Development of a functional bioassay to study the mechanism of action of a novel phenylethylamine alkaloid, schwarzinicine A

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Abstract

Hypertension is the most prevalent risk factor leading to cardiovascular diseases. Resistant or uncontrolled hypertension urges the discovery and development of new drug candidates in treating this health concern. Plant-derived biological active compounds are always the alternative drug candidates for many diseases. This study aims to investigate and characterise the vascular effects of a novel plant phenylethylamine alkaloid, schwarzinicine A from *Ficus schwarzii*. A functional bioassay methodology using four different rat tissues such as trachea, bronchus, aorta and bladder was first optimised at fresh and stored conditions. The functionality of this optimised methodology was proven effective in testing ten different plant alkaloids on rat aorta. This was followed by preliminary testing of schwarzinicine A on four different rat tissues such as trachea, bronchus, aorta and bladder and the results depicted that schwarzinicine A exhibited the highest relaxation efficacy in rat aorta among all tested tissues. The bladder response to schwarzinicine A was inconclusive due to the occurrence of spontaneous contractile response in bladder. Hence, rat aorta was the best candidate for further investigation of schwarzinicine A effect, whether its induced relaxant effect in rat aortic rings was influenced by endothelium and nitric oxide (NO), or was mediated by alpha- and beta-adrenergic receptors, potassium channels, cGMP/cAMP, and calcium channels. The findings showed that the aortic relaxation by schwarzinicine A was not affected by all above-mentioned modulators, with exception that it inhibited calcium channels as suggested from the reduced contraction responses of three tested contractile agents including phenylephrine (alpha-adrenergic receptor agonist), 5-hydroxytryptamine (non-selective serotonin receptor agonist) and potassium chloride (membrane depolarising agent), as well as the contractions by calcium chloride in calcium-free Krebs solution, in the prior treatments with schwarzinicine A. This hypothesis was elucidated in the mechanistic studies using porcine coronary arteries (PCA) and rat dorsal root ganglion (DRG) cells. In porcine coronary arteries, schwarzinicine A also caused the similar relaxant effect in response to U46619 pre-contraction and also the reduced contractions to calcium chloride in calcium-free experiments, as compared to those observed in rat aortic

rings. Cell-based calcium imaging assay using rat DRG cells was to further elucidate the inhibitory role of schwarzinicine A in calcium channels. Schwarzinicine A was revealed to inhibit the calcium entry via dominant voltage-gated calcium channel opening by KCl. All the gathered findings from rat aorta, PCA and rat DRG cells concluded that schwarzincine A exhibited an inhibitory role on calcium channels.

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Publications

Papers:

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2. Lim JL, Sim KS, Yong KT, Loong BJ, Ting KN, Lim SH, Low YY and Kam TS (2015). Biologically active vallesamine, strychnan, and rhazinilam alkaloids from *Alstonia*: Pneumatophorine, a *nor-seco*vallesamine with unusual incorporation of a 3-ethylpyridine moiety. *Phytochemistry*. **117:** 317-324.

3. Yap VA, Loong BJ, Ting KN, Loh HS, Yong KT, Low YY, Kam TS and Lim KH (2015). Hispidacine, an unusual 8,40-oxyneolignan-alkaloid with vasorelaxant activity, and hispiloscine, an antiproliferative phenanthroindolizidine alkaloid, from *Ficus hispida* Linn. *Phytochemistry*. **109:** 96-102.

Abbreviations

$^{\cdot}O_2^-$	superoxide anion
$[Ca^{2+}]_i$	intracellular Ca^{2+}
2-APB	2-aminoethoxydiphenyl borate
4-AP	4-aminopyridine
5'AMP	5' adenosine monophosphate
5'GMP	5' guanosine monophosphate
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AC	adenylyl cyclase
ACE	angiotensin-converting enzyme
ACEI	angiotensin-converting enzyme inhibitor
AM	acetoxymethyl
ARBs	Angiotensin II receptor blockers
AWS	Animal Welfare Act
BB	beta-blocker
B_{KCa}	Large conductance calcium activated potassium channel
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CCB	calcium channel blockers
cDAP	cyclic dinucleotide phosphate
cGMP	cyclic guanosine monophosphate
cGTP	cyclic guanosine triphosphate
CIOMS	Council for International Organisation of Medical Sciences
CNS	central nervous system
COX	cyclooxygenase
CYP450	cytochrome P450
DAG	diacylglycerol
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
EC_{50}	half maximal effective concentration
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
E_{max}	Maximum response
eNOS	endothelial NOS
ER	endoplasmic reticulum
E_{res}	resting membrane potential
FFA	flufenamic acid
FRET	Föster resonance energy transfer
GC	guanylyl cyclase
GDNF	glial cell line-derived neurotrophic factor
GECI	genetically-encoded Ca^{2+} indicator
GEF	guanine nucleotide exchanger factors
GPCR	G protein-coupled receptor
IACUC	Institutional Animal Care and Use Committee
IBMX	isobutylmethylxanthine
IC_{50}	half maximal inhibitory concentration
ICLAS	International Council for Laboratory Animal Science
IK_{Ca}	Intermediate conductance calcium activated potassium channel
iNOS	inducible NOS
IP_3	inositol triphosphate
IUBS	International Union of Biological Sciences
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Calcium-activated potassium channel
K_d	dissociation constant
K_i	inhibitory constant
K_{IR}	inwardly rectifying K^+ channel
K_v	voltage-gated K^+ channel

LCA and RCA	Left and right coronary artery
L-NAME	N^G-Nitro-L-Arginine methyl ester
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
NAADP	nicotinic acid dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal NOS
NO	nitric oxide
NOS	NO synthase
NSCC	non-selective cation channel
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PCA	porcine coronary artery
PDE	Phosphodiesterase
pEC_{50}	negative logarithm of EC_{50}
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PHS	Public Health Service
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PMCA	plasma membrane Ca^{2+}
PPARδ	peroxisome proliferator activated receptor
RAAS	renin-angiotensin-aldosterone system
ROCC	receptor-operated calcium channel

ROK	Rho kinase
SEM	standard error of mean
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SHR	spontaneous hypertensive rat
SK_{Ca}	small conductance calcium activated potassium channel
SMOC	second messenger-operated calcium channel
SNPs	single-nucleotide polymorphisms
SOCC	store-operated calcium channel
TEA	tetraethylammonium
TK	tyrosine kinase
TRP	transient receptor potential
TRPC	transient receptor potential canonical
TRPM3	transient receptor potential melastatin 3
VOCC	voltage-operated calcium channel

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Chapter 1: Literature Review & General Introduction

1.1 Cardiovascular disease

Cardiovascular diseases account for 17.5 million of deaths per year worldwide as documented by the World Health Organisation (WHO, 2014). This figure represents one third of total deaths worldwide and half of the death due to non-communicable diseases (NCDs) besides cancer, diabetes and chronic pulmonary diseases (Mendis *et al.*, 2011). The death from NCDs accounts for 63% of global death (Mendis *et al.*, 2011). Alarmingly, more than 80% of cardiovascular diseases' global burdens occur in the low-income and middle-income countries according to Global Burden of Diseases, Injuries, and Risk Factors Study (GBD, 2013). This figure is primarily based on the development and lifestyle changing among low- and middle-income countries (GBD, 2013).

In the last two decades, the mortality of cardiovascular diseases has been declining in high-income countries, yet it increases rapidly in low- and middle-income countries (Mendis *et al.*, 2011). Cardiovascular disease is preventable (Mendis *et al.*, 2011). The implementation of population-wide measures and the improved of individual health care interventions in high-income countries have reduced the population's health and socioeconomic burdens from cardiovascular risk factors, and hence reduce the cardiovascular mortality (Mendis *et al.*, 2011). In contrast, in low- and middle-income countries, the health care systems are only focussed on the treatment of disease and centered in high-technology hospitals (Mendis *et al.*, 2011). This not only increase the socioeconomic burden within the population, and also leave a large portion of population with high cardiovascular risks being undiagnosed (Mendis *et al.*, 2011). Therefore, this explains the fast increase of cardiovascular mortality in the low- and middle-income countries.

Cardiovascular disease is a broad term for all disorders in the heart and blood vessels with direct effects on the heart (acute coronary syndromes, angina, arrhythmia, cardiomyopathy, congenital heart disease, coronary heart disease, heart failure, inflammatory heart disease, ischaemic heart disease, rheumatic heart disease and valvular disease), brain (cerebrovascular disease/ stroke) and circulatory system (deep vein thrombosis, hypertensive heart disease , peripheral artery disease and pulmonary embolism) (World Heart Federation, 2012). A heart attack or stroke is often the first indication of cardiovascular diseases.

The aetiology of cardiovascular diseases is due to many reasons including genetic (inherited profile), behavioural (tobacco use, inadequate physical activities, unhealthy diet and alcohol use), metabolic (increase in blood pressure, blood sugar, and blood lipids, overweight and obesity), socioeconomic (age, gender, economic and educational status) and psychological (stress and depression) conditions (Mendis *et al.*, 2011). These factors elevate the risk of hypertension, the most prevalent event leading to cardiovascular disease, which is responsible for 9.4 million of total cardiovascular-related deaths across the globe every year (WHO, 2014).

1.2 Hypertension

Hypertension is defined as increase in blood pressure that occurs when systolic blood pressure is measured at ≥140mm/Hg and/or diastolic blood pressure is measured at ≥90mm/Hg (reviewed by Giles and Materson, 2013). Systolic blood pressure measures the arterial pressure during heartbeats (when heart muscle contracts) whereas the diastolic blood pressure measures the arterial pressure between heartbeats (when the heart rests and blood refilling).

More than 90% of hypertension cases are classified as essential hypertension, which represents cases with unknown causes (GBD, 2013). These cases are known as primary hypertension. Those with known causes are classified as secondary hypertension. The occurrence of hypertension raises the risk of cardiovascular

events, prevalently ischaemic heart disease (45%) and stroke (51%) (Mendis *et al.*, 2011). Out of the global 17 million deaths from cardiovascular diseases, 9.4 million of deaths were due to hypertension (WHO, 2014).

1.2.1 Regulation of normal blood pressure

The knowledge of blood pressure regulation is important in understanding the pathogenesis of hypertension. There are two circulation systems in the human body which are systemic and pulmonary circulations (reviewed by Sparks and Rooke, 1987). Systemic circulation refers to the blood circulation from heart to the rest of the body while pulmonary circulation refers to the blood circulation from the heart to the lung. These circulations are mainly modulated via renin-angiotensin-aldosterone system (RAAS), baroreceptors and chemoreceptors (reviewed by Sparks and Rooke, 1987; Atlas, 2007).

Renin-angiotensin-aldosterone system (RAAS) is a main modulator of blood pressure control. This pathway cascade is initiated by the secretion of renin from kidneys. Renin is a rate-limiting enzyme that catalyses the hydrolysis of angiotensinogen into angiotensin I in which angiotensin I is further hydrolysed into angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II plays a major role in RAAS pathway. There are four angiotensin receptor subtypes ($AT_{1\text{-}4}$). Angiotensin II has a direct effect on AT_1 receptors that leads to vasoconstriction, increase in blood pressure, increase in cardiac contractility, vascular and cardiac hypertrophy, renal tubular sodium reabsorption, inhibition of kidney renin release and alterations in sympathetic nervous system (Atlas, 2007). Binding of angiotensin II on AT_2 receptor mediates vasodilation as well as anti-proliferation and apoptosis in vascular smooth muscle cells (to inhibit the growth and remodelling of vascular tissues) (Atlas, 2007). Sequential amino acid removal of angiotensin II by aminopeptidases results in the production of angiotensin III and IV. These two derivatives may have an effect on AT_4 receptor that modulates vascular endothelium function. The functions of AT_3 remain unknown.

Baroreceptors are the stretch-sensing receptors that transport blood pressure information to the central nervous system via neural fibers (Davos *et al.*, 2002; Di Rienzo *et al.*, 2009). This shapes the function of baroreceptors in maintaining mean arterial pressure (MAP). These receptors are found in carotid and aortic bodies (Di Rienzo *et al.*, 2009). Aortic and carotid baroreceptors share the equivalent contribution in controlling arterial baroreflex and heart rate (Kougias *et al.*, 2010). Changes in arterial pressure threshold lead to pressure resetting by baroreceptors. This can be done by either reversing the altered blood pressure back to normal without a change in receptor sensitivity (acute baroreceptor resetting) or reducing the receptor sensitivity (chronic resetting) (Kougias *et al.*, 2010).

Similar to arterial baroreceptors, arterial chemoreceptors are also located at aortic and carotid bodies. Arterial chemoreceptors are oxygen-sensing receptors and maintaining oxygen homeostasis in vascular tissues (Prabhakar and Peng, 2004). The situations such as hypoxemia (deficiency of oxygen in blood) and hypercapnia (retention of carbon dioxide in blood) stimulate the reflex control by chemoreceptors (Schultz *et al.*, 2007). Hypoxia causes vascular inflammation and endothelial dysfunction due to the lack of oxygen supply. This situation is specific to the activation of arterial chemoreceptors hence it is typically used as the stimulus for these receptors (Schultz *et al.*, 2007). The activation of arterial chemoreceptors by hypoxia increases the activity of sympathetic nervous system to redistribute the blood flow to the blood vessels (Schultz *et al.*, 2007).

In addition to RAAS and baroreceptor, blood pressure can be regulated by other factors, such as genetic factors (such as single/multiple gene modulation and inheritable gene factors), sympathetic nervous system, fluid retention, hormonal factors (insulin and aldosterone), age and gender of subjects, and environmental factors (such as stress level). Alterations in these regulators can lead to the pathogenesis of hypertension.

1.2.2 Pathogenesis of hypertension

Hypertension itself is a complex phenotype that can be caused by genetic, environmental, behavioural and social issues (Landsberg *et al.*, 2013). Therefore, any cause of alteration in the pathophysiological mechanisms of maintaining a normal blood pressure could contribute to hypertension (Beevers *et al.*, 2001). These pathophysiological mechanisms include the balance between baroreflex and chemoreflex, renin-angiotensin-aldosterone system, autonomic nervous system and the involvement of other body-produced vasoactive substances that control the sodium transport and vascular tone, such as bradykinin, endothelin and atrial natriuretic peptide (Beevers *et al.*, 2001). In addition, the structural and functional abnormalities in blood vessels (endothelial dysfunction or damage, irregular levels of haemostatic factors, platelet activation and fibrinolysis, as well as irregular blood flow), insulin resistance or insensitivity, inheritable genetic factors and intrauterine influences, could also contribute to hypertension (Beevers *et al.*, 2001). The complex and interactive relationship between some of these factors is compiled and discussed below.

Blood pressure is normally regulated by the blood flow in the blood vessel. Changes in the internal vessel diameter, vessel length and blood viscosity will give rise to a vascular resistance (renal and peripheral) in this blood flow that results in an elevated blood pressure. These changes are affected by vascular reactivity and remodelling. Hypertensive patients experience an increase in vasoconstriction due to a greater sensitivity to vasoconstrictors, as compared to healthy subjects (Oparil *et al.*, 2003). Vascular remodelling refers to a change in the lumen diameter of blood vessel, in which it could either be an inward (reduced of lumen diameter) or outward (increase in lumen diameter) remodelling. Untreated hypertension revealed a reduction in lumen areas, increase in media-lumen ratios (without change in media area) and a decrease in parallel-connected vessels (Oparil *et al.*, 2003).

Vascular remodelling can be caused by an increase in sympathetic nervous system activity. In addition, this increase of sympathetic nervous system can also cause an

imbalance within autonomic nervous system (increase in sympathetic nervous system and decrease in parasympathetic nervous system) that gives rise to the pathogenesis of hypertension. Increase of sympathetic tone is mediated by a complex involvement of baroreflex and chemoreflex pathways (Oparil *et al.*, 2003). Baroreflex and chemoreflex responses are under modulation of baroreceptor and chemoreceptor respectively in controlling the blood pressure and heart rate. In hypertensive patients, the increase of sympathetic stimulation is due to the resetting of arterial baroreflex responses and an increase of chemoreflex response. The reset of baroreflex response in hypertensive condition suppresses the sympathetic inhibition (Albaghdadi, 2007) under an influence by angiotensin II from RAAS (Tan *et al.*, 2007). The arterial baroreflex markedly inhibits the peripheral chemoreflex, in which enhanced peripheral chemoreflex sensitivity in hypertensive patients can be associated with the reduced of baroreflex function (Kara *et al.*, 2003).

The stiffness of arteries due to arteriosclerosis is also one of the causes of hypertension. Arteriosclerosis occurs due to either excessive deposition of collagen and fat on the inner lining of large conduit arteries, or smooth muscle cell hypertrophy, or thinning of elastin fibers in the media layer. These structural abnormalities develop over time and often happen in elderly patients. Aging causes the dysfunction of vascular endothelium, in which leads to a decrease in endothelial nitric oxide (an important vasodilator) synthesis and hence the stiffness of arteries. Increased arterial stiffness results in the wide pulse pressure and increase in pulse wave velocity, as commonly observed in elderly hypertensive patients (Oparil *et al.*, 2003). The measurement of aortic stiffness can also be associated to the risk of cardiovascular mortality in hypertensive patients (Laurent *et al.*, 2001).

Despite of its primary role of in regulating blood pressure, angiotensin II also stimulates oxidative stress in vascular cells by activating an oxidative enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) to promote the formation of oxidant superoxide anion ($^{\cdot}O_2^-$) that later reacts with nitric oxide in forming oxidant peroxynitrite ($ONOO^-$) (Griendling *et al.*, 1994). The stimulated oxidative stress and reduced in nitric oxide bioactivity therefore increase the vasoconstriction

to angiotensin II, vascular lesion and remodelling, thrombosis and inflammation (reviewed by Nguyen Dinh Cat *et al.*, 2013). Due to the detrimental effect of long term angiotensin II infusion, the inhibition of angiotensin II production by ACE inhibitors and angiotensin II receptor blockers (ARBs) has become a therapeutic target of hypertension.

Pharmacogenomics is currently gaining interest in the research of understanding the pathogenesis of hypertension. There are at least 30 genetic variants in association with the increase in blood pressure (Lieb *et al.*, 2013). The renal influence (later gives rise to RAAS) in blood pressure control has provided an insight in discovering the corresponded regulating genes, such as *M235T* variant in angiotensinogen gene and ACE gene (Ortlepp *et al.*, 2003; Srivastava *et al.*, 2012). Inheritable genetic influence in hypertension was revealed from the twin studies (Feinleib *et al.*, 1977) and population studies (Longini *et al.*, 1984). Related to this inheritable genetic influence, the single-nucleotide polymorphisms (SNPs) were found in the promoters of *N*-methyltransferase and catalase gene, coding region of NEDD4L ubiquitin ligase gene and mitochondrial deoxyribonucleic acid (DNA) of hypertensive probands (Kouremenos *et al.*, 2014). The rare and heritable hypertension, such as glucocorticoid-remediable aldosteronism and Liddle's syndrome may be caused by single gene mutations (Conlin, 2008).

1.3 Antihypertensive drug therapy

The aim of antihypertensive drug therapy is to reduce the patients' blood pressure down to below 140/90 mmHg [National Institute for Health and Care Excellence (NICE) guidance, 2011]. For patients with atherosclerotic cardiovascular disease, diabetes or chronic renal failure, an even lower pressure of 130/80mmHg is recommended (NICE guidance, 2011). There are several common antihypertensive drug classes such as diuretics, beta-blockers (BBs), angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs) and calcium channel blockers (CCBs) (Hill and Smith, 2005). Patients with hypertension often receive a

cocktail of medication depending on how well they respond to the drugs (NICE guidance, 2011).

In some cases, the age and ethnic background of patients affects their antihypertensive treatments. For examples, an ACE inhibitor works more effectively in younger Caucasians compared to other drugs, whereas calcium channel blockers work efficiently in Afro-Caribbean patients, as well as in the elderly above 55 years old (NICE guidance, 2011).

According to the recommendation by NICE guidance (2011) the initial treatment for essential hypertension follows the steps of: Step 1, ACEI or ARB for adult aged <55 years old, CCBs for those aged >55 years old and beta-blocker is considered for younger patients; Step 2: diuretics is given for inadequate response and Step 3: alpha/beta-receptor blocker is consider for non-optimal response. The details for each antihypertensive drug class are written in following subsections.

1.3.1 Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs)

Numerous meta-analyses revealed that ACEIs and ARBs significantly reduce the risk of cardiovascular mortality and morbidity (Abuissa *et al.*, 2005; Savarese *et al.*, 2013; Cheng *et al.*, 2014), through their ability to lower blood pressure (Matchar *et al.*, 2008). These two classes of antihypertensive agents both act on renin-angiotensin-aldosterone system (RAAS).

Angiotensin II is a blood-circulating hormone that is normally produced in blood vessels. This hormone causes vasoconstriction of blood vessels, causing an increase in blood pressure. Abnormally high amount of angiotensin II is commonly detected in patients with cardiovascular diseases. To reduce this, ACEIs play a role in inhibiting the production of angiotensin II, whereas ARBs block the binding of angiotensin II to the angiotensin II receptor type 1 (AT_1 receptor).

The drug examples of ACEIs, normally ending in 'pril', include ramipril, captopril, enalapril, benazepril and fosinopril. For ARBs, examples are losartan, irbesartan and valsartan. Unlikely other antihypertensive drug classes, these two classes exhibit less adverse effects such as initiating renal problem and new on-set diabetics (reviewed by Sweitzer, 2003 and Terra, 2003). Some ARBs are specially prescribed to prevent renal and heart failure in patients with hypertension, as well as to patients who cannot tolerate ACEIs (reviewed by Terra, 2003). Some studies suggested the use of ACEIs as the initial treatment (Wing *et al.*, 2003; Cheng *et al.*, 2014), as proven better improvements among older patients, as compared to the treatment with diuretics (Wing *et al.*, 2003).

1.3.2 Calcium channel blockers

Calcium channel blockers (CCBs) are potent vasodilators, thus perceived as effective antihypertensive agents. Initial treatment with CCBs also prevents major forms of cardiovascular diseases and reduces blood pressure across all patient groups, regardless of gender, race, ethnicity, age and sodium intake (reviewed by Elliott and Ram, 2011). CCBs have been found to be more effective in lowering blood pressure when compared to beta blockers (Chen *et al.*, 2010) for example.

This drug class is divided into three groups: dihydropyridine, phenylalkylamine and benzothiazepine. Verapamil from phenylalkylamine group was the first identified substance in this class of CCBs (Trenkwalder, 2004). Dihydropyridine is the largest group with known substances, such as amlodipine, felodipine, isradipine, lacidipine, nicardipine and nifedipine.

CCBs are known to inhibit calcium influx in order to dilate the vascular tissues. Based on the inhibitory effects of these drugs, different classifications were made by different researchers which are (i) Fleckenstein and (ii) Robertson and Robertson (reviewed by Yousef *et al.*, 2005). The classification by Fleckenstein was based on the inhibition percentage of the drugs on calcium current whereas the one by Robertson and Robertson was based on the target sites of the CCB drugs. Despite

the high effectiveness of CCBs across a broad range of patients, the use of CCBs in complex hypertension (hypertension patients with other health issues) is still in debate. Combination of CCBs with other antihypertensive class is recommended (NICE guidance, 2011).

1.3.3 Diuretics

Despite that the initial use of diuretics in renal treatment, its vasodilatory effect was later discovered. Today, diuretics are shown to exhibit both vasodilatory and natriuretic effects. There are four classes of diuretics, which include thiazides, thiazides-related, potassium sparing and loop diuretics. Among all, thiazide diuretics are the most potent vasodilators, even at low doses (reviewed by Shah *et al.*, 2004). Since the first report of JNC in year 1977 and prior to the evaluation of the NICE 2011 evaluation, this diuretic class has always been placed as the first-line treatment for mild to moderate hypertension. For severe hypertension, thiazide diuretics are commonly used in combination with other classes of antihypertensive drugs (ACEIs, ARBs, BBs and CCBs) (NICE guidance, 2011). Examples of the commonly used thiazide-type diuretics are hydrochlorothiazide, clorthalidone and indapamide. In addition to thiazide-type diuretics, loop diuretics, such as furosemide and bumetanide are also used in antihypertensive treatment.

Diuretics are known to lower blood pressure, giving rise to antihypertensive effects. With their nature in renal treatment, diuretics mediate their antihypertensive effects via $Na^+/K^+/Cl^-$ cotransport and chloride-mediated intracellular calcium stores refilling (Greenberg *et al.*, 1994). In addition, diuretics could also cause the relaxation of blood vessels by releasing endothelium-derived relaxing factors (Wiemer *et al.*, 1994) and activating potassium channels (Pickkers *et al.*, 1998). These events eventually lead to an antihypertensive effect.

1.3.4 Beta-adrenergic receptor blockers

Beta-adrenergic receptor blockers better known as beta-blockers, have been used for more than four decades to treating hypertension. They act by inhibiting the activity of the beta-adrenergic receptor and causing vascular relaxation.

A variety of beta-blockers are prescribed according to the physiological conditions, for example drug tolerance and other health complexities in the patients. These include atenolol, metoprolol, propranolol, bisoprolol, nebivolol and carvedilol. The existing beta-blockers are different in terms of their pharmacokinetics and pharmacodynamics (reviewed by Talbert, 2004). Atenolol, metoprolol, bisoprolol and nebivolol are selective to β_1-adrenergic receptors. Carvedilol causes vasodilation by inhibiting β_1-, β_2 and α_1-adrenergic receptors (reviewed by Talbert *et al.*, 2004). This vasodilating activity is also facilitated by the production of nitric oxide (NO) by carvedilol itself (Yoshioka *et al.*, 2000).

Several reviews on numerous meta-analyses studies have stated the unsuitability of beta-blocker as a first-line therapy drug for uncomplicated hypertension (Messerli *et al.*, 2008; De Caterina and Leone, 2010; DiNicolantonio *et al.*, 2015). These studies revealed the multiple adverse effects of beta-blockers and their inefficacy in lowering blood pressure. Beta-blockers are known to promote the risks of new-onset diabetes, dyslipoproteinemia and kidney problem (Fogari *et al.*, 1997; Messerli *et al.*, 2004; Bui, 2010). Among all the beta-blockers, carvedilol is the most effective with less adverse effects.

1.3.5 Other hypertensive drug classes

In addition to the abovementioned commonly prescribed hypertensive drugs, other hypertensive drug classes, such as alpha-adrenergic blockers and potassium channel activators, are also prescribed under certain circumstances. These two drug classes are not first-line treatment in treating hypertension, yet they are used as add-on drugs in treating resistant hypertension. They are often used as a step 3 or

4 option due to inadequate response to the above-mentioned hypertensive drug classes (ACE/ARB inhibitors, CCBs and diuretics).

Alpha-adrenergic blocker (or known as alpha-blocker) acts on postganglionic alpha$_1$-receptors, in which results in vasodilation. Alpha$_1$ adrenergic receptor is commonly known to cause vasoconstriction upon its activation. Some examples of prescribed alpha-blocker are doxazosin, indoramin, prazosin and terazosin (British Hypertension Society, 2011). On the other hand, potassium channel is one of the modulators in modulating the vascular activity in our bodies. Activation of this channel by its channel activators leads to the relaxation of vascular smooth muscles. The clinically used potassium channel activators include aprikalim, bimakalim, cromakalim, nicorandil and pinacidil (Haeusler and Lues, 1994). These activators are selectively target for ATP-sensitive potassium channel (Humphries and Dart, 2015). Sadly, for other potassium channel subtypes, there are very few selective activators are currently licensed (Humphries and Dart, 2015).

1.4 Resistant hypertension

The problem of resistant hypertension occurs when the blood pressure is still uncontrolled even under medication of three or four drug combinations. Estimated 20% of such cases occur within the population hypertensive patients in England (Falaschetti *et al.*, 2009). The precise cause of this problem is not clearly known. About 5% to 10% in patients with resistant hypertension seem to have an underlying secondary cause on top of their hypertensive conditions. These secondary causes, such as primary hyperaldosteronism, thyroid disease and renal parenchymal disease have worsen the treatment of hypertension, hence results in resistant hypertension. This is in addition of the lifestyle factors (such as obesity, excessive alcohol and salt intake and smoking), side effects of other medication and volume overload (such as renal insufficiency and high salt intake). These factors can all contribute to resistant hypertension (Sarafidis *et al.*, 2008; Myat *et al.*, 2012). The older hypertensive patients (aged >75 years old) also tend to experience the

problem of resistant hypertension as compared to the younger patients (Myat *et al.*, 2012).

Sometimes, pseudo (seemingly)-resistant hypertension can be mistaken as the true resistant hypertension. Therefore, certain factors (either related to the patients or physicians) should be validated in order to eliminate this technical problem. In the part of the patients, the factors of white coat effect, arterfact due to arterioslerotic arteries, side effects of antihypertensive treatment, inadequate education, poor memory and cognition, costs of drugs; and the factors of poor blood pressure measurement technique, inadequate drugs for prescription and combination and poor communication with patients for the part of physicians, should all be validated before reaching out for the treatment of true resistant hypertension (Myat *et al.*, 2012).

Once diagnosed, the treatment of resistant hypertension (at step 4 of the treatment of hypertension) is started by considering a further diuretic therapy by prescribing either low-dose spironolactone or higher dose of thiazide-like diuretics, depending on the blood potassium level (NICE, 2011). Alpha- or beta-blocker is considered if the response to this further diuretic therapy is inadequate (NICE, 2011). The greater consumption of antihypertensive drugs by patients with resistant hypertension causes them to experience much severe adverse cardiovascular events, compared to those consume ≤3 antihypertensive drugs (Myat *et al.*, 2012).

1.5 Drug discovery

The problem of resistant hypertension urges the discovery of new antihypertensive drugs. This is essential in broadening the availability of current antihypertensive drugs and to provide more tolerated drugs for treating resistant hypertension.

The pipeline of discovery until its approval takes more than ten years to accomplish. This begins from early drug discovery, preclinical development, Investigational New Drug (IND) filing, clinical development, New Drug Application (NDA) filing and

eventually the Food and Drug Administration (FDA) filing (reviewed by Hughes *et al.*, 2011). In between each phase, numerous evaluations are undertaken to confirm the therapeutic activity and safety of the potential drugs.

For the early drug discovery phase, a few essential steps such as the target identification and validation, compound screening, secondary assays and finally the *in vivo* analysis are undertaken. These steps have to be achieved before the preclinical development phase. The identification and validation of potential drug-like targets are important to determine the following procedures of drug development. In achieving these, there are many useful experimental techniques to be used in terms of the genetic and expression data profiling, analysis of molecular signalling pathways, cell-based and *in vivo* disease models, molecular pharmacology of variants, tool compounds and bioactive molecules, literature survey and competitor information (reviewed by Hughes *et al.*, 2011).

Following the target validation, the 'hit' compound will be identified and proceed to the lead discovery phase. The 'hit' compound is defined as the compound with desired and confirmed activity after compound screening and retesting (reviewed by Hughes *et al.*, 2011). There are some common compound screening assays, including high-throughput screening, focussed screening, fragment screening, virtual screening, physiological screening and nuclear magnetic resonance (NMR) screening. The descriptions of these screening assays are listed in *Table 1.1*.

Screening assays	Descriptions
High-throughput screen	Screening of large amount of compounds
Focussed screen	Screening of compounds with specific 'hit' classes and those with similar structures
Fragment screen	Screening for compounds with low mM activity (built from small compounds in crystal forms), later used as building blocks for larger molecules.
Virtual screen	Integration of X-ray structure of a compound into a virtual compound library to provide a base for building a compound.
Physiological screen	A tissue-based approach to determine the drug activity.
NMR screen	Screening for compounds with low mM activity that are later used to form larger molecules by incorporating small compounds (fragments) into the protein targets of known crystal or NMR structure.

Table 1.1: Screening assays for compounds (adapted from a review by Hughes *et al.*, 2011).

At the present time, the pace of drug discovery is not parallel to the increased knowledge in the biomedical field. Drug productivity is taking place at a modest rate, with an average of 20 to 30 drugs approved by the FDA each year (Fricker, 2013). When antihypertensive drugs are concerned, even fewer numbers of new drugs are approved by FDA each year, as shown in the statistics from year 2010 to 2015 (*Figure 1.1*). This happens when translational and applied science, rather than the basic scientific research are heavily emphasised (Fricker, 2013). A balance between both areas is highly recommended to ensure productive discovery of new drugs.

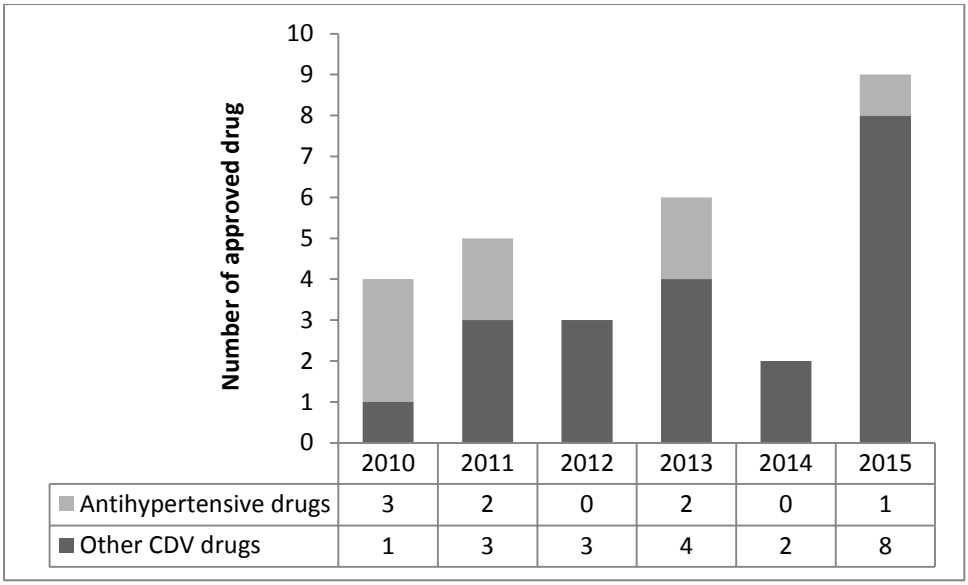


Figure 1.1: Statistics of approved antihypertensive drugs by FDA from year 2010 to 2015 (Fricker, 2013).

1.6 Plant-based products as therapeutic targets

In the field of drug development, natural products have gained in popularity as the alternative source. This is due to the side effects and expensive cost of the current health treatments. Natural products are derived from the sources of higher plants, microbes, fungi and marine lives (reviewed by Dias *et al.*, 2012). Of the stated sources, higher plants are the main natural source of therapeutic products. The plant parts, from roots, barks and leaves of plants, are utilised as the therapeutic sources.

The earliest record of natural products [oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh)] was documented back in Mesopotamia (2600 B.C.) (Cragg and Newman, 2005). During World War II, various plant-based therapeutic drugs were discovered and are still in use today. They include quinine from *Cinchona* bark, morphine and codeine from opium poppy, digoxin from *Digitalis* leaves, as well as atropine and hyoscine from the *Solanaceae* species (reviewed by Phillipson, 2001). The development of plant-based therapeutic drugs was extended through the post-war years, however with limited discovery

breakthroughs (reviewed by Phillipson, 2001). This led to a shift, with an increased interest in synthetic drugs. However, recently the pharmaceutical industry has backtracked, and shifted its focus back to plant-based products (reviewed by Phillipson, 2001).

There are a few reasons for this increased interest in plant-based therapy by the pharmaceutical industry. Firstly, the advances in chemistry development and bioassay technology have facilitated the process of plant-based drug development (Newman *et al.*, 2003; Paterson and Anderson, 2005; Littleton *et al.*, 2005; Rollinger *et al.*, 2006). In addition, a better understanding of biological mechanisms in certain organisms also provides an opportunity for discovering and developing new plant-based drugs (McChesney *et al.*, 2007). Additionally, the shift is also because of the rapid diminishing of biological resources, including certain plant species. This has encouraged research into finding potential pharmaceutical benefits in the nearly-depleted plant species (McChesney *et al.*, 2007).

There are estimated 500,000 plant species in the world. This high figure value requires the need of some selective approaches in finding those with therapeutic potentials. These approaches can be divided into random selection and follow-up approaches. Random selection can either be done following a phytochemical screening of the plants or following a few bioassays in examining the biological activity of the plants (Fabricant and Farnsworth, 2001). For the follow-up approaches, reviews are done on written reports dated back from 1930s to 1970s on the plant biological activity and the ethnomedical uses of the plants (Fabricant and Farnsworth, 2001). Those selected plants are then used for further investigations on their preclinical and clinical functions.

1.6.1 Plant-based products as antihypertensive agents

Newman and colleagues (2003) reported that 65% of antihypertensive drugs (48 out of 74) are derived from natural product structures or their mimics. This marks the significance of natural products in new drug development.

Majority of the world population, especially the ethnic population, rely mainly on plant-derived traditional medicines as their primary health care (Farnsworth *et al.*, 1985). Most plant-derived antihypertensive drugs are developed based on their ethnomedical information, derived from the traditional medicine systems that the ethnic populations practise (Fabricant and Farnsworth, 2001). The biological active compounds are isolated from these traditional medicinal plants and studied. These compounds are either used as a base molecule for other drug synthesis or directly developed into an active drug. The examples of these plant-derived antihypertensive drugs are listed in *Table 1.2*.

Compounds	Plant source	Common name	Pharmacological action
Deserpidine	*Rauwolfia canescens* L.	Snakeroot	Inhibits the sequestering of neurotransmitters by ATP/Mg^{2+} pump[a]
Papaverine	*Papaver somniferum*	Opium poppy	Causes relaxation of vascular smooth muscles[a,b]
Protoveratrines A and B	*Veratrum album* L.	White hellebore	Causes antidiuretic action (Protoveratrines A) and hypotensive activity[c]
(i)Reserpine (ii)Rescinnamine	*Rauwolfia serpentine*	Snakeroot	(i) Reserpine: inhibits the vesicular monoamine transporter 2[d] (ii) Rescinnamine: inhibits the enzymatic conversion of angiotensin I to angiotensin II[d]

Table 1.2: Examples of the plant-based antihypertensive drugs with their common names (adapted from Fabricant and Farnsworth, 2001). [a] DrugBank, [b]Williams, 2008, [c]Winer, 1960, [d]IUPHAR database.

Both deserpidine and reserpine from *Rauwolfia* species are known as the catecholamine-depleting sympatholytics. Papaverine (from opium poppy) is a vasodilator, which provides the basic structure for the calcium channel blocker, verapamil. Both showed to cause vascular smooth muscle relaxation by blocking non-dihydropyridine calcium channels (Hayashi and Toda, 1977). Protoveratrines A and B, both from *Veratrum album*, have antihypertensive properties. Despite the slight structural difference between these two compounds (different by one hydroxyl group), their difference in therapeutic activity was reported (Winer, 1956). When administrated orally in hypertensive men, protoveratrine A exhibited stronger hypotensive activity within a narrower therapeutic dosage range than that of protoveratrine B (Winer, 1960). The exact mechanism of protoveratrines A and B is not clearly known.

1.6.2 Evidence of cardiovascular effects of plant alkaloids

A long history of the medicinal uses of plants is known to exist. Currently, there are only 20% of the total 50,000 plant species known to exist that have been studied and reported to have medicinal value. Considering the great figure of total plant species, the discovery of new plant species that have potential medicinal use is encouraged.

The bioactive compounds in plants can be grouped into primary and secondary metabolites, in which the primary metabolites are used for plant physiological developments while secondary metabolites are the mediators of ecological interactions (reviewed by Mann, 1978). The secondary metabolites have medicinal effects in humans; therefore they hold the realm in drug discovery. There are many plant secondary metabolites, such as tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (reviewed by Mann, 1978 and Edeoga *et al.*, 2005). Amongst all these secondary metabolites, plant alkaloids have been vastly studied and reported to hold the greatest therapeutic potential in cardiovascular disease.

Alkaloids are organic nitrogenous bases mostly found in plants. They consist of heterocyclic nitrogen groups that are derived from the amino acid metabolism. Alkaloids are commonly classified based on their heterocyclic ring system, but some have been classified based on their biosynthetic origins from amino acid metabolism (reviewed by Fester, 2010). Examples of plant alkaloid groups include pyridine, tropane, lupinane, *iso*quinoline, phenanthridine, quinoline and indole groups (reviewed by Henry, 1949).

Traditional medicinal plants are commonly utilised in countries such as China, Japan and India for various diseases and illness. With their diverse medicinal properties, they are always the targets for isolation of active compounds. From those popular medicinal plants, some plant alkaloids have been isolated, for example mesaconitine [from Aconiti tuber (*Aconitum japonicum*)], rhynchophylline, isorhynchophylline, hirsutine and corynantheine (collectively from *Uncaria rhynchophylla*), cassiarin A (from *Cassia siamea*) as well as 10-methoxyaffinisine (from *Tabernaemontana dichotoma*) (Mitamura *et al.*, 2002; reviewed by Shi *et al.*, 2003; Matsumoto *et al.*, 2010; Zaima *et al.*, 2013). These alkaloids have been confirmed to display vasorelaxant activity in various animal models.

