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CHEMICAL INVESTIGATIONS OF PECTIC OLIGOSACCHARIDES FROM VACCINIUM MACROCARPON

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CHEMICAL INVESTIGATIONS OF PECTIC
OLIGOSACCHARIDES FROM *VACCINIUM*

MACROCARPON

BY

ZHIYUAN PENG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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ABSTRACT

Urinary tract infections (UTIs) caused by uropathogenic *Escherichia coli* (UPEC) are a serious problem worldwide, especially for females. Previous research in our laboratory suggests that cranberry pectic oligosaccharides (cPOS) can reverse dormant phenotypes in UPEC and therefore could prove useful in the prevention of recurrent UTIs. This thesis focused on the purification and structure determination of purified pectic oligosaccharides from cranberry hull extract. The research helped established the structures of cPOS, provided high purity materials for biological testing, and enabled further studies on the effect of different degrees of polymerization (DP) in preventing dormant phenotypes in UPEC. More specifically, cPOS was determined to be comprised of galacturonic acid residues by applying chemical derivatization methods. Small quantities of terpene glycosides impurities in previously tested fractions were removed successfully by implementing a trituration method. The bioassay results of terpene glycosides-free cPOS confirmed the impurities were not responsible for the bioactivity of cPOS. Finally, a semi-preparative HPLC method using HILIC chromatography was established for the purification of cPOS components with variable DPs. A preliminary structure-activity relationship based on the variation of cPOS DPs is also described.

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12 and Figure 14) and Dr. Nicholas DaSilva (Figure 15). Their work made this thesis much improved and complete.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	v
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	viii
CHAPTER 1	1
INTRODUCTION	1
CHAPTER 2	4
REVIEW OF LITERATURE	4
CHAPTER 3	8
METHODOLOGY	8
CHAPTER 4	14
RESULTS	14
CHAPTER 5	27
CONCLUSION	27
APPENDIX	29
BIBLIOGRAPHY	39

LIST OF FIGURES

Figure 1. Structures of iridoid glucosides	16
Figure 2. LC-MS analysis of iridoid glucosides.....	16
Figure 3. HILIC HPLC chromatogram of cPOS (Top) and cPOS-t (Bottom) at 235 nm.	17
Figure 4. ¹ H NMR spectrum (D ₂ O, 500 MHz) of standards (purified 4-mer pectic oligosaccharide and iridoids), and cPOS-t (trituated 10% PGC).....	18
Figure 5. HSQC spectrum of cPOS-t (D ₂ O, 500 MHz).....	19
Figure 6. Persister cell viability treating with cPOS-t (CFT073+ampicillin+ cPOS-t)	19
Figure 7. Persister cell reduction following treatment with cPOS and ampicillin.....	20
Figure 8. Uronic acid identification of cPOS.....	22
Figure 9. Analytical HILIC HPLC chromatography of cPOS by using TOSOH Tskgel Amide-80 HR column (7.8 mm I.D x 30 cm, 10 μm), 1.0 mL/min.	24
Figure 10. Preparative HILIC HPLC chromatography of cPOS by using TOSOH Tskgel Amide-80 HR column (7.8 mm I.D x 30 cm, 10 μm), 1.5mL/min.	24
Figure 11. ¹ H NMR spectrum (D ₂ O, 300 MHz) of 11 fractions separated by preparative HILIC HPLC.....	25
Figure 12. CFT073 quiescence assay using glucose-free M9 plates.	26

Figure 13. Modulation of IL-1 β and TNF- α production following treatment with constituents from cranberry juice extract powder.	
.....	33
Figure 14. HPLC separation of one active fraction (70% water/30% ACN portion).	
.....	35
Figure 15. Modulation of IL-6 and TNF- α production following treatment with constituents from cranberry juice extract powder	
.....	36
Figure 16. Cranberry extract significantly attenuates the release of pro-inflammatory TNF α and IL-6 cytokines.	
.....	38

LIST OF ABBREVIATIONS

UTI, urinary tract infection; UPEC Uropathogenic *Escherichia coli*; QIR quiescent intracellular reservoirs; IBC intracellular bacterial communities; NMR, nuclear magnetic resonance; PACs, proanthocyanidins; TFA, trifluoroacetic acid; CFU, colony-forming units; HSQC, heteronuclear single quantum coherence spectroscopy.

CHAPTER 1

INTRODUCTION

Urinary tract infections (UTIs) are one of the most prevalent infectious diseases worldwide, affecting people of all ages.¹ It is estimated that 10.5 million UTI cases occur annually in United states.² Although most UTI patients only suffer from minimally painful symptoms, the Centers for Disease Control and Prevention reports that UTIs are responsible for 13,000 deaths annually.³ About 50% of women will experience a UTI at some point in life, while 25% will experience a second, recurrent UTI within 6 months after treatment of the initial UTI.⁴

Uropathogenic *Escherichia coli* (UPEC) cause nearly 90% of all diagnosed UTIs.⁵ UPEC forms biofilm-like intracellular bacterial communities (IBC) after invasion of superficial facet cells in the bladder. This niche permits bacteria to reside and replicate in intracellular environments that are predominantly protected from host innate immunity and antibiotic treatments. UPEC can spread from IBCs to infect other urethral epithelial cells. This process is proposed to allow UPEC to develop quiescent intracellular reservoirs (QIRs)⁶ in the bladder tissue, resulting in bacteria reservoirs that remain viable for months within the bladder tissue despite the absence of bacteria in the urine.^{7,8} Multiple studies have used *in vitro* and *in vivo*⁹ models for UTIs to verify that UPEC can invade bladder epithelial cells^{10,11,12} and kidney epithelial cells.¹³

Previous data supports that cranberry (*Vaccinium macrocarpon*) consumption may hinder bacterial adherence to uroepithelial cells, potentially easing UTI related

symptoms.¹⁴ The cranberry plant is an evergreen shrub that was first cultivated in North America. Cranberries are a major agricultural product in both the United States and Canada.¹⁵ Additionally, there is positive clinical evidence that cranberry products can be used prophylactically to reduce recurrent infections.¹⁶ Previous investigations of the responsible cranberry constituents have mainly focused on proanthocyanidins, particularly those with A-type linkages,¹⁷ and small organic acids.¹⁸ More recently, researchers have focused on the potential involvement of cranberry complex carbohydrates for beneficial effects against UPEC.^{15,19} Our lab previously discovered that cranberry xyloglucan can interfere with biofilm formation by UPEC.²⁰ More recently, we discovered that cranberry pectic oligosaccharides (cPOS) prevent quiescence in the UPEC strain CFT073.²¹

In previous work,²² iridoid terpene glycosides were identified in fractions containing cPOS following chromatographic separations. The removal of iridoids is necessary to prove the activity of cPOS on UPEC in our biological assays. Our separation approach leveraged the differential solubility of the iridoids and cPOS in ethanol and water. HPLC and NMR spectroscopy were used to measure the residual impurities in the cPOS fraction, and the iridoid-free cPOS was then tested in a persister cell viability assay.

Previously, we used NMR spectroscopy to determine that the structure of cPOS is comprised of repeating galacturonic acid units. However, the NMR approach was insufficient for differentiating between galacturonic and glucuronic acid monosaccharide units. In this thesis, we pursued a chemical derivatization approach to complement the previous NMR studies. Specifically, cPOS was hydrolyzed, modified

by a 1-phenyl-3-methyl-5-pyrazolone (PMP) reaction, and studied by HPLC in comparison to the appropriate standards. These experiments confirmed that cPOS is comprised of galacturonic acid monomers.

Additional studies in this thesis were aimed at optimizing the separation of individual components of cPOS from a complex mixture. cPOS is a mixture of oligosaccharides with varying degrees of polymerization (DP). The effect of DP on the bioactivities of cPOS are currently unknown. The high polarity and water solubility of cPOS makes separation of its individual components challenging. We developed an HPLC method using HILIC chromatography to separate cPOS constituents of varying DPs. These studies may ultimately help resolve structure–activity relationships (SAR) related to cPOS degree of polymerization and reversal of UPEC quiescence.

CHAPTER 2

REVIEW OF LITERATURE

Oligosaccharides have been studied for their effects on various biological processes, including inhibition of bacterial translation and interruption of cell wall biosynthesis.²³ In addition, oligosaccharides are critical mediators of cancer, cellular recognition and microbial pathogenesis.²⁴ Respectively, oligosaccharides may serve as potential treatments for various diseases, ranging from bacterial infections to type II diabetes.²⁵ While oligosaccharide natural products possess a wide spectrum of potential biological activities, they are relatively underrepresented in pharmacopeia.²⁶ Generally, the chemical investigation of oligosaccharides is difficult as compared to other classes of molecules, such as linear peptides and oligonucleotides. The branched structures of oligosaccharides impede amplification methods like polymerase chain reaction (PCR). Therefore, procuring oligosaccharides by direct isolation from natural products is preferred,^{27,28} although this is complicated by their water-soluble nature. Sometimes, to maximize the activity of oligosaccharides, modification such as esterification and glycosylation would be performed.²⁹ Also, these polar, functionalized oligosaccharides can be present at negligible concentrations, and thus are more challenging to detect, isolate and identify than other natural products.²⁶

One of the more well studied examples are human milk oligosaccharides (HMO). These sialylated oligosaccharides and glycans were first discovered as a prebiotic “bifidus factor”,³⁰ which helps shape a healthy intestinal microbiota composition for

infants. HMO promote the proliferation of beneficial bacteria by serving as a metabolic substrate.³¹ Sialylated human milk oligosaccharides enhance the newborn immature immune system and are crucial for the proper development and well-being of the gut.³²

Like other dietary oligosaccharides, cranberry oligosaccharides have shown various bioactivities, such as inhibition of bacterial adhesion,³³ enhanced growth of commensal bacteria,³⁴ and reduced biofilm formation.^{14,35} These soluble oligosaccharides make up to higher than 20% w/w in many cranberry materials.¹⁵ However, the contribution of oligosaccharides to the proposed health benefits of cranberry are largely unresolved.

