Bioassay-guided isolation and biochemical characterisation of vasorelaxant compounds extracted from a Dalbergia species

Sharada Laidlay

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Thesis Abstract

Natural products have been the source of many of our successful drugs providing us with an unrivalled chemical diversity combined with drug-like properties. The search for bioactive compounds can be helped considerably by the phytotherapeutic knowledge held by indigenous communities. In this study solvent extracts from the bark of a Dalbergia species and used by a community in Borneo, will be investigated to isolate, identify and biochemically characterise compounds showing vasorelaxation.

At the core of this study is the hypertensive model, which uses phenylephrine precontracted rat aortic rings as a bioassay to identify solvent extracts, fractions and sub-fractions that cause relaxation. These fractions are generated using chromatographic techniques and solvent systems developed specifically for this purpose. Structural elucidation of the isolated compounds was undertaken by studying extensive data from UV, MS, 1D and 2D ^{1}H and ^{13}C NMR spectra. This study also undertook the pharmacological characterisation of the isolated compounds using the same bioassay together with enzyme and receptor inhibitors to identify the signalling pathways involved.

In this study the plant was identified as a Dalbergia species using DNA profiling techniques and in particular sequences from the *matK* and *rpoC1* barcoding genes to construct a phylogenetic tree. Solvent extracts of the bark showed the presence of compounds that caused both vascular relaxation and contraction. From the hexane extract which showed only relaxation two related bioactive compounds were isolated and identified as 5,7-dihydroxy, 6, 2',4',5'-tetramethoxy isoflavone or Caviunin (S/F4) and its isomer 5,7-dihydroxy, 8, 2',4',5'-tetramethoxy isoflavone or Isocaviunin (S/F3) not previously investigated for there vascular activity.

Both these compounds showed an endothelium and concentration-dependent relaxation of rat aortic rings with $EC_{50}=1.8x10^{-5} \pm 1.898 \times 10^{-6}$ M and relaxation at 10μM of 53.2±6.4% for caviunin and $EC_{50}=1.669x10^{-6} \pm 1.53x10^{-7}$ M and relaxation at 10μM =73.9±7.3% for isocaviunin. The vasorelaxation of these compounds was abolished in the presence of NOS inhibitor L-NAME and soluble guanylyl cyclase inhibitor ODQ. Tetraethylammonium, the non-selective inhibitor of calcium-activated K^+ channels partially attenuated caviunin-induced relaxation only, suggesting that either the EDHF response via IK_{Ca} or SK_{Ca} may also be involved. The involvement of membrane estrogen-receptors ERα and ERβ upstream of eNOS was assessed using the inhibitor ICI 182780. Isocaviunin–induced relaxation was competitively inhibited by ICI 182780 (0.1μM, 1μM, 10μM), but caviunin was only partially, although significantly attenuated at the highest concentration (10μM).

Robustigenin (5-hydroxy, 7, 2',4',5'-tetramethoxy isoflavone) is also a tetra-methoxy isoflavonoid molecule and differs from caviunin and isocaviunin in not having a free 7-OH in the A-ring. It therefore does not have two of the key structural features required for estrogen receptor binding and activity. Despite this robustigenin relaxes rat aortic rings with $EC_{50}=5.744x10^{-6} \pm 6.5x10^{-7}$M and relaxation at 10μM of 68.4±3.55%. L-NAME, ODQ and ICI 182780 attenuate this relaxation significantly.

Isoflavonoids are a class of secondary metabolites with phytoestrogenic activity, known for their beneficial effects in hormone dependent cancers, osteoporosis and CVD. This research has shown that these three isoflavonoids cause acute, non-genomic vasorelaxation via NO/cGMP following stimulation of membrane estrogen receptors. The results suggest these molecules have potential antihypertensive properties by releasing NO, a factor that may also help mitigate the effects of endothelial dysfunction.

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Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
11,12 EET	11,12-epoxyeicosatrienoic acid
ACEI	Angiotensin converting enzyme inhibitors
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
BK_{Ca}	Ca^{2+}-activated large conductance K^+ channel
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium
CC	Column chromatography
CCB	Calcium channel blockers
CAM	Calmodulin
$CDCL_3$	Deuterated chloroform
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase 1 & 2
CV	Cardiovascular
DAG	Diacylglycerol
DAN	Diaminonaphthalene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cells
EDHF	Endothelium-derived hyper-polarising factor

EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ERK/MAPK	Extracellular signal regulated kinase/ Mitogen activated protein kinase
ESI-TOF MS	Electrospray ionisation- time of flight mass spectrometry
FAD	Flavine adenine dinucleotide
FCS	Fetal calf serum
FDA	Food and drug administration
FMN	Flavin mononucleotide
GPCR	G-protein coupled receptors
GPER	G-protein estrogen receptor
GTP	Guanosine 5' triphosphate
Hsp	Heat shock protein
HUVECS	Human umbilical vein endothelial cells
EC_{50}	Concentration of 'drug' where response is halved
IK_{Ca}	Intermediate conductance potassium channels
IP_3	Inositol 1,4,5-triphosphate
K_{ATP}	ATP-activated potassium channels
K_{Ca}	Calcium-activated potassium channels
K_v	Voltage-gated potassium channels
L-NAME	NG-nitro-L-arginine methyl ester
L-NMA	NG-monomethyl-L-arginine
L-NNA	NG-nitro-L-arginine
LDL	Low density lipoprotein

LPA	Lysophosphatidic acid
matK	Maturase K barcoding gene
MLC(K)	Myosin light chain (kinase)
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National centre for biotechnology information
NE	Noradrenaline
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
P2	Purinergic receptors
PCR	Polymerase chain reaction
PAF	Platelet activating factor
PDE	Phosphodiesterase
PE	Phenylephrine
PGI_2	Prostacyclin
PI3-K	Phosphoinositide-3-kinase
PIP2	Phosphatidylinositol 4,5,bisphosphate
PKA or PKB(Akt), PKC, PKG	Protein kinase A, B, C or G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
R&D	Research and Development
RAR	Rat aortic rings
R_f	Retention factor
RNA	Ribonucleic acid

rpoC1/rbcL	Barcoding genes
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SBP	Systolic blood pressure
S.E.M.	Standard error of the mean
Ser	Serine
SERCA	Sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase
SERD	Selective estrogen receptor down-regulator
S/F3 & S/F4	Isocaviunin & caviunin
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rats
SK_{Ca}	Small conductance potassium channels
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TEA	Tetraethylammonium
Thr	Threonine
TK	Tyrosine kinase
TLC	Thin layer chromatography
TRAM-34	(1-[2-chlorophenyl)diphenylmethyl]-1H-pyrazole
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

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Authors declaration

The research contained in this thesis is the original work of the author other than where formally indicated in the text. This thesis has not previously been submitted to this or any other university for a degree.

Chapter 1

General Introduction

1.1 Introduction

There is a crisis in the field of drug discovery resulting from the paucity of new chemical entities coming to market that is impacting on the effective treatment of a number of clinical pathologies. Plants have an unrivalled chemical diversity and have the potential to provide a source for new pharmaceutical lead compounds (Gu et al., 2013). It is estimated that approximately 25% of medicines are derived from plant products (Newman et al., 2012) and it is estimated that only 15% of the world's plant species have been studied to assess their relevance to the drug discovery process (De Luca et al., 2012).

It is often said that the first instance of the isolation of a compound from a plant by a European is that of the extraction of morphine from the poppy by Friederich Serturner in 1805 (Hamilton et al., 2000). Then, in the mid 19th Century Claude Bernard showed that the poison curare, isolated by South American Indians from the vine Strychnos toxifera, interfered with the transmission of nerve impulses. Later Paul Ehrlich developed the idea that these compounds interacted with a specific 'receptor' within the body, leading to the birth of the pharmaceutical industry as we know it today (Rang, 2006). The search for new pharmaceuticals from the natural world continued well into the 20th century giving sway to modern technologically driven approaches based on chemical synthesis of compounds, assessed by high throughput screening techniques, often on a single target, and guided by the financial considerations of the large pharmaceutical companies. The uncertainty inherent in such a precise and clinical approach to drug discovery was that the single interaction of a lead compound on a single target might not be germane to the disease pathogenesis or affect the disease phenotype. Therefore, despite a massive increase in R&D budgets the number of FDA approved drugs remained low. As a result

successful drug validation in phase II clinical trials saw a decline between 2006-7 and 2008-9 from 28% to just 18%, culminating in clinical testing on humans in phase III trials to only half of this number (Arrowsmith 2011a & b). These results combine those of sixteen different pharmaceutical companies, which control 60% of global R&D spending. This attrition rate has continued as seen in a similar audit carried out in 2013 (Arrowsmith et al., 2013). Experts acknowledge that less than 10% of drug candidates in clinical development make it to market, and our knowledge in treating a number of diseases is not complete as a result of not identifying new targets or due to the multiplicity of targets that are involved in effecting a response.

1.1.1 Ethnopharmacy

It is clear that this approach to developing new drugs has not produced the bonanza predicted in a number of pathologies (Brower et al., 2008; Pucci et al., 2013), and this scarcity in new drug leads has coincided with a declining focus on the search for new drug leads from the natural world (Cragg et al., 2013). There is now a general acceptance of the value of traditional herbal medicinal knowledge as witnessed by the resurgence in medicinal plant products and nutraceuticals in the USA (Briskin 2000), and it is clear there is a demand for this knowledge worldwide (WHO, 2013). With the need to increase the pipeline of new drug leads, scientists can be guided by how indigenous communities use plants as medicines or as part of their diet (Jennings et al., 2015). The benefits of this approach are that the plant has a provenance of prior use in humans to elicit a particular response and therefore some of the groundwork has already been achieved. Furthermore, a study of the chemotaxonomy of the plant family can predict the presence of structurally related compounds with similar or improved chemical and pharmaceutical profiles. The interest in drug discovery from

natural products is also now being helped by improvements in screening technologies which upto now has proved to be a major barrier (Li et al., 2009).

1.1.2 Plant identification

There is an issue relating to taxonomic identification of plant materials sourced from remote communities that often use local tribal names. This issue can be addressed by employing DNA barcoding techniques using the genetic material from the plant sample (Palhares et al., 2015). The technique uses a short section of DNA chosen from a region of the genome that spans an evolutionary rate that is high enough to determine interspecies variation but low enough to identify intra-species differences. Several sequences have been identified such as the *matK*, *rpoC1* and *rbcL* gene sequences which are considered suitable for this purpose, although individually can sometimes be inconclusive due to lack of comparative sequences (Hollingsworth et al., 2011). This difficulty can be overcome by using two or more of these barcoding sequences, which together can provide greater certainty as to the identity of the plant. Standard DNA sequencing techniques are used to isolate and compare these gene sequences against a database such as Genbank that provides a repository of these sequences. This technique has proved invaluable in identifying for example species of Moroccan medicinal plants from a market stall (Kool et al., 2012).

1.1.3 Secondary metabolites

Secondary metabolites are grouped by structural characteristics and biosynthetic origins into the alkaloids, terpenoids and the phenolic compounds (Harborne 1998). These compounds although derived from primary metabolic processes are not directly involved in the normal metabolic requirements of the plant such as growth, development or reproduction (Brusotti et al., 2014), but are thought to provide a protective role for the plant allowing them to interact successfully with their

environment (Harborne, 1993). Many of these secondary compound groups are unique to specific taxa and provide a pool of related molecules. They can often provide the start point for drug discovery, one example being that of diosgenin isolated from yams from which a number of steroidal drugs are produced including oral contraceptives, or that of artemisinin from sweet wormwood, which provides the raw material from which antimalarial compounds are developed (Tu et al., 2011). Our shared evolutionary history makes it highly likely that there is a homogeneity between plants and animals that enables us to draw on the resources found within the plant kingdom (Kennedy et al., 2011) and secondary metabolites may hold the key to a number of human health related issues (Russell et al., 2010).

1.1.4 Screening for bioactives

A shift away from a pure target-based screening approach has been necessitated by the paucity of drugs coming to market. There is also an acceptance that an understanding of the complexities and heterogeneity of animal systems is required for treatments to be successful and once again plant sourced drug molecules are more likely to interact with multiple protein targets (Medina-Franco et al., 2013). Phenotypic screening uses organelles, cells, tissues, organs or the whole animal to represent the biological system at the preclinical stage, and this screening technique has provided greater dividends in discovering new chemical entities in the period between 1999-2008 (Swinney et al., 2011). The main consideration in using this technique is the choice of bioassay to represent the physiological process under scrutiny. Although animal bioassays and models may not truly represent the pathology of a disease state in humans, the teratogenicity of the thalidomide drug being a case in point (Kim et al., 2011), it does provide insight into the biological processes that underlie the condition and could act as the starting point in a journey

that leads to the development of a new drug. This approach however is considerably slower since automation is harder, and the lack of knowledge of the molecular mechanism can slow optimization of the drug candidate.

1.1.5 Aortic ring bioassay and cell based screening

The aortic ring bioassay is representative of the systems biology approach involving multiple cell types and which depicts the functional vasorelaxant response to bioactives. The assay can help elucidate the molecular mechanisms responsible for this response by incubating the rings with inhibitors of receptors or enzymes associated with particular biochemical pathways. Rings can be sourced from all stages of development from embryonic through to adult to represent the ageing process, or they can be sourced from models that depict disease states such as hypertension diabetes, and atherosclerosis. Rings can also be sourced from different vascular beds or from a variety of animals to provide a broad picture of the effect of a particular bioactive (Sarikonda et al., 2009).

Antihypertensive models from normotensive animals can be effected using rings pre-contracted by a number of agonists of specific receptors or by depolarization to study different pathways. Typical inhibitors used include α_1-agonists phenylephrine (PE) and noradrenaline (NE) to cause contraction of the vascular smooth muscle. Agonist stimulation of α_1-receptors, result in the inositol triphosphate (IP_3) activation of the sarcoplasmic reticulum resulting in the release of calcium accompanied by a transient contraction. This is followed by a secondary calcium wave due to the influx of extracellular calcium that is followed by a more sustained contraction (Lee et al., 2001). Alternatively contraction can be caused by depolarization of the smooth muscle cells using KCl accompanied by an influx of calcium via L-type calcium channels from the extracellular space (Fransen et al., 2012). These methods of

inducing contraction are clearly able to distinguish between different mechanistic pathways of vasorelaxation. For instance activation by α_1-agonists can more definitively identify bioactive molecules that decrease intracellular calcium, while depolarization induced contraction provides a suitable method for identifying calcium-channel blocking activity. The more traditional cell-based assay models can detect a single response e.g. release of nitric oxide and have enormous potential in terms of cost, speed and the ability to undertake several experiments in parallel.

1.2 Vascular Physiology

The architecture of a typical blood vessel shows that it comprises three distinct layers, the tunica intima, tunica media and the tunica adventitia.

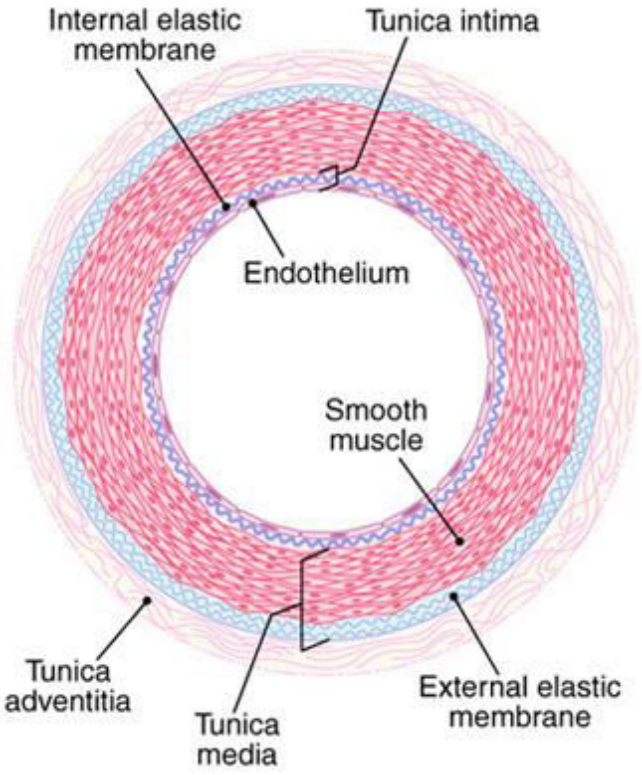

Figure 1 - 1: The cross section of a typical artery http://ice.uthscsa .edu/

The tunica intima is the monolayer of endothelial cells that lines the lumen of the entire vasculature and is subjected to the plethora of chemicals and stresses associated with the flow of blood. It is separated by the internal elastic lamina from the tunica media that is formed primarily of smooth muscle cells and matrix proteins collagen and elastin, the composition of which is determined by the size of the blood vessel. The tunica adventitia is separated by the external elastic lamina and is composed of loose connective tissue made up of fibroblasts, macrophages, has its own blood supply especially in the larger vessels like the aorta and has sympathetic nerve fibres which can release the vasoconstrictor noradrenaline (Levick, 2010).

1.2.1 Vascular tone and the endothelium

Maintenance of vascular tone is a balance between vasorelaxant and vasoconstrictor factors produced by the endothelium in response to the stresses it sustains. The discovery of the endothelium (Furchgott & Zawadzaki 1980), and the endothelium-derived relaxant factor (EDRF) identified as nitric oxide (NO) (Ignarro et al., 1987, Palmer et al., 1987) was pivotal in underlining the importance of the endothelium in maintaining vascular tone. Sheer stress was identified as key to increasing NO together with a number of ligand activated processes (Pittner et al., 2005). Prostacyclin (PGI_2) and the elusive endothelium-derived hyper-polarising factor (EDHF) are the other two major vasorelaxant mediators identified and the effects of these three vasorelaxant mechanisms are highly dependent on the vascular bed (Shimokawa et al., 1996). Opposing these vasorelaxant forces are the contractile mediators endothelin (ET), angiotensin II and thromboxane A_2 (Levick, 2010). Imbalance between these two effects leads to endothelial dysfunction, hypertension and arteriosclerosis both of which have a central role in a number of serious cardiovascular pathologies.

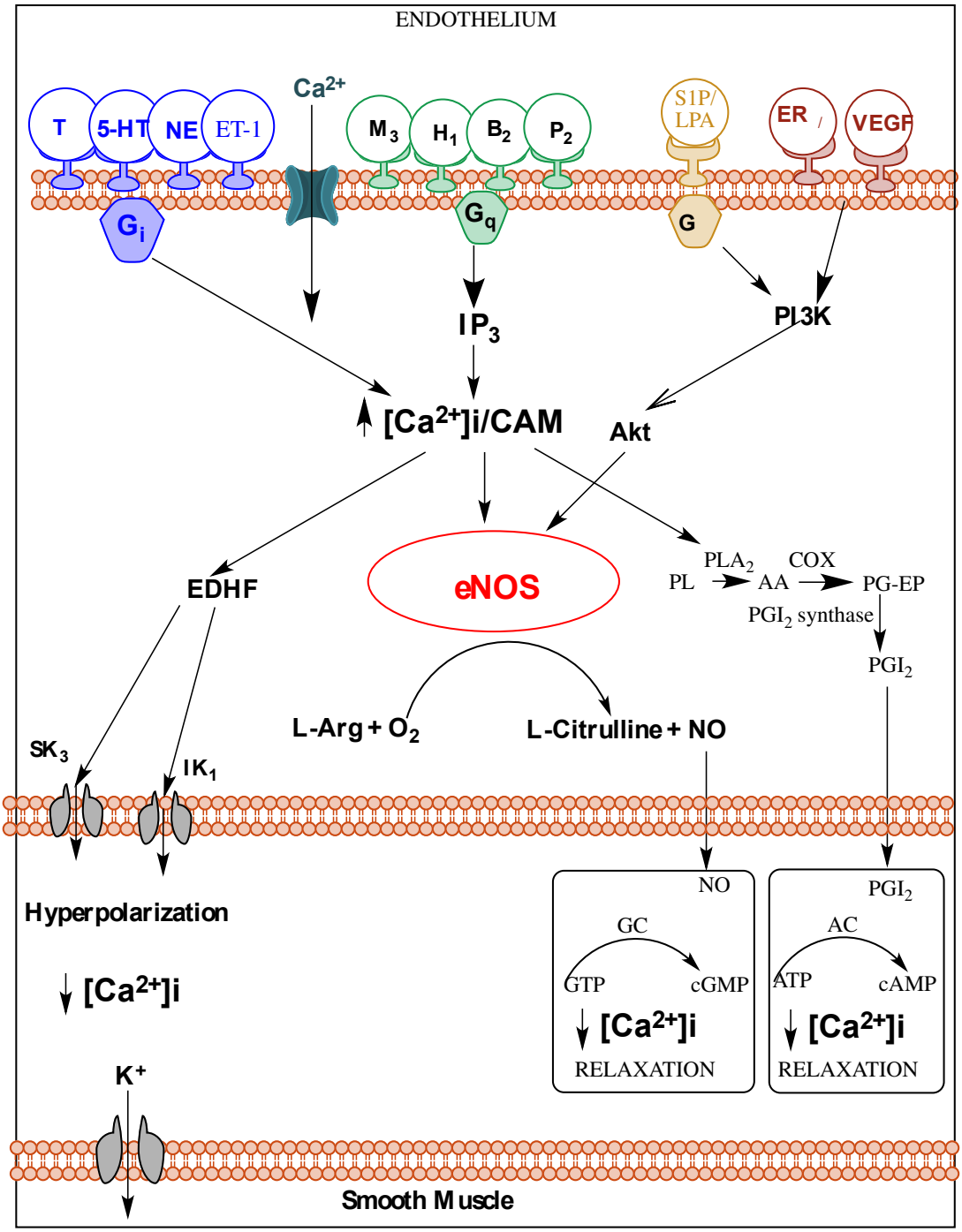

Figure 1 - 2: Mechanisms of endothelium-derived vasorelaxation.

Endogenous mediators thrombin (T), Serotonin (5-HT), Noradrenaline (NE), endothelin-I (ET-1), acetylcholine (M_3), histamine (H_1), bradykinin (B_2), ADP (P_2) act via Gαq and Gαi G-proteins to increase internal Ca^{2+}. Platelet-derived mediators sphingosine-1-P (S1P) and lysophosphatidic acid (LPA) act via Gβγ to increase Ca^{2+}. Increase in Ca^{2+} activates eNOS to increase cGMP nucleotides, prostacyclin pathway to increase cAMP nucleotides and EDHF to activate Ca^{2+}-activated potassium channels to cause relaxation. Hormones estrogen (ER) and vascular endothelial growth factor (VEGF) act directly via PI3K and Akt (PKB) to activate eNOS but are also associated with an increase in Ca^{2+}.

1.2.3 NO/cGMP pathway

This is the single most important mechanism responsible for maintaining vascular tone and vascular wall homeostasis. NO is produced by the action of three different isoforms of nitric oxide synthase (NOS), neuronal nNOS, inducible iNOS and endothelial eNOS, the last being particularly important in the vasculature. All have similar structural properties but varied regulatory properties dependent on the internal $[Ca^{2+}]_{i.}$ Increases in resting $[Ca^{2+}]_i$ following agonist binding, are required for nNOS and eNOS activation but iNOS can bind calmodulin even in resting cells oxidize L-arginine to L-citrulline and NO. (Feletou, 2011).

The enzyme consists of a N-terminal oxygenase domain with binding sites for haem, oxygen, L-arginine and tetrahydrobiopterin (BH_4), and a C-terminal reductase domain with binding sites for flavin adenine dinucleotide (FAD) and Flavin mononucleotide (FMN), NADPH and calmodulin (CAM) (Forstermann et al., 2006). Caveolae are invaginations of the plasma membrane and are abundantly distributed in endothelial cells. Structurally caveolae comprises of cholesterol, sphingolipids and caveolin-1 and these components make the caveolae membranes less fluid but enables protein-protein interactions and protein-membrane interactions (Reeves et al., 2012). The caveolae compartmentalize signal transduction by binding and orchestrating a wide variety of signalling molecules, and being on the interface with the lumen can respond to pressure stresses caused by the flow of blood. Homo-dimerization is a requirement for eNOS functionality and the cofactors BH_4 and haem are essential for this to occur (Cai et al., 2002). Furthermore irreversible myristoylation and reversible palmitoylation target eNOS to the caveolae before binding of substrates together with caveolin-1 from which eNOS must dissociate to become active (Gonzalez et al., 2002; Ju et al., 1997). The C-terminal of caveolin binds G-protein subunits,

phosphatidylinositol-3-OH kinase (PI3-K) and Src family of tyrosine kinase proteins (Dudzinski et al., 2007). Phosphorylation of CAM by CK_2 kinase reverses eNOS activity (Greif et al., 2004).

1.2.3.1 Calcium-dependent control of eNOS and its endogenous mediators

The GPCR superfamily of receptors can bind structurally diverse endogenous ligands that transduce their activity onto the intracellular heterotrimeric G-proteins made up of combinations of α, β or γ subunits. Each subunit in turn has its own variants and there are at least 20Gα, 6Gβ and 12Gγ subunits. Endogenous ligands can activate eNOS via different combinations of these subunits. Ligands such as acetylcholine (M_3), bradykinin (B_2), histamine (H_1) and purines (P_2) amongst others transmit their signals via GPCRs that are coupled via a Gαq mechanism to activate phospholipase C (PLCβ) which cleaves phosphatidylinositol 4,5 bisphosphate (PIP2) resulting in the formation of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3) (Vanhoutte et al., 2009). DAG in turn activates protein kinase C (PKC), and IP_3 activates calcium channels particularly on the sarcoplasmic reticulum to cause an increase in $[Ca^{2+}]_i$ that leads to Ca^{2+}-CAM binding and the activation of eNOS (Isshiki et al., 1998). The localisation of eNOS in the caveolae close to other transduction mechanisms is also perhaps the key to its rapid activity. Ca^{2+}-CAM binding to eNOS is the main but not only trigger that initiates NO synthesis which occurs when electrons are transferred from NADPH via FAD and FMN to the haem group converting L-arginine to L-citrulline with the release of NO.

1.2.3.2 Calcium independent eNOS control and its endogenous mediators

A number of kinase controlled signal transduction cascades regulate eNOS activity by phosphorylation and dephosphorylation at key serine and threonine regulatory sites.

One of these kinase pathways involves the activation of PI3-K (Haynes et al., 2000) and its downstream serine/threonine kinase Akt (or PKB) (Fulton et al., 1999) that mediates the regulation of eNOS by phosphorylation. Important stimulatory loci are found on Ser 1177, Ser 617 and Ser 635, while inhibitory or dephosphorylation loci are found at Thr 495 and Ser 116 all corresponding to the human eNOS (Bauer et al., 2003; Dimmeler et al., 1999). Ser 1177 is an important loci phosphorylated by a number of kinases including Akt while phosphorylation at Thr 495 prevents calmodulin binding (Fleming et al., 2001). S-nitrosylation deactivation of eNOS is also recognized as another receptor mediated method of eNOS regulation and denitrosylation reverses this inhibition of eNOS (Erwin et al., 2005).

Upstream effectors are both receptor and non-receptor activated pathways. For example insulin phosphorylation at serine 1177 (Montagnani et al., 2001) or serine 615 (Ritchie et al., 2010) induced activation of eNOS occurs via its receptor tyrosine kinase associated with caveolin-1 in the caveolae (Repetto et al.,2005) to activate PI3-K/Akt (Zeng et al., 2000). This activation is accompanied by denitrosylation of eNOS (Erwin et al., 2005). The insulin mimetic adiponectin is also thought to activate insulin tyrosine kinase to regulate eNOS via PI3-K and AMPK, but not Akt pathway (Chen et al., 2003). A novel mechanism following VEGF activation of eNOS involves the dephosphorylation at serine 116 to enhance eNOS activity (Kou et al., 2002b). Sphingosine-1-phosphate (S1P) (Igarashi et al., 2008) and lysophosphatidic acid (LPA) (Kou et al., 2002a) released from activated platelets mediate its activities via SIP receptor associated Gβγ subunits of activated GPCR and PI3-Kβ/Akt to phosphorylate serine 1179 on eNOS. The dominant estrogen 17β-estradiol's activity via its membrane ERα estrogen receptor activates eNOS in a calcium-independent manner via PI3K/Akt to release NO (Chambliss et al., 2000; Simoncini et al., 2000).

Calcium spikes that precede eNOS translocation to the nucleus together with concomitant activation of eNOS to release NO have been identified (Goetz et al., 1999). 17β-estradiol can also activate other protein kinase pathways through its ERα receptor such as the ERK/ MAPK pathway to activate eNOS (Razandi et al., 2000). Non-receptor protein TK of the Src family are cytoplasmic kinases (c-Src), can also mediate the activation of eNOS by 17β-estradiol (Li et al., 2007), but at physiological concentrations 17β-estradiol is found to act as a L-type calcium-channel inhibitor (Kitazawa et al., 1997). The protein thrombin, in addition to stimulatory GPCR coupled activation and via PI3K (Motley et al., 2007), can also inhibit Akt mediated eNOS phosphorylation at Ser 1177 via a rho/rho kinase mechanism while heat shock protein (hsp90) binds to eNOS and or Akt to promote eNOS activation (Thors et al., 2003). These interactions all superimpose another level of eNOS regulation.

This discussion on the known regulatory pathways to eNOS activation describes a highly sophisticated picture where a surfeit of structurally diverse endogenous ligands activate both membrane and cytosolic receptors to achieve rapid control of eNOS to manage and maintain vascular tone. It is then unsurprising that the profusion of xenobiotic molecules derived from plants can also trigger these biochemical events by activating a subset of G-proteins or kinase pathways leading to eNOS activation.

Other NOS isoforms found alongside eNOS in the vasculature include nNOS shown to exist in the cytosol fraction only of endothelial and smooth muscle cells from HUVECs and also in adult rat aortas. This is in contrast to eNOS, which was also found in the plasma membrane fraction (Bachetti et al., 2004). These different locations together with the lack of myristoylation sites on nNOS, could account for

the findings that co-transfection studies of Akt, with either eNOS or nNOS, shows Akt only phosphorylates sites on eNOS (Fulton et al., 1999). Clinical studies using nNOS specific inhibitors on healthy individuals has shown that nNOS regulates basal blood flow in human coronary vascular beds and does not inhibit either substance P or acetylcholine stimulated acute vasodilation which is inhibited by L-NMMA (Seddon et al., 2008 & 2009). These studies suggest distinct roles for the NOS isoforms with eNOS responding more dynamically to control vasomotor tone versus nNOS modulation of basal vascular tone. More recently using mesenteric arteries from female rats, 17β estradiol has been shown to phosphorylate nNOS via membrane estrogen receptors to cause acute vascular relaxation, a finding not replicated in male rats (Lekontseva et al., 2011). The implications of the localization of nNOS in the endothelium will therefore require further study. HUVEC cells for example do not possess iNOS at the point of removal from the umbilical veins, but express iNOS in response e.g. by cytokines, a response that occurs over a period of hours and days (Cristine de Assiss et al., 2002). This isoform is unlikely to be involved in mediating acute vasodilatory responses in our rat aortic ring models.

NO is known to be a critical regulator of cellular and tissue function in both physiological and pathophysiological conditions (Ignarro et al., 2002; Thomas et al., 2008). NO induced enhancement of vascular physiology involves anti-atherosclerotic activity, inhibition of leukocyte and platelet aggregation and adhesion, and prevention of smooth muscle cell proliferation and LDL oxidation, the consequence of which is anti-inflammatory activity and protection of the vasculature.

There are a number of NOS inhibitors that can be used to assess the involvement of the NO/cGMP pathway following agonist binding. The NOS inhibitors derived from

arginine are non-specific NOS inhibitors, which include the competitive NG-monomethyl-L-arginine (L-NMA) found naturally in living organisms and the synthetic inhibitors, the poorly soluble NG-nitro-L-arginine (L-NNA) and the highly soluble L-Nω-nitroarginine methyl ester (L-NAME) (Vitecek et al., 2012).

1.2.3.3 Key role for cGMP

Some of the known cytoprotective targets of NO are the ferrous heme proteins such as soluble guanylate cyclase (sGC), oxyhaemoglobin (HbO_2/Hb) and cytochrome C oxidase. Activation of the heme prosthetic group of sGC by NO is seen as the key mechanism by which smooth muscle relaxation occurs. Activated sGC causes the conversion of GTP to cyclic guanosine monophosphate (cGMP), which now activates a multiplicity of mechanisms leading to smooth muscle relaxation. These mechanisms include activation of cGMP-dependent protein kinases such as PKG which decrease intracellular $[Ca^{2+}]_i$, and activate phosphates that dephosphorylate myosin light chain, cGMP-regulation of cation channels again via PKG activation of BK_{Ca} channels to cause hyperpolarization or via cGMP-regulation of phosphodiesterases. The NO/cGMP/PKG pathway is accepted as being the most significant in causing vascular relaxation (Francis et al., 2010).

1.2.4 Prostacyclin pathway

The action of phospholipase A_2 (PLA_2) on membrane phospholipids forms arachidonic acid which is the precursor to prostacyclin (PGI_2) synthesized through the actions of cyclooxygenase 1&2 (COX) and prostacyclin synthase (Moncada et al., 1979). PGI_2 is an inhibitor of platelet aggregation (Saniabadi et al., 1984) and is a potent vasodilator, although it does not regulate basal vascular tone (Nakayama et al., 2006). Its vasorelaxant activity is more prominent in larger mesenteric arteries when compared to its actions in the aorta (Shimokawa et al., 1996), where it is synthesized

concurrently with platelet activating factor (PAF), which signals leukocytes to its cell surface. Arachidonic acid, acetylcholine (M_2 & M_3) (Jaiswal et al., 1991), bradykinin (B_2) (Yamasaki et al., 2000) and 17β-estradiol (Sobrino et al., 2010) cause an increase in endothelial Ca^{2+} concentration amongst other mechanisms, that results in an increase in PGI_2 measured as 6-keto-PGF1α. Noradrenaline (α_1), angiotensin II (Cooper et al., 1984) or K^+ also induce PGI_2. PGI_2 binds to the GPCR-IP receptors on smooth muscle to induce a signalling cascade through Gαs, preferentially activating adenylyl cyclase causing an increase in cAMP that results in vascular relaxation. PKA is activated by cAMP to decrease $[Ca^{2+}]_I$ by causing extrusion and reuptake of calcium via Ca^{2+}-ATPases and prevents phosphorylation of key proteins such as myosin light chain kinase causing relaxation (Majed et al., 2012). PKA also activates calcium-activated potassium channels such as the BK_{Ca} (Tanaka et al., 2004) and SK_{Ca} (Dong et al., 1998) leading to hyperpolarization and vascular relaxation. PGI_2 activity can be assessed by inhibition with non-steroidal anti-inflammatory drugs such as aspirin, indomethacin and ibuprofen that block cyclooxygenase (COX) attenuating PGI_2-induced vascular activity.

1.2.5 Endothelium-dependent hyperpolarising factor

The EDHF response was first observed in studies with acetylcholine-induced relaxation that could not be attenuated by inhibitors of NOS, cGMP or COX. (Chen et al., 1988). It is defined as a non-NO and non-PGI_2 mediated endothelium-dependent relaxation of the vasculature accompanied by smooth muscle hyperpolarization and lowered intracellular calcium concentration (Taylor and Weston, 1988). Distinct calcium-activated potassium (K_{Ca}) channels located in different subcellular compartments are now known to be involved in mediating the EDHF response. These are the intermediate-conductance IK_{Ca} (KCa3.1) channels now shown to exist in the

non-caveolae sections of the cell near the myo-endothelial gap junctions and activated by local IP_3 receptor mediated increase in calcium and locally activated calcium sparks (Ledoux et al., 2008). The small-conductance SK_{Ca} (KCa2.3) that exist within the caveolae in contrast, are predominantly at the inter-endothelial junctions and also controlled by an increase in calcium levels (Absi et al., 2007; Dora et al., 2008). The EDHF response is insensitive to specific inhibitors of the BK_{Ca}, K_v and K_{ATP} channels (Chen et al., 1991).

Venom derived peptides can be used to distinguish between the various K_{Ca} channels. Apamin, a bee venom blocks all three types of SK_{Ca}, while IK_{Ca} channels are blocked by charybdotoxin sourced from the scorpion, though the latter is not specific as it also blocks voltage-gated K^+ channels. TRAM-34 (1-[2-chlorophenyl)diphenylmethyl]-1H-pyrazole) is a more potent and selective blocker of IK_{Ca}, as are arachidonic acid and clotrimazole, but the latter has a limited use as it also blocks P450 enzymes. Studies of pathways by which hyperpolarized endothelial cell currents following agonist activation is transmitted to the smooth muscle has found the myo-endothelial gap junctions (MEGJ) are involved (de Wit & Griffith 2010). 18α-glycyrrhetinic acid (18α-GA) or Gap 27, inhibitors of gap junctions (Chaytor et al., 1998) and ouabain an inhibitor of Na^+/K^+ ATPase on the smooth muscle (Bussemaker et al., 2002) can prevent the transmission of hyperpolarization.

A number of candidates have been put forward as the EDH factor, but the numerous studies undertaken in this area suggest this effect may not be due to a single diffusible substance. Nevertheless hydrogen peroxide and other reactive oxygen species (ROS), the cytochrome P450 regenerated epoxyeicosatrienoic acids (EETs), potassium K^+ ions and others have been put forward as possible candidates (Feletou et al., 2009).

1.2.6 Mechanism of smooth muscle contraction and relaxation

Vascular tone is ultimately mediated by the response of the smooth muscle cells to either endothelium-derived mediators or by direct agonist action on the smooth muscle. The most important factor controlling this process is the modulation of internal $[Ca^{2+}]_i$ which results from agonist binding to a receptor. An increase in internal $[Ca^{2+}]_i$ causes the activation of myosin light chain kinase (MLCK) to phosphorylate MLC leading to the formation of bridges with actin and eventual contraction (Carvajal et al., 2000). There are a number of endogenous and exogenous mediators that can initiate a decrease in internal $[Ca^{2+}]_i$ by a number of mechanisms. These include the calcium-activated potassium channels that lead to hyperpolarization and inhibition of voltage-dependent calcium-channels, extrusion of Ca^{2+} via Ca^{2+}/ATPase activity into the sarcoplasmic reticulum or into the extracellular space and IP_3 receptor inhibition. A decrease in Ca^{2+} activates phosphatase enzymes that dephosphorylate MLC leading to relaxation (Ishida et al., 2005).

1.2.7 Endothelial dysfunction

First observed as impaired vasodilation to acetylcholine it is now associated with the pathogenesis of a number of cardiovascular disease states. It is broadly categorized as a condition with reduced vasodilation, highly susceptible to pro-inflammatory influences and a pro-thrombotic state. In patients with stage 1 essential hypertension it has been shown that approximately 60% of patients exhibited impaired small artery vasodilation (Park et al., 2001), but may be preceded by a period of vascular changes prior to BP increasing, as a 100% of these patients exhibited small artery remodelling (Schiffrin 2012). At the molecular level it is characterized by decreased NO generation and chronic increase in ROS production leading to oxidative stress. The source of ROS may be due to NADPH oxidase, xanthine oxidase or mitochondrial

ubiquinone present in EC and VSMC, where superoxide generation is seen as a separate pathway and not just a consequence of auto-oxidation (Ullrich et al., 2000). But where imbalance occurs it leads to production of cytokines and uncoupling of eNOS in conditions of low BH_4. Electron flows with uncoupled eNOS result in the reduction of molecular oxygen at the prosthetic heme site rather than NO generation (Landmesser et al., 2003). Peroxynitrite is also formed when heightened levels of ROS reacts with NO. These nitrite species are cytotoxic to proteins, oxidizes LDL and degrades BH_4 leading to further uncoupling of eNOS.

The pro-thrombotic state is characterised by an increase in adhesion molecules, chemokines such as macrophages, and production of plasminogen activator inhibitor-1 resulting in inflammation (Endemann et al., 2004). C-reactive protein (CRP) an inflammatory marker has also been shown to decrease eNOS activity (Venugopal et al., 2002). In addition vasoactive peptides such as angiotensin II, endothelin-1 and the accumulation of asymmetric dimethylarginine, an endogenous NO inhibitor, can all cause endothelial dysfunction. This dysfunctional vascular state is prognostic for a number of progressive cardiovascular diseases which if not reversed can lead to increased morbidity and eventually mortality.

Improvement of the endothelial function resulting in the increased production of NO is considered to be key to reversing increasingly poor cardiovascular outcomes for example in heart failure patients. A number of interventions to alleviate endothelial dysfunction such as exercise shown to increase NO and ACE inhibitors that reduce oxidative stress by increasing bradykinin (Rajagopalan et al., 1996) have been identified. Natural product discoveries are also gaining traction in this area e.g. red wine polyphenols have been shown to reduce superoxide production or increase NO output (Lopez-Sepulveda et al., 2012).

1.3 Literature Survey

The plant kingdom currently comprises over 642 families, which contain 17,020 plant genera according to the plantlist.org catalogue, a collaboration undertaken between the Royal Botanical gardens, Kew and the Missouri Botanical gardens. The total number of plant species is estimated to be around 400,000. The angiosperms or flowering plants make up a large part of this having 405 plant families and nearly 14,500 plant genera. The plants identified in this survey as having vasoactive effects are found to reside in over one hundred plant families, that is a quarter of all the known angiosperm plant families. The survey below groups the plants based on their current classification as found in "Tropicos.org. Missouri Botanical Garden. 29 Jan 2016 <http://www.tropicos.org", and four plant species that could not be fully identified using this database, Cinnamomi ramulus, Uncaria villosa, Uncariae ramulus and Xanthoceras sorbifolia were identified using the National plant germplasm system https://npgsweb.ars-grin.gov recommended by Kew.

1.3.1 Search criteria

The aim of the literature review was to recognize the phytomedicinal contribution made by indigenous communities by surveying the literature to identify plants from which solvent extracts or compounds isolated cause relaxation at the level of the vasculature. The survey also focuses on identifying the vascular mechanisms by which this relaxation is transduced and to discover the receptors that initiate this relaxation using aortic ring bioassays. An online search using EMBASE and PUBMED, which includes the MEDLINE database, was undertaken using the MESH search terms "Plant OR Extracts OR Ethno*", to identify the plant source, AND "vasodil* OR vasorel*", truncated search terms to determine the activity, and published between 1995 and 2013. Over 1000 publications were extracted and found

to contain papers with no original data (review articles, letters and editorials), which were excluded. In addition research where the primary focus was on structure-activity studies of previously isolated bioactive compounds with vasorelaxant activity, or work done solely on diabetic or hypertensive disease models were excluded. Studies not published in the English language were excluded. References from these articles were searched to identify additional studies that were missed by the original search.

The plants are grouped in families in order to identify closely related species that may reveal information on the chemotaxonomy of the plants. The plant extracts and any compounds isolated and tested are also documented as are the biochemical pathways identified in the study. The geographical locations known to use the plant in their healthcare system have also been included.

1.3.1.1 Abbreviations used in the table

Solvents – AqE – aqueous extract, BuOH – butanol, $CHCl_3$ – chloroform, DCME – dichloromethane Extract, EtoAc – ethylacetate, EtOH – ethanol; GBE – gingko biloba extract, Hex – hexane, MeOH – methanol, Pet Ether – petroleum ether.

Inhibitors - MTX – Methoxamine; CT – Charybdotoxin; AP – Apamin; TEA-tetraethylammonium; HOE- HOE 140;

Signaling pathways – NE – noradrenaline, PE – phenylephrine, KCl – potassium chloride, NO – nitric oxide, cGMP – cyclic guanosine monophosphate, COX – cyclo-oxygenase, cAMP – cyclic adenosine monophosphate, M_3 – muscarinic receptor, PDE – Phosphodiesterase, B_2 – bradykinin, ER – estrogen receptor, ET – endothelin, EDHF – endothelium-dependent hyper-polarizing factor, H_1 – histamine receptor, α/β - adrenergic receptors, PI3K/Akt – calcium-independent pathway, ROS – reactive oxygen species, E- - endothelium denuded, E+ - endothelium intact, O – other (U44619 or 5-hydroxytryptamine).

Table - √ = Responsible for Relaxation; $\surd_P$ Partially responsible for relaxation;

X = No effect; Blank = Not tested; ↑- Inhibitor potentiates relaxant effect.

