MONASH University

PLANTS AND PURINERGICS: A STUDY OF STINGING NETTLE EXTRACT AND THE MALE RODENT UROGENITAL SYSTEM

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‘It is good to have an end to journey toward; but it is the journey that matters, in the end.’

Ernest Hemingway

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications

The results presented in this thesis have led to the following publications

Conference Abstracts

Oral presentations

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Eise N, Thompson P, Simpson JS, and Ventura S (2014) A polar compound present in stinging nettle leaf extract inhibits the purinergic component of contractility in the rat prostate gland. *Joint ASCEPT-MPGPCR Scientific Meeting.* Melbourne, VIC. December 2014

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Poster presentations

Eise N, Gundani K, Nguyen N, Baell J, Simpson JS, Thompson P, and Ventura S (2015) New horizons for male contraception: A non-hormonal approach via blockade of P2X1-purinoceptors and α_{1A}-adrenoceptors. *Joint APSA-ASCEPT Scientific Meeting*. Hobart, TAS. December 2015

Eise N, Gundani K, Nguyen N, Baell J, Simpson JS, Thompson P, and Ventura S (2015) New horizons for male contraception: A non-hormonal approach via blockade of P2X1-purinoceptors and α_{1A}-adrenoceptors. *7th National Symposium on Advances in Urogenital and Gut Research.* Adelaide, SA. October 2015

Eise N, Thompson P, Simpson JS, and Ventura S (2014) A polar compound present in stinging nettle leaf extract inhibits the purinergic component of contractility in the rat prostate gland. *Joint ASCEPT-MPGPCR Scientific Meeting.* Melbourne, VIC. December 2014

Eise N, Thompson P, Simpson JS, and Ventura S (2014) A polar compound present in stinging nettle leaf extract inhibits the purinergic component of contractility in the rat prostate gland. *9th Annual Postgraduate Research Symposium*. Monash University, Parkville. November 2014

Eise N, Thompson P, Simpson JS, and Ventura S (2014) Bioactive components of commercially available stinging nettle leaf extract that inhibit the purinergic component of contractility in the rat prostate gland is due to an aqueous-soluble polar bioactive. *6th National Symposium on Advances in Urogenital and Gut Research.* Gold Coast, QLD. September 2014

Eise N, Thompson P, Simpson JS, and Ventura S (2014) Identification of a compound in commercially available stinging nettle leaf extract that inhibits the purinergic component of contractility in the rat prostate gland. *17th World Congress of Basic and Clinical Pharmacology.* Cape Town, South Africa. July 2014

Eise N, Simpson JS, Thompson P, and Ventura S (2013) An aqueous-soluble polar bioactive is responsible for the inhibitory effect of stinging nettle leaf extract on the purinergic component of contractility in the rat prostate gland. Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT), *Annual Scientific Meeting.* Melbourne, VIC. December 2013

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Eise N, Simpson JS, and Ventura S (2013) Stinging nettle leaf extract affects smooth muscle contractility in the isolated rat prostate gland by acting within the purinergic system. *4th Annual Meeting of the* International Continence Society (ICS). Barcelona, Spain. August 2013

Eise N, Simpson JS, and Ventura S (2012) Pharmacological investigation of the effects of *Urtica dioica* on the contractility of the isolated rat prostate gland. *4th National Symposium on Advances in Urogenital and Gut Research*. Sydney, NSW. November 2012

Eise N, Simpson JS, and Ventura S (2012) Effects of stinging nettle extract on smooth muscle contractility in the isolated rat prostate gland. *Joint ASCEPT-APSA Annual Scientific Meeting*. Sydney, NSW. December 2012

Other publications

Journal articles

White P J, Larson I, Styles K, Yuriev E, Evans DR, Rangachari PK, Short JL, Exintaris B, Malone DT, Davie B, **Eise N**, and McNamara K (2016) Adopting an active learning approach to teaching in a research intensive higher education context resulted in changes in staff attitudes and behaviours. *Higher Education Research and Development* 35: 619-633.

White P J, Larson I, Styles K, Yuriev E, Evans DR, Rangachari PK, Malone DT, Davie B, Naidu S, and **Eise N** (2015). Using active learning strategies to shift student attitudes and behaviours about learning and teaching in a research intensive educational context. *Pharmacy Education* 15(1): 162-172.

Chua T, **Eise N,** Simpson SJ, and Ventura S (2014) Pharmacological characterization and chemical fractionation of a liposterolic extract of saw palmetto. *Journal of Ethnopharmacology* 152: 283-291.

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Yuriev E, White PJ, Larson I, Styles K, Evans DR, Rangachari PK, Short JL, Exintaris B, Malone DT, Davie B, **Eise, N**, and McNamara K (2014) Concerted implementation of active learning in a research intensive faculty: Changes in staff and students attitudes and behaviors. *248th ACS National Meeting*. San Francisco, California. August 2014

Yuriev E, White PJ, Larson I, Styles K, Evans DR, Rangachari PK, Short JL, Exintaris B, Malone DT, Davie B, **Eise N,** and McNamara K (2014) Concerted implementation of active learning in a research intensive faculty: Changes in staff and students attitudes and behaviors. *RACI National Congress.* Adelaide, SA. December 2014

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As I hear a collective sigh of relief from all who harassed, hassled, badgered, and pestered, I can (almost) officially sign off as ……

Dr Nic

Summary

The aims of this thesis were to 1) examine the effects of commercially available stinging nettle (*Urtica dioica*) root and leaf extracts on contractility of the rat prostate gland, 2) isolate and identify any bioactive compounds from the extract, and 3) investigate the effect of the stinging nettle leaf extract on male mouse fertility.

Plant extracts are commonly used for the treatment of lower urinary tract symptoms associated with benign prostatic hyperplasia (BPH). Given the paucity of research into the effects of plant extracts on the contractility of prostatic smooth muscle, using isolated organ bath studies, Chapter 3 investigated the effect of commercially available stinging nettle root and leaf extracts against electrical field stimulation-induced contraction in the isolated rat prostate gland. The leaf, but not root, extract exhibited smooth muscle relaxant activity, particularly at lower frequencies. Further studies using exogenously administered agonists revealed the leaf extract attenuated adenosine 5'-triphosphate (ATP)- and αβ-methylene ATP-induced contraction but displayed no effect on contraction mediated by either noradrenaline or acetylcholine.

As the leaf extract was found to be active, the purpose of Chapter 4 was to isolate, identify and characterise the bioactive or bioactives responsible. Using reversed-phase chromatographic separation we were able to isolate four active fractions from the stinging nettle leaf extract. These fractions not only retained the ability to attenuate contraction of the isolated rat prostate gland, but also attenuated the αβ-methylene ATP-induced contraction of the isolated rat vas deferens. Mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy revealed the predominant compounds within two of the active fractions to be 4-hydroxycinnamic acid, also known as *p*-coumaric acid, and caffeoylmalic acid. Nonetheless the active compound remains to be identified, as both 4-hydroxycinnamic acid and

caffeoylmalic acid were deemed inactive. The bioactive is believed to be present in low concentrations within these active fractions.

Previous work by our lab led to the suggestion that dual pharmacological blockade of the α_{1A}-adrenoceptors and P2X1-purinoceptors may result in a safe and effective non-hormonal target for male contraception. Pharmacological blockade of the α_{1A}-aderenoceptor is currently available as a pharmacotherapy for BPH, however an effective, selective, and therapeutically active P2X1-purinoceptor antagonist is still to be developed. Currently the known P2X1-purinoceptor antagonists lack both receptor subtype selectivity and drug-like properties limiting their therapeutic use. As the stinging nettle leaf extract was shown to be active *ex vivo* against P2X1-purinoceptor mediated contraction in the vas deferens, Chapter 5 examined the effect of the extract in a preliminary male mice fertility study. Although there was a notable decrease in the degree of impregnation of female mice, further investigation is warranted. As the active component of the extract is yet to be identified, its concentration within the extract may be too low to be effective, or there may be pharmacodynamics and pharmacokinetic limitations. If found to be a small drug-like molecule, its potential as a lead candidate for further development as a male contraceptive justifies further investigation.

Concluding remarks in Chapter 6 indicate that this body of work is clinically relevant. Stinging nettle leaf extract may advance into a novel therapeutic agent in the treatment of BPH and / or for the development of a non-hormonal male contraceptive. The limitations and possible future directions of this research are also discussed.

Abbreviations and units of measurement

Abbreviations

^{13}C-NMR: Carbon 13 nuclear magnetic resonance

2D COSY NMR: 2-dimensional correlated spectroscopy nuclear magnetic resonance

5-HT: 5-hydroxytryptamine

ACN: Acetonitrile

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

ANOVA: Analysis of variance

API-MS: Atmospheric pressure ionisation mass spectrometer

app: apparent

ATP: Adenosine-5`-phosphate

AUA: American Urological Association

bFGF: Basic fibroblast growth factor

bp: Base pair

BPH: Benign prostatic hyperplasia

bpm: Beats per minute

br: broad

B-TURP: Bipolar transurethral resection of the prostate

Ca^{2+}: Calcium

cGMP: Cyclic guanosine monophosphate

CGRP: Calcitonin gene-related peptide

CNS: Central nervous system

d: doublet

DAD: Diode array detector

DCM: Dichloromethane

DHT: Dihydrotestosterone

DIDS: 4,4-diisothiocyantostilbene-2,29-disulfonate

DMAU: Dimethandrolone undecanoate

DMPA: Depot medroxyprogesterone acetate

DNA: Deoxyribonucleic acid

EAU: European Association of Urology

EDTA: Ethylenediaminetetraacetic acid

EFS: Electrical field stimulation

ESI-MS: Electrospray ionisation mass spectrometry

^{1}H NMR: Proton nuclear magnetic resonance

HoLEP KTP: Holmium laser enucleation of the prostate using titanyl-phosphate

HoLEP: Holmium laser enucleation of the prostate

HPLC: High-performance liquid chromatography

HSQC: Heteronuclear single quantum coherence

IFIS: Intraoperative floppy iris syndrome

IPSS: International Prostate Symptom Score

KO: Knockout

LC/MSD: Liquid chromatography/mass selective detector

LC-MS: Liquid chromatography mass spectrometry

LLE: Liquid-liquid extraction

LUTS: Lower urinary tract symptoms

m: multiplet

MARP: Monash Animal Research Platform

MDMF: Murine Disease Model Facility

MeOH: Methanol

MIPS: Monash Institute of Pharmaceutical Sciences

NANC: Non-adrenergic non-cholinergic

NF023: 8,8'-[Carbonylbis(imino-3,1-phenylene carbonylimino)]bis(1,3,5-naphthalene-trisulfonic acid

NO: Nitric oxide

NPY: Neuropeptide Y

NTS: Nucleus tractus solitarius

p: pentet

P5P: Pyridoxal-5-phosphate

PCR: Polymerase chain reaction

PDE5I: Phosphodiesterase 5 inhibitor

Plasmakinetic B-TUVP: Plasmakinetic bipolar transurethral vaporisation of the prostate

PPADS: Pyridoxalphosphate-6-azophenyl-2`,4`-disulphonic acid

ppm: Parts per million

q: quartet

RISUG: Reversible inhibition of sperm under guidance

R_{SHBG}: Sex hormone-binding globulin receptor

s: singlet

SEM: Standard error of the mean

SHBG: Sex hormone-binding globulin protein

SPE: Solid phase extraction

t: triplet

TFA: Trifluoroacetic acid

Tris: Trisaminomethane hydrochloride

TUIP: Transurethral incision of the prostate

TUMT: Transurethral microwave thermotherapy

TURP: Transurethral resection of the prostate

UHPLC/MS: Ultra high-performance liquid chromatography mass spectrometer

UV: Ultraviolet

VIP: Vasoactive intestinal peptide

Units of measurement

°C: Degree Celsius
%: Percent
cm: centimetre
g: gram
g/mol: Molar mass
g.s: Integral (grams.second)
Hz: Hertz
kV: Kilovolt
M: Molar
min: minute
mg: Milligram
MHz: Megahertz
ml: Millilitre
mm: Millimetre
mmHg: Millimetres of mercury
mM: Millimolar
ms: Millisecond
nm: Nanometre
nM: Nanomolar
s: Second
µg: Microgram
µl: Microlitre
µm: Micrometre
µM: Micromolar
V: volts
v/v: volume/volume
x g: centrifugal force

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Chapter 1

Introduction

1.1 The male urogenital tract

The male urogenital system is comprised of both the lower urinary and reproductive tracts. The lower urinary tract, which includes the bladder and the urethra, is involved in the process of micturition. This employs two discrete processes: bladder filling and the storage of urine, and the voiding of urine via the urethra. The reproductive tract is involved in the production, transport, and maintenance of sperm, and makes use of the urethra, vasa deferentia, epididymides, testes, and the accessory glands: the seminal vesicles, the prostate, and the bulbourethral (Cowper's) and urethral (Littre) glands (Figure 1.1).

In a healthy adult, the voiding of urine is voluntary. Urine is produced in the kidneys then transported to the bladder, via the ureters, where it is temporarily stored until voiding occurs. Peristaltic contractions of the ureters transport urine from the kidneys to the bladder. The normal adult bladder is an elastic organ able to passively expand to accommodate maximum storage with a limited increase in intra-vesical pressure (Klevmark, 1974, Wein *et al.*, 2011). Accumulation of urine is able to occur as the striated sphincter surrounding the urethra at the base of the bladder is tonically contracted preventing the passing of urine. Urination involves coordination between bladder contraction and relaxation of the sphincter and urethral smooth muscle. The striated sphincter opens, as the bladder smooth muscle contracts, and the urethral smooth muscle relaxes, allowing the flow of urine out of the bladder and into the urethra. In a human male the urethra traverses the prostate gland, which resides at the base of bladder, through the penis, where the urine is ultimately expelled.

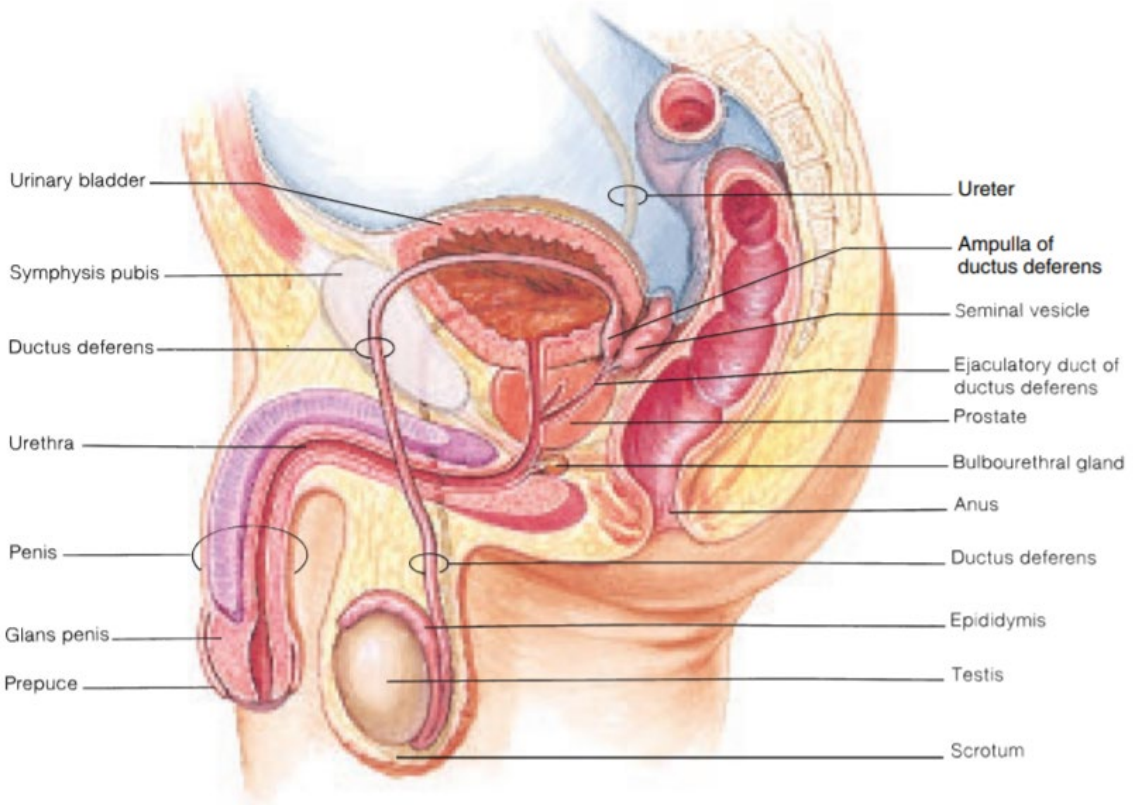


Figure 1.1: The male lower urinary and reproductive systems. Image modified from Human Anatomy 6th ed- Kent Van De Graff.

Within the reproductive tract spermatogenesis begins in the seminiferous tubules of the testes where germ cells produce immature and immotile spermatozoa. Aiding this process are the Sertoli cells, providing nutrients (Walker, 2003, Walker, 2009), and the testosterone-secreting Leydig cells located between the adjacent seminiferous tubules (Shevliuk *et al.*, 1998) (Figure 1.2). Continual production of spermatozoa results in increasing pressure, which in conjunction with cilia, forces them into the efferent ducts and epididymis. As they migrate through the caput and corpus epididymal sections, the spermatozoa undergo maturation. Once they reach the cauda epididymis, they are stored until ejaculation occurs. This process is discussed further in section 1.5.

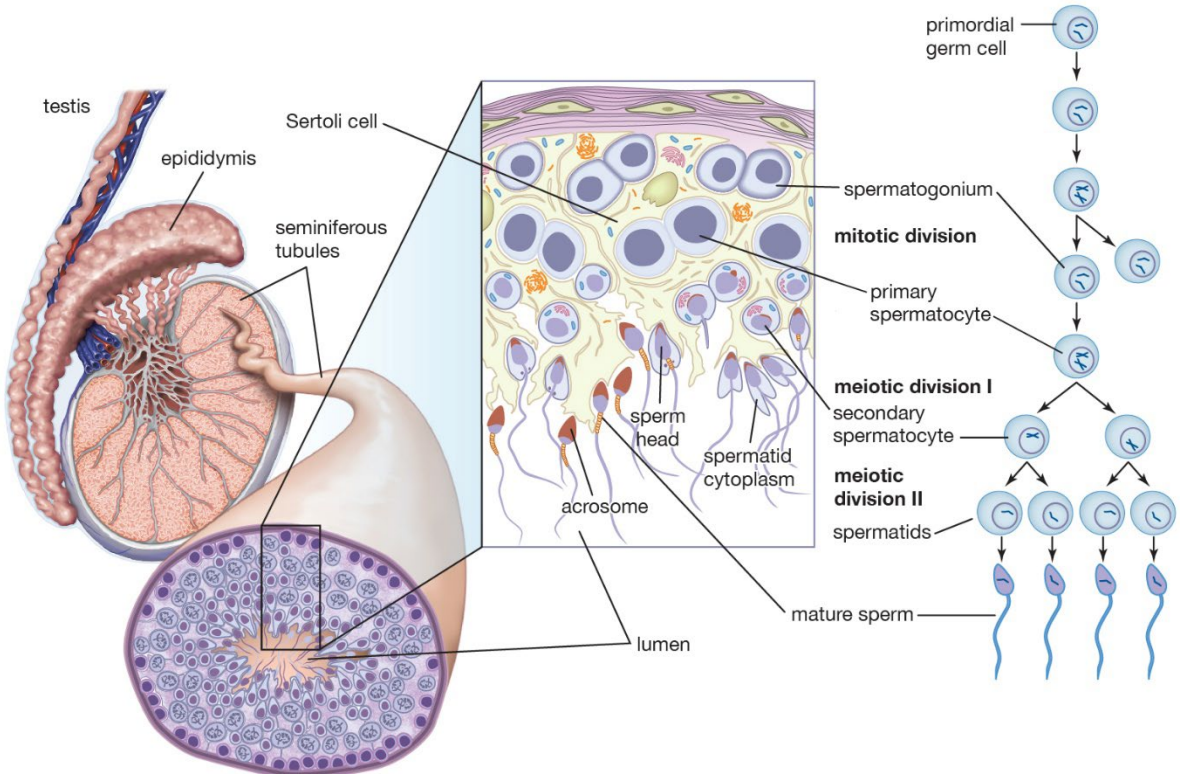


Figure 1.2: The internal structure of the testes and the process of spermatogenesis. Image modified from Encyclopaedia Britannica.

1.2 The prostate gland

A normal human adult prostate is a single organ weighing approximately 18 grams and measuring approximately 3 cm in length, 4 cm in width, and 2 cm in depth (Chung *et al.*, 2012). It has a superior broad base that is contiguous with the base of the bladder (Chung *et al.*, 2012). Anatomically fixed between the symphysis pubis and the rectum (Mahapokai *et al.*, 2000), the prostate is traversed by the prostatic urethra which runs the length of the prostate lying closest to its anterior surface (Berman *et al.*, 2012). The prostate is responsible for the slow accumulation and rapid expulsion of a small volume of fluid, therefore requiring a large storage and low secretory capacity (McNeal, 1988). The prostate completely encapsulates the ejaculatory duct, adding prostatic fluid to semen during ejaculation, as the semen empties into the prostatic urethra at the verumontanum. Prostatic fluid, along with secretions of the other

male accessory reproductive glands, creates an environment suitable for sperm function and survival, as well as facilitating transport, (Aumuller *et al.*, 1982, Wein *et al.*, 2011).

1.2.1 Growth and development

The prostate is a derivative of the primitive endoderm, or gut tube (Berman *et al.*, 2012). Growth of the prostate is regulated by both systemic and locally produced steroid hormones and growth factors. The development of the human prostate depends not only on circulating androgens and an intact androgen-receptor pathway, but also on the conversion of testosterone, produced by the testes, into the more potent biologically active metabolite, dihydrotestosterone (DHT), in peripheral tissue via the enzyme 5α-reductase (Andersson *et al.*, 1991, Mahendroo *et al.*, 1999). The androgen-dependent morphogenesis of the tissue is highly regulated (Cunha *et al.*, 2004). Although testosterone is the primary circulating androgen, DHT has a higher affinity for the nuclear androgen receptor (Fang *et al.*, 1969, Wilson, 2011). Activation of this receptor results in prostatic cell growth and proliferation (Cunha *et al.*, 1987). Epithelial cell differentiation, proliferation, and apoptosis are all involved in the development and maintenance of the prostate. Epithelial growth factor and basic fibroblast growth factor (bFGF) are also involved in the development of the prostate, stimulating the growth of both myofibroblasts and smooth muscle cells. (Zhang *et al.*, 1997).

Under the influence of circulating foetal androgens and DHT stimulation, the urogenital sinus, a subdivision of the cloaca, differentiates, forming solid epithelial outgrowths known as prostatic buds (Cunha *et al.*, 1987, Berman *et al.*, 2012). These prostatic buds invade at stereotyped locations forming distinct zones in the human prostate (Berman *et al.*, 2012). Tubuloalveolar ducts form from basal and luminal epithelium arising from rapidly lengthened, arborised, cannulated and cytodiffentiated prostatic buds (Cunha *et al.*, 1987). The ducts grow and spread throughout the urogenital mesenchyme, differentiating and maturing into the prostatic stroma. At birth, ductal branching is complete (Cunha *et al.*, 1987). At this stage, the

prostate is predominately connective tissue with a relatively small proportion of smooth muscle (Swyer, 1944).

Prostate growth and homeostasis continue to depend on androgens throughout life (Berman *et al.*, 2012). From birth to puberty there is a slow increase in volume until approximately the ninth year when, during puberty, testosterone surges, causing rapid development and characteristic hyperplasia of the epithelium of the ducts, the formation of pseudo-acini, as well as duct proliferation, resulting in a rapid increase in volume. The rate of increase in prostatic size persists until the middle of the third decade of life, when circulating androgen levels stabilise and the corresponding prostatic growth slows and the volume plateaus. At approximately 45 years, growth of the prostate slowly accelerates again commonly resulting in benign prostatic hyperplasia (BPH) (Swyer, 1944, Berry *et al.*, 1984).

Growth of prostate cells has been linked to the human sex hormone-binding globulin protein (SHBG), an allosteric protein discovered in the 1960's (Rosenbaum *et al.*, 1966, Pearlman *et al.*, 1967, Nakhla *et al.*, 1996). The sex hormone-binding globulin receptor (R_{SHBG}), found on human hyperplastic prostatic cell membranes (Hryb *et al.*, 1985) and prostatic cancer cells (Nakhla *et al.*, 1990) binds with SHGB forming the SHGB-R_{SHBG} complex. This complex binds with a high affinity with DHT (Pearlman *et al.*, 1967, Kato *et al.*, 1968, Vermeulen *et al.*, 1968, Forest *et al.*, 1972, Hryb *et al.*, 1990) however, in a separate study, it was the metabolite of DHT, 5α-androstan-3α,17β-diol, which was shown to be an agonist of the SHGB-R_{SHGB} complex (Nakhla *et al.*, 1995) causing an intracellular accumulation of cAMP (Nakhla *et al.*, 1990) and consequently an increase in growth of prostate cells (Nakhla *et al.*, 1996). Plasma concentrations of SHBG have also been shown to increase with age (Nakhla *et al.*, 1994).

In addition to androgens, growth and proliferation of the prostatic stroma has been linked to oestradiol, which is produced locally in the prostate via the conversion of testosterone by

aromatase, and acts at the ERα oestrogen receptor (Prins *et al.*, 2008, Ho *et al.*, 2011). Oestrogen was also noted to be an agonist of the SHGB-R_{SHGB} complex, causing a robust rise in intracellular cAMP (Nakhla *et al.*, 1995).

The prostate itself can double in size in the 50 years following puberty, however different zones of the prostate show distinct differences in the degree of growth.

1.2.2 Zonal anatomy

The human prostate can be broadly characterised into four zones: the peripheral, central, and transition zones, and the anterior fibromuscular stroma (Figure 1.3) (McNeal, 1981). The human prostate is surrounded by a capsule, a thin fibromuscular stroma which encapsulates most of the external surface. It is made up of inner layers of smooth muscle fibres and an outer collagenous membrane (McNeal, 1988). The predominately glandular peripheral, central, and transition zones are differentiated by histology and anatomy suggesting different biological functions. The anterior fibromuscular stroma is entirely non-glandular consisting of connective tissue and smooth muscle (McNeal, 1981, McNeal, 1988).

The peripheral and central zones constitute the majority of the glandular tissue mass at 70% and 25% respectively (McNeal, 1988). The peripheral zone surrounds the central zone, forming the lateral and posterior aspects of the prostate gland. Epithelial cells in the peripheral zone are simple columnar with distinct borders and basally placed small dark-staining nuclei. Within the central zone, there are less distinct membranes, more opaque and granular cytoplasm, larger nuclei which are displaced, and variable cell length (McNeal, 1981).

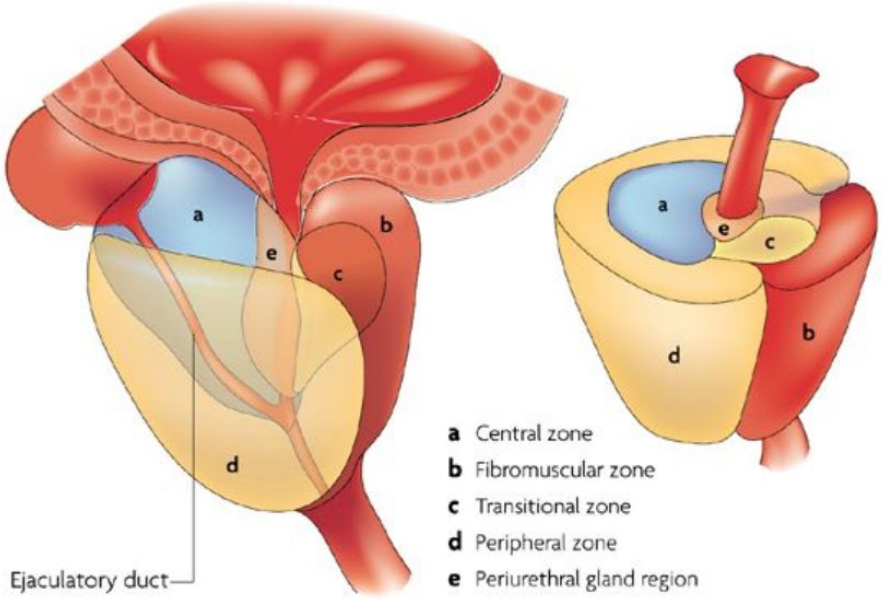


Figure 1.3: The zonal anatomy of the human prostate gland. Image modified from (De Marzo *et al.*, 2007).

Double row ducts track the length of the distal urethra from the verumontanum to the apex of the prostate. These extend and branch laterally before terminating near the capsule (McNeal, 1988). The central zone contains secretory ducts which arise circumferentially from the opening of the ejaculatory ducts and branch directly toward the base of the bladder. This acinar tissue contains large spaces of irregular contour and numerous interluminal ridges, or septa, projecting from the walls, compared with the smooth non-septate walls of the acini of the peripheral zone (McNeal, 1981). Additionally, there is a greater proportion of epithelial cells than stroma cells in comparison to the other zones (McNeal, 1988). Prostate cancer commonly originates in the peripheral zone, with 70% of adenocarcinomas arising from this zone (Wein *et al.*, 2011). Whilst tumours arising from the central zone are rare, accounting for only 2.5% of prostate malignancies, they are highly aggressive (Cohen *et al.*, 2008, Vargas *et al.*, 2012). Following puberty, the central zone may triple in size (Well *et al.*, 2007) whereas the size of peripheral zone remains similar (McNeal, 1968).

The transition zone is found ventral to the urethra and is separated from the other glandular components by a discrete fibromuscular band. It contains the remaining 5-10% of the glandular

tissue mass (McNeal, 1981, McNeal, 1988). Lateral and ventral ducts branch extensively from a single point of the proximal urethra towards the anterior fibromuscular stroma (McNeal, 1978, McNeal, 1988). The preprostatic sphincter, a non-glandular component, is found within the transitional zone. This sphincter surrounds the proximal urethral segment preventing retrograde ejaculation and closes off the urethra to the bladder (McNeal, 1988). Although the smallest, the transition zone is the primary site of origin of BPH (McNeal, 1978, McNeal, 1988, Wein *et al.*, 2011) as growth of this zone continues throughout life (Sampson *et al.*, 2007).

The thick and non-glandular anterior fibromuscular stroma is continuous with the detrusor muscle of the bladder extending from the bladder neck, over the anteromedial surface of the prostate, joining the urethra at the prostate apex (McNeal, 1981). It consists of large, compact bundles of smooth muscle fibres and connective tissue which fuse with the bordering glandular zones and the prostatic capsule (McNeal, 1981, McNeal, 1988).

1.2.3 Innervation and contraction of the prostate

Dual autonomic innervation of the prostate is essential for prostatic contraction, as well as growth, maturation, and function. The prostate is surrounded by a dense nerve plexus, many of which also branch to other areas of the male reproductive tract (Benoit *et al.*, 1994). Both sympathetic and parasympathetic neurones stem from the pelvic plexus and travel through the cavernous nerves to the prostate where they ramify throughout the glandular and stromal elements (Vaalasti *et al.*, 1979, Chung *et al.*, 2012). Preganglionic sympathetic fibres arise from the lumbar cord segments and descend into the pelvis via the hypogastric nerve whereas parasympathetic fibres arise from the brainstem and the sacral regions (McVary *et al.*, 1998).

Contraction and modulation of prostatic smooth muscle tone is largely due to both sympathetic and parasympathetic innervation. In addition, it may also be regulated by numerous other non-adrenergic non-cholinergic mechanisms such as purinergic, nitrergic (Hedlund *et al.*,

1997), and peptidergic, as well as through histamine, thromboxane A_2 (Strittmatter *et al.*, 2011), and cannabinoid CB_1 receptors (Tokanovic *et al.*, 2007) mechanisms.

1.2.3.1 Adrenergic innervation

The prostatic stroma is predominantly innervated by sympathetic neurones (Vaalasti *et al.*, 1980b, Higgins *et al.*, 1989, McVary *et al.*, 1998). Adrenergic innervation is present throughout the capsular smooth muscle and around the ducts of the prostatic glands (Vaalasti *et al.*, 1980a, Vaalasti *et al.*, 1980b). Adrenergic nerve terminals have been reported to lay in close contact with both rat (Vaalasti *et al.*, 1979) and human (Lepor *et al.*, 1994) prostatic smooth muscle cells.

Noradrenaline released from noradrenergic nerves acts at post-junctional G protein-coupled α_1-adrenoceptors. Exogenously applied α-adrenoceptor agonists have been shown to elicit prostatic smooth muscle contraction, which were inhibited or blocked by non-specific α-adrenoceptor antagonists (Raz *et al.*, 1973, Caine *et al.*, 1975) and subsequently by non-specific α_1-adrenoceptor antagonists (Shapiro *et al.*, 1981). Activation of the α_1-adrenoceptors results in smooth muscle contraction due to depolarisation of the membrane potential and subsequent calcium entry through L-type or T-type calcium channels, and intracellular calcium release into the cytosol (Drescher *et al.*, 1994). Additionally, the contractile apparatus may be sensitised to calcium (Chen *et al.*, 1995).

Currently there are three categorised distinct subtypes of α_1-adrenoceptor in the human prostate, the α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor subtypes, all of which are differentially localised. The α_{1A}-adrenoceptor, the most prevalent subtype expressed in prostates of various species, including humans, is primarily localised to the prostatic stroma (Testa *et al.*, 1993, Walden *et al.*, 1997, Michel *et al.*, 2006). α_{1A}-Adrenoceptors have been linked to the active smooth muscle tone of the prostate (Lepor *et al.*, 1995), however more recently the α_{1L}-adrenoceptor, a

functional phenotype of the α_{1A}-adrenoceptor, has been shown to be responsible for mediating adrenergic contractile responses in prostatic smooth muscle contractions of various species including human (Muramatsu *et al.*, 1994, Guh *et al.*, 1995, Ford *et al.*, 1996, Daniels *et al.*, 1999), rat (Hiraoka *et al.*, 1999), and mouse (Gray *et al.*, 2006).

Alongside mediating contraction, adrenergic innervation plays a role in the growth of the prostate. In the rat, prostatic cell proliferation and hyperplasia can be induced by chronic agonist activation of α_1-adrenoceptors (Golomb *et al.*, 1998, Marinese *et al.*, 2003, Kim *et al.*, 2009); additionally a reduction in rat prostatic weight has been shown following sympathectomy of the hypogastric nerve (McVary *et al.*, 1994). The α_{1B}-adrenoceptor and the abundant α_{1D}-adrenoceptor subtypes have little to no involvement in smooth muscle contraction (Forray *et al.*, 1994, Guh *et al.*, 1995, Chapple, 1999, Chess-Williams *et al.*, 2002). The α_{1B}-adrenoceptor is predominantly found in the epithelium (Walden *et al.*, 1999), while the α_{1D}-adrenoceptor, detected in the stroma, and found to be abundant in the blood vessels (Walden *et al.*, 1999), has a predominant role in growth and proliferation (Forray *et al.*, 1994, Guh *et al.*, 1995, Chapple, 1999, Chess-Williams *et al.*, 2002)

1.2.3.2 Cholinergic innervation

Cholinergic innervation of the prostate appears to be less dense when compared to adrenergic nerves (Ventura *et al.*, 2002), however it has been found in various regions of the human prostate including the anterior capsule, peripheral zone, proximal, central, and distal zones (Crowe *et al.*, 1991). The primary transmitter for cholinergic neurones is the endogenous agonist acetylcholine which acts on G protein-coupled muscarinic receptors (for review see (Witte *et al.*, 2008)). Prostatic cholinergic neurones have also been demonstrated to contain co-transmitters such as vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), and nitric oxide (NO) (Higgins *et al.*, 1989, Hedlund *et al.*, 1997, Dixon *et al.*, 2000).

Muscarinic receptors are found predominantly on the glandular epithelium, however, expression of these receptors has also been reported in the prostatic stroma (Hedlund *et al.*, 1985a, James *et al.*, 1989, Lau, Ventura*, et al.*, 1998, Obara *et al.*, 2000, Nadelhaft, 2003, Blanco *et al.*, 2004). Electron microscopy of human prostate sections have also shown nerve terminals to be in close association with the smooth muscle cells in the stroma, and in the glandular epithelium regions (Gosling, 1983, Wang *et al.*, 1991) of the human (Dunzendorfer *et al.*, 1976, Vaalasti *et al.*, 1980a, Higgins *et al.*, 1989, Chapple *et al.*, 1991), guinea pig (Lau, Ventura*, et al.*, 1998), and rat (Nadelhaft, 2003) prostates implying a dual role in both the modulation of smooth muscle tone, as well as prostatic secretion. Adrenergic innervation of the prostate plays a more important role in the contraction of the prostate as cholinergic-mediated contraction has been found to be of lesser magnitude than that of α_1-adrenoceptor stimulation (Ventura *et al.*, 2002). Prostatic secretions are produced upon stimulation of the cholinergic nerves or direct activation of the muscarinic receptors on the glandular epithelium (Smith, 1968, Bruschini *et al.*, 1978, Wang *et al.*, 1991).

Five subtypes of the muscarinic receptor have been identified with high species variability as different muscarinic receptor subtypes are abundant in different species: M_2 in the canine (Fernandez *et al.*, 1998), M_1 in the guinea pig (Lau *et al.*, 2000), and M_3 in the rat (Yazawa *et al.*, 1993, Lau and Pennefather, 1998). The M_2 and M_3 muscarinic receptor subtypes are also present in the guinea pig prostate, (Lau, Ventura*, et al.*, 1998, Lau *et al.*, 2000). Activation of the M_3 muscarinic receptors elicits contraction of the rat prostatic smooth muscle (Lau and Pennefather, 1998) while, in response to nerve stimulation, the M_1 subtype demonstrates a more secretory role (Lau, Ventura*, et al.*, 1998, Lau *et al.*, 2000). In the canine prostate, M_2 muscarinic receptors are found on the peripheral capsule whereas the glandular tissues contain the M_3 subtype (Fernandez *et al.*, 1998). In the rat, there is a lobular distinction between muscarinic receptor subtypes. M_3 muscarinic receptors are found on the ventral lobes of the

prostate, while the M_2 subtype is dominant in the lateral and dorsal lobes (Pontari *et al.*, 1998). The M_4 muscarinic receptor has a greater role in other parts of the male urogenital system as it has been reported in the guinea pig urinary bladder (Alberts, 1995) as well as in the mouse vas deferens where they mediate the potentiation of neurogenic contractions (Matsuno *et al.*, 1992). Additionally, M_4 muscarinic receptor mRNA has been demonstrated to be present in the smooth muscle cells of the human corpus cavernosum (Toselli *et al.*, 1994).

The M_1 muscarinic receptor is the most abundantly expressed subtype in the human prostate and is weakly but significantly associated with prostate size (Witte *et al.*, 2014). Muscarinic receptors are primarily located on prostatic epithelium with the presence of M_1, M_2, and M_5 muscarinic receptor subtypes found on epithelial cell cultures (Yazawa *et al.*, 1994). They are considered to be important in the regulation of prostatic growth (Witte *et al.*, 2008) as evidenced by studies on primary cultures from patients with hyperplastic or malignant prostates. When treated with the non-specific muscarinic agonist carbachol, these cultures exhibited accelerated growth (Rayford *et al.*, 1997).

1.2.3.3 Purinergic Innervation

In 1972 Burnstock proposed the concept of a third, non-adrenergic non-cholinergic component innervating many organs. As the principal active compound was discovered to be a purine nucleotide, it was described as being purinergic (Burnstock, 1972). The co-transmission of adenosine-5`-triphosphate (ATP) with noradrenaline was also proposed (Burnstock, 1972), however, this was not widely accepted for almost two decades.

ATP acts on two families of purinergic receptors, the G protein-coupled P2Y-purinoceptors, and the ligand-gated ion channel P2X-purinoceptors. Currently eight mammalian subtypes of P2Y-purinoceptors have been cloned and characterised, which have been further classified into 2 subgroups (P2Y1,2,4,6- and 11-, and P2Y12,13- and 14-purinoceptors) dependant on their

amino acid sequence and signal transduction pathways (for reviews see (Burnstock, 2007a, von Kugelgen *et al.*, 2016)). They are widely expressed with almost all human cell types expressing P2Y receptors. They exhibit various physiological and pathophysiological roles (Table 1.1) (for reviews see (Ralevic *et al.*, 1998, Burnstock, 2007a, Burnstock, 2011)).

P2X-purinoceptors are fast-response, membrane-bound, cation-selective receptors. Extracellular ATP, from nerve terminals or local tissue (Brake *et al.*, 1994, Valera *et al.*, 1994), acts as a true neurotransmitter in excitable cells such as smooth muscle cells, binding to post-junctional P2X-purinoceptors on the extracellular membrane (Haynes *et al.*, 1997, Lau, Ventura*, et al.*, 1998, Lee *et al.*, 2000b, Ventura *et al.*, 2003, Buljubasich *et al.*, 2004, Buljubasich *et al.*, 2006). The binding of ATP directly activates the ion channels allowing the entry of sodium, potassium, and calcium ions into the cell, mediating membrane depolarisation (Bean, 1992) and initiating contraction.

