EFFECTS OF NATURAL PRODUCTS ON CONTRACTILITY IN THE PROSTATE GLAND

Su Nguok Ngie
BSc. (Hons), MSc.

A thesis submitted for the degree of Doctor of Philosophy in Pharmacology
at Monash University in 2018

Drug Discovery Biology and Medicinal Chemistry
Monash Institute of Pharmaceutical Sciences
Faculty of Pharmacy and Pharmaceutical Sciences
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By

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SUMMARY

Benign Prostatic Hyperplasia (BPH) is a progressive condition characterized by increased prostatic contractility and prostate enlargement. Medicines which are able to relax prostatic smooth muscle are the most effective agents at relieving the lower urinary tract symptoms (LUTS) that accompany such disorders. *Costus speciosus* is widely used as a food source and dietary supplement while traditionally it has been used to treat several illnesses. In particular, it has been used in folklore medicine by Sarawak natives to treat urological disorders. Therefore, the aims for this study are to assess the effectiveness of extracts of *C. speciosus*, in treating urological disorders by investigating their effects on nerve mediated contractility of isolated rat prostates. Isolation and identification of bioactive components may become useful in the development of novel pharmacotherapeutic agents for treating symptoms associated with BPH.

CHAPTER 3

This chapter describes the preliminary screening of a list of plants extracts including *C. speciosus*, that were used by Sarawak indigenous people in treating urological disorders by using isolated organ bath techniques. None of these organic plant extracts affected electrical field stimulation (EFS) induced contractile responses of isolated rat prostates. *C. speciosus* extracts prepared under different water temperatures attenuated EFS-induced contractions of rat prostates with the following order of potency: hot water < decoction < cold-water. The plant part extract that had the highest potency was root ($p < 0.0001$, n = 6), followed by rhizome ($p = 0.0004$, n = 6) and stem ($p = 0.0057$, n = 6). Leaf extract did not have any inhibitory effect ($p = 0.0988$, n = 6). Apart from the inhibitory effect seen by the *C. speciosus* rhizome, stem or root cold-water extract, these extracts also produced a transient tonic contraction on unstimulated rat prostate upon administration to unstimulated isolated rat prostate preparations. These findings suggest that *C. speciosus* rhizome may be suitable to be developed as a new pharmacotherapeutic agent for the relaxation of prostatic smooth muscle in patients suffering LUTS associated with BPH.

CHAPTER 4

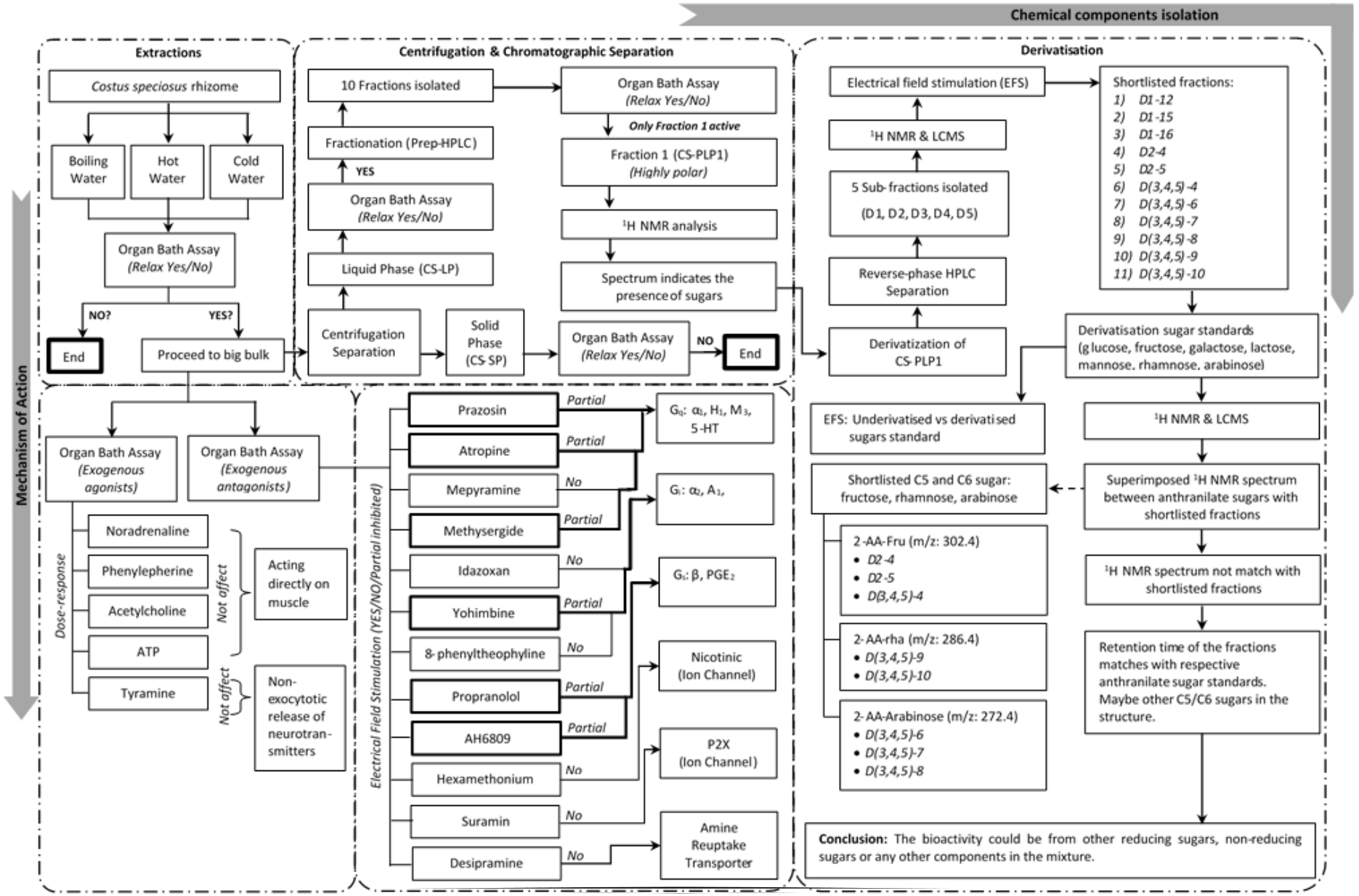
This chapter investigates the pharmacological mechanism of action of *C. speciosus* rhizome cold-water extract. Direct smooth muscle contraction elicited by exogenously administered noradrenaline (1 nM – 100 µM), phenylephrine (1 nM – 100 µM), acetylcholine (1 nM – 100 µM), and ATP (300 nM – 1 mM) were not directly affected by the *C. speciosus* rhizome cold-water extract (2.0 mg/mL). The extract also did not affect rat prostate contractions elicited by tyramine

(10 nM – 100 µM) which is an indirectly acting sympathomimetic agonist. In addition, EFS-induced contractions were still attenuated by the rhizome extract (2.0 mg/mL) in the presence of prazosin (300 nM; $p = 0.0715$, n = 6), suramin (30 nM; $p = 0.0037$, n = 6), yohimbine (1 µM; $p = 0.0042$, n = 6), idazoxan (1 µM; $p = 0.0100$, n = 6), propranolol (1 µM; $p = 0.0110$, n = 6), atropine (1 µM; $p = 0.0271$, n = 6), methysergide (1 µM; $p = 0.0014$, n = 6), mepyramine (1 µM; $p = 0.0010$, n = 8), hexamethonium (10 µM; $p = 0.0135$, n = 6), desipramine (100 nM; $p = 0.0003$, n = 6), 8-phenyltheophylline (10 µM; $p = 0.0090$, n = 6), and AH6809 (10 µM; $p = 0.0040$, n = 4). These findings suggest that the extract inhibits contractility of rat prostatic smooth muscle by an unidentified but indirect pre-junctional mechanism that inhibits exocytotic release of neurotransmitter.

CHAPTER 5

This chapter describes analytical chemistry experiments designed to isolate and identify the bioactive chemical constituents present in the cold-water extract of *C. speciosus* rhizome. Analytical chemistry techniques employed were flash column chromatography, analytical and preparative HPLC separation, thin layer chromatography (TLC), liquid chromatography–mass spectrometry (LCMS), and proton nuclear magnetic resonance (^{1}H NMR). This chapter revealed separation problems encountered by *C. speciosus* rhizome cold-water extract using flash column chromatography. Chromatographic separation and isolated organ bath bioassays of *C. speciosus* rhizome cold-water extract revealed that inhibitory activity of the extract was due to the highly polar soluble components that were present in the liquid phase. Extensive studies on the carbohydrates present in the *C. speciosus* rhizome cold-water extract suggested that the chemical structure of the bioactive components might consist of other reducing sugars or non-reducing sugars. Conversely, the inhibitory effects displayed by the extract might be due to other non-carbohydrate based components which existed as minor peaks on the ^{1}H NMR spectrum of the extract. This study developed a systematic method for the chemical analysis of *C. speciosus* that included extraction, *ex vivo* experimentation, chromatography, and ^{1}H NMR analysis.

A chronology of this study is depicted in detail on page x.


Chemical components isolation
Mechanism of Action
Extractions
Costus speciosus rhizome
Boiling Water
Hot Water
Cold Water
Organ Bath Assay (Relax Yes/No)
NO?
YES?
End
Proceed to big bulk
Centrifugation & Chromatographic Separation
10 Fractions isolated
Organ Bath Assay (Relax Yes/No)
Only Fraction 1 active
Fraction 1 (CS-PLP1) (Highly polar)
1H NMR analysis
Spectrum indicates the presence of sugars
Fractionation (Prep-HPLC)
YES
Organ Bath Assay (Relax Yes/No)
Liquid Phase (CS-LP)
Centrifugation Separation
Solid Phase (CS-SP)
Organ Bath Assay (Relax Yes/No)
NO
End
Derivatisation
Electrical field stimulation (EFS)
1H NMR & LCMS
5 Sub-fractions isolated (D1, D2, D3, D4, D5)
Reverse-phase HPLC Separation
Derivatization of CS-PLP1
Shortlisted fractions:
1) D1-12
2) D1-15
3) D1-16
4) D2-4
5) D2-5
6) D(3,4,5)-4
7) D(3,4,5)-6
8) D(3,4,5)-7
9) D(3,4,5)-8
10) D(3,4,5)-9
11) D(3,4,5)-10
Derivatisation sugar standards (glucose, fructose, galactose, lactose, mannose, rhamnose, arabinose)
1H NMR & LCMS
Superimposed 1H NMR spectrum between anthranilate sugars with shortlisted fractions
1H NMR spectrum not match with shortlisted fractions
Retention time of the fractions matches with respective anthranilate sugar standards. Maybe other C5/C6 sugars in the structure.
EFS: Underivatised vs derivatised sugars standard
Shortlisted C5 and C6 sugar: fructose, rhamnose, arabinose
2-AA-Fru (m/z: 302.4)
D2-4
D2-5
D(3,4,5)-4
2-AA-rha (m/z: 286.4)
D(3,4,5)-9
D(3,4,5)-10
2-AA-Arabinose (m/z: 272.4)
D(3,4,5)-6
D(3,4,5)-7
D(3,4,5)-8
Conclusion: The bioactivity could be from other reducing sugars, non-reducing sugars or any other components in the mixture.
Organ Bath Assay (Exogenous agonists)
Organ Bath Assay (Exogenous antagonists)
Dose-response
Noradrenaline
Phenylepherine
Acetylcholine
ATP
Tyramine
Not affect
Acting directly on muscle
Not affect
Non-exocytotic release of neurotran-smitters
Electrical Field Stimulation (YES/NO/Partial inhibited)
Prazosin
Atropine
Mepyramine
Methysergide
Idazoxan
Yohimbine
8-phenyltheophyline
Propranolol
AH6809
Hexamethonium
Suramin
Desipramine
Partial
Partial
No
Partial
No
Partial
No
Partial
Partial
No
No
No
Gq: α1, H1, M3, 5-HT
Gi: α2, A1,
Gs: β, PGE2
Nicotinic (Ion Channel)
P2X (Ion Channel)
Amine Reuptake Transporter

ABBREVIATIONS AND UNITS OF MEASUREMENT

Abbreviations

^{1}H NMR	Proton Nuclear Magnetic Resonance
2-AA	2-aminobenzoic acid / anthranilic acid
5-HT	5-hydroxytryptamine
8-PT	8-phenyltheophylline
ACh	Acetylcholine
ACN	Acetonitrile
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ANOVA	Analysis of Variance
ATP	Adenosine-5'-triphosphate
BPH	Benign Prostatic Hyperplasia
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
COX	Cyclooxygenase
DAG	Diacyl-glycerol
dH_2O	Distilled water
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
EFS	Electrical field stimulation
EMT	Epithelial-mesenchymal transition
GABA	*Gamma*-Aminobutyric acid
GI	Gastrointestinal
GPCR	G protein-coupled receptor
HPLC	High-Performance Liquid Chromatography
IP3	Inositol-1,4,5-triphosphate
LCMS	Liquid Chromatography-Mass Spectrometry
LUTS	Lower urinary tract symptoms
mACh	Metabotropic muscarinic receptors
MAO	Monoamine oxidase

MeOH	Methanol
MgSO4.7H2O	Magnesium sulfate heptahydrate
NA	Noradrenaline
nACh	Ionotropic nicotinic receptors
NaCl	Sodium chloride
NET	Noradrenaline reuptake transporter
NO	Nitric oxide
SD	Sprague Dawley
TLC	Thin-layer chromatography

Units of Measurement

°C	degrees Celsius
%	Percent
cm	centimetre
g	gram
g.s	Integral (grams.second)
Hr	hour
Hz	hertz
L	litre
mA	milliampere
min	minute
mg	milligram
mL	millilitre
mM	millimolar
ms	millisecond
m/z	Mass to charge ratio
n	Number of animals
nM	nanomolar
pH	Negative logarithm of hydrogen ion concentration
ppm	Parts per million
s	second
µg	microgram
µL	microliter
µM	micromolar
V	Volts

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DECLARATION

I declare that to the best of my knowledge and belief that the material contained within this thesis has not been accepted for the award of any other degree or diploma in any university or other institution, nor does it contain material which has been previously published or written by a third-party, except where due reference is made in the text of the thesis.

Signature : ……  ……

Print Name : Su Nguok Ngie @ Eunice

Date : 11 October 2018 ……

PUBLICATIONS

Results from the studies discussed in this thesis have led to the following publications:

Manuscript in preparation for submission to a scientific journal

Nguok Ngie Eunice Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura. Ethnopharmacological effects of *Costus speciosus* on prostate contractility.

Conference abstracts

i. Oral presentations

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2017). Pharmacological effects of a jungle ginger on rat prostatic smooth muscle. *APSA-ASCEPT 2017 Joint Scientific Meeting – Optimising medicines for optimal patient outcomes*, 5 - 8 December, 2017, Brisbane, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2017). Pharmacological effects of a jungle ginger on rat prostatic smooth muscle. *9th National Symposium on Advances in Gastrointestinal & Urogenital Research*, 4 December, 2017, Gold Coast, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2016). Biological effects on rat prostatic smooth muscle and chemical fractionation of *Costus speciosus* rhizome. *ASCEPT-MPGPCR 2016 Joint Scientific Meeting – Therapeutic targeting: right place, right time, right effect*, 27 - 30 November, 2016, Melbourne, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2016). Biological effects on rat prostatic smooth muscle and chemical fractionation of *Costus speciosus* rhizome. *8th National Symposium on Advances in Gastrointestinal & Urogenital Research*, 25 November, 2016, Melbourne, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2016). Effects of *Costus speciosus* on prostate contractility. *11th Annual Postgraduate Symposium*, 29 September, 2016, Monash Institute of Pharmaceutical Sciences, Parkville, Victoria, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2016). *Costus speciosus*: effects on prostate contractility. *Drug Discovery Biology 4th Annual Student Symposium 2016*, 21 – 22 July, 2016, Monash Institute of Pharmaceutical Sciences, Parkville, Victoria, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2015). Sarawak jungle ginger, *Costus speciosus*, inhibits smooth muscle contraction of the isolated rat prostate gland. *10th Annual Postgraduate Research Symposium*, 1 October, 2015, Monash Institute of Pharmaceutical Sciences, Parkville, Victoria, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2015). A Sarawak jungle ginger – *Costus speciosus* inhibits nerve mediated smooth muscle contraction of the isolated rat prostate gland. *ASPA-ASCEPT 2015*, 29 November – 2nd December, 2015, Hobart, Australia.

ii. Poster presentations

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2018). Chemical fractionation and pharmacological effects of a jungle ginger on prostate contractility. *Monash University 13th Annual Postgraduate Symposium*, 26 September, 2018, Victoria, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2018). Novel pharmacological effects of a jungle ginger on prostate contractility. *18th World Congress of Basic and Clinical Pharmacology (WCP2018)*, 1-6 July, 2018, Kyoto, Japan.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2017). Pharmacological effects of a jungle ginger on rat prostatic smooth muscle. *APSA-ASCEPT 2017 Joint Scientific Meeting – Optimising medicines for optimal patient outcomes*, 5 - 8 December, 2017, Brisbane, Australia.

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Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2016). Biological effects on rat prostatic smooth muscle and chemical fractionation of *Costus speciosus* rhizome. *8th National Symposium on Advances in Gastrointestinal & Urogenital Research*, 25 November, 2016, Melbourne, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2015). A Sarawak jungle ginger - *Costus speciosus* inhibits nerve mediated smooth muscle contraction of the isolated rat prostate gland. *APSA-ASCEPT 2015 Joint Scientific Meeting -- Drugs to medicines: up close and personal*, 29 November – 2 December, 2015, Hobart, Australia.

Eunice NN Su, Jamie S Simpson, Philip Thompson, Sabatino Ventura (2015). Sarawak jungle ginger, *Costus speciosus*, inhibits smooth muscle contraction of the isolated rat prostate gland. *Cancer Therapeutics HDR Student Symposium 2015*, 23rd July, 2015, Victoria, Australia.

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"Have I not commanded you? Be strong and courageous. Do not be afraid; do not be discouraged, for the LORD your God will be with you wherever you go."

(Joshua 1:9)

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1

General Introduction

1.1 DEVELOPMENT OF THE MALE GENITOURINARY SYSTEM

The genitourinary system consists of two components, that service the body's urinary and reproductive systems. However, they are intimately related embryonically and anatomically (Sadler, 2011). In males, the major organs of the male reproductive system are the testes, epididymis, vas deferens, ejaculatory ducts, prostate, seminal vesicles and bulbourethral glands. While the major tissues of the urinary system are the kidneys, ureters and bladder with the urethra being common to both systems (**Figure 1-1**).

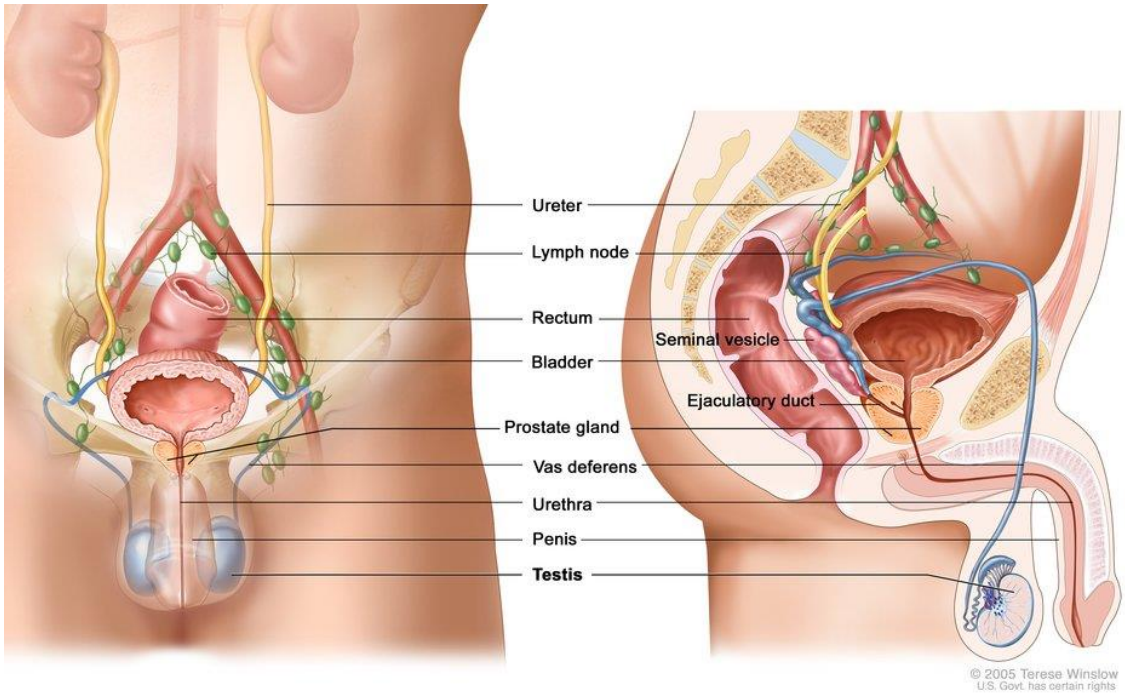


Figure 1-1. Anatomy of the male urinary systems (Winslow, 2008).

During gestation in humans, there are three kidney components that form simultaneously: pronephros, mesonephros and metanephros (Gray, 2000; Sadler, 2015). Embryonically, these all develop from the intermediate mesoderm to form the principal excretory organ during early

gestation period (4-8 weeks) (Park, 2007). Pronephros is a primary and non-functional group of cells that form vestigial excretory units named nephrotomes. The pronephric duct grows caudally until it opens into the ventral part of the cloaca. These cell groups will regress by the end of the fourth week of human embryonic life (Gray, 2000; Sadler, 2015). As soon as pronephros regresses, mesonephros excretory tubules appear (**Figure 1-2A**).

The mesonephros tubules elongate and form glomerulus tubules which then form Bowman's capsule. The tubule will then form the longitudinal collecting duct laterally which is known as the mesonephric or Wolffian duct (Gray, 2000; Sadler, 2015). The urogenital ridge is formed during this stage (**Figure 1-2A**). It is suspended by a common mesentery together with the mesonephros. However, it is gradually separated from the mesonephros when the embryo grows. During the seventh week, the urogenital ridge will develop into either the testes in males or ovaries in females (Gray, 2000) (**Figure 1-2C & D**). Part of the mesonephric duct and caudal tubules will remain and become associated with the formation of the male genital system (i.e. epididymis, ductus deferens, ejaculatory duct, efferent ducts of the testis, ductuli aberrantes, paradidymis) but they disappear in the female (Gray, 2000; Sadler, 2015).

The final stage is metanephros which appears caudal to the mesonephros in the fifth week of human embryonic life. It develops from the intermediate mesoderm and forms the permanent functional kidney (Gray, 2000; Sadler, 2015). Rudiments of the pelvis and calyces of the kidney are formed by the ureteric bud which grow out from the mesonephric duct close to the cloaca. The bud invaginates into the metanephric tissue and ultimately gives rise to the collecting ducts of the kidney and ureter (Gray, 2000; Sadler, 2015). The kidney, therefore, develops from two origins: (1) the metanephric mesoderm that gives rise to excretory units, and (2) the ureteric bud, that forms the collecting system (Gray, 2000; Sadler, 2015) (**Figure 1-2B**).

Both urinary and reproductive structures are developed from the intermediate mesoderm that temporarily connects the paraxial mesoderm and the lateral plate (Gray, 2000; Sadler, 2011; Sadler, 2015). Initially, the excretory ducts of both of these systems share a common cavity known as the cloaca (Sadler, 2015). The cloaca later divides into the urogenital sinus and the anorectal canal (**Figure 1-2C**). During the eighth week of gestation in humans, the ureter has separated from the mesonephric duct when it gradually merges into the wall of the urogenital sinus. The urogenital sinus can be separated into three portions: (1) vesicourethral portion, (2)

pelvic portion, and (3) phallic portion. The vesicourethral portion is the main part of the urogenital sinus that later forms the urinary bladder.

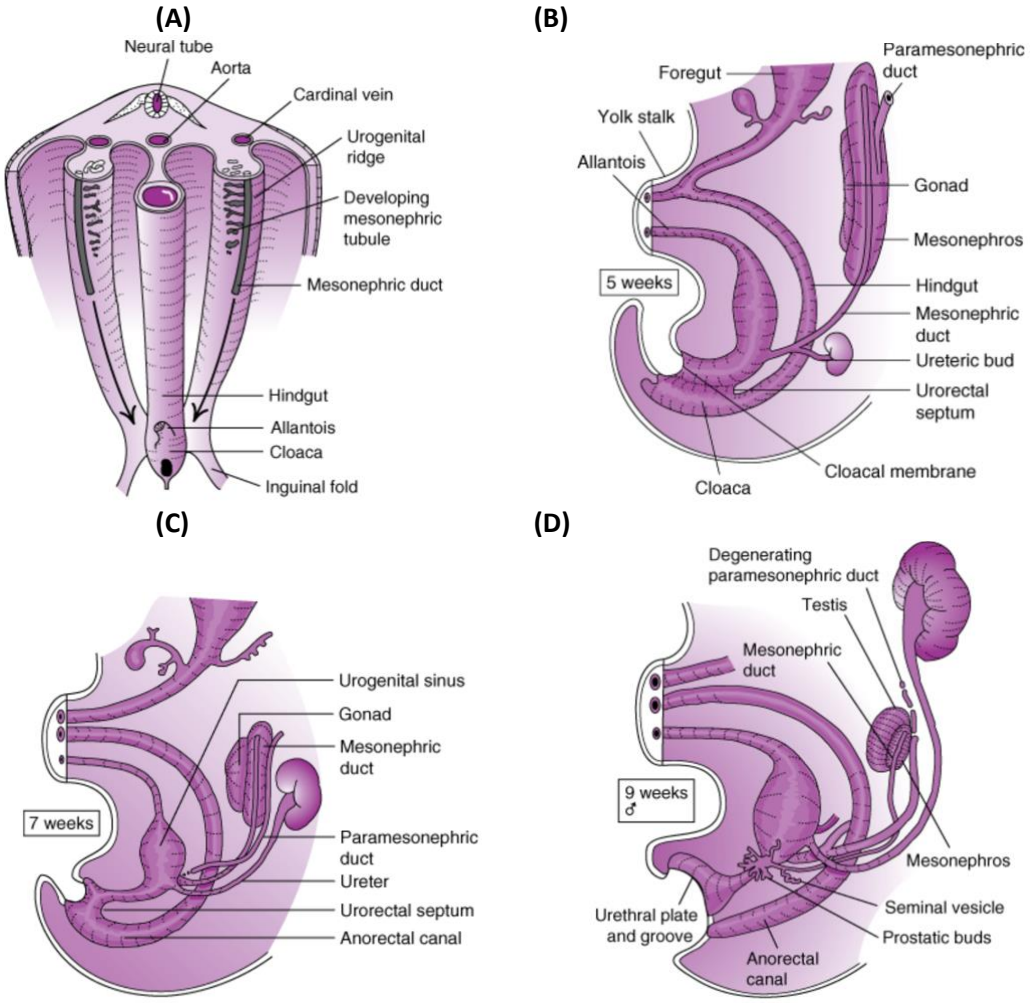


Figure 1-2. Development of the urinary system in the human male embryo. Permanent kidneys begin to develop in the fifth week from the ureteric bud and metanephric mass of mesoderm. Penile urethra is first developed from the definitive urogenital sinus. The buds from the urethra will give rise to the prostate gland, whereas seminal vesicles are developed from the budding of the ductus deferens (DeUgarte, 2013).

When the lumen of the allantois is obliterated, its apex is prolonged forming a thick fibrous cord known as the urachus which connects to the apex of the bladder and becomes the medial umbilical ligament (**Figure 1-5**) in adults. The epithelium of the prostatic urethra continues to proliferate and invaginate into the surrounding mesenchyma to form the prostate gland at the end of the third month of embryonic life in human males. The development of the genital ducts in the male forms from parts of the mesonephric kidney system when stimulated by testosterone (Gray, 2000; Sadler, 2015). The morphogenesis of prostatic epithelial ducts and acini branching out from the mesenchymal cells of the urogenital sinus occurs under the influence of testicular androgens (McNicholas and Mitchell, 2006).

1.2 ANATOMY AND DEVELOPMENT OF THE LOWER URINARY TRACT

The lower urinary tract (LUT) in both sexes consists of a reservoir (bladder), and an outlet (bladder neck, urethra, urethral sphincter, pelvic floor). In men, the bladder outlet is surrounded by the prostate gland (**Figure 1-3**) (Alleemudder and Ballaro, 2016; de Groat and Yoshimura, 2015; Mahadevan, 2016; Mangera et al., 2010). The anatomy of the LUT in males and females is different due to considerable differences in the pelvic anatomy of the sexual organs.

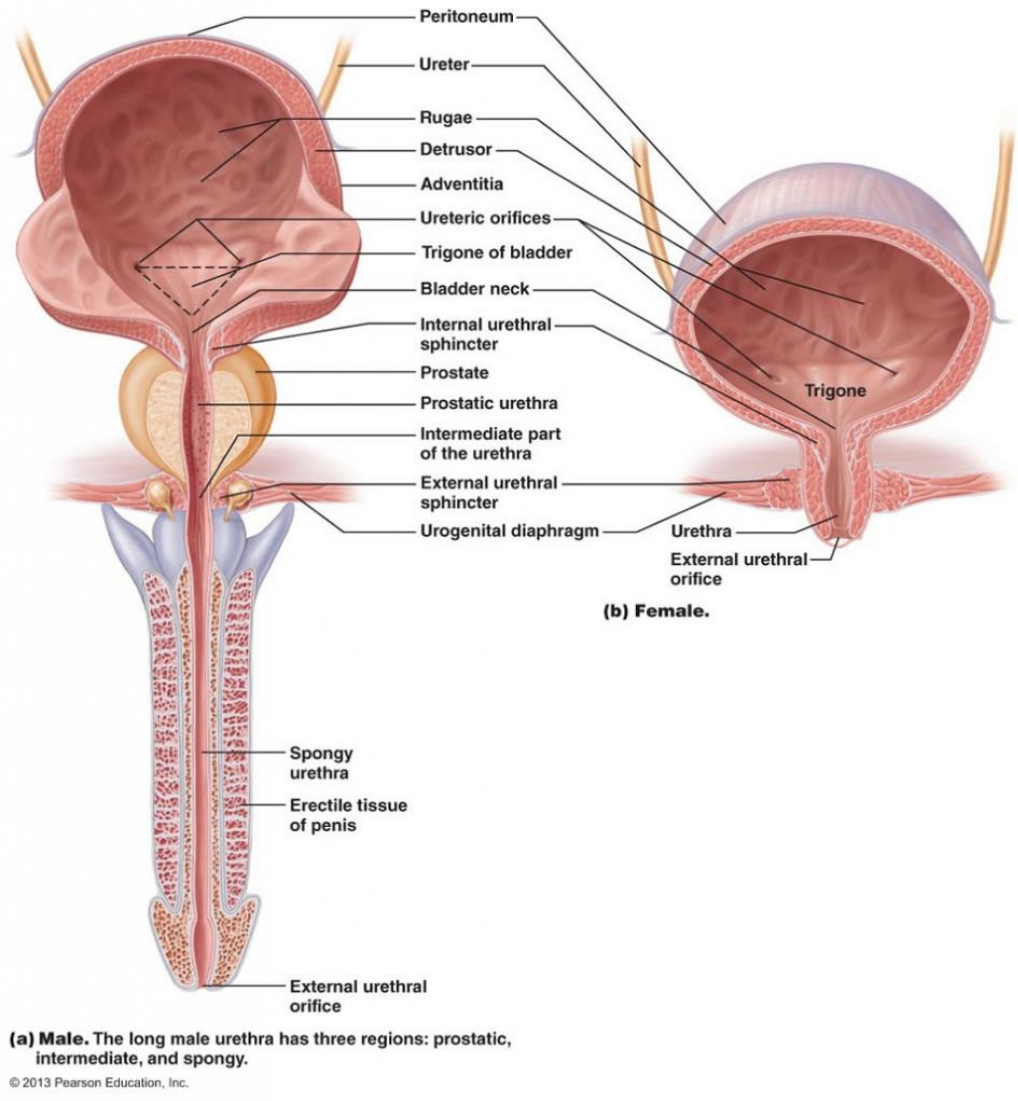


Figure 1-3. Lower urinary tract in both male and female (Pearson Education, 2013).

Generally, the urinary bladder can be divided into four parts: (1) apex, (2) base, (3) superior surface, and (4) inferolateral surfaces (**Figure 1-4**). The urinary bladder is a distensible and extraperitoneal pelvic organ that serves as a temporary reservoir for urine conveyed through ureters from the kidney. The ureters terminate at the bladder posteriorly at its base

(Alleemudder and Ballaro, 2016; de Groat and Yoshimura, 2015; Mahadevan, 2016; Mangera et al., 2010). The distal end of the ureters are covered with a fibromuscular sheath (Waldeyer) that prevents retrograde flow of urine when the bladder fills (Alleemudder and Ballaro, 2016). Micturition of the urinary bladder is under voluntary control and is regulated by a complex neuronal control system located in the brain and spinal cord (de Groat and Yoshimura, 2015). The urinary bladder apex connects the urachus to the anterior abdominal wall and has a base composed of the trigone and bladder neck (Alleemudder and Ballaro, 2016).

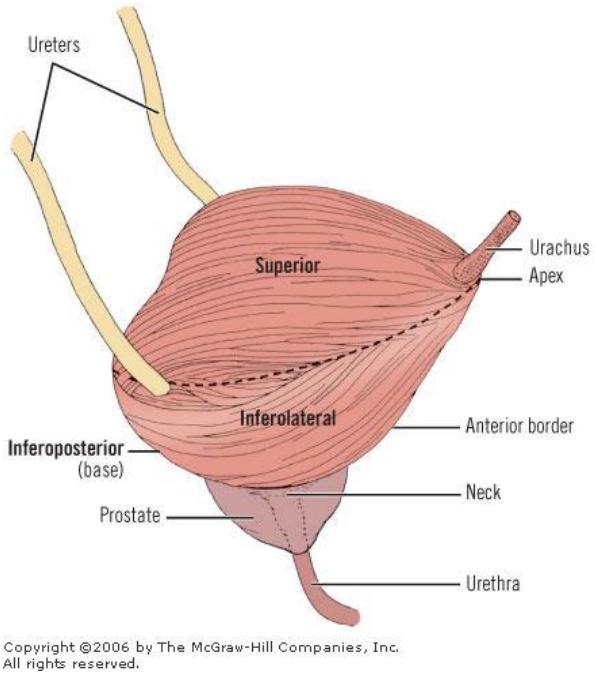


Figure 1-4. Anatomy of urinary bladder (The McGraw-Hill Companies, 2006).

Histologically, the urinary bladder consists of four layers: (1) urothelium (inner), (2) lamina propria, (3) detrusor muscle and (4) serosa (outer). The urothelium (transitional epithelium) which lines the inside of the urinary bladder is made up of three basic layers: basal cell layer, an intermediate layer and a superficial layer that consists of large hexagonal cells known as “umbrella cells” (Birder et al., 2010; Mahadevan, 2016). The umbrella cells are covered by a layer of glycosaminoglycans that protect the bladder wall from harmful agents found in urine such as bacteria and various toxins (Alleemudder and Ballaro, 2016). The base of the bladder in males is intimately associated with the seminal vesicles, vas deferens and terminal ureter (Alleemudder and Ballaro, 2016). The point where inferolateral surfaces intersect with the base of the bladder is the bladder neck which lies directly on the prostate in males. The bladder neck is anchored into position by the puboprostatic ligaments that consist of the fibrous capsule of the prostate (Drake et al., 2015; Mahadevan, 2016; Mangera et al., 2010). As the bladder neck continues directly into the urethra, the inner layer is consistent from the bladder neck through to the

urethra. The middle layer which forms a pre-prostatic ring that circles the bladder neck makes up the internal urinary sphincter (**Figure 1-5**) whilst the outer most layer spans the back of the ureters as well as a loop surrounding the anterior of the bladder neck (Alleemudder and Ballaro, 2016; Mangera et al., 2010).

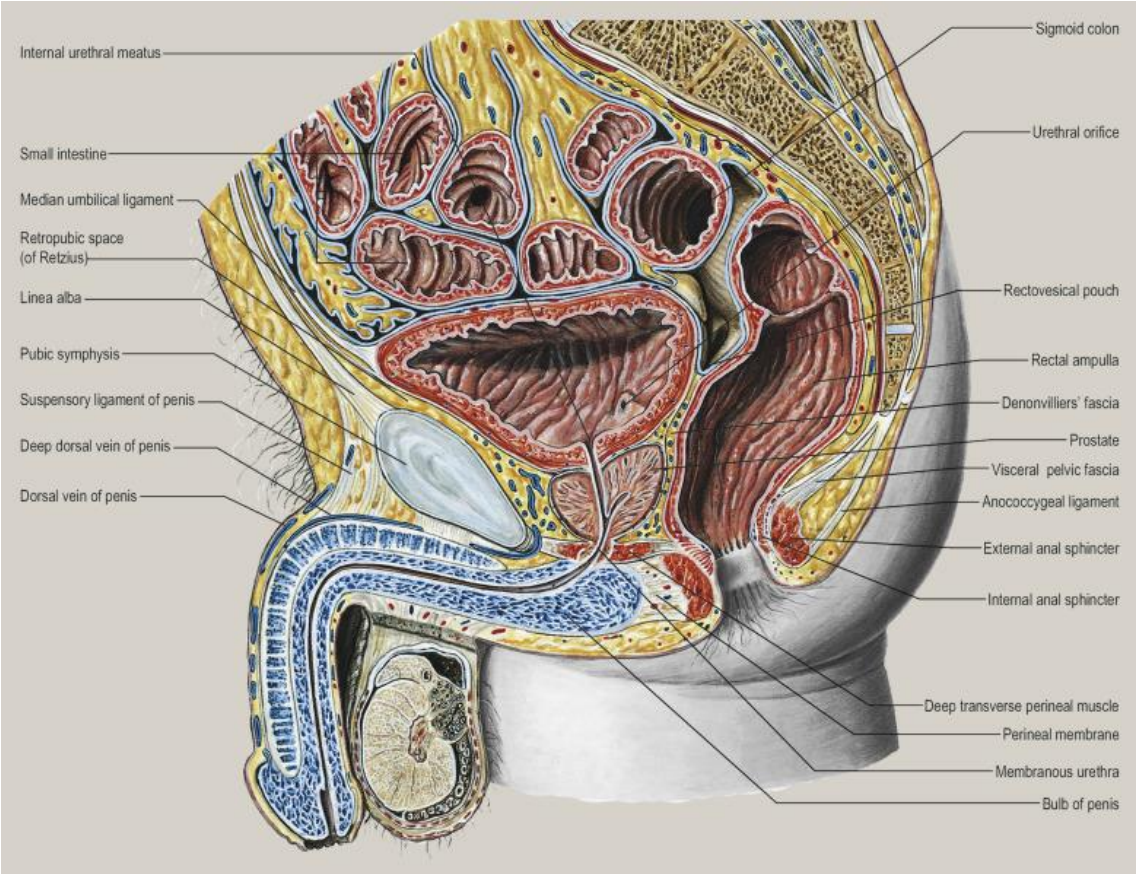


Figure 1-5. Median sagittal section of the male pelvis showing the relationship between prostate gland and the lower urinary tract (Mahadevan, 2016).

A human adult male urethra ranges from 18-20 cm long. It spans from the internal urethral meatus at the bladder neck to the external urethral orifice at the end of the penis. The whole male urethra can be divided into four anatomically distinct segments: the pre-prostatic urethra (bladder neck), prostatic urethra, membranous urethra and the spongiose or penile urethra (**Figure 1-6**) (de Groat and Yoshimura, 2015; Drake et al., 2015; Mahadevan, 2016). In urological nomenclature, the posterior urethra refers to the pre-prostatic, prostatic and membranous segments of the urethra; whilst the anterior urethra refers to the whole spongiose urethra (Mahadevan, 2016). The length of the pre-prostatic urethra is approximately 1 cm in humans and consists of a circular collar of smooth muscle cells which is densely innervated by sympathetic noradrenergic nerves that mediate contraction of the sphincter to prevent retrograde ejaculation. It functions as the genital sphincter and is very distinct from the urinary

sphincter at the bladder neck (Mahadevan, 2016). It spreads out vertically from the internal urethral meatus to the superior aspect or base of the prostate (the internal urethral sphincter) (Drake et al., 2015; Mahadevan, 2016). The sympathetic noradrenergic nerves that innervate the pre-prostatic urethra also innervate and cause contraction of the smooth muscle of the prostatic stroma, seminal vesicles and vas deferens during ejaculation (Mahadevan, 2016).

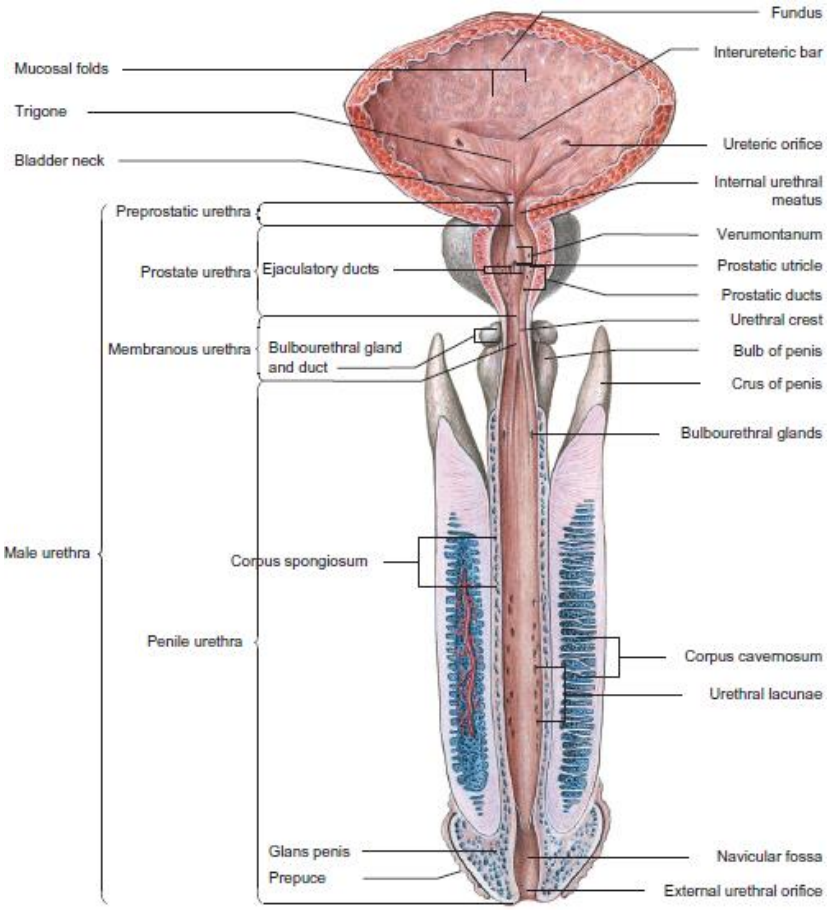


Figure 1-6. Longitudinal section of the male lower urinary tract (Clement and Giuliano, 2015)

The prostatic part of the urethra is about 3 - 4 cm long and is closer to the anterior surface of the prostate extending through the prostate gland to end at its apex. Generally, it can be divided into three segments: proximal, intermediate, and distal (Clegg, 1957). On the posterior surface of the urethral lumen, there is an elevation of the mucous membrane and surrounding tissue forming a narrow median longitudinal ridge termed the urethral crest. The prostatic ducts empty prostatic fluid into the urethra via a shallow longitudinal depression termed the prostatic sinus on each side of the urethral crest (**Figure 1-6**). At the midline of the urethral crest, is a rounded eminence called the seminal colliculus (also known as verumontanum) (**Figure 1-6**) that is important during transurethral transection of the prostate to determine the position of the prostate. At the centre of the seminal colliculus is a slit-like orifice known as the prostatic utricle

(**Figure 1-6**). The ejaculatory ducts of the male reproductive system open on both sides of the prostatic utricle on the verumontanum (**Figure 1-6**) (Alleemudder and Ballaro, 2016; de Groat and Yoshimura, 2015; Drake et al., 2015; Mahadevan, 2016; Mangera et al., 2010).

The urethra enters a deep perineal pouch inferior to the prostatic apex. This segment which spans on average 2 – 2.5 cm is termed the membranous urethra. It has a sphincteric function and is lined with a thin smooth muscle layer (**Figure 1-6**) (Alleemudder and Ballaro, 2016; Brooks, 2007). This segment is also very narrow and inflexible. The two main subdivisions of the trigone (the deep and superficial perineal pouches) are demarcated by a distinct fascial layer called the perineal membrane (Mahadevan, 2016). The membranous urethra, external urethral sphincter, bulbourethral glands (Cowper's gland), the pudendal nerves and the dorsal nerves of the penis lie within the deep perineal pouch. Both the prostatic and membranous urethra are lined with transitional epithelium (Alleemudder and Ballaro, 2016; Brooks, 2007; de Groat and Yoshimura, 2015; Drake et al., 2015; Mahadevan, 2016; Mangera et al., 2010).

The longest part (approximately 15 cm) of the urethra is enclosed entirely within the corpus spongiosum which lies in the ventral groove between the two corpora cavernosa (**Figure 1-6**). It consists of bulbar, penile and glandular sections and is sometimes referred to as the 'spongy' urethra. The spongiose urethra is an extension of the membranous urethra and is lined with pseudostratified columnar epithelium proximally and distally with stratified squamous epithelium. The penile urethra contains numerous mucous glands as well as follicles termed periurethral glands of Littré (Alleemudder and Ballaro, 2016; de Groat and Yoshimura, 2015; Drake et al., 2015; Mahadevan, 2016; Mangera et al., 2010).

1.3 ANATOMY AND DEVELOPMENT OF THE PROSTATE GLAND

The hypothalamic-pituitary-gonadal (HPG) axis is an important part of the endocrine system involved in male reproduction. The hypothalamus produces gonadotropin-releasing hormone (GnRH) which stimulates the pituitary gland to produce the gonadotropins – luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Emanuele and Emanuele, 2001). In males, LH stimulates the production of testosterone from the Leydig cells of the testes; whilst FSH stimulates sperm production in the Sertoli cells of the testes and is crucial for sperm maturation in the epididymis (Emanuele and Emanuele, 2001). Testosterone (**Figure 1-7**) is a cholesterol derivative and the major hormone in the development of the male reproductive system

(Chughtai et al., 2016; DeLay and Kohler, 2016; Roberts, 1995). The majority of testosterone (95%) is produced by Leydig cells and once in the systemic circulation, testosterone exerts negative feedback to the hypothalamus to decrease the release of gonadotropin-releasing hormone (GnRH) and lower the GnRH sensitivity of gonadotropic cells thus inhibiting luteinizing hormone (LH) release. Testosterone is the major (98 %) circulating androgen in blood but is converted to dihydrotestosterone (DHT) in the prostate by the 5α-reductase enzyme and estradiol (E_2) by the aromatase (CYP19A1) enzyme in the testes (Clement and Giuliano, 2015; DeLay and Kohler, 2016; Matsumoto and Bremner, 2016). DHT acts in an autocrine manner in the stromal cells, the paracrine manner by diffusing to adjacent epithelial cells, or endocrine manner when it diffuses from the circulation into the prostate (Chughtai et al., 2016).

Embryonically, the prostate first appears from the endodermal urogenital sinus during the third month of gestation. Its development is stimulated primarily by DHT. Other male phenotypes developed from the urogenital sinus under androgenic hormonal influence include the urethra and periurethral glands. In contrast, development of Wolffian-derived sex accessory glands such as the seminal vesicles, epididymis, vas deferens, ampulla, and ejaculatory ducts is stimulated by fetal testosterone rather than DHT and will be fully developed by week 13 of gestation in humans (McNicholas and Mitchell, 2006; Veltri and Rodriguez, 2007). During the last few months of intra-uterine life, there is an increase in the proliferation and differentiation of the prostatic epithelium, squamous metaplasia, cystic dilatation of the tubules as well as enlargement and dilatation of the ejaculatory ducts (Andrews, 1951). These changes continue until term and continue for one to four weeks after birth before gradually undergoing complete or partial regression over one to four months.

As these changes in the immature prostate occur concurrently with those in the breast, uterus, and vagina of the foetus, it is believed that they are caused by circulating maternal oestrogens during the later months of pregnancy (Andrews, 1951). Prostatic tubules in the later months of pregnancy are formed by two layers of cells, a luminal layer, and a horizontal basal layer. The luminal layers consist of either low columnar or flattened epithelium. From the fourth month after birth until puberty, the size of the prostate remains stagnant with only a minimal increase in the stroma (Andrews, 1951). The gland develops to its adult form with a rapid increases in size and secretory ability at puberty (Franks, 1954).

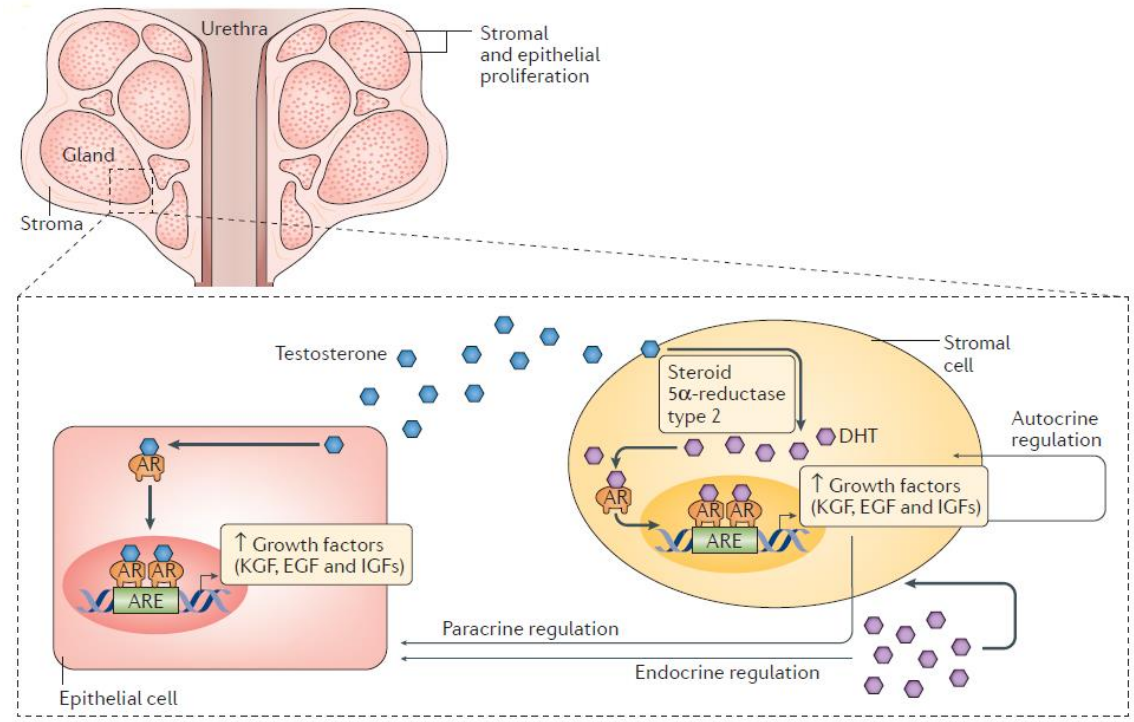


Figure 1-7. Testosterone produced in the Leydig cells of the testes diffuses into prostate stromal and epithelial cells. In the epithelial cells, testosterone binds to the androgen receptor (AR) which binds to the androgen response element (ARE), subsequently upregulating the expression of various growth factor genes such as keratinocyte growth factor (KGF), epidermal growth factor (EGF) and insulin-like growth factors (IGFs). In stromal cells, the testosterone is converted to dihydrotestosterone (DHT) which promotes proliferation in an autocrine manner as well as in a paracrine manner by diffusing into adjacent epithelial cells. DHT that is produced in other tissues such as liver and skin can also diffuse into the prostate from the systemic circulation and act in an endocrine manner (Chughtai et al., 2016).

The prostate is a small but important organ in the male reproductive system. It is also referred to as an accessory sex gland as it is only indirectly involved in procreation. The term 'prostate' was originally derived from 'prohistani', a Greek word, which means 'to stand in front of'. In 335 B.C., Herophilus of Alexandria has used this word to describe the organ which is located 'in front of' the urinary bladder (Chapple, 1994; Lowsley, 1912; Sharma et al., 2017). The prostate is ovoid in shape that is about the size of a walnut in young men. It is made up of 30 % fibromuscular and 70 % glandular tissue. A foetal human prostate weighs approximately 1.5 g and reaches 10 g at puberty (DeLay and Kohler, 2016). An adult normal prostate usually weighs approximately 20 g and is 4 cm in diameter. It continues to grow until age 40 to 45 (**Figure 1-8**) (Brooks, 2007; Mangera et al., 2010; Rous, 2002). The human prostate is comprised of smooth muscle, glandular and fibrous or connective tissue. The only difference between normal and hyperplastic prostate is the latter has more glandular cells (Rous, 2002). The average human prostate weight at autopsy increases after 50 years of age due to increased prevalence of BPH in ageing men.

The two most prevalent diseases in ageing men that arise in the prostate are benign prostatic hyperplasia (BPH) and prostate cancer (PCa). Only BPH will be discussed in this thesis.

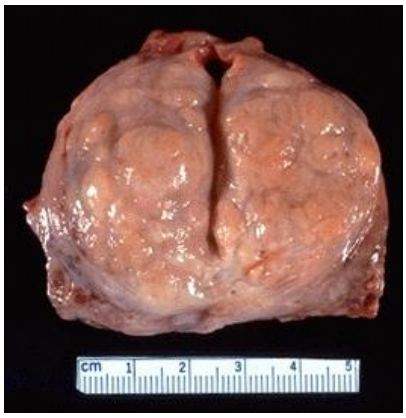


Figure 1-8. This is the gross appearance of benign prostatic hyperplasia (BPH). Size of a normal human prostate is 3 to 4 cm in cross-section, by comparison (Klatt, 2017).

1.3.1 Morphology of the Prostate Gland

The prostate is enclosed within a capsule of strong connective tissue and it lies entirely behind the pubic symphysis (Mangera et al., 2010), inferior to the bladder and anterior to the rectum which allows the prostate to be palpated and biopsied through the rectum (Roberts, 1995). The prostatic capsule has an average thickness of 0.5 mm and is made up of collagen, an abundance of smooth-muscle cells and elastin in high density (Brooks, 2007). The prostate enclosed in the capsule is composed of alveoli lined with columnar glandular epithelium. The alveoli are embedded in the relatively thick fibromuscular stroma. At the apex of the prostate, there is no capsule that separates the bladder from the prostate (Brooks, 2007). The concept of prostatic lobes had been introduced as early as 1912 from the studies of human embryos (Lowsley, 1912). The fibroelastic prostatic capsule gives rise to septa which allow the whole prostate to be anatomically subdivided into five lobes (anterior, posterior, median, and two laterals) based on the location of ducts in the urethra, different pathologic lesions and embryonic origin (Brooks, 2007; Clement and Giuliano, 2015; de Groat and Yoshimura, 2015; Kumar and Majumder, 1995; Lowsley, 1912). These prostatic lobes surround the proximal urethra (prostatic urethra) from the bladder neck and the apex rests on the superior surface of the urogenital diaphragm (Clement and Giuliano, 2015; Lee et al., 2011). The ejaculatory ducts of the male reproductive system appear on each side of the prostatic utricle in the prostatic part of the urethra (**Figure 1-9**).

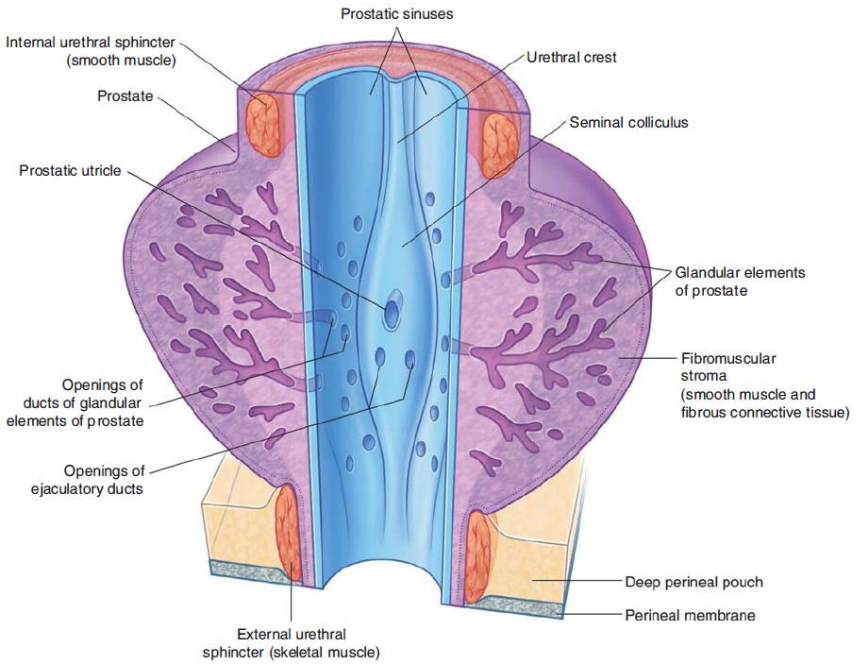


Figure 1-9. Cross section of the prostatic urethra in men (Drake et al., 2015).

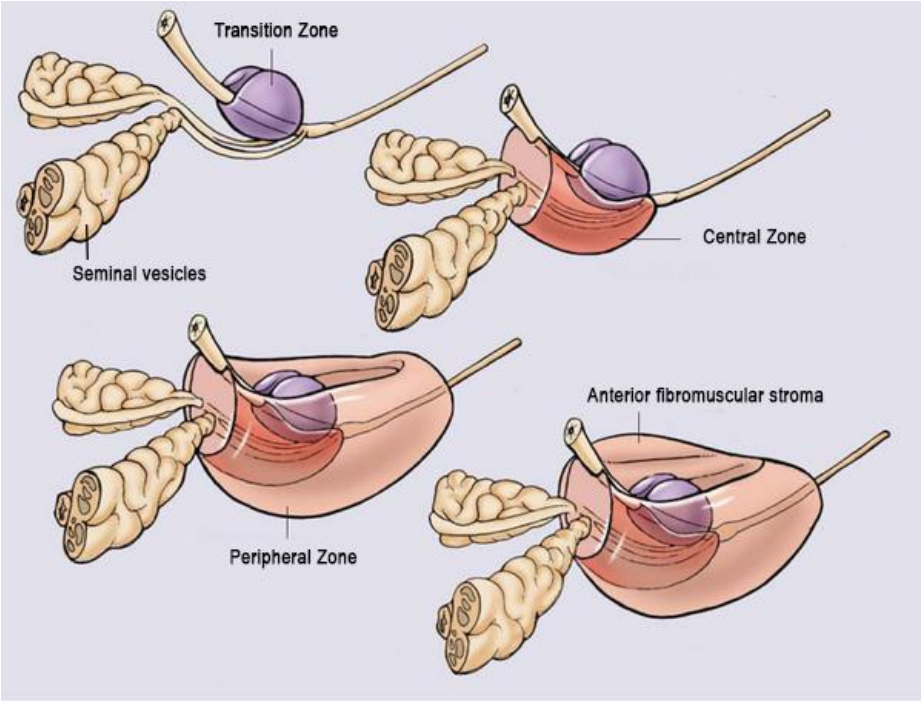


Figure 1-10. Zonal anatomy of the prostate based on the McNeal concept. The central zone comprises about 25 % of the whole prostate volume. The peripheral zone which extends posterolaterally around the distal prostatic urethra and central zone comprises about 70 % of the whole prostate volume which is the largest area. Outer prostate refers to the peripheral and central zones; whilst the inner prostate encompasses the transition zone and anterior fibromuscular stroma (Brooks, 2007; Shah, 2012).

1.3.1.1 McNeals's Zonal Hypothesis

Although the prostatic lobes concept had emerged in 1912 as a result of studies carried out on human embryos (Lowsley, 1912), the most precise exploration of human adult prostate zonal morphology was later proposed by McNeal (McNeal, 1968; McNeal, 1981). McNeal's concept was that there are four basic anatomic regions of the prostate which can be described and distinguished into nonglandular and glandular compartments. A schematic diagram of prostatic zonal anatomy is depicted in **Figure 1-10**. The prostate is divided into: 1) peripheral zone, 2) central zone, 3) preprostatic region and 4) anterior fibromuscular stroma. It has been suggested that benign nodular hyperplasia arises from the inner part of the prostatic gland in later life (Franks, 1954). This portion of the prostate was later termed the transition zone (McNeal, 1978).

1.3.1.1.1 Glandular compartments

There are three major glandular regions in the prostate that differ in biology and histology. These regions are the peripheral, central, and transition zones.

The peripheral zone (PZ) covers the majority of the glandular prostate volume, which is about 75% of the entire prostate (**Figure 1-10**). It is localised posterolaterally to the transition zone and forms a disc of ductal tissue radiating laterally from the prostatic urethra and distal to the base of the verumontanum (**Figure 1-12**) (McNeal, 1981).

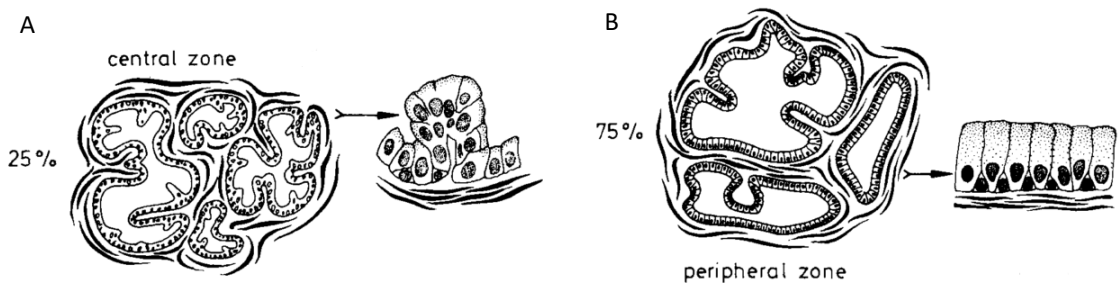


Figure 1-11. Morphology of the central and peripheral zones as described by McNeal (Aumüller, 1983).

The histologic feature of the gland is characterised by simple and small acinar spaces lined by tall columnar secretory epithelial cells. The peripheral zone muscle tissue is normally loosely woven and finely textured (McNeal, 1978). This region is highly susceptible to the occurrence of carcinoma, chronic prostatitis, and post inflammatory atrophy (Hammerich et al., 2009). The morphology of the central and peripheral zones as described by McNeal and depicted by Anumüller (1983) is shown in **Figure 1-11**.

The central zone (CZ) constitutes 25% of the prostatic volume (**Figure 1-10**). It is a cone-shaped-like region that arises from the mesenchyme near to the ejaculatory duct orifices and then it follows these ducts proximally from the convexity of the verumontanum (**Figure 1-12**). It branches laterally close to the prostate base to join their acinar lobules and together makes up most of the prostatic base; it also runs parallel with the most proximal branches of the peripheral zone. The region where these two zones bind together creates a flat disc of tissue which is clearly visible as a distinct boundary in the normal adult prostate gland (**Figure 1-12**) (McNeal, 1981).

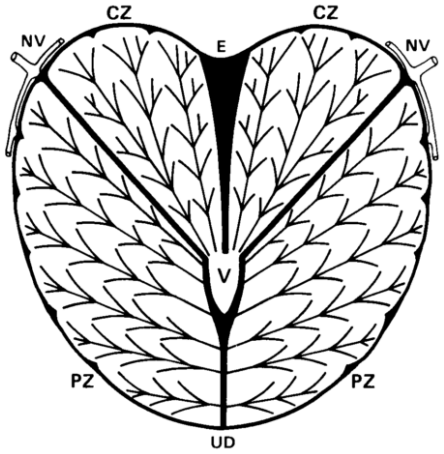


Figure 1-12. Relationships between the central zone (CZ) and the peripheral zone (PZ) with the distal urethral segment (UD), verumontanum (V), and ejaculatory ducts (E) making up the stromal core. The boundary between the central zone and peripheral zone is marked by heavy dark lines. Neurovascular bundle: NV (McNeal, 1988).

Histologically, this second largest region of the prostate is characterised by the existence of relatively large acini of irregular contour, lined by columnar to the cuboidal epithelium. The epithelium of this region is abundant. The cells are more opaque, with granular cytoplasm and less obvious membrane. They appear crowded with larger nuclei lying at different levels from the basement membrane. The central zone has been postulated as the remnant of the Wolffian duct system whereas the peripheral zone and urethra originate from the urogenital sinus (McNeal, 1981). This distinctive morphological and biological difference between the peripheral and central zone is important for the study of different prostatic diseases as most (70 %) carcinomas of the prostate originate from the peripheral zone (McNeal, 1981).

Histologically, the transition zone is similar to the main prostate posteriorly except that its muscle tissue is extremely compact with coarse fibres. This is the smallest zone and comprises only 5% of the total glandular prostate volume in the young human male adult (**Figure 1-10**). The transition zone (TZ) is composed of two symmetrical lobules lying lateral to the proximal prostatic urethra (**Figure 1-13**). Smooth muscle fibres of the transition zone are arranged poorly

around acini. The majority of them form abundant but incomplete concentric rings medially that are parallel to the fibres of the enclosed cylindrical sphincter. Some of the innermost fibres even appear to blend in with the sphincter fibres dorsal to the urethra. Another typical characteristic of this zone is its most medial gland branches often curve and extend inward and pierce into the lateral part of the sphincter. The transition zone is separated from other prostatic glandular tissue by a border which histologically lacks a combination of its concentric muscle with the sphincter (McNeal, 1978). The periurethral gland is a small part of the transition zone at the proximal urethral segment (McNeal, 1988). The periurethral region and transition zone are regions exclusively susceptible to benign prostatic hyperplasia (BPH) (McNeal, 1981). McNeal (1978) has postulated that reawakening of embryonic inductive interactions between prostatic epithelium and stroma cause BPH. The prostatic nodules accumulate with age where gland budding and branching towards a central focus occurs primarily in the transition zone (McNeal, 1978).

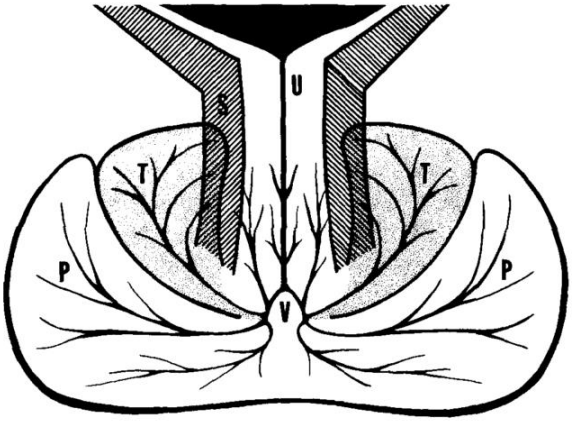


Figure 1-13. Transition zone (T) ducts fan out from the urethra and proximal to the base of the verumontanum (V), spreading between peripheral zone (P) and sphincter (S). Periurethral stroma: U. Bladder: black shading (McNeal, 1981).

1.3.1.1.2 Non-glandular compartments

The nonglandular regions of the prostate are the preprostatic sphincter, the striated sphincter, the anterior fibromuscular stroma, and the prostatic capsule (McNeal, 1988). The anterior fibromuscular stroma forms a thick apron that shields from view the anterior surface of the other three glandular regions and its inner aspect fuses tightly with these three zones (McNeal, 1981). It has no apparent important physiological function in the adult prostate gland. Its thick tissue extends downward from the detrusor muscle at the bladder neck over the anteromedial surface of the prostate and narrows to join the urethra at the prostate apex. Histologically, it

constitutes randomly orientated large compact smooth muscle cells merging with those of the bladder neck (McNeal, 1981).

1.3.2 Histology of the Prostate Gland

Histologically, the prostate gland comprises two major cell types: stromal and epithelial compartments which are oestrogen-dependent as well as androgen-dependent (El-Alfy et al., 2000; Habermann et al., 2001; Kassen et al., 1996; Risbridger et al., 2001). These cells overlying the heterogeneity of the prostatic ducts are regulated by steroid hormones to support different biological activities within the prostate gland (Farnsworth, 1999). The prostatic stroma consists of three major cell types namely smooth muscle cells, fibroblasts and endothelial cells (Hudson, 2004; Kassen et al., 1996). Steroid hormones, as well as α-adrenergic receptor agonists, are important in the differentiation and development of the prostatic epithelial cells. Stromal cells are involved in directing this hormonal process as well as in secreting several growth factors (Schalken and van Leenders, 2003).

The prostatic epithelium is composed of five discrete cell populations: basal cells, secretory luminal cells, stem cells, amplifying cells and neuroendocrine cells. All are believed to be derived from a common ancestral pluripotent stem cell (Bonkhoff and Remberger, 1996; Hudson, 2004; Hudson et al., 2001; Sharma et al., 2017). Secretory cells in the prostate contribute to the seminal plasma. Secreted fluids include prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) into the glandular lumen. The secretory cells in the prostate are divided from the basement membrane and stroma by a layer of basal cells. The basal cells do not have secretory vesicles and are polygonal in shape. Their cellular components comprise of mitochondria, endoplasmic reticulum, free ribosomes, Golgi apparatus, and pinocytic vesicles as well as irregular shaped nuclei (Sharma et al., 2017). These basal cells are not myoepithelial cells but make up the proliferative segment of the prostate epithelium which differentiate and eventually mature into secretory cells. Androgen receptors are virtually absent in the basal cell layer (McNeal, 1988). The unique feature of basal cells in human prostate as compared to other species is the relatively high ratio of basal to luminal cells with gap junctions which probably function to regulate activities between the two cell types (El-Alfy et al., 2000). Neuroendocrine cells appear on the basal cells between secretory cells and are rich in serotonin-containing granules. Some of the neuroendocrine cells also contain peptide hormones such as somatostatin, calcitonin, and bombesin (McNeal, 1988; Sharma et al., 2017). These cells secrete peptides such

as neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP), as well as biogenic amines such as 5-hydroxytryptamine (Martìn et al., 2000).

1.3.3 Physiology and Innervation of the Prostate Gland

1.3.3.1 Physiology of the Prostate Gland

Stimulation of α-adrenergic receptors on the prostatic smooth muscle cells of the stroma produces contraction and expulsion of prostatic secretions from the prostatic acini into the urethra through several orifices in the verumontanum during the emission phase of ejaculation (Clement and Giuliano, 2015). Prostatic secretions are a homogenous and serous fluid that contribute about 30 % of the total seminal fluid volume with the remaining 70 % produced by the seminal vesicles. These secretions are secreted by prostatic epithelial cells and stored in the prostatic acini until required during the emission phase of ejaculation. Prostatic fluid is a milky acidic (pH 6.6) fluid that is high in citric acid, zinc and choline, and includes several secretory proteins such as acid phosphatase, seminin, plasminogen activator and PSA as shown in **Table 1-1**.

Table 1-1. The constituents of human semen (Lawrentschuk and Perera, 2000).

Colour	White, opalescent	
Specific gravity	1.028	
pH	7.35 – 7.50	
Volume	3 mL	
Specific constituents of semen		
Gland/Site	**Volume and % in the ejaculate**	**Features**
Testis/Epididymis	0.15 mL (5 %)	Average approximately 80 million/mL spermatozoa
Seminal Vesical	1.5 – 2.0 mL (50 – 65 %)	Fructose (1.5 – 6.5 mg/mL), phosphorylcholine ergothioneine, ascorbic acid, flavins, prostaglandins, bicarbonate
Prostate	0.6 – 0.9 mL (20 – 30 %)	Prostate, spermine, citric acid, cholesterol, phospholipids, fibrinolysin, fibrinogenase, zinc acid, phosphatase, prostate specific antigen (PSA), enzyme amylase, kallikreins, semenogelin, calcium
Bulbourethral Glands	< 0.15 mL (< 5 %)	Clear mucus

These secretions are essential to provide spermatozoa with higher motility and longevity during and after ejaculation and may involve the liquification of coagulated ejaculate (Clement and Giuliano, 2015; Kumar and Majumder, 1995; Roberts, 1995). However, the exact role of each component is still unknown. The level of PSA in the blood is also an important indicator of any

abnormal growth of the prostate such as prostate cancer. Apart from prostatic secretions, in human prostate, there is also the occurrence of "diacytosis" which gives rise to secretion of prostasomes. Prostasomes are organelle-like structures rich in ATP and various enzymes (Aumüller, 1989).

1.3.3.2 Innervation of the Prostate Gland

As with other smooth muscle cells, the force of contraction in prostatic smooth muscle cells develops from MgATP-dependent cycling to form cross bridges between myosin thick filaments and actin thin filaments. These interactions are phosphorylated by myosin light chain (MLC) kinase regulated by the binding of cytosolic free Ca^{2+} to calmodulin (Abdel-Latif, 1991; Kamm and Stull, 1989). The contraction of the smooth muscle is affected by second messengers such as cyclic AMP (cAMP), cyclic GMP (cGMP), and diacylglycerol (DAG) that alter the concentration of cytoplasmic intracellular Ca^{2+} through activation of specific protein kinases (Kamm and Stull, 1989; Nishimura and van Breemen, 1989).

Binding of the extracellular first messengers such as neurotransmitters, hormones, chemokines, lipid mediators or drugs with a seven transmembrane-spanning G protein-coupled receptor (GPCR) generates a second messenger cyclic adenosine monophosphate (cAMP). This second messenger is catalysed from ATP via the enzyme adenylate cyclase (AC) and degraded by the enzyme phosphodiesterase (PDE) (Serezani et al., 2008). The adenylate cyclase activity is modulated by signal transducing guanosine-binding proteins (G proteins), G_q, G_s or G_i, for either stimulation or inhibition (Casey and Gilman, 1988). Excitation of Ca^{2+}-mobilizing receptors such as muscarinic cholinergic, α_1-adrenergic, histaminergic, peptidergic, or eicosanoids leads to the contraction of muscles; whereas relaxation of smooth muscle is stimulated by β-adrenergic receptors and mediated by cAMP (Abdel-Latif, 1991; Rasmussen et al., 1990). In the prostate, cAMP is also involved in the modulation of the metabolic actions of androgenic steroids (Singhal et al., 1971).

Electrical field stimulation-induced contractile responses of the guinea-pig prostate are sensitive to neurotoxins such as tetrodotoxin. This indicates that the contraction of the prostate by electrical field stimulation at these parameters is mediated via the release of neurotransmitter rather than direct depolarization of the prostatic smooth muscle (Haynes and Hill, 1997). The contraction in the prostate can be categorized into two types based on the pattern of the contracting wave: H-type, and P-type (Watanabe et al., 1988). H-type prostatic contraction is a

prominent tonic contraction; whilst P-type is a much weaker clonic contraction. H-type tonic contraction can be observed typically during hypogastric nerve stimulation that results in secretion of prostatic fluid. Conversely, P-type contraction is typical of pelvic nerve stimulation where no fluid excretion is involved (Watanabe et al., 1988).

The prostate and urethra are anatomically and embryonically intimately related. As such, they both have a large amount of smooth muscle tissue containing similar extracellular receptors. The autonomic nervous system has been shown to play a pivotal role in the growth, maturation, and secretory function of the prostate via α-adrenergic receptors and muscarinic receptors that can be stimulated by the release of neurotransmitters from the numerous nerve fibres found within the prostate gland (McVary et al., 1998). In the prostate, both sympathetic and parasympathetic fibres originating from the thoracolumbar and sacral spinal cord, respectively have been found to be present (Ali et al., 2004; Lepor and Shapiro, 1990). This motor and secretory innervation of the prostate gland is extended from the cavernous nerves in the pelvic plexus as well as from the hypogastric nerve (Kazunori Kihara et al., 1998; Liberman et al., 1989; Macht, 2017). Parasympathetic nerves which end in the glandular acini of the prostate are found to promote the excretion of prostatic fluid (Clement and Giuliano, 2015); whilst sympathetic fibres end in the prostatic smooth muscle cells of the stroma and capsule and control prostatic contractility (Clement and Giuliano, 2015). Apart from parasympathetic and sympathetic fibres, nonadrenergic and noncholinergic (NANC) fibres containing transmitters such as nitric oxide (NO), enkephalins, vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) are also present in the prostate (Clement and Giuliano, 2015). Nitric oxide synthase (NOS) has been found at different levels within peripheral and transition zones of the prostate. Nitrinergic innervation has been found to be less dense in BPH patients (Bloch et al., 1998). It has been found to relax noradrenaline-contracted prostatic stroma in the electrical stimulation. Inhibition of nitric oxide synthesis resulted in the decrease of the electrically stimulated relaxation (Hedlund et al., 1997). These studies suggested that NO may be important in the clinical management of BPH. Furthermore, NO has also been demonstrated to be involved in the regulation of prostatic secretion and excretion (Burnett et al., 1995).