1.3.2 Survey of vascular activity found in plants used in indigenous medicine

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
Acanthaceae	*Andrographis paniculata*, Taiwan	14-deoxy-11, 12-dihydroan-drographolide	(Wu et al., 2008)	X	√											√
		14-deoxy andrographolide	(Zhang and Tan, 1998)	√	√	$√_P$	√	X								√
	Brillantaisia nitens Lindaus, Cameroon	Leaf DCM / MeoH extract	(Dimo et al., 2007)	√	√	X		X							$√_{TEA}$	√
	Hypoestes serpens Madagascar	Serpendione	(Andriamihaja et al., 2001)	√												
	Pseuderanthemum palatiferum, Thailand	Leaf - AqE	(Khonsung et al., 2011)	√		X			X							X
Acoraceae (Araceae)	*Acorus calamus* Pakistan	Rhizome Hexane	(Shah and Gilani, 2009)	√	√	√										√
Alismataceae	*Echinodorus grandiflorus*, China	Leaf - AqE	(Tibiriçá et al., 2007)	√		√	√	X	X						X_{CT}	X
Amaryllidaceae (Alliaceae/Liliaceae)	*Allium fistulosum* China	Onion AqE	(Chen et al., 1999b)	√		√		X								$√_P$
	Allium cepa, Pakistan	Onion peel EtOH 70%	(Naseri et al., 2008)	√	√	X	X	X	X	$√_{PDE}$						√
	Allium sativum L. India	Garlic juice	(Ashraf et al., 2004)	X		X	√	↑								
		AqE Garlic	(Kim-Park and Ku, 2000, Ku et al., 2002)	√		√		X			$√_{ET}$					X
Anacardiaceae	*Anacardium occidentale*, Diet	Leaf MeOH	(Runnie et al., 2004)	√		√										X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Rhus coriaria, Europe	Leaf MeOH	(Beretta et al., 2009)	√		√	√	√								X
	Sclerocarya birrea, South Africa	Stem bark AqE	(Ojewole, 2006)	√		√										
Annonaceae	*Annona squamosa*, Japan	Cyclosquamosin B, Seeds	(Morita et al., 2006b)	√												√
	Annona cherimolia	Liriodenine Norushinsunine	(Chulia et al., 1995)	√	√								$√_P$			√
	Phaeanthus crassipetalus Malaysia	(-) Limacine-bark	(Zaima et al., 2012b)	√		√										X
		Pecrassipine A-bark		√											X_{TEA}	√
		Backebergine-leaf		√											X_{TEA}	√
	Xylopia aethiopica, Africa	Rhizomes Hex, DCME	(Somova et al., 2001)	X	X											√
	Xylopia langsdorffianna, Brazil	Labdane-302	(de Oliveira et al., 2006)	√	√	$√_P$		$√_P$								$√_P$
Apiaceae	*Alepidea amatymbica*, Africa	Rhizomes Hex, DCM Extracts	(Somova et al., 2001)	X	X											√
	Angelica dahurica, China	Imperatorin Isoimperatorin	(Nie et al., 2009) MAR only	√		√p							X			√p
	Angelica Furcijuga, Japan	Root MeOH	(Matsuda et al., 2000)	√	√											√
	Angelica gigas China	Root Ethanol, AqE	(Rhyu et al., 2005)	√		√	√	X	√						$X_{CT/AP}$	X
	Angelica keiskei	Root EtoAc, EtOH	(Matsuura et al., 2001)	√		√	X									√
	Bupleurum fruticosum, China	Root $CHCl_3$	(Testai et al., 2005)	√			X	X/X								√
	Coriandrum sativum	Dietary	(Jabeen et al., 2009)	√	√											√

Family	Scientific Name	Bioactive	Reference	NE/PEE+	KClE+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Foeniculum vulgare, Diet	Fennel oil, Anethole	(Tognolini et al., 2007)	√	√											√
	Ligusticum chuanxiong, China	Butylidene phthalide	(Chan et al., 2006, Chan et al., 2007, Chan et al., 2009, Chen et al., 2011, Kwan, 1994, Liang et al., 2005)	O	√	$√_P$	$√_P$	X/X					X			$√_P$
	Ligusticum wallichi China	Whole plant AqE	(Kim et al., 2004, Kim et al., 2010, Kim and Rhyu, 2010)	√		√	√	X	√						X_{CT}	√
		BuOH-tetramethyl pyrazine	Kim et al., 2010,	X	X										√	√
		Rhizome $CHCl_3$	(Rhyu et al., 2004)	√		√		X	X							X
	Peucedanum japonicum Japan	(+)cis-4'-O-acetyl -3'-O-angeloyl khellactone	(Lee et al., 2002a)	√	√	√	√p	X	X			X	Xβ			√p
	Peucedanum praeruptorun	8-methoxy psoralen	(Zhao et al., 1999)	√												
	Trachyspermum ammi, Pakistan	Thymol	(Aftab et al., 1995)	√	√			X	X				X			√
Apocynaceae	*Alstonia angustiloba* Asia/Africa	Leaf MeOH	(Koyama et al., 2008)	√												
	Alstonia macrophylla, Asia	Vincamedine	(Arai et al., 2012)	√		$√_P$									X_{TEA}	√
	Alstonia scholaris, Pakistan	Leaf EtOH	(Channa et al., 2005)	√		√		X	X				X			X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Apocynum venetum, China	Leaf 70%EtOH	(Kwan et al., 2005) (Tagawa et al., 2004)	√	√	√		X	X				X		$\surd_{TEA}$	X
	Aspidosperma subincanum, Brazil	Bark EtOH	(Bernardes et al., 2013)	$\surd_P$		$\surd_P$	$\surd_P$	$\surd_{P(cAMP)}$							$\surd_{TEA}$	√
	Cynanchum stauntonii	Stauntonine	(Wang et al., 2004)	√	√											
	Hancornia speciosa Gomes, Brazil	Leaf EtOH	(Ferreira et al., 2007a, Ferreira et al., 2007b)	√		√		X	X					√		X
	Himatanthus lancifolius, Brazil	Indole alkaloid fraction	(Rattmann et al., 2005)	√												√
	Hunteria zeylanica, Malaysia	Bisnicalaterine B&C isomers	(Hirasawa et al., 2010)	√												√
	Neisosperma Oppositifolia Tracheabiontam Malaysia	Bark Hex, Oppositinines A & B	(Ahmad et al., 2010)	√		√										X
	Tabernaemontana dichotoma Japan	10methoxyaffinisine, Cathafoline, Alstonisine	(Zaima et al., 2012a)	√	√	$\surd_P$										$\surd_P$
Aquifoliaceae	*Ilex paraguariensis* Brazil	Leaf AqE, BuOH	(Muccillo Baisch et al., 1998), (Stein et al., 2005)	$\surd_{MTx}$		√	X	X								X
Araliaceae	*Acanthopanax Gracilistylu* (Panax notoginseng), Asia	Ginsenoside Rb1, Rg1	(Pan et al., 2012)	√		√								√		
	Eleutherococcus senticosus, China	Root AqE	(Kwan et al., 2004b)	√		√		X	$\surd_P$				X		√	
Arecaceae	*Calamus quiquesetinervius*, Taiwan	Flavonolignans & Flavonoids	(Chang et al., 2010)	√												

Family	Scientific Name	Bioactive	Reference	NE/PEE+	KClE+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Cocos nucifera Malaysia	Fruit EtOH	(Bankar et al., 2011) +HT RAR	√	√	√	√	$\surd_P$	$\surd_P$							
	Elaeis guineensis Malaysia	Palm leaf MeOH	(Ndiaye et al., 2010)	O		√		X						√	$\surd_{P(CT/AP)}$	
	Euterpe oleracea Brazil	Fruit AqE	(Rocha et al., 2007)	√		$\surd_P$	√	X	X		X_{HOE}	X	X		$\surd_{CT/AP}$	
Asteraceae	*Achillea millefolium* Pakistan	Aerial parts MeOH	(Khan and Gilani, 2011)	√	√	$\surd_P$										$\surd_P$
	Artemisia argyi	Plant MeOH - Moxartenolide	(Yoshikawa et al., 1996)	√	√											
	Artemisia herba-alba, Morocco	Aerial part AqE	(Skiker et al., 2010)	√		√	√	X	X						X_{TEA}	$\surd_P$
	Artemisia ludoviciana, Mexico	Plant DCM Extract	(Estrada-Soto et al., 2012)	√	√	X	X		X							$\surd_P$
	Artemisia verlotorum Italy	Leaf AqE	(Calderone et al., 1999)	√		√	√		$\surd_P$	√						
	Bidens pilosa, Cameroon	Leaf MeOH:DCM	(Nguelefack et al., 2005)	√	√			$\surd_P$				$\surd_P$				√
	Calea glomerata Colombia	Aerial parts EtOH	(Guerrero et al., 2002a)	√	√											
	Carthamus tinctorius Diet	N(P-coumaroyl serotonin & feruloylserotonin	(Takimoto et al., 2011)	√												√
	Centaurea corcubionensis	Centuarein & centaureidin	(Orallo et al., 1998)	√	√											√
	Chrysanthemum morifolium, China	Flower EtOAc	(Jiang et al., 2005)	√	√	√	√	X					X		X_{TEA}	$\surd_P$
	Cichorium intybus Japan	Chicoric acid	(Sakurai et al., 2003)	√	X											√
	Cirsium japonicum	Plant AqE	(Kim et al., 2008)	√		√	√	X	X		X_{HO}	√				

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET E	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	De Candole, Korea															
	Helichrysum ceres,		(Musabayane et al., 2008)	$\surd_{MTX}$	√	√	√	$\surd_P$								$\surd_P$
Asteraceae	*Iostephane heterophylla* Mexico	Bisbolene, Xanthorrhizol	(Campos et al., 2000)	√	√	X		X								√
	Petasites formosanus, Taiwan	S-Petasin	(Wang et al., 2001a) (Wang et al., 2010)	O	√	X	X	X								√
	Petasites hybridus Denmark	S-Petasin	(Sheykhzade et al., 2008) -+MAB	O	√	X		X								√
	Spilanthes acmella Thailand	Leaf EtOAc and $CHCl_3$	(Wongsawatkul et al., 2008)	√		$\surd_P$		$\surd_P$								X
	Stevia rebaudiana		(Lee et al., 2001)	X	X											√
	Tanacetum vulgare Morocco	Plant AqE	(Lahlou et al., 2008)	√	√	√										$\surd_P$
	Vernonia liatroides Mexico	Glaucolides D & E	(Campos et al., 2003)	√	√											√
	Viguiera arenaria Brazil	Ent-pimara-8(14),15- diene-19-oic acid	(Tirapelli et al., 2004)	√	√	$\surd_P$	$\surd_P$	$\surd_P$							X_{TEA}	√
	Viguiera robusta, Brazil	Kaurenoic acid + Pimara above	(Tirapelli et al., 2005)	√	√											$\surd_P$
Asparagaceae	*Drimiopsis maculata*	Homo-isoflavonoid	(Fusi et al., 2008)	√	√		$\surd_P$			X						
Berberidaceae	*Berberis vulgaris*, Iran	Fruit AqE	(Fatehi-Hassanabad et al., 2005)	√		$\surd_{RAR}$, X_{MAB}										$\surd_P$
	Epimedium sagittatum, China	Aerial parts BuOH	(Wang et al., 2007a)	√		$\surd_P$										
Bignoniaceae	*Mansoa hirsuta D.C.,* Brazil	Leaf EtOH	(Campana et al., 2009)	√		√		X								X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
Bixaceae	*Cochlospermum vitifolium*, Mexico	Bark MeOH (Naringenin)	(Sánchez-Salgado et al., 2010, Sánchez-Salgado et al., 2007)	√		$√_P$	√	$√_P$	X	√						$√_P$
Boraginaceae	*Borago officinalis* Pakistan	Leaf EtOH 80%	(Gilani et al., 2007a)	√	√											√
Brassicaceae	*Brassica napus*, Japan	Rapakinin	Yamada 2010	√		X		√			X_{HOE}					X
	Raphanus sativus Pakistan	Plant AqE	(Ghayur and Gilani, 2006a)	√		√			√							X
Buxaceae	*Sarcococca saligna* Pakistan	Plant AqE	(Ghayur and Gilani, 2006b)	√	√	X										√
Calophyllaceae	*Mammea Africana* Cameroon	Stem DCME	(Dongmo et al., 2007)	√	√	$√_P$		X								$√_P$
Cannabaceae	*Celtis durandii* Cameroon	Leaf DCM/MeOH	(Dimo et al., 2005)	√	√			√							X_{TEA}	X
	Humulus Lupulus France	Flowers AqE	(Figard et al., 2008)	√		√		√							X_{TEA}	
Capparaceae	*Capparis aphylla* Pakistan	Aerial MeOH	(Shah and Gilani, 2011)	√	√	$√_P$			$√_P$							√
Caprifoliaceae	*Valeriana edulis* Mexico	Rhizome Hexane	(Estrada-Soto et al., 2010)	√	√		X								X_{TEA}	√
	Valeriana prionophylla, Italy	Root EtOH - Prinsepiol	(Piccinelli et al., 2004)	√												X
Caricaceae	*Carica papaya* Malaysia	Shoots MeOH	(Abeywardena et al., 2002) (Runnie et al., 2004)	√		√		$√_{PMAB}$								X
Caryo-phyllaceae	*Stellaria dichotoma*, Japan	Root MeOH Dichotomin J&K	(Morita et al., 2005)	√												
	Vaccaria segetalis, China	cNonapeptide Segetalin A-H	(Morita et al., 2006a)	√												√
Celastraceae	*Maytenus ilicifolia*,	Leaf $CHCl_3$:	(Rattmann et al.,	√		√	√	X	X		X_{HO}	X			X_{TEA}	X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Brazil	MeOH 2:1	2006)								E					
Chryso-balanceae	*Licania pittieri* Venezuela	Pomolic acid	(Estrada et al., 2011)	√	X	√	√	X	X				X			
Cistaceae	*Cistus ladaniferus* Morocco	Leaf AqE	(Belmokhtar et al., 2009)	√		√			X							X
Cleomaceae	*Gynandropsis gynandra*, Diet	Leaf MeOH	(Runnie et al., 2004)	√		√										X
Clusiaceae	*Allanblackia monticola* Cameroon	Leaf	(Azebaze et al., 2007)	√	√											
	Garcinia Kola, Nigeria	Kolaviron	(Adaramoye and Medeiros, 2009)	√	√										$√_{CT}$	√
	Rheedia gardneriana	7-epiclusianone	(Cruz et al., 2006)	√		√		X								X
Combretaceae	*Combretum laxiforum* South Africa	Leaf MeOH	(Nsuadi Manga et al., 2012)	√		√	√	X								X
	Combretum molle, South Africa	Mollic acid	(Ojewole, 2008)	√		√										
	Combretum racemosum, Congo	Leaf & root MeOH	(Nsuadi Manga et al., 2012)	√		√	√	$√_P$								X
	Terminalia superba Congo	Stem DCM:MeOH	(Tom et al., 2010)	√	$√_P$										$√_{CT}$	√
Convolvulaceae	*Ipomoea batatas* Diet	Leaf MeOH	(Runnie et al., 2004)	√		√										X
	Ipomoea stans, Mexico	Root AqE	(Perusquía et al., 1995)	√												
	Ipomoea tyrianthina Mexico	Tyrianthin A&B	(León-Rivera et al., 2009, León-Rivera et al., 2011)	√												X
		Root-Convolvulin	(León-Rivera et al., 2009, León-Rivera	√												X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
			et al., 2011)													
Cornaceae	*Cornus officinalis*, China		(Kang et al., 2007)	√		√	√	X	X				X			X
Costaceae	*Dimerocostus strobilaceus*, Panama	Aerial MeOH/DCM	(Guerrero et al., 2009)	√												X
Cucurbitaceae	*Benincasa hispida*, Diet	Wax gourd juice	(Nakashima et al., 2011)	√		√			X							X
	Gynostemma pentaphyllum, US	Gypenoside triterpenes	(Tanner et al., 1999) PCAR	O		√		X								X
Cupressaceae	*Juniperus excela*, Mediterranean	Aerial part MeOH	(Khan et al., 2012)	√	√	$\surd_P$										√
	Taxodium mucronatum, Mexico	Aerial part AqE	(Perusquía et al., 1995)	√												
Daphni-phyllaceae	*Daphniphyllum teijsmannii*, Japan	Daphniphyllum E & F	(Morita et al., 2006d)	$\surd_P$												
	Daphniphylum macropodum, Japan	Pordamacrines A&B	(Matsuno et al., 2007)	$\surd_P$												
Dicksoniaceae (Pteridophytes)	*Dicksonia sellowiana,* Brazil	Leaf hydroalcohol	(Rattmann et al., 2009) (Rattmann et al., 2012)	√		√	√	$\surd_P$	√					√	$\surd_{CT}$ $\surd_{P(AP)}$	X
Ebenaceae	*Diospyros kaki*, Japan	Leaf AqE Proanthocyanidin	(Kawakami et al., 2011)	√		√	√	X						√		X
Equisetaceae (Pteridophytes)	*Equisetum arvense*, Japan	Dicaffeoyl-meso-tartaric acid	(Sakurai et al., 2003)	√	X											√
Ericaceae	*Arbutus unedo*, Morocco	Leaf AqE-tannins	(Afkir et al., 2008, Ziyyat et al., 2002),	√		√	√		X							X
	Craibiodendron henryi, China	Grayanane	(Huang et al., 2005)	$\surd_P$		$\surd_P$		$\surd_P$								
	Vaccinium angustifolium, USA	Blueberry diet-fed animals	(Kalea et al., 2010, Kalea et al., 2009,	√		√		X								

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
			Kristo et al., 2010)													
	Vaccinium myrtillus	Extract purchased	(Bell and Gochenaur, 2006)	O		√										X
Eucommiaceae	*Eucommia ulmoides Oliv*, China	Leaf AqE	(Jin et al., 2008, Kwan et al., 2004a)	$\surd_{MTX}$	X	X		X	√	√					$\surd_{TEA}$	X
		Bark/Leaf AqE	Kwan et al., 2004a	√	X	√	√		X						$\surd_{TEA}$	X
Euphorbiaceae	*Croton cajucara*, Brazil	Dehydrocrotonin-diterpene	(Silva et al., 2005)	√		√										$\surd_P$
Euphorbiaceae	*Croton celtidifolius* Brazil	Bark EtOH |AqE	(DalBo et al., 2008a, DalBo et al., 2008b) MABS	√		√	√	X	X		X_{HOE}	X			$X_{CT/AP/TEA}$	√
	Croton nepetaefolius	Methyleugenol and a terpineol	(Magalhães et al., 2004, Magalhães et al., 2008)	√		√	√									$\surd_P$
	Croton schiedeanus Schlecht, Colombia	Aerial part EtOH Ayanin	(Guerrero et al., 2002a, Guerrero et al., 2002b, Guerrero et al., 2004)	√		√	√									$\surd_P$
		Quercetin 3,7 dimethyl ether	(Guerrero et al., 2002b)	√		√	√									X
	Croton zambesicus Benin	Ent-18-OH-(-tra-chyloban-3β-ol) or (-isopimara-7,15 diene-3β-ol)	(Baccelli et al., 2005, Baccelli et al., 2007, Baccelli et al., 2010, Martinsen et al., 2010)	$\surd_P$	√	$\surd_P$										√
	Croton zehntneri, Diet	Anethole	(Soares et al., 2007)	√	√	X		X								√
	Jatropha gossypiifolia, Brazil	Stem/Leaf EtOH	(Abreu et al., 2003)	√												√

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
Fabaceae (Leguminosae)	*Albizia inopinata* Brazil	Leaf EtOH	(de S. Pires et al., 2000, Maciel et al., 2004)	√	√	√		X	X							$√_P$
	Astragalus membranaceus China	Calycosin	(Wu et al., 2006), (Zhang et al., 2007)	√	√	X		X								√
		Plant AqE EtOH	(Zhang et al., 2005)	√	X	$√_P$	√									√
		Astralogoside	(Zhang et al., 2007	√	√	$√_P$	$√_P$	$√_P$								$√_P$
	Caesalpinia benthamiana Ivory Coast	Roots AqE	(Zamblé et al., 2008)	√		√										
	Caesalpinia ferrea Mart, Brazil	Bark AqE	(Menezes et al., 2007)	√	√	X			X							√
	Caesalpinia sappan Brazil	Bark MeOH Haemotoxylin	(Xie et al., 2000),	√		√	√									X
		Brazilin	(Sasaki et al., 2010)	X		X										√
			(Hu et al., 2003)	√		√	√									
		Sappanchalcone	(Sasaki et al., 2010)	√		√		$√_P$								X
		Protosappan D	(Sasaki et al., 2010)	$√_{MAB}$		$√_{MAB}$		$√_{MAB/RAR}$								$√_{RAR}$
	Calycotome villosa Morocco	Chrysin glucoside	(Cherkaoui-Tangi et al., 2008)	√		√										X
	Canavalia brasiliensis, Brazil	Seed Lectins	(Assreuy et al., 2009, Ameer et al., 2010a)	√		√		$√_P$							X_{TEA}	X
	Canavalia gladiate Brazil	Seed Lectins	(Assreuy et al., 2009, Ameer et al., 2010a)	√		√		$√_P$							X_{TEA}	
	Canavalia maritime Brazil	Seed Lectins	(Gadelha et al., 2005)	√		√										X
	Cassia siamea	Cassiarin A	(Matsumoto et al.,	√		√	√	X							X_{TEA}	X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Japan		2010)													
	Crotalaria sassiliflora, Korea	Aerial part DCM:MeOH 3:1	(Koh et al., 2007)	√		√	√									X
	Dalbergia odorifera Taiwan	Butein	(Yu et al., 1995)	√		√	√	X		√					X_{CT}	X
	Derris urucu, Brazil	Brazil	(Mendes et al., 2011)	√		√										X
	Dioclea grandiflora Brazil	Floranol	(Lemos et al., 2006, Lemos et al., 1999)	√		X										√
		Dioclein	(Lemos et al., 1999)	√		√	√	X	X							X
Fabaceae (Leguminosae)	*Lespedeza cuneata* Japan	Plant AqE	(Lee et al., 2012)	√		√	√	X	X				$X_{β}$		X_{TEA}	X
	Lysidice rhodostega		(Gao et al., 2004)	√		$√_P$		$√_P$								
	Moldenhawera nutans Queiroz Brazil	Labd-8(17)-en-15-oic acid	(Lahlou et al., 2007)	O	√											√
	Parkia biglobosa Benin	Leaf hydroalcohol EtoAC, BUOH	(Tokoudagba et al., 2010)	√		$+√_P$		$+√_P$							$+√_{CT/AP}$	X
	Pisum arvense Brazil	Lectins	(Assreuy et al., 2011)	√		√		X	X							
	Psoralea corylifolia China	Bakuchicin	(Li et al., 2011)	√		√	√	X	X				X		X_{TEA}	X
	Pueraria lobata Gegen, China	Diadzein, Diadzin	(Deng et al., 2012,)	O		X	X	X/X							$√_{TEA}$	√
		Puerarin	(Deng et al., 2012,)	O		$√_P$	X	X/X			X				$√_{TEA\ in\ E-}$	√
	Securigera securidaca, Iran	Seeds -hydro-alcohol extract	(Garjani et al., 2009)	√												X
	Sophora alopecuroids	Matrine - alkaloid	(Zheng et al., 2009)	√	X								X		X_{TEA}	√

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	Sophora flavescens China	Root EtOH	(Jin et al., 2011),	√		√	↑	X	X						X_{TEA}	X
Gentianaceae	*Gentiana arisanensis,* China	Isovitexin-flavone C-glycoside	(Lin et al., 1997)	√	√											
	Gentiana kochiana Italy	Gentiacaulein & Gentiakochianin	(Chericoni et al., 2003) (Baragatti et al., 2002)	√	√											√
	Halenia elliptica D. Tibet	1-OH-2,3,5 tri-methoxy xanthone	(Wang et al., 2007b)	O		√	√	X/X	X		X	X			X_{TEA}	$√_P$
		1,5 di-OH-2,3 di-methoxy xanthone	Wang et al., 2008	O		X	X								$√_{TEA}$	√
Ginkgoaceae (Gymnosperm)	*Gingko Biloba*, China	GBE includes quercetin	(Dell'Agli et al., 2006, Li et al., 2007, Mansour et al., 2011, Nishida and Satoh, 2004, Kubota et al., 2001, Kubota et al., 2006)	√		√				√				√		X
		EGB 761 special extract	(Koltermann et al., 2007) Animal	√		√								√		
Grossulariaceae	*Ribes nigrum L.*, Diet	Black currant juice	(Nakamura et al., 2002)	√		√	√	X	X			√		√		X
Hernandiaceae	*Hernandia nymphaeifolia*		(Chen et al., 2001)	√	√											
	Illigera luzonensis, Taiwan	Stem MeOH	(Chen et al., 1997)	√	√											
Hypericaceae	*Hypericum oblongifolium*, Pakistan	Aerial part EtOH	(Khan et al., 2010)	√	√											
	Hypericum perforatum, Turkey	Standardized extract	(Tugrul et al., 2011)	√	√	X										√

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Vismia latifolia, Brazil	Euxanthone	(Câmara et al., 2010)	√	√	X		X								√
Juglandaceae	*Juglans regia*, Mexico	Leaf AqE	(Perusquía et al., 1995)	√												
Lamiaceae	*Agastache mexicana* Mexico	Tilianin flavonoid	(Hernández-Abreu et al., 2009, Hernández-Abreu et al., 2011)	√		√	√	X	X						$\sqrt{}_{P\ TEA}$	$\sqrt{}_P$
	Ajuga iva Morocco	Plant AqE	(El-Hilaly et al., 2004)	√		$\sqrt{}_P$										$\sqrt{}_P$
	Leonurus artemisia China	Leonurine (4-guanidino-n-butyl syringate	(Chen and Kwan, 2001)	√	√											√
	Leonurus heterophyllus, Japan	Cycloleonuri peptides E&F	(Morita et al., 2006c)	$\sqrt{}_P$												$\sqrt{}_P$
	Lepechinia caulescens, Mexico	Ursolic acid	(Aguirre-Crespo et al., 2006)	√	X	√	√	$\sqrt{}_P$	X							X
	Marrubium vulgare Morocco	Aerial parts AqE - Marrubenol and marrubium	(El Bardai et al., 2004) (El Bardai et al., 2003)	√	√	$\sqrt{}_P$										√
	Melissa officinalis Turkey	Rosamarinic acid	(Ersoy et al., 2008)	√		√		$\sqrt{}_P$								X
	Mentha villosa Hudson, Brazil	Rotundifoline	(Guedes et al., 2002, Guedes et al., 2004b)	√	√	$\sqrt{}_P$		$\sqrt{}_P$	$\sqrt{}_P$							$\sqrt{}_P$
		Leaf AqE	(Guedes et al., 2004a)	√	√	√		√	X							$\sqrt{}_P$
	Ocimum basilicum Morocco	Leaf AqE	(Amrani et al., 2009)	√												
	Ocimum gratissimum, Brazil	Leaf AqE - eugenol	(Interaminense et al., 2007)	√	√											$\sqrt{}_P$

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Origanum vulgare Mediterranean	Carvacrol	(Earley et al., 2010)	O		X		X							$\surd_{AP/TRAM}$	X
	Orthosiphon aristatus, Indonesia	Methyl ripario-chromene A	(Matsubara et al., 1999)	O	X											√
	Perilla frutescens	Periallaldehyde	(Takagi et al., 2005)	√	√					X			X		X_{TEA}	√
	Salvia miltiorrhiza Bunge Danshen China	Tanshinone II-Phytoestrogen	(Fan et al., 2011)	√	√	√					$\surd_{ER}$					X
		Dihydro-tanshinone	(Lam et al., 2008)	O		X	$\surd_P$	X	X				X			√
	Satureja obovata Lag, Mexico	Eriodictyol-5,7,3',4'-tetra hydroxyl-flavonone	(Ramon Sanchez de Rojas et al., 1999)	X	X											√
	Scutellaria baicalensis, China	Baicalin	(Lin et al., 2010)	O	√	X	√	X/√								√
	Thymus capitatus, diet	Essential oil	(Yvon et al., 2012)	√												
	Vitex cienkowskii Cameroon	Bark DCM:MeOH 1:1	(Dongmo et al., 2011)	√	X	√	√	X/X								X
	Ziziphora clinopodioides, Uyghur	Plant DCME	(Senejoux et al., 2010)	√	√											√
Lauraceae	*Aniba canelilla*, Brazil	Essential oil – 1-nitro-2-phenylethane	(Lahlou et al., 2005), (de Siqueira et al., 2010)	√	√	√			√							√
	Cassytha filiformis Taiwan	Plant EtOH	(Tsai et al., 2008)	√												
	Cinnamomi ramulus, Korea	Plant EtOH	(Kang and Shin, 2011)	√												√
	Laurus nobilis, Diet	Essential oil	(Yvon et al., 2012)	√												
	Litsea lancifolia	N-allylaurolitsine	(Sulaiman et al.,	√												

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	Malaysia		2011)													
	Ocotea duckei Brazil	Reticuline	(Dias et al., 2004)	√		√		X	√							X
	Ocotea quixos, Ecuador	Calices-trans cinnamaldehyde	(Ballabeni et al., 2007)	√												√
	Persea americana South Africa	Leaf AqE	(Ojewole et al., 2007) (Owolabi et al., 2005)	√		√	√	√								X
Linaceae	*Linum usitatissimum*, Diet	Flaxseed supplement	(Dupasquier et al., 2006)	√	√											
Loranthaceae	*Agelanthus dodoneifolius* Burkino Faso	Plant EtOH – dihydropyranone dodoneine	(Ouedraogo et al., 2011)(Ouedraogo et al., 2011)	√		X		X								√
	Loranthus ferrugineus Malaysia	Aerial Part MeOH	(Ameer et al., 2010b)	√	$√_P$	√	√	√	√				X			X
	Psittacanthus calyculatus, Mexico	Plant AqE	(Ibarra-Alvarado et al., 2010)	√			$√_P$									$√_P$
Lythraceae	*Cuphea carthagenensis*, Brazil	Crude hydro-alcoholic extract	(Schuldt et al., 2000)	√		√	√									$√_P$
Magnoliaceae	*Magnolia liliflora*, China	Plant MeOH	(Yin et al., 2005)	√		√										X
	Magnolia obovata China	Honokiol	(Seok et al., 2011)	O												√
	Michelia figo Orient	Leaf MeOH	(Chericoni et al., 2004)	√			X	X/X								$√_R$ yanodine
Malpighiaceae	*Galphimia glauca* Mexico	Leaf AqE	(Perusquía et al., 1995)	√												

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Malvaceae	*Chiranthodendron pentadactylon* Mexico	Flowers AqE	(Perusquía et al., 1995) (Ibarra-Alvarado et al., 2010)	√												
	Guazuma ulmifolia Panama	Bark acetone	(Magos et al., 2008)	√		√		X	X							X
	Hibiscus sabdariffa Malaysia	Calyces BuOH	(Sarr et al., 2009)	√		√	√	X						√		$√_P$
	Sida cordifolia Brazil	Leaf AqE	(Santos et al., 2006)	√		√		√	√						$√_{CT/AP/TEA}$	X
Meliaceae	*Dysoxylum lenticellatum*, China	Dysolenticin	(Huang et al., 2011)	√												
	Ekebergia capensis Sparrm South Africa	Leaf EtOH	(Kamadyaapa et al., 2009)	$√_{MTX}$		√	√	√					X_{Alpha}			$√_P$
Meni-spermaceae	*Chondrondendron platyphylla*, Brazil	Curine–bisbenzyl isoquinoline	(Medeiros et al., 2011), (Dias et al., 2002)	√	√		X									√
	Coscinium fenestratum Thailand	Stem AqE	(Wongcome et al., 2007)	√	$√_P$	√										
Moraceae	*Morus alba*, China	Leaf EtoAC	(Xia et al., 2008) (Xia et al., 2007)	√	√											√
Musaceae	*Musa paradisiaca* Nigeria	Plantain AqE	(Orie, 1997)	$√_P$	√											
Myrtaceae	*Eucalyptus gracilis* Tunisia/France	Aerial part - essential oil	(Yvon et al., 2012)	√												
	Eugenia uniflora Brazil	Leaf hydro-alcoholic	(Wazlawik et al., 1997)	√		√	√									X
	Melaleuca quinquenervia		(Lee et al., 2002b)	√		$√_P$										√
	Psidium guajava	Leaf AqE	(Chiwororo and	√		$√_P$										$√_P$

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	South Africa		Ojewole, 2008)													
	Syzygium guineense Ethiopa	Leaf hydro-alcohol	(Ayele et al., 2010)	O	√	X	X	X	X			X				√
Nitrariaceae	*Nitraria sibirica Pall.* Uyghur	Fruit – hydro-alochol	(Senejoux et al., 2012)	√	$\surd_P$	√		X	√						$\surd_{CT/AP}$	X
	Peganum harmala China	Harmaline	(Berrougui et al., 2006, Berrougui et al., 2002)	√	√	√		√					$\surd_A$			X
Ochnaceae	*Ouratea semiserrata*		(Cortes et al., 2002)	√		√		X	X							X
Oleaceae	*Forsythia suspensa* Japan	Forsythiaside - fruits	(Iizuka and Nagai, 2005)	√	X											√
	Forsythia viridissima, Japan	Forsythide - Leaf	(Iizuka et al., 2009)	√	X											√
	Ligstrum purpurascens		(Wong et al., 2001)	O	√											√
Onagraceae	*Oenothera odorata* Korea	Seeds EtOH	(Kim et al., 2011)	√		√	√							√		X
Orchidaceae	*Laelia autumnalis* Mexico	Root MeOH	(Vergara-Galicia et al., 2008, Vergara-Galicia et al., 2010a, Vergara-Galicia et al., 2010b)	√	√		$\surd_P$		X				X_b		X_{TEA}	√
	Orchis mascula, Pakistan	Root AqE	(Aziz et al., 2009)	√	√											√
	Scaphyglottis livida Mexico	Gigantol & 3,7-dihydroxy-2,4-dimethoxy phenanthrene	(Estrada-Soto et al., 2006)	√		$\surd_P$	√									√
Orobanchaceae	*Cistanche tubulosa* Japan	Echinaroside	(He et al., 2009, Yoshikawa et al.,	√		√	√	X	$\surd_P$							X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
			2006)													
		Stem MeOH phenylethanoids	(Yoshikawa et al., 2006)	√	X											
Paeoniaceae	*Paeonia emodi* Pakistan	Root EtOH 70%	(Ghayur et al., 2008)	√	√	X			X							√
	Paeonia lactiflora	Root EtOH	(Jin et al., 2012),	√		√	√	X	X				X	√	$√_{TEA}$	X
	Paeonia moutan	Root bark MeOH	(Yoo et al., 2006)	√		√										
	Paeonia suffruticosa	Root bark MeOH	(Kang et al., 2005b)	√		√	√	$√_P$								$√_P$
Pandaceae	*Microdesmis keayana* Ivory Coast	Root-keayanidine B and keayanine	(Zamblé et al., 2009)	√		√										
Papaveraceae	*Platycapnos spicata*	Nantenine	(Orallo and Alzueta, 2001)	√	√	X										X
Passifloraceae	*Passiflora edulis*	Scirpusin B dimer of piceatanol	(Sano et al., 2011)	√		√										X
Pedaliaceae	*Sesamum indicum* India	Root Petroleum Ether	(Suresh Kumar et al., 2008)	√	√	$√_P$	$√_P$	X	X				X			$√_P$
	Sesamum radiatum Ivory Coast	Leaf AqE	(Konan et al., 2008)	√		√		$√_P$							$√_{TEA}$	X
Phyllanthaceae	*Phyllanthus acidus*, Thailand	Leaf BuOH	(Leeya et al., 2010)	√	√	X	√	X	X				X			
	Phyllanthus niruri Thailand	Methyl brevifolin carboxylate	(Iizuka et al., 2006)	√	X											X
		Phyllanthin & hypophyllanthin	(Iizuka et al., 2006, Inchoo et al., 2011)	√	√		X						X		X_{TEA}	√
Pinaceae (Gymnosperm)	*Pinus pinaster* Japan	Flavengol	(Kwak et al., 2009)	√		√	√									
Piperaceae	*Piper betle* Malaysia	Plant MeOH	(Runnie et al., 2004)	√		√										$√_P$

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
	Piper methysticum kava	Kavain	(Martin et al., 2002)	√												√
	Piper nigrum Pakistan	Piperine-alkaloid	(Taqvi et al., 2008)	$√_P$	√											√
	Piper truncatum Brazil	Leaf – Eudesmin,	(Raimundo et al., 2009)	√		√	√	$√_P$	X			$√_P$	X			X
Plantaginaceae	*Bacopa monnieri*, Thailand	Bacoside A3 & Bacopaside II	(Kamkaew et al., 2011)	√		√										$√_P$
	Globularia alypa Morocco	Leaf MeOH	(Chokri et al., 2012)	√	√	X		X	√						√	
Poaceae	*Andropogon muricatus Retz* Pakistan	Aerial part 70% MeOH	(Gilani et al., 2007b)	√	√											$√_P$
	Cymbopogon citrati, Malaysia	Leaf MeOH	(Devi et al., 2012)	√		$√_P$		$√_P$								$√_P$
	Phyllostachys nigra China	Friedelin – Bamboo shavings	(Jiao et al., 2007)	√												
	Zea mays South America	Cob hydroalcohol extract	(Moreno-Loaiza and Paz-Aliaga, 2010)	O	√	√										
Polygalaceae	*Polygala caudata* China	Root - Xanthones	(Lin et al., 2005)	O	√											√
	Polygala paniculata Brazil	Plant hydro-alcohol E-Rutin	(da Rocha Lapa et al., 2011)	√		√	√		X			X			$√_{P(CT)}$	X
Polygonaceae	*Fagopyrum tartaricum*, Japan	Wheat AqE acidic	(Ushida et al., 2008)	√	√	√	√									X
	Polygonum aviculare, Korea	Plant Hexane & butanol	(Yin et al., 2005	√		√										X
	Rheum officinale Japan	San'o-Shashin	(Sanae et al., 2001)	√												
	Rheum undulatum Korea	Rhubarb-AqE Rhizome-	(Moon et al., 2006) (Yoo et al., 2007)	√		√		X	X						X_{TEA}	X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
		hydroxy-stilbenes														
Primulaceae	*Lysimachia clethroides,* Korea	Plant hydroxyl-alcohol	(Lee et al., 2010)	√		√	√	X						√	$X_{CT/AP}$	
Ranunculaceae	*Aconitum japonicum,* Japan	Mesaconitine	(Mitamura et al., 2002)	√	√	√			X						$√_{AP}$	X
	Cimicifuga dahurica, Japan	Cimifugic aced D	(Noguchi et al., 1998)	√	X											√
	Coptis chinensis Japan	San'o-Shashin	(Sanae et al., 2001)	√												√
	Coptis chinensis China	Coptisine	(Gong et al., 2012)	√		$√_P$	$√_P$	$√_P$								$√_P$
	Pulsatilla chinensis China	Anemoside A3;	(Zhang et al., 2010)	√	$√_P$										$√_{CT/AP}$	√
Rhamnaceae	*Scutia buxifolia* Brazil	Bark butanol	(Da Silva et al., 2012)	√		√	√		X		X_{B2}	X				$√_P$
	Zizyphus spina, Iran	Leaf EtOH 70%	(Godini et al., 2009)	O	X											√
Rosaceae	*Aronia melanocarpa* Diet	Cyanidin-3-galactosine or arabinoside	(Bell and Gochenaur, 2006)	O		√										X
	Crataegus Monogyna Europe	Crataegus special WS 1442	(Anselm et al., 2009, Chen et al., 1998)	√		$√_P$								√	√	X
		WS1442/F- C	(Brixius et al., 2006)	√		√										X
		Fruit EtOH	(Chen et al., 1998)	√	$√_P$	√	√	X								X
	Crataegus oxyacantha, Europe	Crataegus extract in MeOH	(Kim et al., 2000)	√		√	√	X	X			X	X		$√_{TEA}$	X
	Fragaria ananassa Diet	Fruit	(Edirisinghe et al., 2008b)	√		√								√		X
		Leaf AqE	(Mudnic et al.,	√		√		$√_P$								X

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
			2009)													
	Geum japonicum, China	Plant butanol	(Xie et al., 2007)	√		√	√									X
	Malus pumila Japan	Procyanidin fraction	(Matsui et al., 2009)	√	$\surd_P$	√	√		X						$\surd_{TEA}$	
	Rubus fruticosus Croatia	Wine	(Mudnic et al., 2012)	√												
	Rubus idaeus, UK	Fruit- Sanguiin H6 Lambertianin	(Mullen et al., 2002)	√												
	Sorbus commixta Orient	Plant – n-BuOH	(Yin et al., 2005	√		√										X
	Sorbus commixta Korea	Plant MeOH	(Kang et al., 2005a)	√		√	√	X	X				X		X_{TEA}	X
Rubiaceae	*Alseis yucatanensis* Belize	Bark AqE	(Slish et al., 2004)	√	$\surd_P$											√
	Mitragyna ciliata Cameroon	Bark Hexane	(Dongmo et al., 2004)	√	$\surd_P$											
	Mitragyna inermis West Africa	Bark AqE PCAR	(Ouédraogo et al., 2004)	√	$\surd_P$	√		X								√
	Morinda citrifolia Polynesia	Plant MeoH	(Runnie et al., 2004)	√		$\surd_P$										X MABS $\surd_{RAR}$
		Root EtOH 70%	(Gilani et al., 2010a)	√		X			X							√
	Morinda lucida Nigeria	Leaf AqE	(Ettarh and Emeka, 2004)	√		√	√									$\surd_P$
	Nauclea latifolia Nigeria	Leaf & root AqE	(Akpanabiatu et al., 2005)	√												
	Nauclea officinalis Malaysia	Bark – naucline, and indole	(Liew et al., 2012)	√												

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
		alkaloid														
	Psychotria poeppigiana Panama	Aerial parts MeOH acid	(Guerrero et al., 2010)	$\surd_P$												
	Randia siamensis Thailand	Fruit- n-butanol	(Khwanchuea et al., 2007)	√		√							$\surd_{bot\ h}$			X
	Uncaria macrophylla, China	Dihydrocorynan-theine	(Wang et al., 2011)	√												
	Uncaria rhynchophylla China	Rhynchophylline & isorhynchophy-lline isomers	(Zhang et al., 2004)	$\surd_P$	√	X			X				X			√
	Uncaria villosa (Name found in paper) Malaysia	Villocarines – indole alkaloids	(Matsuo et al., 2011)	√	√	$\surd_P$										$\surd_P$
	Uncariae Ramulus et Uncus, (Name found in paper) China	Geissoschizine methyl ether-indole alkaloids	(Yuzurihara et al., 2002)	√	√	$\surd_P$										√
Rutaceae	*Casimiroa pubescens,* Mexico	Seeds MeOH	(Froldi et al., 2011)	√		√	√		√			X				
	Casimiroa edulis Mexico	Seed AqE & MeOH	(Baisch et al., 2004, Bertin et al., 2011, Magos et al., 1995) (Froldi et al., 2011)	√	X	√	√		√			X	$\surd_a$			
	Cedrelopsis grevei Madagascar	Madagascar	(Mingorance et al., 2008)	√		√		X								
	Citrus natsudaidai Japan	Cyclonatsudamin e A	(Morita et al., 2007)	√		√										X
	Evodia Rutaecarpa China	Dehydro-evodiamine	(Chiou et al., 1996)	√		X	X	X							X_{TEA}	√
		Evodiamine		$\surd_P$	√	$\surd_P$	$\surd_P$	X							X_{TEA}	$\surd_P$

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
		Rutaecarpine		√		√	√	X							X_{TEA}	X
	Zanthoxylum armatum, Pakistan	Stem/Leaf/Seed MeOH	(Gilani et al., 2010b)	√	√											√
	Zanthoxylum integrifoliolum Taiwan	(-) Tetrahydro-berberine	(Chen et al., 1999a)	√	√											
	Zanthoxylum piperitum, Korea	Leaf AqE	(Li et al., 2010)	√		√	√	X	X				Xβ		X_{TEA}	X
Santalaceae	*Viscum album* Nigeria	Leaf AqE	(Mojiminiyi et al., 2008)	√	$√_P$											√
Sapindaceae	*Acer nikoense*, Japan	Heartwood MeOH	(Iizuka et al., 2007)	√			X									√
	Xanthoceras sorbifolia, China	Leaf EtOH	(Jin et al., 2010)	√		√	√	X	X				X	√	$√_{TEA}$	X
Saururaceae	*Saururus chinensis* Korea	Root EtOH	(Ryu et al., 2008)	√											X_{TEA}	
Schisandraceae	*Schisandra chinensis* Korea	Fruit Hexane	(Park et al., 2009)	√		√	√	X							X_{TEA}	$√_P$
		Gomisin-lignan	(Park et al., 2012)	√		√								√		$√_P$
		Fruit AqE	(Lee et al., 2004), (Rhyu et al., 2006)	√		√	√	√			$√_{ER}$					$√_P$ /X
		Root EtOH	(Ryu et al., 2008)	√		√									$√_{TEA}$	$√_P$
Selaginellaceae (Pteridophytes)	*Selaginella tamariscina*, Orient	Amentoflavone	(Kang et al., 2004), (Yin et al., 2005	√		√										X
Solanaceae	*Capsicum frutescenes* Malaysia	Fruit MeOH	(Abeywardena et al., 2002), (Runnie et al., 2004)	√		$√_P$										X
	Hyoscyamus niger Pakistan	Crude extract	(Khan and Gilani, 2008)	√	√	X										√
	Solanum paludosum	Alkaloid fraction	(Monteiro et al., 2012)	√		√	√	X	X						$√_{TEA}$	$√_P$

Family	Scientific Name	Bioactive	Reference	NE/PEE+	KClE+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
Theaceae	*Camellia sinensis*, India	Green tea (EPCG)	(Lorenz et al., 2009)	√		X								√		
		Black tea (Theaflavin)	(Lorenz et al., 2009)	√		X								√		
Thymelaeaceae	*Phaleria macrocarpa* Indonesia	Icariside	(Oshimi et al., 2008)	√												
Ulmaceae	*Ulmus davidiana* Korea	Root EtOH	(Cho et al., 2011)	√	$√_P$	√										X
	Ulmus macrocarpa	Root EtOH	(Oh et al., 2008)	√	$√_P$	√									$√_{TEA}$	X
Urticaceae	*Cecropia lyratiloba* Brazil	Leaf MeOH	(Ramos Almeida et al., 2006)	√		√			X							
	Cecropia obtusifolia, Panama	Leaf MeOH	(Guerrero et al., 2010)	$√_P$												
	Musanga cecropioides, Africa	Leaf AqE	(Dongmo et al., 2002)	√			√									X
	Urtica dioica Morocco	Root AqE, MeOH	(Testai et al., 2002)	√	√	√	√								$√_{TEA}$	X
Violaceae	*Viola odorata* Pakistan		(Siddiqi et al., 2012)	√	√	$√_P$			X			X				$√_P$
Vitaceae	*Vitis labrusca* Europe	Red Wine-alcohol free extract	(Schuldt et al., 2005)	√	X	√	√								$X_{CT/AP/TEA}$	$√_P$
		Grape Skin-alcohol free	(Soares De Moura et al., 2002) MABS	√		√	√	X							X_{TEA}	X
			(Madeira et al., 2005, Madeira et al., 2009) (Dell'Agli et al., 2005	√			√		X	√	X_{HOE}	X	X	√	$√_{CT/AP}$	X
		Grape Juice	(Anselm et al.,			√								√	$X_{CT/AP}$	

Family	Scientific Name	Bioactive	Reference	NE/PE E+	KCl E+	NO	cGMP	COX/cAMP	M_3	PDE	B_2/ERET	H_1	α2/β	Pi3-K/Akt	EDHF	E-
			2007)													
		Grape Seed	(Edirisinghe et al., 2008a) (Aldini et al., 2003)	√ √		√ √	√	$\sqrt_P$						√		X
		Stem - Vitisin C	(Seya et al., 2003)	√		√	√		X							X
	Vitis thunbergii var. taiwaniana	Stem/Leaf – Vitisin A	(Lin et al., 2012)	√		√										
Zingiberaceae	*Alpinia henryi K.* China	Cardomonin, Alpinetin	(Wang et al., 2001b)	√	√	√	√	X	√							√
	Alpinia zerumbet (K. Schum), China	Leaf EtOH/Water Essential oil-1,8-cineole	(de Moura et al., 2005), (Pinto et al., 2009)	√		√	√	X	X		$\sqrt_P$ HOE	X	X		$X_{CT/AP}$ $\sqrt_{TEA}$	X
	Curcuma longa C. West Africa	Rhizome MeOH	(Adaramoye et al., 2009)	√	√	X									X_{TEA}	√
	Kaempferia parviflor, Thailand	5,7-dimethoxy flavone	(Malakul et al., 2011) (Tep-areenan et al., 2010)	√	X	√	√	√/X							$\sqrt_{TEA}$	$\sqrt_P$ X MTX
	Zingiber officinale Pakistan	Ginger MeOH	(Ghayur and Gilani, 2005)	√	√	X			X							√
		Ginger AqE/ gingerols	Ghayur et al., 2005	√	√	$\sqrt_P$			$\sqrt_P$			X				$\sqrt_P$
Zygophyllaceae	*Tribulus terrestris* Kuwait	Kuwait	(Phillips et al., 2006)	√	$\sqrt_P$	$\sqrt_P$		X					X_B			

Table 1 - 1: Survey of plants with ethnomedicinal use and which show vasorelaxant activity

1.3.3 Plant analysis

The results portray a wide spectrum of plant families that represent over a quarter of all known angiosperm families. The five plant families possessing the greatest number of species are listed as Orchidaceae, Asteraceae, Fabaceae, Rubiaceae and the Poaceae or true grasses and two of these families are well represented in this survey. Asteraceae (22), and Fabaceae (24) have the greatest species count followed by Lamiaceae (18) and Rubiaceae (13), although both Orchidaceae and Poaceae are poorly represented. It must be remembered that these are plants identified as having a medicinal purpose by indigenous communities, and the studies represented in the survey confirm a beneficial effect of the plant.

1.3.4 Phytochemical discoveries

The survey has also found that from nearly 40% of the plants represented in the table the compounds responsible for activity were isolated and their vasorelaxant activity elucidated. This amounts to a consideration of well over 150 compounds descriptive of a number of phytochemical types and characterised as having vasorelaxant activity in an *in vitro* bioassay, but with the benefit of prior use in humans. At least a third of these studies have also undertaken some animal work, nearly all confirming the *in vitro* vascular effects in normotensive and/or hypertensive animal models. Within each of the larger family groups one can find a snapshot of the structural diversity of chemical compounds that are representative of the variation seen in the entire survey. Preliminary identification of the plant to be studied in this project was made informally by sources at the Singapore botanical gardens by studying the leaf structure, following which they identified the plant as belonging to the Legume family. The plant family Fabaceae are well represented in the literature survey above.

They include flavonoids such as chrysin glycoside and the minor flavonoids, the isoflavonoids calycosin daidzein, puerarin and daidzin, the chalcones butein, the homoisoflavonoids brazilin and haemotoxylin, a stilbene resveratrol, a dihydroflavonol isotirumalin and a prenylated version, floranol. But although flavonoid compounds dominate this group, alkaloids, steroidal saponins, diterpenes, proteins and the phenylpropanoids furanocoumarin bakuchicin are also present and show vascular activity. This variety and complexity in chemical structure is mirrored across the whole survey.

A recent survey covering the period between 1990-2007, identified 207 bioactive compounds that cause relaxation at the level of the vasculature (Luna-Vazquez et al., 2013). They also found a wide range of phytochemical classes represented in their search. The alkaloids and flavonoid compounds identified consisted of approximately 50% of the bioactive compounds found with chalcones, homo-isoflavonoids, phenolic compounds, stilbenes, lignans, xanthones, coumarins, anthraquinones, phtalides, peptides and terpenes comprising the other half of the compounds identified. Over 98% of these bioactives they found have the ability to activate eNOS to stimulate the NO/cGMP pathway.

1.3.5 Pharmacology of bioactives identified in survey

There has been considerable success in identifying the vascular pathways that cause relaxation in a number of these compounds, but relatively little success in identifying particular upstream receptors that initiate relaxation. What is clear is that individual compounds can activate multiple mechanisms that may be either endothelium-dependent or independent. Despite the focus in this project on endothelium-dependent

signalling pathways a number of these compounds have also shown calcium-channel blocking activity similar to that of verapamil or amlodipine.

1.3.5.1 NOS/cGMP pathway

By far the most dominant mechanism by which plant derived extracts and compounds caused relaxation was through the activation of NOS enzymes to release nitric oxide. The activity of nearly 60% of extracts or compounds were inhibited by the use of L-arginine analogues such as L-NMA and L-NAME, and in a further 30% of studies this pathway had not been tested. Just 10% of all the studies described had confirmation that NOS was not involved in causing relaxation. Although it appears that eNOS activation in particular is the main target of a very large number of chemically diverse compounds of plant origin, it is less clear what the upstream receptors are that initiate this process, or the subsequent signalling transduction pathways that lead to eNOS activation.

1.3.5.1.1 Calcium-dependent pathways to NO release

Just over 6% of plant extracts activate muscarinic receptors but over double that number of studies (>14%) showed that these receptors were not involved in eNOS activation. Studies using the H_1 specific receptor antagonist diphenhydramine or chlorpheniramine have identified just 4 plants out of 17 studies showing the involvement of a histaminergic-type action (Kim et al., 2008; Nguelefack et al., 2005; Raimundo et al., 2009; Nakamura et al., 2002), while using the bradykinin antagonist HOE-140 just 1 out of 8 studies has shown a partial involvement of the bradykinin receptor (deMoura et al., 2005). Adrenergic involvement on smooth muscle using the α_1-receptor antagonist prazosin has been shown on 2 occasions (Magos et al., 1995; Berrougui et al., 2006), but only a single study using the α_2-selective antagonist

yohimbine, showed no effect (deMoura et al., 2005). Studies testing purinergic (P_2) involvement, although not included in the table, are also limited, but the vasorelaxant effect of the triterpenoid Pomolic acid from *Licani pittieri* (Estrada et al., 2011) was inhibited by the P_2 antagonist suramin, while apyrase an enzyme that hydrolyses ATP/ADP could not prevent this effect suggesting the presence of a molecule that can activate a 'purinergic' receptor. By contrast grape polyphenol studies using reactive blue 2, a selective P_2Y antagonist, and apyrase cause inhibition of the relaxant effect showing that ATP/ADP are involved. Studies with $\alpha_1\beta$-methylene ATP, an inhibitor of ecto- ATPases that degrade these nucleotides shifted the anthocyanin relaxation curve to the left, i.e. increasing relaxation. The authors conclude that these studies reflect the increase in nucleotides by the polyphenols, which can act on P_2Y receptors to trigger the release of NO (Mendes et al., 2003).

1.3.5.1.2 Calcium-independent pathways to NO release

Another dominant pathway to eNOS activation is via the redox sensitive activation of the phosphorylation cascade controlled by PI3-K/Akt. Of the 18 plants studied, all showed involvement of this signalling pathway by using either Wortmannin (Ferreira et al., 2007) or LY294002 (Pan et al., 2012), specific inhibitors of PI3-K. It is unclear if these are receptor-mediated events. However potential upstream receptors that activate the PI3K/Akt pathway are the membrane estrogen receptors that can be inhibited using the non-specific estrogen receptor antagonist ICI 182 780. Two studies showed the vasorelaxant activity of the diterpene tanshinone II (Fan et al., 2011) and a specific fraction from *Schizandra chinensis* (Lee et al., 2004) could be blocked by ICI 182 780 suggesting non-genomic estrogenic activity.

