Phytosomal Beta-Caryophyllene for the Treatment of Pain and Inflammation in Interstitial Cystitis

by

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Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

Dedication Page

I dedicate this thesis to my parents. I may not be winning an award, but I'll always remember to thank you first.

Table of Contents

List of Tables

List of Figures

Abstract

Interstitial cystitis (IC) is a chronic bladder condition of unknown etiology characterized by pain and inflammation. This study examined the effects of oral phytosomal beta-caryophyllene (BCP), a cannabinoid type 2 receptor agonist with local anaesthetic properties, in an experimental model of IC. Female BALB/c mice were pre-treated with BCP, then given lipopolysaccharide (LPS) intravesically. After 24 hours, behaviour was evaluated, intravital microscopy was completed, and bladder samples were collected. Oral phytosomal BCP significantly attenuated pain-induced behavioral changes, decreased leukocyte activation in the bladder microvasculature, and improved histology scores. Additionally, 5637 bladder epithelial cells were utilized to investigate BCP's effects on LPS-induced inflammation *in vitro*. Although no significant changes in inflammatory mediator production were observed in the cell culture, changes in cytokine release kinetics cannot be excluded. Overall, oral phytosomal BCP effectively reduces pain and inflammation associated with experimental IC by decreasing leukocyte activation and extravasation in the bladder microvasculature.

List of Abbreviations and Symbols Used

2-AG	2-arachidonoylglycerol
ABHD12	Alpha-beta domain hydrolase 12
ABHD6	Alpha-beta domain hydrolase 6
ATCC	American Type Culture Collection
BCA	Bicinchonic Acid
BCP	Beta-caryophyllene
BPS	Bladder pain syndrome
BSA	Bovine serum albumin
CACF	Carleton Animal Care Facility
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CCK-8	Cell-counting kit-8
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DMSO	Dimethysulfoxide
E. coli	*Escherichia coli*
ECS	Endocannabinoid system
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FAAH	Fatty acid amide hydrolase
FCD	Functional capillary density
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GPCR	G protein-coupled receptor
GRAS	Generally recognized as safe
H&E	Hematoxylin and eosin
HIC	Hunner type interstitial cystitis
i.p.	Intraperitoneal
IC	Interstitial cystitis

ICAM-1	Intercellular adhesion molecule 1
IL-13	Interleukin 13
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IVM	Intravital microscopy
JNK1/2	Jun N-terminal kinase 1 and 2
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MGL	Monoacylglycerol
NAAA	N-acetylethanolamine-hydrolyzing acid amidase
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PBS	Phosphate buffered saline
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activate receptor gamma
PPS	Pentosan polysulfate
RPMI	Roswell Park Memorial Institute
T-PER	Tissue protein extraction reagent
THC	Tetrahydrocannabinol
TLR4	Toll-like receptor 4
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRPV1	Transient receptor potential vanilloid 1
UTI	Urinary tract infection
VCAM-1	Vascular cell adhesion molecule 1

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Chapter 1: Introduction

1.1 Interstitial Cystitis

1.1.1 Definition, Symptoms, and Epidemiology

Interstitial cystitis (IC), sometimes referred to as bladder pain syndrome (BPS) or IC/BPS, is a chronic inflammatory disease of the bladder with unknown etiology. Symptoms of IC/BPS include increased urinary urgency and frequency, as well as chronic pelvic pain and nocturia [1,2]. Pain is described as a pressure or discomfort, and is especially important, as this pressure or discomfort increases as the bladder fills and drives urinary urgency [2]. The pain is typically located in the suprapubic area but can also be felt throughout the pelvis [1].

These symptoms must persist for at least six weeks before a diagnosis can be made [3]. Such a quick turnaround is unusual, as the mean time from first symptoms to diagnosis can range from 5-7 years [3,4]. Patients are typically diagnosed based on the symptoms above combined with exclusion of all other relevant diagnoses, including infection, urinary incontinence, cancer, endometriosis, non-infectious cystitis, vulvar disorders, overactive bladder, pudendal nerve entrapment, prostate-related pain, and pelvic floor disorders [1,3]. However, these conditions may also co-exist with IC/BPS, further complicating diagnosis [1].

Given the difficulties in diagnosing this condition, individual presentation varies between patients. IC/BPS can be broadly classified according to the presence or absence of ulcers called Hunner lesions, resulting in two subtypes: Hunner type IC (HIC) and BPS [5,6]. Hunner lesions are small red lesions on the bladder mucosa with abnormal capillary structure that cover scarring. They are visible by cystoscopy and are important diagnostically due to their association with symptom severity and resolution [6]. Recent sources indicate that HIC is the inflammatory phenotype of the disease, while BPS is typically non-inflammatory, although both forms have the characteristic urinary urgency, urinary frequency, and pelvic pain [7,8].

IC/BPS symptoms have a large impact on daily life. Painful symptoms make daily activities difficult, including work and personal relationships, in addition to having negative impacts on mental and physical health [3,9]. This leads to significantly worse quality of life overall for IC/BPS patients compared to non-diseased controls [9,10].

IC/BPS patients also have reduced quality of life compared to patients with similar conditions like overactive bladder, vulvodynia, and endometriosis [3,11] . These reductions in quality of life were even more profound with the presence of a comorbid condition such as irritable bowel syndrome or fibromyalgia [10].

IC/BPS is often comorbid with other health conditions, including those involved in diagnosis, discussed above. In addition, IC/BPS patients are at a higher risk of so-called functional somatic syndromes, which include but are not limited to conditions like fibromyalgia, chronic fatigue syndrome, and irritable bowel syndrome [12]. Symptoms of irritable bowel syndrome tend to begin around the same time as IC/BPS symptoms for patients with both diseases, while the onset of other commonly comorbid conditions tend to occur after symptom onset [13]. Beyond functional somatic symptoms, many IC/BPS patients have a history of urinary tract infections (UTIs). Women who had recurrent UTIs as children, especially UTIs that required antibiotics, are at increased risk for diagnosis, and up to 50% of patients have a history of UTI at the time of diagnosis [1,14].

Mental health conditions are also common among IC/BPS patients. Many IC patients score higher than healthy controls on measures of mental health-related co-morbidities, including measures of depression and anxiety [15,16]. A recent systematic review found that depression rates were higher among patients with IC/BPS. The same was true of anxiety disorders. Patients were more likely to either have an anxiety disorder prior to diagnosis of IC/BPS or to develop an anxiety disorder after an IC/BPS diagnosis [17]. Given the variability in onset of mental health issues, it is unclear whether they occur in response to IC/BPS symptoms or whether they are the result of a common underlying factor for both conditions [13].

Beyond an IC/BPS patient's usual symptoms, patients can also undergo periods of worsening symptoms called "flares". These flares are often brought on by external factors associated with daily living like seasonal allergies and sex, as well as increased stress [5,18,19]. Many patients report that both exercise and sitting can trigger symptoms. Food triggers are also common, with one study reporting that 94.1% of patients had one or more dietary trigger(s) [19].

In line with the difficulties surrounding diagnosis and comorbidities, the statistics surrounding IC are also not well-defined. Approximately 90% of IC patients are female

[1]. However, it is not clear whether this is a true sex difference or if this condition is simply underdiagnosed in males. In fact, 2013 study estimated the prevalence of IC/BPS in men as 1.9-4.2%, which overlaps with the estimated prevalence of 2.70-6.53% in women [20,21]. There are also differences between sexes regarding when patients are diagnosed. A 2003 study found that the median age of IC diagnosis in females is age 44.5, while for males that age rises to 71.5. Females had a shorter time to diagnosis and sought medical care fewer times before being diagnosed with IC compared to males [22].

Beyond the impacts on the health and personal life of patients, IC/BPS also has a large impact on the healthcare system. IC/BPS treatment has an estimated economic impact of $20-40 billion per year in the United States [23]. These large costs are due in part to the lack of effective, long-term treatment options [24].

1.1.2 Pathophysiology

Much is unknown about the exact pathophysiology of IC, but there are a number of key theories and findings surrounding the disease and its progression. There are important differences between HIC and BPS, likely owing to Hunner lesions and differences in inflammation. Overall, the disease is characterized by urothelial breakdown and a vicious cycle of damage in the bladder, as well as increased inflammatory mediators and processes, both of which lead to increased bladder sensitization and painful symptoms. The primary features of IC pathophysiology can be found in Figure 1.

HIC shows distinct pathophysiologic features compared to BPS [6]. Clearly, the Hunner lesions are an important difference, but the inflammatory differences between these presentations are also important to note. Patients with HIC show significantly increased leukocyte infiltration and loss of epithelial tissue [8]. Specifically, B-cell expansion seems to be a distinct marker of this subtype [6]. Gene expression for inflammatory cytokines and nociceptors tends to be over-expressed in this group, and nitric oxide (NO) levels are upregulated [6,25]. These features combined with the fact that Hunner lesions correlate with symptom severity, and treatments targeting these lesions provide symptom relief, indicate the importance of Hunner lesions in diagnosis and disease characterization [6].

Glomerulations are a frequently cited finding in IC/BPS disease states. These are the result of small submucosal hemorrhages found on the bladder wall and are typically observed after hydrodistension [26]. Although previously thought to be an important diagnostic feature, the number of glomerulations does not correlate with disease or symptom severity. In addition, they are commonly found in both healthy people without IC symptoms and other urological disorders, indicating that they are not an essential feature of the disease [6,26].

The role of the urothelium in IC/BPS is much more evident. The urothelium lines the lumen of the bladder and protects the underlying tissues from the contents of the urine, including ions, water and solute flux, toxins, and various pathogens [8,27]. Changes to the urothelium have been seen in both IC models and patients and include damage to the layers of glycosaminoglycans (GAG) chondroitin sulfate and mucins that cover the urothelium [5,8,27]. These changes are theorized to contribute to the symptoms of the disease by allowing solutes like urea and potassium to reach the neural or muscle layers of the bladder, including the afferent nerve endings that are found throughout the interstitium [5,28,29]. This exposure can activate the nerve endings, leading to pain sensations, and can also cause damage to the bladder [29]. In addition, potassium exposure leads to upregulation of sensory afferent nerves, which in turn leads to increased pain sensations and inflammation [5,30]. When the bladder is exposed to these contents long term, especially in the presence of inflammatory mediators, this can lead to lasting changes in nerve function [28]. Overall, urothelial breakdown leads to a vicious cycle that exacerbates the issues underlying IC/BPS symptoms.

While this urothelial breakdown drives itself, expression of tight junction proteins is another important contributor. Tight junction proteins link the umbrella cells that make up the superficial layer of the uroepithelium and allow for solute exchange between cells while providing a barrier between distinct tissue areas [31]. IC/BPS patients show decreased expression of these proteins, which can lead to a “leaky” urothelium that more easily allows damaging substances to reach sensitive areas, accelerating the cycle of damage [28,29].

Urothelial breakdown leads to exposure of pain-sensing fibers, which is an important contributor to IC/BPS symptoms. C-fibers, which are nociceptive and

mechanosensory nerve fibers found in the urothelium and bladder submucosa, are also implicated in IC/BPS development [32]. These fibers are exposed after a bladder insult leads to uroepithelial damage and are then activated by a variety of stimuli, including potassium leaks into the bladder interstitium, neurotransmitter release, or histamine from mast cells [5,32]. Since the afferent nerves are exposed in IC/BPS, these nerves signal in a hypersensitive manner, leading to changes in urinary function [5]. These changes include increased and more prolonged pain sensations, especially in response to bladder filling, compared to a normal bladder [33]. In fact, in studies that examined the functional consequences of IC/BPS, patients felt pain and the urge to void at lower volumes than those without the disease [34].

In addition to nerve exposure in the bladder, uroepithelial-neural interactions also contribute to IC/BPS symptoms. Urothelial cells release a variety of neurotransmitters, cytokines, and chemokines [7]. IC patients often have altered release of these substances, which indicates increased inflammation, but which is also theorized to contribute to involuntary bladder contractions [28]. For example, NO levels can be used to evaluate treatment response in IC patients, with a decrease in NO correlating with a decrease in IC symptoms [35]. Nerve growth factor and antiproliferative factor are also released in IC, leading to activation of submucosal afferent nerves and mast cells [36]. Antiproliferative factor further increases urothelial permeability by decreasing the expression of both tight junction and adhesion proteins, as well as by slowing the growth of urothelial cells [29,37]. In terms of pro-inflammatory cytokines, IC/BPS patients had higher levels of macrophage-derived chemokine and interleukin-4 in their urine when compared to controls [16]. These patients also show elevated levels of interleukin 6 (IL-6) in urine, which seems to be associated with more severe inflammation [36,38].

An increased number of mast cells and increased mast cell activation, especially in patients with HIC, is another common feature of IC [5,36]. This is thought to occur due to the increased cytokine production and increased sensory afferent nerve proliferation that result from urothelial damage, and contributes to a vicious cycle of urothelial damage, as the activated mast cells release more inflammatory and nociceptive mediators, leading to additional pain and inflammation [5,39]. Specifically, histamine has been identified as an important mast cell-released inflammatory mediator in IC

pathogenesis. Mast cells isolated from IC patients released more histamine in response to inflammatory stimuli and histamine levels are typically increased in IC patient bladders compared to controls [36,40]. This histamine release can lead to additional downstream processes, such as vasodilation, angiogenesis, and pain, as well as further urothelial damage, overactivity of the detrusor, fibrosis, and increased release of nerve growth factor [5,36].

Fibrosis is another indicator of inflammatory damage that can occur in IC/BPS, as it is the result of chronic, sustained inflammation. Fibrosis is an irreversible change that can lead to decreased bladder compliance and is often managed using surgery in IC/BPS patients [5]. IC/BPS patients with fibrosis have significantly reduced urinary capacity and significantly increased urinary frequency compared to IC/BPS patients without [41]. Interestingly, fibrosis is most often reported in BPS patients rather than HIC patients in those studies that differentiate by disease phenotype, which suggests that neurogenic inflammation is involved in BPS pathology [41,42].

While not an infectious condition, microbes could still contribute to IC symptoms. Some studies have observed significant changes in the bladder microbiome in IC/BPS patients compared to healthy controls, including increased expression of certain bacterial genera and decreased overall diversity of species, but results vary widely [43]. For example, one study found that IC/BPS patients have less *Lactobacillus acidophilus*, a commensal antimicrobial microbe whose presence is inversely correlated with symptom severity of IC, while an earlier study found that bacteria in the *Lactobacillus* genus were actually increased in IC/BPS patients [16,44]. Other studies have found no significant differences in the IC/BPS urinary microbiome compared to controls [43]. Beyond the commensal microbes, infectious microbes should also be considered. The more frequent UTIs reported in IC/BPS patients can also lead to increased bladder sensitivity, although it is speculated that this could be due to a shared underlying cause for both conditions [14,45,46]. Evidently, the exact role of microbes in IC/BPS pathology remains unclear.

Chronic stress seems to contribute to IC development, symptoms, and flares. Patients who experience more daily life stressors report more severe urinary urgency and pain compared to patients with lower stress levels [47]. There is evidence that this stress affects the nervous system, leading to a sympathetic nervous system-mediated pain called

"stress-induced hyperalgesia" that is thought to be caused by decreased descending inhibition [7,48]. Stress itself can also drive inflammation, for example by increasing expression of inflammatory cytokines [49], which further contributes to the cycle of bladder damage and pain.

The ongoing inflammation along with persistent activation of pain-sensing fibers in the IC/BPS bladder can lead to central pain amplification. When neurons in the dorsal horn of the spinal cord are constantly activated, the spinal cord becomes hyperexcitable and pain persists even when the disease state or inflammation is resolved [5,7]. This phenomenon also seems to affect higher areas of the brain, as the parts of the brainstem and cortex that control memory, learning, and perception are affected by chronic pain [7]. In fact, IC/BPS is associated with changes in the white matter structure in the brain [50]. Overall, these structural changes lead to changes in how information is processed and could be a driving factor in IC/BPS patients' perception of bladder filling and urinary urgency, as they are correlated with increased pelvic pain, more severe urological symptoms, and worse overall quality of life [50].

Cross-sensitization between various organ systems, especially the bladder and gut, could provide some link between the symptoms and processes discussed above. The bladder-gut-brain axis theory states that excessive stress leads to an increased response to normal stimuli for voiding, which could explain the increased urinary frequency seen in IC/BPS patients [51]. This concept also connects the symptoms seen in IC with common comorbidities like irritable bowel syndrome and chronic stress [52]. It is theorized that bowel issues like irritable bowel syndrome and lower urinary tract issues like IC are related in some capacity due to their similar symptoms, chronic nature, and resistance to treatment [42]. These shared features are purported occur due to the bladder-gut-brain axis, as well as C-fibers and the bowel microbiome [51].

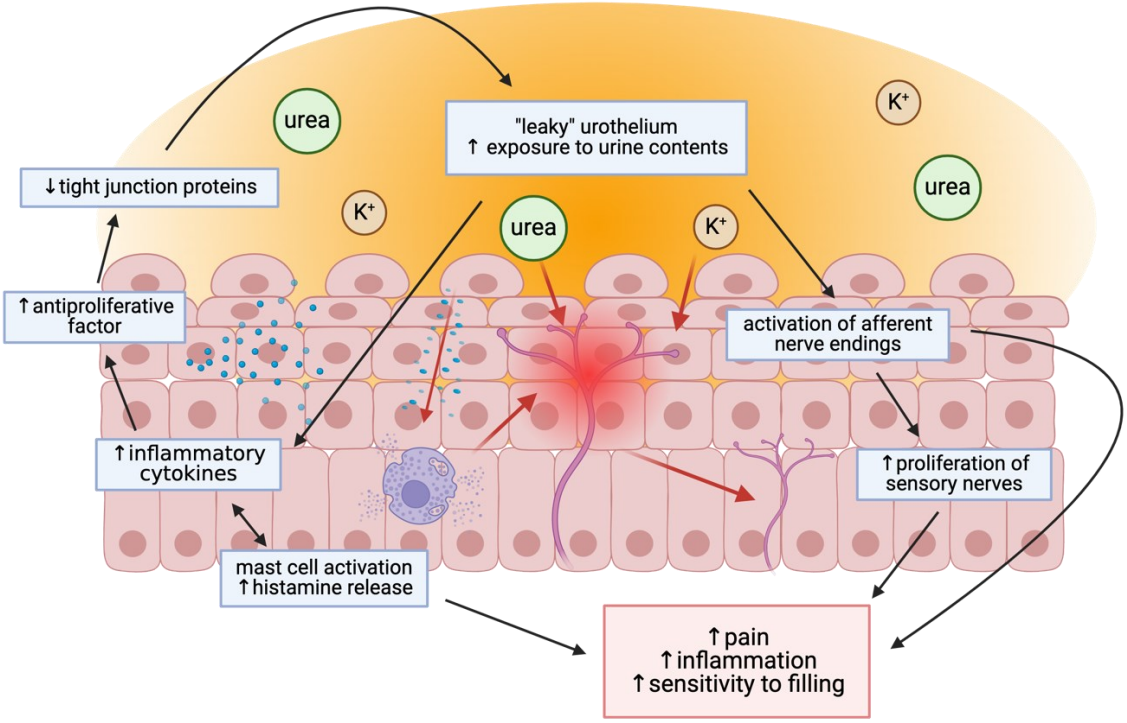

Figure 1. Simplified mechanism of interstitial cystitis pathophysiology. Development of IC is driven by a cycle of damage. “Leaky” umbrella cells allow solutes from the urine to reach deeper layers of the urothelium. These solutes activate afferent nerve endings, which increases pain and sensitivity to filling, and over time leads to increase proliferation of these sensory nerves. Additionally, exposure to urinary solutes increases inflammatory cytokine release. Inflammatory cytokines activate immune cells like mast cells to increase their release of histamine, which in turn increases pain, inflammatory cytokine release, and overall inflammation. Inflammatory cytokines also increase expression of antiproliferative factor, which decreases the expression of tight junction proteins, further exacerbating the “leaky” urothelium. Created with BioRender.com.

1.1.3 Treatment

Given the complications surrounding IC/BPS diagnosis and pathophysiology, treatment of this condition is also difficult. There is no cure, so treatments focus on controlling symptoms and increasing quality of life while minimizing side effects [1]. The exact treatment guidelines vary slightly from country to country, but the specific strategies employed will depend on disease subtype, symptoms, and patient willingness. Treatments vary from lifestyle changes to medications to surgery and are often combined for maximum symptom relief.

First-line treatment typically starts with patient education and dietary modifications [1]. One study found that 50% patients who received advice and support around managing their disease showed significant improvement in their symptoms. The support included validating to the patient that they have IC/BPS, explaining the disease, encouraging water intake, discussing strategies to avoid constipation, providing information on an appropriate diet, discussing strategies to combat sexual dysfunction, discussing stress reduction strategies, and providing resources for additional support. These patients saw the most improvement when they implemented stress reduction techniques and were more careful about their diet [53]. Dietary changes include avoiding citrus fruits, tomatoes, spicy foods, carbonated and alcoholic beverages, coffee, tea, vitamin C, and artificial sweeteners [18].

In tandem with education and diet, bladder training to decrease the frequency of voiding is commonly used [1]. This has been shown to improve functional bladder capacity, increase the interval between voiding, and decrease the number of voids per day. Patients also found that it was an effective strategy, with 88% reporting at least some improvement in their symptoms [54].

Stress management techniques and psychological support are recommended as first-line treatments for the psychosocial-related symptoms of IC/BPS [1]. In addition to worsening symptoms of IC/BPS, untreated psychosocial co-morbidities like anxiety and depression can impact patient adherence to treatment [17]. These psychological treatments would typically be combined with other treatment strategies. For example, cognitive behaviour therapy combined with a patient's normal IC/BPS treatment has been shown to improve anxiety and decrease perception of symptom severity [55].

Various forms of physical therapy can also help IC/BPS symptoms, especially in patients who experience suprapubic tenderness. Physiotherapy and massage are recommended specifically for patients with pelvic floor dysfunction. Acupuncture can be beneficial, although it is unclear if the effect goes beyond placebo. Trigger point injections are sometimes used, but only for patients with trigger point pain [1].

While these less invasive first-line treatments can work for some patients, many patients require additional treatments on top of lifestyle changes. Pharmacological treatments for IC/BPS typically function via mechanisms that disrupt the cycle of damage, whether they target the urothelial GAG layer, block C fiber activation, modulate inflammation, or decrease pain [5]. These treatments are either taken orally or are administered directly into the bladder via a catheter, which is referred to as intravesical installation. A summary of commonly recommended pharmacological treatments can be found in Table 1.

The only pharmacological treatments currently approved by Health Canada to treat IC/BPS are oral pentosan polysulfate (PPS) and intravesical dimethyl-sulfoxide (DMSO) [1]. PPS is a GAG replacement therapy that also has anti-inflammatory effects and is typically recommended for BPS over HIC [1,28]. However, evidence for its ability to improve symptoms varies, especially over time periods longer than a year [1]. Some evidence indicates that long term use of PPS can lead to pigmentary maculopathy, although a recent review indicates that this may be due to IC itself rather than PPS use [56,57]. In addition, patients may not see symptom relief until 6-9 months into therapy, and current guidelines indicate that most patients are unlikely to see significant benefits [1,58]. DMSO is a chemical solvent that has shown both anti-inflammatory and analgesic effects in patients and is recommended to manage the urinary-related symptoms of IC/BPS [1]. It is suspected to work by relaxing bladder smooth muscle, blocking nerves, and dissolving collagen that could otherwise lead to fibrosis in the bladder [59,60]. DMSO tends to have a strong taste and smell, which can be uncomfortable and sometimes intolerable for patients [61]. While DMSO is considered safe, pain during and following its instillation causes patients to discontinue use [1,62]. Clearly, while these medications can be effective for managing IC/BPS, there are gaps in their effectiveness that lead patients to seek other treatment strategies.

There are a number of medications that are prescribed off-label to treat IC/BPS. For example, medications that target the nervous system, such as amitriptyline and gabapentin can be used. Amitriptyline is a tricyclic antidepressant that is commonly used to treat IC/BPS and is thought to work by blocking acetylcholine receptors and preventing reuptake of certain neurotransmitters, therefore dampening hyperactivity in the nervous system [63,64]. It is also known to decrease inflammation by blocking histamine H1 receptors [64]. Amitriptyline is recommended for BPS over HIC, however, due to somewhat varied results from clinical studies and a high rate of side effects, is only recommended after first-line treatments have failed [1]. Gabapentin is an anticonvulsant that is also used to treat chronic pain by modulating the activity of calcium channels in the central nervous system (CNS) [65]. Although it has not been well-studied in IC/BPS, it is effective at reducing pain in neuropathic pain conditions and does seem to effectively reduce pelvic pain [1,65]. Thus, it is primarily recommended for relief of neurologic symptoms [1].

Medications that target histamine release can be used, including cimetidine and hydroxyzine. Cimetidine is known to antagonize histamine H2 receptors and is also recommended after first-line treatment for management of BPS and neurologic symptoms, although there are only a few small studies that show its efficacy in reducing symptoms [1,66]. Hydroxyzine is a histamine H1 receptor antagonist that also blocks acetylcholine receptors [60]. It has more varied evidence for its efficacy, with some studies showing improvement, while others showed no difference, and widespread reports of side effects [1]. However, it was shown to be more effective when combined with PPS and is thus recommended primarily for both BPS and patients with a history of allergy [1,67].

Other medications like cyclosporine A and quercetin target the inflammatory processes underlying IC/BPS in different ways. Cyclosporine A blocks calcineurin to suppress T cell activation and prevent inflammatory cytokine release [60]. It has a somewhat small evidence base but seems to be effective, especially for patients with significant inflammation like HIC patients. This medication does have significant side effects that require close observation, so it is recommended only when many other treatment options have failed [1]. Quercetin is a bioflavonoid with anti-inflammatory

effects that is used to treat chronic pelvic pain syndrome in males [60]. Although its exact mechanism remains unclear, quercetin improved symptoms in the one study that examined its effects in IC/BPS and is recommended for BPS [1].

Intravesical treatments are another common way to manage the urinary-related symptoms of IC/BPS. These treatments are often used in combination, which is referred to as a "cocktail" [1]. Heparin, hyaluronic acid, and chondroitin sulfate are all theorized to target the GAG layer of the bladder, helping to restore its barrier function [60]. Heparin seems to be the most effective out of the three, likely due to its additional anti-inflammatory effects, but all are recommended as part of the treatment strategy rather than as monotherapies [1,60]. PPS, which also targets the GAG layer, can be administered intravesically, but this is recommended in addition to administration of its oral counterpart [1,63]. Oxybutynin is theorized to target the bladder wall, decreasing pain and blocking acetylcholine [1,60]. It also has no reported side effects but does not have a large evidence base [1]. Lidocaine is a local anesthetic that is recommended for short-term treatment of pain, such as during a flare [1,60].

Beyond lifestyle changes and pharmacological treatments, more invasive procedures like hydrodistension, treatment of Hunner lesions, and botulinum toxin A injections can be employed, especially when more conservative treatment options fail. Hydrodistension, where the bladder is stretched with water, is a commonly used treatment for BPS despite varied evidence for its efficacy. This treatment tends to decrease in efficacy over time and has serious side effects, especially with longer sessions, so is only recommended for short-term use at lower filling volumes [1]. Hunner lesions can be treated directly through a variety of functions including transuretheral electrocauterization or laser fulguration [63]. These strategies can produce a significant improvement in symptoms, but also have serious side effects and patients will require additional treatment when the lesions return [1]. Botulinum toxin A is thought to decrease central sensitization and dampen the release of neurological signaling molecules like nerve growth factor in the IC/BPS bladder [60,63]. It can improve symptoms and quality of life in BPS and is safe and effective for multiple injections, but its side effects can be serious and lead to the short-term need of a catheter [1].

Surgical procedures can also be used to treat IC/BPS. However, given their invasive nature and significant side effects, they are only employed after other treatments have been attempted. Sacral neuromodulation, which involves stimulating the nerve pathways that mediate information about bladder sensations, is somewhat effective at reducing neurologic and urinary symptoms for patients who are symptomatic even while on other treatments, but can have serious side effects and has a high rate of revision [1,68]. Radical surgery, which can involve cystectomy, bladder augmentation, and/or urinary diversion, is only considered when all other options have failed and the IC/BPS symptoms are having a significant negative impact on the patient's quality of life [1,63]. However, these procedures do seem to effectively reduce pain and improve urinary function, especially for patients with HIC [1].

There are a number of emerging experimental treatments for IC/BPS which are not yet widely available, but which show great potential to reduce symptoms. Phosphodiesterase-5 inhibitors have been effective at reducing symptoms in BPS without significant side effects. They cause blood vessels to dilate, which relaxes the bladder smooth muscle that tends to contract from the excessive potassium and acetylcholine present in IC/BPS [69]. Monoclonal antibodies like tanezumab, which blocks nerve growth factor, have anti-inflammatory effects and were shown to be effective at decreasing pain in a small study [42]. Intravesical liposomes are small phospholipid bilayers that are thought to create a protective coating over the damaged bladder urothelium, shielding it from further damage [32]. Cannabinoid receptor agonists are another emerging treatment strategy that can effectively decrease pain and inflammation in a variety of conditions through modulation of the endocannabinoid system (ECS) [1].

Table 1. Summary of pharmacological IC/BPS treatments.