1.3.3.2.1 Adrenergic innervation

Adrenergic receptors and the regulation of their expression by androgens are involved in the physiological function, and growth of the prostate (McVary et al., 1998). Adrenergic terminals have been located in the vas deferens as well as most of the accessory genital glands. Adrenergic

innervation is mostly restricted to the smooth musculature of these organs. These adrenergic fibres originate from peripheral adrenergic cell bodies located near, or even within the effector organs which emanate from a special type of "short" adrenergic neuron (Owman and Sjöstrand, 1965). Adrenergic innervation has been found to be less dense in human prostate following hypertrophy of the prostatic tissue, with advancing age (Baumgarten et al., 1968).

In the early 70s, it was demonstrated in a dynamic fashion using *in-vitro* isometric techniques that α-adrenergic receptors are present in great abundance in the ventral prostate of the rat. However, β-adrenergic responses were absent (Raz et al., 1973). Conversely, animal studies showed the presence of an adenylate cyclase system in rat ventral prostate which is sensitive to β-adrenergic stimulation (Tsang and Singhal, 1976). This β-adrenoceptor was found to mediate relaxation of prostatic smooth muscle and a decrease of this receptor in BPH may contribute to urethral obstruction (Tsujii et al., 1992). Animal studies have also demonstrated that the β_2-subtype of adrenergic receptor coupled to adenylate cyclase in the ventral prostate of the rat (Poyet et al., 1986) and guinea-pig (Haynes and Hill, 1997) modulates contraction of the prostate. In contrast, the β_1-subtype's involvement in the inhibition of prostate contraction does not appear to couple to adenylate cyclase but only to voltage-operated calcium channels (VOCCs) to inhibit contractile responses (Haynes and Hill, 1997). Radioligand binding studies further confirmed the abundance of β_1- and β_2- adrenergic receptors expressed in the porcine and human prostate in addition to α_1-adrenoceptors (Goepel et al., 1997). Among the three β-adrenoceptor subtypes, only selective β_3-adrenoceptor agonists do not inhibit the electrical field stimulation-induced contractile responses of the rat prostate which indicated that only the β_1- and β_2-subtypes are involved in the inhibition of prostatic smooth muscle contraction (Kalodimos and Ventura, 2001). However, this subtype specificity appears to be species dependent since β_3-adrenoceptor agonists are effective inhibitors of Ca^{2+} elevation elicited by phenylephrine in human cultured prostatic stromal cells (Haynes, 2007).

The innervation of the prostatic capsule, prostatic adenoma and the bladder neck by α-adrenergic receptors has been widely reported (Caine et al., 1975). In prostatic adenoma, there is moderate innervation of α-adrenergic receptors. Similarly, the adrenergic response is strong in the trigonal muscle of the bladder. Caine *et al.* (1975) were the first to suggest that in patients with enlarged prostates, the prostatic capsule had an increased response to sympathetic stimulation accompanied by increased tension within the prostate leading to an increase in pressure within the partly obstructed urethra causing sudden acute retention in the

patient. They suggested that BPH could be treated with α-adrenoceptor antagonists to relieve this obstruction.

In the late 70s, innervation by adrenergic neuronal inputs was demonstrated in the rat ventral prostate (Vaalasti and Hervonen, 1979). The adrenergic axons formed more intimate contacts with the smooth muscle cells with a synaptic gap of only 15-20 nm. This indicated that the adrenergic innervation played a major role in regulating the contractility of prostatic smooth muscle to expel prostatic fluid into the urethra prior to ejaculation (Burnett et al., 1995; Lau and Pennefather, 1998; Vaalasti and Hervonen, 1979; Wang et al., 1991).

Later studies showed two distinct adrenergic receptor subtypes to be present in the prostate. These were pharmacologically classified as: α_1 (Lepor and Shapiro, 1984) and α_2 (Shapiro and Lepor, 1986), based on their sensitivity to selective agonists and antagonists. The α_1-adrenoceptors are located post-junctionally on the smooth muscle cells and mediate prostatic contractility. They are predominantly localized to the prostatic stroma rather than glandular tissue. Furthermore, within the stromal tissue, they are found in smooth muscle cells but not fibroblasts or connective tissue (Chapple et al., 1989). The contractile response of the prostate smooth muscle is controlled primarily by the α_1-adrenoceptor subtype and is inhibited by the G protein inhibitor pertussis toxin or Ca^{2+} channel (L-type) blockers (Drescher et al., 1994; Haynes and Ventura, 2005; Hieble et al., 1985; Lepor, 2016; Preston and Haynes, 2003; Wang et al., 1991). In contrast, α_2-adrenoceptors are located pre-junctionally on post-synaptic adrenergic nerve terminals. Nerve mediated release of noradrenaline has been found to be up-regulated in symptomatic BPH as compared to asymptomatic BPH and normal prostate (Caine, 1986; Gup et al., 1990; Timmermans and van Zwieten, 1981)

Three subtypes of α_1 adrenoceptor have now been identified (α_{1A} incorporating the subtype previously known as α_{1C}, α_{1B} and α_{1D}) and three for α_2 adrenoceptor (α_{2A}, α_{2B}, α_{2C}) based on genetic origin, pharmacology, structure and functionality (Chapple et al., 1994; Hieble et al., 1995; Lepor, 2016; Lepor et al., 1993). The α_{1A} subtype in urogenital tissues is often putatively referred to as 'α_{1L}' due to its lower affinity for prazosin (Ford et al., 1997); however, in human prostate, it has moderately lower affinity for prazosin hence 'α_{1L}' hypothesis is still under debate (Michel and Vrydag, 2006; Muramatsu et al., 1994). The α_{1A} subtype is also predominantly found in urethra and prostate membranes of different animals including rats, rabbits, pigs, dogs, monkeys and humans (Testa et al., 1993). The α_{1A} subtype had been found to be primarily

localised in the human prostatic smooth muscle (Lepor et al., 1995; Lepor et al., 1994; Michel and Vrydag, 2006) where it mediates contraction (Forray et al., 1994). The same subtype is also observed in prostate adenomas (Lepor and Shapiro, 1984). α_{1B} and α_{1D} subtypes are generally not detected in binding assays (Goepel et al., 1997; Michel and Vrydag, 2006; Testa et al., 1993). Murata *et al*., (2000) found that the tension of vascular smooth muscle is predominantly mediated by the α_{1B} subtype (Murata et al., 2000).

The α_2-adrenoceptor subtype is a G_i-coupled receptor which located entirely pre-junctional at post-synaptic adrenergic nerves terminal (Bylund, 1992; Lanier et al., 1991). Competition radioligand-binding data revealed that α_{2A}-adrenoceptor was predominantly, if not exclusively, located in the human prostate (Goepel et al., 1997). Immunohistochemical staining studies revealed that α_{2A}-adrenoceptor label was predominantly detected in the prostatic epithelium of both young and aged rats (Slater et al., 2000). Although it has been reported to be present in prostatic epithelium instead of prostatic stroma, however, no functional studies on the post-junctional contractile function of prostatic α_2-adrenoceptor is available (Michel and Vrydag, 2006).

1.3.3.2.2 Cholinergic innervation

It has also been demonstrated that the prostate responded to acetylcholine with atropine-sensitive contractions which showed that cholinergic receptors are also present (Raz et al., 1973). Nevertheless, in the human and rat prostate, cholinergic nerve fibres are not as abundant as adrenergic nerve fibres. In the late 70s, non-adrenergic neuronal inputs were demonstrated in the rat ventral prostate (Vaalasti and Hervonen, 1979). Cholinergic axons were less abundant and located at a distance 100-200 nm from the smooth muscle cells (Burnett et al., 1995; Lau and Pennefather, 1998; Vaalasti and Hervonen, 1979; Wang et al., 1991). The cholinergic receptors are later found in several parts of the human prostate such as the anterior capsule, peripheral zone, proximal central prostate and distal central prostate (Crowe et al., 1991).

Muscarinic receptors are predominantly found in the epithelial cells of the human prostate rather than in stromal cells where adrenoceptors predominate (Michel and Vrydag, 2006). As mentioned earlier, cholinergic nerve fibres coursing through the glandular epithelium are suggested to be involved in the secretory function of the prostate. Even so, contractile responses of the human prostatic capsule are mediated by acetylcholine (McVary et al., 1998; Wang et al., 1991; Yazawa and Honda, 1993). Muscarinic acetylcholine receptors subtypes have been shown

to be differentially expressed in different tissues of the male reproductive tract and are mostly involved in modulating contraction of smooth muscle (Avellar et al., 2010). Nevertheless, autoradiography studies showed that muscarinic acetylcholine receptors were localized to the epithelial cells of the prostate gland which is consistent with the neurophysiological control of prostatic secretion (Lepor and Kuhar, 1984). Among all the muscarinic subtypes, M_3 receptors are the most abundant in rat prostate tissue and have been shown to modulate carbachol-induced contractions of the rat ventral prostate (Latifpour et al., 1991; Lau and Pennefather, 1998). In functional experiments, only M_3 muscarinic receptors were involved in the contractile response to carbachol in rat prostatic smooth muscle (Lau and Pennefather, 1998; Lepor and Kuhar, 1984).

The innervation of the prostatic capsule and the bladder neck by cholinergic receptors has been widely reported (Caine et al., 1975). Human BPH prostatic tissue, has high expression of muscarinic cholinergic receptors localized to the epithelial cells (Lepor and Kuhar, 1984). In contrast, the number of muscarinic receptors has been found to be significantly reduced in aged rats (Yazawa and Honda, 1993). Furthermore, in human prostatic adenoma, there are no cholinergic receptors found (Caine et al., 1975).

1.3.3.2.3 Non-adrenergic non-cholinergic innervation

Purinoceptors are present in human prostate (Fang et al., 1992; Longhurst et al., 1996) and smooth muscle contraction of the rat prostate is mediated by ATP released from nerve fibres by electrical field stimulation (Ventura et al., 2003). This mechanism is supported by immunohistochemical studies where P2X1 purinoceptors are predominantly found in the fibromuscular stroma of the rat prostate (Lee et al., 2000; Ventura et al., 2003). In double knockout α_{1A} (-/-)/P2X1 (-/-) male mice, total infertility is seen including a lack of secretions from the seminal vesicles, coagulating gland and prostate gland (White et al., 2013a). This suggests that α_{1A}-adrenoceptors and P2X1 purinoceptors are vital for prostatic contractility and the maintenance of normal male reproductive function.

In the guinea-pig prostate, contractile responses to phenylephrine were not inhibited by the P2X-purinoceptor antagonist suramin. This indicated that prostatic contractions mediated by α_1-adrenoceptors did not cause the release of ATP (Haynes and Hill, 1997). Lau *et al*., (1998) showed that suramin only managed to slightly reduce the field stimulation-induced contractile response of the guinea-pig prostatic smooth muscle (Lau et al., 1998). It was later shown that

electrical field stimulation induced contractile responses of the prostate are monophasic and slow or tonic in contrast to the biphasic contractile responses observed in the rat vas deferens where the fast component of the response was postulated as purinergic, and the slow component was noradrenergic (Ventura, 1998; Ventura et al., 2003). ATP and noradrenaline had been shown to act as cotransmitters in the rat and guinea-pig fibromuscular stroma of the prostate gland mainly at lower frequencies of electrical field stimulation (Buljubasich and Ventura, 2004; Ventura et al., 2003). In addition, Ventura *et al*., (2003) confirmed the presence of P2X1 purinoceptors in the rat prostate smooth muscle layers. In contrast, the nerve-mediated contraction in the mouse prostate is predominantly mediated by α_1-adrenoceptors and a cholinergic component with no purinergic innervation (White et al., 2010). Inhibition of the nerve mediated prostatic contractions by either ATP or adenosine can also be attenuated by the adenosine receptor antagonist, 8-phenyltheophylline indicating that modulation of prostatic contractions can also occur via stimulation of prejunctional A_1 adenosine receptors (Preston et al., 2000).

The prejunctional adenosine/P1 purinoceptors have been demonstrated to modulate the release of noradrenaline from postganglionic sympathetic nerves (Abbracchio and Burnstock, 1994). They had been shown to be present pre-synaptically where their activation contributed to the inhibition of excitatory neurotransmitters such as dopamine, GABA, glutamate, acetylcholine, serotonin, and noradrenaline (Preston et al., 2000; Williams, 2002). Studies have shown that A_1 and A_{2A} adenosine receptors possess modulatory effects on α_1-adrenoceptors to modulate contraction of the prostate. Activation of pre- and post-junctional A_1 receptors are thought to modulate the release of noradrenaline or smooth muscle tone, respectively, to inhibit electrical field stimulation-induced contractile responses of a variety of urogenital organs including the rat prostate (Preston et al., 2000), vas deferens of rats (Hourani et al., 1993; Kurz et al., 1993), guinea pigs (Haynes et al., 1998a) and mice (Kurz et al., 1993) and the cauda epididymis of the rat (Ventura and Pennefather, 1992) and guinea pig (Haynes et al., 1998a). Later, this was suggested to be due to stimulation of α_1-adrenoceptors which inhibit adenylate cyclase (Preston et al., 2004). Similarly, stimulation of post-junctional A_{2A} adenosine receptors inhibit the α_1-adrenoceptor mediated contractile responses of human prostatic stromal cells (Preston et al., 2004), and the cauda epididymis of the guinea-pig (Haynes et al., 1998b). Activation of pre-junctional adenosine receptors can also potentiate rat vas deferens contractions by enhancing the release of noradrenaline (Gonçalves and Queiroz, 1993). It was later shown that apart from modulating the release of noradrenaline, A_{2A} adenosine receptors

are also important in modulating the release of noradrenaline during nerve stimulation (Gray et al., 2008). It is noteworthy that adenosine receptors can be directly activated by adenosine or indirectly by adenosine hydrolysed from released ATP to cause inhibition of rat prostatic contractions.

Tyramine is a biogenic amine. Its receptor is categorized into the Class A GPCR family. It is an indirect acting sympathomimetic amine. It was first suggested to be involved in the storage and release of the adrenergic transmitter by Burn and Rand (Burn and Rand, 1958). The release of endogenous noradrenaline normally occurs via calcium-dependent, exocytotic mechanisms. However, release of noradrenaline by tyramine is neither calcium-dependent nor exocytotic. Indeed, tyramine causes the release of noradrenaline by displacing the transmitter from vesicular binding sites (Arnold et al., 1989; Gray et al., 2007; Langer, 1977). Tyramine has been suggested to be taken up into the nerve terminals via activation of reverse transport of NET to stimulate the release of catecholamines such as noradrenaline from peripheral sympathetic nerve endings (Mandela and Ordway, 2006; Tulane University, 2013). Ingested tyramine is broken down in the liver or GI tract by monoamine oxidase (MAO) to reduce its concentration in the cytoplasm by conversion into inactive metabolites (Trendelenburg, 1972). Tyramine has been reported to be present in saw palmetto ethanol extract and has been suggested to elicit contractile responses of rat prostate via indirect sympathomimetic effects (Cao et al., 2006; Chua et al., 2011).

Serotonin (5-HT) is a monoamine produced in the central nervous system (brain stem) (5 %) and gastrointestinal enterochromaffin cells (95 %) that plays an essential modulatory role in neurotransmission as well as numerous physiological functions and pathological conditions. These include vasoconstriction, gastrointestinal motility, modulation of mood and anxiety, as well as regulation of bone mass (Cui and Kaartinen, 2015; Gainetdinov et al., 2002). Serotonin is stored mainly in the central nervous system, platelets, with small amounts circulating in the peripheral system (Cui and Kaartinen, 2015). Serotonin itself is a receptor agonist at pre- and post-synaptic serotonergic receptors. 5-HT_1-like receptors have been suggested to be associated with inhibition of pre-junctional release of neurotransmitter, smooth muscle relaxation, contraction of certain vascular smooth muscles and tachycardia in the cat (Bradley et al., 1986; Saxena et al., 1998).

In the prostate, serotonin neurotransmitter is present and secreted by the prostatic neuroendocrine cells (Davis, 1987; Di Sant'Agnese, 1998; Kester et al., 2003). It is noteworthy that platelet aggregation in BPH patients were elevated resulting in increased platelet serotonin levels as well as plasma serotonin levels. It has also been associated with prostate cancer cell growth (Yazaki et al., 1987). Later studies have shown that 5-HT_{2A} and maybe 5-HT_{2C} receptors were present in the prostate and mediate the contraction of the prostate. Prostatic serotonin is also suggested to directly or indirectly contribute to rat prostatic adrenergic contractility (Kester et al., 2003; Killam et al., 1995; Langer, 1977). Of note, functional studies showed that in the presence of prazosin, serotonin at higher concentrations activates α_1-adrenoceptors in prostate tissue (Killam et al., 1995). 5-HT_{2A} and 5-HT_{2C} receptors are $G_{\alpha q/11}$-coupled involved in activation of phospholipase C producing inositol triphosphate (IP_3) and diacylglycerol (DAG) resulting in increased intracellular calcium (Roth et al., 1984; Roth et al., 1998). They also regulate $Na^+/K^+/Cl^-$ co-transport in fibroblasts (Mayer and Sanders-Bush, 1994). 5-HT_{2A} receptors have now been identified to mediate smooth muscle contraction both *in vivo* and *in vitro*. Nevertheless, there are also other 5-HT receptor subtypes involved in mediating relaxation effects on different smooth muscle (Feniuk, 1984; Feniuk et al., 1983; Humphrey, 1984).

Histamine is an endogenous biogenic amine involved in the physiological function of several organs such as lungs, skin, and gastrointestinal tract. It mediates physiological changes including neurotransmission, inflammation, smooth muscle contraction, dilatation of capillaries, chemotaxis, cytokine production, and secretion of gastric acid (Panula et al., 2015; Wouters et al., 2016). It has been first shown to be released from the digestive tract and bladder smooth muscle during contraction stimulated by acetylcholine, potassium chloride and pituitary extract (Ambache and Barsoum, 1939). Further, it has also been suggested to stimulate contraction of smooth muscle in gut and bronchi where the effect was able to be blocked by the antihistamine mepyramine (Black et al., 1972; Feldberg and Smith, 1954). Later, it has been demonstrated that the histamine receptors involved in smooth muscle contraction were designated as H_1-receptors (Ash et al., 1997). Evidence showed that histamine has a role in the contractile responses of the human, dog, and guinea-pig urethrovesical smooth muscle which are mediated via H_1-receptors (Khanna et al., 1977). Furthermore, H_1-receptors have been found to mediate contraction of guinea-pig vasa deferentia but not in mice and rats (Vohra, 1981). In addition, histamine has been reported to cause contraction of the human prostate by activating H_1-receptors. These contractions were abolished by the antihistamine diphenhydramine (Kester et al., 2003). The responses of histamine were demonstrated to be neurogenic and not direct as they were

abolished by tetrodotoxin (Kerr, 2006). Apart from human, histamine has also been reported to mediate prostatic smooth muscle contractions in both rabbit (Sudoh et al., 1997) and guinea-pig but not in the rat (Cohen and Drey, 1989). Interestingly, functional studies showed that histamine competitively inhibited α_1-adrenoceptors in human prostate (Kester et al., 2003). In the hyperplastic prostate, histamine has been reported to be present in mast cells and its concentrations increased as men aged due to BPH (Gupta, 1970; Kester et al., 2003). Apart from its role in prostatic smooth muscle contraction, activation of H_1-receptors has been found to couple to phosphoinositide hydrolysis and initiate Ca^{2+} mobilization that led to inhibition of prostate carcinoma DU-145 cell proliferation (Valencia et al., 2001).

Prostanoids consist of three main groups, namely prostaglandins, prostacyclins, and thromboxanes (Antonucci et al., 2007). These prostanoids are synthesised from arachidonic acid from cellular phospholipids by cyclooxygenase (COX) and other synthases which ultimately produced five major prostanoids, prostaglandins E_2 (PGE2), PGI_2, $PGF_{2\alpha}$, PGD_2 and thromboxane A_2 (TXA_2) (Smith, 1992). Prostaglandins (PGs), similar to other neurotransmitters, have both excitatory and inhibitory effects on smooth muscle (Bolton, 1979). It was first identified in the sperm and prostate glands of several species by two independent groups, Goldblatt and von Euler, and was then named as "prostaglandin" by von Euler in 1935 (Bergström et al., 1968; Bergström et al., 1959; Hinman, 1967). Later, the first two prostaglandins, PGE_1 (Bergström and Sjövall, 1960a) and $PGF_{1\alpha}$ (Bergström and Sjövall, 1960b) which were able to lower blood pressure and have prominent physiological activity on smooth muscle were successfully isolated from the prostate gland of sheep in 1957 by Bergström and co-workers. Functional studies have revealed that the blood pressure lowering property were only found with PGE; whereas smooth muscle stimulation effects were noticed with both PGE and PGF on the intestines of rabbit, rat, chicken and guinea-pig, uterus of guinea-pigs, and the iris muscle of the cow (Bergström et al., 1959). Additionally, PGE_2 has been demonstrated to cause concentration-dependent inhibition of field stimulation-induced contractile responses of rat prostate via EP_2 receptors which is consistent with studies by Coleman *et al.* (1994) that EP_2 receptors relaxed other nonvascular smooth muscle preparations (Coleman et al., 1994; Tokanovic et al., 2007; Tokanovic et al., 2010). Tokanovic and co-workers also further demonstrated that EP_3 and EP_4 receptors were not involved in the relaxation of urogenital smooth muscle (Tokanovic et al., 2010). Prostaglandins of the E series had been demonstrated to regulate the inhibition of noradrenaline release by restriction of calcium availability via EP_1 and EP_2 receptors (Hedqvist, 1970; 1976; Stjärne, 1973). In addition, PGE_2 has been suggested to act pre-junctionally to inhibit the contractions of rat

prostatic smooth muscle rather than post-junctionally as it did not inhibit contractions elicited by exogenously administered agonists such as noradrenaline and ATP (Tokanovic et al., 2010). Interestingly, $PGF_{2\alpha}$ has been found to dose-dependently contract the urogenital smooth muscle of rabbits including prostate, urinary bladder body (detrusor muscle), urinary bladder base (trigone), and urethra (Sudoh et al., 1997) but lacks inhibitory effects on field stimulation-induced contractions of rat prostate (Tokanovic et al., 2010). Recently, studies have suggested that by targeting COX/PGE_2/STAT3 signaling using metformin, prostate cancer epithelial-mesenchymal transition (EMT), as well as metastasis, are able to be inhibited (Tong et al., 2017).

1.4 PROSTATE DISEASE: BENIGN PROSTATIC HYPERPLASIA

1.4.1 Aetiology and Epidemiology

Benign prostatic hyperplasia (BPH) is a progressive condition characterised by the enlargement of the prostate in the periurethral and transition zone. This growth leads to the obstruction of the lower urinary tract and is an almost inevitable occurrence in ageing men (Briganti et al., 2009; Hudson et al., 2001). It is characterised by the unregulated proliferation of connective tissue, smooth muscle and glandular epithelium primarily within the transition zone of the prostate (Auffenberg et al., 2009). Often, men with histological BPH will never seek medical treatment until the condition is associated with lower urinary tract symptoms (LUTS) (Bachmann and Rosette, 2011). The obstruction of the urethra also gives rise to prostatism and eventually progresses into renal failure in very severe cases (Braeckman and Denis, 2017). In epidemiologic studies, the most common measures of LUTS used are the American Urological Association Symptom Index (the AUA-SI), International Prostate Symptom Score (IPSS) and urine flow rate measurement using uroflowmetry (normal urine flow (Qmax) is greater than 10 mL/sec) (Arianayagam et al., 2011). In order to establish a universal standard to determine the prevalence of clinical BPH, the IPSS has been recommended by the European Association of Urology (AUA) and the World Health Organisation (WHO) to assess suspected BPH and LUTS patients clinically to find the most suitable treatment (**Figure 1-14**). Patients with an IPSS score ≥ 8, peak flow rate < 15 mL/sec, and prostate volume > 20 cm3, are considered to have clinical BPH (Bachmann and Rosette, 2011; Patel and Parsons, 2014; van Venrooij et al., 1995).

BPH is the second most common cause of surgery in men older than 65 in the United States (Oesterling, 1995). In Australia, transurethral resections of the prostate (TURPs) are the most common form of prostate surgery for the treatment of severe BPH and there are over 25 000

performed annually which cost approximately AUD $90 million (Australian Health Technology Advisory Committee, 1994). In 1992, approximately 2,200,000 BPH patients were treated surgically and pharmacologically in Italy at a cost of more than USD $46 million dollars per year (Di Silverio et al., 1993). Despite the high burden of BPH on public health, the exact aetiology of BPH still remains largely unknown. Generally, potential aetiologies can be categorized into non-modifiable and modifiable risk factors. Non-modifiable risk factors include age and genetics; whereas modifiable risk factors include geography, alcohol consumption, steroid hormone, dietary fat, meat and milk consumption, diabetes, inflammation, metabolic syndrome, cardiovascular disease, low body-mass index, smoking, sexual dysfunction, and tuberculosis history (Araki et al., 1983; Bachmann and Rosette, 2011; Briganti et al., 2009; Chughtai et al., 2016; Ngai et al., 2017; Sidney et al., 1991; Thorpe and Neal, 2003). Of these many mechanisms that appear to be involved in the pathogenesis of BPH, aging and androgens represent the core mechanisms in in its development.

	Not at all	Less than 1 time in 5	Less than half the time	About half the time	More than half the time	Almost always	Score
Incomplete emptying: over the past month, how often have you had a sensation of not emptying your bladder completely after you finish urinating?	0	1	2	3	4	5	
Frequency: over the past month, how often have you had to urinate again less than two hours after you finished urinating?	0	1	2	3	4	5	
Intermittency: over the past month, how often have you found you stopped and started again several times when you urinated?	0	1	2	3	4	5	
Urgency: over the last month, how difficult have you found it to postpone urination?	0	1	2	3	4	5	
Weak stream: over the past month, how often have you had a weak urinary stream?	0	1	2	3	4	5	
Straining: over the past month, how often have you had to push or strain to begin urination?	0	1	2	3	4	5	
Nocturia	None	Once	Twice	Three times	Four times	⩾5 times	Score
Over the past month, how many times did you most typically get up to urinate from the time you went to bed until the time you got up in the morning?	0	1	2	3	4	5	
Total IPSS							
Quality of life (QOL) due to urinary symptoms	Delighted	Pleased	Mostly satisfied	Mixed: about equally satisfied and dissatisfied	Mostly dissatisfied	Unhappy	Terrible
If you were to spend the rest of your life with your urinary condition the way it is now, how would you feel about that?	0	1	2	3	4	5	6
Total IPSS (including QOL score)							
(0–7, mildly symptomatic; 8–19, moderately symptomatic; 20–35 severely symptomatic)							

Figure 1-14. The International Prostate Symptom Score (IPSS) (Woo et al., 2011).

The occurrence of BPH can first be detected in men aged between 31 and 40 years of age (8%). The pathological symptoms are detectable in about 50 % of the men between 51 and 60 years old. By the seventh decade (60-70 years), the prevalence of BPH has rapidly increased to >70 %. The prevalence of pathological BPH can reach as high as 90 % in the ninth decade (**Figure 1-15**) (Berry et al., 1984). In one of the longest and largest surveys of its kind, conducted in Olmsted

County, Minnesota, moderate to severe obstructive symptoms occurred in 13 % of men between 40-49 years old and 28 % of those 70 years old and above (Chute et al., 1993). Similarly, in a global survey, the prevalence of severe LUTS in men reported from four research centres in Auxerre (France), Boxmeer (the Netherlands), Birmingham (UK), and Seoul (Republic of Korea), was seen to increase with age from 5.4 % of men aged 60 – 69 to 7.5 % of men between age 70 to 79. Severity of LUTS had increased with increasing age across all four centres indicating that there is no marked cultural variation (Boyle et al., 2003). In the "Bettering the Evaluation and Care of Health" (BEACH) program from April 2009 to March 2011, around 228 000 cases of BPH were managed by general practitioners in Australia per year nationally. Two-thirds of these cases were men aged 65 years and above (Charles et al., 2011). Histological criteria for BPH has been shown to be very similar globally, regardless of ethnicity (**Figure 1-15**) (Bachmann and Rosette, 2011; Lepor, 2004).

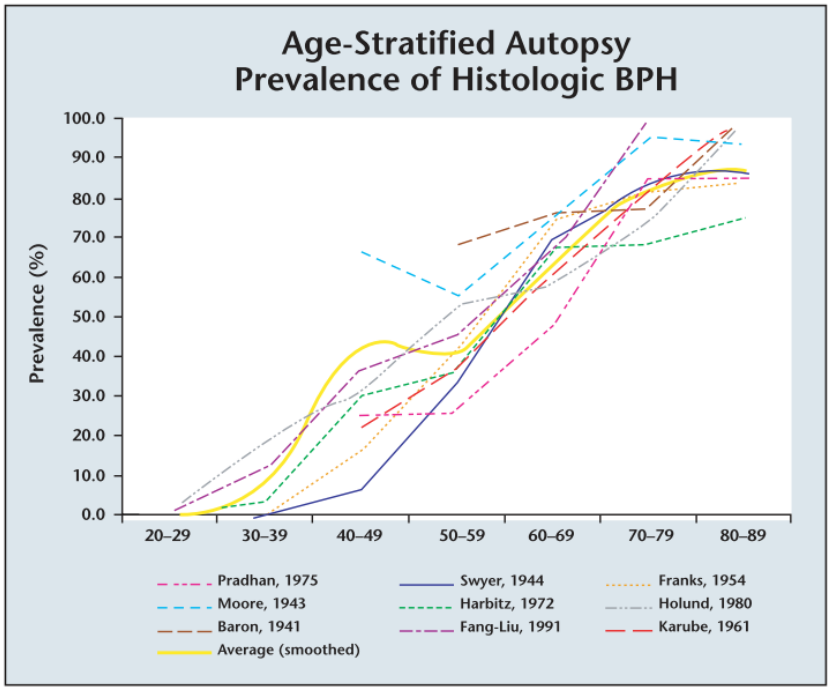


Figure 1-15. Age-stratified autopsy prevalence of benign prostatic hyperplasia (BPH) globally (Lepor, 2004).

Apart from aging, dihydrotestosterones (DHT) also play a pivotal role in the enlargement of the prostate as they are involved in the early development and normal growth of the prostate (Carson and Rittmaster, 2003). It is believed that androgens promote tissue growth by two methods: actively inhibiting cell death and promoting cell proliferation and differentiation in the prostate (McConnell, 1991; Naslund and Coffey, 1986). There are two isozymes of 5α-reductase that convert testosterone to DHT: Type 1 and Type 2 (Andersson et al., 1991). Type 2 is predominantly in the prostate whereas Type 1 is predominately in the liver and skin but also

present throughout the body where DHT is expressed (Iehle et al., 1995). Interestingly, males with 5α-reductase deficiency, do not develop BPH (Walsh et al., 1983). Moreover, males who have been castrated prior to puberty also fail to develop BPH (Carson and Rittmaster, 2003; Lepor, 2004; McConnell, 1992).

Animal studies have shown that castration has led to smaller soma size in certain pelvic autonomic neurons which is mostly due to the loss of circulating testosterone (Keast and Saunders, 1998). Nevertheless, prostatic volume does not directly correlate with DHT levels which suggests that there are other regulatory factors involved in the etiology of BPH (Carson and Rittmaster, 2003; McConnell, 1992; Walsh et al., 1983). Apart from androgens, estrogen also appears to be involved in the stimulation of BPH tissue stromal growth. Estrogen enhances adrenergic urethral smooth muscle contraction by increasing the number of α-adrenoceptors (Larsson et al., 1984). McConnell (1991) suggested that clinical symptoms of BPH are due to its unique anatomic structure apart from being age-related. The human prostate consists of a prostatic capsule which is believed to play a vital role in the development of prostatism. This is because the only other species which are known to spontaneously develop BPH are dogs and chimpanzees. However, the canine prostate lacks a capsule and they rarely develop symptoms of prostatism, whereas chimpanzees whose prostate is also contained within a prostatic capsule also develop prostatism symptoms associated with BPH (McConnell, 1991; Wilson, 1980). The presence of a prostatic capsule, is believed to restrict the expansion of prostatic tissue and forces it to grow inward thus impinging on the urethra. This mechanical mechanism restricts urethral distensibility which ultimately worsens the urinary obstruction (McConnell, 1991).

The prevalence of BPH varies between different countries and this phenomenon is postulated to be due to hormonal, genetic and nutritional factors (Jin et al., 1999). Epidemiological studies have shown that BPH risk is the lowest in Asian-Americans compared with other American ethnic groups most probably due to their diet which is rich in anti-phytoestrogens that are known to inhibit the growth of the prostate. Moreover, moderate alcohol consumption is also suggested to reduce BPH risk (Kang et al., 2004). Animal studies have shown that alcohol consumption suppresses testosterone and depletes testicular gonadotrophin receptors (Emanuele and Emanuele, 2001; Emanuele et al., 1998; Salonen et al., 1992). In addition, prostate volumes and prostate specific antigen (PSA) plasma concentrations have been demonstrated to be lower in Chinese men residing in China compared to Australian non-Chinese men and Chinese migrants

living in Australia which suggests that environmental factors are important in influencing prostate size (Jin et al., 1999).

It has forecast that the world male aging population will reach 1.4 billion by 2030 and 2.1 billion by 2050 (ASDReports, 2016). Consequently, medical treatments for urological disorders in the five largest countries in Europe (the UK, Germany, France, Italy and Spain) and Japan have been forecast to exceed $ 10 billion in 2017 (ASDReports, 2012). Due to the high prevalence of BPH and LUTS incidence and the costs of treatment, there is an urgent need to establish protective treatments that either limit the rate of prostate growth or decrease the consequences of obstruction on the bladder. Although BPH is not a life-threatening disease, it can cause detrimental effects on the quality of life to both the men suffering from the disease and their partners.

In aged rat prostate, density and distribution of prostatic α_1-adrenoceptors that directly mediate smooth muscle contraction has been found to be decreased thus causing reduction in the contractile response mediated by α_1-adrenoceptor agonists (Slater et al., 2000; Yono et al., 2006). The size of the prostate from 12-month-old α_{1A}-adrenoceptor knockout mice were smaller compared to those from wild type controls at the same age. This suggests that α_{1A}-adrenoceptors play an important role in sympathetically mediated prostate growth with age (White et al., 2013b). Interestingly, cholinergic innervation changes in the aged human prostate have not been observed (Hedlund et al., 1985). Nonetheless, $M_{1\text{-}3}$ muscarinic receptor mRNA as well as M_3 muscarinic receptor density decreases in aged rat prostate (Saito et al., 2010; Yazawa and Honda, 1993). In contrast, studies using antibodies have shown that M_2 muscarinic receptors increased with age in rat prostate indicating that cholinergic changes in aged prostate may be more subtype and species dependent (Slater et al., 2000). Mechanisms that inhibit prostate contractility have also been shown to change with age. The density of α_2-adrenoceptors, that inhibit the release of noradrenaline have been found to increase with age in human and rat prostates indicates increase in phosphorylation-activation of proteins and metabolic rate in aged rat epithelium (Chapple et al., 1989; Slater et al., 2000). Another receptor involved in prostatic smooth muscle relaxation, the β-adrenoceptor, was also found to decrease in expression in aged rat prostate (Michel and Vrydag, 2006; Slater et al., 2000). Nitrergic innervation as well as nitric oxide mediated relaxation has also been shown to be reduced in aged guinea-pig prostate (Dey et al., 2012). These studies explain how LUTS due to BPH develops in an age-dependent manner.

1.4.2 Pathophysiology

Based on available biological evidence, BPH appears to be etiologically unrelated to prostatic adenocarcinoma which typically originates in the peripheral zone. Even for prostatic adenocarcinoma originating in the transition zone, the evidence suggests a possible precursor role for BPH either directly or through atypical adenomatous hyperplasia appears weak (Guess, 2001). Nevertheless, there is a possible relationship between BPH and prostate cancer incidence and mortality based on epidemiological evidence, in vitro and autopsy studies (Chang et al., 2012; Orsted and Bojesen, 2013)

The natural history of BPH involves two phases, which are the pathological and clinical phases (Isaacs and Coffey, 1989). The pathological phase has two stages, which are termed microscopic and macroscopic BPH. Microscopic refers to the histologically detectable hyperplastic changes within the prostate that occur as early as the fourth decade of life. Macroscopic refers to the enlargement of the prostate that typically begins during the fifth or sixth decade of life. Nevertheless, not all patients with a macroscopically enlarged prostate will develop clinical BPH. Clinical phases occur only if the enlargement is substantial or becomes complicated by other disorders, such as prostatitis and develops symptomatic dysuria (Dharmananda, 2002; Isaacs and Coffey, 1989). Therefore, the important therapeutic approach is to prevent or reverse the progression of pathological BPH into clinical BPH (Isaacs and Coffey, 1989).

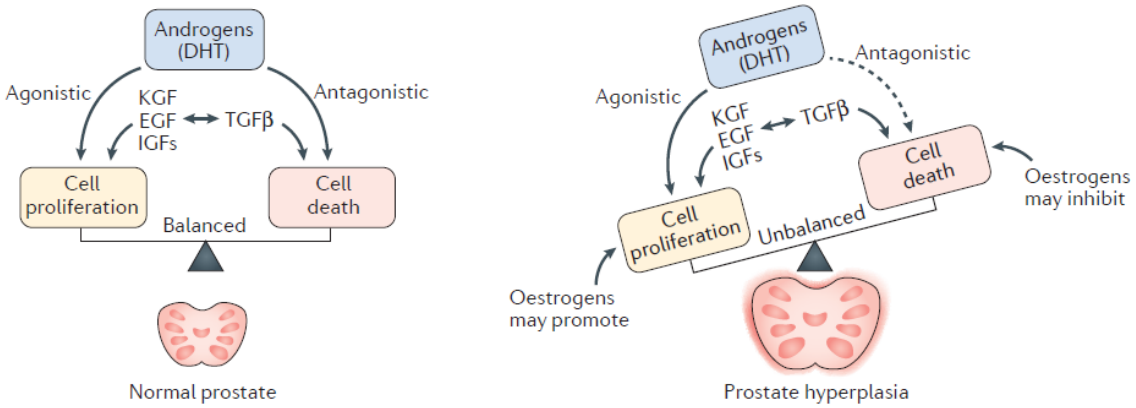


Figure 1-16. DHT is involved in the regulation of growth factors such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), and insulin-like growth factors (IGFs), that are important in cell proliferation as well as transforming growth factor-β (TGFβ) in apoptosis. Cellular homeostasis Imbalance between these regulation mechanisms in the prostate is thought to result in the development of benign prostatic hyperplasia (BPH). The right hand panel depicts the imbalance and development of BPH. The dashed arrow indicates less antagonistic action by DHT as compared to the left hand side with the bold arrow (Roehrborn, 2008).

Despite the fact that the pathophysiological mechanism of BPH is not fully understood, there are hypotheses suggesting that the progression of BPH is influenced by the disruption of the DHT-regulated homeostasis between cell proliferation and cell death (Carson and Rittmaster, 2003; Griffiths et al., 1997). In the cellular proliferation of the human prostate cells, growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), and insulin-like growth factors (IGFs) are modulated by DHT (**Figure 1-16**) (Griffiths et al., 1997).

Apart from those factors, DHT also negatively regulates transforming growth factor-*β* (TGF*β*) expression which is essential for apoptosis (**Figure 1-16**) (Kim et al., 1996; Niu et al., 2001). Therefore, imbalance of the interactions between growth factors and steroid hormones alters cell proliferation and cell death leading to the onset of BPH (**Figure 1-16**) (Chughtai et al., 2016).

1.4.3 Relationships with Lower Urinary Tract Symptoms (LUTS)

The male LUT comprises the bladder, urethra, and the prostate. BPH is the non-cancerous aberrant proliferation of both prostatic stroma and epithelial cells accompanied by lower urinary tract symptoms (LUTS), which can be bothersome and detrimental to quality of life. However, not all men with BPH will develop LUTS and vice versa (Kaplan, 2008). Lower urinary tract symptoms give rise to various prevalent and bothersome symptoms such as the urgent need to urinate frequently (day and night), urgency, nocturia, poor urine flow, intermittency, urinary incontinence, hesitancy and the sensation of incomplete bladder emptying (Abrams et al., 2002; Alleemudder and Ballaro, 2016; Woo et al., 2011).

Aging has been identified as the most significant risk factor for the development of BPH and associated LUTS (Cameron-Strange, 1996; Christopher Ho et al., 2011). In the Olmsted County study, it was suggested that men with moderate to severe LUTS, pathological BPH or impaired flow rates have a greater possibility of developing urinary retention or requiring prostatectomy (Chute et al., 1993). Acute urinary retention is a very painful condition requiring approximately 20 % of men to undergo prostatectomy in the UK alone (Thorpe and Neal, 2003). Although Asian men are generally believed to have good prostate health, a study done amongst nine Asian countries (i.e. Japan, China, Korea, Taiwan, Philippines, Thailand, Singapore, Pakistan, India) showed that there is an age-dependent trend toward increasing prevalence of LUTS from 18%, 29%, 40%, and 56% in the age groups of 40-49, 50-59, 60-69, and 70-79 years, respectively. In 2008, an estimated 45.2 % of the male worldwide population (4.3 billion) was affected by LUTS and this percentage was anticipated to increase to 45.8 % by 2018. The number of individuals

affected by LUTS is greatest in Asia and this is expected to increase very rapidly between 2008 and 2018 by 19.7 %. Similar patterns are expected in Europe, Africa, North America, and South America (Irwin et al., 2011). The prevalence of metabolic syndrome (MetS) in Asian men with BPH is also expected to rise from 26.7 % to 55.4 % (Ngai et al., 2017). As for Australian men, LUTS due to BPH is a very common condition with the corresponding figures for prevalence being less than 20%, 48% and 70% among men aged under 45 years, 65-79 years and 80 years and above, respectively (Homma et al., 1997; Parsons et al., 2008; Pinnock and Marshall, 1997; Woo et al., 2011).

1.4.4 Treatments and Complications

Diagnosis of BPH is usually carried out via physical examination by a physician and self-assessment by the patient to determine a score for their urinary symptom severity (**Figure 1-14**). PSA, also called kallikrein III (Bachmann and Rosette, 2011), is a glycoprotein peptidase secreted by the tall columnar luminal cells of the prostate epithelium and normally present in the blood in low levels but it increases as a man gets older. The physiological function of PSA is to liquefy the semen to aid sperm motility. As serum PSA levels are androgen-dependent, they are normally elevated by increases in testosterone or DHT often associated with the occurrence of prostate diseases such as BPH, prostatitis and prostate cancer (Grummet, 2015; Schalken, 2004).

PSA levels are used as a guideline to determine prostate volume, however, there is variability in PSA levels with respect to prostate volume (Nichols et al., 2012). Collectively, there are four types of treatments used to ameliorate LUTS associated with BPH depending on disease severity, impact on quality of life, patient preference, presence of complications and fitness for surgery. The possible treatments are: active surveillance (watchful waiting), pharmacotherapy, minimally invasive surgical therapies (MISTs) and radical surgery (Woo et al., 2011). Despite the proven effectiveness of these treatments, many patients still prefer using extracts from plants or herbs for the relief of symptoms. In today's modern world, phytotherapy as well as synthetic pharmacological agents (i.e. α-adrenoceptor antagonists, phosphodiesterase-5 inhibitors, 5α-reductase inhibitors) play a pivotal role in the management of BPH, especially in Europe and the Far East (Buck, 1996; Lawrentschuk and Perera, 2000).

Due to the plethora of therapies available, urologists, patients, insurers and government regulators have a hard time determining which treatments are the most suitable for the modern management of BPH. Recently, a collaboration between the American Urological Association

(AUA) and a multidisciplinary expert panel has developed a symptom index for BPH based on studies conducted involving 210 BPH patients and 108 control subjects. They claim that this AUA symptom index is clinically sensible, reliable, valid and responsive in practice (Barry et al., 2017) (**Figure 1-17**).

Question	Not at All	Less Than 1 Time in 5	Less Than Half the Time	About Half the Time	More Than Half the Time	Almost Always
1. During the last month or so, how often have you had a sensation of not emptying your bladder completely after you finished urinating?	0	1	2	3	4	5
2. During the last month or so, how often have you had to urinate again less than 2 hours after you finished urinating?	0	1	2	3	4	5
3. During the last month or so, how often have you found you stopped and started again several times when you urinated?	0	1	2	3	4	5
4. During the last month or so, how often have you found it difficult to postpone urination?	0	1	2	3	4	5
5. During the last month or so, how often have you had a weak urinary stream?	0	1	2	3	4	5
6. During the last month or so, how often have you had to push or strain to begin urination?	0	1	2	3	4	5
7. During the last month, how many times did you most typically get up to urinate from the time you went to bed at night until the time you got up in the morning?	None	1 Time	2 Times	3 Times	4 Times	5 or More times
	0	1	2	3	4	5

AUA symptom score = sum of questions 1 to 7.

Figure 1-17. American Urological Association (AUA) symptom index (Barry et al., 2017).

1.4.4.1 Pharmacotherapy

Urinary obstruction in men with BPH is caused by both mechanical/static and dynamic components (Caine, 1986). The static/mechanical component is due to the anatomical obstruction caused by enlargement of the prostate constricting the prostatic urethra and bladder outlet. In ageing men, there is an increase in prostate weight due to chronic exposure to testosterone which is converted to DHT within the prostate by the 5α-reductase enzyme. DHT has 10 times more affinity for androgen receptors compared to testosterone and is the greatest contributor to the static component of BPH (Thiyagarajan et al., 2002). Apart from its role in static obstruction, testosterone also increases sensitivity of the prostatic tissue to α-adrenoceptor mediated contractile responses leading to LUTS in BPH patients (Thiyagarajan et al., 2002). Dynamic obstruction of the urethra is due to an increase in force of prostatic smooth muscle, generated by increased sympathetic nervous system activity (Caine, 1986).

Generally, the hyperplastic volume consists of approximately 80 % to 20 % stromal to epithelial cells, respectively. The tone of the prostatic smooth muscle is mediated by noradrenaline released from adrenergic nerves to stimulate post-junctional α_1-adrenoceptors located on the prostatic smooth muscle (Lepor, 2016; Wang et al., 1991). Therefore, two types of drugs have been used to treat BPH targeting these two distinct components: (1) α-adrenoceptor antagonists, and (2) 5α-reductase inhibitors (hormone therapy) (**Table 1-2**) (Cameron-Strange, 1996; Lepor,

2016). To a lesser extent, several other drugs such as antimuscarinic/anticholinergic drugs, beta-adrenoceptor agonists, estrogen suppressors, and phosphodiesterase (PDE) 5 inhibitors have also been used to ameliorate LUTS associated with BPH in men (Boehm et al., 1998; Lepor, 2016) (**Table 1-2**).

Table 1-2. Summary of the drugs commonly used to treat lower urinary tract symptoms (Cameron-Strange, 1996).

Drug class	Site of action	Mechanism of action	Effects	Indication
Anticholinergics e.g. propantheline (Pro-Banthine)	Detrusor	Reduces detrusor contractility	Reduced flow; urinary retention	Detrusor instability
Alpha-adrenoceptor antagonists, e.g. prazosin (Minipress)	Bladder neck	Relaxes bladder neck	Improved flow; reduced obstructive symptoms	Bladder neck obstruction
Hormones e.g. finasteride (Proscar)	Prostate	Reduces prostate volume	Improved flow; reduced obstructive symptoms	Prostatic outlet obstruction

1.4.4.1.1 α-adrenoceptor antagonists

This group of drugs is used to relax the bladder neck as well as the smooth muscle of the prostate. They block the postjunctional adrenoceptor activated by noradrenaline thus relieving pressure off the lower urinary tract caused by the dynamic component of BPH without affecting the detrusor muscle of the bladder wall (**Figure 1-18**) (Meenakshi et al., 2017). α-adrenoceptor antagonists were the most commonly prescribed drugs (about 20 % in 2010) for men with BPH or LUTS (Charnow, 2013). The first α-adrenoceptor antagonist to be introduced was dibenamine in 1947 (Nickerson and Goodman, 1947). Later, the first clinical study of prostate medication therapy was performed using phenoxybenzamine, a nonselective α-adrenoceptor antagonist, which was initially used as an antihypertensive agent before being used to treat bladder obstruction caused by BPH (Caine et al., 1978). There are several α_1-adrenoceptor selective antagonists that have since been developed for the treatment of BPH worldwide including several quinazolines (prazosin, alfuzosin, doxazosin, bunazosin and terazosin), an aralkylamine (tamsulosin) and an indoleamine (indoramin) (Hieble, 2004). Selective for α_{1A}-adrenoceptors mean that they have less cardiovascular side effects than those non-selective α blockers. Tamsulosin was the first approved third-generation agent introduced in 1996 and is now the most common α_{1A}-adrenoceptor antagonist prescribed (Cameron-Strange, 1996; Charnow, 2013; Lepor, 1993; Sato et al., 2001). There are some common side effects caused by α-adrenoceptor

antagonists such as hypotension, asthenia, decreased libido, amblyopia, dizziness, tachycardia, blocked nose, and retrograde ejaculation or anejaculation (Caine, 1986; Lepor, 2016). Another more recently FDA-approved third-generation agent, silodosin, is a unique α_{1A} subtype selective adrenoceptor antagonist which has a greater therapeutic effect on LUTS in patients with BPH with less cardiovascular adverse effects (Cantrell et al., 2010; Lepor and Hill, 2010). However, silodosin has a higher incidence of adverse effects on ejaculation. Nevertheless, α-adrenoceptor antagonists remain as the first-line treatment of LUTS caused by BPH as the benefits on urinary symptoms outweigh the risks of side effects due to their use.

1.4.4.1.2 5α-reductase inhibitors

5α-reductase inhibitors are hormonal therapy drugs that are used to shrink the enlarged prostate. Prostate volume can usually be reduced by at least 30 % over a period of two to six months and subsequently improves the flow of urine through the previously obstructed urethra. Dutasteride (Type 1 and Type 2 5α-reductase inhibitor) and finasteride (selective Type 2 inhibitor) are competitive inhibitors of the 5α-reductase enzyme are commonly used to reduce levels of DHT to reduce prostate volume (**Figure 1-18**) (Cameron-Strange, 1996; Carson and Rittmaster, 2003; Lepor, 2004; Roehrborn, 2011).

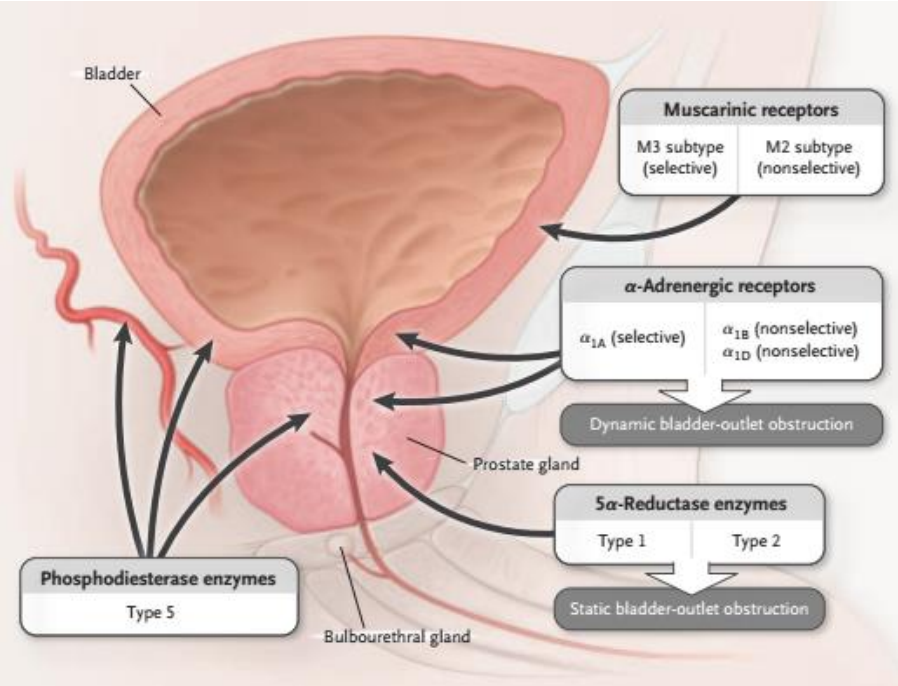


Figure 1-18. Sites of action of drugs acting at adrenergic, cholinergic, and nonadrenergic noncholinergic mechanisms to treat lower urinary tract symptoms (LUTS). Pharmacological treatments use selective drugs targeting receptors or enzymes that are predominantly localized to the bladder or prostate to treat LUTS (Sarma and Wei, 2012).

However, the 5α-reductase inhibitors are associated with several side effects including: loss of libido, erectile dysfunction and ejaculatory disorders (Braeckman and Denis, 2017; Roehrborn, 2011). They also have a delayed onset of symptom relief of up to 6 months and may affect PSA levels masking the early detection of prostate cancer. Both of these are disadvantages which also affect its use (Gleave M. et al., 2006; Thomson, 2005). Due to distinct but complementary mechanisms of 5α-reductase inhibitors and α-adrenoceptor antagonists, these two therapies are often combined to produce their beneficial effects on the static and dynamic components in BPH/LUTS patients diagnosed with enlarged prostate glands with symptoms of bladder outlet obstruction (Meenakshi et al., 2017). The Medical Therapy of Prostatic Symptoms (MTOPS) Study is the first study performed to examine the safety of medical therapy for BPH and its clinical progression. McConnell and colleagues (2003) have shown that combination treatment with both doxazosin and finasteride was safe and had significantly reduced the risk of clinical progression for BPH (McConnell et al., 2003). In addition, combination therapy with 5α-reductase inhibitors and α-adrenoceptor antagonists demonstrated a 25 % decrease in prostate cancer incidence (Fullhase and Schneider, 2016).

1.4.4.1.3 Anticholinergics

Overactive bladder (OAB) symptoms are very common among BPH patients. OAB symptoms are characterized by involuntary detrusor contractions normally caused by detrusor overactivity (Bachmann and Rosette, 2011; Kaplan, 2006). Initially, it was thought that anticholinergic drugs would potentially worsen the LUTS associated with BPH as the bladder is rich in cholinergic neurons, that release acetylcholine on to muscarinic receptors that mediate bladder contraction, that might cause worsening of postvoid residual urine volumes or acute urinary retention (Bachmann and Rosette, 2011; Barry and Roehrborn, 1997). However, the antimuscarinic drug, tolterodine, has been shown to not worsen the postvoid residual volume or urinary retention in men with OAB (Appell, 2006). In Australia, there are five types of antimuscarinic drugs have been approved for the treatment of OAB: oxybutynin, tolterodine, propantheline, solifenacin and darifenacin (Woo et al., 2011). Darifenacin and solifenacin are the selective M_3 muscarinic receptor antagonists (**Figure 1-18**) which primarily target the receptors in the bladder detrusor smooth muscle (Andersson, 2004; Andersson and Michel, 2011; Andersson and Sjögren, 1982; Woo et al., 2011).

In a combination BPH therapy study using both tamsulosin and tolterodine, there was significant increase in bladder capacity, and no patients experienced acute urinary retention

(Athanasopoulos et al., 2003). Some of the typical side effects associated with muscarinic receptor antagonists experienced by patients included dry mouth, dry eyes, and constipation (Sarma and Wei, 2012). Moreover, in the absence or presence of bladder outlet obstruction (BOO), it was shown to be well-tolerated in the treatment of LUTS including those who had not responded to α-adrenoceptor antagonist treatment (Kaplan, 2006).

1.4.4.1.4 Phosphodiesterase (PDE) 5 inhibitors

Phosphodiesterase (PDE) 5 has been found in the male genitourinary tissues including bladder detrusor, transition zone of prostatic tissue, and vascular smooth muscle cells associated with the urinary tract and erectile tissue (**Figure 1-18**) (Andersson et al., 2011; Andersson and Michel, 2011). PDE 5 inhibitors have been predominantly used in the treatment of men with erectile dysfunction. It is an isoenzyme that specifically hydrolyses cyclic nucleotides such as cGMP. cGMP is an intracellular mediator of smooth muscle relaxation that is inactivated by conversion to 5' GMP by PDEs (Mostafa, 2008; Rybalkin et al., 2003). Clinical studies showed that erectile dysfunction and LUTS caused by BPH shared a common pathophysiology (Mostafa, 2008). The PDE5 inhibitors used to treat LUTS due to BPH are the same as those used to treat erectile dysfunction and include sildenafil, tadalafil and vardenafil (Miller, 2013). These inhibitors have been shown to have efficacy in treating LUTS secondary to BPH. Some of the adverse effects are rhinitis, headache, flushing, and gastrointestinal disorders (Miller, 2013). α-adrenergically mediated contraction of human prostate strips in organ bath studies have been shown to be inhibited by PDE5 inhibitors (ÜCkert et al., 2001; Ückert and Oelke, 2011; Ückert et al., 2008). Furthermore, combination therapy with both α-adrenoceptor antagonists and PDE5 inhibitors for the treatment of LUTS and erectile dysfunction (ED), respectively has significantly improved the maximum urinary flow rate as compared to α-adrenoceptor antagonists alone (Gacci et al., 2016; Yan et al., 2014). To date, tadalafil is the most common FDA-approved PDE5 inhibitor used in the treatment of both ED and LUTS secondary to BPH (Warde, 2011).

1.4.4.2 Surgery

Pharmacotherapies are usually only effective to treat mild to moderate BPH symptoms. For severe or complicated BPH, invasive surgeries remain the gold standard. Generally, there are three main groups of surgery options available for BPH patients: compressing the prostate tissue, debulking of the prostate, and removal of the entire prostate or prostatectomy (Chughtai et al., 2016). Compression involves compressing the prostate laterally using a special device to widen the urethral channel. This is a relatively new and non-ablative approach that was approved in

2013 by the US FDA. The name of the treatment is the UroLift® system (Chughtai et al., 2016). Prostate debulking is a surgical technique that uses an endoscopic approach to remove some of the prostate that obstructs the urethral outlet (Chughtai et al., 2016). Transurethral resection of the prostate (TURP) is the most common form of this surgery that has been in use since 1935. As it is a difficult operation requiring highly experienced surgeons, some of the complications associated with TURP are continued infection and bleeding from the prostatic urethra following surgery (Beckmann, 1957). In some of the patients, erectile dysfunction may occur and retrograde ejaculation is very common. However, it has a very low mortality rate of only around 0.2 – 0.4 % (Cameron-Strange, 1996; Thorpe and Neal, 2003). Apart from TURP, several other surgeries including bipolar diathermy (Gyrus), laser and transurethral microwave therapy (TUMT) are also available (Chughtai et al., 2016). Bladder neck incision (BNI) is another surgery option for BPH patients with minor urethral or bladder neck obstruction. The risk and side effects are minimal as compared to TURP. If the prostate is too large to be removed using transurethral procedures, open prostatectomy or adenectomy is an option. However, it is not popular as the morbidity and hospitalization associated is greater than with TURP (Cameron-Strange, 1996). Moreover, open prostatectomy is almost certain to result in erectile dysfunction as the nerves controlling diameter of the arteries supplying the erectile tissue are severed during the procedure. There are several other more refined but less commonly used treatments for BPH including transurethral needle ablation (TUNA), cryotherapy, prostate hyperthermia, and focussed ultrasound ablation (Cameron-Strange, 1996).

1.4.4.3 Phytotherapy

In European countries, such as Germany and Austria, phytotherapy is considered as first line treatment for BPH. Herbal-based products in these countries comprise 90 % of the total treatments for BPH and the majority of them contain Saw Palmetto extract (Goepel et al., 1999; Thiyagarajan, 2002). Several studies have shown that herbal supplements may have beneficial effects on BPH, such as *Phellodendron amurense* (Xu and Ventura, 2010), *Hypoxis rooperi* (Braeckman and Denis, 2017), *Secale cereal* (Rye) (Braeckman and Denis, 2017), *Urtica dioica* (Stinging Nettle) (Braeckman and Denis, 2017), *Cucurbita pepo* (pumpkin seeds) (Braeckman and Denis, 2017), *Trifolium pratense* (Red Clover) (Brandli et al., 2010), Saw Palmetto (*Serenoa repens*) (Tacklind et al., 2012; Wilt et al., 2011), *Cucurbita peponis* (Dvorkin and Song, 2002), and *Pygeum africanum* (Strong, 2004; Wilt et al., 2011) (**Table 1-3**). Saw Palmetto has been extensively studied and is the most popular plant that is used to treat BPH/LUTS throughout the world. It has been found to be a potent 5α-reductase inhibitor in *in vitro* assays (Iehle et al.,

1995). In contrast, in a double-blind clinical trial, there was no significant difference between Saw Palmetto and placebo groups in the change in American Urological Association Symptom Index (AUASI) scores, peak urinary flow rates, prostate volume, residual volume after voiding, quality of life or PSA levels (Bent et al., 2006). In an *in vitro* rat prostate study previously conducted in our lab, Saw Palmetto was shown to contain an amphetamine or tyramine like substance which is likely to worsen BPH symptoms when used acutely and has clinical implications in terms of probable drug interactions (Cao et al., 2006). In a subsequent study, it was confirmed that Saw Palmetto indeed contained tyramine which induces release of noradrenaline from sympathetic neurons and causes indirect α_1-adrenoceptor mediated contractions (Chua et al., 2011).

Table 1-3. Phytotherapeutic agents commonly used in the treatment of benign prostatic hyperplasia (BPH) (Lawrentschuk and Perera, 2000).

Plant extract	Proposed pharmacological effect
Saw Palmetto: fruit (*Serenoa repens*)	Antiandrogenic, anti-inflammatory
African plum: bark (*Pygeum africanum*)	Antiandrogenic, potential growth factor manipulation, anti-inflammatory
Pumpkin: seed (*Cucurbita pepo*)	Active compounds: phytosterols
Cernilton: pollen (Secale cereal, Rye)	α-adrenoceptor antagonist
South African star grass: root (*Hypoxis rooperi*)	Antiandrogenic, effects in detrusor function
Stinging nettle: root	Steroid hormone: reducing prostate growth
Opuntia: flower (Cactus)	Unknown
Pinus: flower (Pine)	Unknown

1.5 RATS AS AN ANIMAL MODEL FOR PROSTATE STUDIES

The rat, mouse, and dog are the most common animal models used in the study of prostatic morphology, while Wistar and Sprague-Dawley rats are now the most widely used and preferable laboratory animals worldwide (Sengupta, 2013). Anatomically, the rodent prostate is slightly different compared to human as it is not merged into one compact anatomical structure. Instead, rat prostate is divided into four distinct lobular structures: (1) anterior or coagulating lobe, (2) dorsal lobe, (3) ventral lobe, and (4) lateral lobe (Hayashi et al., 1991; Jesik et al., 1982; Oliveira et al., 2016; Price, 1936; Sugimura et al., 1986). The rodent prostate exists as pairs on both left and right sides inferior to the bladder (Aaron et al., 2016). The lateral and dorsal lobes resemble each other more as compared to the ventral lobe (**Figure 1-19**). In prostate research,

despite gross anatomical differences between rodents and humans, the prostate gland of the rat has been used extensively as a suitable animal model for studies due to many similarities in its pharmacology and histochemistry with human prostate (Price, 1936).

Adult male rats have been used in studies of the stromal tissue of the prostate gland which reveal that smooth muscle cells and fibroblasts are the two most prominent cell types. Other components in the prostatic stroma include interstitial cells, collagenous fibrils, mast cells, macrophages, undifferentiated cells of low electron density as well as small blood vessels and unmyelinated nerves (Flickinger, 1972). A comparative study of the fine structure of the rat prostate complex revealed the apparent differences between epithelial cells of the different prostate lobes. These epithelial cells have specific ultrastructural characteristics of their own which morphologically distinguish them from other lobes despite having many common features (Dahl et al., 1973). Studies carried out to investigate regional variation in morphological and functional activities of the epithelial cells lining the rat prostatic ductal system showed that the ductal system of the rat can be divided into three segments designated as proximal, intermediate, and distal.

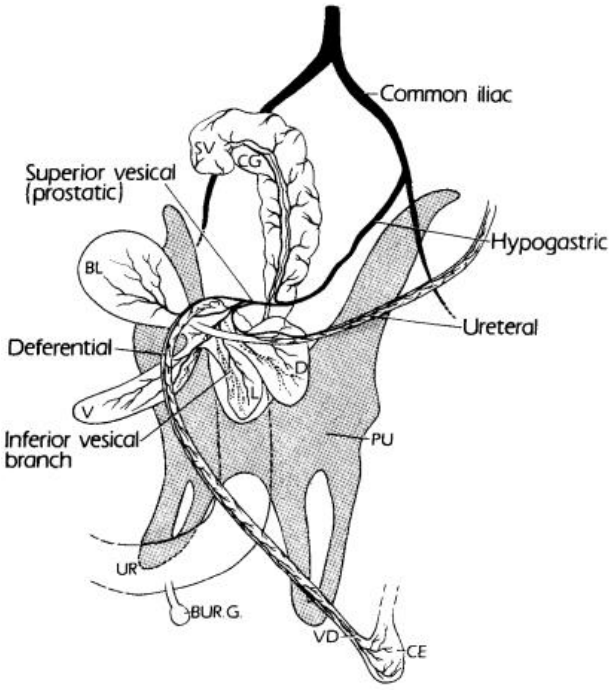


Figure 1-19. Diagram depicting the rat prostatic lobes. V: ventral lobe, prostate; L: lateral lobe, prostate; D: dorsal lobe, prostate; BL: urinary bladder; BUR.G.: bulbourethral gland; CE: cauda epididymis; CG: coagulating or anterior prostate gland; PU: pubis; SV: seminal vesicle; VD: vas deferens; (Jesik et al., 1982)

Noradrenergic nerves are present in the fibromuscular stroma of the prostate in all species including rats, where contractility of the rat prostatic smooth muscle tone as in man is mediated

via release of noradrenaline from these sympathetic nerves acting on α_1-adrenoceptors (Gray and Ventura, 2005; Lau et al., 1998; Pennefather et al., 1999). Cholinergic nerves are also present in both the fibromuscular stroma and epithelial cells in humans (Dunzendorfer et al., 1976), rat, and guinea-pig prostates (Lau et al., 1998). ATP receptors have also been reported in the rat and mouse prostate and mediate nerve-induced contractions of the fibromuscular stroma (Gray and Ventura, 2005; Lee et al., 2000; Ventura et al., 2003). Neurotransmitters and neuromodulators that have been found in human prostate including vasoactive intestinal polypeptide (VIP), neuropeptide Y, calcitonin gene-related peptide (CGRP), and the tachykinins neurokinin A and substance P, have also been reported in rodents (Carvalho et al., 1986; Pennefather et al., 2000). Based on numerous studies on rats, similar patterns of prostate morphology and physiology exist between the rat and human prostate. This indicates that the rat prostate is a suitable model to be used in this study. Therefore, the animal to be used in this study is the Sprague Dawley (SD) rat as it has been used extensively in our laboratory studies and data collected before this can serve as a good comparison for this study.

1.6 RAT MODEL FOR EXPERIMENTAL DESIGN

The Sprague Dawley® (SD) strain of rats is the most widely used rat model across a span of biomedical research disciplines including reproductive, nutritional, toxicological, pharmacological and behavioural research (Erika and Carly, 2008; Janvier, 2017). The SD rat was first developed at Sprague Dawley farms in Madison, Wisconsin, U.S.A. in 1925 (ENVIGO, 2016; Marcia et al., 2015; TACONIC, 2016). It is an outbred strain, bred by crossing the multipurpose breed of albino rat, Wistar to hybrids of laboratory derived and wild stocks of rat (Janvier, 2017; Marcia et al., 2015). Their physical characteristics that differentiate them from Wistar rats is their elongated head (**Figure 1-20**) and a tail which is longer in proportion to their body (Janvier, 2017). Average body weight for adult SD rats is 250-300 g for female and 450-500 g for male. The SD rat is a docile breed and can be handled easily which makes it a popular strain for use in biomedical research (Erika and Carly, 2008).

Figure 1-20. Sprague Dawley® (SD) rat.

In this study, seven to eight weeks old SD rats were used. Rats at the age of 25 days (3-4 weeks) have smaller prostatic acinous diameter and shorter epithelium, however, the histological structure of the prostate is similar of that with an adult male rat (Price, 1936). Rats will reach sexual maturity from 40 to 60 days of age and start mating from 8-10 weeks (Quinn, 2005; Sengupta, 2013). The two lateral prostate lobes from an 8-week-old SD rat is depicted in **Figure 1-21**. Serum testosterone and luteinizing hormone (LH) concentrations are decreased in aged male rats. Furthermore, aged male rats had a reduced response to human chorionic gonadotropin (hCG) or luteinizing hormone-releasing hormone (LHRN) as compared to young adult male rats (Miller, 1983).

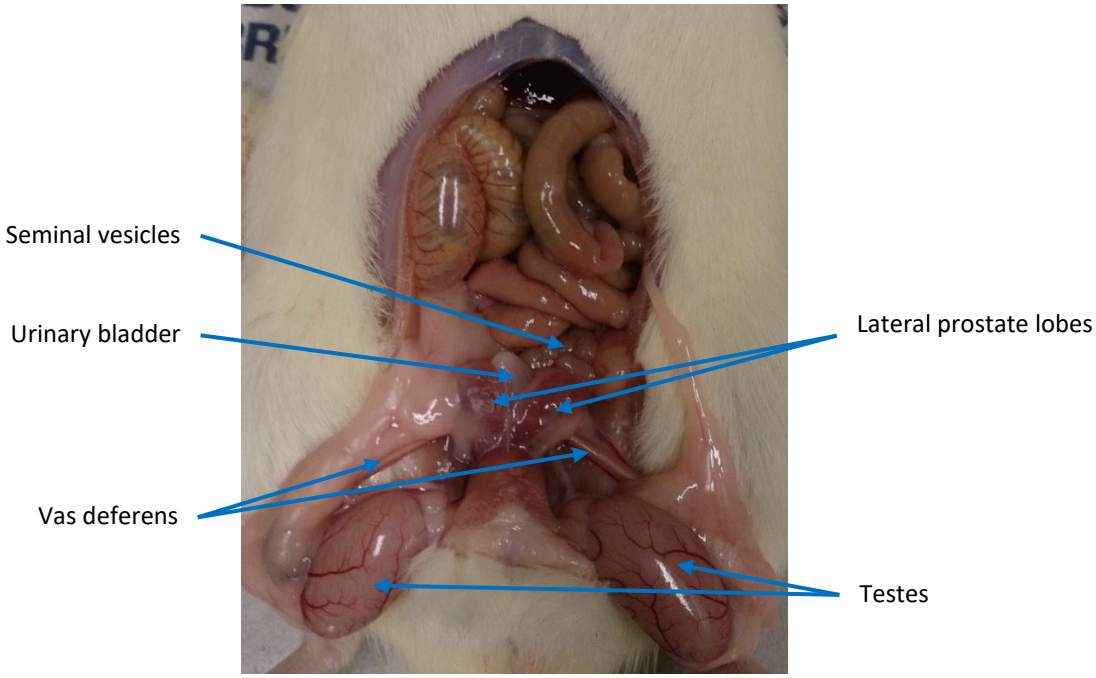


Figure 1-21. The reproductive system of a male Sprague Dawley® (SD) rat.

1.7 Natural Products

The history of the use of botanicals in medicine is as old as human civilization. The first records date back to 2600 BC, where the people of Mesopotamia recorded on clay tablets in cuneiform, the use of different plants in their daily life. One of the best-known records of Egyptian medicinal practice is the "Ebers Papyrus" dating back to 1500 BC that documents approximately 700 drug formulations mostly of plant origin. The Chinese Materia Medica extensively documents the use of herbs from 1100 BC. The Indian Ayurvedic document dates back to 1000 BC and is one of the ancient records that still informs herbal medicine use today (Borchardt, 2002; Cragg and Newman, 2001). The most intriguing evidence was the discovery of medicinal herbs in the personal effects of an "Ice Man" whose body was frozen in the Swiss Alps approximately 5300 years ago. The herb was revealed by palaeobotanical study as the woody fruits of *Piptoporus betulinus* which contains toxic resins and agaric acid that are powerful purgatives. The man was probably taking this herb to treat the parasites found in his intestine (Capasso, 1998). The first Europeans to explore botanical medicines were the Greek physician Hippocrates (fifth century BC), followed by the Romans Galen (second century BC) and Dioscorides (first century BC) (Leicach and Chludil, 2014).

Natural products refer to extracts or mixtures of chemical compounds that are produced naturally by living organisms including plants, bacteria, protozoa, insects, marine life, fungi, endophytes and other animals including humans, that have a pharmacological or biological activity (Mouhssen, 2013). Natural products can be divided into primary and secondary metabolites. Plants are sessile and thus have physical barriers such as cell walls at the cellular level to defend themselves or bark and thorns at the macroscopic level. They also produce a formidable array of bioactive secondary metabolites and defensive proteins that are not essential to growth, as a defensive system to protect themselves from fungal and bacterial pathogens as well as animal herbivores (Polya, 2003). Natural products play a pivotal role in pharmaceutical research. Many of the active ingredients from traditional medicinal products are derived from plants where the extracts of the plants have been extensively studied using various screening methods by pharmaceutical companies, independent research institutions and/or university institutes. Although there are approximately 250,000 plant species in the world, it is estimated that only 10 % of these have been screened for bioactivity in scientific laboratories (Verpoorte, 1998). Drug discovery from plants started at the beginning of the 19th century when analgesic and sleep-inducing agents were isolated from opium by a German apothecary assistant

(Atanasov et al., 2015). Since then, numerous bioactive natural products, primarily alkaloids which are the most important family of secondary metabolites associated with pharmacological activity, have been isolated from natural sources. These include quinine, caffeine, nicotine, atropine, cocaine, capsaicin, and many more (Atanasov et al., 2015; Corson and Crews, 2007).

Traditional or folklore herbal medicines continue to contribute to the development of pharmacotherapy for millions of people globally. According to WHO (2000), traditional or folklore medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness. WHO estimated that in 1985, there were about 65 % of the world population predominately dependent on plant-derived traditional medicines for their primary health care (World Health Organization, 2002). This is probably because traditional herbal medicines have been commonly used over millennia.

1.7.1 Ethnopharmacology and Phytotherapy for BPH: Progress and the Future of Drug Development

Ethnopharmacology is the study of the pharmacological effects of a given culture's traditional medicines derived from animals, fungi, microbes, minerals and plants (**Figure 1-22**) (Ariane Löhnert, 2014; Ríos, 2011). In contrast, ethnobotany studies the interactions between humans and plants as well as different aspects of the use of plants as food, cosmetics, textiles, gardening and medicine (Ríos, 2011). Both ethnopharmacology and ethnobotany have contributed robust medicines over the years particularly by the discovery of many important plant-derived medicines. Indeed natural products have played a dominant role in the discovery of leads for new medicines in treating human diseases (Newman and Cragg, 2016).

Phytotherapy or the use of plant extracts for treatment of LUTS associated with BPH was first described on Egyptian papyrus in the 15th century B.C. (Lowe and Ku, 1996). Most herbal medicines are derived from approximately 40 species of flowering plants with approximately 30 phytotherapeutic compounds having been used in the treatment of symptoms associated with BPH (Wilt et al., 2007). Phytotherapeutic products contain plant extract derived from different plant parts including roots, seeds, bark, flowers, leaves, rhizome or stem. These herbal products are commercially available as single herbs, herbal combinations or a combination of herbs and western medicines.

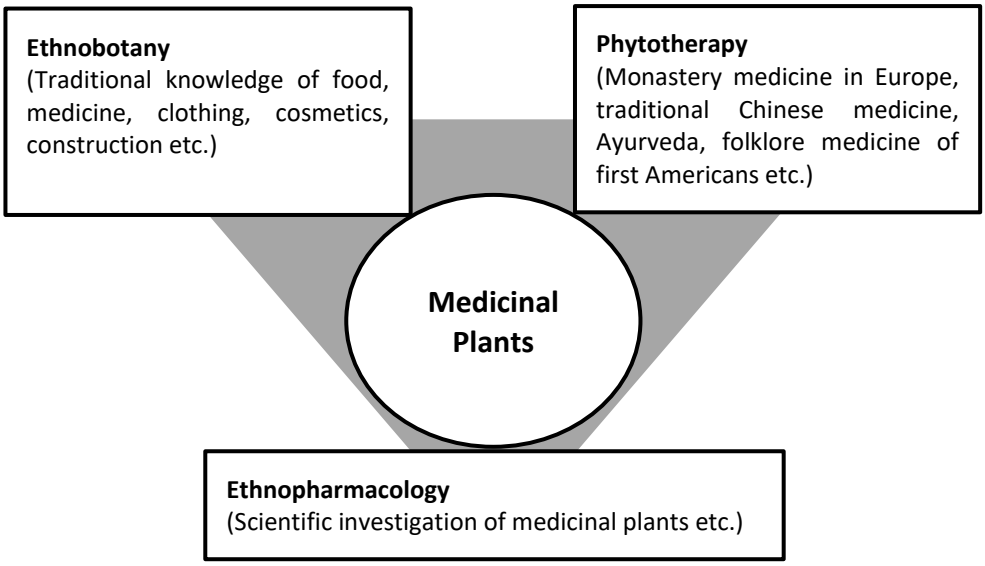


Figure 1-22. Interactions between medicinal plants with ethnobotany, phytotherapy and ethnopharmacology (Ariane Löhnert, 2014).