1.3.5.2 Prostacyclin pathway

A significant proportion of the plants had been tested to determine the involvement of the prostanoid pathway. Of the 37% of studies that tested for prostaglandin activity just 11% showed that the relaxation could be blocked by indomethacin, a COX inhibitor. There were no studies where the relaxation was due solely to prostacyclin activity but a study with a rapeseed protein named Rapakinin from *Brassica napus*, identified the activation of multiple pathways that included attenuation of the relaxant effect by indomethacin and the prostacyclin IP receptor antagonist CAY 10441 (Yamada et al., 2010). Interestingly the effect of the eNOS inhibitor L-NAME only showed a partial attenuation in relaxation. In contrast studies on garlic (Ashraff et al., 2004) and *Echinodorus grandiflorus* (Tibirica et al., 2007) show that incubation with indomethacin did not block the relaxant response, but actually potentiated it suggesting inhibition of contractile prostaglandins. The results of this survey suggest that the prostanoid pathway is only represented in a minor way and is clearly not a significant mode of action of plant-derived bioactives.

1.3.5.3 EDHF modulated relaxation

Plant extracts designated as acting via EDHF mechanisms are distinguished on the basis of their responses to charybdotoxin, Tram 34, apamin or TEA. Of the 74 studies using TEA, exactly half show that this pathway is not involved. There are 23 studies using the other inhibitors mentioned here, of which 11 show involvement in blocking IK_{Ca} or SK_{Ca}. Tram 34 and apamin inhibitors of IK_{Ca} or SK_{Ca} respectively inhibit vascular relaxation by carvacrol from the oregano plant (Earley et al., 2010). A study on Malus pumila induced relaxation shows that the endothelial-dependent relaxation can be completely blocked by a combination of eNOS inhibitor and TEA (Matsui et al., 2009). Transmission of vasorelaxant hyperpolarisation between the endothelium

and smooth muscle caused by red wine can be blocked using Ouabain, a Na^+/K^+-ATPase inhibitor (Schuldt et al., 2005) while the use of the gap junction inhibitor 18α-glycyrrhetinic acid (18α-GA) has similarly shown that the hyperpolarisation response to *Eucommia ulmoides* can be blocked (Jin et al., 2008). Both these mechanisms are thought to be signalling pathways through which the hyperpolarisation is transmitted to the smooth muscle. Cytochrome P450 derived metabolites are known to cause hyperpolarisation by generating epoxyeicosatrienoic acids. Using the cytochrome P450 inhibitor SKF525A, the relaxation due to an extract from *Acorus calamus* (Shah & Gilani, 2009) was blocked. The vasorelaxation due to this extract was uninfluenced by either a NOS or COX inhibitor. Studies to determine vascular activity are becoming more rigorous and this will be required to decode the activity of these bioactives.

1.3.6 Related CV mechanisms of extracts and compounds

Many plants in addition to their vasorelaxant activity have shown their ability to modulate other key pathways important in cardiovascular physiology and pathophysiology. Allied physiological mechanisms identified in addition to the vasorelaxant activity include antiplatelet aggregation effects by the aqueous extract of the herb *Oscimum basilicum* (Amrani et al., 2009); Antithrombotic activity was shown using Foeniculum vulgare (fennel) and its main constituent anethole following thrombosis induced by collagen and adrenaline (Tognolini et al., 2007); Hypocholesterolemic activity using the seed extract from *Securigera securidaca* showed a reduction in triglyceride level and LDL cholesterol and a partial reduction in total serum cholesterol when compared to that of control animals (Garjani et al., 2009). Other studies have shown diuretic activity in rats by coriander seed extracts (Jabeen et al., 2009). These studies are by no means limited to those highlighted here

and a number of studies listed in the survey are progressing research to identify allied beneficial effects of the plants extracts and compounds.

1.3.7 Summary

The results of the survey have identified the potential beneficial effects of a vast number of plant extracts on the vasculature and in a number of cases identified some of the signalling pathways through which this activity is transduced. The predominant endothelium-dependent mechanism by which plant-derived extracts or compound cause vascular relaxation is by activating eNOS to cause relaxation. There is also evidence suggesting the activation of the prostanoid and EDHF pathways are also important in causing vascular relaxation. However none of the studies identified in the survey attributed vascular relaxation solely to these mechanisms suggesting at least in the aorta these mechanisms were less important. Surprisingly few receptor-initiated mechanisms have been identified, with the muscarinic receptor being the most studied. Endothelium-independent mechanisms also dominate with a substantial number of studies identifying calcium-channel blocking (CCB) activity. These mechanisms are however not mutually exclusive and relaxation can be induced by a number of different signalling pathways. However the conclusions especially in terms of the receptors or upstream kinases involved in eNOS activation is determined by the subset of experiments undertaken in each study, and these experiments are highly varied.

Many of these studies have also begun the process of identifying the allied CV beneficial effects of these extracts and compounds in pathophysiology states using various bioassay models, in animal experiments and a few extracts have also undergone human trials.

1.4 Aims

The purpose of this project was to isolate, identify and pharmacologically characterise vasorelaxant compounds from the bark of a plant used in Borneo in a 'tea' preparation. The premise for studying the tea was based on an observation of the perceived effect the tea *may* have had on a friend already on antihypertensive medication and who felt faint on standing after drinking the tea. This observation was purely speculative as other members of our group of 15 suffered no ill effects and does not preclude other explanations such as dehydration or heat exhaustion.

Previous work had shown the presence of a contractile component in the aqueous extract of the bark, while a methanolic extract of the bark showed acute endothelium-dependent vasorelaxant activity in the rat aortic ring bioassay. It was hypothesized therefore that this vascular activity must be due to the presence of vasoactive compounds that interact with the endothelium to cause relaxation.

These goals were to be achieved by -

1. Utilizing the rat aortic ring bioassay or endothelial cell assay to guide isolation of vasoactive extracts, fractions and compounds.
2. Undertaking structure elucidation methods to determine the chemical structures of compounds isolated.
3. Identifing the plant using DNA barcoding techniques to genus or species level.
4. Characterising the biochemical pathways that lead to vascular relaxation.

Chapter 2

Bioinformatics

2.1 Introduction

Indigenous medicine is predominantly plant based and often different parts of the plant are used which in isolation are difficult to identify using morphological features alone. Plants can also be identified on the basis of genetic data and it is proposed to utilize this method to identify the plant bark under investigation in this project. DNA barcoding is the use of a short, standardized region of the genome, which can be used to make comparisons across a number of taxa (Hollingsworth et al., 2011). The technique involves the sequencing of the barcode, for example the plastid chloroplast *matK* or *rpoC1* genes from an unknown plant species and compares the sequences using an algorithm against other *matK* or *rpoC1* sequences in a database such as Genbank (http://www.blast.ncbi.nlm.nih.gov). Only a very small quantity of plant material is required and it can be obtained from any part of the plant. This technique is therefore invaluable where standard morphological features or specialist taxonomical knowledge is in short supply.

The process of choosing a barcode that is representative of the wide variety of plant life is challenging. The boundary between finding a region of the genome that is evolving rapidly to provide the clear distinction between interspecies variation and the low sequence variation demanded to conclusively determine intra-species differences is marginal. DNA barcoding requires only a short region of such a gene for ease of experimental procedures and cost, and the sequences on either side of the barcode should be conservative to assist with the design of universal primers There is therefore still a lot of contention as to the suitability of a particular region or even how many barcodes are required to identify a plant conclusively (www.rbgkew.org.uk/barcoding). Currently a combination of *matK* and *rpoC1* has been proposed by Royal Botanic Gardens, Kew: DNA Barcoding: Phase 2 Update

2013, (http://www.kew.org/barcoding/update.html) as an option to identify unknown plant species and this will be applied in this project. Interpretation of the results therefore can be challenging particularly in distinguishing between closely related species. This method can also be limited by the input of barcoding sequences into the databases, but is invaluable as a tool in studying biodiversity or simply to identify plant material where access to the plant itself is difficult.

2.1.1 DNA *matK* barcode

Group II introns are a class of self-catalytic ribozymes found in mRNA, tRNA and rRNA of chloroplasts and mitochondria. The group II intron for tRNA lysine UUU, (trnK), contains the maturase (*matK)* coding gene between its 5' exon and 3'-exon, and this is found in chloroplasts of most land plants (Neuhaus &Link, 1987). The *matK* gene is roughly 1570 base pairs and is thought it might be the only group II intron maturase encoded in the chloroplast that catalyzes intron removal from RNA (Barthet et al., 2007). This suggests that it might also be involved in post-transcriptional processing. This region has a high evolutionary rate of substitution that is three times higher at the nucleotide level and six times higher at the amino acid level compared to the chloroplast gene Rubisco (rbcL) that encodes the large subunit of the enzyme ribulose-1, 5-biphosphate carboxylase (Johnson & Soltis 1994). Insertions and deletions (indels) occur frequently and do so in groups of three base pairs thereby maintaining the reading frame. These features make it suitable as a barcode for the identification of plant families (Li et al., 2011). However a number of studies show that no single plastid locus is able to resolve species identification alone, and two or three loci showed better resolution (Hollingsworth et al., 2009).

2.1.2 DNA rpoC1 barcode

This is another locus identified only on the plastid chloroplast genome where the RNA polymerase core subunit gene (*rpoC1)* codes for the DNA-directed RNA polymerase subunit beta in photosynthetic higher plants (Serino et al., 1998). The *rpoC1* barcode has been shown to have success in combination with other barcoding genes like psbA-trnH (Kool et al., 2012). A study on Dendrobium species using the *rpoC1, rpoB* and *matK* together resolved the maximum number of species (Singh et al., 2012).

2.2 Aims

The classification of the bark was unlikely to be achieved through conventional taxonomic study and therefore the purpose of this chapter was to undertake a DNA barcoding study using the *matK* and *rpoC1* genes to identify the bark and leaf of a plant collected in Sarawak, Borneo. This process will be achieved by -

- Extract DNA from the bark and leaf of the plant using commercially available kits.
- Amplify the *matK* and *rpoC1* genes using commercially available primers.
- Use external sources to sequence the genes.
- Align sequences and generate a phylogenetic tree to provide information to identify the plant.

2.3 Methods and materials

Leaf and bark material was obtained from two locations, approximately 15km apart.

In the lab the plant material was prepared by freezing samples in liquid nitrogen and pulverizing frozen samples in a mortar before DNA was extracted using a commercially available kit (Qiagen DNeasy Plant Mini Kit), and following the manufacturer's instructions. The DNA concentration and purity were assessed using Thermo Scientific^{TM}, Nanodrop before amplification.

The *matK* and rpoC1 regions of the chloroplast were amplified using the degenerate primers *matK*472F (5'-CCC RTY CAT CTG GAA ATC TTG GTT C-3'); *matK*1248R (5'-GCT RTR ATA ATG AGA AAG ATT TCT GC-3') (Yu et al, 2011) and *LP1*F (5'- TATGAAACCAGAATGGATGG-3'); *LP5*R (5'-CAAGAAGCATATCTTGASTYGG-3') (Hollingsworth et al, 2009).

The PCR reaction mixture consisted of 1× PCR buffer, 0.2 mM of each dNTP, 0.5 μM each primer, 1 U Taq polymerase, and 5–50 ng template DNA. Thermal cycling conditions for *matK* and rpoC1 were: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 48°C for 40 s, 72°C for 1min, and a final extension at 72°C for 10 min. The PCR products were verified by electrophoresis in 2% agarose gels stained with SafeView (NBS Biologicals). PCR products are 776bp (*matK*) and 533bp (*rpoC1*). PCR products were sent to Source Bioscience (www.sourcebioscience.com) for sequencing.

Sequence data were checked for double peaks and noise before inputting into NCBI GenBank's MEGABLAST (Basic Local Alignment Search Tool) to identify the most

similar sequences in the Genbank library (McGinnis et al., 2004). The top 50 sequences with a minimum 98% identity and >95% sequence coverage were retrieved for *matK* only, as sequence coverage for *rpoC1* was much more limited. These sequences included a number of duplicates and *matK* sequences of variable lengths requiring the sequences to be trimmed to 697 nucleotides to reduce the impact of having missing data at the terminal ends of the sequence. In order to provide a reference point for the clade that included the unknown species, an outgroup was selected. Pterocarpus acapulcensis and Tipuana tipu were selected for this purpose and their sequences retrieved from Genbank (Cardoso et al., 2013). This resulted in the file now containing a total of 36 sequences including that of the unknown bark sequence. Sequence alignments were performed using CLC Sequence Viewer (Version 7) employing progressive alignment where multiple alignments are generated through the successive construction of pairwise alignments. The *matK* phylogenetic tree was inferred using Bayesian inference and implemented in MrBayes (version 3.2) using the default parameters (Ronquist et al., 2012). The Bayesian output tree was then visualized and edited using FigTree (Version 1.4.2) (Rambaut 2012 http://tree.bio.ed.ac.uk/software/figtree/).

2.5 Results

2.5.1 *matK*

2.5.1.1 Comparison of the aligned *matK* sequence from leaf and bark material

```
     486
Leaf:AAATCTTGGTTCAAAGTCTTCGATACTGGGTGAAAGATCCCCCCTTCTTTCA
Bark:**************************************************
     538
Leaf:TTTATTAAGATTGTTTATTTATGAGTATTGTAATTGGAATAGTCTTATTACT
Bark:**************************************************
     590
Leaf:AAAAAAAAACTTATTTCTACTTTTTCAAAAAGTAATCCAAGAATTCTCTTGT
Bark:**************************************************
     642
Leaf:TCCTATTTAATTTTTATGTATGTGAACACGAATCCATCTTCCTTTTTCTACG
Bark:**************************************************
     694
Leaf:TAAGAGATCCTCTTATTTACGATTAAACTCTTTTATCGTTATTTTTGAGCGA
Bark:**************************************************
     746
Leaf:ATCTATTTCTATGCAAAAATCGAACATCTTGTGGAAGTCTTTTCTAAGAATT
Bark:**************************************************
     798
Leaf:TTTCGTCTACCTTATCATTCTTCAAGGATCCTTTGATTCATTATGTTAGATA
Bark:**************************A***********************
     850
Leaf:TCAAGGAAAAGCCATTCTGGCTTCAAAGAATGCGCCTCTTTTGATGAATAAA
Bark:**************************************************
     902
```

```
Leaf:TGGAAACACTATCTCATCTATTTCTGGCAATGTAATTTTGATGTTTGGTCTC
Bark:**************************************************
     954
Leaf:AACCTGGAACGATCCATATAAATCCATTATTATCCGAGAATTCATTTCACTT
Bark:**************************************************
     1006
Leaf:TTTTTGGGGGGGCTATCTTTCAAATGTGCGGCTCAATTTTTCAGTGGTCCGG
Bark:**************************************************
     1058
Leaf:AATCAAATGCTAGAAAATTCATTTCTAATCGAAATTCTTATGAAAAAGCTTG
Bark:**************************************************
     1110
Leaf:ATACAATAGTTCCAATTATTCCTTTAATTAGATCTTTGGCTAAAGCGAAATT
Bark:**************************************************
     1162
Leaf:TTGTAATATATTAGGGCATCCCATTAGTAAGCCTGTTTGGGCCGATTCATCC
Bark:**************************************************
     1214
Leaf:GATTTTGATATTATTAACCGATTTTTGCGGATATGCAGAAATCTTTCTCATT
Bark:**************************************************
     1266
```

Figure 2 - 1: *matK* sequence from leaf and bark material showing the single variant at 824G/A

2.5.1.2 Sequence plots.

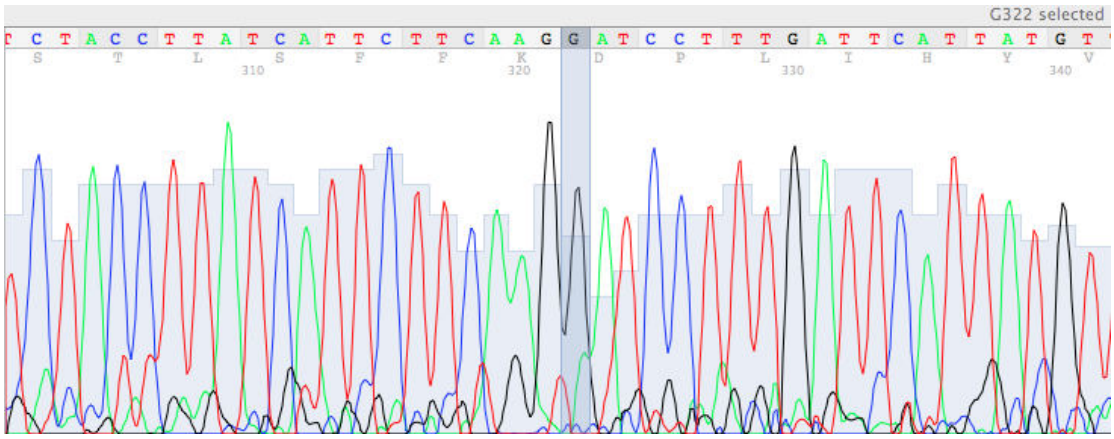


Figure 2 - 2: *matK* sequence from leaf material confirming the site of variant 824G/A shown on Figure 2-1.

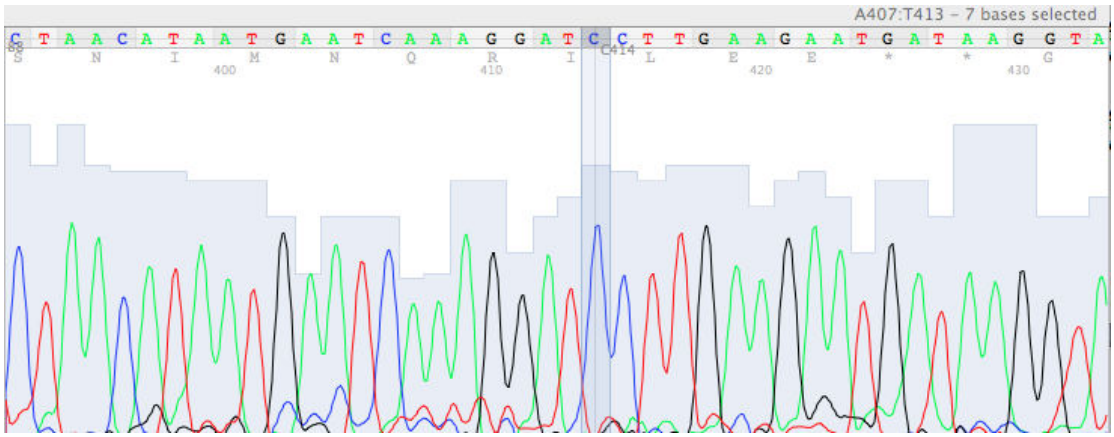


Figure 2 - 3: *matK* reverse sequence from leaf material confirming the site of variant 824G/A shown on Figure 2-1.

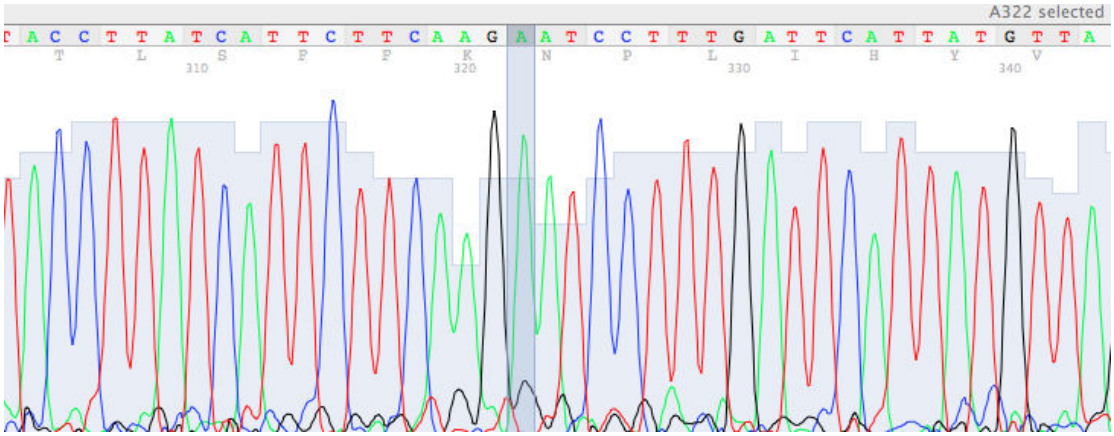


Figure 2 - 4: *matK* sequence from bark material confirming the site of variant 824G/A shown on Figure 2-1.

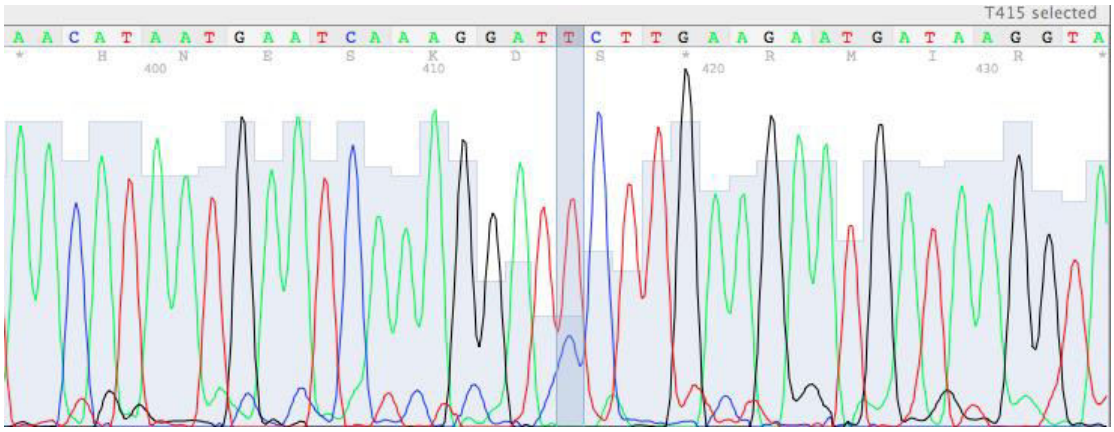
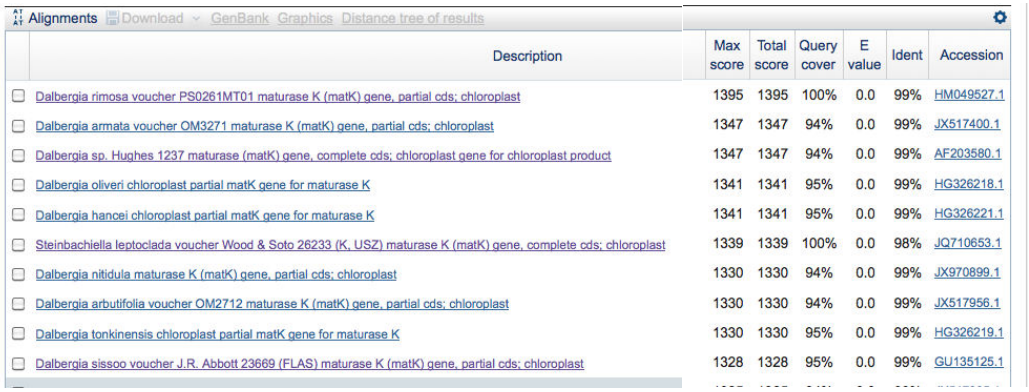


Figure 2 - 5: *matK* reverse sequence from bark material confirming the site of variant 824G/A shown on Figure 2-1.

2.5.1.3 Top ten BLAST hits for the 776bp sequence.



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Dalbergia rimosa voucher PS0261MT01 maturase K (matK) gene, partial cds; chloroplast	1395	1395	100%	0.0	99%	HM049527.1
Dalbergia armata voucher OM3271 maturase K (matK) gene, partial cds; chloroplast	1347	1347	94%	0.0	99%	JX517400.1
Dalbergia sp. Hughes 1237 maturase (matK) gene, complete cds; chloroplast gene for chloroplast product	1347	1347	94%	0.0	99%	AF203580.1
Dalbergia oliveri chloroplast partial matK gene for maturase K	1341	1341	95%	0.0	99%	HG326218.1
Dalbergia hancei chloroplast partial matK gene for maturase K	1341	1341	95%	0.0	99%	HG326221.1
Steinbachiella leptoclada voucher Wood & Soto 26233 (K, USZ) maturase K (matK) gene, complete cds; chloroplast	1339	1339	100%	0.0	98%	JQ710653.1
Dalbergia nitidula maturase K (matK) gene, partial cds; chloroplast	1330	1330	94%	0.0	99%	JX970899.1
Dalbergia arbutifolia voucher OM2712 maturase K (matK) gene, partial cds; chloroplast	1330	1330	94%	0.0	99%	JX517956.1
Dalbergia tonkinensis chloroplast partial matK gene for maturase K	1330	1330	95%	0.0	99%	HG326219.1
Dalbergia sissoo voucher J.R. Abbott 23669 (FLAS) maturase K (matK) gene, partial cds; chloroplast	1328	1328	95%	0.0	99%	GU135125.1

Figure 2 - 6: Top ten *matK* BLAST hits.

2.5.1.4 Comparison of leaf sequence with Dalbergia rimosa

Leaf material (Query) and Dalbergia rimosa (subject "*") is detailed below (Figure 2.7) and shows a total of nine SNP in a 776 base pair sequence.

```
Query  121  CTACTTTTTCAAAAAGTAATCCAAGAATTCTCTTGTTCCTATTTAATTTTTATGTATGTG  180
Sbjct  158  ****************************************A**************A**  217
Query..421..AACACTATCTCATCTATTTCTGGCAATGTAATTTTGATGTTTGGTCTCAACCTGGAACGA  480
Sbjct  458  ****************************C*****T**********************  517
Query  541  CAAATGTGCGGCTCAATTTTTCAGTGGTCCGGAATCAAATGCTAGAAAATTCATTTCTAA  600
Sbjct  578  **************************************************G*********  637
Query  601  TCGAAATTCTTATGAAAAAGCTTGATACAATAGTTCCAATTATTCCTTTAATTAGATCTT  660
Sbjct  638  **********G*******A****************************************  697
Query  721  ATTCATCCGATTTTGATATTATTAACCGATTTTTGCGGATATGCAGAAATCTTTCTCATT  780
Sbjct  758  G**************************************************T*********  817
```

Figure 2 - 7: Sequence variation between leaf material and *Dalbergia rimosa*

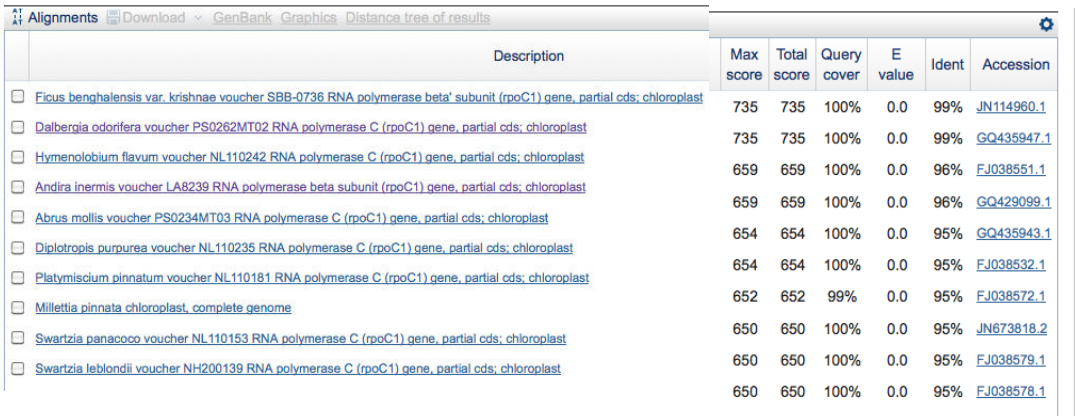
2.5.2 *rpoC1*

2.5.2.1 Leaf sequence

GACTCTGCTTGGCAAACGGGTTGATTATTCGGGACGTTCTGTTATTGTAGT
AGGTCCATCACTTTCATTACATCGATGTGGATTACCTCGCGAAATAGCAAT
AGAGCTTTTCCAGACATTTGTAATTCGTGGTCTAATTCGAAAACATTTTGC
TTCGAACATGGGAATTGCTAAGAGTAAGATTAGGGAAAAAGAACCGATTG
TATGGGAACTACTTCAAGAAGTTATGCAGGGGCATCCCGTATTGCTAAAT
AGAGCGCCTACTCTGCATAGATTAGGTATACAGGCATTCCAACCCATTTTA
GTAGAAGGGCGTGCTAtttGTTTACATCCATTAGTTTGTAAAGGATTCAATG
CAGACTTTGATGGAGACCAAATGGCTGTTCATGTGCCTTTATCTTTGGAAG
CTC

Figure 2 - 8: Barcode *rpoC1* sequence from leaf material.

2.5.2.2 Top ten Blast hits of 410base pair edit of the sequence.



Description	Max score	Total score	Query cover	E value	Ident	Accession
Ficus benghalensis var. krishnae voucher SBB-0736 RNA polymerase beta' subunit (rpoC1) gene, partial cds; chloroplast	735	735	100%	0.0	99%	JN114960.1
Dalbergia odorifera voucher PS0262MT02 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	735	735	100%	0.0	99%	GQ435947.1
Hymenolobium flavum voucher NL110242 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	659	659	100%	0.0	96%	FJ038551.1
Andira inermis voucher LA8239 RNA polymerase beta subunit (rpoC1) gene, partial cds; chloroplast	659	659	100%	0.0	96%	GQ429099.1
Abrus mollis voucher PS0234MT03 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	654	654	100%	0.0	95%	GQ435943.1
Diplotropis purpurea voucher NL110235 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	654	654	100%	0.0	95%	FJ038532.1
Platymiscium pinnatum voucher NL110181 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	652	652	99%	0.0	95%	FJ038572.1
Millettia pinnata chloroplast, complete genome	650	650	100%	0.0	95%	JN673818.2
Swartzia panacoco voucher NL110153 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	650	650	100%	0.0	95%	FJ038579.1
Swartzia leblondii voucher NH200139 RNA polymerase C (rpoC1) gene, partial cds; chloroplast	650	650	100%	0.0	95%	FJ038578.1

Figure 2 - 9: Top ten *rpoC1* BLAST hits.

2.5.2.3 Sequence variation in rpoC1 between leaf and Dalbergia odorifera

The variation in the leaf sequence (query) and Dalbergia odorifera (subject) showed a total of one SNP in a 410 base pair sequence.

Query 181

```
GATTAGGGAAAAAGAACCGATTGTATGGGAACTACTTCAAGAAGTTATGCAGGGGCATCC  240

Sbjct 184

A**********************************************************  243
```

Figure 2 - 10: Site of sequence variation between leaf material and *Dalbergia odorifera*

2.5.2.4 Phylogenetic tree of 36 closely related Dalbergia sequences

Bayesian inference was used to estimate the phylogenetic trees using the subset of Dalbergia species identified on Genbank as being closely related to our bark sample based on the results of the BLAST search. The bark sample was named Dalbergia Biddayuh in recognition of the community from which the sample was sourced and aligned together with the other sequences.

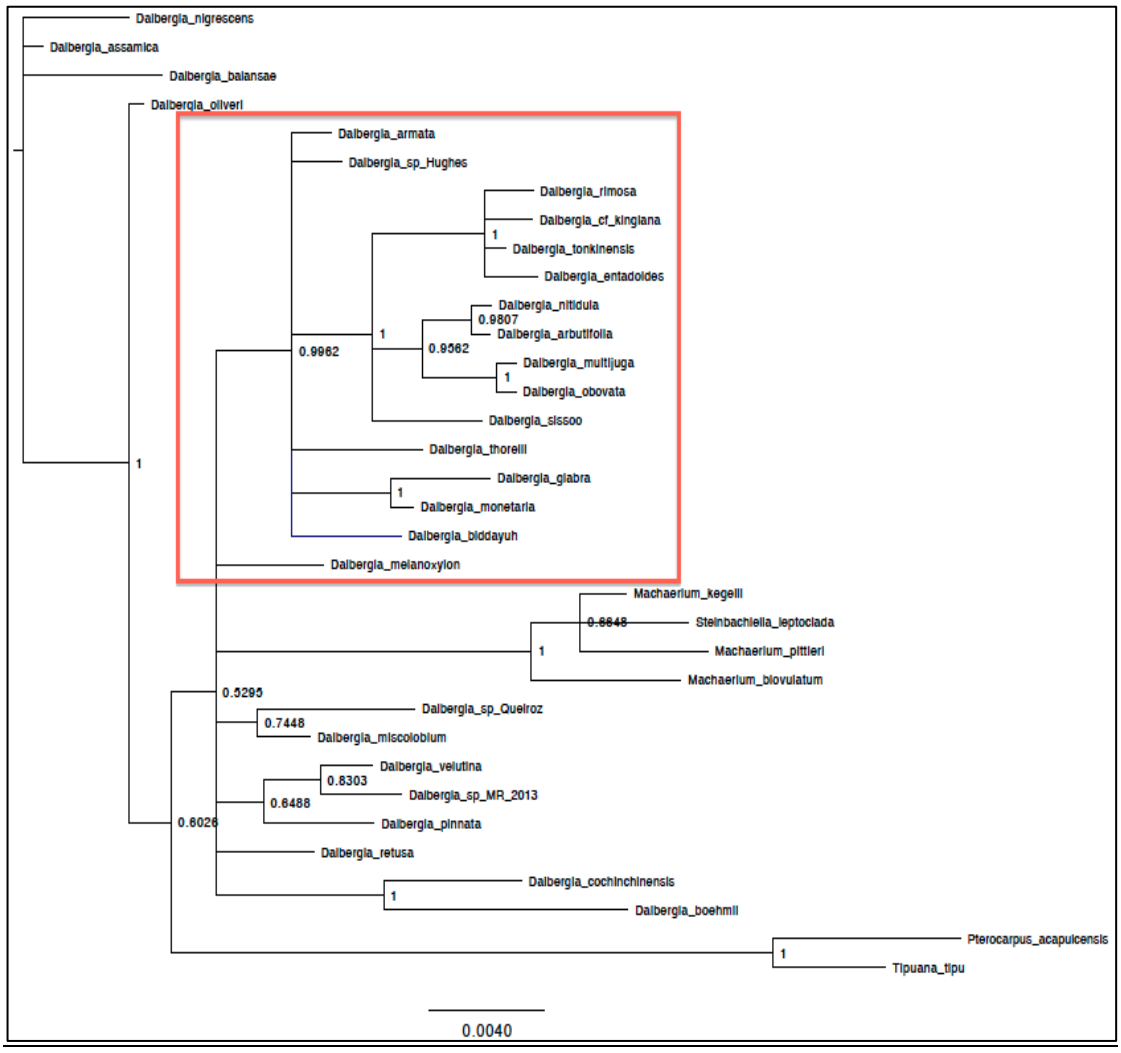


Figure 2 - 11: The consensus tree resulting from Bayesian analysis of the *matK* gene of Dalbergia species closely related to that of the unknown sample. Numbers besides nodes are posterior probabilities.

Bayesian inference analysis was repeated using the *matK* species sequences highlighted in the red box (Figure 2.11) to rule out any ambiguous placement of the unknown sample. The standard deviation of split frequencies achieved after 30,000 generations was 0.0114 for the 1st experiment and in the 2nd experiment using only a subset of sequences 0.011113 was achieved (Figure 2.12) below.

2.5.2.5 Phylogenetic analysis of subset of 16 sequences highlighted above

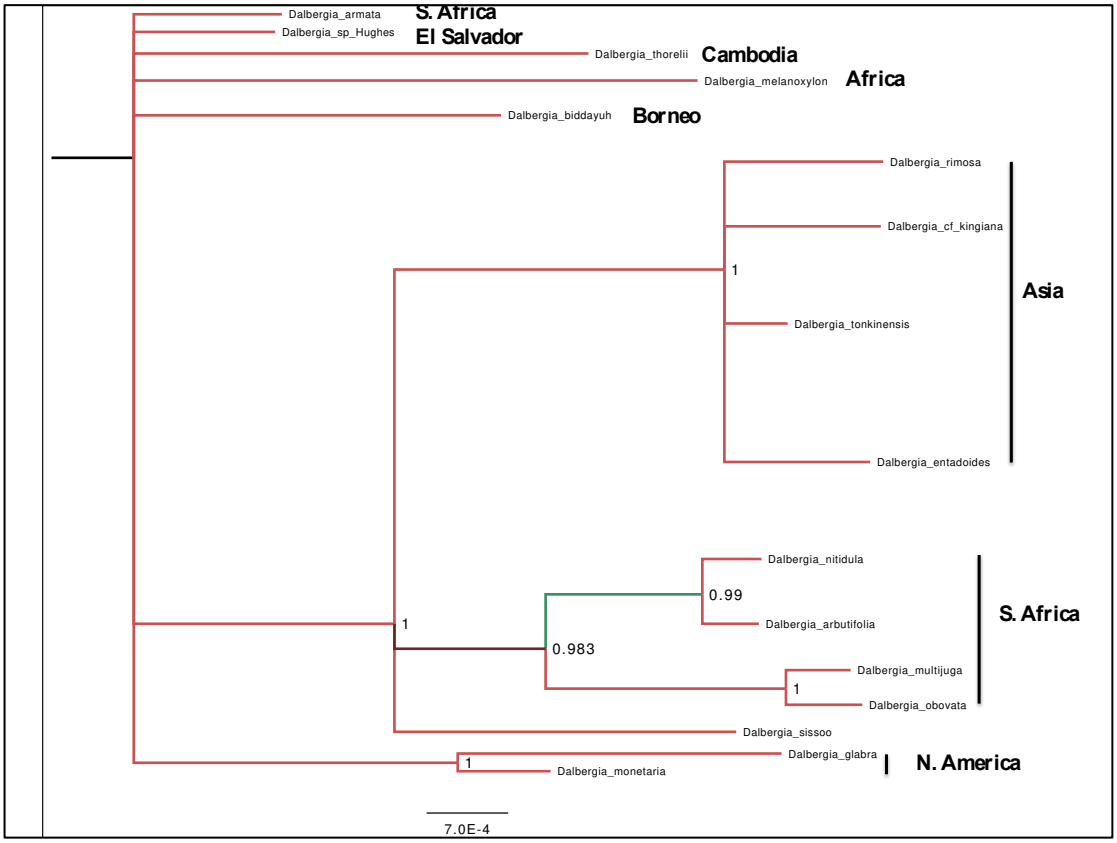


Figure 2 - 12: Consensus tree derived from the *matK* Bayesian analysis of the subset of Dalbergia sequences from Figure 2.9 highlighted in red and including biogeographic location.

The tree presented in Figure 2.11 & 2.12 consists of branches that connect nodes, the internal nodes representing hypothetical ancestors. The length of the branches represents the mutational change that is calculated to have occurred between a pair of nodes, and the figures depicted at each node is the probability of a node being correct, that is estimating the uncertainty of a node and is termed the posterior probability (Hall et al., 2013).

2.6 Discussion

The *matK* sequence identified a close similarity (99% identity) between our unknown sample and *Dalbergia rimosa*, a shrub common throughout Asia. The NCBI (The National Center for Biotechnology Information) database has good coverage of the plant kingdom sequenced at the *matK* barcode gene (84,776 sequences), with the top 100 BLAST hits belonging to the *Dalbergia* genus. However, the fact that there are ten SNP variants between the query plant and *D. rimosa* suggests that the plant in question is a separate species. The *rpoC1* sequence has less coverage on the database (9,030 sequences). A Blast search of the full *rpoC1* sequence of 533bp did not achieve close hits, with the highest identity being 95%, belonging to *Andira inermis* (a tree native to South America). However, the majority of the database is composed of rpoC1 sequences that are shorter (410bp) than the 533bp sequence initially submitted. A Blast search of the edited sequence identified two close hits (99% identity): *Ficus benghalensis* and *Dalbergia odorifera*. *F. benghalensis* is a large fig tree native to the Indian subcontinent and *D. odoriferai* a small-medium sized fragrant rosewood tree found throughout Asia. Taken together, it seems that there is reasonable support for the notion that the query species belongs to the *Dalbergia* genus, but belongs to a species not yet identified on the basis of either *matK* or *rpoC1* barcoding genes. The leaf and bark sequences varied by a single base at the *matK* gene, but considering these two samples were collected several km apart, it is likely that this represents intraspecific variation.

In this tree a number of these nodes are supported as having a posterior probabilities of ‘1’ or very close to ‘1’ indicating a high probability that the sequences to the right of the node are exceptionally well supported and correctly grouped together to the exclusion of others, but there are also several nodes with low posterior probability and

which are not clarified on the basis of the current data. To further investigate the support for the relationships in Figure 2.11, the area highlighted in red that also includes *Dalbergia biddayuh* was subjected to further Bayesian analysis and depicted in Figure 2.12 along with the geographical location from which the *matK* sequences were obtained. The species identified from the Americas and South Africa are particularly well resolved into balanced monophyletic clades showing bifurcating nodes supported as having posterior probabilities of '1' or very close to '1'. This is unsurprising as the geographic location is restricted. The Indochina /Asian species despite showing a common ancestor with a high probability factor is not well resolved, but gives rise to a polytomy despite the close geographical locations. It also includes the sequence from Dalbergia rimosa identified as being the most closely related species to Dalbergia biddayuh in Genbank and both sequences are from the Indochina region. Nevertheless these two species are in separate clades to each other. The five species at the top of the tree that includes Dalbergia biddayuh also depicts a polytomy suggesting that there isn't enough data to resolve these lineages further without added information. Nevertheless the bark can be reliably identified as belonging to the Dalbergia genus based on the analysis above, but using the *matK* sequences currently input into Genbank it cannot currently be identified to species level.

The Dalbergia genus has a pantropical geographic distribution comprising 47 genera and is predominantly found in the Neotropics and Africa (Cardoso et al., 2013). Only six of these genera with around 100 plant species are thought to occur in the tropical Asia with the majority belonging to the Dalbergia genus (Vatanparast et al., 2013). Work is on-going to identify the species using taxonomy specialists in this field.

Chapter 3

Isolation, identification and preliminary pharmacological characterisation of bioactive compounds

3.1 Introduction

Dalbergia species are prominent as phytomedicines used by indigenous communities in their healthcare systems (Saha et al., 2013). Flavonoid-type compounds dominate this genus and in particular many studies have identified isoflavonoid, neoflavonoids, cinnamylphenols, furans and other compound types to a lesser extent (Vasudeva et al., 2009). The diversity found in secondary metabolites in plants is immense but they can be broadly categorized into three major groups based on their biosynthetic origins, the terpenoids, the alkaloids and phenolic compounds that contain the flavonoid compounds (Hussain et al., 2012). The beneficial effects of a number of these secondary metabolites are currently under much scrutiny and in this chapter experiments to determine the identity of the compounds responsible for vasorelaxation will be undertaken using bioassay-guided experiments to isolate compounds with vascular activity and to begin preliminary pharmacological characterisation. Furthermore, studies will be undertaken to elucidate any structures isolated.

3.1.1 Rat aortic ring bioassay

Isolated organ bath experiments are used in all the published studies identified in the literature survey to assess the functional responses of bioactive molecules on the vasculature. This assay can be further exploited to assess this functional response in the presence of inhibitors specific for a defined target such as a receptor. At the core of the study is the rat aortic ring bioassay protocols used to identify extracts and compounds that have vasoactive properties. The rings are contracted with phenylephrine (1μM), which causes an initial phasic contraction due to a transient increase in intracellular Ca^{2+} released from the sarcoplasmic reticulum following IP_3-

induced activation. This is followed by a tonic contraction due to Ca^{2+}-influx from the buffer solution via L-type calcium-channels that are inhibited by calcium-channel blockers (Fransen et al.,2012) The bioassay response to this contraction is indicative of the presence of either a single compound or is the effect of several compounds that may work together synergistically. This bioassay-guided approach together with the development of solvent systems and separation methods to identify fractions and sub-fractions should lead to the successful isolation of the compound(s) responsible for this functional response.

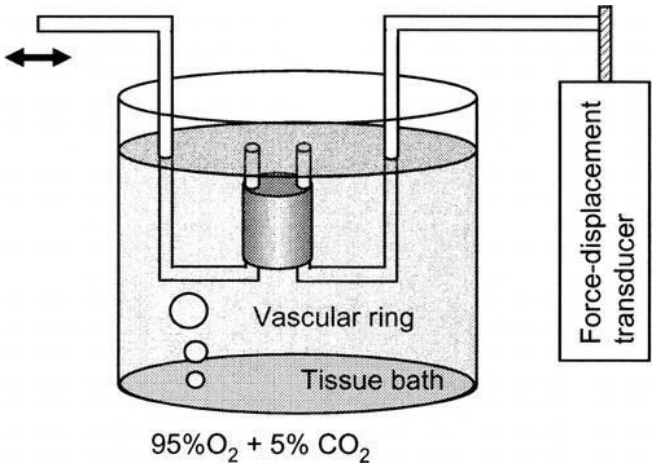


Figure 3 - 1: Illustration of an individual organ bath showing the rings attached to the force-displacement transducer

3.1.2 Extraction techniques

Cold or hot, aqueous or solvent extraction of natural products that have been prepared by sun drying and milling have been undertaken since biblical times and earlier. Many of these techniques are still used today refined by an understanding of the phytochemistry of plants, the range of solvents available and processes that can be

employed to increase yield or limit destruction. The principle of like begets like can be used to isolate particular compounds based on solubility, for example using hexane to isolate fatty compounds or aqueous extracts to isolate glycosides. A range of solvents can be found that can extract compounds preferentially dependent on lipophilicity ranging from polar protic solvents such as acetic acid and methanol through to the non-polar solvents such as pentane and hexane. In between is a range of solvents both borderline aprotic such as dichloromethane and ethylacetate that can be used successfully for extraction. These solvents combined with methods that can increase yield such as heat or reduce solvent consumption such as soxhlet extraction methods can prove useful in this study (Bucar et al., 2013). A number of these solvent systems have been used to identify extracts with vasorelaxant activity and are detailed in the literature survey.

3.1.3 Separation techniques

Chromatographic methods are analytical tools originally discovered by the Russian botanist Mikhail Tswett (1872-1919), and is used to separate and identify individual compounds in a multicomponent systems. Thin layer chromatography (TLC), column chromatography (CC) and high performance liquid chromatography (HPLC) are used extensively to separate compounds. The system relies on a stationary phase onto which the analyte is adsorbed, and is usually comprised of polar, inorganic compounds such as silica or alumina. The degree of adsorption will depend on the chemical nature of the stationary phase and its surface area, and the nature of the solute and solvent. Mobile liquid phase optimisation is key to separation of extracts, which more often than not comprise a mixture of lipophilic and hydrophilic constituents. Repeated CC and TLC are often used first due to cost benefits and these results often each feed into the next series of experiments to refine the active fraction.

CC provides a means of separating large volumes while TLC provides a quick and cheap way of fingerprinting fractions.

HPLC is a high-definition separation method which when coupled with UV detection produces a spectrum of the eluting peaks. It relies on the mechanisms of adsorption and partitioning on a solid stationery column, which is encased in a stainless steel metal case. It is eluted with a solvent system using either a gradient elution system or an isocratic solvent mixture. It has many advantages such as speed, high resolution and high sensitivity detecting compounds down to the nanogram level. It is also highly reproducible and the elution process can be automated. There are two main types of HPLC, normal and reverse-phased chromatography. The latter applies the principles of hydrophobicity, as the stationary phase is non-polar comprising silica with carbon chains attached to it of various lengths, the most common of which is the alkyl chain $C_{18}H_{37}$. The particle size, shape, porosity and packing of the column need to be uniform to provide reproducible and consistent results. The mobile phase is now polar and usually comprises of water and a less polar solvent such as methanol or acetonitrile (Willow, 2011).

3.1.4 Structure elucidation techniques

There are a number of techniques, which can be employed to decode chemical structures isolated from natural products, and these need to be used in a complementary fashion as each technique has different strengths and weaknesses. Mass spectrometry (MS), ultraviolet (UV, infrared (IR), Nuclear magnetic resonance (NMR) spectra and X-ray crystallography will all be required. Of these NMR is the most important spectral technique for structure elucidation.

3.1.4.1 Mass spectroscopy

MS is used to determine the molecular weight of a compound. Different techniques exist such as electron impact techniques that can ionise and fragment the molecule giving invaluable information of the structure, or gentler techniques such as electrospray ionization (ESI) can be used to ionize the entire molecule without fragmenting it thereby providing information on the complete structure. Accurate mass measurements can also provide information on the number of carbon, hydrogen and oxygen atoms that are included in the molecule, from which a molecular formula can be predicted.

3.1.4.2 UV spectroscopy

UV techniques are used extensively with CC, TLC and HPLC at 254nm and 360nm to identify the double bond in phenolic compounds and other compounds such as aldehydes and ketones. Diagnostic spray reagents can also be used in conjunction with UV to detect a wide variety of compounds (Wagner H & Bladt S, Springer 1996).

3.1.4.3 NMR spectroscopy

^{1}H and ^{13}C are the most commonly measured nuclei in NMR as a nucleus containing an odd number of protons is required. Tetramethylsilane (TMS) is used as an internal standard as it is inert and shows a single signal from which all other signals are defined. The three most important parameters in NMR spectra are the chemical shift (δ), which shows how shielded or deshielded a proton is in the molecule, coupling which can result in splitting patterns identifying close neighbours, and the peak area, which is proportional to the number of protons detected. The information provided by these 1D spectra to elucidate structures can be further enhanced by undertaking 2D correlation spectroscopy with either a single type of nucleus or two different nuclei.

The former is called homonuclear e.g. COSY spectra, and the latter heteronuclear correlation spectroscopy e.g. HSQC or HMBC spectra. A COSY spectrum provides information between protons that are close together and no more than two, three or four bonds distant from each other. HSQC spectra identifies the protons that are attached to carbons and HMBC spectra show distances between protons and carbon atoms that are two to three bonds apart. These spectra taken together can provide a picture of the molecule that is generally unambiguous.

3.1.4.5 X-ray crystallography

X-ray crystallography provides the most comprehensive of these techniques to identify the 3-D structure of chemical compounds. This requires the synthesis of a crystal of the correct proportions through which an X-ray is passed. The diffraction pattern generated, which is representative of the 3-D molecule, can be interpreted using computer programs.

3.2 Aims

The aim of this chapter is to undertake the rat aortic ring bioassay-guided identification of solvent extracts from the bark of this Dalbergia species, from which fractions and sub-fractions will be identified using chromatographic techniques developed specifically for this purpose. This process will be repeated until the compounds responsible for the vasorelaxant functional response are revealed.

The compounds isolated will be subjected to extensive structure elucidation work by recording and analysing MS, UV and detailed 1D and 2D ^{1}H and ^{13}C NMR spectra. In addition it is intended to perform X-ray crystallography work on any crystal structures isolated.

