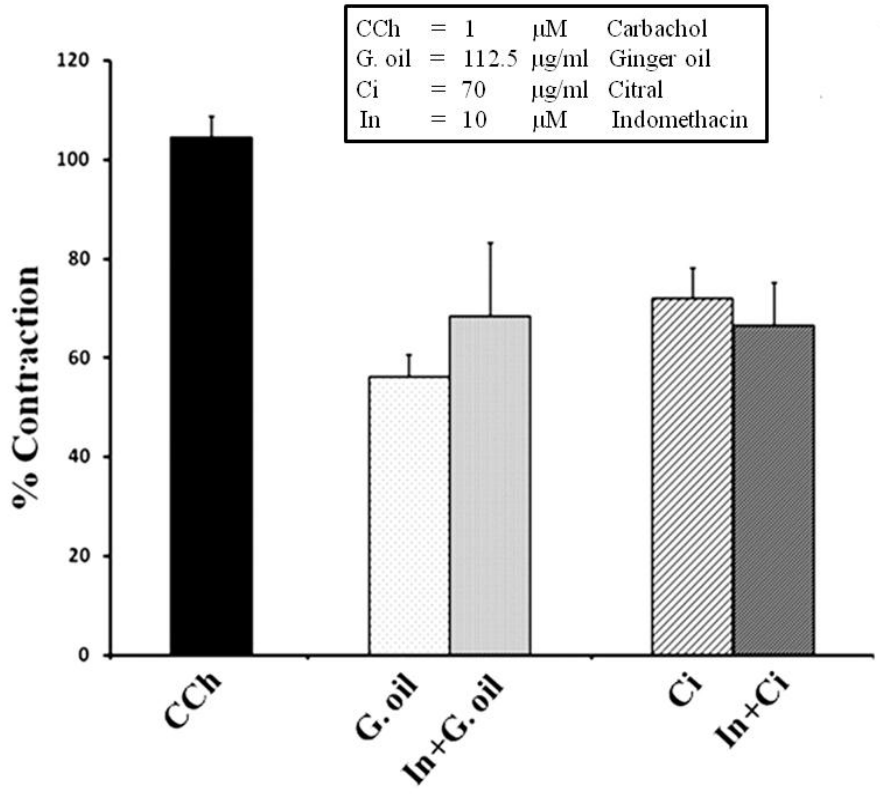


Figure 4.9 Effects of COX inhibitors on bronchodilatory activity of ginger oil and citral. The effects of ginger oil (112.5 µg/ml) and citral (70 µg/ml) were evaluated in the presence of indomethacin (10 µM; for 30 min) on sustained contraction elicited by CCh (1 µM) in isolated rat trachea. Indomethacin did not significantly alter the relaxation of trachea smooth muscle rings induced by ginger oil and citral. Data are reported as mean ± S.E.M. $p>0.05$ (n = 5-8).

CHAPTER V

DISCUSSION AND CONCLUSION

In recent years, ginger rhizomes have been increasingly used as alternative medicine in several ailments such as gastrointestinal tract disorders (Apariman *et al*., 2006; Willetts *et al*., 2003; Yamahara *et al*., 1989), hypertension (Ghayur *et al*., 2005), inflammation and pain (Altman and Marcussen, 2001). Ginger oil is one of several essential oils that have been used in aromatherapy as an alternative treatment for short-term knee pain relief (Yip and Tam, 2008). Buddhakala and co-workers (Buddhakala *et al*., 2008) found that ginger oil inhibited uterine contraction induced by $PGF_{2\alpha}$. In terms of respiratory system, methanolic and aqueous crude extracts of ginger have been reported to relax rat tracheal smooth muscle (Ghayur and Gilani, 2007), reduce rat airway hyperreactivity and remodeling (Aimbire *et al*., 2007; Kuo, Hsu, Huang, Tsai, and Ko, 2011) and relieve bronchospasm in asthmatic patients (Rouhi *et al*., 2006). The experiments reported herein were undertaken with the aims of providing scientific evidence to find active compounds of ginger oil on its bronchodilatory effect with possible mechanisms.

