EVALUATION OF THE CARDIOVASCULAR ACTIVITY AND TOXICITY STUDY OF *ALSTONIA SCHOLARIS* BARK EXTRACTS

IDRIS BELLO

UNIVERSITI SAINS MALAYSIA

March 2016

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By

IDRIS BELLO

Thesis submitted in fulfillment of the requirements

for the degree of Master of Science

March 2015

ACKNOWLEDGEMENT

I will like to start with showing my gratitude to almighty Allah (S.W.A) for the strength, wisdom and health He bestowed on me which has been the guiding force that has helped me to accomplish this phase in my journey of knowledge seeking.

The period of my studying for this M.Sc. degree has given me vast experiences and knowledge which I believe will be valuable in my future endeavors and will help me substantially to achieve greater heights. A number of individuals have been instrumental in my study.

My immense gratitude goes to my supervisor Prof. Mohd. Zaini Bin Asmawi for his innumerable and unquantifiable support, understanding and perseverance with my shortcomings in guiding me through the tasking process of learning the art of research and all the dynamics involve. Prof. Mohd. Zaini's influence stretch beyond the confines of academic field; the warmness and comfort level he creates between him and his students, his listening ears, persuasion to achieve excellence while understanding our limits, subtle correction of errors and leniency beyond our expectations are attributes that endears both his students and others towards him. His contribution towards my study cannot be overemphasized.

I am also indebted to my co-supervisor, Dr. Roziahanim Mahmud for her unflinching interest in the progress of my research and constant encouragement and invaluable advises she has offered me. Her readiness to offer any help she can is overwhelming and I am grateful for that.

My appreciation goes to Dr. Yam Mun Fei for his guidance in the initial stages of my candidature. Thank you for the advices and hospitality you espoused. A name that cannot be left out is Dr. Abubakar Kabiru. I will like to thank him for his benign worm reception upon our arrival, his timely advices and all the help he renders. Thank you for providing the convivial atmosphere you espoused in the lab. My appreciation also goes to all my lab colleagues; Nasiba Usman Salisu, Adlin Yussof, Yasser Tabana, Fatim Khairudeen, Dr. Tri widyati, Abdulmunem Bakkouri, Majed Al-mansoubb, Bassel Al-Hindi, Raghdaa Al-zouhri, Mir Reza and Faramarz for their support during my candidature.

I am much grateful to my respected elder brother, Alh. Usman Salisu Zuru for his moral and financial support throughout my studies. This will not have been possible without his vehemence and passion for education and his willingness to make the selfless sacrifices needed to empower these under his wing. I will forever be grateful.

Last but not the least are the most influential people in my life, my parents; for the trouble, patience, encouragement, sacrifice, moral lessons and education they have strived to impact on my life cannot be overemphasize or express with words. Thank you for your guidance throughout my life and may Allah reward you with Jannatul Firdaus.

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March
2016

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LIST OF ABBREVIATIONS

%	Percent
(GPx)	Glutathione peroxidase
±	Plus minus
°C	Degree celsius
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
5-HT	5-hydroxy tryptamine
ACE	Angiotensin-converting enzyme
Ach	Acetylcholine
AChE	Acetylcholinesterase
ADH	Antidiuretic hormone
ADV	Adenovirus
AMP	Adenosine Monophosphate
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANP	Arterial natriuretic peptide
ASME	Methanol extract of *Alstonia scholaris*
ASWE	Water extract of *Alstonia scholaris*
AT-1	Angiotensin type I receptor
ATP	Adenosine triphosphate
ATPase	Adenyl triphosphatase
AV	Atrioventricluar
AVP	Arginine vasopressin
BK_{Ca}	Large-conductance calcium-sensitive potassium channel
BP	Blood pressure
Ca^{2+}	Calcium ion
CAT	Catalase
cGMP	Cyclic guanosine monophosphate

CHD	Coronary heart disease
CHF	Congestive heart failure
Cl^-	Chloride ion
Cl_{Ca}	Calcium-activated chloride channel
Cl_{VR}	Volume-regulated chloride channel
cm	Centimeter
CNS	Central nervous system
CO	Carbon monoxide
COX	Cyclooxygenase enzymes
CVD	Cardiovascular disease
d	Day
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DCF-ASME	Dichloromethane fraction of Methanol extract of *Alstonia scholaris*
DG	diacylglycerol
DMPP	1,1-dimethyl-4-phenylpiperazinium iodide
DNA	Deoxyribonucleic Acid
EAF-ASME	Ethyl acetate fraction of *Alstonia scholaris* methanol extract
EC	Endothelium cell
EC_{50}	Median effective concentration
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxation factor
eNOS	Endothelial nitric oxide synthase
et al.	And others
ET	Endothelin
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
F	Fraction
g	Gram
GC	Gas chromatography
GIT	Gastrointestinal tract

GMP	Guanosine Monophosphate
GSH	reduced glutathione form
GSSG	oxidized glutathione
h	Hour
H_2O_2	Hydrogen peroxide
HbO_2	Oxyhemoglobin
HO	hemeoxygenases
$HO^{\bullet}$	Hydroxyl racial
$HO_2\bullet$	hydroperoxyl radical
HOCl	Hypochlorous acid
HPLC	High-performance liquid chromatography
HR	Heart Rate
HSV	Herpes simplex virus
i.p.	Intraperitoneal
i.v.	Intravenous
IK_{Ca}	Intermediate-conductance calcium-sensitive potassium channel
iNOS	Inducible nitric oxide synthase
IP	PGI_2 receptor
IP_3	1, 4, 5-inositoltriphosphate
K^+	Potassium ion
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Calcium-activated potassium channel
kg	Kilogram
K_{IR}	Inward rectifier potassium channel
K_{to}	Transient outward current K^+ channel
K_V	Delayed rectifier potassium channel
L	Liter
L-NAME	N^{ω}-nitro-L-arginine methyl ester
m	Meter
M	Molar
MAP	Mean arterial pressure
mg	Milligram

MI	Myocardial infarction
mL	Milliliter
N_2O_3	Dinitrogen trioxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	nitric oxide synthase
OLF	Oubain-like factor
OONO-	Peroxynitrite
PGI_2	Prostacyclin
PNS	Parasympathetic nervous system
RAAS	Renin Angiotensin aldosterone system
RNS	Nitrogen species
RO_2•	Peroxyl
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SMCs	Smooth Muscle Cells
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
T80	Tween 80
TPR	Total Peripheral Resistance
TXA2	Thromboxane
VSM	Vascular Smooth Muscle
VSMC	Vascular Smooth Muscle Cells

