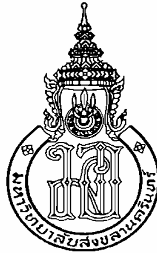

Cardiovascular Effects of Crude Extract and Substances Isolated from Leaves of *Phyllanthus acidus* in Rats

Yuttapong Leeya

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences

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ชื่อวิทยานิพนธ์	ผลต่อหัวใจและหลอดเลือดของสารสกัดอย่างหยาบ และสารที่แยกได้จากใบมะยมในหนูแร็ท
ผู้เขียน	นายยุทธพงศ์ หลี้ยา
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บทคัดย่อ

มะยม (*Phyllanthus acidus*) เป็นสมุนไพรชนิดหนึ่งตามตำรายาไทยมีสรรพคุณในการรักษาโรคผิวหนัง, แก้ไข้ และแก้ความดันโลหิตสูง การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผล และกลไกในการลดความดันโลหิตของสารสกัดหยาบด้วย n-butanol จากใบมะยมสด พร้อมทั้งแยกสารที่ออกฤทธิ์ดังกล่าว การศึกษาพบว่าสารสกัดหยาบมีฤทธิ์ลดความดันโลหิต และลดอัตราการเต้นของหัวใจในหนูแร็ทสลบ ฤทธิ์ดังกล่าวไม่สามารถยับยั้งด้วย atropine หรือ propranolol นอกจากนี้พบว่าสารสกัดหยาบมีผลทำให้หลอดเลือด thoracic aorta ที่กระตุ้นให้หดตัวก่อนด้วยทั้ง Phe หรือ KCl เกิดการคลายตัว โดยผลดังกล่าวแสดงฤทธิ์ได้เป็นระยะเวลาค่อนข้างนาน พบว่า LNA หรือการกำจัด endothelium ไม่สามารถลบล้างฤทธิ์ดังกล่าวได้ สำหรับผลการคลายตัวของหลอดเลือด aorta ที่กระตุ้นให้หดตัวก่อนด้วย Phe ไม่อาจยับยั้งด้วย atropine, propranolol หรือ indomethacin พบว่า TEA, glybenclamide หรือ ODQ สามารถยับยั้งผลดังกล่าวได้ในหลอดเลือด aorta ที่ไม่มี endothelium อย่างมีนัยสำคัญ nifedipine หรือภาวะ Ca^{2+}-free มีผลให้ทำหลอดเลือด aorta ตอบสนองต่อการกระตุ้นให้หดตัวด้วย Phe ลดลงและจะยิ่งลดลงเมื่อให้สารสกัดหยาบร่วมด้วย การแยกสารสกัดหยาบโดยวิธี column chromatography สามารถแยกสารบริสุทธิ์ที่แสดงฤทธิ์ได้ 5 ชนิดได้แก่ adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, และ kaempferol โดยสารแต่ละชนิดมีผลลดความดันโลหิต และลดอัตราการเต้นของหัวใจในหนูแร็ทสลบ และมีผลทำให้หลอดเลือด aorta ที่กระตุ้นให้หดตัวก่อนด้วย Phe เกิดการคลายตัว โดย LNA หรือการกำจัด endothelium มีผลให้การคลายตัวของหลอดเลือดลดลง สำหรับฤทธิ์การคลายตัวหลอดเลือดของ adenosine ยับยั้งได้ด้วย ODQ และ TEA ในขณะที่ glybenclamide ยับยั้งฤทธิ์ของ hypogallic acid จากการศึกษาสรุปได้ว่า ผลในการลดความดันโลหิตของสารสกัดหยาบเกิดจากการแสดงฤทธิ์ร่วมกันของสารที่แยกได้ทั้ง 5 ชนิด โดยออกฤทธิ์โดยตรงที่หลอดเลือดทำให้หลอดเลือดเกิดการคลายตัว และออกฤทธิ์โดยอ้อมโดยกระตุ้นให้มีการหลั่ง nitric oxide จาก endothelium พร้อมทั้งกระตุ้นเอนไซม์ soluble guanylate cyclase รวมถึงกระตุ้นให้มีการเปิดของ K_{ATP} และ K_{Ca} channels ที่กล้ามเนื้อเรียบของหลอดเลือด นอกจากนี้ยังอาจแสดงฤทธิ์ไปยับยั้งการเปิดของ Ca^{2+}-channel หรือยังยั้งการหลั่งของ Ca^{2+} จากภายในเซลล์ และไม่ได้ออกฤทธิ์ผ่านทาง muscarinic หรือ β-adrenergic receptors

Thesis Title Cardiovascular effects of crude extract and substances isolated from leaves of *Phyllanthus acidus* in rats

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ABSTRACT

Phyllanthus acidus is used in Thai Folkloric Medicine for dermatitis, antipyretic and antihypertensive treatment. The present study aimed to investigate the effects, identify the active substances and establish the mechanisms involved in the hypotensive activity of n-butanol extract from leaves of *Phyllanthus acidus* (PA extract). PA extract caused a decrease in blood pressure and heart rate of anesthetized rats which were not inhibited by atropine or propranolol. PA extract caused a prolonged dilatation of thoracic aortic rings preconstricted with either Phe or KCl, and this effect was not inhibited by LNA or removal of vascular endothelium. For Phe-preconstricted aortic rings, the dilatation activity of PA extract was not inhibited by atropine, propranolol or indomethacin. TEA, glybenclamide or ODQ significantly inhibited this activity on endothelium-denuded aortic rings. Nifedipine or Ca^{2+}-free medium reduced in contractile responsiveness of the aortic rings to Phe, and that was further potentiated by PA extract. Using hypotensive-guided fractionations by column chromatography, 5 active substances: adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, and kaempferol were isolated. Each of these substances caused a decrease in blood pressure and heart rate of anesthetized rats, and caused dilatation of thoracic aortic rings preconstricted with Phe. LNA or removal of the endothelium reduced this activity. ODQ and TEA attenuated the vasodilatation activity of adenosine where as glybenclamide and ODQ attenuated the effect of hypogallic acid. These results suggest that the hypotensive activity of PA extract would be a synergistic effect of these five substances to act directly at the blood vessels to cause vasodilatation, and indirectly via the vascular endothelium to stimulate release of nitric oxide, as well as at the smooth muscle to stimulate soluble guanylate cyclase, and to open the K_{ATP} and K_{Ca} channels. In addition PA extract may also play a role as a Ca^{2+}-channel inhibitor or may involve inhibition of Ca^{2+} mobilization from the intracellular store, but do not act through the muscarinic or β-adrenergic receptors.

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Yuttapong Leeya

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LIST OF ABBREVIATIONS AND SYMBOLS

AC	=	adenylate cyclase
ACh	=	acetylcholine
ANS	=	autonomic nervous system
ATP	=	adenosine triphosphate
Atro	=	atropine
AV	=	atrioventricular
BP	=	blood pressure
bpm	=	beat per minute
^{o}C	=	degree Celsius
Ca^{2+}	=	calcium
$CaCl_2$	=	calcium chloride dihydrate
cAMP	=	cyclic adenosine monophosphate
$CDCl_3$	=	chloroform-d_6
cGMP	=	cyclic guaosine monophosphate
$CHCl_3$	=	chloroform
CO_2	=	cabondioxide
^{13}C NMR	=	carbon-13 Nuclear Magnetic Resonance
CNS	=	central nervous system
C-R	=	concentration-response
DAG	=	diacylglycerol
DMSO	=	dimethyl- d_6 sulfoxide
DOC	=	depolarization-operated channels
DP	=	diastolic pressure
endo	=	endothelium
eNOS	=	endothelial nitric oxide synthase
g	=	gram
GC	=	guanylate cyclase
glyben	=	glybenclamide
G-protein	=	guanine nucleotide binding protein
GTP	=	guanosine triphosphate
hrs	=	hour

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

^1H NMR	=	proton Nuclear Magnetic Resonance
HPLC	=	High Performance Liquid Chromatography
HR	=	heart rate
H_2O	=	water
Hz	=	hertz
IDM	=	indomethacin
iNOS	=	inducible nitric oxide synthase
IP_3	=	inositol triphosphate
i.p.	=	intraperitoneal
i.v.	=	intravenous
K^+	=	potassium
K_{ATP}	=	ATP-sensitive K^+ channels
K_{Ca}	=	Ca^{2+}-sensitive K^+ channels
KCl	=	potassium chloride
KH_2PO_4	=	potassium dihydrogen orthophosphate
kg	=	kilogram
LNA	=	N^G-nitro-L-arginine
M	=	Molar
MAP	=	mean arterial blood pressure
MeOH	=	methanol
mg	=	milligram
$MgSO_4$	=	magnesium sulphate
min	=	minute
ml	=	milliliter
MLCK	=	myosin light chain kinase
mm	=	millimeter
mM	=	milli Molar
mmHg	=	millimeters of mercury
MLC	=	myosin light chain
MLCK	=	myosin light chain kinase
MPLC	=	Moderate Pressure Liquid Chromatography

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

n	=	number
Na^+	=	sodium
NaCl	=	sodium chloride
Na_2EDTA	=	disodium etylenediaminetetraacetic acid
$NaHCO_3$	=	sodium hydrogen carbonate
NE	=	norepinephrine
nm	=	nanometer
NMR	=	Nuclear Magnetic Resonance
NO	=	nitric oxide
NOS	=	nitric oxide synthase
O_2	=	oxigen
ODQ	=	1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one
PA extract	=	*Phyllanthus acidus* extract
PGI_2	=	prostacyclin
Phe	=	phenylephrine
PIP_2	=	phosphatidyl inositol bisphosphate
PKC	=	protein kinase C
PKG	=	protein kinase G
PLC	=	phospholipase C
ROC	=	receptor-operated channels
RP	=	reversed phase
SAC	=	stretch-activated cation channel
SA	=	sinoatrial
S.E.	=	standard error
SP	=	systolic pressure
SR	=	sarcoplasmic reticulum
TEA	=	tetraethylammonium
TFA	=	trifluoroacetic acid
TLC	=	Thin Layer Chromatography
UV	=	ultraviolet
VOC	=	voltage-operated channels

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

%	=	percent
μM	=	micro Molar
α	=	alpha
β	=	beta
δ	=	chemical shift

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Traditional medicine is generally defined as the sum total of all knowledge and practices, whether explicable or not, used in diagnosing, preventing, and eliminating physical, mental, or societal imbalances. It relies exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing (WHO, 1978). Sometimes traditional medicine involves a sophisticated theory or system, though the knowledge of traditional medicine is often passed on, verbally or otherwise, from generation to generation (Zhang, 2000).

Traditional herbal medicine refers to medicinal products whose active ingredients are derived from aerial or underground parts of plants or other plant material or the combination of them, whether in the crude state or as a plant preparation. Plant material includes such substances as juices, gums, and oils. Herbal medicines may contain plant materials other than the active ingredients and may even contain other non-plant organic or inorganic active ingredients. Traditional herbal medicine is the oldest known type of medical treatment and has been practiced in virtually every culture worldwide (Hills et al., 2006). Humans have used plant materials as herbal medicines since prehistoric times. In some countries such as China documentary evidence shows that herbal medicines have been used for at least 7000 years, while in Europe there is also a rich history in the use of herbal plants (Wilkinson, 1998). In Thailand, traditional medicine draws from Chinese and Indian traditions.

Interest in herbal medicines began to decline in the 1700's when the reduction approach to science began to dominate. Plants were extracted and pure compounds were isolated, produced and synthesized. This eventually led to the development of the pharmaceutical industry where the synthetic approach to drug designs still dominates today. Although there is a large amount of anecdotal evidence to support the use of herbal medicines, the scientific studies to support these claims are in most cases in their infancy. Therefore there is interest in the scientific community to conduct research to purify the active constituents from the herbal medicine and prove that the constituents are effective

clinically and to clarify their potential use as drugs. In 1994, The National Institute of Thai Traditional Medicine, Ministry of Public Health was established to undertake research on Thai traditional medicine and herbs, to promote their use in the health services (Pornsiripongse, 2003).

Cardiovascular diseases, defined as diseases of the heart and circulatory system are the main cause of mortality, morbidity and hospitalisation. Cardiovascular disease, and its complications, is the main cause of death for both males and females among all racial and ethnic groups in almost countries of the world. It has now become a major public health problem in less-developed countries. It is usually in the top five causes of death (Akinkugbe, 1990; Dodu, 1984). About two-thirds of the estimated 14.3 million annual cardiovascular disease deaths occur in the developing world (Bonita and Beaglchole, 1994).

Because cardiovascular disease has now become the main cause of mortality in the history of mankind, most breakthroughs that have discovered medical uses for natural products have been in the area of cardiovascular research. Cardiovascular agents account for approximately 15% of all prescriptions filled each year, and a large number of these are based on naturally occurring substances (Culter and Cutler, 2005). The drugs: digitalis, reserpine, ajmaline, quinidine, ergotamine, atropine and lovastatin, are a few examples of important therapeutic agents isolated from plants (Gilani, 1998).

However, modern medicine is now beginning to accept the use of standardized plant extracts. Therefore, it is of interest to find new cardiovascular drugs from Thai traditional plants by extracting them and isolating and purifying cardiovascular active substances using current scientific methods.

1.2 Cardiovascular system

The cardiovascular system consists of two anatomical components: the heart and the blood vessels (Fox, 2002). The heart contains four muscular chambers double pump: two upper, right and left atria, which receive venous blood and fill the blood into the ventricles, and two lower, right and left ventricles, which receive blood from atria and eject blood into arteries (Van De Graaff and Fox, 1995). The blood vessels form a tubular network that can be divided into two main circuits: the pulmonary circuit, which carries blood to the lungs, and the systemic circuit, which transports blood to the rest of the body

(Matini, 2001). These four chambers of the heart and two circuits of the blood vessels are arranged in series, which mean that the blood must flow through them in sequence as shown in figure 1 (Rhoades and Pflanzer, 2003).

The function of the heart and the vascular system (blood vessels) are mainly controlled by the autonomic nervous system, which consists of sympathetic and parasympathetic nervous system, acting via adrenergic receptors and muscarinic recepetor (Opie, 1998). These components: the heart, the blood vessel and the autonomic nervous system, interact in a complex manner to control blood flow to organs throughout the body by transporting and distributing essential substances to the tissues and removing the by-products of metabolism.

1.2.1 The Heart

The heart is a hollow, four chambers muscular organ. It is located within the thoracic cavity between the lungs. About two-thirds of the heart is left of the midline, with its apex, or cone-shaped end, pointing downward in contact with the diaphragm. The base of the heart is the broad superior end, where the large vessels attach. The heart is enclosed and protected by a loose fitting, serous sac of dense regular connective tissue called the parietal pericardium, or pericardial sac (figure 2). The parietal pericardium separates the heart from the other thoracic organs and forms the wall of the pericardial cavity, which contains a watery, lubricating pericardial fluid (Van De Graaff and Fox, 1995).

Heart wall

The wall of the heart is composed of three distinct layers: outer layer, middle layer and inner layer (figure 3).

- The outer layer is the epicardium, also called visceral pericardium that covers the outer surface of the heart. This serous membrane consists of an exposed mesothelium and an underlying layer of loose connective tissue that is attached to the myocardium. The space between this layer and the parietal pericardium is the pericardial cavity.

- The thick middle layer of the heart is the myocardium, or muscular wall of the heart, forms both atria and ventricles. This layer contains cardiac muscle tissue,

blood vessels and nerves. The thickness of the myocardium varies in different chambers, depending on the force needed to eject blood. The thickest portion therefore surrounds the left ventricle. The atrial walls are relatively thin.

- The inner layer of the heart is the endocardium, is simple squamous epithelium that is continuous with the endothelium of the attached blood vessels. The endocardium also forms part of the valves of the heart (Matini, 2001).

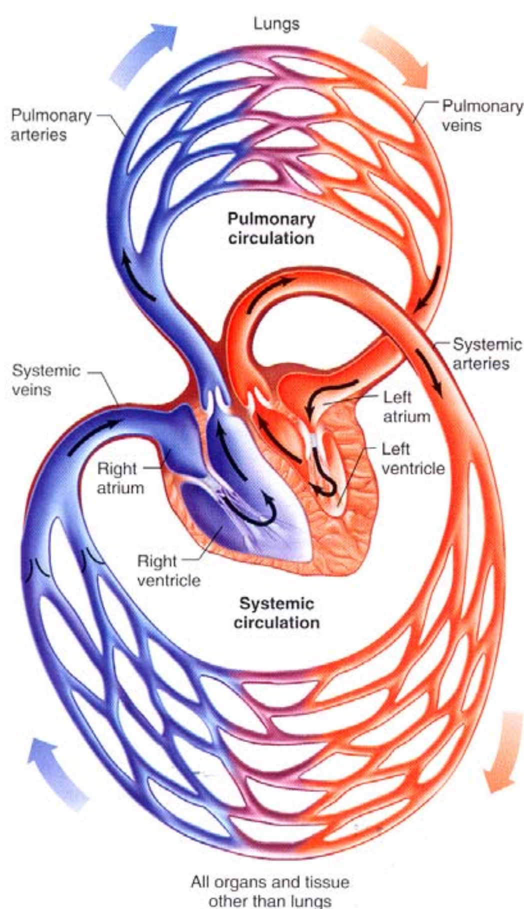


Figure 1 Schematic diagram of the cardiovascular system (Widmaier et al., 2004).

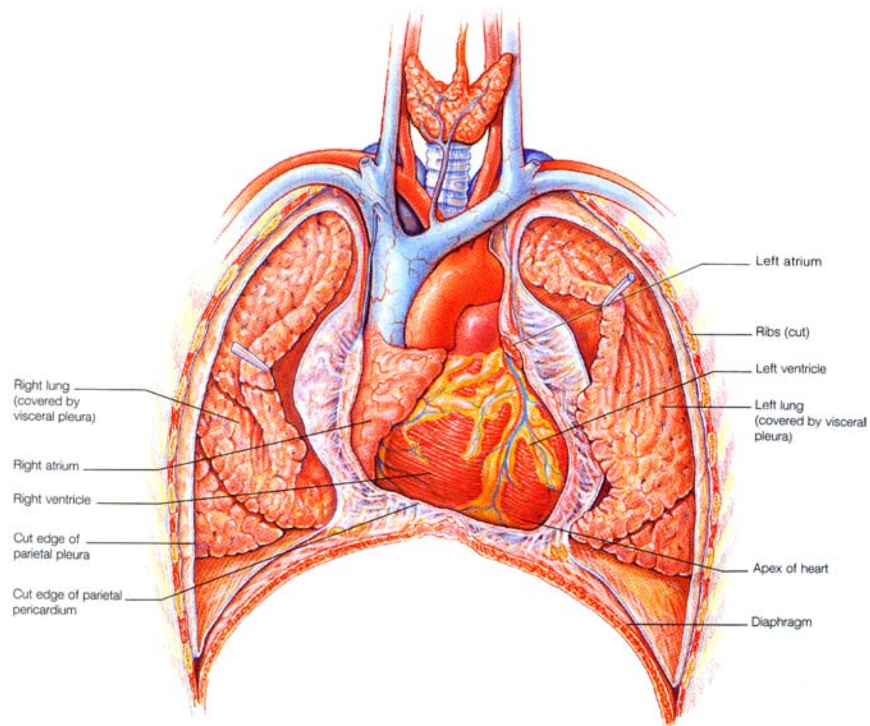


Figure 2 Position of the heart and associated serous membranes within the thoracic cavity (Van De Graaff and Fox, 1995).

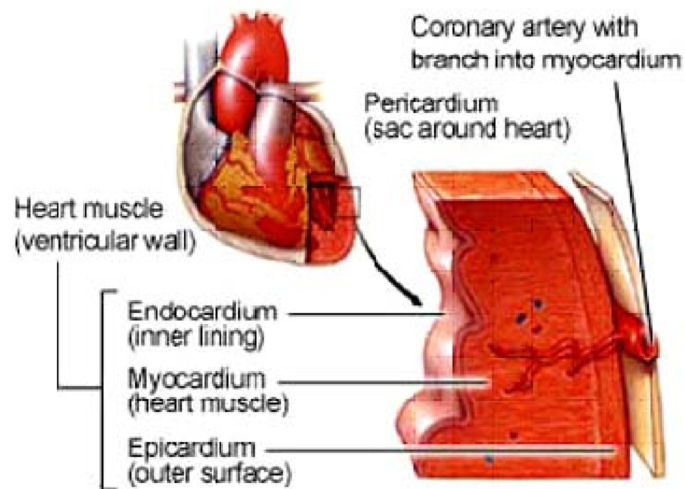


Figure 3 Layers of the heart wall (http://www.ohiohealth.com)

Cardiac muscle

The cardiac muscle cells of the myocardium are arranged in layer that are tightly bound together and completely encircle the blood-filled chambers. The properties of the cardiac muscle are combines between the skeletal and the smooth muscle. The cells are striated as the result of an arrangement of thick myosin and thin actin filaments similar to the skeletal muscle. Cardiac muscle cells are shorter than skeletal muscle fibers, and have several branching processes. Adjacent cells of cardiac muscle cells are interconnected by intercalated discs (figure 4) which are held together by desmosomes and linked by gap junctions, similar to the smooth muscles (Widmaier et al., 2004).

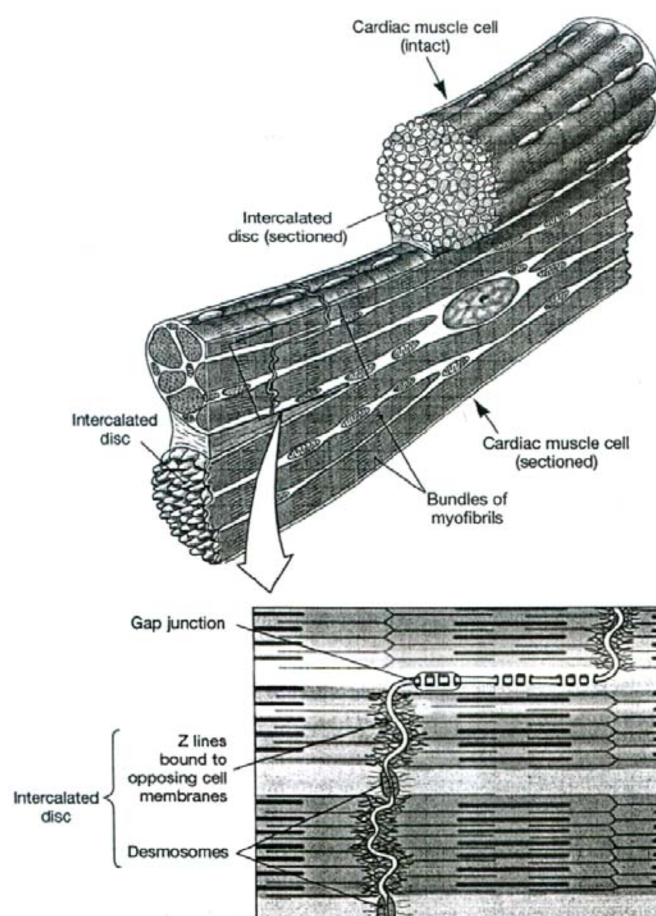


Figure 4 Diagrammatic views of cardiac muscle tissue (a) and structure of an intercalated disc (Matini, 2001).

Heart chambers

The interior of the heart is divided into four chambers: upper right and left atrium and lower right and left ventricles. The right atrium communicates with the right ventricle, and the left atrium with the left ventricle (figure 5). The atria are separated by the thin muscular interatrial septum, while the ventricles are separated by the thick muscular interventricular septum (Matini, 2001).

- The right atrium receives venous blood from the systemic circuit through the two great veins: the superior vena cava, which drain the upper portion of the body, and the inferior vena cava, which drain the lower portion of the body.

- The right ventricle receives blood from the right atrium through the tricuspid valve. Ventricular contraction causes the tricuspid valve close and blood exit from the right ventricle through the pulmonary trunk and enter to the lungs through the right and left pulmonary arteries. When the right ventricle relaxes, the pulmonary semilunar valve closes to prevent backflow of blood into the ventricle.