The International Consultation on Benign Prostatic Hyperplasia (Paris, June 1991) had recommended that, except where surgery is the recommended treatment, the choice of therapy should be a decision between the physician and the patient. Due to perceived low side effect risks, phytotherapeutic agents have been extensively prescribed for the treatment of symptoms related to BPH (Di Silverio et al., 1993). Germany is one country that extensively prescribes plant-derived medicines for a number of indications (Buck, 1996). As both surgery and medicinal treatments for BPH give rise to many undesirable side effects, phytotherapy has gained more interest throughout the world especially in Germany, Switzerland, Austria, Spain, Italy, France and Japan (Buck, 1996). This can be seen in > 90 % of all the prescriptions for BPH management in Germany being phytotherapeutic agents, and 49 % in Italy (Buck, 1996; Di Silverio et al., 1993). In Germany, 50 % of urologists prefer to prescribe plant-based active agents as the first-line treatment for milder to moderate LUTS as compared to chemically derived agents (Lowe and Ku, 1996; Pagano et al., 2014).

From the phytotherapeutic agents used for the treatment of LUTS/BPH, a variety of active components have been suggested based on their biological mechanisms of action. Mechanisms of action include: 5α-reductase inhibition, anti-inflammatory, interference with growth factors, antiandrogenic, estrogenic, aromatase inhibition, decrease of sex hormone-binding globulin, alteration of cholesterol, effects on α-adrenergic receptors, free radical scavenging, alterations in lipid peroxidation, modulation of prolactin-induced prostatic growth or protection of bladder and detrusor function (Meenakshi et al., 2017). The number of active constituents of the plant

extracts used to treat BPH/LUTS is constantly increasing. The most prominent chemicals are phytosterols, *β*-sitosterols, fatty acids and lectins. Other reported components include campesterol, stigmasterol, lupenone, lupeol, terpenoids, plant oil, polysaccharides, flavonoids, phytoestrogens, coumestrol, genistein, δ5-sterols, and δ7-sterols (Carbin et al., 1990; Fagelman and Lowe, 2002; Madersbacher et al., 2007).

Herbal dietary supplement total retail sales in the United States have experienced consecutive yearly growth since 2002 and achieved an estimated 6.8 % growth in 2014 with roughly $US 6.4 billion spent on dietary supplements. Saw Palmetto is among the top 10 selling herbal dietary supplements recording $US 17,990,612 in sales in 2014. The sales data reflects increased consumer preference towards herbal dietary compared to prescription medications (Tyler Smith et al., 2015). This preference is mostly due to the undesirable side effects experienced by LUTS/BPH patients. Despite the advancement of organic chemistry over the past two centuries which produced an enormous number of semi-synthetic and synthetic compounds with improved potency in treating LUTS/BPH, the role of herbal medicines as phytotherapies is still preferred by many patients.

1.7.2 Ethnomedicinal Plant: *Costus speciosus*

The world is endowed with rich biodiversity that provides at least 7,000 edible plant species, including both wild and cultivated plants that have medicinal, nutraceutical, pharmaceutical and other health value. These plant species can be good sources of phenolic phytochemicals such as flavonoids, tannins, pectins and saponins (Lim, 2012). Apart from fruits, flowers, or leaves, there are other edible plant parts including modified storage subterranean stems (corms, rhizomes, stem tubers) and unmodified subterranean stem stolons, above-ground swollen stems and hypocotyls, storage roots and bulbs. These are eaten as vegetables, herbal teas, or used as spices, food additives or nutraceuticals. The plant being investigated in this study, *Costus speciosus*, consists of a modified subterranean stem made up of rhizomes that grow underground producing roots and shoots. Rhizomes normally serve as a storage compartment for the plant to survive but also play a role in the plant's life cycle (Lim, 2015).

Costus speciosus (Koenig) Sm. (*C. speciosus*), is a tropical herbaceous plant belonging to the Costaceae family under the order Zingiberales. This plant order contains high concentrations of steroidal saponins that exhibit a variety of biological activities. Costaceae was previously included as a subfamily with the Zingiberaceae family due to similarities such as their capitulum

and floral characteristics (Specht and Stevenson, 2006). Nevertheless, *C. speciosus* has the distinct characteristic of having a one-sided spiral arrangement of leaves on its stem (**Figure 1-23**).

The genus *Costus* contains up to 175 species (Ariharan et al., 2012; Specht and Stevenson, 2006). *C. speciosus* grows in primary or secondary forests especially those with moist soil or clay loam soil with shady areas in Southeast Asia. It is commonly known as crêpe ginger and has simple leaves that are spirally arranged along a twisted stem (Chai, 2006; Daisy et al., 2008; Pawar and Pawar, 2012). It is an erect, succulent, perennial herb that is able to grow to a height of 3 metres (Burkill, 2002; Chai, 2006). Its leaves are elliptic to oblong in shape, usually dark green in colour but with some species variations. The leaves are thick and silky beneath, and spirally arranged 15 – 35 cm X 6 – 10 cm with stem clasping sheaths up to 4 cm. It has white flowers about 5 - 6 cm long with a cup-shaped labellum and crest yellow stamens. The flower petals look like crêpe paper, thus the name crêpe ginger. The plant has large inflorescence, thick and cone-like terminal spikes with bright red bracts (**Figure 1-23**).

Figure 1-23. *Costus speciosus* with its special physical characteristic of one-sided spiral arrangement of leaves and stem clasping sheaths up to 4 cm. It has large white flowers in thick and cone-like terminal spikes with bright red leaf-like.

It also has seeds which are black in colour. The rhizomes are tuberous and clothed with sheaths consisting of a brownish epidermis or cork buried underground, normally 10 – 30 cm in length and 1.5 – 3.5 cm in diameter. The rhizomes are sub-cylindrical in shape and seldom branching (**Figure 1-24**). (Choudhury and Sarma, 2016; Rani et al., 2012). In Sarawak, the plant is widely distributed in the wild and in recent years has become very popular as an ornamental plant.

Figure 1-24. The rhizome of the *Costus speciosus.*

1.7.2.1 Folklore Use

C. speciosus has been widely used as traditional medicine in Southeast Asia. It has been used in the Ayurveda system of medicine to treat diverse ailments in India. Its rhizomes are edible and are a famine food in India (Burkill, 2002). This plant is also an important source of diosgenin (Daisy et al., 2008). Diosgenin has been used for the commercial synthesis of cortisone, pregnenolone, progesterone and other steroid products. In Ayurveda, the rhizome and roots of this plant are described as bitter, astringent and acrid but with therapeutic potential due to the following actions: aphrodisiac, laxative, anthelmintic, detoxifying, antipyretic, expectorant, tonic, digestive, and stimulant. The juice of the rhizome has been applied to the head for cooling and relief from headache while leaves are given to treat mental disorders and fever. A decoction of the rhizome has also been used orally for treatment of kidney and gallstones (Bijauliya et al., 2017), while decoction of the stem has been used to treat fever and dysentery (Srivastava et al., 2012). Moreover, an alkaloid extract isolated from the rhizome is known to display papaverine-like smooth muscle relaxant and anti-spasmodic activities (Bhattacharya et al., 1972).

In Bangladesh, the roots of *C. speciosus* have been used for treating urinary tract infection (UTI) symptoms such as leucorrhoea in women and to improved urine clearance. Furthermore, the leaves and roots are also used to treat the sexually transmitted diseases (STD) such as gonorrhoea and syphilis. Apart from treating STDs and UTIs, natives of Bangladesh also use *C. speciosus* as a contraceptive as well as to treat smallpox, worm infestation, leprosy, baldness, malaria, bronchitis, gout, muscle weakness, dysentery, eczema, paralysis and gonorrhoea (Hossan et al., 2010). In other countries, it had also been used traditionally to treat alopecia, inflammation of the eye, dysentery, and syphilis in Java, oral cancer in Europe, and cough, fever, leprosy, constipation and smallpox in Malaysia (Hossan et al., 2010).

Likewise, this species of plant has many medicinal uses in the indigenous community of Sarawak, Malaysia to treat fevers (Kedayan community), rheumatism, pneumonia, cough, inflammation, helminthic infection, toxicity, constipation, indigestion, urinary symptoms and skin diseases (Burkill, 2002; Chai, 2006; Vijayalakshmi and Sarada, 2008). Moreover, the Bidayuh community from the island of Borneo also grew plants in their rice fields to protect the crop against pests and diseases ensuring a bountiful harvest (Chai, 2006).

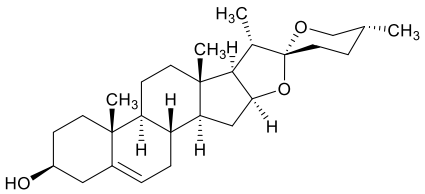
1.7.2.2 Phytochemical Properties

The curative properties of medicinal plants are attributed to the presence of various secondary metabolites including carbohydrates, alkaloids, flavonoids, saponin glycosides, phenols, saponins, essential oils, coumarins, carboxylic acids, steroids, ferulic acid, tannins, vitamin E and C, sesquiterpenes and sterols. Phytochemical analysis in *C. speciosus* has indicated the occurrence of many of these micronutrients in the rhizome including carbohydrates, vitamin C, vitamin E, flavonoids, phenols, glycosides, saponins, alkaloids and minerals such as Zn, Cu, Mn, Se, and Fe. The pharmacological and medicinal potential of this plant is associated with the presence of these microelements and minerals (El-Far et al., 2018; Gavillan-Suarez et al., 2015; Jagtap and Satpute, 2014; Karthikeyan et al., 2012; Saraf, 2010; Srivastava et al., 2011b).

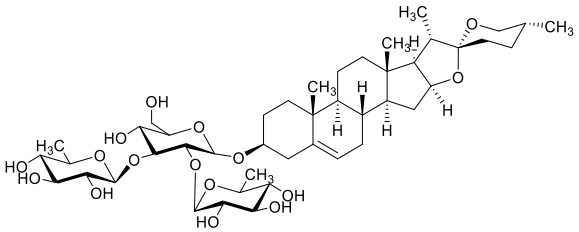
Specific phytoconstituents that have been isolated from this species include: diosgenin (Dasgupta and Pandey, 1970), dihydrophytylplastoquinone (Mahmood et al., 1984), *α*-tocopherol (Devi and Asna, 2010), 5*α*-stigmast-9(11)-en-3*β*-ol (Gupta et al., 1981b; Gupta et al., 1986; Mahmood et al., 1984), dioscin (Mahato et al., 1980; Tschesche and Pandey, 1978), tetradecyl-11-methyltridecanoate (Gupta et al., 1986), methyl ester of *p*-coumaric acid (Bandara et al., 1988). For details of other phytoconstituents isolated from *C. speciosus* refer to **Appendix 1**.

Diosgenin is a steroidal sapogenin that is a common raw material used as a precursor for the synthesis of a number of steroidal drugs such as corticosteroids, sex hormones, oral contraceptives, and anabolic agents (Gupta et al., 1981a). Studies have shown that it can be absorbed through the gut and plays an important role in the control of cholesterol metabolism (Roman et al., 1995). Diosgenin strongly enhances activation of the *p53* enzyme and it causes cell cycle arrest associated with apoptosis in contrast to the actions of the related compounds hecogenin and tigogenin (Corbiere et al., 2003). Cell cycle arrest at the G2/M phase and apoptosis by diosgenin has also been demonstrated in human leukemia K562 cells. The results

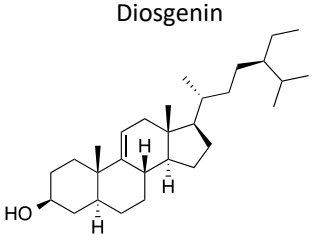
revealed the association between Ca^{2+} homeostasis disruption, mitochondrial dysfunction, reactive oxygen species (ROS) production and caspase activation (Liu et al., 2005). The antiapoptotic Bcl-2 and Bcl-xL proteins were downregulated, whereas the proapoptotic Bax protein was upregulated. Furthermore, diosgenin has been found to show estrogenic action on the mammary epithelium of ovariectomized mice (Aradhana et al., 1992).



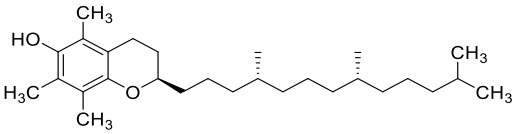
Diosgenin



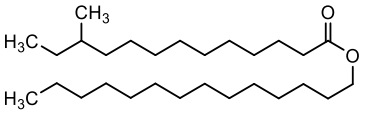
Dioscin



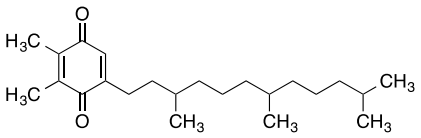
5*α*-stigmast-9(11)-en-3*β*-ol



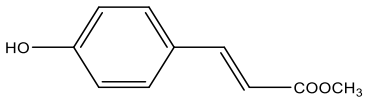
α-tocopherol



Tetradecyl-11-methyltridecanoate



Dihydrophytylplastoquinone



Methyl ester of *p*-coumaric acid

Diosgenin is commercially obtained from the flowering plant genus *Dioscorea* but due to increased demand, pharmaceutical companies have searched for alternative sources. Since the presence of diosgenin in the rhizome of *C. speciosus* was first reported, many follow up studies have been done on this plant due to the commercial value of diosgenin. Diosgenin has since been isolated from the rhizomes of *C. speciosus* with a yield of 2.12 % total sapogenins (Dasgupta and Pandey, 1970; De et al., 1982).

1.7.2.3 Pharmacological Activity

Alkaloids isolated from *C. speciosus* rhizome have been implicated to have papaverine-like smooth muscle relaxant and spasmolytic or anticholinesterase activities in lab animals (Bhattacharya et al., 1972; Bhattacharya et al., 1973). These alkaloids caused red tears (chromodacryorrhea) response in rat in the dacryorrhea test and anticholinesterase activity in dog blood pressure. *C. speciosus* extracts also exhibited nonspecific spasmolytic activity on guinea pig ileum (Banerji et al., 1983; Srivastava et al., 2011b). *C. speciosus* has also been shown to have a number of other therapeutic actions, namely: anti-inflammatory (Binny et al., 2010; Srivastava et al., 2012), antipyretic (Binny et al., 2010), analgesic (Srivastava et al., 2013), anti-nociceptive (Bhattacharya and Nagaich, 2010), antidiabetic (Daisy et al., 2008), antihyperglycemic and hypolipidemic activity (Bavarva and Narasimhacharya, 2008; Daisy et al., 2008; Eliza et al., 2009; Gavillan-Suarez et al., 2015), treatment of bronchial asthma (Srivastava et al., 2012), antifertility (Tewari et al., 1973), uterine stimulant (Lijuan et al., 2011a), anti-abortifacients (Lijuan et al., 2011b; Tewari et al., 1973), hepatoprotective activity (Bhuyan and Zaman, 2008), antioxidant activity (Bavarva and Narasimhacharya, 2008; Jha et al., 2010; Vijayalakshmi and Sarada, 2008), antibacterial activity (Ariharan et al., 2012; Saraf, 2010), antifungal activity (AL-Ameri and Azeez, 2013; Bandara et al., 1988; Suzan Khayyat and AL-Kattan, 2017), anthelmintic activity (Srivastava et al., 2011a), acaricide activity (Ismail, 2017), dietary supplement (El-Far and Abou-Ghanema, 2013), larvicidal activity (Muniyandi et al., 2013; Surendra Kumar et al., 2013), diuretic activity (Dubey Subodh et al., 2010), central nervous system depressant action (Sekiduka-Kumano et al., 2013; Verma and Khosa, 2009; Wolf et al., 1985) and anticancer activity (Choudhury and Sarma, 2016; Selim and Al Jaouni, 2016). Details of some of these activities are shown as following.

1.7.2.3.1 Anti-inflammatory, antipyretic, analgesic and anti-nociceptive actions

C. speciosus possesses anti-inflammatory and anti-arthritic effects (Srivastava et al., 2012). The rhizome itself has been used to treat pneumonia, gout rheumatism, dropsy, urinary diseases, bronchial asthma, and jaundice (Srivastava et al., 2012). Srivastava *et al.*, (2012) reported that an alcoholic extract of *C. speciosus* gave a positive result in controlling inflammation in an adjuvant induced arthritic model in rats. It was concluded that the extract has promise as an anti-arthritic agent and in the general treatment of inflammatory disorders. High doses of *C. speciosus* showed minimal antipyretic activity in yeast-induced pyrexia in rats, but significant anti-inflammatory activity has been observed in carrageenan induced edema and cotton pellet granuloma in rats (Binny et al., 2010).

Another study showed that the flavonoid compounds found in *C. speciosus* could act at prostaglandins that are involved in the late phase of acute inflammation and pain perception (Srivastava et al., 2013). Hence, these flavonoids may mediate the anti-inflammatory and analgesic properties of *C. speciosus*. *C. speciosus* has also been reported to have significant anti-nociceptive properties in Swiss albino mice (Bhattacharya and Nagaich, 2010). Both the aqueous and ethanol extract of the stem bark demonstrated significant peripheral anti-nociceptive actions against acetic acid induced writhing in mice. The ethanol extract was shown to be active both centrally and peripherally on anti-nociceptive mechanisms in the mouse tail flick test compared to the aqueous extract which only inhibited the peripheral pain mechanism.

1.7.2.3.2 Antihyperglycemic, antihyperlipemic and antidiabetic actions

C. speciosus is commonly known as the "insulin" plant in Puerto Rico and Mexico (Benny, 2004; Gavillan-Suarez et al., 2015). Traditionally, diabetic patients consumed one leaf of *C. speciosus* to regulate blood glucose levels. The roots and rhizome were found to have hypoglycemic or antidiabetic (Bavarva and Narasimhacharya, 2008; Daisy et al., 2008; Eliza et al., 2009; Gavillan-Suarez et al., 2015) and antihyperlipemic (Bavarva and Narasimhacharya, 2008; Daisy et al., 2008; Eliza et al., 2009; Gavillan-Suarez et al., 2015) actions. The root reversed the hyperlipidemia by reducing plasma total lipid, cholesterol, and triglyceride levels and thus improved hepatic antioxidant enzyme activity (Bavarva and Narasimhacharya, 2008). Furthermore, the methanolic and aqueous extracts of *C. speciosus* rhizomes show optimum effects on high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (Rajesh et al., 2009). The antidiabetic and antihyperglycemic activity was confirmed by demonstration that administration of the *C. speciosus* rhizome hexane extract in streptozotocin (STZ)-induced male diabetic Wistar rats caused a significant increase in high density lipoprotein (HDL) cholesterol as well as plasma insulin, tissue glycogen, and serum protein levels. The hexane extract of the rhizome also remarkably reduced glycosylated haemoglobin, serum total cholesterol and triglyceride, urea, uric acid and creatinine levels. In addition, the extract also normalized plasma enzyme aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and acid phosphate (ACP) levels (Daisy et al., 2008).

The ability of *C. speciosus* to modulate diabetic diseases have further been demonstrated by both methanol and aqueous extracts of the leaves causing decreases in peripheral insulin resistance resulting in the correction of adipose tissue insulin resistance. In addition, methanol leaf extract exhibits hypotriglyceridemic effects in Wistar male rats induced with an insulin

resistant diet (Subasinghe Hewa Walpola Amila Sewwandi et al., 2014). It was also shown that in some *in vivo* studies, lower doses of *C. speciosus* aqueous extract complemented with insulin had a better glucose modulating effect, probably due to a synergistic or additive effect of the plant with insulin (Gavillan-Suarez et al., 2015).

1.7.2.3.3 Antifertility, uterine stimulant, anti-abortifacients

Traditionally, *C. speciosus* has been widely used for fertility control in women by the rural people of Rangia, a sub-division of the Kamrup District in Assam, India (Najma et al., 2012). Studies showed that the methanolic rhizome extract significantly decreased the ovarian weight and increased uterine weight in gonado-intact female adult mice. They suggested that this plant has potential endocrine active agents (Najma et al., 2012). This phenomenon was also observed in the studies using an alcoholic extract of *C. speciosus* that significantly increased vaginal cornification due to the estrogenic activity of the rhizome (Lijuan et al., 2011b). The estrogenic activity of the *C. speciosus* rhizome had been previously reported to be due to the saponin component that has been shown to increase the uterine weight of ovariectomized (OVE) albino rats (Singh et al., 1972) as well as terminate pregnancy in rats (Tewari et al., 1973)

The effects of *C. speciosus* rhizome extract has also been examined on uterine contractions which may be useful in slowly progressing labour. It was demonstrated that *C. speciosus* rhizome ethanol extract exerted significant stimulation of rat uterine contractility largely due to its constituent, *β*-sitosterol. The uterotonic effect displayed by the extract was determined to be via a non-estrogen receptor mediated mechanism. The transient force produced by the extract was abolished in the presence of the L-type calcium channel antagonist, nifedipine or the myosin light chain kinase (MLCK) inhibitor, wortmannin. As uterine contractility involves Ca^{2+}-calmodulin-MLCK, it was concluded that the increased contractility by this rhizome extract occurred via a Ca^{2+}-calmodulin-MLCK-dependant pathway (Lijuan et al., 2011a). Based on these studies, it has been suggested that *C. speciosus* rhizome extract may be useful to induce labour, protect against miscarriage, regulate the menstrual cycle or enhance fertility (Lijuan et al., 2011b; Tewari et al., 1973).

1.7.2.3.4 Diuretic activity

Alcoholic and aqueous extracts of *C. speciosus* have been evaluated for diuretic activity in Wistar albino rats using furosemide as a standard (Dubey Subodh et al., 2010). The study showed that

both extracts significantly increased urine output and urinary electrolyte concentration indicating the diuretic potential of *C. speciosus*.

1.7.2.3.5 Central nervous system (CNS) depressant action

Interestingly, *C. speciosus* ethanolic extract has been revealed to possess antidepressant activity and adaptogenic potential (Verma and Khosa, 2009). When stress was used to induce alterations in metabolism and neurotransmitter release in the CNS and peripheral nervous system, rats treated with extract had an increase in monoamine oxidase (MAO) activity and thus decreased the elevated 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels caused by cold immobilization stress (Sekiduka-Kumano et al., 2013; Verma and Khosa, 2009; Wolf et al., 1985).

1.7.2.3.6 Antioxidant

Antioxidant activity of petroleum ether, cyclohexane, benzene, ethyl acetate, chloroform, acetone, methanol and aqueous extracts of *C. speciosus* have been widely investigated. Benzene extract has been shown to exhibit the most significant antioxidant action suggesting that the antioxidant activity observed is due to the total phenolic content found in the plant. This includes tannins, lignans, coumarins, quinones, xanthones, phenolic acids, flavones, flavonols, stilbenes, catechins, and anthocyanins, all of which provide protection for living organisms or delay the damage caused by harmful environmental radicals (Jha et al., 2010; Nehete et al., 2010). Polyphenol content was also high in methanolic extracts of the root and peel of the stem as compared to leaf (Vijayalakshmi and Sarada, 2008). Methanolic extract of *C. speciosus* rhizome also exhibited concentration dependent antioxidant activity which indicated that the methanolic extracts of *C. speciosus* exhibited hydroxyl radical scavenging activity and free radical quenching ability (Jha et al., 2010; Vijayalakshmi and Sarada, 2008). Higher amounts of total phenol content as well as ascorbic acid have also been found in the methanolic extract of *C. speciosus* leaves and stems which significantly eliminate reactive oxygen species (ROS) (Sarma et al., 2015). Flavonoids identified in methanol and aqueous extracts of *C. speciosus* using high-performance thin-layer chromatography (HPTLC) also showed significant antioxidant activity (Jagtap and Satpute, 2014). Furthermore, high doses of *C. speciosus* have been found to have greater antioxidant activity when compared with glibenclamide (Bavarva and Narasimhacharya, 2008).

1.7.2.3.7 Anticancer activity

In the progression of cancers, angiogenesis plays a pivotal role. Diosgenin isolated from rhizome extract of *C. speciosus* had been demonstrated to exhibit remarkable anticancer activity through an antiangiogenesis mechanism. Anticancer assay results demonstrated that diosgenin significantly inhibited the growth of hepatocellular (HepG2) carcinoma as well as caspase-3 deficient breast adenocarcinoma (MCF-7) cell lines (Selim and Al Jaouni, 2016). Anticancer activity was also seen in methanolic extracts of *C. speciosus* rhizome which showed cytotoxic activity for lung carcinoma (NCI-H460) and MCF-7 cancer cell lines (Su et al., 2009). Three semi-pure compounds have been isolated through bioassay-guided fractionation (Othman et al., 2011). Apart from diosgenin, costunolide which is a sesquiterpene lactone isolated from *C. speciosus*, also showed remarkable anticancer activity at micromolar concentrations against two breast cancer cell lines (MCF-7 and MDA-MB-231). The compound effectively reduced the viability of both breast cancer cell lines through mediating cell cycle arrest at the G2/M phase (Roy and Manikkam, 2015).

1.8 Aims

In Western medicine, the most effective drugs for the treatment of LUTS caused by BPH are those which can relax prostatic smooth muscle tone such as α-adrenoceptor antagonists (Ventura et al., 2011).

As men age, a balanced testosterone to estrogen (T:E) synergism is necessary for integrity and normal growth of the prostate. With aging, the T:E ratio drops, often drastically. Testosterone normally will be converted to estrogen by the aromatase enzyme, which eventually becomes prevalent in aging men. Progesterone inhibits the conversion of testosterone to DHT by the enzyme 5α-reductase and also maintains the balance of the T:E ratio (Krieg et al., 1993). It also stimulates the activity of the tumour suppressor gene *p53*, which is important in the prevention of prostate cancer progression. Diosgenin has been used for the commercial synthesis of cortisone, pregnenolone, progesterone and other steroid products. *C. speciosus* has been proven to contain diosgenin and this might be important in overcoming prostate diseases.

To our knowledge, no reports exist on *C. speciosus* activity in relation to prostate gland contractility. In view of its potential in treating urological disorders and as a smooth muscle

relaxant, we evaluate its pharmacological activities in the contractility of the prostate gland with a view to its therapeutic potential in the treatment of LUTS associated with BPH.

The great impetus for research into *Costus speciosus* on the contractility of the prostate gland has been its traditional medicinal use by the Sarawak Lun Bawang indigenous people to treat urinary diseases indicating that this plant may possess pharmacological smooth muscle relaxant properties which can be exploited therapeutically.

The aims of this project are, therefore to:

1. To assess and characterize the bioactivity of *C. speciosus* on prostate contractility since this is the most effective mechanism for treating urinary symptoms.

2. To isolate and identify active fractions and individual compounds which may become useful for future drug discovery in the pharmacotherapy of BPH.

2

Materials and Methods

2.1 ANIMAL AND DISSECTION

2.1.1 Sprague Dawley® Rat

Male Sprague-Dawley® (SD) rats aged 7 – 8 weeks old were purchased from the Monash Animal Research Platform (MARP) Rodent Breeding Facility (Monash University, Clayton). Rats were then transported to The Faculty of Pharmacy and Pharmaceutical Sciences (PPS) (Monash University, Parkville) Animal Facility prior to experimentation.

2.1.2 Animal Housing and Ethics

Rats were housed at 22^{o}C with Fibrecycle or Aspen Chip bedding and exposed to a photoperiod cycle of 12 h light/12 h dark. Rats were allowed access to food and water *ad libitum*. Ethical approval for the use of animals in research was obtained from the Monash Institute of Pharmaceutical Sciences (MIPS) Animal Ethics Committees (AEC) (Ethics numbers MIPS.2013.15, MIPS.2016.36). All studies conformed to the requirements of the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition 2013 (the Code) and Prevention of Cruelty to Animals Act (1986) and Regulations (2008).

2.1.3 Animal Tissues Preparation

Rats were placed in a CO_2 chamber and euthanased by inhalation of CO_2 gas. An abdominal incision along the midline of the abdomen was made, exposing the male urogenital tract. Excess fat and connective tissue were cut away to reveal the prostates lobes. The left and right lobes of the prostate were carefully dissected out providing two prostate preparations from each rat. The prostates were separately placed in specimen jars containing Krebs-Henseleit solution, pH7.4 (mM: NaCl 118.1, KCl 4.69, KH_2PO_4 1.2, $NaHCO_3$ 25.0, D(+)glucose 11.7, $MgSO_4.7H_2O$ 1.1, $CaCl_2$ 2.5).

2.2 PLANT MATERIAL PREPARATION

C. speciosus was collected from villages in Sarawak, Malaysia. Plant materials were kept fresh and protected against microbial and enzymatic degradation in zip lock bags filled with water and 70 % ethanol during the time between sample collection and processing. Plants were identified and authenticated by Sarawak Biodiversity Centre (SBC) botanists. A voucher specimen was deposited in the SBC herbarium. The following preparation process was carried out at the SBC. Fresh rhizomes, roots, leaves, and stems were carefully separated and washed thoroughly under running tap water to clear the plant materials of soil, epiphyte, and microbial contamination. The plant materials were cut into small pieces (approximately 0.5 cm thickness) and air-dried in the shade with occasional shifting. After several days when the plant materials were totally dry, they were transferred into a drying oven at 45 ^{o}C for approximately two weeks depending on the different plant parts. The completely dry plant materials were ground into a coarse powder using a mechanical grinder and vacuum packed before shipping to Monash University, Parkville Campus, Australia.

2.3 *C. SPECIOSUS* CRUDE EXTRACT PREPARATION

2.3.1 Experimental Scale Extraction for Preliminary Screening

In general, the overall procedures to obtain the crude extract from the *C. speciosus* plant parts were as follows:

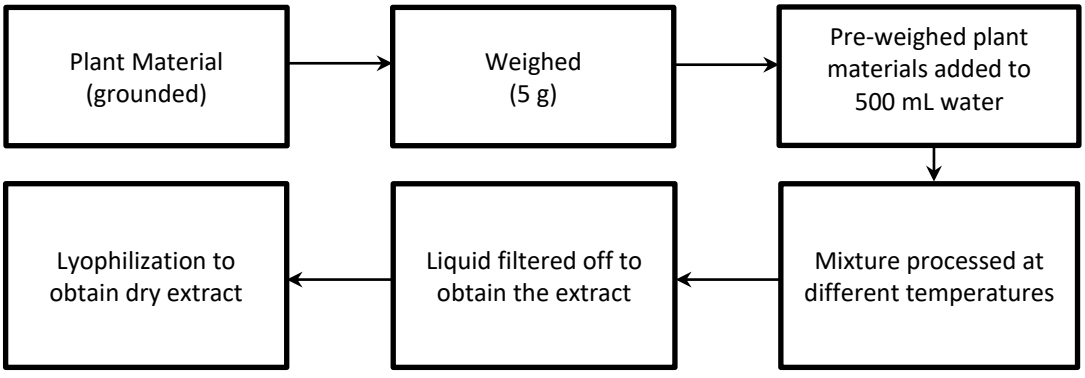


Figure 2-1. General experimental procedures for *C. speciosus* experimental scale extraction.

Five grams of dried and ground rhizome and root were weighed separately. To obtain the extract at different temperatures (i.e decoction, warm water, room temperature), distilled water with different temperatures (70 – 100 ^{o}C) was then added to the plant materials. The extract was

separated from the marc (plant materials residues) by filtering through a porcelain perforated funnel layered with filter paper (Whatman 1 or 4, 90 mm Ø). 100 mL of distilled water at different temperatures (70 – 100 ^{o}C) was re-added into the marc followed by filtration to ensure all the biological components in the rhizome had been retrieved. Liquid chromatography-mass spectrometry (LCMS) and High-performance liquid chromatography (HPLC) analyses were performed on the extract obtained from different temperatures to compare their respective chemical profiles. Extract was frozen by submerging in liquid nitrogen. The frozen extract was then transferred to a 1200 mL Fast-Freeze flask (Labconco) and dried using lyophilizers (FreeZone®, Labconco; Dynavac Freeze Drier, Model FD405).

2.3.1.1 Decoction Extraction

2.3.1.1.1 *C. speciosus* rhizome and root

2.3.1.1.1.1 Without lyophilization

140 mL of distilled water was heated in a beaker and brought to boil on a hot plate. The beaker was removed and allowed to cool for one minute after boiling. The plant materials were added into the boiling water and mixed gently using a spatula. Subsequently, the beaker was returned to the hot plate and boiled for another 5 minutes. The mixture was filtered using a Büchner vacuum filtration funnel with glass sintered disc. The extract collected was used in organ bath studies without lyophilization.

2.3.1.1.1.2 With lyophilization

Weighing, filtration and lyophilisation steps were as described in **Figure 2-1**. Plant materials weighed were slowly added to 200 mL of boiling distilled water on hot plate set at 250 ^{o}C and stirred well using a spatula. The mixture was then boiled for a further 5 minutes.

2.3.1.1.2 *C. speciosus* rhizome

Weighing, filtration and lyophilisation steps were as described in **Figure 2-1**. The plant material was slowly added to 200 mL of boiling distilled water heated on hot plate set at 250 ^{o}C and stirred well using a spatula. The mixture was then boiled for a further 5 minutes.

2.3.1.2 Hot Water Extraction of the *C. speciosus* Rhizome and Root

Weighing, filtration and lyophilisation steps were as described in **Figure 2-1**. Distilled water was heated on hot plate until boiling. The boiling distilled water was then removed from the hot

plate and cooled down to approximately 70 °C. 200 mL of the 70 °C distilled water was added to the plant material mixture and stirred well using a spatula for five minutes.

2.3.1.3 Maceration Extraction for Different *C. speciosus* Plant Parts

Weighing, filtration and lyophilisation steps were as described in **Figure 2-1**. Plant materials were added to 200 mL of ambient temperature distilled water. The mixture was stirred using a magnetic stirrer for different duration depended on the plant parts used:

i. Rhizome and root (45 min)
ii. Rhizome (90 min)
iii. Root (90 min)

2.3.1.4 Methanol Extraction

Weighing, filtration and lyophilisation steps were as described in **Figure 2-1**. 250 mL of methanol (ACD Grade, Merck) was added to a flask containing 10 g of air-dried and ground rhizome. The rhizome methanol mixture was sonicated for 30 min and filtered using filter paper (diameter 330 mm, Whatman 1). 150 mL of methanol was then added to the marc and sonicated for another 30 min followed by filtration. This step was repeated following the addition of 100 mL methanol. Lastly, the marc was submerged in 100 mL methanol for two days to ensure all the biological components in the rhizome had been retrieved and the extract appeared colourless. The extract was then filtered and concentrated using a rotary evaporator, re-dissolved in Milli-Q water and then transferred to 50 mL centrifuge tubes and frozen in liquid nitrogen.

2.3.2 Large Scale Extraction of the *C. speciosus* Rhizome, Stem, Leaf and Root using Maceration Extraction Method

Approximately 123 g of dried and ground rhizome/stem/leaf/root was weighed and carefully transferred into two 2 L conical flasks equally. One litre of Milli-Q water was then added to each of the flasks. The flasks were then secured on a shaker and shaken at 160 rpm for 48 hours at room temperature. For initial filtration, the extract was vacuum filtered through a porcelain perforated funnel. The filtered extract was then vacuum filtered using a porcelain perforated funnel layered with filter paper (diameter 90 mm, Whatman 1 or 4) and Celite® 545 (VWR), respectively to obtain marc free extract. After filtration, 1 L of Milli-Q water was re-added to the marc and shaken at 160 rpm for another 48 hours at room temperature. After 48 hours, the filtration process was repeated. Extracts obtained were frozen in liquid nitrogen. The frozen

extract was then transferred to a 1200 mL Fast-Freeze flask (Labconco) and dried using lyophilizers (FreeZone®, Labconco; Dynavac Freeze Drier, Model FD405).

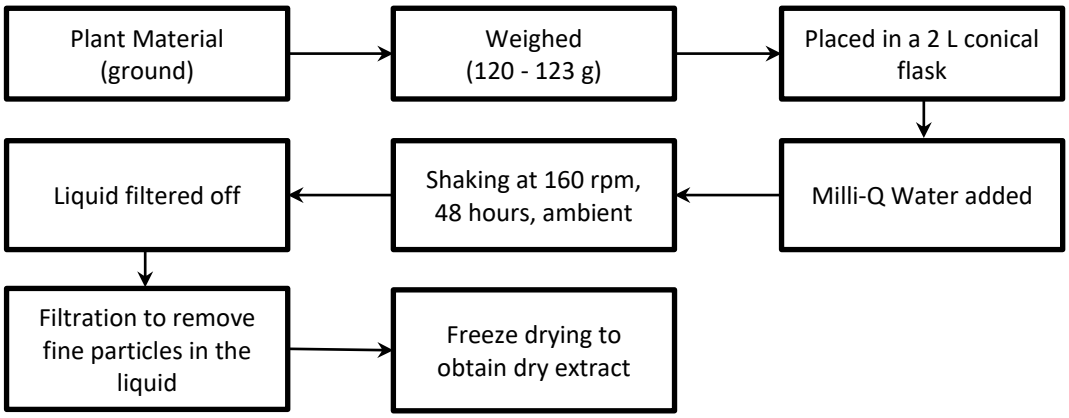


Figure 2-2. General experimental procedures for large scale extraction for *C. speciosus* rhizome, stem, leaf, and root.

2.4 PLANTS USED TRADITIONALLY BY SARAWAK INDIGENOUS COMMUNITY TO TREAT URINARY DISEASES

Twenty-three plant extracts collected from the tropical forest of Sarawak, Malaysia, were selected from the Sarawak Biodiversity Centre Natural Product Library based on their traditional use in treating urinary disorders. These extracts were originally extracted with dichloromethane and methanol (DCM:MeOH) in a 1:1 (v/v) ratio or 100 % methanol, and subsequently concentrated using a rotary evaporator.

Table 2-1. Plant extracts of plants used traditionally by Sarawak indigenous communities for the treatment of various urinary diseases.

No.	Plant barcode	Plant Part & Extract Barcode	Solvent System	Vehicle
1	*C. speciosus* A004669	Rhizome	MeOH	DMSO
2		Leaf; A004669010103	DCM:MeOH (1:1)	DMSO
3		Stem; A004669020103	DCM:MeOH (1:1)	DMSO
4	A002889	Whole plant; A002889010102	DCM:MeOH (1:1)	DMSO
5		Root; A002889020102	DCM:MeOH (1:1)	DMSO
6		Leaf; A002889030102	DCM:MeOH (1:1)	DMSO
7		Stem; A002889040102	DCM:MeOH (1:1)	DMSO
8	A002923	Whole plant; A002923010102	DCM:MeOH (1:1)	DMSO
9		Leaf; A002923020102	DCM:MeOH (1:1)	DMSO
10		Stem; A002923030102	DCM:MeOH (1:1)	DMSO
11	A001014	Whole plant; A001014010103	DCM:MeOH (1:1)	DMSO
12	A004737	Leaf; A004737010103	DCM:MeOH (1:1)	DMSO
13		Stem; A004737020103	DCM:MeOH (1:1)	DMSO
14	A000466	Whole plant; A000466020301	DCM:MeOH (1:1)	DMSO
15		Root; A000466010301	DCM:MeOH (1:1)	DMSO
16		Leaf; A000466030103	DCM:MeOH (1:1)	DMSO
17	A004804	Whole plant; A004804010103	DCM:MeOH (1:1)	DMSO
18	A004674	Whole plant; A0004674010103	DCM:MeOH (1:1)	DMSO
19	A000389	Whole plant; A000389010201	DCM:MeOH (1:1)	DMSO
20	A000417	Stem; A000417010301	DCM:MeOH (1:1)	DMSO
21	A001374	Whole plant; A0001374010103	DCM:MeOH (1:1)	DMSO
22	A004666	Whole plant; A0004666010103	DCM:MeOH (1:1)	DMSO
23	A004533	Whole plant; A004533010103	DCM:MeOH (1:1)	DMSO

2.5 ISOLATED ORGAN BATH STUDIES

The dissected prostate lobes were placed on gauze moistened with Krebs-Henseleit solution. The prostatic capsule, excess fat and connective tissue were carefully removed. The two prostate lobes were carefully separated and one of the prostate lobes was kept submerged in the Krebs-Henseleit solution while the other prostate lobe was prepared for mounting in an isolated organ bath.

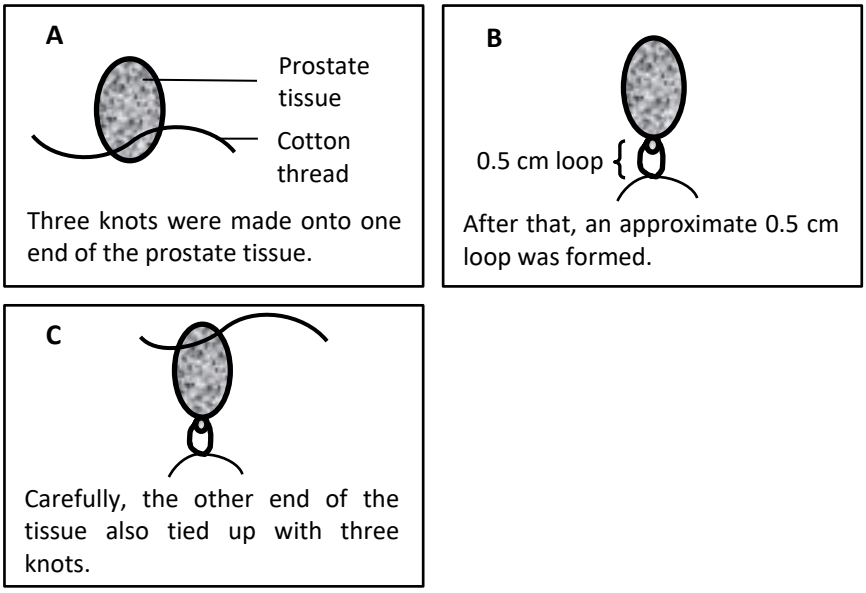


Figure 2-3. Steps to tie up prostate tissue before mounting to a tissue holder.

Before the prostate tissue was mounted to the tissue holder, the tissue was tied according to the steps depicted in **Figure 2-3**. The end with the loop was attached to a perspex tissue holder incorporated with two parallel platinum electrodes. The tissue holder with the isolated prostate was then placed in a 10 mL water jacketed organ bath containing Krebs-Henseleit solution bubbled with 95 % O_2 / 5 % CO_2, and maintained at 37 ^{o}C. The other end of the prostate tissue was attached to an isometric Grass FT03 force-displacement transducer (Grass Instrument, Quincy, MA) connected to a PowerLab 4/SP data acquisition system (ADInstruments Pty. Ltd., Castle Hill, NSW) run on a personal computer with LabChart software (Version 5, ADInstruments Pty. Ltd.) for the measurement and recording of isometric contractions. The isolated prostates were equilibrated for a period of 1 hour under a resting force of approximately 1.0 g prior to experimentation. During the 1 hour equilibration period, the isolated prostates were electrically field stimulated with electrical pulses of 0.5 ms duration, 60 V, at 0.01 Hz generated by a Grass S88 stimulator (Grass Instruments, Quincy, MA) through the platinum electrodes embedded in

the tissue holder. The tension was re-adjusted to 0.7 g prior to construction of discrete agonist concentration-response curves and frequency-response curves.

2.6 FREQUENCY-RESPONSE CURVE

Frequency-response curves to electrical field stimulation (EFS) were constructed using frequencies of 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 Hz (0.5 ms duration, 60 V), via 10 s trains (or 10 pulses) of electrical stimulation delivered at 10 min intervals (**Table 2-2**). The trains of pulses delivered at lower frequencies (< 1 Hz) were for 10 pulses and for 10 s at higher frequencies (> 1 Hz). An initial control frequency-response curve was constructed to determine the contractile response of the tissue at each frequency. A second frequency response curve was then constructed after the tissue had been exposed to the test extract for 30 min. Following this 30 min exposure to the test extract, the tissue was washed with Krebs-Henseleit solution for 20 s before re-administration of extract into the organ bath and construction of the second frequency response curve. The volume of test extract added to the organ bath was ≤ 200 µL. The tissues were allowed 10 min to recover before stimulation at each frequency. Tissues were washed and extract re-added before each train of electrical field stimulation (EFS) measured, to avoid sporadic disturbances caused by frothing from prostatic tissue secretions. An appropriate parallel time control graph was also constructed in which the isolated rat prostates were not exposed to *C. speciosus* extracts during the second frequency-response curve.

Table 2-2. Electrical field stimulation frequencies and their respective duration.

Frequency	Train duration required to generate 10 pulses
0.1	100 sec
0.2	50 sec
0.5	20 sec
1	10 sec
2	10 sec
5	10 sec
10	10 sec
20	10 sec

2.7 AGONIST CONCENTRATION-RESPONSE CURVE

After the isolated rat prostates were equilibrated for a period of 1 hour under a resting force of approximately 1.0 g and stimulated with electrical pulses of 0.5 ms duration, 60 V, at 0.01 Hz,

the tissue was exposed to a priming dose of 10 µM noradrenaline or 100 µM acetylcholine to ensure subsequent reproducible responses. As for tyramine and ATP, priming dose is not needed. For each concentration of agonist, the prostate was allowed to reach maximum contraction or plateau before washing for 20 sec and allowing 10 min to recover. The tension was re-adjusted to 0.7 g prior to construction of discrete agonist concentration-response curves in the absence and presence of *C. speciosus* rhizome methanol or cold-water extract. Initial concentration-response curves to exogenously administered noradrenaline (1 nM – 100 µM), phenylephrine (1 nM – 100 µM), acetylcholine (1 nM – 100 µM), ATP (300 nM – 1 mM), or tyramine (10 nM – 100 µM) were constructed, with a concentration progression ratio of half a log molar unit. The response to each agonist concentration was allowed to reach its highest contractile response or plateau and before washing for approximately 20 secs, and was allowing the tissue to rest for 10 min before continuing with the next agonist concentration. If no response was observed after 20 sec, the tissue was washed and allowed 10 min for recovery. The peak maximum contractile response observed after addition of each concentration was used for data calculations.

During the second concentration response curve in the presence of plant extract, a thorough washout was performed every time after the highest contractile response had been reached or after 20 secs if no response was produced for the concentration of noradrenaline, phenylephrine, acetylcholine, ATP, or tyramine added. After the washout, extract was re-added and the tissue was again allowed 10 min to recover in the presence of extract before addition of the next agonist concentration. An appropriate time control curve was also constructed in parallel in which the isolated rat prostates were not exposed to *C. speciosus* extracts during the second concentration-response curve.

2.8 DRUGS AND VEHICLE SOLUTIONS

2.8.1 Agonists and Antagonists Used

Table 2-3. Components used to prepare catecholamine diluent

Chemical	NaCl	NaH_2PO_4	Ascorbic Acid
mM in 1 L Milli-Q water	154	1.2	0.2

Table 2-4. Summary of the concentration, mechanism of action, and vehicle used for dissolving and diluting the agonists used in the study.

Drug/Abbreviation	Chemical Name	Catalogue Number & Supplier	Mechanism of Action	Dissolving Vehicle	Experimental Concentration
Acetylcholine (ACh)	Acetylcholine chloride	A6625; Sigma, St Louis, U.S.A	Muscarinic receptor agonist	Milli-Q water	1 nM – 100 µM
ATP	Adenosine 5'-triphosphate magnesium salt Adenosine 5'-triphosphate, disodium salt	A9187; Sigma, St Louis, U.S.A A2383; Sigma, St Louis, U.S.A	P2X1 ligand-gated ion channel agonist	Milli-Q water	300 nM – 1 mM
Histamine	Histamine dihydrochloride	H7250; Sigma, St Louis, U.S.A	Histamine (H) receptor agonist	Milli-Q water	10 µM
Noradrenaline	(-)-Arterenol / (±)-Norepinephrine (+)-bitartrate salt	A9512, A0937; Sigma, St Louis, U.S.A	Non-selective adrenoceptor agonist	Catecholamine diluent	1 nM – 100 µM
Phenylephrine	L-phenylephrine hydrochloride	P6126; Sigma, St Louis, U.S.A	α-adrenoceptor agonist	Milli-Q water	1 nM – 100 µM
Serotonin	Serotonin creatinine sulfate monohydrate	H7752; Sigma, St Louis, U.S.A	Serotonin (5-HT) receptor agonist	Milli-Q water	10 µM
Tyramine	Tyramine hydrochloride	T2879; Sigma, St Louis, U.S.A	Indirectly acting sympathomimetic	Milli-Q water	10 nM – 100 µM

Table 2-5. Summary of the concentration, mechanism of action, and vehicle used for dissolving and diluting the antagonists used in the study.

Drug/abbreviation	Chemical name	Catalogue Number & Supplier	Mechanism of Action	Dissolving vehicle	Experimental Concentration
8-phenyltheophylline	1,3-Dimethyl-8-phenylxanthine	P2278; Sigma, St Louis, U.S.A	Adenosine (A_1) receptor antagonist	80 % MeOH v/v 20 % 0.2 M NaOH	10 µM
AH6809	6-Isopropoxy-9-oxoxanthene-2-carboxylic acid	A1221; Sigma, St Louis, U.S.A	Prostaglandin receptor antagonist	Milli-Q water	10 µM
Atropine	Atropine sulphate	A0257; Sigma, St Louis, U.S.A	Muscarinic receptor antagonist	Milli-Q water	1 µM
Desipramine	Desipramine hydrochloride	D3900; Sigma, St Louis, U.S.A	Selective noradrenaline reuptake inhibitor	Milli-Q water	100 nM
Hexamethonium	Hexamethonium bromide	H0879; Sigma, St Louis, U.S.A	Ganglionic nicotinic (nACh) receptor antagonist	Milli-Q water	10 µM
Idazoxan	Idazoxan HCl	I115; Research Biochemicals International (RBI)	Selective α_2-adrenoceptor antagonist	Milli-Q water	1 µM
Methysergide	Methysergide-hydrogenmaleinate	Sandoz, Basel, Switzerland	Serotonin (5-HT) receptor antagonist	Milli-Q water	1 µM
Mepyramine	Pyrilamine maleate	P5514; Sigma, St Louis, U.S.A	Histamine (H_1) receptor antagonist	Milli-Q water	1 µM
Prazosin	Prazosin hydrochloride	P7791; Sigma, St Louis, U.S.A	Selective α_1-adrenoceptor antagonist	Milli-Q water	300 nM
Propranolol	(±)-Propranolol hydrochloride	P0884; Sigma, St Louis, U.S.A	β-adrenoceptor antagonist	Milli-Q water	1 µM
Suramin	Suramin sodium salt	S2671; Sigma, St Louis, U.S.A	P2-purinoceptor antagonist	Milli-Q water	30 nM
Yohimbine	Yohimbine hydrochloride	Y3125; Sigma, St Louis, U.S.A	α_2-adrenoceptor antagonist	Milli-Q water	1 µM

2.9 ANALYSIS OF DATA

2.9.1 Net Peak Contractile Force

The net peak contractile force (g) of the prostate in response to electrical field stimulation or agonist-mediated contraction was measured at each frequency or concentration. Baseline variance was removed from the peak contractile response of each frequency or concentration by subtraction of the baseline height from the contractile response peak height (maximum-minimum). Mean frequency or mean log concentration-response curves (normalized) in the presence of *C. speciosus* extracts were compared with the previously obtained control mean frequency or mean log concentration-response curves (normalized) (no extract) using Prism version 7.0 software for Windows (GraphPad Software, La Jolla California USA) and analysed using two-way repeated measures analysis of variance (ANOVA). The mean curves were constructed by pooling data from *n* experiments. Results are expressed as the mean ± standard error of the mean (S.E.M.). The value of *n* represents the number of animals used. The *p*-values stated were used to evaluate the statistical significance of any difference between concentration and treatment, $p < 0.05$ was considered significant in all cases.

2.9.2 Integral Forces

In some experiments, integral forces [force x time (g.s.) where s = 40 seconds (0.5 Hz) and 60 seconds (1 – 20 Hz)] of the prostate contraction elicited by electrical field stimulation in the presence and absence of antagonists were measured and expressed as percentage of the control contraction. Mean curves of the percentage of contraction remaining were constructed by pooling data from *n* experiments using Prism version 7.0 software for Windows (GraphPad Software, La Jolla California USA) and analysed using one-way ANOVA followed by Dunnett's multiple comparisons test, Uncorrected Fisher's LSD, or Bonferroni's multiple comparisons test.

Where indicated, integral force (g.s. where s = 40 seconds) was also measured to generate concentration-response curves showing tonic contraction of unstimulated isolated rat prostates elicited during administration of i) *C. speciosus* rhizome, stem, leaf, or root cold-water extract, ii) *C. speciosus* rhizome cold-water extract in the absence and presence of different pharmacological agents, and 3) liquid phase and solid phase extract isolated from centrifugation separation of *C. speciosus* rhizome cold-water extract. Means of the force of tonic contraction were constructed by pooling data from *n* experiments using Prism version 7.0 software for

Windows (GraphPad Software, La Jolla California USA) and analysed using paired t-test (two-tailed) or one-way ANOVA followed by Uncorrected Fisher's LSD to determine the difference between tonic contractions elicited during administration of the aforementioned extracts on unstimulated isolated rat prostate with control.

Results are expressed as the mean ± standard error of the mean (S.E.M.). The value of *n* represents the number of animals used. The *p*-values stated were used to evaluate the statistical significance of any difference between concentration and treatment, $p < 0.05$ was considered significant in all cases.

2.10 CHEMICAL ANALYSIS

2.10.1 Chromatography

2.10.1.1 Reverse-phase Flash Chromatography

Reverse-phase (RP) flash chromatography was used to separate the chemical components from *C. speciosus* water extract based on hydrophobicity. The column used was 90 mm in diameter, while stationary phase used was C18 silica gel (DAVISIL® 633NC18E; Grace, USA). Approximately 700 g of C18 silica was mixed with 100 % MeOH (HPLC grade; Scharlau, Spain) and the slurry was carefully poured into the column to give a height of approximately 15 cm. The eluent was drained out and one litre of 50 % MeOH/H_2O (HPLC grade; Scharlau, Spain) was added and eluted. This procedure was repeated for 30 % and 10 % MeOH/H_2O (HPLC grade; Scharlau, Spain), respectively. The column was allowed to stand in 10 % MeOH/H_2O overnight to allow settling and equilibration, and was then flushed with 1 L of 10 % MeOH/H_2O. The cold-water extract of the *C. speciosus* rhizome (18 g) was dissolved in 60 mL of Milli-Q water and 6 mL of 10 % MeOH/H_2O before being slowly applied to the top of the column. Elution of the column was carried out using 1 L of 10 %, 30 %, 50 %, 70 %, 90 % MeOH/H_2O and 100 % MeOH, followed by 10 % DCM/MeOH and 100 % DCM (LCMS grade; Merck, Germany), respectively.

2.10.1.2 Thin Layer Chromatography

Thin layer chromatography (TLC) was performed on normal phase silica gel 60 F_{254} pre-coated plates (0.25 mm; Merck) and reverse phase silica gel 60 RP-18 F_{254S} pre-coated plates (0.20 mm; Merck).

2.10.1.3 Liquid Chromatography-Mass Spectrometry

Fractions collected from the analytical and preparative HPLC were analysed by LC-MS system (LCMS-2020; Shimadzu), equipped with the ultra-high-speed LC/MS Prominence UFLC system including pumps, UV, and microwave system (LC-20AD, SIL-20A HT, CBM-20A, SPD-M20A, CTO-20A; Shimadzu). Approximately 20 µL of sample was aliquoted to a LCMS screw vial. Solvent system used were 0.05 % TFA/H_2O (Buffer A), 0.05 % Acetonitrile/H_2O (Buffer B), and water:acetonitrile (50:50) for needle wash. Method used for the analysis was "LCMS 0-80B 15 min Pos". Two microliters of the sample were injected through Luna® 3 µm C8(2) 100Å, RP LC column (100 x 2.0 mm) (00D-4248-B0; Phenomenex). Gradient took 15 minutes to archive 80 % ACN, maintained for 1 minute and returned to 100 % water at 20 minutes, and terminated at 25 minutes. Data was managed and analysed using LabSolutions, Version 5.53 (Shimadzu) software.

2.10.1.4 Analytical High-Performance Liquid Chromatography

Samples separation were analysed using an analytical high-performance liquid chromatography (analytical HPLC) (2795 Alliance®, Waters) equipped with dual wavelength absorbance detector (2487, Waters), Alliance® 2795 Separations Module, and Alliance® HPLC High Throughput (HT) System. HPLC separation was performed with different columns, including Luna® 5 µm C8(2) 100Å, (150 x 4.60 mm) (00F-4249-E0; Phenomenex), Zorbax Eclipse Plus C18 Rapid Resolution 3.5-Micron column (4.6 X 100 mm) (Phenomenex), Synergi™ 4 µm Hydro-RP 80 Å, (250 x 10.0 mm) (00G-4375-N0, Phenomenex), Synergi™ 4 µm Fusion-RP 80 Å, (150 x 4.60 mm) (00F-4424-E0, Phenomenex), and Synergi™ 4 µm Hydro-RP 80 Å (50 x 21.20 mm) (00B-4375-P0-AX, Phenomenex). 200 µL of samples were aliquoted into a 1.5 mL microcentrifuge tube. Solvent system used were 0.1 % TFA/H_2O (Buffer A), and 0.1 % TFA/Acetonitrile (Buffer B). The injected volume ranged between 2 – 20 µL and the flow rate was 1 mL/min. Methods used for the separation including "Method A", "Method B", "Method C" and "Method D" (**Table 2-6**). Total run time was 20-30 min. Data were managed and analysed using Empower™ 2 software. Unless otherwise specified, the UV detector was set at 254 nm for all separations.

Table 2-6. Method A, B, C and D gradient tables.

Method A			
Time	**Flow**	**% Buffer A**	**% Buffer B**
	1.00	100.0	0.0
14.00	1.00	80.0	20.0
20.00	1.00	0.0	100.0
21.00	1.00	0.0	100.0
22.00	1.00	100.0	0.0
30.00	0.05	100.0	0.0

Method B			
Time	**Flow**	**% Buffer A**	**% Buffer B**
	1.00	100.0	0.0
10.00	1.00	60.0	40.0
11.00	1.00	20.0	80.0
12.00	1.00	100.0	0.0
22.00	1.00	100.0	0.0
23.00	0.05	100.0	0.0

Method C			
Time	**Flow**	**% Buffer A**	**% Buffer B**
	1.00	100.0	0.0
10.00	1.00	80.0	20.0
11.00	1.00	20.0	80.0
12.00	1.00	100.0	0.0
22.00	1.00	100.0	0.0
23.00	0.05	100.0	0.0

Method D			
Time	**Flow**	**% Buffer A**	**% Buffer B**
	1.00	100.0	0.0
10.00	1.00	20.0	80.0
11.00	1.00	20.0	80.0
12.00	1.00	100.0	0.0
22.00	1.00	100.0	0.0
23.00	0.05	100.0	0.0

2.10.1.5 Fractionation by Preparative High-Performance Liquid Chromatography

Preparative-scale HPLC has been used to fractionate chemical components of the samples. Fractionation of the samples was performed on Waters™ Prep LC Preparative Chromatography System equipped with Waters™ 486 Tunable Absorbance Detector, and Waters™ Prep LC Controller. Solvent system used were 0.1 % TFA/H_2O or 100 % Milli-Q water (Buffer A), and 0.1 % TFA/Acetonitrile or 100 % Acetonitrile (Buffer B). Samples between 0.5 mL – 10 mL were manually injected using syringe into the system through sample injector. The separation of the sample chemical components was performed via column Luna® 10 µm C8(2) 100Å, AX (50 x 21.20 mm) (00B-4250-P0-AX; Phenomenex), or Luna® 10 µm C8(2) 100 Å, A X 1A P, (250 x 21.2 mm) (00G-4250-P0-AX; Phenomenex). Data were managed and analysed using Empower™ 2 software.

2.10.2 Nuclear Magnetic Resonance Spectroscopy Data Acquisition and Processing

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance III Nanobay 400 MHz NMR spectrometer coupled to the BACS 60 automatic sample changer. The spectrometer is equipped with a 5 mm PABBO BB – 1H/D Z – GRD probe (Bruker, 2017). The NMR experiment was run in Bruker's TOP-SPIN interface which consisted of ICON-NMR component. Routine proton (^{1}H) NMR was performed by dissolving the sample in an appropriate deuterated NMR solvent such as deuterium oxide (D_2O) (DLM-4-100; Cambridge Isotope Laboratories, Inc.), methanol-d_4 (CD_3OD) (151947-10G-GL; Sigma), and Chloroform-d ($CDCl_3$) (DLM-7-100S; Cambridge Isotope Laboratories, Inc.). The sample was fully dissolved and 300 –

500 µL of the solution was then transferred into a clean NMR tube. Number of scans performed was between 64 and 128. NMR data was analysed using Mestrelab MNova Version 6.0.2-5475 software. For ^{1}H spectra, solvent peak reference for D_2O is 4.79 ppm, CD_3OD is 3.31 ppm, and $CDCl_3$ is 7.26 ppm. The chemical shifts were expressed in parts per million (ppm) as δ values and the coupling constants (*J*) in Hertz (Hz).

2.10.3 Centrifugation Separation

Centrifugation separation method was used to separate insoluble particles present in the cold-water extract of the *C. speciosus* rhizome. 1 mL of Milli-Q water was added to 200 mg of cold-water extract of the *C. speciosus* rhizome. The mixture was vortexed and sonicated to ensure the dried extract was fully dissolved. The dissolved extract was then centrifuged at 11,000 rpm for 15 minutes. Centrifugation of the extract successfully separated undissolved particles (namely, solid phase) of the extract from the extract liquid (namely, liquid phase). The liquid phase extract was carefully transferred to a pre-weighed 1.5 mL microcentrifuge tube via pipetting. Both solid phase and liquid phase were frozen using liquid nitrogen and lyophilised to obtain the dried extract mass.

2.10.4 Derivatization Method

Derivatization of the carbohydrates was performed according to the method described by Anumula (1994) with some modifications (Anumula, 1994). Reductive amination of carbohydrates with anthranilic acid was performed in methanol-acetate-borate medium. 1 mL of methanol-acetate-borate medium [4 % sodium acetate trihydrate (10236.4Q; Merck) and 2% boric acid (Ajax, Australia) in 5 mL methanol] was added to a glass vial containing 30 mg of anthranilic acid (Ajax, Australia) and 20 mg of sodium cyanoborohydride (15615-9; Aldrich). The mixture was vortexed vigorously to prepare anthranilic acid (ABA) reagent. 20 µL of freshly prepared 1 % sodium acetate trihydrate was added to 1.5 mL microcentrifuge tube which contained 14 mg of the sugar [glucose (D(+)-Glucose anhydrous, Merck, Germany), fructose (Univar, Australia), galactose (BDH, England), lactose (New Zealand), mannose (D(+)-Mannose, Ajax Chemicals, Ohio), rhamnose (L-Rhamnose monohydrate, Sigma, US), or arabinose (L-(+)-Arabinose, Sigma, US)] or CS-PLP1 of the liquid phase extract isolated from centrifugation separation of the *C. speciosus* rhizome cold-water extract. The mixture was carefully mixed and 50 µL of ABA reagent was then added. The microcentrifuge tube was heated at 80 ^{o}C for 1 hour on a heater block (Multi-Blok®, Lab-line). During the heating process, the lid of the tube was carefully opened to release hydrogen gas produced from the reductive amination process. After

1 hour, the tube was cooled to room temperature and proceed to chemical separation using preparative RP-HPLC or bioactivity validation using isolated organ bath.

3

Bioactivity of Plant Extracts on Isolated Rat Prostate Gland

3.1 INTRODUCTION

The history of the use of botanicals in medicines is as old as human civilization and traditional or folklore herbal medicines continue to contribute to the development of pharmacotherapy for millions of people globally. As defined by WHO (2000), traditional or folklore medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness. In 1985, WHO estimated that approximately 65 % of the world's population predominately depended on plant-derived traditional medicines for their primary health care (World Health Organization, 2002). This is probably because traditional herbal medicines have been used over millennia and due to their perceived safety, more countries have embraced the use of traditional herbal medicines for the maintenance of health and well-being in their people. The number of countries regulating the use of herbal medicines has increased from 65 in 1999 to 119 in 2012 (World Health Organization, 2013). In European countries alone, there are over 100 million people who are traditional herbal medicine users (European Information Centre for Complementary & Alternative Medicine, 2008).

Phytotherapy or the use of plant extracts for the treatment of LUTS associated with BPH was first described on Egyptian papyrus in the 15th century B.C. (Lowe and Ku, 1996). Most of the herbal medicines are derived from approximately 40 species of flowering plants and there are about 30 phytotherapeutic compounds which have been used for the treatment of symptoms associated with BPH (Wilt et al., 2007). In many cases, most of these are developed directly or

indirectly based on traditional knowledge which had been passed from generation to generation. “Phytotherapeutic products” are defined as products containing extracts derived from different plant parts including roots, seeds, bark, flowers, leaves, rhizome, or stem. These herbal products are available as single herbs, herbal combinations, or combinations of herbs and drugs. Despite the advancement of organic chemistry over the past two centuries, which has produced an enormous number of semi-synthetic and synthetic compounds with improved potency in treating LUTS/BPH, the use of herbal medicines as phytotherapy is still widespread.

3.1.1 Traditional Medicines in Sarawak

Sarawak is located in one of the 25 global biodiversity hotspots, having mega biodiversity resulting from its tropical rainforest (Long, 2014). Due to this mega biodiversity, different ethnic groups in Sarawak have practiced folklore or traditional medicines for generations and more than 1,000 local herb have been used in medical treatment (Borneo Post Online, 2011). A Natural Product Library (NPL) was established by the Sarawak Biodiversity Centre (SBC) consisting of extracts derived from plants used as traditional medicines by the indigenous communities of Sarawak. The NPL library also contains extracts from fermentation products of biodiverse microbial isolates (actinomycetes and fungi) from various sites in Sarawak.

For the purpose of this study, plant extracts in the SBC NPL, reported to be used by the Sarawak indigenous communities to treat urinary diseases, were shortlisted. *C. speciosus* is locally known as daun setawar hutan or siluk, and apart from being consumed as a vegetable, it has also been used by the Lun Bawang indigenous community to treat urinary diseases. Traditionally, the folklore medicine arises from rhizomes, leaves or stems prepared by direct burning on a steel plate, wood or gas stove until it becomes ashes. The ashes are then applied as a traditional remedy topically for alleviating illnesses such as haematuria, difficulty in urinating, painful urination, and diabetes. However, to date, there is no scientific evidence justifying the use of *C. speciosus* extracts for this or any other medical purpose.

3.1.2 Aim

In this study, the effect of plant extracts on the contractile responses of the isolated rat prostatic smooth muscle are evaluated using *in vitro* isometric tension studies. The plant extract with the most prominent effects on the contraction of the prostatic smooth muscle will be pursued for the isolation of bioactive compounds.

3.2 MATERIALS AND METHODS

General methods for animal husbandry, dissection and animal ethics are detailed in Chapter 2, Section 2.1. Details for the plant collection, preparations, processing as well as extraction methods can be found in Chapter 2, Section 2.2 and 2.3. Plant extracts used in the preliminary screening are shown in Chapter 2, **Table 2-1**.

3.2.1 Isolated Organ Bath Studies

General methods on performance of organ bath studies including tissue preparations, initial period of equilibrium as well as parameters of the experiments are detailed in Chapter 2, Section 2.5 and 2.7, otherwise stated.

3.2.1.1 Preliminary Screening of Extracts

Information on the plants extracts used traditionally by Sarawak indigenous community to treat urinary diseases can be found in Chapter 2, **Table 2-1**. Preliminary screening was performed to assess the effects of extracts on the contractile responses elicited to nerve-stimulation by electrical field stimulation. As minimal quantities of the extracts were used, only the frequencies 0.2, 2.0, and 20 Hz were measured. Final concentration in the bath was 0.1 mg/mL.

3.2.1.2 Effects of *C. speciosus* Extracts from Different Small-Scale Extraction Methods

Details for each of the extraction methods were described in Chapter 2, Section 2.3, otherwise stated.

3.2.1.2.1 Methanol extraction

The effects of *C. speciosus* rhizome methanolic extract prepared from Chapter 2, Section 2.3.1.4 were investigated on EFS-induced contractile responses of isolated rat prostate. *C. speciosus* rhizome dried methanolic extract was weighed and dissolved in DMSO to the required concentrations that produce final concentrations of 0.1 mg/mL, 0.5 mg/mL and 2.0 mg/mL.

3.2.1.2.2 Decoction extraction

3.2.1.2.2.1 *Effects of* C. speciosus *rhizome and root (without lyophilise) extract on nerve-mediated contraction*

The effects of fresh (without going through lyophilise) *C. speciosus* rhizome and root decoction extract obtained from Chapter 2, Section 2.3.1.1.1.1 were investigated on EFS-induced contractile responses of isolated rat prostate. 20 µL of fresh extract with unknown concentration was added directly into the organ bath near to the isolated rat prostate tissue to investigate its bioactivity.

3.2.1.2.2.2 *Effects of* C. speciosus *rhizome and roots (with lyophilize) extract on nerve-mediated contraction*

Nerve-mediated contractions were conducted to investigate dose-dependent effect of *C. speciosus* rhizome and root decoction extract obtained from Chapter 2, Section 2.3.1.1.1.2 on EFS-induced contractile responses of isolated rat prostate. *C. speciosus* rhizome and root decoction dried extract was weighed and dissolved in Milli-Q water to the required concentrations to produce final concentrations 0.1 mg/mL, 0.2 mg/mL, 0.6 mg/mL, 1.5 mg/mL, and 2.0 mg/mL.

3.2.1.2.2.3 *Effects of* C. speciosus *rhizome extract on nerve-mediated contraction*

Nerve-mediated contractions were conducted to investigate effect of *C. speciosus* rhizome decoction extract obtained from Chapter 2, Section 2.3.1.1.2 on EFS-induced contractile responses of isolated rat prostate. *C. speciosus* rhizome dried decoction extract was weighed and dissolved in Milli-Q water to the required concentration to produce final concentration 2.0 mg/mL.

3.2.1.2.3 Hot water extraction

3.2.1.2.3.1 *Effects of* C. speciosus *rhizome and root extract on nerve-mediated contraction*

Nerve-mediated contractions were conducted to investigate dose-dependent effect of *C. speciosus* rhizome and root hot water extract obtained from Chapter 2, Section 2.3.1.2 on EFS-induced contractile responses of isolated rat prostate. *C. speciosus* rhizome and root hot water dried extract was weighed and dissolved in Milli-Q water to the required concentration to produce final concentration 0.1 mg/mL.

3.2.1.2.4 Maceration extraction

3.2.1.2.4.1 *Effects of* C. speciosus *rhizome and root extract on nerve-mediated contraction*

Nerve-mediated contractions were conducted to investigate dose-dependent effect of *C. speciosus* rhizome and root cold-water extract obtained from Chapter 2, Section 2.3.1.3(i) on EFS-induced contractile responses of isolated rat prostate. *C. speciosus* rhizome and root cold-water dried extract was weighed and dissolved in Milli-Q water to the required concentrations to produce final concentrations 0.1 mg/mL, 0.6 mg/mL, 1.5 mg/mL, and 2.0 mg/mL.

3.2.1.2.4.2 *Effects of* C. speciosus *rhizome/leaf/stem/root cold-water extract on nerve-mediated contraction*

Nerve-mediated contractions were conducted to investigate the effect of *C. speciosus* rhizome/leaf/stem/root cold-water extract obtained from Chapter 2, Section 2.3.1.3(ii) and (iii), and Section 2.3.2, on EFS-induced contractile responses of isolated rat prostate. *C. speciosus* rhizome/leaf/stem/root dried cold-water extract was weighed and dissolved in Milli-Q water to the required concentration to produce final concentration 2.0 mg/mL.

3.2.2 Frequency Response Curves

General information on construction of mean frequency response curve is described in Chapter 2, Section 2.6.

3.2.3 Analysis of Data

General information on data analysis was described in Chapter 2, Section 2.9. For n = 2, it is not valid to do statistic, therefore we only looking for larger effects.

3.3 RESULTS

3.3.1 Identification of Bioactive Plant Extracts

Trains of EFS (0.5 ms, 60 V, 0.2, 2 and 20 Hz) evoked frequency-dependent contractions of the isolated rat prostate that were reproducible over the time course of the experiment and were not affected by the vehicles used. Preliminary screening results showed that none of the plant organic extracts listed in Chapter 2, **Table 2-1**, were able to affect contractile responses to EFS at 0.1 mg/mL (n = 2).

