Exploring the Effects of Medicinal Fungi on Isolated Bladder Strips (ex vivo) and *Caenorhabditis elegans* (in vivo): Focus on the Caterpillar Fungus, *Ophiocordyceps sinensis*

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Publications / Awards

Publications

1. **Pang, L.-Y.**, Then, S.-M., Lim, K.-H., et al. (2023) "Biopharmaceutical Potential of Ophiocordyceps sinensis for Human Health." In *Mushrooms with Therapeutic Potentials*. Singapore: Springer Nature Singapore. pp. 189–219. doi:10.1007/978-981-19-9550-7_6.

2. **Pang, L.-Y.**, Lee, M.-K., Kong, B. H., Fung, S.-Y., Ng, S. T., Tan, C. S., Then, S.-M., Lim, K.-H., Roberts, R., & Ting, K.-N. (2022). Chinese Caterpillar Mushroom, *Ophiocordyceps sinensis* (Ascomycota), Cold-Water Extract Relaxes the Rat Isolated Bladder. *International Journal of Medicinal Mushrooms*, 24(3), 1–14. doi:10.1615/IntJMedMushrooms.2022043097

3. Shamsul Anuar, N. A., **Pang, L.-Y.**, Selvam, S. B., Gibbins, C. N., & Ting, K.-N. (2022). Polyethylene microplastics adversely affect airway patency. *Life Sciences, Medicine and Biomedicine*, 6(1). doi:10.28916/lsmb.6.1.2022.93

4. **Pang, L.-Y.**, Sonagara, S., Oduwole, O., Gibbins, C., & Ting, K.-N. (2021). Microplastic - an emerging silent menace to public health. *Life Sciences, Medicine and Biomedicine*, 5(10). doi:10.28916/lsmb.5.10.2021.72

5. **Pang, L.-Y.**, Tan, C.-S., Ng, S.-T., Lim, K.-H., Then, S.-M., & Ting, K.-N. (2020). *Ophiocordyceps sinensis*: a prominent source of bioactive components for alleviating overactive bladder. *Life Sciences, Medicine and Biomedicine*, 4(9). doi:10.28916/lsmb.4.9.2020.67

Presentations

1. **21st World Congress of Food Science & Technology (2022) in Singapore**
   Poster Presentations: *Ophiocordyceps sinensis* improves the healthspan in *Caenorhabditis elegans;* Use of *Ophiocordyceps sinensis* for overactive bladder

2. **The 2nd Joint SWU/UNM Annual Postgraduate Symposium 2022 in Bangkok, Thailand**
   Oral Presentation: The bladder relaxant effect of *Lignosus rhinocerus* and its synergistic effect with *Ophiocordyceps sinensis*

3. **University of Nottingham Malaysia Postgraduate Showcase 2021**
   Poster Presentation: Insight into the mechanism behind *Ophiocordyceps sinensis*-induced bladder relaxation

4. **Three Minute Thesis competition (3MT®) 2021**
   Using medicinal mushrooms to relieve overactive bladder

5. **6th UNM Pharm/Biomed Annual Postgraduate Research Symposium 2021**
   Poster presentation: The role of urothelium underlying the bladder relaxant effect of *Ophiocordyceps sinensis* cold water extract

6. **Mushroom Science Virtual 2020 by the Malaysian Society for Biochemistry and Molecular Biology**
   Oral presentation: *Ophiocordyceps sinensis* cold water extract relaxes rat detrusor muscle

7. **Faculty of Science and Engineering Postgraduate Research Showcase 2020**
   Poster presentation: *Ophiocordyceps sinensis*: A medicinal mushroom for overactive bladder

8. **UNM Pharm/AAPS-NUS PharmSci @ Asia Symposium: Pharmaceutical & Biomedical Science in an Evolving Healthcare Landscape**
   Poster presentation: Can medicinal mushrooms alleviate overactive bladder?

Awards

1. Winner for the Tri Campus Award 2022: UNM Research Team Award for Most Supportive/Inclusive Research Environment
2. Winner for the Tri Campus Award 2022: UNM Postgraduate Assistant Award
3. Second runner-up Oral Presentation at the 2nd Joint SWU-UNM Postgraduate Symposium 2022
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Abstract

Medicinal fungi have been traditionally used to treat many medical conditions. Today, they remain a major source of new pharmaceutical products due to the presence of a plethora of bioactive components. In China and several East Asian countries, *Ophiocordyceps sinensis*, one of the most sought-after medicinal fungi, is widely used for alleviation of urinary symptoms such as frequent urination and nocturia – the two most common symptoms of overactive bladder syndrome (OAB). Despite this, there is no credible scientific evidence that links the effect of *O. sinensis* to bladder function. Therefore, this study was initiated to explore the therapeutic potential of medicinal fungi in OAB due to the limitations of the current conventional treatment options, and to provide scientific validation of *O. sinensis* in alleviating OAB symptoms. Specifically, the principal aim of this study was to investigate the pharmacological actions of *O. sinensis* extract in bladder by using the isolated tissue bath technique. The secondary aim was to provide evidence on its health benefits, given its established antioxidant properties and that OAB is highly prevalent in the geriatric population. A cultivated strain of *O. sinensis* (OCS02®) that has been authenticated by genetic identification was utilised in this study.

In bladder strips pre-contracted with carbachol (muscarinic receptor agonist), the hot water extract of *O. sinensis* (OCS02-HWE) produced a transient contraction whereas the cold water extract of *O. sinensis* (OCS02-CWE) elicited a biphasic response that consists of a transient contraction followed by a sustained relaxation response. To elucidate the bioactive components responsible for the bladder relaxant effect, OCS02-CWE was fractionated using size-exclusion chromatography. All the

fractions exhibited different effects on the carbachol-pre-contracted bladder strips in which the high molecular weight fraction (OCS02-HMW) demonstrated a weak but sustained relaxation response; the medium molecular weight fraction (OCS02-MMW) produced a marked but non-sustained relaxation response; the low molecular weight fraction (OCS02-LMW) elicited only a transient contractile response, like OCS02-HWE. Based on these results as well as their respective carbohydrates and protein contents, we postulated that the contractile effect produced by *O. sinensis* could be attributed to heat-stable, carbohydrate components of low molecular weight. In contrast, the potent and sustained relaxant effect could be contributed by heat-labile components that present in both HMW and MMW fractions. To ensure that the maximum relaxation response of *O. sinensis* is achieved, OCS02-CWE was used for subsequent investigations.

Through a series of mechanistic studies and the use of pharmacological tools, it appeared that the contractile and relaxant effects of OCS02-CWE were mediated via different pathways and did not involve muscarinic receptors, purinergic receptors, adrenoceptors, 5-hydroxytrptamine receptors and cyclic nucleotides. The relaxant effect of OCS02-CWE is partly urothelium-dependent, likely to be mediated via production of NO and regulation of intracellular Ca^{2+}. This is evidenced by the attenuation of OCS02-CWE-induced relaxation response following the removal of urothelium and in the presence of L-NAME (nitric oxide synthase inhibitor) or thapsigargin (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor). In those conditions, the transient contraction induced by OCS02-CWE was not significantly altered. It was nevertheless absent in KCl-pre-contracted bladder strips, suggesting that the transient contraction was a result of membrane depolarisation. Moreover, the transient contraction was potentiated by TEA (non-selective K^+ channel

blocker) and attenuated by membrane permeable Ca^{2+} blockers including nifedipine, verapamil, SKF-96365 and schwarzinicine A. TEA is known to increase the duration and amplitude of an action potential, and membrane depolarisation leads to Ca^{2+} influx via membrane permeable Ca^{2+} channels. The transient contraction of OCS02-CWE was also attenuated in the presence of the cold water extract of *Lignosus rhinocerus* (TM02-CWE), a medicinal fungus that exhibits potent Ca^{2+} inhibitory activity in smooth muscle.

To offer an insight into the use of medicinal fungi to treat OAB, we assessed the combined effect of OCS02-CWE and TM02-CWE in bladder relaxation. Through response-additive model, we showed that these extracts produced synergistic bladder relaxant effect when they are used at equal proportion and at concentrations below 4 mg/mL. They also appeared to extend lifespan and promote health in *Caenorhabditis elegans*. In summary, medicinal fungi contain bioactive components that could be further developed to treat OAB and improve health. For this purpose, *O. sinensis* that exhibits bladder relaxant effect via the regulation of NO and Ca^{2+} represents a promising source that is worthy of further investigation.

Declaration

I declare that all experiments throughout this thesis were carried out by myself. This excludes:

1. OCS02 powder was supplied by LiGNO Sdn Bhd (LiGNO), Malaysia.
2. Cold water extraction of OCS02 was performed by Ms Ng Min Jia and Dr Kong Boon Hong from the Department of Molecular Medicine, Universiti Malaya **(section 2.3.2)**
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6. Lifespan assay of *C. elegans* treated with TM02-CWE was performed by Ms Booi Han Ni from the School of Pharmacy, University of Nottingham Malaysia **(section 6.3.5)**

Abbreviations

$[Ca^{2+}]_i$	intracellular calcium concentration
2-APB	2-aminoethyl diphenylborinate
3Rs	Replacement, Reduction and Refinement
5-HT	5-hydroxytryptamine
8-PT	1,3-dimethyl-8-phenyltheophylline
AC	adenylyl cyclase
ACE	angiotensin-converting enzyme
ADP	adenosine 5'-diphosphate
ANOVA	Analysis of Variance
AR	adenosine receptor
ARB	angiotensin receptor blocker
ATP	adenosine triphosphate
AWERB	Animal Welfare and Ethics Review Body
BK_{Ca} channel	large-conductance Ca^{2+}-activated K^+ channel
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CAT	cysteine aminotransferase
CBS	cystathionine β-synthase
CDC	Centers for Disease Control and Prevention
CGC	Caenorhabditis Genetics Centre
cGMP	cyclic guanosine monophosphate
CO	carbon monoxide
CO_2	carbon dioxide
COX	cyclooxygenases
CRC	concentration-response curve
CSE	cystathionine γ-lyase
CWE	cold water extract

DAG	1,2 diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSM	detrusor smooth muscle
E. coli	*Escherichia coli*
EC_{50}	half-maximal effective concentration
EFS	electrical field stimulation
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
eNOS	endothelial nitric oxide synthase
ENPP	ectonucleotide pyrophosphatase
ENTPDase	ectonucleoside triphosphate diphosphohydrolase
GC	guanylyl cyclase
GPCR	G-protein-coupled receptor
H&E	haematoxylin and eosin
H_2O	water
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
HMW	high molecular weight
HO-1	haem oxygenase-1
HWE	hot water extract
IBMX	3-isobutyl-1-methylxanthine
ICS	International Continence Society
IK	intermediate conductance calcium-activated potassium channel
iNOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate (inositol triphosphate)
IP_3R	inositol 1,4,5-triphosphate (inositol triphosphate) receptor
IPTG	isopropyl β-D-1-thiogalactopyranoside
K^+	potassium ion
K_2HPO_4	dipotassium phosphate
K_{2P} channel	two-pore-domain potassium channel
K_{ATP} channel	inward-rectifying ATP-sensitive potassium channel

K_{Ca} channel	Ca^{2+}-activated potassium channel
KCl	potassium chloride
kDa	kilodalton
KH_2PO_4	potassium dihydrogen phosphate
KPO_4	potassium phosphate
K_V channel	voltage-gated potassium channel
L. rhinocerus	*Lignosus rhinocerus*
L-NAME	NG-nitro-L-arginine methyl ester
L-NOARG	NG-nitro-L-arginine
LB	Luria Bertani
LMW	low molecular weight
LUTS	lower urinary tract symptoms
MDA	malondialdehyde
$MgSO_4$	magnesium sulphate
min	minute
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMW	medium molecular weight
mN	milliNewton
MPST	3-mercaptopyruvate sulfurtransferase
mRNA	messenger ribonucleic acid
MT	metallothionein
MW	molecular weight
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaHS	sodium hydrosulfide
NANC	non-adrenergic, non-cholinergic
NaOH	sodium hydroxide
NCHS	National Center for Health Statistics
NGM	nematode growth media
NHANES	National Health and Nutrition Examination Survey

NLRP3	nucleotide binding and oligomerisation domain-like receptor family pyrin domain-containing 3
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
Nrf2	nuclear factor erythroid-derived 2-like 2
NT5E (CD73)	5'nucleotidases
O. sinensis	*Ophiocordyceps sinensis*
O_2	oxygen
OAB	overactive bladder
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PDE	phosphodiesterase
PG	prostaglandin
PGI_2	prostacyclin
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMC	pontine micturition centre
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt
ppm	pump per minute
PTNS	posterior tibial nerve stimulation
Rho A	Ras homolog family member A
RNA	ribonucleic acid
ROCC	receptor-operated calcium channel
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
RuBPY	cis-[Ru(bpy)2(py)(NO2)](PF6)
RyR	ryanodine receptors
SD	Sprague-Dawley
SEM	standard error of mean
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

sGC	soluble guanylyl cyclase
SHR	Spontaneously Hypertensive Rat
SIN	linsidomine chlorhydrate
SK	small-conductance calcium-activated potassium channels
SNM	sacral neuromodulation
SNP	sodium nitroprusside
SOCC	store-operated calcium channel
SOD	superoxide dismutase
SPE	Saw Palmetto Extract
SQW	Suo-Quan-Wan
SR	sarcoplasmic reticulum
TCM	traditional Chinese medicine
TDX	tetrodotoxin
TEA	tetraethylammonium
TMM	Tiger's milk mushroom
TRPA	transient receptor potential cation channel subfamily A
TRPC	transient receptor potential canonical
TRPV	transient receptor potential vanilloid
TXA_2	thromboxane
UDP	uridine diphosphate
UKM	Universiti Kebangsaan Malaysia
UM	Universiti Malaya
UNM	University of Nottingham Malaysia
UPM	Universiti Putra Malaysia
UTP	uridine triphosphate
Ve	elution volume
VGCC	voltage-gated calcium channel
Vo	void volume

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1 Introduction

1.1 Urinary bladder

1.1.1 Morphology of the bladder

The urinary bladder or simply bladder, is a distensible, hollow smooth muscle organ of the urinary system that serves as a temporary reservoir for urine conveyed through ureters from the kidney (Sam and LaGrange, 2019). It is located in the lesser pelvis when it is empty and extends into the abdominal cavity when it is full. The bladder is divided into two parts: the upper part is composed of the apex and body, while the lower part is composed of the fundus, trigone and neck. Histologically, the bladder consists of a highly impermeable urothelium, an intermediate vascularised suburothelium, a thick smooth muscle coat, known as the detrusor smooth muscle (DSM), and serosa or adventitia (Figure 1.1).

Urothelium, the innermost layer of the bladder, consists of three layers of stratified epithelial cells: the superficial layer that comprised of umbrella cells, the intermediate cells and the basal cells (Winder et al., 2014). The number of intermediate cells varies between species. For example, there is only one intermediate cell layer in the bladder of rodents whereas in humans', there are five layers of intermediate cells (Jost et al., 1989; Winder et al., 2014). Beneath the urothelium, there is the suburothelium which is also known as lamina propria. It consists of various cell types including nerve cells, interstitial cells and myofibroblasts. The DSM is responsible for contraction and relaxation of the bladder, allowing urine excretion and storage, the two important functions of a bladder. It is made up of three layers of smooth muscle

cells in which the outer and inner layers tend to be oriented longitudinally, and those of the middle layer are oriented circularly (Andersson and Arner, 2004). The bladder dome is covered by a thin connective tissue layer, known as serosa. It contains blood vessels of various sizes. In areas of the bladder where there is no serosa, adventitia serves as the bladder's outer layer (Ajalloueian et al., 2018) (Figure 1.1).

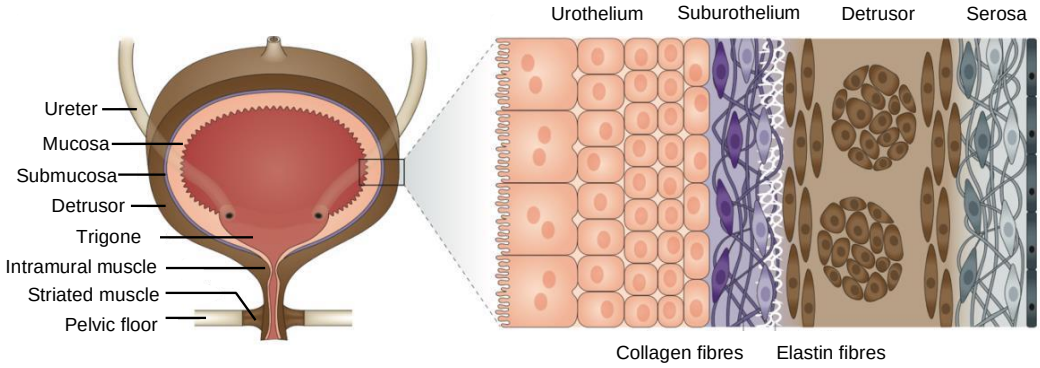

Figure 1.1 Bladder anatomy and muscle layers. *The bladder wall comprises of four layers: the mucosal layer (urothelium), submucosal layer (suburothelium or lamina propria), the muscular layer (detrusor smooth muscle) and the serosa layer. The urothelium is multi-layered and includes basal cells, intermediate cells and umbrella cells. The suburothelium is composed of collagens, interstitial cells, fibroblasts and nerve cells. The muscular component of the bladder comprises 60 – 70% of the normal bladder wall and consists of inner, middle and external layers. Adapted from Ajalloueian et al. (2018).*

In the DSM layer, bundles of muscle cells vary extensively in size and tend to arrange in all directions. Within the main bundles, the smooth muscle cells may exist in groups of small functional units, or fascicles. The orientation and interaction between the smooth muscle cells then determine the shape of the bladder and how it reacts in response to the presence of urine. The individual DSM cells are typical smooth muscle cells, like other smooth muscle organs such as trachea and blood vessels (Sam and LaGrange, 2019). They are long, spindle-shaped cells with a central nucleus. The cytoplasm is packed with the normal myofilaments, and there are also

mitochondria and sarcoplasmic reticulum (SR). In the bladder of smaller animals, muscle bundles and arrangement patterns of DSM are less complex than humans. These differences, along with varied number of urothelial cells are thought to contribute to the micturition patterns, contractile properties and regulation that vary between species (Andersson and Arner, 2004).

While bladder contraction and relaxation are mainly controlled by the DSM and its local signalling pathways, bladder filling and emptying is regulated by external nervous and hormonal control system (Andersson and Arner, 2004). These complex interactions ensure the bladder remains relax during the filling stage with no leakage, and to fully expel the urine while voiding with no urine retention. Disturbances of the storage function may lead to lower urinary tract symptoms (LUTS) such as incontinence and urinary urgency. On the other hand, disturbances of the voiding function can result in symptoms like weak stream, feeling of incomplete bladder emptying and post-micturition dribble. In addition to contractions that void urine, DSM in normal bladder exhibits non-voiding contractions in vivo during filling (Drake et al., 2018). These activities are usually termed as spontaneous contraction, transient contraction, micromotions or spontaneous myogenic contraction, (Chakrabarty et al., 2019; Drake et al., 2017; Fry et al., 2004; Drake et al., 2018; Wang et al., 2018b). They are also present in isolated preparations to reflect local smooth muscle contractions in the bladder wall, making isolated preparations an ideal way to study the bladder function (Heppner et al., 2016).

1.1.2 Physiology and innervation of the bladder

1.1.2.1 Excitation-contraction coupling

Like all smooth muscles, the DSM contraction is resulted by the sliding movements between actin and myosin, the two major contractile protein filaments found in smooth muscles (Andersson and Arner, 2004). In general, this process is stimulated by intracellular Ca^{2+} binding to regulatory protein calmodulin (CaM) (Horowitz et al., 1996). Formation of Ca^{2+}/CaM complex then activates myosin light chain kinase (MLCK) that catalyses phosphorylation of myosin light chain (MLC). It involves the conversion of adenosine triphosphate (ATP) to adenosine 5'-diphosphate (ADP), and attachment of the free phosphate group onto MLC. The phosphorylated MLC then attaches to the actin filament to form cross bridges and initiate DSM contraction (Kamm and Stull, 1985). This process can be reversed by dephosphorylation of MLC with the catalytic action of myosin light-chain phosphatase (MLCP) in response to a decrease in $[Ca^{2+}]_i$. As $[Ca^{2+}]_i$ decreases, Ca^{2+} dissociates from CaM. Dephosphorylation of the MLC uncouples the cross-bridge formation between actin and myosin, resulting in DSM relaxation. The balance between activities of MLCP and MLCK and the concentration of intracellular Ca^{2+}, $[Ca^{2+}]_i$, thus regulates the smooth muscle tone.

Increase in $[Ca^{2+}]_i$ can be stimulated by intracellular Ca^{2+} release from the intracellular Ca^{2+} store, SR, and/or extracellular Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCC), store-operated Ca^{2+} channels (SOCC), receptor-operated Ca^{2+} channels (ROCC), triggered by different upstream signalling pathways (Andersson and Arner, 2004). Following an elevated $[Ca^{2+}]_i$ level, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) comes into play by promoting Ca^{2+} reuptake into the SR to maintain Ca^{2+} homeostasis in the cell (Wray and Burdyga, 2010). There is

also a part of the molecular mechanism for DSM contraction and relaxation that does not rely on Ca^{2+} (Yoshimura and Yamaguchi, 1997). The different signalling pathways of DSM contraction and relaxation are explained in the following subsections.

1.1.2.2 Membrane excitation

The electrophysiological properties of the detrusor of various species have been studied. In general, action potential in the DSM can be stimulated by multiple factors, including neurotransmitters, circulating hormones and mechanical stretch (Montgomery and Fry, 1992; Poley et al., 2008; Thorneloe et al., 2005; Valeri et al., 2009). Upon stimulation, depolarisation begins with an inward flow of Ca^{2+} current, evidenced by its sensitivity towards L-type VGCCs blocker but not tetrodotoxin (TTX), a Na^{+} blocker (Montgomery and Fry, 1992). The elevated $[Ca^{2+}]_i$ causes DSM contraction following formation of the Ca^{2+}/CaM complex as described in **section 1.1.2.1**.

While both L-type VGCCs and T-type VGCCs are expressed in the DSM, the role of T-type VGCCs appears to be much less important than the L-type channels (Fry et al., 2006). The extracellular Ca^{2+} influx in the DSM is mainly regulated by L-type VGCCs, which can be inhibited by drugs such as nifedipine, verapamil or diltiazem (Triggle, 1998). In isolated bladder tissues, blockade of L-type VGCCs has been shown to abolish spontaneous action potentials, Ca^{2+} transients, and phasic contractions (Badawi et al., 2006). Knockdown of L-type VGCCs in mice also resulted in severely reduced micturition, increased bladder mass and absence of spontaneous phasic contractions (Wegener et al., 2004). In accordance with these studies that suggested the importance of extracellular Ca^{2+} in the generation of action potential in DSM, Hashitani et al. (2001) showed that the spontaneous action potentials cannot be prevented by the inhibition of intracellular Ca^{2+} release. However, Ca^{2+} release from

SR may amplify the increase in $[Ca^{2+}]_i$ associated with the action potentials (Hashitani et al., 2001).

After the cell reaches its highest voltage from depolarisation, repolarisation happens as Ca^{2+} current is inactivated (Berridge, 2008). It involves initiation of an outward K^+ current, mediated by activation of K^+ channels that leads to a decrease of intracellular Ca^{2+} and subsequent DSM relaxation (Hashitani et al., 2001). The DSM express several families of K^+ channels, including voltage-gated K^+ (K_v) channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, inward-rectifying ATP-sensitive K^+ (K_{ATP}) channels and two-pore-domain K^+ (K_{2P}) channels (Petkov, 2012). Among the K^+ channels, BK channel which belongs to the K_{Ca} channels family is the most important K^+ channels in the regulation of DSM function (Petkov, 2014). This is largely because of its unique characteristic as the only member of the K^+ channel family that is activated by both voltage and Ca^{2+}. The activation of BK channel depends on both Ca^{2+} entry through the L-type VGCCs and Ca^{2+} release from SR (Function et al., 2011; Herrera et al., 2001; Ohi et al., 2001).

Overall, studies showed that the physiological function of BK channel is to reduce membrane excitability and oppose both myogenic and neurogenic DSM contractions. Iberiotoxin and charybdotoxin are BK channel blockers commonly used in pharmacological studies (Petkov, 2012). Charybdotoxin also blocks IK channels and several K_V ($K_V1.2$, $K_V1.3$, $K_V1.6$ and $K_V1.8$) channels (Petkov, 2012). Tetraethylammonium (TEA) is also a powerful pharmacological tool used in the studies of K^+ channels (Petkov, 2012). It is capable of blocking BK and SK channels as well as all K_V channels. It is noteworthy that although IK channels are expressed at the mRNA and protein levels, they may not have a role in DSM excitability and contractibility (Afeli et al., 2012). This appears to be the same for the role of ATP-

sensitive K^+ channels (K_{ATP} or $K_{ir}6$ channels) in the DSM (Kajioka et al., 2011; Imai et al., 2001). Supposedly, activation of K_{ATP} channels leads to membrane hyperpolarisation and closure of the L-type Ca^{2+} channels and results in DSM relaxation. However, it has been shown that glibenclamide, a specific K_{ATP} channel inhibitor did not affect DSM excitability (Buckner et al., 2002). Petkov (2012) also reported that DSM strips isolated from various knock-out mouse models lacking different K_{ATP} channel subunits showed no difference in contractility when compared with DSM strips from mice with functional K_{ATP} channels. While these studies suggest that K_{ATP} channels are negligible in the bladder, density of K_{ATP} channels appear to be significantly higher in DSM than other tissues (Shieh et al., 2001). The role of these K_{ATP} channels in the bladder thus remains a baffling question.

1.1.2.3 Muscarinic receptors

In a normal bladder, the contraction is predominated by the activation of G-protein-coupled muscarinic receptors (Wang et al., 1995). All muscarinic receptors subtypes including M_1, M_2, M_3, M_4 and M_5 are expressed in the bladder in which M_1, M_3 and M_5 are coupled to the α subunits of $G_{q/11}$, and M_2 and M_4 are coupled to the α subunits of $G_{i/o}$ (Chess-Williams, 2002). There is a predominance of M_2 and M_3 receptors in which the density of M_2 receptors is far greater than the density of M_3 receptors. The M_2:M_3 ratio being 3:1 in most species including human (Uchiyama and Chess-Williams, 2004). Despite the predominance of M_2 receptors, studies have shown that M_3 receptors are the major contributor of DSM contraction (Chess-Williams et al., 2001). The importance of M_3 receptors has been demonstrated in M_2 and M_3 receptor knockout animal models. Based on the contractile response of bladder strips isolated from M_2 and M_3 knockout mice towards carbachol, a non-selective muscarinic agonist, Matsui et al. (2002) reported that M_2 and M_3 receptors contribute to 5% and 95% of

DSM contraction, respectively, Besides, M_3 knockout mice has shown severe consequences on the bladder functions including longer voiding intervals, larger micturition volumes and bladder capacity, compared to M_2 knockout or normal mice (Igawa et al., 2004).

The classic downstream signalling of $G_{q/11}$ involves activation of phospholipase C (PLC) that hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) into 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Mizuno and Itoh, 2009). IP_3 then binds to IP_3 receptors (IP_3Rs) embedded on the membrane of SR and promotes the release of stored Ca^{2+}. A localised increase of $[Ca^{2+}]_i$ then triggers the activation of ryanodine receptors (RyRs) further increases Ca^{2+} efflux from the SR. To maintain cellular Ca^{2+} homeostasis, the depletion of Ca^{2+} in the SR triggers the opening of SOCC to facilitate transfer of Ca^{2+} ions from the extracellular space (McFadzean and Gibson, 2002). Transient receptor potential canonical (TRPC) channels that are closely coupled to RyRs have been suggested as a potential candidate of the SOCC (Kajioka et al., 2005).

In parallel, the other product of PIP_2 hydrolysis, DAG, remains membrane bound and promotes the translocation of protein kinase C (PKC) from the cytoplasm to the membrane (Mizuno and Itoh, 2009). PKC plays a role in phosphorylating calponin, a protein associated with actin filaments, preventing its ability to inhibit myosin ATPase. It also inhibits MLCP to indirectly promote DSM contraction by facilitating additional Ca^{2+} influx into the cells through activation of ROCC, possibly via the TRPC channels (Earley and Brayden, 2015; Venkatachalam et al., 2003).

The role of TRPC channels in muscarinic receptor-mediated DSM contraction has only been validated recently. Griffin et al. (2016) showed that ML204, a selective TRPC4 and TRPC5 inhibitor, inhibited carbachol-induced contraction in the murine

bladder. However, the effect was suggested to be solely caused by TRPC4 inhibition as PCR experiments showed that DSM cells do not express TRPC5 channels (Andersson, 2019). Thus, it was postulated that following stimulation of $G_{q/11}$-coupled M_3 receptors, TRPC4 channels were activated, leading to membrane depolarisation and activation of VGCCs. This finding was further supported by the impaired spontaneous and muscarinic receptors-mediated bladder contraction in TRPC4 deficient mice (Griffin et al., 2018).

While the interaction between TRPC and L-type VGCCs in the bladder has not been established, findings of Griffin et al. (2016) and Griffin et al. (2018) correlate with previous reports that showed the importance of L-type VGCCs in M_3-receptor-mediated contraction. Wegener et al. (2004) has demonstrated that mice deficient in the smooth muscle L-type VGCCs have a severely reduced micturition and dilated bladder. Their bladder tissues also exhibited a markedly reduced DSM contraction in response to carbachol. Moreover, neither rho-kinase pathways nor Ca^{2+} release from the SR can compensate the lack of L-type VGCCs (Wegener et al., 2004). Similarly, in DSM tissues isolated from various species such as humans, rats and guinea pigs, L-type VGCC blockers markedly suppress acetylcholine or carbachol-induced contractions (Schneider et al., 2004; Rivera and Brading, 2006; Wu et al., 2002). Interestingly, the concentration of VGCC blockers needed to inhibit bladder contraction are much lower than those typically needed to inhibit contraction of vascular smooth muscle (Badawi et al., 2006). Apart from the possible involvement of TRPC4 channels, more investigations are needed to uncover the coupling between muscarinic receptors and L-type VGCCs.

M_3 receptors are also coupled to $G_{12/13}$ protein which activates the monomeric G protein Rho A and its downstream effector Rho-associated protein kinase (ROCK)

(Burstein et al., 1997). ROCK, in turn, inhibits phosphorylation of MLC through inactivation of MLCP, leading to DSM contraction. This pathway is often referred to as the Ca^{2+}-sensitising pathway, or the Ca^{2+}-independent pathway, as an increase in $[Ca^{2+}]_i$ is not needed for the contraction. This pathway is considered as less important in normal bladder, but recent studies have suggested its exacerbation in local and systemic pathological conditions that affect the bladder (Peters et al., 2006).

As aforementioned, 5% of muscarinic receptor-mediated contraction is contributed by the $G_{i/o}$-coupled M_2 receptors. Activation of $G_{i/o}$ inhibits activity of adenylyl cyclase (AC), leading to the reduced production of second messenger cyclic adenosine monophosphate (cAMP) and prevention of DSM relaxation (Uchiyama and Chess-Williams, 2004). The role of cAMP in detrusor regulation is explained in **section 1.1.2.5**.

1.1.2.4 Purinergic receptors

Purinergic signalling induced by ATP in the bladder was proposed around 50 years ago after determining ATP as a main non-adrenergic, non-cholinergic (NANC) transmitter in the gut and bladder (Burnstock et al., 1972a, 1972b). Generally, ATP modulates a wide range of cell and organ functions via activation of purinergic P2 receptors. These receptors are divided into two distinct families, that are, P2X receptors, which are ligand-gated cation-channels and P2Y receptors, which are GPCRs. Currently, seven subtypes of P2X receptors ($P2X_{1-7}$) and eight subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) have been recognised in the mammalian cells (Kügelgen, 2008). In the bladder, all 7 P2X receptors as well as $P2Y_1$, $P2Y_2$, and $P2Y_4$ are expressed on both urothelial and DSM cells. Immunohistochemical experiments showed that $P2X_1$, $P2Y_2$ and $P2Y_4$ are the most abundant P2 receptors in the bladder (Burnstock, 2014).

Functional contributions of these receptors to the bladder have been demonstrated using transgenic animal models, isolated bladder tissues with and without urothelium and a series of pharmacological agent such as α,β-methylene ATP (selective P2X agonist), suramin (non-selective purinergic antagonist), pyridoxalphosphate-6-azophenyl-2'4'-disulfonic acid or PPADS (selective P2X antagonist) (Aronsson et al., 2010; Boland et al., 1993; Lee et al., 2018c; Hao et al., 2019). Generally, activation of P2X receptors by ATP leads to membrane depolarisation and extracellular Ca^{2+} influx, resulting in DSM contraction. On the other hand, activation of the P2Y receptors by ATP induces DSM relaxation. The downstream signalling of P2Y receptors that results in DSM relaxation remains unclear, but it is likely to involve elevation of cAMP and/or activation of K^+ channels (Burnstock, 2014). It is also worth noting that the purinergic component contributes to less than 3% of DSM contraction in healthy individuals, but in pathological conditions, it is increased to 40% (O'Reilly et al., 2002). This phenomenon indicates that the expression of purinergic receptors in the bladder may be altered in disease state.

There is another group of purinergic receptors known as the P1 purinoceptors or adenosine receptors (ARs) expressed on the DSM and urothelial cells (Aronsson et al., 2010; Yu et al., 2006). They are a class of GPCRs and are divided into four subtypes i.e., A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 receptors are coupled to G_i family that inactivates AC and decrease production of cAMP whereas A_{2A} and A_{2B} receptors are coupled to G_s family and stimulate cAMP production (Yu et al., 2006). While activation of different AR subtypes leads to both smooth muscle contraction and relaxation, adenosine mainly exhibits relaxation responses in DSM, likely because the expression of A2 receptors are higher in the DSM and urothelial cells (Zhou et al.,

2017b). The DSM relaxant effect of adenosine has been proven through its sensitivity towards 1,3-dimethyl-8-phenyltheophylline, 8-PT (non-selective A_2 receptor antagonist), PSB1115 (selective A_{2B} receptor antagonist) and istradefylline (selective A_{2A} receptor antagonist), in isolated tissue preparations (Aronsson et al., 2010; Boland et al., 1993; Kitta et al., 2014). Besides, a recent study reported that deletion of A_{2B} receptors in mice has a significant reduction of DSM contractile force (Hao et al., 2019).

1.1.2.5 Adrenoceptors

The expression of α_1-, α_2- and β-adrenoceptors have been detected in the bladder (Michel and Vrydag, 2006). In most animal species, α_1-adrenoceptors are poorly expressed and have limited functional role in the bladder (Michel and Vrydag, 2006). For instance, α_1-adrenoceptor agonist, phenylephrine, produced only weak contractions in rat bladder (Roedel et al., 2018; Bouchelouche et al., 2005). Currently, the functional role of α_2-adrenoceptors in the bladder remains inadequate. Santicioli et al. (1983) has suggested a possible role of α_2-adrenoceptors in pre-junctional neurotransmitter release as clonidine, an α_2-adrenoceptor agonist, inhibited the EFS-induced contraction in rat bladder, but not TDX. However, the post-junctional function of α_2-adrenoceptors in the bladder has not been established and they do not produce bladder responses in vitro (Michel & Vrydag, 2006).

Among the three β-adrenoceptor subtypes (β_1, β_2 and β_3) that are expressed in the bladder, β_3-adrenoceptors are the most abundant subtype and account for more than 95% of all β-adrenoceptor mRNA in the human bladder (Nomiya and Yamaguchi, 2003). β-adrenoceptors are coupled to G_s protein in which its activation augments DSM relaxation. It is well recognised that activation of G_s protein leads to activation of AC that facilitates conversion of cAMP from ATP (Billington and Penn, 2003). In

turn, cAMP causes activation of protein kinase A (PKA) which targets MLCK and hinders phosphorylation of MLC, resulting in DSM relaxation. Apart from increasing the production of cAMP, compelling evidence suggests that β-adrenoceptors may activate BK channels to promote efflux of K^+ ions and hyperpolarisation (Ghatta et al., 2006). For instance, inhibition of BK channel significantly reduced β-adrenoceptor-mediated DSM relaxation in mice, and isoprenaline, a non-selective β-adrenoceptor agonist markedly modulated K^+ conductance in human DSM (Brown et al., 2008; Takemoto et al., 2008).

1.1.2.6 5-hydroxytryptamine receptors

5-hydroxytryptamine (5-HT), also known as serotonin has long been implicated in the central control of micturition (Ramage, 2006). Over the past decade, the functional role of 5-HT on the bladder has received substantial attention due to the discovery of 5-HT receptors expression on the DSM and urothelial cells. However, the downstream mechanism of 5-HT is complex due to the multitude of sites of action, receptor subtypes and species-specific actions (Matsumoto-Miyai et al., 2015). 5-HT receptors are categorised into seven classes (5-HT_1 to 5-HT_7) in which 5-HT_1 and 5-HT_5 subtypes are coupled to $G_{i/o}$ protein and suppress production of cAMP; 5-HT_4, 5-HT_6 and 5-HT_7 subtypes are coupled to G_s protein that stimulate production of cAMP; 5-HT_2 subtypes are $G_{q/11}$-coupled receptor that activate PLC; and 5-HT_3 subtypes are the only 5-HT ligand-gated cation channels. In normal urinary bladders, the expression of both 5-HT_2 and 5-HT_7 mRNA predominates. While 5-HT_2 mediates contraction and 5-HT_7 mediates relaxation, exogenous application of 5-HT produces appear to elicit only contractile response in bladder tissues, suggesting the dominant role of 5-HT_2 in DSM regulation (Kim and Lee, 2001; Hernández et al., 2003; Mbaki and Ramage, 2008; Lychkova and Pavone, 2013). Studies have also suggested the slight

role of 5-HT_3 and 5-HT_4 receptors in the regulation of DSM. They may indirectly cause DSM contraction by facilitating the release of acetylcholine and ATP (Matsumoto-Miyai et al., 2015). Although urothelial cells do not release 5-HT, urothelial 5-HT receptors may have a role in controlling distension-induced ATP release from the urothelium (Matsumoto-Miyai et al., 2016).

1.1.3 Urothelial-derived signalling molecules

In the past, urothelium was thought to act only as a passive barrier to ions and solutes. Following extensive research over the past two decades, its roles in the regulation of bladder are revealed. Urothelial cells can sense changes in their extracellular environment, and respond to chemical, mechanical and thermal stimuli by releasing various factors such as acetylcholine, ATP, adenosine, nitric oxide, hydrogen sulfide (H_2S), prostaglandins and neuropeptides (Winder et al., 2014).

1.1.3.1 Acetylcholine

The basal release of acetylcholine in the bladder originated from the urothelium was first reported by Yoshida et al. (2006). Before this, neurons were thought to be the only source of acetylcholine in the bladder. This finding was then substantiated by Hanna-Mitchell et al. (2007). The authors demonstrated that the urothelial cells release acetylcholine via polyspecific organic cation transporter (OCT_3), unlike neurons which release acetylcholine via vesicular storage and exocytosis (Hanna-Mitchell et al., 2007). Muscarinic receptors are also expressed on the urothelial cells, allowing the binding of acetylcholine in addition to the DSM, afferent nerve terminals and myofibroblasts, for muscarinic modulation of the bladder function (Hawthorn et al., 2000). The downstream signalling of activation of muscarinic receptors by acetylcholine are detailed in **section 1.1.2.3**. It is noteworthy that acetylcholine

released from the urothelial cells may initiate negative feedback mechanism to inhibit its own release in the urothelium once the muscarinic receptors on the urothelial cells are occupied (Hanna-Mitchell et al., 2007). It may also indirectly facilitate the release of other substances such as ATP, nitric oxide, and prostaglandins from the urothelial cells and thus, alter neural excitability of the tissues (Winder et al., 2014).

1.1.3.2 Adenosine triphosphate

Pivotal to the sensory role of urothelium is the release of ATP during mechanical deformation in response to physical stimuli such as shear stress, stretch, osmotic swelling, hypoxia, and chemical stimuli (Winder et al., 2014). Several pathways underlying the release of ATP and other nucleosides (adenosine diphosphate - ADP, uridine triphosphate – UTP, and uridine diphosphate - UDP) from the urothelial cells into both mucosal and serosa compartments of the bladder wall have been suggested: (1) through vesicular transport or exocytosis (Nakagomi et al., 2016); (2) through pannexin hemichannel conductive efflux (Timóteo et al., 2014); (3) negative feedback control of ATP release following A1 receptor activation by adenosine (Dunning-Davies et al., 2013); (4) initiated by the increase of $[Ca2^{+}]_i$ (Dunning-Davies et al., 2013; Wang et al., 2005). Recently, a study revealed the functional circadian system of the urothelium which regulates diurnal ATP release through connexin 43 hemichannels, suggesting its role in the regulation of day-night changes in functional bladder capacity (Sengiku et al., 2018). Like neuronal released ATP, ATP released from the urothelium may bind to P2X or P2Y receptors, localised on the urothelial cells and DSM cells. See **section 1.1.2.4** for the downstream signalling pathways of P2X and P2Y receptors.

1.1.3.3 Adenosine

Growing evidence indicates the importance of adenosine in regulating bladder health. In addition to acting as a component of DNA and RNA, it functions as an endogenous transmitter in the extracellular space (Sachdeva and Gupta, 2013). The main understanding on the formation of adenosine is through the extracellular hydrolysis of ATP by ectonucleotidases including ectonucleoside triphosphate diphosphohydrolase (ENTPDase, CD39) and ectonucleotide pyrophosphatase (ENPP). These ectonucleotidases hydrolyse ATP and ADP to AMP which in turn, was further hydrolysed by 5'nucleotidases (NT5E, CD73) to adenosine (Winder et al., 2014). Following the establishment of the mechanosensory properties of urothelium, studies showed that adenosine is also released from the luminal and basolateral surfaces of urothelium in response to stretch of the bladder wall during filling and the contraction of the DSM cells (Yu et al., 2006). Adenosine then exhibits its effects by activating adenosine receptors, as explained in **section 1.1.2.4.**

1.1.3.4 Nitric oxide

Nitric oxide (NO) is a NANC gasotransmitter that plays a role in the regulation of bladder. Like in endothelial cells and epithelial cells, it is formed in the urothelial cells from natural amino acid L-arginine through nitric oxide synthase (NOS) (Fernandes and Hernández, 2016). It is then translocated into the DSM cells by simple diffusion. There are three distinct isoforms of NOS: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS). eNOS and nNOS are considered to be constitutively expressed while iNOS is usually expressed upon certain intracellular signalling (Smet et al., 1996). For instance, in mammalian cell culture, the expression of iNOS can be induced by lipopolysaccharide (LPS) treatment (Kim et al., 2005b). The expression of both eNOS and nNOS have

been detected on the urothelial cells (Smet et al., 1996). While elucidating the functional roles of NOS, NOS inhibitors such as NG-nitro-L-arginine (L-NOARG) and NG-nitro-L-arginine methyl ester (L-NAME) are commonly used (Pfeiffer et al., 1996). There are several hypotheses surrounding the release of NO from the urothelium. First, NO can be released from urothelial cells upon mechanical force or stretch (Birder et al., 1998, 2002a). Second, NO can be released from the urothelium upon activation of muscarinic receptors, possibly M_5 receptors (Andersson et al., 2012, 2008). Third, activation of β-adrenoceptors can lead to a Ca^{2+}-dependent release of NO (Birder et al., 2002b; Persyn et al., 2016). The roles of NO on DSM contraction and relaxation are elaborated in **section 4.1 (Chapter 4)**.

1.1.3.5 Prostaglandins

The involvement of prostaglandins in bladder physiology was noticed following the detection of increased prostaglandins level in the urine of patients suffering from bladder diseases (Suh et al., 2015; Kim et al., 2006, 2005a). Recent studies found that prostaglandins are synthesised in both urothelium and suburothelium by cyclooxygenases (COX-1 or COX-2) in response to stretch, nerve stimulation and inflammatory mediators (Stromberga et al., 2020a). Therefore, the production of prostaglandin is usually low in healthy tissues but can be immediately elevated following inflammation. Through COX, prostaglandins are converted into five primary prostanoids including PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane (TXA_2). These five primary prostaglandins have been shown to stimulate contractions and phasic activity of the bladder (Stromberga et al., 2020b). However, there are significant differences in the DSM contractile responses between species for different prostaglandin receptor subtypes. For example, PGE_2 contracted rat, macaque and human bladder strips, but $PGF_{2\alpha}$ has a lower potency in rat (Root et

al., 2015). Overall, PGE_2 is thought to be the major contributor to the modulation of bladder function (Stromberga et al., 2020b). Interestingly, the main contractile effects of PGE_2 in both urothelium and DSM are mediated via FP receptor, with no contribution from EP1 – 4 receptors, the prototypical receptors of PGE_2 (Stromberga et al., 2020a). Activation of FP receptors leads to G_q-dependent PLC activation, resulting in the increase of $[Ca^{2+}]_i$ and activation of MAPK as well as Rho kinase.

1.1.3.6 Hydrogen sulfide

Hydrogen sulfide (H_2S) is a newly discovered gas transmitter in mammals, after carbon monoxide (CO) and NO. More recently, it is found to be endogenously produced in the bladder of several species, such as trout, rat, pig, and human, implying its role in the control of bladder function (D'Emmanuele di Villa Bianca et al., 2016; Fernandes et al., 2013; Dombkowski et al., 2006; Patacchini et al., 2005). In the cells, H_2S can be synthesised by two mechanisms. The first involves cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) that are responsible for the metabolism of L-cysteine, while the second involves the combined action of 3-mercaptopyruvate sulfurtransferase (MPST) and cysteine aminotransferase (CAT) (Zou et al., 2018). In the bladder, the biosynthesis of H_2S is more likely to depend on MPST and CAT as these enzymes are detected in the urothelium and DSM, but neither CBS nor CSE was detected (Zou et al., 2018). Production of H_2S in the bladder results in DSM relaxation, but its mechanistic pathways vary between species. In rat and human DSM, H_2S-mediated relaxation seemed to involve the inhibition of TRPC channels and activation of K_{ATP} channels, whereas in pig bladder neck, activation of $TRPA_1$ and/or $TRPV_1$ cation channels is implicated (Fernandes et al., 2013; Mitidieri et al., 2022; Zou et al., 2018; Patacchini et al., 2005).

1.2 Bladder disorder: Overactive bladder

Overactive bladder (OAB) is one of the most common conditions that affect the bladder. It is defined by the International Continence Society (ICS) as a condition characterised by urinary urgency, with or without urge incontinence, usually with increased daytime frequency and nocturia, if there is no proven infection or other obvious pathology (Abrams et al., 2003). It is a devastating chronic condition that substantially affects the social, occupational, domestic, physical, sexual and psychological aspects of those affected ones (Abrams et al., 2000). The epidemiologic study specific to OAB has only begun in the late 2000s in developed countries due to the imprecise definitions of OAB in the past. Some studies examined the prevalence of OAB solely based on the occurrence of urge incontinence, daytime frequency or only in either gender. The prevalence of OAB also appears to be varied in different countries. According to one of the largest population-based surveys that studied the prevalence of LUTS and OAB in Canada, Germany, Italy, Sweden and the UK, the overall OAB prevalence was 11.8%, with similar rates in men and women (Irwin et al., 2006). In the USA, OAB also represents a significant public health burden, affecting one out of seven adults (Reynolds et al., 2016). Over the past few years, more epidemiologic data of OAB in Asian countries have been made available. In China, Taiwan and South Korea, OAB symptoms were found to affect 20% of individuals aged 40 years old and above (Chuang et al., 2019). On the other hand, the prevalence of OAB in Malaysia ranges from 19% to 42% (Ho et al., 2012; Ahmad et al., 2015). While the overall prevalence of OAB reported in these studies varies, they consistently demonstrated that its prevalence increases with age, suggesting that age is a major risk factor of OAB. It is also concerned that patients with OAB are significantly frailer than individuals seeking care for other non-oncologic urologic disease (Suskind et al.,

2017). Moreover, there is a positive correlation between OAB and mortality risk in adult population (Cho et al., 2021).

1.2.1 Pathophysiology of OAB

The aetiology and pathophysiology of OAB are complex and multifactorial. Three main theories have been proposed regarding its aetiology: myogenic, neurogenic and urotheliogenic (Brading, 1997; De Groat, 1997; Tyagi, 2011). Recent studies also revealed a strong association between OAB and metabolic syndrome as a result of inflammation and oxidative stress (He et al., 2016). There is also a group of patients suffering from idiopathic OAB in which the cause of OAB cannot be identified and this represents one of the main hurdles in managing this condition (Hanna-Mitchell et al., 2014).

1.2.1.1 Myogenic theory

The myogenic theory of OAB was originally explained by Brading (1997) who stated that the urgency is initiated from the involuntary contraction of the DSM due to an unstable elevation in intravesical pressure during bladder filling. The changes in properties, structure and innervation of DSM are initiated by abnormal electrical coupling of the cells and instability of membrane potential through cell depolarisation, that in turn interrupt the coordinated contraction of the DSM (Brading, 1997). This theory was later substantiated by Sui et al. (2009). Using DSM cells from overactive human bladders, the authors demonstrated that the autonomous contraction of the DSM in OAB was a result of spontaneous and transient increase of $[Ca^{2+}]_i$. Furthermore, the spontaneous Ca^{2+} oscillations were Ca^{2+}-dependent, sensitive to L-type VGCC antagonist and SERCA inhibitor, suggesting that defective Ca^{2+} regulation by either membrane permeable Ca^{2+} channels or SR contributes to OAB (Sui et al.,

2009). The involuntary DSM contraction may also cause periodic bladder ischaemia that in turn, damage the intrinsic neurons in the bladder and promote secondary changes in the smooth muscle properties (Christ and Hodges, 2006).

1.2.1.2 Neurogenic theory

The neurogenic theory suggests that OAB arise from either increased afferent input from the bladder, abnormal central processing of afferent input leading to the reduced pontine micturition centre (PMC) inhibition or impaired micturition reflex organised in spinal cord (De Groat, 1997). In the lumbosacral cord, excitatory glutamatergic and inhibitory glycinergic neurons influence both the afferent and efferent limbs of the micturition reflex (Sugaya et al., 2005). In paediatric populations, damage often results from congenital and perinatal defects such as cerebral palsy and sacral agenesis (Truzzi et al., 2022). On the other hand, neurogenic OAB is commonly seen in adult patients who have neurological disorders such as multiple sclerosis, Parkinson's disease, spinal cord injury and post-cerebrovascular events (Meng et al., 2012; Tyagi, 2011). Recently, studies have demonstrated that the underlying mechanism of neurogenic OAB could be caused by the upregulation of $TRPV_1$ receptors on the C-fibre nerve endings, following damage to the brain or spinal cord, or upregulation of nicotinic acetylcholine receptors in pelvic ganglion neurons that potentiate ganglionic transmission (Chung et al., 2015; Park et al., 2018; de Groat and Yoshimura, 2012).

1.2.1.3 Urotheliogenic theory

Following the recent discovery of the functional role of urothelium, a new etiological paradigm of OAB has emerged: the urotheliogenic theory. Given the ability of the urothelium in releasing relaxing factors such as adenosine, NO and H_2S, damage of the urothelium may result in involuntary contraction of the DSM. The interaction

between neurons and urothelial cells also plays an important role in the regulation of bladder function. Apodaca et al. (2003) reported that spinal cord injury results in a significant disruption of the urothelium which contribute to OAB symptoms. The importance of urothelium in controlling bladder function has also been demonstrated by Shioyama et al. (2008) in which rats with chronic urothelial injury showed an increase in urinary frequency and a decrease in voided volume. The authors suggested that the effect was likely to be caused by an elevation of PGE_2 production. Consistently, higher levels of PGE_2 have been detected in patients with OAB (Hegele et al., 2014). Thus, the underlying mechanism of urotheliogenic OAB could involve inflammation in the bladder. Another factor of urotheliogenic OAB has been described recently, that is, uropathogenic *Escherichia coli* colonisation in the urothelial cells (Mansfield et al., 2022). Several studies have identified large numbers of bacteria from bladder biopsies or urothelial cells from OAB patients and there is a positive correlation between density of intracellular bacteria and severity of OAB symptoms in patients (Ognenovska et al., 2021). Currently, bacterial infection remains an underappreciated contributor to the pathophysiology of OAB.

1.2.1.4 Metabolic syndrome and OAB

In the third National Health and Nutrition Examination Survey (NHANES III) conducted by the National Center for Health Statistics (NCHS), part of the Centers for Disease Control and Prevention (CDC), it was discovered that men with diabetes, hypertension and hypercholesterolemia have an increased risk of nocturia, incomplete bladder emptying, weak steam and hesitancy (Rohrmann et al., 2005). Recently, the association between obesity and OAB has also been demonstrated (Hagovska et al., 2020; Kim et al., 2021). These studies thus suggest that there is a link between metabolic syndrome and OAB. Metabolic syndrome is a cluster of conditions that

occur together, including obesity, insulin resistance, hypercholesterolaemia, dyslipidaemia and hypertension, as a result of overnutrition, sedentary lifestyles and excess adiposity. Recent findings suggest that the pathophysiology of OAB in patients with metabolic syndrome might be different as they are often resistant to the usual OAB treatment. In diabetes, OAB is thought to be caused by osmolarity diuresis effect, metabolic perturbation and neuropathy (Kirschner-Hermanns et al., 2012). Exacerbation of muscarinic activation and influx of extracellular Ca^{2+} are also implicated due to upregulation of M_3 receptors and L-type VGCCs in diabetic bladders (Leiria et al., 2011). On the other hand, OAB in obesity could be a result of the increased intra-abdominal pressure and intravesical pressure due to accumulation of body fat and visceral fat in the abdominal cavity (Hagovska et al., 2020). Urothelium injury in hypercholesterolemia and hyperlipidaemia may also exacerbate the development of OAB. It is well-recognised that metabolic syndrome evokes stress reactions in the body system such as oxidation and inflammation (Cornier et al., 2008). These reactions may promote chronic bladder ischaemia, leading to DSM dysfunction and incomplete voiding (He et al., 2016). The resultant OAB further aggravates the disturbances and inflammatory response in the body system, creating a vicious cycle.

1.2.2 Management of OAB and treatment limitations

Currently, the management of OAB is divided into three levels of care, ranging from most conservative to most specialised (Willis-Gray et al., 2016). In general, this stepwise approach applies to all kinds of OAB. The first-line treatment is behavioural therapy that involves lifestyle modification such as limiting consumption of water, avoiding alcohol or caffeine intake, bladder retraining and pelvic floor muscle training (PFMT).

Second line treatment is initiated if patients are unable to perform conservative

therapy or if symptoms do not improve after six months (National Institute for Health and Care & Excellence, 2019). Currently, antimuscarinic agents such as oxybutynin, solifenacin and tolterodine which have an efficacy of 65 – 70% in reducing major OAB symptoms, remain the mainstay of treatment in OAB (Arnold et al., 2012). Despite of their efficacy, antimuscarinic agents are associated with many unpleasant effects that often lead to non-compliance and treatment failure. Dry mouth and constipation are the most common side effects experienced by antimuscarinic users due to the abundance of muscarinic M_2 and M_3 receptors at the salivary glands and gastrointestinal tract (Athanasopoulos and Giannitsas, 2011). They may also block muscarinic receptors in the brain, causing blurred vision, dizziness and cognitive impairment (Moga et al., 2017). Therefore, there are concerns regarding anticholinergic burden, especially in elderly patients and those who are taking concurrent medications with antimuscarinic effects such as antiemetics, antipsychotics, and bronchodilators. More recently, it is established that the use of non-selective antimuscarinics (oxybutynin, tolterodine, trospium and fesoterodine) is associated with a 50% increase in mortality risk among elderly patients with dementia and OAB (Kachru et al., 2020).

Apart from antimuscarinic agents, mirabegron, a β_3-adrenoceptor agonist, is commonly prescribed to OAB patients since 2012. It has demonstrated sustained improvements in urinary frequency and incontinence in OAB patients and has a similar clinical efficacy with antimuscarinics. Mirabegron is usually initiated in patients who cannot or poorly tolerate antimuscarinics. Whilst not contributing to the muscarinic burden, mirabegron may cause dizziness, back pain and cardiovascular-related adverse effects such as hypertension and tachycardia (Yamaguchi et al., 2015). Currently, several novel β_3-adrenoceptor agonists including vibegron, solabegron and

ritobegron are undergoing pre-clinical or clinical trials and have shown promising therapeutic effects (Joseph et al., 2022).

In patients with refractory OAB who exhibit inadequate response to first-line and second-line therapies, third line-treatment including intra-detrusor injection of botulinum toxin, posterior tibial nerve stimulation (PTNS) and sacral neuromodulation (SNM). Briefly, botulinum toxin is a purified neurotoxin complex that acts by inhibiting Ca^{2+}-mediated release of acetylcholine at the pre-synaptic neuromuscular junction in peripheral nerve endings, resulting in decreased muscle contractility and muscle atrophy at the injection site. The effect of botulinum toxin is reversible thus most patients require repeat injection every 3 to 12 months, depending on the patients' condition. Following repeated dosing, some patients may develop resistance to the toxin, resulting in treatment failure. It is also contra-indicated in patients with pre-existing neurologic disorders affecting transmission at the neuromuscular junction. SNM requires a two-stage approach in which a percutaneous electrode is placed into the sacral foramen to stimulate the S3 or S4 nerve roots. Subsequently, a permanent electrical pulse generator is implanted if the patient shows >50% improvement in symptoms. While SNM has improved OAB symptoms in many, it is associated with a high rate of adverse events. It has been reported that about 30% of patients required surgical modification to relocate or remove the device due to pain at the stimulation site, infection or lead migration (Sukhu et al., 2016). Unlike SNM, PTNS delivers electrical stimulation to the sacral micturition centres via a fine needle placed just above the medial aspect of the ankle (Painter and Suskind, 2019). It requires maintenance therapy every two to three weeks, for up to three years. However, studies have shown that it does not provide statistically significant benefit when compared to tolterodine (de Wall and Heesakkers, 2017).

Augmentation cystoplasty is also available as a last resort to refractory OAB. It is a surgical procedure to increase the size of the bladder and reduce the contractility of the bladder by joining a patch of gut (usually ileum) to the bladder. Although can be effective, this procedure can cause many complications such as chronic inflammation, diarrhoea, thromboembolism, the need for intermittent self-catherization, formation of bladder stones which happens in up to 40% of patients, and fatal perforation (Veeratterapillay et al., 2013).

Noteworthily, recent clinical studies suggest that combination therapy may provide better treatment outcome in patients with OAB by increasing effectiveness of treatment and reducing the overall side effects. This treatment approach is elaborated in **section 5.1.1** (**Chapter 5)**. Overall, while the current treatments for OAB do provide relief in some patients, they are accompanied by a range of adverse effects that may eventually outweigh their benefits. The treatment options for elderly patients, patients with comorbidities or those with refractory OAB are particularly limited. Therefore, there is undoubtedly a need for new and better medicine to treat OAB. Since the early 2000s, scientists have been suggesting a shift from drug discovery approach of finding 'new entity drugs' to 'combining existing agents' or drug repurposing (Patwardhan and Mashelkar, 2009; Sleigh and Barton, 2010). Therefore, drug discovery based on traditional medicines and ethnopharmacology are being considered as an attractive option (Pirintsos et al., 2022). In the following subsection, several traditional preparations or natural products used for OAB are reviewed.

1.2.3 Traditional preparations and natural products used for OAB

In the USA, about 74.6% of adults with OAB have tried to relieve their urinary symptoms using complementary and alternative medicines including traditional Chinese medicine (TCM), herbal remedies and natural products (Chughtai et al., 2013). Kajiwara & Mutaguchi (2008) reported that Gosha-jinki-gan, a TCM formula comprised of 10 different herbs, significantly improved daytime urination and improving the quality of life in females with OAB. It has also shown clinical efficacy in alleviating nocturia in patients who were unresponsive to antimuscarinic agents (Yagi et al., 2016). However, the use of Gosha-jinki-gan could cause poor appetite, epigastric distress and diarrhoea (Kajiwara and Mutaguchi, 2008).

In another study, Lai et al. (2015) has delineated the mechanism of action of Suo-Quan-Wan (SQW), a TCM formula which consists of three ingredients, in slowing the progression of OAB and improving bladder function. It is likely to modulate the expression of $TRPV_1$ receptors in the bladder (Lai et al., 2015). While the exact role of $TRPV_1$ in healthy bladders is not established, scientists have shown that there is an increased expression of $TRPV_1$ in both bladder nerves and urothelium (Zhang et al., 2019; Li et al., 2011a). Recently, Xu et al. (2017) demonstrated that SQW increased the sensitivity and expression of β_3-adrenoceptors in ageing rat, suggesting its potential therapeutic effects in DSM relaxation dysfunction. It is currently not clear whether these effects were contributed by a single active ingredient or combination of the ingredients present in Gosha-jinki-gan and SQW.

In many European countries, Saw Palmetto Extract (SPE) is widely used for the treatment of LUTS, and its effects have been validated through modern scientific research (Suzuki et al., 2009). Intraduodenal administration of SPE significantly improved the urodynamic symptoms in OAB rats by increasing bladder capacity and

prolonging micturition interval (Oki et al., 2005). In a recent randomised, double-blind and placebo-controlled study, daily consumption of 320 mg of SPE significantly alleviated the symptoms of daytime frequency and nocturia in OAB women after 12 weeks (Yamada et al., 2022). It is likely to mediate via inhibition of α_1-adrenoceptors and muscarinic receptors (Suzuki et al., 2007). Besides, nobiletin-containing shekwasha extract that is commonly used in Japan to ease urinary symptoms also exhibited inhibitory properties on muscarinic receptors (Ito et al., 2018). Therefore, the use of SPE and shekwasha extract could contribute to cholinergic burden, which is a major concern of the use of conventional antimuscarinics.

These studies manifest the importance of comprehensive scientific validation of natural or traditional remedies. In addition to substantiating their use in clinical conditions, scientific investigation also minimises the risk of adverse events, drug-drug or disease state interaction, through characterisation of their mechanisms of action. Nonetheless, many herbs and medicinal fungi used for OAB or to relieve symptoms of OAB in various countries or regions have not undergone scientific validation. One important example is *Ophiocordyceps sinensis* (Berk.) G.H. Sung, Hywel-Jones & Spatafora (*O. sinensis*), a popular medicinal fungus used in China and some East Asian countries, which is also the focus of this study.

1.3 Medicinal fungi and drug discovery

Fungi have been used for centuries for their exquisite flavours, nutritional values, and medicinal properties. Out of the 14,000 known species of mushrooms worldwide, only 5% of them have been identified to possess medicinal properties and they are termed medicinal fungi (Hrudayanath and Sameer, 2014). Medicinal fungi can be defined as macroscopic fungi that are used in the form of extracts or powder for the prevention, alleviation of diseases, and/or for nutritional purposes (Wasser 2014). They inevitably

represent a major source of pharmaceutical compounds worthy of scientific investigation.

In general, new drug discovery from natural sources including medicinal fungi is established based on their traditional uses as documented in the literature, or sometimes, anecdotal record. For example, Krestin (PSK), a protein-bound glycan was isolated from *Trametes versicolor*, a medicinal fungus that is used traditionally to boost immune system. Following years of extensive research, the clinical use of PSK in cancer treatment was approved by the Japanese National Health Registry in 1977 (Fujii, 1996). Today, more than 130 pharmacological effects of medicinal fungi have been identified, such as anticancer, antibacterial, antiviral, antidiabetic, antioxidant, antihypertensive, immunomodulatory and smooth muscle relaxation (Venturella et al., 2021; Wasser, 2014).

These activities can be attributed to the bioactive components present in the fungi, such as β-glucans, polysaccharide-protein complexes, triterpenes, lactones, and alkaloids (Bains et al., 2021). They also contain enzymes that have critical roles in the normal physiology of human health and several disease pathogeneses, such as superoxide dismutase, glucose oxidase and peroxidase (Wasser 2014). Generally, these bioactive components are obtained following extraction, fractionation and isolation. Nowadays, bioactivity-guided fractionation is considered as an efficient approach during early drug discovery to retrieve active chemical constituents and pure compounds from medicinal fungi (Katiyar et al., 2012). In the process, bioassays with pharmacological relevance are performed to expedite the screening process of their bioactivities. Some assays of relevance to this project are described in **section 1.5** and the process of extraction and bioactivity-guided fractionation is discussed at a greater length in **section 2.1.2** (**Chapter 2)**.

1.4 *Ophiocordyceps sinensis*

O. sinensis was first described scientifically by a British mycologist, Dr. Miles Berkeley, in 1843 as *Sphaeria sinensis* Berk. It was renamed *Cordyceps sinensis* by an Italian mycologist Pier Andrea Saccardo in 1878; where the name *Cordyceps* originated from two Latin words, 'cord' meaning club and 'ceps' meaning head, implying a club fungus fruiting out of the head of a caterpillar (Kumar Bhandari *et al.* 2010). Following a molecular phylogenetic classification in 2007, *C. sinensis* was transferred to the genus *Ophiocordyceps* and was renamed *Ophiocordyceps sinensis* (Sung *et al.* 2007). Its taxonomy is as follows: Fungi (Kingdom), Ascomycota (Phylum), Sordariomycetes (Class), Hypocreales (Order), Ophiocordycipitaceae (Family), and *Ophiocordyceps* (Genus).

O. sinensis is mainly distributed on the Tibetan Plateau between 3000 and 5000 m a.s.l. and its surrounding regions, including the eastern Tibet, eastern Qinghai, western Sichuan, northern Yunnan, and southwestern Gansu provinces (Li et al., 2011b). It is also found in other Himalayan countries such as Nepal, Bhutan, and India at altitudes above 3800 m. Due to its confined geographical distribution and overexploitation for medicinal and financial purposes, *O. sinensis* has been listed as an endangered species under the second class of state protection since 1999 (State Council of the People's Republic of China, 2000). As a consequence of climate change over the years, the distribution of natural *O. sinensis* has further decreased and moved towards the central part of the Tibetan Plateau (Yan et al., 2017). Recently, it has been included as an endangered species on the China Biodiversity Red List 2018 (Wei et al., 2021).

O. sinensis is well-known for its interesting, rather long, and unusual life cycle. In late autumn, the fruiting bodies of *O. sinensis* disperse spores and scatter on the soil

surface, developing into infective conidia. These infective conidia then parasitise the underground larvae of ghost moths within the family Hepialidae (Zhang et al., 2012). It is fascinating to note that the infection rates are highest in the fourth and fifth instar larvae that are shedding old cuticles and forming new ones, and this stage in the larval life cycle coincides with the release of spores by *O. sinensis*. Once entering the moth's body, the fungus multiplies by yeast-like budding and fills the hemocoel with threadlike hyphae (Guo et al., 2017). Then, each infected larva moves gradually into a position ideal for the growth of the fungus; usually, 2 – 5 cm below the soil surface, it becomes rigid and dies with the head facing upward. The fungus emerges from the dead host and forms a small stroma bud right before winter. More than 90% of the contents of the larva are eventually replaced by the sclerotium, turning into a nutrient store for the fungus. At the same time, the intact exoskeleton serves as a support for the sclerotium throughout winter (Guo et al. 2017). In the following spring, the fungus ruptures the host larva, forming a sexual sporulating structure known as the stroma or fruiting body, and emerges above the soil surface (Figure 1.2). This interesting life cycle is the origin of its name, "Dong Chong Xia Cao," in Chinese, which means "winter worm, summer grass."

Figure 1.2 Wild Ophiocordyceps sinensis.

1.4.1 Ethnomedicinal uses

The exotic medicinal properties of *O. sinensis* were discovered over 2000 years ago when local herders in Tibet used *O. sinensis* to improve reproductive capacity and increase the milk production of their livestock (Panda and Swain, 2011). Its medicinal values propagated to mainland China during the Qing dynasty and were recorded in the "Essentials of the Materia Medica: *Ben Cao Bei Yao*," 1694 by Wang Ang (Lin and Li, 2011). "*Ben Cao Bei Yao*" was thought to be the first official documentation of *O. sinensis* in the history of TCM in China. In this piece of record, Wang Ang emphasised its medicinal values in haemostasis, lung protection, and kidney invigoration. Kidney invigoration is comprehended to cure frequent urination in the history of TCM (Liao et al., 2022). According to the anecdotal record, the uses and methods of preparation of *O. sinensis* appear to be based on the empirical trial-and-error methods by the local healers. Thus, they may vary in different areas and countries.

A survey conducted in the Northern Yunnan provinces reported that the local communities used *O. sinensis* to improve eyesight and treat calcium deficiency, indigestion, hypertension and diabetes (Chen *et al*. 2010). The methods of preparation include steaming the ground stroma powder with eggs, serving it as a chicken stew, boiling it with *Po Cha* (local butter tea), or having it raw. *O. sinensis* is also consumed as a general tonic to enhance vitality and promote endurance and life expectancy. For this purpose, the Bhutanese community would consume it each morning and evening after brewing it in a cup of *chang* (locally made alcohol) or hot water for an hour. Mixing it with Ginseng root decoction has also been recorded to treat severe diseases such as cancer (Panda and Swain 2011). In North Sikkim, aborigines consumed *O. sinensis* with milk to enhance their libido and sex performance (Panda 2015). Thus, it is commonly regarded as the "Himalayan Viagra" in Tibet and Nepal.

Recently, a patent pertained to a method in preparing *O. sinensis* for treating nocturia has been filed in China (Liang, 2013). It describes the oral use of *O. sinensis* decoction together with Eucommia bark, *Herba Epimedii* and *Herba Cistanche*.

1.4.2 Pharmacological activities

While *O. sinensis* has been used in traditional Chinese and Tibetan medicine for centuries, the interest in scientific research towards its medicinal properties only surged in the early 1990s after a group of female athletes revealed that *O. sinensis* extracts were part of their diet regime, during the 1993 National Games in Beijing, China. Over the past three decades, a broad spectrum of pharmacological activities of *O. sinensis* has been reported, including antioxidative (Yamaguchi et al., 2000a; Li et al., 2001, 2003; Nguyen et al., 2021; Tong and Guo, 2022; Singh et al., 2013; Kong et al., 2021; Ko and Leung, 2007; Dong and Yao, 2008; Wang et al., 2012), anticancer (Mei et al., 2014; Sang et al., 2020; Yoshikawa et al., 2011), immunomodulatory (Kuo et al., 2001; Cheung et al., 2009; Wang et al., 2011; Yap et al., 2020a; Wang et al., 2015b; Qian et al., 2012; Sun et al., 2018; Li et al., 2009; Jung et al., 2019), smooth muscle relaxant effects (Chiou et al., 2000; Wang et al., 2016; Yu et al., 2019), antidiabetic (Qi et al., 2013; El Ashry et al., 2012; Kan et al., 2012; Luo et al., 2015; Wang et al., 2018a), cholesterol-lowering (Kim, 2010; Yamaguchi et al., 2000b), antimicrobial (Mamta et al., 2015; Ren et al., 2014; Kaushik et al., 2019; Mishra et al., 2019), antiviral (Zhu et al., 2016), osteoprotective (Qi et al., 2012; Zhang et al., 2014; Qi et al., 2013), radiation protective (Lin et al., 2007; Nakamura et al., 2003), antifibrotic (Peng et al., 2014), antidepression and memory enhancing (Dong et al., 2014; Zhang et al., 2022). These properties are demonstrated in both in vitro and in vivo assays, and mostly attributed to the aqueous extracts containing polysaccharides

and protein. Some of these activities are discussed in the following subsections.

1.4.2.1 Antioxidant activities

Due to the long-standing use of *O. sinensis* as a tonic and energy booster, the antioxidative effect is one of its most extensively studied properties. Y. Yamaguchi et al. (2000a) pioneered the investigation in this area by demonstrating the suppression of low-density lipoprotein oxidation in macrophages exposed to hot water extract of *O. sinensis*. Subsequent investigation proposed that the antioxidative effects of *O. sinensis* could be attributed to the presence of polysaccharides, proteins and peptides. For instance, the antioxidant activity of a partially purified polysaccharide fraction of *O. sinensis* is 10 – 30 times more potent than its water extracts (Li et al., 2001). To substantiate this finding, the authors isolated the pure polysaccharide fraction and demonstrated its strong protective effect against H_2O_2-induced oxidative stress in rat pheochromocytoma cells (Li et al., 2003).

Besides, daily administration of Cs-C-Q80 (an *O. sinensis* formula listed in the Pharmacopoeia of the People's Republic of China, containing 39.4 % of carbohydrate and 14.8% protein) at 1.5 g/kg for four weeks markedly suppressed the malondialdehyde (MDA) levels in liver and cardiac tissues of doxorubicin-treated rats (Wu et al., 2015). MDA level is an indicator of the degree of membrane lipid peroxidation and oxidative damage in liver tissue. Similarly, Nguyen et al. (2021) recently reported the hepatoprotective effect of exopolysaccharide purified from cultivated *O. sinensis* by increasing glutathione levels and reducing MDA levels.

More recently, over 8000 putative antioxidant peptides have been identified in wild and cultured *O. sinensis* (Tong and Guo, 2022). Gene ontology analysis showed that most of these peptides shared similar genes with the typical antioxidant peptides involved in biological processes such as cell redox homeostasis, anti-oxidative stress,

and cellular transport. Some peptides are essential in organising cellular components (Tong and Guo 2022). Remarkably, these antioxidant peptides in *O. sinensis* are thought to contribute to its ability in high-altitude adaptation. *O. sinensis* has been proven to attenuate hypoxia-induced ROS generation, oxidation of lipids and proteins in human lung epithelial cells via induction of antioxidant genes, including heme oxygenase-1 (HO-1), metallothionein (MT), and nuclear factor erythroid-derived 2-like 2 (Nrf2) (Singh et al., 2013). It is important to note that while antioxidant is not currently classified as a therapeutic class, oxidative stress and generation of reactive oxygen species (ROS) are implicated in the pathophysiology of many medical conditions including cancer, autoimmune diseases, cardiovascular diseases and OAB, the focus of this study. Therefore, the therapeutic potential of antioxidants should be further explored.

1.4.2.2 Anticancer activities

Several studies have revealed the broad-spectrum anticancer properties of *O. sinensis* and its mechanisms of action involve inhibition of cell proliferation, induction of apoptosis, inhibition of metastasis and immunomodulation. Yoshikawa et al. (2011) proposed that the proliferation inhibitory effect of *O. sinensis* could be attributed to the activation of adenosine A_3 receptor. In B16-BL6 mouse melanoma, mouse Lewis lung cancer cells, human fibrosarcoma cells HT1080, and human colon cancer cells CW-2, the proliferation inhibitory effect of *O. sinensis* was inhibited by MRS11191, a selective adenosine A_3 receptor agonist, and promoted by an adenosine deaminase inhibitor (Yoshikawa et al., 2011).

In another study, heteroglycan from *O. sinensis* inhibited the proliferation of S180 murine sarcoma cells in a concentration-dependent manner and promoted the apoptosis of the cells at a rate higher than that of 5-fluorouracil, a cytotoxic

chemotherapy medication (Mei et al., 2014). Moreover, *O. sinensis*-derived volatile oil exhibited an overt inhibitory effect against paclitaxel-resistant lung cancer cell line A549 and ovarian cancer cell line A2780 (Sang et al., 2020). These findings suggest that *O. sinensis* may contain a potential lead for the discovery of therapeutic agents against paclitaxel-resistant cancer cells. Moreover, the anticancer activities of *O. sinensis* have been liked to its immunomodulatory actions. Yap, Li, Yap, et al. (2020) showed that the cold water extract and polysaccharides fractions derived from cultured *O. sinensis* reduced the production of IL-6 and IL-9 in murine macrophages. These interleukins have tumour-promoting abilities and are responsible in the proliferation and differentiation of cancer cells (Yap et al., 2020a).

Notwithstanding the great therapeutic potential of *O. sinensis* in multiple cancer types, it may not be suitable in some cancers. The safety of *O. sinensis* consumption by patients with prostate cancer has been questioned as administration of *O. sinensis* extract at a dose of 50 mg/g in mice for 24 days raised the serum testosterone level and caused enlargement of the prostate glands (Ma et al., 2018). Interestingly, Alamoudi et al. (2022) recently showed that cordycepin, one of the bioactive components in *O. sinensis*, prevented the increase in prostate weight and testosterone production in rats with benign prostatic hyperplasia. Therefore, there is a pressing need to clarify the bioactive component(s) responsible for the anticancer effects of *O. sinensis*, in order to identify lead compounds for the development of new anticancer drugs, and to reduce the occurrence of disease-state interaction in consumers or patients.

1.4.2.3 Smooth muscle relaxant effects

The smooth muscle relaxant effects of *O. sinensis* have been demonstrated in several studies. In rat aortic rings, *O. sinensis* protein extracts exhibited an endothelium-dependent relaxation response that was L-NAME- and TEA-sensitive, suggesting that *O. sinensis* may produce NO and activate K^+ channels to promote vasorelaxation (Chiou et al., 2000). In the same study, the authors also demonstrated the reduction of mean arterial pressure (MAP) of the rats following administration of the extracts. At the highest dose of 32 mg/kg, the MAP was reduced from 107 ± 6 mmHg to 49 ± 3 mmHg. Recently, Xiang et al. (2016) demonstrated the blood pressure lowering effects of a purified *O. sinensis* polysaccharide fraction in spontaneously hypertensive rats (SHR). It also markedly enhanced the production of NO and decreased the levels of endothelin-1, adrenaline, noradrenaline and angiotensin-II in SHR (Xiang et al., 2016). Endothelin-1 is a potential marker of endothelial dysfunction and is usually elevated in hypertensive subjects, whereas adrenaline and noradrenaline are neurotransmitters that increase blood pressure, heart rate, and blood sugar levels (Floras, 1992; Akter et al., 2015). Furthermore, Luitel et al. (2020) demonstrated the potent vasodilatory effect of *O. sinensis* in an ex vivo, isolated, ventilated, and buffer-perfused mouse lungs. These studies thus suggest that *O. sinensis* may promote vasorelaxation and provide beneficial effects on both systemic and pulmonary circulation. The bronchodilatory effect of *O. sinensis* has also been demonstrated recently by our research group (unpublished data) and this property could be linked to its traditional use in treating lung and respiratory conditions.

1.4.2.4 Antidiabetic, antilipidemic and anti-cholesterol activities

The beneficial effects of *O. sinensis* in lowering blood sugar, cholesterol, and lipids have been established in several studies. In general, *O. sinensis* exerts its antidiabetic effects via promotion of glucose metabolism and insulin release by preserving pancreatic β-cells. In an in vivo study, treatment with polysaccharide extract of *O. sinensis* at a dose of 200 mg/kg for 7 days significantly increased the serum insulin level and reduced the blood glucose level in streptozotocin-induced diabetic rats and alloxan-induced diabetic mice (Zhang et al., 2006). In another study, similar effects were observed when *O. sinensis* was administered to the streptozotocin-induced diabetic rats at a lower dose and for a longer treatment period (100 mg/kg for 21 days) (El Ashry et al., 2012). These data suggest that this fungus could be useful in treating both acute and chronic conditions. In addition, the reduction of blood glucose level was comparable to glibenclamide, a sulfonylurea antidiabetic medication (El Ashry et al., 2012).

O. sinensis has also been shown to increase the viability of pancreatic β-cells, decrease the body weight gain and improve the cholesterol profile in KK/HIJ diabetic mice fed with a high-fat diet (Kan et al., 2012). Preservation of pancreatic β-cells is an important aspect in the treatment of diabetes as these cells secrete insulin and are often damaged or become dysfunctional due to high glucose or lipid levels, inflammatory mediators released from the adipose tissue, or oxidative stress. In China, *O. sinensis* is in clinical use for diabetic nephropathy since the early 2000s (Luo et al., 2015). Combining the results from 60 randomised controlled trials involving 4288 participants, a meta-analysis showed that *O. sinensis* significantly improved the therapeutic effect of angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) in diabetic nephropathy (Luo et al., 2015). The underlying mechanisms of *O. sinensis* in diabetic nephropathy are likely to involve inhibition of

the purinergic $P2X_7$ receptors and NLRP3 inflammasome (NLRP3: nucleotide binding and oligomerisation domain-like receptor family pyrin domain-containing 3) (Wang et al., 2018a).

When there is persistently high glucose in the blood, endothelial lining of the blood vessels may be damaged, making the blood vessels more susceptible to fats and cholesterol build-up, leading to atherosclerosis. A heteropolysaccharide isolated from *O. sinensis* has exhibited strong inhibitory activity against cholesterol esterase that in turn, prevent the absorption of dietary cholesterol esters (Kim, 2010). *O. sinensis* may also inhibit platelet activation, which is involved in the recruitment of inflammatory cells towards the lesion site in atherosclerosis (Lu et al., 2014; Chang et al., 2015).

1.5 Research approaches

1.5.1 Rat as an animal model to study bladder function

Rodents such as mice and rats, and non-human primates such as rabbits and pigs are the most used animal models in the study of bladder function. While none of these models completely mimics the bladder functions in humans as the complexity in humans differs, they do share similar anatomical structures and thus allow the analysis of specific risks factors or elements that contribute to bladder function. Among these animal models, Wistar and Sprague-Dawley (SD) rats are the most widely used and preferred laboratory animals worldwide. In this study, the choice of adult male SD rats was based on the several factors. First, adult male SD rats have been used extensively in our laboratory. Therefore, data collected prior to this study can serve as a valid comparison. Second, the amplitudes of spontaneous myogenic contraction in neonatal rats are much larger and may create greater variability in results (Kullmann et al., 2014). Third, older rats have impaired bladder function and thus may limit the

interpretation of results obtained (Zhao et al., 2010). Lastly, while new findings suggest that females do not significantly increase variability in rodent research (Beery, 2018), current understanding of the signal transduction in rat bladders mainly derived from male models. Thus, usage of male rats in the relevant experiments allow us to interpret the results more comprehensively.

1.5.2 Isolated tissue bath

To study bladder function, several in vivo and in vitro methods to have been developed. Cystometry is the primary in vivo approach as it allows measurement of detrusor pressure during the continuous filling of the bladder and testing of bladder sensation. While this intact preparation provides information under close to physiological conditions, the use of bladder strips in organ bath or isolated tissue bath provides greater flexibility when surgical and/or pharmacological manipulations would affect the survival and stability of the in vivo preparation, or when the studies require the use of human tissue (Kullmann et al., 2014).

Organ bath or the isolated tissue bath is a classical in vitro experimental technique in physiology and pharmacology. This technique was discovered by Rudolf Magnus in 1904 when he observed that an isolated ileum strip retained its spontaneous movements after being suspended in warm oxygenated Ringer-Locke solution. Over the years, this technique has been improved and used extensively to suit various research purposes surrounding drug development and basic research as they allow the tissue to function as a whole tissue, with a physiological outcome (contraction or relaxation) (Jespersen et al., 2015). This experimental method is thus particularly useful in the investigation of the effects of drug or preparation of interest, on a myriad of contractile tissues such as airway, vascular, gastrointestinal, urinary and reproductive tissues.

By adopting this technique in the study of bladder function, each component of the bladder i.e., DSM, urothelium and nerves, that is involved in bladder contraction and relaxation, can be studied. For instance, the role of urothelium can be examined by removing the urothelial layer on the bladder strips and the involvement of nerves can be investigated through electrical field stimulation. These methods can thus be utilised in the study of urotheliogenic OAB and neurogenic OAB, respectively. Another important element is that bladder strips can be used to evaluate changes in the spontaneous myogenic contractions by measuring their amplitude or frequency. Moreover, by exposing the bladder strips to pharmacological tools of relevance, the ion channels, receptors and/or intracellular pathways that are involved in either relaxation or contraction of the DSM can be identified.

Depending on the research focus, the isolated tissue bath setup may vary across research institutions. However, they do share several features. In general, the tissues of interest are excised into rings or strips of fixed lengths and mounted to a rod before placing into a double-layered water-jacketed glass baths that connects to a water bath. In the bath, there is a constant volume of physiological salt solution such as the Krebs-Ringer or Krebs-Henseleit solution and continuous supply of carbogen to maintain the tissues' viability. Tissues in the bath would be connected to an isometric force transducer which will capture the force generated by the tissue and convert it into an electrical signal (Jespersen et al., 2015). Finally, a data acquisition system is used to detect the changes in the electrical signal, allow visualisation of the experiment and analysis of the captured data (Jespersen et al., 2015). The experimental setup for the present study is described in detail in **section 2.3.5** (**Chapter 2)**.

1.5.3 *Caenorhabditis elegans*

In early drug discovery, elucidation of pharmacological effects of nature-derived preparations and drugs involves a series of in vitro and in vivo experiments. However, many of the typically used in vivo models are costly, time-consuming and have complicated genetics. Moreover, there are significant ethical arguments against in vivo testing in recent years. For these reasons, *Caenorhabditis elegans* (*C. elegans*) is being widely employed as an in vivo model in the field of biomedical sciences.

Genome analysis revealed that these nematodes have approximately 20,000 protein-coding genes and at least 83% of its proteome have human homologous genes (Lai et al., 2000). This characteristic has made them exceptionally useful for translational research. In addition, they are relatively inexpensive and easy to manipulate. The use of *C. elegans* also promotes animal welfare as it acts as one of the best "replacement" models as described in the 3Rs principle (replacement, reduction and refinement) in animal research. Over the years, *C. elegans* has shed lights on a wide of cellular signalling pathways such as anti-obesity mechanisms (Xiao et al., 2020; Zhu et al., 2021), anti-ageing mechanisms(Wang et al., 2020; Zeng et al., 2019; Peng et al., 2021; Edwards et al., 2015; Lee et al., 2015), antioxidative pathways (Sun et al., 2021; Guerrero-Rubio et al., 2021), and muscle physiology (Brozovich et al., 2016; Chow et al., 2006; Trojanowski et al., 2016; Zwaal et al., 2001; Shtonda and Avery, 2005). In this study, *C. elegans* is used to evaluate the effect of *O. sinensis* in improving lifespan and health, given that age is a major risk factor of OAB. Further in-depth information related to the physiological aspects of *C. elegans*, and assays conducted in this study are depicted in **Chapter 6**.

1.6 Research aim

Modern research has demonstrated the antioxidant, anti-inflammatory and vasorelaxant properties of *O. sinensis*. These pharmacological activities could provide beneficial effects in OAB due to its multifactorial pathophysiology that involves muscle or nerve dysfunction, damage to the urothelium, inflammation and chronic oxidative stress. Currently, no scientific evidence is available on the effect of *O. sinensis* on bladder function albeit its traditional use in alleviating urinary symptoms. Thus far only two studies reported the possible clinical efficacy of *O. sinensis* in improving nocturia in elderly patients (Zhang et al., 1997; Bao et al., 1994). However, these studies did not provide any physiological evidence that *O. sinensis* could promote bladder relaxation or how *O. sinensis* improved nocturia in the patients. Therefore, there is a need to substantiate the medicinal use of *O. sinensis* in relieving OAB-related symptoms.

This research aimed to expand the knowledge on medicinal fungi, with a focus on *O. sinensis*, and to explore its potential as a new or adjunct treatment in the management of OAB. In order to achieve the aim, several objectives have been set and these include:

i. To investigate the functional activity of *O. sinensis* extracts and fractions of different molecular weight in isolated rat bladder strips (**Chapter 2**)

ii. To elucidate the bioactive components in *O. sinensis* accounting for the bladder relaxant effect based on the efficacies, protein and polysaccharide contents of the fractions (**Chapter 2**)

iii. To elucidate the mechanism(s) of action of *O. sinensis* extract-induced bladder relaxation (**Chapter 3 and 4**)

iv. To investigate the combined effects of *O. sinensis* and another medicinal

fungus (*Lignosus rhinocerus*) in bladder relaxation. This will provide insight into combination therapy in OAB (**Chapter 5**)

v. To examine the effects *O. sinensis* on the lifespan and pharyngeal pumping activity of *C. elegans* (**Chapter 6**)

2 Bioactivity of *Ophiocordyceps sinensis* Extracts in Rat Bladder

2.1 Introduction

2.1.1 Composition of OCS02 cultivar

Following the declaration of *O. sinensis* as an endangered species, growing worldwide demand, and incidents of poisoning due to heavy metal contamination in wild *O. sinensis*, artificial cultivation of this fungus has been taking place since late 1990s (Wu et al., 1996; State Council of the People's Republic of China, 2000; Yue et al., 2013). After several decades of efforts and attempts, cultivation methods of the pure mycelium or fruiting bodies of *O. sinensis* have been successfully developed. These novel approaches do not only provide an alternative source for consumers and researchers to substantiate the medicinal uses of *O. sinensis* but also protect the limited natural resources for its sustainable utilisation (Li et al., 2019).

Many studies have revealed the bioactivities and pharmacological actions of cultivated *O. sinensis*, showing that it is equally potent, if not more, than its natural counterpart in health-promoting functions. For example, Yao et al. (2014) showed that both wild and preparations derived from cultured *O. sinensis* mycelia antagonised the activity of the pro-fibrotic cytokine in renal epithelial cells with similar potency. Then, another study demonstrated that the water extracts of cultured *O. sinensis* have a stronger antioxidant effect than the wild fungus (Wang et al., 2015a).

In this study, we utilised OCS02® cultivar (LiGNO Biotech, Selangor, Malaysia), abbreviated as OCS02 throughout this thesis, to study the pharmacological

effects of *O. sinensis* in the bladder. It was cultivated on a rice-based solid medium from the fruiting body of the fresh wild-type *O. sinensis* originated from Shangri-La, Yunnan, China (Figure 2.1) (Fung et al., 2018). Following its successful cultivation, OCS02 has been authenticated with polymerase chain reaction amplification of the partial small subunit of its ribosomal DNA (Fung et al., 2017).

Figure 2.1 Fruiting bodies of Ophiocordyceps sinensis (OCS02 cultivar) cultivated on a rice-based solid medium (Fung et al., 2018).

Following the authentication of its genome, the composition analysis of OCS02 cultivar was carried out and compared with the wild types and other cultivars (Fung et al., 2018). Although it is explicable that its chemical composition may differ from the wild-type and other cultivars due to differences in the growth substrate and culture conditions, OCS02 cultivar has demonstrated comparable characteristics to the natural *O. sinensis*. It contains large amounts of polysaccharides, amino acids and nucleosides. The presence of adenosine and cordycepin in OCS02 cultivar is an important feature as these nucleosides could be used as biomarkers to differentiate genuine wild-type and cultivated *O. sinensis* from counterfeits and mimics (Hsu et al.,

2002). Quality assurance and safety are among the reasons that drive researchers to begin artificial cultivation due to heavy metals contamination of wild *O. sinensis* (Xiao et al., 2021; Zhou et al., 2018). In OCS02, toxic elements of concern such as lead, arsenic, cadmium and mercury were not detected. The results of the chemical analysis of OCS02 cultivar performed by Fung et al. (2018) were summarised in Table 2.1.

Table 2.1 Chemical analysis of OCS02, adapted from Fung et al. (2018).