- The left atrium receives oxygenated blood from the pulmonary circuit through the four branches of the pulmonary veins, two right and two left pulmonary veins, after gas exchange has occurred within the capillaries of the lungs

- The left ventricle has thicker walls than the right ventricle, receives blood from the left atrium. These two chambers are separated by bicuspid valve. When the left ventricle is relaxed, the valve is open and allows blood to flow from the left atrium to the left ventricle. When the left ventricle contracts, the valve closes to prevent backflow of blood into the atrium. Oxygenated blood exits the left ventricle through the ascending portion of the aorta. As a result of the pressure of the blood when the left ventricle relaxes, the aortic semilunar valve closes to prevent backflow of ejected blood into the relaxed ventricle (Van De Graaff and Fox, 1995).

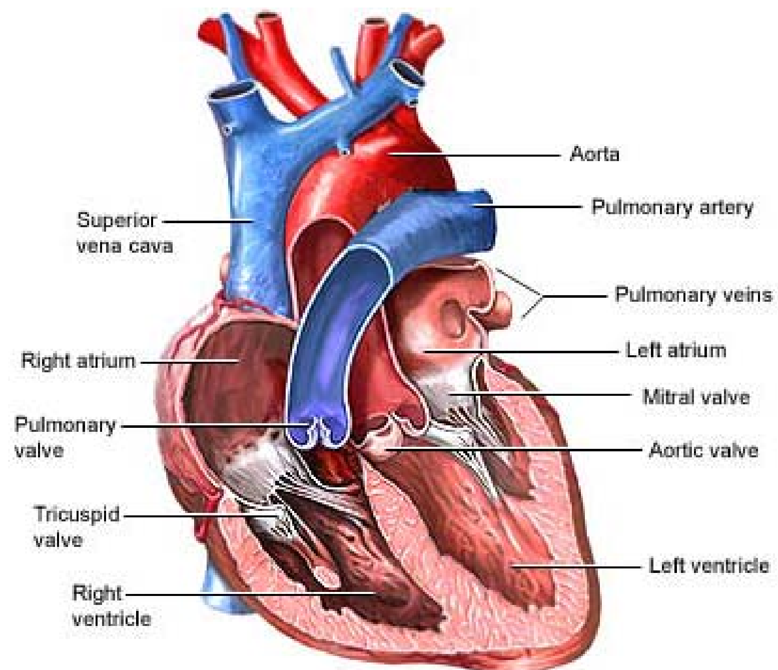


Figure 5 Diagrammatic frontal sections through the heart (http://www.besthealth.com)

Heart valves

Efficient pumping action of the heart requires a minimum of reflux as the blood is transported. This is achieved through two sets of unidirectional, reciprocating valves: the semilunar valves consist of the pulmonary and the aortic valves and the atrioventricular valves consist of the tricuspid and bicuspid valves as shown in figure 6 (Smith and Kampine, 1990).

- The semilunar valves are located at the exit of the right and left ventricles and open and close passively. They prevent the backflow of blood from the pulmonary trunk and aorta into the right and left ventricles respectively.

- The atrioventricular (AV) valves are located between atria and ventricles. They prevent the backflow of blood from the ventricles to atria when the ventricles are contracting which have the chordae tendineae and papillary muscles play and important role in the function of AV valves (Matini, 2001).

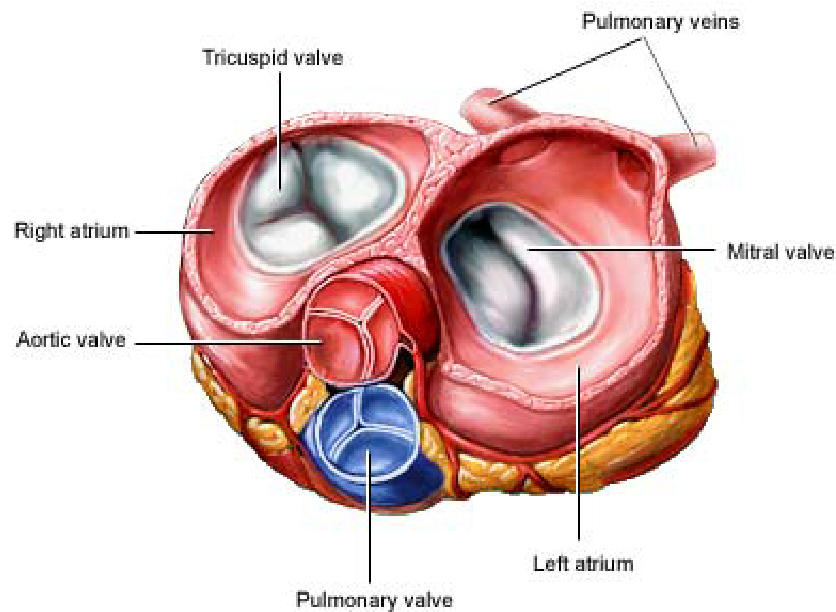


Figure 6 Valves of the heart (http://www.besthealth.com)

Blood supply

The heart works continuously, and cardiac muscle cells require reliable supplies of oxygen and nutrients. The coronary circulation supplies blood to the muscle tissues of the heart (Matini, 2001). They receive there blood supply like the cells of all other organs via arteries that branch from the aorta. The arteries supplying the myocardium are the coronary arteries, and the blood flowing through them is the coronary blood flow (figure 7). The coronary arteries exit from the very first part of the aorta and lead to a branching network of small blood vessels similar to those in other organs (Widmaier et al., 2004).

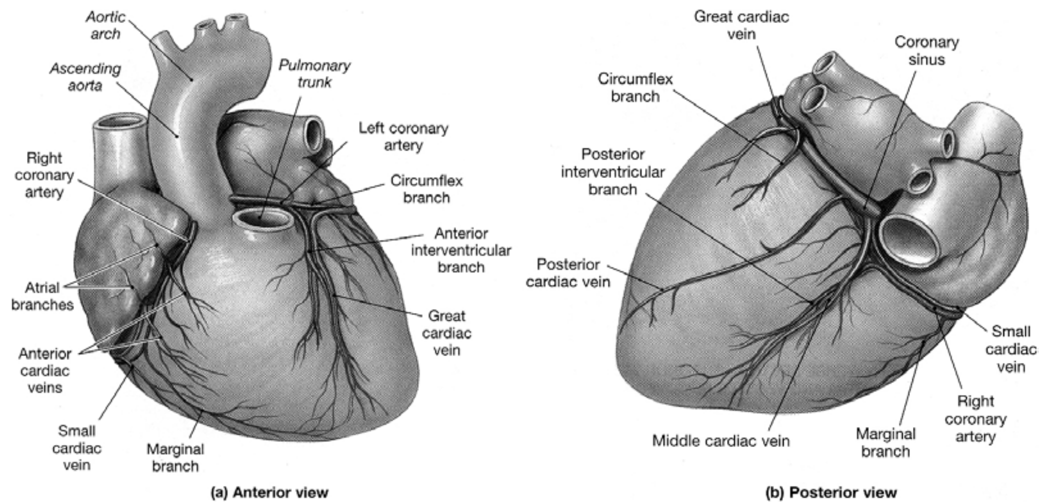


Figure 7 Coronary vessels supplying the anterior surface (a) and posterior surface (b) of the heart (Matini, 2001).

Heart function, in terms of contractile force (inotropy) and beating frequency (heart rate) relies on a three-tiered control system: (1) an immediate and fast feedback response of the cardiac tissue to the actual mechanical load, (2) regulation of cardiac performance by the autonomic nervous system involving humoral primary messengers affecting the intracellular signaling systems, and (3) long-term adaptation to altered physiological and pathological conditions produced by changes in gene expression. However, the first two modes of regulation partially overlap and primarily depend on the sympathetic nervous system (Zaugg and Schaub, 2004).

1.2.2 The blood vessels

The blood vessels of the body are tubular networks which form a closed delivery system that begin and end at the heart. Although the blood vessels sometimes compare to a system of pumping pipes within which blood circulates. Unlike rigid pipes, blood vessels are dynamic structures that pulsate, constrict and relax, and even proliferate, as demanded by the changing needs of the body (Marieb, 2001).

There are two main circuits of the blood vessels network: the smaller pulmonary circuit, which carries blood to the lungs, and the much larger systemic circuit, which supply and drain all the organs and tissues of the body. Each circuit consists of a pump, a distributing system, an exchange system, and a collecting system (Figure 8). Whereas the two circuits function in a generally similar manner, they have some important differences; the pulmonary circuit has a much lesser volume, its vessels are shorter and thinner walled, and it operates under lower pressure and with less resistance to flow (Smith and Kampine, 1990).

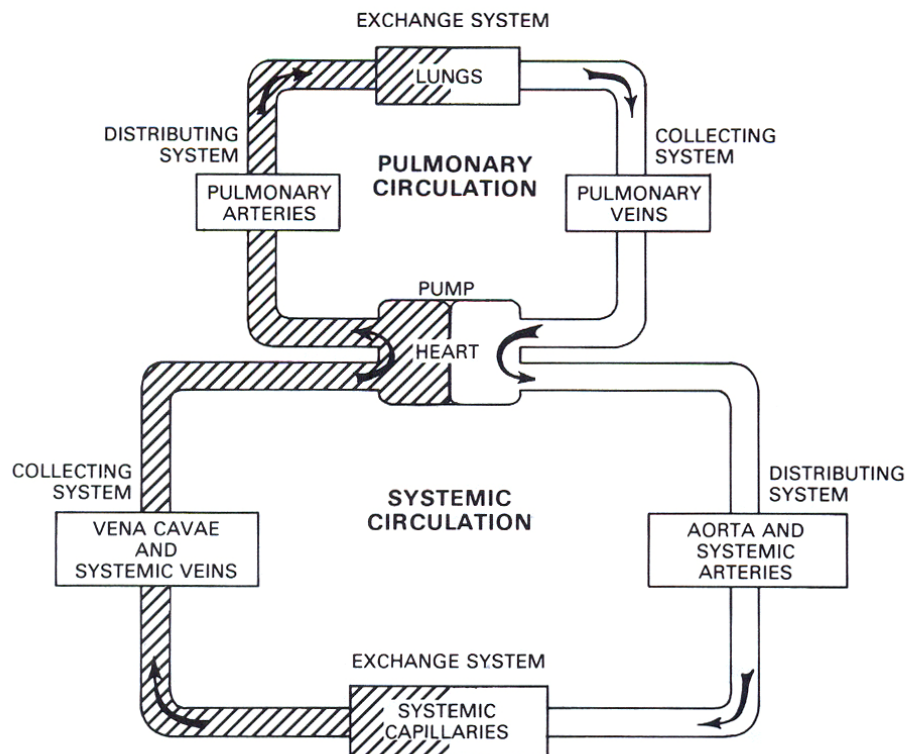


Figure 8 Functional division of the circulation (Smith and Kampine, 1990)

Structure of blood vessel walls

The wall of all blood vessels, except the very smallest, are composed of three distinct layers, or tunics: tunica interna, tunica media and tunica externa (Figure 9). These tunics surround a central blood containing space, the vessel lumen.

- Tunica interna, or tunica intima, the innermost layer of the blood vessel, which exposed to the blood. It consists of a simple squamous endothelium overlying a basement membrane and a sparse layer of fibrous tissue

- Tunica media, the middle layer, it contains concentric sheets of smooth muscle, collagen, and sometimes elastic tissue. This layer, especially the smooth muscles, is responsible for the vasoconstriction (smooth muscles contract) and vasodilatation (smooth muscles relax) of blood vessels.

- Tunica externa, or tunica adventitia, the outermost layer of the blood vessel. This layer is composed largely of loosely woven collagen fibers that protect and reinforce the blood vessels, and anchor it to surrounding structures. The tunica externa is infiltrated with nerve fibers, lymphatic vessels and a network of elastin fibers.

Their layered walls give arteries and veins considerable strength. However, the walls of arteries and veins are too thick to allow diffusion between the blood stream and surrounding tissues, or even between the blood and the tissues of the vessels itself. For this reason, the walls of large vessels contain small arteries and veins that supply the smooth muscle cells and fibroblasts of the tunica media and tunica externa. These blood vessels are called vasa vasorum (Matini, 2001; Saladin, 2004).

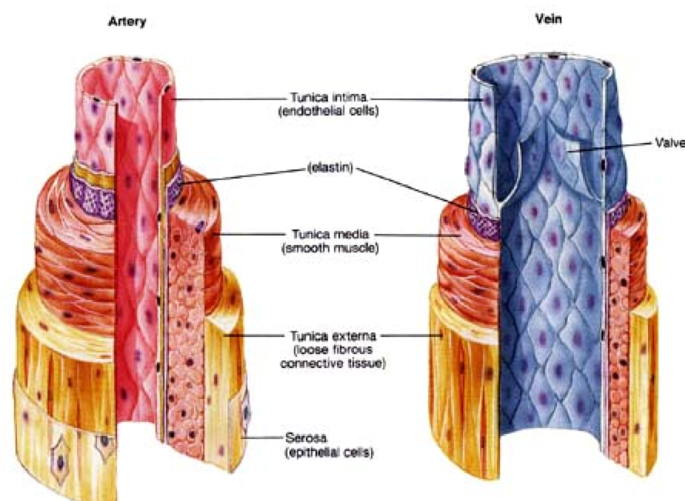


Figure 9 Structure of the blood vessel walls (http://tlhung.blogspot.com)

Classification of blood vessels

The three major types of blood vessels are arteries, capillaries and veins (Marieb, 2001). Arteries and veins simply act as conduits for blood. Arteries carry blood away from heart; by contrast veins carry blood toward the heart. Only the capillaries have intimate contact with tissue cells and directly serve cellular needs. Exchanges between the blood and tissue cells occur primarily through the thin capillary walls.

Blood vessels are functionally classified into 5 types (figure 10).

- Conducting (elastic) arteries are the largest artery. Some examples are the pulmonary artery, aorta and major branches. They have very distensible walls because their tunica media consists of numerous sheets of elastic tissue. This allows larges arteries to expand and receive the stroke volume during ventricular ejection, and recoil during diastole. This lessens the fluctuations in blood pressure exerted on smaller arteries downstream.

- Distributing (muscular) arteries are the medium to small arteries such as cerebral, femoral and coronary arteries. Their tunica media is thicker relative to the lumen diameter than in conducting arteries, and they contain more smooth muscle cells constituting about three-quarters of the wall thickness. The distributing arteries act as low-resistance conduits and their thick walls help prevent collapse at sharp bends like the knee joint.

- Resistance (small) arteries are the smallest, terminal arteries and arterioles. Their tunica media is thicker in proportion to the lumen than that of lager arteries. The high resistance of the arterioles and smallest arteries is caused by their narrow lumen. When they dilate the resistance to flow falls, so blood flow increase. By contracting hard it can temporarily prevent blood from flowing through the group of capillaries that it feeds, and can thus influence capillary exchange.

- Exchange vessels or capillaries are the end of circulatory system where materials are exchanged between the blood and tissue fluid. They consist only of endothelium and a basement membrane. Capillaries are organized in groups called capillary beds which total cross-sectional area is very large.

- Capacitance vessels are the vessels that act as a variable reservoir of blood because of their large number and size, venules and veins contain about two-third of the circulating blood at any one instant. The wall of these vessels is thin and comprises an intima, a thin media composed of smooth muscle, collagen and an adventitia (Levick, 2000; Saladin, 2004; Wynsberghe et al., 1995).

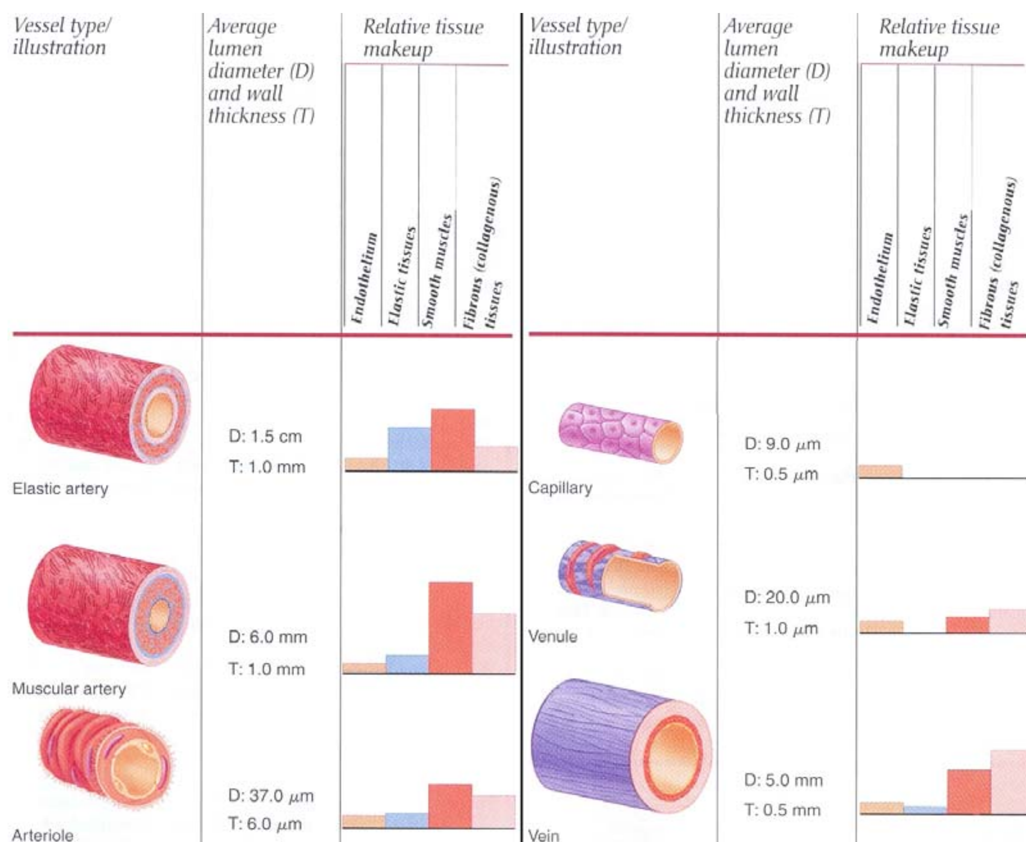


Figure 10 Functional types of the blood vessels (Marieb, 2001)

Biology of the endothelial cells

The endothelial cells are the crucial structure in the tunica interna layer of blood vessel and rest on a basement membrane, separated from smooth muscle cells (Opie, 1998). They consist of a single cell monolayer of a polygonal, flattened cells, which highly active in the control of the circulation (figure 11). Endothelial cells play an

important role in the regulation of blood vessels by produce powerful vasodilators: prostacyclin (Moncada, 1976) and endothelium-derived relaxing factors, very likely to be identical with nitric oxide (Furchgott and Zawadzki, 1980), as well as the metabolic vasoactive substances: catecholamine, angiotensin and bradykinin (Vane, 1969).The endothelial cells also release the vasoconstrictor substances: endothelin and thromboxane A2 (Opie, 1998).

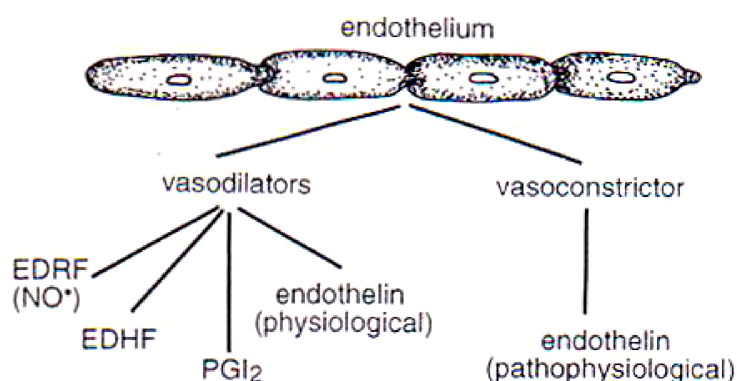


Figure 11 Role of endothelium in vascular regulation (Opie, 1998)

Nitric oxide

Nitric oxide (NO) is a lipophilic, freely, diffusible gas. It travels to the vascular smooth muscle from the endothelium to stimulate guanylate cyclase in vascular smooth muscles to produce cyclic guaosine monophosphate (cGMP), leading to vasodilatation (Murad, 1996; Rapoport et al., 1983). NO has several important cardiovascular actions: it is a powerful vasodilator, inhibits platelet aggregation, inhibits the nuclear transcription of leucocyte-binding adhesion molecules in endothelium, or inhibits vascular smooth muscle proliferation (Levick, 2000). Because its half-life is very short, measure in seconds or even less, so NO must be generated continuously to be effective. NO is synthesized in the endothelial cells from L-arginine, which enter the cells via an amino acid transporter in the cell membrane. L-arginine is changed to NO by enzyme endothelial nitric oxide synthase (eNOS) and has L-citrulline as a co-product (Boulanger et al., 1998). There are three main factors affecting nitric oxide synthase (NOS) activity:

pharmacological agonist, shear stress due to blood flow and formation of inducible nitric oxide synthase (iNOS) (Levick, 2000).

The secretion of NO to regulate vasodilatation is induced by two pathways (figure 12): mechanical and pharmacological pathways. (1) Mechanical pathway: shear stress of blood flow activates stretch-activated cation channel (SAC), which allows a flux of extracellular Ca^{2+} ion into the cells. (2) Pharmacological pathway: agonists, such as acetylcholine, bradykinin or substance P, raise cytosolic Ca^{2+} by two processes: (a) they activate the receptor-operated channels (ROC) resulting in an influx of extracellular Ca^{2+} ion into the cells. (b) The agonist-guanine nucleotide binding protein (G-protein) complex activates a membrane bound enzyme called phospholipase C (PLC). This catalyses the production of a cytoplasmic messenger, inosital triphosphate (IP_3), which activates the Ca^{2+} release from the store membrane. All of these three processes induce a transient increase in intracellular Ca^{2+}, which then binds to calmodulin and form Ca^{2+}-calmodulin complex. This complex activates eNOS to produce NO. Then NO diffuses out of endothelium to vascular smooth muscle cells, where it combines with the haem group in the enzyme guanylate cyclase (GC) to activate the enzyme. The activation of GC produces cGMP from guanosine triphosphate (GTP). The cGMP activates protein kinase G (PKG) by a number of phosphorylating actions to reduce the cytosolic Ca^{2+} concentration in the vascular myocyte leading to vasodilatation (Levick, 2000).

In some vessels and species, NO also cause hyperpolarization of vascular smooth muscle, e.g. in the coronary artery. Hyperpolarization is a well known mechanism for inducing relaxation of blood vessels. Myocyte hyperpolarization occurs when K^+ channels open, and it appears that at high concentration NO can directly activate Ca^{2+}-sensitive K^+ channels (K_{Ca}) in the myocyte membrane (Cohen and Vanhoutte, 1995).

Contraction and relaxation of vascular smooth muscle

The contractile activity of vascular smooth muscle is depending on the concentration of cytosolic Ca^{2+}. The increasing or reducing of cytosolic Ca^{2+} is the result from the opening of Ca^{2+} channels through voltage-gated Ca^{2+} channels (electromechanical coupling) or receptor-mediated Ca^{2+} channels (pharmacomechanical coupling) (Milnor, 1990). However, the pharmacomechanical coupling is predominate mechanism for eliciting contraction of vascular smooth muscle.

