

In silico and *In vitro* Discovery of Plant-Based Pro-Inflammatory Cytokine Inhibitors

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Purpose: Dry eye disease (DED) is a prevalent condition range between 44.2% to 62.9% of the population, with female sex and aging being the greatest risk factors. The pathogenesis of DED is based on the dysregulation of tear film homeostasis, resulting in a vicious circle of desiccating stress, inflammation, and tissue damage. Mild DED is managed with artificial tears and warm compresses, whereas moderate-to-severe DED require additional anti-inflammatory therapies to break the vicious circle. Anti-inflammatory drugs such as corticosteroids are effective treatments but are associated with adverse side effects including increased risk of cataracts and glaucoma. Discovering novel anti-inflammatory therapeutics with minimal adverse side effects would greatly benefit DED patients who depend on anti-inflammatory treatment to mitigate symptoms. Flavonoids are a class of chemicals found in all vegetation; some are known for their anti-inflammatory effects. The numerous members of the flavonoid class could be leveraged to discover novel inhibitors against inflammatory DED targets. The purpose of this thesis was to discover a novel flavonoid that could act as an anti-inflammatory agent to treat DED.

Methods: This thesis employed a hybrid approach using network analysis, computational modelling, and in vitro validation. Network analysis was employed to select DED-associated protein targets and candidate flavonoids. “Dry Eye Syndrome” proteins were extracted from the STRING-DISEASE database and were cross-referenced with drug databanks using the functional enrichment analysis tool, ToppFun, for flavonoids. A network analysis tool, Cytoscape (v 3.9.1.), was utilized to visualize and rank DED-associated proteins and flavonoids by number of edge interactions. Two proteins were selected for their distinct roles in the immune response, and two flavonoids were selected for their association with multiple pro-inflammatory

DED-associated proteins. Molecular docking and molecular dynamic (MD) simulations modelled and simulated the protein-ligand complex of both protein targets with each flavonoid molecule. Four protein-ligand complexes were assessed for stability and specific residue-ligand interactions. The inhibitory effects of both flavonoids were assessed in vitro with ELISAs against both protein targets. The biocompatibility of the most effective flavonoid inhibitor was assessed with human corneal epithelial cells (HCEC) using an Alamar Blue metabolic activity assay; viability was assessed by evaluating the proportion of live, apoptotic, and dead cells.

Results: The network analysis generated a protein-protein interaction network of 64 DED-associated proteins and a drug-protein network of 108 flavonoids. Tumour necrosis factor (TNF) α (21 interactions) and interleukin (IL) 17A (14 interactions) were amongst the 5 highly ranked proteins and selected for their roles in the innate and adaptive immune response, respectively. Luteolin (19 interactions) and rutin (17 interactions) were the highest ranked flavonoids associated with DED-associated proteins. Molecular docking and MD simulations predicted that both flavonoids could bind directly to the receptor binding sites of both cytokines. However, ELISAs showed that neither luteolin nor rutin could inhibit TNF- α and TNF receptor interaction (all $p \geq 0.07$). On the other hand, both luteolin ($IC_{50} = 16.54$ mM) and rutin ($IC_{50} = 8.73$ mM) had inhibitory effects against IL-17A and IL-17A receptor interaction (all $p < 0.01$). Metabolic activity and viability of HCEC remained high (all $\geq 85.95\%$) below 3.0 mM rutin; no significant difference in metabolic activity and viability were detected in all concentrations of rutin compared to the control (all $p > 0.06$).

Conclusion: This thesis identified rutin as a novel flavonoid inhibitor of IL-17A. These findings suggest that rutin may play a role in anti-inflammatory therapeutics for DED.

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List of Abbreviations

ADDE	Aqueous Deficient Dry Eye
AMD	Age-related Macular Degeneration
APC	Antigen Presenting Cells
CAD	Canadian Dollars
CCL	Chemokine Ligand
CD4 or Th	T Helper Cell
CD8 or CTL	Cytotoxic T Cell
CID	Compound Identification
CTD	Comparative Toxicogenomics Database
CXCL	C-X-C Motif Chemokine Ligand
DAMP	Damage-Associated Molecular Patterns
DED	Dry Eye Disease
DMEM/F12	Dulbecco's Minimal Essential Medium/Nutrient Mixture F12
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDE	Evaporative Dry Eye
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
HCEC	Human Corneal Epithelial Cell
HPV	Human Papillomavirus
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MD	Molecular Dynamics
MGD	Meibomian Gland Dysfunction
MHC	Major Histocompatibility Complex
MM-PBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
MMP	Matrix Metalloproteinase
NF-κB	Nuclear factor κ -light-chain-enhancer of activated B cells
NSSDE	Non-Sjögren Syndrome Dry Eye
OCT	Optical Coherence Tomography
OPC	Optimal Point Charge
OSDI	Ocular Surface Disease Index
PAMP	Pathogen-Associated Molecular Patterns
PDB	Protein Data Bank
PPI	Protein-Protein Interaction