Composition	**Amounts (g/kg)**
Total polysaccharides	482.800
α-Glucan	29.750
β-1,3/1,6-Glucan	342.500
Others (not specified)	110.550
Total amino acids	237.030
Arginine	45.200
Lysine	20.300
Serine	18.600
Threonine	18.200
Valine	16.000
Leucine	16.000
Alanine	15.600
Proline	13.900
Aspartic acid	12.200
Tyrosine	10.100
Isoleucine	9.580
Glutamic acid	9.160
Phenylalanine	8.620
Glycine	7.100
Histidine	6.450
Cysteine	6.140
Methionine	2.370
Tryptophan	1.500
Hydroxyproline	ND (< 0.05)
Hydroxylysine	ND (< 0.05)

Nucleosides and their derivatives	
Cordycepin	0.655
Adenosine	0.364
Adenine	0.015
Hydroxyethyl-adenosine	0.126
Ethyl-adenosine	0.091
Mineral and heavy metal	
Potassium	5.140
Calcium	0.703
Magnesium	0.676
Sodium	0.121
Chromium	0.0025
Lead	ND (< 0.001)
Arsenic	ND (< 0.001)
Cadmium	ND (< 0.0002)
Mercury	ND (< 0.00005)

(Abbreviation: ND = not determined)

2.1.2 Extraction and fractionation of natural substances

Extraction is a primary step in the investigation of the bioactive constituents from natural sources to further characterise their pharmacological activities (Azwanida NN, 2015). Extraction methods include solvent extraction, distillation method, pressing and sublimation according to the extraction principle (Zhang et al., 2018; Azwanida NN, 2015). Solvent extraction is the most used extraction method and the solvents used include water and organic solvents like dimethyl sulfoxide (DMSO), methanol, ethanol and chloroform. There is an increasing use of modern extraction methods such as microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction and pressurised liquid extraction, to reduce the use of organic solvents and increase efficiency (Zhang et al., 2018; Azwanida NN, 2015).

The choice of extraction method and solvent are the main determinants of the efficiency of the extraction and the composition of the resultant crude extracts. Based

on the law of similarity (like dissolves like), solvents are likely to dissolve solute with a similar polarity (Zhang et al., 2018). Therefore, the polarity of targeted compounds should be considered during the process of solvent selection. Besides, given that studies involving bioactivities of natural medicines are usually based on their traditional uses, the choice of extraction method and solvent should ideally mirror the traditional methods of preparation (Fabricant and Farnsworth, 2001).

Crude extracts are known to contain a variety of compounds that may or may not elicit bioactivity. Fractionation is a useful technique to reduce the complexity of the extracts to assist in identification of the bioactive compounds (Weller, 2012). When the biological activity of an extract is established, the extract can be fractionated, and the resulting fractions are further tested for their activities. The most bioactive fraction will be subjected to subsequent chemical analysis including isolation/purification and structure elucidation of the bioactive compounds.

Fractionation can be performed using several methods including chromatography, precipitation, crystallisation, liberation, distillation and electrophoresis (Houghton and Raman, 1998; Abubakar and Haque, 2020). Chromatography is the most used technique for fractionation. In general, there are five different types of chromatography: a) adsorption chromatography, b) partition chromatography, c) affinity chromatography, d) ion chromatography and e) size exclusion chromatography (Abubakar and Haque, 2020). Recently, there are several novel bio-separation technologies such as membrane chromatography and three-dimensional gas chromatography (Chen et al., 2022; Abdulhussain et al., 2021). The choice of fractionation method is usually based on several factors including the nature of the extract (its chemical and physical properties), availability of equipment in research facility, budget, purpose of the fraction, procedure following fractionation

and safety (Houghton and Raman, 1998).

It is noteworthy that on many occasions, the bioactivity of the fractionated extracts would be weaker than the crude extracts. For example, the crude ethanolic extract of *Pothomorphe umbellatum* exhibited a stronger antioxidant activity against exogeneous cytoplasmic ROS than its fractions (Lopes et al., 2013). Abu Lafi et al. (2018) also reported that the crude extract of *Inula viscosa* produced a marked antimalarial activity but not its fractions. These studies suggest that the components present in the crude extract may work synergistically to produce the respective biological effects. All in all, fractionation remains indispensable in drug discovery involving medicinal plants and fungi to provide insight into future work in elucidating the mechanism of action using crude extract or fractions and the isolation of bioactive compounds. Thus, the present study was designed based on the concept of bioassay-guided fractionation, as illustrated in Figure 2.2.

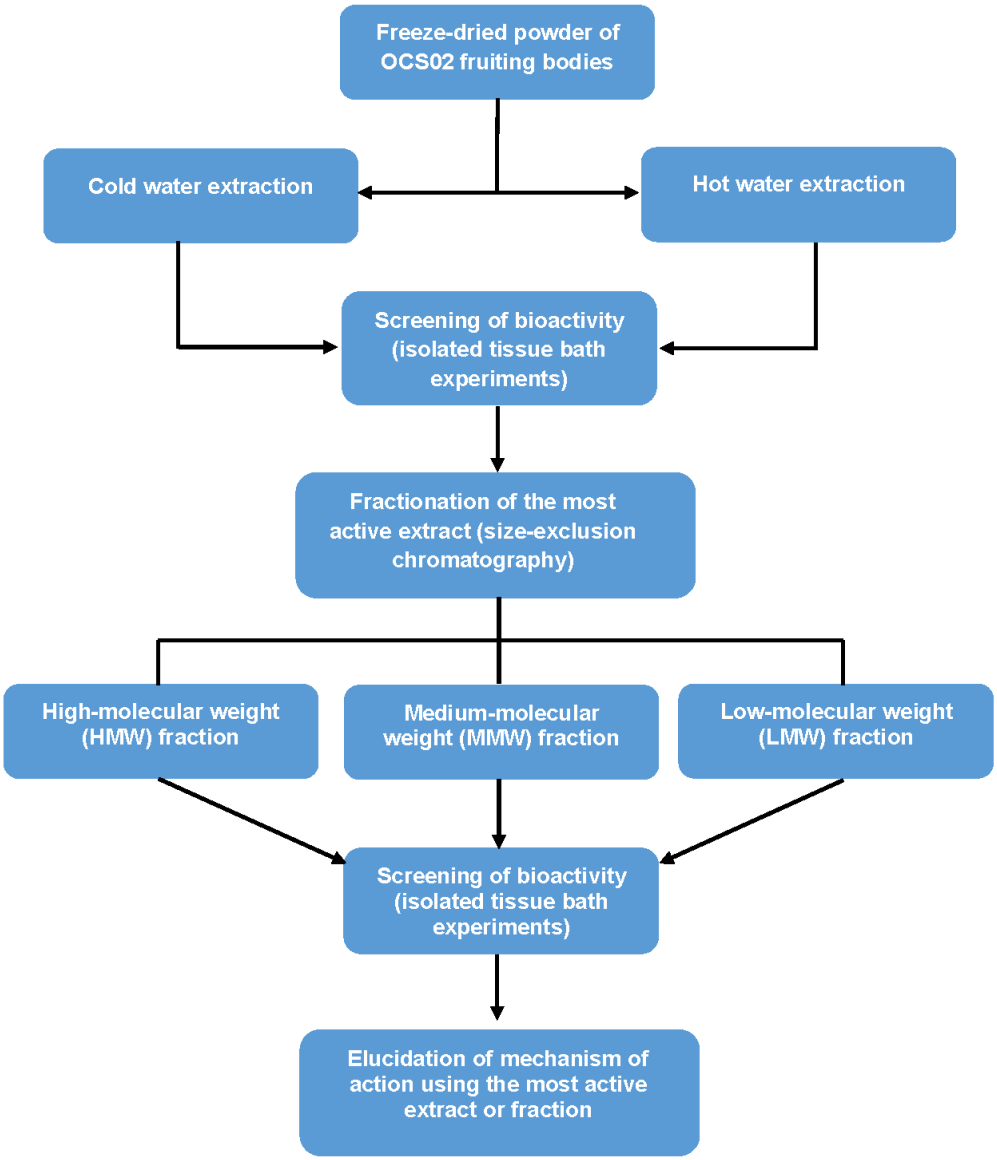

Figure 2.2 Extraction and fractionation procedure of OCS02 cultivar prior to its pharmacological investigation.

2.2 Aim and objectives

Despite the traditional use of *O. sinensis* in relieving frequent urination, there is no credible scientific evidence that shows its effect on the bladder. In this chapter, we aimed to optimise the aqueous extraction method of *O. sinensis* fruiting bodies (OCS02 cultivar) and evaluate their bioactivities in rat bladder using the isolated tissue bath technique. The extract with the most prominent relaxant effect against the contraction of the bladder strips will be fractionated and used in subsequent mechanistic studies. The objectives within this chapter were:

i. To perform hot and cold water extraction of OCS02 cultivar
ii. To determine the carbohydrate and protein content of the hot and cold water extracts (i.e., OCS02-HWE and OCS02-CWE respectively)
iii. To investigate the effect of OCS02-HWE and OCS02-CWE in pre-contracted bladder strips
iv. To fractionate the most active extract by size exclusion chromatography
v. To evaluate the bioactivity of the molecular weight-based fractions in pre-contracted bladder strips
vi. To study the effects of oxybutynin and mirabegron in the bladder as positive controls

2.3 Materials and methods

2.3.1 Preparation of Krebs-Henseleit bicarbonate solution and drugs

Standard Krebs-Henseleit bicarbonate solution (referred to as Krebs solution throughout this thesis) at pH 7.4 was freshly prepared on the day of experiment with the following composition (mM): sodium chloride (NaCl) 120, potassium chloride (KCl) 5.4, magnesium sulphate ($MgSO_4$) 1.2, potassium dihydrogen phosphate (KH_2PO_4) 1.2, sodium bicarbonate ($NaHCO_3$) 25, *D*-glucose 11.7, and calcium chloride ($CaCl_2$) 1.26. All the salts used for the preparation of Krebs solution were purchased from Chemiz, Malaysia. After dissolving all the salts in purified water, Krebs solution was aerated with 95% O_2 and 5% CO_2 for at least 15 minutes. Type II purified water of Milli-Q quality (> 15 MΩ) was used throughout the experiments. Drugs used in this chapter and their details are listed in Table 2.2.

Table 2.2 Drugs and their details.

Drugs	**Company, Country**	**Mechanism of action**	**Solvent (Stock concentration)**
Carbamylcholine or carbachol	Nacalai Tesque, Japan	Muscarinic receptors agonist	Purified H_2O (0.1 M)
Mirabegron	Sigma Aldrich, USA	β_3-Adrenoceptor agonist	DMSO (0.1 M)
Oxybutynin	Sigma Aldrich, USA	Muscarinic receptors antagonist	Purified H_2O (0.1 M)

2.3.2 Preparation of *O. sinensis* extracts

The extraction methods were adapted from previous papers (Yap et al., 2020a, 2020b). Extractions of the freeze dried powder of OCS02 were performed using water with a mass:volume ratio of 1:20 (w/v). For hot water extraction, the powder was suspended in water at 95 – 100 °C (not boiling) for two hours, whereas for cold water extraction, the powder was suspended in water and stirred for 24 hours using a magnetic stirrer at 4 °C. Following the extraction, insoluble substances were removed by centrifugation at 8000 *g* for 30 minutes and the supernatants were filtered through Whatman No.1 filter paper. The hot water and cold water filtrates were collected and freeze dried for at least 48 hours to produce the hot water extract and cold water extract (Freeze-dryer Alpha 1-2 LDplus, Martin Christ, Germany). The hot and cold water extracts of OCS02 are termed OCS02-HWE and OCS02-CWE, respectively, throughout this thesis. The extracts were kept at -80 °C for long-term storage and dissolved in purified water prior to the experiments.

2.3.3 Determination of protein and carbohydrate contents of *O. sinensis* extracts

Total protein content of the extracts was determined using Bradford assay with bovine serum albumin (BSA) used as a standard (Bradford, 1976). Total carbohydrate content of the extracts was determined using the phenol-sulfuric acid assay with *D*-glucose used as a standard (Dubois et al., 1956).

2.3.4 Fractionation of *O. sinensis* cold water extract

The fractionation method has been described in previous studies (Ng et al., 2023; Yap et al., 2020a). Briefly, one gram of OCS02-CWE was dissolved in 10 mL of purified water. The dissolved OCS02-CWE was put through a convectional column packed with Sephadex G50 fine [40 cm × (1.25 cm)2 × π] and eluted using 50 mM of ammonium acetate by gravity. The pooled fractions were classified according to their molecular weight (MW): high molecular weight (HMW; > 30 kDa), medium molecular weight (MMW; 7 – 30 kDa), and low molecular weight (LMW; < 7 kDa). Molecular mass of the pooled fractions was determined by comparing their ratio of elution volume (Ve) and void volume (Vo) to the Ve/Vo of selected protein standards including albumin bovine (66 kDa), albumin egg (45 kDa), pepsin (34.7 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa). The pooled fractions were then freeze-dried and stored at -80°C.

2.3.5 Isolated tissue bath and tissue preparation

Ethics approvals were obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB) (UNMC12 and UNMC26, Appendix A). This research also complies with the European guidelines for laboratory animal use and care (86/609/EEC). Experiments were conducted with healthy male SD rats (235 – 502 g; 2 – 3 months old) obtained from the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) or Universiti Kebangsaan Malaysia (UKM). The animals were euthanised on the day of experiment by asphyxiation from CO_2. Then, they were kept in an ice box and transported to our laboratory for dissection and excision of tissues. The rat bladder was isolated and immediately immersed in a Sylgard plate filled with Krebs solution. The bladder body was trimmed into longitudinal strips of 8 mm × 2 mm (length × width) (Kullmann et al., 2014). Figure 2.3A shows an isolated rat

bladder and Figure 2.3B shows the bladder after being divided in half in the sagittal plane.

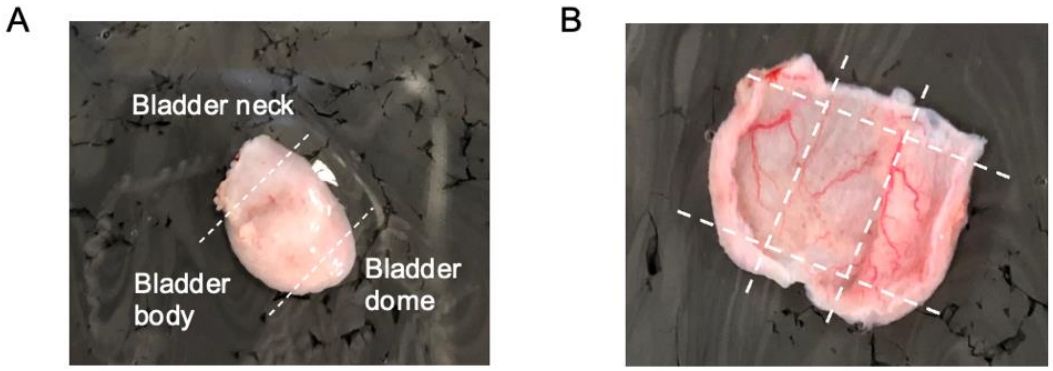

Figure 2.3 Bladder isolated from a healthy adult male Sprague Dawley rat. *(A) Anatomy of a rat bladder. The bladder neck is a small region which consists of the trigone and extends from the ureters to the urethra. The bladder body mainly consists of detrusor smooth muscle whereas bladder dome is the roof of the bladder. (B) The dashed lines illustrate how the bladder was trimmed into longitudinal strips.*

Once the bladder strip was trimmed, one end of the strip was hooked onto a metal rod using a surgical silk thread and placed in a glass chamber filled with 5 mL of Krebs solution. The glass chamber was aerated with 95% O_2 and 5% CO_2 and the bath temperature was maintained at 37.5 °C throughout the experiments. The other end of the strip was connected to a force transducer (MLTF050/ST, ADInstruments, USA) which detects the changes in isometric tension produced by tissue contraction and relaxation. Data was recorded by a PowerLab data acquisition programme (LabChart v7.3.4) and a laptop (Hewlett-Packard, USA). The isolated tissue bath setup in our laboratory is depicted in Figure 2.4.

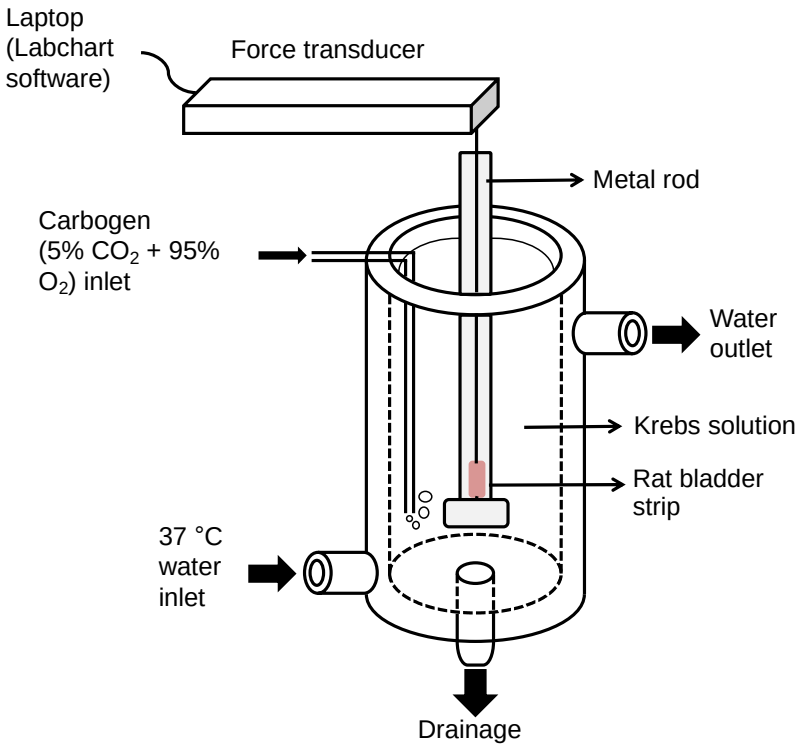

Figure 2.4 Illustration of the isolated tissue bath setup and the method of tying bladder strip to a metal rod.

After the application of a passive tension of 9.8 mN (equivalent to 1.0 g), the tissues were left to equilibrate to bath condition for 30 minutes. Then, all tissues were exposed twice to 60 mM of KCl to assess their viability and to provide reference contracture (average of the two tonic responses induced by KCl) for subsequent data analysis (Loong et al., 2015). Tissues with an average contraction of less than 3.9 mN (0.4 g) were deemed not viable thus discarded and excluded from data analysis. Following the tissue viability test, tissues were left to return to resting tension for at least 15 minutes before the performance of experimental protocols.

2.3.6 Isometric tension recordings

2.3.6.1 Construction of cumulative concentration-response curves to OCS02-CWE

Bladder strips were pre-contracted with 1 μM of carbachol to obtain a stable submaximal contraction size between 70% and 80% of the maximum carbachol contraction. Upon establishing a stable tonic contraction, cumulative concentration-response curves (CRCs) to OCS02-CWE were constructed from 0.5 mg/mL to 8.0 mg/mL. Vehicle control was performed in parallel using purified water.

2.3.6.2 Construction of non-cumulative concentration-response curves to OCS02-CWE or OCS02-HWE

Bladder strips were pre-contracted with 1 μM of carbachol. Upon establishing a stable tonic contraction, non-cumulative CRCs to OCS02-CWE or OCS02-HWE were constructed from 0.5 mg/mL to 5.0 mg/mL. Once the response induced by the extract plateaued, the bath content was washed out and replaced with fresh Krebs solution. The tissues were then allowed to rest for 15 minutes before the next addition of carbachol for pre-contraction and the samples. On average, this protocol required 6.5 hours to complete, excluding the time taken during tissue preparation. Due to long experimental duration, non-cumulative CRCs to higher concentrations of OCS02-CWE (6.0 mg/mL to 8.0 mg/mL) were tested in a different set of preparation in parallel. Vehicle control was performed in parallel using purified water of equivalent volume for each concentration.

2.3.6.3 Effects of OCS02-CWE and fractions on pre-contracted bladder strips

Bladder strips were pre-contracted with 1 μM of carbachol. Once a stable tonic contraction was established, 5 mg/mL of OCS02-CWE or fractions was added into the bath in a randomised manner. This concentration (5 mg/mL) was used as it produced

the greatest relaxation response during the construction of non-cumulative CRCs (see section 2.4.4) The bath contents were washed out and replaced with fresh Krebs solution once the response plateaued. After 15 minutes of resting time, tissues were stimulated with carbachol again prior to the addition of another fraction. Vehicle control was performed in parallel using 250 µL of purified water. This volume, 250 µL, is equivalent to the volume of the extracts used to achieve a final concentration of 5 mg/mL in the bath).

2.3.6.3 Effects of the conventional treatment for OAB on bladder strips pre-contracted with carbachol

To serve as positive controls, cumulative CRCs to oxybutynin, a non-selective antimuscarinic, or mirabegron, a β_3-adrenoceptor agonist, were constructed in bladder strips pre-contracted with 1 µM of carbachol. Vehicle control (DMSO) was performed in parallel.

2.3.7 Data analysis

Mean KCl-induced tone of each tissue was obtained by averaging the value ‘x’ and ‘y’ (Figure 2.5). The value was used to calculate carbachol-induced contractions in the experiments.

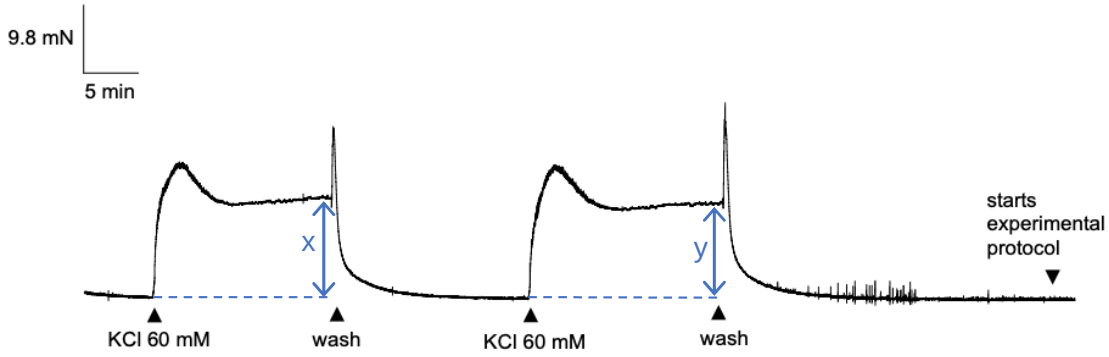

Figure 2.5 Representative isometric tension trace recording of KCl-induced contraction in the bladder strips. *The difference in magnitude of tonic responses induced by both KCl additions were averaged to serve as the reference contracture (reference KCl-induced tone). (Abbreviations: KCl = potassium chloride; mN = milliNewton; min = minute).*

The pre-contractile tone induced by carbachol was obtained by measuring the difference of bladder tone from the baseline ‘a’ (Figure 2.6) and displayed in mN. The contractile and relaxation responses induced by the extracts were expressed as a percentage contraction or relaxation of carbachol-induced tone where 100% contraction indicates that the contraction has doubled from the pre-contractile tone whereas 100% relaxation indicates that the tissue tension has returned to the baseline tension (Figure 2.6). The contraction or relaxation responses induced by the extracts obtained from the respective non-cumulative CRCs were presented using bar graphs; with standard bar graphs showing tissue contraction and reversed bar graphs showing tissue relaxation.

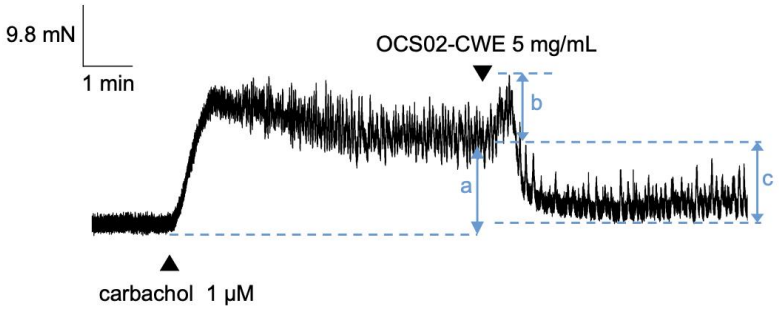

Figure 2.6 Illustration of a representative trace recording showing the contractile tone induced by carbachol and the responses induced by OCS02-CWE. *The differences in the magnitudes were measured and used to calculate tissue contraction or relaxation induced by the extract or fraction.*

The percentage of tissue contraction or relaxation was calculated using the following equations:

$$Tissue\ contraction = \frac{Magnitude\ of\ extract{-}induced\ contraction\ (b)}{Magnitude\ of\ carbachol{-}induced\ contraction\ (a)} \times 100\%$$

$$Tissue\ relaxation = \frac{Magnitude\ of\ extract{-}induced\ relaxation\ (c)}{Magnitude\ of\ carbahol{-}induced\ contraction\ (a)} \times 100\%$$

From oxybutynin and mirabegron CRCs, the respective relaxation responses were expressed as a percentage of the carbachol-induced tone. The percentage concentration-responses were presented in a non-linear regression curve using three parameters equation in GraphPad Prism (version 9.0 for macOS, GraphPad Software, La Jolla California USA). All data were expressed as mean ± standard error of mean (SEM), with *n* refers to the number of animals used in each group. Maximum tissue response (E_{max}) and pEC_{50} were derived from nonlinear regression analysis of the obtained CRC. pEC_{50} is the negative logarithm of EC_{50} where EC_{50} is the concentration of drug that produces 50% of its maximum response.

Statistical analysis was performed using Student's *t*-test to compare between

control group and a treatment group. Analysis of Variance (ANOVA) was performed to compare the effects of three or more groups. *Post hoc* tests were performed when a significance was detected ($p < 0.05$). Dunnett's *post hoc* test was used to compare between vehicle control and treatment groups. Two-way ANOVA followed by Sidak's *post hoc* test was performed to compare the effects between treatment groups across different concentrations. Results were considered statistically significant if *p*-value was less than 0.05. Individual significance was indicated in asterisk symbol in table or graph, where '****' or '$^{\#\#\#\#}$' indicates $p < 0.0001$, '***' or '$^{\#\#\#}$' indicates $p < 0.001$, '**' or '$^{\#\#}$' indicates $p < 0.01$ and '*' or '$^{\#}$' indicates $p < 0.05$.

2.4 Results

2.4.1 Yield from the extraction of OCS02 cultivar

The resultant OCS02-HWE and OCS02-CWE appeared in the form of light milky flakes. The percentage yields of OCS02-HWE and OCS02-CWE were 20% and 10%, respectively (w/w). Both extracts were soluble in water, but OCS02-HWE solution was more viscous than OCS02-CWE solution. Therefore, stock concentrations of 100 mg/mL and 50 mg/mL of OCS02-CWE and OCS02-HWE, respectively, were used for the isolated tissue bath experiments.

2.4.2 Protein and carbohydrate contents of *O. sinensis* extracts

The total content of protein and carbohydrate in both extracts are shown in Table 2.3. The total carbohydrate in OCS02-HWE was 2.3 times greater than OCS02-CWE whereas its protein content was 1.6 times lower.

Table 2.3 Protein and carbohydrate contents of O. sinensis hot water extract and cold water extract.

Sample	Protein (% w/w)	Carbohydrate (% w/w)
OCS02-CWE	2.12 ± 0.20	41.47 ± 3.52
OCS02-HWE	1.65 ± 0.44	93.87 ± 3.33

Protein and carbohydrate contents were estimated based on dry weight (w/w)(n = 3).

2.4.3 Cumulative addition of OCS02-CWE did not produce significant relaxation response in bladder

During our initial testing, the CRCs to OCS02-CWE were performed in a cumulative manner. Results showed that the responses induced by OCS02-CWE was inconsistent as intermittent contractions and relaxations were observed. Figure 2.7 shows a representative trace recording of the cumulative CRC to OCS02-CWE. In the analysis

of this set of data, only the maximum reduction in tension (tissue relaxation) induced by each concentration of OCS02-CWE was measured as some did not induce a contractile tone (Figure 2.7). The maximum relaxation response induced by OCS02-CWE was not significantly different from the vehicle control at the highest concentration tested, 8 mg/mL (48.64 ± 7.13% vs 23.03 ± 4.57%, $p = 0.0715$) (Figure 2.8).

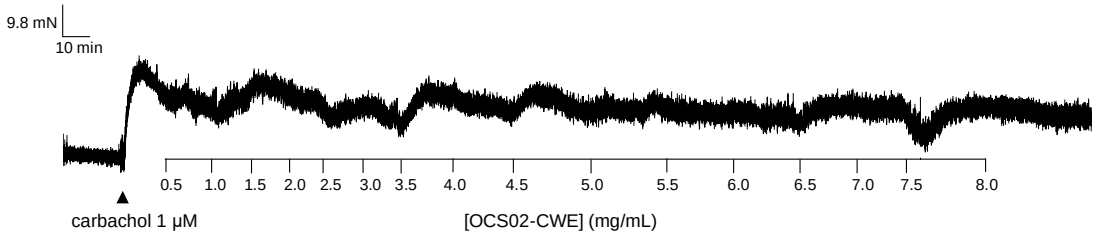

Figure 2.7 Representative trace recording of a cumulative addition of OCS02-CWE. *The bladder strip was pre-contracted with 1 µM of carbachol prior to the construction of cumulative CRC to OCS02-CWE from 0.5 mg/mL to 8.0 mg/mL. OCS02-CWE was added into the bath every 10 minutes or until the response plateaued. (Abbreviation: CWE = cold water extract; CRC = concentration-response curve; min = minute; mN = milliNewton).*

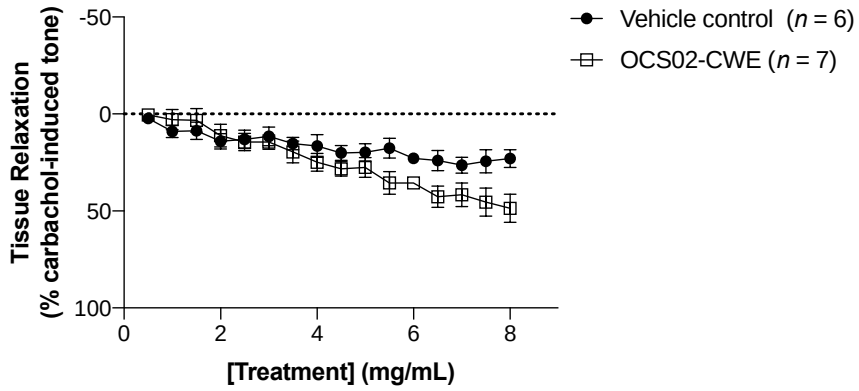

Figure 2.8 Effect of cumulative addition of OCS02-CWE on carbachol-pre-contracted bladder strips. *Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviation: CWE = cold water extract).*

2.4.4 OCS02-CWE produced a biphasic relaxation response in pre-contracted bladder whereas OSC02-HWE was devoid of relaxant effect

Following the above observations (Figures 2.7 and 2.8), OCS02-CWE were added non-cumulatively to examine its effects on the bladder strips. The effect of OCS02-HWE was also studied. Each of the non-cumulative CRC to the extracts was constructed from 0.5 mg/mL to 5.0 mg/mL in which 10 repeated applications of carbachol to pre-contract the tissues prior to the addition of extracts, were required. To confirm that the magnitudes of the pre-contractile tones were consistent throughout the construction of the CRCs, carbachol-induced tones prior to the addition of the extracts were measured. Figure 2.9 and Table 2.4 depict the contractions induced by carbachol throughout the CRCs in the respective groups.

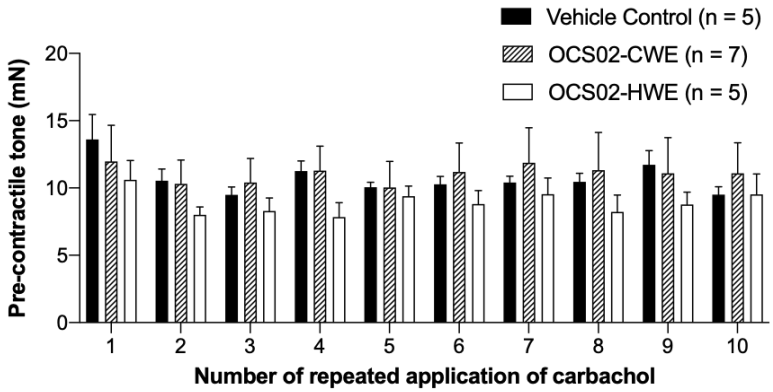

Figure 2.9 Repeated applications of carbachol produced consistent tonic contraction. *Data are shown as mean ± SEM of n number of animals. Two-way ANOVA where comparison of means between all the groups was made, p > 0.05. (Abbreviations: CWE = cold water extract; HWE = hot water extract; mN = milliNewton).*

Table 2.4 Tissue contraction elicited by carbachol throughout the non-cumulative addition of OCS02 extracts from 0.5 mg/mL to 8.0 mg/mL versus vehicle control.

Sequence of carbachol pre-contraction prior to the addition of extracts	**Pre-contractile tone induced by carbachol (mN)**		
	Vehicle control (*n* = 5)	**OCS02-CWE (*n* = 7)**	**OCS02-HWE (*n* = 5)**
1	13.60 ± 1.86	11.97 ± 2.69	10.60 ± 1.43
2	10.54 ± 0.86	10.30 ± 1.77	8.02 ± 0.58
3	9.49 ± 0.60	10.42 ± 1.78	8.30 ± 0.97
4	11.25 ± 0.76	11.28 ± 1.83	7.84 ± 1.07
5	10.05 ± 0.37	10.04 ± 1.94	9.39 ± 0.75
6	10.27 ± 0.59	11.19 ± 2.16	8.80 ± 1.01
7	10.41 ± 0.46	11.87 ± 2.60	9.55 ± 1.19
8	10.47 ± 0.62	11.33 ± 2.80	8.23 ± 1.25
9	11.72 ± 1.05	11.09 ± 2.65	8.88 ± 0.91
10	9.51 ± 0.58	11.09 ± 2.27	9.53 ± 1.51

Data are shown as mean ± SEM of n number of animals. Two-way ANOVA where comparison of means between all the groups was made, p > 0.05. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; HWE= hot water extract; mN = milliNewton)

In carbachol-pre-contracted bladder strips, both OCS02-CWE and OCS02-HWE produced an immediate transient contraction (Figure 2.6 and Figure 2.10). OCS02-HWE-induced transient contraction was observed from 1 mg/mL onwards and peaked at 4 mg/mL (maximum contractile response: 91.21 ± 16.53%). The transient spike of the contraction induced by OCS02-CWE was only prominent at concentrations above 2 mg/mL and peaked at 4 mg/mL (maximum contractile response: 40.06 ± 8.95%) (Figure 2.11). The vehicle control (purified water) did not

exert any contractile effect on the carbachol-pre-contracted bladder strips (data not shown).

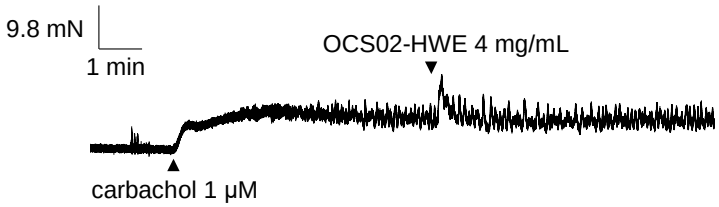

Figure 2.10 A representative isometric tension trace recording of the effect of OCS02-HWE on carbachol-pre-contracted bladder strips. *(Abbreviations: HWE = hot water extract; min = minute; mN = milliNewton)*

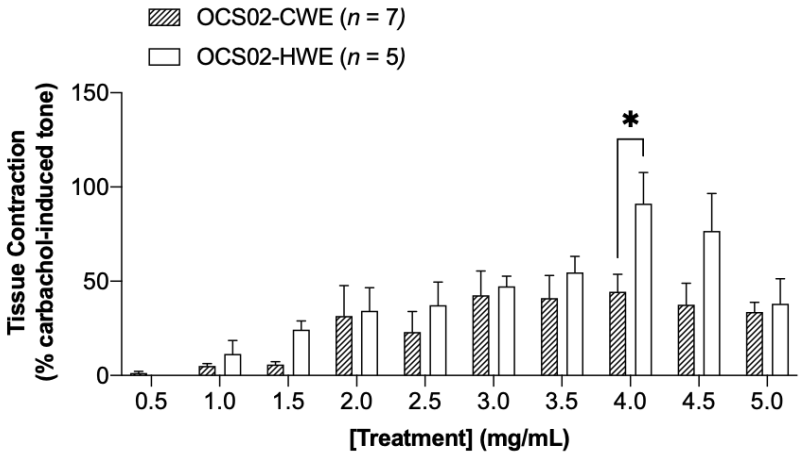

Figure 2.11 The immediate transient contraction induced by OCS02 extracts in pre-contracted bladder strips. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA with Sidak's post hoc test was made between the groups, *p < 0.05. (Abbreviations: CWE = cold water extract; HWE = hot water extract).*

In the OCS02-HWE-treated group, the tissue tension returned to a level which was similar to the carbachol-induced tone following the transient contraction, showing that HWE was devoid of any relaxant effect (Figure 2.10). In contrast, OCS02-CWE produced a significant relaxation response following the transient contraction (Figure

2.6). The greatest relaxation response (56.12 ± 3.78%) was achieved at 5 mg/mL with a clear trend of concentration-dependent relaxation, $p = 0.0002$ when compared to the vehicle control (2.41 ± 0.12%) (Figure 2.12).

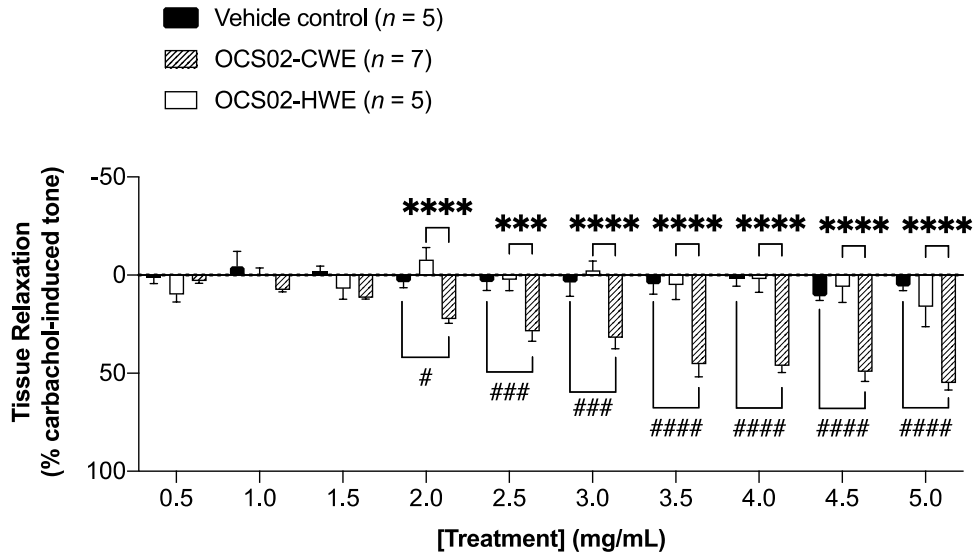

Figure 2.12 The relaxation response induced by OCS02 extracts in carbachol pre-contracted bladder strips. *Tissue responses have been expressed as a percentage of carbachol-induced contraction. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA with Sidak's post hoc test was made between all the groups: vehicle control vs OCS02-HWE, $p > 0.05$; vehicle control vs OCS02-CWE, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$; OCS02-CWE vs OCS02-HWE, $^{***}p < 0.001$, $^{****}p < 0.0001$. (Abbreviations: CWE = cold water extract; HWE = hot water extract).*

The biphasic response consisting of the transient contraction and relaxation response induced by OCS02-CWE from Figure 2.11 and Figure 2.12, respectively, were incorporated in Figure 2.13. Concentrations between 6 mg/mL and 8 mg/mL examined in a separate set of experiments did not produce a greater relaxation response in the carbachol-pre-contracted bladder strips compared to 5 mg/mL of OCS02-CWE. The relaxation responses were between 39.53 ± 1.10% to 49.21 ± 2.00%. Therefore, 5 mg/mL of OCS02-CWE was used in subsequent mechanistic studies.

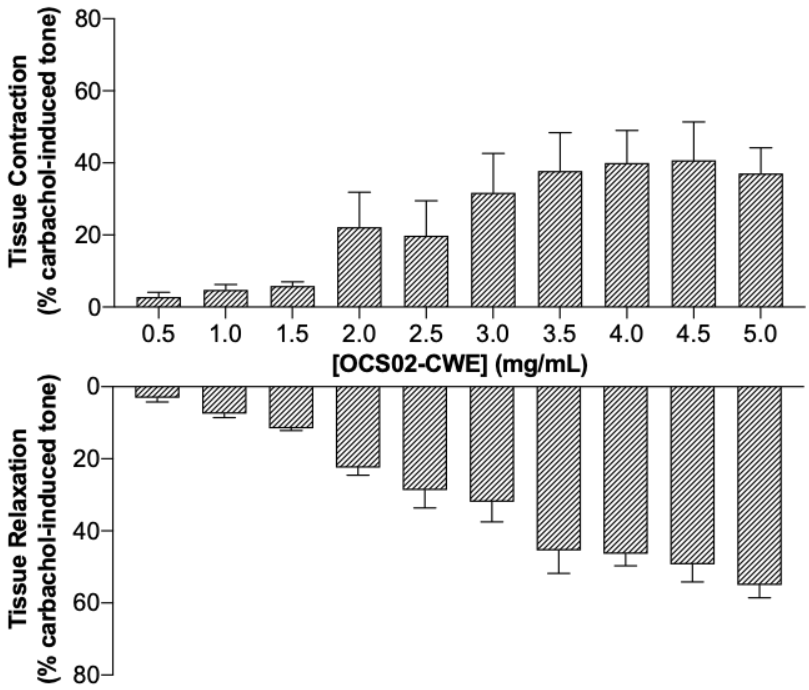

Figure 2.13 The biphasic response induced by OCS02-CWE in bladder strips pre-contracted with carbachol. *Tissue responses have been expressed as a percentage of carbachol-induced contraction. Data are shown as mean ± SEM of 5 – 7 animals. (Abbreviation: CWE = cold water extract).*

2.4.5 OCS02-MMW fraction elicited the greatest relaxation magnitude

To compare the bioactivities of OCS02-CWE with its fractions (OCS02-HMW, OCS02-MMW and OCS02-LMW) in bladder strips, a single concentration of 5 mg/mL of OCS02-CWE or fractions were added to the bladder strips pre-contracted with carbachol. In this set of experiment, OCS02-CWE produced a transient contraction of 39.62 ± 4.95% and a relaxation response of 56.91 ± 5.40%. Among the fractions, OCS02-MMW elicited the greatest relaxation response (46.50 ± 3.87%, $p > 0.05$ as compared to OCS02-CWE) but it was short-lived (Figure 2.14 and Figure 2.15). The greatest relaxation response was observed at 1 – 2 min after the application. The relaxation response induced by OCS02-HMW was 32.91 ± 2.08%, $p = 0.0008$ whereas OCS02-LMW produced the lowest relaxation response, 15.64 ± 4.56%, $p < 0.0001$ (Figure 2.15). Interestingly, among the fractions, only OCS02-LMW elicited an immediate transient contraction prior to its relaxation response as seen in OCS02-CWE, but with a lower magnitude (19.45 ± 5.70%, $p = 0.0283$).

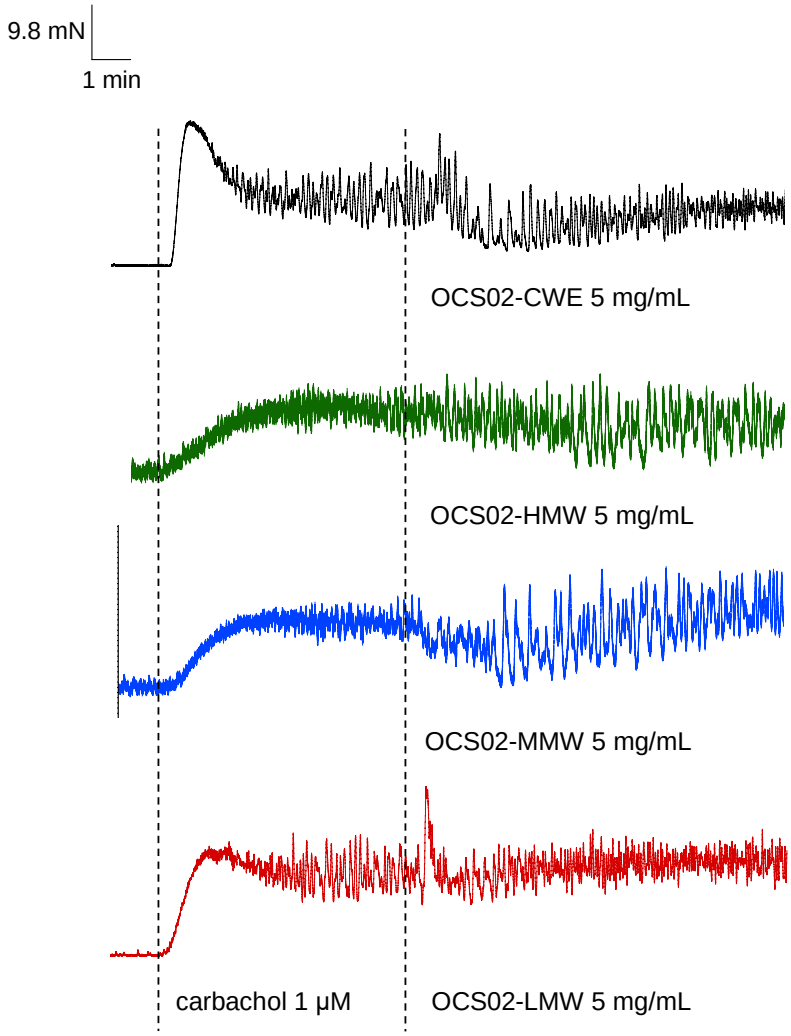

Figure 2.14 Representative trace recordings of the effects of OCS02-CWE and its fractions in bladder strips pre-contracted with carbachol. *(Abbreviations: CWE = cold water extract; LMW = low molecular weight; MMW = medium molecular weight; HMW = high molecular weight; min = minute; mN = milliNewton)*

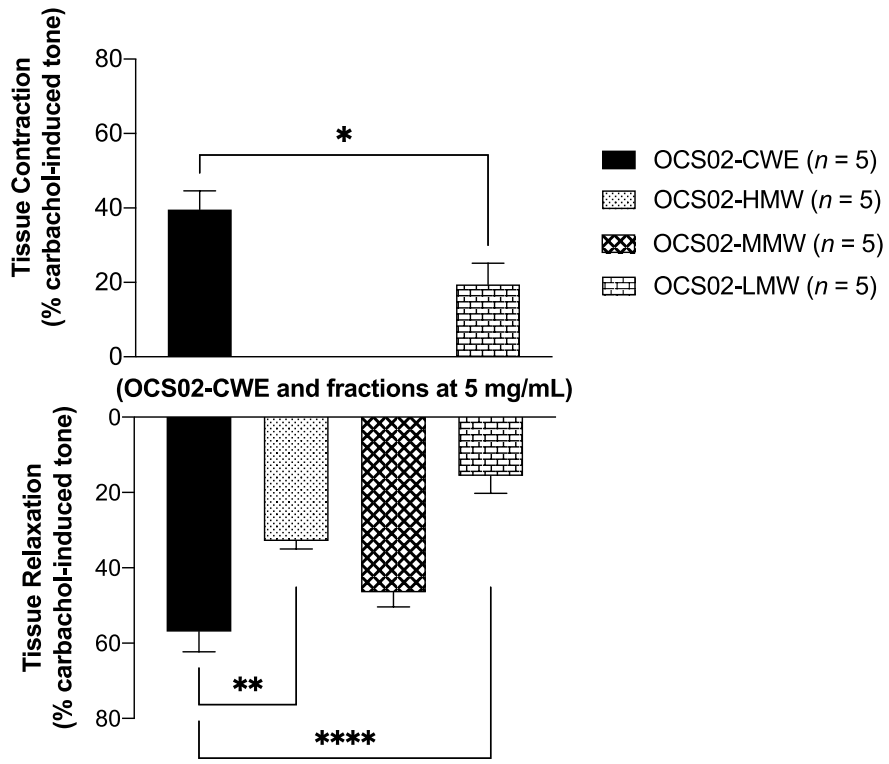

Figure 2.15 Effects of OCS02-CWE and its fractions on pre-contracted bladder. *Tissue responses have been expressed as a percentage of carbachol-induced contraction. Data are shown as mean ± SEM of 5 animals. Student's t-test was used to compare the difference between the contractile response induced by OCS02-CWE and OCS02-LMW, *p < 0.05. One-way ANOVA with Dunnett's post hoc test was performed to compare the difference between the relaxation response induced by OCS02-CWE and the fractions, ***p < 0.001, ****p < 0.0001. (Abbreviations: CWE = cold water extract; LMW = low molecular weight; MMW = medium molecular weight; HMW = high molecular weight)*

2.4.6 Oxybutynin and mirabegron relaxed pre-contracted bladder strips

CRCs to oxybutynin and mirabegron were constructed as positive controls. In bladder strips pre-contracted with carbachol, both oxybutynin and mirabegron elicited significant relaxation responses (E_{max} = oxybutynin, 110.5 ± 5.93 %, $p < 0.0001$; mirabegron, 100.7 ± 2.72 %, $p < 0.0001$) when compared to the vehicle control (E_{max} = 0.97 ± 8.59%) (Figure 2.16).

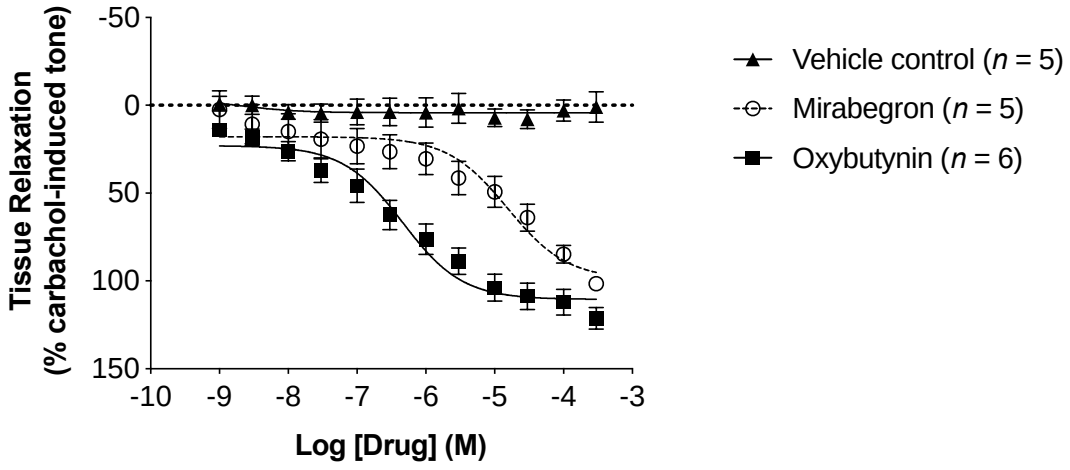

Figure 2.16 Effects of oxybutynin and mirabegron on pre-contracted bladder strips. *Tissue responses have been expressed as a percentage of carbachol-induced contraction. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves)*

2.5 Discussion

O. sinensis is traditionally consumed by drinking the decoction, eating it raw with water or milk, or mixing it with other herbal preparations such as texus leaf and Ginseng root (Panda and Swain, 2011). In order to mimic these traditional consumption methods, aqueous extraction method was adopted in our study. Another consideration was that OCS02 contains more water-soluble compounds with higher polarity, as evidenced by the fact that the methanol extract has lower yield compared to the water extract. Besides, OCS02 methanol extract did not elicit any biological response in the preliminary experiments (personal communication with Prof. Fung Shin Yee, UM). Our results showed that hot water extraction produced a higher yield than cold water extraction. Together with the higher amount of carbohydrates detected in OCS02-HWE compared to OCS02-CWE, the results imply that high temperature aids the solubility of carbohydrates from the OCS02 cultivar. Likewise, Benito-Román et al. (2016) showed that β-glucans are more soluble at higher temperatures.

In order to study the possible relaxant effect of *O. sinensis*, the bladder strips were pre-contracted with carbachol, a muscarinic agonist. This mimics the physiological regulation of bladder during micturition where parasympathetic control mediated by activation $G_{q/11}$-coupled M_3 receptors predominates (Yamanishi et al., 2001). In the initial experiments, the CRCs to OCS02-CWE was constructed in a cumulative manner. It was observed that OCS02-CWE caused intermittent tissue contractions throughout the CRCs and did not produce significant relaxation response when compared to the vehicle control. This suggests that cumulative addition of OCS02-CWE may induce tachyphylaxis in the bladder strips. Therefore, a different approach was used by exposing the tissues to OCS02-CWE non-cumulatively.

During the construction of non-cumulative CRCs to OCS02-CWE, the effects

induced by OCS02-CWE were consistent in which a biphasic response was observed at all concentrations tested. It is apparent that OCS02-CWE contains constituents that are able to elicit both contraction and relaxation in the bladder. These effects could be contributed by a single constituent or a group of constituents that mediate via different biological mechanisms of action. Moreover, the effects of OCS02-CWE are likely to be reversible as it did not alter the magnitude of carbachol-induced contractions throughout the experiment.

Extraction parameter such as temperature is known to vary the magnitudes of bioactivity of the extracted compounds. For instance, high extraction temperature increased the antioxidant activity of soluble flaxseed gum extracts (Vieira et al., 2019). In another study, the cold water extract of *L. rhinocerus* exhibited a marked relaxant effect in airway tissues compared to its hot water extract (Lee et al., 2018b). Interestingly, OCS02-HWE also produced an immediate transient contraction in carbachol-pre-contracted bladder strips, but it did not elicit any relaxant effect. The maximum contraction induced by OCS02-HWE was about two-times greater than that of OCS02-CWE. Through the phenol-sulphuric assays, it is revealed that the ratio of carbohydrates in OCS02-HWE to OCS02-CWE was about 2.3 to 1.0. In this regard, carbohydrates may be accountable for the contractile effect of the extracts.

The lack of relaxant effect of OCS02-HWE suggests that the main active constituent contributing to the bladder relaxation effect could be heat-sensitive, such as proteins and protein-carbohydrates complexes (Chen et al., 2016). This postulation could be supplemented by the relatively higher protein content in OCS02-CWE compared to OCS02-HWE. In an earlier study, a macromolecule protein extract of *O. sinensis* elicited a significant vasorelaxation response in rat aortic rings pre-contracted with phenylephrine, an α_1-adrenergic agonist (Chiou et al., 2000). The vasorelaxant

effect of *O. sinensis* reported by Chiou et al. (2000) was straightforward and did not involve a contractile response, as seen in our study.

As outlined in the introduction, the identification of pure bioactive compound(s) in traditional medicines is one of the most crucial steps to validate its bioactivity and safety for its development into pharmaceutical products. However, the isolation and purification of bioactive components is a tedious process due to the complexity of crude extract mixture in addition to the varying stability and sensitivity of the constituents. For OCS02-CWE, its complex biological actions, the bulk mass (carbohydrates) of the extract, being thermo-sensitive, have all added to this challenge. Therefore, size exclusion chromatography was adopted in our study to fractionate OCS02-CWE, allowing the separation of larger molecules from smaller molecules.

The fractionated extracts of OCS02-CWE exhibited different characteristics in the pre-contracted bladder and their relaxation effects were inferior compared to OCS02-CWE. This phenomenon is not uncommon in natural medicines. Several studies have shown that the overall effect of nature-derived extracts are caused by the synergistic effects between their components (Sanhueza et al., 2017; Rutkowska et al., 2019). Among the fractions, OCS02-MMW fraction produced the greatest relaxation response. However, its relaxant effect was transient, with the maximum response occurring 1 – 2 min after application. On the other hand, OCS02-HMW fraction produced a sustained relaxation response, but the magnitude was significantly lower than that of OCS02-CWE. Another important observation was that only OCS02-LMW fraction elicited a transient contractile effect like OCS02-CWE, but with a significantly lower magnitude. Based on the activities induced by the fractions, we speculate that the relaxation response induced by OCS02-CWE could be contributed by the constituents present in both high and medium MW fractions, and its contractile

effect could be caused by the components present in the low MW fraction.

The carbohydrates and protein contents of these fractions have been previously reported (Yap et al., 2020a). In OCS02-LMW, no protein was detected, and it has the highest carbohydrate contents among the three fractions. Then, the protein and carbohydrate contents in OCS02-MMW were 2.0 times higher and 1.1 times lower, respectively, than that of OCS02-HMW. Therefore, following our previous speculation, the constituent(s) in OCS02-CWE that is responsible for the contractile effect could be thermo-stable carbohydrates with low MW. In contrast, the constituent(s) in OCS02-CWE that produces a relaxant effect may contain proteins that are heat-sensitive and present in the high and medium MW fractions. However, OCS02-CWE may contain other bioactive components that are neither proteins nor carbohydrates such as nucleosides, as well as secondary metabolites. These components may also contribute to the bioactivities observed in our experiments.

While OCS02-CWE produced a significant bladder relaxation response, its efficacy appeared to be weaker than oxybutynin and mirabegron, the conventional therapeutic agents for OAB. Both oxybutynin and mirabegron were capable in causing full relaxation of the carbachol-pre-contracted bladder strips. Nevertheless, it may not be practical to compare their efficacies as such because both oxybutynin and mirabegron are pure compounds with a single mechanism of action that have been fully characterised. Oxybutynin acts as a competitive antagonist at the muscarinic receptor on the DSM, resulting in bladder relaxation. Mirabegron, on the other hand, is a β_3-adrenoceptor agonist that increases production of cAMP in the DSM and promotes DSM relaxation. From our results and the clinical use of these therapeutic agents in OAB treatment, it is certain that antagonism of muscarinic receptors and activation of β_3 adrenoceptors are both important pathways in bladder relaxation. The

ability of OCS02-CWE to relax carbachol-induced contractions also implies that these pathways could be implicated in its mechanism of action. Given that Ca^{2+} ions are the main determinant of smooth muscle contraction or relaxation, the regulation of Ca^{2+} may also be involved in the actions of OCS02-CWE. Moreover, urothelium and urothelial-derived relaxing factors including ATP, NO and H_2S may play a role in OCS02-CWE-induced responses.

Due to the fast and transient characteristics of OCS02-CWE-induced contraction, the activation of neurons may be implicated (Berridge, 2008; Heppner et al., 2016). Similar observation has been reported in which the crude extract of *Chamaemelum nobile* induced an immediate transient contraction via the activation of cholinergic neurons of the gut wall (Sándor et al., 2018). Regrettably, the neurogenic origin of bladder contraction or the effect of OCS02-CWE in nerve-induced bladder contraction cannot be investigated in our study. When performing experiments on nerve-induced contractions via electrical field stimulation (EFS), the use of TDX that blocks Na^+ channel, is required to verify the selectivity of EFS for neural transmission (Kullmann et al., 2014). Due to the prohibition on the use of TDX by the Malaysian Dangerous Drug Act 1952, we were unable to procure and use this compound. Nevertheless, other possible mechanistic pathways of OCS02-CWE-induced contraction including potentiation of muscarinic activation, involvement of urothelial-derived stimulating factor such as H_2O_2, activation of purinergic receptors and regulation of Ca^{2+} will be investigated in the subsequent mechanistic studies.

2.6 Chapter summary

In summary, this chapter presented the bioactivity of *O. sinensis* (OCS02 cultivar) in regulating bladder contraction and relaxation responses. OCS02-HWE was devoid of bladder relaxant effect but possessed transient contractile effect. On the other hand, OCS02-CWE demonstrated a biphasic bladder relaxant effect that consists of a transient contraction followed by a sustained relaxation response. The contractile effect of OCS02-CWE is likely to be caused by heat-stable carbohydrates with low MW (< 7.0 kDa), while its relaxant effect is likely to be contributed by heat-sensitive proteins or protein-carbohydrate complexes with MW > 7.0 kDa. Current results do not exclude the possibility of other constituents such as nucleosides and secondary metabolites as bioactive components in the extract. Based on the findings in this chapter, OCS02-CWE is used in the subsequent mechanistic studies to elucidate its mechanism of action in the bladder.

3 Elucidation of the Mechanisms of Action of *Ophiocordyceps sinensis* in Rat Bladder

3.1 Introduction

In Chapter 2, we have evaluated the bioactivity of OCS02-HWE, OCS02-CWE and the fractions of OCS02-CWE in rat bladder strips. This chapter thus describes the experimental protocols carried out to elucidate the mechanism(s) of action of OCS02-CWE. The rationale for selecting OCS02-CWE in these mechanistic studies includes: 1) OCS02-HWE and OCS02-LMW fraction produced transient contraction and were lacking in relaxant effect; 2) the relaxation response induced by OCS02-HMW and OCS02-MMW fractions was feeble and less sustained compared to OCS02-CWE; and 3) OCS02-HMW and -MMW fractions were lacking in contractile effect that may provide useful information in the process of elucidating the mechanism of action of *O. sinensis* in bladder. Given that OCS02-CWE altered the muscarinic response manifested by carbachol, it may potentiate muscarinic receptors activation to produce the transient contraction or inhibit muscarinic receptors to elicit its relaxant effect. OCS02-CWE is also likely to exert its effect through other contractile and relaxation pathways in the DSM such as activation of β_3 adrenoceptors, modulation of purinergic signalling, regulation of urothelial-derived relaxing factors, production of cyclic nucleotide second messengers, inhibition of MLCK and Rho kinase, and regulation of Ca^{2+} through K^+ channels, membrane permeable Ca^{2+} channels and intracellular store.

3.2 Aim and objectives

This study aimed to elucidate the mechanism(s) of action of OCS02-CWE in bladder using the isolated tissue bath technique. The specific objectives of this chapter were as follows:

i. To examine the effect of OCS02-CWE on depolarisation-induced bladder contraction
ii. To evaluate the effect of OCS02-CWE on the bladder tone and spontaneous myogenic activity induced by different contractile agents
iii. To examine the role of urothelium in OCS02-CWE-induced responses
iv. To study the effects of ATP, adenosine and cordycepin in both pre-contracted and resting bladder strips
v. To investigate the main bladder relaxation mechanisms of OCS02-CWE
vi. To investigate the main bladder contractile mechanisms of OCS02-CWE
vii. To determine the role of OCS02-CWE in the regulation of Ca^{2+}

3.3 Materials and methods

3.3.1 Drugs and Krebs-Henseleit bicarbonate solution

All drugs were dissolved in the respective solvents to make stock solutions and were further diluted to achieve the desired concentration. Details on the drugs including brands, mechanism of action, solvent used, and stock concentrations are summarised in Table 3.1. Krebs solution was prepared as described in **section 2.3.1**. In some experiments, Ca^{2+}-free Krebs solution was used, and it was prepared in the similar composition as normal Krebs solution but without the inclusion of $CaCl_2$ in the solution. OCS02-CWE was prepared as described in **section 2.3.2**.

Table 3.1 Summary of drugs and their details.

Drug	Company, Country	Mechanism of Action	Solvent	Stock concentration
2-APB	Tocris, UK	IP_3 receptor antagonist	DMSO	0.1 M
5-HT	Nacalai Tesque, Japan	5-HT receptor agonist	H_2O	0.1 M
8-PT	Sigma-Aldrich, USA	A_1 and A_{2A} receptor antagonist	80% Methanol in 0.2M NaOH	0.1 M
Adenosine	Nacalai Tesque, Japan	Adenosine receptors agonist	DMSO	1.0 M
ATP	Sigma-Aldrich, USA	P2 receptor agonist	H_2O	1.0 M
Atropine	Nacalai Tesque, Japan	Muscarinic antagonist	H_2O	0.1 M
Carbachol	Nacalai Tesque, Japan	Muscarinic agonist	H_2O	0.1 M
Cordycepin	Tocris, UK	Adenosine analogue	DMSO	0.1 M
Forskolin	Tocris, UK	AC activator	H_2O	0.1 M

IBMX	Sigma-Aldrich, USA	Non-selective PDE inhibitor	H_2O	0.1 M
L-NAME	Sigma-Aldrich, USA	NOS inhibitor	H_2O	0.1 M
NaHS	Sigma-Aldrich, USA	H_2S donor	H_2O	1.0 M
Nifedipine	Nacalai Tesque, Japan	Dihydropyridine L-type VGCC blocker	DMSO	0.1 M
ODQ	Tocris, UK	Soluble GC inhibitor	DMSO	0.1 M
PPADS	Sigma-Aldrich, USA	Non-selective P2 antagonist	H_2O	0.1 M
Rolipram	Tocris, UK	PDE5 inhibitor	DMSO	0.01 M
Ryanodine	Tocris, UK	RyR blocker	DMSO	0.1 M
Schwarzinicine A	University of Nottingham Malaysia	L-type VGCC, TRPC 3 ,4, 5, 6 channel inhibitor	DMSO	0.1 M
SKF-96365	Tocris, UK	Non-selective TRPC channel blocker, SOCC blocker, L-type VGCC blocker	DMSO	0.1 M

Suramin sodium	Santa Cruz, USA	Non-selective P2 antagonist	H_2O	0.1 M
S-(-)-propranolol hydrochloride	Santa Cruz, USA	Non-selective β-adrenoceptor antagonist	H_2O	0.1 M
SNP	Sigma-Aldrich, USA	NO donor	H_2O	0.1 M
TEA	Sigma-Aldrich, USA	Non-selective K^+ channel blocker	H_2O	0.1 M
Thapsigargin	Tocris, UK	SERCA inhibitor	DMSO	0.1 M
Verapamil	Tokyo Chemical Industry, UK	Phenylalkylamine VGCC blocker	DMSO	0.1 M

(Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; 5-HT = 5-hydroxytyramine; 8-PT = 8-phenyltheophylline; ATP = Adenosine triphosphate; IBMX = 3-isobutyl-1-methylxanthine; IP_3 = inositol triphosphate; AC = adenylate cyclase, DMSO = dimethyl sulfoxide; GC = guanylate cyclase; H_2S = hydrogen sulfide; L-NAME = N^G-nitro-L-arginine methyl ester; NaHS = sodium hydrosulfide; NO = nitric oxide; NOS = nitric oxide synthase; ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE = phosphodiesterase; PPADS = pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SNP = sodium nitroprusside; TEA = tetraethylammonium; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)

3.3.2 Tissue preparation

Ethics approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB) (UNMC12 and UNMC26, Appendix A). The experiments were conducted with male SD rats (260 – 503 g; 2 – 3 months old) purchased from UPM or UKM. The animals were sacrificed on the day of experiment by asphyxiation from CO_2. The bladder was isolated, prepared and mounted to the isolated tissue bath set up according to the steps detailed in **section 2.3.5**. In experiments to study the role of urothelium, the urothelium of bladder strips was removed by three gentle swabs using cotton bud in a single direction, as previously described (Moro et al., 2012; Munoz et al., 2010).

3.3.3 Histological investigation

Prior to the performance of experimental protocol described in **section 3.3.4.5,** the complete removal of the urothelium was assessed by histological investigation. Following dissection and isolation of the bladder, the bladder strips were kept in cold Krebs solution and sent to the Veterinary Laboratory Services Unit, UPM for slides preparation. The histology slides were prepared, and the sections were stained with Harris's haematoxylin and eosin (H&E) as previously described (Pauzi et al., 2020). The sections were then examined and photographed under a light microscope (Olympus CX21, Japan).

3.3.4 Isometric tension recordings

3.3.4.1 Effect of OCS02-CWE on bladder strips pre-contracted with KCl

To investigate the effect of OCS02-CWE on depolarisation-induced contraction, the bladder strips were pre-contracted with 60 mM of KCl. Upon establishing stable contractions, non-cumulative CRCs to OCS02-CWE from 0.5 mg/mL to 5.0 mg/mL were constructed. Purified water of equivalent amount for each concentration was used as vehicle control. Then, the relaxation response was compared with those elicited by OCS02-CWE in carbachol-induced pre-contraction.

3.3.4.2 Effect of OCS02-CWE on bladder contraction induced by different contractile agents

Bladder strips were pre-incubated with 5 mg/mL of OCS02-CWE or purified water of equivalent volume for 30 minutes, prior to the construction of cumulative CRCs to carbachol (1 nM to 300 µM), KCl (1 mM to 3 M), 5-HT (1 nM to 300 µM) or H_2O_2 (3 µM to 1 M). This concentration of OCS02-CWE (5 mg/mL) was used as it elicited the greatest relaxation response in earlier experiments (**section 2.4.4**). Carbachol, KCl, 5-HT or H_2O_2 was added at intervals of 5 minutes or until the response plateaued.

3.3.4.3 Effect of muscarinic antagonist on the relaxation response induced by OCS02-CWE

Bladder strips were pre-incubated with 1 µM of atropine (Wang et al., 1995) or vehicle control for 30 minutes, prior to the addition of 60 mM of KCl to pre-contract the tissues. Once a stable contraction was achieved, 5 mg/mL of OCS02-CWE was added into the bath. Each bladder strip was only used once for atropine pre-incubation and a single concentration of OCS02-CWE (5 mg/mL) as repeated application of atropine may interfere with the Ca^{2+} mobilisation to produce irreversible inhibition (Araki et al., 1976).

3.3.4.4 Effects of β-adrenoceptor antagonist and K^+ channel blocker on OCS02-CWE-induced responses

In order to investigate the involvement of β-adrenoceptors in OCS02-CWE-induced responses, a reversible, non-selective β-adrenergic receptor antagonist, propranolol (100 μM; Altunkaynak-Camca, 2020) was used to pre-incubate the bladder strips. To study the involvement of K^+ channels, a reversible, K_V and K_{Ca} channel blocker, TEA (1 mM; Han et al., 2012), was used to pre-incubate the tissues.

Following the 30-minute-pre-incubation period, bladder strips were pre-contracted with 1 μM of carbachol. Then, OCS02-CWE was added into the bath. The CRCs to OCS02-CWE in the presence of pre-incubation compound were constructed in a non-cumulative and randomised manner from 2 mg/mL to 5 mg/mL. Tissues were washed at least three times after each addition of OCS02-CWE and allowed to return to their resting tension for at least 15 minutes, before re-addition of the respective pre-incubation compounds and the next concentration of OCS02-CWE.

3.3.4.5 The role of urothelium in OCS02-CWE-induced responses

To study the role of urothelium in the actions of OCS02-CWE, non-cumulative CRCs to OCS02-CWE were constructed in carbachol-pre-contracted, urothelium-denuded bladder strips from 0.5 mg/mL to 5.0 mg/mL. The results were compared with those obtained from the non-cumulative CRCs constructed in urothelium-intact bladder strips in **section 2.4.4**.

3.3.4.6 Effects of ATP, adenosine and cordycepin on the bladder strips

To examine the effect of ATP on bladder, a single concentration of ATP (2 mM; Kim et al., 2003) was added to carbachol-pre-contracted bladder strips. Only one concentration was used in this protocol because cumulative addition of ATP may produce tachyphylaxis (Aronsson et al., 2010). When ATP was added non-cumulatively, the bladder tissues did not respond to concentrations below 2 mM and repetitive addition promotes tachyphylaxis in the tissues. ATP also did not produce any response in resting bladder strips (data not shown). Purified water was used as vehicle control in this experiment.

Then, to study the effects of adenosine and cordycepin on the bladder strips, cumulative CRCs to adenosine or cordycepin were constructed at basal tone (without drug-induced pre-contraction) and in carbachol-induced tone. Adenosine or cordycepin was added at intervals of 5 minutes or until response plateaued. Vehicle control (DSMO) was performed in parallel.

To confirm the suitability of the inhibitors to study the role of purinergic receptors in OCS02-CWE-induced responses, the effects of purinergic antagonists were examined on ATP-, adenosine- and cordycepin-induced responses. The bladder strips were pre-incubated with non-selective P2 purinoceptor antagonists, suramin sodium (100 µM; Hernández et al., 2009) or PPADS (30 µM; Hernández et al., 2009) for 30 minutes. Then, 1 µM of carbachol was used to pre-contract the tissues prior to the addition of 2 mM of ATP. A final concentration of DMSO 0.225% v/v was used as the vehicle control.

Adenosine receptor antagonist, 8-phenyltheophylline (8-PT) (10 µM; Boland et al., 1993) was used to evaluate the role of ARs in adenosine- and cordycepin-induced relaxation. Following an incubation period of 30 minutes, adenosine or cordycepin CRCs were constructed in either resting bladder strips or carbachol-pre-

contracted bladder strips. Methanol 0.02% v/v in 4 mM NaOH was used as the vehicle control for 8-PT.

3.3.4.7 Effect of purinergic antagonists on OCS02-CWE-induced responses

Subsequently, the role of purinergic receptors including P1 (adenosine receptors) and P2 (ATP receptors) on OCS02-CWE-induced responses was investigated. The bladder strips were pre-incubated with either suramin sodium 100 µM, PPADS 30 µM, 8-PT 10 µM or vehicle control for 30 minutes. Then, the bladder tissues were pre-contracted with 1 µM of carbachol prior to the addition of 5 mg/mL of OCS02-CWE. Only one concentration of OCS02-CWE (5 mg/mL) was used in this protocol as incomplete removal of PPADS following wash out has been suggested (Brown et al., 2001). DMSO 0.225% v/v and methanol 0.02% v/v in 4 mM NaOH was used as the vehicle control in the respective groups.

In another experiment, the bladder strips were pre-incubated with 5 mg/mL OCS02-CWE for 30 minutes. Then, 1 µM of carbachol was used to pre-contract the tissue prior to the addition of 2 mM of ATP. Purified water of equivalent volume was used as the vehicle control.

3.3.4.8 Effect of OCS02-CWE on H_2S-induced bladder relaxation

The participation of H_2S-mediated pathway in OCS02-CWE-induced relaxation was investigated in subsequent experiments. Bladder strips were pre-incubated with 5 mg/mL of OCS02-CWE or vehicle control (250 µL of purified water) for 30 minutes. Then, 1 µM of carbachol was used to pre-contract the tissues prior to the construction of cumulative CRCs to NaHS.

3.3.4.9 The role of NO in OCS02-CWE-induced responses

To investigate the involvement of NO in OCS02-CWE-induced responses, the bladder strips were pre-incubated with a NOS inhibitor, L-NAME (100 µM; Bassiouni et al., 2019) or NO donor, SNP 10 µM (Moro et al., 2012), for 30 minutes. Then, the tissues were pre-contracted with 1 µM of carbachol prior to the application of OCS02-CWE. The CRCs to OCS02-CWE in the presence of L-NAME were constructed non-cumulatively in a randomised manner, as described in **section 3.3.4.4**.

3.3.4.10 The role of cyclic nucleotides in OCS02-CWE-induced responses

To study the involvement of cAMP and cGMP pathways, the bladder strips were pre-incubated with an AC activator, forskolin (10 µM, Michel & Sand, 2009); a soluble GC inhibitor, ODQ (30 µM, Kaneda et al., 2018); a non-specific PDE inhibitor, IBMX (10 µM, Kaneda et al., 2018), a PDE4 inhibitor, rolipram (10 µM, Xin et al., 2014) for at least 30 minutes. A final concentration of DMSO 0.225% v/v was used as the vehicle control. Following the 30-minute-incubation period, the bladder tissues were pre-contracted with 1 µM of carbachol. Then, OCS02-CWE was applied to the tissues. The addition of drugs and extracts were performed in a non-cumulative and randomised manner, as described in **section 3.3.4.4**.

3.3.4.11 The role of intra- and extracellular Ca^{2+} in OCS02-CWE-induced bladder relaxation

The role of Ca^{2+} in OCS02-CWE-induced relaxation response was investigated. First, the bladder strips were pre-incubated with OCS02-CWE or vehicle control (purified water) in either normal or Ca^{2+}-free Krebs solution supplemented with 1 mM of EGTA for 30 minutes. Then, a single concentration of carbachol (100 µM) was added to induce contractile tone and tissues were left in the bath for 1 hour. In another set of experiments, the bladder strips with or without urothelium were immersed in Ca^{2+}-

free Krebs solution for 30 minutes. Then, the tissues were pre-incubated with either 5 mg/mL of OCS02-CWE, an L-type Ca^{2+} channel blocker, nifedipine (10 µM, Maggi et al., 1989), or vehicle control (250 µL of purified water), for another 30 minutes. Following a single application of 60 mM of KCl to induce membrane depolarisation, cumulative CRCs to $CaCl_2$ were constructed to stimulate tissue contraction.

3.3.4.12 Effect of Ca^{2+} regulators on the biphasic responses induced by OCS02-CWE

The bladder strips were pre-incubated with a series of Ca^{2+} modulators including L-type Ca^{2+} channel blocker nifedipine 100 nM and verapamil 100 nM (Somogyi et al., 1997), non-selective TPRC channel blocker and SOCC blocker, SKF-96365 (10 µM, Yang et al., 2018), non-selective TRPC inhibitor and putative L-type VGCC blocker, schwarzinicine A (30 µM, Mak et al., 2022), SERCA inhibitor, thapsigargin (1 µM, Wuest et al., 2007), ryanodine blocker, ryanodine (10 µM, Fritz et al., 2007) or IP_3 receptor antagonist, 2-APB (10 µM, Hashitani et al., 2012). A lower concentration of nifedipine and verapamil was used in this protocol to allow sufficient contractile tone to be elicited by carbachol (Somogyi et al., 1997). A final concentration of DMSO 0.15% v/v was used as the vehicle control. After an incubation period of 30 minutes, 1 µM of carbachol was used to pre-contract the tissues prior to application of 5 mg/mL of OCS02-CWE.

3.3.4.13 Effect of OCS02-CWE on resting bladder strips

Non-cumulative CRCs to OCS02-CWE were constructed in resting bladder strips from 1 mg/mL to 5 mg/mL. Between each addition of OCS02-CWE, the bath content was washed out and replaced with fresh Krebs solution. Tissues were left to return to resting tension for about 15 minutes before the application of the next concentration of OCS02-CWE. Purified water of equivalent volume was used as the vehicle control in this experiment.

3.3.4.14 Effect of muscarinic receptor antagonist and Ca^{2+} channel blockers on OCS02-CWE-induced contraction at resting bladder strips

Following tissue viability test, 4 mg/mL of OCS02-CWE was applied to the bladder strips to elicit a response. This concentration (4 mg/mL) was used as it produced the greatest contractile response in previous experiments (**section 2.3.6.2** and **section 3.4.14**). Then, the bath content was washed out, replaced with fresh Krebs solution and tissues were left to return to resting tension. After 15 minutes, the bladder tissues were pre-incubated with 1 µM of verapamil, 1 µM of nifedipine, 10 µM of SKF-96365, 30 µM of schwarzinicine A or vehicle control (DMSO 0.15% v/v). After 30 minutes, tissues were exposed to 4 mg/mL OCS02-CWE for the second time. In another set of experiments, the bladder tissues were pre-incubated with Ca^{2+}-free Krebs solution instead of Ca^{2+} channel blockers. Similar protocol was carried out in the presence of atropine 1 µM or vehicle control (purified water) to investigate if the activation of muscarinic receptors is involved in the transient contraction induced by OCS02-CWE. OCS02-CWE-induced tone during the second addition was expressed as a percentage normalised to the first OCS02-CWE-induced tone.

3.3.5 Data analysis

Data were analysed and graphs were drawn using PRISM version 9.0 (GraphPad software). All data were presented as mean ± SEM of *n* number of animals. Concentrations of drugs were presented in graph in logarithm of concentrations in molar (M) whereas concentrations of OCS02-CWE were shown in mg/mL due to tight concentration range used. In Figure 3.5A, Figure 3.5B, Figure 3.13 and Figure 3.14, E_{max} and pEC_{50} were derived from the non-linear regression curve. In Figure 3.5C, Figure 3.5D, Figure 3.17 and Figure 3.22, the E_{max} was taken from the response produced at the highest concentration tested as the response has not plateaued and pEC_{50} was not calculated. The percentage of tissue contraction and relaxation induced by OCS02-CWE was measured as described in **section 2.3.7** and were expressed either in KCl-induced tone or carbachol-induced tone, depending on the pre-contractile agents. In Figure 3.6 the spontaneous myogenic contraction induced by contractile agents (carbachol, KCl, H_2O_2 and 5-HT) were displayed and expressed in milliNewton (mN). The measurements of the amplitude were obtained as illustrated in Figure 3.1.

Statistical analysis was performed using unpaired Student's *t*-test to compare between control and treatment group. One-way ANOVA was used to compare the means of more than two groups and *post hoc* tests were used when there is an interaction between the groups. Dunnett's *post hoc* test was used to compare vehicle control with all other treatment groups whereas Sidak's *post hoc* test was used to compare all groups. Two-way ANOVA was used to compare all possible combinations within groups and *post hoc* tests were used when there is an interaction between the groups. Results with $p < 0.05$ were considered statistically significant. Individual significance was indicated in asterisk symbol in table or graph, where '****' indicates $p < 0.0001$, '***' indicates $p < 0.001$, '**' indicates $p < 0.01$ and '*' indicates

$p < 0.05$.

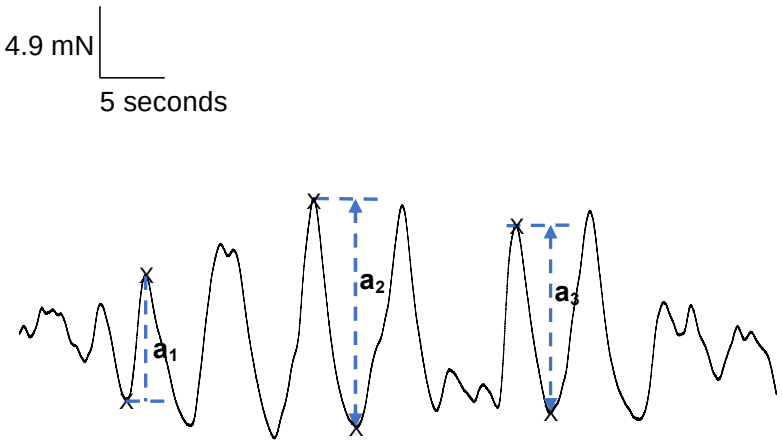

Figure 3.1 Illustration of the spontaneous myogenic contractions induced by carbachol in the bladder strips. *Contractile agents including carbachol, KCl, H_2O_2 and 5-HT induced spontaneous myogenic contractions in the bladder strips. The magnitude of the spontaneous myogenic contractions induced by each concentration of the contractile agent were measured within the last 30 seconds time frame before the next concentration. Three points (difference between crests and their respective troughs) were measured and averaged to obtain the readings for each concentration. Amplitude of the spontaneous myogenic contraction = $(a_1+a_2+a_3)/3$.*

3.4 Results

3.4.1 OCS02-CWE caused small but significant relaxation response against KCl-pre-contracted bladder strips

The contractile tones induced by 60 mM of KCl were consistent throughout the construction of the non-cumulative CRCs to OCS02-CWE from 0.5 mg/mL to 8.0 mg/mL, and vehicle control (Figure 3.2 and Table 3.2).

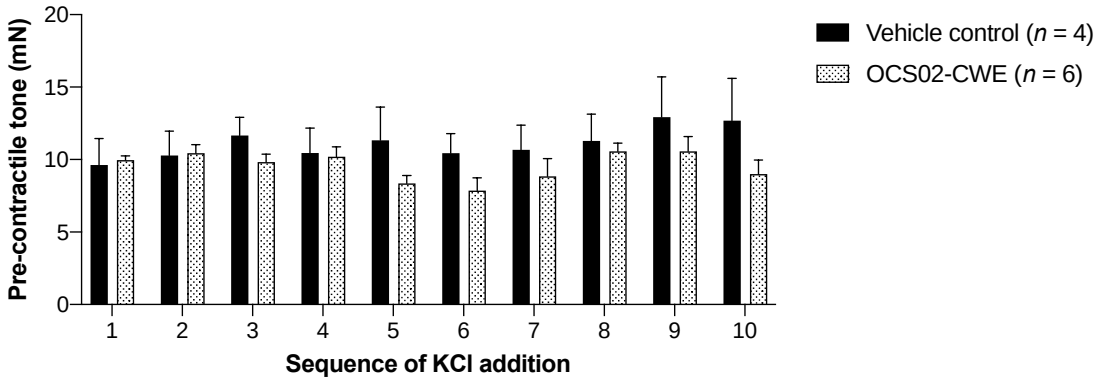

Figure 3.2 Repeated application of KCl produced consistent tonic contraction. *Data are shown as mean ± SEM of n number of animals. Two-way ANOVA where comparison of means between all the groups was made, p > 0.05. (Abbreviations: CWE = cold water extract; KCl = potassium chloride; mN = milliNewton)*

Table 3.2 Tissue contraction elicited by KCl throughout the non-cumulative addition of OCS02-CWE from 0.5 mg/mL to 8.0 mg/mL versus vehicle control.

Sequence of KCl pre-contraction prior to the addition of OCS02-CWE	**Pre-contractile tone induced by KCl (mN)**	
	Vehicle control (*n* = 4)	**OCS02-CWE (*n* = 6)**
1	9.63 ± 1.83	9.98 ± 0.37
2	10.29 ± 1.67	9.94 ± 0.40
3	11.66 ± 1.25	9.88 ± 0.67
4	10.46 ± 1.70	9.86 ± 0.70
5	11.34 ± 2.28	8.29 ± 0.66
6	10.44 ± 1.36	7.45 ± 0.94
7	10.68 ± 1.70	8.10 ± 1.15
8	11.29 ± 1.84	10.17 ± 0.45
9	12.94 ± 2.78	10.76 ± 1.23
10	12.69 ± 2.92	8.43 ± 0.97

Two-way ANOVA where comparison of means between all the groups was made, $p > 0.05$. Tissue contraction was measured from the baseline tension and expressed in mN. Data are mean ± SEM of n number of animals. (Abbreviations: CWE = cold water extract; KCl = potassium chloride; mN = milliNewton)

In KCl-pre-contracted bladder strips, OCS02-CWE produced a straightforward relaxation response without causing a transient contraction (Figure 3.3). The relaxation response produced at the highest concentration tested, 5 mg/mL, was 32.59 ± 3.51%, $p = 0.0068$ when compared to the vehicle control (14.02 ± 3.35%) (Figure 3.4A). When comparing the relaxation response elicited by OCS02-CWE in carbachol-induced contraction and KCl-induced contraction, OCS02-CWE was less effective in relaxing KCl-pre-contracted bladder strips. For the ease of visualisation,

the relaxation response induced by OCS02-CWE in carbachol-pre-contracted bladder strips in Figure 2.12 was incorporated in Figure 3.4B to compare the effects of OCS02-CWE in carbachol- and KCl-pre-contracted bladder strips.

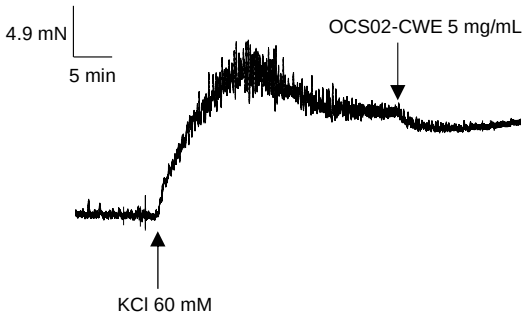

Figure 3.3 Representative trace recording of the effect of OCS02-CWE on KCl-pre-contracted bladder strips. *Non-cumulative CRCs to OCS02-CWE were constructed in bladder strips pre-contracted with 60 mM of KCl. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; KCl = potassium chloride; min = minute; mN = milliNewton)*

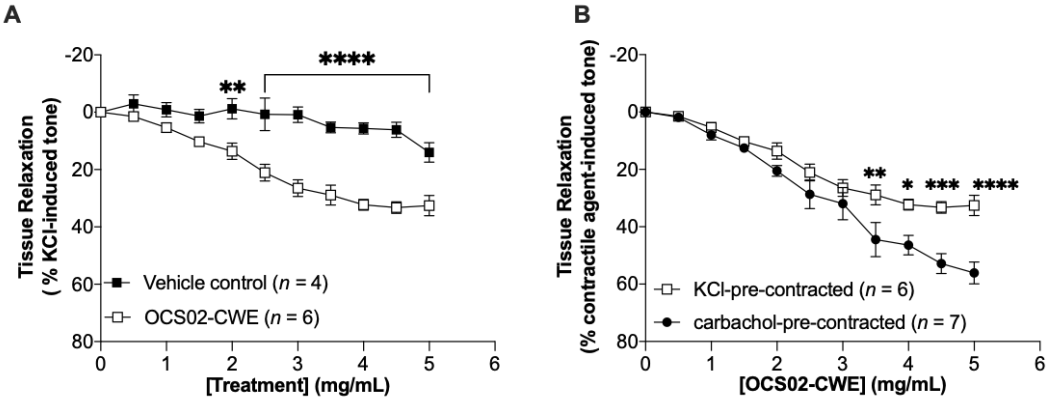

Figure 3.4 Effect of OCS02-CWE on bladder strips pre-contracted with KCl or carbachol. *(A) Non-cumulative CRCs to OCS02-CWE or vehicle control in bladder strips pre-contracted with 60 mM of KCl. (B) Non-cumulative CRCs to OCS02-CWE in bladder strips pre-contracted with KCl or carbachol. Tissue responses have been expressed as a percentage of KCl- or carbachol-induced contraction. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA with Sidak's post hoc test was made between the groups, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.(Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; KCl = potassium chloride)*

3.4.2 OCS02-CWE significantly suppressed carbachol-induced contraction, including spontaneous myogenic contractions

Pre-treatment of OCS02-CWE at 5 mg/mL has significantly suppressed the E_{max} of carbachol-induced contraction. However, the potency of carbachol (pEC_{50}) was not affected (Figure 3.5A). Compared to carbachol, 5-HT induced a relatively small magnitude of contractile tone, and its maximum contraction is only about 10% of those produced by carbachol. The E_{max} and pEC_{50} of 5-HT-induced contraction were not affected by the presence of OCS02-CWE (Figure 3.5B). Pre-treatment of OCS02-CWE has also significantly attenuated the E_{max} of KCl (Figure 3.5C) and H_2O_2 (Figure 3.5D). The pEC_{50} values of KCl and H_2O_2 were not determined because the tissue contraction has not plateaued. The E_{max} and pEC_{50} (when applicable) values of the contractile agents in the presence of vehicle control or OCS02-CWE were summarised in Table 3.3.