Treatment	Mechanism of Action
Amitriptyline (oral)	Analgesic: blocks acetylcholine receptors and prevents neurotransmitter reuptake
Chondroitin sulfate (intravesical)	GAG replacement
Cimetidine (oral)	Anti-inflammatory: antagonizes histamine H2 receptors
Cyclosporine A (oral)	Anti-inflammatory: blocks calcineurin to suppress T cell activation and prevent inflammatory cytokine release
Dimethyl sulfoxide (intravesical)	Analgesic: blocks nerves and relaxes smooth muscle Anti-inflammatory: dissolves collagen
Gabapentin (oral)	Analgesic: modulates the activity of calcium channels in the central nervous system
Heparin (intravesical)	Anti-inflammatory GAG replacement
Hyaluronic acid (intravesical)	GAG replacement
Hydroxyzine (oral)	Anti-inflammatory: antagonizes histamine H1 receptor Analgesic: blocks acetylcholine receptors
Lidocaine (intravesical)	Analgesic: local anaesthetic
Oxybutynin (intravesical)	Analgesic: blocks acetylcholine
Pentosan polysulfate (oral/intravesical)	Anti-inflammatory GAG replacement
Quercetin (oral)	Anti-inflammatory

1.2 Cannabinoid Receptor Agonists

1.2.1 The Endocannabinoid System (ECS)

The ECS is a biological body system that plays an important role in many homeostatic mechanisms, including those in the nervous system, respiratory system, and cardiovascular system, among others. This leads to a wide range of effects, such as differences in mood, cognition, appetite, fertility, immune function, and pain [70,71]. The ECS is activated by ligands called cannabinoids, which can be further characterized based on their source. Endocannabinoids are synthesized within the body, phytocannabinoids are sourced from the cannabis plant, and synthetic cannabinoids are developed in a laboratory setting [72]. All of these cannabinoids mediate their effects through a variety of receptors, enzymes, and signalling pathways that make up the ECS.

The two main receptors in the ECS are cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), both of which are G protein-coupled receptors (GPCRs) with seven transmembrane domains [73,74]. These receptors share about 44% homology overall, with 68% homology in the transmembrane domains [74]. Both cannabinoid receptors are coupled to heterotrimeric Gi/o proteins [73]. CB1 receptors are more common in the CNS, especially on neural axons and axon terminals, where they mediate functions in memory, cognition, motor skills, and analgesia [75,76]. However, CB1 receptors are also expressed at lower levels in the periphery [75]. In contrast, CB2 receptors are typically found in the immune system and periphery, including on immune cells like mast cells and macrophages, as well as in the spleen, lung, and bladder [75,77,78]. CB2 receptors are upregulated in response to inflammation and tend to mediate effects on the immune response [75].

Beyond the main cannabinoid receptors, additional players, including nuclear receptors like peroxisome proliferator activated receptors (PPARs) and ion channels like transient receptor potential (TRP) channels can be activated by certain cannabinoids [79]. PPARs are located virtually everywhere in the body and mediate functions in metabolism, energy balance, and inflammation [80]. TRP channels are located on sensory neurons. They signal in response to physical sensations like pain and temperature, but additionally assist in immune function [81].

The ECS can be activated by a variety of endocannabinoids. The most well-known endocannabinoids include anandamide and 2-arachidonoylglycerol (2-AG). These ligands are synthesized on-demand from their membrane-bound precursor components upon receptor activation by certain neurotransmitters or by neuronal depolarization and increased intracellular calcium, where they are quickly released [73,76,79]. Anandamide and 2-AG activate both CB1 and CB2 receptors, which is a common feature of many cannabinoids, although anandamide has a higher affinity for CB1 receptors while 2-AG has similar affinity for both receptors [79,82,83]. These endocannabinoids also activate additional receptors beyond CB1 and CB2, leading to a variety of non-specific effects [75]. Endocannabinoids have a short half-life and are quickly taken up into cells and degraded by enzymes like fatty acid amide hydrolase (FAAH), cyclooxygenase-2 (COX-2), N-acetylethanolamine-hydrolyzing acid amidase (NAAA), monoacylglycerol lipase (MGL), and alpha-beta domain hydrolases 6 and 12 (ABHD6 and 12) [73,79,84–87].

In addition to the wide range of endocannabinoid-mediated effects, exogenous ligands, such as those phytocannabinoids found in the plant *Cannabis sativa*, activate the ECS in a similar manner. Ligands will often preferentially bind to either CB1 or CB2, although some, such as tetrahydrocannabinol (THC) will bind to both [73]. Activation of the ECS causes a variety of effects throughout the body, which are mediated by downstream signalling from mitogen-activated protein kinase (MAPK) activation and adenyl cyclase inhibition [76].

The ECS and CB2 receptors in particular are of interest in the treatment of painful inflammatory conditions like IC. CB2 receptors lack the undesirable cardiovascular, immune, and CNS-related side effects seen with CB1 receptor activation [70,72,88]. Their anti-inflammatory effects combined with their presence on both bladder and immune cells mean that they are well-positioned to mitigate the inflammatory dysfunction that drives IC. In addition, CB2 receptor activation has shown analgesic effects, particularly in models of inflammatory and neuropathic pain [88–90].

1.2.2 Cannabinoid Receptor 2 Activation

As a GPCR, CB2 receptor activation leads to signalling pathways mediated by G proteins. CB2 agonist binding causes signalling through coupled Gαi/o proteins, leading

to inhibition of adenylyl cyclase and decreased cAMP production [91]. This activation can also activate Gβγ subunits, leading to inhibition of voltage-gated calcium channels, activation of phosphoinositide-3-kinase, and activation of G protein-gated inwardly rectifying potassium channels. These G proteins also signal through the MAPK pathway, which leads to the extracellular signal-regulated kinase 1 or 2 (ERK1/2) activation that is commonly seen with CB2 receptor signalling [92]. This activation and subsequent signalling pathway lead to a number of downstream effects, including alteration of cytokine and/or chemokine expression, changes to adenosine signalling, modulation of cellular adhesion molecule expression, and modulation of inflammatory cell migration, proliferation, and apoptosis, all of which contribute to CB2's anti-inflammatory effects [93].

CB2 activation has a distinct advantage over CB1 for therapeutic purposes. Since CB2 receptors are not abundant in the CNS, their activation does not produce the psychoactive effects seen with CB1 receptor activation [88,93]. This makes CB2 receptors an excellent therapeutic target for conditions affecting peripheral organ systems like IC/BPS.

CB2 receptor activation has great potential for applications in pain and inflammation, as this receptor plays an important role in the immune response. CB2-knockout mice show excessive inflammation compared to controls [92]. CB2 receptor protein and mRNA expression is upregulated in the bladder mucosa and detrusor during animal models of acute and chronic bladder inflammation [94,95], indicating that CB2 receptors are a viable target for IC/BPS. CB2, but not CB1, agonists showed anti-inflammatory activity in a model of IC by reducing endothelial infiltration of leukocytes and the mRNA expression of inflammatory cytokines. These effects were shown to be CB2-specific, as administration of the specific CB2 receptor antagonist AM-630 blocked the anti-inflammatory activity [95].

CB2 receptors can be activated by a number of ligands, including those from the endocannabinoid, phytocannabinoid, synthetic cannabinoid, and terpene families. Some of the most well-known CB2 receptor agonists are the synthetic cannabinoids HU-308 and AM1241, which are highly selective for the receptor [75,88]. The phytocannabinoid

beta-caryophyllene (BCP) is a sesquiterpene that activates CB2 to produce therapeutic effects in a variety of inflammatory models [89,96].

1.2.3 Beta-Caryophyllene (BCP)

BCP is a non-psychoactive sesquiterpene found in many plants, including cloves, oregano, cinnamon, black pepper, various essential oils, and *Cannabis sativa* [96,97]. Given that it is found in many plant sources, this compound is widely consumed in food, but it is also used in cosmetic manufacturing [96,98]. BCP is also an emerging anti-inflammatory and analgesic agent and functions primarily through activation of CB2 receptors [89,96]. The chemical structure of BCP is shown in Figure 2.

In addition to being widely consumed, BCP is generally recognized as safe (GRAS) by the FDA, meaning that it is approved for use in food. However, it has also shown safety at therapeutic doses. BCP shows low cytotoxic activity *in vitro* [99]. This safety is also reflected in animal studies, with doses of 700 mg/kg for 90 days (in rats) and 2000 mg/kg for 28 days (in mice) showing no toxic or adverse effects [100,101]. Despite its relative safety, there is some evidence that BCP inhibits CYP3A in the liver, which should be taken into consideration if combining BCP with other drugs that are metabolized by CYP3A [102].

BCP is typically considered to be a selective CB2 receptor agonist, with a much higher affinity for CB2 (Ki = 155 nM) than for CB1. Activation of CB2 by BCP leads to suppression of Erk1/2 and JNK1/2 signaling and inhibition of pro-inflammatory cytokine expression [96]. However, in recent years there has been evidence suggesting that this compound can affect additional receptors. BCP has been shown to bind peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activate receptor gamma (PPARγ) [103,104]. This activation could contribute to BCP's pain-relieving effects, as CB2 and PPARα were shown to mediate analgesia in various tests of acute pain [105]. The activation of these additional receptors is involved in the anti-inflammatory effects of BCP, with both CB2 and PPARγ contributing to suppression of vascular inflammation [106]. In addition to its anti-inflammatory effects, PPARγ activation could contribute to urothelial wound repair [29,107]. The effects of BCP by activation of either PPARα or PPARγ in the absence of CB2 is typically not seen,

indicating that these receptors must work together in some capacity, although the mechanism of this effect and exact contribution of each receptor is unclear.

Opioid receptors are also implicated in BCP signalling. Various forms of the opioid receptor antagonist naloxone have been shown to reverse BCP's analgesic effects in animal models [108,109]. It is hypothesized that CB2 receptor activation leads to release of β-endorphin, which leads to activation of opioid receptors in sensory neurons and antinociception [109,110].

In addition to activation of various receptors, BCP has also shown local anesthetic activity. This has been demonstrated both *in vitro* and *in vivo*, although the effects are short-lived [111]. While the exact mechanism of this effect is unknown, local anaesthetic activity typically occurs through blocking the action of voltage-gated ion channels [112], and BCP has been shown affect some ion channels, including transient receptor potential vanilloid type-1 (TRPV1). TRPV1 is a nociceptor and cation channel found on primary sensory neurons and in the urothelium whose expression is affected by inflammatory mediators [77,113]. BCP differentially modulated TRPV1 expression in a rat model of neuroinflammation depending on the experimental condition [113]. In acute models of pain, black pepper seed extract containing at least 30% BCP produced an analgesic effect that was partially mediated by TRPV1, along with contributions from PPARα and CB2 [105].

BCP has effects on immune cell adhesion in the vasculature, which is an important step in the inflammatory process for peripheral tissues. BCP affects the innate immune system by decreasing the expression of adhesion molecules like E-selectin, P-selectin, and intercellular adhesion molecule 1 (ICAM-1), leading to decreased neutrophil infiltration into tissues [114,115]. BCP also downregulates expression of vascular cell adhesion molecule 1 (VCAM-1), which is an important endothelial adhesion protein in the vasculature that is upregulated in response to oxidative stress [106,116]. Taken together, the evidence shows that BCP acts at a number of steps in the leukocyte adhesion cascade to prevent immune cell infiltration into tissues.

In addition to its effects on immune cells, BCP also reduces inflammation by normalizing oxidative stress. One study found that oral administration of copaiba oil, of which BCP is the main constituent, was effective at reducing oxidative stress in arthritic

rats [117]. In addition, BCP administration during vascular inflammation increased endothelial nitric oxide synthase (eNOS) while decreasing inducible nitric oxide synthase (iNOS), returning the balance to homeostatic levels [106].

BCP is effective at restoring the balance of pro- and anti-inflammatory cytokines to homeostatic levels. BCP's effects on pro-inflammatory cytokines have been well-studied. BCP has been shown to reduce LPS-induced inflammatory cytokine expression of both interleukin 1 beta (IL-1β) and tumor necrosis factor (TNF) *in vitro* [96]. It also reduces both mRNA and protein expression of IL-6 and can reduce expression of interleukin 8 (IL-8) [118,119]. However, BCP can also increase expression of anti-inflammatory cytokines, including interleukin 13 (IL-13) [118].

Given these anti-inflammatory mechanisms, it should be no surprise that BCP has shown both analgesic and anti-inflammatory effects in models of inflammatory pain. For example, orally administered BCP reduced chronic, but not acute, inflammatory pain in mice [97]. Local BCP injection had analgesic effects in a mouse model of neuropathic pain [90], which could have beneficial effects for the nervous system-mediated aspects of IC. In fact, BCP has been shown to be effective at reducing IC/BPS symptoms in a mouse model. One study from our group using an IC model found that intraperitoneal, intravesical, and oral administration of BCP all reduced inflammation. In addition, the intravesical administration of BCP was superior to intravesical DMSO at reducing inflammation and the oral administration of BCP also reduced IC-related pain [89]. Studies administering BCP over several days showed no tolerance to its analgesic effects, demonstrating BCP's potential to treat chronic pain conditions [97].

Despite BCP's therapeutic potential, its applications are limited by its low solubility in aqueous media (including in many bodily fluids), as this results in low oral bioavailability [120]. While other routes of administration are possible, oral drug delivery is more convenient and less invasive for patients. Therefore, novel formulations that increase BCP's oral bioavailability would allow this compound to have a stronger effect on target tissues and systems.

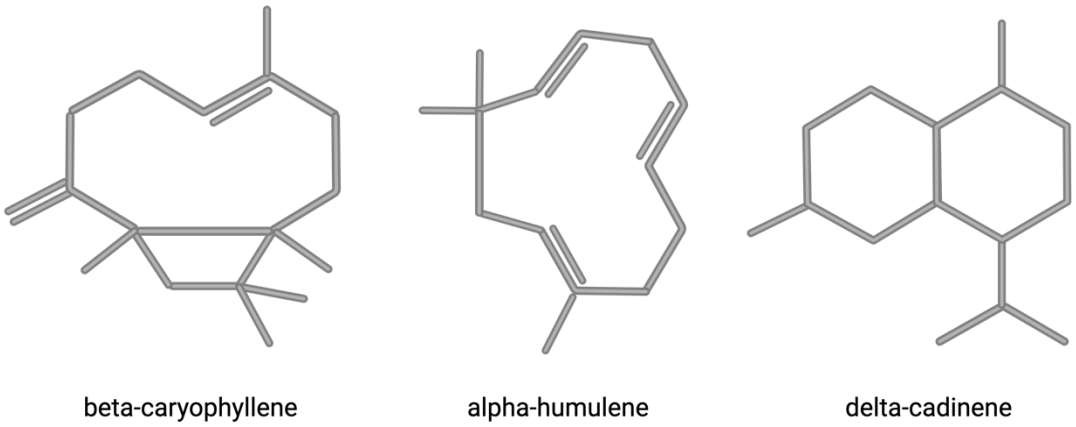

Figure 2. Chemical structure of the sesquiterpene beta-caryophyllene compared to other sesquiterpenes alpha-humulene and delta-cadinene.
Sesquiterpenes are 15-carbon compounds that consist of three isoprene units and are found in various plant sources. Created with BioRender.com.

1.3 Nanocarriers for Drug Delivery

1.3.1 Definition, Advantages, and Applications

Nanomedicine refers to the application of nanotechnologies for medical use and encompasses the use of all particles with at least one dimension less than 1000 nm, often referred to as nanocarriers [121]. Beyond this unifying characteristic, nanocarriers vary in their composition and their specific applications. Examples include organic-based nanocarriers like liposomes, micelles, dendrimers, phytosomes, nanoemulsions, solid lipid nanoparticles, as well as inorganic-based nanocarriers like gold or silver nanoparticles [122,123]. The most appropriate nanocarrier for a particular substance depends on a number of factors, such as the substance's size, its lipophilicity, the target organ or tissue, and the desired route of administration.

Nanoparticle drug formulations have great potential in the pharmaceutical industry. In general, they can increase the bioavailability of a particular compound, allowing for decreased loss to first-pass metabolism and more efficient delivery [124]. These effects are especially important for oral formulations, as entry though the gut provides unique challenges to drug delivery. Compounds must withstand the wide pH gradients, penetrate through the mucous layers, and then be taken up by the cells of the intestinal epithelium to reach the systemic circulation [123]. Nanocarrier formulations provide protection from this environment, increase permeability of the compound across membranes, and can even inhibit drug efflux transporters, all of which improve bioavailability [124]. Beyond the inherent challenges of reaching the circulation, many potential drugs do not dissolve well in aqueous solutions or do not permeate the gastrointestinal mucosa effectively [124,125]. The use of a nanocarrier protects the compound from degrading in the gut, which allows it more time to absorb into the circulation [124].

There are nanocarrier formulations currently available for a variety of applications. Doxil is a liposomal formulation of doxorubicin that was approved by the FDA in 1995 and allowed for improved bio-distribution and reduced side effects [126]. The Pfizer and Moderna Covid-19 vaccines used a lipid nanoparticle formulation to aid stability and ensure that the mRNA reached the immune system [127]. Phytosomal formulations are commercially available for phytochemicals like curcumin [128].

Although not yet commercially available, a variety of nanocarrier formulations have been studied for BCP delivery, including solid lipid nanoparticles, nanoemulsions, and liposomes [120].

1.3.2 Phytosomes

A phytosome is a type of nanoparticle that incorporates phytochemicals, which are plant-derived bioactive compounds, into the nanocarrier structure. These nanocarriers are made from phospholipids, most often phosphatidylcholine [128]. The phytochemicals are linked directly to the phosphate group of the phospholipid by hydrogen bonds and are on the outside of the structure rather than being contained within the nanoparticle [122,129]. This creates a phytosome-phospholipid complex, which is typically combined with cholesterol and a solvent to create a lipid bilayer in solution [130]. This lipid bilayer structure mimics the cell membrane and allows the phytosome to pass through biological tissues [128].

Since phytochemicals can have difficulty crossing biological membranes, are often not soluble in biological fluids, and have poor bioavailability overall, they are difficult to develop into drug formulations [131]. However, phytosomes have important characteristics that allow them to improve oral drug delivery. They protect the phytochemical from the digestive system, ensuring that the active constituent is not destroyed by digestive enzymes or gut bacteria [132]. The phospholipids are amphipathic, meaning they can dissolve in both hydrophobic and hydrophilic environments, and are an essential component of cellular membranes, allowing for more efficient transport across these membranes [131,133]. These factors allow the phytosome to increase the bioavailability of the phytochemical and reduce the dose required to produce a therapeutic effect [128]. In addition, the bond between the phytochemical and the phospholipid improves stability and entrapment efficiency [132], ensuring that as much of the active constituent reaches the region of interest as possible.

Beyond their advantages in drug delivery, phytosomes are also advantageous compared to other nanocarriers due to their safety. Certainly, factors like biocompatibility and bioaccumulation should be evaluated for every nanocarrier formulation, even if the phytochemical has been proven to be safe. However, the phytosome itself is made of

components that are normally found in the body, decreasing the likelihood of an immune response [128]. In addition, being made from biological materials found in the cell membrane means the phytosome is less likely to negatively impact the plasma membrane's function, which is a commonly cited cause of nanocarrier toxicity [134].

A number of phytosomal formulations are available on the market already, demonstrating that these formulations are both safe and effective. These phytosomes are varied in both their composition and their applications. Among other uses, anti-inflammatory, immunomodulatory, and antioxidant activity are frequently listed [128]. Phytosomal formulations of a variety of phytochemicals have shown safety and efficacy in clinical trials for cancer, nonalcoholic fatty liver disease, and chronic kidney disease [135–138].

Given the unique properties and advantages of phytosomes, a phytosomal formulation of BCP would be advantageous when considering oral drug delivery. A phytosome would provide increased protection from and stability in the gastrointestinal tract, allowing more of the compound to reach the systemic circulation. This is especially important for BCP, which has low solubility in aqueous media and is sensitive to acidic environments, both of which are characteristic of the gastrointestinal environment [120]. This formulation has the potential to greatly improve oral delivery of BCP, allowing for more convenient drug administration for the treatment of chronic inflammatory conditions like IC/BPS.

1.5 Hypothesis

Overall, we expect that BCP will show potential as an effective treatment for IC by decreasing inflammation and pain. First, we hypothesize that phytosomal BCP decreases pain and inflammation in an acute murine model of IC. Second, we hypothesize that BCP decreases measures of LPS-induced inflammation, including NO and inflammatory cytokine production, in 5637 bladder cell culture.

1.6 Objectives

Building on previous work in our lab [89], the overarching objective of this study was to examine the therapeutic potential of a novel BCP formulation for the treatment of IC.

First, we used a murine model of IC to examine BCP's anti-inflammatory and analgesic effects *in vivo*. We compared local inflammatory changes in the mouse bladder after LPS administration with and without BCP treatment using intravital microscopy (IVM) as well as H&E staining of the bladder. We assessed differences in inflammatory tissue cytokine levels in IC in treated vs untreated mouse bladders using an enzyme-linked immunosorbent assay (ELSIA) for IL-6. We then assessed the analgesic effects of BCP on IC *in vivo* using von Frey aesthesiometry and a behaviour scoring scale.

We then studied BCP's effects on the cellular inflammatory mediators released by bladder cells *in vitro*. We examined viability, NO production, and release of the cytokines IL-6, IL-8, and TNF. This allowed us to gain insight into some of BCP's potential mechanisms of action for the effects we saw *in vivo*.

Chapter 2: Materials and Methods

2.1 *in vivo* Work

2.1.1 Animals

All experimental procedures were approved by the University Committee on Laboratory Animals (protocol 21-032), in accordance with the guidelines developed by the Canadian Council on Animal Care. *in vivo* experiments used healthy female BALB/c mice from Charles River Laboratories International (Wilmington, Mississippi, USA) that were 8-12 weeks old and weighed 18-24 g. Mice were housed in the Carleton Animal Care Facility (CACF) in ventilated, climate-controlled rack cages rack cages on a 12-hour light/dark cycle with *ad libitum* access to water and standard mouse chow. All mice were acclimatized to the CACF for a minimum of one week before experimental procedures.

2.1.2 Timelines

The *in vivo* experiments were primarily completed over a period of about one day. Mice were pre-treated before IC induction and final measurements were made 24 hours after IC induction. However, mice in the pain and behaviour studies were acclimatized to the behaviour apparatus on two days preceding IC induction, for 15 minutes each day. On the day directly preceding induction, these mice were also acclimatized to the von Frey aesthesiometer. The timeline for these experiments can be found in Figure 3.

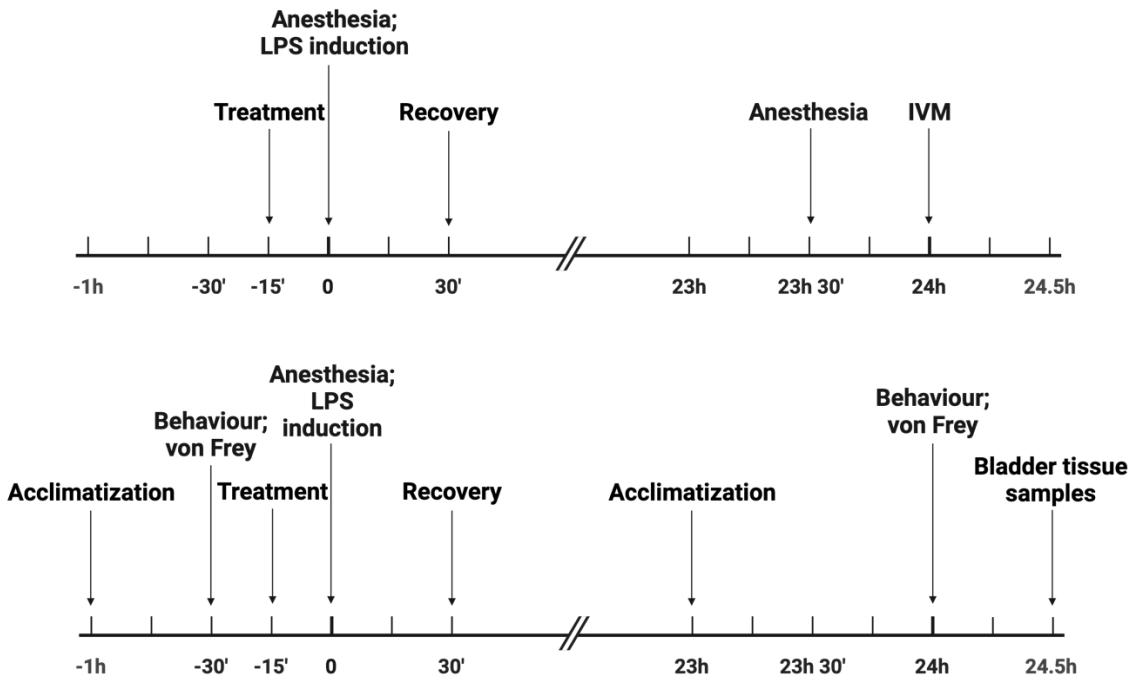

Figure 3. Experimental timelines for *in vivo* experiments.

Intravital microscopy (IVM) experiments (top) and pain and behaviour studies (bottom). Created with BioRender.com.

2.1.3 Experimental Groups

For *in vivo* experiments, animals were pre-treated with corn oil vehicle (VEH), empty phytosome in corn oil (PHY), or BCP phytosome in corn oil (PHY+BCP). They were then given an intravesical instillation of either normal saline for the sham groups (SHAM) or LPS for the IC groups (LPS). This resulted in six experimental groups: SHAM VEH, SHAM PHY, SHAM PHY+BCP, LPS VEH, LPS PHY, LPS PHY+BCP, as outlined in Table 2.

Table 2. *in vivo* experimental groups.

Group	Oral Treatment	Intravesical Instillation
SHAM VEH	Corn oil	Saline
SHAM PHY	Empty phytosome in corn oil	Saline
SHAM PHY+BCP	BCP phytosome in corn oil	Saline
LPS VEH	Corn oil	LPS
LPS PHY	Empty phytosome in corn oil	LPS
LPS PHY+BCP	BCP phytosome in corn oil	LPS

2.1.4 Interstitial Cystitis Induction

Mice were pre-treated via oral gavage with 100 mg/kg body weight (5 µl/g body weight) of phytosomal BCP (Indena, Milan, Italy) in corn oil, or the appropriate volume of corn oil (density: 0.92 g/cm^3; Sigma-Aldrich, ON, Canada) or empty phytosome (Indena, Milan, Italy) in corn oil. The phytosomal BCP and empty phytosome vehicle were reconstituted in corn oil before use. Each phytosomal BCP vial contained 12.5 µg of BCP and 40.5 µg of phytosome in 568 µl of corn oil, while the reconstituted phytosome vials contained 40.5 µg of phytosome in 581 µl of corn oil. Reconstituted vials were stored at 4ºC and discarded after 48 hours.

Mice were anesthetized using 5% isoflurane. Once the mouse was under anesthesia, it was transferred to a nose cone and anesthesia was maintained at 1.8-2.2% isoflurane. The mouse was situated on a heating pad in a supine position, with a piece of tape securing the position of the nose cone. A drop of tear gel was applied to both of the mouse's eyes using a clean cotton tip applicator. The mouse was monitored every five minutes via pedal reflex to ensure surgical depth of anesthesia.

Once the mouse was under anesthesia, a sterilized catheter could be inserted. The suprapubic area was gently pressed to empty any remaining urine from the bladder. Then, the urethral opening was sanitized using an alcohol swab. The urethral meatus was held using curved tweezers, and serrated tweezers were used to guide a sterile catheter into the urethra. Vaseline was used to lubricate the catheter. Catheters consisted of approximately 4 cm of gas-sterile polyethylene P10 tubing (inner diameter: 0.28mm, BD Intramedic, Sparks, MD, USA) on the head of a 30-gauge needle (0.3 mm x 13 mm, BD PrecisionGlide, Franklin Lakes, NJ, USA).

Once the catheter reached the bladder, mice were given 50 µl of either normal saline or *Escherichia coli* (*E. coli*) LPS (150 µg/ml, 0.375 mg/kg; serotype O26:B6, L8274; Sigma-Aldrich, ON, Canada) through the catheter using a 1 ml syringe [89]. This was administered over a period of ten seconds to minimize vesicoureteral reflux. When the full volume of liquid had been administered, the catheter was carefully removed, and the urethral opening was held shut with an aneurysm clip. The mouse was kept under anesthesia with the liquid in its bladder for 30 minutes.

After the hold period, the aneurysm clip was removed and the mouse was woken up. Mice were placed back in their cage, which was placed halfway on a heating pad, and given a mix of water and crushed food pellets (mash) during recovery. Mice recovered for approximately 24 hours before endpoint measurements were taken.

2.1.5 Intravital Microscopy

2.1.5.1 Anesthesia and Surgery

Approximately 23 hours and 30 minutes after IC induction, mice were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (90 mg/kg, 27.3 mg/ml; Ceva Sante Animale, Montreal, QC, Canada) using a 25-gauge 5/8-inch-long needle on a 1 mL syringe. Mice were placed on a heating pad to maintain their body temperature at 37ºC for the duration of imaging. Depth of anesthesia was confirmed by pedal reflex approximately five minutes after administration of pentobarbital. 0.1-0.2 mL of 5.47 mg/ml pentobarbital was used to maintain the level of anesthesia for the duration of the procedure.