Seven subunit isoforms of P2X-purinoceptor subtypes have been identified which may form homo- or hetero-oligomers (North, 2002). In the last decade the subunit compositions of functional P2X-purinoceptor channels have been elucidated, along with an understanding of their biophysical characteristics such as ion selectivity, permeability and kinetics of activation and inactivation (Burnstock, 2007a). They have been classified as P2X1 through to P2X7 dependant on their chronological order of discovery (Burnstock, 2007b). They have been characterised on smooth muscle cells as well as autonomic and sensory cells (Table 1.1) (Bean, 1992, Burnstock, 2011).

Table 1.1: P2X-purinoceptors and P2Y-purinoceptors and their main distributions. Adapted from (Burnstock, 2011).

Receptor	Main distribution
P2X-purinoceptors:	
P2X1	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurones
P2X2	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia
P2X3	Sensory neurones, NTS, some sympathetic neurones
P2X4	CNS, testis, colon
P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord
P2X6	CNS, motor neurones in spinal cord
P2X7	Apoptotic cells in immune cells, pancreas, skin, etc.
P2Y-purinoceptors	
P2Y1	Epithelial and endothelial cells, platelets, immune cells, osteoclasts
P2Y2	Immune, epithelial and endothelial cells, kidney tubules, osteoblasts
P2Y4	Endothelial cells
P2Y6	Some epithelial cells, placenta, T-cells, thymus
P2Y11	Spleen, intestine, granulocytes
P2Y12	Platelets, glial cells
P2Y13	Spleen, brain, lymph nodes, bone marrow
P2Y14	Placenta, adipose tissue, stomach, intestine, discrete brain regions

The homomeric P2X1-purinoceptor is a cation-selective channel with a relatively high permeability to calcium, and little selectivity for sodium over potassium (Burnstock, 2007b). They are predominantly expressed in smooth muscle where they regulate smooth muscle contractility, and in platelets where they exhibit various prothrombotic functions, as well as in the cerebellum and dorsal horn spinal neurons (Burnstock, 2011). In smooth muscle cells, the

binding of ATP to the receptor mediates the influx of cytosolic calcium ions and subsequent membrane depolarisation.

Using immunohistochemical techniques P2X1-purinoceptors have been observed in the membranes of the smooth muscle layer of much of the male urinary and sexual reproductive tracts (Lee *et al.*, 2000b). P2X1-purinoceptors are abundant in smooth muscle within the detrusor, in the submucosal and serosal layers of the bladder, of the rat (Lee *et al.*, 2000b, Lee *et al.*, 2000a) and guinea pig (Burnstock *et al.*, 2000), and are associated with the smooth muscle of the rat ureter (Lee *et al.*, 2000a).

They have been shown to be present in the cavernous smooth muscle of the rat trabeculae and corpus cavernosum, as well as the vascular smooth muscle of the penis, suggesting a role in detumescence (Lee *et al.*, 2000b). They have also been postulated to play a role in the transport of sperm as they are found densely populating the outer smooth muscle membrane localised in clusters in the rat vas deferens, with larger clusters apposing nerve varicosities (Burnstock, 2007a). These are primarily the P2X2- and P2X6-purinoceptors (Lee *et al.*, 2000b). Additionally, P2X1-purinoceptors have been observed in the smooth muscle of the epididymal duct and are associated with the vascular smooth muscle in rat seminal vesicles and urethra (Lee *et al.*, 2000b), although, in the seminal vesicles, it is the P2X2-purinoceptors which predominate (Lee *et al.*, 2000b).

In the rat prostate P2X1-purinoceptors predominate the smooth muscle of the connective tissue between the tubules, P2X3-purinoceptors are associated with the nerve fibres in the interstitial connective tissue, and P2X7-purinoceptors are found within the glandular epithelium (Lee *et al.*, 2000b). The P2X2- and P2X6-purinoceptors are found in the smooth muscle of the epididymal ducts (Lee *et al.*, 2000b). P2X7-purinoceptors have also been demonstrated in the fibromuscular stroma of the guinea pig prostate (Buljubasich *et al.*, 2004)

ATP co-transmits with other neurotransmitters in both the parasympathetic and sympathetic nervous systems. Within the bladder, in response to parasympathetic nerve stimulation, ATP, released with acetylcholine (Kasakov *et al.*, 1982), acts on P2X1-purinoceptors (Lee *et al.*, 2000a) in the detrusor muscle, in the submucosal and serosal layers of the bladder, while acetylcholine primarily acts on M_3 receptors (Wang *et al.*, 1995).

Endogenous ATP is released concurrently with noradrenaline from terminal varicosities of electrically stimulated sympathetic neurones (Stjarne *et al.*, 1985, Lew *et al.*, 1987, Kasakov *et al.*, 1988, Vizi *et al.*, 1988) during high frequency stimulation (Buljubasich *et al.*, 2004). ATP is metabolised into adenosine which has a modulatory role. Acting at the prejunctional A_1 adenosine receptor, electrically evoked contractile responses in the isolated rat prostate are attenuated via inhibition of noradrenaline release (Preston *et al.*, 2000). Concentrated and uniform distributions of P2X1-purinoceptors, co-localised with α-actin, alongside α-adrenoceptors, are found in the smooth muscle cells of the fibromuscular stroma of the rat (Ventura *et al.*, 2003) and guinea-pig prostate (Buljubasich *et al.*, 2004).

Co-transmission of noradrenaline and ATP causes biphasic contraction of smooth muscle, which has been observed in the vasculature (Bulloch *et al.*, 1990), vas deferens (Stjarne *et al.*, 1985), and rat prostate gland (Ventura *et al.*, 2003). Observed in the vas deferens, biphasic contraction is comprised of two phases of contraction, a rapid first contraction, with a concurrent slower contractile phase (Major *et al.*, 1989). ATP has been shown to be responsible for the first rapid contraction, and noradrenaline responsible for the slower contractile phase (Sneddon *et al.*, 1984) allowing a synergistic effect on the biphasic contractile response of the prostate gland (Stjarne *et al.*, 1985, Lew *et al.*, 1987, Bulloch *et al.*, 1990).

The functional role is to produce fast, non-adrenergic non-cholinergic excitatory neurotransmission from sympathetic neurones (Bean, 1992). Within the guinea-pig prostate

gland, residual contraction was observed in the presence of adrenergic blockade (Lau, Ventura, *et al.*, 1998), which is attenuated by suramin and αβ-methylene-ATP (Buljubasich *et al.*, 2006). ATP and noradrenaline are released concurrently from varicosities of sympathetic nerves causing contraction of the rat prostate gland (Ventura *et al.*, 2003). While noradrenaline causes an intracellular calcium release into the cytosol of smooth muscle cells (Drescher *et al.*, 1994), ATP results in an influx of calcium from the extracellular environment (Egan *et al.*, 2004). This effect appears to be both age and species dependent as nerve mediated contractile responses in the young mouse prostate are not mediated by ATP (Gray *et al.*, 2005, White *et al.*, 2010). In the mouse, contraction may occur due to the metabolism of ATP to adenosine by ecto-nucleotidases, which can activate pre-junctional A_{2A} adenosine receptors which facilitate noradrenaline release (Gray *et al.*, 2008). However, in the rat prostate, activation of the pre-junctional A_1 adenosine receptors results in inhibition of excitatory neurotransmitter release (Preston *et al.*, 2000).

1.2.3.4 Other non-adrenergic non-cholinergic (NANC) innervation and contributors to prostate contraction

Other neural inputs such as vasoactive intestinal polypeptide (VIP), enkephalins, neuropeptide Y (NPY), nitric oxide (NO), and calcitonin gene-related peptide (CGRP) have been discovered in NANC nerves (Vaalasti *et al.*, 1980a, Burnett *et al.*, 1995, Ventura *et al.*, 2002).

In the human prostate VIP has been identified in nerve fibres located beneath the prostatic epithelium, whereas dense NPY innervation has been shown to be closely associated with the stroma and blood vessels (Higgins *et al.*, 1989, Chapple *et al.*, 1991, Hedlund *et al.*, 1997, Iwata *et al.*, 2001). Additionally, both VIP and NPY have been demonstrated to be present in neuroendocrine cells (Martin *et al.*, 2000).

VIP co-localises predominantly to cholinergic nerves innervating the prostatic glandular epithelium (Vaalasti *et al.*, 1980a, Hedlund *et al.*, 1996, Wanigasekara *et al.*, 2003, Rodriguez

et al., 2005, Keast, 2006), while NPY is co-localised in all adrenergic nerves in the pelvic ganglia and in some cholinergic nerves (Keast, 2006) including those innervating the prostatic acini (Ventura *et al.*, 2002). NPY innervation is also seen in the prostates of guinea pigs, rats (Pennefather *et al.*, 2000), and mice (Wanigasekara *et al.*, 2003, Gray *et al.*, 2005).

The physiological role of the NANC transmitters is unclear; they are proposed to have an important function in the regulation of prostatic contraction. Despite dense NPY innervation, the distribution of NPY receptors in human (Davis *et al.*, 2000) and rat prostates (Kopp *et al.*, 1997, Matsuda *et al.*, 2002) is not clearly defined. It is probable NPY is involved in prostatic growth (Ruscica *et al.*, 2006) rather than in the regulation or mediation of contraction of the prostate (Watts *et al.*, 1991, Davis *et al.*, 2000, Lau *et al.*, 2000).

Located predominantly in the smooth muscle stroma and in the epithelial layers of the human prostate (Hedlund *et al.*, 1997) (Crowe *et al.*, 1991) VIP and NO are involved in smooth muscle relaxation, blood flow and prostatic secretion (Hedlund *et al.*, 1997) (Stjernquist *et al.*, 1983, Said, 1984). High concentrations of exogenous NO inhibits nerve-mediated contractions and relaxes noradrenaline pre-contracted human prostate preparations (Hedlund *et al.*, 1997). VIP does not appear to be involved in contraction but rather in secretion or prostatic growth (Smith *et al.*, 1984, Gkonos *et al.*, 1996, Juarranz *et al.*, 2001).

GCRP is found in nerves within the prostatic stroma and glandular epithelium (Crowe *et al.*, 1991, Jen *et al.*, 1996, Hedlund *et al.*, 1997, Gray *et al.*, 2005, Walden *et al.*, 2005), as well as neuroendocrine cells in the human prostate (Abrahamsson *et al.*, 2000). It has been shown to relax phenylephrine mediated contractions (Watts *et al.*, 1991) or inhibit nerve mediated contractions (Ventura *et al.*, 2000) in the rat prostate suggesting a physiological role in the relaxation of the prostate. CGRP containing nerve fibres also play a role in the regulation of prostatic secretions (Tainio, 1995, Pennefather *et al.*, 2000, Arciszewski, 2004).

Dense innervation of nitric oxide synthase-containing nerves can be found localised throughout the prostatic stroma and glandular epithelium (Burnett *et al.*, 1995). The role of nitrergic neuronal mechanisms is to regulate prostatic smooth muscle tone through the relaxation of prostatic smooth muscle. NO is commonly co-localised with both noradrenergic and cholinergic nerves (Jen *et al.*, 1996, Bloch *et al.*, 1997, Hedlund *et al.*, 1997).

1.3 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a prolific disease, which is increasing due to an aging male population (United Nations. Dept. of Economic and Social Affairs. Population Division., 2015). The disease will affect the majority of men within their lifetime, making it the predominant neoplastic disease. The prevalence of BPH is low until the fourth decade of life, after which there is a rapid and continuous increase (Isaacs *et al.*, 1989). By the age of 60 years approximately 50% of men will be affected by BPH. This increases to 88% of males showing signs after the age of 80 (Berry *et al.*, 1984, Isaacs *et al.*, 1989, Napalkov *et al.*, 1995). BPH is the result of both an increase in the proliferation of the stroma resulting in an increase in the size and volume of the prostate, the static component, and an increase in the contractility attributable to an increase in the smooth muscle tone, known as the dynamic component. These two clinically distinct, but equally important elements, together with the location of the prostate, makes males vulnerable to the lower urinary tract symptoms (LUTS) observed in the clinical form of the disease. LUTS can be characterised as related to urine storage (irritative) and urine voiding (obstructive) symptoms. These are interrelated yet distinct phases of lower urinary function. Although the condition is not considered to be life-threatening, associated symptoms can severely affect the quality of life of both sufferers as well as their families (Barry, 2001).

In the normal prostate stromal tissue constitutes 55% of the inner, and 45% of the outer, component with glandular tissue contributing the other 45% and 55% respectively (Bartsch *et al.*, 1980). In BPH there is a statistical increase in the proportion of stromal tissue and a concurrent decrease in the glandular tissue (Bartsch *et al.*, 1980).

The exact cause of BPH remains elusive. Both age and androgens are required for the development of BPH. Age represents the central mechanism as age-related tissue modifications increase the severity of symptoms (Isaacs *et al.*, 1989, Napalkov *et al.*, 1995, Briganti *et al.*, 2009). Although androgens do not directly cause BPH, their presence is required as androgen-dependent growth is essential for the development and progression of the disease (Briganti *et al.*, 2009). A change in androgen levels with age is thought to be primarily responsible for the growth of the prostate. Men who are castrated prior to puberty have mutated, or a lack of, 5α-reductase isoenzymes producing malformed prostates, and BPH does not develop (Imperato-McGinley *et al.*, 1974, Steers, 2001, Carson *et al.*, 2003, Ho *et al.*, 2011).

Although the presence of DHT is required in the development of BPH, by itself is not sufficient to cause LUTS as it has been shown there is not a significant difference in DHT between hyperplastic prostates and normal prostatic tissue (Walsh *et al.*, 1983, Isaacs *et al.*, 1989). Appropriate concentrations of testosterone and oestradiol are important for the normal regulated growth of the prostate (Suzuki, Takezawa*, et al.*, 1994). Baseline levels and rates of change in hormone levels are associated with rates of change in LUTS, maximum urinary flow, and prostate volume, over time. High baseline oestradiol levels and rapid declines in oestradiol over time are associated with rapid increases in LUTS and rapid decreases in maximum flow rate (St Sauver *et al.*, 2011), while lower baseline and rapid declines in bioavailable testosterone levels were associated with more rapid increases in prostate volume (St Sauver *et al.*, 2011). High testosterone levels, oestradiol levels and the ratio of DHT to testosterone are associated with a reduced BPH risk, which may reflect a decreased activity of 5α-reductase

(Kristal *et al.*, 2008). In the human prostate, levels of circulating testosterone decrease in the aging prostate (Carson *et al.*, 2003), whereas levels of DHT, which is produced locally in the prostate by 5α-reductase remain relatively consistent with age (van der Sluis *et al.*, 2012).

In a healthy prostate androgens mediate cell proliferation and growth as well as prevent apoptosis (Marcelli *et al.*, 1999, Carson *et al.*, 2003). Hyperplasia occurs either as a focal proliferation of smooth muscle cells and basal epithelial cells (Sampson *et al.*, 2007, Well *et al.*, 2007), or as a decrease in apoptosis, which leads to benign enlargement of the prostate. There is a shift in androgen metabolism favouring the accumulation of DHT, shown to induce prostatic growth (Baulieu *et al.*, 1968, Siiteri *et al.*, 1970).

Stromal nodules, with altered cellular morphological composition, initially appear in the transition and periurethral zones of the human prostate (McNeal, 1978, Untergasser *et al.*, 2005). Increases in both the glandular and stromal elements in the transition and periurethral zones (Steers *et al.*, 1995) increase the ratio of stroma to epithelium from 2:1 in a normal prostate to 5:1 in BPH (Bartsch *et al.*, 1979).

The hyperplastic growth compresses the prostate gland against the serous capsule, forming a muscular pseudocapsule, causing the prostate to press inwards and onto the urethra (Mahapokai *et al.*, 2000). This obstruction impairs the flow of urine resulting in obstructive symptoms such as hesitancy, slow or poor stream, post-micturition dribble, and incomplete emptying (Barry *et al.*, 1997). The presence of the prostatic capsule is an important factor in the development of LUTS in BPH. Aged dogs, which lack a prostatic capsule, develop histological BPH however LUTS and urethral obstruction do not develop (Wein *et al.*, 2011).

Oestrogen has also been implicated in the development of BPH (Prins *et al.*, 2008, Ho *et al.*, 2011). As previously discussed, oestrogen induces proliferation in the prostate via ERα receptors located on the prostatic stroma (Prins *et al.*, 2008). While testosterone levels decline

with age, oestrogen levels remain constant throughout life, resulting in an increase in the oestrogen/testosterone level ratio and an oestrogen dominant environment (Roberts *et al.*, 2004, Prins *et al.*, 2008). Additionally, there is an increase in ERα receptors in hyperplasic prostates (Royuela *et al.*, 2001). Studies have shown oestrogen acts synergistically with androgens to promote prostatic hyperplasia (Walsh *et al.*, 1976).

Other factors, including stromal-epithelial interactions (Marker *et al.*, 2003, Cunha *et al.*, 2004), embryonic awakening (McNeal, 1978), prostatic stem cells (Isaacs, 2008), changes in growth factors under hormonal control, and inflammation (Kramer *et al.*, 2007), are thought to play a role in, or are required for, the development of BPH.

The relationship between symptoms, prostate size, and urinary flow is not pronounced (Andersen *et al.*, 1979, Isaacs *et al.*, 1989, Girman *et al.*, 1995); heterogeneity is a recognised factor in BPH. There is an age-related increase in the risk of the development of moderate to severe symptoms, however the correlation of symptoms with an increase in the size of the prostate is modest at best (Girman *et al.*, 1995). Up to half of adult males with an enlarged prostate display severe dysuria, however a third of males presenting with an increase in prostate volume do not display clinical symptoms (Isaacs *et al.*, 1989). Additionally, up to 43% of men without BPH will develop LUTS (Isaacs *et al.*, 1989).

An α_{1A}-adrenoceptor mediated increased tone of the hyperplastic prostatic smooth muscle plays a more significant role in the prostatic obstruction of the urethra (Caine *et al.*, 1976, Roehrborn *et al.*, 2004). This increase in the muscular tone is caused by an increase in the proportion of smooth muscle compared to the glandular component in the stroma (Bartsch *et al.*, 1979, Shapiro *et al.*, 1992), and subsequent alterations in the neural control (Lepor *et al.*, 1991). It has been shown there may be both an increase in the adrenoceptor mediated contractile response of the prostate in BPH and an increase in the α_{1A}-adrenoceptor density (Hedlund *et*

al., 1985a, Lepor *et al.*, 1991, Kondo *et al.*, 1993, Yamada *et al.*, 1994). This suggests an up regulation of adrenergic contraction may play a role in the development of irritative symptoms such as urgency, frequency and nocturia (Barry *et al.*, 1997). A concurrent decrease in nitrergic innervation in the prostate causing a decrease in nitric oxide mediated relaxation (Bloch *et al.*, 1997, Aikawa *et al.*, 2001) may also contribute to the increase in the tone of the prostatic smooth muscle. Oestradiol has also been shown to increase the expression of smooth muscle cell markers in human prostatic cell culture and to slow down the transformation of smooth muscle cells to myofibroblasts (Zhang *et al.*, 1997).

1.3.1 Treatment

A rise in the incidence of BPH, and therefore LUTS, results in significant costs associated with treatment (Kirby *et al.*, 2010). In 2000, in the United States alone, BPH was responsible for $1.1 billion in direct health costs per year (Wei *et al.*, 2005). Although it is more prevalent in older males, BPH can similarly affect working age males, with over two million 45 to 64 year old men in the workforce receiving treatment for LUTS leading to additional indirect costs with a loss of 2 million working days, costing employers up to $500 million dollars per year (Saigal *et al.*, 2005). In the older generation, almost 25% of men with BPH in their 80's receive treatment (Jacobsen *et al.*, 1999). A greater incidence of moderate to severe LUTS requiring treatment is seen in Europe compared to Australia, with up 20% of European men aged 50 to 59 years increasing to 40% of men older than 70 years requiring treatment compared with 19% of men 45 years and older in Australia (Berges *et al.*, 2001, Latz *et al.*, 2013). Conventional treatment relies on pharmacological intervention which reduces the size of the prostate, decreases the contractility, or a combination of both.

In those patients who have a small prostate volume, and mild symptoms, watchful waiting or active surveillance is the preferred strategy (McVary *et al.*, 2011). In these patients it may be useful to adopt lifestyle interventions, such as fluid intake alterations if storage symptoms

predominate (McVary *et al.*, 2011). As weight gain and obesity appear to impact LUTS, weight control may be considered in an attempt to prolong the onset of symptoms requiring conventional pharmacotherapy (McVary *et al.*, 2011). BPH is a progressive disease with a moderate risk of mild symptoms progressing clinically and requiring pharmacological intervention (Djavan *et al.*, 2004). Medication is prescribed for over a third of Australian men presenting to their general practitioners with symptoms of BPH (Charles *et al.*, 2011).

Reduction of the size and volume of the prostate can be achieved using the 5α-reductase inhibitors such as dutasteride and finasteride. Two isoenzymes of 5α-reductase have been identified, type 1 and type 2 (Russell *et al.*, 1994). Type 2 is predominantly found in the prostate. Finasteride acts preferentially on type 2 5α-reductase, whereas the newer dutasteride inhibits both enzymes. By preventing the conversion of testosterone to DHT, prostatic DHT levels decrease and activation of the androgen receptor is reduced. Proliferation is inhibited, and apoptosis is induced, thereby reducing the size of the prostate (Carson *et al.*, 2003, Gravas *et al.*, 2010). Regardless of the baseline size of the prostate, long term use of 5α-reductase inhibitors consistently reduces the total prostate volume in men by approximately 25% (Kaplan *et al.*, 2008) and can reduce the risk of disease progression (McConnell *et al.*, 1998, McConnell *et al.*, 2003, Roehrborn, 2008). They have a delayed onset of action, taking up to 6 months to reach full effectiveness and require continuous administration to maintain their effect on the size of the prostate (Nickel *et al.*, 1996, Naslund *et al.*, 2007, Gravas *et al.*, 2010, McVary *et al.*, 2011). Although prostate volume is reduced with finasteride, a clinically relevant improvement of micturition only occurs in a third of treated BPH patients, and then only after 6 months of treatment at the earliest (Bach *et al.*, 1997).

α-Adrenoceptor antagonists were found to be superior at increasing peak urinary flow when compared to the 5α-reductase inhibitors (Gravas *et al.*, 2010). Early studies with the non-selective, irreversible phenoxybenzamine, found it to be useful in the symptomatic

treatment of LUTS associated with BPH, showing a highly significant improvement in both the peak flow rate and the mean flow rate, however there was no decrease in the amount of residual urine (Caine *et al.*, 1978). The main pharmacological action of α-adrenoceptor antagonists is to facilitate a decrease in smooth muscle tone in the prostate and urethra. Onset is rapid in contrast to the endocrinological action of the 5α-reductase inhibitors. They are recommended for mild to moderate cases of LUTS associated with BPH regardless of the size of the prostate and considered to be the best tolerated of the treatments currently available (Oesterling, 1995, Lepor, 2007, Roehrborn, 2009). α-Adrenoceptor antagonists are used to treat the majority of patients with LUTS associated with BPH with tamsulosin being the most commonly prescribed overall, but great variance is seen across countries (Hutchison *et al.*, 2007). Of the prescribed medication for BPH in Australian men, almost 80% constitute the α_1-adrenoceptor antagonists with tamsulosin and prazosin comprising 13.6% and 13.3%, respectively (Charles *et al.*, 2011). Comparable efficacy in the improvement of symptoms and maximum urinary flow rate is seen across the α-adrenoceptor antagonists, tamsulosin, alfuzosin, doxazosin, and terazosin (Milani *et al.*, 2005), however the most effective single drug over the other α-adrenoceptor antagonists was found to be tamsulosin (Hutchison *et al.*, 2007). They do not, however, halt the progression of the disease nor do they affect the prostate size (Souverein *et al.*, 2003, Boyle *et al.*, 2004).

The combination therapy of 5α-reductase inhibitors and α-adrenoceptor antagonists is superior over monotherapy, with significant improvements in the clinical progression or worsening of symptoms (McConnell *et al.*, 2003, Greco *et al.*, 2008, Roehrborn, 2009). Greater improvements in the maximal urinary flow rates are seen with combination therapy compared with either drug alone (McConnell *et al.*, 2003). Combination therapy is useful in BPH with moderate to severe symptoms with enlarged prostates (McVary *et al.*, 2011). The most common and effective combination of finasteride and tamsulosin resulted in a significant

increase in efficacy and improvement in the majority of patients (Hutchison *et al.*, 2007), however adverse events are more frequently reported (McConnell *et al.*, 2003, Roehrborn, 2009). Although the correlation between changes in prostate volume, and maximum flow rates and urinary symptoms are modest, the relationship becomes stronger in those men experiencing rapid annual increases in prostate volumes, and rapid increases in urinary symptoms (Kaplan *et al.*, 2008). The overall risk of acute urinary retention and the use of invasive therapy is reduced with 5α-reductase inhibitor monotherapy, or in combination with an α-adrenoceptor antagonist, whereas monotherapy with an α-adrenoceptor antagonist such as doxazosin, only delays the time to these events without a reduction in risk (McConnell *et al.*, 2003).

The commonly seen adverse events of therapy causes approximately a quarter of patients taking monotherapy, and almost a fifth of those taking combination therapy to discontinue treatment (McConnell *et al.*, 2003). The 5α-reductase inhibitors are well tolerated, with side effects primarily of a sexual or reproductive nature including erectile dysfunction, reduced libido (Carbone *et al.*, 2003), and abnormal ejaculation, as well as a reduction in semen volume and sperm motility (Amory *et al.*, 2007). Side effects of α-adrenoceptor antagonism are typical, including postural hypotension, fatigue and dizziness, as well as ejaculatory dysfunction (Carbone *et al.*, 2003, Lepor, 2007). The specificity of the different α-adrenoceptor antagonists may result in the differing propensity of side effects. Abnormal ejaculation and retrograde ejaculation, but not blood pressure effects, are associated with tamsulosin and silodosin, (Lepor, 1998, Marks *et al.*, 2009). More recently, a strong association between the development of intraoperative floppy iris syndrome (IFIS) was reported during cataract surgery and the use of tamsulosin (Chang *et al.*, 2005). This increased risk appears to be associated with all α_1-adrenoceptor antagonists, however severe IFIS is statistically more likely with tamsulosin (Chatziralli *et al.*, 2011, Chang *et al.*, 2014).

Muscarinic receptor antagonists, and more recently the β_3-adrenoceptor agonists such as mirabegron, improve the symptoms of overactive bladder in men including frequency and urgency (Roehrborn *et al.*, 2008, Yokoyama *et al.*, 2009, Chapple, Amarenco*, et al.*, 2013). They are recommended in moderate to severe LUTS where the main symptoms are associated with bladder storage (Gratzke *et al.*, 2015). The β_3-adrenoceptor agonists are better tolerated than the muscarinic antagonists (Chapple, Kaplan*, et al.*, 2013).

Tadalafil, an orally available phosphodiesterase 5 inhibitor (PDE5I), used for the treatment of erectile dysfunction, has been approved to treat LUTS in men with erectile dysfunction. Although the exact mechanism of action of the PDE5I's remains unclear, they reduce smooth muscle tone in the detrusor, prostate and urethra by regulating the cyclic guanosine monophosphate (cGMP) degradation and increasing intracellular cGMP thus enhancing the NO/cGMP signalling pathway (Giuliano *et al.*, 2013). Younger men with a low body mass index and more severe LUTS benefit most from the treatment (Gacci *et al.*, 2012), however long-term treatment, efficacy, tolerability, and effect on disease progression have not been established (Gratzke *et al.*, 2015).

For those who have not responded to pharmacological intervention, for those with severe LUTS, or in patients with large prostates, there are several surgical options available including transurethral needle ablation, laser therapy, transurethral microwave thermotherapy (TUMT), transurethral resection of the prostate (TURP), transurethral incision of the prostate (TUIP) and open prostatectomy by a suprapubic, retropubic or perineal approach. The lifetime probability of surgical treatment for BPH is 29%, that is, a third of men showing symptoms will require surgery (Napalkov *et al.*, 1995). TURP remains the benchmark therapy and is considered the reference standard of surgical treatment. It is the most common accounting for almost 95% of all prostatectomies in the US, with a success rate of 85%, and, up until 2008, 96% of all surgical treatment in Australia (Concato *et al.*, 1992, Patel *et al.*, 2019). Although very effective at

improving urinary flow and relieving voiding symptoms associated with BPH, post-operative complications include urinary incontinence, impotence, retrograde ejaculation, urethral stricture, chronic urinary infection, and haemorrhage (Arai *et al.*, 2000, Deliveliotis *et al.*, 2004, Frieben *et al.*, 2010). Almost 15% of patients will require a secondary procedure, including a subsequent TURP procedure after 8 years of follow up (Madersbacher *et al.*, 2005). TUIP is effective and safe for those patients with a smaller prostate, with a favourable morbidity profile, however there is little evidence on the long-term effectiveness (Madersbacher *et al.*, 2004, Reich *et al.*, 2006, Gratzke *et al.*, 2015). Open prostatectomy is the only validated, effective treatment, in men with large prostates where conservative treatment has failed, however it is the most invasive (Gratzke *et al.*, 2015). Other newer alternatives include modifications to TURP, such as bipolar TURP (B-TURP), and plasmakinetic bipolar transurethral vaporisation of the prostate (plasmakinetic B-TUVP), as well as laser therapies such as holmium laser enucleation of the prostate (HoLEP) and holmium laser resection of the prostate using potassium titanyl-phosphate (HoLRP KTP). However, there is limited long-term follow up data of these methods (Gratzke *et al.*, 2015). In recent years, the introduction of these newer procedures, predominantly photoselective vaporisation, have reduced the use of TURP in Australia, yet it remains the common procedure in the older age groups (Patel *et al.*, 2019).

1.3.2 Phytotherapy

Phytotherapy has been used for centuries as an alternative to conventional therapies for the treatment and symptomatic relief of the management and symptomatic relief of LUTS associated with BPH. Treatment is dependent on the severity of symptoms. Phytotherapy is commonly used in those with a lower IPSS (International Prostate Symptom Score), in young patients with mild to moderate symptoms, and for those with smaller prostates (Hutchison *et al.*, 2007, Fourcade *et al.*, 2008). Overall, conventional pharmacotherapy remains the primary choice for the treatment of LUTS associated with BPH, with the α-adrenoceptor antagonists

and 5α-reductase inhibitors constituting just over two thirds of treatments used, however phytotherapies are considered to be a viable alternative (Fourcade *et al.*, 2008). Phytotherapy prescribing differs markedly across different countries with over a third of medicated patients in Germany being treated solely with phytotherapeutics compared with less than 4% in Italy (Hutchison *et al.*, 2007). In France, almost half of patients use plant extracts for their symptoms of BPH, slightly more than the α-antagonists (Fourcade *et al.*, 2008). In Germany and Austria phytotherapeutic agents are used as first-line treatments for patients with mild to moderate symptoms of BPH, accounting for greater than ninety percent of all drugs prescribed for the medical management of BPH (Blumenthal *et al.*, 1998). Despite their extensive use, there is a paucity of published high-quality clinical trials.

Increasingly, patients are seeking complementary medicines as an adjunct or replacement for their treatment of symptoms of BPH. Although the European Association of Urology (EAU) and American Urological Association (AUA) guidelines do not recommend the use of complementary medicines or plant extracts for the treatment of the lower urinary tract symptoms associated with BPH (Madersbacher *et al.*, 2004, Gratzke *et al.*, 2015), they are continually prescribed at high rates, particularly in countries such as Hungary, and Belgium, constituting almost half of the prescriptions for BPH (Cornu *et al.*, 2010). Phytotherapeutics have demonstrated some efficacy with three quarters of patients showing symptom reduction and almost half demonstrating significant improvements (Hutchison *et al.*, 2007).

Serenoa ripens, commonly known as saw palmetto, and *Pygeum africanum*, the African cherry tree, are the two most commonly used plant extracts for the symptomatic treatment of BPH (Dedhia *et al.*, 2008, Fourcade *et al.*, 2008). *Urtica dioica*, commonly known as stinging nettle, is available as an extract in at least 16 commercially available products and is particularly popular in Germany (Madersbacher *et al.*, 2007).

1.4 Stinging nettle

Stinging nettle is a dioecious perennial herb found in temperate and tropical climates such as Europe, Asia, North America and northern Africa. The root has been used in traditional medicine for various conditions (Sezik *et al.*, 2001, Pradhan *et al.*, 2008, Tagarelli *et al.*, 2010, Mustafa *et al.*, 2012), and commercially used for the treatment of symptomatic BPH as it is a constituent found in Australian over the counter supplements such as Blackmores Prostate Health Formula and Swisse Ultiboost Prostate (Blackmores Products Prostate Health Formula, n.d., Swisse Ultiboost Prostate, n.d.). Aerial parts of the plant are also used in traditional medicine with the leaf featured in various cultures for a diverse range of ailments, including diabetes, asthma, and hypertension (Ziyyat *et al.*, 1997, Sezik *et al.*, 2001, Ghorbani, 2005, Tahraoui *et al.*, 2007, Pradhan *et al.*, 2008, Cavender *et al.*, 2009, Pieroni *et al.*, 2009, Mustafa *et al.*, 2012) while in Turkey, the young shoots of the plant are commonly consumed for the treatment of nocturia (Sezik *et al.*, 2001).

1.4.1 Chemistry and Pharmacology

Chemically, stinging nettle is a complex mixture of both water and alcohol-soluble compounds including sterols, phenolic acids, flavonoids and their glycosides, coumarins, lectins, polysaccharides, proanthocyanidins, carotenoids, fatty acids, as well as various vitamins and minerals. Concentrations of the constituents differ not only between parts of the plant, but abiotic and biotic factors can cause variations between plants. The main components found in stinging nettle were all found to vary with the harvesting month and degree of maturation, with younger leaves showing the highest content of polyphenols (Nencu *et al.*, 2012). Total phenolic acid and flavonoid concentration was found to be significantly reduced with high nitrogen levels in the soil, while harvest time results in a significant change in the flavonoid composition (Grevsen *et al.*, 2008, Nencu *et al.*, 2012).

Stinging nettle leaves predominantly contain the hydroxycinnamic acid, caffeic acid, as well as other acids such as ferulic acid, and *p*-coumaric acid (Figure 1.4A). Conjugates formed from reactions with quinic, malic, benzoic, tartartic acid, and glucose (Budzianowski, 1991, Kavtaradze *et al.*, 2001, Grevsen *et al.*, 2008, Pinelli *et al.*, 2008, Nencu *et al.*, 2012, Orcic *et al.*, 2014), are common, with the predominant conjugates being chlorogenic acid (3-*O*-caffeoylquinic acid) (Budzianowski, 1991, Yildiz *et al.*, 2008), neochlorogenic acid (5-*O*-caffeoylquinic acid), 5-*O*-feruloylquinic acid, and 2-*O*-caffeoylmalic acid (Budzianowski, 1991, Pinelli *et al.*, 2008, Yildiz *et al.*, 2008). The tartaric conjugate of caffeic acid has also been described (Nencu *et al.*, 2012, Orcic *et al.*, 2014). Stinging nettle leaf extracts have been shown to contain ferulic acid, in the free form (Fiamegos *et al.*, 2004), in the glycosidic and esterified forms (chinic-, tartaric-, or malic-esters), and as derivatives (Kraus *et al.*, 1991, Nencu *et al.*, 2012, Orcic *et al.*, 2014). *p*-Coumaric acid, possibly existing as a derivative, has also been identified in stinging nettle leaves (Fiamegos *et al.*, 2004, Pinelli *et al.*, 2008, Nencu *et al.*, 2012, Orcic *et al.*, 2014).

Hydroxybenzoic acid derivatives (Figure 1.4B), *p*-hydroxybenzoic acid, gentisic acid, and protocatechuic acid have been found in low concentrations, as has quinic acid, and the coumarin derivative scopoletin (Nencu *et al.*, 2012, Orcic *et al.*, 2014).

Believed to be bioactive, the flavonol glycosides (Figure 1.4C), (Akbay *et al.*, 2003, Gulcin *et al.*, 2004), generally occur as sugar conjugates, principally as *O*-glycosides (Chaurasia *et al.*, 1987). Of the flavonoids, quercetin-3-*O*-rutinoside (rutin), quercetin 3-*O*-glucoside (isoquercitrin), kaempferol 3-*O*-rutinoside, and isorhamnetin 3-*O*-rutinoside were all detected in stinging nettle leaves (Kavtaradze *et al.*, 2001, Akbay *et al.*, 2003, Grevsen *et al.*, 2008, Pinelli *et al.*, 2008, Nencu *et al.*, 2012, Orcic *et al.*, 2014) , with the anthocyanin glycosides limited to the stalks (Pinelli *et al.*, 2008).

β-Sitosterol, one of several phytosterols commonly found in the root of the stinging nettle plant (Sajfrtova *et al.*, 2005, Nahata *et al.*, 2012), has also been detected in the leaf (Kavtaradze *et al.*, 2001).

Nutritionally beneficial compounds have also been identified in the leaves of the stinging nettle plant, including the essential fatty acids, both saturated fatty acids such as palmitic and stearic acids, and the more prevalent polyunsaturated fatty acids, such as linoleic and α-linoleic acids (Guil-Guerrero *et al.*, 2003, Farag *et al.*, 2013). Other oxygenated fatty acids such as 9-hydroxy-10,12-octadecadienoic acid, demonstrated to inhibit aromatase, have been identified in the roots (Gansser *et al.*, 1995a), alongside other octadecanedioic, octadecatrienoic and dodecenedioic acids (Farag *et al.*, 2013).

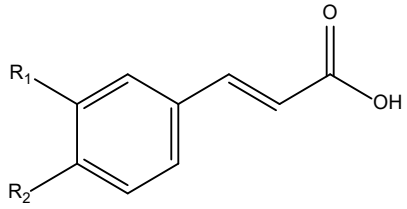
The pigmented carotenoids, present in most fruit and vegetables, provide a source of dietary pro-vitamin A. Predominantly identified carotenoids in the stinging nettle plant include *β*-carotene, lutein and lutein isomers (Guil-Guerrero *et al.*, 2003, Sovova *et al.*, 2004). Lycopene, lycopene isomers, violaxanthin, neoxanthin and trace amounts of *β*-cryptoxanthin and zeaxanthin have also been detected (Guil-Guerrero *et al.*, 2003).

Leaves contain high amounts of bound aromatic norisoprenoid constituents, normally glycosidically bound, with the α-ionol derivatives being the major compounds (Neugebauer *et al.*, 1994, Neugebauer and Schreier, 1995, Neugebauer, Winterhalter*, et al.*, 1995).

Other various compounds described include enzymes such as polyphenol oxidase copper protein responsible for the enzymatic browning reaction by oxidising the cinnamic acids. (Gulcin *et al.*, 2005), glycoproteins such as acid invertases both in the cytosol and ionically bound to the cell wall (Fahrendorf *et al.*, 1990), and a highly branched carbohydrate-protein polymer water soluble cell wall glycoprotein (Andersen *et al.*, 1978). Reported in root extracts (Schottner *et al.*, 1997), dimeric phenylpropane lignans such as neoolivil glycoside have also

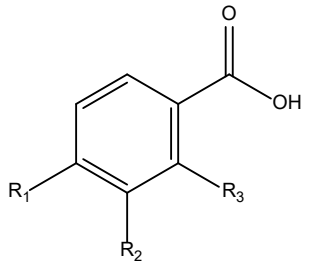
been detected in the leaf (Kavtaradze *et al.*, 2001). Saponins, amino acids, coumarins, and N-containing compounds have all been identified in trace amounts (Kavtaradze *et al.*, 2001). Finally, proteins, polysaccharides, neutral sugars and uronic acids (Lichius *et al.*, 1999) have been isolated from the leaves.

[A]



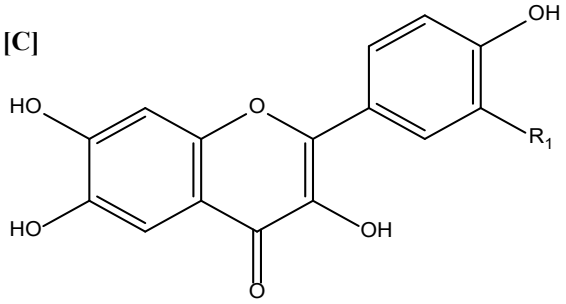
Hydroxycinnamic acids	**R_1**	**R_2**
Caffeic acid	-OH	-OH
Ferulic acid	$-OCH_3$	-OH
p-Coumaric acid	-H	-OH

[B]



Hydroxybenzoates	**R_1**	**R_2**	**R_3**
p-hydroxybenzoic acid	-OH	-H	-H
Gentisic acid	-H	-OH	-OH
Protocatechuic acid	-OH	-OH	-H

[C]



Flavonoids	**R_1**
Quercetin	-OH
Kaempferol	-H
Isorhamnetin	$-OCH_3$

Figure 1.4: Structures of [A] the hydroxycinnamic acids, [B] the hydroxybenzoates, and [C] the flavonoids found in stinging nettle extracts.

Although many of the compounds found in stinging nettle extracts have been claimed to have activity, a definitive pharmacological pathway has not been elucidated. Extracts of stinging nettle appear to have spasmolytic and smooth muscle relaxant activity, as well as the pathways contributing to the growth of the prostate, indicating stinging nettle may alleviate symptoms by inhibiting both the dynamic and static components of BPH.