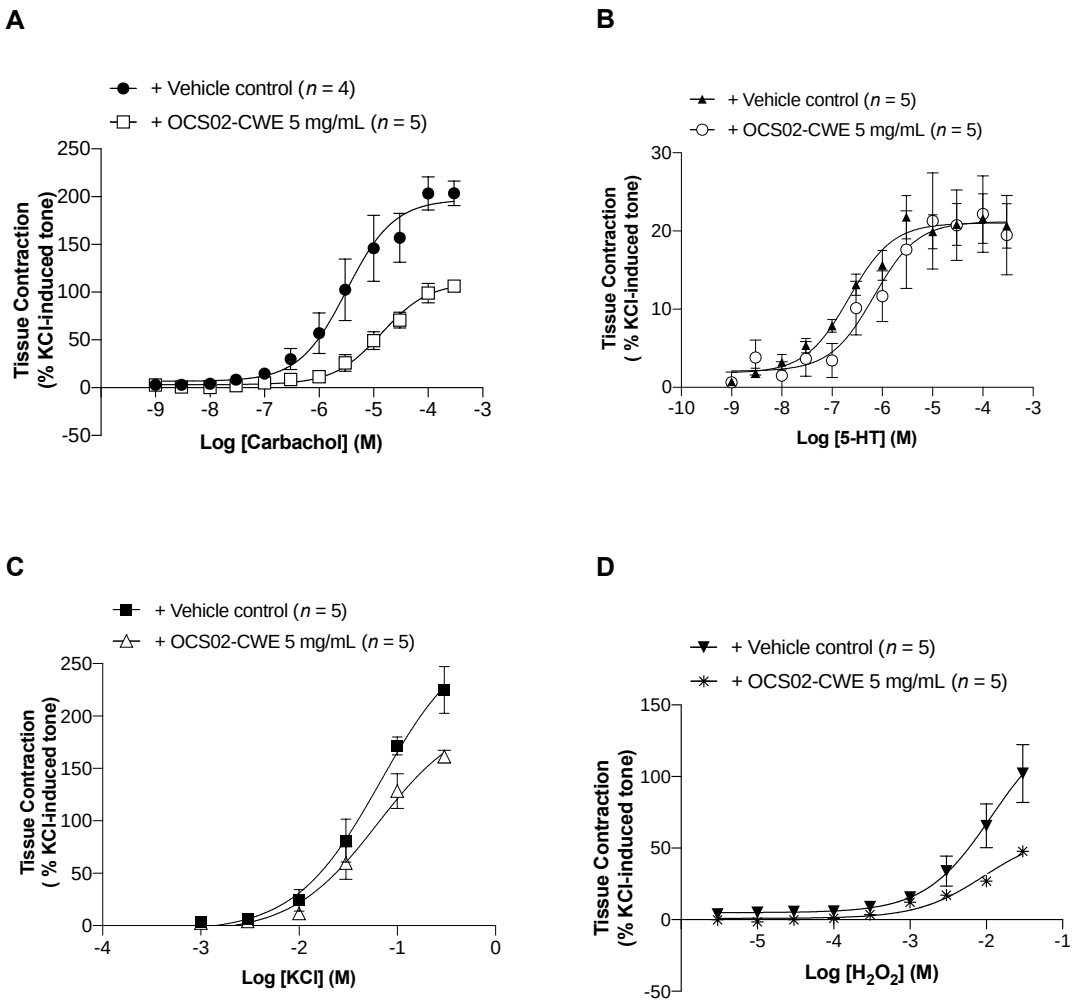

Figure 3.5 Effect of OCS02-CWE on the contractile responses induced by different contractile agents. *In the presence of 5 mg/mL of OCS02-CWE or vehicle control, CRCs to (A) carbachol; (B) 5-HT; (C) KCl; and (D) H_2O_2 were constructed. Tissue responses have been expressed as a percentage of KCl-induced contraction. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; KCl = potassium chloride)*

Table 3.3 Summary of the maximum tissue responses (E_{max}) and potency (pEC_{50}) of the contractile response of carbachol, 5-HT, KCl and H_2O_2 in the presence of OCS02-CWE or vehicle control.

Contractile agent	Pre-incubation treatment	E_{max}	pEC_{50}
Carbachol	Vehicle control	218.40 ± 14.90%	5.33 ± 0.39
	OCS02-CWE	112.10 ± 6.11% ****(p = 0.0002)	4.85 ± 0.16
5-HT	Vehicle control	21.72 ± 4.98%	6.08 ± 0.26
	OCS02-CWE	21.31 ± 2.78%	6.76 ± 0.27
KCl	Vehicle control	224.8 ± 22.27%	ND[a]
	OCS02-CWE	161.30 ± 6.04% **(p = 0.0093)	
H_2O_2	Vehicle control	102.10 ± 20.18%	ND[a]
	OCS02-CWE	47.81 ± 6.12%, *(p = 0.0330)	

Data are shown as the mean ± SEM of 4 – 5 animals. Student's t-test was used to compare OCS02-CWE to vehicle control in their respective group. Individual p-values were stated in the respective row when $p < 0.05$. (Abbreviations: 5-HT = 5-hydroxytryptamine; CWE = cold water extract; H_2O_2 = hydrogen peroxide; KCl = potassium chloride; ND = not determined). [a]pEC50 was not determined as the maximum contraction was not achieved at the highest concentration tested.

In addition, the effect of OCS02-CWE on the spontaneous myogenic contractions induced by these contractile agents were analysed. Pre-treatment of OCS02-CWE has significantly diminished the spontaneous myogenic contractions induced by carbachol but not 5-HT, KCl and H_2O_2 (Figure 3.6).

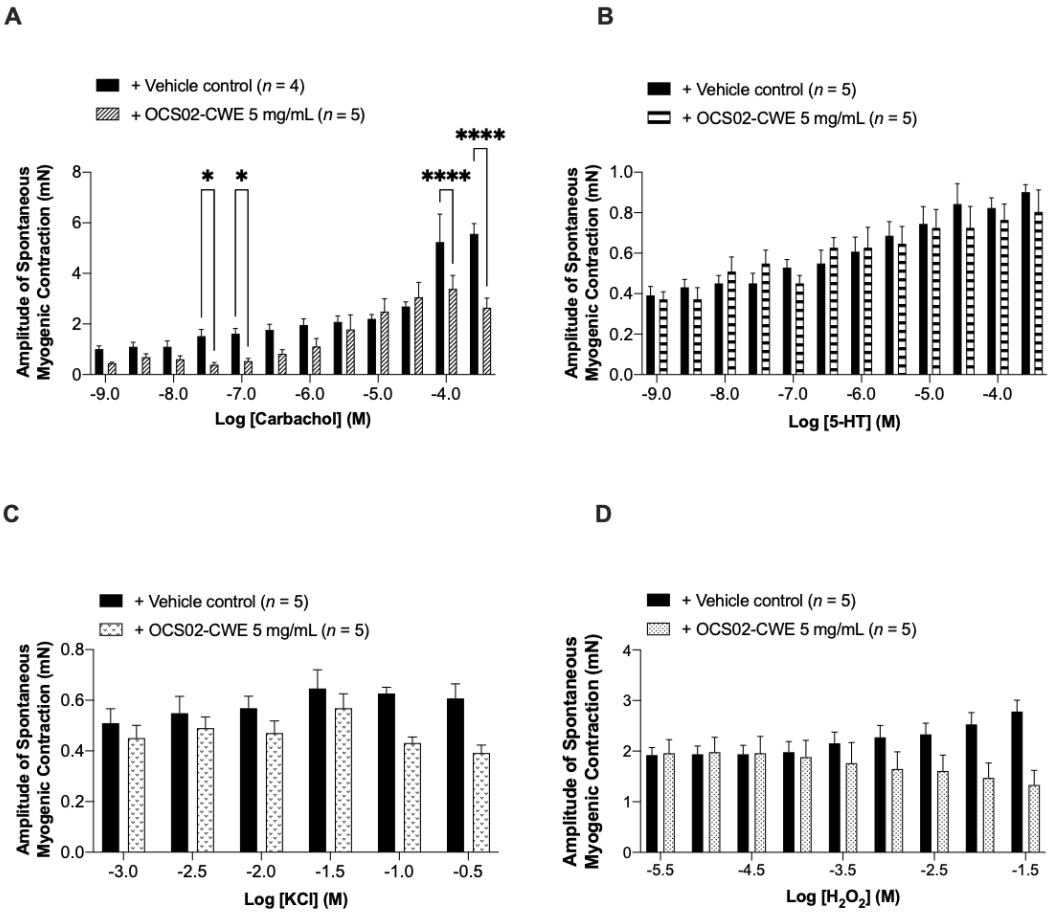

Figure 3.6 Effect of OCS02-CWE on the spontaneous myogenic contractions induced by different contractile agents. *Amplitude of spontaneous myogenic contraction induced by (A) carbachol; (B) 5-HT; (C) KCl; and (D) H_2O_2 in the presence of OCS02-CWE or vehicle control. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA where comparison of means was made between vehicle control and OCS02-CWE in the respective group, (A) $p < 0.0001$; (B) $p > 0.05$; (C) $p > 0.05$ and (D) $p > 0.05$. Sidak's post hoc test was performed in (A) where *p < 0.05, ****p < 0.0001. (Abbreviations: 5-HT = 5-hyroxytryptamine; CWE = cold water extract; H_2O_2 = hydrogen peroxide; KCl = potassium chloride; mN = milliNewton)*

3.4.3 OCS02-CWE-induced relaxation was not affected by muscarinic receptor antagonist

Atropine was used to investigate the participation of muscarinic receptors in OCS02-CWE-induced bladder relaxation. The relaxation response induced by 5 mg/mL of OCS02-CWE in KCl-pre-contracted bladder strips was not affected by atropine (Tissue relaxation = 22.46 ± 3.07%; vehicle control, 20.32 ± 4.58%, $p > 0.05$) (Figure 3.7).

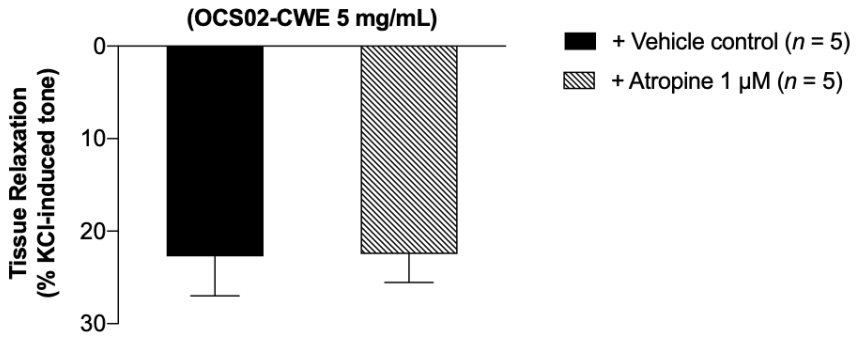

Figure 3.7 Effect of muscarinic receptor antagonist, atropine, on OCS02-CWE-induced relaxation response. *Tissue responses have been expressed as a percentage of KCl-induced tone. Data are shown as mean ± SEM of n number animals. Student's t-test was performed to compare the difference between treatment group and vehicle control, p > 0.05. (Abbreviations: CWE = cold water extract; KCl = potassium chloride).*

3.4.4 OCS02-CWE-induced responses were not affected by β-adrenoceptors antagonist and non-selective K^+ channels blocker

The roles of β-adrenoceptors and K^+ channels were investigated by pre-incubating the bladder strips with propranolol and TEA, respectively. The presence of propranolol did not alter the biphasic responses induced by OCS02-CWE, but its transient contraction was marginally potentiated (not statistically significant, $p > 0.05$) in the presence of TEA (Figure 3.8). The maximum contractile response induced by OCS02-CWE was achieved at 4 mg/mL whereas the maximum relaxation response was achieved at 5 mg/mL in all treatment groups. The values of the maximum responses were summarised in Table 3.4.

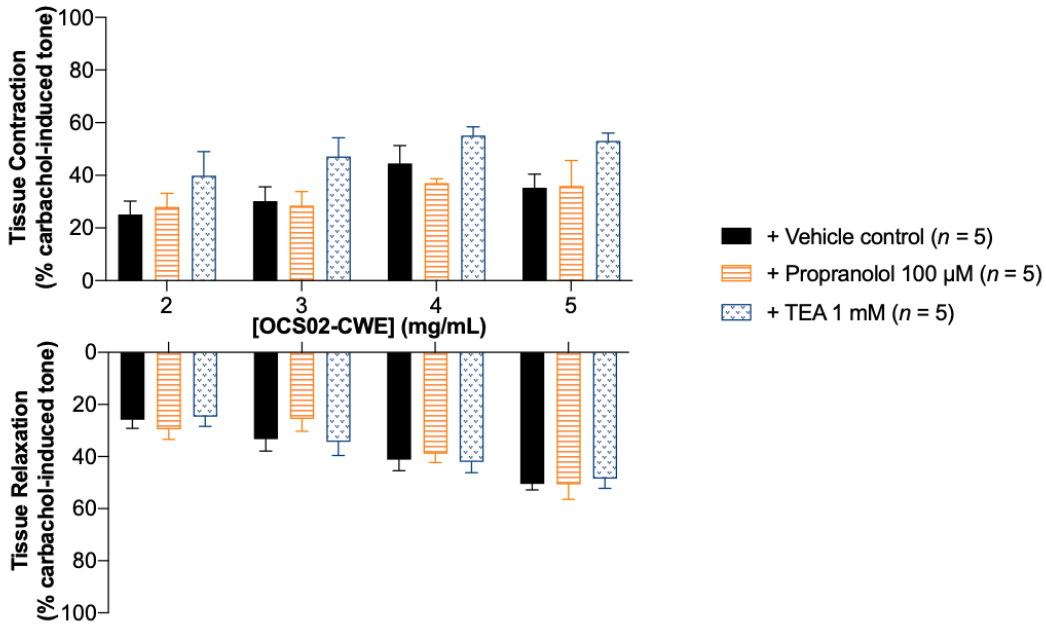

Figure 3.8 Effect β-adrenoceptor antagonist (propranolol) and non-selective K^+ channel blocker (TEA) on OCS02-CWE-induced responses. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA where comparison between vehicle control and each treatment group was made separately for the contractile response and relaxation response induced by OCS02-CWE, $p > 0.05$. (Abbreviations: CWE = cold water extract; TEA = tetraethylammonium)*

Table 3.4 The maximum tissue contraction and relaxation response induced by OCS02-CWE in the presence of propranolol and TEA.

Pre-incubation compounds	Maximum contraction (%)	Maximum relaxation (%)
Vehicle control (purified water)	44.57 ± 6.73	50.52 ± 2.30
Propranolol 100 µM	37.11 ± 1.60	50.66 ± 5.77
TEA 1 mM	53.07 ± 2.96	48.45 ± 3.76

Data are shown as mean ± SEM. One-way ANOVA where comparison of the means was made between vehicle control and the treatment groups, $p > 0.05$. (Abbreviations: CWE = cold water extract; TEA = tetraethylammonium).

3.4.5 Removal of urothelium attenuated OSC02-CWE-induced relaxation response

To confirm the complete removal of urothelium via gentle swabbing, histological investigation was performed. Figure 3.9 shows that the urothelial layer was successfully removed without damaging the suburothelium.

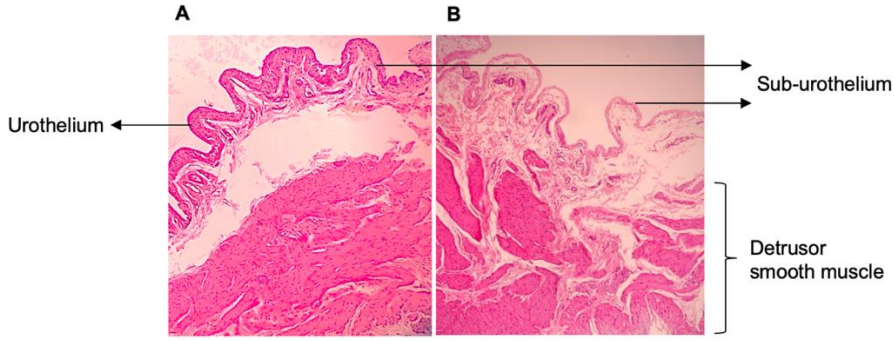

Figure 3.9 Representative slide of the histology of rat bladder section. *(A) Urothelium-intact bladder strip and (B) urothelium-denuded bladder strip. The urothelium was removed by three gentle swabs in a single direction using cotton buds (n = 3 for both urothelium-intact and urothelium-denuded bladder strips)*

Non-cumulative CRCs to OCS02-CWE were constructed in the urothelium-denuded bladder strips and the results were compared with those obtained from the urothelium-intact strips (**section 2.4.4**). The pre-contractile tone induced by 1 µM of carbachol in the urothelium-denuded bladder strips prior to each addition of OCS02-CWE was 7.63 ± 0.29 mN (76.38 ± 2.35% of KCl-induced tone). Similarly, OCS02-CWE elicited a biphasic response in urothelium-denuded bladder strips. The transient contraction was only noticeable at concentrations > 2 mg/mL and peaked at 4 mg/mL in the urothelium-denuded bladder strips. Although the removal of urothelium did not statistically change the magnitude of the transient contraction induced by OCS02-CWE (maximum tissue contraction = urothelium-intact, 40.06 ± 8.95%; urothelium-denuded, 23.81 ± 3.08%, $p > 0.05$), there was a consistent trend of a lower response across the concentration range (Figure 3.10). On the contrary, the relaxation response

induced by OCS02-CWE was significantly attenuated in urothelium-denuded bladder strips (maximum tissue relaxation = urothelium-intact, 56.12 ± 3.78%; urothelium-denuded, 30.56 ± 6.39%, $p = 0.0043$) (Figure 3.10).

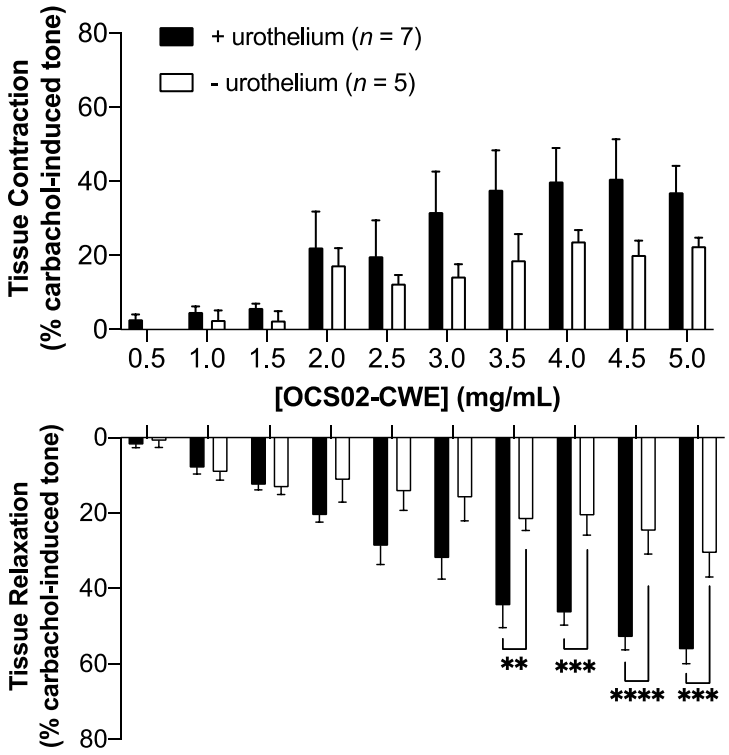

Figure 3.10 Effect of OCS02-CWE on urothelium-intact and urothelium-denuded bladder strips precontracted with carbachol. OCS02-CWE was added non-cumulatively to carbachol-pre-contracted bladder strips with or without urothelium. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA followed by Sidak's post hoc test where comparison was made between urothelium-intact and urothelium-denuded group, separately for the contractile response ($p > 0.05$) and relaxation response ($p < 0.05$), **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (Abbreviations: CWE = cold water extract).*

3.4.6 ATP produced a biphasic response in carbachol-pre-contracted bladder strips

In bladder strips pre-contracted with carbachol, 2 mM of ATP produced a biphasic relaxation response that consists of a transient contraction followed by a sustained relaxation response (Figure 3.11). This effect is comparable to the responses produced by OCS02-CWE. Therefore, subsequent experiment investigated whether OCS02-CWE and ATP share a similar mechanism of action.

The contractile response induced by ATP was significantly attenuated in the presence of the non-selective P2 receptor antagonists (PPADS and suramin) (+vehicle control, 64.78 ± 14.43 %; +PPADS, 3.27 ± 1.10 %, p = 0.0009; +suramin, 23.97 ± 6.22 %, p = 0.0148) (Figure 3.12). However, the reduction in relaxation response induced by ATP in the presence of PPADS or suramin was not statistically significant (+vehicle control, 38.18 ± 9.42%; +PPADS, 28.29 ± 8.73%, $p > 0.05$; +suramin, 28.81 ± 7.37%, $p > 0.05$) (Figure 3.12).

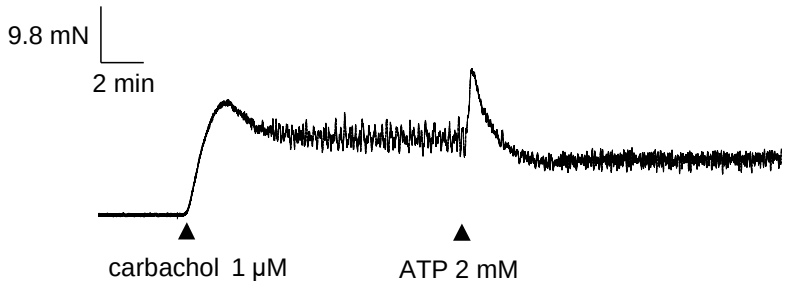

Figure 3.11 ATP elicited a transient contraction and a sustained relaxation response in carbachol-pre-contracted bladder strips. *Once a stable contractile tone was achieved by 1 µM of carbachol, 2 mM ATP was added into the bath and left until the response has plateaued.*

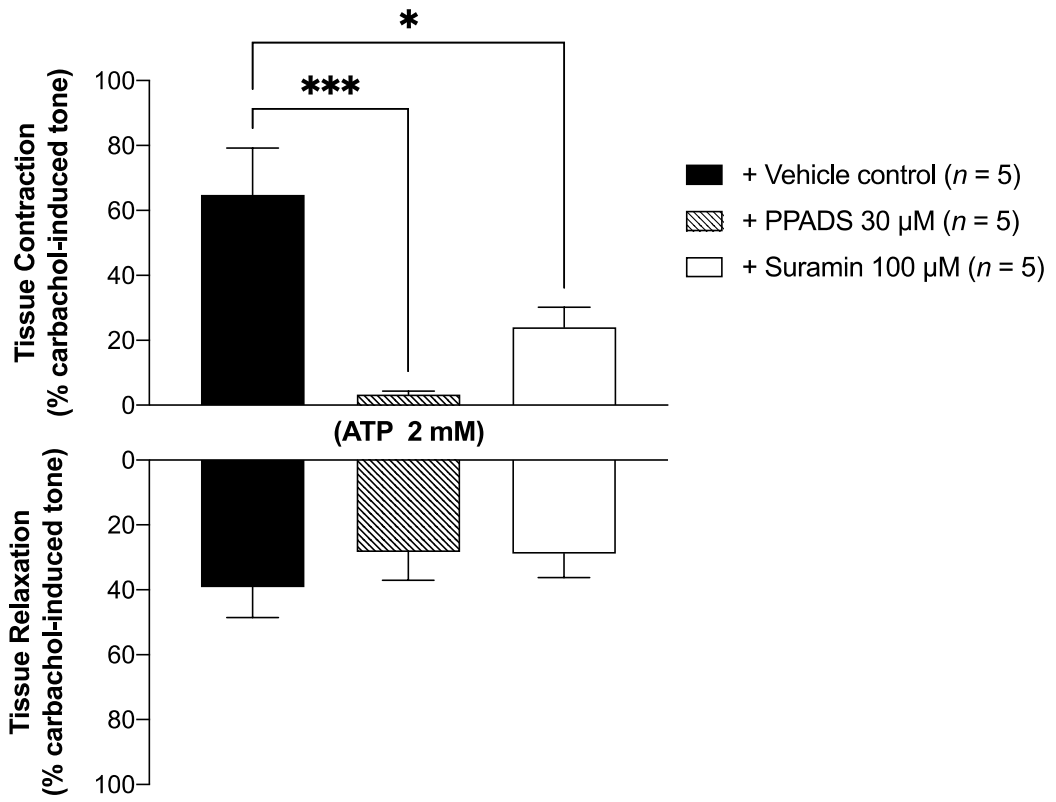

Figure 3.12 Effect of ATP on carbachol-pre-contracted bladder strips in the presence of non-selective purinergic antagonists. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. One-way ANOVA with Dunnett's post hoc test where comparison between vehicle control and each treatment group was made separately for the contractile and relaxation responses (ATP-induced contraction, *p < 0.05, ***p < 0.001; ATP-induced relaxation, p > 0.05). (Abbreviations: ATP = adenosine triphosphate; PPADS = pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt)*

3.4.7 Adenosine and cordycepin elicited significant bladder relaxation response

Next, the effects of adenosine and cordycepin were investigated. At basal tone, adenosine significantly relaxed the bladder strips compared to the vehicle control (E_{max} = 29.36 ± 7.28%; vehicle control, -0.89 ± 1.79%, p = 0.0037) (Figure 3.13A). In pre-contracted bladder strips, adenosine produced a more pronounced relaxation response (E_{max} = 80.52 ± 9.03 %; vehicle control, 22.49 ± 3.31%, p = 0.0040) (Figure 3.13B). Then, pre-incubation of the tissues with 8-PT significantly attenuated the maximum relaxation response induced by adenosine at carbachol-pre-contracted tone (+vehicle control, 61.44 ± 5.10%; +8-PT, 37.45 ± 7.86%, p = 0.047) but its effect at basal tone was not significantly altered (+vehicle control, 14.53 ± 1.79%; +8-PT, 4.29 ± 2.42%; p > 0.05) (Figure 3.13C, D).

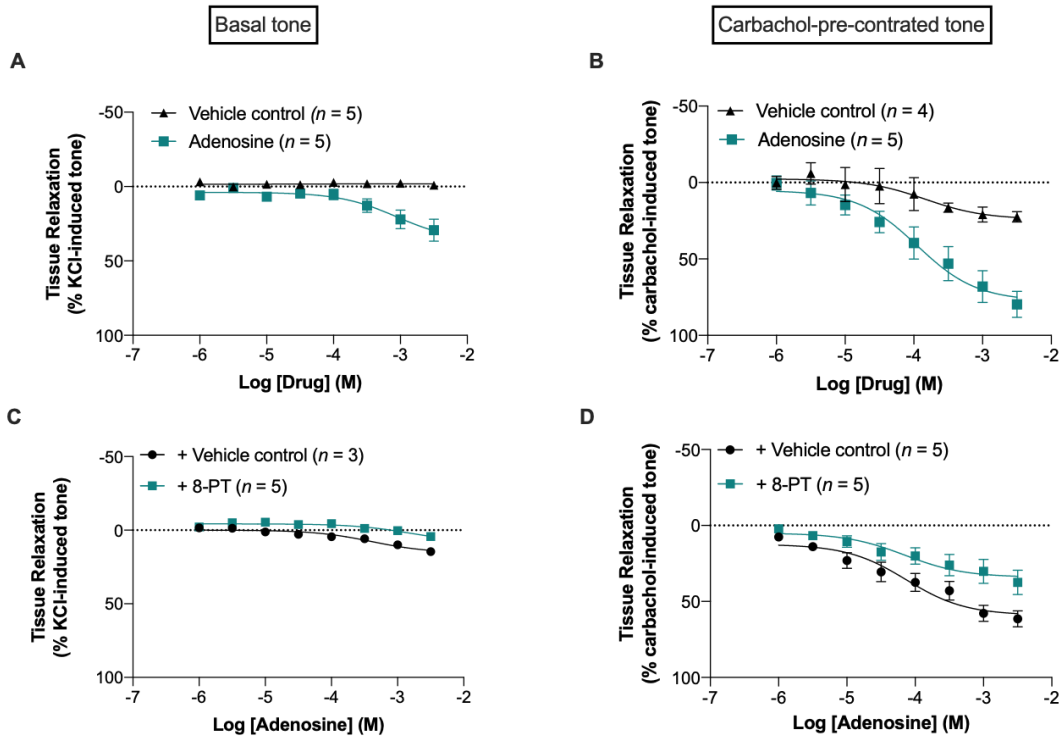

Figure 3.13 Effect of adenosine on the bladder. *CRCs to adenosine or vehicle control (A) at basal tone; (B) in carbachol-pre-contracted tone; CRCs to adenosine (C) in the presence of 8-PT at basal tone and (D) in the presence of 8-PT in carbachol-pre-contracted tone. Tissue responses have been expressed as the percentage of carbachol-induced tone or KCl-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: 8-PT = 8-phenyltheophylline; KCl = potassium chloride).*

Cordycepin did not elicit any response at basal tone ($p > 0.05$) but significantly relaxed the bladder strips pre-contracted with carbachol (E_{max} = 48.20 ± 2.69%, p = 0.0001) when compared to the vehicle control (E_{max} = 15.93 ± 3.72%) (Figure 3.14A, B). In the presence of 8-PT, its effect in basal tone was unaltered +vehicle control, 12.64 ± 4.66%; (+8-PT, 10.30 ± 3.26 %, $p > 0.05$) (Figure 3.14C) whereas in carbachol-pre-contracted bladder strips, its maximum relaxation response was significantly attenuated (+vehicle control, 45.65 ± 3.37%; +8-PT, 22.17 ± 5.21%, p = 0.0189) (Figure 3.14D).

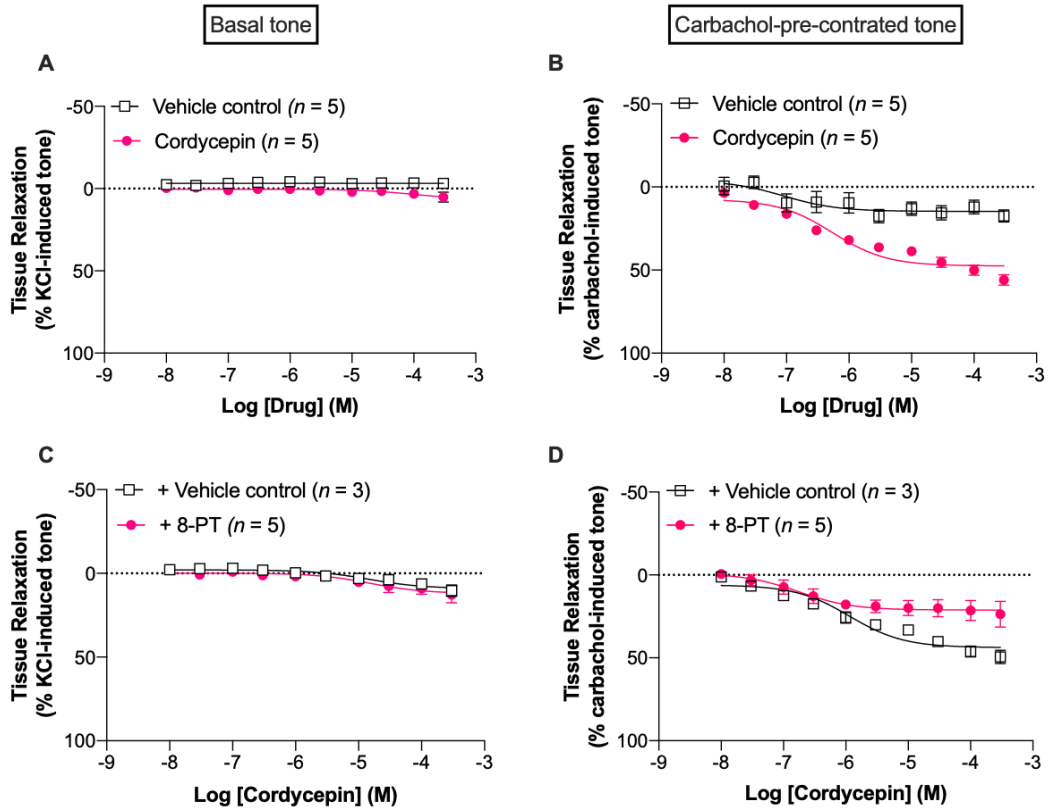

Figure 3.14 Effect of cordycepin on bladder. *CRCs to cordycepin or vehicle control (A) at basal tone; (B) in carbachol-pre-contracted tone; CRCs to cordycepin (C) in the presence of 8-PT at basal tone; and (D) in the presence of 8-PT in carbachol-pre-contracted tone. Tissue responses have been expressed as the percentage of carbachol-induced tone or KCl-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: 8-PT = 8-phenyltheophylline; KCl = potassium chloride)*

3.4.8 OCS02-CWE-induced responses were not altered by purinergic receptor antagonists

The presence of suramin or PPADS did not affect the transient contraction induced by OCS02-CWE (+vehicle control, 28.68 ± 5.44%; +suramin, 30.50 ± 12.51%, p = 0.4252; +PPADS, 45.66 ± 3.64%, $p > 0.05$) as well as its relaxation response (+vehicle control, 62.69 ± 6.52%; +suramin, 61.55 ± 5.50%, p = 0.9902; +PPADS, 48.30 ± 7.56%, $p > 0.05$) (Figure 3.15A). In the reverse experiment, OCS02-CWE did not affect ATP-induced transient contraction (+vehicle control, 37.04 ± 12.75%; +OCS02-CWE, 45.94 ± 7.62%, $p > 0.05$) and relaxation response (+vehicle control, 52.08 ± 5.30%; + OCS02-CWE, 55.44 ± 5.09%, $p > 0.05$) (Figure 3.15B). Inhibition of adenosine receptors by 8-PT also did not affect the contractile response (+8-PT, 53.29 ± 4.03%; +vehicle control, 50.93 ± 2.78%, $p > 0.05$) and relaxation response (+8-PT, 47.49 ± 3.90%; +vehicle control, 48.62 ± 2.14%, $p > 0.05$) elicited by OSC02-CWE (Figure 3.16).

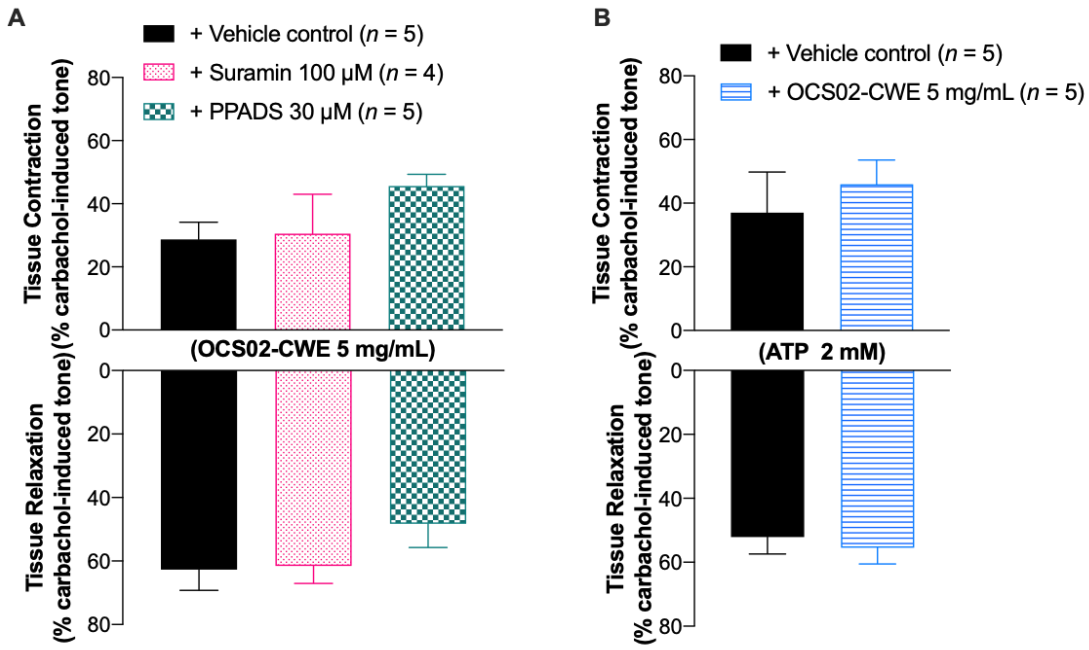

Figure 3.15 Role of P2 purinergic receptors on OCS02-CWE-induced responses. *(A) Effect of PPADS or suramin on OCS02-CWE-induced biphasic response. (B) Effect of OCS02-CWE on ATP-induced biphasic response. Tissue responses have been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. For A, one-way ANOVA where comparison between vehicle control and each treatment group was made separately in OCS02-CWE-induced contraction and relaxation response ($p > 0.05$). For B, Student's t-test was used to compare the difference between vehicle control and OCS02-CWE in the contraction and relaxation response of ATP ($p > 0.05$). (Abbreviations: 8-PT = 8-phenyltheophylline; ATP = adenosine triphosphate; CWE = cold water extract; PPADS = pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt)*

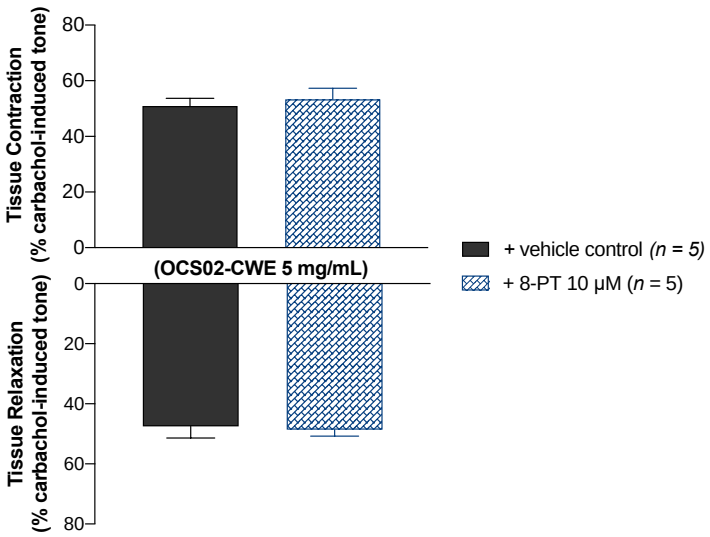

Figure 3.16 Effect of adenosine receptor antagonist, 8-PT, on OCS02-CWE-induced responses. *Tissue responses have been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. Student's t-test where comparison between vehicle control and 8-PT was made separately for the contractile and relaxation response ($p > 0.05$). (Abbreviations: 8-PT = 8-phenyltheophylline, CWE = cold water extract).*

3.4.9 OCS02-CWE-induced bladder relaxation was not mediated through the H_2S-mediated pathway

To investigate whether the relaxing pathway of H_2S was involved in OCS02-CWE-induced responses, cumulative CRCs to NaHS were constructed either in the presence of 5 mg/mL of OCS02-CWE or vehicle control. Pre-treatment of OCS02-CWE at 5 mg/mL did not affect the relaxation response elicited by NaHS (E_{max}: + vehicle control, 73.04 ± 3.83 %; + OCS02-CWE, 83.32 ± 9.92 %; $p > 0.05$) (Figure 3.17).

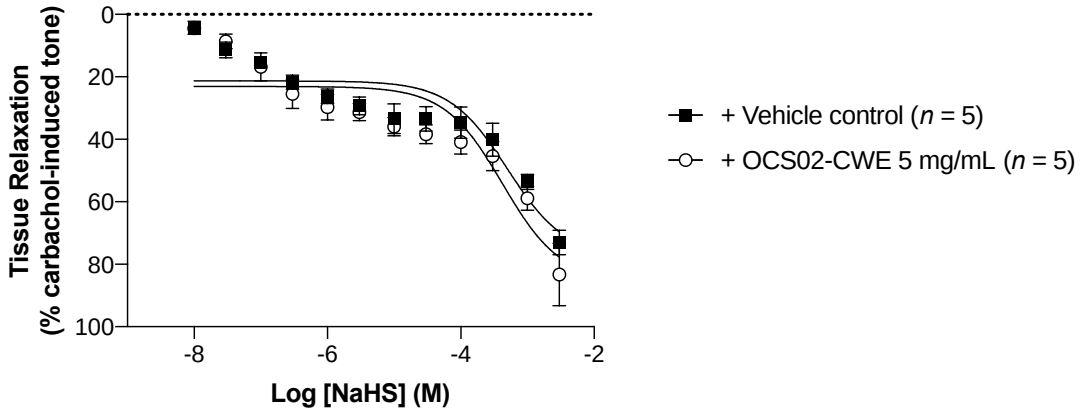

Figure 3.17 Effect of OCS02-CWE on H_2S-induced bladder relaxation. *Tissue responses have been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract, NaHS = sodium hydrosulfide)*

3.4.10 OCS02-CWE-induced relaxation appears to be mediated via the regulation of NO

Pre-treatment of L-NAME and SNP altered OCS02-CWE-induced immediate transient contraction and relaxation response. The contractile response elicited by OCS02-CWE was significantly potentiated in the presence of SNP but not L-NAME. However, there was a clear trend observed across the concentration range tested (Figure 3.18). On the other hand, the relaxation response induced by OCS02-CWE was significantly attenuated in the presence of L-NAME and SNP. The values of the maximum tissue contraction and relaxation were summarised in Table 3.5.

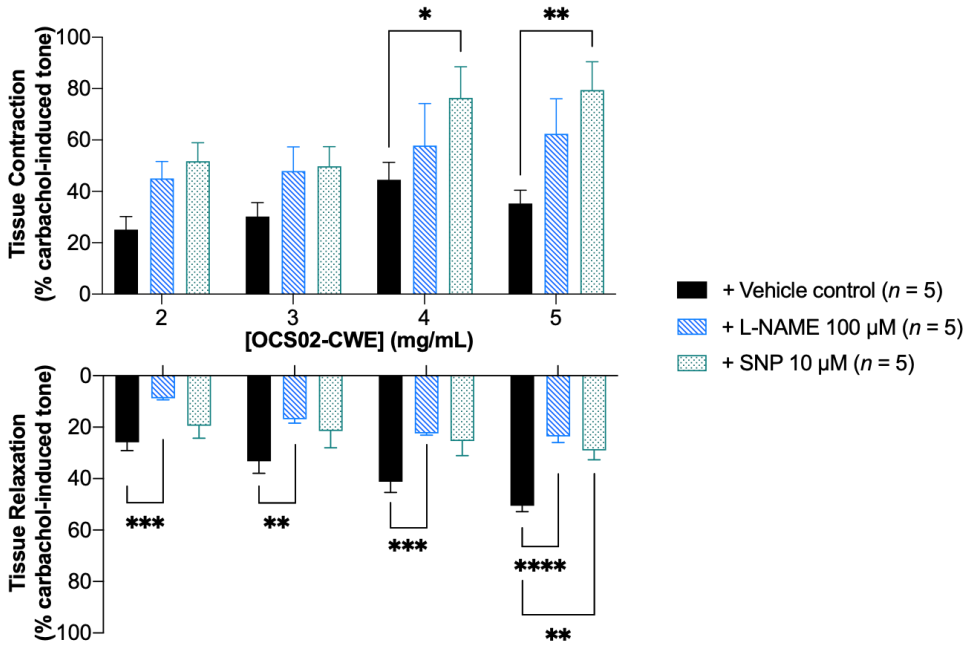

Figure 3.18 Effect of NOS inhibitor (L-NAME) and NO donor (SNP) on OCS02-CWE-induced responses*. Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA followed by Sidak's post hoc test where comparison between vehicle control and each treatment group was made separately for the contractile response and relaxation response induced by OCS02-CWE, *p < 0.05, ** p < 0.01, ***p < 0.001, **** p < 0.0001. (Abbreviations: CWE = cold water extract; L-NAME = N_G-nitro-L-arginine methyl ester; NOS = nitric oxide synthase; SNP = sodium nitroprusside)*

Table 3.5 The maximum tissue contraction and relaxation induced by OCS02-CWE in the presence of NOS inhibitor (L-NAME) and NO donor (SNP).

Pre-incubation compounds	**Maximum contraction (%)**	**Maximum relaxation (%)**
Vehicle control (H_2O)	44.57 ± 6.73	50.52 ± 2.30
L-NAME 100 µM	62.43 ± 13.63	23.58 ± 2.37 **(p = 0.0033)
SNP 10 µM	79.43 ± 11.04 *(p = 0.0244)	29.05 ± 3.60 *(p = 0.0360)

Data are shown as mean ± SEM of 5 animals. One-way ANOVA where comparison of the means was made between vehicle control and the treatment groups. Individual p-values were stated in the respective rows when $p < 0.05$. (Abbreviations: CWE = cold water extract; L-NAME = N_G-nitro-L-arginine methyl ester; NOS = nitric oxide synthase; SNP = sodium nitroprusside)

3.4.11 OCS02-CWE-induced responses were not affected by PDE inhibitors, cAMP activator and sGC inhibitor

To study the role of cyclic nucleotides (cAMP and cGMP) in OCS02-induced responses, forskolin, rolipram, IBMX and ODQ were used. Rolipram and ODQ did not significantly alter the contractile and relaxation responses induced by OCS02-CWE ($p > 0.05$) (Figure 3.19A). IBMX slightly enhanced OCS02-CWE-induced transient contraction but did not affect its relaxation response, when compared to the vehicle control ($p > 0.05$) (Figure 3.19A). The presence of forskolin appeared to enhance the maximum relaxation response elicited by OCS02-CWE although the change was not statistically significant ($p > 0.05$) (Figure 3.19B). The values of maximum contractile and relaxation responses induced by OCS02-CWE in the presence of each pre-incubation compound were summarised in Table 3.6.

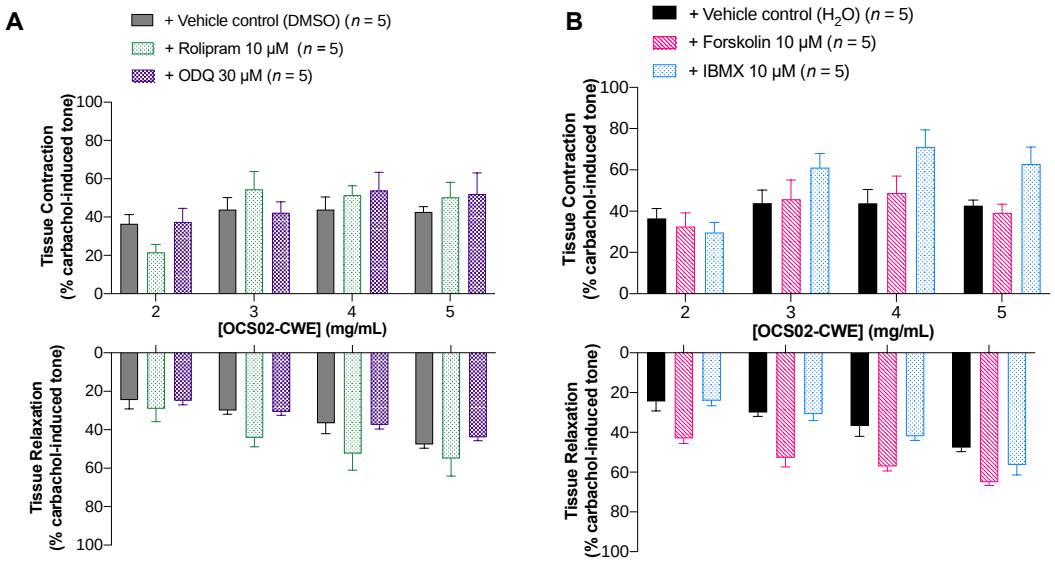

Figure 3.19 Effect of PDE inhibitors, cAMP activator and sGC inhibitor on OCS02-CWE-induced responses. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA where comparison between vehicle control and each treatment group was made separately for the contractile response and relaxation response induced by OCS02-CWE, $p > 0.05$. (Abbreviations: CWE = cold water extract; IBMX = 3-isobutyl-1-methylxanthine; ODQ =1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one).*

Table 3.6 The maximum tissue contraction and relaxation induced by OCS02-CWE in the presence of forskolin, ODQ, IBMX or rolipram.

Pre-incubation compounds	Maximum contraction (%)	Maximum relaxation (%)
Vehicle control (DMSO)	43.91 ± 6.26	47.65 ± 1.95
ODQ 30 µM	53.92 ± 9.49	43.89 ± 1.83
Rolipram 10 µM	51.48 ± 4.91	54.99 ± 9.14
Vehicle control (H_2O)	44.57 ± 6.73	50.52 ± 2.30
Forskolin 10 µM	48.73 ± 8.30	65.07 ± 1.63
IBMX 10 µM	71.11 ± 8.26	56.30 ± 5.10

One-way ANOVA followed by Dunnett's post hoc test was used to compare between ODQ, rolipram with vehicle control (DMSO), $p > 0.05$ or forskolin, IBMX with vehicle control (H_2O), $p > 0.05$. Data are shown as mean ± SEM of 5 animals.

3.4.12 OCS02-CWE appears to have a role in both extracellular Ca^{2+} influx and intracellular Ca^{2+} regulation

In the presence of external Ca^{2+}, carbachol elicited a large phasic contraction followed by a tonic contraction. Pre-incubation with OCS02-CWE significantly reduced the magnitude of phasic contraction induced by carbachol but did not affect its tonic contraction. Then, in the absence of external Ca^{2+}, the phasic contraction induced by carbachol was abolished but the tonic contraction increased gradually over time. When the bladder strips were pre-incubated with OCS02-CWE in Ca^{2+}-free Krebs solution, the initial contraction induced by carbachol was potentiated. Figure 3.20 shows the representative trace recordings of carbachol-induced contraction in the respective conditions. While the change in tissue contraction was not statistically significant, the potentiation effect of OCS02-CWE on carbachol-induced contraction in Ca^{2+}-free condition can be observed from the isometric tension recording traces during the experiments (Figure 3.20). OCS02-CWE did not affect the tonic contraction induced by carbachol over time in Ca^{2+}-free condition (Figure 3.21). The values of tissue contraction induced by carbachol over time were summarised in Table 3.7.

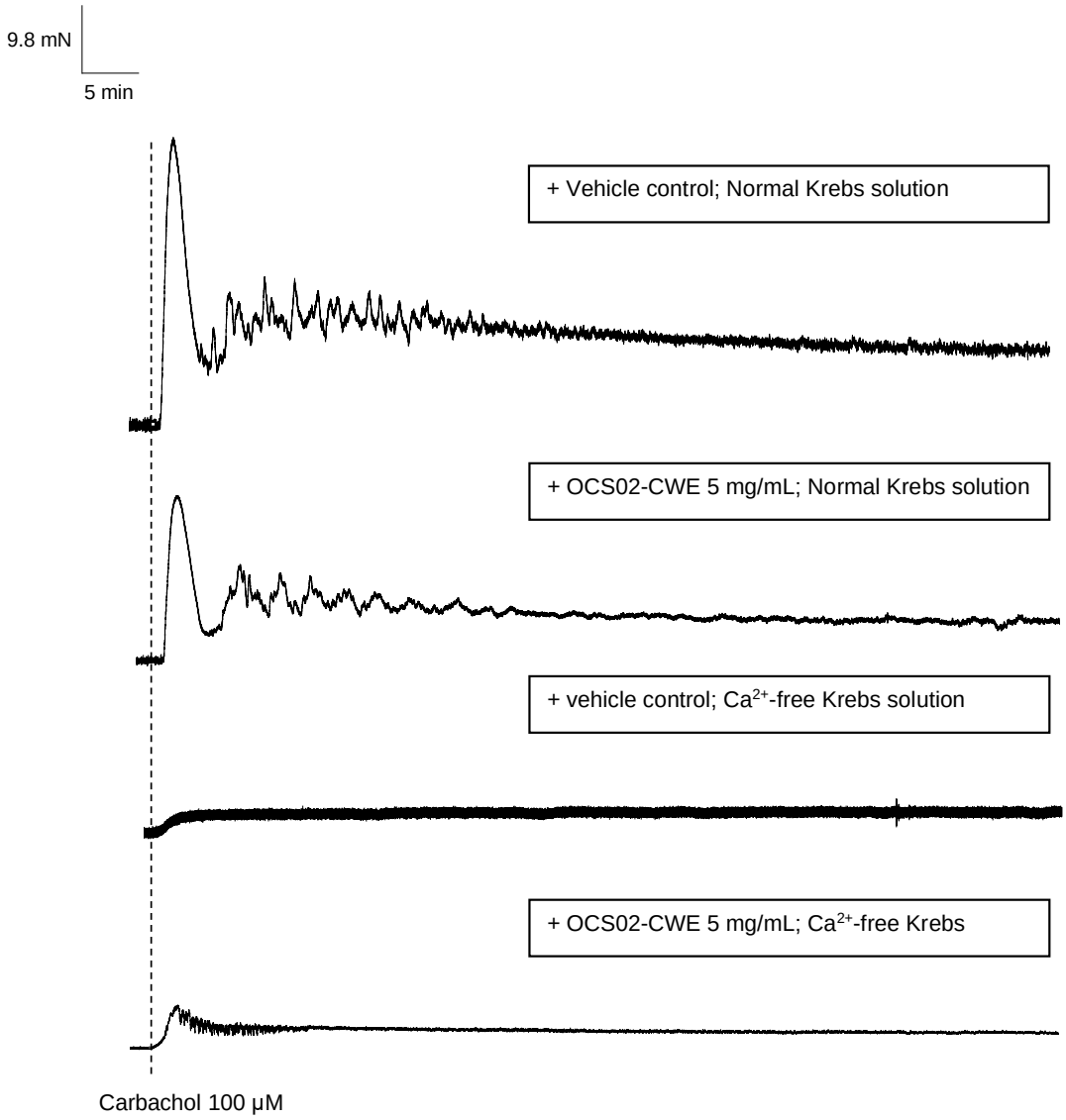

Figure 3.20 Representative trace recordings of carbachol-induced contraction in normal and Ca^{2+}-free Krebs solution, with or without OCS02-CWE.

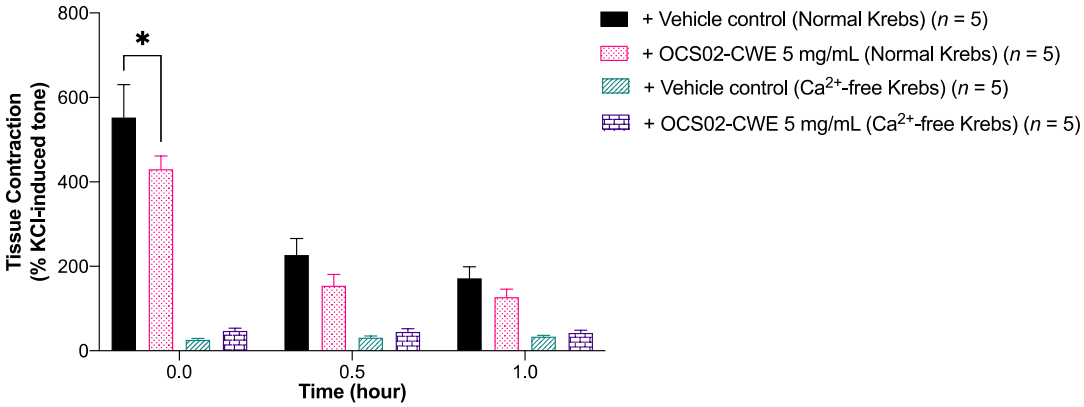

Figure 3.21 Effect of OCS02-CWE on carbachol-induced contraction in normal and Ca^{2+}-free Krebs solution. *Tissue responses were recorded at 0, 0.5 and 1.0 hour after the addition of carbachol 100 µM and were expressed as the percentage of KCl-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA followed by Tukey's post hoc test was performed for differences between the groups, *p < 0.05. (Abbreviations: CWE = cold water extract, KCl = potassium chloride)*

Table 3.7 Carbachol-induced contraction in normal or Ca^{2+}-free Krebs solution with or without the presence of OCS02-CWE.

Treatment	Time (hour)	Tissue contraction (%)	
		Vehicle control	OCS02-CWE 5 mg/mL
Normal Krebs	0	552.50 ± 77.86	429.80 ± 31.74 *(p = 0.0262)
	0.5	226.9 ± 38.99	154.2 ± 26.33
	1.0	171.50 ± 27.78	127.00 ± 18.92
Ca^{2+}-free Krebs	0	25.81 ± 3.52	47.26 ± 6.39
	0.5	31.33 ± 3.86	44.96 ± 7.33
	1.0	33.55 ± 3.10	42.51 ± 6.31

Data are shown as mean ± SEM of 5 animals. Two-way ANOVA with Tukey's post hoc test was performed to compare between vehicle control and OCS02-CWE in either normal or free Krebs separately at each time point. Individual p-values were stated in the respective columns when p < 0.05. (Abbreviations: CWE = cold water extract, KCl = potassium chloride)

To substantiate the above findings, effects of OCS02-CWE on Ca^{2+}-induced contractions were examined. Removal of urothelium did not affect the contractile response induced by Ca^{2+}. In contrast, pre-incubation with 5 mg/mL of OCS02-CWE significantly suppressed the Ca^{2+}-induced contraction as compared to the vehicle control (Figure 3.22). The Ca^{2+} inhibitory effect of OCS02-CWE was comparable to the L-type VGCC blocker, nifedipine. Moreover, the suppression effect of OCS02-CWE on Ca^{2+}-induced contraction was reduced in urothelium-denuded bladder strips, but the difference was not statistically significant ($p > 0.05$). The values of maximum tissue contraction induced by $CaCl_2$ in each treatment group were summarised in Table 3.8.

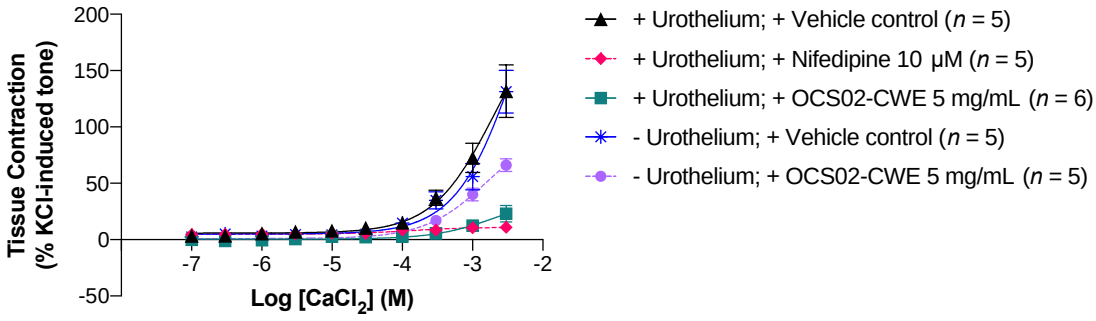

Figure 3.22 Effects of OCS02-CWE on $CaCl_2$-inudced contractions in Ca^{2+}-free Krebs solution. *Tissue contraction has been expressed as the percentage of 60 mM KCl-induced contraction. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CWE = cold water extract; KCl = potassium chloride).*

Table 3.8 The maximum tissue contraction induced by $CaCl_2$ in each treatment group.

Treatment	Maximum contraction (%)	
	Urothelium-intact	**Urothelium-denuded**
Vehicle control	131.70 ± 23.37	131.30 ± 19.10
OCS02-CWE 5 mg/mL	22.93 ± 7.32 ***(p = 0.0002)	66.11 ± 5.60 *(0.0423)
Nifedipine 10 µM	10.86 ± 2.48 ***(p = 0.0002)	ND

Data are shown as mean ± SEM of 5 animals. One-way ANOVA followed by Sidak's post host test was made to compare the means between each treatment group. Individual p-values between vehicle control and the treatment group were stated in the respective column. (Abbreviations: CWE = cold water extract; ND = not determined).

3.4.13 OCS02-CWE-induced responses in carbachol-pre-contracted bladder strips were altered by Ca^{2+} blockers

The effects of Ca^{2+} blockers on the biphasic response of OCS02-CWE were investigated. For the ease of visualisation, the results were categorised in different panels according to the mechanism of action of the pre-incubation compounds. This arrangement does not affect the statistical analysis as the comparison was made altogether against the vehicle control. In the presence of nifedipine, verapamil, SKF-96365 and schwarzinicine A, OCS02-CWE-induced transient contraction was significantly attenuated but its relaxation response was not affected (Figure 3.23A and Figure 3.23B). Ryanodine and 2-APB did not affect OCS02-CWE-induced responses (Figure 3.23C). On the contrary, thapsigargin significantly reduced OCS02-CWE-induced relaxation response but did not alter its contractile response (Figure 3.23D). The values of tissue contraction and relaxation responses were summarised in Table 3.9.

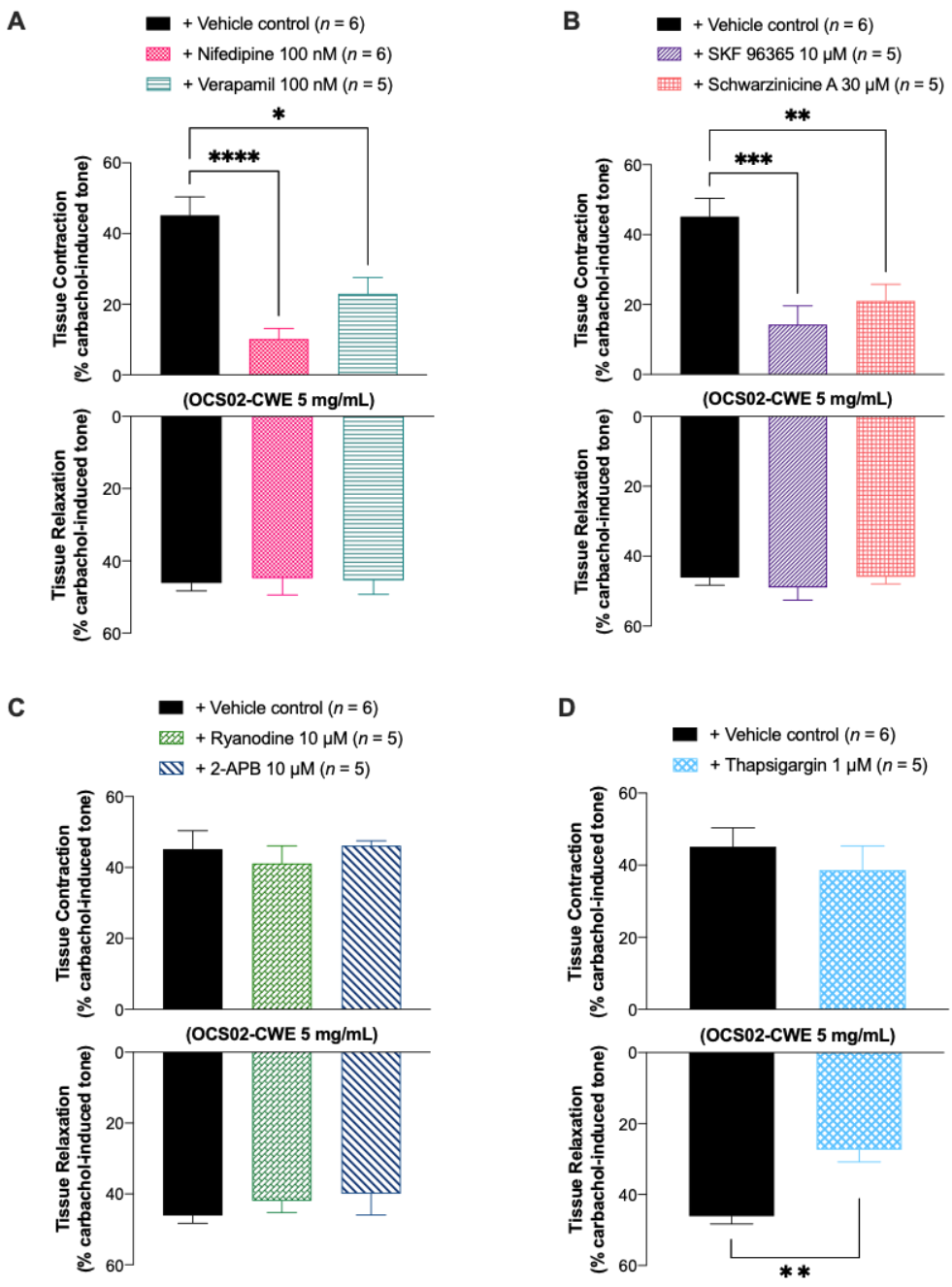

Figure 3.23 Role of membrane permeable Ca^{2+} channels and intracellular Ca^{2+} regulators on the biphasic response induced by OCS02-CWE in carbachol-pre-contracted bladder. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. One-way ANOVA followed by Dunnett's post hoc test where comparison between vehicle control and each treatment group was made, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; CWE = cold water extract)*

Table 3.9 OCS02-CWE-induced transient contraction and relaxation response in the presence of Ca^{2+} channel blockers.

Treatment	Tissue contraction (%)	Tissue relaxation (%)
Vehicle control	45.12 ± 5.26	46.12 ± 2.18
Nifedipine 100 nM	10.18 ± 2.95 ***($p < 0.0001$)	44.78 ± 4.62
Verapamil 100 nM	28.69 ± 3.93 *($p = 0.0125$)	45.42 ± 3.86
SKF-96365 10 µM	14.26 ± 5.38 ***($p = 0.0003$)	48.97 ± 3.61
Schwarzinicine A 30 µM	21.07 ± 4.73 ***($p = 0.0038$)	45.92 ± 2.01
2-APB 10 µM	46.15 ± 1.38	39.96 ± 5.96
Ryanodine 10 µM	41.11 ± 5.01	41.92 ± 3.35
Thapsigargin 1 µM	38.63 ± 6.70	27.41 ± 3.41 **($p = 0.0049$)

Data are shown as mean ± SEM of 5 – 6 animals. One-way ANOVA followed by Dunnett's post hoc test was used to compare the mean between vehicle control and each treatment group, separately for the contraction and relaxation response. Individual p-values were stated in the respective columns when $p < 0.05$.

3.4.14 OCS02-CWE elicited a sustained contractile response in resting bladder strips

OCS02-CWE produced sustained contraction in resting bladder strips. However, the contraction could only be observed in 5 out of 8 of the bladder strips i.e., 3 out of the 8 strips did not respond to OCS02-CWE. Representative trace recordings are shown in Figure 3.24. The maximum contraction induced by OCS02-CWE was achieved at 4 mg/mL (25.79 ± 3.04%, p = 0.0002) compared to vehicle control (4.05 ± 1.46%) (Figure 3.25). The data were analysed from the tissues which consistently elicited contractile response (n = 5).

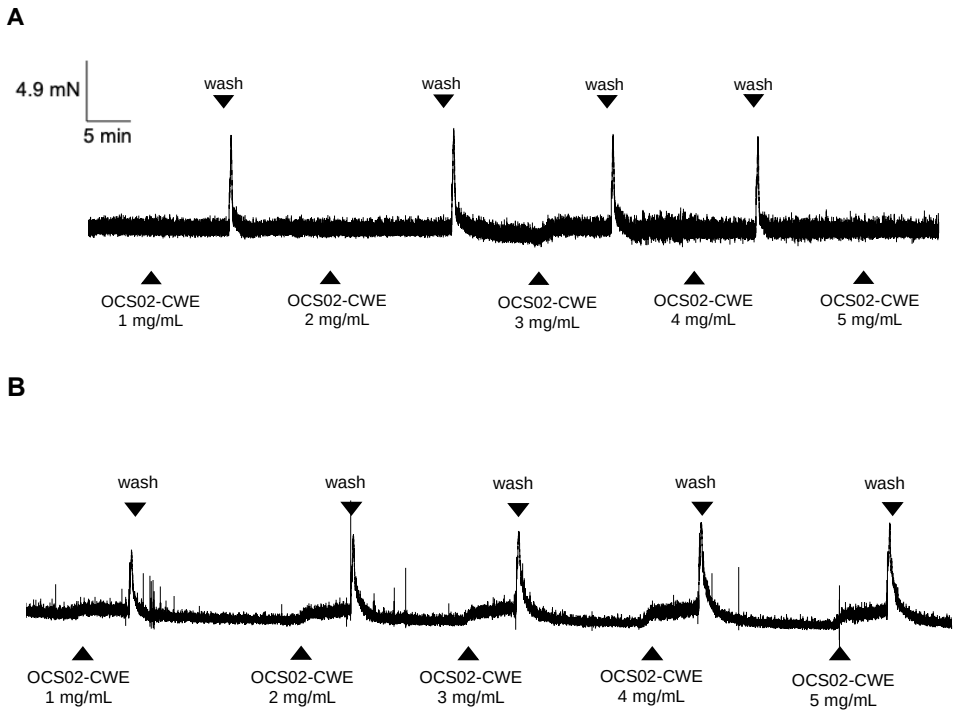

Figure 3.24 Representative isometric tension trace recordings of the non-cumulative CRCs to OCS02-CWE in resting bladder strips. *Non-cumulative CRCs to OCS02-CWE were constructed in resting bladder strips from 1 mg/mL to 5 mg/mL. (A) In 3 out of 8 resting bladder strips, OCS02-CWE did not elicit any response, or the contractile response was not consistent. (B) In 5 out of 8 resting bladder strips, OCS02-CWE consistently produced sustained contractions and the effects were reversible after washed out.*

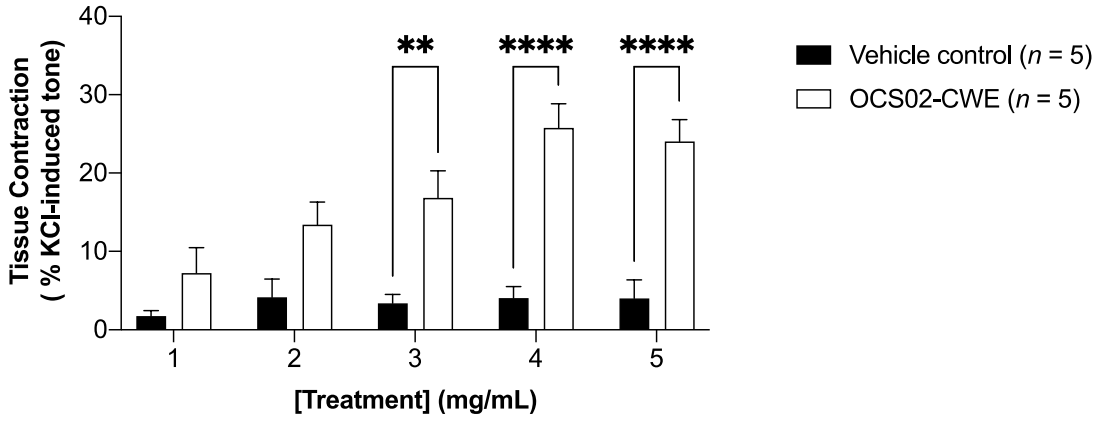

Figure 3.25 Effect of OCS02-CWE on resting bladder strips. *Tissue contractions have been expressed as a percentage of KCl-induced tone. Data are shown as mean ± SEM of 5 animals out of 8 animals tested. OCS02-CWE did not produce any response in 3 out of 8 animals. Two-way ANOVA followed by Sidak's post hoc test where comparison between vehicle control and each treatment group at each concentration was made, **p < 0.01; ****p < 0.0001.*

3.4.15 Transient contraction induced by OCS02-CWE at resting tone requires extracellular Ca^{2+}

In resting bladder strips, pre-treatment of atropine did not affect the contraction induced by OCS02-CWE (Figure 3.26A). In contrast, OCS02-CWE-induced transient contraction was abolished in Ca^{2+}-free Krebs solution and significantly attenuated in the presence of nifedipine, verapamil and SKF-96365. However, schwarzinicine A did not suppress OCS02-CWE-induced contraction in the bladder strips at basal tone (Figure 3.26B). The values of tissue contraction induced by OCS02-CWE in each treatment group were depicted in Table 3.10.

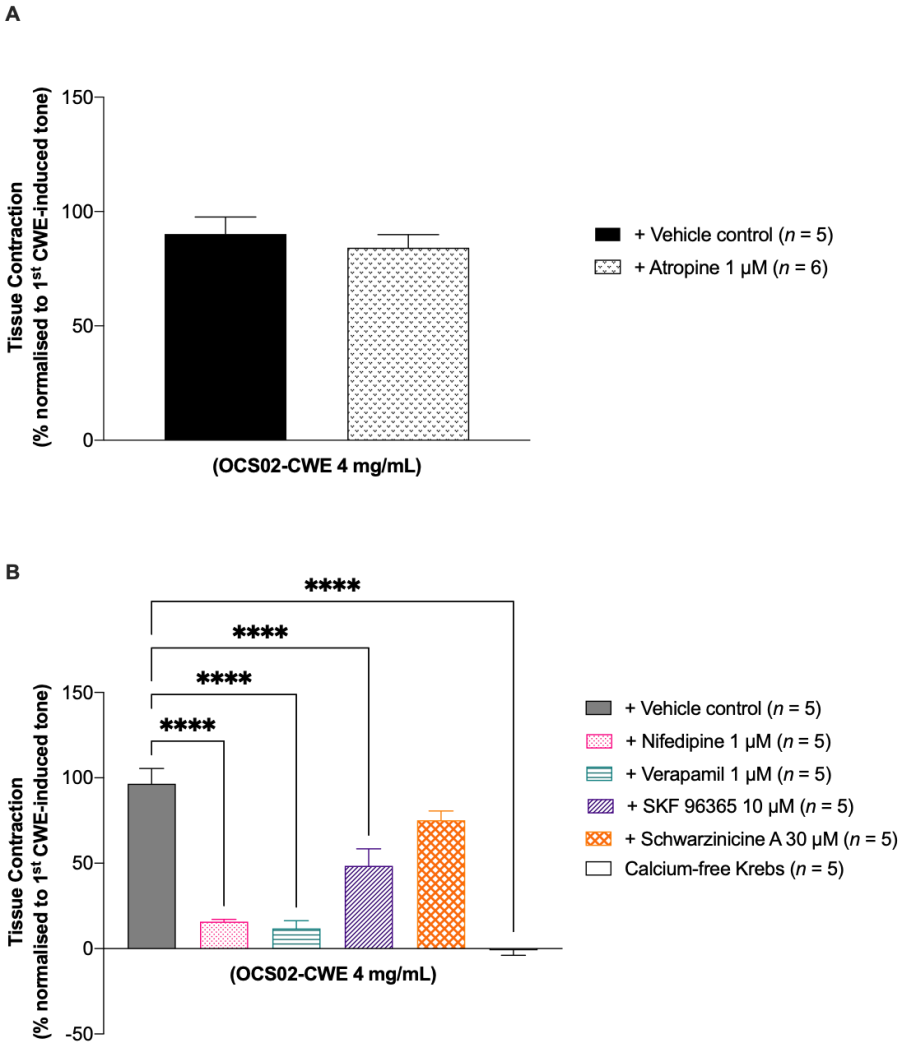

Figure 3.26 Effect of OCS02-CWE-induced contraction in the presence of atropine or different Ca^{2+} channel blockers. *The bladder strips were exposed twice to OCS02-CWE before and after the pre-incubation of vehicle control or (A) non-selective muscarinic receptor antagonist, atropine, or (B) VGCC blockers, nifedipine and verapamil, SOCC/non-selective TRPC inhibitors, SKF-96365, L-type and non-selective TRPC inhibitor, schwarzinicine A, or Ca^{2+}-free Krebs solution. Tissue response induced by second OCS02-CWE addition was normalised to the response induced by first OCS02-CWE (represents 100%). Data are shown as mean ± SEM of n number of animals. For A, student's t-test was performed to compare between vehicle control and atropine, $p > 0.05$. For B, one-way ANOVA followed by Dunnett's post hoc test was performed to compare between vehicle control and treatment groups, ****$p < 0.0001$. (Abbreviations: CWE = cold water extract; SOCC = store operated Ca^{2+} channel; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel).*

Table 3.10 Effect of various pre-incubation compounds on OCS02-CWE-induced contraction in resting bladder strips.

Treatment	1st OCS02-CWE-induced contraction (%)	2nd OCS02-CWE-induced contraction (%)	% Normalised to 1st OCS02-CWE-induced contraction
Vehicle control (H_2O)	21.62 ± 2.94	19.68 ± 3.21	84.19 ± 5.71
Atropine 1 µM	16.78 ± 3.15	13.93 ± 2.43	90.18 ± 7.41
Vehicle control (DMSO)	20.59 ± 3.36	18.88 ± 2.03	96.59 ± 8.93
Nifedipine 1 µM	23.84 ± 5.61	F3.27 3.54 ± 0.78	15.78 ± 1.38 ****($p < 0.0001$)
Verapamil 1 µM	14.68 ± 2.30	1.63 ± 0.70	11.81 ± 4.50 ****($p < 0.0001$)
SKF-96365 10 µM	14.95 ± 3.75	7.64 ± 2.92	48.47 ± 10.01 ****($p < 0.0001$)
Schwarzinicine A 30 µM	10.52 ± 0.92	8.61 ± 1.19	75.16 ± 5.47 ($p = 0.0844$)
Ca^{2+}-free Krebs	18.88 ± 6.68	-0.59 ± 0.84	-0.93 ± 2.97 ****($p < 0.0001$)

*Data are shown as mean ± SEM of 5 – 6 animals. Student's t-test was performed to compare between vehicle control and atropine, p > 0.05. One-way ANOVA followed by Dunnett's post hoc test was performed to compare between vehicle control and treatment groups, ****p < 0.0001. Individual p-values were stated in the respective columns. (Abbreviations: CWE = cold water extract)*

3.5 Discussion

In **Chapter 2**, OCS02-CWE was shown to elicit a biphasic response in carbachol-pre-contracted bladder strips that involves a transient contraction followed by a sustained relaxation response. To elucidate its mechanism of action, the bladder strips were exposed to an array of compounds with affinity for muscarinic, β-adrenergic, purinergic and serotonergic receptors to assess whether they would alter the relaxation or contractile response of OCS02-CWE. The involvement of K^+ channels, Ca^{2+} channels and the role of urothelium in the action of OCS02-CWE were also investigated. Our results suggest that the contractile and relaxation responses elicited by OCS02-CWE were mediated through different signalling pathways. To gain a clearer understanding, the relaxation and contractile mechanism of OCS02-CWE were explained separately in **section 3.5.1** and **section 3.5.2**, respectively.

3.5.1 The relaxation mechanism of OCS02-CWE

3.5.1.1 Role of contractile agents

KCl or a high concentration of K^+ has long been used to activate smooth muscle by a highly reproducible mechanism that bypasses GPCR (Ratz et al., 2005). Upon stimulation by KCl, extracellular Ca^{2+} enters the cells via the opening of VGCCs. The increase in intracellular Ca^{2+} level then leads to Ca^{2+}/CaM-dependent MLC kinase activation, MLC phosphorylation and smooth muscle contraction. Our results showed that OCS02-CWE significantly relaxed KCl-pre-contracted bladder strips, implying that OCS02-CWE may not specifically antagonise muscarinic receptors. Instead, it could interrupt the contractile mechanism of membrane depolarisation by inhibiting Ca^{2+} influx and/or promoting Ca^{2+} desensitisation. However, OCS02-CWE was less effective in relaxing KCl-induced contraction compared to muscarinic receptor-mediated contraction in the bladder strips, wherein the maximum relaxation response

elicited by OCS02-CWE in carbachol-pre-contracted bladder strips is 2-fold greater than its response in KCl-pre-contracted bladder strips. These results suggest that OCS02-CWE-induced bladder relaxation are likely to involve both voltage-dependent and -independent mechanisms. To confirm this, reverse experiments were conducted by pre-incubating the tissues with OCS02-CWE before constructing cumulative CRCs to carbachol or KCl. The effects of OCS02-CWE on other non-cholinergic stimuli including 5-HT and H_2O_2 were also examined.

OCS02-CWE significantly attenuated the maximum contraction induced by carbachol, KCl- and H_2O_2, but did not alter 5-HT-induced contraction. The differences in the effects of OCS02-CWE are likely to be caused by the varied mechanisms of these contractile agents. In DSM, the contractile response to carbachol is mediated predominantly by the $G_{q/11}$-coupled M_3 muscarinic receptor (Yamanishi et al., 2001). Our results showed that OCS02-CWE overtly suppressed the maximum contraction induced by carbachol but did not affect its potency, implying that OCS02-CWE inhibited carbachol-induced contractions in a non-competitive manner. Thus, OCS02-CWE was unlikely to interact with muscarinic receptor per se. In addition to suppressing the carbachol-induced bladder tone, OCS02-CWE diminished the spontaneous myogenic contraction induced by carbachol. Given that the generation of spontaneous myogenic contraction largely relies on extracellular Ca^{2+} influx (Drake et al., 2018), OCS02-CWE may have a role in preventing Ca^{2+}-influx. Recently, it is found that muscarinic receptor-mediated contraction of the DSM also leads to the activation of TRPC4 channels (Griffin et al., 2018). Therefore, involvement of TRPC channels could be implicated.

The contraction induced by 5-HT in smooth muscles is mediated by the activation of $G_{q/11}$-coupled-5-HT_{2A} receptors on both postjunctional and prejunctional

sites (Recio et al., 2009). In our experiments, 5-HT elicited a relatively weak bladder contraction in which its E_{max} was only about 10% of that of carbachol. This finding is comparable to a previous finding where the maximum contraction induced by 5-HT was about 12.5% of carbachol's (Sakai et al., 2013). The low magnitude of contraction and negligible spontaneous myogenic contraction induced by 5-HT suggest that its role in the regulation of bladder contraction is relatively minimal, compared to muscarinic receptor-mediated contractions. Besides, pre-treating the tissues with OCS02-CWE did not affect the contraction induced by 5-HT, inferring that 5-HT receptors may not be involved its mechanism of action.

In this study, we also investigated the effect of OCS02-CWE on H_2O_2, a urothelial-derived stimulating factor, that has been shown to induce OAB in mouse model (Homan et al., 2013). Han et al. (2010) demonstrated that H_2O_2-induced contraction in rat bladder is associated with extracellular Ca^{2+} influx, activation of rho kinase following stimulation of $G_{12/13}$ protein, and anti-oxidative pathways. Given that OCS02-CWE significantly suppressed H_2O_2-induced contraction, these pathways could participate in its mechanism of action. In parallel, M_3 receptors are also coupled to $G_{12/13}$ protein which activates Rho A and its downstream effector ROCK (Braverman et al., 2006).

When the effects of OCS02-CWE on the responses induced by these contractile agents (carbachol, 5-HT, KCl and H_2O_2) are compared as a whole, OCS02-CWE appears to have an overt suppression effect on carbachol-induced contraction. While it similarly attenuated the E_{max} of carbachol and H_2O_2 by nearly 2-fold, only the spontaneous myogenic contractions induced by carbachol was significantly diminished. Furthermore, the concentrations needed by KCl and H_2O_2 to elicit a similar level of contractile tone with carbachol is 1000-fold and 3000-fold higher,

respectively. Considering the known and putative mechanisms of action of all these contractile agents as above-mentioned and as depicted in Figure 3.27, OCS02-CWE is likely to have a role in both voltage-dependent and -independent regulation of Ca^{2+}.

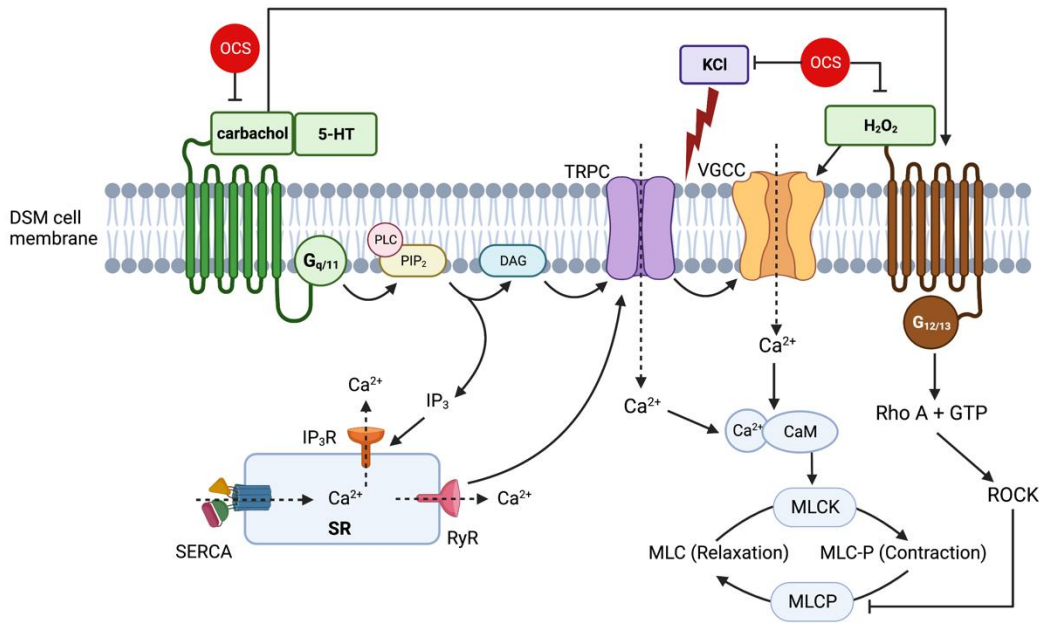

Figure 3.27 Schematic diagram depicting the regulation of Ca^{2+} by carbachol, KCl, 5-HT and H_2O_2 to induce bladder contraction and the effect of OCS02-CWE-pre-incubation on these contractile agents. *Activation of muscarinic M_3 receptors by carbachol and 5-HT_{2A} receptors by 5-HT initiates $G_{q/11}$ protein signal transduction. M_3 receptors are also coupled to $G_{12/13}$ protein. KCl causes membrane depolarisation and activates VGCC. The putative contractile mechanisms of H_2O_2 in bladder involve activation of VGCC and Rho A via $G_{12/13}$ protein activation. The solid pointed arrows denote downstream effectors activation; blunt arrows denote inhibitory effect; and dashed arrows denote flow of Ca^{2+}. (Abbreviations: 5-HT = 5-hydroxytryptamine; CaM = calmodulin; DAG = diacylglycerol; DSM = detrusor smooth muscle; H_2O_2 = hydrogen peroxide; IP_3R = inositol triphosphate receptor; KCl = potassium chloride; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; OCS = Ophiocordyceps sinensis (OCS02 cold water extract); PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; ROCK = rho kinase; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR = sarcoplasmic reticulum; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

3.5.1.2 Role of muscarinic receptors

The therapeutic effects of antimuscarinics have been established since the late 1900s and remain the most important treatment group in the management of OAB. In the previous experiment, it was shown that OCS02-CWE inhibited carbachol-induced contractions in a non-competitive manner, suggesting that it is unlikely to act on muscarinic receptors. To substantiate this finding, the relaxation response induced by OCS02-CWE was investigated in the presence of atropine, a non-selective muscarinic antagonist, in KCl-pre-contracted bladder strips. The results confirmed that OCS02-CWE-induced bladder relaxant effect has no muscarinic effect as the inhibition of muscarinic receptors by atropine did not affect the bladder relaxant effect exhibited by OCS02-CWE.

3.5.1.3 Role of adrenoceptors

As outlined in **section 1.1.2.5** (**Chapter 1**), α-adrenoceptors appear to have a negligible role in the DSM regulation. While we attempted to study the role of α-adrenoceptors in OCS02-CWE-induced responses, the addition of phenylephrine, an α_1-adrenoceptor agonist did not produce any response in the bladder strips (data not shown). Therefore, this pathway was not further investigated in our study. Subsequently, our results demonstrated that OCS02-CWE has no β-adrenergic effect as the presence of propranolol, a non-selective β-adrenoceptors antagonist, did not affect the bladder relaxant effect exhibited by OCS02-CWE. This observation agrees with the previous finding that OCS02-CWE was less effective in relaxing KCl-induced contraction as smooth muscle relaxation stimulated by the activation of β-adrenoceptors is associated with hyperpolarisation (Garland et al., 2011). β-adrenoceptor agonist, isoproterenol, has been shown to exhibit a 100-fold higher potency in relaxing bladder strips pre-contracted with KCl compared to carbachol

(Longhurst and Levendusky, 1999). Therefore, it is unlikely that OCS02-CWE's mechanism of action involves the activation of β-adrenoceptors as it was less effective in relaxing KCl-pre-contracted tissues.

3.5.1.4 Role of K^+-channels

K^+ channels are one the most important regulators of DSM contraction and relaxation. The opening of K^+ channels hyperpolarises the plasma membrane through efflux of K^+ and limits Ca^{2+} influx via L-type VGCCs, resulting in DSM relaxation (Petkov, 2012). Conversely, closure of K^+ channels reduces the hyperpolarising current and leads to DSM contraction. This mechanistic pathway was tested using TEA, a non-selective K^+ channel blocker. Our results showed that pre-incubation with TEA did not affect OCS02-CWE-induced bladder relaxation, indicating that the activation of K^+ channels was not involved in its mechanism of action. If OCS02-CWE promotes DSM relaxation by activating K^+ channels, the blockade of K^+ channels would prevent the relaxant effect OCS02-CWE.

3.5.1.5 Role of urothelium and urothelial-derived relaxing factors

It is now well recognised that the urothelium does not only provide barrier function but participates in maintaining bladder homeostasis (Winder et al., 2014). Currently, there is no standardised method to remove urothelium from isolated bladder preparations. In this study, we adopted the methods introduced by Munoz et al. (2010) and Moro et al. (2012), that is, by swabbing the inner layer of the bladder strips three times using a cotton bud. Through this method, the urothelium of the bladder strips was successfully removed, leaving the sub-urothelium intact. The role of sub-urothelium has not been fully characterised but it is thought to have a role in bladder mechano-sensation (Roosen et al., 2009). On the other hand, urothelial cells can

release several urothelial-derived relaxing factors such as NO, ATP, adenosine and H_2S and regulate bladder contractility (D'Emmanuele di Villa Bianca et al., 2016; Dunning-Davies et al., 2013; Hawthorn et al., 2000).

Our results showed that the relaxant effect of OCS02-CWE was significantly attenuated in urothelium-denuded bladder strips, suggesting that the urothelium is essential for its relaxant effect. However, the presence of urothelium is not an absolute condition for OCS02-CWE to exhibit its relaxant effect because the relaxation response was not eliminated in urothelium-denuded strips. Therefore, OCS02-CWE may also exert its effect directly on the DSM possibly through the activation or inhibition of the same group of receptors or enzymes expressed on both urothelial and DSM cells, or through a different mechanistic pathway.

Subsequently, the involvement of the urothelial-derived relaxing factors was investigated. Since purinergic P1 and P2 receptors are expressed on both urothelial and DSM cells, modulating these receptors may influence both urothelium and DSM layers. ATP, adenosine and cordycepin that have affinities for purinergic receptors were used. However, our results indicated that neither inhibition of P1 receptors (adenosine receptors) nor P2 receptors altered the relaxant effect of OCS02-CWE. Hence, OCS02-CWE-induced relaxation response was unlikely to be mediated through purinergic signalling. These results also imply that adenosine and cordycepin may not be the bioactive components in OCS02-CWE that elicit relaxant effect albeit their presence in the OCS02 cultivar (Fung et al., 2018).