Finally preliminary pharmacological characterisation of the isolated compound to compare the activity with that of the original extract will also be undertaken.

3.3 Materials

Fisher Scientific (UK)

Acros Organics Silica gel mesh size 0.035-0.07mm 60Å

Analytical grade hexane, ethylacetate, methanol, dichloromethane, chloroform, Sulphuric acid and Vanillin

HPLC grade methanol and deuterated chloroform

All general lab reagents and all tissue culture plastics

Whatman cellulose soxhlet extraction thimble (100mm)

Fortis Technologies Ltd (UK)

HPLC analytical column 5μM C18, (150 x 4.6mm) GE Healthcare

Merck (UK)

Thin layer chromatography plates – Silica gel $60F_{254}$ Aluminium backed

Phenomenex (UK)

Preparative HPLC column – Gemini 5μM C18, (250 x 21.2mm)

Sigma (Poole, UK)

Acetylcholine, Phenylephrine, L-NG-Nitro-L-arginine methyl ester (CAS 51298-62-5) (L-NAME), Dimethyl sulphoxide, Sodium nitroprusside

Plant material was purchased from Borneo

Batch 1 – July 2009

Batch 2 – November 2013

Batch 3 – February 2014

Batch 4 August 2014

3.4 Methods

3.4.1 Preparation of plant material

3.4.1.2 Collection

Collection of the bark occurs throughout the year by older villagers. It is the outer bark that includes dead tissue that is harvested.

3.4.1.2 Drying

Once harvested the bark is sun dried. Samples were stored in a freezer and thawed and dried in a fume cupboard as required.

3.4.1.3 Milling

The dried bark was ground in a Culatt C213 Swing Hammer Mill. The milling produced a mixture of finely shredded and some powdered material. The increase in surface area of the particulate matter improves the results of the extraction process.

3.4.2 Extraction methods

A number of different solvent extraction methods were employed to isolate vasoactive compounds. In all cases the extract was concentrated under vacuum in a rotary evaporator VWR (RV10 digital FLEX) by IKA, after which the extract was resolubilized in either water only or with less than 3% DMSO where required before testing on rat aortic rings. The concentration of all extracts used reflected the equivalent of 1g of bark in 6mls of water.

3.4.2.1 Aqueous extract preparation

An extract was prepared by adding bark to hot water for 10 minutes or using a method that mimicked the indigenous preparation of the bark. This involved the preparation of a 'tea', made by boiling 10g of bark in approximately 100mls water for 30minutes to produce a red wine coloured liquid.

3.4.2.2 Cold solvent extraction

Hexane, chloroform, dichloromethane and methanol extracts were prepared by placing bark in a cellulose soxhlet extraction thimble stoppered with cotton wool and left in a beaker with enough solvent to cover the plant material for an hour.

3.4.2.3 Soxhlet extraction

This involves the continuous cycling of fresh solvent into the reflux condenser bearing plant material encapsulated in a cellulose thimble. The fresh solvent reaching the condenser on each cycle is therefore able to further extract the bark on each cycle to achieve a greater concentration of extract than would be possible on a single cycle. A sequence using solvents of increasing polarity was used to extract the plant material thus simplifying the composition according to solubility. The sequence used below involved approximately 20 cycles per hour and was used to extract the bark.

Extraction process	**Solvent**	**Time (Hrs)**	**Extract characteristics**	**Yield per 1kg**
Cold (defatting)	Hexane	1	Pale yellow	0.3g
Soxhlet	Hexane	20	Green-yellow	8.81g
Soxhlet	Ethylacetate	3	Pale orange	3.4g
Soxhlet	Methanol	3	Wine red	15.2g

Table 3 - 1: Bark solvent extraction sequence

3.4.3 Separation of extracts

3.4.3.1 Hypersep-Pak LC-18 cartridges

In order to separate the components in the aqueous extract, the 'tea' was applied to a Hypersep-Pak LC-18 cartridge used for reverse-phase extraction. This was expected to give good separation of moderately polar to non-polar compounds with strong retention of the less polar components of the tea. Four cartridges were prepared with a 1g equivalent 'tea'. Each column was eluted with 20mls of increasing concentrations of aqueous methanol (20% (control), 40%, 60% and 80%), evaporated to dryness and resolubilized in water before testing on endothelium-intact rings. The tea components not retained on the column was also tested for activity. Fifteen aliquots of 50µl each, representing a concentration range of 0.261mg/ml – 3.915mg/ml was added at 1.5 min intervals to each bath.

3.4.3.2 Chromatographic separation of crude soxhlet hexane extract (Table 3-2)

A large glass column was plugged with glass wool, over which 0.5cm layer of chromatography grade sand was poured. Hexane was poured onto this to release any air bubbles that may have been trapped. A slurry containing 80g of silica gel mixed with 100mls of hexane was poured in and more sand added on top to protect the column. The slurry had previously been placed on a sonicator to remove any air bubbles. When the solvent was approximately 1-2ml from the top of the slurry, the soxhlet hexane extract (0.5g) suspended in hexane was pipetted gently on top of the layer of sand. In total a solvent gradient of 450mls of eluent comprising hexane : ethylacetate + 1% glacial acetic acid in 50ml combinations of increasing polarity was added as in the following ratios 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. This was followed by 150ml ethylacetate : methanol + 1% glacial acetic

acid combinations 90:10, 75:25 and 50:50. In total 80, 7-8ml fractions were obtained from each column. Each of these fractions was subjected to TLC and grouped.

3.4.3.3 Thin Layer Chromatography

A TLC method was developed for the separation and identification of fractions from the hexane extract of the bark. A number of solvent combinations were tested on 10x10cm silica plates backed with aluminium and the hexane-ethylacetate combination was selected for further development and tested with and without 1% glacial acetic acid or 1% ammonium hydroxide solution. In total eleven grouped fractions (A-K) were identified from the 80 fractions isolated above. This solvent system provided an R_f of the active fraction components between 0.2 and 0.4.

a) TLC Detection Methods

i) UV detection at 254nm and 360nm

ii) 20% concentrated sulphuric acid in 50% methanol was used to spray TLC plates. The plates were heated at 100°C in an oven for 5-10mins until the spots developed. Charring showed the presence of compounds.

iii) Vanillin-concentrated H_2SO_4 spray used for terpenoids and phenylpropanoids, the latter having been identified in Dalbergia species, was prepared by combining 0.25g vanillin and 50mls concentrated sulphuric acid–ethanol (80:20) on ice. The plates were sprayed in a fume cupboard and heated at 100°C in an oven for 5-10mins until the spots developed. Purple spots showed the presence of compounds.

3.4.3.4 Column Chromatography method (2) for separation of fraction H&I

The combined active fractions (H&I) were further separated using a second silica gel column and a solvent combination comprising 50ml dichloromethane-methanol 96:4 with the following ratios 100:0, 96:4, 90:10 and 80:20. A smaller glass column

(1.5x30cms) was prepared with 30g of silica gel using the method in section 3.4.3.1 above, and using dichloromethane to prepare the slurry.

A total of 40 fractions obtained were combined to form four groups (H1, H2, H3, H4) following TLC studies and these were tested on rat aortic rings.

3.4.3.5 High Pressure Liquid Chromatography techniques

a) Analytical HPLC

The method identified here used an HP 1050 HPLC system together with a Fortis HPLC analytical reverse-phase C18 column 5µm C18, (150 x 4.6mm) eluted with an isocratic solvent mixture of methanol-HPLC grade water 50:50 using a flow rate of 1ml/min and UV detection at 254nm. This method achieved the separation of the two isomeric compounds found in fraction H2 and H3 combined from the second CC method.

b) Preparative HPLC

This method was initially scaled up using a preparative HPLC column, the Phenomenex Gemini 5µm C18, (250 x 21.2mm) on a Waters HPLC. Solvent system was as above, an isocratic methanol : water 50:50 with a flow rate of 20mls/min and UV detection at 254nm. Unfortunately after a short use the machine was irreparable and separation was only possible on the analytical.

3.4.4 Structure elucidation methods

3.4.4.1 Mass spectrometry

Molecular Mass spectra were obtained using ESI – TOF techniques on a Bruker microTOF HCT PLUS.

3.4.4.2 UV/visible spectra

UV spectra of the grouped fractions containing either S/F4 or S/F3 were prepared in methanol and obtained on the UV/Vis Perkin Elmer Lambda 45.

3.4.4.3 NMR spectra

^{1}H and ^{13}C NMR spectra were run at 25°C on a Bruker Avance 400 spectrometer at 400MHz and 100MHz respectively, by Dr Howard Dodd, at the University of Brighton. The NMR solvent was deuterated chloroform or DMSO, and chemical shifts are given in δ (ppm). ^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC and NOE spectra were acquired using standard pulse sequences and parameters supplied by Bruker.

3.4.4.4 X-ray crystallography

Samples of S/F4 (caviunin) were prepared in hexane, cyclohexane, diethyl ether, chloroform, dichloromethane, ethylacetate, ethanol and methanol by heating the compound until it dissolved and slow-cooled in the vials and left in the fridge for three months. Crystals were identified in the ethanol solvent only and sent to the X-ray lab at Sussex University where it was tested on an Agilent Gemini Ultra machine.

3.4.5 Pharmacology : Rat aortic ring bioassay preparation

3.4.5.1 Vascular tissue preparation

Male Sprague-Dawley rats (180-220g) were sacrificed according to Home Office rules, dissected and the thoracic aorta removed, and cleared of peri-adventitial fat and connective tissue. Rings, 2-3mm were cut and in some the endothelium was left intact and in others the endothelium was removed. The tissues were immediately mounted in 8ml tissue baths filled with Krebs solution, with a composition (in mmol/L) - $CaCl_2$ 1.6, $MgSO_4$ 1.17, EDTA 0.026, NaCl 130, $NaHCO_3$ 14.9, KCl 4.7, KH_2PO_4 1.18, and glucose 5, and kept warmed (37°C) and gas-equilibrated (95%O_2, 5% CO_2).

3.4.5.2 Measurement of ring tension

Isometric tension of the rings was measured with isometric transducers (DMT), digitised using PowerLab and displayed on a Macintosh laptop with LabChart™ software. A preload tension of 1.5g was applied during a 40 minutes equilibrium period, during which time Krebs solution was replaced at 10minute intervals. Dose response curves to phenylephrine (1nM to 100μM) were first obtained, followed by subsequent washing with Krebs solution. The rings were pre-contracted with 1μM phenylephrine and left for 10 minutes to achieve a stable contraction prior to testing the plant extracts.

3.4.5.3 Experimental protocols

All extracts were assayed using ten cumulative concentrations of 50μl at 90sec intervals representing 0.261mg/ml – 2.61mg/ml of bark. In some experiments the concentration range was extended to 0.261mg/ml – 3.915mg/ml. This was the equivalent of 1g of extract solubilised in 6mls water. DMSO was used to solubilise some components at less than 3% volume. Removing the endothelium by gently

abrasion with a matchstick tested for endothelium-dependency. The integrity of the rings was tested with acetylcholine (1μM) or sodium nitroprusside (1μM) at the end of an experiment. The assessment of signaling was undertaken using the rat aortic rings in conjunction with inhibitors. The inhibitors were incubated for 20 minutes prior to the addition of phenylephrine to precontract the rings.

3.4.6 Endothelial cell culture assay

A number of endothelial cell lines were investigated for their ability to release NO following agonist stimulation. Cell culture techniques used and details of treatment of cells with a number of known agonists are given, together with the NO detection methods employed (Appendix 1). NO was not detected consistently from the cell lines studied to provide a reliable assay to identify vasoactive fractions. Primary cell culture lines were not investigated due to cost.

3.4.7 Statistical analysis

The results are from 'n' animals expressed as the mean ± S.E.M of the percentage relaxation induced taking the maximal phenylephrine (1μM) – induced contraction as 100%. To test if the transformed biological activity data for each "drug" was normally distributed or not, the D'Agostino-Pearson test to compute a single P value was undertaken where $P > 0.05$ indicates a Gaussian distribution. Concentration response curves were analysed by non-linear regression using GraphPad Prism to calculate the $EC_{50,}$ the median effective concentration. Analysis of variance between groups was assessed by 2-way ANOVA to determine statistical significance followed by Bonferroni post hoc tests using GraphPad Prism version 5.Oc for AppleMac (www.graphpad.com) where $p \leq 0.05$ was considered statistically significant.

3.5 Results

3.5.1 Schematic showing solvent extraction and isolation sequence

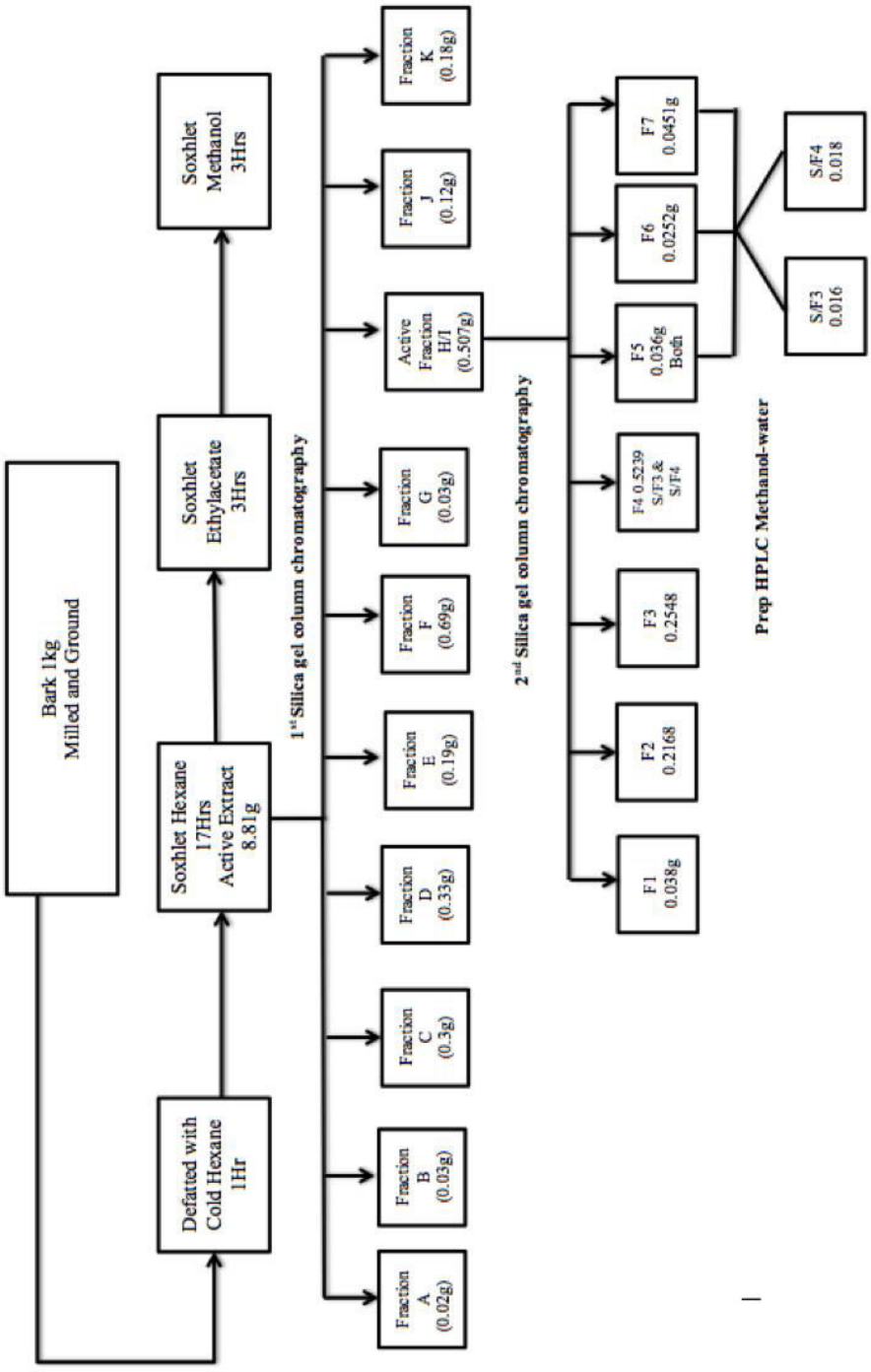


Table 3 - 2: Simplified diagram for extraction and isolation of vasorelaxant compounds

3.5.2 Aqueous extract

The key results from the extensive extraction experiments undertaken are presented here.

3.5.2.1 Vascular effects of the aqueous extract and in the presence of L-NMA

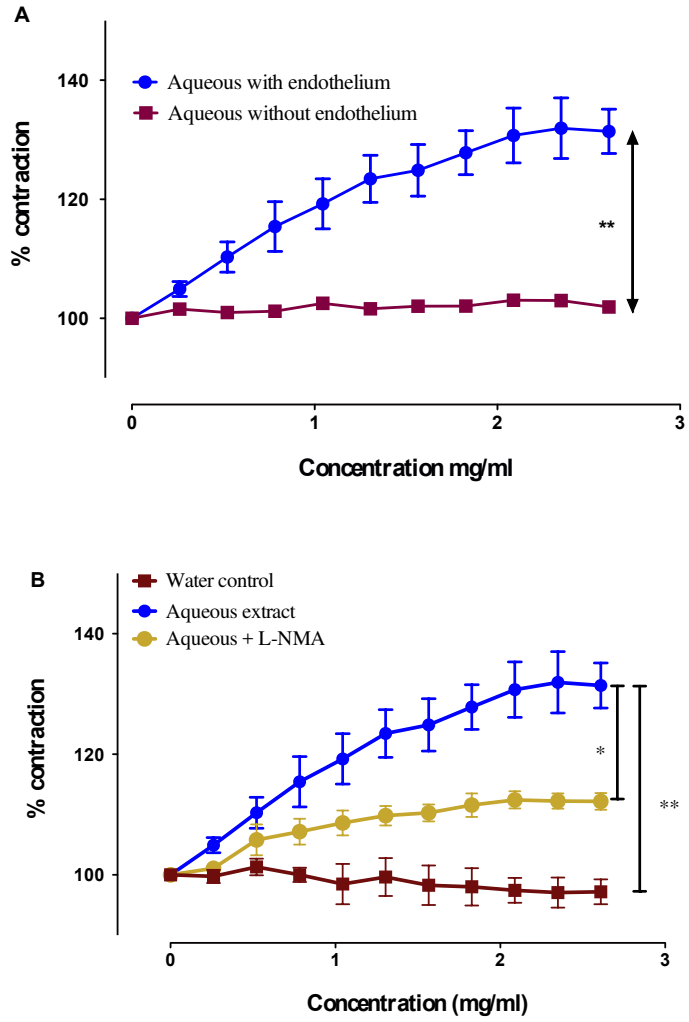


Figure 3 - 2: (A) shows the vascular response of the aqueous extract of bark on endothelium-intact (control) and denuded rings precontracted with phenylephrine (1μM). (B) shows the effect of L-NMA on the aqueous extract on endothelium-intact rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus control (*p≤0.05, **p≤0.01).

The graph above represents the response obtained by adding the bark to water for ten minutes. The contractile response of approximately 30% is endothelium-dependent rising steeply with the addition of the initial concentration. By the 8th aliquot the curve begins to plateau suggesting it has reached its maximal response. By preparing a 'tea' by boiling the bark in water for 30 min to mimic the local use, we obtained a contractile response of over 100%. (Results not shown here). Surprisingly incubation with L-NMA, a non-selective inhibitor of NOS showed a significant attenuation in the contractile response. This response was of the aqueous extract and will require further experiments and isolation of the component compounds to understand this response.

3.5.2.2 Experiments show the presence of vasorelaxant components in the aqueous extract

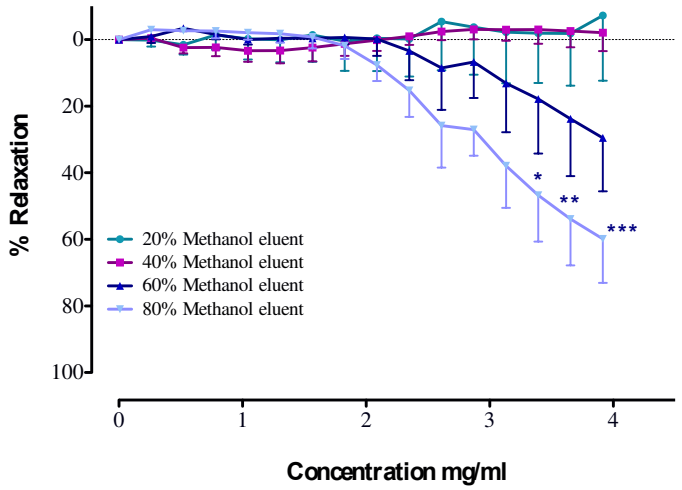


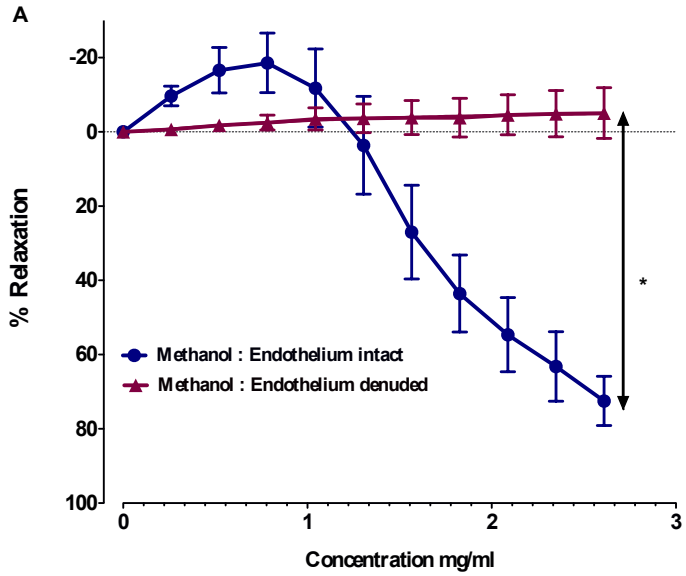
Figure 3 - 3: Vasorelaxant effects following elution with increasing methanol concentrations on phenylephrine (1μM) pre-contracted endothelium intact rat aortic rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results did not show overall significance but was significant on specific points between 80% and 20% elution as shown (*p≤0.05, **p≤0.01, (*p ≤ 0.001).**

Elution with increasing concentrations of methanol showed the relaxant effect only at higher methanol concentrations. The cumulative concentration range was increased to

3.915mg/ml to identify the vasorelaxant effect. Despite this, elution with 20% or 40% methanol did not show any vascular effects. The experiment using 60% methanol gave a maximal relaxant response of approximately 30% that was not significant compared to that with 20% methanol, while using 80% methanol a 60% relaxation was shown that was significant at the maximum concentration. This suggests the vasorelaxant component has lipophilic characteristics as it was preferentially extracted in methanol. In contrast the contractile component was clearly hydrophilic in nature as it remained in the aqueous solution and was not retained on the column (Data not shown).

3.5.3 Solvent extracts

3.5.3.1 Comparison of solvent extracts in inducing vascular responses



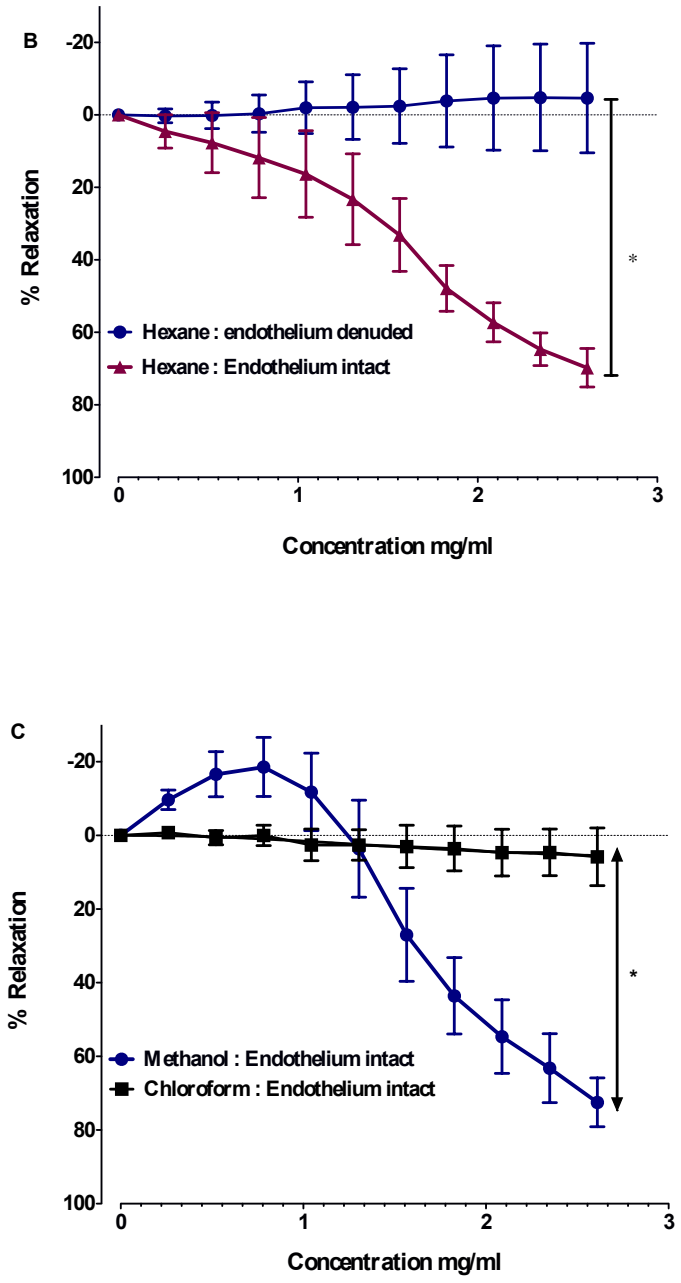


Figure 3 - 4: Comparison of the vascular effects of (A) methanol and (B) hexane extracts on endothelium intact (control) and denuded rings. (C) Chloroform extract is on endothelium-intact rings alone and is compared to methanol extract (control). Each point represents the mean ± S.E.M. (n=4-5) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus control (*p≤0.05)

The above results show that the aqueous extract in Figure 3-4 shows only a contractile effect, the methanol extract shows a biphasic response and the hexane extract shows

only a vasorelaxant response and was chosen for subsequent fractionation due to the lack of any contractile effect that would simplify the isolation processes by not compromising the identification of the relaxant response and the determination of statistical significance.

3.5.3.2 Preliminary pharmacological investigation of the methanol extract

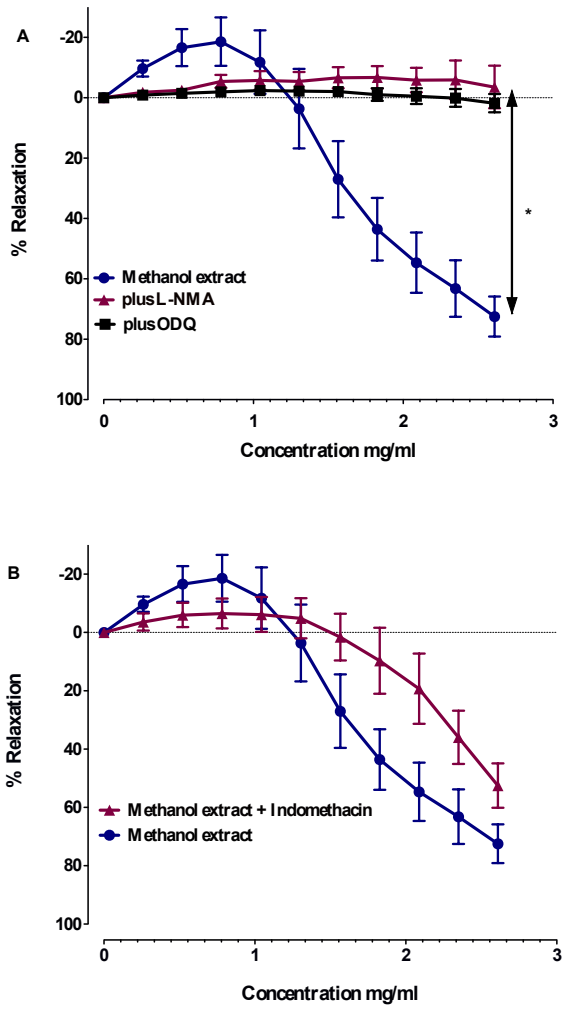


Figure 3 - 5: (A) Effects of L-NMA and ODQ inhibitors of NOS and cGMP and (B) indomethacin inhibitor of COX, on methanol extract (control) - induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus control in (A) only (*p≤0.05).

These results show that the vasorelaxant and contractile effect can be attenuated in the presence L-NMA and ODQ inhibitors of NOS and cGMP, but is not significantly attenuated in the presence of indomethacin, an inhibitor of COX.

3.5.4 Fractionation of the soxhlet hexane extract

3.5.4.1 Table of TLC results (see Table of extraction & isolation - Table 3.2)

The majority of the spots appeared as a black spot on a green background when viewed at UV 254nm. The fluorescent blue colour in fractions C and D were obtained by viewing at UV360, yellow colours shown were after spraying with methanolic sulphuric acid and heating at 100°C and the pink colour after spraying with vanillin.

Rf Value	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E	Fraction F	Fraction G	Fraction H&I combined	Fraction J	Fraction K
		0.94								
			0.88							
			0.8							
			0.75	0.75						
				0.73	0.73					
					0.64	0.64				
						0.56				
						0.49				
						0.45	0.45			
							0.39			
							0.33			
							0.26	**0.26**		
								0.2		
								0.15	0.15	
									0.06	0.06

Table 3 - 3: Depicts the TLC R_f values of grouped fractions following separation using the first column chromatographic method

3.5.4.2 Comparison of the vascular effects of Fractions A-K tested on endothelium-intact rat aortic rings

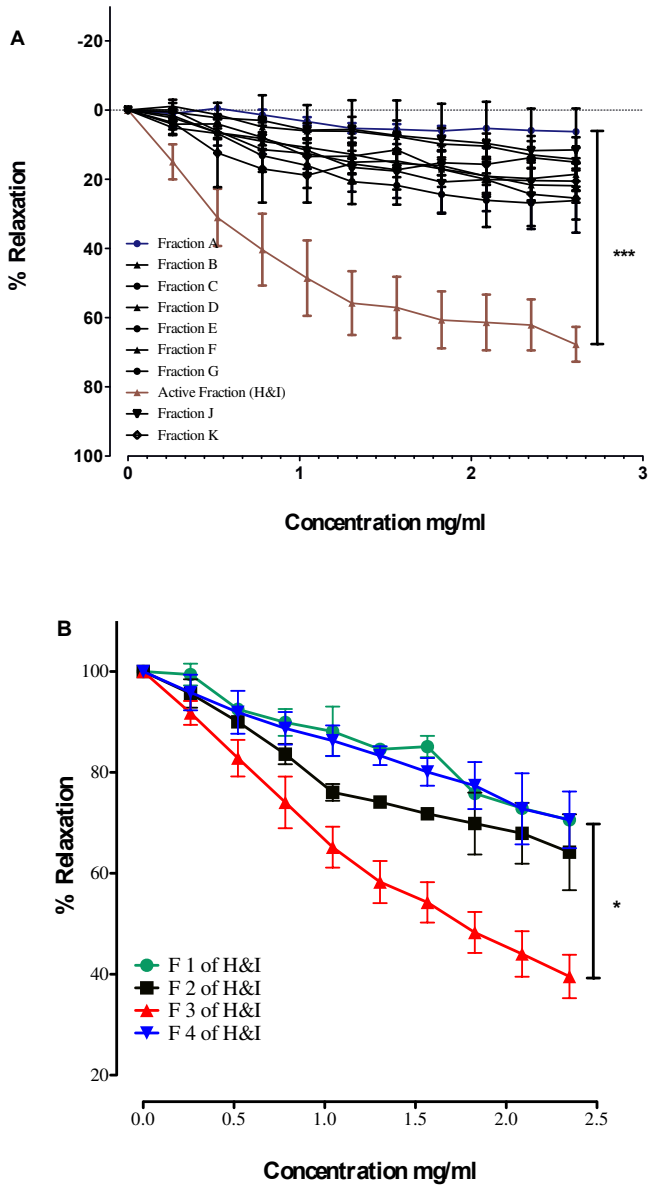


Figure 3 - 6: (A) Comparison of the vasorelaxant effect of hexane extract fractions A (control)- K, and (B) comparison of fractions following separation of active fraction H&I, fraction 1 (control) – 4, on phenylephrine (1μM) pre-contracted endothelium intact rat aortic rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus control (*p ≤0.05, ***p ≤ 0.001).

The results in Figure 3-6A shows a significant separation of the vasorelaxant active components found in fraction H&I after the first column chromatographic process. Fraction H&I was further fractionated by a second column chromatographic method to provide fraction 3 of H&I (Figure 3-6B) that was now subjected to HPLC separation to isolate two compounds as shown below.

3.5.4.3 HPLC of Fraction 3 of H&I

The method described in section 3.4.3.5a provided the following separation.

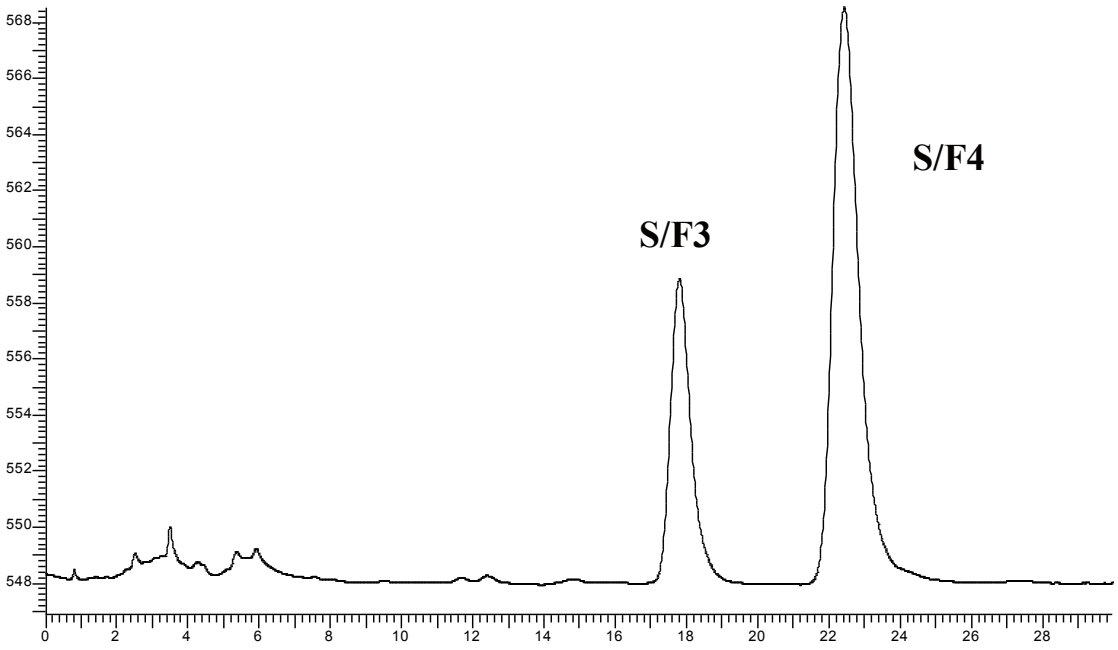


Figure 3 - 7: Representative example of HPLC profile showing separation of the active fraction into S/F3 and S/F4

The two largest peaks labelled S/F3 eluted at 17 minutes and S/F4 eluted at 23 minutes in the example shown above. This profile was fairly consistent across a number of runs. Although this technique proved the best for separating the active fraction it was an onerous process to collect enough material for testing.

3.5.5 Pharmacology of S/F3 fraction

3.5.5.1 Endothelium-dependent and independent effects of S/F3

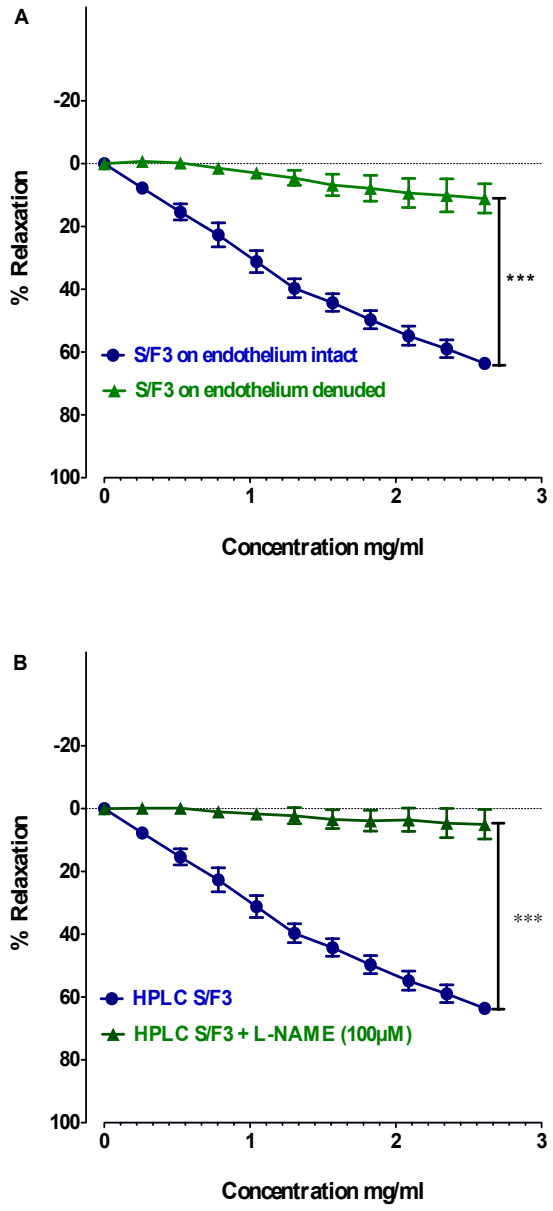


Figure 3 - 8: (A) Comparison of S/F3 on endothelium-intact (control) (n=6) and denuded (n=4) rings precontracted with phenylephrine (1µM). (B) Effect of S/F3 induced relaxation in the presence of L-NAME (n=5). Each point represents the mean ± S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus control (*$p \leq 0.001$).**

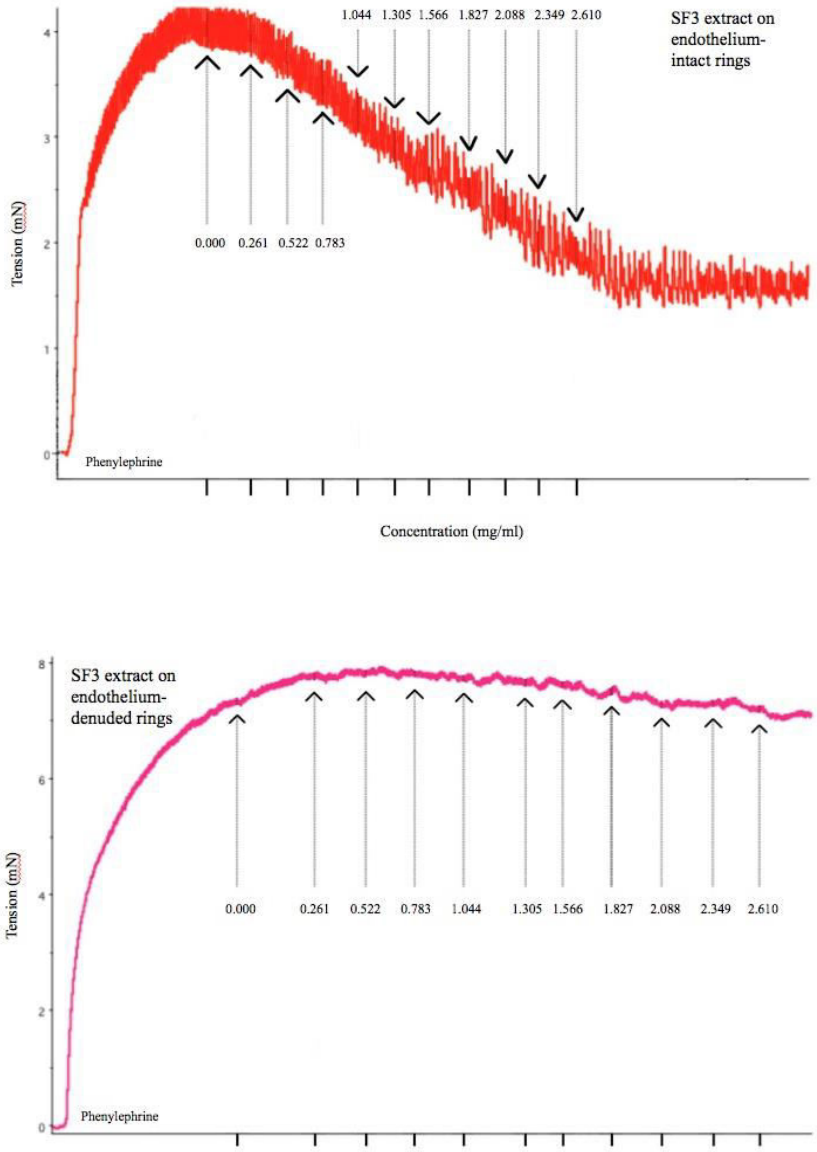


Figure 3 - 9: Representative trace of S/F3 on endothelium intact rat aortic rings above and endothelium denuded rat aortic rings below precontracted with phenylephrine (1μM).

Figure 3-8 (A) shows that S/F3 induces a vasorelaxant effect that is concentration- and endothelium-dependent on rat aortic rings precontracted with phenylephrine showing a maximal relaxation of 63.62 ± 1.84%. This vasorelaxant effect is attenuated by endothelium denudation. Figure 3-8 (B) shows this vasorelaxant effect is also attenuated following inhibition by L-NAME. Figure 3-9 shows actual representative traces of S/F3 on endothelium-intact rings and endothelium-denuded rings. The traces show the rapid contractile effect of phenylephrine (1μM) on aortic rings. It reaches a maximum just before eight minutes at which point the 1st concentration of S/F3 is added. In rings where the endothelium is intact the vasorelaxant effect following addition of S/F3 can be clearly seen. This effect stops after the addition of the last dose allowing the trace to plateau.

3.5.6 Pharmacology of S/F4 fraction

3.5.6.1 Endothelium-dependent and independent effects of S/F4

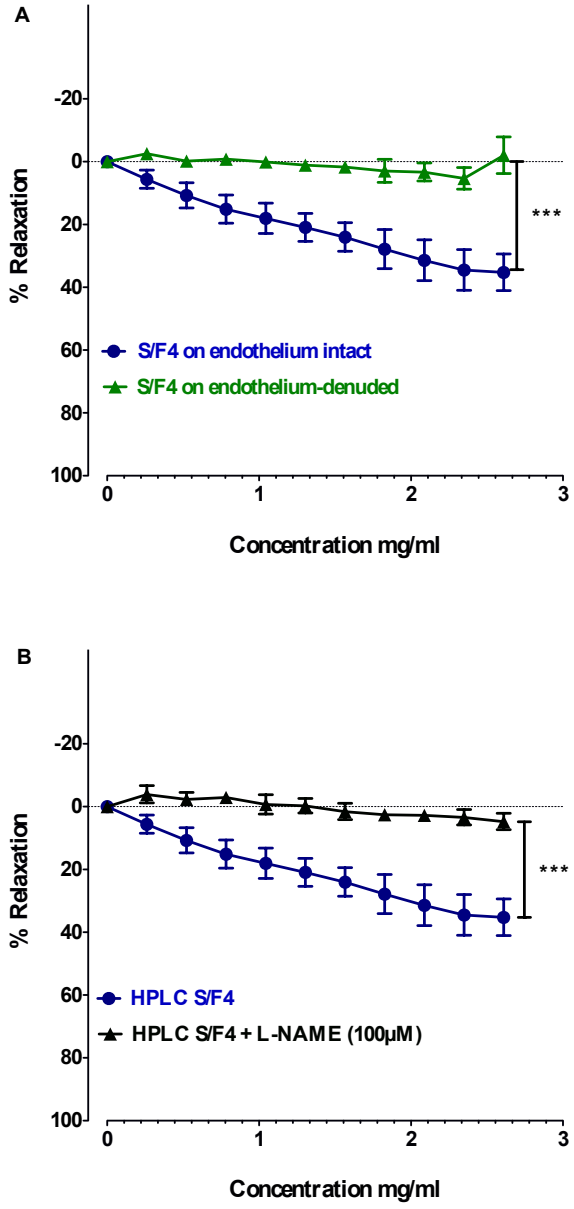


Figure 3 - 10: (A) Comparison of S/F4 on endothelium-intact (control) and denuded rings precontracted with phenylephrine (1µM). (B) Effect of S/F4 induced relaxation in the presence of L-NAME. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus control (*$p \leq 0.001$).**

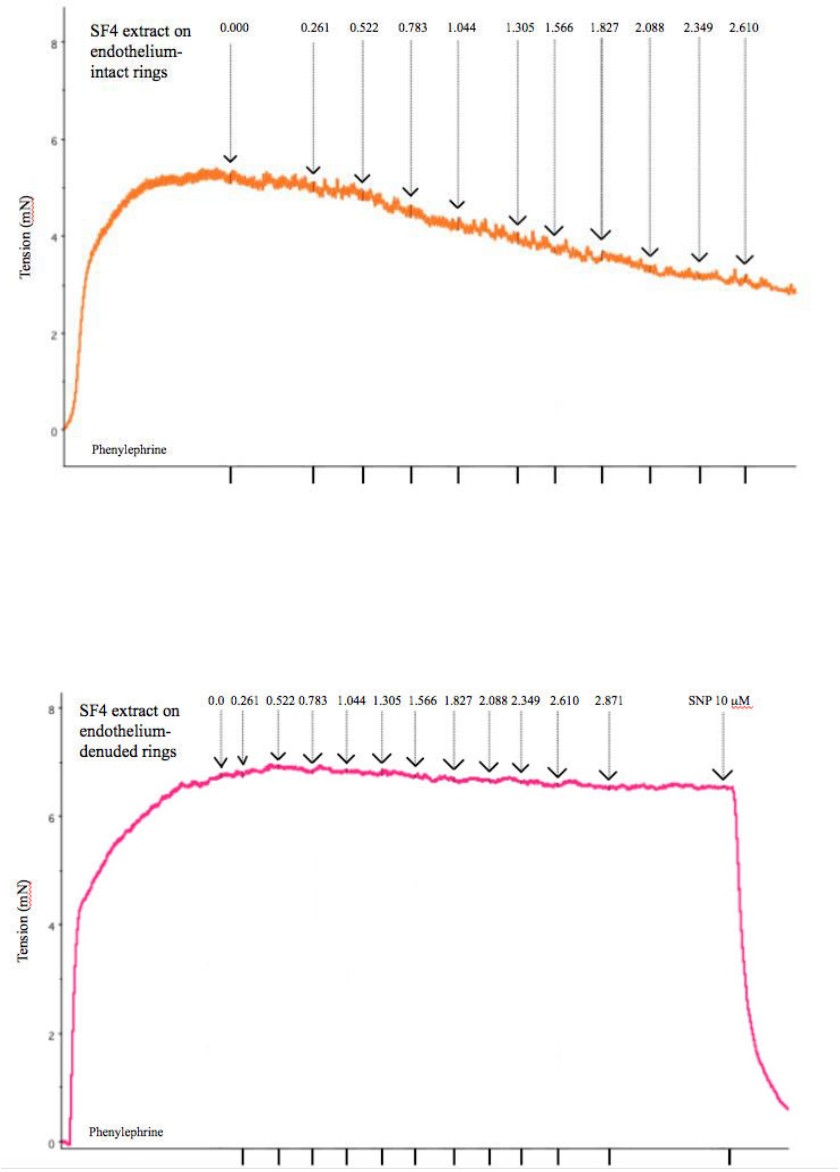


Figure 3 - 11: Representative trace of S/F4 on endothelium intact rat aortic rings above and endothelium denuded rat aortic rings below precontracted with phenylephrine (1µM).

Figure 3-10 (A) shows that S/F4 induces a vasorelaxant effect that is concentration- and endothelium-dependent on rat aortic rings precontracted with phenylephrine showing a maximal relaxation of 35.27 ± 5.8%. This vasorelaxant effect is attenuated by endothelium denudation. Figure 3-10 (B) shows this vasorelaxant effect is also attenuated following inhibition by L-NAME. Figure 3-11 shows actual representative traces of S/F4 on endothelium-intact rings above and endothelium-denuded rings below. The traces show the rapid contractile effect of phenylephrine (1μM) on aortic rings. It reaches a maximum just before eight minutes at which point the 1st concentration of S/F4 is added. In rings where the endothelium is intact the vasorelaxant effect of S/F4 is less than that of S/F3 (63.62 ± 1.84%).

3.5.7 Mass spectra of S/F4

Mass spectroscopy studies used the continuous ion source techniques of electrospray ionization (ESI) coupled with time of flight (TOF) mass analyser. The ESI-MS spectra of S/F4 showed a pseudo molecular ion peak $(M+Na)^+$ at m/z 397.08331, $(M+H)^+$ at m/z 375.101 and a related peak at $(2M+Na)^+$ at m/z 771.18723.