Since cranberry is well known for its contributions to urinary tract health, understanding the potential contributions of each constituent may aid in optimizing cranberry products. Oligosaccharides may play a role in UTI prevention since these may be found in the urine. Coleman et al. demonstrated that an octasaccharide arabinoxyloglucan, a water soluble type of hemicellulose oligosaccharide isolated from cranberry, could be isolated from the urine of swine that were fed a cranberry product.¹⁹ Moreover, this molecule demonstrated antiadhesion properties against bacteria. This suggests that dietary oligosaccharides may be similarly excreted into human urine.

Pectic oligosaccharides (POS) are the hydrolysis products of pectin. Pectins are high molecular weight (MW) structural polysaccharides found inside the lamella of primary cell walls within almost all higher plant tissues.³⁷ Pectin is a complex of acidic polymers which is comprised of homogalacturonic and branched rhamnogalacturonic regions. These pectins are broken down by natural enzymes during fruit ripening and softening, resulting in lower molecular weight (MW) oligomers with different degrees

of polymerization (DP).³⁸ Pectin is abundant in whole fresh cranberries, and with the addition of heat and sugar during the juice making process, pectins can induce gel formation in the juice.³⁹ Therefore, commercial cranberry juice is treated with pectinase macerations before filtering to increase juice yields.⁴⁰ This suggests the inclusion of significant pectic oligosaccharides in consumer cranberry products such as juice.

POS can be produced from agricultural byproducts, further enhancing the potential for developing biomedicines and/or functional foods. For instance, the low-calorie and odorless POS can be used in anti-obesity diets⁴¹ and the non-digestible property enables POS to selectively affect bacteria flora in the colon.⁴² Notably, pectic oligosaccharides also have immunomodulatory and anti-inflammatory^{43,44} activity. POS possess antioxidant activity⁴⁵ and are reported to provide potential protection against cardiovascular diseases.⁴⁶ Individual cPOS molecules from cranberry are generated from cranberry pectin by various treatment methods, such as enzymatic,⁴⁷ chemical⁴⁸ and physical⁴¹ methods. Among them, enzymatic treatment for POS degradation is considered as the most common due to the minimum chemical modifications as compared to other pretreatment methods.⁴⁹ It has been reported that endo-polygalacturonase can randomly cleave the glycosidic bond of the α -(1–4)-polygalacturonan in pectin.⁴⁹ Also, pectin methyl esterase and pectin acetyl esterase can remove methyl esters and acetyl groups on galacturonic acid residues.⁵⁰

As mentioned previously, the pectinase enzyme mixtures are used to degrade cell wall structures biochemically to increase juice production. Pectinase includes pectin lyase and pectate lyase, both of which are able to cleave the smooth region of pectin, a region of α -(1–4) linked D-galacturonic acid residues, and form α -(1–4)-D-GalpA

residues containing a 4,5-unsaturated galacturonic acid methyl ester at the non-reducing end.⁵¹ De-esterified products treated with of Klerzyme 150 pectinase, a pectinase enzyme mixture, resulted in unsaturated oligosaccharides after heat treatment.⁵² Interestingly, our group previously isolated unsaturated methyl-esterified galacturonic acid from a cranberry fraction that had been treated with same pectinase.²¹

Further research is required to understand the possible roles and uses of cPOS in UTI prevention. While cPOS has been shown to inhibit UPEC from residing in a quiescent state,⁵³ the impact of oligosaccharide degree of polymerization is unknown. Furthermore, studies of cPOS on UPEC persister cells have been hindered by impurities that remain following various chromatographic separations. Also, the monosaccharide composition of cPOS has yet to be proven using chemical derivatization methods. The research presented in this thesis helps overcome these current limitations in our knowledge regarding the chemical nature of cPOS and its biological effects against UPEC.

CHAPTER 3

METHODOLOGY

3.1 Equipment

1D and 2D NMR (HSQC) experiments were collected on an Agilent NMR 500 MHz spectrometer (Varian VNMRs 500MHz, Agilent Technologies) in D₂O (99.99%, Sigma-Aldrich) at 25 °C. LC/MS analysis was performed on a Shimadzu Prominence UFLC system coupled to an AB Sciex QTRAP 4500 mass spectrometer with electrospray ionization source in the negative ion mode. Flash chromatography used a Combiflash Rf200 equipped with a 100 g C18 RediSepRf High Performance Gold column (Teledyne ISCO). Semi-preparative HPLC experiments were performed on a Shimadzu Prominence i-series HPLC, using TSKgel Amide-80, 7.8 mm I.D x 30 cm, 10 µm column. Analytical HPLC experiments were also performed on the Shimadzu Prominence i-series HPLC using a 4.6 × 250 mm, 5 µm TSKgel Amide-80 HR HILIC column. Centrifugation was conducted with an Eppendorf 5804 centrifuge. All solvents used were HPLC grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3.2 Fractionation of cranberry (*V. macrocarpon*) materials

A pectinase (Klerzyme 150, DSM Food Specialties, IN) degraded cranberry hull extract (1.0 g) was dissolved in 20 mL distilled water and fractionated by reversed phase flash chromatography. After purging and pre-conditioning a Combi-Flash chromatography instrument, a C18 column (100 g C18 RediSepRf High Performance

Gold) was loaded with the cranberry hull extract sample and sequentially eluted with 500 mL of distilled H₂O (Fraction Cranf1W, 752 mg, 37.6%), 500 mL of 15% methanol/water (Fraction Cranf1b, 480 mg, 24.0%), and finally 500 mL of MeOH (Fraction Cranf1M, 564 mg, 28.2%) at 35 mL/min. The three fractions were concentrated by rotary evaporation and lyophilization and stored at -20 °C.

3.3 Trituration of active fractions

Trituration of fraction Cranf1b to remove iridoid terpenes was accomplished by sonicating 200 mg Cranf1b in 30 mL 95% ethanol/water and then centrifuging the solids at 17,000 x g for 8 min. The collected solids were then dissolved in deionized (DI) H₂O and lyophilized. The trituration step was repeated twice prior to further separation by PGC chromatography to provide sample cPOS-t (91.7%, w/w dry). ¹H NMR data and analytical HPLC analysis of the resulting products were assessed to determine the iridoid terpenes were successfully removed by trituration. HPLC was accomplished on a Prominence i-series system (Shimadzu) using a TSKgel Amide-80 HR HILIC column (4.6 × 250 mm, 5 µm, TOSOH Bioscience, Tokyo, Japan) with a 1.0 mL/min flow rate and 35 °C while monitoring at UV 235 nm (20 µL injections of a 10 mg/mL sample). The column was first eluted with 80% ACN/water (+0.1% formic acid) for 2 min, followed by a linear gradient to 40% ACN/water (+0.1% formic acid) for 20 min and then held for 5 min.

3.4 cPOS separation by chromatography on porous graphitized carbon

Cranflb was further fractionated using porous graphitized carbon (PGC) chromatography (1 g cartridges, ThermoFisher Scientific). The PGC cartridge was first eluted with 50% ACN/water for cleaning and then equilibrated with distilled water for conditioning. A total of 40 mg of Cranflb was dissolved in 2 mL distilled water, loaded onto the PGC cartridge, and eluted sequentially with 18 mL H₂O, 12 mL 10% ACN/water (+0.1% TFA), and 18 mL 30% ACN/water (+0.1% TFA). All fractions were lyophilized and stored at -20 °C.

3.5 Structure determination of cPOS

All the individually collected fractions from step 3.3 were analyzed by LC/MS and 1D and 2D NMR spectroscopy for structure determination. The LC/MS analyses were performed on an Amide-80 column using the HPLC conditions described above. LC/MS were operated in negative ion mode and while scanning in a range from m/z 200 to m/z 2000. For NMR analysis, samples were deuterium exchanged twice by dissolving in D₂O (99.99%, Sigma-Aldrich) and shaking for 5 min. ¹H and HSQC NMR experiments were conducted on a 500 MHz NMR spectrometer at 25 °C, with compounds dissolved at 10 mg/mL. ¹H NMR spectra were acquired with 16 scans. HSQC spectra were obtained with 4 scans per increment and 128 t1 increments.