PENILAIAN AKTIVITI KARDIOVASKULAR DAN KAJIAN KETOKSIKAN EKSTRAK KULIT KAYU *ALSTONIA SCHOLARIS*

ABSTRAK

Tujuan kajian ini adalah untuk memeriksa kesan antihipertensi ekstrak kulit kayu *Alstonia scholaris* (pulai) dan mekanisme tindakan farmakologinya. Pengekstrakan berturut-turut telah dijalankan untuk mendapatkan ekstrak metanol (ASME) dan air (ASWE) yang diperolehi selepas penyahlemakan dengan eter petroleum. Kesan antihipertensi ekstrak ini telah dinilai pada tikus hipertensi spontan (SHR). Pemberian ASME secara oral setiap hari (1000 mg/kg selama 2 minggu) mempamerkan penurunan ketara tekanan darah ($p < 0.05$) tikus berbanding SHR kawalan. Melalui teknik fraksinasi cecair-cecair, ASME telah difraksikan berturut-turut menggunakan diklorometana, etil asetat dan n-butanol. Semua fraksi (0.125 – 4 mg/mL) menunjukkan vasorelaksi bergantung dos pada sediaan cincin aorta yang telah diprakontraksikan dengan fenileferina (PE; 1 μM) atau kalium klorida (80 mM). Fraksi n-butanol (NBF-ASME) merupakan fraksi yang paling poten ($R_{max} = 106.4 \pm 0.045\%$). Prainkubasi cincin aorta dengan NBF-ASME (0.5, 1 dan 2 mg/mL) merencat tindak balas kontraksi cincin aorta yang diaruhkan PE dengan ketara ($p < 0.05$-0.001). Pembuangan endotelium dan inkubasi dengan L-NAME, indometasin, atropina dan propranolol tidak menjejaskan dengan ketara relaksi oleh NBF-ASME. Tambahan pula, penghalang saluran K^+, TEA menunjukkan sedikit aktiviti perencatan manakala glibenklamida tidak menunjukkan kesan perencatan. Walau bagaimanapun, cincin aorta yang diprarawat dengan ODQ menunjukkan perencatan keluk relaksi NBF - ASME dengan ketara ($p < 0.01$-0.001). Dalam larutan bebas Ca^{2+}, NBF-ASME merencat pembebasan Ca^{2+} dalam sel dari retikulum

sarkoplasmik. NBF-ASME juga merencat kontraksi cincin aorta tidak berendotelium aruhan-$CaCl_2$ bergantung kepada kepekatan. Keputusan percubaan menunjukkan bahawa *A. scholaris* bertindak dengan menghalang saluran kalsium, mengaktifkan secara langsung enzim guanilat siklase dan mungkin juga merencat pembentukan inositol 1,4,5–trifosfat. Ketoksikan akut dan subakut ekstrak metanol *A. scholaris* (ASME) turut dinilai. Dalam kajian ketoksikan akut, dos tunggal ASME 2,000 mg/kg didapati tidak toksik. Manakala dalam kajian ketoksikan subakut tikus SD dari keuda-dua jantina telah diberikan tiga dos ASME (250, 500 dan 1000 mg/kg/hari) selama 28 hari. ASME 250 mg/kg tidak menunjukkan sebarang perbezaan yang signifikan dalam semua parameter berbanding dengan tikus kawalan. Beberapa perubahan ketara dari segi berat badan, hematologi dan parameter biokimia diperhatikan berlaku pada kumpulan tikus percubaan pada dos 500 dan 1000 mg/kg dengan dua kematian berlaku pada dos yang tertinggi. Kajian histopatologi menunjukkan berlaku sedikit degenerasi (lesion) dan nekrosis sentrilobular pada hati. Keputusan ini menunjukkan bahawa pemberian ASME secara oral setiap hari pada dos 500 mg/kg keatas adalah toksik dan mungkin fatal kepada haiwan terutamanya kerana kerosakan hati.

EVALUATION OF THE CARDIOVASCULAR ACTIVITY AND TOXICITY STUDY OF *ALSTONIA SCHOLARIS* BARK EXTRACTS

ABSTRACT

The aim of the present study was to investigate the antihypertensive effect of *Alstonia scholaris* (pulai) bark extracts and its pharmacological mechanism of actions. Successive extraction was carried out to obtain methanol (ASME) and water (ASWE) extract of the bark after defatting with petroleum ether. The antihypertensive effect of these extracts were evaluated on spontaneous hypertensive rats (SHR). Daily oral administration of ASME (1000 mg/kg for 2 weeks) exhibited a significant decrease in the blood pressure ($p < 0.05$) of the rats compared to control SHR. By means of liquid-liquid fractionation technique, the aqueous ASME solution was successively fractionated using dichloromethane, ethyl acetate and *n*-butanol. All the fractions (0.125 – 4 mg/mL) gave a dose-dependent vasorelaxation on aortic ring preparations pre-contracted with phenylephrine (PE; 1 μM) or potassium chloride (80 mM). The n-butanol fraction (NBF-ASME) was the most potent fractions (R_{max} = 106.4 ± 0.045 %). Pre-incubation of aortic rings with NBF-ASME (0.5, 1 and 2 mg/mL) significantly inhibit the contractile response of the aortic rings to PE-induced contraction ($p<0.05$-0.001). Removal of endothelium and incubation with L-NAME, indomethacin, atropine, and propranolol did not significantly affect the relaxation effect of NBF-ASME. Furthermore, of the K^+ channel blockers, TEA showed slight inhibitory activity while glibenclamide showed no inhibitory effect. However, aortic rings pretreated with ODQ showed a significant suppression of the relaxation curve of NBF-ASME ($p<0.01$-0.001). In Ca^{2+}-free solution, NBF-ASME inhibits the release of intracellular Ca^{2+} from the sarcoplasmic reticulum. NBF-ASME also inhibits $CaCl_2$-induced contraction in endothelium-denuded aortic rings in a

concentration-dependent manner. The result suggested that *A. scholaris* act via blocking calcium channels, direct activation of soluble guanylate cyclase and possibly by also inhibiting the formation of inositol 1, 4, 5-triphosphate. The acute and subacute toxicity of methanol extract of *A. scholaris* (ASME) was also evaluated. In the acute toxicity study, single dose of ASME 2,000 mg/kg was found to be non-toxic. In subacute toxicity study, SD rats of either sex were administered three doses of ASME (250, 500 and 1000 mg/kg/day) for 28 days. ASME 250 mg/kg, did not produce any significant difference in all the parameters when compared to control rats. Some significant changes in body weight, hematological and biochemical parameters were observed in experimental groups of rats at the dose of 500 and 1000 mg/kg with two animals died at the highest dose. Histopathological study revealed slight degeneration (lesion) and centrilobular necrosis in the liver. These results demonstrated that daily oral administration of ASME at 500 mg/kg and above is toxic and may be fatal to the animal primarily due to liver damage..

CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview of the Cardiovascular System

The cardiovascular system is the body's transport system through which substances are conveyed and distributed from one part of the body to another via the circulation of the blood. The cardiovascular system, also referred to as circulatory system plays a vital role in sustaining life. It is responsible for the distribution of oxygen and nutrients to cells while removing carbon dioxide and other nitrogenous waste product. Its proper functioning is also responsible for maintaining an optimum pH, and the mobility of the elements, proteins and cells of the immune system.

1.2 Components of the Cardiovascular System

The cardiovascular system consists of the heart which serves as the pump, the arteries, veins and the capillaries that serve as the conducting tubes (blood vessels) and the circulating blood.

1.2.1 The Heart

The human heart, a hollow organ with a muscular structure, is situated slightly to the left of the thoracic cavity and is roughly the size of a man's closed fist. It is responsible for propelling blood through the blood vessels by repeated contractions and relaxation synchrony. The heart is composed of a unique type of an involuntary muscle tissue called cardiac muscle, that is found only in this organ (Guyton and Hall, 2006). It is comprised of two pairs of chambers on the left and right side. Each side consists of an upper chamber known as atrium (plural; atria) and a lower chamber known as ventricle (plural; ventriculus) and functions as an independent pump. The two pumps are separated by a thick muscular

wall called septum which prevents the mixing of blood in the left and right sides of the heart. Connecting the atriums and ventricles are atrioventricular openings with valves that allow the passage of circulating blood from the atriums to the ventricles (Figure 1.1). The valves in the right atrium are referred to as tricuspid valves while those of the left are known to as bicuspid valves (Saladin, 2003).