Aqueous extracts of stinging nettle roots have demonstrated vasorelaxant activity on aortic preparations precontracted with potassium chloride and noradrenaline (Testai *et al.*, 2002). The suggested involvement of NO and elevation of cGMP (Testai *et al.*, 2002) may indicate a PDE5I-like activity. Other studies using aqueous extracts of the leaf have demonstrated reversible acute hypotensive effects (Tahri *et al.*, 2000, Legssyer *et al.*, 2002) postulated to be due to a non-adrenergic, non-cholinergic pathway (Legssyer *et al.*, 2002).

Caffeic acid and its derivatives have demonstrated vasorelaxant properties (Long *et al.*, 2009, Leeya *et al.*, 2010), as well as inhibitory effects on the contractility of the rat intestine (Aviello *et al.*, 2010). Although the mechanism of action has not been determined, Suzuki *et al.* postulated the derivatives chlorogenic acid and 5-caffeoylquinic acid inhibit the excessive production of reactive oxygen species, increasing the bioavailability of NO resulting in vasodilation (Suzuki *et al.*, 2006). Furthermore, flavonoids found in stinging nettle also exhibit vasodilatory action (Duarte *et al.*, 1993). This activity has been attributed to various pathways, including a decrease in the transmembrane uptake of calcium (Duarte *et al.*, 1993).

A comparable improvement in symptoms of BPH has been shown with a combination containing both saw palmetto and stinging nettle root extract compared to finasteride, with more reported adverse effects seen with finasteride (Sokeland, 2000). This effect was unrelated to prostate volume. An aqueous extract of nettle root exhibited a dose related inhibition of the binding of SHBG to receptors on human prostatic membranes (Hryb *et al.*, 1995). This may be due to lignans (Yarnell, 2002). A methanolic extract demonstrated a statistically significant inhibition of hyperplasia in a urogenital sinus implanted into the ventral prostatic gland of adult mice (Lichius *et al.*, 1997). The responsible compound or compounds has not been clearly identified. The same methanolic extract demonstrated an antiproliferative effect on human prostatic epithelial cells, but not on stromal cells (Konrad *et al.*, 2000).

β-Sitosterol, is thought to play a role in the symptomatic treatment of BPH via inhibition of 5α-reductase (Hartmann *et al.*, 1996, Cabeza *et al.*, 2003, Nahata *et al.*, 2012, Aggarwal *et al.*, 2014). In 2012 Nahata and colleagues demonstrated stinging nettle to have an ameliorative effect against testosterone induced BPH in rats (Nahata *et al.*, 2012). More recently an aqueous, ethanolic, and petroleum extract of stinging nettle was shown to be a potent inhibitor of 5α-reductase (Nahata *et al.*, 2014).

1.5 The male reproductive tract

The lower urogenital organs are essential for reproduction. Crucial factors are sperm emission and ejaculation, the process of transporting sperm from the testes to the urethra and then propelling the sperm, suspended in semen, into the female reproductive system. This procedure primarily involves the vas deferens and the urethra.

Sperm is transported from its storage site in the cauda epididymis to the urethra via the vas deferens, a tubular organ which connects the epididymis to the ejaculatory duct. Surrounded by an outer layer of connective tissue, lies a coat of circular smooth muscle between an inner and outer layer of longitudinal muscle (Gosling *et al.*, 1972). Epithelial cells make up a glandular layer which surrounds the lumen (Steers, 1994).

The prostate works in tandem with the seminal vesicles. The prostatic fluid, along with the seminal fluid produced by the seminal vesicles, nurtures, protects, and facilitates sperm transport for reproduction (Aumuller *et al.*, 1982, Wein *et al.*, 2011, Berman *et al.*, 2012). Most of the semen comes from the seminal vesicles, whilst the prostate plays a minor role (Berman *et al.*, 2012).

1.5.1 Process of ejaculation

The normal ejaculation process involves two consecutive phases, emission and expulsion. During emission, sperm are transported through the vas deferens into the pelvic urethra where

they are mixed with seminal and prostatic secretions. Expulsion involves the passage of sperm suspended in the pooled seminal and prostatic fluid through the penile urethra (Hib *et al.*, 1982).

Both sympathetic and parasympathetic nerves innervate the vas deferens. Transport through the vas deferens into the urethra during emission occurs through sympathetically mediated smooth muscle contraction (Kimura *et al.*, 1975, Alm, 1982, McConnell *et al.*, 1982, Kolbeck *et al.*, 1992). The smooth muscle layer receives a rich supply of short adrenergic nerves from the inferior hypogastric ganglion that mediate contraction. The sympathetic adrenergic nerves release noradrenaline and ATP as co-transmitters. Noradrenaline and ATP are co-localised with the neurotransmitter neuropeptide Y in sympathetic nerve terminals innervating the vas deferens (Keast, 2006).

Contraction of the smooth muscle is biphasic. ATP acting on P2X1-purinoceptors results in a fast-acting twitch while noradrenaline acting on α_{1A}-adrenoceptors is responsible for the slower tonic component (Westfall *et al.*, 2001, Burnstock, 2011). These co-ordinated contractions of the vas deferens propel the sperm from the epididymis to the ejaculatory duct (Steers, 1994).

These strong contractile responses are only partially blocked by α_1-adrenergic antagonists (Stjernquist *et al.*, 1983, Kolbeck *et al.*, 1992). A substantial non-adrenergic component of contraction exists in the prostatic portions of the human vas deferens (Hedlund *et al.*, 1985b). As both noradrenaline and ATP are released from the sympathetic neurons, it is believed the purinergic component is responsible for the rapid emission of sperm into the urethra prior to ejaculation with noradrenaline preventing reflux into the vas deferens during ejaculation (Dunn, 2000). In the pre-ejaculatory stage, the pre-prostatic and internal sphincters are progressively contracted, shutting off the prostatic urethra from the bladder, preventing retrograde ejaculation (Blacklock, 1974). Expulsion of sperm through the urethra occurs due

to rhythmic contractions of both the pelviperineal striated muscles, primarily the bulbospongiosus muscle (Clement *et al.*, 2016) and the parasympathetically innervated smooth muscles of the urethra (Watanabe *et al.*, 1979, Kluck, 1980, Giuliano *et al.*, 2005).

1.5.2 Oral male contraception

Since the advent of the condom in the 18th century (Youssef, 1993) and the subsequent development of surgical vasectomy in the late 19th century (Drake *et al.*, 1999), there have been no new male-directed contraceptives. While effective, with typical use, condoms have a high failure rate (Sundaram *et al.*, 2017) while vasectomies are invasive, costly, and not easily reversible (Patel *et al.*, 2016). Over the last 40 years there has been extensive work in both novel hormonal and non-hormonal methods. Hormonal methods are at the forefront, where testicular steroidogenesis is inhibited in an attempt to block spermatogenesis. Non-hormonal targets are an emerging area.

Spermatogenesis is a complex process requiring approximately ten weeks to completion and relies on the presence of high concentrations of intra-testicular testosterone (Matsumoto *et al.*, 1985, Coviello *et al.*, 2004). Male hormonal contraception relies on exogenous male hormones which suppress testosterone production and consequently sperm production.

Hormonal male contraceptives are greater than 97% effective when they limit the sperm concentrations to less than 1 million per ml of ejaculate (World Health Organization Task Force on Methods for the Regulation of Male, 1996). Early efficacy studies using testosterone enanthate at twice the physiologic replacement dose resulted in 70% of men achieving azoospermia and only one pregnancy (World Health Organization Task Force on Methods for the Regulation of Male, 1990) . When severe oligozoospermic levels are achieved, contraceptive efficacy is high, comparable to the female oral contraceptive pill, with only a 1.4% failure rate (World Health Organization Task Force on Methods for the Regulation of

Male, 1996). The weekly injection schedule required to reach this level of contraception has proven to be arduous, and with androgenic side effects, such as changes in mood and libido, acne, and weight gain, has resulted in premature discontinuation (World Health Organization Task Force on Methods for the Regulation of Male, 1996).

Subsequent studies into hormonal contraception involved three monthly testosterone implants (McLachlan *et al.*, 2000), T-pellets with intramuscular depot medroxyprogesterone acetate (DMPA) (Turner *et al.*, 2003), and transdermal testosterone in combination with oral progesterone (Soufir *et al.*, 2011). Lower doses of testosterone were shown to reduce androgenic side effects; however, efficacy was found to be low (McLachlan *et al.*, 2000). When adequate suppression of sperm production was achieved, unacceptable adverse effects were experienced (Turner *et al.*, 2003). Effective contraception in all cases was delayed (McLachlan *et al.*, 2000, Turner *et al.*, 2003, Soufir *et al.*, 2011). A phase 2 efficacy study using a combination of intramuscular testosterone udeconate with progestin norethisterone enanthate resulted in acceptable contraception, however the study was terminated prematurely due to moderate to severe depression and androgenic side effects (Behre *et al.*, 2016).

An oral delivery of testosterone following the development of dimethandrolone undecanoate (DMAU), a derivative of 19-nortestosterone, showed promise with hormonal changes consistent with effective male contraception using a daily oral dosing regimen (Ayoub *et al.*, 2017, Thirumalai *et al.*, 2019), however, androgenic side effects persisted, and adequate sperm suppression was not demonstrated (Thirumalai *et al.*, 2019).

Currently studies are underway to develop, and optimise dose and delivery, of alternative derivatives of 19-nortestosterone (Kumar *et al.*, 1992, von Eckardstein *et al.*, 2003, Walton *et al.*, 2007, Hild *et al.*, 2010, Attardi *et al.*, 2011, Wu *et al.*, 2019), as well as alternate progestins

(Mahabadi *et al.*, 2009, Sitruk-Ware *et al.*, 2010, Ilani *et al.*, 2012) with early results showing promising results.

Contraception that disrupts spermatogenesis involves long pre- and post-contraceptive wait times, that may result in an increase in the risk of failure as well as a decrease in compliance. Non-hormonal methods are a valid and potential alternative.

Vaso-occlusive contraceptive methods, such as reversible inhibition of sperm under guidance (RISUG) using an occlusive polymer or styrene maleic anhydride dissolved in DMSO have been successful in humans resulting in azoospermia with minimal side effects (Guha *et al.*, 1993, Guha *et al.*, 1997), however reversibility in humans is yet to be ascertained. The procedure involves the RISUG to be administered into the lumen of the vas deferens which may potentially deter patients. Additionally, local carcinogenicity, potential teratogenicity in pregnancies, and the restoration of normal vas deferens anatomy is yet to be demonstrated (Manivannan *et al.*, 1999, Manivannan *et al.*, 2005). Although promising results have been seen using styrene-alt-maleic acid in rabbits (Waller *et al.*, 2016, Waller *et al.*, 2017), studies in humans have not been undertaken as full restoration of sperm function has not been demonstrated in animal studies.

Preliminary studies have been undertaken targeting surface proteins on spermatozoa (O'Rand M *et al.*, 2004, O'Rand *et al.*, 2009, Mitra *et al.*, 2010) and on altering the expression of proteins in Sertoli cells in the cynomolgus monkey (Hild *et al.*, 2007) and stallions (Pozor *et al.*, 2013). These have resulted in infertility, a loss of forward or progressive sperm motility (O'Rand M *et al.*, 2004, O'Rand *et al.*, 2009, Mitra *et al.*, 2010), and, additionally, a reversible decrease in the sperm concentration (Hild *et al.*, 2007, Pozor *et al.*, 2013). A reversible reduction in the sperm motility with minimal non-gonadal side effects was achieved in both *in vitro* studies (Silva *et al.*, 2013, O'Rand *et al.*, 2016) and in non-human primates (O'Rand *et al.*, 2018),

however, further studies are required to investigate the long term effects, and to optimise delivery options.

Non-hormonal reversible suppression of spermatogenesis has been achieved in rodents using derivatives of lonidamide (Grima *et al.*, 2001, Cheng *et al.*, 2005, Tash *et al.*, 2008). Problematic adverse effects such as liver inflammation and skeletal muscle dystrophy, and fatalities at higher doses have occurred (Mruk *et al.*, 2006, Tash *et al.*, 2008).

Retinoic acid receptor antagonists, such as WIN 18,446, a compound known to inhibit retinoic acid biosynthesis required for the initiation and maintenance of spermatogenesis (Koubova *et al.*, 2006, Vernet *et al.*, 2006), have demonstrated reversible, severe oligozoospermia (Heller *et al.*, 1961, Roth *et al.*, 2016). These antagonists are showing promise as studies in rodents are proving them to be efficacious, reversible with normal, healthy, fertile progeny (Chung *et al.*, 2011, Chung *et al.*, 2016). Unfortunately, disulfiram reactions were commonly observed (Heller *et al.*, 1961, Roth *et al.*, 2016). Knockout technology disrupting male specific genes has shown male-specific infertility through the loss of the function of the testis or epididymal specific proteins (Dix *et al.*, 1996) (Kastner *et al.*, 1996, Sonnenberg-Riethmacher *et al.*, 1996). These all result in the malformation or reduction in production of spermatozoa, raising the issue of reversibility.

An alternative approach is to inhibit the transport of sperm through the vas deferens which relies on α_{1A}-adrenoceptor and P2X1-purinoceptor mediated contraction. Early studies using the nonselective α_1-adrenoceptor antagonist prazosin, demonstrated a decrease in fertility, initially in rats (Paz *et al.*, 1984), and later in adult human males, with minimal side effects and considerable acceptability (Homonnai *et al.*, 1984). Contradictory to these early promising results, a subsequent study was unable to confirm the acceptability or efficacy of prazosin as a male contraceptive (Kjaergaard *et al.*, 1988). Later studies using specific α_{1A}-adrenoceptor

antagonists such as tamsulosin showed potent antifertility effects in male rats (Ratnasooriya *et al.*, 1994). Although the antifertility effect was rapid in onset, rapidly reversible, and with virtually no effect on libido or mating performance, there was significant impairment in ejaculatory competence (Ratnasooriya *et al.*, 1994). As the movement of spermatozoa also relies on P2X1-purinoceptor mediated contraction, the development of a pharmacological P2X1-purinoceptor antagonist is imperative for the development of a non-hormonal male contraceptive. Mulryan *et al* demonstrated the vital role the P2X1-purinoceptor plays in fertility showing a reduced vas deferens contraction and infertility in male mice lacking the P2X1-purinoceptor (Mulryan *et al.*, 2000). The paradigm of male contraception via antagonism of both the α-adrenoceptor and P2X1-purinoceptor was demonstrated by White *et al* through double knockout mice demonstrating inhibition of male fertility without an effect on spermatozoa (White *et al.*, 2013). Using reversible pharmacological compounds would grant this avenue for male contraception as a viable option.

1.5.2.1 α_{1A}-Adrenoceptor antagonists

As previously discussed, the α_1-adrenoceptor antagonists are currently used therapeutically for the treatment of BPH. Several studies have demonstrated the use of the α_1-adrenoceptor antagonists in the treatment of BPH to be associated with adverse effects of ejaculation dysfunction (Caine *et al.*, 1976, Abrams *et al.*, 1995, Roehrborn, 2006, Lepor, 2007) characterised by low ejaculate volume (Hisasue *et al.*, 2006). These adverse effects may be exploited in the development of a male oral contraceptive.

Abnormal ejaculation associated with the α_{1A}-adrenoceptor antagonist tamsulosin was suggested to be due to its action on α_{1A}-adrenoceptors present in the seminal vesicles resulting in a decrease in emissions (Hisasue *et al.*, 2006). In a small study, markedly decreased sperm concentration was observed with the administration of tamsulosin with azoospermia occurring in 83% of participants (Hisasue *et al.*, 2006) potentially due to the predominance of

α_{1A}-adrenoceptors in the vas deferens (Moriyama *et al.*, 1997). Tamsulosin has also demonstrated similar affinities for D_2-like dopamine and 5-HT_{1A} receptors as for the α_{1A}-adrenoceptors (Andersson *et al.*, 2003). As these receptors located in the CNS have been shown to inhibit ejaculation (Ahlenius *et al.*, 1980, Fernandez-Guasti *et al.*, 1992, Matuszewich *et al.*, 1999, Clement *et al.*, 2006), these off target effects may also contribute to the anejaculation observed with tamsulosin. The highly selective α_{1A}-adrenoceptor antagonist silodosin, shown to have high tissue selectivity for the vas deferens compared with other α_{1A}-adrenoceptor antagonists (Noguchi *et al.*, 2008), resulted in dry ejaculation and a loss of seminal emission (Shimizu *et al.*, 2010). Even though orgasm is retained with the α_{1A}-adrenoceptor antagonists, the loss of seminal emission can be the source of great dissatisfaction in men (Kobayashi *et al.*, 2009). In contrast, a reduction in ejaculate may help to curb the transmission of sexually transmitted diseases as many viruses responsible for sexually transmitted diseases, such as hepatitis B 45-48, HIV (Ho *et al.*, 1984, Zagury *et al.*, 1984), *Neisseria.gonorrhoeae* (Jennings *et al.*, 1977, Garcia *et al.*, 1981, Toth, 1987) and *Chlamydia trachomatis* (Bruce *et al.*, 1981, Wolner-Hanssen *et al.*, 1984) have been shown to be present in semen. Although retrograde ejaculation has been reported with tamsulosin (Debruyne, 2000) and silodosin (Marks *et al.*, 2009), this has been disputed with tamsulosin showing abnormal ejaculation is not retrograde, but rather emission failure (Hisasue *et al.*, 2006); this may also apply to silodosin.

The α_1-adrenoceptor antagonists have demonstrated safety and excellent long-term tolerability in the treatment of BPH (Narayan *et al.*, 2003, Narayan *et al.*, 2005). They have been shown to be rapid-acting with effective blockade of ejaculation in three to five days, which is fully reversible upon its withdrawal (Hellstrom *et al.*, 2006, Hisasue *et al.*, 2006). Testicular function and sperm quality are not affected, even after long-term use (Sanbe *et al.*, 2007). Furthermore, they exhibit minimal effect on libido (van Dijk *et al.*, 2006).

Ejaculation dysfunction associated with α_{1A}-adrenoceptor antagonism is dose related (Lepor, 2007), therefore the development of a pharmacological P2X1-selective antagonist would potentially decrease side effects while retaining contraceptive efficacy.

1.5.2.2 P2X1-purinoceptor antagonists

Although not used therapeutically as a P2X-purinoceptor antagonist, the pharmaceutically available suramin, (8-(3-benz-amido-4-methylbenzamido)-napthalene-1,3,5-trisulfonic acid), and its derivatives, as well as pyridoxal-5-phosphate (P5P), the active metabolite of vitamin B6, have all exhibited activity at purinoceptors.

Showing reversible antagonism at P2-purinoceptors in the mouse vas deferens, (Dunn *et al.*, 1988), and characterised as slowly equilibrating and slowly reversible at P2X1-purinoceptors (Leff *et al.*, 1990), suramin does not discriminate between P2X and P2Y purinoceptors demonstrating activity at P2Y-purinoceptors (Charlton *et al.*, 1996b, Charlton *et al.*, 1996a). Suramin is a promiscuous antagonist showing activity at other receptor types such as GABA (Nakazawa *et al.*, 1995, Luo *et al.*, 2018), glutamate (Nakazawa *et al.*, 1995), A_1 adenosine and D_2 dopamine receptors (Beindl *et al.*, 1996).

The suramin analogue, NF023 (8,8'-[Carbonylbis(imino-3,1-phenylene carbonylimino)] bis(1,3,5-naphthalene-trisulfonic acid), is a moderately selective, competitive, and reversible antagonist of P2X-purinoceptors receptors. It demonstrates a greater selectivity for P2X1-purinoceptos when compared to other P2X-purinoceptors (Soto *et al.*, 1999) as well as P2Y-purinoceptors (Bultmann, Wittenburg*, et al.*, 1996, Ziyal *et al.*, 1997).

Both suramin and NF023 inhibit ecto-nucleotidase activity, however NF023 has a higher P2X1-purinoceptor selectivity and potency (Beukers *et al.*, 1995, Bultmann, Wittenburg*, et al.*, 1996, Ziyal *et al.*, 1997). Comparable to suramin, NF023 also exhibits direct antagonism of A_1 adenosine and D_2 dopamine receptors (Beindl *et al.*, 1996).

P5P is a P2X-purinoceptor antagonist (Trezise, Bell*, et al.*, 1994), however is not specific for P2X1-purinoceptors as it also antagonises expressed homomeric P2X2- and heteromeric P2X2/3- purinoceptors (Theriault *et al.*, 2014). P5P may be useful as a starting compound for the synthesis of more P2X-purinoceptors-selective antagonists as, compared to suramin, it is selective for purine receptors over non-purine receptors and is competitive at P2X1-purinoceptors in rat vas deferens (Trezise, Bell*, et al.*, 1994).

Similarly, PPADS (Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid), a derivative of P5P, is a selective P2-purinoceptor antagonist at both pre- and post-junctional sites, without activity at α_1-adrenoceptors, M_2 and M_3 muscarinic receptors, H_1 histamine, and A_1 adenosine receptors (Lambrecht *et al.*, 1992). Although it is an antagonist at P2X-purinoceptors (Ziganshin *et al.*, 1993, Ziganshin *et al.*, 1994), it is not selective as it has demonstrated activity at P2Y-purionoceptors in both cloned (Charlton *et al.*, 1996b) and native cells (Brown *et al.*, 1995). Iso-PPADS (pyridoxalphosphate-6-azopenyl-2`,5`-disulfonic acid), an isomer of PPADS, has also been shown to be a competitive, slowly equilibrating, slowly reversible non-selective antagonist for P2X-purinoceptors (Khakh *et al.*, 1994, Trezise, Kennedy*, et al.*, 1994).

The dyes, reactive blue 2, reactive red, trypan blue, and Evans blue, have all been investigated for activity within the purinergic system. Reactive blue 2, an anthraquinone-sulfonic acid derivative, has shown non-competitive antagonism at P2 purinoceptors with low selectivity between P2X-purinoceptors and P2Y-purinoceptors (Bultmann *et al.*, 1994b), and a narrow effective concentration range and time of exposure (Burnstock *et al.*, 1987, Hopwood *et al.*, 1987, Houston *et al.*, 1987). It has not shown selectivity, acting at various receptors in the rat vas deferens (Bultmann *et al.*, 1994b) and urinary bladder (Choo, 1981, McMillian *et al.*, 1993, Wiley *et al.*, 1993, Suzuki and Kokubun, 1994), including P2X2- (Brake *et al.*, 1994) and P2X4-purinoceptors (Seguela *et al.*, 1996).

The chloride transport blocker 4,4-diisothiocyanatostilbene-2,29-disulfonate (DIDS), an anion exchange inhibitor, is a non-competitive pseudo-irreversible antagonist of P2X1-purinoceptors in guinea pig and rat vas deferens, and selective when compared to P2Y1-purinoceptors of guinea pig taenia coli (Fedan *et al.*, 1990, Bultmann *et al.*, 1994a, Bultmann, Pause*, et al.*, 1996). It is also non-selective in its inhibition of ecto-nucleotidase activity (Bultmann, Pause*, et al.*, 1996).

1.6 Considerations of the rat and mouse as experimental models

1.6.1 The prostate

There are obvious differences in the anatomy of the human and both the rat and mouse prostate. The human prostate is a single compact lobular structure consisting of merged internal glandular zones surrounded by a capsule whereas both the rat and mouse prostates consist of four distinct lobes, a ventral, two lateral and a dorsal lobe, with the dorsal and lateral lobes resembling each other more closely than the ventral (Jesik *et al.*, 1982, Roy-Burman *et al.*, 2004).

Both human and rat prostates display heterogeneity of anatomy and function between the lobes (McNeal, 1981, McNeal, 1988, Steidle *et al.*, 1989). Fibromuscular tissue makes up approximately a third of the bulk of the tissue within the capsule of the human prostate (McNeal, 1978). Rat prostatic tissue is tubuloalveolar, consisting of epithelium-lined acini surrounded by a stromal matrix. Each acinus is surrounded by a single cell layer of smooth muscle. In contrast to the human prostate where the stromal and epithelial cells are approximately equal in number (Bartsch *et al.*, 1980), both the adult rat and mouse prostate have a low content of fibromuscular stroma (Mori *et al.*, 2009), with the epithelial to stromal ratio at approximately 5:1 (DeKlerk *et al.*, 1978). In normal rats the prostatic tissue is mostly comprised of ducts and luminal epithelial cells (Sugimura *et al.*, 1986, Mori *et al.*, 2009).

Although anatomical differences exist, the rat prostate remains a suitable *in vitro* model of human prostate contractility as nerve-mediated contractile responses are sympathetic in nature. In both rat and human prostates, the α_{1A}-adrenoceptor subtype predominates in the prostatic smooth muscle (Scofield *et al.*, 1995, Walden *et al.*, 1997) mediating contraction of the prostate both by the endogenous release of noradrenaline through electrical field stimulation, and the addition of adrenergic agonists. In the rat prostate, although all lobes contribute to the contractility of the gland, the maximal contraction to α_1-adrenergic stimulation is in the ventral lobe (Steidle *et al.*, 1989). Additionally, as seen in the human prostate (Longhurst *et al.*, 1996), the expression of P2X1-purinoceptors have been demonstrated with the rodent prostate gland, (Lee *et al.*, 2000b) with purinergic pathways contributing to the contraction of the rat prostate (Ventura *et al.*, 2003).

The major species difference is the encapsulation of the human prostate within a well-formed prostatic fascia whereas the rodent prostate lacks a well-developed capsule. In the human prostate the capsule plays a role in the overall contractility (Caine *et al.*, 1975), however in the rat ventral prostate there is a paucity of smooth muscle, and no significant contribution by the capsule to contractile responsiveness (Steidle *et al.*, 1989). In both human (McNeal, 1988) and rat prostates, the smooth muscle fibres are random in orientation. This, and the lack of contractile response in the capsule of the rat prostate, implies it is not necessary to orient the prostate for contractility experiments (Steidle *et al.*, 1989). It also results in a lower maximal tension for the isolated prostate.

1.6.2 The vas deferens

Like in humans, both rat and mouse vas deferens are innervated by the sympathetic nervous system with both α_{1A}-adrenoceptors (Stjarne *et al.*, 1985, Scofield *et al.*, 1995), and P2X1-purinoceptors (Liang *et al.*, 2000, Mulryan *et al.*, 2000, Liang *et al.*, 2001, Banks *et al.*, 2006) present and responsible for contraction. As seen in human vas deferens, the biphasic

response to electrical stimulation is also observed in the rat and mouse vas deferens (Swedin, 1971). Although it is believed a functional role exists for the both the circular and longitudinal muscle in the vas deferens (Amobi *et al.*, 2012) both human and rat vas deferens show contractile responses predominantly in the longitudinal muscle (Amobi *et al.*, 2012, Donoso *et al.*, 2014) with the noradrenergic component predominant in the epididymal segment and purinergic-mediated contractility more potent in the prostatic segment (Sneddon *et al.*, 1992).

1.6.3 Genetically modified receptor knockout mice

The development and availability of genetically modified receptor knockout mice has proven to be an invaluable tool in fertility studies. Dual genetic deletion of α_{1A}-adrenoceptors and P2X1-purinoceptors enabled the proof of concept that pharmacological antagonism of both the α_{1A}-adrenoceptor and the P2X1-purinoceptor is a potential target for a male contraceptive (White *et al.*, 2013) as well as a potential benefit in the treatment of BPH. As pharmacological antagonists of the α_{1A}-adrenoceptor are commercially and readily available, the investigation into orally bioavailable P2X1-purinoceptor antagonists is required.

1.7 Aims

The aims of this project are to:

1. Examine the effects of commercially available stinging nettle (*Urtica dioica*) root and leaf extracts on contractility of the rat prostate gland. By using known contractile agonists in isolated rat prostates, both stinging nettle root and leaf extracts will be tested for dose dependent relaxation.
2. To isolate and identify any bioactive compounds from the extract. The extract exhibiting relaxation in the precontracted prostates will be chemically separated into fractions in an attempt to elucidate the active compound.

3. To investigate the effect of the stinging nettle leaf extract on male mouse fertility. Wild-type mice will be dosed daily with stinging nettle leaf extract to ascertain if there is a pharmacological effect *in vivo* on fertility when compared to both wild-type control mice and to genetically modified P2X1-purinceptor knockout mice.

Chapter 2

Materials and Methods

2.1 Animals and tissue

2.1.1 Rats

Male wild-type Sprague-Dawley rats were obtained at 7-9 weeks of age from the Monash Animal Research Platform (MARP) (Monash University, Clayton). Prior to experimentation they were housed at the Monash Institute of Pharmaceutical Sciences (MIPS) Animal Facility (Monash University, Parkville).

2.1.2 Wild-type mice

Male wild-type C57Bl/6 mice were obtained at 7-8 weeks of age from the Monash Animal Research Platform (MARP) (Monash University, Clayton) and housed at the Murine Disease Model Facility (MDMF) Holding Room (Monash University, Parkville) prior to experimentation.

2.1.3 Knockout mice

Breeding pairs of P2X1-purinoceptor knockout mice were obtained from Prof R.J. Evans (Department of Cell Physiology & Pharmacology, University of Leicester, UK). Colonies were housed at the Murine Disease Model Facility (MDMF) Holding Room (Monash University, Parkville) and were maintained on a C57Bl/6 background by heterozygous breeding pairs.

2.1.4 Animal housing and ethics

All animals were exposed to a photoperiod of 12 hours light and 12 hours dark and housed under standard conditions at 22 °C; food and water were accessed *ad libitum*. All animals were euthanized by asphyxiation through exposure to CO_2 gas.

Prior approval for animal experimentation was obtained from the Monash University Standing Committee of Animal Ethics in Animal Experimentation; ethics numbers VCPA 2009/15 and MIPS 2013/15 for the use of Sprague-Dawley rats, MIPS 2014-04 and MIPS 2015-06 for the use of genetically modified and wild-type C57Bl/6 mice, respectively.

2.2 Genotyping

Polymerase chain reaction (PCR) was used to routinely determine the genotype of individual mice in the knockout breeding colonies.

2.2.1 DNA extraction

A 2-5 mm tail sample was collected from each mouse after weaning, immediately frozen on dry ice, and stored at -80 °C. Genomic DNA was extracted by the addition of NaOH 50 mM (600 µL) to each sample and incubated at 95 ºC with constant mixing for 10 minutes. Tris HCl 1 M (150 µL) at pH 8 was added to each sample and shaken vigorously before centrifugation at 15,000 *x g* for 5 minutes.

2.2.2 DNA amplification by polymerase chain reaction (PCR)

Supernatant (1 µl) from freshly prepared DNA extract was added to a PCR reaction mixture (24 µl) comprising of GoTaq Green Master Mix (Promega), MgCl (25 µM), gene specific primers and nuclease free water. A thermal cycler (2720 Thermal Cycler, Applied Biosystems) was used to amplify the DNA sequence of interest. The cycling conditions were as follows: initial denaturation at 94 ºC for 2 min, followed by 30 cycles of denaturation at 94 ºC for 10 s, annealing at 60 ºC for 30 s, and synthesis at 72 ºC for 45 s. Primers were initially dissolved in TE buffer (Tris 10 mM, EDTA 1 mM; adjusted to pH 8 with HCl), and made up to a 200 mM stock solution. This was further diluted with nuclease free water to a working concentration.

2.2.3 Gel electrophoresis

Following amplification, 15 µl of each sample was loaded onto a 2% TAE (Tris acetate 40 mM, EDTA 1 mM) agarose gel containing 0.1 µL/ml SYBR safe DNA stain 10,000x (Invitrogen), and immersed into an electrophoresis tank (Mini Sub Cell, Bio-Rad) containing TEA buffer. The samples were run at 80 V for 30-45 mins against 5 µL of a 50 bp DNA ladder (Fermentas GeneRuler, 0.1 µg/µL). The subsequent bands were viewed and photographed under UV light using a GelCapture imaging system (DNR Bio-Imaging Systems).

Table 2.1: Primer nucleotide sequences and concentrations used for DNA amplification and genotype determination

Gene[1]	PCR primer nucleotide sequences (5`-3`)	Primer	Final primer concentration	Detection	
				Genotype	Product length (bp)
P2rx1	AAC CCA GAT CCC ACC AAC GAA C	Forward	0.4 µM	Wild-type	317
	TCA CCC AAT GAC GTA GAC CAG A	Reverse	0.4 µM		
	GGT TCT CCG GCC GCT TGG GTG G	Forward	0.4 µM	Mutant	519
	GCG CGC CTT GAG CCT GGC GAA C	Reverse	0.4 µM		

1. Mouse gene name: *P2rx1*, P2X1-purinoceptor

2.3 Dissection

2.3.1 Rat prostate gland and vasa deferentia

Rats were killed by asphyxiation using CO_2 gas. An incision in the lower abdomen exposed the male urogenital tract. The left and right prostate lobes and the prostatic portion of the vasa deferentia were carefully dissected out for use in isolated organ bath studies. Excess fat and connective tissue were removed. Tissues were then placed in Krebs-Henseleit solution (NaCl 118.1 mM, KCl 4.7 mM, $MgSO_4.7H_2O$ 1.1 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, glucose 11.7 mM, $CaCl_2$ 2.5 mM; freshly made each morning).

2.3.2 Mice

Male mice were weighed and then killed by asphyxiation using CO_2 gas. An incision was made along the midline of the abdomen. The prostate, vasa deferentia, bladder, testes, seminal vesicles, kidneys, liver, spleen, and heart, were all carefully dissected out and weighed; the length of the vasa deferentia were measured.

Female mice were killed using the above procedure. An incision was made along the midline of the abdomen to reveal the uterine tubes. The presence or absence of foetuses were noted, and foetal number recorded.

2.4 Isolated organ bath studies

2.4.1 Organ bath setup

Following dissection, each rat prostate lobe or vas deferens was mounted in a 10 ml water-jacketed glass organ bath containing Krebs-Henseleit solution. One end of the tissue was attached to a Perspex tissue holder incorporating two vertical parallel platinum electrodes connected to a Grass S88 stimulator. The other end of the tissue was attached to an isometric Grass FT03 force displacement-transducer for the recording of smooth muscle contractions.

The tissue was maintained at 37 °C and bubbled with 5% CO_2 in O_2 throughout the experiment. Developed force was recorded via a PowerLab data acquisition system (chart 5.1) run on a personal computer. Each isometric Grass FT03 force displacement transducer was calibrated prior to individual experiments to ensure precise and reproducible observations. The PowerLab 4/SP data acquisition system and LabChart software version 5.1 used an inbuilt function to generate a linear curve from the forces generated by 0 g, as well as standard 1 or 2 g weights initially observed in millivolts, enabling the analysis of force in the subsequent experiment. Prior to experimentation the tissues were equilibrated for 60 minutes under a resting force of 0.7 - 1.0 g. To ensure tissue viability, nerve terminals within the tissue were field stimulated during the equilibration period using electrical pulses of 0.5 ms duration and 60 V at 0.01 Hz.

In all prostatic experiments, the organ bath medium was periodically replaced when secretions caused frothing to occur. When necessary the test extract or vehicle was replaced after bath washes.

2.4.2 Electrical field stimulation

Following equilibration, frequency-response curves to electrical field stimulation (0.5 ms pulse duration, 60 V, 0.1 – 20 Hz) were constructed using a frequency progression ratio of approximately one third of a log unit. Trains of pulses were delivered at 10 minute intervals. Each train consisted of 10 pulses for frequencies up to 1 Hz, or 10 second duration for frequencies greater than or equal to 1 Hz. At the completion of the frequency-response curve, the tissues were washed with three to five times the bath volume.

A control curve without the presence of the test sample was initially performed. A second frequency-response curve was performed after the tissue was exposed to the test sample for a period of thirty minutes.

2.4.3 Exogenously administered agonists

In a separate set of experiments, following the equilibration period, noradrenaline (1 nM – 0.1 mM), acetylcholine (1 nM – 0.1 mM), ATP (10 nM – 1 mM), or αβ-methylene ATP (3 nM – 10 μM) was used to construct discrete concentration-response curves on unstimulated tissue. Only one agonist was added to each tissue. A concentration progression ratio of half a log unit was employed. Once the contractile plateau had been reached, or if no response was observed after 20 seconds, the tissue was washed with three to five times the bath volume and allowed a 10 minute recovery period before the next concentration was applied.

A second agonist-induced curve was performed after the tissue was exposed to the test sample for a period of thirty minutes.

2.4.4 Statistical analysis of isolated organ bath experiments

The number of experimental animals used is represented by *n*. The prostatic force (g) at the peak height of contraction, or for vas deferens preparations, integral force (g.s where s = 20 seconds) as area under the curve, were measured in response to discrete frequencies of electrical simulation or concentrations of agonists, in the absence or presence of the test extract.

All data were analysed by two-way repeated measures analysis of variance (ANOVA) and if required with Bonferroni post-test correction for multiple comparisons using GraphPad Prism version 5.0. The *p*-value for the interaction between treatment and frequency was used. This enabled a comparison of the differences between the control and treatment groups at all frequencies and concentrations on the frequency and concentration-response curve. If the *p*-value for the interaction was greater than 0.05 then the *p*-value for treatment was used to see whether there was a constant effect across all frequencies and concentrations. Measurements from each tissue sample were pooled and expressed as the mean ± the standard error of the

mean (SEM). *P*-values were used to represent the probability of the observed changes being due to chance. A value of $p < 0.05$ was considered statistically significant.

2.5 Extracts and reagents

Stinging nettle root and leaf extracts (500 mg dried plant material/ml in 25% alcohol) were obtained from MediHerb® Pty. Ltd. (Warwick, Queensland). These extracts are available in Australia for practitioner dispensing.

Noradrenaline bitartrate salt (Arterenol®, Sigma) was dissolved and diluted to correct concentrations using a catecholamine diluent (NaCl 154 mM, NaH_2PO_4 1.2 mM, ascorbic acid 0.2 mM in distilled water). Acetylcholine chloride (Sigma), adenosine 5'-triphosphate magnesium salt (ATP) (Sigma), and αβ-methylene ATP lithium salt (Sigma) were all dissolved and diluted to required concentrations using distilled water. All drugs were made fresh on the morning of experimentation. Water used in all experiments was distilled using the MilliPore system (MA, U.S.A.).

Ferulic acid (AK Sci), and coumarin (AK Sci), were dissolved in deuterated water, and *p*-coumaric acid (Aldrich) was dissolved in d_4-methanol, and all were analysed using NMR as per section 2.7.1.

2.6 Chemical separation

2.6.1 Liquid-liquid partitioning

Initially undissolved solids in the sample were removed from stinging nettle leaf extract (100 ml) by vacuum filtration (Whatman filter paper 55) and ethanol vehicle evaporated off via rotavap. Aqueous residue was extracted with distilled ethyl acetate (30 ml) three times, and the resulting organic layers combined. Approximately 5 ml of saturated sodium chloride solution was added to the combined organic phase to remove leftover aqueous material. The

resulting organic layer was then dried with magnesium sulfate, filtered and the solvent evaporated. The remaining aqueous extract was lyophilised to dryness.

2.6.2 Reversed-phase flash chromatography

Reversed-phase flash chromatography was carried out using Davisil 633NC_{18}E silica gel (Grace, Rowville, VIC) in a glass column of diameter 5 cm and a silica length of approximately 15 cm, under nitrogen gas (Still *et al.*, 1978).

Stinging nettle leaf extract (160 ml), diluted with distilled water (80 ml) was loaded onto the column. The sample was eluted sequentially with 1000 ml each of 10%, 40%, 60% methanol in distilled water, 100% methanol and 50% methanol in dichloromethane. The solvents were evaporated from the eluents and the aqueous fractions lyophilised, and made up to concentration in distilled water, 25% ethanol, or dimethyl sulfoxide for bioactivity testing in isolated organ bath experiments.

2.6.3 Preparative HPLC

Preparative high-performance liquid chromatography (HPLC) was carried out on the Waters PrepLC System with a tuneable absorbance detector (Waters 486) using a Synergi C_{18} RP Hydro column (50 mm x 21.2 mm x 4µm). To achieve separation, either a gradient elution with a flow rate of 10 ml/min, consisting of isocratic 100% A for the first 5 minutes increasing to 70% B over 20 minutes, increasing to 100% B over 2 minutes, held at 100% B for 3 minutes, then decreasing to 0% B over 2 minutes, and finally an isocratic elution at 0% B for 3 minutes, or an isocratic elution of 20% B and a flow rate of 5 ml/min were used; where A was 0.1% TFA/H_2O and B was 80% ACN/0.1% TFA/H_2O.

2.7 Chemical analysis

2.7.1 Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were recorded at 400 MHz, using a Bruker Advance III Nanobay 400 MHz NMR spectrometer. Data acquisition and processing was managed using XWINNMR (Bruker) software package version 3.5 and plotting was managed using MestReNova v6.0.2. Chemical shifts for all ^{1}H spectra were measured in parts per million (ppm) and referenced to an internal standard of residual proteo-solvent. In reporting spectroscopic data, the following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; br, broad; app, apparent.

2.7.2 Liquid chromatography mass spectrometry (LC-MS)

An Ultra high-performance liquid chromatography mass spectrometer (UHPLC/MS) (Agilent 1260/6120) was used to separate the extracts and fractions and to detect the compounds. Separation was achieved using a Poroshell 120 EC-C18 column (3.0 mm x 5.0 mm; 2.7 µm) at column temperature of 35 °C. The mobile phase consisted of a binary solvent system using aqueous formic acid (0.1%) and ACN/formic acid (99.9:0.1 v/v) as solvents A and B respectively. Elution was performed using a gradient over 5 minutes, beginning with a gradient from 5% B to 100% B over 2 mins 30 s, isocratic for 1 min 18 s, then decreased back to 5% B over 1 min 12 s. An injection volume of 1 µl and a constant flow rate of 0.5 ml/min were used. The flow from the UHPLC was directed into the electrospray ionisation mass spectrometer (API-MS). The data was acquired using LC/MSD Chemstation Rev.B.04.03 coupled with Masshunter Easy Access Software in negative and positive ion mode with the following conditions: capillary voltage, 3.0 kV for both positive and negative modes; drying gas temperature, 350 °C. Detection was monitored at 254 nm and 214 nm using a 1290 Infinity G4212A 1290 DAD.