3.5.7.1 Mass spectrometry results for S/F4

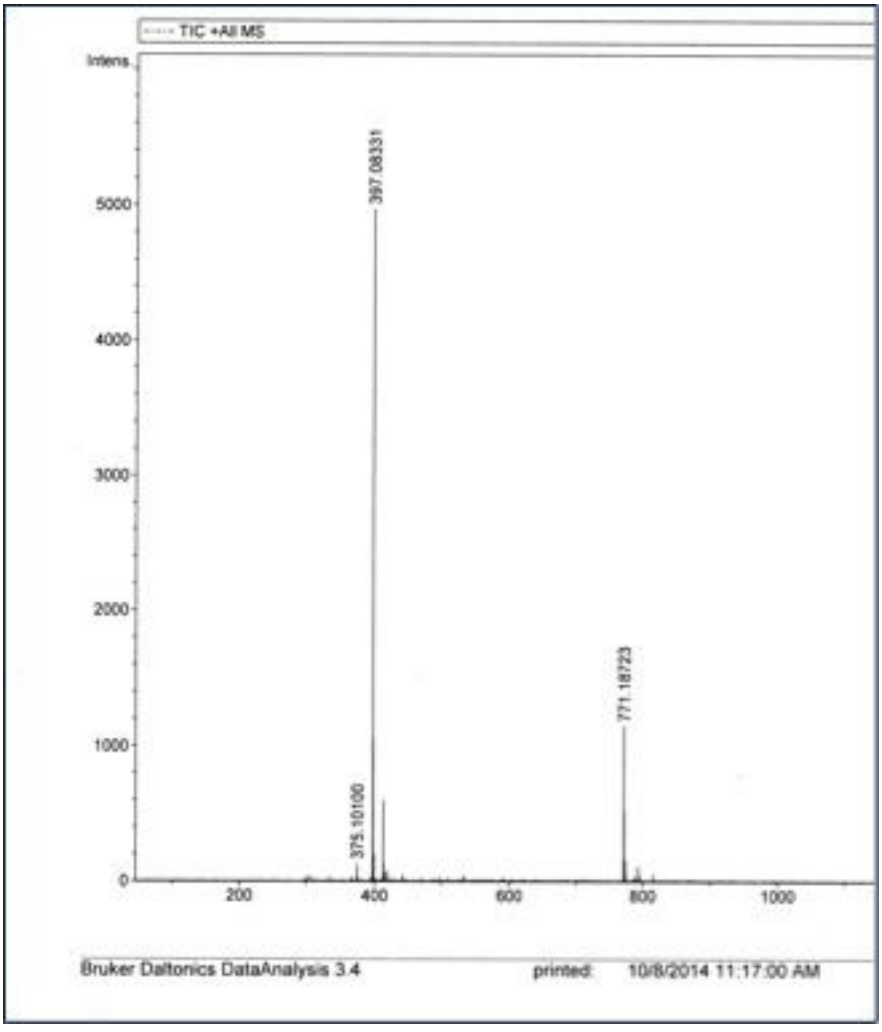


Figure 3 - 12: ESI-MS results show the molecular mass of S/F4

3.5.8 Mass spectra of S/F3

The ESI-MS spectra of S/F3 showed a pseudo molecular ion peak $(M+Na)^+$ at m/z 397.0880, $(M+H)^+$ at m/z 375.10735 and a related peak at $(2M+Na)^+$ at m/z 771.18878.

3.5.8.1 Mass spectrometry results for S/F3

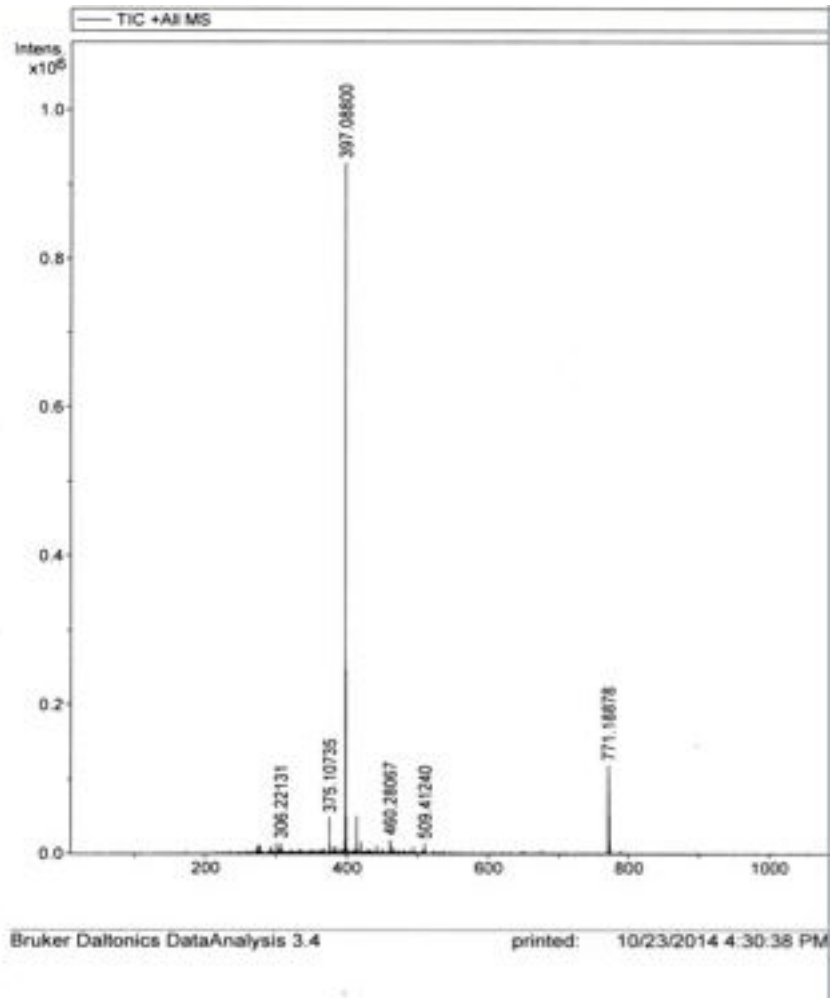


Figure 3 - 13: ESI-MS results show S/F3 mass as the main peak together with trace compounds

3.5.9 UV Spectra

S/F3 showed peaks at 267.07nm and 293.21nm shown below and S/F4 showed similar peaks at 267.07nm and 307.78nm (spectrum not shown).

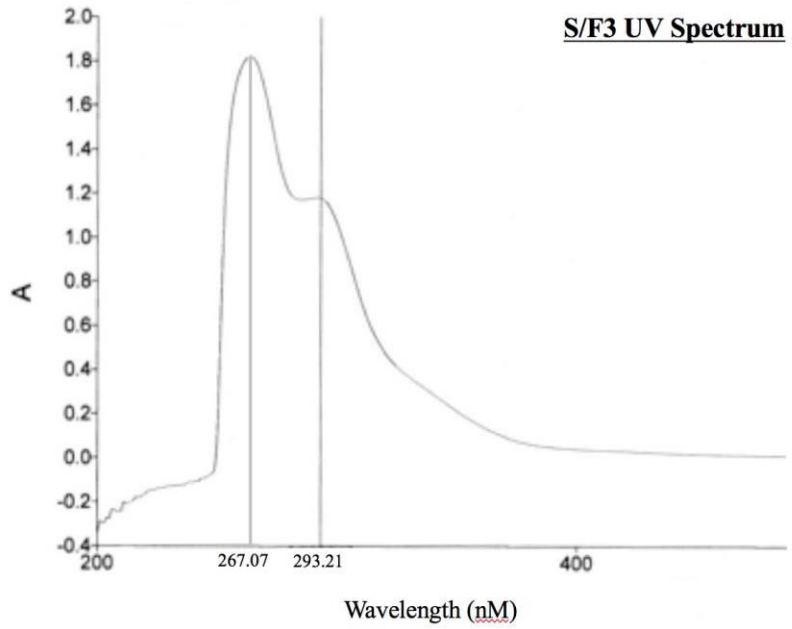


Figure 3 - 14: UV spectrum of S/F3 showing isoflavonoid features

3.5.10 Suggested compound structures

Based on the mass spectrometry data and the molecular formula identified as $C_{19}H_{18}O_8$, UV and NMR data, together with extensive comparison of published NMR data and taxonomic identification of the bark as a Dalbergia species, it is suggested that the two compounds are most likely to be the isomers caviunin (S/F4) and isocaviunin (S/F3). These structures are shown below.

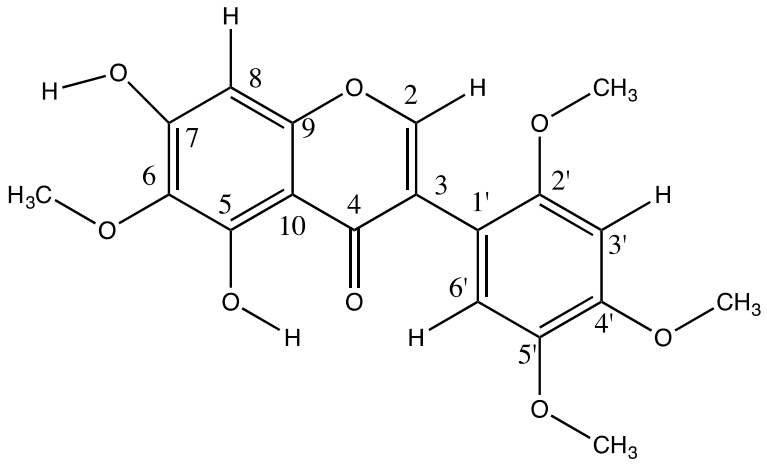


Figure 3 - 15: Molecular structure of 5, 7-dihydroxy, 6, 2', 4', 5'-tetramethoxy isoflavone or caviunin

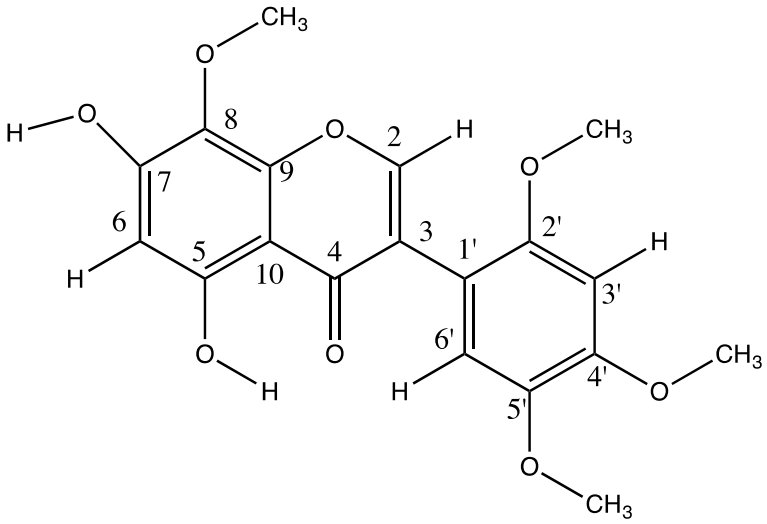


Figure 3 - 16: Molecular structure of 5, 7-dihydroxy, 8, 2', 4', 5'-tetramethoxy isoflavone or Isocaviunin

3.5.11 S/F4 NMR studies

Detailed structural confirmation of the isomers shown above required the study of the ^{1}H, ^{13}C, DEPT 135, HSQC, NOE and HMBC data presented below. Structural assignments detailed alongside the shifts will be substantiated below after presentation of the HMBC data. Due to the lack of coupling patterns much of the structural assignments required HMBC data and a number of these positions were substantiated in S/F4 by NOE data too.

3.5.11.1 S/F4 ^{1}H NMR in $CDCl_3$

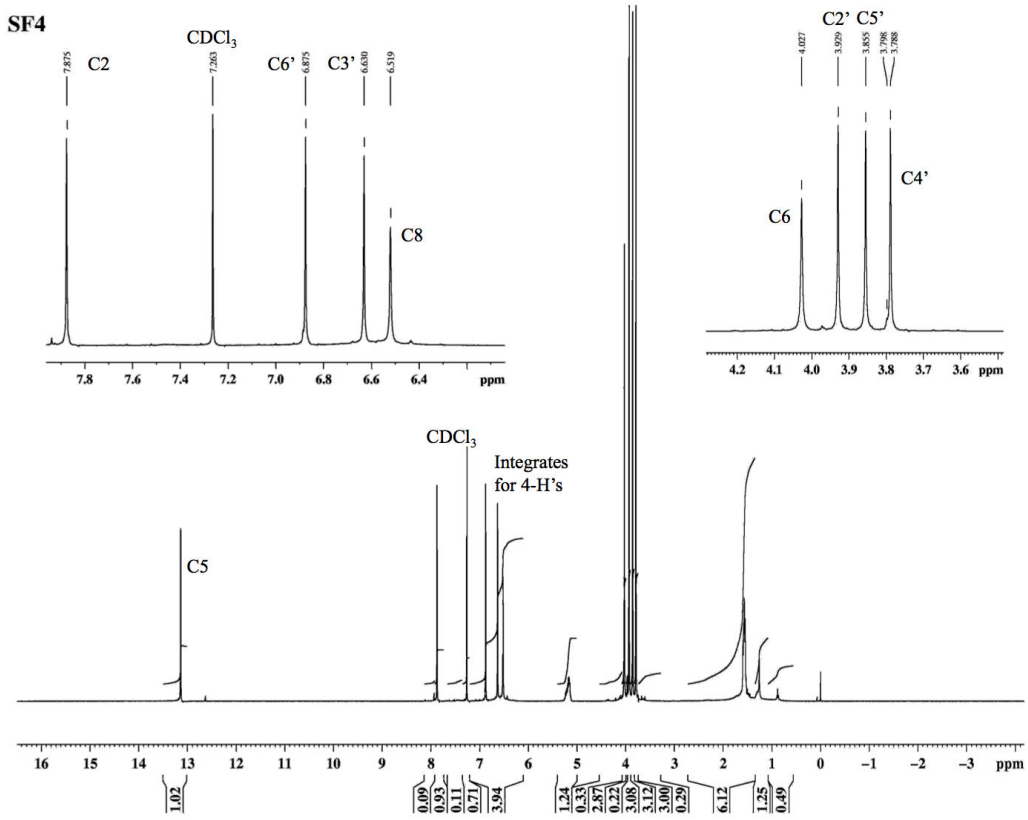


Figure 3 - 17: ^{1}H NMR spectra for caviunin (S/F4)

Inspection of the expanded annotated ^{1}H spectra above shows the presence of signals integrating for 18 observable protons. The four protons that occur in the aromatic region (6-8ppm) are single protons that lack coupling. The first three of these

aromatic protons integrate for four protons and include an exchangeable hydroxyl proton beneath. Each of the four tall singlet signals around 4ppm integrates for three protons identifying four methoxy groups. The final, deshielded proton at 13.13 is identified as the chelated hydroxyl group. Taken together the 18 protons correspond to the number of protons identified in the molecular formula using MS. The signals are identified as ^{1}H NMR δ (ppm) (400MHz, $CDCL_3$): 3.786 (3H, s, - OCH_3 – 2'), 3.854 (3H, s, - OCH_3 – 5'), 3.973 (3H, s, - OCH_3 – 4'), 4.030 (3H, s, - OCH_3 – 6), 6.519 (1H, s, – C-H – 8), 6.631 (1H, s, – C-H – 3'), 6.878 (1H, s, – C-H – 6'), 7.881 (1H, s, – C-H – 2), 13.13 (1H, s, – OH – 5).

Comparison of the proton signals in $CDCl_3$ and a solvent composed of $CDCL_3$ + DMSO 90:10 (Figure 3-18) has identified a broad peak at δ - 10.15 in the latter with the loss of the broad peak below the aromatic hydrogen signals δ - 6.519, 6.631 and 6.878 which when taken together integrates now for 3 protons only. This suggests this signal is due to an exchangeable proton from a hydroxyl group.

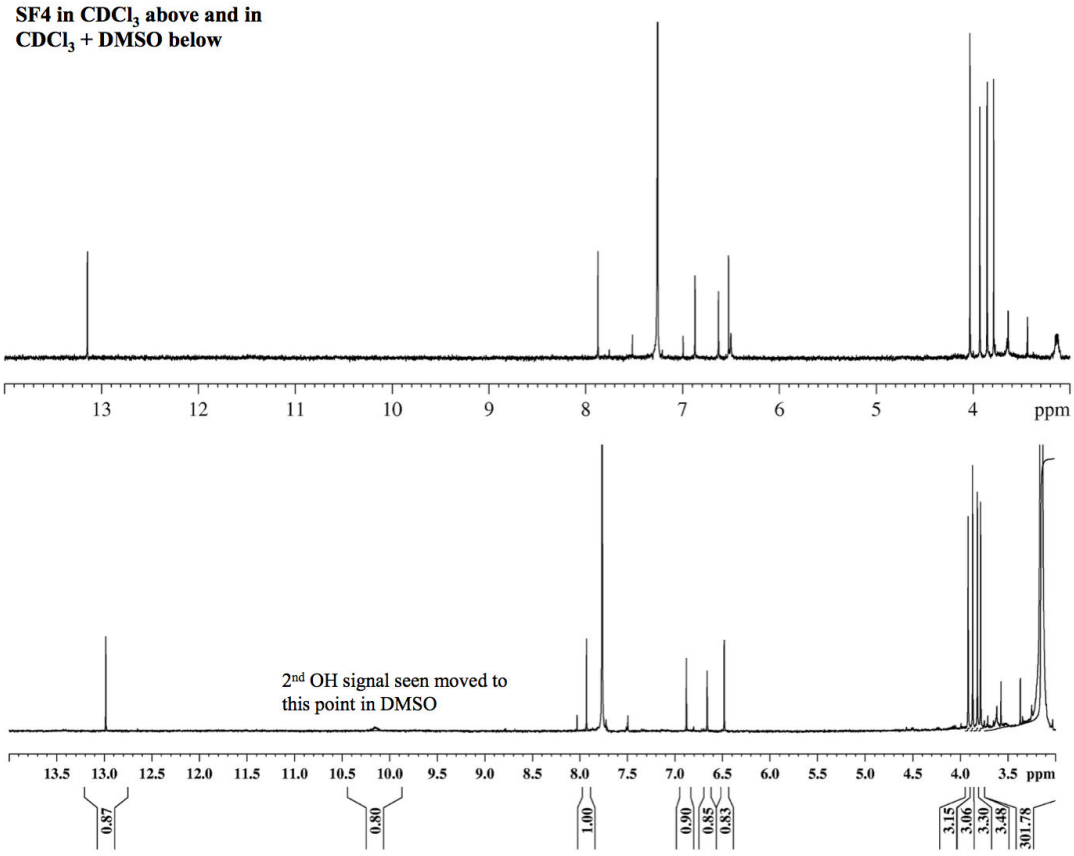


Figure 3 - 18: Comparison of ^{1}H NMR spectra in $CDCl_3$ above and a combination of $CDCl_3$ and DMSO below for caviunin (S/F4)

3.5.11.2 S/F4 ^{13}C NMR in $CDCl_3$

The ^{13}C spectrum shows the presence of 19 carbons which appear as singlets due to proton decoupling. ^{13}C NMR δ (ppm) (100MHz, $CDCL_3$): four methoxy carbons can be found at 56.2 (OCH_3, C-4'), 56.62 (OCH_3, C-5'), 56.83 (OCH_3, C-2') and 60.86 (OCH_3, C-6). Fifteen aromatic singlet carbons are found at 93.18 (C-8), 98.24 (C-3'), 106.52 (C-10), 110.75 (C-1'), 115.20 (C-6'), 120.04 (C-3), 130.34 (C-6), 143.14 (C-5'), 150.18 (C-4'), 152.04 (C-2'), 152.54 (C-9), 153.47 (C-5), 154.91 (C-2), 155.04 (C-7), 181.27 (C-4).

3.5.11.3 S/F4 ^{13}C DEPT-135 spectra

The DEPT-135 spectrum shows only the carbons attached to hydrogen atoms. The spectrum for S/F4 shows the 4-methoxy carbon signals at 56.20, 56.62, 56.83 and 60.86, while the 4 aromatic C-H are found at 93.18 (C-8), 98.24 (C-3'), 115.2 (C-6') and 154.91 (C-2). This allows the identification of the other eleven as quaternary carbons, as there are no CH_2 groups as none of signals in the ^{1}H spectrum integrate for two hydrogens.

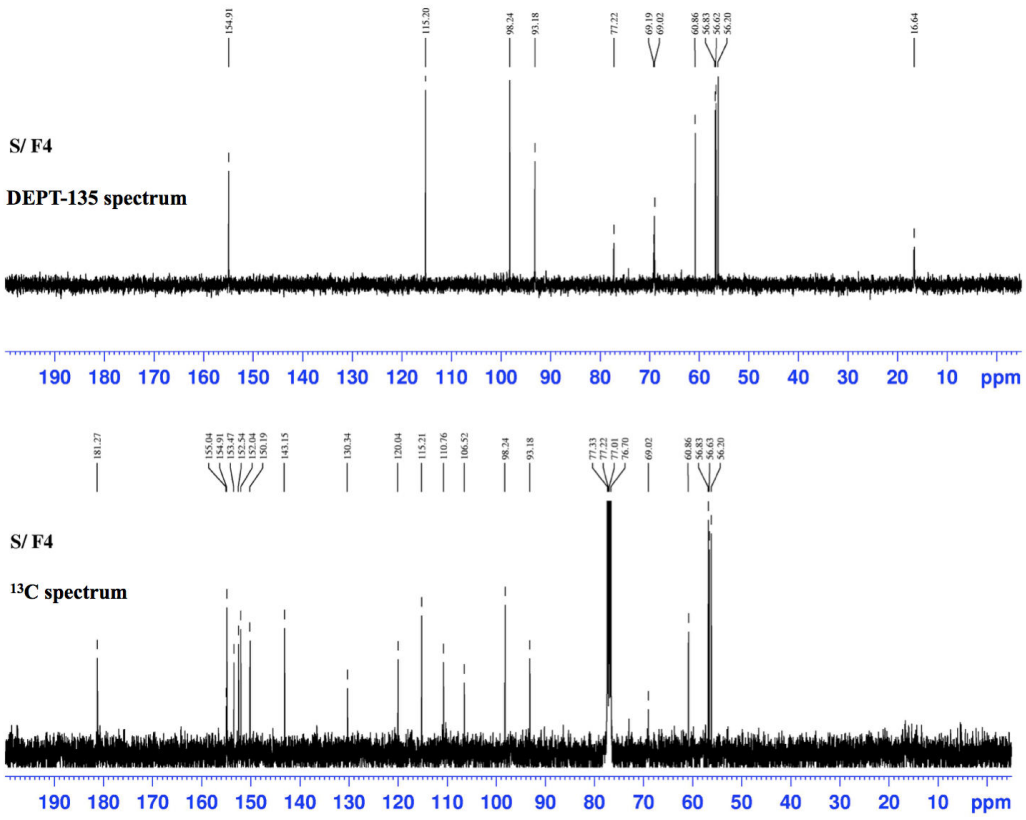


Figure 3 - 19: Comparison of DEPT-135 and ^{13}C NMR for caviunin (S/F4)

3.5.11.4 S/F4 2D COSY spectra

COSY- ^{1}H-^{1}H-correlation spectroscopy shows couplings between protons that are 2-4 bonds distant from each other. No significant correlation was noted.

3.5.11.5 S/F4 2D HSQC spectra

The heteronuclear single quantum coherence (HSQC) for S/F4 shows direct $^{1}H - ^{13}C$ coupling information: the 4-methoxy signals show the following coupling C2'-56.83⇔ H-3.786, C5'-56.62⇔ H-3.854, C4'-56.20⇔ H-3.973 and C6-60.86⇔ H-4.03. The aromatic hydrogen signals show the following coupling information: C8-93.18⇔ H-6.519, C3'-98.24⇔ H-6.631, C6'-115.20⇔ H-6.878 and C2-154.91⇔ H-7.881 confirming the DEPT-135 spectrum.

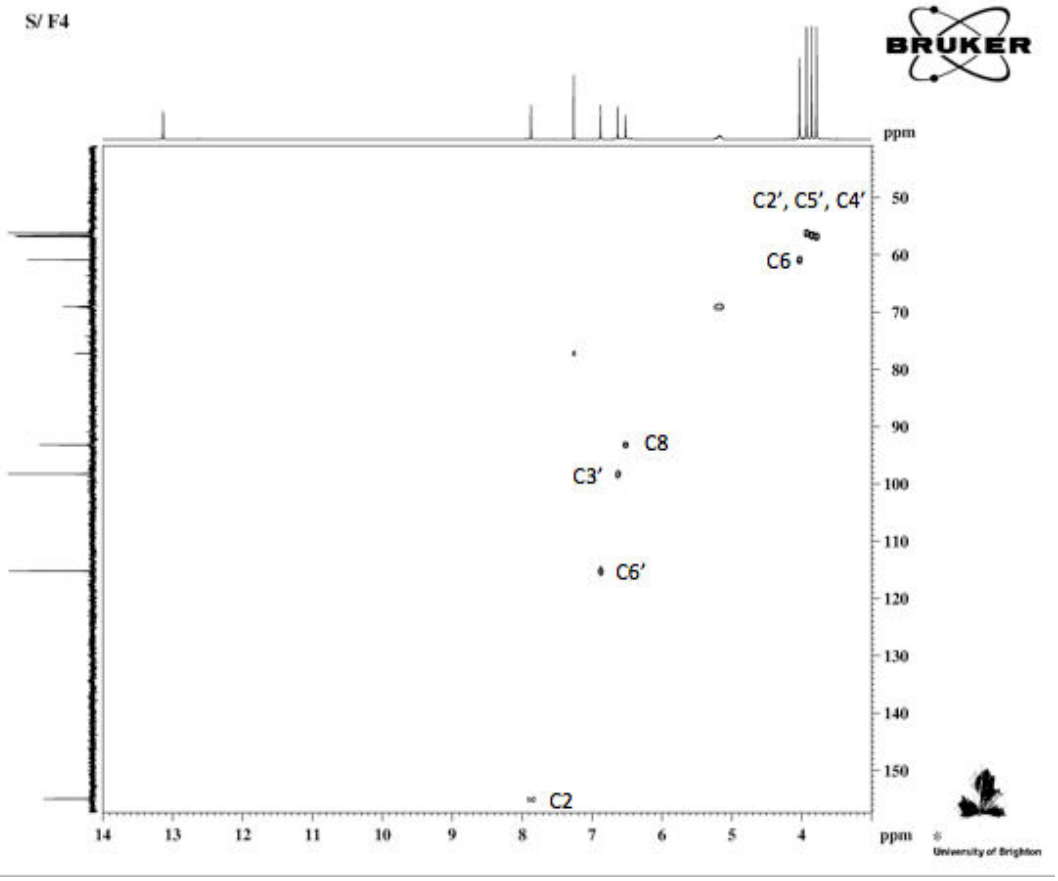


Figure 3 - 20: HSQC correlations for caviunin (S/F4)

3.5.11.6 S/F4 1D NOE spectra

The Nuclear Overhauser Effect (NOE) provides information on connectivity of atoms through space unlike that of the NMR techniques used up to this point and which provide information only on connectivity through a few bonds. By irradiating a single ^{1}H nucleus the change in intensity of other ^{1}H nuclei that are close in space can be identified.

3.5.11.6.1 NOE Spectra

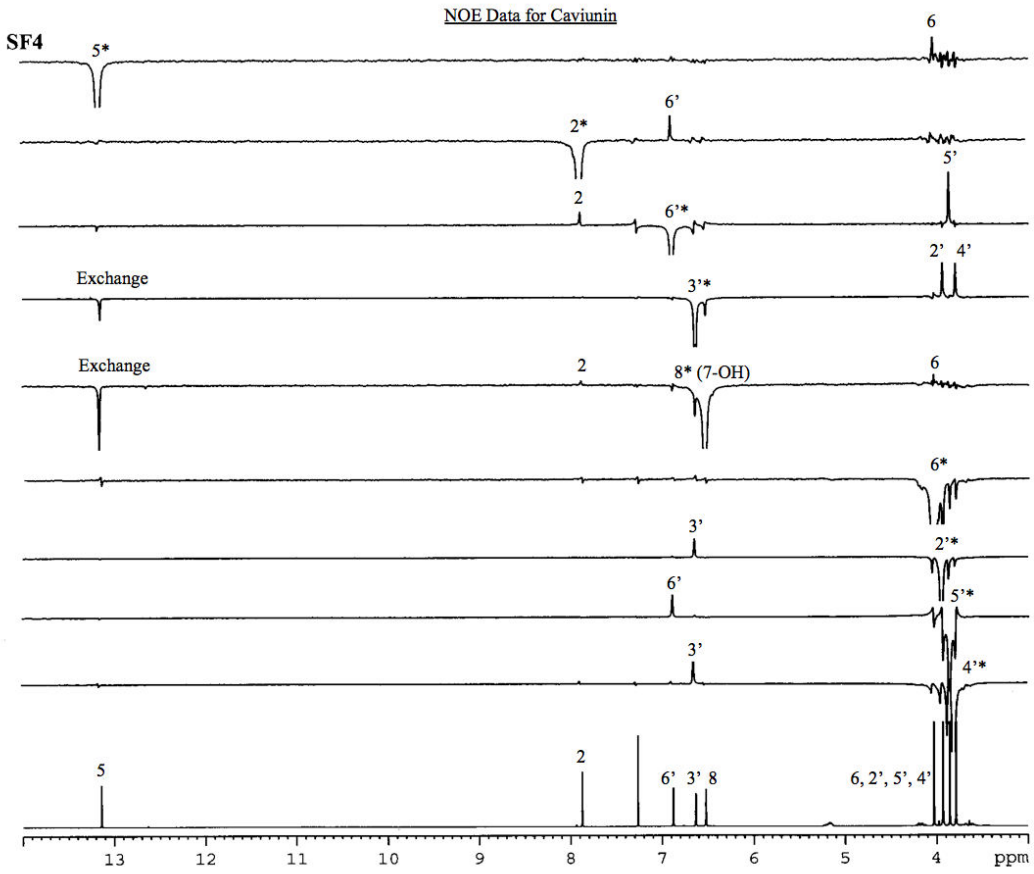


Figure 3 - 21: 1D NOE connectivity's for caviunin

The NOE results are tabulated below. The numbers correspond to the position of the proton nuclei in caviunin, with the asterisk (*) indicating the proton that is being irradiated.

3.5.11.6.2 NOE connectivity information

***H-atom irradiated**	**Shift (ppm)**	**NOE**
H5	13.1364	H6
H2	7.8743	H6’
$CHCl_3$	7.2616	-
H6’	6.8755	H2, H5’
H3’	6.6304	H2’, H4’
H8 (7-OH)	6.5209	H6, Exchange
H6	4.0295	-
H2’	3.9292	H3’
H5’	3.8551	H6’
H4’	3.7878	3’

Table 3 - 4: NOE connectivity data for caviunin (S/F4)

3.5.11.7 S/F4 2D HMBC spectra

Heteronuclear multiband coherence (HMBC) shows correlations between protons and carbon atoms that are two-three bonds distant. It has provided much of the key correlation data in decoding the structures of these isoflavonoids due to the lack of proton coupling. The correlations for S/F4 are tabulated in 3-4 below and key correlations are diagrammatically presented below. The 2', 4', 5' $-OCH_3$ positions on the B-ring can be seen clearly on the HMBC spectra, and the same connectivity's are also seen in NOE data presented below.

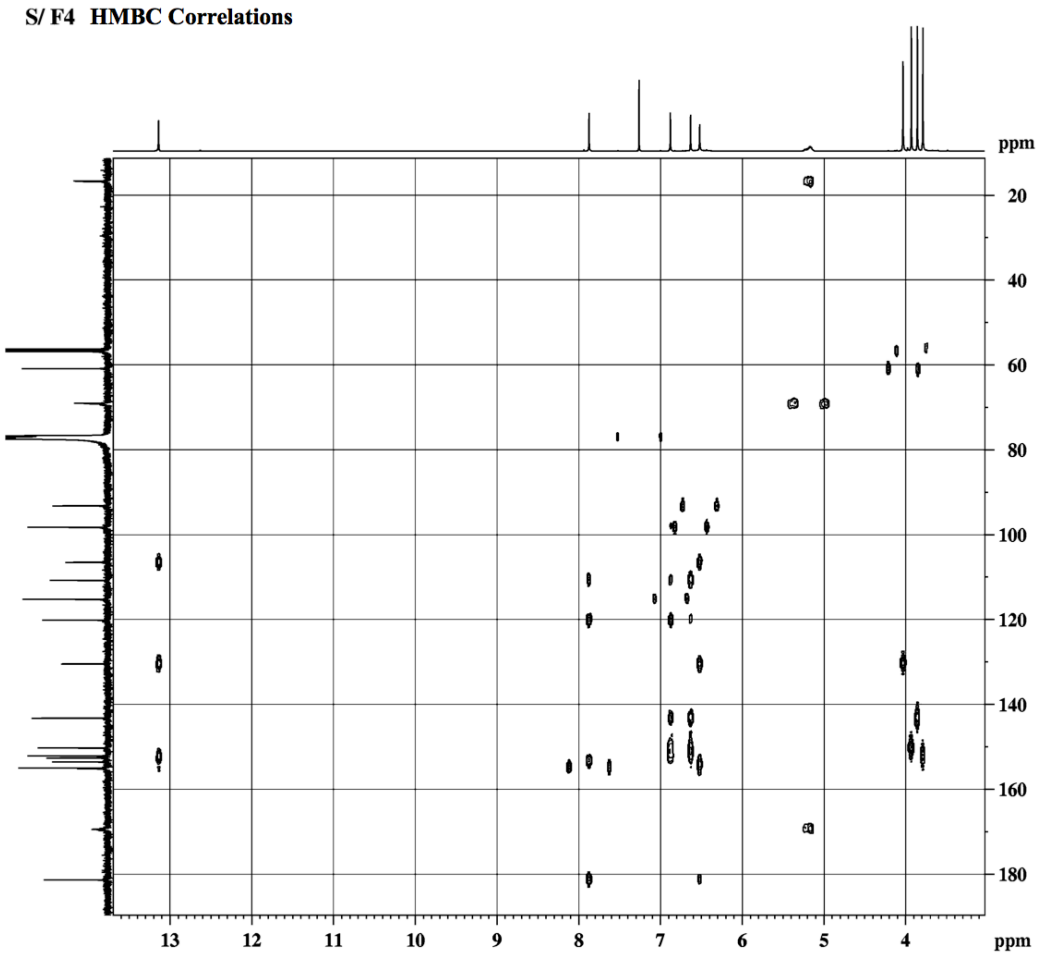


Figure 3 - 22: HMBC correlations for caviunin (S/F4)

3.5.11.8 S/F4 2D HMBC spectral correlations

S/F4 $CDCl_3$ 400MHz			
Position	**δ_H**	**δ_C**	**HMBC correlations**
1			
2	7.881	154.91	C3 (120.04), C1' (110.75), C4 (181.27), C9 (152.54)
3		120.04	
4		181.27	
5	13.13(OH)	153.47	C5 (153.47), C6 (130.34), C10 (106.8)
6		130.34	
7		155.04	
8	6.519	93.18	C6 (130.34), C7 (155.04), C9 (152.54), C10 (106.52)
9		152.54	
10		106.52	
1'		110.75	
2'		152.04	
3'	6.631	98.24	C1' (110.75), C2'(152.04), C4'(150.18), C5'(143.14)
4'		150.18	
5'		143.14	
6'	6.878	115.2	C1' (110.75), C3 (120.04), C5'(143.14), C2'(152.04), C4' (150.18)
6-OCH_3	4.03	60.86	C6 (130.34)
2'-OCH_3	3.786	56.83	C2' (152.04)
4'-OCH_3	3.973	56.2	C4' (150.18)
5'-OCH_3	3.854	56.62	C5' (143.14)

Table 3 - 5: HMBC correlations for caviunin

3.5.12 S/F3 NMR studies

S/F3 was found in considerably smaller quantities than that of S/F4.

3.5.12.1 S/F3 ^{1}H spectra in $CDCl_3$

^{1}H NMR spectrum for S/F3 is similar to that of S/F4. The key difference in the spectra is found in the position of the C-5 hydroxyl group, which appears at δ (ppm) 12.633. The rest of the assignments for S/F3 are as follows: δ (ppm) (400MHz, $CDCL_3$): 3.799 (3H, s, - OCH_3 – 2'), 3.859 (3H, s, - OCH_3 – 5'), 3.934 (3H, s, - OCH_3 – 4'), 3.979 (3H, s, - OCH_3 – 8), 6.444 (1H, s, – C-H – 6), 6.636 (1H, s, – C-H – 3'), 6.885 (1H, s, – C-H – 6'), 7.936 (1H, s, – C-H – 2), 12.633 (1H, s, – OH – 5).

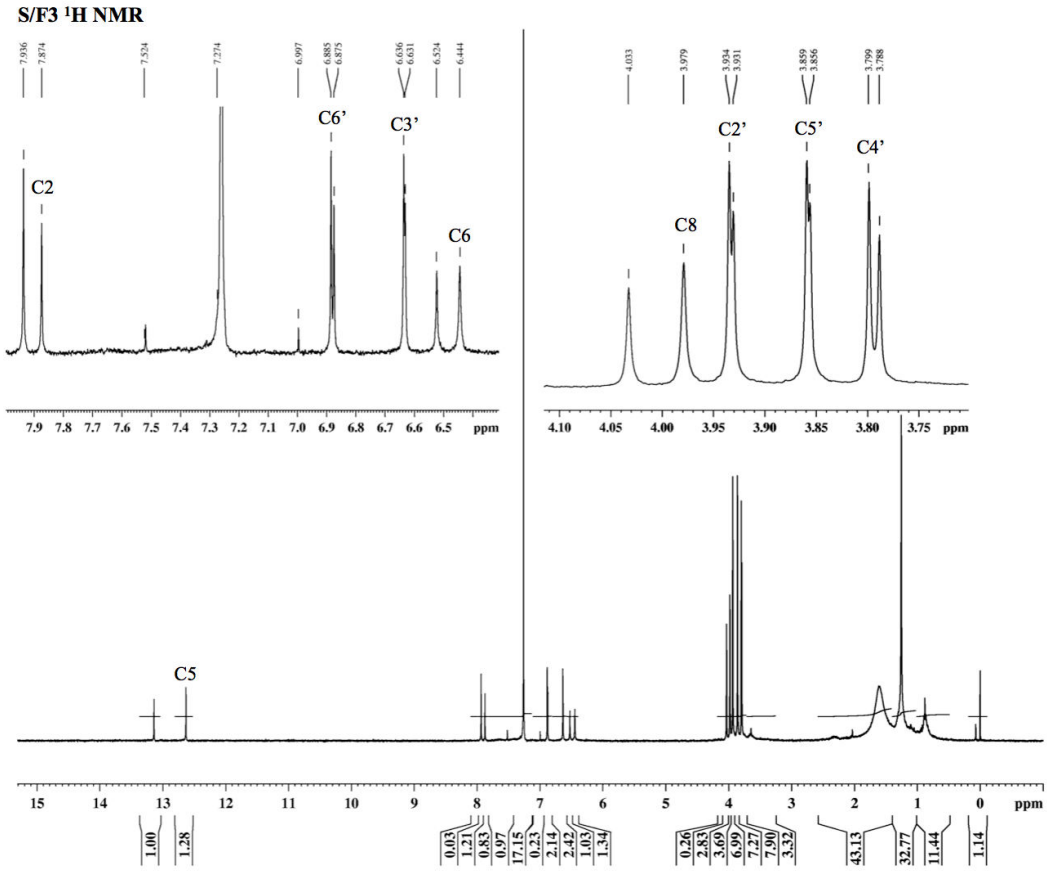


Figure 3 - 23: ^{1}H NMR spectra for isocaviunin (S/F3)

3.5.12.2 S/F3 ^{13}C spectra in $CDCl_3$

The spectrum for S/F3 is virtually identical to that of S/F4 and is shown below together with the DEPT-135 spectrum. The shifts are tabulated in Table 3-5 below.

3.5.12.3 S/F3 ^{13}C DEPT-135 spectra

The DEPT-135 spectrum for S/F3 shows the 4-methoxy carbon signals are found at 56.20, 56.62, 56.83 and 60.86, while the 4 aromatic C-H are found at 98.24 (C-6), 93.18 (C-3'), 115.2 (C-6') and 154.91 (C-2).

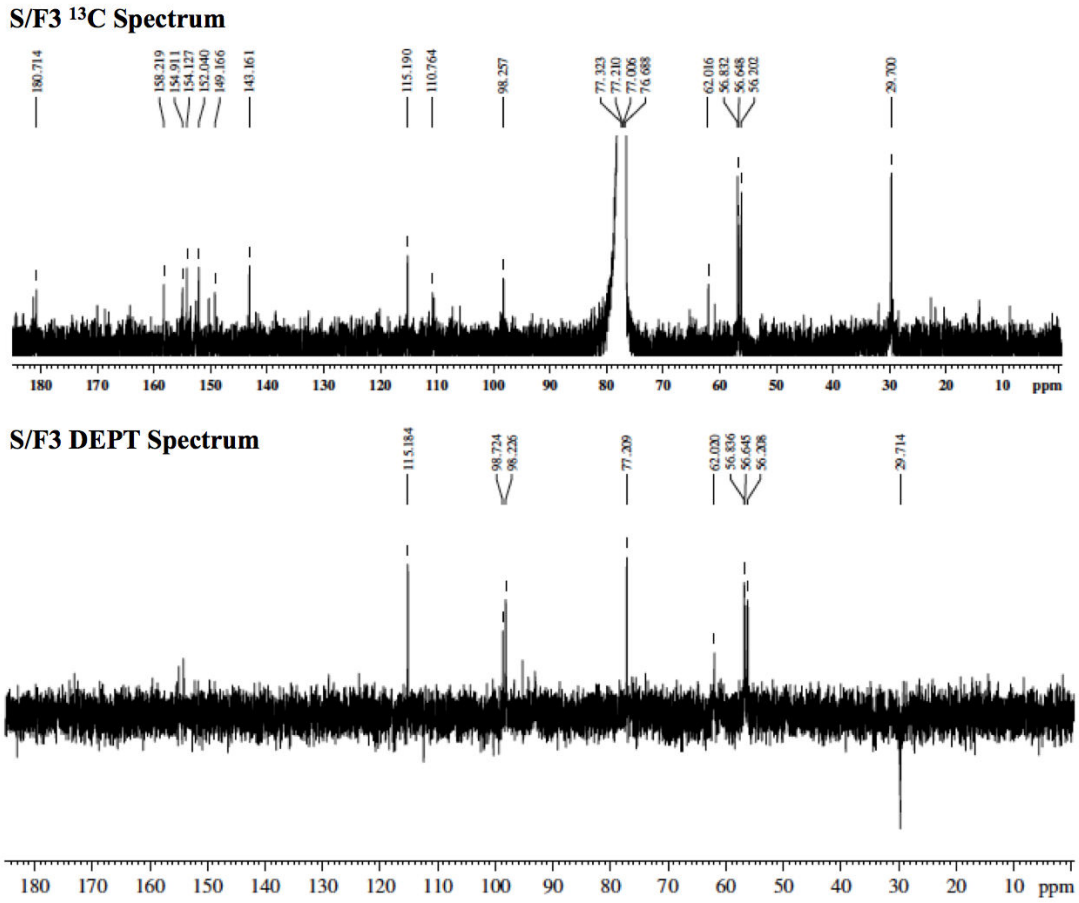


Figure 3 - 24: ^{13}C spectra above and the DEPT-135 below for isocaviunin (S/F3)

3.5.12.4 S/F3 2D HSQC spectra

The HSQC spectrum for S/F3 shows direct ^{1}H – ^{13}C coupling information: the 4-OCH_3 signals show the identical coupling at C-56.83⇔ H-3.786, C-56.62⇔ H-3.854, C-56.20⇔ H-3.973 and C-60.86⇔ H-4.03. The hydrogen signals show coupling at 93.245 (C-3'), 98.207 (C-6), 115.198 (C-6') and 154.134 (C-2).

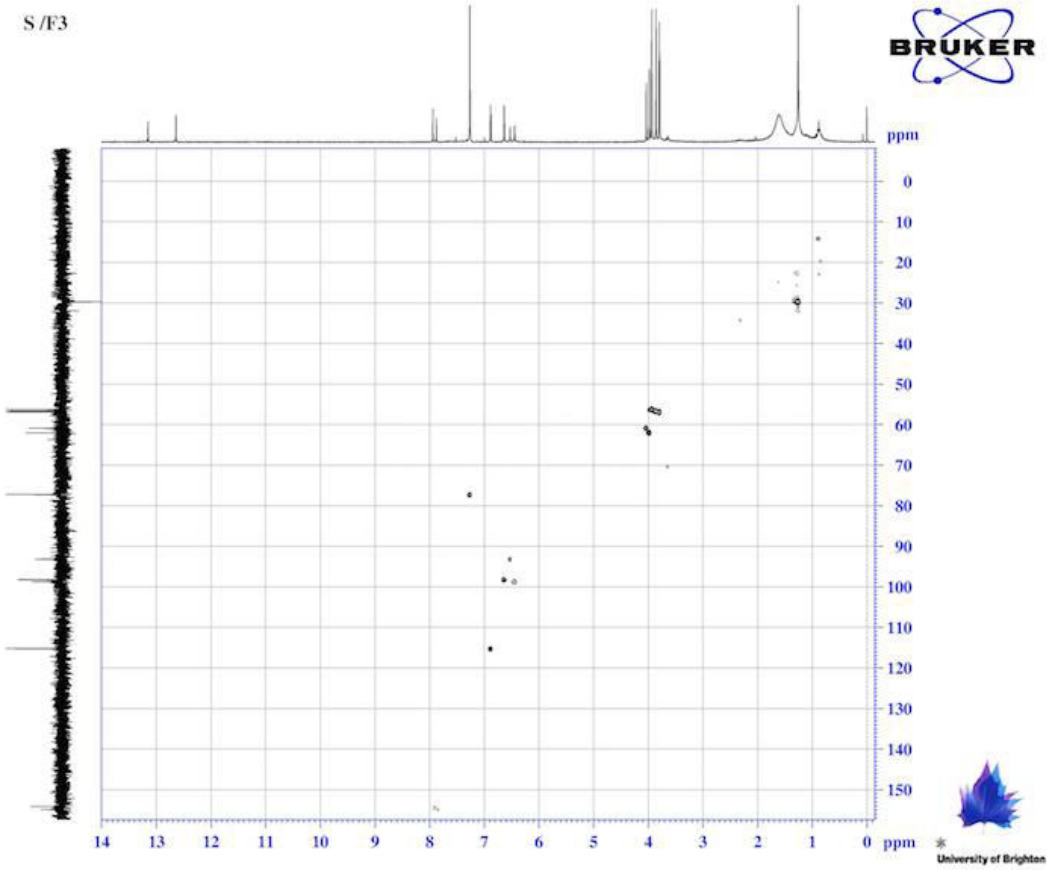


Figure 3 - 25 : HSQC correlations for isocaviunin (S/F3)

3.5.12.5 S/F3 HMBC spectra

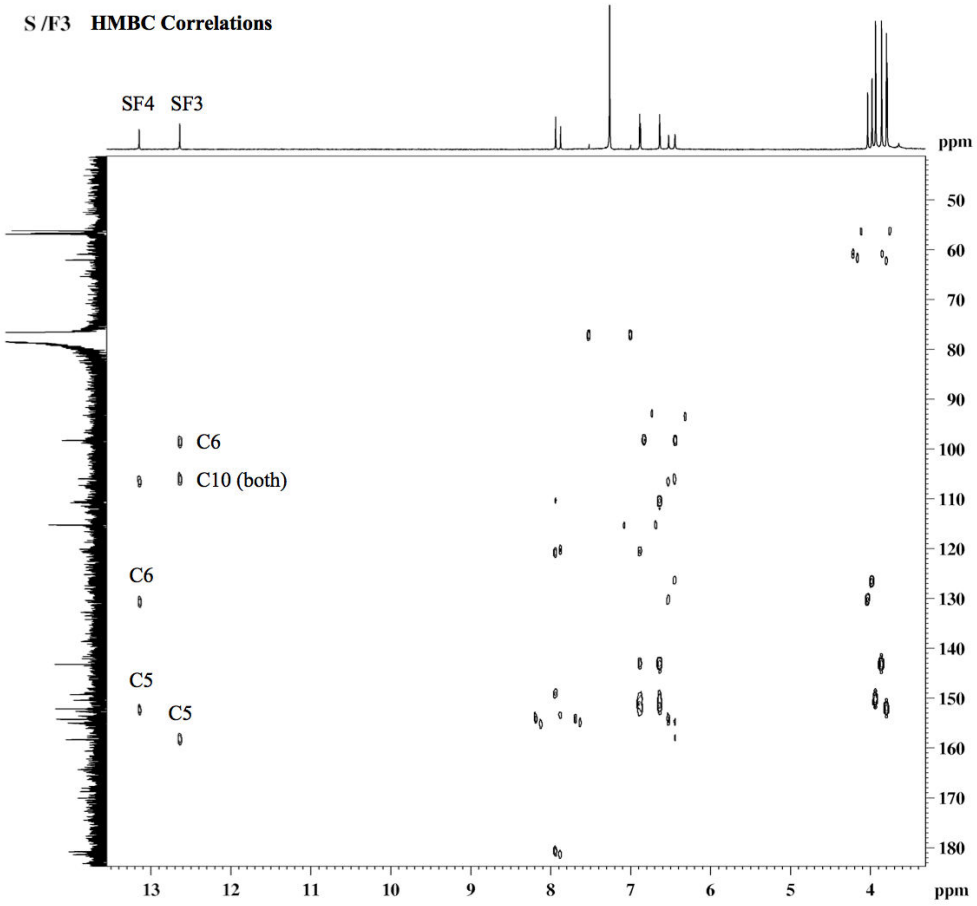


Figure 3 - 26: HMBC correlations for isocaviunin (S/F3)

This HMBC spectrum is of both compounds and clearly shows the key differences in the HMBC correlations between the two compounds as identified.

3.5.12.6 S/F3 2D HMBC spectral correlations

S/F3 $CDCl_3$			
Position	**δ_H 400MHz**	**δ_C 100MHz**	**HMBC correlations**
1			
2	7.936	154.134	C3 (120.855), C1' (110.844), C4 (180.718), C9 (154.909)
3		120.855	
4		180.718	
5	12.633(OH)	158.278	C5 (158.278), C6 (98.207), C10 (106.5)
6	6.444	98.2	C10 (106.5), C7 (153.5), C5 (158.278), C8 (126.826)
7		157.5	
8		126.826	
9		154.909	
10		106.5	
1'		110.844	
2'		152.555	
3'	6.636	93.245	C2'(152.555), C4'(150.291), C5'(143.21)
4'		150.291	
5'		143.21	
6'	6.885	115.198	C1' (110.844), C3(120.855), C5'(143.21), C2'(152.555), C4' (150.291)
8-OCH_3	3.979	62.02	C8 (126.826)
2'-OCH_3	3.934	56.648	C2' (152.555)
4'-OCH_3	3.799	56.204	C4' (150.291)
5'-OCH_3	3.859	56.832	C5' (143.21)

Table 3 - 6 HMBC correlation for isocaviunin (S/F3)

3.5.13 X-ray crystallography

Although crystals were found in the ethanol vial, analysis showed that these were not single crystals, instead they were made up of many very small needle crystals packed together. These are unsuitable for X-Ray analysis as the data would be un-interpretable.