More than sixty constituents in ginger oil have been identified and mostly are monoterpenoids. However, the contents previously reported of these monoterpenoids varied presumably due to the difference in cultivated places (Gupta *et al*., 2011; Sasidharan, Venugopal, and Menon, 2011). In this study, the results from GC/MS analysis revealed that ginger oil was composed of citral, eucalyptol, and camphene as

its major constituents. It should be noted that a number of compounds, such as α-pinene, β-phellandrene and zingiberene were also detected in the ginger oil preparation based on the data from the NIST library. In this study, based on the area of peaks from GC/MS, it was showed that citral was the major component in the ginger oil preparation. The pharmacological activities of citral such as bactericide, fungicide, insecticide, antihistamine, and anti-tumor agent have been reported (Dudai *et al*., 2000; Farah, Trimble, Ndebele, and Mawson, 2010; Rodov, Ben-Yehoshua, Fang, Kim, and Ashkenazi, 1995; Somolinos, García, Condón, Mackey, and Pagán, 2010); however its bronchodilatory activity has never been described.

In the present study, *in vitro* myorelaxant properties of ginger oil and citral were examined in rat isolated trachea contracted with the muscarinic agonist carbachol (CCh). As seen in Figures 4.3B and 4.5, the results showed that both ginger oil and citral inhibited CCh-induced brochocontraction in dose-response manner. Figure 4.4 showed that ginger oil at 225 µg/ml practically abolished the CCh-evoked tracheal contraction whereas 140 µg/ml citral (the concentration corresponding to its content in ginger oil used) reduced the contraction to about 25 % of control. This suggested that there might be other compounds which were responsible for relaxing effect of ginger oil, such as eucalyptol. Its inhibitory effect of on rat airway smooth muscle has been observed: however, the EC_{50} of eucalyptol from previous report was 408.9 µg/ml (Nascimento *et al*., 2009) which was much higher than its content in ginger oil. The concentration of ginger oil required for 100% airway relaxation (225 µg/ml) contained only 16 µg/ml of eucalyptol. At this concentration, eucalyptol did not significantly reduce the amplitude of tracheal contraction compared to control (Figure 4.4). It was concluded that eucalyptol was not the major active compound responsible for the brochodilatory effect of ginger oil, but partly contributed in this effect. In contrast, the

essential oil extracted from *Artemisia maritima* L. has been found to contain about 40% of eucalyptol (Shah *et al*., 2011). It is likely that eucalyptol could be one of the major active compounds for the bronchodilatory activity of the essential oil extract from *Artemisia maritima.*

Currently, the use of medicinal plants for bronchodilatory effects has received increasing attention from researchers. Both active ingredients and mechanisms of action underlying the antispasmodic and bronchodilatory activities have been studied. It is well known that the relaxation of respiratory smooth muscle is caused by two crucial mechanisms which are the activation of β_2-adrenergic receptor (Delmotte, Ressmeyer, Bai, and Sanderson, 2010) and the release of endothelium-derived relaxant factors such as nitric oxide (Munakata *et al*., 1990) and prostaglandins (Ismailoglu, Sahin-Erdemli, Sungur, and Ilhan, 2004). Mechanisms that have been reported to participate in the bronchodilatory action of medicinal plants include β_2-adrenergic receptor (Chaudhary *et al*., 2012), cholinomimetic pathways and blockade of calcium channel (Khan and Gilani, 2009). Moreover, de Sousa and colleagues (de Sousa *et al.*, 2010) have found the participation of prostaglandin E_2 and nitric oxide in the relaxant effect of *Mentha piperita* essential oil.

In view of its use in airway disorders, ginger oil and citral were subjected to pharmacological investigation in order to validate the medicinal uses and explore the possible mechanism in its bronchodilator activity. Carbacol is cholinergic agonist which induced tracheal contraction via muscarinic M_3 receptor (Janbaz *et al*., 2013). Stimulation of M_3 receptor activates phospholipase C and increases IP_3 synthesis which in turn, promotes calcium release from sarcoplasmic reticulum and at the same time, Ca^{2+} influx from the extracellular space, causing elevation in the intracellular

Ca^{2+} concentration which causes contraction (Pereira, Marques, Sudo, Kaplan, and Zapata-Sudo, 2013).