2.7.3 Analytical HPLC

Analytical HPLC was achieved using a Synergi C_{18} RP Hydro column (250 mm x 4.6 mm x 4 µm) on a HPLC system (Waters 2690) with a photodiode array detector (Waters 996) An injection volume of 50 µl with an isocratic elution of 20% B and a flow rate of 1 ml/min was used; where A was 0.1% TFA/H_2O and B was 80% ACN/0.1% TFA/H_2O.

2.8 In vivo fertility experiments

2.8.1 Administration of stinging nettle leaf extract

Wild-type C57B1/6 male mice were administered daily oral doses of either vehicle (100 µl 25% ethanol) or stinging nettle leaf extract (100 µl of 500 mg/ml). Following the accrual of control resting blood pressure and heart rate measurements, two days of rest were allowed before dosing commenced and continued throughout the mating period. Dosing occurred post experimental procedures.

2.8.2 Blood pressure and heart rate analyses

Prior to measuring cardiovascular parameters, eight-week-old male P2X1-purinoceptor knockout and wild-type mice were housed in a reverse light-cycle facility (12 hours light/dark; 7:00 am off, 7:00 pm on) for three days to acclimatise. Resting blood pressure (mmHg) and heart rate measurements (beats per minute (bpm)) were obtained using the tail cuff method with a non-invasive blood pressure analysis system (SC1000, Hatteras Instruments, Cary North Carolina) connected to a personal computer, using the parameters described in Table 2.2. Over a period of five consecutive days, analysis comprised of 20 repeat blood pressure and heart rate measurements per day. After two days of rest, the mice were orally dosed daily with either vehicle (100 µl 25% ethanol) or stinging nettle leaf extract (100 µl of 500 mg/ml). The measurement means were averaged, and standard deviations calculated to determine the final blood pressure and pulse rate.

Table 2.2: Instrument and analysis parameters for blood pressure and pulse rate analyses.

Parameter	Setting
Preliminary cycles	5 cycles
Measurement cycles	20 cycles
Time between measurements	5 seconds
Pulse time out	30 seconds
Measurement time out	60 seconds or 180 mmHg
Consecutive peaks for pulse rate	70
Systolic measurement threshold	12%
Diastolic measurement threshold	50%
Platform temperature	40 °C
Measurement cycles needed to avoid exclusion	15 cycles

2.8.3 Breeding observations

Subsequent to analysis of cardiovascular parameters, P2X1-purinoceptor knockout and treated wild-type male mice were mated with seven to eight-week-old female mice to test their fertility. Wild-type female mice were housed in a reverse light-cycle facility (12 hours light/dark; 7:00 am off, 7:00 pm on) for three days to acclimatise. The female was then placed in the cage of the male in a dark behavioural room for two hours per day for up to nine days or until copulation to the point of ejaculation was confirmed. A B/W CCD camera (VIDO) and infrared lights were used to observe the mice, and video files were recorded using Windows movie maker (Microsoft) for subsequent analysis. Once copulation had been confirmed the female remained in her cage where she was allowed to gestate for 14 days before being sacrificed and examined to determine whether pregnancy had occurred, and the number of implanted foetuses were recorded. Each male was mated with two individual wild-type female mice.

Chapter 3

Pharmacological Investigation of *Urtica dioica* (Stinging Nettle) Extracts on the Contractility of the Rat Prostate Gland

Abstract

Stinging nettle root extract is commercially used for the symptomatic treatment of BPH, and although not typically used for BPH, the leaf extract has been used in traditional medicine for both nocturia and hypertension suggesting it may engender a relaxant effect on prostatic smooth muscle. Both stinging nettle root and leaf extracts were tested for their effect on prostatic smooth muscle contractility on isolated rat prostate glands in organ bath studies. Contraction was induced by either electrical field stimulation or the exogenously administered agonists noradrenaline, acetylcholine, adenosine 5`-triphosphate (ATP), or αβ-methylene ATP. The root extract did not elicit an effect on electrical field stimulation-induced contraction (p = 0.135; n = 6). The leaf extract, though, attenuated contraction by electrical field stimulation (p = 0.023; n = 6), and subsequently ATP (p = 0.004; n = 6), and αβ-methylene ATP (p = 0.019; n = 6), without effect on noradrenaline- or acetylcholine-induced contraction (p = 0.907; n = 6, and p = 0.362; n = 6, respectively). It is concluded that stinging nettle leaf extract reduces the contractility of the prostatic smooth muscle by acting as an antagonist on postjunctional P2X1-purinoceptors. As there are currently no suitable *in vivo* antagonists for the P2X1-purinoceptor, these findings indicate stinging nettle leaf extract, or its bioactive ingredient, would potentially have a significant role in the development of more effective therapies for the symptomatic treatment of BPH.

3.1 Introduction

Stinging nettle root extract has been shown to alleviate the lower urinary tract symptoms associated with BPH by reducing the size of the prostate through various mechanisms as described in section 1.4.1. The contractile response of prostatic smooth muscle is of equal, if not greater, importance in the aetiology of symptomatic BPH. The effect of phytotherapeutics on prostatic smooth muscle contractility is therefore a significant consideration in the treatment of lower urinary tract symptoms associated with BPH.

Although stinging root extract has not yet demonstrated an effect on prostatic smooth muscle contractility, it has been shown to possess vasorelaxant activity mediated by both endothelial nitric oxide release, and a negative inotropic action (Testai *et al.*, 2002). *In vivo*, a reduction in blood pressure in spontaneously hypertensive Wistar rats was observed after the administration of either quercetin, a flavonoid, (Duarte *et al.*, 2001, Perez-Vizcaino *et al.*, 2009) or chlorogenic acid (Suzuki *et al.*, 2006), both of which have been identified in stinging nettle plants (Budzianowski, 1991, Akbay *et al.*, 2003, Suzuki *et al.*, 2006, Grevsen *et al.*, 2008, Pinelli *et al.*, 2008). Quercetin has also demonstrated vasorelaxant activity on aortic preparations with an intact endothelial layer pre-contracted with KCl or noradrenaline (Duarte *et al.*, 1993). Both chlorogenic acid (Iwahashi *et al.*, 1990, Ohnishi *et al.*, 1994) and flavonoids (Bors *et al.*, 1990, Chen *et al.*, 1990, Rice-Evans *et al.*, 1995, Rice-Evans *et al.*, 1996, Rice-Evans *et al.*, 1997) are known to act as antioxidants. It has been postulated chlorogenic acid may exert its effect through increasing the availability of nitric oxide by inhibiting the excessive production of reactive oxygen species, thereby increasing vasodilation (Suzuki *et al.*, 2006). Quercetin was also found to enhance the endothelium-dependent relaxation to acetylcholine (Duarte *et al.*, 2001). It has been suggested that the main vasodilatory mechanism of action of quercetin is by

the inhibition of protein kinase C, and a decrease in calcium (Ca^{2+}) uptake may also contribute to its effects (Duarte *et al.*, 1993).

Stinging nettle leaf extract, although not used commercially for the symptomatic treatment of BPH, warrants investigation as the aerial parts of the plant are decocted as a tea in Turkey for the treatment of nocturia (Sezik *et al.*, 2001), a common symptom of BPH, as well as in the alpine regions of Italy, to increase urinary output (Pieroni *et al.*, 2009). Additionally, the leaves have been used in traditional and folk medicines for the treatment of hypertension (Ziyyat *et al.*, 1997, Tahraoui *et al.*, 2007, Tagarelli *et al.*, 2010). An aqueous extract of the aerial parts of stinging nettle produced an acute dose-dependent reduction in arterial blood pressure in male Wistar rats (Tahri *et al.*, 2000) as well as exerted a hypotensive action associated with a decreased heart rate both *ex vivo* (Legssyer *et al.*, 2002) and *in vivo* (Dizaye *et al.*, 2013) in the rat. Non-cholinergic and non-adrenergic pathways were found to induce a strong bradycardia thought to account for the hypotensive effect *in vivo* (Legssyer *et al.*, 2002).

Current studies have not indicated a specific effect of either stinging nettle root or leaf extract on prostatic smooth muscle contractility, however the potential vasorelaxant effect of these extracts may transfer to the prostate. Although a reduction of symptoms can be obtained by reducing the size of the prostate, a greater alleviation of LUTS is associated with a decrease in the contractility of the prostate (for review see (Bach *et al.*, 1997)). This chapter therefore investigates the effects of both stinging nettle root and leaf extracts on rat prostatic smooth muscle contractility *in vitro* using isolated organ bath experiments.

3.2 Methods

3.2.1 Animals and tissues

Sprague-Dawley rats were obtained, and housed, as described in Chapter 2, sections 2.1.1 and 2.1.4. Details of animal ethics approval are as per Chapter 2, section 2.1.4.

Rats were euthanized by asphyxiation through exposure to CO_2 gas. Prostate tissue was removed as described in Chapter 2, section 2.3.1.

3.2.2 Isolated organ bath studies

Isolated preparations of rat prostate glands were set up for isolated organ bath studies and statistically analysed using the procedure outlined in Chapter 2, section 2.4.

Within time control experiments, the tissue was not exposed to any test extracts during the 30 minute latency period between frequency-response or concentration response-curves.

For subsequent test extract experiments, stinging nettle root extract (500 mg plant material/ml in 25% alcohol) and stinging nettle leaf extract (500 mg plant material/ml in 25% alcohol) at bath concentrations of either 5 mg/ml or 15 mg/ml were tested separately using the protocol as described in Chapter 2, section 2.4.2. Contralateral pairs of prostate lobes served as internal controls.

3.3 Results

3.3.1 Contractile responses to electrical field stimulation

Electrical field stimulation of the nerve terminals (60 V, 0.5 ms, 0.1 - 20 Hz for ten pulses or 10 s at 10 min intervals) elicited frequency-dependent contractions in isolated rat prostates in all experiments.

3.3.2 Effects of stinging nettle root extract on responses to electrical field stimulation

Contractile responses of the isolated rat prostate gland to electrical field stimulation were not significantly affected over the time course of the experiment ($p = 0.105$; Figure 3.1).

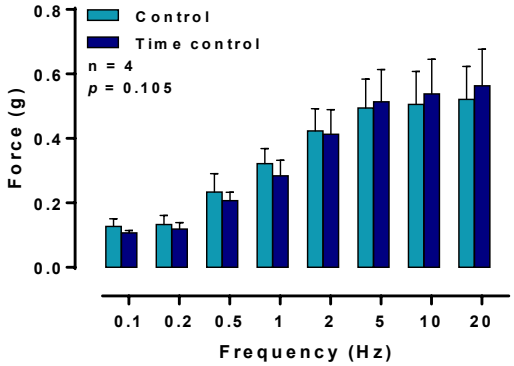


Figure 3.1: Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.1 Hz to 20 Hz for 10 pulses or 10 seconds) in isolated rat prostate gland preparations ($p = 0.105$). Following the first curve the tissue was allowed to rest for thirty minutes without stimulation before the second curve was constructed. Bars represent the mean force generated by prostates taken from four rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures of ANOVA and represent the probability of time causing a significant change in the contractile response.

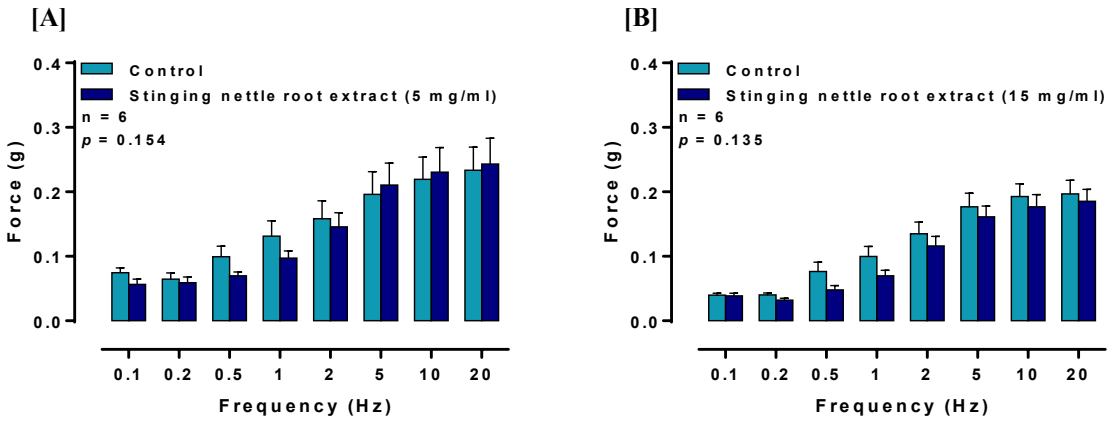


Figure 3.2: Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.1 Hz to 20 Hz for 10 pulses or 10 seconds) of isolated rat prostate gland preparations prior to and after the addition of [A] stinging nettle root extract (5 mg/ml) ($p = 0.154$) and [B] stinging nettle root extract (15 mg/ml) ($p = 0.135$). Bars represent the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

Incubation of isolated rat prostate glands in stinging nettle root extract (5 mg/ml) for 30 min did not exert any effect on contractions in response to electrical field stimulation ($p = 0.154$;

Figure 3.2A). Increasing the root extract concentration to 15 mg/ml did not ameliorate the response to electrical field stimulation-induced contraction (p = 0.135; Figure 3.2B).

3.3.2.1 Effects of stinging nettle leaf extract on responses to electrical field stimulation

As was observed with stinging nettle root extract, contractions in response to electrical field stimulation were not attenuated in the presence of the lower concentration of leaf extract (5 mg/ml) (p = 0.998; Figure 3.3A). However, at the higher concentration of 15 mg/ml, significant attenuation of electrical field stimulation-induced contraction was observed (p =0.023; Figure 3.3B).

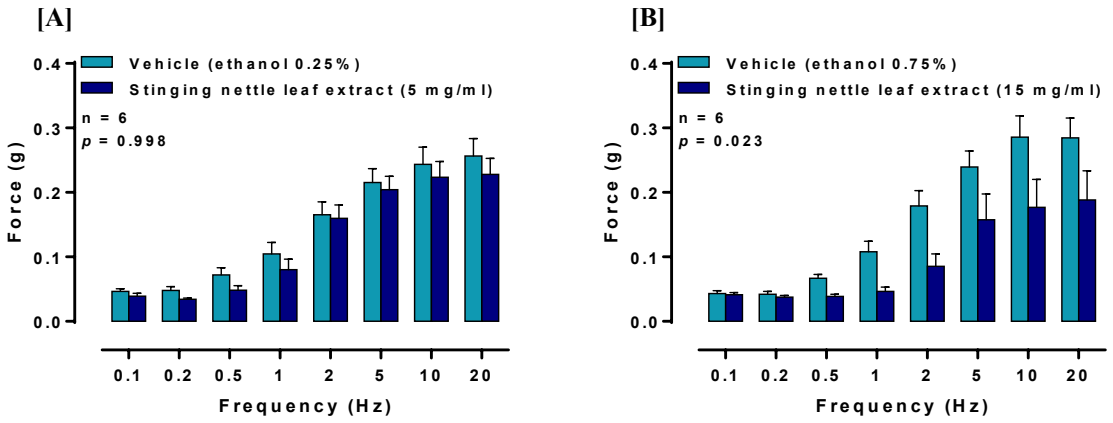


Figure 3.3: Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.1 Hz to 20 Hz for 10 pulses or 10 seconds) of isolated rat prostate gland preparations after the addition of [A] vehicle (ethanol 0.25% v/v) or stinging nettle leaf extract (5 mg/ml) (p = 0.998) and [B] vehicle (ethanol 0.75% v/v) or stinging nettle leaf extract (15 mg/ml) (p = 0.023). Bars represent the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

3.3.3 Effects of stinging nettle leaf extract on contractile responses to exogenously administered agonists

Concentration-dependent contractions of the rat prostatic tissue were observed to each of the exogenously administered agonists.

3.3.3.1 Contractile responses to exogenously administered noradrenaline

After construction of the first concentration-response curve to noradrenaline and following a stimulation free rest time of thirty minutes, a discernible attenuation of the magnitude of noradrenaline-induced contractions was seen ($p < 0.001$; Figure 3.4) as indicated by an approximately 10-fold rightward shift in the concentration-response curve.

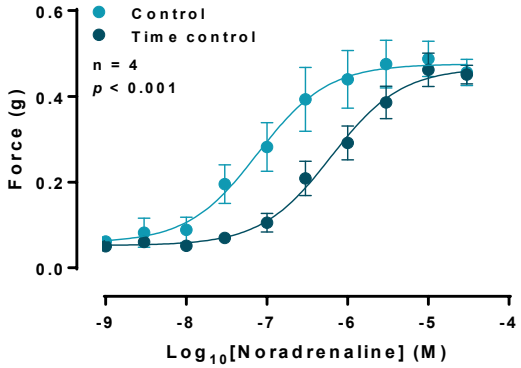


Figure 3.4: Mean contractile responses to exogenously administered noradrenaline (1 nM – 30 µM in half log unit increments) in isolated rat prostate gland preparations ($p < 0.001$). Following the first curve the tissue was allowed to rest for thirty minutes without stimulation before the second curve was constructed. Each point represents the mean force generated by prostates taken from four rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures of ANOVA and represent the probability of time causing a significant change in the contractile response.

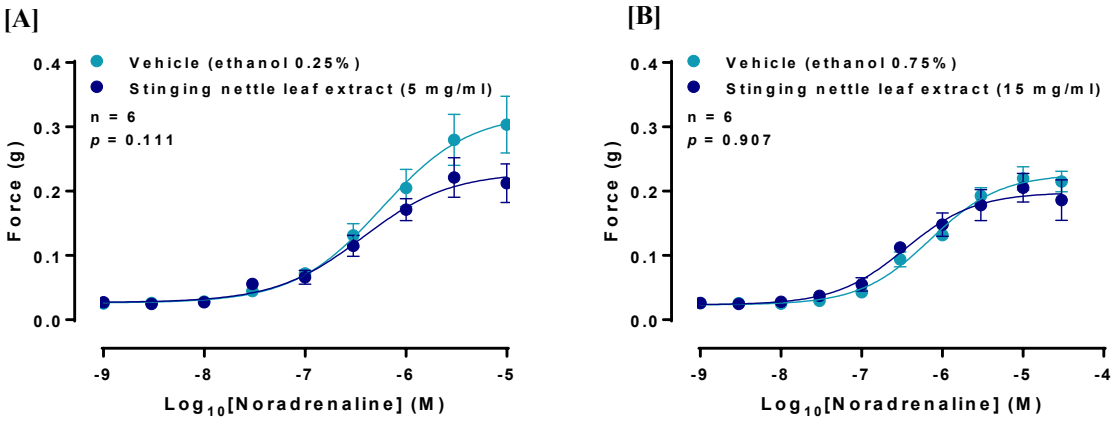


Figure 3.5: Mean contractile responses to exogenously administered noradrenaline (1 nM – 10 µM or 30 µM in half log unit increments) of isolated rat prostate gland preparations after the addition of [A] vehicle (ethanol 0.25% v/v) or stinging nettle leaf extract (5 mg/ml) ($p = 0.111$) and [B] vehicle (ethanol 0.75% v/v) or stinging nettle leaf extract (15 mg/ml) ($p = 0.907$). Each point represents the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

Noradrenaline-induced contraction was not attenuated in the presence of either 5mg/ml or 15 mg/ml of leaf extract (p = 0.111; Figure 3.5A and p = 0.907; Figure 3.5B, respectively) when compared to paired vehicle controls which were run in parallel.

3.3.3.2 Contractile responses to exogenously administered acetylcholine

Time-control curves conducted for acetylcholine-induced contraction exhibited a similar significant rightward shift over the time course of the experiment (p = 0.010; Figure 3.6) as was previously observed with noradrenaline (Figure 3.4).

Stinging nettle leaf extract, at either a lower concentration (5 mg/ml) or at a higher concentration (15 mg/ml), was unable to attenuate acetylcholine-induced contractions (p = 0.596; Figure 3.7A, and p = 0.362; Figure 3.7B, respectively).

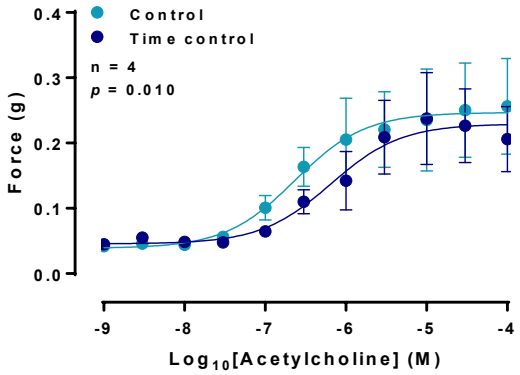


Figure 3.6: Mean contractile responses to exogenously administered acetylcholine (1 nM – 100 µM in half log unit increments) in isolated rat prostate gland preparations (p = 0.010). Following the first curve the tissue was allowed to rest for thirty minutes without stimulation before the second curve was constructed. Each point represents the mean force generated by prostates taken from four rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that changes in the contractile response over time were due to chance.

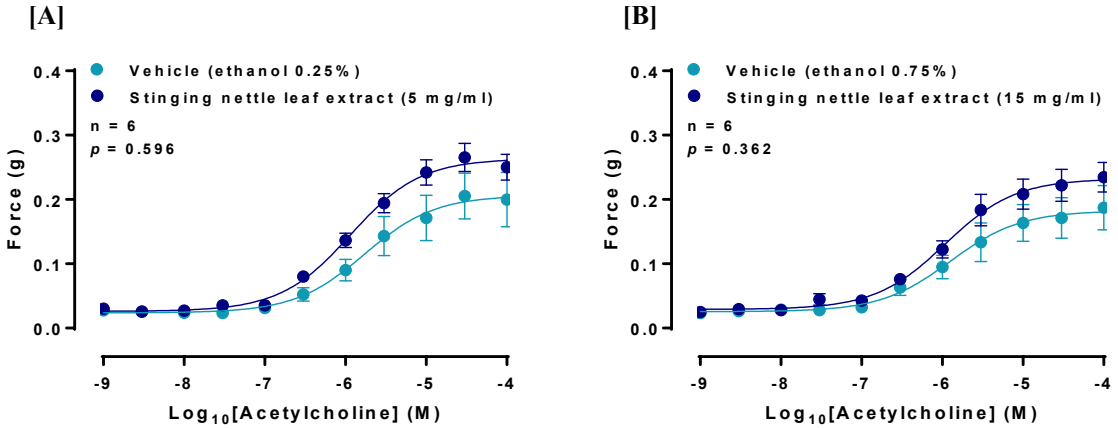


Figure 3.7: Mean contractile responses to exogenously administered acetylcholine (1 nM – 100 µM in half log unit increments) of isolated rat prostate gland preparations after the addition of [A] vehicle (ethanol 0.25% v/v) or stinging nettle leaf extract (5 mg/ml) (p = 0.596) and [B] vehicle (ethanol 0.75% v/v) or stinging nettle leaf extract (15 mg/ml) (p = 0.362). Each point represents the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

3.3.3.3 Contractile response to exogenously administered ATP

There was no observable shift in the curve over the duration of a time control experiment (p = 0.840; Figure 3.8) when ATP was used to contract isolated rat prostate glands.

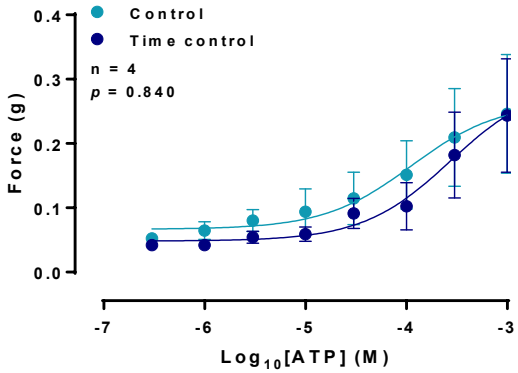


Figure 3.8: Mean contractile responses to exogenously administered ATP (300 nM – 10 µM in half log unit increments) in isolated rat prostate gland preparations (p = 0.840). Following the first curve the tissue was allowed to rest for thirty minutes without stimulation before the second curve was constructed. Each point represents the mean force generated by prostates taken from four rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that changes in the contractile response observed over time were due to chance.

The addition of 5 mg/ml of stinging nettle leaf extract did not affect ATP-induced contraction in the isolated rat prostate (p = 0.634; Figure 3.9A). As observed with electrical field stimulation-induced contraction the higher concentration of stinging nettle leaf extract (15 mg/ml) attenuated ATP-induced contraction in the isolated rat prostate gland (p = 0.004; Figure 3.9B). No significant difference was observed in the contractions of different tissues in the absence of either concentration of extract or vehicle (p = 0.812 and p = 0.880, respectively).

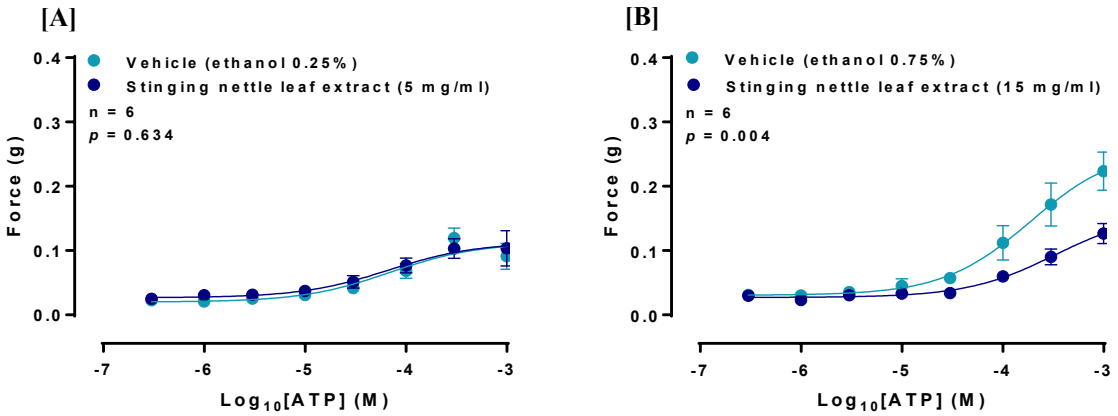


Figure 3.9: Mean contractile response to exogenously administered ATP (300 nM – 10 µM in half log unit increments) of isolated rat prostate gland preparations after the addition of [A] vehicle (ethanol 0.25% v/v) or 5 mg/ml stinging nettle leaf extract (p = 0.634), and [B] vehicle (ethanol 0.75% v/v) or 15 mg/ml stinging nettle leaf extract (p = 0.004). Each point represents the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

3.3.3.4 Contractile response to exogenously administered αβ-methylene ATP

As was observed with ATP, time did not have an effect on the contractility of the isolated rat prostate gland to exogenously administered αβ-methylene ATP as there was no apparent shift of the curve over the duration of the experiment (p = 0.116; Figure 3.10).

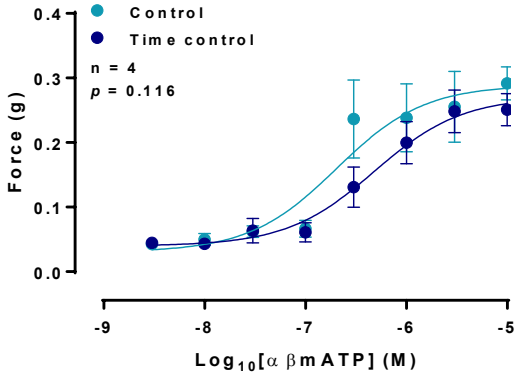


Figure 3.10: Mean contractile responses to exogenously administered αβ-methylene ATP (3 nM – 10 µM in half log unit increments) in isolated rat prostate gland preparations (p = 0.116). Following the first curve the tissue was allowed to rest for thirty minutes without stimulation before the second curve was constructed. Each point represents the mean force generated by prostates taken from four rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that changes in the contractile response observed over time were due to chance.

Attenuation of αβ-methylene ATP-induced contraction was observed in the presence of 5 mg/ml of stinging nettle leaf extract (p = 0.019; Figure 3.11). Prior to the addition of either vehicle or extract, the different tissues responded similarly (p = 0.884).

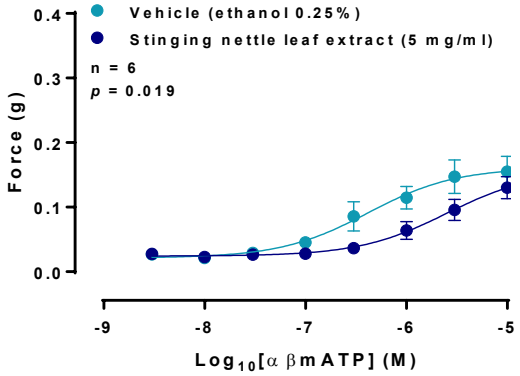


Figure 3.11: Mean contractile responses to exogenously administered αβ-methylene ATP (3 nM – 10 µM in half log unit increments) of isolated rat prostate gland preparations after the addition of vehicle (ethanol 0.25% v/v) or 5 mg/ml stinging nettle leaf extract (p = 0.019). Each point represents the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

In all experiments, prior to the addition of vehicle or stinging nettle leaf extract, the contractile responses of the isolated rat prostates to electrical field stimulation or exogenous agonist were similar (Table 3.1).

Table 3.1: *p*-values comparing the contractile responses of rat prostates to either electrical field stimulation or agonist-induced contraction prior to the addition of either vehicle or stinging nettle leaf extract. ANOVA *p*-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance. In all experiments n = 6.

Contraction induced by	*p*-value comparing the contractile response prior to the addition of 100 µl of either ethanol 25% or 500 mg/mL stinging nettle leaf extract	*p*-value comparing the contractile response prior to the addition of 100 µl of either ethanol 25% or 500 mg/mL stinging nettle leaf extract
Electrical field stimulation	0.998	0.837
Noradrenaline	0.202	0.986
Acetylcholine	0.736	0.663
ATP	0.812	0.880
αβ-methylene ATP	0.884	N/A

3.4 Discussion

This chapter describes the effects of stinging nettle root and leaf extracts on electrical field simulation-induced, and subsequently, agonist-induced contraction of the isolated rat prostate gland. The root extract was initially tested, as this part of the plant is found in commercially available stinging nettle preparations for the symptomatic treatment of BPH. Traditional medicine also utilises the roots of the stinging nettle plant for the treatment of urinary problems including prostatitis, nocturia, frequency, and dysuria (Kultur, 2007, Rodriguez-Fragoso *et al.*, 2008). Although not known to be commercially used for lower urinary tract symptoms, the leaf extract was investigated as, in traditional medicine, teas are brewed from the aerial parts of the plant to increase diuresis (Saric-Kundalic *et al.*, 2010) as well as lower blood pressure (Kultur, 2007, Calvo *et al.*, 2011, Menendez-Baceta *et al.*, 2014). The increased urinary flow, particularly in elderly men, may be due to a relaxation of prostatic tissue surrounding the urethra thereby removing obstruction. Additionally, the reduction of blood pressure suggests a relaxant effect on vascular smooth muscle, innervated by sympathetic nerves similar to prostatic smooth muscle. This implies the leaf extract may potentially engender an effect on

prostatic smooth muscle. To avoid vehicle effects, 15 mg/ml was chosen as it was the highest concentration possible where the vehicle had a minimal effect on isolated tissue preparations. The lower concentration of 5 mg/ml, a one third log unit lower concentration, was also used to demonstrate a concentration-response relationship.

Electrically evoked contraction of rat prostatic smooth muscle using the parameters described in this chapter occurs primarily through activation of sympathetic and parasympathetic nerve fibres causing the respective release of noradrenaline, acetylcholine, and the co-transmitter ATP (Lau, Ventura*, et al.*, 1998, Ventura *et al.*, 2003). The proportion of each neurotransmitter released varies with different frequencies. At low frequencies ranging from 0.1 – 2 Hz, a proportionally greater amount of ATP is released (Ventura *et al.*, 2003), more acetylcholine is released at frequencies ranging from 1 – 5 Hz (White *et al.*, 2010), whereas at higher frequencies of 10 and 20 Hz, noradrenaline is the primary neurotransmitter (White *et al.*, 2010). Therefore, frequencies ranging from 0.1 Hz to 20 Hz was used to contract the tissue and to determine the effect of the stinging nettle extracts.

Stinging nettle root extract did not attenuate electrical field stimulation-induced contraction, indicating it is not active along the sympathetic, parasympathetic, or purinergic pathways. It is unlikely, but still possible, that the root extract may have some effect on the contractility of smooth muscle through alternate mechanisms of action, as it has been shown to alleviate LUTS associated with BPH (for review see (Chrubasik *et al.*, 2007)). Nitric oxide (NO), vasoactive intestinal polypeptide (VIP) and/or neuropeptide Y (NPY) have been found to be co-localised with either noradrenergic or cholinergic nerve fibres supplying the prostate (Jen *et al.*, 1996, Hedlund *et al.*, 1997). Both NPY and VIP have been found to be localised within the prostatic stroma and smooth muscle layers (Lange *et al.*, 1990, Hedlund *et al.*, 1997). As the functional effects of VIP on prostatic smooth muscle were found to be relatively small when compared to

smooth muscle from other urogenital regions, the role of VIP on prostatic smooth muscle activity is likely to be minor (Hedlund *et al.*, 1997). Nitric oxide synthase, VIP and NPY are all found within the dense network of cholinesterase-staining fibres supplying both the prostate epithelium and stroma, implying a role in smooth muscle tone as well as the modulation of prostatic secretions (Higgins *et al.*, 1989, Hedlund *et al.*, 1997, Ventura *et al.*, 2002). The root extract may be acting along one of these alternate pathways modifying the contractility of the prostatic smooth muscle and therefore exhibiting the therapeutic effects seen when it is used for the alleviation of LUTS associated with BPH.

Stinging nettle leaf extract was shown to attenuate electrical field stimulation-induced contraction in a concentration-dependent manner. This establishes the dose-dependency of the inhibitory action of stinging nettle leaf extract on prostatic smooth muscle. Further experiments were subsequently conducted to determine if the attenuation was due to a prejunctional or postjunctional effect of the leaf extract on the prostatic smooth muscle, and whether it was acting via a specific neurotransmitter mechanism arising from either the sympathetic or parasympathetic nervous systems.

Rat and human prostatic smooth muscle receive predominantly noradrenergic innervation (Lau, Ventura*, et al.*, 1998, McVary *et al.*, 1998). Contraction is mediated through the release of noradrenaline acting at α_{1A}-adrenoceptors (Raz *et al.*, 1973, Caine *et al.*, 1975, Hieble *et al.*, 1985, Gup *et al.*, 1989) causing a distinct rise in prostatic smooth muscle tone (Raz *et al.*, 1973, Caine *et al.*, 1975). Stinging nettle leaf extract, however, did not attenuate noradrenaline-induced contraction.

Electrical field stimulation causes the release of acetylcholine which acts on M_3 muscarinic receptors in the smooth muscle of the prostate (Lau, Ventura*, et al.*, 1998) eliciting contraction.

Whilst the cholinergic system is primarily localised to the glandular epithelium in the human prostate (Ruggieri *et al.*, 1995), acetylcholinesterase-positive fibres are present in the stroma of the rat prostate (Lau, Ventura*, et al.*, 1998). Activation of muscarinic receptors elicits a contractile response, suggesting the cholinergic system has a role in both smooth muscle tone, as well as the modulation of prostatic secretions (Raz *et al.*, 1973). As prostate muscarinic receptors are predominantly epithelial it has been suggested the major role of acetylcholine is secretomotor (Ventura *et al.*, 2002). It is believed, though, acetylcholine may enhance noradrenaline-mediated contraction via synergism with other contractile agents (Roosen *et al.*, 2009).

As with noradrenaline-induced contraction, acetylcholine-induced contraction was associated with a rightward shift in concentration-response curves subsequent to the initial concentration-response curve indicating a diminishing sensitivity of the tissue to acetylcholine over the time course of the experiment. Nonetheless, the leaf extract did not attenuate acetylcholine induced contraction.

The purinergic system constitutes part of the sympathetic nervous system. ATP is co-localised within postganglionic sympathetic nerve fibres, acting as a co-transmitter alongside noradrenaline (Abbracchio *et al.*, 2009). ATP is released concurrently with noradrenaline from terminal varicosities of sympathetic nerves in the rat prostate gland during stimulation *in vivo*, as well as *in vitro* during low frequency nerve stimulation by electrical field stimulation (Ventura *et al.*, 2003). The predominant P2X1-purinoceptors are activated in the fibromuscular stroma of the rat prostate resulting in contraction of the smooth muscle (Khakh *et al.*, 1995, Soler *et al.*, 2013).

The leaf extract attenuated ATP-induced contraction suggesting an effect at P2X1-purinoceptors. The experiments were therefore repeated using the more potent and stable purinergic receptor agonist, αβ-methylene ATP. Extracellular 5`-nucleotidases rapidly hydrolyse ATP and terminate its activation of purinoceptors. The methylene group present between the α and β phosphate moieties in the structure of αβ-methylene ATP (Figure 3.12) renders greater resistance to this hydrolysis and eventual breakdown. Sequential hydrolyses of the triphosphate tail by extracellular 5`-nucleotidases in the neuroeffector junction of the prostate (Burnstock, 1972, Windscheif, 1996, Konrad *et al.*, 1998) produces, ADP, AMP, and finally adenosine, each of which have a decreasing affinity for P2X1-purinoceptors (Burnstock, 1972). Although similar results were achieved with αβ-methylene ATP, the rapid breakdown of ATP to lower affinity products at the P2X1-purinoceptor, and the greater stability of αβ-methylene ATP, may in part explain the difference in potency of the leaf extract at inhibiting ATP-induced contraction compared to contraction elicited by αβ-methylene ATP. Depending on the type of antagonism, a higher concentration of stinging nettle leaf extract may have resulted in a decrease in the maximum response or alternatively, shifted the curve further to the right for αβ-methylene ATP-induced contraction. Future experiments to investigate this possibility would be both prudent and interesting.

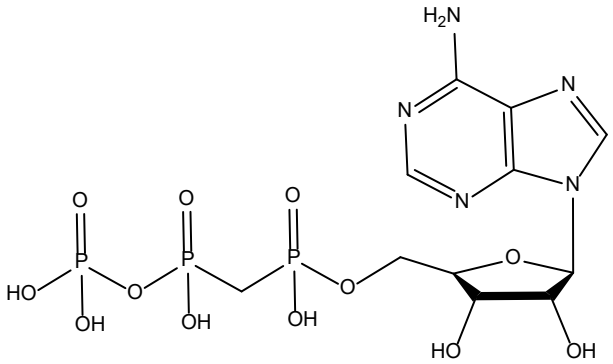


Figure 3.12: Structure of αβ-methylene ATP

P2X1-purinoceptors are desensitised by both ATP and to a greater degree, αβ-methylene ATP. Although this property can be exploited as a valuable tool in investigating the identity of transmitters released from nerves (Kasakov *et al.*, 1982), avoidance of desensitisation was essential in our experiments. P2X1-purinoceptors exposed for twenty seconds to low concentrations of αβ-methylene ATP will initially result in a decrease in contraction (Kasakov *et al.*, 1982) ultimately progressing to an abrogation of excitatory responses (Kasakov *et al.*, 1982). Timely removal of the agonists was of paramount importance to guarantee desensitisation did not occur. Moreover, desensitisation is reversible. Full activity of P2X1-purinoceptors is restored after an appropriate resting period following washout (Kasakov *et al.*, 1982, MacKenzie, Kirkpatrick*, et al.*, 1988). The timely removal of the agonists and an appropriate resting period were implemented during the study, and therefore time did not impact on the ATP- or αβ-methylene ATP-induced contraction over the time course of each experiment.

Our experimental findings infer stinging nettle leaf extract is acting postjunctionally as a P2X1-purinoceptor antagonist. Aqueous stinging nettle extracts have previously been shown to result in non-cholinergic, non-adrenergic bradycardia with a concurrent hypotensive response both *in vitro* and *in vivo* (Legssyer *et al.*, 2002, Dizaye *et al.*, 2013). P2X1-purinoceptor activation in the heart (Hansen, Bennett*, et al.*, 1999) and blood vessels (Hansen, Dutton*, et al.*, 1999) results in both a negative chronotropic effect as well as dilation of coronary and other blood vessels (Drury *et al.*, 1929). The activity of stinging nettle leaf extract may potentially be due to an enhanced inhibitory action on the P2X1-purinoceptor mediated contractile response rather than a direct antagonism of the receptors. The activity, however, was selective for ATP- and αβ-methylene ATP-induced contractions, with a lack of activity observed with noradrenaline- and acetylcholine-induced contraction. These findings

support the suggestion aqueous stinging nettle leaf extract acts postjunctionally on P2X1-purinoceptors.

As the contractile response of prostatic smooth muscle is important in the aetiology of symptomatic BPH, the attenuation of ATP-induced contraction by stinging nettle leaf extract would theoretically have an important role in reducing the contractility of the prostatic smooth muscle in BPH and therefore be a significant consideration in the treatment of lower urinary tract symptoms associated with BPH.