Once surgical depth of anesthesia was achieved, fluorochromes were administered. This occurred about 23 hours and 45 minutes after IC induction. The tail was sanitized using an alcohol swab. A mix of rhodamine 6G (1.5 ml/kg, 0.75 mg/kg body weight; Sigma-Aldrich, ON, Canada), used to visualize leukocytes, and fluorescein isothiocyanate (FITC)-albumin (1 ml/kg, 50 mg/kg; Sigma-Aldrich, ON, Canada), used to visualize capillaries, were administered via tail vein injection using a 37-gauge 0.5 in needle head and a 1 mL syringe.

Upon successful injection of fluorochromes, the bladder was then exteriorized. To begin, the mouse was placed on its back. Scissors were used to make a midline incision of the skin of the lower abdomen, then the abdominal wall was cut to expose the abdominal cavity. The gut and surrounding tissues were moved aside using wet cotton tip applicators to fully expose the bladder. Care was taken to minimize damage to surrounding tissues, including avoiding cutting excess fat whenever possible. Any remaining urine was emptied by gently squeezing the bladder with wet cotton tip applicators. The bladder was kept moist by frequent administration of saline.

The mouse was then catheterized using a sterilized catheter. Vaseline was applied to the end of the tubing. Curved tweezers were used to hold the ureteral meatus open, while ridged tweezers were used to slowly guide the catheter up the urethra and into the bladder. 100 µl of saline was slowly administered into the bladder, then the catheter was carefully removed, and an aneurysm clip was immediately used to hold the ureteral opening shut for the duration of imaging.

With the bladder exteriorized and filled, final preparations for imaging could be made. The bladder was moistened with saline and an 18 mm round glass coverslip was placed on top to facilitate imaging. The coverslip was held in place with the end of a pair of curved forceps placed on the glass. The mouse and heating pad were transferred over to the microscope stage.

2.1.5.2 Microscopy

Imaging took place 24 hours after IC induction using an epifluorescent microscope (Leica, DM LM, Wetzlar, Germany). Bladders were visualized using the 20x objective (Leica, Germany) with a 10x eyepiece (HC Plan, Leica, Germany).

Green light, passing through a 530-550 nm bandpass excitation filter was used to excite rhodamine (emission wavelength 515 nm) and visualize leukocyte trafficking in venules. Blue light passing through a 460-490 nm bandpass excitation filter was used to excite FITC (emission wavelength 520 nm), which allowed visualization of functional capillary density (FCD) in the microcirculation. A minimum of six visual fields per light colour were captured for a period of 30 seconds each using Volocity software (Perkin Elmer, Waltham, MA, USA).

Once imaging was complete, animals were sacrificed by a pentobarbital overdose, which was confirmed by cervical dislocation.

2.1.5.3 Video Analysis

IVM videos were captured using a digital EM-CCD camera C9100-02 with AC-adapter A3472-07 (Hamamatsu, Herrsching, Germany). These videos were then loaded onto a hard drive to be analyzed offline using ImageJ software (NIH, USA). All videos were analyzed in a blinded fashion.

Leukocyte adhesion was determined by outlining a section of a submucosal venule which was calculated as a cylindrical surface area. The number of leukocytes within that area that did not move over the 30 second capture period (defined as "adherent leukocytes") were counted and divided by the total area to give a measurement of number of adherent leukocytes per mm^2.

Leukocyte rolling was determined by quantifying the number of leukocytes that passed a set point over the 30 second capture period. A random submucosal venule was chosen, and a line was drawn across its lumen using ImageJ software. The number of leukocytes passing that line was counted and converted to a value of number of rolling leukocytes per minute. Representative images for leukocyte activation can be found in Figure 4.

FCD was determined by measuring capillary perfusion over a set area. A rectangular section of the video area was marked, and the area measured. Within that area, the distance each capillary that perfused over the 30 second capture period was marked using the ImageJ software and the area measured. The distance each capillary perfused was summed and divided by the total area observed to give a measurement of FCD in cm/cm^2. Representative FCD images can be found in Figure 5.

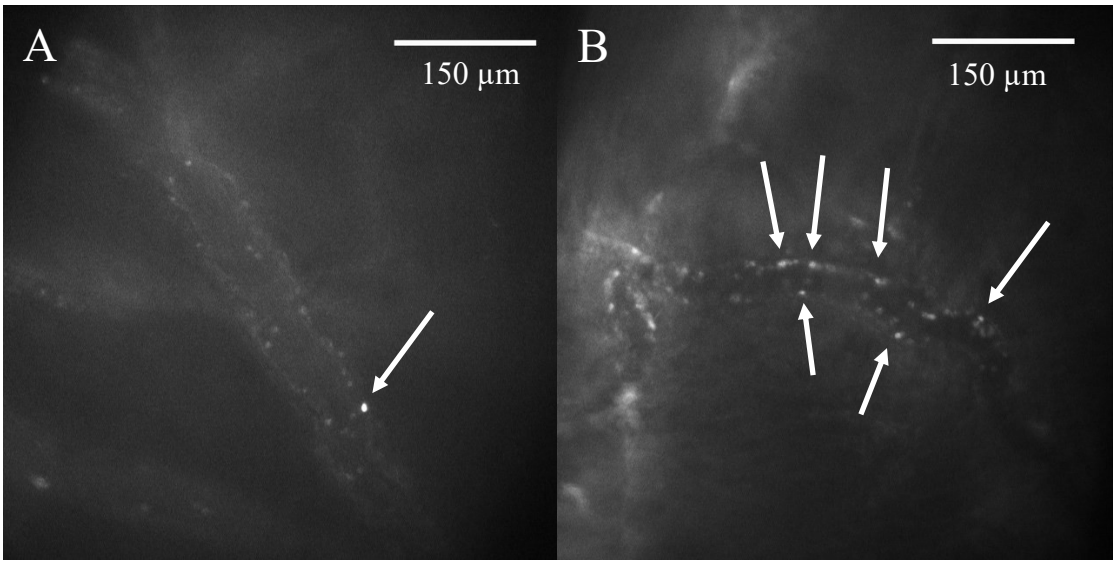

Figure 4. Still-frame intravital microscopy images of activated leukocytes within the submucosal venules of the bladder microcirculation in female BALB/c mice. Images represent (A) a control animal and (B) an untreated LPS animal at a magnification of 200x. Scale bars indicate 150 µm. Arrows indicate adherent leukocytes.

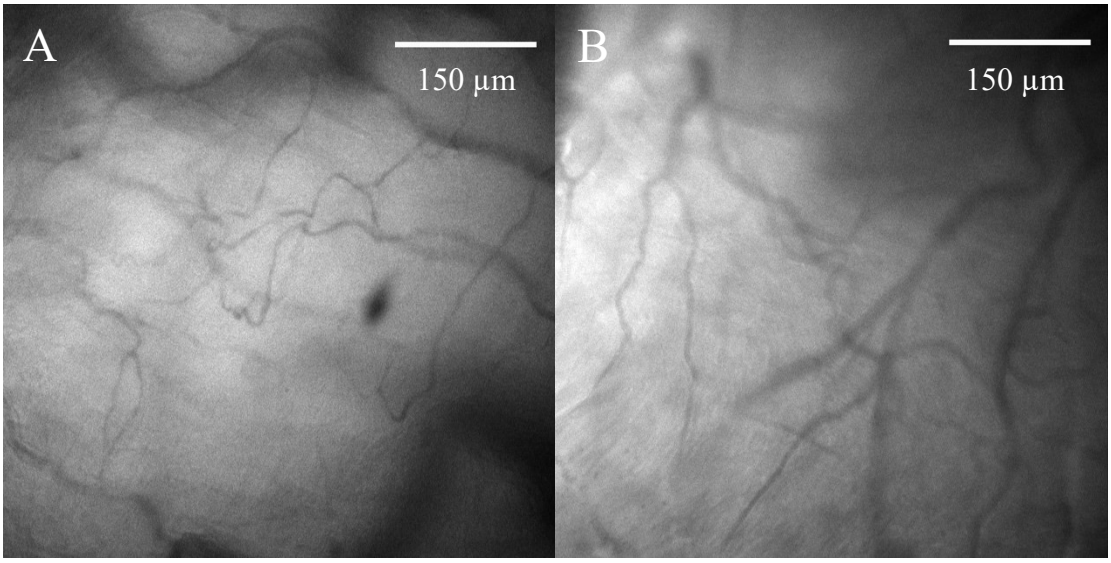

Figure 5. **St**ill-frame intravital microscopy images of capillary perfusion within the bladder microcirculation of female BALB/c mice.

Images represent (A) a control animal with dense microvasculature and (B) an untreated LPS animal with reduced number of perfused capillaries at a magnification of 200x. Scale bars indicate 150 µm.

2.1.6 Pain Assessment

2.1.6.1 Acclimatization

Mice were acclimatized to a plexiglass enclosure with a mesh floor (IITC Life Sciences, Woodland Hills, CA, USA) for 15 minutes per day on the two days leading up to the experimental procedures. They were placed in the von Frey enclosure, consisting of an elevated mesh floor and a clear plexiglass separator with enough room for each mouse. A paper towel was placed below the mesh floor to ensure cleanliness. The enclosure was in a dim, quiet room with only the observer was present. On the second day of acclimatization, mice were poked in the lower abdomen three times using the von Frey filament.

Directly preceding the induction of IC and endpoint tissue collection, mice were placed in the enclosure for a baseline pain assessment. They were allowed to acclimatize to the enclosure for a minimum of one hour, with the observer entering the room at least 15 minutes before making the first measurements.

2.1.6.2 Behaviour Scoring

A blinded examiner scored the mice separately on eye opening, posture, and motoric activity. Scores for each individual parameter ranged from zero to 10, with zero indicating no evidence of pain and 10 being maximum evidence of pain, as outlined in Table 3. The three scores were then added together into a single score out of 30. Scores taken directly preceding the endpoint were subtracted from initial scores to give the change in behaviour score over the experimental timeframe for each mouse.

Table 3. Behaviour scoring scale.

Individual parameters for each mouse were scored before and after cystitis induction according to the description, then totaled to give a score out of 30. Levels of eye opening and posture that fell between the extremes were given intermediate, whole number scores.

Parameter	Description
Eye opening	0: eyes fully open 5: eyes halfway open 10: eyes fully closed
Posture	0: normal posture 10: fully rounded back or limp posture
Motoric activity	Level of activity within 20 seconds; every 2 seconds spent not moving adds one point to the score (2s of stillness/20s = score of 1/10)

2.1.6.3 von Frey Aesthesiometry

Following the behaviour assessment, evoked pain was measured using a 2390 series IITC Life Science Electronic von Frey aesthesiometer (Woodland Hills, California, USA). Using the rigid tip, the von Frey aesthesiometer was applied to the lower abdomen of the mouse with increasing force until the mouse withdrew. The maximum force tolerated before withdrawal (in grams) was recorded. In the case of the tip hitting the mesh or missing the lower abdomen, the measurement was discarded and a new measurement was taken in its place. We collected a minimum of five measurements per mouse, with a minimum of 30 seconds between measurements. Measurements were averaged for each timepoint, and then the average of the scores taken directly preceding the endpoint were subtracted from the average of the initial scores to give the change in pain tolerance over the experimental timeframe for each mouse. Once the measurements were complete, mice were returned to their cages and we proceeded with either IC induction or endpoint tissue collection.

2.1.6.4 Euthanasia and Tissue Collection

Mice were euthanized with a pentobarbital overdose approximately 24 hours and 30 minutes after IC induction. The lower abdominal cavity was cut open, first through the skin, then through the muscle and fat to expose the bladder. The bladder was drained of any remaining urine by squeezing with clean cotton swabs. The bladder was then held in place with tweezers and detached from the surrounding tissue, including from the ureters, by cutting with scissors.

Bladder samples were placed on a clean surface and sliced in half using a small razor blade. One half of the bladder was placed in a histology cassette and stored in 10% neutral buffered formalin for histological scoring, while the other half was placed in a 1.5 mL Eppendorf and flash frozen in liquid nitrogen for cytokine analysis via ELISA.

2.1.7 Histology

2.1.7.1 Tissue Processing, Staining and Cutting

Bladder samples were kept in 10% neutral buffered formalin for at least 24 hours before being transferred into 70% ethanol.

Tissue samples were processed, embedded, sliced, and stained using hematoxylin and eosin (H&E) by the IWK Department of Pathology and Laboratory Medicine. Two 5 µm cross-sections of each sample were cut from each paraffin-embedded tissue block, which were then H&E stained and scored.

2.1.7.2 Bladder Histopathology Scoring

Bladder histopathology slides were viewed by a an Optika B-290TB brightfield microscope (Optika Microscopes, Ponterica, Italy) on the 40x objective. The sample with the most clearly visible tissue structures was chosen for scoring. If both samples on the slide were similarly easy to visualize, the sample was chosen at random. Samples were scored based on a scoring system adapted from Hopkins et al. 1998 [139], with modifications based on suggestions from Dr. Cheng Wang (Departments of Pathology and Urology, Dalhousie University, Halifax, Nova Scotia, Canada), as seen in Table 4.

For scoring, samples were divided into four quadrants, with the middle of the bladder lumen as the center point of the quadrants. Each sample was scored individually based on the scale above, then the individual scores were averaged to produce an overall score for the sample.

Table 4. Histology scoring scale.

Score	Criteria
0	No signs of inflammation; inflammatory cells primarily contained within vessels.
1	Focal infiltration of inflammatory cells into the subepithelium.
2	Criteria of score 1 plus multifocal infiltration of inflammatory cells in the subepithelium; may have signs of mild edema.
3	Criteria of score 2 plus diffuse infiltration of inflammatory cells in the subepithelium and edema; may have signs of neutrophil necrosis.
4	Criteria of score 3 plus infiltration of inflammatory cells into the mucosa.
5	Criteria of score 4 plus infiltration of inflammatory cells into the muscle layer.
6	Criteria of score 6 plus loss of surface epithelium.

2.1.8 Tissue Cytokine Evaluation

2.1.8.1 Tissue Processing

Before the ELISA and BCA were performed, bladder samples were homogenized. Samples were removed from the -80ºC freezer and thawed on ice in tubes containing 100 µl of Tissue protein extraction reagent (T-PER; ThermoFisher, Grand Island, NY, USA) before the start of the assay.

Samples were homogenized in 2 ml tubes filled with 2.8 mm ceramic beads (model 19-628; Omni International, Kennesaw, GA, USA) using a PowerLyzer 24 bead mill homogenizer (Qiagen Sciences, MA, USA) at 5 m/s for three cycles of 30 seconds, with 10 seconds between cycles. After homogenization, an additional 100 µl of T-PER containing a protease inhibitor (10 ml T-PER per 1 protease inhibitor tablet; ThermoFisher, Grand Island, NY, USA) was added. Samples were then centrifuged for 15 minutes at 4ºC and 5000 g. Samples were then stored in 1.5 ml Eppendorf tubes at -80ºC until needed.

2.1.8.2 Bicinchonic Acid (BCA) Protein Assay

Total protein content was quantified in the bladder samples using the Pierce Bicinchonic Acid (BCA) Protein Assay Kit (ThermoFisher, Grand Island, NY, USA) before the ELISA was performed. The assay was performed according to manufacturer's instructions.

First, the standards, reagents, and samples were prepared. While the samples thawed on ice, the bovine serum albumin (BSA) standards were prepared. A 1 ml ampule of 2 mg/ml Albumin standard that serially diluted in T-PER to give eight standards at concentrations of 2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, and 25 µg/ml. A blank standard of only T-PER was also prepared. The working reagent was prepared by mixing 50 parts of BCA Reagent A with one part of BCA Reagent B. The samples were diluted 1:8 using T-PER.

25 µl of each standard or sample dilution was pipetted in duplicates into a 96-well plate. 200 µl of working reagent was added to each well and the plate was placed on a plate shaker at room temperature for 30 seconds. The plate was then covered and

incubated for 30 minutes at 37ºC. After the incubation period, the plate was cooled to room temperature and the absorbance was measured at 562 nm.

The absorbance value from the blank well was subtracted from each of the standard and sample wells to provide a corrected mean absorbance for each sample. The mean absorbance was then multiplied by the dilution factor to calculate the total protein concentration.

2.1.8.3 ELISA

Tissue levels of IL-6 were then quantified using a mouse IL-6 ELISA kit (Invitrogen, Waltham, MA, USA) over a three-day period according to manufacturer's instructions.

The first day of the procedure was used to coat the plate. The coating buffer was diluted 1:10 in distilled water. The capture antibody was then diluted 1:250 in this diluted coating buffer. 100 µl of this solution was added to each well of a 96-well plate. The plate was then covered and incubated at 4ºC on a plate shaker set to 200 RPM overnight. The assay diluent was also prepared by diluting the concentrate 1:5 in distilled water.

The samples were thawed on ice while the plate was prepared. The wells were emptied and washed out three times using wash buffer, which was 0.05% Tween-20 in PBS. Each wash required filling the wells with wash buffer (300 µl per well) and agitating the plate for one minute. After the final wash, the plate was inverted and tapped against a paper towel to remove any remaining buffer without allowing the wells to dry fully. 200 µl of assay diluent was then added and the plate was incubated for one hour at room temperature and 200 RPM to block the wells.

While the plate incubated, the standards and samples were prepared. The standard was reconstituted by adding the recommended volume of distilled water (as per manufacturer's instructions) to the vial and gently swirling. The standard was allowed to rest for 10-30 minutes for full reconstitution. The set of eight standards was prepared by serial dilution with assay diluent, where the top standard was 500 pg/ml and each subsequent standard was half the concentration of the previous standard. A blank standard containing only assay diluent was also prepared. The thawed samples were centrifuged for ten minutes at 4ºC and 2000 g before being returned to ice.

After the incubation period, the plate was washed as described above. 50 µl of either sample or standard was added to each well in duplicates. The plate was covered again and incubated overnight at 4ºC and 200 RPM.

On the third day, the plate could be visualized. The detection antibody and Avidin-HRP enzyme concentrate were both diluted 1:250 in assay diluent. The plate was washed again as described above. 100 µl of detection antibody was added per well, then the plate was covered and incubated for one hour at room temperature and 200 RPM. After the incubation, the plate was washed again and 100 µl of enzyme was added per well. The plate was again incubated at room temperature and 200 RPM, this time for 30 minutes.

The plate was washed again, this time for five washes, and 95 µl of tetramethylbenzidine (TMB) substrate solution was added to each well. The plate was covered again and incubated for 10-20 minutes at room temperature and 200 RPM. The wells were monitored for colour change. Once the standards were clearly differentiated, 100 µl of 1M HCl stop solution was added to each well.

The plate was read at 450 nm within ten minutes to avoid precipitation. The sample data points were adjusted based on the sample dilutions. The adjusted ELISA values were then normalized for total IL-6 protein levels by dividing them by the calculated BCA value for each sample.

2.2 *in vitro* Work

2.2.1 Cell Culture

5637 bladder epithelial carcinoma cells from American Type Culture Collection (ATCC; Cedarlane Labs, Burlington, ON, Canada), which were isolated from the urinary bladder of a 68-year-old white man with grade II carcinoma, were used for all *in vitro* experiments. All procedures were completed using aseptic technique. Cells were grown under standard conditions (37ºC and 5% CO_2 with a humidified atmosphere) in complete Roswell Park Memorial Institute (RPMI) media, which consisted of RPMI 1640 containing 10% fetal bovine serum (ThermoFisher, Grand Island, NY, USA). Media was refreshed as required.

Once cells were 80% confluent, they were split into separate flasks. The media was removed and the flask was rinsed with about 5 ml of warmed 0.25% trypsin

(ThermoFisher, Grand Island, NY, USA). Enough trypsin to cover the bottom of the flask (about 3 ml) was placed on the cells and the flask was incubated under standard conditions until the cells detached from the flask, which typically took ten minutes. Once the majority of the cells detached, the trypsin was neutralized by adding the same volume of complete RPMI media to the flask. The cells were then counted using trypan blue exclusion dye and the media containing the cells was then split between multiple flasks, each containing additional complete RPMI media.

2.2.2 Viability Assay

Cells were seeded in a 96 well plate at a density of 10 000 cells per well and incubated under standard conditions in serum-free RPMI media overnight. Media was removed from the cells and discarded. Cells were washed with about 100 µl of sterile phosphate buffered saline (PBS). Cells were then incubated for one or three hour(s) with serum-free RPMI media and either LPS or BCP.

For the LPS groups, dilutions of 5 µg/ml, 10 µg/ml, 50 µg/ml, and 100 µg/ml of LPS were made. Cells were given 10 µl of the appropriate LPS dilution and 90 µl of media, which produced final LPS concentrations of 0.5 µg/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml. Control cells were given 10 µl of saline in place of LPS.

For BCP, final concentrations of 50, 100, 250, and 500 µg/ml were used. Each well was given the appropriate volumes of media containing either BCP or vehicle control (Tween-80) outlined in Table 5 in addition to 50 µl of serum-free RPMI media.

Once the incubation period was complete, the media was removed and cells were washed again with PBS. They were then given 90 µl of serum free RPMI media and 10 µl of Cell-Counting Kit-8 (CCK-8) dye (APExBIO, Houston, TX, USA). Cells were then incubated for an hour under standard conditions.

After the incubation, the level of cell death was quantified using a Cytation 5 plate reader set to a wavelength of 450 nm using Gen5 software (BioTek Instruments Inc, Winooski, VT, USA). Absorbance under each condition was normalized to give a percent viability compared to the positive control. Each experiment was run in duplicates and was completed a minimum of three separate times.

Table 5. *in vitro* experimental setup for BCP viability experiments.

Group	BCP Media Volume (µl)	Tween-80 Media Volume (µl)
Control	0	50
50 µg/ml BCP	5	45
100 µg/ml BCP	10	40
250 µg/ml BCP	25	25
500 µg/ml BCP	50	0

2.2.3 Nitric Oxide Assay

Before the assay could be completed, samples were collected. Each experiment was run in duplicates and was completed a minimum of three separate times. Cells were seeded in a 24-well plate at a density of 250 000 cells per well. They were incubated overnight at standard conditions and serum starved to synchronize the cell cycles.

Media was removed from the cells and discarded. Cells were washed with 500 µl of warmed D-PBS, which was also discarded. Cells were then incubated for three hours at 37ºC and 5% CO_2 with a mixture of LPS and serum-free RPMI media. LPS stock was diluted to 100 µg/ml. Cells were then given the appropriate volume of LPS and PBS according to Table 6, resulting in final LPS concentrations of 0.5 µg/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml. Cells also received 450 µl of serum-free RPMI media.

Samples were collected after one or three hours of incubation. Cells were washed twice with D-PBS as above before being lysed with 100 µl of ice-cold assay buffer from the assay kit (Abcam Nitric Oxide Assay Kit (ab65328); Waltham, MA, USA), which was pipetted up and down to facilitate lysis. Samples were centrifuged for 5 minutes at 10000 RPM and 4ºC. The duplicates were pooled for sample collection and samples were stored at -80ºC until the date of the assay.

The NO assay was completed as per manufacturer's instructions. Standards were prepared fresh at the appropriate concentrations (ranging from 2-10 nMol) while samples were thawed on ice. Standards and samples were added to a flat-bottom 96 well plate in duplicate, with assay buffer making up the remaining volume to a total of 85 µl. Blank wells containing only assay buffer were also added. 5 µl each of nitrate reductase and enzyme co-factor were added to each standard and sample well. The plate was then incubated in the dark at room temperature for one hour. 5 µl of enhancer was added to each standard and sample well and the plate was incubated at room temperature for 10 minutes. 50 µl each of Griess Reagent I and II were added to each standard and sample well immediately before measurement. The concentration of NO was quantified using a plate reader at an optical density of 540 nm. The absorbance for each well was adjusted based on blank controls and the standard curve was then used to determine the concentration of NO in each sample.

Table 6. *in vitro* experimental setup for the viability assay.

Group	LPS Volume (µl)	PBS Volume (µl)
Control	0	50
0.5 µg/ml LPS	2.5	47.5
1 µg/ml LPS	5	45
5 µg/ml LPS	25	25
10 µg/ml LPS	50	0

2.2.4 ELISA

Cells were treated according to section 2.2.3, with collection of supernatant directly following the one or three-hour incubation. Duplicates were pooled and samples were stored at -80ºC until the ELISA was run.

Cell serum levels of IL-6, IL-8, and TNF were quantified using human ELISA kits for their respective cytokines (Invitrogen, Waltham, MA, USA) according to manufacturer's instructions, following the same protocol in section 2.1.8.3. The kits varied slightly in standard concentration and the enzyme used depending on the cytokine examined. The top standard was 200 pg/ml for the IL-6 kit, 250 pg/ml for the IL-8 kit, and 500 pg/ml for the TNF kit. The IL-6 and TNF kits used streptavidin-HRP for the enzyme, while the IL-8 kit used avidin-HRP for the enzyme.

The plates were all read using a plate reader at 450 nm. The standard wells were used to generate a curve, which was then used to calculate the concentration of IL-6, IL-8, or TNF in each sample well.

2.3 Statistical Analysis

All statistical analysis was completed using GraphPad Prism 10 (GraphPad Software, La Jolla, CA, USA). Data was first analyzed for normality using the Kolmogorov-Smirnov Test. Outliers were removed using the ROUT method. Data was analyzed for significance using a one-way ANOVA with Dunnett's test for post-hoc analysis. Comparisons between two groups were analyzed using a one- or two-tailed t-test. A p-value of less than 0.05 was considered statistically significant and data was expressed as mean plus or minus standard deviation.

Chapter 3: Results

3.1 The Effect of Phytosomal Beta-Caryophyllene on Lipopolysaccharide-Induced Bladder Inflammation in Female BALB/c Mice

3.1.1 Intravital Microscopy

Phytosomal BCP was effective at reducing inflammation within the bladder microvasculature as evaluated using our primary IVM parameter, leukocyte adhesion (Figure 6). 0.375 mg/kg of LPS significantly increased leukocyte adhesion ($p<0.05$) in the bladder microvasculature of mice compared to control when observed 24 hours after experimental IC induction. When mice were pre-treated with 100 mg/kg of phytosomal BCP, leukocyte adhesion was significantly decreased ($p<0.05$) compared to untreated LPS controls. IC mice treated with the empty phytosome vehicle control showed decreased leukocyte adhesion in the bladder microvasculature compared to untreated mice, but this difference was not statistically significant. No significant differences were observed between sham groups.

For our secondary IVM parameters, no significant differences were observed between groups. There were no differences between sham groups or with untreated LPS compared to both control and treatment groups with either leukocyte rolling (Figure 7) or FCD (Figure 8).

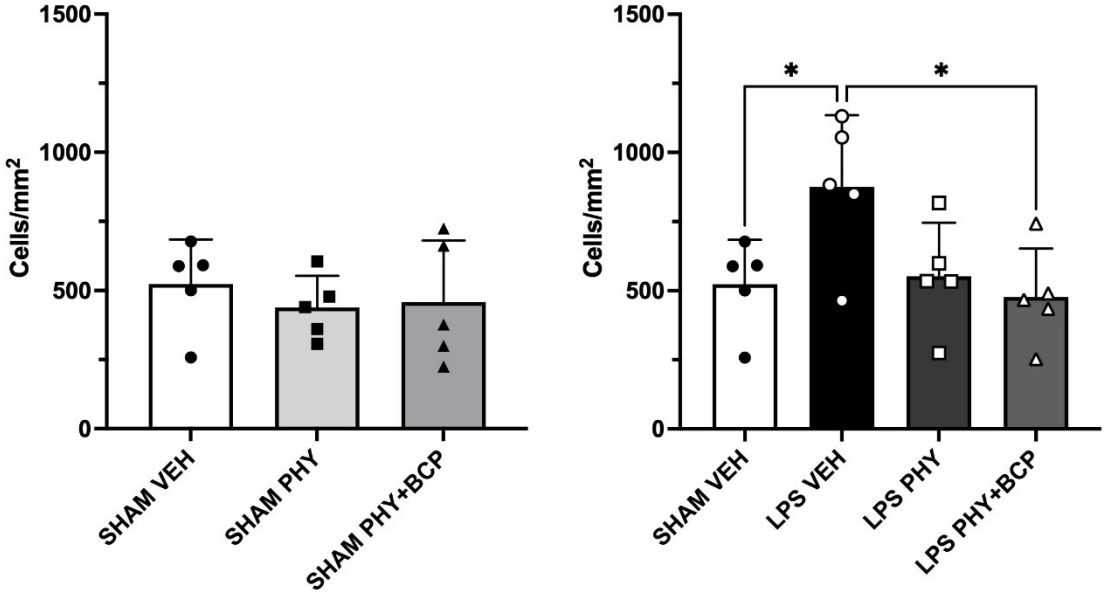

Figure 6. 100 mg/kg of phytosomal BCP reduces leukocyte adhesion in submucosal bladder venules of female BALB/c mice 24 hours after IC induction.

Leukocyte adhesion was assessed using intravital microscopy in the following groups: sham IC with vehicle control (SHAM VEH), sham IC with empty phytosome (SHAM PHY), sham IC with phytosomal BCP (SHAM PHY+BCP), IC with vehicle control (LPS VEH), IC with empty phytosome (LPS PHY), and IC treated with phytosomal BCP (LPS PHY+BCP). Data is in cells/mm^2 and is represented as mean ± SD, n=5. Statistical analysis by one-way ANOVA with multiple comparisons, *$p<0.05$.