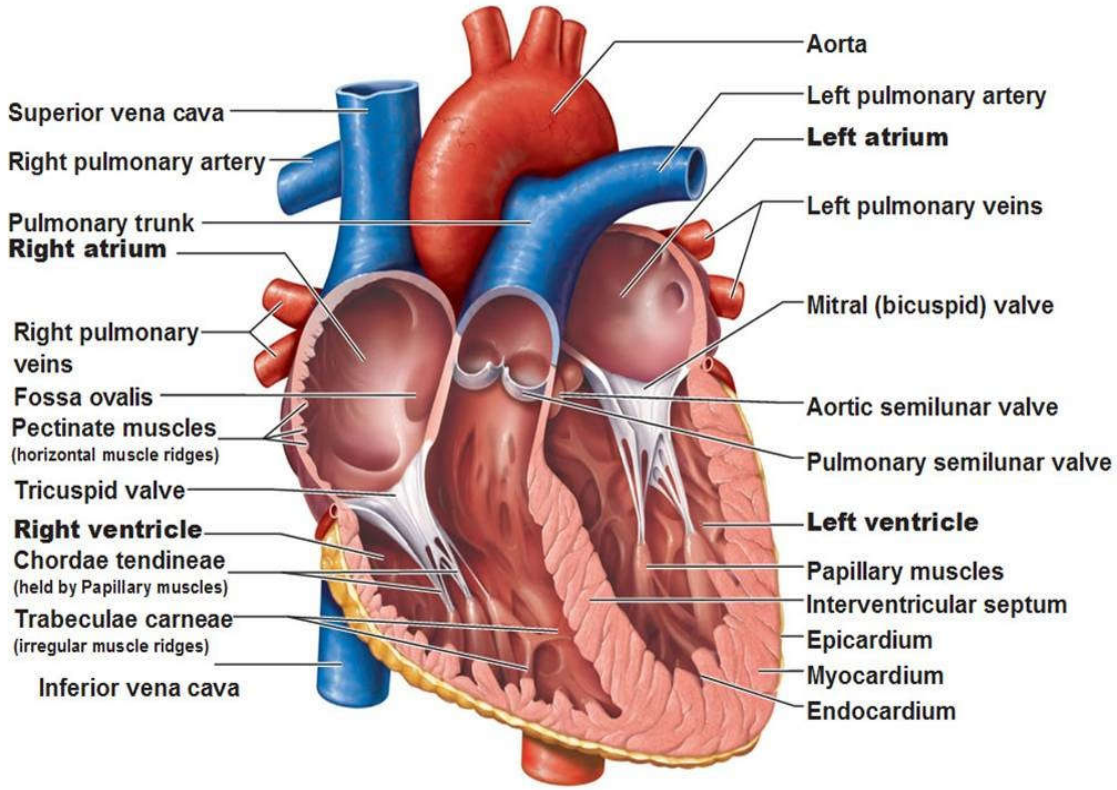


Figure 1.1: Anatomy of the frontal section of the heart's cross-sectional view (Diagram adapted from McGraw-Hill companies.inc).

The atrioventricular valves ensure the one-directional flow of blood from the atriums into the ventricles, thereby avoiding the backflow of circulating blood (Pappano and Wier, 2013). The right atrium receives blood coming into the heart from the blood vessels via the superior

and inferior vena cava and the left ventricle pumps blood out from the heart via the pulmonary arteries into the blood vessels.

1.2.2 Conducting fluid (Blood)

Blood consists of cellular elements (red blood cells, white blood cells, and platelets), plasma as well as the fluid in which the blood cells are suspended. The total normal circulating blood volume is approximately 8% of body weight (Guyton and Hall, 2006). Red blood cells are the most numerous cellular elements in the blood. They are produce from the bone marrow of the femur and are biconcave in shape. An integral structural component of the red blood cell is the hemoglobin, which confers on it the ability to bind oxygen molecules for transportation. The fluid portion of the blood, the plasma, is about 90% water and accounts for 55 to 60% of total blood volume. Proteins content of the blood is about 10% while other substances including hormones, enzymes, nutrient molecules, gases, electrolytes, and excretory products covers the remaining 2%. All of these substances are dissolved in the plasma (e.g., oxygen) or are colloidal materials (dispersed solute materials that do not precipitate out, e.g., proteins) (Tortora and Darrickson, 2006).

1.2.3 Blood Vessels

The blood vessels serve as the conducting tubules of the cardiovascular system. They form a close network of tubes, conveying the blood to all parts of the body organs in a unidirectional manner (Guyton and Hall, 2006).

1.2.3.1 Structure of blood vessels

A typical blood vessel consists of three layers of membrane tissues adapted for their biological function (Figure 1.2). The internal layer of tissue, called the tunica intima,

comprises of single layer of simple squamous epithelial cells with a small amount of supporting connective tissue. The middle layer of tissue, the tunica media, is made up of smooth muscle organized in a circular pattern with different layers of elastic tissue. The outer region of the blood vessel is made of collagen and elastic fibers and is called the tunica adventitia. Both arteries and veins have all three layers of tissue. However, the proportions of the three layers between arteries and veins differ dramatically (Saladin, 2003).

1.2.3.2 Arteries and Arterioles

Arteries are muscular blood vessels that convey both oxygenated and deoxygenated blood away from the heart. Blood flows from the right atrium into the right ventricle of the heart. From there it is pumped through the pulmonary arteries. The pulmonary arteries carries deoxygenated blood to the lungs where it is oxygenated. The oxygenated blood from the lungs returns into the left atrium of the heart through the pulmonary vein and advance to enter the right ventricle. The oxygenated blood from the right ventricle is pumped into large arteries called aorta which conveys the blood to the rest of the body. Arteries have a thick elastic wall that enable them withstand high pressure blood coming from the heart.