3.6 Discussion

Initial experiments concentrated on the aqueous extraction of the bark to brew a tea by either immersing the bark in hot water briefly or by boiling to mimic the indigenous preparation. The first extract obtained from the bark of this plant showed the presence of a component in the aqueous extract that caused a concentration and endothelium-dependent contraction of the rat aortic ring bioassay. Interestingly this contractile effect was attenuated by removal of the endothelium or partially attenuated by prior treatment with L-NAME a NOS inhibitor. Furthermore preparation of the tea in a manner akin to the indigenous method showed a greater contractile effect indicating that the components were heat-stable. Nevertheless this contractile component was highly hydrophilic being retained predominantly in the aqueous fraction following application of the tea to a Hypersep-pak LC-18 cartridge that retains moderately polar compounds.

The focus of this project was however to identify compounds causing vascular relaxation and to this end a number of solvent extractions of the bark were studied. A methanol extract was shown to possess compounds that caused a biphasic contractile and vasorelaxant response in endothelium intact rings, and both these effects were attenuated following incubation with L-NAME. The hexane soxhlet extract in contrast only showed a vasorelaxant effect on endothelium-intact rings and was chosen for fractionation to prevent any complications arising from the presence of a contractile component. Repeated fractionation of the hexane extract yielded 0.038% S/F4 and 0.0016% S/F3. Both compounds were isolated as an amorphous, off-white solids and HRESIMS studies identified both compounds as having the same molecular weight of 374, corresponding to a molecular formula $C_{19}H_{18}O_8$ and revealing they were isomers of each other. The results of the literature survey undertaken at the beginning of this

project, the survey of bioactives (Luna Vazquez et al., 2013) and further research has not identified the study of these molecules in this context.

The UV absorption maxima showed a major peak at 267 and a shoulder peak at 307.8nm for S/F4, and 267 and 293nm for S/F3 indicative of an isoflavonoid nucleus, compounds known to be characteristic of the Dalbergia genus. UV flavonoid spectra are composed of two absorption maxima in the ranges 240-285nm (band II) due to the A-ring and 300-550nm (band I) due to the B-ring. Isoflavone type compounds generally show only the absorption associated with band II of the A-ring due to the lack of conjugation between the A and B ring structures. However band I is sometimes seen as a shoulder peak between 300-330nm (Markham, K.R. 1982) which is seen in these spectra.

The ^{1}H NMR spectrum of S/F4 shows no proton coupling, but four single aromatic proton signals, four methoxy ($–OCH_3$) group signals and two hydrogens from hydroxyl groups are described. From the ^{13}C spectrum nineteen carbon signals descriptive of the groups to which they are attached can also be identified. The spectra obtained from DEPT-135, and 2D HSQC correlation confirmed the positions of those carbons attached directly to either a C-H or $C-H_3$. The proton observed at 7.881 is characteristic of the downfield signal seen at position 2 of the isoflavonoid nucleus. The proton signal at 13.13 is typical of a chelated hydroxyl at position 5, which sits next to the carbonyl group at position 4. Work done on the 5-OH signal has shown that $6-OCH_3$ or 6-prenylation de-shielding effects leads to the 5-OH signal being shifted to lower fields consistently >13ppm. The signal shift of 5-OH is key in distinguishing between isoflavones that are 6-substituted or lack 6-substitution (Tahara et al., 1991) indicating that in S/F4, position 6 is occupied by $–OCH_3$ group. The exchangeable signal for the 2^{nd} hydroxyl proton can be seen at 10.15 when the

sample is run in solvent composed of $CDCL_3$ + DMSO 90:10, while when using only $CDCL_3$ as solvent, the hydroxyl signal resides beneath protons at δ 6.519, 6.631 and 6.878 which when taken together integrates for 4 protons (Figure 3-17 & 3-18).

The NOE connectivity data proved conclusively that the methoxy positions in the B-ring were at the 2', 4' and 5' positions typical of isoflavones from Dalbergia species (Veitch et al., 2007). The A-ring connectivity's for S/F4 also identified key relationship. By irradiating the 6-OCH_3 no significant response was observed suggesting it was not next to an individual hydrogen atom and the same response was observed when hitting the 8-H. In both these experiments evidence for the presence of exchangeable protons was observed. Further evidence for the presence of the hydrogen from the 2nd hydroxyl group presenting below the three hydrogens H8 – 6.519, H3' – 6.631 and H6' – 6.878 and integrating for four protons (Figure 3-17) is shown in the NOE spectra (Figure 3-21) with signals that suggest proton exchange. This presents as a downfield phase signal due to exchange between the two-hydroxyl groups.

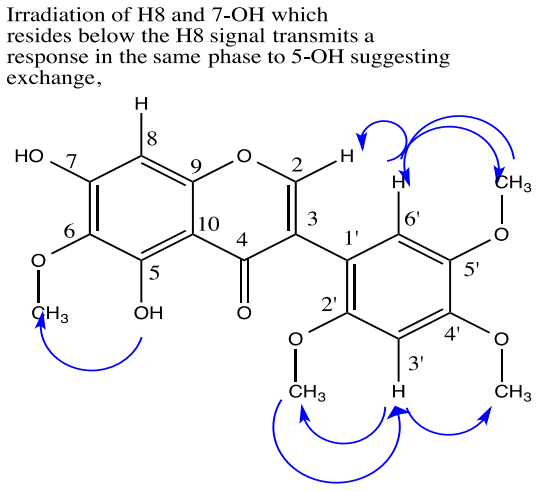


Figure 3 - 27: Shows the key NOE connectivity information

Due to the lack of coupling between any of the proton signals much of the structure determination had to be undertaken using the HMBC correlations to identify cross peak connectivity and substantiate the complete assignments. Cross peaks associated with the methoxy groups can be clearly identified on the HMBC spectrum and positions attributed to these are as detailed in Table 3-4. These positions for the methoxy groups in the B-ring conform to the common 2',4',5'-O-substitution found in Dalbergia legumes (Veitch et al., 2007) and confirm the NOE data obtained above. The cross peaks identified here are comparable with those of earlier studies (Kite et al., 2010; Dixit et al., 2012) and therefore the structure of S/F4 was identified as the isoflavonoid Caviunin or 5,7-dihydroxy, 6, 2',4',5'-tetramethoxy isoflavone.

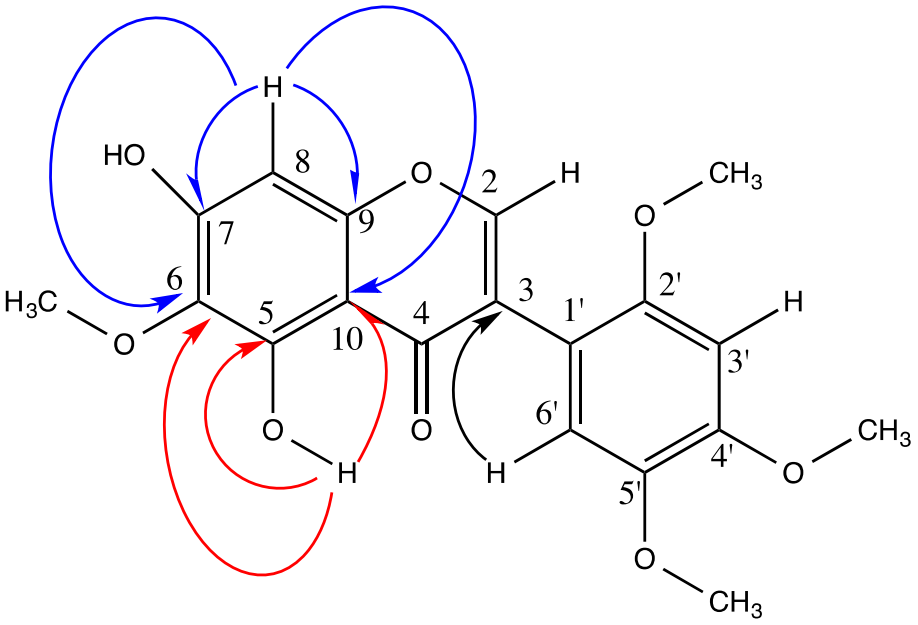


Figure 3 - 28: S/F4 or caviunin showing the key HMBC correlations

^{1}H NMR and ^{13}C signal assignments for S/F3 complement the data obtained for caviunin (S/F4). The major differences were identified in the A-ring and seen in the 5-OH signal, which was markedly lower at δ-12.633 and lies in the range predicted in

the study (Tahara et al., 1991) of a molecule that lacks 6-substitution. ^{13}C NMR signals were supported from information obtained from the DEPT-135, HSQC spectrum and the HMBC spectrum. Once again the key to decoding the structure of S/F3 was found in the HMBC spectra, which clearly showed the changes in the A-ring correlations compared to caviunin. Of significance was the 5-OH signal (δ-12.633), which correlates with the C-6 signal at δ 98.2 of S/F3, compared to the same 5-OH signal in caviunin (S/F4) that correlates with the downfield methoxylated carbon C-6 (δ-130.34) signal. The key HMBC correlations of S/F3 are shown above in Table 3-5 and are descriptive of the caviunin isomer isocaviunin or 5,7-dihydroxy, 8, 2',4',5'-tetramethoxy isoflavone. The detailed spectra for isocaviunin have been identified here for the first time.

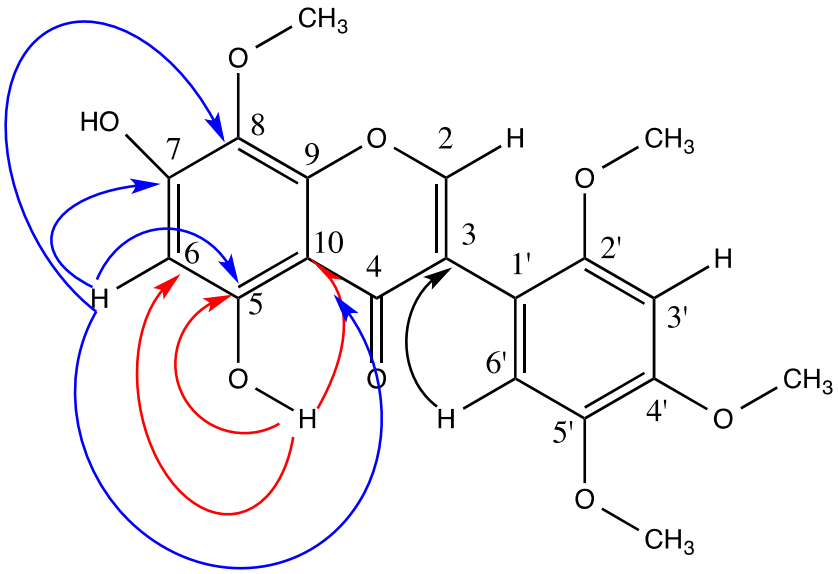


Figure 3 - 29: S/F3 or isocaviunin showing the key HMBC correlations

Caviunin has been isolated from a number of Dalbergia species, the first instance appears to be from *Dalbergia nigra*, identified chemically (Gottlieb et al., 1961). Since then caviunin has been isolated from *Dalbergia riparia* (Filho et al., 1973), *Dalbergia retusa* (Manners et al., 1974), *Dalbergia inundata* (de Almeida et al., 1974, *Dalbergia spruceana* (Cook et al., 1978) and *Dalbergia paniculata* from which caviunin has been shown to be present in almost every organ (Adinarayana et al., 1971). Glycosidic forms which are 'O'-substituted have also been isolated as caviunin 7-O-rhamnoglucoside from the root of *D. paniculata* (Rajulu et al., 1980), caviunin 7-O-[β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside from *Dalbergia sissoo* (Dixit et al., 2012) and from *D. paniculata* (Rajulu et al., 1980), and a 7-O-glycosylcaviunin from *D. riparia* where the sugar was not identified have been discovered (Filho et al., 1973). A 'C'- substituted glycoside 8-C-β-D-gluco-pyranosy1-5,7-dihydroxy-2',4',5',6-tetramethoxyisoflavone (8-C-glucosyl- caviunin), also called dalpaniculin has been isolated from *D.paniculata* seeds (Rao et al., 1991). Of the Dalbergia species identified here two are represented as close neighbours in the phylogenetic tree (Figure 2-11). There are no studies where caviunin is isolated from a plant outside the Dalbergia genus (Veitch et al., 2007; 2009; 2013) and may be considered to be indicative of this genus.

In contrast there is limited information on isocaviunin aglycone and a single paper provided ^{1}H NMR data that alone was inconclusive. These signals obtained in $CDCl_3$ (90MHz) for the glycosylated molecule and identified as isocaviunin 7-β-(1-6) gentiobioside from *D. sissoo* showed four-methoxy and four single proton assignments that were in agreement with the signals identified in this study, although assignments to positions had not been made in this paper (Sharma et al., 1980). A

study of *D. paniculata* seeds (Rao et al., 1991) mentions the isolation of isocaviunin 7-O-glucoside but no significant data is given.

The presence of compounds causing both contraction and relaxation in a 'tea' preparation is not unusual. Application of Lipinski's "rule of fives" that are widely applied in drug discovery to understand membrane permeability and absorption in the body of compounds following oral absorption, requires a partition coefficient (Log P) value less than five, a molecular weight less than five hundred and fewer than five H-bond donors and ten H-bond acceptors. Caviunin and isocaviunin meet all these criteria with predicted Log P values of 1.53 and 1.62 respectively. In contrast the preliminary information on the contractile components suggests they are distinctly hydrophilic and may not be absorbed or that the balance between contractile and relaxant effects is in favour of the latter following consumption. However this will only be verifiable once the contractile compounds are isolated.

In conclusion the compounds isolated as S/F3 and S/F4 from the bark of this Dalbergia species have been identified as the isoflavonoid isomers caviunin and isocaviunin. Both compounds show dose and endothelium-dependent vasorelaxant activity, as endothelium removal or incubation with L-NAME significantly attenuates relaxation in the rat aortic ring bioassay. Isocaviunin was found to be more potent than that of caviunin, and the vasorelaxant activity of both compounds was mediated by the release of NO.

In the next chapter detailed pharmacological experiments will be undertaken in an attempt to reveal the biochemical mechanisms by which each of the compounds isolated cause vascular relaxation.

Chapter 4

Endothelium-dependent mechanisms of Isocaviunin and Caviunin induced vascular relaxation

4.1 Introduction

In chapter 3 we have identified S/F3 and S/F4 as the non-steroidal phytoestrogens isocaviunin and caviunin respectively, and shown these compounds to have predominantly endothelium-dependent activity as endothelium removal or incubation of rat aortic rings with the NOS inhibitor L-NAME, attenuates vascular relaxation. These studies have also shown isocaviunin to cause greater relaxation when compared with its isomer caviunin.

Pharmacological characterisation of these compounds so far is limited to studies on the osteogenic potential of caviunin-based compounds from Dalbergia sissoo Roxb (Dixit et al., 2012; Kushwaha et al., 2014). Isoflavonoid compounds have structural features similar to estrogen (Fang et al., 2001; Simons et al., 2012) implying estrogenic activity. Work done on the more common isoflavones such as genistein and daidzein have identified rapid, non-genomic vasorelaxant activity similar to 17β-estradiol (Deng et al., 2012; Joy et al., 2006; Mishra et al., 2000).

4.2 Aims

In this chapter experiments are undertaken to characterize the endothelium-dependent acute functional responses to isocaviunin and caviunin using the rat aortic ring bioassay. The involvement of biochemical mechanisms upstream of NOS by which caviunin and isocaviunin induce rapid endothelium-dependent vascular relaxation will be studied using a range of receptor and enzyme inhibitors. This is the first study to be undertaken which assesses the vascular potential of these compounds and begins the process of elucidating the underlying mechanisms by which this occurs.

4.3 Materials

Sigma (UK)

Atropine sulphate

Tetraethylammonium

1H-[1,2,4] oxadiazolo[4,3-a]quinoxaline-1-one (ODQ)

Indomethacin

17β-estradiol

LY294002

Tocris Bioscience (USA)

ICI 182 780 (17α, 17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol)

Caviunin and isocaviunin were isolated in this project

All other chemicals used are listed in Chapter 3, p108.

4.4 Methods

4.4.1 Preparation of rat aortic rings

All the experiments detailed in this chapter use rat aortic rings to assess endothelium-dependent signalling pathways for all test compounds. The procedures used to prepare the aortic rings are fully described in Chapter 3, section 3.4.5.

4.4.2 Experimental protocols

At the core of these studies is the hypertensive model created using the α_1-receptor agonist phenylephrine (1μM) to pre-contract rat aortic rings. After a stable contraction is achieved cumulative doses of the compound are added to reverse this contraction and determine the level of relaxation that can be induced by each compound. Acetylcholine (1μM) or sodium nitroprusside (10μM) was added at the end of each experiment to confirm the integrity of the endothelium or smooth muscle respectively. All inhibitor studies were conducted by pre-incubating the rings for 20 minutes prior to repeating concentration response curves, apart from TEA, which was added just prior to precontraction.

4.4.3 Isocaviunin vascular studies

4.4.3.1 Construction of concentration-response curves

The cumulative addition of half log increments of isocaviunin (0.0003μM - 10μM) to phenylephrine (1μM) precontracted endothelium-intact rat aortic rings at 90-second intervals or when relaxation plateaued was used assess the relaxant response.

4.4.3.2 Determining the mechanism of endothelium-dependent relaxation by isocaviunin

The contribution of the signalling pathways via 1) NO/cGMP, 2) prostanoid or 3) involvement of potassium channels were assessed by repeating CRC in the presence of L-NAME (100μM), ODQ (10μM), indomethacin (10μM) or (TEA) (5mM) respectively.

4.4.3.3 Determination of receptor pathways activated by isocaviunin

Concentration response curves to isocaviunin were repeated in the presence of atropine (1μM) the non-selective inhibitor of muscarinic receptors, ICI 182 780 (0.1μM, 1μM, 10μM) the non-selective membrane estrogen receptor antagonist and G15 (3μM) the selective antagonist of the intracellular G-protein coupled estrogen receptor (GPER), to assess the involvement of these receptors in inducing relaxation.

4.4.3.4 Evaluation of PI3K inhibitor LY294002

LY294002 (10μM) is a cell permeable inhibitor of PI3-K and acts on the ATP binding site of this enzyme. Incubation of the rings with the inhibitor prior to the addition of PE (1μM) prevented contraction of the rings, while addition after contraction with PE (1μM) caused relaxation.

4.4.3.5 Comparison of isocaviunin-induced relaxation with acetylcholine

The cumulative addition of half log increments of acetylcholine (0.0003μM - 10μM) to phenylephrine (1μM) precontracted endothelium-intact rat aortic rings at 90-second intervals or when relaxation plateaued was used assess the relaxant response. These experiments were repeated again in the presence of L-NAME (100μM) and ODQ (10μM) to assess the NO/cGMP pathway and also by atropine (1μM) to determine the importance of the muscarinic (M_3) receptor in inducing activity.

4.4.4 Caviunin vascular studies

4.4.4.1 Construction of concentration-response curves

The cumulative addition of half log increments of caviunin (0.001μM - 30μM) to phenylephrine (1μM) precontracted endothelium-intact rat aortic rings at 90-second intervals or when relaxation plateaued was used to assess the relaxant response.

4.4.4.2 Determining the mechanism of endothelium-dependent relaxation by caviunin

Contribution of NO/cGMP and involvement of potassium-channels in caviunin-induced relaxation was assessed using the non-selective inhibitor L-NAME (100μM), the selective inhibitor ODQ (10μM), and the non-selective inhibitor of calcium-activated potassium channels TEA (5mM).

4.4.4.3 Determination of receptor pathways activated by caviunin

Concentration response curves to caviunin were repeated in the presence of ICI 182 780 (10μM) the non-selective membrane estrogen receptor antagonist and G15 (3μM) the selective antagonist of GPER receptors to assess the involvement of these receptors in inducing relaxation.

4.4.5 17β-estradiol vascular studies

4.4.5.1 Construction of concentration-response curves

The cumulative addition of half log increments of 17β-estradiol (0.01μM - 10μM) to phenylephrine (1μM) precontracted endothelium-intact rat aortic rings at 90-second intervals or when relaxation plateaued was used to assess the relaxant response.

4.4.5.2 Mechanism of endothelium-dependent relaxation by 17β- estradiol

Contribution of NO/cGMP or the involvement of potassium channels in 17β-estradiol-induced relaxation was assessed using the non-selective inhibitor L-NAME (100μM), selective inhibitor ODQ (10μM), or TEA (5mM) respectively. The combination of TEA (5mM) and ICI 182 780 (10μM) was also assessed together.

4.4.5.3 Determination of receptor pathways activated by 17β-estradiol

Concentration response curves with 17β-estradiol were repeated in the presence of ICI 182 780 (10μM) the non-selective membrane estrogen receptor antagonist and G15 (3μM) the selective antagonist of GPER receptors.

4.4.7 Statistical analysis

Statistical methods employed are detailed on p116.

4.5 Results

4.5.1 Isocaviunin results

4.5.1.1. Concentration response curve for isocaviunin on endothelium-intact rat aortic rings

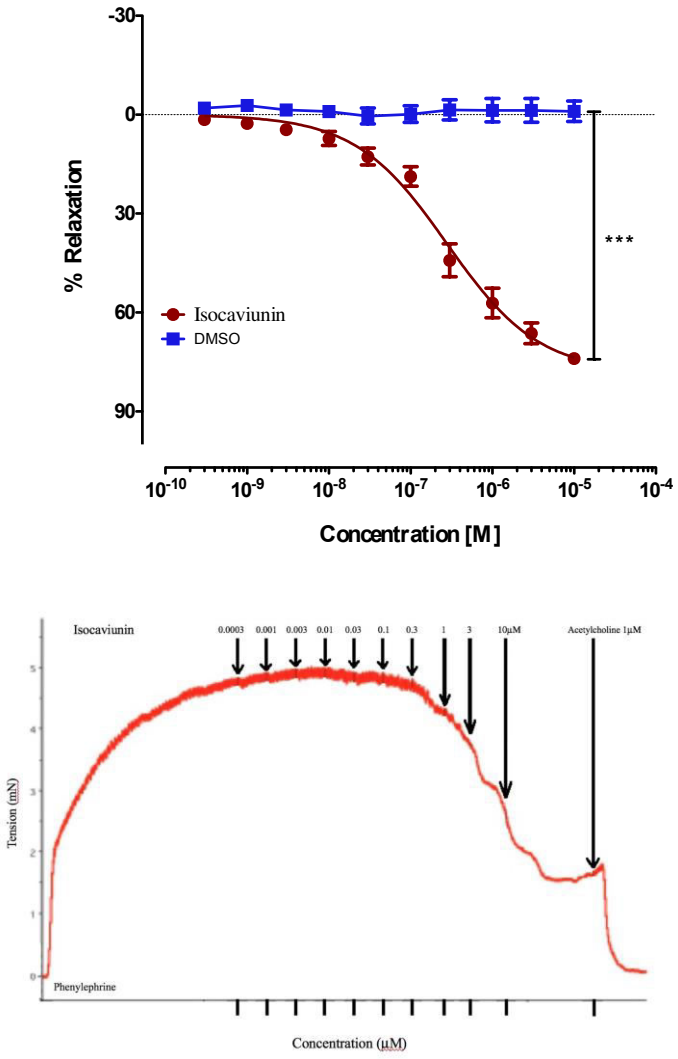


Figure 4 - 1: Dose response curve for isocaviunin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=15) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results show overall significance versus DMSO alone (* p≤0.001).**
Below is a representative trace of isocaviunin-induced relaxation.

Figure 4-1 shows the cumulative addition of isocaviunin caused a concentration-dependent relaxation in male endothelium-intact rat aortic rings with an $EC_{50}=1.669x10^{-6} \pm 1.53x10^{-7}$ M and relaxation of 73.9% ± 7.3% at 10μM, the highest concentration used. The concentration range was limited to 1nM to 10μM due to the limited quantity of compound that was available. The results of the D'Agostino-Pearson normality test with isocaviunin ($P>0.05$) suggest the data are not inconsistent with a Gaussian distribution.

The representative trace shown below in Figure 4-1 shows the increase in the contractile response over a time course of 8-10 minutes following the addition of phenylephrine (1μM), and the subsequent relaxation following addition of cumulative concentrations of isocaviunin (30nM–10μM). Acetylcholine added at the end of the experiments relaxes the tissue further and confirms the integrity of the endothelium.

4.5.1.2 Comparative effects of L-NAME and ODQ on isocaviunin and acetylcholine-induced relaxation

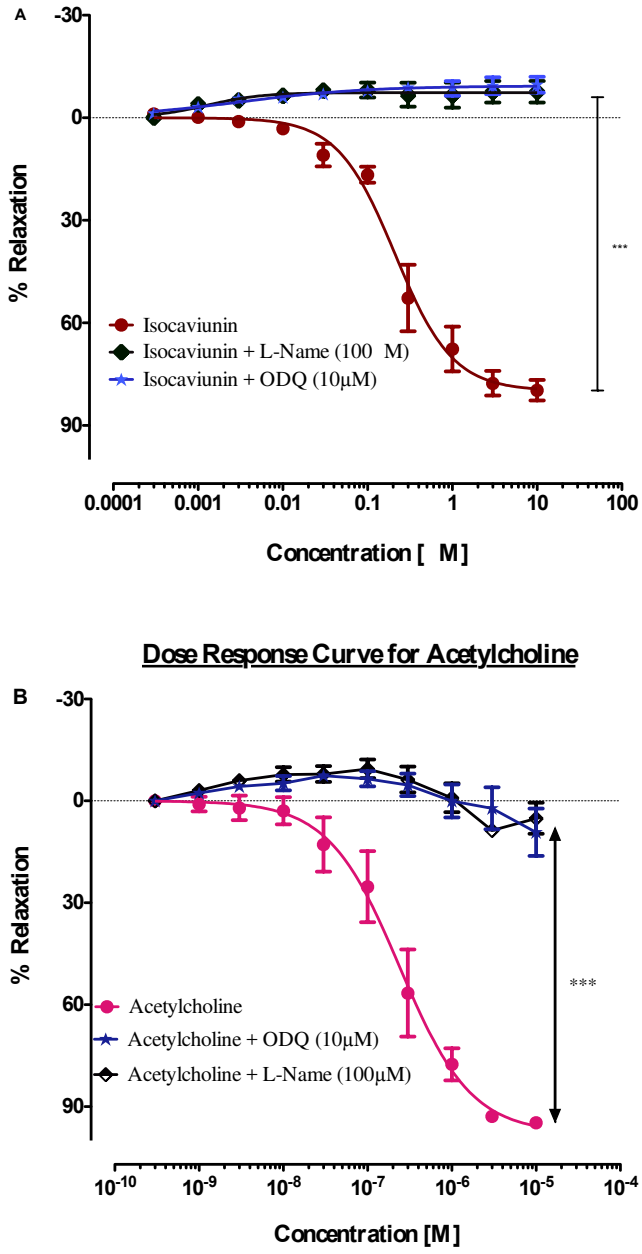


Figure 4 - 2: Inhibitory effect of L-NAME AND ODQ on (A) isocaviunin-induced relaxation and (B) acetylcholine-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean (n=4) ± S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results show overall significance versus drug alone (*p≤0.001).**

4.5.1.3 Effect of Indomethacin and TEA on isocaviunin-induced relaxation

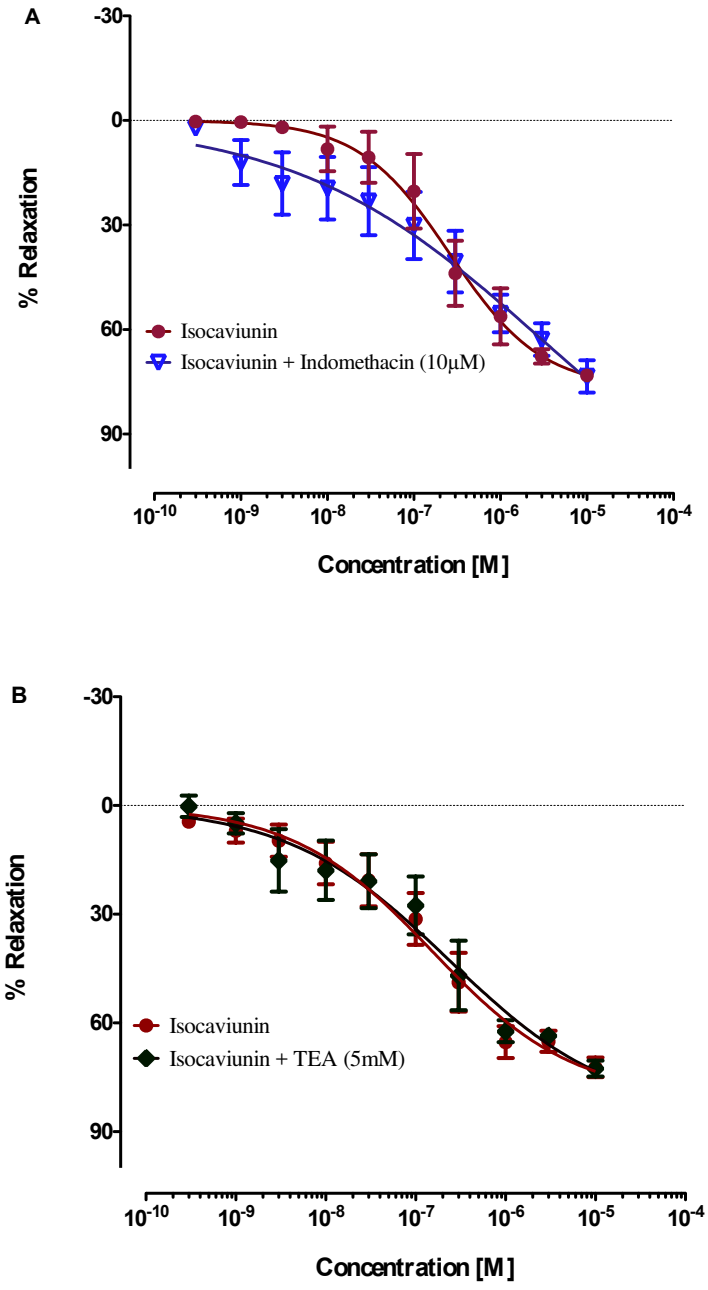


Figure 4 - 3: Effect of (A) indomethacin and (B) TEA on isocaviunin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean (n=4) ± S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. The results above are not significant.

The concentration response curve for acetylcholine was undertaken using the same dose-range as isocaviunin (1nM– 10μM). The acetylcholine-induced relaxation was more potent than that seen with isocaviunin with an EC_{50}= $2.671x10^{-7}$ ± $2.479\ x10^{-8}$M and relaxation of 94.8% ± 1.81% at 10μM. The results of the D'Agostino-Pearson normality test with acetylcholine (P>0.05) suggest the data are not inconsistent with a Gaussian distribution.

Incubation of rings with L-NAME, a non-selective inhibitor of NOS, and ODQ, the irreversible but NO competitive inhibitor of soluble guanylyl cyclase attenuated both isocaviunin and acetylcholine-induced relaxation. The contribution of the prostanoid pathway to the vasodilator effects of isocaviunin was evaluated using indomethacin, a non-selective inhibitor of COX to determine the influence of prostacyclin (PGI_2). Indomethacin did not significantly reduce the relaxation induced by isocaviunin and neither did TEA, the non-selective inhibitor of calcium-activated K^+ channels. These results suggest that isocaviunin-induced relaxation is mediated predominantly via the NO/cGMP pathway.

4.5.1.4 Comparative effects of atropine on isocaviunin and acetylcholine-induced relaxation

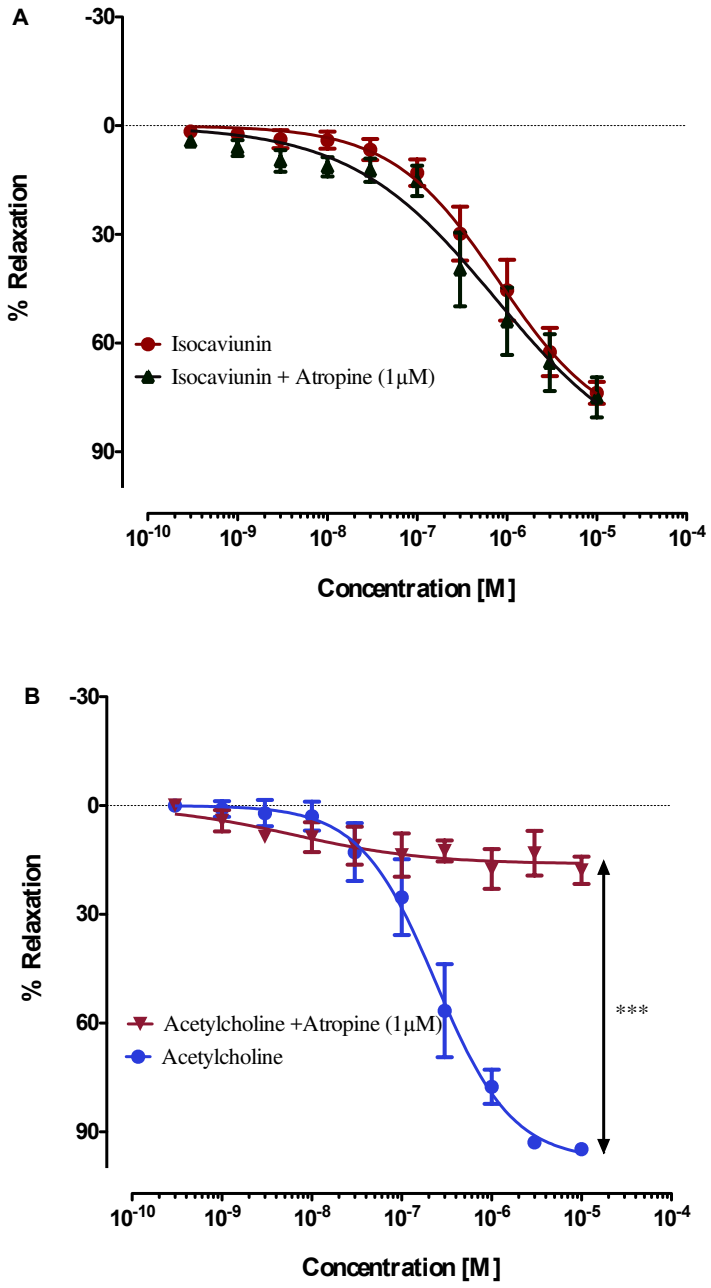


Figure 4 - 4: Comparison of the inhibitory effect of atropine on (A) isocaviunin (n=7) and (B) acetylcholine (n=3) induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results show overall significance versus acetylcholine alone (*p≤0.001).**

Pre-treatment of rat aortic rings with atropine (1μM) the non-selective muscarinic agonist attenuated acetylcholine-induced relaxation but showed no effect on vascular relaxation caused by isocaviunin. The computed results found for isocaviunin alone $EC_{50}=1.669x10^{-6} \pm 1.53x10^{-7}$ M and relaxation of 73.9% ± 7.3% at 10μM, and following incubation with atropine $EC_{50} = 1.784x10^{-6}$ M ± $2.46x10^{-7}$ M and relaxation of 77.6% ± 5.29% at 10μM, suggests that the muscarinic receptors are not involved in initiating isocaviunin-induced vasorelaxation.

4.5.1.5 Effect of membrane estrogen receptor inhibitor ICI 182 780 and G-protein coupled estrogen receptor inhibitor G15 on isocaviunin-induced relaxation

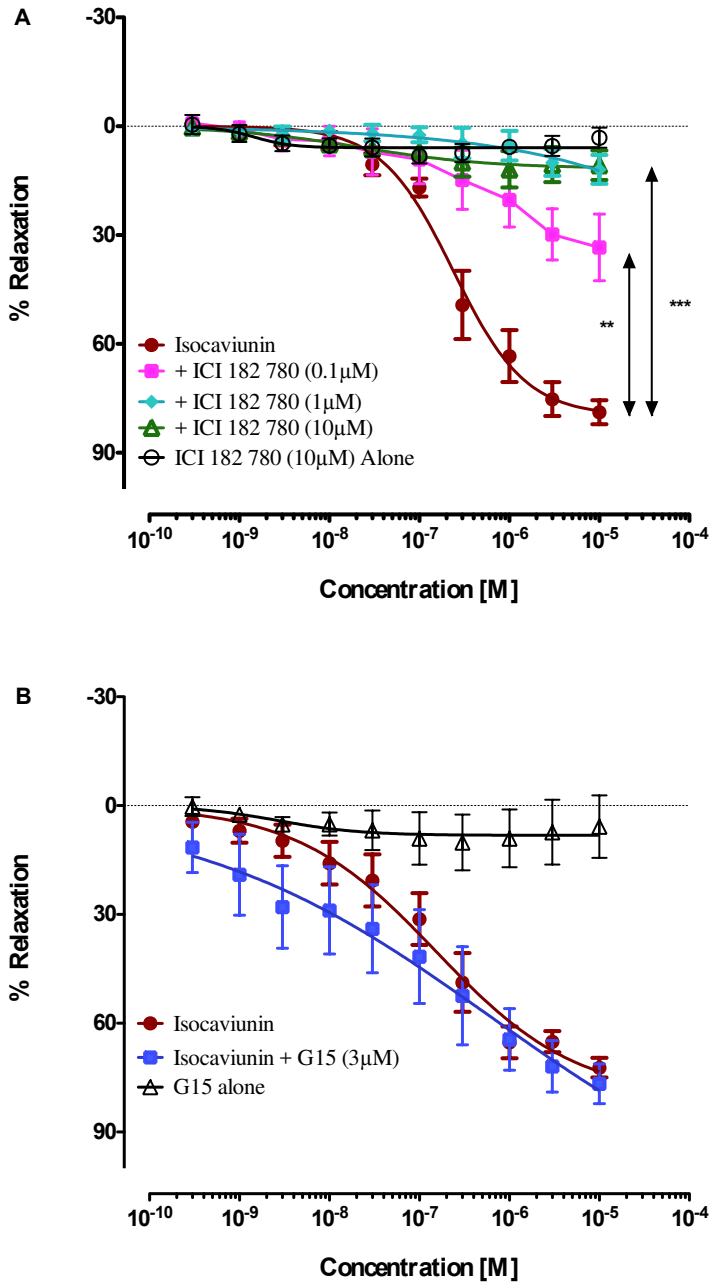


Figure 4 - 5: Effects of (A) estrogen receptor inhibitors ICI 182 780 and (B) G15 on isocaviunin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus drug alone in (A) (p≤0.01, ***p≤0.001).**

Figure 4-5 (A) above shows the inhibitory effects of the competitive membrane estrogen receptor inhibitor ICI 182 780 on isocaviunin-induced relaxation. The inhibitor showed a dose-dependent attenuation in isocaviunin-induced relaxation. This suggests that ERα and ERβ membrane estrogen receptors most likely mediate the rapid non-nuclear vasorelaxation of isocaviunin. Incubation of rat aortic rings with ICI 182 780 (10μM) alone did not have any significant vascular effects.

In contrast in Figure 4-5(B) the specific GPER selective antagonist G15 did not show attenuation of isocaviunin-induced relaxation in rat aortic rings. Experiments undertaken with G15 (3μM) alone did not show any significant vascular effects. It is therefore unlikely that the intracellular receptor GPER is involved in initiating isocaviunin vasorelaxation.

4.5.1.6 Effect of PI3K inhibitor LY294002 on rat aortic rings

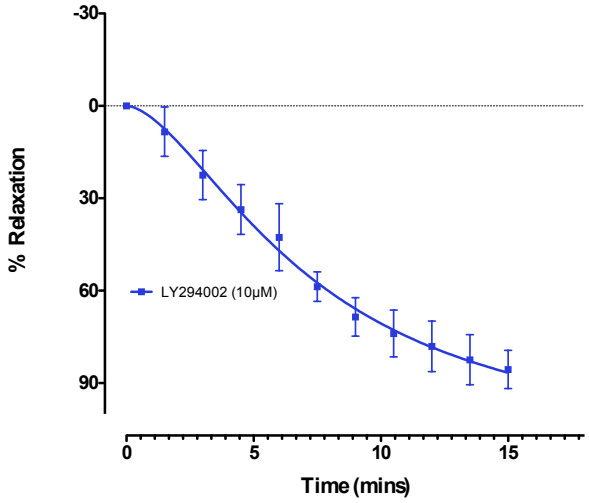


Figure 4 - 6: Effects of PI3K inhibitor LY294002 (10μM) alone on phenylephrine precontracted rat aortic rings. Results (n=3) are the mean ((±S.E.M.) responses expressed as a percentage of the maximum contraction of the inhibitor left for the duration of a typical experiment.

LY294002 alone, a PI3K inhibitor used to assess the involvement of the PI3K/Akt kinase pathway upstream of eNOS caused relaxation of the rat aortic rings. This prevented any further experiments being undertaken.

4.5.2 Caviunin results

4.5.2.1. Concentration response curve for caviunin on endothelium-intact rat aortic rings

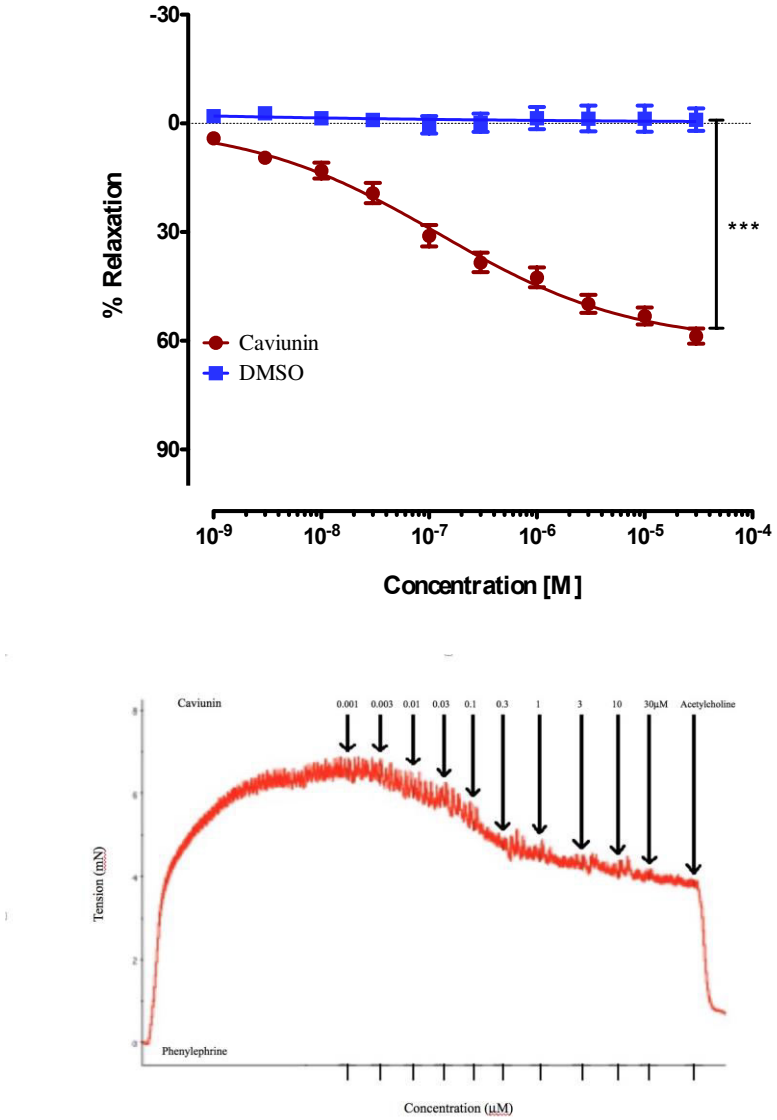


Figure 4 - 7: Dose response curve for caviunin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=15) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results show overall significance versus DMSO alone (* p≤0.001).**
Below is a representative trace of caviunin-induced relaxation.

Figure 4 -7 shows the effects of the cumulative addition of caviunin on rat aortic rings causes a concentration-dependent relaxation with an EC_{50} = 1.8 x 10^{-5} M ± 1.898 x 10^{-6} M and relaxation of 53.2 ± 6.4% at 10μM, of the maximum phenylephrine induced contraction. The concentration range was limited to 1nM to 30μM due to the limited quantity of compound that was available. The results of the D'Agostino-Pearson normality test with caviunin (P>0.05) suggest the data are not inconsistent with a Gaussian distribution.

Figure 4-7 shows a representative trace of caviunin-induced relaxation, identifying the increase in contraction following the addition of phenylephrine 1μM and the subsequent relaxation following addition of cumulative concentrations of caviunin (1nM to 30μM). Acetylcholine added at the end of the experiment shows further relaxation and confirms the integrity of the endothelium.

4.5.2.2 Effect of L-NAME, ODQ and TEA on caviunin-induced relaxation

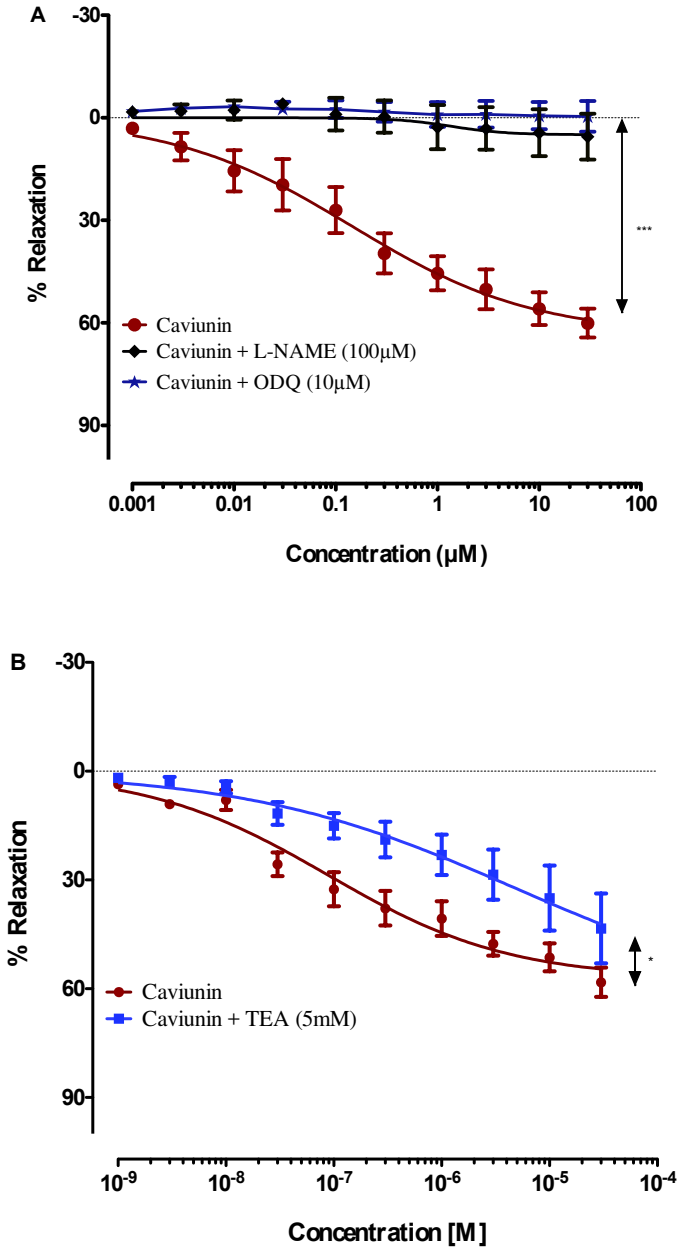


Figure 4 - 8: Effect of (A) L-NAME, ODQ (n=5) and (B) TEA (n=7) on caviunin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results show overall significance versus caviunin alone (*p≤0.05, ***p≤0.001).

Figure 4-8 (A) shows the caviunin-induced relaxation was abolished following incubation of rings with L-NAME, a non-selective inhibitor of NOS and ODQ the irreversible but NO competitive inhibitor of soluble guanylyl cyclase. Figure 4-8 (B) shows TEA, the non-selective inhibitor of calcium-activated K^+ channels also showed attenuation in relaxation of caviunin-induced relaxation that was significant. These results suggest that caviunin-induced relaxation is mediated predominantly via the NO/cGMP pathway but EDHF response may also be involved albeit to a lesser extent.

4.5.2.3 Effect of membrane estrogen receptor inhibitor ICI 182 780 and G-protein coupled estrogen receptor inhibitor G15 on caviunin-induced relaxation

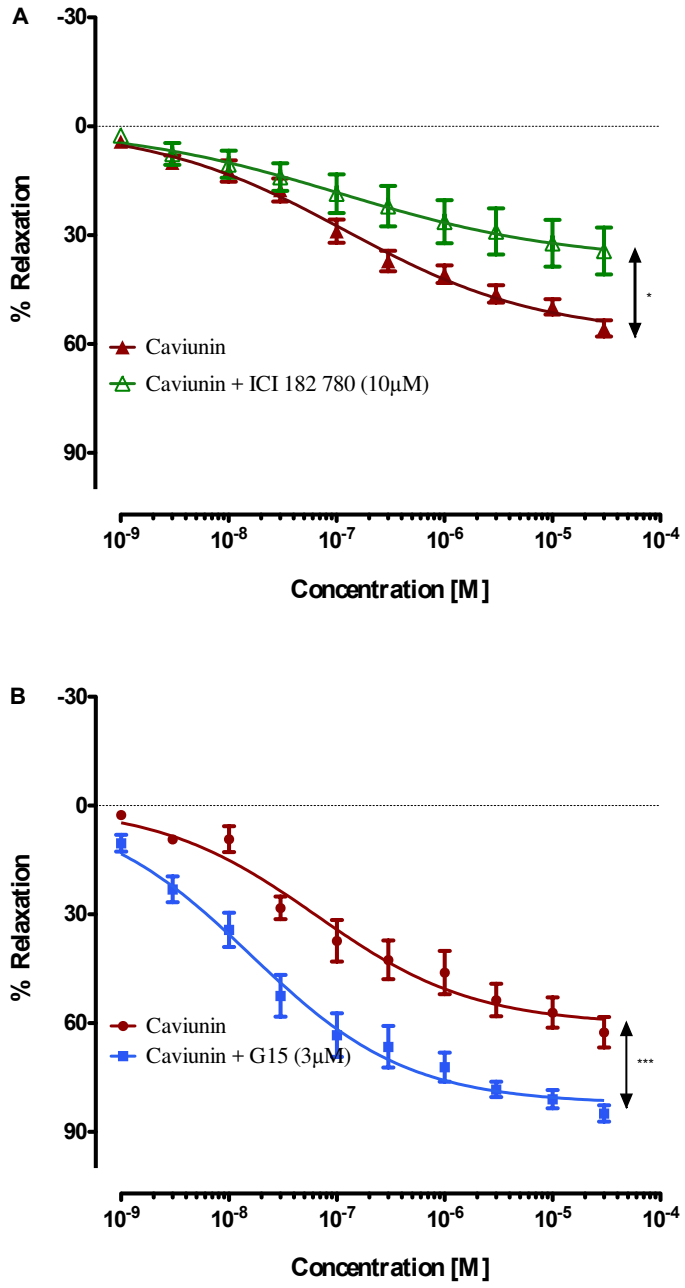


Figure 4 - 9: (A) Inhibition of caviunin-induced relaxation by membrane estrogen receptor inhibitor ICI 182 780 and (B) potentiation of caviunin-induced relaxation by GPER inhibitor G15 on phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (ICI 182 780 (n=11), G15 (n=6)) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus caviunin alone (*p≤0.05, ***p≤0.001).