PRR	Pattern Recognition Receptors
RCSB	Research Collaboratory for Structural Bioinformatics
RMSD	Root-Mean-Squared Deviation
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SSDE	Sjögren Syndrome Dry Eye
TACE	TNF- α Converting Enzyme
TBUT	Tear Break-up Time
TFOS DEWS	Tear Film and Ocular Surface Society Dry Eye Workshop
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
USD	US Dollars
VCAM	Vascular Cell Adhesion Molecule

Chapter 1 Literature Review

1.1 Literature Review

1.1.1 Ocular Surface Anatomy and Physiology

The ocular surface consists of the corneal and conjunctival epithelium, the lacrimal glands, the meibomian glands, and the tear film. The epithelium of the ocular surface primarily consists of epithelial cells and goblet cells. The epithelial cells are tightly connected by tight and adherens junctions serving as a physical barrier against the external environment and resisting shear stress.^{1,2} Aquaporins are water channels distributed across the epithelium which regulates water movement across cornea/conjunctival epithelial layers.³ Goblet cells are found in the epithelium and produces glycoproteins called mucins which contribute to various functional aspects of the tear film.^{4,5} From the naked eye, the corneal and conjunctival epithelium appears to be smooth, however, microscopic projections called microvilli cover the ocular surface.¹

The main lacrimal gland is located in the lacrimal fossa on the temporal side of the eye, and the accessory gland is located in the upper eyelid on the temporal side.⁶ The lacrimal glands secrete tears composed of 98.2% water, electrolytes, and other organic molecules (i.e. glucose, urea, and amino acids).^{7,8} Three cell types make up the gland: acinar, ductal epithelial and myoepithelial cells.⁷ Acinar cells produce and store water, electrolytes, and antibacterial proteins.⁷ As the fluid enters the duct, the ductal epithelium modifies the fluid by absorbing or secreting water and electrolytes.⁷ Myoepithelial cells along the ductal epithelium directs tear fluid secreted from the glands to the ocular surface.⁷ The lacrimal gland is innervated primarily

by the parasympathetic nervous system, where corneal stimulus eventually lead to tear secretion.⁷ The lacrimal gland supplies the majority of the mucoaqueous phase of the tear film.⁸

Modified sebaceous glands called meibomian glands contribute the superficial lipid layer of the tear film. Approximately 20-40 glands in the upper lid and 20-30 glands in lower lid are found within the tarsal plate of the eye lid; the orifice of the glands line the eyelid margin.⁹ The gland's shape is described as a "chain of onions", where a central duct is lined by sac-shaped acini.⁹ The acinus produce meibum lipids through holocrine secretion.¹⁰ Basal epithelial cells (immature meibocytes) making up the outer lining of the acinus differentiate and migrate centripetally towards the center of the acinus, accumulating meibum lipids over its lifespan.^{9,11,12} Upon holocrine lysis, meibum is pushed out of the acinus into the central duct, where striated fibers of Riolan's muscles control the release of meibum through contraction and relaxation.¹³ The meibum plays a role to prevent evaporation and acts as a barrier against debris.¹⁴

1.1.2 The Tear Film

The tear film maintains ocular surface health,¹⁵ and functions to provide a smooth optical surface for refraction, lubrication between the ocular surface and eyelids, transportation of nutrients and metabolic by-products to and from the cornea, and to act as a barrier against pathogens and debris.^{15,16} The tear film is made up of two different layers: the lipid layer and mucoaqueous layer.^{16,17}