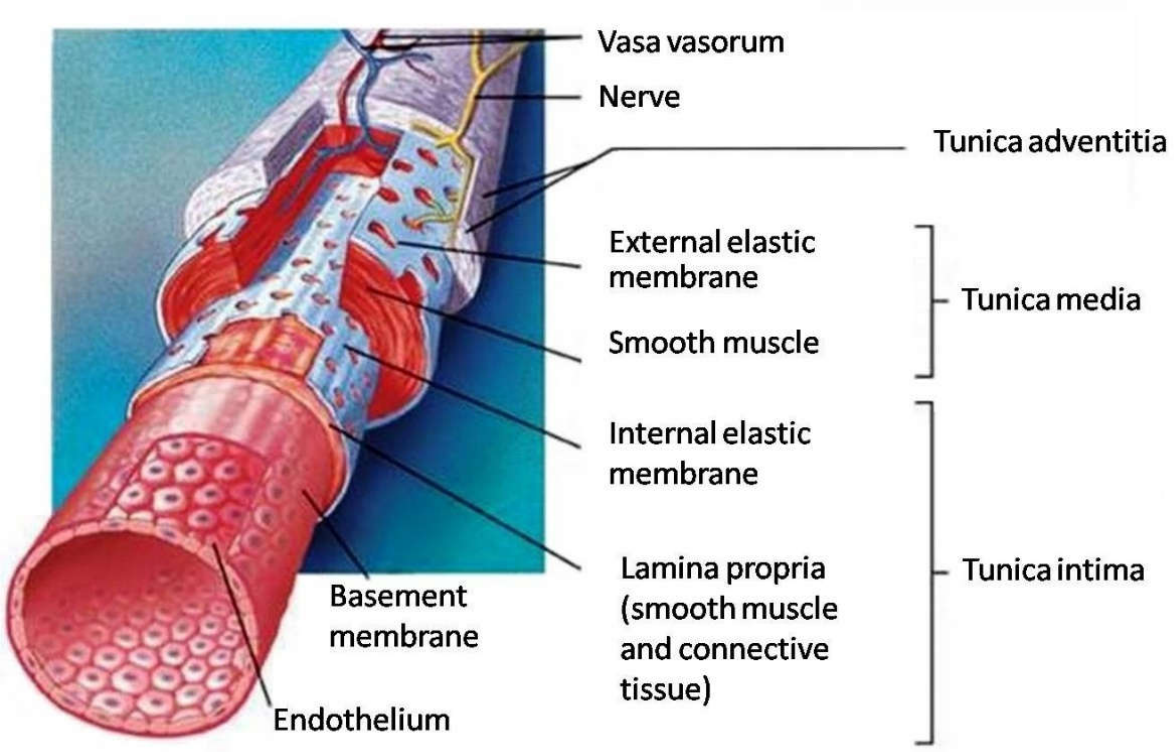


Figure 1.2: Structure and the layers of a typical blood vessel in the circulatory system (Diagram adapted from McGraw-Hill companies.inc).

An arteriole is a small artery that extends and leads to capillaries. Arterioles have thick smooth muscular walls. These smooth muscles are able to contract (causing vessel constriction) and relax (causing vessel dilation). This contracting and relaxing affects blood pressure; the higher number of vessels dilated, the lower blood pressure will be. Arterioles are just visible to the naked eye (Pappano and Wier, 2013).

1.2.3.3 Capillaries

Capillaries are the smallest of a body's vessels in the circulatory system; they connect arteries and veins, and most closely interact with tissues. They are very widespread in the body and have a total surface area is approximately 6,300 square meters (Wang and Xing, 2010). Because of this, no cell is more than 50 micrometers away from capillaries. The walls of

capillaries are composed of a single layer of endothelium cells, which is lines the inner wall of all the vessels. Due to the very thin layer, molecules such as oxygen, water and lipids can move across the tissues by the process of diffusion. Waste products such as carbon dioxide and urea can diffuse back into the blood to be carried away for removal from the body (Masuda et al., 2012).

1.2.3.4 Veins and venules

Veins are the vessels that carry blood from the capillaries back into the heart. The small veins are called venules. The venules form the initial proximal part of the veins adjoined with the capillaries end. The venule branches link to form larger veins which eventually join to form the vena cava, the largest vein that supplies the heart.

Structurally, the vein have the same three tissue layers as present in the arterial walls, however there are minor differences when compared to the arterial layers (Lawson and Weinstein, 2002). The inner endothelium layer of veins is smooth with protrusions at intervals that folded to form valves. The valves function to prevent backflow of blood and are more numerous in veins of the legs, where blood must often return to the heart against the gravitational force (Golledge and Quigley, 2003; Rutherford et al., 2000). The middle layer of veins consists of a thin layer of smooth muscle opposed to the thick layer found in arteries. It is thin because veins are less involve in the regulation of blood pressure and blood flow compare to the arteries. The outer layer of veins is also thin; not as much fibrous connective tissue is necessary because blood pressure in veins is very low (Scanlon and Sanders, 2007).

1.2.4 Vascular Smooth Muscle Cells (VSMC).

The vascular smooth muscle cell (VSMC) forms an integral structural element of the blood vessels essentially involve in the regulatory processes of the vascular system (Aziz et al., 2014). Smooth muscle arises from the mesenchyme at various sites in the embryo where smooth muscle ordinarily will occur in the adult (Furchgott, 2013; Worth et al., 2001). The undifferentiated cells in these areas continue to divide until they become confluent, after which individual cells, the myoblasts, differentiate. The myoblasts elongate and take on the fusiform shape characteristic of adult smooth muscle cells (Guo and Chen, 2012; Krause, 2005). Smooth muscle cells are typically spindle shaped and vary in size from 20 μm (small blood vessels) to 400–500 μm (uterus), depending on site in the body. Each cell has a single, centrally located nucleus which is elongated or elliptical in shape. The VSM provides strategic vicinity in the blood vessels upon which both external and internal blood pressure regulatory factors exert their organic function. Impairment in this structure affects the overall cardiovascular system and is associated with numerous cardiovascular disease and dysfunction (Al-Sadi, 2012).

1.2.4.1 Physiological Function of VSMC

The vascular smooth muscle cell (VSMC) in mature animals is a highly specialized cell that perform the principal function of regulating the of blood vessel tone-diameter, blood pressure, and blood flow distribution (Aziz, et al., 2014). The smooth muscle cells (SMCs) constitute the predominant cellular component of the vascular media which respond to both external and internal physiological or pharmacological stimuli leading to vasoconstriction or dilation, syntheses of extracellular matrix (collagen, elastin and proteoglycans), elaboration and migration of growth factors and cytokines into the intima and cell proliferation and

regeneration after vascular injury (Curcio et al., 2011; Rzucidlo et al., 2007). These activities of SMCs are important in both normal vascular repair and pathological processes. The vascular system immensely depends on the modulation of the SMCs vasculature tone.

1.2.4.2 The Contractile Elements in Smooth Muscles

Over the past decade, an acceptable understanding on the structural components involved in the contractile process of smooth muscle has been arrived at. Pile of experimental evidence has identified three major structural protein elements that are involve in the contractile process of the smooth muscle. The components include two types of filaments.

1.2.4.2.1 Thick filaments: Myosin and the cross-bridge formation

Myosin is the main protein of smooth muscle thick filaments. Each thick filament is made up of more than 200 myosin molecules. These are fibrous structures with globular ends. Myosin is composed of two heavy chain subunits, each about 200 kDa. The heavy subunits have two types of light chain subunits (20 and 17 kDa, respectively) protruding at given intervals. Phosphorylation of the 20 kDa light chain is vital in the initiation of the contractile process; the 17 kDa light chain function is not as clearly defined (Levine et al., 2001).

The SMCs motor response is controlled by the sliding of myosin and actin filaments over each other (Cooke, 2004). The energy requirement for this process is provided by the hydrolysis of ATP. ATP is utilized by the myosin filament to cause molecular conformational changes in its own structure, which in turn facilitate its interaction with actin filaments, leading to the formation of cross-bridges (Hartel et al., 2007; Matsumura et al., 1999). The cross-bridge occurs when the extended globular heads in the myosin filaments interact with actin filaments. These heads lean on the actin filament and drag along to produce contraction.

The cross-bridges is halted when the actin and myosin filament detached from one another and returning to their original conformation which result in relaxation. This process is known as cross-bridge cycling and is considered to be common to all smooth muscles (Smolock et al., 2007).

1.2.4.2.2 The Thin Filament: Actin and Tropomyosin

Actin, which is the major component of the thin filament, is found in all mammalian cells. The chemical composition of actin from different sources may differ slightly. Actin is a fibrous molecule that forms the thin filament and is composed of two strands of 42 kDa globular actin molecules. The two functions of actin in the contractile process are: (1) transmission of force by the movement of the bridge of the myosin molecule against the actin and (2) activation of the myosin ATPase to generate free energy required for contraction (Regnier et al., 2004). Like actin, tropomyosin is a fairly ubiquitous molecule in all types of muscle with a molecular weight of about 70 kDa and is an alpha helix that is closely attached to the actin molecule in the thin filament. Furthermore, the ratio of tropomyosin to actin molecules (approximately 1:3) is similar in all types of muscle (Marston and El-Mezgueldi, 2008).