Figure 4-9 (A) shows the effect of the membrane estrogen receptor inhibitor ICI 182 780 (10μM) on caviunin-induced relaxation of rat aortic rings precontracted with phenylephrine (1μM) that was significant, but did not completely abolish the vasorelaxant effect unlike that seen with isocaviunin Figure 4-5 (A). This suggests that other vasorelaxant pathways are involved in causing acute caviunin-induced relaxation.

In Figure 4-9 (B) shows in contrast, caviunin-induced relaxation following incubation with G15, the selective inhibitor of GPER, was significantly potentiated. This effect was observed immediately, increasing with the addition of each cumulative concentration of caviunin and resulted in nearly a 50% increase in relaxation to 81.9% at 10μM and EC_{50} = 2.61 x 10^{-7} M ± 7.52 x 10^{-8} M compared to relaxation of 53.2% and EC_{50} 1.8 x 10^{-5} M ± 1.898 x 10^{-6} M with caviunin alone.

4.5.2.4 Effect of a combination of estrogen receptor inhibitor ICI 182 780 and TEA, inhibitor of calcium-activated K^+ channels on caviunin-induced relaxation

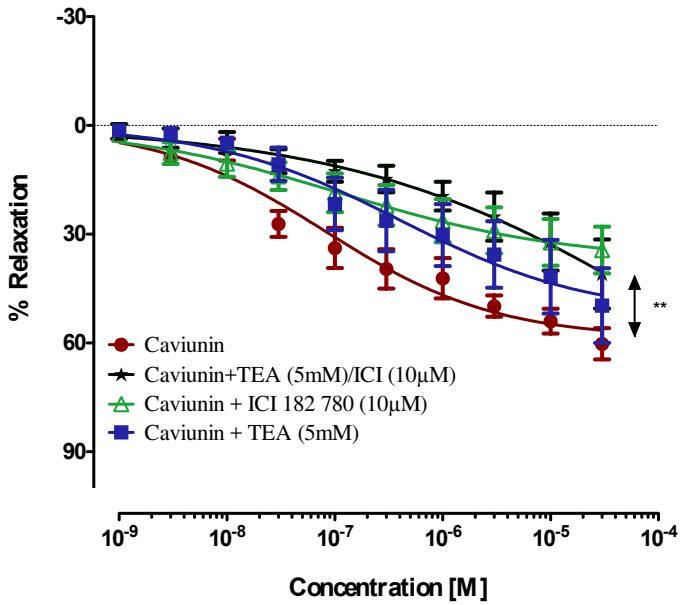
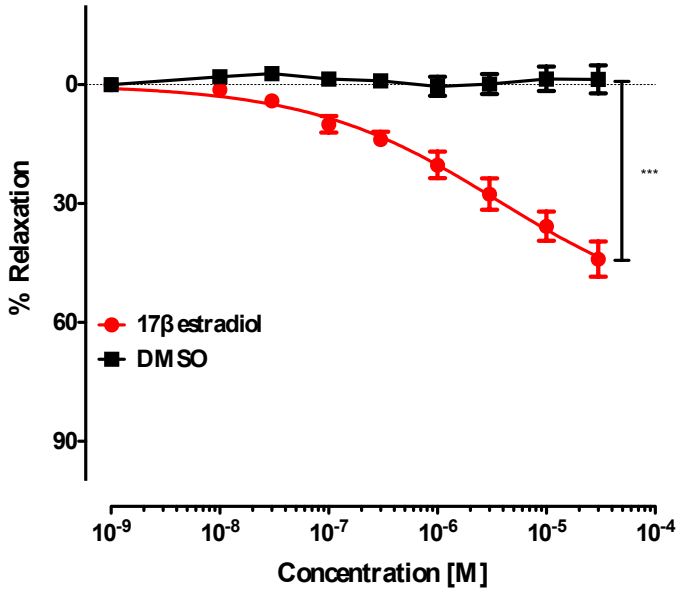


Figure 4 - 10: Comparison of the effects of a combination of ICI 182 780 and TEA and with the inhibitors individually on caviunin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus caviunin alone (p≤0.01).**

The combination of ICI 182 780 and TEA was used to inhibit both membrane estrogen receptors and calcium-activated potassium channels together. This combination attenuated the relaxation induced by caviunin significantly (**p≤0.01), but this effect was not significantly different when compared with either ICI 182 780 or TEA alone on caviunin-induced relaxation. It is unclear at this stage which other biochemical pathways are involved in causing caviunin-induced relaxation.

4.5.3 17β-estradiol results

4.5.3.1 Concentration response curve for 17β-estradiol on endothelium-intact rat aortic rings



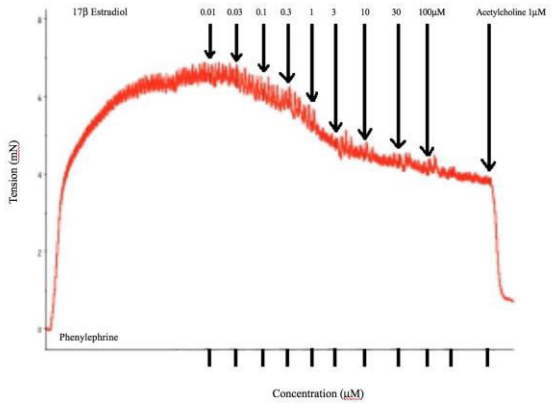


Figure 4 - 11: Dose response curve for 17β-estradiol-induced relaxation of phenylephrine (1μM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=8) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. The results show overall significance versus DMSO alone (* p≤0.001).**
A representative trace is shown below.

Figure 4-11 shows the effects of the cumulative addition of 17β-estradiol on rat aortic rings causes a concentration-dependent relaxation with an EC_{50} = 3.13 x 10^{-5} M ± 2.88 x 10^{-6} M and relaxation of 36.05 ± 3.78% at 10μM, of the maximum phenylephrine induced contraction. The concentration range used ranged from 10nM to 100μM for the dose-response curve only. The results of the D'Agostino-Pearson normality test with 17β-estradiol (P>0.05) suggest the data are not inconsistent with a Gaussian distribution.

Figure 4-11 also shows a representative trace of 17β-estradiol-induced relaxation of rat aortic rings, identifying the increase in contraction following the addition of phenylephrine 1μM and the subsequent relaxation following addition of cumulative concentrations of 17β-estradiol. Acetylcholine added at the end of the experiment shows further relaxation and confirms the integrity of the endothelium.

4.5.3.2 Effect of L-NAME, ODQ and TEA on 17β-estradiol-induced relaxation

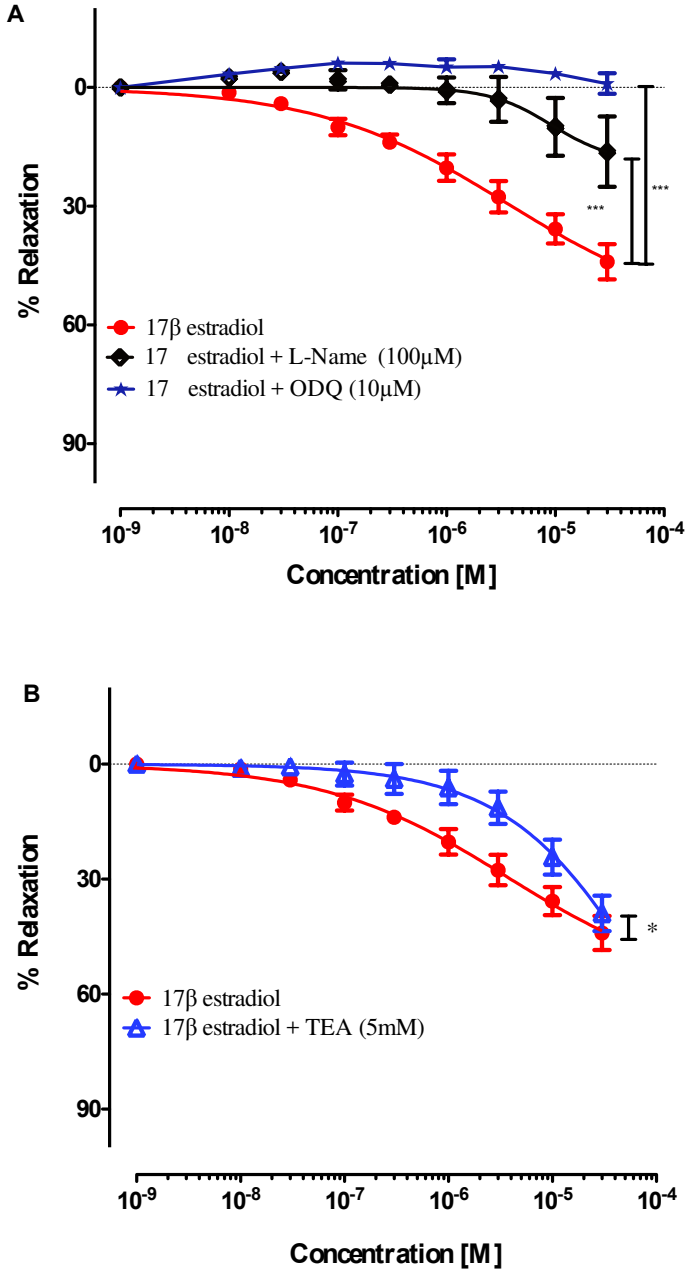


Figure 4 - 12: Effects of (A) L-NAME and ODQ and (B) TEA on 17β-estradiol-induced relaxation of phenylephrine (1μM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ±S.E.M. (L-NAME & ODQ n=4, TEA n=7 & 17β-estradiol n=8) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus 17β-estradiol alone (*p≤0.05, ***p≤0.001).

Figure 4-12 (A) shows the 17β-estradiol-induced relaxation was abolished following incubation of rings with L-NAME, a non-selective inhibitor of NOS and ODQ the irreversible but NO competitive inhibitor of soluble guanylyl cyclase. Figure 4-12 (B) shows TEA, the non-selective inhibitor of calcium-activated K^+ channels also showed attenuation in relaxation of caviunin-induced relaxation that was significant. These results suggest that caviunin-induced relaxation is mediated predominantly via the NO/cGMP pathway but EDHF response or BK_{Ca} activation may also be involved albeit to a lesser extent.

4.5.3.3 Effect of membrane estrogen receptor inhibitor ICI 182 780 and G-protein coupled estrogen receptor inhibitor G15 on estrogen-induced relaxation

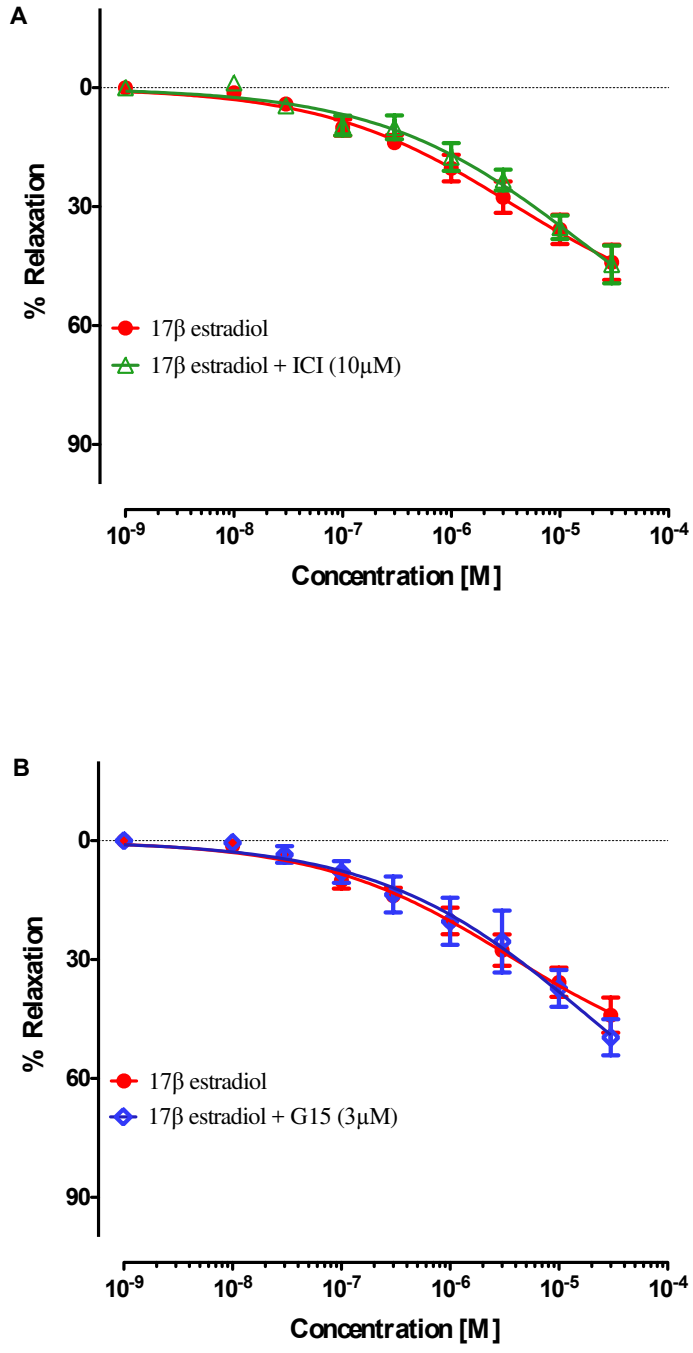


Figure 4 - 13: Comparison of effects (A) ICI 182 780 and (B) G15 on 17β-estradiol (n=8) induced relaxation on phenylephrine (1μM) precontracted endothelium-intact rat aortic rings. Results are the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results do not show significance.

Figure 4-13 (A) and 4-13 (B) shows that 17β-estradiol induced acute vasorelaxation on rat aortic ring precontracted with phenylephrine (1μM), is not attenuated following incubation with either ICI 182 780 (10μM), an inhibitor of membrane estrogen receptors or G15 (3μM), the intracellular GPER inhibitor.

4.5.3.4 Effect of a combination of estrogen receptor inhibitor ICI 182 780 and TEA, inhibitor of calcium-activated K^+ channels on caviunin-induced relaxation

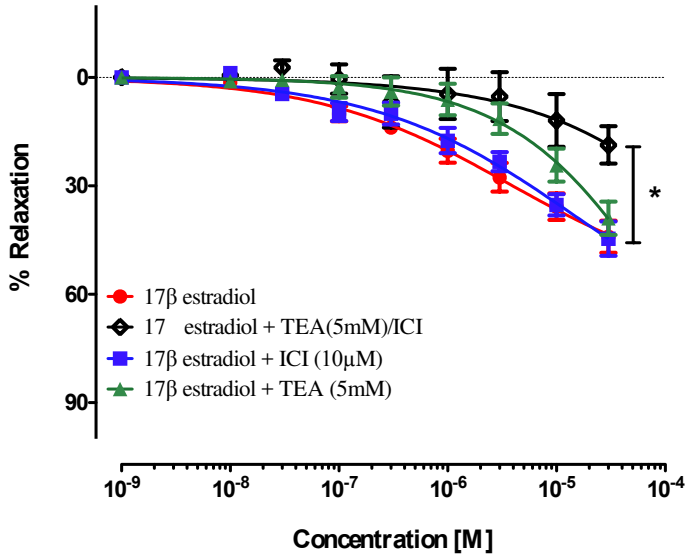


Figure 4 - 14: Comparison of the effects of a combination of ICI 182 780 and TEA and individually on 17β-estradiol-induced relaxation of phenylephrine (1μM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ± S.E.M. (n=4) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus 17β-estradiol alone (*p≤0.05).

Figure 4-14 above shows the combination of ICI 182 780 and TEA was used to inhibit both membrane estrogen receptors and calcium-activated potassium channels together. This combination attenuated the relaxation induced by 17β-estradiol significantly, but this effect was not significantly different when compared with either ICI 182 780 or TEA individually on 17β-estradiol-induced relaxation. These results compare with caviunin above Section 4.5.2.4.

4.6 Discussion

The results identified in the last chapter show for the first time that the phytoestrogens isocaviunin and caviunin can induce acute vasorelaxation in a dose and endothelium-dependent manner in the rat aortic ring bioassay. Although these two compounds are structurally highly similar, differing only in the position of a single methoxy group, there are key differences in biochemical pathways activated as detailed below.

4.6.1 Isocaviunin

Isocaviunin has been identified as having potent acute vasodilatory effects that are endothelium-dependent involving the NO/cGMP pathway predominantly, as vasorelaxant activity is significantly attenuated by L-NAME and ODQ. Neither the prostanoid pathway nor calcium-activated potassium channels were found to be responsible for isocaviunin-induced activity, as relaxation was not attenuated following incubation with either indomethacin or TEA. Parallel experiments undertaken in this project with acetylcholine and the endogenous 17β-estradiol-induced vascular relaxation were also shown to be inhibited following incubation with L-NAME and ODQ. 17β-estradiol acting via ERα has also been shown to cause vascular relaxation by increasing prostacyclin (Burger et al., 2009; Sobrino et al., 2010) and by activating BK_{Ca} in the smooth muscle (Abou-Mohamed et al., 2003), vascular effects that can be inhibited by indomethacin (10μM) or TEA (1mM) respectively. Endothelium-derived hyperpolarising effects via the SK_{Ca} and IK_{Ca} have also been shown to be important in 17β-estradiol mediated vasorelaxation (Liu et al., 2001). It is unlikely these mechanisms are involved in isocaviunin-induced relaxation. Known upstream receptor pathways that activate NOS were investigated using atropine, the non-selective inhibitor of muscarinic receptors. The concentration of atropine used was shown to attenuate the more potent acetylcholine-induced vascular

relaxation, but the same concentration of atropine did not attenuate isocaviunin-induced relaxation, demonstrating that muscarinic receptors are not activated by isocaviunin. In contrast studies with the competitive membrane estrogen receptor inhibitor ICI 182 780 significantly attenuated relaxation at all three-inhibitor concentrations trialled. The vasorelaxant response induced by isocaviunin is therefore initiated by activation of the population of membrane estrogen receptors on the endothelium. Studies with the recently identified intracellular G-protein coupled estrogen receptor (GPER) inhibitor G15 however did not attenuate relaxation. Experiments were also undertaken to determine the involvement of the PI3K/Akt enzyme pathway using the PI3K inhibitor LY294002. Despite a number of attempts LY294002 alone was found to cause relaxation of phenylephrine precontracted aortic rings, which prevented further study. Overall these results have shown that isocaviunin induces relaxation by singularly activating membrane estrogen receptors to activate eNOS, resulting in the release of NO, which activates cGMP in the smooth muscle leading to relaxation.

4.6.2 Caviunin

Caviunin-induced relaxation was also shown to be endothelium-dependent occurring predominantly via the NO/cGMP pathway as L-NAME and ODQ significantly attenuate relaxation. However the vasorelaxant effect induced is less potent than isocaviunin. It also differs from isocaviunin in that TEA attenuates relaxation significantly suggesting the partial involvement of calcium-activated potassium channels in inducing relaxation. TEA was also found to significantly attenuate 17β-estradiol-induced relaxation to a similar degree as that of caviunin. To study the involvement of estrogen receptors, ICI 182 780 and G15 inhibitor responses to caviunin were studied. Whereas ICI 182 780 marginally but significantly attenuated

relaxation, G15 markedly potentiated caviunin-induced relaxation. A combination of ICI 182 780 and TEA together also significantly attenuated relaxation of caviunin, but this attenuation in relaxation was not significantly different from experiments using either ICI 182 780 or TEA alone. Analogous experiments undertaken to assess membrane estrogen receptor and GPER activation by 17β-estradiol using ICI 182 780 and G15 respectively showed no significant attenuation in relaxation by either of these inhibitors in the rat aortic ring model.

These results show the similarities and subtle differences in the functional response of isocaviunin and caviunin. They both cause acute relaxation predominantly via NO/cGMP pathway most likely by activating eNOS in preference to nNOS as these studies were undertaken on male rats (Lekontseva et al., 2011), following activation of either ERα or ERβ or both membrane estrogen receptors albeit to varying degrees. Either an EDHF component via SK_{Ca} or IK_{Ca} potassium channels or BK_{Ca} activation is also important in caviunin-induced relaxation, mechanistic pathways also exploited by 17β-estradiol.

4.6.3 Estrogen receptor-induced vascular activity

Stimulation of the membrane estrogen receptors was found to be solely responsible for isocaviunin-induced relaxation, partially involved with caviunin-induced relaxation and not seen in studies with 17β-estradiol in the rat aortic ring model. The schematic below depicts the likely pathways following activation of membrane estrogen receptors.

4.6.3.1 Schematic of eNOS activation by membrane estrogen receptors

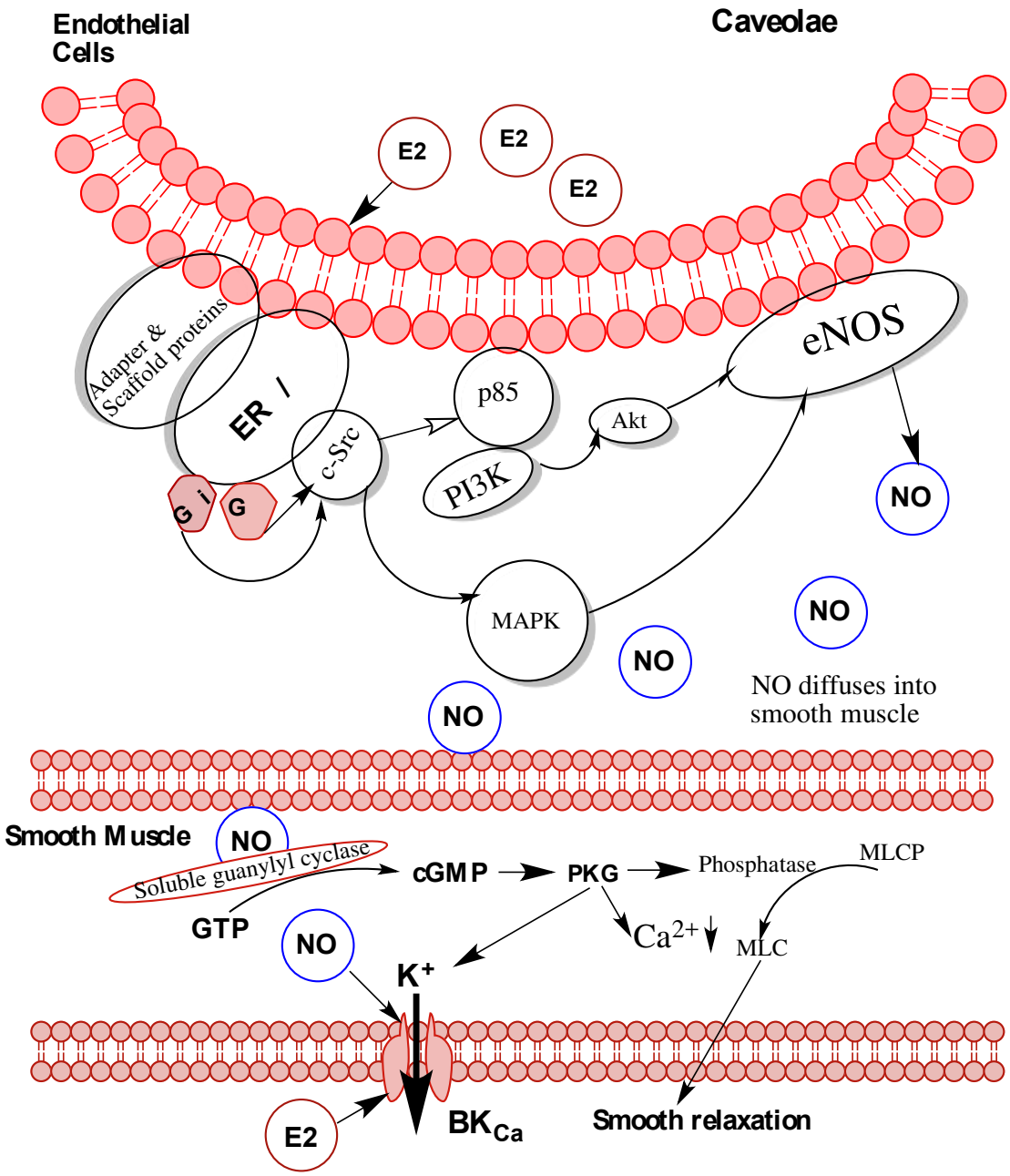


Figure 4 - 15: Known mechanisms of rapid, non-genomic estrogen ERα and ERβ receptor signalling in caveolae of endothelial cells following agonist binding. (Compiled from Chambliss et al., 2010; Fulton et al., 1999; Guo et al., 2005; Haynes et al., 2003; Kumar et al., 2007; Mendelsohn et al., 2010; Razandi et al., 2000; Simoncini et al., 2000)

The schematic above depicts the transduction pathways identified to date following ERα or ERβ activation, and now shown to be associated with isocaviunin and caviunin-induced relaxation. The ERα and ERβ receptors associated with inducing acute vasorelaxation were initially identified following studies with endogenous 17β-estradiol. Isoflavonoid compounds like genistein that mimic key estrogen binding motifs are also known to bind to estrogen receptors (Kuiper et al., 1998). These receptors have a distinct tissue distribution and are shown to mediate effects in the immune, cardiovascular, CNS, skeletal and reproductive systems (Gustaffson et al., 2003; Kuiper et al., 1996). ERα and ERβ receptors are found localized in the caveolae of endothelial cell membranes bound to scaffold and adaptor proteins (Chambliss et al., 2000), and on agonist binding they combine with low molecular weight Gαi and Gβγ G-proteins (Kumar et al., 2007; Chambliss et al., 2002; Chambliss et al., 2010) to initiate a multiplicity of pathways to activate eNOS (Guo et al., 2005). This occurs via the downstream tyrosine kinase Src enzyme to activate the p85 subunit of the PI3-kinase pathway (Haynes et al., 2003), which can be inhibited by LY294002 or Wortmannin (Bucci et al., 2002), and leads in turn to PKB/Akt phosphorylation of eNOS at serine 1177 with the release of NO (Chambliss et al., 2010; Mendelsohn et al., 2010). Mitogen activated protein kinases (MAPK) (Chen et al., 1999) are the other major pathway leading directly to eNOS activation.

ICI 182 780 is a selective estrogen receptor downregulator (SERD) and is a non-selective inhibitor of both ERα and ERβ, but it is also known to activate like other classical ER antagonists e.g. tamoxifen, the intracellular GPER receptor (Filardo et al., 2000; Thomas et al., 2005) and BK_{Ca} channel on smooth muscle (Dick et al.,

2002) to cause vascular relaxation. No significant effects were identified in our control experiments with ICI 182 780 or G15 alone.

The contribution of these membrane receptors in inducing the isocaviunin-induced response is therefore straightforward as ICI 182 780 shows a clinical and highly specific attenuation in relaxation. It is tempting then to suggest that isocaviunin-induced relaxation is solely due to membrane estrogen receptors. The enormous potential of identifying a compound with acute estrogenic activity that may not have the associated nuclear estrogenic effects has obvious therapeutic advantages, for example in breast cancer treatment (Wu et al., 2011). The results of the studies here with caviunin-induced relaxation, like that of 17β-estradiol is less conclusive and suggest activation of multiple pathways resulting in eNOS activation.

17β-estradiol differentially activates membrane estrogen receptors and the more recently discovered intracellular GPER receptor that resides on the endoplasmic reticulum (Haas et al., 2007). The results of 17β-estradiol-induced relaxation of rat aortic rings observed here complement ring experiments using female rats (Bolego et al., 2005) and male rats (Bucci et al., 2002). However ICI 182 780 (10μM) could not attenuate 17β-estradiol-induced relaxation in these experiments and others (Mishra et al., 2000). However using a higher concentration of ICI 182 780 (20μM), Bucci et al., showed a significant attenuation of E2-induced relaxation. There is the possibility that the concentration of ICI 182 780 used in the experiments here was not high enough, although the 10μM concentration used here did abolish isocaviunin-induced relaxation. This comparison adds weight to the fact that membrane estrogen receptors are the primary mechanism by which isocaviunin induces relaxation and that this is

not the case for either caviunin or 17β-estradiol. It would be interesting to repeat experiments with 17β-estradiol using higher concentrations of ICI 182 780.

The signalling pathway following isocaviunin and caviunin activation of membrane estrogen receptors is most likely transduced via a kinase pathway to activate eNOS. 17β-estradiol is known to activate eNOS via the PI3K/Akt kinase induced phosphorylation of eNOS at physiologically sub-maximal Ca^{2+} concentrations (Simoncini et al., 2000). Recent findings show that PI3K mediates E2 activity by increasing association of ERα via Src to p85α, the regulatory subunit of PI3K (Simoncini et al., 2003), leading to Akt activation of eNOS (Fulton et al., 1999). Tyrosine kinase (TK) and mitogen-activated protein kinase (MAPK) pathways have also been explored to understand 17β-estradiol -induced vasorelaxation. Inhibition of these pathways prevented the activation of eNOS suggesting that 17β-estradiol induces non-genomic activity via either TK or MAPK or both pathways (Chen et al., 1999).

Studies with the PI3K inhibitor LY294002 undertaken in this project proved challenging. It has been shown that LY294002 is not selective for PI3K and binds to targets unrelated to the PI3K family (Gharbi et al., 2007). Nevertheless using higher concentrations of LY294002 (25μM), a significant reduction in 17β-estradiol mediated relaxation after 6 hours of incubation was reported confirming reports that PI3K is involved in 17β-estradiol -mediated relaxation of rat aortic rings (Bucci et al., 2002).

4.6.3.2 Schematic of intracellular GPER induced activation of eNOS

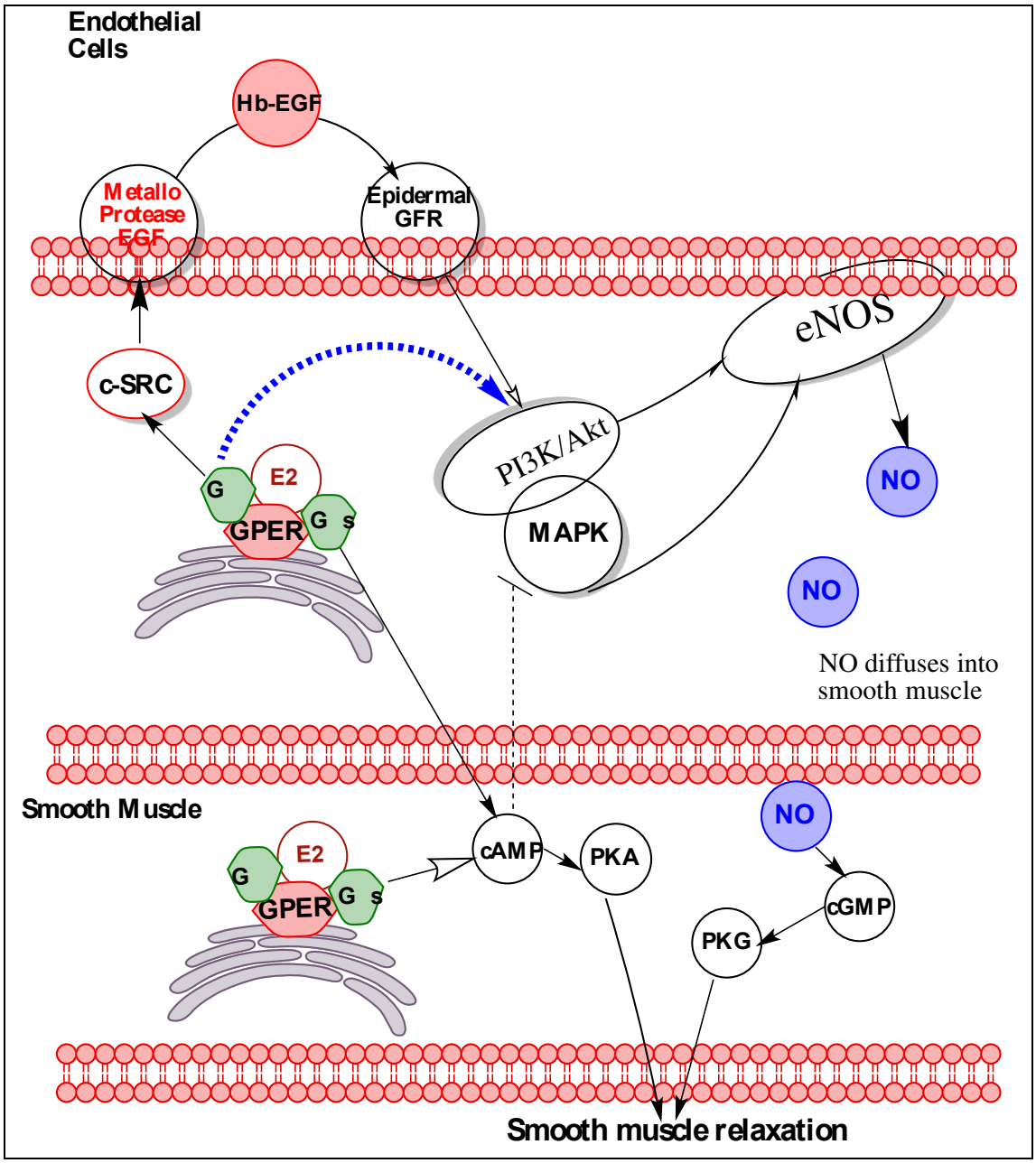


Figure 4 - 16: Schematic of known mechanisms of intracellular G-protein coupled estrogen receptor GPER induced vascular relaxation (Compiled from Broughton et al., 2010; Jang et al., 2013; Lindsey et al., 2014; Meyer et al., 2014; Revankar et al., 2005; Yu et al., 2014)

The schematic above depicts the complexity of the transduction pathways following GPER binding and activation by 17β-estradiol, mostly elucidated using the GPER specific agonist G-1. The dashed lines are suggested pathways not yet confirmed by experiment. Much of the early work on GPER activation was undertaken using non-vascular cell lines that identified the intracellular location of GPER (Revankar et al., 2005) or on the plasma membrane (Filardo et al., 2007). In these studies GPER Gβγ induced transduction via c-SRC activation of metallo-proteases to cause activation of epidermal growth factor receptor and MAPK (Filardo et al., 2000) and a second pathway via Gαs to activate adenylyl cyclase with the subsequent elevation of cAMP, which opposes MAPK, were identified (Filardo et al., 2002).

In the vasculature a number of these GPER mechanisms are also being elucidated following the discovery of GPER in a number of vessels (Haas et al., 2007). The importance of the GPER receptor in decreasing mean arterial pressure was shown using G-1 in normotensive Sprague-Dawley rats (Haas et al., 2009) and ovariectomized rats (Lindsey et al., 2009 and 2011). In rat carotid arteries GPER was present to a greater extent in endothelial cells than in VSMC, and aortic ring studies show that both endothelium-denudation and L-NAME abolish G-1-induced vasorelaxation (Broughton et al., 2010). Deletion of GPER in mice increases COX-derived prostanoid contractile factors resulting in endothelial vasoconstriction of VSMC (Meyer et al., 2012). G-1 mediated phosphorylation of Ser-1177 on eNOS occurs via Akt and is also shown to be sensitive to PI3K inhibition (Meyer et al., 2014), although the GPER effect was assessed to be less important than that of ERα or ERβ (Ding et al., 2015). Increase in cAMP following G-1, ICI182 780 or tamoxifen activation of GPER in porcine coronary smooth muscle is also found to be involved in vascular relaxation by activating PKA to dephosphorylate MLCP

(Lindsey et al., 2014; Yu et al., 2014) while vascular endothelium-intact and denuded rat aortic ring studies have been used to essentially confirm activation of the extracellular EGFR-pathway to PI3K/Akt activation first shown by Filardo et al., 2000 and 2002 (Jang et al., 2013). These results highlight the central role of the GPER receptor in mediating vascular tone.

In this study the influence of the intracellular GPER receptor was assessed using the GPER selective inhibitor G15. G15 did not significantly attenuate isocaviunin-induced relaxation. In contrast the effect of G15 on caviunin-induced relaxation was pronounced resulting in marked potentiation of the relaxant response that occurs immediately on addition of the first concentration of caviunin. This resulted in the significant increase in relaxation by nearly 50% of the caviunin-induced relaxation. If it can be proven that the cAMP inhibitory effects on MAPK first identified in cancer cells (Filardo et al., 2002) also occurs in the vasculature, it can provide a mechanism to explain caviunin-induced enhancement of vasorelaxation. G15 is a relatively recent discovery and therefore it is not surprising that there is minimal literature on its use as a pharmacological tool. Available information on G15 ligand binding studies or pharmacology investigations determining it as a selective inhibitor of GPER do not describe inhibition of contractile effects of for example, contractile prostaglandins (Dennis et al., 2009) which may provide a mechanism to describe this potentiation. Our data obtained here with 17β-estradiol, following incubation with G15 showed no significant difference.

4.6.4 Endothelium-dependent hyperpolarization effects

Caviunin-induced relaxation is not explained completely by membrane estrogen receptor activation unlike isocaviunin. Like 17β-estradiol however caviunin is

significantly inhibited by TEA the non-selective inhibitor of calcium-activated potassium channels. This involves endothelium-dependent hyperpolarization (EDHF), via the small (SK_{Ca}) and intermediate (IK_{Ca}) potassium channels that reside mainly on the endothelial cells. TEA also blocks BK_{Ca} channels on the smooth muscle not associated with the EDHF response, but also inhibiting the direct action of NO (Feletou et al., 2006). EDHF mediated responses can be distinguished, as they are generally insensitive to iberiotoxin or glibenclamide, specific inhibitors of BK_{Ca} and K_{ATP} channels respectively (Gluais et al., 2005). A contribution of outward K^+ current movement due to endothelium-derived hyper-polarisation may be involved in causing relaxation in caviunin or 17β-estradiol-induced relaxation, and this could be in addition to the eNOS mediated effects as L-NAME and ODQ abolishes vasorelaxation. 17β-estradiol is also known to directly activate BK_{Ca} on vascular smooth muscle (Maher et al., 2013), but further experiments would be required to establish this pathway in caviunin-induced relaxation. So although it is clear that inhibition of K^+ channels can contribute to caviunin-induced relaxation, it is less clear which K^+ channels are involved.

4.6.5 Vasorelaxant activity of common isoflavonoids

A survey of isoflavonoid compounds has found that the most abundant isoflavonoids in soy products genistein and daidzein show endothelium-dependent vasorelaxation at concentrations up to 10μM albeit with markedly differing efficacies (Mishra et al., 2000). Above this concentration these compounds only showed endothelium-independent relaxation (Figtree et al., 2000; Mishra et al., 2000; Rajah et al., 2012). The maximum concentrations used in this project were caviunin (30μM) and isocaviunin (10μM), which showed only endothelium-dependent activity. There is then the possibility that at higher concentrations isocaviunin and caviunin may have

endothelium-independent effects. Both genistein and daidzein like 17β- estradiol have been shown to activate multiple vasorelaxant pathways. Acute non-genomic endothelium-dependent effects identified with daidzein and genistein include phosphorylation of ERK1/2 with a consequent increase in intracellular cGMP (Joy et al., 2006), via PI3K/Akt phosphorylation of eNOS at Ser 1179 in porcine aortic endothelial cells (Yang et al., 2010) or using apamin shown to have an EDHF component (Nevala et al., 2001). Genistein and daidzein also show a number of endothelium-independent activity via the BK_{Ca} channel (Sun et al., 2007) and calcium inhibitory activity in smooth muscle and in the sarcoplasmic reticulum (Speroni et al., 2009), or attenuation of vascular contraction via inhibition of a rhoA/rhoK pathway (Seok et al., 2008). In addition they have genomic effects, for example daidzein in endothelial cells increases the expression of calmodulin and decreases expression of caveolin-1 thereby increasing eNOS activity (Woodman et al., 2004). These compounds also potentiate acetylcholine-induced relaxation in spontaneously hypertensive rats by increasing NO or protecting it from superoxide (O^{2-}) (Vera et al., 2005).

How these vascular effects of genistein and daidzein are mediated is a harder question. Studies have shown now that ICI 182 780, the non-selective inhibitor of both membrane estrogen receptors could not block the endothelium-dependent activity of genistein and daidzein (10μM) (Figtree et al., 2000; Mishra et al., 2000; Vera et al., 2005) and it has been presumed that genistein does not have estrogenic activity via ERα or ERβ. However the selective ERα estrogen receptor antagonist MPP significantly attenuated the genistein induced relaxation in rat aortic rings suggesting that genistein has a high affinity for the ERα receptor to induce activity (Lin et al., 2011). Many of these results with commonly found isoflavonoids are

therefore at variance with our studies with ICI 182780, which did attenuate relaxation significantly of both isocaviunin and caviunin.

4.6.6 Conclusion

In this chapter experiments to assess the vasorelaxant functional response to isocaviunin and caviunin in the rat aortic ring bioassay were undertaken to elucidate the mechanisms involved. In conclusion these two isomeric isoflavonoids have similarities in that endothelium-dependent vascular activity is the result of activation of eNOS to release NO. The key difference between them is the apparent simplicity through which isocaviunin initiates relaxation and the downstream restricted signalling pathway involved. The results for caviunin show that functional responses are the result of partial activation of the same signalling pathways as isocaviunin but this does not present the complete picture. Experiments suggest that either the EDHF response or BK_{Ca} channel activation is also involved and that this molecule is more like 17β-estradiol having the potential to activate multiple pathways to induce acute vasorelaxation. Nevertheless both these compounds are significantly more potent than 17β-estradiol in inducing vasorelaxation.

The structural differences between the two isomers studied and other isoflavonoid compounds discussed here clearly result in different functional responses most probably due to activation of different signalling pathways or due to the degree of activation of a particular pathway. The structural motifs necessary for vascular activity is explored in the next chapter using another isoflavonoid robustigenin that is structurally highly similar to isocaviunin and caviunin but differs in the absence of known key binding motifs.

Chapter 5

Comparison of vascular structure activity relationships between Robustigenin, Caviunin and Isocaviunin

5.1 Introduction

Robustigenin was isolated from the seeds of *Derris robusta* and the genus like Dalbergia is placed in the family Leguminosae, sub-family papilionoideae known for the restricted occurrence of isoflavonoid secondary metabolites (Veitch 2007). The chemical structure of robustigenin was elucidated as 5-hydroxy-7,2',4',5'-tetramethoxy isoflavone (Chibber et al., 1979).

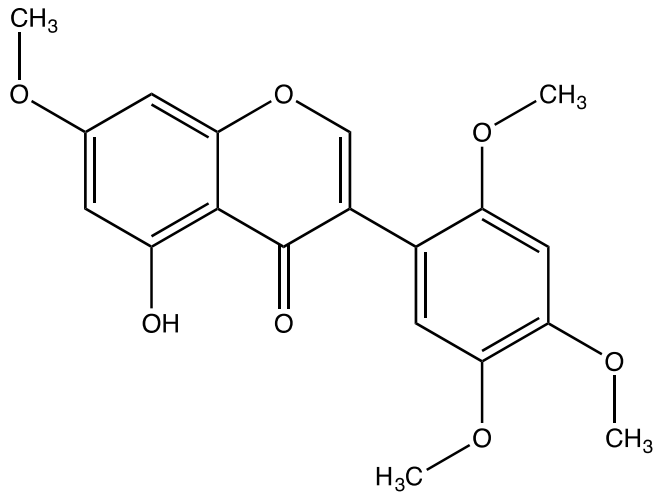


Figure 5 - 1: Structure of robustigenin

The structure of robustigenin is very similar to both caviunin and isocaviunin in that they all have the 3-phenyl chromene-4-one structure, with an identical substitution pattern in the B-ring and a 5-hydroxyl group in the A-ring. Robustigenin differs in the 7-position, which has a methoxy group instead of the hydroxyl group that is considered important for binding to membrane estrogen receptors and thereby demonstrate estrogenic activity. It was chosen for this reason to assess the vasorelaxation of a compound that did not have two of the key structural motifs

considered necessary for ligand binding to membrane estrogen receptors. Like 17β-estradiol this isoflavonoid was only soluble in DMSO.

5.1.1 17β-estradiol Structure Activity Relationships (SARS)

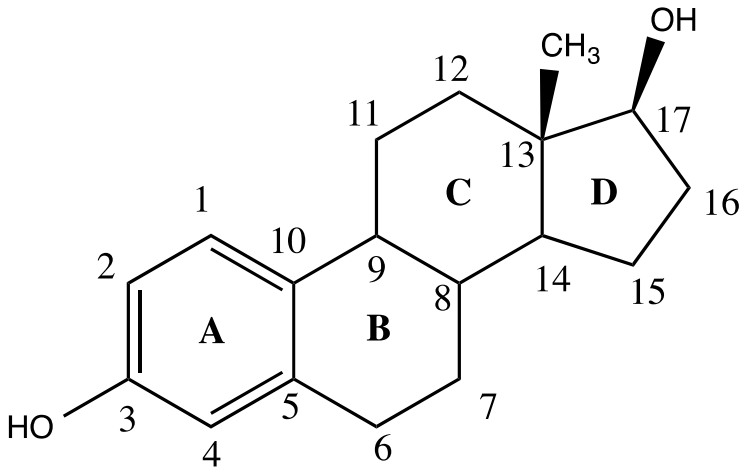


Figure 5 - 2: Structure of 17β-estradiol

17β-estradiol is the most active of the endogenous estrogens in the body and its pleiotropic activities particularly in relation to cardiovascular effects are well known (Kelly et al., 2011). It has also now been shown to have direct acute effects acting via membrane estrogen receptors (Kumar et al., 2007) and via the intracellular GPER (Thomas et al., 2005). The essential structural motifs have been identified for 17β-estradiol binding to its receptor in competitive binding assays, together with X-ray crystallography studies on the ligand-binding site (Anstead et al., 1997; Brzozowski et al., 1997). These studies have shown that H-bonding of the 3-OH in the phenolic A-ring and 17β-OH in the D-ring at the other end of a hydrophobic molecule is essential for binding. The distance of 11 angstrom between the O-O of the two-hydroxyl groups is another key design feature of the molecule required for binding and activity,

as is having a ring structure. Steric hydrophobic centres at 7α and 11β also play a part in bonding. Greater activity is seen in molecules possessing more of these structural features (Fang et al., 2001).

5.1.2 Comparison of structural characteristics of isoflavonoids and 17β-estradiol

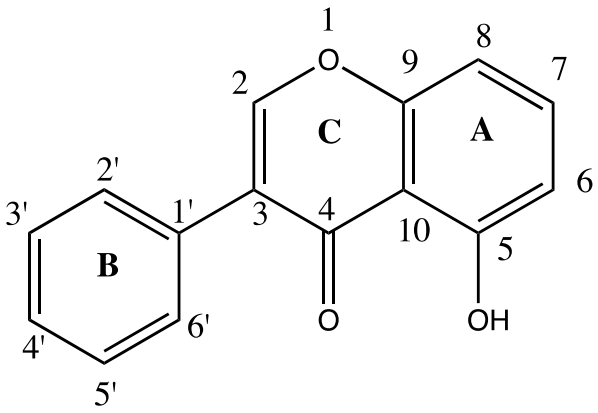


Figure 5 - 3: Structure of the isoflavonoid backbone

The isoflavonoids have a number of key structural and biological similarities to their endogenous counterpart 17β-estradiol. Genistein, 5,7,4'-trihydroxy isoflavone is an isoflavonoid found in soybeans. It features a 7-OH group in a position akin to the 17β in estradiol, and a 4'-OH in the B–ring which is positioned to mimic the 3-OH (Manas et al., 2004). The distance between the O-O (7-OH – 4'-OH) is slightly greater but does not affect binding but studies do show that some isoflavonoids have a preference for the ERβ receptor (Kuiper et al., 1998; Pike et al., 2000). They do not however possess the equivalent steric hydrophobic centres as at 7α and 11β of 17β-estradiol. Robustigenin has methoxy groups as opposed to hydroxyl groups in both the 7 and 4' positions and it was hypothesized that this molecule may not have the

same degree of estrogenic activity that manifests in the vasorelaxation induced by other isoflavonoids in the rat aortic ring bioassay.

5.2 Aims

Robustigenin is an isoflavonoid that is structurally similar to isocaviunin, caviunin and 17β-estradiol, but it has key differences such as the absence of 4'-OH or 7-OH phenolic groups. These differences can potentially preclude activation of membrane estrogen receptors, which presents as either reduced or minimal vasorelaxation. The aim of this chapter was to investigate whether the lack of these structural features will alter this activity. The vasorelaxant activity of robustigenin would be studied in the rat aortic ring bioassay model and receptor activated signalling pathways responsible for vascular activity would be assessed against a number of inhibitors. The structure activity relationships between isocaviunin, caviunin, robustigenin and 17β-estradiol induced vasorelaxation of phenylephrine (1μM) precontracted rat aortic rings can then be compared.

5.3 Materials

All reagents used are listed under the materials section in Chapter 4.3 except for Robustigenin that was purchased from Plantec UK Ltd.

5.4 Methods

5.4.1 Preparation of Rat aortic rings

All the experiments detailed in this chapter use rat aortic rings to assess endothelium-dependent signalling pathways for robustigenin. The procedures used to prepare the aortic rings are fully described in section 3.4.5.