3.6 Determination of monosaccharides composition by PMP-derivatization

The composition of the poly-galacturonic acid backbone of cPOS was determined by acid hydrolysis of cPOS followed by derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP). The strategy followed a previously published protocol as

follows.^{54,55} 10 mg cPOS was hydrolyzed in 1 mL of 2 M TFA at 90 °C for 5 h. After cooling to room temperature, the reaction mixture was centrifuged at 3000 rpm for 5 min. The supernatant was evaporated *in vacuo* to remove residual TFA. The hydrolysate mixture was dissolved in 1 mL distilled water and treated with 30 μ L NaOH (0.3 M) and 20 μ L PMP solution (0.5 M in methanol) for derivatization. The mixture was incubated at 70 °C for 60 min, cooled to room temperature and neutralized with 30 μ L of HCl (0.3 M). The mixture was then extracted with 1 mL chloroform. The aqueous layer was filtered by passing through a 0.45 μ m syringe filter. Glucuronic acid (2.0 mM), galacturonic acid (2.0 mM) and a distilled water blank were subjected to the identical reaction conditions. HPLC analyses of PMP-labeled samples were conducted on a Prominence i-series system (Shimadzu) using a BAKERBOND C18 column (4.6 mm $\times$ 250 mm, 5 μ m, J.T. Baker Inc., Phillipsburg, NJ, USA). The injection volume was 20 μ L with a flow rate of 1.0 mL/min at 35 °C. Mobile phase A was 10% ACN/water with 0.045% KH_2PO_4 –0.05% triethylamine buffer (pH 7.5) and mobile phase B was 100% ACN. The column was eluted with 6% B for 4 min, a gradient of 6–12% B for 1 min and then 12% B for 20 min. The UV detection wavelength was 254 nm.

3.7 Purification of oligosaccharides with various degrees of polymerization

Oligosaccharides with different DPs in 10% PGC fraction were sequentially purified by HPLC using a TOSOH Tskgel Amide-80 HR column (7.8 mm I.D $\times$ 30 cm, 10 μ m) (TOSOH Bioscience) with a 1.6 mL/min flow rate and 35 °C while monitoring at UV 235 nm (100 μ L injections of a 20 mg/mL sample). The column was initially eluted with 80% ACN/water (+0.1% formic acid) for 2 min, followed by a linear

gradient to 40% ACN/water (+0.1% formic acid) for 20 min and then held for 5 min. Fractions corresponding to cPOS with different DPs were collected based on UV absorbances and/or retention time and concentrated by lyophilization.

3.8 Persister cell viability assay and quiescence lawn assay

The persister cell viability assay was performed following a previously published protocol.⁶ Briefly, *E. coli* strain CFT073 was streaked from *cryo*-stocks onto LB agar plates and incubated overnight at 37 °C. A loopful of cells from the plate were added to 10 mL 0.4% glucose M9 minimal media in a 125 mL culture flask and incubated overnight at 37 °C and 200 rpm. Cultures were diluted in fresh 0.2% glucose M9 minimal media to an optical density (OD₆₀₀) of 0.1 (~10⁸ CFU/mL) and grown in the presence of ampicillin sodium (0.1 mg/mL) to generate persister cell fractions. The cultures were incubated shaking (200 rpm) at 37 °C and viable counts were measured at 0 h, 4 h, and 24 h by plating on LB media. Test cultures were treated with cPOS fractions at 1-2 mg/mL. Control experiments included cultures without antibiotic (cPOS only) and cultures without antibiotic or cPOS.

The quiescence lawn assay also followed a published protocol.⁶ Briefly, overnight cultures of CFT073 were prepared in 0.4% glucose M9 minimal media as described above for the persister cell viability assay. Bacteria from this culture were diluted to a final concentration of 10⁵ CFU in 4 mL of liquid overlay media (0.2% glucose M9 minimal media with 0.9% noble agar at 45 °C). Each 4 mL overlay inoculum was poured over a prewarmed (37 °C) 0.2% glucose M9 minimal media agar plate immediately after inoculation. These plates were allowed to solidify at room temperature with lids slightly

ajar. Test solutions were added to the overlay media and allowed to dry before incubating the plate upside down at 37 °C for 24 h or 48 h. No growth of *E. coli* in the overlay is considered to be quiescent if it is able to be reversed with the positive control (3 co-spots of 5 µL of each tyrosine, lysine, and methionine, each at 0.1 mg/mL in H₂O).

CHAPTER 4

RESULTS

4.1 Separation of iridoid terpene glycosides from cPOS

In previous work, three iridoid terpene glucosides (Figure 1) were identified as 6,7-dihydromonotropein (**a**), monotropein (**b**) and deacetylasperulsidic acid (**c**) in fractions containing cPOS following chromatographic separations. Iridoids were separated and analyzed by ESI-TOF-MS in the negative ion mode (Figure 2). The major peak showed two ions in negative mode at m/z 391.1204 and 389.1050, suggesting two co-eluted molecules which were subsequently further separated by C18 column chromatography as **a** and **b**, respectively. The second peak showed one negative ion at m/z 389.1050 (**c**).

Although these iridoid glucosides were minor impurities and inactive in the *in vitro* quiescence inhibition assay (data not shown), the removal of iridoids was necessary to prove the activity of cPOS on UPEC in our persister cell assay.

The separation approach described here was based on the differential solubility of the iridoids and cPOS in ethanol and water. We found that these impurities could be removed from the cPOS fraction by exploiting their differential solubility in ethanol. Cranflb was triturated with 95% aqueous ethanol prior to PGC separation, producing a cPOS fraction devoid of iridoid glycosides (cPOS-t, 91.7%, w/w dry) as determined by HPLC analysis (Figure 3) and ^1H NMR analysis (Figure 4). 10 mg/mL solutions of cPOS fraction and iridoid-deficient cPOS fraction (cPOS-t) were analyzed by HILIC HPLC using UV-monitoring (235 nm). Figure 2 shows a comparison of pre- and post-triturated samples and demonstrates the successful removal of iridoid glucosides.

Moreover, the absence of typical ^1H NMR signals corresponding to the double bonds on the cyclopentanopyran scaffold of iridoid glucosides in cPOS-t further supports the successful removal of iridoid terpenes from cPOS by the trituration method (Figure 4). HSQC NMR analysis of the cPOS-t cranberry fraction revealed proton and carbon resonances with chemical shifts characteristic for poly-uronic acids. Additional structural features included a methoxy group at δ_{H} 3.70/ δ_{C} 52.8 and a vinylic methine at δ_{H} 6.00/ δ_{C} 111.8 (Figure 5). UV absorption maxima at 235 nm was consistent with the presence of an α, β -unsaturated carboxylic acid.

The cPOS-t fraction was tested in a persister cell viability assay and found to maintain activity similar to cPOS (Figure 6,7). Treatment of *E. coli* CFT073 with both cPOS and ampicillin resulted in >1000-fold fewer persister cells versus treatment with ampicillin alone.²⁰ A similar reduction of persister cells was obtained when conducting the assay with cPOS-t (Figure 6). The viable cell counts in the *E. coli* CFT073 cultures decreased slightly during the first 4 h of incubation in both the presence and absence of ampicillin. By 24 h, *E. coli* CFT073 had grown to just over 10^9 CFU/mL in the absence of ampicillin. In contrast, the presence of ampicillin reduced the viable counts an additional ~1000-fold over the next 20 h, to about 10^6 CFU/mL. As shown by the results in Figure 5, in the presence of 1 mg/mL cPOS and absence of ampicillin, *E. coli* CFT073 viable counts increased rapidly, reaching stationary phase within 4 h. Importantly, in the presence of both the cPOS and ampicillin, *E. coli* CFT073 viable counts decreased rapidly within 4 h, and reached a level of about 3×10^2 CFU/mL after 24 h.

Figure 1. Structures of iridoid glucosides: 6,7-dihydromonotropein (**a**), and deacetylasperulsidic acid (**b**) and monotropein (**c**).

Figure 2. LC-MS analysis in the negative ion mode of iridoid glucosides showing extracted ions of m/z 391 and m/z 389.

Figure 3. HILIC HPLC chromatogram of cPOS (Top) and cPOS-t (Bottom) at 235 nm. 6,7-dihydromonotropein (**a**), deacetylasperulsidic acid (**b**) and monotropein (**c**). Only trace amount of iridoid glucosides remained in cPOS-t. Adapted from Sun et al.²¹

Figure 4. ^1H NMR spectrum (D_2O , 500 MHz) of standards (purified 4-mer pectic oligosaccharide and iridoids), and cPOS-t (triturated 10% PGC). The ^1H NMR analysis indicated the triturated 10% PGC was similar to 4-mer pectic oligosaccharide and signals corresponding to the iridoids were absent.

Figure 5. HSQC spectrum of cPOS-t (D_2O , 500 MHz). The 1H and HSQC NMR spectra of the active cranberry fraction revealed proton and carbon resonances with chemical shifts characteristic for poly-uronic acids.