In addition, some underutilised medicinal plants or plants that are used as folk medicine also provide a great opportunity for discovering novel active compounds. These plants are sometimes used as medicine in rural areas without any indigenous knowledge. Some examples of the bioactive alkaloids from these plants include the kopsingine from *Kopsia teoi* (Mok *et al.*, 1997), villocarines A to D from *Uncaria villosa* (Matsuo *et al.*, 2011), harmine and harmaline from wild Syrian rue (Moloudizargari *et al.*, 2013), as well as mucronines F to H from *Ziziphus mucronata* (reviewed by Mokgolodi *et al.*, 2011). The isolated kopsingine (Mok *et al.*, 1998) and villocarines A to D from *Uncaria villosa* (Matsuo *et al.*, 2011) showed vasorelaxant activity in rat aorta.

1.6.3 A local fig plant: *Ficus schwarzii*

Ficus schwarzii is the plant of interest in this study. The plant genus *Ficus* consists of around 900 species of woody trees, shrubs and vines that belongs to family Moraceae (Encyclopædia Britannica). *Ficus* plant species are commonly known as fig plants. Majority of *Ficus* species are distributed in Southeast Asia region from Myanmar to Sulawesi (Berg, 2010).

Ficus schwarzii are found near rivers, streams or rainforest at altitudes up to 1900m in the countries of Thailand, Indonesia and Malaysia (Global Biodiversity Information Facility; Berg and Culmsee, 2011). The collection of this *Ficus* species was initially known as *Ficus miquelii* King, which later the name of *Ficus schwarzii* was introduced by Corner in year 1960 (Berg, 2010). The appearance of this *Ficus* species is comprehensively described by Berg and Culmsee (2011) as a 15 meter-tall fig tree with hairy and leafy twigs and lamina that are partly brownish, whereas the globose figs fruit in clusters up to 8cm long at the lower part of the trunk. A better view of *Ficus schwarzii* plants is shown in *Figure A* (Appendix A).

In wild, fig fruit is an important food source for insects in which a mutualism relationship is formed between fig wasps (or ants) and fig plants (Harrison, 2014). The small fig clusters and rapid crop turnover of *Ficus schwarzii* allows a high density of fig wasps populates, hence making it a suitable model in studying this ecology (Harrison, 2014). In local population, this *Ficus* species are also commonly used as food source and medicines. Traditionally, the latex of *Ficus schwarzii* is used externally against ringworm infection (Asian Plant). In a recent study, the alkaloid extracts of this *Ficus* species exhibited an anti-proliferative effect on human cancer cells (Abubakar *et al.*, 2015).

Other well-known *Ficus* species, such as *Ficus carica*, *Ficus thonningii* and *Ficus religiosa* are extensively used in treating the ailments of gastrointestinal, endocrine, reproductive, respiratory and central nervous systems (Singh *et al.*, 2011; Dangarembizi *et al.*, 2012; Badgujar *et al.*, 2014). In addition, the young leaves, leaf buds and figs of some less-known wild edible *Ficus* species, such as *Ficus virens* var. *sublanceolata* and *Ficus auriculata* are commonly consumed in the ethnic diet in

China (Shi *et al.*, 2011; Shi *et al.*, 2014). Antioxidant properties were found in the leaves of these *Ficus* species (Shi *et al.*, 2011). All above-mentioned therapeutic effects are yet to be discovered in *Ficus schwarzii*, which warrants the pharmacological investigation of this *Ficus* species.

1.7 Pharmacological screening for potential drug compound

High-throughput screening assays are commonly employed to screen for a large number of compound samples efficiently. However, before this, a simple two-step screening process is employed (reviewed by Iversen *et al.*, 2006). As mentioned in **Section 1.5** above, first the 'hits' or active compounds are identified in a selected screening assay. Using the same screening assay, a step-two screening on these 'hits' in their concentration-response mode is performed to determine the estimated values of IC_{50} (half maximal inhibitory concentration), EC_{50} (half maximal effective concentration) or K_i (inhibitory constant). These estimated values determine the potency of a drug. The two common screening assays are described in the following **Section 1.7.1** and **1.7.2**.

The selection of screening assays of potential drug compound is based on their pharmacological relevance, reproducibility, costs, and quality. Most compounds are dissolved in organic solvents [for example ethanol and dimethyl sulfoxide (DMSO)]. Optimisation of the chosen assay is necessary to avoid the interruptive effects of the solvents.

1.7.1 Organ bath technique

Organ bath technique is commonly employed for drug testing in tissues of interest. The commonly tested tissues includes vascular, airway, gastrointestinal and urinary tissues. The design of the organ bath apparatus is to replicate the physiological condition in an external environment.

The technique of organ bath was started back in 1904 when Magnus observed a retained rhythmic contraction in an isolated intestinal strip that was suspended in warm oxygenated Ringer-Locke solution (a type of physiological salt solution) (reviewed by Gryglewski and Mackiewicz, 2010). Since then, many pharmacologists have described the design of functional organ bath bioassays using various kinds of tissues and active substances (Finkleman, 1930; Chen *et al.*, 1948; Gaddum, 1953; Vane and Williams, 1974). Gaddum (1953) introduced the term of 'superfusion' describing the technique where physiological fluids flow over the tissues.

The design of the organ bath apparatus has been modified and improved since the early days. In general, the organ bath apparatus consists of a series of double-layered glass baths that are connected to a water bath that is always set at 37^{o}C. A constant flow of water is maintained across the glass baths to constantly keep the bath temperature at 37^{o}C. The physiological salt solution, such as Krebs-Ringer bicarbonate solution is then filled into the bath and gassed at 95% O_2 and 5% CO_2.

The tissues of interest are mounted to a fixed rod and connected to an isometric force transducer (Jespersen *et al.*, 2015). Different mounting devices are used depending on the nature of tissues used, whether they are in the form of vessels or strips (refer to *Figure 1.2*) and the purpose of experiment. For neuropharmacology, tissues will be attached to an electrode rod to stimulate the connective nervous activity of the tissues (refer to *Figure 1.3*). Recordings of their generated isometric forces are translated and interpreted using suitable computer software. The overview of the organ bath setup is illustrated in *Figure 1.2*.

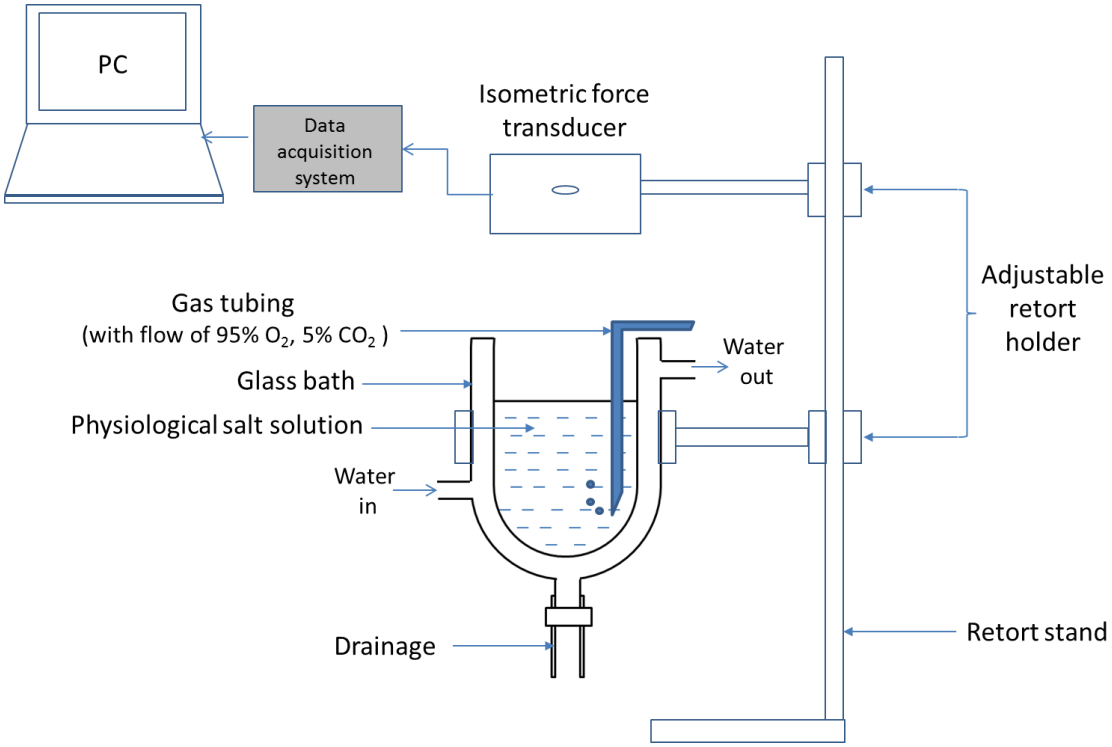


Figure 1.2: Schematic diagram of an organ bath set up that is connected to an isometric force transducer to a data acquisition system and finally to a computer (Adapted from Krajniak and Klohr, 2001; Jespersen *et al.*, 2015).

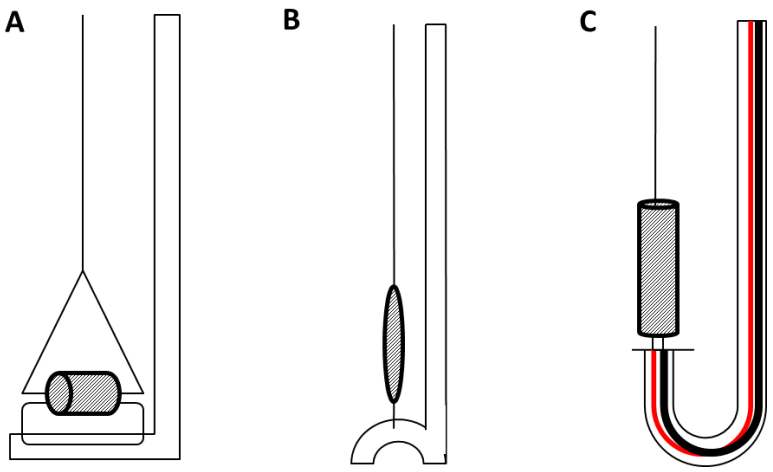


Figure 1.3: Schematic diagram of a variety of mounting setups for tissues in the form of vessel rings and strips as well as those for electrical stimulation. *Figure 1.3A* illustrates the horizontal mounting of vessel rings to a glass rod and a triangle wire that is connected to a force transducer via silk thread. *Figure 1.3B* illustrates the vertical mounting of a tissue strip in which one end of strip is attached to a metal rod while another end is to a force transducer via silk thread. *Figure 1.3C* illustrates the vertical mounting of tissue rings used for electrical stimulation in which one end of tissue is attached to an electrode rod (consisting electrodes within) while the other end to a force transducer via silk thread (Adapted from Cairns *et al.*, 2007; Ribeiro-Filho *et al.*, 2012; Montgomery *et al.*, 2016).

1.7.1.1 Functional studies of smooth muscle tissues using organ bath technique

In general, muscle contraction means the shortening of muscle cells that generates force. Shortening of muscle cells is as a result of cross-bridge between the two contractile proteins, actin and myosin. There are four types of muscle contraction, such as concentric, eccentric, isometric contraction, as well as passive stretch. In an organ bath assembly, isometric contraction of the muscle tissues is of concern (Jespersen *et al.*, 2015). Isometric contraction occurs when muscle is held at a constant length, hence the name of isometric force that is generated during the contraction. The generation of isometric force is highly dependent on the length of muscle.

The general overview of organ bath assembly is previously described in previously mentioned **Section 1.7.1**. During organ bath experiment, an initial tension is applied to the mounted tissues of interest depending on the length of cut tissues. This initial tension determines the optimal resting tension of the tissues (Blanc *et al.*, 1999). An increase of this initial tension indicates the contraction of tissues and *vice versa*. For relaxant studies, the tissues must first be contracted by a contractile stimulus to establish a submaximal tension, followed by a concentration-response curve of relaxant.

Organ bath technique is a great pharmacological method to investigate the contractile/ relaxant ability of smooth muscle tissues, such as vascular, airway, bladder, gastrointestinal and intestinal smooth muscles. Vascular smooth muscle is of the main focus of this study, therefore a brief knowledge of the contractile and relaxation mechanisms is essential for organ bath experiments. The contraction of smooth muscle occurs when intracellular calcium ion binds with calmodulin and this complex phosphorylates myosin light chain kinase to cause the shortening of smooth muscle cells. The level of intracellular calcium ions is dependent on the the pathways of calcium entry across the plasma membrane and calcium release from the internal stores. These two pathways are described in details in **Section 1.8.1.1**.

Depending on the design of experiments, there are some known vasoconstrictors and vasodilators that are used to stimulate the contraction and relaxation of vascular smooth muscles respectively. To stimulate a contractile response in vascular smooth muscles, noradrenaline and phenylephrine (Eckly-Michel *et al.*, 1999), endothelin-1 (ET-1) and U46619 are commonly used. Noradrenaline and phenylephrine are both α_1-adrenergic agonists that linked to G_q protein and stimulates the vascular contraction via inositol triphosphate signal transduction pathways (Eckly-Michel *et al.*, 1999). Similarly, ET-1 which is a hormone that acts on endothelin receptor ET_A also mediates vasoconstriction via inositol triphosphate transduction signal ing pathways (Vignon-Zellweger *et al.*, 2012). U46619 acts on thromboxane A_2 agonist that can either coupled to a G_q or a $G_{12/13}$ proteins that stimulates vascular contraction via inositol triphosphate signalling pathway or Rho kinase-mediated pathway (Shenker *et al.*, 1991; Wilson *et al.*, 2005)

There are many pathways leading to the relaxation of vascular smooth muscles (refer to **Section 1.8.2**). The direct vasodilators such as isoprenaline and dobutamine (both β-adrenergic agonists; Brunton *et al.*, 2006), forskolin (activator of adenylyl cyclase) (Hisajima *et al.*, 1986), sodium nitroprusside (nitric oxide donor) and acetylcholine (cholinergic receptor agonist; Grześk *et al.*, 2014) are used in organ bath studies. Cyclic nucleotides [cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP)] are the common downstream modulators after the activation of the respective target sites by above-mentioned vasodilators (Lincoln *et al.*, 2001; Sassone-Corsi, 2012; Hottinger *et al.*, 2014).

1.7.2 Cell-based assays

Cell-based assays are the basis of high-throughput screening for active biological substances. There exists a variety of cell-based assays depending on the cell lines and detection methods of interest. The other considerations for selecting a suitable cell-based assay are the accuracy, reproducibility and cost-effectiveness.

Many different types of cell lines have been used for cell-based assays. They include immortalised cell lines, primary cells, human cancer cell lines, cancer stem cells, mesenchymal stem cells, embryonic stem cells and induced pluripotent stem cells (reviewed by Zang *et al.*, 2012). Among all, immortalised cell lines are the most widely employed for screening assays because they are cheap and rapid to grow (reviewed by Zang *et al.*, 2012). The choice of cell lines depends on the pharmacological relevance, for example, myocardial cell lines are used for screening of cardiac-related drugs, smooth/skeletal muscle cells for corresponded muscle-targeted drugs and neuronal cells (such as dorsal root ganglion cells) are used for neurotransmitter drugs (reviewed by Allen *et al.*, 2005).

There are four major detection methods in cell-based assays which employ reporter genes, fluorescence/bioluminescent resonance energy transfer (F/BRET), calcium mobilisation and label-free detection methods (reviewed by Zang *et al.*, 2012). These detection methods allow the qualification and quantification of the activity of

biological active substances based on the target gene/protein expression. Fluorescent and bioluminescent proteins are commonly employed in the detection methods due to their rapid detection of stimulated activity.

Numerous cell-based high-throughput assays have been described in screening plant products and developing new drugs. Galietta and colleague (2001) demonstrated a cell-based screening of agonists for cystic fibrosis whereas Swevers and colleagues (2004) detected ecdysteroid agonists and antagonists in plant extracts using a reporter cell line. Some commercial drugs were successfully developed using cell-based screening assays. For example, Eltrombopag (Promacta/ Revolade, GlaxoSmithKline) for treating thrombocytopenia, was developed using a cell-based luciferase reporter screening system; Bortezomib (Velcade®) for treating myeloma as well as BMS-790052, a developed clinical candidate for inhibiting hepatitis C virus replication using a cell-based system by O'Boyle and colleagues (2005) (reviewed by Zang *et al.*, 2012).

1.8 Blood vessels

The ability of blood vessels to contract and relax regulates the blood flow and circulation in the body, in addition to maintain the blood pressure. Blood vessels are made up of three main layers, including tunica intima, tunica media and tunica adventitia (Li, 2004). *Tunica intima* is located at the innermost with presence of a single layer of vascular endothelium, followed by *tunica media* as the middle layer that consists of elastic fibers and smooth muscle, and the outermost *tunica adventitia* consists entirely with connective tissues (Li, 2004).

The vascular activity takes place as a result of a complex interplay between endothelium and smooth muscle, as described in **Section 1.8.1** and **1.8.2** below.

1.8.1 Vascular contraction

Vascular contraction occurs in the smooth muscle during an increase of intracellular calcium. The calcium serves as an activator to bind with a calcium-modulated protein, calmodulin (CaM). This complex will then phosphorylate the light chain of myosin to allow the cross-bridge cycling between myosin and actin, and eventually the shortening of smooth muscle cells (reviewed by Murphy and Rembold, 2005). Shortening of vascular smooth muscle cells is collectively named as the contraction in vascular tissues.

The contraction of vascular smooth muscle is highly dependent on the level of intracellular calcium. This intracellular calcium level is mediated by two pathways, which are calcium entry across the cell plasma membrane and calcium release from the internal stores.

1.8.1.1 Calcium entry and calcium release

The entry of extracellular calcium is usually via calcium-specific channels across the cell membrane. The opening of these calcium channels is stimulated by either the change of membrane potential (i.e. depolarisation) or in the presence of external calcium stimuli. There are a variety of calcium channels that have been characterised and categorised into voltage-operated calcium channels (VOCCs), receptor-operated calcium channels (ROCCs), second messenger-operated calcium channels (SMOCs) and store-operated calcium channels (SOCCs) (Berridge, 2007).

The stimulation of these calcium channels is differentiated by their specific stimuli and involved signalling molecules. For VOCCs, the stimulation is mainly mediated by the membrane potential of the cell. There are five identified VOCCs found in different cell types, including L-, N-, T-, P- and Q-type VOCCs (reviewed by Dolphin, 2003). Among all, L-type VOCC is the best studied subtype in many cell types, such as skeletal, cardiac and smooth muscle cells (reviewed by Lipscombe *et al.*, 2004).

The other calcium channels, ROCCs, SMOCs and SOCCs are closely linked to one another (reviewed by McFadzean and Gibson, 2002). ROCCs is activated by specific external stimuli that trigger the activation of transmembrane receptors in producing second messengers that leads to the calcium mobilisation in cells. Most transmembrane receptors in the cells belong to G protein-coupled receptor (GPCR) superfamily. Some examples of the major GPCR receptors in regulating the vascular contraction include alpha-adrenergic, thromboxane A_2 and serotonin receptors. These receptors stimulate the hydrolysis of phospholipase C in producing two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) (Mckune and Watts, 2001). These two second messengers play an essential role in directing the mobilisation of calcium through activation of IP_3 receptor and protein kinase C (PKC). This is in part of the SMOC signalling pathway, in addition to the other involved second messengers, such as ryanodines, nicotinic acid dinucleotide phosphate (NAADP), cyclic dinucleotide phosphate (cDAP) and calcium ion itself (Berridge, 2007). These second messenger can also trigger the opening of SOCCs and calcium release from the internal stores (sarcoplasmic/ endoplasmic reticulum).

Numerous studies characterised that the SOCC properties of transient receptor potential (TRP)-mediated channels (reviewed by McFadzean and Gibson, 2002). In vascular tissues, TRP-mediated vascular contraction is also proven in many studies (Jung *et al.*, 2002; Welsh *et al.*, 2002; Albert and Large, 2003). In addition to SOCC, there are other components which also mediate the release of calcium from the internal stores. They are sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, plasma membrane Ca^{2+} (PMCA) pumps and sodium-calcium exchangers (reviewed by Periasamy and Kalyanasundaram, 2007; Brini and Carafoli, 2011).

1.8.2 Vascular relaxation

Relaxation of vascular tissues occurs when either the contractile stimulus is removed or the contraction mechanisms are inhibited. During this, reduced of intracellular calcium and increase of myosin light chain phosphatase activities are

observed. These events are evoked by some substances that are either endothelium-dependent or –independent.

A downstream signalling pathway by cyclic nucleotides, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) is often elicited to cause vascular relaxation. cAMP is claimed to be in conjunction with the presence of cGMP (Lamping 2001). Cyclic GMP is related to endothelial NO-mediated pathways, while cAMP is mediated by agonist-activated pathways. Cyclic GMP and cAMP are produced by the activation of guanylyl cyclase and adenylyl cyclase respectively (Lincoln *et al.*, 2001; Sassone-Corsi, 2012). These productions will then lead to the respective stimulations of protein kinase G (PKG) and protein kinase A (PKA) that cause the relaxation of vascular tissues (Lincoln *et al.*, 2001; Sassone-Corsi, 2012). Phosphodiesterases (PDEs) play a regulatory role in controlling the amount of intracellular cyclic nucleotides by hydrolysing them into their inactive forms (5'GMP or 5'AMP) (Omori and Kotera, 2007).

1.8.2.1 Endothelium-derived mechanisms

The endothelium plays an important role in regulating the relaxation of vascular tissues. The presence of endothelium is essential for many endothelium-derived relaxing factors (EDRFs) to function, including nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factors (EDHFs). These EDRFs were first introduced and described by Furchgott and Zawadzki (1980).

Nitric oxide is a key signalling molecule in many biological systems. The biosynthesis of NO from L-arginine is catalysed by three different NO synthase (NOS) isoforms such as neuronal NOS (type I, nNOS/ncNOS), inducible NOS (type II, iNOS/mNOS) and endothelial NOS (type III, eNOS/ecNOS) (Rang *et al.*, 2000). These isoforms are expressed at different locations: nNOS is mostly found in central and peripheral neuronal cells as well as some endothelial cells, iNOS is expressed in macrophages, endothelial cells, vascular smooth muscle cells and cardiac myocytes in response to immune activation, and eNOS is expressed mainly in endothelial cells (Viaro *et al.*,

2000). The nNOS and eNOS are constitutively expressed and generate smaller amount of NO compared to that of iNOS which has much higher expression (Rang *et al.*, 2000).

The constitutive NOS (nNOS and eNOS) is regulated by the presence of intracellular free calcium and calcium-calmodulin complexes, whereas iNOS do not require calcium to be activated (Viaro *et al.*, 2000). The synthesized NO stimulates relaxation by activating guanylate cyclase to increase concentration of cyclic guanosine monophosphate (cGMP) converted from cyclic guanosine triphosphate (cGTP). Cyclic GMP later activates cGMP-dependent protein kinases (PKGs) to stimulate a series of cellular responses, including vascular relaxation.

Another endothelium-dependent vasodilator, prostacyclin (PGI_2) is identified (Shimokawa *et al.*, 1988) with anti-aggregation properties (Radomski *et al.*, 1987). Prostacyclin also induces the differentiation of vascular smooth muscle cells (VSMCs) that promotes its cardiovascular protective effect (Fetalvero *et al.*, 2006). Prostacyclin is a 20-carbon derivative of arachidonic acid (Moncada and Vane, 1978). Arachidonic acid is either released from membrane phospholipids by phospholipase A_2 or from endothelial phospholipids by phospholipase D. This molecule is later converted to prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by cyclooxygenase (COX), finally to PGI_2 by PGI synthase. The PGI_2 exhibits local effect and is rapidly converted to an inactive metabolite, prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$).

Prostanoids are short-lived and fragile which challenges the investigations of their biological events. Therefore, stable PGI_2 analogues such as beraprost, iloprost and cicaprost, are developed and used for the investigations. The PGI_2 is primarily mediated by prostacyclin receptor IP (International Union of Pharmacology Receptor classification). IP receptor is a G-protein coupled receptor that generates cAMP upon activation. Some studies demonstrated that PGI_2 also acts by peroxisome proliferator activated receptor (PPARδ)-mediated pathway that activates adenylyl cyclase to increase cAMP level (Gupta *et al.*, 2000; Lim and Dey, 2002).

In addition, the endothelium-derived hyperpolarising factors (EDHFs) cause vascular relaxation by activating potassium (K^+) channels and resulting in membrane hyperpolarisation (Busse *et al.*, 2002; Bryan *et al.*, 2005; Ozkor and Quyyumi, 2011). This occurs prior to the increase in the agonist-induced intracellular free calcium (Ozkor and Quyyumi, 2011). Calcium-activated potassium channels (K_{Ca}) was suggested as the activated-channels in porcine (Fisslthaler *et al.*, 1999) and human vascular beds (Miura and Gutterman, 1998; Coats *et al.*, 2001; Archer *et al.*, 2003). Later, these were confirmed as EDHFs found to activate a simultaneous activity of intermediate- and small-conductance K_{Ca} channels in rat mesenteric arteries (Hinton and Langton, 2003), and large-conductance K_{Ca} channels in human internal mammary artery (Archer *et al.*, 2003).

Despite the roles of cytochrome P450 (CYP450) metabolites, hydrogen peroxide, gap junctions and potassium ion in eliciting EDHF responses, however the nature of EDHFs is yet unclear due to the controversial findings of each substance. Taken together, there is one or more than one EDHF is involved in the NO- and prostanoid-independent, endothelium-dependent hyperpolarisation.

1.8.2.2 Potassium channel activation

Potassium (K^+) channels are important regulator for vascular tone. The activation of K^+ channels is determined by the change in cell membrane potential. When a cell is depolarised (membrane potential is more positive than resting membrane potential, E_{res}), K^+ will be activated to repolarise the cell to return to its E_{res} level. Depolarisation of cells can be either caused by elevated intracellular calcium level or elevated extracellular potassium level, resulting in vascular constriction. However, a mild increase of extracellular K^+ will hyperpolarise the cell and cause vasodilation (Knot *et al.*, 1996). These vascular events will therefore stimulate the opening of different K^+ channels, with three to-date identified potassium channels, such as voltage-gated K^+ channels (K_v), calcium-activated K^+ channels (K_{Ca}) and inwardly rectifying K^+ channels (K_{IR}).

Potassium channels are classified according to their physiological functions and pharmacological properties. All K^+ channels share a conserved sequence, named TTVGYGD that act as an 'ion selectivity filter' (Heginbotham *et al.*, 1994). Voltage-gated and calcium-activated K^+ channels are both activated by membrane depolarisation while the latter is also affected by the change in intracellular calcium concentration (Ko *et al.*, 2008). In contrast, inwardly rectifying K^+ channels is activated by the K^+-induced membrane hyperpolarisation and release of K^+ from adjacent cells. Interestingly, this channel type allows the potassium flow into rather than out of the cell and maintains E_{res}.

In addition, activation of K^+ channels is also affected by the phosphorylation of vasodilation-related kinases, which are protein kinase A (PKA) and protein kinase G (PKG). Phosphorylation of PKA and PKG is activated by cAMP and cGMP respectively. Studies revealed that the kinase phosphorylation stimulates K^+ channels in cerebral (Robertson *et al.*, 1993), coronary (White *et al.*, 2000) and mesenteric resistance (Yang *et al.*, 2008) arteries, that eventually leads to vasodilation. Quinn and colleagues (2004) stated that multiple phosphorylation sites at K^+ channels are required for the modulation by PKA kinase to enhance the channel activity.

The presence and subtypes of K^+ channels can be determined pharmacologically using known commercial channel inhibitory drugs. For K_v channels, 4-aminopyridine (4-AP) is the most selective inhibitors (Nelson and Quayle, 1995). Other drugs such as phencyclidine, tedisamil, quinidine, tetraethylammonium (TEA) and glibenclamide also possess K_v-inhibiting properties (Nelson and Quayle, 1995). TEA and glibenclamide, however are not selectively inhibit K_v channels as the TEA inhibit K_{Ca} channels at high concentration and glibenclamide also inhibits K_{ATP} channels. For K_{Ca} channels, different inhibitory drugs are used: B_{KCa} channels are inhibited by TEA, iberiotoxin and charybdotoxin; SK_{Ca} channels are sensitive to apamin, scyllatoxin, biccuculline, dequalinium and UCL1684 (Strøbæk *et al.*, 2000) and; IK_{Ca} channels are also inhibited by charybdotoxin as well as clotrimazole and TRAM-34 as the more specific inhibitors (Ledoux *et al.*, 2006). K_{IR} channels are generally inhibited by barium ion, whereas K_{ATP} channels which contain K_{IR} subunits are selectively inhibited by chlorpropamide, tolbutamide and glibenclamide.

1.9 Translational studies from animal into human

Animal research has existed throughout history from the time of ancient Greece. During that time, animals were dissected for anatomical studies however; this did not raise any issues on animal ethics. The moral issues of animal ethics only came into light in the seventeenth century. Today, all animal experimentations are required to follow the framework on 'The Principle of Three R's-*Replacement, Reduction and Refinement*' that was introduced by Russell and Burch (1959).

With the restriction of direct human experimentation, animal research has played an important role in translating the biomedical knowledge. Animal experimentations can be performed either *in vivo* or *ex vivo*. *In vivo* experiments refer to those that are done on living animals, whereas *ex vivo* experiments refer to those that are done on the isolated tissues from a euthanised animal. These experiments provide a lot of useful information in terms of human diseases and their underlying mechanisms. The translational of animal research into clinical human trials forms a big part of the drug development phase. There is a controversial debate on the benefits of animal research to humans (Pound *et al.*, 2004; Martić-Kehl *et al.*, 2012). However, the lessons from animal research have undoubtedly provided insight into developing therapies to manage various human diseases (Hackam and Redelmeier, 2006).

The high figure of animal sacrifice every year has always urged an alternative option in translational studies (Taylor *et al.*, 2008). Alternative to living animals, mammalian cells is another reliable candidate in translation studies. The emergence of cell-based assays has primarily fulfilled the principles of replacement, reduction and refinement in animal research.

1.9.1 Translational studies in hypertension

Hypertension is a complex health condition with many possible causes. The genetic and phenotypic changes in hypertension have been evaluated in different animal

models. This not only enhances the understanding of the underlying mechanisms, and also facilitates the discovery and development of new antihypertensive drugs.

A variety of animal species have been used as models for experimental hypertension, including rat, mice, monkeys and pigs (reviewed by Leong *et al.*, 2015). Among all, rat is the preferential animal species because of its small size and its physiology is easier to monitor (Iannaccone and Jacob, 2009). In addition, pig is one of the favourable non-rodent animal models due to its close physiological and organ structural homogeneity to human. Because of these, pig serves as an excellent model in cardiovascular studies (reviewed by Bassols *et al.*, 2014). To control the genetic diversity and environmental factors, laboratory pigs (or known as miniature pigs) and genetically-modified pigs are employed as human pathology models (Kobayashi *et al.*, 2012; Bassols *et al.*, 2014).

Targeting different types of hypertension, a few animal-based experimental models have been developed. Spontaneously hypertensive rats (SHRs), Dahl salt-sensitive rats and transgenic rats are commonly used to investigate genetic hypertension (Pinto *et al.*, 1998). Among all, SHRs are the most widely used in screening for antihypertensive agents. Other types of experimental hypertension, such as endocrine hypertension, renal hypertension, environmental hypertension (i.e. stress-induced models) and pharmacological hypertension (i.e. nitric-oxide deficient and endothelial dysfunction models) models are also developed in any animal species (reviewed by Leong *et al.*, 2015).

1.10 Aims of the thesis

The problem of resistant hypertension has justified the discovery and development of new antihypertensive drugs for widening the availability of current drug selection. The richness of biological compounds in plants has provided a great source for new drug discovery. The main aim of this study was to optimise a functional bioassay methodology to characterise the pharmacological effects of a novel plant alkaloid, schwarzinicine A, from local fig plant species, *Ficus schwarzii*, on different bioassays

using isolated rat aorta, porcine coronary arteries and rat dorsal root ganglion cells. There is little knownledge on the biological effect of schwarzinicine A. Therefore, the choice of these biological samples allows a diverse investigation of this compound. In addition, shifting of investigation from rat aorta to porcine coronary arteries and rat dorsal root ganglion is also an act of 'replacing' as described in the principle of 'Three Rs' (the details of this principle will be stated in the next chapter) to reduce the use of laboratory animals (rats in this case). To achieve this, several steps were carried out as listed below:

Step I: Optimisation of an organ bath methodology using four different rat tissues (aorta, trachea, bronchus and bladder) in fresh and stored condition **(Chapter 2)**,

Step II: Preliminary testing of schwarzinicine A on four different tissues (aorta, trachea, bronchus and bladder) **(Chapter 3)**;

Step III: Elucidation of schwarzinicine A-induced mechanisms on (i) isolated rat aorta **(Chapter 4)**, (ii) isolated porcine coronary arteries **(Chapter 5)** and (iii) rat dorsal root ganglion primary cell line **(Chapter 6)**.

All experiments involving animal tissues were performed using organ bath technique **(Chapter 1 to 5)**. Calcium imaging study was performed on dorsal root ganglion cell line to elucidate the mechanism of schwarzinicine A **(Chapter 6)**. All experiments are discussed in further details in their respective chapters.

Chapter 2: Optimisation of organ bath methodology using fresh and stored rat tissues

2.1 Introduction

2.1.1 Animal research and ethics

Animal experiments have existed for centuries, due to the banning of human dissection. The debate on animal ethics became increasingly aggressive in the nineteenth- and twentieth-century. This urged a proper framework for animal research, leading to the inception of the 'Three Rs' principle by Russell and Burch in year 1959, which depicted a decline of expected animals used during 1970s (Franco, 2013). The principle of 'Three Rs' consists of 'Replacement', 'Reduction' and 'Refinement' in which they are defined as replacing animals with non-sentient materials, reducing the number of animals used to obtain precise information, and refining the experimental method to minimise pain and suffering on the animals respectively (Russell and Burch, 1959).

This principle of 'Three Rs' was later either implemented into regulations or adapted by many regulatory and oversight bodies. These regulations and governing bodies regulate the humane use and care of animals for research studies. In United States, animal research are governed by the laws in Animal Welfare Act (AWS) and Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals, as well as reviewed by Institutional Animal Care and Use Committee (IACUC) and Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) (National Research Council (US) Committee, 2004). For countries from European Union and United Kingdom, the main legislations that regulates animal use for experiments are Council Directive 86/609/EEC (issued by European Communities in 1986) and Animals (Scientific

Procedures) Act 1986 (or known as ASPA 1986) respectively (National Research Council (US) Committee, 2004). In addition, other regulatory bodies such as International Council for Laboratory Animal Science (ICLAS), Council for International Organisation of Medical Sciences (CIOMS), International Union of Biological Sciences (IUBS) and International Committee on Laboratory Animals (ICLAS) also provide guidance for many research countries in ensuring the humane use of animals for experiments (reviewed by Saraf and Kumaraswamy, 2013).

Despite of the existence of animal ethics regulations and governing bodies, there are yet more than one million animals killed each year for research purposes, including an additional of 21.1% killed mainly for their organs and tissues (Taylor *et al.*, 2008). Breeding and killing of animals as surplus is also commonly encountered in laboratory (Taylor *et al.*, 2008). The lacking of uniformity, transparency and accountability in the existing regulations is claimed to be the main cause of this problem (Latham, 2012). However, some are also questioning whether the principle of 'Three Rs' is fully implemented by researchers, review boards and funders (reviewed by Ferdowsian and Beck, 2011).

2.1.2 Cold storage of tissues

Storage of tissues at cold temperature is a way of preserving tissues for limited period of time. Generally, cold storage of tissues is referred to storage at 4 to 10^{o}C in appropriate storage solution. The period of storage is the key limiting factor of the stored tissue viability (van Buskirk *et al.*, 2004).

In organ bath studies, cold storage of tissues is commonly applied for the purpose of shipping or prolonging the lifespan of tissues. This storage method is derived from method used in transporting donated organs. The technique of cold storage has been used in a variety of tissues from any animal model, such as aorta (Chow and Zhang, 2011; Ghosn *et al.*, 2011), bronchus (Bäck *et al.*, 2006) and bladder (Schneider *et al.*, 2011). These studies have demonstrated the unchanged structural integrity of aortic tissues (Ghosn *et al.*, 2011) and their functionalities after cold

storage. The mechanical and physiological functions of stored tissues are most preserved within 24 hours of storage which as followed by a decline in function with time (Bäck *et al.*, 2006; Chow and Zhang, 2011).

2.1.3 Assessment of smooth muscle activity in animal tissues

Organ bath technique is effective in evaluating smooth muscle activity of animal and human tissues. Smooth muscles are found in blood vessels, respiratory and gastrointestinal tracts and urinary bladder. The activity of these smooth muscles can be accessed by exposing the tissues to a variety of contractile and relaxation agents. In some tissues, for example blood vessels, endothelium-derived factors/ mediators are known to regulate the activity of their smooth muscles. Therefore, to study the primary role of smooth muscle activity, the endothelium layer will be removed either mechanically or chemically.

The structural arrangement of most (smooth) muscle-containing tissues is nearly similar (*Figure 2.1*). There are three to four layers of specialised tissues that surround the tissue lumen. These layers are termed differently in different tissues. For example, in airway and bladder tissues, from the lumen outwards, there are layers of mucosa, submucosa and adventitia (Krstic, 1991). In the same order, these layers are named *tunica interna*, *tunica media* and *tunica externa* in vascular tissues (Li, 2004). Smooth muscle is sandwiched between the layer of mucosa and adventitia (Li, 2004). It can also be referred as either *tunica media* or submucosa layer in vascular and airway tissues respectively.

In vascular tissues, the endothelium layer resides at *tunica interna*, in which surrounded by *tunica media* that consists of smooth muscle layer (Li, 2004). Similarly, the airway epithelium and bladder urothelium resides at the mucosa layer and are surrounded by their respective smooth muscle layers (Krstic, 1991). This structural arrangement has provided an insight in understanding the interaction of endothelium (or epithelium or urothelium) with smooth muscle activity.

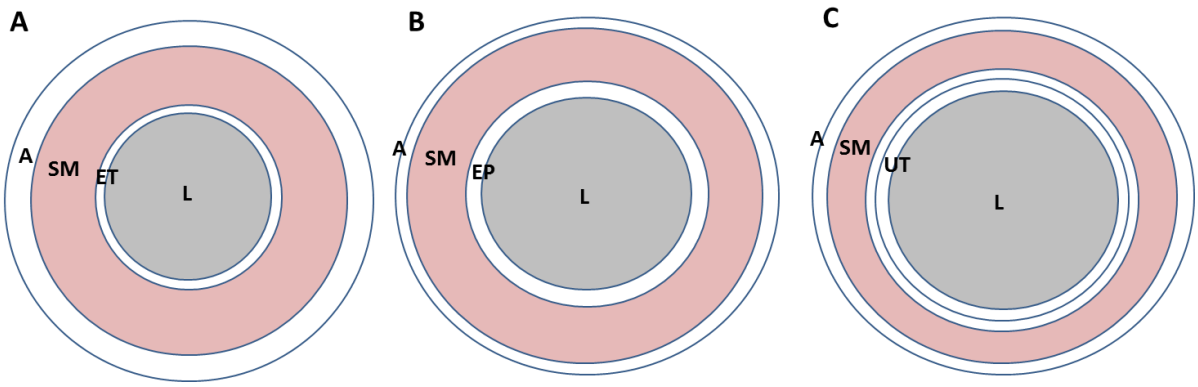


Figure 2.1: Schematic diagram illustrates the relative structures of (A) artery, (B) airway and (C) bladder tissues. Area in grey indicates the luminal space of each tissue, whereas area in pink indicates the smooth muscle layer of each tissue. L, lumen; ET, endothelium; EP, epithelium; UT, urothelium; SM, smooth muscle; A, adventitia. (Adapted from Krstic, 1991; Li, 2004)

2.1.4 Significances of study

The purpose of this chapter was to optimise a functional bioassay methodology to test the effects of cold storage (overnight) on four different smooth muscle tissues (aorta, trachea, bronchus and bladder) isolated from Sprague-Dawley rats. The information derived from this chapter would inform future animal research in fulfilling 'The principle of Three R's'. The specific objectives of this chapter were described, as below:

(i) to investigate the contractile responses of all tested tissues before and after storage using phenylephrine (α_1-adrenergic agonist, for aortic tissues) and carbachol (muscarinic receptor agonist, for trachea, bronchus and bladder);

(ii) to investigate the influence of cold storage on the relaxation of aortic rings. Aortic rings were pre-contracted with phenylephrine, and relaxed with three relaxant agents: isoprenaline (non-selective β-adrenergic agonist), forskolin (adenylyl cyclase activator) and carbachol (muscarinic receptor agonist) in a cumulative concentration manner.