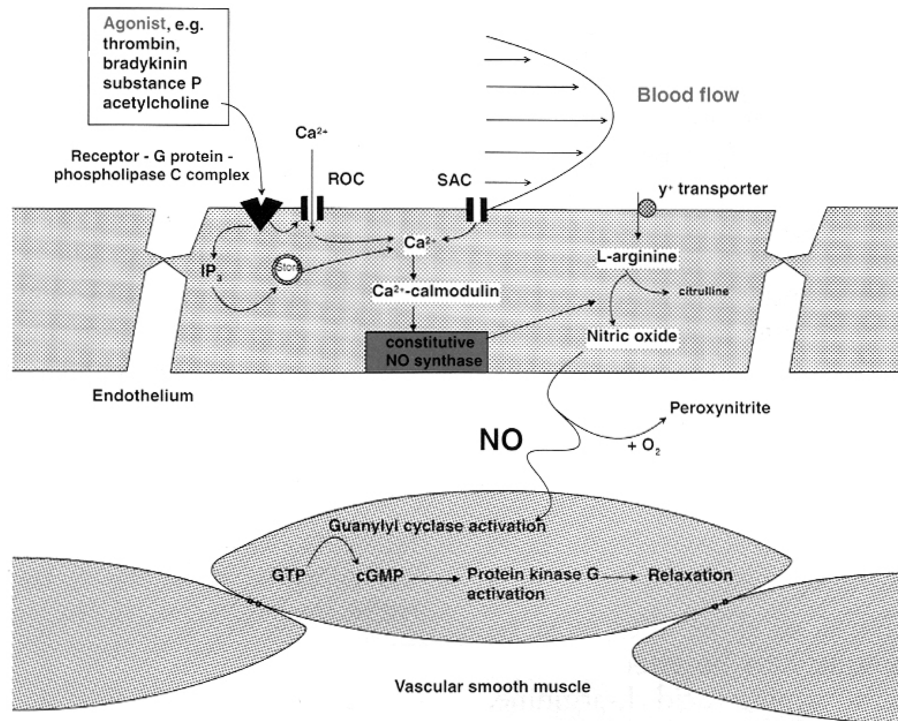


Figure 12 The regulation of nitric oxide production by endothelium (Levick, 2000)

Contraction of vascular smooth muscle was acted by releasing calcium from the sarcoplasmic reticulum (SR). For example, stimulation of receptor in membrane of vascular smooth muscle by vasoconstrictors leads to increased activity of PLC in a reaction coupled to G-protein. PLC splits phosphatidyl inositol biphosphate (PIP_2) into 2 messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 promotes the release of Ca^{2+} from SR. This Ca^{2+} bind to calmodulin, which in turn binds to myosin light chain kinase (MLCK). The latter phosphorylates the myosin light chain (MLC). The phosphorylated myosin heads form crossbridges with actin filament, producing shortening and tension (figure 13). Vasoconstriction also occurs in response to enhanced activity of the Ca^{2+} channels which either receptor-operated channels (ROC) or depolarization-operated channels (DOC) (Berne and Levy, 1998; Levick, 2000; Opie, 1998).

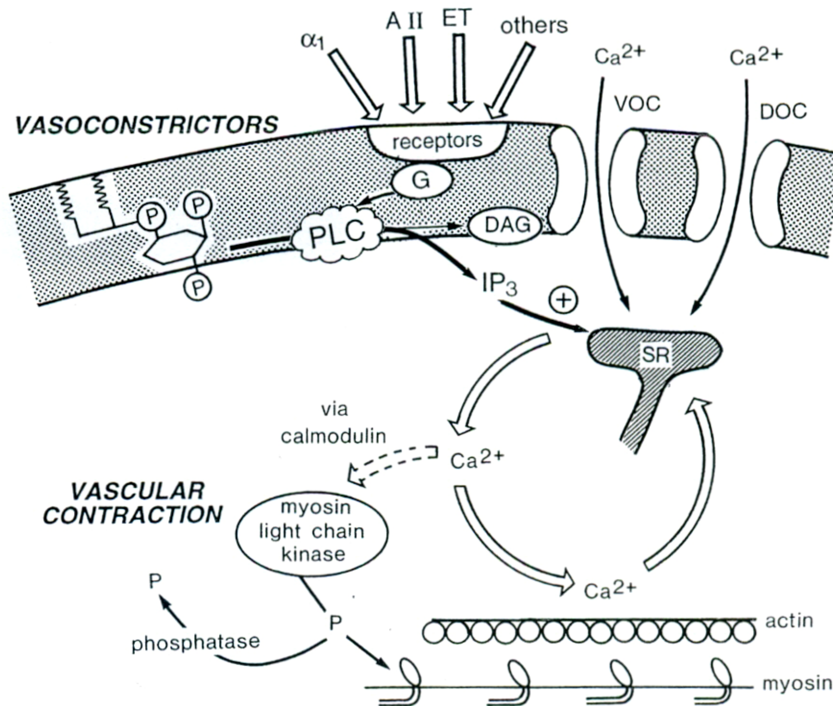


Figure 13 Vasoconstrictory mechanisms (Opie, 1998)

Relaxation of vascular smooth muscle most act by the formation of cyclic nucleotides; cyclic adenosine monophosphate (cAMP) and cyclic guaosine monophosphate (cGMP). These are both vasodilatory, possibly by inhibition of MLCK or reduce cytosolic Ca^{2+} (figure 14). Vasodilatory cAMP is formed by stimulation of adenylate cyclase (AC) in response to adenosine or β_2-stimulation, or by prostacyclin (PGI_2). cGMP is the messenger for GC, which in turn stimulated by NO (Levick, 2000; Opie, 1998).

Control of the contraction and relaxation of vascular smooth muscle is mainly regulated by the cytosolic Ca^{2+} through synthesis of the second messengers, cAMP and cGMP, as well as by acting on the protein kinase: MLCK, Protein kinase C (PKC), cAMP dependent protein kinase, and cGMP dependent protein kinase (Silver, 1985)

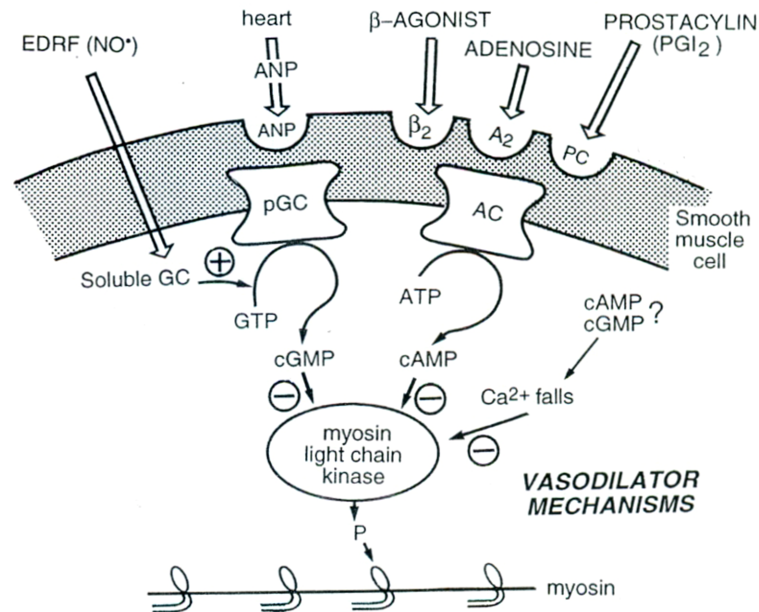


Figure 14 Vasodilatory mechanisms (Opie, 1998)

Role of ion channels in vascular smooth muscle

- Ca^{2+}channels. In vascular smooth muscle there are 2 types of Ca^{2+} channels, voltage-operated channels (VOC) and receptor-operated channels (ROC), while there is only one type of VOC in heart. VOC response to adrenergic stimulation (norepinephrine: NE), to K^{+} induced depolarization or to spontaneous automaticity. ROC response to α_1, angiotensin II or endothelin receptor stimulation. The result is increased opening probability of the Ca^{2+} channels to cause contraction. The receptors that enhance Na^{+} opening also stimulate PLC with formation of vasoconstrictory IP_3. This dual mechanism of action promotes powerful vasoconstriction (figure 15).

- ATP-sensitive K^{+} channels (K_{ATP}). These channels can be opened by receptor stimulation, adenosine or acetylcholine, or by a vasodilator drugs or by metabolic factors, such as a decrease in intracellular adenosine triphosphate (ATP) to a very low level. When channels open, the transferred K^{+} from inside to outside cell increase the state of polarization, so that they induce hyperpolarization. Such hyperpolarization closed the Ca^{2+} channels, then cytosolic Ca^{2+} decrease and vascular tone decrease (figure 16).

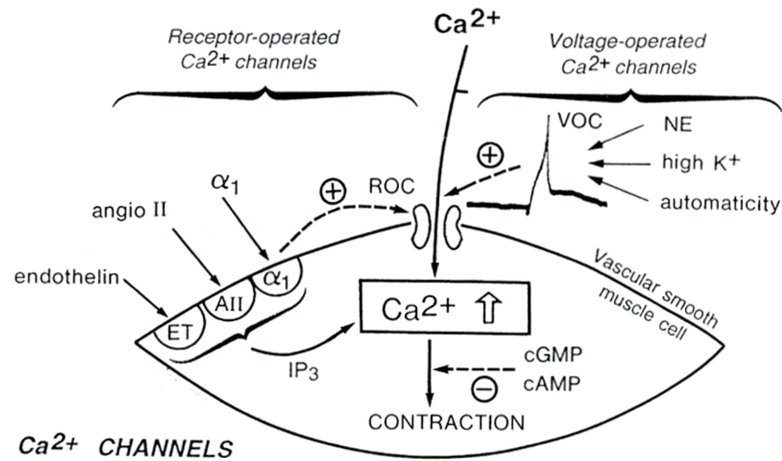


Figure 15 Two types of calcium channel regulation (Opie, 1998)

- Ca^{2+}-sensitive K^{+} channels (K_{Ca}). The existence of this channel can no longer be challenged. They are activated by intracellular Ca^{2+} and by depolarization. Since open this channels cause hyperpolarization, and are activated by depolarization, they exert a stabilizing, negative feedback on the membrane potential. They provide feedback from an excess cytosolic Ca^{2+}. When internal Ca^{2+} is high, this channels open, and there is hyperpolarization and vasodilation (figure 16) (Levick, 2000; Opie, 1998).

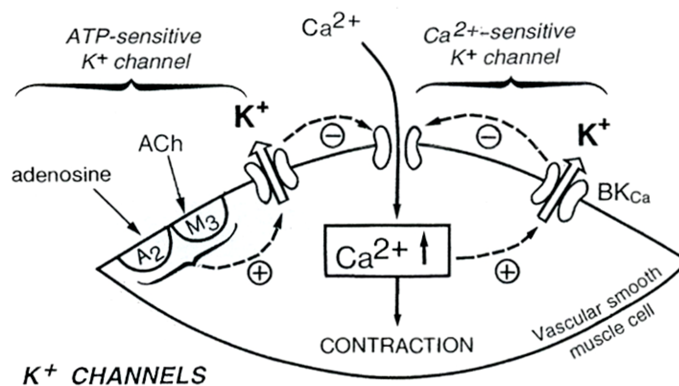


Figure 16 Vasodilatory role of potassium channel activity (Opie, 1998)

Blood Pressure

Blood pressure (BP) is the force per unit area exerted against the wall of the blood vessel by its contained blood. Blood pressure is expressed in terms of millimeters of mercury (mmHg). The term blood pressure means systemic arterial blood pressure in the largest arteries near the heart. The differences in blood pressure within the vascular system provide the driving force that keeps blooding always from a higher to a lower pressure area through the body (Marieb, 2001). The maximum pressure reached during peak ventricular ejection is call systolic pressure (SP) and the minimum pressure occurring just before ventricular ejection begins is called diastolic pressure (DP) (figure 17). The difference between the systolic and diastolic pressure is called the pulse pressure. Because aortic pressure fluctuates up and down with each heart beat, the important pressure figure to consider is the mean arterial blood pressure (MAP). MAP is the pressure driving blood into the tissues average over the entire cardiac cycle. It is approximately equal to the sum of diastolic pressure and one-third of the pulse pressure (Saladin, 2004). This can be shown in equation form as: MAP = DP + 1/3(SP-DP). This MAP is not specific to any artery, because the aorta and other large arteries have such large diameters that they offer only negligible resistance to flow, and the mean pressure is therefore similar everywhere in the large arteries (Vander et al., 1994).

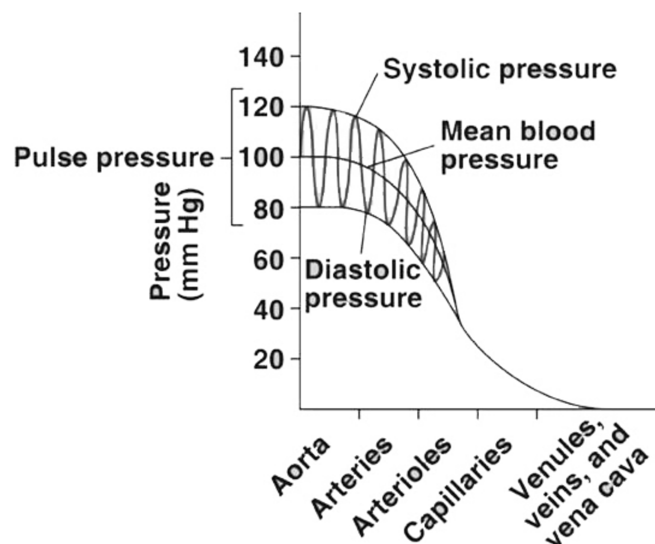


Figure 17 Blood pressure in various blood vessels of the systemic circulation (http://www.rci.rutgers.edu)

The method by which arterial pressure is regulated depends upon whether short-term or long-term adaptation is required. Short-term adjustments (over minutes and hours) are intended to correct temporary imbalances of pressure. The reaction is a combination of (1) adaptation of the local blood vessels to the altered volumes and pressure (auto-regulation) and (2) rapid autonomic neural responses, intended to return the arterial pressure toward normal. On the other hand, long-term arterial pressure adaptation (weeks and months) usually accomplished through changes in extracellular fluid and blood volume and renal mechanism (Smith and Kampine, 1990).

1.2.3 The autonomic nervous system

The autonomic nervous system (ANS) is response for integrating and modulating the function of all the autonomous organs of the body, including the cardiovascular system. The ANS is composed of the sympathetic and parasympathetic nervous system. In the ANS, two neurons in series extend from the central nervous system (CNS) to the effector organs. The first is preganglionic neuron and the second is postganglionic neuron, these two neurons are synapse in autonomic ganglia outside the CNS (figure 18). An exception is the preganglionic neurons that extend to the adrenal gland. There, the postganglionic neurons are actually the hormone-secreting cells of the adrenal medulla (Van Putte et al., 2010).

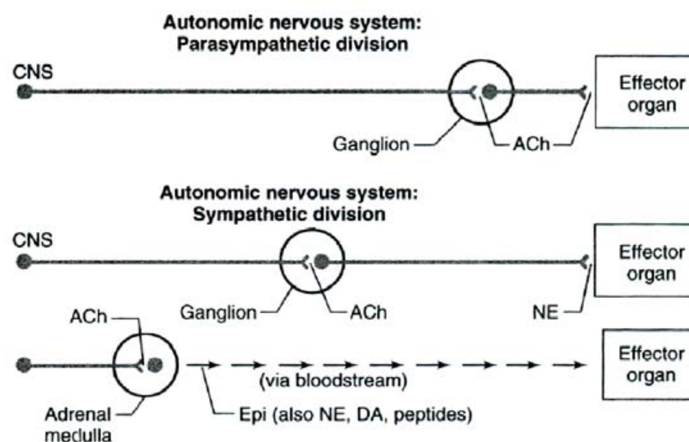


Figure 18 Neurotransmitter release of the sympathetic and parasympathetic (Vander et al., 2001)

In both sympathetic and parasympathetic nervous system, acetylcholine is the major neurotransmitter released between pre- and postganglionic fibers in autonomic ganglia. In the sympathetic nervous system, NE is usually the major transmitter between the postganglionic fiber and the effector cells. In the parasympathetic nervous system, acetylcholine (ACh) is the major transmitter between the postganglionic fiber and the effector cells (Vander et al., 2001). In addition, the adrenal medulla is also a component of the sympathetic nervous system that is analogous to postganglionic sympathetic nerve fiber. Instead of NE, the adrenal medulla release epinephrine (E) (Hoffman and Taylor, 2001).

The target organ of the sympathetic nervous system are the receptors for NE and E, which known as adrenergic receptor. The parasympathetic nervous system has the receptor for ACh to be the target group, which known as muscarinic receptors (Johnson et al., 1998).

Sympathetic nervous system at heart and blood vessels

Descending fibers from the cardiac and vasomotor centers of the medulla synapse with cells of the intermediolateral cell column of the spinal cord: preganglionic fibers from the cord travel via the anterior spinal roots to the thoracolumbar sympathetic chain (figure 19). Descending impulses from the cerebral cortex and hypothalamus may also, by way of these spinal pathways, induce sympathetic vascular response. The postgaglionic fibers from the cervical and upper four thoracic sympathetic ganglia supply the heart and the entire peripheral circulation (Smith and Kampine, 1990).

At heart, cardiac sympathetics are widely distributed to the sinoatrial (SA) and atrioventricular (AV) nodes and to the myocardium. Stimulation of these fibers activates β-adrenergic receptors and causes increases in rate and force of contraction of the heart (figure 20). These mechanisms are stimulated by acting via (1) right stellate ganglion to increase release of NE to area of SA and AV nodes, (2) the left stellate ganglion to increase release of NE to left ventricle and (3) adrenal medulla to release E to all part of the heart. The receptors stimulated are the β-adrenergic receptors. As a sequence, the heart rate increases, as dose the rate of conduction of the electrical impulse through the AV node and the conduction system. At the same time, the force of contraction increases. (Opie, 1998)

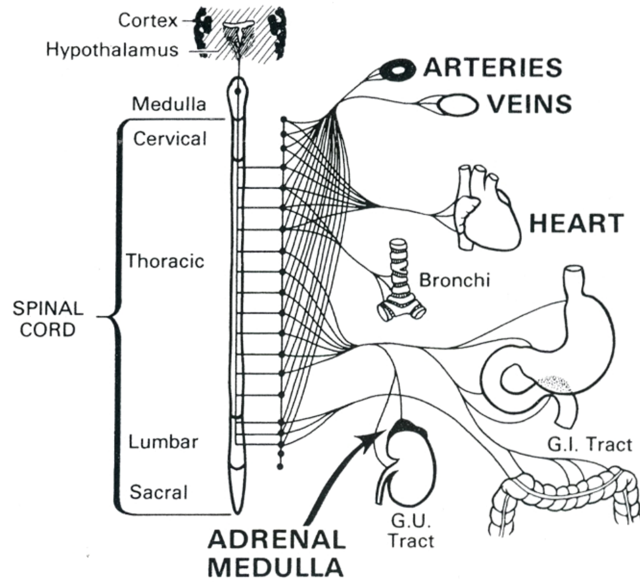


Figure 19 Sympathetic pathways to the heart and vasculature (Smith and Kampine, 1990)

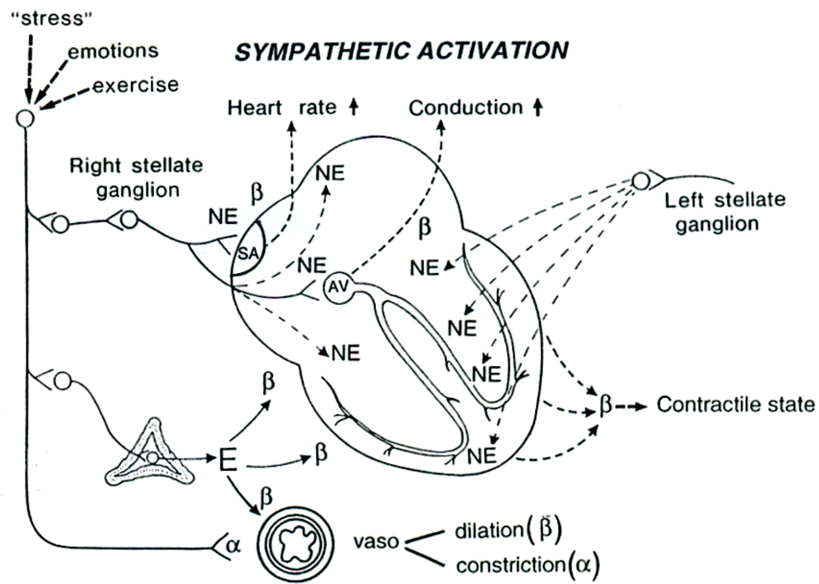


Figure 20 Mechanism of sympathetic stimulation (Opie, 1998)

At blood vessel, sympathetic nerve fibers innervate in the adventitial layer and which have the varicosities that release NE as a neurotransmitter, which acts on the smooth muscle and endothelial cells to regulate vascular tone (Kanagy, 2005). These fibers activate α-adrenergic receptors to produce a general vasoconstriction. Sympathetic stimulation may also activate peripheral β-adrenergic receptors, which will tend to produce vasodilation, particularly in skeletal muscle. However the β-adrenergic dilator tendency in muscle is overshadowed by much stronger α-constrictor action (Smith and Kampine, 1990).

α-Adrenergic receptors in blood vessels

The α_1- and α_2-adrenergic receptors are both found in vascular smooth muscel cells of many blood vessels. Their binding by agonists leads to muscle contraction, the α_1-adrenergic receptors play a crucial role in the regulation of vascular tone. α-Adrenergic receptors have approximately the same affinity for the adrenally released catecholamine E as for NE (Milnor, 1990).

α_1-Adrenergic receptors produce a vascular contraction by activating phosphoinositide turnover and calcium signaling (Hieble et al., 1995). NE or E binds to α_1-adrenergic receptors that interact with G-proteins, allows the operation of two mechanisms. First mechanism is the opening of the receptor-operated Ca^{2+} channels, causing extracellular Ca^{2+} to flow into the cell. The second mechanism involves a chain of biochemical reactions that involves activation of α_1-adrenergic receptors leading to the dissociation of α and $\beta\gamma$ subunits of G-proteins that, in turn, activate PLC. This enzyme hydrolyses PIP_2 into IP_3 and DAG (Zhong and Minneman, 1999). IP_3 binds on the SR to release stored intracellular Ca^{2+}. In addition, when the cell depolarizes, the extracellular Ca^{2+} ions enter through the voltage-sensitive Ca^{2+} channels. These mechanisms lead to a large increase of intracellular Ca^{2+}, which then binds to calmodulin to form the calcium-calmodulin complex. This complex activates MLCK to phosphorylate MLC, causing crossbridge formation and contraction. On the other hand, DAG activates protein kinase C rising in sensitivity to Ca^{2+}. This inhibits MLC phosphatase to promote vascular smooth muscle contraction (Levick, 2000).

β-Adrenergic receptors in blood vessels

Two types of β-adrenergic receptors, β_1 and β_2, are found in most blood vessels, where they inhibit contraction and thus relax the smooth muscle. β-Adrenergic receptors not only share the same neurotransmitter, NE, with α-Adrenergic receptors, but also often coexist in the same vessel. In such case, the net effect depends on the relative affinity and numbers of the two classes of receptors, the alpha effect usually predominates. β-Adrenergic receptors have a greater affinity for E than NE (Milnor, 1990).

E mediates vasodilatation by acting via β_2-adrenergic receptor by binding to β_2-adrenergic receptors that couple to G_S-protein. It activates AC to convert ATP to cAMP. The cAMP induces protein kinase A (PKA) activation and causes vasodilatation by three pathways. First, the cytosolic Ca^{2+} concentration falls due to stimulation of the Ca^{2+}-ATPase pumps in the surface membrane and SR. Second, a decrease in cytosolic Ca^{2+} concentration is promoted by a hyperpolarization, reduce the opening of Ca^{2+} voltage-sensitive channels, that is brought about by phosphorylation of K^+ channels by PKA. Third, the sensitivity of the contractile process to Ca^{2+} is attenuated, because PKA phosphorylates MLCK to inhibit its action (Levick, 2000).

The relaxant responses of the rat aorta to a non-specific β-adrenergic receptor agonist, isoproterenol, could be inhibited by methylene blue and hemoglobin, indicating that the endothelium-dependent NO/cGMP system may be activated by stimulation of β-adrenergic receptors (Gray and Marshall, 1992; Iranami et al., 1996).

Parasympathecic nervous system at heart and blood vessels

Preganglionic fibers of the craniosacral division of the ANS arise either in motor nuclei of the brain stem and exit with the cranial nerves, or in the sacral division of the spinal cord (figure 21). Preganglionic fibers of this division run to or almost to the innervated organs before synapsing, so that parasympathetic effects tend to be more discrete and specific than sympathetic effects (Smith and Kampine, 1990).

The heart is supplied by the vagus cranial nerve, which are distributed to the cardiac pacemakers and to the myocardium, particularly that of the atrium (figure 22). Vagal innervation to the SA node and AV junctional region is particularly important. Vagal stimulation activates cholinergic muscarinic receptors in the heart, which exert strong

inhibitory effects by slowing heart and AV conduction velocity and by decreasing cardiac contractility, primarily of the heart (Smith and Kampine, 1990).