Figure 6. Persister cell viability treating with cPOS-t (CFT073+ampicillin+ cPOS-t) is significantly different at 4 h (**- $P \leq 0.01$) and 24 h (**- $P \leq 0.01$), in comparison to CFT073+ampicillin. CFT073 cell viability with (CFT073+cPOS-t) or without cPOS-t treatment (CFT073) were evaluated as controls. cPOS-t: 2 mg/mL; ampicillin: 0.1 mg/mL. Adapted from Sun et al.²¹

Figure 7. Persister cell viability treating with cPOS (CFT073+ampicillin+ cPOS) is significantly different at 4 h (**- $P \leq 0.01$) and 24 h (***- $P \leq 0.01$), in comparison to CFT073+ampicillin. CFT073 cell viability with (CFT073+cPOS) or without cPOS treatment (CFT073) were evaluated as controls. cPOS: 2 mg/mL; ampicillin: 0.1 mg/mL. Adapted from Sun et al.²¹

4.2 Verification of the cPOS monosaccharide composition by chemical derivatization

Previously, our lab used NMR spectroscopy to determine the structure of cPOS is comprised of repeating galacturonic acid units.²² However, the NMR approach is limited in its ability to differentiate between galacturonic and glucuronic acid monosaccharide units, since the two anomeric proton peaks generally used to differentiate between different monosaccharide residues are very similar in the case of these two sugars. Therefore, a chemical derivatization approach was applied to complement the previous NMR studies. Specifically, cPOS was hydrolyzed, modified by a 1-phenyl-3-methyl-5-pyrazolone (PMP) reaction, and analyzed by reversed-phase HPLC with UV detection in comparison to the appropriate standards. By comparing

HPLC traces, the monosaccharide composition of cPOS was determined to be comprised of galacturonic acid units. Figures 8B and 8C show the retention times for PMP reaction products with glucuronic acid and galacturonic acid, respectively. Comparison of retention time between cPOS hydrolyzed products (Figure 8D, $t_R = 6.0$ min) and standard derivatives (PMP, $t_R = 3.3$ min; glucuronic acid, $t_R = 4.6$ min; galacturonic acid, $t_R = 6.0$ min) confirms that the cranberry POS oligosaccharides were comprised of galacturonic acid units.

Figure 8. Uronic acid identification of cPOS. cPOS, glucuronic acid and galacturonic acid were derivitized with PMP and analyzed by HPLC as described in the Methods. The stacked chromatograms show the results for reactions conducted with PMP without an added carbohydrate (**A**); Glucuronic acid (**B**); Galacturonic acid (**C**); and cPOS hydrolysate (**D**). The peak with a retention time at 6.0 min in **D** indicates that cPOS is comprised of galacturonic acid. Adapted from Sun et al.²¹

4.3 Purification of cPOS for determining the effect of DP on UPEC phenotypes

With the active cPOS monosaccharide composition verified, we next sought to enable structure-activity relationships (SAR) of cPOS components with respect to the observed reversal of CFT073 quiescence. It is well recognized that DP is one of the crucial factors with regard to the bioactivities of oligosaccharides.^{15,27} To assess the effect of cPOS DP on reversal of CFT073 quiescence, a separation method was developed for isolation of cPOS components with different DPs. Good separation was ultimately achieved using hydrophilic interaction chromatography (HILIC) with hydrophilic (amide) stationary phases and reversed-phase type eluents. In a HILIC system, the surface forms a water-rich layer on the polar stationary phase, leaving a water-deficient mobile phase, forming a liquid/liquid extraction system. The analytes interact between these two layers. Compounds with higher polarity will elute later since they have higher interaction with the stationary aqueous layer. As for oligosaccharides, the oligomers with higher DPs have relatively higher polarity, which elute later while oligomers with lower DPs elute earlier.

Taking previous analytical HILIC HPLC separation results into consideration,⁵⁶ a similar separation method with slight modifications was used to isolate cPOS. A solvent

system comprised of acetonitrile and water with 0.1% TFA was the best mobile phase for cPOS separation. The presence of TFA in eluting solvents increased the acidity which resulted in a better separation for acidic compounds. In our case, since the molecules of interest consisted of galacturonic acid oligomers, ensuring the molecules were fully protonated was expected to give better separation, as well as increase the consistency of each separation batch (Figure 9). A 1.5 mL/min flowrate with a semi-preparative HILIC column provided chromatographic separation similar to using the analytical column at 1.0 mL/min (Figure 9).

Figure 9. Analytical HILIC HPLC chromatography of cPOS by using TOSOH Tskgel Amide-80 HR column (7.8 mm I.D x 30 cm, 10 μ m), 1.0 mL/min.

The collection of cPOS components from the semipreparative separation was based on the chromatographic peaks. Considering the non-polar impurities observed previously, fractions were collected starting from fraction 1 (t_R = 15 min) to fraction 10 (t_R = 27 min) for bioassay analysis (Figure 10). All the combined eluents prior to 14.7 min were combined as fraction 0. By injecting 40 mg of cPOS-t, a total of 11 fractions were obtained with sufficient material for testing in the quiescence bioassay.

Figure 10. Preparative HILIC HPLC chromatography of cPOS by using TOSOH Tskgel Amide-80 HR column (7.8 mm I.D x 30 cm, 10 μ m), 1.5mL/min. For each 40 mg of cPOS-t, the certain amount of fractions 0–10 were obtained (fraction 0: 4.7 mg, fraction 1: 1.4 mg, fraction 2: 1.6 mg, fraction 3: 1.0 mg, fraction 4: 1.6 mg, fraction 5: 1.7 mg, fraction 6: 2.9 mg, fraction 7: 1.9 mg, fraction 8: 3.0 mg, fraction 9: 3.5 mg and fraction 10: 3.8 mg).

Figure 11. ^1H NMR spectrum (D_2O , 300 MHz) of 11 fractions separated by preparative HILIC HPLC.

The semipreparative HILIC HPLC not only gave a good separation of impurities and cPOS, but also separation of cPOS with different DPs. As shown in Figure 10, the analysis of ^1H NMR spectra of all 11 fractions indicated that fractions 0–2 were impurities with only trace amounts of oligosaccharides while fractions 3–10 showed resonances consistent with cPOS. This includes the typical proton signals of a vinylic methine around δ_{H} 6.10 and methoxy group around δ_{H} 3.70. The successful separation method using HILIC HPLC set the stage for preliminary structure–activity relationship (SAR) analysis. According to the activity result (Figure 12), the activity variation of each fraction suggested that the DPs of cPOS plays a role in reversing UPEC quiescence. Only fraction 4 showed activity (5 μL spot; 10 mg/mL) while no obvious positive effects of other obtained fractions were observed, indicating that only cPOS with a specific and relatively low DP might be responsible for the observed activity. Further characterization of the active fraction and comparison to the inactive ones will provide further clues for the structural requirements for biological activity in this assay.

Figure 12. CFT073 quiescence assay using glucose-free M9 plates. A colony of actively growing CFT073 toothpicked into the agar plate as positive control; Sample A: cPOS-t, (10 mg/mL); Sample B: Cranflb, (10 mg/mL); Fractions 1 to 10 were obtained from HPLC separation of cPOS-t, (10 mg/mL).

CHAPTER 5

CONCLUSION

Polysaccharides exhibit a variety of biological functions and characterizing their monosaccharide composition is a critical first step to understanding their structure and function. Previously, our lab used ^1H and HSQC NMR spectroscopy to deduce the structure of cPOS, revealing chemical shifts for the active cranberry fraction consistent with polyuronic acids. In this study, chemical derivatization was used to confirm that cPOS is comprised of galacturonic acid residues. The smooth region of pectin is called galacturonans which are linear chains of α -(1–4)-linked D-galacturonic acid. Hence, cPOS is hypothesized to be the hydrolysis product of pectin.

Acid hydrolysis of cPOS followed by PMP derivatization required optimization. Temperature and pH value are two crucial parameters for both the hydrolysis and derivatization reactions. Galacturonic acid oligosaccharides are relatively more stable than other saccharides.⁵⁰ However, higher reaction temperatures will lead to degradation of oligosaccharides. We used a moderate temperature (90 °C versus 120 °C) and longer reaction time (5 hours compare to 2 hours)⁵⁷ to successfully hydrolyze our product. During the derivatization step, carbanions are required to form a Schiff base and subsequent reactions to generate derivatives, which means the reaction should be conducted under basic conditions. Since uronic acids are more acidic than other sugars due to their carboxylic acid, the addition of NaOH was necessary to adjust the pH and facilitate the reaction.

Three minor impurities, all iridoid glucosides, were previously identified by our lab in the active 10% cPOS fraction obtained using porous graphitized carbon chromatography. Specifically, these were identified as 6,7-dihydromonotropein, deacetylasperulsidic acid and monotropein and found to be inactive in the quiescence assay. Previous studies have shown that the iridoid glucosides monotropein and 6,7-dihydromonotropein also lack antiadhesive effects against *Escherichia coli*.^{58,59} We sought to remove these impurities to provide a more highly purified cranberry pectic oligosaccharide sample for biological study. Based on the differential solubility in ethanol of iridoid glucosides and cPOS, iterative trituration with 95% aqueous ethanol proved successful for removing the iridoid impurities before PGC fractionation. HPLC and ¹H NMR analyses verified the removal of iridoid glucosides, and the persister cell bioassay again confirmed that these were not responsible for the bioactivity of cPOS.