(iii) to study the influence of cold storage on the function of aortic endothelium. Aortic tissues were denuded of endothelium mechanically and treated with a nitric oxide inhibitor, N^G-Nitro-L-Arginine methyl ester (L-NAME).

2.2 Materials and Methods

2.2.1 Drugs and Krebs-Ringer bicarbonate solution

(R)-(−)-phenylephrine hydrochloride (Tocris Bioscience, USA), isoproterenol hydrochloride (isoprenaline) (TCI, Japan), forskolin (Merck, USA), carbamylcholine chloride (carbachol) (Nacalai Tesque, Japan) and N^G-Nitro-L-Arginine methyl ester (L-NAME) hydrochloride (ChemSln, USA) were purchased. All drugs, except forskolin, were dissolved in distilled water to prepare a 10mM stock concentration. Forskolin was first dissolved in 100% dimethyl sulfoxide (DMSO) before being further diluted in distilled water to make final concentration of <0.2 % (v/v) (in bath). The Krebs-Ringer bicarbonate solution was freshly prepared daily following the composition (in mM): NaCl 120, KCl 5.4, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2$ 1.26 and gassed with 95 % O2, 5% CO_2.

2.2.2 Preparation of tissues

Male Sprague–Dawley rats (240–480 g) were used in all experiments. They were purchased from the animal house in University Putra Malaysia (UPM), Malaysia. Ethics approval was obtained from the University of Nottingham's Animal Welfare Ethics Committee (UNMC#2kn, refer to Appendix C). Rats were anesthetized with diethyl ether and euthanized by cervical dislocation. The entire respiratory tree, aorta and bladder were immediately excised and transferred into fresh Krebs-Ringer bicarbonate solution. All tissues were cleaned of their connective tissues. The cleaned tissues were cut into rings or strips of a desired length. Trachea and bronchus were cut into 2-mm rings; aorta into 4-mm rings and bladder into 5×2 mm (length × width) strips.

2.2.3 Organ bath assembly

In the tissue baths, the airway and aorta rings were suspended on metal wire triangles. Bladder strips were tied longitudinally to a metal hook. These preparations were then connected to an isometric force transducer (MLTF050/ST, ADInstruments, USA) via a long surgical suture thread. The measurement of tension was recorded using a PowerLab data acquisition system (LabChart v7.3.4) that was connected to a computer. All tissues were maintained in 20ml Krebs-Ringer bicarbonate solution at 37°C and continuously gassed with 95 % O_2, 5 % CO_2. All tissues were allowed to equilibrate for at least 30 min before the application of tension [1 g wt (9.81 mN) for trachea, bronchus and bladder and 2 g wt (19.62 mN) for aortic rings]. Another 30 min of equilibration was given after all tissues were loaded with tension. All tissues were then stimulated twice with 60mM KCl to check their reactivity and viability (van der Graaf *et al.*, 2013).

2.2.4 Organ bath experiments

Data were collected from tissues that were (i) used on the day of surgical removal (denoted as fresh) and (ii) stored in 4°C Krebs solution for 24 hours (denoted as stored). The response rates of all tested tissues from both conditions were documented. The *n* number is referred as the number of animals used in an experiment.

2.2.4.1 Aortic rings

To investigate the changes of contractile responses in fresh and stored aortic rings, a cumulative concentration-response curve of phenylephrine (1nM to 30µM) was performed. For endothelium denudation, the endothelium of aortic rings was removed by gently rubbing the intimal surface of the rings using the blunt tip of stainless forceps. This was followed by the testing of endothelium integrity by using 1µM carbachol after the rings were pre-constricted with 0.1µM phenylephrine (to

establish a submaximal contraction). Successful denudation was confirmed if the tissues produced less than 30% relaxation (Kaya *et al.*, 2003). Some preparations were treated with L-NAME for 30 minutes to inhibit the activity of nitric oxide synthase (NOS). The vasorelaxant effects of aortic tissues were studied using isoprenaline (0.1nM to 3μM), forskolin (0.1nM to 30μM) and carbachol (0.1nM to 30μM) after pre-contraction with 0.1μM phenylephrine.

2.2.4.2 Airway smooth muscles (trachea and bronchus) and bladder

The contractile responses of airway smooth muscles (trachea and bronchus) and bladder from fresh and stored conditions were tested. The muscarinic receptor agonist, carbachol was added cumulatively to the airway smooth muscle (ASM) rings and bladder strips with different concentration ranges: 0.1nM to 30 μM and 0.1nM to 3μM respectively.

2.2.5 Data analysis

The response rates of tissues from fresh and stored conditions were calculated as (number of responsive tissue/ total number of tissues used) × 100 %. For aortic tissue and bladder strips, their respective contraction to phenylephrine and carbachol was expressed as a percentage of the KCl (60mM)-induced contraction. For airway smooth muscles (trachea and bronchus), their contraction to carbachol was expressed in mN force (=tension weight x 9.81) due to their low contractile magnitude to 60mM KCl. The relaxation responses of aortic tissues to both carbachol and forskolin were expressed as a percentage relaxation to the phenylephrine-induced contraction. All data were expressed as mean ± standard error of mean (SEM) of *n* number of animals. All data were analysed using PRISM Version 6 (Graph Pad Software). Maximum response (E_{max}) and pEC_{50} values were obtained in which pEC_{50} is the negative logarithm of EC_{50}. EC_{50} is the concentration of drug that produces 50% of its maximum response.

The differences between mean values of E_{max} and pEC_{50} were analysed using either unpaired t tests (two-tailed) or one-way ANOVA analysis as appropriate. Unpaired

t-test was used to compare the statistical differences from two experimental groups: one from a control condition while another from a treatment condition (PRISM Version 6; Graph Pad Software). For comparing the statistical differences from more than two experimental groups (between control and different treatment conditions), one-way ANOVA followed by Dunnett's multiple comparisons test was applied (PRISM Version 6; Graph Pad Software). A probability of less than 0.05 ($P<0.05$) was considered to be statistically significant.

2.3 Results

2.3.1 Tissue response rate

The response rate of all tested tissues was determined by their contractile response to respective contractile agonists. Any tissue with contractile magnitude more than 3.9mN was considered as responsive. A 100% response rate was achieved for all tested tissues (aorta n=10, trachea n=5 and bladder n=4) after storage, with the exception of bronchial rings. Of the 13 bronchial rings investigated, only 10 (76.9%) and 5 rings (38.5%) were responsive before and after storage respectively. A 60% reduction in response was observed for bronchial rings after storage.

2.3.2 Phenylephrine-induced contraction in aortic tissues after storage

Phenylephrine produced a concentration-dependent contraction in aortic tissues. A small but insignificant change of phenylephrine-induced contraction was observed in stored aortic tissues ($P>0.05$) (see *Figure 2.2* and *Table 2.1*).

From the concentration-response curves, 0.1µM phenylephrine was selected to induce tone in the aortic rings to about 70% for the subsequent relaxation experiments. At this concentration, the contraction to phenylephrine was unaltered in endothelium-intact (fresh: 8.9 ± 0.75 mN vs stored: 7.1 ± 0.53 mN, both at n=10) and endothelium-denuded aortic tissues before and after storage (fresh, denuded: 7.9 ± 1.37 mN vs stored, denuded: 6.1 ± 0.55 mN, both at n=7). In addition to these,

the contractions of control and L-NAME treated aortic tissues (both at fresh conditions) to 0.1µM phenylephrine were also of similar magnitude.

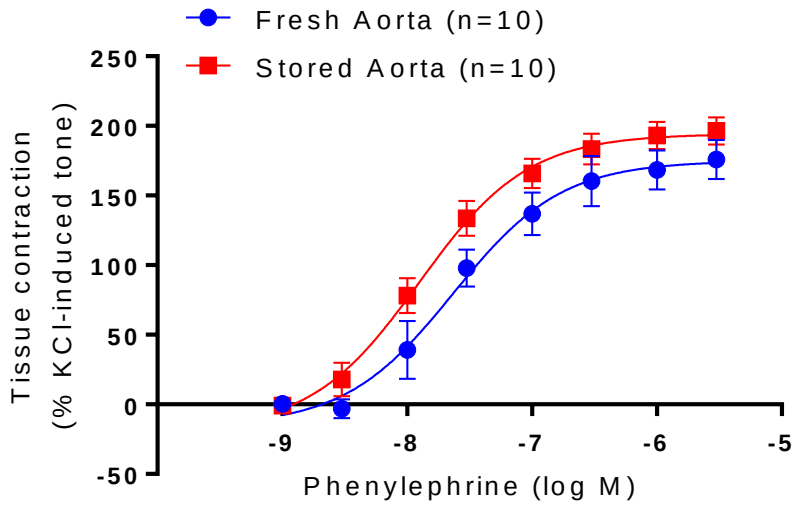


Figure 2.2: Effects of phenylephrine on fresh and stored aortic tissues (n=10). The contractions are expressed as a percentage of 60mM KCl-induced contraction. The data represents the mean values ± SEM of *n* number of animals. The unpaired t test showed no significant difference (pEC_{50} and E_{Max}) in the contraction of aortic tissues to phenylephrine in fresh and stored conditions.

2.3.3 Isoprenaline-, forskolin- and carbachol-induced relaxation in aortic tissues after storage

The relaxations of aortic tissues to isoprenaline, forskolin and carbachol were expressed as a percentage of phenylephrine-induced contraction (at 0.1µM). Fresh endothelium-intact aortic tissues were used as control tissues for all three experiments. For isoprenaline-induced relaxation, the response was unaltered between the fresh and stored conditions (see *Figure 2.3* and *Table 2.1*). Unaltered relaxant responses to isoprenaline were also observed in fresh tissues that were treated with L-NAME, and following endothelium removal (P>0.05) (see *Figure 2.3* and *Table 2.1*).

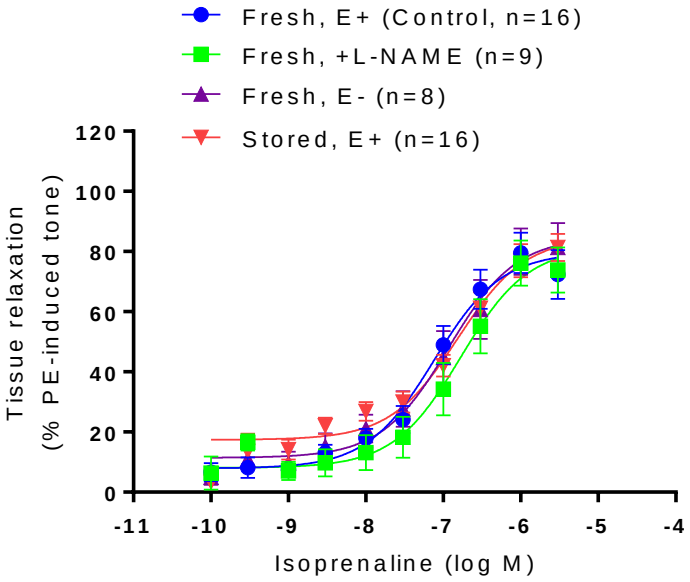


Figure 2.3: Effects of isoprenaline in fresh and stored aortic tissues (both at n=16), as well as in L NAME-treated (n=9) and endothelium-denuded (E-) fresh tissues (n=8). Fresh endothelium-intact aortic tissues (E+) served as the control tissues. All aortic tissues were pre-constricted with 0.1µM phenylephrine. The data represents the mean values ± SEM of *n* number of animals. The one-way ANOVA followed by Dunnett's multiple comparisons test showed no significant difference in the aortic relaxation to isoprenaline in all stated conditions, as compared to the control tissues (fresh, E+).

A small but significant rightward shift ($P<0.05$, refer to *Figure 2.4* and *Table 2.1*) was observed for the forskolin-induced relaxation curve in fresh endothelium-denuded (E-) tissues, as compared to the control tissues (control E+ 7.37 ± 0.10, n=5; fresh E- 7.13± 0.08, n=5). Unchanged maximal relaxations to forskolin were observed in these fresh endothelium-denuded aortic tissues (see *Figure 2.4* and *Table 2.1*). The forskolin-induced relaxation was also unaltered in stored aortic tissues (maximum relaxation 117.4 ± 4.0%; pEC_{50} 7.09± 0.11, n=5) (see *Figure 2.4* and *Table 2.1*).

In response to carbachol, a significant rightward shift and a reduction of almost 2-fold in the maximum relaxation observed after storage in endothelium-intact segments (fresh 111.6 ± 3.9%, n=9; stored 67.6 ± 3.6%, n=9) (see *Figure 2.5* and *Table 2.1*).

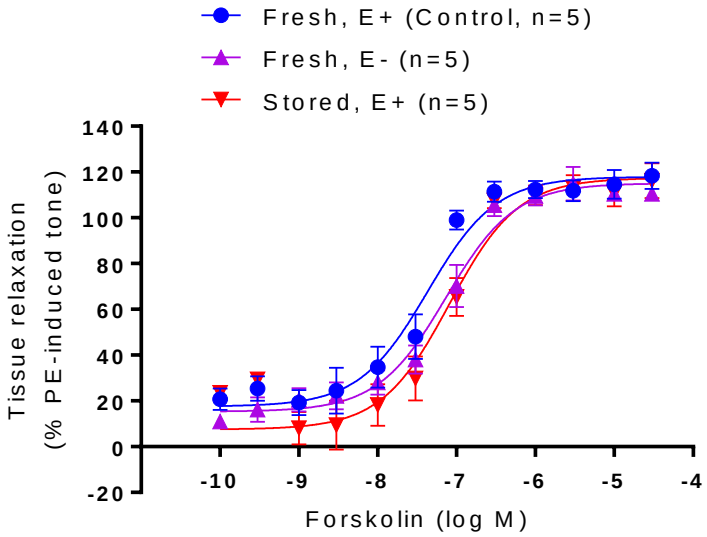


Figure 2.4: Effects of forskolin in endothelium-intact (both at n=5) and –denuded aortic tissues (fresh: n=5, stored: n=5) from fresh and stored conditions. Fresh endothelium-intact aortic tissues (E+) served as the control tissues. All aortic tissues were pre-constricted with 0.1µM phenylephrine. The data represents the mean values ± SEM of *n* number of animals. The one-way ANOVA followed by Dunnett's multiple comparisons test showed a small but significant rightward shifting of forskolin-induced relaxation curve in fresh endothelium-denuded tissues and no significant change in stored tissues (E+), as both compared to the control tissues (fresh, E+).

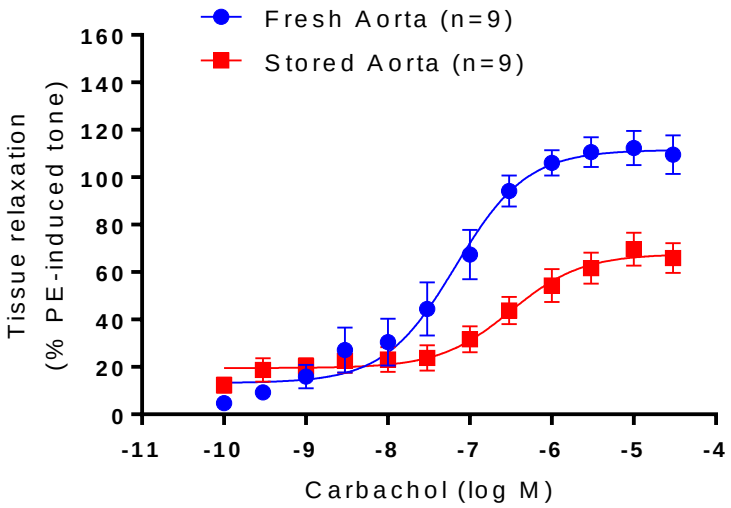


Figure 2.5: Effects of carbachol in fresh and stored aortic tissues (both at n=9) that were pre-constricted with 0.1µM phenylephrine. The data represents the mean values ± SEM of *n* number of animals. The unpaired t-test showed a significant difference in both E_{max} and pEC_{50} mean values of carbachol-induced relaxation curve in stored aortic tissues, as compared to the fresh tissues ($P<0.05$).

Treatment with		Fresh			*p* value		Stored			*p* values	
		n **(number of animals)**	E_{max}	pEC_{50}	E_{max}	pEC_{50}	***n*** **(number of animals)**	E_{max}	pEC_{50}	E_{max}	pEC_{50}
(A)	**Phenylephrine**	10	174.9 ± 8.8	7.63 ± 0.14	Control		10	194.3 ± 5.97	7.90 ± 0.10	0.3160[a]	0.1073[a]
(B)	**Isoprenaline**										
	E+	16	79.7± 4.18%	7.12 ± 0.13	Control		16	84.8 ± 3.9 %	6.82± 0.11	0.6455[b]	0.1317[b]
	+L-NAME	9	81.6± 6.52%	6.78± 0.17	0.9942[b]	0.4143[b]					
	E-	8	84.8± 5.69%	6.93± 0.16	0.9453[b]	0.7120[b]					
(C)	**Forskolin**										
	E+	5	117.9 ± 3.26%	7.37 ± 0.10	Control		5	117.4 ± 4.0%	7.09± 0.11	0.9998[c]	0.0522[c]
	E-	5	115.1 ± 2.89%	7.13± 0.08	0.9341[c]	* (0.0340)[c]					
(D)	**Carbachol**	9	111.6 ± 3.89 %	7.18 ± 0.11	Control		9	67.6 ± 3.56%	6.5± 0.18	** (0.0014)[d]	0.0561[d]

Table 2.1: Summary of all data obtained from contraction and relaxation experiments in aortic rings. The data represented the mean values ± SEM of *n* number of animals. The contraction data in response to phenylephrine is presented in percentage (%) of KCl-induced contraction. The relaxation data (in response to isoprenaline, forskolin and carbachol) is presented in % of phenylephrine-induced contraction. [a] Unpaired t-test comparing between fresh and stored aortic tissues with phenylephrine treatment.

[b] One-way ANOVA followed by Dunnett's multiple comparisons test comparing control aortic tissues (fresh, E+) with (i) fresh endothelium-denuded (fresh, E-), (ii) L-NAME (fresh, +L-NAME) treated aortic tissues, and (iii) stored tissues (stored, E+) in response to isoprenaline.

[c] One-way ANOVA followed by Dunnett's multiple comparisons test comparing control aortic tissues (fresh, E+) with (i) fresh endothelium-denuded (fresh, E-) and (ii) stored tissues (stored, E+) in response to forskolin.

[d] Unpaired t-test comparing control aortic tissues in response to carbachol before and after storage.

2.3.4 Carbachol-induced contraction in airway smooth muscle and bladder tissues

Majority of airway segments did not respond to the addition of 60mM KCl whether in fresh or stored conditions. However, contractile responses were observed during the cumulative addition of carbachol (fresh trachea, E_{max} 9.93 ± 0.62 mN; fresh bronchus, E_{max} 8.99 ± 0.43 mN) (see *Figure 2.6*). Overnight storage did not affect the magnitude of contractions of these airway smooth muscles (stored trachea, E_{max} 9.54 ± 0.6 mN; stored bronchus, E_{max} 7.70 ± 0.41 mN) (see *Figure 2.6*). For bladder tissues, contractions to 60mM KCl was observed (fresh, E_{max} 234.6± 16.8 %, n=4). Overnight storage did not affect the contractions of bladder tissues to carbachol (stored, E_{max} 233.0± 18.0 %, n=4) (see *Figure 2.7*). The contractile responses of airway smooth muscle and bladder tissues are summarised in *Table 2.2*.

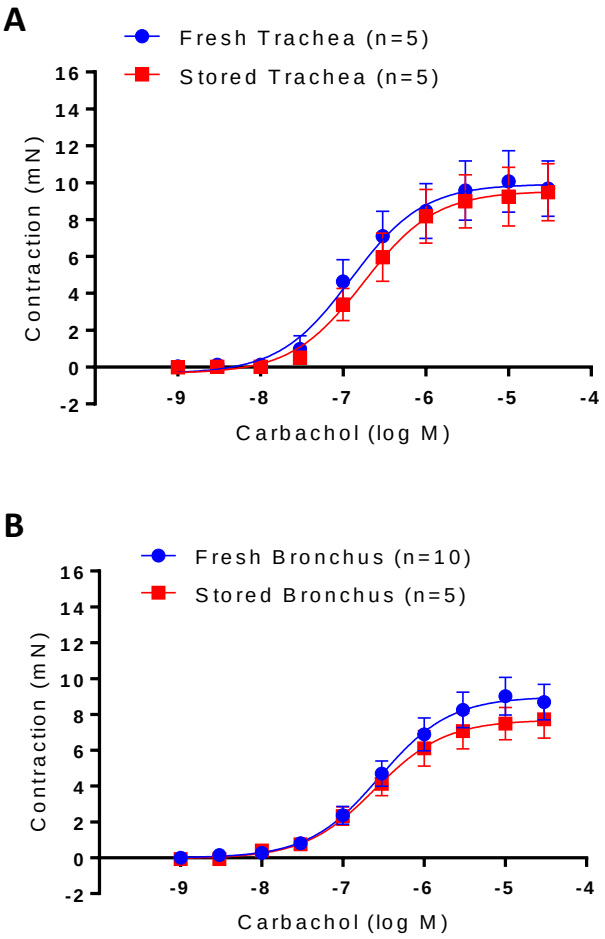


Figure 2.6: Effects of carbachol on fresh and stored (A) trachea (both at n=5) (B) bronchus segments (fresh: n=10, stored: n=5). The contractions are expressed in contraction force (in unit mN). The data represents the mean values ± SEM of *n* number of animals. The unpaired t-test showed no significant difference in the contraction to carbachol in airway smooth muscles between fresh and stored conditions.

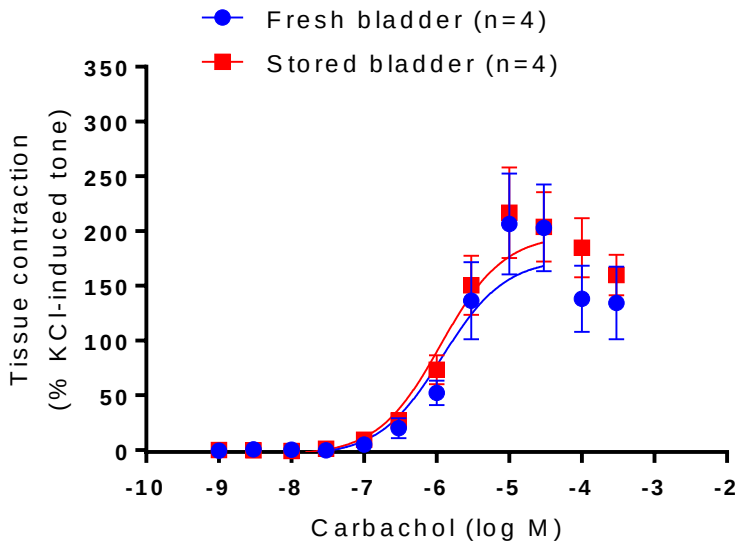


Figure 2.7: Effects of carbachol on fresh and stored bladder strips (both at n=4). The contractions are expressed as a percentage of KCl-induced contraction. The data represents the mean values ± SEM of *n* number of animals. The unpaired t-test showed no significant difference in the contraction to carbachol in airway smooth muscles between both fresh and stored conditions.

Tissues	Fresh			Stored			p value	
	n **(number of animals)**	E_{max} **(mN or %)**	pEC_{50}	***n*** **(number of animals)**	E_{max} **(mN or %)**	pEC_{50}	**Emax**	pEC_{50}
Trachea	5	9.93 ± 0.62 mN	6.9 ± 0.17	5	9.54 ± 0.6 mN	6.75 ± 0.17	0.8675	0.1810
Bronchus	10	8.99 ± 0.43 mN	6.55 ± 0.12	5	7.7 ± 0.41 mN	6.6 ± 0.14	0.3881	0.8555
Bladder	4	234.6 ± 16.8 %	5.62 ± 0.17	4	233.0 ± 18.0 %	5.76 ± 0.14	0.6503	0.9223

Table 2.2: Summary of contractile responses (E_{max} and pEC_{50}) of airway (trachea and bronchus) and bladder tissues that were induced by carbachol. The data represents the mean values ± SEM of *n* number of animals. The contraction data of trachea and bronchus is presented in unit mN while the contraction data of bladder tissues is presented in % of KCl-induced contraction. The unpaired t-test comparing the contractile responses (E_{max} and pEC_{50}) of fresh and stored tissues showed no significant difference in the contraction to carbachol in these muscles for both conditions, as shown in the *p* values.

2.4 Discussion

The present chapter demonstrated the functional studies of cold storage by using four different tissues including aorta, trachea, bronchus and bladder, from the same animal. This was useful as a means of differentiating which tissues display an optimal response when fresh, as well as which retained functionality after 24-hour of storage in Krebs-Ringer bicarbonate solution at 4^{o}C. Overnight storage did not alter the contractility of tested tissues, as well as their response rate, with the exception of the bronchi rings in which only 40% of the tissues responded following storage. This is a new reported observation on the effect of storage on the reduced bronchi responsiveness, with no known explanation of this phenomenon at present.

Phenylephrine was used to stimulate the contraction of aortic tissues. This contractile agent acts on α_1-adrenergic receptors that reside on the vascular smooth muscle. The unaltered phenylephrine-induced contraction of aortic tissues after storage suggested their unaltered smooth muscle function. Similarities to this phenylephrine response were also reported in a previous study carried out in mice (Ebner *et al.*, 2012) and rabbit aorta (Török *et al.*, 1993) after two and eight days of cold storage at 4^{o}C.

The influence of storage on the aorta endothelium was also investigated using different relaxant agents (forskolin and carbachol). Aortic endothelium is known and well-studied for its role in regulating contractility of vascular smooth muscles. The present chapter showed that isoprenaline-induced relaxation was not mediated by endothelium and nitric oxide, corroborating previous studies (Satake *et al.*, 1996; Rascado and Bendhack, 2005). Isoprenaline is a non-selective beta-adrenergic agonist that causes relaxation of vascular tissues. Beta-adrenergic receptors belong to G_s protein coupled receptor family. The activation of this G protein subtype family activates enzyme adenylyl cyclase in producing cyclic adenosine monophosphate (cAMP) that triggers the phosphorylation by protein kinase A. This eventually leads to the relaxation of smooth muscles. Adenylyl cyclase is found in both vascular endothelium (Schmitz *et al.*, 2014) and smooth muscles (Ostrom *et al.*, 2002). Nitric oxide is revealed to mediate adenylyl cyclase

signalling cascade (Bassil and Anand-Srivastava, 2006). In present chapter, isoprenaline may have activated adenylyl cyclase isoforms that are located in the aortic smooth muscles and this activation is independent of nitric oxide mediation.

Carbachol acts on muscarinic receptors and mediates endothelium-dependent relaxation (Furchgott and Zawadzki, 1980; Vanhoutte *et al.*, 1986; Jiang *et al.*, 2000). In response to carbachol, the rightward shift of relaxation curves and the decrease of maximum relaxation after storage suggested that storage caused a reduced function of the endothelium or loss of endothelium-dependent relaxing factors. A gradual destruction of endothelial cells after cold storage may have occurred as shown in previous study under electron microscopy observation (Török *et al.*, 1993). The relaxant response to carbachol is found predominantly mediated by the activation of muscarinic M_3 receptor subtypes (Lamping *et al.*, 2004). The activation of M_3 receptor by carbachol produced an endothelium-dependent relaxation that is mediated by endothelial nitric oxide (eNO) and endothelium-dependent hyperpolarising factors (EDHFs) (Jiang *et al.*, 2000). Therefore in present chapter, it is convincing that the reduced effect to carbachol after storage indicates that storage results in the reduced function of endothelium and hence the reduced of eNO and EDHFs in mediating the aortic relaxation.

Forskolin is a direct activator of adenylyl cyclase. It is often used to examine the expression and regulation of adenylyl cyclase in cells (Insel and Ostrom, 2003). There are at least nine isoforms of enzyme adenylyl cyclase expressed in different cell types (Asbóth *et al.*, 2001). Five adenylyl cyclase isoforms (types II to VI) mRNA were found expressed in rat aortic endothelial cells (Manolopoulos *et al.*, 1995). However, the expression level of adenylyl cyclase in aortic endothelial cells differed from the parent tissues (whole aorta) in which isoforms V and VII were expressed abundantly in endothelial cells but in minute in whole aorta (Manolopoulos *et al.*, 1995). Forskolin stimulates all isoforms of adenylyl cyclase, except for isoform IX (Asbóth *et al.*, 2001). In present chapter, a slight right-shifting of forskolin-induced relaxation curve was observed in the endothelium-denuded aortic tissues without a change in its maximum relaxation. Therefore in an intact aortic ring, the distinct

activation of forskolin in aortic smooth muscle is revealed with little potentiation effect by endothelium or endothelium-derived relaxing factors.

In contrast, the unchanged relaxation to forskolin after storage suggests that forskolin-induced activation of adenylyl cyclase in smooth muscle was retained with potentiation effect by endothelium or endothelium-derived relaxing factors. However, the unchanged endothelium function in this experiment can be argued due to a weak indication by a significance value (p) of 0.0522 (close to 0.05) obtained from the mean potency difference of forskolin-induced relaxation curve between fresh and stored intact tissues. Comparing both relaxant effect to carbachol and forskolin in stored tissues, the reduced relaxation was much evidently observed in response to carbachol, in which such effect is due to a reduced endothelium function. Therefore, forskolin appears as a weaker indicator in showing the status of endothelial function after storage, as compared to carbachol.

The contractions of trachea and bladder tissues were retained even after 24-hour of cold storage at 4^{o}C. These observations were consistent to canine trachea (Janssen *et al.*, 2001) and human bladder (Schneider *et al.*, 2011) using the same storage conditions. Schneider and colleagues (2011) also reported that the contraction ability of human bladder strips remained unaltered even after two days of cold storage. The retention of contractility was also demonstrated by stored bronchus. However, the use of stored bronchus is limited by its reduced reactivity to drug after storage.

The roles of the epithelial and urothelial cells in regulating the respective contractile response of airway smooth muscles and bladder are contradictory. There is evidence of showing the independent effects of tracheal epithelium and bladder urothelium in affecting smooth muscle contractions (Sadeghi-Hashjin *et al.*, 1996; Michel, 2015). Sadeghi-Hashjin and colleagues (1996) reported that the minute amount of the released relaxing factors (nitric oxide and prostaglandin E_2, at 2.39 ± 0.38 pmol.mg^{-1} and 0.20 ± 0.1 ng.mg^{-1} wet weight of tracheal tissues respectively) from bovine tracheal epithelium was inadequate to inhibit histamine-

induced contractions. The role of urothelium in mediating the detrusor contraction has been quantified either with or without prior endothelium removal. The inhibitions of detrusor contraction (evoked by muscarinic agonists) by urothelium-derived mediators were seen in mouse, pig and human bladder but not in rat bladder (Michel, 2015). This may suggest the species selectivity of urothelium-derived inhibition, as well as the absence of urothelial function in present chapter to mediate the contraction of rat bladder to carbachol.

An optimal storage condition is essential to maintain the functional responses of the tissues. A study by Michel (2014) showed an attenuated bladder response when tissues were stored for 24 hours at 37^{o}C, as compared to those stored for 24 hours at 4^{o}C (Schneider *et al.*, 2011). This justifies the need of identifying an optimal storage conditions for the tested tissues, in addition to reducing animal use in research. The storing of bladder strips at 37^{o}C was to meet the study requirement by Michel (2014) in order to desensitise the isoprenaline response in bladder strips. Therefore, the responsiveness of stored tissues should be examined for each study based on the purpose of studies and storage conditions.

The present chapter has successfully demonstrated the retention of smooth muscle contractility of isolated rat aorta, trachea, bronchus and bladder tissues, after 24-hour of cold storage at 4^{o}C. The use of stored tissues can be summarised as (i) stored aortic tissues can still be used in smooth muscle studies, but not in endothelium-related studies; (ii) stored trachea and bladder can still be used without any change in contractile response; (iii) the use of stored bronchus is limited and not advisable due to its reduced responsiveness after storage. In addition, the experimental design in this chapter was proven to be effective in testing the functionality of different tissues.

Chapter 3: Preliminary testing of schwarzinicine A on rat tissues

3.1 Introduction

3.1.1 Schwarzinicine A from *Ficus schwarzii*: Origin and structure

A local fig plant species, *Ficus schwarzii* is used traditionally against ringworm infection (Asian Plant). The therapeutic uses of this plant species are yet to be discovered. From this plant species, a novel plant compound, named schwarzinicine A has recently been isolated and characterised by Dr Kuan Hon Lim and Mr Premanand Krishnan from University of Nottingham Malaysia Campus, Malaysia. Hence, this is the first study in exploring the potential therapeutic effect of this novel plant compound on different bioassays.

Schwarzinicine A is a novel phenylethylamine alkaloid isolated from the leaves of *Ficus schwarzii* plant. The scientific name of this plant compound is N-(3,4-dimethoxyphenethyl)-1,4-bis(3,4-dimethoxyphenyl)butan-2-amine, with the chemical structure shown in *Figure 3.1*.

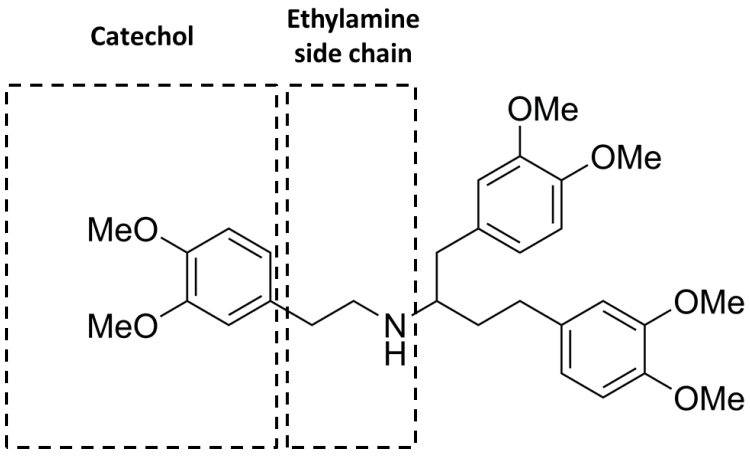


Figure 3.1: Chemical structure of schwarzinicine A.

Schwarzinicine A shares similar structural features with known catecholamines (for example noradrenaline, isoprenaline and dopamine) that consist of a catechol moiety with an ethylamine side chain (see *Figure 3.1*). A catechol moiety is a phenyl ring with hydroxyl substituents at positions 3 and 4 (see *Figure 3.1*). Since schwarzinicine A is a novel phenylethylamine alkaloid, its biological activity is unknown. In order to determine the putative bioactivity of schwarzinicine A, the structure-activity relationship of the various catecholamines was referred to (Brunton *et al.*, 2006).

In catecholamines, substitution at aromatic ring confers the maximal receptor affinity of the drug (Brunton *et al.*, 2006). Substitution at α-carbon atom produces drugs, for example *d*-amphetamine that blocks the oxidation of monoamine oxidase (MAO) and potent in central nervous system (Miller *et al.*, 1980). Substitution at β-carbon atom produces two naturally occurring catecholamines, adrenaline and noradrenaline, by which the substitution confers to their agonist activity to respective α- and β-adrenergic receptors. The size of akyl substituents at amino group affects the selectivity of α- and β-activity. The increasing size of akyl substituent increases the β-receptor activity, as seen in isoprenaline. The general structure of a catecholamine, consisting of an aromatic ring, α- and β-carbon atoms, and the amino group, is illustrated in *Figure 3.2*.

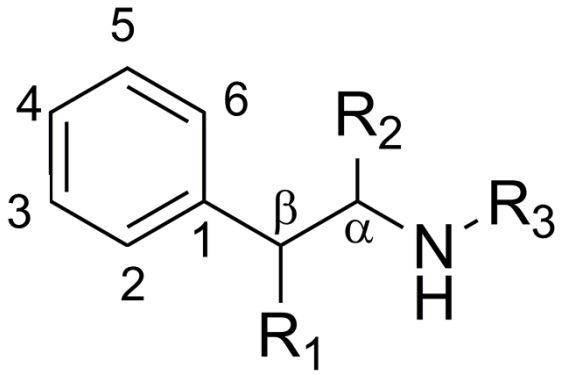


Figure 3.2: General chemical structure of catecholamines (Adapted from Brunton *et al.*, 2006).

Schwarzinicince A possesses methoxy (-OCH_3) groups at both positions 3 and 4 of the phenyl ring and a complex R_3 substituent at the amino group (see *Figure 3.2*). The structure of schwarzinicine A has no substitution at α- and β-carbon atoms. Amongst all well-studied catecholamines, schwarzinicine A shares the closest similarity to dobutamine, a β-adrenergic agonist which has similar substitution pattern compared to that of schwarzinicine A with regards to the aromatic ring of the phenylethylamine partial structure (see *Figure 3.3*). The two phenolic OH groups in the catechol moiety of dobutamine have been replaced with two methoxy groups in schwarzinicine A. However, the R_3 substituent in schwarzinicine A is slightly more elaborate compared to that in dobutamine (see *Figure 3.3*).

Phenylethylamine is the parent of all catecholamines in appearing as their partial structure (as shown in *Figure 3.3*). Phenylethylamine is used clinically as psychoactive drug that acts selectively on trace amine-associated receptor 1 (TAAR1) subtype (Kleinau *et al.*, 2011). TAAR receptor family is often related to neurological disorders, such as schizophrenia and Parkinson's disease (Berry, 2007). Recently, phenylethylamine was revealed to exhibit a partial allosteric antagonism effect of on β_1/β_2-adrenergic receptors in human embryonic kidney cells (HEK293) (Kleinau *et al.*, 2011).

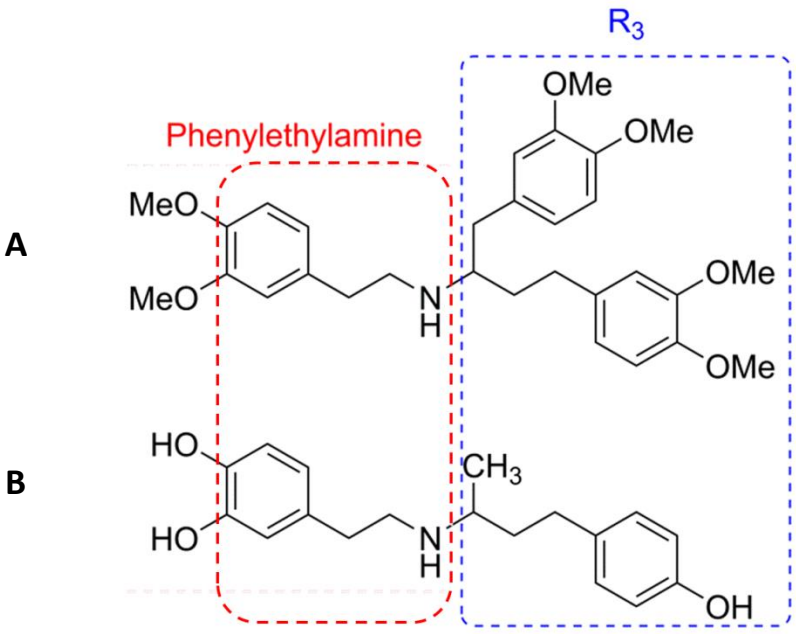


Figure 3.3: Chemical structures of (A) schwarzinicine A and (B) dobutamine.

3.1.2 Dobutamine and its pharmacological actions

Dobutamine is a sympathomimetic amine that was synthesised by Tuttle and Mills (1975) using isoprenaline as the parent chemical structure. It is commonly used as a potent inotropic agent in congestive heart failure and causes vasodilatory effect in cardiovascular system (Tuttle and Mills, 1975; Ruffolo, 1987).

Clinically, a racemic mixture of dobutamine is used. In this racemic mixture, the levo- and dextro-isomers of dobutamine are present in an asymmetrical manner. The levo-isomer is a partial α_1-adrenergic agonist, whereas dextro-isomer is a potent β_1- and β_2-adrenergic agonist (Brunton *et al*, 2006). At this mean, dobutamine is capable of activating α_1-, β_1-and β_2-adrenergic receptors. A combined stimulation of α_1- and β_1-adrenergic receptor was found in dobutamine-induced inotropic activity in myocardium (Ruffolo, 1987). However, in vasculature, the vasoconstriction effect of α_1-adrenergic receptor is counterbalanced by the vasodilatory effect of β-adrenergic receptor (Ruffolo, 1987; Kern, 2003). The pK_i values of β-adrenergic receptor stimulation by dobutamine is summarised in *Table 3.1*. As a result, dobutamine is generally considered as a β-adrenergic selective agonist in the vascular system (Brunton *et al.*, 2006).