This is a significant finding as although P2X1-purinoceptors are known to be functionally important in a number of physiological systems, there are currently no P2X1-purinoceptor antagonists which are suitable for use *in vivo*. Given the anecdotal evidence suggesting that stinging nettle is a therapeutically useful herbal remedy for a number of varying disorders, this extract may indeed contain a P2X1-purinoceptor antagonist which is viable in intact physiological systems.

Chapter 4

Identification of the Chemical Components of the Bioactive Fractions of *Urtica dioica* (Stinging Nettle) Leaf Extract

Abstract

This study endeavoured to identify chemical components in bioactive fractions of stinging nettle leaf extract. Whole leaf extract (50 ml of 500 mg/ml) was filtered then separated into its aqueous and organic components using distilled ethyl acetate, yielding 59.02 mg and 3.49 g respectively. Each component was tested for bioactivity in isolated organ bath studies. Contractions of isolated rat prostatic tissue induced by electrical field stimulation (0.5 ms, 60 V, 0.1 Hz to 20 Hz for 10 pulses or 10 seconds) and αβ-methylene ATP (0.3 μM – 1 mM) were attenuated by the aqueous (n =6; p = 0.02 and p < 0.0001, respectively), but not the organic, component. Reversed-phase flash chromatography resulted in five fractions of decreasing polarity, of which the most polar fraction attenuated electrical field stimulation-induced contraction (n = 6; p = 0.005). ^{1}H-NMR of this fraction revealed a large number of complex signals consistent with a mixture of mono and disaccharides, as well as numerous minor signals. Further separation using preparative HPLC with a gradient elution, resulted in two adjacent fractions, fractions D1 (n = 4; p = 0.048) and D2 (n = 4; p = 0.033) showing activity against the contractile response of the isolated rat prostate to 1 μM αβ-methylene ATP. Further separation of D1 yielded two fractions, D1.7/8 and D1.9, which exhibited activity against αβ-methylene ATP induced contraction in both the isolated rat prostate (n = 6; p = 0.030 and 0.002 respectively) and the isolated rat vas deferens (n = 6; p = 0.042 and 0.023, respectively). Caffeoylmalic acid was identified within D1.7/8 as the predominant compound, whereas D1.9 was determined to contain 4-hydroxycinnamic acid. Fraction D2 was also separated further yielding two active fractions, D2.5 and D2.6. ^{1}H-NMR spectra and HR-MS indicated the presence of a derivative or isomer of ferulic acid. Although activity of the fractions was observed and various compounds identified, the bioactive responsible for the observed activity is yet to be elucidated.

4.1 Introduction

Commonly containing thousands of compounds, plants are a valuable source of novel treatments to a myriad of disease states. As the use of alternative medication for the symptomatic treatment of disease is increasing (MacLennan *et al.*, 2006, Williamson *et al.*, 2008, Frass *et al.*, 2012), not only is the validation of these therapies progressively becoming more important, but also the identification of biologically active compounds is imperative for the discovery of new medications.

To date, a comprehensive chemical characterisation of the composition of stinging nettle leaf extracts has not been achieved. Characterising the chemical composition of any plant can be difficult. Various biotic and abiotic factors affect the chemical profile with differences occurring due to the cultivation region (Pinelli *et al.*, 2008), plant maturity (Guil-Guerrero *et al.*, 2003), the month of harvest (Grevsen *et al.*, 2008, Nencu *et al.*, 2012), and soil concentrations of nitrogen (Grevsen *et al.*, 2008). Extraction methods may similarly affect the chemical composition. The two main techniques, solid phase extraction (SPE) and liquid-liquid extraction (LLE) have been associated with variations in the recovery of the individual components (for review see (Carrasco-Pancorbo *et al.*, 2005)). Potential biologically active components may be lost both during the extraction process and storage due to oxidation and degradation (Sovova *et al.*, 2004). Likewise, the solvents used for sample separation may affect the reproducibility, quality and recovery of metabolites from plant extracts resulting in up to a 10 to 20-fold difference in the levels of flavonoids and phenolic acids (Farag *et al.*, 2013).

Various techniques have been developed for the analytical processes of separation and elucidation of compounds within plant extracts. Reversed-phase HPLC, using both isocratic and gradient elution procedures, is used preferentially to normal phase HPLC for the separation of compounds as it provides greater reproducibility of retention times and separation of the

most polar compounds (Pirisi *et al.*, 1997, Esti *et al.*, 1998). The coupling of liquid chromatography to mass spectrometry (LC-MS) is a valuable tool in the metabolic profiling of plant extracts. A mass spectrometer is a universal detector and can offer high sensitivity allowing the acquisition of molecular mass data and structural information (Ryan *et al.*, 1999). Negative mode electrospray ionisation mass spectrometry (ESI-MS) is particularly useful and effective in the identification of phenolic compounds offering sensitivity and selectivity particularly for the qualitative and quantitative identification of compounds in complex matrices (Savarese *et al.*, 2007). Nuclear magnetic resonance (NMR) has advanced over the past decade, improving in mass-sensitivity. Today it is indispensable in the structure elucidation and quantification of the low concentrations of compounds within plants with the advent of microcoil and microtube probes (Molinski, 2010a, Molinski, 2010b).

Although there remain numerous unidentified compounds within stinging nettle leaves, many have been detected and characterised. The highest concentration of acetylcholine known to be found in any plant (greater than 10 mg/ml) is found in the hairs present on the leaves (Emmelin *et al.*, 1947). These hairs also contain histamine and 5-hydroxytryptamine (5-HT) (Emmelin *et al.*, 1947, Collier *et al.*, 1956, Oliver *et al.*, 1991). While these three compounds can cause prostatic smooth muscle contraction (Cohen *et al.*, 1989, Ventura *et al.*, 2002) (Kester *et al.*, 2003), the likelihood of them being present in a commercially available extract is low as the activity of all of these compounds decline when stored in extracted liquids at room temperature (Collier *et al.*, 1956, Sletten *et al.*, 2005).

Phenolic compounds are predominant within stinging nettle leaves, both insoluble, found in the cell walls, and soluble, compartmentalised within the plant vacuoles. A minor fraction are present in the free acid form, whereas most present as esters, ethers, or are bound to cellulose, proteins, lignans, flavonoids, glucose, or terpenes. The phenolic acids derived from

hydroxycinnamic acid and hydroxybenzoic acid, as well as derivatives, esterified and glycosidic forms, have all previously been identified in stinging nettle leaves (Budzianowski, 1991, Kraus *et al.*, 1991, Fiamegos *et al.*, 2004, Pinelli *et al.*, 2008, Yildiz *et al.*, 2008, Nencu *et al.*, 2012, Orcic *et al.*, 2014). Bioactive flavonol glycosides, commonly occurring as sugar conjugates, are also present in the leaves (Chaurasia *et al.*, 1987, Kavtaradze *et al.*, 2001, Akbay *et al.*, 2003, Grevsen *et al.*, 2008, Pinelli *et al.*, 2008, Nencu *et al.*, 2012, Orcic *et al.*, 2014) whereas the anthocyanin glycosides are limited to the stalks (Pinelli *et al.*, 2008). Lignans (Kavtaradze *et al.*, 2001) proteins, polysaccharides, neutral sugars and uronic acids (Lichius *et al.*, 1999), aromatic norisoprenoid constituents (Neugebauer *et al.*, 1994, Neugebauer and Schreier, 1995, Neugebauer, Winterhalter*, et al.*, 1995), pigmented carotenoids (Guil-Guerrero *et al.*, 2003, Sovova *et al.*, 2004) and saturated fatty acids (Gansser *et al.*, 1995a, Guil-Guerrero *et al.*, 2003, Farag *et al.*, 2013) have also been detected. Commonly found in the root, *β*-sitosterol, (Sajfrtova *et al.*, 2005, Nahata *et al.*, 2012), thought to play a role in the symptomatic treatment of BPH (Hartmann *et al.*, 1996, Nahata *et al.*, 2012) via inhibition of 5α-reductase (Cabeza *et al.*, 2003, Nahata *et al.*, 2012, Aggarwal *et al.*, 2014), has also been detected in the leaf (Kavtaradze *et al.*, 2001). For a comprehensive summary refer to Chapter 1: General Introduction: section 1.4.1.

This chapter aimed to chemically separate and analyse a commercially available stinging nettle leaf extract to identify and characterise compounds within the extract including those responsible for the effects observed on smooth muscle contractility demonstrated in Chapter 3.

4.2 Methods

4.2.1 Chemical separation and identification

Extracts were chemically separated and analysed as described in Chapter 2, sections 2.6 and 2.7.

4.2.2 Animals and Tissue

Sprague-Dawley rats were obtained as described in Chapter 2, section 2.1.1. They were housed and animal ethics were obtained as per Chapter 2, section 2.1.4.

Rats were euthanized by asphyxiation through exposure to CO_2 gas. Prostate tissue and vasa deferentia and were removed as described in Chapter 2, section 2.3.1.

4.2.3 Isolated organ bath studies

Isolated preparations of rat vas deferens and prostatic tissue were set up for isolated organ bath studies and statistically analysed using the procedure outlined in Chapter 2, section 2.4.

Organic and aqueous phases from liquid-liquid partitioning of the stinging nettle leaf extract were dissolved in 25% ethanol, and made up to an equivalent bath concentration as the original extract tested in Chapter 3, calculated on the mass obtained from the liquid-liquid partitioning (see Appendix A for an example calculation). Using the protocol as described in Chapter 2, section 2.4.2, both the organic and aqueous phases were tested separately in isolated organ bath studies, and frequency-response curves were constructed.

The aqueous phase was tested in a subsequent set of experiments against ATP- and αβ-methylene ATP-induced contractions using the procedure outlined in Chapter 2, section 2.4.3.

The fractions obtained from reversed-phase flash chromatography were made up to four times the equivalent concentration of the whole extract using distilled water. Each fraction was tested separately against electrical field stimulation-induced contraction as per the procedure described in Chapter 2, section 2.4.2 using frequencies 0.5, 1 and 2 Hz only.

The fractions obtained from preparative HPLC were diluted to the required concentration, calculated from the fraction mass (see Appendix A for an example calculation), using 25% ethanol and tested separately in isolated organ bath studies as per the protocol in Chapter 2, section 2.4.3 against a single bath concentration of 1 μM αβ-methylene ATP. Following testing against the agonist, tissues were washed with three to five times the bath volume to remove the test fraction, and the tissue was allowed to rest for thirty minutes before the addition of a single bath concentration of 1 μM αβ-methylene ATP to assess whether the effects of the fractions were reversible.

4.3 Results

4.3.1 Liquid-liquid partitioning

Initially 50 ml of MediHerb stinging nettle leaf extract (500 mg/ml) was filtered and separated into its organic and aqueous components yielding 59.02 mg and 3.49 g respectively. The organic fraction did not exhibit activity (p = 0.978; Figure 4.1A) whereas the aqueous component was found to attenuate electrical field stimulation-induced contractions of isolated rat prostate gland (p = 0.028; Figure 4.1B). In both experiments there was no discernible difference in the contractility of the tissues prior to the administration of either vehicle or test compound (p = 0.996 and p > 0.999, respectively).

To ascertain whether the bioactive or bioactives retained activity at the purinoceptors after partitioning, the aqueous fraction was tested against both ATP- and αβ-methylene ATP-induced contraction. No effect was observed when tested against ATP-induced contraction (p = 0.566; Figure 4.2A). However, attenuation of contraction was apparent when the fraction was tested against αβ-methylene ATP (p < 0.0001; Figure 4.2B).

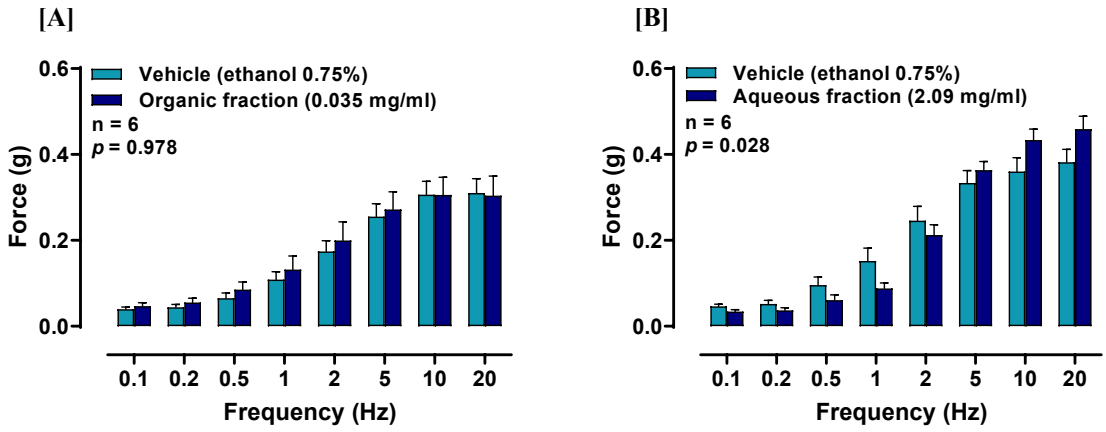


Figure 4.1: Mean contractile responses to electrical field stimulation (0.5 ms, 60 V, 0.1 Hz to 20 Hz for 10 pulses or 10 seconds) of isolated rat prostate gland preparations after the addition of [A] vehicle (ethanol 0.75% v/v) or organic component (35 µg/ml) (p = 0.978) and [B] vehicle (ethanol 0.75% v/v) or aqueous component (2.09 mg/ml) (p = 0.028). Bars represent the mean force generated by prostates taken from six rats. Error bars represent SEM. p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

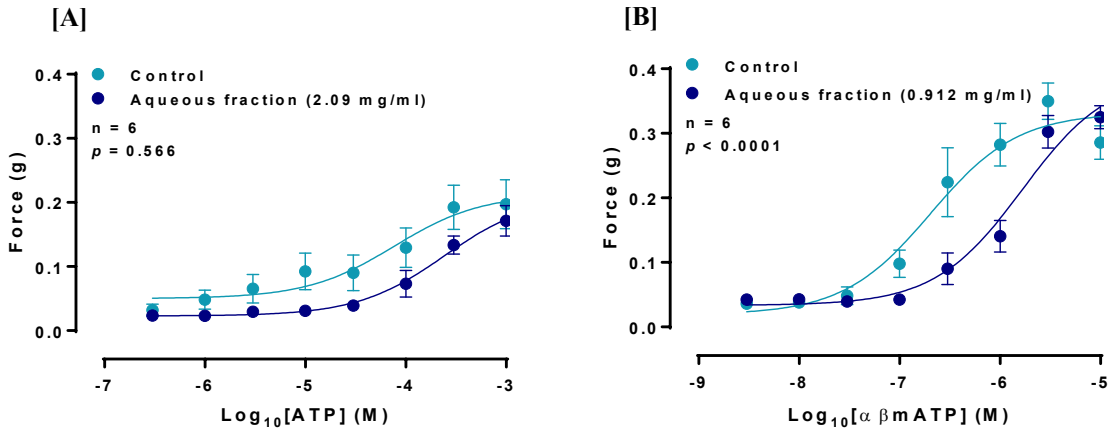


Figure 4.2: Mean contractile response to exogenously administered [A] ATP (0.3 µM – 10 µM in half log unit increments) of isolated rat prostate gland preparations prior to and after the addition of aqueous fraction (2.09 mg/ml) (p = 0.566) and [B] αβ-methylene ATP (3 nM – 10 µM in half log unit increments) prior to or after the addition of aqueous fraction (0.912 mg/ml) (p < 0.0001). Each point represents the mean force generated by prostates taken from six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

4.3.2 Chromatographic fractions

4.3.2.1 Reversed-phase flash chromatography

Stinging nettle leaf extract (160 ml; 500 mg/ml) was eluted sequentially with 10%, 40% and 60% methanol/distilled water, 100% methanol, and 50% methanol/DCM to produce 10.24 g, 0.580 g, 0.334 g, 0.160 g, and 0.352 g respectively of crude fractions (Figure 4.3). Fractions 1 and 3, eluted with 10% and 60% methanol respectively, attenuated electrical field stimulation-induced contraction of isolated rat prostates in organ bath experiments (p = 0.005; Figure 4.4A and p = 0.008; Figure 4.4C, respectively). The remaining fractions, fractions 2, 4, and 5, did not show activity (p = 0.850; Figure 4.4B, p = 0.465; Figure 4.4D, and p = 0.135; Figure 4.4E).

Figure 4.3: Flow chart of separation by reversed-phase column chromatography and resultant fractions.

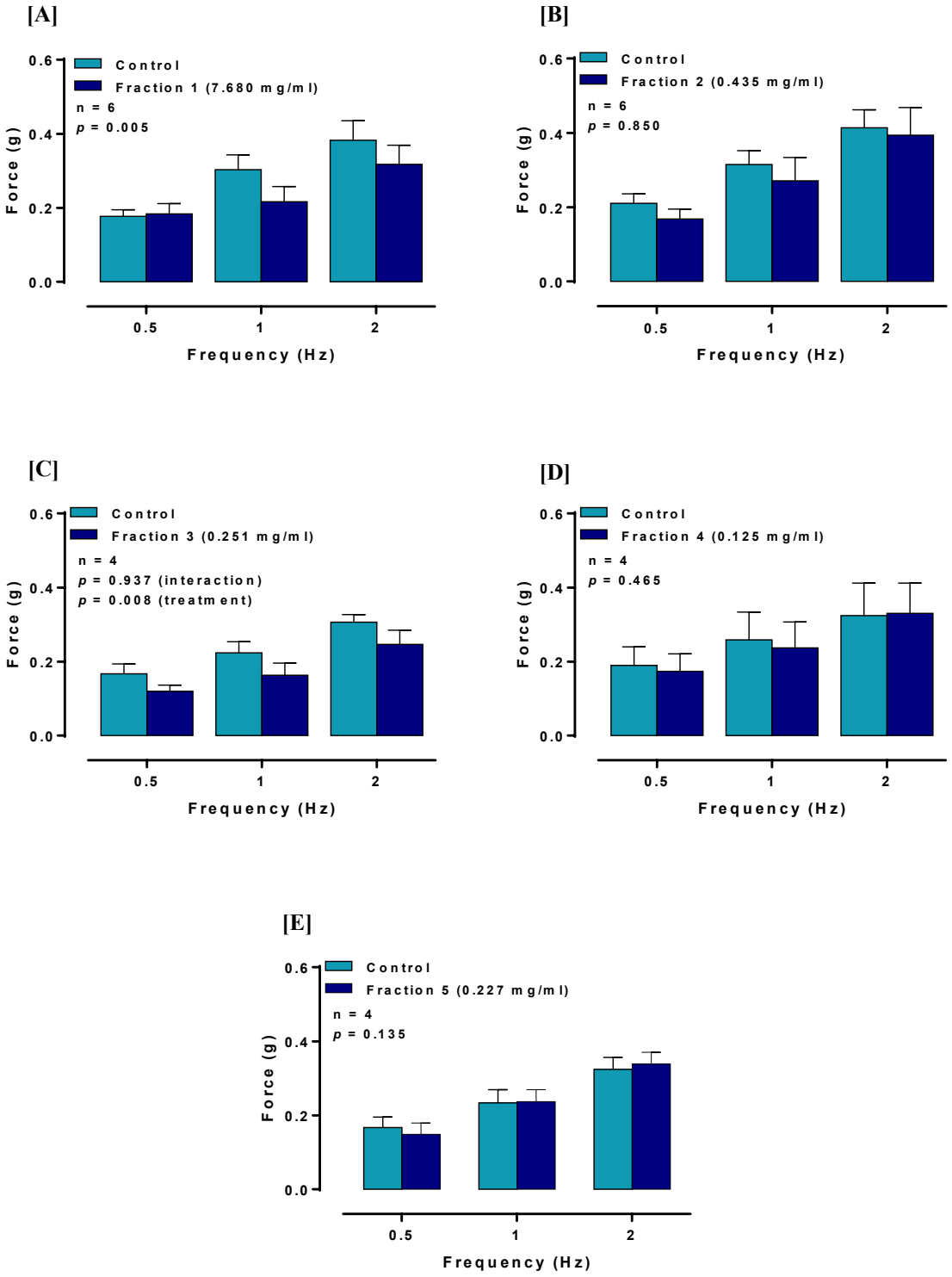


Figure 4.4: Mean contractile response to electrical field stimulation (0.5 ms, 60 V, 0.5 Hz to 2 Hz for 10 pulses) of isolated rat prostate gland preparations prior to and after the addition of [A] fraction 1 (7.680 mg/mL) (p = 0.005), [B] fraction 2 (0.435 mg/mL) (p = 0.850), [C] fraction 3 (0.251 mg/mL) (p = 0.937), [D] fraction 4 (0.125 mg/mL) (p = 0.465), and [E] fraction 5 (0.227 mg/mL) (p = 0.135) Bars represent the mean force generated by prostates taken from four or six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

The ^{1}H NMR spectrum of all the fractions were recorded (for fractions 2-5 see Appendix B). A large number of complex signals between δ 3.22 and 4.05, and an anomeric proton at δ 5.19 (d, J = 3.7 Hz, 1H) (Figure 4.5), consistent with the presence of mono and disaccharides, were present in fraction 1. Numerous minor signals were also observed throughout the spectrum.

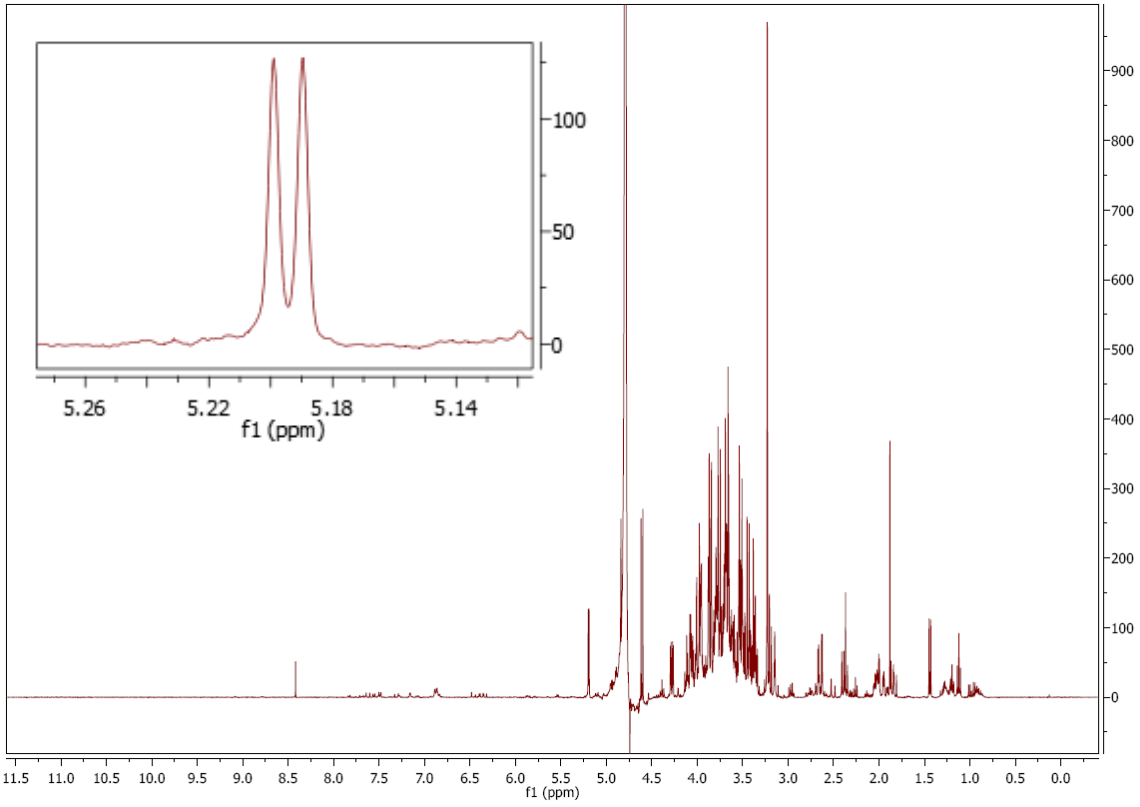


Figure 4.5: Expanded ^{1}H NMR spectra of the active crude fraction 1 of stinging nettle leaf extract in deuterated water. Inset: Anomeric proton at δ 5.19

Preparative HPLC, optimised using a gradient elution, resulted in twelve timed fractions each collected for 2.5 minutes. Two of the fractions, D1 and D2, which eluted between 15 and 17.5 minutes, and 17.5 and 20 minutes, respectively, were found to be active in isolated rat prostates against contraction elicited by a single concentration (1 μM) of αβ-methylene ATP (p = 0.048; Figure 4.6A and p = 0.033; Figure 4.6B respectively). The contractility of the tissues prior to the addition of vehicle or test compound was similar for both fraction D1 (p = 0.764) and D2 (p = 0.981). Inhibition was reversed after washout (p < 0.001 and p = 0.003, respectively).

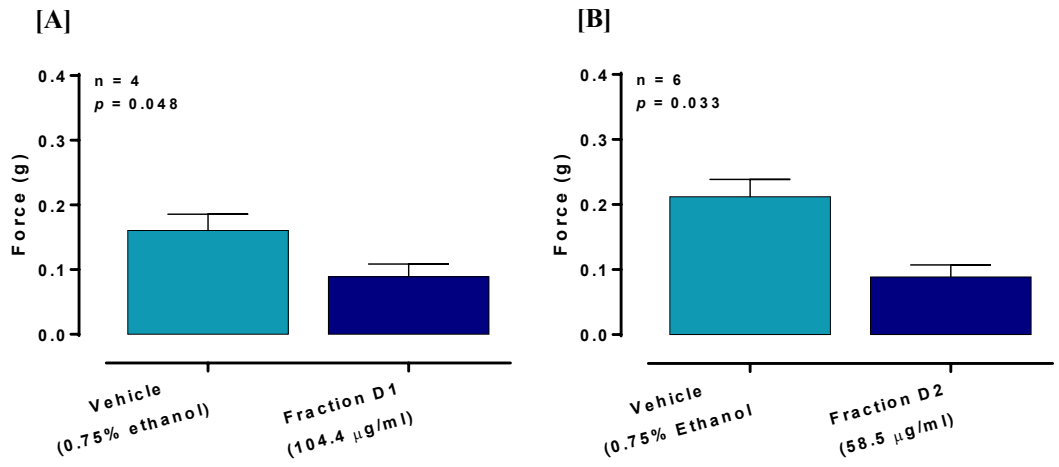


Figure 4.6: Mean contractile response to exogenously administered αβ-methylene ATP (1 µM) of isolated rat prostate gland preparations after the addition of [A] vehicle (0.75% ethanol) or fraction D1 (104.4 µg/mL) (p = 0.048) and [B] vehicle (0.75% ethanol) or fraction D2 (58.5 µg/ml) (p = 0.033). Bars represent the mean force generated by prostates taken from four or six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

An overlay of the NMR spectra for both D1 and D2 in deuterated methanol revealed the presence of overlapping signals (Figure 4.7). Analytical HPLC chromatograms of the fractions displayed a common peak eluting at 11.935 minutes.

The active fraction D1 was further separated using preparative HPLC with an isocratic elution. Twelve fractions were collected either as timed fractions or as eluted peaks.

Two fractions, D1.7, D1.8, which eluted between 14 and 16 minutes, and 16 and 18 minutes, respectively, produced ^{1}H NMR spectra displaying the same major signals. Consequently, the two fractions were combined and treated as a single fraction D1.7/8.

Fraction D1.7/8 was found to be active, displaying inhibition of contraction induced by a single concentration (1 µM) of αβ-methylene ATP in both the isolated rat prostate (p = 0.030; Figure 4.8A) and the isolated vas deferens (p = 0.042; Figure 4.8B). There was no difference in the contractility of the tissues prior to the addition of vehicle or test compound in either the prostate

or the vas deferens ($p = 0.679$; $p = 0.372$, respectively). Inhibition was found to be reversible after washout in both experiments ($p = 0.002$ and $p = 0.007$, respectively).

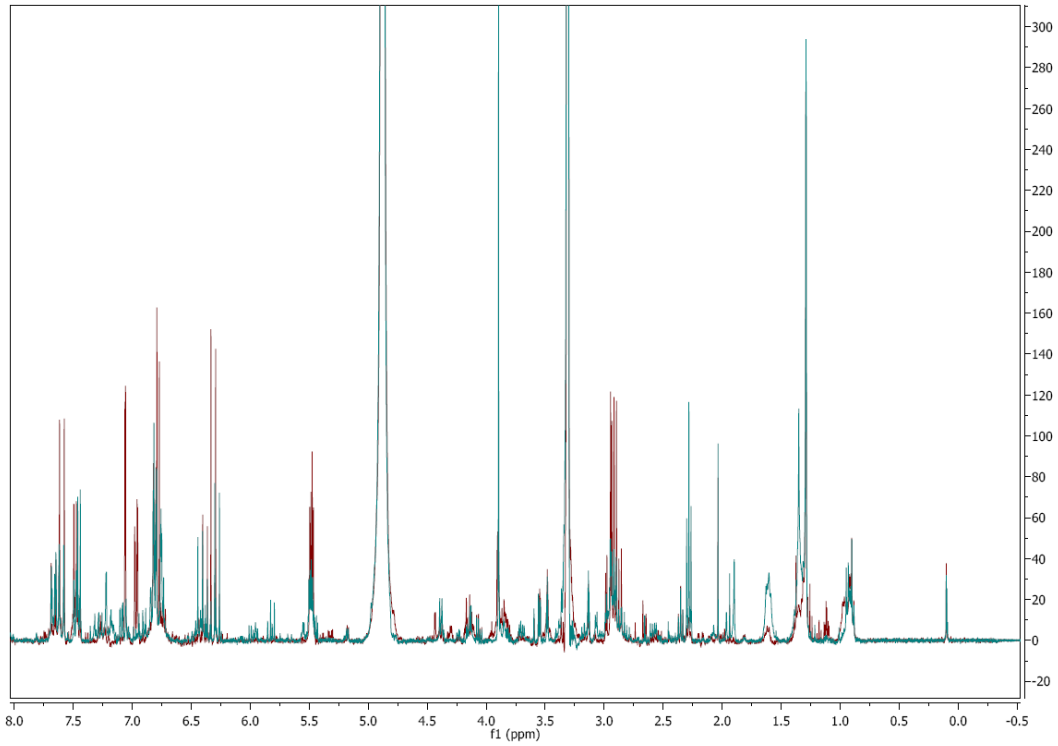


Figure 4.7: Superimposed expanded ^{1}H NMR spectra of the overlaid active fractions D1 (red) and D2 (blue) in d_4-methanol.

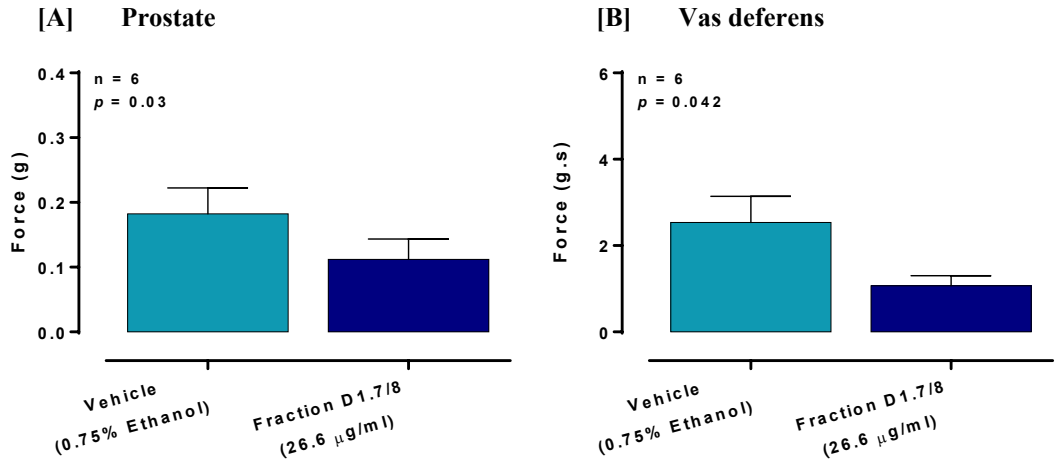


Figure 4.8: Mean contractile response to exogenously administered αβ-methylene ATP (1 µM) of isolated rat [A] prostate gland and [B] vas deferens preparations after the addition of vehicle (0.75% ethanol) or fraction D1.7/8 (26.6 µg/ml) ($p = 0.030$ and $p = 0.042$, respectively). Bars represent the mean force generated by prostates or vasa deferentia taken from six rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

Within fraction D1.7/8, high resolution mass spectrometry (HR-MS) in negative mode revealed peaks at *m/z* 295, *m/z* 179, *m/z* 135, and *m/z* 133 for a compound with a molecular mass of 296.0541 g/mol and a suggested a molecular formula of $C_{13}H_{12}O_8$. These peaks are characteristic of the pseudomolecular ion and fragmentation pattern of caffeoylmalic acid (Harbaum *et al.*, 2007) (Figure 4.9).

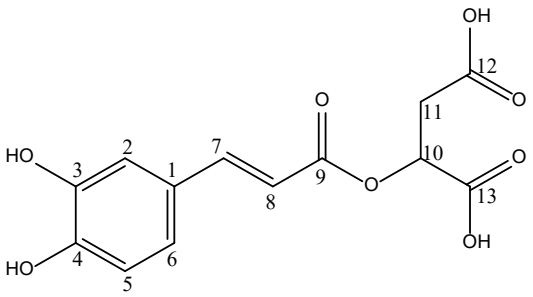


Figure 4.9: Structure of caffeoylmalic acid.

A postulated fragmentation scheme for caffeoylmalic acid *m/z* 295 $[M-H]^-$ is the formation of the pseudomolecular ion for caffeic acid *m/z* 179 $[M-H-A]^-$ from the loss of a neutral fragment $C_4H_4O_4$ (Figure 4.10A). The loss of CO_2 from caffeic acid gives rise to a characteristic peak at *m/z* 135 (Savarese *et al.*, 2007, Abu-Reidah *et al.*, 2013). The fragmentation of caffeoylmalic acid produces a peak at *m/z* 133 (Abu-Reidah *et al.*, 2013). This suggests caffeoylmalic $[M-H]^-$ may undergo a second fragmentation pathway forming the pseudomolecular ion for malic acid *m/z* 133 $[M-H-B]^-$ and the neutral molecule $C_9H_6O_3$ (Figure 4.10B). Both the fragmentation of caffeoylmalic acid and malic acid give rise to a peak at *m/z* 115 (Abu-Reidah *et al.*, 2013). Although a peak at *m/*z 115 was not observed with our sample, this reported fragment further supports the generation of the malic acid pseudomolecular ion in the proposed fragmentation pathway of caffeoylmalic acid (Figure 4.10B).

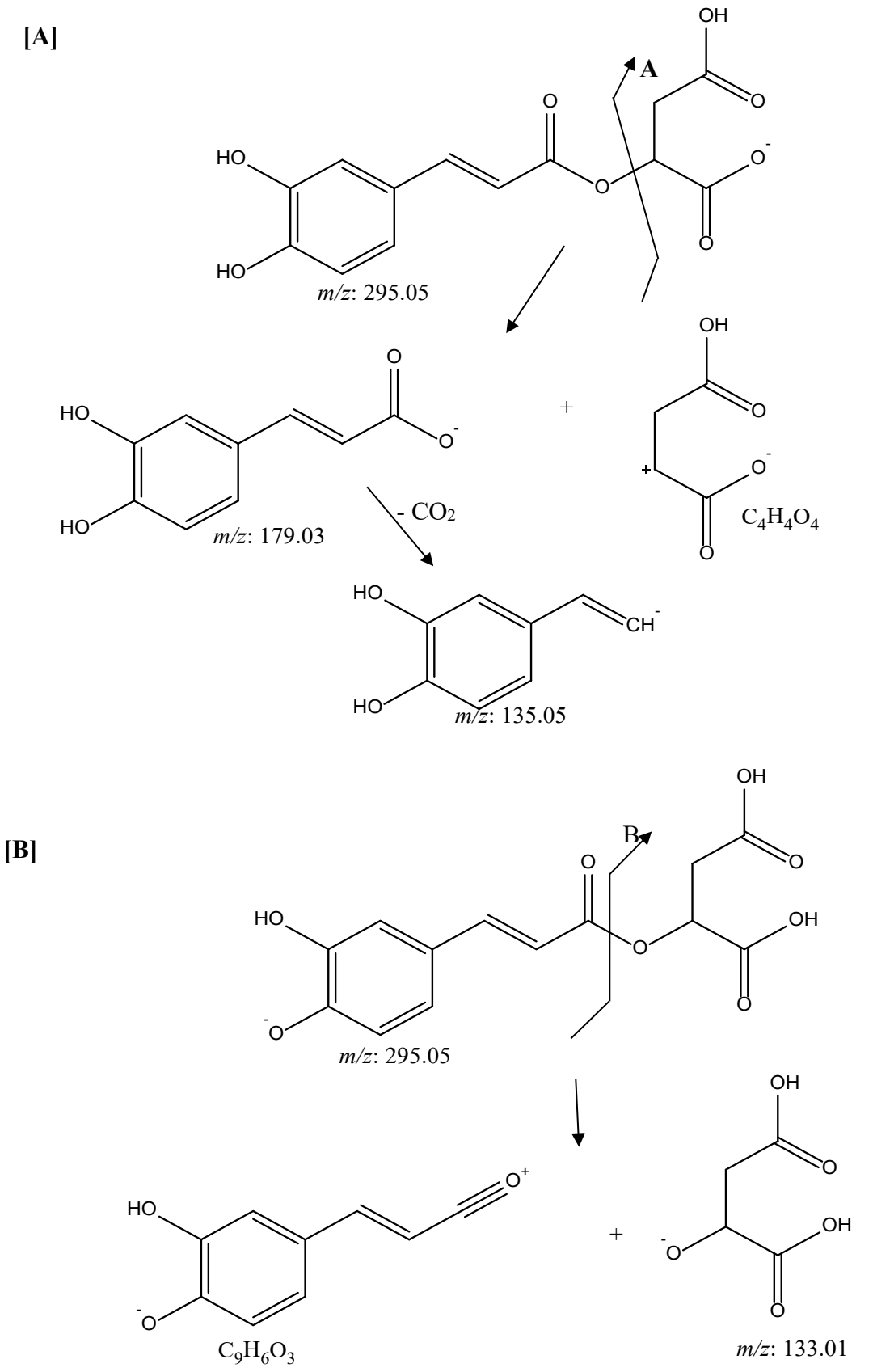


Figure 4.10: Proposed fragmentation patterns for caffeoylmalic acid in the negative mode. [A] The molecular ion *m/z* 295 $[M-H]^-$ gives rise to *m/z* 179 $[M-H-A]^-$ by the loss of the neutral fragment $C_4H_4O_4$. The subsequent loss of CO_2 results in *m/z* 135 $[M-H-A-CO_2]^-$. [B] The molecular ion *m/z* 295 $[M-H]^-$ gives rise to *m/z* 133 $[M-H-B]^-$ and the neutral molecule $C_9H_6O_3$.

A ^{1}H NMR spectrum of fraction D1.7/8 indicated one major compound and several minor compounds (Figure 4.11).

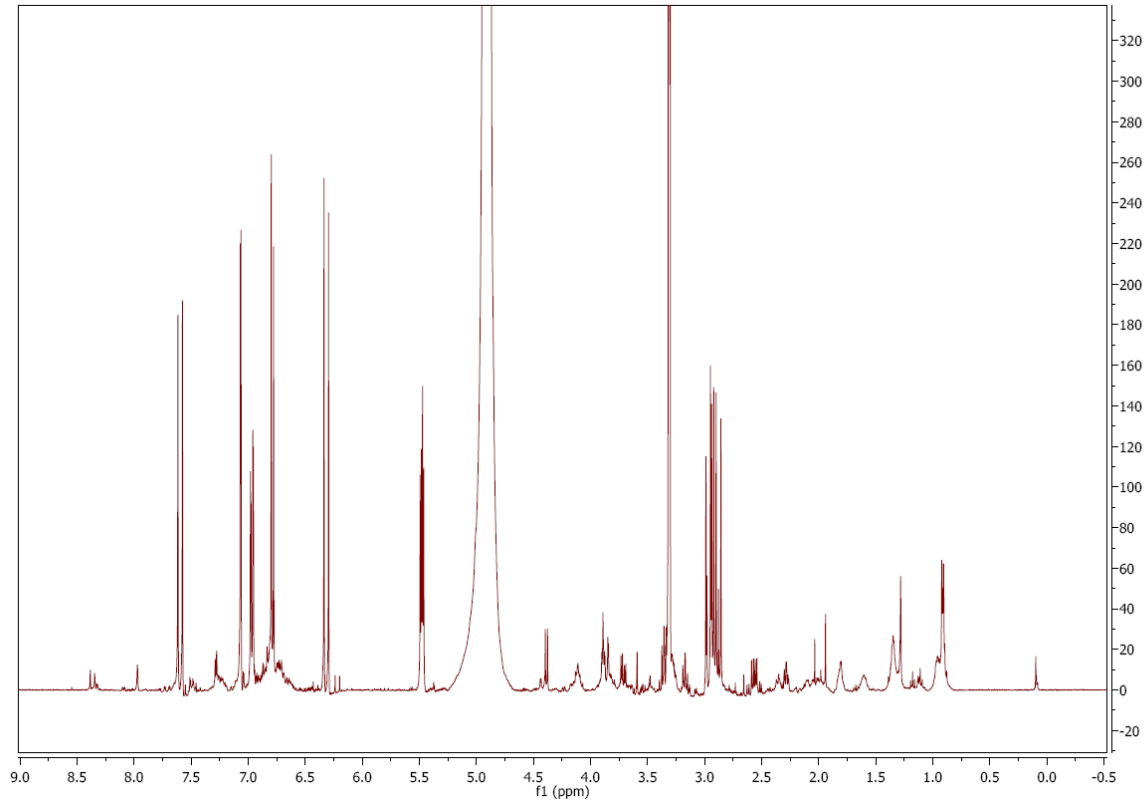


Figure 4.11: Expanded ^{1}H NMR spectrum of the active fraction D1.7/8 in d_4-methanol.