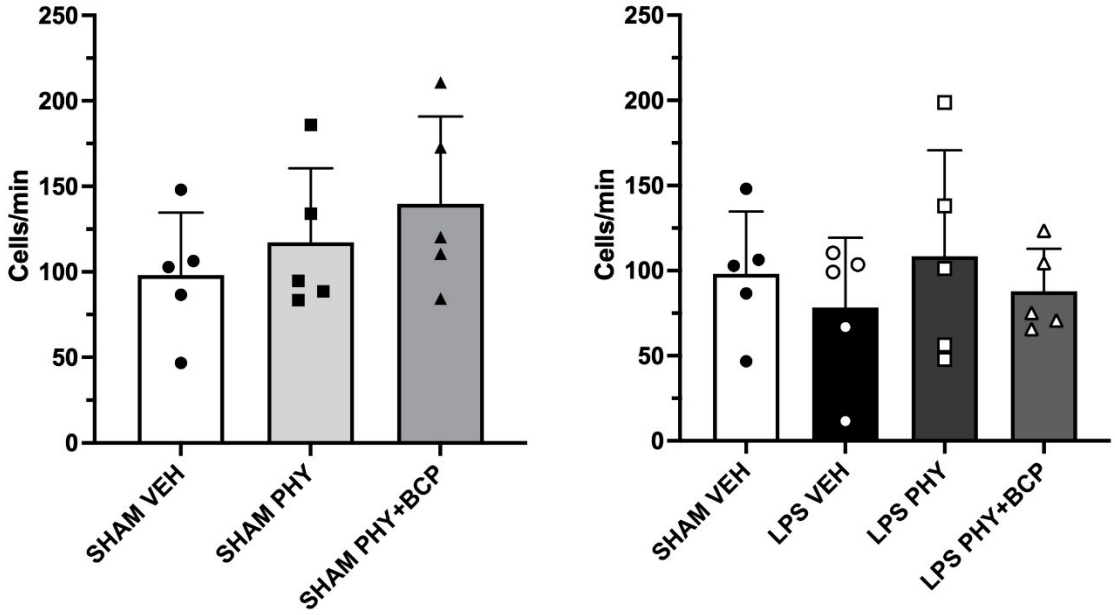

Figure 7. 0.375 mg/kg of LPS does not alter leukocyte rolling in submucosal bladder venules of female BALB/c mice after 24 hours.

Leukocyte rolling was assessed using intravital microscopy in the following groups: sham IC with vehicle control (SHAM VEH), sham IC with empty phytosome (SHAM PHY), sham IC with phytosomal BCP (SHAM PHY+BCP), IC with vehicle control (LPS VEH), IC with empty phytosome (LPS PHY), and IC treated with phytosomal BCP (LPS PHY+BCP). Data is in cells/min and is represented as mean ± SD, n=5.

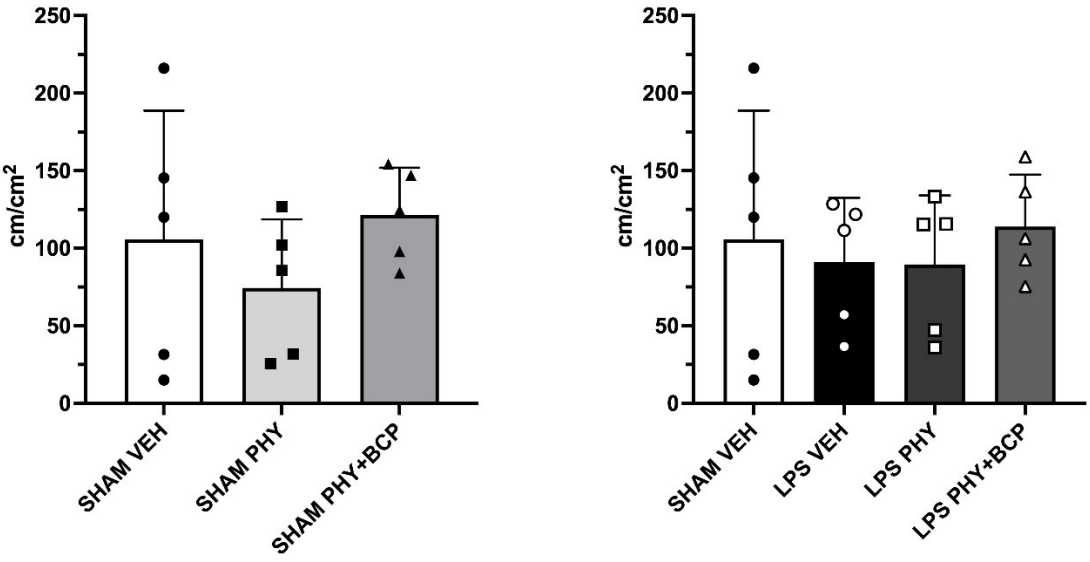

Figure 8. 0.375 mg/kg of LPS does not alter functional capillary density in submucosal bladder venules of female BALB/c mice after 24 hours.

Functional capillary density was assessed using intravital microscopy in the following groups: sham IC with vehicle control (SHAM VEH), sham IC with empty phytosome (SHAM PHY), sham IC with phytosomal BCP (SHAM PHY+BCP), IC with vehicle control (LPS VEH), IC with empty phytosome (LPS PHY), and IC treated with phytosomal BCP (LPS PHY+BCP). Data is in cm/cm^2 and is represented as mean ± SD, n=5.

3.1.2 Bladder Histopathology

Phytosomal BCP was effective at reducing histological signs of inflammation within the mouse bladder (Figure 9). 0.375 mg/kg of LPS significantly increased histology scores ($p<0.01$) in mouse bladders 24 hours after experimental IC induction. When mice were pre-treated with 100 mg/kg of phytosomal BCP, histology scores were significantly decreased ($p<0.05$) compared to untreated LPS controls using a one-tailed t-test. Bladders from the IC mice treated with the empty phytosome vehicle control had histology scores that fell between the scores for untreated and phytosomal BCP treated groups, but the difference was not statistically significant from either group. No significant differences were observed between sham groups. Representative histology images showing normal and inflamed bladders are shown in Figure 10.

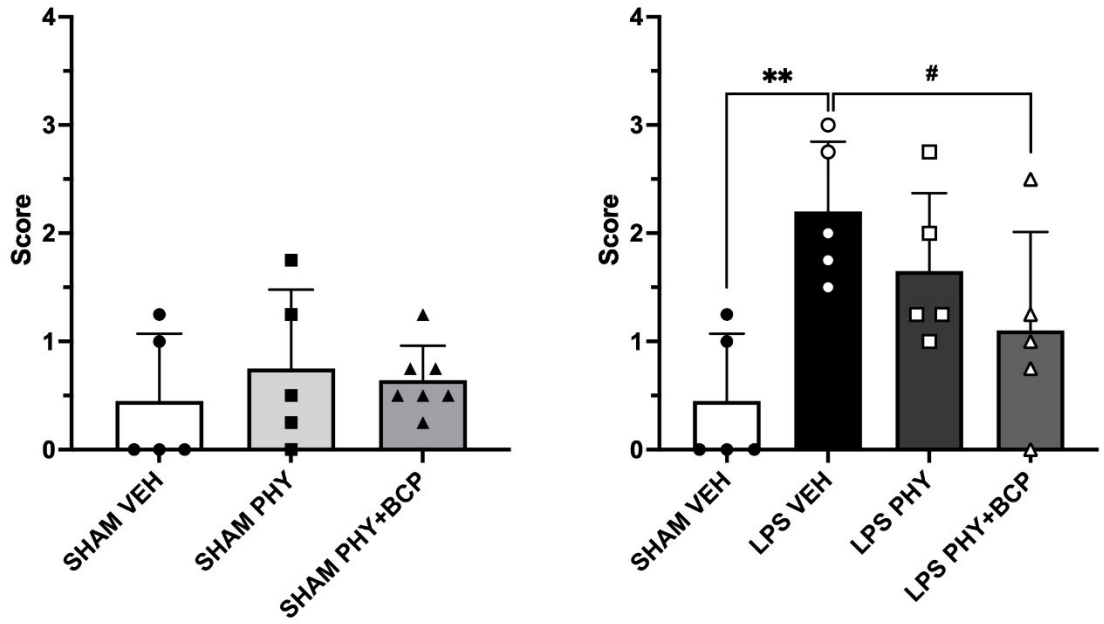

Figure 9. Phytosomal BCP normalizes histological signs of inflammation in female BALB/c mouse bladders 24 hours after IC induction.

Histological changes were assessed in the following groups: sham IC with vehicle control (SHAM VEH; n=5), sham IC with empty phytosome (SHAM PHY; n=5), sham IC with phytosomal BCP (SHAM PHY+BCP; n=7), IC with vehicle control (LPS VEH; n=5), IC with empty phytosome (LPS PHY; n=5), and IC treated with phytosomal BCP (LPS PHY+BCP; n=5). Data is represented as mean ± SD. Statistical analysis by one-way ANOVA with multiple comparisons, **$p<0.01$, and one-tailed t-test, #$p<0.05$.

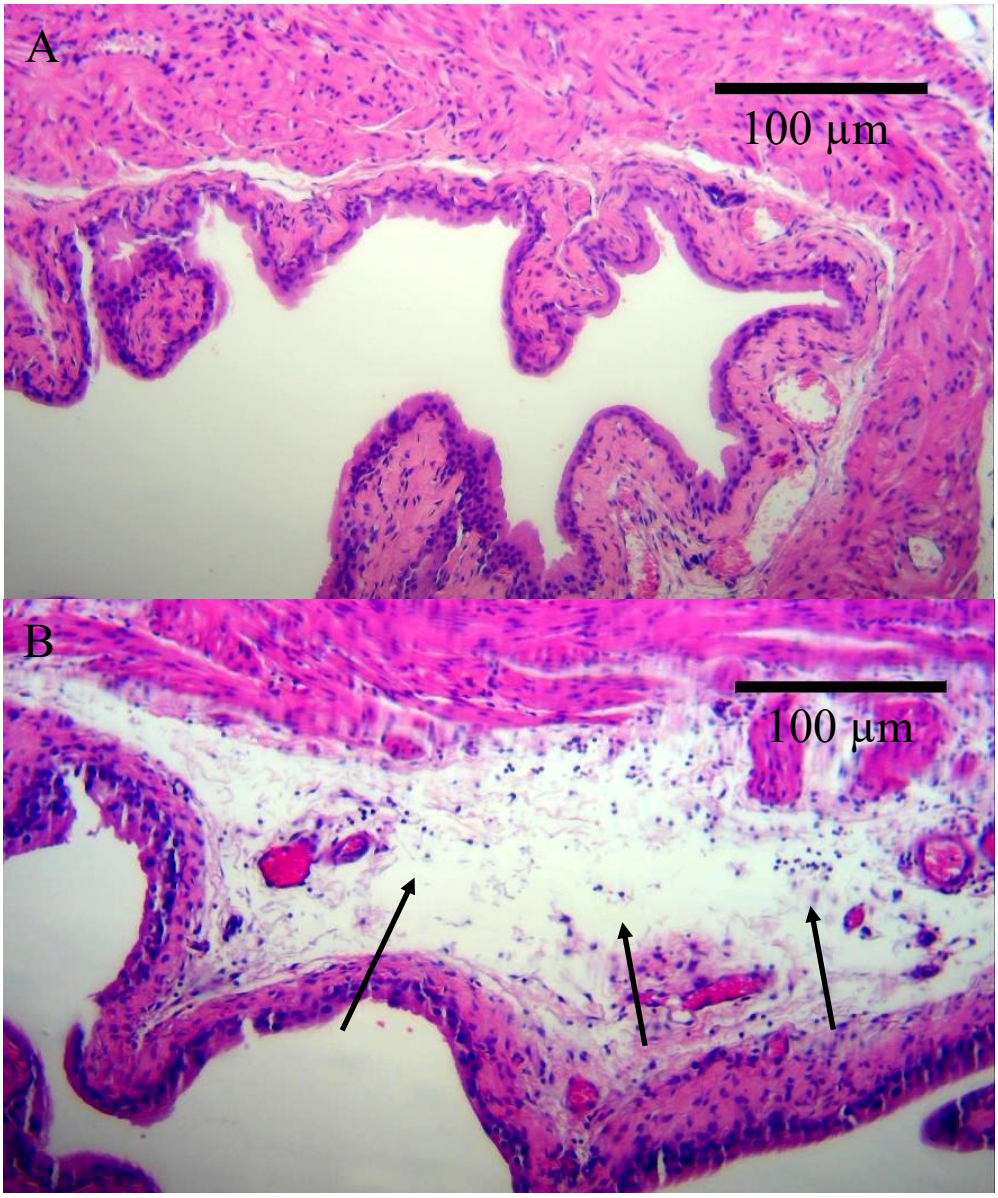

Figure 10. Representative histology images of female BALB/c mouse bladders. Images represent (A) a control animal (representative score of 0) and (B) an untreated LPS animal (arrow indicates edema with diffuse immune cell infiltration in the subepithelium; representative score of 3) at a magnification of 100x. Scale bars indicate 100 µm.

3.1.3 ELISA

No changes in Il-6 cytokine levels were observed in mouse bladder tissue samples 24 hours after IC induction (Figure 11). There were no differences between sham groups or with untreated LPS compared to both control and treatment groups, and overall cytokine expression was near the lower limit of detection for most groups.

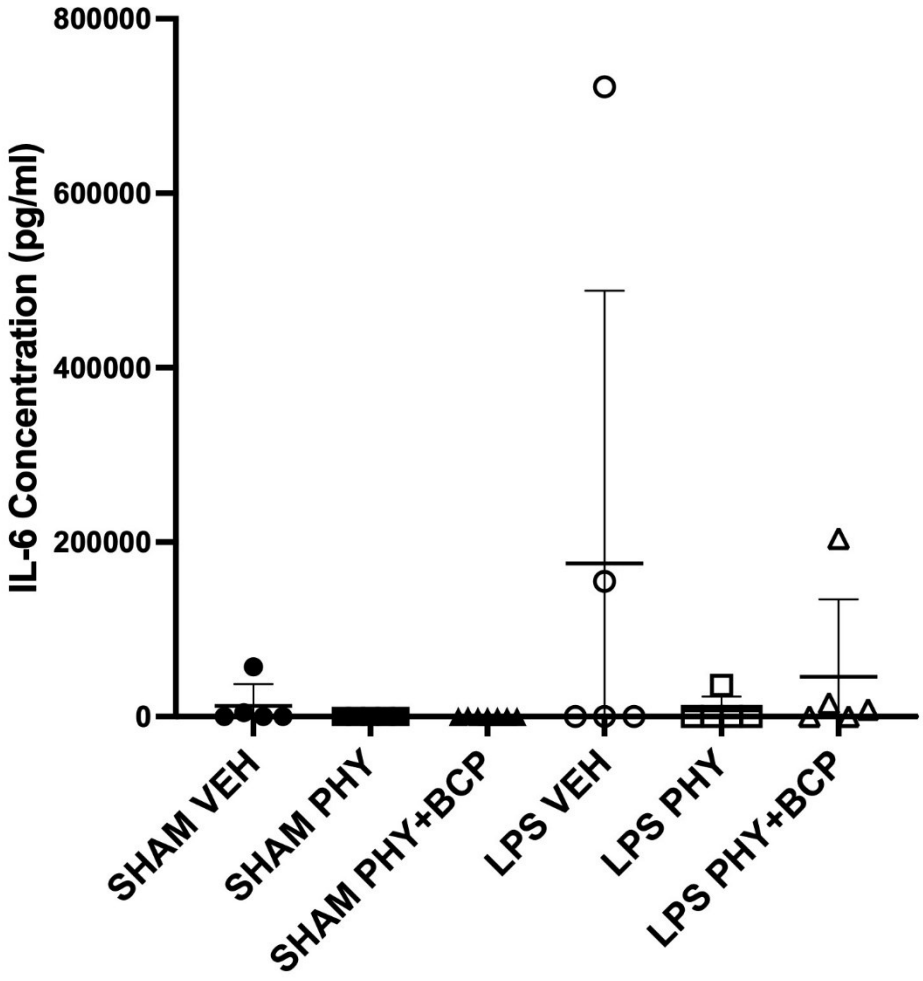

Figure 11. 0.375 mg/kg LPS does not alter IL-6 levels in female BALB/c mouse bladders after 24 hours.

IL-6 protein levels were assessed using an ELISA in the following groups: sham IC with vehicle control (SHAM VEH; n=4), sham IC with empty phytosome (SHAM PHY; n=5), sham IC with phytosomal BCP (SHAM PHY+BCP; n=7), IC with vehicle control (LPS VEH; n=3), IC with empty phytosome (LPS PHY; n=4), and IC treated with phytosomal BCP (LPS PHY+BCP; n=4). Data is in pg/ml and is represented as mean ± SD.

3.2 The Effect of Phytosomal Beta-Caryophyllene on Pain produced by Lipopolysaccharide-Induced Bladder Inflammation in Female BALB/c Mice

3.2.1 Behaviour Scoring

Phytosomal BCP was effective at reducing behavioural signs of pain (Figure 12). 0.375 mg/kg of LPS significantly increased behaviour scores ($p<0.01$) compared to control 24 hours after experimental IC induction. When mice were pre-treated with 100 mg/kg of phytosomal BCP, behaviour scores were significantly decreased ($p<0.05$) compared to untreated LPS controls. While the IC mice treated with empty phytosome vehicle control appeared to have elevated behaviour scores compared to control, this difference was not statistically significant. No significant differences were observed between sham groups.

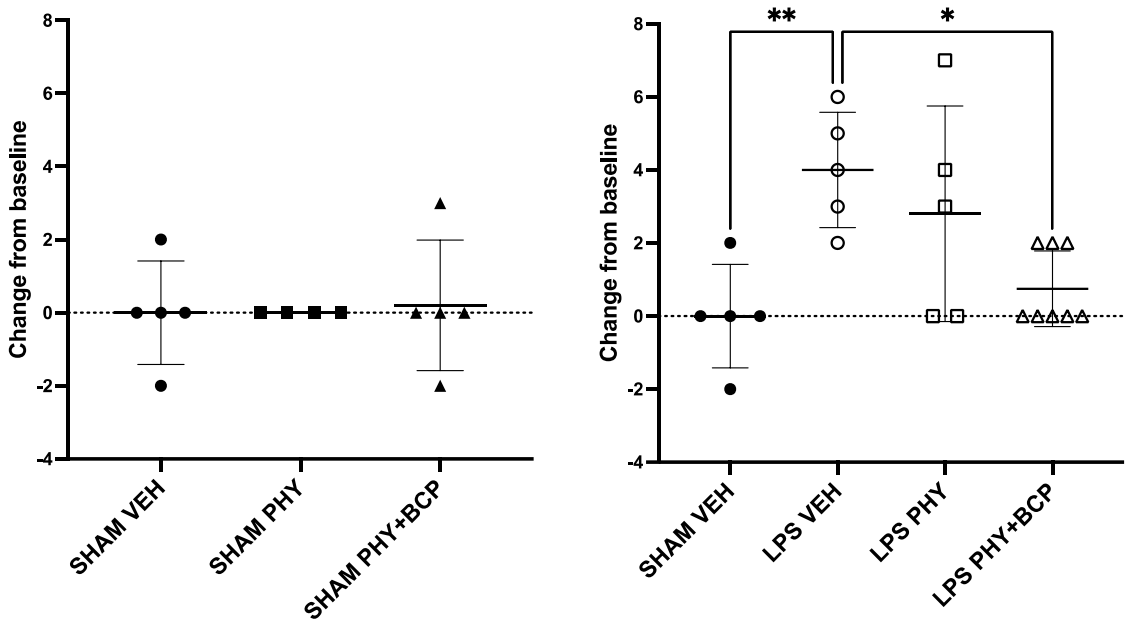

Figure 12. Phytosomal BCP reduces behavioural signs of pain in female BALB/c mice 24 hours after IC induction.

Behavioural signs of pain were assessed in the following groups: sham IC with vehicle control (SHAM VEH; n=5), sham IC with empty phytosome (SHAM PHY; n=4), sham IC with phytosomal BCP (SHAM PHY+BCP; n=5), IC with vehicle control (LPS VEH; n=5), IC with empty phytosome (LPS PHY; n=5), and IC treated with phytosomal BCP (LPS PHY+BCP; n=8). Each data point represents the change in behaviour score 24 hours after IC induction compared to the individual mouse's initial behaviour score. Data represented as mean ± SD. Statistical analysis by one-way ANOVA with multiple comparisons, *$p<0.05$, **$p<0.01$.

3.2.2 von Frey Aesthesiometry

Overall, there were no significant differences in evoked pain response when evaluated by one-way ANOVA (Figure 13). 0.375 mg/kg of LPS did induce a significant reduction in force tolerated on the lower abdomen (*$p<0.05$) when compared to control using a two-tailed t-test. However, treatment with phytosomal BCP did not significantly alter this change. The phytosomal BCP treated group seemed to have decreased evoked pain tolerance after IC induction compared to control, while the LPS treated with empty phytosome appeared to have a higher evoked pain tolerance compared to the other LPS groups, but these differences were not statistically significant. There were no differences between sham groups.

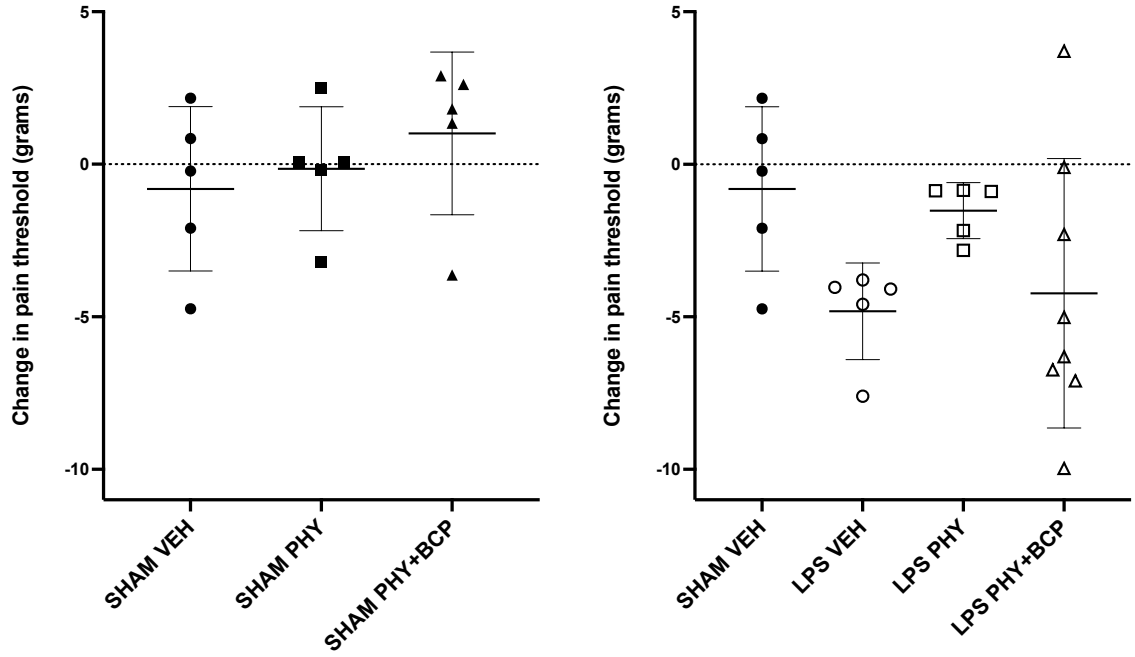

Figure 13. 0.375 mg/kg of LPS does not alter evoked pain tolerance of female BALB/c mice 24 hours after IC induction.

Behavioural signs of pain were assessed in the following groups: sham IC with vehicle control (SHAM VEH; n=5), sham IC with empty phytosome (SHAM PHY; n=5), sham IC with phytosomal BCP (SHAM PHY+BCP; n=5), IC with vehicle control (LPS VEH; n=5), IC with empty phytosome (LPS PHY; n=5), and IC treated with phytosomal BCP (LPS PHY+BCP; n=8). Each data point represents the change in force tolerated before withdrawal 24 hours after IC induction compared to the individual mouse's initial tolerance. Data is in grams and is represented as mean ± SD.

3.3 The Effect of Beta-Caryophyllene on LPS-Induced Inflammation in 5637 Bladder Epithelial Cells

3.3.1 1-hour Model

3.3.1.1 Viability

To determine the appropriate LPS concentration and BCP dose for *in vitro* experiments, viability assays were performed to ensure that these substances were not significantly impacting the number of cells per well. There were no significant differences in viability at any of the LPS concentrations examined after 1 hour, with all groups ending up at or above control levels of viability (Figure 14). The same was true of the BCP doses examined at this timepoint (Figure 15).

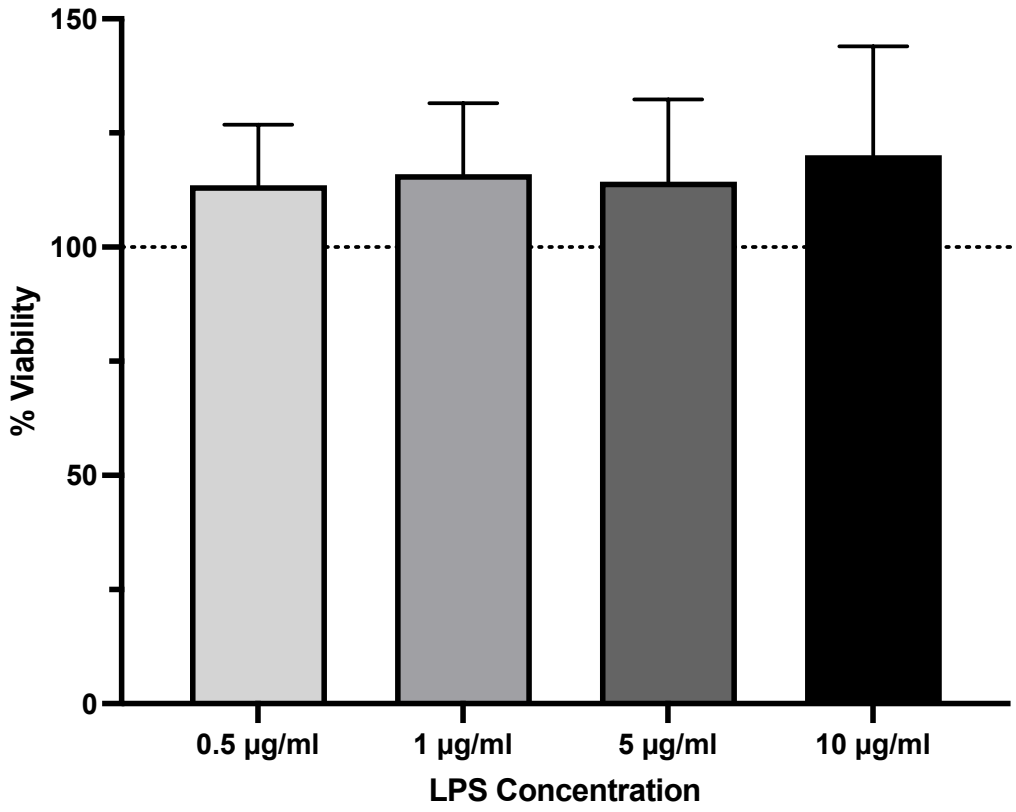

Figure 14. 0.5-10 µg/ml of LPS does not alter 5637 bladder epithelial cell viability after 1 hour.

Viability was evaluated using CCK-8 dye after cells were incubated with 0.5, 1, 5, or 10 µg/ml of LPS or saline (control). Data was normalized as a percentage of control viability and is represented as mean ± SD, n=4.

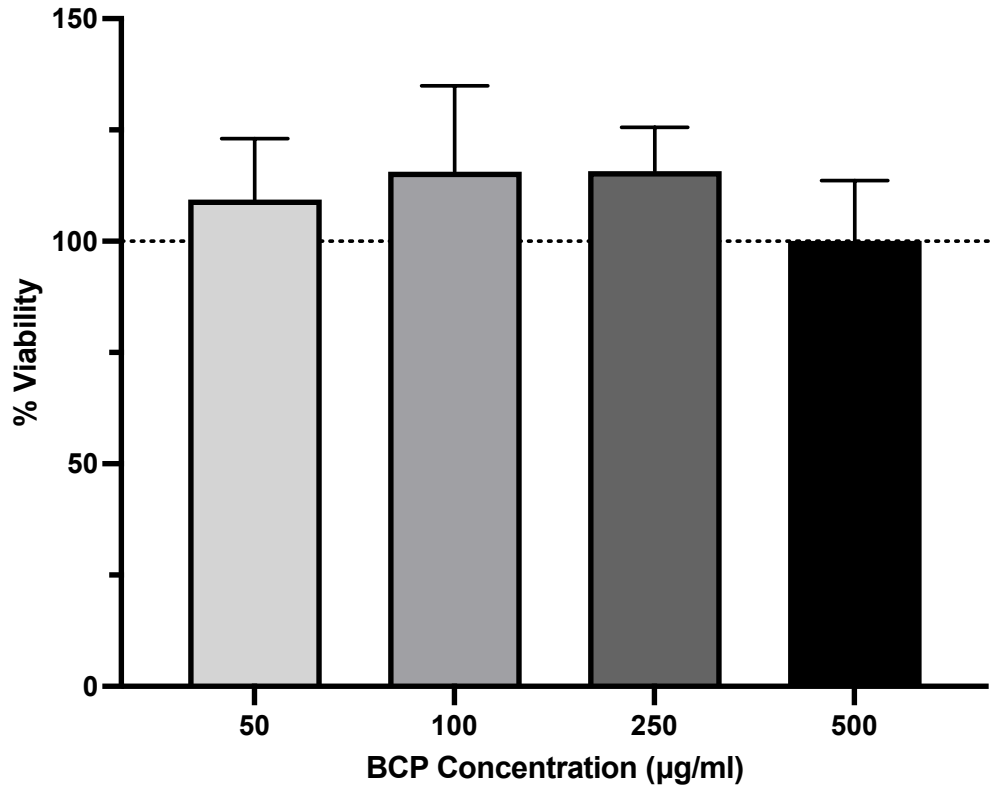

Figure 15. 50-500 µg/ml of BCP does not alter 5637 bladder epithelial cell viability after 1 hour.