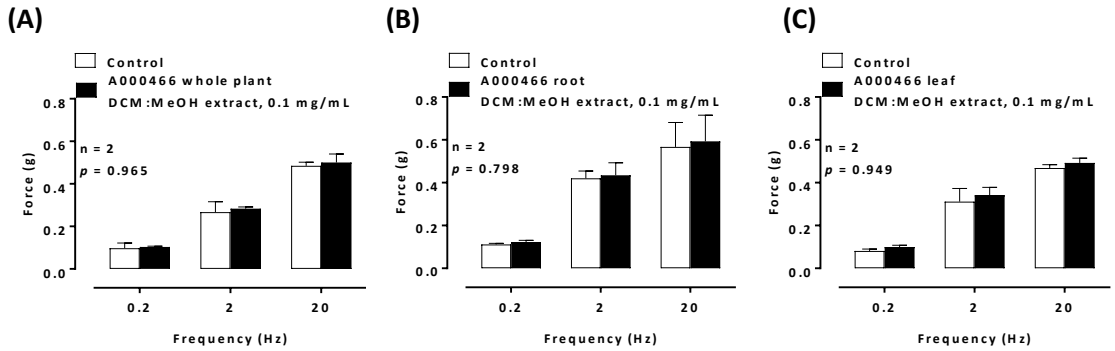


Figure 3-1. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostate after administration of A000466: (A) whole plant, (B) root, and (C) leaf DCM:MeOH extracts, respectively. Columns represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

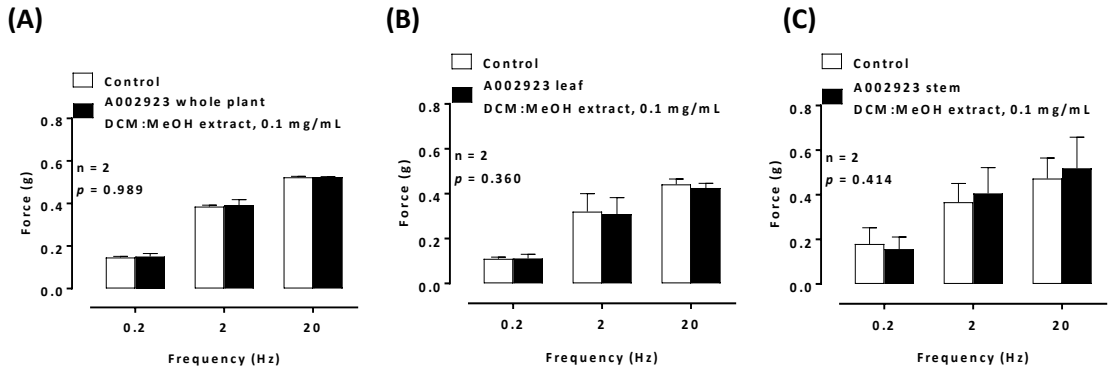


Figure 3-2. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostates after administration of A002923: **(A)** whole plant, **(B)** leaf and **(C)** stem DCM:MeOH extracts, respectively. Columns represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

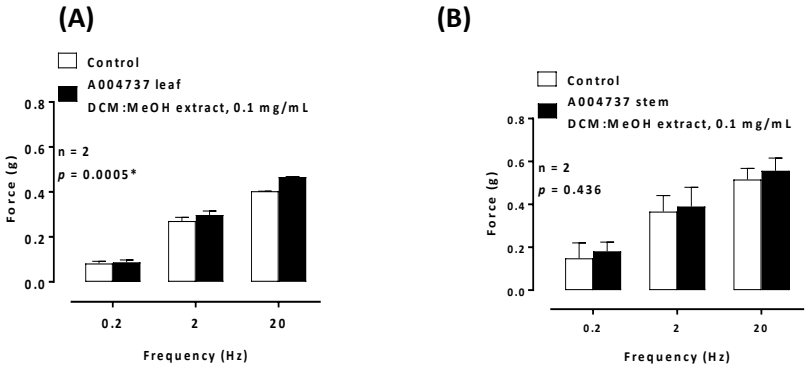


Figure 3-3. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostates after administration of A004737: **(A)** leaf and **(B)** stem DCM:MeOH extracts, respectively. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

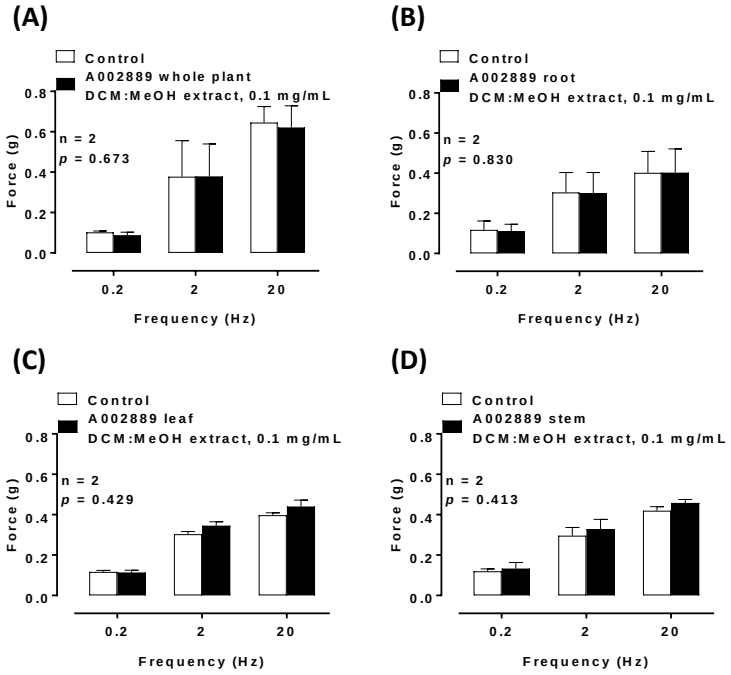


Figure 3-4. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostates after administration of A002889: **(A)** whole plant, **(B)** root, **(C)** leaf, and **(D)** stem DCM:MeOH extracts, respectively. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

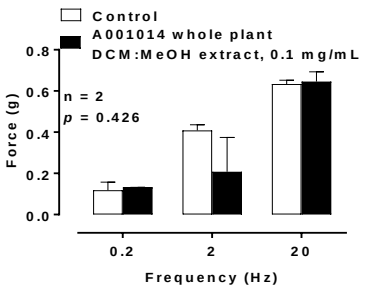


Figure 3-5. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostates before and after administration of A001014 whole plant DCM:MeOH extract. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

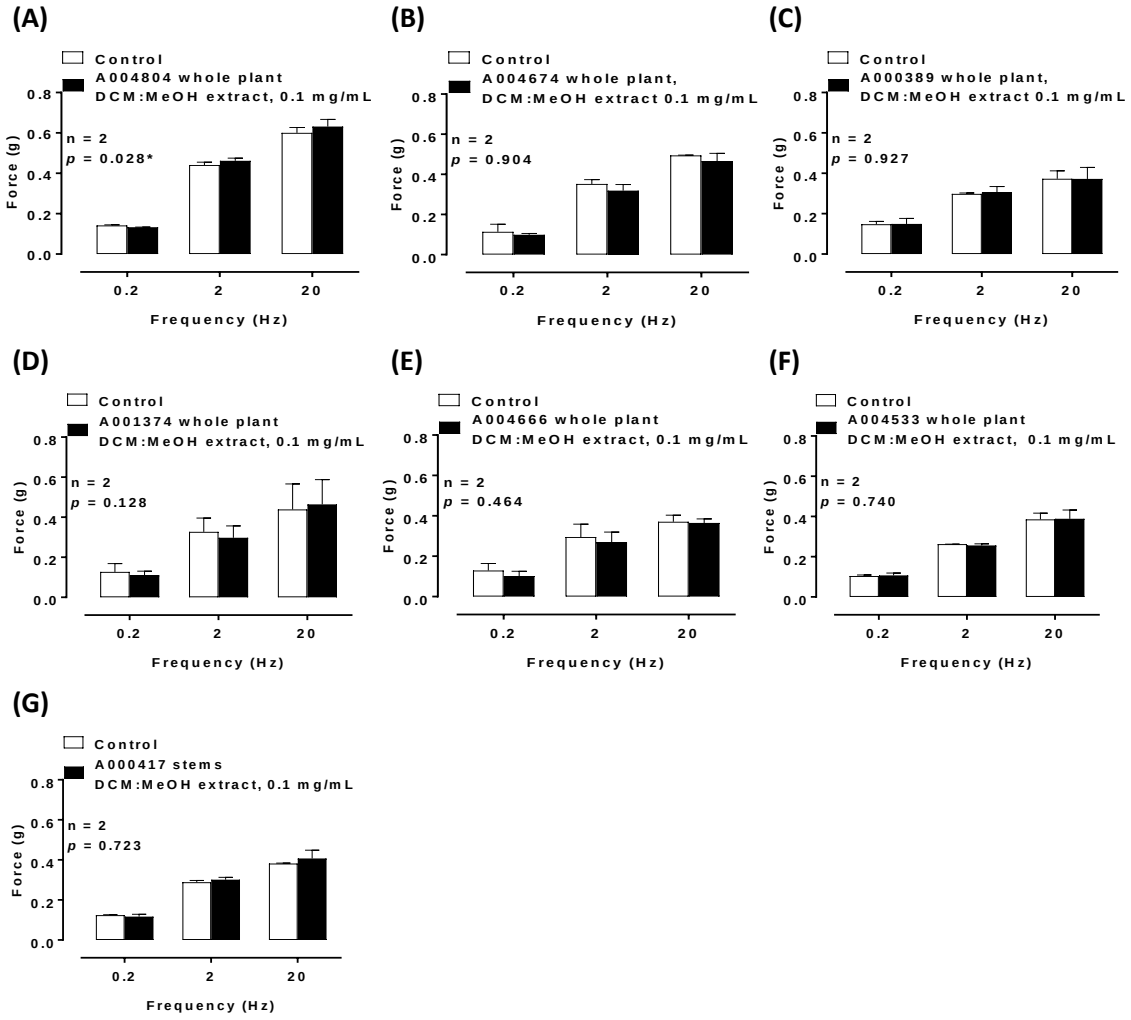


Figure 3-6. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostates before and after administration of **(A)** A004804, **(B)** A004674, **(C)** A000389, **(D)** A001374, **(E)** A004666 and **(F)** A004533 whole plant DCM:MeOH extract, and **(G)** A000417 stem DCM:MeOH extract. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

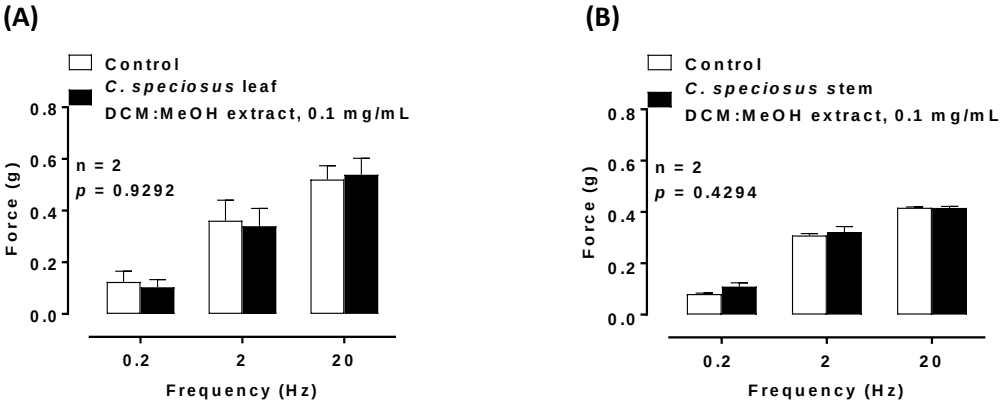


Figure 3-7. Comparison of mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.2, 2.0 and 20 Hz) in isolated rat prostates after administration of *C. speciosus* **(A)** leaf and **(B)** stem DCM:MeOH extracts at 0.1 mg/mL. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

3.3.2 Effects of *C. speciosus* Extracts from Different Extraction Methods on Isolated Rat Prostate Gland

3.3.2.1 Methanol Extraction

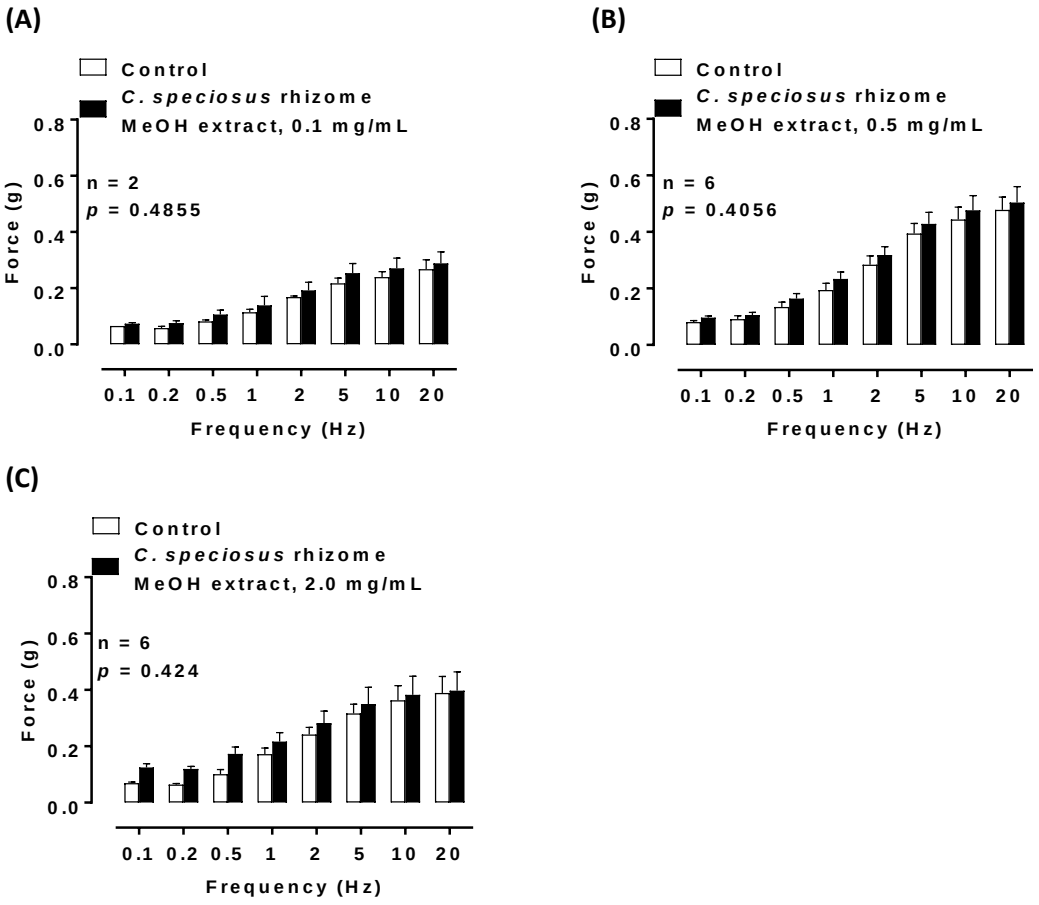


Figure 3-8. Comparisons of mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostate before and after administration of *C. speciosus* rhizome methanolic extract at 0.1 mg/mL, 0.5 mg/mL, and 2.0 mg/mL respectively. Bars represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

Nerve-mediated contractions of the isolated rat prostates treated with increasing concentrations of *C. speciosus* rhizome methanolic extract (0.1 mg/mL, 0.5 mg/mL, 2.0 mg/mL) in independent experiments were not affected when compared to the control (**Figure 3-8**).

3.3.2.2 Decoction Extraction

3.3.2.2.1 Effects of *C. speciosus* rhizome and root extract (without lyophilisation) on isolated rat prostate

Thirty minutes pre-incubation of isolated rat prostates with 20 µL fresh *C. speciosus* rhizome and root decoction extract had no effect on nerve-mediated prostatic contractility (**Figure 3-9**; $p = 0.9849$, n = 6).

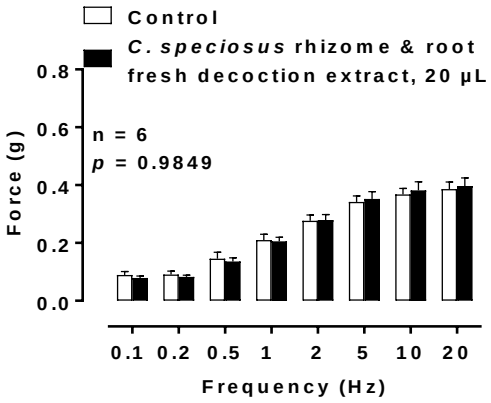


Figure 3-9. Mean contractile responses of isolated rat prostate to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostate before and after administration of *C. speciosus* rhizome and root fresh decoction extract. Columns represent mean force from six prostate preparations. Error bars represent S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

3.3.2.2.2 Effects of *C. speciosus* rhizome and root (with lyophilisation) decoction extract on isolated rat prostate

The *C. speciosus* rhizome and root decoction extract showed a significant concentration-dependent effect on nerve-mediated prostatic contractility (**Figure 3-10**). Nerve-mediated contraction of isolated rat prostate using EFS were not affected by *C. speciosus* rhizome and root decoction extract at lower concentrations as compared to the control (**Figure 3-10A, B, C**). At 1.5 mg/mL concentration, EFS-induced contractile responses of the isolated rat prostate were slightly attenuated (**Figure 3-10D**). In the experiment when isolated rat prostates were incubated with the highest concentration, 2.0 mg/mL, contractile responses mediated by EFS were significantly attenuated as compared to the control (**Figure 3-10E**; $p = 0.0161$, n = 4).

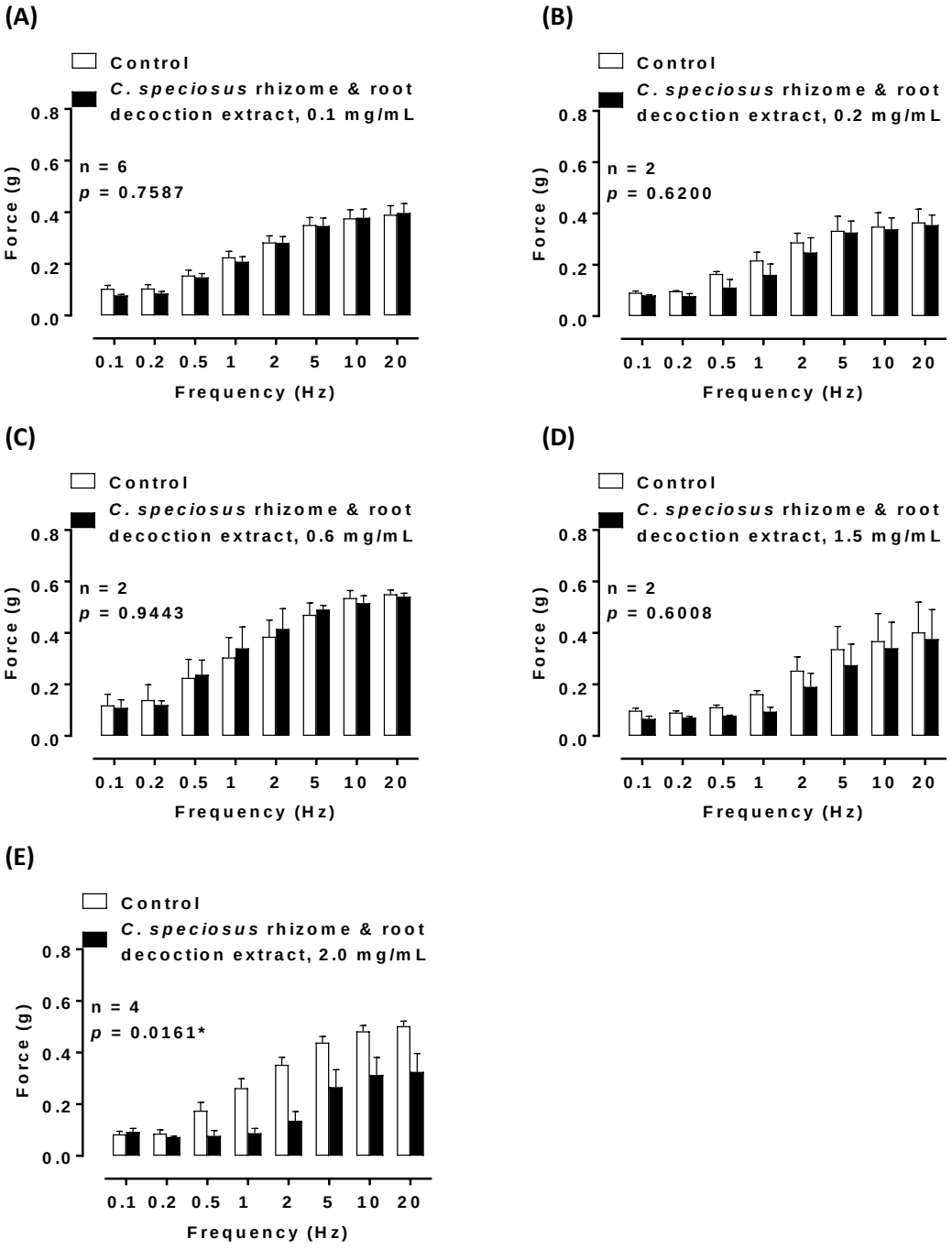


Figure 3-10. Mean contractile responses of isolated rat prostate before and after administration of *C. speciosus* rhizome and root crude extract by decoction extraction. Extract concentration: **(A)** 0.1 mg/mL, **(B)** 0.2 mg/mL, **(C)** 0.6 mg/mL, **(D)** 1.5 mg/mL, and **(E)** 2.0 mg/mL. Columns represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

3.3.2.2.3 Effects of *C. speciosus* rhizome decoction extract on isolated rat prostate

Incubation of isolated rat prostate with *C. speciosus* rhizome decoction extract at 2.0 mg/mL concentration attenuated EFS-induced contractile responses particularly at 0.5, 1.0, and 2.0 Hz frequency, as compared to the control (**Figure 3-11**).

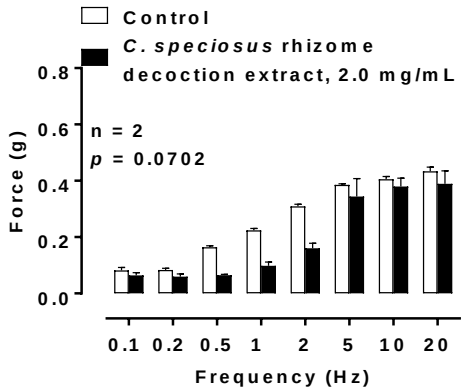


Figure 3-11. Mean contractile responses of isolated rat prostate before and after administration of *C. speciosus* rhizome decoction extract. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

3.3.2.3 Hot Water Extraction

3.3.2.3.1 Effects of *C. speciosus* rhizome and root hot water extract on isolated rat prostate

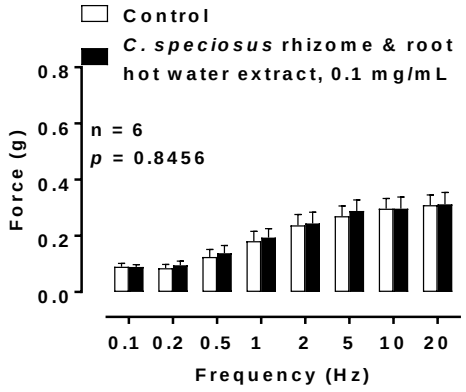


Figure 3-12. Mean contractile responses of isolated rat prostate before and after administration of *C. speciosus* rhizome hot water extract. Graph bars represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

C. speciosus rhizome hot water (70 ^{o}C) extract at 0.1 mg/mL concentration did not show any inhibitory effect on EFS-induced contractile responses of isolated rat prostate as compared to the control (**Figure 3-12**).

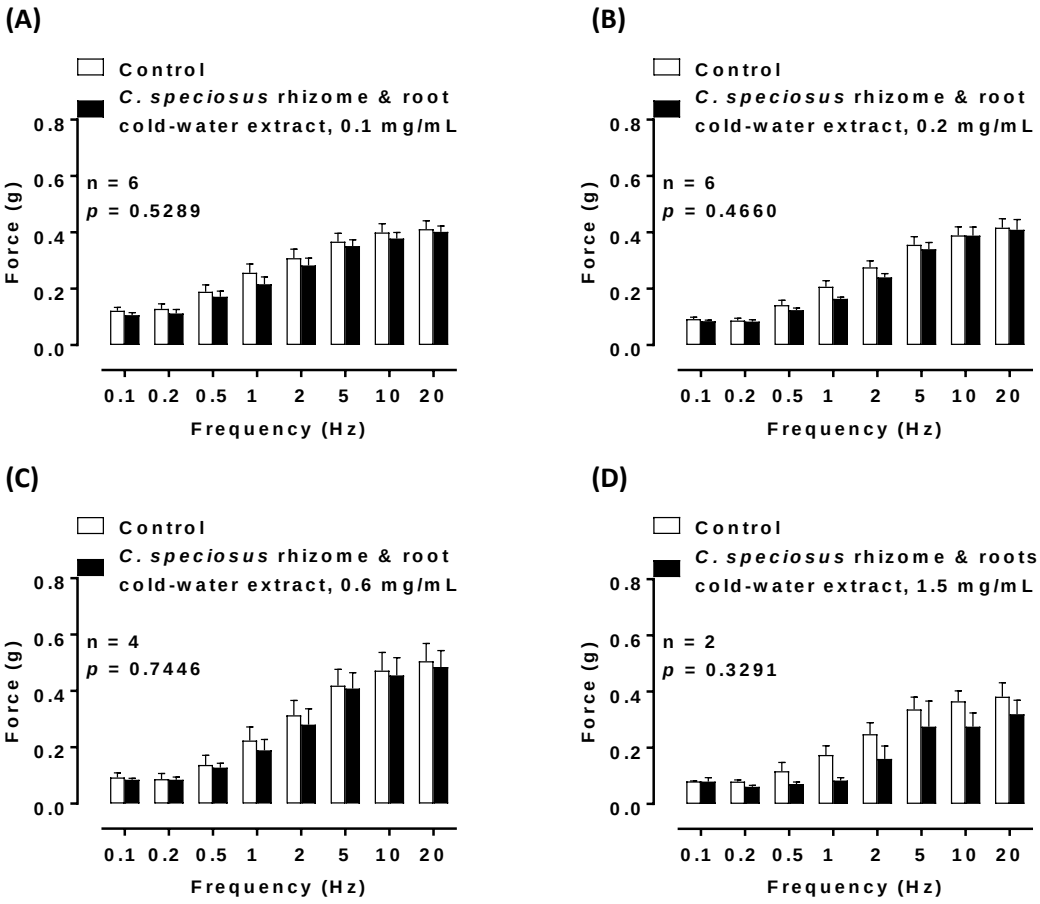
3.3.2.4 Maceration Extraction

3.3.2.4.1 Effects of *C. speciosus* rhizome and root cold-water extract on isolated rat prostate

C. speciosus rhizome and root cold-water extract (0.5 mg/mL – 2.0 mg/mL) caused a significant concentration-dependent relaxation of the EFS-induced contractile responses of isolated rat

prostate, as compared to the vehicle (**Figure 3-13**). A large amount of frothing was also observed during incubation of isolated rat prostates with *C. speciosus* rhizome, stem, leaf or root cold-water extract.

Nerve-mediated contraction of isolated rat prostate in response to EFS was not affected by *C. speciosus* rhizome and root cold-water extract at lower concentrations as compared to the control (**Figure 3-13A, B, C**). At 1.5 mg/mL, EFS-induced contractile responses of the isolated rat prostate were slightly attenuated (**Figure 3-13D**). In the experiment when isolated rat prostates were incubated with a higher concentration of extract 2.0 mg/mL, nerve-mediated contractions of the isolated rat prostate were significantly attenuated (**Figure 3-13E**; p = 0.0034, n = 4), as compared to other concentrations tested (**Figure 3-13A, B, C, D**).



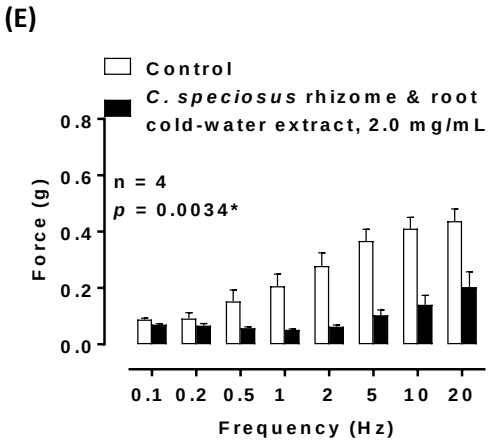
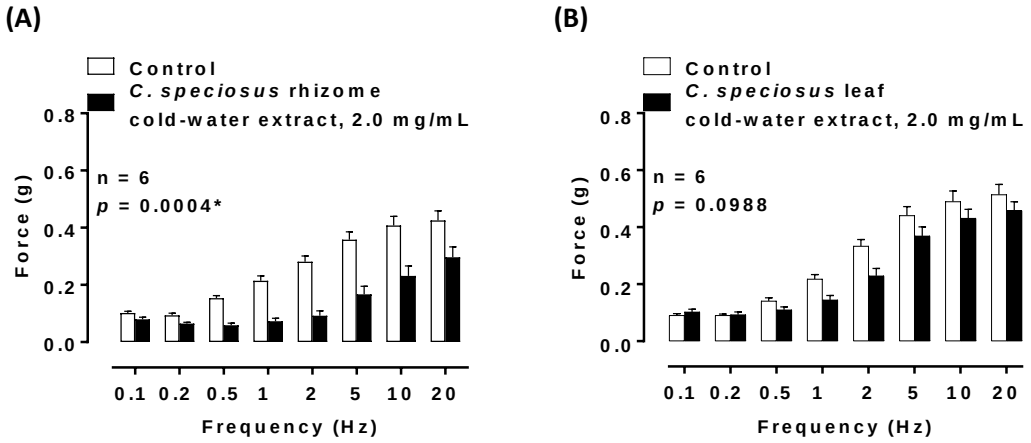


Figure 3-13. Mean contractile responses of isolated rat prostate before and after administration of *C. speciosus* rhizome and root crude extract from cold-water extraction. Extract concentration: **(A)** 0.1 mg/mL, **(B)** 0.2 mg/mL, **(C)** 0.6 mg/mL, **(D)** 1.5 mg/mL, and **(E)** 2.0 mg/mL. Columns represent mean force ± S.E.M. (**p* < 0.05; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

3.3.2.4.2 Effects of *C. speciosus* rhizome, leaf, stem, and root cold-water extracts on isolated rat prostate

C. speciosus rhizome (**Figure 3-14A**; *p* = 0.0004, n = 6), stem (**Figure 3-14C**; *p* = 0.0057, n = 6), and root (**Figure 3-14D**; *p* < 0.0001, n = 6) cold-water extract at concentration 2.0 mg/mL caused inhibition of electrically evoked contractile responses of the isolated rat prostate as compared to the control. The same inhibitory effect was not observed in the *C. speciosus* leaf cold-water extract (**Figure 3-14B**; *p* = 0.0988, n = 6).



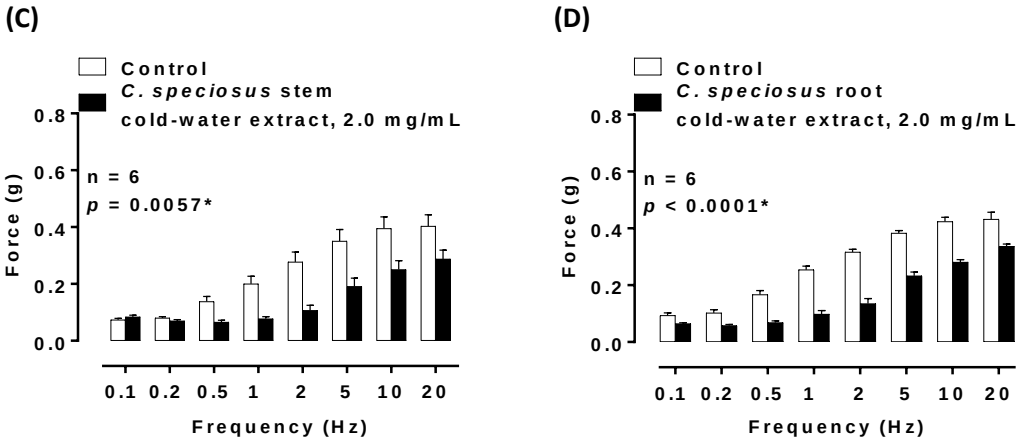


Figure 3-14. Mean contractile responses of isolated rat prostate before and after administration of *C. speciosus*: **(A)** rhizome (2.0 mg/mL; $p = 0.0004$, n = 6), **(B)** leaf (2.0 mg/mL; $p = 0.0988$, n = 6), **(C)** stem (2.0 mg/mL; $p = 0.0057$, n = 6), and **(D)** root (2.0 mg/mL; $p < 0.0001$, n = 6) cold-water crude extracts. Columns represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of the treatment causing a significant change in the contractile responses.

3.3.2.4.3 *C. speciosus* extracts caused tonic contraction on isolated rat prostate

In addition to the inhibitory effect displayed by *C. speciosus* rhizome/stem/root cold-water extract on EFS-induced contractile responses of the isolated rat prostates, the extracts also produced a tonic contraction of isolated rat prostates upon administration to the organ bath (**Figure 3-16**). The tonic contraction generated was transient and the force returned to baseline levels after 10 – 30 seconds depending on the type of extract administered (**Figure 3-15**). From the four *C. speciosus* extracts, leaf cold-water extract elicited the smallest tonic contraction magnitude on isolated rat prostates (**Figure 3-16B**). Rhizome and stem cold-water extracts both produced comparable tonic contractions of approximately 2.8 g.s force (**Figure 3-16A, C**). It is worth noting that, although root cold-water extract possessed a greater inhibitory effect, the extract produced a slightly weaker tonic contraction when compared to rhizome and stem (**Figure 3-16D**).

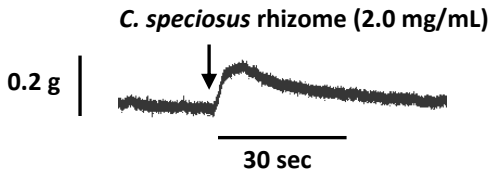


Figure 3-15. Representative trace of the tonic contractile responses of the isolated rat prostate upon administration of *C. speciosus* rhizome cold-water extract.

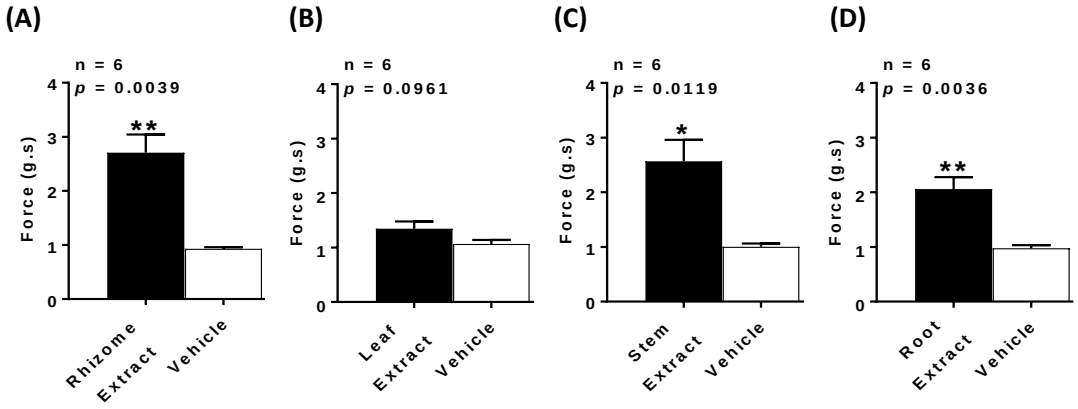


Figure 3-16. Mean area under the curve (force x time) of tonic contractions elicited by unstimulated isolated rat prostates after administration of *C. speciosus* **(A)** rhizome (p = 0.0085, n = 6), **(B)** leaf (p = 0.0961, n = 6), **(C)** stem (p = 0.0119, n = 6), and **(D)** root (p = 0.0036, n = 6), respectively at 2.0 mg/mL. Columns represent mean force ± S.E.M. (*p < 0.05). p-values represent the probability of the difference between extract and vehicle causing a significant change in the contractile responses (paired t-test).

Overall vehicle control result for electrical field stimulation-induced contractions of rat prostatic smooth muscle indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle **Figure 3-17**.

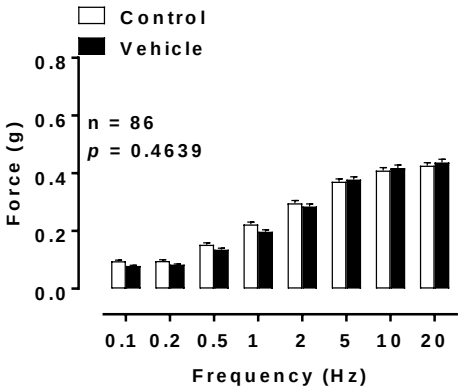


Figure 3-17. Mean contractile responses to electrical field stimulation on isolated rat prostate in the absence (open bars) and presence (closed bars) of vehicle is reproducible over the time course of the experimental protocol and not affected by vehicle (p = 0.4639, n = 86). Columns represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of the treatment causing a change in the contractile responses.

Table 3-1. Summary of the bioactivity of *C. speciosus* extracts. Efficacy levels were based on the comparative inhibitory effects on EFS-induced contractility depicted in **Figure 3-7** to **Figure 3-14**.

Plant part	Extraction Method	Temperature	Vehicle	Concentration (mg/mL)	Efficacy
Rhizome	Methanol	N/A	DMSO	0.1	-
	Methanol	N/A	DMSO	0.5	-
	Methanol	N/A	DMSO	2.0	-
	Decoction	100 °C	Milli-Q water	2.0	Low
	Maceration	R.T	Milli-Q water	2.0	Medium
Leaf	DCM:MeOH	N/A	DMSO	0.1	-
	Maceration	R.T	Milli-Q water	2.0	Low
Stem	DCM:MeOH	N/A	DMSO	0.1	-
	Maceration	R.T	Milli-Q water	2.0	Medium
Root	Maceration	R.T	Milli-Q water	2.0	Medium
Rhizome & Root	Decoction	100 °C	Milli-Q water	0.1	-
			Milli-Q water	0.2	-
			Milli-Q water	0.6	-
			Milli-Q water	1.5	Low
			Milli-Q water	2.0	Medium
Rhizome & Root	Maceration	R.T	Milli-Q water	0.1	-
			Milli-Q water	0.2	-
			Milli-Q water	0.6	-
			Milli-Q water	1.5	Medium
			Milli-Q water	2.0	High
Rhizome & Root	Hot water	70 °C	Milli-Q water	0.1	-

Notes:
R.T : Room Temperature
N/A : Not Available
No inhibition : -
Efficacy level 1 : Low (10 – 30 %)
Efficacy level 2 : Medium (30 – 60 %)
Efficacy level 3 : High (60 – 80 %)

3.4 DISCUSSION

Preliminary screening of 12 plant extracts shortlisted (Chapter 2, **Table 2-1**, No. 4 – 23) from the SBC NPL, did not attenuate EFS-induced contractile responses of the isolated rat prostates (**Figure 3-1** - **Figure 3-6**). Nevertheless, A001014 whole plant DCM:MeOH extract seems to attenuate contractile response induced at 2 Hz electrical field stimulation (**Figure 3-5**). However, due to insufficient amount of extract, repetition of this experiment was not possible. Traditional preparations of these twelve plant species were different from the extraction method performed in the laboratory. Laboratory extract samples were extracted using a mixture of dichloromethane/methanol (DCM:MeOH) in a ratio 1:1 solvent system. Plant extracts extracted using this solvent system contained more lipophilic compounds (Sasidharan et al., 2011) than extracts prepared via traditional techniques. There are two explanations as to why these twelve plant species extracts did not affect contractile responses of the isolated rat prostate induced by electrical field stimulation; a) potential active constituents are lost, distorted or destroyed during the extraction process using the DCM:MeOH solvent system; b) the plant extracts did not contain bioactive constituents that affect nerve-mediated contractions of the isolated rat prostate.

In a cell-based assay, methanolic extracts of *C. speciosus* rhizome were found to exhibit anticancer activity, showing increased cytotoxic activity with a 50 % inhibition of the total cell growth (GI_{50}) value from 11.5 µg/mL (first column chromatography separation) to 0.7 µg/mL (third column chromatography separation) for lung carcinoma (NCI-H460) and 10.5 µg/mL (first column chromatography separation) to 0.7 µg/mL (third column chromatography separation) for MCF-7 cancer cell lines (Su et al., 2009).

Traditionally, *C. speciosus* rhizome or stem has been grilled on a steel plate then apply topically at the bladder area as traditional medicine to treat urinary disease. Alternatively, the rhizome and stem were burned and eaten as traditional medicine to treat urinary diseases by women of the Lun Bawang indigenous community in Sarawak. However, concentration-dependent results showed that methanolic extract of *C. speciosus* rhizome did not affect the nerve-mediated smooth muscle contraction of the isolated rat prostates induced by electrical field stimulation (**Figure 3-8**).

Traditionally, medicinal plants are prepared either singly or as part of a combination treatment in the form of an aqueous extract. Since there was no effect caused by methanolic extracts of *C. speciosus* rhizome (**Figure 3-8**), as well as DCM:MeOH extracts of *C. speciosus* leaf (**Figure 3-7A**) and stem (**Figure 3-7B**), alternative extraction approaches were performed. These alternative approaches were designed to mimic as much as possible traditional preparations to ensure potential active constituents were not lost, destroyed or changed during the extraction. These extraction approaches used water as the solvent system, at varied temperatures. The extracts extracted using water, contained hydrophilic compounds. The extraction methods performed were, a) decoction extraction, b) hot water extraction, and c) maceration/cold water (room temperature) extraction.

The rationale for preparing *C. speciosus* extract using the decoction method was to mimic the traditional preparation method of intensely heating the rhizome and stem on a grill. However, administration of fresh decoction extract of *C. speciosus* rhizome and root combination did not show any effect on EFS-induced contractions of isolated rat prostate (**Figure 3-9**). It is worth noting that the fresh extract has a short storage lifespan due to its susceptibility to bacteria or fungus contamination, hence, the extract was dried using a lyophilization process. Although there is evidence to suggest lyophilization does not perfectly preserve medicinal compounds (Abascal et al., 2005), in our hands lyophilized extracts when stored at -80 ^{o}C in the dark, were more potent than fresh decoction extracts, while still maintaining reproducible results.

The lyophilised decoction extracts of the *C. speciosus* rhizome and root combination, attenuated EFS-induced contractile responses of isolated rat prostates in a concentration-dependent manner. A similar but weaker activity, was also observed with the *C. speciosus* rhizome decoction extract. This indicates that the *C. speciosus* root extract might just have higher concentration of bioactive constituents compared to the rhizome, thus producing higher potency. Decoction extraction was not performed for *C. speciosus* root, due to the limited quantity of plant material. Hot water extract of *C. speciosus* rhizome and root combination also did not show any inhibitory effect on nerve-mediated contraction induced by EFS on isolated rat prostates. The results for both decoction and hot water extracts indicated that potential bioactive constituents in *C. speciosus* rhizome and root may be thermally labile as high boiling extraction temperatures often lead to degradation of heat-sensitive compounds. Therefore, the maceration approach at room temperatures was applied to prepare extract from different plant parts of *C. speciosus*. Results observed from cold-water extracts of *C. speciosus* further

supported the suggestion that the bioactive compounds that inhibited EFS-induced contractile responses of rat prostates are thermally labile.

Among the different parts of *C. speciosus* screened in this study, root cold-water showed the highest inhibitory potency on isolated rat prostate contractile responses induced by EFS. This was followed by rhizome and then stem. *C. speciosus* leaf cold-water extract showed the least inhibitory effect on nerve-mediated contractions of isolated rat prostate. This indicates that the bioactive constituents that attenuate contractile responses of isolated rat prostate induced by EFS are only minimally present in the leaf of *C. speciosus*. This is consistent with the traditional medicine practices of the Lun Bawang community who tend to use the rhizome, stem and root of the *C. speciosus* plant.

The optimum effective concentration of *C. speciosus* rhizome, stem or root cold-water extract that significantly attenuated EFS-induced contractile responses of isolated rat prostates was 2.0 mg/mL. One of the important challenges related to the use of plants as a source for bioactive compounds is insufficient compound quantities needed for thorough characterization of the pharmacological activity of its bioactive compounds. Herein therefore, rhizome of the *C. speciosus* has been chosen to be used in this study although root has higher bioactivity potency. The effective concentration was subsequently used to determine the mechanism of action of *C. speciosus* rhizome cold-water extract on isolated rat prostatic smooth muscle contractility.

Interestingly, apart from an inhibitory effect seen with the *C. speciosus* rhizome, stem or root cold-water extract, these extracts also produced a transient tonic contraction of rat prostate upon administration into the isolated organ bath. Hence, it is likely that other bioactive constituents are present in the extract which elicit the tonic contraction via a different biological mechanism of action.

C. speciosus methanol extract had no response on EFS induced contractions of isolated rat prostates. Since noradrenaline and ATP are released during EFS, it is assumed that the extract does not directly affect these contractions. Therefore, the extract was only tested on contractions mediated by acetylcholine to determine whether it had an effect on some other post-junctional mechanism

The quantity of the compounds present in the plant extract is variable owing to the variability in the extraction procedures, as well as natural variability in plants (Lowe and Fagelman, 1999). This study demonstrates that different extraction methods can greatly influence the chemical composition of plant extracts, thus affecting their bioactive profiles. In this case, only aqueous extracts of *C. speciosus* showed an effect on nerve-mediated contractile responses of isolated rat prostate. It is possible that the *C. speciosus* extracts might contain saponin chemical compounds, as the extracts produced soap-like bubbles during extractions. Furthermore, this study also revealed that cold-water extract of *C. speciosus* rhizome, stem, or root, were optimal for maintaining efficacy in attenuating rat prostatic smooth muscle contractility induced by EFS. Medicines that can relax or diminish prostatic smooth muscle tone have been shown to be beneficial in treating LUTS symptoms associated with BPH, thus it is possible that extracts from this plant may have benefits in treating urinary symptoms.

3.5 CONCLUSION

The twelve plant extracts of plants used traditionally by Sarawak indigenous communities for the treatment of various urinary diseases showed no response on electrically induced contraction of isolated rat prostates. This study demonstrated that only aqueous extracts particularly the cold-water extract of *C. speciosus* rhizome, stem, and root, showed an effect on nerve-mediated contractile responses of isolated rat prostate.

4

Identification of Biological Mechanisms of Action of *Costus speciosus* on Isolated Rat Prostate using Pharmacological Tools

4.1 INTRODUCTION

Cold-water extract of *C. speciosus* rhizome, stem, and root, was demonstrated to possess significant inhibitory effects on nerve-mediated contractile responses of isolated rat prostate induced by electrical field stimulation in this study - Effects of natural products on contractility in the prostate gland. To our knowledge, there are no studies that report *C. speciosus* activity relating to the prostate gland including functional studies to determine the pharmacological effects of *C. speciosus* on prostatic smooth muscle contractility.

4.1.1 Aim

The aim of this study was to identify mechanism of actions for the *C. speciosus* rhizome cold-water extract on isolated rat prostate by using different pharmacological agents acting on membrane bound receptors or extracellular messengers which have been suggested to play a role in mediating the contractility of prostate smooth muscle (for review of the signaling pathways in detail, refer to (Ventura et al., 2011) (**Figure 4-1**).

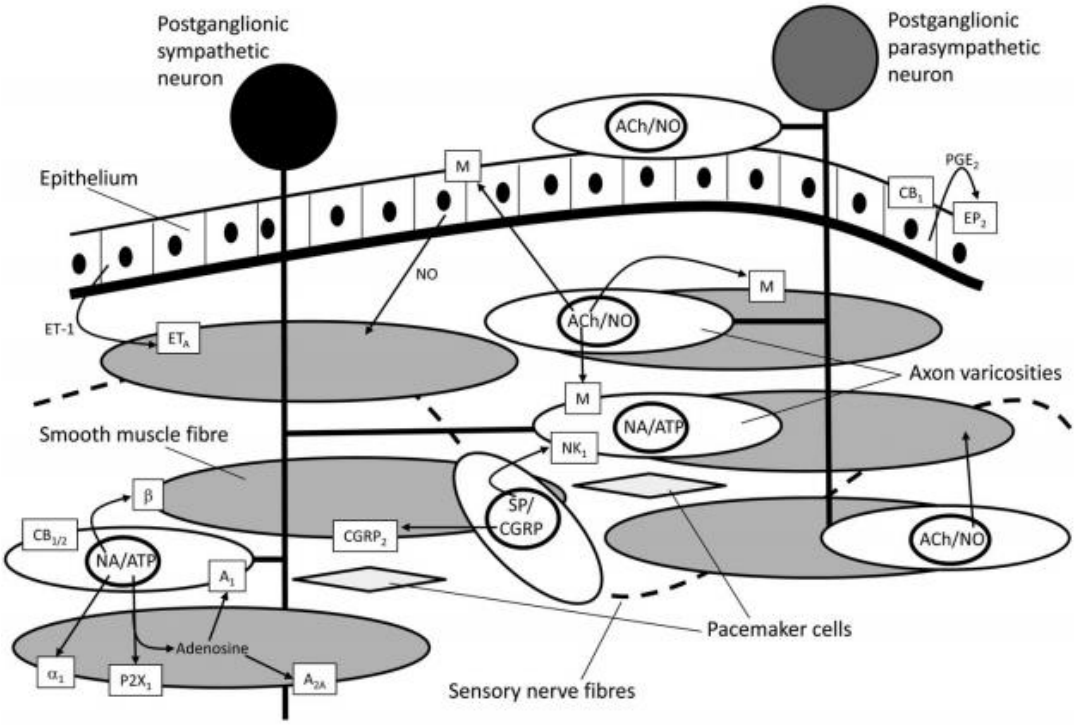


Figure 4-1. Schematic representative diagram depicting the membrane bound receptors and extracellular messengers involved in the contractility of prostate smooth muscle based on experimental data derived from different species (Ventura et al., 2011).

4.2 MATERIALS AND METHODS

General methods for animal husbandry, dissection and animal ethics are detailed in Chapter 2, Section 2.1. Details for the plant collection, preparations, as well as processing can be found in Chapter 2, Section 2.2.

4.2.1 Isolated Organ Bath studies

General methods on performance of organ bath studies including tissue preparations, initial period of equilibrium as well as parameters of the experiments are detailed in Chapter 2, Section 2.5.

4.2.2 Frequency Response Graph

General information on construction of mean frequency response curves are described in Chapter 2, Section 2.6.

4.2.3 Agonist Concentration-response Curve

General information in construction of mean agonist concentration-response curves are described in Chapter 2, Section 2.7.

4.2.4 Plant Materials

Details of the extraction methods to prepare *C. speciosus* rhizome methanol extract and cold-water extract were described in Chapter 2, Section 2.3.1.4, and Section 2.3.2, respectively.

4.2.5 Drugs and Solutions

Details of all agonists and antagonists including vehicles used were described in Chapter 2, Section 2.8, **Table 2-3**, **Table 2-4**, and **Table 2-5**.

4.2.6 Effects of *C. speciosus* Rhizome Cold-water Extract on Stimulation-induced Contractions in the Presence of Receptor Antagonists

The mechanism of action of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on electrical field stimulation-induced contractile responses of isolated rat prostatic smooth muscle was investigated in the presence of various receptor antagonists. After initial equilibration for 30 minutes, isolated rat prostates were pre-incubated with one or a combination of the pharmacological tool listed in **Table 2-5**, for another 30 minutes. These drugs were present in the isolated organ bath for the remainder of the duration of the experiment to block their respective target receptors.

4.2.7 Analysis of Data

General information on data analysis was described in Chapter 2, Section 2.9.

4.3 RESULTS

4.3.1 Effects of *C. speciosus* Rhizome Cold-water Extract on Contractile Responses to Exogenous Noradrenaline and Phenylephrine

Exogenously administered noradrenaline (1 nM – 100 µM) elicited reproducible concentration-dependent contractions of isolated rat prostate preparations. Cold-water extract of *C. speciosus* rhizome (2.0 mg/ml) caused a small shift to the right in the mean noradrenaline concentration-response curve. This was also associated with a slight reduction in the maximum response to noradrenaline (**Figure 4-2A**). However, this was not different compared to vehicle (**Figure 4-2C**). There was no effect of the extract to the contractile responses elicited by exogenous administration of the agonist phenylephrine (**Figure 4-2B**).

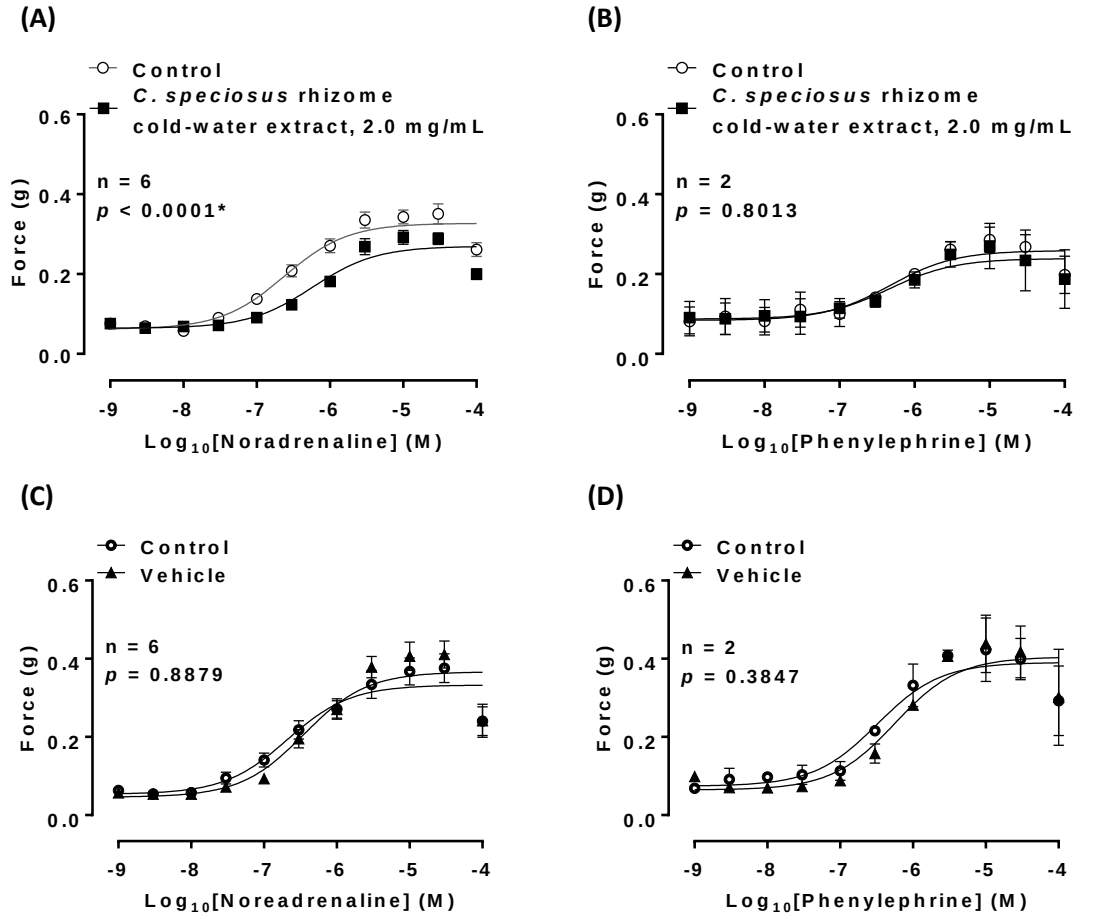


Figure 4-2. Mean log concentration-response curves to **(A)** noradrenaline and **(B)** phenylephrine on isolated rat prostate in the presence (■) and absence (○) of *C. speciosus* rhizome cold-water extract (2.0 mg/mL). Concentration-response curves to **(C)** noradrenaline and **(D)** phenylephrine, on isolated rat prostate in the presence (▲) and absence (O) of vehicle. Symbols and error bars represent mean ± S.E.M. *p*-values represent the probability of a significant interaction between treatment and concentration ($p < 0.05$; two-way repeated measures ANOVA).

4.3.2 Effects of *C. speciosus* Rhizome Methanol and Cold-water Extract on Contractile Responses to Exogenous Acetylcholine

Exogenously administered acetylcholine (1 nM – 100 µM) elicited reproducible concentration-dependent contractions of isolated rat prostate. The methanol extract of *C. speciosus* rhizome had no effect on contractile responses elicited by acetylcholine in isolated rat prostates (0.5 mg/mL) (**Figure 4-3A**; $p = 0.0555$, n = 6). Similar effects were observed in the vehicle control, indicating that there was a time-course effect over the duration of this experiment (**Figure 4-3C**). Cold-water extract of *C. speciosus* rhizome (2.0 mg/mL), on the other hand, caused a small shift to the right (**Figure 4-3B**; $p = 0.0020$, n = 6). It is noteworthy that there was a slight reduction in the maximum response to acetylcholine in the presence of *C. speciosus* rhizome cold-water extract. The reduction was significant ($p = 0.0020$, n = 6) as compared to methanol extract of *C. speciosus* rhizome (0.5 mg/mL). Vehicle control indicated that the concentration-response curves were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-3D**).

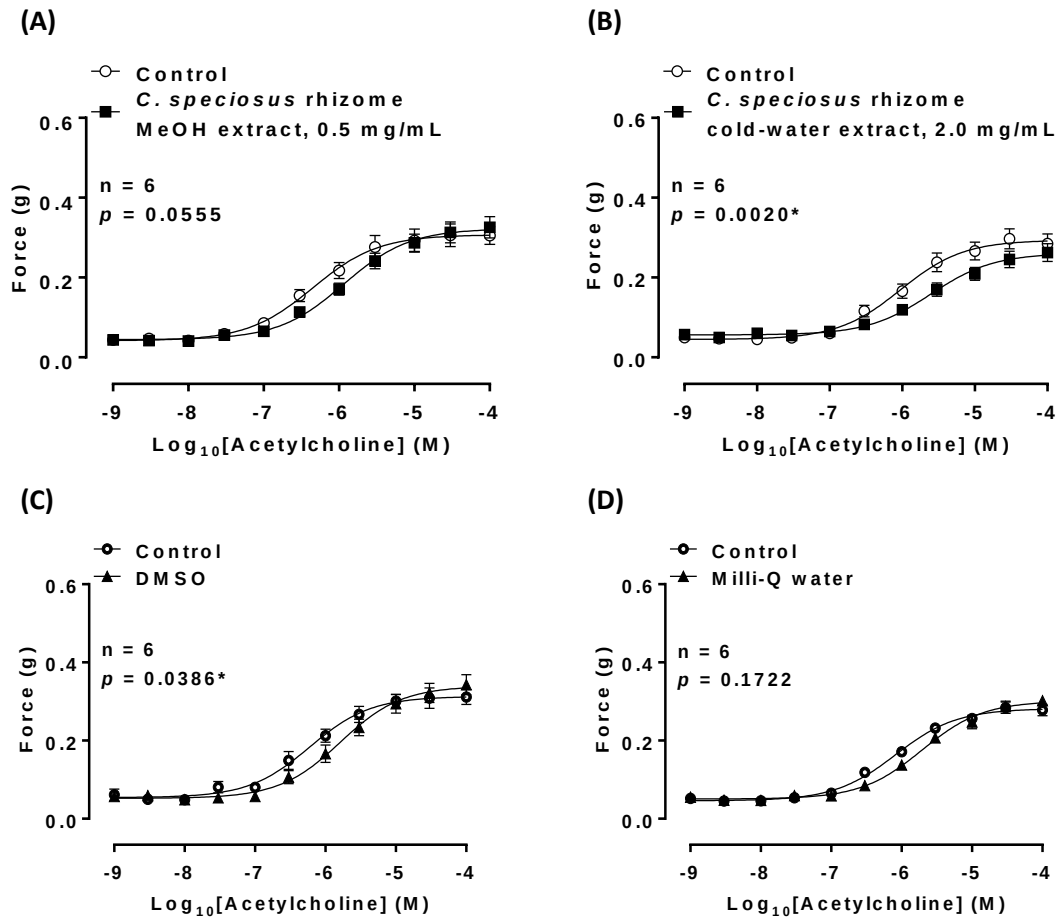


Figure 4-3. Mean log concentration-response curves to acetylcholine on isolated rat prostate in the presence (■) and absence (○) of *C. speciosus* rhizome **(A)** methanol extract (0.5 mg/mL) (p = 0.0555, n = 6) and **(B)** cold-water extract (2.0 mg/mL) (p = 0.0020, n = 6). Concentration-response curves to acetylcholine on isolated rat prostate in the presence (▲) and absence (O) of **(C)** DMSO (p = 0.0386, n = 6) or **(D)** Milli-Q water (p < 0.0001, n = 6). Symbols and error bars represent mean ± S.E.M. p-values represent the probability of a significant interaction between treatment and concentration (p < 0.05; two-way repeated measures ANOVA).

4.3.3 Effects of *C. speciosus* Rhizome Cold-water Extract on Contractile Responses to Exogenous ATP

Exogenously administered ATP (300 nM – 1 mM) elicited concentration-dependent contractions of isolated rat prostate. The cold-water extract of *C. speciosus* rhizome (2.0 mg/mL) was also found to cause a slight shift to the right in the mean ATP concentration-response curve in isolated rat prostates (**Figure 4-4A**; p < 0.0010, n = 6). It is noteworthy that there was a slight reduction in the maximum response to ATP seen in the presence of *C. speciosus* rhizome cold-water extract. However similar effects were observed in the vehicle control, indicating that there was a time-course effect over the duration of this experiment (**Figure 4-4B**).

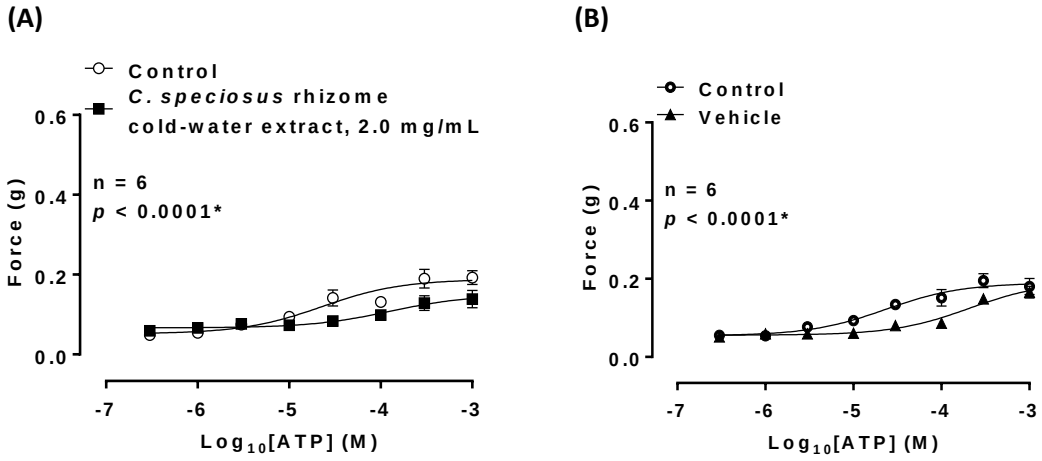


Figure 4-4. Mean log concentration-response curves to ATP on isolated rat prostate **(A)** in the presence (■) and absence (○) of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) (p < 0.0001, n = 6). **(B)** Mean log concentration-response curves to ATP on isolated rat prostate in the presence (▲) and absence (O) of vehicle. Symbols and error bars represent mean ± S.E.M. p-values represent the probability of a significant interaction between treatment and concentration (p < 0.05; two-way repeated measures ANOVA).

4.3.4 Effects of *C. speciosus* Rhizome Cold-water Extract on Contractile Responses to Exogenous Tyramine

Exogenously administered tyramine (10 nM – 100 µM) elicited reproducible concentration-dependent contractions of isolated rat prostate over the time course of the experimental

protocol (**Figure 4-5B**; $p = 0.6650$, n = 4). Of note, the concentration-response curve to tyramine in the presence of *C. speciosus* rhizome (2.0 mg/mL) was shifted to the right by a similar magnitude to that caused by vehicle. Therefore, the cold-water extract of *C. speciosus* rhizome (2.0 mg/mL) was found to have no effect on contractile responses elicited by tyramine in isolated rat prostate. (**Figure 4-5A**; $p = 0.0256$, n = 4).

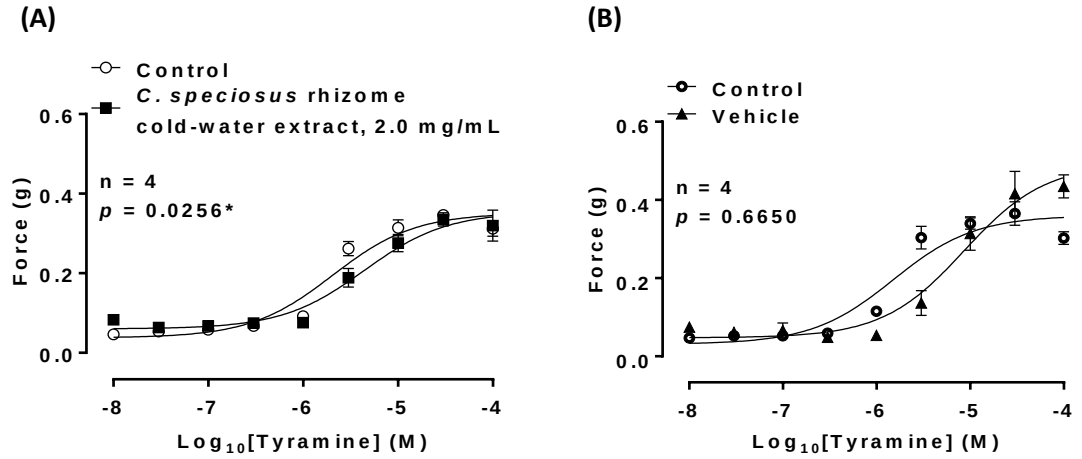


Figure 4-5. Mean log concentration-response curves to tyramine on isolated rat prostate **(A)** in the presence (■) and absence (○) of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) ($p = 0.0256$, n = 4). **(B)** Mean log concentration-response curves to tyramine on isolated rat prostate in the presence (▲) and absence (**O**) of vehicle ($p = 0.6650$, n = 4). Symbols and error bars represent mean ± S.E.M. *p*-values represent the probability of a significant interaction between treatment and concentration ($p < 0.05$; two-way repeated measures ANOVA).

4.3.5 Effects of Prazosin

In the presence of the α_1-adrenoceptor selective antagonist, prazosin (300 nM) *C. speciosus* rhizome cold-water extract (2.0 mg/mL) was still able to inhibit the contractile response of isolated rat prostates to electrical field stimulation, however the inhibition was reduced (**Figure 4-6A**; $p = 0.0715$, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-6B**; $p = 0.6990$, n = 6).

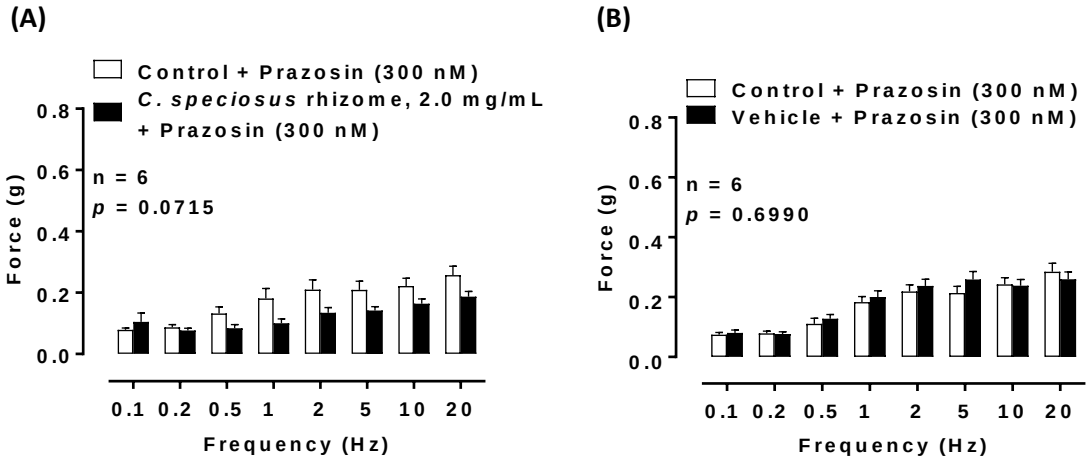


Figure 4-6. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) of isolated rat prostates in the presence of prazosin (300 nM) before and after administration of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) (p = 0.0715, n = 6) **(A)**. Mean contractile responses to electrical field stimulation of isolated rat prostate in the presence of prazosin (300 nM) before (open bars) and after (solid bars) vehicle over the time course of the experimental protocol (p = 0.6990, n = 6). Bars represent mean force ± S.E.M. p-values represent the probability of a significant interaction between treatment and frequency ($p < 0.05$; two-way repeated measures ANOVA).

4.3.6 Effects of Yohimbine and Idazoxan

Mean frequency-response curves to electrical field stimulation in isolated rat prostates after administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of yohimbine (1 µM) were not different compared to control (**Figure 4-7A**; p = 0.0042, n = 6).

Idazoxan (1 µM) is also a potent and non-subtype selective α_2-adrenoceptor antagonist (Lladó et al., 1996). Pre-incubation of isolated rat prostate with idazoxan (1 µM) did not decrease the inhibitory effect exhibited by the *C. speciosus* rhizome cold-water extract on contractile responses mediated by electrical field stimulation (**Figure 4-7C**; p = 0.0100, n = 6).

Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle for yohimbine (**Figure 4-7B**; p = 0.8167, n = 6) and idazoxan (**Figure 4-7D**; p = 0.6025, n = 6).

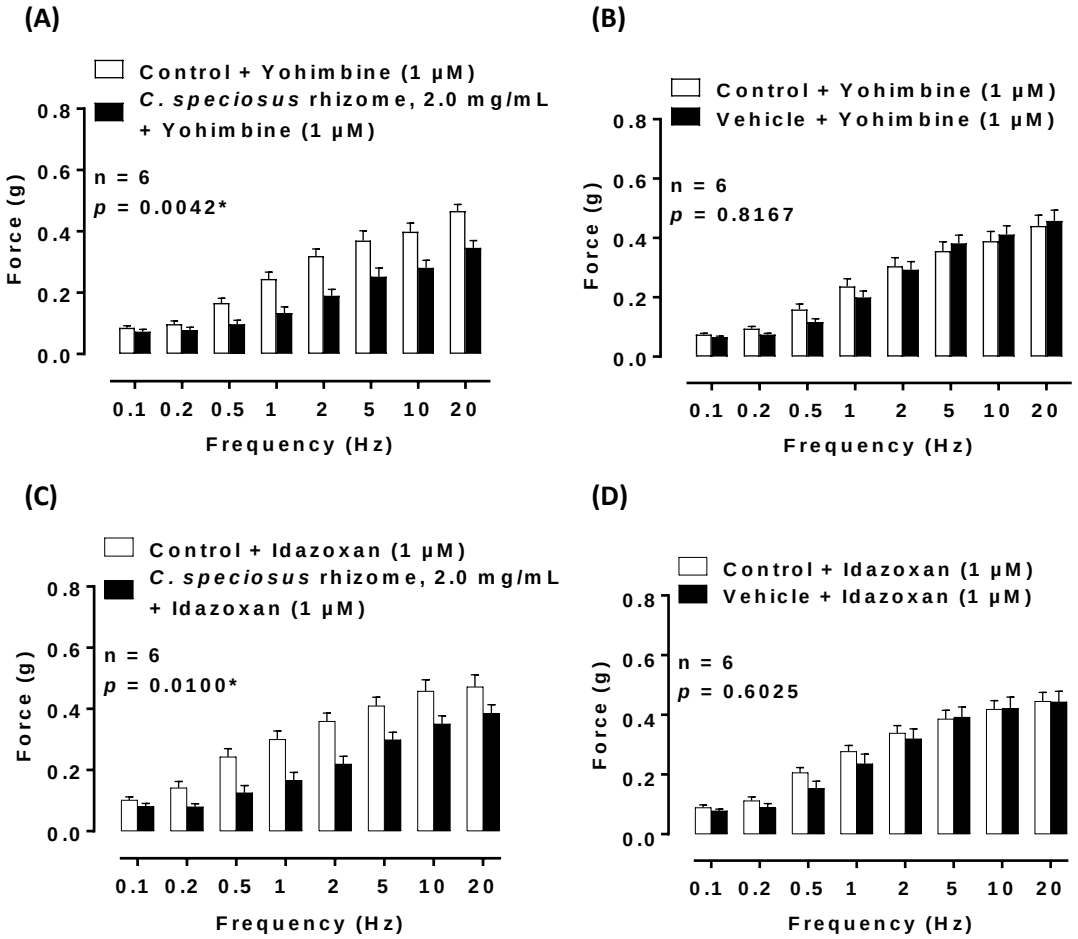


Figure 4-7. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of **(A)** yohimbine (1 µM) (p = 0.0042, n = 6) and **(C)** idazoxan (1 µM) (p = 0.0100, n = 6), respectively. Mean contractile responses to electrical field stimulation on isolated rat prostate before (open bars) and after (solid bars) administration of vehicle is reproducible over the time course of the experimental protocol and not affected by vehicle. Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.7 Effects of Desipramine

Preincubation of isolated rat prostates with the selective noradrenaline reuptake transporter (Uptake 1) inhibitor, desipramine (100 nM), had no effect on the inhibitory effect exerted by *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on the electrical field stimulation-induced contractile responses of isolated rat prostates (**Figure 4-8A**; p = 0.0003, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-8B**; p = 0.4965, n = 6).

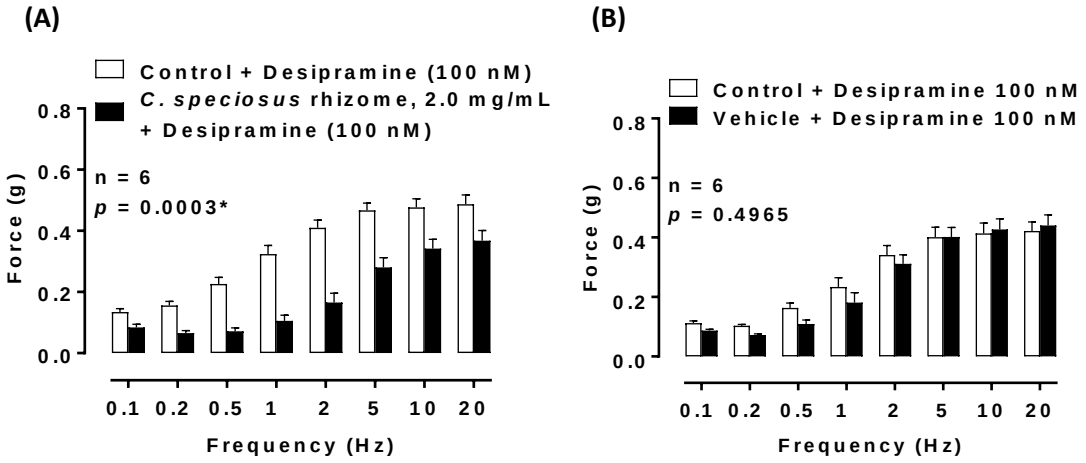


Figure 4-8. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of desipramine (100 nM) (p = 0.0003, n = 6). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate before (open bars) and after (solid bars) administration of vehicle (p = 0.4965, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). The p-values represent the probability of a significant interaction between treatment and frequency.

4.3.8 Effects of Propranolol

The nonselective *β*-adrenoceptor antagonist, propranolol (1 µM) did not change the inhibitory effect of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on electrical field stimulation-induced contractile responses of isolated rat prostates (**Figure 4-9A**; p = 0.0110, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-9B**; p = 5350, n = 6).

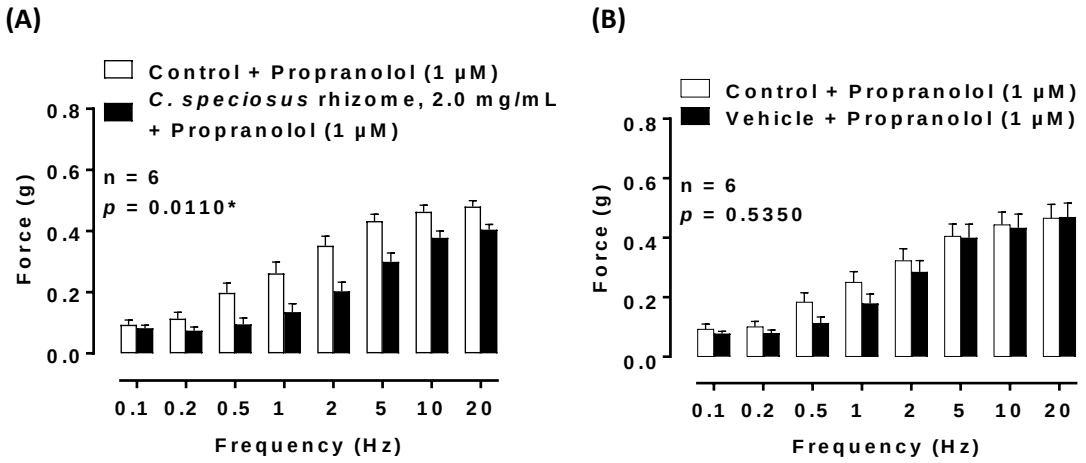


Figure 4-9. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) of isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of propranolol (1 µM) (p = 0.0110, n = 6). **B,** Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.5350, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.9 Effects of Atropine

Pre-treatment with the non-selective muscarinic receptor antagonist, atropine (1 µM), did not affect the inhibitory effects caused by *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on contractile responses induced by electrical field stimulation of isolated rat prostates (**Figure 4-10A**; $p = 0.0271$, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-10B**; $p = 4081$, n = 6).

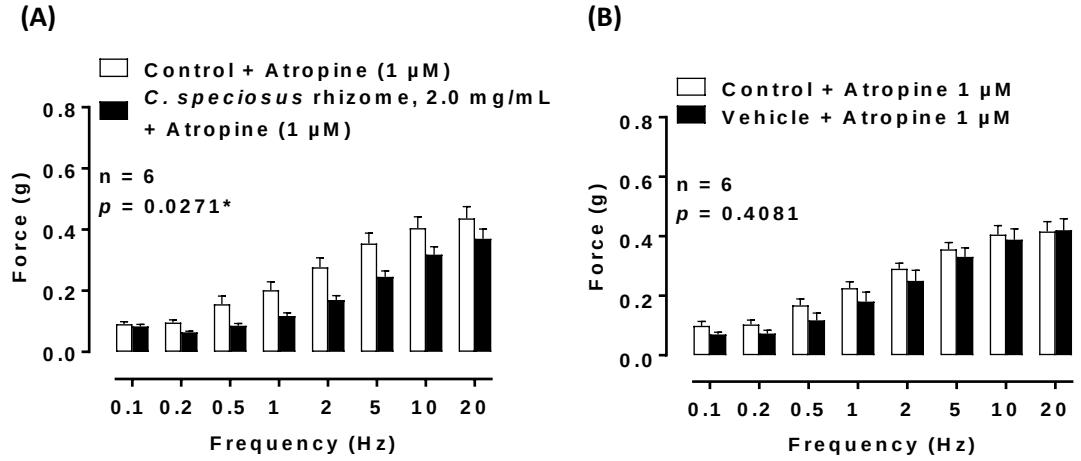


Figure 4-10. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of atropine (1 µM) ($p = 0.0271$, n = 6). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle ($p = 0.4081$, n = 6). Bars represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of a significant interaction between treatment and frequency.