Agent	**Peripheral vascular receptors**					
	α_1		**β_1**		**β_2**	
	Effect	**pK_i value**	**Effect**	**pK_i value**	**Effect**	**pK_i value**
Dobutamine	Offset by β-adrenergic stimulation	-	Peripheral vasodilation	6.2	Peripheral vasodilation	5.5

Table 3.1: Dobutamine-stimulated adrenergic receptors in peripheral vascular system and their respective agonist affinity in pK_i determinants (IUPHAR database).

3.1.3 Adrenergic receptors and their stimulations

Adrenergic receptors (ARs) are literally found in almost all cell types in balancing the physiological and pathological states of our bodies. Adrenaline and noradrenaline are the two endogenous catecholamines that act on adrenergic receptors in sympathetic nervous system. Adrenergic receptors are divided into two main groups, which are α- and β-ARs. Two main groups of α-adrenergic receptors, α_1- and α_2-ARs and their respective subtypes: α_{1A}, α_{1B} and α_{1D}; α_{2A}, α_{2B} and α_{2C} are identified pharmacologically (Granneman, 2001). Conversely, three β-adrenergic receptors, β_1-, β_2-, and β_3-ARs are also identified. In addition to these, a fourth β-AR named β_4 is also proposed by some researchers. This β-adrenergic subtype is a novel atypical β-AR and its precise mechanisms of action remain unclear (Granneman, 2001)

There are a large number of adrenergic agonists and antagonists available for research and clinical uses. They serve as useful pharmacological tool in identifying the localisation and distribution of any adrenergic subtypes in certain tissues. Some examples of experimentally and clinically used α-AR agonists are methoxamine, phenylephrine, oxymetazoline, clonidine and guanabenz (IUPHAR database). Prazosin, phentolamine and tolazoline are the antagonists for α-ARs (IUPHAR database). For β-ARs, the commonly used agonists are isoprenaline, dobutamine and salmeterol, while the antagonists include timolol, propranolol, nebivolol and carvedilol (IUPHAR database).

The distribution of adrenergic subtypes can vary between tissue types and animal species. For example, high expression of α-ARs are found in human vascular tissues, however, the distribution of subtypes varies with different type of vascular tissues. Many studies reported the predominant role of α_{1D}AR subtype in mediating the contraction of various vascular beds in humans (Jensen *et al.*, 2009) and rats (Kenny *et al.*, 1995; Hussain and Marshall, 1997). However, this receptor subtype does not express in all human vascular tissues, such as carotid artery, saphenous vein, mesenteric artery and renal artery (Rudner *et al.*, 1999), indicating the role of the other α-AR subtypes in these vascular tissues. In other tissues, weak expression of

α-ARs are detected in human urinary bladder and airway systems, but these tissues express high expression level of β-ARs in mediating their smooth muscle relaxation (Michel and Vrydag, 2006; Barisione *et al.*, 2010). Based on the expression level of adrenergic receptor subtypes in various tissues, vascular tissues are the best candidate for studying α-ARs while urinary bladder and airway tissues are the best for β-AR studies.

Stimulations of α- and β-adrenergic receptors give rise to the contraction and relaxation of smooth muscles respectively. Adrenergic receptors are the members of G-protein coupled receptor family. Their functional roles are based on their specific coupling via different G-proteins such as G_q, G_s and $G_{o/i}$. Commonly, α_1ARs couple via G_q proteins and causes phospholipase C hydrolysis to produce second messengers, IP_3 and DAG, resulting in muscle contraction. Despite of the same functional role as α_1ARs in mediating muscle contraction, α_2ARs mediate their role via G_i proteins that inhibit enzyme adenylyl cyclase that produces cyclic adenosine monophosphate (cAMP). The production of cAMP causes the relaxation of smooth muscle. In contrast, β-ARs that mediate muscle relaxation couple via G_s proteins resulting in activation of enzyme adenylyl cyclase and produces cAMP.

3.1.5 Significances of study

The biological activity of schwarzinicine A is unknown due to its novelty. Due the structural similarity of this compound with dobutamine (a racemic drug exhibits both α- and β-adrenergic effects), the adrenergic effect of schwarzinicine A was postulated and this was investigated on four different isolated rat tissues (trachea, bronchus, aorta and bladder). These four tissues are known to express adrenergic receptors, in which aorta expresses both α- and β-adrenergic receptors (Rudner *et al.*, 1999; Brawley *et al.*, 2000) while trachea, bronchus and bladder express high level of β-ARs with low expression level of α-ARs. The response magnitudes of schwarzinicine A in these tissues were compared. The effect of schwarzinicine A in aorta was also compared with that of dobutamine as the control.

3.2 Materials and methods

3.2.1 Drugs and Krebs-Ringer bicarbonate solution

Schwarzinicine A was first dissolved in 100% DMSO to achieve 0.1M stock concentration, followed by ten-fold dilutions with descending DMSO content from 50% (v/v), 25% (v/v) to pure distilled water. Drugs including phenylephrine (ChromaDex, USA), dobutamine hydrochloride (Santa Cruz Biotech, USA) and carbamylcholine chloride (carbachol; Nacalai Tesque, Japan) were used. Phenylephrine and carbachol were dissolved in distilled water, while dobutamine was dissolved in 100% DMSO to make 10mM stock concentration. Solutions were later diluted with distilled water following a series of 10-fold dilutions. The Krebs-Ringer bicarbonate solution was prepared as described in **Section 2.2.1** (Chapter 2).

3.2.2 Preparation of tissues and organ bath assembly

Refer to **Section 2.2.2** (Chapter 2).

3.2.3 Organ bath experiment

3.2.3.1 Effects of schwarzinicine A in different rat tissues

Four different tissues, trachea, bronchus, aorta and bladder were used to investigate the relaxant effect of schwarzinicine A. Trachea, bronchus and bladder were pre-constricted with 1µM carbachol, while aorta was pre-constricted with 0.1µM phenylephrine. The concentration of contractile agents used elicited at least 70% submaximal of contraction. Once a stable tone was established, schwarzinicine A (0.1nM to 30µM) concentration-response curve was constructed for each tissue type.

3.2.3.2 Effects of dobutamine and schwarzinicine A on isolated rat aorta

Schwarzinicine A was most efficacious and potent in the isolated rat aorta compared to all tissues used. Therefore, it was the tissue of choice for subsequent investigations. Cumulative response curves of dobutamine (0.1nM to 30µM) and schwarzinicine A (0.1nM to 30µM) were generated from aortic tissues that were pre-constricted with 0.1µM phenylephrine.

3.2.4 Data analysis

The relaxant responses to schwarzinicine A was calculated as a percentage inhibition of the respective contractile agonist-induced contraction. All data were expressed as mean ± SEM of *n* number of animals. All data were analysed using PRISM Version 6 (Graph Pad Software). Maximum response (E_{max}) and pEC_{50} values were obtained in which pEC_{50} is the negative logarithm of EC_{50} where EC_{50} is the concentration of drug that produces 50 % of its maximum response. Statistical analyses were performed using unpaired t tests (two-tailed) for comparing the mean differences between schwarzinicine A and dobutamine in aortic tissues (PRISM Version 6; Graph Pad Software). A probability of less than 0.05 ($P<0.05$) was considered to be statistically significant.

3.3 Results

3.3.1 Different relaxant profile produced by schwarzinicine A in different tissues

Schwarzinicine A induced relaxant effects in trachea, bronchus and aorta. In trachea and bronchus, schwarzinicine A-induced relaxations were only detected at higher concentrations (10 and 30µM). Therefore, there were insufficient data points to construct a complete concentration-response curve and to calculate the pEC_{50}. Aortic rings relaxed at a higher potency with pEC_{50} value of 6.04 ± 0.07 (n=5). Schwarzinicine A-induced responses in different tissues were compared at the concentration of 30µM (the last concentration used). At 30µM, aorta relaxed at

110.8 ± 7.8% whereas trachea and bronchus relaxed at 70.66 ± 15.38% and 71.73 ± 9.1% respectively (see *Figure 3.4*). Bladder strips developed a spontaneous contractile oscillation after the addition of carbachol and this effect was sustained throughout the additions of schwarzinicine A (see *Figure 3.5*). Hence, no meaningful data was recorded for this tissue.

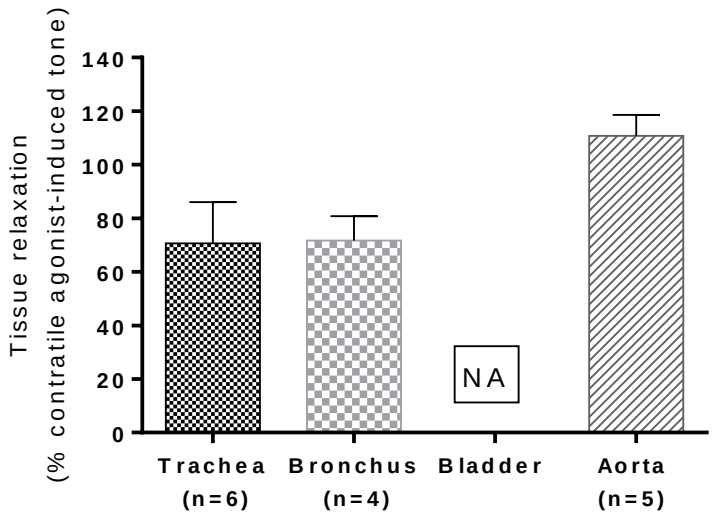


Figure 3.4: Schwarzinicine A-induced relaxation at 30µM in isolated rat trachea, bronchus, bladder and aorta. Relaxations are expressed as a percentage of the respective contraction induced by the pre-contractile agent. The data represented the mean values ± SEM of *n* number of animals. Isolated rat aorta relaxed at the highest magnitude compared to the other tissues.

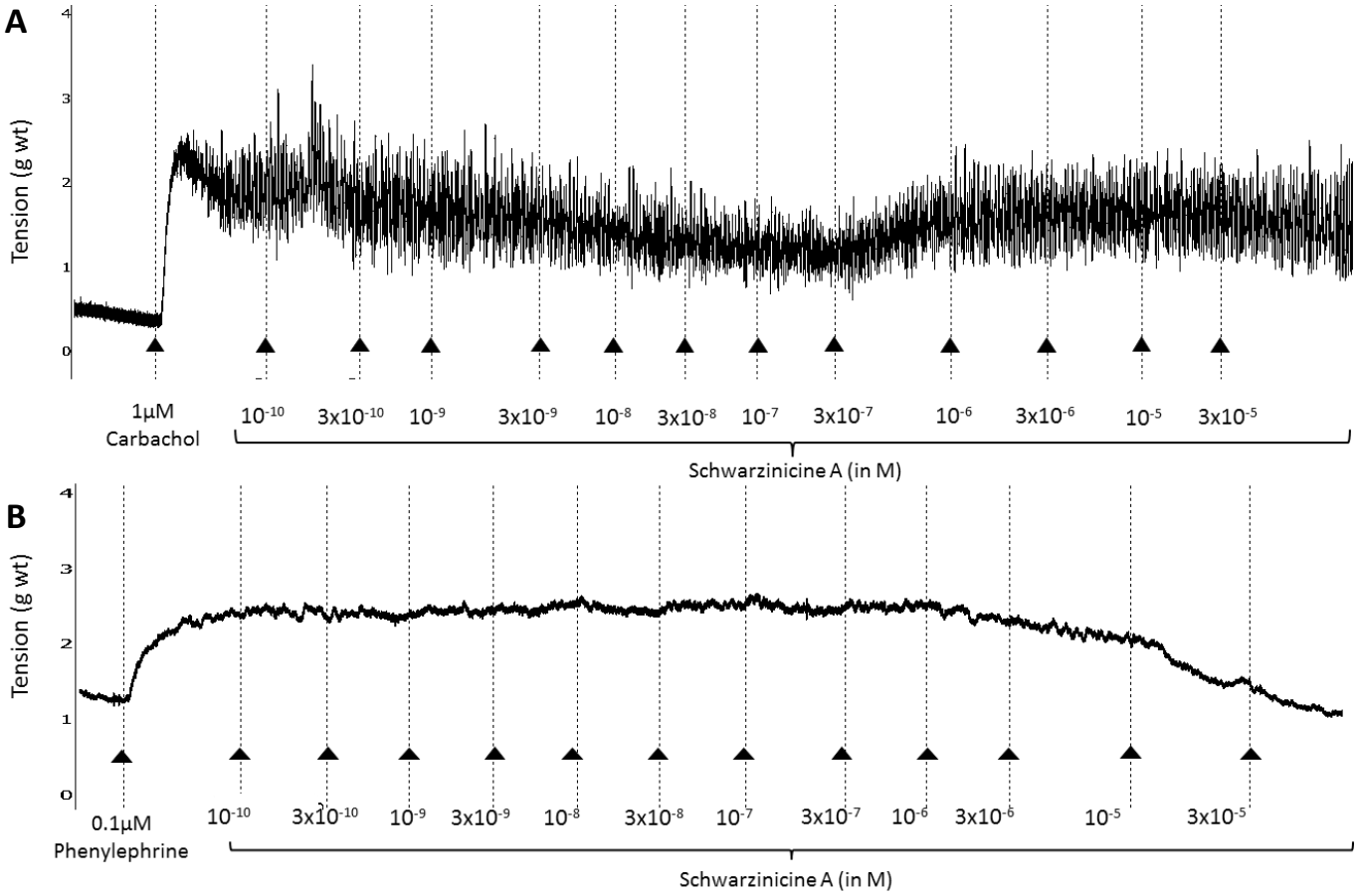


Figure 3.5: Representative trace recordings of rat (A) bladder and (B) aortic activities in response to their respective pre-contraction agents (carbachol and phenylephrine respectively) and schwarzinicine A. *Figure 3.5A* illustrates in rat bladder tissues, spontaneous contractile activity started to occur during the contraction to carbachol and this effect was not altered upon the addition of schwarzinicine A. *Figure 3.5B* illustrates in rat aortic tissues, stable phenylephrine-induced contraction was observed and schwarzinicine A relaxed the tissues in a concentration-dependent manner.

3.3.2 Aorta relaxed at same efficacy to schwarzinicine A and dobutamine

In aortic tissues that were pre-constricted with phenylephrine, a similar relaxation response was observed for schwarzinicine A (E_{max}: 114.3 ± 3.69%, n=5) and dobutamine (100.9 ± 5.86 %, n=3) (P>0.05; see *Figure 3.6*). However, dobutamine was more potent than schwarzinicine A (pEC_{50} 7.37 ± 0.23, n=3 vs 6.04 ± 0.07, n=5) (P<0.05).

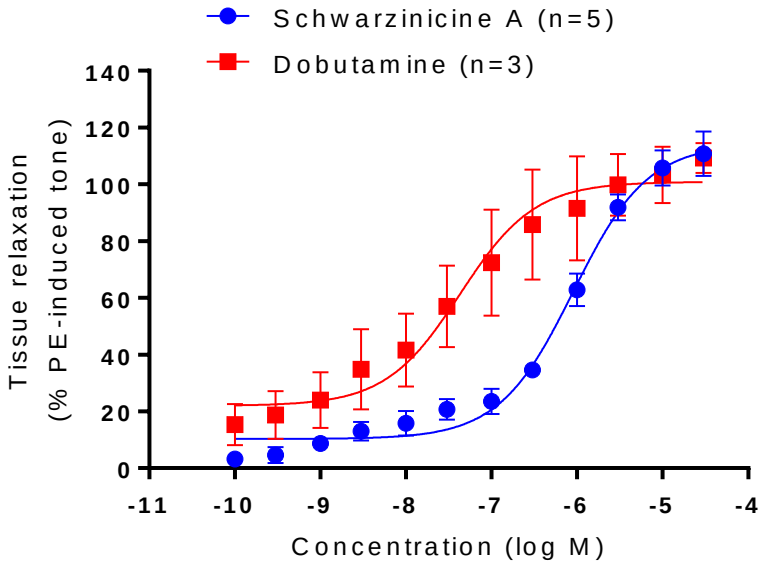


Figure 3.6: The relaxant effects of schwarzinicine A (n=5) and dobutamine (n=3) in aortic tissues that were pre-constricted with phenylephrine (PE). The relaxations were expressed as a percentage of phenylephrine-induced contraction. The data represents the mean values ± SEM of *n* number of animals. The schwarzinicine A-induced relaxation was at the same efficacy as that to dobutamine but at a lower potency (unpaired t-test, P<0.05).

3.4 Discussion

The observed contractile responses of all tested tissues were as a result of extracellular calcium entry and intracellular calcium release via activation of muscarinic subtype 3 (M_3) (airways and bladder) and α-adrenergic receptors (aorta) respectively. Spontaneous phasic activity was observed during the carbachol-induced contractile response in bladder tissues (see *Figure 3.5*). This event is not

unusual and has been reported in other studies (Akino *et al*., 2008; Anderson *et al.*, 2013).

The spontaneous phasic activity in isolated bladder strips is myogenic and caused by the longitudinal stretch (Levin *et al.*, 1986). In a whole bladder, this spontaneous activity appears to maintain the bladder shape during the filling phase (Brading, 2006). Evidence demonstrated that calcium dynamics in the detrusor muscle has regulated the spontaneous contractile activity (the upstroke of action potential) (Petkov *et al.*, 2001; Hashitani *et al.*, 2004). Modulation of spontaneous phasic activity can be mediated by potassium channels (Buckner *et al.*, 2002), partly to be BK_{Ca} channels (Heppner *et al.*, 1997), cyclic nucleotides and Rho kinases (Hashitani *et al.*, 2004). These modulators may be related to urothelium and suburothelium (Buckner *et al.*, 2002; Akino *et al.*, 2008; Meng *et al.*, 2008). Due to this spontaneous contractile activity, the effect of schwarzinicine A in bladder tissues was non-quantifiable, rendering the tissue unsuitable for further characterisation of relaxant mechanisms possessed by schwarzinicine A.

It was previously predicted of the adrenergic effects of schwarzinicine A due to its similar core chemical structure to dobutamine. Schwarzinicine A has a methoxy (-OCH_3) group substituted at both position 3 and 4 of the aromatic benzene ring whereas dobutamine has a hydroxyl group at the same positions. The former also has a larger substitution at the terminal amino side than that of dobutamine. In present study, both airway (trachea and bronchus) and aortic tissues have exhibited relaxant response to schwarzinicine A. This may suggest a possible β-adrenergic agonist role of schwarzinicine A in mediating cAMP-dependent relaxation in both tissue types. In addition to this, the partial allosteric antagonism effect of phenylethylamine on β-adrenergic receptors (Kleinau *et al.*, 2011) suggests that the core phenylethylamine structure in catecholamines, for example in isoprenaline and dobutamine, may somehow contribute to their respective β-adrenergic activities. Therefore, it is worth investigating the potential effect of schwarzinicine A on β-adrenergic receptors, taking into consideration the partial structure of phenylethylamine in schwarzinicine A.

Among all tested tissues, the highest schwarzinicine A-induced relaxation magnitude and potency was observed in the aortic tissues. This suggests the highest distribution of schwarzinicine A-targeted receptor in this tissue type, which preferably to be β-ARs. This relaxant response in aortic tissues is also similar to that of dobutamine but at a much lower potency. There is a possibility that schwarzinicine A may act on β- adrenergic receptors thus justifying further investigations. In addition, schwarzinicine A could also be an antagonist of the α-AR as it reduced the induced contraction magnitude by phenylephrine, which is a α_1-AR agonist.

In the general aspect of smooth muscle relaxation mechanisms, there are other possibilities that can explain these relaxant effects to schwarzinicine A. First, schwarzinicine A could play a role in inhibiting the associated receptors (α-adrenergic receptor for aorta and muscarinic subtype 3 for trachea, bronchus and bladder) and causes a decrease in the intracellular concentration of calcium. Inhibition of calcium channels (for example voltage-gated calcium channels), activation of potassium channel (for example calcium-activated potassium channels) and sodium exchangers (Knox and Tattersfield, 1995) are also the relevant mechanisms that decrease the intracellular calcium content. These mechanisms can be investigation target of schwarzinicine A.

Second, smooth muscle relaxation can also be caused by a non βAR-induced cAMP and cGMP-dependent pathways. There is a possibility that schwarzincine A involved in these pathways. Both cAMP and cGMP are second cell messengers that contribute to the relaxant effect in vascular and airway tissues (Knox and Tattersfield, 1995). The synthesis of cAMP is associated with the activation of the enzyme adenylyl cyclase that could be linked to G_s protein-coupled receptors. The production of cGMP by soluble guanylyl cyclase is either activated by nitric oxide or the nitro group of relaxant agents (Knox and Tattersfield, 1995). These two cell messengers induce the relaxant effect by activating the cAMP- and cGMP-mediated protein kinases (PKA and PKG respectively).

There are numerous studies confirming nitric oxide as a potent relaxant agent in vascular tissues (Ignarro *et al.*, 1987; Vanheel *et al.*, 1994; Cohen *et al.*, 1999). This raises the possibility of its involvement in scharwazinicine A-induced vascular relaxation. There are three nitric oxide synthases (NOS) including neuronal, inducible and endothelial NOS that generate a potent vasodilator, nitric oxide. Among all, endothelial NOS is often studied and found to play a main role in vascular relaxation, thus an interest in the influence of the endothelium in relaxation. Such an influence on the relaxation by schwarzinicine A was investigated and discussed in Chapter 4.

In summary, the highest schwarzinicine A-induced relaxation magnitude in aortic tissues (among all tested tissues) has rendered its suitability for further investigation. The influence of endothelium in mediating schwarzincine A was tested following the previously optimised methodology that utilises the endothelium-denuded aortic tissues (in Chapter 2). Other possible contributing factors to the mechanism of the schwarzinicine A-induced relaxant effects were investigated and discussed in Chapter 4.

Chapter 4: Pharmacological evaluation of schwarzinicine A-induced relaxation on rat aorta

4.1 Introduction

Previous findings in Chapter 3 demonstrated that schwarzinicine A elicited relaxation in different isolated rat airway (trachea and bronchus) and aortic tissues, by which the later exhibited the highest relaxation magnitude with highest potency among all tested tissues. These results allow the evaluation of schwarzinicine A-induced relaxation mechanisms in the present experiments.

4.1.1 Modulation of vascular relaxation

Intracellular calcium plays a central role in mediating the vascular activity by initiating the phosphorylation of myosin light chain (MLC) by enzyme myosin light chain kinase (MLCK) to result in vascular contraction. However, a decrease of this intracellular calcium will lead to the opposite effect, which is the vascular relaxation. With this knowledge, the relaxant response of vascular tissues is experimentally accessible using a commercial drug or experimental compound if their contraction is stimulated beforehand. An understanding on the relaxation mechanisms is important in elucidating the underlying mechanisms of one relaxant agent possesses.

Relaxation of vascular tissues is known to be mediated by a variety of modulators that reduce the level of intracellular calcium and sensitivity, via different signalling pathways. These modulators include endothelium-dependent relaxing factors (EDRFs), agonist or antagonists of G protein-coupled receptors (GPCRs), potassium channel activators and calcium channel inhibitors. The overall relaxation mechanisms in vascular tissues by these modulators are depicted in *Figure 4.1*.

The relaxation of vascular smooth muscles is often related to the EDRFs that are derived from the vascular endothelium. The most studied EDRF is endothelial nitric oxide. The synthesis of this nitric oxide is by activation of endothelial nitric oxide synthase (eNOs) that resides at the endothelium. With the close proximity between endothelium and its adjacent smooth muscle layer, the diffusion of EDRFs from endothelium into smooth muscles has been confirmed in many studies (Bassenge *et al.*, 1987; Keitoku *et al.*, 1988 and Condorelli and George, 2002). This supports the essential role of endothelium in vascular relaxation.

G-protein coupled receptors (GPCRs) are the most abundant transmembrane receptors in the cells. This receptor superfamily consists of some major regulatory receptors in vascular activity, such as adrenergic, serotonin, thromboxane, muscarinic receptors. These receptors can be classified according to their specific coupling to different G protein subtypes, including G_q, $G_{12/13,}$ $G_{i/o}$ and G_s proteins, in which coupling via G_q or $G_{12/13}$ or $G_{i/o}$ proteins results in the vasoconstriction, whereas coupling via G_s protein results in vasodilation (reviewed by Wettschureck and Offermanns, 2005). Therefore pharmacologically, the relaxation of vascular smooth muscles can be achieved by using the agonists of G_s protein-coupled receptors and the antagonists of G_q- or $G_{12/13}$ -or $G_{i/o}$- protein-coupled receptors. Phenylephrine, histamine and U46619 are the commonly used G_q protein-coupled receptor agonists in evoking vascular contraction, whereas isoprenaline as the G_s protein-coupled receptor agonist, is used to elicit vascular relaxation.

Ion channel play an important role in transporting essential cations, such as potassium ions, calcium ions and sodium ions across the cell membrane to maintain the homeostasis of cell physiological functions. The potassium and sodium ions are the two main cations in regulating the vascular activity. The transport of potassium and calcium ions via their respective channels is highly dependent on the membrane potential of the cells that giving rise to the depolarising (by calcium ion) or hyperpolarising (by potassium ion) state of the cell membrane. Vascular relaxation occurs either when the cell membrane is hyperpolarised with the activation of potassium channels, or when the calcium channel is inhibited and reduces the intracellular calcium content.

Many modulators, such as EDRFs, agonists of G_s protein-coupled receptors and potassium channel activators, mediate the vascular relaxation via the downstream cyclic nucleotide-regulated signalling pathways. The cyclic nucleotides, cAMP and cGMP are the regulatory proteins for vascular relaxation. The cAMP and cGMP are generated from activation of enzyme adenylyl cyclase and guanylyl cyclase respectively. These cyclic nucleotides inhibit the phosphorylation of MLC by its enzyme MLCK, in which resulting in vascular relaxation. This cAMP-dependent vascular relaxation does not occur constitutively due to its regulation by endogenous phosphodiesterases (PDEs). Pharmacologically, this reaction can be reversed by inhibiting the regulation by PDEs in order to promote constitutive vascular relaxation. There are PDE inhibitors, such as isobutylmethylxanthine (IBMX), rolipram, roflumilast and cilomilast that available commercially for therapeutic purpose (IUPHAR database).

Experimentally, a drug or an experimental compound must first be in its dissolved state in order to perform any pharmacological activity (reviewed by Stegemann *et al.*, 2007). Solvents (for example distilled water, dimethyl sulfoxide, ethanol and methanol) are used to dissolve the drug or experimental compound, in which they are known as the vehicles (reviewed by Johnson and Besselsen, 2002). To determine the effect of vehicle in an experiment design, the vehicle is administered in the same manner as the drug or experimental compound (reviewed by Johnson and Besselsen, 2002).

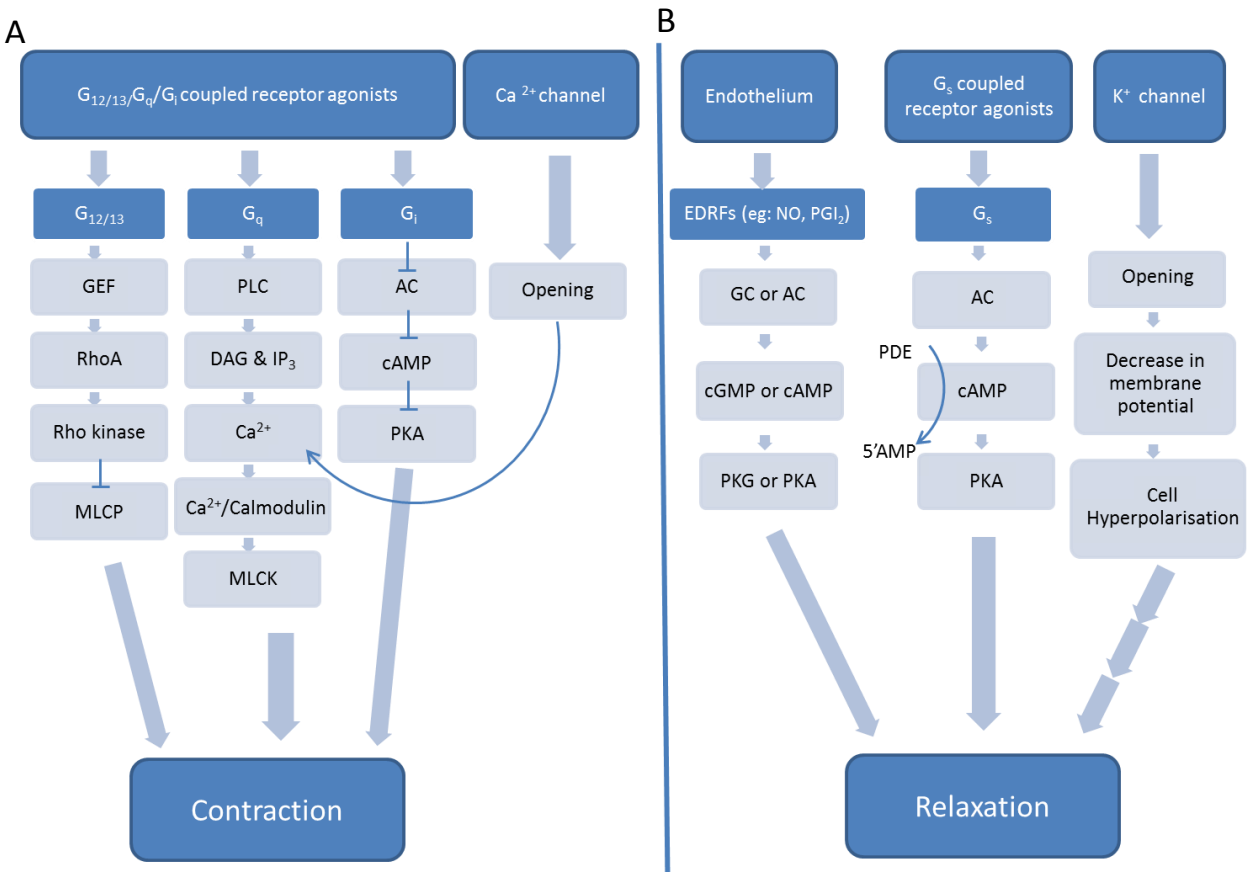


Figure 4.1: Schematic diagram illustrates the (A) contraction and (B) relaxation mechanisms in vascular tissues. Vascular contraction can be triggered by the activation of either $G_{12/13}$ or G_q or G_i protein-coupled receptors and the opening of calcium channels via different signalling pathways (as shown in *Figure 4.1A*). The inhibition of these signalling pathways using either the respective G protein-coupled receptor antagonists or calcium channel blockers, can lead to the relaxation of vascular tissues. The relaxation of vascular tissues can also be triggered by (i) endothelium-derived relaxing factors (EDRFs), for example nitric oxide (NO) and prostaglandin (PGI_2), (ii) activation of G_s protein-coupled receptors and (iii) opening of potassium channels. These modulators elicit the downstream cyclic nucleotides- (cGMP or cAMP)-mediated signalling pathways (as shown in *Figure 4.1B*). Phosphodiesterase (PDE) can convert cAMP into its inactive form (5'AMP), hence reducing the vascular relaxation. This event can be reversed by PDE inhibitors. GEF: guanine nucleotide exchanger factors; MLCP: myosin light chain phosphatase; PLC: phospholipase C; DAG: diacylglycerol; IP3: inositol triphosphate; MLCK: myosin light chain kinase; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; GC: guanylyl cyclase; cGMP: cyclic guanosine monophosphate; PKG: protein kinase G; PDE: phosphodiesterase; 5'AMP: 5' adenosine monophosphate. (Adapted from Furchgott and Zawadzki, 1980; Knot *et al.*, 1996; Lincoln *et al.*, 2001; Wettschureck and Offermanns, 2005; Omori and Kotera, 2007; Berridge, 2007; Sassone-Corsi, 2012)

4.1.2 Significances of study

The relaxant effect of schwarzinicine A was observed in isolated rat aorta, as well as in airway smooth muscles (trachea and bronchus) albeit at a lower extent as described in previous chapter (Chapter 3). To understand the underlying mechanisms of schwarzinicine A elicited, below were the experimental objectives:

(i) to investigate the effects of schwarzinicine A in response to different contractile agents (phenylephrine, KCl and U46619),

(ii) to investigate the possible adrenergic effect of schwarzinicine A in α- and β-adrenergic pathways using their relevant antagonists: α-AR, rauwolscine and prazosin; β-AR timolol and propranolol;

(iii) to investigate the role of endothelium and endothelium-derived relaxing factors by endothelium removal and using a NO inhibitor, L-NAME;

(iv) to study the role of potassium channel in schwarzinicine A effect using potassium channel blocker, TEA;

(v) to study the role of cAMP in schwarzinicine A-induced relaxation by inhibiting its regulatory pathway using phosphodiesterase inhibitors, IBMX and rolipram; and

(vi) to study the effects of schwarzinicine A on calcium entry in calcium-free Krebs solution and calcium release by concentration-dependent contraction agents (phenylephrine, KCl, 5-hydroxytryptamine and U46619).

4.3 Materials and Methods

4.2.1 Drugs and Krebs-Ringer bicarbonate solution

Schwarzinicine A was prepared as described in **Section 3.2.1** (Chapter 3). Drugs used in this section are listed and purchased as displayed in *Table 4.1*. All drugs were dissolved in 100% DMSO, except for N^G-Nitro-L-Arginine methyl ester (L-NAME) and phenylephrine that were dissolved in distilled water, and prazosin that was dissolved in 50% methanol. All dissolved drugs were made into stock concentration of 10mM, except for tetraethylammonium (TEA) in stock

concentration of 5M. A serial of ten-fold dilution was made for all dissolved drug solutions. Details on the solvents used and stock concentration for each drug are summarised in *Table 4.1*. Calcium-containing Krebs solution (or normal Krebs solution) was prepared as described in **Section 2.2.1** (Chapter 2). Calcium-free Krebs solution was prepared with $CaCl_2$ omitted and gassed in the same manner as normal Krebs solution.

Drugs	Company, Country	Solvent	Stock concentration (mM)
N^G-Nitro-L-Arginine methyl ester (L-NAME) hydrochloride	ChemSln, USA	Distilled water	10
5-Hydroxytryptamine hydrochloride	Nacalai Tesque, Japan	100% DMSO	10
Nifedipine	Nacalai Tesque, Japan	100% DMSO	10
S-(-)-propranolol hydrochloride	Santa Cruz, USA	100% DMSO	10
Timolol maleate	Santa Cruz, USA	100% DMSO	10
Prazosin hydrochloride	Sigma, USA	50% Methanol	10
Tetraethylammonium (TEA) chloride	Sigma, USA	100% DMSO	5 (in M)
Rolipram	Tocris Bioscience, UK	100% DMSO	10
U46619	Tocris Bioscience, UK	100% DMSO	10
IBMX	Tocris Bioscience, UK	100% DMSO	10
Rauwolscine hydrochloride	Tocris Bioscience, UK	100% DMSO	10
(R)-(-)-phenylephrine hydrochloride	Tocris Bioscience, USA	Distilled water	10

Table 4.1: Drugs used in the present experiments, with their company of purchase and types of solvents used.

4.2.2 Preparation of tissues and organ bath assembly

Refer to **Section 2.2.2** (Chapter 2).

4.2.3 Organ bath experiments

The design of experimental protocols in this chapter centred on investigating the putative relaxation mechanism(s) of schwarzinicine A.

4.2.3.1 Effects of schwarzinicine A on aorta pre-constricted with different contractile agents

Aortic tissues were pre-constricted with (i) 0.1µM phenylephrine (ii) 0.1µM U46619 or (iii) 60mM KCl. These concentrations of contractile agents elicited at least 70% contraction of their respective maximum contractions. Once a stable contractile tone was achieved, schwarzinicine A (10nM to 30µM) was applied cumulatively. Comparisons on the schwarzinicine A-induced relaxant response in rat aortic tissues obtained from these three pre-contractile agents were undertaken.

4.2.3.2 Effects of alpha- and beta-adrenergic antagonists on schwarzinicine A induced relaxation

To investigate whether schwarzincine A was antagonistic to alpha-adrenergic receptors, rauwolscine (10µM) and prazosin (10µM) were applied for 30 minutes prior to the cumulative addition of schwarzinicine A to aortic tissue pre-constricted with 60mM KCl. To investigate whether the schwarzincine A-induced relaxant response is influenced by the activation of β-adrenergic receptors, of the β_2-selective adrenergic receptor antagonist, timolol (30µM) and non-selective β-adrenergic receptor antagonist, propranolol (30µM) were applied before the cumulative application of schwarzinicine A (10nM to 30µM) on aortic tissues pre-constricted with phenylephrine.

4.2.3.3 Effects of endothelium and nitric oxide on schwarzinicine A-induced relaxation

To investigate the possible involvement of the endothelium, schwarzinicine A (10nM to 30μM) was applied cumulatively on endothelium-denuded aortic tissues that were pre-constricted with phenylephrine. The successful denudation of endothelium was confirmed following the described steps in Chapter 2. In combination with endothelium denudation, the nitric oxide inhibitor, the aortic tissues were pre-treated with L-NAME (0.1 mM) with an incubation period of 30 minutes preceding the cumulative addition of schwarzinicine A following pre-constriction with phenylephrine. The L-NAME served to confirm elimination of residual endothelial nitric oxide within the system, with considerations towards the possible involvement of nitric oxide in schwarzinicine A-induced relaxant responses.

4.2.3.4 Effect of potassium channel blocker on schwarzinicine A-induced relaxation

A potassium channel blocker, tetraethylammonium (TEA, 1mM) was added into the bath containing the aortic tissues for 30 minutes. Phenylephrine (0.1μM) was then applied to pre-constrict the tissues, followed by cumulative application of schwarzinicine A (10nM to 30μM).

4.2.3.5 Effects of phosphodiesterase inhibitors on schwarzinicine A-induced relaxation

Non-selective phosphodiesterase (PDE) and a phosphodiesterase type IV (PDE IV)-selective inhibitors: isobutylmethylxanthine (IBMX) and rolipram were used to investigate the involvement of cGMP/cAMP in the relaxation of schwarzinicine A. After a 30-minute incubation period of IBMX (1μM) and rolipram (10μM), schwarzinicine A was added cumulatively into the bath with aortic tissues pre-constricted with either 0.1μM phenylephrine or 60mM KCl.

4.2.3.6 Effect of schwarzinicine A on contractile response of different agonists

Concentration-response curves of four contractile agonists including phenylephrine (10nM to 30μM), 5-hydroxytryptamine (0.1μM to 0.3mM), U46619 (1nM to 10μM) and KCl (0.1mM to 0.1M) were constructed after incubation of schwarzinicine A (30μM) for 30 minutes. This concentration of schwarzinicine A was chosen based on the ability that it produced maximum relaxation in the concentration-response curve (refer to **Section 4.3.1**, *Table 4.2* and *Figure 4.2*). Calcium channel blocker, nifedipine (10μM) was included in the concentration-response curve of KCl to examine the role of calcium in the KCl-induced contraction.

4.2.3.7 Effects of schwarzinicine A on $CaCl_2$-induced contraction in calcium-free Krebs solution

Once viability of the aortic tissues was confirmed (refer to Chapter 2), normal Krebs solution was replaced with calcium-free Krebs solution. Tissues were equilibrated for 30 minutes in the calcium-free Krebs, before incubating the tissues further with concentrations of schrwazinicine A (3, 10, 30μM) for a duration of 30 minutes. These three concentrations of schwarzinicine A were chosen based on their cumulative concentration-dependent relaxant effect in pre-contricted aorta (refer to **Section 4.3.1**, *Table 4.2* and *Figure 4.2*) and to investigate a concentration-dependent effect of schwarzinicine A on $CaCl_2$-induced contraction. This was followed by a single application of 60mM KCl to confirm the viability of tissues in calcium-free Krebs solution. Once the KCl-induced contractile response plateaued, a concentration response curve to calcium chloride (1μM to 3mM) was constructed.

4.2.3 Data analysis

The contractile responses were either expressed in mN force or in percentage of 60mM KCl-induced contraction tone. The relaxation responses were expressed as percentage inhibition of the respective contractile agonist-induced contraction. All data were expressed as mean ± SEM of *n* number of animals. All data were analysed using PRISM Version 6 (Graph Pad Software). Maximum response (E_{max}) and pEC_{50} values were obtained in which pEC_{50} is the negative logarithm of EC_{50} where EC_{50} is the concentration of drug that produces 50% of its maximum response. The mean differences of both E_{max} and pEC_{50} mean values were analysed between the control concentration-response curves (without prior drug treatment) and sample concentration-response curves (with prior drug treatment) using unpaired t test (two-tailed). For comparing more than two treatment groups, their mean differences were tested using one-way ANOVA followed by Dunnett's multiple comparisons test (PRISM Version 6; Graph Pad Software). A probability of less than 0.05 ($P<0.05$) was considered to be statistically significant.