The role of H_2S, a gasotransmitter released from the urothelium, in the regulation of DSM contractility has recently been introduced (Fernandes and Hernández, 2016). It is synthesised endogenously from L-cysteine, mainly by the action CSE and/or CBS. NaHS is an exogenous source of H_2S. Currently, the

molecular site of action of H_2S remains unknown, but in the human bladder, K_{ATP} channels may serve a role in its relaxation pathway as glibenclamide prevented H_2S-induced relaxation (Gai et al., 2013). Besides, CSE-derived H_2S has been suggested to modulate cAMP levels thus contribute to β_3-adrenoceptors-mediated relaxation (Mitidieri et al., 2022). Our results showed that OCS02-CWE did not affect NaHS-induced relaxation response, suggesting that OCS02-CWE and H_2S do not share similar mechanism in the bladder.

NO is another important gasotransmitter derived from the urothelium. It is synthesised from L-arginine via NOS and diffuses across the DSM cell membranes to elicit its effect in the DSM cells. To test the possible involvement of NO in OCS02-CWE-induced relaxation response, L-NAME, a false substrate of NOS, was used to block its activity in the bladder strips. In the presence of L-NAME, OCS02-CWE-induced relaxation was attenuated, inferring that OCS02-CWE may stimulate production of NO to produce its relaxant effect. This result ties well with a previous study done by Chiou et al. (2000) in which the protein extract of *O. sinensis* elicited an endothelium-dependent, L-NAME-sensitive vasorelaxation response. From the composition analysis of OCS02, L-arginine, the precursor of NO, is found to be a major component (45.2 g/kg) (Fung et al., 2018). Thus, it could be the bioactive component in OCS02-CWE that exhibits relaxant effect.

Besides, it is plausible that OCS02-CWE prolongs the half-life of NO. In biological system, the half-life of NO is only about 1 to 10 seconds as it is efficiently removed by reacting with oxyhaemoglobin (Beckman and Koppenol, 1996). However, in aqueous buffer and in vitro preparations, its half-life is usually longer, ranging from a few minutes and up to 900 minutes (Kelm, 1999). The half-life of NO is also inversely proportional to its concentration i.e., when NO in the system becomes more

dilute, its half-life becomes much longer. Therefore, when the concentration of NO is reduced in the presence of L-NAME, the remaining relaxant effect caused by OCS02-CWE could be due to its effect in prolonging NO's half-life. To support this postulation, the effect of NO donor, SNP, on OCS02-CWE-induced relaxation response was studied.

Our results showed that OCS02-CWE-induced relaxation response was also attenuated in the presence of SNP. In smooth muscle cells, SNP catabolises to NO^+ redox form and reacts with sulfhydryl-containing molecules to make S-nitrosothiols (SNO), which in turn yields NO in the system (Tepavcevic et al., 2008). Therefore, if OCS02-CWE extends the half-life of NO, it would have an added effect with SNP in relaxing the bladder strips. The attenuation of OCS02-CWE by SNP thus indicates that OCS02-CWE is more likely to involve production of NO rather than extending its half-life. Previous studies have shown that endogenously synthesised NO (through L-arginine) and exogenously applied NO (via NO donors) may mediate via different pathways due to the formation of different NO redox forms (Peralta et al., 2001; Qian and Fulton, 2012). It has also been demonstrated that L-arginine antagonised the relaxing effect of SNP in rat renal arteries by producing only NO-radical (Tepavcevic et al., 2008; Oreščanin and Milovanović, 2006). Hence, if OCS02-CWE stimulates endogenous production of NO in the urothelium, presumably acting as a substrate of NOS, SNP could antagonise the relaxation response induced by OCS02-CWE.

However, the exact mechanism of NO-induced bladder relaxation is yet to be clarified as previous studies have reported contradicting information. NO-mediated bladder relaxation has been reported in rat, sheep and goat (Chung et al., 1996; Thornbury et al., 1992; Vijayraj et al., 2011). In contrast, in the bladder of guinea pig and mice, NO has elicited an excitatory effect (Fujiwara et al., 2000; Yanai et al.,

2008). In isolated human bladder strips, NO donor displayed complex responses that involved relaxation, contraction or a transient relaxation followed by contraction (Moon, 2002). These studies proposed that the effect of NO is different across species and may involve cGMP-dependent and/or cGMP-independent pathways. Thus, it is essential to investigate the role of cGMP in OCS02-CWE-induced relaxation to clarify its NO-related relaxant mechanism. The downstream signalling of urothelium-derived relaxing factors is illustrated in Figure 3.28.

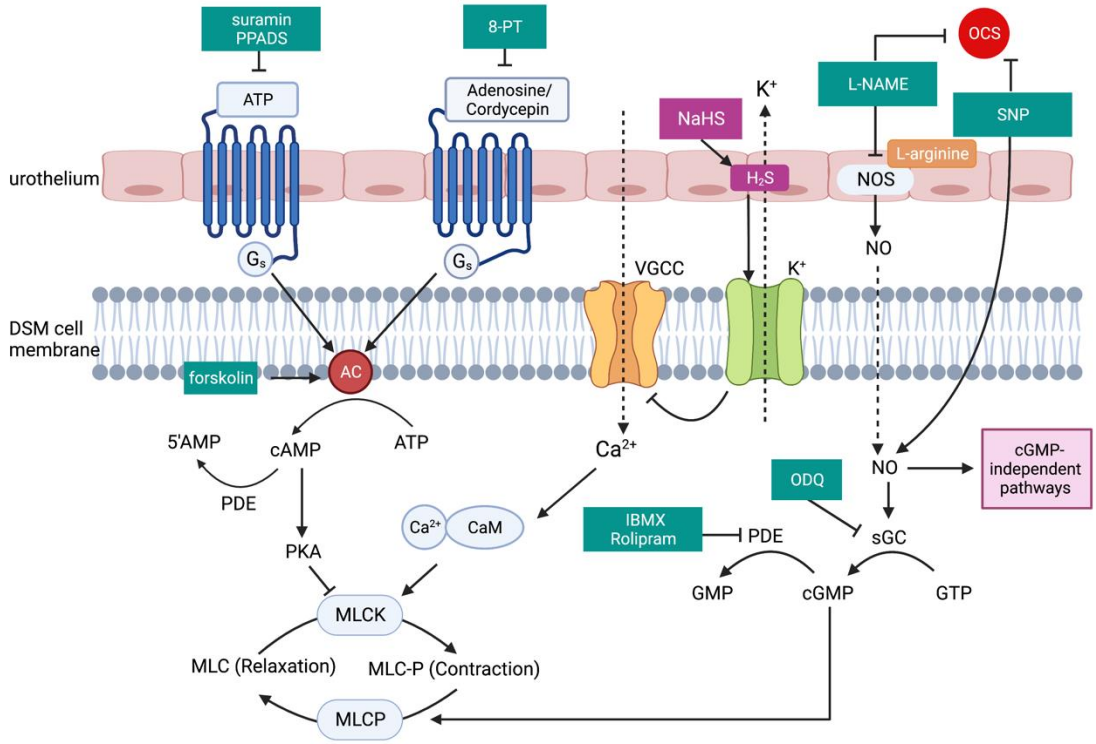

Figure 3.28 Schematic diagram depicting the downstream signalling of urothelium-derived relaxing factors and the inhibitory effect of L-NAME and SNP on OCS02-CWE-induced relaxation. *The exact mechanistic pathway of H_2S remains unclear but it is likely to involve activation of K_{ATP} channels that leads to hyperpolarisation and closure of VGCCs. L-arginine is converted to NO in the urothelium via NOS and diffuses across the DSM cell membrane. NO-induced relaxation may mediate via cGMP-dependent and cGMP-independent pathways. Solid pointed arrows denote downstream effectors activation; blunt arrows denote inhibitory effect; and dashed arrows denote flow of ions or molecules. (Abbreviations: 8-PT = 8-phenyltheophylline; AC = adenylate cyclase; ATP = adenosine triphosphate; CaM = calmodulin; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; DSM = detrusor smooth muscle; GMP = guanosine monophosphate; GTP = guanosine triphosphate; IBMX = 3-isobutyl-1-methylxanthine; IP_3 = inositol triphosphate; L-NAME = NG-nitro-L-arginine methyl ester; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; NO = nitric oxide; NOS = nitric oxide synthase; OCS = Ophiocordyceps sinensis (OCS02 cold water extract); ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE = phosphodiesterase; PKA = protein kinase A; PPADS = pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt; sGC = soluble guanylate cyclase; SNP = sodium nitroprusside; VGCC = voltage-gated Ca^{2+} channel)*

3.5.1.6 Role of cyclic nucleotides

cAMP and cGMP are both cyclic nucleotide second messengers that participate in the regulation of smooth muscle contractility. As detailed in **section 1.1.2.5 (Chapter 1)**, activation of β_3-adrenoceptors promotes cAMP production and results in DSM relaxation. Forskolin is an established AC activator that potentiates the production of cAMP via the conversion of ATP. The production of cAMP can also be increased through the inhibition of PDE that catalyses hydrolysis of cAMP to 5'AMP (Figure 3.28).

In the present study, OCS02-CWE-induced relaxation response was enhanced in the presence of forskolin, but the difference was not statistically significant. It is difficult to explain such results, but two interpretations can be considered: 1) OCS02-CWE may prevent the breakdown of cAMP through PDE inhibition; 2) OCS02-CWE may mediate its relaxant effect via a cAMP-independent pathway that elicits an additive effect with forskolin. As rolipram (cAMP-selective PDE4 inhibitor) did not affect OCS02-CWE-induced relaxation, and the activation of β_3-adrenoceptors that stimulates cAMP-downstream signalling in the action of OCS02-CWE has been excluded in previous experiments, the effect of OCS02-CWE on PDE can be ruled out. Considering the involvement of NO in the action of OCS02-CWE, the potentiation effect on OCS02-CWE-induced relaxation by forskolin could be attributed to its NO-mediated pathway. Hashimoto et al. (2006) has demonstrated that forskolin increased the production of NO induced by cilostazol, a selective PDE3 inhibitor, in human aortic endothelial cells. On the other hand, it could involve a NO-independent mechanism that acts synergistically with cAMP, given that the relaxation response induced by OCS02-CWE was only partially removed following the removal of urothelium, or in the presence of L-NAME or SNP.

As indicated above, NO-mediated relaxation may involve both cGMP-

dependent and -independent pathways. We first examined the possible involvement of cGMP by exposing the tissues to ODQ, a soluble GC inhibitor, or IBMX, a non-selective PDE inhibitor. Our results revealed that neither IBMX nor ODQ affects the relaxation response elicited by OCS02-CWE, implying that its NO-mediated relaxant effect does not require formation of cGMP. Considering this, the experiments in **Chapter 4** were designed to clarify the mechanistic pathway of NO-mediated relaxation in rat bladder.

3.5.1.7 Role of intra- and extracellular Ca^{2+}

Knowing that Ca^{2+} is the main determinant in smooth muscle contraction and relaxation, we then investigated whether the urothelium-independent relaxation of OCS02-CWE was due to its interaction with other DSM components that inhibits intracellular Ca^{2+} release or extracellular Ca^{2+} influx. In the absence of external Ca^{2+}, the initial phasic contraction induced by carbachol was abolished, but the tonic contraction increased gradually over time. It suggests that the contraction induced by carbachol when there was no external Ca^{2+} was caused by intracellular Ca^{2+} release. Our results agree with previous reports in which $G_{q/11}$-mediated responses of the bladder are highly dependent on Ca^{2+} influx via VGCCs (Frazier et al., 2007; Rivera and Brading, 2006; Schneider et al., 2004). In the presence of OSC02-CWE, the phasic contraction induced by carbachol in normal (Ca^{2+}-containing) Krebs was attenuated, implying that OCS02-CWE can prevent extracellular Ca^{2+} influx. Interestingly, in Ca^{2+}-free Krebs solution, OCS02-CWE potentiated the initial contraction of carbachol. While the change was not statistically significant, there was an obvious difference in the pattern of initial contraction induced by carbachol, as shown in the representative trace recordings. This observation suggests that OCS02-CWE may have a role in Ca^{2+} mobilisation by increasing the level of Ca^{2+} in the intracellular store to be utilised by

carbachol.

Since OCS02-CWE suppressed phasic contraction induced by carbachol which is highly dependent on extracellular Ca^{2+}, its effect on Ca^{2+}-induced contractions were investigated. In Ca^{2+}-free Krebs solution, $CaCl_2$ was added cumulatively to the tissues to stimulate bladder contraction following membrane depolarisation by KCl and opening of L-type VGCCs. Our results showed that OCS02-CWE mirrored the inhibitory effect of nifedipine, a dihydropyridine L-type VGCC blocker on Ca^{2+}-induced contraction, suggesting that OCS02-CWE may inhibit extracellular Ca^{2+} influx. Moreover, it is observed that the inhibitory effect of OCS02-CWE against Ca^{2+}-induced contractions was less pronounced in urothelium-denuded bladder tissues, but it remains significantly different from vehicle control. This finding corroborates our previous statement that OCS02-CWE acts on both urothelial and DSM layers.

3.5.1.8 Role of membrane permeable Ca^{2+} channels

After having gathered observations from the inhibitory effect of OCS02-CWE on carbachol-, KCl-, H_2O_2 and Ca^{2+}-induced contractions, it is essential to clarify the role of membrane-permeable Ca^{2+} channels in the action of OCS02-CWE. The involvement of L-type VGCCs was assessed by dihydropyridine nifedipine and phenylalkylamine verapamil. Other than blocking the L-type VGCCS, verapamil also blocks N-type, P/Q-type, R-type and T-type VGCCs (Dobrev et al., 1999). SKF-96365 is a known non-selective TRPC channel blocker that also inhibits Ca^{2+} entry via SOCC and L-type VGCC (Jezior et al., 2001). Schwarzinicine A, on the other hand, is a novel phenylethylamine analogue isolated from the leaf extract of *Ficus schwarzii* Koord in our laboratories (Krishnan et al., 2020). It has demonstrated significant inhibitory effects on TRPC3, TRPC4, TRPC5 and TRPC6 channels, and L-type VGCCs to

promote vasorelaxation (Mak et al., 2022). To confirm that schwarzinicine A exhibits bladder relaxant activity, some experiments have been performed and the results were displayed in Appendix B. The effects of nifedipine, verapamil, SKF-96365 and schwarzinicine A on carbachol-induced contractions were also elucidated in **Chapter 5** to validate the choice of these pharmacological tools.

Our results showed that none of these Ca^{2+} channel blockers altered the relaxant effect of OCS02-CWE, but they inhibited its contractile effects, detailed in **section 3.5.2**. It is intriguing to understand how OCS02-CWE prevented Ca^{2+} influx but inhibition of the Ca^{2+} channels that regulate Ca^{2+} influx did not change its relaxation response. Several explanations are proposed to address the discrepancies in our results. First, the inhibition of extracellular Ca^{2+} entry by OCS02-CWE was mediated through other pathway(s) different from nifedipine, verapamil, SKF-96365 and schwarzinicine A. Second, the concentration of nifedipine and verapamil used in this protocol (100 nM) may not be sufficient to inhibit OCS02-CWE-induced relaxation. If this postulation were true, it suggests the limitation of using isolated tissue bath technique in confirming the Ca^{2+}-inhibitory pathway of OCS02-CWE because a higher concentration of nifedipine or verapamil would prevent sufficient pre-contractile tone induced by carbachol. Third, the loss of the contractile effect of OCS02-CWE in the presence of these Ca^{2+} channel inhibitors could have changed the intracellular signalling that results in its relaxation response. This is further explained in **section 3.5.2**. The other possible explanation is that the OCS02-CWE-induced relaxation involves a complex mechanism that is different at resting and raised (pre-contracted) bladder tone. In experiments that demonstrated Ca^{2+} inhibitory effect of OCS02-CWE, the bladder strips were pre-treated with OCS02-CWE for 30 minutes at resting tension, whereas when studying the effect of Ca^{2+} blockers, OCS02-CWE

was added to carbachol-pre-contracted strips to elicit a response. If the Ca^{2+} inhibitory effect of OCS02-CWE was due to the production of NO, the mechanism could be varied based on the origin of the Ca^{2+} pools that it utilises. For example, NO-induced vasorelaxation can be mediated via desensitisation of the contractile apparatus to Ca^{2+} thus preventing Ca^{2+} influx via VGCC, and/or decrease of intracellular Ca^{2+} via a cGMP-independent stimulation of SERCA (van Hove et al., 2009). Currently, there is no information available on the interaction between NO and Ca^{2+} pools in the bladder. At this point, our results do not provide substantial evidence to support the involvement of membrane permeable Ca^{2+} channels in OCS02-CWE-induced bladder relaxation.

3.5.1.9 Role of sarcoplasmic reticulum

IP_3Rs and RyRs are two Ca^{2+} release channels located on the SR. The Ca^{2+} signal emanating from IP_3 generation following activation of muscarinic receptors may be amplified by further Ca^{2+} release from RyRs (Chambers et al., 1999). Our results showed that the relaxant effect of OCS02-CWE was unaffected by both 2-APB, an IP_3R antagonist and ryanodine, an RyR antagonist. In coherence with previous inference, these findings suggest that the relaxation response induced by OSC02-CWE was unlikely to be mediated via activation of muscarinic receptors and inhibition of intracellular Ca^{2+} release.

The SERCA pump, which is also embedded on the SR membrane, is responsible for transporting Ca^{2+} into the SR at the expense of ATP where one free Ca^{2+} ion was pumped into the SR per molecule of ATP consumed (Rohrmann, 2004) (Figure 3.29). Functionally, it expedites the removal of Ca^{2+} from the cytoplasm to maintain Ca^{2+} homeostasis and promote smooth muscle relaxation. The activity of SERCA pump can be inhibited by thapsigargin, leading to depletion of Ca^{2+} in the

store (van Hove et al., 2009). The inhibition of OCS02-CWE-mediated relaxation by thapsigargin suggests that OCS02-CWE-induced relaxation could be mediated by activating SERCA that reduces intracellular Ca^{2+} concentration.

However, the reduction in OCS02-CWE-induced relaxation by thapsigargin can also be interpreted as it inhibits SERCA, like thapsigargin. While inhibition of SERCA increases intracellular Ca^{2+} concentration, thapsigargin has been shown to produce endothelium-dependent relaxation in rat mesentery arteries and rat thoracic aorta (Fukao et al., 1995; Moritoki et al., 1994; Takahashi et al., 2000). These studies suggest that thapsigargin may stimulate NO by a Ca^{2+}-dependent mechanism. When thapsigargin inhibits SERCA pump and depletes Ca^{2+} stores, SOCCs were activated to promote extracellular Ca^{2+} influx, leading to the activation of NOS and production of NO (Moritoki et al., 1994). Then, NO release induced by thapsigargin causes hyperpolarisation to promote relaxation (Fukao et al., 1995). These mechanisms have not been investigated in the bladder.

Wuest et al. (2007) demonstrated that thapsigargin concentration-dependently increased the basal tension of mouse, porcine and human bladders but reduced carbachol-induced contraction. Elevation of baseline tension with thapsigargin suggests that it blocks Ca^{2+} reuptake into SR and raises the Ca^{2+} level in the cytoplasm. Then, in the presence of thapsigargin, SR will eventually be depleted and no longer provide enough Ca^{2+} for full contraction induced by carbachol. Additionally, the loss of SERCA function cannot be compensated by Ca^{2+} influx through L-type VGCC as they utilise different Ca^{2+} pools (Wuest et al., 2007). Based on this evidence, we are inclined to suggest that OCS02-CWE promotes activation of SERCA rather than inhibiting it, because carbachol-induced contraction was potentiated by OCS02-CWE in Ca^{2+}-free Krebs solution. When OCS02-CWE activates SERCA and increases Ca^{2+}

level in the intracellular store, addition of carbachol in Ca^{2+}-free bath triggered a transient and rapid release of Ca^{2+} from the intracellular store. If OCS02-CWE inhibits SERCA, the intracellular store will be depleted following the long incubation period and eventually not providing sufficient Ca^{2+} to be utilised by carbachol to elicit contraction. The role of membrane permeable Ca^{2+} channel and SR in the regulation of bladder contraction is illustrated in Figure 3.29.

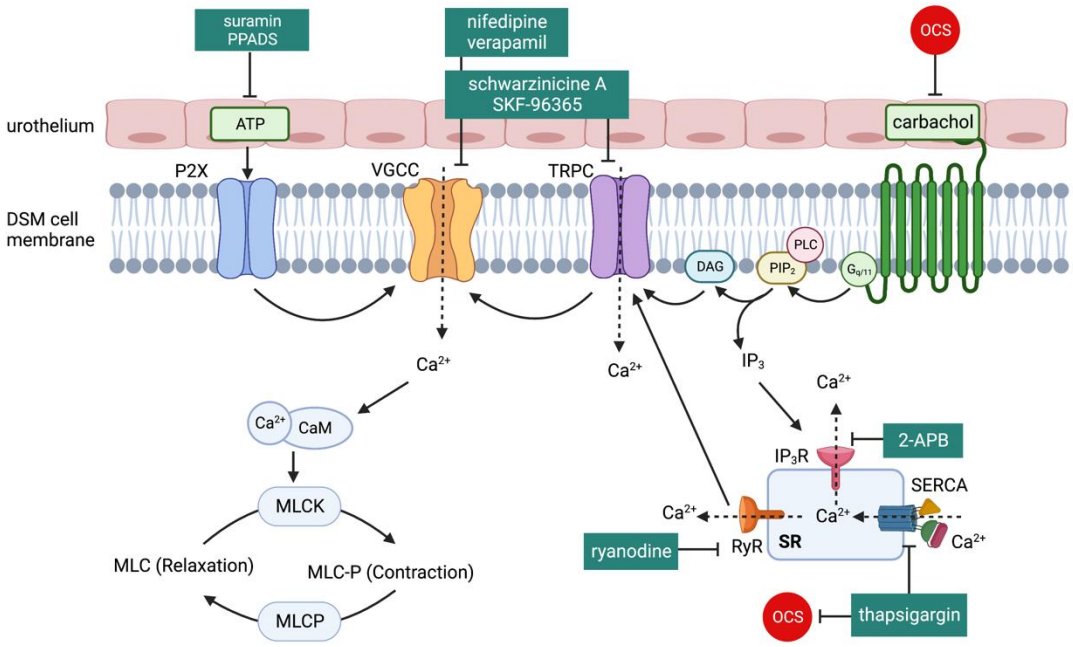

Figure 3.29 Schematic diagram depicting the role of Ca^{2+} regulators in the DSM, inhibitory effect of OCS02-CWE on carbachol, and the inhibitory effect of thapsigargin on OCS02-CWE-induced relaxation. *Solid pointed arrows denote downstream effectors activation; blunt arrows denote inhibitory effects; and dashed arrows denote flow of Ca^{2+} ions. (Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; ATP = adenosine triphosphate; CaM = calmodulin; DAG = diacylglycerol; DSM = detrusor smooth muscle; IP_3 = inositol triphosphate; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; OCS = Ophiocordyceps sinensis (OCS02 cold water extract); P2X = purinergic P2X receptor; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR = sarcoplasmic reticulum; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

3.5.2 Contractile pathway of OCS02-CWE

When exposed to KCl-pre-contracted bladder strips, OCS02-CWE-induced transient contraction was absent, suggesting that OCS02-CWE may induce membrane depolarisation. In a previous study, similar observation was reported in the effect of ATP where it elicited transient contraction in carbachol-pre-contracted but not in KCl-pre-contracted bladder strips (Boland et al., 1993). Application of ATP evokes a rapid depolarisation through the activation of P2X receptors and triggers extracellular Ca^{2+} influx. As P2X receptors desensitised following rapid activation, ATP stimulates the activation of GPCR-mediated P2Y receptors to promote relaxation (Burnstock, 2014). Therefore, in membrane depolarisation-induced pre-contraction, ATP elicits only relaxation response (Boland et al., 1993).

O. sinensis has been shown to drive ATP synthesis in cells to produce immunomodulatory and antioxidative effects (Bai et al., 2020; Chen et al., 2013; Ko and Leung, 2007). Thus, we hypothesised that OCS02-CWE might stimulate production of ATP in the DSM cells and result in the biphasic response. Our results showed that non-selective P2 receptor antagonists, PPADS and suramin, blocked the transient contraction induced by ATP but not OCS02-CWE, implying that the activation of purinergic receptors was not involved in the contractile effect of OCS02-CWE. Pre-incubation of the bladder strips with OCS02-CWE also did not affect ATP-induced transient contraction.

While investigating the role of Ca^{2+} channels in the action of OCS02-CWE, it was found that OCS02-CWE-induced transient contraction in carbachol-pre-contracted bladder strips was sensitive to the membrane permeable Ca^{2+} channel blockers including nifedipine, verapamil, SKF-96365 and schwarzinicine A. In contrast, thapsigargin, 2-APB and ryanodine did not affect OCS02-CWE-induced

transient contraction. Based on these findings, OCS02-CWE-induced contraction is likely to be caused by extracellular Ca^{2+} influx, which supports the idea that OCS02-CWE causes membrane depolarisation. Since L-type VGCCs are also expressed on the urothelium, the results also explain the slight reduction of transient contraction induced by OCS02-CWE in urothelium-denuded bladder strips.

When OCS02-CWE was applied to bladder at resting tension, it exhibited a small, sustained contraction in 73% of the bladder strips. This observation indicates that the relaxant effect of OCS02-CWE may overcome its contractile effect, providing an explanation to the transient contractile effect in pre-contracted bladder strips. To allow a fair comparison, the effects of pharmacological tools on OCS02-CWE were studied in the same bladder strip by exposing the strips with OCS02-CWE twice (before and after the respective treatments). The bladder strip was discarded if contractile response was not observed in the first OCS02-CWE application. In total, 4 bladder strips were discarded.

While examining the role of muscarinic receptors OCS02-CWE's relaxation mechanism, KCl was used as the pre-contractile agent in the presence of atropine. This protocol did not allow us to exclude the possibility of OCS02-CWE in potentiating muscarinic activation to produce transient contraction. Therefore, the effect of atropine on OCS02-CWE-induced transient contraction in basal tone was investigated. The results confirmed that OCS02-CWE did not act on muscarinic receptors. Then, in Ca^{2+}-free Krebs solution, OCS02-CWE-induced contraction was abolished whereas in the presence of nifedipine, verapamil and SKF-96365, its contractile effect was significantly attenuated. These observations imply that OCS02-CWE promotes Ca^{2+} influx to elicit contractile response, likely via L-type VGCCs following membrane depolarisation. Figure 3.30 depicts the mechanistic pathway of bladder contraction

discussed herein.

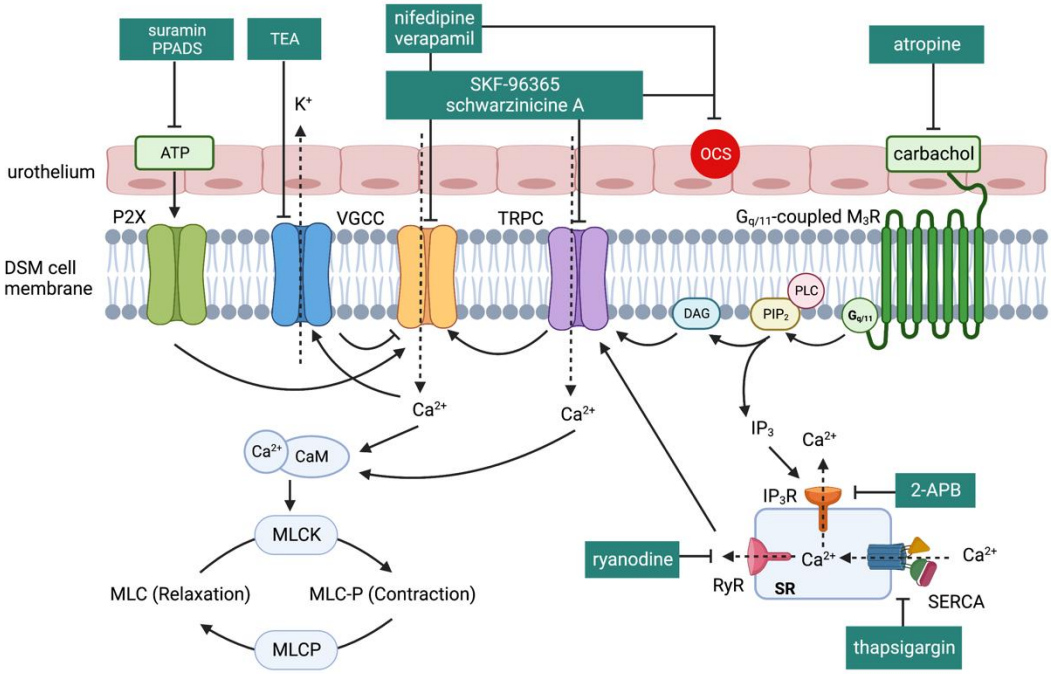

Figure 3.30 Schematic diagram depicting the role of membrane permeable Ca^{2+} channels, purinergic (P2X) receptor and $G_{q/11}$-coupled muscarinic receptor in DSM contraction and the effects of membrane permeable Ca^{2+} channels blocker on OCS02-CWE. *Solid pointed arrows denote downstream effectors activation; blunt arrows denote inhibitory effects; and dashed arrows denote flow of Ca^{2+} ions. (Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; ATP = adenosine triphosphate; CaM = calmodulin; DAG = diacylglycerol; DSM = detrusor smooth muscle; IP_3 = inositol triphosphate; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; OCS = Ophiocordyceps sinensis (OCS02 cold water extract); PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR = sarcoplasmic reticulum; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

3.6 Chapter summary

The findings in this chapter show that the contractile and relaxation responses of OCS02-CWE were mediated via different pathways as the responses were not inhibited simultaneously by a single pharmacological tool, probably because they utilise different Ca^{2+} pools in the cells and were derived from different bioactive components. However, the relaxant effect may overcome the contractile effect of OCS02-CWE. This is evidenced by the enhanced transient contraction in the presence of L-NAME and SNP that suppresses the relaxation response. Besides, OCS02-CWE elicited transient contraction in pre-contracted bladder strips but a small, sustained contraction in resting bladder strips. Membrane depolarisation, absence of external Ca^{2+} or the presence of membrane permeable Ca^{2+} blockers removed the transient contraction induced by OCS02-CWE, indicating that it utilises extracellular Ca^{2+} to elicit bladder contraction. Then, its relaxant effect was attenuated following the removal of urothelium or in the presence of L-NAME, SNP or thapsigargin. The responses can be described as a sequential event as follows: Upon membrane depolarisation induced by OCS02-CWE, external Ca^{2+} fluxed into the cells and results in bladder contraction. Then, tissue relaxation occurs as OCS02-CWE stimulates production of NO, activates SERCA and promotes Ca^{2+} reuptake into SR. Further investigation is needed to find out if the activation of SERCA was due to production of NO or they were independent of each other. To substantiate the findings in this chapter, the experiments in the following chapter (**Chapter 4**) were designed to elucidate the mechanism of action of NO in bladder relaxation.

4 Mechanistic Pathways of Nitric Oxide-induced Bladder Relaxation

4.1 Introduction

In Chapter 3, we proposed that the transient contraction and sustained relaxation elicited by OCS02-CWE in carbachol-pre-contracted bladder strips is a sequential event that involves the regulation of Ca^{2+} and NO. Following these findings, this chapter focuses on understanding the role of NO in regulating bladder function. The role of NO as an NANC neurotransmitter in the vascular smooth muscle (VSM) was first established in the early 1900s (Sanders and Ward, 1992), but its contribution in the bladder physiology has not been fully established. Evidence of NO-mediated response in the DSM came by when James et al. (1993) described an L-NOARG-sensitive but TDX-resistant response to EFS in human bladder strips. Later, Birder et al. (1998) discovered that NO release from the bladder was not only mediated by nerves but largely depends on non-neural mechanisms in the lamina propria. This finding contributed substantially to the subsequent research regarding the role of urothelium. NO formed in the urothelial cells diffuses into the DSM cells to elicit its effect (Winder et al., 2014). Currently, there is no direct evidence to show that NO is produced by the DSM cells.

Unlike in the VSM where NO elicits only relaxation response, NO has been shown to produce both excitatory and inhibitory responses in the bladder (Chung et al., 1996; Moon, 2002; Moro et al., 2012; Fujiwara et al., 2000; Yanai et al., 2008). In an earlier study, EFS- evoked relaxation response in pig bladder strips pre-contracted with endothelin-1 in the presence of α, β-methyl ATP and atropine, but when pre-

contracted with K^+, EFS did not produce any response (Persson and Andersson, 1992). The element that produced EFS-induced relaxation was not revealed by the authors, but it is likely to be caused by EFS-induced NO as the origin of the effect was neither purinergic nor cholinergic. Subsequently, Chung et al. (1996) demonstrated photo-induced adequate nitric oxide (PIANO)-mediated relaxation in rat bladder strips. The DSM relaxation induced by PIANO was accompanied by an increase of cGMP levels in the tissues (Chung et al., 1996). The study suggested that PIANO-mediated bladder relaxation involves the prototypical pathway of NO signalling, that is, via a cGMP-dependent pathway. Besides, Vignozzi et al. (2012) reported that SNP-induced bladder relaxation is more pronounced in the bladder of female rats compared to male rats, likely because PDE5 activity is more pronounced in males.

On the other hand, several studies suggested that NO-mediated bladder relaxation was independent of cGMP. In human isolated bladder strips, SNP and dibutyl-cGMP (a cell-permeable cyclic nucleotide analogue) produced a complex response that involved relaxation, contraction and biphasic (Moon, 2002). It is suggested that NO-mediated contraction involves cGMP production as it was inhibited by methylene blue, a soluble GC inhibitor. In contrast, inhibition of sGC did not affect the relaxant response produced by SNP, implying that NO and cGMP may act independently in the human bladder via different pathways (Moon, 2002). In a recent study, L-arginine elicited relaxation response in goat bladder, and it could not be inhibited by methylene blue (Baruah and Deka, 2021). Instead, the bladder relaxation response induced by L-arginine was attenuated by L-NAME and TEA. The authors thus concluded that L-arginine relaxed goat detrusor via a cGMP-independent pathway that activates K^+ channels.

The findings obtained by Moon (2002) were consistent with those reported by

Fujiwara et al. (2000), that the accumulation of cGMP in the cells did not contribute to DSM relaxation but enhanced EFS-induced contractions (Fujiwara et al., 2000). In another study, SNP and SIN-1 enhanced the spontaneous contractions in guinea pig bladders by accelerating spontaneous action potentials (Yanai et al., 2008). However, the enhancement of spontaneous contractions was thought to be mediated via cGMP-independent mechanisms that involve the release of Ca^{2+} from intracellular stores. These studies indicate that both cGMP-dependent and -independent pathways are implicated in NO-induced DSM contraction. Noteworthily, the effect of NO in bladder could be species-specific as the current evidence of NO-induced contraction or relaxation was obtained from a wide range of species, including human (Moon, 2002; James et al., 1993), goat (Vijayraj et al., 2011; Baruah and Deka, 2021), guinea pig (Yanai et al., 2008), rat (Chung et al., 1996; Vignozzi et al., 2012) and mouse (Fujiwara et al., 2000).

Additionally, interstitial cells could contribute to the modulation of DSM contractility via cGMP. According to Smet et al. (1996), DSM did not express cGMP-immunoreactivity following stimulation with SNP, but interstitial cells exhibited an intense induction of cGMP-immunoreactivity. Bladder interstitial cells are morphologically like those of Cajal and have specialised pace-making properties. They are responsible for signal transmission between DSM bundles, from efferent nerves to DSM and from the urothelium to afferent nerves (Kubota et al., 2011). However, in studies using isolated tissues, it is impossible to eliminate interstitial cells during tissue preparation to examine their roles as they lie along the lamina propria and within the DSM bundles (Figure 4.1).

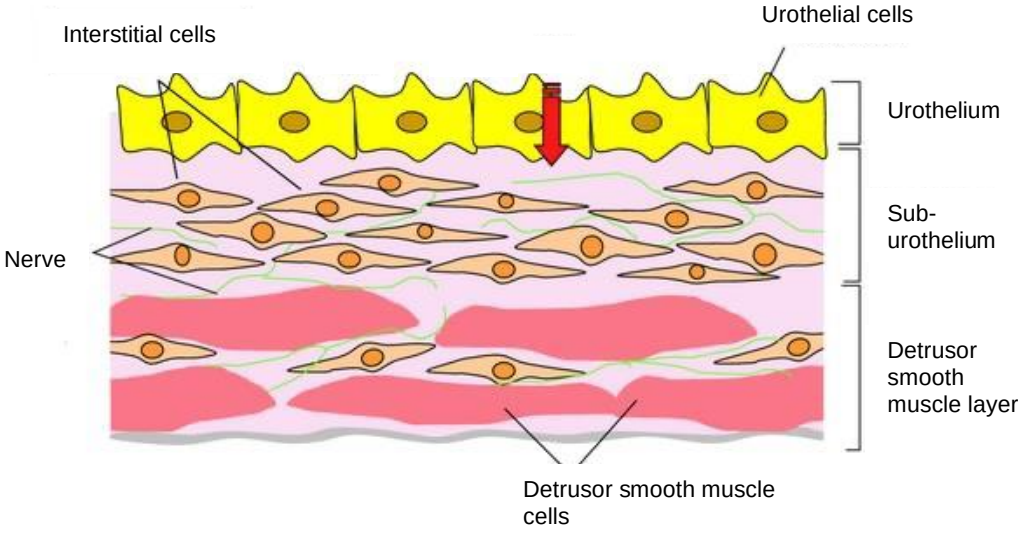

Figure 4.1 Distribution interstitial cells in the bladder and their interaction with urothelium, nerves and smooth muscle cells. *Adapted from Kubota et al. (2011).*

Several in vivo studies have been conducted to evaluate the role of NO in bladder. Long-term NO deficiency induced by L-NAME treatment increased the contractile responses of rat bladder towards carbachol and reduced β_3-adrenoceptor-mediated relaxation (Mónica et al., 2008). Besides, the administration of nicorandil, an NO donor and ATP-sensitive K^+ channel opener, has been shown to significantly reduce the micturition frequency in spontaneously hypertensive rats (SHRs) (Saito et al., 2012). These studies indicate that NO involves in the regulation of bladder contractility. The establishment of the role of NO in the bladder also provides a basis for therapeutic intervention in LUTS.

4.2 Study aim and objectives

This study aimed to elucidate the mechanistic pathway of NO-mediated relaxation in rat bladder using SNP. This will provide further evidence to substantiate the proposed mechanisms of action of *O. sinensis* (OCS02-CWE) as elucidated in **Chapter 3**. The specific objectives of this chapter were as follows:

i. To investigate the effects of SNP in carbachol- and KCl-pre-contracted bladder strips
ii. To examine the role of urothelium in SNP-induced bladder relaxation
iii. To investigate the role of cGMP in SNP-induced bladder relaxation
iv. To assess the role of K^+ channels in SNP-induced bladder relaxation
v. To investigate the role of intra- and extracellular Ca^{2+} in the relaxation pathway of SNP
vi. To investigate the effect of OCS02-CWE and L-arginine on SNP-induced bladder relaxation

4.3 Materials and methods

4.3.1 Preparation of Krebs-Henseleit bicarbonate solution and drugs

Krebs solution was prepared as described in **section 2.3.1**. When Ca^{2+}-free Krebs solution was used, it was prepared in the similar composition as normal Krebs solution but without the inclusion of $CaCl_2$ in the solution. OCS02-CWE was prepared as described in **section 2.3.2**. The preparation of drugs used in this chapter including 2-APB, carbachol, IBMX, ODQ, SKF-96365, SNP, TEA, thapsigargin and ryanodine, were the same as listed in **section 3.3.1**. Their details including brands, mechanism of action, solvent used, stock concentration were listed in Table 3.1. L-arginine (Sigma Aldrich, USA) was dissolved in purified water to make a stock concentration of 1.0 M and glibenclamide (TCI, UK) was dissolved in 100% DMSO to make a stock concentration of 0.1 M.

4.3.2 Tissue preparation

Ethics approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB) (UNMC 12 and UNMC26, Appendix A). The experiments were conducted with male Sprague-Dawley rats (216 – 547g; 2 – 3 months old) purchased from UPM and UKM. The animals were sacrificed on the day of experiment by asphyxiation from CO_2. The bladder was isolated, prepared and mounted to the isolated tissue bath set up according to the steps detailed in **section 2.3.5**. In experiments to study the role of urothelium, the urothelium of bladder strips was removed as described in **section 3.3.3**.

4.3.3 Isometric tension recordings

4.3.3.1 Effect of SNP against KCl- and carbachol-induced pre-contraction

The bladder strips were pre-contracted with either 1 µM of carbachol or 60 mM of KCl. Upon establishing stable contractions, cumulative CRCs to SNP were constructed from 10 nM to 300 µM. SNP was added to the bath every 5 minutes or until the response plateaued. Purified water was used as vehicle control.

4.3.3.2 Role of urothelium in SNP-induced bladder relaxation

To investigate the role of urothelium in SNP-induced bladder relaxation, CRCs to SNP were constructed in urothelium-denuded strips pre-contracted with 1 µM of carbachol. The results were then compared with those obtained from **section 4.3.3.1**.

4.3.3.3 The role of cGMP in SNP-induced bladder relaxation

To study the involvement of cGMP in SNP-mediated relaxation, the bladder strips were pre-incubated with a soluble GC inhibitor, ODQ (30 µM), a non-selective PDE inhibitor, IBMX (10 µM) or vehicle control (DMSO 0.225% v/v) for 30 minutes. Then, the bladder strips were pre-contracted with 1 µM of carbachol prior to the construction of CRCs to SNP.

4.3.3.4 Effect of K^+ channel blockers on SNP-induced relaxation

To investigate the role of K^+ channels, the bladder strips were pre-incubated with a non-selective K^+ channel blocker, TEA (1 mM), and a K_{ATP} channel blocker, glibenclamide (10 µM, Deka & Brading, 2004) for 30 minutes. Then, the bladder strips were pre-contracted with 1 µM of carbachol prior to the construction of CRCs to SNP.

4.3.3.5 The role of intra- and extracellular Ca^{2+} in bladder relaxation mediated by SNP

The bladder strips were pre-incubated with 10 µM or 30 µM of SNP in Ca^{2+}-free Krebs solution supplemented with 1 mM of EGTA for 30 minutes. These concentrations were chosen based on the SNP cumulative CRCs in carbachol-pre-contracted bladder strips, where they achieved 80% – 90% of the maximum relaxation response. Then, a single concentration of carbachol (100 µM) was added into the bath to induce a contractile tone. Tissues were left in the bath for 1 hour to study the magnitude of contraction over time.

In another set of experiments to study the role of extracellular Ca^{2+}, the bladder strips were immersed in Ca^{2+}-free Krebs solution 30 minutes. Then, the tissues were pre-incubated with either 10 µM, 30 µM of SNP or vehicle control for another 30 minutes. 60 mM of KCl used to induce membrane depolarisation prior to the construction of cumulative CRCs to $CaCl_2$.

4.3.3.6 Effect of Ca^{2+} modulators on SNP-induced relaxation

The bladder strips were pre-incubated with either 100 µM of 2-APB, an IP_3R antagonist, 10 µM of SKF-96365, a non-selective TRPC channel blocker, 1 µM of thapsigargin, a SERCA pump inhibitor, and 10 µM of ryanodine, a RyR antagonist, for 30 minutes. DMSO 0.1% v/v was used as the vehicle control. Then, the bladder strips were pre-contracted with 1 µM of carbachol prior to the construction of CRCs to SNP.

4.3.3.7 Effect of OCS02-CWE and L-arginine on SNP-induced relaxation

In Chapter 3, we proposed that endogenous production of NO would antagonise the effect of exogenously supplied NO as SNP attenuated the bladder relaxant effect of OCS02-CWE. To verify this hypothesis, the effect of OCS02-CWE and L-arginine on SNP-induced relaxation was studied. The bladder strips were pre-incubated in either 5 mg/mL of OCS02-CWE, 1 mM of L-arginine (Baruah and Deka, 2021) or vehicle control (purified H_2O) for 30 minutes. Then, the bladder strips were pre-contracted with 1 µM of carbachol prior to the construction of CRCs to SNP.

4.3.4 Data analysis

Data were analysed and graphs were drawn using PRISM version 9.0 (GraphPad software). The data were presented as mean ± SEM of *n* number of animals. Concentration of drugs were presented in logarithm of concentration in molar (M) in all graphs. The percentage of tissue relaxation induced by SNP were expressed in carbachol-induced tone (Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.8 and Figure 4.9) whereas the percentage of tissue contraction induced by carbachol and $CaCl_2$ (Figure 4.6 and Figure 4.7) was expressed in KCl-induced tone. E_{max} and pEC_{50} were derived from the non-linear regression curves. When the response has not plateaued, the E_{max} was taken from the response produced at the highest concentration tested and pEC_{50} was not calculated (Figure 4.7). Statistical analysis was performed using Student's *t*-test to compare the mean between control and treatment group. One-way ANOVA was used to compare the means of three or more groups and *post hoc* test was performed when a significance is detected, $p < 0.05$. Two-way ANOVA followed by Tukey's *post hoc* test was used to compare all possible combinations within the groups. Results with $p < 0.05$ were considered statistically significant. Individual significance was indicated in asterisk symbol in table or graph, where '****' indicates $p < 0.0001$, '***' indicates $p < 0.001$, '**' indicates $p < 0.01$ and '*' indicates $p < 0.05$.

4.4 Results

4.4.1 SNP significantly relaxed carbachol-pre-contracted but not KCl-pre-contracted bladder strips

The pre-contractile tones induced by carbachol and KCl were 12.64 ± 2.23 mN and 15.05 ± 2.61 mN, respectively ($p > 0.05$). As shown in Figure 4.2, SNP significantly relaxed carbachol-pre-contracted bladder strips when compared to vehicle control (E_{max} = SNP, 50.91 ± 1.58%; vehicle control, 3.48 ± 5.97%, $p < 0.0001$). However, SNP did not significantly relax the bladder strips pre-contracted with KCl (E_{max} = 18.19 ± 3.48%, $p = 0.0621$ vs vehicle control). When comparing the E_{max} of SNP in carbachol-induced pre-contraction and KCl-induced pre-contraction, the maximum tissue relaxation induced by SNP was significantly different from each other (carbachol vs KCl; $p = 0.0003$).

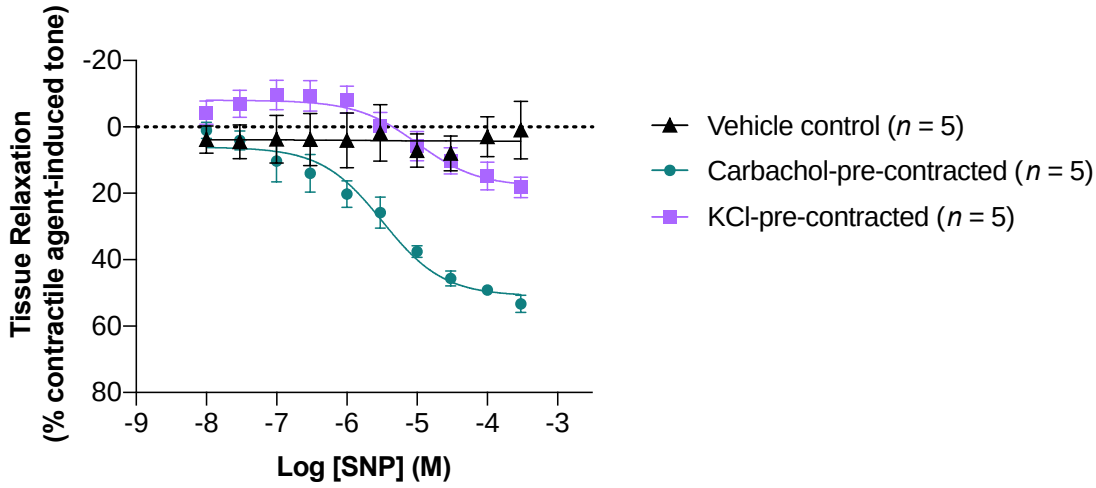

Figure 4.2 Effect of SNP on the bladder strips pre-contracted with carbachol or KCl. *Tissue relaxation has been expressed as the percentage of the respective contractile agent-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviation: KCl = potassium chloride; SNP = sodium nitroprusside)*

4.4.2 SNP-induced bladder relaxation was independent of urothelium

To confirm that SNP mediates its effect directly on the DSM, the relaxation response elicited by SNP was compared between urothelium-intact and -denuded bladder strips (Figure 4.3). Removal of urothelium did not alter the relaxation profile of SNP (E_{max} = urothelium-intact, 50.91 ± 1.58%; urothelium-denuded, 42.46 ± 5.28%, p = 0.1639; pEC_{50} = urothelium-intact, 5.63 ± 0.23; urothelium-denuded, 5.99 ± 0.15, p = 0.2358).

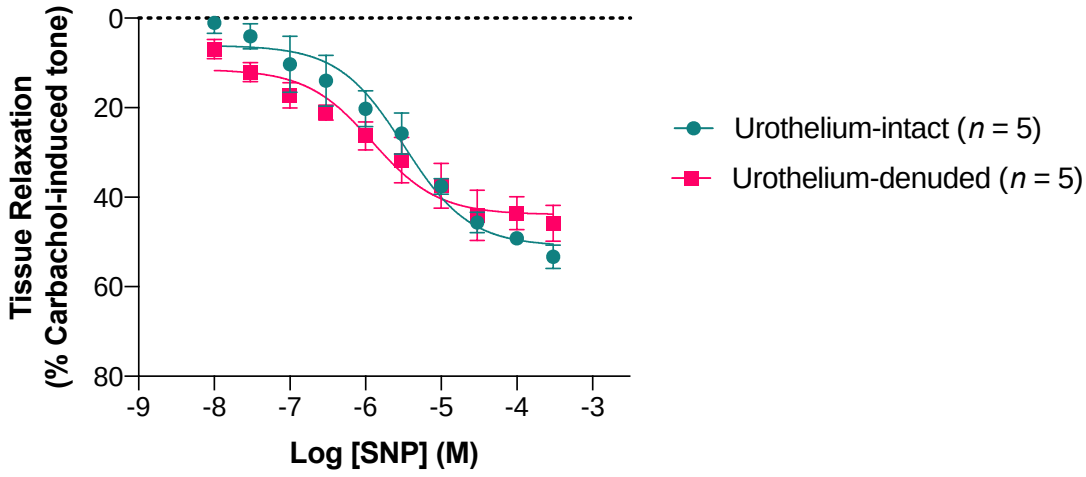

Figure 4.3 Effect of SNP in urothelium-intact and -denuded bladder strips pre-contracted with carbachol. *Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviation: SNP = sodium nitroprusside)*

4.4.3 ODQ and IBMX did not alter SNP-induced relaxation

Subsequently, to determine the involvement of cGMP in the action of SNP, the bladder strips were pre-incubated with either ODQ or IBMX. Our results showed that pre-incubation with these compounds slightly shifted the CRCs to the left, suggesting that ODQ and IBMX increased the potency of SNP. However, the change was not statistically significant (Figure 4.4). The maximum response of SNP was not affected by ODQ and IBMX. The values of E_{max} and pEC_{50} of SNP in the presence of vehicle control, ODQ and IBMX were summarised in Table 4.1.

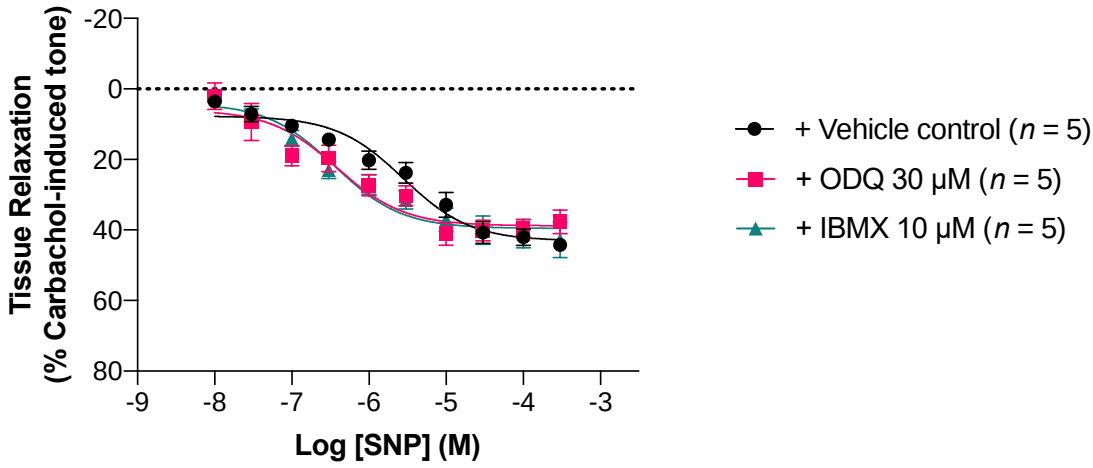

Figure 4.4 Effect of sGC inhibitor (ODQ) and PDE inhibitor (IBMX) on SNP-induced bladder relaxation. *Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviation: IBMX = 3-isobutyl-1-methylxanthine; ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SNP = sodium nitroprusside)*

Table 4.1 Maximum tissue relaxation (E_{max}) and potency (pEC_{50}) of SNP-induced bladder relaxation in the presence of sGC inhibitor or non-selective PDE inhibitor.

Treatment	E_{max} (%)	pEC_{50}
Vehicle control (DMSO 0.225% v/v)	43.16 ± 0.97	5.93 ± 0.29
ODQ 30 µM	38.94 ± 2.45	6.47 ± 0.18
IBMX 10 µM	40.06 ± 4.14	6.42 ± 0.15

Data are shown as mean ± SEM of 5 number of animals. One-way ANOVA where comparison of the means were made between vehicle control and the treatment groups, $p > 0.05$. (Abbreviation: IBMX = 3-isobutyl-1-methylxanthine; ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SNP = sodium nitroprusside)

4.4.4 SNP-induced relaxation was diminished by TEA but not glibenclamide

K^+ channel blockers glibenclamide and TEA were used to investigate the involvement of K^+ channels in SNP-induced bladder relaxation. Presence of glibenclamide did not alter the relaxant effect of SNP but TEA significantly suppressed its relaxation response (Figure 4.5). The values of Emax and pEC_{50} of SNP in the presence of vehicle control and K^+ channel blockers were summarised in Table 4.2.

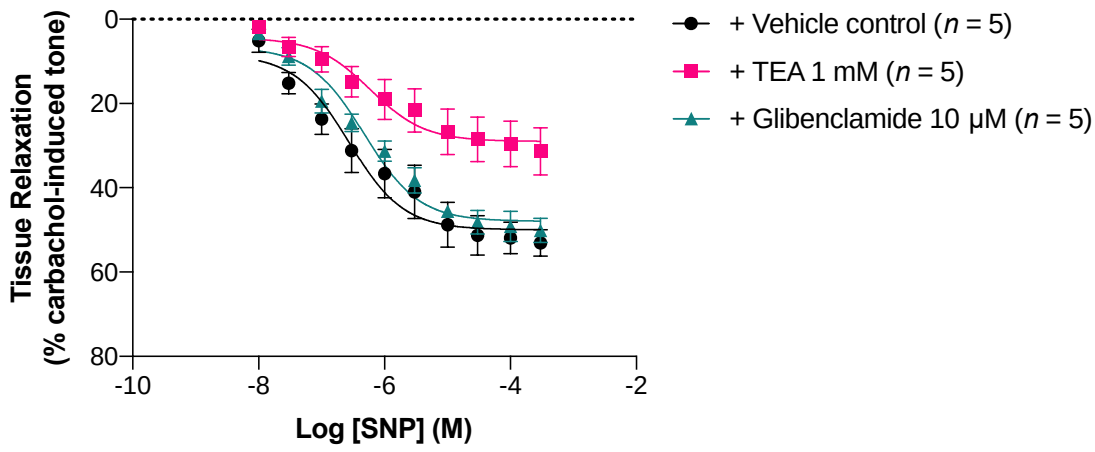

Figure 4.5 Effect of the non-selective K_{Ca} channel blocker, TEA or K_{ATP} channel blocker, glibenclamide, on SNP-induced bladder relaxation. *Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: TEA = tetraethylammonium; SNP = sodium nitroprusside).*

Table 4.2 Maximum tissue relaxation (E_{max}) and potency (pEC_{50}) of SNP-induced bladder relaxation in the presence of K^+ channel blockers.

Treatment	E_{max} (%)	pEC_{50}
Vehicle control (DMSO 0.1% v/v)	50.98 ± 4.23	6.44 ± 0.23
TEA 1 mM	30.31 ± 5.06 **(p = 0.0083)	6.03 ± 0.35
Glibenclamide 10 µM	48.14 ± 3.00	6.38 ± 0.21

Data are shown as mean ± SEM of 5 – 6 animals. One-way ANOVA followed by Dunnett's post hoc test was used to compare the mean of E_{max} or pEC_{50} between vehicle control and each treatment group. Individual p-values were stated in the respective columns when $p < 0.05$. (Abbreviations: TEA = tetraethylammonium; SNP = sodium nitroprusside)

4.4.5 SNP significantly suppressed carbachol-induced contraction in Ca^{2+}-free Krebs solution

In **section 3.4.12**, we have demonstrated that carbachol-induced contraction in Ca^{2+}-free Krebs solution supplemented with EGTA was largely caused by intracellular Ca^{2+} release. Based on the finding, tissues were pre-incubated with SNP at 10 µM and 30 µM to examine if they affect the intracellular Ca^{2+} release induced by carbachol. Results showed that in the presence of SNP, carbachol-induced contraction in the absence of external Ca^{2+} was significantly attenuated (Figure 4.6). The values of carbachol-induced contraction at each time point were summarised in Table 4.3.

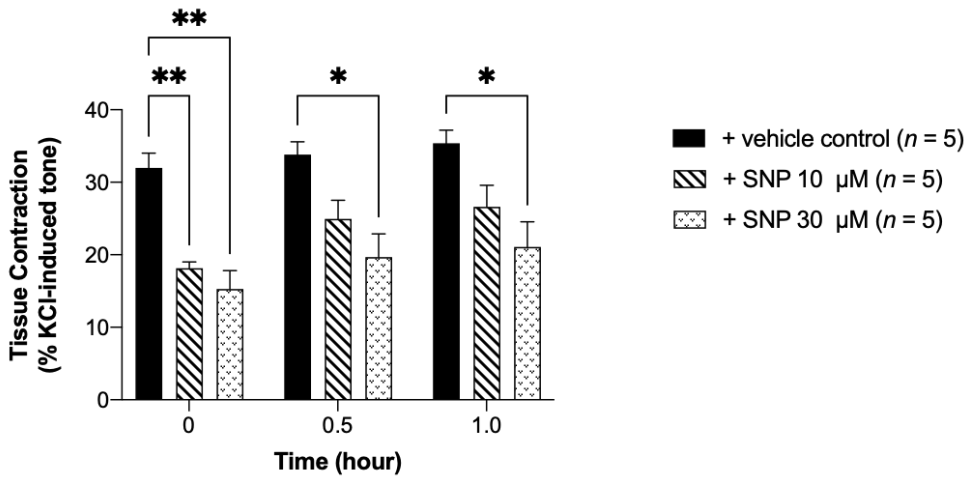

Figure 4.6 Effect of SNP on carbachol-induced contraction in Ca^{2+}-free Krebs solution supplemented with EGTA over time. *Tissue contraction has been expressed as a percentage of KCl-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA followed by Tukey's post hoc test was performed to compare between vehicle control and SNP at each time point, *p < 0.05, **p < 0.01. (Abbreviations: EGTA = Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid; KCl = potassium chloride; SNP = sodium nitroprusside)*

Table 4.3 Tissue contraction induced by carbachol in Ca^{2+}-free Krebs solution supplemented with EGTA.

Treatment/ Time (hour)	Tissue contraction (%)		
	Vehicle control	**SNP 10 µM**	**SNP 30 µM**
0	31.99 ± 2.03	18.15 ± 0.87 **(p = 0.0027)	15.28 ± 2.55 **(p = 0.0026)
0.5	33.82 ± 1.76	24.96 ± 2.56	19.69 ± 3.18 *(p = 0.0177)
1.0	35.40 ± 1.78	26.60 ± 2.99	21.08 ± 3.45 *(p = 0.0240)

Data are shown as mean ± SEM of 5 animals. Two-way ANOVA with Tukey's post hoc test was performed to compare between vehicle control and OCS02-CWE in either normal or free Krebs separately at each time point. Individual p-values were stated in the respective columns when $p < 0.05$. (Abbreviations: CWE = cold water extract, KCl = potassium chloride; SNP = sodium nitroprusside)

4.4.6 SNP marginally inhibited Ca^{2+}-induced bladder contraction

Then, the role of extracellular Ca^{2+} in SNP-induced relaxation was investigated. Pre-incubation with SNP at 10 µM and 30 µM marginally reduced the maximum contraction induced by Ca^{2+} compared to vehicle control (E_{max} = +vehicle control, 172.70 ± 15.02%; +SNP 10 µM, 126.80 ± 12.20%, p = 0.0932; +SNP 30 µM, 109.80 ± 14.77%, p = 0.0207) (Figure 4.7).

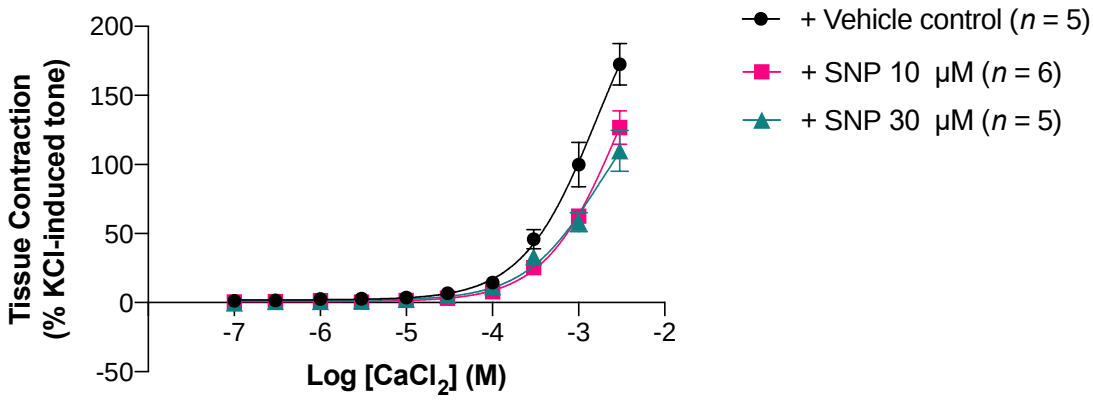

Figure 4.7 Effect of SNP on $CaCl_2$-induced bladder contraction in Ca^{2+}-free Krebs solution. *Tissue contraction has been expressed as the percentage of 60 mM KCl-induced contraction. Data are shown as mean ± SEM of n number of animals. (Abbreviations: KCl = potassium chloride; $CaCl_2$ = calcium chloride; SNP = sodium nitroprusside).*

4.4.7 SNP-induced relaxation was attenuated by thapsigargin

Following the above findings where both intracellular and extracellular Ca^{2+} play a role in the action of SNP, the effects of Ca^{2+} modulators on SNP-induced relaxation were investigated. For the ease of visualisation, the results were categorised in different panels according to the mechanism of action of drugs. This arrangement does not affect the statistical analysis as the comparison was made altogether against the vehicle control. RyR blocker, ryanodine, did not alter SNP-induced relaxation in the bladder strips (Figure 4.8A). In the presence of IP_3 receptor antagonist, 2-APB, SNP-induced relaxation seemed to be reduced but the effect was not statistically significant (Figure 4.8A). In contrast, SERCA inhibitor, thapsigargin, significantly reduced the relaxation response induced by SNP (Figure 4.8B). Then, SKF-96365 was used to investigate the involvement of TRPC channels, L-type VGCCs and SOCCs. Following pre-incubation with SKF-96365, SNP-induced relaxation appeared to be potentiated but the change was not statistically significant (Figure 4.8C). The values of E_{max} and pEC_{50} of SNP in the presence of each treatment were summarised in Table 4.4.

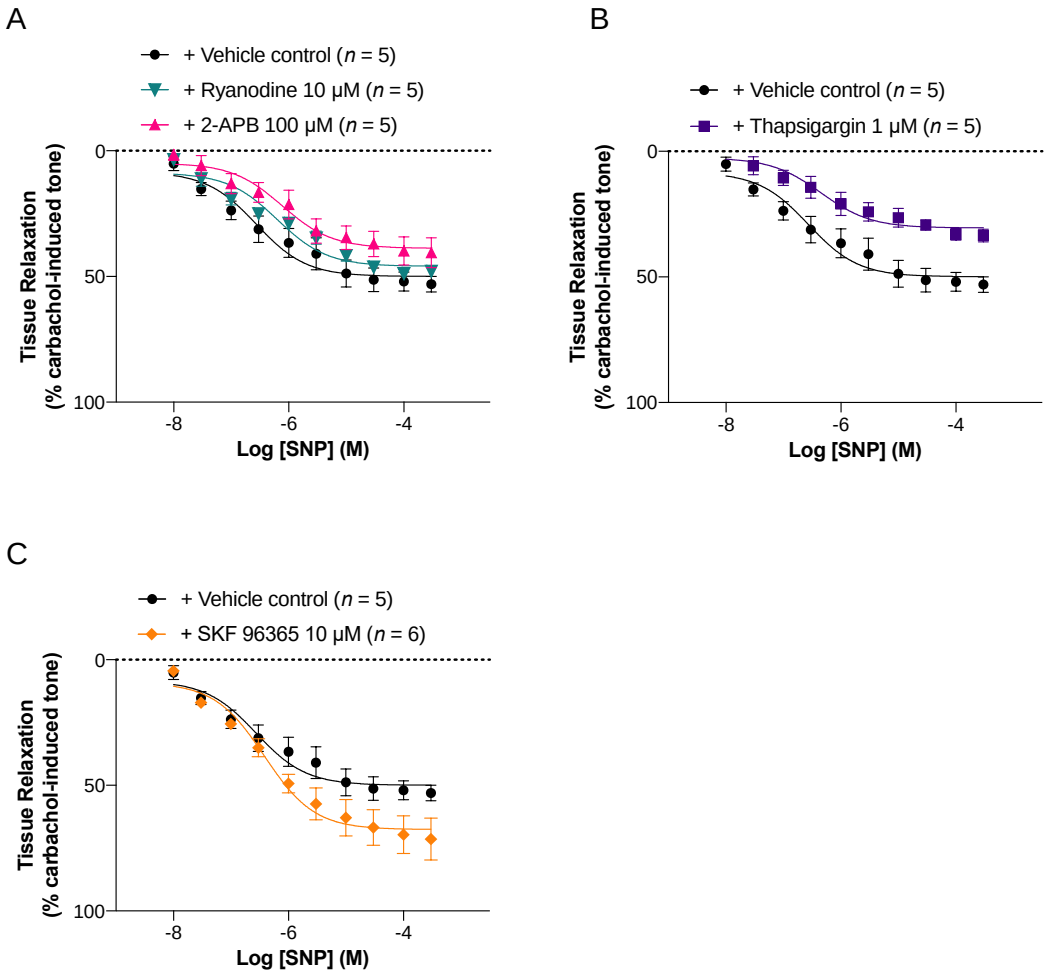

Figure 4.8 Effect of Ca^{2+} modulators on SNP-induced relaxation. *CRCs to SNP were constructed in the presence of (A) RyR blocker, ryanodine and IP_3R antagonist, 2-APB; (B) SERCA inhibitor, thapsigargin; (C) TRPC channel, SOCC and L-type VGCC blocker, SKF-96365. Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; CRC = concentration-response curve; IP_3R = inositol triphosphate receptor; RyR = ryanodine receptor; SERCA = sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SNP = sodium nitroprusside; SOCC = store-operated Ca^{2+} channel; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

Table 4.4 Maximum tissue relaxation (E_{max}) and potency (pEC_{50}) of SNP-induced relaxation in the presence of Ca^{2+} modulators.

Treatment	E_{max} (%)	pEC_{50}
Vehicle control (DMSO 0.1% v/v)	50.98 ± 4.23	6.44 ± 0.23
2-APB 100 µM	39.55 ± 5.26	6.20 ± 0.25
Ryanodine 10 µM	47.03 ± 1.74	6.12 ± 0.26
SKF-96325 10 µM	67.75 ± 7.59	6.45 ± 0.08
Thapsigargin 1 µM	31.32 ± 1.93 *(p = 0.0435)	6.33 ± 0.27

Data are shown as mean ± SEM of 5 – 6 animals. One-way ANOVA followed by Dunnett's post hoc test was used to compare the mean between vehicle control and each treatment group. Individual p-values were stated in the respective column when $p < 0.05$. (Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; SNP = sodium nitroprusside)

4.4.8 OCS02-CWE and L-arginine attenuated SNP-induced relaxation

When the bladder strips were pre-incubated with OCS02-CWE, the relaxation response induced by SNP was significantly attenuated (Figure 4.9). The effect was mirrored by L-arginine. The values of E_{max} and pEC_{50} of SNP in the presence of vehicle control, OCS02-CWE and L-arginine were summarised in Table 4.5.

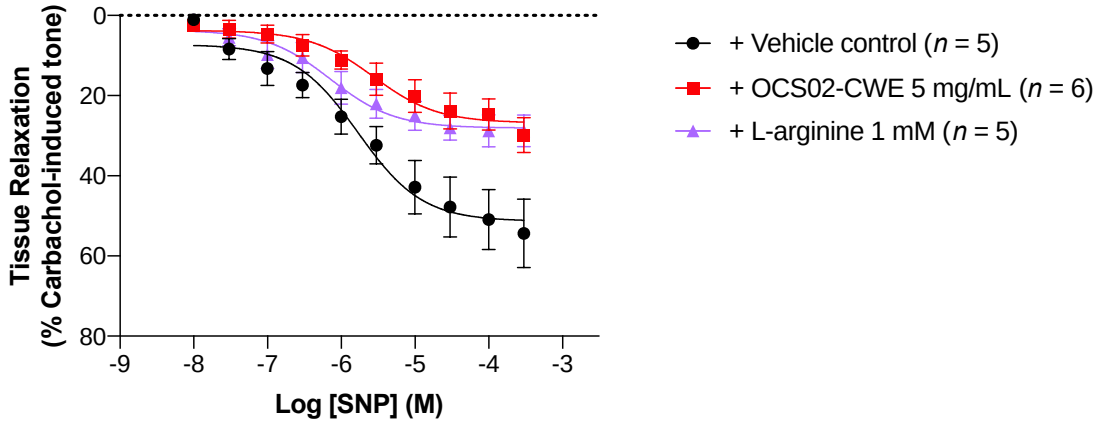

Figure 4.9 Effect of OCS02-CWE and L-arginine on SNP-induced bladder relaxation. *Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CWE = cold water extract; SNP = sodium nitroprusside).*

Table 4.5 Maximum tissue relaxation (E_{max}) and potency (pEC_{50}) of SNP-induced bladder relaxation in the presence of vehicle control, OCS02-CWE or L-arginine.

Treatment	E_{max} (%)	pEC_{50}
Vehicle control (purified water)	51.62 ± 7.88	5.93 ± 0.29
OCS02-CWE 5 mg/mL	28.30 ± 4.23 *(p = 0.0153)	6.47 ± 0.18
L-arginine 1 mM	27.97 ± 3.31 *(p = 0.0182)	6.42 ± 0.15

Data are shown as mean ± SEM of 5 – 6 animals. One-way ANOVA followed by Dunnett's post hoc test was used to compare the mean between vehicle control and each treatment group, $p = 0.0131$ for E_{max} and $p = 0.0290$ for pEC50. Individual p-values were stated in the respective columns when $p < 0.05$.

4.5 Discussion

In recent years, there has been an increased interest in the role of NO in regulating bladder function. Nonetheless, the effects of NO in bladder and its mechanisms of action remain controversial due to its varied responses in different species. Both excitatory and inhibitory effects of NO have been demonstrated in the bladder of various species as outlined in **section 4.1**. The present study was designed to elucidate the mechanistic pathway of SNP, an established NO donor, in the rat isolated bladder. Further understanding of the role of NO was required to provide evidence to substantiate the mechanism of action of OCS02-CWE in bladder relaxation through the regulation of NO as described in **section 3.5.1.5** (**Chapter 3)**.

4.5.1 Role of contractile agents

SNP produced a marked relaxation response in carbachol-pre-contracted but not in KCl-pre-contracted bladder strips, implying that the relaxant effect of NO was dependent on the contractile agent. This points to the cellular mechanism of Ca^{2+} mobilisation or G-protein subunits mobilisation following activation of the muscarinic receptor. Since 60 mM of KCl and 1 µM of carbachol produced similar pre-contractile tones, the difference in the relaxation capacity of SNP was not likely to be caused by the levels of contraction. It is well-documented that KCl-induced contraction is caused by depolarisation of the cell membrane that led to the opening of VGCCs, allowing the influx of extracellular Ca^{2+}. Therefore, SNP-induced relaxation response is unlikely to involve direct inhibition of the VGCCs. In contrast, SNP may have a role in intracellular Ca^{2+} mobilisation. While the contraction induced by $G_{q/11}$ activation was largely dependent on extracellular Ca^{2+}, release of Ca^{2+} from intracellular stores remains essential in the maintenance of its tonic contraction (Oh et al., 1999; Schneider et al., 2004).

In line with our findings, van Hove et al. (2009) demonstrated that both endogenous and exogenous NO produced significantly greater vasorelaxant response in mouse aortic segments pre-contracted with phenylephrine than those pre-contracted with KCl. In VSM cells, when NO reduces KCl-induced contractions, the relaxation was mainly mediated via activation of sGC and subsequent cGMP-mediated effects, which were independent of intracellular Ca^{2+} levels. In turn, when recruitment of Ca^{2+} from intracellular stores was involved during pre-contraction, NO-induced relaxation was cGMP-independent (van Hove et al., 2009).

4.5.2 Role of urothelium

To our knowledge, no study on the role of urothelium in SNP-induced response has been published when this experiment was carried out. Our results showed that the removal of urothelium did not affect the relaxation response elicited by SNP in rat bladder, suggesting that the bladder relaxant effect of SNP is urothelium-independent and is attributed to its direct action on the DSM. This agrees with the general understanding on the action of SNP where it releases NO to evoke smooth muscle relaxation (Bates et al., 1991) and confirms the notion that SNP-induced relaxation is a direct response of NO on the DSM.

4.5.3 Role of cGMP

Regardless of the origin of NO, either endogenously or exogenously produced, there is limited information regarding the role of cGMP in NO-induced bladder relaxation. In our experiments, the sGC inhibitor, ODQ, did not alter the bladder relaxant effect of SNP, implying that SNP-induced relaxation in rat bladder does not require the activation of sGC that synthesises cGMP from GTP. This finding is in line with a previous study where inhibition of sGC using methylene blue did not affect SNP-induced relaxation response in human bladder strips (Moon, 2002). In smooth muscle

cells, PDE hydrolyses cGMP to inactive 5'-GMP. Thus, inhibition of PDE retains a high level of cGMP in these cells to promote or maintain the relaxation response. If SNP promotes bladder relaxation via generation of cGMP, the presence of PDE inhibitor (such as IBMX) would potentiate SNP-induced relaxation. The inability of IBMX, to alter the SNP-induced relaxation thus confirms that its signalling pathway is independent of cGMP. Comparably, Fujiwara et al. (2000) demonstrated that an increased cGMP level in the DSM did not contribute to the bladder relaxation.

However, our findings opposed those reported by Artim et al. (2009) who demonstrated that the inhibition of sGC and the presence of 8-bromo-cGMP (a membrane-permeable cGMP analogue) diminished the relaxant effect of NO donor *S*-nitroso-*N*-acetyl-$_{DL}$-penicillamine (SNAP). Other than the choice of NO donor, age of the animals used may also contribute to the discrepancies in both studies. Artim et al. (2009) studied the effects of SNAP in neonatal rat bladder strips while adult rat bladder strips were utilised in the present study. Adult rodents have been shown to have a lower expression of PDE mRNA. Sanchez et al. (1998) reported that the levels of PDE5 mRNA levels were the most abundant in the lungs of neonatal rats but were significantly lower in the adult lungs. The expression of PDE3A in adult rat bladder was also significantly lower compared to the neonatal rat bladder (Zhu et al., 2017).

4.5.4 Role of K^+ channels

K^+ channels have been implicated in the role of NO-induced vasorelaxation but their roles in the NO-mediated bladder relaxation remains scarce. BK channels, belonging to the group of K_{Ca} channels, are the most physiologically relevant K^+ channel in the DSM (Petkov, 2014). However, several studies have suggested the involvement of K_{ATP} in NO-mediated responses. In the smooth muscle of rabbit mesenteric arteries, NO induces hyperpolarisation by activating K_{ATP} channels following increase of cGMP level (Murphy and Brayden, 1995). Similarly, Deka & Brading (2004) showed that NO activates K_{ATP} channels in guinea-pig bladder myocytes via a cGMP-dependent mechanism. Glibenclamide and ODQ inhibited the SIN-activated inward current in the whole cell patch clamp experiments (Deka and Brading, 2004). These studies suggest that the activation of K_{ATP} channels by NO is accompanied by the rise of cGMP levels in the cells.

To examine the possible role of K_{ATP} channels in the bladder relaxation induced by SNP, the CRCs to SNP were constructed in the presence of glibenclamide. Our results showed that the SNP-induced relaxation was not affected by the presence of glibenclamide, thus, excluding the possible participation of K_{ATP} channels. In contrast, the SNP-induced bladder relaxation was substantially reduced in the presence of TEA. The reduction of SNP-induced relaxation by TEA implies that SNP may activate K_{Ca} channels and generate hyperpolarisation. As a result, Ca^{2+} influx via L-type VGCCs is limited.

4.5.5 NO-sensitive Ca^{2+} pools

At this point, results in the present study indicate that SNP elicits rat bladder relaxation via cGMP-independent pathway that may involve the activation of K_V or K_{Ca} channels. The evidence that we gathered include its limited relaxation response in high K^+ and in the presence of TEA. Similar observations have been reported in several studies that investigated the mechanism of NO in smooth muscle relaxation. In non-pregnant human myometrium, charybdotoxin-sensitive relaxation induced by NO donor CysNO was not augmented by the GC inhibitor methylene blue (Bradley et al., 1998). In goat bladder strips, TEA also inhibited NO-induced relaxation that is insensitive to methylene blue (Baruah and Deka, 2021). The participation of K_{Ca} channels in SNP-mediated relaxation indicates that it plays a role in Ca^{2+} mobilisation. Therefore, subsequent experiments investigated the effect of SNP on intracellular Ca^{2+} release and extracellular Ca^{2+} influx.

In Chapter 3, we have shown that carbachol-induced contraction was greatly attenuated in the absence of external Ca^{2+} so the remaining contractile tone elicited by carbachol in Ca^{2+}-free Krebs supplemented with EGTA was thought to be due to Ca^{2+} release from the intracellular store initiated by the downstream signalling of $G_{q/11}$ protein. In the presence of SNP, carbachol-induced contraction in the Ca^{2+}-free environment was markedly reduced. This finding corroborates the involvement of K_{Ca} channels in the mechanism of action of SNP because activation of K_{Ca} channels may antagonise muscarinic receptors (Kume et al., 1995).

The results also suggest that SNP may inhibit Ca^{2+} release from the intracellular store as activation of IP_3R by $G_{q/11}$ activation also triggers Ca^{2+} release via RyRs (Fritz et al., 2007). In a previous study, SNAP was shown to reduce acetylcholine- and caffeine-induced Ca^{2+} increment in porcine tracheal smooth muscle

cells by inhibiting Ca^{2+} release through both IP_3 and RyR (Kannan et al., 1997). Likewise, Perez-Zoghbi et al. (2010) demonstrated that NO donor NOC-5 inhibited the increase of intracellular Ca^{2+} and airway contraction induced by IP_3. In the present study, while ryanodine and 2-APB seemed to reduce the magnitude of SNP-induced relaxation response in the bladder strips, the reduction in the relaxation response was not statistically significant.

On the contrary, thapsigargin significantly lessened SNP-induced bladder relaxation. Therefore, SNP may activate SERCA to promote reuptake of Ca^{2+} into the SR and lead to bladder relaxation. While this finding presents obvious disagreements with those reported by Kannan et al. (1997) and Perez-Zoghbi et al. (2010), it substantiates the findings obtained by Cogolludo et al. (2001) and van Hove et al. (2009). In isolated piglet pulmonary and mesenteric arteries, SNP reduced the intracellular Ca^{2+} level and causes relaxation through the activation of SERCA (Cogolludo et al., 2001). It is also noted that the experimental setup of Cogolludo et al. (2001) was similar to ours and the tissues were pre-incubated with thapsigargin in the low micromolar range for at least 30 minutes. Cogolludo et al. (2001) also confirmed this finding using Fura-2-loaded arteries. During the Ca^{2+}-imaging experiments, thapsigargin markedly inhibited the relaxation and reduction of the intracellular Ca^{2+} induced by SNP. Therefore, the discrepancies in the results among these studies could be due to the incubation time and concentration of thapsigargin, the choice of tissue types and the selection of NO donors. A recent study compared the effect between the SNP and cis-[Ru(bpy)2(py)(NO2)](PF6) (RuBPY) in mesenteric resistance arteries obtained from renal hypertensive (2K-1C) and normotensive (2K) rats (Araújo et al., 2019). The results showed that SNP and RuBPY released comparable amounts of NO in the rats but only the SNP-induced relaxation

in normotensive 2K rat mesenteries arteries was sensitive to thapsigargin. In contrast, RuBPY-induced relaxation did not involve SERCA activation in both normotensive and hypertensive arteries (Araújo et al., 2019).

While preventing the rise of $[Ca^{2+}]_i$, this study also questioned whether SNP has a role in regulating the extracellular Ca^{2+} influx, since it relaxed carbachol-induced tone and activated K_{Ca} channels. It is well-established that Ca^{2+} influx through VGCCs plays a major role in mediating contractions in the DSM; and activation of K_{Ca} channels lead to closure of VGCCs. Our results showed that SNP only marginally inhibited Ca^{2+}-induced contractions, corresponds to its attenuated relaxant effect in KCl-induced contracted bladder strips. Although it shows similarity in extracellular Ca^{2+} inhibitory effect as reported in goat bladder goat bladder (Vijayraj et al., 2011), it did not exhibit a clear concentration-dependent effect. Therefore, it is unlikely that SNP causes direct inhibition on the membrane permeable Ca^{2+} channels, as suggested by Vijayraj et al. (2011). This speculation can also be supported by the slight potentiation of its relaxation response by SKF-96365 (not statistically significant). It implies that SNP mediates relaxation through a different pathway initiated by SKF-96365. When SKF-96365 inhibits L-type VGCCs and TRPC channels and limits Ca^{2+} influx into the cells, carbachol utilises stored Ca^{2+} to elicit contraction (Andersson and Arner, 2004). Subsequently, as K_{Ca} channel and SERCA were activated by SNP-derived NO, the concentration of intracellular Ca^{2+} was reduced and resulted in relaxation response. These purported mechanisms of action of SNP are depicted in Figure 4.10.

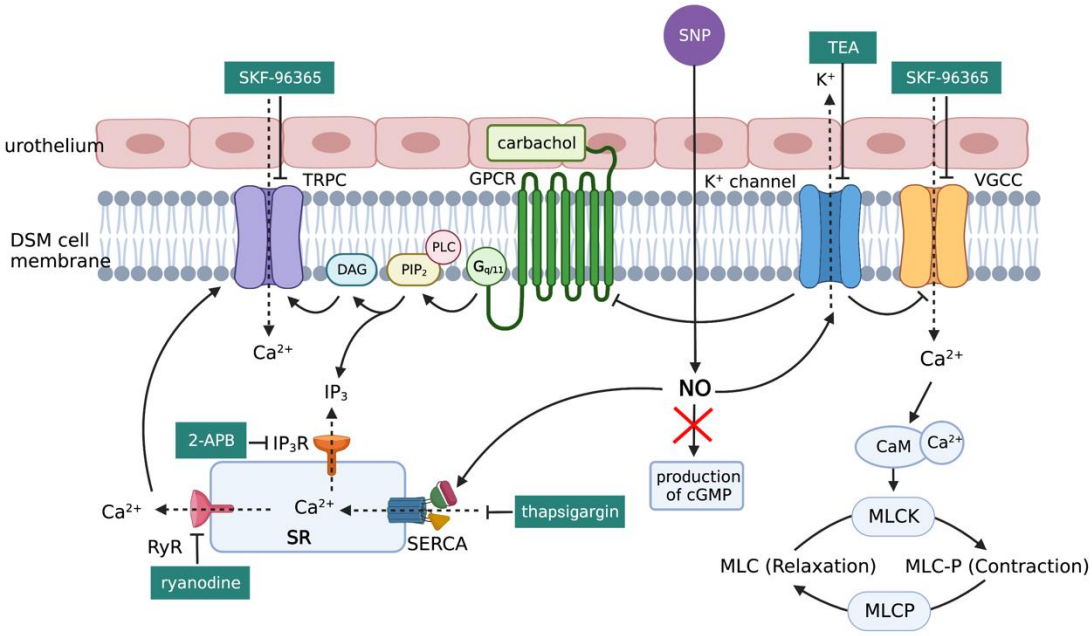

Figure 4.10 Schematic diagram illustrating the purported mechanisms of action of SNP in rat bladder. *Solid pointed arrows denote downstream effectors activation; blunt arrows denote inhibitory effects; and dashed arrows denote flow of Ca^{2+} or K^{+} ions. (Abbreviations: 2-APB = 2-aminoethyl diphenylborinate; CaM = calmodulin; cGMP = cyclic guanosine monophosphate; DAG = diacylglycerol; DSM = detrusor smooth muscle; IP_3 = inositol triphosphate; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; NO = nitric oxide; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SNP = sodium nitroprusside; SR = sarcoplasmic reticulum; SOCC = store-operated Ca^{2+} channel; TEA = tetraethylammonium; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

4.5.6 Effect of OCS02-CWE and L-arginine

Findings in Chapter 3 suggested that OCS02-CWE may induce membrane depolarisation and activate VGCCs to evoke the DSM transient contraction. The follow-on sustained relaxation response by OCS02-CWE is explained through its role in NO regulation and activation of SERCA. However, pre-incubation with SNP reduced the relaxation response induced by OCS02-CWE. This led to the speculation that SNP which supplies NO exogenously may interfere with the urothelial-derived NO. To substantiate this finding, we examined the effect of OCS02-CWE pre-incubation on SNP-induced relaxation. The relaxant effect of SNP was markedly attenuated in the presence of OCS02-CWE. L-arginine also exhibited similar inhibitory effect on SNP-induced relaxation response although it did not produce significant relaxation response at the same concentration tested (data not shown). L-arginine has also been shown to antagonise SNP-induced relaxation in renal arteries of normotensive rats but not in renal arteries of spontaneously hypertensive rats (Oreščanin and Milovanović, 2006). By modifying the chemical versatility of NO into redox active forms, $NO^{\bullet}$, NO^{+} and NO^{-}, L-arginine may affect the role of NO^{+} formed by SNP. Detailed explanation cannot be specified as elucidation of mechanistic pathways of different NO forms was beyond the scope of this study. However, if the postulated mechanism of SNP that involves activation SERCA were true, it is sensible that the stimulation of Ca^{2+} reuptake by OCS02-CWE during pre-incubation would diminish the effect of SNP that acts on the same target protein.

4.6 Chapter summary

In this chapter, evidence gathered point to a different relaxing capacity of SNP in depolarisation- or carbachol-induced contraction which is dependent on the pathways of Ca^{2+} mobilisation during the generation of contractile tone. SNP-induced relaxation in carbachol-pre-contracted bladder strips (muscarinic receptor-mediated contraction) is proposed to be through a cGMP-independent pathway. It is likely to mediate via activation of TEA-sensitive K^+ channels and hyperpolarisation. Besides, activation of SERCA that reduces intracellular Ca^{2+} level may be involved in its mechanism of action. Since neither TEA nor thapsigargin completely eliminated SNP-induced relaxation, it is speculated that both pathways are involved in the SNP-induced relaxation in the rat bladder. In addition to highlighting the role of NO in bladder physiology, these findings allow a better understanding on the NO-mediated relaxation pathway of OCS02-CWE, the focus of this thesis. While the effect of OCS02-CWE cannot be portrayed by SNP alone due to their obvious differences such as chemical and physical properties, and the way they generate NO, these data suggest that the activation of SERCA is involved in NO-mediated relaxation, particularly in rat bladder.

5 The Combined Effects of *Ophiocordyceps sinensis* and Another Medicinal Fungus, *Lignosus rhinocerus,* in Bladder Relaxation

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5.1 Introduction

5.1.1 Combination therapy in OAB

In many medical conditions, combination therapy that can be defined as the use of more than one method or one drug to treat a disease, often deliver better efficacy, decrease toxicity and reduce development of drug resistance. For example, in hypertension, combination therapy using drugs in different therapeutic class such as α-blockers, β- blockers, Ca^{2+} channel blocker, angiotensin-converting enzyme (ACE) inhibitor and angiotensin II receptor blocker (ARB), have been proven to provide a better treatment outcome (Richards and Tobe, 2014; Düsing et al., 2017). Recent clinical trials have also recommended the use of low-dose combination therapy for more effective blood pressure lowering effects and to reduce the occurrence of adverse reactions (Atkins and Chow, 2020).

Due to the limitations in the current treatment of OAB, the therapeutic effects of combining treatment modalities are also being investigated. In **section 1.2.2**, the existing therapeutic options for the treatment of OAB have been outlined, including the first-line therapies that focus on behavioural modifications, second-line therapies that are pharmacologic (muscarinic antagonists and β_3-adrenoceptor agonists), third-

line therapies such as peripheral tibial nerve stimulation (PTNS), sacral neuromodulation (SNS) and botulinum toxins (BTX) intradetrusor injection, and fourth-line therapies that involve augmentation cystoplasty or urinary diversion in severe, refractory and complicated OAB.

Several studies have demonstrated the efficacy of several combined therapies including concomitant use of two or more antimuscarinic agents, an antimuscarinic agent with a β_3-adrenoceptor agonist, bladder training with medications, and PTNS on top of medications. Nardulli et al. (2012) compared the effects of oxybutynin plus trospium chloride versus oxybutynin plus solifenacin in a randomised, double-blind, controlled trials for 12 weeks. It was found that both groups of patients experienced significantly fewer episodes of incontinence with improved bladder compliance, bladder capacity and volume voided. However, patients receiving oxybutynin and solifenacin experienced more side effects (Nardulli et al. 2012). Likewise, combined use of propiverine with either tolterodine, solifenacin or oxybutynin, has significantly reduced urgency and daytime voiding, as compared to monotherapy (Yi et al., 2013).

Although the combined use of antimuscarinic agents provides desirable treatment outcome, the occurrence of side effects is generally higher than baseline antimuscarinic monotherapy. Therefore, when drugs that target the same receptors are used, in this case, muscarinic receptors, the outcome may not be desirable due to the increased side effects. Combination therapy should thus focus on targeting complementary receptors with a lower dose to achieve the therapeutic desire with lower risk of side effects. For instance, the three largest clinical trials that investigated the use of mirabegron (β_3-adrenoceptor agonist) and solifenacin (antimuscarinic) i.e., SYMPHONY (randomised, double-blind, parallel-group, factorial design phase 2 study), BESIDE (randomised, double-blind, parallel-group, phase 3B study) and

SYNERGY (randomised, double-blind, parallel-group, placebo- and active-controlled phase 3 study) reported that the combination use of these two therapeutic agents significantly improved OAB symptoms when compared to mirabegron or solifenacin monotherapy (Abrams et al., 2015; Drake et al., 2016; Herschorn et al., 2017).