4.3.10 Effects of Hexamethonium

The nonselective nicotinic acetylcholine receptor (nAChRs) antagonist, hexamethonium (10 µM) did not affect the inhibitory effect of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on electrical field stimulation-induced contractile responses of isolated rat prostates (**Figure 4-11A**; $p = 0.0135$, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-11B**; $p = 0.4097$, n = 6).

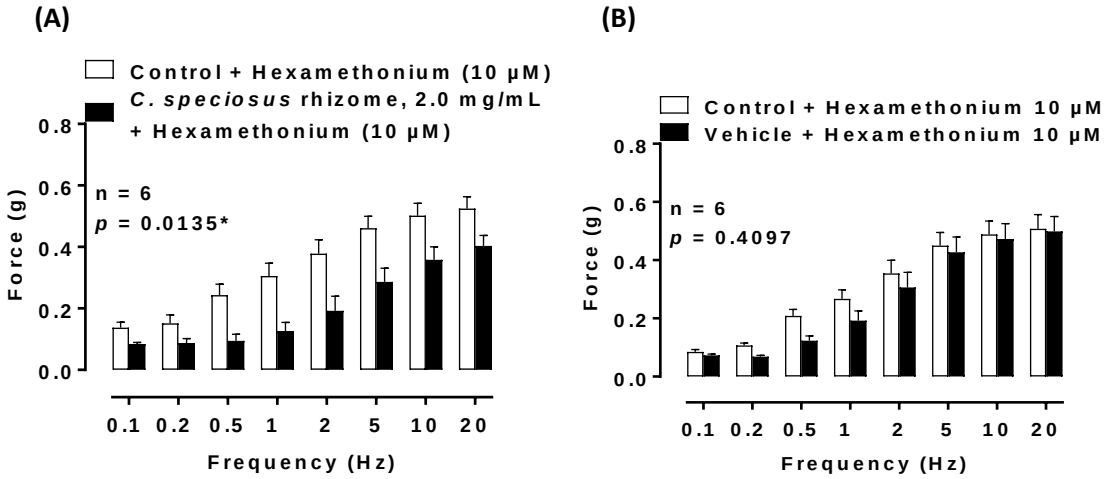
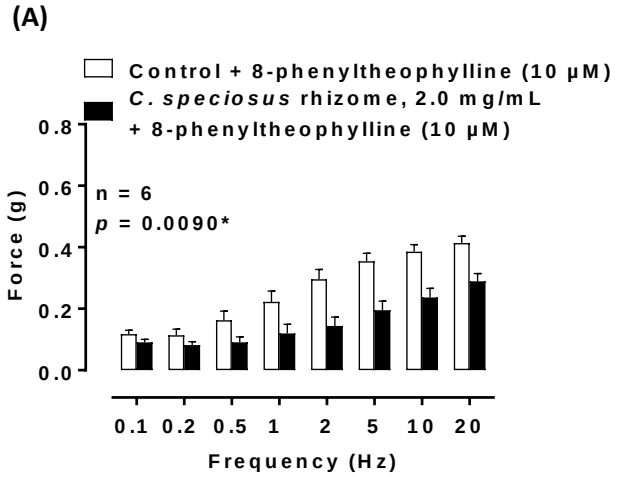


Figure 4-11. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of hexamethonium (10 µM) (p = 0.0135, n = 6). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.4097, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.11 Effects of 8-phenyltheophylline

Preincubation of isolated rat prostates with the non-selective A_1-adenosine receptor antagonist, 8-phenyltheophylline (8-PT) (10 µM) had no effect on the inhibitory effect exerted by *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on the electrical field stimulation-induced contractile responses of isolated rat prostates (**Figure 4-12A**; p = 0.0090, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-12B**; p = 8509, n = 6).



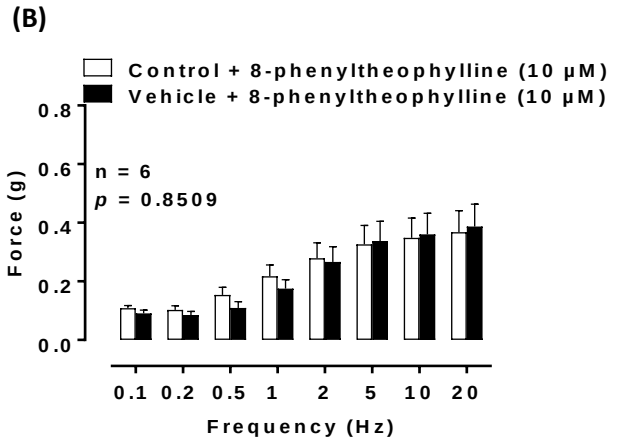


Figure 4-12. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of 8-phenyltheophylline (10 µM) (p = 0.0090, n = 6). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.8509, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.12 Effects of Suramin

Preincubation of isolated rat prostates with the non-selective P2 purinoceptor antagonist suramin (30 µM) had no effect on the inhibitory effect exerted by *C. speciosus* rhizome cold-water extract (2.0 mg/mL) on the electrical field stimulation-induced contractile responses of isolated rat prostates (**Figure 4-13A**; p = 0.0037, n = 6). Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-13B**; p = 0.2783, n = 6).

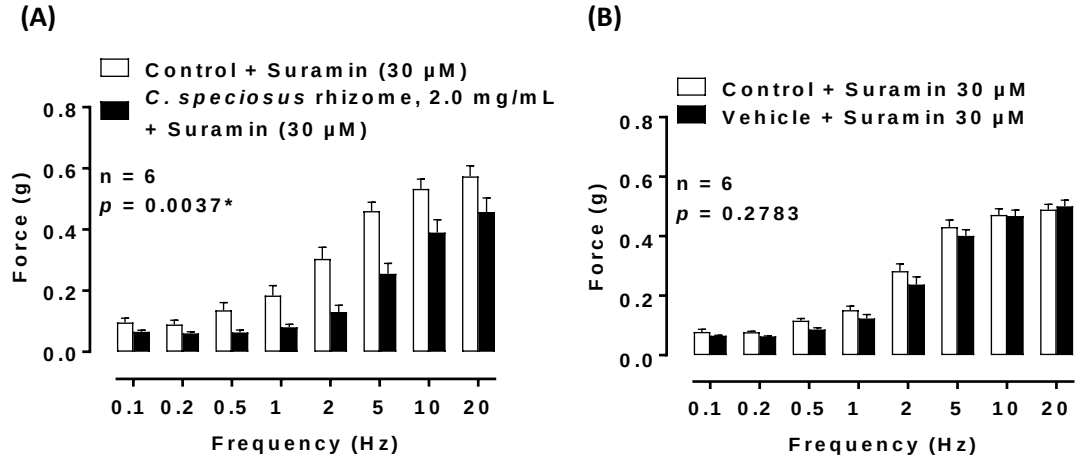


Figure 4-13. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) isolated rat prostates before (open bars) and after (solid bars) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of suramin (30 µM) (p = 0.0037, n = 6). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.2783, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.13 Effects of Methysergide

The competitive serotonin (5-HT) receptor antagonist, methysergide (1 µM) did not affect the inhibitory effect of *C. speciosus* rhizome cold-water extract on electrical field stimulation-induced contractile responses of isolated rat prostates (**Figure 4-14A**; $p = 0.0014$, n = 6)**.** Vehicle control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-14B**; $p = 4918$, n = 6).

There were no significant differences in electrical field stimulation-induced contractions between serotonin (10 or 100 µM) incubated isolated rat prostates and control (**Appendix 2A**; $p = 0.6058$, n = 4) and 100 µM (**Appendix 2B**; $p = 0.4392$, n = 4), respectively.

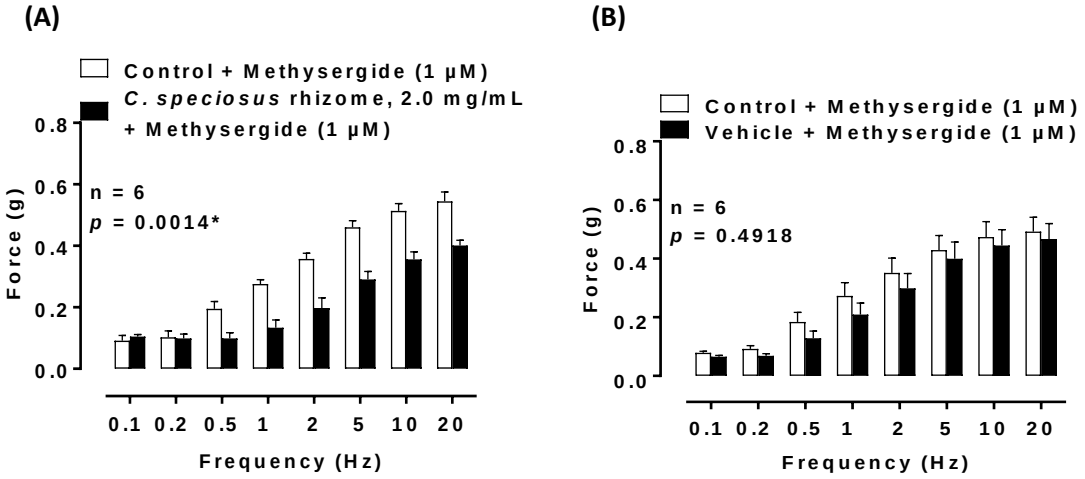


Figure 4-14. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open columns) and after (solid columns) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of methysergide (1 µM) ($p = 0.0014$, n = 6). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle ($p = 0.4918$, n = 6). Bars represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent the probability of a significant interaction between treatment and frequency.

4.3.14 Effects of Mepyramine

C. speciosus rhizome cold-water extract (2.0 mg/mL) caused relaxation to the nerve-mediated electrical field stimulation induced contractions of the isolated rat prostates in the presence of the histamine (H_1) receptor antagonist, mepyramine (1 µM) (**Figure 4-15A**; $p = 0.0010$, n = 8). There were no significant differences in the field stimulation-induced contractions for the vehicle/time controls (**Figure 4-15B**; $p = 0.7414$, n = 6).

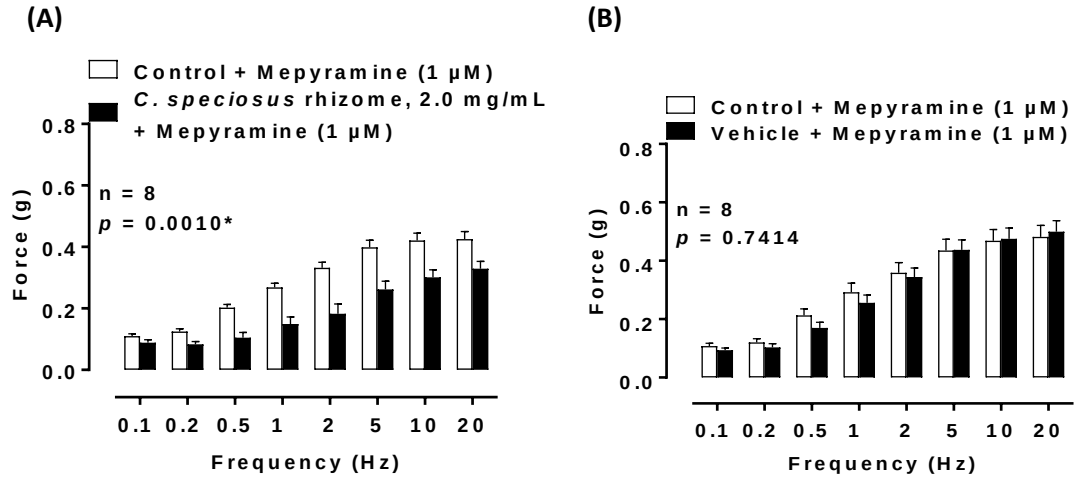
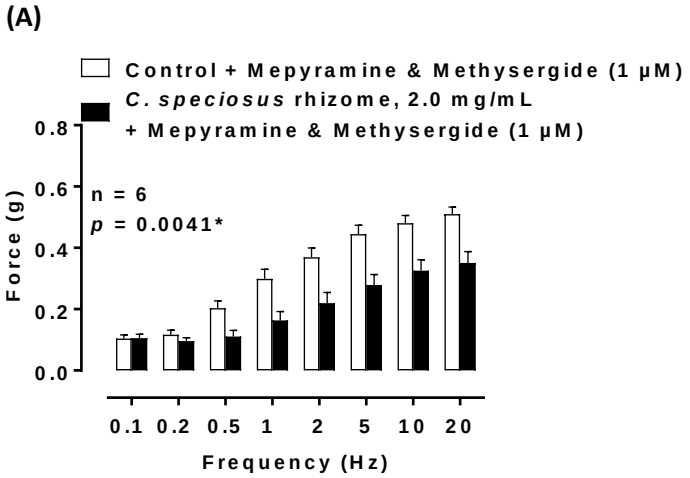


Figure 4-15. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) of isolated rat prostates before (open columns) and after (solid columns) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of mepyramine (1 µM) (p = 0.0010, n = 8). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle in the presence of mepyramine (1 µM) (p = 0.7414, n = 8). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

There were no significant differences in electrical field stimulation-induced contractions between histamine (10-100 µM) incubated isolated rat prostates and control (**Appendix 3A**; p = 0.7262, n = 4) and (100 µM) (**Appendix 3B**; p = 0.8860, n = 4), respectively.

4.3.15 Effects of Serotonin and Histamine Receptor Antagonists

The combination of mepyramine (1 µM) (serotonin antagonist) and methysergide (1 µM) (histamine antagonist) did not affect the inhibitory activity of the *C. speciosus* rhizome cold-water extract (2.0 mg/mL). The extract still attenuated electrically evoked contractions of isolated rat prostates (**Figure 4-16A**; p = 0.0041, n = 6). There were no differences in the field stimulation-induced contractions for the vehicle/time controls (**Figure 4-16B**).



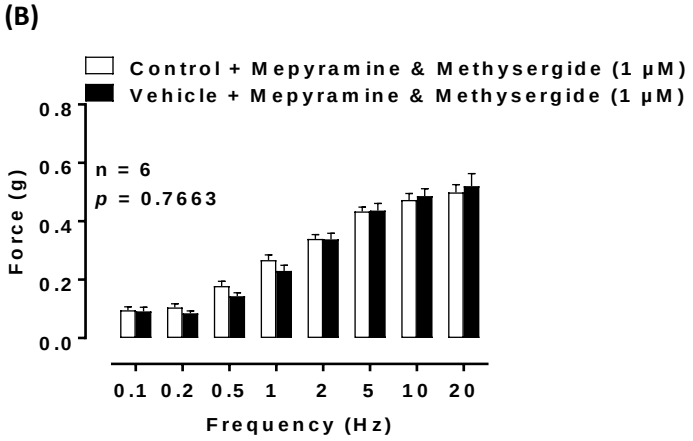


Figure 4-16. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) of isolated rat prostates before (open columns) and after (solid columns) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of both mepyramine (1 µM) and methysergide (1 µM) (p = 0.0041, n = 6). **B**, Mean contractile responses to electrical field stimulation on isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.7663, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.16 Effects of a Prostanoid Receptor Antagonist

The combined EP_1 and EP_2 prostanoid receptor antagonist, AH6809 (10 µM) did not affect the inhibitory activity of the *C. speciosus* rhizome cold-water extract (2.0 mg/mL). The extract still attenuated electrically evoked contractions of isolated rat prostates (**Figure 4-17A**; p = 0.0040, n = 4). There were no differences in the electrical field stimulation-induced contractions for the vehicle / time controls (**Figure 4-17B**).

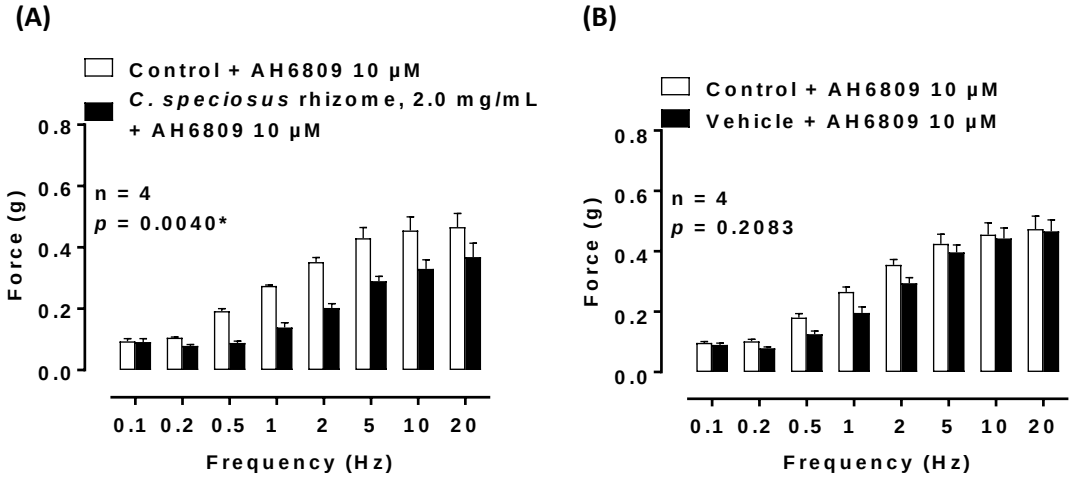
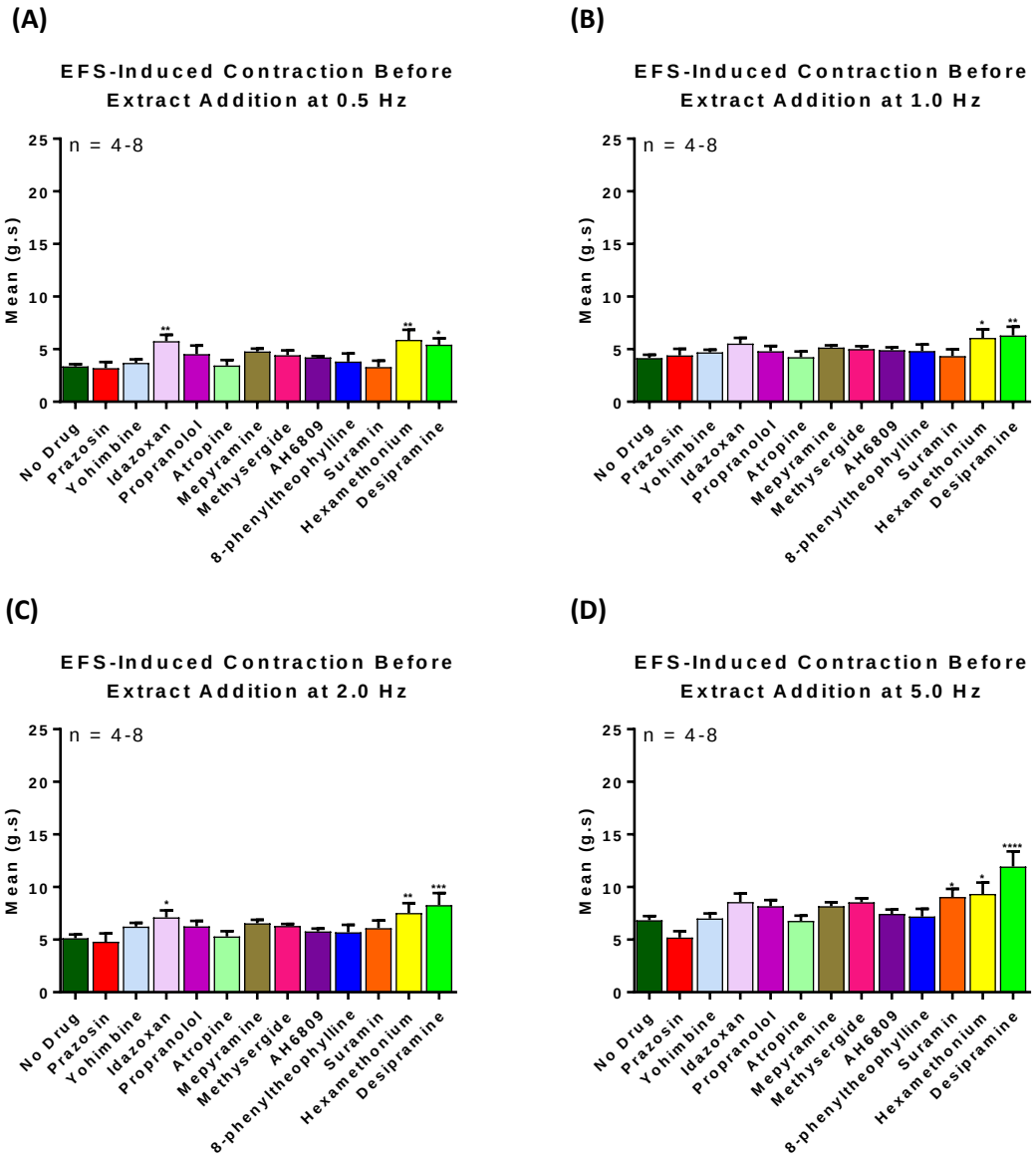


Figure 4-17. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) of isolated rat prostates before (open columns) and after (solid columns) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of AH6809 (10 µM) (p = 0.0040, n = 4). **B**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.2083, n = 4). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency.

4.3.17 Degree of inhibition by *C. speciosus* Rhizome Cold-water Extract in the Presence of the Different Pharmacological Agents

EFS-induced contractions of isolated rat prostates before administration of *C. speciosus* rhizome cold-water extract in the presence of the aforementioned pharmacological agents were generally quite consistent in term of magnitude (g.s) throughout different frequencies. Nevertheless, prazosin tended to reduce the magnitude of the EFS-induced contractions while desipramine and to a lesser extent hexamethonium tended to increase the size of the EFS-induced contractions (**Figure 4-18**). Similarly, EFS-induced contractions of isolated rat prostates when measured as peak height (g) in the presence of pharmacological agents remained generally consistent except for prazosin which tended to reduce the magnitude (**Appendix 4**).



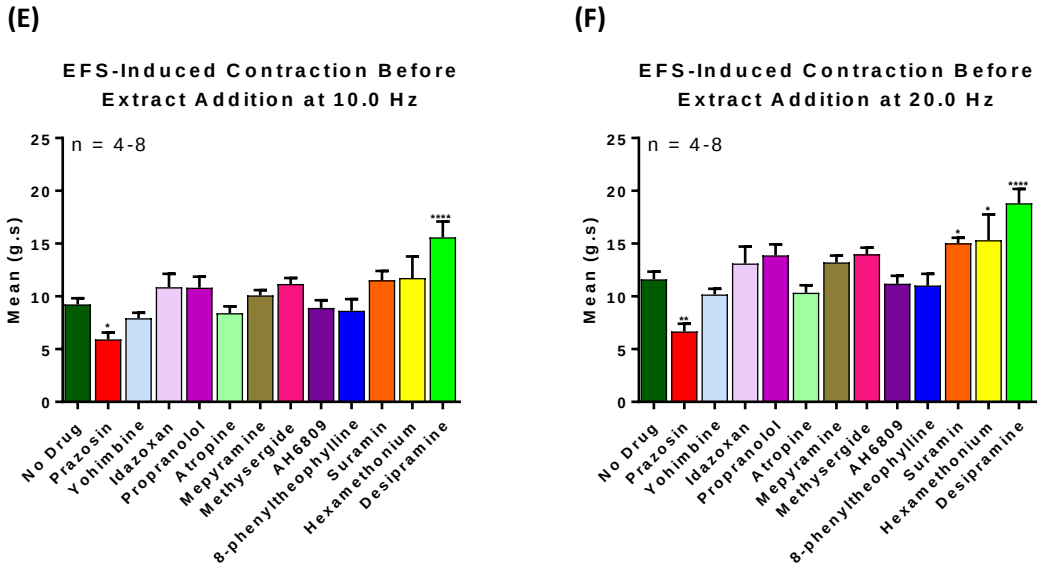
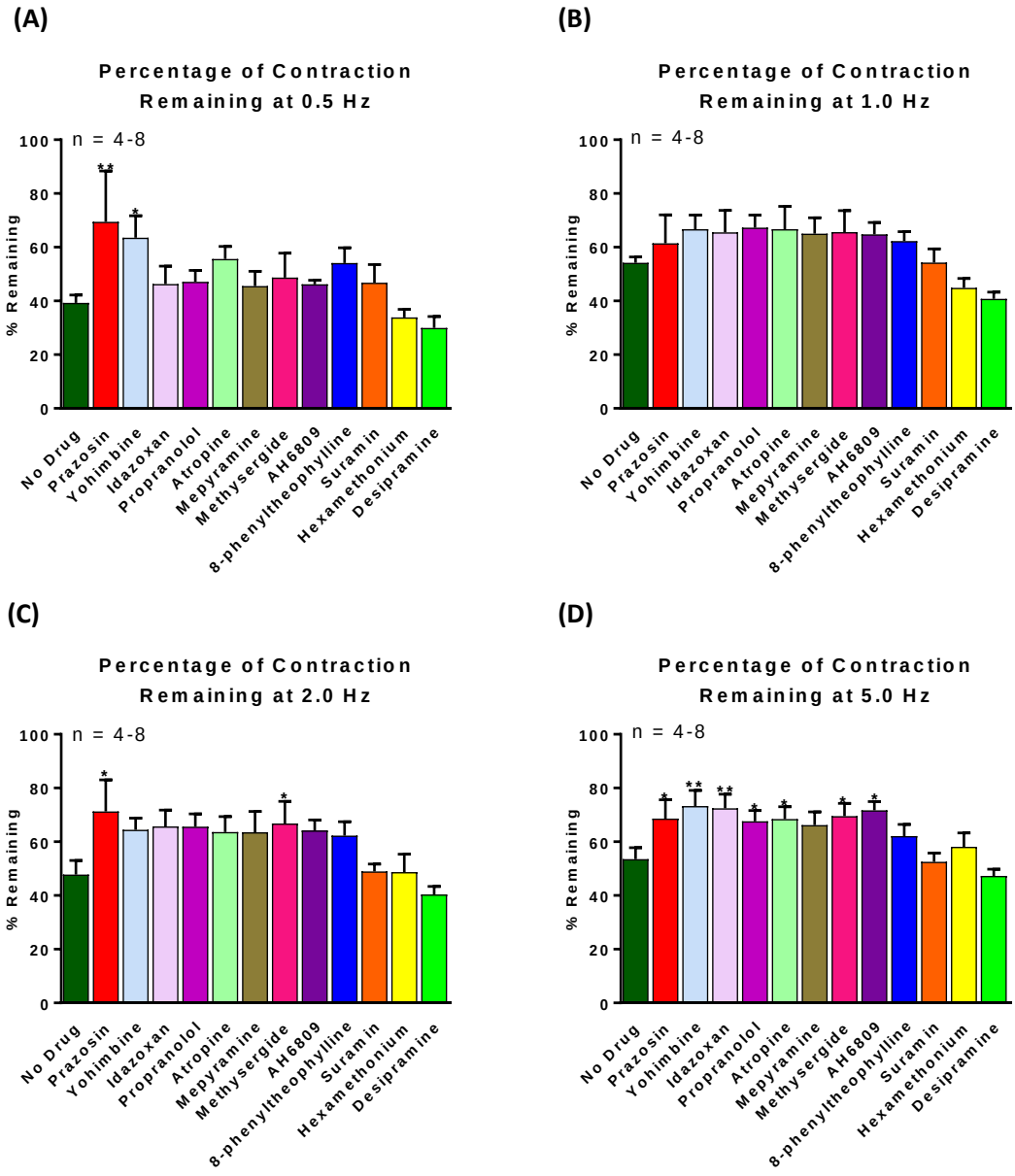


Figure 4-18. Mean area under the curve of contraction to electrical field stimulation induced nerve-mediated contraction of rat prostates before treated with *C. speciosus* rhizome cold-water extract (2.0 mg/mL) in absence (no drug) and presence of the following pharmacological agents: prazosin (300 nM), suramin (30 nM), yohimbine (1 µM), idazoxan (1 µM), propranolol (1 µM, n=6), atropine (1 µM, n=6), methysergide (1 µM, n=6), mepyramine (1 µM, n=8), hexamethonium (10 µM, n=6), desipramine (100 nM, n=6), 8-phenyltheophylline (10 µM, n=6), and AH6809 (10 µM, n=4). Results are expressed as means ± S.E.M. (*$p < 0.1$, **$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for statistical evaluation. *p*-values represent probability of any change in the magnitude of contraction at each frequency being due to chance.

In general, *C. speciosus* rhizome cold-water extract (2.0 mg/mL) markedly attenuated electrical field-stimulation induced nerve-mediated contractions of isolated rat prostates in the presence of the aforementioned pharmacological agents. However, the degree of relaxation caused by *C. speciosus* rhizome cold-water extract (2.0 mg/mL) varied in the presence of each pharmacological agent when expressed as a percentage of the control contraction that remained after administration of the *C. speciosus* rhizome cold-water extract (2.0 mg/mL) from 0.5 Hz – 20 Hz (**Figure 4-19**). The degree of relaxation caused by the extract in the presence of each pharmacological agent calculated using peak height differences also displayed similar patterns (**Appendix 5**). Differences in the amount of reversal of the inhibitory effect exhibited by *C. speciosus* rhizome cold-water extract on electrical field stimulation induced nerve-mediated contraction of rat prostate induced by pharmacological agents were analysed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test, and are shown in **Figure 4-19**. Prazosin (300 nM, n = 6) reversed the inhibitory effect exhibited by *C. speciosus* rhizome cold-water extract on electrical field stimulation induced nerve-mediated contraction of rat prostate by approximately 70 % at the different frequencies. Similarly, the non-selective antagonist of α_2-adrenoceptors, yohimbine (1 µM, n = 6), reversed the inhibitory effect exhibited by *C. speciosus* rhizome cold-water extract on nerve-mediated contraction of rat prostate by

approximately 70% at the different frequencies. Interestingly, idazoxan (1 µM, n = 6), a similarly potent and non-subtype selective α_2-adrenoceptor antagonist, reversed the inhibitory effect exhibited by *C. speciosus* rhizome cold-water extract on nerve-mediated contraction of rat prostate to a lesser extent as did exposure of rat prostatic preparations to propranolol (1 µM, n = 6). Atropine (1 µM, n = 6) but not hexamethonium also reversed the inhibitory effect of *C. speciosus* rhizome cold-water extract on nerve-mediated contraction of rat prostate to a similar degree at the different frequencies. The inhibitory effect of *C. speciosus* rhizome cold-water extract on electrical stimulation-induced contractions of rat prostate was also reversed in the presence of methysergide (1 µM, n = 6) and AH6809 (10 µM, n = 4), but were not significantly affected in the presence of suramin, 8-phenyltheophylline, mepyramine, and desipramine (**Table 4-1**).



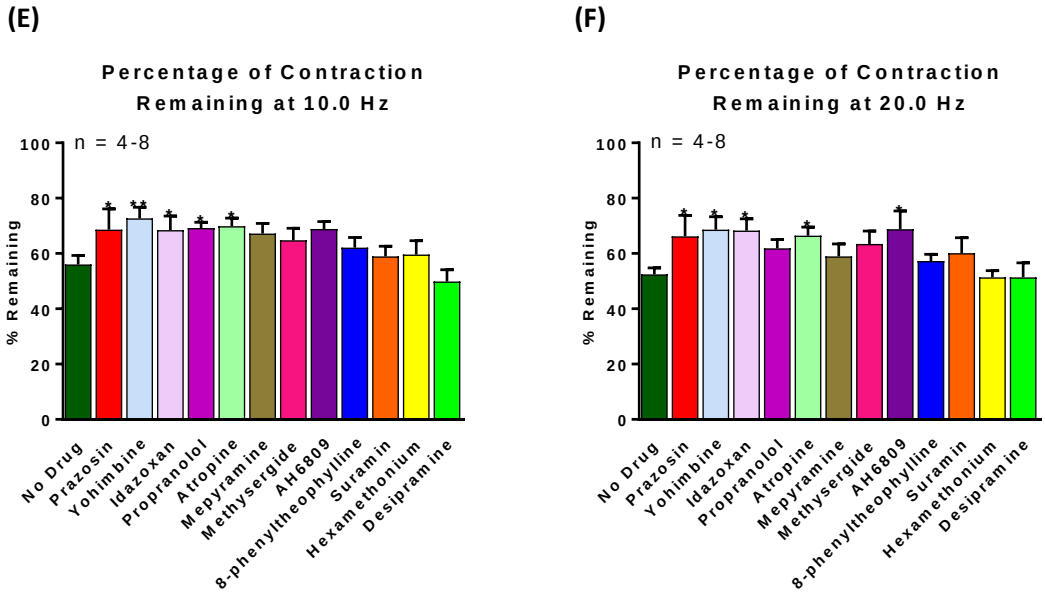


Figure 4-19. Mean percentage of contraction remaining to electrical field stimulation induced nerve-mediated contraction of rat prostates treated with *C. speciosus* rhizome cold-water extract (2.0 mg/mL) in absence (no drug) and presence of the following pharmacological agents: prazosin (300 nM), suramin (30 nM), yohimbine (1 µM), idazoxan (1 µM), propranolol (1 µM, n=6), atropine (1 µM, n=6), methysergide (1 µM, n=6), mepyramine (1 µM, n=8), hexamethonium (10 µM, n=6), desipramine (100 nM, n=6), 8-phenyltheophylline (10 µM, n=6), and AH6809 (10 µM, n=4). Results are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for statistical evaluation. (*$p < 0.05$, **$p < 0.01$). *p*-values represent probability of any change in the degree of inhibition being due to chance.

The shortlisted antagonists that showed some ability to reverse the inhibitory activity of *C. speciosus* rhizome cold-water extract on nerve-mediated contractile responses of isolated rat prostates were combined as an antagonist cocktail. This was done to investigate whether their effects were additive and whether such a combined effect of multiple mechanisms of action might explain the observed inhibitory effect of *C. speciosus* rhizome cold-water extract on nerve-mediated contractile responses of isolated rat prostates. Yohimbine and Idazoxan are both antagonists for α_2-adrenoceptors, therefore, only yohimbine was selected to be part of the cocktail as it had a greater effect on extract inhibition. AH6809 was not incorporated into the cocktail due to an insufficient amount of the material being available. Frequency-response curves indicated that the antagonist cocktail only partially blocked the inhibitory effect exhibited by *C. speciosus* rhizome cold-water extract on nerve-mediated contractions of rat prostate, and this was more markedly visible at lower frequencies (0.1 – 0.5 Hz) (**Figure 4-20A**; $p = 0.2250$, n = 6). This attenuation of the *C. speciosus* rhizome cold-water extract inhibition was of a similar magnitude as observed for each pharmacological agent in isolation. The antagonist cocktail reversed the inhibitory effect exhibited by *C. speciosus* rhizome cold-water extract on nerve-mediated contraction of rat prostate by approximately 80% at the different frequencies. Vehicle

control indicated that the frequency-dependent contractions were reproducible over the time course of the experimental protocol and not affected by vehicle (**Figure 4-20B**; p = 7078, n = 6). In summary, it appears as though there was no additive effect of the different antagonists. EFS-induced contractions (g) of isolated rat prostates in the presence of antagonist cocktail alone tended to reduce the magnitude probably due to the presence of prazosin (**Appendix 4**).

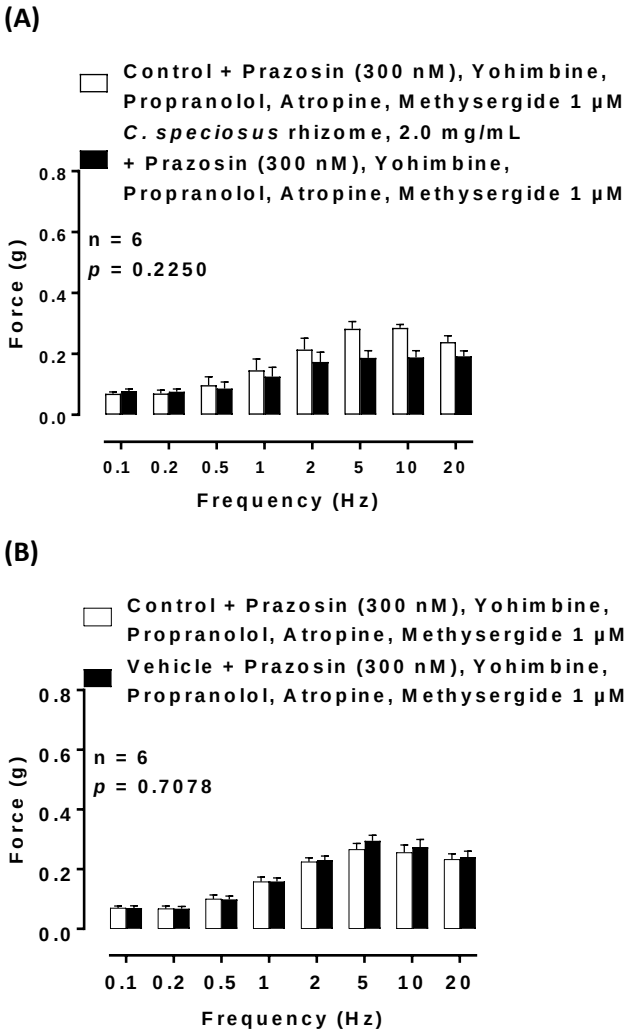


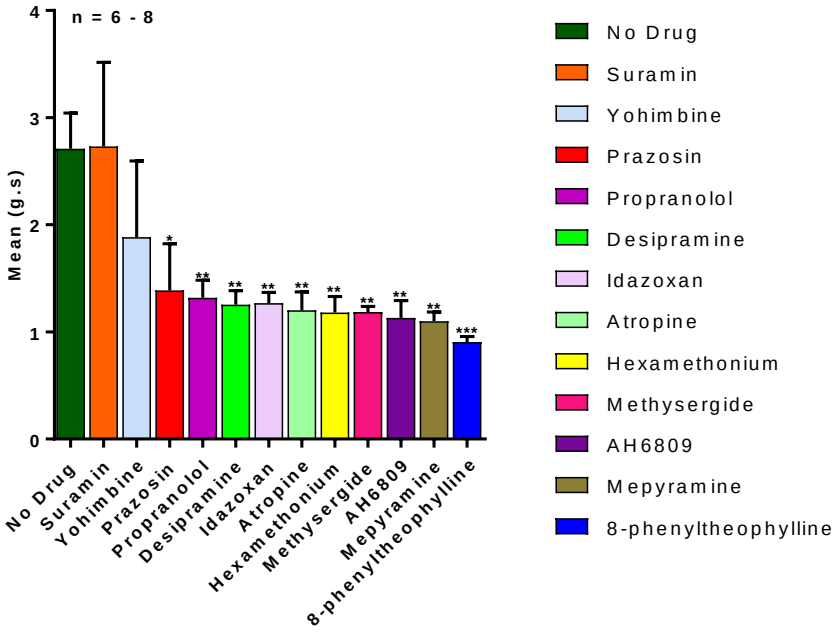
Figure 4-20. A, Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open columns) and after (solid columns) administration of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL in the presence of an antagonist cocktail containing: prazosin (300 nM), propranolol (1 µM), atropine (1 µM), methysergide (1 µM), and yohimbine (1 µM) (p = 0.2250, n = 6). **B**, Mean contractile responses to electrical field stimulation on isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.7078, n = 6). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a difference in the contractile responses being due to chance.

4.3.18 Tonic Contraction Produced by *C. speciosus* Rhizome Cold-water Extract on Isolated Rat Prostate in Presence of Different Pharmacological Agents

In addition to the inhibitory effects produced by *C. speciosus* rhizome cold-water extract on electrically evoked contractile responses of the isolated rat prostate, a transient tonic contraction on unstimulated rat prostates was observed upon direct administration to the isolated organ baths at the beginning of the 30 min incubation period. The magnitude of this transient tonic contraction varied in the presence of different pharmacological agents. The force generated, generally returned to base line within 30 s.

The mean magnitude of the tonic contraction produced by the extract in the absence of other drugs was 2.70 ± 0.35 g.s. (p = 0.0039, n = 6) (**Figure 4-21A**). The magnitude of the tonic contraction produced by the extract in the presence of other pharmacological agents decreased in the following rank order of efficacy, prazosin > propranolol, desipramine, Idazoxan, atropine, hexamethonium, methysergide, AH6809, mepyramine > 8-phenyltheophylline. Both suramin and yohimbine did not affect magnitude of the tonic contraction produced by the extract **Table 4-1**.

(A)



(B)

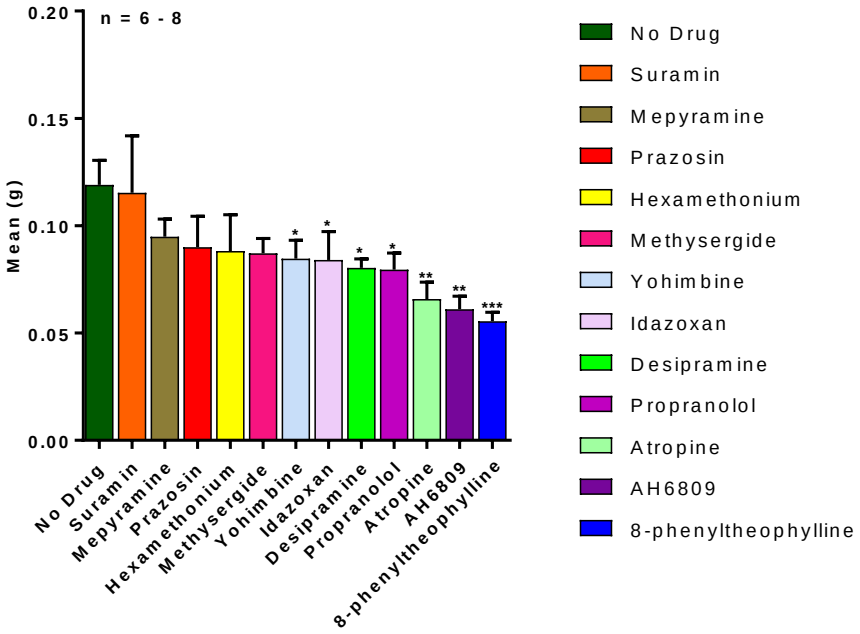
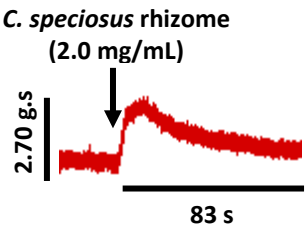


Figure 4-21. (A) Mean area under the curve of transient tonic contractile responses of unstimulated isolated rat prostates to administration of *C. speciosus* rhizome cold-water extract (2.0 mg/mL). **(B)** Mean magnitude maximum to minimum difference of contractile responses of unstimulated rat prostates to administration of *C. speciosus* rhizome cold-water extract (2.0 mg/mL). Columns represent mean force ± S.E.M. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for the statistical evaluations. *p*-values represent probability of differences from control (no drug) being due to chance.

The tonic contraction elicited by the *C. speciosus* rhizome cold-water extract (**Figure 4-22A**) was moderately abolished in the presence of mepyramine (1 µM) (**Figure 4-22B**). However, histamine did not elicit a tonic contraction when administered exogenously (**Figure 4-22D**) (**Appendix 3**). It is noteworthy that serotonin has elicited tonic contraction of rat prostate of greater magnitude as compared to the *C. speciosus* rhizome cold-water extract (**Figure 4-22E**) (**Appendix 2**). Furthermore, in the presence of the 5-HT receptor antagonist, methysergide (1 µM), the magnitude of the tonic contraction elicited by the extract was moderately abolished (**Figure 4-22C**).

(A)



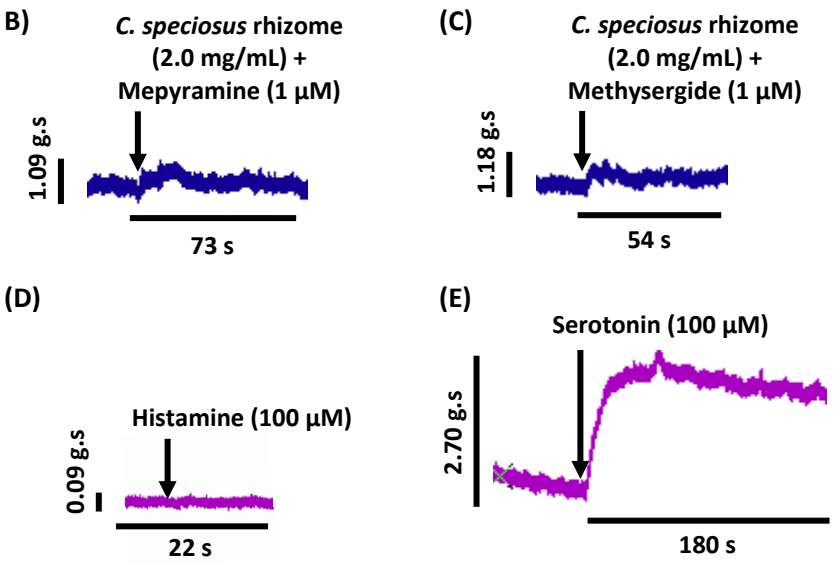


Figure 4-22. Comparisons of the traces for tonic contractions elicited by **A,** *C. speciosus* rhizome cold-water extract (2.0 mg/mL) alone, **B,** *C. speciosus* rhizome cold-water extract (2.0 mg/mL) in the presence of mepyramine (1 µM), **C,** *C. speciosus* rhizome cold-water extract (2.0 mg/mL) in the presence of methysergide (1 µM), **D,** histamine (100 µM), and **E,** serotonin (100 µM).

Table 4-1 gives a summary of the effects and the mechanisms of action of the pharmacological tools used to investigate the biological mechanisms of action of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) including the receptor/transporter involved, its signaling pathway (G protein) and efficacy.

Table 4-1. Summary of the sites of action of the antagonists used to investigate the mechanisms of action of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) including their signaling pathway (G protein), degree of reversal of inhibition (**Figure 4-19**) and effects on the tonic contraction (**Figure 4-21**).

Antagonist	Receptor	Signaling Pathway (G-protein)	Effect on Inhibition of EFS by Extract	Effect on Tonic Contraction Elicited by Extract
Prazosin	α_1	G_q	-	↓↓
Yohimbine	α_2	G_i	-	↓
Idazoxan	α_2	G_i	-	↓↓
Desipramine	Uptake 1	Amine reuptake transporter	ns	↓↓
Propranolol	β	G_s	-	↓↓
Atropine	M_3	G_q	-	↓↓
Hexamethonium	N	Ion channel	ns	↓↓
8-phenyltheophylline	A_1	G_i	ns	↓↓↓
Suramin	P2X	Ion Channel	ns	≈
Methysergide	5-HT	G_q & Ion channel	-	↓↓
Mepyramine	H_1	G_q	ns	↓↓
AH6809	PGE	G_s	-	↓↓

Notes:

In each case, n = 6-8
ns : *The antagonist did not affect the inhibitory effect of the extract*
"-" : *The antagonist attenuated the inhibitory effect of* C. speciosus *rhizome cold-water extract on EFS-induced contractions*
↓ (30 %) : *Slightly decreased tonic contraction elicited by* C. speciosus *rhizome cold-water extract*
↓↓ (45 – 60 %) : *Moderately decreased tonic contraction elicited by* C. speciosus *rhizome cold-water extract*
↓↓↓ (70 %) : *Strongly decreased tonic contraction elicited by* C. speciosus *rhizome cold-water extract*
"≈" : *Antagonist did not affect tonic contraction elicited by the* C. speciosus *rhizome cold-water extract*

4.4 DISCUSSION

In the previous chapter, *C. speciosus* rhizome cold-water extract (2.0 mg/mL) was demonstrated to markedly attenuate nerve-mediated contractile responses of isolated rat prostate elicited by electrical field stimulation. Relaxation of the prostatic smooth muscle tone by *C. speciosus* rhizome extract may be useful in the treatment of BPH. Therefore, to gain insight into the mechanism of action underlying the inhibitory effect of the *C. speciosus* rhizome cold-water extract, isolated rat prostates were incubated with an array of agonists and antagonists with affinity for adrenergic, cholinergic, purinergic, tyramine, serotonergic, histaminergic, and prostanoid receptors to see whether they would produce additive effects on the bioactivity of the extract.

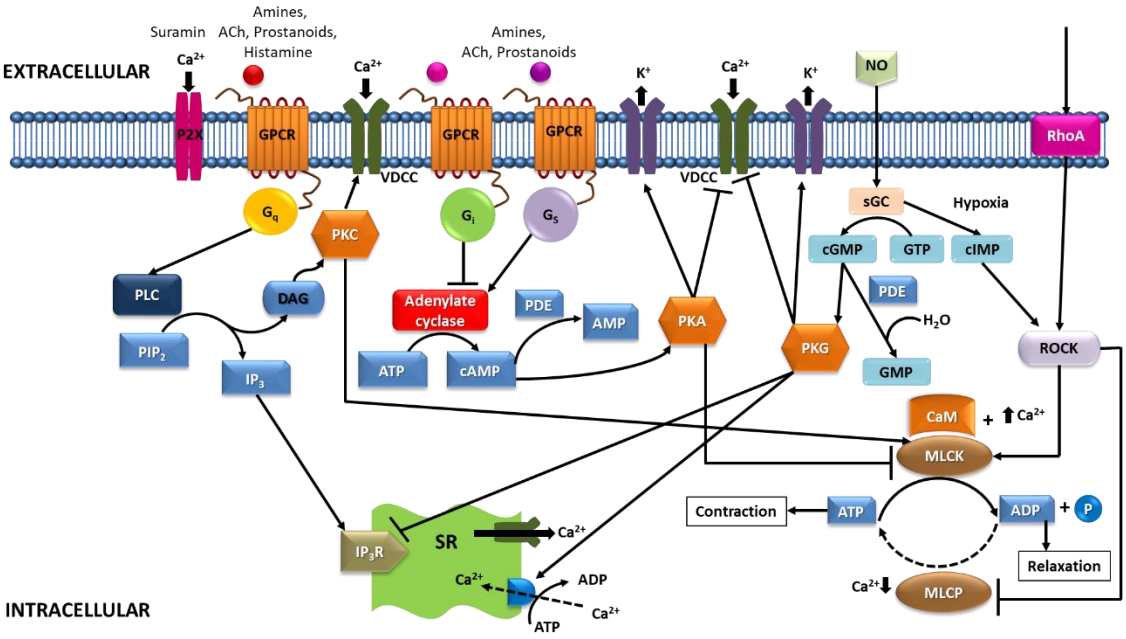


Figure 4-23. Schematic diagram of the intracellular signaling pathways modulating contractility of prostatic smooth muscle following activation of receptors by extracellular mediators. ACh: acetylcholine; AMP: adenosine monophosphate; ATP: adenosine 5'-triphosphate; CaM: Calmodulin; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; cIMP: inosine cyclic 3',5'-monophosphate; DAG: diacylglycerol; GMP: guanosine monophosphate; GPCR: G protein-coupled receptor; IP_3R: inositol triphosphate receptor; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; NO: nitric oxide; PDE: phosphodiesterase; PIP_2: phosphatidylinositol bisphosphate; PK: protein kinase; PLC: Protein kinase C; RhoA: Ras homolog gene family, member A; ROCK: Rho-associated, coiled-coil-containing protein kinase 1; sGC: soluble guanylyl cyclase; SR: sarcoplasmic reticulum.

4.4.1 Adrenergic Receptors

4.4.1.1 α_1-adrenoceptors

It is well accepted that the prostate gland is innervated by sympathetic nerves and the contractile response of the prostatic smooth muscle is known to be mediated primarily by the post-junctional α_{1A} subtype of adrenergic receptor in response to activation by noradrenaline (Pennefather et al., 1999; Pennefather et al., 2000). Contractions mediated by the α_1-adrenoceptor are inhibited by the G_q protein inhibitor pertussis toxin as well as Ca^{2+} channel (L-type) blockers (Drescher et al., 1994; Furuya et al., 1982; Haynes and Ventura, 2005; Hieble et al., 1985; Kunisawa et al., 1985; Lepor, 2016; Preston and Haynes, 2003; Wang et al., 1991). Binding sites of α_1-adrenoceptors have been found mainly in the prostatic stroma (Chapple et al., 1989).

Noradrenaline is the main catecholamine present in the male sex organs and it has been shown as the major endogenous neurotransmitter that activates post-junctional α_1-adrenoceptors during electrical field stimulation to elicit contractions of the prostatic smooth muscle (Holzbauer and Sharman, 1972; Lau et al., 1998). In the male lower urinary tract, α_1-adrenoceptors are predominantly located in the prostate, bladder neck and urethra. This has led to the development of therapeutic uses for α-adrenergic blockers as they block the post-junctional adrenoceptor activated by noradrenaline and subsequently produce relief from the symptoms caused by bladder outlet obstruction due to BPH, without affecting the detrusor muscle of the bladder wall (Meenakshi et al., 2017).

Prazosin, the selective short-acting α_1-adrenoceptor antagonist, has 1000-fold greater affinity for α_1 than for α_2-adrenoceptors, blocking the post-junctional α_1-adrenoceptors to inhibit the contraction of the prostatic smooth muscle (Meenakshi et al., 2017). Its lack of effect on the inhibitory action of the extract suggests that the extract does not act through the post-junctional α_1-adrenoceptors.

The noradrenaline reuptake transporter (NET) (also known as Uptake 1) is a neuronal transporter that recaptures noradrenaline in the vicinity of the outer surface of the nerve ending into the cytoplasm of the nerve terminal (**Figure 4-25**). Recaptured noradrenaline is subsequently either deaminated or recycled back into storage vesicles (Langer, 1977). Desipramine is a tricyclic antidepressant that is a relatively selective noradrenaline reuptake inhibitor primarily used to treat depression as well as other psychiatric disorders such as

attention-deficit hyperactivity disorder (ADHD) (Zhou, 2004). It blocks the noradrenaline transporter thus increasing extracellular concentrations of noradrenaline released by nerve stimulation. Its lack of effect on the inhibitory action of *C. speciosus* rhizome cold-water extract indicates that the extract does not act through the Uptake 1 transporter.

4.4.1.2 α_2-adrenoceptors

α_2-adrenoceptors regulate the negative feedback mechanism for exocytotic noradrenaline release during nerve stimulation, but not for the release of noradrenaline elicited by tyramine as the feedback mechanism regulates the influx of calcium ions (Langer, 1977; Starke and Montel, 1974). In the human prostate, α_2-adrenoceptor agonists and antagonists are able to inhibit or increase the pre-junctional release of noradrenaline, respectively (**Figure 4-25**) (Guh et al., 1995; Hedlund et al., 1985). However, α_2-adrenoceptors do not directly participate in the contractile role (Michel and Vrydag, 2006). Nevertheless, an age-related decline of prejunctional α_2-adrenoceptor sensitivity has been suggested to contribute to the increase in noradrenaline plasma levels of ageing men (Docherty, 1990; MacGilchrist et al., 1989; Ziegler et al., 1976). In symptomatic BPH, α_2-adrenoceptors have been found to be up-regulated as compared to asymptomatic BPH and normal prostate (Berthelsen and Pettinger, 1977; Caine, 1986; Gup et al., 1990; Timmermans and van Zwieten, 1981).

Yohimbine is an indole alkaloid which acts as a non-selective antagonist with high affinity (pK_i = 7.9 – 9.5) at α_2-adrenoceptors (Starke et al., 1975). It also has moderate affinity for α_1-adrenoceptors, several serotonin receptor subtypes and dopamine receptor subtypes (predominantly of the D_2 type) (Scatton et al., 1980). Antagonism of α_2-adrenoceptors by yohimbine was unable to reverse the inhibitory effects of *C. speciosus* rhizome cold-water extract on electrical field stimulation-induced contractile responses of rat prostates. Due to yohimbine's known non-selectivity, the relaxant effect of the extract was also studied using the more potent and selective α_2-adrenoceptor antagonist, idazoxan (Bowes et al., 1992; Doxey et al., 1983). Idazoxan is also an agonist at 5-HT_{1A} autoreceptors in the rat brain (Lladó et al., 1996). However, pre-incubation of isolated rat prostate with idazoxan did not reverse the inhibitory effect exhibited by the *C. speciosus* rhizome cold-water extract on contractile responses of isolated rat prostates mediated by electrical field stimulation**.** These findings indicate that *C. speciosus* rhizome cold-water extract did not act via pre-junctional α_2-adrenoceptors to exert its relaxant effects on nerve-mediated contractile responses of isolated rat prostates.

4.4.1.3 *β*-adrenoceptors

β-adrenoceptors are activated by the endogenous agonists, adrenaline and noradrenaline (Lands et al., 1967a; Lands et al., 1967b). It has been demonstrated that relaxation of prostatic smooth muscle cells can be mediated through *β*-adrenoceptors, with the predominant subtype varying among species (Haynes and Hill, 1997; Kalodimos and Ventura, 2001; Langer, 1977; Matsumoto et al., 2013; Suzuki et al., 2016). Propranolol is a nonselective antagonist that blocks both β_1- and β_2-adrenoceptors but not β_3-adrenoceptors. Propranolol did not hamper the inhibitory effect of *C. speciosus* rhizome cold-water extract on electrical field stimulation-induced contractile responses of isolated rat prostates. This finding indicates that the inhibitory effects exerted by *C. speciosus* rhizome cold-water extract on nerve-mediated contractile responses of isolated rat prostate was not through pre- and/or post-synaptic modulation of β_1- and/or β_2-adrenoceptors. Both β_1- and β_3-adrenoceptors have been implicated in the relaxation of nerve-mediated contraction of human prostate (Suzuki et al., 2016). It is noteworthy that β_3-adrenoceptors are resistant to blockade by propranolol. Therefore, it remains possible that the extract exerted its inhibitory effect on nerve-mediated contractions of rat prostate via β_3-adrenoceptors.

4.4.2 Cholinoceptors

Acetylcholine (ACh), binds and activates two structurally and functionally related cell surface receptors. Cholinoceptors are either pre- or post-synaptically located and mediate both excitatory and inhibitory actions throughout the central and peripheral nervous systems (**Figure 4-25**). The receptors that are activated by ACh are the ionotropic nicotinic receptors (nAChR) (ACh-gated cation channel) and metabotropic muscarinic receptors (mAChR) (GPCR) (Birdsall and Hulme, 1983; Caulfield and Birdsall, 1998; Hulme et al., 1990). ACh has also been found to elicit prostatic smooth muscle contraction of several species including rats and mice although the response is less than that elicited by α-adrenoceptor agonists (Ventura et al., 2002; White et al., 2010). Nevertheless, ACh has minimal contractile effects on the human prostate stroma although it contracts the prostatic capsule (Caine et al., 1975; Hedlund et al., 1985). It is noteworthy that prostatic smooth muscle contraction among different species is mediated by different muscarinic subtypes. Studies have demonstrated that M_1 is the main muscarinic receptor subtype in both human and guinea-pigs as compared to, mice (M_3), canines (M_2/M_3) and rats (M_3) (Fernández et al., 1998; Lau et al., 2000; Lau et al., 1998; Najbar-Kaszkiel et al., 1997; Raz et al., 1973; Ruggieri et al., 1995; Ventura et al., 2002; White et al., 2011).

4.4.2.1 Muscarinic Receptors

The muscarinic receptors (mAChR) are GPCRs that directly modulate homeostasis of phospholipase C, inositol triphosphate, cAMP, as well as calcium (Albuquerque et al., 2009) (**Figure 4-23**). As depicted in **Figure 4-23**, some muscarinic subtypes couple to G_q whereas others to $G_{i/s}$ as shown in **Figure 4-24**.

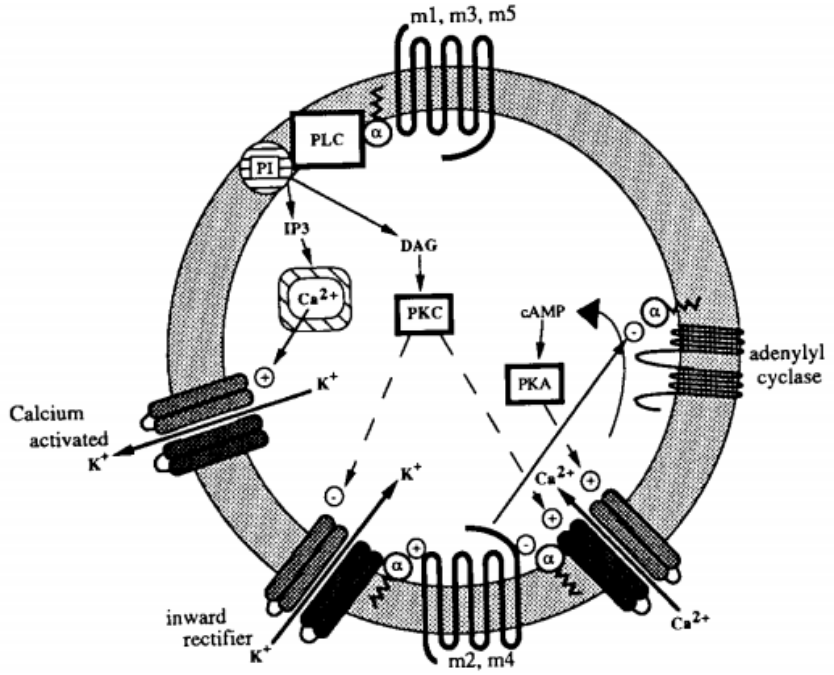


Figure 4-24. Model summarising intracellular signaling of five muscarinic receptor subtypes. PKC: protein kinase C; PKA: protein kinase A; DAG: diacylglycerol (Jones, 1993).

There are five distinct mAChR subtypes encoded by five distinct related genes which have been identified in mammals (**Figure 4-24**). M_1, M_3, and M_5 mAChRs are coupled to the $G_{\alpha q/11}$ family of GPCRs to mediate stimulation of phosphoinositide hydrolysis, whilst M_2 and M_4 mAChRs are coupled with $G_{\alpha i/0}$ family of GPCRs to inhibit accumulation of cAMP (**Figure 4-23**) (Caulfield and Birdsall, 1998; Eglen, 2005; Eglen, 2006; Ehlert et al., 1997). The mAChR subtypes have been shown to be differentially expressed in different tissues of the male reproductive tract and are frequently involved in modulating contraction of smooth muscle (Avellar et al., 2010). However, it has also been suggested that they are not primarily involved in the control of smooth muscle contractions (Hedlund et al., 1985). Several studies have shown that mAChRs are profoundly localized to the glandular epithelium of the prostate (Hedlund et al., 1985; Lepor and Kuhar, 1984), indicating a secretory rather than a contractile role. Among all the muscarinic subtypes, M_3 has been reported to be predominantly found in rat prostate tissue (Yazawa and Honda, 1993) which is not surprising for a glandular tissue. This indicates that muscarinic M_3 subtype receptors are important in the modulation of carbachol-induced contractions of the rat ventral

prostate (Latifpour et al., 1991; Lau and Pennefather, 1998; Lepor et al., 1994). In functional experiments, M_1, M_2, and M_4 were not involved in the contractile response to carbachol in the rat prostatic smooth muscle (Lau and Pennefather, 1998; Lepor and Kuhar, 1984). Human prostate contraction induced by endogenous acetylcholine (released from either parasympathetic or sympathetic nerve fibres) via muscarinic cholinergic receptors has been observed and the contractions were blocked by atropine which is a competitive antagonist of the muscarinic acetylcholine receptor. The muscarinic receptor subtype that was found to be predominantly present in human prostate was M_1 (Kester et al., 2003; Luthin et al., 1997; Ruggieri et al., 1995). In this study, concentration-response curves to exogenously administered acetylcholine (1 nM – 100 µM) on isolated rat prostates were not affected by *C. speciosus* rhizome methanol extract. Similarly, nerve-mediated contraction of isolated rat prostates in the presence of atropine was unaffected by the *C. speciosus* rhizome cold-water extract, implying that the extract did not act through muscarinic receptors to cause relaxation of the rat prostatic smooth muscle.

4.4.2.2 Nicotinic Receptors

The nicotinic acetylcholine receptors, are also activated by the neurotransmitter acetylcholine and have been categorized into several subtypes. Notably, nicotinic acetylcholine receptors are subdivided into two major subtypes, the muscle type and the neuronal type based on the expression of subunits on these major sites. Unlike muscarinic receptors, nicotinic acetylcholine receptors are ligand-gated, non-selective cation channels (Albuquerque et al., 2009; Brammar, 1996). The secretory innervation of the prostate is mainly parasympathetic in nature (Farrell, 1938). Electrical stimulation of the hypogastric nerve has been shown to induce secretion of the canine prostate gland which could be blocked by atropine as well as hexamethonium (Smith and Lebeaux, 1970). These findings suggested that activation of preganglionic cholinergic fibers which synapse with postganglionic parasympathetic cholinergic fibers induced secretion of the prostate gland (Owman and Sjöstrand, 1965; Smith and Lebeaux, 1970).

Investigations into the effect of *C. speciosus* rhizome cold-water extract on nicotinic cholinergic receptors were conducted using hexamethonium, a nicotinic cholinergic receptor antagonist that blocks synaptic transmission at autonomic ganglia, skeletal neuromuscular junctions and in the central nervous system (Milne and Byrne, 1981; Slater et al., 1986). Findings revealed that pre- and post-junctional nicotinic acetylcholine receptors are not involved in the inhibitory effect exerted by *C. speciosus* rhizome cold-water extract.

4.4.3 Purinoceptors

Purinoceptors were originally distinguished into two primary group, P1 and P2 that were activated by either adenosine or ATP, respectively (Burnstock, 1972). Of note, ATP had been shown to be broken down to adenosine very quickly in the extracellular space by sequential dephosphorylation from ATP→ADP (by ecto-ATPases), ATP & ADP→AMP (by ectoapyrases), and AMP→adenosine (by ecto-5'-nucleotidase) (Cusack, 1993; Edwards, 1994; Zimmermann and Braun, 1999). The P1 nomenclature for adenosine receptors has since been replaced to "A" for adenosine receptors.

4.4.3.1 Adenosine Receptors

Preincubation of isolated rat prostates with the non-selective A_1-purinoceptor antagonist, 8-PT had no effect on response to the inhibitory effect of *C. speciosus* rhizome cold-water extract at 2.0 mg/mL on nerve-mediated contractions. This implies that the extract did not affect pre-junctional A_1-adenosine receptors to inhibit rat prostate contractile responses mediated by electrical field stimulation.

4.4.3.2 P2 Purinoceptors

ATP is a cotransmitter with noradrenaline, acetylcholine, glutamate, γ-aminobutyric acid (GABA), vasoactive intestinal peptide, calcitonin gene-related peptide, and neuropeptide Y in different tissues throughout the body (Burnstock, 1999; Edwards and Gibb, 1993; Williams, 2002). In particular, it has been demonstrated as an excitatory cotransmitter with noradrenaline by eliciting Ca^{2+} influx and releasing cytosolic Ca^{2+}, respectively in rat and guinea-pig prostatic smooth muscle, particularly at low-frequency stimulation (Abbracchio and Burnstock, 1998; Buljubasich and Ventura, 2004; Ventura et al., 2003).

In this study, concentration-response curves to ATP demonstrated that *C. speciosus* rhizome cold-water extract only slightly caused a shift to the right of the ATP concentration-response curves to contractions of the isolated rat prostate compared to control. The response was not as dramatic as seen with the inhibition of electrical field stimulation induced contractions, suggesting an inhibitory effect on transmitter release at a prejunctional site of action. The mechanism of action of the extract inhibitory activity was further investigated using suramin, a non-subtype selective ATP receptor antagonist (Dunn and Blakeley, 1988; Hoyle, 1990). The results with suramin implied that the extract did not exert its inhibitory effect via post-junctional P2X ionotropic channels.

4.4.4 Tyramine

Tyramine, has been reported to be present in saw palmetto ethanol extracts and is known to elicit contractile responses of rat prostate via indirect sympathomimetic effects (Cao et al., 2006; Chua et al., 2011). It is noteworthy that isolated rat prostate contractions elicited by exogenous tyramine were not visibly affected by the cold-water extract of the *C. speciosus* rhizome in this study. This indicated that the extract only affects exocytotic release of transmitters that is dependent on the influx of Ca^{2+} ions into the nerve terminal, as seen with electrical field stimulation.

4.4.5 5-HT Receptors

Methysergide is a potent competitive antagonist of serotonin (5-HT) receptors which has higher binding affinity at the 5-HT_{2C} (pK_i = 8.6 – 9.1) (Egan et al., 2000; Knight et al., 2004), 5-HT_{2A} (pK_i = 8.4) (Knight et al., 2004) and 5-HT_{1D} (pK_i = 8.4 – 8.9) (Leysen et al., 1996; Weinshank et al., 1992) subtypes in the CHO-K1, NIH-3T3 and HEK-293 cell lines. It also has reasonable affinity for the human 5-HT_1 receptor (Mylecharane, 1989). In rats, methysergide is a highly potent, competitive antagonist at 5-HT_{2C} (pK_i = 9.3) as well as 5-HT_{2A} (pK_i = 8.4-9.5) (Egan et al., 1998; Herrick-Davis et al., 1997) subtypes of 5-HT receptor. *C. speciosus* rhizome cold-water extract continued to attenuate nerve-mediated contraction of isolated rat prostates in the presence of methysergide, indicating that the relaxant effects on isolated rat prostates exerted by the extract were not through 5-HT receptors.

4.4.6 Histaminergic Receptors

To investigate whether *C. speciosus* rhizome cold-water extract modulates the prostatic smooth muscle tone via the H_1-receptor, the effects of extract were studied in the presence of mepyramine. Mepyramine did not alter the inhibitory effect of *C. speciosus* rhizome cold-water extract on rat prostates). These findings imply that the extract did not exert its inhibitory effect via H_1-receptors in rat prostate.

4.4.7 Prostanoid Receptors

Prostanoid receptors have been categorised into EP (EP_1 – EP_4), IP, FP, DP and TP, respectively (Coleman et al., 1984; Kennedy et al., 1982). These receptors have been further grouped into three clusters: Cluster 1 consists of relaxant receptors, EP_2, EP_4, IP and DP; Cluster 2 consists of contractile receptors, EP_1, FP and TP; and Cluster 3 consists of the inhibitory receptor EP_3. PGE_1

and PGE_2 are the ligands for EP receptors (Narumiya et al., 1999). Studies have demonstrated that contraction of guinea-pig trachea can be induced via the EP_1 receptor through PGE_2 induced Ca^{2+} mobilization that is partially blocked by AH6809, a competitive EP_1 receptor antagonist. AH6809 also has affinity for EP_2 receptors (Coleman et al., 1985; Coleman and Kennedy, 1985; Coleman et al., 1994; Creese and Denborough, 1982; Narumiya et al., 1999; Smith, 1992). Furthermore, interaction of PGE_2 with EP_2 receptors has been reported to stimulate cAMP formation (Honda et al., 1993; Jumblatt and Paterson, 1991; Sonnenburg and Smith, 1988).

Our functional studies demonstrated that the *C. speciosus* rhizome cold-water extract continued to attenuate electrically evoked contractions of isolated rat prostates in the presence of AH6809. Nevertheless, AH6809 altered the inhibitory effect of *C. speciosus* rhizome cold-water extract on electrical stimulation-induced contractions of rat prostate. These findings indicated that the extract might not directly exert its relaxant effect on rat prostatic smooth muscle via EP_1 or EP_2 receptors. However, the extract might partially exert its relaxant effects through the inhibition of noradrenaline release by restriction of calcium availability via EP_1 or/and EP_2 receptors as reported in other studies (Hedqvist, 1970; 1976; Stjärne, 1973).

4.4.8 Percentage of Contraction Remaining after treatment with *C. speciosus* Rhizome Cold-water Extract in the Presence of Pharmacological Agents

It is noteworthy that prazosin, yohimbine, idazoxan, propranolol, atropine, mepyramine, methysergide, AH6809, and 8-PT are all antagonists of GPCRs (**Table 4-1**). Since the relaxation effect caused by the *C. speciosus* rhizome cold-water extract on EFS-induced contractions of rat prostate was partially attenuated by all of these receptor antagonists, the extract inhibition might be the result of the extract blocking G proteins. Conversely, results also suggested that the mechanism of actions of the extract is unlikely to be via ligand-gated ion channels (i.e P2X1 and nicotinic acetylcholine receptors) or transporters (e.g. Uptake 1). These findings indicate a complex nature to the observed inhibition exerted by the extract which might require the contribution of many different components present in the extract that have little effect on their own but result in a combined effect that results in the relaxation of rat prostatic smooth muscle.

4.4.9 Effects of *C. speciosus* Rhizome Cold-water Extract on Rat Prostate in the Presence of GPCR Antagonist Cocktail

Antagonism with a combination of α_1-, α_2-, β-adrenoceptors, M_3-adrenoceptors, and 5-HT receptors with prazosin, yohimbine, propranolol, atropine, and methysergide, respectively, did

not completely hamper the inhibitory effects exerted by *C. speciosus* rhizome cold-water extract on contractions of isolated rat prostates induced by electrical field stimulation. However, these findings do not preclude that the inhibitory effects exerted by the extract were possibly influenced by many different components present in the extract which have a small effect on different receptors and result in an additive relaxation of rat prostatic smooth muscle.

4.4.10 Tonic Contraction Produced by *C. speciosus* Rhizome Cold-water Extract on Isolated Rat Prostate in Presence of Different Pharmacological Agents

C. speciosus rhizome, leaf, root, and stem extracts produced a tonic contraction of unstimulated isolated rat prostates upon administration to the organ baths. Interestingly, magnitudes of the tonic contraction elicited by *C. speciosus* rhizome cold-water extract varied in the presence of different pharmacological agents**.** The tonic contraction elicited by the extract was reduced in the presence of prazosin (α_1-adrenoceptors), idazoxan (α_2-adrenoceptors), propranolol (β-adrenoceptors), atropine (M_3-receptors), hexamethonium (nicotinic acetylcholine receptors), desipramine (Uptake 1, noradrenaline transporter), AH6809 (prostaglandin-receptors), 8-phenyltheophylline (A_1-receptors), mepyramine (H_1-receptors) and methysergide (5-HT receptors) (**Table 4-1**).

Of note, prazosin, propranolol, idazoxan, atropine, AH6809, and methysergide were all demonstrated to slightly attenuate the inhibitory effects of *C. speciosus* rhizome cold-water extract to field stimulation-induced contractions of rat prostates. Furthermore, the tonic contractions elicited by the extract were not affected by suramin. The extract induced inhibition of nerve-mediated contractile responses of rat prostates was also unaffected by suramin.

Tonic contractions have been observed in isolated rat prostates following administration of serotonin which indicates that serotonin directly stimulates the rat prostatic smooth muscle to contract. This observation is consistent with other studies that 5-HT neurons were present and directly or indirectly contribute to the rat prostatic adrenergic contractility (Kester et al., 2003; Killam et al., 1995; Langer, 1977). Of note, tonic contractions stimulated by *C. speciosus* rhizome cold-water extract were moderately abolished by methysergide which indicated that there might be constituents present in the extract that activate 5-HT receptors. Furthermore, inhibitory effects of the extract on nerve-mediated contractions of rat prostates were attenuated in the presence of methysergide. These may be due to the same or different

constituents present in the extract that affect rat prostatic smooth muscle tone through pre- or postjunctional mechanisms.

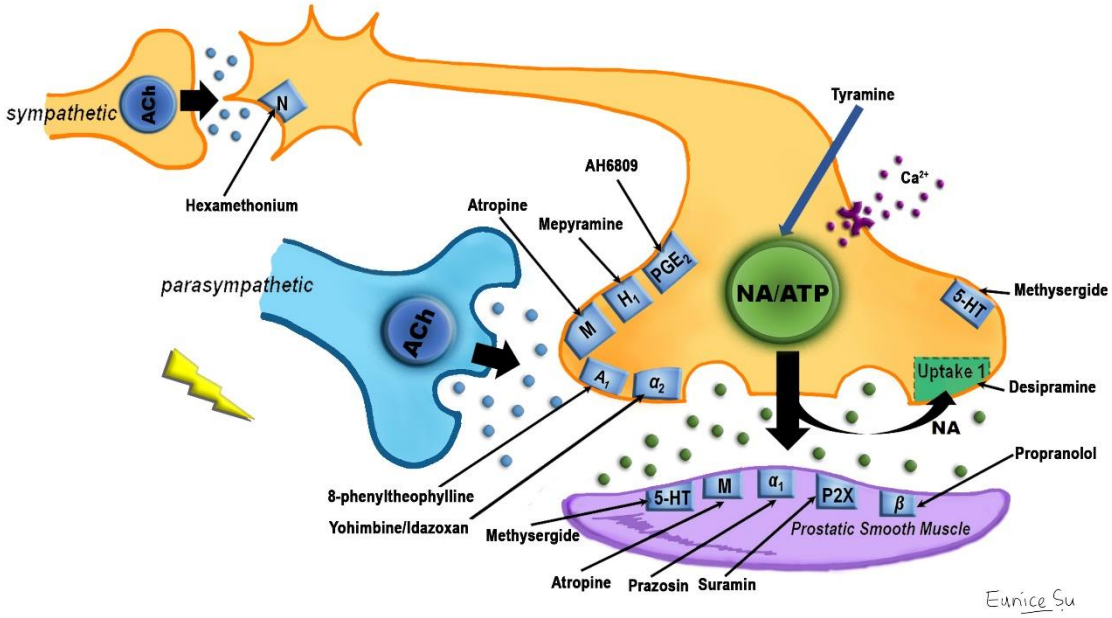


Figure 4-25. Schematic diagram depicting the pharmacological tools that have been used to study the effect of *C. speciosus* rhizome cold-water extract on membrane bound receptors which are involved in the contractility of prostate smooth muscle. Sites of action are also indicated.