4.4 Results

4.3.1 Different schwarzinicine A-induced relaxation profile produced in response to pre- contraction agents

To investigate the effects of different pre-contractile agents on schwarzinicine A, phenylephrine, KCl and U46619 were used. Phenylephrine (0.1µM, n=9), KCl (60mM, n=6) and U46619 (0.1µM, n=5) produced similar contractile tones (10.91 ± 0.88, 10.3 ± 1.35 and 10.91 ± 0.93 mN respectively) preceding treatment with schwarzinicine A (see *Table 4.2*). The vehicle (< 0.165% DMSO v/v in bath) did not affect in the relaxation tones that were induced by schwarzinicine A in response to all pre-contraction agents (see *Table 4.2*).

Pre-exposure with phenylephrine caused relaxation to schwarzincine A with maximum relaxation of 122.9 ± 5.41 % of induced contractile tone and pEC_{50} of 5.86 ± 0.1. At the same concentration range of schwarzinicine A, pre-exposures to KCl

and U46619 have shifted the schwarzinicine A-induced relaxation curves to the right (see *Figure 4.2*). The rightward shift of relaxation curves with pre-exposure to KCl and U46619 did not allow the determination of their pEC_{50} values. Hence these pre-contractile agents were compared at their responses to 30µM schwarzinicine A.

At the highest concentration of schwarzinicine A (30µM), aortic tissues that were pre-constricted with phenylephrine and KCl exhibited comparable relaxation tone (119.3 ± 10.59 and 110.0 ± 11.54 % of respective induced contraction), while those pre-constricted with U46619 produced the lowest tone (62.49 ± 3.36 % of induced contraction) (see *Table 4.2* and *Figure 4.2*). Therefore, phenylephrine and KCl were used as the pre-contractile agents in the subsequent experiment.

Pre-contraction agents	**n number**	**Contraction tone (mN)**	**Relaxation tone (% of respective induced contraction tone)**		
			30µM schwarzinicine A	**Vehicle control of 30µM schwarzinicine A**	***p* value**
Phenylephrine	9	10.91 ± 0.88	119.3 ± 10.59	42.78 ± 10.08	*** (0.0003)
KCl	6	10.3 ± 1.35	110.0 ± 11.54	18.74 ± 7.64	**** (<0.0001)
U46619	5	10.91 ± 0.93	62.49 ± 3.36	42.85 ± 5.24	* (0.0149)

Table 4.2: The contraction tone (in mN) produced by pre-exposure of phenylephrine, KCl and U46619, and their respective relaxation tone that was induced by 30µM schwarzinicine A. The relaxation tone is expressed as the percentage of respective pre-contractile agents. The data represents the mean values ± SEM. The *p* value from unpaired t-test analysis indicates the significant difference between the relaxation tones that were induced by 30µM schwarzinicine A and its respective vehicle control (0.165% DMSO v/v) for all three pre-contraction conditions.

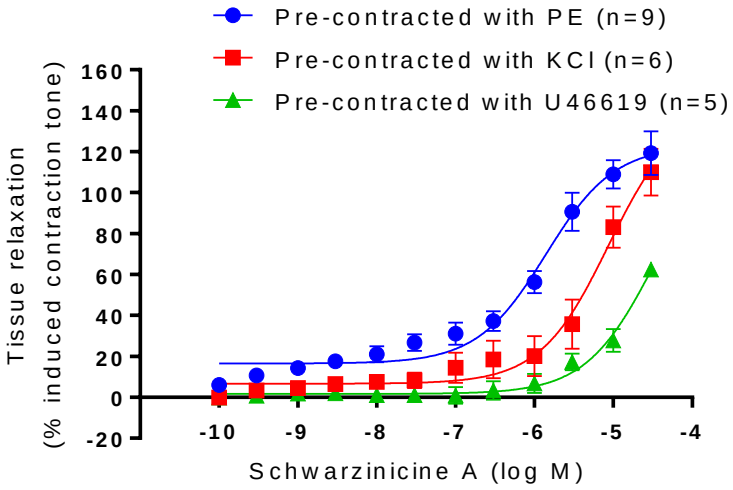


Figure 4.2: Effects of schwarzinicine A (10nM to 30µM) on aorta pre-constricted with 0.1µM phenylephrine (PE, n=5), 60mM KCl (n=6) and 0.1µM U46619 (n=5). The relaxations are expressed as a percentage of respective contractile agent-induced contractions. The data represents the mean values ± SEM of *n* number of animals.

4.3.2 Schwarzinicine A-induced relaxation was not mediated by adrenoceptors

The effects of alpha- and beta-adrenergic receptor antagonists on schwarzinicine A-induced relaxation were tested. Aortic tissues without prior treatment with adrenergic receptor antagonists served as controls. In response to KCl-induced contraction, the relaxation to schwarzinicine A was also not affected by the alpha-adrenergic receptor antagonists, rauwolscine (10µM) and prazosin (10µM) (*Figure 4.3A*). The beta-adrenergic receptor antagonists, timolol (3µM) and propranolol (3µM), did not affect the relaxation to schwarzinicine A in response to phenylephrine-induced contraction (see *Table 4.3* and *Figure 4.3B*).

Pre-contractile agents	Antagonists		Maximum response (%)	pEC_{50}
KCl	*Alpha-adrenergic*	Control	141.7 ± 16.71	5.06 ± 0.15
		Rauwolscine (10µM)	106.7 ± 22.35	5.17 ± 0.29
		Prazosin (10µM)	147.2 ± 20.86	5.28 ± 0.21
Phenylephrine	*Beta-adrenergic*	Control	127.1 ± 8.58	5.86 ± 0.09
		Timolol (3µM)	131.2 ± 9.25	5.48 ± 0.13
		Propranolol (3µM)	119.8 ± 6.22	5.72 ± 0.12

Table 4.3: Summary of the effects of alpha-adrenergic receptor antagonists (rauwolscine and prazosin, 10µM) and beta- (timolol and propranolol, 3µM) on schwarzinicine A-induced relaxation with respective pre-contractile agonists (KCl and phenylephrine). The data represents the mean values ± SEM of *n* number of animals. Maximum response was measured as the percentage of contraction tone induced by pre-contractile agonists.

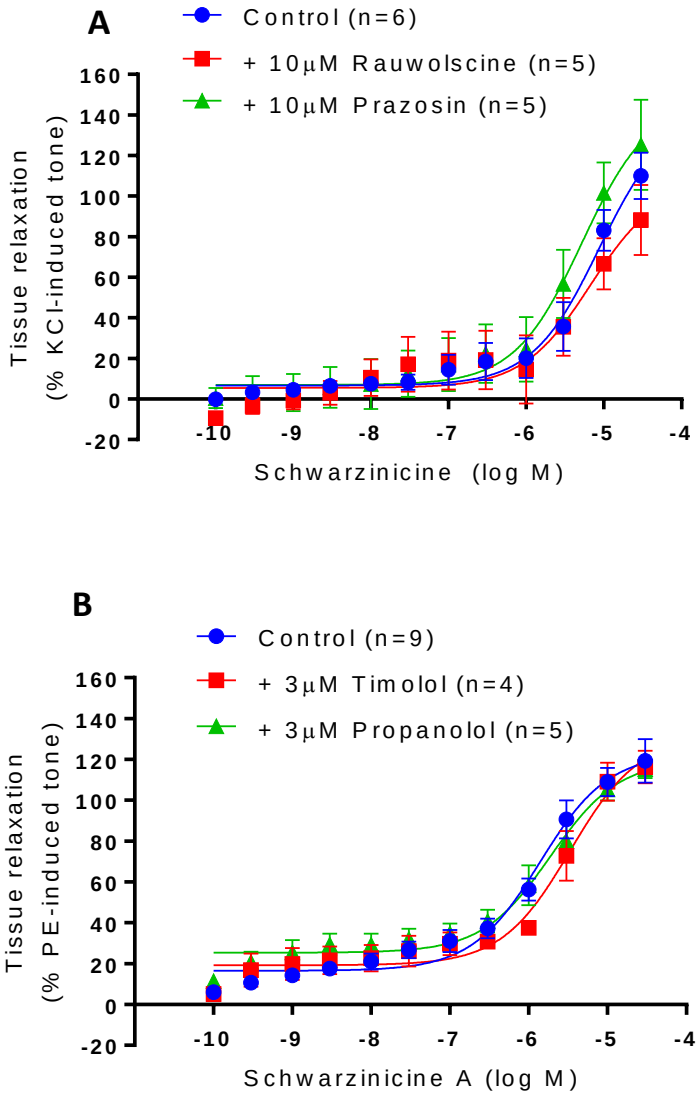


Figure 4.3: Effects of (A) alpha-adrenergic antagonists (rauwolscine and prazosin, 10µM) and (B) beta-adrenergic antagonists (timolol and propranolol, 3µM) on schwarzinicine A-induced relaxation. The experiment in *Figure 4.3A* was performed on aorta pre-constricted with KCl while *Figure 4.3B* on aorta pre-constricted with phenylephrine. Controls are the aortic tissues without prior treatment with alpha- and beta-adrenergic receptor antagonists. The relaxations were expressed as a percentage of respective KCl- and phenylephrine-induced contraction. The data represents the mean values ± SEM of *n* number of animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the schwarzinicine A-induced relaxations between control and those with prior treatments with adrenergic antagonists, showing no significant difference between control and treated aortic tissues.

4.3.3 Schwarzinicine A-induced relaxation was endothelium- and nitric oxide-independent

Phenylephrine was used to contract the aortic tissues. Endothelium-intact aortic tissues (E+) without prior treatment with nitric oxide synthase inhibitor, L-NAME served as controls. The endothelium removal (E-) (maximum response: 121.1 ± 4.88%, pEC_{50}: 5.79 ± 0.08; n=5) did not affect the relaxation to schwarzinicine A (control, maximum response: 122.9 ± 5.41 %, pEC_{50}: 5.86 ± 0.09; n=9). When in combination with L-NAME, endothelium-denuded aortic tissues relaxed the same as control (pEC_{50}: 5.41 ± 0.07, n=6) (see *Figure 4.4*). These results are based on the analysis from one-way ANOVA followed by Dunnett's multiple comparison tests that compared the relaxations to schwarzinicine A after endothelium removal and in combined with L-NAME treatment with the control endothelium-intact aortic tissues.

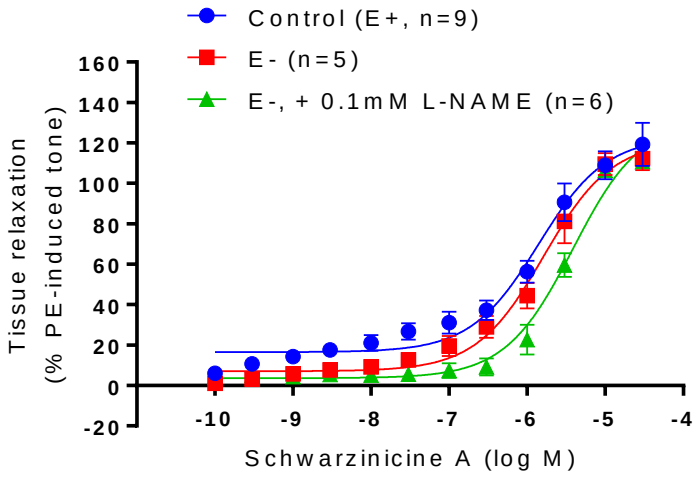


Figure 4.4: Effects of endothelium removal (E-, n=5) and in combination with L-NAME (E- + L-NAME, n=6) on schwarzinicine A-induced relaxation. Controls are the endothelium-intact aortic tissues (E+) without prior L-NAME treatment. The relaxations are expressed as a percentage of phenylephrine-induced contraction. The data represents the mean values ± SEM of *n* number of animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared the relaxations to schwarzinicine A after endothelium removal and in combined with L-NAME treatment with the control endothelium-intact aortic tissues, and confirmed that these two treatments (E- and E- +L-NAME) had no significant effect on schwarzinicine A-induced relaxation.

4.3.4 Schwarzinicine A-induced relaxation was not mediated by potassium channel

Phenylephrine was used to contract the aortic tissues. The influence of schwarzinicine A in K^+ channels was investigated. Incubation of 30 minutes with a non-selective potassium channel blocker, TEA did not alter the relaxation to schwarzinicine A (maximum response 127.1 ± 8.58 %, pEC_{50} 5.73 ± 0.13 vs Control: maximum response 122.9 ± 5.41 %, pEC_{50}: 5.86 ± 0.09) (see *Figure 4.5*). Aortic tissues without prior treatment with TEA served as the controls.

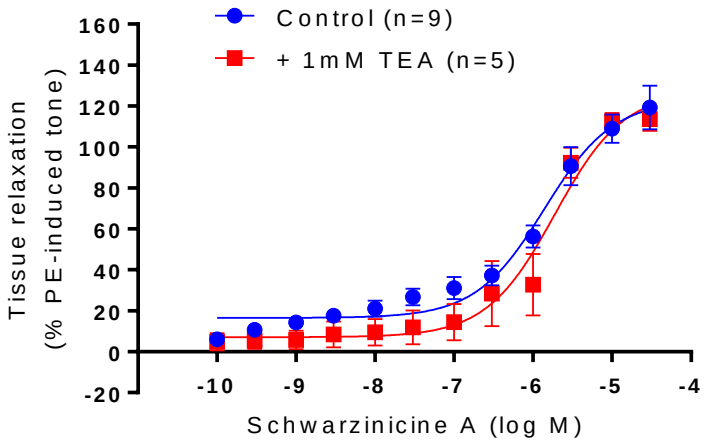


Figure 4.5: Effect of potassium channel blocker, TEA on aorta pre-constricted with phenylephrine (n=5). Controls are the aortic tissues without prior treatment with TEA. The relaxations were expressed as a percentage of phenylephrine-induced contraction. The data represents the mean values ± SEM of *n* number of animals. The unpaired t-test compared schwarzinicine A-induced relaxations between control and that with prior TEA treatment, showing no significant difference in between control and TEA-treated aortic tissues (P>0.05).

4.3.5 Schwarzinicine A-induced relaxation was not regulated by cGMP or cAMP

Phenylephrine and KCl were used to contract the aortic tissues. To investigate the effects of phosphodiesterase inhibitors on schwarzinicine A-induced relaxation, rolipram and IBMX were used. Aortic tissues without prior treatment with phosphodiesterase inhibitors served as the controls. Pre-treatment with rolipram (10µM) or IBMX (1µM) did not alter the relaxation curve of schwarzincine A in

response to both pre-exposures to phenylephrine (0.1 µM) and KCl (60mM) (see *Figure 4.6*).

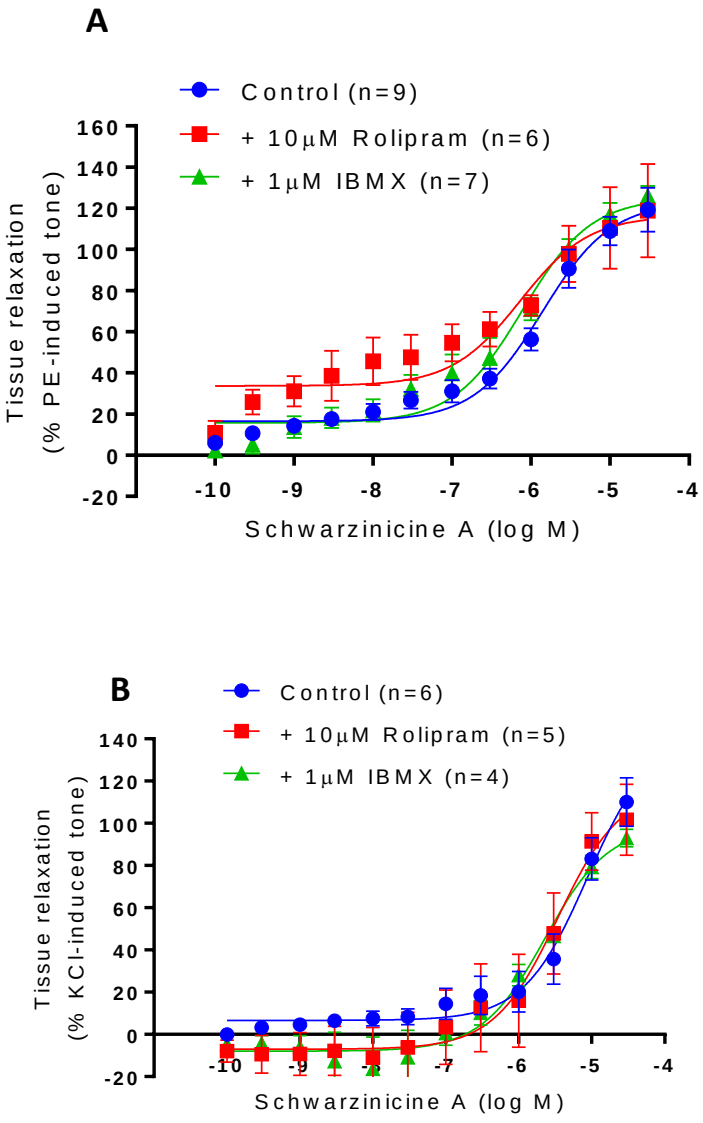


Figure 4.6: Effects of phosphodiesterase inhibitors, rolipram (10µM) and IBMX (1µM) on schwarzinicine A-induced relaxation elicited by aortic tissues pre-constricted with (A) phenylephrine (PE) and (B) KCl respectively. Controls are the aortic tissues without prior treatment with phosphodiesterase inhibitors. The relaxations were then expressed as a percentage of phenylephrine- and KCl-induced contraction respectively. The data represents the mean values ± SEM of *n* number of animals. The one-way ANOVA followed by Dunnett's multiple comparison test compared schwarzinicine A-induced relaxations between control and those with prior treatment with phosphodiesterase inhibitors, showing no significant difference between control and treated aortic tissues.

4.3.6 Schwarzinicine A displayed different characteristics with different contractile agents

This experiment investigated the effect of schwarzinicine A on the contractile response of aorta to different contractile agents (phenylephrine, U46619, 5-hydroxytryptamine and KCl) in a cumulative concentration-dependent manner. Vehicle controls (0.165% v/v DMSO) were included for each contraction experiment.

The contraction curve of 5-hydroxytryptamine was shifted to the right by the DMSO vehicle (0.165% v/v) in which pEC_{50} value could not be determined (see *Figure 4.7C*). Hence, the comparison was done on the maximum response of 5-hydroxytryptamine at 0.3mM between control (without prior treatment with vehicle and schwarzinicine A), vehicle-treated and schwarzinicine A-treated aortic rings (one way ANOVA followed by Dunnett's multiple comparison test). At 0.3mM, the response to 5-hydroxytryptamine was significantly reduced by schwarzinicine A treatment but not affected by DMSO vehicle (see *Figure 4.7C* and *Table 4.4*).

DMSO vehicle however, did not affect the responses to phenylephrine and KCl. Schwarzinicine A shifted the contractile curves of phenylephrine and KCl to the right but has little effect on U46619-induced contraction (see *Figure 4.7A*&*B* and *Table 4.4*). The rightward shift of KCl-induced contraction by schwarzinicine A could not allow the determination of pEC_{50}, hence the response comparison between control (without prior treatment with vehicle and schwarzinicine A), vehicle-treated and schwarzinicine A-treated aortic rings, was done at the last induced concentration of 300mM (*see Figure 4.7D* and *Table 4.4*). Both vehicle and schwarzinicine A did not affect the response of KCl at 300mM (*see Figure 4.7D* and *Table 4.4*). Nifedipine, on the other hand, significantly reduced the response to KCl at 300mM (see *Figure 4.7D* and *Table 4.4*).

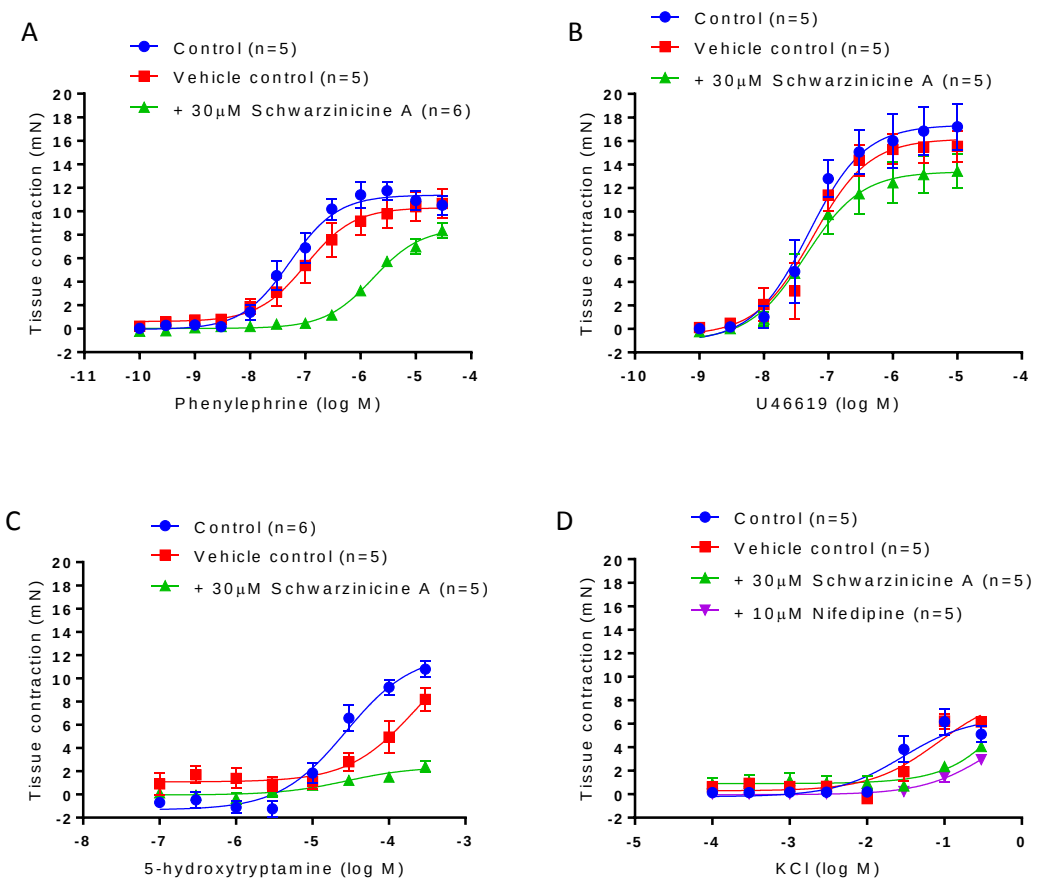


Figure 4.7: Effects of 30µM schwarzinicine A on the contractile response induced by (A) phenylephrine, (B) U46619, (C) 5-hydroxytryptamine and (D) KCl. Controls are the aortic tissues without prior treatments with schwarzinicine A and DMSO vehicle. The contractions were expressed as a force of contraction in force mN and data represented the mean values ± SEM of *n* number of animals. The corresponding vehicle control to 30µM schwarzincine A (0.165% DMSO v/v) was included for each graph. In *Figure 4.7D*, nifedipine was added as reference for comparing the relaxant effect of schwarzincine A in response to KCl-induced contraction tone. There was no significant effect of vehicle in phenylephrine, U46619 and KCl-induced contractions, except for 5-hydroxytryptamine. One-way ANOVA followed Dunnett's multiple comparison test comparing the contractions between control and those with prior treatments with schwarzinicince A and DMSO vehicle (for all contractile agents) as well as with nifedipine (for KCl-induced contraction only in *Figure 4.7D*) showed that schwarzinicine A significantly reduced the cumulative contraction to 5-hydroxytryptamine- and significantly shifted the contraction curves of phenylephrine and KCl to the right ($P<0.05$).

Contractile agonists	Control		Vehicle-treated		Schwarzinicine A-treated		Nifedipine-treated	
(A)	**Maximum response (mN)**	**pEC_{50}**	**Maximum response (mN)**	**pEC_{50}**	**Maximum response (mN)**	**pEC_{50}**		
(i) Phenylephrine	11.38 ± 0.40	7.28 ± 0.10	10.30 ± 0.52	6.99 ± 0.15	8.59 ± 0.30	5.77 ± 0.06 ****		
(ii) U46619	17.37 ± 0.98	7.31 ± 0.16	16.18 ± 0.80	7.24 ± 0.14	13.39 ± 0.74	7.36 ± 0.16		
(B)	**Maximum response at last concentration (mN)**		**Maximum response at last concentration (mN)**		**Maximum response at last concentration (mN)**		**Maximum response at last concentration (mN)**	
(i) KCl	5.10 ± 0.66		6.14 ± 0.29		4.06 ± 0.36		2.94 ± 0.22 **	
(ii) 5-hydroxytryptamine	10.79 ± 0.73		8.18 ± 0.98		2.41 ± 0.45 **			

Table 4.4: Summary of the contractile responses of all tested agents (phenylephrine, U46619, KCl and 5-hydroxytryptamine) in presence of DMSO vehicle and schwarzinicine A, as well as nifedipine for KCl-induced contraction only. Controls are the aortic tissues without prior treatment with schwarzinicine A and DMSO vehicle. This table is divided into two sections in which (A) depicting the maximum response (in unit mN) and pEC_{50} of phenylephrine- and U46619-induced contraction in the stated conditions, while (B) depicting the maximum response (in unit mN) of KCl- and 5-hydroxytryptamine-induced contraction at 300mM and 0.3mM respectively as pEC_{50} values could not be determined (explained in **Section 4.3.6**). The data represents the mean values ± SEM of *n* number of animals. The significant difference between the control aortic tissues and those with prior treatment was interpreted with ** ($p<0.01$) and **** ($p< 0.0001$) as stated in the table (one way ANOVA followed by Dunnett's comparison tests).

4.3.7 Schwarzinicine A inhibited the contractile response to $CaCl_2$ in calcium-free Krebs solution

Aortic tissues without pre-treated with schwarzinicine A and nifedipine served as the controls. Removal of extracellular calcium has reduced the contraction of aortic tissues to KCl with no further alteration in the presence of schwarzinicine A or nifedipine (see *Table 4.5*). In calcium-free Krebs solution, the calcium channel blocker nifedipine totally abolished the contractile responses upon re-addition of $CaCl_2$ to the system (see *Table 4.5* and *Figure 4.8*). The contractile response to $CaCl_2$ following pre-treatment with schwarzinicine A (3, 10 and 30μM) was concentration-dependent, with total abolishment observed in the presence of 30μM schwarzinicine A, which was similar to the reported nifedipine response (see *Table 4.5* and *Figure 4.8*). Vehicle did not affect the inhibitory effect of schwarzinicine A (see *Table 4.5*).

Treatment		*n* number	(i) KCl-induced contraction		(ii) $CaCl_2$-induced contraction	
			In calcium-free Krebs solution (%)	*p* value	Maximum contraction (%)	*p* value
Control		6	9.86 ± 4.02		92.38 ± 17.86	
+ Vehicle control						
	0.084%	6	9.70 ± 6.40	>0.9999	132.1 ± 11.53	0.2547
	0.055%	5	8.28 ± 6.30	0.9997	144.6 ± 17.53	0.1218
	0.165%	6	1.86 ± 9.07	0.8758	134.4 ± 20.46	0.2178
+Schwarzinicine A						
	3μM	6	13.24 ± 6.43	0.9979	72.40 ± 12.23	0.4857
	10μM	6	10.66 ± 6.48	0.9999	25.73 ± 5.65	*** (0.0006)
	30μM	6	8.71 ± 4.70	0.9998	2.22 ± 4.85	****(<0.0001)
+10μM Nifedipine		6	11.65 ± 4.31	0.9997	Abolished	****(<0.0001)

Table 4.5: Summary of the effects of schwarzinicine A (3, 10 and 30 μM) and nifedipine (10μM) on (i) KCl- and (ii) $CaCl_2$-induced maximum contraction, in calcium-free Krebs solution. Vehicle controls (DMSO) at 0.084%, 0.055% and 0.165% v/v represent schwarzinicine A at 3, 10 and 30 μM respectively. The contractions

were expressed as percentage of the KCl-induced contraction in normal Krebs solution. Data represents the mean values ± SEM of *n* number of animals. The significance difference between the contractions that were induced in the control and those with prior treatment with schwarzincince A (3, 10 and 30µM) or nifedipine is interpreted in stated *p* value. A *p* value of >0.05 is considered no significant difference.

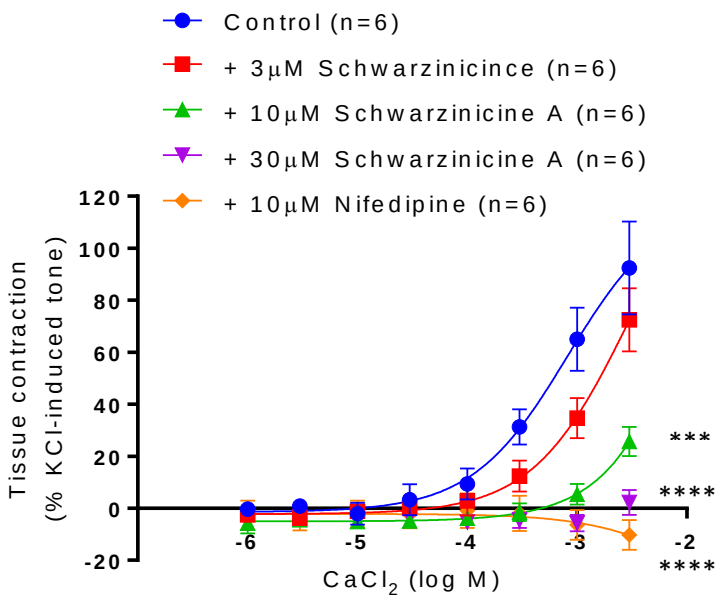


Figure 4.8: Effects of schwarzinicine A (3, 10 and 30µM) on $CaCl_2$-induced contraction in calcium-free Krebs solution. The contractile responses were expressed as the percentage to 60mM KCl-induced contraction tone. The data represents the mean values ± SEM of *n* number of animals. Significant reductions in $CaCl_2$-induced contractions were shown on those aortic tissues treated with 10 µM and 30µM of schwarzinicine A and 10µM nifedipine [one-way ANOVA followed by Dunnett's multiple comparison test, *** (p <0.001) and **** (p< 0.0001)].

4.4 Discussion

This study aimed to characterise the relaxant-induced mechanism of the novel plant indole alkaloid, schwarzinicine A on isolated rat tissues. Previous findings in Chapter 3 demonstrated that the relaxant effect of this compound was most potent and

efficient in isolated rat aorta (determined by its highest E_{max} and lowest pEC_{50} values), thus directing the subsequent experiments undertaken. With no knowledge of this compound in biological samples, a step-wise approach was applied to investigate and better understand its vascular effects.

Pre-exposure to the contractile agents, phenylephrine, KCl and U46619 in isolated rat aorta resulted in the generation of different relaxation tones to schwarzinicine A, in which the pre-contractile response to phenylephrine produced the most potent relaxation response as determined from the pEC_{50}. Pre-exposure to KCl induced a similar maximum relaxant response compared to phenylephrine, making KCl an alternative pre-contractile agent. In deducing the mechanism by which schwarzinicine A induced its relaxant response in rat aortic tissue, the present findings demonstrated that the relaxation to schwarzinicine A was independent of the influence from endothelium, NO, cGMP/cAMP and no involvement of potassium channel. It was also clearly demonstrated that schwarzinicine A-induced relaxation did not appear to be mediated via α- and β-adrenoceptors directly as the prior treatment of antagonists to these receptors did not affect its responses. In contrast, schwarzinicine A attenuated the cumulative concentration-contraction of phenylephrine, KCl and 5-hydroxytryptamine. In a calcium-free Krebs solution, schwarzinicine A also markedly suppressed the contractile response to $CaCl_2$. Therefore, it appears that schwarzinicine A affects the calcium movements into the aortic tissues in the presence of contractile agents.

Pre-exposure to contractile agents may evoke some underlying mechanisms that affect the following vascular relaxation (Eckly-Michel *et al.*, 1999) which may explain the different schwarzinicine A-induced relaxation profile exposed to different pre-contractile agents (phenylephrine, KCl and U46619). The present study showed the absence of endothelium and NO influence in schwarzinicine A-induced relaxation following pre-contraction to the α-adrenergic receptor agonist, phenylephrine. This addresses that the contribution of endothelial NO in potentiating the vascular relaxation is unlikely. With pre-exposure to KCl, a similar maximum schwarzinicine-induced response was observed to that of phenylephrine, but with a lower potency. In contrast, pre-exposure to U46619 (a thromboxane

angiotensin-II, TXA-II mimetic) produced a minimal relaxation to schwarzinicine A. Similarly, schwarzinicine A did not affect U46619-induced contraction. This shows that the relaxation by schwarzinicine A is selective and only affected the contractions mediated by α-adrenergic receptor (induced by phenylephrine) and depolarisation (induced by KCl).

Adrenergic receptor (α- and β-adrenergic receptors) signalling pathway is one of the major pathways in mediating vascular activity. In vascular tissues, α-adrenergic receptor is responsible for vasoconstriction while beta-adrenergic receptor is for vasodilation. An inhibition of α-adrenergic receptor will in turn result in vasodilation. The reduced phenylephrine-induced contraction and comparable relaxation efficacy as dobutamine (refer to Chapter 3) have raised the possible roles of schwarzinicine A in mediating α- and β-adrenergic receptor signalling pathways. However, this possibility is ruled out with no observed influence of α- (rauwolscine and prazosin) and β-adrenergic (timolol and propranolol) antagonists in schwarzinicine A-induced relaxation. This infers that schwarzinicine A does not act via both α- and β-adrenergic receptors.

Cyclic nucleotides (cAMP and cGMP) play a central role in mediating the relaxation of vascular tissues. These cyclic nucleotides are synthesised from enzyme adenylyl cyclase and guanylyl cyclase respectively. Mediators, such as nitric oxide, β-adrenergic receptor and potassium channel could activate the synthesis of these cyclic nucleotides. In present study, these mediators did not appear to affect the relaxation to schwarzinicine A, thus questioning the possible involvement of cAMP/cGMP in schwarzinicine A-induced relaxation. Phosphodiesterase plays a role in regulating the amount of intracellular cAMP or cGMP, thus the deactivation of this enzyme would retain the amount of active cAMP or cGMP in mediating the vascular relaxant response. However in the present study, a pre-treatment with phosphodiesterase inhibitors (rolipram and IBMX) did not affect schwarzinicine A-induced relaxation. The gathered evidence indicates that schwarzinicine A-induced vascular relaxation is not mediated via cAMP- or cGMP-dependent pathways.

From the above results, the focus was thereafter diverted to investigate whether schwarzinicine A exhibited its relaxant effect by reducing the calcium mobilisation induced by the contractile agents. In control samples, comparable concentration-response curve magnitudes were induced by all tested contractile agents (phenylephrine, U46619, KCl and 5-hydroxytryptamine), with KCl displaying the lowest magnitude. High KCl induces contraction via membrane depolarisation that allows the opening of VOCCs. In contrast, GPCR agonists mediate contraction by activating calcium entry through multiple calcium channels (such as VOCC, ROCC and SMOC) and calcium release from the internal stores through the opening of SOCC by IP_3 (Perez and Sanderson, 2005; Ratz *et al.*, 2005). The multiplicity of GPCR agonist-mediated contraction mechanisms may have explained the higher contraction magnitudes induced by GPCR agonists as compared to that by KCl.

In terms of the inhibitory effect of schwarzinicine A, the present study demonstrated a significant reduction in the phenylephrine, 5-hydroxytryptamine and KCl-induced contractile response, with no effect on the U46619-induced contractile response. The contractions to 5-hydroxytryptamine appeared more sensitive to the effect of DMSO vehicle. A study by Srivastava and co-workers (1998) reported that the reduced of 5-hydroxytryptamine -induced contractions by DMSO were as a result of the interaction of intracellular hydroxyl radicals (scavenged by DMSO) and endothelial NOS. Despite the inhibitory effect of DMSO on 5-hydroxytryptamine -induced contraction, a significant and further attenuation of contractile response was observed in schwarzinicine A-treated aortic tissues. The absence of a vehicle effect in phenylephrine-, KCl- and U46619-induced contractile responses suggested the absence of endothelial NOS-DMSO interactive effect.

Phenylephrine induces contraction by acting on α_{1D}- adrenergic receptor that activates phospholipase C (PLC) to hydrolyse phosphatidylinositol and produces two cell messengers, IP_3 and DAG. This leads to the opening of receptor-operated calcium channel (ROCCs). The IP_3 and DAG produced by noradrenaline stimulation were reported to mediate the activation of *transient receptor potential* subtype, TRPC6 in rabbit portal vein myocytes (Albert and Large, 2003), in which noradrenaline is also α- adrenergic receptor agonist. In addition, increasing

evidence also shows the dependence of receptor-operated calcium channel (ROCCs) to TRPC6 protein in regulating vasoconstriction (Boulay *et al.*, 1997; Inoue *et al.*, 2001; Jung *et al.*, 2002). The TRPC6 is found in Sprague-Dawley rat resistance arteries (Welsh *et al.*, 2002) and embryonic rat thoracic aortic smooth muscle cells (A7r5 cells) (Jung *et al.*, 2002). TRPC6 activation can also trigger an opening of a non-selective cation channel (NSCCs) downstream of DAG-mediated protein kinase C that leads to cation influx (Fuchs *et al.*, 2011; Inoue *et al.*, 2001; Albert & Large, 2006). DAG was also found directly activates mammalian TRPC3/6/7 channels (Harteneck and Gollasch, 2011; Fuchs *et al.*, 2011).

From this, the involvement of TRP channels via IP_3 and DAG in phenylephrine-stimulated contraction is hypothesised in present study. In addition, an involvement of TRP channel was also revealed in KCl-depolarised vascular smooth muscles in rabbit femoral and renal arteries (Ratz and Berg, 2006). Hence, a potential role of schwarzinicine A in inhibiting TRP channels is again suggested with regards of its induced changes in phenylephrine- and KCl-induced contractions. However, no such hypothesis to be made for the effects of schwarzinicine A in 5-hydroxytryptamine (in explaining the reduced of maximum contraction) and U46619-induced contractions (in explaining the absence of effect) due to the limited knowledge of TRP channel involvement in these two agonist activities.

The 5-hydroxytryptamine is a non-selective serotonin receptor agonist. Serotonin subtype, 5-HT (2A) is the predominant subtype that mediates the contraction in aorta as observed in rat (Villazón *et al.*, 2002) and mouse (Mckune and Watts, 2001). The contraction by this serotonin subtype is via the activation of phospholipase C and L-type calcium channel (Mckune and Watts, 2001). These mechanisms are characteristic to the activation of all G_q protein-coupled receptors. Phenylephrine, 5-hydroxytryptamine and U46619 are agonists to receptors that are coupled to G_q proteins. However, the absence effect of schwarzinicine A on the contraction to U46619 suggests that U46619 may display a different contraction signalling pathway other than G_q protein-coupled receptor signalling pathway in rat aorta. In addition to G_q protein, thromboxane receptor can also couple to different G proteins, such as $G_{12/13}$ protein (Gohla *et al.*, 2000). The coupling to this G-protein

leads to RhoA/Rho-kinase-mediated pathway that inhibits myosin light chain phosphates which indirectly promotes smooth muscle contractions (Gohla *et al.*, 2000). As an agonist to thromboxane receptor, U46619 may have elicited $G_{12/13}$ protein-mediated pathway in rat aortic rings in present chapter in which its response is insensitive to schwarzinicine A. This can be supported by Grann and colleagues (2015) addressing that the stimulation of thromboxane receptor by U46619 results in a direct MLCP phosphorylation via Rho kinase activity.

In the presence of calcium-free Krebs solution, schwarzinicine A reduced the potency and maximum response of $CaCl_2$ re-addition without interference of the vehicle. This suggests that schwarzinicine A caused a reduction in the calcium sensitivity and calcium influx in the rat aortic tissue. In summary, calcium channels appear to be the common calcium signalling mechanism exhibited by phenylephrine, KCl and 5-hydroxytryptamine. Therefore, schwarzinicine A may play role in inhibiting this signalling channels, but as yet the full underlying pathway is to be discovered.

CHAPTER 5: Pharmacological evaluation of schwarzinicine A-induced relaxation on porcine coronary arteries

5.1 Introduction

Schwarzinicine A appears to inhibit calcium mobilisation in isolated rat aorta. This activity will be explored in another vascular type, which is porcine coronary artery (PCA). The choice of this vascular type is to replace the need of euthanizing living laboratory animals (refer to Sprague-Dawley rats in this study) in meeting the principle of 'Three Rs' (European Pharmaceutical Industry Update, 2015) in addition to explore the effect of schwarzinine A on different vascular bed. The PCA is also favoured in pre-clinical testing due to its close resemblance to human counterparts (Suzuki *et al.*, 2011). This characteristic is essential in the future translational studies. Therefore, it serves as a good model in this experiment to investigate the effect of schwarzinicine A.