Viability was evaluated using CCK-8 dye after cells were incubated with 50, 100, 250, or 500 µg/ml of BCP or saline (control). Data is normalized as a percentage of control viability and is represented as mean ± SD, n=3.

3.3.1.2 Nitric Oxide Assay

A nitric oxide assay was used to determine if any of the LPS concentrations (0.5, 1, 5, or 10 µg/ml) altered this inflammatory parameter. However, the nitric oxide levels for all groups were below the limit of detection specified by the kit, so a graph of these values cannot be shown. The standard curve is shown in Figure 16.

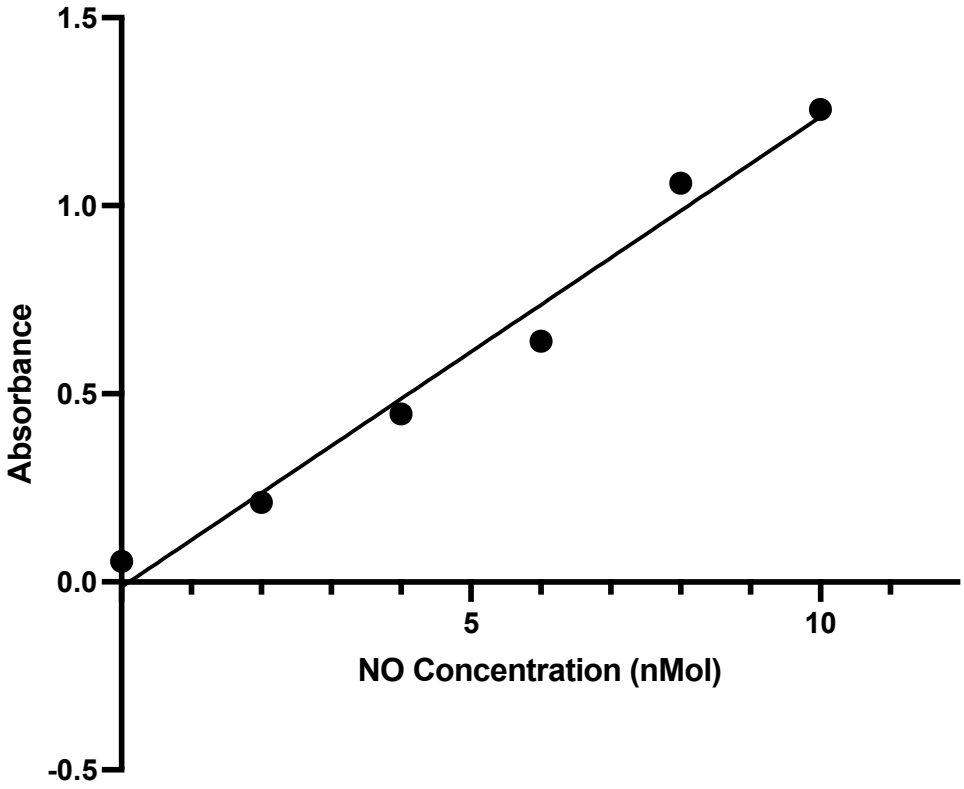

Figure 16. Nitric oxide assay standard curve for 1-hour *in vitro* experiments. Each point represents the absorbance of the kit's nitric oxide standards, whose concentration ranges from 0-10 nMol. Nitric oxide levels from 5637 bladder epithelial cells were below the kit's limit of detection after 1 hour for all of the groups examined, which were saline (control), and 0.5, 1, 5, and 10 μg/ml of LPS. N=3 for all groups.

3.3.1.3 ELISA

An ELISA that examined IL-6 levels in cell supernatant was used to determine if any of the LPS concentrations (0.5, 1, 5, or 10 μg/ml) altered cellular release this inflammatory parameter. However, the IL-6 levels for all groups were below the limit of detection specified by the kit, so the values cannot be shown. The standard curve is shown in Figure 17.

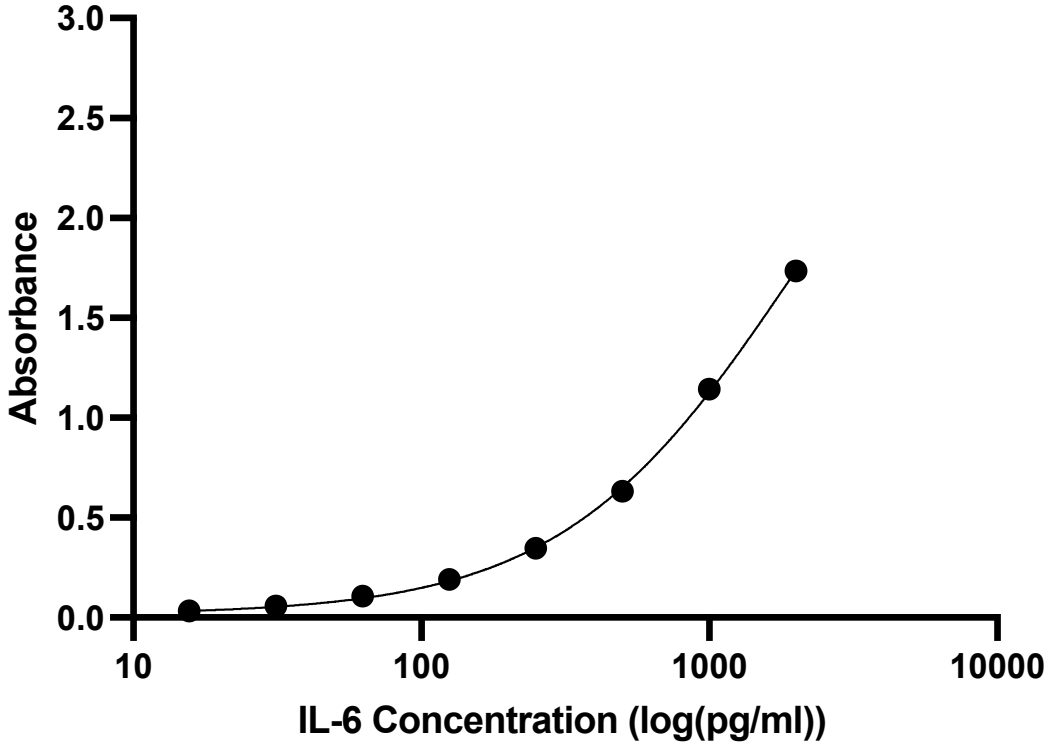

Figure 17. IL-6 ELISA standard curve for 1-hour *in vitro* experiments.
Each point represents the absorbance of the kit's IL-6 standards, whose concentration ranges from 15.625-2000 pg/ml. IL-6 levels from 5637 bladder epithelial cells were not detectable by ELISA after 1 hour for any of the groups examined, which were or saline (control) and 0.5, 1, 5, and 10 µg/ml of LPS. N=3 for all groups.

3.3.2 3-hour Model

3.3.2.1 Viability

Since the levels of the inflammatory parameters examined were undetectable after 1 hour, we decided to examine the effects of LPS and BCP *in vitro* after 3 hours. Again, viability assays were performed first to ensure that these substances were not significantly impacting the number of cells per well at this new timepoint.

There were no significant differences in viability at any of the LPS concentrations examined after 3 hours (Figure 18). Although the viability for the 10 µg/ml dose appeared reduced compared to the other groups, this difference was not statistically significant and remained above the threshold of 80% viability.

There was a significant decrease in viability ($p<0.001$) for the 250 and 500 µg/ml BCP doses after 3 hours, while 50 and 100 µg/ml doses were not altered compared to control (Figure 19). Therefore, the 250 and 500 µg/ml BCP doses were excluded from further study at this timepoint.

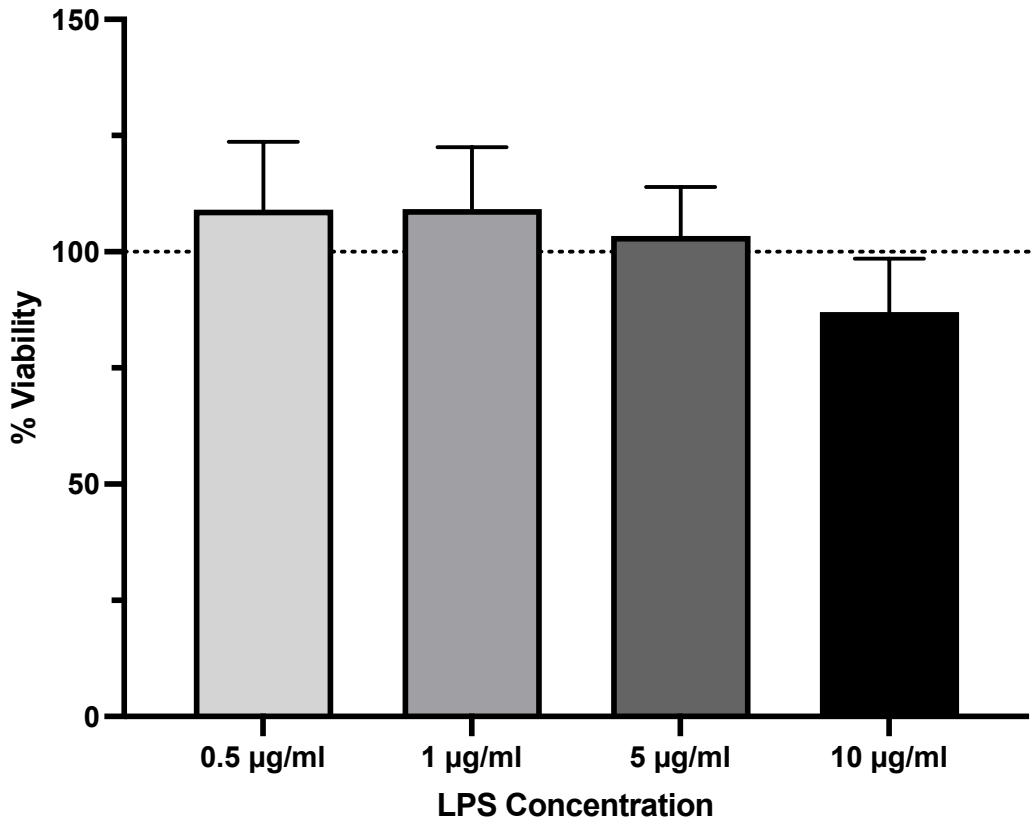

Figure 18. 0.5-10 µg/ml of LPS does not alter 5637 bladder epithelial cell viability after 3 hours.

Viability was evaluated using CCK-8 dye after cells were incubated with 0.5, 1, 5, or 10 µg/ml of LPS or saline (control). Data is normalized as a percentage of control viability and is represented as mean ± SD, n=4.

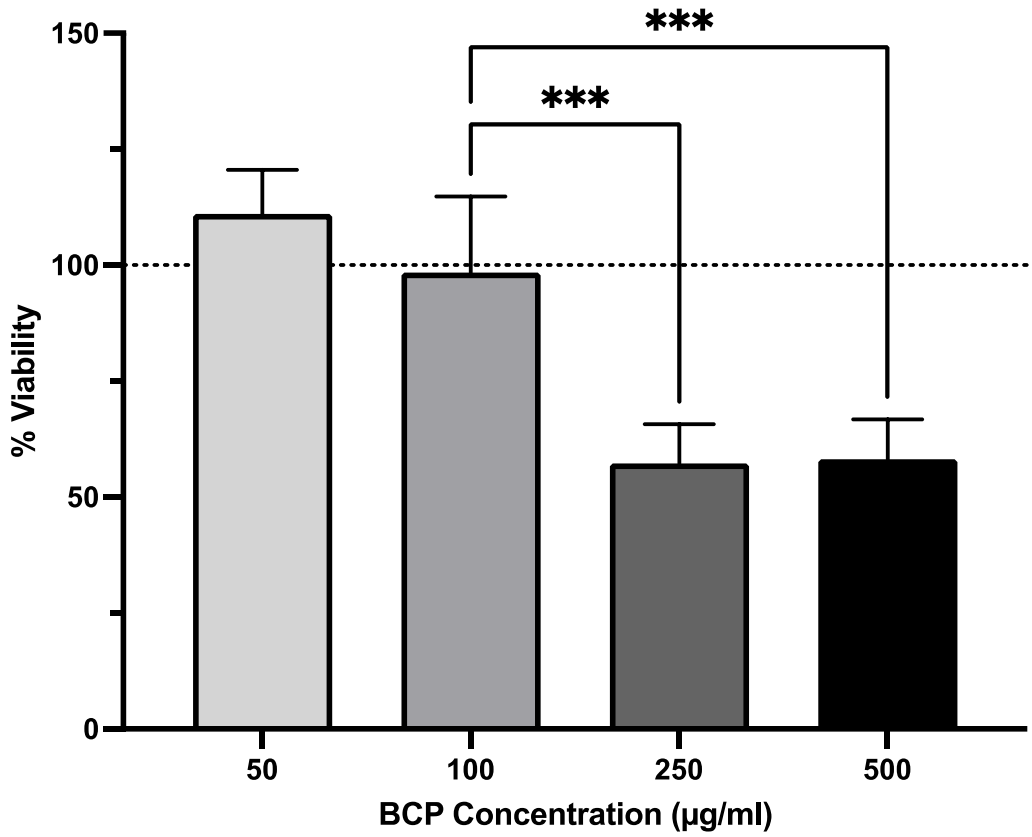

Figure 19. 250 and 500 µg/ml of BCP decrease viability in 5637 bladder epithelial cells compared to control after 3 hours.

Viability was evaluated using CCK-8 dye after cells were incubated with 50, 100, 250, or 500 µg/ml of BCP or saline (control). Data is normalized as a percentage of control viability and is represented as mean ± SD, n=4. Statistical analysis by one-way ANOVA with multiple comparisons, ***$p<0.001$.

3.3.2.2 Nitric Oxide Assay

The nitric oxide levels for all groups (control, and 0.5, 1, 5, or 10 µg/ml of LPS) were again below the limit of detection specified by the kit for this timepoint. The standard curve is shown in Figure 20. Given the low levels of nitric oxide produced by 5637 bladder epithelial cells at the timepoints used in this study, this parameter was excluded from further study.

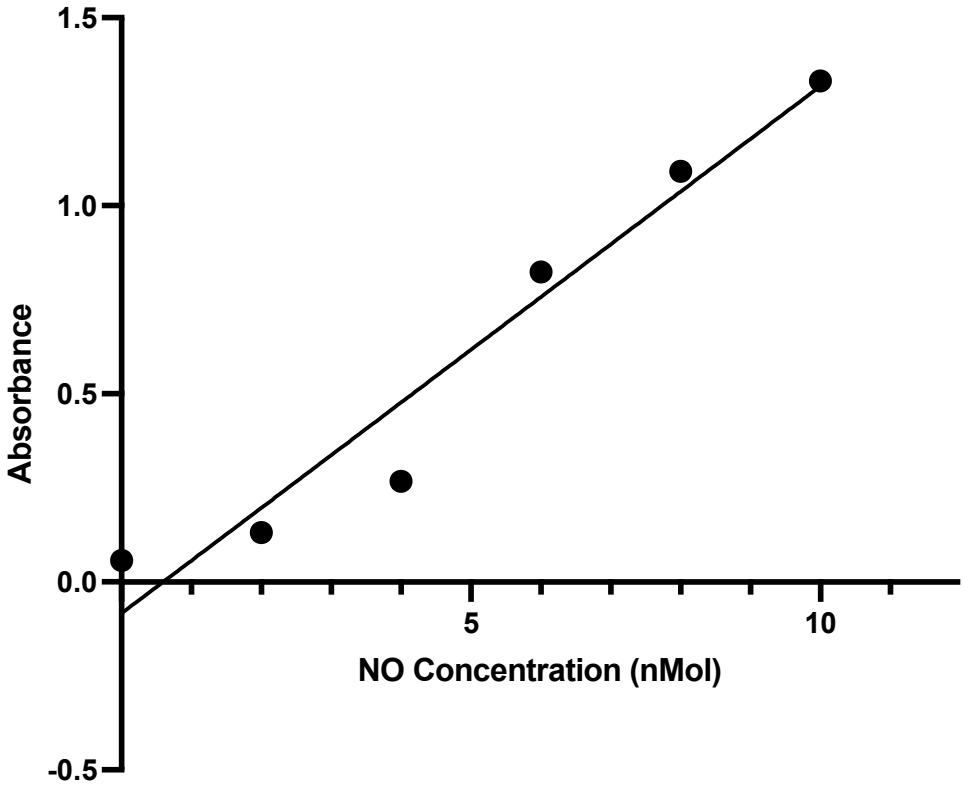

Figure 20. Nitric oxide assay standard curve for 3-hour in vitro experiments. Each point represents the absorbance of the kit's nitric oxide standards, whose concentration ranges from 0-10 nMol. Nitric oxide levels from 5637 bladder epithelial cells were below the kit's limit of detection after 3 hours for all of the groups examined, which were saline (control), and 0.5, 1, 5, and 10 μg/ml of LPS. N=3 for all groups.

3.3.2.3 ELISA

Again, an ELISA for IL-6 levels in cell supernatant was used to determine if any of the LPS concentrations altered cellular release this inflammatory parameter at the longer 3-hour timepoint. LPS concentrations of 5 and 10 µg/ml significantly increased IL-6 levels ($p<0.001$; Figure 21). 0.5 and 1 µg/ml of LPS appeared to increase the levels of IL-6, but this difference was not statistically significant. Therefore, for subsequent assays examining the effect of BCP on cellular mediators of inflammation, the 10 µg/ml concentration of LPS was used.

For the assays looking at BCP's effects on inflammatory cytokine release, we did not see any significant differences with 10, 50, or 100 µg/ml of BCP on LPS-stimulated cells. For IL-6, there was again a significant increase in cytokine release ($p<0.05$) in the untreated LPS-stimulated cells compared to control when examined using a two-tailed t-test. However, there were no significant differences when cells were treated with BCP (Figure 22). The results show a similar pattern for IL-8, where LPS significantly increased IL-8 release using a two-tailed t-test ($p<0.01$), but none of the BCP doses tested were able to reverse this finding (Figure 23). For both the IL-6 and IL-8 ELISAs, the mean cytokine release for the 50 and 100 µg/ml doses of BCP appeared elevated compared to the untreated and 10 µg/ml BCP groups. However, these differences were not statistically significant. The TNF levels for all groups were below the limit of detection specified by the kit. The standard curve is shown in Figure 24.

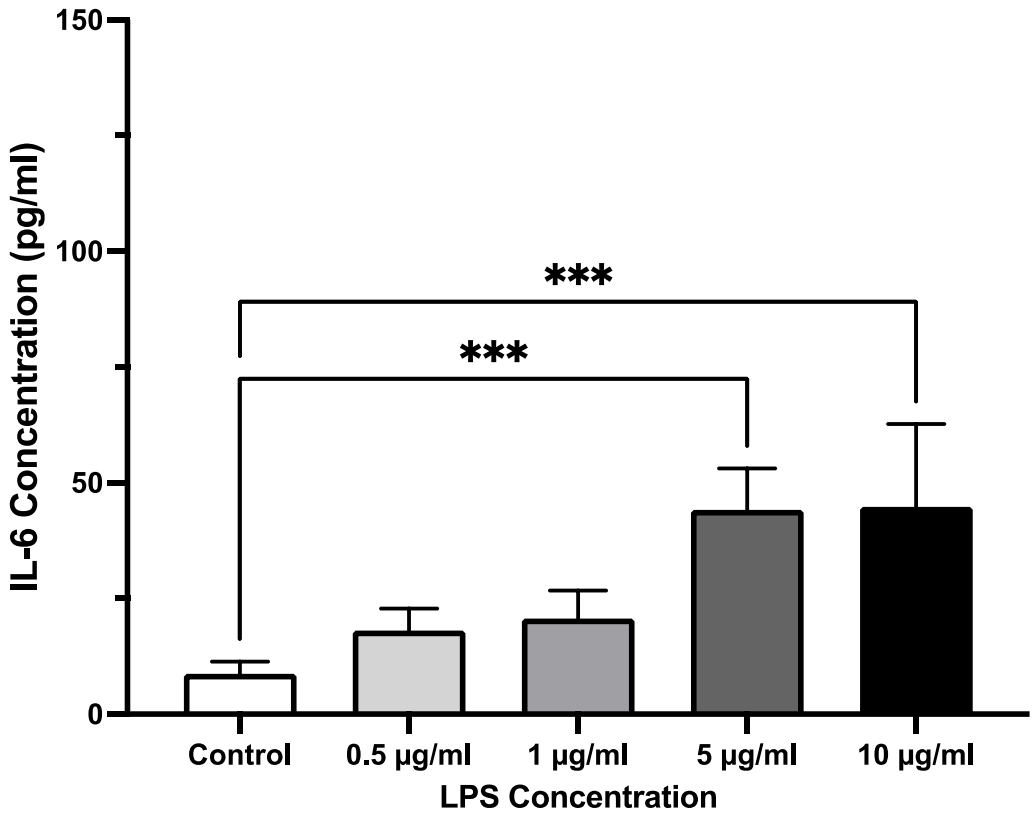

Figure 21. 5 and 10 µg/ml of LPS increase IL-6 release in 5637 bladder epithelial cells after 3 hours.

IL-6 protein levels were assessed using an ELISA after cells were incubated with saline (control; n=3), or 0.5 (n=4), 1 (n=4), 5 (n=3), or 10 µg/ml (n=3) of LPS. Data represented as mean ± SD. Statistical analysis by one-way ANOVA with multiple comparisons, ***$p<0.001$.

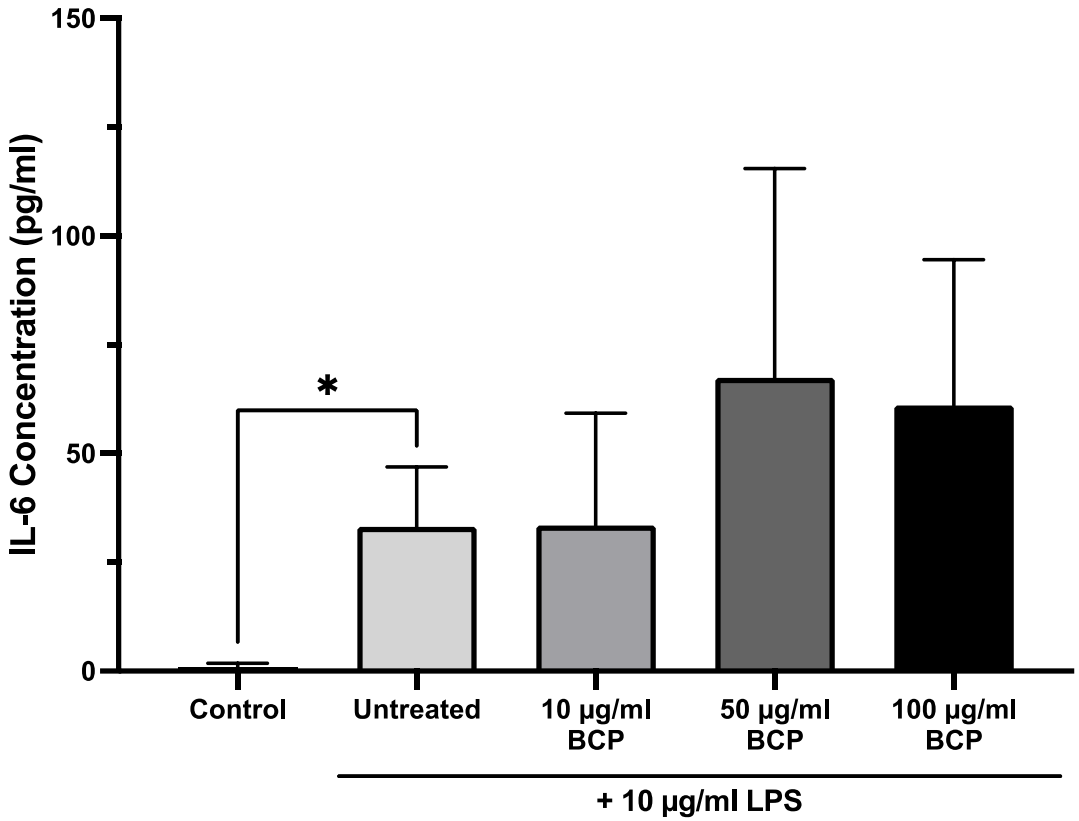

Figure 22. 10-100 µg/ml of BCP does not alter IL-6 release in LPS-stimulated 5637 bladder epithelial cells after 3 hours.

IL-6 protein levels were assessed using an ELISA for the following groups: saline and tween-80 (control), 10 µg/ml LPS and tween-80 (untreated), and 10 µg/ml LPS treated with 10, 50, or 100 µg/ml BCP (10, 50, and 100 µg/ml BCP, respectively). Data represented as mean ± SD, n=3. Statistical analysis by two-tailed t-test *$p<0.05$.

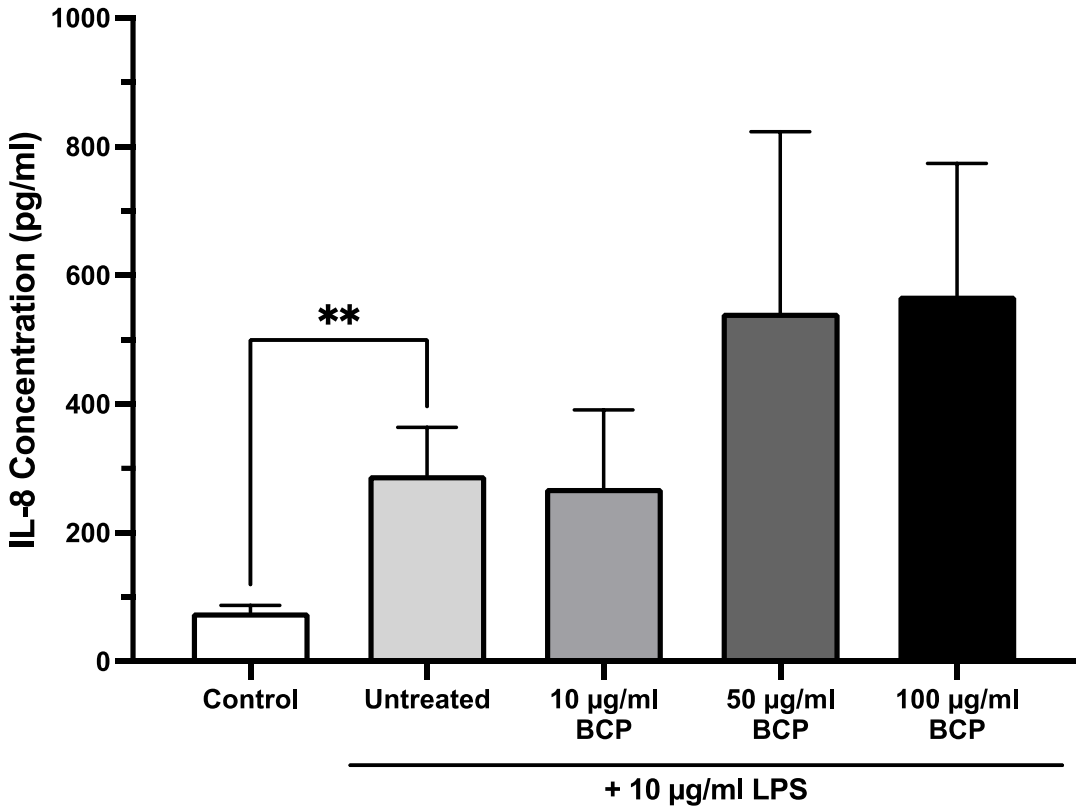

Figure 23. 10-100 µg/ml of BCP does not alter IL-8 release in LPS-stimulated 5637 bladder epithelial cells after 3 hours.

IL-6 protein levels were assessed using an ELISA for the following groups: saline and tween-80 (control), 10 µg/ml LPS and tween-80 (untreated), and 10 µg/ml LPS treated with 10, 50, or 100 µg/ml BCP (10, 50, and 100 µg/ml BCP, respectively). Data represented as mean ± SD, n=3. Statistical analysis by two-tailed t-test **$p<0.01$.