The outermost layer of the tear film is the lipid layer,¹⁴ composing of meibum secreted by the meibomian glands, and its thickness ranging from 15 nm - 157 nm .^{9,14,18} The composition of meibum consists of mainly (95%) non-polar lipids (i.e. wax esters, cholesteryl esters and triacylglycerides), and 5% polar lipids (i.e. (O-acyl)-omega-hydroxy fatty acids and <0.1%

phospholipids).^{15,19} The polar lipids allow non-polar lipids to interface with the hydrophilic mucoaqueous layer of the tear film.²⁰ The lipid layer functions to slow the rate of evaporation and stabilizes the shear-thinning of the tear film as it is spread across the ocular surface.^{14,18}

Below the lipid layer is the mucoaqueous layer, a gel composed of aqueous tear fluid and mucin glycoproteins.^{14,15,21} Approximately 3-4 μm thick,¹⁵ this layer acts as a reservoir for proteins that maintain ocular surface health and protection. The aqueous component is supplied by the lacrimal glands, whereas the mucins make up a large portion of the mucoaqueous layer.^{4,14} Water-soluble mucins, produced by goblet cells (i.e. MUC2, MUC5AC, MUC5B, MUC6 and MUC19), form a gel-like fluid with the aqueous layer and coats the ocular surface for lubrication.^{4,5,15} Transmembrane mucins (i.e. MUC1, MUC4, MUC16) form the glycocalyx layer between the epithelium and tear film to ensure adhesion of the mucoaqueous layer to the corneal and conjunctival surface.^{1,14,15}

The proteome of the tear film is diverse, encompassing over 1500 different proteins.²² Davidson and Kuonen¹⁶ highlighted a set of antimicrobial proteins in the tear film. Enzymes such as lysozyme, lactoferrin, and immunoglobulins such as IgA are some of the defense mechanisms employed against external pathogens.^{8,16,23,24} The pathogens undergo bacteriolysis through lysosomal degradation of the cell wall and membrane.²⁴ The primary immunoglobulin, IgA, is produced by plasma cells and transported to the tear film by mucosal epithelial cells in the lacrimal glands and conjunctiva.²⁵ The secretion of immunoglobulins is regulated by hormones, immune factors, and neural responses.¹⁶ IgA prevents the attachment of microorganisms to the epithelial layer by binding to the organism itself.^{16,24} Other than IgA, IgG and IgM are also found in lower concentrations.¹⁶ Notably, many of the proteins identified can be used as biomarkers to differentiate dry eye disease (DED) subtypes.¹⁵ Willcox et al.,¹⁵

summarized some notable biomarkers for DED such as interleukin (IL) 6, IL-1, epidermal growth factor, and matrix metalloproteinase (MMP) 9.

1.1.3 Pathways and Roles of the Immune Response

The role of the immune response is to protect the body from infections and damage due to bacteria, toxins, and viruses.²⁶ The ocular surface, being warm and moist, is an environment that is ideal for microbial growth; therefore, robust immune surveillance within ocular surface tissues is required.²⁷ While the immune response is a crucial part of eliminating foreign pathogens, the regulation of the response is also important to prevent unnecessary damage from occurring.²⁸ Failure to appropriately regulate and resolve immune activity results in persistent inflammatory damage to tissues, an underpinning mechanism that drives DED.⁴ Overall, the immune response could be further subcategorized into two aspects, the innate and adaptive immune responses.^{29,30}

The innate response is quick, non-specific, and is the first line of defence activated within minutes to hours to control and eliminate pathogens or damaged cells.²⁶ The response is initiated by the detection of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).²⁶ PAMPs are unique, highly conserved structures found on bacterial and fungi organisms such as lipopolysaccharides, RNA, DNA, or flagellin.²⁶ Similarly, DAMPs are molecules released by damaged or dead cells such as metabolites, double stranded DNA, heat shock proteins and monosodium urate.²⁶ These patterns are detected by pattern recognition receptors (PRR) found on epithelial and effector cells (e.g. neutrophils, and antigen presenting cells (APC) (i.e. macrophages and dendritic cells)).^{26,31,32} Alongside PRRs, neutrophils also have fragment, crystallizable (Fc) receptors that recognize the Fc portion of