In order to assess the possible mechanisms underlying the bronchodilatory activities of ginger oil and citral, their inhibitory effects were studied in the presence of Ca^{2+} channel blockers, β-adrenergic receptor antagonist, NOS and COX inhibitors on the CCh-induced contractile behavior of the rat isolated trachea smooth muscle.

It is widely known that the Ca^{2+} channel blockers have been found to be useful in bronchospastic disorders. In trachea, both intracellular and extracellular Ca^{2+} sources are important in development of muscle tension. Ca^{2+} enters the cell through at least two pathways: voltage-operated calcium channels (VOCCs) (L-type, mainly) and receptor-operated cation channels (ROCC) (Ersoy *et al*., 2008; Pereira *et al*., 2013). In addition to these mechanisms, Leung and colleagues (Leung, Yung, Yao, Laher, and Huang, 2008) demonstrated that Ca^{2+} influx in airway smooth muscle occurs via store-operated Ca^{2+} (SOC) channels in response to sarcoplasmic reticulum (SR) Ca^{2+} depletion, thus allowing for replenishment of intracellular Ca^{2+} stores. Specifically, in vascular smooth muscle, removal of extracellular Ca^{2+}, the use of inhibitors of SR Ca^{2+} uptake and blockade of L-type voltage-operated Ca^{2+} chanels are important tools to distinguish which source is providing Ca^{2+} to cell for functional responses (Fleckenstein and Fleckenstein-Grün, 1988; Toque, Monica, Morganti, De Nucci, and Antunes, 2010). This strategy can be used in other types of smooth muscle as well.

In the present study, the entry of Ca^{2+} through L-type voltage-operated Ca^{2+} channel was studied using verapamil as pharmacological tool. Verapamil is a standard competitive Ca^{2+} channel antagonist (Fleckenstein, 1977). Basically, in the presence of a competitive antagonist, higher concentrations of agonist are required to produce a given effect. Thus, the agonist concentration (in this experiment, it refers to Ca^{2+}) is

required for a given effect in the presence of concentration of an antagonist is shifted to the right. This is not the case with an irreversible (or noncompetitive) antagonist, which reduces the maximal effect the agonist can achieve, although it may not change its EC_{50}. In contrast, a competitive antagonist increases EC_{50} of agonist which was observed with the dose response curves of Ca^{2+} in the presence of ginger oil and citral in this study. These results suggested that myorelaxant effects of ginger oil and the active compound ctiral may possible be mediated via L- type Ca^{2+} channel blockade.

β_2-adrenoceptor agonists are common in the management of asthma and airway hyperresponsivness (Ge, Dai, Wan, Liu, and Mei, 2013). It is widely known that stimulation of β_2-adrenergic receptor by agonist leads to an increase of adenylyl cyclase activity, thus resulting in an increase of intracellular cAMP levels. The traditional mechanism for cAMP action is via the stimulation of protein kinase A (PKA) to phosphorylate a variety of target proteins to induce airway smooth muscle cell relaxation. The current study was aimed to study a possible mechanism of action of ginger oil through β-adrenergic pathway. It was found that its bronchodilator activity, as well as citral, was inhibited by the β_2-adenergic antagonist propranolol. These results suggested that a stimulatory effect on β_2-adrenoceptor also play a role on the relaxant effect of ginger oil and citral.

Interestingly, Ca^{2+} channel blockers are known to be useful as tracheal relaxant in disorders characterized by hyperresponsiveness of the respiratory tract (Janbaz, Nisar, Ashraf, and Qadir, 2012). Bronchodilatory activities mediated by Ca^{2+} channel blockade have been studied in other plant extracts. For example, the aqueous-methanolic extract of *Amaranthus spinsus* was tested on CCh-induced bronchospam in rabbit tracheal preparation. It shifted Ca^{2+} concentration response curves constructed in rabbit trachea towards right, similar to diltiazem (a Ca^{2+} channel blocker). Moreover,