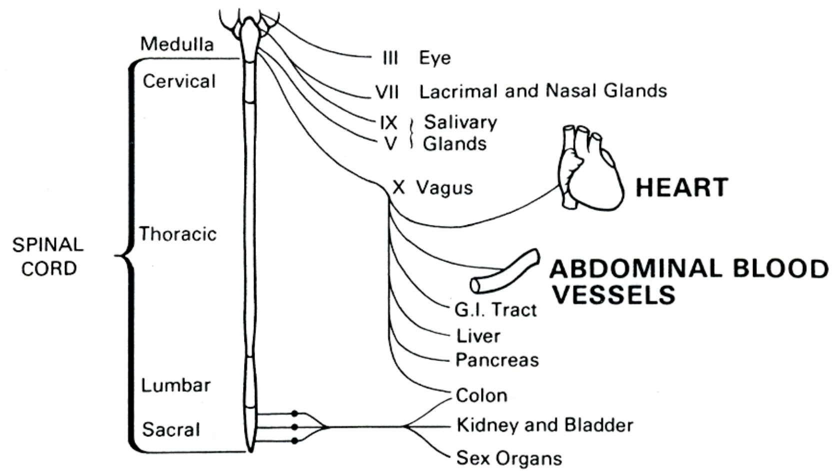


Figure 21 Parasympathetic pathways to the heart and vasculature (Smith and Kampine, 1990)

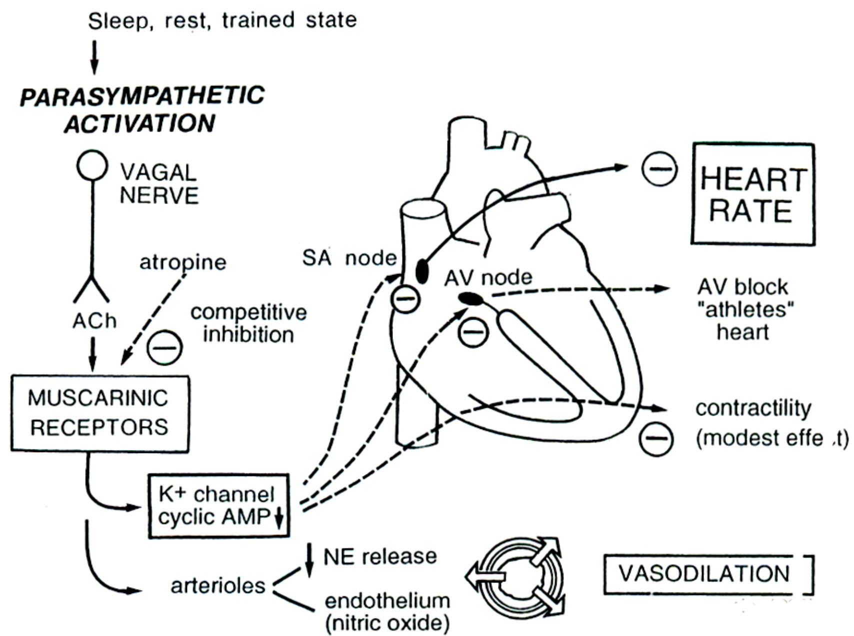


Figure 22 Mechanism of parasympathetic stimulation (Opie, 1998)

On blood vessels, only a small proportion of the resistence vessels of the body receive parasympathetic fibers. In the vascular endothelium, ACh acts via cholinergic M_3 muscarinic receptors causing the release of endothelium-derived relaxing factor (EDRF) that dilates vascular smooth muscle (Opie, 1998).

Muscarinic receptors in blood vessels

Cholinergic receptors of the muscarinic type are found in many parts of the vascular tree. Their action is complicated by the fact that they appear in endothelial as well as smooth muscle cells. Binding of ACh to such receptors in endothelial cells releases a substance that causes muscle relaxation. It acts via M_3 receptors on the surface of the endothelial cells, causing an increasing of intracellular Ca^{2+}. The Ca^{2+} activates the enzyme NOS to produce NO, causing vascular smooth muscle relaxation (Eglen and Watson, 1996; Caulfield and Birdsall, 1998). In contrast, binding to cholinergic receptors of smooth muscle cells causes contraction. The messenger system employed by cholinergic receptors to invoke contraction of smooth muscle is believed to involve phosphoinositides (Milnor, 1990).

CHAPTER 2

HYPOTENSIVE ACTIVITY OF AN N-BUTANOL EXTRACT FROM LEAVES OF *PHYLLANTHUS ACIDUS* (L.) SKEELS IN RATS

2.1 Abstract

The present study aimed to investigate the effects and establish the mechanisms that would be involved in the hypotensive activity of n-butanol extract from leaves of *Phyllanthus acidus* (PA extract) in rats. The PA extract (0.3-100 mg/kg, i.v.) caused a decrease in both mean arterial blood pressure (MAP) and heart rate (HR) of anesthetized rats in a dose dependent manner. These effects were not inhibited by atropine, a muscarinic receptor antagonist, (0.6 mg/kg) or propranolol, a β-adrenergic receptor antagonist, (0.6 mg/kg). In the isolated thoracic aortic rings, PA extract caused a prolonged an endothelium-independent relaxation of the aortic rings preconstricted with either phenylephrine (Phe, 3 μM) or KCl (40 mM). The relaxant activity was not abolished by N^G-nitro-L-arginine (LNA, 0.3 mM), a nitric oxide synthase inhibitor, or removal of the vascular endothelium. For the aortic rings preconstricted with Phe, the vasorelaxant activity of the PA extract was not inhibited by atropine (10^{-7} M), propranolol (10^{-7} M) or indomethacin, a non specific cyclo-oxygenase inhibitor, (10^{-6} M), whether the endothelium presence or not. Tetraethylammonium (TEA, 1 mM), a non-specific Ca^{2+} sensitive K^+ channel (K_{Ca}) inhibitor, or glybenclamide (10^{-5} M), a specific ATP sensitive K^+ channel (K_{ATP}) inhibitor, caused a significant decrease in vasodilator responses of the PA extract on aortic rings without endothelium. 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M), a specific soluble guanylate cyclase (sGC) inhibitor, caused a significant decrease in vasodilator responses to the PA extract of aortic rings whether the endothelium present or not. When the aortic rings were pre-incubated with both glybenclamide and ODQ, the concentration-response (C-R) curve to PA extract were parallel shift to the right. Nifedipine, a Ca^{2+}-channel inhibitor, (1 μM) or Ca^{2+}-free Krebs solution depressed the aortic rings constrictor response to Phe for endothelium-intact and denuded-thoracic aortic rings. When the PA extract was also added into the preparation, the vasoconstrictor responses to Phe were much further depressed.

These results suggested that the PA extract has hypotensive and negative chronotropic activities in rats. The active components act directly at the blood vessels to cause vasodilatation as a K_{Ca} opener, K_{ATP} opener and as a sGC stimulator. In addition PA extract may also play a role as a Ca^{2+}-channel inhibitor or may involve inhibition of Ca^{2+} mobilization from the intracellular store, but do not act through the muscarinic or β-adrenergic receptors.

2.2 Introduction

The hypertension remains inadequately managed everywhere, and in spite of the large number of antihypertensive drugs and combinations, most people in developing countries have poor access to modern health care. Therefore, pharmacological validation of medicinal plants or ethnomedical treatment methods could greatly benefit the populations with poor economic resources.

Phyllanthus acidus (L.) Skeel (*P. acidus*), synonymous with *P. acidissimus* (Blanco) Mull. Arg., *P. distichus* (L.) Mull. Arg., *Cicca diaticha* L., *C. acidissima* Blanco, *C. acida* (L.) Merr. and *Averrho acida* L., is a small tropical tree which is native to probably to the coast region of North-East Brazil, belongs to the family Euphorbiaceae (figure 23). Its common name is Tahitian Gooseberry, Otaheiti Gooseberry or Star Gooseberry, and was named "Mayom" in Thailand. It is widely cultivated throughout the country (Van Welzen and Chayamarit, 2007). In Thai Folkloric medicine, almost every part of this plant has been used for many purposes. For example, the roots have been used as an antipyretic and dermatitis, the stem barks are claimed to treat menstruation fever, the fruits have been used to nourish blood and the leaves are used for antihypertension (Pongboonrod, 1959, Teingburanathum, 1999, www.tungsong.com).

Only a few studies concerning the chemical constituents and the biological activities of this plant have been investigated. The phytochemical studies carried out with *P. acidus* stem bark have led to the isolation of lupeol (Dekker, 1908), phyllanthus acidus sterol (Ultee, 1933), beta-amyrin and phyllanthol (Sengupta and Mukhopdhyay, 1966). The root part contains phyllanthusol A and phyllanthusol B (Vongvanich et al., 2000; Durham et al., 2002). For the biological studies of the plant: Mokkhammit et al. (1971) reported that an ethanolic extract from dried wood did not show any toxicity to mouse. The decoction of leaves and branches has very weak antibacterial activity (Haicour, 1974)

while the whole plant except root extract with 50 % ethanol has no effect *in vitro* against Ranikhet Virus or Vaccinia Virus (Aswal et al., 1984). By using a fungal-feeding assay, the methanolic extract from fresh fruits shows strong antinematodal activity against *Bursaphelenchus xylophilus* (Muhammad et al., 1997). The ethanolic extract from dried barks has no inhibitory effect on the test of fish and shrimp bacterial pathogens (Direkbusarakom et al., 1998). In 2000 Vongvanich et al. found phyllanthusol A and B, which were isolated from dried roots of the plant exhibit cytotoxic activity on BC and KB cell lines. The methanolic extract from dried fruits possesses strong in vitro antibacterial activity against the bacteria tested (Melendoz and Capriles, 2006). Lee et al. (2006) reported that a methanolic extract from *P. acidus* had a hepatoprotective effect on rats with acute liver damage induced by carbon tetrachloride. Recently, the n-butanol extract from fresh leaves has been investigated as a potential treatment for the cystic fibrosis lung disease (Sousa et al., 2007).

However, there is no scientific investigation of this plant on the hypotensive activity. Thus, we did a preliminary study of crude extract from leaves of this plant, and found that intravenous injection of crude extract caused a decrease in both MAP and HR in anesthetized rats. And it caused a vasodilatation of isolated thoracic aortic rings which were preconstricted with phenylephrine.

2.3 Objective

To investigate the hypotensive effects of an n-butanol extract from leaves of *Phyllanthus acidus* in the rats, as well as establish the mechanisms that would be involved in the hypotensive activity.

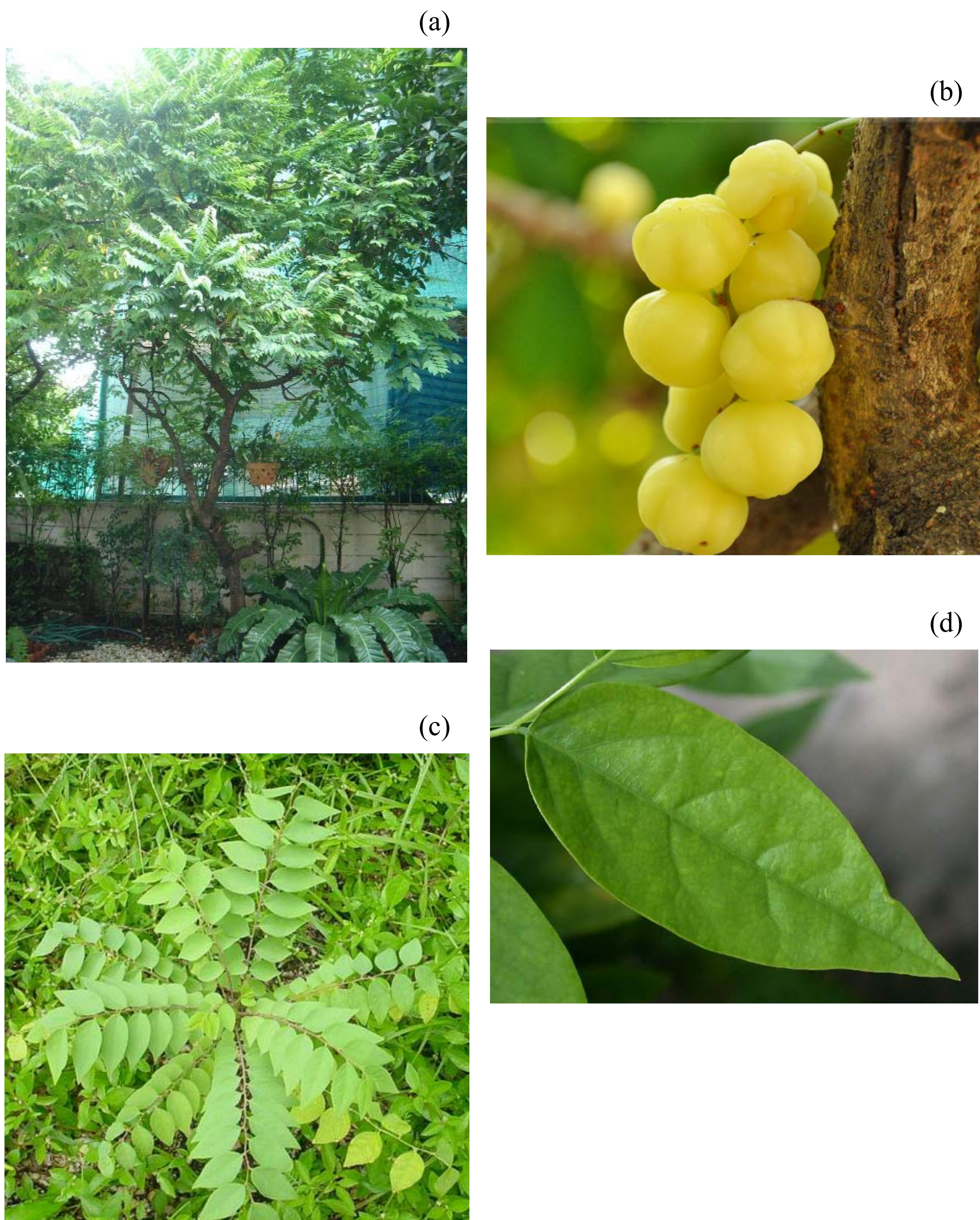


Figure 23 Tree (a), fruits (b) and leaves (c and d) of *Phyllanthus acidus*

2.4 Material and Methods

2.4.1 Plant material

Fresh leaves of *P. acidus* were collected in Songkhla Province, Thailand. Authentication was achieved by comparison with herbarium specimens in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Songkhla Province, Thailand, where a voucher specimen (Collecting No. 2548-01) of the plant material has been deposited.

2.4.2 Preparation of *Phyllanthus acidus* extract

Fresh leaves of *P. acidus* (100 kg) were simmered in hot filtered water for a period of 3 hours. Only clear solution was collected and heated at 50°C to reduced the volume to 50%. The concentrated solution was partition extracted with water-saturated n-butanol. The n-butanol phase was collected and evaporated under reduce pressure to dryness *in vacuo*, and the residue was lyophilized to obtain a yellow brown powder (506 g) of *P. acidus* extract (PA extract). The diagram of PA extract preparation is shown in figure 24.

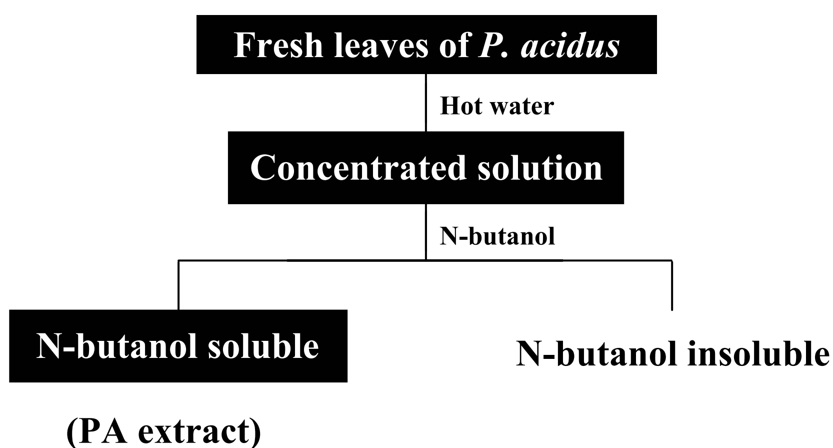


Figure 24 Flow chart showing preparation of n-butanol extract from leaves of *Phyllanthus acidus* (PA extract)

2.4.3 Pharmacological studies of the PA extract

Adult female Wistar rats in estrus (210-250 g) were supplied from the Animal House, Faculty of Science, Prince of Songkla University. The animals were housed in controlled environmental conditions at 25 ^{o}C on a 10 hrs dark and 14 hrs light cycle and allowed freely access to standard food and tap water *ad libitum*. The methods employed in this study were approved by the Prince of Songkla University Animal Care and Use Committee. The investigation conformed to the Guide for the Care and Use of Laboratory Animals.

***In vivo* studies**

Adult female Wistar rats (210-250 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg, i.p.). The tracheal tube was cannulated with a polyethylene tube to maintain airway patency and the animals breathed room air spontaneously. A polyethylene catheter was cannulated through the left common carotid artery which was connected to a pressure transducer (P23 ID, Gould Statham Instrument, Hato Rey, Puerto Rico) and connected to a Grass polygraph (model 7D, Grass Instrument, Quincy, MA, U.S.A.) for systemic blood pressure monitoring, and the heart rate was recorded by using a tachograph driven by the blood pressure wave. Another polyethylene tube was cannulated through the right jugular vein for intravenous administration of drugs. The animal was then equilibrated for at least 40 min before the experiments were started (figure 25). Each rat was used for only one agonist.

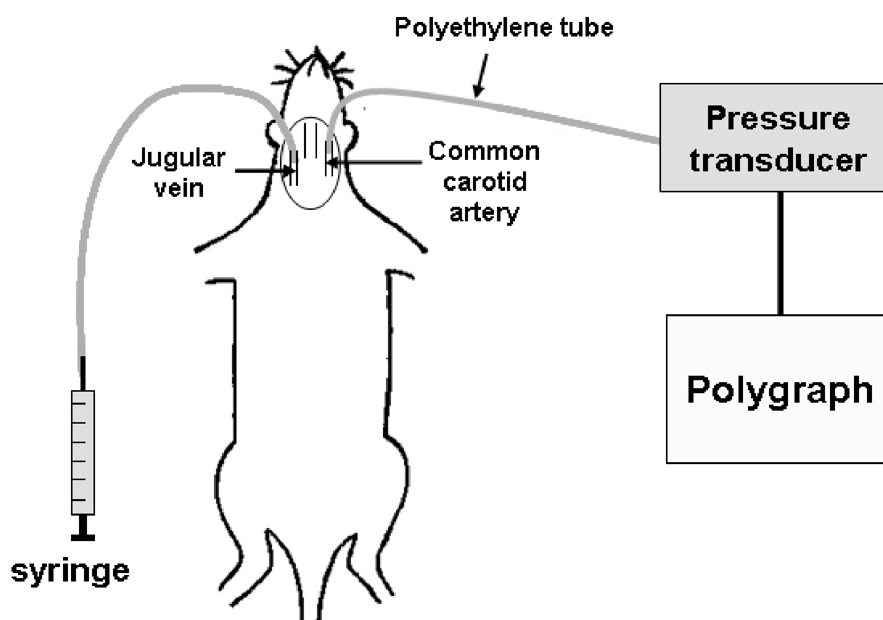


Figure 25 *In vivo* preparation of anesthetized rat

Effects of the PA extract on the mean arterial blood pressure and heart rate of anesthetized rats

After the equilibration period and the systemic blood pressure reached steady state, the dose-response relationship to the PA extract (0.3-100 mg/kg) was determined by injection of the drug through the right jugular vein of a volume not exceeding 0.1 ml for each dose and flushed with 0.1 ml normal saline.

Using another sets of animals, after equilibration of the animals for 40 min, atropine (0.6 mg/kg, i.v.) or propranolol (0.6 mg/kg, i.v.) were injected through the right jugular vein. After 20 min re-equilibration, the dose-response relationship to PA extract was determined.

***In vitro* preparation**

Adult female Wistar rats in estrus (210-250 g) were killed by decapitation with a guillotine. Thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue. Two adjacent rings of 5 mm in length from thoracic

aorta were cut, and the endothelium was removed mechanically from one ring by gently rubbing of the intimal surface of ring with a stainless steel rod, using the method of Jansakul et al. (1989). The aortic rings with or without functional endothelium were mounted horizontally between two parallel stainless steel hooks with extremely care not to damage the endothelium for the endothelium-intact aortic rings, and suspended in a 20 ml organ bath containing Kerbs-Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4.7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37°C and continuously bubbled with 95% O_2 and 5% CO_2 mixture. One of the hooks was fixed at the bottom and the other was connected to a force displacement transducer which connected to a Grass polygraph for the monitoring of changes in isometric tension. Prior to addition of drugs, tissues were equilibrate under a resting tension of 1 g and the bath solution was replaced with pre-warmed and oxygenated Kerbs-Henseleit solution every 15 min (figure 26).

The preparations was allowed to equilibrate for 60 min, the presence or absence of a functional endothelium of the thoracic aortic ring was assessed in all preparation as follows: the aortic rings with and without endothelium were preconstricted with 3 μM phenylephrine until the contraction reached a steady state (7-10 min), in order to test their contractility, and the dilatory response to 30 μM acetylcholine was recorded. The experiments were continued only when acetylcholine induces more than 80% vasodilatation for the endothelium-intact aortic rings and there was no dilatory response to acetylcholine for the endothelium-denuded aortic rings. Preparations were then washed several times with drug-free Krebs-Henseleit solution, and allow relaxing fully for 45 min before the experimental protocol began.

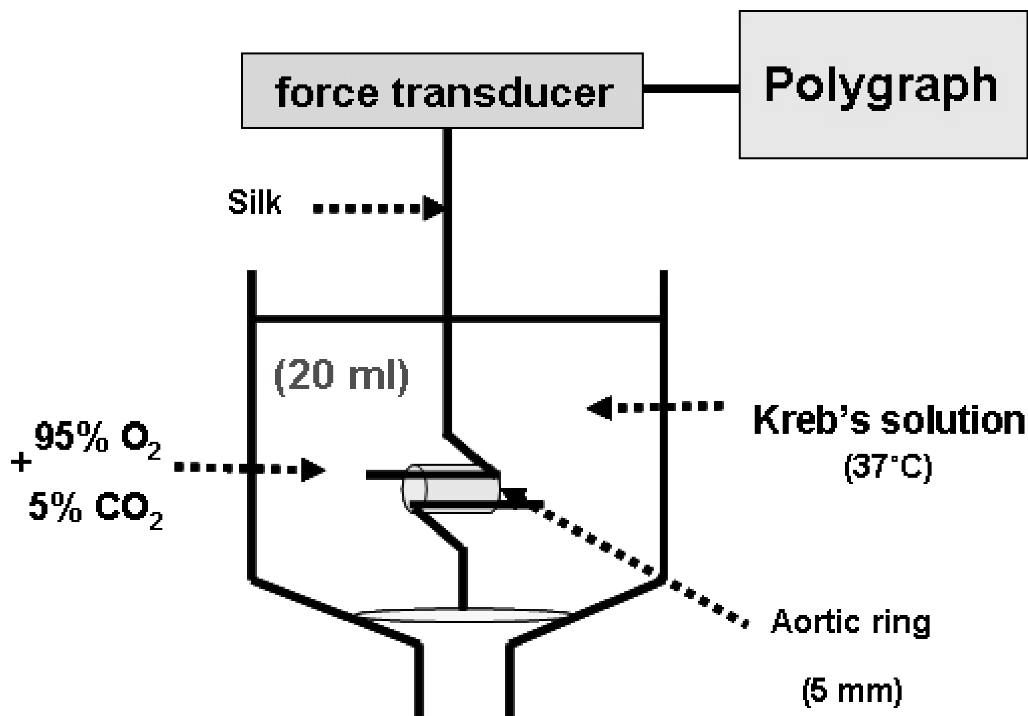


Figure 26 *In vitro* preparation of the thoracic aortic ring

Effects of the PA extract on thoracic aortae in vitro

After the re-equilibration, the thoracic aortic rings with and without endothelium were again preconstricted with 3 μM phenylephrine or 40 mM KCl for 10 min. When the contraction reached a steady state, a cumulative concentration-response curve (C-R curve) was constructed to the PA extract (0.1-30 mg/ml) or to the vehicle (10 % DMSO). Then after several washing and a re-equilibration period of 45 min, the thoracic aortic rings were challenged with 3 μM phenylephrine or 40 mM KCl for 10 min by which time the maximal contraction has been reached and followed by several washing. This procedure was repeated every 45 min until its contractile responses to phenylephrine or KCl returned to the same magnitude as that of the control one (before challenging with PA extract), but not more than 4 consecutive repeats.