antibodies.²⁹ The two sub-types of PRRs are transmembrane toll-like receptors (TLR) and cytosolic receptors (i.e. NOD-like receptors, cytosolic DNA sensors, and RIG-like receptors).^{26,31} Triggered TLRs activate transcription factor NF- κ B and stimulate the release of proinflammatory cytokines (i.e. tumour necrosis factor α (TNF- α) and IL-1 β), and chemokines (i.e. chemokine ligand (CCL) 2 and C-X-C motif chemokine ligand (CXCL) 8).²⁶ The mechanism of cytosolic receptors is similar to TLRs.²⁶ Upon binding, the receptors are activated, which eventually leads to the activation of caspase-1, which then cleaves preformed pro-IL-1 β , pro-IL-18, and gasdermin D, releasing inflammatory mediators from the cell (i.e. IL-1 β , IL-18, TNF- α , IL-6, IL-8).^{26,31} IL-1 and TNF- α increases the expression of cell adhesion molecules (i.e. intercellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1) and chemokines (i.e. IL-8 and CCL2) to promote migration of leukocytes (B cells and T cells) to the affected tissues.²⁶ Neutrophils and macrophages phagocytose microbes and use noxious agents (i.e. reactive oxygen species, nitric oxide, and proteolytic enzymes) to destroy or disable the pathogen.²⁶

The adaptive immune response supports the innate response when the innate response is unable to eliminate the foreign pathogen.³⁰ Compared to the innate response, the adaptive response is more specific and long-lasting but will require a few days to activate.^{26,30} The two subcategories of the adaptive immune response are the humoral and cell-mediated responses.²⁶ The humoral response primarily involve B lymphocyte cells to eliminate extracellular microbes.²⁶ Naive B cells are either activated in a T cell-independent or dependent fashion.³³ The B cell contains B cell receptors and TLRs,³³ where stimulation of both receptors by an antigen will lead to the maturation of B cells into short-lived plasma cells.³³ Alternatively, T cell-dependent activation involves presenting the antigen on the major histocompatibility complex

(MHC) II to CD4 T helper (Th) cells for approval.²⁶ This mode of activation initiates the maturation and differentiation of B cells into long-lived plasma cells and memory B cells.^{26,33} Plasma cells produce antibodies (i.e. IgM, IgG, IgA, and IgE) that bind to a corresponding antigen on the microbe, marking it for phagocytes (i.e. neutrophils and macrophages) and also activating the complement system for elimination.²⁶ Memory cells remain in the tissues post-infection to allow a more rapid response upon repeated exposure in the future.²⁶ The cell-mediated immune response attacks cells that have been infected with intracellular microbes and mainly involves cytotoxic T cells (CD8 or CTL).²⁶ The response involves APCs (i.e. dendritic cells and macrophages)²⁶ presenting fragments of a microbe on its MHC II in lymphoid organs.²⁶ The antigens are presented to naive T lymphocytes (T cell), stimulating clonal expansion and differentiation.²⁶ Three types of T cells will be activated: T helper (Th) cells, CD8s and T regulatory (Treg) cells.²⁶ Three main subtypes of Th cells will emerge depending on the cytokines present: Th1, Th2, or Th17.^{26,34} Other T helper cell types may also emerge including Th3, Th9, and Th22.³⁴ Th1 and Th2 mediate the cell-mediated and humoral response. Th1 cells mainly express Interferon (IFN) γ , activating other APCs aimed to eliminate the microbe (i.e. macrophages, dendritic cells and CD8s).^{26,35} Th2 cells will express cytokines (i.e. IL-4, IL-5, IL-13) to activate a specialized white blood cell, eosinophils, and upregulate the production of IgE antibodies for larger pathogens.²⁶ Th17 mediates the neutrophil and epithelial response by the expression of IL-17, which upregulates neutrophil activation and inflammation, and IL-22, which is signals simulates inflammation via upregulation of chemokines.³⁴ The pathogenesis of some inflammatory diseases involve the dysregulation of Th17 cells, such as DED (described in section 1.2.3).^{34,36} Finally, Tregs inhibit the production of pro-inflammatory mediators (i.e. IFN- γ and IL-17), express apoptotic enzymes and downregulate proinflammatory Th and CD8 cells.³⁷

Some T cells are turned into memory T cells which will remain in tissue for long-term, allowing for quick activation of the immune response upon repeated infection.²⁶