1.2.4.3 Excitation-Contraction Coupling in Smooth Muscle

The mechanism of excitation-contraction coupling in smooth muscle differs from that of skeletal muscle. While in skeletal muscle binding of actin and myosin is permitted when Ca^{2+} binds troponin C; in smooth muscle, there is no troponin. Rather, the interaction of actin and myosin is controlled by the binding of Ca^{2+} to another protein, calmodulin. In turn, Ca^{2+}-

calmodulin regulates myosin-light-chain kinase, which regulates cross-bridge cycling (Costanzo, 2013).

1.2.4.3.1 Smooth Muscle Contraction

The steps involved in excitation-contraction coupling in smooth muscle bring about the contraction and relaxation of the smooth muscle cells and occur as follows:

- Depolarization of smooth muscle opens voltage-gated Ca^{2+} channels in the sarcolemmal membrane. With the Ca^{2+} channels open, Ca^{2+} currents pass into the cell down its electrochemical gradient. This influx of Ca^{2+} from the extracellular fluid (ECF) causes an upsurge in intracellular Ca^{2+} concentration. In contrast to skeletal muscle, where action potentials are required to produce contraction, in smooth muscle, sub-threshold depolarization (which does not lead to an action potential) can open these voltage-gated Ca^{2+} channels and cause a rise in intracellular Ca^{2+} concentration. If the depolarization of the smooth muscle membrane reaches threshold, then action potentials can occur, causing even greater depolarization and even greater opening of voltage-gated Ca^{2+} channels.
- Ca^{2+} that enters the smooth muscle cells through voltage-gated Ca^{2+} channels and stimulate the releases of additional Ca^{2+} from the sarcoplasmic reticulum (SR; called Ca^{2+}-induced Ca^{2+} release). Consequently, the increase in intracellular Ca^{2+} is partly due to Ca^{2+} entry across the sarcolemmal membrane through either the voltage-gate and partly due to the release of Ca^{2+} from the intracellular SR stores.
- Two additional mechanisms may contribute to the increase in intracellular Ca^{2+} concentration: ligand-gated Ca^{2+} channels and inositol 1,4,5-triphosphate (IP_3)–gated Ca^{2+} release channels on the plasma membrane of SR. Ligand-gated

Ca^{2+} channels in the sarcolemmal membrane may be opened by various hormones and neurotransmitters, permitting the entry of additional Ca^{2+} from the ECF. IP3-gated Ca^{2+} release channels in the membrane of the sarcoplasmic reticulum may be opened by hormones and neurotransmitters. Either of these mechanisms may augment the rise in intracellular Ca^{2+}concentration caused by depolarization.

- The rise in intracellular Ca^{2+} concentration causes Ca^{2+} to bind to calmodulin. Like troponin C in skeletal muscle, calmodulin binds four ions of Ca^{2+} in a cooperative fashion. The Ca^{2+}-calmodulin complex binds to and activates myosin-light-chain kinase.
- The myosin-light-chain kinase, when activated, phosphorylates myosin light chain. When myosin light chain is phosphorylated, the conformation of the myosin head is altered, greatly increasing its ATPase activity. (In contrast, skeletal muscle myosin ATPase activity is always high.) The increase in myosin ATPase activity allows myosin to bind to actin, thereby initiating cross-bridge cycling and production of tension. The amount of tension is proportional to the intracellular Ca^{2+} concentration.
- Ca^{2+}-calmodulin, in addition to the effects on myosin described earlier, also has effects on two thin filament proteins, calponin and caldesmon. At low levels of intracellular Ca^{2+}, calponin and caldesmon bind actin, inhibiting myosin ATPase and preventing the interaction of actin and myosin. When the intracellular Ca^{2+} increases, the Ca^{2+}-calmodulin complex leads to phosphorylation of calponin and caldesmon, releasing their inhibition of myosin ATPase and facilitating the formation of cross-bridges between actin and myosin.

1.2.4.3.2 Smooth muscle relaxation

Relaxation of smooth muscle occurs when the intracellular Ca^{2+} concentration falls below the level needed to form Ca^{2+}-calmodulin complexes. A fall in intracellular Ca^{2+} concentration can occur by a variety of mechanisms including hyperpolarization (which closes voltage-gated Ca^{2+} channels); direct inhibition of Ca^{2+} channels by ligands such as cyclic AMP and cyclic GMP; inhibition of IP_3 production and decreased release of Ca^{2+} from sarcoplasmic reticulum; and increased Ca^{2+} ATPase activity in sarcoplasmic reticulum. Additionally, relaxation of smooth muscle can involve activation of myosin-light-chain phosphatase, which dephosphorylates myosin light chain, leading to inhibition of myosin ATPase (Figure 1.3).