4.5 CONCLUDING REMARKS ON THE MECHANISM OF ACTION OF *C. SPECIOSUS* RHIZOME COLD-WATER EXTRACT

The current functional studies revealed that direct smooth muscle contraction elicited by exogenously administered noradrenaline (1 nM – 100 µM, n = 6), phenylephrine (1 nM – 100 µM, n = 2), acetylcholine (1 nM – 100 µM, n = 6), and ATP (300 nM – 1 mM, n = 6) were not greatly affected by the cold-water extract of *C. speciosus* rhizome. Furthermore, rat prostate contractions elicited by tyramine (10 nM – 100 µM, n = 4), the indirectly acting sympathomimetic agonist, were also not abolished by *C. speciosus* rhizome cold-water extract. The precise mechanism of action of *C. speciosus* rhizome cold-water extract on isolated rat prostate is at presently obscure, although it is likely that *C. speciosus* rhizome cold-water extract inhibits contractility of rat prostatic smooth muscle by an indirect pre-junctional mechanisms that inhibit exocytotic release of neurotransmitter as the greater effect of the extract was seen on responses that were elicited by electrical nerve stimulation as compared to agonist-induced contractions mediated by post-junctional receptors. For instance, the extract may block N-type Ca^{2+} channel

permeability which modulates influx of free ionized extracellular Ca^{2+} that leads to neurotransmitter release during electrical field stimulation (Minneman, 1988).

Nevertheless, there also might be non-specific mechanisms of action post-junctionally such as G protein inhibition, or myosin light chains kinase inhibition, or modulation of other proteins involved in smooth muscle contraction such as modulation of intracellular Ca^{2+} concentration through L-type Ca^{2+} channels (Abdel-Latif, 1991; Preston et al., 2004; Preston and Haynes, 2003). Contraction of smooth muscle is mediated by second messengers such as cyclic AMP (cAMP), cyclic GMP (cGMP), and diacylglycerol (DAG) that alter the concentration of intracellular Ca^{2+} through activation of specific protein kinases (Kamm and Stull, 1989; Nishimura and van Breemen, 1989). In addition, potassium channels have been suggested to play a pivotal role in the regulation of prostate smooth muscle contraction via activation of cGMP-dependent protein kinase (PKG) (Francis and Corbin, 1994) to induce opening of ATP sensitive K^+ (K_{ATP}) channels that subsequently caused hyperpolarization to the cellular membrane and lead to inhibition of intracellular Ca^{2+} influx through L-type Ca^{2+} channels (Haynes and Cook, 2006). In addition to these voltage-operated Ca^{2+} channels, the extract might affect receptor-operated Ca^{2+} channels (Bolton, 1979; Van Breemen et al., 1978).

Nitrergic innervation has been reported to be found in the human prostate stroma. Relaxation of prostatic smooth muscle cell contraction has been found to be attributed to the stimulation of nitrergic nerves that release NO. Studies show that NO has inhibited noradrenaline-mediated contraction of the prostatic stroma. Furthermore, inhibition of NO synthesis has interfered with relaxation induced by electrical field stimulation. It is noteworthy that nitrergic innervation density has been found to be less dense in BPH patients which suggest the importance of NO in the treatment of BPH (Bloch et al., 1997; Hedlund et al., 1997; Takeda et al., 1995). In addition, NO has also been reported to be involved in inflammatory diseases of the urinary bladder (Lundberg et al., 1996; Smith et al., 1996). Recently, it has been demonstrated that activation of the *β*-adrenoceptor modulates production and release of NO from human urothelial cells (Winder et al., 2017). In addition, extracts of *C. speciosus* have been shown to possess anti-inflammatory activity (Al-Attas et al., 2015; Binny et al., 2010; Selim and Al Jaouni, 2016), therefore, *C. speciosus* rhizome cold-water extract's relaxant effects on electrically-induced contractions of rat prostate might involve modulation of a NO pathway. This study could be investigated by performing the experiments in the presence of a nitric oxide synthase inhibitor such as L-NAME or a NO donor like sodium nitroprusside (Dey et al., 2012).

Hitherto, the *C. speciosus* rhizome cold-water extract might not be affecting one specific receptor, but it could be affecting several receptor mechanisms via a pathway which is common to them. Of course, the possibility also still remains that there are multiple components in the extract that affect multiple mechanisms and pathways.

5

Chemical characterisation of a bioactive extract from *Costus speciosus* Rhizome

5.1 INTRODUCTION

Prostatic smooth muscle contractility is important for the relief of LUTS associated with BPH. As mentioned in Chapter 3, a cold-water extract from *C. speciosus* rhizome inhibited the EFS-induced contractile response of isolated rat prostates. Plant extracts usually consist of a combination of various types of compounds or phytochemicals with a range of properties, which makes isolation of the biologically active components a major challenge. This chapter will describe the fractionation of the extract and the use of isolated organ bath studies to isolate and identify bioactive fractions of the cold-water extract from *C. speciosus* rhizome that exert relaxant effects on isolated rat prostate glands *ex vivo*. A range of chemical separation, chemical derivatisation and analysis techniques used in natural products research were examined, such as thin layer chromatography (TLC), flash column chromatography, high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LCMS) and nuclear magnetic resonance (NMR) analysis (Sasidharan et al., 2011). It is noteworthy that the ^{1}H NMR spectrum of the *C. speciosus* rhizome cold-water extract showed an abundance of signals consistent with carbohydrates.

Carbohydrates are the most abundant compounds in Nature. However, the quantitative and qualitative study of saccharides is a very intricate and challenging area. In recent years, as attention has focused on glycobiology, carbohydrates have become important molecules to contemplate as they have complementary interactions with neighbouring cells resulting in

transmission of biological information (Gabius, 2009). This has inspired several carbohydrate-processing enzymes to be developed as important targets for therapeutic intervention (Dwek et al., 2002; Tasneem and Robert, 2003) such as influenza virus inhibition (Babu et al., 2006; Democratis et al., 2006), glycosidase inhibitors against HIV (Gruters et al., 1987), Gaucher's disease, hepatitis and cancer (Maria et al., 2006).

Carbohydrates, including oligosaccharides, polysaccharides and glycoconjugates, are a relevant subclass of bioactive compounds isolated from plants which have been widely used in therapeutics, diagnostics, food additives, and biomaterials (Courtois, 2009; Delattre et al., 2005; Morris and Morris, 2012; Ramberg et al., 2010; Thakur et al., 2012). Several carbohydrates isolated from medicinal plants and fungi have been reported to possess biological activities (Li et al., 2013). The water-soluble polysaccharides isolated from medicinal plants and fungi have been recognised for their significant role in pharmaceutical applications such as antitumor (Zong et al., 2012), antiviral (Shi et al., 2017; Wang et al., 2012), immunoregulatory (Jiang et al., 2010), biomineralization (Arias and Fernández, 2008) , and hypolipidemic effects (Gunness and Gidley, 2010). Recent years, herbal glycomics has evolved as a trend in herbal medicine research.

Some unique challenges in the analysis of carbohydrates in natural products as compared to other classes include that in general, carbohydrates do not absorb UV light above 200 nm, making detection in chromatographic separations more difficult. Equally, the common carbohydrates as mono-, di or polysaccharides exist as geometric or stereoisomers, which means that many molecules will have a common molecular formula and thus give similar primary mass spectra.

Attaching chromophores to the carbohydrate molecule via chemical derivatisation with UV absorptivity at selected wavelengths during chromatographic analysis has been a widely used technique to analyse carbohydrates (Herbreteau, 1992). Several derivatization reagents for saccharides are reported in the literature, including 3-methyl-1-phenyl-2-pyrazoline (Honda et al., 1989; Honda et al., 1991), 8-aminopyrene,1,3,6-trisulfonate (Guttman, 1997), benzamidine (Koimur et al., 1996), Fmoc-hydrazine (Zhang et al., 1991), phenylisocyanate (Rakotomanga et al., 1992), 2-aminobenzoic acid (Anumula, 1994), *p*-aminobenzoic acid ethyl ester (ABEE) (Akiyama, 1991; Kwon and Kim, 1995), and aminopyrazine (Wu et al., 2000). Among these, 2-aminobenzoic acid was selected for the derivatization of carbohydrates in this study.

5.1.1 Aim

The aim of this chapter is to isolate and identify fractions and individual compounds which may become useful for future drug discovery in the pharmacotherapy of BPH.

5.2 MATERIALS AND METHODS

General methods for animal husbandry, dissection and animal ethics are detailed in Chapter 2, Section 2.1. Details for the plant collection, preparations, as well as processing can be found in Chapter 2, Section 2.2. Extraction methods to prepare *C. speciosus* rhizome methanol extract and cold-water extract were described in Chapter 2, Section 2.3.1.4, and Section 2.3.2, respectively. General methods to perform isolated organ bath studies including tissue preparations, initial period of equilibrium as well as parameters of the experiments and results analysis are detailed in Chapter 2, Section 2.5, 2.6 and 2.9.

5.2.1 Chemistry

General procedures for flash column chromatography setup, packing, solvent systems used, and sample preparation were described in Chapter 2, Section 2.10. Details for centrifugation separation method were described in Chapter 2, Section 2.10.3.

5.2.1.1 Derivatization Method

Details of the method, reagents preparation and parameters were described in Chapter 2, Section 2.10.4.

5.2.1.1.1 Derivatization of sugar standards with anthranilic acid (2-aminobenzoic acid, 2-AA)

20 µL of freshly prepared 1 % sodium acetate trihydrate was added to a 1.5 mL microcentrifuge tube which contained 14 mg of the sugar (glucose, fructose, galactose, lactose, mannose, rhamnose, or arabinose). The mixture was carefully mixed and 50 µL of ABA reagent was then added. The microcentrifuge tube was heated at 80 ^{o}C for 1 hour on a heater block (Multi-Blok®, Lab-line). During the heating process, the lid of the tube was carefully opened to release hydrogen gas produced from the reductive amination process. After 1 hour, the tube was cooled to room temperature and the reaction mixture was subjected to LCMS analysis, chemical separation using preparative RP-HPLC or bioactivity validation using isolated organ bath. For a large scale reductive amination reaction of these saccharides, 800 mg was added with proportional volumes of reagents as mentioned above.

5.2.1.1.2 Derivatization of CS-PLP1 isolated from liquid phase extract obtained from centrifugation separation of *C. speciosus* rhizome cold-water extract with anthranilic acid (2 aminobenzoic acid, 2-AA)

20 µL of freshly prepared 1 % sodium acetate trihydrate was added to three 1.5 mL microcentrifuge tubes which respectively contained 3 mg, 7 mg, and 14 mg of CS-PLP1 isolated from the liquid phase extract obtained from centrifugation separation of the *C. speciosus* rhizome cold-water extract. The mixture in each tube was carefully mixed and 50 µL of ABA reagent was then added. The microcentrifuge tubes were heated at 80 ^{o}C for 1 hour on heater block (Multi-Blok®, Lab-line). During the heating process, the lids of the tubes were carefully opened to release hydrogen gas produced from the reductive amination process. After 1 hour, the reaction mixtures were cooled to room temperature and proceeded to LCMS analysis, chemical separation using analytical and preparative RP-HPLC or bioactivity validation using isolated organ bath.

5.2.1.2 Nuclear Magnetic Resonance Spectroscopy Data Acquisition and Processing

General information on NMR and data analysis was mentioned in Chapter 2, Section 2.10.2.

5.2.1.3 Flash Column Chromatography

5.2.1.3.1 Recovery of the *C. speciosus* rhizome cold-water extract from reverse-phase flash column chromatography

The cold-water extract of the *C. speciosus* rhizome clogged the RP-column (C-18) due to its high viscosity. The 10 % MeOH/H_2O (HPLC grade; Scharlau, Spain) flowthrough was slow even under positive air pressure. The experiment was terminated.

The extract remaining on top of the column was carefully scraped out and the remaining solvent was evaporated using a rotary evaporator. The dried mixture of extract and C-18 was then transferred to a 2 L Büchner filtration funnel with glass sintered disc layered with Celite® 545 (VWR) and a filter paper (Whatman, 110 mm Ø). The solvent system used for the filtration or extract recovery were 1 L of each 10 %, 30 %, 50 %, 70 %, 90% MeOH/H_2O, and 100 % MeOH (HPLC grade; Scharlau, Spain), followed by 10 % DCM/MeOH and 100 % DCM (LCMS grade; Merck, Germany) (**Appendix 6**). Each of the filtrates was collected and solvent was evaporated using a rotary evaporator. The C18 Silica was then recycled by sieving through a 355 µm

microplate siever (ISO 3310-1; Endecotts, London) to remove any extract particles which remained.

Additionally, approximately 500 mL of 10 % MeOH/H_2O, 50 % MeOH/H_2O, 100 % MeOH, and 100 % DCM solvent systems, respectively, were used to elute the extract residues that had remained in the column (**Appendix 6**). Each of the eluates was collected, concentrated using a rotary evaporator and subsequently lyophilized. Of note, eluates of 50 % MeOH/H_2O, 100 % MeOH, and 100 % DCM were combined with their respective fractions yielded from filtration through Celite® 545. LCMS and HPLC analyses were performed on all the fractions obtained.

Bioactivity of fractions obtained from both filtration & elution were selectively validated by investigating their effects on EFS-induced contractile responses of isolated rat prostates.

5.2.1.4 Analytical High-Performance Liquid Chromatography

General methods and columns information were mentioned in Chapter 2, Section 2.10.1.4.

5.2.1.4.1 Fraction 1 eluted from flash column chromatography

10 µL of F1 was injected and analysed using an analytical high-performance liquid chromatography (analytical HPLC) (2795 Alliance®, Waters). To find a suitable column for optimal separation, two columns have been used. The columns used were Luna® 5 µm C8(2) 100 Å, (150 x 4.60 mm) (00F-4249-E0; Phenomenex), and Synergi™ 4 µm Fusion-RP 80 Å, (150 x 4.60 mm) (00F-4424-E0, Phenomenex), operated in solvent system 0.1 % TFA/H_2O (Buffer A), and 0.1 % TFA/ACN (Buffer B) at a flow rate of 1 mL/min. Method used for the separation was "Method A" as described in **Table 2-6**, Chapter 2, Section 2.10.1.4 and the total run time was 30 min. Gradient of the chromatography took 14 min to reach 80 % Buffer A (0.1 % TFA/H_2O) and 20 % Buffer B (0.1 % TFA/ACN), then it was maintained for 6 min before reaching 100 % of Buffer B at 20 min, and finally returned to 100 % Buffer A at 21 min which then ended at 30 min. Data was managed and analysed using Empower™ 2 software.

5.2.1.4.2 Liquid phase extract isolated from the centrifugation separation of *C. speciosus* rhizome cold-water extract

121 milligrams of dried liquid phase extract was dissolved in 1 mL of Milli-Q water. 30 µL of the dissolved liquid phase extract was transferred to a sample vial and mixed with 30 µL of ACN:H_2O with ratio 50:50. Injection volumes ranged from 5 – 30 µL and method used was "Method D" as

described in **Table 2-6**, Chapter 2, Section 2.10.1.4. Separation of the sample chemical components was performed via column Synergi™ 4 µm Fusion-RP 80 Å, (150 x 4.60 mm) (00F-4424-E0, Phenomenex) operated in solvent system 100 % Milli-Q water (Buffer A) and 100 % ACN (Buffer B) at a flow rate of 1 mL/min. Total run time was 20 min.

5.2.1.4.3 Anthranilate sugar standards

70 µL of anthranilate sugar standards (i.e. fructose, galactose, glucose, mannose, and lactose) was subjected to analytical RP-HPLC analysis on Synergi™ 4 µm Fusion-RP 80 Å, (150 x 4.60 mm) (00F-4424-E0, Phenomenex) operated in solvent system 0.1 % TFA/H_2O (Buffer A) and 0.1 % TFA/ACN (Buffer B) at a flow rate of 1 mL/min. Injection volume was 2 µL and method used was "Method B" as described in **Table 2-6**, Chapter 2, Section 2.10.1.4.

5.2.1.4.4 Anthranilate CS-PLP1 isolated from liquid phase extract obtained from centrifugation separation of *C. speciosus* rhizome cold-water extract

70 µL of anthranilate CS-PLP1 fraction contained different masses prepared in Section 5.2.5.2.2 were subjected to analytical RP-HPLC analysis on Synergi™ 4 µm Fusion-RP 80 Å, (150 x 4.60 mm) (00F-4424-E0, Phenomenex) operated in solvent system 0.1 % TFA/H_2O (Buffer A) and 0.1 % TFA/ACN (Buffer B) at a flow rate of 1 mL/min. Injection volume was 2 µL and method used was "Method B" as described in **Table 2-6**, Chapter 2, Section 2.10.1.4.

5.2.1.4.5 Comparison between anthranilate CS-PLP1 with anthranilate sugar standards

10 µL of the anthranilate CS-PLP1 was spiked with 10 µL of the anthranilate sugar standards (i.e. fructose, galactose, glucose, mannose, and lactose) with a mixture of 40 µL of ACN:H_2O with ratio 50:50. Anthranilic acid, with a concentration of 0.1 mg/mL, was used as a control. Injection volume was 10 µL and method used was "Method C" as described in **Table 2-6**, Chapter 2, Section 2.10.1.4. Separation of the sample chemical components was performed via column Synergi™ 4 µm Fusion-RP 80 Å, (150 x 4.60 mm) (00F-4424-E0, Phenomenex) operated in solvent system 0.1 % TFA/H_2O (Buffer A) and 0.1 % TFA/ACN (Buffer B) at a flow rate of 1 mL/min. Total run time was 20 min.

5.2.1.5 Thin Layer Chromatography Separation for Fraction 1

General information on the aluminium TLC plates were described in Chapter 2, Section 2.10.1.2.

The Fraction 1 (eluted from RP flash column chromatography) was analysed using analytical HPLC and revealed highly polar compounds (**Figure 5-3**); therefore, normal-phase (NP) TLC was used to determine a suitable solvent system to separate chemical components from Fraction 1 effectively. Mixtures of high polarity solvents (such as ethyl acetate (EtOAc) (Merck, Germany), formic acid (FA) (Merck, Germany), *n*-butanol (*n*-BuOH), methanol (MeOH) (HPLC grade; Scharlau, Spain), glacial acetic acid (GAA) (Merck, Germany), acetic acid (AcOH) (VWR, France), water (Milli-Q)), and non polar solvents (such as hexane (Hex) (Merck, Germany), dichloromethane (DCM) (LCMS grade; Merck, Germany)) with additive triethylamine (TEA) (Aldrich, USA) at different percentage ratios were used. Furthermore, reverse-phase (RP) (C18) TLC was also used for the separation of F1 with a high polar solvent (**Table 5-1**). Different intensities of F1 were spotted on both the NP and RP pre-coated silica TLC plate, i.e. six drops and three drops of F1 on left and right spots on each TLC plate, respectively. As the chemicals separated from F1 were colourless, both RP and NP TLC plates were dipped in phosphomolybdic acid and heated with a heat gun which allowed the visualization of spots under UV-C light (254 nm). The absorbent layer will fluoresce light-green under UV-C (254 nm), whereas the compounds that absorb UV-C will quench this fluorescence yielding dark purple or blue spots on the TLC plate.

Table 5-1. Solvent system used for normal-phase and reverse-phase thin layer chromatography for Fraction 1, eluted from reverse-phase flash column chromatography.

	Normal-phase - TLC	
	Solvent system	**Ratio (v/v)**
A.	MeOH:DCM	5:95
B.	MeOH:DCM	10:90
C.	MeOH:DCM	30:70
D.	Hex:EA:GAA:FA (Verma and Khosa, 2012)	2:1:1:0.75
E.	*n*-BuOH:AcOH: H_2O (BAW)	4:1:1
F.	*n*-BuOH:AcOH: H_2O (BAW)	5:4:1
G.	MeOH	100
H.	MeOH:TEA	95:5
I.	MeOH:AcOH	95:5
J.	H_2O:MeOH	50:50
K.	H_2O:MeOH	95:5
L.	H_2O:MeOH:AcOH	15:80:5
M.	H_2O:MeOH:AcOH	30:65:5
N.	H_2O:MeOH:AcOH	47.5:47.5:5
O.	H_2O:MeOH:TEA	47.5:47.5:5
	Reverse-phase - TLC	
	Solvent system	**Ratio (v/v)**
P.	H_2O:MeOH	95:5

5.2.1.6 Fractionation by Preparative High-Performance Liquid Chromatography

General methods and columns information were mentioned in Chapter 2, Section 2.10.1.5.

5.2.1.6.1 *C. speciosus* rhizome cold-water extract

200 mg of dried *C. speciosus* rhizome cold-water crude extract was dissolved in 3 mL of Milli-Q H_2O. The mixture was vortexed vigorously until fully dissolved. The extract was then filtered using a syringe filter to eliminate undissolved particles that will cause clogging to the column. Undissolved extract/suspended particles that were trapped in the syringe filter were flushed with 20 % MeOH/H_2O. The collected suspension was frozen using liquid nitrogen and lyophilized to obtain the dry extract. Subsequently, approximately 2.5 mL of the filtrate was injected into the system through sample injector. Chromatographic separation of the extract was operated through column Luna® 10 µm C8(2) 100 Å, AX (50 x 21.20 mm) (00B-4250-P0-AX; Phenomenex) at a flow rate of 10 mL/min. Solvent systems used were 100 % Milli-Q H_2O (Buffer A) and 100 % ACN (Buffer B). The method used a total run time of 40 min and the gradient of the chromatography started with 100 % Buffer A for 10 min. Then, it took 20 min to reach 60 % Buffer A and 40 % Buffer B. The next minute the gradient increased to 80 % Buffer B and 20 % Buffer A. Finally, returned to 100 % Buffer A at 32 min which then ended at 40 min. Data were managed and analysed using Empower™ 2 software. LCMS and ^{1}H NMR analyses were performed on all the fractions obtained.

Bioactivity of these fractions was validated by investigating their effects on EFS-induced contractile responses of isolated rat prostates.

5.2.1.6.2 Liquid phase extract of the *C. speciosus* rhizome cold-water extract

Liquid phase extract (121 mg) was dissolved in 1 mL of Milli-Q H_2O and injected for chemical components separation. Method used an isocratic gradient where it was maintained at 100 % Buffer A (100 % Milli-Q H_2O) for 10 min. Then it took 15 min to reach 80 % Buffer B (100 % ACN) and 20 % Buffer A (100 % Milli-Q H_2O). It was maintained for 6 min and back to 100 % Buffer A. The run was terminated at 40 min. The flow rate was 6 mL/min through column Luna® 10 µm C8(2) 100 Å, A X 1A P, (250 x 21.2 mm) (00G-4250-P0-AX; Phenomenex). The elution for each peak was collected otherwise every 30 mL. Fractions isolated were frozen using liquid nitrogen and lyophilized to obtain the dry mass. LCMS and ^{1}H NMR analyses were performed on all the fractions obtained.

Bioactivity of these fractions was validated by investigating their effects on EFS-induced contractile responses of isolated rat prostates.

5.2.1.6.3 Anthranilate sugar standards

For pure derivatives, a reverse-phase HPLC column Luna® 10 µm C8(2) 100 Å, A X 1A P, (250 x 21.2 mm) (00G-4250-P0-AX; Phenomenex) was used. 500 µL of Milli-Q H_2O were added into each anthranilate sugar standards. All the separations of large scale 2-AA derivatives of saccharides were performed using a method at a flow rate of 10 mL/min. Buffer A consisted of 0.1 % TFA in H_2O and Buffer B consisted of 0.1 % TFA in ACN. The column was equilibrated for 43 min with the same running method and 7 min with the initial condition of 100 % Buffer A. The derivatives were separated using 60 % Buffer A and 40 % Buffer B for 40 min followed by a linear gradient reaching 80 % Buffer B at 42 min and then a linear decrease to 0 % Buffer B at 43 min, ending at 60 min.

5.2.1.6.4 Anthranilate CS-PLP1 fraction isolated from liquid phase extract obtained from centrifugation separation of *C. speciosus* rhizome cold-water crude extract

140 µL of anthranilate CS-PLP1 was subjected to preparative RP-HPLC on Luna® 10 µm C8(2) 100 Å, A X 1A P, (250 x 21.2 mm) (00G-4250-P0-AX; Phenomenex). The mobile phase consisted of Buffer A (0.1 % TFA/H_2O) and Buffer B (0.1 % TFA/ACN) and was pumped at a flow rate 10 mL/min. Method used a total run time of 60.0 min. Gradient of the chromatography took 40 min to get to 60 % Buffer A (0.1 % TFA/H_2O) and 40 % Buffer B (0.1 % TFA/ACN), maintained for 2 min and changed to 20 % Buffer A and 80 % Buffer B. It got back to the original 100 % Buffer A at the next minute and ended at 60 min. The elution for each peak was collected otherwise every 30 mL. Fractions isolated were frozen using liquid nitrogen and lyophilized to obtain the dry mass. LCMS and ^{1}H NMR analyses were performed on all the fractions obtained.

5.2.1.6.5 Anthranilate CS-PLP1 sub-fractions

Sub-fractions isolated from the anthranilate CS-PLP1 was subjected to preparative RP-HPLC on Luna® 10 µm C8(2) 100 Å, A X 1A P, (250 x 21.2 mm) (00G-4250-P0-AX; Phenomenex). The mobile phase consisted of Buffer A (0.1 % TFA/H_2O) and Buffer B (0.1 % TFA/ACN) and were pumped at a flow rate 10 mL/min. Method used was “Slow Prep 254 Eunice Hydrophil” and total run time was 60.0 min. Gradient of the chromatography took 40 min to get to 60 % Buffer A (0.1 % TFA/H_2O) and 40 % Buffer B (0.1 % TFA/ACN), maintained for 2 min and change to 20 % Buffer A and 80 % Buffer B. It got back to the original 100 % Buffer A at the next minute and ended at

60 min. The elution for each peak was collected otherwise every 30 mL. Fractions isolated were frozen using liquid nitrogen and lyophilized to obtained the dry mass. LCMS and ^{1}H NMR analyses were performed on all the fractions obtained.

5.3 RESULTS

5.3.1 Method Development

5.3.1.1 Reverse-phase Flash Column Chromatography

The first attempt to fractionate the *C. speciosus* rhizome cold water crude extract utilized reverse-phase (RP) chromatography. In RP chromatography, polar components elute before non-polar components. Unfortunately, the attempt to fractionate a bulk (18 g) sample of the extract failed, as the C18 stationary phase of the RP flash column was blocked by particulate matter in the concentrate. However, the top of the stationery phase was retrieved and some partitioning was achieved from the unblocked column using filtration and washing, which yielded eight fractions (F1 – F8). The colour of the fractions changed from dark brown (F1 and F2) → yellow (F3 and F4) → pale yellow (F5 and F6) → colourless (F7) → pale yellow (F8). The stationary phase was washed to elute compounds that remained on the column and yielded four fractions: F*1 (10 % MeOH/H_2O), F*3 (50 % MeOH/H_2O), F*6 (100 % MeOH), and F*8 (100 % DCM). These fractions were analysed using LCMS and combined with the corresponding F3, F6, and F8 from the recovery filtration (**Appendix 6**). A flowchart of the procedure is outlined in **Appendix 6**.

While not an optimal separation, the collected fractions were analysed in organ bath studies. The EFS-induced contractile responses of the isolated rat prostates showed that bioactivity was found in the initial eluted fraction (10 % MeOH) prior to the column blocking (**Figure 5-2A**) and when the stationary phase was washed with 30 % MeOH (**Figure 5-2B**). There was no bioactivity found in fractions retrieved after washing with 50 % and 90 % MeOH, and 10 % and 100 % DCM (**Appendix 7**). A ^{1}H NMR spectrum of F*1 (eluted from column) is shown in **Figure 5-1**. Analytical HPLC analysis further confirmed that the F*1 contained mainly polar components that eluted with the void (**Figure 5-3**).

We learned that the C18 RP flash column was not suitable for the isolation of bioactive components from the *C. speciosus* rhizome cold water extract as the stationary phase was blocked by the extract. However, we also learned that the bioactivity resided in the most polar fractions as other fractions retrieved using less polar organic solvents did not affect the EFS-induced contractile responses of the isolated rat prostates.

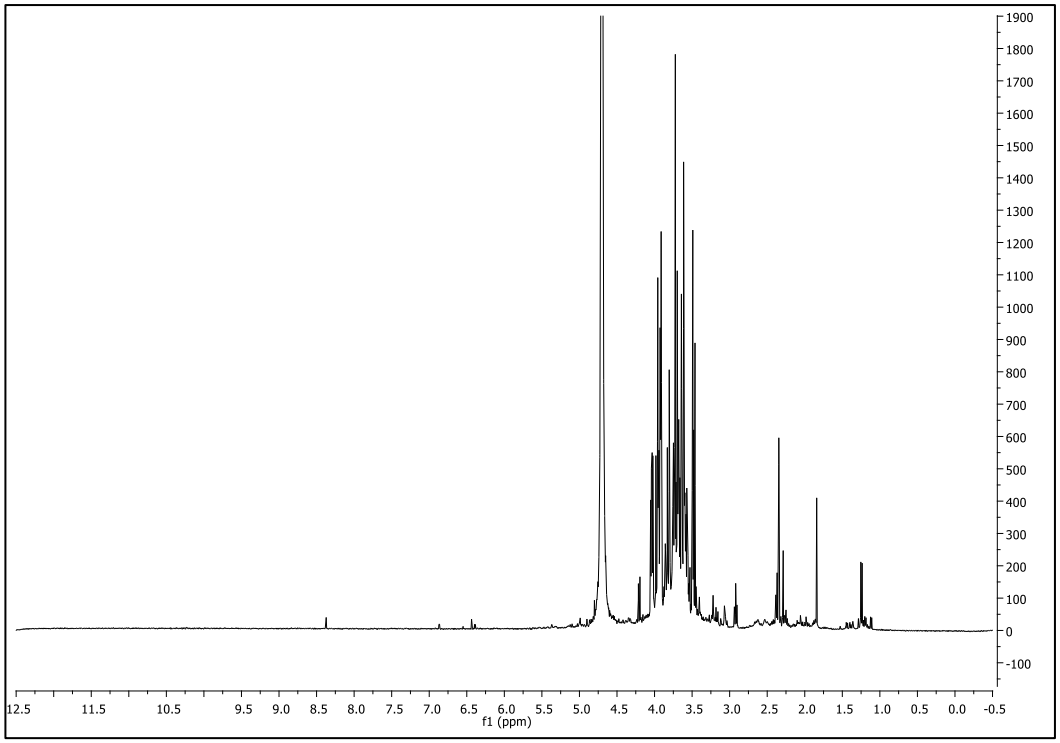
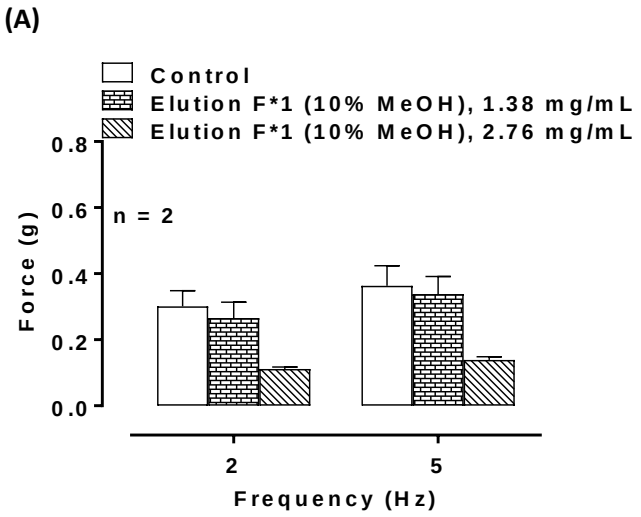


Figure 5-1. ^{1}H NMR spectrum: Fraction 1 (F*1) (10 % MeOH) eluted from RP flash column diluted in D_2O.



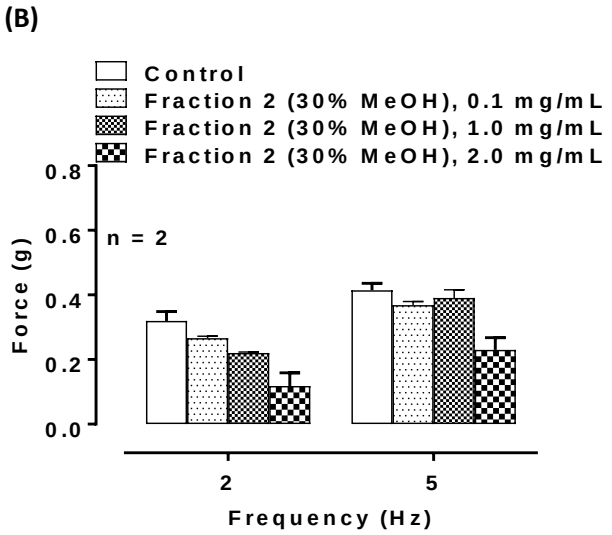


Figure 5-2. Bioactivity validation for selected fractions. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 2 and 5 Hz) in isolated rat prostates after administration (**A**) Fraction 1 (F*1) eluted from column (10 % MeOH/H_2O) (1.38 mg/mL, n = 2; 2.76 mg/mL, n = 2) and (**B**) Fraction 2 (30 % MeOH/H_2O) (0.1 mg/mL, n = 2; 1.0 mg/mL, n = 2, 2.0 mg/mL, n = 2). Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA, followed by Tukey's multiple comparisons test).

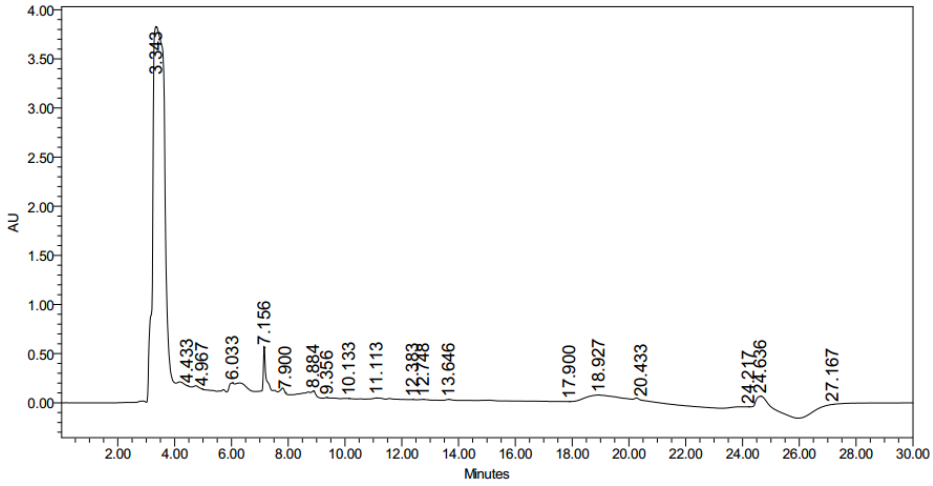


Figure 5-3. Analytical HPLC of F*1 eluted from RP flash column chromatography. Flow rate is 1 mL/min. Method used for the separation had a total run time of 30 min. Mobile phase: Buffer A (0.1 % TFA/H_2O) and Buffer B (0.1 % TFA/ACN).

5.3.1.2 Thin Layer Chromatography Separation for Fraction 1

An attempt was made to analyse the bioactive fraction by TLC to determine a suitable solvent system for the isolation of bioactive components from the extract. On normal phase TLC plates, 15 solvent conditions were examined but were unable to resolve the components present in F*1 (Traces **A – O, Figure 5-4**).

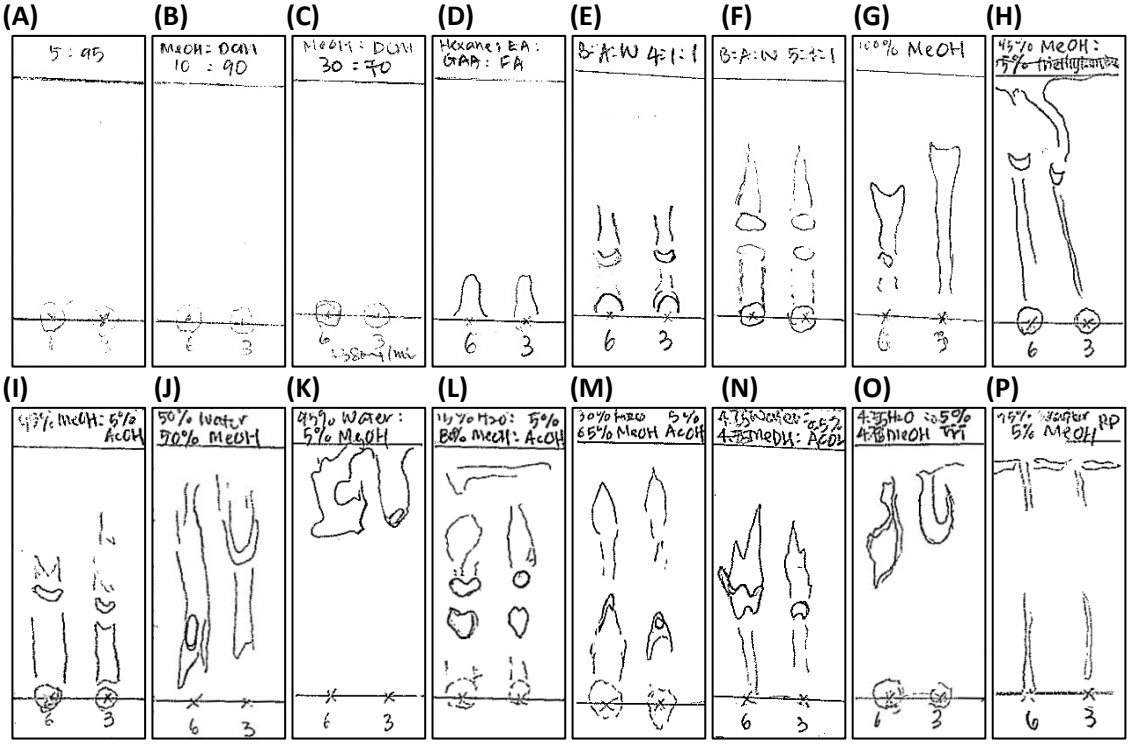


Figure 5-4. (A – O), normal-phase and **(P),** reverse-phase thin layer chromatography of F1 using solvent system as described in **Table 5-1**. Intensity on left side spot consisted of six drops of F1, whereas right side consisted of three drops of F1.

Similarly, an attempt to use RP-TLC gave no useful results (Trace **P, Figure 5-4).** There were clearly multiple components present in F*1 based on elution and resolution on **Figure 5-4** that were beyond the resolving power of TLC.

5.3.1.3 Fractionation *C. speciosus* Rhizome Cold-water Extract via Preparative High Performance Liquid Chromatography

We next turned to RP-HPLC to fractionate the sample. First, a 200 mg sample of the extract was suspended in water and filtered. Chromatographic fractionation of the filtrate yielded 20 fractions (CS-PF1 – CS-PF20) (**Appendix 8**).

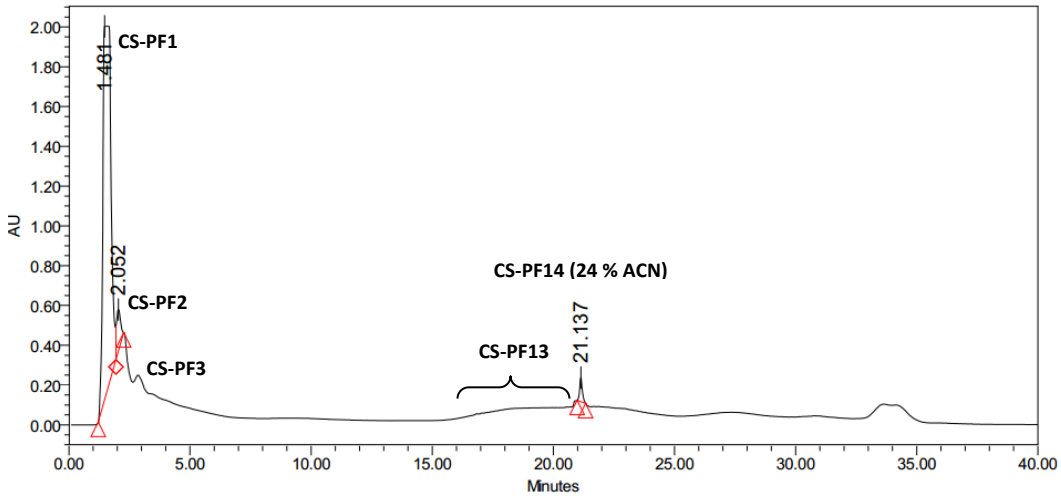


Figure 5-5. Preparative HPLC fractionation of filtered *C. speciosus* rhizome cold-water crude extract. Flow rate was 10 mL/min. Method used a total run time of 40 min. Mobile phase: Buffer A (100 % Milli-Q H_2O) and Buffer B (100 % ACN). Twenty fractions were collected. The four major peaks were designated as CS-PF1, CS-PF2, CS-PF3, and CS-PF4, respectively.

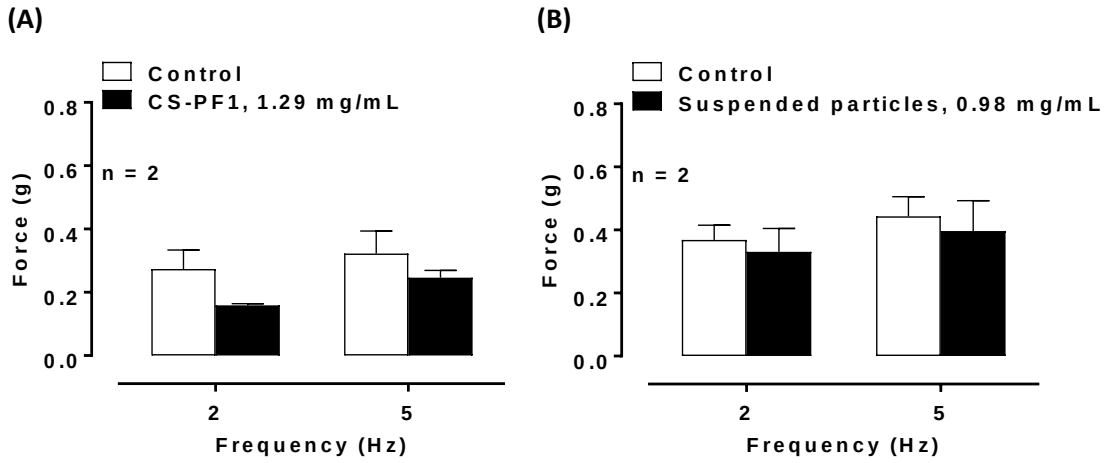


Figure 5-6. Mean contractile responses of fractions isolated from preparative HPLC fractionation of filtered *C. speciosus* rhizome cold-water crude extract. **(A),** CS-PF1, 1.29 mg/mL; **(B),** Suspended particles, 0.98 mg/mL. Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA).

EFS results showed that the first eluted fraction, CS-PF1, attenuated contractile responses of isolated rat prostates at 1.29 mg/mL (**Figure 5-6A**). The rest of the fractions did not affect electrically evoked contractile responses of isolated rat prostates (**Appendix 9**) as compared to the control. The insoluble residue was re-suspended (0.98 mg/mL; **Figure 5-6B**) and interestingly it showed moderate inhibition on the EFS-induced contractions of rat prostates. We hypothesised that this may be due to the residual polar components.

5.3.1.4 Centrifugation Separation

In order to improve the separation of liquid and solid phases, a 200 mg sample of the extract was centrifuged at 11,000 rpm for 15 minutes. The clear brown liquid (**Figure 5-7**) was carefully aspirated and then the liquid and solid phases were lyophilized to dryness. Dried mass yielded for liquid phase was 121 mg and solid phase was 58 mg (**Appendix 10**).

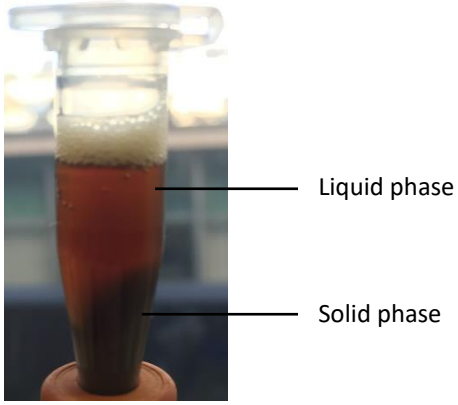


Figure 5-7. Centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Upper layer opaque brownish colour: Liquid phase; Bottom layer undissolved dark brown colour: Solid phase.

Frequency-response graphs of the liquid and solid phase effect on isolated rat prostates are shown in **Figure 5-8**. The graphs show that the liquid phase extract (1.3 mg/mL) (**Figure 5-8A;** p = 0.052 mg/mL, n = 4) strongly attenuated EFS-induced contractile responses of rat prostates, with percentage of the contraction remained was 58±7 % (**Figure 5-9A**; p = 0.0448, n = 4), 66±5 % (**Figure 5-9B**; p = 0.0128, n = 4), and 75±6 % (**Figure 5-9C**; p = 0.0177, n = 4), respectively at 2.0 Hz, 5.0 Hz, and 10 Hz, as compared to solid phase extract (0.5 mg/mL) (**Figure 5-8B**; p = 0.4030, n = 4) and the vehicle. Differences between the liquid and solid phase extracts with the vehicle were tested using one-way analysis of variance (ANOVA) followed by Fisher's LSD test for the statistical evaluations (**Figure 5-9**).

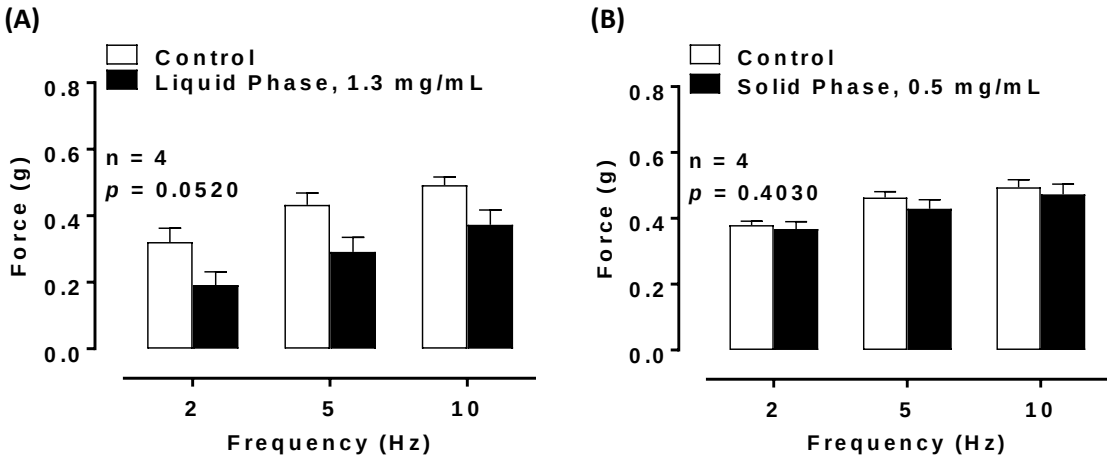


Figure 5-8. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 2 Hz, 5 Hz, and 10 Hz, 10 s pulses) in isolated rat prostates administered with **(A)** liquid phase (1.30 mg/mL; $p = 0.0520$, n = 4) and **(B)** solid phase (0.5 mg/mL; $p = 0.4030$, n = 4) extracts obtained from the centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Bars represent mean force ± S.E.M. (*$p < 0.05$; two-way repeated-measures of ANOVA). *p*-values represent probability of the treatment causing a significant change in the contractile responses.

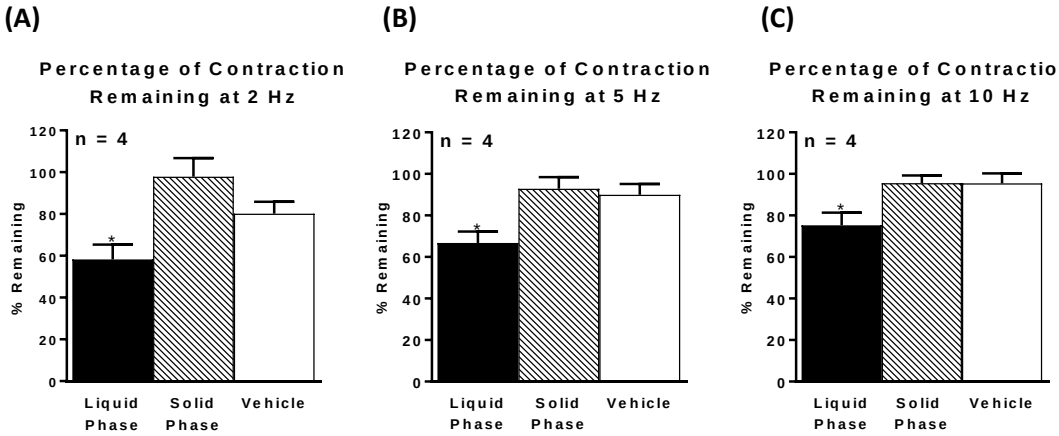


Figure 5-9. Mean percentage of contraction remained to nerve-mediated contraction (2 Hz, 5 Hz, 10 Hz) in rat prostates administered with liquid phase (1.3 mg/mL) and solid phase (0.5 mg/mL) extracts obtained from the centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Liquid phase extract has significantly attenuated EFS-induced contractions of isolated rat prostate as compared to the vehicle in **(A)** 2 Hz ($p = 0.0448$, n = 4), **(B)** 5 Hz ($p = 0.0128$, n = 4), **(C)** 10 Hz ($p = 0.0177$, n = 4). Results are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for the statistical evaluations.

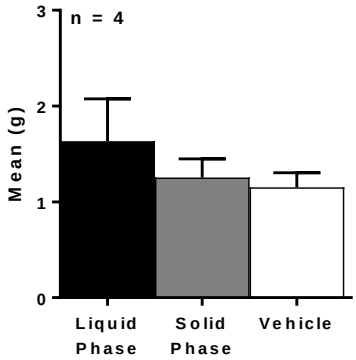


Figure 5-10. Mean area under the curve of tonic contraction, elicited on unstimulated isolated rat prostates after administration of liquid phase (1.3 mg/mL) and solid phase (0.5 mg/mL) extracts obtained from the centrifugation separation of *C. speciosus* rhizome cold-water crude extract. The extract produced tonic contraction on isolated rat prostates upon administration prior to 30 min incubation period. Graph bars represent mean force ± S.E.M. (*$p < 0.05$). One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for the statistical evaluations. *p*-values represent probability of the treatment causing a significant change in the contractile responses.

Interestingly, the tonic contraction effect on the unstimulated isolated rat prostates was only produced by the liquid phase extract but not by the solid phase extract (**Figure 5-10**). The magnitude of the tonic contraction produced by the liquid phase extract was greater than the

solid phase extract as compared to the vehicle. Tonic contraction generated was transient and the force returned to the base line after 5 – 10 seconds, which was shorter and smaller as compared to *C. speciosus* rhizome cold-water crude extract as mentioned in Chapter 3, Section 3.3.2.4.3.

Analytical reverse-phase chromatographic analysis revealed a major peak with high absorbance intensity at a relatively early retention time of 7.1 min (**Figure 5-11**).

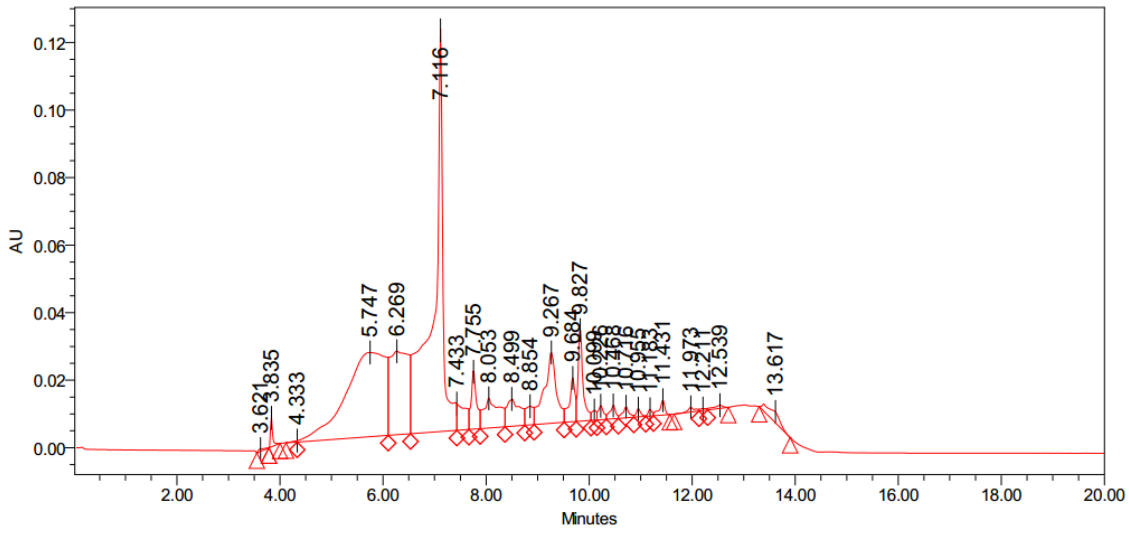


Figure 5-11. Analytical RP-HPLC analysis of the liquid phase extract obtained from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Mobile phase: Buffer A (100 % Milli-Q H_2O) and Buffer B (100 % ACN). UV detection wavelength at 254 nm. Flow rate was 1 mL/min. Method used a total run time of 20 min.

5.3.1.5 Preparative RP-HPLC Fractionation of Liquid Phase Extract from Centrifuged *C. speciosus* Rhizome Cold-water Extract

Preparative RP-HPLC separation of the soluble portion of the centrifuged extract yielded 10 fractions (i.e. CS-PLP1 – CS-PLP10) (**Appendix 10**). The first peak eluted was CS-PLP1 at retention time 9.1 min (**Figure 5-12**) with the highest absorbance reading among all the 10 fractions retrieved. Electrical field stimulation results showed that the CS-PLP1 attenuated contractile responses of isolated rat prostates at 0.97 mg/mL (**Figure 5-13**). Other fractions did not inhibit EFS-induced contractile responses of isolated rat prostates as compared to the control **(Appendix 11)**.

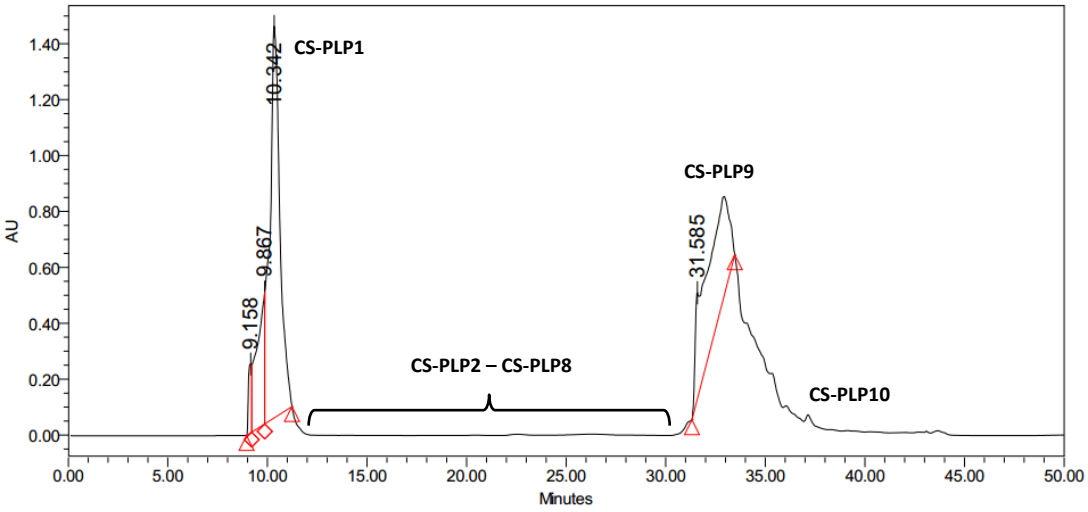


Figure 5-12. Preparative RP-HPLC of the liquid phase extract obtained from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Mobile phase: Buffer A (100 % Milli-Q H_2O) and Buffer B (100 % ACN). UV detection wavelength at 254 nm. Flow rate was 6 mL/min. Method used a total run time of 40 min.

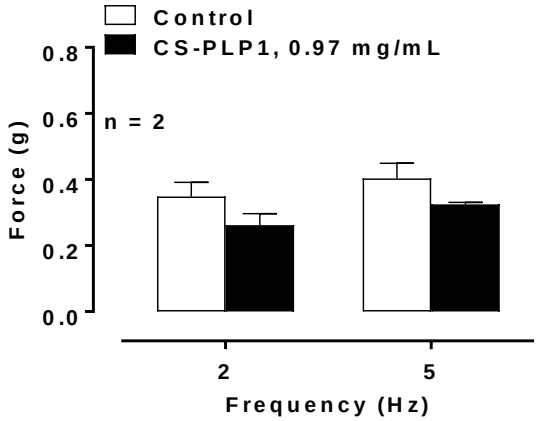


Figure 5-13. Mean contractile responses of fraction CS-PLP1 (1.29 mg/mL) isolated from preparative RP HPLC fractionation of liquid phase extract isolated from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA).

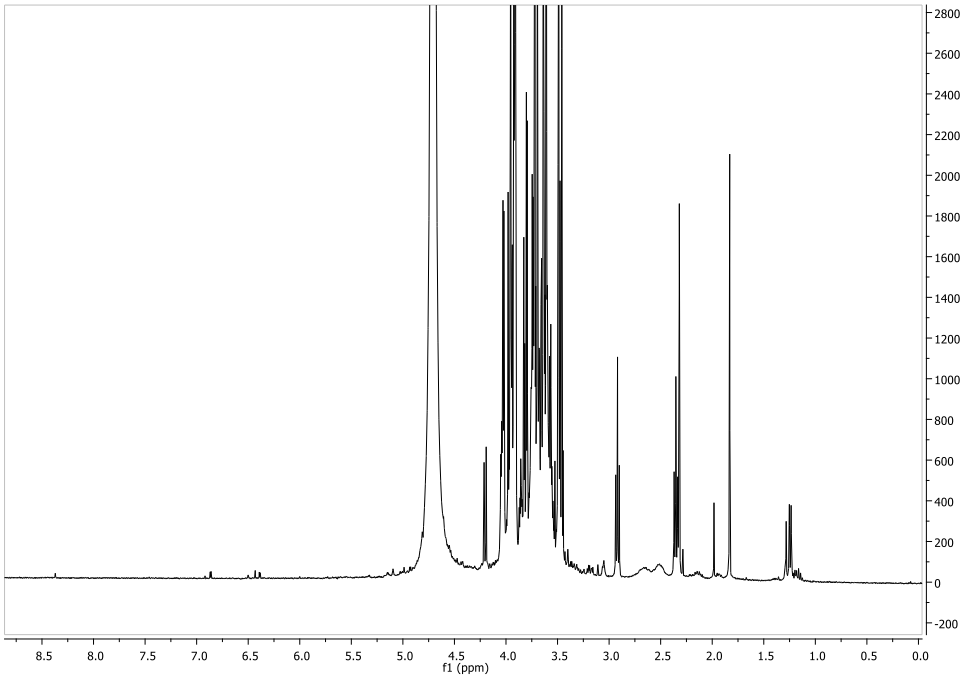


Figure 5-14. ^{1}H NMR spectrum: CS-PLP1 isolated from liquid phase extract from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. The fraction was dissolved in D_2O.

^{1}H NMR spectrum of CS-PLP1 (**Figure 5-14**) showed that the fraction still consisted of carbohydrates. However, the spectrum is less complex compared to the NMR of Fraction 1 (F*1) in **Figure 5-1**. Centrifugation separation followed by preparative RP-HPLC managed to remove some inactive constituents, leaving a simpler sample for analysis but one that still contained multiple components. In summary, an active component from 200 mg of the cold-water extract of *C. speciosus* rhizome could be concentrated into a 58 mg sample (**Appendix 10**). As summarised in **Figure 5-15,** the crude extract was fractionated into three major parts and the bioactivity of the extract has been successfully identified in the soluble portion that eluted with the solvent from RP-HPLC.

While further isolation of the bioactive components from the fraction may be possible, it would require techniques not available in our laboratories. These include a better RP stationary phase, such as those with good carbohydrate selectivity.

Given the likelihood that the sample was carbohydrate based, we instead sought to derivatise the extract to see if we could positively identify the major components of the extract, either by comparison to authentic samples or *de novo* characterisation.

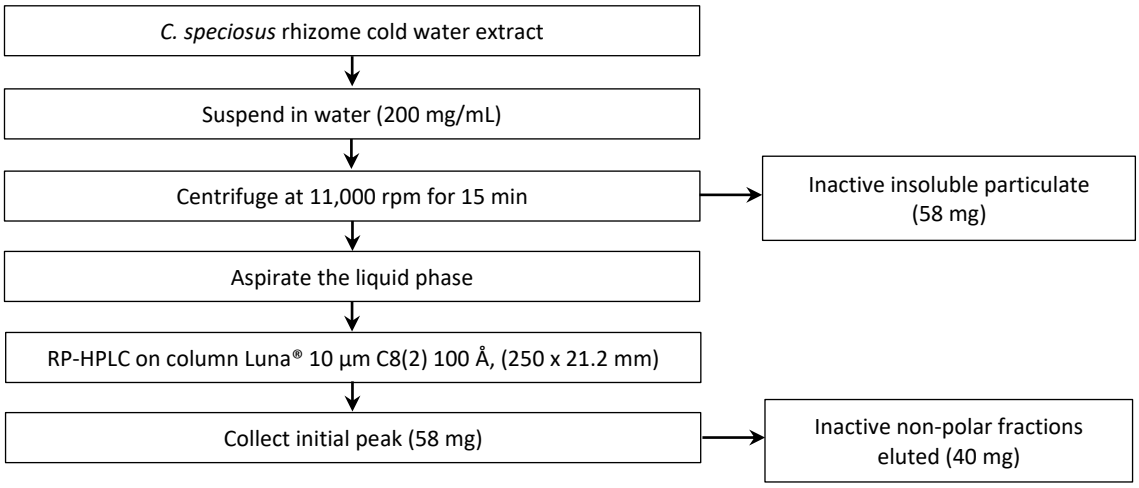


Figure 5-15. Procedures used to isolate bioactive fraction from the *C. speciosus* rhizome cold water crude extract (200 mg).

5.3.2 Derivatization

5.3.2.1 Derivatized Sugar Standards

5.3.2.1.1 Preparation and analysis of derivatized standards

The sugar standards were derivatized with anthranilic acid. Analytical reverse-phase chromatography of anthranilic acid (0.1 mg/mL) produced a single peak at retention time 4.3 min, which served as the control. The saccharide derivatives were prepared on 800 mg scale and purified by preparative HPLC, with UV detection at 254 nm and were eluted at 18 min for arabinose (2-AA-Ara) and 28 min for fructose (2-AA-Fru); whereas for smaller scale (14 mg), saccharide derivatives were detected with a UV detector at 254 nm and eluted at 31 min for glucose (2-AA-Glu), 29.5 min, 30.0 min, 31.2 min for fructose (2-AA-Fru), 27.0 min for lactose (2-AA-Lac), 29.4 min for mannose (2-AA-Man), 33.4 min for arabinose (2-AA-Ara), 29.0 min for galactose (2-AA-Gal), and 36.4 min for rhamnose (2-AA-Rha).

Table 5-2. Percentage yield of anthranilate sugars after purification.

Anthranilate sugar	Percentage yield (%)
2-AA-Glu	9
2-AA-Fru	14
2-AA-Lac	29
2-AA-Man	7
2-AA-Ara	18
2-AA-Gal	22
2-AA-Rha	25

5.3.2.1.2 LCMS of anthranilate sugar standards

LCMS spectra for anthranilate sugar standards, which used a UV detector at 214 nm, demonstrated that 2-AA-Fru was eluted at 10.55 min, 10.89 min, and 10.95 min with mass peaks 373.55, 302.4 and 373.6, respectively. 2-AA-Gal, 2-AA-Glu, and 2-AA-Man, which were eluted at 10.79 min, 10.88 min and 10.88 min, respectively, all contained a mass peak at 302.4. 2-AA-Lac, which is a disaccharide, was eluted at 10.57 min with mass peak 464.5. Arabinose, which is an aldohexose, its anthranilate derivatives (2-AA-Ara) was eluted at 11.10 min with mass peak 272.4. Furthermore, rhamnose, which is normally known as 6-deoxy-hexose, its anthranilate derivatives (2-AA-Rha) (**Figure 5-16**) was eluted at 11.32 min with mass peak 286.4.

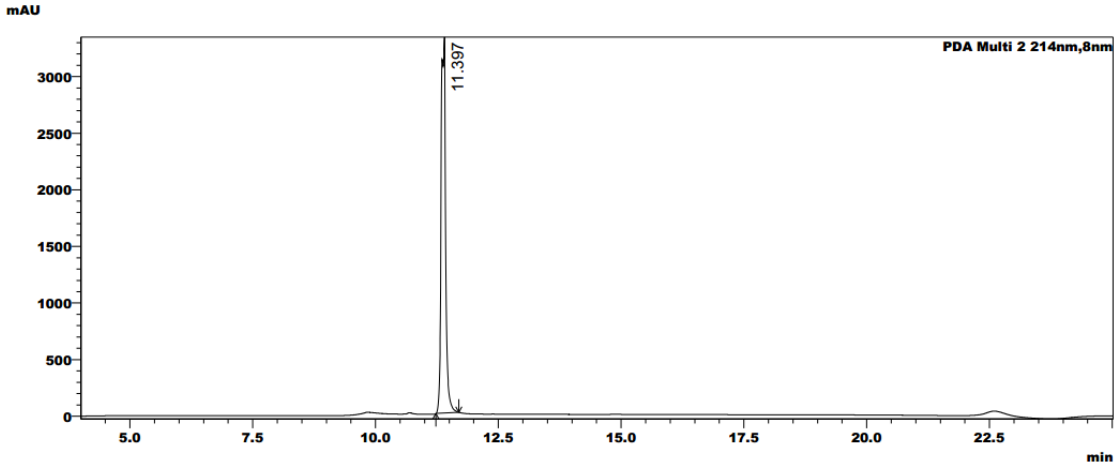


Figure 5-16. LCMS analysis: 2-AA-Rha.

5.3.2.2 Derivatisation of CS-PLP1 with Anthranilic Acid

5.3.2.2.1 Preparation and analysis of derivatized CS-PLP1

LCMS spectra for anthranilate CS-PLP1 (**Figure 5-17**), which used a UV detector at 214 nm, revealed the fraction consisted of five peaks (**Table 5-3**).

Table 5-3. LCMS analysis of anthranilate CS-PLP1.

Peak	Retention time (min)	m/z	Inference
1	10.53	220.0 233.0 243.0 255.0 261.4 272.0 273.0 287.0 301.0 362.3 464.5 523.6	Same m/z as 2-AA-Ara Same m/z as 2-AA-Lac
2	10.93	272.4 286.4 302.4 316.4 448.5 464.5	Same m/z as 2-AA-Ara Same m/z as 2-AA-Rha Same m/z as 2-AA-Fruc, 2-AA-Glu, 2-AA-Gal, 2-AA-Man Same m/z as 2-AA-Lac
3	11.11	272.4 302.4 316.4 387.5	Same m/z as 2-AA-Fruc, 2-AA-Glu, 2-AA-Gal, 2-AA-Man
4	11.29	286.4 316.4 448.5	Same m/z as 2-AA-Rha
5	13.23	268.4	

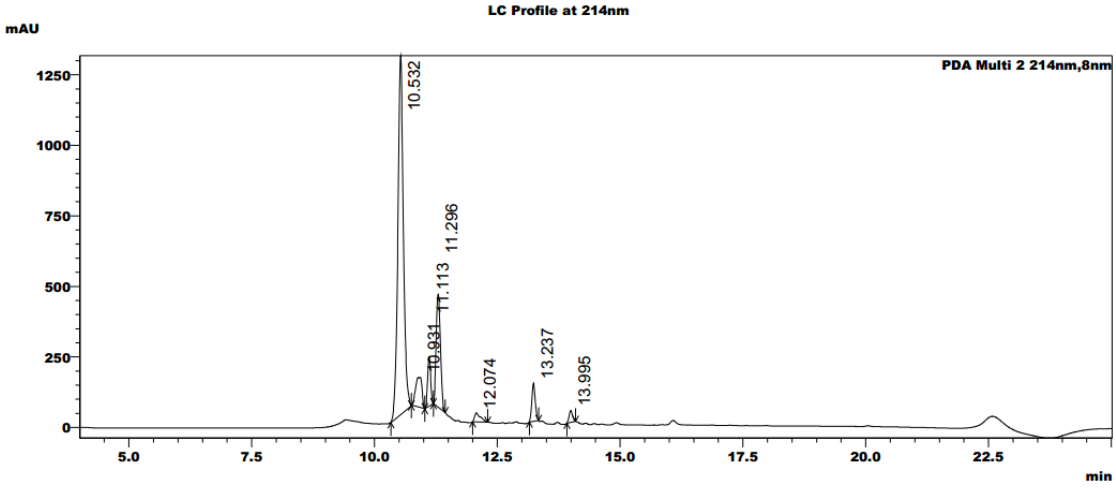


Figure 5-17. LCMS spectrum: Anthranilate CS-PLP1.

5.3.2.3 Comparison between Anthranilate CS-PLP1 with Anthranilate Sugar Standards

Anthranilate CS-PLP1 was spiked with anthranilate sugar standards. Analytical RP-HPLC separation demonstrated that anthranilate CS-PLP1 had several peaks overlapping with the sugar standard derivatives (**Appendix 13**), which indicated that CS-PLP1 may consist of those

standards. This result led to an attempt to isolate the derivatised components on a preparative scale for ^{1}H NMR analysis.

5.3.2.4 Preparative RP-HPLC Fractionation of Anthranilate CS-PLP1

The anthranilate CS-PLP1 was purified through a preparative RP-HPLC column and five fractions were collected as depicted (**Figure 5-18**). First fraction (D-1) was eluted at 4 min, followed by a second fraction (D-2), which eluted at 5 min. Third (D-3) and fourth (D-4) fractions were eluted at 6 min and 7 min, respectively. Last fraction (D-5) was eluted at 8.16 min (**Figure 5-18**). Dried CS-PLP1-A1 was pale yellow in colour (**Figure 5-19A**), whereas other fractions were off-white powders (**Figure 5-19B, C, D, E**).

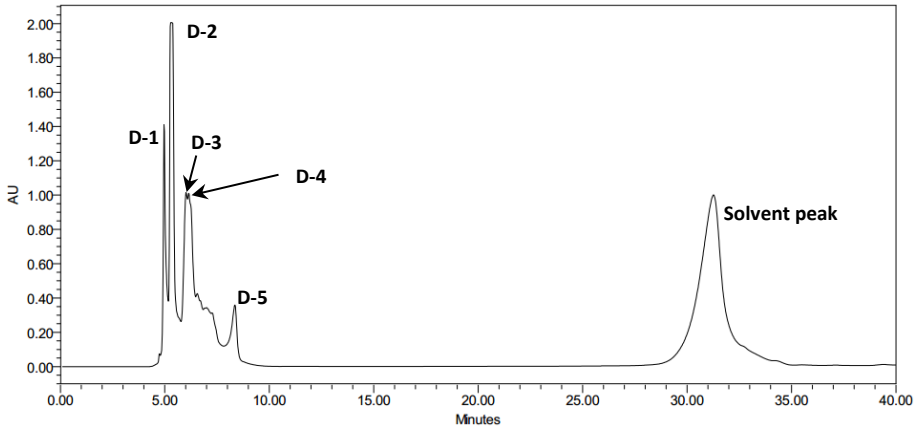


Figure 5-18. Preparative RP-HPLC: Anthranilate CS-PLP1. Five peaks have been isolated: D-1 to D-5. All the peaks were eluted between 5 – 9 min. Mobile phase: Buffer A (0.1 % TFA/H_2O) and Buffer B (0.1 % TFA/ACN). UV detection at 254 nm. Flow rate was 10 mL/min. Method used a total run time of 40 min.

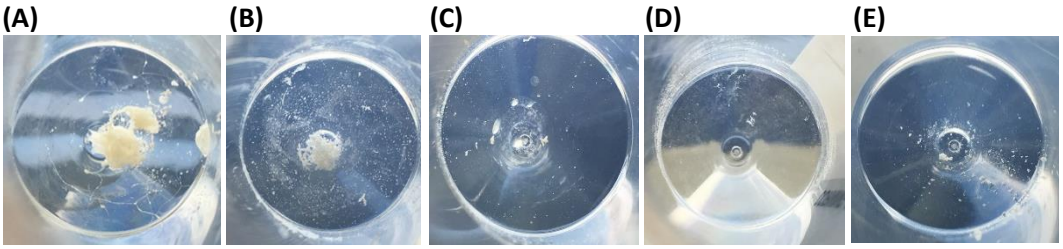


Figure 5-19. Physical characteristic of fractions yielded from preparative RP chromatographic separation of anthranilate CS-PLP1. **(A)** D-1; **(B)** D-2; **(C)** D-3; **(D)** D-4; **(E)** D-5.

Analysis of these fractions (D-1, D-2, D-3, D-4, and D-5) by LCMS revealed each fraction contained several peaks which eluted in the UV 214 nm profile (**Appendix 14**). This indicates the fractions collected were not pure.

5.3.2.4.1 Preparative RP-HPLC fractionation of anthranilate CS-PLP1 sub-fractions

To obtain purer fractions, further preparative RP-HPLC fractionation of anthranilate CS-PLP1 sub-fractions was performed (**Appendix 15**). The last three sub-fractions (D-3, D-4 and D-5) were combined for the isolation. Preparative RP chromatographic separation of these sub-fractions is depicted in **Figure 5-20.** LCMS analysis revealed that D-1-12 was eluted at 10.39 min, 10.72 min, 11.13 min, 11.30 min, 11.96 min, and 12.09 min with mass peaks 262.4, 464.5, 316.4, 316.4, 298.4 and 298.4, respectively. D-1-15 was eluted at 11.79 min with mass peak 373.6. D-1-16 was eluted at 12.11 min with mass peak 298.4. D-2-4 and D-2-5, which were eluted at 10.93 min and 10.02 min respectively, contained the same mass peak 302.4. D-(3,4,5)-4 was eluted at 11.01 min and contained the mass peak 302.4. Furthermore, D-(3,4,5)-6 and D-(3,4,5)-7, which eluted at 11.25 min and 11.18 min respectively, contained the same mass peak 272.4. D-(3,4,5)-8 eluted at 11.12 min and 11.24 min with same mass peak 272.4, and 11.34 min with mass peak 272.3. D-(3,4,5)-9 was eluted at 11.33 min with mass peak 286.4. Lastly, D-(3,4,5)-10 was eluted at 11.33 min and 11.46 min, consisting of mass peaks 242.3 and 286.4 respectively. Other fractions which did not show any peaks in the LC spectra are not mentioned here (**Figure 5-20**).

5.3.2.5 ^{1}H NMR Spectra Comparison between Anthranilate CS-PLP1 Isolated Sub-fractions with Anthranilate Sugar Standards

With several pure fractions, D-2-4, D-2-5, D-(3,4,5)-4, D-(3,4,5)-6, D-(3,4,5)-7, D-(3,4,5)-8, D-(3,4,5)-9 and D-(3,4,5)-10, which in our hands matched the HPLC RT and the m/z ion of our standards, a comparison of their NMR spectra was made to unambiguously confirm identity with the standards. We hypothesized that 2-AA-Fruc might match to fractions D-2-4, D-2-5, or D-(3,4,5)-4. Superimposition of the ^{1}H NMR spectrum of these samples showed that their proton chemical shifts were very different although D-2-4, D-2-5, and D-(3,4,5)-4 showed quite similar resonances with 2-AA-Fru.