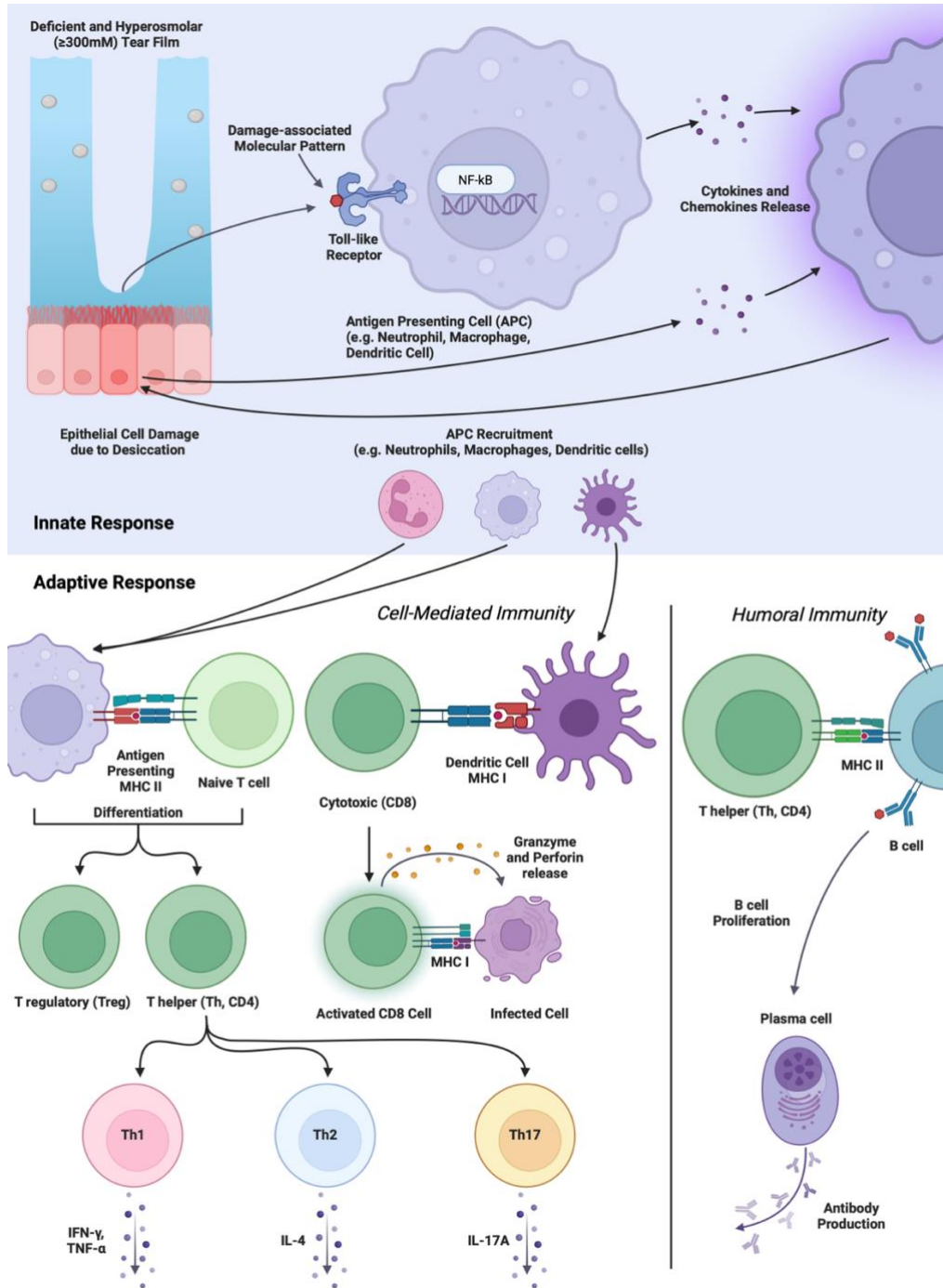


Figure 1. Immune Response in DED: Innate and Adaptive Response. Created with BioRender.com.

1.2 Dry Eye Disease: Etiology, Pathogenesis, and Treatment

DED, as defined by the Tear Film and Ocular Surface Society Dry Eye Workshop III (TFOS DEWS III), is “...*a multifactorial, symptomatic disease characterized by a loss of homeostasis of the tear film and/or ocular surface, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities are etiological factors*”.³⁸