The positions of protons for the proposed compound in fraction D1.7/8 refer to those given on the structure in Figure 4.9, and the ^{1}H NMR signals refer to those shown in the expansions in Figure 4.12, Figure 4.13, and Figure 4.14. The pair of doublets at δ 7.60 (d, J = 15.9 Hz, 1H) and δ 6.31 (d, J = 15.9 Hz, 1H) are consistent with trans double bond protons of cinnamic acids such as those at positions 7 and 8. Characteristically large J values are seen with these protons. The signal at δ 7.60 is indicative of a proton at position 7, as a proton in this position would undergo a greater downfield shift due to the proximity of the phenyl group. Subsequently the signal at δ 6.31 denotes a proton in position 8. The signals at δ 7.07 (s, J = 2.0 Hz, 1H), δ 6.97 (d, J = 8.2, 2.0 Hz, 1H) and δ 6.79 (d, J = 8.2 Hz, 1H) are consistent with aromatic protons at positions 2, 5, and 6 respectively. The small J value of 2 Hz of the signal at δ 7.07 is indicative

of *J*-coupling by a proton in the para position. This coupling causes a fine splitting of the singlet. The doublets at δ 6.79 and δ 6.97 with *J* values of 8.2 Hz are typical of ortho-coupling. The additional splitting of the doublet at δ 6.97 with the *J* value of 2 Hz indicates a proton coupled to protons in both the ortho and para positions.

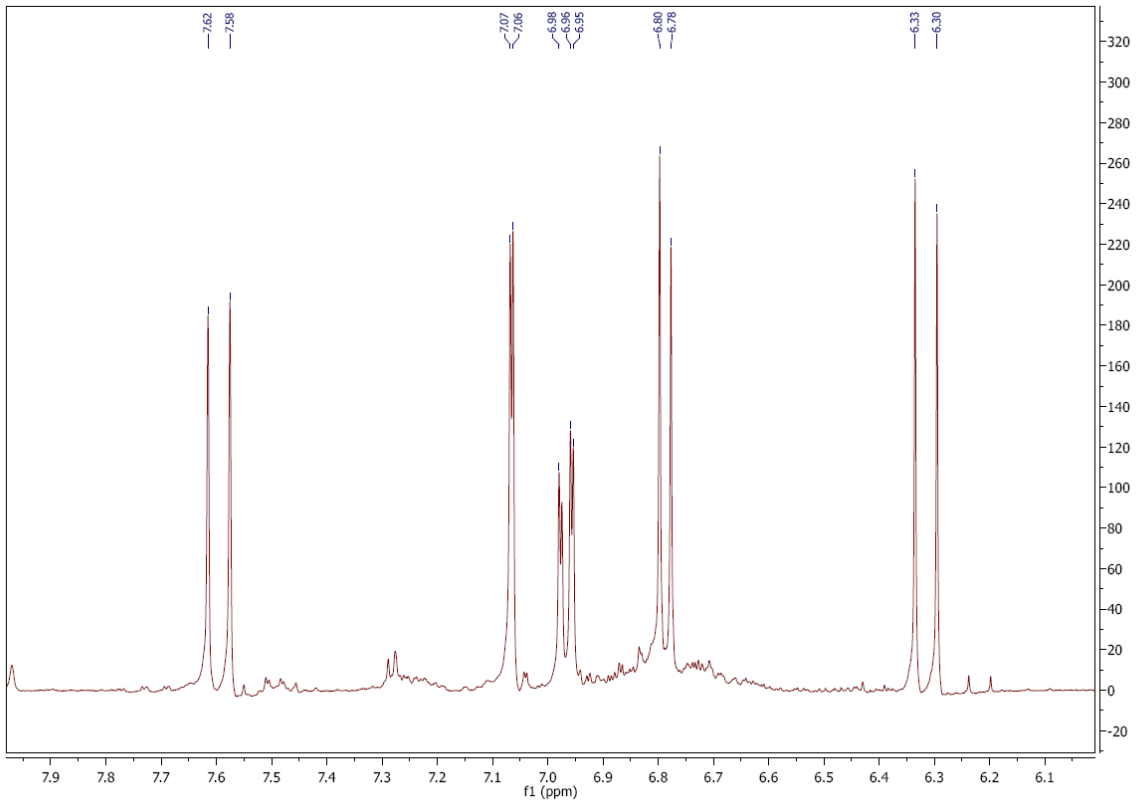


Figure 4.12: Expanded ^{1}H NMR spectrum of the active fraction D1.7/8 in d_4-methanol from δ 6.00 to δ 8.00.

The doublet of doublets at δ 5.48 (*J* = 8.4 Hz and 4.2 Hz, 1 H) may be attributed to a 3-bond coupling to two different protons, with typical *J* values ranging between 4 and 8 Hz. These signals correlate to a proton at position 10 within the malic acid moiety. As a proton at this position is attached to a chiral carbon, the time-weighted average environment of the two protons at position 11 will be slightly different. Although internal rotation helps to equalise the two protons, as it is a diastereotopic CH_2 group, they are never fully equivalent. A proton at position 10 would couple with both these protons resulting in a doublet of doublets.

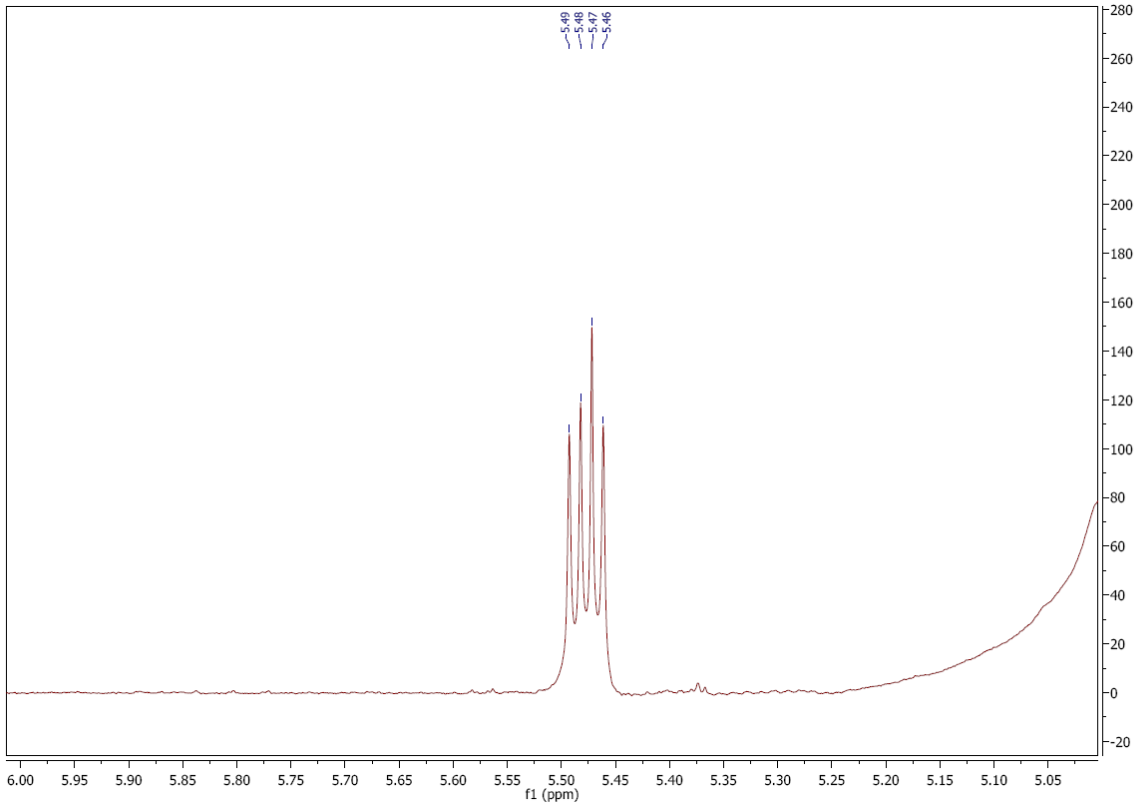


Figure 4.13: Expanded ^{1}H NMR spectrum of D1.7/8 in d_4-methanol from δ 5.00 to δ 6.00.

The signals observed at δ 2.93 (J = 16.2 Hz and 3.8 Hz, and J = 16.7 Hz and 8.4 Hz, 2 H) have J values commonly seen with both geminal coupling where high J values of 15 to 16 Hz are seen, and 3-bond coupling where J values are typically between 4 and 8 Hz. Diastereotopic protons, such as those at position 11 couple to each other. Additional coupling with a proton at position 10 will result in eight peaks composed of two doublets of doublets. Proton-proton couplings were confirmed with 2D COSY NMR. These major signals support the proposal that the predominant compound within this fraction is caffeoylmalic acid.

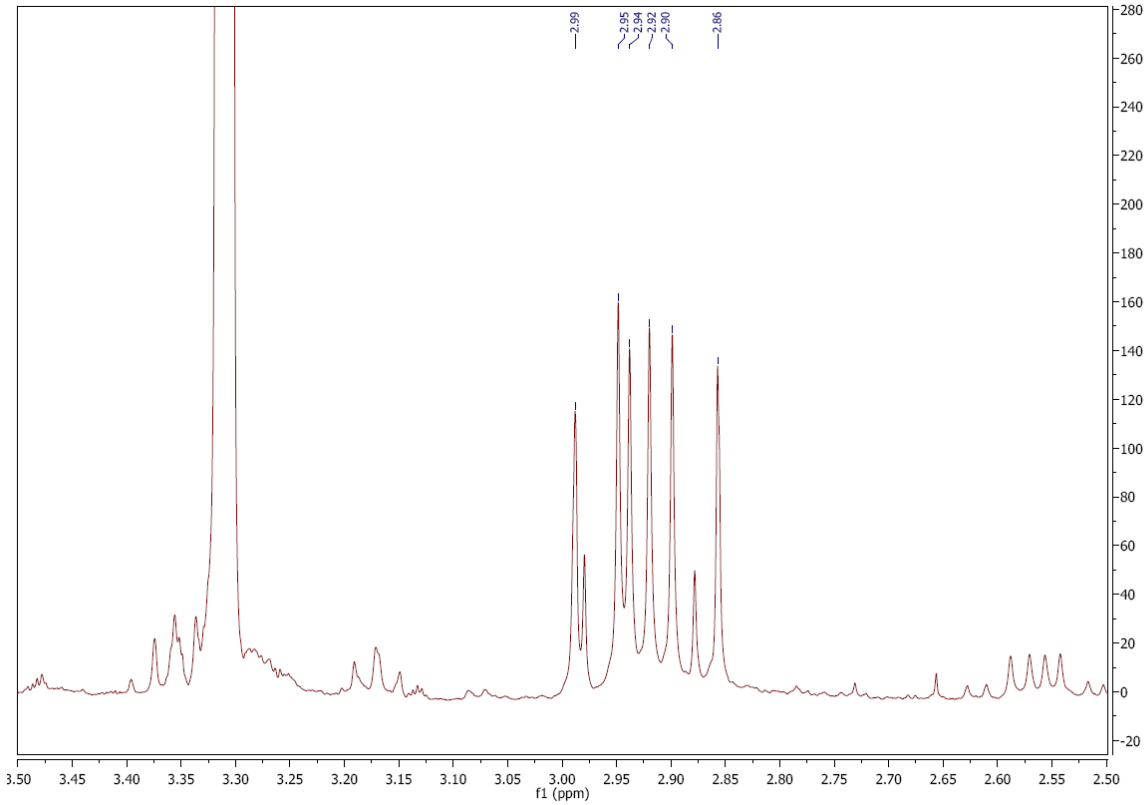


Figure 4.14: Expanded ^{1}H NMR spectrum of D1.7/8 in d_4-methanol from δ 2.50 to δ 3.50.

The structure of caffeoylmalic acid was further supported by ^{13}C NMR (Figure 4.15), and HSQC. Signals at δ 127.6, δ 115.2, δ 147.9, δ 146.8, δ 116.5, and δ 123.2, are indicative of benzene carbons (1-6, respectively), while signals at δ 149.8 and δ 114.2 are typical of the carbons found at positions 7 and 8, respectively. The chemical shift of esters and carboxylic acids commonly lie between δ 155 and δ 185 (Pavia *et al.*, 2001). Of the three signals within this range, an ester carbon at position 9 is most likely to have a signal of δ 173.1. The presence of the two oxygens and adjacent alkene carbon at position 8 would move this signal downfield with the greatest chemical shift. The two carboxylic carbons at positions 12 and 13 are both attached to secondary carbons (C11 and C10, respectively) however the signal at δ 172.8 may be assigned to a carbon at position 13 as the oxygen attached to C10 will shift the signal slightly downfield. The remaining signal at δ 168.1 may be assigned to the carbon at position 12. The saturated carbon at position 10 is in the presence of an attached electronegative oxygen which

will slightly shift the signal downfield so may be assigned to the signal at δ 70.0, leaving the saturated carbon without any electronegative effects at position 11 to be allocated the signal at δ 37.1. The assigned carbons and protons were further supported by HSQC where the protons were observed to be connected to their respective carbons as per the positions allocated.

Table 4.1: Proton and carbon assignments for caffeoylmalic acid.

Position assignment[a]	ppm (H)	Multiplicity	*J* (Hz)	Integration	ppm (C)
1	-	-	-	-	127.6
2	7.07	s	$J_{2,5} = 2.0$	1H	115.2
3	-	-	-	-	147.9
4	-	-	-	-	146.8
5	6.97	d	$J_{5,6} = 8.2$ $J_{5,2} = 2.0$	1H	116.5
6	6.79	d	$J_{6,5} = 8.2$	1H	123.2
7	7.60	d	$J_{7,8} = 15.9$	1H	149.8
8	6.31	d	$J_{8,7} = 15.9$	1H	114.2
9	-	-	-	-	173.1
10	5.48	dd	$^{3}J_{10,11} = 8.4$ $^{3}J_{10,11'} = 4.2$	1H	70.0
11	2.93	dd	$^{2}J_{11,11'} = 16.7$ $^{2}J_{11',11} = 16.2$ $^{3}J_{11,10} = 8.4$ $^{3}J_{11',10} = 3.8$	2H	37.1
12	-	-	-	-	168.1
13	-	-	-	-	172.8

[a] Positions refer to Figure 4.9

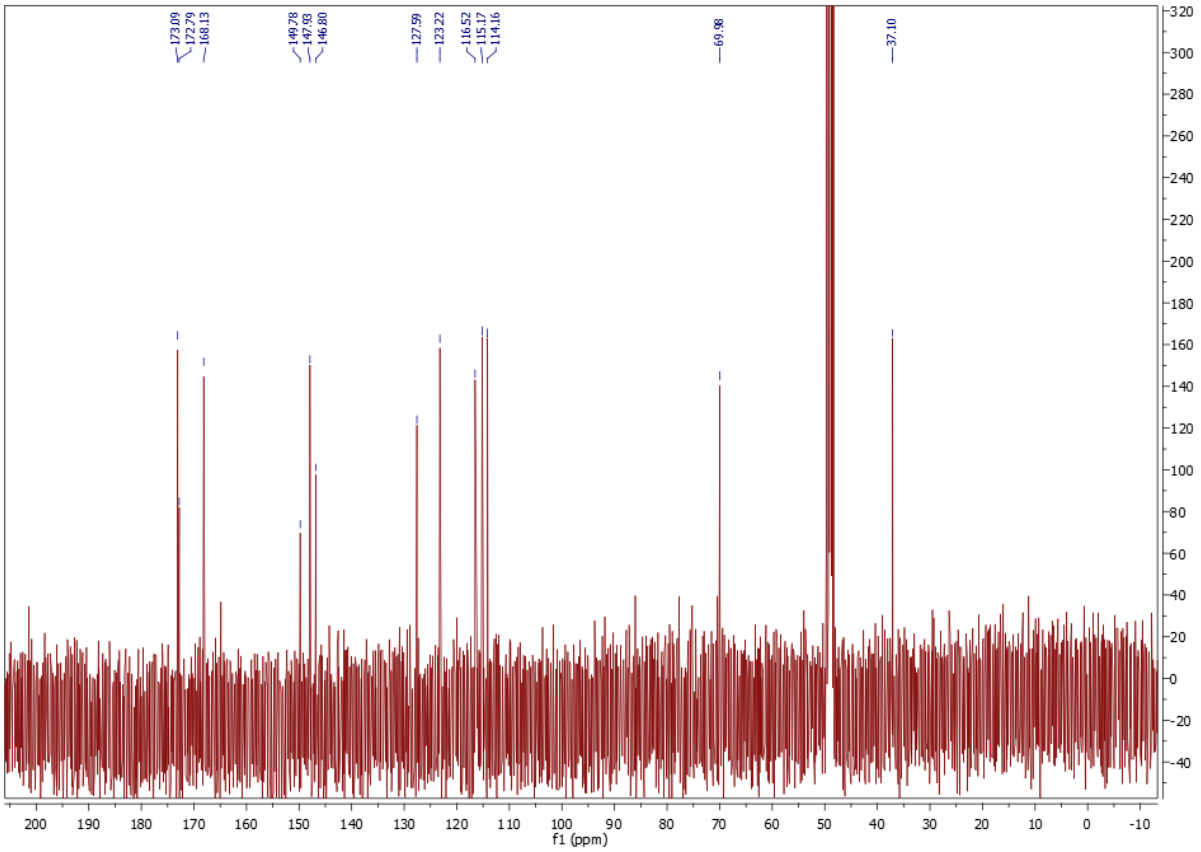


Figure 4.15: Expanded ^{13}C NMR spectrum of D1.7/8 in *d*$_4$-methanol from δ 20.00 to δ 180.00.

A second fraction, D1.9, eluted between 18 and 22 minutes, was also found to be active against contraction elicited by a single concentration (1 μM) of αβ-methylene ATP in both the isolated rat prostate and isolated vas deferens (p = 0.002; Figure 4.16A and p = 0.023; Figure 4.16B). For both fractions, in both the prostate and the vas deferens, the contractility of the tissue before addition of vehicle or fraction was found to be similar (p = 0.949; p = 0.636 respectively). Reversal of inhibition was observed after washout in the vas deferens (p = 0.05) but not prostate (p = 0.139).

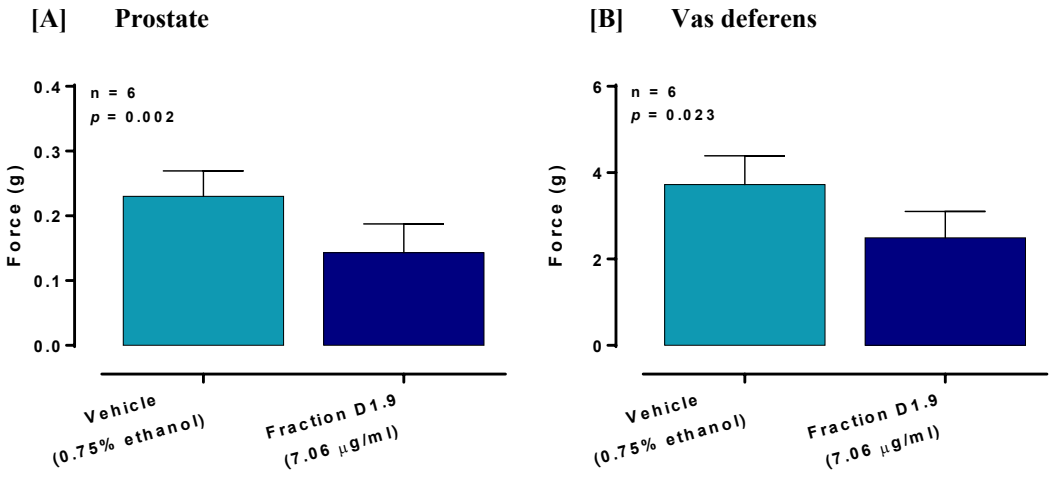


Figure 4.16: Mean contractile response to exogenously administered αβ-methylene ATP (1 µM) of isolated rat [A] prostate gland and [B] vas deferens preparations after the addition of vehicle (0.75% ethanol) or fraction D1.9 (7.06 µg/ml) (p = 0.002 and p = 0.023, respectively). Bars represent the mean force generated by prostates or vasa deferentia taken from six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

A ^{1}H NMR spectrum of fraction D1.9 also indicated this fraction contained one major and several minor compounds (Figure 4.17).

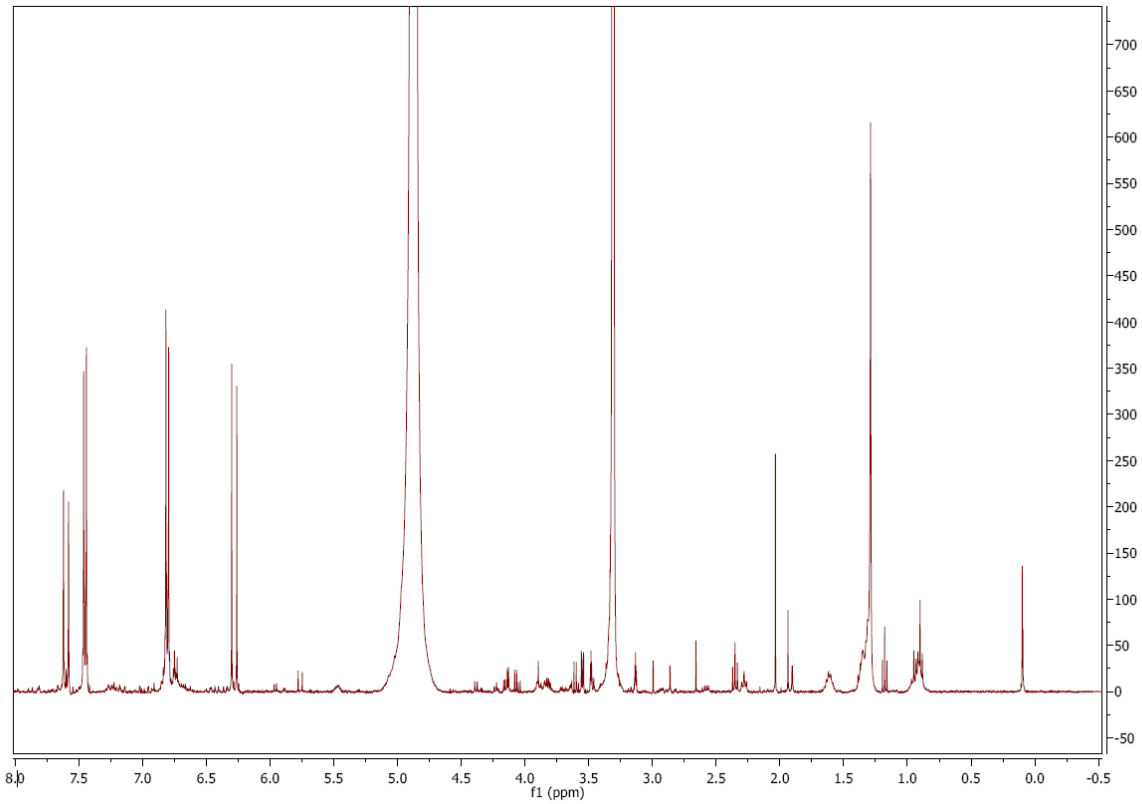


Figure 4.17: Expanded ^{1}H NMR spectrum of the active fraction D1.9 in d_4-methanol.

The signals observed are indicative of a hydroxycinnamic acid such as *p*-coumaric acid (Figure 4.18) with signals exhibited at δ 7.60 (d, *J* = 15.9 Hz, 1H) and δ 6.28 (d, *J* = 15.9 Hz, 1H) (Figure 4.19) commonly observed with protons at positions 7 and 8 respectively. Signals at δ 6.81 (d, *J* = 8.6 Hz, 2H) and δ 7.45 (d, *J* = 8.5 Hz, 2H) are typically seen from protons at positions 2 and 3, and 5 and 6, respectively on the phenyl ring (Figure 4.18). The shape of the doublets (Figure 4.18 inset) are characteristic of a para-disubstituted phenyl ring. Although each signal retains the gross doublet shape predicted by first-order analysis, additional peaks are apparent.

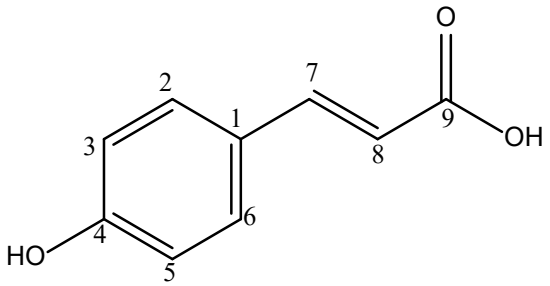


Figure 4.18: Structure of 4-hydoxycinnamic acid or *p*-coumaric acid.

Superimposition of a ^{1}H NMR spectrum of a commercially available authentic sample of *p*-coumaric acid and our sample, demonstrated the signals to be equivalent (Appendix C).

HR-MS analysis supported the presence of *p*-coumaric acid in fraction D1.9. A compound of molecular mass of 164.0174 g/mol, with a suggested molecular formula of $C_9H_8O_3$, a pseudomolecular ion peak at *m/z* 163, and a peak at *m/z* 119, likely due to a loss of CO_2 (-44), were all observed.

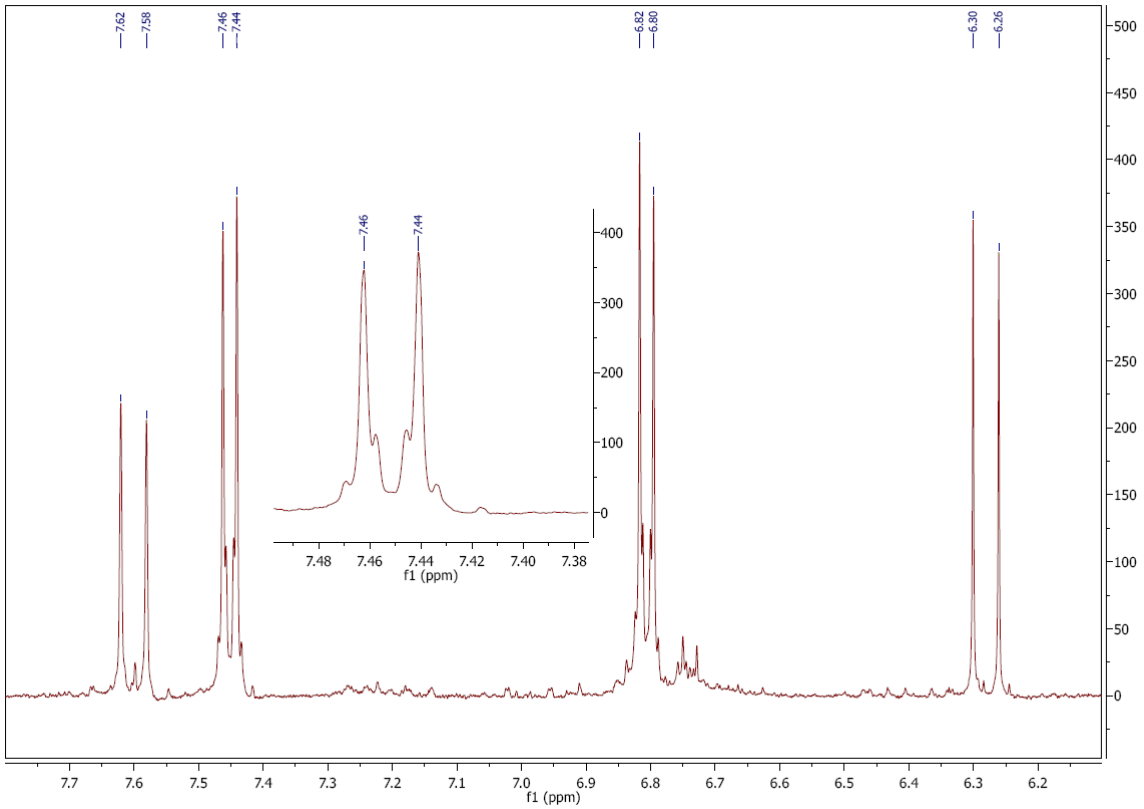


Figure 4.19: Expanded ^{1}H NMR spectrum of the active fraction D1.9 in d_4-methanol from δ 6.00 to δ 7.80. Inset: expansion of doublet at δ 7.45 showing the characteristic shape of a para-disubstituted phenyl ring.

Although the predominant compound within this fraction was found to be *p*-coumaric acid, a commercial sample at increasing concentrations (1 nM to 0.1 mM) did not exhibit inhibitory activity against αβ-methylene ATP- induced contraction (1 μM) in isolated rat prostate glands or vas deferens (Appendix D).

The second active fraction D2, was also separated using preparative HPLC resulting in fifteen fractions, of which two, D2.5 and D2.6, were found to be active against a single bath concentration of 1 μM αβ-methylene ATP-induced contraction in the isolated rat prostate gland (p = 0.017; Figure 4.20A, and p = 0.010; Figure 4.20B). Inhibition was reversed after washout for D2.6 (p = 0.021).

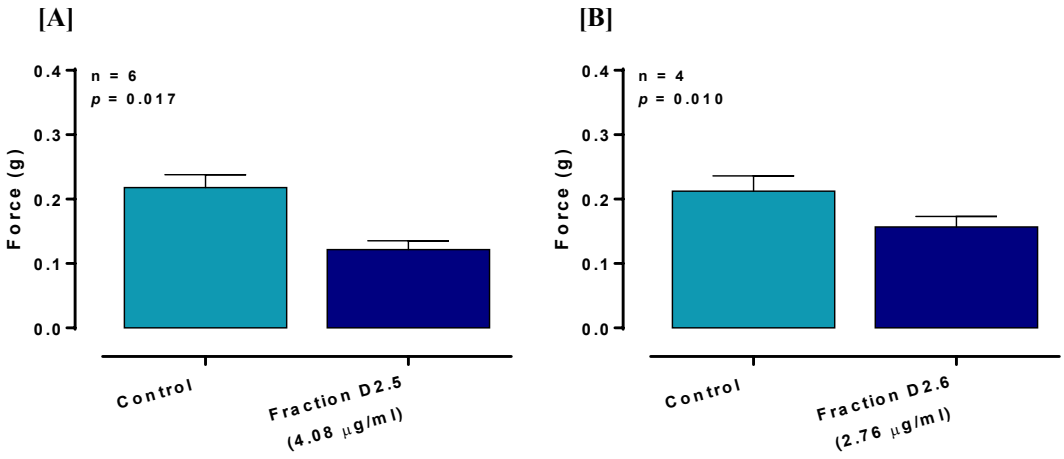
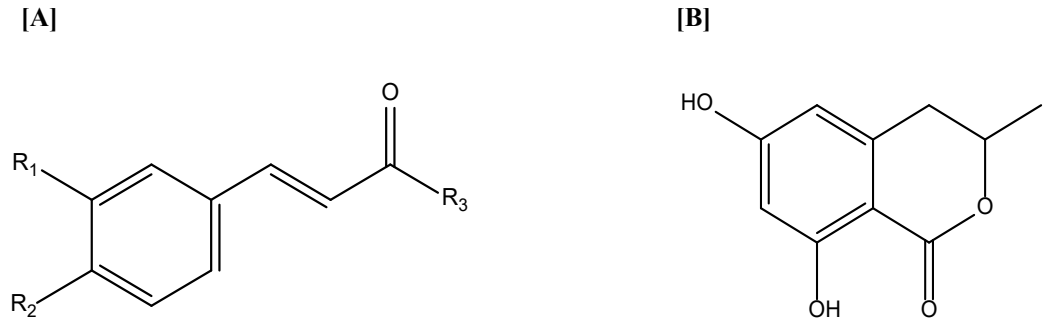


Figure 4.20: Mean contractile response to exogenously administered αβ-methylene ATP (1 µM) of isolated rat prostate gland preparations prior to and after the addition of [A] fraction D2.5 (4.08 µg/ml) (p = 0.026) and [B] fraction D2.6 (2.76 µg/mg) (p = 0.010). Bars represent the mean force generated by prostates taken from four or six rats. Error bars represent SEM. ANOVA p-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.



Compound	R_1	R_2	R_3
3-OH, 4-MeO cinnamic acid	-OH	$-OCH_3$	-OH
Methyl caffeate	-OH	-OH	$-OCH_3$

Figure 4.21: Structures of [A] cinnamic acid derivatives [B] 6-hydroxymellein.

HR-MS data for both fractions revealed the presence of a compound with a molecular weight of 194.058 g/mol and a suggested molecular formula of $C_{10}H_{10}O_4$. These data are consistent with the presence of possible structures related to substituted cinnamic acids including ferulic

acid, 3-hydroxy,4-methoxy-cinnamic acid, and methyl caffeate, or a dihydroisocoumarin such as 6-hydroxymellein (Figure 4.21).

The ^{1}H NMR spectra of D2.5 and D2.6 indicated the presence of two or more separate compounds for each fraction. Signals at δ 3.88 (s,3H) and at δ 3.87 (s,3H) for fractions D2.5 and D2.6, respectively, indicated the presence of a methoxy group. Signals at δ 7.72 (d, J = 16.01 Hz, 1H) and δ 6.44 (d, J = 16.0 Hz, 1H), and δ 7.65 (d, J = 16.0 Hz, 1H) and δ 6.36 (d, J = 15.9 Hz, 1H) respectively in the D2.5 spectrum, are consistent with trans double bond protons in two separate compounds. Similar peaks at δ 6.43 (d, J = 16.3 Hz, 1H) and δ 7.67 (d, J = 16.3 Hz, 1H) were observed in the ^{1}H NMR spectrum for D2.6. Additionally, a 2D COSY on fraction D2.6 indicated the presence of two ortho, meta di-substituted phenyl compounds.

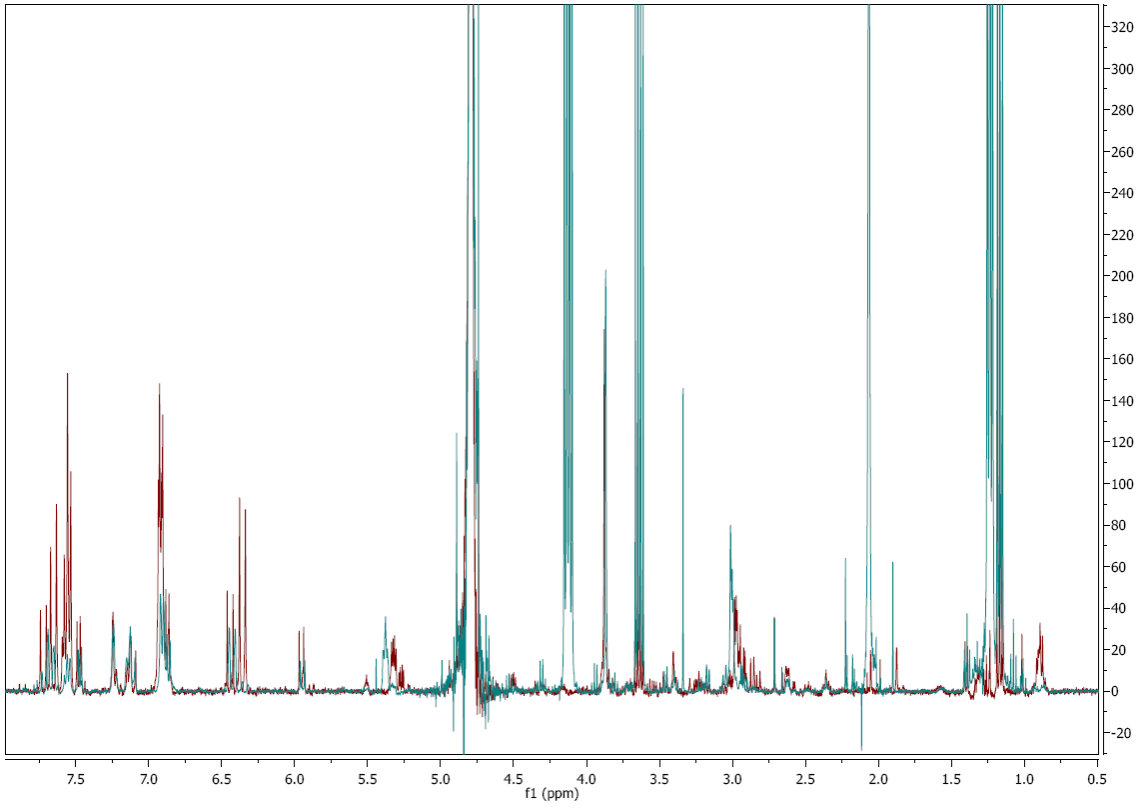


Figure 4.22: Superimposed expanded ^{1}H NMR spectra of the overlaid active fractions D2.5 (red) and D2.6 (blue) in d_4-methanol.

When the ^{1}H NMR spectrum of an authentic sample of ferulic acid was compared with spectra from both fractions (Appendix C), differences in chemical shifts were apparent, however overall the number and types of signals were very similar. This suggests the presence of isomers and/or derivatives of ferulic acid.

HR-MS analysis of both fractions revealed a compound with a molecular weight of 146.0372 g/mol and a molecular formula of $C_9H_6O_2$. This would be consistent with a coumarin isomer or analogue. An observed mass of 176.0471 g/mol with a suggested molecular formula of $C_{10}H_8O_3$ was also present in fraction D2.6 consistent with a methoxy analogue or isomer of, coumarin (Figure 4.23).

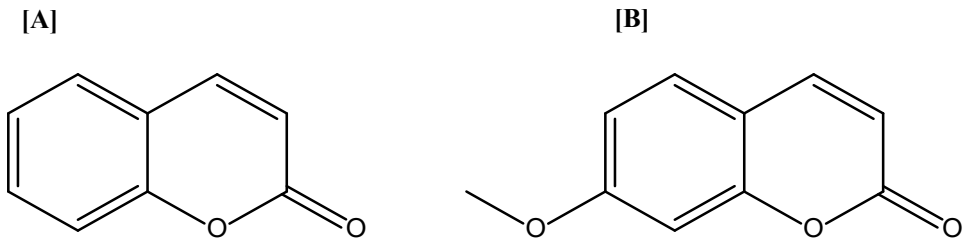


Figure 4.23: Structures of [A] coumarin and [B] 7-methoxy coumarin – an example of a methoxy analogue of coumarin.

4.4 Discussion

The determination and characterisation of the active components within plant extracts such as stinging nettle leaf, can be challenging. Many molecules are still unidentified; therefore, it is difficult to compare with data in the literature. To date, only a limited number of active compounds in stinging nettle leaf found to be beneficial in the symptomatic treatment of BPH, have been identified and characterised. The compounds that have been identified as active appear to affect the hyperplasia associated with BPH (Lichius *et al.*, 1997, Lichius *et al.*, 1999). Although the mechanism of action of this remains unclear, it is likely the effect of stinging nettle extracts is through activity on sex hormone binding globulin (SHBG) (Gansser *et al.*,

1995b, Hryb *et al.*, 1995, Schottner *et al.*, 1997, Safarinejad, 2005), aromatase (Gansser *et al.*, 1995a, Hartmann *et al.*, 1996), epithelial growth factor (EGF) (Wagner *et al.*, 1994, Wagner *et al.*, 1995), and/or 5α-reductase (Hartmann *et al.*, 1996, Nahata *et al.*, 2014).

There is a paucity of literature identifying compounds from stinging nettle leaf which have an effect on the contractility of prostatic smooth muscle. In Chapter 3, stinging nettle leaf extract was found to attenuate αβ-methylene ATP-induced contraction in the isolated rat prostate gland. In this chapter separation and analytical chemical techniques were used in an attempt to elucidate the active compound responsible for the observed activity. Isolated organ bath experiments were undertaken to monitor bioactivity throughout the separation and isolation processes.

Liquid-liquid partitioning of the leaf extract revealed over 98% of the recovered mass to be in the aqueous phase, with less than 2% in the organic. Traditional medicine commonly prepares barks, leaves and plant materials as teas (Akbay *et al.*, 2003, Calvo *et al.*, 2011, Hayta *et al.*, 2014, Menendez-Baceta *et al.*, 2014), therefore it is logical to predict bioactives may be contained in aqueous extracts. The aqueous phase was found to be pharmacologically active indicating the bioactive is hydrophilic.

The aqueous fraction was observed to attenuate αβ-methylene ATP-, but not ATP-, induced contraction in the isolated rat prostate. Extracellular ATPases and 5'-nucleotidases cause the rapid breakdown of ATP to ADP, AMP, and adenosine. The presence of a methylene group between the α and β phosphate groups renders the more stable agonist, αβ-methylene ATP, resistant to enzymatic breakdown. This implies higher concentrations of ATP, compared to αβ-methylene ATP, are required to achieve equipotent contraction of the smooth muscle. It has been shown higher concentrations of ATP are required to attain the same threshold for action potential discharge and concomitant contraction (MacKenzie, Manzini*, et al.*, 1988). The

presence of the methylene group and resultant resistant to enzymatic breakdown enables a discernible effect of an antagonist against the induced contraction to be more easily seen.