5.4.2 Experimental protocols

At the core of these studies is the hypertensive model created using the α_1-receptor antagonist phenylephrine (1μM) to pre-contract rat aortic rings. After a stable contraction is achieved cumulative doses of robustigenin in half log-increments and in the dose-range (10nM – 10μM) is added to reverse this contraction and determine the level of relaxation that can be induced. Acetylcholine (1μM) was added at the end of an experiment to confirm the integrity of the endothelium. All inhibitor studies were conducted by pre-incubating the rings for 20 minutes prior to reassessment of concentration response curve in the presence of inhibitors.

5.4.3 Assessment of vascular reactivity

5.4.3.1 Construction of concentration response curves

The cumulative addition of half log increments of robustigenin (0.01μM - 10μM) to phenylephrine (1μM) precontracted rings was used to determine the potency and maximum relaxation.

5.4.3.2 Evaluation of robustigenin concentration response curves in the presence of L-NAME & ODQ

The contribution of eNOS and guanylyl cyclase to the vascular effects mediated by robustigenin was evaluated, by repeating concentration response curves in the presence of L-NAME (100μM) and ODQ (10μM), respectively.

5.4.3.3 Evaluation of robustigenin concentration response curves in the presence of ICI 182 780

The contribution of membrane estrogen receptors in initiating the vascular effects of robustigenin was evaluated, by repeating concentration response curves in the presence of ICI 182 780 (10μM).

5.4.4 Statistical analysis

Statistical methods employed are detailed on p116.

5.5 Results

5.5.1 Robustigenin results

5.5.1.1 Concentration response curve for robustigenin on endothelium-intact rat aortic rings

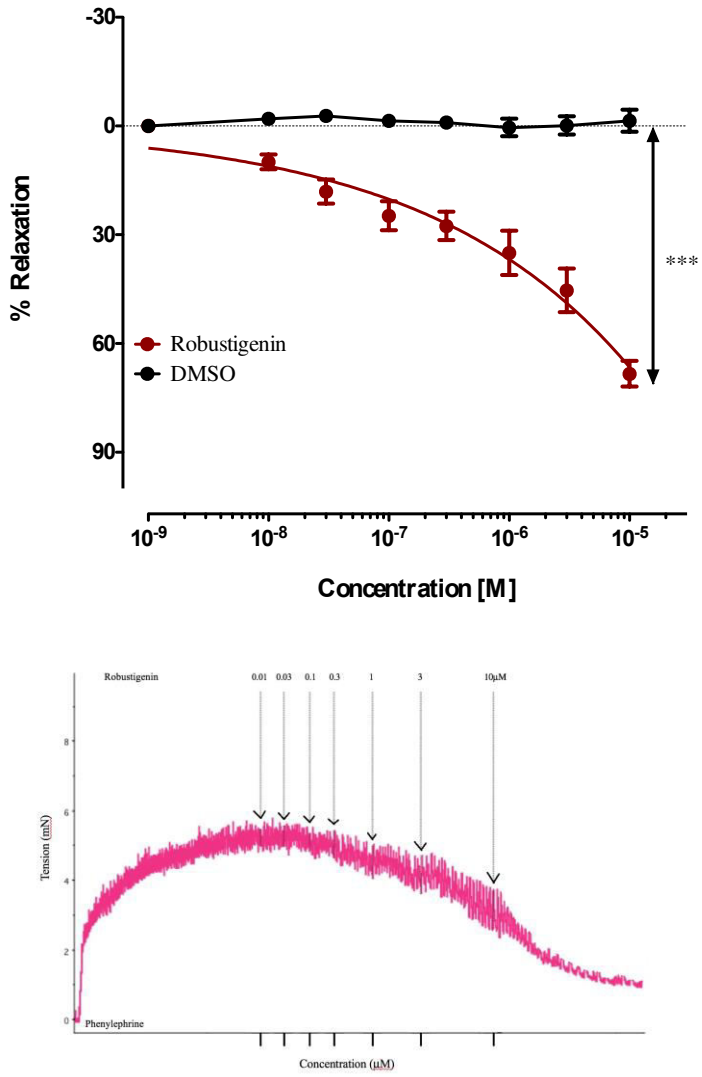


Figure 5 - 4: Dose response curve for robustigenin-induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Each point represents the mean ±S.E.M. (n=7) responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results show overall significance versus DMSO alone (*p≤0.001).**
Below is a representative trace of robustigenin-induced relaxation.

5.5.1.2 Effect of L-NAME and ODQ on robustigenin-induced relaxation

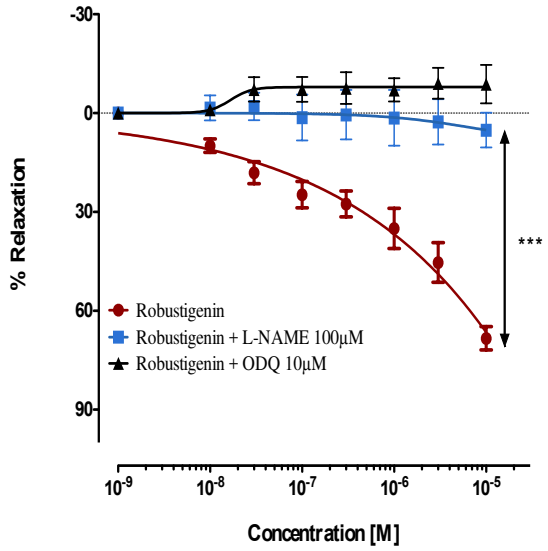


Figure 5 - 5: Effects of L-NAME (n=4) and ODQ (n=4) on robustigenin (n=7) induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Results presented here are the mean ±S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus robustigenin alone (*p≤0.001).**

Figure 5-4 shows the cumulative addition of robustigenin caused a concentration-dependent relaxation in male endothelium-intact rat aortic rings with an EC_{50} = 5.744 x 10^{-6} M ± 6.50 x 10^{-7} M and relaxation of 68.4% ± 3.55% at 10µM of the maximum phenylephrine induced contraction. The results of the D'Agostino-Pearson normality test (P≥0.05) suggest the data are not inconsistent with a Gaussian distribution. Figure 5-4 below shows a representative trace of robustigenin-induced relaxation on rat aortic rings precontracted with phenylephrine (1µM), identifying the increase in contraction which occurs over a period of approximately 8 minutes and the subsequent relaxation with of cumulative doses of robustigenin. Figure 5-5 shows the significant attenuation in relaxation of robustigenin-induced relaxation following incubation of aortic rings with L-NAME or ODQ. These results suggest that robustigenin-induced relaxation is mediated exclusively via the NO/cGMP pathway.

5.5.1.3 Effect of membrane estrogen receptor inhibitor ICI 182 780 on robustigenin-induced vasorelaxation

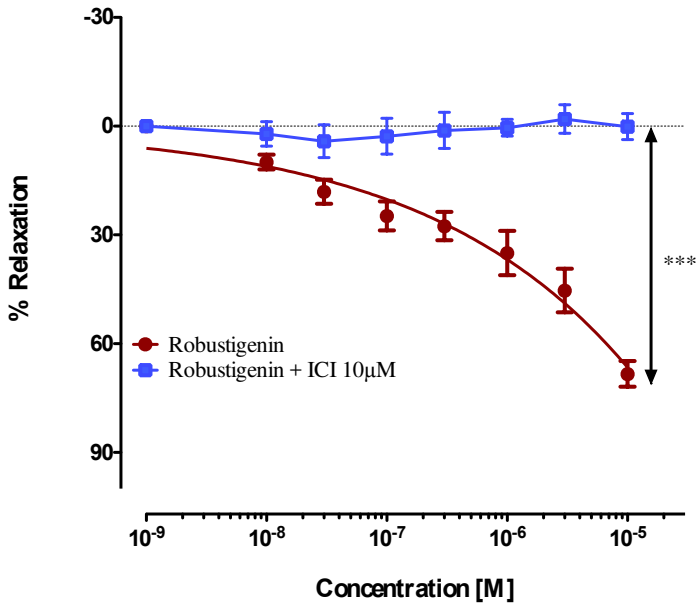


Figure 5 - 6: Effects of ICI 182 780 (10µM) on robustigenin (n=7) induced relaxation of phenylephrine (1µM) precontracted endothelium-intact rat aortic rings. Results presented here are the mean ±S.E.M. responses expressed as a percentage of the maximum phenylephrine contraction and compared by two-way ANOVA followed by Bonferroni post hoc tests. Results showed overall significance versus robustigenin alone (*p≤0.001).**

Figure 5-6 shows the attenuation in robustigenin-induced relaxation following incubation of the rat aortic rings with membrane estrogen receptor inhibitor ICI 182 780. These results once again suggest the membrane estrogen receptors are the predominant mechanism by which robustigenin activates eNOS resulting in vascular relaxation.

5.6 Discussion

5.6.1 Robustigenin

The results identified in this chapter show for the first time that the phytoestrogen robustigenin can induce acute vasorelaxation in a concentration and endothelium-dependent manner in the phenylephrine (1μM) precontracted rat aortic ring bioassay. In particular the studies show: –

i) Concentration response curves for robustigenin-induced relaxation gives an EC_{50} = 5.744 x 10^{-6} M ± 6.50 x 10^{-7} M and relaxation of 68.4% ± 3.55% at 10μM.

ii) NO/ cGMP activation is predominantly responsible for robustigenin-induced relaxation as L-NAME and ODQ inhibitors of eNOS and soluble guanylyl cyclase respectively significantly attenuated relaxation.

iii) Membrane estrogen receptors ERα and ERβ are predominantly responsible for initiating robustigenin-induced relaxation as membrane estrogen receptor inhibitor ICI 182 780 significantly inhibits these receptors.

These results show that robustigenin like isocaviunin and caviunin activates membrane estrogen receptors to initiate a cascade of events that leads to vascular relaxation. These results are not significantly different from isocaviunin at 10μM (EC_{50} = 1.669 x 10^{-6} ± 1.53x10^{-7} M and relaxation of 73.9 ± 7.3%), but it is more potent than caviunin (EC_{50} = 1.80 x 10^{-5}± 1.898 x 10^{-6} M and relaxation of 53.2 ± 6.4%). This is despite there being some key structural differences between these compounds known to be important for receptor activation.

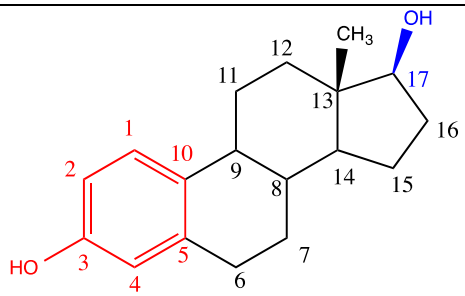
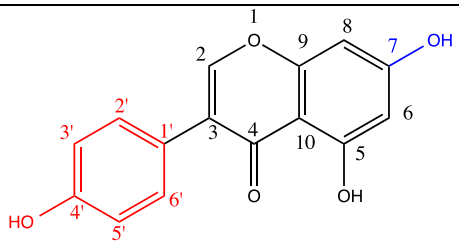
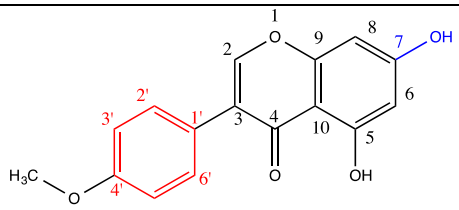
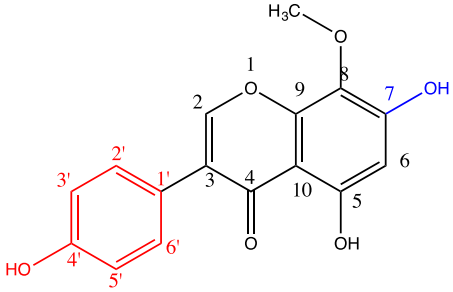
The structural characteristics of isoflavonoids predict estrogenic activity. A study using a yeast transcription activation assay expressing ERα and ERβ (Bovee et al.,

2004) or using a ligand-binding assay with both receptors together with mRNA expression (Kuiper et al., 1997) show genistein and other isoflavonoids have estrogenic activity, but unlike 17β-estradiol, these isoflavonoids show a preference for the ERβ receptor. Competitive binding assays using receptor proteins have shown that although genistein binds with the same affinity as 17β-estradiol, the concentration required for transcription was 10^4 times greater for genistein but shows only half the activity assessed by growth of MCF-7 breast cancer cells (Morito et al., 2001). Despite these studies comparing the genomic activity of endogenous estrogens and isoflavonoids there is little information that addresses the issue of structure activity relationships that predict rapid non-genomic vascular activity between these compounds.

A search of the literature has identified just eight isoflavonoids that have been assessed for their vascular reactivity in the rat aortic ring bioassay and these are presented in Table 5-1 and 5-2, based on the presence or not of the 5-OH group. There are a number of differences in each of these studies but particularly in the manner in which the potency is presented. In some they are quoted as IC_{50} and in others as EC_{50} values as seen on the tables. It is difficult with so few studies to identify patterns of structure-activity-relationships, but a few broad generalisations are discussed here.

All the isoflavones identified in the literature as having vasorelaxant activity show the O-substituted pattern on the isoflavone skeleton, which includes hydroxylation, methoxylation and methylene-dioxy substitutions, or include O-glycosylation or C-glycosylation patterns. All these compounds that have been identified are found in Dalbergia species except for robustigenin.

5.6.2 Isoflavonoids with the 5-OH group

Compound	Structure	Endothelium intact	Endothelium removed	Reference:
17β-estradiol		EC_{50} = 31.3 µM	-	Chapter 4
Genistein		IC_{50} = 5.7µM (4.3-7.7)	IC_{50} = 13.4µM (11.8-15.2)	Mishra et al., 2000
Biochanin A		SHR EC_{50} = 14µM,	EC_{50} = 54 µM	Wang et al., 2006
Tectorigenin		Not studied	IC_{50} = 27±4 µM	Filipeanu et al., 1995

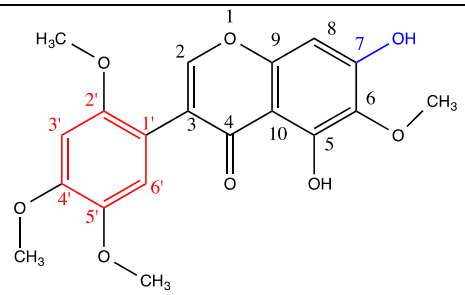
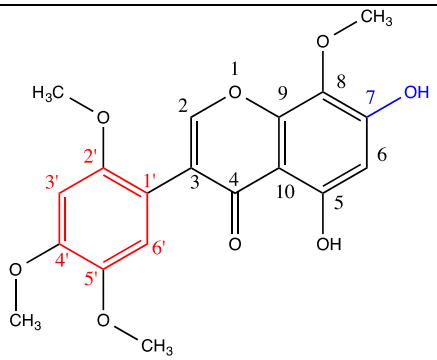
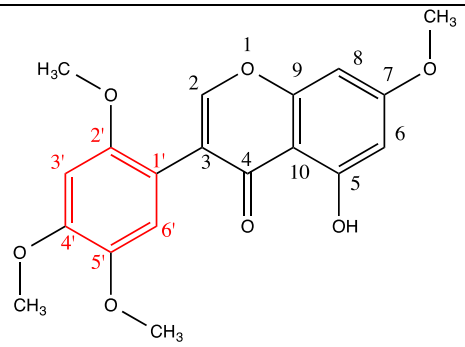
Caviunin		EC_{50} = 18.0μM	Not found	Chapter 4
Isocaviunin		EC_{50} = 1.67μM	Not found	Chapter 4
Robustigenin		EC_{50} = 5.74μM	Not found	Chapter 5

Table 5 - 1: Comparison of the potency and structural features of 5-OH isoflavonoid compounds compared with 17β-estradiol. Colour coded sections are considered important for membrane estrogen receptor binding and lack of these motifs can be seen in some structures.

5.6.3 Isoflavonoids without a 5-OH group

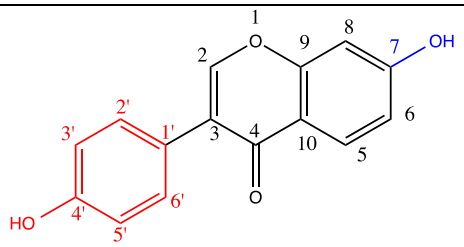
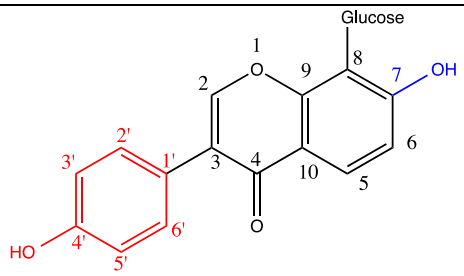
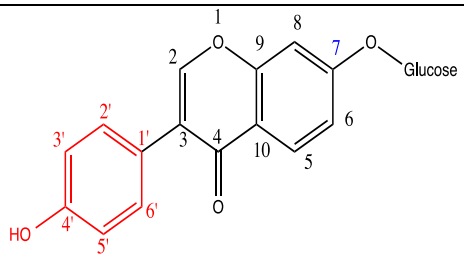
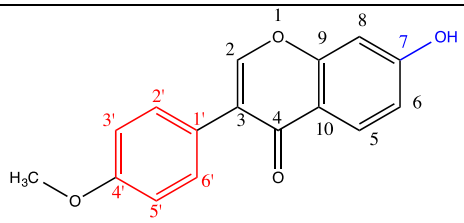
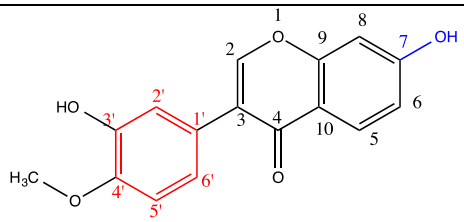
Isoflavonoid	Structure	Endothelium intact	Endothelium removed	Reference
Daidzein		IC_{50} = 33.6 µM	IC_{50} = 54.4µM[1] or 20µM[2]	[1]Mishra et al., 2000, [2]Deng et al 2012
Puerarin		Partial	IC_{50} = 304 ± 49µM	Deng et al., 2012
Daidzin		Not found	IC_{50} = 140± 21µM	Deng et al., 2012
Formononetin		Only E_{max}= 93.5% at 300µM	Only E_{max}= 48.4% at 300µM	Zhao et al., 2012
Calycosin		EC_{50} = 34.6µM	EC_{50} = 34.6µM	Wu et al., 2006

Table 5 - 2: Comparison of the potency and structural features of isoflavonoid compounds lacking 5-OH group, compared with 17β-estradiol. Colour coded sections are considered important for membrane estrogen receptor binding and lack of these motifs can be seen in some structures

The structures are aligned in the above tables in such a way that structural motifs on the isoflavonoids that mimic that on 17β-estradiol can be identified. The phenolic A-ring of 17β-estradiol with the 3-OH group and the corresponding phenolic B-ring on isoflavonoids with the equivalent 4'-OH group is coloured red, while the D ring 17β-OH group and its equivalent the A-ring 7-OH on isoflavonoid compounds is coloured blue. These structures show a decrease in the colour-coded areas as you progress down the table, and this corresponds to fewer structural features that are considered important for binding and subsequent activity (Pike et al., 2006).

Extensive ligand binding studies undertaken with 230 natural xenoestrogens in a rat uterine bioassay show that removal or modification of these hydroxyl groups' results in a decrease in binding affinity, and in particular changes at the 3-OH group (4'-OH in isoflavonoids) have a greater impact (Fang et al., 2001). This is most probably due to the fact that the 3-OH group H-bonds to three residues Glu 353, Arg 394 and a water molecule, while 17β-OH binds to a single residue His 524 in the ERβ ligand-binding site and similar residues are seen in the ERα ligand-binding site albeit with slightly differing spatial positions (Pike et al., 1999). This lack of binding motifs in caviunin, isocaviunin and robustigenin do not appear to adversely affect acute endothelium-dependent vasorelaxant responses, in fact it appears to enhance it.

The majority of the 5-OH isoflavonoids show endothelium-dependent vasorelaxant activity but with tectorigenin this effect was not studied. Comparison between isoflavonoids with equivalent structures such as 5-OH genistein, and daidzein which lacks the 5-hydroxy group, show a significant difference in potency in stimulating the transcriptional activity of membrane estrogen receptors (Miksicek et al., 1994;

Miksicek et al., 1995) and on inducing rapid vascular relaxation on rat aortic rings (Mishra et al., 2000). Both genistein and daidzein have the two-hydroxyl groups considered important for binding and activity similar to 17β-estradiol with the key difference being the absence of the 5-OH group in daidzein. Clearly the presence of the 5-OH group increases activity in this bioassay and in the rat uterine assay and this increase in activity is attributed to intra-molecular bonding between the 5-OH and 4-carbonyl group, which extends the electron-withdrawing capacity of the carbonyl group, resulting in an improved H-bond donor at the 7-OH position (Fang et al., 2001). The complementary comparison between biochanin A with the 5-OH group and the equivalent formononetin without the 5-OH group has also been studied and show differences that could be attributed to the presence or absence of the 5-OH group. They also show significantly lower potency when compared to genistein and daidzein most likely due to the loss of the 4'-OH to methoxylation. Calycosin with a structure similar to formononetin but with an additional hydroxyl group at 3'-OH shows endothelium-independent activity only. Puerarin and daidzin analogues of daidzein, which have glycosylated isoflavonoid A-rings show significantly, lower potency in the rat aortic ring bioassay, and glycosides of isoflavonoids have been shown to bind poorly to ERα and ERβ (Morito et al., 2001). ICI 182 780 does not attenuate the relaxation of any of the compounds discussed in this paragraph suggesting that activity is independent of membrane estrogen receptors or that they have a greater affinity for the receptor than the inhibitor. Genistein has been shown to have tyrosine kinase inhibitory activity (Nakashima et al., 1991) and genistein and biochanin A have been shown to have calcium-channel blocking activity (Figtree et al., 2000) that may also be responsible for activity.

This is in contrast to the results shown here with robustigenin and that of isocaviunin and caviunin in Chapter 4. Both isocaviunin and robustigenin-induced relaxation is almost completely abolished by ICI 182 780 while that of caviunin is partially but significantly inhibited. This suggests that membrane estrogen receptors are responsible in large part for the acute vascular activity shown by these compounds, following binding to these receptors. Isocaviunin and caviunin have only one of these hydroxyl groups at the less active 7-OH position (17β-OH, 17β-estradiol) as the 4'-OH (3-OH; 17β-estradiol) is methoxylated. They both also have a higher number of methoxy substituents thought to compromise activity but despite this the activity is significantly greater than that of 17β-estradiol. The methoxy groups at either the 6-OCH_3 or 8- OCH_3 may account for the difference between caviunin and isocaviunin. The 6-OCH_3 substitution clearly affects caviunin-induced relaxation more dramatically having a 10-fold lower EC_{50} than its isoform and with a maximal relaxation that is considerably lower. With robustigenin both key hydroxyl groups have been methoxylated and in terms of motifs for optimal binding at 17β-estradiol sites it is poor. It however does not possess the 6-OCH_3, and induces vascular relaxation that is comparable to isocaviunin and considerably greater than 17β-estradiol and caviunin. These studies show that the heavier substitution patterns do not affect endothelium-dependent relaxation adversely.

The table below summarises the results of our vascular experiments on rat aortic rings with 17β-estradiol, isocaviunin, caviunin and robustigenin and highlights the structural variation of the compounds. The results of rat aortic ring experiments with acetylcholine the benchmark muscarinic agonist used to assess endothelial dysfunction is also included for comparison.

5.6.4 Comparison of structure-activity-relationships of compounds studied in this project

Isoflavonoid/ Agonist	A ring positions				B ring	EC_{50} (μM)	% relax 10μM
	5	6	7	8	4'		
Isocaviunin	OH	H	OH	OCH_3	OCH_3	1.669	73.9
Robustigenin	OH	H	OCH_3	H	OCH_3	5.744	68.4
Caviunin	OH	OCH_3	OH	H	OCH_3	18.0	53.2
17β-estradiol			17β-OH		3-OH	31.3	36.05
Acetylcholine (muscarinic)						0.247	94.8

Table 5 - 3: Summary of the structural features of the isoflavonoid compounds studied, together with details of the vasorelaxant activity induced in rat aortic rings

These isoflavonoids therefore have only a limited selection of the 5 key features considered necessary for membrane estrogen receptor binding. Nevertheless all these isoflavonoid compounds cause vasorelaxation to a significantly greater degree at 10μM than 17β-estradiol and all of them show significant attenuation of this relaxation in the presence of membrane estrogen receptor inhibitor ICI 182 780.

In conclusion robustigenin exhibits rapid endothelium- and dose-dependent relaxation of rat aortic rings following stimulation of estrogen membrane receptors. Structural comparisons of robustigenin with 17β-estradiol show that the lack of the hydroxyl motifs considered a key structural binding requirement to estrogen receptors does not create a barrier to robustigenin-induced activity or caviunin and isocaviunin. It has been found that physiological concentrations of genistein range from 1nM - 1μM (Rajah et al., 2012) compared to the physiological concentration of 17β-estradiol of 1nM (Castillo et al., 2006). All phytoestrogens studied in this project showed greater endothelium-dependent relaxation compared to 17β-estradiol and at concentrations close to that of the physiological concentration of genistein. In contrast high concentrations of 17β-estradiol e.g. 100nM and above can have no clinical relevance. These compounds shown here have potential to act as selective estrogen receptor modulators with beneficial physiological effects.

Chapter 6

General Discussion

6.1 Final discussion

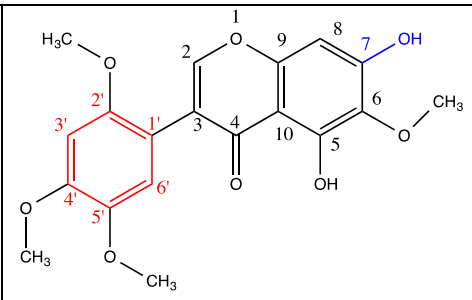
This project has undertaken a multidisciplinary approach to isolate, identify and biochemically characterize vasoactive compounds found in the bark of a plant, identified in this project as a Dalbergia species using DNA barcoding techniques. The core methodology used in this project is the bioassay-guided isolation and pharmacological characterization of extracts and compounds using the phenylephrine (1μM) precontracted rat aortic ring hypertensive model. The vasorelaxant compounds isolated in this project, and subjected to extensive NMR studies, are identified as the isoflavonoid isomers caviunin and isocaviunin, not previously studied for their potentially beneficial cardiovascular effects. Caviunin is well known having been isolated from a number of Dalbergia plant species and is considered a taxonomic marker for this genus. The presence of caviunin therefore further confirms the taxonomic identity of the bark. The isomer isocaviunin is considerably less well known and this project has for the first time obtained the 1D ^{1}H and ^{13}C NMR spectra and the 2D HSQC and HMBC spectra that conclusively differentiate between the two isomers (Fig 3-26). This project has also identified a heat-stable extract that causes vascular contraction in the rat aortic ring assay that may be of future interest.

The pharmacological characterization of these two compounds has shown them both to possess acute, non-genomic endothelium-dependent vasorelaxant activity. Either endothelium removal or incubation with L-NAME abolishes relaxation suggesting these isoflavonoids do not activate smooth muscle vasorelaxant pathways. There are however considerable dissimilarities in how these two molecules transduce signalling activity and these differences may be of pharmacological interest.

Isocaviunin is unique amongst isoflavonoids studied to date in that vascular relaxation is predominantly and perhaps solely dependent on activation of membrane estrogen receptors to initiate vascular pathways that lead to NOS stimulation. Caviunin, in contrast, activates multiple mechanisms via membrane estrogen receptors as ICI 182 780 partially but significantly attenuates relaxation, but it also induces a vasorelaxant response via calcium activated potassium channels as relaxation can be partially inhibited by TEA. It is unclear at this stage whether the potassium channels involved are those on the endothelial cells and associated with the EDHF response or the BK_{Ca} channels residing on the smooth muscle. This acute vascular activity is more in line with responses seen with 17β-estradiol or the phytoestrogen genistein, which causes relaxation via a number of mechanisms including EDHF and BK_{Ca} channel activation.

In order to further characterize the activity of these two isomers, vascular studies were undertaken using G15, the selective antagonist of the intracellular 17β-estradiol receptor, GPER. G15 did not attenuate 17β-estradiol or isocaviunin-induced relaxation in our experiments, but unexpectedly was found to potentiate the vasorelaxant effect of caviunin substantially. There is a complexity in GPER-induced signalling activity (Figure 4-16), and one explanation may involve the inhibitory effects of G15 on GPER Gαs-induced activation of cAMP, which has an inhibitory effect on MAPK, opposing GPER-induced stimulatory effects via Gβγ. This pathway will obviously require further study as it is currently speculative. G15 is a relatively new inhibitor and to date no studies have been identified involving isoflavonoids and G15. Neither does it feature in the literature survey conducted at the start of this project although a number of isoflavonoid structures have been identified.

The apparent ability of the heavily substituted isoflavonoid isomers caviunin and isocaviunin, compared to genistein or 17β-estradiol, to activate the structurally well characterized membrane estrogen receptors, with limited estrogenic ligand binding features compelled further investigation. Robustigenin is an isoflavonoid isolated from Derris robusta that has even fewer structural features required for membrane estrogen receptor binding. Compared to caviunin and isocaviunin it lacks the 7-OH group in the A- ring, which is methoxylated but retains the aromatic ring in the key position. Robustigenin was also found to induce endothelium-dependent relaxation to a similar degree as isocaviunin, and to a greater extent than caviunin, by activating NOS. Furthermore this activity was attenuated following incubation of the rings with ICI 182 780, once again emphasizing the central role of the membrane estrogen receptors in inducing activity. Therefore despite the paucity of estrogenic structural features isocaviunin, caviunin and robustigenin induce vascular activity albeit to varying degrees but to a greater extent than 17β-estradiol, by activating membrane estrogen receptors to stimulate eNOS resulting in NO-dependent relaxation. These results also suggest that methoxylation at the 6-OCH_3 position is the least effective in inducing relaxation compared to methoxylation at the 7-OCH_3 or 8-OCH_3 position in the A-ring.

Caviunin	

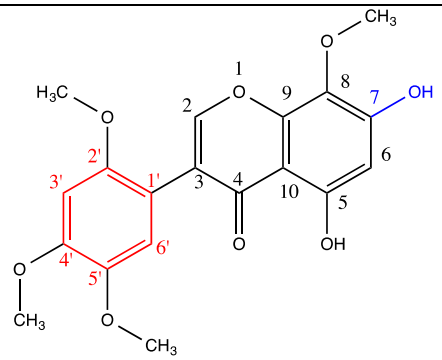
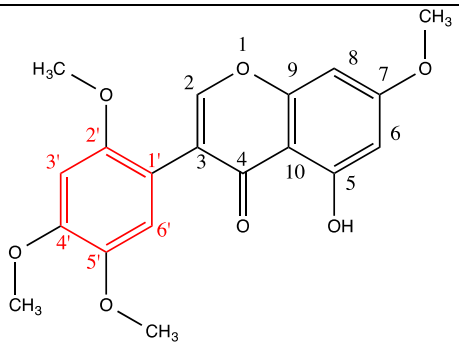
Isocaviunin	
Robustigenin	

Figure 6 - 1: Structure comparison of caviunin, isocaviunin and robustigenin

The diagram below describes the vascular pathways shown in this project to cause acute vascular relaxation following binding by the isoflavonoids isocaviunin, caviunin and robustigenin to membrane estrogen receptors. The red arrows on the diagram are descriptive of the most likely pathway to NOS activation following binding to membrane estrogen receptors, although we were not able to verify the involvement of the PI3K/Akt pathway. The blue arrows describe the possible pathways attributed to caviunin activation of the potassium-channels. The prostanoid pathway was not found to be involved in isocaviunin-induced activity.

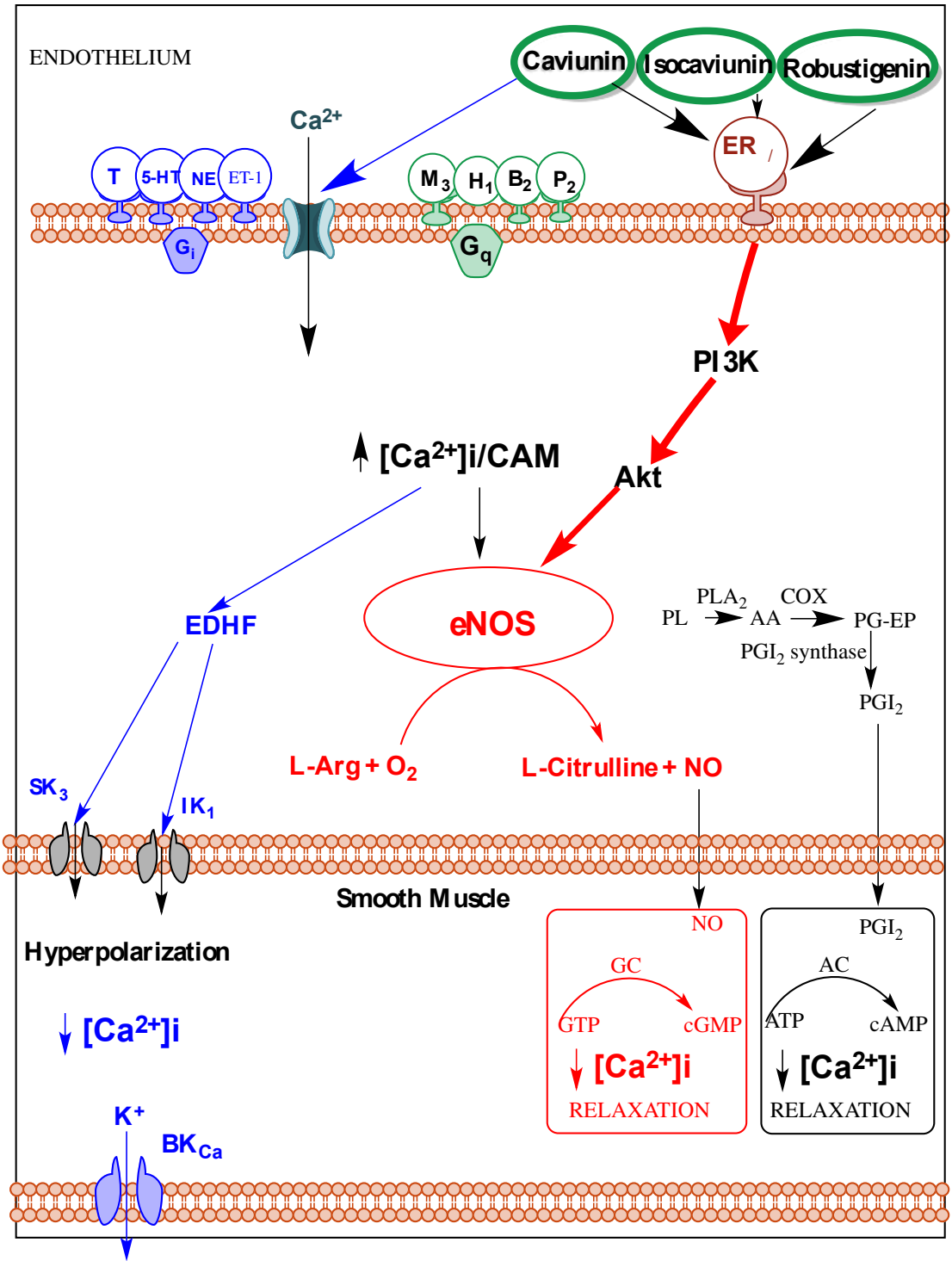


Figure 6 - 2: Diagram of known pathways activated by isocaviunin, caviunin and robustigenin (red) and caviunin (blue)

6.2 Summary of results on isoflavonoid compounds compared to endogenous 17β-estradiol

Mechanism	Isocaviunin	Caviunin	Robustigenin	17β-estradiol
Endothelium-dependent	√	√	√	√
Endothelium-independent	X	X	Not tested here	Not tested here
NOS/L-NAME (100μM)	√	√	√	√
cGMP/ODQ (10μM)	√	√	√	√
Muscarinic (M_3)/atropine	X	Not tested	Not tested	Not tested
Membrane estrogen receptors ICI 182780(10μM)	√	√	√	X
GPER/G15 (3μM)	X	Potentiates	Not tested	X
COX/Indomethacin (10μM)	X	Not tested	Not tested	Not tested
EDHF or BK_{Ca}/ TEA (5mM)	X	√	Not tested	√

Table 6 - 1: Comparison of the pharmacological mechanisms of isocaviunin, caviunin and robustigenin and 17β-estradiol

√ = Activity-dependent X = Activity-independent

There is an inalienable connection between animals and plants due to our shared evolutionary history, and therefore it shouldn't be surprising that plant-synthesized compounds and particularly secondary metabolites can have significant beneficial effects to human health. Societies that have a history of using plants as medicines can guide our search to discover novel compounds that already have a prior use in humans. The literature review undertaken at the beginning of this project provides irrefutable proof of the benefits of this approach and we must acknowledge this contribution. This contribution was found to be immense, the survey identifying a quarter of angiosperm plant families with genera having vascular activity. The predominant endothelium-dependent mechanisms by which plant extracts and compounds caused relaxation involved the NO/cGMP pathway. However the endothelium-independent relaxation mechanism involving calcium-channel blocking activity was also significant. These activities were not mutually exclusive with a number of these compounds showing activity via multiple biochemical mechanisms that involved both endothelium-dependent and independent pathways.

17β-estradiol has always been known to have central role in the reproductive physiology of premenopausal women (Ling et al., 2006; Mendelsohn, 2002). There is now a growing body of evidence of the beneficial effects of 17β-estradiol elsewhere in the body including on the vasculature in premenopausal women. Despite these protective effects they also have a number of other physiological effects that can preclude their use as pharmacological agents (Rossouw et al., 2002). Isoflavonoids are phytoestrogens, known from epidemiological studies (Miadokova et al., 2009; Ososki et al., 2003) to be beneficial to human health (Khalil, 2010; Lagari et al., 2014), or worthy of further investigation for example in postmenopausal vasomotor symptoms

(Lethaby et al., 2013). These beneficial effects of isoflavones include lower risk of coronary heart disease following consumption of a soy diet (Zhang et al., 2003) in improving lipid profiles (Zhan et al., 2005) and decreasing either systolic or diastolic BP (Hooper et al., 2008). Small clinical trials have identified increased NO-dependent dilation of forearm vasculature by genistein (Walker et al 2001), improvement in endothelial dysfunction (Colacurci et al., 2005) and the decrease in endothelin-1 (Squadrito et al., 2002).

6.3 Presence of caviunin and isocaviunin in the tea

HPLC separation of the aqueous extract shows presence of isocaviunin and caviunin.

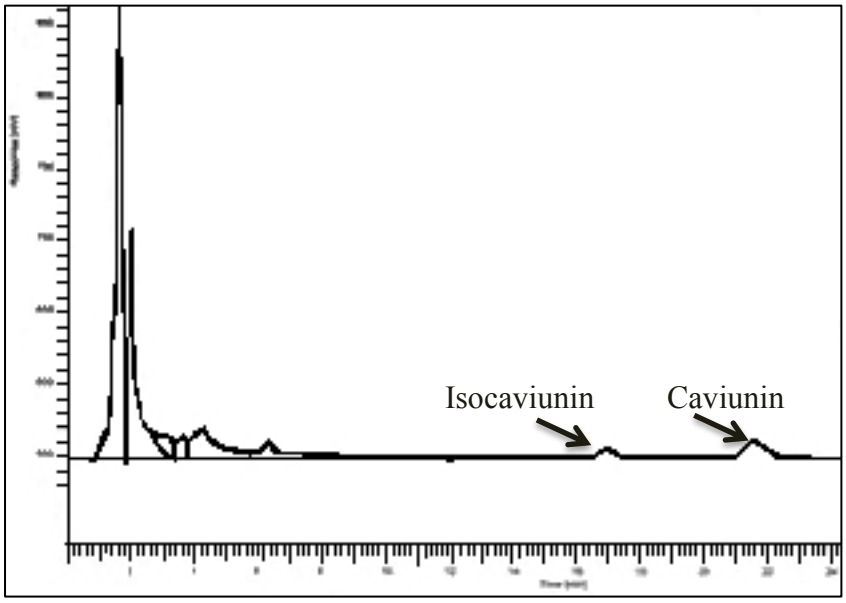


Figure 6 - 3: HPLC separation of the 'tea' shows the presence of isocaviunin and caviunin

The discovery of isocaviunin and caviunin in this project, compounds that already have a prior alleged beneficial use in humans, has provided insights into isoflavonoid research that can help with the development of selective estrogen receptor modulators with unique pharmacological profiles.

The plant world is an untapped resource for the discovery of new drug leads. It is a resource which holds an abundant diversity in scaffold structures sympathetic to humans, provides compounds with the ability to interact with multiple targets within the cell, and many plants come with the benefit of prior therapeutic insight held by indigenous communities, giving us a platform from which to approach drug discovery. The intellectual property rights of these communities should be acknowledged in any commercial exploitation of such knowledge. The success of Youyou Tu's Nobel prize (2015) in Physiology or Medicine for the discovery of the antimalarial compound Artemisinin, which has fundamentally changed the treatment of malaria, is an inspiration, not just by what nature has to offer but to the ingenuity of humans in deciphering these secrets without the 21st century toolbox. We owe it then to future generations to safeguard both these resources and reinvigorate the search for new drug leads from plants.

6.4 Main limitations

Although the major goals of isolating vasoactive compounds, structurally identifying two compounds and pharmacologically characterizing these compounds to identify key mechanisms have been achieved, there remains several unanswered questions.

- Despite much effort and resources being employed in identifying the bark, it has only been identified to genus level.
- The development of the endothelial cell bioassay was not successful and as a result it has not been possible to quantify the release of NO in response to activation of NOS by the compounds isolated.
- Despite the use of these compounds in humans it would be of interest to study the toxicity profile of these compounds.
- Lack of suitable preparative HPLC equipment made it possible for only small quantities of material to be isolated and the effects of the compounds were limited to low concentrations.
- It has not been possible to show the involvement of PI3K/Akt pathway following stimulation of membrane estrogen receptors and eNOS activation.

6.5 Future work

Discussions are on-going with the Legume team at Kew Botanical Gardens through whom it is hoped contact with the Asian Dalbergia specialist will be made to identify this plant to species level.

These compounds have the potential to function as SERMS in the human body and to this end a number of pharmacological experiments to characterize these compounds in different cell line populations should be undertaken. This should include effects of the compounds on different tissue beds e.g. breast cancer cell lines, vascular effects on the mesenteric vascular beds where the EDHF response is more significant or evaluation of the effects of these compounds on hypertensive models.

Studying crystal structures of particularly isocaviunin and robustigenin bound to a receptor would also be of interest as these compounds cause significant vasorelaxation despite lacking several key ligand-binding features.

Further characterization of the combined effects of caviunin and G15 are also worthy of further investigation.

Appendix 1

A1.1 Endothelial cell culture assay

Development of a cell culture assay was undertaken to detect NO release following incubation with known agonists acetylcholine, glutamate, histamine, ATP and L-arginine. The following cell lines were studied:

1. Brain endothelial cell line - passage 23 upwards

2. EA HY926 cells – passage 10-40

3. Mouse Endothelial Cells ($MEC^{+/+}$)- passage 20-25

A1.2 Cell culture techniques

The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS), supplemented with 1% penicillin/streptomycin, sodium pyruvate and 1% non-essential amino acids. The cells were incubated at 37°C in a humidified incubator (Haraeus) atmosphere of 95% air and 5% CO_2. Cells were grown to confluence prior to treatment with the agonists detailed below.

A1.2.1 Cell passaging

When cells have reached a sub-confluent growth stage they were passaged every 3-4 days depending on the cells to prevent differentiation. The cells were in the $25cm^3$ Nunc culture flasks and treated with 3mls of 0.1% trypsin solution ensuring the bottom of the flask was covered and left for 5 minutes at 37°C. Looking under a microscope the cells were checked to ensure they had detached from the flask surface and the trypsin was neutralised with 7ml culture medium containing 10% FCS. The solution was transferred to a centrifuge tube, balanced with an equivalent tube and centrifuged for 5 minutes at 400g. The supernatant was aspirated and the pellet was initially resuspended for counting in a haemocytometer following which it was resuspended in 12mls of culture medium ensuring the cells were evenly distributed.

The solution was divided into 3 flasks of 4mls each and to each flask a further 10mls of culture medium was added giving a total of 14mls per flask. One flask was stored for future use while the others were maintained in the incubator and the cells plated in 96 or 48-well plates.

A1.2.2 Treatment of Cells

On the day of the experiment the cells were washed with Hepes buffer (20mM Hepes, 133mM NaCl, 6.5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5.5mM glucose, 50μM L-arginine and 0.1% w/v BSA (not more than 5mls in 100mls) and pH 7.4. Following washing of the cells, they were incubated in Hepes buffer or PBS; 100μl in the 96 well-plates at a density of $3.5x10^4$ cells per well using the central 60 wells only, or 200μl in the 48-well plates at an equivalent cell density in the absence (control) or treated with agonists. The experiments are detailed in the table below and the cells allowed to incubate for 1, 2, 4 and 6 hours after the addition of agonist.

A1.2.3 Agonist concentrations used

Agonist	Receptor activated	Agonist Concentration		
5-HT/DMEM		1μM	3μM	10μM
Acetylcholine/DMEM	Muscarinic	1μM	3μM	10μM
Acetylcholine/PBS	Muscarinic			
Glutamate/DMEM	Glutamate (NMDA)	5mM	10mM	20mM
Histamine/DMEM	H_2		3μM	10μM
ATP/PBS	P_2Y	10μM	50μM	100μM
Arginine		100μM added to half the wells on each plate.		

Table: Agonists experiments

Each experiment was performed in replicates (n=6 for) each agonist concentration and incubation time. In addition into half the wells from each row 100µm Arginine was added while the rest were not treated.

In order to assess the effect of the agonist on NO-release 75µl from each 96-well plate and 100µl from each 48-well plate were collected for assay.

A1.3 NO Detection Methods

A1.3.1 Griess Reagent – Colorimetric assay

The organic chemist Johann Peter Griess discovered that nitrites could be detected by reacting with sulphanilic acid to form the diazonium ion which then couples to N-(1-napthyl)ethylenamidiamine to form a chromophoric azo derivative which can be detectable by absorbance at 540 nm. The sensitivity of this method has a nitrite limit of detection of 1µM- 100µM.

Equal quantities of 1% sulphanilamide in 5% phosphoric acid and 0.1% α-napthylamine ethylenediamine in distilled water were combined together just prior to use to form the reagent. The supernatant sample collected from each well was then added to an equal measure of reagent and the colour change obtained was observed on an ASYS-UVM 340 Spectrophotometer.

A1.3.2 Nitrite/Nitrate Reductase assay

NO reacts rapidly with O_2 to form nitrite and nitrates among other NOx. The assay done in two parts, use nitrate reductase to increase the nitrite concentration produced by converting nitrate back to nitrite after which it is tested with the Griess reagent.

50µl of test sample was added to each 96-well plate along with 25µl of nitrate reductase solution prepared fresh on ice by adding 10 enzyme unit in 16.4ml of

40mM Tris buffer at pH 7.6. This was followed by adding 25µl NADPH solution also prepared fresh on ice by using 6.7mg NADPH2 dissolved in 50ml of 40mM Tris buffer pH 7.6. To this 100µl Griess reagent was added and allowed to incubate for 10mins. The plate was read on a spectrophotometer at 550nm.

A1.3.3 Diaminonaphthalene (DAN) Fluorimetric method

This method is selective for NO and relies on the ability of NO to form N-nitrosating agents even under neutral conditions. DAN at a concentration of 0.05mg/ml in 0.62M HCL was added to test samples in a 96-well plate to equal 10% sample volume. The plates were covered in foil and incubated for 10mins. 2.8M NaOH was added to stop the reaction after 10mins and the fluorescence was measured at 365nm on a Fluorescent plate reader (Biotek Synergy T), (DAN), which can be used to detect down to 10nM nitrite, (Kleinhenz et al 2003).

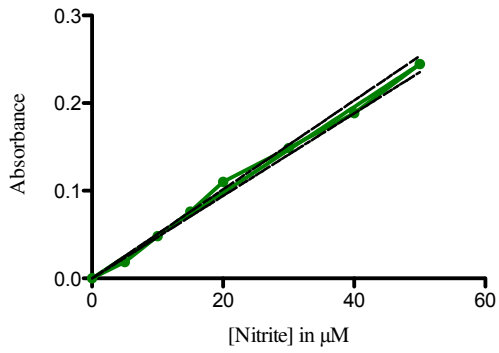
A1.3.4 Preparation of a Nitrite standard curve

A 1mM stock solution of sodium nitrite was used to prepare the following solutions - 50, 40, 30, 20, 15, 10, 5, and 0µM (blank). Using a 96-well plate 100µl of each solution was added in duplicate. To each well 10µl of DAN was added and the plates incubated for 10mins after which 5µl of 2.8M NaOH was added to each well to stop the reaction and the fluorescence detected at 365nm.

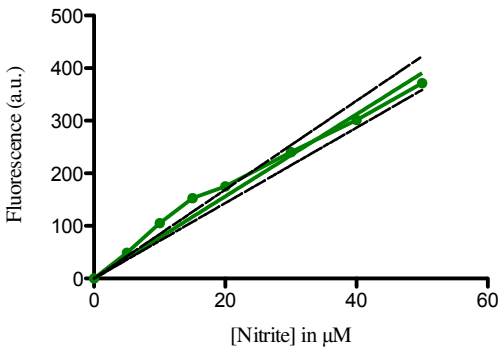
A1.4 Statistical analysis

The results expressed here are the means ± SEM of 4-6 experiments. Statistical significance was determined through a two-way analysis of variance (ANOVA) performed using GraphPad Prism 5 for Mac OS 10. Values of $p<0.05$ were statistically significant.

A1.4.1 Standard curves



Standard curve using absorbance readings from a Griess Assay



Standard curve using absorbance reading from DAN assay

A1.6 Conclusion

NO release was not detected from any of the cell lines studied in the above experiments.

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