Using another set of animals, only the endothelium-intact thoracic aortic rings were pre-incubated with N^G-nitro-L-arginine (LNA, 0.3 mM) for 30 min, then the aortic rings were preconstricted with 3 μM phenylephrine until the contraction reached a steady state (10-15 min), and the cumulative concentration-response relationship to PA extract was obtained.

Effects of calcium on thoracic aortae in vitro

Using another set of animals, the endothelium-intact thoracic aortic rings in the presence or absence of LNA were challenged with 3 μM phenylephrine for 10 min (plateau reached) followed by several washings, and re-equilibration for 45 min. The thoracic aortic rings were then pre-incubated with nifedipine (1 μM), or the suspending solution replaced with Ca^{2+}-free Krebs solution for 30 min, and then challenged with 3 μM phenylephrine in the presence of nifedipine or in the Ca^{2+}-free Krebs solution for 10 min, followed by several washings, and re-equilibration for 45 min. Then the same procedures were repeated by adding the PA extract into the incubation medium together with the nifedipine or into the Ca^{2+}-free Krebs solution and incubating for 30 min before adding 3 μM phenylephrine into the incubating medium and recording the isometric tension developed over 10 min.

Effects of atropine, propranolol, indomethacin, TEA, glybenclamide or ODQ on responses to PA extract on thoracic aortae in vitro

Using another set of animals, the thoracic aortic rings with or without endothelium were pre-incubated with atropine (10^{-7} M), propranolol (10^{-7} M), indomethacin (10^{-6} M), Tetraethylammonium (TEA, 1mM), glybenclamide (10^{-5} M) or 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M) for 30 min, then each thoracic aortic ring was preconstricted with 3 μM phenylephrine for 10-15 min (plateau reached), and the cumulative concentration-response relationship to PA extract was obtained in the presence of those corresponding blockers.

2.4.4 Drugs and Chemicals

The following drugs were used: acetylcholine chloride, atropine sulphate, glybenclamide, indomethacin, nifedipine, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), phenylephrine hydrochloride, propranolol hydrochloride, N^G-nitro-L-arginine (LNA), tetraethylammonium (TEA). All drugs were purchased from Sigma, USA. The organic solvents for plant extraction, n-butanol, were purchased from Merck, Germany.

PA extract, LNA and TEA were dissolved in distilled water, the remainders were dissolved in a solution (g/L): NaCl 9.0, NaH_2PO_4 0.19 and ascorbic acid 0.03.

2.4.5 Statistic analysis

Results were expressed as mean ± S.E. of 6 experiments (n=6). Changes in blood pressure and heart rate were recognized as the difference between the steady pressure or heart rate before and the lowest pressure or heart rate after injection. The blood pressure were recorded in mmHg as systolic pressure (SP) and diastolic pressure (DP) and were expressed as mean arterial blood pressure (MAP), which was calculated as DP+1/3 (SP-DP). Vasodilator responses of thoracic aortic rings were expressed as percentage relaxation of vessels from maximal contraction of 3 µM phenylephrine preconstriction levels. Statistical differences were determined by the Student's paired or unpaired t-test or one way ANOVA. The P value < 0.05 was considered to be significant in all experiments.

2.5 Results

2.5.1 Effects of the PA extract on the mean arterial blood pressure and heart rate of anesthetized rats

The effect of PA extract on mean arterial blood pressure (MAP) and heart rate (HR) were shown in figure 28. The typical recordings of blood pressure and heart rate to PA extract in anesthetized rats were shown in figure 27. An intravenous injection of the PA extract (0.3-100 mg/kg) caused a decrease in both mean arterial blood pressure and heart rate in anesthetized female rats in a dose-dependent manner. The hypotensive and

negative chronotropic effects of the PA extract were not modified by pretreatment of the animals with atropine (0.6 mg/ml), a muscarinic receptor antagonist (figure 28a) or propranolol (0.6 mg/ml), a β -adrenergic receptor antagonist (figure 28b).

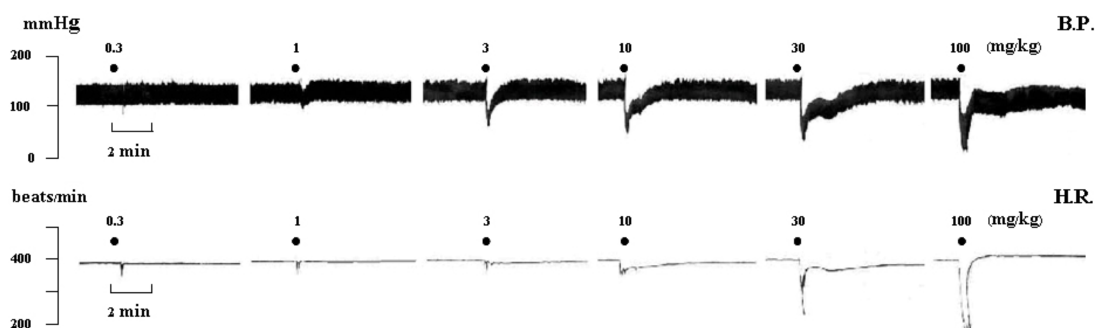


Figure 27 Typical recording showing effects of intravenous injection of the PA extract on blood pressure and heart rate

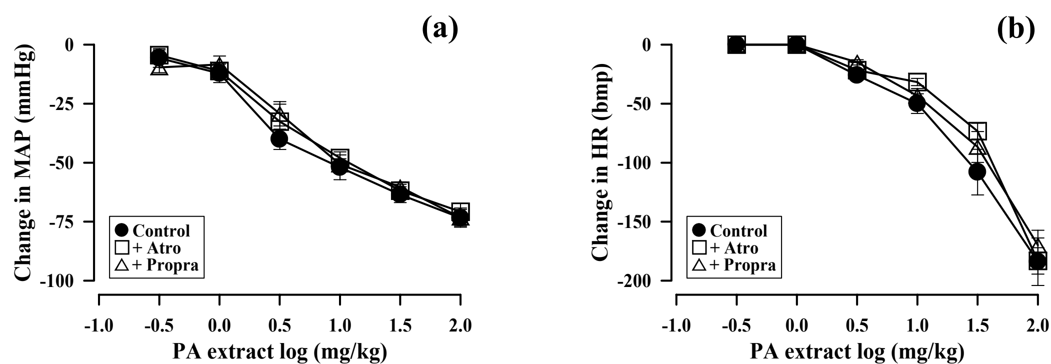


Figure 28 Effect of atropine (Atro) or propranolol (Propra) on the decrease in mean arterial blood pressure (a) and the decrease in heart rate (b) of PA extract in anesthetized rats. Each point represents mean ± S.E. mean of 6 experiments (n=6).

2.5.2 Effects of the PA extract on thoracic aortae in vitro

The PA extract (0.1-30 mg/ml) caused an endothelium-independent dilatation of thoracic aortic rings preconstricted with either phenylephrine or with KCl in a dose-dependent manner, whereas the vehicle (10 % DMSO) did not show any effect (data not shown). Typical recording of the vasodilator responses to PA extract is shown in figure 29. N-nitro-L-arginine (LNA), a nitric oxide synthase inhibitor did not modify the vasodilator activity of the PA extract on the aortic rings whether they were pre-constricted with phenylephrine or with KCl (figure 30). In addition, the vasodilator activity of the PA extract on the thoracic aortic ring was persisted for at least 2 hrs after completing the PA extract cumulative concentration-response (C-R) curve performing. This was tested by performing the contractile response to 3 μM phenylephrine or with 40 mM KCl after several washing of the aortic ring with Kreb's solution at 45 min interval up to 180 min by which the contractile response to these two agonists were slowly recovered to normal responsiveness whereas with the vehicle control groups the aortic rings had recovered to be normally responsiveness to the phenylephrine or KCl induced vasoconstriction after only 45 min (figure 31).

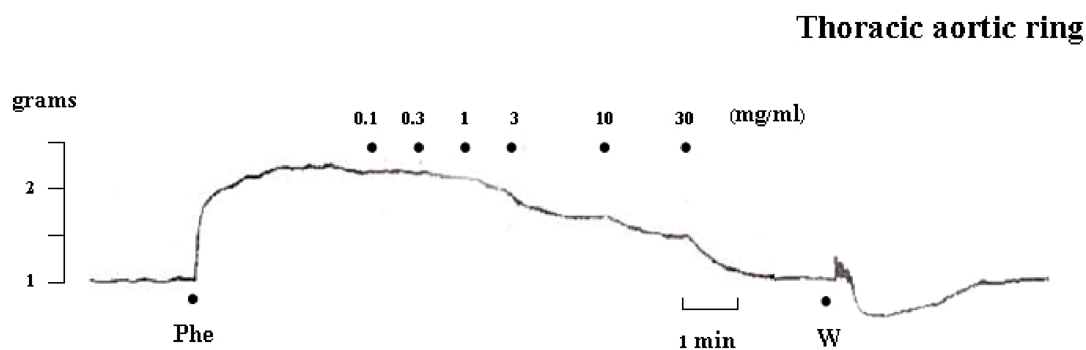


Figure 29 Typical recording showing effect of the PA extract on vasodilatation of thoracic aortic ring

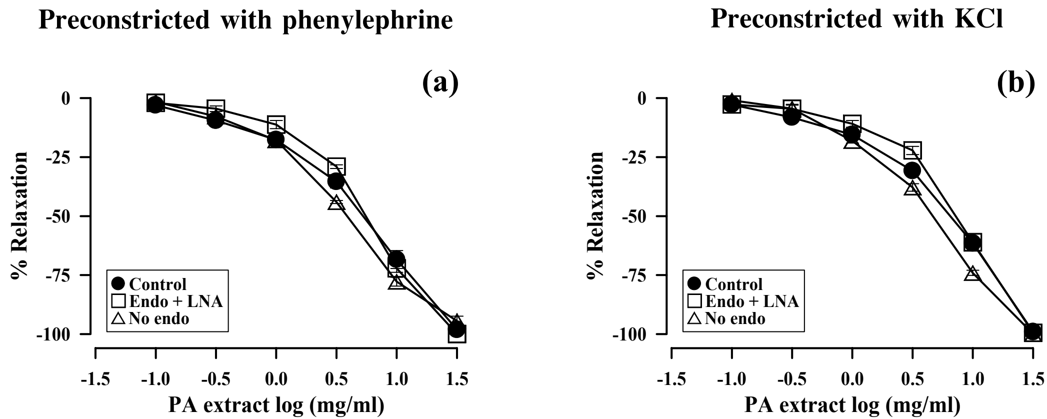


Figure 30 Effect of LNA or removal of endothelium (No endo) on the dilatation of the thoracic aortic rings which had preconstricted with phenylephrine (a) or with KCl (b) to PA extract. Each point represents mean ± S.E. mean of 6 experiments (n=6).

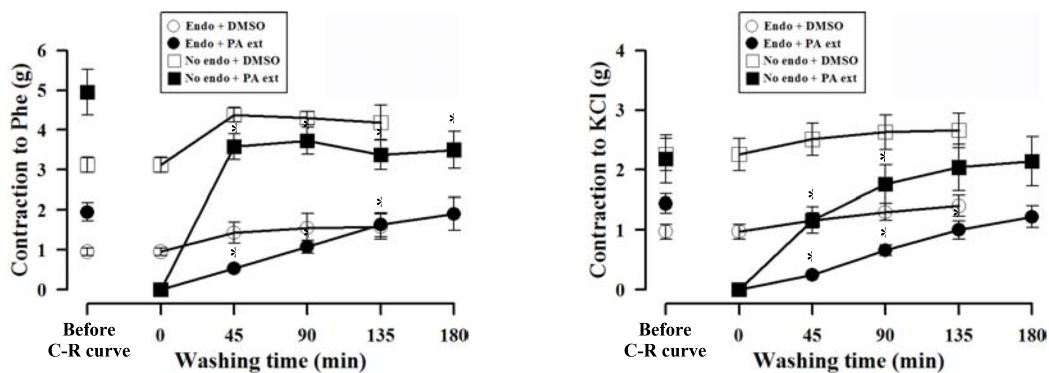


Figure 31 Maximal contractile responses of the endothelium-intact (Endo) or denuded (No endo) thoracic aortic rings to phenylephrine (left) or to KCl (right) at 45 min interval after washing of the first challenge (0 min) with Phe or KCl alone or of the one which had also been performing a dose-dilator response relationship to PA extract. Each point represents mean ± S.E. mean of 6 experiments (n=6). * Significantly lower than that produced at the 0 min of their corresponding groups.

2.5.3 Effects of calcium on thoracic aortae in vitro

Nifedipine, a Ca^{2+}-channel inhibitor (figure 32a), or challenged in the Ca^{2+} free Krebs solution (figure 32b), caused a reduction in contractile responsiveness of the thoracic aortic rings to phenylephrine with or without the presence of LNA. In addition, when the PA extract was also added into the incubation medium, the contractile responsiveness of the thoracic aortic rings to phenylephrine was much further depressed.

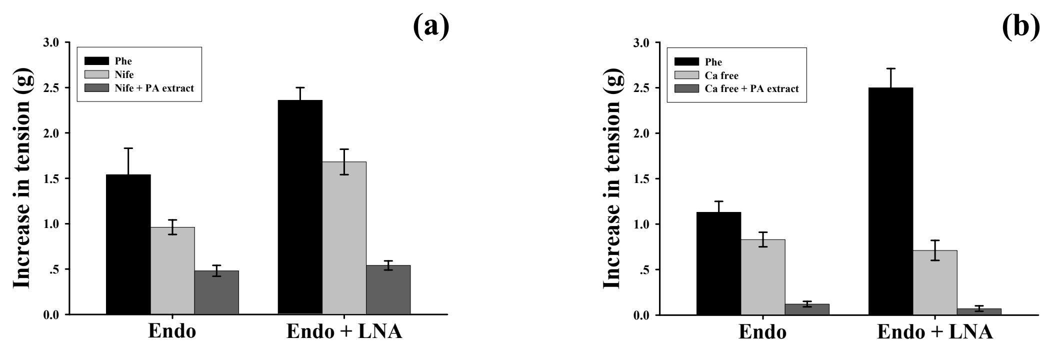


Figure 32 Effect of nifedipine, nifedipine and PA extract, calcium free solution, or calcium free solution and PA extract on contractile responses of the endothelium-intact aortic rings (Endo), or incubation of aortic rings with LNA (Endo+LNA) to phenylephrine. Each point represents mean ± S.E. mean of 6 experiments (n=6).

2.5.4 Effects of atropine, propranolol, indomethacin, TEA, glybenclamide or ODQ on responses to PA extract on thoracic aortae in vitro

As shown in figure 33, atropine, a muscarinic receptor antagonist, propranolol, a β-adrenergic receptor antagonist or indomethacin, a non specific cyclo-oxygenase inhibitor did not have any effects on the C-R curves to PA extract of the thoracic aortic rings pre-constricted with phenylephrine. TEA, a non-specific Ca^{2+} sensitive K^{+} channel inhibitor, showed a slight inhibition of the vasodilatory effect at low concentrations of the PA extract but this was not statistically significant. On the other hand, a slight potentiating effect was observed when the concentration of the PA extract was progressively increase, however this effect disappeared after incubating the aortic ring with LNA. In addition, when the vascular endothelium was removed, TEA caused a small inhibition of the vasodilatory activity of the thoracic aortic ring (figure 34a and b). Glybenclamide, an ATP sensitive K^{+}-channel inhibitor, did not modify the vasodilator activity of the PA extract on the thoracic aortic rings with endothelium-intact. Whereas those endothelium-denuded aortic rings, glybenclamide caused a significant rightward shift of the PA extract C-R curve to the right (figure 34c and d). ODQ, a guanylate cyclase inhibitor, caused a significantly decrease in sensitivity of the vasodilator responses to PA extract on phenylephrine constricted thoracic aortic rings whether the endothelium presence or not (figure 34e and f). In addition, these effects were more pronounced rightward shift of the C-R curve when both glybenclamide and ODQ were together pre-incubated in the endothelium-denuded aortic rings (figure 34g and h).

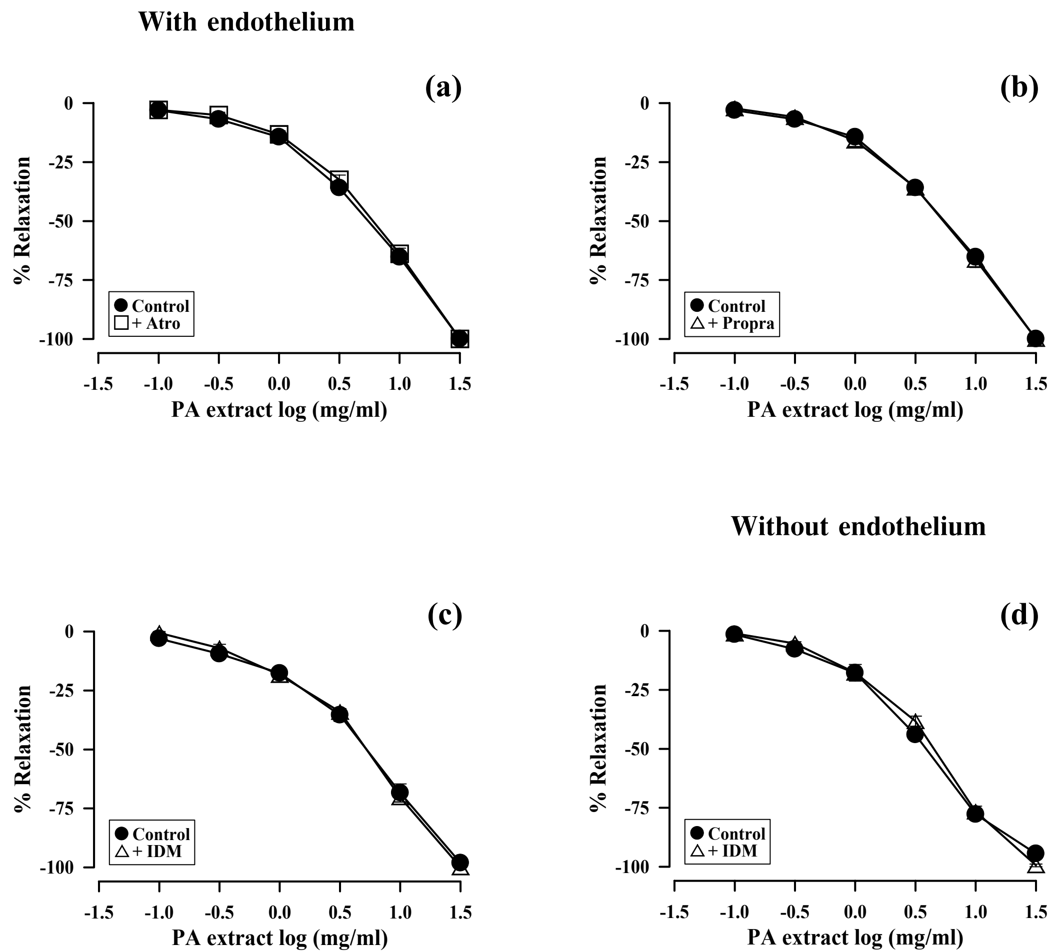


Figure 33 Effect of atropine (Atro), propranolol (Propra) or indomethacin (IDM) on the dilatation of the endothelium-intact (a and c) or -denuded (b and d) thoracic aortic rings preconstricted with phenylephrine to the PA extract. Each point represents mean ± S.E. mean of 6 experiments (n=6).

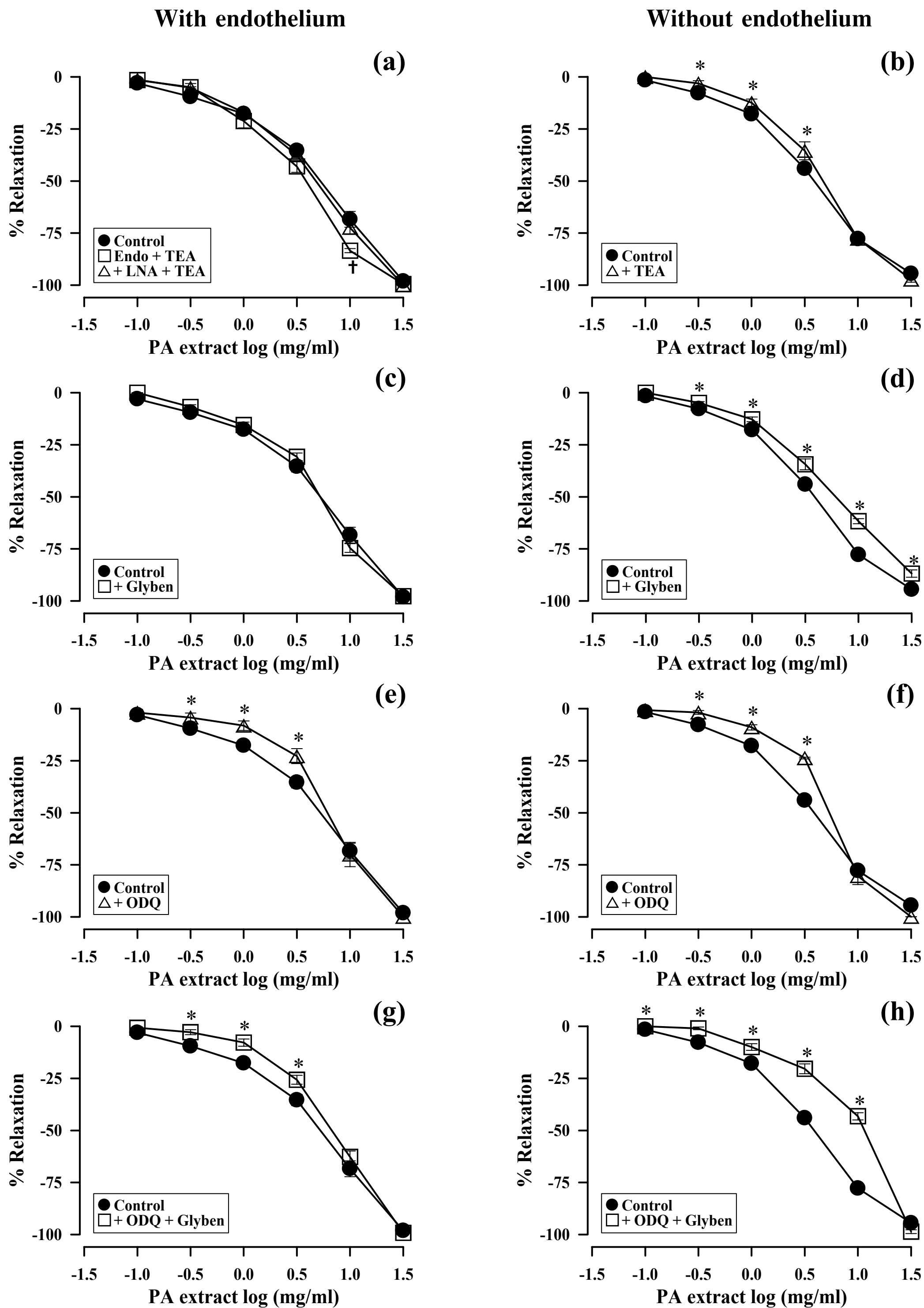


Figure 34 Effect of TEA, glybenclamide (glyben) and/or ODQ on the dilatation of the endothelium-intact (a, c, e and g) or -denude (b, d, f and h) thoracic aortic rings preconstricted with phenylephrine to the PA extract. Each point represents mean ± S.E. mean of 6 experiment (n=6).

2.6 Discussion

In Thai herbal medicine, traditional use of *Phyllanthus acidus* (*P. acidus*) leaves is prepared as a decoction, which would be contained both organic and inorganic substances. The present study we aim to investigate the hypotensive activity of the organic part of the *P. acidus* decoction. Thus, the decoction or the concentrated water soluble part from the fresh leaves of the plant was partition extracted with n-butanol, the most polar water insoluble organic solvent. The n-butanol soluble part was collected, evaporated and lyophilized respectively to obtain the n-butanol extract of *P. acidus* (PA extract).

PA extract exerts a concentration-dependent hypotensive and negative chronotropic activities in anesthetized rats. The finding that atropine, a muscarinic receptor antagonist, or propranol, a β-adrenergic receptor antagonist, the same dosages as reported by Hopkins and Hodgson (1998), did not modified the C-R curve of the PA extract on mean arterial blood pressure and heart rate, indicating that the hypotensive and negative chronotropic activities of the PA extract are unlikely due to the active component acting through the muscarinic or β-adrenergic receptors of the cardiovascular system.