The observed reduction in the maximum contraction in the control curve for αβ-methylene ATP may be attributable to the desensitization of the P2X1-purinoceptors. P2X1-purinoceptors typically undergo rapid desensitization when exposed to either ATP or αβ-methylene ATP (Burnstock, 2007a). The stabilisation of αβ-methylene ATP against enzymatic breakdown renders persistent agonist present, resulting in a greater probability of desensitization and hence a decrease in the maximum contraction of the tissue.

The effect of the ethanolic vehicle may also be exaggerated. Acute exposure to ethanol *in vitro* in organ bath studies has been shown to reduce the contractile response to ATP and to electrical field stimulation in the rat bladder (Yokoi *et al.*, 1996), to phenylephrine in the corpus cavernosum and rat aorta (Strickland *et al.*, 1988, Saito *et al.*, 1994), and to potassium chloride and acetylcholine in the gall bladder (Masui *et al.*, 1993). This has been attributed to the suppression of the influx of calcium into the cell (Twombly *et al.*, 1990, Briner *et al.*, 1993) by the inhibition of ATP-gated channel function (Weight *et al.*, 1999) on P2X-purinoceptors (Davies *et al.*, 2002). As the aqueous component was found to be freely soluble in distilled water, which did not show any effect on agonist induced contraction, the vehicle was changed to remove any influence ethanol would potentially exhibit.

Reversed-phase column chromatography was used in the first step of fractionating the extract. Material can be lost during column fractionation; therefore, a higher concentration of the resulting fractions was pharmacologically tested. Two of the fractions, fraction 1, the most polar fraction, and fraction 3, were found to be active. As fraction 1 yielded the greatest amount of material, further investigation into this fraction was conducted initially. ^{1}H NMR spectroscopic analysis indicated this sample contained a mixture of carbohydrates, seen

between δ 3.22 and 4.05, as well as a large number of minor compounds indicated by the many signals downfield between δ 6.00 and 8.00.

Preparative HPLC produced two active fractions which were subsequently separated into fractions, four of which retained activity. Within the four active fractions, the presence of two compounds caffeoylmalic acid, and the hydroxycinnamic acid, *p*-coumaric acid was indicated using NMR and HR-MS data. *p*-Coumaric acid (Nencu *et al.*, 2012) and 2-*O*-caffeoylmalic acid (Grevsen *et al.*, 2008, Pinelli *et al.*, 2008, Nencu *et al.*, 2012), have previously been reported in stinging nettle leaves. Although the activity of caffeoylmalic acid has not been previously reported, caffeic acid and its phenethyl ester have been previously shown to dilate preconstricted rat (Leeya *et al.*, 2010) and porcine coronary aortic rings (Long *et al.*, 2009) and to relax myogenic contractions of the rat ileum (Aviello *et al.*, 2010). Numerous mechanisms of action have been postulated including increasing the release of nitric oxide (Long *et al.*, 2009, Leeya *et al.*, 2010), stimulation of soluble guanylate cyclase, and the opening of K_{ATP} and K_{Ca} channels (Leeya *et al.*, 2010), as well as activity at β-adrenergic receptors (Long *et al.*, 2009) or L-type calcium channels (Aviello *et al.*, 2010) indicating the mechanism of action may be multifactorial, and may incorporate activity within the purinergic system.

Various pharmacological and biological properties have been associated with *p*-coumaric acid including anti-oxidant activity (Lim *et al.*, 1999, Abdel-Wahab *et al.*, 2003), neuroprotection (Vauzour *et al.*, 2010), inhibition of cell proliferation (Janicke *et al.*, 2005) and in vitro antiplatelet activity against ADP-induced aggregation (Luceri *et al.*, 2007). Derivatives have demonstrated a non-specific relaxant effect on the guinea pig isolated trachea (Marcucci *et al.*, 2001) thought to be through control of calcium mobilisation (Paulino *et al.*, 2003). Literature pertaining to the activity of *p*-coumaric acid on smooth muscle contractility or the purinergic system, has been elusive.

Spectroscopic data for the active fractions D2.5 and D2.6 was consistent with isomers or derivatives of ferulic acid, although not for ferulic acid itself. While ferulic acid and its derivatives are found in stinging nettle extracts (Kraus *et al.*, 1991, Nencu *et al.*, 2012, Orcic *et al.*, 2014) investigation into its activity on smooth muscle has centred around its ability to act as an antihypertensive. Its vasodilating effect is considered to be multifactorial (Mancuso *et al.*, 2014) partially explained by an enhancement in the bioavailability of nitric oxide both *in vitro* (Suzuki *et al.*, 2002, Suzuki *et al.*, 2007), and *in vivo* (Choi *et al.*, 2012) as well as an endothelium-independent non-selective mechanism of action (Chen *et al.*, 2009).

Activity may be attributed to a coumarin or a coumarin isomer such as isocoumarin or chromone, or to a methyl or methoxy derivative of the 7-hydroxy coumarin, umbelliferone. Greater than 1300 coumarins have been identified in natural sources (Hoult *et al.*, 1996). Of these, various derivatives and isomers have exhibited activity in smooth muscle, so it is possible more than one isomer or derivative may exhibit the observed bioactivity. It is also worth noting *p*-coumaric acid is involved in the biosynthetic pathway of umbelliferone (Brown *et al.*, 1964), therefore the detection of *p*-coumaric acid may not indicate the identification of the active compound, but rather a precursor. The active fractions may contain very similar compounds as they eluted adjacent to each other and it is difficult to separate such structurally similar isomers. Coumarins including scopoletin, previously found in stinging nettle (Sajfrtova *et al.*, 2005, Nahata *et al.*, 2012), scoparone, and osthol, possess a non-specific spasmolytic effect, decreasing the influx of calcium into smooth muscle cells (Kozawa *et al.*, 1981, Ojewole *et al.*, 1983, Hoult *et al.*, 1996, Oliveira, Romero*, et al.*, 2001). Hypotensive effects of coumarins and their glycosides are believed to occur through a calcium antagonist-like action at voltage-gated calcium channels (Gilani *et al.*, 2000). Furthermore, dihydropyranocoumarins and dihydrofuranocoumarins have been shown to have both coronary vasodilator and spasmolytic activity (Thastrup *et al.*, 1983). Alongside scoparone, and osthol, they have also

exhibited an ability to inhibit platelet aggregation by inhibition of calcium entry into the platelets (Thastrup *et al.*, 1985, Hoult *et al.*, 1996). This may be consistent with activity on P2X1-purinoceptors as these receptors are known to be present on platelets and generate significant increases in intracellular calcium which is required for the secondary phase of aggregation (Mahaut-Smith *et al.*, 2011). Although coumarins have not been shown to have activity at purinoceptors, they are believed to inhibit some of the calcium signalling elements involved in the smooth muscle contraction event therefore it is possible a coumarin analogue or derivative may be responsible for the effects seen.

The elucidation of bioactive compounds using NMR is complex and complicated in the absence of suitable reference compounds. NMR is a useful tool in the confirmation of compound identification; however, a significant limitation of NMR is the requirement for substantial amounts of the compounds to be isolated (Carrasco-Pancorbo *et al.*, 2005). The minor compounds in the fractions were identified using NMR, however the smaller peaks indicating less predominant compounds are difficult to analyse using NMR, as there is much overlap, and the required time and fraction quantities for further separation and identification prevented further analysis in this study.

The bioactive, responsible for the attenuation of αβ-methylene ATP-induced contraction in the isolated prostate gland, is yet to be conclusively identified. The proprietary sample of *p*-coumaric acid did not exhibit activity. It is uncertain whether caffeoylmalic acid, ferulic acid or its isomers are active as the fractions are not yet sufficiently pure and without comparison to an authentic sample, we cannot be absolutely sure of the activity. NMR data indicates the presence of a possible derivative of caffeic acid present in fraction 3 (Appendix B), therefore similar compounds within the different fractions may all possess some level of activity. Alternatively, a more minor component may be responsible for the activity, but as this

component may be eluting over many fractions, it is probable it is poorly behaved under chromatography.

Chapter 5

Effects of oral *Urtica dioica* (Stinging Nettle) Leaf Extract on Fertility in Male Mice: A Comparison with P2X1-purinoceptor Knockout Mice

Abstract

Both the α_{1A}-adrenoceptor and the P2X1-purinoceptor are essential in the male urogenital system for the transport of sperm from its storage site in the cauda epididymis into the ejaculate through contraction of the vas deferens. Previous studies have shown male α_{1A}-adrenoceptor knockout mice, (Sanbe *et al*., 2007) and more markedly P2X1-purinoceptor knockout mice (Mulryan *et al*., 2000) to be sub-fertile, due to lower numbers of sperm in the ejaculate resulting from a reduction in vas deferens contractility. Furthermore, simultaneous knockout of both receptors results in complete male infertility (White *et al* 2013). Pharmacological blockade of the α_{1A}-adrenoceptor is possible as antagonists are readily available and commonly used for the treatment of benign prostatic hyperplasia. However, an effective, selective, and therapeutically active, P2X1-purinoceptor antagonist is still to be developed. As stinging nettle leaf extract was shown to pharmacologically antagonise P2X1-purinoceptors in the prostate (Chapter 3), and its bioactive fractions exhibited activity against αβ-methylene ATP induced contraction in the vas deferens (Chapter 4), this chapter tested for its effect on fertility in male mice. P2X1-purinoceptor knockout mice resulted in a 91.7% decrease in pregnancies with a mean foetal number of 0.25 ± 0.35 per pregnancy. Stinging nettle leaf extract (100 µl of 500 mg/ml daily) reduced male fertility by 50% (50% pregnancy rate and mean foetal number 3.25 ± 1.45) when compared to vehicle-treated male mice (83% pregnancy rate and mean foetal number of 6.08 ± 1.43). Cardiovascular function was not affected by either knockout of the P2X1-purinoceptor, or by the administration of stinging nettle leaf extract. There was no difference between the resting systolic blood pressure ($p = 0.072$) or in the pulse rate ($p = 0.057$). The mice were functionally unaffected with normal mating behaviour. The vasa deferentia were elongated and heavier in the P2X1-purinoceptor knockout mice compared with wild-type and stinging nettle leaf extract treated mice. The prostate was also greater in mass in these mice. The bladder and the testes were found to be of significantly lower weight in the stinging nettle leaf extract treated mice. All other organs and total body weight were unaffected.

5.1 Introduction

In rodents and humans, noradrenaline and ATP released from sympathetic neurons act on α_{1A}-adrenoceptors and P2X1-purinoceptors, respectively, to contract the vas deferens and transport sperm from its storage site in the cauda epididymis to the urethra during the ejaculation process (Amobi *et al.*, 1999, Banks *et al.*, 2006).

The physiological importance of ATP and P2X1-purinoceptors in the vas deferens during the emission phase of the ejaculation process has been demonstrated through the generation of P2X1-purinoceptor deficient mice (Mulryan *et al.*, 2000). Phenotypically normal heterozygous male and female mice exhibited no selective fertilisation or mortality in utero producing homozygous mice at a normal Mendelian distribution. However homozygous females did not produce offspring when mated with homozygous males (Mulryan *et al.*, 2000). Furthermore, reduced fertility was found to be specific to the male P2X1-purinoceptor knockout mice as homozygous males mated with wild-type females resulted in only a 13.7% pregnancy rate, and a 30% decrease in litter size compared to heterozygous males (Mulryan *et al.*, 2000). α_1-Adrenoceptors are also important for male fertility as α_{1A}-adrenoceptor knockout mice have also been shown to be sub-fertile (Sanbe *et al.*, 2007). Male α_{1A}-adrenoceptor knockout mice mated with wild-type females resulted in a 50% reduction in the pregnancy rate (Sanbe *et al.*, 2007). Simultaneous knockout of both the α_{1A}-adrenoceptors and P2X1-purinoceptors results in complete male infertility with no apparent physiological or behavioural effects (White *et al.*, 2013). Across all knockout genotypes, no differences were observed in mating behaviour, when compared to wild-type mice, but vaginal plugs were absent in wild-type female mice mated with dual α_{1A}-adrenoceptor and P2X1-purinoceptor knockout mice. Nonetheless, sperm recovered from the cauda epididymis were motile and viable as demonstrated by their ability to fertilise ova *in vitro* (Mulryan *et al.*, 2000, Sanbe *et al.*, 2007, White *et al.*, 2013). In all three

studies, the decrease in male fertility was determined to be through a reduction of sperm in the ejaculate associated with an attenuation in neurogenic vas deferens contractility (Mulryan *et al.*, 2000, Sanbe *et al.*, 2007, White *et al.*, 2013).

As α_{1A}-adrenoceptor and P2X1-purinoceptor double knockout mice resulted in complete male infertility (White *et al.*, 2013), pharmacological blockade of both the α_{1A}-adrenoceptor and P2X1-purinoceptor may result in an effective, novel, oral form of male contraception. Early studies using the α-adrenoceptor antagonists, phenoxybenzamine (Homonnai *et al.*, 1984, Paz *et al.*, 1984) and prazosin (Ratnasooriya *et al.*, 1984), resulted in inhibition of ejaculate volume with a reversible decrease in fertility in humans (Homonnai *et al.*, 1984) and rats (Paz *et al.*, 1984, Ratnasooriya *et al.*, 1984). More recently, therapeutically used selective α_{1A}-adrenoceptor antagonists such as tamsulosin have been shown to effectively reduce male fertility in rats (Ratnasooriya *et al.*, 1994) and total functional sperm count in men (Goktas *et al.*, 2006, Hellstrom *et al.*, 2006, Wang *et al.*, 2012). To test this combination therapy as an effective oral form of male contraception, a P2X1-purinoceptor antagonist is required for use in combination with tamsulosin. To date, however, there is a lack of potent and selective P2X1-purinoceptor antagonists. They are either low potency, large, polyanionic molecules, or rapidly metabolised acidic nucleotides, making them unsuitable for *in vivo* use. As shown in Chapter 3, an extract of stinging nettle was able to attenuate αβ-methylene ATP-induced smooth muscle contractions *ex vivo*. As it is a commercially available oral therapeutic herbal preparation for the treatment of various ailments, as well as administered orally in traditional medicine for nocturia (Sezik *et al.*, 2001) and hypertension (Ziyyat *et al.*, 1997, Tahraoui *et al.*, 2007, Tagarelli *et al.*, 2010), it may prove to be orally active *in vivo* at inhibiting the purinergic contractile response of the vas deferens smooth muscle. This chapter therefore

investigates the *in vivo* effects of stinging nettle leaf extract on the fertility of male mice following oral administration.

5.2 Methods

5.2.1 Animal housing and ethics

Wild-type C57Bl/6 male and female mice, and P2X1-purinoceptor knockout male mice were obtained and housed as described in Chapter 2, sections 2.1.2, 2.1.3 and 2.1.4. Details of animal ethics approval are as described in Chapter 2, section 2.1.4.

Mice were euthanased by asphyxiation through exposure to CO_2 gas and organs dissected as per the procedure outlined in Chapter 2, section 2.3.2.

5.2.2 Blood pressure and heart rate analyses

Cardiovascular parameters were measured in eight-week-old male P2X1-purinoceptor knockout and wild-type mice as described in Chapter 2, section 2.8.2.

5.2.3 Breeding observations

P2X1-purinoceptor knockout and treated wild-type male mice were mated with seven- to eight-week-old female mice, and mating was observed as describe in Chapter 2, section 2.8.3.

5.3 Results

5.4 Behaviour

Normal libido was maintained with breeding observations showing normal mounting and mating in both groups with copulation occurring to the point of ejaculation. This was observed by typical post-copulation behaviour by the male mice.

5.4.1 Effects of P2X1-purinoceptor receptor deletion and stinging nettle leaf extract on the cardiovascular system

Resting systolic blood pressure did not differ between P2X1-purinoceptor knockout and wild-type male mice. Similarly, treatment with stinging nettle leaf extract or vehicle did not affect the blood pressure of wild-type male mice over the five-day dosing period (p = 0.072; Figure 5.1A).

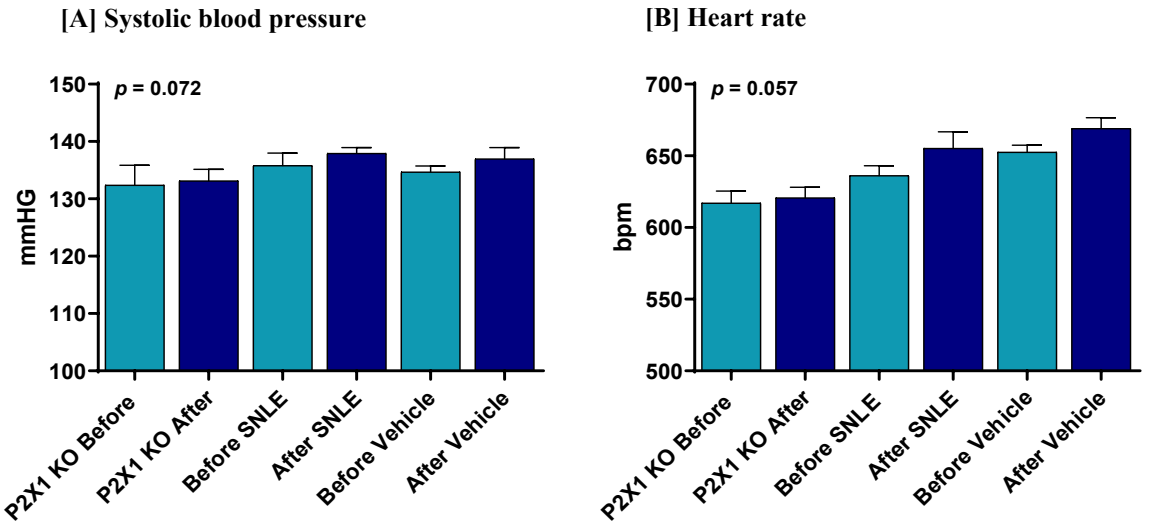


Figure 5.1: Resting systolic blood pressure [A] and heart rate [B] measured by the tail cuff method in age matched P2X1-purinoceptor knockout (KO) (n = 6) and wild-type male mice, prior to and subsequent to daily oral dosing with stinging nettle leaf extract (SNLE) (100 µl of 500 mg/ml) or vehicle (100 µl 20% ethanol), where n = 6 for each treatment variable. Bars represent the mean blood pressure or heart rate over 5 consecutive days following 3 days of acclimatisation. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures of ANOVA and represent the probability of genotype or treatment causing a significant change in blood pressure or pulse rate.

Additionally, the pulse rate did not differ between the P2X1-purinoceptor knockout and wild-type male mice. The addition of stinging nettle leaf extract or vehicle, again did not change the pulse rate in wild-type mice over the five-day dosing period (p = 0.057; Figure 5.1B).

5.4.2 Effect of P2X1-purinoceptor receptor deletion and stinging nettle leaf extract on body and organ weights

No differences were noted in the body weights among the P2X1-purinoceptor knockout and differently treated wild-type mice or in the weights of their spleen, kidney, liver, or heart.

Within the lower urogenital tract, there were changes in the vas deferens, prostate, bladder and testes, but not in the seminal vesicles. Although the length of the vasa deferentia were not significantly different between the three groups, (Figure 5.2A) there was an increase in weight in the vas deferens of the P2X1-purinoceptor knockout mice when compared to vehicle-treated wild-type mice but not a significant difference when compared to the stinging nettle leaf extract-treated wild-type mice (Figure 5.2B). Additionally, no difference was seen in vas deferens weight between the vehicle-treated and stinging nettle leaf extract-treated wild-type mice. The prostate was found to be heavier in the P2X1-purinoceptor knockout mice when compared to both the vehicle-treated and the stinging nettle leaf extract-treated wild-type mice (Figure 5.2C).

Differences were observed in the weights of both the testes and the bladder, with stinging nettle leaf extract-treated mice presenting with significantly lower weights than the P2X1-purinoceptor knockout (Figure 5.2D and $p < 0.05$; Figure 5.2E, respectively). There were no observed differences between the weights of either the testes or the bladder between vehicle-treated wild-type mice and either stinging nettle leaf extract- treated wild-type mice, or P2X1-purinoceptor knockout mice.

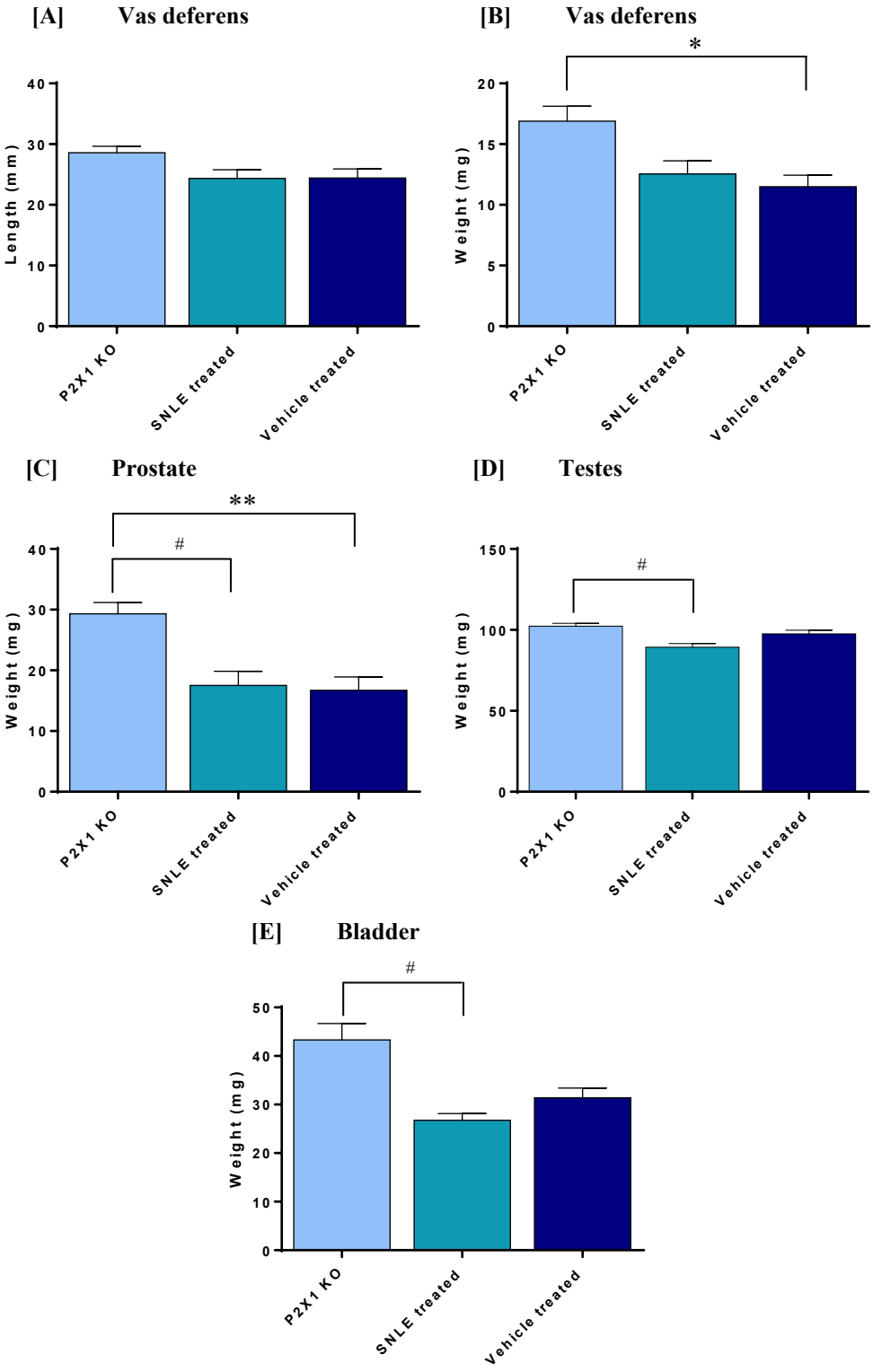


Figure 5.2: Effect of P2X1-purinoceptor knockout (KO) and treatment with 100 µl of stinging nettle leaf extract (SNLE) (500 mg/ml) or vehicle (25% ethanol) in wild-type male mice on the length [A] and weight [B] of the vas deferens, and the weight of the [C] prostate, [D] bladder, and [E] testes in age matched male mice where n = 12 for each treatment variable. Bars represent the mean weight or length of the respective organ. Error bars represent SEM. * $p < 0.05$ and ** $p < 0.001$ represent the probability of a significant difference in the weight between vehicle-treated wild-type male mice and P2X1-purinoceptor knockout male mice. $^{\#}p < 0.05$ represents the probability of a significant difference in the weight between stinging nettle leaf extract-treated wild-type mice and P2X1-purinoceptor knockout mice. ANOVA *p*-values were calculated by a one-way ANOVA test with a Bonferroni correction where n = 12.

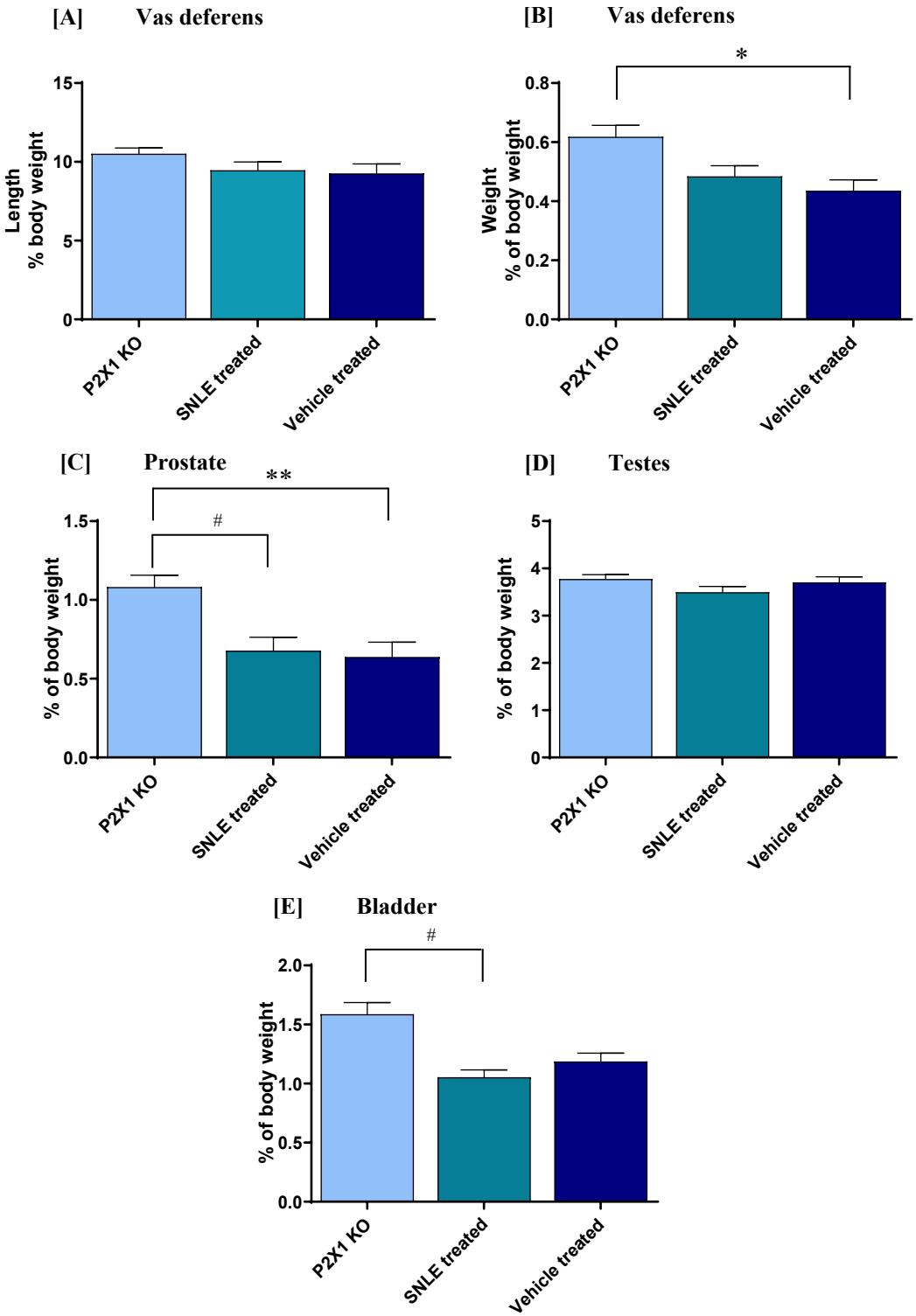


Figure 5.3: Effect of P2X1-purinoceptor knockout (KO) and treatment with 100 µl of stinging nettle leaf extract (SNLE) (500 mg/ml) or vehicle (25% ethanol) in wild-type male mice on the length as a percent of the body weight [A] and weight as a percent of the body weight [B] of the vas deferens, and the percent of the body weight of the [C] prostate, [D] bladder, and [E] testes in age matched male mice where n = 12 for each treatment variable. Bars represent the mean weight or length of the respective organ. Error bars represent SEM. * $p < 0.05$ and **$p < 0.001$ represent the probability of a significant difference in the weight between vehicle-treated wild-type male mice and P2X1-purinoceptor knockout male mice. $^{\#}p < 0.05$ represents the probability of a significant difference in the weight between stinging nettle leaf extract-treated wild-type male mice and P2X1-purinoceptor knockout male mice. ANOVA *p*-values were calculated by a one-way ANOVA test with a Bonferroni correction where n = 12.

5.4.3 Effect of P2X1-purinoceptor receptor deletion and stinging nettle leaf extract on fertility of male mice

Following gestation, the positive pregnancy rate and foetal number was determined in each wild-type female mate to ascertain the effect of either genotype, or treatment with either stinging nettle leaf extract or vehicle on fertility in male mice.

Table 5.1: Pregnancy rate and mean foetal number per mating for P2X1-purinoceptor knock-out male mice, and wild-type male mice treated with 100 µl of either stinging nettle leaf extract (500mg/ml) or vehicle (25% ethanol).

Mouse	Mouse Number	Mating 1 Foetal Number	Mating 2 Foetal Number	Pregnancy rate (%)	Mean foetal number ± SEM
P2X1-KO	1	0	0	**8.3**	**0.25 ± 0.35**
	2	0	0		
	3	0	0		
	4	0	3		
	5	0	0		
	6	0	0		
Stinging nettle leaf extract-treated wild-type	1	7	0	**50**	**3.25 ± 1.45**
	2	4	8		
	3	6	0		
	4	6	8		
	5	0	0		
	6	0	0		
Vehicle-treated wild-type	1	0	10	**83**	**6.08 ±1.43**
	2	9	9		
	3	0	9		
	4	5	6		
	5	3	7		
	6	6	9		

Both the pregnancy rate and the mean foetal number in females mated with males treated with stinging nettle leaf extract or P2X1-KO male mice, were reduced. Of the twelve wild-type females mated with P2X1-purinoceptor KO male mice only one pregnancy occurred (8.3%; mean foetal number 0.25 ± 0.35 per mating), for stinging nettle leaf extract treated

wild-type males, pregnancy occurred in six of the twelve mated wild-type females (50%; mean foetal number 3.25 ± 1.45 per mating), whereas 10 out of the 12 mated wild-type females were found to be pregnant (83%; mean foetal number 6.08 ± 1.43 per mating) after mating with vehicle treated wild-type male mice (Table 5.1). A significant difference was observed between the mean foetal number of the P2X1-purinoceptor knockout mice and the vehicle-treated wild-type mice ($p < 0.001$; Figure 5.4). Although there appears to be an effect on foetal number in wild-type mice when treated with stinging nettle leaf extract, this was not found to be significant when compared to either P2X1-purinoceptor knockout mice and vehicle-treated wild-type mice.

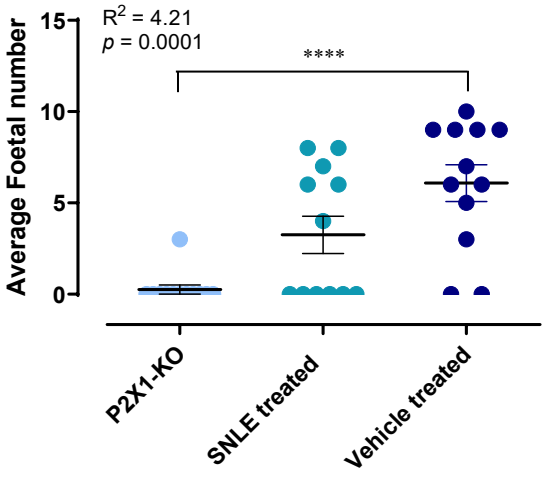


Figure 5.4: Foetal number resulting from wild-type female mice mated with age matched P2X1-purinoceptor knockout (n = 12), and wild-type male mice treated with an oral daily dosing of stinging nettle leaf extract (SNLE) (100 µl of 500 mg/ml) or vehicle (100 µl 25% ethanol); where n = 12 for each treatment variable. The centre line represents the mean foetal number. Error bars represent SEM. ****$p < 0.0001$. ANOVA *p*-values were determined by one-way ANOVA and represent the probability of genotype or treatment significantly affecting male fertility represented by the resultant foetal number.

5.5 Discussion

The results of this chapter compare the effects of genetic deletion of P2X1-purinoceptors, with those of pharmacological antagonism of these receptors by the oral administration of stinging nettle leaf extract, on fertility in male mice.

Wild-type male mice treated with stinging nettle leaf extract were found to be sub-fertile while fertility was more markedly reduced by genetic deletion of P2X1-purinoceptors. Overall in P2X1-purinoceptor knockout male mice, fertility was reduced to 8.3%, which is comparable to previous studies showing almost a 90% decrease in male fertility (Mulryan *et al.*, 2000).

It is possible that decreases in fertility rates may be attributed to defective sperm. Purinergic signalling has been suggested to play a role in spermatogenesis and to modulate the steroidogenic process in the testes increasing testosterone secretion in the mouse Leydig cells (Koles *et al.*, 2007). P2X2, P2X3, P2X5, and P2X7-purinoceptors were found to be expressed in the various germ cell types throughout the different stages of the cycle of the seminiferous epithelium (Glass *et al.*, 2001), while the P2X2-purinoceptor subtype is expressed in the Leydig cells (Koles *et al.*, 2007) and in the Sertoli cells (Veitinger *et al.*, 2011). While other subtypes may be involved in sperm development, P2X1-purinoceptor immunoreactivity, although detected in the blood vessels of the mouse (Mulryan *et al.*, 2000) and rat testis (Glass *et al.*, 2001), has not been detected in the seminiferous tubules (Mulryan *et al.*, 2000), indicating these receptors are not likely to be involved in normal spermatogenetic processes. Furthermore, male mice lacking P2X1-purinoceptors maintain normal sperm quality, motility, and count; these spermatozoa were effective at fertilizing ova in vitro (Mulryan *et al.*, 2000). Additionally, sperm from dual knockout male mice retrieved from the epididymis were normal in number and motility, testes appeared normal, and normal litters were produced after *in-vitro*

fertilisation of wild-type females following intracytoplasmic sperm injection (White *et al.*, 2013).

Normal function of the genitourinary organs in various male species including human males relies on sympathetic activation of both the α_{1A}-adrenoceptors (Sadraei *et al.*, 1995, Namasivayam *et al.*, 1999, Silva *et al.*, 1999), and the P2X1-puinoceptors (Baumgarten *et al.*, 1968, Burnstock *et al.*, 2000, Lee *et al.*, 2000a, Schwinn *et al.*, 2000, Gur *et al.*, 2007, Burnstock, 2014).

The weight of the testes was not found to be significantly different in the P2X1-purinoceptor knockout mice, and in either of the treated wild-type groups. Oestrogens have been found to play a role in the normal function and development of the male reproductive organs. Atrophy of the testes has been observed in oestrogen receptor knockout male mice, as well as a disruption of the reabsorption of the luminal fluid in the head of the epididymis, causing the sperm to enter the epididymis diluted and, interestingly, a subsequent decrease in fertility (Hess *et al.*, 1997, Oliveira, Carnes*, et al.*, 2001). Although stinging nettle root extract has been found to interfere with the conversion of testosterone to oestrogens via the inhibition of aromatase (Gansser *et al.*, 1995a, Koch, 2001), these compounds may not be present in the leaf extract, or may be present in concentrations too small to have an effect.

P2X1-purinoceptors may play a role in the development of the male reproductive tract. The length of the vas deferens was not affected in P2X1-purinceptor knockout mice nor in the mice administered pharmacological antagonism via stinging nettle leaf extract. Genetic deletion of P2X1-purinoceptors in male mice previously resulted in a decrease in fertility, reportedly due to an absence of sperm in the ejaculate as observed by uterine lavage following copulation (Mulryan *et al.*, 2000). Both P2X1-purinoceptor- and α_{1A}-adrenoceptor-mediated responses are responsible for the contraction of the vas deferens during ejaculation (Banks *et al.*, 2006, White

et al., 2010). Activation of these receptors on the smooth muscle of the vas deferens facilitates the unidirectional transit of sperm from the cauda epididymis, through the vas deferens to the urethra from where the sperm is expelled. Contractility of the vas deferens has been found to be compromised in P2X1-purinoceptor knockout mice (Sanbe *et al.*, 2007) and contractile responses were lost following chemical sympathectomy (Carvalho *et al.*, 1993). In P2X1-purinoceptor knockout mice there was a decrease in the pregnancy rate without an observed effect on vas deferens length. White *et al* previously reported an observable difference in the length of vas deferens in double knockout α_{1A}-adrenoceptor/P2X1-purinoceptor mice (White *et al.*, 2013). The absence of P2X1-purinoceptors, causes a decrease in contractility, however the presence of the α_{1A}adrenoceptors may negate the effect on the length of the vas deferens.

The weight of the vas deferens, however, was significantly increased in P2X1-purinoceptor knockout mice when compared to vehicle-treated wild-type mice. A loss in contractility of the vas deferens in P2X1-purinoceptor knockout mice may result in the retention of secretions and fluids within the lumen resulting in an increase in weight. Although the weight of the vas deferens of the stinging nettle leaf extract-treated wild-type mice appeared to be greater, this was not found to be significant. Interestingly, there was also not a significant difference between the P2X1-purinoceptor knockout mice and stinging nettle leaf extract wild-type mice. The dose of the stinging nettle leaf extract may not have been sufficient to enable a significant decrease in the contractility, and therefore increase in the weight of the vas deferens.

The deletion of P2X1-purinoceptor genes, is non-lethal as differences were not observed in the body weights between wild-type and P2X1 knockout mice nor in the weights of the vital organs such as the heart, lungs and liver. Oral administration of stinging nettle leaf extract is similarly

not detrimental to mice as the extract did not affect the body or vital organ weights. The mice, as a whole, maintained normal growth, physiology and behaviour.

Although overall body or vital organ weight were not found to be different, between wild-type, wild-type treated, and P2X1-purinoceptor knockout mice, the bladder was observed to be significantly increased in mass in the knockout mice, when compared to the stinging nettle leaf extract-treated wild-type mice. P2X1-purinoceptor immunoreactivity has been strongly detected in the mouse detrusor, apparently localised on the membranes of the smooth muscle cell (Vial *et al.*, 2000). Denervation of the rat bladder can increase its mass (Tuttle *et al.*, 1994) due to increases in myocyte mass or extracellular matrix production, from tissue oedema, or a combination of all three (Levin *et al.*, 1990, Gabella *et al.*, 1992). Additionally, increases in muscle stretch or afterload can induce dramatic tissue hypertrophy (Levin *et al.*, 1990). Although a substantial component of the nerve evoked bladder contraction is mediated through P2X1-purinoceptors with M_3 muscarinic receptors accounting for the residual response, it has been reported there is no effect of P2X1-purinoceptor deficiency on bladder function or size (Vial *et al.*, 2000), or bladder filling (Mulryan *et al.*, 2000). It is therefore difficult to assign a functional cause for the hypertrophy seen in P2X1-purinoceptor knockout mice. It is possible that the same effect was not seen in stinging nettle leaf extract-treated mice as the duration of treatment may not have been sufficient to observe a similar effect. Denervation only caused significant effects after three to six weeks (Tuttle *et al.*, 1994), therefore in this study, two weeks of treatment may not be sufficient to result in hypertrophy of the bladder. It may be that the increase in mass of the bladder in P2X1-purinoceptor knockout mice is due to developmental effects, where the bladder is increased in size due to a decreased contractility, with a resulting increase in the storage of urine. A similar increase in the weight of the bladder may become apparent with a longer duration of treatment with stinging nettle leaf extract.