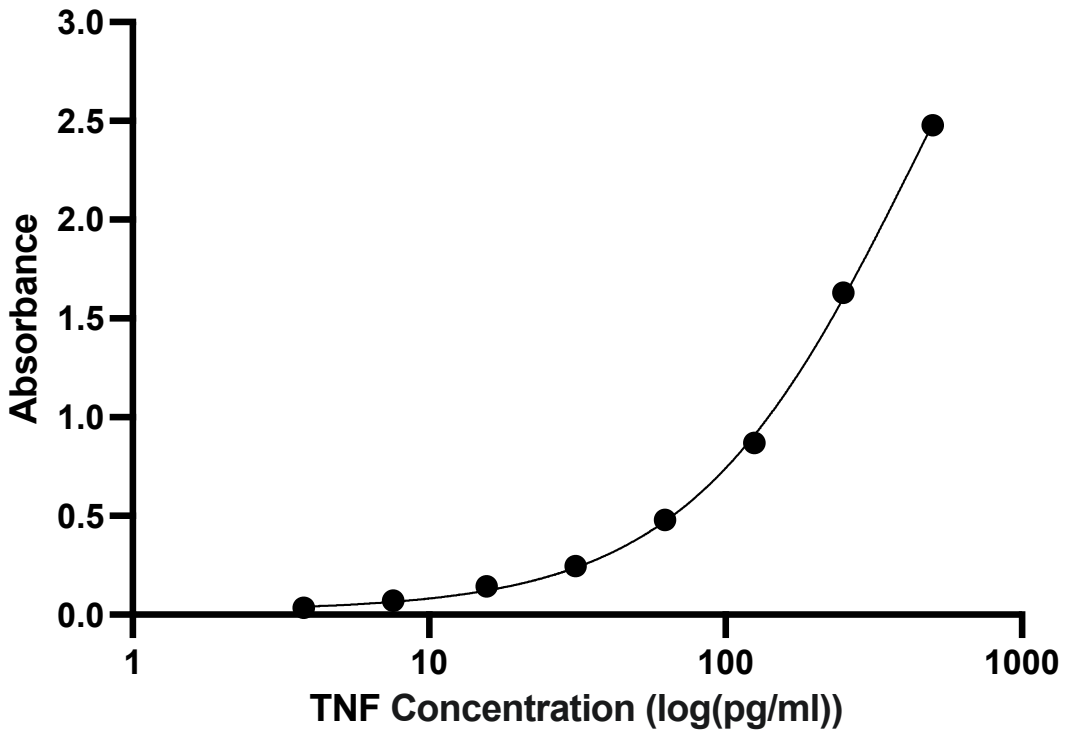

Figure 24. TNF ELISA standard curve for 3-hour *in vitro* experiments.
Each point represents the absorbance of the kit's TNF standards, whose concentration ranges from 1.5625-200 pg/ml. TNF levels from 5637 bladder epithelial cells were not detectable by ELISA after 3 hours for any of the groups examined, which were saline and tween-80 (control), 10 µg/ml LPS and tween-80 (untreated), and 10 µg/ml LPS treated with 10, 50, or 100 µg/ml BCP (10, 50, and 100 µg/ml BCP, respectively). N=3 for all groups.

3.4 Summary of Key Results

Overall, we found that oral phytosomal BCP reduced signs of inflammation and pain *in vivo* (Figure 25). Leukocyte adhesion and histology scores in IC mice were significantly reduced 24 hours after treatment, while leukocyte rolling and functional capillary density were not altered by either LPS or BCP. Behaviour scores (non-evoked pain) were also significantly reduced in IC mice 24 hours after treatment, while evoked pain tolerance was not altered by either LPS or BCP.

BCP had fewer effects in the 5637 cells. In terms of viability, LPS did not induce any changes after 1 or 3 hours of incubation, while higher doses of BCP significantly reduced viability, but only after 3 hours. For inflammatory cytokines, IL-6 was undetectable after 1 hour, but was significantly increased after 3 hours with LPS. IL-8 was also increased after 3 hours with LPS, but treatment with BCP did not alter IL-6 or IL-8 levels. TNF was not detectable after 3 hours, and NO levels were below the limit of detection at both timepoints.

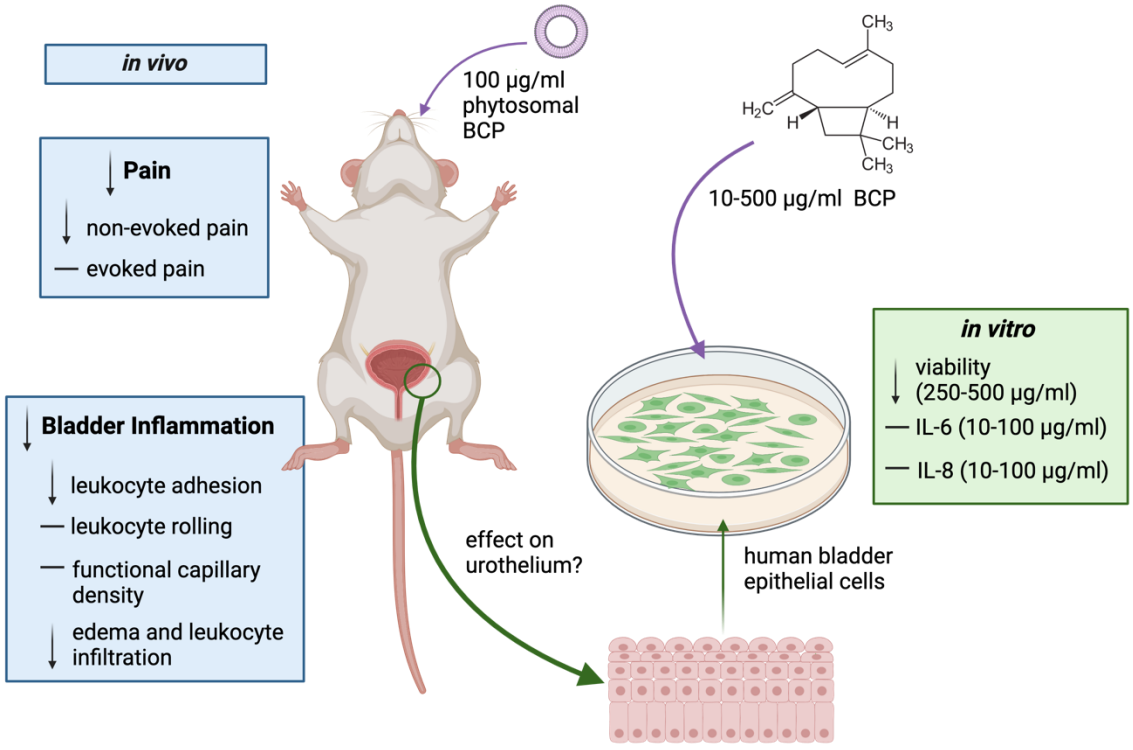

Figure 25. Key findings.

Treatment with phytosomal BCP reduced behaviour scores (non-evoked pain), leukocyte adhesion, and histology scores (edema and leukocyte infiltration) *in vivo* over a 24-hour period. *in vitro*, treatment with 250-500 µg/ml of BCP reduced viability after 3 hours, and treatment with 10-100 µg/ml of BCP did not alter cytokine release. Created with BioRender.com.

Chapter 4: Discussion

4.1 Phytosomal Beta-Caryophyllene as a Treatment for Bladder Inflammation

The first objective of this study was to examine the effects of phytosomal BCP on bladder inflammation *in vivo*. With IVM, we observed decreased leukocyte adhesion in the microvasculature of the bladder wall in BCP-treated IC mice when compared to untreated IC mice, indicating decreased immune cell activation when treated with phytosomal BCP. However, there were no differences between any of the groups for both leukocyte rolling and capillary perfusion, indicating that 0.375 mg/kg of LPS was not sufficient to cause alterations in these parameters. The same was true of tissue IL-6 levels for all groups. Taken together, these findings indicate that intravesical administration of 0.375 mg/kg of LPS induces mild inflammation *in vivo*, and that this inflammation is reversed with oral pre-treatment of 100 mg/kg phytosomal BCP.

The decreased inflammation, as observed by decreased leukocyte adhesion in IVM and decreased histology scores, supports the growing body of evidence that BCP is an effective anti-inflammatory agent in the bladder. In a study of murine UTI, Dickson et al. found that BCP effectively reduced inflammation 24 hours after infection by observing leukocyte adhesion and capillary perfusion [140]. However, BCP is not only anti-inflammatory in infectious models. In a study by Berger et al., 100 mg/kg of intravesical or oral BCP was effective at reducing signs of inflammation, including leukocyte adhesion, in a 2-hour model of IC [89].

Given that leukocyte adhesion and histology scores specifically were decreased by BCP, there are a few potential mechanisms by which this compound may be exerting its anti-inflammatory effects. Since immune cells are known to express CB2 receptors [78], BCP could be acting directly on these cells, which would explain the changes observed in leukocyte adhesion with BCP treatment. A similar effect was seen in a study of leukocyte infiltration across the blood brain barrier, where CB2-activated leukocytes that were injected into mice showed decreased adhesion and infiltration into the CNS [141]. While the blood brain barrier is a more stringent barrier than that of the bladder, this study by Rom et al. highlights the fact that direct CB2 activation of leukocytes, rather than CB2 activation in the tissue, can impact their adhesion to and infiltration across physiological barriers. This change in leukocyte infiltration could be due to changes in

the expression of cellular adhesion molecules, as BCP treatment decreases the levels of E-selectin, P-selectin, ICAM-1, and VCAM-1 in various inflammatory models [106,114]. Further supporting this hypothesis, immune cell infiltration is an important part of our histology scoring criteria. Other studies have used CB2 activation to decrease immune cell infiltration and histology scores in the bladder, with Tambaro et al. reporting a significant decrease in leukocytes when IC mice were treated with the CB2 agonist JWH015. This effect was CB2-dependent, as administration of the CB2 receptor antagonist AM630 reversed this effect [95]. Given this evidence and the results of the current study, it is likely that activation of the leukocyte CB2 receptor is a major contributor to the anti-inflammatory effects of phytosomal BCP.

BCP could also be acting directly on the bladder epithelial cells, as these cells are also known to express CB2 receptors [77]. CB2 activation is known to affect bladder function, including improving the ability to contract, decreasing bladder pressure, and increasing the time between voids [77,142]. However, in contrast to the present study, both of these functionality studies used intravesical administration of their respective CB2 agonists, rather than oral administration like the present study. In addition, these functionality studies do not examine BCP's anti-inflammatory effects directly.

Other studies that have characterized BCP's pharmacokinetic effects can provide some insight as to how much BCP reaches the urothelium to exert anti-inflammatory effects. One study that examined the pharmacokinetics of orally administered BCP in rats found that from an oral dose of 50 mg/kg, the maximum plasma concentration (C_{max}) of BCP was 0.12 µg/ml, which occurred 3.5 hours after administration (T_{max}) [143], however, this study did not characterize how much BCP reaches specific organ systems. In addition, this pharmacokinetic study did not characterize the metabolism of phytosomal BCP, so it is not clear how much BCP reaches the bladder after 24 hours in the present model. It is known that BCP's metabolites, 14-hydroxycarophyllene and 14-hydroxycaryophyllene oxide, are eliminated in the urine [144]. This means that BCP's hydroxylated metabolites can easily reach the urothelium, but the anti-inflammatory activity of these metabolites has not yet been characterized. Therefore, phytosomal BCP could be acting on the urothelium to mediate its anti-inflammatory effects, but given the uncertainty surrounding its pharmacokinetics and the clear effect of BCP on the immune

cells, it is unlikely that the urothelium is the only site of phytosomal BCP's anti-inflammatory actions.

Pre-treatment with BCP is also known to interfere with the signalling of toll-like receptor 4 (TLR4) in various inflammatory models. While this effect has not yet been observed in the bladder, it has been demonstrated in a murine cerebral ischemia-reperfusion model and an *in vitro* model of LPS-induced liver injury [145,146]. TLR4 is key in the present model because LPS activates TLR4 to mediate its inflammatory effects, including in bladder epithelial cells [147]. BCP is thought to interfere with TLR4 signaling by suppressing nuclear factor-κB (NF-κB), a transcription factor that increases the expression of proinflammatory cytokines, while also activating the MAPK pathway, as is commonly seen with CB2 receptor activation [145].

Beyond CB2, there could be additional receptors contributing to the anti-inflammatory effects of BCP, such as PPARγ. As discussed in the introduction, BCP has shown anti-inflammatory effects in a model of vascular inflammation through activation of PPARγ in conjunction with CB2 [106]. PPARγ involvement in BCP's anti-inflammatory effects has also been observed on diet-induced neuroinflammatory parameters, including TNF and iNOS [148]. While PPARγ involvement has not been confirmed in cystitis, a study examining the anti-inflammatory effects of BCP in a model of UTI indicated that BCP reduced signs of inflammation in the bladder, but that these changes were not reversed with administration of AM630, indicating that mechanisms other than CB2 activation must be contributing to BCP's anti-inflammatory effects in bacterial cystitis [140]. However, it is important to consider that in an infectious model like UTI, BCP could also be mediating anti-bacterial effects, which are not a factor in LPS-induced inflammation. Evidently, more work is necessary to fully characterize all the receptors contributing to BCP's effects on inflammation.

It is also possible that the components of the phytosome are contributing to and even enhancing these anti-inflammatory effects. As noted in the results, the IC animals that received the empty phytosome had leukocyte adhesion levels and histology scores that fell between, but did not differ significantly from, both corn oil-treated and phytosomal BCP-treated IC. If the phytosomal components were simply inert, we would expect to see very similar results to the corn oil-treated IC group, but this was not the

case. This effect could be due to one of the primary components of the phytosome, phosphatidylcholine [128]. Orally administered phosphatidylcholines have demonstrated anti-inflammatory effects, including a reduction in leukocyte adhesion, in a rat model of arthritis. This arthritis study also found a reduction in leukocyte infiltration into the knee, which was accompanied by a decrease in ICAM-1 expression [149]. These results align with what we saw in the empty phytosome-treated groups, although our effect was not significant. Beyond their effects on immune cells, phosphatidylcholines have also restored intestinal barrier function after liver injury in rats [150], which is an important consideration given that reduced barrier function is an important driver of IC pathophysiology. In terms of the present study, the ability to repair organs and restore barrier function would be reflected in histology scores, which appeared lower when IC mice were given the empty phytosome. Overall, phosphatidylcholines have demonstrated anti-inflammatory effects similar to those we observed and merit further study for their applications in IC/BPS.

We did not see changes in leukocyte rolling and capillary perfusion, which were our secondary IVM parameters, but this does not necessarily contradict the anti-inflammatory effects discussed above. The lack of change to both leukocyte rolling and capillary perfusion is in line with other studies that used IVM to evaluate LPS-induced changes in the bladder microcirculation. Kowalewska et al. did not see any changes in leukocyte rolling 4 hours after administration of 1 mg/kg of LPS [151]. While this is a shorter timepoint than the present study, these authors also used a larger concentration of LPS. Although Berger et al. saw changes in capillary perfusion with the same concentration of LPS as the present study, their endpoint was only 2 hours after LPS administration [89], which is considerably shorter than our 24-hour endpoint. Conversely, Hagn et al. did not see any significant changes in capillary perfusion using the same LPS concentration and timepoint as Berger et al. [152]. Since the present study found that 0.375 mg/kg of LPS was not enough to induce a significant change in leukocyte rolling and capillary perfusion after 24 hours, there was no effect in these parameters for BCP to treat.

We also did not see any changes in bladder IL-6 levels, including in untreated IC animals. IL-6 is an important parameter in a model of IC because it is a pro-inflammatory

cytokine whose levels are associated with more severe inflammation in IC/BPS patients [38]. Since not all of the inflammatory parameters examined in the present study were altered upon LPS administration, it is possible that this is a true effect, and that 0.375 mg/kg of LPS is not sufficient to induce changes in IL-6 levels after 24 hours. However, the findings in the literature on IL-6 expression in the bladder tissue after inflammatory stimuli would suggest otherwise. Li et al. found that protein levels of IL-6 in the bladder were shown to be upregulated in protamine/LPS-induced IC after 24 hours [153]. Conversely, using a model of UTI rather than LPS-induced inflammation, Dickson et al. were unable to detect IL-6 in bladder tissue after 24 hours [140]. However, their lack of IL-6 in the bladder tissue could be due to the choice of model, as the strain of uropathogenic *E. coli* the authors used is known to suppress IL-6 expression, especially when compared to LPS-induced inflammation [154]. Therefore, it is unexpected that we would not see a change in IL-6 levels in the bladder upon stimulation with LPS.

It is important to note that the experimental conditions for bladder tissue processing in this project were not ideal. Adding a protease inhibitor to the extraction buffer is recommended as standard protocol [155], however in the present study, a protease inhibitor was added following tissue homogenization rather than preceding it. Although the samples were kept on ice between steps, the protein could have degraded during or directly following the homogenization step. Given the variation in protein levels within groups and the findings from other, similar literature discussed above, the late addition of the protease inhibitor seems to be the most likely explanation for the low bladder IL-6 levels we observed.

4.2 Phytosomal Beta-Caryophyllene as a Treatment for Pain Associated with Bladder Inflammation

The second main objective of this study was to evaluate the analgesic effects of phytosomal BCP. This was examined using behaviour scoring, which evaluated non-evoked pain, and von Frey aesthesiometry, which evaluated evoked pain tolerance. We saw a significant increase in behaviour score, indicating increased pain, 24 hours after LPS administration. This effect was reversed when mice were pre-treated with phytosomal BCP. We saw a reduction in evoked pain tolerance with von Frey

aesthesiometry in the untreated LPS group, but this reduction was not altered with administration of phytosomal BCP. This difference in evoked pain tolerance was significant when comparing only the untreated LPS group to the sham group, indicating that overall pain levels were low and that perhaps our method of von Frey is not ideal for evaluating pain in this model.

The decreased pain demonstrated by phytosomal BCP-treated mice via behaviour scoring indicates that BCP decreases inflammatory pain. Overall pain levels were not particularly high, as untreated IC mice demonstrated an average score increase of 4 out of a total possible increase of 30. However, pain levels were significantly increased compared to both sham and treated mice, indicating that phytosomal BCP was still able to treat this modest level of pain. Additionally, these pain levels make sense considering the modest, but significant, signs of inflammation demonstrated in the IVM and histology studies.

The decrease in behaviour score with BCP treatment we observed is also reflected in the literature that examined BCP's effects on pain in other models. For example, 5 mg/kg of oral BCP was effective at reducing behavioural signs of formalin-induced inflammatory pain in the hindpaw [97]. In a study similar to the present work, researchers found that oral BCP significantly reduced behavioural signs of pain compared to control 2 hours after LPS administration. While this study seemed to produce higher pain levels after IC induction compared to the present study, BCP was also not able to fully reverse the LPS-induced pain, as the BCP-treated group still had significantly increased pain levels compared to non-IC controls [89].

One potential mechanism for the pain-relieving effects of BCP we observed is CB2 receptor activation. As discussed in the introduction, BCP is known to activate CB2 to decrease inflammation, which leads to analgesic effects. For example, BCP was shown to attenuate neuropathic pain in a CB2-dependent manner, as administration of AM630 reversed BCP's effects on mechanical allodynia [90]. Another study found that the pain-relieving effects of BCP were not CB2-dependent in the bladder, but considering that this was in a murine model of UTI, BCP's antibacterial effects are likely contributing to its analgesia [140]. Therefore, although CB2 activation is one potential mechanism, it is not clear whether this is the primary source of BCP's analgesic effects

BCP also mediates local anaesthetic effects [111], which could contribute to the analgesia observed in the present study, as systemic administration of local anaesthetics can be an effective strategy for pain relief. For example, intravenous administration of the local anaesthetic lidocaine is an effective strategy to reduce postoperative pain [156]. While BCP's local anaesthetic effects are not as well-characterized as lidocaine's, both of these substances have been reported to act on sodium channels, which is one mechanism by which local anaesthetics can produce pain-relieving effects [105,156].

As discussed in the introduction, it is also possible that TRPV1 and/or PPARα could be contributing to BCP's analgesic effects. Black pepper seed extract containing 30% BCP effectively reduced pain through CB2, TRPV1, and PPARα activation [105]. These findings were supported by molecular docking studies that predicted strong binding affinity with the agonist-binding sites of all three of these receptors. Overall, CB2 activation is a known mechanism by which BCP mediates its analgesic effects, but the potential contribution of additional receptors requires further study.

Beyond the effects of BCP alone, it is important to consider how the phytosome may have contributed to the analgesic effects. We saw an insignificant reduction in pain in the empty phytosome group, mirroring the effect seen when examining inflammation. Evidently, the phytosome could have helped facilitate the delivery of BCP into the circulation, as we hypothesized. However, again, it is important to consider that the phytosomal components may be contributing to this effect. The analgesic effects of phosphatidylcholines alone are largely unclear. One study into local anaesthetics found that their empty liposome vehicle, made of phosphatidylcholines from an egg, showed no analgesic effect when evaluated by aversive response to rat upper lip pinching [157]. However, another study that examined the effect of oral phosphatidylcholines in arthritis found that pain tolerance, as measured by hindlimb von Frey aesthesiometry and the thermal paw withdrawal, was significantly increased with treatment [149]. Given this evidence in conjunction with the results of the present study, it seems that the analgesic effect of the phytosome alone is simply a side effect from the small reduction in inflammation observed with this treatment condition, but further research is required to confirm the exact mechanism.

Despite seeing a clear effect on behaviour scoring, the results were less straightforward when we examined evoked pain tolerance using von Frey aesthesiometry. We saw no significant differences in the force tolerated between groups with a one-way ANOVA. We did see a modest reduction in force tolerated upon LPS administration, which indicates an increase in pain, but we did not see any significant differences between any of the other groups. In addition, we saw a large amount of variation within groups. This could be due to a number of reasons. First, it is possible that there was not enough inflammation to cause a large enough difference in pain tolerance to treat. Although there was a significant increase in inflammatory measures, these changes were not particularly large. We observed a significant reduction in evoked pain tolerance in the untreated LPS group using a t-test, which does not take into account the results from the empty phytosome or phytosomal BCP groups. This is in contrast to results seen by Berger et al., who used the same LPS dose as the present study but a shorter timepoint. Their study did not find a significant decrease in force tolerated with LPS administration as measured by von Frey, but they did find that oral administration of BCP in IC mice significantly increased the force tolerated to the bladder compared to untreated IC controls [89]. Interestingly, another study found that IL-6 stimulation in a model of IC resulted in increased sensitivity to abdominal stimulation by von Frey [158]. We did not see an increase in IL-6 levels in the present study and we also did not see a change in evoked pain tolerance, which fits with this association.

Our particular method of von Frey aesthesiometry could also be contributing to the lack of difference seen between most groups. We were particularly interested in whether the pain was localized to the bladder, and thus decided to apply force to the lower abdomen rather than employing the more widely used hindpaw method of von Frey [159–161]. Since the lower abdomen is a relatively large area, it is difficult to ensure that the same spot is poked each time, which could be contributing to the variability seen within groups. This is similar to results seen in the UTI study by Dickson et al., where abdominal von Frey results were somewhat variable within groups, especially 6 hours after infection [140]. However, earlier studies found that abdominal von Frey was a reliable measure of pain, as both Berger et al. and Hagn et al. used the same method and observed consistent results within groups [89,152].

Finally, it is possible that the pain in our model was not localized directly to the bladder. Since both Berger and Hagn used a 2-hour model of IC, perhaps by 24 hours after induction the pain sensation is not concentrated directly over the bladder. This is not what we expected since our model used a local instillation of LPS, however, this would explain the discrepancies seen between the behaviour scoring and von Frey. Supporting this, the localization of visceral pain generally tends to be diffuse [162]. Alternatively, if the pain is truly localized to the bladder, given the difficulties with abdominal von Frey discussed above, perhaps we are not able to accurately hit the small area where the pain is located each time. However, this accuracy explanation does not explain why other models of IC found this technique to be reliable. Therefore, the true reason for the variation in von Frey data is likely a combination of all the theories discussed above.

4.3 Effect of Beta-Caryophyllene and Lipopolysaccharide on 5637 Bladder Epithelial Cells

4.3.1 Cell Viability

Since the *in vivo* experiments indicated that phytosomal BCP decreased bladder inflammation and associated pain, the final objective was to examine *in vitro* how BCP was acting on bladder epithelial cells, as they are an important player in driving bladder inflammation. We saw no changes in viability after one hour in the selected cell line for all of the LPS and BCP concentrations tested, indicating that all of the concentrations tested would be suitable for further study.

The 3-hour timepoint produced more varied results in terms of viability. Like the 1-hour timepoint, none of the LPS concentrations significantly altered viability, which indicated that they were all suitable for future assays. However, the higher BCP doses (250 and 500 µg/ml) did significantly reduce viability and were thus excluded from further study, as a reduced number of cells would be a confounding factor for the quantity of inflammatory mediators released. BCP is well-tolerated *in vivo*, with an estimated LD50 of over 5000 mg/kg body weight in rats [100]. 500 µg/ml of BCP has shown some cytotoxic activity in fibroblasts, however the authors only observed about 18% cytotoxicity after 24 hours [99]. This is a much shorter timeline and far less cell death

than the present study, albeit in a different cell line. Therefore, cell death in response to BCP was not a result that we anticipated.

BCP primarily acts through CB2 activation, but the role of CB2 activation in cell death varies depending on the study. For example, the CB2 receptor agonist AM1241 increased expression of apoptotic proteins in the liver (and this effect was reversed with administration of AM630), while THC-mediated cytotoxic effects were shown to be independent of both CB1 and CB2 activation in leukemia cell lines [163,164]. Interestingly, despite their contradictory findings regarding the role of CB2 activation in cell death, both of these studies found that the MAPK pathway was involved, and that specifically decreased expression of ERK was associated with apoptosis. BCP is also known to mediate anticancer effects in a number of cancer cell lines, including the 5637 bladder cells used in the present study [99,165,166]. In a study that examined the effects of 5-80 μg/ml of BCP on 5637 cell viability, researchers found that all of the BCP doses tested reduced viability after 24 hours. This reduction in viability occurred because BCP increased the expression of reactive oxygen species and induced apoptosis [165]. Since the cell line used in the present work is from a bladder carcinoma and has shown vulnerability to the anticancer effects of BCP, it is likely impacted by these effects when sufficient BCP is present (such as at doses of 250 and 500 μg/ml), which would cause the clear reduction in viability we saw at higher BCP doses after 3 hours.

4.3.2 Inflammatory Mediator Release

After we determined which concentrations of LPS and BCP did not impact viability at our chosen timepoint, we moved forward to determine which of these LPS concentrations altered NO and IL-6 release, as this would provide an inflammatory signal for BCP to alter. However, none of the LPS concentrations tested significantly changed the cells' release of these inflammatory mediators after one hour, which prompted us to extend the timepoint. Although there is a fair amount of variation in endpoint time and inflammatory trigger used between studies, since LPS is a widely used inflammatory trigger, we expected to see some change in NO or IL-6 release. Since we did not, we decided that moving to a longer timepoint made the most sense, as most likely our cells did not have sufficient time to produce a detectable quantity of inflammatory mediators.

Many studies take the first measurement of inflammatory mediators at least 2-4 hours after administration of the inflammatory stimulus [167–169], so we selected 3 hours as our timepoint for subsequent studies.

Unlike the 1-hour timepoint, we did see changes in inflammatory cytokine levels after 3 hours. In particular, IL-6 and IL-8 were increased in response to LPS, which was expected. 5637 cells have been shown to produce IL-6 and IL-8 2-3 hours after infection with *E. coli* [169]. More importantly, these cells also produce significant amounts of IL-6 and IL-8 after 3 hours with varying concentrations of *E. coli* LPS [167,169], so our findings fit with the existing literature. This response is also a good model for IC presentation in patients, as they tend to show elevated levels of IL-6 and IL-8 in their urine and blood [38,170].

In our cytokine studies at the 3-hour timepoint, BCP treatment did not significantly impact the levels of IL-6 and IL-8. BCP is known to decrease levels of IL-6 in LPS-stimulated human oral mucosa epithelial cells through a CB2-dependent mechanism [171]. BCP's effect on IL-8 is less well-characterized, but one study found that extract from the plant *Helichrysum odoratissimum*, which contains BCP as one of its major constituents, effectively reduced IL-8 levels in an *in vitro* model of acne [172].

Although not significant, treatment with 50 and 100 μg/ml of BCP appeared to increase the release of IL-6 and IL-8. It is possible that the unexpected results in cytokine levels are due to the fact that these cells are a carcinoma cell line rather than a primary cell line, so their cytokine secretion could be altered compared to primary cells. In fact, 5637 cells have shown increased release of colony-stimulating factors compared to primary urothelial cells [173]. However, as discussed above, other studies using 5637 cells have been able to detect inflammatory cytokines like IL-6 and IL-8 after stimulation with LPS [167,169]. Alternatively, the difference in cytokine levels could also be due to differences in the timing rather than the quantity of cytokine release, as BCP could have altered the cytokine release kinetics in these cells (Figure 26). Since we captured cytokine levels at a single timepoint, we do not have the full picture of cytokine release over time in these cells. Perhaps BCP treatment shifted the kinetics of IL-6 and IL-8 release so that their levels peaked earlier, giving the appearance of increased cytokine release, when in fact only the timing of cytokine release was altered. The opposite effect could have also

occurred, as BCP treatment could have also delayed cytokine release. Evidently, further study is needed to fully characterize BCP's effect on IL-6 and IL-8 release in 5637 cells.

In contrast to IL-6 and IL-8, we were unable to detect TNF in any of the groups after three hours. TNF is an important inflammatory cytokine that has been shown to be elevated in the urine of IC patients [174]. We expected that TNF would be elevated upon stimulation with LPS, as Wang et al. found that 10 μg/ml of LPS stimulation in 5637 cells resulted in elevated TNF levels compared to control [175]. TNF is one of the earliest cytokines released in the inflammatory process and drives the secretion of other pro-inflammatory factors like IL-6 and IL-8 [176,177], so it should be elevated in response to an inflammatory stimulus like LPS. However, is possible that any TNF that was produced was already degraded after 3 hours, as it *in vivo* studies have found that this cytokine has a half-life of less than an hour in the circulation [178]. In addition, given the unexpected results we observed for IL-6 and IL-8, it is possible that TNF signalling is altered in this cell line. Contradicting both of these theories, Wang et al. found that TNF levels were detectable in all groups and significantly increased in response to LPS after 24 hours using the same cell line and LPS concentration as the present study [175]. However, that study did not specify the species or serotype of LPS used, and the bacterial source of LPS is known to affect the inflammatory response [151,179], which could account for the discrepancies with the present work.