Drugs which possess hypotensive activity may act centrally via the autonomic nervous systems or periphery via the vascular system to cause vasodilatation which then decreased peripheral resistance. In the present study we aimed to concentrate on the peripheral effects of the PA extract, especially on the vasodilatation. To examine this possibility, isolated preparation of the thoracic aortic rings from rats were studied.
As shown in the result section, PA extract caused a concentration-dependent vasodilatation of the thoracic aortic rings preconstricted with 3 μM phenylephrine or 40 mM KCl. This effect persisted after removal of the vascular endothelium or pre-incubation the aortic rings with LNA, a nitric oxide synthase inhibitor. These results suggested that the vasodilator activity of the PA extract on the thoracic aortic rings is an endothelium-independent and it is not modified by nitric oxide from the vascular endothelium. Although LNA could not modify the PA extract C-R curve of the endothelium-intact aortic rings, the role of nitric oxide (NO) on the vasodilator activity of the PA extract can not be excluded. The reason is that the PA extract may contain several active substances that might have different vasodilatory potency and/or its ability to stimulate release of the NO. The finding that PA extract also caused vasodilatation in the aortic rings preconstricted with KCl, indicated that the vasorelaxant effect of the PA extract is non-specific, it could antagonized via

α_1-adrenergic receptor-mediated vasoconstriction, as well as by a voltage-mediated vasoconstriction. In addition we also found that the inhibitory effect of the PA extract to the contractile response of the endothelium-intact and endothelium-denuded aortic rings to phenylephrine and KCl persisted for at least 2 hrs, although it gradually recovered to their normal responsiveness after a periodic washing and re-equilibration of every 45 min. This effect was not found in the vehicle control group which had never been challenged with the PA extract. These results suggested that the vasodilatory effect of the PA extract is a long lasting activity. In order to prevent any misleading results due to the post-effects of the PA extract, in any experiment, a separate set of thoracic aortic rings were used: one set for the control experiment and the other one for the PA extract experiment.

In order to confirm that the hypotensive effect of the PA extract did not involve the muscarinic receptors and/or the β-adrenergic receptors at the blood vessel, experiments were performed by incubating the aortic rings with atropine, a muscarinic receptor antagonist, or with propranolol, a β-adrenergic receptor antagonist, before performing the C-R curve to the PA extract (in the presence of these corresponding antagonist). As shown in the result section, atropine or propranolol did not modified the C-R curve vasodilator effect of the PA extract confirming that this activity of the PA extract did not involve the muscarinic receptors or the β-adrenergic receptors on the blood vessel.

Ca^{2+} plays a pivotal role in controlling vascular reactivity. In the rat thoracic aortic rings, the α_1-adrenergic receptor agonist, phenylephrine induced an initial phasic contraction followed by a tonic contraction. The initial contraction is mediated by intracellular Ca^{2+} release, where as the sustained tonic contraction results from Ca^{2+} influx via voltage-calcium channels (Nelson et al., 1988, Abebe et al., 1991, Akata, 2007). In the present study, PA extract completely antagonized the phenylephrine-induced constriction of the thoracic aortic rings. Thus it is possible that PA extract may also play a role as a Ca^{2+} channel inhibitor. To examine this possibility, we used nifedipine, a Ca^{2+}-channel inhibitor, to block the voltage Ca^{2+}-channel before performing the constrictor response to phenylephrine in the presence of the PA extract. In this situation, nifedipine attenuated the constrictor response to phenylephrine. When both nifedipine and PA extract were added together into the preparation medium, a further attenuation was found, suggesting that the PA extract may play a role as an inhibitor of voltage Ca^{2+}-channel. Further experiments were performed in the Ca^{2+}-free Krebs solution to assess the effect of

the PA extract on the intracellular Ca^{2+}release. A similarly effect was found, the constrictor responses of the thoracic aortic rings to phenylephrine in the Ca^{2+}-free Krebs solution was attenuated compares to those obtained in the normal Krebs solution. When the PA extract was added, a further attenuation was found, indicating that the PA extract may involve the inhibition of Ca^{2+}mobilization from the intracellular store, since the adrenergic receptor-induced contraction in the Ca^{2+}-free Krebs solution is responsible by the Ca^{2+}release from the intracellular store, sarcoplasmic reticulum (Noguer, D'Ocon, 1992).

Some prostaglandins such as prostacyclin or prostaglandin E which normally produced by the cyclo-oxygenase pathway can relax the vascular smooth muscle. Thus, it is possible that the vasodilator activity of the PA extract may be due to the active substances in the PA extract stimulate release of these vasodilatory prostaglandins.
To investigate this possibility, thoracic aortic rings were pre-incubated with indomethacin, a non specific cyclo-oxygenase inhibitor, before obtaining the C-R curve of the PA extract on the thoracic aortic rings either endothelium-intact or endothelium-denuded, the result suggesting that vasodilatory prostaglandins may not involve in the vasodilator effect of the PA extract.

A possible role of K^{+} channels and a cyclic nucleotide, cGMP, which participated in the vasodilatory activity of the PA extract were also studied by pretreatment the aortic rings with TEA, a non-specific Ca^{2+} sensitive K^{+} channel inhibitor, or glybenclamide, a specific ATP sensitive K^{+} channel inhibitor, and/or ODQ, a specific soluble guanylate cyclase inhibitor before performing the C-R curve of the PA extract to the aortic rings. TEA inhibited the vasodilatory activity of the PA extract on the endothelium-denuded thoracic aortic rings, whereas a potentiating effect was found on the endothelium-intact aortic rings and this effect was abolished by LNA. These findings indicate that the PA extract could play a role in the opening of the Ca^{2+} sensitive K^{+} channels in the vascular smooth muscle but then this activity is overcome by NO generated from the vascular endothelium. However, further specific investigation would need to clarify this possibility. Glybenclamide caused a significant inhibition on the vasodilatory activity of the PA extract only on the endothelium-denuded aortic rings. This indicated that the PA extract may also have a secondary effect on the smooth muscle of the blood vessel by opening the ATP sensitive K^{+} channels. ODQ inhibited relaxation on both the endothelium-intact and endothelium-denuded thoracic aortic rings, indicating that the

PA extract may also have a secondary effect on the blood vessel by activating the soluble guanylate cyclase which then increased the cGMP level to promote its direct vasodilatory activity. In addition to the finding that when both glybenclamide and ODQ were added together there was an increased inhibitory effect of the PA extract on the vasodilatory activity but only for the endothelium-denuded thoracic aortic rings, confirming that the PA extract could act via the smooth muscle to open the ATP sensitive K^+ channels.

2.7 Conclusion

The present study has demonstrated that *P. acidus* extract has a hypotensive and a negative chronotropic activity in rats. The hypotensive effect of the PA extract is mediated directly at the vascular smooth muscle of blood vessel as a soluble guanylate cyclase stimulator, as an ATP-sensitive K^+ channel opener and/or Ca^{2+}-sensitive K^+ channel opener to promote the vasodilatatory activity. In addition PA extract may also play a role as a Ca^{2+}-channel inhibitor or may involve inhibition of Ca^{2+} mobilization from the intracellular store, but do not act through the muscarinic or β-adrenergic receptors. However, further study is required to identify the active substance(s) responsible for these activities. These findings provide scientific support for the traditional uses of decoction from *Phyllanthus acidus* leaves in the treatment of hypertension in man.

CHAPTER 3
BIOACTIVE SUBSTANCES ISOLATED FROM FRESH LEAVES OF *PHYLLANTHUS ACIDUS*

3.1 Abstract

The present study aimed to identify the active substance(s) from fresh leaves of *Phyllanthus acidus* and establish the mechanisms that would be involved in the hypotensive activity in rats. Using hypotensive- and vasodilator-guided fractionation, 5 hypotensive substances: adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol were isolated from the *P. acidus* (PA extract). Each substance caused a decrease in mean arterial blood pressure (MAP) and heart rate (HR) in anesthetized rats, and caused relaxation of the thoracic aortic rings preconstriced with phenylephrine (Phe, 3 μM). The vasorelaxant effects of these 5 substances were attenuated by N^{G}-nitro-L-arginine (LNA, 0.3 mM), a nitric oxide synthase inhibitor, or removal of the endothelium. 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M), a specific soluble guanylate cyclase inhibitor, or tetraethylammonium (TEA, 1 mM), a non-specific Ca^{2+} sensitive K^{+} channel inhibitor attenuated the vasodilatory activity of adenosine whereas glybenclamide (10^{-5} M), a specific ATP sensitive K^{+} channel inhibitor or ODQ attenuated the effect of hypogallic acid. In addition, the vasorelaxant activity produced by kaempferol persisted for 2 hrs.

These results suggested that the active substances which responsible for the hypotensive and vasorelaxant activities are adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol which act directly at the blood vessel to cause vasodilatation and act indirectly via the vascular endothelium to stimulate release of nitric oxide, as well as at the vascular smooth muscle to stimulate the soluble guanylate cyclase or open the ATP-sensitive K^{+} channel or open the Ca^{2+}-sensitive K^{+} channel.

3.2 Introduction

In chapter 2, it was found that an n-butanol extract from fresh leaves of *Phyllanthus acidus* (PA extract) produced a hypotensive and a negative chronotropic effects in rats, which indicated that at least two different activities were found in the n-butanol extract from fresh leaves of *P. acidus*. Thus it is of interest to isolate the pure compounds and find out whether those activities are responsible by one compound or one for each activity. The knowledge obtained would be useful for folkloric therapy, as well as provided an opportunity to develop the pure or partial pure compounds as a cardiovascular drug.

3.3 Objectives

To identify the active substance(s) responsible for the hypotensive and negative chronotropic activities in rats from an n-butanol extract from fresh leaves of *Phyllanthus acidus* (PA extract) and establish the mechanisms that would be involved.

3.4 Materials and Methods

3.4.1 Extraction and Isolation

Fresh leaves of *P. acidus* (100 kg) were simmered in hot filtered water for a period of 3 hrs. Only clear solution was collected and heated at 50°C to reduce the volume to 50 %. The concentrated solution was partition extracted with water-saturated n-butanol. The n-butanol phase was collected and evaporated under reduce pressure to dryness *in vacuo*, and the residue was lyophilized to obtain a yellow brown powder (506 g) of *P. acidus* extract (PA extract).

Isolation of active substance(s) from the PA extract follows the diagram in figure 35 and figure 36. Using hypotensive guide fractionation, the PA extract (500 g) was partition extracted with chloroform ($CHCl_3$) and followed by ethyl acetate. The ethyl acetate soluble part and insoluble part were collected and evaporated. The ethyl acetate insoluble part was subjected to column chromatography over silica gel 100 (0.063-0.200 mm, 850 g) and eluted with a gradient of $CHCl_3$: MeOH from 100 % $CHCl_3$ to 100 %

MeOH, yielding 3 fractions (A1-A3) on the basic of thin layer chromatography (TLC: gel 60 F_{254} A1 sheet, Merck, detection at 254 and 356 nm, $CHCl_3$: MeOH = 8 : 2 as a mobile phase). The hypotensive fraction, A3, was re-chromatographed on silica gel 60 (0.040-0.063 mm) and eluted with a gradient of $CHCl_3$: MeOH from 100 % $CHCl_3$ to 100 % MeOH, yielding 4 fractions (B1-B4). The hypotensive fraction, B3, was further fractionated by silica gel reversed phase C_{18} column chromatography using gradient elution of MeOH-H_2O: from 10 % MeOH to 80 % MeOH increasing each step by 10 % MeOH and using 2.5 liter of each concentration. This yielded 2 hypotensive compounds, which were identified as adenosine (355.9 mg) and caffeic acid (40.8 mg) respectively.

The ethyl acetate soluble part was subjected to column chromatography over silica gel 100 (0.063-0.200 mm, 850 g) and eluted with a gradient of $CHCl_3$: MeOH from 100 % $CHCl_3$ to 100 % MeOH, yielding 4 fractions (C1-C4) on the basic of thin layer chromatography (TLC: gel 60 F_{254} A1 sheet, Merck, detection at 254 and 356 nm, $CHCl_3$: MeOH = 8 : 2 as a mobile phase). The hypotensive fraction, C1, was further separated by medium pressure liquid chromatography (MPLC) on a Lichroprep® RP_{18} column (70X460 mm, 40-63 μM, Merck), eluted with a gradient of MeOH-H_2O-0.05% trifluoroacetic acid (TFA) at a flow rate of 10 ml/min, UV 254 (Buchi 681 pump equipped with a Knauer UV detector), yielding 3 hypotensive compounds: 4-hydroxybenzoic acid (1,004.6 mg), hypogallic acid (355.9 mg) and kaempferol (697.4 mg).

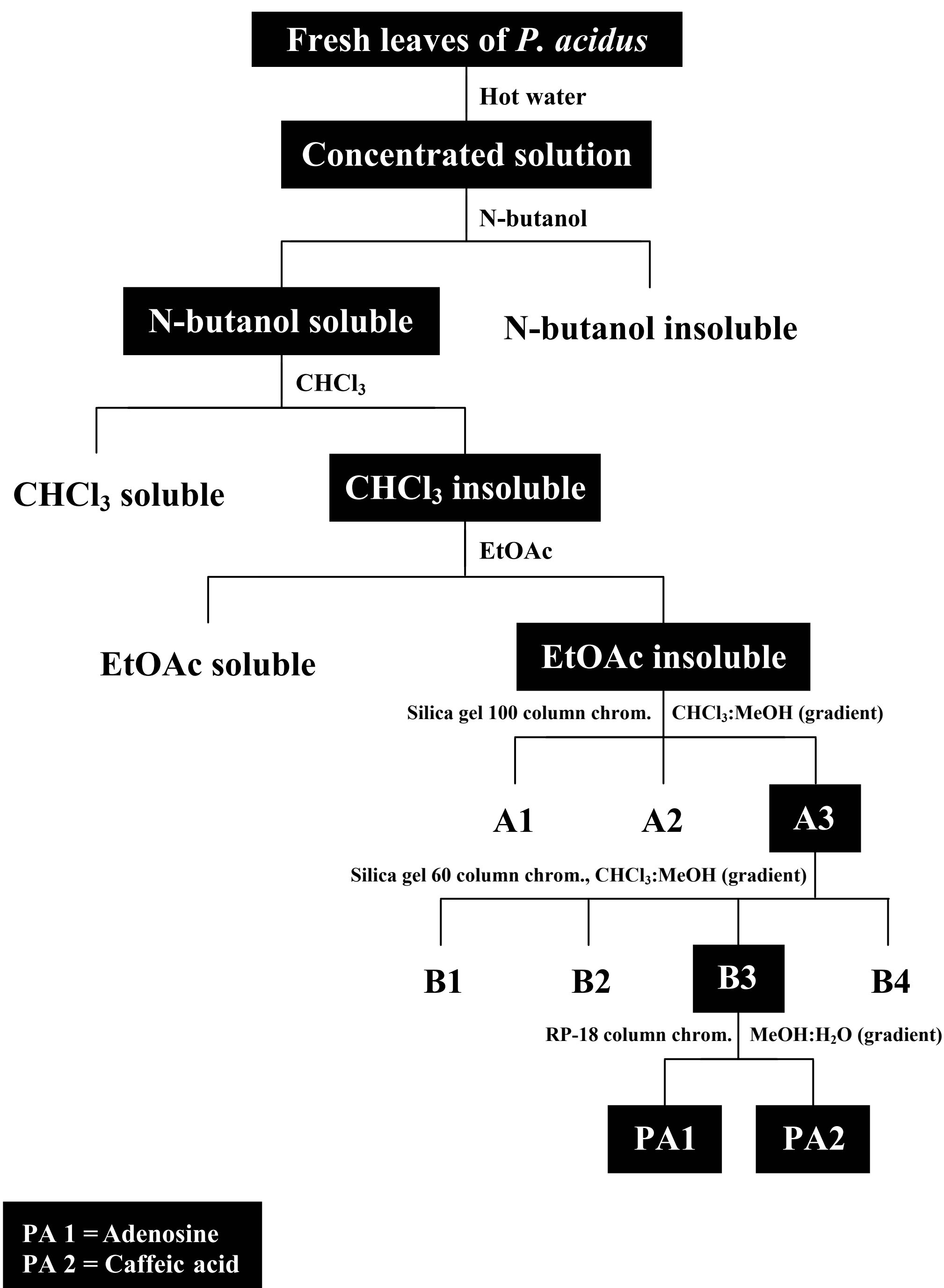


Figure 35 Flow chart showing the isolation of constituents from ethyl acetate insoluble part of the PA extract

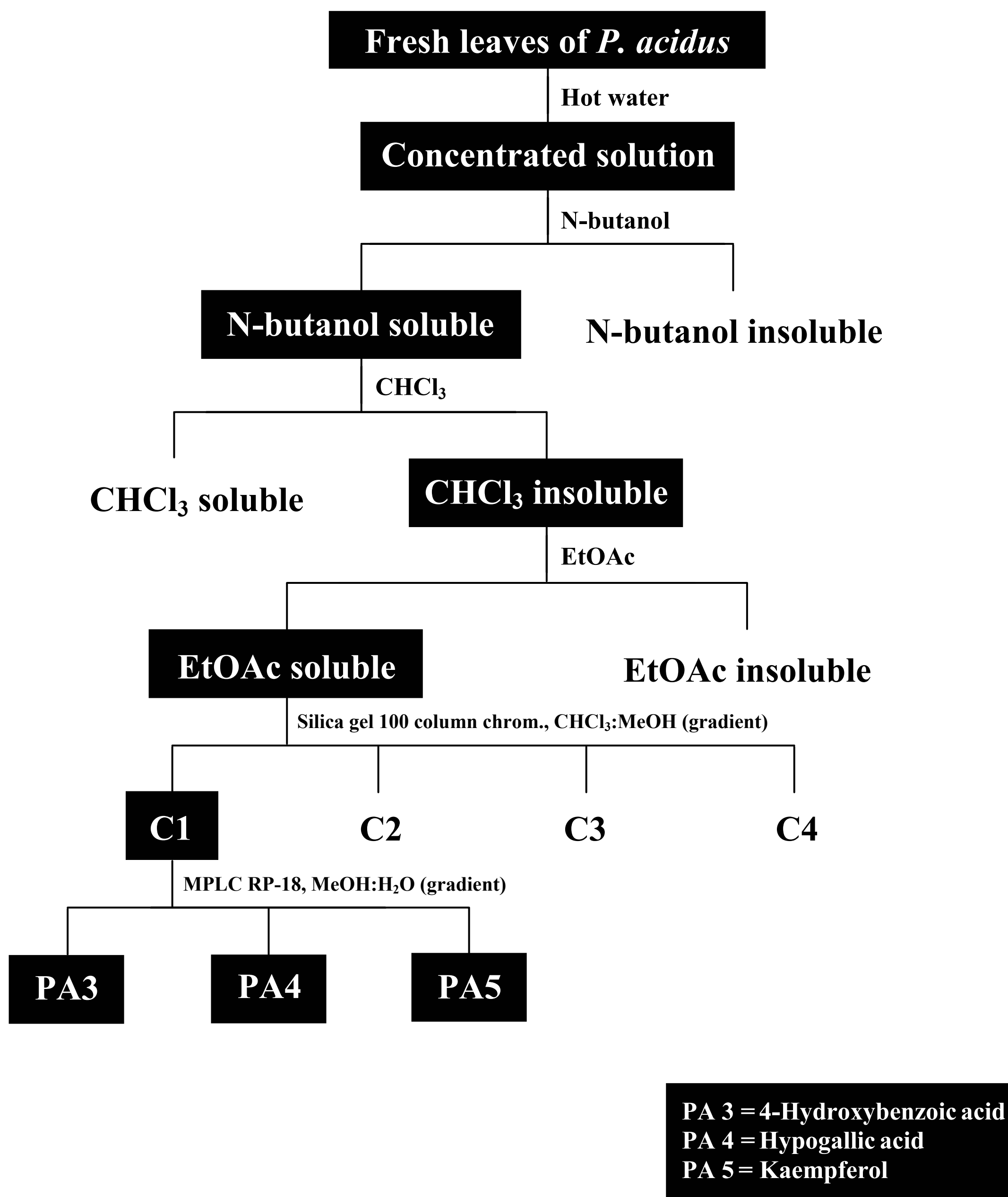


Figure 36 Flow chart showing the isolation of constituents from ethyl acetate soluble part of the PA extract

3.4.2 Structural determination

Chemical structures of the compounds isolated from the *P. acidus* extract were elucidated by Prof. Kurt Hostettmann and his colleges, University of Lausanne, Switzerland, using spectroscopic and chemical methods.

The pure compounds were characterized by mass spectroscopy on a TSQ-700 triple stage quadrupole instrument (Finnigan MAT, San Jose, CA, USA), and ^{1}H and ^{13}C NMR spectra which were recorded on a Varian Inova 500 spectrometer (Varian, Palo Alto, CA, USA) (500 MHz and 125 MHz, respectively) in either DMSO-d_6 or $CDCl_3$; chemical shifts in ppm as δ rel. to Me_4Si (internal standard).

The PA extract, as well as the isolated active substances were analyzed by high performance liquid chromatography (HPLC) in order to obtain a chemical profile. Analytical HPLC was carried on a HP 1100 system equipped with a photo diode array detector (Agilent Technologies). The extract was analyzed on a symmetry ®C_{18} column (5μm, 3.9x150 mm i.d.; Waters), with a gradient of MeOH : H_2O + 0.05 % of TFA (10 : 90 to 100 : 0). The flow rate was 1 ml/min; the UV traces were measured at 210 and 254 nm and UV spectra (DAD) were recorded between 200 and 500 nm.

3.4.3 Pharmacological studies of the isolated substances

Effects on mean arterial blood pressure and heart rate *in vivo*

Adult female Wistar rats in estrus (210-250 g) were supplied from the Animal House, Faculty of Science, Prince of Songkla University. The animals were housed in controlled environmental conditions at 25 °C on a 10 hrs dark and 14 hrs light cycle and allowed access to standard food and tap water *ad libitum*. The methods employed in this study were approved by the Prince of Songkla University Animal Care and Use Committee. The investigation conformed to the Guide for the Care and Use of Laboratory Animals.

Effect of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on the mean arterial blood pressure and heart rate of anesthetized rats

Adult female Wistar rats (210-230 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg, i.p.). The tracheal tube was cannulated with a polyethylene tube to maintain airway patency and the animals breathed room air spontaneously. A polyethylene catheter was cannulated through the right common carotid artery which was connected to a pressure transducer (P23 ID, Gould Statham Instrument, Hato Rey, Puerto Rico) and connected to a Grass polygraph (model 7D, Grass Instrument, Quincy, MA, U.S.A.) for systemic blood pressure monitoring, and the heart rate was recorded by using a tachograph driven by the blood pressure wave. Another polyethylene tube was cannulated through the left jugular vein for intravenous administration of drugs. The animal was then equilibrated for at least 40 min before the experiments were started. Each rat was used for only one agonist.

After the equilibration period and the systemic blood pressure reached steady state, the dose-response relationship to adenosine (0.03-0.3 mg/kg), 4-hydroxybenzoic acid (1-10 mg/kg), caffeic acid (1-10 mg/kg), hypogallic acid (1-10 mg/kg), or kaempferol (1-10 mg/kg) were determined by injection of the drug through the left jugular vein of a volume not exceeding 0.1 ml for each dose and flushed with 0.1 ml normal saline.

Effect of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on thoracic aortae *in vitro*

Adult female Wistar rats in estrus (230-250 g) were killed by decapitation with a guillotine. Thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue. Two adjacent rings of 6-7 mm in length from thoracic aorta were cut, and the endothelium was removed mechanically from one by gently rubbing of the intimal surface of ring with a stainless steel rod. The aortic rings with or without functional endothelium were mounted horizontally between two parallel stainless steel hooks with extremely care not to damage the endothelium for the endothelium-intact aortic rings, and suspended in a 20 ml organ bath containing Kerbs-Henseleit solution of the following

composition (mM): Nacl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ท$7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37°C and continuously bubbled with 95 % O_2 and 5 % CO_2 mixture. One of the hooks was fixed at the bottom and the other was connected to a force displacement transducer which connected to a Grass polygraph for the monitoring of changes in isometric tension. Prior to addition of drugs, tissues were equilibrate under a resting tension of 1 g and the bath solution was replaced with pre-warmed and oxygenated Kerbs-Henseleit solution every 15 min.