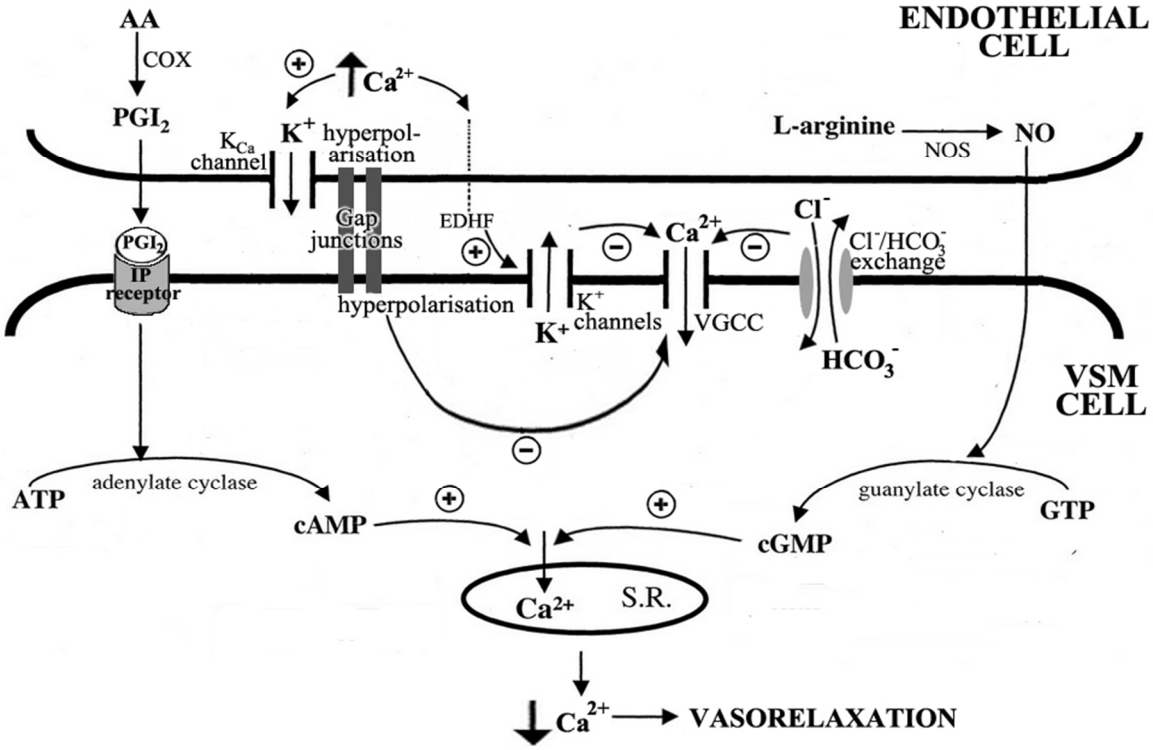


Figure 1.3: Schematic illustration of the mechanisms contributing to vasorelaxation of vascular smooth muscle (VSM) cells. In VSM, hyperpolarisation causes closure of VGCC, decreasing intracellular Ca^{2+} and eliciting vasorelaxation. Various mechanisms lead to hyperpolarisation of VSM, including efflux of K^+ through VSM K^+ channels and EDHF. The EDHF pathway consists of an initial increase in intracellular calcium in endothelial cells which causes VSM hyperpolarisation via various pathways, including transfer of hyperpolarisation via gap junctions, or release of diffusible factors from endothelium which activate BKCa and K_{ATP} channels on VSM. NO and PGI_2, are produced in endothelial cells before diffusing to adjacent smooth muscle cells to increase cGMP and cAMP levels, respectively, thus causing vasorelaxation. Activation of Cl-/HCO3- exchange on VSM causes intracellular acidification, which reduces the open probability of VGCC, causing vasorelaxation. [AA, arachidonic acid; VSM, vascular smooth muscle; VGCC, voltage-gate calcium channels; EDHF, endothelial derived hyperpolarizing factor; NO, nitric oxide; PGI_2, prostacyclin; NOS, nitric oxide synthase; COX, cyclo-oxygenase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; ATP, adenosine triphosphate VSM; vascular smooth muscle].

1.3 Regulation of blood pressure

The multivariable mechanisms controlling the blood pressure can be categorized into intrinsic and extrinsic control mechanisms, which can be further subdivided into local control mechanisms and hormonal control mechanisms. Cardiac performance and vascular tone at any time are the result of the integration of all the control mechanisms. To some extent, this categorization can be described as artificial because each categories affects the other.

1.3.1 Extrinsic Mechanisms

The extrinsic control systems consist of the sympathetic nervous system (SNS), parasympathetic nervous system (PNS), and the circulating hormones which contribute to the regulation of BP and blood volume.

1.3.1.1 Nervous System

The nervous system plays a vital role in the short-term regulation of blood pressure BP. The BP regulatory center is located in the medulla. Previous findings have shown that autonomic innervations of blood vessels provide a tight regulation of blood pressure within a narrow range (Joyner et al., 2008). Blood vessels are primarily innervated with sympathetic nerve fibers while the heart receives both sympathetic and parasympathetic innervations (Thomas and Segal, 2004). Activation of the sympathetic nervous system (SNS) leads to an increase in the vascular smooth muscle (VSM) tone resulting in vasoconstriction of both the resistance and capacitance vessels. This leads to an increase in total peripheral resistance (TPR). SNS activation also increases the heart rate (HR) and force of contraction, and also causes renin release (McCorry, 2007). Current researches have indicated hyperactivity of the SNS as a

possible index and pathogenesis on the development of essential hypertension (Svarstad et al., 2001) and other cardiovascular diseases (Malpas, 2010; Parati and Esler, 2012).

1.3.1.2 Humoral Systems

The humoral system regulates BP mainly through the renin angiotensin aldosterone system (RAAS), and other mediators described below.

1.3.1.2.1 Renin Angiotensin Aldosterone System

RAAS is an endocrine system that plays an integral role in controlling the hemodynamic state of the body and arterial blood pressure primarily via the regulation of body fluid. Changes in the pump function, coronary blood flow and systemic arterial compliance underlie the role of the RAAS on the cardiovascular system in maintaining circulatory integrity (Brewster et al., 2003). Decrease in renal perfusion in the kidney due to low BP leads to the release of the proteolytic enzyme called Renin from the juxtaglomerular cells (Atlas, 2007; Rao, 2010). Renin cleaves an inactive peptide angiotensinogen (alpha 2 globulin circulating in the blood) to derive angiotensin I (Ang I). Ang I is converted to angiotensin II (Ang II) by an enzyme found in lung capillaries called angiotensin-converting-enzyme (ACE). Ang II is an effective vasoconstrictor. Ang II acts as an agonist in an endocrine, paracrine, autocrine and intracranial fashion (Ellis et al., 2012). Ang II stimulates the secretion of aldosterone from the adrenal cortex. Aldosterone influences the kidney tubules to retain sodium and water. This increases the blood volume which contributes to an increase in BP. RAAS hormonal system responds to low blood volume or a fall in BP (Gwathmey et al., 2012).

1.3.1.2.2 Arginine Vasopressin System

Arginine vasopressin (AVP), also called antidiuretic hormone (ADH), is a nonapeptide protein which is derived from a prehormone synthesized in hypothalamus and stored in the posterior pituitary gland (Birnbaumer, 2000). AVP plays a pivotal role in the reabsorption of water from the collecting ducts in the kidney via activation of V_2 receptors (Nakamura et al., 2006). At pharmacological doses, AVP exerts a profound vasoconstriction by activating V_1 receptors found on vascular smooth muscle cells (VSMC). Stimulation of AVP release is triggered by an increase in osmolality (Robertson, 2006), rise in arterial blood pressure, fall in blood volume, and decrease in venous return .

1.3.1.2.3 Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is a 28 amino acid polypeptide hormone produced and secreted by cardiomyocytes of the atria of the heart (Horio et al., 2000; Kumar and Pandey, 2009). It is released in response to atrial stretch, stretching of the vessel walls, stimulation of beta receptors, hypervolemia, hypernatremia, elevation in Ang II and following exercise. It exerts both, a natriuretic and a vasodilatory effect. The overall effect of ANP is to counter RAAS mediated increase in BP (Park et al., 2015).

1.3.1.2.4 Oubain like Factor

Oubain-like factor (OLF) is an endogenous substance that is indistinguishable from the digitalis glycoside, oubain. It has been found circulating in blood (Manunta et al., 2001). This substance inhibits Na^+/K^+ activated ATPase and induces natriuresis and vasoconstriction.

OLF is released in response to high sodium intake, hypervolemia and in conditions of cardiovascular disease state such as hypertension (Manunta et al., 2009).

1.3.2 Intrinsic Mechanisms

Intrinsic system regulates blood flow mainly within the tissue/organ or remains restricted to specific regional vascular beds. This mechanism includes myogenic mechanism which refers to the property of the muscle tissue to respond to stretch. It is likely that metabolic and humoral mechanisms contribute to the regulation of blood flow as an intrinsic control system (Guyenet, 2006). In addition to the extrinsic and the intrinsic regulatory mechanisms, the endothelium is now widely recognized as a one of the major regulators of BP and blood flow. The endothelium can be considered as an endocrine organ in its own right and can be considered as a part of the humoral systems of regulation or as a separate system.