1.2.1 Epidemiology and Socioeconomic Impact

The TFOS DEWS III: Digest estimates that between 44.2% - 62.9% of a population suffers from DED, depending on the definition of disease. The prevalence of the disease is more common in women (30%) compared to men (20%) between 40-80 years.³⁹ Multiple population studies were considered and Stapleton et al. (2025)³⁹ identified consistence evidence of several risk factors increasing the risk of DED including autoimmune disorders (e.g. Sjögren’s disease), ophthalmic disorders (e.g. meibomian gland dysfunction (MGD)), and demographic factors (e.g. increasing age and female sex) to name a few. The risk of developing DED in populations aged over 40 years old is 25% in women and 17.3% in men with odds increasing 1.2x every 10 years.⁴⁰ Thus, increasing age is a major risk factor for developing DED, with additional risk for the female population.^{40,41} An aging population has been an increasing concern for many nations throughout the 21st century.⁴² For example, Canada has a rapidly aging population where the projected proportion of seniors over 65 years is expected increase from 18.5% (2021) to between 21.6% and 22.9% in 2030 and between 21.6% and 29.8% in 2068.⁴³ As aging is a major factor in DED pathogenesis,⁴ the prevalence of the disease is expected increase over the next few decades.

The symptoms of DED can negatively impact daily life through socioeconomic means and impeding the routine tasks of daily living. In the United States, the average direct medical cost due to DED is \$783 USD per patient annually, with more severe conditions costing up to \$1267 USD.⁴⁴ This total cost includes medical visits, supplements, and treatments for DED.⁴⁴ DED-related loss of productivity is reported to cost on average of \$11,302 USD per patient.⁴⁴ Chan et al.⁴⁵ conducted an economic impact study based in Canada, and found that overall annual DED-related economic loss was on average \$24,331 CAD per patient due to loss of productivity (\$22,007 CAD), and direct medical costs (\$2,324 CAD). The study also surveyed for quality of life factors and found that DED negatively impacted mental health, the ability to perform daily tasks (i.e. work responsibilities, home tasks, etc.), and driving.⁴⁵

1.2.2 Etiology

DED may stem from multiple etiologies resulting in the loss of tear film homeostasis.⁴ DED can be further classified into two etiological subtypes: aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE).⁴⁶ ADDE is characterized by the lack of tear production on to the ocular surface due to dysfunction of the lacrimal gland. ADDE can be further subdivided into Sjögren syndrome dry eye (SSDE) and non-Sjögren syndrome dry eye (NSSDE).⁴ As the name suggests, SSDE is a result of Sjögren syndrome, an autoimmune disease where immune cells target the lacrimal and salivary glands.⁴⁷ Eventually, these glands are destroyed leading to DED and dry mouth.⁴⁷ On the other hand, NSSDE is due to factors not related to Sjögren syndrome. Aging, defined as “... *the accumulated changes in structure and function that occur in a tissue or organism over its lifespan*”,⁴ is the most common driver of NSSDE. Over time, corneal sensitivity is decreased, reducing the sensory drive to the lacrimal gland to induce tear secretion.⁴⁸ Other age-related factors include decreased function of the lacrimal gland itself,

decreased size of the glands, loss of innervation, and infiltration by lymphocytes (e.g. Th cells and CD8),⁴⁹ which further damages and reduces output of tears.⁵⁰ The accumulation of T cells may be due to the dysregulation of regulatory mechanisms (e.g. increased expression of pro-inflammatory mediators IFN- γ , and IL-2),⁵¹ and accumulation of memory T cells.^{52,53} Other than aging, other systemic conditions involved in chronic inflammation and/or obstruction or damage of the lacrimal gland may lead to the development of NSSDE.⁴

EDE is characterized by an excessively rapid evaporating tear film despite a normal and functional lacrimal gland.⁴ MGD is the leading etiology of EDE,⁵⁴ as a result of reduced or altered delivery of meibum to the ocular surface.⁹ A main driver of MGD is hyperkeratinisation and obstruction of the meibomian gland orifices, causing meibum produced within the gland to stagnate and become thick and paste-like in consistency, further promoting obstruction.⁵⁵ This blockage leads to increased intraglandular pressure and can induce hyperplasia and acinar atrophy.⁵⁶ Furthermore, as age increases, the proliferation rate meibocytes also decrease, reducing meibum production and secretion.⁵⁷

Other than age and female sex, other common risk factors of general DED include but are not limited to allergies, certain medications and treatments (e.g. antihistamines, corneal refractive surgery, estrogen therapy, etc.), other systemic diseases (e.g. hepatitis C, diabetes mellitus, etc.), and the environment (i.e. low humidity).⁵⁸