Furthermore, superimposition of the ^{1}H NMR spectrum of 2-AA-Ara with fractions D-(3,4,5)-6, D-2-7, and D-(3,4,5)-8 also failed to match. Of note, D-(3,4,5)-9 and D-(3,4,5)-10 despite having the same m/z ion as 2-AA-Rha, their ^{1}H NMR spectra also did not match (**Appendix 16**). We speculate that the fractions might contain other C5 and C6 sugars in the chemical structures. However, there are also other possible factors which might affect the NMR spectra, such as some reagents from the derivatization process, pH, concentrations, etc. As the peak positions and multiplicity of the NMR spectra is too similar for a lot of them to be a coincidence.

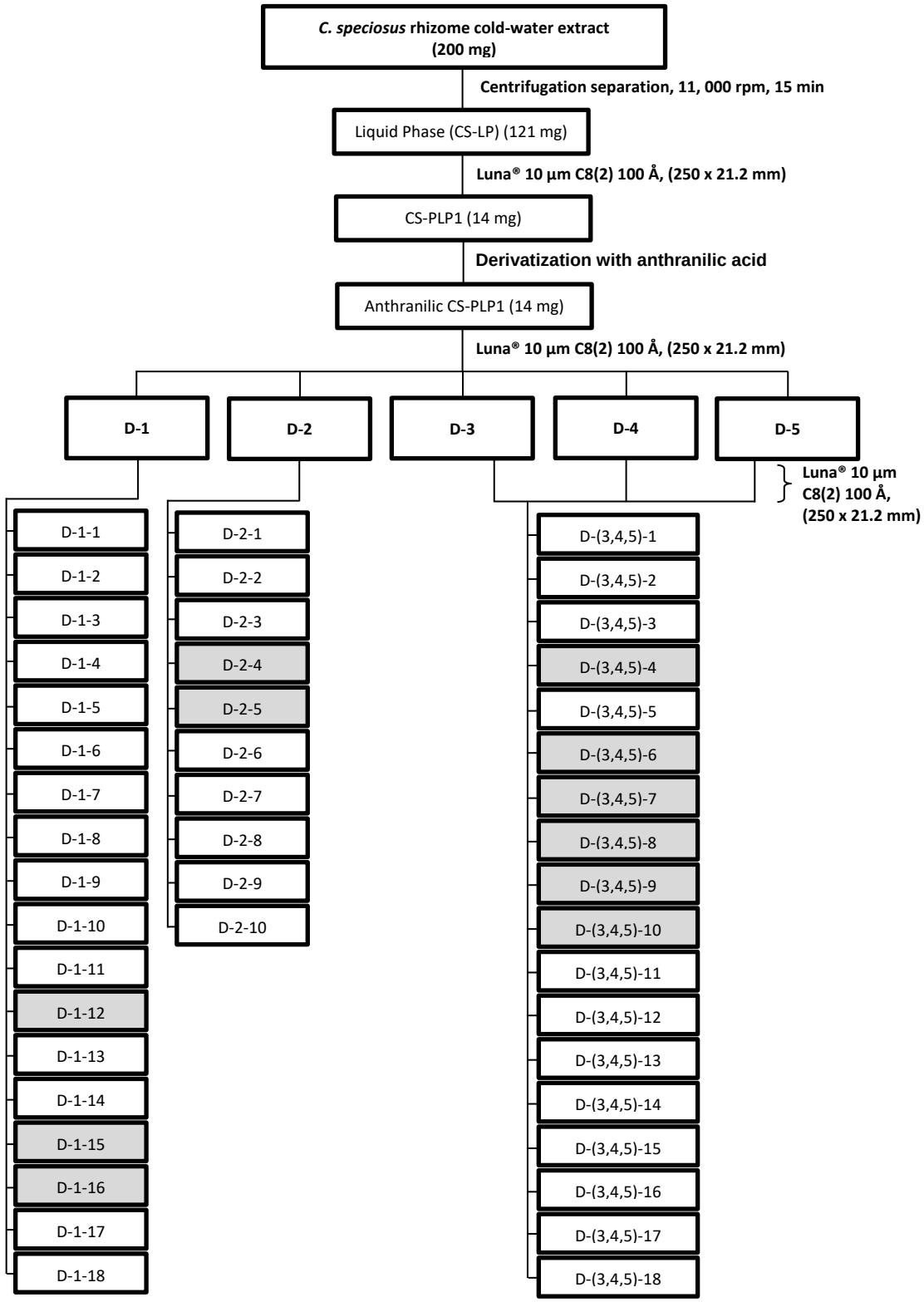
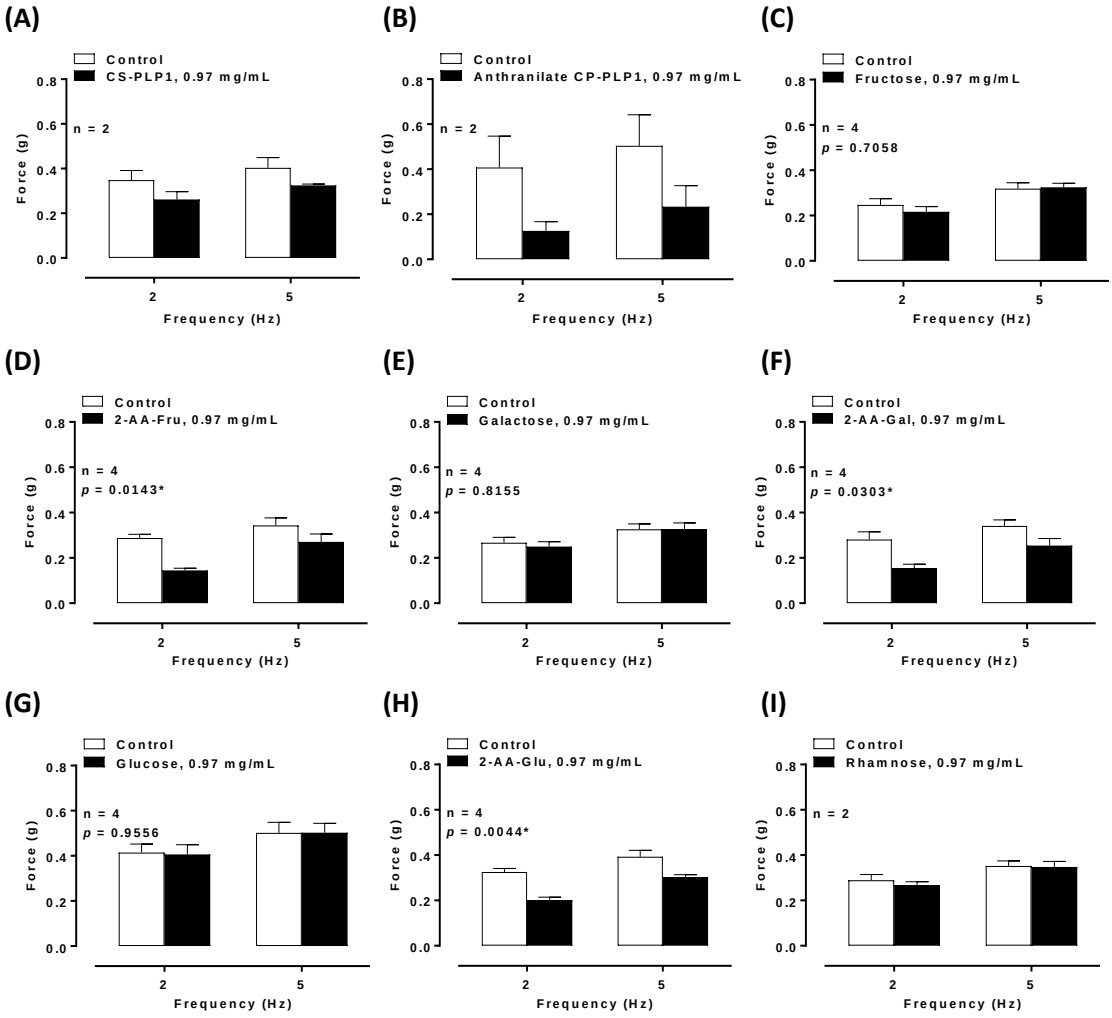


Figure 5-20. Chronology showing isolation of liquid phase (CS-LP) from centrifugation separation of *C. speciosus* rhizome cold-water crude extract followed by preparative reverse-phase chromatographic fractionation. Derivatization was performed to the first fraction (CS-PLP1) and further fractionation of anthranilate CS-PLP1 yielded five fractions (D-1, D-2, D3, D4, D-5). Finally, preparative reverse-phase HPLC fractionation of anthranilate CS-PLP1 sub-fractions yielded 18 fractions for D-1, 10 fractions for D-2, and 18 fractions for D-(3,4,5) combined mixture. Fractions that showed peaks in the LCMS 214 nm UV profile are highlighted with grey colour.

5.3.3 Bioactivity Validation for Anthranilate CS-PLP1 Using Electrical Field Stimulation

Frequency-response graphs demonstrate that greater inhibition activity was observed in anthranilate CP-PLP1 (**Figure 5-21B**) as compared to the underivatized CP-PLP1 (**Figure 5-21A**). Furthermore, EFS-induced contractile responses of isolated rat prostates were not affected by fructose (**Figure 5-21C**), galactose (**Figure 5-21E**), glucose (**Figure 5-21G**), rhamnose (**Figure 5-21I**), and arabinose (**Figure 5-21K**). In contrast, all their respective derivatives show inhibition on EFS-induced contractions of rat prostates (**Figure 5-21D, F, H, J, L**). Of note, the derivatization reagent did not have an effect on the EFS-induced contractions.



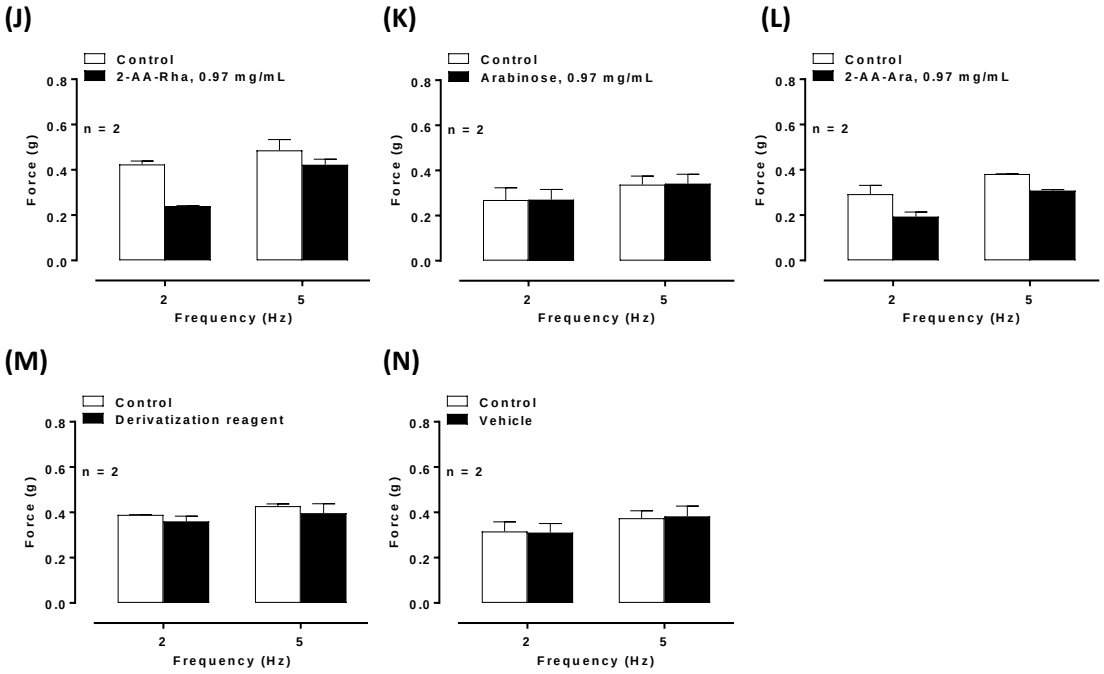
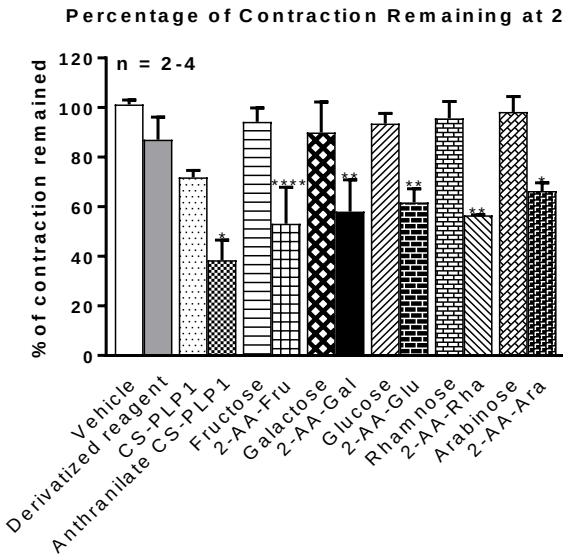


Figure 5-21. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 2 and 5 Hz) in isolated rat prostates before (open columns) and after (solid columns) administration of **(A)** CS-PLP1 (0.97 mg/mL; n = 2), **(B)** Anthranilate CS-PLP1 (0.97 mg/mL; n = 2), **(C)** Fructose (0.97 mg/mL; p = 0.7058, n = 4), **(D)** 2-AA-Fru (0.97 mg/mL; p = 0.0143, n = 4), **(E)** Galactose (0.97 mg/mL; p = 0.8155, n = 4), **(F)** 2-AA-Gal (0.97 mg/mL; p = 0.0303, n = 4), **(G)** Glucose (0.97 mg/mL; p = 0.9556, n = 4), **(H)** 2-AA-Glu (0.97 mg/mL, p = 0.0044, n = 4), **(I)** Rhamnose (0.97 mg/mL; n = 2), **(J)** 2-AA-Rha (0.97 mg/mL; n = 2), **(K)** Arabinose (0.97 mg/mL; n = 2), **(L)** 2-AA-Ara (0.97 mg/mL; n = 2), **(M)** Derivatization reagent (n = 2), and **(N)** Vehicle (Milli-Q H_2O). Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA) (*p < 0.05). The p-values represent the probability of a significant interaction between treatment and frequency.

Comparison of the mean percentage remained for EFS-induced contractions at 2 Hz (**Figure 5-22A**; p = 0.0300, n = 2) and 5 Hz (**Figure 5-22B**; p = 0.0055, n = 2), between CS-PLP1 and anthranilate CS-PLP1, revealed that the latter has significantly inhibited EFS-induced contractions, as compared to CS-PLP1. Interestingly, results also showed that all the reductive sugars (i.e. 2-AA-Fru, 2-AA-Gal, 2-AA-Glu, 2-AA-Rha, 2-AA-Ara) significantly attenuated EFS-induced contractions of rat prostates as compared to the non-derivatized counterpart. These inhibitory effects were not contributed by the derivatization reagent that was present in the derivatized samples.

(A)



(B)

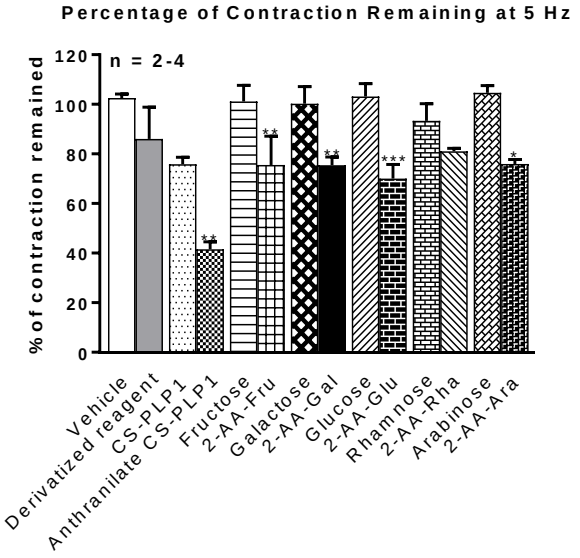


Figure 5-22. (A) Comparisons of mean percentage of contraction remained to EFS-induced contractions in rat prostates between CS-PLP1 and anthranilate CS-PLP1 (0.97 mg/mL; $p = 0.0300$, n = 2), derivatized reagent and vehicle (Milli-Q H_2O) (0.97 mg/mL; $p > 0.9999$, n = 2), fructose and 2-AA-Fru (0.97 mg/mL; $p < 0.0001$, n = 4), galactose and 2-AA-Gal (0.97 mg/mL; $p = 0.0018$, n = 4), glucose and 2-AA-Glu (0.97 mg/mL; $p = 0.0018$, n = 4), rhamnose and 2-AA-Rha (0.97 mg/mL; $p = 0.0077$, n = 2), arabinose and 2-AA-Ara (0.97 mg/mL; $p = 0.0418$, n = 2) at 2 Hz. **(B)** Comparisons of mean percentage of contraction remained to EFS-induced contractions in rat prostates between CS-PLP1 and anthranilate CS-PLP1 (0.97 mg/mL; $p = 0.0055$, n = 2), derivatized reagent and vehicle (Milli-Q H_2O) (0.97 mg/mL; $p = 0.5512$, n = 2), fructose and 2-AA-Fru (0.97 mg/mL; $p = 0.0033$, n = 4), galactose and 2-AA-Gal (0.97 mg/mL; $p = 0.0046$, n = 4), glucose and 2-AA-Glu (0.97 mg/mL; $p = 0.0001$, n = 4), rhamnose and 2-AA-Rha (0.97 mg/mL; $p > 0.9999$, n = 2), arabinose and 2-AA-Ara (0.97 mg/mL; $p = 0.0267$, n = 2) at 5 Hz. Results are expressed as means ± S.E.M (*$p < 0.05$ **, $p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$). One-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparisons test were used for the statistical evaluations. *p*-values represent probability of the treatment causing a significant change in the contractile responses.

5.4 DISCUSSION

Identifying the pure bioactive compounds from medicinal plants is crucial to validating their efficacy and safety as pharmacotherapeutics. With hindsight, the main challenge of the chemical isolation of the *C. speciosus* rhizome cold-water extract was finding an effective method to separate the active components from inactive ones' due to their polarity, and probably their chemical similarity (as carbohydrates) to the bulk mass of the extract. Likewise, as we are working with small quantity of starting material, samples obtained from different chemical isolation approaches are insufficient to have n = 6 sample size for electrical field stimulation experiments. The EFS experiments performed were mainly as brief indicative of the samples' bioactivity obtained although the low number of observations may contribute to interpretational uncertainties.

5.4.1 Chromatographic Isolation

A range of chromatographic techniques were attempted, with none successfully resolving the active component. Flash RP-HPLC was unsuitable but yielded the polar fraction F*1 which, by TLC, gave us a clue that there were multiple components present **Figure 5-4**. One of the solvent conditions tested has been reported as an optimised solvent system consisting of toluene, ethyl acetate, glacial acetic acid, and formic acid (2:1:1:0.75), which can effectively separate components from *C. speciosus* rhizome ethanol extract on HPTLC plate (Verma and Khosa, 2012). Nevertheless, this solvent condition only slightly separated components from F*1, suggesting that it is not suitable for this study (**Figure 5-4D**).

5.4.2 Centrifugation Separation and Fractionation Using Preparative RP-HPLC

Due to no suitable available facilities, our initial intention to separate bioactive components from 18 g of crude extract was not possible. Hence, a smaller portion of the extract (200 mg) was used. We further found out that the insoluble particulates of the extract were inactive and were eliminated using centrifugation. Functional studies revealed that the inhibitory effect showed by the *C. speciosus* rhizome cold-water extract was observed only by the liquid phase but not by the solid phase. Typical yields of liquid phase extract amounted to 58 - 60 % (w/w) of the dried cold-water crude extract of *C. speciosus* rhizome, whereas the solid phase only yielded 25 - 29 % (w/w) of the dried *C. speciosus* rhizome cold-water extract (**Appendix 10**). Subsequent fractionation of the liquid phase extract using preparative RP-HPLC successfully isolated and

identified an active polar fraction. Optimisation of the attempt to isolate bioactive components from the *C. speciosus* rhizome cold water extract has successfully removed inactive components which amounted to over half of the amount of the starting material.

Further chromatographic fractionation of the active polar fraction initially eluted (CS-PLP1) seemed to be difficult despite several modifications on the gradients, as the fraction has a high polar characteristic. Given that the extract was an aqueous extract, the high polar characteristic of the active CS-PLP1 fraction isolated was not surprising. Alternatively, since the ^{1}H NMR spectrum of CS-PLP1 indicated that carbohydrates (either monosaccharides, disaccharides or polysaccharides) were present in the *C. speciosus* rhizome cold-water extract, the carbohydrates might play a role in the relaxant effects possessed by the extract on electrically evoked contractile responses of isolated rat prostates. Therefore, to confirm whether the carbohydrates present in CS-PLP1 were involved in the relaxant effects exhibited on electrically evoked contractions of rat prostate, the derivatization approach from Anumula (1994) was modified to determine the possible presence of sugar structures in the bioactive component(s) of CS-PLP1 (Anumula, 1994).

5.4.3 Derivatization

The derivatization approach utilized anthranilic acid (2-aminobenzoic acid, ABA or 2-AA) (Townsend, 1993) for quantitative determination of oligosaccharides found in CS-PLP1. Anthranilic acid is an aldose-specific reagent and did not react with sialic acid (Anumula, 1994). Glycans labelled with anthranilic acid have better separation and peak shape on C_{18} chromatography (Higel et al., 2013). Reverse phase HPLC has been commonly used for the separation of glycans and glycoconjugates. Derivatization of the glycans with anthranilic acid enables the retention of glycans in RP stationary phases (Kailemia et al., 2014). The weakness of this approach is that it only provides information on whether there are any reducing sugars present in the sample.

The reductive amination of monosaccharides with anthranilic acid (ABA or 2-AA) is exemplified in **Figure 5-23**. In the reductive amination reaction, the amino group of 2-AA reacts with the anomeric carbon to form an intermediate imine. In the presence of a reducing agent, sodium cyanoborohydride ($NaBH_3CN$), the imine will be converted to the amine, producing anthranilate glucose (2-AA-Glu) as the end product in this example.

Anthranilic acid peak was only present in the chromatographic spectrum of 2-AA-Fru, and not 2-AA-Gal, 2-AA-Glu, and 2-AA-Man which indicated that the reductive animation was incomplete in 2-AA-Fru. Furthermore, chromatogram of 2-AA-Fru revealed the presence of doubling peaks with same intensities at 5.6 min and 5.8 min, respectively, despite the control peak. Fructose is a ketose and not an aldose sugar, where the ketose group makes the anomeric carbon less reactive to reductive amination. A ketose group consists of a hemiketal group and is prone to tautomerization, which possibly explains the doubling peaks.

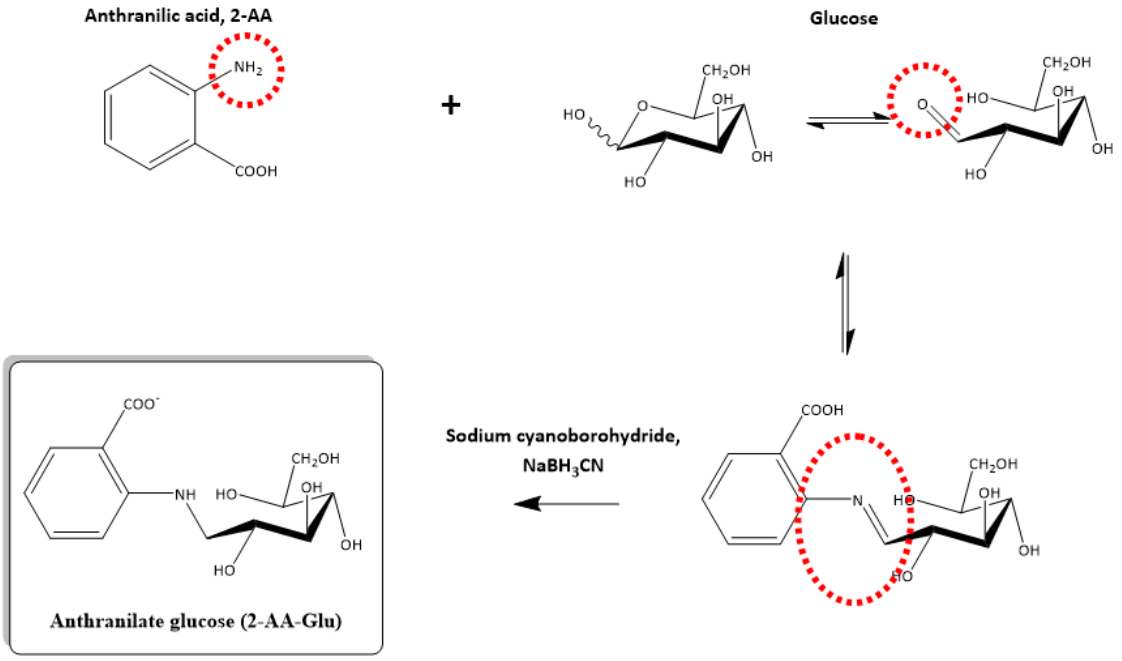


Figure 5-23. Derivatization reaction of glucose with anthranilic acid (2-AA).

Derivatization efficiency was investigated by using several masses of CS-PLP1, i.e. 3 mg, 7 mg, and 14 mg. Analytical RP-HPLC analysis demonstrated that CS-PLP1 had been successfully derivatized with anthranilic acid and the derivatization reaction was concentration-dependent (**Appendix 12**). The anthranilate CS-PLP1 was spiked with sugar references to identify the possible presence of sugars in CS-PLP1 (**Appendix 13**). Chromatograms demonstrated that all the anthranilate sugars derivatives peak were present in the anthranilate CS-PLP1. Furthermore, LCMS analysis of anthranilate sugar standards also corresponded to the m/z shown in LCMS spectrum of anthranilate CS-PLP1. This indicated the possible presence of reducing sugars in CS-PLP1.

Preparative RP-HPLC separation of anthranilate CS-PLP1 yielded five fractions, designated as D-1, D-2, D-3, D-4, and D-5 (**Figure 5-18**). LCMS analysis of the sub-fractions isolated from these

fractions revealed that some of these fractions consisted of eluted peaks having the same m/z with the anthranilate sugar standards including fructose, arabinose, rhamnose, and lactose but not galactose, mannose or glucose. Of note, D-1-12 has the same mass with 2-AA-Lac (*i.e.* 464.5 m/z). Lactose is a disaccharide sugar that is composed of galactose and glucose. Fructose, which is a ketohexose, the LCMS UV profile showed that the fractions contained a mass peak of 2-AA-Fru derivatives. D-1-15 eluted a peak that has the same retention time as one of the 2-AA-Fru peaks that has the mass 373.6 m/z. Furthermore, a peak eluted from D-2-4, D-2-5, and D-(3,4,5)-4, consisted of a mass peak the same as one of the peaks eluted from 2-AA-Fru at 10.98 min, which was 302.4 m/z. Arabinose is one of the aldohexoses. Interestingly, peaks eluted from both D-(3,4,5)-6, D-(3,4,5)-7, and D-(3,4,5)-8 showed the same mass peak as 2-AA-Ara, which is 272.4 m/z. Fractions 9 and 10 separated from D-(3,4,5) combination have the same mass peak as 2-AA-Rha, 286.4 m/z. LCMS analysis of these fractions separated from D-1, D-2, and D-(3,4,5) suggested the presence of hexose and pentose.

5.4.4 ^{1}NMR Analysis

However, superimposed ^{1}H NMR spectrums of aforementioned fractions with the anthranilate sugar standards indicated that the chemical shifts were different, although there were some similarities in the spectrum patterns (**Appendix 16**). These findings suggest that there is possible presence of other types of hexose, ketohexose or pentose, of the bioactive compounds chemical structure, since LCMS analysis demonstrated the presence of these anthranilate sugars mass ions in the fractions isolated from anthranilate D-1, D-2, and D-(3,4,5). Saccharides such as glucose, sucrose, and fructose have been previously reported to have no apparent physiological activity on the isolated rat prostate (Chua et al., 2011). Functional studies on glucose, fructose, arabinose, and rhamnose were consistent with Chua et al. (2011) (**Figure 5-21**). Any effects seen on the EFS-induced contractions of isolated rat prostates treated with anthranilate CS-PLP1 were produced by the anthranilate CS-PLP1 instead of the derivatization reagents present.

Interestingly, all the anthranilate sugars showed inhibition on the EFS-induced contractions of rat prostates. This has further complicated the isolation and characterisation process due to glycans chemical heterogeneity and diversity (Raman et al., 2005). Of note, anthranilate CS-PLP1 also demonstrated greater inhibitory effect compared to underivatized CS-PLP1. The reductive animation reaction might have produced CS-PLP1 derivatives with greater inhibition ability.

5.5 CONCLUSION

Overall, this chapter has described the systematic development of a method to isolate and validate bioactive compounds present in the *C. speciosus* rhizome cold-water crude extract. The aim was to obtain purified bioactive components from the extract. **Figure 5-24** outlines a summary of the procedures used. Pure bioactive compounds are yet to be obtained but further experiments aimed at improving extraction and characteristic determination are no longer needed. We do not have good capacity and suitable equipment to isolate the bioactive compounds. However, the fractionation of the active polar fraction might be more efficient if we could use a better RP stationary phase, such as those with good carbohydrate selectivity.

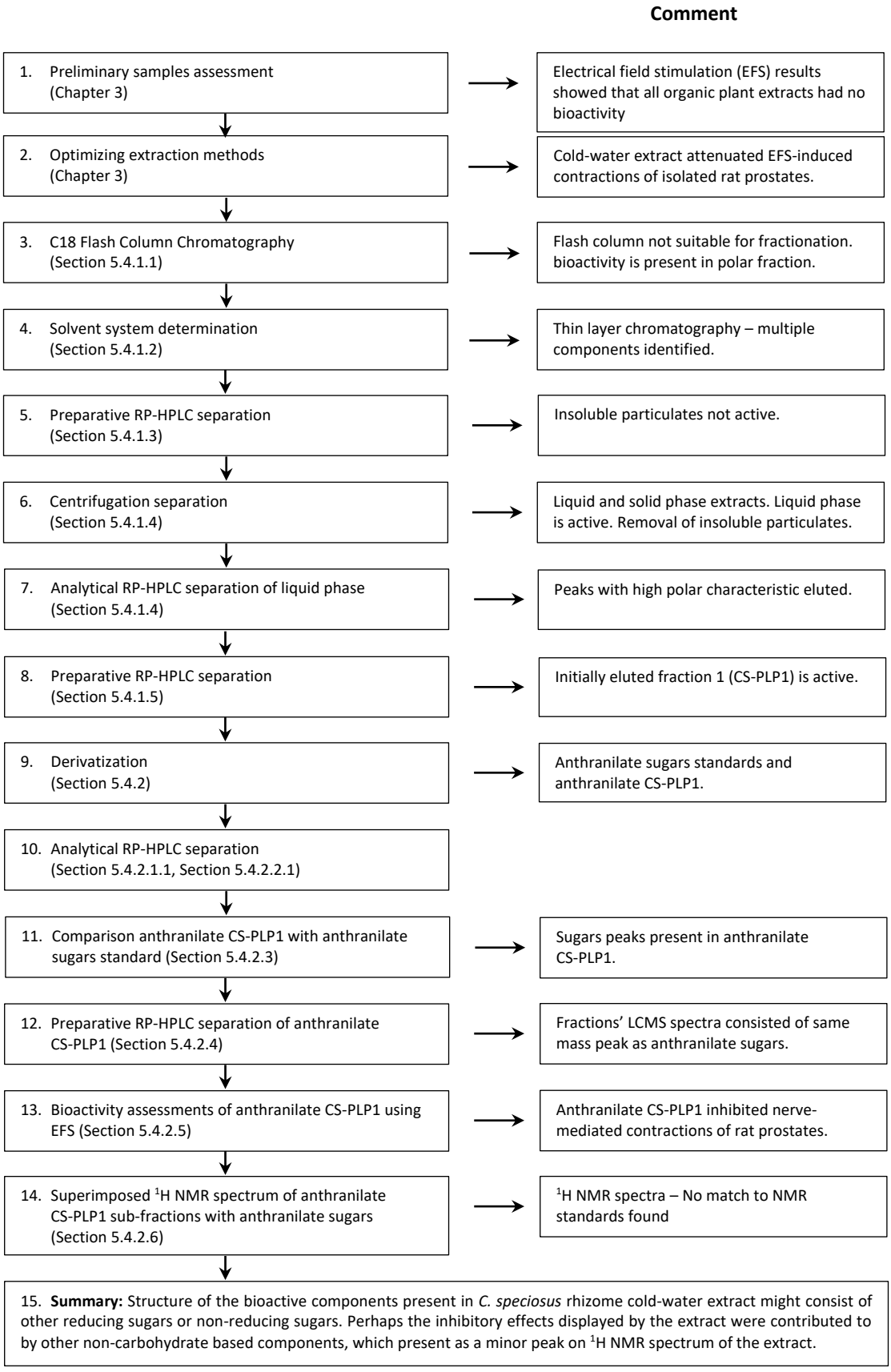


Figure 5-24. Method development for extraction, isolation, and separation of *C. speciosus* rhizome cold-water extract.

6

Concluding Remarks

Phytotherapy is defined as the use of plants or plant extracts which are not part of a normal healthy diet, for medicinal purposes. In most cases, it originates from traditional or folklore medicines also known as botanical medicine, herbalism, medical herbalism, herbal medicine and herbology (Horák et al., 2014). Traditional knowledge of medicinal plants that was passed down from previous generations formed the foundation of the ethnopharmacological approach for natural products drug discovery (Buenz et al., 2017) and has strongly influenced the development of the modern pharmaceutical industry (Dias et al., 2012). Ethnomedical knowledge has remained an important component in healthcare systems globally, and a growing amount of scientific evidence supports the pivotal role of complementary and alternative healthcare practices in improving the health and well-being of patients.

In 1994, the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA), declaring that herbal medicines are not drugs and would be called “botanicals”. Since then the medicinal herbal markets have received a tremendous boost in sales and marketing (Goldman, 2001). Herbal medicines were then called “dietary supplements” and classified along with vitamins, minerals and other health products which allowed companies to make “health” or “structure/function” claims and exempted them from the regulations imposed by the FDA on regular drugs (Institute of Medicine (US) Committee on the Use of Complementary and Alternative Medicine by the American Public, 2005).

Sarawak is located at latitude 0^{o} 50‘ and 5^{o} N and longitude 109^{o} 36‘ and 115^{o} 40‘ E (**Figure 6-1**) (Long, 2014) and has been designated as one of the world’s 25 hot spots by the Critical Ecosystem Partnership Fund (CEPF) having mega biodiversity within its tropical rainforest (Fund, 2018; Sodhi et al., 2010). Due to its mega biodiversity, different ethnic groups in Sarawak have practiced folklore or traditional medicine using its natural products since long ago to treat different illnesses. *C. speciosus* which is locally known as daun setawar hutan or siluk, apart from

being consumed as a vegetable, has also been used traditionally by the Lun Bawang indigenous community to treat urinary diseases. *C. speciosus* has been extensively studied in many pharmacological tests, however, none have studied prostatic smooth muscle contraction. Therefore, to our knowledge, this study is the first to investigate the effect of *C. speciosus* extracts on isolated rat prostatic contractility.

Figure 6-1. Map shows the location of Sarawak, Malaysia (Long, 2014).

There are many aspects to the development of BPH and prostatic inflammation may contribute to prostate growth either in terms of BPH or neoplastic changes (Robert et al., 2009; Royuela et al., 2000; Sciarra et al., 2008). There is now a growing body of evidence suggesting that inflammation plays an important role in the pathogenesis of BPH (Bostanci et al., 2013; De Nunzio et al., 2016; Kramer and Marberger, 2006; Kramer et al., 2007). Hence, drugs with anti-inflammatory effects could be used in the management of BPH. *C. speciosus* has been shown to have anti-inflammatory activity (Srivastava et al., 2012). Studying and characterising the bioactivity of *C. speciosus* on prostate contractility could lead to the discovery of multiple biological effects that may be beneficial for the therapy of BPH e.g. smooth muscle relaxant, anti-inflammatory, diuretic etc. This may lead to the discovery of novel pharmacotherapies for

BPH with multiple sites of action and fewer side effects as compared to other drug treatments on the market. Anti-inflammatory treatment certainly seems to be part of the future treatment of not only BPH but also other diseases associated with the lower urinary tract. Perhaps, the next step in the studies of *C. speciosus* includes examining its anti-inflammatory properties in an animal model of BPH.

In Chapter 3, functional studies showed that bioactivity of the extracts of *C. speciosus* was temperature-dependent with more potent inhibition observed with cold-water extracts compared to decoction and warm water extracts. Moreover, extracts obtained from rhizome, stem, and root, respectively possessed smooth muscle relaxant effects; however, this activity was not observed in the leaf.

In Chapter 4, direct smooth muscle contraction elicited by exogenously administered noradrenaline, phenylephrine, acetylcholine, and ATP was only slightly affected by the *C. speciosus* rhizome cold-water extract, as were rat prostate contractions elicited by tyramine which is an indirectly acting sympathomimetic agonist. This study was unable to determine a pharmacological mechanism of action for the observed inhibitory effect of the abstract on isolated rat prostate but it appears to work by inhibiting an as-yet unidentified, but indirect pre-junctional mechanism that inhibits exocytotic release of transmitters, as a larger effect of the extract was seen with electrical field stimulation compared to agonist-induced contractions that act at post-junctional sites. We speculate the extract might have blocked or negatively modulated the N-type Ca^{2+} channel permeability leading to inhibition of the influx of free ionized extracellular Ca^{2+} thus resulting in inhibition of transmitter release (Minneman, 1988).

In addition, a non-specific mechanism of action may also be present post-junctionally within the rat prostatic smooth muscle. This site may inhibit G-proteins, myosin light chain kinase, other protein kinases or L-type Ca^{2+} channels. These possible sites of action are involved in smooth muscle contraction through a number of pathways (Abdel-Latif, 1991; Preston et al., 2004; Preston and Haynes, 2003). In addition to the voltage-operated Ca^{2+} channels, the extract might have affected receptor-operated Ca^{2+} channels (Bolton, 1979; Van Breemen et al., 1978). Furthermore, the inhibitory effects of the extract might also be attributed to cotransmission of neurotransmitters that have synergistic actions on pre- and post-junctional effector cells. Therefore, we speculate that *C. speciosus* extracts might have multiple mechanisms of action that affect prostate contractility. The components present in the extracts that might be beneficial in the treatment of BPH symptoms could also work additively or even synergistically

through there different sites of action, which is a common trait of traditional medicines (Buenz et al., 2017).

Chemical separation of *C. speciosus* rhizome cold-water extract showed that the inhibitory activity of the extract was due to the soluble components that were present in the highly polar fraction of the liquid phase but not the undissolved particles. Due to the limitations of available facilities, further fractionation of this highly polar fraction was not feasible despite several modifications to the experimental gradients. Therefore, a derivatization method was adapted and modified (Anumula, 1994) to target carbohydrates that were present in the extract based on their ^{1}H NMR spectra. The approach aimed to derivatize the reducing sugars that may have been present in the fractions isolated from the highly polar fraction (CS-PLP1), and analyse them with LCMS and ^{1}H NMR. It was hoped that by using these approaches, we would be able to deduce the chemical structures of the bioactive components. Despite results from LCMS analysis indicating that the sub-fraction m/z matched with the derivatized standards (i.e. 2-AA-Fru, 2-AA-Ara, 2-AA-Rha), superimposition of ^{1}H NMR spectra did not match although there were some similarities in the spectral pattern.

Further, functional studies also revealed that anthranilate CS-PLP1 demonstrated greater inhibitory effects compared to the underivatized CS-PLP1. This means that a reductive animation reaction might have produced anthranilate CS-PLP1 with greater inhibitory ability. Surprisingly, anthranilate sugars also showed inhibition on the electrical field stimulation-induced contractions of rat prostates as compared to their underivatized counterparts. Anthranilate sugars and CS-PLP1 might have caused some structural disturbance by blocking the interaction between the cell surface receptors that modulate rat prostatic smooth muscle tone. This may have further complicated the isolation and characterisation process.

It is important to determine the active compound(s) responsible for the muscle relaxation activity of *C. speciosus* rhizome cold-water extract and whether the crude extract might be preferable to the activity of the purified active compound(s) alone. Isolation and elucidation of active components from *C. speciosus* rhizome cold-water extract and their mechanisms of action are important, as they will provide the basic guidelines for the safety and efficacy of its use in the pharmacotherapy of BPH. After extensive study of carbohydrates present in the *C. speciosus* rhizome cold-water extract, the possible involvement of reducing sugars or non-reducing sugars in the extract's inhibitory activity was still not clearly delineated. Furthermore, the inhibitory

effects displayed by the extract might also be due to other non-carbohydrate based components, which are present as minor peaks in the ^{1}H NMR spectra of the extract. For future work, employing a column specific for carbohydrate separation would be recommended as standard reverse-phase columns have poor retention properties for highly polar components (Liu and Rochfort, 2013) such as those in the *C. speciosus* rhizome cold-water extract. Nevertheless, this study has developed a systematic method for the chemical analysis of *C. speciosus* from extraction, *in vitro* experiment, chromatography, and ^{1}H NMR analysis.

For the next step in the investigation of the biological mechanism of action, the effect of *C. speciosus* rhizome cold-water extract on other mechanisms which have been reported to be involved in the prostate contraction (Ventura et al., 2011) such as CGRP and cannabinoid receptors or NO pathway could be studied. Further, studies could also be carried out to investigate the effect of the extract on pre-junctional (neuronal) calcium channels by using cultured neuronal cells that express N-type Ca^{2+} channels since contraction elicited by tyramine is not affected by the extract. Horsfall *et al.* (1994) suggested that guinea-pig prostatic stroma was similar to the prostatic stroma found in men with BPH, hence it would be good to study the effects of *C. speciosus* rhizome cold-water extract in modulating the prostatic smooth muscle tone of aged guinea-pig (Horsfall et al., 1994) or alternatively using human prostatic tissue if available.

Toxicology experiments could be carried out to investigate safe use of the extract in animal models. Although *C. speciosus* rhizome has been used by indigenous people of Sarawak to treat urinary disorders for centuries, which implies a certain lack of toxicity, there may still be some ingredients which are not suitable for long-term administration (Yuan and Lin, 2000). Such safety data will be important to support whether *C. speciosus* rhizome cold-water extract is suitable to be developed as a complementary medicine. A small and well-designed clinical trial (approximately 20 men with age between 50 – 90 years old who have been diagnosed with BPH associated LUTS) that adheres to Australia Therapeutic Goods Administration (TGA) regulations could be performed to investigate the efficacy of *C. speciosus* rhizome cold-water extract in relieving LUTS.

Furthermore, results of the mechanism of action studies in Chapter 4 have suggested that *C. speciosus* rhizome cold-water extract inhibition appeared to act through several different GPCRs **Table 4-1**. Therefore, inhibition of G_q proteins and some G_i and G_s proteins could be

investigated as a means of determining a more specific site and mechanism of action. Pertussis toxin (PTX) and UBO-QIC are both inhibitors of G_i/G_s and G_q proteins, respectively. Nonetheless, these inhibitors are difficult to use in tissue-based experiments. Hence, validation studies of the extract's effect on G_i/G_s- and G_q-regulated biological processes (**Figure 4-23**) by using these inhibitors could be performed using cell-based assays in future (Heximer et al., 1997; Matthey et al., 2017; Schrage et al., 2015; Silva Junior et al., 2017; Takasaki et al., 2004; Wenzel et al., 2016).

In Chapter 3 and 4, *C. speciosus* root and stem extracts along with rhizome extract were shown to possess similar inhibitory effects as well as causing a transient tonic contraction. Therefore, it would be interesting to study and compare the pharmacological activities of both root and stem extracts with rhizome in future to find out which parts of the plant has greater biological effects or the highest concentration of bioactives.

Of interest, apart from the prostate contractility studies, other studies could be carried out to investigate the effect of *C. speciosus* on urinary tract infection (UTI) in women, and the possibility of bioactive compound isolation to treat UTIs in women. Furthermore, as most of the urogenital organs are sharing similar receptors and neurotransmitters, the effects of *C. speciosus* on other urogenital organs would be of huge interest.

6.1 LIMITATIONS

Isolation, analysis, and validation of potential phytotherapeutic compounds from natural resources such as plants or herbs is still a challenging, laborious and time-consuming process (Atanasov et al., 2015; Sarkar, 2015).

6.1.1 Target Identification

Pharmacological target identification of the *C. speciosus* rhizome cold-water extract on rat prostates contractility was carried out by using isolated organ bath studies throughout this PhD study. Selection of suitable antagonists and agonists to study the extract's activities was carefully and extensively planned. However, to find an absolutely specific target receptor is not always possible as the nature of crude plant extracts means that they contain many chemical constituents unless a pure active compound can be isolated which in this case was not possible.

6.1.2 The Challenge of Working with Natural Products

One of the important challenges related to the use of plants as a source for bioactive compound isolation is insufficient crude material quantities needed for the thorough characterization of the pharmacological activity of bioactive compounds (Atanasov et al., 2015). The capacity and suitable equipment for the large scale extraction of the *C. speciosus* rhizome to produce enough extract for both chemical isolation and pharmacological validation was not at our disposal. And the current laboratory scale of extraction and lyophilization processing was overly time consuming (Zhang et al., 2018).

6.1.3 Lead Identification

The main challenge in the chemical isolation of the *C. speciosus* rhizome cold-water extract was finding an effective method to separate the active components from inactive ones due to their polarity, and probably their chemical similarity (as carbohydrates) formed the bulk mass of the extract.

A range of chromatographic techniques were attempted, with none successfully resolving the active component. Due to a lack of suitable and available facilities, initial big bulk fractionation via flash RP-HPLC to separate bioactive components was not possible. Hence, a smaller portion of the extract was used. Natural product isolation usually ends up with a decreased amount of active compounds that limit downstream bioactivity validation (Atanasov et al., 2015; Bucar et al., 2013; Zhang et al., 2018). Working with small amounts of starting material/extract causes downstream bioactivity validation to become impossible due to the small amounts of working experimental material.

For the purposes of this study, the capacity and suitable equipment to isolate the bioactive compounds was not adequate. However, the fractionation of the active polar fraction might be more efficient if we could use a more robust RP stationary phase, such as those with good carbohydrate selectivity. Furthermore, it was not possible to repeat the flash RP-HPLC using a larger portion of liquid phase extract with better solubility due to the limited availability of *C. speciosus* rhizome cold-water crude extract. The crude extract that contains insoluble particles has a sticky characteristic and often blocked the flash RP-HPLC resulting in the mobile phase being unable to be flushed through the stationary phase even under nitrogen or compressed air.

Knowledge of traditional medicines has re-emerged as a crucial contemporary resource that contributes to the augmentation of the drug discovery process via ethnopharmacology, which ultimately leverages the identification of novel therapeutic targets (Ariane Löhnert, 2014; Buenz et al., 2017; Fabricant and Farnsworth, 2001; Sarkar, 2015). With the isolation and identification of bioactive components from *C. speciosus*, we hope that it will lead to a new phytotherapeutic agent that can be used to treat urinary symptoms associated with BPH and improve the quality of life for ageing men. Nevertheless, in this study, it has been shown that extracts from this plant show great efficacy in their ability to relax prostatic smooth muscle which is the most effective way to treat LUTS resulting from BPH.

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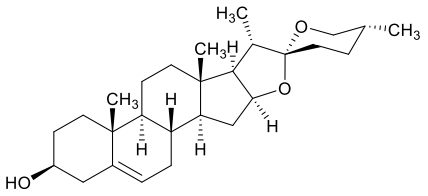
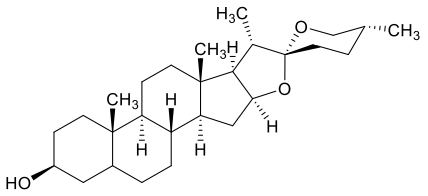
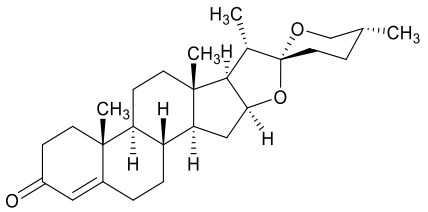
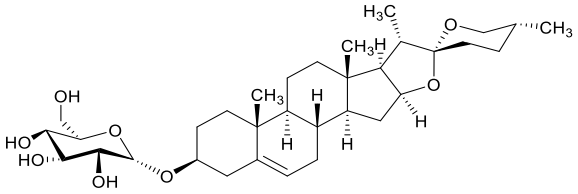
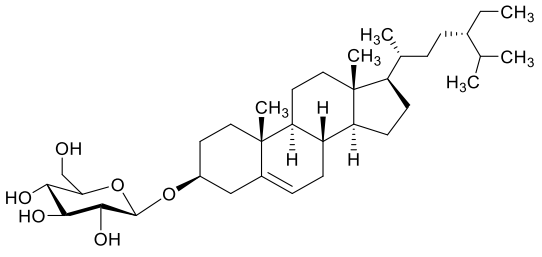
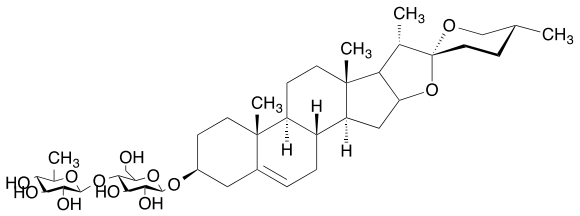
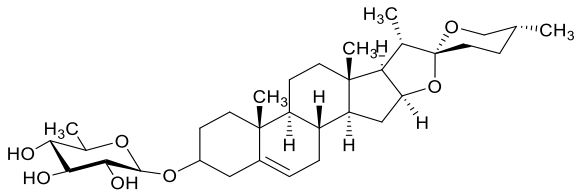
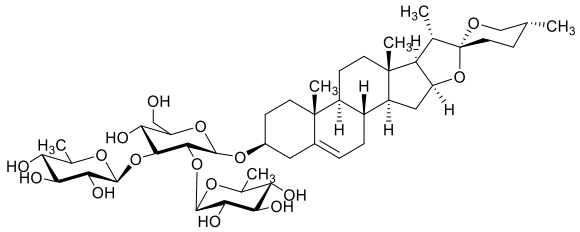
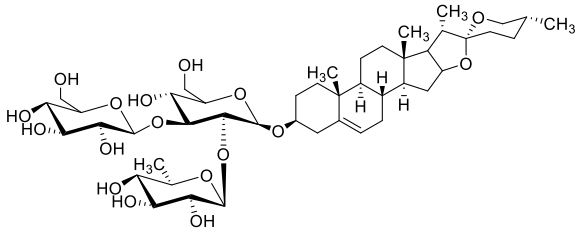
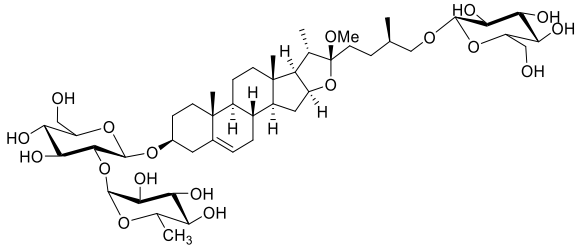
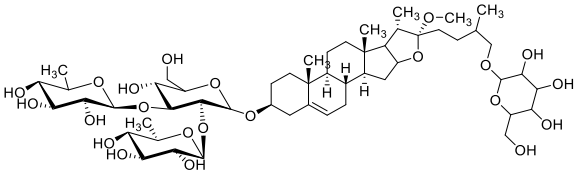
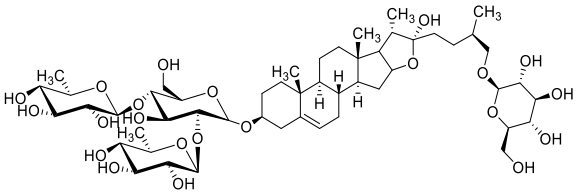
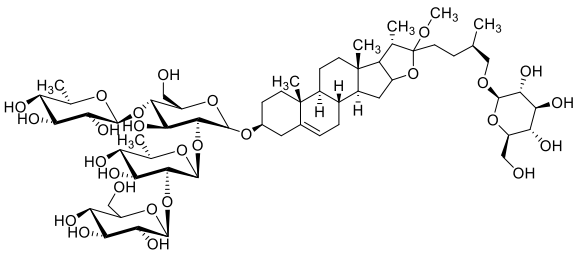
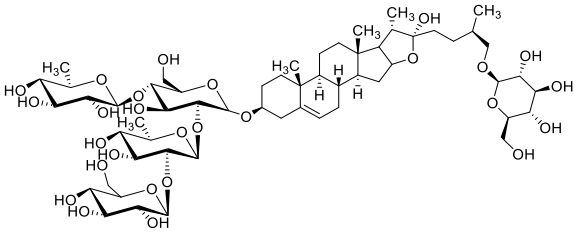
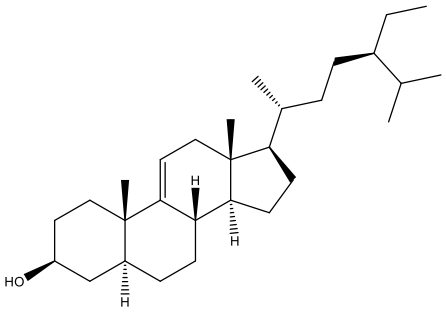
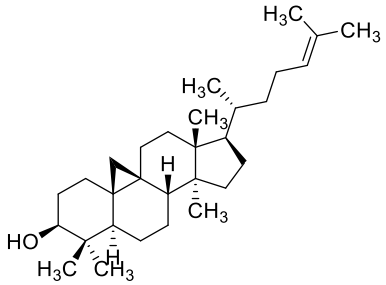
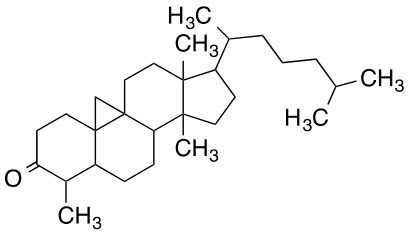
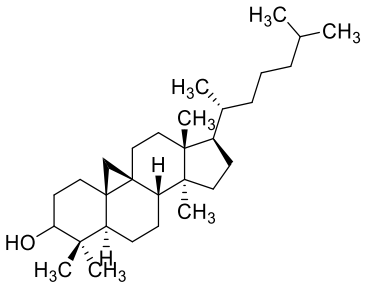
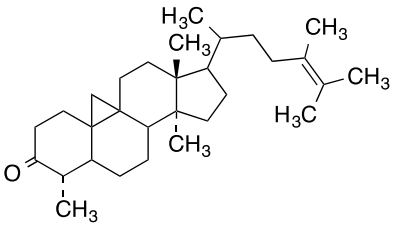
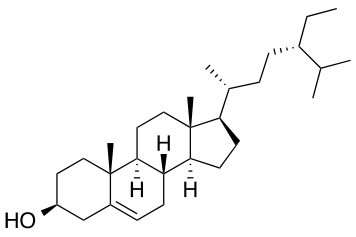
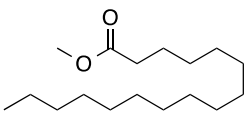
APPENDIX

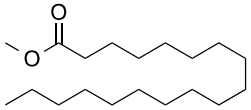
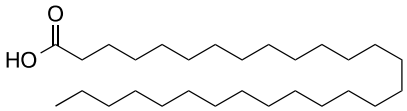
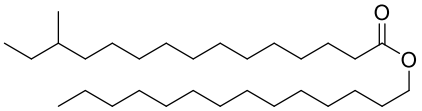
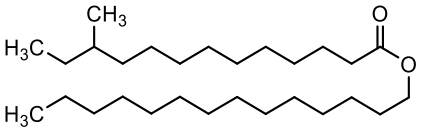
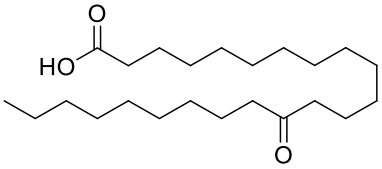
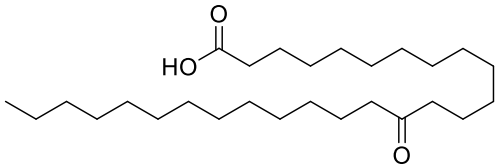
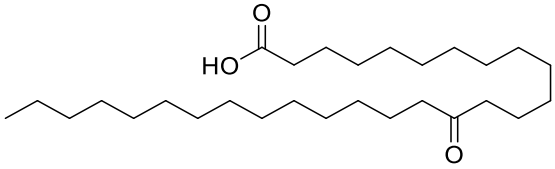
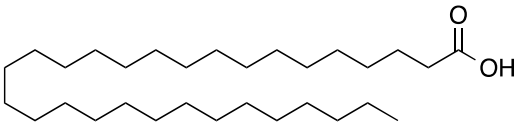
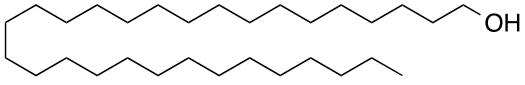
Appendix 1. Reported compounds isolated from different plant parts of *C. speciosus*.

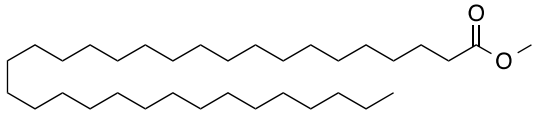
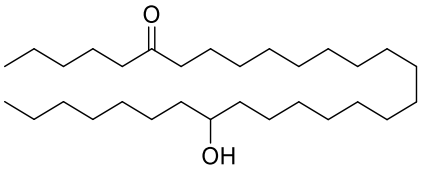
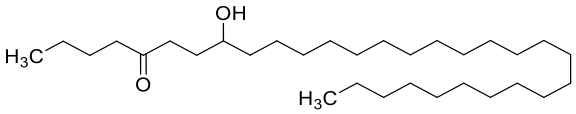
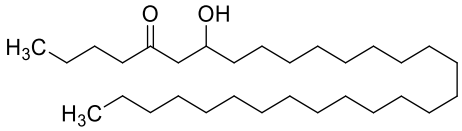
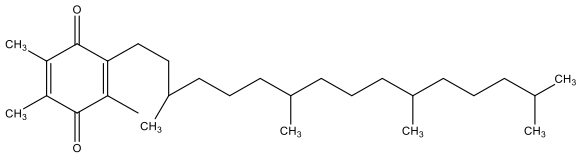
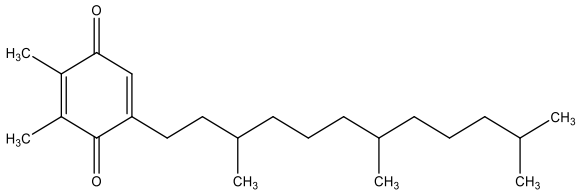
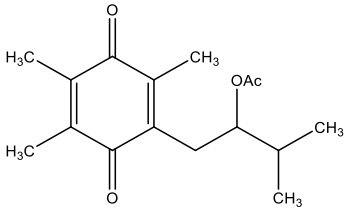
Class	Compounds	Plant Part
Sapogenin	1. Diosgenin 	Rhizome, Seed, Stem, Root, Flower, Leave (Dasgupta and Pandey, 1970)
	2. Tigogenin 	Rhizome (Jagtap and Satpute, 2014)
Saponins	1. Diosgenone 	Root (Qiao et al., 2002)
	2. Trillin (diosgenin-3-*O*-*β*-D-glucopyranoside) 	Seed (Singh and Thakur, 1982a)
	3. Costucoside A (*β*-sitosterol-*β*-D-glucopyranoside) 	Seed, Root (Singh and Thakur, 1982b)

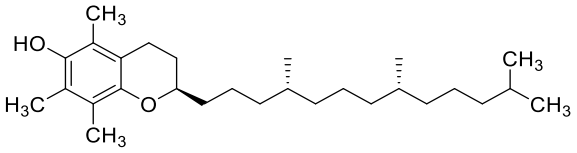
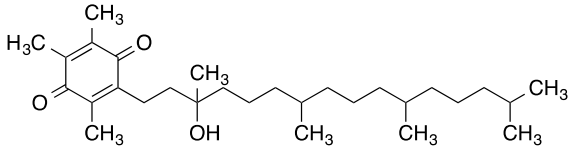
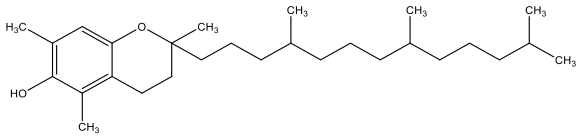
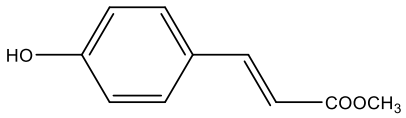
Saponins	4. Costucoside B / Prosapogenin B of dioscin (Diosgenin-3-*O*-*α*-L-Rhamnopyranosyl (1→4)-*β*-D-glycopyranoside 	Seed, Root (Gupta et al., 1983; Singh and Thakur, 1982b)
	5. Costucoside C / Prosapogenin A of dioscin (Diosgenin-3-*O*-*α*-L-Rhamnopyranosyl) 	Seed, Root (Gupta et al., 1983; Singh and Thakur, 1982b)
	6. Costucoside D / Dioscin (Diosgenin-3-*O*-*α*-L-Rhamnopyranosyl (1→2)-[*α*-L-rhamnopyranosyl (1→4)]-*β*-D-glucopyranoside 	Seed, Root (Gupta et al., 1983; Mahato et al., 1980; Singh and Thakur, 1982b; Tschesche and Pandey, 1978)
	7. Costucoside E or Gracillin (3-*O*-[*α*-L-Rhamnopyranosyl (1→2)-[*β*-D-glucopyranosyl (1→3)]-*β*-D-glucopyranoside 	Rhizome, Root, Seed (Gupta et al., 1983; Mahato et al., 1980; Singh and Thakur, 1982b)
	8. Costucoside F (3-*O*-[*α*-L-Rhamnopyranosyl (1→2)-*β*-D glucopyranosyl]-26-*O* (*β*-D-glucopyranosyl)-22*α*-methoxy-(25*R*)-furost-5-en-3*β*,26-diol 	Seed (Singh and Thakur, 1982a)

Saponins	9. Costucoside G / methyl protodioscin (3-*O*-*α*-L-Rhamnopyranosyl (1→2)[*α*-L-rhamnopyranosyl(1→4)]-*β*-D-glucopyranosyl-22*α* methoxyl-25*R*)-furost-5-en-3*β*, 26-diol) 	Seed (Agrawal et al., 1984; Singh and Thakur, 1982a)
	10. Costucoside H / Protodioscin (22*α*-hydroxy compound of substance G (above)) 	Seed (Singh and Thakur, 1982a; Singh and Thakur, 1982b)
	11. Costucoside I (3-*O*-*β*-D-glucopyranosyl (1→2) [*α*-L-Rhamnopyranosyl (1→4)]*β*-D-Glucopyranosyl-26-*O*-(*β*-D-glucopyranosyl-22*α* metoxyl-25*R*)-furost-5-en-3*β*, 26 diol 	Seed (Singh and Thakur, 1982b)
	12. Costucoside J (22*α*-hydroxy compounds of costucoside I) 	Seed (Singh and Thakur, 1982b)
Steroids / Triterpene	1. 5*α*-Stigmast-9(11)-en-3*β*-ol 	Root, Rhizome, Seed (Gupta et al., 1981b)

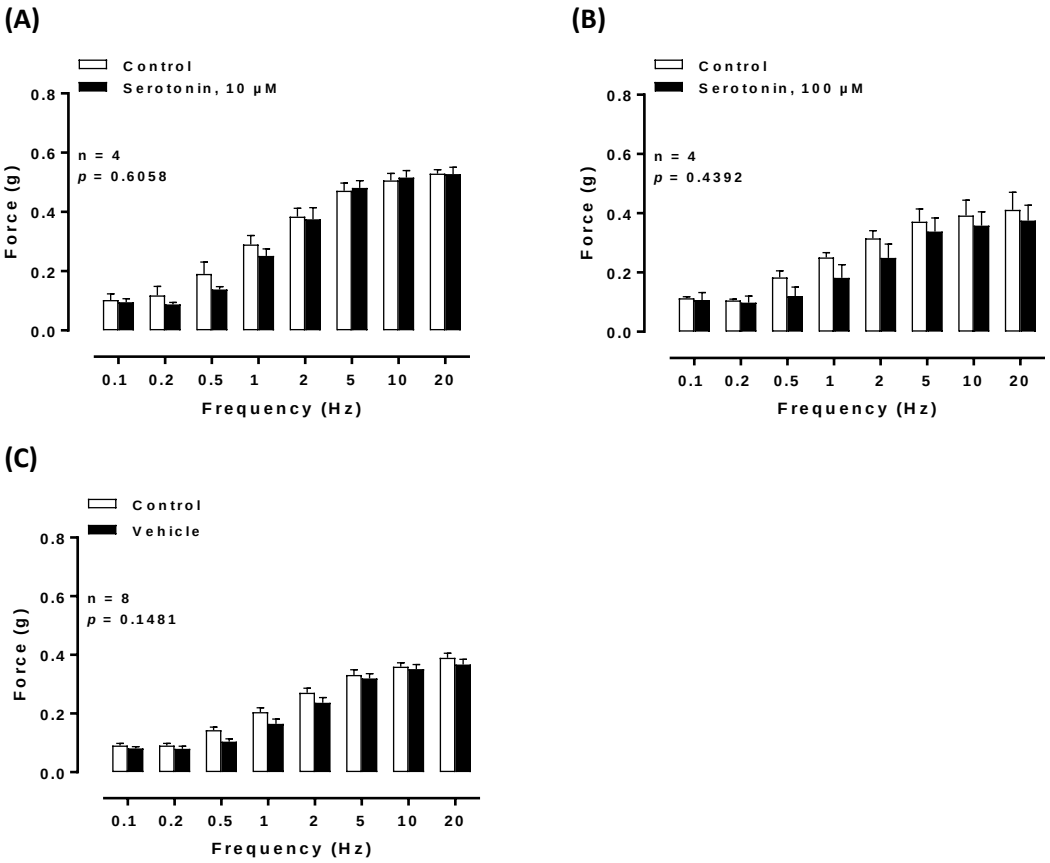
	2. Cycloartenol / Cycloartenyl acetate 	Root (Gupta et al., 1988)
	3. 31-norcycloartanone 	Root (Gupta et al., 1988)
	4. Cycloartanol / Cycloartanyl acetate 	Root (Gupta et al., 1988)
	5. Cycloaudenyl acetate / Cyclolaudenol 	Root (Gupta et al., 1988)
	6. Sitosterol 	Rhizome, Root (Gupta et al., 1981c)
Aliphatic Esters, Oxo Acids and Branched Fatty Acid Esters	1. Methyl hexadecanoate 	Seed (Singh, 1984)

	2. Methyl octadecanoate 	Seed (Singh, 1984)
	3. Octacosanoic acid / Tetracosanyl octadecanoate 	Seed, Rhizome (Jagtap and Satpute, 2014; Qiao et al., 2002; Singh, 1984)
	4. Tetradecyl 13-methylpentadecanoate 	Rhizome (Gupta et al., 1986)
	5. Tetradecyl-11methyltridecanoate 	Rhizome (Gupta et al., 1986)
	6. 14-Oxotricosanoic acid 	Rhizome (Gupta et al., 1986)
	7. 14-Oxoheptacosanoic acid 	Rhizome (Gupta et al., 1986)
	8. 14-Oxo-octacosanoic acid 	Rhizome (Gupta et al., 1986)
	9. Triacontanoic acid 	Rhizome, Root (Gupta et al., 1981c)
	10. Triacontanol 	Rhizome (Gupta et al., 1986)

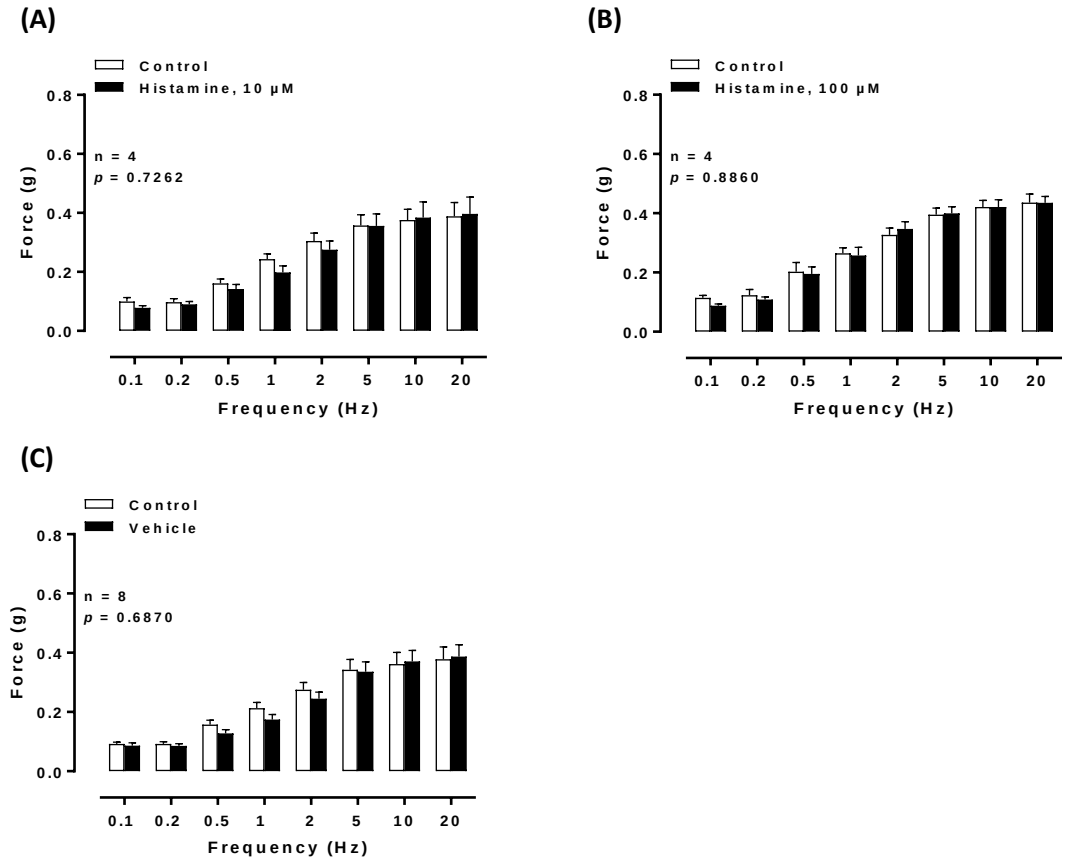
	11. Methyl tritriacontanoate 	Root (Gupta et al., 1981c)
Fatty Acids	1. Palmitic, stearic, oleic, linoleic, arachidic	Seed (Srivastava et al., 2011b)
Hydroxyl Ketones	1. 8-Hydroxytriacontan-25-one 	Root (Gupta et al., 1982)
	2. 24-hydroxyhentriacontan-27-one 	Root (Gupta et al., 1981c)
	3. 24-hydroxytriacontan-26-one 	Root (Gupta et al., 1981c)
Benzoquinones	1. 6-methyl dihydrophytylplasto-quinone 	Seed (Mahmood et al., 1984)
	2. Dihydrophytylplastoquinone 	Seed (Mahmood et al., 1984)
	3. 2,3,6-trimethyl-5-(2-acetyliospentyl)-1,4-benzoquinone 	Seed (Mahmood et al., 1989)

Tocopherol	1. *α*-Tocopherol 	Seed (Devi and Asna, 2010)
	2. *α*-Tocopherolquinone 	Seed (Mahmood et al., 1984)
	3. ξ_2-tocopherol 	Seed (Mahmood et al., 1985)
Benzopyrone	1. Methyl ester of *p*-coumaric acid 	Rhizome (Bandara et al., 1988)

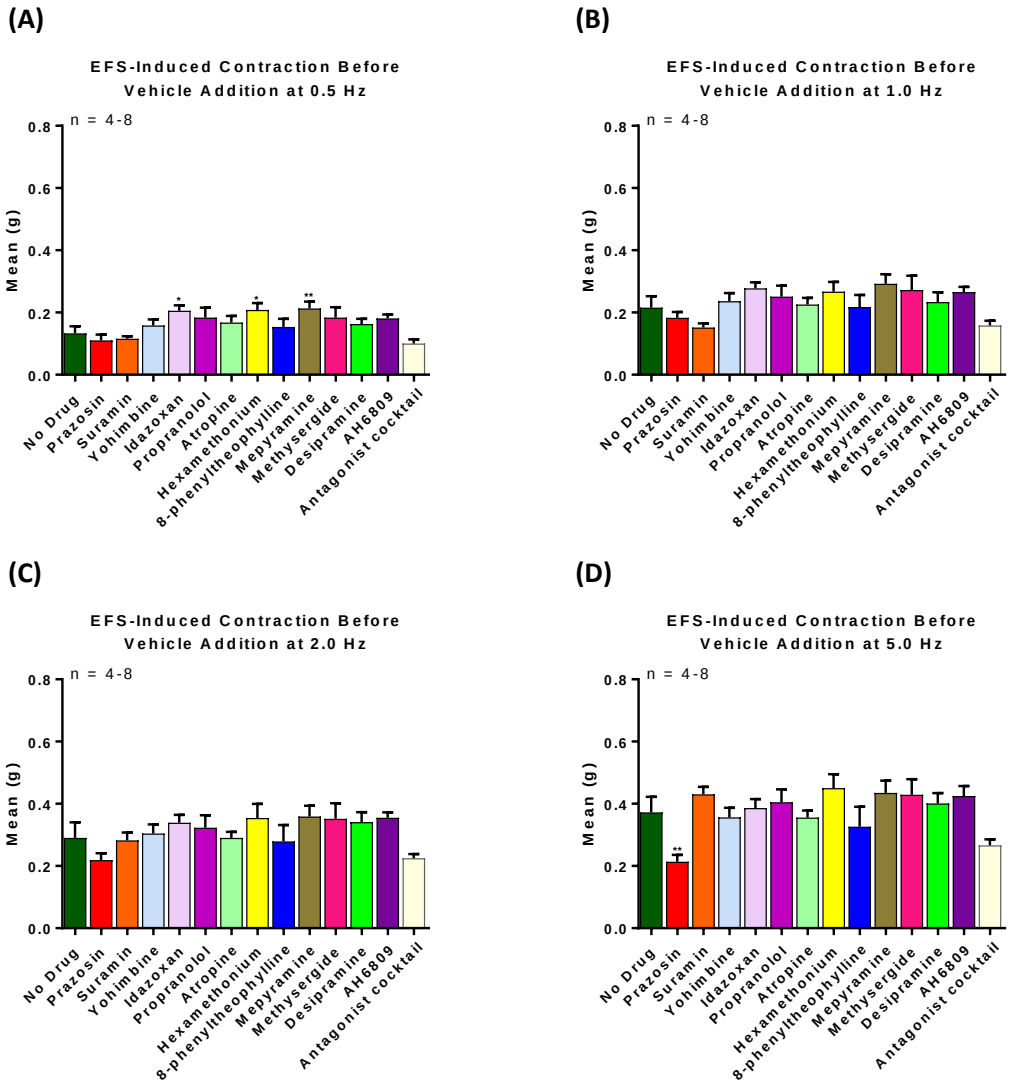
Appendix 2. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) of isolated rat prostates before (open columns) and after (solid columns) administration of serotonin (10 µM, p = 0.6058, n = 4) **(A)** and (100 µM, p = 0.4392, n = 4) **(B)**. **C**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.1481, n = 8). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency (two-way repeated measures ANOVA).



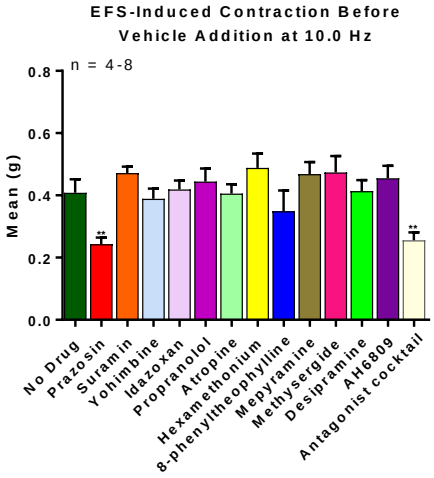
Appendix 3. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 1-20 Hz, 10 s pulses) in isolated rat prostates before (open columns) and after (solid columns) administration of histamine (10 µM) (p = 0.7262, n = 4) **(A)** and 100 µM (p = 0.8860, n = 4) **(B)**. **C**, Mean contractile responses to electrical field stimulation of isolated rat prostate in the absence (open bars) and presence (solid bars) of vehicle (p = 0.6870, n = 8). Bars represent mean force ± S.E.M. (*p < 0.05; two-way repeated-measures of ANOVA). p-values represent the probability of a significant interaction between treatment and frequency (two-way repeated measures ANOVA).