While behavioural therapy is regarded as the first-line therapy for OAB, many patients do not fully understand or apply lifestyle modifications before starting pharmacotherapy (Alkis et al., 2022). According to SOLAR (SOLifenacine Alone and with simplified bladder Re-training), a prospective, randomised, parallel-group, open-label study in patients with OAB, bladder training plus solifenacin has significantly improved urinary frequency and overall treatment satisfaction (Mattiasson et al., 2010). However, the combined treatment did not improve patients' episodes of urge incontinence.

Investigations on combination therapy with third-line treatments (PTNS, SNM and BTX injection) remain scarce and involve only a small number of participants. From a randomised controlled trial involving 105 women, the combination of PTNS and an antimuscarinic agent (solifenacin) showed greater effectiveness in relieving OAB symptoms than solifenacin alone or PTNS alone (Vecchioli-Scaldazza and Morosetti, 2018). Currently, the University of Alabama, USA is undertaking a study to explore the efficacy of PTNS and mirabegron combined treatment (ClinicalTrials.gov identifier: NCT04907032). To date, there is no robust data available on the combined use of SNM or BTX injection with conventional OAB medications.

Traditional medicines also represent a great example of combination therapy. For example, in TCM, several herbs, fungi or other ingredients are combined according to strict rules to form prescriptions. In the management of OAB, Gosha-

jinki-gan which contain ten ingredients and SQW which contain 3 ingredients are commonly used (**section 1.2.3**, **Chapter 1**). In the light of these studies, combination therapy is a promising treatment for OAB by enhancing treatment effectiveness and/or decreasing side effects induced by medications. The utilisation of combination therapy should focus on the process of discovering new OAB treatments and therapeutic targets.

5.1.2 Synergism, additive and antagonism in combination therapy

During the development of a combination therapy, examination of interactions between the drugs is often the first step. The interactions can be categorised into synergistic (positive interaction), additive (non-interactive) and antagonistic (negative interaction) (Tallarida, 2006). Synergism is a coordinated or correlated action of two or more substances so that the combined action is greater than the sum of each acting separately. Additive effect indicates that the combined effect of substances is a pure summation effect of the substances taken separately whereas antagonistic indicates that a combined effect is less than the additive effect.

Drug interactions can be examined at different levels ranging from molecular targets, disease pathways, cellular processes, tissue responses to patient responses, through in vitro, in vivo studies, pre-clinical studies and clinical research (Foucquier and Guedj, 2015). The outcomes of the OAB combination therapies are mostly examples of measurement of patient responses through clinical studies. However, it is often challenging to determine the effects of drug combinations in vivo and clinical trials due to high variability in each species and individuals, particularly when the drugs have not been characterised. In vitro assays including the isolated tissue bath set up used in the present study, is one of the most versatile methods in studying

combination therapy as they are the assays of choice for measuring drug effect in native tissue (Hoare, 2021).

Many mathematical and pharmacological models have been developed to analyse the effects of combination therapy, including the effect-based approach and dose-effect-based approach. An effect-based approach compares the effect resulting from the combination of two drugs directly to the effects of its individual component. Four main strategies are commonly used in the effect-based approach, including (1) Combination Subthresholding, (2) Highest Single Agent, (3) Response Additivity, and (4) Bliss Independence Approach (Reviewed by Foucquier and Guedj 2015). Their principles and limitations are summarised in Table 5.1 and illustrated in Figure 5.1.

Table 5.1 The four main strategies of effect-based approach in analysing drug interaction.

Effect-Based Approach	**Principle**	**Limitation**
Combination Subthresholding	The combination of non-effective doses of drugs yields significant effect.	Does not necessarily imply a convincing difference between the effect of the drug combination and the effects of its individual components.
Highest Single Agent (Gaddum's non-interaction effect)	The resulting effect of drug combination is greater than the effects produced by the most efficacious components in the drug combination.	Does not demonstrate an improved drug combination effect compared to the expected additive effect of its individual components unless one of the components is not effective at all concentrations tested.
Response Additivity (Linear interaction effect)	To show that a positive drug combination effect occurs when the observed combination effect is greater than the expected additive effect given by the sum of the individual effects ($E_A + E_B$).	Only applicable for linear dose-response curves
Bliss Independence Model	Drug combination effects are the outcomes of probabilistic processes and assumes that drugs act independently to contribute a common result.	Requires understanding of the mechanism(s) of action of the drugs and assumes drugs have exponential dose-effect curves.

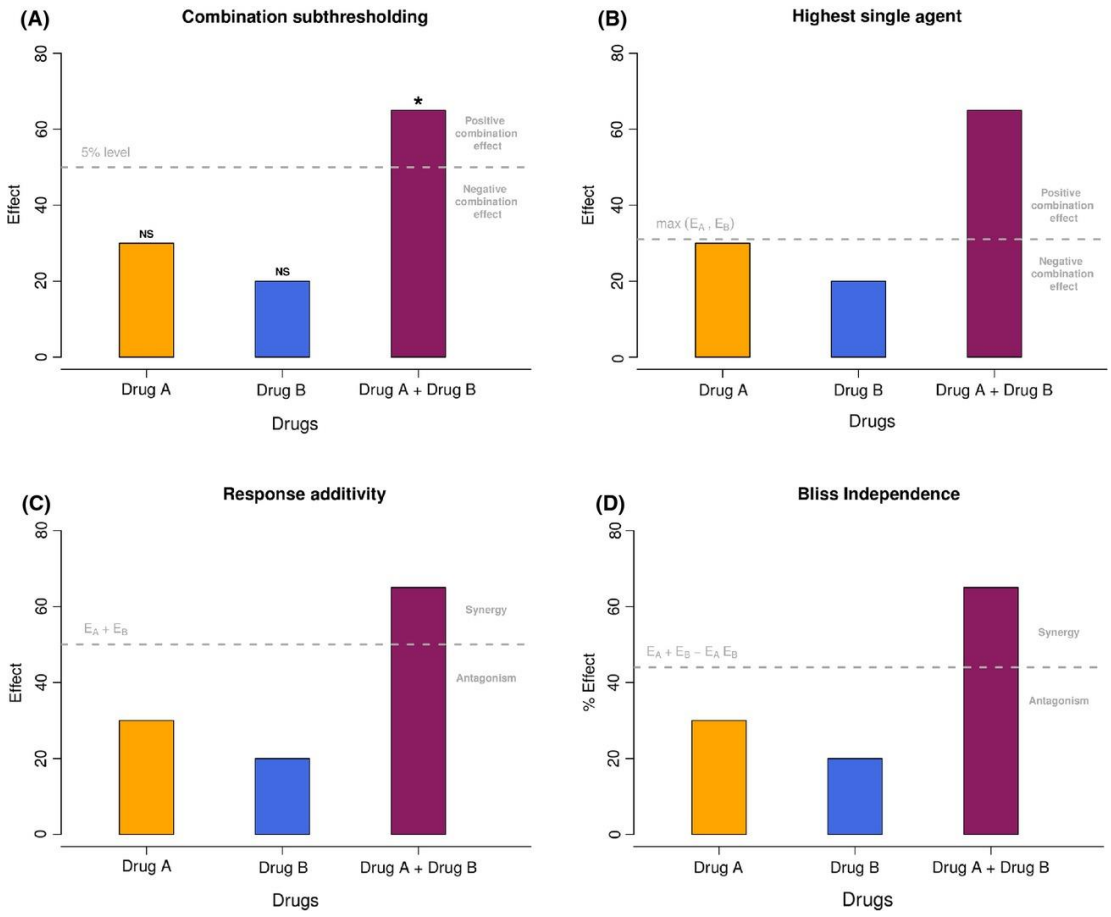


Figure 5.1 Illustration of the four effect-based approaches. *(A) Combination Subthresholding, (B) Highest Single Agent, (C), Response Additivity, and (D) Bliss Independence. Based on E_A = 30, E_B = 20, and E_{AB} = 65. NS, Non-significant; *, Significant at the 5% level. (Adapted from Foucquier and Guedj 2015).*

To many, dose-effect-based approach is considered as a more appropriate way to compare different agents having non-linear dose-/concentration-response curve (Foucquier and Guedj 2015). It relies on the mathematical framework known as Loewe Additivity, that was built on the concepts of dose equivalence and sham combination. The dose equivalence principle assumes that both substances that reach the same effect can be interchanged whereas sham combination assumes that a compound dose not interact with itself. Loewe model can be expressed in a graphical method (isobolographic analysis) that produces a curve called an isobole, a plot in rectangular coordinates when the axes represent the doses or concentrations of

substance A and substance B. The points that constitute the isobole are doses/concentrations that represent the amount of each substance expected to yield an effect of specified magnitude when they are given together (Tallarida, 2006). Generally, this method requires substances that are being used in combination to have a constant potency ratio (parallel log-dose scale response curve) and have equal individual drug maximum effects.

As isobolographic method allows simple and straightforward analytical considerations of results, it has been widely used by researchers to analyse the dose/concentration-effect of combination treatment, even when the data do not fit the assumption of the model. For example, the combined effects of quercetin and catechin were studied and interpreted using isobolographic analysis although the two compounds did not achieve similar maximum effect and did not have a constant potency ratio (Menendez et al., 2011). The inappropriate use of Loewe additivity and the linear isoboles may result in an inaccurate interpretation of the experimental data (Tallarida 2006). Hence, it is crucial to choose the most appropriate model to analyse the experimental data to avoid misinterpretation. Additionally, the study of combined therapy using traditional medicines is often more challenging due to their immense complexity and variability. Despite the challenges, they are proven to provide synergistic effects in treating many conditions or reduce the occurrence of side effects (Fu et al., 2018).

In the previous chapters, we have demonstrated the mechanisms of action of OCS02-CWE in the bladder. It appears that the contractile and relaxant effect of OCS02-CWE were mediated via different pathways, and the contractile effect may hinder its relaxant effect when the extract was added cumulatively. Given that the contraction induced by OCS02-CWE was caused by influx of extracellular Ca^{2+}, it is

possible that preventing Ca^{2+} influx may enhance its relaxation response during cumulative application. Because of this, *Lignosus rhinocerus*, a valuable medicinal fungus, has captured our interest due to its overt Ca^{2+} blocking activity in promoting airway and vascular relaxation (Lee et al., 2018b, 2018a; Govindaraju et al., 2018). It has also exhibited bladder relaxant effect in our preliminary experiments. In the following sections, the ethnomedicinal uses and chemical composition of *L. rhinocerus* are reviewed.

5.1.3 *Lignosus rhinocerus*

5.1.3.1 A brief history of *L. rhinocerus*

Lignosus rhinocerus (Cooke) Ryvarden (*L. rhinocerus*), commonly known as the tiger's milk mushroom (TMM), is a rare and valuable medicinal mushroom mainly distributed in South China, Thailand, Malaysia, Indonesia, Philippines, Sri Lanka, Papua New Guinea, Australia and New Zealand (Nallathamby et al., 2018). It is traditionally consumed by the indigenous communities in Southeast Asia and South China for various medicinal purposes. Belonging to the Polyporaceae family (Basidiomycota), *L. rhinocerus* consists of pileus (cap), stipe (stem) and sclerotium, as illustrated in Figure 5.2. It is also known for its unusual morphology in which the fruiting body (cap and stem) raises from the sclerotium under the ground, rather than from woody substrate like other polypores (Nallathamby et al., 2018). The fruiting body, however, has a woody texture and the sclerotium is a compacted mass of fungal mycelium in white that provides nutrients to support the growth and development of the mushroom. According to the local folklore, *L. rhinocerus* often emerges on the spot where a tigress had accidentally dribbled during lactation and the white sclerotium resembles the congealed white milk; hence the name (tiger's milk mushroom).

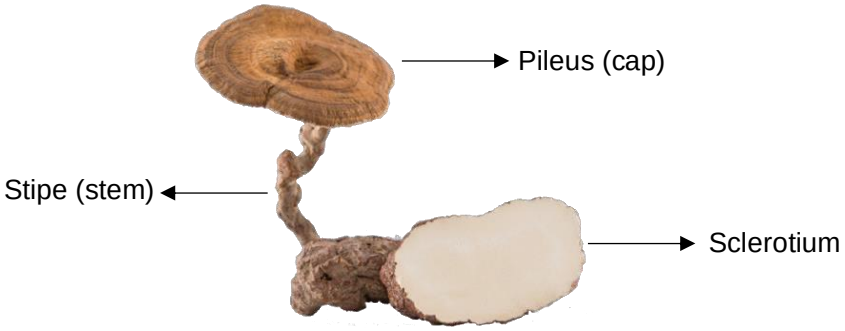


Figure 5.2 The morphology of L. rhinocerus (tiger's milk mushroom).

5.1.3.2 Ethnomedicinal uses

The medicinal usage history of *L. rhinocerus* can be dated back to 1664 when it was first recorded as "*Lac Tygridis*", meaning tiger's milk, in the "The Diary of John Evelyn". The scientific documentation of this mushroom was not available until two centuries later when it was recorded as *Fomes rhinocerus* Cooke in 1879. It was also taxonomically narrated under other genera, including *Scindalma*, *Polystictus* and *Microporus* by different authors before it was correctly renamed as *Lignosus*. Over the years, the scientific names that have been used for *L. rhinocerus* include *Polyporus rhinocerus* Cooke (1879), *Fomes rhinocerus* Cooke (1879), *Scindalma rhinocerus* (Cooke) Kuntze (1898), *Polyporus sacer var. rhinocerotis* (Cooke) Llyod (1921), *Polystictus rhinocerus* (Cooke) Boedjin (1940), *Polystictus rhinocerotis* (Cooke) Boedjin (1940), *Microporus rhinocerus* (Cooke) Imazeki (1952), *Microporus rhinocerotis* (Cooke) Imazeki (1952). Finally, in 1972, it has been officially named as *Lignosus rhinocerus* (Cooke) Ryvarden (1972) (Reviewed by Nallathamby et al. 2018).

In various tribes of the aboriginals in Malaysia (the Semai, Temuan and Jakun), *L. rhinocerus* was used to relief asthma, cough, food poisoning, joint pain, liver

ailments and as a general tonic. The consumption methods of *L. rhinocerus* are found to be varied in each area, probably affected by the local culture and traditions. Although most reports point to the sclerotium as the only part with medicinal value, some tribes have used the "whole plant" to treat tuberculosis and colds (Lau et al., 2015). This practice is, however, considered unfavourable, possibly due to the woody nature of the fruiting body and hard fructification.

According to an ethnomycological survey conducted in Selangor, Malaysia, The Temuan, one of the eighteen tribes of aborigines in Peninsular Malaysia, generally consumed the sclerotium of *L. rhinocerus* by drinking the decoction to treat cough, cold and asthma. They also took *L. rhinocerus* with *Polyalthia bullata* (a medicinal plant, locally known as Tongkat Ali) for postpartum recovery (Azliza et al., 2012). The Mah Meri, another indigenous community in Peninsula Malaysia, often consumed *L. rhinocerus* with *Piper betle* L., *Areca catechu* L., and slaked lime for asthma and breathlessness. It was also recorded that in Songkok Village (Hulu Selangor, Malaysia), the sclerotium was grated and left to dew. After that, it was mixed with *burung lilin*'s (a hornbill species) beak before consumption (Azliza, 2013). In Sungai Jang (Hulu Selangor, Malaysia), the sclerotium was used to increase the production of breast milk after delivery. For this purpose, the juice was squeezed from the sclerotium and applied topically. Moreover, Kensiu, the aborigines in Lubuk Ulu Legong (Kedah, Malaysia) would burn the sclerotium and apply the ash on chest to treat asthma. They also applied the shredded sclerotium on chest to treat breast cancer.

The ethnomedicinal study also revealed that *L. rhinocerus* has been used as an energy-boosting food to revitalise the body and to stave off hunger. Some suggested that the *L. rhinocerus*-infused water can improve vitality, increase alertness and overall wellness of the individual (Sabaratnam et al., 2013). For a very long time, the

medicinal uses of *L. rhinocerus* were circulated only within the indigenous communities. With advances in digital technologies and information sharing, it has become one of the most popular medicinal mushrooms and functional food among the urban populations in Malaysia, Singapore, Thailand and China in recent years. In Hong Kong, *L. rhinocerus* is often prescribed by Chinese physicians to treat liver cancer, gastric ulcers and chronic hepatitis (reviewed by Lau et al. 2015). The outbreak of COVID-19 has also boosted the market of *L. rhinocerus* due to its traditional uses in respiratory conditions.

5.1.3.3 Chemical composition of *L. rhinocerus*

The chemical composition of wild, authenticated *L. rhinocerus* collected from Negeri Sembilan, Malaysia has been analysed (Lau et al., 2013). According to the report, the fruiting body and sclerotium of *L. rhinocerus* were rich in carbohydrates and dietary fibres but low in fat. It also contains a moderate amount of proteins and contains all essential amino acids except tryptophan. The nutrient composition of the sclerotium was similar to that of the fruiting body and mycelium (Lau et al., 2013).

The chemical composition analysis of the cultivated *L. rhinocerus* (TM02) has also been carried out and compared with its wild counterpart collected from Pahang, Malaysia (Yap et al., 2013). Generally, the sclerotium of TM02 has a higher nutritional value than the wild mushrooms. The protein and essential amino acid contents of TM02 were nearly 4 times higher than that of the wild type. The mineral contents including calcium, potassium, sodium and magnesium, in TM02 were also higher.

Subsequently, TM02 was further analysed by Lee et al. (2018b) in the attempt to determine its bioactive components. Apart from β-glucans, polysaccharides and glycoproteins, trace amounts of adenosine and its derivatives have been detected, as listed in Table 5.2. Several bioactive compounds such as linoleic acid and octadecane

have also been proposed following the gas chromatography-mass spectrometry (GC-MS) analysis carried out by (Johnathan et al., 2016).

Table 5.2 Summary of the composition analysis of Lignosus rhinocerus sclerotial powder TM02 (Lee et al., 2018b).

Analyte	Content (mg/g)
Total polysaccharides	401.4
Beta-1,3/1,6-glucan	286.8
Glycoprotein	186.1
Alpha-glucan	29.91
3′-deoxyadenosine	0.873
Ethyl-adenosine	0.399
Adenosine	0.282
Hydroxyethyl-adenosine	0.268
Adenine	0.024

5.2 Study aim and objectives

This study aimed to investigate the combined effects between *L. rhinocerus* and *O. sinensis* in bladder relaxation. Thus, the experiments in this chapter were planned to first, characterise the bladder relaxant effect of *L. rhinocerus* cold water extract, and then, to study its possible interaction with *O. sinensis* (OCS02-CWE). The cold water extract of *Lignosus rhinocerus* (TM02 cultivar) was termed as "TM02-CWE" in this chapter and throughout this thesis. The specific objectives of this chapter were:

i. To investigate the relaxant effect of TM02-CWE in carbachol- pre-contracted bladder strips
ii. To compare the effect of TM02-CWE and fractions in carbachol-pre-contracted bladder strips
iii. To study the role of urothelium in TM02-CWE-induced bladder relaxation
iv. To determine the role of TM02-CWE in regulating Ca^{2+} homeostasis
v. To investigate the role of Ca^{2+} channels in carbachol-induced contractions
vi. To assess the Ca^{2+}-inhibiting mechanism of TM02-CWE using known and experimental Ca^{2+} channel blockers
vii. To evaluate the in vitro interaction between TM02-CWE and OCS02-CWE in bladder relaxation

5.3 Materials and methods

5.3.1 Drugs and solutions

Krebs solution was prepared as described in **section 2.3.1**. In some experiments, Ca^{2+}-free Krebs solution in the absence or presence of EGTA 1 mM was used. All drugs were dissolved in respective solvents and made into a stock concentration of 0.1 M. Calcium chloride ($CaCl_2$) was made into a stock concentration of 1.0 M. Drugs including carbachol, nifedipine, verapamil, SKF-96365, schwarzinicine A were used in this study. Their details were listed in Table 3.1 **(Chapter 3**).

5.3.2 Preparation, extraction and fractionation of TM02-CWE

The cold water extraction was performed in accordance to the preparation of OCS02-CWE as detailed in **section 2.3.2**. The fractionation of TM02-CWE was performed by Miss Ng Min Jia, under the supervision of Prof Fung Shin Yee at UM, as described in **section 2.3.4**.

5.3.3 Tissue preparation and isolated tissue bath setup

Ethics approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB) (UNMC12 and UNMC26, Appendix A). All experiments were conducted with male Sprague Dawley rats (218 – 463 g; 2 – 3 months old) purchased from UKM or UPM. Rats were sacrificed on the day of experiment by CO_2 inhalation. The bladder was isolated, prepared and mounted to the isolated tissue bath set up according to the steps detailed in **section 2.3.5**. In experiments to study the role of urothelium, the urothelium of bladder strips was removed according to steps described in **section 3.3.4.5**.

5.3.4 Isometric tension recordings

5.3.4.1 Effect of TM02-CWE and fractions on carbachol-pre-contracted bladder strips

Following tissue viability tests, the bladder strips were exposed to 1 µM of carbachol to induce a submaximal contractile tone of 70 – 80% of KCl-induced tone. Once a stable contraction was established, TM02-CWE and its fractions including TM02-HMW, TM02-MMW or TM02-LMW were added cumulatively into the bath every 10 minutes or until the response plateaued. The CRCs to TM02-CWE or its fractions were conducted from 0.5 mg/mL to 8.0 mg/mL. Vehicle control using purified water was performed in parallel.

5.3.4.2 The role of urothelium in TM02-CWE-induced relaxation

To study the role of urothelium in the bladder relaxant effect of TM02-CWE, cumulative CRCs to TM02-CWE were constructed in urothelium-denuded bladder strips pre-contracted with 1 µM of carbachol. The results obtained were compared with the results obtained from **section 5.3.4.1.**

5.3.4.3 Effect of TM02-CWE on intracellular Ca^{2+} release induced by carbachol

To assess if TM02-CWE blocks intracellular Ca^{2+} release, the bladder strips were pre-incubated either with 5 mg/mL of TM02-CWE or purified water (vehicle control) in normal Krebs solution or Ca^{2+}-free Krebs solution containing 1 mM of EGTA for at least 30 minutes. Then, a single concentration of carbachol (100 µM) was added into the bath to induce a contractile tone and tissues were left in the bath for 1 hour to study the contractile response over time.

5.3.4.4 Effect of TM02-CWE on the contractile responses to Ca^{2+} re-introduction

Following the tissue viability test, the bladder strips were submerged in Ca^{2+}-free Krebs solution for 30 minutes. Then, the tissues were incubated with 1.0 mg/mL, 2.5 mg/mL or 5.0 mg/mL of TM02-CWE. These concentrations were chosen to study the concentration-dependent effect of TM02-CWE as they reversed 10%, 25% and 50% of carbachol-induced tone. Purified water of equivalent volume to 5.0 mg/mL of TM02-CWE was used as vehicle control. After an incubation period of 30 minutes, 60 mM of KCl was used to induce membrane depolarisation. Then, cumulative CRCs to $CaCl_2$ were constructed.

5.3.4.5 Effect of TM02-CWE and Ca^{2+}-channel blockers on carbachol-induced contractions

To investigate the role of extracellular Ca^{2+}, CRCs to carbachol were constructed in either Ca^{2+}-containing Krebs solution or Ca^{2+}-free Krebs solution. In another set of experiments, the bladder strips were pre-incubated with 5 mg/mL of TM02-CWE or Ca^{2+} channel blockers including 100 nM of nifedipine, 100 nM of verapamil, 10 µM of SKF-96365 or 30 µM of schwarzinicine A for 30 minutes. Then, cumulative CRCs to carbachol were constructed.

5.3.4.6 Effect of Ca^{2+}-channel blockers on TM02-CWE-induced relaxation

To identify the Ca^{2+}-mediated mechanism(s) of TM02-CWE-induced relaxation in the bladder, the bladder strips were pre-incubated with either 100 nM of nifedipine, 100 nM of verapamil, 10 µM of SKF-96365, 30 µM of schwarzinicine A for 30 minutes prior to carbachol pre-contraction. DMSO 0.225% v/v was used as the vehicle control. Once a stable contractile tone was achieved, cumulative CRCs to TM02-CWE were constructed.

5.3.4.7 Effect of TM02-CWE on OCS02-CWE-induced biphasic responses

In **Chapter 3**, we demonstrated that OCS02-CWE induced transient contraction in carbachol-pre-contracted bladder strips by stimulating extracellular Ca^{2+} influx. Thus, subsequent experiments assessed the effect of TM02-CWE on OCS02-CWE-induced biphasic response in the bladder strips. Bladder tissues were pre-incubated with either 1.0 mg/mL or 2.5 mg/mL of TM02-CWE for 30 minutes. Then, 1 µM of carbachol was used to elicit a contractile tone. Once a stable contraction was achieved, OCS02-CWE 5 mg/mL was added into the bath and left until the response plateaued. Purified water of equivalent volume to 2.5 mg/mL of TM02-CWE was used as the vehicle control. A maximum of 2.5 mg/mL of TM02-CWE was used in this experiment to ensure similar magnitude of carbachol-induced tone. Only one concentration of OCS02-CWE (5.0 mg/mL) was tested in each bladder strip as initial experiments showed that repeated addition of TM02-CWE would desensitise the tissues (data not shown).

5.3.4.8 Effect of TM02-CWE and OCS02-CWE combination on carbachol-pre-contracted bladder strips

To investigate the relationship between TM02-CWE and OCS02-CWE in bladder relaxation, CRCs to TM02-CWE and OCS02-CWE at a ratio of 1:1 were constructed in the bladder strips pre-contracted with 1 µM of carbachol. The combined samples were prepared by mixing the stock solutions of TM02-CWE and OCS02-CWE before adding into the bath cumulatively. The CRCs to the combined extract were constructed from 0.5 mg/mL to 8.0 mg/mL, i.e., from 0.25 mg/mL of TM02-CWE plus 0.25 mg/mL of OCS02-CWE to 4.0 mg/mL of TM02-CWE plus 4.0 mg/mL of OCS02-CWE. The responses were compared to the effect of OCS02-CWE and TM02-CWE derived from their individual CRCs.

5.3.5 Data analysis

Data were analysed and graphs were drawn using GraphPad Prism v9.0. All data were presented as mean ± SEM of *n* number of animals. Concentrations of drugs were presented in graph in logarithm of concentrations in molar (M) (Figure 5.7 and Figure 5.8) whereas concentrations of TM02-CWE and OCS02-CWE were shown in mg/mL due to tight concentration range used (Figure 5.3, Figure 5.4, Figure 5.9, Figure 5.10 and Figure 5.12). In Figure 5.7, E_{max} was taken from the response produced at the highest concentration tested as the response has not plateaued and pEC_{50} was not calculated. In Figure 5.8, E_{max} and pEC_{50} were derived from the non-linear regression curve. Statistical analysis was performed using unpaired Student's *t*-test to compare between control and treatment group. One-way ANOVA followed by Dunnett's *post hoc* test was used to compare the effects of three or more groups. Two-way ANOVA followed by Tukey's *post hoc* test was used to compare all possible combinations in the groups. Results with $p < 0.05$ were considered statistically significant.

To determine the responses derived from the combined OCS02 and TM02 extract, a response additivity graph was constructed for each effect level at the maximum relaxation response, 50% relaxation response and 25% relaxation response. The graphs illustrate the proportional relaxant effect induced by the individual extracts (OCS02-CWE and TM02-CWE), in addition to the combination effect of the extract mixture. The additive line on the graph was generated by summation of the relaxation response achieved by the individual extract, representing the expected level of relaxation response if the response induced by the extract mixture was simply the sum of the individual proportional responses (Figure 5.13).

5.4 Results

5.4.1 TM02-CWE elicited the superior bladder relaxation response compared to its fractions

TM02-CWE significantly relaxed carbachol-pre-contracted bladder strips (maximum relaxation response = vehicle control, 23.03 ± 4.57%; TM02-CWE, 106.30 ± 10.82%, $p < 0.0001$) (Figure 5.3A). Compared to the vehicle control, TM02-HMW fraction and TM02-MMW fraction also produced significant relaxation response of 92.92 ± 5.74% ($p < 0.0001$) and 67.75 ± 3.47% ($p = 0.0005$), respectively (Figure 5.3A). The bladder relaxant effect of TM02-CWE was not statistically different from TM02-HMW fraction ($p > 0.05$) (Figure 5.3B). TM02-LMW fraction, on the other hand, was lack of relaxant effect and produced a maximum relaxation response of 22.36 ± 5.30% at the highest concentration tested, similar to vehicle control, $p > 0.9999$ (Figure 5.3A, B). The rank order of maximum relaxation responses from the tested samples is: TM02-CWE ≥ TM02-HMW fraction > TM02-MMW fraction > TM02-LMW fraction. To ensure the maximum relaxant effect of *L. rhinocerus* was achieved in the process of elucidating its mechanism(s) of action, subsequent experiments were performed using TM02-CWE.

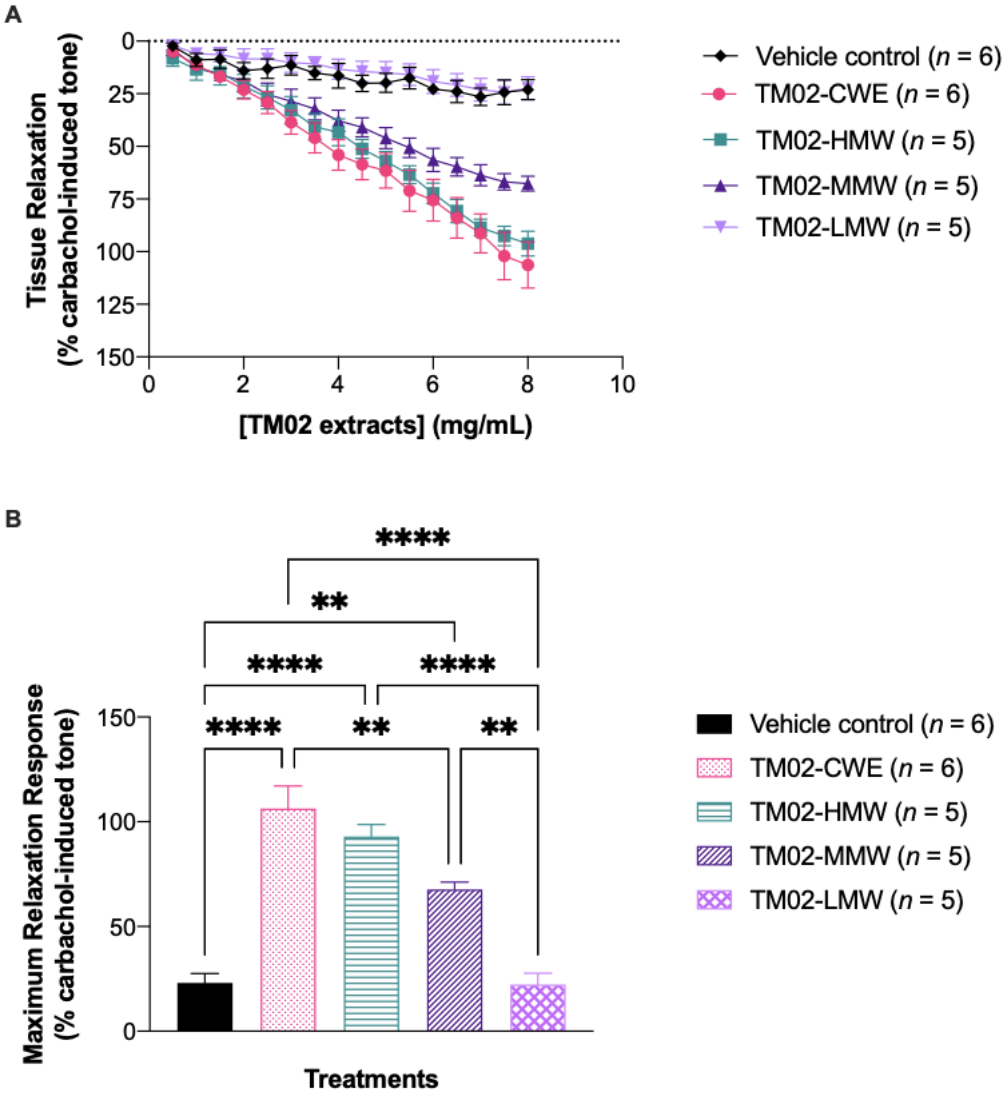


Figure 5.3 Effect of TM02 cold water extract and its fractions on bladder strips pre-contracted with carbachol. *(A) Cumulative CRCs to TM02-CWE and its fractions (TM02-HMW, TM02-MMW and TM02-LMW) were constructed in bladder strips pre-contracted with 1 µM of carbachol. (B) Maximum tissue relaxation response produced by TM02-CWE and fractions at 8.0 mg/mL. Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. One-way ANOVA with Tukey's post-hoc test where comparison of means between all the groups was made:** p < 0.01; ****p < 0.0001. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; HMW = high-molecular-weight; MMW = medium-molecular-weight; LMW = low-molecular-weight)*

5.4.2 TM02-CWE -induced bladder relaxation was independent of urothelium

To determine whether TM02-CWE-induced bladder relaxation is urothelium-dependent, the reduction of carbachol-induced tone by TM02-CWE was compared between urothelium-intact and urothelium-denuded bladder strips. Our results showed that TM02-CWE elicited a similar bladder relaxation profile in both urothelium-intact and urothelium-denuded bladder strips (maximum relaxation response = urothelium-intact, 106.30 ± 10.82%; urothelium-denuded, 88.52 ± 5.50%, p = 0.2033) (Figure 5.4).

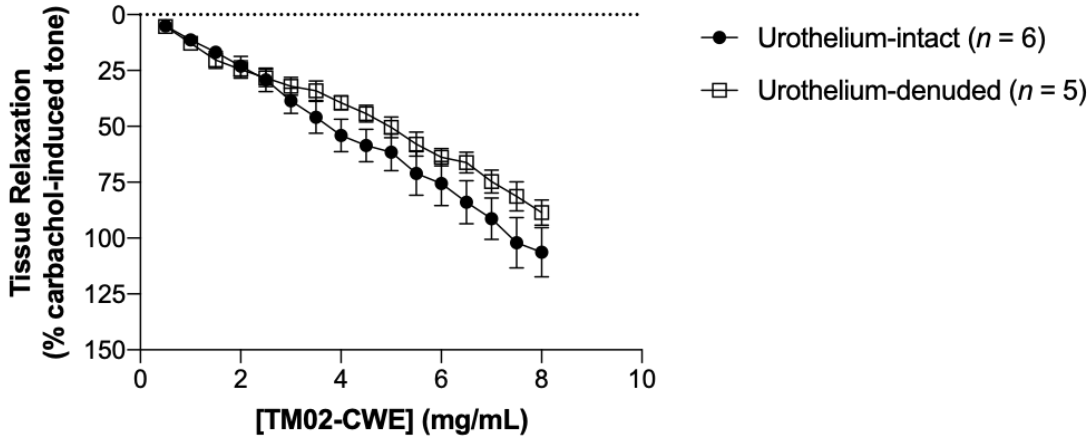


Figure 5.4 The role of urothelium in TM02-CWE-induced bladder relaxation. *Cumulative CRCs to TM02-CWE were constructed in urothelium-intact bladder strips or urothelium-denuded bladder strips. Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviation: CRCs = concentration-response curves; CWE = cold water extract)*

5.4.3 TM02-CWE inhibited muscarinic receptor-induced extracellular Ca^{2+} influx but not intracellular Ca^{2+} release

To investigate the role of TM02-CWE in Ca^{2+} regulation, the bladder strips were pre-incubated with TM02-CWE in either Ca^{2+}-free or normal Krebs solution. The results showed that in the presence of TM02-CWE, both phasic and tonic contraction induced by carbachol in the presence of Ca^{2+} was significantly suppressed. Figure 5.5 shows the representative trace recordings of carbachol-induced contraction in the presence of vehicle control or TM02-CWE. Then, in Ca^{2+}-free Krebs solution, TM02-CWE did not alter carbachol-induced contraction (Figure 5.6). The values of tissue contraction induced by carbachol over time were summarised in Table 5.3.

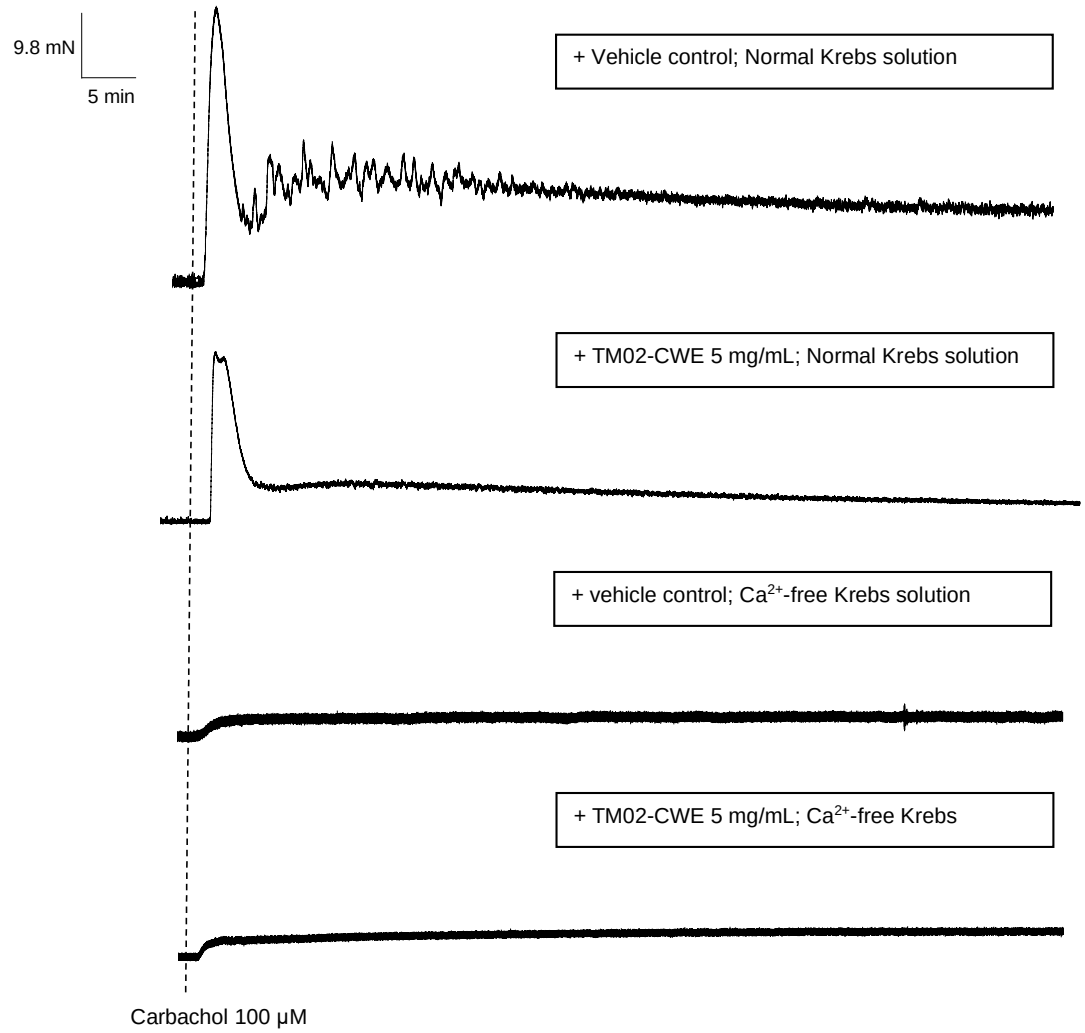


Figure 5.5 Representative trace recordings of carbachol-induced contraction in normal and Ca^{2+}-free Krebs solution, with or without TM02-CWE.

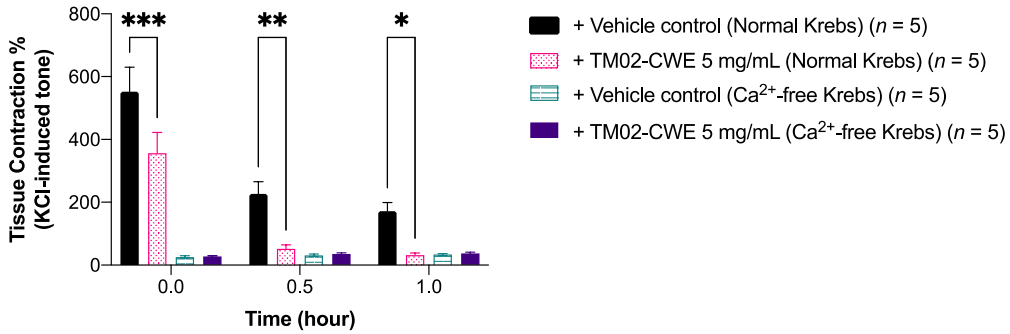


Figure 5.6 Effect of TM02-CWE on carbachol-induced contraction in normal and Ca^{2+}-free Krebs solution. *Tissue responses were recorded at 0, 0.5 and 1.0 hour after the addition of carbachol 100 µM and have been expressed as the percentage of KCl-induced tone. Data are shown as mean ± SEM of n number of animals. Two-way ANOVA followed by Tukey's post hoc test was used to compare vehicle control and TM02-CWE treatment in either normal or Ca^{2+}-free Krebs at each time point, *p<0.05, **p<0.01, ***p<0.001. (Abbreviations: KCl= potassium chloride; CWE = cold water extract).*

Table 5.3 Carbachol-induced contraction in normal or Ca^{2+}-free Krebs solution with or without the presence of TM02-CWE.

Treatment	Time (hour)	Tissue contraction (%)	
		Vehicle control	TM02-CWE 5 mg/mL
Normal Krebs	0	552.50 ± 77.86	356.70 ± 65.64 ***(p =0.0006)
	0.5	226.9 ± 38.99	51.86 ± 12.27 **(p =0.0024)
	1	171.50 ± 27.78	31.38 ± 6.72 *(p =0.0204)
Ca^{2+}-free Krebs	0	25.81 ± 3.52	26.82 ± 2.61
	0.5	31.33 ± 3.86	34.76 ± 3.40
	1	33.55 ± 3.10	36.57 ± 4.00

Data are shown as mean ± SEM of 5 animals. Two-way ANOVA with Tukey's post hoc test was performed to compare between vehicle control and TM02-CWE in either normal or free Krebs separately at each time point. Individual p-values were stated in the respective columns when $p < 0.05$. (Abbreviations: CWE = cold water extract, KCl = potassium chloride) (Abbreviations: CWE = cold water extract, KCl = potassium chloride)

5.4.4 TM02-CWE suppressed extracellular Ca^{2+}-induced contraction in a concentration-dependent and urothelium-independent manner

Subsequently, the effect of TM02-CWE on Ca^{2+}-induced contraction was investigated to substantiate its role in inhibiting extracellular Ca^{2+} influx. Pre-incubation with TM02-CWE at 1.0 mg/mL, 2.5 mg/mL and 5.0 mg/mL significantly suppressed the contraction induced by the cumulative addition of $CaCl_2$ as compared to vehicle control (Figure 5.7). The attenuation of tissue contraction by TM02-CWE was concentration-dependent and at 5 mg/mL, $CaCl_2$-induced contraction was nearly abolished. Removal of urothelium did not affect the suppression effect of TM02-CWE on Ca^{2+}-induced contractions in the bladder strips (Figure 5.7B). The maximum contractile responses induced by $CaCl_2$ were summarised in Table 5.5.

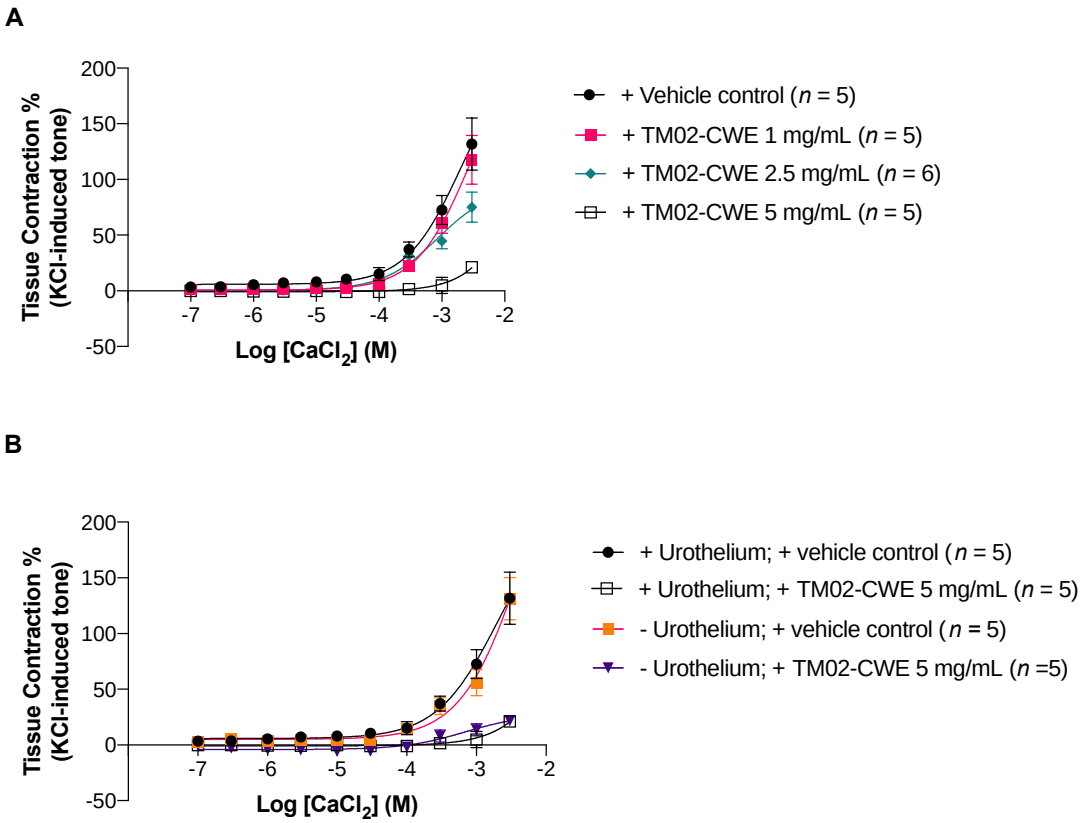


Figure 5.7 Effect of TM02-CWE on $CaCl_2$-induced bladder contraction. *(A) Concentration-dependent effect of TM02-CWE pre-incubation on $CaCl_2$-induced contraction in urothelium-intact bladder strips; (B) Effect of 5 mg/mL of TM02-CWE on $CaCl_2$-induced contraction in urothelium-intact and urothelium-denuded bladder strips. Tissue contraction has been expressed as the percentage of reference KCl-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; KCl = potassium chloride)*

Table 5.4 Effect of TM02-CWE on $CaCl_2$-induced bladder contraction. *Maximum tissue responses of urothelium-intact bladder strips induced by $CaCl_2$ following pre-incubation with vehicle control or TM02-CWE (1.0 mg/mL, 2.5 mg/mL or 5.0 mg/mL) and the maximum tissue responses of urothelium-denuded bladder strips induced by $CaCl_2$ following pre-incubation with vehicle control or 5.0 mg/mL of TM02-CWE.*

Treatment group	**Maximum tissue contraction (% KCl-induced tone)**	
	Urothelium-intact	**Urothelium-denuded**
+ Vehicle control	131.70 ± 23.37	131.30 ± 19.10
+ TM02-CWE 1.0 mg/mL	117.60 ± 21.99	ND
+ TM02-CWE 2.5 mg/mL	75.20 ± 13.56	ND
+ TM02-CWE 5.0 mg/mL	21.18 ± 4.62 ***(p = 0.0010)	21.83 ± 3.92 ****(p = 0.0004)

Data are shown as mean ± SEM of 5 – 6 animals. One-way ANOVA followed by Sidak's post host test was made to compare the means between each treatment group. Individual p-values between vehicle control and the treatment group were stated in the respective column. (Abbreviations: CWE = cold water extract; ND = not determined).

5.4.5 Carbachol-induced bladder contraction was attenuated by Ca^{2+}-channel blockers

To investigate the Ca^{2+} channels involved in carbachol-induced contraction, CRCs to carbachol in the presence of Ca^{2+} channel blockers were constructed. For the ease of visualisation, CRCs of carbachol in the presence of TM02-CWE and Ca^{2+} channel blockers were categorised in different panels according to their putative mechanisms of action. The CRCs to carbachol were also constructed in Ca^{2+}-free Krebs solution to act as a positive control.

Comparing to the vehicle control, carbachol-induced bladder contraction was significantly diminished in the presence of TM02-CWE (Figure 5.8B) and all other Ca^{2+} channel blockers tested in this study (Figure 5.8C, D). TM02-CWE at 5 mg/mL appeared to have a similar suppression effect on the maximum contraction induced carbachol with 100 nM of nifedipine, and it was 1.5- to 2.0-fold greater than 30 µM of schwarzinicine A, 10 µM of SKF-96365 and 100 nM of verapamil. The potency of carbachol was not affected by nifedipine but it is significantly increased by TM02-CWE, schwarzinicine A, SKF-96365 and verapamil (Figure 5.8D). The values of the E_{max} and pEC_{50} of carbachol in the presence of the respective pre-incubation compound were summarised in Table 5.5.

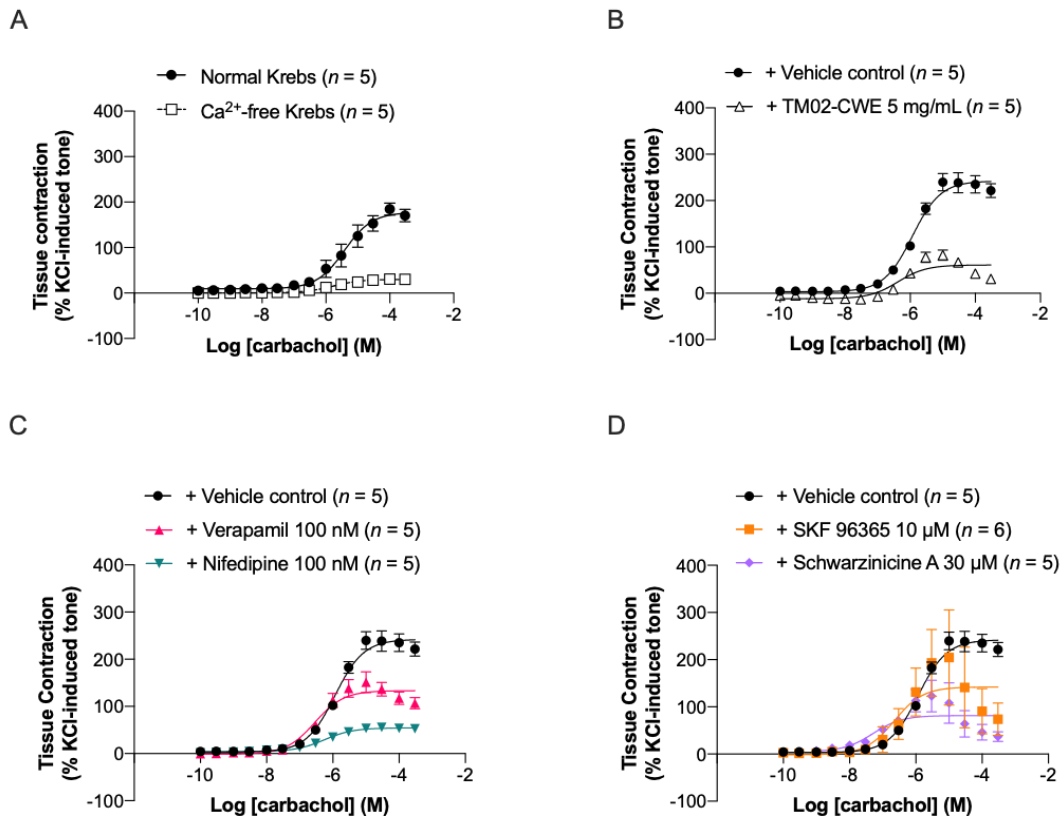


Figure 5.8 Effect of Ca^{2+} removal and Ca^{2+} channel blockers on carbachol-induced bladder contraction. *CRCs to carbachol were constructed (A) in the presence or absence of external Ca^{2+}; (B) in the presence of TM02-CWE 5 mg/mL; (C) VGCC inhibitors (nifedipine and verapamil); and (D) non-selective TRPC inhibitors (SKF-96365 and Schwarzinicine A). Tissue contraction has been expressed as the percentage of KCl-induced tone in Ca^{2+}-containing Krebs solution. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract; KCl = potassium chloride; SOCC = store-operated Ca^{2+} channel; VGCC = voltage-gated Ca^{2+} channel).*

Table 5.5 The maximum contractile response (E_{max}) and pEC_{50} values of carbachol derived from the respective concentration-response curve in the presence or absence of external Ca^{2+} and in the presence of different Ca^{2+} channel blockers.

Treatment group	E_{max}	pEC_{50}
Normal Krebs solution	194.30 ± 12.11	5.30 ± 0.31
Ca^{2+}-free Krebs solution	30.21 ± 1.97 (**** $p < 0.0001$)	5.76 ± 0.12
+ Vehicle control	242.80 ± 18.73	5.95 ± 0.07
+ TM02-CWE 5 mg/mL	61.90 ± 8.21 (**** $p < 0.0001$)	6.38 ± 0.12 (* $p = 0.0435$)
+ Verapamil 100 nM	133.5 ± 15.59 (** $p = 0.0021$)	6.54 ± 0.04 (** $p = 0.0036$)
+ Nifedipine 100 nM	54.62 ± 8.18 (**** $p < 0.0001$)	6.27 ± 0.16 ($p = 0.1750$)
+ SKF 96365 10 µM	143.10 ± 26.56 (** $p = 0.0034$)	6.66 ± 0.09 (*** $p = 0.0004$)
+ Schwarzinicine A 30 µM	96.08 ± 25.85 (*** $p = 0.0002$)	7.25 ± 0.14 (**** $p < 0.0001$)

*Data are shown as mean ± SEM of 5 – 6 animals. Student's t-test was made between the normal Krebs solution and Ca^{2+}-free Krebs solution group (****$p < 0.0001$) whereas one-way ANOVA with Dunnett post-hoc test where comparison of the means was made between the vehicle control and treatment groups (****$p < 0.0001$). Individual p-value was stated in the respective row. (Abbreviations: CWE = cold water extract).*

5.4.6 TM02-CWE-induced bladder relaxation was attenuated in the presence of nifedipine

Subsequently, a series of experiments were performed to examine the effect of Ca^{2+} channel blockers on the bladder relaxant effect of TM02-CWE. Nifedipine significantly attenuated the maximum relaxation response induced by TM02-CWE as compared to the vehicle control (Figure 5.9). In the presence of verapamil, SKF-96365 and Schwarzinicine A, the maximum relaxation response induced by TM02-CWE was not significantly affected although appeared to be potentiated. The values of the maximum relaxation response produced by TM02-CWE were summarised in Table 5.6.

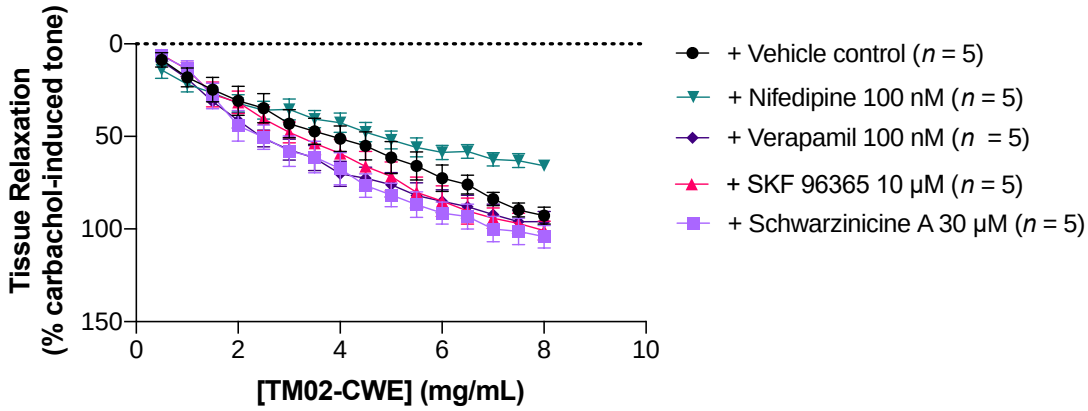


Figure 5.9 Effect of TM02-CWE-induced bladder relaxation in the presence of different Ca^{2+} channel blockers. *CRCs to TM02-CWE were constructed in the presence of SKF-96365, schwarzinicine A, nifedipine or verapamil. Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract)*

Table 5.6 Maximum relaxation response of TM02-CWE in the presence of different Ca^{2+} channel blockers.

Treatment	Maximum relaxation response (% carbachol-induced tone)
+ Vehicle control	92.71 ± 4.38
+ Nifedipine 100 nM	65.81 ± 2.77 **(0.0029)
+ Verapamil 100 nM	96.06 ± 5.44
+ SKF-96365 10 µM	100.90 ± 4.96
+ Schwarzinicine A 30 µM	104.10 ± 5.92

Data are shown as mean ± SEM of 5 animals. One-way ANOVA with Dunnett post-hoc test where comparison of the means between control and treatment groups was made and the respective p-values stated in the table (Abbreviation: CWE = cold water extract).

5.4.7 TM02-CWE attenuated OCS02-CWE-induced transient contraction

To assess the interaction between TM02-CWE and OCS02-CWE, a series of protocols were performed. Figure 5.10 shows a representative trace recording of the effect of TM02-CWE 2.5 mg/mL on the biphasic response of OCS02-CWE. Pre-incubation of the bladder strips with TM02-CWE suppressed the immediate transient contractile response induced by OCS02-CWE in a concentration-dependent manner (Figure 5.11). However, it did not affect the relaxation response induced by OCS02-CWE. The values of OCS02-CWE-induced contraction and relaxation responses following pre-incubation with TM02-CWE were summarised in Table 5.7.

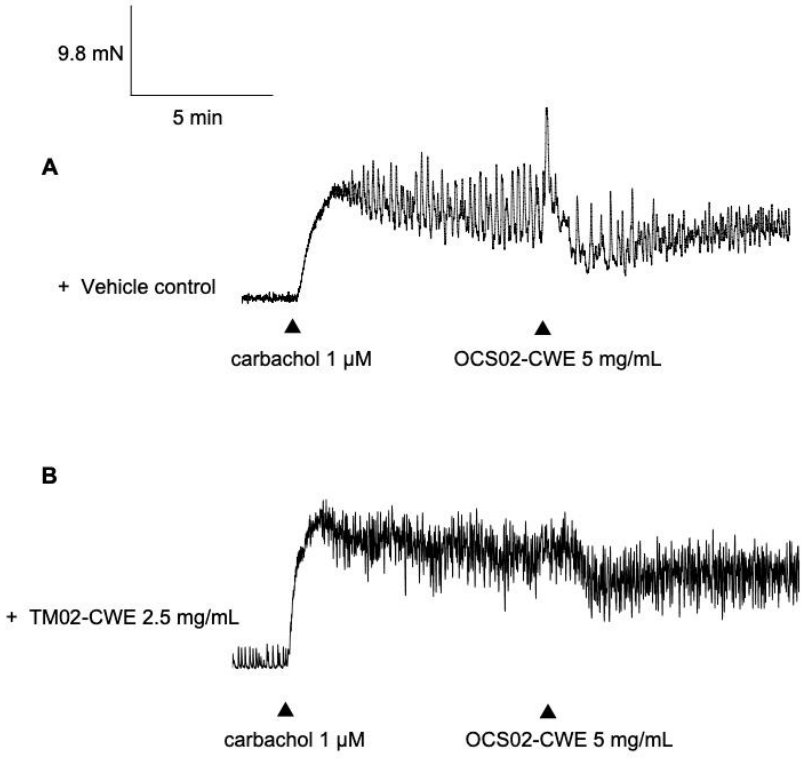


Figure 5.10 Effect of TM02-CWE on the biphasic response induced by OCS02-CWE. *Representative trace recordings of (A) vehicle control and (B) 2.5 mg/mL of TM02-CWE on the biphasic response induced by OCS02-CWE. (Abbreviation: CWE = cold water extract; min = minute; mN = milliNewton).*

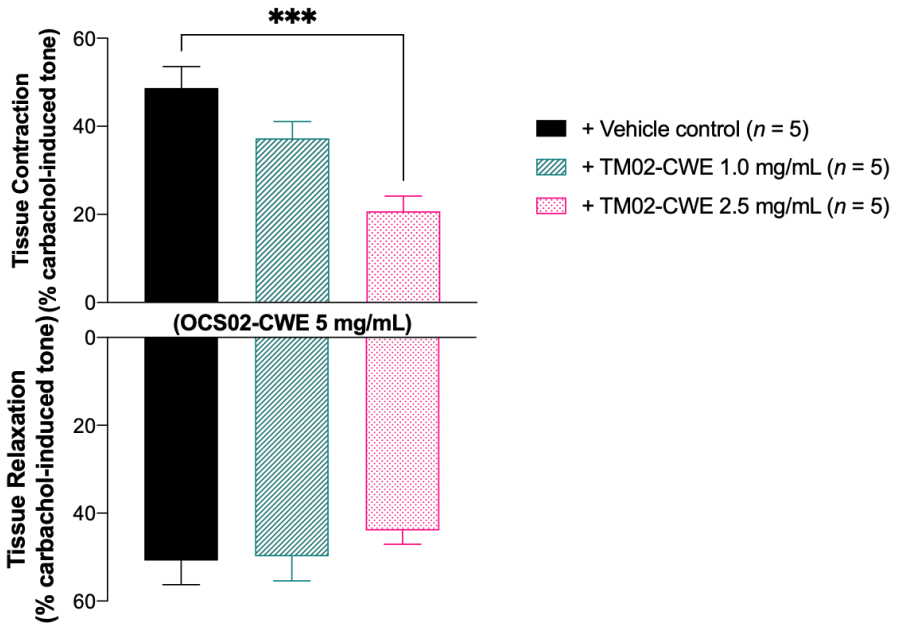


Figure 5.11 Effect of TM02-CWE on the biphasic response induced by OCS02-CWE. *Tissue responses have been expressed as a percentage of carbachol-induced tone. Data are shown as mean ± SEM of 5 animals. One-way ANOVA with Dunnett post-hoc test was performed for differences between vehicle control and the presence of TM02-CWE,***p < 0.001. (Abbreviation: CWE = cold water extract).*

Table 5.7 Effect of TM02-CWE on OCS02-CWE-induced biphasic response.

Treatment	OCS02-CWE-induced immediate transient contraction (%)	OCS02-CWE-induced relaxation (%)
+ Vehicle control	48.70 ± 4.85	50.76 ± 5.49
+ TM02-CWE 1.0 mg/mL	37.27 ± 3.80	49.84 ± 5.55
+ TM02-CWE 2.5 mg/mL	20.71 ± 3.44 ***(p = 0.0008)	43.93 ± 3.13

*Data are shown as mean ± SEM of 5 animals. One-way ANOVA with Dunnett post-hoc test was performed for differences between vehicle control and the presence of TM02-CWE separately for OCS02-CWE-induced transient contraction ($p < 0.05$) and OCS02-CWE-induced relaxation ($p > 0.05$) (Abbreviations: CWE = cold water extract)***.

5.4.8 Low concentrations of TM02-CWE and OCS02-CWE produced synergistic effects

OCS02-CWE CRC in **section 2.4.3 (Chapter 2)** and TM02-CWE CRC in Figure 5.3 were incorporated in Figure 5.12 to compare with the CRC of the combined extract. The combined extract produced a straightforward relaxation response in the bladder strips, without exhibiting transient or intermittent contraction as seen in the cumulative CRCs of OCS02-CWE. At the highest concentration tested (4 mg/mL of OCS02-CWE and 4 mg/mL of TM02-CWE), the combined extract produced a greater maximum relaxation response when compared to 8 mg/mL of OCS02-CWE alone but lower than 8 mg/mL of TM02-CWE alone (Figure 5.12).

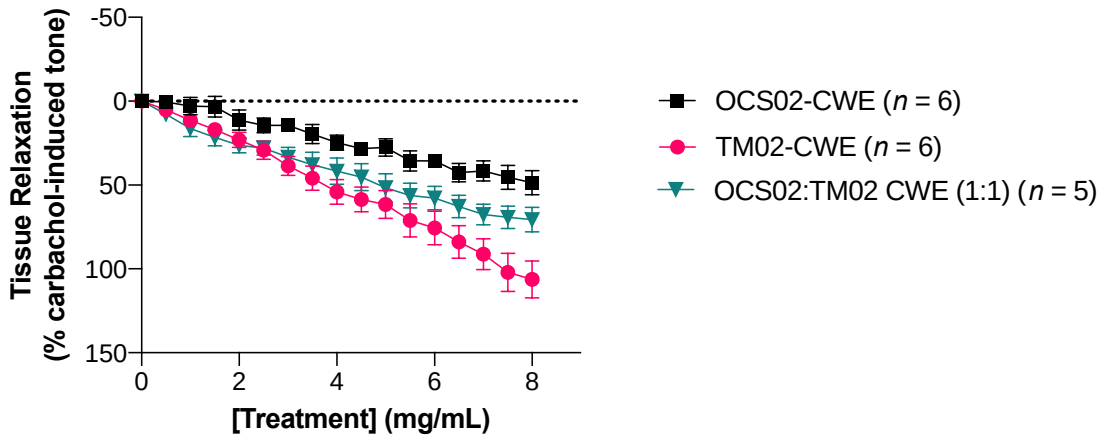


Figure 5.12 The combined effect of TM02-CWE and OCS02-CWE in relaxing carbachol-pre-contracted bladder strips. *CRCs to the combined extract containing OCS02-CWE and TM02-CWE at a ratio of 1:1 were constructed. The resultant concentration of the extract mixture was summation of each extract at the respective concentration i.e., 2 mg/mL of the 1:1 combined extract contained 1 mg/mL of OCS02-CWE and 1 mg/mL of TM02-CWE. Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. (Abbreviations: CRCs = concentration-response curves; CWE = cold water extract)*

To analyse the interaction between the two extracts, the response additivity graphs were constructed at three different effect levels: 30% and 50% of the maximum relaxation response and the maximum relaxation response, derived from the CRCs to the combined extract (Figure 5.12). According to Figure 5.13, the synergistic effects between OCS02-CWE and TM02-CWE reduces as the concentration increases. From the response additivity graphs at these three levels, concentrations beyond 4 mg/mL of each extract may produce antagonistic effect. At 30% and 50% of the maximum relaxation response, the effect of the extract mixture was greater than that of the additive line, indicating a positive interaction (Figure 5.13A – B). However, the maximum relaxation response elicited by the combined effect falls slightly below the line of additivity, indicating a negative interaction (Figure 5.13C). Thus, the results suggest that the synergistic effect between the two extracts was concentration-dependent.

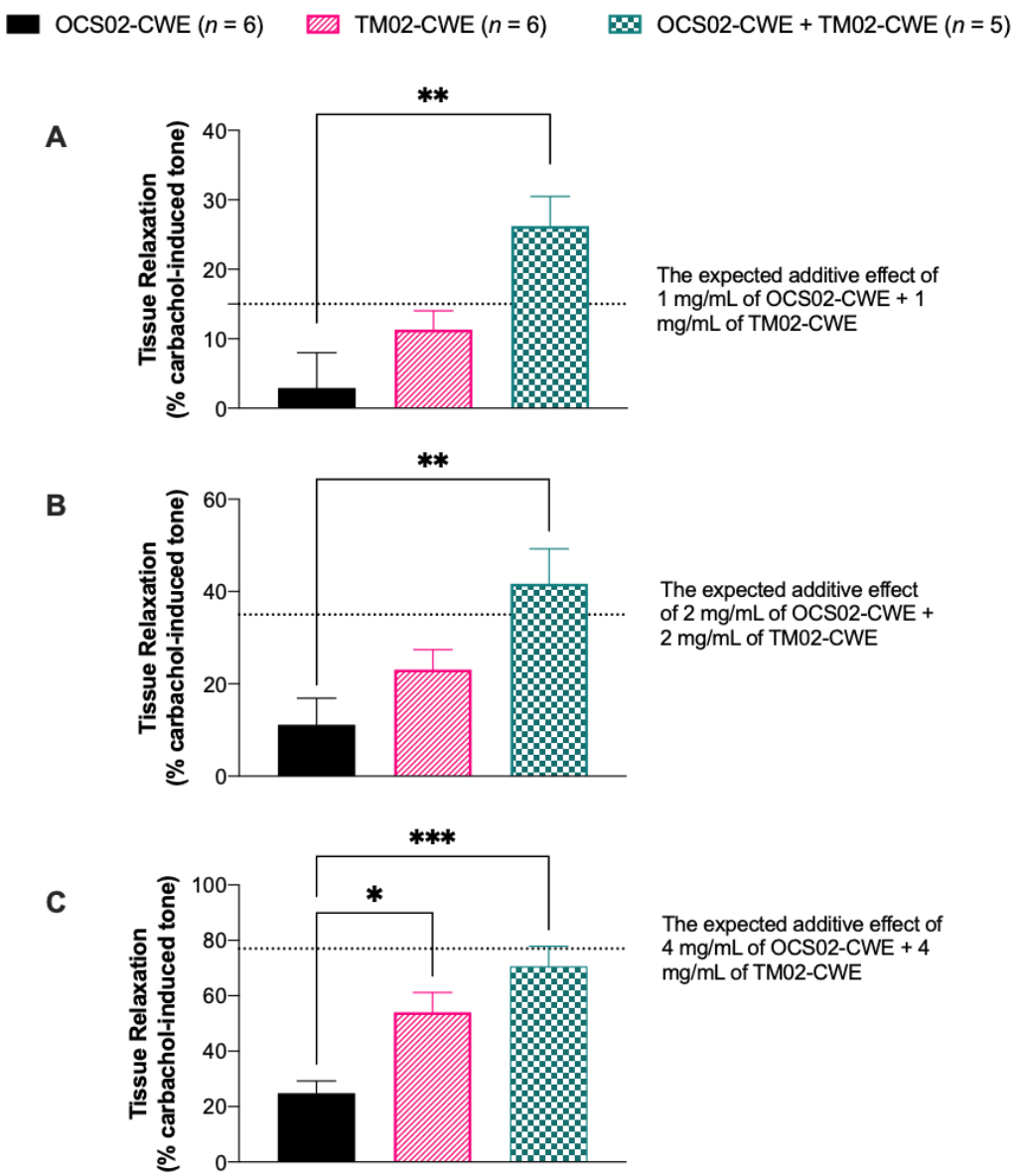


Figure 5.13 Effect-based response additivity for bladder relaxation response induced by OCS02-CWE and TM02-CWE. *The dashed line represents the expected additive effect if the response derived from the extract mixture was simply the sum of the individual responses of OCS02-CWE and TM02-CWE at the respective concentration (A) 30% relaxant response was achieved by the resultant concentration of 2 mg/mL. (B) 50% relaxation response was achieved by the resultant concentration of 4 mg/mL. (C) The maximum relaxation response achieved at the highest concentration tested, 8 mg/mL. A response above or below the additive line indicates a positive interaction or a negative interaction between the extracts, respectively. Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as mean ± SEM of n number of animals. One-way ANOVA with Tukey's post-hoc test where comparison of means between the treatment groups was made: *p < 0.05, **p < 0.01, ***p < 0.001. (Abbreviations: CWE = cold water extract)*

5.5 Discussion

Our findings in **Chapter 3** have demonstrated the pharmacological actions of OCS02-CWE in the bladder. It promotes extracellular Ca^{2+} entry to induce transient bladder contraction and relaxes the bladder via regulation of NO and activation of SERCA. To further explore the potential of medicinal fungi in OAB treatments, the experiments in this chapter were designed to investigate the pharmacological properties of *L. rhinocerus* (TM02) in the bladder and to assess its interaction with *O. sinensis* (OCS02-CWE) in bladder relaxation.

5.5.1 Bioactivity of TM02-CWE and its fractions

As mentioned in **Chapter 2**, fractionation is one of the most crucial steps in the process of identifying bioactive components. The composition analysis of *L. rhinocerus* revealed that it consists of mainly carbohydrates (α- and β-glucans), followed by proteins, and several nucleosides (adenosine and its derivatives) (Lee et al., 2018b). Therefore, size exclusion chromatography was used to fractionate the crude extract as it allows a good separation of large molecules from the small molecules. In airway smooth muscles, the HMW fraction of TM02 has elicited the greatest relaxation response as compared to CWE and other fractions (Lee et al., 2018a). Hence, it is hypothesised that the bladder relaxant effect of TM02-CWE may also vary in fractions of different molecular weight. The results in this study showed that the relaxation response induced by TM02-CWE was greater than all the fractions. Among the fractions, TM02-HMW fraction has the highest efficacy in relaxing the carbachol-pre-contracted bladder, followed by TM02-MMW fraction. TM02-LMW fraction, on the other hand, was lack of relaxant effect.

According to Lee et al. (2018a), the protein contents of TM02-CWE, -HMW, -MMW and -LMW were 2%, 4%, 1% and 0.3%, respectively, whereas the

carbohydrate contents were 68%, 71%, 35% and 22%, respectively. Thus, the bladder relaxant effect of TM02-CWE could be attributed to the large-molecular-weight substances such as carbohydrates, proteins, and/or carbohydrate-protein complexes that are present in TM02-CWE and TM02-HMW at a relatively larger proportion. To ensure the maximum relaxant effect of *L. rhinocerus* was achieved in the process of elucidating its mechanism of action, subsequent experiments were performed using TM02-CWE.

5.5.2 Role of urothelium

In previous chapters, the role of urothelium in bladder relaxation has been explicitly explained and demonstrated. In this study, our results showed that the removal of urothelium has no significant impact on TM02-CWE-induced relaxation response. Thus, unlike OCS02-CWE that may act on both urothelium and the DSM layer, TM02-CWE acts only on the DSM and its action is unlikely to involve urothelial-derived relaxing factors such as NO.

5.5.3 Role of intra- and extracellular Ca^{2+}

Lee et al. (2018b) has demonstrated that the airway smooth muscle relaxant effect of TM02-CWE is associated with the regulation of Ca^{2+}. Although the underlying mechanism remains elusive, evidence pointed that it does not involve the modulation of muscarinic receptors, β-adrenoceptors, K^+-channels, regulation of cyclic nucleotide second messengers nor NO production, in the smooth muscles (Lee et al., 2018b). Therefore, this study focused on the elucidation of Ca^{2+}-mediated relaxation pathways of TM02-CWE.

In the absence of external Ca^{2+}, carbachol-induced contraction is presumed to be solely dependent on intracellular Ca^{2+} release from the SR. Under this condition, pre-incubation of the bladder tissues with TM02-CWE did not affect the contractile

tone induced by carbachol. It suggests that the mechanism of action of TM02-CWE is unlikely to involve inhibition of Ca^{2+} release from the SR through IP_3Rs or RyRs, or reuptake of Ca^{2+} through SERCA. On the contrary, in the presence of external Ca^{2+}, TM02-CWE overtly suppressed the phasic and tonic contraction induced by carbachol. This observation indicates that TM02-CWE inhibits extracellular Ca^{2+} influx to prevent DSM contraction. To confirm this postulation, the effect of TM02-CWE on Ca^{2+}-induced contraction was investigated. Our results showed that pre-incubation with TM02-CWE significantly suppressed Ca^{2+}-induced contraction in a concentration-dependent manner. The suppression effect of TM02-CWE on Ca^{2+}-induced contraction was enhanced by increasing concentrations of TM02-CWE. At 5 mg/mL of TM02-CWE, the Ca^{2+}-induced contraction was abolished, even after the removal of urothelium. These findings further support that TM02-CWE acts directly on the DSM by inhibiting extracellular Ca^{2+} influx.

5.5.4 The contractile mechanism of carbachol

To substantiate the claim that TM02-CWE inhibits extracellular Ca^{2+} influx, subsequent experiments were designed to study the role of membrane permeable Ca^{2+} channels in TM02-CWE-mediated relaxation response. Given that carbachol is used as the main contractile agent in the present study and throughout this thesis, it is essential to clarify the effect of the selected membrane permeable Ca^{2+} channels blockers in the contractile mechanism of carbachol. As a comparison, CRCs to carbachol were constructed in Ca^{2+}-free Krebs solution. Without external Ca^{2+}, the maximum contraction induced by carbachol was dramatically attenuated, confirming that extracellular Ca^{2+} was the main source of Ca^{2+} in carbachol-induced contraction. Ca^{2+} channel blockers have exerted varied degree of inhibition on the contraction induced by carbachol. Among all the Ca^{2+} channel blockers tested, nifedipine has the

greatest suppression effect on carbachol, inferring a major role of L-type VGCCs in contraction caused by $G_{q/11}$ activation. The rank of the suppression effect on carbachol-induced contraction is: nifedipine > TM02-CWE > schwarzinicine A > verapamil > SKF-96365.

From the carbachol CRCs in the presence of these Ca^{2+} blockers, it is observed that TM02-CWE, schwarzinicine A, verapamil and SKF-96365 produced a similar effect on carbachol-induced contraction. Following the maximum tissue contraction that was achieved at 10 μM of carbachol, the contractile tone dropped sharply, and the data points resembled a bell-shaped curve. This phenomenon is likely to be caused by receptor desensitisation (Tanahashi et al., 2021). Moreover, these Ca^{2+} blockers, including TM02-CWE, have enhanced the potency of carbachol, implying that they increased the tissue sensitivity to carbachol at low concentrations. In contrast, nifedipine did not affect the potency of carbachol. Based on these results, TM02-CWE may have a similar mechanism of action to that of schwarzinicine A, verapamil and SKF-96365.

While nifedipine and verapamil are both L-type VGCC blockers, they exhibited different degree of suppressing effect on carbachol-induced bladder contraction. This finding is in line with a previous study where nifedipine exhibited a greater relaxation response than verapamil in tracheal tissues pre-contracted with carbachol (Hirota et al., 2003). This difference could be explained by the dissimilar molecular structures and the variation in binding sites between dihydropyridines (nifedipine) and phenylalkylamines (verapamil). A recent crystallographic analysis revealed that dihydropyridines block the channel pore by interacting with its external, lipid-facing surface whereas phenylalkylamines bind in the central cavity of the pore of the intracellular side of the selectivity filter and block the ion-conducting pathway

(Tang et al., 2016).

Currently, there is no information regarding the binding site of SKF-96365 or schwarzinicine A on the L-type VGCCs. Based on the results obtained in the present study, it is possible that SKF-96365 and schwarzinicine A behave in a similar way to verapamil. The chemical structures of these three compounds (verapamil, SKF-96365 and schwarzinicine A) exhibit similarities i.e., they consist of at least two methoxy aromatic rings and their flexible alkyl skeleton chains contain a central electronegative atom (Singh et al., 2010; Mak et al., 2022). The chemical structures of these compounds are shown in Appendix C. The suppression effect of SKF-96365 and schwarzinicine A on carbachol-induced contraction could also be attributed to the blockade of TRPC channels. TRPC4 channels have been revealed to have a role in triggering extracellular Ca^{2+} influx following muscarinic receptors-induced DSM contraction (Griffin et al., 2018, 2016). Therefore, inhibition of TRPC channels may also prevent Ca^{2+} influx induced by carbachol. In summary, these data provide a better understanding on the role of membrane permeable Ca^{2+} channels in bladder contraction induced by carbachol through the activation of $G_{q/11}$-coupled muscarinic receptors.

5.5.5 Relaxation profile of TM02-CWE

Subsequently, our results demonstrated that nifedipine significantly attenuated TM02-CWE-induced bladder relaxation whereas verapamil, SKF-96365 or schwarzinicine A appeared to potentiate the relaxation response (not statistically significant). These results suggest that nifedipine but not verapamil, SKF-96365 or schwarzinicine A prevents the binding of TM02-CWE. In general, dihydropyridines such as nifedipine have a preferential inhibition on inactive or resting VGCCs. They bind directly to the

inactive VGCCs and stabilise their inactive conformation (Joyner and Johnson, 2008; Singh et al., 2015). Conversely, phenylalkylamines that bind from the intracellular loop of the L-type VGCCs, do not bind to the rested, or closed state of VGCCs (Bergson et al. 2011). Therefore, the binding of verapamil is likely to take place after the activation of L-type VGCCs following $G_{q/11}$ activation. The attenuation of TM02-CWE-relaxation response in the presence of nifedipine thus suggests that TM02-CWE requires activated L-type VGCCs to elicit its effect. This also infers that TM02-CWE may permeate into the plasma membrane before blocking the ion channel, like verapamil.

Besides, since verapamil, SKF-96365 and schwarzinicine A enhanced the relaxation response of TM02-CWE, it supports our postulation that they may have similar mechanisms of action. Assuming TM02-CWE binds only to the activated L-type VGCCs, its effect may take place at the same time when verapamil starts to act on the channel. As a result, both TM02-CWE and verapamil occupy the available L-type VGCCs in a shorter period and produce an enhanced relaxant effect on the carbachol-pre-contracted bladder strips. Likewise, this could also explain the potentiation effect of SKF-96365 and schwarzinicine A on TM02-CWE-induced relaxation response, due to their structural similarity described above. It is noteworthy that our current data is unable to completely exclude the involvement of TRPC in the relaxation mechanism of TM02-CWE. However, this mechanism of action is less likely as the concentration of SKF-96365 (10 µM) and schwarzinicine A (30 µM) are presumably the optimum concentration used to inhibit TRPC channels (Yang et al., 2018; Mak et al., 2022). Therefore, if TM02-CWE blocks TRPC channels, the presence of the SKF-96365 or schwarzinicine A could have prevented the relaxation response induced by TM02-CWE. The potentiated relaxant effect of TM02-CWE by

these non-selective TRPC channels blockers thus infers that Ca^{2+} influx through both L-type VGCCs and TPRC channels that contribute to carbachol-induced contraction was disrupted. To gain a better understanding, more experiments are required to determine the respective potency and affinity of schwarzinicine A and SKF-96365 on L-type VGCCs and TRPC channels in the DSM cells. The postulated mechanism of action of TM02-CWE is illustrated in Figure 5.14.

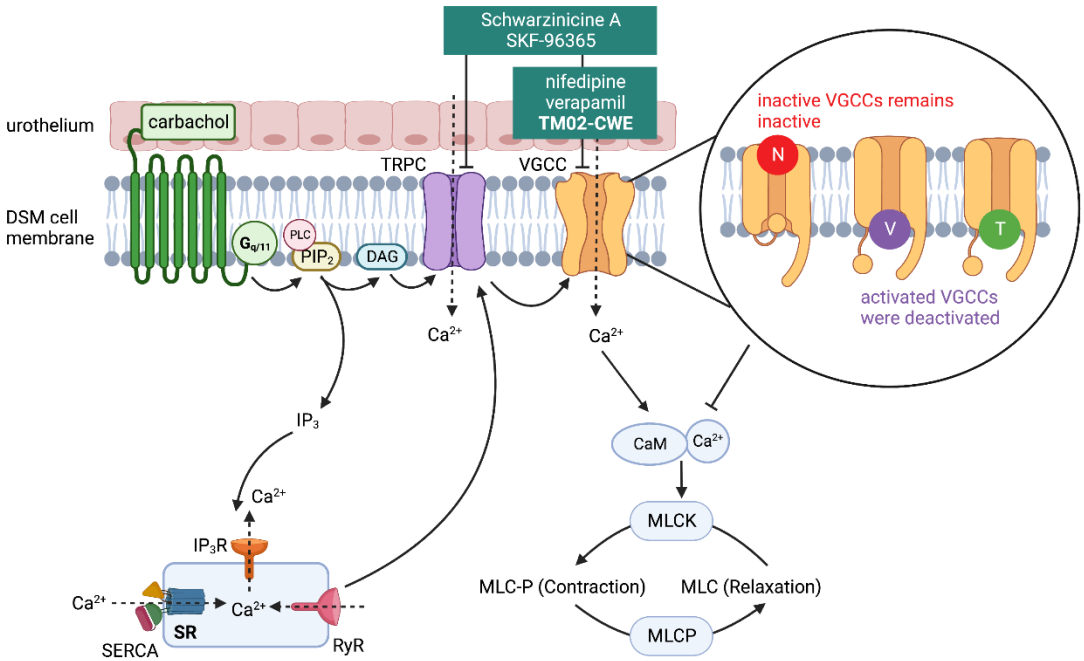


Figure 5.14 Schematic diagram illustrating the purported relaxation mechanism of TM02-CWE. *Activation of $G_{q/11}$-coupled muscarinic receptors by carbachol leads to its downstream signalling that results in intracellular Ca^{2+} release and activates VGCCs and TRPCs to promote extracellular Ca^{2+} influx. Nifedipine (represented by letter "N") and verapamil (represented by letter "V") are both L-type VGCCs but nifedipine prefers to bind to inactivated VGCCs whereas verapamil binds only to the activated VGCCs. Both resulted in VGCCs' blockade, prevented extracellular Ca^{2+} influx and led to DSM relaxation. TM02-CWE (represented by letter "T") is postulated to bind to activated VGCCs. Solid pointed arrows denote downstream effectors activation; blunt arrows denote downstream effectors inhibition; and dashed arrows denote flow of Ca^{2+}. (Abbreviations: CaM = calmodulin; CWE = cold water extract; DAG = diacylglycerol; DSM = detrusor smooth muscle; IP_3 = inositol triphosphate; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR = sarcoplasmic reticulum; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

5.5.6 Combined effects of OCS02-CWE and TM02-CWE

Following the above findings, we showed that pre-incubation with TM02-CWE would suppress the transient contraction induced by OCS02-CWE in a concentration-dependent manner. These results supplemented the mechanism of action of OCS02-CWE in bladder contraction as well as providing further support that TM02-CWE promotes bladder relaxation via the inhibition of extracellular Ca^{2+} influx. Moreover, consistent with the previous findings, inhibition of the transient contraction of OCS02-CWE would not affect its relaxation response.

To enrich our findings on the combined effect of both medicinal fungi extracts on bladder relaxation, a response-additivity analysis was performed to evaluate the interaction between TM02-CWE and OCS02-CWE. The simultaneous addition of TM02-CWE and OCS02-CWE has eliminated the intermittent contraction caused by OCS02-CWE during cumulative addition, as reported in **Chapter 2**. From the CRCs, it was also noticed that the combined effects of OCS02-CWE and TM02-CWE were greater at the lower concentration range. Therefore, three effect levels were chosen for the response-additive analysis to ensure the results were considered and interpreted comprehensively.

Based on the response-additivity analysis and at the ratio of 1:1, TM02-CWE and OCS02-CWE exhibited synergistic effects at low concentrations (< 4 mg/mL). These synergistic effects could be attributed to their different mechanisms of action in mediating DSM relaxation. TM02-CWE, has been shown in this study, to elicit bladder relaxation through blockade of L-type Ca^{2+} channels whereas OCS02-CWE mediated through regulation of NO. The synergistic effect of NO and Ca^{2+} channel blockers in vasodilation have been reported where nifedipine increases the bioavailability of endothelial NO (Berkels et al., 2001). On the other hand, the loss of

synergistic effect at high concentrations (≥ 4 mg/mL) could be explained as follows. In **Chapter 2** and **Chapter 3**, we reported that the maximum bladder relaxation of OCS02-CWE (50% to 60% reduction in carbachol-induced tone) was achieved at 5 mg/mL when being added non-cumulatively and it induced transient contraction at concentrations above 2 mg/mL. Therefore, the contractile effect of OCS02-CWE at higher concentrations that is mediated by extracellular Ca^{2+} influx could compete with TM02-CWE and reduce the overall relaxant effect of the combined extract.

5.6 Chapter summary

Findings from this chapter have demonstrated the modulation of Ca^{2+} by TM02-CWE in rat bladder. Its mechanism of action in bladder relaxation involves inhibition of extracellular Ca^{2+} influx via the L-type VGCCs. It is likely that through this mechanistic pathway, TM02-CWE attenuated OCS02-CWE-induced transient contraction in the bladder strips pre-contracted with carbachol. Besides, low concentrations of TM02-CWE and OCS02-CWE (< 4 mg/mL) seemed to provide synergistic bladder relaxant effect. These results cast a new light on the positive interaction between Ca^{2+} blockade and production of NO in smooth muscle relaxation which may constitute the object of future studies.

6 Effects of *Ophiocordyceps sinensis* on *Caenorhabditis elegans*: A Comparison Study with *Lignosus rhinocerus*

6.1 Introduction

Caenorhabditis elegans, a tiny and non-parasitic nematode, is one of the most useful model organisms for nearly every aspect of biology. As the first multicellular organism with a complete genome sequence, *C. elegans* has shed light on the molecular identification of many key genes in developmental and cell biological processes (Howe, 2019). Genome analysis revealed that *C. elegans* has approximately 20,000 protein-coding genes and at least 83% of its proteome has human homologous genes (Lai et al., 2000). In contrast, only 11% or less of the *C. elegans* proteome contains nematode-specific genes (Lai et al., 2000). Its genome also contains orthologs of about 40% of genes related to human diseases (Culetto and Sattelle, 2000).

The original strain (N2) of this fascinating organism was first isolated from mushroom farm compost in the 1950s in Bristol, UK (Félix and Braendle, 2010). Then, in 1966, it was adopted as a research model by Sydney Brenner, a biologist and a geneticist, due to the ease of its genetic manipulation. Since then, it has been used in a diverse research area including studies of the basic functions and interactions of eukaryotic cells, host-parasite interactions and evolution (Corsi et al., 2015). In recent years, *C. elegans* has been used extensively in the research of neurobiology, developmental biology and genetics. The N2 strain is used as a reference wild type in

nearly all of *C. elegans* research and has resulted in thousands of fully characterised mutants (Félix and Braendle, 2010).

In the laboratory, *C. elegans* can be grown in either liquid or solid culture at temperatures ranging from 16 °C to 25 °C. Temperature beyond 25 °C is not possible as its fertility decreases with increasing temperature, and sterility is reached at 27 °C (Petrella, 2014). Regarding its dietary needs in the laboratory setting, *Escherichia coli* strain OP50 is the most used bacterial food source. The use of *E. coli* was introduced by Sydney Brenner as it grows in thin lawns, allowing easier visualisation and mating of the nematodes (Brenner, 1974). Other strains of *E. coli* such as POS-1 and K12 have also been used, depending on the experimental requirements. Another feature that makes *C. elegans* a powerful research model is that it can be cryopreserved and revived when needed for experimental procedures.