1.2.3 Pathogenesis

The core pathogenesis of DED revolves around an insufficient or unstable tear film and desiccation of the ocular surface.¹ An unstable and rapidly evaporating tear film leads to tear hyperosmolarity (>300 mOsm/L),¹⁵ inducing tissue damage and inflammation.⁴ Under chronic

hyperosmolar stress, the immune response is perpetually activated, leading to further tissue damage.⁵⁹ Indeed, excessive Th17 activity are known to be involved in DED.^{60,61}

Upon initiation, the innate immune response is triggered, and a cascade of pro-inflammatory signals are released including TNF- α , IL-1, and IL-6.^{62,63} Subsequently, the adaptive immune response activates as exposure to the stimulus (i.e. desiccation) continues. Naive T cells undergo differentiation and maturation into three effector T cell types: Th cells, Treg cells, and CD8 cells.⁶⁴ The proliferating T cells amplify the immune response and secrete additional pro-inflammatory factors (i.e. MMPs, pro-inflammatory cytokines, and cell adhesion molecules).^{36,60} In healthy, non-DED patients, the activity of Treg and Th cells are balanced to appropriately regulate the immune response. However, excessive inflammatory activity is linked loss of T cell regulation, including Treg resistance in Th17 cells.⁶¹ Dysregulation of the immune response leads to chronic uncontrolled inflammation where immune-mediated damage occurs to the corneal and conjunctival epithelium.⁴ This damage disrupts ocular surface function, leading to loss of homeostasis and further desiccation occurs.⁴

1.2.4 Diagnosis and Diagnostic Tests

The first step of DED diagnosis involves thorough examination of the patient's medical history and risk factors. Some pertinent information include environment associated with symptoms occurring, medication history, and associated systemic diseases.⁵⁸ As previously discussed, factors such as environment, medication, and systemic disease may increase the risk of developing DED.⁵⁸ Symptom evaluation may be conducted using standardized questionnaires (e.g. Ocular Surface Disease Index (OSDI)), where the patient is queried about frequency and/or severity of visual disturbances, ocular discomfort, or impact of DED to their quality of life.^{65,66}

The OSDI is a commonly utilized questionnaire, where scores greater than 12 are considered symptomatic; severity of symptoms may be determined by an increasing score: mild (13-22), moderate (23-32), severe (33-100).⁶⁷

Loss of tear film stability (i.e. tear break up time (TBUT)) is a fundamental diagnostic criterion.^{68,69} TBUT measures the duration before the tear film begins to destabilize after a complete blink.⁷⁰ The test is typically conducted with sodium fluorescein (invasive tear stability) or observing for a disruption in light reflected off the tear film (non-invasive).^{71,72} A TBUT of <10 seconds is considered an unstable tear film.⁶⁷

Ocular surface staining is conducted by instilling sodium fluorescein onto the ocular surface to evaluate the extent of epithelial damage.⁶⁹ The dye highlights areas of compromised cells on the ocular surface (e.g. loss of tight junctions, loss of glycocalyx, and cell death).⁷³ The pattern of the stain may provide etiological clues, e.g., chronic exposure due to incomplete blinking or eyelid closure would typically present in the inferior third of the cornea.⁶⁷

Tear meniscometry determines the volume of tears on the ocular surface by measuring the height of the tear meniscus either via photographic or optical coherence tomography (OCT) imaging.⁷⁴ Alternatively, the Schirmer's test involves inserting a paper strip between the inferior eyelid and the eye; the length of wetting on the strip after 5 minutes is measured.^{75,76} Schirmer's scores that are at least 10 mm of wetting after 5 minutes are considered normal.⁶⁷

Eyelid margin examination, meibomian gland expression, and imaging are all methods for evaluating MGD.⁷⁷ Visual inspection of the eyelids include the assessment of eyelid architecture irregularities and the degree of obstruction of the orifice of the glands.⁷⁷ Physical force may be applied to the eyelid to evaluate the viscosity, volume, and quality of the expressed

meibum.⁷⁷ Meibography may also be utilized to observe and image the glands directly to evaluate atrophy.⁷⁷

While DED may coexist amongst other ocular conditions, both signs and symptoms of DED are required to effectively diagnose the symptomatic disease.⁷⁸ The signs of DED are identified through diagnostic tests such as TBUT or ocular surface staining to establish the tear film's dysregulation.⁷⁸ The symptoms of DED are detected through patient history and standardized questionnaires (e.g. OSDI).⁵⁸ The use of multiple diagnostic methods not only detect DED but may also rule out other ocular diseases (e.g. allergies, etc.), as many of them share similar signs/symptoms with DED.⁷⁸ The diagnosis of DED is crucial for patients, since it serves to acknowledge their symptoms and the attention of their healthcare providers. This creates opportunities for proper treatment and education to improve symptoms and quality of life.