the bronchodilator effect of the crude extract of *A. spinsus* was also examined. Like ginger oil and citral in this study, the results indicated that its bronchodilatory activity was mediated through β_2-adrenergic receptor stimulation and calcium channel blockade (Tiwari, Dwivedi, and Kakkar, 2010). Recently, Janbaz and co-workers (Janbaz *et al*., 2013) reported that the extract of *Tephrosia purpurea*, Linn. produced a relaxant effect on CCh-induced contraction of isolated rabbit tracheal preparations in a manner similar to verapamil. The observed bronchodilator response suggested that its possible mechanism be mediated through Ca^{2+} channel blocker. In addition, the view of its use in disorders associated with increased airway resistance, the crude extract of *Fumaria parviflara* was studies for possible bronchodilator effect against CCh-induced bronchoconstriction in guinea-pig and rabbit tracheal preparations. It have been demonstrated that *F. parviflara* relaxes airway smooth muscle through dual pathways, calcium channel blocker and muscarinic receptor blockade (Najeeb ur, Bashir, Al-Rehaily, and Gilani, 2012).

The relaxation effect on smooth muscle of citral, a major constituent of several plants including ginger oil, was found mediated through calcium channel. Recently, it has been demonstrated that the essential oil of *Pectis brevipedunculata* and it major constituents, citral (81.9%) elicited vasorelaxation on thoracic aorta by affecting the Ca^{2+} influx through voltage-dependent L-type Ca^{2+} chanels, (Pereira *et al*., 2013). It has been reported that citral is one of the major constituents of *Cymboagon citratus* and its inhibitory effect on rat aorta smooth muscle was highly comparable to verapamil, a standard VOCC antagonist. Moreover, the study in isolated rabbit ileum showed that citral shifted the calcium concentration-response curve to the right, suggesting that it may affect the intracellular calcium concentration by blocking the

Ca^{2+} influx from extracellular space possibly via receptor-operated calcium channels (Devi, Sim, and Ismail, 2011).

It is well known that the airways epithelium layer plays an important role in modulating the basal tone and reactivity of smooth muscle (Butler, Adler, Evans, Morgan, and Szarek, 1987; Murlas, 1986). In the airway, the muscle tone is modified by balance between synthesis and release of epithelium-derived relaxing factors such as nitric oxide (Buga, Gold, Wood, Chaudhuri, and Ignarro, 1989; Nijkamp, van der Linde, and Folkerts, 1993) and prostaglandins (Farmer, Hay, Raeburn, and Fedan, 1987; Tschirhart, Frossard, Bertrand, and Landry, 1987).

Nitric oxide has been shown to play an important role in airway functions under physiological and pathological conditions. In animals and human airways, nitric oxide has been shown to be the primary relaxant transmitter (Belvisi *et al*., 1992; Hwang, Wu, and Teng, 1998). Increased levels of nitric oxide can be found in certain spasmodic conditions, for example, allergic rhinitis, adult respiratory distress syndrome and asthma inmediate and late phase (de Sousa *et al*., 2010). Accordingly, the soluble guanylyl cyclase is expressed in airway smooth muscle (Hamad, Range, Holland, and Knox, 1999), and agents that stimulate this enzyme activity induced airway smooth muscle relaxation *in vitro* (Ijioma, Challiss, and Boyle, 1995; Lin *et al*., 2006; Nakahara *et al*., 2002).

Inflammation is associated with a large range of mediators that initiates inflammatory response for arachidonic acid (AA) release by cell biosynthesis of prostaglandins (PGs) via the cyclooxygenase (COX) pathway. COX-1 and COX-2 are the targets of widely used non-steroidal anti-inflammatory drugs (NSAIDs) and essential for such physiological processes as maintenance of the gastrointestinal tract, renal function and fever. COX-1 is expressed constitutively in all tissues, but COX-2 is