Cumulative studies have reported that the relaxation of substances may differ by the selectivity of pre-contractile agents [as seen in the relaxation of taurine in response to the contraction to KCl and α-adrenoceptor agonist (Franconi *et al.*, 1982; Ristori and Verdetti, 1991; Li *et al.*, 1996)]. Consistently, by referring to **Section 3.3.1** (Chapter 3), schwarzinicine A depressed the pre-contraction to phenylephrine and KCl, but produced a low relaxation to U46619 in rat aorta. However, the effect of schwarzinicine A on porcine coronary artery is yet to be explored. Depending on the vascular types and species, the effect of schwarzinicine A to pre-contractile agents may vary. Therefore, this study is extended to study the effect of schwarinicine A on the pre-contraction to U46619 in PCA tissues, in addition to explore the relaxant effect of schwarzinicine A in other vascular tissues, besides of rat aorta. U46619 is a commonly used pre-contractile agent for PCA tissues as it was reported to produce higher and more stable contraction, as

compared to other agents, such as noradrenaline, phenylephrine and prostaglandin $F_{2\alpha}$ in PCA tissues (Horst and Robinson, 1985; Stahl *et al.*, 1995).

A brief introduction on PCA, the calcium-mediated contraction in PCA and the regulation of thromboxane A_2 receptor by the thromboxane A_2 analogue, U46619 in vascular tissues are stated in the context.

5.1.1 Coronary arteries

Coronary arteries are the first branching off arteries from the root aorta. There are two coronary arteries, namely left and right coronary arteries (LCA and RCA respectively), that supplying oxygenated blood to the heart and transporting the blood from the heart to the body. These two coronary arteries reside at the left and right side of the heart respectively. Right coronary arteries pump blood to the lung while the left coronary arteries pump blood to the rest of the body.

5.1.1.1 Coronary arteries in porcine

In translational studies, pig is one of the non-rodent animal species choices, besides that of the dog and monkey (Swindle *et al.*, 2012). The pig shares similar anatomic and physiologic features as humans, for example in coronary artery systems (Swindle *et al.*, 2012).

Many studies have reported the close resemblance between the coronary artery systems of pig and man (Weaver *et al.*, 1986; Rodrigues *et al.*, 2005; Sahni *et al.*, 2008). Coronary artery systems from both species are similar in terms of the origin and branching pattern, as well as the blood distribution (Rodrigues *et al.*, 2005). Both coronary artery systems are made up of LCA and RCA. Additionally, studies have shown that the RCA in both species were dominant in their blood distribution (Weaver *et al.*, 1986; Sahni *et al.*, 2008; Swindle *et al.*, 2012). However, they also found subtle differences where minority of the pig hearts possessed left dominance supply by LCA and/or with a balanced blood supply (Weaver *et al.*, 1986; Crick *et al.*,

1998). These dominances were determined by the blood distribution routes of the coronary arteries.

Like any other blood vessel type, porcine coronary arteries have the same vascular structural arrangement that consists of tunica intima (endothelium), tunica media (elastic fibers and smooth muscle) and tunica adventitia (connective tissues) layers (Reese *et al.*, 2002). It is often difficult to construct a study that involves human tissues because of the limited sources, safety and ethical concerns, as well as consent issues. Therefore, the close resemblance between the pig and human tissues allows the use of porcine tissues as an alternative route to understand their physiological and pharmacological responses. In vascular studies, porcine coronary arteries are commonly used and their arterial dimensions (in term of external diameter and arterial wall thickness) are comparable to human arterial tissues (van Andel *et al.*, 2003).

5.1.2 Pharmacological analysis of calcium-mediated PCA contraction

The vascular tone of porcine coronary arteries (PCA) is mediated by calcium signalling pathways. The calcium signalling pathways involve the calcium entry from extracellular space and calcium release from the internal stores. Calcium enters the cell across the plasma membrane via voltage-dependent calcium channels (VOCCs), receptor-operated calcium channels (ROCCs) and second messenger-operated calcium channels (SMOCs). These calcium channels are activated by their distinct modulators, in which VOCCs are mediated by the change of membrane potential; ROCCs are mediated by the activation of abundant G protein-coupled receptors (GPCRs) whereas SMOCs are mediated by the presence of diffusible second messengers, such as inositol triphosphate (IP_3), nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic adenine nucleotide (cADP) and calcium ion itself (Berridge, 2007).

In response to the calcium channel activations, the downstream mechanisms including IP_3 and DAG release, protein kinase activation and MLCK phosphorylation

occur towards the smooth muscle contraction. Each component of the pathways is important for normal contraction of PCAs.

There is a wide variety of experimental drugs that modulates the signalling pathways. These drugs are not only used for understanding the Ca^{2+} pathways but also facilitating the characterisation of therapeutic products. For example, to investigate the presence of VOCCs in PCAs, the known calcium-entry agonists such as Bay K-8644, YC-170 and CGP 28392 (Sasaguri *et al.*, 1987) as well as the calcium channel blockers such as nifedipine, verapamil and diltiazem (Vaghy *et al.*, 1987) are commonly used. For downstream mechanisms, Y27632 and calphostin C are used to inhibit Rho-kinase and protein kinase C (PKC) respectively to study the calcium sensitisation in porcine coronary arteries (Nobe and Paul, 2001).

5.1.3 Regulation of thromboxane A_2 receptors in vascular tissues

Thromboxane A_2 receptor, or known as TP receptor belongs to G protein-coupled receptor superfamily. This receptor is responsible in platelet aggregation and vascular contraction. The mRNA of two splice variants of this receptor, TPα and TPβ, are found in vascular smooth muscles (Hirata *et al.*, 1996; Miggin and Kinsella, 1998). Quantitative *in vitro* receptor autoradiography also revealed the presence of thromboxane A_2 receptors in human coronary arteries (Katugampola and Davenport, 2001).

In vascular smooth muscles, TP receptors stimulate the contraction of smooth muscles via coupling to different G- proteins. In early study, it was revealed that the stimulation of TP receptors hydrolysed phospholipase C and caused intracellular calcium mobilisation (Houslay *et al.*, 1986). Later, this is confirmed by the coupling of TP receptors with G_q proteins (Shenker *et al.*, 1991). In rat coudal arterial smooth muscle, the coupling of TP receptors with $G_{12/13}$ proteins was also revealed in mediating calcium entry and calcium sensitisation (Wilson *et al.*, 2005). Another G protein, G_h was also found in coupling with TP receptors to activate phospholipase C-δ (Feng *et al.*, 1996).

In experimental studies, a TXA_2 analogue, U46619 is used extensively to study the mechanisms of TP receptors. The U46619-induced contraction involves the activation of Ca^{2+}/calmodulin/MLCK pathway (Turek *et al.*, 2002; Ding and Murray, 2005). U46619 is also known to phosphorylate many different protein kinases, such as protein kinase C (PKC), protein kinase A (PKA), tyrosine kinase (TK), Rho kinase (ROK) and mitogen-activated protein kinase (MAPK) (Kinsella *et al.*, 1994; Turek *et al.*, 2002, Bolla *et al.*, 2002; Ding and Murray 2005). The kinase phosphorylation by U46619 is selective between species and smooth muscle types. For example, in canine pulmonary vascular smooth muscle, activation of TK and ROK kinases, but not PKC and MAPK kinases were involved in U46619-induced contraction (Janssen *et al.*, 2001). In contrast, in rat mesenteric resistance arteries, only activation of p38MAPK by U46619 was observed (Bolla *et al.*, 2002).

5.1.4 Significances of study

The PCA tissues serve as a good model to examine the effects of putative therapeutic compounds due to its close resemblance to humans. The thromboxane A2 analogue, U46619 is commonly used to stimulate contraction in porcine coronary artery as shown in some studies by Nakayama and colleagues (2001), Nobe and Paul (2001) as well as Kaneda and colleagues (2010). This analogue is used in this study to pre-contract PCA before the cumulative administration of schwarzinicine A.

The objectives of this study is

(i) to investigate the relaxant effect of schwarzinicine A on isolated porcine coronary arteries that were pre-contracted with U46619 and

(ii) to study the effects of schwarzinicine A on calcium entry in calcium-free Krebs solution.

These examinations were in conjunction with the previous findings in Chapter 4 to compare the pharmacological responses of schwarzinicine A in a different species

and vascular tissue types. The experimental protocol was also designed to further elucidate the role of schwarzinicine A on calcium mobilisation.

5.2 Materials and Methods

5.2.1 Drugs and solutions

Schwarzinicine A, U46619, nifedipine, KCl were prepared as described **Section 3.2.1** and **Section 4.2.1** (Chapter 3 and 4 respectively). Calcium chloride ($CaCl_2$) was dissolved in distilled water and made up to 1M stock concentration. The calcium-containing and calcium-free Krebs solutions were prepared as described in **Section 3.2.1** (Chapter 3).

5.2.2 Preparation of tissues

Hearts were collected from freshly-slaughtered pigs from a local abattoir. The hearts were transported back to the laboratory in Krebs solution that was pre-gassed with O_2-CO_2 (95:5) gas mixture. The anterior coronary artery was dissected from each heart and stored overnight at 4^{o}C in pre-gassed Krebs solution. In the following day, tissues were finely dissected and cleaned from attached connective and fatty tissues. Cleaned tissues were cut into 4mm rings in length from each coronary artery. The porcine coronary artery (PCA) rings were then mounted on metal wire triangles and placed in 5ml-sized tissue baths filled with Krebs solution that was continuously gassed with O_2-CO_2 (95:5) gas mixture. All tissues were allowed to equilibrate for at least 30 mins before tension was applied at 68.67-78.48mN. A subsequent 30mins of equilibration was applied after all tissues were loaded with tension. All tissues were then stimulated with 60mM KCl (twice) to check their reactivity.

5.2.3 Organ bath experiments

All PCA rings were pre-constricted with U46619 (1 to 30nM) until a stable submaximal contraction was achieved (approximately 70% of the second KCl-induced contraction). This was followed by a cumulative application of schwarzinicine A (0.1nM to 30µM).

5.2.4 Calcium-free experiment

After the reactivity of PCA was confirmed, the calcium-containing Krebs solution was replaced with calcium-free Krebs solution and allowed to equilibrate for at least 30 mins. This was followed by single dosing with schwarzinicine A into the bath solution, which was thereafter incubated for another 30 mins. In present study, the effects of these two concentrations of schwrazincine A (10 and 30µM) were tested on PCA tissues. These two concentrations of schwarzinicine A were chosen based on their cumulative concentration-dependent relaxant effect in pre-contricted PCA tissues (refer to **Section 5.3.1**, *Figure 5.2*) and to investigate a concentration-dependent effect of schwarzinicine A on $CaCl_2$-induced contraction. A single addition of 60mM KCl was added after the schwarzinicine A treatment and subsequently allowed to plateau. Re-addition of $CaCl_2$ (1µM to 1mM) was then applied. The effect of the calcium antagonist, nifedipine was also tested in some experiments by replacing schwarzinicine A with 10µM nifedipine. The experimental procedure is summarised in *Figure 5.1*.

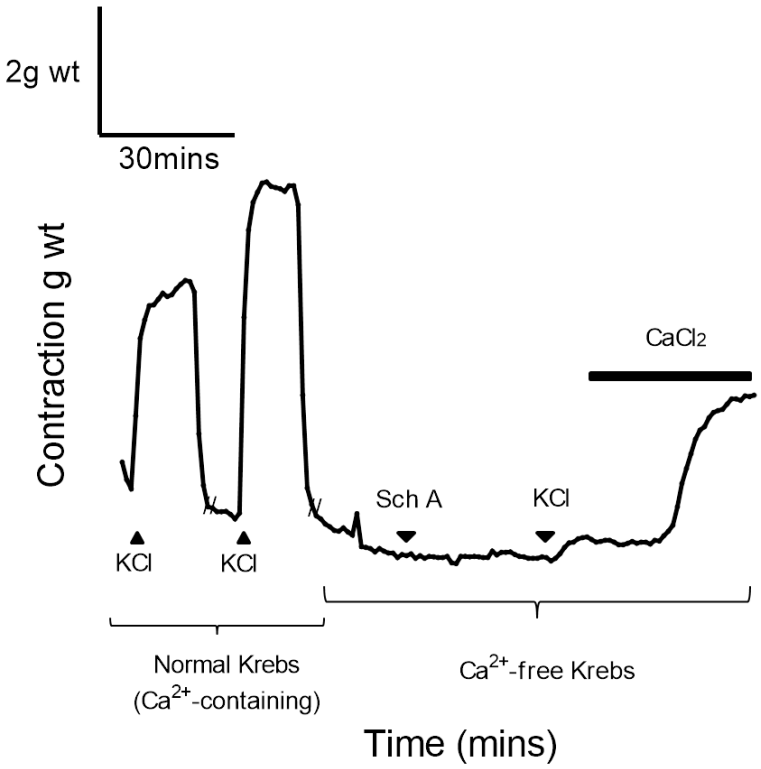


Figure 5.1: The representative experimental procedure for calcium re-addition experiments. Sch A: schwarzinicine A.

5.2.5 Data analysis

The relaxation response of PCA rings was normalised with the pre-contraction tone by U46619. For calcium-free experiment (**Section 5.2.4**), controls were the PCA rings without treatment. The PCA contraction to KCl in normal Krebs solution was used to normalise KCl- and $CaCl_2$-induced in calcium-free Krebs solution. The significance difference of KCl-induced contraction between control and treated PCA rings in calcium-free Krebs solution was compared using one-way ANOVA followed by Dunnett's multiple comparisons test. Same analysis test was also used to compare the significant difference of maximum $CaCl_2$-induced contraction between control and treated PCA rings in calcium-free Krebs solution. The overall experimental results on PCA rings from present chapter were compared and discussed with the findings in Chapter 4 (refer to **Section 4.3.1** and **Section 4.3.7**).

5.3 Results

5.3.1 Vasorelaxation effect of schwarzinicine A in U46619-induced contraction

Schwarzinicine A induced a significant relaxation (maximum response 141.4 ± 17.04%, pEC_{50} 4.47 ± 0.13, n=6) in PCA rings that were pre-constricted with U46619 (see *Figure 5.2*). There was no effect of vehicle (≤0.165% DMSO v/v) detected.

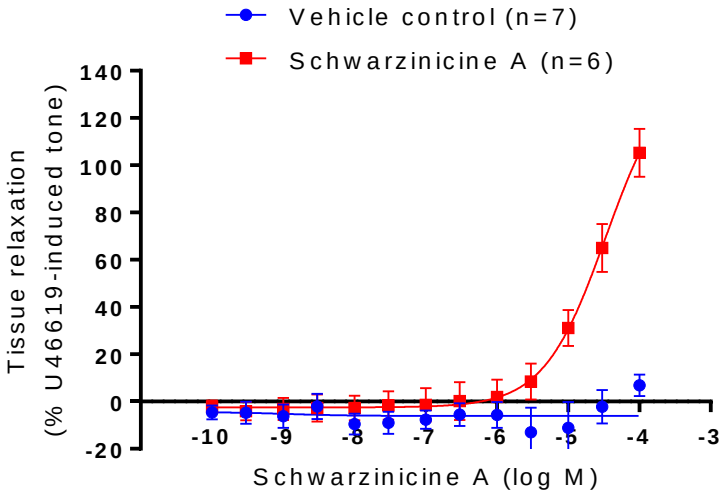


Figure 5.2: Effect of schwarzinicine A on PCA rings pre-constricted with 0.1µM U46619 (n=6). The relaxations were expressed as a percentage of U46619-induced contraction. The data represents the mean values ± SEM of *n* number of animals.

5.3.2 Schwarzinicine A inhibited the contractile response to $CaCl_2$ in calcium-free Krebs solution

In normal Krebs solution (calcium-containing), same contraction magnitude to KCl was observed in all PCA rings from all treatment groups. The KCl-induced contraction in normal Krebs solution was used to normalise the subsequent KCl- and $CaCl_2$-induced contractions in calcium-free Krebs solution. Removal of extracellular calcium (in calcium-free Krebs solution) has markedly reduced KCl-induced contraction (control, 27.6 ± 6.94%; see *Table 5.1*). Significant further reductions in KCl-induced contraction by both 30µM schwarzinicine A and 10µM nifedipine were observed (see *Table 5.1*). Schwarzinicine A at 10µM caused a

further but insignificant reduction in the induced contraction (see *Table 5.1*). Re-addition of $CaCl_2$ subsequently restored the contractile ability of the arteries.

Two concentrations of schwarzinicine A (10 and 30µM) markedly reduced the $CaCl_2$-induced contraction (see *Figure 5.3*). Maximum contraction was detected at 1mM $CaCl_2$ addition. The maximum $CaCl_2$-induced contraction (control, 153.6 ± 15.8%) was reduced by 58.2% under treatment of 10µM schwarzinicine A (64.28 ± 3.76%, n=6), and further reduced by 81.5% under treatment of 30µM schwarzinicine A (28.38 ± 7.13%, n=6). The calcium channel blocker, 10µM nifedipine (n=4) almost abolished the $CaCl_2$-mediated contractile response (7.74 ± 4.34%, n=4) at 1mM $CaCl_2$ (see *Table 5.1*). There was no significant influence of vehicles in the maximum $CaCl_2$-induced contractions, in corresponding to all treatments (see *Table 5.1*).

PCA rings with treatment of	*n* number	(i) KCl-induced contraction		(ii) $CaCl_2$-induced contraction	
		In calcium-free Krebs solution (%)	*p* value	Maximum $CaCl_2$-induced contraction (%)	*p* value
Control	6	27.6 ± 6.94		153.6 ± 15.8	
10µM schwarzinicine A	6	10.69 ± 4.66	0.0514	64.28 ± 3.76	*** (<0.0001)
30µM schwarzinicine A	7	3.79 ± 1.12	** (0.0025)	28.38 ± 7.13	*** (< 0.0001)
10µM Nifedipine	4	6.87 ± 2.71	* (0.0286)	7.74 ± 4.34	*** (< 0.0001)
Vehicle (0.055% DMSO v/v)	6	20.22 ± 4.89	0.6704	163.9 ± 12.88	0.9646
Vehicle (0.165% DMSO v/v)	7	20.72 ± 4.16	0.6964	122.2 ± 17.04	0.2422

Table 5.1: Summary of the effects of schwarzinicine A and nifedipine on the contractions to (i) KCl and (ii) $CaCl_2$ in calcium-free Krebs solution. Controls are PCA rings without treatments. Both KCl- and $CaCl_2$-induced contractions in calcium-free Krebs solution are expressed as the percentage of the contraction to KCl in calcium-containing Krebs solution. Data is presented as mean ± SEM values of *n* number of animals. A *p* value of <0.05 indicates a significant difference between the response of control and treated PCA rings to (i) KCl and (ii) $CaCl_2$ in calcium-free Krebs solution (one-way ANOVA analysis followed by Dunnett's multiple comparisons test). There was no significant influence of vehicles in the maximum $CaCl_2$-induced contractions.

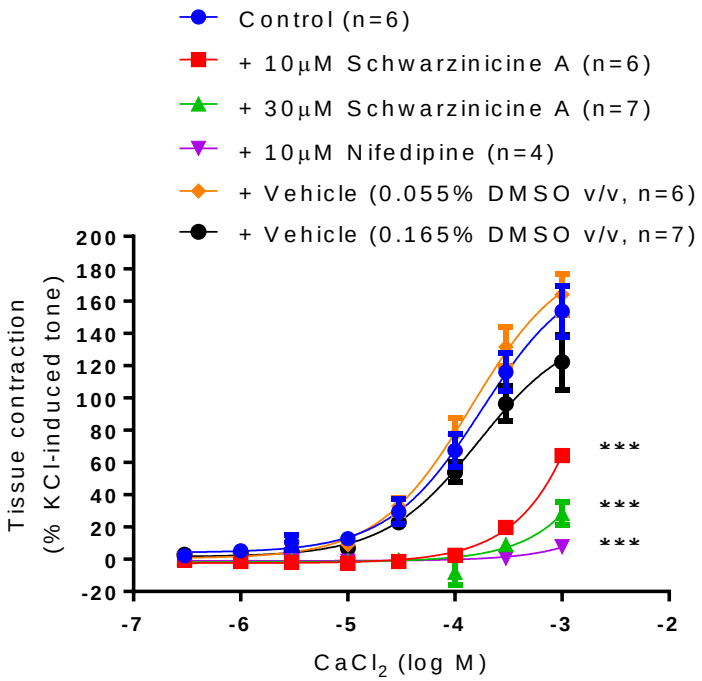


Figure 5.3: Effects of schwarzinicine A (10µM and 30µM, both at n=6) and nifedipine (10µM) on cumulative $CaCl_2$-induced contraction in calcium-free Krebs solution. The respective vehicles for 10µM (0.055% DMSO v/v, n=6) and 30µM schwarzinicine A (0.165% DMSO v/v, n=7) were included. There was no significant influence of vehicles in the maximum $CaCl_2$-induced contractions.The contractions were expressed as the percentage of the contraction to KCl in calcium-containing Krebs solution. The data represents the mean values ± SEM of *n* number of animals. The $CaCl_2$-induced contractions were markedly reduced by schwarzinicine A at both concentrations of 10µM and 30µM [one-way ANOVA analysis followed by Dunnett's multiple comparisons test, *** ($p < 0.001$)].

5.3.3 Comparison of schwarzinicine A-induced effects on isolated PCA arteries and rat aorta

Similar to rat aorta (refer to Chapter 4, **Section 4.3.1** and **Section 4.3.7**), schwarzinicine A also induced relaxation in isolated PCA rings and inhibited $CaCl_2$-induced contraction.

In calcium-free Krebs solution, a markedly reduction in KCl-induced contraction was observed in both PCA and rat aortic tissues (PCA, 27.6 ± 6.94% vs rat aorta, 9.86 ± 4.02% of their respective KCl-induced contractions in normal Krebs solution). This KCl-induced contraction was further reduced significantly by 30µM schwarzinicine A

and 10μM nifedipine in PCA rings. Such observation did not occur in the rat aortic tissues. The maximum $CaCl_2$-induced contraction data in PCA and rat aortic tissues were investigated at 1mM and 3mM $CaCl_2$ respectively. The control $CaCl_2$-induced contraction in isolated PCA arteries was much higher (153.6 ± 15.8% of respective KCl-induced contraction) than that of rat aorta (92.38 ± 17.86% of respective KCl-induced contraction), in which explained the different data collection points (refer to *Figure 4.8* and 5.3, and *Table 5.2*). In both tissues, a concentration dependent decrease in the maximum contractile response to $CaCl_2$ re-addition was observed with increasing concentrations of schwarzinicine A (refer to *Table 5.2*). Nifedipine also abolished the contraction to $CaCl_2$ in rat aorta and almost abolished the contraction in PCA tissues (see *Table 5.2*).

Tissues	**Maximum $CaCl_2$-induced contraction (%)**			
	Control	**10μM schwarzinicine A**	**30μM schwarzinicine A**	**10μM nifedipine**
Rat aorta	92.38 ± 17.86	25.73 ± 5.65	2.22 ± 4.85	Abolished
PCA arteries	153.6 ± 15.8	64.28 ± 3.76	28.38 ± 7.13	7.74 ± 4.34

Table 5.2: Comparison of the effects of schwarzinicine A in isolated rat aorta and PCA arteries. Schwarzinicine A caused similar inhibition on $CaCl_2$-induced contraction at 10 and 30μM of schwarzinicine A. Potency value (indicated by pEC_{50}) for schwarzinicine A-induced relaxation curves in isolated rat aorta was unavailable due to insufficient data points. Data is presented as mean ± SEM values. Maximum $CaCl_2$-induced contraction is expressed in the percentage of KCl-induced contraction.

5.4 Discussion

Pharmacological responses between tissues may vary between species and tissues types, thus a justification for investigating schwarzinicine A in PCA and comparing the respective responses with rat aortic tissues in this study. The present experiments demonstrated that schwarzinicine A induced a relaxant response in isolated PCA arteries that were pre-contracted with the thromboxane A_2 mimetic,

U46619. Additionally, schwarzinicine A was confirmed to inhibit the cumulative contraction of $CaCl_2$ at 10 and 30μM. These results are consistent with the previously reported findings investigating similar properties of schwarzinicine A on isolated rat aorta (refer to *Figure 4.2* and *Figure 4.8*).

It is generally known that one of the contractions of smooth muscle is due to calcium mobilisation. This calcium mobilisation has been observed to be mediated by either the activation of calcium channels, depolarisation of the membrane or inhibition of calcium-activated potassium channels (K_{Ca}). Moreover, the relaxation of smooth muscle may be due to the reduction or inhibition of calcium mobilisation and activation of potassium channels. Many studies have reported that U46619 mediated contraction results from activation of calcium channels that allow calcium influx and release into the cells (Tosun *et al.*, 1998; Turek *et al.*, 2002; Ding and Murray, 2005) via the activation thromboxane receptors. Thromboxane receptors are known to couple to either G_q or $G_{12/13}$ proteins (Shenker *et al.*, 1991; Wilson *et al.*, 2005). Therefore in the present study, the schwarzinicine A-induced relaxation was proposed as occurring from the inhibition of calcium channels that limit calcium mobilisation, evidence found in both rat aorta and porcine coronary artery.

KCl is a contractile stimulus that bypasses G protein-coupled receptors (GPCRs) in stimulating membrane depolarisation and L-type VOCCs. Therefore, it has been used as a comparative tool in evaluating the complex contraction/relaxation mechanisms of other drugs in smooth muscles. Generally, KCl induces membrane depolarisation and calcium entry via L-type VOCCs lead to a downstream calcium/calmodulin/MLCK-mediated contraction pathway. In addition to this, KCl is also found to trigger a calcium sensitisation process by inhibiting MLCP via Rho kinase activation (Sakurada *et al.*, 2003), in which this process was initially thought to be selective for $G_{12/13}$ protein-coupled receptor activations (Somlyo and Somlyo, 2000). The MLCP dephosphorylates the myosin light chain, which relaxes the smooth muscle. Calcium sensitisation inhibits this activity and occurs in conjunction with MLCK activity which is agonist-activated and calcium-dependent, in order to produce a sustained contraction (Mizuno *et al.*, 2008).

In calcium-free experiments, removal of extracellular calcium and addition of nifedipine (L-type VOCC inhibitor) were included to eliminate the factor of calcium entry from extracellular space. However, removal of extracellular calcium has reduced but not abolished the KCl-induced contraction in PCA and rat aortic tissues, suggesting an alternative role of KCl in inducing vascular contraction, potentially via calcium sensitisation pathway. Consistent to this, in rabbit aortic smooth muscle, KCl-induced calcium sensitisation via Rho activation was abolished upon the removal of extracellular calcium (Sakurada *et al.*, 2003). In addition to its VOCC blockage, nifedipine was also known to inhibit KCl-induced calcium sensitisation in rabbit femoral and renal arteries (Ratz and Miner, 2009). Present study showed that the KCl-induced contraction in PCA but not in rat aorta was further reduced by nifedipine in calcium-free Krebs solution. This suggests a potential role of KCl-induced calcium sensitisation in PCA but little or absence in rat aortic tissues. Schwarzinicine A also caused a similar reduction in KCl-induced contraction in PCA rings, as shown by nifedipine, may also indicate a possible role of schwarzinicine A in calcium sensitisation pathway.

Both aorta and coronary arteries belong in the class of large arteries and share the same vessel structures. However, the size difference of PCA and rat aortic tissues may affect the relative comparison of their pharmacological responses. Therefore, in obtaining a relative comparison, a normalisation is required for the contractions of PCA and rat aortic tissues to KCl and $CaCl_2$ in calcium-free Krebs solution. These contractions were normalised with their respective contractions to KCl in normal Krebs solution. The contribution of Rho kinase/MLCP-dependent calcium sensitisation pathway in vascular contraction is shown higher in large arteries class (thoracic aorta) compared to the small arteries class (small mesenteric artery) (Kitazawa and Kitazawa, 2012). Considering the same arterial class PCA and rat aorta belong, the KCl-induced calcium sensitisation is likely present. However, KCl-induced contraction in PCA rings was relatively higher compared to that in rat aorta, suggesting a higher contributory role of KCl-induced calcium sensitisation in PCA tissues than that in rat aortic tissues. Greater $CaCl_2$-induced contraction was also observed in PCA compared to that in rat aortic tissues. This induced contraction in

PCA tissues also appeared to be more sensitive to schwarzinicine A than that in rat aortic tissues.

Both aorta and coronary arteries share the same structural arrangement, consisting of *tunica* intima, *tunica* media and *tunica* adventitia (Li, 2004). *Tunica* media is the thickest layer as seen in guinea pigs and Wistar rats (de Mello *et al.*, 2004). The amounts of elastic fibers and smooth muscle cells within this layer are proportional to the layer thickness (de Mello *et al.*, 2004; Alberts *et al.*, 2002). Theorectically, vascular response can be enhanced by the greater amount of smooth muscle cells within the layer. There is no direct evidence comparing the thickness of *tunica* media between PCA tissues and Sprague-Dawley rat aorta. However, a study (de Mello *et al.*, 2004) showed that there was a significant difference in the thickness of *tunica* media between guinea pig and Wistar rat aortic tissues (218.1 µm vs 87.3 µm), in relative to their body weight (450g and 300g respectively). Therefore, the greater body weight of a pig may suggest a thicker *tunica* media within its coronary artery, as compared to that of rat aorta, hence the higher $CaCl_2$-induced contractions and sensitivity to schwarzinicine A. However, further evaluation is recommended to investigate the relationship between the thickness of *tunica* media and drug sensitivity.

In summary, the relaxant effect of schwarzinicine A was proven in both PCA and rat aortic tissues. The KCl-induced calcium sensitisation in calcium-free solution was proposed and this appeared to be inhibited by schwarzinicine A in both PCA and rat aortic tissues. The role of schwarzinicine A in calcium-dependent mechanisms is further elucidated in Chapter 6 using calcium-rich rat dorsal root ganglion (DRG) cells.

Chapter 6: Calcium imaging on schwarzinicine A-induced effect using rat dorsal root ganglion cells

6.1 Introduction

In complementing the previous results on isolated rat aortic and porcine coronary arterial tissues, the potential inhibition of schwarzinicine A on calcium signalling pathway is further investigated on rat dorsal root ganglion (DRG) cells, as described and discussed in the present chapter. This assay is selected due to the cell's variety of calcium signalling pathways.

6.1.1 Calcium imaging

Calcium imaging technology was developed to monitor calcium dynamics in living cells. Calcium dynamics can be visualised with the implementation of calcium indicators and appropriate imaging instrumentation. The development of calcium imaging is an ongoing process with parallel development and improvement of calcium indicators and imaging systems.

The development of calcium imaging technology evolved from development of Ca^{2+}-sensitive dyes and their acetoxymethyl ester forms (Pozzan *et al.*, 1982, Grynkiewics *et al.*, 1985), introduction of genetically-encoded calcium indicators (GECIs), development of calcium indicators that utilise Föster resonance energy transfer (FRET) (for example FIP-CB_{SM} and cameleon) (Romoser *et al.*, 1997; Miyawaki *et al.*, 1999) to numerous improvement strategies to enhance the performance of calcium imaging. These improvement strategies included the development of calcium sensors with single fluorophore (for example camgaroos, pericams, GCaMP) (Nagai *et al.*, 2001; Nakai *et al.*, 2001) and improvement of FRET-based GECIs by introduction of circularly-permuted fluorophores (Baird *et al.*, 1999; Nagai *et al.*, 2004), replacement of calcium-sensing moiety (Heim and Griesbeck

2004) and structural remodelling of their calmodulin-peptide interface (Palmer *et al.*, 2006).

In general, calcium indicators are classified into two groups, chemical calcium indicators and GECIs. Comparing both, the application of chemical calcium indicators is much more user-friendly and does not require cell transfection in which it is necessary for GECIs (Paredes *et al.*, 2008). Chemical calcium indicators are also more commercially available with a broader range of calcium affinities than the genetically-encoded fluorescent proteins (Paredes *et al.*, 2008). However, the cellular localisation of chemical calcium indicators is difficult to control, thus and compartmentalisation of indicators may happen during long hours of experimentation (Paredes *et al.*, 2008). These problems can be overcome by the use of genetically-encoded fluorescent proteins that allow precise dye targeting and thus favouring a longer working period.

Calcium indicators are chosen based on their calcium-binding affinity that is determined by their dissociation constant (K_d). The K_d of indicators is dependent on the concentration of calcium ion by which the indicators respond to the calcium ion concentration range between 0.1 to 10 times of their K_d values (Paredes *et al.*, 2008). Upon binding with calcium ion, different calcium indicators respond differently, by shifting their absorption or emission spectrum, or even changing their emitted fluorescence intensities (Takahashi *et al.*, 1999).

For the ease of visualisation, calcium indicators can be loaded to the living cells using the three common approaches, which are single cell loading, ester loading and GECI expression. Single cell loading is achieved by using sharp electrode, whole-cell patch clamp and single cell electroporation (reviewed by Grienberger and Konnerth, 2012). Ester loading is applicable for those fluorescent calcium indicators with attached acetoxymethyl ester that are cell permeable (Tsien *et al.*, 1982). For GECI expression, calcium indicators can be inserted into the cells via viral transduction, *in utero* electroporation and generation of transgenic mouse lines (reviewed by Grienberger and Konnerth, 2012).

Once the indicator is loaded into the cells, the calcium dynamics can then be monitored using optical imaging systems such as multi-parameter digitized video microscopy, confocal laser scanning microscopy, two-photon excitation laser scanning microscopy, pulsed-laser imaging, photomultiplier tube and time-resolved fluorescence imaging microscopy (reviewed by Takahashi *et al.*, 1999). The choice on the imaging system is dependent on the calcium indicators used and the purpose of experiments undertaken. Fang and colleagues (2009) detailed the factors of imaging system considerations based on the frequency of calcium measurement, speed of acquisition, UV-transmission properties of indicators, quantum efficiency and spatial resolution of camera.

6.1.2 Fura-2 and its acetoxymethyl (AM) ester derivative, fura-2/AM

Fura-2 is a fluorescence calcium indicator (Grynkiewics *et al.*, 1985). When bound to Ca^{2+}, shifting of the absorption peak by Fura-2 is observed (Grynkiewics *et al.*, 1985). Fura-2 has a maximal absorption peak range of 335 to 363nm, and an emission peak of 500nm. This dye also has a dual excitation wavelength (340 and 380nm) that allows a ratiometric measurement of intracellular calcium content.

Studies have reported the broad use of Fura-2 in many living cells and tissues, such as in rat hepatocytes primary cultures (Rooney *et al.*, 1989), neurons cells from frog and chick (Lipscombe *et al.*, 1988; O'Donovan *et al.*, 1993); plant mitochondria (Zottini and Zannoni, 1993) and pancreas acini (Betzenhanser *et al.*, 2013). In its original form, fura-2 impermeable to the cell membrane; however, this is overcome with an acetoxymethyl (AM) ester bound. This fura-2 derivative is known as fura-2/AM that is membrane permeable (Grynkiewics *et al.*, 1985). Once fura-2/AM enters the cells, the bond between fura-2 and AM ester is cleaved off by intracellular esterase. The free fura-2 can now bind with the intracellular calcium.

For some calcium indicators, a limited range of calcium can be measured. The dual excitation wavelengths of fura-2 allow a wide concentration range of calcium to be imaged (Szmacinski and Lakowics, 1995). Fura-2, even when not bound to calcium

(calcium-free), can still be excited at a wavelength of ≥390nm, thus providing some reference for a calcium-free state (Szmacinski and Lakowics, 1995).

6.1.3 Dorsal root ganglion neurons

Dorsal root ganglion (DRG) is a peripheral sensory ganglion that is present between the dorsal root and spinal cord. This ganglion is made of a permeable connective tissue capsule and contains pseudounipolar neurons. These neurons are responsible for conveying neuronal information from peripheral tissues to the central nervous system (CNS). They are also sheathed by satellite glial cells with resident receptors that respond to neuroactive agents and signals from neighbouring cells.

The neuronal cell size is one of the main criteria to differentiate DRG neurons. The heterogenous subpopulations of DRG neurons have been identified and classified into two main populations, which are small dark and large light neurons, based on their cell size and cytoplasmic appearance (Price, 1985). In general, there are four types of neurons which are Aα-, Aβ-, Aδ and C-type neurons. Harper and Lawson (1985) correlated the size of DRG neurons with their axonal conduction velocities; in which large cell bodies contain rapidly-conducting Aα- and Aβ- fibers whereas the smaller cell bodies contain the slower-conducting Aδ and C-type fibers. In a later study (Petruska *et al.*, 2000), DRG neuronal populations were further classified into nine subtypes based on their current responses, cell size, action potential, as well as their responses to proton, capsaicin and ATP.

6.1.3.1 Calcium signalling in DRG neurons

Similar to other cell types, neuronal cells also exhibit calcium signalling to perform relative cellular functions. In DRG neurons, several types of voltage-gated calcium channels including T-, L-, N-type calcium channels, have been identified in chicks (Tsien *et al.*, 1988) and rats (Scroggs and Fox, 1992). Other new P/Q- and R- type

calcium channels were also identified in rat DRG neurons (Mintz *et al.*, 1992; Fang *et al.*, 2010). These channels were categorised based on their pharmacological and electrophysiological properties.

Neurons respond to external and internal calcium stimuli by implementing the concept of 'neuron-within-a-neuron' (Berridge, 1997). This concept is based on a binary membrane system that involves the plasma membrane and intracellular endoplasmic reticulum (ER) in a neuronal cell (Berridge, 1997). The plasma membrane responds to the external stimuli and generates fast propagating action potentials via voltage-dependent sodium and calcium channels (Berridge, 1997). In contrast, ER responds to the internal signals and generates slower propagating and regenerative calcium signal via the receptor activation of IP_3 receptors and ryanodine receptors (Berridge, 1997). Despite of the individual roles of plasma membrane and ER in calcium signalling, the reciprocal interaction between these two systems is crucial for neuronal functions (Berridge, 1997).

6.1.4 Significances of study

The main aim of this chapter was to investigate the effect of schwarzinicine A on calcium dynamics using DRG neuronal cells. The dominant neuronal L-type calcium channel is the primary focus. Using a L-type calcium channel blocker, nifedipine, the potential inhibitory effect of schwarzinicine A on KCl-induced calcium mobilisation was compared and evaluated. DRG neuron cells were loaded with fura 2/AM and their calcium dynamics were monitored using a digital microscopic system.

6.2 Materials and Methods

6.2.1 Drugs and solutions

All chemicals and reagents were purchased from Sigma-Aldrich (UK) unless stated otherwise. Superfusion buffer was prepared following composition (in mM): NaCl 145; KCl 5; $CaCl_2$ 2; $MgSO_4$ 1; HEPES 10; glucose 10 at pH 7.4. Buffer was kept

constantly at 37^{o}C during use. Fura 2-AM was dissolved in anhydrous DMSO to make a stock concentration of 5µM. Schwarzinicine A and nifedipine were prepared as stated in Chapter 3.

6.2.2 Glass coverslip preparation and coating

Glass coverslips (19mm diameter) were sterilised following the steps described: soaked in 10% Decon 90% (Decon Laboratories Ltd, UK) overnight, rinsed twice with double distilled water (ddH_2O), rinsed under flowing tap water for 2 hours, rinsed twice again in ddH_2O, soaked in concentrated HCl for 30 minutes, rinsed twice with ddH_2O, rinsed under flowing tap water for 2 hours, rinsed three times with ddH_2O and finally autoclaved. Autoclaved glass coverslips were stored in sterile distilled water. For coating, 100µl of poly-L-lysine was added at the centre of coverslips, then left to dry for 20 minutes at room temperature. The coverslip was subsequently washed once with sterile water and left to dry. This was followed by another coating with laminin (1mg/ml) and allowed to dry for at least 45 minutes. The coverslips were then washed once with sterile water and left to dry before use.