The preparations was allowed to equilibrate for 60 min, the presence or absence of a functional endothelium of the thoracic aortic ring was assessed in all preparation as follows: the aortic rings with and without endothelium were preconstricted with 3 μM phenylephrine until the contraction reached a steady state (7-10 min), in order to test their contractility, and the dilatory response to 30 μM acetylcholine was recorded. The experiments were continued only when acetylcholine induces more than 80% vasodilatation for the endothelium-intact aortic rings and there was no dilatory response to acetylcholine for the endothelium-denuded aortic rings. Preparations were then washed several times with drug-free Krebs-Henseleit solution, and allow relaxing fully for 45 min before the experimental protocol began.

After the re-equilibration, the thoracic aortic rings with and without endothelium were again preconstricted with 3 μM phenylephrine for 10 min. When the contraction reached a steady state, a cumulative concentration-response relationship curve (C-R curve) to adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, or kaempferol were constructed.

Using another set of animals, only the endothelium-intact thoracic aortic rings were pre-incubated with N^{G}-nitro-L-arginine (LNA, 0.3 mM) for 30 min, then the aortic rings were preconstricted with 3 μM phenylephrine until the contraction reached a steady state (10-15 min), and the cumulative concentration-response relationship to adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, or kaempferol were obtained.

Using another set of animals, the thoracic aortic rings with or without endothelium were pre-incubated with Tetraethylammonium (TEA, 1 mM), glybenclamide (10^{-5} M) or 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M) for

30 min, then each thoracic aortic ring was preconstricted with 3 μM phenylephrine for 10-15 min (plateau reached), and the cumulative concentration-response relationship to adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, or kaempferol were obtained in the presence or absence of those corresponding blockers.

Another set of thoracic aortic rings with and without endothelium were preconstricted with 3 μM phenylephrine for 10-15 min (plateau reached), followed by a cumulative concentration-response relationship to the 5 pure compound-cocktail (adenosine 14.41 %, 4-hydroxybenzoic acid 15.00 %, caffeic acid 1.65 %, hypogallic acid 40.69 % and kaempferol 28.25 %).

3.4.4 Drugs and chemicals

The following drugs were used: acetylcholine chloride, glybenclamide, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), phenylephrine hydrochloride, N^G-nitro-L-arginine (LNA), tetraethylammonium (TEA), adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol. All drugs were purchased from Sigma, USA. The organic solvents for plant extraction, n-butanol, were purchased from Merck, Germany.

LNA and TEA were dissolved in distilled water, acetylcholine chloride and phenylephrine hydrochloride were dissolved in a solution (g/L): NaCl 9.0, NaH_2PO_4 0.19 and ascorbic acid 0.03 and the remaining stock solutions were initially dissolved in 10% DMSO but their further serial dilution were made in distilled water.

3.4.5 Statistic analysis

Results were expressed as mean $\pm$ S.E. of 6 experiments (n=6). Changes in blood pressure and heart rate were recognized as the difference between the steady pressure or heart rate before and the lowest pressure or heart rate after injection. The blood pressure were recorded in mmHg as systolic pressure (SP) and diastolic pressure (DP) and were expressed as mean arterial blood pressure (MAP), which was calculated as DP+1/3 (SP-DP). Vasodilator responses of thoracic aortic rings were expressed as percentage relaxation of vessels from maximal contraction of 3 μM phenylephrine preconstruction levels. Statistical differences were determined by the Student's paired or

unpaired *t*-test or one way ANOVA. The *P* value < 0.05 was considered to be significant in all experiments.

3.5 Results

3.5.1 Isolated compounds

As shown in figure 37, five compounds were isolated from *P. acidus* extract are as follows:

Adenosine (1, PA 1) is a white amorphous powder. For MS, ^{1}H and ^{13}C NMR, see Aldrich, 1992.

4-Hydroxybenzoic acid (2, PA 3) is a white amorphous powder. For MS, ^{1}H and ^{13}C NMR, see Li et al, 2003.

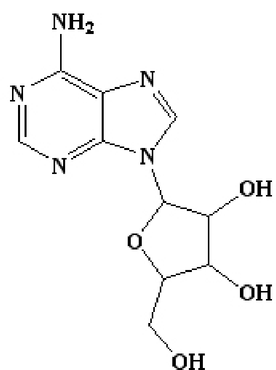
Caffeic acid (3, PA 2) is a white amorphous powder. For MS, ^{1}H and ^{13}C NMR, see Schmutz et al., 1993.

Hypogallic acid (4, PA 4) is a white amorphous powder. For MS, ^{1}H and ^{13}C NMR, see Choudhary et al., 2008.

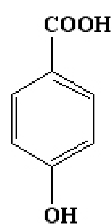
Kaempferol (5, PA 5) is a Yellow amorphous powder. For MS, ^{1}H and ^{13}C NMR, see Reddy et al., 2009.

The chemical structures of the above substances were elucidated by Prof. Hostettman and his colleges

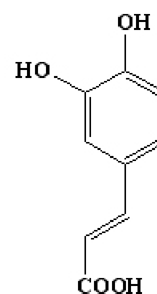
The HPLC chromatograms together with the corresponding UV spectra of the PA extract and the five pure substances are shown in figure 38.



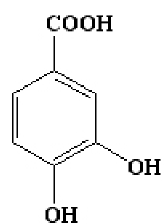
Adenosine
(Compound 1, PA 1)



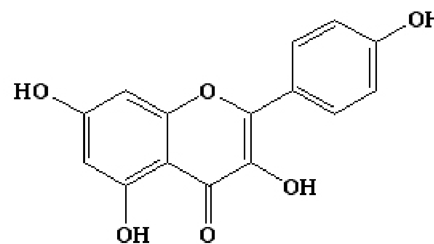
4-Hydroxybenzoic acid
(Compound 2, PA 3)



Caffeic acid
(Compound 3, PA 2)



Hypogallic acid
(Compound 4, PA 4)



Kaempferol
(Compound 5, PA 5)

Figure 37 Structures of pure compounds isolated from the *P. acidus* extract

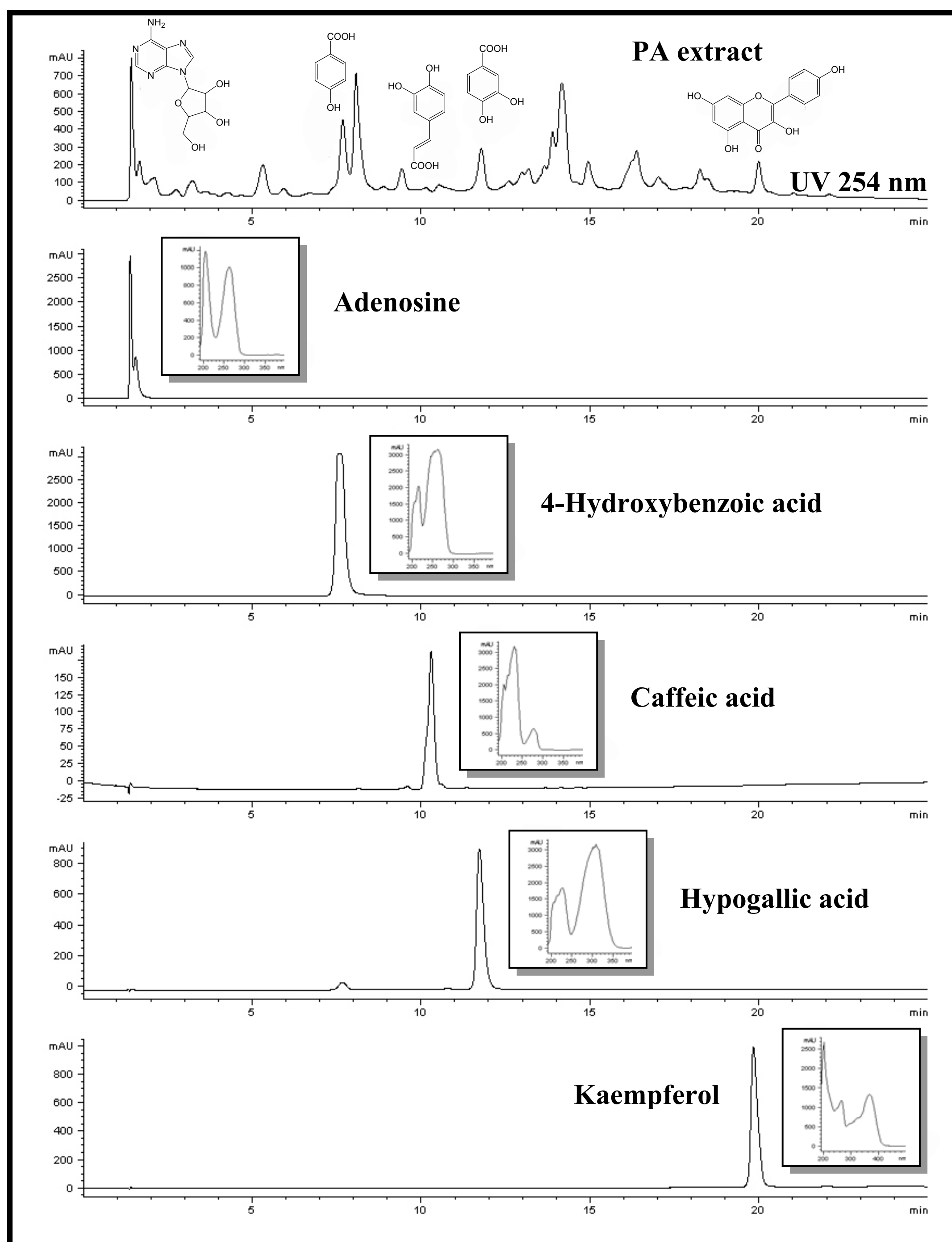


Figure 38 HPLC chromatogram of *P. acidus* extract (PA extract), and adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol, isolated from the PA extract. The column eluant from the PA extract and the isolated compounds were scanned at the wavelengths 254 nm.

3.5.2 Effect of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on the mean arterial blood pressure and heart rate of anesthetized rats

An intravenous injection of adenosine (0.03-0.3 mg/kg) caused a marked decrease in the MAP and HR in a dose-dependent manner, whereas 4-hydroxybenzoic acid (1-10 mg/kg) caused only a slight decrease. In the case of caffeic acid (1-10 mg/kg), hypogallic acid (1-10 mg/kg) and kaempferol (1-10 mg/kg) only the highest dose (10 mg/kg) caused a decrease in MAP and HR of anesthetized rats (figure 39).

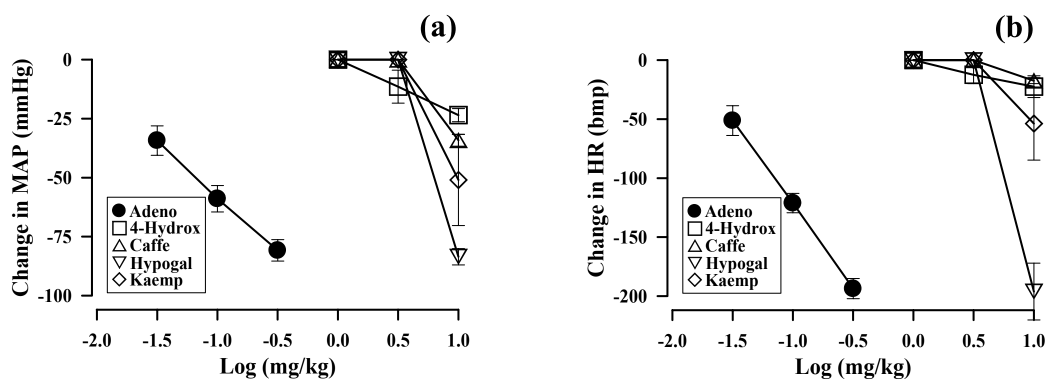


Figure 39 Effect of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on mean arterial blood pressure (MAP) and heart rate (HR) in anesthetized rats. Each point represents mean $\pm$ S.E. mean of 6 experiments (n=6).

3.5.3 Effect of adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol on thoracic aortae *in vitro*

As shown in figure 40-44, adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol caused a dilatation of thoracic aortic rings preconstricted with 3 μM phenylephrine in a concentration-dependent manner. Removal of the vascular endothelium, or pre-incubating the thoracic aortic rings with LNA, caused a significant rightward shift of the concentration-response curves with an increase in the EC_{50} values, except that of the kaempferol on the endothelium-denuded aortic rings (figure 44b). The vasodilatory activity of these compounds returned to normal within 45 min (data not show), except that for kaempferol when the effect persisted for up to 3 hrs (figure 44e).

Glybenclamide did not modify the vasodilatory activity of these 5 pure compounds on the thoracic aortic rings preconstricted with phenylephrine, except the ones without endothelium that glybenclamide could significant decreased the vasodilator effect of hypogallic acid (figure 43d).

ODQ caused a significant rightward shift of the concentration-response curves with an increase in EC_{50} values to adenosine, 4-hydroxybenzoic acid, caffeic acid and hypogallic acid on the endothelium-intact aortic rings preconstricted with phenylephrine. When the endothelium of the thoracic aortic rings was removed, the inhibitory effect of the ODQ persisted with adenosine and the hypogallic acid (figure 40f and 43f), but not with the 4-hydroxybenzoic acid and caffeic acid.

TEA did not modify the vasodilatory effect of these 5 pure compounds on the thoracic aortic rings preconstricted with phenylephrine, except that for adenosine where it caused a parallel rightward shift of the vasodilator curve of the endothelium-denuded ones (figure 40h). When the 5 pure compounds were mixed together in the same proportion to that found in the PA extract, the cocktail compounds also caused an endothelium independent dilatation of the thoracic aortic rings preconstricted with phenylephrine and its effect persisted for up to 3 hrs (figure 45).

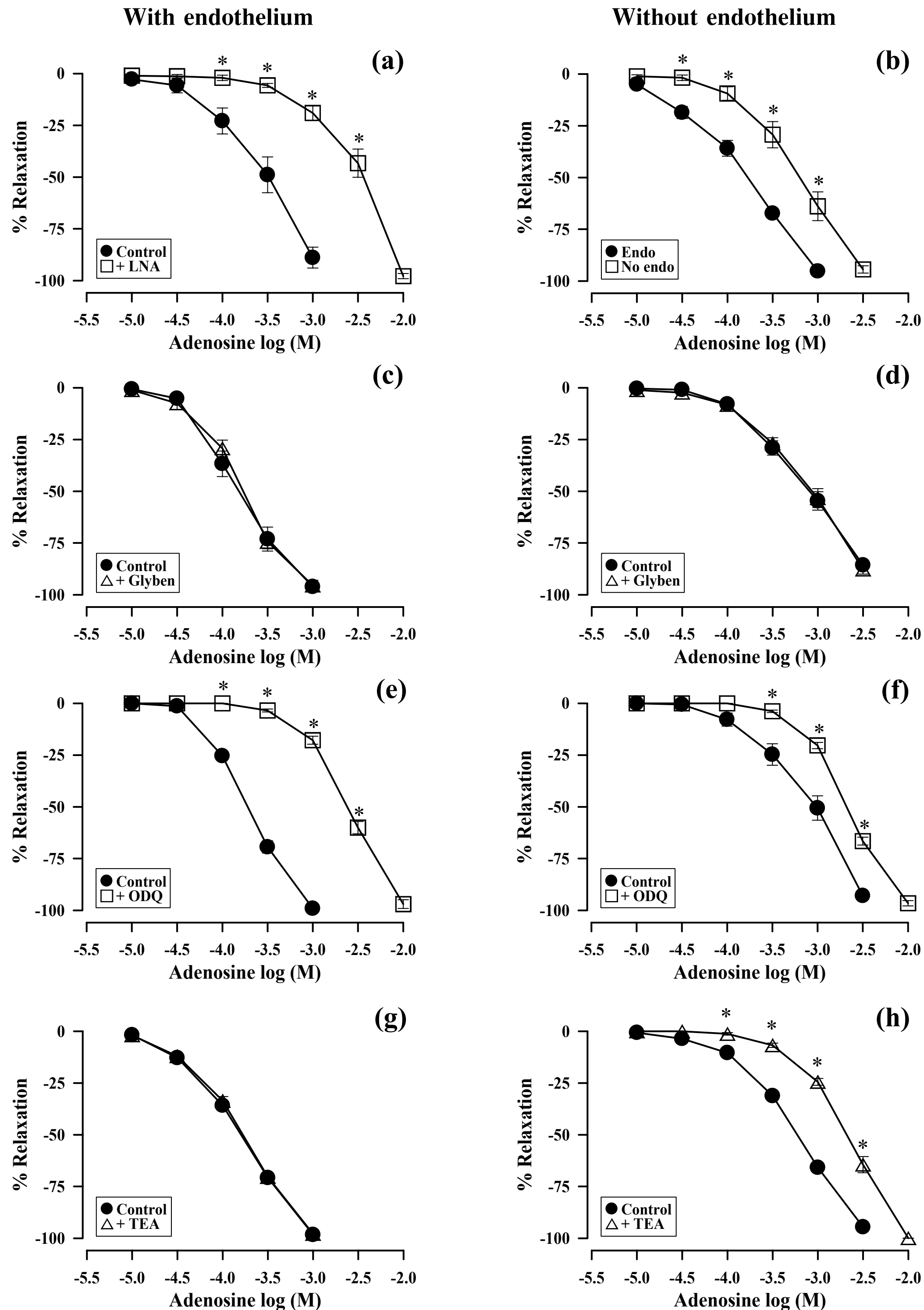


Figure 40 Effect of LNA, endothelium, glybenclamide (glyben), ODQ, or TEA on the dilatation of the endothelium-intact (a, c, e and g) or denude (b, d, f and h) thoracic aortic rings preconstricted with phenylephrine to adenosine. Each point represents mean ± S.E. mean of 6 experiments (n=6).

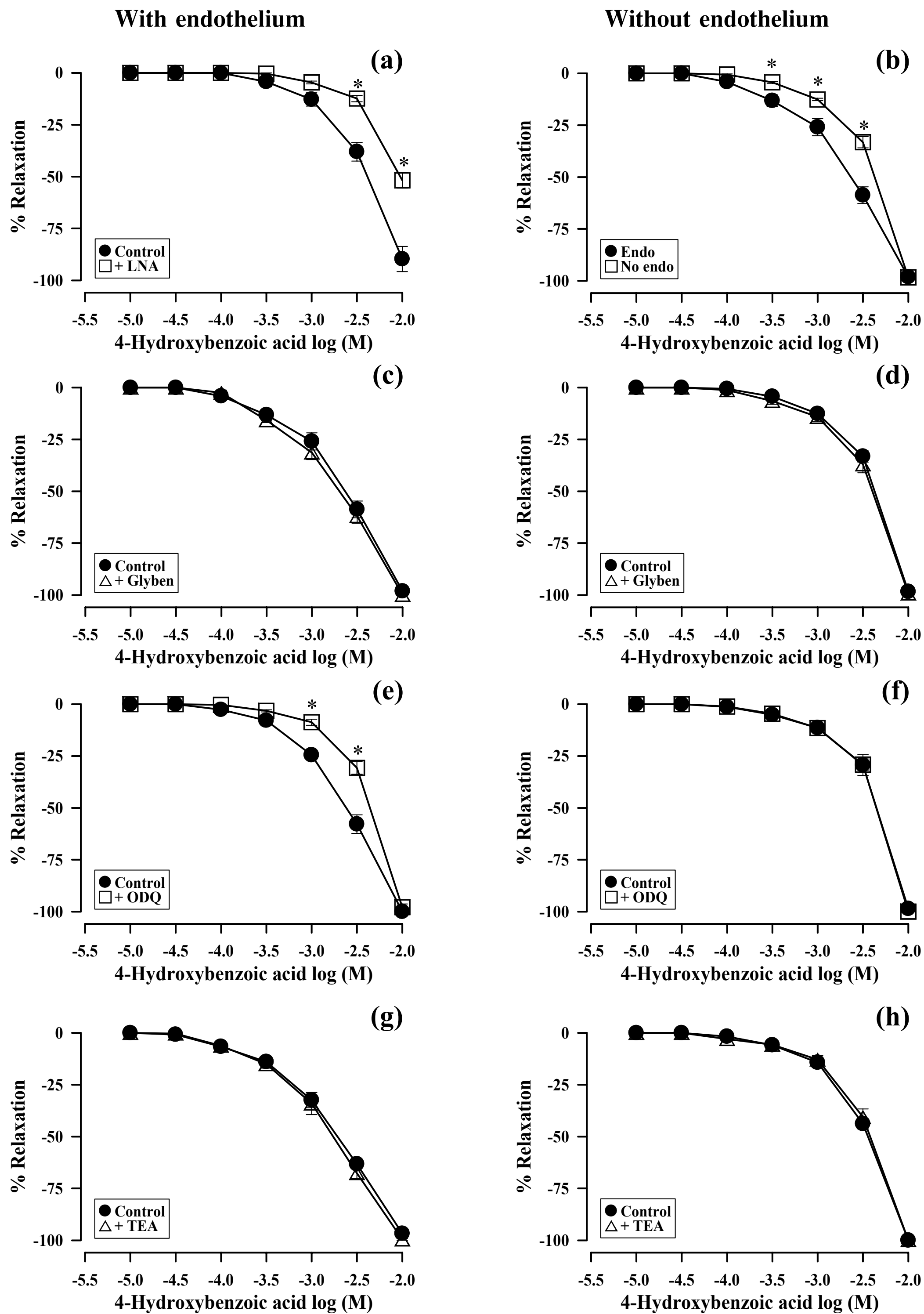


Figure 41 Effect of LNA, endothelium, glybenclamide (glyben), ODQ, or TEA on the dilatation of the endothelium-intact (a, c, e and g) or denude (b, d, f and h) thoracic aortic rings preconstricted with phenylephrine to 4-hydroxybenzoic acid. Each point represents mean $\pm$ S.E. mean of 6 experiments (n=6).

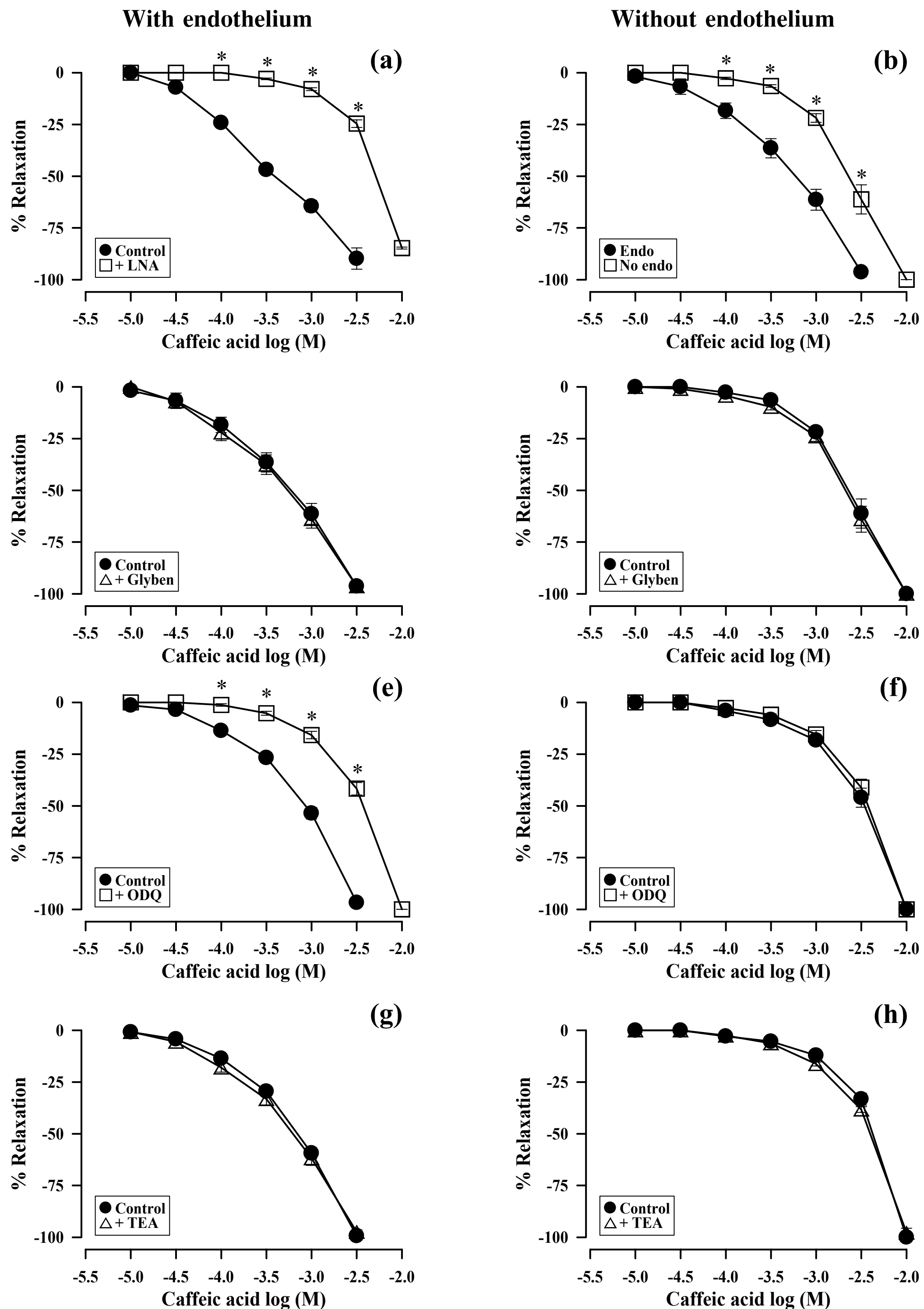


Figure 42 Effect of LNA, endothelium, glybenclamide (glyben), ODQ or TEA on the dilatation of the endothelium-intact (a, c, e and g) or denude (b, d, f and h) thoracic aortic rings preconstricted with phenylephrine to caffeic acid. Each point represents mean ± S.E. mean of 6 experiments (n=6).