Similar to the results from the one-hour model, the levels of NO after three hours were again far below what the kit would detect. However, our findings contrast with results from other similar studies. Lin et al. found that nitrite, the stable product of NO oxidation, was both detectable in 5637 cells and elevated in response to *E. coli* infection after 2 hours [180]. Other studies using LPS on different bladder cell lines used longer timepoints, where T24 cells showed detectable levels of NO 24 hours post-LPS stimulation and frog bladder epithelial cells showed detectable levels beginning about 5 hours post-LPS stimulation [181,182]. Some studies have even seen changes in response to BCP using 5637 cells, with 40 μg/ml of BCP producing an increase in reactive oxygen species after 24 hours [165]. While BCP and LPS act through different receptors and typically mediate opposing effects, perhaps we would have seen increased NO production in response to LPS with a longer timepoint.

A different LPS concentration may have also increased NO production. None of the LPS concentrations we tested reduced viability at either timepoint. Therefore, it is possible that a larger concentration of LPS could be used without negative repercussions on viability, and a larger concentration of LPS could be enough to induce a detectable amount of NO release. Nikolaeva et al. did find that nitrite released increased with increasing LPS concentrations [182]. In addition, Wang et al. observed an increase in reactive oxygen species in 5637 cells after 24 hours of incubation with 10 µg/ml of LPS [175]. While this is the same LPS concentration as the present study with a longer observation period, if 5637 cells remained viable for a 24-hour period using our largest concentration of LPS, they would likely remain viable for a 3-hour period using an even larger concentration of LPS. Overall, when considering our NO results in conjunction with the existing literature, it seems that 5637 bladder epithelial cells do not release large amounts of NO in response to 0.5-10 µg/ml of LPS after 1-3 hours, but that perhaps a longer timepoint or larger LPS concentration is required to produce detectable results.

Taken together, the results in the *in vitro* model indicate that although release of these inflammatory mediators are important indicators of disease in IC, the overall inflammatory response is low *in vitro*, and it is unlikely that the therapeutic principle of the *in vivo* effects is due to BCP is acting directly on the urothelial cells.

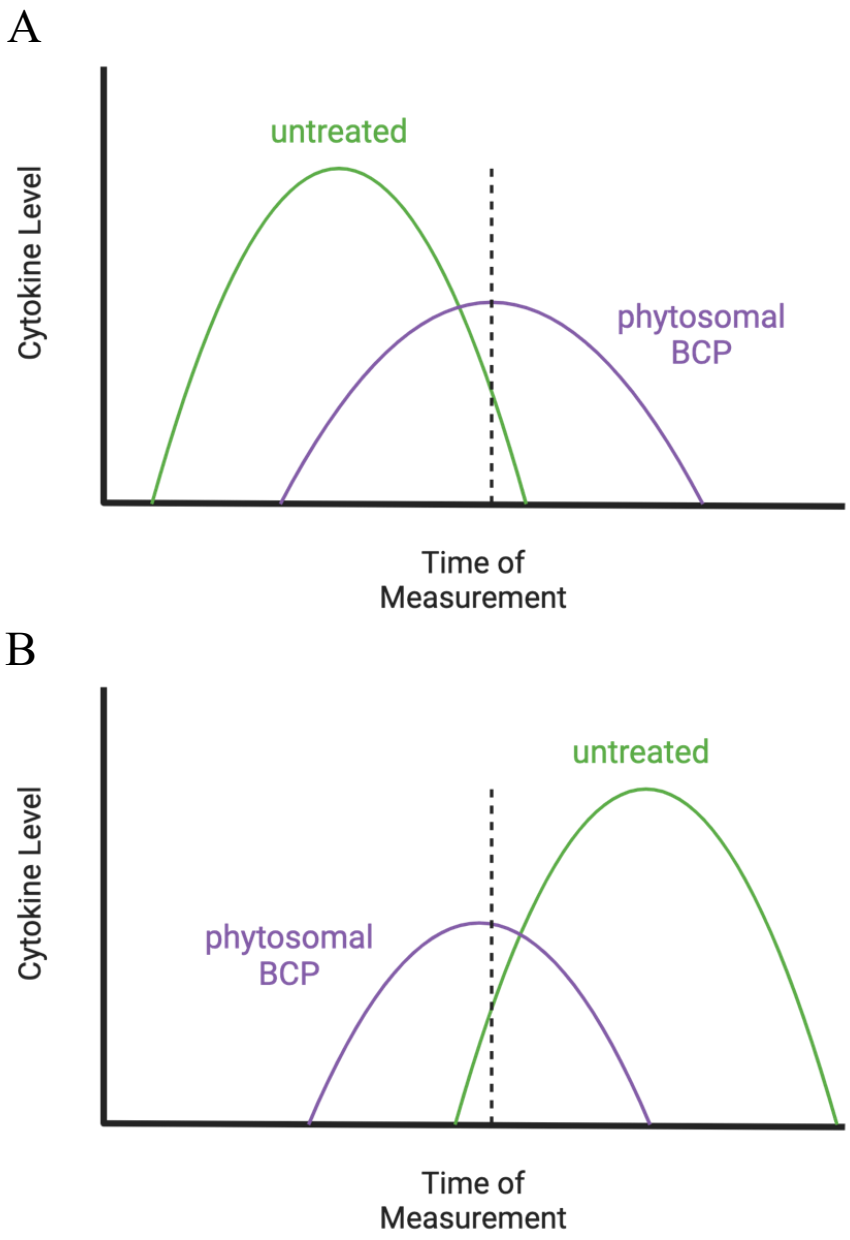

Figure 26. Potential impact of phytosomal BCP on cytokine release kinetics in 5637 urothelial cells.

In both panels, treatment with phytosomal BCP reduces the cytokine levels. However, the timing of the measurement makes it appear as though treatment increases cytokine levels, as the treatment also shifts the timing of peak cytokine levels to later (panel A) or earlier (panel B) than the untreated group. Created with BioRender.com.

4.4 Limitations

This study had some limitations. The main limitations are related to the ability to model IC *in vivo*, the generalizability of a 24-hour timepoint to a chronic disease, the ability to evaluate pain responses in an animal model, the generalizability of the *in vivo* findings, and the ability to create an *in vitro* model that can be easily correlated with the results *in vivo*. While these limitations do not negate the results of the present study, they are important to consider when interpreting its results.

As with any animal model, it is impossible to fully capture the presentation and pathophysiology of the disease. IC is an especially difficult disease to model, as its exact cause is unknown, so it can be difficult to incorporate every important aspect in one model. While the present model is able to show the inflammation and pain associated with the disease, the shorter timepoint and choice of inflammatory trigger mean that the nervous system adaptations seen in patients do not have time to develop. Our mice also do not experience the same social stressors that contribute to the mental health issues that IC/BPS patients experience. Therefore, this model can be used only to make conclusions about BCP's potential to improve the inflammation and pain-related symptoms of IC/BPS.

The timepoint of this particular model also makes extrapolating the results to a chronic disease difficult. This study was intended as a preliminary investigation into the effectiveness of a novel formulation of BCP for oral delivery, so the 24-hour endpoint was suitable for this purpose. However, symptoms of the target disease persists for months and years. Therefore, although we saw anti-inflammatory and analgesic effects over the study's duration, further work is needed to ensure that these effects continue for the longer time periods required to treat IC.

The accurate evaluation of pain responses in an animal model can also limit the generalizability of pre-clinical work. Mice are unable to communicate relative pain levels the way a person can, which means that we must rely on outward responses to capture an internal experience. While we tried to capture this by using multiple strategies to evaluate pain, there are a multitude of other methods that could have been used instead of the methods chosen, and these alternative methods could have been more sensitive at capturing the pain response. In addition, while care was taken to minimize bias, the

nature of completing the experiments and analysis independently makes this difficult. Especially with the more subjective criteria for the pain and behaviour studies, it is nearly impossible to entirely eliminate unconscious biases. However, to combat this as much as possible, behaviour videos were recorded, and a random sample of these videos was verified by an independent evaluator.

Since this study was completed using only female mice, the results can also only be generalized to a female population. As with most research, sex-specific effects are potentially relevant and should be closely examined before generalizing the results to a male population. Although IC/BPS is more common in women than in men, both sexes are impacted by the disease and are in need of novel treatment strategies [1,20,21]. While examining in a female population allowed us to generalize to most of the IC/BPS patient population, future work should be done to ensure that the effect of BCP on bladder inflammation and pain holds true in males.

While the *in vitro* studies aimed to understand BCP's effects on the bladder tissue directly, again, this model had some limitations. The particular cell line we used is isolated from a patient with carcinoma. While this is generally suitable for modelling, it is possible that this cell line has altered signaling behaviour compared to a primary urothelial cell line. Various tumor cell lines have shown differences in their internal signalling pathways, including pathways related to cell communication and metabolism [183]. As discussed above, we saw some unexpected results when examining the release of pro-inflammatory mediators, including a lack of NO release at either timepoint. Altered signalling could also explain why BCP was ineffective at reducing the production of pro-inflammatory cytokines, as the concentrations of LPS and BCP used in the treated assays did not reduce viability at the same timepoint.

Even with an ideal cell line to model the bladder, determining the correct dose of BCP that correlates with the *in vivo* experiments is difficult. Although we know how much BCP is given to each mouse, we do not know how much of this actually reaches the bladder, as this would require an in-depth study of phytosomal BCP's pharmacokinetics after oral administration, which has not yet been completed. In addition, while we wanted to understand BCP's effects on the urothelium, our model is missing the potential contribution from the phytosome. As discussed above, an *in vitro* model that most

accurately incorporated the phytosomal components would require a clear understanding of the pharmacokinetics of phytosomal BCP, but even a preliminary model would be beneficial in understanding the mechanism of phytosomal BCP.

4.5 Future Directions

This research could be taken in a number of directions moving forward. Future work is needed to characterize the absorption and metabolism of the phytosomal formulation, to examine the effects of phytosomal BCP over a longer time period, to examine potential sex differences, and to explore additional applications of the formulation beyond an oral route of administration. In addition, the path to clinical use should be considered so that this treatment can reach patients as soon as possible.

Importantly, the pharmacokinetics of phytosomal BCP should be definitively examined. While we saw clear anti-inflammatory and analgesic effects *in vivo*, the contribution of the phytosome should not be ignored, as even the empty phytosome showed a similar, albeit non-significant, effect. A clear understanding of how the phytosomal formulation is absorbed, distributed, metabolized, and excreted would help clarify how the phytosome contributes to these effects observed in the present study. This understanding would then facilitate appropriate dosing of phytosomal BCP in patients and would additionally aid in applying this strategy to other phytochemicals.

A longer timepoint or chronic model is an important next step, as this would produce findings that are more applicable to IC patients. While shorter timepoints like that used the present study are necessary for preliminary investigations, additional pre-clinical studies are needed before phytosomal BCP can be given to patients. Studying this treatment at a longer timepoint would likely require a different model of IC induction than the present study, as the inflammation would need to persist for the entire study duration. One way to ensure prolong inflammation is to administer multiple instillations of LPS, as Yoshizumi et al. found that daily intravesical instillation of LPS over a period of four days in rats produced detectable signs of pain and inflammation for up to 21 days after the first instillation [184]. Alternatively, intravesical hyaluronidase could be used, as this inflammatory stimulus produced significant bladder inflammation in rats when administered multiple times per week over a period of one-to two months [185]. i.p.

injections of ketamine can also be used for long-term cystitis models, as Li et al. saw an increase in bladder inflammation after 8-12 weeks of daily injections [186].

As mentioned in the limitations section, expanding this study to male mice would also be important in translating this research into patients. While much of the older literature posits that IC/BPS is overwhelmingly found in women, more recent sources indicate that the number of men with this disease is much closer to the number of women than previously anticipated [9,20,21,187]. Beyond the need to ensure that treatments can be applicable to multiple patient populations, there is always the potential for sex differences in response to a particular disease or compound, and sex differences have been observed in studies examining BCP's effects. In a model of inflammatory pain, males experienced greater analgesia with BCP treatment compared to females [188]. However, in a model of skin wound healing, females treated with BCP showed a significant increase in re-epithelialization, while males showed no change compared to untreated controls [189]. Given these conflicting results regarding BCP's effectiveness between sexes, sex differences are important to consider when designing future studies into BCP's effects in IC.

Examining phytosomal BCP as an intravesical treatment could also prove to be a promising avenue. Intravesical liposomes are an emerging treatment avenue for IC/BPS and are thought to both protect and repair the damaged urothelium [32]. Since phytosomes are structurally similar to liposomes and are made of an important component of the cell membrane [128], it stands to reason that the phytosome itself could provide benefits in addition to acting as a vehicle for BCP. As discussed in the introduction, many common IC/BPS treatments are administered intravesically, so this is considered a standard route of administration of treatments for this condition. In addition, intravesical and oral treatments can be combined, as is the case with PPS [1]. Therefore, the anti-inflammatory and analgesic effects of oral phytosomal BCP could be enhanced by additionally administering the formulation intravesically.

Finally, it is important to consider what steps are needed get this potential treatment to patients. Additional pre-clinical studies, such as those discussed above, would be necessary to further characterize phytosomal BCP's effects. However, it would also be important to connect with those involved with the commercialization of research,

such as industry partners, manufacturers, and regulatory bodies, to ensure that this treatment can become accessible to clinicians and patients as quickly as possible.

4.6 Conclusions

In conclusion, we found that the novel phytosomal formulation of BCP is effective at reducing leukocyte adhesion and extravasation in the microvasculature of the bladder wall and attenuating behavioural signs of pain in experimental IC *in vivo*. The observed anti-inflammatory and analgesic effects of oral phytosomal BCP represent a potential novel avenue for the treatment of patients with IC.

References

1 Cox A, Golda N, Nadeau G, Nickel JC, Carr L, Corcos J, et al. CUA guideline: Diagnosis and treatment of interstitial cystitis/bladder pain syndrome. Can Urol Assoc J. 2016 May;10(5–6):E136–E135.

2 Hanno P. International Consultation on IC – Rome, September 2004 / Forging an International Consensus: Progress in Painful Bladder Syndrome / Interstitial cystitis. Int Urogynecol J. 2005 Jun;16(S1):S2–5.

3 Hanno PM, Erickson D, Moldwin R, Faraday MM. Diagnosis and Treatment of Interstitial Cystitis/Bladder Pain Syndrome: AUA Guideline Amendment. J Urol. 2015 May;193(5):1545–53.

4 Curhan GC, Speizer FE, Hunter DJ, Curhan SG, Stampfer MJ. Epidemiology of Interstitial Cystitis: A Population Based Study. J Urol. 1999 Feb;161(2):549–52.

5 Grover S, Srivastava A, Lee R, Tewari AK, Te AE. Role of inflammation in bladder function and interstitial cystitis. Ther Adv Urol. 2011 Feb;3(1):19–33.

6 Homma Y. Interstitial cystitis, bladder pain syndrome, hypersensitive bladder, and interstitial cystitis/bladder pain syndrome – clarification of definitions and relationships. Int J Urol. 2019 Jun;26(S1):20–4.

7 Birder LA. Pathophysiology of interstitial cystitis. Int J Urol. 2019 Jun;26(S1):12–5.

8 Maeda D, Akiyama Y, Morikawa T, Kunita A, Ota Y, Katoh H, et al. Hunner-Type (Classic) Interstitial Cystitis: A Distinct Inflammatory Disorder Characterized by Pancystitis, with Frequent Expansion of Clonal B-Cells and Epithelial Denudation. PLoS One. 2015 Nov;10(11):e0143316.

9 Clemens JQ, Link CL, Eggers PW, Kusek JW, Nyberg LM, Mckinlay JB. Prevalence of Painful Bladder Symptoms and Effect on Quality of Life in Black, Hispanic and White Men and Women. J Urol. 2007 Apr;177:1390–4.

10 Suskind AM, Berry SH, Suttorp MJ, Elliott MN, Hays RD, Ewing BA, et al. Health-related quality of life in patients with interstitial cystitis/ bladder pain syndrome and frequently associated comorbidities. Quality of life research. 2013 Sep;22(7):1537–41.

11 Berry SH, Hayes RD, Suttorp M, Nyberg LM, Clemens JQ. Health-Related Quality of Life Impact of Interstitial Cystitis/Painful Bladder Syndrome and Other Symptomatic Pelvic Disorders. J Urol. 2009 Apr;181(4S):90–1.

12 Warren JW, Langenberg P, Clauw DJ. The number of existing functional somatic syndromes (FSSs) is an important risk factor for new, different FSSs. J Psychosom Res. 2013 Jan;74(1):12–7.

13 Clemens JQ, Elliott MN, Suttorp M, Berry SH. Temporal Ordering of Interstitial Cystitis/Bladder Pain Syndrome and Non-bladder Conditions. Urology. 2012 Dec;80(6):1227–32.

14 Peters KM, Killinger KA, Ibrahim IA. Childhood Symptoms and Events in Women With Interstitial Cystitis/Painful Bladder Syndrome. Urology. 2009 Feb;73(2):258–62.

15 Goldstein HB, Safaeian P, Garrod K, Finamore PS, Kellogg-Spadt S, Whitmore KE. Depression, abuse and its relationship to interstitial cystitis. Int Urogynecol J. 2008 Dec;19(12):1683–6.

16 Abernethy MG, Rosenfeld A, White JR, Mueller MG, Lewicky-Gaupp C, Kenton K. Urinary Microbiome and Cytokine Levels in Women With Interstitial Cystitis. Obstet Gynecol. 2017 Mar;129(3):500–6.

17 McKernan LC, Walsh CG, Reynolds WS, Crofford LJ, Dmochowski RR, Williams DA. Psychosocial co-morbidities in Interstitial Cystitis/Bladder Pain syndrome (IC/BPS): A systematic review. Neurourol Urodyn. 2018;37(3):926–41.

18 Bassaly R, Downes K, Hart S. Dietary Consumption Triggers in Interstitial Cystitis/Bladder Pain Syndrome Patients. Female Pelvic Med Reconstr Surg. 2011 Jan;17(1):36–9.

19 Lai HH, Vetter J, Song J, Andriole GL, Colditz GA, Sutcliffe S. Management of Symptom Flares and Patient-reported Flare Triggers in Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS)—Findings From One Site of the MAPP Research Network. Urology. 2019 Apr;126:24–33.

20 Suskind AM, Berry SH, Ewing BA, Elliott MN, Suttorp MJ, Clemens JQ. The Prevalence and Overlap of Interstitial Cystitis/Bladder Pain Syndrome and Chronic Prostatitis/Chronic Pelvic Pain Syndrome in Men: Results of the RAND Interstitial Cystitis Epidemiology Male Study. J Urol. 2013 Jan;189(1):141–5.

21 Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, et al. Prevalence of Symptoms of Bladder Pain Syndrome/Interstitial Cystitis Among Adult Females in the United States. J Urol. 2011 Aug;186(2):540–4.

22 Roberts RO, Bergstralh EJ, Bass SE, Lightner DJ, Lieber MM, Jacobsen SJ. Incidence of physician-diagnosed interstitial cystitis in Olmsted County: a community-based study. BJU Int. 2003 Feb;91(3):181–5.

23 Pierce AN, Christianson JA. Stress and Chronic Pelvic Pain. Progress in Molecular Biology and Translational Science. Elsevier; 2015; pp 509–35.

24 Riedl C, Engelhardt P, Schwarz B. Treatment Costs of Bladder Pain Syndrome/Interstitial Cystitis in Austria: A Pharmacoeconomic Approach Following Current Guidelines. Clin Drug Investig. 2013 Oct;33(10):737–42.

25 Logadottir Y, Ehren I, Fall M, Wiklund N, Peeker R, Hanno P. Intravesical Nitric Oxide Production Discriminates Between Classic and Nonulcer Interstitial Cystitis. J Urol. 2004 Mar;171(3):1148–51.

26 Wennevik GE, Meijlink JM, Hanno P, Nordling J. The Role of Glomerulations in Bladder Pain Syndrome: A Review. J Urol. 2016 Jan;195(1):19–25.

27 Lavelle JP, Meyers SA, Ruiz WG, Buffington CAT, Zeidel ML, Apodaca G. Urothelial pathophysiological changes in feline interstitial cystitis: a human model. Am J Physiol Renal Physiol. 2000 Apr;278(4):F540–53.

28 Grundy L, Caldwell A, Brierley SM. Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome. Front Neurosci. 2018 Dec;12:931.

29 Birder L, Andersson K-E. Urothelial Signaling. Physiol Rev. 2013 Apr;93(2):653–80.

30 Parsons C, Greenberger M, Gabal L, Bidair M, Barme G. The role of urinary potassium in the pathogenesis and diagnosis of interstitial cystitis. J Urol. 1998 Jun;159(6):1862–7.

31 Acharya P, Beckel J, Ruiz WG, Wang E, Rojas R, Birder L, et al. Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium. Am J Physiol Renal Physiol. 2004 Aug;287(2):F305–18.

32 Janicki JJ, Gruber MA, Chancellor MB. Intravesical liposome drug delivery and IC/BPS. Transl Androl Urol. 2015;4(5):572.

33 Yoshimura N, Oguchi T, Yokoyama H, Funahashi Y, Yoshikawa S, Sugino Y, et al. Bladder afferent hyperexcitability in bladder pain syndrome/interstitial cystitis. Int J Urol. 2014 Apr;21(S1):18–25.

34 Kim SH, Kim B, Kim SW, Oh S-J. Urodynamic Findings of the Painful Bladder Syndrome/Interstitial Cystitis: A Comparison With Idiopathic Overactive Bladder. J Urol. 2009 Jun;181(6):2550–4.

35 Hosseini A, Ehrén I, Wiklund NP. Nitric oxide as an objective marker for evaluation of treatment response in patients with classic interstitial cystitis. J Urol. 2004 Dec;172(6):2261–5.

36 Theoharides TC, Kempuraj D, Sant GR. Mast cell involvement in interstitial cystitis: a review of human and experimental evidence. Urology. 2001 Jun;57(S6A):47–55.

37 Graham E, Chai TC. Dysfunction of bladder urothelium and bladder urothelial cells in interstitial cystitis. Curr Urol Rep. 2006 Dec;7(6):440–6.

38 Lotz M, Villiger P, Hugli T, Koziol J, Zuraw BL. Interleukin-6 and Interstitial Cystitis. J Urol. 1994 Sep;152(3):869–73.

39 Sant GR, Kempuraj D, Marchand JE, Theoharides TC. The Mast Cell in Interstitial Cystitis: Role in Pathophysiology and Pathogenesis. Urology. 2007 Apr;69(4):S34–40.

40 Frenz AM, Christmas TJ, Pearce FL. Does the mast cell have an intrinsic role in the pathogenesis of interstitial cystitis? Agents Actions. 1994 Jun;41(S1):C14–5.

41 Kim A, Han JY, Ryu CM, Yu HY, Lee S, Kim YH, et al. Histopathological characteristics of interstitial cystitis/bladder pain syndrome without Hunner lesion. Histopathology. 2017 Sep;71(3):415–24.

42 Yu W, Kuo H. Multimodal therapies and strategies for the treatment of interstitial cystitis/bladder pain syndrome in Taiwan. Low Urin Tract Symptoms. 2024 Jan;16(1):e12508.

43 Bschleipfer T, Karl I. Bladder Microbiome in the Context of Urological Disorders—Is There a Biomarker Potential for Interstitial Cystitis? Diagnostics. 2022 Jan;12(2):281.

44 Siddiqui H, Lagesen K, Nederbragt AJ, Jeansson SL, Jakobsen KS. Alterations of microbiota in urine from women with interstitial cystitis. BMC Microbiol. 2012 Dec;12:205.

45 Dickson K, Zhou J, Lehmann C. Lower Urinary Tract Inflammation and Infection: Key Microbiological and Immunological Aspects. J Clin Med. 2024 Jan;13(2):315.

46 Arya LA, Northington GM, Asfaw T, Harvie H, Malykhina A. Evidence of Bladder Oversensitivity in the Absence of an Infection in Premenopausal Women with a History of Recurrent Urinary Tract Infections. BJU Int. 2012 Jul;110(2):247–51.

47 Rothrock NE, Lutgendorf SK, Kreder KJ, Ratliff T, Zimmerman B. Stress and symptoms in patients with interstitial cystitis: a life stress model. Urology. 2001 Mar;57(3):422–7.

48 Gil DW, Wang J, Gu C, Donello JE, Cabrera S, Al-Chaer ED. Role of sympathetic nervous system in rat model of chronic visceral pain. Neurogastroenterol Motil. 2016 Mar;28(3):423–31.

49 Rohleder N. Stress and inflammation – The need to address the gap in the transition between acute and chronic stress effects. Psychoneuroendocrinology. 2019 Jul;105:164–71.

50 Farmer MA, Huang L, Martucci K, Yang CC, Maravilla KR, Harris RE, et al. Brain white matter abnormalities in female interstitial cystitis/bladder pain syndrome: A MAPP network neuroimaging study. J Urol. 2015 Jul;194(1):118–26.

51 Panicker JN, Marcelissen T, von Gontard A, Vrijens D, Abrams P, Wyndaele M. Bladder-bowel interactions: Do we understand pelvic organ cross-sensitization? International Consultation on Incontinence Research Society (ICI-RS) 2018. Neurourol Urodyn. 2019 Dec;38(S5):S25–34.

52 Leue C, Kruimel J, Vrijens D, Masclee A, Van Os J, Van Koeveringe G. Functional urological disorders: A sensitized defence response in the bladder-gut-brain axis. Nat Rev Urol. 2017 Mar;14(3):153–63.

53 Bosch PC. Examination of the significant placebo effect in the treatment of interstitial cystitis/bladder pain syndrome. Urology. 2014 Aug;84(2):321–6.

54 Chaiken DC, Blaivas JG, Blaivas ST. Behavioral Therapy for the Treatment of Refractory Interstitial Cystitis. J Urol. 1993 Jun;149(6):1445–8.

55 Yu W-R, Jhang J-F, Chen B-Y, Ou S-R, Li H-M, Kuo H-C. Multimodal Treatment with Cognitive Behavioral Therapeutic Intervention Plus Bladder Treatment Is More Effective than Monotherapy for Patients with Interstitial Cystitis/Bladder Pain Syndrome—A Randomized Clinical Trial. J Clin Med. 2022 Oct;11(20):6221.

56 Pearce WA, Chen R, Jain N. Pigmentary Maculopathy Associated with Chronic Exposure to Pentosan Polysulfate Sodium. Ophthalmology. 2018 Nov;125(11):1793–802.

57 Proctor JG. Pentosan polysulfate and a pigmentary maculopathy: causation versus correlation? Can J Urol. 2023 Dec;30(6):11732–9.

58 Evans RJ, Moldwin RM, Cossons N, Darekar A, Mills IW, Scholfield D. Proof of Concept Trial of Tanezumab for the Treatment of Symptoms Associated With Interstitial Cystitis. J Urol. 2011 May;185(5):1716–21.

59 Sant GR, LaRock DR. Standard Intravesical Therapies for Interstitial Cystitis. Urol Clin North Am. 1994 Feb;21(1):73–83.

60 Giusto LL, Zahner PM, Shoskes DA. An evaluation of the pharmacotherapy for interstitial cystitis. Expert Opin Pharmacother. 2018 Jul;19(10):1097–108.

61 Dimitrakov J, Kroenke K, Steers WD, Berde C, Zurakowski D, Freeman MR, et al. Pharmacologic Management of Painful Bladder Syndrome/Interstitial Cystitis. Arch Intern Med. 2007 Oct;167(18):1922–9.

62 Tutolo M, Ammirati E, Castagna G, Klockaerts K, Plancke H, Ost D, et al. A prospective randomized controlled multicentre trial comparing intravesical DMSO and chondroïtin sulphate 2% for painful bladder syndrome/interstitial cystitis. Int Braz J Urol. 2017 Feb;43(1):134–41.

63 Jhang J-F, Jiang Y-H, Kuo H-C. Current Understanding of the Pathophysiology and Novel Treatments of Interstitial Cystitis/Bladder Pain Syndrome. Biomedicines. 2022 Sep;10(10):2380.

64 Henry Lai H. Management of Interstitial Cystitis/Bladder Pain Syndrome with Tricyclic Antidepressants. Urological and Gynaecological Chronic Pelvic Pain. Cham: Springer International Publishing; 2017; pp 107–17.

65 Lee JW, Han DY, Jeong HJ. Bladder Pain Syndrome Treated with Triple Therapy with Gabapentin, Amitriptyline, and a Nonsteroidal Anti-Inflammatory Drug. Int Neurourol J. 2010 Dec;14(4):256–60.

66 Thilagarajah R, O'N Witherow R, Walker MM. Oral cimetidine gives effective symptom relief in painful bladder disease: a prospective, randomized, double-blind placebo-controlled trial. BJU Int. 2001 Feb;87(3):207–12.

67 Sant GR, Propert KJ, Hanno PM, Burks D, Culkin D, Diokno AC, et al. A Pilot Clinical Trial of Oral Pentosan Polysulfate And Oral Hydroxyzine in Patients With Interstitial Cystitis. J Urol. 2003 Sep;170(3):810–5.