6.2.3 Dorsal root ganglia (DRG) cell preparation

Dorsal root ganglia (DRG) neurons were isolated from adult male Sprague-Dawley rats following the method descriptions by Lindsay (1988). Two rats (250-300g) were killed by CO_2 exposure and then cervical dislocation. The skin of the rat dorsal surface (back) was lifted and cut, and the underlying vertebral column was isolated. The isolated vertebral column was then placed onto a petri dish containing Hank's balanced saline solution with HEPES (0.1M, pH7.4) and 10% horse serum. Any bones and connective tissues were removed from the column. A midline incision was made along the ventral plate of the vertebral column, exposing the inner DRGs. The DRGs were removed carefully and placed in HEPES-supplemented HBSS and washed once under gravity. DRGS were incubated in 5ml neurobasal media (Invitrogen, UK) (containing 2.5mg/ml collagenase and supplemented with 10%

horse serum) at 37^{o}C, 5% CO_2 for 90 minutes. Ganglia were then washed twice with Dulbecco's calcium- and magnesium-free phosphate buffered saline (PBS), followed by incubation in 5ml PBS with 2.5mg/ml porcine trypsin. The DRGs were washed twice with PBS and placed in 1ml of neurobasal medium without serum before being triturated several times with plastic pastettes to form a cell suspension. The cell suspension was layered carefully on top of 4ml HBSS containing 15% bovine serum albumin solution, forming a bilayer. This bilayer was centrifuged at 500g, 20^{o}C for 5 minutes. The cell pellet was collected and resuspended in 1ml neurobasal medium supplemented with 10% horse serum, which later filled up to 1.5ml neurobasal medium with 50ng/ml glial cell line-derived neurotrophic factor (GDNF), 2mM L-glutamine, 200U/ml penicillin and 200ng/ml streptomycin. Cell suspension of 150µl was pipetted onto a poly-L-lysine- and laminin-coated glass coverslip, and incubated at 37^{o}C, 5% CO_2 overnight. The incubated cells on a glass coverslip were then ready for intracellular calcium imaging experiments.

6.2.4 Fura 2-AM cell loading

Before imaging, a fluorescent calcium dye, Fura 2-AM was loaded into the cells to allow the visualisation of intracellular calcium dynamics. The cells were washed three times with superfusion buffer to remove any residual serum. A Fura 2-AM solution (2.5µl of 5µM Fura 2-AM in 450µl superfusion buffer and 50µl horse serum) was added to the cells, followed by 30-minute incubation in the dark. Cells were washed three times with superfusion buffer and left in the buffer for 15 minutes.

6.2.5 Calcium imaging of DRG neurons

In a field of 30-40 neuronal cells, the intracellular Ca^{2+} ($[Ca^{2+}]_i$) of individual neurons were estimated as the change in the ratios of peak fluorescence intensities (measured at 500 nm) at excitation wavelengths of 340 and 380 nm respectively (Improvision System). The mean diameter of the cells (n=535) was 27.5 ± 0.2 mm. Coverslips were fixed on a Perspex chamber using vacuum grease. DRG neurones

were superfused (2ml/min) with 30mM KCl (in superfusion buffer, 60s) to evoke a depolarisation-induced Ca^{2+} influx and this was represented as the control response. This was followed by a 45-minute washout period, before a direct addition of 1ml schwarzinicine A (30µM) for 4 minutes and later an addition of combined 30mM KCl and 30µM schwarzinicine A. Similar experiments were repeated by replacing schwarzinicine A with 30µM nifedipine.

6.2.6 Data analysis

Neurones that exhibited an increase <0.1 from basal ratio after the superfusion with KCl, were treated as non-viable and excluded from data collection. The *n* number represents the number of neuron cells tested. The mean 340/380nm ratio represents the $[(Ca^{2+})_i]$ while the changes in $[(Ca^{2+})_i]$ were expressed as the percentage of the initial KCl response (first addition at 30mM). Data were expressed in mean values ± SEM. Neurones that displayed <10% of KCl responses were treated as non-responsive to drug. Statistical analysis was performed using one way ANOVA with any P value <0.05 considered statistically significant.

6.3 Results

High potassium (30mM KCl) produced a sharp peak mean 340/380nm ratio increase of 0.36 ± 0.01 (n=271) from basal level. Washing with superfusion buffer reversed this response from a depolarising state to basal level. The response of second KCl addition was comparable as the initial KCl response (81.81 ± 0.76%, n=271). This response is regarded as the control response. During the four-minute infusion of schwarzinicine A (30µM), a slight decrease of 340/380nm ratio was observed (refer to the circle in red in *Figure 6.1A*). In the preceding combination with schwarzinicine A, KCl peak response was significantly reduced to 55.16 ± 1.34% compared to that of the control KCl response (P<0.0001, n=183).

The presence of L-type calcium channel upon depolarisation with high KCl was investigated using the channel blocker, nifedipine. During the infusion with nifedipine, a gradual and slow increase of 340/380nm ratio was observed (refer to the circle in red in *Figure 6.1B*). This was followed by a further sharp peak increase during the preceding combination of KCl and nifedipine (refer to *Figure 6.1B*). In the overall result, no influence of vehicle (0.033% DMSO v/v) was observed (refer to *Figure 6.2*).

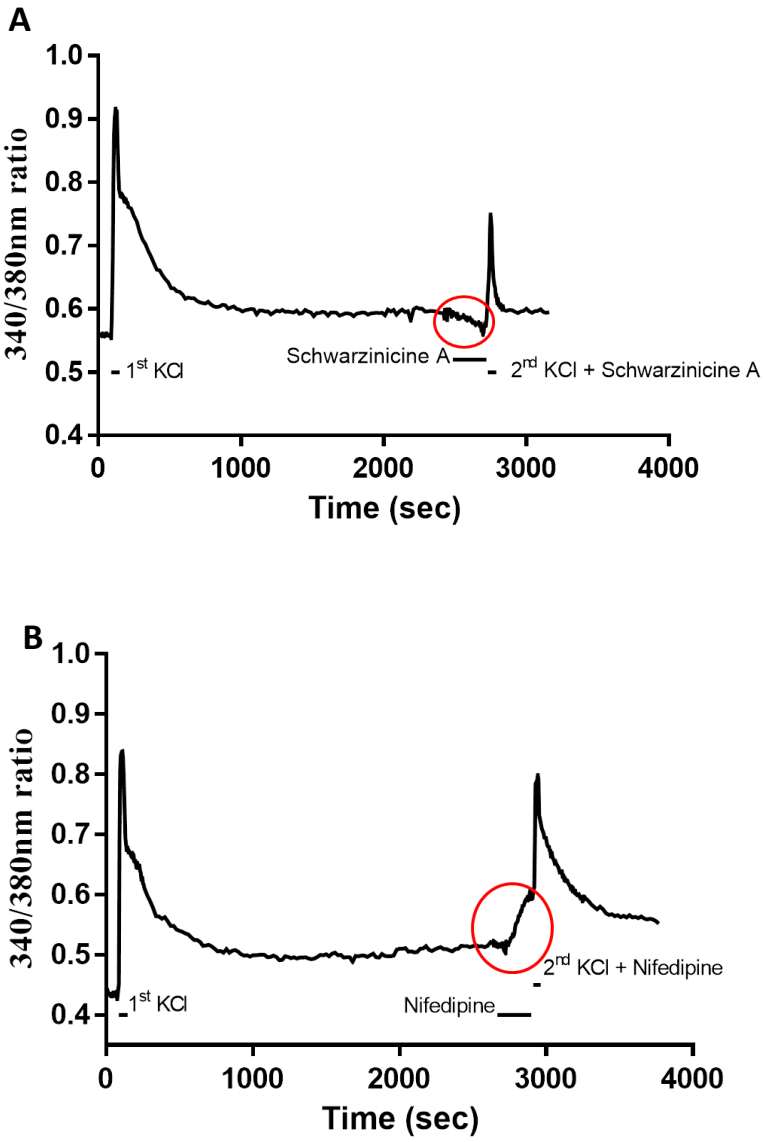


Figure 6.1: Representative calcium imaging traces of (A) schwarzinicine A- and (B) nifedipine-treated neuron cells for the entire experimental period. The addition of drugs is indicated by the black bars below the traces. Circles in red in *Figure 6.1A* and *B* illustrate the effects of four-minute infusion of schwarzinicine A and nifedipine on DRG neuron cells respectively. There was no influence of vehicle (0.033% DMSO v/v) observed.

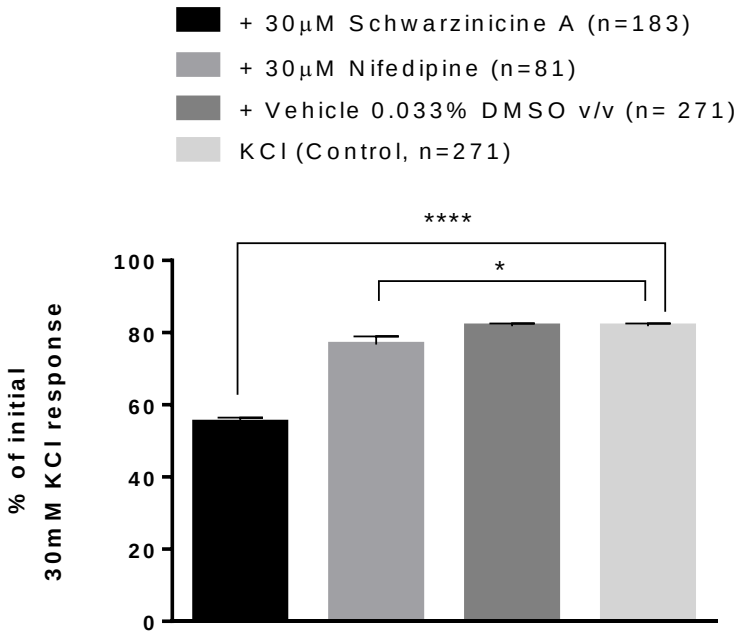


Figure 6.2: Effects of schwarzinicine A (n=183) and nifedipine (n=81) on KCl peak response, along with their effects during infusion. All data are expressed as a percentage of the initial KCl response. The data represents the mean values ± SEM of *n* number of neurons. KCl peak response was significantly reduced in neurons treated with schwarzinicine A (P<0.001) and nifedipine. There was no influence of vehicle (0.033% DMSO v/v) observed. * (p< 0.05) and **** (p< 0.0001)

	Percentage of the initial KCl response	***n* (number of neurons)**	***p* value**
Control (KCl alone)	81.81 ± 0.76	271	
KCl + 30µM Schwarzinicine A	55.16 ± 1.34	183	**** (<0.0001)
KCl + 30µM Nifedipine	75.77 ± 2.37	81	* (0.0181)

Table 6.1: Summary of the effects of schwarzinicine A and nifedipine on KCl peak response. The responses are presented in the percentage of the initial KCl response. The data represents mean values ± SEM of *n* number of neurons. A p value of <0.05 indicates a significant difference in the peak response between control and treated neurons.

6.4 Discussion

The changes of intracellular calcium, [$(Ca^{2+})_i$] are indicated by the change in 340/380nm ratio. Elevated K^+ (30mM) stimulated a reversible and reproducible increase of [$(Ca^{2+})_i$], that was indicated by a sharp peak of 340/380nm ratio. This is commonly reported as the result of membrane depolarisation by elevated K^+ stimulation. Membrane depolarisation also triggers the opening of voltage-dependent calcium channels, in which L- and N-type are the dominant channels for calcium entry in neurons (Fuchs *et al.*, 2007).

KCl was added to introduce a depolarising state in DRG cells. By washing (removal of extracellular calcium), the KCl-induced depolarising state was reversed to a basal level. The calcium clearance by reversing depolarising state to basal level is modulated by sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) pumps, plasma membrane calcium ATPase (PMCA) pumps and sodium-calcium exchangers, as seen in cerebellar Purkinje cell somata (Fierro *et al.*, 1998) and pancreatic β-cells (Chen *et al.*, 2003). SERCA, PMCA and sodium-calcium exchangers act cooperatively in maintaining the level of cystolic calcium in the cells. In present study, 30-minute recovery duration was allowed after the depolarisation by KCl. This gave a sufficient time for the clearance of calcium load from the cells. After the peak of first KCl response, a sharp followed by a slow gradual recovery indicates the possible participation of SERCA, PMCA and sodium-calcium exchangers at different time points (Chen *et al.*, 2003). Schwarzinicine A appears to reduce this recovered basal level (second basal level) and therefore suggesting that schwarzinicine A may play an antagonism role with the calcium clearance components.

In the present study, nifedipine was initially used as L-type VOCC calcium entry blocker. However, the infusion of nifedipine has caused a delayed increase of basal [$(Ca^{2+})_i$] in the absence of external calcium stimuli. This is an interesting observation in which most studies using the same DRG cell type reported that the infusion of L-type calcium channel blocker did not affect the basal [$(Ca^{2+})_i$] level (Sun *et al.*, 2003; Fuchs *et al.*, 2007).

An elevation of intracellular calcium by calcium antagonists (nifedipine) was evidently reported in gingival fibroblasts (Hattori *et al.*, 2011), complementary to the present study. This observation in gingival fibroblasts was proposed to occur via non-selective cation channels (NSCCs) that are independent of voltage-dependent calcium channel pathways (Hattori and Wang, 2006), in addition to calcium release from the endoplasmic reticulum (Hattori and Wang, 2005). It is proposed that calcium-sensing receptors (CaSRs) are the first to be stimulated by the calcium antagonists, thus inducing IP_3-linked PKC activation to trigger calcium entry by NSCCs and calcium release from ER (Hattori *et al.*, 2011). There is increasing evidence confirming the transient receptor potential (TRP) family as members of NSCCs (Qian *et al.*, 2002; Launay *et al.*, 2002; Lee *et al.*, 2003). The activation of nifedipine on a TRP subtype, TRPM3 has been revealed (Wagner *et al.*, 2008; Drews *et al.*, 2014). TRPM3 is highly expressed in the sensory system while its subtype, TRPM3α2 is found in the rat DRG (Vriens *et al.*, 2011). From these, the interesting observation of nifedipine-elevated basal calcium can be potentially linked to an activation of TRPM channel by nifedipine that results in an influx of calcium.

The basal calcium elevation during the infusion of nifedipine was gradual and slow, while a sharp increase of intracellular calcium occurred during the subsequent addition of combined KCl and nifedipine. When combined, nifedipine did not seem to alter the response of KCl, indicating that the sharp increase of intracellular calcium was solely induced by KCl in spite of the previous basal calcium elevation by nifedipine. These observations may suggest that nifedipine and KCl elicit different calcium signalling pathways. The unaltered effect of nifedipine (blocking of L-type calcium channel) on KCl-induced response, suggests a dominant effect of KCl. Nifedipine may have potentially activated TRPM channel whereas KCl stimulated voltage-dependent calcium channel via membrane depolarisation. The latter mechanism by KCl appears to be more dominant in elevating calcium increase in DRG cells as compared to that mediated by nifedipine-activated TRPM channel.

In contrast, infusion of schwarzinicine A decreased the basal calcium level and suppressed the calcium-elevating response of KCl. As mentioned previously, the basal calcium level is cooperatively maintained by SERCA pumps, PMCA pumps and

sodium-calcium exchanges via two pathways: sequestering calcium into intracellular stores and transporting calcium towards the extracellular space (Fierro *et al.*, 1998). However, calcium from ER and cytosol stores in resting neurons is lost continuously across the plasma membrane, suggesting a more prominent mechanism in maintaining the calcium level at rest (Samtleben *et al.*, 2015). Samtleben and colleagues (2015) reported that in hippocampal neurons, store-operated calcium entry (SOCE) route also maintains the resting calcium level in both ER and cytosol stores, in conjunction with SERCA activity. This event is a continuous process without activation of any signalling cascade.

Without the presence of calcium stimuli, a decrease of basal calcium level by schwarzinicine A can be explained by the reduction of cytosolic calcium under the influence of the calcium homeostasis mechanisms (SERCA pumps, PMCA pumps and sodium-calcium exchangers) and SOCEs. Considering a potential activation of TRPM3 channel by nifedipine during the infusion period, schwarzinicine A-reduced basal calcium level at this time point A may also suggest a potential role of schwarzinicine A in attenuating the TRP channels. In addition, the antagonism effect of schwarzinicine A on KCl-elevated calcium level indicates that this compound also inhibits calcium entry.

In summary, the present finding has confirmed the inhibitory effect of schwarzinicine A was potentially mediated via cation/calcium channels which may include VOCC, TRP and SOCE channels in rat DRG cells. This further supports our previous functional data reported in isolated rat aortic and porcine coronary arterial tissues (Chapter 4 and 5) that the relaxation effect of this novel compound was mediated through the inhibition of calcium entry into the vascular smooth muscle cells.

Chapter 7: General discussion

Hypertension accounts for one of the most prevalent health complications worldwide (GBD, 2013) and costing £1 billion in the related healthcare management (NICE, 2011). Despite the current availability of antihypertensive drugs, resistant or uncontrolled hypertension is of great concern. Resistant hypertension refers to uncontrolled high blood pressure following treatment with at least three antihypertensive drugs. An estimated 20% of such cases occur within the hypertensive population in England (Falaschetti *et al.*, 2009).

The abundance of higher plants and their potential beneficial effects in cardiovascular therapy have encouraged the manipulation of the Malaysian flora in discovering new therapeutic molecules to treat cardiovascular diseases including hypertension. To reduce the time taken during the phase of target identification, a reliable screening assay for 'hit' compounds is highly useful. This study has employed the techniques of organ bath pharmacology and calcium imaging to evaluate the vascular effects of a novel plant alkaloid, schwarzinicine A, from a local fig plant species, *Ficus schwarzii*.

7.1 Optimisation of a functional bioassay methodology in pharmacological evaluation of plant alkaloids

This study successfully optimised the organ bath assay in testing (i) the smooth muscle activity of isolated rat aorta, trachea, bronchus and bladder tissues, and (ii) the biological effects of plant alkaloids on isolated rat aortic tissues. These data supported the following studies in evaluating the vascular effects and mechanism of schwarzinicine A.

Using the organ bath technique, the contractile responses of four tested tissues from the rat (aorta, trachea, bronchus and bladder) together with the relaxant

response in aortic tissues, were replicated consistently. With the objective of reducing the number of animals used overall, this study applied 'The Principle of Three R's', whereby the cold storage of tissues at 4^{o}C for 24 hours was employed, with their functional responses investigated thereafter. The outcomes from this study were that there was a partial loss of endothelial derived relaxation factors in stored aortic rings and reduced reactivity in stored bronchus tissue, suggesting that stored aorta can only be used in smooth muscle studies. Additionally, the display of reduced reactivity in stored bronchus suggests its limited use in functional studies. This information is useful in optimising the potential use of a single sacrificed animal, as well as avoiding the wastage of animal tissues and other resources.

To assess the robustness of our functional assay, we started by testing the vascular effects of different plant alkaloids isolated from two local plants, *Alstonia pneumatophora* and *Alstonia rostrata.* Relaxant effects in aortic tissues were demonstrated by all tested plant alkaloids with no evidence of any contractile effect from these compounds (refer to Appendix B). A number of these compounds are novel and we are the first to report of their vasorelaxant activity (Lim *et al.*, 2015). The information on this finding allowed the subsequent investigation of a novel phenylethylamine-derived alkaloid, schwarzinicine A, as described below.

7.2 Pharmacological and mechanism of action of schwarzinicine A in rat tissues

The main focus of this thesis is to investigate the potential pharmacological action of schwarzinicine A. This is a novel plant alkaloid that is isolated from a local tree, *Ficus schwarzii*, by our research group. [All publication on this compound is currently embargoed as we are awaiting more data for the potential of a patent.] Traditionally, *Ficus schwarzii* is used against ringworm infection (Asian Plant). Recently, the alkaloid extracts from this plant have been shown to have an anti-proliferative effect on human cancer cells (Abubakar *et al.*, 2015). To date, there is no study reporting the biological effects of schwarzinicine A. Therefore, this is the first study that exploring the effects of this novel compound on different bioassays.

Due to the occurrence of carbachol-induced spontaneous contractile responses in bladder tissue, the effect of schwarzinicine A was observed to be non-conclusive. Schwarzinicine A exhibited the highest efficacy of relaxation in the aortic tissues where its maximum relaxation was comparable to the beta agonist, dobutamine. With this exciting and novel finding, subsequent experiments were undertaken in trying to deduce the mechanism by which schwarzinicine A induced its relaxant response.

There are a number of possibilities in explaining the relaxation mechanisms of schwarzinicine A. We first scrutinised at its chemical structure. A close structural similarity of schwarzincine A was ascribed to a known catecholamine dobutamine, an agonist of the β_2-adrenoceptor, where its vascular relaxation is known to be mediated via the elevation of cAMP. We thus hypothesised that schwarzinicine A possessed a similar mechanism of action as dobutamine. Subsequent experiments were designed to test this hypothesis but our findings refuted any involvement of the β_2-adrenoceptor, cAMP or PDEs (refer to *Figure 7.1*). Further investigations did confirm that schwarzinicine A-induced relaxation was not mediated by endothelium-derived relaxation factors including nitric oxide, antagonism of alpha-adrenergic receptors or potassium channels (refer to *Figure 7.1*).

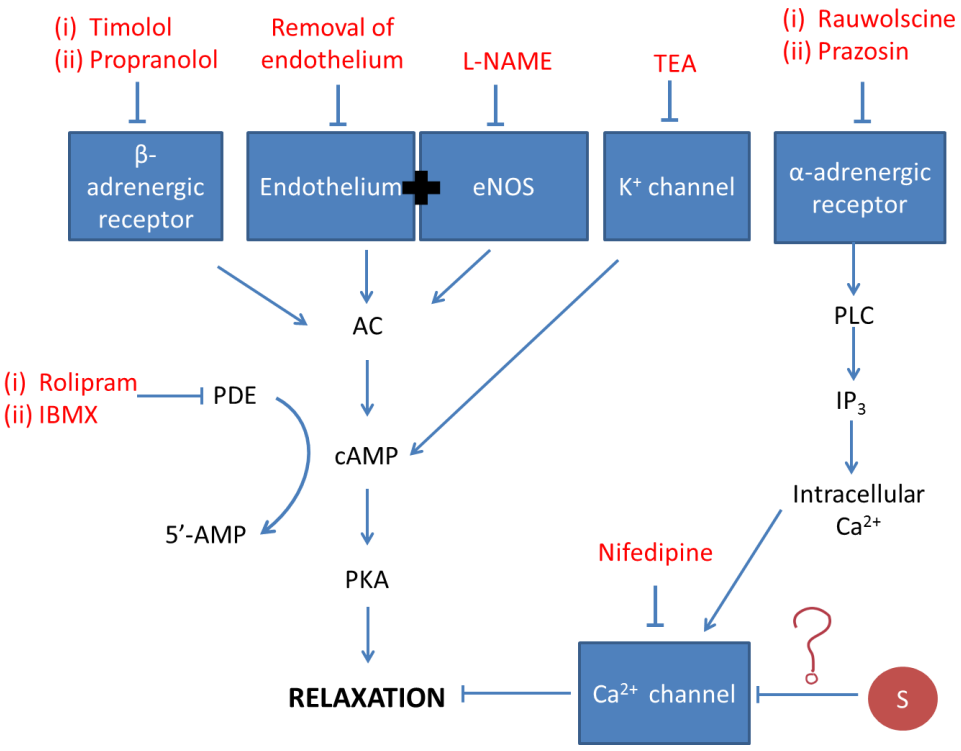


Figure 7.1: The summary of proposed relaxation mechanisms that may be elicited by schwarzinicine A (S). The observed relaxant responses of schwarzinicine A eliminated the above proposed mechanisms as a target with conclusions drawn of independence from endothelium, endothelial nitric oxide, α- and β-adrenergic receptors, potassium (K^+) channel and PDEs mechanisms. An inhibitory role of schwarzinicine A on calcium channels was possible from its inhibition on calcium influx in calcium-free Krebs solution (discussed in context below). Inhibitor drugs for each proposed mechanisms are shown in red. eNOS, endothelial nitric oxide; PDE, phosphodiesterase; AC, adenylyl cyclase; cyclic adenosine monophosphate; PKA, protein kinase A.

Interestingly, schwarzinicine A was shown to affect calcium entry in the calcium-free Krebs protocol similar to nifedipine, a clinically used calcium channel blocker. Additionally, schwarzinicine A clearly affected the contractions induced by phenylephrine, 5-hydroxytryptamine and KCl, but not U46619-induced contractions. Potassium chloride causes contraction through the activation of voltage-operated calcium channels (VOCCs) by depolarising the cells. Whilst phenylephrine and 5-hydroxytryptamine are selective α_{1D}-adrenoceptor agonist and non-selective serotonin receptor agonists respectively, both receptors are coupled to the G_q protein. Contraction mediated through the G_q protein is generally described as via

the release of two sources of calcium. Firstly, the release of intracellular calcium via IP_3 activation from the internal stores and secondly through the influx of extracellular calcium or via cation or calcium channels (see *Figure 7.2A* in **Section 7.4**). Thus this led to the hypothesis that schwarzincine A could potentially be affecting calcium mobilisation.

In addition, there are evidences stated that the production of IP_3 and DAG from α_1-adrenergic receptor activation by phenylephrine (Albert and Large, 2003; Thebault *et al.*, 2006; Harteneck and Gollasch, 2011; Fuchs *et al.*, 2011) and the depolarisation by KCl have stimulated TRP channels in vascular smooth muscles (Ratz and Berg, 2006). TRP channels are described to be parts of receptor-operated calcium channel (ROCC) and store-operated calcium channel (SOCC) (Earley and Brayden, 2015). Therefore, in this study, the involvement of TRP channel is hypothesised and affected by schwarzinicine A based on the reduced potency (pEC_{50}) of phenylephrine and KCl by this compound. However, this hypothesis remains speculative and requires further investigations.

Both phenylephrine and 5-hydroxytryptamine are G_q-protein coupled receptor agonists. The contractile responses to these two drugs was reduced by schwarzinicine A, in which suggests the inhibitory action of schwarzinicine A on the signalling pathways by G_q-protein coupled receptor activation. In contrast, the activation of thromboxane A_2 receptor by U46619 was not affected by schwarzinicine A in spite of the fact that thromboxane A_2 receptor is also commonly known as a G_q-protein coupled receptor. This suggests that U46619 may have elicited a different contractile mechanism, other than G_q protein receptor-mediated pathway. In some studies, the couplings of thromboxane A_2 receptor to both G_q and $G_{12/13}$ proteins as well as to the constitutive forms of these two G-proteins, $G_{\alpha12/13}$ protein are shown (Gohla *et al.*, 2000; Zhang *et al.*, 2006; Zhang *et al.*, 2009). The activation of G_q and $G_{12/13}$ protein leads to the respective inositol phospholipid hydrolysis and Rho/Rho kinase-mediated pathway (Shenker *et al.*, 1991; Baldassare *et al.*, 1993; Gohla *et al.*, 2000). Therefore, it can be suggested that U46619 have stimulated a dominant $G_{12/13}$-protein coupled thromboxane A_2 receptor and subsequent Rho kinase/MLCP-mediated pathway (see *Figure 7.2A* in **Section 7.4**)

In addition to aortic tissues, schwarzinicine A-induced relaxation was also observed in airway smooth muscle tissues (trachea and bronchus), but not conclusive in bladder tissues. This may indicate that schwarzinicine A potentially inhibits the same contractile mechanism in both aortic and airway smooth muscle tissues. However, the relaxation in airway smooth muscle tissues was observed to be at a lower efficacy as compared to that in the aortic tissues. This comparison results in a few hypotheses, proposed as: (i) different receptor expression or density in airway smooth muscles that is sensitive to schwarzinicine A, as compared to aortic tissues; and (ii) airway smooth muscles exhibit different contraction pathways, for example via different G protein-coupled receptors (other than G_q protein-coupled receptors) or cation/calcium channels that are less sensitive to schwarzinicine A. In contrast, despite using the same contractile agonist (carbachol) in both airway smooth muscle and bladder tissues, the schwarzinicine A-induced effect on bladder tissues is inconclusive due to the interference of the spontaneous contraction response (as discussed in **Section 3.4**, Chapter 3) on the schwarzinicine A-induced response.

Carbachol was used as a contractile agonist in airway smooth muscle. This airway-induced contractile response is mainly mediated by the carbachol-activated muscarinic type 3 (M_3) receptor (Brown *et al.*, 2013). Similar to the α_1-adrenergic receptor (as previously mentioned), M_3 receptor is also a G_q protein-coupled receptor and its activation leads to IP_3- and DAG-mediated mechanisms (see *Figure 7.2A* in **Section 7.4**). Therefore, it is unlikely that different G-proteins, other than G_q proteins, are coupled to M_3 receptor. This is supported by the relaxant effect of schwarzinicine A on aortic rings and trachea in response to phenylephrine and carbachol respectively in spite of their difference in magnitudes of response. The involvement of TRP channels in carbachol-induced contraction is not clearly known, however the expression of different TRP channel subtypes are widely reviewed and reported in airway smooth muscles (Ong and Barritt, 2004; Moran *et al.*, 2011; Grace *et al.*, 2014). The presence of TRP channels in trachea and bronchus are worth to be explored, in further elucidating the role of schwarzinicine A.

7.3 Mechanistic studies of schwarzinicine A on PCA tissues and rat DRG cells

The investigation of schwarzincine A on rat aorta was a preliminary study in identifying the potential calcium-inhibiting effect of this compound. To further elucidating this effect, another vascular tissue type which was PCA tissues was chosen. Both coronary artery and aorta belong to the large arteries and share similarity in structure and functions. In addition, the close resemblance of PCA to human coronary arteries has made this investigation of schwarzinicine A on PCA tissues excellent for the future translation studies onto human vascular tissues. Astonishingly, schwarzincine A has not only relaxed the PCA tissues in a cumulative concentration-response manner and also inhibited the calcium entry by $CaCl_2$ addition in the same tissues. These responses mirrored those that were observed in rat aorta.

Following this, calcium imaging technique using rat DRG cells was perform to further validate the inhibitory effect of schwarzinicine A on calcium mobilisation. The richness in variety of VOCCs (L-, T-, P/Q- and N-type) in neuronal cells initiated this investigation. The KCl-induced depolarisation in DRG cells confirmed calcium entry through VOCCs. Among all VOCCs, L-type calcium channels are the majority in modulating neuronal calcium activity. In the present study, nifedipine was used as the L-type calcium channel inhibitor. However, KCl-induced depolarisation in DRG cells was not sensitive to the inhibitory effect of nifedipine, suggesting the activation of non-L-type VOCCs by KCl. A similar observation was reported in primary cultures of cerebellar granule cells (Toescu, 1999). This insensitivity to nifedipine may be due to interference of its induced potential SOCC, or TRPM3-induced intracellular calcium increase in DRG cells (as observed in the present study) with KCl activity as discussed below.

Nifedipine is typically used in the treatment of hypertension by inhibiting L-type calcium channels in vascular tissues. The inhibition of calcium entry by nifedipine on the restoration of extracellular calcium was observed in rat aortic and porcine coronary arterial tissues. However, the opposite response was observed in DRG cells, in which a slow and delayed elevation of intracellular calcium was induced by

nifedipine. In addition to the previously suggested SOCC activation by nifedipine, this phenomenon is also suggested to be related to an activation of transient receptor potential melastatin 3 (TRPM3) channel, consistent to a study of nifedipine-activated TRPM3 channel in TRPM3-transfected HEK293 and HEK293 cells (Drews *et al.*, 2014). However, when DRG cells were exposed to a combination of nifedipine and KCl, the elevation of intracellular calcium was observed to produce a similar increase in amplitude to that of KCl exposure alone. This suggests that KCl-induced intracellular calcium increase is dominant to the nifedipine-sensitive TRPM3-mediated calcium influx.

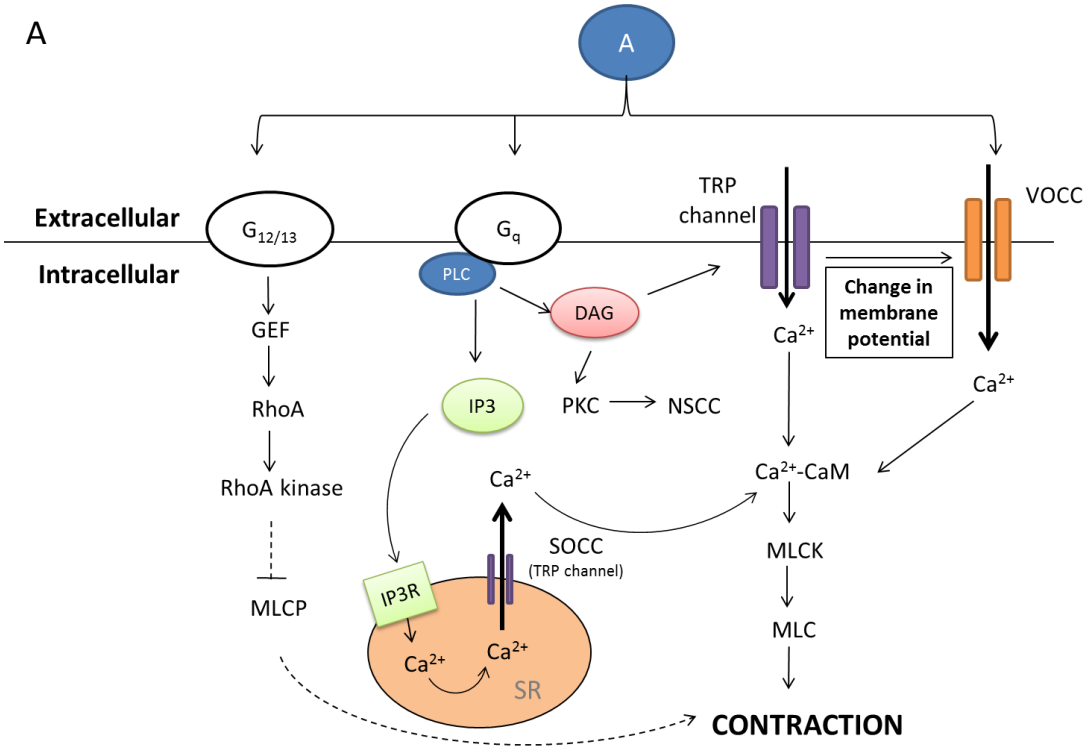
TRPM channels are generally linked to the detection of heat responses (Vriens *et al.*, 2011). However, it was recently revealed to enhance the contraction of vascular smooth muscle cells (Naylor *et al.*, 2010). TRPM family members, such as TRPM2, TRPM3, TRPM4, TRPM7 and TRPM8, were found to be expressed in rat intralobar pulmonary arteries and aorta (Yang *et al.*, 2006). In the present study, in contrast to the nifedipine effect, a reduced intracellular calcium level was observed in DRG cells exposed to schwarzinicine A alone. In relating this with the abundant expression of TRPM3 in sensory cells (including DRG cells), and the activation of this channel by nifedipine, TRPM channels may be a potential target for schwarzinicine A in addition to the non L-type calcium channel activated by KCl.

7.4 Summary and findings

Overall, schwarzinicine A has reduced the potency of phenylephrine and KCl, the efficacy of 5-hydroxytryptamine and the calcium entry by $CaCl_2$ in calcium-free medium. Similarly, in PCA tissues, schwarzinicine A also elicited a concentration-dependent relaxation and inhibited the calcium entry by $CaCl_2$ in calcium-free medium, as well as reducing the depolarisation to KCl in rat DRG cells. These results suggest the calcium-inhibiting effects of schwarzinicine A. Based on these results, some potential mechanisms of schwarzinicine A, via VOCC, ROCC and SOCC channels are proposed and presented in *Figure 7.1B*. The potential role of schwarzinicine A on TRP channels is also proposed as many studies have identified this channel type as part of ROCC and SOCC (reviewed by Earley and Brayden, 2015).

Many TRP subfamilies, including TRPC, TRPV and TRPM that have been found to be expressed in different vascular tissues (Inoue *et al.*, 2001, Jung *et al.*, 2002; Welsh *et al.*, 2002, Yang *et al.*, 2006; Kochukov *et al.*, 2013; Loga *et al.*, 2013). The activation of TRP channels by phenylephrine and KCl in some studies (Albert and Large, 2003; Thebault *et al.*, 2006; Ratz and Berg, 2006; Harteneck and Gollasch, 2011; Fuchs *et al.*, 2011) have raised the interest of investigating the role of schwarzinicine A on TRP channels in the future.

A


A
Extracellular
Intracellular
$G_{12/13}$
G_q
PLC
DAG
TRP channel
VOCC
Change in membrane potential
GEF
RhoA
RhoA kinase
MLCP
IP3
PKC
NSCC
Ca^{2+}
Ca^{2+}-CaM
MLCK
MLC
IP3R
SOCC
(TRP channel)
SR
CONTRACTION

B

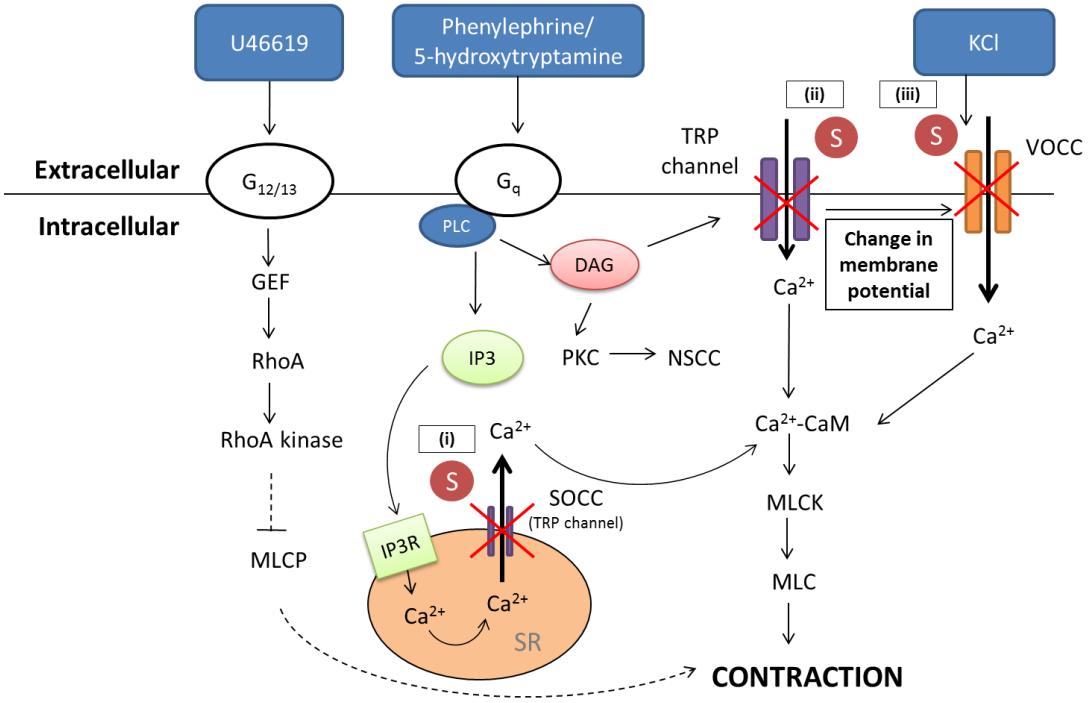

U46619
Phenylephrine/
5-hydroxytryptamine
KCl
Extracellular
Intracellular
$G_{12/13}$
G_q
PLC
DAG
TRP channel
(ii)
(iii)
S
VOCC
Change in membrane potential
GEF
RhoA
RhoA kinase
MLCP
IP3
PKC
NSCC
Ca^{2+}
Ca^{2+}-CaM
(i)
MLCK
MLC
IP3R
SOCC
(TRP channel)
SR
CONTRACTION

Figure 7.2: Schematic diagrams illustrating the contractile mechanisms elicited by contractile agonist and that in the presence of schwarzinicine A. *Figure 7.2A* illustrates the potential contractile mechanisms by contractile agonists (stated in A). *Figure 7.2B* illustrates the contractile mechanisms by phenylephrine, 5-hydroxytryptamine, KCl and U46619 along with the proposed mechanisms of schwarzinicine A (stated in S). Schwarzinicine A elicits its inhibitory action on G_q protein-coupled receptor-mediated pathways that are activated by phenylephrine and 5-hydroxytryptamine, and possibly involves (i) SOCC (TRP channels) at sarcoplasmic reticulum (SR), (ii) TRP channels at plasma membrane (directly activated by DAG; Harteneck and Gollasch, 2011; Fuchs *et al.*, 2011) and (iii) VOCC. Schwarzinicine A also inhibits the depolarisation by KCl via VOCC. TRP channel activation causes a change in membrane potential that opens VOCC (reviewed by Earley and Brayden, 2015) as shown in a study by Ratz and Berg, 2006. Schwarzincine A does not appear to affect the RhoA kinase pathway upon activation of $G_{12/13}$ protein-coupled receptor by U46619. PLC: phospholipase C; IP3: inositol trisphosphate; DAG: diacylglycerol; TRP: transient receptor potential; SOCC: store-operated calcium channel; VOCC: voltage-operated calcium channel; PKC: protein kinase C; NSCC: non-selective cation channel; GEF: guanine nucleotide exchange factors; MLC: myosin light chain; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; CaM: calmodulin; A: agonist; S: schwarzinicine A.

7.5 Limitations of study

The present study successfully met the initial aims of optimising a functional bioassay methodology to investigate the pharmacological effects of a novel plant-derived compound using an organ bath technique. In spite of this success, some limitations still present during this study, as discussed below.