Degree of polymerization is another crucial factor to be determined when characterizing the activity of cPOS against UPEC. Due to the limitation of column size, our group previously only separated small quantities and determined the structures of two pectic oligosaccharides, namely uG3^{m2} and UG4^{m3}.²¹ In this thesis work, a semi-preparative HPLC method using HILIC chromatography was developed for the purification of cPOS components with variable DPs. ¹H NMR results further confirmed that cPOS with variable DPs could be separated from the complex mixture of oligosaccharides. This method will be useful for future efforts to further investigate the biological properties of cPOS oligosaccharides.

APPENDIX

Anti-inflammatory effects of cPOS

1. Introduction

Cranberry is the fruit of the evergreen shrub *Vaccinium macrocarpon* and has been extensively studied for benefits to human health, including urinary tract health¹⁵ and anti-inflammation effects.^{60,61} Inflammation is an important process for protecting against infections and healing wounds. However, inflammation is also implicated in numerous chronic diseases, including cancer.⁶² Dietary sources of anti-inflammatory compounds could therefore provide beneficial health outcomes. In previous investigations of the anti-inflammatory properties of cranberry constituents, investigators have focused on proanthocyanidins,⁶³ especially A-type proanthocyanidins,⁶⁴ neglecting the potential contributions of cranberry oligosaccharide constituents.

Yet, polysaccharides from botanical sources have previously shown various effects on inflammation pathways. Some polysaccharides have been shown to increase the expression of cytokines and chemokines, including tumor necrosis factor (TNF- α), Interleukin-1 β (IL-1 β)⁶⁵ and IL-6.⁶⁶ On the contrary, some citrus pectins can exert anti-inflammatory action. Also, bupleurum polysaccharides were found to up-regulate phagocytic activities⁶⁷ and inhibit secretion of inflammation related cytokines.⁶⁸ This multi-function can be referred as biological response modifiers (BRMs). Previously, our lab found xyloglucan and pectic oligosaccharides in cranberry extract subfraction Cranf1b.²² Xyloglucan, a hemicellulose, has been reported to attenuate inflammation⁶⁹

and activate macrophages.⁶⁵ However, how the effect of xyloglucans and pectic oligosaccharides on the secretion of cytokines is unknown.

In this study, we tested the effects of cranberry extracts enriched in oligosaccharides for modulation of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6. An attempt to identify the active components was pursued using bioactivity guided isolation.

2. Anti-inflammatory evaluation of oligosaccharide-rich cranberry extracts by measuring TNF- α and IL-1 β secretion by THP-1 cells

To evaluate the anti-inflammatory properties of cranberry extract, a model was applied initially to examine effects against TNF- α and IL-1 β . THP-1 cells were seeded at a density of 5×10^4 cells per well in 48 well plate. PMA (25 ng/ml) was added to induce differentiation for 48 hours. Next, the PMA was removed and cells were incubated with PMA free medium for another 24 hours. LPS was added for 4 hours, then samples were added and incubated for another 1 hour. Nigericin was then added for another 1 hour. The supernatant was collected and IL-1 β and TNF- α were measured by ELISA. Nigericin is an inducer for inflammasome activation and boosts IL-1 β secretion. Inflammasome activation needs two inducers: LPS and nigericin. MCC is a positive control small molecule that specifically inhibits inflammasome activation. Fraction Cranf1b its PGC subfractions (Scheme 1) were tested and showed positive results for the anti-inflammatory activity by IL-1 β and TNF- α assays (Figure 13).

LPS stimulated the release of TNF- α (114.87 ± 5.98 pg/mL) and IL-1 β (303.30 ± 40.38 pg/mL) whereas the blank control did not produce significant levels (12.53 ± 0.98

pg/mL and 135.37 ± 26.39 pg/mL, respectively). LPS and nigericin treatment also resulted in elevated TNF- α (53.59 ± 9.68 pg/mL) and IL-1 β (583.35 ± 72.52 pg/mL) concentrations. Fraction S2 (70% water/30% ACN portion) at a concentration of 0.01 μ g/mL was the most effective at reducing the secretion of TNF- α by 67% (37.97 ± 3.92 pg/mL), while none of the samples demonstrated a reduction in IL-1 β secretion.

Scheme 1. Sample preparation for TNF- α and IL-1 β assay. Sample preparation method was detailed in Sections 3.2 and Section 3.4 of this thesis. Fraction S1: Cranf1b portion and fraction S2: 70% water/30% ACN portion, highlighted in red, were determined to be positive in TNF- α and IL-1 β assay.

Figure 13. Results of IL-1 β and TNF- α assay for fractions obtained from sample preparation of cranberry juice extract powder. Fraction S1: Cranflb portion; fraction S2: 70% water/30% ACN portion; fraction S3: 90% water/10% ACN portion; fraction S4: 100% water portion. Data shown as mean $\pm$ SE.

3. Anti-inflammatory evaluation of cranberry extracts for modulating TNF- α and IL-6 secretion

The previous experiments suggested that fraction S2 showed the most promise for impacting pro-inflammatory cytokine production. This fraction had been generated using porous graphitized carbon chromatography PGC 30% ACN fraction (Scheme 2). To further separate this active fraction, an HPLC method was devised to for the separation of oligosaccharides. The conditions were as follows: UV detector was set at 254 nm (Figure 14, pink trace), flow rate 1.5 mL/min. The TSKgel Amide-80 column (10 μ m, 7.8 mm I.D x 30 cm) was initially eluted with 60% ACN/water (+0.1% formic acid) for 2 min, followed by a linear gradient to 40% ACN/water (+0.1% formic acid) for 28 min and then held for 5 min.

Similar to the previously described method, the anti-inflammation effects of HPLC separation fractions from active fraction (70% water/30% ACN portion) was tested using assays that measure IL-6 and TNF- α . THP-1 cells were seeded into 48 well plates at a density of 50,000 cells/well in medium with PMA (25 nM) for 48 hours. Following this period, cells were visually checked for adherence and proper morphology before exchanging differentiation medium to serum free medium for 24 hours prior to sample treatment. Test samples were weighed and dissolved in FBS free medium before being diluted in cell culture medium at concentrations of 100 and 10 $\mu\text{g/mL}$. CE at these concentrations were added to each respective set of wells for 1 hour before being exposed to LPS at 1 $\mu\text{g/mL}$ for 24 hours. Following LPS exposure, medium was collected, centrifuged and assayed for IL-6 and TNF- α using respective ELISA kits.

LPS was able to stimulate the release of TNF- α (289.56 ± 25.97 pg/mL) and IL-6 (753.32 ± 12.33 pg/mL) into culture media whereas the blank control did not produce significant levels (30.51 ± 2.16 pg/mL and 278.51 ± 36.42 pg/mL respectively). Fraction S4 (first subfraction obtained from 70% water/30% ACN portion) at a concentration of 100 $\mu\text{g/mL}$ was the most effective at reducing the secretion of TNF- α by 94% (17.13 ± 3.88 pg/mL) and at 100 $\mu\text{g/mL}$, IL-6 secretion was significantly attenuated by approximately 73% (206.19 ± 7.44 pg/mL).

Scheme 2. Further separation of active fraction (70% water/30% ACN portion). All subfractions were tested for TNF- α and IL-6 assay. The active subfraction (S4) was highlighted in red.

Figure 14. HPLC separation of the active S3 fraction (70% water/30% ACN portion). A total of four subfractions (S4–S7) were obtained by HPLC separation. Fractions were collected based on retention time (fraction S4: 2.0 min–7.0 min; fraction S5: 7.0 min–12.0 min; fraction S6: 12.0 min–17.0 min; fraction S7: 17.0 min–22.0 min).

Figure 15. Results of IL-6 and TNF- α assays for fractions obtained from sample preparation of cranberry juice extract powder. Fraction S1: Cranf1b portion; fraction S2: Cranf1M; fraction S3: 70%water/30% ACN portion; S4–S7: four subfractions obtained from HPLC separation of S3; S8: iridoid-rich fraction. Data shown as mean $\pm$ SE.

4. Anti-inflammatory evaluation of subfractions of cranberry extract in THP-1 monocytes

To further evaluate the anti-inflammatory properties of Cranberry Extract, LPS sensitive, phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 monocytes were used. THP-1 monocytes were differentiated to macrophages according to previously described methodologies.⁷⁰ In brief, THP-1 cells were seeded into 24 well plates at a

density of approximately 200,000 cells/mL and allowed to incubate for at least 24 hours prior to PMA exposure. PMA was then added directly to each well at final concentration of 25 nM for 48 hours. Following this period, cells were visually checked for adherence and proper morphology before exchanging existing differentiation media to serum free media for at least 24 hours prior to compound treatment.

Cranberry Extract (CE) was initially weighed and dissolved in PBS before being diluted in cell culture medium at concentrations of 100 and 0.01 $\mu\text{g/mL}$. CE at these concentrations were added to each respective set of wells for 1 hour before being exposed to LPS at 1 $\mu\text{g/mL}$ for 6 hours. Following LPS exposure, media was collected, centrifuged and assayed for IL-6 and TNF- α using respective ELISA kits (Biolegend, San Diego CA USA). We observed that LPS is able to stimulate the release of TNF- α (1027 ± 27.4 pg/mL) and IL-6 (229.6 ± 12.69 pg/mL) into culture media whereas the vehicle control did not produce significant levels (50.75 ± 0.05 pg/mL and 11.67 ± 0.18 pg/mL respectively). Resveratrol (RESV) was used at 20 μM and was found to decrease concentrations of both TNF- α (944.8 ± 25.7 pg/mL) and IL-6 (44.3 ± 4.63 pg/mL). CE at a concentrations of 0.01 $\mu\text{g/mL}$ was the most effective at reducing the secretion of TNF- α by 32% (694.7 ± 88.16 pg/mL) and at 0.1 $\mu\text{g/mL}$, IL-6 secretion was significantly attenuated by approximately 72% (63.50 ± 3.5 pg/mL).