68 Gajewski JB, Al-Zahrani AA. The long-term efficacy of sacral neuromodulation in the management of intractable cases of bladder pain syndrome: 14 years of experience in one centre. BJU Int. 2011 Apr;107(8):1258–64.

69 Chen H, Wang F, Chen W, Ye xue ting, Zhou Q, Shao F, et al. Efficacy of Daily Low-dose Sildenafil for Treating Interstitial Cystitis: Results of a Randomized, Double-blind, Placebo-controlled Trial—Treatment of Interstitial Cystitis/Painful Bladder Syndrome With Low-dose Sildenafil. Urology. 2014 Jul;84(1):51–6.

70 Lowe H, Toyang N, Steele B, Bryant J, Ngwa W. The Endocannabinoid System: A Potential Target for the Treatment of Various Diseases. Int J Mol Sci. 2021 Sep;22(17):9472.

71 Battista N, Di Tommaso M, Bari M, Maccarrone M. The endocannabinoid system: an overview. Front Behav Neurosci. 2012 Mar;6:9.

72 Chiou L-C, Hu SS-J, Ho Y-C. Targeting the cannabinoid system for pain relief? Acta Anaesthesiol Taiwan. 2013 Dec;51(4):161–70.

73 Kilaru A, Chapma KD. The endocannabinoid system. Essays Biochem. 2020 Sep;64(3):485–99.

74 Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature. 1993 Sep;365(6441):61–5.

75 Howlett AC, Abood ME. CB 1 and CB 2 Receptor Pharmacology. Adv Pharmacol. 2017 Jun;80:169–206.

76 Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat. 2002 Aug;68–69:619–31.

77 Gratzke C, Streng T, Park A, Christ G, Stief CG, Hedlund P, et al. Distribution and Function of Cannabinoid Receptors 1 and 2 in the Rat, Monkey and Human Bladder. J Urol. 2009 Apr;181(4):1939–48.

78 Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, et al. Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. Eur J Biochem. 1995 Aug;232(1):54–61.

79 Lu HC, MacKie K. An introduction to the endogenous cannabinoid system. Biol Psychiatry. 2016 Apr;79(7):516–25.

80 Tyagi S, Gupta P, Saini A, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J Adv Pharm Technol Res. 2011 Oct;2(4):236–40.

81 Silverman HA, Chen A, Kravatz NL, Chavan SS, Chang EH. Involvement of Neural Transient Receptor Potential Channels in Peripheral Inflammation. Front Immunol. 2020 Oct;11.

82 Savinainen JR, Jaè T, Laine K, Laitinen JT. Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB1 receptor-dependent G-protein activation in rat cerebellar membranes. Br J Pharmacol. 2001 Oct;134:664–72.

83 Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW. Endocannabinoid 2-Arachidonyl Glycerol Is a Full Agonist through Human Type 2 Cannabinoid Receptor: Antagonism by Anandamide. Mol Pharmacol. 2000 Feb;57:1045–50.

84 Blankman JL, Simon GM, Cravatt BF. A Comprehensive Profile of Brain Enzymes that Hydrolyze the Endocannabinoid 2-Arachidonoylglycerol. Chem Biol. 2007 Dec;14(12):1347–56.

85 Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature. 1996;384(6604):83–7.

86 Woodward DF, Liang Y, Krauss AHP. Prostamides (prostaglandin-ethanolamides) and their pharmacology. Br J Pharmacol. 2008 Feb;153(3):410–9.

87 Tsuboi K, Sun YX, Okamoto Y, Araki N, Tonai T, Ueda N. Molecular Characterization of N-Acylethanolamine-hydrolyzing Acid Amidase, a Novel Member of the Choloylglycine Hydrolase Family with Structural and Functional Similarity to Acid Ceramidase. J Biol Chem. 2005 Mar;280(12):11082–92.

88 Makriyannis A, Mechoulam R, Piomelli D. Therapeutic opportunities through modulation of the endocannabinoid system. Neuropharmacology. 2005 Jun;48(8):1068–71.

89 Berger G, Arora N, Burkovskiy I, Xia Y, Chinnadurai A, Westhofen R, et al. Experimental Cannabinoid 2 Receptor Activation by Phyto-Derived and Synthetic Cannabinoid Ligands in LPS-Induced Interstitial Cystitis in Mice. Molecules. 2019 Nov;24(23):4239.

90 Kuwahata H, Katsuyama S, Komatsu T, Nakamura H, Corasaniti MT, Bagetta G, et al. Local Peripheral Effects of β-Caryophyllene through CB2 Receptors in Neuropathic Pain in Mice. Pharmacol Pharm. 2012 Oct;3(4):397–403.

91 Demuth DG, Molleman A. Cannabinoid signalling. Life Sci. 2006 Jan;78(6):549–63.

92 Ibsen MS, Connor M, Glass M. Cannabinoid CB1 and CB2 Receptor Signaling and Bias. Cannabis Cannabinoid Res. 2017 Jan;2(1):48–60.

93 Pacher P, Bátkai S, Kunos G. The Endocannabinoid System as an Emerging Target of Pharmacotherapy. Pharmacol Rev. 2006 Sep;58(3):389–462.

94 Merriam FV, Wang Z, Guerios SD, Bjorling DE. Cannabinoid receptor 2 is increased in acutely and chronically inflamed bladder of rats. Neurosci Lett. 2008 Nov;445(1):130–4.

95 Tambaro S, Casu MA, Mastinu A, Lazzari P. Evaluation of selective cannabinoid CB1 and CB2 receptor agonists in a mouse model of lipopolysaccharide-induced interstitial cystitis. Eur J Pharmacol. 2014 Apr;729:67–74.

96 Gertsch J, Leonti M, Raduner S, Racz I, Chen J-Z, Xie X-Q, et al. Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci U S A. 2008 Jul;105(26):9099–104.

97 Klauke A-L, Racz I, Pradier B, Markert A, Zimmer AM, Gertsch J, et al. The cannabinoid CB2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. Eur Neuropsychopharmacol. 2014 Apr;24(4):608–20.

98 Sköld M, Karlberg AT, Matura M, Börje A. The fragrance chemical β-caryophyllene—air oxidation and skin sensitization. Food Chem Toxicol. 2006 Apr;44(4):538–45.

99 Neta MCS, Vittorazzi C, Guimarães AC, Martins JDL, Fronza M, Endringer DC, et al. Effects of β-caryophyllene and Murraya paniculata essential oil in the murine hepatoma cells and in the bacteria and fungi 24-h time-kill curve studies. Pharm Biol. 2017 Jan;55(1):190–7.

100 Schmitt D, Levy R, Carroll B. Toxicological Evaluation of β-Caryophyllene Oil. Int J Toxicol. 2016 Jun;35(5):558–67.

101 Oliveira GL da S, Machado KC, Machado KC, da Silva AP dos SCL, Feitosa CM, de Castro Almeida FR. Non-clinical toxicity of β -caryophyllene, a dietary cannabinoid: Absence of adverse effects in female Swiss mice. Regul Toxicol Pharmacol. 2018 Feb;92:338–46.

102 Nguyen LT, Myslivečková Z, Szotáková B, Špičáková A, Lněničková K, Ambrož M, et al. The inhibitory effects of β-caryophyllene, β-caryophyllene oxide and α-humulene on the activities of the main drug-metabolizing enzymes in rat and human liver in vitro. Chem Biol Interact. 2017 Dec;278:123–8.

103 Wu C, Jia Y, Lee JH, Jun HJ, Lee HS, Hwang KY, et al. trans-Caryophyllene is a natural agonistic ligand for peroxisome proliferator-activated receptor-α. Bioorg Med Chem Lett. 2014 Jul;24(14):3168–74.

104 Scandiffio R, Bonzano S, Cottone E, Shrestha S, Bossi S, De Marchis S, et al. Beta-Caryophyllene Modifies Intracellular Lipid Composition in a Cell Model of Hepatic Steatosis by Acting through CB2 and PPAR Receptors. Int J Mol Sci. 2023 Mar;24(7):6060.

105 Venkatakrishna K, Sundeep K, Sudeep HV, Gouthamchandra K, Shyamprasad K. ViphyllinTM, a Standardized Black Pepper Seed Extract Exerts Antinociceptive Effects in Murine Pain Models via Activation of Cannabinoid Receptor CB2, Peroxisome Proliferator-Activated Receptor-Alpha and TRPV1 Ion Channels. J Pain Res. 2022 Feb;Volume 15:355–66.

106 Youssef DA, El-Fayoumi HM, Mahmoud MF. Beta-caryophyllene protects against diet-induced dyslipidemia and vascular inflammation in rats: Involvement of CB2 and PPAR-γ receptors. Chem Biol Interact. 2019 Jan;297:16–24.

107 Varley CL, Stahlschmidt J, Smith B, Stower M, Southgate J. Activation of Peroxisome Proliferator-Activated Receptor-γ Reverses Squamous Metaplasia and Induces Transitional Differentiation in Normal Human Urothelial Cells. Am J Pathol. 2004 May;164(5):1789–98.

108 Hernandez-Leon A, González-Trujano ME, Narváez-González F, Pérez-Ortega G, Rivero-Cruz F, Aguilar MI. Role of β-Caryophyllene in the Antinociceptive and Anti-Inflammatory Effects of Tagetes lucida Cav. Essential Oil. Molecules. 2020 Feb;25(3):675.

109 Katsuyama S, Mizoguchi H, Kuwahata H, Komatsu T, Nagaoka K, Nakamura H, et al. Involvement of peripheral cannabinoid and opioid receptors in β-caryophyllene-induced antinociception. Eur J Pain. 2013 May;17(5):664–75.

110 Ibrahim MM, Porreca F, Lai J, Albrecht PJ, Rice FL, Khodorova A, et al. CB2 cannabinoid receptor activation produces antinociception by stimulating peripheral

release of endogenous opioids. Proc Natl Acad Sci U S A. 2005 Feb;102(8):3093–8.

111 Ghelardini C, Galeotti N, Di L, Mannelli C, Mazzanti G, Bartolini A. Local anaesthetic activity of b-caryophyllene. Il Farmaco. 2001;56(5–7):387–9.

112 Taylor A, McLeod G. Basic pharmacology of local anaesthetics. BJA Educ. 2020 Feb;20(2):34–41.

113 Serra MP, Boi M, Carta A, Murru E, Carta G, Banni S, et al. Anti-Inflammatory Effect of Beta-Caryophyllene Mediated by the Involvement of TRPV1, BDNF and trkB in the Rat Cerebral Cortex after Hypoperfusion/Reperfusion. Int J Mol Sci. 2022 Mar;23(7):3633.

114 Varga Z V., Matyas C, Erdelyi K, Cinar R, Nieri D, Chicca A, et al. β-Caryophyllene protects against alcoholic steatohepatitis by attenuating inflammation and metabolic dysregulation in mice. Br J Pharmacol. 2018 Jan;175(2):320–34.

115 Bui TM, Wiesolek HL, Sumagin R. ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. J Leukoc Biol. 2020 Sep;108(3):787–99.

116 Kong D-H, Kim Y, Kim M, Jang J, Lee S. Emerging Roles of Vascular Cell Adhesion Molecule-1 (VCAM-1) in Immunological Disorders and Cancer. Int J Mol Sci. 2018 Apr;19(4):1057.

117 Ames-Sibin AP, Barizão CL, Castro-Ghizoni C V., Silva FMS, Sá-Nakanishi AB, Bracht L, et al. β-Caryophyllene, the major constituent of copaiba oil, reduces systemic inflammation and oxidative stress in arthritic rats. J Cell Biochem. 2018 Dec;119(12):10262–77.

118 Baradaran Rahimi V, Askari VR. A mechanistic review on immunomodulatory effects of selective type two cannabinoid receptor β-caryophyllene. BioFactors. 2022 Jul;48(4):857–82.

119 Wei C, Huang L, Zheng Y, Cai X. Selective activation of cannabinoid receptor 2 regulates Treg/Th17 balance to ameliorate neutrophilic asthma in mice. Ann Transl Med. 2021;9(12):1015.

120 Santos PS, Oliveira TC, R. Júnior LM, Figueiras A, Nunes LCC. β-caryophyllene Delivery Systems: Enhancing the Oral Pharmacokinetic and Stability. Curr Pharm Des. 2018 Sep;24(29):3440–53.

121 Wong HL, Wu XY, Bendayan R. Nanotechnological advances for the delivery of CNS therapeutics. Adv Drug Deliv Rev. 2012 May;64(7):686–700.

122 Alharbi WS, Almughem FA, Almehmady AM, Jarallah SJ, Alsharif WK, Alzahrani NM, et al. Phytosomes as an Emerging Nanotechnology Platform for the Topical Delivery of Bioactive Phytochemicals. Pharmaceutics. 2021 Sep;13(9):1475.

123 Plaza-Oliver M, Santander-Ortega MJ, Lozano MV. Current approaches in lipid-based nanocarriers for oral drug delivery. Drug Deliv Transl Res. 2021 Apr;11(2):471–97.

124 Zhang L, Wang S, Zhang M, Sun J. Nanocarriers for oral drug delivery. J Drug Target. 2013 Jul;21(6):515–27.

125 Mrsny RJ. Oral drug delivery research in Europe. J Control Release. 2012 Jul;161(2):247–53.

126 Barenholz Y. Doxil® — The first FDA-approved nano-drug: Lessons learned. J Control Release. 2012 Jun;160(2):117–34.

127 Khurana A, Allawadhi P, Khurana I, Allwadhi S, Weiskirchen R, Banothu AK, et al. Role of nanotechnology behind the success of mRNA vaccines for COVID-19. Nano Today. 2021 Jun;38:101142.

128 Barani M, Sangiovanni E, Angarano M, Rajizadeh MA, Mehrabani M, Piazza S, et al. Phytosomes as Innovative Delivery Systems for Phytochemicals: A Comprehensive Review of Literature. Int J Nanomedicine. 2021 Oct;16:6983–7022.

129 Gnananath K, Nataraj KS, Rao BG. Phospholipid Complex Technique for Superior Bioavailability of Phytoconstituents. Adv Pharm Bull. 2017 Apr;7(1):35–42.

130 Chen R-P, Chavda VP, Patel AB, Chen Z-S. Phytochemical Delivery Through Transferosome (Phytosome): An Advanced Transdermal Drug Delivery for Complementary Medicines. Front Pharmacol. 2022 Feb;13:850862.

131 Teja PK, Mithiya J, Kate AS, Bairwa K, Chauthe SK. Herbal nanomedicines: Recent advancements, challenges, opportunities and regulatory overview. Phytomedicine. 2022 Feb;96:153890.

132 Gupta MK, Sansare V, Shrivastava B, Jadhav S, Gurav P. Comprehensive review on use of phospholipid based vesicles for phytoactive delivery. J Liposome Res. 2022 Jul;32(3):211–23.

133 Agrawal V, Amresh G, Chaturvedi S, Agrawal VK, Gupta A. Improvement in bioavailability of class-III drug: Phytolipid delivery system. Int J Pharm Pharm Sci. 2012 Jan;4(S1):37–42.

134 Bastús NG, Puntes V. Nanosafety: Towards Safer Nanoparticles by Design. Curr Med Chem. 2017 Apr;25(35):4587–601.

135 Flaig TW, Gustafson DL, Su LJ, Zirrolli JA, Crighton F, Harrison GS, et al. A phase I and pharmacokinetic study of silybin-phytosome in prostate cancer patients. Invest New Drugs. 2007 Apr;25(2):139–46.

136 Pastorelli D, Fabricio ASC, Giovanis P, D'Ippolito S, Fiduccia P, Soldà C, et al. Phytosome complex of curcumin as complementary therapy of advanced pancreatic cancer improves safety and efficacy of gemcitabine: Results of a prospective phase II trial. Pharmacol Res. 2018 Jun;132:72–9.

137 Loguercio C, Andreone P, Brisc C, Brisc MC, Bugianesi E, Chiaramonte M, et al. Silybin combined with phosphatidylcholine and vitamin E in patients with nonalcoholic fatty liver disease: A randomized controlled trial. Free Radic Biol Med. 2012 May;52(9):1658–65.

138 Pivari F, Mingione A, Piazzini G, Ceccarani C, Ottaviano E, Brasacchio C, et al. Curcumin Supplementation (Meriva®) Modulates Inflammation, Lipid Peroxidation and Gut Microbiota Composition in Chronic Kidney Disease. Nutrients. 2022 Jan;14(1):231.

139 Hopkins WJ, Gendron-Fitzpatrick A, Balish E, Uehling DT. Time course and host responses to Escherichia coli urinary tract infection in genetically distinct mouse strains. Infect Immun. 1998;66(6):2798–802.

140 Dickson K, Scott C, White H, Zhou J, Kelly M, Lehmann C. Antibacterial and Analgesic Properties of Beta-Caryophyllene in a Murine Urinary Tract Infection Model. Molecules. 2023 May;28(10):4144.

141 Rom S, Zuluaga-Ramirez V, Dykstra H, Reichenbach NL, Pacher P, Persidsky Y. Selective Activation of Cannabinoid Receptor 2 in Leukocytes Suppresses Their Engagement of the Brain Endothelium and Protects the Blood-Brain Barrier. Am J Pathol. 2013 Nov;183(5):1548–58.

142 Kim SD, Cho KJ, Kim JC. Expression of cannabinoid 1 and, 2 receptors and the effects of cannabinoid 1 and, 2 receptor agonists on detrusor overactivity associated with bladder outlet obstruction in rats. BMC Urol. 2017 Dec;17(1):121.

143 Liu H, Yang G, Tang Y, Cao D, Qi T, Qi Y, et al. Physicochemical characterization and pharmacokinetics evaluation of-caryophyllene/-cyclodextrin inclusion complex. Int J Pharm. 2013;450:304–10.

144 Asakawa Y, Ishida T, Toyota M, Takemoto T. Terpenoid biotransformation in mammals. IV Biotransformation of (+)-longifolene, (-)-caryophyllene, (-)-caryophyllene oxide, (-)-cyclocolorenone, (+)-nootkatone, (-)-elemol, (-)-abietic acid and (+)-dehydroabietic acid in rabbits. Xenobiotica. 1986 Aug;16(8):753–67.

145 Cho HI, Hong JM, Choi JW, Choi HS, Hwan Kwak J, Lee DU, et al. β-Caryophyllene alleviates d-galactosamine and lipopolysaccharide-induced hepatic injury through suppression of the TLR4 and RAGE signaling pathways. Eur J Pharmacol. 2015 Oct;764:613–21.

146 Yang M, Lv Y, Tian X, Lou J, An R, Zhang Q, et al. Neuroprotective Effect of β-Caryophyllene on Cerebral Ischemia-Reperfusion Injury via Regulation of Necroptotic Neuronal Death and Inflammation: In Vivo and in Vitro. Front Neurosci. 2017 Oct;11:583.

147 Bäckhed F, Meijer L, Normark S, Richter-Dahlfors A. TLR4-dependent recognition of lipopolysaccharide by epithelial cells requires sCD14. Cell Microbiol. 2002 Aug;4(8):493–501.

148 Youssef DA, El-Fayoumi HM, Mahmoud MF. Beta-caryophyllene alleviates diet-induced neurobehavioral changes in rats: The role of CB2 and PPAR-γ receptors. Biomed Pharmacother. 2019 Feb;110:145–54.

149 Hartmann P, Szabó A, Erős G, Gurabi D, Horváth G, Németh I, et al. Anti-inflammatory effects of phosphatidylcholine in neutrophil leukocyte-dependent acute arthritis in rats. Eur J Pharmacol. 2009 Nov;622(1–3):58–64.

150 Chen M, Huang H, Zhou P, Zhang J, Dai Y, Yang D, et al. Oral Phosphatidylcholine Improves Intestinal Barrier Function in Drug-Induced Liver Injury in Rats. Gastroenterol Res Pract. 2019 Sep;2019:1–6.

151 Kowalewska PM, Burrows LL, Fox-Robichaud AE. Intravital Microscopy of the Murine Urinary Bladder Microcirculation. Microcirculation. 2011 Nov;18(8):613–22.

152 Hagn G, Holbein B, Zhou J, Lehmann C. Anti-inflammatory iron chelator, DIBI, reduces leukocyte-endothelial adhesion and clinical symptoms of LPS-induced interstitial cystitis in mice. Clin Hemorheol Microcirc. 2021 Dec;79(3):395–406.

153 Li J, Luo H, Dong X, Liu Q, Wu C, Zhang T, et al. Therapeutic effect of urine-derived stem cells for protamine/lipopolysaccharide-induced interstitial cystitis in a rat model. Stem Cell Res Ther. 2017 Dec;8(1):107.

154 Hilbert DW, Pascal KE, Libby EK, Mordechai E, Adelson ME, Trama JP. Uropathogenic Escherichia coli dominantly suppress the innate immune response of bladder epithelial cells by a lipopolysaccharide- and Toll-like receptor 4-independent pathway. Microbes Infect. 2008 Feb;10(2):114–21.

155 ELISA sample preparation guide [Internet]. Abcam. 2024 [cited 2024 May 8]. Available from: https://www.abcam.com/protocols/elisa-sample-preparation-guide-1

156 Chu R, Umukoro N, Greer T, Roberts J, Adekoya P, Odonkor CA, et al. Intravenous Lidocaine Infusion for the Management of Early Postoperative Pain: A Comprehensive Review of Controlled Trials Intravenous Lidocaine Infusion for the Management of Early Postoperative Pain. Psychopharmacol Bull. 2020 Oct;50(4 Suppl 1):216–59.

157 de Araujo DR, Cereda CMS, Brunetto GB, Vomero VU, Pierucci A, Neto HS, et al. Pharmacological and local toxicity studies of a liposomal formulation for the novel local anaesthetic ropivacaine. J Pharm Pharmacol. 2008 Nov;60(11):1449–57.

158 Zheng Z, Zhang J, Zhang C, Li W, Ma K, Huang H, et al. The study on the function and cell source of interleukin-6 in interstitial cystitis/bladder painful syndrome rat model. Immun Inflamm Dis. 2021 Dec;9(4):1520–8.

159 Wang Z-Y, Wang P, Bjorling DE. Treatment with a Cannabinoid Receptor 2 Agonist Decreases Severity of Established Cystitis. J Urol. 2014 Apr;191(4):1153–8.

160 Cunha TM, Verri Jr. WA, Vivancos GG, Moreira IF, Reis S, Parada CA, et al. An electronic pressure-meter nociception paw test for mice. Braz J Med Biol Res. 2004 Mar;37(3):401–7.

161 Martinov T, Mack M, Sykes A, Chatterjea D. Measuring Changes in Tactile Sensitivity in the Hind Paw of Mice Using an Electronic von Frey Apparatus. J Vis Exp. 2013 Dec;82(82):e51212.

162 McMahon SB, Dmitrieva N, Koltzenburg M. Visceral pain. Br J Anaesth. 1995 Aug;75(2):132–44.

163 Powles T, Te Poele R, Shamash J, Chaplin T, Propper D, Joel S, et al. Cannabis-induced cytotoxicity in leukemic cell lines: the role of the cannabinoid receptors and the MAPK pathway. Blood. 2005 Feb;105(3):1214–21.

164 Wu Y, Ma R, Long C, Shu Y, He P, Zhou Y, et al. The protective effect of cannabinoid type II receptor agonist AM1241 on ConA-induced liver injury in mice via mitogen-activated protein kinase signalling pathway. Int J Immunopathol Pharmacol. 2021 Jan;35:1–13.

165 Yu X, Liao B, Zhu P, Cheng S, Du Z, Jiang G. β-Caryophyllene induces apoptosis and inhibits cell proliferation by deregulation of STAT-3/mTOR/AKT signaling in human bladder cancer cells: An in vitro study. J Biochem Mol Toxicol. 2021 Oct;35(10):e22863.

166 Dahham SS, Tabana YM, Iqbal MA, Ahamed MBK, Ezzat MO, Majid ASA, et al. The anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of Aquilaria crassna. Molecules. 2015 Jul;20(7):11808–29.

167 Li D, Zhang X, Chen B. SIGIRR participates in negative regulation of LPS response and tolerance in human bladder epithelial cells. BMC Immunol. 2015 Dec;16:73.

168 Ying H, Da L, Yu-Xiu S, Yu X, Li-Xia L, Li-Mei X, et al. TLR4 mediates MAPK-STAT3 axis activation in bladder epithelial cells. Inflammation. 2013 Oct;36(5):1064–74.

169 Hunstad DA, Justice SS, Hung CS, Lauer SR, Hultgren SJ. Suppression of Bladder Epithelial Cytokine Responses by Uropathogenic Escherichia coli. Infect Immun. 2005 Jul;73(7):3999–4006.

170 Kumar P, Oster RA, Assimos DG, Ness TJ, Mitchell T. Bioenergetic profiles of peripheral mononuclear cells and systemic inflammation in women with Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS). PLoS One. 2024 Feb;19(2):e0298981.

171 Picciolo G, Pallio G, Altavilla D, Vaccaro M, Oteri G, Irrera N, et al. β-Caryophyllene Reduces the Inflammatory Phenotype of Periodontal Cells by Targeting CB2 Receptors. Biomedicines. 2020 Jun;8(6):164.

172 De Canha MN, Komarnytsky S, Langhansova L, Lall N. Exploring the Anti-Acne Potential of Impepho [Helichrysum odoratissimum (L.) Sweet] to Combat Cutibacterium acnes Virulence. Front Pharmacol. 2020 Jan;10:1559.

173 Zinzar SN, Svet-Moldavsky GJ, Fogh J, Mann PE, Arlin Z, Iliescu K, et al. Elaboration of granulocyte-macrophage colony-stimulating factor by human tumor cell lines and normal urothelium. Exp Hematol. 1985 Jul;13(6):574–80.

174 Jiang Y-H, Jhang J-F, Kuo H-C. Can We Use Urinary Cytokine/Chemokine Analysis in Discriminating Ulcer-Type Interstitial Cystitis/Bladder Pain Syndrome? Diagnostics. 2022 Apr;12(5):1093.

175 Wang X, Ji T, Jiang Z, Wang J, Su X, Shan L. Tolterodine ameliorates inflammatory response and ferroptosis against LPS in human bladder epithelial cells. J Biochem Mol Toxicol. 2024 Jan;38(1):e23517.

176 Fong Y, Tracey KJ, Moldawer LL, Hesse DG, Manogue KB, Kenney JS, et al. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin 6 appearance during lethal bacteremia. J Exp Med. 1989 Nov;170(5):1627–33.

177 Sommer C, Kress M. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett. 2004 May;361(1–3):184–7.

178 Müller N, Schneider B, Pfizenmaier K, Wajant H. Superior serum half life of albumin tagged TNF ligands. Biochem Biophys Res Commun. 2010 Jun;396(4):793–9.

179 Batista CRA, Gomes GF, Candelario-Jalil E, Fiebich BL, de Oliveira ACP. Lipopolysaccharide-Induced Neuroinflammation as a Bridge to Understand Neurodegeneration. Int J Mol Sci. 2019 May;20(9):2293.

180 Lin AE, Beasley FC, Olson J, Keller N, Shalwitz RA, Hannan TJ, et al. Role of Hypoxia Inducible Factor-1α (HIF-1α) in Innate Defense against Uropathogenic Escherichia coli Infection. PLoS Pathog. 2015 Apr;11(4):e1004818.

181 Chen C, Li J, Wang J, Zhang M, Zhang L, Lin Z. Oxybutynin ameliorates LPS-induced inflammatory response in human bladder epithelial cells. J Biochem Mol Toxicol. 2024 Jan;38(1):e23584.

182 Nikolaeva S, Bachteeva V, Fock E, Herterich S, Lavrova E, Borodkina A, et al. Frog urinary bladder epithelial cells express TLR4 and respond to bacterial LPS by increase of iNOS expression and L-arginine uptake. Am J Physiol Regul Integr Comp Physiol. 2012 Nov;303(10):1042–52.

183 Ertel A, Verghese A, Byers SW, Ochs M, Tozeren A. Pathway-specific differences between tumor cell lines and normal and tumor tissue cells. Mol Cancer. 2006 Dec;5(1):55.

184 Yoshizumi M, Watanabe C, Mizoguchi H. Gabapentin reduces painful bladder hypersensitivity in rats with lipopolysaccharide-induced chronic cystitis. Pharmacol Res Perspect. 2021 Feb;9(1):e00697.

185 Lv YS, Yao YS, Rong L, Lin ME, Deng BH, Xie Y, et al. Intravesical hyaluronidase causes chronic cystitis in a rat model: A potential model of bladder pain syndrome/interstitial cystitis. Int J Urol. 2014 Jun;21(6):601–7.

186 Li M, Yang K, Wang X, Xu X, Zhu L, Wang H. Mast cells infiltration and decreased E-cadherin expression in ketamine-induced cystitis. Toxicol Rep. 2015 May;2:205–9.

187 Rudick CN, Pavlov VI, Chen MC, Klumpp DJ. Gender Specific Pelvic Pain Severity in Neurogenic Cystitis. J Urol. 2012 Feb;187(2):715–24.

188 Ceccarelli I, Fiorenzani P, Pessina F, Pinassi J, Aglianò M, Miragliotta V, et al. The CB2 Agonist β-Caryophyllene in Male and Female Rats Exposed to a Model of Persistent Inflammatory Pain. Front Neurosci. 2020 Aug;14:850.

189 Koyama S, Purk A, Kaur M, Soini HA, Novotny M V., Davis K, et al. Beta-caryophyllene enhances wound healing through multiple routes. PLoS One. 2019 Dec;14(12):e0216104.