Overall, a diverse investigation of schwarzinicine A was carried out on rat aorta, porcine coronary arteries (PCA) and rat dorsal root ganglion (DRG) cells. Not only schwarzinicine A showed inhibitory effect on calcium mobilisation in these biological samples and the uses of PCA and DRG cells have also met the principle of 'Three Rs'. However, a question concerning the localisation of mechanism is raised. Despite the similar expression of various calcium channels in rat DRG cells as vascular smooth muscle cells, the effect of schwarzinicine A on vascular smooth muscle cells is yet to be explored. Therefore, further investigation is recommended.

Of initial concern was the variation of tissue responsiveness to drugs within different batches of animals (Sprague-Dawley male laboratory rat in the case of the present study). Tissue segments with a low drug response (<0.4g stimulated tension force) were discarded and replaced with a new tissue segment; in return, this resulted in some wastage of tissues. This variation was observed in spite of the controlled laboratory environment (in the aspect of habitat temperature, habitat lighting, animal food and disease control) where the animals were kept. The contribution of genetic variation is unlikely in the batches of Sprague-Dawley rats as this variation is minimised by using inbreeding and linebreeding approaches (reviewed by Paigen, 2003).

Another limitation noted was the concern of the influence of the vehicle on tissue contractility, or its possible interference with the schwarzinicine A activity. A slight though insignificant relaxation effect by the vehicle (DMSO) was observed in the rat aortic tissues but not in porcine coronary arterial tissues. DMSO was used to dissolve schwarzinicine A as it is a common solvent used in biological studies. However, rat aortic tissues showed higher sensitivity to DMSO than porcine coronary arterial tissues. However, we can with confidence conclude that interference of DMSO on schwarzinicine A-induced responses in rat aortic tissues is negligible.

Unavoidable wastage of tissues did occur during this study. The present study demonstrated that the smooth muscles functionality remains intact even after overnight storage at 4^{o}C. This indeed has provided an insight into reducing the sacrifice of animals and wastage of animal tissues. However, without any prior knowledge on the effects of novel plant compounds on certain tissues, the use of stored tissues was not recommended until optimisation of the storage conditions is confirmed. For example, only fresh aortic tissues were treated with schwarzinicine A in the present study, to avoid any potential influence of storage in the relaxant effect of schwarzinicine A. Despite this, the aim of reducing animal use was still fulfilled by maximising the use of all required fresh tissues from each sacrificed animal, in addition to preserving the limited source of schwarzinicine A. At the point of the experimental period, additional tissue baths were installed to

accommodate the use of all fresh aortic tissues and hence avoid the storage of tissues.

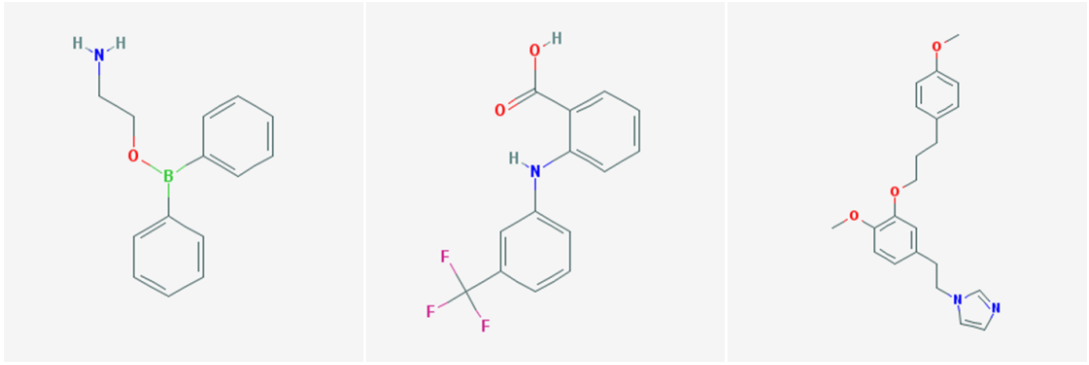
7.6 Future studies

The concern of localisation of mechanism in this study has urged a further investigation in investigating the potential calcium-inhibiting effect of schwarzinicine A on freshly isolated smooth muscle cells from either rat aorta or porcine coronary arteries. This could be achieved using calcium imaging technology.

Future studies in investigating the inhibitory role of schwarzinicine A on TRP channels are warranted. The emerging role of TRP channels in mediating vascular smooth muscle contraction has provided a new therapeutic site for hypertension treatments. Many studies have identified TRPC1, TRPC3 and TRPC6 as the molecular candidate for vascular smooth muscle TRP channels (Inoue *et al.*, 2001, Jung *et al.*, 2002; Welsh *et al.*, 2002, Kochukov *et al.*, 2013; Loga *et al.*, 2013). These TRPC channels, as well as TRPM channel could potentially be the target of investigation. However, at this current time, only broad-range TRP channel inhibitors such as 2-aminoethoxydiphenyl borate (2-APB), flufenamic acid (FFA) and SKF96365 (Bencze *et al.*, 2015) are available and commonly used. Hence, a discovery of new selective TRP channel inhibitors is highly needed.

In further investigating the potential role of schwarzinicine A as TRP channel inhibitor, several future experiments can be carried out, as stated below:

(i) Comparative studies comparing the vascular effects of schwarzinicine A with those by the widely used TRP channel inhibitors, such as 2-aminoethoxydiphenyl borate (2-APB), flufenamic acid (FFA) and SKF96365. Schwarzinicine A appears to share the closest similarity to SKF96365 among all (refer to *Figure 7.3*), in which this TRP channel inhibitor is the best candidate to be used in the comparative studies.



2-aminoethoxydiphenyl borate (2-APB)

Flufenamic acid (FFA)

SKF96365

Figure 7.3: The chemical structures of TRP channel inhibitors, 2-aminoethoxydiphenyl borate (2-APB), flufenamic acid (FFA) and SKF96365 (obtained from PubChem database).

(ii) Isolated aortic tissues from spontaneous hypertensive rats (SHRs) can be used to elucidate the vascular effects of schwarzinicine A, whether it is at better potency and efficacy. Upregulation of TRP subtypes, TRPC1 and TRPC6, was observed in SHRs (Lin *et al.*, 2015).

(iii) Assessment of western blot experiment in checking the expression of TRP channels in both Sprague-Dawley rats and SHRs.

7.7 Conclusion statements

The chemical diversity and versatility of plants have provided a great opportunity in discovering and developing new antihypertensive drugs. Plant phytochemicals are often the candidate target for new drug discovery. Most of these phytochemicals have no prior knowledge on their biological effects. A blind testing of them on any biomedical interest would be a head start of knowing their potential effects. In meeting this, this study has successfully achieved the study objectives (refer to **Section 1.10**, Chapter 1) and concluded that:

(i) A functional bioassay methodology using organ bath technique was successfully optimised and confirmed the retained contractile activity of four different rat smooth muscles (aorta, trachea, bronchus and bladder) in fresh and stored condition.

(ii) Using the same functional bioassay, the relaxant effect of a novel plant alkaloid, schwarzinicine A from local fig plant, *Ficus schwarzii* was clearly revealed in rat aortic tissues and porcine coronary arteries, as well as in airway smooth muscles but in a lower extent.

(iii) The relaxation mechanisms by schwarzinicine A in rat aortic tissues was demonstrated to be independent of the influences of endothelium and nitric oxide as well as not mediated by alpha- and beta-adrenergic receptors, potassium channels and cAMP. The inhibition of calcium channels by schwarzinicine A was revealed.

(iv) The inhibitory role of schwarzinicine A in calcium channel was successfully shown from the gathered results on rat aortic tissues, porcine coronary arteries and dorsal root ganglion cells. Schwarzinicine A was revealed to inhibit calcium signalling pathways. Future studies will focus on investigating the effect of schwarzinicine A on TRP channels, as it is proposed in **Section 7.6**.

Appendix A: *Ficus schwarzii*

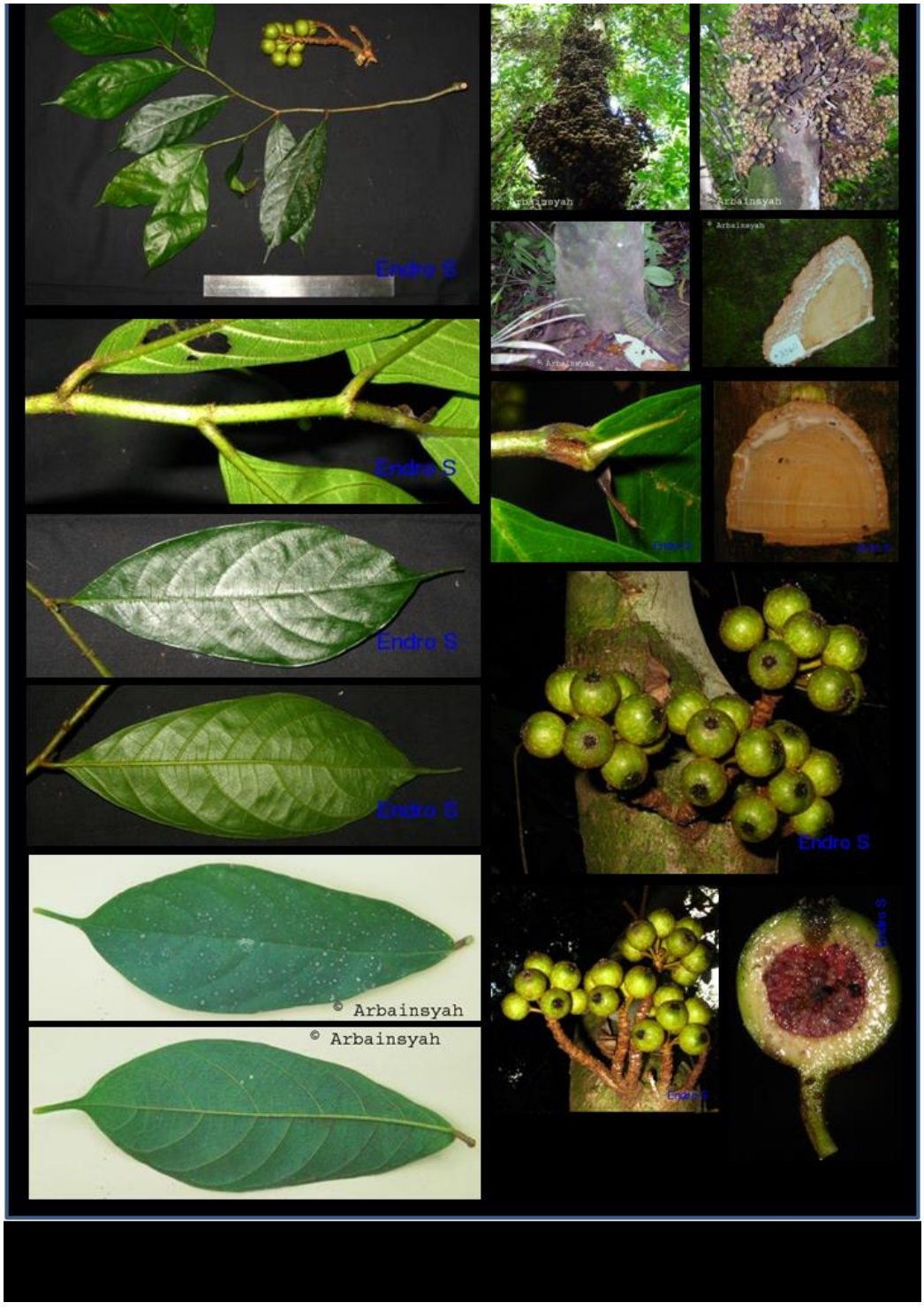


Figure A: The images of leaves, trunk, figs and twigs of *Ficus schwarzii*.

(Adapted from Asian Plant: *Ficus schwarzii* Koord.).

Appendix B: Investigation of the effects of alkaloids from local plants on rat aorta

1.1 Introduction

In many studies, plant bioactive compounds, including plant alkaloids have been isolated and characterised using the organ bath assay. These findings are useful in drug discovery as an approach to broadening the availability of therapeutic drugs. Therefore, the present study was to investigate the potential effects of novel plant alkaloids (isolated and characterised from *Alstonia pneumatophora* and *Alstonia rostrata* plants) on the rat aorta.

1.1.1 *Alstonia pneumatophore* and *Alstonia rostrate*: Botany and medicinal uses

Alstonia is a plant genus that is derived from Apocynaceae family. *Alstonia* plant species are mainly grown in African and Asian countries (reviewed by Pratyush *et al.*, 2011). More than 40 plant species from this genus have been discovered and studied (reviewed by Pratyush *et al.*, 2011). In Malaysia, *Alstonia* species are commonly known as 'pulai' and mainly used in the wood industry while the bark and latex are also used for medicinal purposes (FAO, Regional Office for Asia and the Pacific).

Alstonia pneumatophora is generally found in mixed peat-swamp forest on shoallow peat and mouth of large rivers. It is a tree that can grow up to 45 meters tall with pneumatophore roots (Asian Plant). Other features includes the leaves in whorls with short or no petiole (0-7mm long), long and narrow fruits that are in pairs and seeds with two tufts of hairs (Markgraf, 1974; Asian Plant).

Figure A: The images of leaves, fruits and flowers of *Alstonia pneumatophore* (Adapted from Asian Plant: *Alstonia pneumatophora* Backer ex Den Berger).

Alstonia rostrata is found in evergreen or open-degraded forest at altitude range from 500 to 1300 meters (Biotik). The tree of *Alstonia rostrata* can grow up to 30-meter tall with bole fluted at the tree base (Biotik). The characteristic botanical features of this species are the white latex produced by trees, leaves in the arrangement of whorls as well as recurved and overlapping white flower petal lobes

with puberulent at both sides (refer to *Figure B*) (Biotik; BGO Plant Database, The Botanical Garden Organization).

Figure B: The images illustrate (a) the white latex on tree, (b) leaves in whorl and (c) recurved and overlapping white flower petal lobes in *Alstonia rostrata* (Adapted from Biotik and BGO Plant Database).

Traditionally, the latex from the bark of *Alstonia pneumatophora* and *Alstonia rostrata* is used for wound healing (Plant Use) and anti-septic medicines (Biotik). The leaves of *Alstonia rostrata* are also used for wound cleaning (Biotik). Studies have shown the crude extracts of *Alstonia* species to possess antibacterial (Khyade and Valkos, 2009; Opoku and Akoto, 2015), antioxidant (Obiagwu *et al.*, 2014) and antihypertensive properties (Bello *et al.*, 2015). This knowledge has allowed the further investigation of bioactive compounds in these plants.

Alstonia species are known to be rich in alkaloid content. To date, a variety of novel alkaloids are isolated from *Alstonia pneumatophora* and *Alstonia rostrata*. Some examples includes alpneumines A-H (Koyama *et al.*, 2010a) and alsmaphorazines A-B from *Alstonia pneumatophora* (Koyama *et al.*, 2010b), as well as alstrostines A-F from *Alstonia rostrata* (Cai *et al.*, 2011; Bao *et al.*, 2012). Alpneumines A-H from *Alstonia pneumatophore* have shown anti-melanogenic properties on mouse melanoma cells (Koyama *et al.*, 2010a) while the biological effects of *Alstonia rostrata* alkaloids are yet unknown. Therefore, the pharmacological investigation of these alkaloids is an ongoing process.

1.1.2 Significances of study

The main purpose of this experiment was to utilise the organ bath technique to investigate the vascular effects of bioactive compounds from various plants. Lowering blood pressure is one of the therapeutic potentials of *Alstonia* species (Bello *et al.*, 2015). Considering this and the richness of alkaloids in *Alstonia* species, the vascular relaxant effects of ten isolated alkaloids from two local *Alstonia* species (found in Peninsular Malaysia), *Alstonia pneumatophora* Backer ex Den Berger and *Alstonia rostrata* C.E.C. Fisch, were of interest. These ten alkaloids included 19-epi-echitamidine (**N1**), pneumatophorine (**N2**), echitamidine (**N3**), N(4)-demethylalstogastine (**N4**), rostracine (**N5**), undulifoline (**N6**), 20(S)-tubotaiwine (**N7**), 19,20(E)-vallesamine (**N8**), 15-hydroxyangustilobine A (**N9**) and 6,7-secoangustilobine B (**N10**). The relaxant effects of these alkaloids were investigated, with concentration-response curves generated of each alkaloid on isolated rat aorta that was pre-constricted with the contractile agonist, phenylephrine. A positive control using isoprenaline as relaxant agent was included for comparison. The functionality of this experiment design was previously confirmed in Chapter 2.

1.2 Materials and Methods

1.2.1 Plant alkaloids and their aqueous solution preparations

Ten plant alkaloids were isolated from the bark extracts of *A. pneumatophora* and *A. rostrata*, and characterised by Professor Kam and his team in University of Malaya, Malaysia (some of these alkaloids were then published in Lim *et al.*, 2015). The full list of these alkaloids was listed in *Table A*, along with their molecular weight. The chemical structures of these alkaloids were included in *Figure A*. All plant alkaloids were dissolved in 100% DMSO to make a stock concentration of 0.1M and further diluted with 50% DMSO (v/v), 25% DMSO (v/v) and distilled water in sequence. The final DMSO concentration in the bath was <0.165% (v/v).

Alkaloids	Name of alkaloids	Molecular weight (MW)	Plant source
N1	19-epi-echitamidine	340	*A. pneumatophore*
N2	Pneumatophorine	433	*A. pneumatophore*
N3	Echitamidine	340	*A. rostrata*
N4	N(4)-demethylalstogastine	340	*A. pneumatophore*
N5	Rostracine	206	*A. rostrata*
N6	Undulifoline	340	*A. rostrata*
N7	20(S)-tubotaiwine	324	*A. pneumatophore & A. rostrata*
N8	19,20(E)-vallesamine	340	*A. pneumatophore & A. rostrata*
N9	15-hydroxyangustilobine A	368	*A. pneumatophore*
N10	6,7-*seco*angustilobine B	340	*A. pneumatophore*

Table A: The list of ten alkaloids (**N1**-**N10**) that were isolated from two local *Alstonia* species, *Alstonia pneumatophora* and *Alstonia rostrata* with their molecular weight (MW) included. These are the gifts from Professor Kam (University of Malaya, Malaysia).

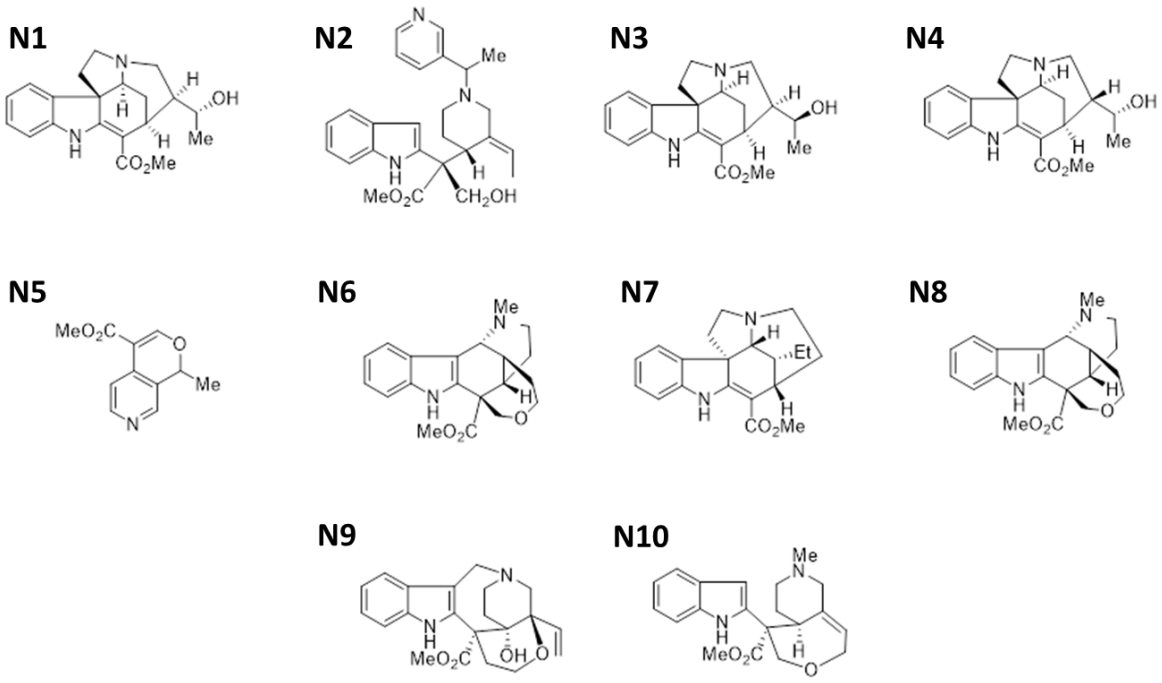


Figure C: The chemical structures of alkaloids **N1** to **N10** (respective name referred to *Table A*).

1.2.2 Drugs and Krebs-Ringer bicarbonate solution

Phenylephrine, isoprenaline and Krebs solution were used in this study and prepared as previously stated in **Section 2.2.1** (in Chapter 2).

1.2.3 Preparation of tissues

Aortic tissues were isolated from male Sprague-Dawley rats and prepared as mentioned in **Section 2.2.2** (in Chapter 2).

1.2.4 Organ bath experiment

The organ bath setup was assembled as mentioned in **Section 2.2.3** (Chapter 2). The aortic rings were mounted and let to equilibrate for at least 30 minutes. The viability of each aortic ring was tested with 60mM KCl twice, followed by a 30-minute resting period After the 30-minute resting period, aortic rings were constricted with 0.1µM phenylephrine to establish a submaximal contraction of the tissues (as obtained and described in Chapter 2). This was followed by a cumulative concentration-response curve of each plant alkaloid (0.1nM to 0.1mM). A concentration-response curve of isoprenaline on pre-constricted aortic tissues with 0.1µM phenylephrine was also constructed.

1.2.5 Data analysis

Data analysis of this study was as described as that for the aortic tissues in **Section 2.2.5** (in Chapter 2).

1.3 Results

All alkaloids (**N1** to **N10**) displayed relaxant effects on rat aortic tissues. Alkaloid **N2**, **N8** and **N10** elicited relaxant responses that were considerably less than that

produced by isoprenaline (maximum relaxation: 79.74 ± 4.18%), following the ascending sequence of **N10** < **N2** < **N8** < Isoprenaline (refer to *Table B*). In contrast, the remaining alkaloids produced relaxant responses that were greater in magnitude than that of isoprenaline, following the ascending sequence of isoprenaline < **N5** < **N1** < **N6** < **N3** < **N7** < **N4** < **N9** (refer to *Table B*). As a range, alkaloid **N10** and **N9** produced the lowest and highest maximum relaxation magnitudes respectively (maximum relaxation: 18.02 ± 1.31 % vs 185.1 ± 78.5 %). Taken together, seven out of the ten tested alkaloids elicited complete relaxation (≥100% of phenylephrine-induced submaximal contraction).

In terms of potency, alkaloid **N10** and **N9** elicited the highest and lowest potency (pEC_{50}: 6.92 ± 0.22 vs 4.12 ± 0.39) respectively, but yet less potent than isoprenaline. The representative relaxation curves of alkaloid **N9** and **N10** was shown in *Figure B*.

Alkaloids	**Name of alkaloids**	***n* number**	**Maximum relaxation, E_{max} (%)**	**EC_{50} (in M)**	**pEC_{50}**
N10	6,7-*seco*angustilobine B	3	18.02 ± 1.31	1.19×10^{-7}	6.92 ± 0.22
N2	Pneumatophorine	3	63.01 ± 9.32	3.15×10^{-5}	4.50 ± 0.23
N8	19,20(E)-vallesamine	3	77.44 ± 12.07	1.50×10^{-5}	4.83 ± 0.24
Control	Isoprenaline	16	79.74 ± 4.18	7.57×10^{-8}	7.12 ± 0.13
N5	Rostracine	3	110.4 ± 14.61	6.98×10^{-6}	5.16 ± 0.26
N1	19-epi-echitamidine	3	125.0 ± 5.43	9.38×10^{-6}	5.03 ± 0.08
N6	Undulifoline	3	125.5 ± 16.44	9.16×10^{-7}	6.04 ± 0.37
N3	Echitamidine	3	128.4 ± 14.06	1.33×10^{-5}	4.88 ± 0.19
N7	20(S)-tubotaiwine	3	132.4 ± 4.79	3.25×10^{-6}	5.49 ± 0.07
N4	N(4)-demethylalstogastine	3	138.8 ± 9.65	4.49×10^{-6}	5.35 ± 0.15
N9	15-hydroxyangustilobine A	3	185.1 ± 78.5	7.65×10^{-5}	4.12 ± 0.39

Table B: The maximum response, EC_{50} and pEC_{50} by all tested alkaloids (**N1** to **N10**) in comparison with control isoprenaline-induced relaxation. Data represents mean± SEM of *n* number of animals. Table is arranged according to the efficacy of alkaloids (maximum relaxation, E_{max}) in an ascending order.

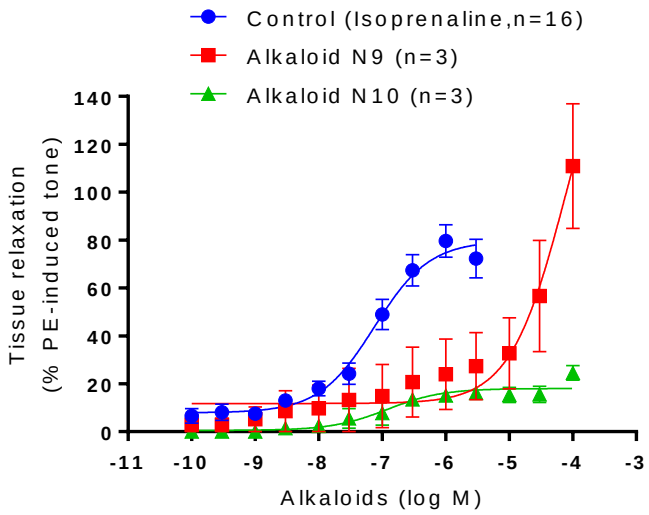


Figure D: The representative relaxant effects of alkaloid **N9** (n=3) and **N10** (n=3) in rat aortic rings pre-constricted with phenylephrine, compared to control isoprenaline-induced relaxations (n=16). Data represents mean ± SEM of *n* number of animals. Among all tested alkaloids, alkaloid **N10** elicited the lowest relaxation magnitude but with highest potency (maximum relaxation: 18.02 ± 1.31%, pEC_{50}: 6.92 ± 0.22), whereas alkaloid **N9** elicited the highest relaxation magnitude with lowest potency (maximum relaxation: 185.1 ± 78.5 %, pEC_{50}: 4.12 ± 0.39).

1.4 Discussion

The present study successfully demonstrated the vasorelaxant effects of isolated plant alkaloids from *A. pneumatophora* and *A. rostrata* on rat aortic tissues. All tested alkaloids elicited concentration-dependent relaxant effects on the aortic tissues in different potency and efficacy.

Isoprenaline is a potent β-adrenergic agonist that was previously shown to produce relaxant effect on rat aortic tissues (refer to Chapter 2). In the present study, isoprenaline elicited ~80% vascular relaxation following pre-constriction with phenylephrine. In contrast, majority of the tested alkaloids exhibited complete relaxation (≥100) of the aortic tissues. Comparing these, *Alstonia* alkaloids appeared to be more effective in eliciting relaxation on rat aortic tissues than that of isoprenaline.

In pharmacology, the measures of maximum responses and pEC_{50} (the negative logarithm of concentration of a drug/compound that produces 50% of maximum response) determine the efficacy and potency of a drug/compound respectively. The present study showed that all tested alkaloids appeared to be less potent as relaxant agents compared to the control isoprenaline. However, the potency of a drug/ product does not reflect their efficacy in evoking a response, as shown by the tested alkaloids. For example, despite a display of the highest maximum relaxation, 15-hydroxyangustilobine A (**N9**) displayed the lowest potency value among all alkaloids. In contrast, 6,7-*seco*angustilobine B (**N10**) that elicited lowest maximum relaxation, has the highest potency value (pEC_{50}). The efficacy and potency ranks of all tested alkaloids are presented in *Figure E*.

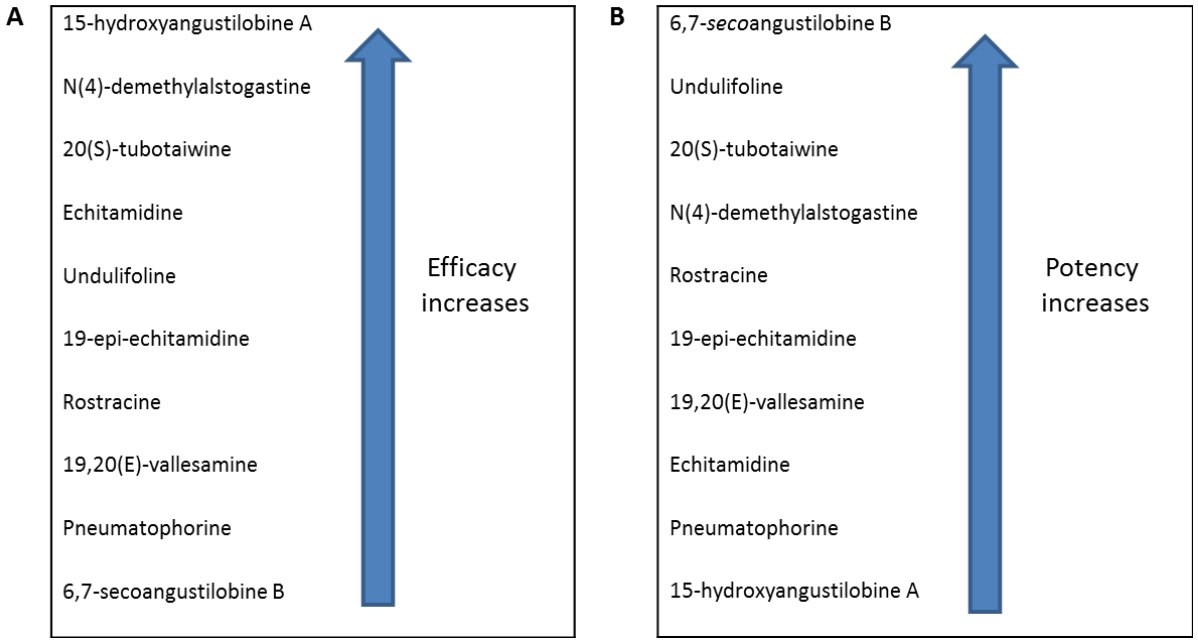


Figure E: The (A) efficacy and (B) potency ranks of isolated alkaloids from *Alstonia pneumatophora* and *Alstonia rostrata*.

Both compounds **N9** and **N10** are indole-derived alkaloids. These two alkaloids contain a characteristic indole group that is made up of a six-membered benzene ring that fused to a five-membered nitrogen-containing pyrrole ring (as shown in *Figure F*). This indole group can also be found in other clinically used drug molecules, such as vincamine, reserpine, perindopril, pindolol, yohimbine and serotonin in

treating hypertension (Kaushik *et al.*, 2013; see *Figure G*). This suggests the contributory role of indole group in compounds **N9** and **N10** in eliciting relaxant effect on rat aortic rings. By this, the different relaxant effects elicited by these two compounds (in terms of potency and efficacy) may therefore depend on the substituents that were attached to their respective indole groups.

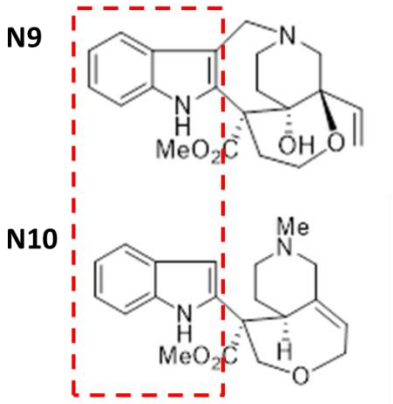


Figure F: Structural comparison between compound **N9** and **N10**. Box in red illustrates the similar indole structure in these two compounds.

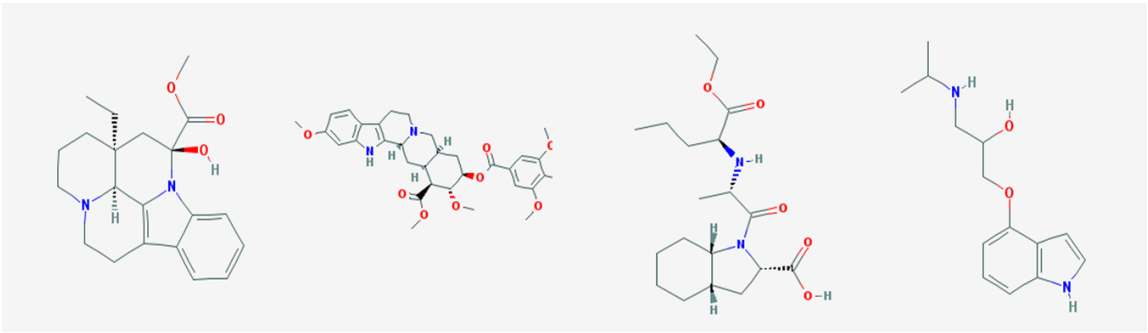


Figure G: Some examples of indole-derived drug molecules that are clinically used in treating hypertension (structures obtained from PubChem database). These drug molecules exhibit antihypertensive properties by causing vascular relaxation (Kaushik *et al.*, 2013).

The phenylephrine-induced contractile response in the aorta is due to an increase in intracellular calcium via extracellular calcium influx and/or calcium release from the internal stores. Conversely, a decrease in intracellular calcium would result in the relaxation of smooth muscle. Considering the relaxant effects of the alkaloids, one can speculate that their effect is mediated from a decrease in intracellular calcium by either directly inhibiting the calcium mobilisation pathway or coupled to other relaxation modulators that eventually cause relaxation. Such modulators leading to vascular smooth muscle relaxation include, nitric oxide, cyclic nucleotides (cAMP and cGMP) and potassium channels activators to name a few. For example, reserpine and yohimbine (refer *Figure G*), which are both plant-derived indole alkaloids from *Rauwolfia serpentine* and *Yohimbe* bark extract respectively. Despite they are from the same alkaloid group, however they elicit vascular relaxation via different mechanisms, in which reserpine inhibits the uptake of adrenergic catecholamine while yohimbine inhibits alpha-adrenergic receptors. Therefore, further pharmacological evaluation is needed to fully understand the vascular relaxant effect elicited by a novel plant compound.

For further pharmacological evaluation, a suitable pharmacological tool is needed. Among all, organ bath assembly is the best candidate due to its robust testing on selective tissues in addition to its handy operation. With advancements in pharmacology, many selective chemicals (either agonists or antagonists) are available commercially for the testing of possible mechanisms of which the compounds of interest eliciting.

The present study has demonstrated the organ bath as the best pharmacological tool to examine the effects of novel products on rat aortic tissues. Using organ bath assembly, the relaxant effects of plant novel alkaloids from two local *Alstonia* plant species, *Alstonia pneumatophora* and *Alstonia rostrata* were revealed on rat aortic tissues. This is the first reported evidence of the potential antihypertensive properties of these *Alstonia* alkaloids. The chemical structures of these alkaloids provide an insight in depicting their potential elicited vasodilating mechanisms. This gathered information is useful for the future pharmacological evaluation of other novel plant alkaloids on animal tissues.

Appendix C

Animal ethics approval:

Obtained from University of Nottingham's Animal Welfare Ethics Committee (UNMC#2kn)

Animal Welfare and Ethical Review Body

Cover Form applicable to the use of animals in non-regulated procedures

Title of Study: **Investigation on the effect of natural product extracts and compounds on the isolated rat smooth muscles**

Name of Applicant: **Kang Nee TING**

School: **Life Sciences (Malaysia Campus)**

AWERB REF: UNMC2kn

1. Requests to use animals in non-regulated procedures must incorporate the information required below in the format requested for project licence applicants. The completed form should be returned electronically to the bsu@nottingham.ac.uk. When approved a protocol reference number will be provided to the applicant and must be used when ordering animals for use in the non-regulated study.

Its purpose is to highlight the key points in the proposed programme of work and to provide an executive summary in non-technical language accessible to the lay members of the Animal Welfare and Ethical Review Body.

Your application must be accompanied by this summary written in non-technical terms. You should complete this summary template. We expect that, for all but the most complex of studies, you will be able to provide a satisfactory summary using 500 to 1,000 words.

<table>
<tr><td>Study Title (max. 50 characters)</td><td colspan="3">In-vitro organ bath experiments investigating effects of natural product extracts and isolated compounds on rat smooth muscles.</td></tr>
<tr><td>Key Words (max. 5 words)</td><td colspan="3">Isolated tissues; aorta; bladder; airway</td></tr>
<tr><td>Expected duration of the study (yrs)</td><td colspan="3">4 years (this is formed part of PhD projects, MPharm year 4 research projects and a grant application submitted recently to the Malaysia government)</td></tr>
<tr><td rowspan="6">Purpose of the study</td><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td></td><td>No</td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td></td><td>No</td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
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<tr><td>Higher education or training</td><td>Yes</td><td></td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
<tr><td>Maintenance of colonies of genetically altered animals[1]</td><td></td><td>No</td></tr>
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</td></tr>
<tr><td>Describe the objectives of the study (e.g. the scientific unknowns or scientific/clinical needs being addressed)</td><td>This application is divided into two main objectives:
1. MPharm year 4 research project: To enable final year students to further understand the pharmacology of smooth muscles including understanding the process of research in natural product drug discovery.
2. PhD projects: This forms part of the research skills training. In vitro organ bath technique will be one of the methodology to be included in the thesis.</td></tr>
<tr><td>What are the potential benefits likely to derive from this study (how science could be advanced or humans or animals could benefit from the project)?</td><td>1. Students will gain a better understanding of the pharmacology of the smooth muscles in different vascular beds where technical skills will be gained as part of their training in undergraduate or postgraduate level.
2. These experiments will provide new information on the effect of active extracts and compounds isolated from the natural flora on isolated smooth muscles from the airways, bladder and aorta.
3. In Malaysia, the skills on isolated organ bath are limited and this forms part of an important technique for drug discovery.</td></tr>
<tr><td>What species and approximate numbers of animals do you expect to use over what period of time?</td><td>Adult rats will be utilised for these experiments. With the current set up, it is estimated about 2 to 3 animals will be used weekly.
An estimate of 120 rats will be utilised per year.</td></tr>
<tr><td>What procedures will be conducted?</td><td>All animals will be sacrificed by an experienced animal technician according to the methods described in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.</td></tr>
<tr><td>In the context of what you</td><td>No treatments will be carried out on living</td></tr>
</table>

propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	animals. After humane killing, the tissues will be removed for isolated organ bath experiments.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	The study of some of the pharmacology can only be meaningful when carried out in complex integrated tissues from animals. We have tried carrying out some experiments on primary smooth muscle cell lines and to date this has not been successful. Cell lines only provide limited information on the effects of the compounds and extracts that we have been working with. Answering a direct question, for example whether a compound would dilate the airway is only possible from investigations on the isolated smooth muscle rings.
2. Reduction Explain how you will assure the use of minimum numbers of animals	The maximum number of different organs from each animal will be utilised. For example, the aorta, bladder and airways (bronchus and trachea) will be used from each rat.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	At present no other sources of animal tissues are available but, for future planning, sourcing animal tissues from sheep obtained from local slaughter houses will be considered to minimise the use of rats in these experiments.
4. Housing and Care Please confirm that animals will be housed and cared for according to the requirements of the UK Animals (Scientific Procedures) Act 1986 draft	Animals will be purchased from the animal facilities in a local university (University Putra Malaysia) This animal facility is governed by its own institutional animal care and use committee (IACUC). They adhere to the Malaysian code of practice for the care and use of animals for scientific purposes which are

Code of Practice for the care and accommodation of animals (February 2013) **or** provide details and justification for alternative housing and care standards.	consistent with the UK Home Office regulations. Information on the facility can be found here http://www.vet.upm.edu.my/eiacuc.html
5. Humane Killing Please confirm that animals will be humanely killed using an appropriate method of humane killing detailed in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 **or** provide details and justification for alternative methods of killing.	Animals will be killed by concussion of the brain and/or exposure to carbon dioxide gas. Death is confirmed by dislocation of the neck.
For Office Use Only	
Will the study be subject to Retrospective Assessment?	Yes | No | Date due:

Committee use only

Comments by NACWO:

The proposed project appears appropriate with no welfare issues providing animals are humanely killed by trained and competent staff.
Neil Yates
05.09.13

Comments by NVS:

This would appear to be satisfactory, although an approximation for the total number of rats to be used per year or over the lifetime of the project should be given. Users should be trained to a level of competency that will ensure humane euthanasia is achieved at all times.

Ewan McNeill 05-09-13

Comments by primary reader for the Committee (if applicable):

Comments by Lay Person (If applicable):

Committee decision:

Communicated to applicant (date):

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