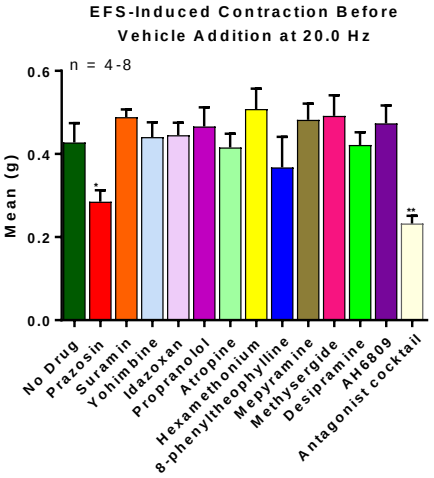
Appendix 4. Mean contractile response to electrical field stimulation of rat prostates of the vehicle control in the absence (no drug) and presence of the following pharmacological agents: prazosin (300 nM), suramin (30 nM), yohimbine (1 µM), idazoxan (1 µM), propranolol (1 µM, n=6), atropine (1 µM, n=6), methysergide (1 µM, n=6), mepyramine (1 µM, n=8), hexamethonium (10 µM, n=6), desipramine (100 nM, n=6), 8-phenyltheophylline (10 µM, n=5), AH6809 (10 µM, n=4), and antagonist cocktail (n = 6). Results are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for statistical evaluation. (*$p < 0.05$, **$p < 0.01$). p-values represent probability of any change in the degree of inhibition being due to chance.



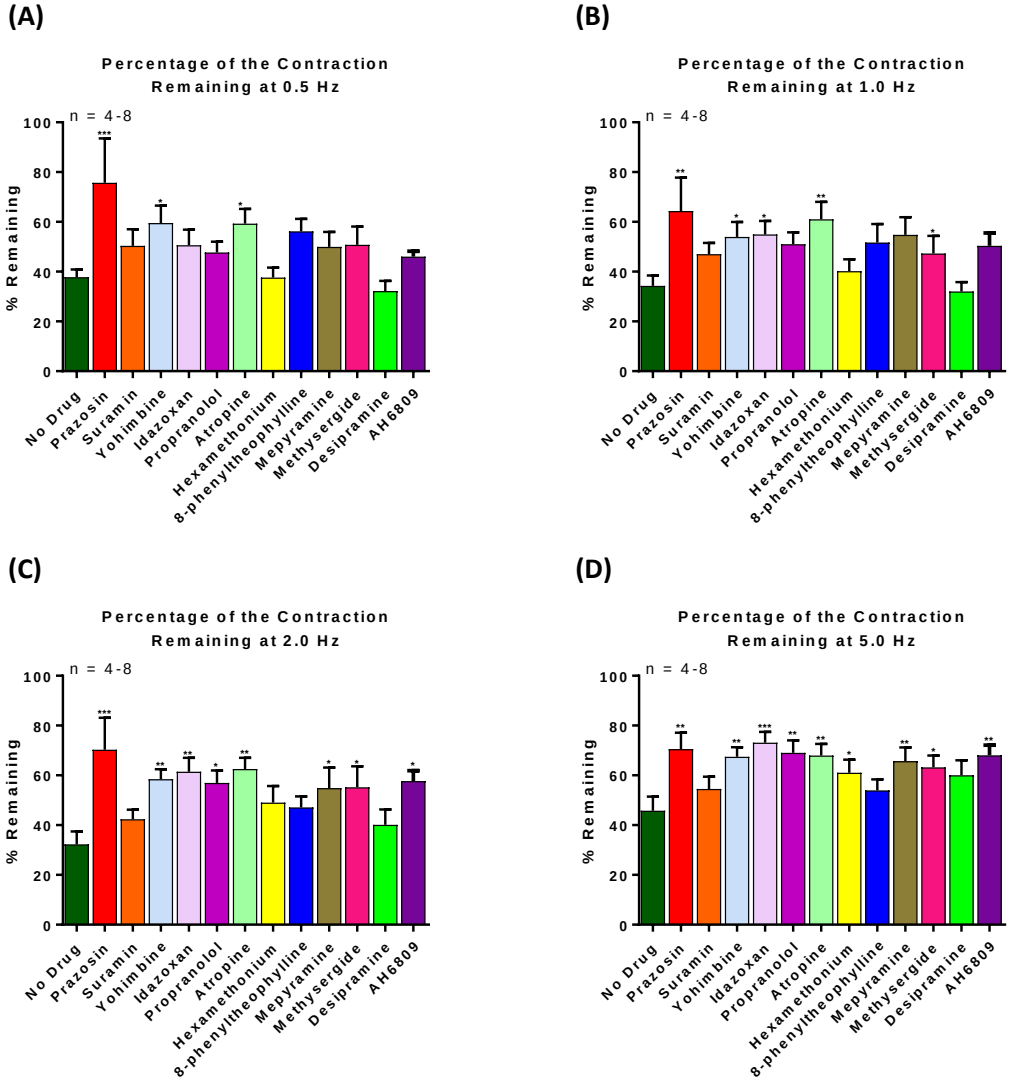
(E)


EFS-Induced Contraction Before
Vehicle Addition at 10.0 Hz
n = 4-8
Mean (g)
0.0
0.2
0.4
0.6
0.8
**
**
No Drug
Prazosin
Suramin
Yohimbine
Idazoxan
Propranolol
Atropine
Hexamethonium
8-phenyltheophylline
Mepyramine
Methysergide
Desipramine
AH6809
Antagonist cocktail

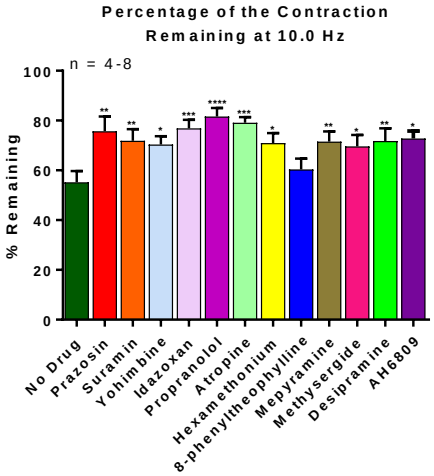
(F)


EFS-Induced Contraction Before
Vehicle Addition at 20.0 Hz
n = 4-8
Mean (g)
0.0
0.2
0.4
0.6
*
**
No Drug
Prazosin
Suramin
Yohimbine
Idazoxan
Propranolol
Atropine
Hexamethonium
8-phenyltheophylline
Mepyramine
Methysergide
Desipramine
AH6809
Antagonist cocktail

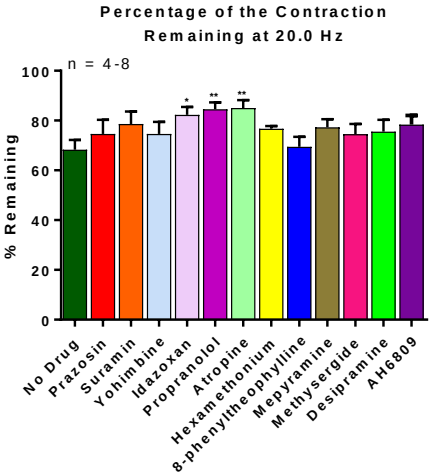
Appendix 5. Mean percentage of control contractile response to electrical field stimulation of rat prostates remaining after administration of *C. speciosus* rhizome cold-water extract (2.0 mg/mL) in the absence (no drug) and presence of the following pharmacological agents: prazosin (300 nM), suramin (30 nM), yohimbine (1 µM), idazoxan (1 µM), propranolol (1 µM, n=6), atropine (1 µM, n=6), methysergide (1 µM, n=6), mepyramine (1 µM, n=8), hexamethonium (10 µM, n=6), desipramine (100 nM, n=6), 8-phenyltheophylline (10 µM, n=5), AH6809 (10 µM, n=4), and antagonist cocktail (n = 6). Results are expressed as means ± S.E.M. (**p* < 0.05, ***p* < 0.005, ****p* < 0.0005, *****p* < 0.0001). One-way analysis of variance (ANOVA) followed by Fisher's LSD test were used for statistical evaluation. *p*-values represent probability of any change in the degree of inhibition being due to chance.



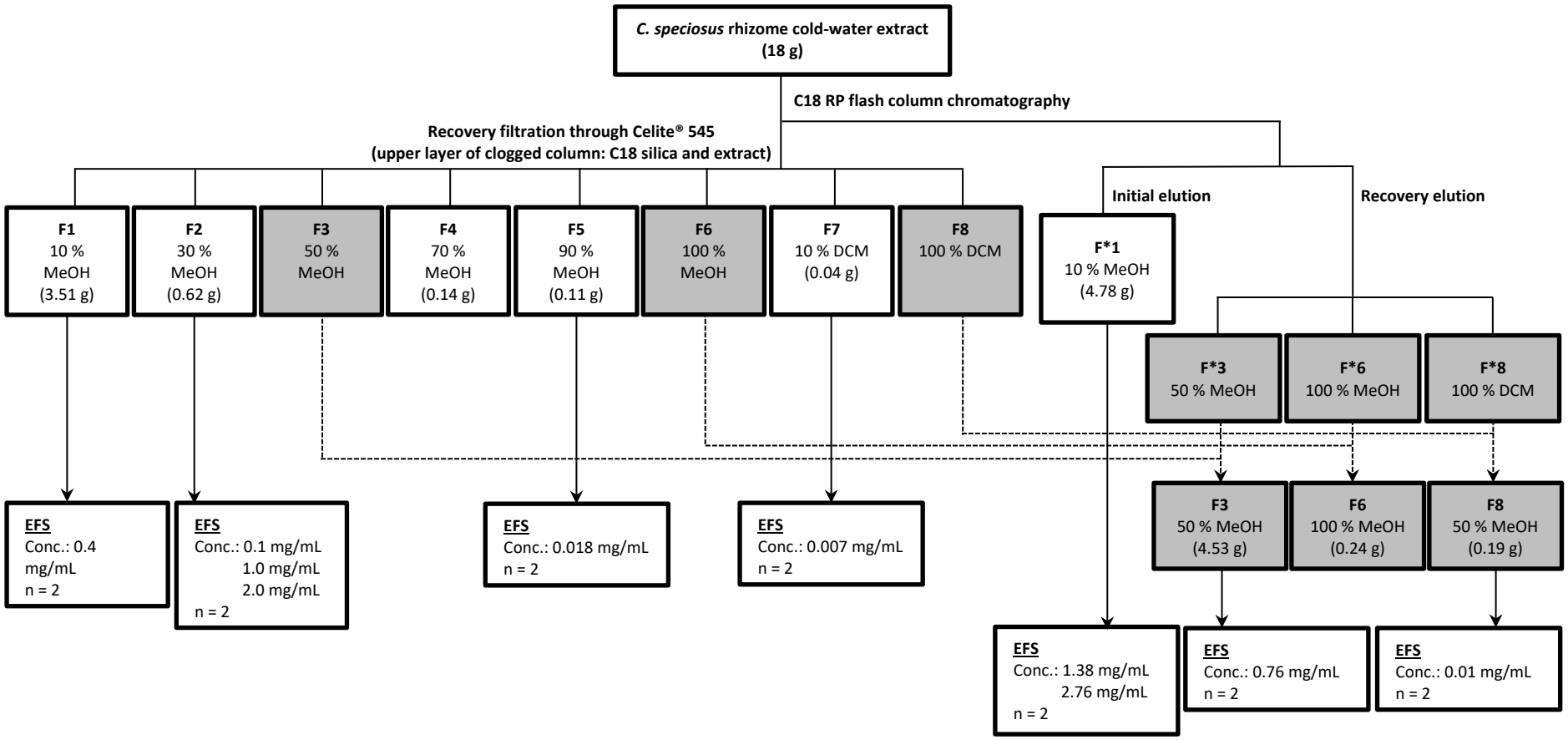
(E)


Percentage of the Contraction
Remaining at 10.0 Hz
n = 4-8
% Remaining
100
80
60
40
20
0
No Drug
Prazosin
Suramin
Yohimbine
Idazoxan
Propranolol
Atropine
Hexamethonium
8-phenyltheophylline
Mepyramine
Methysergide
Desipramine
AH6809

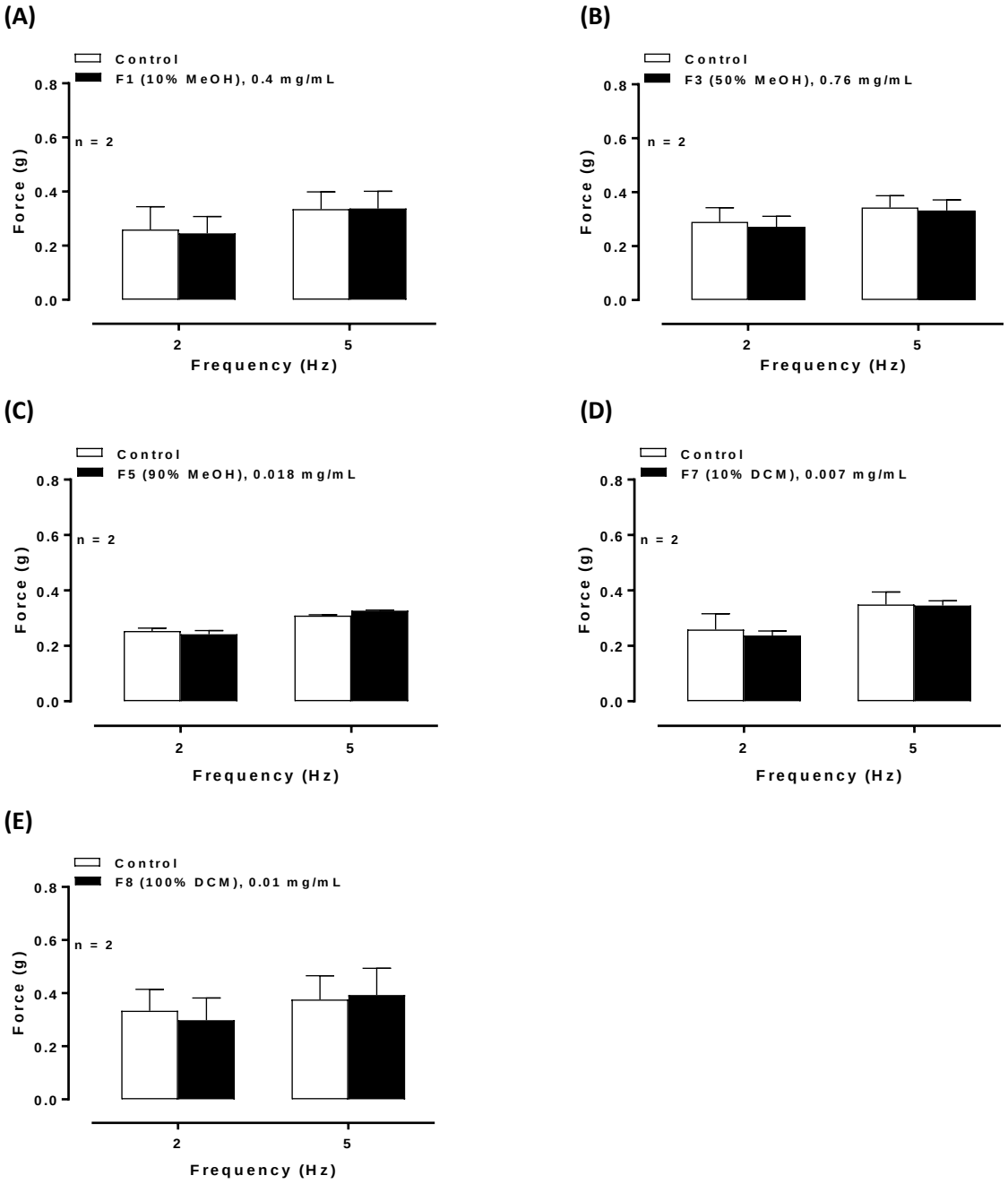
(F)


Percentage of the Contraction
Remaining at 20.0 Hz
n = 4-8
% Remaining
100
80
60
40
20
0
No Drug
Prazosin
Suramin
Yohimbine
Idazoxan
Propranolol
Atropine
Hexamethonium
8-phenyltheophylline
Mepyramine
Methysergide
Desipramine
AH6809

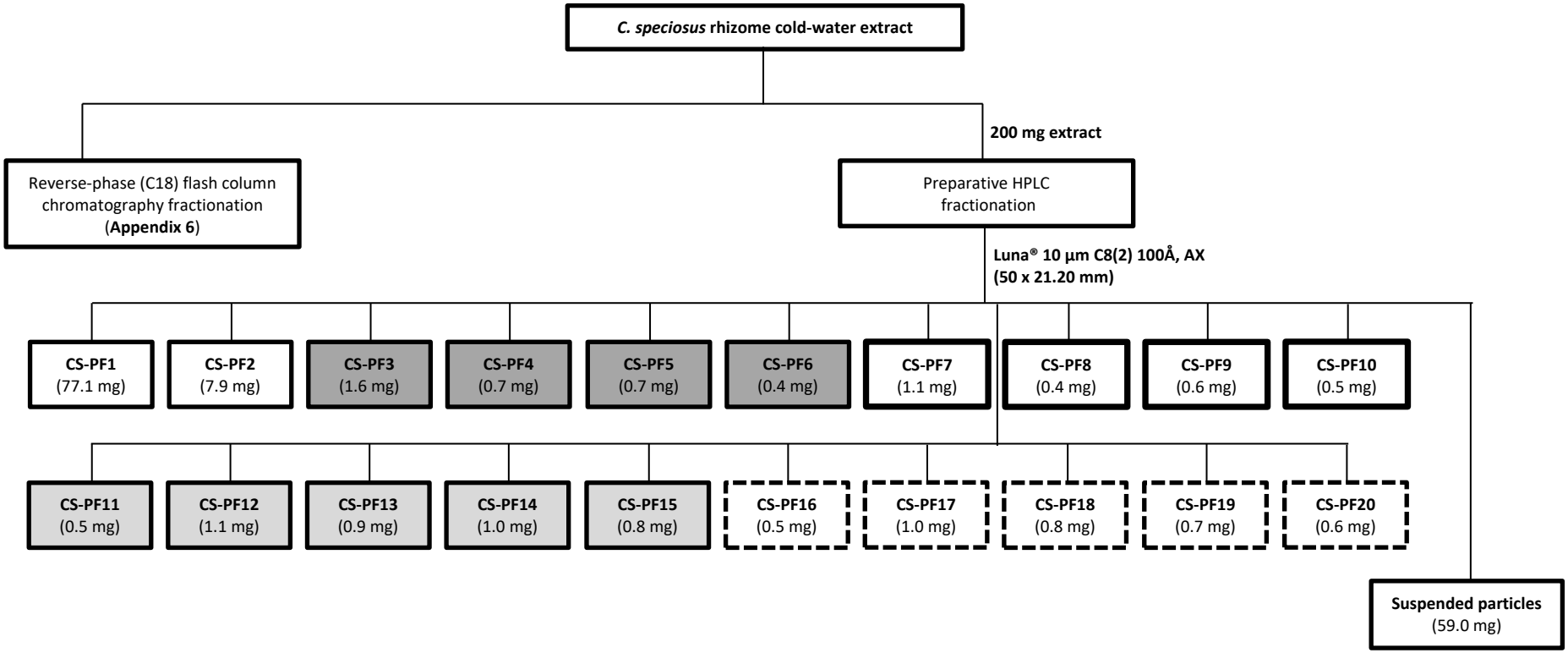
Appendix 6. Chronology of reverse-phase (C18) flash column chromatography fractionation. Elution from the column only collected one fraction (F1). As the column was clogged by the extract, the column was then directly eluted with 50 % MeOH/H_2O (F*3), 100 % MeOH (F*6), and 100 % DCM (F*8), respectively. The extract that clogged the column was removed and recovered by filtrating it through a 2 L Büchner filtration funnel with 1 L of each 10 % (F1), 30 % (F2), 50 % (F3), 70 % (F4), 90 % (F5) MeOH/H_2O, and 100 % (F6) MeOH, 10 % DCM/MeOH (F7) and 100 % DCM (F8), respectively. Bioactivity of F1 (both filtration & elution), F2, F5, F6, F7, F8 were selectively validated by investigating their effects on EFS-induced contractile responses of isolated rat prostates. The grey boxes indicate corresponding solvent systems.



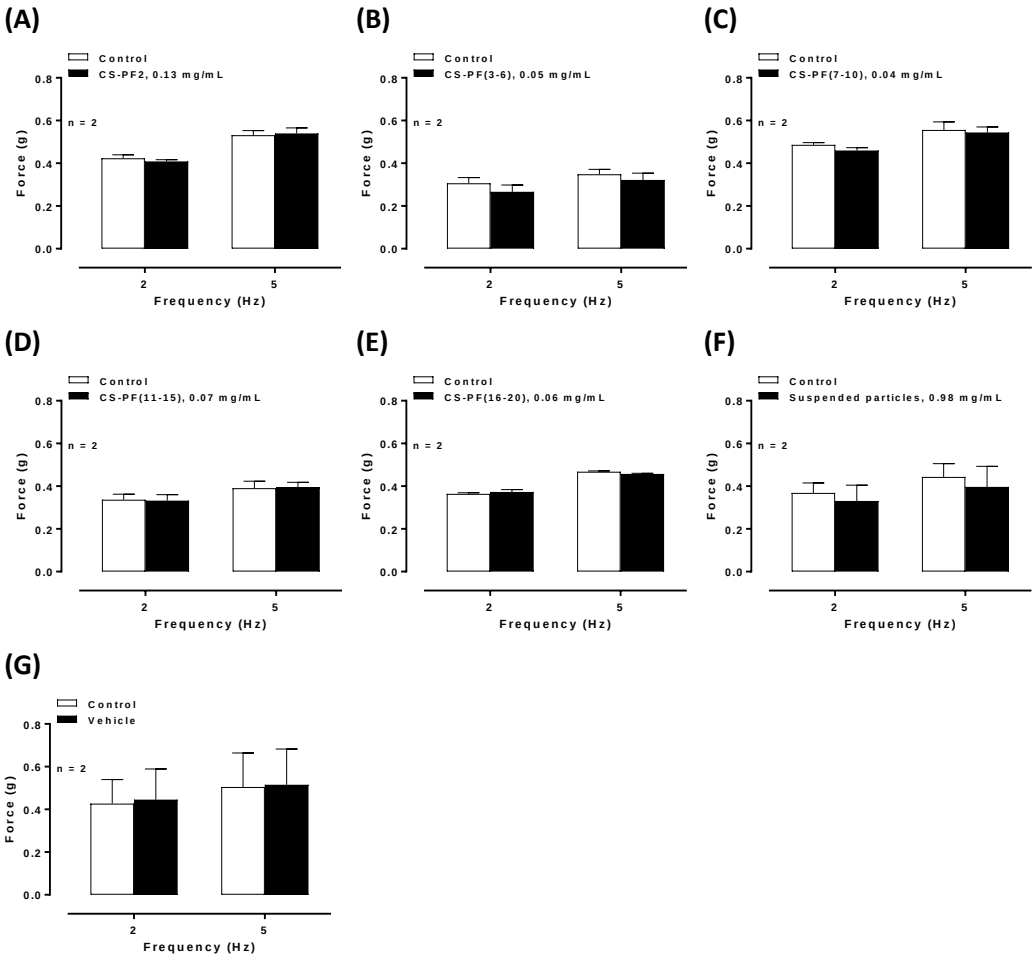
Appendix 7. Bioactivity validation for selected fractions. Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 2 and 5 Hz) in isolated rat prostates after administration (**A**) Fraction 1 (10 % MeOH/H_2O) (0.4 mg/mL, n = 2), (**B**) Fraction 3 (50 % MeOH/H_2O) (0.76 mg/mL, n = 2), (**C**) Fraction 5 (90 % MeOH/H_2O) (0.018 mg/mL, n = 2), (**D**) Fraction 7 (10 % DCM) (0.007 mg/mL, n = 2), and (**E**) Fraction 8 (100 % DCM) (0.01 mg/mL, n = 2), respectively. Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA, followed by Tukey's multiple comparisons test).



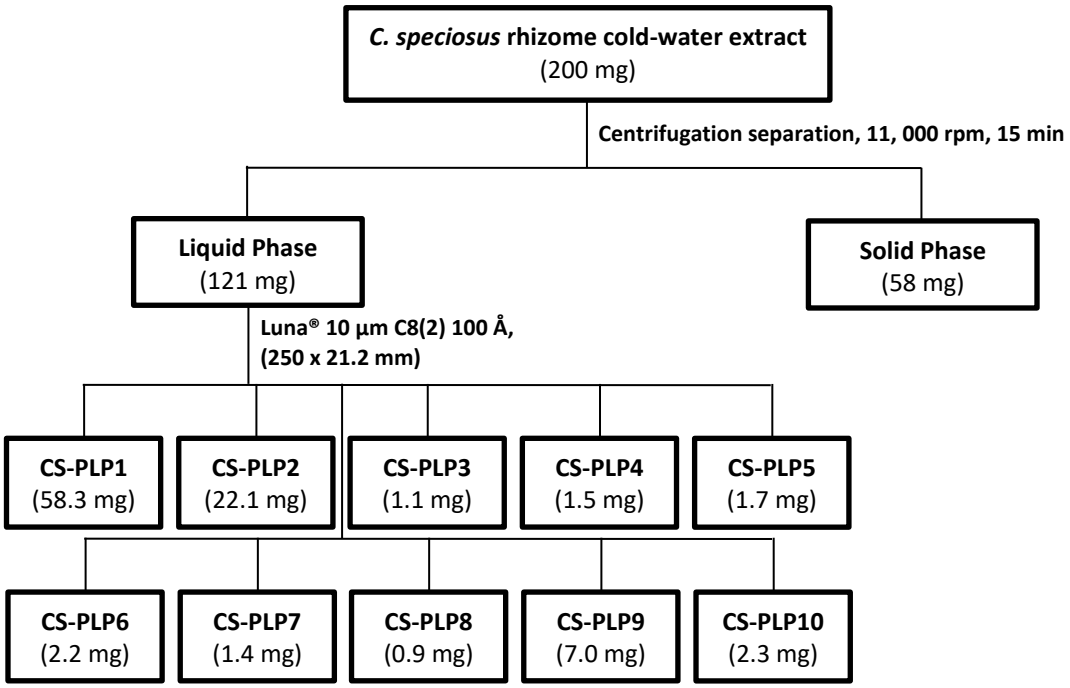
Appendix 8. Preparative HPLC fractionation of *C. speciosus* rhizome cold-water crude extract using preparative HPLC. Chromatographic fractionation yielded 20 fractions. Suspended particles were eluted by flushing of the syringe filter with 20 % MeOH/H_2O. Dark grey boxes: fractions were combined; Boxes with thick outline: fractions were combined; Light grey boxes: fractions were combined; Boxes with dotted outline: fractions were combined.



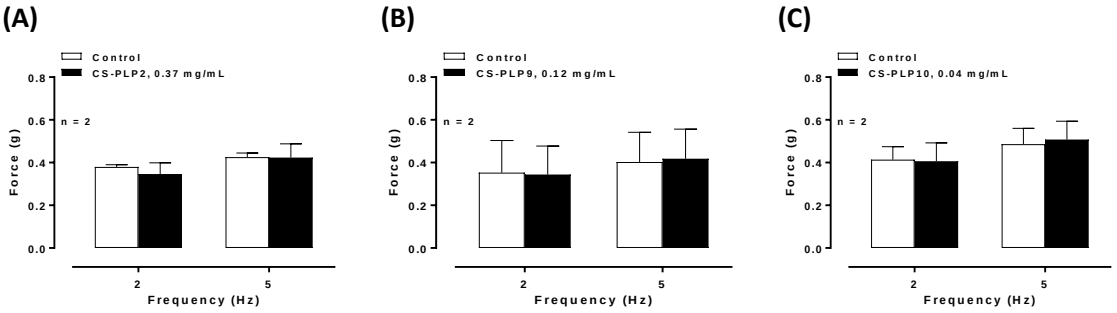
Appendix 9. Mean contractile responses of fractions isolated from preparative HPLC fractionation of filtered *C. speciosus* rhizome cold-water crude extract. **(A),** CS-PF2, 0.13 mg/mL; **(B),** CS-PF(3-6), 0.05 mg/mL; **(C),** CS-PF(7-10), 0.04 mg/mL; **(D),** CS-PF(11-15), 0.07 mg/mL; **(E),** CS-PF(16-20), 0.06 mg/mL; **(F),** Suspended particles, 0.98 mg/mL; **(G),** time control. Mean contractile responses to electrical field stimulation on isolated rat prostate did not affected by vehicle. Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA).



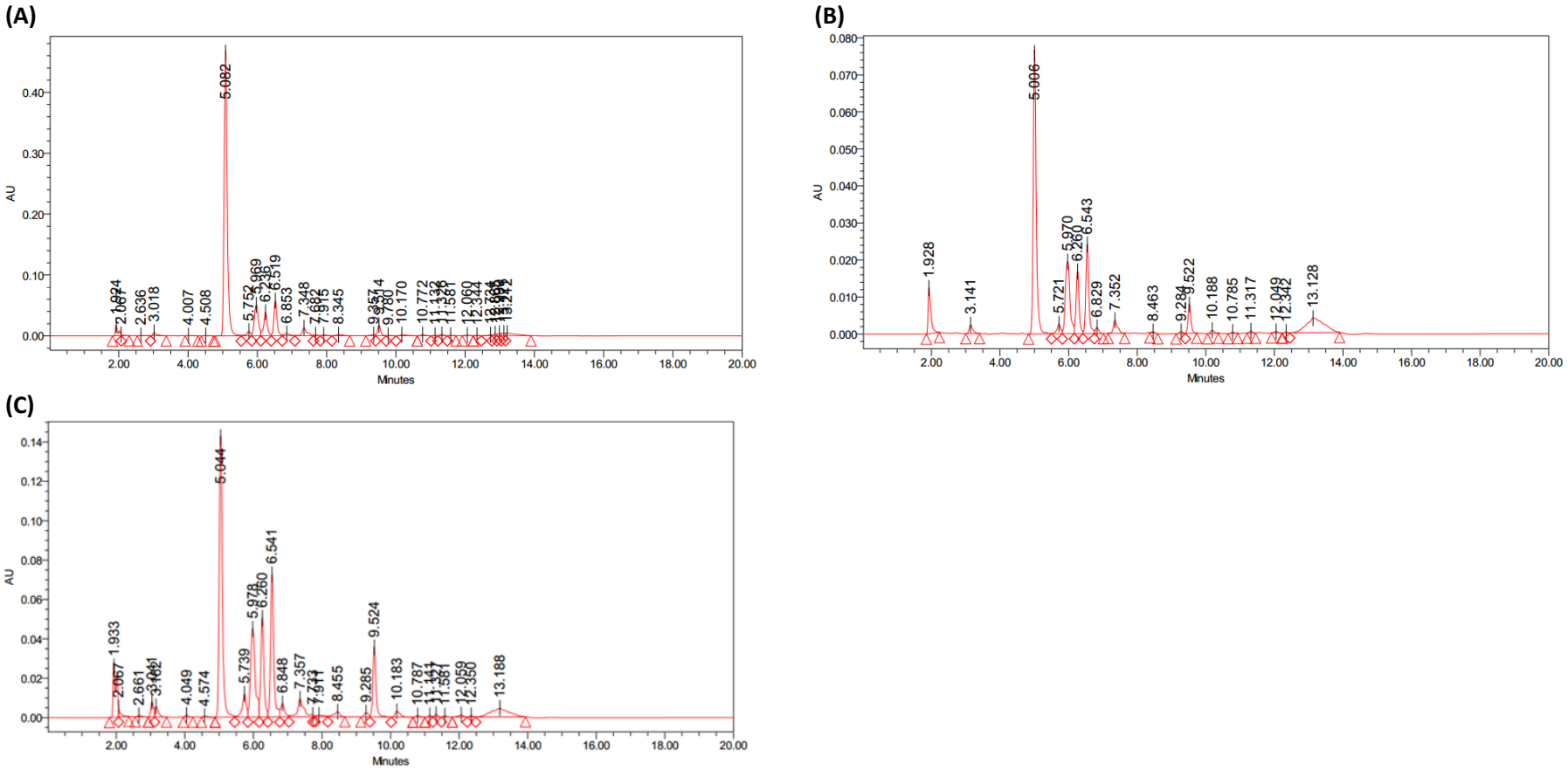
Appendix 10. Chronology of isolation of liquid phase and solid phase extract from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Preparative RP-HPLC fractionation of liquid phase yielded 10 fractions (CS-PLP1 – CS-PLP10).



Appendix 11. Mean contractile responses of fractions isolated from preparative RP-HPLC fractionation of liquid phase extract isolated from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. Fractions with the higher absorbance reading were selected for bioactivity validation using EFS-induced contractions of isolated rat prostates. **(A),** CS-PLP2, 0.37 mg/mL; **(B),** CS-PLP9, 0.12 mg/mL; and **(C),** CS-PLP10, 0.04 mg/mL. Bars represent mean force ± S.E.M. (two-way repeated-measures of ANOVA).

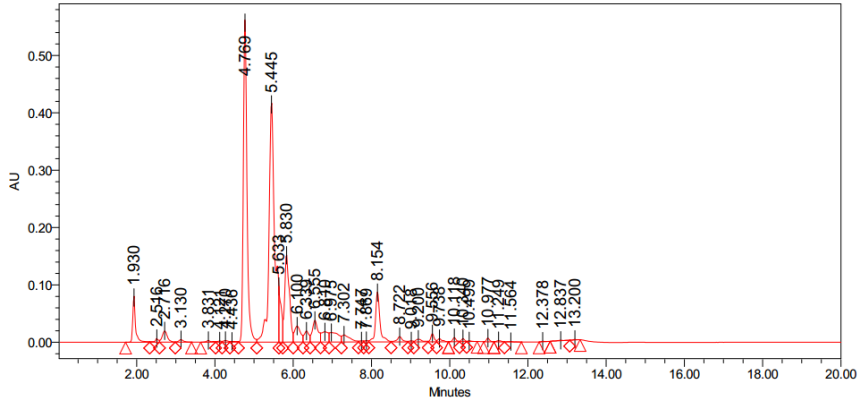


Appendix 12. Analytical RP-HPLC analysis of derivatized CS-PLP1 isolated from liquid phase extract obtained from centrifugation separation of *C. speciosus* rhizome cold-water crude extract. **(A),** 3 mg derivatized CS-PLP1; **(B),** 7 mg derivatized CS-PLP1; and **(C),** 14 mg derivatized CS-PLP1. Mobile phase: Buffer A (0.1 % TFA/H2O) and Buffer B (0.1 % TFA/ACN). UV detection at 245 nm. Flow rate was 1 mL/min.

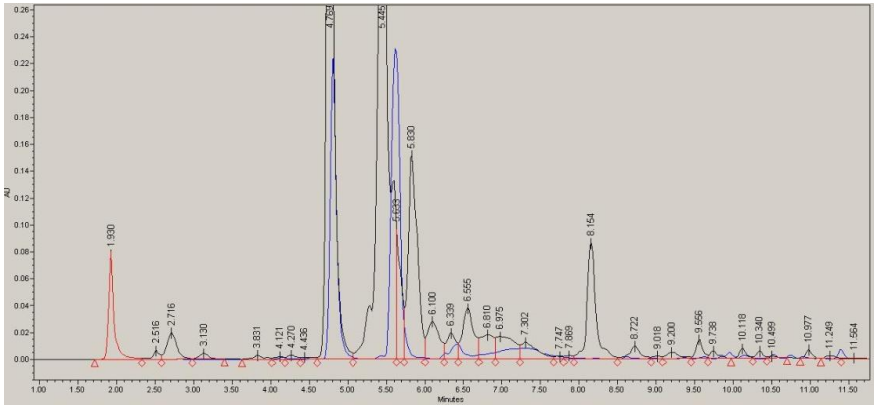


Appendix 13. Analytical RP-HPLC analysis: **(A),** 2-AA-Fru spiked with anthranilate CS-PLP1; **(B),** 2-AA-Gal spiked with anthranilate CS-PLP1; **(C),** 2-AA-Glu spiked with anthranilate CS-PLP1; **(D),** 2-AA-Lac spiked with anthranilate CS-PLP1; **(E),** 2-AA-Man spiked with anthranilate CS-PLP1; and **(F),** Anthranilic acid (0.1 mg/mL) (Control). The blue chromatograms depicted in **(A-2), (B-2), (C-2), (D-2),** and **(E-2)** represent anthranilate fructose, galactose, glucose, lactose, and mannose, respectively. The black line chromatograms represent anthranilate CS-PLP1.

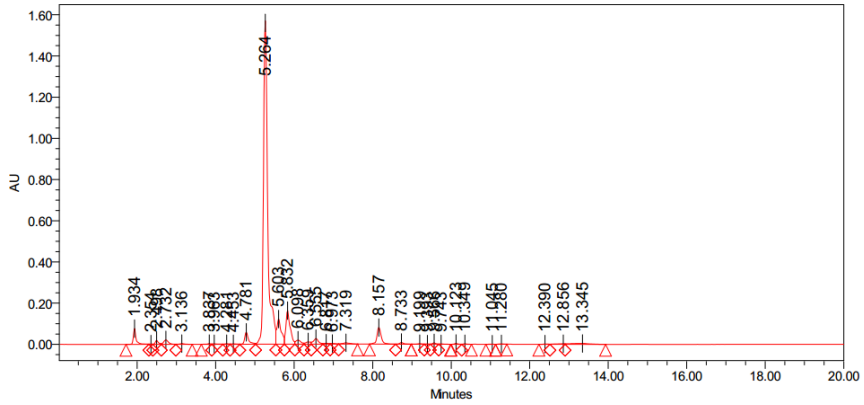
(A-1) 2-AA-Fru vs anthranilate CS-PLP1



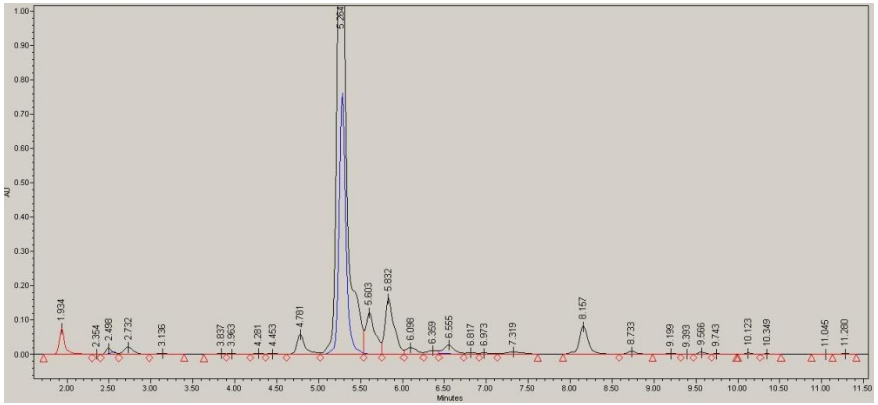
(A-2)



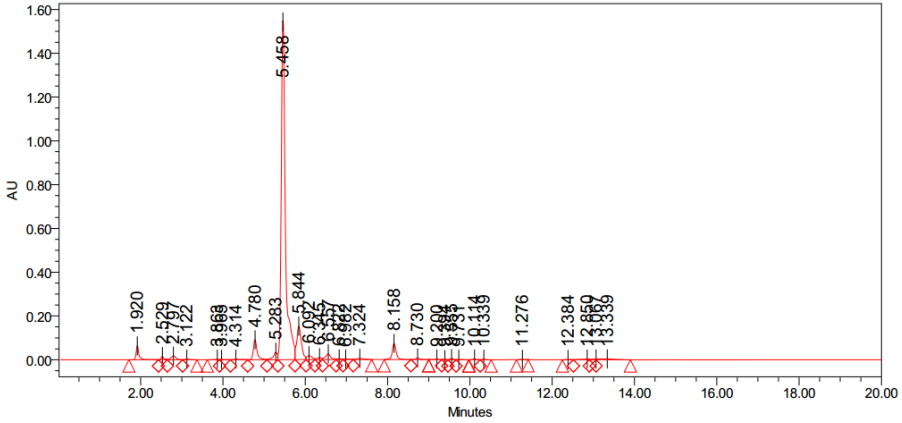
(B-1) 2-AA-Gal vs anthranilate CS-PLP1



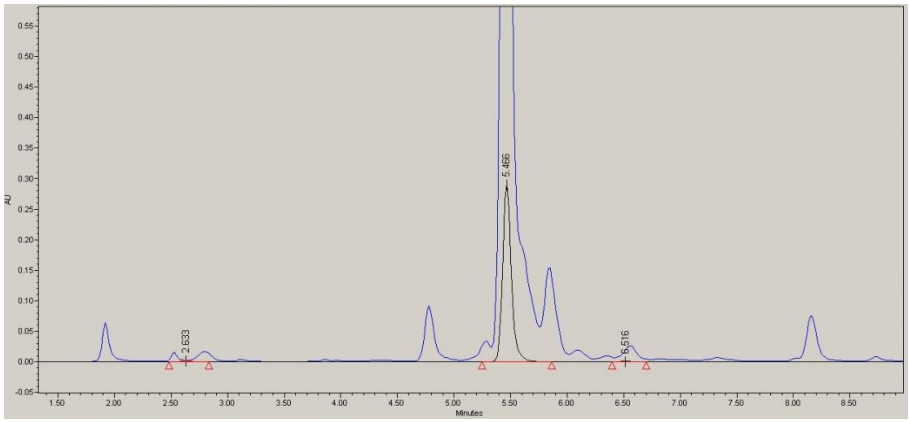
(B-2)



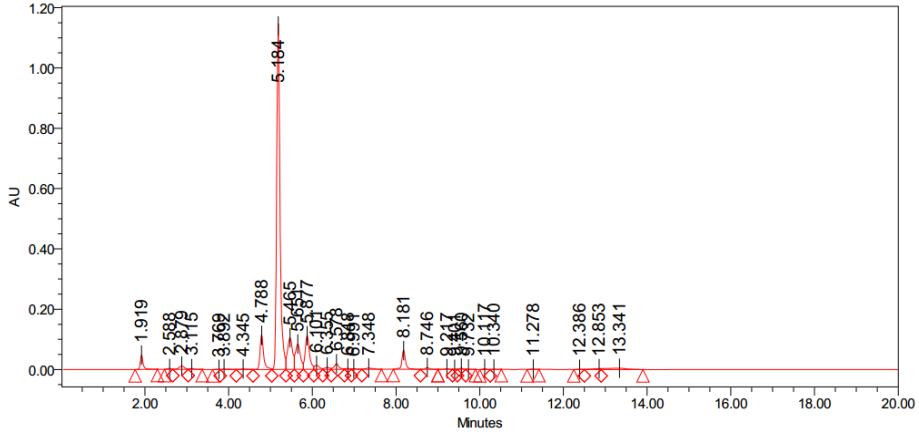
(C-1) 2-AA-Glu vs anthranilate CS-PLP1



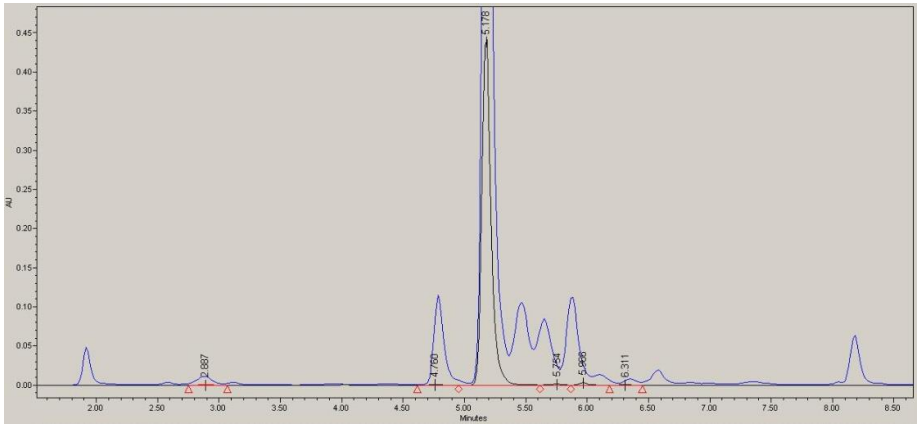
(C-2)



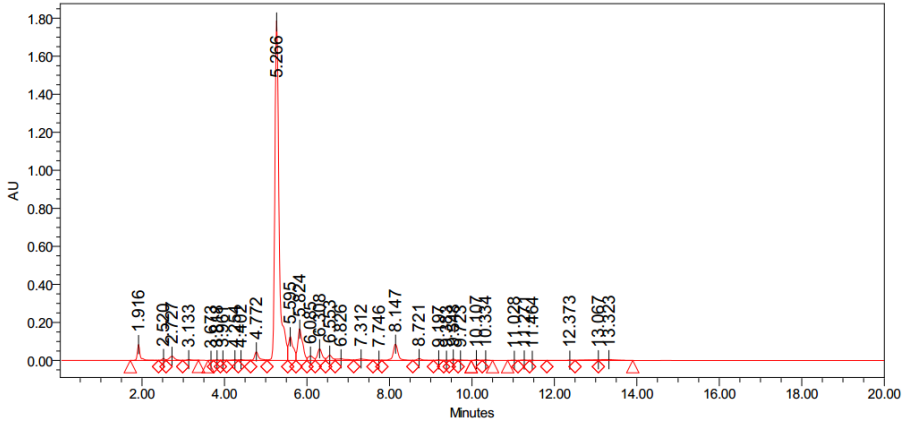
(D-1) 2-AA-Lac vs anthranilate CS-PLP1



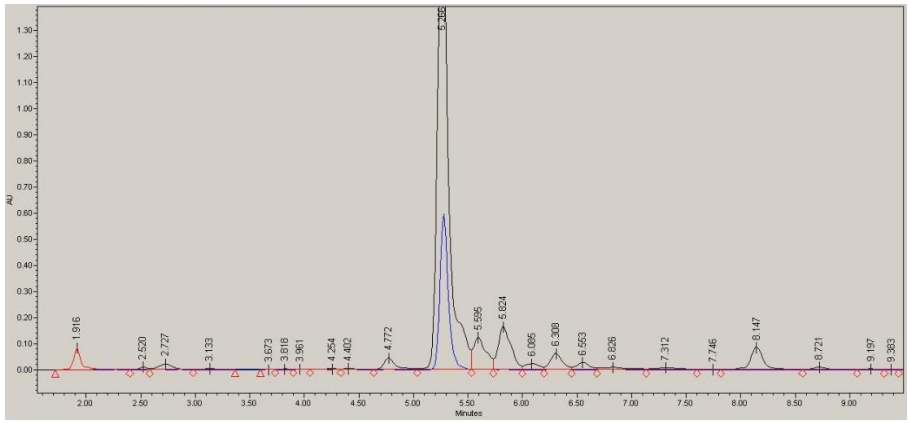
(D-2)



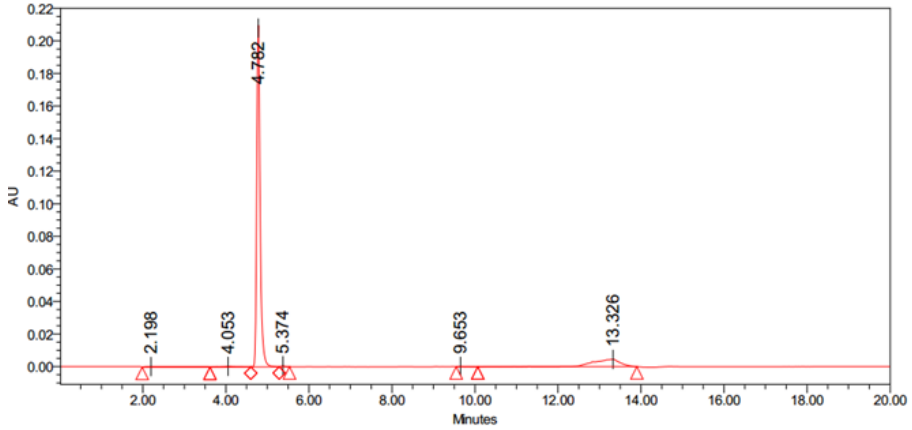
(E-1) 2-AA-Man vs anthranilate CS-PLP1



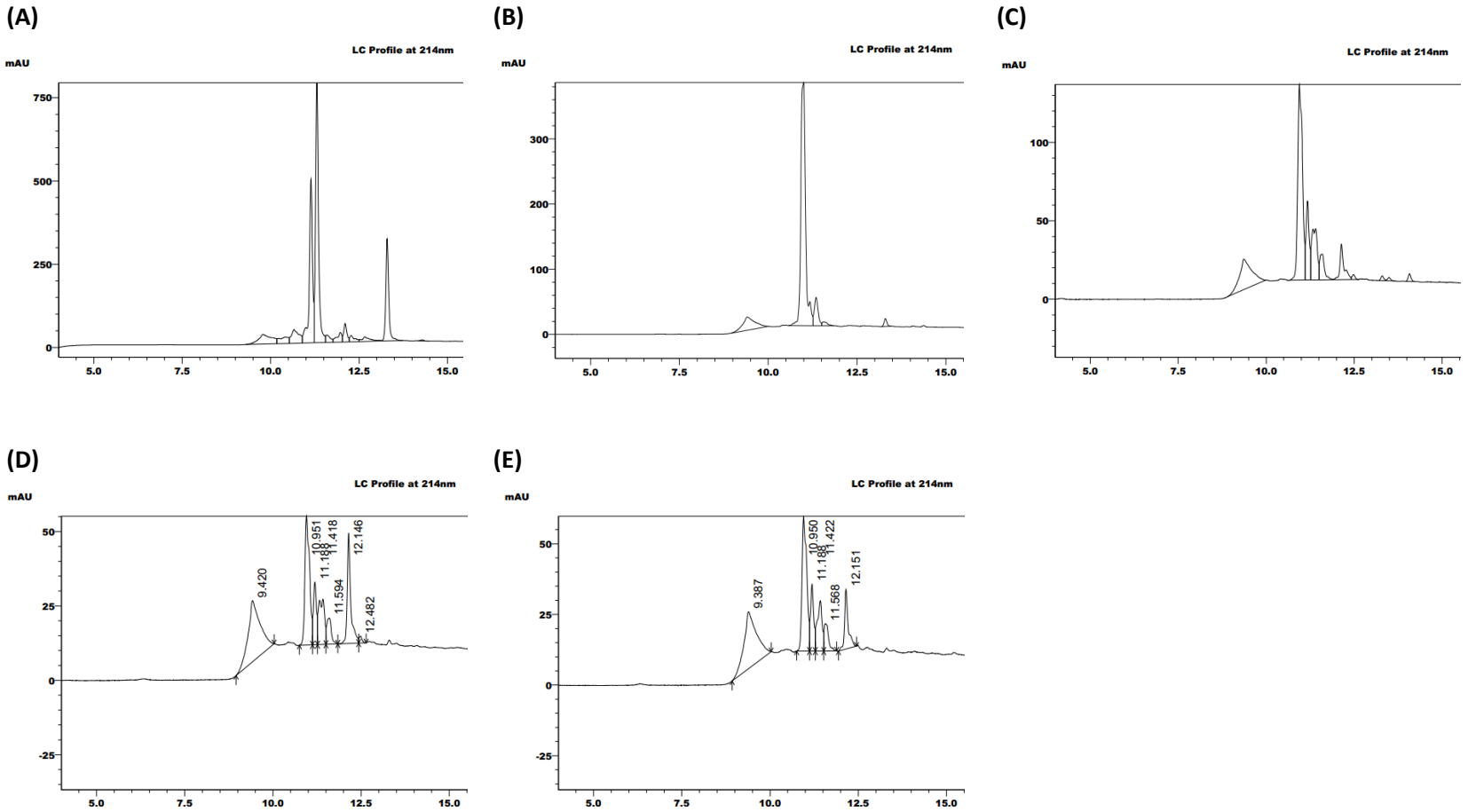
(E-2)



(F) Anthranilic acid 0.1 mg/mL control

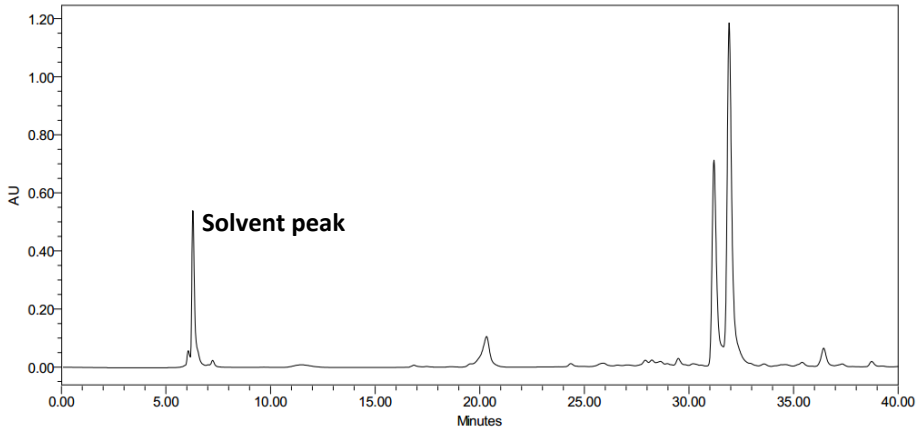


Appendix 14. LCMS analysis: **(A)** D-1, **(B)** D-2, **(C)** D-3, **(D)** D-4, and **(E)** D-5.

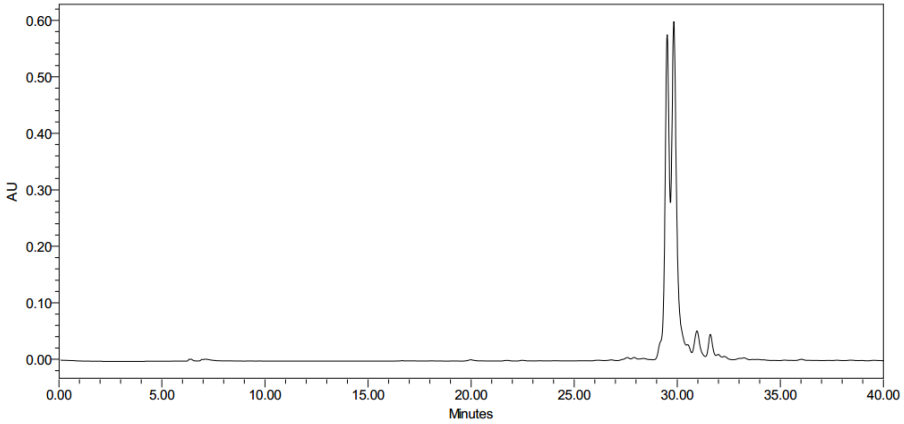


Appendix 15. Preparative RP-HPLC: **(A)** D-1, **(B)** D-2, and **(C)** D-3,4,5. Preparative reverse-phase HPLC fractionation of D-1 yielded 18 fractions; 10 fractions for D-2 and 18 fractions for D-3,4,5 combination. Mobile phase: Buffer A (0.1 % TFA/H2O) and Buffer B (0.1 % TFA/ACN). UV detection at 245 nm. Flow rate was 10 mL/min. Method used a total run time of 40 min.

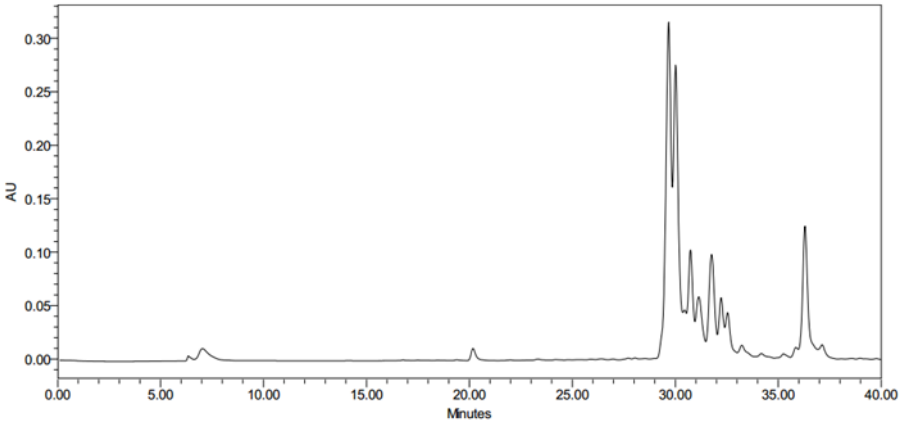
(A) D-1



(B) D-2

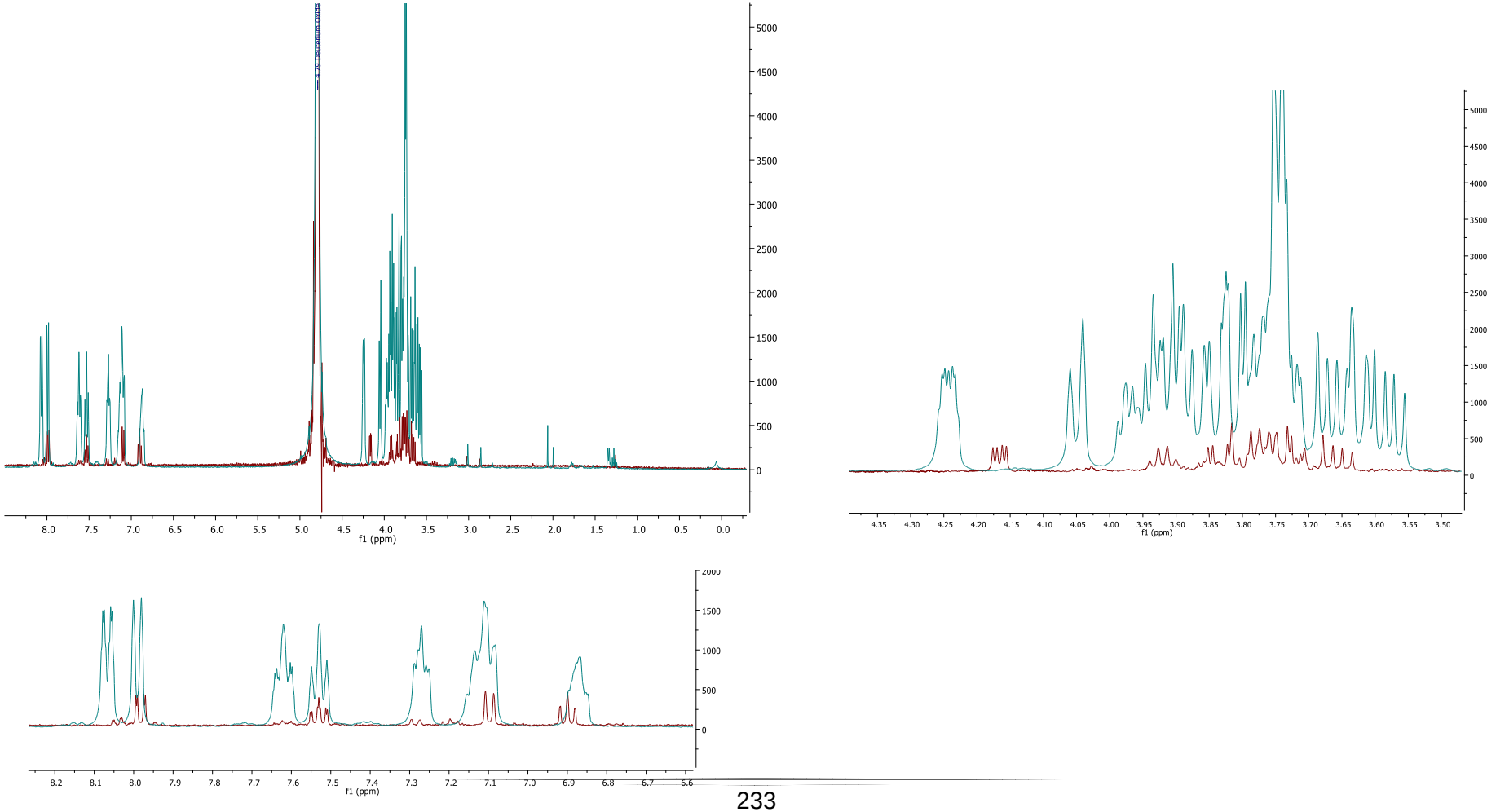


(C) D-3,4,5

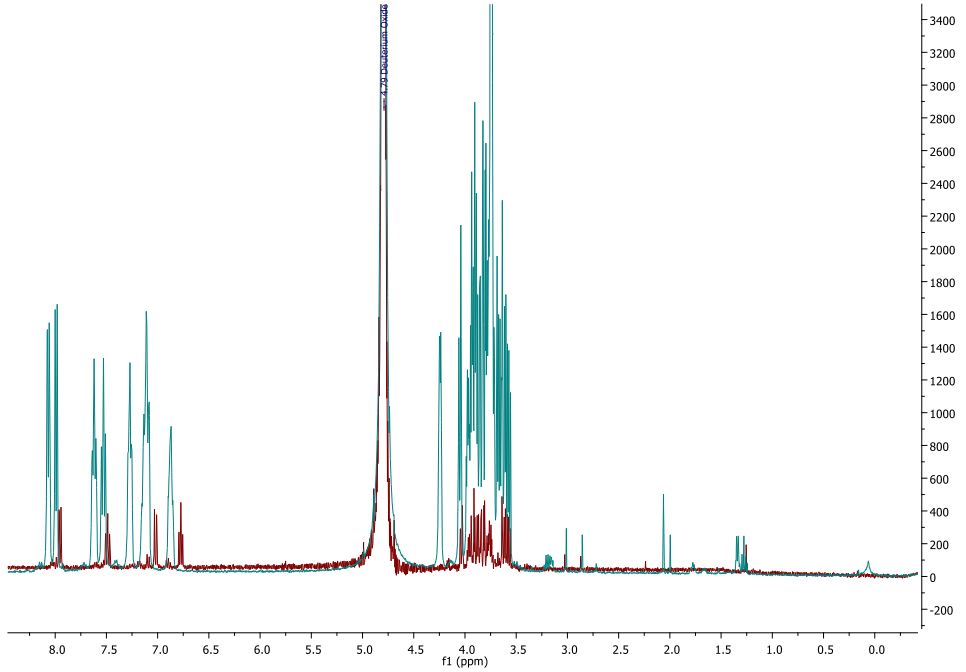


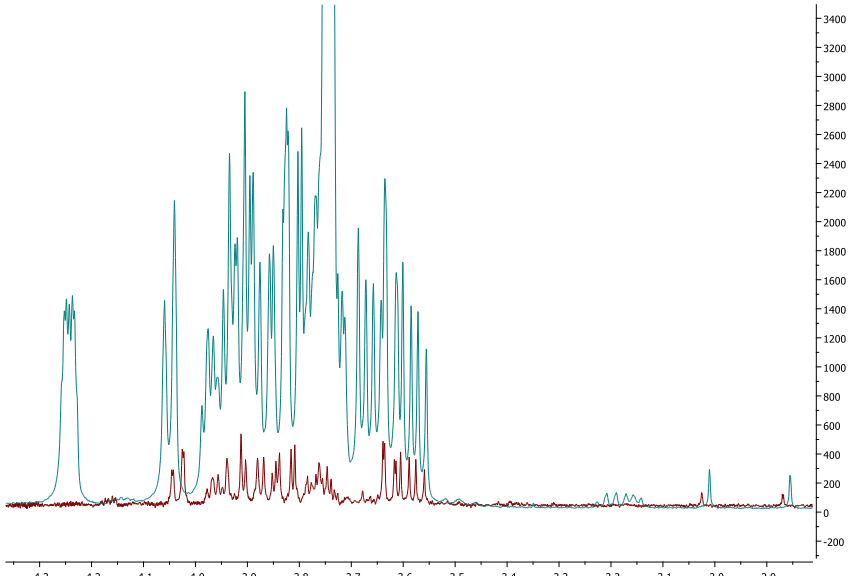
Appendix 16. Superimposed ^{1}H NMR spectra of sub-fractions isolated from anthranilate CS-PLP1 with anthranilate sugar standards. Red spectra: Sub-fraction of anthranilate CS-PLP1; Turquoise spectra: Anthranilate sugar standard.

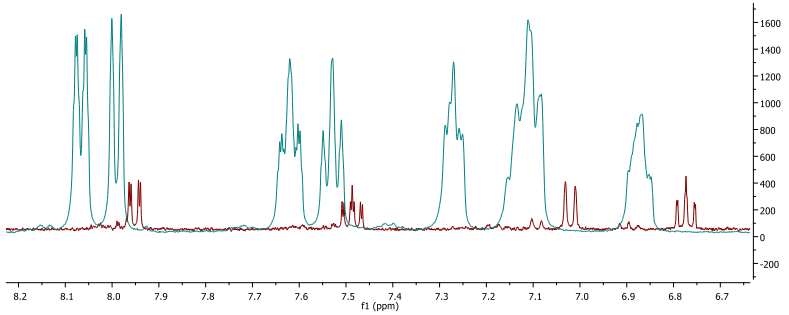
(A) D-2-4 vs 2-AA-Fru



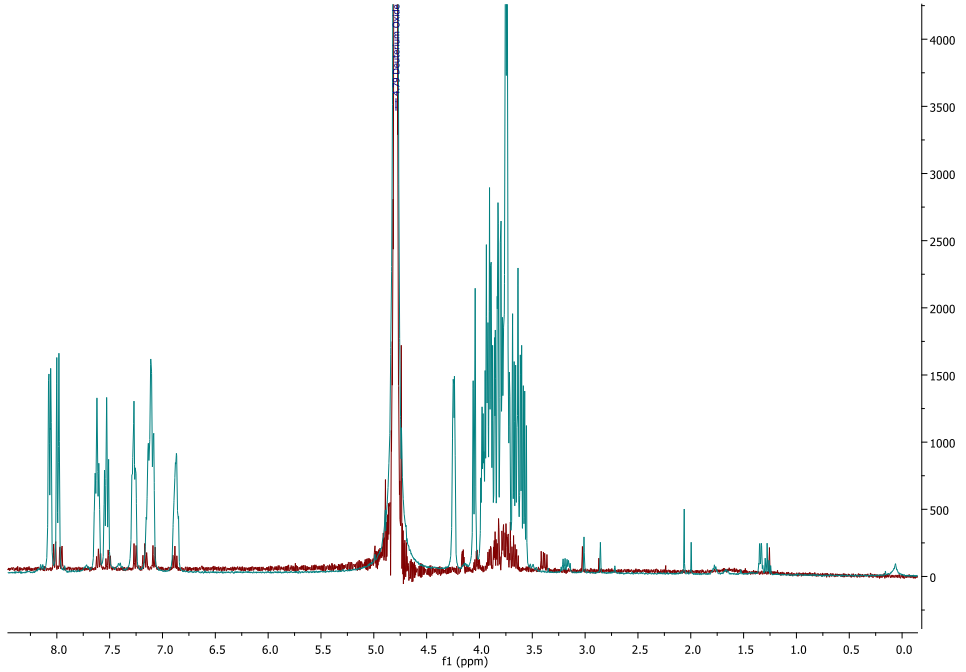
(B) D-2-5 vs 2-AA-Fru

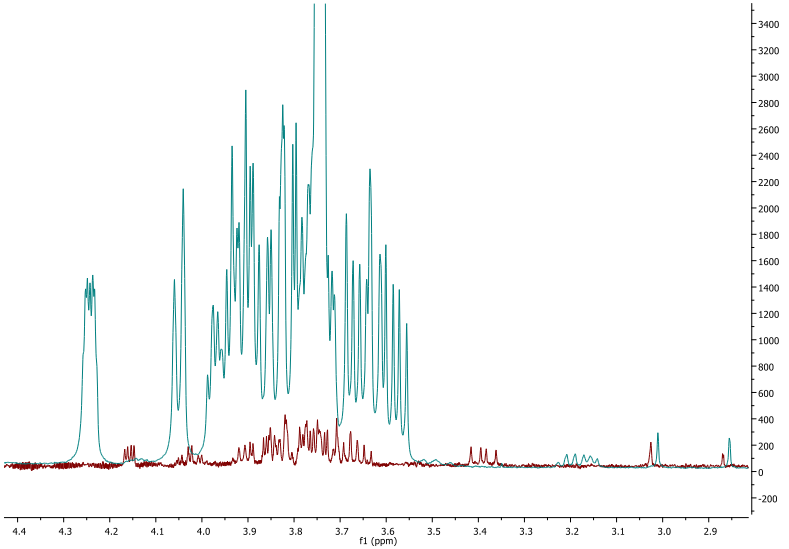


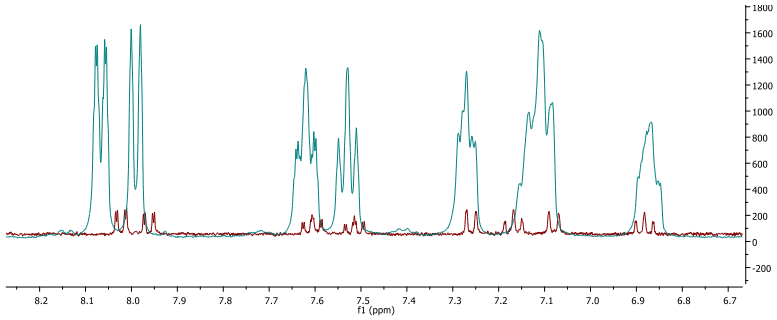




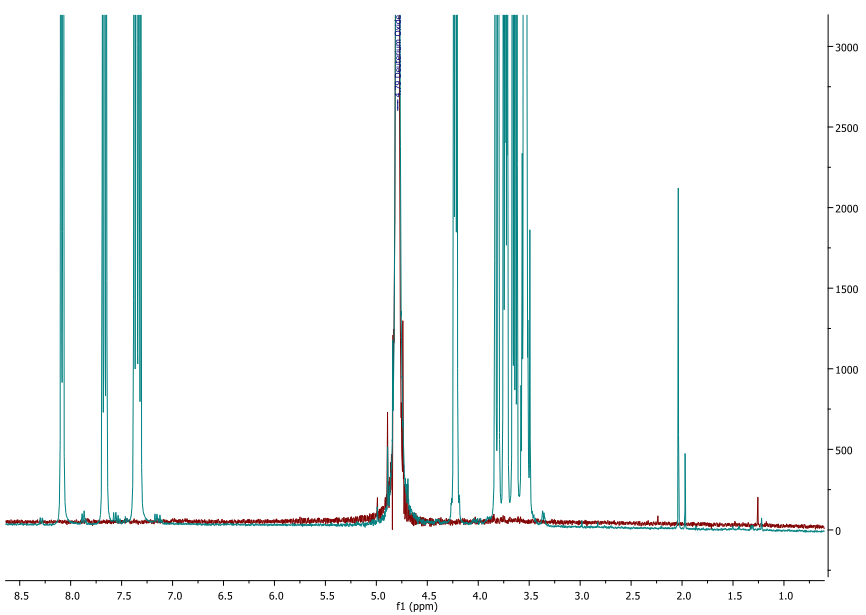
(C) D-3,4,5-4 vs 2-AA-Fru



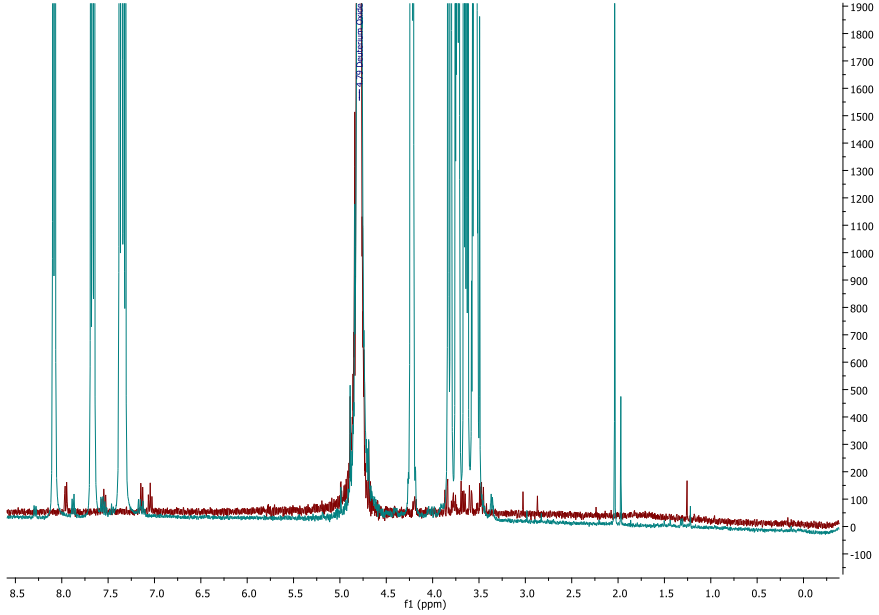




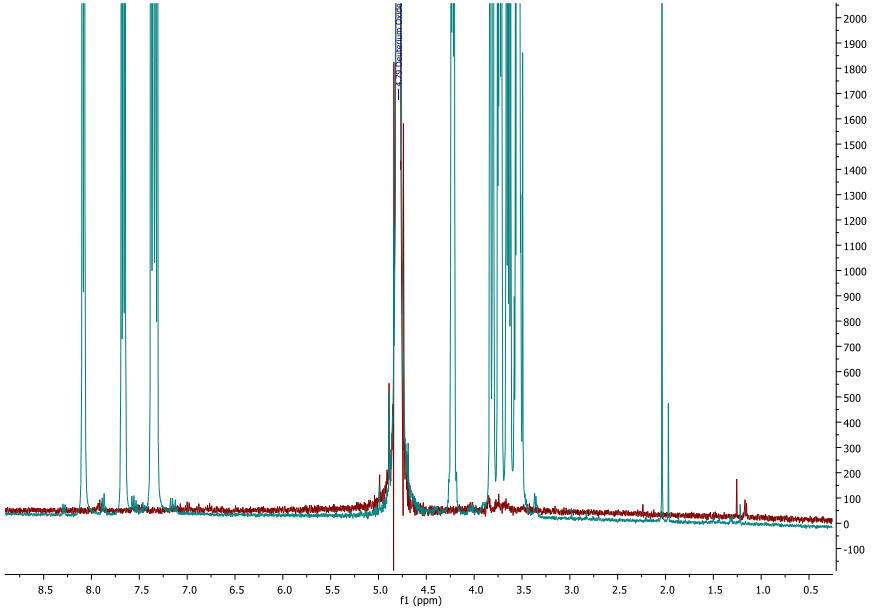
(D) D-3,4,5-6 vs 2-AA-Ara



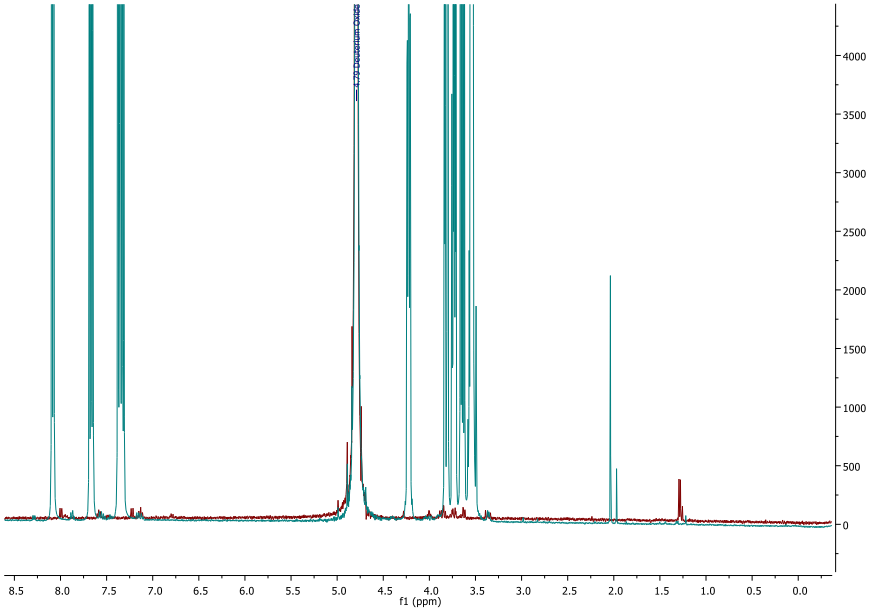
(E) CS-PLP1-A3,4,5-7_30.65 min vs 2-AA-Ara



(F) D-3,4,5-8 vs 2-AA-Ara



(G) CS-PLP1-A3,4,5-9_31.54 min vs 2-AA-Rha



(H) D-3,4,5-10 vs 2-AA-Rha