1.3.2.1 Endothelium

The endothelium is the thin layer of cells that forms the inner intima lining of the blood vessels (Aird, 2007a). Thus, the endothelium is an interface between the lumen and the rest of the vessel wall. These cells are present in the circulatory system, beginning from the heart to the smallest capillaries. Endothelial cells (EC) are specialized epithelium called simple squamous epithelium. EC are very important for the regulation of various functions such as vasodilatation, vasoconstriction, thrombosis, fibrinolysis, angiogenesis, inflammation and edema (Aird, 2007b). All of these factors contribute to the overall BP regulation by the endothelium. Transit of white blood cells into and out of the blood is controlled by the EC The endothelium synthesizes and secretes a number of vasodilator such as EDHF, PGI_2 and NO (Martin, 2009). Carbon monoxide (Thorup et al., 1999) and hydrogen sulfide (H2S)

(Yang et al., 2008) have also been added to the list of vasodilator mediators released from the endothelium, and vasoconstrictor; Endothelin (Sugden, 2003) and thromboxane A2 (Félétou et al., 2010). This vasoactive mediators play a role in the cardiovascular regulatory functions of endothelium. Thus, the endothelium plays an important role in the regulation of vascular tone (Sandoo et al., 2010) and by extension the regulation of blood pressure.

Moreover, other blood borne mediators and mediators released from nerves act on the endothelium, platelets and vascular smooth muscle to regulate vascular tone, platelet function, capillary permeability and other cardiovascular functions. These mediators include angiotensin II (Ang II), norepineprine, histamine, bradykinin, 5-hydroxytryptamine (5-HT) thromboxane and acetylcholine. In a physiological state there is a balance between these vasoconstrictor and vasodilator agents. In disease states such as hypertension and diabetes, this balance is disturbed due to altered bioavailability of one or more of the endothelial mediators such as NO (Rajendran et al., 2013). .

1.4 Phytochemical and pharmacological review of *Alstonia scholaris*

Alstonia scholaris (L.) R. Br., a genus of the family apocynaceae, is a glabrous, evergreen tree native of the Indian subcontinent and Southeast Asia. It has been well documented for its folklore uses in the prevention and treatment of various diseases (Baliga, 2012; Bhanu et al., 2013; Dey, 2011).

1.4.1 Plant Profile

Botanical name → *Alstoniascholaris*
Kingdom → Plantae
Order → Gentianale
Family →Apocynaceae
Genus → *Alstonia*
Species → *Alstonia scholaris*

Alstonia scholaris is widely used in traditional medicinal systems of India, Thailand, Malaysia, Philippines, Australia, China and Africa (Cunningham and Read, 2003; Laidlaw et al., 2007; Rai, 1994). The genus *Alstonia* comprises of more than 60 species all over the world.

Alstonia scholaris is known with numerous names such as devil's tree, white cheese wood, mill wood, milkwood pines, kilky pine, black board tree and dita bark (English). It was initially named *Echites scholaris* by Linnaeus in 1767 (Khyade et al., 2014). In the year 1811, the plant was renamed as Alstonia by Robert Brown in the memory of Professor Charles Alston. The name of species scholaris was derived from the use of its wood in making blackboards for schools in South East Asia (Baliga, 2012).

1.4.2 Morphological characteristics

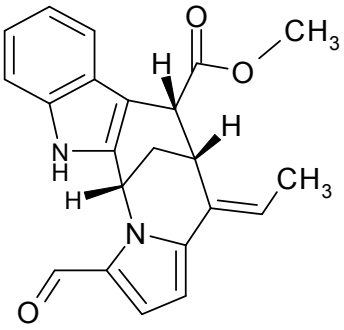
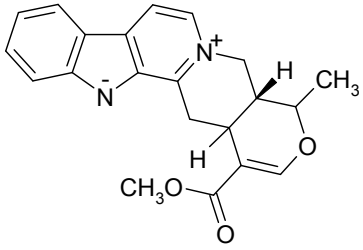
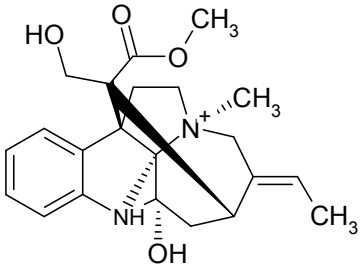
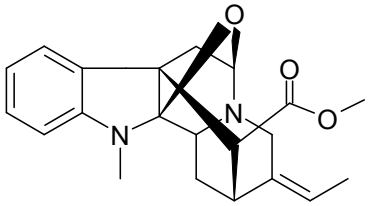
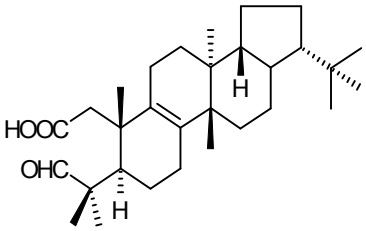
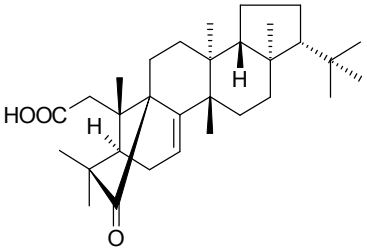
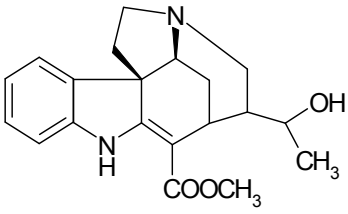
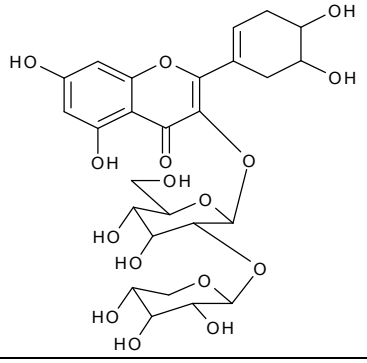
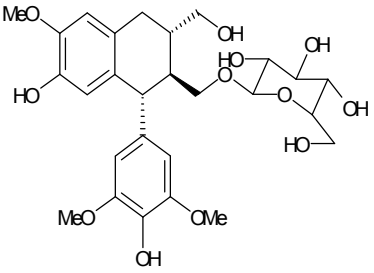
Alstonia scholaris is a medium to large tree of about 40 m height with somewhat tessellated corky grey to grey-white bark with bitter milky juice. The outer blaze is cream yellowish in color with abundant, milky latex that flows rapidly when cut. The Leaves are whorls of 4-8 in the upper axils, obtuse or sometimes shortly and bluntly acuminate, dark green above, pale and covered with a whitish bloom beneath (Figure 1.4). The Flowers are white, in umbellately branched many-flowered pubescent capitates cymes; peduncles 2.5-5 cm long; pedicels very short; bracts oblong, pubescent. Seeds are 6 mm long, linear, flattened, rounded and with a fringe of hairs at both ends, the hairs longer than the seed.