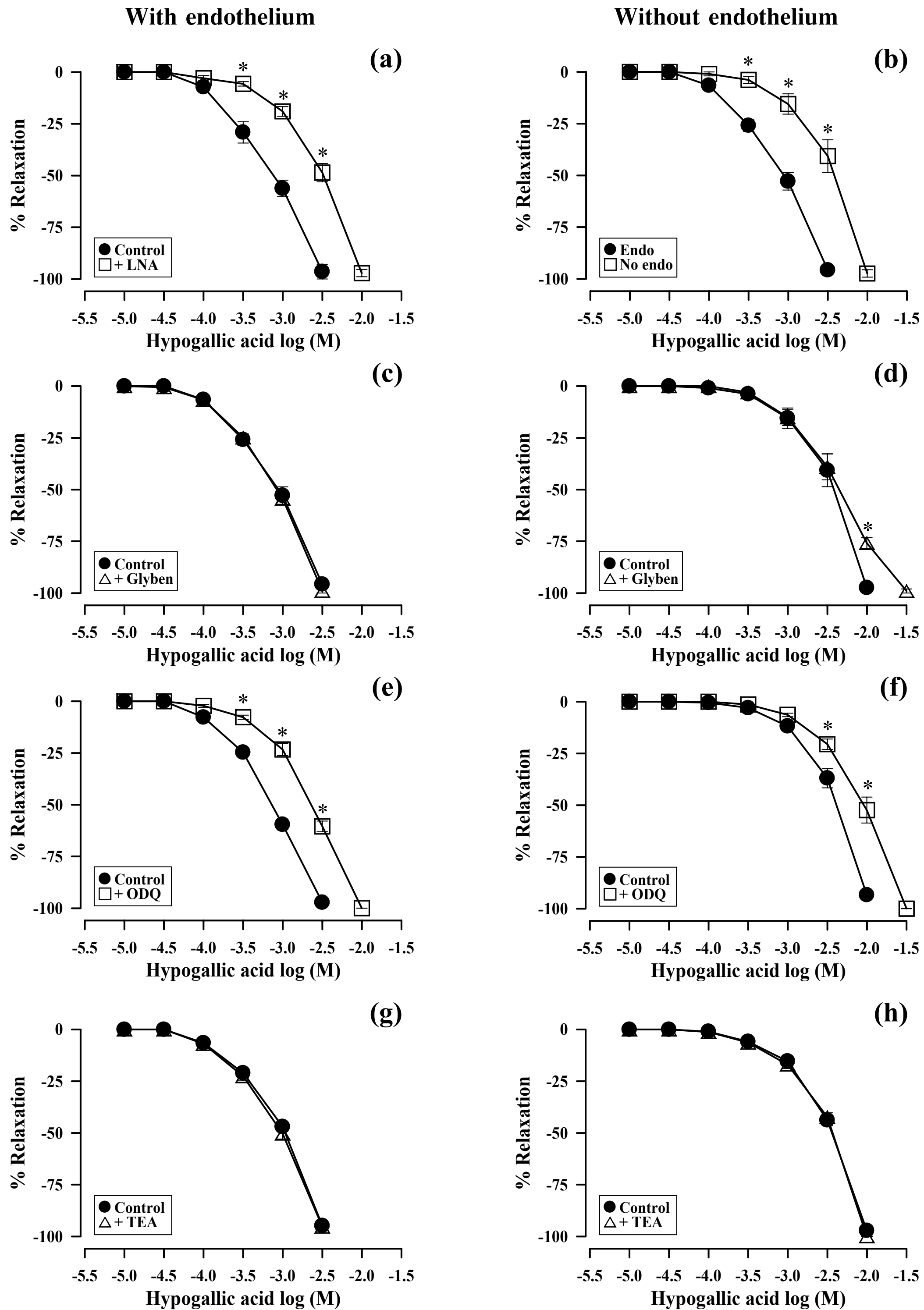


Figure 43 Effect of LNA, endothelium, glybenclamide (glyben), ODQ or TEA on the dilatation of the endothelium-intact (a, c, e and g) or denude (b, d, f and h) thoracic aortic rings preconstricted with phenylephrine to hypogallic acid. Each point represents mean $\pm$ S.E. mean of 6 experiments (n=6).

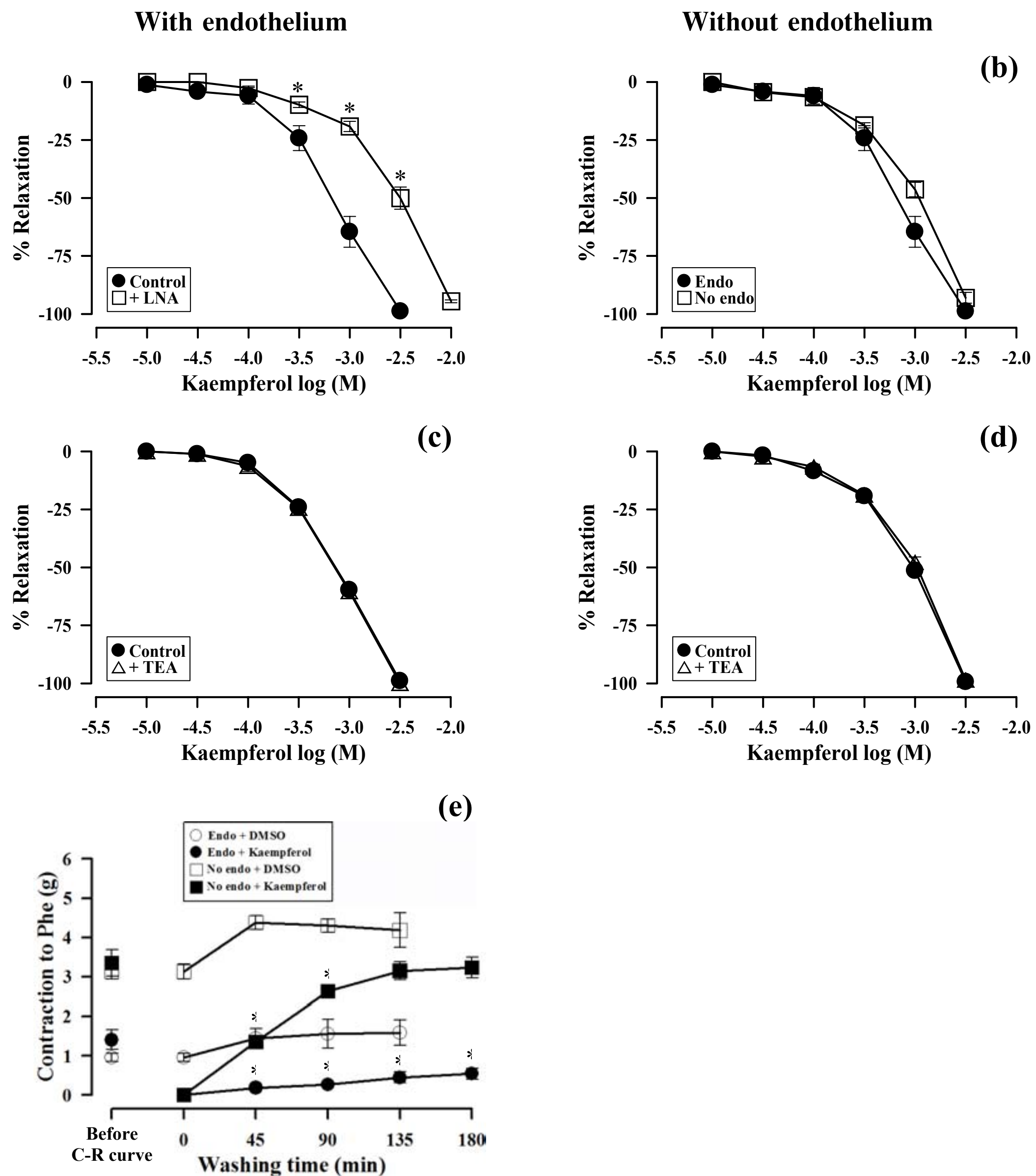


Figure 44 Effect of LNA, endothelium or TEA on the dilatation of the endothelium-intact (a and c) or denude (b and d) thoracic aortic rings preconstricted with phenylephrine to kaempferol. And maximal contractile responses (e) of the thoracic aortic rings to phenylephrine before performing the concentration-response (C-R) curve, at the end of the C-R curve prior washing (0 min) and after a 45 min interval washing period after performing a concentration-dilatory response relationship to DMSO or kaempferol and challenged with phenylephrine. Each point represents mean $\pm$ S.E. mean of 6 experiments (n=6).

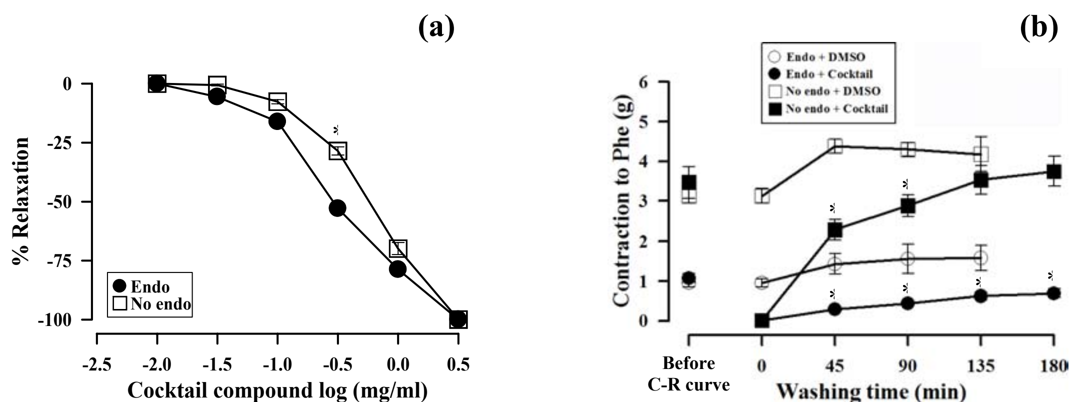


Figure 45 Dilatory responsiveness of a cocktail of the five ingredients (adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol, in the same proportion to that found in the PA extract) on endothelium-intact (Endo) and denuded (No endo) thoracic aortic rings preconstricted with phenylephrine (a). And maximal contractile responses (b) of the thoracic aortic rings to phenylephrine before performing the concentration-response (C-R) curve, at the end of the C-R curve prior washing (0 min) and after a 45 min interval washing period after performing a concentration-dilatory response relationship to DMSO or cocktail of the five ingredients and challenged with phenylephrine. Each point represents mean $\pm$ S.E. mean of 6 experiments (n=6).

3.6 Discussion

In order to identify the active substance(s) of the PA extract, hypotensive- and vasodilatation-guided fractionation of the PA extract was carried out by chemical method using different types of column chromatography. As shown in the method section, five active compounds were identified by classical spectroscopic methods: adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol. The chemical constituents of these compounds in the PA extract are in the following proportion: hypogallic acid : kaempferol : 4-hydroxybenzoic acid : adenosine : caffeic acid = 24 : 17 : 9 : 8 : 1. Although these compounds are not new discoveries, this is the first report that

identifies them as bioactive chemical constituents of the leaves of *Phyllanthus acidus*, as well as elucidation the mechanisms responsible for the hypotensive activity of these plant.

As shown in figure 39 these five pure compounds exert hypotensive and negative chronotropic activity in anesthetized rats, and adenosine has the highest potency for decreasing the blood pressure and heart rate. This result is consistent with the literature (Barraco et al., 1987, Stella et al., 1993, Suzuki et al., 2002). These finding indicate that the hypotensive and negative chronotropic activity of the PA extract is the result of the synergy between these five compounds. Among them, adenosine has been extensive studied for its effect on blood pressure and heart rate by many researchers, and it is well-known that adenosine produced hypotension and bradycardia. These effects are thought to be mediated at adenosine receptors localized at the cardiovascular regulation regions of the hindbrain, including the nucleus tractus solitarius and in the periphery through different adenosine receptor subtypes (Barraco et al., 1988, Shryock and Belardinelli, 1997).

All of these compounds caused a concentration-dependent dilatation of both endothelium-intact and endothelium-denuded thoracic aortic rings preconstricted with phenylephrine (figure 40-44). Adenosine is the most potent vasodilator which is about 3 fold more potent than caffeic acid, hypogallic acid and kaempferol, and about 12 fold more potent than 4-hydroxybenzoicacid (table 1). The vasodilatory activity of these compounds is in the same range as previous reports (Andriambeloson et al., 1998, Cicala et al., 2003, Hourani et al., 2001, Moritoki et al., 1990, Padilla et al., 2005, Perez-vizcaino et al., 2002, Xu et al., 2007), except that this is the first report on the hypotensive and vasodilatory activity of hypogallic acid. The finding that LNA or removal of the vascular endothelium causes a rightward shift of the curve produced by these compounds indicates that these compounds act directly on the blood vessels to cause vasodilatation, as well as indirectly by stimulating the release of nitric oxide from the vascular endothelium to promote vasodilatation. This is also consistent with previous reports (Andriambeloson et al., 1998, Benkhalti et al., 2003, Moritoki et al., 1990, Taubert et al., 2002).

Further experiments were performed to investigate whether adenosine, 4-hydroxybenzoic acid, caffeic acid and hypogalic acid have any secondary effects as an ATP-sensitive K^+ channel and/or stimulation of the soluble guanylate cyclase at the blood vessel. To reveal these possibilities, the C-R curves of each compound on the thoracic aortic rings were performed in the presence of glybenclamide or ODQ. Glybenclamide

pretreatment significantly inhibited only the hypogallic acid-induced vasorelaxtion, but not by the other pure compounds. In addition, the inhibitory effect of glybenclamide occurred only on the endothelium-denuded thoracic aortic rings (figure 43d). These together indicate that hypoallic acid can also play a role in opening the K_{ATP} channels of the vascular smooth muscle of the thoracic aortic rings. ODQ caused a parallel right shift of the concentration-response curves for all of these pure compounds. This implies that the soluble guanylate cyclase pathway would be involved on the vasodilatation produced by these compounds. However, the soluble guanylate cyclase might be generated from nitric oxide perhaps released by these agonists, or be stimulated directly by the agonist.
To unravel these possibilities, further experiments were carried out on the endothelium-denuded thoracic aortic rings, to remove the endothelium nitric oxide generation. In these preparation, ODQ did not cause a shift in the vasodilator concentration response curve of 4-hydroxybenzoic acid and caffeic acid, whereas for adenosine and hypogallic acid, the shift still persisted although it was about two fold less than those obtained from the endothelium-intact thoracic aortic rings. These results indicate that only adenosine and hypogallic acid, but not 4-hydroxybenzoic acid or caffeic acid could stimulate the soluble guanylate cyclase of the vascular smooth muscle to promote their direct effect on vasodilatation. The finding that TEA caused a rightward parallel shift of the relaxation curve for adenosine, but not for the other 4 pure compounds, and only on the endothelium-denuded thoracic aortic rings, indicated that adenosine would also cause an opening of the K_{Ca} channels of the vascular smooth muscle.

Kaempferol is a common flavonoid found in many plants. However, this is the first report of substantial amounts of kaempferol in the leaves of *Phyllanthus acidus*. Kaempferol exerted an endothelium-independent vasodilatation of the thoracic aortic rings, as well as stimulating the release of the nitric oxide from the vascular endothelium. These results are analogous to those reported by Taubert et al. (2002) and also Perez-Vizcaino et al. (2002) who each suggested that the vasodilatory effect of the kaempferol was not associated with the soluble guanylate cyclase since ODQ had no effect on the concentration-response curve of quercetin, by the un-metabolised form of kaempferol.

From chapter 2, the vasodilatatory effects of the PA extract on the thoracic aortic rings are endothelium-independent, and are not modified by LNA or by the removal of the vascular endothelium. This is different from the effects produced by the five individual isolated compounds. This could be explained if these compounds separately

possess differences in (1) their vasodilatatory potency both directly and indirectly via stimulation of the nitric oxide release, opening of the K_{ATP} channels, opening of the K_{Ca} channels and/or stimulation of the soluble guanylate cyclase, and (2) the relative amounts of the individual compounds present in the PA extract. These amount when mixed together in the PA extract or as a cocktail of the five ingredients result in there being no difference in the vasodilatation between the endothelium-intact and endothelium-denuded thoracic aortic rings and also the effect lasted for up 3 hrs. The finding that only kaempferol produced a long-lasting inhibitory effect on the phenylephrine-induced thoracic aortic rings constriction, indicates that the persistent vasodilatatory activity of the PA extract is likely due to kaempferol. Other differences observed between the cocktail of the five isolated compounds and the PA extract can be possibly attributed to other unidentified compounds present in the extract.

3.7 Conclusion

The bioactive principles identified as being responsible for the hypotensive and negative chronotropic activities are most likely to be adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol. These five bioactive principles act directly on the vascular smooth muscle to cause vasodilatation, and indirectly by stimulating the release of nitric oxide from the vascular endothelium, as well as behaving as a soluble guanylate cyclase stimulator, as an ATP-sensitive K^+ channel opener and/or as a Ca^{2+}-sensitive K^+ channel opener to promote the vasodilatatory activities of the compounds.

Table 1 EC50 in vasodilator responses to adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol of endothelium-intact or denuded thoracic aortic ring preconstricted with phenylephrine

Compounds	Endothelium-intact					Endothelium-denuded			
	EC 50 (mM) : 95 % confidential limit					EC 50 (mM) : 95 % confidential limit			
	Control	+ LNA	+ Glyben	+ ODQ	+ TEA	Control	+ Glyben	+ ODQ	+ TEA
Adenosine	0.15	2.47*	0.15	2.38*	0.13	0.53	0.56	2.07*	1.6*
	(0.13 - 0.18)	(1.64 - 3.71)	(0.13 - 0.18)	(2.18 - 2.59)	(0.11 - 0.15)	(0.45 - 0.61)	(0.48 - 0.65)	(1.82 - 2.36)	(1.32 - 1.98)
4-Hydroxybenzoic acid	1.78	3.28*	1.62	2.99*	1.29	2.81	2.68	2.99	2.42
	(1.49 - 2.13)	(2.09 - 5.15)	(1.41 - 1.87)	(2.23 - 4)	(1.07 - 1.55)	(2.15 - 3.68)	(2.06 - 3.5)	(2.19 - 4.07)	(1.66 - 3.54)
Caffeic acid	0.4	3.05*	0.37	2.51*	0.42	1.96	1.82	2.52	2.37
	(0.32 - 0.49)	(2.25 - 4.14)	(0.3 - 0.45)	(2.02 - 3.12)	(0.35 - 0.5)	(1.65 - 2.31)	(1.54 - 2.14)	(2 - 3.18)	(1.66 - 3.38)
Hypogallic acid	0.62	2.23*	0.64	1.92*	0.65	2.53	3.64	6.8*	2.46
	(0.54 - 0.72)	(1.79 - 2.78)	(0.56 - 0.72)	(1.7 - 2.18)	(0.57 - 0.76)	(1.93 - 3.31)	(3.06 - 4.34)	(5.65 - 8.2)	(1.99 - 3.03)
Kaempferol	0.58	2.08*	-	-	0.61	0.7	-	-	0.71
	(0.49 - 0.7)	(1.65 - 2.63)	-	-	(0.53 - 0.69)	(0.56 - 0.87)	-	-	(0.57 - 0.89)

Values were obtained from 6 experiments (n=6) for each groups. * Significantly higher than their corresponding control group.

CONCLUSION

The present study has demonstrated that *P. acidus* extract (PA extract) has a hypotensive and a negative chronotropic activity in rats. These effects were not mediated via the muscarinic receptors or the β-adrenergic receptors of the vascular system. The hypotensive effect of the PA extract is probably mediated directly at the blood vessel to cause vasodilatation. The bioactive principles identified as being responsible for the hypotensive and negative chronotropic activities are most likely to be adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol. The hypotensive activity of the PA extract would be a synergistic effect of these substances. These five bioactive principles act directly on the vascular smooth muscle to cause vasodilatation, and indirectly by stimulating the release of nitric oxide from the vascular endothelium, as well as behaving as a soluble guanylate cyclase stimulator, as an ATP-sensitive K^+ channel opener and/or as a Ca^{2+}-sensitive K^+ channel opener to promote the vasodilatatory activities of the compounds, as proposed in figure 46. In addition PA extract may also play a role as a Ca^{2+}-channel inhibitor or may involve inhibition of Ca^{2+} mobilization from the intracellular store. These findings provide scientific support for the traditional uses of decoction from *Phyllanthus acidus* leaves in the treatment of hypertension in man.

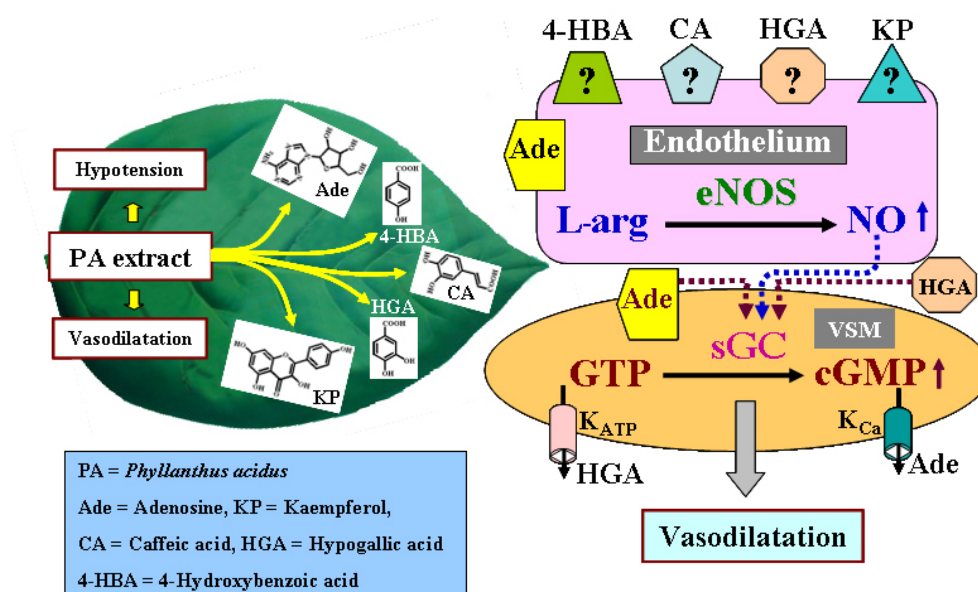


Figure 46 The mechanisms of an n-butanol extract from leaves of *Phyllanthus acidus* that would be involved in the hypotensive activity

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