Figure 16. Cranberry extract significantly attenuates the release of pro-inflammatory TNF- α and IL-6 cytokines. Differentiated THP-1 Cells were pre-treated with vehicle (DMSO), RESV (20 μ M), or CE (100 to 0.01 μ g/mL) for 1 hour before being exposed to LPS (1 μ g/mL) for 6 hours. Media was then collected and saved for cytokine analysis with respective ELISAs. Data shown as mean $\pm$ SE. All comparisons made to LPS $p < 0.05$ * , $p < 0.01$ ** , $p < 0.001$ ***, $p < 0.0001$ ****.

To model the systemic exposure of dietary oligosaccharides from cranberry, THP-1 monocytes, an immortalized human cell line was exploited for its ability to react to various endogenous and exogenous stimuli. Moreover, these cells have been optimally differentiated to macrophages effectively increasing their sensitivity to these stimuli. Lipopolysaccharide is typically used to elicit an immune response by engaging TLR4 pathway further causing a release of pro-inflammatory/pro-oxidant signaling molecules. Specifically, TNF- α and IL-6 are major drivers in this pathway and co-regulate each other.

The cytokines measured only represent a small portion of other possible signaling molecules that interact with other monocytes, adipocytes and dendritic cells and other surrounding tissues. More work is needed to construct a signaling network of cytokines and chemokines. Gene expression data of these inflammation-based molecules has already been explored by another group⁷¹ using a similar treatment methodology; however, the composition of their extract is markedly different to this oligosaccharide and terpenoid enriched fraction. Moreover, these macrophages respond to molecular patterns from pathogen and damaged cells therefore, it is not unlikely that these oligosaccharides may prevent the interaction of LPS or truly act upon a transcription factor or protein target(s) within the cell. Further experiments are also warranted to ascertain the extent of permeability and confirm which major constituents are responsible for the possible anti-inflammatory effects of this cranberry extract.

BIBLIOGRAPHY

- (1) Jamil, I.; Zafar, A.; Qamar, M. U.; Ejaz, H.; Akhtar, J.; Waheed, A. Multi-Drug Resistant *Klebsiella Pneumoniae* Causing Urinary Tract Infections in Children in Pakistan. *Afr. J. Microbiol. Res.* **2014**, *8*, 316–319. <https://doi.org/10.5897/AJMR2013.6409>.
- (2) Foxman, B. Urinary Tract Infection Syndromes: Occurrence, Recurrence, Bacteriology, Risk Factors, and Disease Burden. *Infect. Dis. Clin. North Am.* **2014**, *28* (1), 1–13. <https://doi.org/10.1016/j.idc.2013.09.003>.
- (3) Kumar, M. S.; Das, A. P. Emerging Nanotechnology Based Strategies for Diagnosis and Therapeutics of Urinary Tract Infections: A Review. *Adv. Colloid Interface Sci.* **2017**, *249*, 53–65. <https://doi.org/10.1016/j.cis.2017.06.010>.
- (4) Foxman, B. Epidemiology of Urinary Tract Infections: Incidence, Morbidity, and Economic Costs. *Dis.--Mon. DM* **2003**, *49* (2), 53–70. <https://doi.org/10.1067/mda.2003.7>.
- (5) Flores-Mireles, A. L.; Walker, J. N.; Caparon, M.; Hultgren, S. J. Urinary Tract Infections: Epidemiology, Mechanisms of Infection and Treatment Options. *Nat. Rev. Microbiol.* **2015**, *13* (5), 269–284. <https://doi.org/10.1038/nrmicro3432>.
- (6) Leatham-Jensen, M. P.; Mokszycki, M. E.; Rowley, D. C.; Deering, R.; Camberg, J. L.; Sokurenko, E. V.; Tchesnokova, V. L.; Frimodt-Møller, J.; Krogfelt, K. A.; Leth Nielsen, K.; et al. Uropathogenic *Escherichia Coli* Metabolite-Dependent Quiescence and Persistence May Explain Antibiotic Tolerance during Urinary Tract Infection. *mSphere* **2016**, *1* (1). <https://doi.org/10.1128/mSphere.00055-15>.

- (7) Rosen, D. A.; Hooton, T. M.; Stamm, W. E.; Humphrey, P. A.; Hultgren, S. J. Detection of Intracellular Bacterial Communities in Human Urinary Tract Infection. *PLoS Med.* **2007**, *4* (12), e329. <https://doi.org/10.1371/journal.pmed.0040329>.
- (8) Hunstad, D. A.; Justice, S. S. Intracellular Lifestyles and Immune Evasion Strategies of Uropathogenic *Escherichia Coli*. *Annu. Rev. Microbiol.* **2010**, *64*, 203–221. <https://doi.org/10.1146/annurev.micro.112408.134258>.
- (9) Hannan, T. J.; Hunstad, D. A. A Murine Model for *Escherichia Coli* Urinary Tract Infection. *Methods Mol. Biol. Clifton NJ* **2016**, *1333*, 159–175. https://doi.org/10.1007/978-1-4939-2854-5_14.
- (10) Berry, R. E.; Klumpp, D. J.; Schaeffer, A. J. Urothelial Cultures Support Intracellular Bacterial Community Formation by Uropathogenic *Escherichia Coli*. *Infect. Immun.* **2009**, *77* (7), 2762–2772. <https://doi.org/10.1128/IAI.00323-09>.
- (11) Terada, N.; Ohno, N.; Saitoh, S.; Saitoh, Y.; Fujii, Y.; Kondo, T.; Katoh, R.; Chan, C.; Abraham, S. N.; Ohno, S. Involvement of Dynamin-2 in Formation of Discoid Vesicles in Urinary Bladder Umbrella Cells. *Cell Tissue Res.* **2009**, *337* (1), 91–102. <https://doi.org/10.1007/s00441-009-0804-z>.
- (12) De Nisco, N. J.; Neugent, M.; Mull, J.; Chen, L.; Kuprasertkul, A.; de Souza Santos, M.; Palmer, K. L.; Zimmern, P.; Orth, K. Direct Detection of Tissue-Resident Bacteria and Chronic Inflammation in the Bladder Wall of Postmenopausal Women with Recurrent Urinary Tract Infection. *J. Mol. Biol.* **2019**, *431* (21), 4368–4379. <https://doi.org/10.1016/j.jmb.2019.04.008>.
- (13) Pichon, C.; Héchar, C.; Merle, L. D.; Chaudray, C.; Bonne, I.; Guadagnini, S.; Vandewalle, A.; Bouguénec, C. L. Uropathogenic *Escherichia coli* AL511 Requires

Flagellum to Enter Renal Collecting Duct Cells. *Cell. Microbiol.* **2009**, *11* (4), 616–628.
<https://doi.org/10.1111/j.1462-5822.2008.01278.x>.

(14) Samarasinghe, S.; Reid, R.; AL-Bayati, M. The Anti-Virulence Effect of Cranberry Active Compound Proanthocyanins (PACs) on Expression of Genes in the Third-Generation Cephalosporin-Resistant *Escherichia coli* CTX-M-15 Associated with Urinary Tract Infection. *Antimicrob. Resist. Infect. Control* **2019**, *8*.
<https://doi.org/10.1186/s13756-019-0637-9>.

(15) Coleman, C. M.; Ferreira, D. Oligosaccharides and Complex Carbohydrates: A New Paradigm for Cranberry Bioactivity. *Molecules* **2020**, *25* (4).
<https://doi.org/10.3390/molecules25040881>.

(16) Mantzorou, M.; Giaginis, C. Cranberry Consumption Against Urinary Tract Infections: Clinical Stateof- the-Art and Future Perspectives. *Curr. Pharm. Biotechnol.* **2018**, *19* (13), 1049–1063. <https://doi.org/10.c2174/1389201020666181206104129>.

(17) Gupta, K.; Chou, M. Y.; Howell, A.; Wobbe, C.; Grady, R.; Stapleton, A. E. Cranberry Products Inhibit Adherence of P-Fimbriated *Escherichia coli* to Primary Cultured Bladder and Vaginal Epithelial Cells. *J. Urol.* **2007**, *177* (6), 2357–2360.
<https://doi.org/10.1016/j.juro.2007.01.114>.

(18) Jensen, H. D.; Struve, C.; Christensen, S. B.; Krogfelt, K. A. Cranberry Juice and Combinations of Its Organic Acids Are Effective against Experimental Urinary Tract Infection. *Front. Microbiol.* **2017**, *8*, 542. <https://doi.org/10.3389/fmicb.2017.00542>.