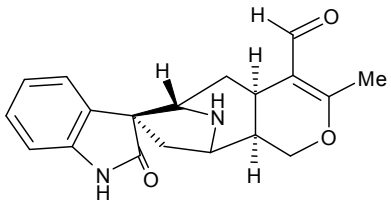
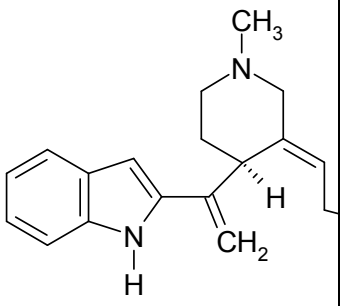
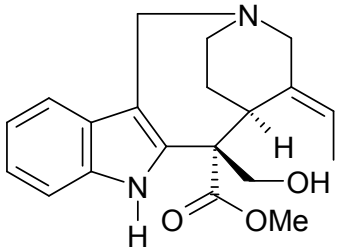
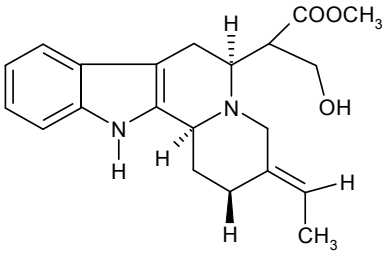
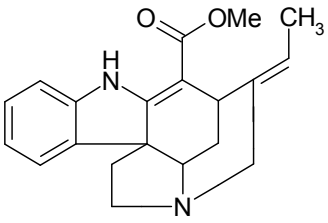
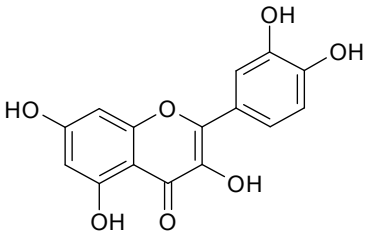
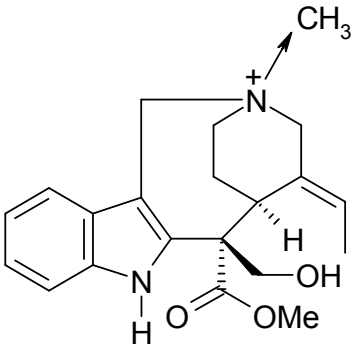
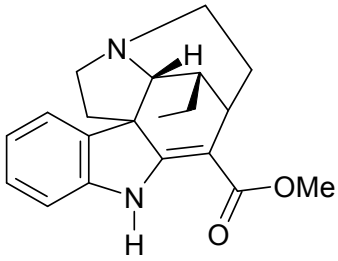
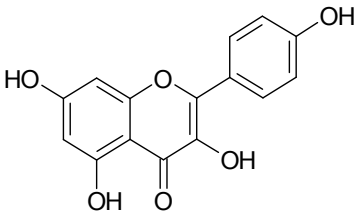
Figure 1.4: Depiction of *Alstonia scholaris*. (A) Leave (B) flower (C) the trunk Bark and (D) the fruit.

1.4.3 Phytochemical studies

Over the past decades, different extracts of *Alstonia scholaris* have been studied extensively for their numerous phytochemical constituents that play a crucial role in its pharmacological and medicinal properties. An estimate of more than four hundred important phytoconstituents have been isolated and characterized from different part of the plant. Alkaloids, iridoids, coumarins, flavonoids, leucoanthocyanines, reducing sugars, simple phenolics steroids, saponins and tannins were documented as the chief chemical constituents. *Alstonia scholaris* contains some of the important alkaloids such as echitamine, tubotaiwine (stem and root bark), akuammine (root bark) and so on (Khyade, et al., 2014; Pratap et al., 2013b).

Table 1.1: Chemical structures of some important phytochemical constituents isolated from *Alstonia scholaris* L.

E-Alstoscholarine 	Alstonine 	Echitamine 
Picrinine 	Alstonic acid A 	Alstonic acid B 
Scholaricine 	Quercetin3-O-β-D-xylopyranosyl (1→2)-β-D-galactopyanoside 	(-)-Lyoniresinol3α-O-β-D-glucopyranoside 

N(1)-demethylalstonal 	Manilamine 	Vallesamine 
Akuammidine 	Akuamicine 	Quercetin 
N^4-methyl angusilobine B 	20 (s)-tubotaiwine 	Kaempferol 

1.4.4 Ethnopharmacological properties

Alstonia scholaris has been widely used for treatment of a wide range of ailments and extensive pharmacological studies have been documented to explore, evaluate and validate its vast pharmacological potentials. The plant has been investigated for its anti-inflammatory and analgesic effects (Shang et al., 2010a); anticancer (Jagetia and Baliga, 2004a; 2006; Jahan et al., 2009; Manjeshwar Shrinath, 2010); antimalarial ; antimicrobial (Bonvicini et al., 2014; Mahapatra and Banerjee, 2010); antifungal and antiviral (Antony et al., 2014; Antony et al., 2012); antifertility (Gupta et al., 2002); antibacterial (Ganjewala and Gupta, 2013; Khan et al., 2003; Molly et al., 2014); anti-arthritic (Arulmozhi et al., 2011; 2014); antihyperglycemic (El-Askary et al., 2013; Khatun et al., 2009; Ragasa et al., 2013); antidiabetic and antihyperlipidemic (Arulmozhi et al., 2010b; Jong-Anurakkun et al., 2007); antidiarrhoeal and antispasmodic (Keawpradub et al., 1999; Shah et al., 2010); antimutagenic (Jagetia et al., 2003); anti-anxiety (Arulmozhi et al., 2008); Antineoplastic (Jagetia and Baliga, 2003a; 2005), anti-ulcerogeni (Ashok et al., 2006); wound healing (Ramachandra et al., 2012); nitric oxide scavenging (Jagetia and Baliga, 2004b); immunostimulating effect (Atmaram et al., 2012; Iwo et al., 2000); radioprotective (Gupta et al., 2008; Jagetia and Baliga, 2003b; Jahan and Goyal, 2010); antiaging effects on the skin (Lee et al., 2012; Saikia et al., 2006), anti-tussive, anti-asthmatic and expectorant activities (Shang et al., 2010b), hepatoprotective effect ; neuroleptic activity (Jash and Chowdary, 2014), broncho-vasodilatory activity (Channa et al., 2005). Kulkarni and Juvekar (2009) reported the anti-stress (adaptogenic) and nootropic activities of methanolic extract of bark of *Alstonia scholaris*. The antioxidant and free radical scavenging profile has also been documented (Arulmozhi et al., 2007; 2010c; Asadujjaman et al., 2013; Kumar et al., 2010).

Some phytochemical constituents isolated from *Alstonia scholaris* have been assayed for their pharmacological activity. Macabeo et al. (2008) studied the inhibitory effect of four alkaloids of *Alstonia scholaris* against myobacterium tuberculosis. Strictamine has been reported to have neuropharmacological and antidepressive effect (Bhattacharya et al., 1979). The allelopatic effect of pantacyclictriterpenoids such as betulinic acid, oleanolic acid, and ursolic acid has also been investigated (Wang et al., 2014). 17-nor-excelsinidine, a novel alkaloid isolated from the twigs and leaves of *Alstonia scholaris* alongside strictamine showed significant inhibitory activity against herpes simplex virus (HSV) and adenovirus (ADV) (Zhang et al., 2014). Echitamine from *Alstonia scholaris* has shown significant antitumor activity (Kamarajan et al., 1991). In Ayurveda, the bitter and astringent concoction of the leave is used as a herb for treating skin disorders, malarial fever, urticaria, chronic dysentery, diarrhea and snake bite. The bark contains alkaloids, ditamine, echitenine and echitamine and used to serve as an alternative to quinine. Bark is use as appetizer, laxative, anthelmintic and galactagogue. The tenders' leaves roasted and pulverized is also useful in ulcers with foul discharge (Ashok Kumar et al., 2014).

1.4.5 Rationale and objectives of the present study

Despite the large volume of pharmacological data on the medicinal properties of *Alstonia scholaris* available, many of the diseases treated traditionally using the plant have not been validated in the laboratory. This provide an opportunity to study the specie both pharmacologically and phytochemically. Therefore, ethnopharmacology can bridge the gap between the traditional use and actual pharmacological veracity and efficacy of the medicinal plant. The objectives of this research is outlined as follows;