6.1.1 The anatomy and sexual forms of *C. elegans*

C. elegans has a simple anatomy which made up of about 1000 somatic cells and often described as a series of concentric tubes. Figure 6.1 shows a picture of an adult *C. elegans* taken in our laboratory and Figure 6.2 shows its body plan with cross sections from head to tail. The outermost layer i.e., the skin of *C. elegans* is composed of a simple epidermal epithelium and overlying cuticle, referred to as the 'epidermis-cuticle complex' (Chisholm and Hsiao, 2012). The cuticle, consisting primarily of collagen, lipids and glycoproteins, determines the shape of the body and provides anchoring points for muscle contraction. This epidermis-cuticle complex encloses the pseudocoelomic fluid-filled cavity that houses the main organ systems of *C. elegans*. The pseudocoelomic cavity consists of 6 coelomocytes, functioning as scavenger cells that endocytose fluid from the pseudocoelom.

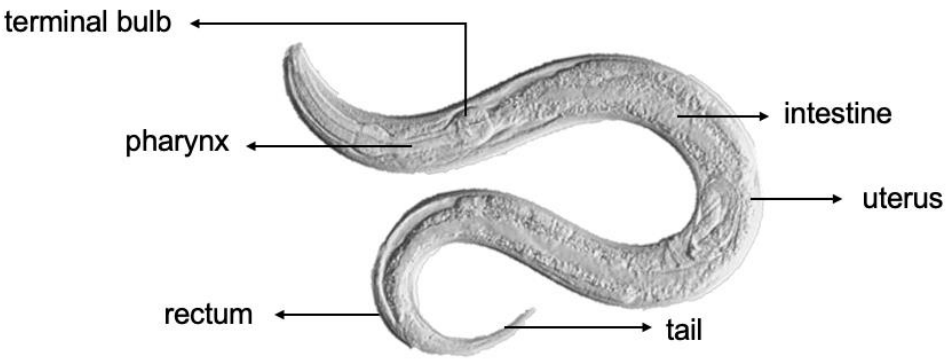


Figure 6.1 An adult C. elegans.

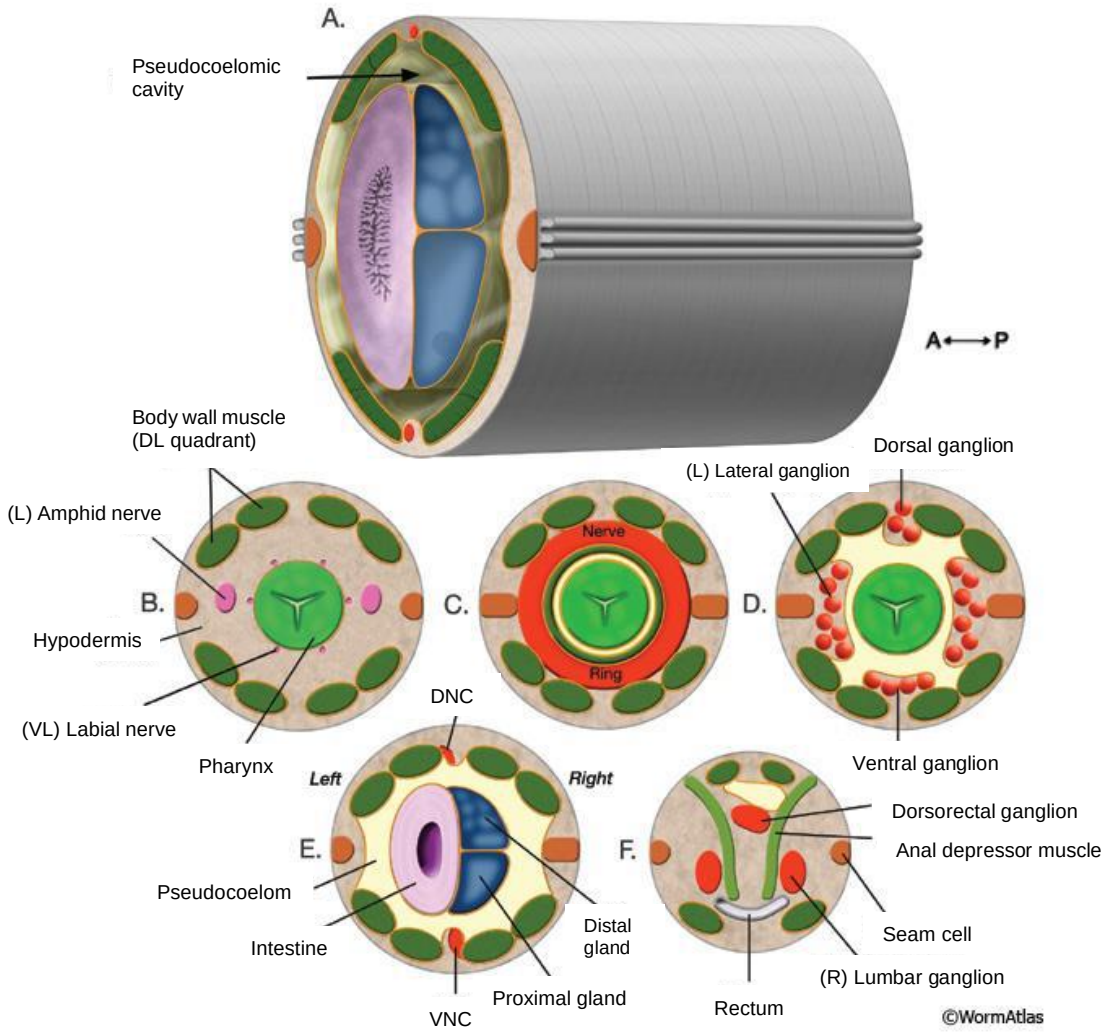


Figure 6.2 Nematode body plan with cross sections from head to tail. *A. Posterior body region. Body wall (outer tube) is separated from the inner tube (alimentary system, gonad) by a pseudocoelom. B. Section through anterior head. C. Section through the middle of head. D. Section through posterior head. E. Section through posterior body. F. Section through tail, rectum area. (Abbreviations: A = anterior; DNC = dorsal nerve cord; NR = nerve ring; P = posterior; VNC = ventral nerve cord) (Adapted from Altun & Hall, 2006)*

In the body, there is a continuous row of neurons (302 neurons in total) that made up the ventral and dorsal nerve cords (VNC and DNC, respectively) which innervate the muscles (Figure 6.2). Majority of the neurons are in the head around the pharynx while others are arranged in a few ganglia in the VNC and in the tail. The neurons have a relatively simple structure with one or two neurites exiting from the

soma. Except for sensory dendrites, most of the neurites give and receive synapses. Thus, they cannot be distinguished as axons or dendrites. The neurites form synapses to each other in four major areas i.e., the nerve ring which encircles the pharynx, the VNC, the DNC and the neuropil of the tail. In addition, *C. elegans* has 50 glial cells derived from neuronal progenitors and 6 glial cells derived from the mesodermal. Glial processes are found at neuronal junctions at every level and are involved in intercellular information transfer (Oikonomou and Shaham, 2011).

Neurons and epidermis are separated from the musculature by a thin basal lamina. The body-wall muscles, arranged into strips in four quadrants (95 cells) with two dorsal and two ventral, receive input from the neurons by sending extensions to motor neuron processes that run along the nerve cords (Figure 6.2). The somatic muscle cells are striated and mononucleated with multiple sarcomeres per cell. In addition to the body-wall muscle, *C. elegans* has pharyngeal muscles that control feeding; vulval and uterine muscles that control egg-laying; enteric muscles that facilitate defecation; and tail-muscles that allow mating in male.

The wild-type *C. elegans* has two sexual forms: self-fertilising hermaphrodites and males (Figure 6.3) (Zarkower, 2006). The gonad of hermaphrodites forms an ovotestis that first produces haploid amoeboid sperms that are stored in the spermatheca. Then, when it reaches adulthood, the germ line switches to produce oocytes. To simplify, hermaphrodites are females whose gonads temporarily produce sperm before they produce oocytes. Hermaphrodites can produce up to 300 self-progenies that are fertilised by the stored sperm. However, if mated with males, hermaphrodites can produce as many as 1400 offspring (Kimble and Ward, 1988).

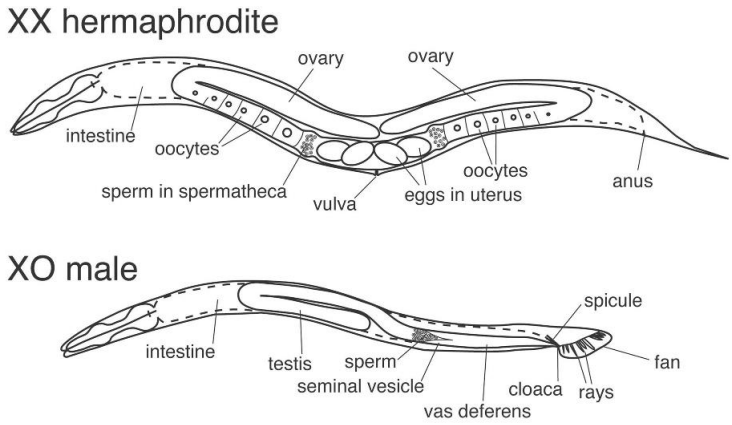


Figure 6.3 The two sexes of C. elegans. *The hermaphrodites and males differ strikingly in overall body size and structures such as the somatic gonad and tail (Adapted from* Zarkower, 2006).

The sexes of *C. elegans* are genetically distinguished by their X chromosome complement as they have no Y chromosomes (Hodgkin, 1987). Hermaphrodites have a pair of identical X chromosome (XX) whereas males have only one X chromosome (XO). Both sexes are diploid with 5 pairs of autosomal chromosomes. Most offspring produced by self-fertilisation are hermaphrodites and only 0.1 – 0.2% of the progeny are males due to occurrence of X-chromosomal non-disjunction during meiosis. As studies found that hermaphrodites favoured self-fertilisation rather than outcrossing, this rare spontaneous production of males has been thought as one of the main factors that avoided the extinction of males *C. elegans* (Chasnov, 2013). Males *C. elegans* are also important for the exchange of genetic material to generate offspring with different genetic compositions and to map genes (Corsi et al., 2015).

6.1.2 The life cycle of *C. elegans*

At the ideal temperature for the growth of *C. elegans* i.e., 20 °C, the embryogenesis takes approximately 16 hours. After fertilisation, an impermeable eggshell is made, and the embryos are usually retained within the hermaphrodite until they reach at the 24-cell stage. The embryo may also develop completely independent of the mother (Altun and Hall, 2006). The hermaphrodite embryo hatches with 558 nuclei and becomes a first stage larva (L1). Then, the larvae begin to eat and develop through four larval stages (L1 – L4) over a period of two and a half days. The L1 stage takes approximately 16 hours whereas each of the other stages takes approximately 12 hours. Each stage ends with a sleep-like period of inactivity called lethargus in which a new cuticle is made. Then, lethargus ends with the moulting of old cuticle. Approximately 12 hours after the L4 *C. elegans* moulted, adult hermaphrodites begin to produce progeny for a period of two to three days until they have utilised all their self-produced sperm. Additional progenies can be generated if the sperm-depleted hermaphrodite mates with a male. After the reproductive period, hermaphrodites can live several more weeks before dying of senescence. The mean lifespan of wild-type *C. elegans* is around 23 days at 16 °C; 14 days at 20 °C; and 9 days at 25 °C (Klass, 1977).

6.1.3 Lifespan regulation of *C. elegans*

Ageing is a progressive and inevitable biological process that is experienced by almost all organisms. It is associated with an increased probability of death due to a spectrum of changes that molecules, cells, organs and organisms undergo over time, from relatively structural deterioration to severe functional impairment (Harman, 1991). Lifespan, on the other hand, is a measurable trait that signifies the period for which an organism lives. Although the terms anti-ageing and lifespan extension are often used interchangeably, it is important to note that lifespan is a single measurable parameter

that defines the length of one's life and does not reflect the ageing process. Nevertheless, the measurement of lifespan of *C. elegans* has been recognised as a useful method to study ageing, age-related diseases and anti-ageing mechanisms because of their short lifespan that can be manipulated via ageing-related signalling pathways or genes (Zhang et al., 2020).

According to current literature, the lifespan of *C. elegans* is regulated by several signalling pathways and epigenetic modifications. These include the insulin/IGF (insulin-like growth factor)-1 signalling pathway, TOR (target of rapamycin) signalling pathway and AMPK signalling pathway (reviewed by Uno & Nishida, 2016). The insulin/IGF-1 signalling pathway is the first pathway implicated in the regulation of ageing in *C. elegans*. It has also been shown to modulate ageing in other species such as flies, mice and humans, implying that the effects of the ageing pathway are conserved (Lapierre and Hansen, 2012). In *C. elegans*, the *daf-2* or *age-1* genes are responsible in the insulin/IGF-1 signalling pathway. Mutations that decrease the activity of *daf-2* have been shown to double the lifespan of *C. elegans* and it is dependent on the activity of another gene, *daf-16*. Both *daf-2* and *daf-16* are also involved in the formation of dauer larva, which is a stress-resistant stage of *C. elegans* that can live several months (Kenyon et al., 1993). On the other hand, mutations that affect the activity of *age-1* may increase the mean lifespan of *C. elegans* by 40% (Friedman and Johnson, 1988). Dietary restriction also extends the lifespan of *C. elegans* by multifactorial processes that involve inhibition of insulin/IGF-1 signalling and reduction of metabolic rate (Wu et al., 2019).

In excess food consumption, TOR can be activated, and it is implicated in disease states where growth and homeostasis are deregulated and compromised. Recent studies suggest that TOR signalling controls cellular senescence and inhibition

of TOR signalling may increase longevity (Zoncu et al., 2011). In *C. elegans*, inhibition of TOR activates *skn-1*, the homolog of nuclear factor erythroid-2-related factor (NRF) proteins, and *pha-4*, the homolog of human forkhead box (FOX) A transcription factor. NRF is a key mediator of genes involved in oxidative phosphorylation and mitochondrial biogenesis whereas FOXA has essential roles in early development, organogenesis, metabolism and age-related metabolic deterioration (Li et al., 2018; Ma et al., 2014). TOR inhibition also stimulates autophagy, a ubiquitous cellular process that is responsible for the degradation of cytoplasmic components and suppress cellular senescence. This pathway is thus one of the most promising avenues of anti-ageing and lifespan extension in *C. elegans* (Chang et al., 2017).

AMPK protein, encoded by *aak-2*, is another important cellular sensor that regulates the lifespan of *C. elegans* (Apfeld et al., 2004). It restores energy homeostasis when cellular energy is compromised by stimulating catabolic processes and preventing energy-consuming processes. Given that the function of mitochondria is impaired in ageing and ATP consumption is increased in older animals to enhance survival, the reduction of AMPK stimulates ageing. Therefore, removal of *aak-2* decreases the lifespan of *C. elegans* and increased expression of *aak-2* increases its lifespan (Apfeld et al., 2004).

The anti-ageing and lifespan extension effects of medicinal fungi has gained interest in recent years due to their potentials to be developed as nutraceutical. *Ganoderma lucidum* water extract has been shown to extend the median lifespan of wild-type *C. elegans* by 45% and its effect is likely to be associated with autophagy induction and stress resistance (Peng et al., 2021). Bioactive components such as protocatechuic acid, caffeic acid and lignan that are present in *Flammulina velutipes*,

Schizophyllym commune and *Anthrodia camphorate*, respectively, have also been shown to extend the lifespan of *C. elegans* (Rahman et al., 2017).

6.1.4 Pharyngeal pumping in *C. elegans*

In *C. elegans*, feeding is achieved through a well-characterised behaviour i.e., pharyngeal pumping that is regulated by the pharynx. The pharynx is a neuromuscular pump at the anterior end of the alimentary tract (Figure 6.1; Figure 6.2). It is 100 µm long with a diameter of 20 µm and consists of a contractile element of 20 muscle cells, 20 neurons that comprise the pharyngeal nervous system and 22 other cells including 4 gland cells, 9 marginal cells and 9 epithelial cells. These cells constitute three functional parts, the corpus, the isthmus and the terminal bulb. During feeding, bacteria are taken up by the mouth and transported to the intestine via the pharynx. The movement of bacteria from outside the *C. elegans* to the intestine is achieved by two pharyngeal movements, that are, pharyngeal pumping and isthmus peristalsis. A pharyngeal pump is defined by one complete cycle of synchronous contraction and relaxation of the corpus and the terminal bulb (Raizen et al., 2012). Isthmus peristalsis occurs to transport the bacteria trapped in the anterior isthmus to the grinder in the terminal bulb. The grinder crushes the bacteria before being digested in the intestine (Song and Avery, 2013).

While isthmus peristalses are coordinated with pumps, the two motions are often studied separately due to several reasons (Song and Avery, 2013). First, isthmus peristalsis does not occur in the absence of pumping and every isthmus peristalsis follows a pump after about 150 msec, regardless of the pharyngeal pumping rate. Second, not every pump is followed by isthmus peristalsis. The ratio of the frequencies of isthmus peristalsis to pharyngeal pumping was 1:3.4, in average. Third, both motions can be activated by the same neurotransmitter such as serotonin. For most

experimental purposes, pharyngeal behavioural analysis requires only a routine stereomicroscope and a pair of eyes. This simple method makes it a versatile model to investigate the feeding behaviour of *C. elegans* and the effect of certain treatment on the pharynx. In recent years, advanced methods such as tracking microscope, fluorescence imaging and PharaGlow (an image analysis tool), have been introduced to measure pharyngeal pumping activity to increase the accuracy and reduce unintentional biases in analysis (Bonnard et al., 2022).

The pharyngeal muscle system coordinates myogenic contractions to achieve feeding and regulates the pharynx in response to environmental stimuli. Albeit being a neuromuscular pump, the pharyngal nervous system is not essential for pumping in *C. elegans*. Feeding may continue even after ablation of all pharyngeal neurons, suggesting that the pumping capacity is probably intrinsic to the muscle cells (Dallière et al., 2016). However, the pharynx pumps in a slow and uncoordinated manner without the regulation of nervous system. Efficient pumping and trapping of bacteria by the pharynx require functional neural circuits that is controlled by a pair of pharyngeal motor neurons, MC and M3 (Ortiz et al., 2018). Each pump cycle corresponds to the propagation of a single pharyngeal muscle action potential, initiated by MC and terminated by M3.

The action potential of pharyngeal muscle can be activated by a variety of neurotransmitters and neuromodulators, and largely relies on T-type and L-type VGCCs, encoded by *cca-1* and *egl-19*, respectively. T-type and L-type VGCCs shape the rise- and plateau phases of the pharyngeal action potential (Kwok et al., 2006). Briefly, T-type VGCCs are activated in response to the small membrane depolarisations (EPSPs) resulting from MC neuron via stimulation of nicotinic receptors. Then, Ca^{2+} influx through T-type VGCCs and the membrane potential is

increased to the threshold for the activation of L-type VGCCs. As a result, intracellular Ca^{2+} increases and results in pharyngeal muscle contraction. Then, repolarisation is modulated by glutamate-gated Cl^- channels and executed by voltage-gated K^+ channels, encoded by *exp-2* (Shtonda and Avery, 2005). As the voltage-dependent K^+ channels are activated, VGCCs are closed, restoring the low resting Ca^{2+} levels. The pharyngeal muscle also expresses RyRs that are encoded by *unc-68*, and the SR Ca^{2+} storage protein, CSQ-1 (Maryon et al., 1996). However, the RyRs are not involved in the regulation of pharyngeal muscle contraction of *C. elegans.*

Generally, the pharyngeal pumping rate of *C. elegans* reaches a maximum of around 300 pumps per minute (ppm) in two-day-old-adults (Alvarez-Illera et al., 2016). Consistent with the idea that organ's function declines with advanced chronological age, the pharynx function of *C. elegans* declines with age. Thus, the pharyngeal pumping rate is commonly used as an indicator of ageing. This age-related change can however be altered through treatments or gene mutations. Comparing to the wild-type *C. elegans,* longer-lived mutants such as *daf-2 (e1370)* and *hsf-1*maintained relatively youthful pharynx function at advanced ages (Russell et al., 2019). In contrast, the shorter-lived *hsf-1* mutant lost function before all other genotypes. The mechanism behind this age-related change remains vague. Alvarez-Illera et al. (2016) attributed the phenomenon to the inhibition of ATP-dependent Ca^{2+}-pumps upon energy depletion. This is because prolonged Ca^{2+} influx was more frequently observed in young nematodes and less frequently observed in energy-deficient mitochondrial respiratory chain *nuo-6* mutants. Despite of the paradoxical observation, the overall Ca^{2+} oscillations were unaltered in young and old nematodes (Alvarez-Illera et al., 2016). Therefore, monitoring of pharyngeal muscle Ca^{2+} dynamic could also be used a novel tool to study cellular energy availability (Alvarez-Illera et al., 2016).

6.2 Aim and objectives

In previous chapters, we have elucidated the pharmacological actions of *O. sinensis* (OCS02-CWE) in the bladder, corroborating its possible therapeutic effects in OAB. Given that OAB is highly prevalent in the geriatric population, this study aimed to investigate the possible benefits of OCS02-CWE in ageing and age-related changes of physiological process using *C. elegans* as an in-vivo model. Measurement of pharyngeal pumping activity of *C. elegans* also allows evaluation of its Ca^{2+} modulating properties. As a comparison, the effects of *L. rhinocerus* (TM02-CWE) which has overt Ca^{2+}-inhibitory property, were also investigated. The objectives of this study were:

i. To investigate the effect of OCS02-CWE and TM02-CWE on the lifespan of *C. elegans*
ii. To examine the effect of OCS02-CWE and TM02-CWE on the pharyngeal pumping rate of *C. elegans*
iii. To assess the relationship between lifespan and pharyngeal pumping rate of *C. elegans*

6.3 Methods and materials

6.3.1 Cultivation of *C. elegans*

Wild-type *C. elegans* (N2 strain) was obtained from the Caenorhabditis Genetics Centre (CGC), University of Minnesota and maintained at 16 °C to propagate freely. They were cultured on 6 cm petri dishes containing nematode growth media (NGM) seeded with a lawn of standard laboratory food source, *E. coli* strain OP50, and were transferred to new plates every 4 to 6 days when the food source has depleted to prevent contamination.

E. coli OP50 were maintained on Luria Bertani (LB) (Friendemann Schimdt, USA) agar plates at 4 °C. To prepare the food source, a single colony of bacteria was inoculated into LB broth supplemented with 100 µg/mL of streptomycin (Nacalai Tesque, Japan) in a conical flask. Then, the flask was incubated overnight at 37 °C with gentle shaking at 180 rpm. After 16 – 20 hours, the *E. coli* culture were transferred into falcon tubes and centrifuged at 4000 rpm for 25 minutes at 4 °C to precipitate the bacterial cells. After centrifugation, the supernatant was discarded, and the bacterial pellets were made concentrated to 25-fold by adding 2 mL of LB broth. The mixture was suspended using a vortex mixer until the bacterial cells were fully suspended in the LB broth. The concentrated *E. coli* were then seeded onto NGM agar plates.

6.3.2 Preparation of NGM agar

All salts used in the preparation of NGM agar and M9 solution (section 6.3.3) were obtained from Chemiz, Malaysia, unless otherwise stated. Before preparing NGM agar, a series of solution was prepared in advance. KPO_4 was prepared by slowly adding 1 M of K_2HPO_4 to 1 M of KH_2PO_4 to reach a pH of 6.0. $MgSO_4$ and $CaCl_2$ were dissolved in purified water to make stock solutions of 1 M. Then, KPO_4, $MgSO_4$ and $CaCl_2$ were autoclaved at 121 °C for 20 minutes. Cholesterol (Sigma Aldrich, USA)

was dissolved in 95% ethanol to make a stock solution of 5 mg/mL. Streptomycin was dissolved in sterile purified water to make a stock solution of 100 mg/mL. Cholesterol and streptomycin were sterilised by filtering through a membrane filter of 0.2 µM pore size. Sterile membrane filtration technique was used as cholesterol and the antibiotic would be denatured if exposed to high temperature and that cholesterol was dissolved in ethanol. These stock solutions were kept at 4 °C for up to a month. To prepare fresh NGM, 2.5 g of bacterial peptone, 3 g of NaCl and 20 g of bacterial agar were dissolved in 1 L of purified water and autoclaved for 1 hour at 121 °C. After autoclaving, 1 mL of 1 M $MgSO_4$, 1 mL of 1 M $CaCl_2$, 1 mL of 5 mg/mL of cholesterol, 1 mL of 100 mg/mL streptomycin, and 25 mL of 1 M KPO_4 were added to the mixture in a biosafety cabinet. The mixtures were swirled gently and pour into 6 cm petri dishes. Then, each NGM plate was seeded with a lawn of *E. coli* OP50. For 6 cm plates, 300 µL of bacteria were seeded whereas for 3.5 assay plates, 50 µL of bacteria were seeded.

To prevent eggs from hatching and interfere the measurement during the lifespan assays and pharyngeal pumping rate assays, the N2 *C. elegans* were subjected to RNAi treatment through feeding (Conte et al., 2015). This was achieved by incorporating 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Merk Millipore, USA) to the NGM agar. The *C. elegans* were fed with *E. coli* expressing dsRNA for a portion of the pos-1 (*E. coli* strain POS-1) instead of *E. coli* OP50. N2 *C. elegans* grown on this bacteria strain show complete embryonic lethality, laying only unhatched eggs (Tijsterman et al., 2004). Carbenicillin (Fisher Scientific, USA) was used to replace streptomycin in the NGM agar as *E. coli* POS-1 are sensitive to streptomycin.

6.3.3 Preparation of age synchronised *C. elegans* for assays

M9 buffer and lysis buffer were required for the preparation of age-synchronised *C. elegans*. The M9 buffer (KH_2PO_4 3 g/L, Na_2HPO_4 6 g/L, NaCl 5g/L and $MgSO_4$ 1 mM) was prepared by dissolving 3 g of KH_2PO_4, 6 g of Na_2HPO_4 and 5 g NaCl in 1 L of purified water. The solution was then autoclaved at 121 °C for 20 minutes. After autoclaving, sterile $MgSO_4$ was added to the solution. M9 buffer was stored at 4 °C for up to a month. Lysis buffer was freshly prepared every time age-synchronisation of *C. elegans* was performed using sodium hypochlorite (household bleach Clorox®), NaOH and sterile purified water. For every 5 mL of lysis buffer, 2 mL of Clorox®, 0.5 mL of 10 M NaOH and 2.5 mL of purified water were required. The solution was mixed gently in a falcon tube.

Plates with mainly gravid *C. elegans* with eggs in the uterus and a lot of laid eggs were chosen for egg preparation (Figure 6.4). To wash the egg-containing plates, 5 mL M9 buffer solution was transferred into the plate. The eggs were washed down from the surface by gently pipetting the solution. The washed mixture was then transferred into a falcon tube.

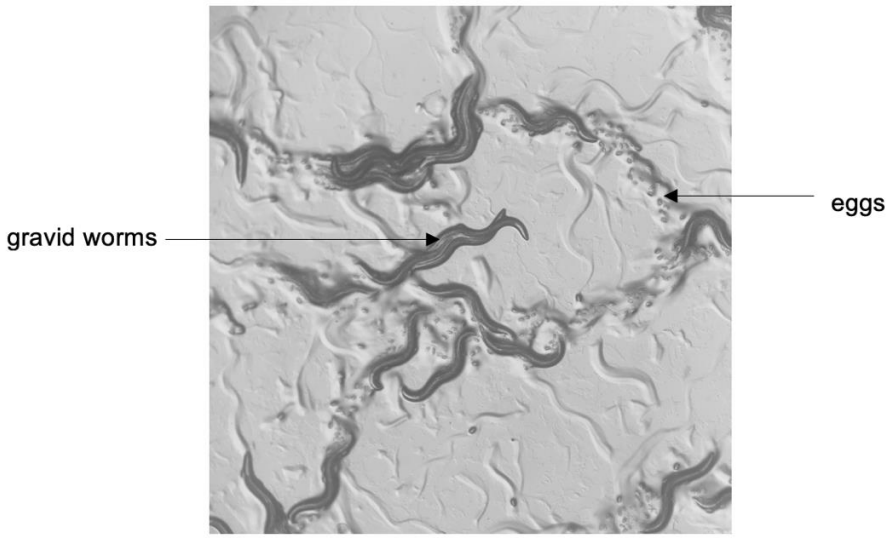


Figure 6.4 Gravid C. elegans and eggs.

Then, equivalent amount of lysis buffer (5 mL) was added into the falcon tube containing eggs and gravid *C. elegans* in M9 buffer. The mixture was then vortexed thoroughly for 5 minutes to break the cuticles to release the eggs which are covered by chitin shell and resistant to bleach. Following thorough mixing, the mixtures were then centrifuged to pellet the eggs. The tubes were spun at 1800 rpm for 1 minute at 25 °C. The supernatant was discarded and 5 mL of M9 buffer was re-added. The centrifugation and re-addition of M9 buffer were repeated twice to ensure complete removal of the bleach. Finally, the supernatant was discarded and the remaining eggs in the pellet were seeded onto fresh NGM-IPTG plates around the *E. coli* POS-1 lawn. The presence of eggs and the correct placement of eggs were checked under a microscope. The egg droplets were allowed to dry and kept at 16 °C. Figure 6.5 shows an egg droplet that was seeded on the NGM plate.

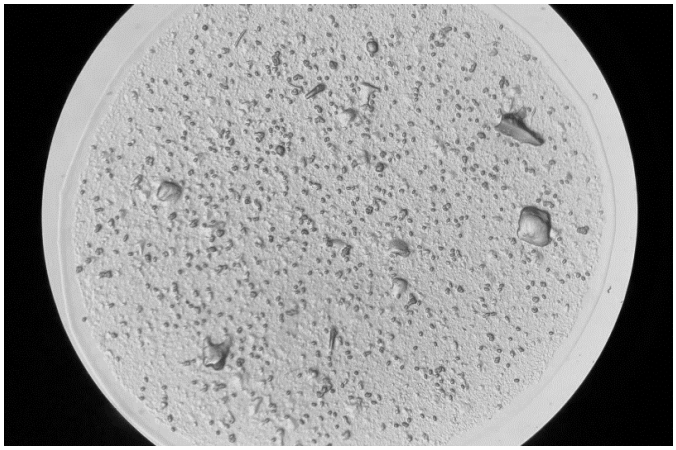


Figure 6.5 C. elegans eggs seeded on NGM agar observed under a stereomicroscope (40x).

6.3.4 Preparation of extracts, drugs and assay plates

OCS02-CWE and TM02-CWE were prepared as described in **section 2.3.2** and **section 5.3.2**. Autoclaved purified water was used to dissolve the freeze-dried extract to minimise risk of contamination and autoclaved purified water was used as the vehicle control. The positive controls used in pharyngeal pumping assays, nifedipine (Nacalai Tesque, Japan) and nemadipine A (Sigma Aldrich, USA) were dissolved in DMSO 100% to make a stock solution of 0.1 M. DMSO 5% was used as the vehicle control for nifedipine and nemadipine A in the pharyngeal pumping rate assays. The stock solutions of the extracts and drugs were sterilised by filtering through a membrane filter with a pore size of 0.2 µM (Sartorius Stedim, Germany). Table 6.1 displays the reconstitution of DMSO, nifedipine, nemadipine A, OCS02-CWE and TM02-CWE in heat-killed *E. coli*, to make up to a final volume of 50 µL and be seeded on one 3.5 cm NGM-containing plate. Heat-killed *E. coli* was used to ensure they do not contribute any unnecessary variables to the experiment.

Table 6.1 Details on stock and final concentration, concentration of DMSO, and volume of stock solution and heat-killed E. coli required for each 3.5 cm assay plate.

Compound	Stock concentration	Final concentration	Volume of stock solution (µL)	Volume of heat-killed *E. coli* (µL)
Blank control	-	-	-	50
DMSO	100%	5%	2.5	47.5
Nifedipine	10 µM	1 µM (0.5% DMSO)	5	45
Nemadipine A	100 µM	20 µM (5% DMSO)	10	40
H_2O	-	-	10	40
OCS02-CWE and TM02-CWE	100 mg/mL	1 mg/mL	0.5	49.5
		5 mg/mL	2.5	47.5
		10 mg/mL	5	45
		20 mg/mL	10	40

(Abbreviations: CWE = cold water extract; DMSO = dimethyl sulfoxide)

6.3.5 Lifespan assay

The lifespan assay of *C. elegans* was performed as previously described by Sutphin and Kaeberlein (2009) with some modifications suggested by Dr Alice Kong, UNM. Age synchronised, *pos-1* RNAi-treated wild-type *C. elegans* at the L4 stage were utilised for a lifespan assay on solid agar medium (NGM agar). They were transferred manually to the assay plates using a platinum wire picker and were divided into experimental groups including a blank control group, a vehicle control group and four OCS02-CWE-treated groups at concentrations of 1 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL. The nematodes were maintained at 16°C throughout the assay period and were scored every other day as alive or dead; those that failed to respond to a gentle touch with platinum wire were considered dead and removed from the plates. They

are censored if become desiccated, missing, have crawled off, exhibiting death unrelated to the treatment (vulval rupture or death due to injury from picking). The experiments were run in three replicates with 30 individual *C. elegans* in each treatment group in each replicate, giving a total of 90 worms for each group. Similar experimental procedure was performed to investigate the effect of TM02-CWE at the same concentration range (1 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL).

6.3.6 Pharyngeal pumping rate assay

The measurement of pharyngeal pumping was performed as described by Raizen et al. (2012) with some modifications suggested by Dr Alice Kong, UNM. Age synchronised, *pos-1* RNAi-treated wild-type *C. elegans* at the L4 stage were utilised for the pharyngeal pumping rate assays. They were transferred manually to the assay plates using a platinum wire picker and were divided into experimental groups that include a blank control group, two vehicle control groups (DMSO 5% and purified water), two positive control groups using 20 µM of nemadipine A and 10 µM of nifedipine (Kwok et al., 2006), four OCS02-CWE-treated groups and four TM02-CWE-treated groups at 1 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL. The nematodes were maintained at 16°C throughout the assay period. Each assay plate contained 5 individual *C. elegans* and their pharyngeal pumping rates were counted for 10 seconds under a light microscope (Olympus CX21), 10x magnification. The pharyngeal pumping rates were counted for each group at the 4^{th}, 24^{th}, 36^{th} and 72^{nd} hour of the assay period. A single pharyngeal pump was defined as one contraction-relaxation cycle of the terminal bulb of the pharyngeal muscle (Raizen et al., 2012).

6.3.7 Combined lifespan and pharyngeal pumping rate assay

To enhance our understanding on the relationship between lifespan and pharyngeal pumping activity, the experiments were modified to run both assays simultaneously using the same batch of *C. elegans*. They were divided into a vehicle control group, an OCS02-CWE (10 mg/mL)-treated group and a TM02-CWE (10 mg/mL)-treated group. The concentration of 10 mg/mL was chosen as it significantly affects the lifespan and pharyngeal pumping in both treatment groups. They were scored live or dead every other day until the last nematode was dead. At the same time, their pharyngeal pumping rates were recorded until the pumps cannot be observed. The experiments were run in three replicates with 10 nematodes per treatment group per replicate.

6.3.8 Data analysis

For all experiments, three replicates for each treatment group were performed with a total of 90 nematodes for lifespan assay, 15 nematodes for pharyngeal pumping rate assay and 30 nematodes for the combined assay. The survival curves were plotted as a percentage of the survival (Figure 6.6, Figure 6.7 and Figure 6.11A). The statistical differences in the lifespan of the treatment and control group were assessed by Kaplan-Meier non-parametric analysis and log-rank (Mantel-Cox) test in Prism version 9.0 (GraphPad software, USA). The overall survival of *C. elegans* has been expressed as mean lifespan ± SEM. Results were considered statistically significant if $p < 0.05$.

For pharyngeal pumping rate assay, data were analysed and compared using two-way ANOVA in Prism version 9.0. Results were considered statistically significant if $p < 0.05$. The pharyngeal pumping rate was expressed as mean number of pumps per minute ± SEM (Figure 6.8, Figure 6.9, Figure 6.10 and Figure 6.11B).

6.4 Results

6.4.1 OCS02-CWE overtly prolonged the lifespan of wild-type *C. elegans*

In order to investigate the effect of OCS02-CWE on the lifespan *C. elegans*, they were treated with various concentrations of the extract. The vehicle control did not show any difference in the mean and maximum lifespan when compared to the blank control group (Figure 6.6A, Table 6.2). Comparing to the vehicle control, OCS02-CWE treatment caused a right shift on the survival curve and the maximum lifespan of *C. elegans* was significantly lengthened in the groups treated with 5 mg/mL and above (Figure 6.6B, Table 6.2). Their mean and maximum lifespan were summarised in Table 6.2.

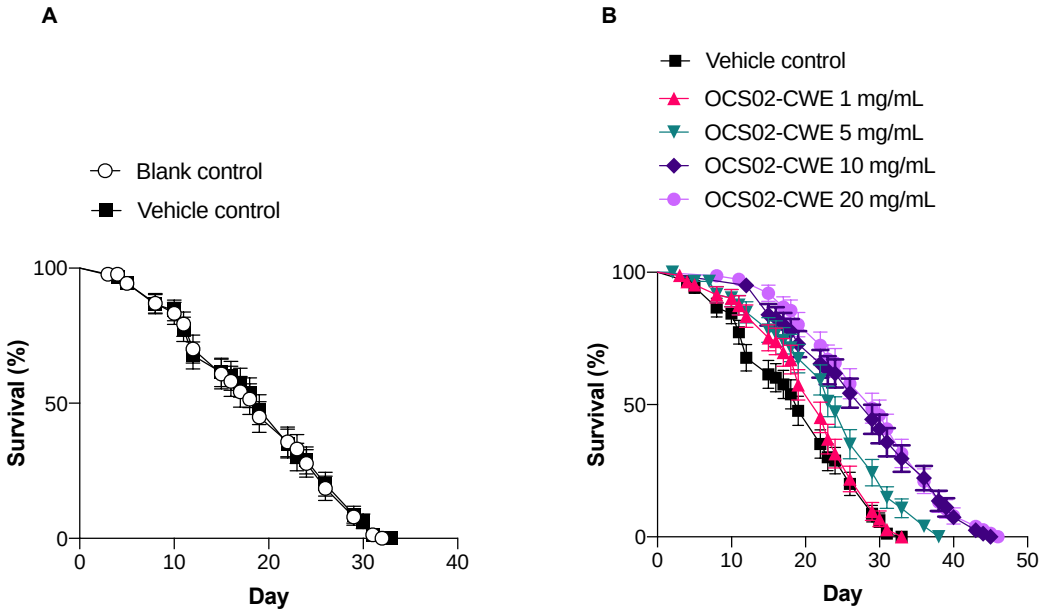


Figure 6.6 Effect of OCS02-CWE on the lifespan of N2 wild-type C. elegans. *Day 1 adult nematodes at the L4 stage were treated with varied concentrations of OCS02-CWE and incubated at 16 °C throughout the assay period. The nematodes were scored every other day from day 1 of adulthood to death. OCS02-CWE significantly increased the survival rate of C. elegans compared to the control and vehicle control groups. The graph represents mean ± SEM of 3 replicates with 30 nematodes per treatment group per replicate. (Abbreviation: CWE = cold water extract)*

Table 6.2 Summary of Kaplan-Meier survival statistics for the treatment groups.

Group	Mean Lifespan (Day)	Maximum Lifespan (Day)	% Increase in Maximum Lifespan
Blank control	18.60 ± 0.92	29.00 ± 2.52	-
Vehicle control	18.66 ± 0.89 (p = 0.9112)	31.67 ± 1.33	9.21 %
OCS02-CWE 1 mg/mL	20.51 ± 0.88 (p = 0.3658)	31.33 ± 0.88	1.07 %
OCS02-CWE 5 mg/mL	23.19 ± 1.02 (p = 0.0002)	37.33 ± 0.67	17.87 %
OCS02-CWE 10 mg/mL	27.29 ± 1.04 (p < 0.0001)	42.00 ± 2.08	32.62 %
OCS02-CWE 20 mg/mL	28.96 ± 1.02 (p < 0.0001)	43.00 ± 2.08	35.78 %

Data are mean ± SEM of 3 replicates with 30 nematodes per treatment group per replicate. Log-Rank (Mantel-Cox) test p-values for the groups: blank control vs vehicle control; vehicle control vs OCS02-CWE groups. Maximum lifespan was obtained by averaging the maximum lifespan in the three replicates. (Abbreviation: CWE = cold water extract)

6.4.2 TM02-CWE extended the lifespan of *C. elegans* but at high concentration

In another set of experiments, the effect of TM02-CWE on the lifespan of *C. elegans* was investigated. The mean and maximum lifespan of the nematodes in the blank control and vehicle control group were similar (Figure 6.7A). Comparing to the vehicle control, 10 mg/mL and 20 mg/mL of TM02-CWE significantly increased the mean lifespan of *C. elegans* (Figure 6.7B). Their mean and maximum lifespan were summarised in Table 6.3.

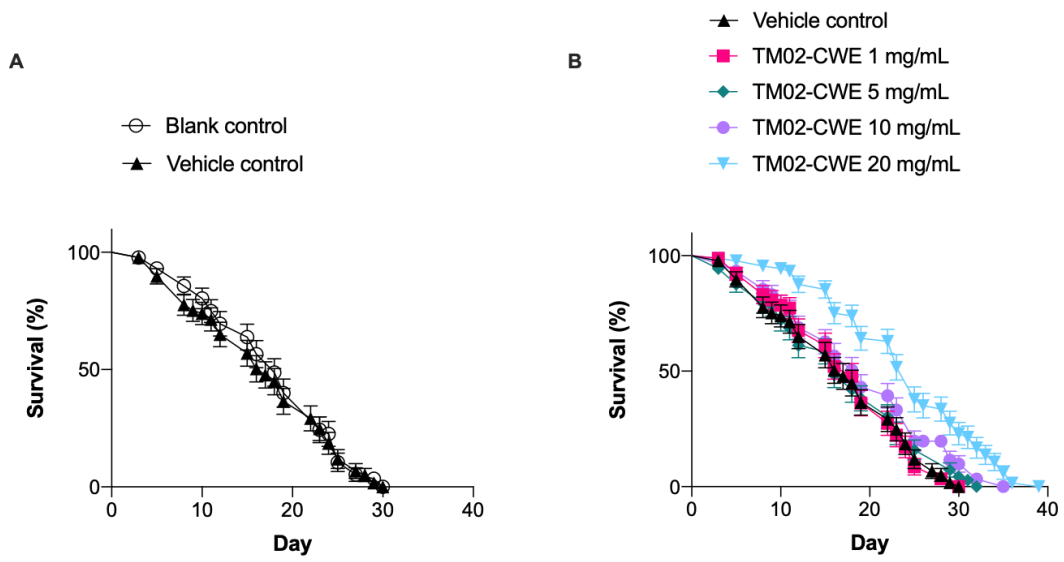


Figure 6.7 Effect of TM02-CWE on the lifespan of N2 wild-type C. elegans. *Day 1 adult nematodes at the L4 stage were treated with varied concentrations of TM02-CWE and incubated at 16 °C throughout the assay period. The nematodes were scored every other day from day 1 of adulthood to death. TM02-CWE significantly increased the survival rate of C. elegans compared to the control and vehicle control groups. The graph represents mean ± SEM of 3 replicates with 30 nematodes per treatment group per replicate. (Abbreviation: CWE = cold water extract)*

Table 6.3 Summary of Kaplan-Meier survival statistics for the treatment groups.

Group	Mean Lifespan (Day)	Maximum Lifespan (Day)	% Increase in Maximum Lifespan
Blank control	16.77 ± 0.91	27.67 ± 1.33	-
Vehicle control	16.04 ± 0.87 (p = 0.4332)	29.33 ± 0.67	6.00 %
TM02-CWE 1 mg/mL	16.12 ± 0.83 (p = 0.7348)	29.00 ± 0.58	-1.13 %
TM02-CWE 5 mg/mL	16.29 ± 0.95 (p = 0.3400)	29.67 ± 0.33	1.16 %
TM02-CWE 10 mg/mL	17.97 ± 0.95 (p = 0.0194)	31.67 ± 0.88	7.98 %
TM02-CWE 20 mg/mL	22.86 ± 0.94 (p < 0.0001)	35.00 ± 0.58	19.33 %

Data are mean ± SEM of 3 replicates with 30 nematodes per treatment group per replicate. Log-Rank (Mantel-Cox) test p-values for the groups: blank control vs vehicle control; vehicle control vs TM02-CWE groups. Maximum lifespan was obtained by averaging the maximum lifespan in the three replicates. (Abbreviation: CWE = cold water extract)

6.4.3 Ca^{2+}-channel blockers decreased the pharyngeal pumping rate of *C. elegans*

In this experiment, Ca^{2+}-channel blockers including nifedipine and nemadipine A were used as positive controls whereas DMSO 5% served as the vehicle control. The pharyngeal pumping rates of *C. elegans* decreased gradually over a period of 72 hours and DMSO did not seem to affect the pharyngeal pumping rate (Figure 6.8A). Comparing to the vehicle control, nifedipine and nemadipine A significantly potentiated the reduction in the pharyngeal pumping rate of *C. elegans* after 48 hours and 72 hours of treatment (Figure 6.8B).

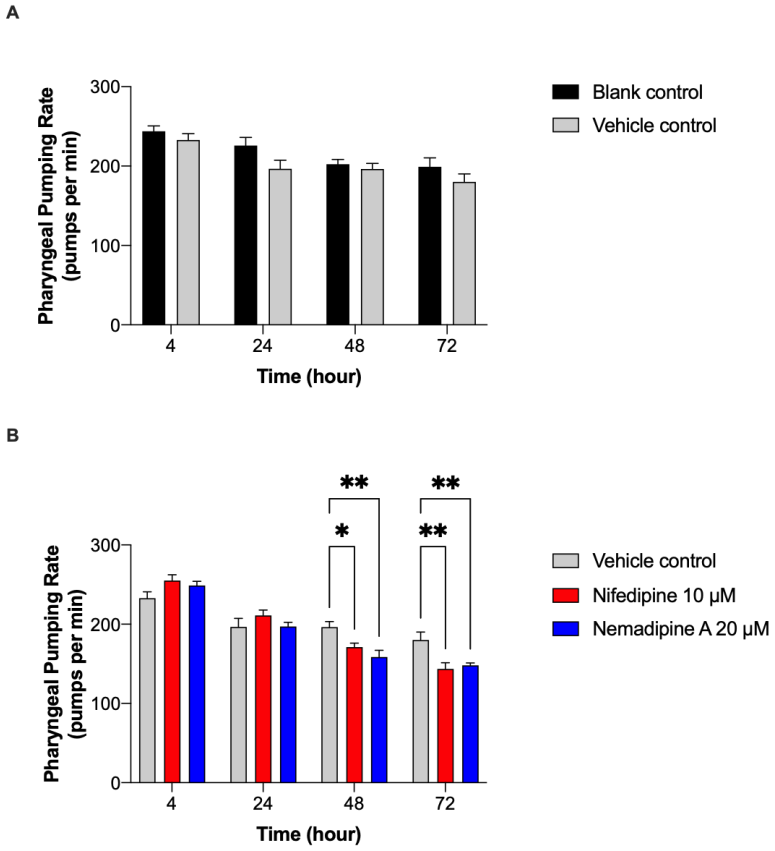


Figure 6.8 Pharyngeal pumping rate of C. elegans. *(A) Pharyngeal pumping rate of C. elegans without any treatment (blank control) and C. elegans treated with vehicle control (DMSO 5%); (B) Pharyngeal pumping rate of C. elegans treated with nifedipine 10 µM and nemadipine A 20 µM. Data are expressed as mean ± SEM of three replicates with five nematodes per replicate, *p < 0.05, **p < 0.01, two-way ANOVA followed by Dunnett's post hoc test.*

6.4.4 OCS02-CWE improved the pharyngeal pumping activity of *C. elegans*

The results showed that OCS02-CWE prevented the reduction of pharyngeal pumping rate of *C. elegans*. From Figure 6.9, it can be observed that the pharyngeal pumping rate in the control group gradually reduced over the period of 72 hours. However, in the groups treated with OCS02-CWE, the pharyngeal pumping rates remained constant over time.

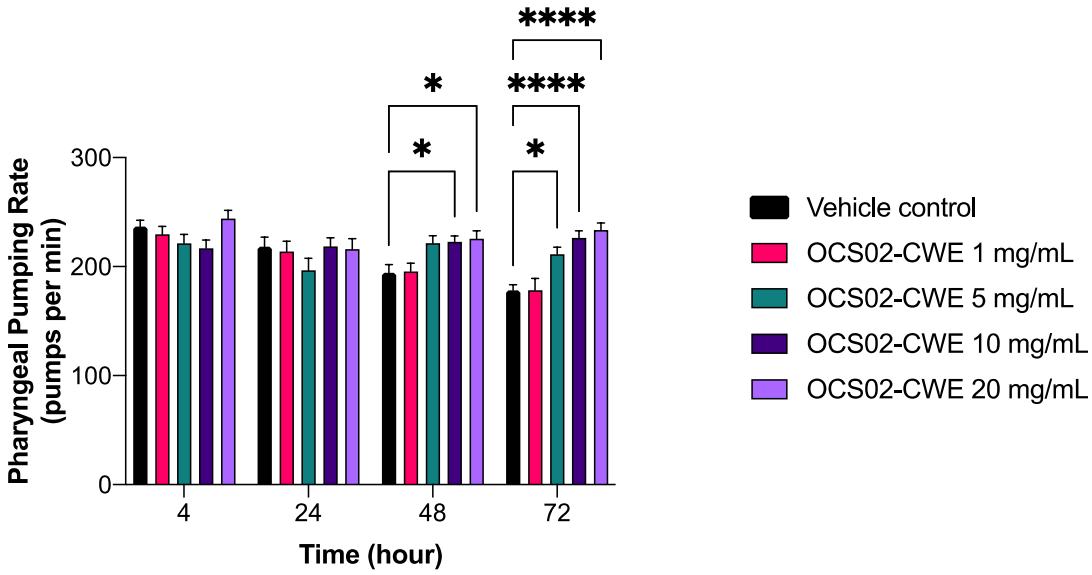


Figure 6.9 Effects of OCS02-CWE on the pharyngeal pumping rate of C. elegans. *Data are expressed as mean ± SEM of three replicates with five nematodes per replicate, *p < 0.05, ****p < 0.0001, two-way ANOVA followed by Dunnett's post hoc test. (Abbreviation: CWE = cold water extract)*

6.4.5 TM02-CWE reduced the pharyngeal pumping rate of *C. elegans*

Unlike OCS02-CWE, TM02-CWE appeared to potentiate the reduction of pharyngeal pumping rate of *C. elegans* over time. The effect was similar to that of the positive controls. At 10 mg/mL, the pharyngeal pumping rates were significantly reduced at the 24th and 72nd hour of treatment period (Figure 6.10).

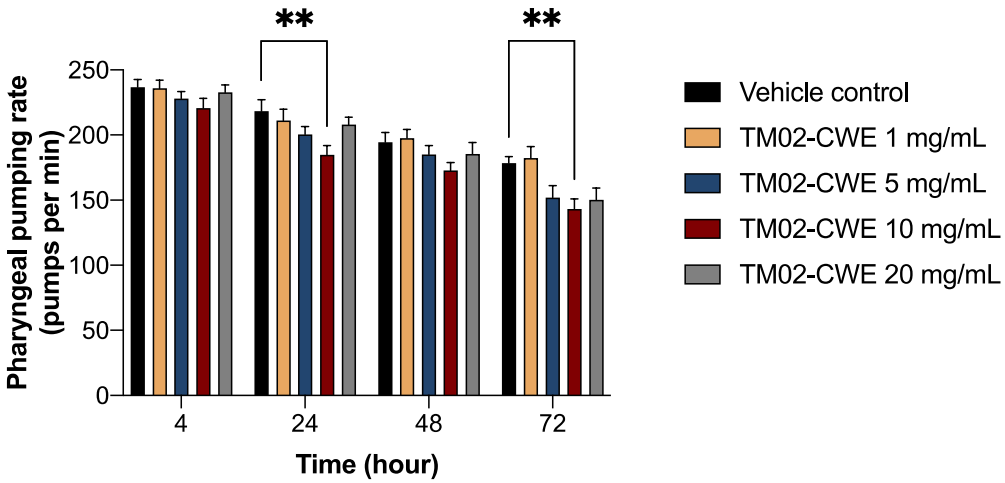


Figure 6.10 Effects of TM02-CWE on the pharyngeal pumping rate of C. elegans. *Data are expressed as mean ± SEM of three replicates with five nematodes per replicate, **p < 0.01, two-way ANOVA followed by Dunnett's post hoc test. (Abbreviation: CWE = cold water extract)*

6.4.6 Pharyngeal pumping rate of *C. elegans* declines over time

From the lifespan assays and pharyngeal pumping rate assays, both OCS02-CWE and TM02-CWE extended the lifespan of *C. elegans* but produced different effects on the pharyngeal pumping activity over 72 hours. To substantiate these findings, experiment was performed to measure the lifespan and pharyngeal pumping rate in the same batch of *C. elegans*. In this experiment, OCS02-CWE and TM02-CWE increased the mean lifespan of the nematodes from 18.47 ± 1.55 days to 23.40 ± 1.90 days (p = 0.0113) and 20.20 ± 1.54 days (p = 0.5003), respectively. The maximum lifespan of the wild-*C. elegans* was increased by 26.38% and 2.21%, respectively (Figure 6.11A).

The pharyngeal pumping rate of *C. elegans* decreased gradually over their lifespan and in the vehicle control group, the pharyngeal pumping activity cannot be observed from day 17 onwards. Consistently, the pharyngeal pumping rates of those treated with OCS02-CWE remained constant from day 1 to 5 and declined gradually over time. Pharyngeal pumping activity was noticeable up to day 25 in the OCS02-CWE-treated group (Figure 6.11B). In contrast, TM02-CWE treatment gradually reduced the pharyngeal pumping rate of the *C. elegans* across day 1 to day 15 (Figure 6.11B). Interestingly, TM02-CWE improved pharyngeal pumping activity near the end of the nematodes' lifespan and the pharyngeal pumping rate can be counted to day 19. The results obtained from this experiment are comparable to those obtained from the individual lifespan and pharyngeal pumping rate assays in the previous sections.

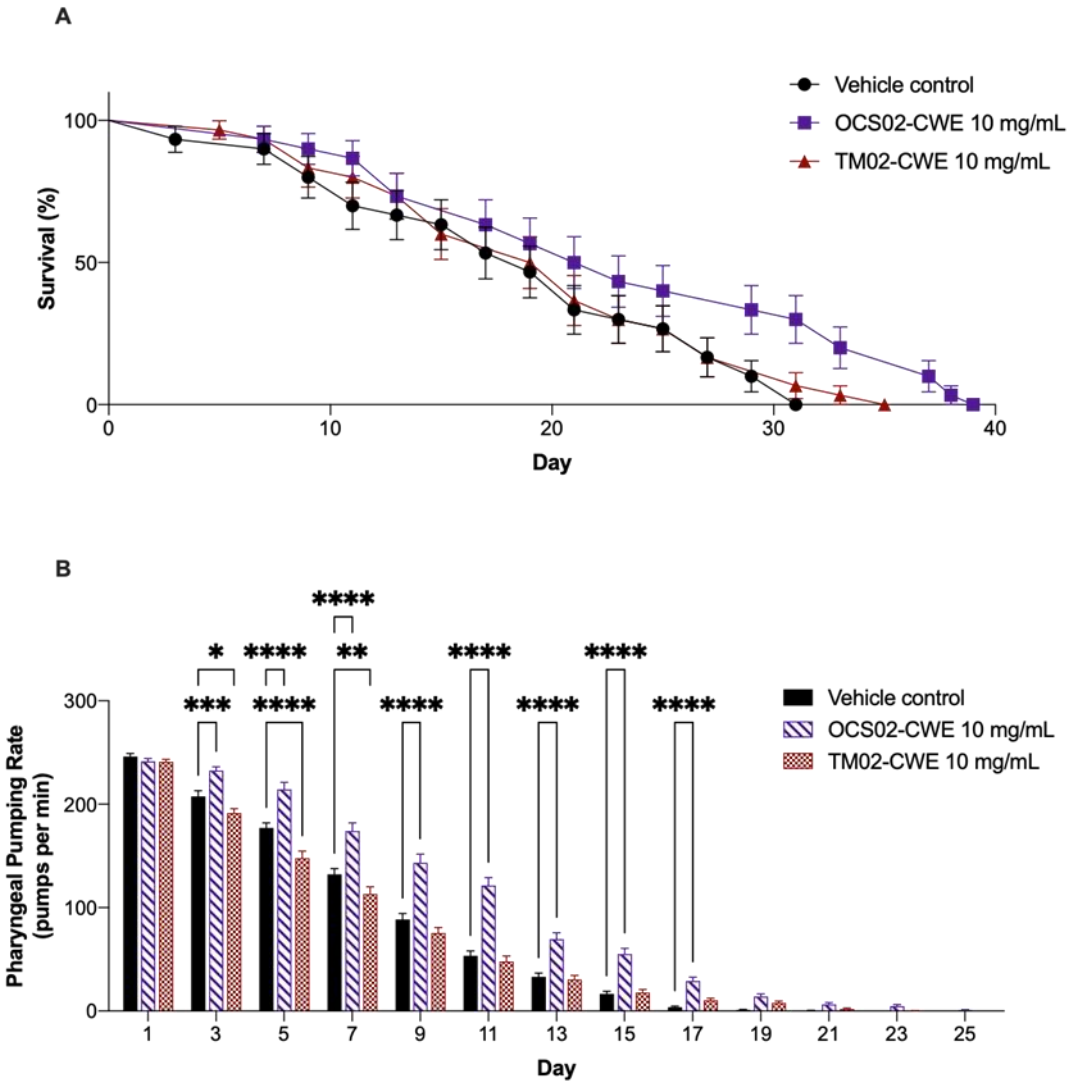


Figure 6.11 Effect of OCS02-CWE and TM02-CWE on the lifespan and pharyngeal pumping rate of C. elegans. *The pharyngeal pumping rate and lifespan were measured in the same batch of nematodes. Data are expressed as mean ± SEM of three replicates with ten nematodes per replicate, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA followed by Tukey's post hoc test. (Abbreviation: CWE = cold water extract)*

6.5 Discussion

In previous chapters, the mechanisms of action of OCS02-CWE and TM02-CWE in bladder relaxation were elucidated, uncovering their potential therapeutic effects in OAB syndrome. Considering that ageing is the main risk factor of OAB, and anti-ageing therapeutics may improve health and ameliorate age-related diseases (Li et al., 2021), the present study investigated the effects of *O. sinensis* and *L. rhinocerus* on the lifespan and pharyngeal pumping rate of *C. elegans*. Due to the similarity of *C. elegans* with humans in certain physiological traits, such as reduced physiological indexes with age, it is an established animal model to evaluate lifespan and age-related diseases. Our results showed that both *O. sinensis* (OCS02-CWE) and *L. rhinocerus* (TM02-CWE) exhibited lifespan extension effects. However, the extracts produced different effects on the pharyngeal pumping activity, one of the commonly used health markers of *C. elegans* (Bansal et al., 2015).

6.5.1 Effects of *O. sinensis* (OCS02-CWE) in *C. elegans*

OCS02-CWE extended the lifespan of *C. elegans* in a dose-dependent manner where the mean lifespan increases with increasing concentration of OCS02-CWE. This result ties well with a previous study performed by Zou et al. (2015). They have demonstrated that *O. sinensis* extract significantly prolonged the lifespan of *Drosophila melanogaster* (fruit flies) by approximately 15% through an antioxidative stress pathway that involves upregulation of SOD-1 and catalase activity, and inhibition of lipofuscin accumulation (Zou et al., 2015). SOD-1 is an antioxidant enzyme that catalyses the dismutation of superoxide into H_2O_2 and O_2, which are less toxic. In *C. elegans*, overexpression of SOD-1 is associated with increased lifespan via the *daf-16* signalling pathway (Cabreiro et al., 2011). Therefore, further

investigation may be conducted with mutant *C. elegans* such as strain CF1038 (lacking *daf-16*) to examine the role of *daf-16* in the *O. sinensis*' mechanism of action in lifespan extension.

While lifespan extension is widely used to study anti-ageing effects of a treatment, it is important to note that anti-ageing does not correlate with therapeutic effect to age-related diseases. In other words, prolonging one's lifespan does not necessarily suggest good quality of life of that individual. It is possible that extending lifespan also extends a period of frailty with increased risk of age-associated diseases (Bansal et al., 2015). In *C. elegans*, age-associated phenotypes such as lipofuscin accumulation and pharyngeal pumping have been used as parameters to evaluate health effects of a treatment. As mentioned earlier, *O. sinensis* has been shown to inhibit lipofuscin accumulation in *D. melanogaster*, suggesting its potential in promote healthy ageing. Regrettably, the scope of current study does not allow the investigation of OCS02-CWE on lipofuscin accumulation in *C. elegans*. Further study is encouraged to address this aspect.

According to previous studies, the change in pharyngeal pumping rate can be observed following a treatment period as short as 24 hours (Lee et al., 2015; Helmcke et al., 2009; Peng et al., 2021). Therefore, our initial experiments studied the effect of OCS02-CWE at different concentrations on the pharyngeal pumping rate over 72 hours. In the blank control group, the pharyngeal pumping rate decreased by about 20% from day 1 to day 4 of adulthood, in accordance with previous studies (Bansal et al., 2015; Calvert et al., 2016). *C. elegans* treated with vehicle control also demonstrated similar pharyngeal pumping rate, suggesting that the purified water used to dissolve OCS02-CWE would not affect the pharyngeal pumping activity of the nematodes. In contrast, OCS02-CWE markedly enhanced the pharyngeal pumping rate of *C. elegans*

after 48 hours. Together with its lifespan extension properties, 10 mg/mL of OCS02-CWE appeared to be the most effective concentration in providing health benefits in *C. elegans*. Therefore, this concentration was used in subsequent experiment to confirm the relationship between pharyngeal pumping activity and lifespan in the same batch of *C. elegans*. Consistently, OCS02-CWE prolonged the lifespan of *C. elegans* and improved their pharyngeal pumping rate. The results imply that the prolongation of lifespan by OCS02-CWE was unlikely to be caused by dietary restriction which is a known factor that causes life extension in *C. elegans* (Kaeberlein et al., 2006). If the nematodes avoid consuming OCS02-CWE-containing food, they would have shown a reduced rate of pharyngeal pumping, which is an indicator of reduced food consumption.

In accordance with previous studies that reported the decline in pharyngeal pumping rate in wild-type *C. elegans* (Bansal et al., 2015; Calvert et al., 2016; Duangjan and Curran, 2022), we were unable to observe any pharyngeal pumping activity from day 17 onwards in the control group. The underlying mechanism that causes the functional decline of the pharynx remains unclear but could be related to progression loss of pharyngeal muscle (Chow et al., 2006). Interestingly, the pharyngeal pumping activities of *C. elegans* were still noticeable up to day 23 in the OCS02-CWE-treated group. These data indicate that OCS02-CWE may have a role in preserving pharyngeal pumping activity or delaying the age-related functional declines of the pharyngeal pump.

Since the present study utilises only wild-type N2 *C. elegans*, we are unable to clarify the gene associated with the changes in pharyngeal pumping rate by OCS02-CWE. However, similar with other animal species and other types of muscle cells, Ca^{2+} triggers the contraction of the pharyngeal muscles of *C. elegans* and it is mainly

caused by Ca^{2+} influx through T- and L-type VGCCs (Trojanowski et al., 2016). The reduction of pharyngeal pumping rate by nifedipine and nemadipine A in the present study confirmed the importance of L-type VGCCs in the contraction of pharyngeal muscle. Nifedipine is an established phenylalkylamine L-type VGCC whereas nemadipine A is a dihydropyridine that antagonises the α_1-subunit of L-type VGCCs that is encoded by *egl-19* gene (Kwok et al., 2006).

Based on the mechanistic studies in the rat bladder, we proposed that OCS02-CWE activates VGCC to stimulate Ca^{2+} entry to elicit bladder contraction (Chapter 3). Therefore, it is plausible that OCS02-CWE affects the pharyngeal muscle in a similar way, that is, by stimulating Ca^{2+} influx via the VGCC. Given that both tonic and phasic stimulation of the pharyngeal muscles are individually sufficient to drive rapid pumping (Trojanowski et al., 2016), it remains questionable whether OCS02-CWE stimulates tonic or phasic contraction, or both, in the pharyngeal muscle. The activation of VGCCs by OCS02-CWE on the neurons cannot be excluded as they are also expressed in the neurons (Frøkjær-Jensen et al., 2006).

It is worth noting that *C. elegans* do not express NOS as they lack an NOS gene. Therefore, the regulation of NO that involves in the bladder relaxant effect of OCS02-CWE may not be applicable to the changes observed in the lifespan and pharyngeal pumping rate of *C. elegans*. Moreover, in Chapter 3 and 4, we demonstrated that OCS02-CWE may activate SERCA to promote bladder relaxation following the production of NO. While SERCA has been shown to regulate pharyngeal pumping activity in *C. elegans* (Zwaal et al., 2001), this mechanism may also not be applicable as current data suggests that the activation of SERCA by OCS02-CWE was linked to production of NO.

6.5.2 Effect of *L. rhinocerus* (TM02-CWE)

The experimental outcomes in Chapter 5 have shown that TM02-CWE relaxes the bladder with a mechanism of action that is different from OCS02-CWE, i.e., inhibition of Ca^{2+} influx via the L-type VGCCs. As a comparison, the effects of TM02-CWE on the lifespan and pharyngeal pumping rate of wild-type *C. elegans* were also investigated using similar concentrations. Results showed that TM02-CWE also extended the lifespan of *C. elegans*.

In a recent study, it was suggested that *L. rhinocerus* increased the lifespan of wild-type *C. elegans* via antioxidative pathway that involves activation of daf-16/FOXO signalling pathway (Kittimongkolsuk et al., 2021). In their study, the concentrations used were markedly lower, that is, from 100 µg/mL to 300 µg/mL. However, the concentrations less than 10 mg/mL in the present study did not affect the mean lifespan of the *C. elegans*. It is difficult to discuss the differences as there were some obvious differences in both studies. For example, the lifespan assays were conducted in a liquid culture system at 20 °C by Kittimongkolsuk et al. (2021) whereas the *C. elegans* were grown in solid NGM plates at 16 °C in the present study. Therefore, the variable in results could be due to the differences in experimental set-up.

Subsequently, TM02-CWE appeared to reduce the pharyngeal pumping rate of *C. elegans* over time, suggesting that TM02-CWE may diminish the contraction of pharyngeal muscle. Due to the reduced pharyngeal pumping activity, the *C. elegans* could have reduced food intake that would possibly contribute to its lifespan extension (Kaeberlein et al., 2006). However, this outcome, again, does not agree with those reported by Kittimongkolsuk et al. (2021) that showed increased pharyngeal pumping in *L. rhinocerus*-treated *C. elegans*. It could be that *L. rhinocerus* increases pharyngeal pumping rate at low concentrations but reduces the rate at high concentrations. Further

investigation is needed to identify the bioactive component that elicits these different responses. Another dissimilarity in Kittimongkolsuk et al. (2021)'s study was that the pharyngeal pumping rates of *C. elegans* in the blank control and vehicle control group maintained at above 200 ppm throughout the assay period of 15 days; but in the present study and other previous studies (Calvert et al., 2016; Bansal et al., 2015; Wang et al., 2020), the pharyngeal pumping rate of *C. elegans* declines over time and cannot be observed starting day 12 to day 17 of adulthood.

As discussed in the previous section, the contraction of pharyngeal muscles relies on Ca^{2+} influx through T- and L-type VGCC. Therefore, TM02-CWE could reduce the pharyngeal pumping rate by disrupting Ca^{2+} activity at either pharyngeal muscle or at the neuromuscular junctions. In Chapter 5, we demonstrated that the relaxation profile of TM02-CWE in bladder was similar to that of a L-type VGCC blocker. The similar efficacy of TM02-CWE and nifedipine in reducing the pharyngeal pumping rate thus corroborates this finding. Both nifedipine and nemadipine A antagonise egl-19 in *C. elegans* and inhibited L-type VGCCs at the same potency (Kwok et al., 2006).

It is also interesting to note that while 10 mg/mL of TM02-CWE reduced the pharyngeal pumping rate of the nematodes during their early adulthood (day 3 to day 11), it improved the pharyngeal pumping activity near the end of their lifespan, on day 15 to day 19. The present study is not able to clarify this phenomenon, but comparing with the effect of OCS02-CWE, the slight improvement of pharyngeal pumping by TM02-CWE in the late adulthood could contribute to its lifespan extension effect. Nevertheless, TM02-CWE and OCS02-CWE could mediate via different pathways in prolonging the life of the nematodes. A recent study showed that inhibition of VGCCs by verapamil extended the lifespan of *C. elegans* by promoting autophagy process

downstream of calcineurin (Liu et al., 2020). Further study is warranted to clarify the role of TM02-CWE in lifespan extension and whether it is associated with the reduction in the pharyngeal pumping activity of *C. elegans*, that may involve promotion of autophagy due to diet restriction.

6.6 Chapter summary

In summary, both OCS02-CWE and TM02-CWE exhibited life extension properties in *C. elegans* but affected the pharyngeal pumping activity in different ways. OCS02-CWE prevented the age-related decline of the pharyngeal pumping rate of *C. elegans* throughout the adulthood. In contrast, the pharyngeal pumping rate of *C. elegans* treated with TM02-CWE declined gradually until day 7 of adulthood. These effects resembled their mechanisms of action in the DSM where OSC02-CWE activates L-type VGCCs to cause contraction and TM02-CWE inhibits the channels to produce relaxation response. While the neuromuscular pharyngeal pump of the *C. elegans* has a simple structure, the pumping activity is a complex process that requires interplay among the nervous system, muscles and environment. Therefore, our results in this study are insufficient to suggest the mechanistic pathways of the extracts in lifespan extension and pharyngeal pumping rate alteration. These results, nevertheless, provide a basis for further investigation into the correlation between Ca^{2+} and associated genetic pathways in improving health of *C. elegans*.

7 General Discussion

7.1 Introduction

OAB is a highly prevalent condition that has far-reaching consequences for both physical and mental health. Due to its complex pathophysiology that may involve myogenic, neurogenic, urotheliogenic and as a result of metabolic disorder, the management of OAB is challenging, with the need to tailor treatment options to patients' condition. The management is even more difficult if patient suffers from idiopathic OAB i.e., when a specific metabolic or pathologic condition that leads to OAB cannot be identified. Currently, regardless of its aetiology, antimuscarinics and β-adrenoceptor agonist are the main therapeutic agents used to relieve OAB by preventing bladder contraction or promoting bladder relaxation, to increase the bladder capacity and reduce episodes of urge incontinence. Generally, they are considered effective in alleviating OAB symptoms but are associated with many undesirable effects. Moreover, OAB is more prevalent in geriatric population who are at greater risk of adverse drug reactions due to polypharmacy (Moga et al., 2017; Kachru et al., 2020). Development of drug resistance in OAB patients has also been reported (Leron et al., 2018). Although several new therapies such as Botox injection and neuromodulation, and surgical implantation or bladder augmentation are available to treat resilient OAB, they are invasive, expensive or require prolonged convalescence. In this study, we investigated the possible therapeutic effects of *O. sinensis* in OAB due to its traditional use in relieving frequent urination. Through isolated tissue bath experiments, our results provide a direct physiological evidence of

its bladder relaxant effect, corroborating its traditional use. We also compared its pharmacological actions with *L. rhinocerus* (TM02-CWE), a medicinal fungus that has elicited substantial smooth muscle relaxant effect, to highlight the potential therapeutic effects of medicinal fungi in OAB. An in-vivo study in *C. elegans* has also been carried out, revealing the health benefits of *O. sinensis* and *L. rhinocerus*.

7.2 Bioassay-guided fractionation and mechanism of action of OCS02-CWE

It is generally accepted that natural extracts should ideally be prepared by mimicking the traditional methods of preparation. According to the ethnomedicinal records, *O. sinensis* was often served in hot soup, as infused water or eaten raw (Chen et al., 2010; Panda and Swain, 2011). Therefore, cold and hot water extraction were performed to test the bladder relaxant effect of these extracts (OCS02-CWE and OCS02-HWE). In our initial attempt to study the effects of OCS02-CWE through cumulative CRCs in bladder strips pre-contracted with carbachol, intermittent transient contraction and relaxation responses were observed (see section 2.5.2, **Chapter 2**). This observation suggests that OCS02-CWE consists of components that could contract and relax the DSM, and the contractile response may override the relaxation response during cumulative application. Besides, the non-sustained nature of the contractile and relaxation responses may be due to tachyphylaxis. We thus adopted a non-cumulative protocol to study the effects of OCS02-CWE.

During the non-cumulative CRCs, OCS02-CWE elicited a biphasic response, involving a transient contractile response that precedes a significant and sustained relaxation response. In contrast, OCS02-HWE produced only a transient contraction and was lacking relaxant effect (see 2.4.4, **Chapter 2**). The results indicate that the

water-soluble bioactive substances in OCS02 may be heat-labile. Then, size-exclusion fractionation of OCS02-CWE was performed to identify the most active fraction that contributes to its bladder relaxant effect. In the isolated tissue bath assay, the fractions of OCS02-CWE exhibited different activities. OCS02-LMW produced a transient contraction and was devoid of relaxant effect, like OCS02-HWE. On the other hand, OCS02-MMW produced a significant yet short-lived relaxant effect. The relaxation response produced by OCS02-HMW was able to sustain but the magnitude was much lower compared to OCS02-CWE (see 2.4.5, **Chapter 2**). Based on these results and their respective protein and carbohydrate contents, the contractile effect of OCS02-CWE could be attributed to heat-stable components with low MW (< 7.0 kDa) whereas the relaxant effect could be contributed by heat-sensitive proteins or protein-carbohydrate complexes with MW > 7.0 kDa. Given that OCS02-CWE produced superior bladder relaxant effect, it was used in the subsequent mechanistic studies.

In the next experiment, it was observed that the transient contraction induced by OCS02-CWE was absent in KCl-pre-contracted bladder strips, and its relaxation response was diminished when compared to carbachol (see section 3.4.1, **Chapter 3**). Therefore, we postulated that OCS02-CWE may stimulate membrane depolarisation and cause transient contraction. Its relaxant mechanism, on the other hand, may involve both voltage-dependent and -independent pathways. Comparing the effect of OCS02-CWE's pre-incubation on the contraction induced by different contractile agents including carbachol, KCl, 5-HT and H_2O_2, OCS02-CWE showed a more pronounced suppression effect in the contraction and spontaneous myogenic activities induced by carbachol. The ability of OCS02-CWE to eliminate the spontaneous myogenic contraction suggests its possible therapeutic effect in OAB, given that bladder strips isolated from humans and animals with OAB have exhibited higher

levels of spontaneous myogenic contractions (Andersson, 2010; Fry et al., 2004). Besides, the effect of OCS02-CWE on carbachol appeared to be non-competitive, implying that it did not block muscarinic receptors (see section 3.4.2, **Chapter 3**). The participation of muscarinic receptors in its mechanism of action was further excluded using atropine, a non-selective muscarinic receptors antagonist (see section 3.4.3 and section 3.4.15, **Chapter 3**).

In the DSM, activation of β-adrenoceptors upregulates AC which converts ATP to cAMP and leads to smooth muscle relaxation. The plausible effects of OCS02-CWE affecting the β-adrenoceptors have been ruled out using propranolol and the result was supported by the unaltered responses of OCS02-CWE in the presence of IBMX, a PDE inhibitor that prevents hydrolysis of cAMP to 5'AMP (see sections 3.4.4 and 3.4.11, **Chapter 3**). Then, subsequent experiments investigated the involvement of K^+ channel, role of urothelium and regulation of Ca^{2+}. In the presence of TEA, a non-selective K^+ channel blocker, the transient contraction induced by OCS02-CWE was potentiated but the relaxation response was not affected (see section 3.4.4, **Chapter 3**). This finding supports the earlier postulation that OCS02-CWE causes membrane depolarisation as TEA increases the duration and amplitude of an action potential by blocking depolarisation-activated delayed rectifier K^+ channels (Barrett et al., 1988; Yim et al., 2013). Nonetheless, it is noteworthy that the effect of TEA is reversible, and it decreases when depolarisation is complete (Hermann and Gorman, 1981). Therefore, the results may not be correctly interpreted if OCS02-CWE elicits its relaxant effect through activation of TEA-sensitive K^+ channel.

Subsequently, the role of urothelium and urothelium-derived relaxing factors were investigated. Removal of the urothelium significantly attenuated the relaxant effect of OCS02-CWE (see section 3.4.5, **Chapter 3**). It is important to note that the

relaxant effect was not completely removed in the absence of urothelium, suggesting that the mechanisms of action of OCS02-CWE involve both urothelium-independent and urothelium-dependent pathways. The participation of purinergic receptors which are expressed on both urothelial cells and DSM cells was first investigated. The results showed that neither P1 nor P2 receptors antagonists alter the responses induced by OCS02-CWE, declining their possible role in the action of OCS02-CWE (see section 3.4.8, **Chapter 3**). The results also infer that adenosine and cordycepin that act on P1 adenosine receptors may not be the bioactive components in OCS02-CWE. It is possible that cold water extraction of OCS02 have removed these small molecules as adenosine is more extractable at high temperature or with the use of organic solvent (Yoswathana et al., 2010). The possible participation of H_2S-mediated pathway in the mechanism of action of OCS02-CWE has also been ruled out (see section 3.4.9, **Chapter 3**).

Next, our findings demonstrated the involvement of NO in OCS02-CWE's mechanism of action. In the presence of L-NAME, an inhibitor of NOS, OCS02-CWE-induced relaxation was significantly attenuated (see section 3.4.10, **Chapter 3**). As a false substrate of the NOS, L-NAME binds to the NOS and prevents the conversion of L-arginine to NO. While it is possible that OCS02-CWE mediate its effect by prolonging the half-life of NO, we are inclined to propose that OCS02-CWE stimulates production of NO through the urothelium. This is because SNP, a NO donor, also attenuated the relaxant effect of OCS02-CWE, likely due to the antagonistic effects between endogenous NO and exogenous NO (Rico Lemus, 2014; Oreščanin and Milovanović, 2006). This postulation was supported by the reduction of SNP-induced relaxation response in the presence of OCS02-CWE and L-arginine (see section 4.4.8, **Chapter 4**). Although NO-mediated relaxation is often associated with

the rise of cGMP level in the smooth muscle cells, several studies have shown that accumulation of cGMP in the DSM cells does not contribute to bladder relaxation (Fujiwara et al., 2000; Artim et al., 2009). Likewise, OCS02-CWE- and SNP-induced relaxation response was independent of cGMP, suggesting that the NO-mediated bladder relaxation may involve regulation of Ca^{2+}.

Therefore, we shifted the focus to examine the role of OCS02-CWE in Ca^{2+} regulation. In the absence of external Ca^{2+}, carbachol-induced contraction is exclusively dependent on Ca^{2+} release from the intracellular store i.e., the SR. Under this condition, OCS02-CWE appeared to potentiate the initial contraction induced by carbachol, suggesting that it may have a role in regulating Ca^{2+} release from the SR (see section 3.4.12, **Chapter 3**). This suggestion was supported by our subsequent observation in which thapsigargin, a SERCA inhibitor, diminished the relaxation response of OCS02-CWE (see section 3.4.13, **Chapter 3**). It suggests that OCS02-CWE activates SERCA, sequestering cytoplasmic Ca^{2+} into SR which is then utilised by carbachol. The participation of IP_3Rs and RyRs in the action of OCS02-CWE has been ruled out by using 2-APB and ryanodine. Compatibly, the relaxation response induced by NO via SNP was also weakened by thapsigargin. From these observations, we propose that as OCS02-CWE stimulates release of NO from the urothelium, it diffuses into the DSM cells and activates SERCA. As a result, the concentration of intracellular Ca^{2+} was reduced, leading to DSM relaxation.

In addition to the modulation of intracellular Ca^{2+}, the findings in this study showed that OCS02-CWE could inhibit extracellular Ca^{2+} influx. The evidence includes its suppression effect on $CaCl_2$-induced contraction in urothelium-denuded bladder strips and the reduction in phasic contraction induced by carbachol in the presence of OCS02-CWE. Phasic contraction induced by carbachol is highly

dependent on extracellular Ca^{2+} influx (Schneider et al., 2004; Uchida et al., 1994; Wuest et al., 2007) (also see section 3.4.12, **Chapter 3**). Therefore, the role of membrane permeable Ca^{2+} channels including TRPC channels and VGCCs, that are implicated in carbachol's signal transduction was investigated. A few TRPC channels and VGCCs blockers were selected in this experiment, including, SKF-96365, schwarzinicine A, verapamil and nifedipine (see section 3.4.13, **Chapter 3**). These Ca^{2+} channel blockers suppressed the transient contractile effect of OCS02-CWE, confirming the contractile mechanism of OCS02-CWE through extracellular Ca^{2+} influx. However, they did not affect the relaxation response induced by OCS02-CWE.

It is worth mentioning that the downstream signalling mechanisms upon receptor activation in the smooth muscle cells are complex as they interact with one another in the tissue to generate diverse functional responses. Looking at the effects of OCS02-CWE, it inhibited Ca^{2+} influx when it was used to pre-incubate the bladder strips at resting tension for a period of 30 minutes; but, when it was exposed to pre-contracted bladder strips, inhibition of membrane Ca^{2+} channels did not prevent its relaxation response. It is possible that OCS02-CWE generates different downstream signalling pathways, either voltage-independent or dependent, before or after $G_{q/11}$ activation. In summary, we have demonstrated that OCS02-CWE elicits both contractile and relaxant effect in rat bladder. It causes membrane depolarisation and opening of L-type VGCCs to promote Ca^{2+} influx, followed by production of NO production and activation of SERCA to initiate bladder relaxation. Besides, it may act directly on the DSM to inhibit extracellular Ca^{2+} influx but the underlying mechanism could not be identified based on the current findings.

7.3 Bioassay-guided fractionation and mechanism of action of TM02-CWE

In bladder strips pre-contracted with carbachol, TM02-CWE elicited significant relaxation response at 3 mg/mL and above (see section 5.4.1, **Chapter 5**). In our attempt to identify its most active fraction, TM02-CWE was fractionated by molecular weight to produce the HMW, MMW and LMW fractions. From the cumulative CRCs, TM02-HMW effectively relaxed the pre-contracted bladder strips, but the maximum relaxation response was slightly lower than TM02-CWE. TM02-MMW elicited a moderate relaxation response while TM02-LMW was devoid of relaxant effect. Based on the analysis of their protein and carbohydrate contents, we speculated that the bioactive component(s) of TM02-CWE that contribute to its relaxant effect is likely to be proteins or protein-carbohydrate complexes present in the HMW fraction. Our results do not exclude the presence of bioactive components in the MMW fraction due to its moderate relaxation response. The subsequent mechanistic studies were performed using TM02-CWE to ensure the maximum relaxation response induced by *L. rhinocerus* was achieved.

In our laboratories, Lee et al. (2018) has ruled out the participation of β-adrenoceptors, muscarinic receptors, K^+ channels, cAMP and cGMP in the smooth muscle relaxant effect of TM02-CWE and highlighted its effect in the regulation of Ca^{2+}, while investigating its bronchodilatory effect. As a follow-up study, we focused on the role of TM02-CWE in Ca^{2+}-mediated responses to explore its possible benefits in the management of OAB. First, the plausible effects of TM02-CWE affecting the urothelium were excluded as it similarly relaxed the urothelium-intact and -denuded bladder strips pre-contracted with carbachol (see section 5.4.2, **Chapter 5**).

Subsequently, our results demonstrated that TM02-CWE has negligible effect

on intracellular Ca^{2+} release from the SR but it exhibited overt suppression effect on extracellular Ca^{2+} influx (see section 5.4.4, **Chapter 5**). This observation correlates well with the previous findings where TM02-CWE relaxed carbachol-pre-contracted bladder strips and tracheal rings (Lee et al., 2018b). Therefore, TM02-CWE could mediate its effect via the membrane permeable Ca^{2+} channels L-type VGCCs and TRPC channels which are implicated in the signal transduction of carbachol. Based on these findings, a series of reverse protocols were performed by pre-incubating the tissues with TM02-CWE prior to constructing the CRCs of carbachol. VGCCs blockers including verapamil and nifedipine, and non-selective TRPC channels blockers including SKF-96365 and schwarzinicine A were used as positive controls in this experiment. These Ca^{2+} channel blockers and TM02-CWE suppressed carbachol-induced contraction at different magnitude (see section 5.4.5, **Chapter 5**). TM02-CWE significantly attenuated the maximum contraction induced by carbachol. Like verapamil, SKF-96365 and schwarzinicine A, it also enhanced the potency of carbachol and potentiated carbachol-induced desensitisation. These findings confirmed the involvement of L-type VGCCs and TRPCs in contraction induced by muscarinic receptors activation as reported in previous studies (Griffin et al., 2016; Hirota et al., 2003). In addition to demonstrating the complexity of cellular Ca^{2+} and the interplay between L-type VGCCs and TRPC channels in regulating Ca^{2+} influx induced by $G_{q/11}$ activation, these findings suggest that TM02-CWE could share a similar mechanism of action with verapamil, SKF-96365 and schwarzinicine A.

Following the above findings, the Ca^{2+} blockers were used to characterise the relaxant effect of TM02-CWE on carbachol-pre-contracted strips. Our results demonstrated that nifedipine significantly diminished the relaxation response induced by TM02-CWE, but verapamil, SKF-9365 and schwarzinicine A, potentiated the

relaxant effect of TM02-CWE (see section 5.4.6, **Chapter 5**). It is established that nifedipine (dihydropyridine) binds to inactive L-type VGCCs but the block of the channels by verapamil (phenylalkylamine) required channel activation (Zhao et al., 2019). Hence, when nifedipine was used to pre-incubate the tissues, it may bind immediately to the inactive L-type VGCCs and stabilise their inactive state. Therefore, the attenuation of TM02-CWE-induced relaxation response in the presence of nifedipine implies that it mediates via the blockade of L-type VGCCs. Moreover, the potentiation of TM02-CWE-induced relaxation response by verapamil implies that it is likely to bind to activated L-type VGCCs. After the addition of carbachol to stimulate pre-contractile tone, VGCCs were activated, allowing the binding of verapamil to inhibit Ca^{2+} influx through the channel. At the same time, TM02-CWE was added cumulatively onto the tissues, occupying the VGCCs together with verapamil at a faster rate, thus, resulting in the potentiated relaxation response. It is noteworthy that while SKF-96365 is commonly used as a non-selective TRPC channel blocker, it also exhibits inhibitory effect on VGCCs. Mak et al. (2022) also suggested the possible inhibitory effect of schwarzinicine A on L-type VGCCs. While the binding site of SKF-96365 and schwarzinicine A on L-type VGCCs have not been reported, it is possible that they share a common binding site with verapamil i.e., the inner pore of the channel, due to their structure similarities. This could also explain their potentiation effects on TM02-CWE-induced relaxation. In summary, TM02-CWE inhibits extracellular Ca^{2+} influx and may prefer binding to the L-type VGCCs in their open state. However, the inhibition of TRPC channels could not be entirely ruled out based on the current findings.

7.4 The combined effects of OCS02- and TM02-CWE in bladder relaxation and their therapeutic potential

Table 7.1 summarises the composition analysis of OCS02 and TM02 reported by Yap et al. (2013), Fung et al. (2018), and Lee et al. (2018). Comparing the composition analysis of OCS02 and TM02, OCS02 contains a greater amount of polysaccharides and amino acids but a lower amount of nucleosides. These differences could contribute to their different mechanisms of action in bladder relaxation. Due to the complex nature of the medicinal fungi and the preferred extraction condition of their components, it is difficult to deduce the bioactive components based on our current results. The evidence in the present study, however, strongly suggest that the bioactive components in both OCS02 and TM02 that contribute to their relaxant effects are heat-labile and may present in the medium and high MW fractions.

Table 7.1 The composition of OCS02 and TM02. *Adapted from Yap et al. (2013), Fung et al. (2018) and Lee et al. (2018)*

Composition	**Amounts (g/kg dry weight)**	
	OCS02	**TM02**
Total polysaccharides	482.800	401.400
α-Glucan	29.750	29.910
β-1,3/1,6-Glucan	342.500	286.800
Others (not specified)	110.550	84.690
Total amino acids	237.030	0.839
Arginine	45.200	0.204
Lysine	20.300	0.046
Serine	18.600	0.085
Threonine	18.200	0.091
Valine	16.000	0.114
Leucine	16.000	0.133
Alanine	15.600	0.114
Proline	13.900	0.017
Aspartic acid	12.200	0.187
Tyrosine	10.100	0.065
Isoleucine	9.580	0.093
Glutamic acid	9.160	0.232
Phenylalanine	8.620	0.085
Glycine	7.100	0.089
Histidine	6.450	0.034
Cysteine	6.140	0.042
Methionine	2.370	0.039
Tryptophan	1.500	ND
Hydroxyproline	ND	ND
Hydroxylysine	ND	ND
Nucleosides and their derivatives		
Cordycepin	0.655	0.873
Adenosine	0.364	0.282
Adenine	0.015	0.024
Hydroxyethyl-adenosine	0.126	0.268
Ethyl-adenosine	0.091	0.399

(Abbreviation: ND = not determined)

Based on the mechanistic studies in **Chapter 3** and **Chapter 5**, it is apparent that OCS02- and TM02-CWE have distinct mechanisms of action in bladder relaxation, but they do share several similarities. The pharmacological properties of OCS02- and TM02-CWE in the bladder were summarised in Table 7.2 and illustrated in Figure 7.1. In brief, both OCS02- and TM02-CWE do not cause modulation of muscarinic receptors, representing an advantage of using *O. sinensis* or *L. rhinocerus* to supplement the use of antimuscarinics such as oxybutynin, solifenacin and tolterodine, in OAB, as they would not create anticholinergic burden in patients. Their mechanisms of action also did not involve β-adrenoceptors, thus, unlikely to interfere with the use of β_3-adrenoceptor agonists. The main relaxation mechanism of OCS02-CWE is partly urothelium-dependent, via production of NO and activation of SERCA. The urothelium-independent pathway of OCS02-CWE involves inhibition of Ca^{2+} influx but its signalling pathway remains unclear. TM02-CWE, on the other hand, acts directly on the DSM by inhibiting extracellular Ca^{2+} influx via L-type VGCCs. The ability of OCS02-CWE and TM02-CWE to act directly on the DSM suggests that they could be useful in OAB patients with injured or disrupted urothelium. Conditions such as spinal cord injury and diabetes which are associated with an increased risk of OAB may also alter the urothelium structure and function (Wu et al., 2022; Apodaca et al., 2003; Lee et al., 2018c). While the therapeutic effect of NO in OAB has not been extensively studied, the administration of a herbal supplement containing 460 mg of L-arginine, the precursor of NO, has been shown to significantly improve OAB symptoms in men (Yagi et al., 2017).