(19) Coleman, C. M.; Auker, K. M.; Killday, K. B.; Azadi, P.; Black, I.; Ferreira, D. Arabinoxylglucan Oligosaccharides May Contribute to the Antiadhesive Properties of

- Porcine Urine after Cranberry Consumption. *J. Nat. Prod.* **2019**, 82 (3), 589–605. <https://doi.org/10.1021/acs.jnatprod.8b01043>.
- (20) Sun, J.; Marais, J. P. J.; Khoo, C.; LaPlante, K.; Vejborg, R. M.; Givskov, M.; Tolker-Nielsen, T.; Seeram, N. P.; Rowley, D. C. Cranberry (*Vaccinium Macrocarpon*) Oligosaccharides Decrease Biofilm Formation by Uropathogenic *Escherichia coli*. *J. Funct. Foods* **2015**, 17, 235–242. <https://doi.org/10.1016/j.jff.2015.05.016>.
- (21) Sun, J.; Deering, R. W.; Peng, Z.; Najia, L.; Khoo, C.; Cohen, P. S.; Seeram, N. P.; Rowley, D. C. Pectic Oligosaccharides from Cranberry Prevent Quiescence and Persistence in the Uropathogenic *Escherichia coli* CFT073. *Sci. Rep.* **2019**, 9 (1), 1–9. <https://doi.org/10.1038/s41598-019-56005-w>.
- (22) Sun, J. Investigations on Biologically Active Carbohydrates from Natural Sources. *Diss. Masters Theses Campus Access* **2016**, 1–190.
- (23) Huber, G.; Neemann, G. Moenomycin, an Inhibitor of Cell Wall Synthesis. *Biochem. Biophys. Res. Commun.* **1968**, 30 (1), 7–13. [https://doi.org/10.1016/0006-291X\(68\)90704-3](https://doi.org/10.1016/0006-291X(68)90704-3).
- (24) Jones, D. J.; Mowrey, D. H.; Anderson, D. B.; Wellenreiter, R. H. Effect of Various Levels of Avilamycin on the Performance of Growing-Finishing Swine. *J. Anim. Sci.* **1987**, 65 (4), 881–885. <https://doi.org/10.2527/jas1987.654881x>.
- (25) Mahmud, T. The C7N Aminocyclitol Family of Natural Products. *Nat. Prod. Rep.* **2003**, 20 (1), 137–166. <https://doi.org/10.1039/b205561a>.
- (26) McCranie, E. K.; Bachmann, B. O. Bioactive Oligosaccharide Natural Products. *Nat. Prod. Rep.* **2014**, 31 (8), 1026–1042. <https://doi.org/10.1039/C3NP70128J>.

- (27) *Essentials of Glycobiology*, 3rd ed.; Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY), 2015.
- (28) Seeberger, P. H.; Werz, D. B. Synthesis and Medical Applications of Oligosaccharides. *Nature* **2007**, *446* (7139), 1046–1051. <https://doi.org/10.1038/nature05819>.
- (29) Alebeek, G.-J. W. M. van; Zabotina, O.; Beldman, G.; Schols, H. A.; Voragen, A. G. J. Esterification and Glycosylation of Oligogalacturonides: Examination of the Reaction Products Using MALDI-TOF MS and HPAEC. *Carbohydr. Polym.* 2000, *1*
- (30) Gauhe, A.; György, P.; Hoover, J. R. E.; Kuhn, R.; Rose, C. S.; Ruelius, H. W.; Zilliken, F. Bifidus Factor. IV. Preparations Obtained from Human Milk. *Arch. Biochem. Biophys.* **1954**, *48* (1), 214–224. [https://doi.org/10.1016/0003-9861\(54\)90326-4](https://doi.org/10.1016/0003-9861(54)90326-4).
- (31) Bode, L. Human Milk Oligosaccharides: Every Baby Needs a Sugar Mama. *Glycobiology* **2012**, *22* (9), 1147–1162. <https://doi.org/10.1093/glycob/cws074>.
- (32) Lis-Kuberka, J.; Orczyk-Pawłowicz, M. Sialylated Oligosaccharides and Glycoconjugates of Human Milk. The Impact on Infant and Newborn Protection, Development and Well-Being. *Nutrients* **2019**, *11* (2). <https://doi.org/10.3390/nu11020306>.
- (33) Cm, C.; Km, A.; Kb, K.; P, A.; I, B.; D, F. Arabinoxylglucan Oligosaccharides May Contribute to the Antiadhesive Properties of Porcine Urine After Cranberry Consumption <http://pubmed.ncbi.nlm.nih.gov/30873836/> (accessed May 20, 2020). <https://doi.org/10.1021/acs.jnatprod.8b01043>.

- (34) E, Ö.; J, S.; Dc, R.; Da, S. A Human Gut Commensal Ferments Cranberry Carbohydrates To Produce Formate <http://pubmed.ncbi.nlm.nih.gov/28667113/> (accessed May 31, 2020). <https://doi.org/10.1128/AEM.01097-17>.
- (35) Sun, X.; Wu, J. Food Derived Anti-Adhesive Components against Bacterial Adhesion: Current Progresses and Future Perspectives. *Trends Food Sci. Technol.* **2017**, *69*, 148–156. <https://doi.org/10.1016/j.tifs.2017.09.002>.
- (36) Sobota A. E. Inhibition of bacterial adherence by cranberry juice: potential use for the treatment of urinary tract infections. *J. Urol.* **1984**, *131*(5), 1013–1016. [https://doi-org.uri.idm.oclc.org/10.1016/s0022-5347\(17\)50751-x](https://doi-org.uri.idm.oclc.org/10.1016/s0022-5347(17)50751-x)
- (37) *Food Polysaccharides and Their Applications*, 2nd ed.; Stephen, A. M., Phillips, G. O., Williams, P. A., Eds.; Food science and technology; CRC/Taylor & Francis: Boca Raton, FL, 2006.
- (38) Diaz-Garcia, L.; Rodriguez-Bonilla, L.; Phillips, M.; Lopez-Hernandez, A.; Grygleski, E.; Atucha, A.; Zalapa, J. Comprehensive Analysis of the Internal Structure and Firmness in American Cranberry (*Vaccinium Macrocarpon* Ait.) Fruit. *PLOS ONE* **2019**, *14* (9), e0222451. <https://doi.org/10.1371/journal.pone.0222451>.
- (39) Anderson, E. E.; Hampton, W. F.; Anti, A. W. Process for Forming a Jellied Cranberry Sauce. US3142577A, July 28, 1964.
- (40) Mantius, H.; Rose, L. Process for Producing Sugars and Acids-Rich Juice and Phytochemical-Rich Juice. US20060177560A1, August 10, 2006.
- (41) Øbro, J.; Harholt, J.; Scheller, H. V.; Orfila, C. Rhamnogalacturonan I in *Solanum Tuberosum* Tubers Contains Complex Arabinogalactan Structures.

Phytochemistry **2004**, 65 (10), 1429–1438.

<https://doi.org/10.1016/j.phytochem.2004.05.002>.

(42) Chen, J.; Liang, R.; Liu, W.; Li, T.; Liu, C.; Wu, S.; Wang, Z. Pectic-Oligosaccharides Prepared by Dynamic High-Pressure Microfluidization and Their in Vitro Fermentation Properties. *Carbohydr. Polym.* **2013**, *91* (1), 175–182. <https://doi.org/10.1016/j.carbpol.2012.08.021>.

(43) Zhang, S.; Hu, H.; He, W.; Muhammad, Z.; Wang, L.; Liu, F.; Pan, S. Regulatory Roles of Pectin Oligosaccharides on Immunoglobulin Production in Healthy Mice Mediated by Gut Microbiota. *Mol. Nutr. Food Res.* **2019**, *63* (14), 1801363. <https://doi.org/10.1002/mnfr.201801363>.

(44) Popov, S. V.; Ovodov, Yu. S. Polypotency of the Immunomodulatory Effect of Pectins. *Biochem. Mosc.* **2013**, *78* (7), 823–835. <https://doi.org/10.1134/S0006297913070134>.

(45) Garthoff, J. A.; Heemskerk, S.; Hempenius, R. A.; Lina, B. A. R.; Krul, C. A. M.; Koeman, Jan. H.; Speijers, G. J. A. Safety Evaluation of Pectin-Derived Acidic Oligosaccharides (PAOS): Genotoxicity and Sub-Chronic Studies. *Regul. Toxicol. Pharmacol.* **2010**, *57* (1), 31–42. <https://doi.org/10.1016/j.yrtph.2009.12.004>.

(46) Mao, X.; Xiao, X.; Chen, D.; Yu, B.; He, J.; Chen, H.; Xiao, X.; Luo, J.; Luo, Y.; Tian, G.; et al. Dietary Apple Pectic Oligosaccharide Improves Gut Barrier Function of Rotavirus-Challenged Weaned Pigs by Increasing Antioxidant Capacity of Enterocytes. *Oncotarget* **2017**, *8* (54), 92420–92430. <https://doi.org/10.18632/oncotarget.21367>.