induced specifically during inflammatory degenerative, and neoplastic processes (Janbaz *et al*., 2012). On the other hand, prostaglandins play major role in regulating diverse physiological processes (Narumiya, Sugimoto, and Ushikubi, 1999). Prostaglandin E_2 (PGE_2) is a metabolite of AA, synthesized by the action of cyclooxygenase and PGE synthase. In addition PGE_2 mainly release from epithelial cells, airway smooth muscle or lung parenchyma is also a potent airway smooth muscle relaxant (Ersoy *et al*., 2008). Meanwhile, the relaxation effect of PGE_2 is directly obtained through the EP_2 receptor activation. This activation triggers the coupling between the EP_2 receptor with Gs protein, and produces relaxation effect (Penn and Benovic, 2008). PGE_2 is produced at increased levels by the airway smooth muscle cells, brochial and alveolar epithelial cells, fibroblasts, and lung inflammatory cells. Because of the relaxant effect of PGE_2 in the airway smooth muscle, this prostanoid has been considered to play a bronchoprotective role in this tissue (Profita *et al*., 2003; Taha *et al*., 2000). However, the various kinds of drugs has been developed to inhibit these enzyme (COX-1, COX-2), non-steroidal anti-inflammatory drugs (NSAIDs) and recently developed selective COX-2 inhibitors. COX inhibitors include aspirin, diclofenac, particularly indomethacin (Howes, 2007).

In this study, pre-treatment of the trachea rings with L-NAME, a nitric oxide synthase inhibitor did not change the bronchodilatory effects of ginger oil and citral on the CCh-induced contraction, suggesting that nitric oxide is not involved on ginger oil-induced relaxation. Similarly, this effect was not changed in trachea rings pretreated with indomethacin, an inhibitor of the cyclooxygenase enzyme and thus the bronchodilatory effects of ginger oil and citral seem not be due to the release of prostaglandins.

In contrast to the result obtained in this study, it was reported that the mechanism underlying the antispasmodic activity displayed on the rabbit ileum spontaneous contraction of citral (a major constituent of *Cymbopogon citratus*) was attenuated by L-NAME (Devi *et al*., 2011). Moreover, in endothelium-aorta, it has been postulated that the essential oil of *Pectis brevipedunculata* and its major constituent citral (up to 80%) elicit vasorelaxation by affecting the nitric oxide/cGMP (Pereira *et al*., 2013). Similarly, Devi and coworkers (Devi *et al*., 2011) have study the relaxant effect of citral on vascular smooth muscle and found that it partially act through nitric oxide pathway. A possible explanation for this discrepancy might be some different contraction mechanisms among smooth muscle in various tissues.

In term of COX pathway, the study of citral as a major constituent of *C. citratus* showed that indomethacin did not affect its relaxant effect on vascular smooth muscle of the isolated thoracic rat aorta (Devi *et al*., 2011). Thus, the result was consistent to the bronchodilatory effects of citral in this study that its mechanism underlying did not involve the release of prostaglandins. The explanation could be the experimental designs of the present study were corresponded with the early-phase response of airway in asthma (pre-incubation periods with indomethacin for 30 minutes). When compare to the late-phase response, the result may be different. The early-phase is defined by a symptom development in 10-15 minutes and maximum peak within 30 minutes and production of IgE antibodies leading to the coating of tissue mast cells and circulating basophils. On the other hand, the late-phase response reaches its maximum at 6-12 hours later and resolves after 24 hours. This phase is characterized by influx of inflammatory cell line such eosinophils, CD4+ TH2 cells, mononuclear cells and basophils, recruited from the circulation being activated by different mediators as for instance cytokines and PGD_2 (Holgate, Church, and

Lichtenstein, 2006). It was suggested that citral, the major component of *C. citratus*, exerted an anti-inflammatory action may be involved in its inhibitory effects on cytokines production of the transcription factor NF-κB by murine macrophage (Bachiega and Sforcin, 2011; Tamaoki *et al*., 2000). The mechanisms involved inflammation which is responsible for late-phase response in asthma of citral and ginger oil need further elucidation.

In conclusion, the brochodilatory effects of ginger oil and citral, its major active ingredient, were reported. The possible mechanism underlying this relaxing effect appeared to involve β_2-adrenergic receptor and Ca^{2+} channel blocking. This study provides the pharmacological basis supporting therapeutic potential of *Z. officinale* rhizomes as a bronchodilator.

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