It is noteworthy that albeit the remarkable smooth muscle relaxant effect of TM02-CWE, its use in OAB may be disputed due to its overt Ca^{2+} inhibitory actions. Clinical use of Ca^{2+} channel blockers has been shown to precipitate or exacerbate

LUTS by increasing residual volume or promoting urine retention (Yu, 2022). The increased residual volume may then increase voiding frequency due to the reduced space of bladder to store urine. Correspondingly, L-type VGCC agonist, Bay k8644, has been shown to increase voiding pressure and reduce voiding frequency despite potentiating bladder contraction (Salman et al., 2017). Nonetheless, beneficial outcomes on the use of Ca^{2+} channel blockers to alleviate OAB have been reported. Following a prospective study, Ani et al. (2021) reported that nifedipine significantly reduced the number of voids and leakage episodes in women with urge incontinence. It could also potentially be used to complement a lower dose of antimuscarinics (Ani et al. 2021). Besides, propiverine hydrochloride, an antimuscarinic agent with Ca^{2+} blocking activity, has shown significant benefits in patients with OAB who were resistant to antimuscarinics, and in paediatric patients (Lapointe et al., 2022; Masumori et al., 2011). Propiverine thus represents an ideal example that multiple targets could provide better outcome in OAB due to its multifactorial pathophysiology. From these studies, it remains a challenge to recommend or decline the use of Ca^{2+} channel blockers in OAB.

As outlined in **Chapter 5**, clinical trials have suggested that combined therapy using medications from different classes provide the most promising treatment outcome. To optimise the potential use of medicinal fungi in OAB, we investigated the combined effects of OCS02- and TM02-CWE. It was shown that the transient contraction induced by OCS02-CWE was diminished in the presence of TM02-CWE. This observation substantiates their respective mechanisms of action whereby TM02-CWE inhibited the extracellular Ca^{2+} influx stimulated by OCS02-CWE (see section 5.4.7, **Chapter 5**). Like the previous observations in which OCS02-CWE-induced relaxation response was reserved in the presence of Ca^{2+} channel blockers, TM02-

CWE did not affect the relaxation response of OCS02-CWE.

Our results also revealed that when the OCS02-CWE and TM02-CWE were used in equal proportion (1:1), they produced synergistic effects at low concentrations (< 4 mg/mL of each extract) (see section 5.4.8, **Chapter 5**). The reduction of the synergistic effect at higher concentrations may be due to their interaction on the L-type VGCCs. The synergistic interaction between different compounds is an important subject in biomedical research. It is a recognised approach to shorten total duration of treatment, improve treatment outcome or decrease treatment-related side effects by using lower doses (Yuan and Chen, 2019; Yi et al., 2013). If we extrapolate these functional data into clinical context, the concomitant use of *L. rhinocerus* (TM02-CWE) could enhance the treatment outcome of *O. sinensis* (OCS02-CWE) by removing the transient contractile effect of *O. sinensis* (OCS02-CWE). This suggestion assumes that the contractile effect of OCS02-CWE is a potential side effect as bladder relaxation is the treatment goal of OAB. From another perspective, given that the administration of Ca^{2+} channel blockers has been shown to increase the risk of urinary retention in patients, combining these medicinal fungi may allow the use of *L. rhinocerus* at a lower dose and reduce the chance of urinary retention due to blockade of Ca^{2+} channels. Besides, Ca^{2+} channel blocker has been thought to increase the bioavailability of NO (Berkels et al., 2001). Hence, incorporating a low concentration of *L. rhinocerus* could possibly enhance the treatment outcome of *O. sinensis*. Their combined effects should be further explored with different proportion of the extract. Nevertheless, *O. sinensis* that elicits dual mechanisms of actions i.e., promotes Ca^{2+} influx to induce contraction and stimulates NO production to initiate relaxation could be a more promising therapeutic agent, because overt relaxation of the bladder leads to urine retention.

Table 7.2 Pharmacological properties of OCS02-CWE and TM02-CWE.

	OCS02-CWE	**TM02-CWE**
Concentration-response curves	Non-cumulative	Cumulative
Effect on carbachol-pre-contracted bladder	Biphasic (transient contraction followed by sustained relaxation)	Straightforward relaxation response
Reversibility	Reversible	Irreversible
Bioactivity of fractions	MMW > HMW > LMW	HMW > MMW > LMW
Maximum relaxation response	50-60% of carbachol-induced tone	100% carbachol-induced tone
Involvement of urothelium	Yes	No
Direct action on DSM	Yes	Yes
Contractile mechanism	Extracellular Ca^{2+} influx stimulated by membrane depolarisation	N/A
Relaxation mechanism	Regulation of NO Activation of SERCA Possible inhibition of extracellular Ca^{2+}	Inhibition of extracellular Ca^{2+} influx via L-type VGCCs

(Abbreviations: CWE = cold water extract; HMW = high molecular weight; LMW = low molecular weight; MMW = medium molecular weight; NO = nitric oxide; SERCA = sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; VGCC = voltage-gated Ca^{2+} channel)

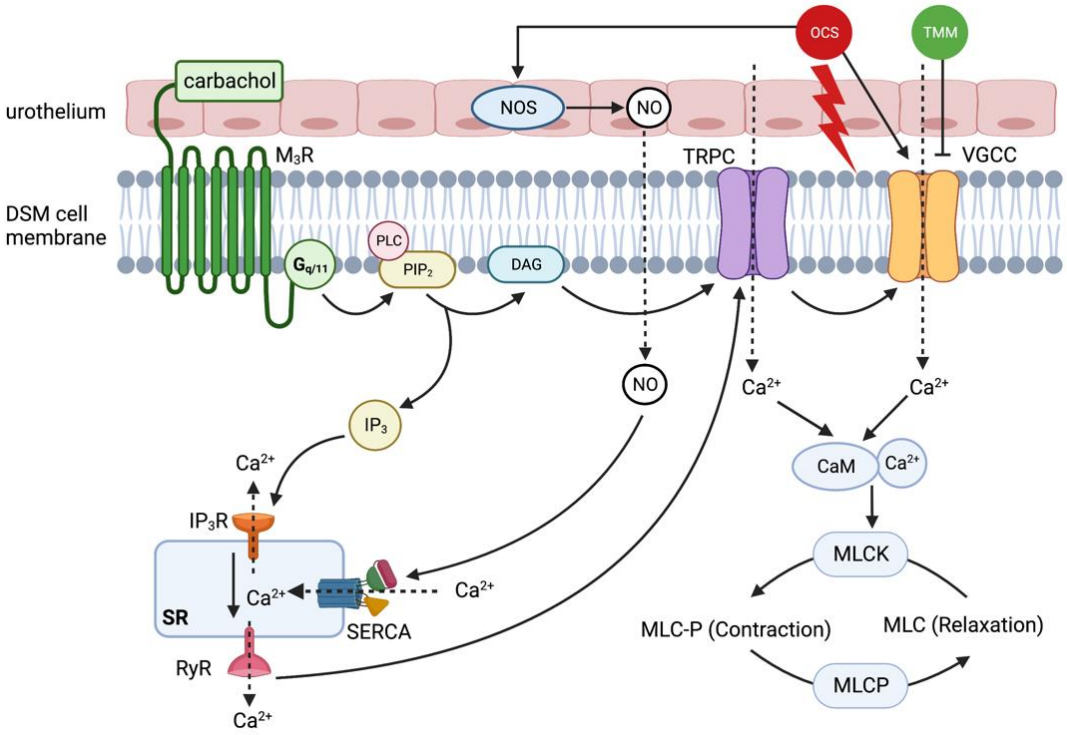


Figure 7.1 Schematic diagram of the proposed mechanisms mediated by cold water extract of Ophiocordyceps sinensis (OCS02-CWE) and Lignosus rhinocerus (TM02-CWE) in detrusor smooth muscle contraction and relaxation. *OCS02-CWE is represented as 'OCS' in whereas TM02-CWE is represented as 'TMM'. Solid pointed arrows denote downstream effectors activation; blunt arrows denote downstream effectors inhibition; and dashed arrows denote flow of Ca^{2+} or NO. OCS02-CWE induces membrane depolarisation, causing the opening of L-type VGCCs and Ca^{2+} influx, resulting in transient bladder contraction. Then, it stimulates NO production in the urothelium that in turn activates SERCA and leads to bladder relaxation. On the other hand, TM02-CWE inhibits L-type VGCCs and prevents extracellular Ca^{2+} influx, leading to bladder relaxation. (Abbreviations: CaM = calmodulin; DAG = diacylglycerol; DSM = detrusor smooth muscle; IP_3 = inositol triphosphate; MLC =myosin light chain; MLCK = myosin light chain kinase; MLC-P = myosin light chain phosphatase; NO = nitric oxide; NOS = nitric oxide synthase; OCS = Ophiocordyceps sinensis (OCS02 cold water extract); PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; RyR = ryanodine receptor; SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR = sarcoplasmic reticulum; SOCC = store-operated Ca^{2+} channel; TMM = Tiger milk mushroom-Lignosus rhinocerus TM02 cold water extract; TRPC = transient receptor potential canonical; VGCC = voltage-gated Ca^{2+} channel)*

7.5 In vivo investigation of OCS02-CWE and TM02-CWE in *C. elegans*

Following the elucidation of their mechanisms of action through isolated tissue bath assays, the in vivo effects of OCS02-CWE and TM02-CWE in wild-type *C. elegans* (N2 strain) were investigated. In addition to revealing their health benefits, the data obtained from this study supports the respective pharmacological actions of the extracts. Both OCS02-CWE and TM02-CWE significantly prolonged the lifespan of *C. elegans*, but OCS02-CWE showed a greater efficacy (see sections 6.4.1 and 6.4.2, **Chapter 6**). At 5 mg/mL, OCS02-CWE prolonged the mean lifespan of *C. elegans* by 24%. On the contrary, 20 mg/mL of TM02-CWE was required to produce similar effect. These results suggest that OCS02-CWE and TM02-CWE contain bioactive components which are capable in initiating downstream signalling responsible for lifespan extension. In recent years, research has started to emphasise on promoting healthspan because extending lifespan does not guarantee a good quality of life. In this study, the pharyngeal pumping rate of *C. elegans* that naturally declines with age was used as a health marker to evaluate the effects of OCS02- and TM02-CWE.

From the measurements of pharyngeal pumping rate across three consecutive days, OCS02-CWE treatment appeared to increase the pharyngeal pumping rate of *C. elegans* while those treated with TM02-CWE showed a decline in the activity (see sections 6.4.4 and 6.4.5, **Chapter 6**). The contraction of the pharyngeal muscle is due to repetitive Ca^{2+} transients induced by rhythmic action potentials. Activation of the motor neuron triggers cell depolarisation, which induces Ca^{2+} entry through L-type and T-type VGCCs, regulated by *cca-1* and *egl-1*, respectively (Trojanowski et al., 2016). The pharyngeal pumping facilitates the feeding process of *C. elegans*. Due to the absence of NOS expression gene in *C. elegans*, the regulation of NO by OCS02-

CWE is not possible in this model. Therefore, the increase of pharyngeal pumping rate is likely to be caused by the stimulatory effect of OCS02-CWE on Ca^{2+} influx. On the other hand, the reduction in pharyngeal pumping rate by TM02-CWE was similar to VGCC blockers nifedipine and nemadipine A, that is, by preventing Ca^{2+} influx into pharyngeal muscle cells. These results corroborate the inhibitory effect of TM02-CWE on Ca^{2+}-mediated contraction in vertebrates (rat airway, vascular and bladder smooth muscles).

To enhance our understanding on the effect of OCS02-CWE and TM02-CWE in improving health, we redesigned the experimental protocol and measured the lifespan and pharyngeal pumping rate simultaneously in the same batch of *C. elegans* (see section 6.4.6, **Chapter 6**). The assessments showed that OCS02-CWE prevents the age-related decline of pharyngeal pumping activity throughout their lifespan, while TM02-CWE potentiates the reduction rate on the first seven days. Interestingly, although these extracts exhibit different effects on the pharyngeal pumping activity, they similarly extend the lifespan of the nematodes. Based on the current results, the lifespan extension effect of OCS02-CWE is unlikely to be caused by caloric deficient or dietary restriction in *C. elegans* as pharyngeal pumping rate is positively correlated with food consumption. It is possible to involve *daf-16* signalling pathway that is implicated in the lifespan extension effect of *O. sinensis* in *D. melanogaster*. On the other hand, TM02-CWE could extend the lifespan of *C. elegans* by promoting autophagy, which had been demonstrated by verapamil (Liu et al., 2020). In summary, OCS02-CWE and TM02-CWE improved health in *C. elegans*, and the results have strengthened our understanding on the Ca^{2+} regulation of OCS02- and TM02-CWE. Considering that age is a major risk of OAB, the use of *O. sinensis* or *L. rhinocerus* could provide additional benefits in patients with OAB.

7.6 Limitations and recommendations on future studies

7.6.1 Functional assays

The findings of this study were mainly obtained from measuring the isometric tension of rat bladder strips via the isolated tissue bath technique. With the standard preparation, the results obtained from isolated tissue bath experiments are highly reproducible and reliable. In this study, it allows us to characterise the mechanisms of action of OCS02-CWE and TM02-CWE. Despite this success, it is not without limitations. The isolated tissue bath system limits the evidence by observing only the effects of the extracts on the contractility of the bladder and is unable to provide insight into responses in a fully integrated multi-cell type system. The physiology of the bladder and micturition is controlled by a complex mechanism involving interaction between the structural parts of the bladder and the autonomic nervous system. We attempted to minimise the variation by using carbachol to mimic the overactivation of muscarinic receptors, that is implicated in OAB.

However, due to the complex downstream signalling pathway of $G_{q/11}$ activation that may involve IP_3Rs, RyRs, SERCA, TRPCs, VGCCs, ROCK, it is difficult to pinpoint the Ca^{2+} channels responsible for the observed effects. These channels or receptors are interlinked with one another, and the absence of specific channel blocker have increased the challenge. For instance, SKF-96365 is commonly used as a non-selective TRPC channel inhibitor but it also exhibits inhibitory effect on VGCCs and SOCCs. Besides, 2-APB which is frequently used to block IP_3R has also demonstrated TRPC inhibitory actions. The measurements of isometric tissue contraction also cannot confirm the involvement of the target proteins if the effect is reversible. For example, the block of TEA could be reversed by OCS02-CWE that stimulates membrane depolarisation. Therefore, if K^+ channels are involved in

OCS02-CWE's mechanisms of action, the effects would not be observed. This speculation could be confirmed by exposing the tissues to both TEA and Ca^{2+} channel blocker but this combination would eventually prevent carbachol-induced pre-contraction. In future study, the patch clamp technique could be employed to study the membrane currents in DSM cells and provide more evidence to confirm the effect of OCS02-CWE against L-type VGCCs or K^+ channels (Tomita, 1988).

The inhibition of TM02-CWE on Ca^{2+} influx was postulated to be via extracellular binding on the L-type VGCCs from the inner pore because nifedipine that binds to inactive VGCCs prevents its relaxation response. However, this suggestion was based on the functional data, and we do not provide molecular evidence. A computational binding study is also not possible before the exact bioactive substance can be identified. Therefore, another area to be explored is the isolation of the exact substances responsible for the pronounced bladder relaxant effect. This could be challenging as our results showed that CWE of both OCS02 and TM02 have superior effects than their fractions. This phenomenon is not uncommon in natural extracts as different compounds may work together to provide a single bioactivity. However, this study has narrowed down the possible bioactive components of OCS02-CWE and TM02-CWE to be temperature-sensitive polysaccharides or protein-polysaccharides complexes. Future protein expression study such as western blot analysis can be performed to investigate the effect of OCS02-CWE or TM02-CWE on the RhoA/ROCK pathway.

Finally, future studies may utilise bladder tissues from other species such as porcine and rabbit to investigate if the effects are species-specific. Then, research can be continued to study their effects in diseased models such as diabetic rats and SHR rats as they have shown to have an enlarged bladder due to reduced elasticity of the

DSM. The urothelial layer of the bladder in diabetic rats and SHR rats may also be damaged due to high blood glucose and constricted vasculature. Transgenic animal models could also be utilised to confirm the proposed mechanisms of action. These studies would provide better extrapolation to clinical use in humans. Following that, future studies could move on to conducting clinical trials in OAB patients to assess the effects of *O. sinensis* in managing urinary symptoms.

7.6.2 In vivo assays using *C. elegans*

Lifespan assays and pharyngeal pumping assays of *C. elegans* have provided insight on the health effects OCS02-CWE and TM02-CWE. However, there are limitations associated with our study. First, *C. elegans* were exposed to food source incorporated with the extract or compound instead of injecting it directly into them. Therefore, a higher concentration is used in our study as conducting lifespan assay on solid culture cannot quantify the exact amount ingested by *C. elegans*. While administration of compound via microinjection in *C. elegans* has been introduced, it is labour-intensive, technically challenging and expensive. It also does not ensure reproducibility of the protocol (Rieckher and Tavernarakis, 2017).

In the lifespan assays, dead *C. elegans* were detected by gentle touch on their heads. It is possible that they were accidentally killed during the process as they become frail at old age. Video recordings or automated experimental setups such as WormScan or Lifespan Machine can be used to detect dead *C. elegans* (García Garví et al., 2021). Besides, manual counting of the pharyngeal pumping is prone to human error and research bias. While we have reduced the chance of error by video recordings and counting on slow-motioned videos, there were occasions that videos were not clear, and the process is time-consuming. Recent studies have introduced fluorescent imaging and scalable methods to measure pharyngeal pumping rate (Bauer and Golden,

2020; Scholz et al., 2016). Therefore, these methods are worth exploring in the future to improve reliability of the results especially when a bigger sample size is used. Moreover, pharyngeal pumping activity can only be observed and measured up to day 15 – 23 of adulthood and cannot provide information for the whole lifespan of the animal. Therefore, other parameters such as growth, motility and reproduction can be evaluated in future studies to expand healthspan benefits profile of both extracts. As current data suggest that OCS02-CWE increased pharyngeal pumping rate and TM02-CWE decreased pharyngeal pumping rate, these assays can be repeated in mutant strains such as *egl-19* (mutation in α_1-subunit of L-type VGCCs), *unc-36* (mutation in α_2-subunit of L-type VGCCs) to confirm their respective mechanisms of action. To elucidate the mechanisms of their lifespan extension effects, *C. elegans* with mutations in *daf-16* and *daf-2* could be utilised as these genes are implicated in lifespan extension of *C. elegans*.

7.6.3 Cell-based assays

To complement the organ bath data on the regulation of Ca^{2+} by OCS02- and TM02-CWE, we attempted to perform Ca^{2+} imaging experiments using human bladder smooth muscle cells. However, we were unable to proceed with the experiments due to autofluorescence induced by the extracts and they have broad fluorescence bands that coincide with that of Fluo-4 AM, the Ca^{2+} dye used in our experiments. The excitation and emission wavelength of Fluo-4 AM are 490 nm and 515 nm, respectively. The fluorescence spectra of OCS02- and TM02-CWE are shown in Appendix D. In future experiments, a different dye such as Fluo-2 AM could be used as it has a lower excitation wavelength of 340 nm. We did not attempt to use Fluo-2 AM as the fluorescent microscope in our laboratory (Axio Observer 7, ZEISS, German) was not equipped with the required optical filter set.

7.7 Concluding statements

For the first time, this study investigated the pharmacological actions of *O. sinensis* in the bladder and compared it with *L. rhinocerus*, another medicinal fungus with proven smooth muscle relaxant effects. Our experiments have successfully characterised the mechanisms of action of these fungi and provided answers to our research questions as follows:

i. The CWE of *O. sinensis* exhibited significant relaxant effect in carbachol-pre-contracted bladder tissues, while its HWE was devoid of relaxant effect. The bioactive components responsible for the bladder relaxant effect of OCS02-CWE are thought to be heat-labile. OCS02-CWE also contains heat-stable components with low molecular weight that stimulate bladder contraction via stimulation of extracellular Ca^{2+} influx.

ii. Gathered evidence from the isolated tissue bath experiments, OCS02-CWE-induced relaxation response is unlikely to involve blockade of muscarinic receptors. It also does not activate β-adrenoceptors and its downstream signalling i.e., cAMP production.

iii. One of the mechanisms of action of OCS02-CWE in mediating its relaxation response was urothelium-dependent, via production of NO that leads to activation of SERCA. Its effect was independent of cGMP production. The urothelium-independent mechanism of OCS02-CWE is likely to involve inhibition of Ca^{2+} influx.

iv. TM02-CWE produced straightforward relaxation response in carbachol-pre-contracted bladder by inhibiting extracellular Ca^{2+} influx via L-type VGCCs. The bladder relaxant effect of *L. rhinocerus* could be attributed to heat-sensitive, high-molecular-weight components such as polysaccharide-protein

complexes and proteins.

v. Low concentrations of equivalent proportion of OCS02- and TM02-CWE (< 4 mg/mL each) produced synergistic bladder relaxation response. Future studies should focus on investigating the effect of the combined extracts at different proportions. A higher proportion of OCS02 than TM02 is suggested due to the promising effects of OCS02-CWE contributed by its dual mechanisms of action.

vi. Treatment with OCS02-CWE and TM02-CWE prolonged the lifespan of *C. elegans* and altered the pharyngeal pumping rate in different ways. OCS02-CWE improved their pharyngeal pumping rate throughout the lifespan whilst TM02-CWE caused reduction in their pharyngeal pumping rate in early adulthood. These responses resembled their effects in the DSM where OCS02-CWE activates VGCCs and TM02-CWE inhibits VGCCs.

Appendix A

The University of Nottingham

Animal Welfare and Ethical Review Body

Cover Form applicable to the use of animals in non-regulated procedures

Title of Study:	**Investigation on the effect of natural product extracts and compounds on the isolated rat smooth muscles**
Name of Applicant:	**Kang Nee TING**
School:	**Life Sciences (Malaysia Campus)**

AWERB REF: UNMC12

1. Requests to use animals in non-regulated procedures must incorporate the information required below in the format requested for project licence applicants. The completed form should be returned electronically to the bsu@nottingham.ac.uk. When approved a protocol reference number will be provided to the applicant and must be used when ordering animals for use in the non-regulated study.

Its purpose is to highlight the key points in the proposed programme of work and to provide an executive summary in non-technical language accessible to the lay members of the Animal Welfare and Ethical Review Body.

Your application must be accompanied by this summary written in non-technical terms. You should complete this summary template. We expect that, for all but the most complex of studies, you will be able to provide a satisfactory summary using 500 to 1,000 words.

<table>
<tr><td>Study Title (max. 50 characters)</td><td colspan="3">In-vitro organ bath experiments investigating effects of natural product extracts and isolated compounds on rat smooth muscles.</td></tr>
<tr><td>Key Words (max. 5 words)</td><td colspan="3">Isolated tissues; aorta; bladder; airway</td></tr>
<tr><td>Expected duration of the study (yrs)</td><td colspan="3">4 years (this is formed part of PhD projects, BSc Biomedical Sciences, MPharm year 4 research projects and a grant application to be submitted to the Malaysia government)</td></tr>
<tr><td rowspan="10">Purpose of the study</td><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td></td><td>No</td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td></td><td>No</td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
<tr><td>Higher education or training</td><td>Yes</td><td></td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
<tr><td>Maintenance of colonies of genetically altered animals[1]</td><td></td><td>No</td></tr>
</table>

Describe the objectives of the study (e.g. the scientific unknowns or scientific/clinical needs being addressed)	This application is divided into two main objectives: 1. MPharm year 3 and BSc Biomedical Sciences research projects: To enable students to further understand the pharmacology of smooth muscles including understanding the process of research in natural product drug discovery. 2. PhD projects: This forms part of the research skills training. In vitro organ bath technique will be one of the methodology to be included in the thesis. There are currently 3 students under my supervision using this technique of experiments
What are the potential benefits likely to derive from this study (how science could be advanced or humans or animals could benefit from the project)?	1. Students will gain a better understanding of the pharmacology of the smooth muscles in different vascular beds where technical skills will be gained as part of their training in undergraduate or postgraduate level. 2. These experiments will provide new information on the effect of active extracts and compounds isolated from the natural flora on isolated smooth muscles from the airways, bladder and aorta. We are moving to test new compounds synthesize based on the skeletal structure of novel compounds isolated from local plant species. 3. In Malaysia, the skills on isolated organ bath are limited and this forms part of an important technique for drug discovery.
What species and approximate numbers of animals do you expect to use over what period of time?	Adult rats will be utilised for these experiments. With the current set up, it is estimated about 3 to 4 animals will be used weekly.
What procedures will be conducted?	All animals will be sacrificed by an experienced animal technician according to the methods described in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	No treatments will be carried out on living animals. After humane killing, the tissues will be removed for isolated organ bath experiments.

Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	The study of some of the pharmacology can only be meaningful when carried out in complex integrated tissues from animals. We have tried carrying out some experiments on primary smooth muscle cell lines and to date this has not been successful. Cell lines only provide limited information on the effects of the compounds and extracts that we have been working with. Answering a direct question, for example whether a compound would dilate the airway is only possible from investigations on the isolated smooth muscle rings.
2. Reduction Explain how you will assure the use of minimum numbers of animals	The maximum number of different organs from each animal will be utilised. For example, the aorta, bladder and airways (bronchus and trachea) will be used from each rat.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	At present no other sources of animal tissues are available but, for future planning, sourcing animal tissues from sheep obtained from local slaughter houses will be considered to minimise the use of rats in these experiments.
4. Housing and Care Please confirm that animals will be housed and cared for according to the requirements of the UK Animals (Scientific Procedures) Act 1986 draft Code of Practice for the care and accommodation of animals (February 2013) **or** provide details and justification for alternative housing and care standards.	Animals will be purchased from the animal facilities in University Putra Malaysia (UPM) and University Kebangsaan Malaysia (UKM) Both of these animal facilities are governed by their own institutional animal care and use committee (IACUC). They adhere to the Malaysian code of practice for the care and use of animals for scientific purposes which are consistent with the UK Home Office regulations. Information on the facility can be found here http://www.vet.upm.edu.my/eiacuc.html (UPM) http://www.kkl.ukm.my:8080/laru/wp-content/uploads/2013/03/UKMAEC-GUIDELINE-2007.pdf (UKM)

5. Humane Killing Please confirm that animals will be humanely killed using an appropriate method of humane killing detailed in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 **or** provide details and justification for alternative methods of killing.	Animals will be killed by concussion of the brain and/or exposure to carbon dioxide gas. Death is confirmed by dislocation of the neck.
For Office Use Only	
Will the study be subject to Retrospective Assessment?	Yes | No | Date due:

Committee use only

Comments by NACWO:

The proposed project appears appropriate with no welfare issues providing animals are humanely killed by trained and competent staff.
Neil Yates
05.09.13

Amendment 07/12/16
The request to source animals from an additional University is reasonable and confirmation has been received that both animal providers adhere to the Malaysian Code of Practice. However additional information on actual standards of animal care has been requested as the standards in place are not clear from the web links provided.
Neil Yates
07/12/16

Confirmation received - OK to proceed.
NY 13/03/17

Comments by NVS:

This would appear to be satisfactory, although an approximation for the total number of rats to be used per year or over the lifetime of the project should be given. Users should be trained to a level of competency that will ensure humane euthanasia is achieved at all times.

Ewan McNeill 05-09-13

I am happy with the information provided.
E. McNeil 13/3/17

We have contacted the animal house at UPM. The technician confirms that the rats are kept between 18 and 20 C. Reply from Kang Nee Ting 15/2/17

The University of Nottingham

Dear Annemarie

Please find attached the requested information on benefits accrued from this work.

From our record we have used 318 rats between September 2014 until now. Many thanks.
Best wishes
Kang Nee

Update on research output following the approval of UNMC2kn

Publication list

PhD thesis of Bi Juin Loong 2016: Development of a functional bioassay to study the mechanism of action of a novel phenylethylamine alkaloid, schwarzinicine

BJ Loong, KH Lim, Y Mbaki and KN Ting: Calcium mobilisation and relaxant properties of a novel phenyethylamine alkaloid, schwarzinicine A (In preparation)

Mei-Kee Lee, Kuan-Hon Lim, Chon-Seng Tan, Szu-Ting Ng, Sue-Mian Then, Suresh Kumar Mohankumar, Kang-Nee Ting: Bronchodilator effects of *Lignosus rhinocerotis* extract on rat isolated airways is linked to the blockage of calcium entry (Submitted to Journal of Phytomedicine).

Jun-Lee Lim, Kae-Shin Sim, Kien-Thai Yong, Bi-Juin Loong, Kang-Nee Ting, Siew-Huah Lim, Yun-Yee Low, Toh-Seok Kam: Biologically active vallesamine, strychnan, and rhazinilam alkaloids from Alstonia: Pneumatophorine, a nor-secovallesamine with unusual incorporation of a 3-ethylpyridine moiety. *Phytochemistry* 09/2015; 117.

Suet-Pick Wong, Chew-Yan Gan, Kuan-Hon Lim, Kang-Nee Ting, Yun-Yee Low, Toh-Seok Kam: Arboridinine, a Pentacyclic Indole Alkaloid with a New Cage Carbon–Nitrogen Skeleton Derived from a Pericine Precursor. *Organic Letters* 07/2015; 17(14):3628-3631.

B J Loong, J H Tan, K H Lim, Y Mbaki, K N Ting: Contractile function of smooth muscle retained after overnight storage. *Naunyn-Schmiedeberg's Archives of Pharmacology* 06/2015; 388(10):1061-1067.

Veronica Alicia Yap, Bi-Juin Loong, Kang-Nee Ting, Sandy Hwei-San Loh, Kien-Thai Yong, Yun-Yee Low, Toh-Seok Kam, Kuan-Hon Lim: Hispidacine, an unusual 8,4'-oxyneolignan-alkaloid with vasorelaxant activity, and hispiloscine, an antiproliferative phenanthroindolizidine alkaloid, from Ficus hispida Linn. *Phytochemistry* 01/2015; 109:96-102.

Choy-Eng Nge, Chew-Yan Gan, Kuan-Hon Lim, Kang-Nee Ting, Yun-Yee Low, Toh-Seok Kam: ChemInform Abstract: Criofolinine and Vernavosine, New Pentacyclic Indole Alkaloids Incorporating Pyrroloazepine and Pyridopyrimidine Moieties Derived from a Common Yohimbine Precursor. *Organic Letters* 12/2014; 46(21).

Students trained

Bi Juin Loong PhD, graduated 2016
Mei Kee Lee, 3rd year PhD
Kayatri Govindaraju, 2nd year PhD
Fong Kai Lee, 1st year PhD
Undergraduates (final year projects for MPharm and BSc Biomedical Sciences & summer placements) > 10

Justification for extension of the work

At present, we have 3 active PhD students (see the list above) using the post mortem tissues for their organ bath experiments. We have also completed a grant from the Ministry of Higher Education entitled "Mechanism of action of Lignosus rhinocerus (Tiger Milk Mushroom) on airway smooth muscle contractility and mast cells degranulation". We are currently preparing two proposals to be submitted to Newton Senior Fellowship and Wellcome Fellowship for the extension of the Tiger Milk Mushroom work. In addition, we have just submitted two grant

applications to the Ministry of Higher Education to study a series of novel compounds with vasorelaxation effects.
These are our justification for requesting extension of the ethics application for another 4 years from Sept 2017 to August 2021.

Comments by primary reader for the Committee (if applicable):

Comments by Lay Person (If applicable):

Committee decision: Approved

Communicated to applicant (date): 13/3/17

University of Nottingham
UK | CHINA | MALAYSIA

AWERB Cover Form (third party establishments)

Application to use animals in studies conducted at third party establishments or overseas

Name of UoN applicant	Kang Nee Ting, Mei-Kee Lee, Kuan-Hon Lim
Title of study	In vitro organ bath experiments investigating effects of natural product extracts and isolated compounds on rat smooth muscles
University Campus	Malaysia
School/Department	Pharmacy
Approval reference no.	

The Animal Welfare & Ethical Review Body (AWERB) exists to ensure that the use of all animals at the University of Nottingham is carefully considered and justified and that proper account is taken of all possibilities for reduction, refinement, and replacement (the 3Rs). To enable this, requests to use animals in studies conducted overseas or at third-party institutions in the UK must be submitted to AWERB and information provided below. The completed form should be submitted to AWERB via bsu@nottingham.ac.uk.

For studies conducted at University of Nottingham's Malaysia and China campuses, an initial scientific review process should be undertaken locally before this form is submitted to AWERB. The review by AWERB will focus on animal welfare and methods to reduce, replace, and refine animal research (the 3Rs). Please provide information in non-technical language accessible to the lay members of AWERB.

For the use of animals overseas this process is in accordance with the requirements of the UK-based research councils; see page 14 of Responsibility in the use of animals in bio-science research: expectations of major research councils and charitable funding agencies. You may also find the ARRIVE guidelines helpful when designing your study; the guide also highlights information that many journals now require for all animal experiments.

Study details

Name and location of the institution where animal studies will take place	University of Nottingham Malaysia	
Name & location of lead investigator	Kang Nee Ting	
Key words (max 5)	Isolated tissues; aorta; bladder; airway	
Expected duration of the study	4 years (this is formed part of PhD projects, BSc Biomedical Sciences, MPharm and BSc research, approved research grants). A similar application was approved in 2017 under #UNMC12.	
Species and expected number of animals to be used in total	Adult rats will be utilised for these experiments. With the current set up, it is estimated about 4 to 6 animals will be used weekly.	
Category of study *Indicate 'Yes' against each category that applies.*	Basic research	X
	Translational and applied research	
	Regulatory use and routine production	

	Protection of the natural environment in the interests of health or welfare of humans or animals	
	Preservation of species	
	Higher education or training	X
	Forensic enquiries	
Has the study been scientifically reviewed? *Please state source of funding if the study has been externally reviewed. Alternatively, give details of any internal review process.*	Yes (Ref: FRGS/1/2020/SKK0/UNIM/01/2) Elucidating the relaxant effects of Ophiocordyceps sinensis (Berk.) on airways and bladder smooth muscles to substantiate its traditional uses in alleviating respiratory and overactive bladder symptoms. Ongoing (Ref: FRGS/1/2017/SKK10/UNIM/01/1) Schwarzinicine A, a novel phenylethylamine alkaloid, as an inhibitor of the transient receptor potential channels in vascular smooth muscle. Ongoing (FOSE Internal grant) Investigation of the bronchodilator effect and underlying mechanisms of Lignosus rhinocerotis aqueous extract. Ongoing	
Scientific background *Briefly summarise the current position in this research area that justifies why this work is being done (information given here should relate only to this study).*	This application is to cover for a number of approved projects and some ongoing small projects where we will share the tissues from the rats. Hence the scientific background will cover a number of areas from testing the smooth muscle reactivity of new compounds synthesised, extracts from plants and medicinal mushrooms, microplastics and other substrates that we are interested in studying the mechanism of actions. Some will focus on the relaxation mechanism and some on potential toxicity due to the exposure of the microplastics on these soft smooth muscle tissues. For further specific information we have to write a separate appendix listing the justification of each project.	
Objective of study *State succinctly the primary objective or research question or hypothesis that will be addressed in this study.*	This application is divided into two main objectives: 1. MPharm & BSc and BSc Biomedical Sciences research projects: To enable project students to further understand the pharmacology of smooth muscles including understanding the process of research in natural product drug discovery. 2. PhD projects: This forms part of the research skills training. In vitro organ bath technique will be one of the methodology to be included in the thesis. There are currently 3 students under my supervision using this technique of experiments	

Study plan *Give a description of the steps in the study. A flowchart or timeline is often helpful to illustrate steps involved. This should focus on what will happen to the animals to allow AWERB readers to identify potential harms.* *Please attach a study protocol if helpful.*	No treatments will be carried out on living animals. After humane killing at the animal facility of either Monash University (Malaysia), University Putra Malaysia (UPM) or University of Kebangsaan Malaysia (UKM), the tissues will be removed for isolated organ bath experiments. These tissues will be used for in vitro organ bath experiments.
What are the potential benefits likely to derive from this study? *Explain how this study will help advance current knowledge or meet a clinical need (this should relate to information provided in the 'Scientific background').* *Please include a description of the primary data outputs.*	1. Students will gain a better understanding of the pharmacology of the smooth muscles in different vascular beds where technical skills will be gained as part of their training in undergraduate or postgraduate level. 2. These experiments will provide new information on the effect of active extracts, compounds isolated from the natural flora, novel synthetic analogues on isolated smooth muscles from the airways, bladder and aorta. We are moving to test new compounds synthesize based on the skeletal structure of novel compounds isolated from local plant species. 3. In Malaysia, the skills on isolated organ bath are limited and this forms part of an important technique for drug discovery
In the context of what you propose to do to the animals, what are the expected adverse effects? What are the likely/expected level of severity? *Identify the expected adverse effects of each procedure you will perform (e.g. the adverse effects of administering substances, blood sampling, etc.).* *Severity limits signify the maximum level of harm an animal might experience. The limits are: non-recovery, sub-threshold, mild, moderate, and severe. See Appendix G of the Guidance on the Operation of ASPA for more information.*	No treatments will be carried out on living animals. After humane killing at the animal facility of either Monash University (Malaysia), University Putra Malaysia (UPM) or University of Kebangsaan Malaysia (UKM), the tissues will be removed for isolated organ bath experiments.
Housing & care *Please confirm that animals will be housed and cared for according to the ASPA Code of Practice or provide details and justification for alternative housing and care standards (e.g. relevant DEFRA guides).*	Animals will be purchased from the animal facility in either in University Putra Malaysia (UPM)and University Kebangsaan Malaysia (UKM) (UPM), University Kebangsaan Malaysia (UKM) or Monash University (MU) based on the availability of the rats at these facilities. All the above animal facilities are governed by their own institutional animal care and use committee (IACUC). They adhere to the Malaysian code of practice for the care and use of animals for scientific purposes which are consistent with the UK Home Office regulations. MU adheres to the guidelines prepared by the Expert Working Group of the Animal Welfare Committee of the National Health and Medical Research Council, Australia.

	All the three animal facilities have been approved previously by AWERB in these applications: #UNMC12, #UNMC18, #UNMC2kn.
What will happen to the animals at the end of the study? *If the animals are to be killed please state the ASPA schedule 1 method of humane killing and confirmation that will be used (see Appendix D of the Guidance on the Operation of ASPA) or provide justification for an alternative method.*	All animals will be sacrificed by an experienced animal technician according to the methods described in Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. Animals will be killed by concussion of the brain and/or exposure to a rising concentration of carbon dioxide gas. Death is confirmed by dislocation of the neck.

Application of the 3Rs

Replacement *Please outline why it is not possible to achieve your objectives without using animals protected under ASPA.* *Have you considered alternative approaches (including the use of non-ASPA protected species such as nematodes or drosophila) and identified why they are not suitable?* *Please explain how any in silico, in vitro or ex vivo techniques have contributed to the background research leading to the work proposed here. Will any of these techniques be used alongside the research using animals and how do they integrate with each other?*	The study of some of the pharmacology can only be meaningful when carried out in complex integrated tissues from animals. We have tried carrying out some experiments on primary smooth muscle cell lines and to date this has not been successful. Cell lines only provide limited information on the effects of the compounds and extracts that we have been working with. Answering a direct question, for example whether a compound would dilate the airway is only possible from investigations on the isolated smooth muscle rings.
Reduction *Please explain how you have determined your animal numbers (using a power calculation for example) and what steps you are taking to ensure you are adhering to the principles of good experimental design to minimise variability (design advice available here).* *Have you applied any methods to reduce the number of animals required for your studies (e.g. the use of live imaging techniques) or sought to gather more data from the same number of animals (e.g. tissue sharing with colleagues)?* *Please also take note of the ARRIVE guidelines which the University has endorsed for all publications involving the use of animals.*	The maximum number of different organs from each animal will be utilised. For example, the aorta, bladder and airways (bronchus and trachea) will be used from each rat.
Refinement *Please note that the words 'refinement' or 'refined' refer to the experience of the animal, NOT a refinement to the science/data.* *Explain your choice of animal(s), model(s) and method(s), focussing on why they are the most refined for the intended purpose. Also define the scientific and humane endpoints that will be used.*	At present no other sources of animal tissues are available but, for future planning, sourcing animal tissues from sheep obtained from local slaughter houses will be considered to minimise the use of rats in these experiments. We are also working on pilot experiments using C elegans as a replacement of in vivo model for very early stages of toxicity study.

Please explain any steps you will take to minimise any the pain, distress or lasting harm that the animals will experience. This includes refinements to both the husbandry approaches (e.g. avoiding single housing social species where possible) and to procedures (e.g. ensuring reagents are administered via the least painful whilst scientifically justified route).	

Outcomes from previous non-ASPA studies

The information below is collected between Sept 2017 and 12 Mar 2021

1. No. of rats - 381 rats

2. No. of UG project students trained - 10 students

3. No. of summer placement students/ RA trained - 5 students

4. PhD students - 8 students
   - Lee Mei Kee (graduated in 2018)
   - Kayatri Govindaraju (graduated in 2020)
   - Lee Fong Kai (graduated in 2020)
   - Chan Zi Yang (passed thesis, graduation in July 2021)
   - Mak Yin Ying (thesis pending)
   - Pang Li Yin (second year)
   - Nathaniel Chan (registered Feb 2021)
   - Booi Han Ni (will register in Apr 2021)

Publication list

Schwarzinicines A–G, 1,4-Diarylbutanoid–Phenethylamine Conjugates from the Leaves of Ficus schwarzii
P Krishnan, FK Lee, VA Yap, YY Low, TS Kam, KT Yong, KN Ting, KH Lim
Journal of natural products 83 (1), 152-158, 2020

Vasorelaxation effects of a newly discovered phenylethylamine (FK244) in hypertensive rat isolated aortae
F Lee, Y Mak, K Lim, C Kong, Y Mbaki, S Then, K Ting
BRITISH JOURNAL OF PHARMACOLOGY 176 (16), 2993-2993, 2019

FK244 (N-phenethyl-1, 2, 3, 4-tetrahydronaphthalen-2-amine), a newly discovered phenylethylamine as a calcium channel blocker
Y Mak, F Lee, K Lim, C Kong, Y Mbaki, S Then, K Ting
BRITISH JOURNAL OF PHARMACOLOGY 176 (16), 2992-2992, 2019

Diphenethylpiperidine alkaloids with tracheal smooth muscle relaxation activity from Hippobroma longiflora (L.) G. Don
ZY Chan, K Govindaraju, P Krishnan, YY Low, KW Chong, KT Yong, …
Phytochemistry Letters 30, 93-98, 2019

Alstoscholactine and Alstolaxepine, Monoterpenoid Indole Alkaloids with γ-Lactone-Bridged Cycloheptane and Oxepane Moieties from Alstonia scholaris
P Krishnan, FK Lee, KW Chong, CW Mai, A Muhamad, SH Lim, YY Low, …
Organic letters 20 (24), 8014-8018, 2018

Co-administration of conjugated linoleic acid and rosiglitazone increases atherogenic co-efficient and alters isoprenaline-induced vasodilatation in rats fed high fat diet
BK Chai, YS Lau, BJ Loong, MM Rais, KN Ting, DM Dharmani, …
Physiological research 67 (5), 729-740, 2018

Airway relaxation effects of water-soluble sclerotial extract from Lignosus rhinocerotis
MK Lee, X Li, ACS Yap, PCK Cheung, CS Tan, ST Ng, R Roberts, …
Frontiers in pharmacology 9, 461, 2018

Govindaraju, K., Lee, M. K., Mbaki, Y., & Ting, K.N.. Intracellular stored calcium plays a minor role in Gq-coupled receptor-mediated contraction in rat airway smooth muscle. Life Sciences, Medicine and Biomedicine 2018, 2(2), 1–6.

Bronchodilator effects of Lignosus rhinocerotis extract on rat isolated airways is linked to the blockage of calcium entry
MK Lee, KH Lim, P Millns, SK Mohankumar, ST Ng, CS Tan, SM Then, …
Phytomedicine 42, 172-179, 2018

Grant/Award obtained by the group

(Ref: FRGS/1/2017/SKK10/UNIM/01/1) Schwarzinicine A, a novel phenylethylamine alkaloid, as an inhibitor of the transient receptor potential channels in vascular smooth muscle. Completed

Schachter Award a travel grant from the British Pharmacological Society GBP1800 in Aug 2019. Completed

An industry grant entitled "Pharmacological investigation of Ophiocordycep sinensis in smooth muscle tissues" was awarded for 2 years at RM28,000 started 1 March 2020 to 28 Feb 2022. Ongoing

FRGS/1/2017/STG01/UNIM/02/3 Synthesis and Structure-Activity Relationship Study of Schwarzinicine A Analogues as Vasorelaxant Agents. Completed

(Ref: FRGS/1/2020/SKK0/UNIM/01/2)
Elucidating the relaxant effects of Ophiocordyceps sinensis (Berk.) on airways and bladder smooth muscles to substantiate its traditional uses in alleviating respiratory and overactive bladder symptoms. Ongoing

(Ref: FRGS/1/2017/SKK10/UNIM/01/1)
Schwarzinicine A, a novel phenylethylamine alkaloid, as an inhibitor of the transient receptor potential channels in vascular smooth muscle. Ongoing

Investigation of the bronchodilator effect and underlying mechanisms of Lignosus rhinocerotis aqueous extract. Ongoing

Local review

If the study has undergone local scientific or ethical review, please attach or insert comments here
n/a

To be signed by the head of the research group

I confirm that welfare standards consistent with the principles of UK legislation (ASPA) will be applied and maintained.

NOTE: International research should also be compliant with all relevant national and local regulatory systems in the host country. Where significant deviations are identified, prior approval must be sought and agreed with both the funding body and the University of Nottingham AWERB.

Name: Kang Nee Ting

Date: 15/3/21

Committee use only

Comments by NACWO:
This application is to continue with a programme of teaching and research that has been previously undertaken since 2017 at UNMC using isolated rat tissue. The outcomes of this programme have been evidenced by the publications listed above. All animals will be obtained from previously approved third-party providers. Providing that the Schedule 1 method of humane killing is undertaken competently, then no additional welfare concerns. Please confirm that the necessary flow valve and chamber will be available and that a rising concentration of CO2 gas will be used to kill the rats according to recommended best practice. See this link on the NC3Rs website regarding outcomes from a consensus meeting on the use of carbon dioxide euthanasia. It is stated that between 43 and 46 animals will be used weekly. Please explain why these numbers were chosen is required to ensure that due regard has been given to any opportunity to reduce animals used and ensure statistically significant data will be obtained. Subject to satisfactory responses to the above points, I consider that the request can be approved. *Neil Yates, 21/03/2021* **APPLICANT RESPONSE:** By email: "I think there is a typo on my part pertaining to this statement of yours. It is stated that between 43 and 46 animals will be used weekly. Instead, it should state 'Adult rats will be utilised for these experiments. With the current set up, it is estimated about 4 to 6 animals will be used weekly.' I have updated the form which I attached it here. Apology for the confusion.

Pertaining to this statement "Please confirm that the necessary flow valve and chambers will be available and that a rising concentration of CO2 gas will be used to kill the rats according to recommended best practice." we have checked and confirmed that all 3 animal facilities that we planned to purchase the rats from have this set up.
Comments by NVS:
n/a
Comments by primary reader (if applicable):
Many thanks for providing the additional information requested. I am happy to now approve the application. *Ian Devonshire, 30/03/21*
Comments by lay person (if applicable):
n/a

Committee decision:	APPROVED
Date communicated to applicant:	30/3/21

Appendix B

Preliminary testing on the effect of Schwarzinicine A on bladder

The effect of schwarzinicine A on the bladder was investigated as previously described (Mak et al., 2022). In bladder strips pre-contracted with carbachol, schwarzinicine A elicited a significant relaxation response (E_{max} = schwarzinicine A, 94.08 ± 2.36%; vehicle control, 17.13 ± 4.63%, $p < 0.0001$) (Figure B-1).

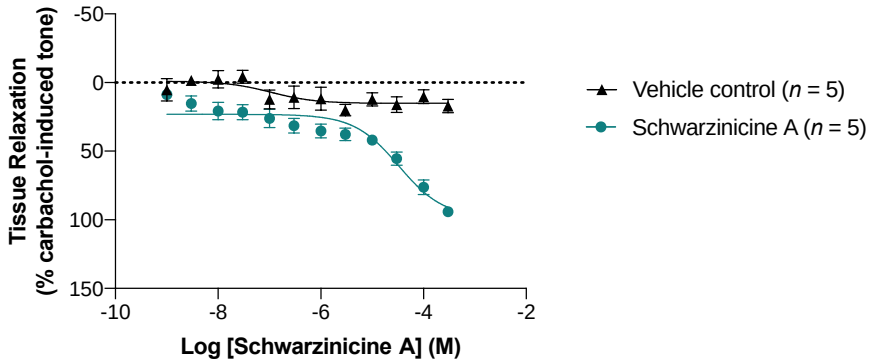


Figure B-1 Effect of schwarzinicine A on carbachol-pre-contracted bladder. *Tissue relaxation has been expressed as the percentage of carbachol-induced tone. Data are shown as the mean ± SEM of n number of animals.*

Then, in Ca^{2+}-free Krebs solution, pre-incubation with schwarzinicine A significantly suppressed Ca^{2+}-induced contractions in the bladder strips. At both 10 µM and 30 µM, the contractile response towards $CaCl_2$ was almost abolished (Figure B-2). The values of tissue contraction induced by $CaCl_2$ were summarised in Table B-1.

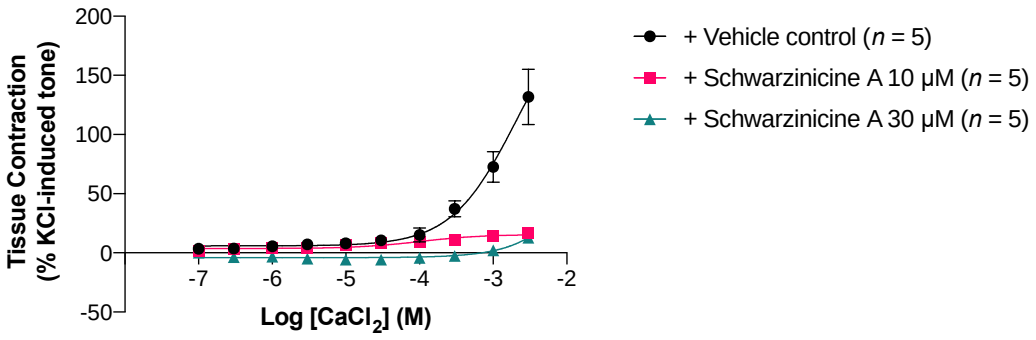


Figure B-2 Effect of Schwarzinicine A on $CaCl_2$-induced contractions. *Isolated rat bladder strips were pre-incubated with either vehicle control, 10 µM or 30 µM of schwarzinicine A. Tissue responses have been expressed as the percentage of carbachol-induced tone. Data are shown as the mean ± SEM of n number of animals.*

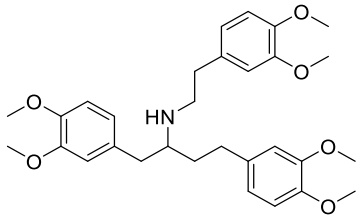
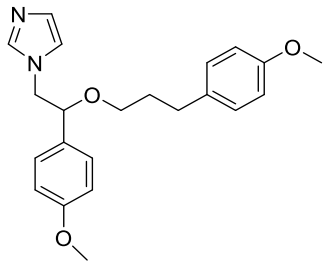
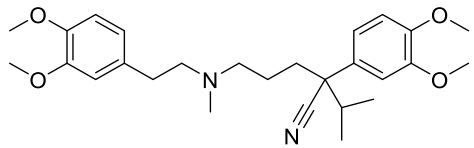
Table B-1 Tissue contraction induced by $CaCl_2$ in isolated rat bladder.

Treatment	Tissue contraction (%)	*p*-value
Vehicle control	131.70 ± 23.27	-
Schwarzinicine A 10 µM	16.31 ± 1.48	****0.0002
Schwarzinicine A 30 µM	12.89 ± 3.59	****0.0001

One-way ANOVA followed by Dunnett's post hoc test was performed to compare the means between vehicle control and the treatment groups, $p < 0.0001$. Tissue responses have been expressed as the percentage of carbachol-induced tone. Data are shown as the mean ± SEM of n number of animals.

While these results are insufficient to delineate the mechanism of action of schwarzinicine A in the bladder, it is apparent that schwarzinicine A also exhibits potent bladder relaxant effect. Its mechanism of action involves inhibition of extracellular Ca^{2+} influx, as demonstrated by Mak et al. (2022).

Appendix C

Compound	**Schwarzinicine A**	**SKF-96365**	**Verapamil**
Chemical structure			
Schwarzinicine A and SKF-96365 share a few structural similarities with verapamil that may contribute to their inhibitory effect on L-type VGCCs. They consist of at least two aromatic rings, at least two methoxy groups and flexible alkyl skeleton chains. All the alkyl skeleton consists of a central electronegative atom.			
Aromatic rings	Three 3,4-dimethoxybenzyl groups	Two 4-methoxybenzyl groups and one imidazole group	Two 3,4-dimethoxybenzyl groups
Central electronegative atom	Nitrogen	Oxygen	Nitrogen
Number of methoxy groups	Six	Two	Four

Appendix D

Absorbance of OCS02-CWE and TM02-CWE was measured using UV-Vis Spectroscopy and their λ_{max} was determined at 300 nm.

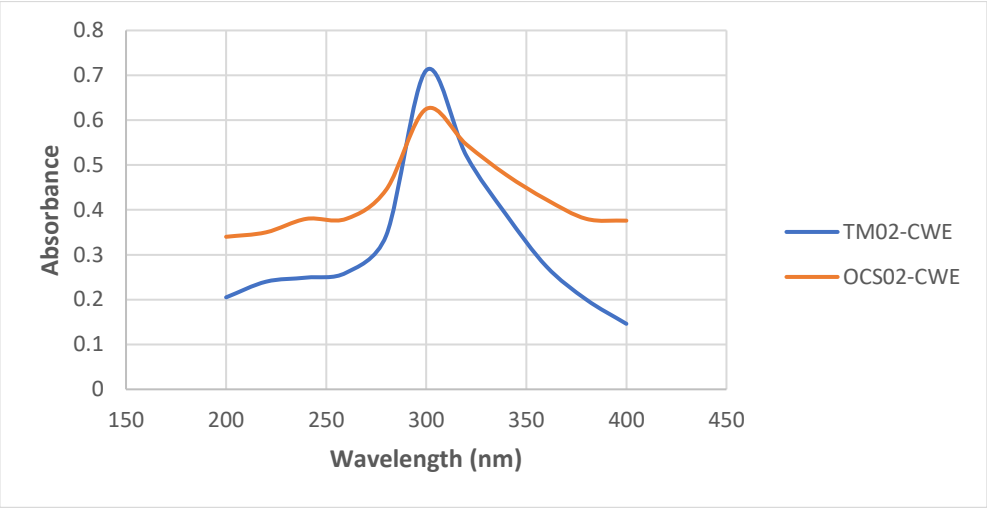


Figure D-1 UV-visible absorption spectra of OCS02-CWE and TM02-CWE.

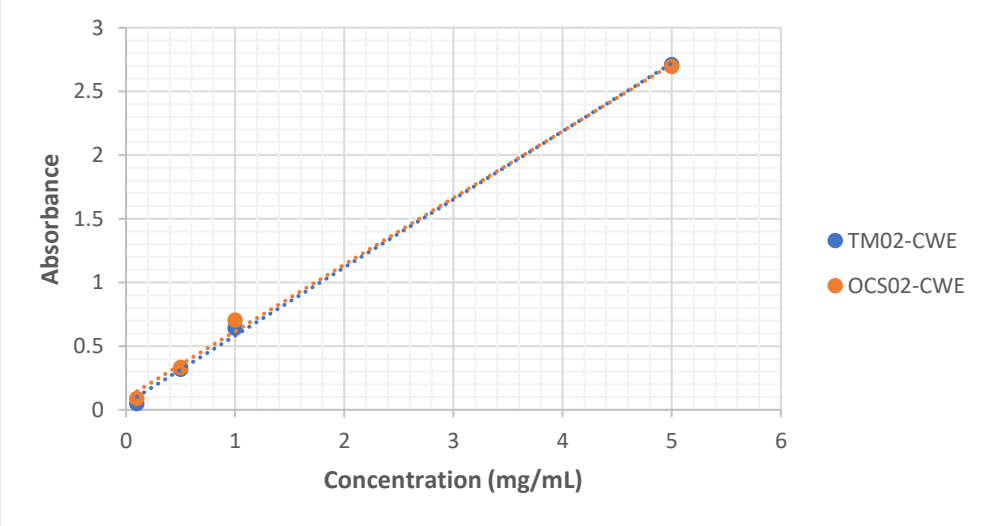


Figure D-2. Plot of absorbance vs concentration (mg/mL) for OCS02-CWE and TM02-CWE.

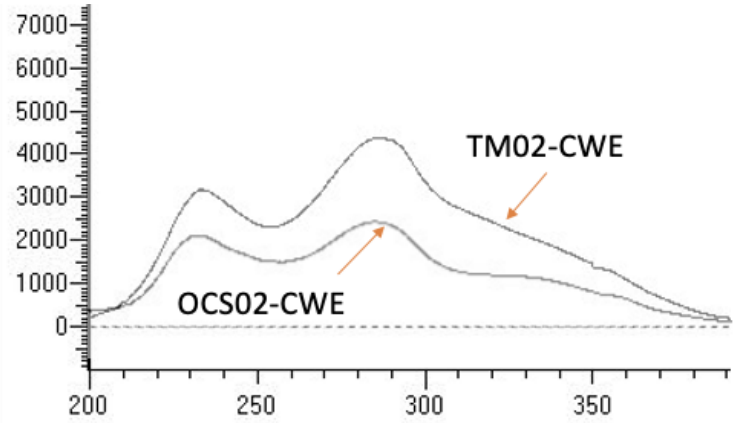


Figure D-3 Fluorescence excitation spectra of OCS02-CWE and TM02-CWE.

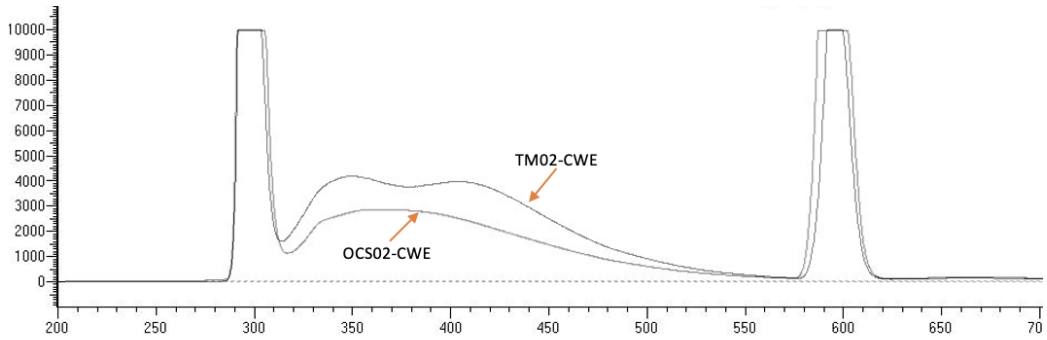


Figure D-4 Fluorescence emission spectra of OCS02-CWE and TM02-CWE.

References

Abdulhussain, N., Nawada, S. and Schoenmakers, P. (2021) Latest Trends on the Future of Three-Dimensional Separations in Chromatography. *Chemical Reviews*. 121 (19). doi:10.1021/acs.chemrev.0c01244.

Abrams, P., Cardozo, L., Fall, M., et al. (2003) The standardisation of terminology in lower urinary tract function: Report from the standardisation sub-committee of the International Continence Society. *Urology*. 61 (1). doi:10.1016/S0090-4295(02)02243-4.

Abrams, P., Kelleher, C., Staskin, D., et al. (2015) Combination treatment with mirabegron and solifenacin in patients with overactive bladder: Efficacy and safety results from a randomised, double-blind, dose-ranging, phase 2 study (symphony). *European Urology*, 67 (3): 577–588. doi:10.1016/j.eururo.2014.02.012.

Abrams, P., Kelleher, C.J., Kerr, L.A., et al. (2000) "Overactive bladder significantly affects quality of life." In *American Journal of Managed Care*. 2000. Available at: https://pubmed.ncbi.nlm.nih.gov/11183901/.

Abu Lafi, S., Akkawi, M., Abu-Remeleh, Q., et al. (2018) Pure Isolates and Preparative HPLC Fractions or Crude Extract of Inula viscosa: Effect on β-hematin Inhibition in vitro. *Pharmacy & Pharmacology International Journal*, 6 (1). doi:10.15406/ppij.2018.06.00145.

Abubakar, A.R. and Haque, M. (2020) Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. *Journal of Pharmacy and Bioallied Sciences*. 12 (1). doi:10.4103/jpbs.JPBS_175_19.

Afeli, S.A.Y., Rovner, E.S. and Petkov, G. V. (2012) SK but not IK channels regulate human detrusor smooth muscle spontaneous and nerve-evoked contractions. *American Journal of Physiology - Renal Physiology*, 303 (4). doi:10.1152/ajprenal.00615.2011.

Ahmad, S.M., Syed Aznal, S.S. and Tham, S.W. (2015) Prevalence of overactive bladder syndrome (OABS) among women with gynaecological problems and its risk factors in a tertiary hospital, Negeri Sembilan, Malaysia: Implication

for primary healthcare providers. *Malaysian Family Physician*, 10 (2): 2–8.

Ajalloueian, F., Lemon, G., Hilborn, J., et al. (2018) Bladder biomechanics and the use of scaffolds for regenerative medicine in the urinary bladder. *Nature Reviews Urology*. 15 (3). doi:10.1038/nrurol.2018.5.

Akter, S., Jesmin, S., Iwashima, Y., et al. (2015) Higher circulatory level of endothelin-1 in hypertensive subjects screened through a cross-sectional study of rural Bangladeshi women. *Hypertension Research*, 38 (3). doi:10.1038/hr.2014.160.

Alamoudi, A.J., Alessi, S.A., Rizg, W.Y., et al. (2022) Cordycepin Attenuates Testosterone-Induced Benign Prostatic Hyperplasia in Rats via Modulation of AMPK and AKT Activation. *Pharmaceutics*, 14 (8): 1652. doi:10.3390/pharmaceutics14081652.

Alkis, O., Ozlu, A., Kartal, I.G., et al. (2022) How effectively do we apply first-line treatment in overactive bladder? *International Urogynecology Journal*, 33 (8): 2299–2306. doi:10.1007/s00192-022-05279-2.

Altun, Z.F. and Hall, D.H. (2006) WormAtas Hermaphrodite Handbook - Introduction. *WormAtlas*. doi:10.3908/wormatlas.1.1.

Altunkaynak-Camca, H.O. (2020) Effect of Nebivolol on the Isolated Rat Bladder Strips Precontracted with Carbachol or Potassium Chloride. *Clinical and Experimental Health Sciences*. doi:10.33808/clinexphealthsci.731484.

Alvarez-Illera, P., Sanchez-Blanco, A., Lopez-Burillo, S., et al. (2016) Long-term monitoring of Ca^{2+} dynamics in C. elegans pharynx: An in vivo energy balance sensor. *Oncotarget*, 7 (42). doi:10.18632/oncotarget.12177.

Andersson, K.E. (2010) Detrusor myocyte activity and afferent signaling. *Neurourology and Urodynamics*. 29 (1). doi:10.1002/nau.20784.

Andersson, K.-E. (2019) TRP Channels as Lower Urinary Tract Sensory Targets. *Medical Sciences*, 7 (5). doi:10.3390/medsci7050067.

Andersson, K.E. and Arner, A. (2004) Urinary bladder contraction and relaxation: Physiology and pathophysiology. *Physiological Reviews*, 84 (3): 935–986. doi:10.1152/physrev.00038.2003.

Andersson, M., Aronsson, P., Doufish, D., et al. (2012) Muscarinic receptor subtypes involved in urothelium-derived relaxatory effects in the inflamed rat urinary bladder. *Autonomic Neuroscience: Basic and Clinical*, 170 (1–2). doi:10.1016/j.autneu.2012.06.004.

Andersson, M.C., Tobin, G. and Giglio, D. (2008) Cholinergic nitric oxide release from the urinary bladder mucosa in cyclophosphamide-induced cystitis of the anaesthetized rat. *British Journal of Pharmacology*, 153 (7). doi:10.1038/bjp.2008.6.

Ani, M.A.A., Al-Tamimi, Z., Haddad, R.A., et al. (2021) Use of calcium channel blockers and nsaids as alternative to anticholinergics in the treatment of urge urinary incontinence in women. *Archivos Venezolanos de Farmacologia y Terapeutica*, 40 (4). doi:10.5281/zenodo.5227566.

Apfeld, J., O'Connor, G., McDonagh, T., et al. (2004) The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans. *Genes and Development*, 18 (24). doi:10.1101/gad.1255404.

Apodaca, G., Kiss, S., Ruiz, W., et al. (2003) Disruption of bladder epithelium barrier function after spinal cord injury. *American Journal of Physiology - Renal Physiology*, 284 (5 53-5). doi:10.1152/ajprenal.00359.2002.

Araki, H., Cheng, J.T., Ogino, K., et al. (1976) Irreversible inhibitory effect of atropine on contractile responses to drugs in isolated rabbit ileum. *The Japanese Journal of Pharmacology*, 26 (6). doi:10.1254/jjp.26.737.

Araújo, A. v., Andrade, F.A., Paulo, M., et al. (2019) NO donors induce vascular relaxation by different cellular mechanisms in hypertensive and normotensive rats. *Nitric Oxide - Biology and Chemistry*, 86. doi:10.1016/j.niox.2019.02.004.

Arnold, J., McLeod, N., Thani-Gasalam, R., et al. (2012) Overactive bladder syndrome: Management and treatment options. *Australian Family Physician*, 41 (11).

Aronsson, P., Andersson, M., Ericsson, T., et al. (2010) Assessment and characterization of purinergic contractions and relaxations in the rat urinary bladder. *Basic and Clinical Pharmacology and Toxicology*. doi:10.1111/j.1742-7843.2010.00554.x.

Artim, D.E., Kullmann, F.A., Daugherty, S.L., et al. (2009) Activation of the nitric oxide-cGMP pathway reduces phasic contractions in neonatal rat bladder strips via protein kinase G. *American Journal of Physiology - Renal Physiology*, 297 (2). doi:10.1152/ajprenal.00207.2009.

El Ashry, F.E.Z.Z., Mahmoud, M.F., El Maraghy, N.N., et al. (2012) Effect of Cordyceps sinensis and taurine either alone or in combination on streptozotocin induced diabetes. *Food and Chemical Toxicology*, 50 (3–4). doi:10.1016/j.fct.2011.12.020.

Athanasopoulos, A. and Giannitsas, K. (2011) An overview of the clinical use of antimuscarinics in the treatment of overactive bladder. *Advances in Urology*. doi:10.1155/2011/820816.

Atkins, E.R. and Chow, C.K. (2020) Low-Dose Combination Therapy for Initial Treatment of Hypertension. *Current Hypertension Reports*. 22 (9). doi:10.1007/s11906-020-01069-7.

Azliza, M.A. (2013) *A Comparison of Ethnobiological Knowledge Between the Mah Meri and Temuan Tribes in Selangor*. University of Malaya.

Azliza, M.A., Ong, H.C., Vikineswary, S., et al. (2012) Ethno-medicinal resources used by the Temuan in Ulu Kuang village. *Studies on Ethno-Medicine*, 6 (1). doi:10.1080/09735070.2012.11886415.

Azwanida NN (2015) A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Medicinal & Aromatic Plants*, 4 (3). doi:10.4172/2167-0412.1000196.

Badawi, J.K., Li, H., Langbein, S., et al. (2006) Inhibitory effects of L- and T-type calcium antagonists on contractions of human detrusor muscle. *European Journal of Clinical Pharmacology*, 62 (5): 347–354. doi:10.1007/s00228-006-0100-8.

Bai, X., Tan, T.Y., Li, Y.X., et al. (2020) The protective effect of cordyceps sinensis extract on cerebral ischemic injury via modulating the mitochondrial respiratory chain and inhibiting the mitochondrial apoptotic pathway. *Biomedicine and Pharmacotherapy*, 124. doi:10.1016/j.biopha.2020.109834.

Bains, A., Chawla, P., Kaur, S., et al. (2021) Bioactives from mushroom: Health

attributes and food industry applications. *Materials*. 14 (24). doi:10.3390/ma14247640.

Bansal, A., Zhu, L.J., Yen, K., et al. (2015) Uncoupling lifespan and healthspan in caenorhabditis elegans longevity mutants. *Proceedings of the National Academy of Sciences of the United States of America*, 112 (3). doi:10.1073/pnas.1412192112.

Bao, Z.D., Wu, Z.G. and Zheng, F. (1994) Amelioration of aminoglycoside nephrotoxicity by Cordyceps sinensis in old patients. *Zhongguo Zhong xi yi jie he za zhi Zhongguo Zhongxiyi jiehe zazhi = Chinese journal of integrated traditional and Western medicine / Zhongguo Zhong xi yi jie he xue hui, Zhongguo Zhong yi yan jiu yuan zhu ban*, 14 (5).

Barrett, E.F., Morita, K. and Scappaticci, K.A. (1988) Effects of tetraethylammonium on the depolarizing after-potential and passive properties of lizard myelinated axons. *The Journal of Physiology*, 402 (1). doi:10.1113/jphysiol.1988.sp017194.

Baruah, R.K. and Deka, D.K. (2021) Relaxation of Goat Detrusor Muscle by L-arginine Involves NO-dependent but Guanylyl Cyclase Independent Activation of KCa Channels. *Indian Journal of Animal Research*, 56 (6): 680–684. doi:10.18805/IJAR.B-4292.

Bassiouni, W., Senbel, A., Norel, X., et al. (2019) Sildenafil corrects the increased contractility of rat detrusor muscle induced by alprostadil in vitro. *Pharmacological Reports*, 71 (4). doi:10.1016/j.pharep.2019.03.004.

Bates, J.N., Baker, M.T., Guerra, R., et al. (1991) Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. *Biochemical Pharmacology*, 42 (SUPPL. 1). doi:10.1016/0006-2952(91)90406-U.

Bauer, R. and Golden, A. (2020) Pharyngeal pumping phenotypes of common fluorescently-tagged C. elegans strains. *microPublication Biology*, (2020).

Beckman, J.S. and Koppenol, W.H. (1996) Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *American Journal of Physiology - Cell Physiology*. 271 (5 40-5). doi:10.1152/ajpcell.1996.271.5.c1424.

Beery, A.K. (2018) Inclusion of females does not increase variability in rodent research studies. *Current Opinion in Behavioral Sciences*. 23. doi:10.1016/j.cobeha.2018.06.016.

Benito-Román, Ó., Martín-Cortés, A., Cocero, M.J., et al. (2016) Dissolution of (1-3),(1-4)- β -Glucans in Pressurized Hot Water: Quantitative Assessment of the Degradation and the Effective Extraction . *International Journal of Carbohydrate Chemistry*, 2016. doi:10.1155/2016/2189837.

Berkels, R., Egink, G., Marsen, T.A., et al. (2001) Nifedipine increases endothelial nitric oxide bioavailability by antioxidative mechanisms. *Hypertension*, 37 (2 I). doi:10.1161/01.HYP.37.2.240.

Berridge, M.J. (2008) Smooth muscle cell calcium activation mechanisms. *Journal of Physiology*. 586 (21). doi:10.1113/jphysiol.2008.160440.

Billington, C.K. and Penn, R.B. (2003) Signaling and regulation of G protein-coupled receptors in airway smooth muscle. *Respiratory Research*. 4. doi:10.1186/1465-9921-4-2.

Birder, L.A., Apodaca, G., De Groat, W.C., et al. (1998) Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. *American Journal of Physiology - Renal Physiology*, 275 (2 44-2). doi:10.1152/ajprenal.1998.275.2.f226.

Birder, L.A., Nakamura, Y., Kiss, S., et al. (2002a) Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1. *Nature Neuroscience*, 5 (9): 856–860. doi:10.1038/nn902.

Birder, L.A., Nealen, M.L., Kiss, S., et al. (2002b) β-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. *Journal of Neuroscience*. doi:10.1523/JNEUROSCI.22-18-08063.2002.

Boland, B., Himpens, B., Paques, C., et al. (1993) ATP induced-relaxation in the mouse bladder smooth muscle. *British Journal of Pharmacology*, 108 (3): 749–753. doi:10.1111/j.1476-5381.1993.tb12872.x.

Bonnard, E., Liu, J., Zjacic, N., et al. (2022) Automatically tracking feeding behavior in populations of foraging C. elegans. *eLife*, 11. doi:10.7554/eLife.77252.

Bouchelouche, K., Andersen, L., Alvarez, S., et al. (2005) Increased contractile

response to phenylephrine in detrusor of patients with bladder outlet obstruction: Effect of the α1a and α1D-adrenergic receptor antagonist tamsulosin. *Journal of Urology*, 173 (2). doi:10.1097/01.ju.0000143198.16610.84.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72 (1–2): 248–254. doi:10.1016/0003-2697(76)90527-3.

Brading, A.F. (1997) "A myogenic basis for the overactive bladder." In *Urology*. 1997. pp. 57–67. doi:10.1016/S0090-4295(97)00591-8.

Bradley, K.K., Buxton, I.L.O., Barber, J.E., et al. (1998) Nitric oxide relaxes human myometrium by a cGMP-independent mechanism. *American Journal of Physiology - Cell Physiology*, 275 (6):44 – 46. doi:10.1152/ajpcell.1998.275.6.c1668.

Braverman, A.S., Tibb, A.S. and Ruggieri, M.R. (2006) M2 and M3 muscarinic receptor activation of urinary bladder contractile signal transduction. I. Normal rat bladder. *Journal of Pharmacology and Experimental Therapeutics*, 316 (2). doi:10.1124/jpet.105.097303.

Brenner, S. (1974) The genetics of Caenorhabditis elegans. *Genetics*, 77 (1). doi:10.1093/genetics/77.1.71.

Brown, S.G., Kim, Y.C., Kim, S.A., et al. (2001) Actions of a series of PPADS analogs at P2X1 and P2X3 receptors. *Drug Development Research*, 53 (4). doi:10.1002/ddr.1197.

Brown, S.M., Bentcheva-Petkova, L.M., Liu, L., et al. (2008) β-Adrenergic relaxation of mouse urinary bladder smooth muscle in the absence of large-conductance Ca^{2+}-activated K^{+} channel. *American Journal of Physiology - Renal Physiology*, 295 (4). doi:10.1152/ajprenal.00440.2007.

Brozovich, F.V., Nicholson, C.J., Degen, C.V., et al. (2016) Mechanisms of Vascular Smooth Muscle Contraction and the Basis for Pharmacologic Treatment of Smooth Muscle Disorders. *Pharmacological Reviews*, 68 (2): 476–532. doi:10.1124/pr.115.010652.

Buckner, S.A., Milicic, I., Daza, A. V., et al. (2002) Spontaneous phasic activity of the pig urinary bladder smooth muscle: Characteristics and sensitivity to potassium channel modulators. *British Journal of Pharmacology*, 135 (3). doi:10.1038/sj.bjp.0704499.

Burnstock, G. (2014) Purinergic signalling in the urinary tract in health and disease. *Purinergic Signalling*. doi:10.1007/s11302-013-9395-y.

Burnstock, G., Dumsday, B. and Smythe, A. (1972a) Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *British Journal of Pharmacology*, 44 (3). doi:10.1111/j.1476-5381.1972.tb07283.x.

Burnstock, G., Satchell, D.G. and Smythe, A. (1972b) A comparison of the excitatory and inhibitory effects of non-adrenergic, non-cholinergic nerve stimulation and exogenously applied ATP on a variety of smooth muscle preparations from different vertebrate species. *British Journal of Pharmacology*, 46 (2). doi:10.1111/j.1476-5381.1972.tb06868.x.

Burstein, E.S., Bräuner-Osborne, H., Spalding, T.A., et al. (1997) Interactions of muscarinic receptors with the heterotrimeric G proteins G(q) and G12: Transduction of proliferative signals. *Journal of Neurochemistry*, 68 (2). doi:10.1046/j.1471-4159.1997.68020525.x.

Cabreiro, F., Ackerman, D., Doonan, R., et al. (2011) Increased life span from overexpression of superoxide dismutase in Caenorhabditis elegans is not caused by decreased oxidative damage. *Free Radical Biology and Medicine*, 51 (8). doi:10.1016/j.freeradbiomed.2011.07.020.

Calvert, S., Tacutu, R., Sharifi, S., et al. (2016) A network pharmacology approach reveals new candidate caloric restriction mimetics in C. elegans. *Aging Cell*, 15 (2). doi:10.1111/acel.12432.

Chakrabarty, B., Bijos, D.A., Vahabi, B., et al. (2019) Modulation of bladder wall micromotions alters intravesical pressure activity in the isolated bladder. *Frontiers in Physiology*, 10 (JAN). doi:10.3389/fphys.2018.01937.

Chambers, P., Neal, D.E. and Gillespie, J.I. (1999) Ryanodine receptors in human bladder smooth muscle. *Experimental Physiology*, 84 (1).

doi:10.1111/j.1469-445X.1999.tb00070.x.

Chang, J.T., Kumsta, C., Hellman, A.B., et al. (2017) Spatiotemporal regulation of autophagy during Caenorhabditis elegans aging. *eLife*, 6. doi:10.7554/eLife.18459.

Chang, Y., Hsu, W.-H., Lu, W.-J., et al. (2015) Inhibitory Mechanisms of CME-1, a Novel Polysaccharide from the Mycelia of Cordyceps sinensis, in Platelet Activation. *Current Pharmaceutical Biotechnology*, 16 (5). doi:10.2174/1389201016666150303152237.

Chasnov, J.R. (2013) The evolutionary role of males in C. elegans . *Worm*, 2 (1). doi:10.4161/worm.21146.

Chen, J., Lee, S., Cao, Y., et al. (2010) Ethnomycological use of medicinal chinese caterpillar fungus, Ophiocordyceps sinensis (Berk.) G. H. Sung et al. (ascomycetes) in Northern Yunnan Province, SW China. *International Journal of Medicinal Mushrooms*, 12 (4): 427–434. doi:10.1615/IntJMedMushr.v12.i4.100.

Chen, J., Yu, B., Cong, H., et al. (2022) Recent development and application of membrane chromatography. *Analytical and Bioanalytical Chemistry*. doi:10.1007/s00216-022-04325-8.

Chen, P.H., Weng, Y.M., Yu, Z.R., et al. (2016) Extraction temperature affects the activities of antioxidation, carbohydrate-digestion enzymes, and angiotensin-converting enzyme of Pleurotus citrinopileatus extract. *Journal of Food and Drug Analysis*. doi:10.1016/j.jfda.2016.02.005.

Chen, P.X., Wang, S., Nie, S., et al. (2013) Properties of Cordyceps Sinensis: A review. *Journal of Functional Foods*, 5 (2): 550–569. doi:10.1016/j.jff.2013.01.034.

Chess-Williams, R. (2002) Muscarinic receptors of the urinary bladder: Detrusor, urothelial and prejunctional. *Autonomic and Autacoid Pharmacology*. 22 (3). doi:10.1046/j.1474-8673.2002.00258.x.

Chess-Williams, R., Chapple, C.R., Yamanishi, T., et al. (2001) The minor population of M3-receptors mediate contraction of human detrusor muscle in vitro. *Journal of Autonomic Pharmacology*, 21 (5). doi:10.1046/j.1365-2680.2001.00231.x.

Cheung, J.K.H., Li, J., Cheung, A.W.H., et al. (2009) Cordysinocan, a polysaccharide isolated from cultured Cordyceps, activates immune responses in cultured T-lymphocytes and macrophages: Signaling cascade and induction of cytokines. *Journal of Ethnopharmacology*, 124 (1). doi:10.1016/j.jep.2009.04.010.

Chiou, W.F., Chang, P.C., Chou, C.J., et al. (2000) Protein constituent contributes to the hypotensive and vasorelaxant activities of Cordyceps sinensis. *Life Sciences*, 66 (14): 1369–1376. doi:10.1016/S0024-3205(00)00445-8.

Chisholm, A.D. and Hsiao, T.I. (2012) The Caenorhabditis elegans epidermis as a model skin. I: Development, patterning, and growth. *Wiley Interdisciplinary Reviews: Developmental Biology*, 1 (6). doi:10.1002/wdev.79.

Cho, S.T., Moon, S., Kim, S.J., et al. (2021) MP02-07 Is there an association between nocturia and cardiovascular disease and mortality? Data from the National Health and Nutrition Examination Survey. *Journal of Urology*, 206 (Supplement 3). doi:10.1097/ju.0000000000001963.07.

Chow, D.K., Glenn, C.F., Johnston, J.L., et al. (2006) Sarcopenia in the Caenorhabditis elegans pharynx correlates with muscle contraction rate over lifespan. *Experimental Gerontology*, 41 (3). doi:10.1016/j.exger.2005.12.004.

Christ, G.J. and Hodges, S. (2006) Molecular mechanisms of detrusor and corporal myocyte contraction: Identifying targets for pharmacotherapy of bladder and erectile dysfunction. *British Journal of Pharmacology*, 147 (SUPPL. 2). doi:10.1038/sj.bjp.0706627.

Chuang, Y.C., Liu, S.P., Lee, K.S., et al. (2019) Prevalence of overactive bladder in China, Taiwan and South Korea: Results from a cross-sectional, population-based study. *LUTS: Lower Urinary Tract Symptoms*, 11 (1). doi:10.1111/luts.12193.

Chughtai, B., Kavaler, E., Lee, R., et al. (2013) Use of herbal supplements for overactive bladder. *Reviews in urology*, 15 (3): 93–6.

Chung, B.H., Choi, S.K. and Chang, K.C. (1996) Effects of nitric oxide on detrusor relaxation. *Journal of Urology*, 155 (6). doi:10.1016/S0022-5347(01)66115-9.

Chung, H.C., Lee, C.K., Park, K.H., et al. (2015) Bladder outlet obstruction causes up-regulation of nicotinic acetylcholine receptors in bladder-projecting pelvic

ganglion neurons. *Brain Research*, 1602 (C): 111–118. doi:10.1016/j.brainres.2015.01.026.

Cogolludo, A.L., Pérez-Vizcaíno, F., Zaragozá-Arnáez, F., et al. (2001) Mechanisms involved in SNP-induced relaxation and $[Ca^{2+}]_i$ reduction in piglet pulmonary and systemic arteries. *British Journal of Pharmacology*, 132 (4). doi:10.1038/sj.bjp.0703894.

Conte, D., MacNeil, L.T., Walhout, A.J.M., et al. (2015) RNA Interference in *Caenorhabditis elegans*. *Current Protocols in Molecular Biology*, 109 (1). doi:10.1002/0471142727.mb2603s109.

Cornier, M.-A., Dabelea, D., Hernandez, T.L., et al. (2008) The Metabolic Syndrome. *Endocrine Reviews*, 29 (7): 777–822. doi:10.1210/er.2008-0024.

Corsi, A.K., Wightman, B. and Chalfie, M. (2015) A transparent window into biology: A primer on *Caenorhabditis elegans*. *Genetics*, 200 (2). doi:10.1534/genetics.115.176099.

Culetto, E. and Sattelle, D.B. (2000) A role for *Caenorhabditis elegans* in understanding the function and interactions of human disease genes. *Human Molecular Genetics*, 9 (6). doi:10.1093/hmg/9.6.869.

Dallière, N., Bhatla, N., Luedtke, Z., et al. (2016) Multiple excitatory and inhibitory neural signals converge to fine-tune *Caenorhabditis elegans* feeding to food availability. *FASEB Journal*, 30 (2). doi:10.1096/fj.15-279257.

Deka, D.K. and Brading, A.F. (2004) Nitric oxide activates glibenclamide-sensitive K+ channels in urinary bladder myocytes through a c-GMP-dependent mechanism. *European Journal of Pharmacology*, 492 (1). doi:10.1016/j.ejphar.2004.03.057.

D'Emmanuele di Villa Bianca, R., Mitidieri, E., Fusco, F., et al. (2016) Urothelium muscarinic activation phosphorylates CBS Ser227 via cGMP/PKG pathway causing human bladder relaxation through H 2 S production. *Scientific Reports*, 6. doi:10.1038/srep31491.

Dobrev, D., Milde, A.S., Andreas, K., et al. (1999) The effects of verapamil and diltiazem on N-, P- and Q-type calcium channels mediating dopamine release in rat striatum. *British Journal of Pharmacology*, 127 (2).

doi:10.1038/sj.bjp.0702574.

Dombkowski, R.A., Doellman, M.M., Head, S.K., et al. (2006) Hydrogen sulfide mediates hypoxia-induced relaxation of trout urinary bladder smooth muscle. *Journal of Experimental Biology*, 209 (16). doi:10.1242/jeb.02376.

Dong, C.H. and Yao, Y.J. (2008) In vitro evaluation of antioxidant activities of aqueous extracts from natural and cultured mycelia of *Cordyceps sinensis*. *LWT - Food Science and Technology*, 41 (4): 669–677. doi:10.1016/j.lwt.2007.05.002.

Dong, Y., Yang, J., Zhang, Y., et al. (2014) *Cordyceps sinensis* Cs-5 improves memory and learning abilities in a dysmnesia model (684.1) . *The FASEB Journal*, 28 (S1). doi:10.1096/fasebj.28.1_supplement.684.1.

Drake, M.J., Chapple, C., Esen, A.A., et al. (2016) Efficacy and Safety of Mirabegron Add-on Therapy to Solifenacin in Incontinent Overactive Bladder Patients with an Inadequate Response to Initial 4-Week Solifenacin Monotherapy: A Randomised Double-blind Multicentre Phase 3B Study (BESIDE). *European Urology*, 70 (1). doi:10.1016/j.eururo.2016.02.030.

Drake, M.J., Fry, C.H., Hashitani, H., et al. (2018) What are the origins and relevance of spontaneous bladder contractions? ICI-RS 2017. *Neurourology and Urodynamics*, 37. doi:10.1002/nau.23485.

Drake, M.J., Kanai, A., Bijos, D.A., et al. (2017) The potential role of unregulated autonomous bladder micromotions in urinary storage and voiding dysfunction; overactive bladder and detrusor underactivity. *BJU International*. 119 (1). doi:10.1111/bju.13598.

Duangjan, C. and Curran, S.P. (2022) Oolonghomobisflavans from *Camellia sinensis* increase Caenorhabditis elegans lifespan and healthspan. *GeroScience*, 44 (1). doi:10.1007/s11357-021-00462-7.

Dubois, M., Gilles, K.A., Hamilton, J.K., et al. (1956) Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, 28 (3): 350–356. doi:10.1021/ac60111a017.

Dunning-Davies, B.M., Fry, C.H., Mansour, D., et al. (2013) The regulation of ATP release from the urothelium by adenosine and transepithelial potential. *BJU*

International, 111 (3). doi:10.1111/j.1464-410X.2012.11421.x.

Düsing, R., Waeber, B., Destro, M., et al. (2017) Triple-combination therapy in the treatment of hypertension: A review of the evidence. *Journal of Human Hypertension*. 31 (8). doi:10.1038/jhh.2017.5.

Earley, S. and Brayden, J.E. (2015) Transient receptor potential channels in the vasculature. *Physiological Reviews*, 95 (2). doi:10.1152/physrev.00026.2014.

Edwards, C., Canfield, J., Copes, N., et al. (2015) Mechanisms of amino acid-mediated lifespan extension in *Caenorhabditis elegans*. *BMC Genetics*, 16 (1). doi:10.1186/s12863-015-0167-2.

Fabricant, D.S. and Farnsworth, N.R. (2001) The Value of Plants Used in Traditional Medicine for Drug Discovery. *Environmental Health Perspectives*, 109 (Supplement 1): 69–75. doi:10.1289/ehp.01109s169.

Félix, M.A. and Braendle, C. (2010) The natural history of *Caenorhabditis elegans*. *Current Biology*. 20 (22). doi:10.1016/j.cub.2010.09.050.

Fernandes, V.S. and Hernández, M. (2016) The Role of Nitric Oxide and Hydrogen Sulfide in Urinary Tract Function. *Basic and Clinical Pharmacology and Toxicology*. 119. doi:10.1111/bcpt.12565.

Fernandes, V.S., Ribeiro, A.S.F., Barahona, M.V., et al. (2013) Hydrogen sulfide mediated inhibitory neurotransmission to the pig bladder neck: Role of KATP channels, sensory nerves and calcium signaling. *Journal of Urology*, 190 (2). doi:10.1016/j.juro.2013.02.103.

Floras, J.S. (1992) Epinephrine and the genesis of hypertension. *Hypertension*. 19 (1). doi:10.1161/01.HYP.19.1.1.

Foucquier, J. and Guedj, M. (2015) Analysis of drug combinations: current methodological landscape. *Pharmacology Research and Perspectives*. 3 (3). doi:10.1002/prp2.149.

Frazier, E.P., Braverman, A.S., Peters, S.L.M., et al. (2007) Does phospholipase C mediate muscarinic receptor-induced rat urinary bladder contraction? *Journal of Pharmacology and Experimental Therapeutics*, 322 (3). doi:10.1124/jpet.107.125393.

Friedman, D.B. and Johnson, T.E. (1988) A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics*, 118 (1). doi:10.1093/genetics/118.1.75.

Fritz, N., Morel, J.L., Jeyakumar, L.H., et al. (2007) RyR1-specific requirement for depolarization-induced Ca^{2+} sparks in urinary bladder smooth muscle. *Journal of Cell Science*, 120 (21). doi:10.1242/jcs.009415.

Frøkjær-Jensen, C., Kindt, K.S., Kerr, R.A., et al. (2006) Effects of voltage-gated calcium channel subunit genes on calcium influx in cultured C. elegans mechanosensory neurons. *Journal of Neurobiology*, 66 (10). doi:10.1002/neu.20261.

Fry, C.H., Sui, G. and Wu, C. (2006) T-type Ca2+ channels in non-vascular smooth muscles. *Cell Calcium*, 40 (2). doi:10.1016/j.ceca.2006.04.027.

Fry, C.H., Sui, G.P., Severs, N.J., et al. (2004) "Spontaneous activity and electrical coupling in human detrusor smooth muscle: Implications for detrusor overactivity?" In *Urology*. 2004. doi:10.1016/j.urology.2003.11.005.

Fu, B., Wang, N., Tan, H.Y., et al. (2018) Multi-component herbal products in the prevention and treatment of chemotherapy-associated toxicity and side effects: A review on experimental and clinical evidences. *Frontiers in Pharmacology*. 9 (NOV). doi:10.3389/fphar.2018.01394.

Fujii, M. (1996) Krestin (PSK). *Biotherapy*, 10 (3).

Fujiwara, M., Andersson, K.E. and Persson, K. (2000) Nitric oxide-induced cGMP accumulation in the mouse bladder is not related to smooth muscle relaxation. *European Journal of Pharmacology*, 401 (2): 241–250. doi:10.1016/S0014-2999(00)00457-X.