- (47) Li, T.; Li, S.; Du, L.; Wang, N.; Guo, M.; Zhang, J.; Yan, F.; Zhang, H. Effects of Haw Pectic Oligosaccharide on Lipid Metabolism and Oxidative Stress in Experimental Hyperlipidemia Mice Induced by High-Fat Diet. *Food Chem.* **2010**, *121* (4), 1010–1013. <https://doi.org/10.1016/j.foodchem.2010.01.039>.
- (48) Sabater, C.; Ferreira-Lazarte, A.; Montilla, A.; Corzo, N. Enzymatic Production and Characterization of Pectic Oligosaccharides Derived from Citrus and Apple Pectins: A GC-MS Study Using Random Forests and Association Rule Learning. *J. Agric. Food Chem.* **2019**, *67* (26), 7435–7447. <https://doi.org/10.1021/acs.jafc.9b00930>.
- (49) Martínez, M.; Gullón, B.; Schols, H. A.; Alonso, J. L.; Parajó, J. C. Assessment of the Production of Oligomeric Compounds from Sugar Beet Pulp. *Ind. Eng. Chem. Res.* **2009**, *48* (10), 4681–4687. <https://doi.org/10.1021/ie8017753>.
- (50) Kim, S.-K.; Rajapakse, N. Enzymatic Production and Biological Activities of Chitosan Oligosaccharides (COS): A Review. *Carbohydr. Polym.* **2005**, *62* (4), 357–368. <https://doi.org/10.1016/j.carbpol.2005.08.012>.
- (51) Cameron, R.; Luzio, G.; Savary, B.; Nuñez, A.; Goodner, K. Digestion Patterns of Two Commercial Endopolygalacturonases on Polygalacturonate Oligomers with a Degree of Polymerization of 7 to 21. *Proc Fla State Hortic Soc* **2009**, *122*. <https://doi.org/10.1016/j.foodchem.2010.01.039>
- (52) Alebeek, G.-J. W. M. van; Christensen, T. M. I. E.; Schols, H. A.; Mikkelsen, J. D.; Voragen, A. G. J. Mode of Action of Pectin Lyase A of *Aspergillus niger* on Differently C6-Substituted Oligogalacturonides. *J. Biol. Chem.* **2002**, *277* (29), 25929–25936. <https://doi.org/10.1074/jbc.M202250200>.
- (43), 39–46.

- (53) Deering, R. W. Using Natural Products to Treat Resistant and Persistent Bacterial Infections. *Diss. Masters Theses Campus Access* **2017**, 1–230.
- (54) Honda, S.; Akao, E.; Suzuki, S.; Okuda, M.; Kakehi, K.; Nakamura, J. High-Performance Liquid Chromatography of Reducing Carbohydrates as Strongly Ultraviolet-Absorbing and Electrochemically Sensitive 1-Phenyl-3-Methyl-5-Pyrazolone Derivatives. *Anal. Biochem.* **1989**, *180* (2), 351–357. [https://doi.org/10.1016/0003-2697\(89\)90444-2](https://doi.org/10.1016/0003-2697(89)90444-2).
- (55) Wang, W.; Chen, F.; Wang, Y.; Wang, L.; Fu, H.; Zheng, F.; Beecher, L. Optimization of Reactions between Reducing Sugars and 1-Phenyl-3-Methyl-5-Pyrazolone (PMP) by Response Surface Methodology. *Food Chem.* **2018**, *254*, 158–164. <https://doi.org/10.1016/j.foodchem.2018.02.001>.
- (56) Z, H.; B, X.; N, W. Impact of Column Temperature and Mobile Phase Components on Selectivity of Hydrophilic Interaction Chromatography (HILIC) http://pubmed.ncbi.nlm.nih.gov/18435508/?from_term=+HILIC+column&from_pos=3 (accessed May 31, 2020). <https://doi.org/10.1002/jssc.200700624>.
- (57) Ai, Y.; Yu, Z.; Chen, Y.; Zhu, X.; Ai, Z.; Liu, S.; Ni, D. Rapid Determination of the Monosaccharide Composition and Contents in Tea Polysaccharides from Yingshuang Green Tea by Pre-Column Derivatization HPLC. *J. Chem.* **2016**, *2016*, 1–5. <https://doi.org/10.1155/2016/6065813>.
- (58) Pappas, E.; Schaich, K. M. Phytochemicals of Cranberries and Cranberry Products: Characterization, Potential Health Effects, and Processing Stability. *Crit. Rev. Food Sci. Nutr.* **2009**, *49* (9), 741–781. <https://doi.org/10.1080/10408390802145377>.

- (59) Jensen, H. D.; Krogfelt, K. A.; Cornett, C.; Hansen, S. H.; Christensen, S. B. Hydrophilic Carboxylic Acids and Iridoid Glycosides in the Juice of American and European Cranberries (*Vaccinium Macrocarpon* and *V. Oxycoccos*), Lingonberries (*V. Vitis-Idaea*), and Blueberries (*V. Myrtillus*). *J. Agric. Food Chem.* **2002**, *50* (23), 6871–6874. <https://doi.org/10.1021/jf0205110>.
- (60) Bodet, C.; Chandad, F.; Grenier, D. Cranberry Components Inhibit Interleukin-6, Interleukin-8, and Prostaglandin E2 Production by Lipopolysaccharide-Activated Gingival Fibroblasts. *Eur. J. Oral Sci.* **2007**, *115* (1), 64–70. <https://doi.org/10.1111/j.1600-0722.2007.00415.x>.
- (61) La, V. D.; Howell, A. B.; Grenier, D. Cranberry Proanthocyanidins Inhibit MMP Production and Activity. *J. Dent. Res.* **2009**, *88* (7), 627–632. <https://doi.org/10.1177/0022034509339487>.
- (62) Hoesel, B.; Schmid, J. A. The Complexity of NF-KB Signaling in Inflammation and Cancer. *Mol. Cancer* **2013**, *12* (1), 86. <https://doi.org/10.1186/1476-4598-12-86>.
- (63) Bodet, C.; Chandad, F.; Grenier, D. Anti-Inflammatory Activity of a High-Molecular-Weight Cranberry Fraction on Macrophages Stimulated by Lipopolysaccharides from Periodontopathogens. *J. Dent. Res.* **2006**, *85* (3), 235–239. <https://doi.org/10.1177/154405910608500306>.
- (64) La, V. D.; Howell, A. B.; Grenier, D. Anti-*Porphyromonas Gingivalis* and Anti-Inflammatory Activities of A-Type Cranberry Proanthocyanidins. *Antimicrob. Agents Chemother.* **2010**, *54* (5), 1778–1784. <https://doi.org/10.1128/AAC.01432-09>.
- (65) Martín-Sánchez, F.; Diamond, C.; Zeitler, M.; Gomez, A. I.; Baroja-Mazo, A.; Bagnall, J.; Spiller, D.; White, M.; Daniels, M. J. D.; Mortellaro, A.; et al.

Inflammasome-Dependent IL-1 β Release Depends upon Membrane Permeabilisation. *Cell Death Differ.* **2016**, 23 (7), 1219–1231. <https://doi.org/10.1038/cdd.2015.176>.

(66) do Rosário, M. M. T.; Kangussu-Marcolino, M. M.; do Amaral, A. E.; Noleto, G. R.; Petkowicz, C. L. de O. Storage Xyloglucans: Potent Macrophages Activators. *Chem. Biol. Interact.* **2011**, 189 (1), 127–133. <https://doi.org/10.1016/j.cbi.2010.09.024>.

(67) Cheng, X.-Q.; Li, H.; Yue, X.-L.; Xie, J.-Y.; Zhang, Y.-Y.; Di, H.-Y.; Chen, D.-F. Macrophage Immunomodulatory Activity of the Polysaccharides from the Roots of *Bupleurum Smithii* Var. *Parvifolium*. *J. Ethnopharmacol.* **2010**, 130 (2), 363–368. <https://doi.org/10.1016/j.jep.2010.05.019>.

(68) Zhang, Z.-D.; Li, H.; Wan, F.; Su, X.-Y.; Lu, Y.; Chen, D.-F.; Zhang, Y.-Y. Polysaccharides Extracted from the Roots of *Bupleurum Chinense* DC Modulates Macrophage Functions. *Chin. J. Nat. Med.* **2017**, 15 (12), 889–898. [https://doi.org/10.1016/S1875-5364\(18\)30004-9](https://doi.org/10.1016/S1875-5364(18)30004-9).

(69) Periasamy, S.; Lin, C.-H.; Nagarajan, B.; Sankaranarayanan, N. V.; Desai, U. R.; Liu, M.-Y. Mucoadhesive Role of Tamarind Xyloglucan on Inflammation Attenuates Ulcerative Colitis. *J. Funct. Foods* **2018**, 47, 1–10. <https://doi.org/10.1016/j.jff.2018.05.035>.

(70) Lund, M. E.; To, J.; O'Brien, B. A.; Donnelly, S. The Choice of Phorbol 12-Myristate 13-Acetate Differentiation Protocol Influences the Response of THP-1 Macrophages to a pro-Inflammatory Stimulus. *J. Immunol. Methods* **2016**, 430, 64–70. <https://doi.org/10.1016/j.jim.2016.01.012>.

- (71) Hannon, D. B.; Thompson, J. T.; Khoo, C.; Juturu, V.; Vanden Heuvel, J. P. Effects of Cranberry Extracts on Gene Expression in THP-1 Cells. *Food Sci. Nutr.* **2017**, *5* (1), 148–159. <https://doi.org/10.1002/fsn3.374>.