Secretions from the seminal vesicles and the coagulating glands play an important role in fertility as they are involved in the facilitation and maintenance of sperm as well as sperm motility (Aumuller *et al.*, 1992). In mice, secretions of the seminal vesicles and coagulating glands form the coagulum plug facilitating the entry of sperm into the uterus, vital for fertility (Williams-Ashman, 1984). During ejaculation, these secretions are expelled via α_{1A}-adrenoceptor- (Silva *et al.*, 1999) and P2X1-purinoceptor-mediated contraction in the guinea pig (Meldrum *et al.*, 1985, Pinna *et al.*, 1997) and rodent (Chin *et al.*, 1988, Wali *et al.*, 1989, Lee *et al.*, 2000b). In double knockout mice, the seminal vesicles were reported to be heavier, thought to be due to fluid engorgement caused by a loss of function of the seminal vesicles, demonstrated by a decrease in contraction to electrical field stimulation (White *et al.*, 2013). The coagulum plug was also absent in wild-type females after copulation with double knockout male mice (White *et al.*, 2013) whereas the coagulation plug weight was not found to be affected in P2X1-knockout mice (Mulryan *et al.*, 2000). In this study the weight of the seminal vesicles did not differ between the different groups of male mice. The contraction and function of these organs may rely primarily on noradrenergic as opposed to purinergic mediated responses as noradrenaline evokes concentration-dependent contractions (Vial *et al.*, 2001). Although P2X1-purinoceptors are present in the seminal vesicles of mice (Chin *et al.*, 1988, Lee *et al.*, 2000b), αβ-methylene ATP has failed to induce contraction (Vial *et al.*, 2001).

Organ weights were measured using wet weight measurements. This portrays an accurate observation of the effect of a compound on an organ for most tissues, however for secretory organs, such as the prostate gland, which partially depend on the P2X1-purinoceptor for the expulsion of secretions (White *et al.*, 2015), the differences in mass may be exaggerated due to a change in the mass of secretions held within the organ rather than the organ mass itself. In this study the mass of the prostate was found to be significantly greater in P2X1-purinoceptor knockout mice when compared to either stinging nettle leaf extract treated or vehicle treated

wild-type mice. However, in double genetic deletion of both the α_{1A}-adrenoceptor and P2X1-purinoceptor in male mice, the prostate weight was found to be similar (White *et al.*, 2013). Denervation of the rat hypogastric nerve results in a lower prostatic weight, a decrease in cell heights and reduced secretory activity of the prostate (Wang *et al.*, 1991). This may explain the difference in prostatic weight when both receptor types are genetically deleted.

Blood pressure homeostasis is maintained in the cardiovascular system of rodents predominantly by noradrenaline-mediated regulation of contraction of the arteries via α_1-adrenoceptors (Guilmard *et al.*, 1996, Rokosh *et al.*, 2002, Docherty, 2010), through the α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors (for review see (Piascik *et al.*, 1995, Cavalli *et al.*, 1997, Docherty, 1998)), as well as the α_2-adrenoceptor subtypes, predominantly the $\alpha_{2A/D}$-adrenoceptors (Docherty, 1998). Other receptor types involved in the regulation of blood pressure, include the β_1- and β_2-adrenoceptors (Chruscinski *et al.*, 2001), as well ATP acting on P2X1-purinoceptors (Burnstock, 1990, Vial *et al.*, 2002, Burnstock, 2004, Lamont *et al.*, 2006). Genetic knockout in male mice of either the α_{1A}-adrenoceptor or both receptor types, results in a marked reduction in resting systolic blood pressure (Rokosh *et al.*, 2002, White *et al.*, 2013). A slightly increased systolic blood pressure has been observed in P2X1-purinoceptor knockout mice (Mulryan *et al.*, 2000). In the present study, cardiovascular parameters were not affected in either P2X1-purinoceptor knockout mice or following the oral administration of stinging nettle leaf extract in wild-type mice. Although vascular tone is associated with purinergic signalling, at rest α_{1A}-adrenoceptor mediated responses have been shown to be sufficient to maintain the sympathetic regulation of blood pressure as a parallel compensatory mechanism (Vial *et al.*, 2002). Furthermore, an increased sensitivity to noradrenaline has been observed in P2X1-purinoceptor knockout mice (Mulryan *et al.*, 2000). Alternatively, although homomeric P2X1-purinoceptor subtypes are highly expressed (Vulchanova *et al.*, 1996), other subtypes have been found to be expressed in the arterial smooth muscle (Nori *et al.*, 1998,

Phillips *et al.*, 1998). These alternate subtypes may also compensate for the lack of P2X1-purinoceptor control. The blood pressure and heart rate measurements were measured by a non-invasive cuff method. Although this enables the use of conscious non-anaesthetised animals, the process requires the animal to be restrained, and a degree of warming to retain sufficient tail blood flow. Both restraint and an increase in temperature have been shown to significantly alter the basal level of an animal's blood pressure (Van Vliet *et al.*, 2000). The recorded blood pressure and heart rate may therefore not accurately reflect solely the effects of the experimental parameters. Therefore, the recorded measurements may be a cumulative response.

On the days of exposure to females for mating, the daily dosing of the male mice occurred after exposure to the females to minimise the effects of oral dosing on mating behaviour. Blood levels were therefore potentially low at the time of mating. Consequently, the effect of stinging nettle leaf extract on fertility may be underestimated in this study.

Differences observed between fertility rates of P2X1-purinoceptor knockout and treatment with stinging nettle leaf extract may be due to various factors. As the responsible compound for the pharmacological antagonism of the P2X1-purinoceptors has not yet been elucidated, the dosage is unknown as are the pharmacokinetic and pharmacodynamic factors. The dose administered may be subtherapeutic, therefore the full extent of activity on fertility may not have been seen. Mice were dosed with 100 µL of 500 mg/ml stinging nettle leaf extract as this is the maximum oral dose we could give a mouse.

Stinging nettle leaf extract is known to contain phenols such as chlorogenic acid (Budzianowski, 1991, Yildiz *et al.*, 2008), and quercetin -3-O-rutinoside (Akbay *et al.*, 2003, Pinelli *et al.*, 2008, Nencu *et al.*, 2012) which have been shown to be metabolized extensively *in vivo* into hippuric acid as well as other phenyl-C_1 and phenyl-C_3 metabolites, and

phenylacetic acids respectively (Olthof *et al.*, 2001) (Olthof *et al.*, 2003). Degradation and conjugation with other compounds such as glucuronic acid and sulphates by colonic microflora reduce bioavailability and result in a proportion of the ingested phenols never reaching the peripheral circulation (Olthof *et al.*, 2003). Once absorbed, further metabolism occurs in the liver and kidneys (Olthof *et al.*, 2003). The activity *in vitro* and *ex vivo* may not reflect what occurs *in vivo* due to extensive metabolism.

This study shows the effect of genetic deletion of the P2X1-purinoceptor as well as administration of stinging nettle leaf extract on the fertility of male mice. The mice appear normal with no difference in body or vital organ weights. As purinergic mechanisms are important throughout the male urogenital system, knocking out P2X1-purinoceptors and the administration of stinging nettle leaf extract not only affected these organs but decreased the fertility which is most likely due to a decrease in vas deferens smooth muscle contractility.

This study therefore confirms that stinging nettle leaf extract may contain a component that is an orally viable P2X1-purinoceptor antagonist. Once isolated and identified, this may have potential as a lead compound for use in combination with tamsulosin for the development of a male contraceptive.

Chapter 6

Concluding Remarks

BPH remains the most prevalent benign neoplastic disease in aging men resulting in a financial burden on society and loss of quality of life due to the associated LUTS. Current accepted treatments rely on conventional pharmacological or surgical intervention. Treatment targets both the growth of the prostate and the contractility of the gland. Reducing the size of the prostate results in a reduced risk of progression of the disease (McConnell *et al.*, 1998, McConnell *et al.*, 2003, Roehrborn, 2008), however those medications that target the contractility of the prostate have a quicker onset of action, and are more effective at alleviating symptoms.

Historically, phytotherapeutics have been utilised by many cultures for various ailments including lower urinary tract symptoms (Sezik *et al.*, 2001). There is however a paucity in the understanding of the mechanism of action with the majority of evidence being anecdotal, with minimal robust studies and clinical trials conducted. The overarching aims of this thesis were to investigate the activity of commercially available stinging nettle extracts on the contractility of the rat prostate gland, and to determine the compound responsible.

Stinging nettle root extracts have been used commercially for the treatment of LUTS associated with BPH with some evidence of effectiveness (for review see (Chrubasik *et al.*, 2007)). In Chapter 3, it was shown that these extracts did not exhibit an effect on the contractility of the prostate. Its activity may therefore be attributable to its effect on the hyperplasia associated with BPH rather than acting on the contractility of the prostate gland. Stinging nettle leaf extract however, demonstrated an ability to reduce the contractility of both electrical field stimulation induced contraction as well as ATP and αβ-methylene ATP mediated contraction. Although the exact mechanism of action was not fully elucidated, the results suggest that the extract

works postjunctionally to inhibit the purinergic pathway, most likely as a purinoceptor antagonist.

Conventional treatment of the LUTS associated with BPH act by either inhibiting 5α-reductase enzymes decreasing the hyperplasia or using α_{1A}-adrenoceptor antagonists to reduce the contractile tone of smooth muscle within the gland. Previous studies have demonstrated a residual contraction to nerve stimulation in both the guinea pig and rat prostate after α-adrenoceptor has antagonists have been administrated (Lau, Ventura*, et al.*, 1998, Ventura *et al.*, 2003). This residual contraction was attributed to the purinergic pathway and ATP acting at P2X1-purinoceptors (Buljubasich *et al.*, 2006). Targeting both the α-adrenoceptors as well as the P2X1-purinoceptors may potentially reduce the overall contractility of the prostate gland and may result in greater symptom control. There may also be the possibility of reducing the doses by using a combination, which may allow a reduction in side effects of the original components, however adverse drug interactions may arise due to the combination itself.

Currently, the known P2X1-purinoceptor antagonists are not selective for the receptor subtype, as well as being promiscuous across other neurogenic and intracellular pathways and receptors. Furthermore, compounds such as suramin, a large polysulfonated molecule, has physical and chemical properties that suggest they are not necessarily suitable for progression as drug candidates. Aside from suramin, which is used as a trypanocide, and the active metabolite of vitamin B6, pyridoxal-5-phosphate, most P2X1-purinoceptor antagonists exist as pharmacological tools. The discovery of an orally bioavailable P2X1-purinoceptor selective compound would be beneficial in future, better treatments of BPH. The purinoceptor subtype selectivity of stinging nettle leaf extract was not investigated during this study. Considering botanical extracts contain a multitude of compounds, stinging nettle leaf extract itself may prove to be promiscuous across various purinergic subtypes as well as other neurogenic

pathways. However, the isolation of a single active component may prove to be a good lead candidate for development as a P2X1-purinoceptor antagonists.

Chapter 4 attempted to isolate the bioactive responsible for the activity seen in Chapter 3. The isolation and identification of bioactive constituents of plant extracts involves several consecutive steps beginning with chromatographic separation followed by activity-guided fractionation and finally structure elucidation and verification of the purity of the isolated compound. Although the compounds found in plants show great structural variety from simple structures consisting of single aromatic rings, to highly complex polymeric compounds, they exist in various conjugates of differing chemical and spectroscopic nature, often eluting at similar times, resulting in overlapping peaks. This makes separation challenging. The complexity of the compounds, and of the matrix they reside within, render samples difficult to accurately analyse. Separation of the active compound from its environs may minimise its activity. Furthermore, although activity was seen within isolated organ bath studies, plant compounds are often not readily absorbed *in vivo* requiring conversion into easily absorbed metabolites (Olthof *et al.*, 2003). The active compounds observed may therefore be metabolised upon oral administration, suggesting the activity *in vitro* and *ex vivo* may not accurately reflect what occurs *in vivo*.

The activity of stinging nettle leaf extract against the purinergic component of contraction in the prostate demonstrated in Chapter 3 prompted a study into its effect on fertility in male mice. P2X1-purinoceptors, alongside α_{1A}-adrenoceptors, are responsible for the contractility of the vas deferens and subsequently the transport of sperm during the ejaculation process. Previously shown in two separate studies, the knockout of P2X1 purinoceptors (Mulryan *et al.*, 2000) and dual α_{1A}-adrenoceptor/P2X1-purinoceptor knockout in male mice (White *et al.*, 2013), resulted in a significant reduction of the fertility of male mice, without detrimental effects on male development or sperm viability. The reduction in the contractility of the vas deferens impedes

the transport of sperm during the ejaculation process, resulting in a reduced rate of pregnancy. As these mice were seen to be phenotypically normal, it can be proposed the use of antagonists required for male contraception would be well tolerated.

Chapter 5 explored the effect of pharmacological blockade of the P2X1-purinoceptor, by stinging nettle leaf extract, on fertility in wild-type male mice, compared to male P2X1-purinoceptor knockout mice. Fertility was reduced, when compared to untreated wild-type male mice, using short-term chronic oral administration of a single concentration of stinging nettle leaf extract. Furthermore, the degree of infertility was similar to that observed in P2X1-purinoceptor knockout mice. Higher concentrations of stinging nettle leaf extract may have led to a more distinct, reduction in fertility in male mice, while the identification, extraction, and purification of the active compound may similarly yield a more significant result.

For a successful oral male contraception, it appears as though pharmacological blockade of both the α_{1A}-adrenoceptor and the P2X1-purinoceptor is required. Pharmacological antagonists of α_1-adrenoceptors are currently available, well-tolerated, and effective, however interestingly the α-adrenoceptor antagonist used, and route of administration determines the effect on the presence of spermatozoa in the ejaculate and ejaculation function. Prazosin delivered directly to the vas deferens or epididymis of healthy adult male rats, by way of silastic formulations constructed in the form of rods or collars, reduced fertility with no effect on mating or courting behaviour, or on the motility of spermatozoa (Ratnasooriya *et al.*, 1984). Conversely, oral prazosin was found to be ineffective on healthy adult human male fertility (Kjaergaard *et al.*, 1988). Selective therapeutic blockade of the α_{1A}-adrenoceptor with tamsulosin has been shown to inhibit the fertility of male rats (Ratnasooriya *et al.*, 1994). At normal doses used in the symptomatic treatment of BPH, tamsulosin significantly reduced total functional sperm count

in semen in healthy men with an associated marked decrease in ejaculate volume and occasional anejaculation (Goktas *et al.*, 2006, Hellstrom *et al.*, 2006, Wang *et al.*, 2012). However, at therapeutic doses, alfuzosin, did not demonstrate the same effect in decreasing total sperm count or ejaculation dysfunction (Hellstrom *et al.*, 2006, Roehrborn, 2006). Differences in the response of the epididymal and prostatic portions of the human vas deferens to exogenous noradrenaline, as well as differences in the activity of various α_1-adrenoceptor antagonists on the two distinct segments, may go some way to explaining these findings (Colabufo *et al.*, 2007). The introduction of a P2X1-purinoceptor antagonist along with an effective α_1-adrenoceptor antagonist may augment the efficacy and reduce side effects associated with doses required to achieve male contraception. Stinging nettle leaf extract may provide a drug-like lead compound that enables the synthesis of an orally active, potent small molecule compound that may be used in the development of a male contraceptive.

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Appendix A

Sample calculation of Equivalent Bath Concentration

Separating the aqueous and organic phases from 50 ml of stinging nettle leaf extract (SNLE) resulted in 3.49 g of material from the aqueous phase.

Electrical Field Stimulation (EFS) induced contraction

For EFS induced contractions 300 µl aliquots are used for each of the following frequencies: 0.1, 0.2, 0.5, 1, 2, 5, and 10 Hz as 300 µl gave significant results

SNLE was added, incubated for 30 minutes, washed out due to frothing caused by prostatic secretions, then re-added. It was also potentially washed out just before each subsequent frequency (again due to frothing that usually occurs within the 10 minute rest period between tests).

Per prostate, 8 x 300 µl (2.4 ml) was required. As n=6, this equated to 14.4 ml. 20 ml of sample was prepared to allow for additional washes that arose.

Separating the aqueous and organic phases from 50 ml of SNLE resulted in 3.49 g of material from the aqueous phase.

To test the aqueous phase against EFS-induced contractions an equivalent dose was prepared as below:

- 50 ml yielded 3.49 g; therefore 20 ml requires 1.396 g of aqueous material resulting in 0.0698 g/ml concentration solution.
- 300 µl aliquots in a 10 ml organ bath were used resulting in a bath concentration of 2.09 mg/ml

Appendix B

^{1}H NMR Spectra of Fractions 2-5

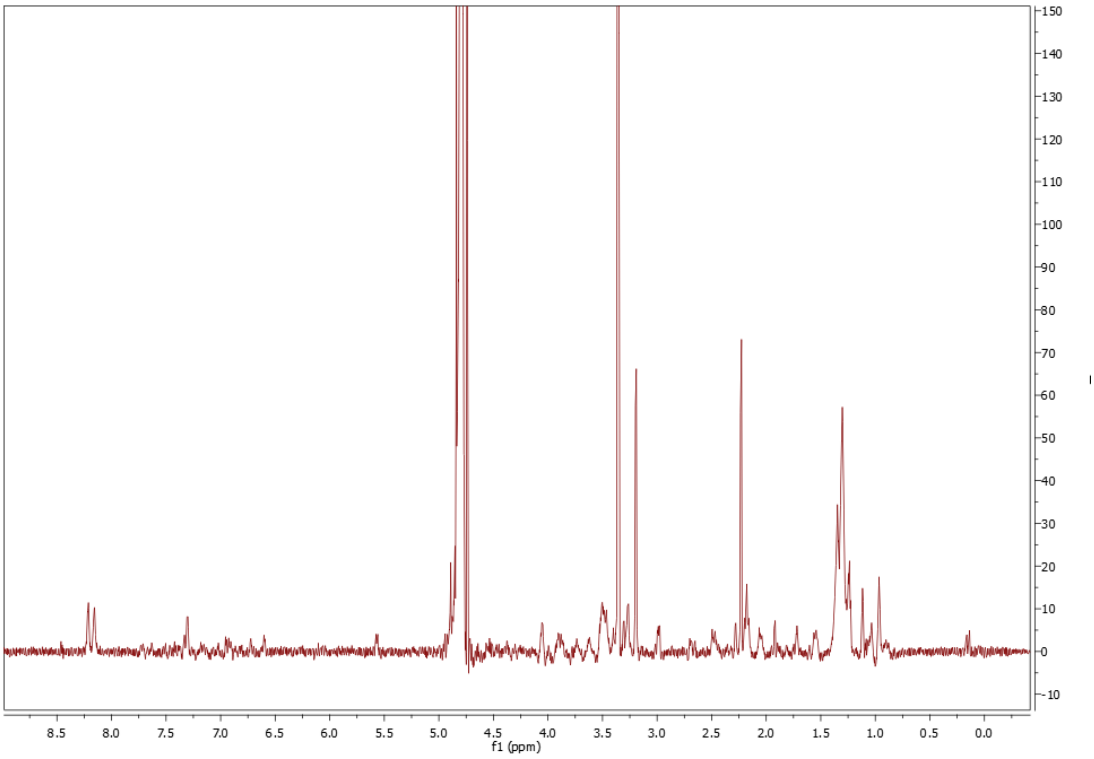

Figure B.1: Expanded ^{1}H NMR spectrum of the active fraction 2 in deuterated water from δ -0.05 to δ 9.00.

A ^{1}H NMR spectrum of inactive fraction 2 (Figure B.1) did not show the characteristic signals for the hydroxycinnamic acids, typically observed between δ 5.50 and δ 7.50, indicating a lack of these compounds within this fraction.

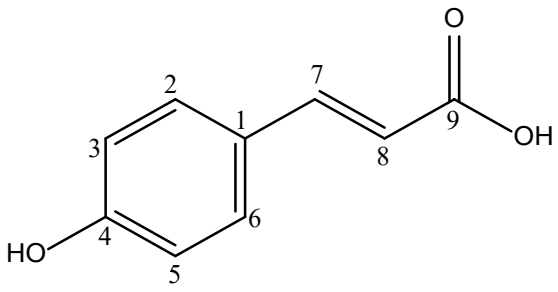

Figure B.2: Structure of 4-hydoxycinnamic acid or *p*-coumaric acid.

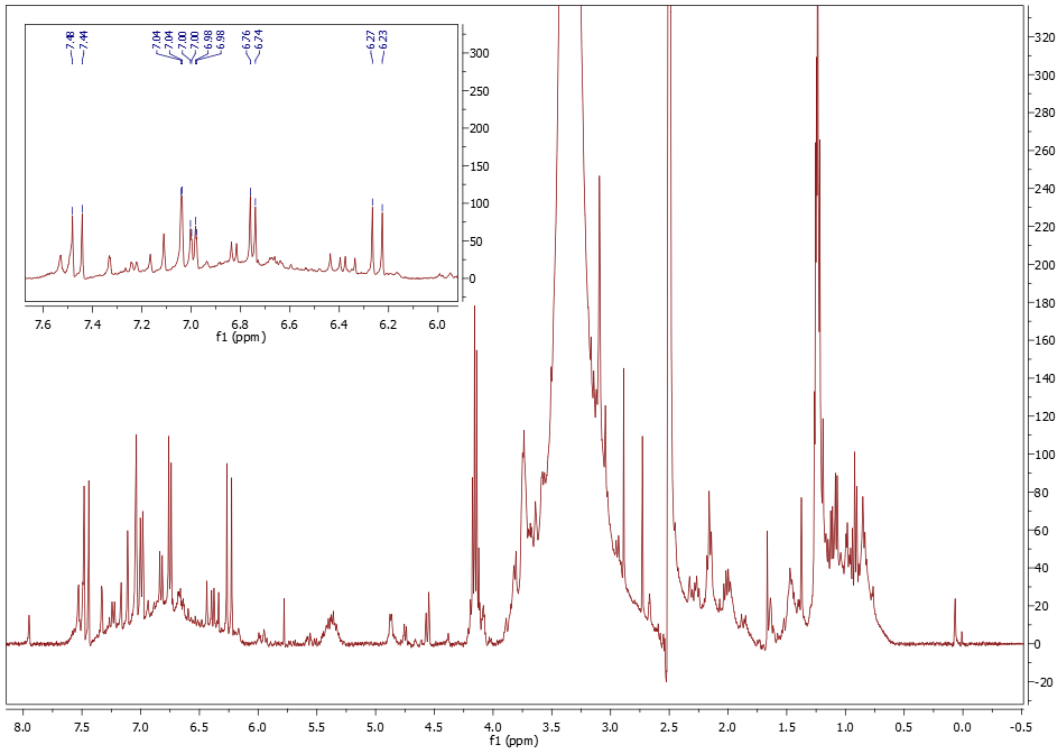

Figure B.3: Expanded ^{1}H NMR spectrum of the active fraction 3 in *d*-dimethyl sulfoxide from δ -0.05 to δ 8.00. Inset: expansion of δ 6.00 to δ 7.60.

A ^{1}H NMR spectrum of fraction 3 (Figure B.**3**) indicated the presence of a hydroxycinnamic acid, possibly a derivative of caffeic acid. The positions of protons refer to those in Figure B.2. The pair of doublets at δ 7.46 (d, J = 15.9 Hz, 1H) and δ 6.25 (d, J = 15.9 Hz, 1H) with characteristically large J values are common to trans double bond protons of cinnamic acids such as those at positions 7 and 8. The signal at δ 7.46 can be attributed to a proton in position 7 as it would undergo a greater downfield shift due to the proximity of the phenyl group. Subsequently the signal at δ 6.25 denotes a proton in position 8. Aromatic protons at positions 2, 5, and 6 respectively would be observed as signals such as those at δ 7.04 (s, J = 1.9 Hz, 1H), δ 6.99 (d, J = 8.1, 1.9 Hz, 1H) and δ 6.75 (d, J = 8.1 Hz, 1H). The small J value of 1.92 Hz of the signal at δ 7.04 is indicative of J-coupling by a proton in the para position. This coupling causes a fine splitting of the singlet. The doublets at δ 6.75 and δ 6.99 with J values of 8.1 Hz are typical of ortho-coupling. The additional splitting of the doublet at δ 6.97 with

the *J* value of 1.9 Hz indicates a proton coupled to protons in both the ortho and para positions. Additional substitution at the carboxylic end of the compound may be present as indicated by the peaks present upfield. This fraction, though, is not pure enough for a definitive identification.

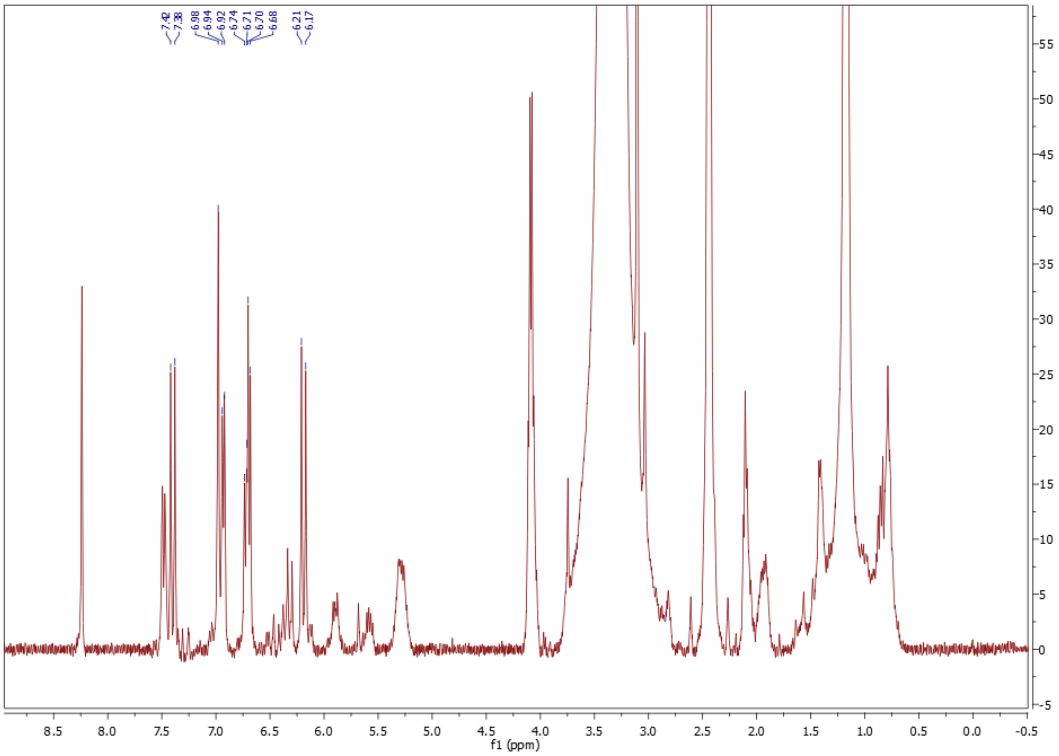

Figure B.4: Expanded ^{1}H NMR spectrum of the inactive fraction 4 in *d*-dimethyl sulfoxide from δ -0.05 to δ 9.00.

Although fraction 4 (Figure B.4) indicates the presence of a hydroxycinnamic acid due to the presence of the pair of doublets at δ 7.40 (d, *J* = 15.8 Hz, 1H) and δ 6.19 (d, *J* = 15.9 Hz, 1H) characteristic of trans double bond protons, the exact structure is not conclusive. The signals at δ 6.93 (d, *J* = 8.4 Hz, 1H), δ 6.73 (d, *J* = 8.9 Hz, 1H), and δ 6.69 (d, *J* = 8.2 Hz, 1H) are all consistent with phenyl protons. This fraction, although contains a hydroxycinnamic acid derivative, was found to be inactive.

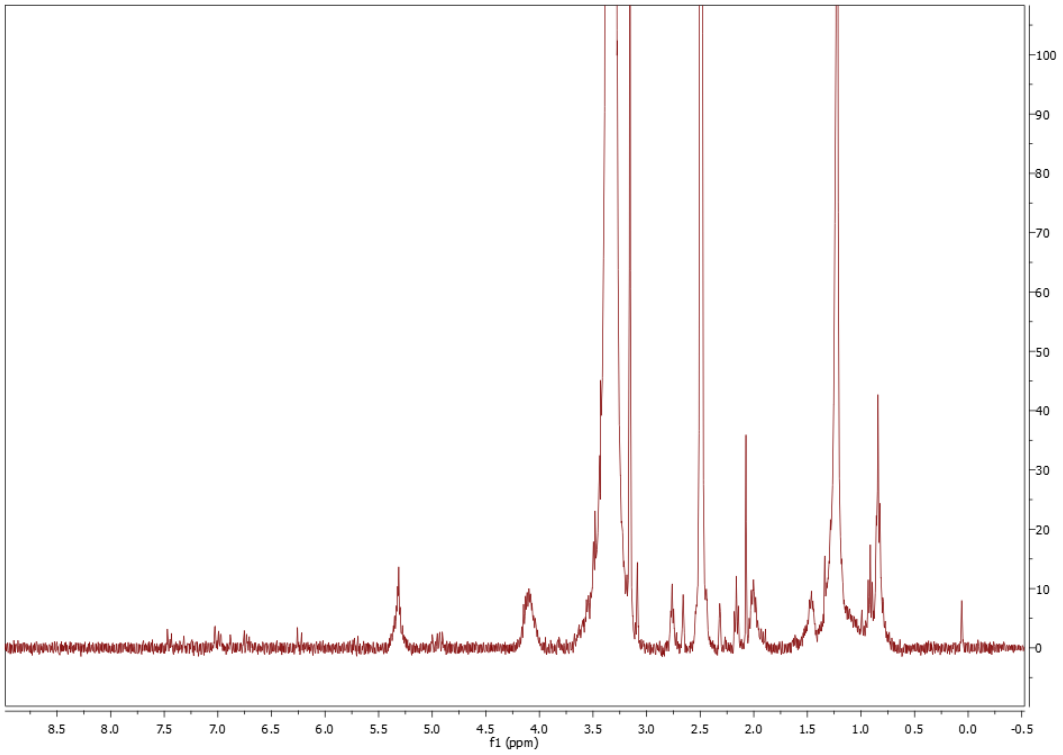

Figure B.5: Expanded ^{1}H NMR spectrum of the inactive fraction 5 in *d*-dimethyl sulfoxide from δ -0.05 to δ 9.00.

Although a ^{1}H NMR spectrum of inactive fraction 5 (Figure B.5) reveals very small signals characteristic signals for the hydroxycinnamic acids between δ 5.50 and δ 7.50, this fraction was deemed to be inactive. This may be due to an active compound being present in only trace amounts, or trace amounts of inactive hydroxycinnamic acids.

Appendix C

Comparisons of ^{1}H NMR Spectra of Commercially Available Samples and Experimental Fractions

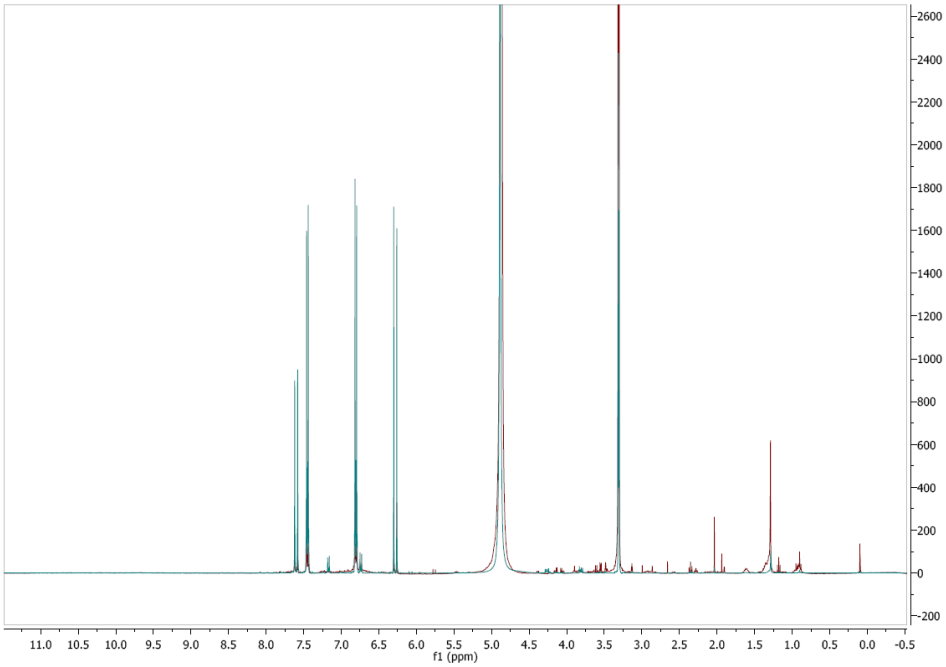

Figure C.1: Superimposed ^{1}H-NMR spectra of Fraction D1.9 and *p*-coumaric acid in methanol-*d*4 from δ -0.50 to δ 12.00.

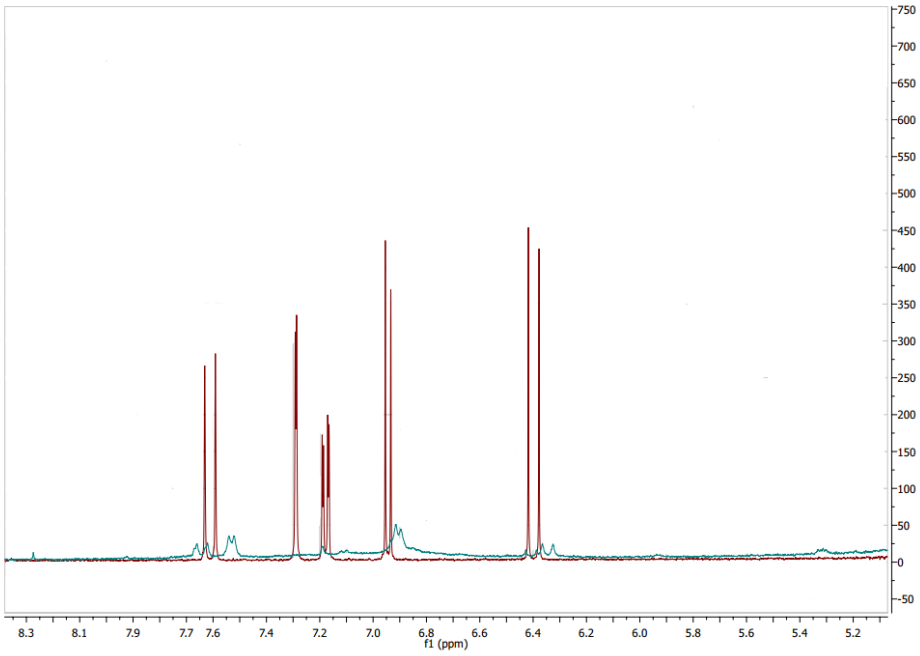

Figure C.2: Superimposed ^{1}H-NMR spectra of Fraction D2.4 and ferulic acid in deuterated water from δ 5.10 to δ 8.30.

Appendix D

Inhibitory Activity of Commercially Available *p*-Coumaric Acid

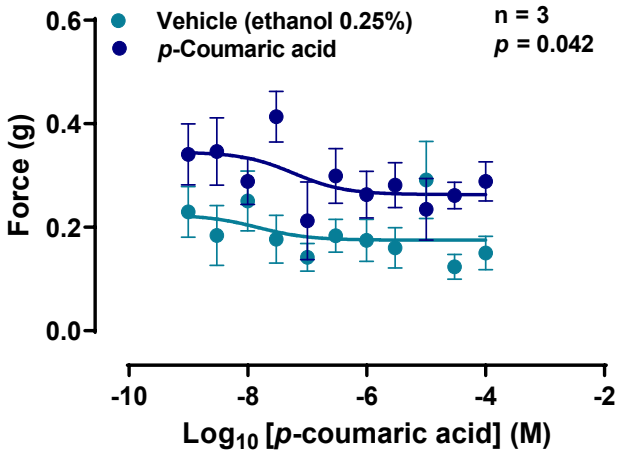

Figure D.1: Mean contractile responses to exogenously administered *p*-coumaric acid (1 nM – 0.1 mM in half log unit increments), or vehicle (ethanol 0.25 % v/v) of isolated rat prostate gland preparations in the presence of 1 µM αβ-methylene ATP (p = 0.042). Each point represents the mean force generated by prostates taken from three rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

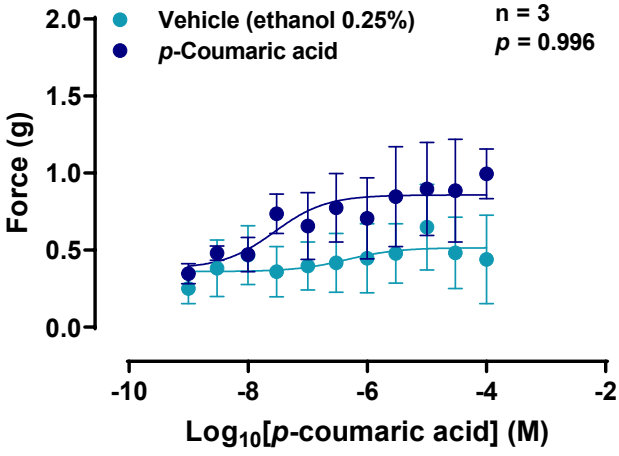

Figure D.2: Mean contractile responses to exogenously administered *p*-coumaric acid (1 nM – 0.1 mM in half log unit increments), or vehicle (ethanol 0.25 % v/v) of isolated rat vas deferens preparations in the presence of 1 µM αβ-methylene ATP (p = 0.042). Each point represents the mean force generated by vasa deferentia taken from three rats. Error bars represent SEM. ANOVA *p*-values were determined by two-way repeated-measures ANOVA and represent the probability that the observed differences were due to chance.

Appendix E

^{1}H NMR of Fraction 3

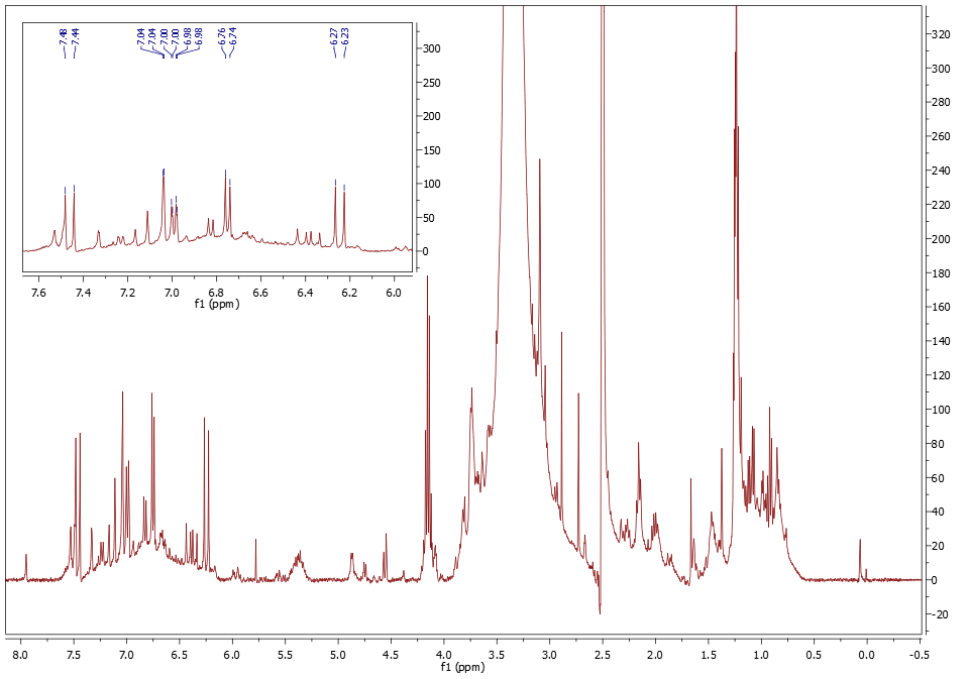

Figure E.1: Expanded ^{1}H NMR spectrum of the active fraction 3 in *d*-dimethyl sulfoxide from δ -0.05 to δ 8.00. Inset: expansion of δ 6.00 to δ 7.60.

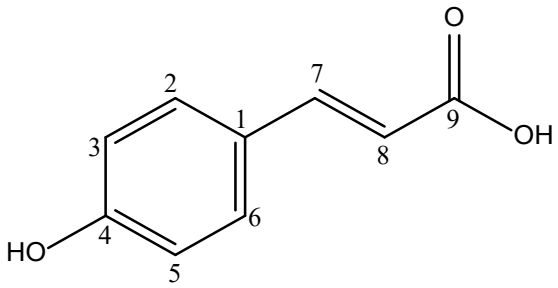

Figure E.2: Structure of 4-hydoxycinnamic acid or *p*-coumaric acid.

A ^{1}H NMR spectrum of fraction 3 (Figure E.1) indicated the presence of a hydroxycinnamic acid, possibly a derivative of caffeic acid. The positions of protons refer to those in Figure E.2. The pair of doublets at δ 7.46 (d, J = 15.9 Hz, 1H) and δ 6.25 (d, J = 15.9 Hz, 1H) with

characteristically large *J* values are common to trans double bond protons of cinnamic acids such as those at positions 7 and 8. The signal at δ 7.46 can be attributed to a proton in position 7 as it would undergo a greater downfield shift due to the proximity of the phenyl group. Subsequently the signal at δ 6.25 denotes a proton in position 8. Aromatic protons at positions 2, 5, and 6 respectively would be observed as signals such as those at δ 7.04 (s, *J* = 1.9 Hz, 1H), δ 6.99 (d, *J* = 8.1, 1.9 Hz, 1H) and δ 6.75 (d, *J* = 8.1 Hz, 1H). The small *J* value of 1.92 Hz of the signal at δ 7.04 is indicative of *J*-coupling by a proton in the para position. This coupling causes a fine splitting of the singlet. The doublets at δ 6.75 and δ 6.99 with *J* values of 8.1 Hz are typical of ortho-coupling. The additional splitting of the doublet at δ 6.97 with the *J* value of 1.9 Hz indicates a proton coupled to protons in both the ortho and para positions. Additional substitution at the carboxylic end of the compound may be present as indicated by the peaks present upfield. This fraction, though, is not pure enough for a definitive identification.