Fukao, M., Hattori, Y., Kanno, M., et al. (1995) Thapsigargin- and cyclopiazonic acid-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *British Journal of Pharmacology*, 115 (6). doi:10.1111/j.1476-5381.1995.tb15908.x.

Function, D.S.M., Hristov, K.L., Chen, M., et al. (2011) Large-conductance voltage- and Ca 2+-activated K + channels regulate human. *American Journal of Physiology - Cell Physiology*, 301 (4). doi:10.1152/ajpcell.00495.2010.

Fung, S.Y., Cheong, P.C.H., Tan, N.H., et al. (2018) Nutrient and chemical analysis of fruiting bodies of a cultivar of the Chinese caterpillar mushroom, Ophiocordyceps sinensis (Ascomycetes). *International Journal of Medicinal Mushrooms*, 20 (5): 459–469. doi:10.1615/IntJMedMushrooms.2018026252.

Fung, S.Y., Lee, S.S., Tan, N.H., et al. (2017) Safety assessment of cultivated fruiting body of Ophiocordyceps sinensis evaluated through subacute toxicity in rats. *Journal of Ethnopharmacology*, 206: 236–244. doi:10.1016/j.jep.2017.05.037.

Gai, J.W., Wahafu, W., Guo, H., et al. (2013) Further evidence of endogenous hydrogen sulphide as a mediator of relaxation in human and rat bladder. *Asian Journal of Andrology*, 15 (5). doi:10.1038/aja.2013.32.

García Garví, A., Puchalt, J.C., Layana Castro, P.E., et al. (2021) Towards Lifespan Automation for Caenorhabditis elegans Based on Deep Learning: Analysing Convolutional and Recurrent Neural Networks for Dead or Live Classification. *Sensors (Basel, Switzerland)*, 21 (14). doi:10.3390/s21144943.

Garland, C.J., Yarova, P.L., Jiménez-Altayó, F., et al. (2011) Vascular hyperpolarization to β-adrenoceptor agonists evokes spreading dilatation in rat isolated mesenteric arteries. *British Journal of Pharmacology*, 164 (3). doi:10.1111/j.1476-5381.2011.01224.x.

Ghatta, S., Nimmagadda, D., Xu, X., et al. (2006) Large-conductance, calcium-activated potassium channels: Structural and functional implications. *Pharmacology and Therapeutics*. 110 (1). doi:10.1016/j.pharmthera.2005.10.007.

Govindaraju, K., Lee, M.K., Ting, K.N., et al. (2018) Lignosus rhinocerotis water-soluble sclerotial extract relaxes rat isolated aortae. *Frontiers in Pharmacology*, 9. doi:10.3389/conf.fphar.2018.63.00083.

Griffin, C.S., Bradley, E., Dudem, S., et al. (2016) Muscarinic Receptor Induced Contractions of the Detrusor are Mediated by Activation of TRPC4 Channels. *Journal of Urology*, 196 (6). doi:10.1016/j.juro.2016.05.108.

Griffin, C.S., Thornbury, K.D., Hollywood, M.A., et al. (2018) Muscarinic receptor-induced contractions of the detrusor are impaired in TRPC4 deficient mice. *Scientific Reports*, 8 (1). doi:10.1038/s41598-018-27617-5.

De Groat, W.C. (1997) “Neurologic basis for the overactive bladder.” In *Urology*. 1997. pp. 36–52. doi:10.1016/S0090-4295(97)00587-6.

de Groat, W.C. and Yoshimura, N. (2012) Plasticity in reflex pathways to the lower urinary tract following spinal cord injury. *Experimental Neurology*. 235 (1) pp. 123–132. doi:10.1016/j.expneurol.2011.05.003.

Guerrero-Rubio, M.A., Hernández-García, S., García-Carmona, F., et al. (2021) Biosynthesis of a novel polymeric chitosan-betaxanthin and characterization of the first sugar-derived betalains and their effects in the in vivo model *Caenorhabditis elegans*. *Carbohydrate Polymers*, 252: 117141. doi:10.1016/j.carbpol.2020.117141.

Guo, L.X., Hong, Y.H., Zhou, Q.Z., et al. (2017) Fungus-larva relation in the formation of Cordyceps sinensis as revealed by stable carbon isotope analysis. *Scientific Reports*, 7 (1). doi:10.1038/s41598-017-08198-1.

Hagovska, M., Švihra, J., Buková, A., et al. (2020) The Relationship between Overweight and Overactive Bladder Symptoms. *Obesity Facts*, 13 (3). doi:10.1159/000506486.

Han, J.H., Chang, I.H., Myung, S.C., et al. (2012) A novel pathway underlying the inhibitory effects of melatonin on isolated rat urinary bladder contraction. *Korean Journal of Physiology and Pharmacology*, 16 (1). doi:10.4196/kjpp.2012.16.1.37.

Han, J.H., Lee, M.Y., Lee, S.Y., et al. (2010) Effect of low concentrations of hydrogen peroxide on the contractile responses of rat detrusor smooth muscle strips. *European Journal of Pharmacology*, 638 (1–3). doi:10.1016/j.ejphar.2010.02.042.

Hanna-Mitchell, A.T., Beckel, J.M., Barbadora, S., et al. (2007) Non-neuronal acetylcholine and urinary bladder urothelium. *Life Sciences*, 80 (24–25). doi:10.1016/j.lfs.2007.02.010.

Hanna-Mitchell, A.T., Kashyap, M., Chan, W.V., et al. (2014) Pathophysiology of idiopathic overactive bladder and the success of treatment: A systematic review from ICI-RS 2013. *Neurourology and Urodynamics*. 33 (5) pp. 611–617. doi:10.1002/nau.22582.

Hao, Y., Wang, L., Chen, H., et al. (2019) Targetable purinergic receptors P2Y12 and A2b antagonistically regulate bladder function. *JCI Insight*. doi:10.1172/jci.insight.122112.

Harman, D. (1991) The aging process: Major risk factor for disease and death. *Proceedings of the National Academy of Sciences of the United States of America*, 88 (12). doi:10.1073/pnas.88.12.5360.

Hashimoto, A., Miyakoda, G., Hirose, Y., et al. (2006) Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. *Atherosclerosis*, 189 (2). doi:10.1016/j.atherosclerosis.2006.01.022.

Hashitani, H., Fukuta, H., Takano, H., et al. (2001) Origin and propagation of spontaneous excitation in smooth muscle of the guinea-pig urinary bladder. *Journal of Physiology*, 530 (2). doi:10.1111/j.1469-7793.2001.0273l.x.

Hashitani, H., Mitsui, R., Shimizu, Y., et al. (2012) Functional and morphological properties of pericytes in suburothelial venules of the mouse bladder. *British Journal of Pharmacology*, 167 (8). doi:10.1111/j.1476-5381.2012.02125.x.

Hawthorn, M.H., Chapple, C.R., Cock, M., et al. (2000) Urothelium-derived inhibitory factor(s) influences on detrusor muscle contractility in vitro. *British journal of pharmacology*, 129 (3): 416–419. doi:10.1038/sj.bjp.0703068.

He, Q., Wang, Z., Liu, G., et al. (2016) Metabolic syndrome, inflammation and lower urinary tract symptoms: Possible translational links. *Prostate Cancer and Prostatic Diseases*, 19 (1): 7–13. doi:10.1038/pcan.2015.43.

Hegele, A., Knippschild, S., Frohme, C., et al. (2014) Changes in prostaglandin E2 in patients with idiopathic overactive bladder syndrome after botulinum toxin type A treatment: Is there a clinical benefit? *BMC Urology*, 14 (1). doi:10.1186/1471-2490-14-85.

Helmcke, K.J., Syversen, T., Miller, D.M., et al. (2009) Characterization of the effects of methylmercury on Caenorhabditis elegans. *Toxicology and Applied Pharmacology*, 240 (2). doi:10.1016/j.taap.2009.03.013.

Heppner, T.J., Tykocki, N.R., Hill-Eubanks, D., et al. (2016) Transient contractions of urinary bladder smooth muscle are drivers of afferent nerve activity during

filling. *Journal of General Physiology*, 147 (4). doi:10.1085/jgp.201511550.

Hermann, A. and Gorman, A.L.F. (1981) Effects of tetraethylammonium on potassium currents in a molluscan neuron. *Journal of General Physiology*, 78 (1). doi:10.1085/jgp.78.1.87.

Hernández, M., Barahona, M.V., Simonsen, U., et al. (2003) Characterization of the 5-hydroxytryptamine receptors mediating contraction in the pig isolated intravesical ureter. *British Journal of Pharmacology*, 138 (1). doi:10.1038/sj.bjp.0705019.

Hernández, M., Knight, G.E., Wildman, S.S.P., et al. (2009) Role of ATP and related purines in inhibitory neurotransmission to the pig urinary bladder neck. *British Journal of Pharmacology*, 157 (8). doi:10.1111/j.1476-5381.2009.00314.x.

Herrera, G.M., Heppner, T.J. and Nelson, M.T. (2001) Voltage dependence of the coupling of Ca^{2+} sparks to BKCa channels in urinary bladder smooth muscle. *American Journal of Physiology - Cell Physiology*, 280 (3 49-3). doi:10.1152/ajpcell.2001.280.3.c481.

Herschorn, S., Chapple, C.R., Abrams, P., et al. (2017) Efficacy and safety of combinations of mirabegron and solifenacin compared with monotherapy and placebo in patients with overactive bladder (SYNERGY study). *BJU International*, 120 (4). doi:10.1111/bju.13882.

Hirota, K., Hashiba, E., Yoshioka, H., et al. (2003) Effects of three different L-type Ca^{2+} entry blockers on airway constriction induced by muscarinic receptor stimulation. *British Journal of Anaesthesia*, 90 (5). doi:10.1093/bja/aeg118.

Ho, C.C.K., Teo, C.Y., Phang, L.F., et al. (2012) Prevalence and Risk Factors Associated with Overactive Bladder. *UroToday International Journal*, 5 (1): 88. doi:10.3834/uij.1944-5784.2012.02.06.

Hoare, S.R.J. (2021) The Problems of Applying Classical Pharmacology Analysis to Modern In Vitro Drug Discovery Assays: Slow Binding Kinetics and High Target Concentration. *SLAS Discovery*. 26 (7). doi:10.1177/24725552211019653.

Hodgkin, J. (1987) Primary sex determination in the nematode *C. elegans*. *Development*, 101 (SUPPL.). doi:10.1242/dev.101.supplement.5.

Homan, T., Tsuzuki, T., Dogishi, K., et al. (2013) A novel mouse model of chronic inflammatory and overactive bladder by a single intravesical injection of hydrogen peroxide. *Journal of Pharmacological Sciences*, 121 (4). doi:10.1254/jphs.12265FP.

Horowitz, A., Menice, C.B., Laporte, R., et al. (1996) Mechanisms of smooth muscle contraction. *Physiological Reviews*, 76 (4): 967–1003. doi:10.1152/physrev.1996.76.4.967.

Houghton, P.J. and Raman, A. (1998) *Laboratory Handbook for the Fractionation of Natural Extracts*. Boston, MA: Springer US. doi:10.1007/978-1-4615-5809-5.

Hrudayanath, T. and Sameer, K.S. (2014) Diversity, nutritional composition and medicinal potential of Indian mushrooms: A review. *African Journal of Biotechnology*, 13 (4). doi:10.5897/ajb2013.13446.

van Hove, C.E., van der Donckt, C., Herman, A.G., et al. (2009) Vasodilator efficacy of nitric oxide depends on mechanisms of intracellular calcium mobilization in mouse aortic smooth muscle cells. *British Journal of Pharmacology*, 158 (3). doi:10.1111/j.1476-5381.2009.00396.x.

Howe, K.L. (2019) A new reference genome sequence for Caenorhabditis elegans? *Lab Animal*. 48 (9). doi:10.1038/s41684-019-0371-1.

Hsu, T.H., Shiao, L.H., Hsieh, C., et al. (2002) A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom DongChongXiaCao, its counterfeit and mimic, and fermented mycelium of Cordyceps sinensis. *Food Chemistry*, 78 (4): 463–469. doi:10.1016/S0308-8146(02)00158-9.

Igawa, Y., Zhang, X., Nishizawa, O., et al. (2004) Cystometric findings in mice lacking muscarinic M_2 or M_3 receptors. *Journal of Urology*, 172 (6 I). doi:10.1097/01.ju.0000138054.77785.4a.

Imai, T., Okamoto, T., Yamamoto, Y., et al. (2001) Effects of different types of K^+ channel modulators on the spontaneous myogenic contraction of guinea-pig urinary bladder smooth muscle. *Acta Physiologica Scandinavica*, 173 (3). doi:10.1046/j.1365-201X.2001.00908.x.

Irwin, D.E., Milsom, I., Hunskaar, S., et al. (2006) Population-Based Survey of

Urinary Incontinence, Overactive Bladder, and Other Lower Urinary Tract Symptoms in Five Countries: Results of the EPIC Study. *European Urology*, 50 (6): 1306–1315. doi:10.1016/j.eururo.2006.09.019.

Ito, Y., Woo, J.T., Nishijima, S., et al. (2018) Nobiletin, a flavone from shekwasha (Citrus depressa), alleviates hypertensive bladder response. *Proceedings for Annual Meeting of The Japanese Pharmacological Society*, WCP2018 (0). doi:10.1254/jpssuppl.wcp2018.0_po2-4-13.

James, M., Birmingham, A. and Hill, S. (1993) Partial mediation by nitric oxide of the relaxation of human isolated detrusor strips in response to electrical field stimulation. *British Journal of Clinical Pharmacology*, 35 (4). doi:10.1111/j.1365-2125.1993.tb04152.x.

Jespersen, B., Tykocki, N.R., Watts, S.W., et al. (2015) Measurement of smooth muscle function in the isolated tissue bath-applications to pharmacology research. *Journal of Visualized Experiments*, (95). doi:10.3791/52324.

Jezior, J.R., Brady, J.D., Rosenstein, D.I., et al. (2001) Dependency of detrusor contractions on calcium sensitization and calcium entry through LOE-908-sensitive channels. *British Journal of Pharmacology*, 134 (1). doi:10.1038/sj.bjp.0704241.

Johnathan, M., Gan, S.H., Ezumi, M.F.W., et al. (2016) Phytochemical profiles and inhibitory effects of Tiger Milk mushroom (*Lignosus rhinocerus*) extract on ovalbumin-induced airway inflammation in a rodent model of asthma. *BMC Complementary and Alternative Medicine*, 16 (1). doi:10.1186/s12906-016-1141-x.

Joseph, S., Maria, S.A. and Peedicayil, J. (2022) Drugs Currently Undergoing Preclinical or Clinical Trials for the Treatment of Overactive Bladder: A Review. *Current Therapeutic Research*, 96: 100669. doi:10.1016/j.curtheres.2022.100669.

Jost, S.P., Gosling, J.A. and Dixon, J.S. (1989) The morphology of normal human bladder urothelium. *Journal of anatomy*, 167.

Joyner, M.J. and Johnson, B.D. (2008) Iron lung? New ideas about hypoxic pulmonary vasoconstriction. *Journal of Physiology*. 586 (24).

doi:10.1113/jphysiol.2008.166249.

Jung, S.J., Jung, E.S., Choi, E.K., et al. (2019) Immunomodulatory effects of a mycelium extract of Cordyceps (Paecilomyces hepiali; CBG-CS-2): A randomized and double-blind clinical trial. *BMC Complementary and Alternative Medicine*, 19 (1). doi:10.1186/s12906-019-2483-y.

Kachru, N., Holmes, H.M., Johnson, M.L., et al. (2020) Risk of Mortality Associated with Non-selective Antimuscarinic medications in Older Adults with Dementia: a Retrospective Study. *Journal of General Internal Medicine*, 35 (7). doi:10.1007/s11606-020-05634-3.

Kaeberlein, T.L., Smith, E.D., Tsuchiya, M., et al. (2006) Lifespan extension in Caenorhabditis elegans by complete removal of food. *Aging Cell*, 5 (6). doi:10.1111/j.1474-9726.2006.00238.x.

Kajioka, S., Nakayama, S., Asano, H., et al. (2005) Involvement of ryanodine receptors in muscarinic receptor-mediated membrane current oscillation in urinary bladder smooth muscle. *American Journal of Physiology - Cell Physiology*, 288 (1 57-1). doi:10.1152/ajpcell.00161.2004.

Kajioka, S., Shahab, N., Asano, H., et al. (2011) Diphosphate regulation of adenosine triphosphate sensitive potassium channel in human bladder smooth muscle cells. *Journal of Urology*, 186 (2). doi:10.1016/j.juro.2011.03.153.

Kajiwara, M. and Mutaguchi, K. (2008) Clinical efficacy and tolerability of Gosha-jinki-gan, japanese traditional herbal medicine, in females with overactive bladder. *Acta Urologica Japonica*.

Kamm, K.E. and Stull, J.T. (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annual Review of Pharmacology and Toxicology*. VOL. 25. doi:10.1146/annurev.pharmtox.25.1.593.

Kan, W.C., Wang, H.Y., Chien, C.C., et al. (2012) Effects of extract from solid-state fermented cordyceps sinensis on type 2 diabetes mellitus. *Evidence-based Complementary and Alternative Medicine*, 2012. doi:10.1155/2012/743107.

Kaneda, T., Sasaki, N., Urakawa, N., et al. (2018) Effects of chlorogenic acid on carbachol-induced contraction of mouse urinary bladder. *Journal of Pharmacological Sciences*. doi:10.1016/j.jphs.2017.12.006.

Kannan, M.S., Prakash, Y.S., Johnson, D.E., et al. (1997) Nitric oxide inhibits calcium release from sarcoplasmic reticulum of porcine tracheal smooth muscle cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 272 (1 16-1). doi:10.1152/ajplung.1997.272.1.l1.

Katiyar, C., Kanjilal, S., Gupta, A., et al. (2012) Drug discovery from plant sources: An integrated approach. *AYU (An International Quarterly Journal of Research in Ayurveda)*, 33 (1). doi:10.4103/0974-8520.100295.

Kaushik, V., Arya, A., Sindhu, A., et al. (2019) Identification, optimization of culture conditions, and bioactive potential of chinese caterpillar mushroom Ophiocordyceps sinensis (Ascomycetes) mycelium isolated from fruiting body. *International Journal of Medicinal Mushrooms*, 21 (9). doi:10.1615/IntJMedMushrooms.2019031841.

Kelm, M. (1999) Nitric oxide metabolism and breakdown. *Biochimica et Biophysica Acta - Bioenergetics*. 1411 (2–3). doi:10.1016/S0005-2728(99)00020-1.

Kenyon, C., Chang, J., Gensch, E., et al. (1993) A C. elegans mutant that lives twice as long as wild type. *Nature*, 366 (6454). doi:10.1038/366461a0.

Kim, H.J. and Lee, S.J. (2001) Serotonin(5-HT): can it be a cause in overactive bladder. *Journal of the Korean Continence Society*, 5 (1). doi:10.5213/jkcs.2001.5.1.39.

Kim, H.J., Yang, H.M., Kim, D.H., et al. (2003) Effects of Ylang-Ylang Essential Oil on the Relaxation of Rat Bladder Muscle in vitro and White Rabbit Bladder in vivo. *Journal of Korean Medical Science*, 18 (3): 409–414. doi:10.3346/jkms.2003.18.3.409.

Kim, J.C., Park, E.Y., Hong, S.H., et al. (2005a) Changes of urinary nerve growth factor and prostaglandins in male patients with overactive bladder symptom. *International Journal of Urology*, 12 (10). doi:10.1111/j.1442-2042.2005.01140.x.

Kim, J.C., Park, E.Y., Seo, S. Il, et al. (2006) Nerve Growth Factor and Prostaglandins in the Urine of Female Patients With Overactive Bladder. *Journal of Urology*, 175 (5). doi:10.1016/S0022-5347(05)00992-4.

Kim, J.K., Lee, Y.G., Han, K., et al. (2021) Obesity, metabolic health, and urological disorders in adults: a nationwide population-based study. *Scientific Reports*,

11 (1). doi:10.1038/s41598-021-88165-z.

Kim, S.D. (2010) Isolation, structure and cholesterol esterase inhibitory activity of a polysaccharide, PS-A, from cordyceps sinensis. *Journal of Applied Biological Chemistry*, 53 (6). doi:10.3839/jksabc.2010.118.

Kim, S.J., Ha, M.S., Choi, E.Y., et al. (2005b) Nitric oxide production and inducible nitric oxide synthase expression induced by Prevotella nigrescens lipopolysaccharide. *FEMS Immunology and Medical Microbiology*, 43 (1). doi:10.1016/j.femsim.2004.07.001.

Kimble, J. and Ward, S. (1988) "Germ-line development and fertilisation." In Wood, W.B. (ed.) *The nematode Caenorhabditis elegans*. Cold Spring Harbor Monograph Series 17. pp. 191–214.

Kirschner-Hermanns, R., Daneshgari, F., Vahabi, B., et al. (2012) Does diabetes mellitus-induced bladder remodeling affect lower urinary tract function?: ICI-RS 2011. *Neurourology and Urodynamics*. 31 (3). doi:10.1002/nau.22228.

Kitta, T., Chancellor, M.B., de Groat, W.C., et al. (2014) Roles of adenosine A1 and A2A receptors in the control of micturition in rats. *Neurourology and Urodynamics*, 33 (8): 1259–1265. doi:10.1002/nau.22487.

Kittimongkolsuk, P., Roxo, M., Li, H., et al. (2021) Extracts of the tiger milk mushroom (Lignosus rhinocerus) enhance stress resistance and extend lifespan in caenorhabditis elegans via the daf-16/foxo signaling pathway. *Pharmaceuticals*, 14 (2). doi:10.3390/ph14020093.

Klass, M.R. (1977) Aging in the nematode Caenorhabditis elegans: Major biological and environmental factors influencing life span. *Mechanisms of Ageing and Development*, 6 (C). doi:10.1016/0047-6374(77)90043-4.

Ko, K.M. and Leung, H.Y. (2007) Enhancement of ATP generation capacity, antioxidant activity and immunomodulatory activities by Chinese Yang and Yin tonifying herbs. *Chinese Medicine*. 2. doi:10.1186/1749-8546-2-3.

Kong, B.H., Yap, C.S.A., Razif, M.F.M., et al. (2021) Antioxidant and cytotoxic effects and identification of ophiocordyceps sinensis bioactive proteins using shotgun proteomic analysis. *Food Technology and Biotechnology*, 59 (2). doi:10.17113/ftb.59.02.21.7151.

Krishnan, P., Lee, F.K., Yap, V.A., et al. (2020) Schwarzinicines A-G, 1,4-Diarylbutanoid-Phenethylamine Conjugates from the Leaves of Ficus schwarzii. *Journal of Natural Products*, 83 (1). doi:10.1021/acs.jnatprod.9b01160.

Kubota, Y., Kojima, Y., Shibata, Y., et al. (2011) Role of KIT-positive interstitial cells of Cajal in the urinary bladder and possible therapeutic target for overactive bladder. *Advances in Urology*. doi:10.1155/2011/816342.

Kügelgen, I. von (2008) Pharmacology of mammalian P2X- and P2Y-receptors. *Biotrend Reviews*, 3 (3).

Kullmann, F.A., Daugherty, S.L., de Groat, W.C., et al. (2014) Bladder smooth muscle strip contractility as a method to evaluate lower urinary tract pharmacology. *Journal of Visualized Experiments*, (90). doi:10.3791/51807.

Kume, H., Mikawa, K., Takagi, K., et al. (1995) Role of G proteins and KCa channels in the muscarinic and beta-adrenergic regulation of airway smooth muscle. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 268 (2): L221–L229. doi:10.1152/ajplung.1995.268.2.L221.

Kuo, Y.C., Tsai, W.J., Wang, J.Y., et al. (2001) Regulation of bronchoalveolar lavage fluids cell function by the immunomodulatory agents from Cordyceps sinensis. *Life Sciences*, 68 (9). doi:10.1016/S0024-3205(00)01011-0.

Kwok, T.C.Y., Ricker, N., Fraser, R., et al. (2006) A small-molecule screen in C. elegans yields a new calcium channel antagonist. *Nature*, 441 (1). doi:10.1038/nature04657.

Lai, C.H., Chou, C.Y., Ch'ang, L.Y., et al. (2000) Identification of novel human genes evolutionarily conserved in Caenorhabditis elegans by comparative proteomics. *Genome Research*, 10 (5). doi:10.1101/gr.10.5.703.

Lai, H., Tan, B., Liang, Z., et al. (2015) Effect of the Chinese traditional prescription Suo Quan Wan on TRPV1 expression in the bladder of rats with bladder outlet obstruction. *BMC Complementary and Alternative Medicine*. doi:10.1186/s12906-015-0898-7.

Lapierre, L.R. and Hansen, M. (2012) Lessons from C. elegans: Signaling pathways for longevity. *Trends in Endocrinology and Metabolism*. 23 (12).

doi:10.1016/j.tem.2012.07.007.

Lapointe, É., Singbo, N., Naud, É., et al. (2022) First North American experience of propiverine use in children with overactive bladder. *Canadian Urological Association Journal*, 16 (10). doi:10.5489/cuaj.7811.

Lau, B.F., Abdullah, N. and Aminudin, N. (2013) Chemical Composition of the Tiger's Milk Mushroom , *Lignosus rhinocerus*. *Journal of Agricultural and Food Chemistry*, 61 (20).

Lau, B.F., Abdullah, N., Aminudin, N., et al. (2015) Ethnomedicinal uses, pharmacological activities, and cultivation of Lignosus spp. (tigeŕs milk mushrooms) in Malaysia - A review. *Journal of Ethnopharmacology*. 169. doi:10.1016/j.jep.2015.04.042.

Lee, J., Kwon, G. and Lim, Y.H. (2015) Elucidating the Mechanism of Weissella-dependent Lifespan Extension in *Caenorhabditis elegans*. *Scientific Reports*, 5. doi:10.1038/srep17128.

Lee, M.K., Li, X., Yap, A.C.S., et al. (2018a) Airway relaxation effects of water-soluble sclerotial extract from *Lignosus rhinocerotis*. *Frontiers in Pharmacology*, 9 (MAY): 461. doi:10.3389/fphar.2018.00461.

Lee, M.K., Lim, K.H., Millns, P., et al. (2018b) Bronchodilator effects of *Lignosus rhinocerotis* extract on rat isolated airways is linked to the blockage of calcium entry. *Phytomedicine*, 42 (2018): 172–179. doi:10.1016/j.phymed.2018.03.025.

Lee, S., Rose'meyer, R., McDermott, C., et al. (2018c) Diabetes-induced alterations in urothelium function: Enhanced ATP release and nerve-evoked contractions in the streptozotocin rat bladder. *Clinical and Experimental Pharmacology and Physiology*, 45 (11). doi:10.1111/1440-1681.13003.

Leiria, L.O.S., Mõnica, F.Z.T., Carvalho, F.D.G.F., et al. (2011) Functional, morphological and molecular characterization of bladder dysfunction in streptozotocin-induced diabetic mice: Evidence of a role for L-type voltage-operated Ca^{2+} channels. *British Journal of Pharmacology*, 163 (6): 1276–1288. doi:10.1111/j.1476-5381.2011.01311.x.

Leron, E., Weintraub, A.Y., Mastrolia, S.A., et al. (2018) Overactive Bladder

Syndrome: Evaluation and Management. *Current Urology*. 11 (3). doi:10.1159/000447205.

Li, M., Sun, Y., Simard, J.M., et al. (2011a) Increased transient receptor potential vanilloid type 1 (TRPV1) signaling in idiopathic overactive bladder urothelial cells. *Neurourology and Urodynamics*, 30 (4). doi:10.1002/nau.21045.

Li, S.P., Li, P., Dong, T.T.X., et al. (2001) Anti-oxidation activity of different types of natural *Cordyceps sinensis* and cultured Cordyceps mycelia. *Phytomedicine*, 8 (3): 207–212. doi:10.1078/0944-7113-00030.

Li, S.P., Zhao, K.J., Ji, Z.N., et al. (2003) A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. *Life Sciences*, 73 (19): 2503–2513. doi:10.1016/S0024-3205(03)00652-0.

Li, X., Liu, Q., Li, W., et al. (2019) A breakthrough in the artificial cultivation of Chinese cordyceps on a large-scale and its impact on science, the economy, and industry. *Critical Reviews in Biotechnology*, 39 (2): 181–191. doi:10.1080/07388551.2018.1531820.

Li, Y., Wang, X.L., Jiao, L., et al. (2011b) A survey of the geographic distribution of *Ophiocordyceps sinensis*. *Journal of Microbiology*, 49 (6): 913–919. doi:10.1007/s12275-011-1193-z.

Li, Y., Xue, W.J., Tian, P.X., et al. (2009) Clinical Application of *Cordyceps sinensis* on Immunosuppressive Therapy in Renal Transplantation. *Transplantation Proceedings*, 41 (5): 1565–1569. doi:10.1016/j.transproceed.2009.02.085.

Li, Z., Cogswell, M., Hixson, K., et al. (2018) Nuclear Respiratory Factor 1 (NRF-1) Controls the Activity Dependent Transcription of the GABA-A Receptor Beta 1 Subunit Gene in Neurons. *Frontiers in Molecular Neuroscience*, 11. doi:10.3389/fnmol.2018.00285.

Li, Z., Zhang, Z., Ren, Y., et al. (2021) Aging and age-related diseases: from mechanisms to therapeutic strategies. *Biogerontology*. 22 (2). doi:10.1007/s10522-021-09910-5.

Liang, F. (2013) *Method for preparing Chinese traditional medicine preparation for treating frequent urination at night through Cordyceps Sinensis*

(CN103655729A)

Liao, Y., Du, X., Fu, Y., et al. (2022) Mechanism of traditional Chinese medicine in treating overactive bladder. *International Urology and Nephrology*, 55 (3): 489–501. doi:10.1007/s11255-022-03434-8.

Lin, B.Q. and Li, S.P. (2011) "Cordyceps as an herbal drug." In *Herbal Medicine: Biomolecular and Clinical Aspects: Second Edition*. pp. 73–105. doi:10.1201/b10787-6.

Lin, C.C., Pumsanguan, W., Koo, M.M.O., et al. (2007) Radiation protective effects of cordyceps sinensis in blood cells. *Tzu Chi Medical Journal*, 19 (4). doi:10.1016/S1016-3190(10)60020-1.

Liu, W., Lin, H., Mao, Z., et al. (2020) Verapamil extends lifespan in Caenorhabditis elegans by inhibiting calcineurin activity and promoting autophagy. *Aging*, 12 (6). doi:10.18632/aging.102951.

Longhurst, P.A. and Levendusky, M. (1999) Pharmacological characterization of β-adrenoceptors mediating relaxation of the rat urinary bladder in vitro. *British Journal of Pharmacology*, 127 (7). doi:10.1038/sj.bjp.0702709.

Loong, B.J., Tan, J.H., Lim, K.H., et al. (2015) Contractile function of smooth muscle retained after overnight storage. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 388 (10): 1061–1067. doi:10.1007/s00210-015-1140-3.

Lopes, A.P., Bagatela, B.S., Rosa, P.C.P., et al. (2013) Antioxidant and cytotoxic effects of crude extract, fractions and 4-nerolidylcathecol from aerial parts of *Pothomorphe umbellata* L. (Piperaceae). *BioMed Research International*, 2013. doi:10.1155/2013/206581.

Lu, W.J., Chang, N.C., Jayakumar, T., et al. (2014) Ex vivo and in vivo studies of CME-1, a novel polysaccharide purified from the mycelia of *Cordyceps sinensis* that inhibits human platelet activation by activating adenylate cyclase/cyclic AMP. *Thrombosis Research*, 134 (6): 1301–1310. doi:10.1016/j.thromres.2014.09.023.

Luitel, H., Novoyatleva, T., Sydykov, A., et al. (2020) Yarsagumba is a Promising Therapeutic Option for Treatment of Pulmonary Hypertension due to the Potent Anti-Proliferative and Vasorelaxant Properties. *Medicina*, 56 (3): 131.

doi:10.3390/medicina56030131.

Luo, Y., Yang, S.K., Zhou, X., et al. (2015) Use of *Ophiocordyceps sinensis* (syn. *Cordyceps sinensis*) combined with angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blockers (ARB) versus ACEI/ARB alone in the treatment of diabetic kidney disease: A meta-analysis. *Renal Failure*, 37 (4). doi:10.3109/0886022X.2015.1009820.

Lychkova, A.E. and Pavone, L.M. (2013) Role of serotonin receptors in regulation of contractile activity of urinary bladder in rabbits. *Urology*, 81 (3). doi:10.1016/j.urology.2012.11.029.

Ma, M. wei, Gao, X. shu, Yu, H. liang, et al. (2018) Cordyceps sinensis Promotes the Growth of Prostate Cancer Cells. *Nutrition and Cancer*, 70 (7): 1166–1172. doi:10.1080/01635581.2018.1504091.

Ma, X., Xu, L., Gavrilova, O., et al. (2014) Role of forkhead box protein A3 in age-associated metabolic decline. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (39). doi:10.1073/pnas.1407640111.

Maggi, C.A., Giuliani, S., Patacchini, R., et al. (1989) Multiple sources of calcium for contraction of the human urinary bladder muscle. *British Journal of Pharmacology*. doi:10.1111/j.1476-5381.1989.tb14634.x.

Mak, Y., Loong, B., Millns, P., et al. (2022) Schwarzinicine A inhibits transient receptor potential canonical channels and exhibits overt vasorelaxation effects. *Phytotherapy Research*, 36 (7): 2952–2963. doi:10.1002/ptr.7489.

Mamta, Mehrotra, S., Amitabh, et al. (2015) Phytochemical and antimicrobial activities of Himalayan *Cordyceps sinensis* (Berk.) Sacc. *Indian Journal of Experimental Biology*, 53 (1): 36–43.

Mansfield, K.J., Chen, Z., Moore, K.H., et al. (2022) Urinary Tract Infection in Overactive Bladder: An Update on Pathophysiological Mechanisms. *Frontiers in Physiology*, 13. doi:10.3389/fphys.2022.886782.

Maryon, E.B., Coronado, R. and Anderson, P. (1996) unc-68 encodes a ryanodine receptor involved in regulating *C. elegans* body-wall muscle contraction. *Journal of Cell Biology*, 134 (4). doi:10.1083/jcb.134.4.885.

Masumori, N., Miyamoto, S., Tsukamoto, T., et al. (2011) The Efficacy and Safety of Propiverine Hydrochloride in Patients with Overactive Bladder Symptoms Who Poorly Responded to Previous Anticholinergic Agents. *Advances in Urology*, 2011: 1–4. doi:10.1155/2011/714978.

Matsui, M., Motomura, D., Fujikawa, T., et al. (2002) Mice lacking M_2 and M_3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. *Journal of Neuroscience*, 22 (24). doi:10.1523/jneurosci.22-24-10627.2002.

Matsumoto-Miyai, K., Yamada, E., Shinzawa, E., et al. (2016) Serotonergic regulation of distention-induced ATP release from the urothelium. *American Journal of Physiology - Renal Physiology*, 310 (7). doi:10.1152/ajprenal.00024.2015.

Matsumoto-Miyai, K., Yoshizumi, M. and Kawatani, M. (2015) Regulatory Effects of 5-Hydroxytryptamine Receptors on Voiding Function. *Advances in Therapy*. 32. doi:10.1007/s12325-015-0240-2.

Mattiasson, A., Masala, A., Morton, R., et al. (2010) Efficacy of simplified bladder training in patients with overactive bladder receiving a solifenacin flexible-dose regimen: Results from a randomized study. *BJU International*, 105 (8). doi:10.1111/j.1464-410X.2009.08910.x.

Mbaki, Y. and Ramage, A.G. (2008) Investigation of the role of 5-HT 2 receptor subtypes in the control of the bladder and the urethra in the anaesthetized female rat. *British Journal of Pharmacology*, 155 (3). doi:10.1038/bjp.2008.273.

McFadzean, I. and Gibson, A. (2002) The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. *British Journal of Pharmacology*. 135 (1). doi:10.1038/sj.bjp.0704468.

Mei, Y.X., Yang, W., Zhu, P.X., et al. (2014) Isolation, Characterization, and Antitumor Activity of a Novel Heteroglycan from Cultured Mycelia of *Cordyceps sinensis*. *Planta Medica*, 80 (13): 1107–1112. doi:10.1055/s-0034-1382960.

Menendez, C., Jimenez, R., Moreno, L., et al. (2011) Lack of synergistic interaction between quercetin and catechin in systemic and pulmonary vascular smooth

muscle. *British Journal of Nutrition*, 105 (9). doi:10.1017/S0007114510004952.

Meng, En, Lin Wei-Yu, Lee Wei-Chia, C.Y.-C. (2012) Pathophysiology of overactive bladder. *Lower urinary tract symptoms*, 4 (4): 48–55. doi:10.1007/s00192-012-1682-6.

Michel, M.C. and Sand, C. (2009) Effect of pre-contraction on β-adrenoceptor-mediated relaxation of rat urinary bladder. *World Journal of Urology*, 27 (6): 711–715. doi:10.1007/s00345-009-0416-y.

Michel, M.C. and Vrydag, W. (2006) "α 1-, α 2- and β-adrenoceptors in the urinary bladder, urethra and prostate." In *British Journal of Pharmacology*. 2006. doi:10.1038/sj.bjp.0706619.

Mishra, J., Rajput, R., Singh, K., et al. (2019) Antioxidant-rich peptide fractions derived from high-altitude chinese caterpillar medicinal mushroom *Ophiocordyceps sinensis* (Ascomycetes) inhibit bacterial pathogens. *International Journal of Medicinal Mushrooms*, 21 (2). doi:10.1615/IntJMedMushrooms.2019030013.

Mitidieri, E., Pecoraro, A., Esposito, E., et al. (2022) β3 Relaxant Effect in Human Bladder Involves Cystathionine γ-Lyase-Derived Urothelial Hydrogen Sulfide. *Antioxidants*, 11 (8): 1480. doi:10.3390/antiox11081480.

Mizuno, N. and Itoh, H. (2009) Functions and regulatory mechanisms of Gq-signaling pathways. *NeuroSignals*. 17 (1). doi:10.1159/000186689.

Moga, D.C., Abner, E.L., Wu, Q., et al. (2017) Bladder antimuscarinics and cognitive decline in elderly patients. *Alzheimer's and Dementia: Translational Research and Clinical Interventions*. doi:10.1016/j.trci.2017.01.003.

Mónica, F.Z.T., Bricola, A.A.O., Báu, F.R., et al. (2008) Long-term nitric oxide deficiency causes muscarinic supersensitivity and reduces β 3-adrenoceptor-mediated relaxation, causing rat detrusor overactivity. *British Journal of Pharmacology*. doi:10.1038/bjp.2008.39.

Montgomery, B.S.I. and Fry, C.H. (1992) The action potential and net membrane currents in isolated human detrusor smooth muscle cells. *Journal of Urology*, 147 (1). doi:10.1016/S0022-5347(17)37192-6.

Moon, A. (2002) Influence of nitric oxide signalling pathways on pre-contracted human detrusor smooth muscle in vitro. *BJU International*, 89 (9): 942–949. doi:10.1046/j.1464-410X.2002.02795.x.

Moritoki, H., Hisayama, T., Kondoh, W., et al. (1994) Thapsigargin, A Ca^{2+}-ATPase inhibitor, relaxes rat aorta via nitric oxide formation. *Life Sciences*, 54 (9). doi:10.1016/0024-3205(94)00875-2.

Moro, C., Leeds, C. and Chess-Williams, R. (2012) Contractile activity of the bladder urothelium/lamina propria and its regulation by nitric oxide. *European Journal of Pharmacology*, 674 (2–3). doi:10.1016/j.ejphar.2011.11.020.

Munoz, A., Gangitano, D.A., Smith, C.P., et al. (2010) Removal of urothelium affects bladder contractility and release of ATP but not release of NO in rat urinary bladder. *BMC Urology*, 10 (10). doi:10.1186/1471-2490-10-10.

Murphy, M.E. and Brayden, J.E. (1995) Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *The Journal of Physiology*, 486 (1). doi:10.1113/jphysiol.1995.sp020789.

Nakagomi, H., Yoshiyama, M., Mochizuki, T., et al. (2016) Urothelial ATP exocytosis: Regulation of bladder compliance in the urine storage phase. *Scientific Reports*. 6. doi:10.1038/srep29761.

Nakamura, K., Konoha, K., Yamaguchi, Y., et al. (2003) Combined effects of Cordyceps sinensis and methotrexate on hematogenic lung metastasis in mice. *Receptors and Channels*, 9 (5): 329–334. doi:10.3109/713745176.

Nallathamby, N., Phan, C.W., Seow, S.L.S., et al. (2018) A status review of the bioactive activities of tiger milk mushroom Lignosus rhinocerotis (Cooke) Ryvarden. *Frontiers in Pharmacology*. doi:10.3389/fphar.2017.00998.

Nardulli, R., Losavio, E., Ranieri, M., et al. (2012) Combined Antimuscarinics for Treatment of Neurogenic Overactive Bladder. *International Journal of Immunopathology and Pharmacology*, 25. doi:10.1177/03946320120250S106.

National Institute For Health and Care and Excellence (2019) *Urinary incontinence and pelvic organ prolapse in women: management*. Available at: https://www.nice.org.uk/guidance/ng123 (Accessed: 8 March 2023).

Ng, M.J., Kong, B.H., Teoh, K.H., et al. (2023) In vivo anti-tumor activity of Lignosus

rhinocerus TM02® using a MCF7-xenograft NCr nude mice model. *Journal of Ethnopharmacology*, 304: 115957. doi:10.1016/j.jep.2022.115957.

Nguyen, Q.V., Vu, T.T., Tran, M.T., et al. (2021) Antioxidant Activity and Hepatoprotective Effect of Exopolysaccharides From Cultivated Ophiocordyceps Sinensis Against CCl4-Induced Liver Damages. *Natural Product Communications*, 16 (2). doi:10.1177/1934578X21997670.

Nomiya, M. and Yamaguchi, O. (2003) A quantitative analysis of mRNA expression of α1 and β-adrenoceptor subtypes and their functional roles in human normal and obstructed bladders. *Journal of Urology*, 170 (2 I). doi:10.1097/01.ju.0000067621.62736.7c.

Ognenovska, S., Chen, Z., Mukerjee, C., et al. (2021) Bacterial colonization of bladder urothelial cells in women with refractory Detrusor Overactivity: The effects of antibiotic therapy. *Pathogens and Disease*, 79 (6). doi:10.1093/femspd/ftab031.

Oh, S.J., Ahn, S.C., Kim, S.J., et al. (1999) Carbachol-induced sustained tonic contraction of rat detrusor muscle. *BJU International*. doi:10.1046/j.1464-410x.1999.00122.x.

Ohi, Y., Yamamura, H., Nagano, N., et al. (2001) Local Ca^{2+} transients and distribution of BK channels and ryanodine receptors in smooth muscle cells of guinea-pig vas deferens and urinary bladder. *Journal of Physiology*, 534 (2). doi:10.1111/j.1469-7793.2001.t01-3-00313.x.

Oikonomou, G. and Shaham, S. (2011) The glia of caenorhabditis elegans. *GLIA*, 59 (9). doi:10.1002/glia.21084.

Oki, T., Suzuki, M., Nishioka, Y., et al. (2005) Effects of saw palmetto extract on micturition reflex of rats and its autonomic receptor binding activity. *Journal of Urology*, 173 (4). doi:10.1097/01.ju.0000146273.26591.f5.

O'Reilly, B.A., Kosaka, A.H., Knight, G.F., et al. (2002) P2X receptors and their role in female idiopathic detrusor instability. *Journal of Urology*, 167 (1). doi:10.1016/S0022-5347(05)65403-1.

Oreščanin, Z. and Milovanović, S.R. (2006) Effect of L-arginine on the relaxation caused by sodium nitroprusside on isolated rat renal artery. *Acta Physiologica*

Hungarica, 93 (4): 271–283. doi:10.1556/APhysiol.93.2006.4.3.

Ortiz, B., Hincks, J., Vayndorf, E., et al. (2018) Pharyngeal Pumping in the Nematode C. elegans : Resolving Treatment Effects through Variability Assessment . *The FASEB Journal*, 32 (S1). doi:10.1096/fasebj.2018.32.1_supplement.862.5.

Painter, C.E. and Suskind, A.M. (2019) Advances in Pharmacotherapy for the Treatment of Overactive Bladder. *Current Bladder Dysfunction Reports*. 14 (4). doi:10.1007/s11884-019-00562-3.

Panda, A.K. and Swain, K.C. (2011) Traditional uses and medicinal potential of Cordyceps sinensis of Sikkim. *Journal of Ayurveda and Integrative Medicine*, 2 (1): 9–13. doi:10.4103/0975-9476.78183.

Park, J.S., Jung, H. Do, Cho, Y.S., et al. (2018) Neonatal bladder irritation is associated with vanilloid receptor TRPV1 expression in adult rats. *International Neurourology Journal*, 22 (3): 169–176. doi:10.5213/inj.1836020.101.

Patacchini, R., Santicioli, P., Giuliani, S., et al. (2005) Pharmacological investigation of hydrogen sulfide (H_2S) contractile activity in rat detrusor muscle. *European Journal of Pharmacology*, 509 (2–3). doi:10.1016/j.ejphar.2005.01.005.

Patwardhan, B. and Mashelkar, R.A. (2009) Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward? *Drug Discovery Today*. 14 (15–16). doi:10.1016/j.drudis.2009.05.009.

Pauzi, N.A., Mohamad, N., Azzam-Sayuti, M., et al. (2020) Antibiotic susceptibility and pathogenicity of Aeromonas hydrophila isolated from red hybrid tilapia (*Oreochromis niloticus*×*Oreochromis mossambicus*) in Malaysia. *Veterinary World*, 13 (10): 2166–2171. doi:10.14202/vetworld.2020.2166-2171.

Peng, H.H., Wu, C.Y., Hsiao, Y.C., et al. (2021) Ganoderma Lucidum Stimulates Autophagy-Dependent Longevity Pathways in *Caenorhabditis Elegans* and Human Cells. *Aging*, 13 (10). doi:10.18632/aging.203068.

Peng, Y., Tao, Y., Wang, Q., et al. (2014) Ergosterol is the active compound of cultured mycelium cordyceps sinensis on antiliver fibrosis. *Evidence-based Complementary and Alternative Medicine*, 2014. doi:10.1155/2014/537234.

Peralta, C., Rull, R., Rimola, A., et al. (2001) Endogenous nitric oxide and exogenous

nitric oxide supplementation in hepatic ischemia-reperfusion injury in the rat. *Transplantation*, 71 (4). doi:10.1097/00007890-200102270-00008.

Perez-Zoghbi, J.F., Bai, Y. and Sanderson, M.J. (2010) Nitric oxide induces airway smooth muscle cell relaxation by decreasing the frequency of agonist-induced Ca^{2+} oscillations. *Journal of General Physiology*, 135 (3). doi:10.1085/jgp.200910365.

Persson, K. and Andersson, K. -E (1992) Nitric oxide and relaxation of pig lower urinary tract. *British Journal of Pharmacology*, 106 (2). doi:10.1111/j.1476-5381.1992.tb14349.x.

Persyn, S., De Wachter, S., Wyndaele, J.J., et al. (2016) The actions of isoprenaline and mirabegron in the isolated whole rat and guinea pig bladder. *Autonomic Neuroscience: Basic and Clinical*, 198. doi:10.1016/j.autneu.2016.05.006.

Peters, S.L.M., Schmidt, M. and Michel, M.C. (2006) Rho kinase: a target for treating urinary bladder dysfunction? *Trends in Pharmacological Sciences*. 27 (9). doi:10.1016/j.tips.2006.07.002.

Petkov, G. V. (2012) Role of potassium ion channels in detrusor smooth muscle function and dysfunction. *Nature Reviews Urology*. 9 (1). doi:10.1038/nrurol.2011.194.

Petkov, G. V. (2014) Central role of the BK channel in urinary bladder smooth muscle physiology and pathophysiology. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 307 (6): R571–R584. doi:10.1152/ajpregu.00142.2014.

Petrella, L.N. (2014) Natural variants of C. elegans demonstrate defects in both sperm function and oogenesis at elevated temperatures. *PLoS ONE*, 9 (11). doi:10.1371/journal.pone.0112377.

Pfeiffer, S., Leopold, E., Schmidt, K., et al. (1996) Inhibition of nitric oxide synthesis by NG-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. *British Journal of Pharmacology*, 118 (6): 1433–1440. doi:10.1111/j.1476-5381.1996.tb15557.x.

Pirintsos, S., Panagiotopoulos, A., Bariotakis, M., et al. (2022) From Traditional Ethnopharmacology to Modern Natural Drug Discovery: A Methodology

Discussion and Specific Examples. *Molecules*, 27 (13): 4060. doi:10.3390/molecules27134060.

Poley, R.N., Dosier, C.R., Speich, J.E., et al. (2008) Stimulated calcium entry and constitutive RhoA kinase activity cause stretch-induced detrusor contraction. *European Journal of Pharmacology*, 599 (1–3). doi:10.1016/j.ejphar.2008.09.045.

Qi, W., Yan, Y.B., Lei, W., et al. (2012) Prevention of disuse osteoporosis in rats by Cordyceps sinensis extract. *Osteoporosis International*, 23 (9). doi:10.1007/s00198-011-1842-4.

Qi, W., Zhang, Y., Yan, Y.B., et al. (2013) The protective effect of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*, on diabetic osteopenia in alloxan-induced diabetic rats. *Evidence-based Complementary and Alternative Medicine*, 2013. doi:10.1155/2013/985636.

Qian, G.M., Pan, G.F. and Guo, J.Y. (2012) Anti-inflammatory and antinociceptive effects of cordymin, a peptide purified from the medicinal mushroom Cordyceps sinensis. *Natural Product Research*, 26 (24): 2358–2362. doi:10.1080/14786419.2012.658800.

Qian, J. and Fulton, D.J.R. (2012) Exogenous, but not endogenous nitric oxide inhibits adhesion molecule expression in human endothelial cells. *Frontiers in Physiology*, 3 JAN. doi:10.3389/fphys.2012.00003.

Rahman, M.A., Abdullah, N. and Aminudin, N. (2017) Corroborative assessment of mushroom as the graceful ageing and lifespan promoting agent. *Biointerface Research in Applied Chemistry*, 7 (3).

Raizen, D., Song, B.M., Trojanowski, N., et al. (2012) Methods for measuring pharyngeal behaviors. *WormBook : the online review of C. elegans biology*. doi:10.1895/wormbook.1.154.1.

Ramage, A.G. (2006) "The role of central 5-hydroxytryptamine (5-HT, serotonin) receptors in the control of micturition." In *British Journal of Pharmacology*. 2006. doi:10.1038/sj.bjp.0706504.

Ratz, P.H., Berg, K.M., Urban, N.H., et al. (2005) Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *American Journal*

of Physiology - Cell Physiology. 288 (4 57-4). doi:10.1152/ajpcell.00529.2004.

Recio, P., Barahona, M.V., Orensanz, L.M., et al. (2009) 5-hydroxytryptamine induced relaxation in the pig urinary bladder neck. *British Journal of Pharmacology*, 157 (2). doi:10.1111/j.1476-5381.2009.00144.x.

Ren, L., Hemar, Y., Perera, C.O., et al. (2014) Antibacterial and antioxidant activities of aqueous extracts of eight edible mushrooms. *Bioactive Carbohydrates and Dietary Fibre*, 3 (2). doi:10.1016/j.bcdf.2014.01.003.

Reynolds, W.S., Fowke, J. and Dmochowski, R. (2016) The Burden of Overactive Bladder on US Public Health. *Current Bladder Dysfunction Reports*. 11 (1). doi:10.1007/s11884-016-0344-9.

Richards, T.R. and Tobe, S.W. (2014) Combining other antihypertensive drugs with β-blockers in hypertension: A focus on safety and tolerability. *Canadian Journal of Cardiology*. 30 (5 S). doi:10.1016/j.cjca.2013.08.012.

Rico Lemus, M. (2014) SNP as an Effective Donor of Nitric Oxide for in vitro Plant Cell and Tissue Culture. *Journal of Plant Biochemistry & Physiology*, 02 (02). doi:10.4172/2329-9029.1000e127.

Rieckher, M. and Tavernarakis, N. (2017) Caenorhabditis elegans Microinjection. *Bio-protocol*, 7 (19).

Rivera, L. and Brading, A.F. (2006) The role of Ca^{2+} influx and intracellular Ca^{2+} release in the muscarinic-mediated contraction of mammalian urinary bladder smooth muscle. *BJU International*, 98 (4). doi:10.1111/j.1464-410X.2006.06431.x.

Roedel, M., Ravens, U., Kasper, M., et al. (2018) Contractile responses in intact and mucosa-denuded human ureter—a comparison with urinary bladder detrusor preparations. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 391 (8). doi:10.1007/s00210-018-1505-5.

Rohrmann, D. (2004) "Calcium metabolism and detrusor failure in the aging bladder." In *The Aging Bladder*. doi:10.1007/978-3-7091-0631-0_6.

Rohrmann, S., Smit, E., Giovannucci, E., et al. (2005) Association between markers of the metabolic syndrome and lower urinary tract symptoms in the Third National Health and Nutrition Examination Survey (NHANES III).

International Journal of Obesity, 29 (3). doi:10.1038/sj.ijo.0802881.

Roosen, A., Datta, S.N., Chowdhury, R.A., et al. (2009) Suburothelial Myofibroblasts in the Human Overactive Bladder and the Effect of Botulinum Neurotoxin Type A Treatment. *European Urology*, 55 (6). doi:10.1016/j.eururo.2008.11.009.

Root, J.A., Davey, D.A. and Af Forselles, K.J. (2015) Prostanoid receptors mediating contraction in rat, macaque and human bladder smooth muscle in vitro. *European Journal of Pharmacology*, 769. doi:10.1016/j.ejphar.2015.11.030.

Russell, J.C., Burnaevskiy, N., Ma, B., et al. (2019) Electrophysiological measures of aging pharynx function in *C. elegans* reveal enhanced organ functionality in older, long-lived mutants. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 74 (8). doi:10.1093/gerona/glx230.

Rutkowska, M., Olszewska, M.A., Kolodziejczyk-Czepas, J., et al. (2019) Sorbus domestica Leaf Extracts and Their Activity Markers: Antioxidant potential and synergy effects in scavenging assays of multiple oxidants. *Molecules*, 24 (12). doi:10.3390/molecules24122289.

Sabaratnam, V., Kah-Hui, W., Naidu, M., et al. (2013) Neuronal health - Can culinary and medicinal mushrooms help? *Journal of Traditional and Complementary Medicine*, 3 (1). doi:10.4103/2225-4110.106549.

Sachdeva, S. and Gupta, M. (2013) Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharmaceutical Journal*. 21 (3). doi:10.1016/j.jsps.2012.05.011.

Saito, M., Ohmasa, F., Tsounapi, P., et al. (2012) Nicorandil ameliorates hypertension-related bladder dysfunction in the rat. *Neurourology and Urodynamics*, 31 (5). doi:10.1002/nau.21213.

Sakai, T., Kasahara, K.I., Tomita, K.I., et al. (2013) 5-hydroxytryptamine-induced bladder hyperactivity via the 5-HT2A receptor in partial bladder outlet obstruction in rats. *American Journal of Physiology - Renal Physiology*, 304 (7). doi:10.1152/ajprenal.00365.2012.

Salman, M., Khan, A.H., Syed Sulaiman, S.A., et al. (2017) Effect of Calcium Channel Blockers on Lower Urinary Tract Symptoms: A Systematic Review. *BioMed*

Research International, 2017. doi:10.1155/2017/4269875.

Sam, P. and LaGrange, C.A. (2019) *Anatomy, Abdomen and; Pelvis, Bladder Detrusor Muscle*. In StatPearls [Internet]. Treasure Island (FL) (Updated 2022 Aug 8). Available from https://www.ncbi.nlm.nih.gov/books/NBK482181/

Sanchez, L.S., de La Monte, S.M., Filippov, G., et al. (1998) Cyclic-GMP-binding, cyclic-GMP-specific phosphodiesterase (PDE5) gene expression is regulated during rat pulmonary development. *Pediatric Research*, 43 (2). doi:10.1203/00006450-199802000-00002.

Sanders, K.M. and Ward, S.M. (1992) Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 262 (3 25-3). doi:10.1152/ajpgi.1992.262.3.g379.

Sándor, Z., Mottaghipisheh, J., Veres, K., et al. (2018) Evidence supports tradition: The in vitro effects of Roman Chamomile on smooth muscles. *Frontiers in Pharmacology*, 9 (APR). doi:10.3389/fphar.2018.00323.

Sang, Q., Pan, Y., Jiang, Z., et al. (2020) HPLC determination of massoia lactone in fermented Cordyceps sinensis mycelium Cs-4 and its anticancer activity in vitro. *Journal of Food Biochemistry*, 44 (9). doi:10.1111/jfbc.13336.

Sanhueza, L., Melo, R., Montero, R., et al. (2017) Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against Staphylococcus aureus and Escherichia coli. *PLoS ONE*, 12 (2). doi:10.1371/journal.pone.0172273.

Santicioli, P., Maggi, C.A. and Meli, A. (1983) The Effects of Clonidine on Electrically-Induced Contractions of Rat Detrusor Strips in vitro. *Journal of Autonomic Pharmacology*, 3 (3). doi:10.1111/j.1474-8673.1983.tb00531.x.

Schneider, T., Fetscher, C., Krege, S., et al. (2004) Signal transduction underlying carbachol-induced contraction of human urinary bladder. *Journal of Pharmacology and Experimental Therapeutics*, 309 (3). doi:10.1124/jpet.103.063735.

Scholz, M., Lynch, D.J., Lee, K.S., et al. (2016) A scalable method for automatically measuring pharyngeal pumping in C. elegans. *Journal of Neuroscience*

Methods, 274. doi:10.1016/j.jneumeth.2016.07.016.

Sengiku, A., Ueda, M., Kono, J., et al. (2018) Circadian coordination of ATP release in the urothelium via connexin43 hemichannels. *Scientific Reports*, 8 (1). doi:10.1038/s41598-018-20379-0.

Shieh, C.C., Feng, J., Buckner, S.A., et al. (2001) Functional implication of spare ATP-sensitive K^+ channels in bladder smooth muscle cells. *Journal of Pharmacology and Experimental Therapeutics*, 296 (3).

Shioyama, R., Aoki, Y., Ito, H., et al. (2008) Long-lasting breaches in the bladder epithelium lead to storage dysfunction with increase in bladder PGE2 levels in the rat. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*. doi:10.1152/ajpregu.00788.2007.

Shtonda, B. and Avery, L. (2005) CCA-1, EGL-19 and EXP-2 currents shape action potentials in the *Caenorhabditis elegans* pharynx. *Journal of Experimental Biology*, 208 (11). doi:10.1242/jeb.01615.

Singh, A., Hildebrand, M.E., Garcia, E., et al. (2010) The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels. *British Journal of Pharmacology*, 160 (6). doi:10.1111/j.1476-5381.2010.00786.x.

Singh, M., Tulsawani, R., Koganti, P., et al. (2013) *Cordyceps sinensis* increases hypoxia tolerance by inducing heme oxygenase-1 and metallothionein via Nrf2 activation in human lung epithelial cells. *BioMed Research International*, 2013: 1–13. doi:10.1155/2013/569206.

Singh, S.K., Naik, P.K., Vishwakarma, N.K., et al. (2015) Identification and Ligand-based Virtual screening of 1 , 4-Dihydropyridine Analogues as Novel Calcium Channel Blockers. *International Journal of Research Studies in Biosciences*, 2015:107–114.

Sleigh, S.H. and Barton, C.L. (2010) Repurposing strategies for therapeutics. *Pharmaceutical Medicine*. 24 (3). doi:10.1007/BF03256811.

Smet, P.J., Jonavicius, J., Marshall, V.R., et al. (1996) Distribution of nitric oxide synthase-immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea-pig and human urinary bladder by cGMP

immunohistochemistry. *Neuroscience*, 71 (2). doi:10.1016/0306-4522(95)00453-X.

Somogyi, G.T., Zernova, G. v., Tanowitz, M., et al. (1997) Role of L- and N-type Ca2+ channels in muscarinic receptor-mediated facilitation of ACh and noradrenaline release in the rat urinary bladder. *Journal of Physiology*, 499 (3). doi:10.1113/jphysiol.1997.sp021957.

Song, B. and Avery, L. (2013) The pharynx of the nematode C. elegans . *Worm*, 2 (1). doi:10.4161/worm.21833.

State Council of the People's Republic of China (2000) *Decree No. 4 of the State Forestry Administration and the Ministry of Agriculture*. Available at: http://www.gov.cn/gongbao/content/2000/content_60072.htm (Accessed: 30 April 2020).

Stromberga, Z., Chess-Williams, R. and Moro, C. (2020a) Prostaglandin E2 and F2alpha Modulate Urinary Bladder Urothelium, Lamina Propria and Detrusor Contractility via the FP Receptor. *Frontiers in Physiology*, 11. doi:10.3389/fphys.2020.00705.

Stromberga, Z., Chess-Williams, R. and Moro, C. (2020b) The five primary prostaglandins stimulate contractions and phasic activity of the urinary bladder urothelium, lamina propria and detrusor. *BMC Urology*, 20 (1). doi:10.1186/s12894-020-00619-0.

Sugaya, K., Nishijima, S., Miyazato, M., et al. (2005) Central nervous control of micturition and urine storage. *Journal of Smooth Muscle Research*. 41 (3). doi:10.1540/jsmr.41.117.

Suh, Y.S., Kim, T.H., Sung, H.H., et al. (2015) Potential biomarkers for diagnosis and prediction of treatment efficacy in overactive bladder patients: Urinary nerve growth factor, prostaglandin E2, and adenosine triphosphate. *Neurourology and Urodynamics*, 34.

Sui, G., Fry, C.H., Malone-Lee, J., et al. (2009) Aberrant Ca^{2+} oscillations in smooth muscle cells from overactive human bladders. *Cell Calcium*, 45 (5): 456–464. doi:10.1016/j.ceca.2009.03.001.

Sukhu, T., Kennelly, M.J. and Kurpad, R. (2016) Sacral neuromodulation in

overactive bladder: A review and current perspectives. *Research and Reports in Urology*. 8. doi:10.2147/RRU.S89544.

Sun, X., Dong, Z., Li, N., et al. (2018) Nucleosides isolated from ophiocordyceps sinensis inhibit cigarette smoke extract-induced inflammation via the SIRT1–nuclear factor-κB/p65 pathway in RAW264.7 macrophages and in COPD mice. *International Journal of COPD*, 13: 2821–2832. doi:10.2147/COPD.S172579.

Sun, X., Wu, X., Chen, X., et al. (2021) Casein-maltodextrin Maillard conjugates encapsulation enhances the antioxidative potential of proanthocyanidins: An in vitro and in vivo evaluation. *Food Chemistry*, 346: 128952. doi:10.1016/j.foodchem.2020.128952.

Sutphin, G. L. and Kaeberlein, M. (2009) Measuring Caenorhabditis elegans life span on solid media. *Journal of Visualised Experiments*, (27). doi:10.3791/1152.

Suskind, A.M., Quanstrom, K., Zhao, S., et al. (2017) Overactive Bladder Is Strongly Associated with Frailty in Older Individuals. *Urology*, 106. doi:10.1016/j.urology.2017.03.058.

Suzuki, M., Ito, Y., Fujino, T., et al. (2009) Pharmacological effects of saw palmetto extract in the lower urinary tract. *Acta Pharmacologica Sinica*. 30 (3). doi:10.1038/aps.2009.1.

Suzuki, M., Oki, T., Sugiyama, T., et al. (2007) Muscarinic and Alpha 1-Adrenergic Receptor Binding Characteristics of Saw Palmetto Extract in Rat Lower Urinary Tract. *Urology*, 69 (6). doi:10.1016/j.urology.2007.02.038.

Takahashi, N., Aizawa, H., Fukuyama, S., et al. (2000) Thapsigargin, a Ca2+-ATPase inhibitor, relaxes guinea pig tracheal smooth muscle by producing epithelium-dependent relaxing factors. *European Journal of Pharmacology*, 410 (1). doi:10.1016/S0014-2999(00)00840-2.

Takemoto, J., Masumiya, H., Nunoki, K., et al. (2008) Potentiation of potassium currents by beta-adrenoceptor agonists in human urinary bladder smooth muscle cells: A possible electrical mechanism of relaxation. *Pharmacology*, 81 (3). doi:10.1159/000114719.

Tallarida, R.J. (2006) An overview of drug combination analysis with isobolograms. *Journal of Pharmacology and Experimental Therapeutics*. 319 (1).

doi:10.1124/jpet.106.104117.

Tanahashi, Y., Komori, S., Matsuyama, H., et al. (2021) Functions of muscarinic receptor subtypes in gastrointestinal smooth muscle: A review of studies with receptor-knockout mice. *International Journal of Molecular Sciences*. 22 (2). doi:10.3390/ijms22020926.

Tang, L., El-Din, T.M.G., Swanson, T.M., et al. (2016) Structural basis for inhibition of a voltage-gated Ca2+ channel by Ca2+ antagonist drugs. *Nature*, 537 (7618). doi:10.1038/nature19102.

Tepavcevic, S., Isenović, E.R., Varagić, V.M., et al. (2008) Sodium nitroprusside regulates the relaxation of the longitudinal muscle in the gut. *Pharmazie*, 63 (2). doi:10.1691/ph.2008.7649.

Thornbury, K.D., Hollywood, M.A. and McHale, N.G. (1992) Mediation by nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in sheep. *The Journal of Physiology*, 451 (1). doi:10.1113/jphysiol.1992.sp019157.

Thorneloe, K.S., Meredith, A.L., Knorn, A.M., et al. (2005) Urodynamic properties and neurotransmitter dependence of urinary bladder contractility in the BK channel deletion model of overactive bladder. *American Journal of Physiology - Renal Physiology*, 289 (3 58-3). doi:10.1152/ajprenal.00060.2005.

Tijsterman, M., May, R.C., Simmer, F., et al. (2004) Genes Required for Systemic RNA Interference in Caenorhabditis elegans. *Current Biology*, 14 (2). doi:10.1016/j.cub.2003.12.029.

Timóteo, M.A., Carneiro, I., Silva, I., et al. (2014) ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors. *Biochemical Pharmacology*, 87 (2). doi:10.1016/j.bcp.2013.11.007.

Tomita, T. (1988) Ionic Channels in Smooth Muscle Studied with Patch-Clamp Methods. *The Japanese Journal of Physiology*, 38 (1). doi:10.2170/jjphysiol.38.1.

Tong, X. and Guo, J. (2022) High Throughput Identification of the Potential Antioxidant Peptides in Ophiocordyceps sinensis. *Molecules*, 27 (2): 438. doi:10.3390/molecules27020438.

Triggle, D.J. (1998) The physiological and pharmacological significance of

cardiovascular T-type, voltage-gated calcium channels. *American Journal of Hypertension*, 11 (4 SUPPL. 2). doi:10.1016/S0895-7061(98)00004-1.

Trojanowski, N.F., Raizen, D.M. and Fang-Yen, C. (2016) Pharyngeal pumping in Caenorhabditis elegans depends on tonic and phasic signaling from the nervous system. *Scientific Reports*, 6. doi:10.1038/srep22940.

Truzzi, J.C., de Almeida, F.G., Sacomani, C.A., et al. (2022) Neurogenic bladder – concepts and treatment recommendations. *International Braz J Urol*, 48 (2). doi:10.1590/S1677-5538.IBJU.2021.0098.

Tyagi, P. (2011) Pathophysiology of the urothelium and detrusor. *Canadian Urological Association Journal*, 5 (5): S128–S130. doi:10.5489/cuaj.11181.

Uchida, W., Masuda, N., Shirai, Y., et al. (1994) The role of extracellular Ca2+ in carbachol-induced tonic contraction of the pig detrusor smooth muscle. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 350 (4): 398–402. doi:10.1007/BF00178958.

Uchiyama, T. and Chess-Williams, R. (2004) Muscarinic receptor subtypes of the bladder and gastrointestinal tract. *Journal of Smooth Muscle Research*. 40 (6). doi:10.1540/jsmr.40.237.

Uno, M. and Nishida, E. (2016) Lifespan-regulating genes in c. Elegans. *npj Aging and Mechanisms of Disease*, 2 (1). doi:10.1038/npjamd.2016.10.

Valeri, A., Brain, K.L., Young, J.S., et al. (2009) Effects of 17β-oestradiol on rat detrusor smooth muscle contractility. *Experimental Physiology*, 94 (7). doi:10.1113/expphysiol.2009.047118.

Vecchioli-Scaldazza, C. and Morosetti, C. (2018) Effectiveness and durability of solifenacin versus percutaneous tibial nerve stimulation versus their combination for the treatment of women with overactive bladder syndrome: A randomized controlled study with a follow-up of ten months. *International Braz J Urol*, 44 (1). doi:10.1590/S1677-5538.IBJU.2016.0611.

Veeratterapillay, R., Thorpe, A.C. and Harding, C. (2013) "Augmentation cystoplasty: Contemporary indications, techniques and complications." In *Indian Journal of Urology*. 2013. pp. 322–327. doi:10.4103/0970-1591.120114.

Venkatachalam, K., Zheng, F. and Gill, D.L. (2003) Regulation of canonical transient

receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. *Journal of Biological Chemistry*, 278 (31). doi:10.1074/jbc.M302751200.

Venturella, G., Ferraro, V., Cirlincione, F., et al. (2021) Medicinal mushrooms: Bioactive compounds, use, and clinical trials. *International Journal of Molecular Sciences*. 22 (2). doi:10.3390/ijms22020634.

Vieira, J.M., Mantovani, R.A., Raposo, M.F.J., et al. (2019) Effect of extraction temperature on rheological behavior and antioxidant capacity of flaxseed gum. *Carbohydrate Polymers*, 213. doi:10.1016/j.carbpol.2019.02.078.

Vignozzi, L., Filippi, S., Morelli, A., et al. (2012) Testosterone/Estradiol Ratio Regulates NO-Induced Bladder Relaxation and Responsiveness to PDE5 Inhibitors. *Journal of Sexual Medicine*, 9 (12). doi:10.1111/j.1743-6109.2012.02946.x.

Vijayraj, D., Roy, D.C. and Deka, D.K. (2011) Nitric oxide donors inhibit calcium stimulated contraction in detrusor muscle of goat. *Indian Journal of Animal Sciences*, 81 (12): 1208–1211.

de Wall, L.L. and Heesakkers, J.P.F.A. (2017) Effectiveness of percutaneous tibial nerve stimulation in the treatment of overactive bladder syndrome. *Research and Reports in Urology*. 9. doi:10.2147/RRU.S124981.

Wang, C., Hou, X. xia, Rui, H. liang, et al. (2018a) Artificially cultivated Ophiocordyceps sinensis alleviates diabetic nephropathy and its podocyte injury via inhibiting P2X7R expression and NLRP3 inflammasome activation. *Journal of Diabetes Research*, 2018. doi:10.1155/2018/1390418.

Wang, E.C.Y., Lee, J.M., Ruiz, W.G., et al. (2005) ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *Journal of Clinical Investigation*, 115 (9). doi:10.1172/JCI24086.

Wang, J., Deng, N., Wang, H., et al. (2020) Effects of orange extracts on longevity, healthspan, and stress resistance in Caenorhabditis elegans. *Molecules*, 25 (2). doi:10.3390/molecules25020351.

Wang, J., Kan, L., Nie, S., et al. (2015a) A comparison of chemical composition, bioactive components and antioxidant activity of natural and cultured

Cordyceps sinensis. *LWT*, 63 (1). doi:10.1016/j.lwt.2015.03.109.

Wang, J., Liu, Y.M., Cao, W., et al. (2012) Anti-inflammation and antioxidant effect of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*, in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. *Metabolic Brain Disease*, 27 (2). doi:10.1007/s11011-012-9282-1.

Wang, M., Xing, N., Wu, L., et al. (2018b) Regulation of Spontaneous Contractions in Intact Rat Bladder Strips and the Effects of Hydrogen Peroxide. *BioMed Research International*, 2018. doi:10.1155/2018/2925985.

Wang, N., Li, J., Huang, X., et al. (2016) Herbal Medicine Cordyceps sinensis Improves Health-Related Quality of Life in Moderate-to-Severe Asthma. *Evidence-based Complementary and Alternative Medicine*, 2016 (6134593). doi:10.1155/2016/6134593.

Wang, P., Luthin, G.R. and Ruggieri, M.R. (1995) Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. *Journal of Pharmacology and Experimental Therapeutics*, 273 (2).

Wang, Y., Wang, Y., Liu, D., et al. (2015b) Cordyceps sinensis polysaccharide inhibits PDGF-BB-induced inflammation and ROS production in human mesangial cells. *Carbohydrate Polymers*, 125: 135–145. doi:10.1016/j.carbpol.2015.02.012.

Wang, Z.M., Peng, X., Lee, K.L.D., et al. (2011) Structural characterisation and immunomodulatory property of an acidic polysaccharide from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. *Food Chemistry*, 125 (2): 637–643. doi:10.1016/j.foodchem.2010.09.052.

Wasser, S.P. (2014) Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. *Biomedical Journal*. 37 (6). doi:10.4103/2319-4170.138318.

Wegener, J.W., Schulla, V., Lee, T., et al. (2004) An essential role of Ca v 1.2 L-type calcium channel for urinary bladder function . *The FASEB Journal*, 18 (10). doi:10.1096/fj.04-1516fje.

Wei, Y., Zhang, L., Wang, J., et al. (2021) Chinese caterpillar fungus (*Ophiocordyceps sinensis*) in China: Current distribution, trading, and futures under climate

change and overexploitation. *Science of the Total Environment*, 755. doi:10.1016/j.scitotenv.2020.142548.

Weller, M.G. (2012) A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. *Sensors (Switzerland)*. 12 (7). doi:10.3390/s120709181.

Willis-Gray, M.G., Dieter, A.A. and Geller, E.J. (2016) Evaluation and management of overactive bladder: Strategies for optimizing care. *Research and Reports in Urology*, 8: 113–122. doi:10.2147/RRU.S93636.

Winder, M., Tobin, G., Zupančič, D., et al. (2014) Signalling molecules in the urothelium. *BioMed Research International*, 2014. doi:10.1155/2014/297295.

Wray, S. and Burdyga, T. (2010) Sarcoplasmic reticulum function in smooth muscle. *Physiological Reviews*. 90 (1). doi:10.1152/physrev.00018.2008.

Wu, C., Sui, G. and Fry, C.H. (2002) The role of the L-type Ca^{2+} channel in refilling functional intracellular Ca^{2+} stores in guinea-pig detrusor smooth muscle. *Journal of Physiology*, 538 (2). doi:10.1113/jphysiol.2001.013191.

Wu, R., Gao, J.P., Wang, H.L., et al. (2015) Effects of fermented *Cordyceps sinensis* on oxidative stress in doxorubicin treated rats. *Pharmacognosy Magazine*, 11 (44): 724–731. doi:10.4103/0973-1296.165562.

Wu, S.Y., Jiang, Y.H., Jhang, J.F., et al. (2022) Inflammation and Barrier Function Deficits in the Bladder Urothelium of Patients with Chronic Spinal Cord Injury and Recurrent Urinary Tract Infections. *Biomedicines*, 10 (2). doi:10.3390/biomedicines10020220.

Wu, T.N., Yang, K.C., Wang, C.M., et al. (1996) Lead poisoning caused by contaminated Cordyceps, a Chinese herbal medicine: Two case reports. *Science of the Total Environment*, 182 (1–3). doi:10.1016/0048-9697(96)05054-1.

Wu, Z., Isik, M., Moroz, N., et al. (2019) Dietary Restriction Extends Lifespan through Metabolic Regulation of Innate Immunity. *Cell Metabolism*, 29 (5). doi:10.1016/j.cmet.2019.02.013.

Wuest, M., Hiller, N., Braeter, M., et al. (2007) Contribution of Ca^{2+} influx to carbachol-induced detrusor contraction is different in human urinary bladder

compared to pig and mouse. *European Journal of Pharmacology*, 565 (1–3). doi:10.1016/j.ejphar.2007.02.046.

Xiang, F., Lin, L., Hu, M., et al. (2016) Therapeutic efficacy of a polysaccharide isolated from *Cordyceps sinensis* on hypertensive rats. *International Journal of Biological Macromolecules*, 82: 308–314. doi:10.1016/j.ijbiomac.2015.09.060.

Xiao, X., Tan, C., Sun, X., et al. (2020) Fermented barley β -glucan regulates fat deposition in *Caenorhabditis elegans*. *Journal of the Science of Food and Agriculture*, 100 (8): 3408–3417. doi:10.1002/jsfa.10375.

Xiao, Y., Li, C., Xu, W., et al. (2021) Arsenic Content, Speciation, and Distribution in Wild *Cordyceps sinensis*. *Evidence-based Complementary and Alternative Medicine*, 2021. doi:10.1155/2021/6651498.

Xin, W., Li, N., Cheng, Q., et al. (2014) BK channel-mediated relaxation of urinary bladder smooth muscle: A novel paradigm for phosphodiesterase type 4 regulation of bladder function. *Journal of Pharmacology and Experimental Therapeutics*, 349 (4). doi:10.1124/jpet.113.210708.

Xu, Y.F., Liang, Z.J., Kuang, Z.J., et al. (2017) Effect of suo quan wan on the bladder function of aging rats based on the β-adrenoceptor. *Experimental and Therapeutic Medicine*, 13 (6). doi:10.3892/etm.2017.4394.

Yagi, H., Nishio, K., Sato, R., et al. (2016) Clinical efficacy and tolerability of Gosha-jinki-gan, a Japanese traditional herbal medicine, for nocturia. *Journal of Traditional and Complementary Medicine*, 6 (1). doi:10.1016/j.jtcme.2014.11.021.

Yamada, S., Shirai, M., Ono, K., et al. (2022) Beneficial Effects of Saw Palmetto Fruit Extract on Urinary Symptoms in Japanese Female Subjects by a Multicenter, Randomized, Double-Blind, Placebo-Controlled Study. *Nutrients*, 14 (6). doi:10.3390/nu14061190.

Yamaguchi, O., Kakizaki, H., Homma, Y., et al. (2015) Safety and efficacy of mirabegron as “add-on” therapy in patients with overactive bladder treated with solifenacin: A post-marketing, open-label study in Japan (MILAI study). *BJU International*, 116 (4): 612–622. doi:10.1111/bju.13068.

Yamaguchi, Y., Kagota, S., Nakamura, K., et al. (2000a) Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. *Phytotherapy Research*, 14 (8): 647–649. doi:10.1002/1099-1573(200012)14:8<647::AID-PTR670>3.0.CO;2-W.

Yamaguchi, Y., Kagota, S., Nakamura, K., et al. (2000b) Inhibitory effects of water extracts from fruiting bodies of cultured *Cordyceps sinensis* on raised serum lipid peroxide levels and aortic cholesterol deposition in atherosclerotic mice. *Phytotherapy Research*, 14 (8). doi:10.1002/1099-1573(200012)14:8<650::AID-PTR675>3.0.CO;2-0.

Yamanishi, T., Chappie, C.R. and Chess-Williams, R. (2001) Which muscarinic receptor is important in the bladder? *World Journal of Urology*, 19 (5): 299–306. doi:10.1007/s003450100226.

Yan, Y., Li, Y., Wang, W.J., et al. (2017) Range shifts in response to climate change of Ophiocordyceps sinensis, a fungus endemic to the Tibetan Plateau. *Biological Conservation*, 206. doi:10.1016/j.biocon.2016.12.023.

Yanai, Y., Hashitani, H., Hayase, M., et al. (2008) Role of nitric oxide/cyclic GMP pathway in regulating spontaneous excitations in detrusor smooth muscle of the guinea-pie bladder. *Neurourology and Urodynamics*, 27 (5): 446–453. doi:10.1002/nau.20517.

Yang, S., Wang, D., Cao, X., et al. (2018) Store operated calcium channels are associated with diabetic cystopathy in streptozotocin-induced diabetic rats. *Molecular Medicine Reports*, 17 (5). doi:10.3892/mmr.2018.8723.

Yao, X., Meran, S., Fang, Y., et al. (2014) *Cordyceps sinensis*: In vitro anti-fibrotic bioactivity of natural and cultured preparations. *Food Hydrocolloids*, 35: 444–452. doi:10.1016/j.foodhyd.2013.06.023.

Yap, A.C.S., Li, X., Yap, Y.H.Y., et al. (2020a) Immunomodulatory properties of water-soluble polysaccharides extracted from the fruiting body of Chinese caterpillar mushroom, *ophiocordyceps sinensis* cultivar OCS02® (Ascomycetes). *International Journal of Medicinal Mushrooms*, 22 (10): 967–977. doi:10.1615/IntJMedMushrooms.2020036351.

Yap, A.C.S., Li, X., Yeng, Y.Y.H., et al. (2020b) The immunomodulating properties

of tiger milk medicinal mushroom, *lignosus rhinocerus* tm02® cultivar (Agaricomycetes) and its associated carbohydrate composition. *International Journal of Medicinal Mushrooms*, 22 (8). doi:10.1615/IntJMedMushrooms.2020035658.

Yap, Y.H.Y., Tan, N., Fung, S., et al. (2013) Nutrient composition, antioxidant properties, and anti-proliferative activity of *Lignosus rhinocerus* Cooke sclerotium. *Journal of the science of food and agriculture*, 93 (12): 2945—2952. doi:10.1002/jsfa.6121.

Yi, J., Jeong, S.J., Chung, M.S., et al. (2013) Efficacy and tolerability of combined medication of two different antimuscarinics for treatment of adults with idiopathic overactive bladder in whom a single agent antimuscarinic therapy failed. *Journal of the Canadian Urological Association*, 7 (2). doi:10.5489/cuaj.269.

Yim, P.D., Gallos, G., Perez-Zoghbi, J.F., et al. (2013) Chloride channel blockers promote relaxation of TEA-induced contraction in airway smooth muscle. *Journal of smooth muscle research = Nihon Heikatsukin Gakkai kikanshi*, 49. doi:10.1540/jsmr.49.112.

Yoshida, M., Inadome, A., Maeda, Y., et al. (2006) Non-neuronal cholinergic system in human bladder urothelium. *Urology*, 67 (2). doi:10.1016/j.urology.2005.08.014.

Yoshikawa, N., Nishiuchi, A., Kubo, E., et al. (2011) <i>Cordyceps sinensis</i> Acts As an Adenosine A₃ Receptor Agonist on Mouse Melanoma and Lung Carcinoma Cells, and Human Fibrosarcoma and Colon Carcinoma Cells. *Pharmacology & Pharmacy*, 02 (04): 266–270. doi:10.4236/pp.2011.24034.

Yoshimura, Y. and Yamaguchi, O. (1997) Calcium independent contraction of bladder smooth muscle. *International Journal of Urology*, 4 (1). doi:10.1111/j.1442-2042.1997.tb00142.x.

Yoswathana, N., Eshtiaghi, M.N. and Ebadi, A.G. (2010) Extraction of adenosine from Ganoderma lucidum using novel extraction technologies. *Asian Journal of Chemistry*, 22 (3).

Yu, W. (2022) Reviving Cav1.2 as an attractive drug target to treat bladder dysfunction. *FASEB Journal*, 36 (1). doi:10.1096/fj.202101475R.

Yu, W., Zacharia, L.C., Jackson, E.K., et al. (2006) Adenosine receptor expression and function in bladder uroepithelium. *American Journal of Physiology - Cell Physiology*, 291 (2). doi:10.1152/ajpcell.00025.2006.

Yu, X., Mao, Y., Shergis, J.L., et al. (2019) Effectiveness and Safety of Oral Cordyceps sinensis on Stable COPD of GOLD Stages 2-3: Systematic Review and Meta-Analysis. *Evidence-based Complementary and Alternative Medicine*, 2019. doi:10.1155/2019/4903671.

Yuan, S. and Chen, H. (2019) Mathematical rules for synergistic, additive, and antagonistic effects of multi-drug combinations and their application in research and development of combinatorial drugs and special medical food combinations. *Food Science and Human Wellness*, 8 (2). doi:10.1016/j.fshw.2019.01.003.

Yue, K., Ye, M., Lin, X., et al. (2013) The artificial cultivation of medicinal caterpillar fungus, *Ophiocordyceps sinensis* (Ascomycetes): A review. *International Journal of Medicinal Mushrooms*, 15 (5): 425–434. doi:10.1615/IntJMedMushr.v15.i5.10.

Zarkower, D. (2006) Somatic sex determination. *WormBook : the online review of C. elegans biology*. doi:10.1895/wormbook.1.84.1.

Zeng, L., Yang, Z., Yun, T., et al. (2019) Antiaging effect of a Jianpi-yangwei formula in *Caenorhabditis elegans*. *BMC Complementary and Alternative Medicine*, 19 (1). doi:10.1186/s12906-019-2704-4.

Zhang, D. wei, Wang, Z. lin, Qi, W., et al. (2014) The effects of *Cordyceps sinensis* phytoestrogen on estrogen deficiency-induced osteoporosis in Ovariectomized rats. *BMC Complementary and Alternative Medicine*, 14 (1). doi:10.1186/1472-6882-14-484.

Zhang, G., Huang, Y., Bian, Y., et al. (2006) Hypoglycemic activity of the fungi Cordyceps militaris, *Cordyceps sinensis*, Tricholoma mongolicum, and Omphalia lapidescens in streptozotocin-induced diabetic rats. *Applied Microbiology and Biotechnology*, 72 (6). doi:10.1007/s00253-006-0411-9.

Zhang, J., Yi, Q.T., Gong, M., et al. (2019) Upregulation of TRPV1 in spinal dorsal root ganglion by activating NGF-TrkA pathway contributes to pelvic organ cross-sensitisation in rats with experimental autoimmune prostatitis. *Andrologia*, 51 (8). doi:10.1111/and.13302.

Zhang, P., Li, S., Li, J., et al. (2018a) Identification of ophiocordyceps sinensis and its artificially cultured ophiocordyceps mycelia by ultra-performance liquid chromatography/orbitrap fusion mass spectrometry and chemometrics. *Molecules*, 23 (5). doi:10.3390/molecules23051013.

Zhang, Q.-W., Lin, L.-G. and Ye, W.-C. (2018b) Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese Medicine*, 13 (1): 20. doi:10.1186/s13020-018-0177-x.

Zhang, S., Li, F., Zhou, T., et al. (2020) *Caenorhabditis elegans* as a Useful Model for Studying Aging Mutations. *Frontiers in Endocrinology*. 11. doi:10.3389/fendo.2020.554994.

Zhang, X., Wang, M., Qiao, Y., et al. (2022) Exploring the mechanisms of action of *Cordyceps sinensis* for the treatment of depression using network pharmacology and molecular docking. *Annals of Translational Medicine*, 10 (6). doi:10.21037/atm-22-762.

Zhang, Y., Li, E., Wang, C., et al. (2012) *Ophiocordyceps sinensis*, the flagship fungus of china: Terminology, life strategy and ecology. *Mycology*, 3 (1): 2–10. doi:10.1080/21501203.2011.654354.

Zhang, Z.J., Luo, H.L. and Li, J.S. (1997) Clinical and experimental studies on elimination of oxygen free radical of jinshuibao capsule in treating senile deficiency syndrome and its deoxyribonucleic acid damage repairing effects. *Zhongguo Zhong xi yi jie he za zhi Zhongguo Zhongxiyi jiehe zazhi = Chinese journal of integrated traditional and Western medicine / Zhongguo Zhong xi yi jie he xue hui, Zhongguo Zhong yi yan jiu yuan zhu ban*, 17 (1): 35–38.

Zhao, W., Aboushwareb, T., Turner, C., et al. (2010) Impaired Bladder Function in Aging Male Rats. *Journal of Urology*, 184 (1). doi:10.1016/j.juro.2010.03.004.

Zhao, Y., Huang, G., Wu, J., et al. (2019) Molecular Basis for Ligand Modulation of a Mammalian Voltage-Gated Ca^{2+} Channel. *Cell*, 177 (6).

doi:10.1016/j.cell.2019.04.043.

Zhou, L., Hao, Q.X., Wang, S., et al. (2017a) Study on distribution of five heavy metal elements in different parts of Cordyceps sinensis by microwave digestion ICP-MS. *Zhongguo Zhongyao Zazhi*, 42 (15).

Zhou, L., Wang, S., Hao, Q., et al. (2018) Bioaccessibility and risk assessment of heavy metals, and analysis of arsenic speciation in Cordyceps sinensis. *Chinese Medicine (United Kingdom)*, 13 (1). doi:10.1186/s13020-018-0196-7.

Zhou, Y., Chu, X., Deng, F., et al. (2017b) The adenosine A2b receptor promotes tumor progression of bladder urothelial carcinoma by enhancing MAPK signaling pathway. *Oncotarget*, 8 (30). doi:10.18632/oncotarget.17835.

Zhou, Y., Wang, M., Zhang, H., et al. (2019) Comparative study of the composition of cultivated, naturally grown Cordyceps sinensis, and stiff worms across different sampling years. *PLoS ONE*, 14 (12). doi:10.1371/journal.pone.0225750.

Zhu, X., Zhai, K., Mi, Y., et al. (2017) Expression and function of phosphodiesterases (PDEs) in the rat urinary bladder. *BMC Urology*, 17 (1). doi:10.1186/s12894-017-0244-0.

Zhu, Y., Bai, J., Zhou, Y., et al. (2021) Water-soluble and alkali-soluble polysaccharides from bitter melon inhibited lipid accumulation in HepG2 cells and Caenorhabditis elegans. *International Journal of Biological Macromolecules*, 166: 155–165. doi:10.1016/j.ijbiomac.2020.10.128.

Zhu, Y., Ma, L., Hu, Q., et al. (2016) In Vitro Anti-HIV-1 Activity of Cordyceps sinensis Extracts. *Bing du xue bao = Chinese journal of virology*, 32 (4).

Zoncu, R., Efeyan, A. and Sabatini, D.M. (2011) MTOR: From growth signal integration to cancer, diabetes and ageing. *Nature Reviews Molecular Cell Biology*. 12 (1). doi:10.1038/nrm3025.

Zou, S., Shimizu, T., Shimizu, S., et al. (2018) Possible role of hydrogen sulfide as an endogenous relaxation factor in the rat bladder and prostate. *Neurourology and Urodynamics*, 37 (8). doi:10.1002/nau.23788.

Zou, Y., Liu, Y., Ruan, M., et al. (2015) *Cordyceps sinensis* oral liquid prolongs the lifespan of the fruit fly, *Drosophila melanogaster*, by inhibiting oxidative

stress. *International Journal of Molecular Medicine*, 36 (4). doi:10.3892/ijmm.2015.2296.

Zwaal, R.R., Van Baelen, K., Groenen, J.T.M., et al. (2001) The Sarco-Endoplasmic Reticulum Ca^{2+} ATPase Is Required for Development and Muscle Function in Caenorhabditis elegans. *Journal of Biological Chemistry*, 276 (47). doi:10.1074/jbc.M104693200.