1.2.5 Management

The TFOS DEWS II Management and Therapy Report outlined three stages of DED management and treatment, based on increasing severity, as follows.⁷⁹ The first stage focuses on patient education, prevention (e.g. environment modification), artificial tears and warm compresses. The second stage involves on use of anti-inflammatories. The third stage includes more intensive treatments with therapeutic contact lenses or serum eye drops. The final stage involves surgical solutions to treat DED (e.g. punctal occlusion, membrane grafts, etc.).

Mild cases of DED are typically managed with artificial tears and warm compresses.⁸⁰ Artificial tears act to supplement the unstable tear film.^{80,81} Formulations between artificial tears vary greatly to fulfill the differing needs of each DED patient, depending on the etiology of the

disease (i.e. viscosity-enhancing agents, electrolytes, osmo-protectants, lipids, antioxidants).⁸⁰

Warm compresses involve applying heat to the eyelids to improve mobilization of meibum from the meibomian glands to the tear film.⁸¹

Moderate-to-severe DED require another layer of therapy, characterized by use of anti-inflammatory drugs.^{28,82} The main four therapeutics currently in use to mitigate inflammation are topical corticosteroids, cyclosporine, lifitegrast, and low-dose oral antibiotics.^{28,82} Corticosteroids are effective in downregulating expression of pro-inflammatory factors including MMP-9, IL-1 α , IL-1 β and TNF- α .⁸³ However, long-term use of steroids increase the risk for developing glaucoma (increased intraocular pressure⁸⁴) and cataracts.⁸⁵ Oral low-dose antibiotics (e.g., doxycycline) also reduce pro-inflammatory factors (i.e. MMP-9, IL-1 α , IL-1 β and TNF- α).⁸³ However, long-term use was associated with gradual loss in efficacy, and can negatively affect the gut microbiome.⁸⁶ Cyclosporine is an immunomodulator and is common for treating moderate-to-severe DED.^{87,88} A systematic review conducted by De Paiva et al.⁸⁹ found inconsistent results regarding the efficacy of the drug relative to artificial tears, and that cyclosporine comes with significant adverse effects (i.e. burning and stinging). Lifitegrast acts as an anti-inflammation by acting as a lymphocyte function-associated antigen 1 antagonist.^{90,91} A meta-analysis conducted by Nichols et al.⁹⁰ found that clinical trials mainly reported mild-to-moderate adverse effects upon instillation (i.e. irritation), but studies were only reported up to 360 days, thus long-term studies are needed to fully understand the impact of this therapeutic. A meta-analysis conducted by Haber et al.⁹² determined lifitegrast to be effective for short-term therapy compared to placebo, although the use of lifitegrast in combination with other therapeutics is not well established. Recently, Wu et al.⁹³ conducted a comprehensive safety analysis of lifitegrast after its market release in 2016. The study found that the majority of

reported adverse events (e.g. irritation upon instillation) were expected but identified 24 unexpected and rare adverse events. The pharmacovigilance study emphasized the need for more long-term studies to understand the safety of the therapeutic.

1.3 Study Rationale

DED is a chronic disease where an ideal therapeutic must be effective and safe for long-term use. While topical steroids are effective at inhibiting excessive inflammation, the increased risk of developing cataracts and glaucoma with long-term use is not acceptable.⁹⁴⁻⁹⁶ Similarly with antibiotics, the drug is found to gradually lose efficacy over time and negatively affects the gut microbiome.⁸⁶ Additionally, the long-term effect of cyclosporine and lifitegrast are also not known, with many patients discontinuing their treatment within 12 months due to adverse side effects such as burning or stinging sensations upon administration.⁹⁷ Thus, due to the chronic nature of DED, the management of inflammation must be effective and be well-tolerated for long-term use.

Flavonoids are a subset of molecules found within the polyphenol class and are characterized as two or more 6-member rings connected by a heterocyclic pyran and a hydroxyl group.⁹⁸ Flavonoids are naturally produced in plants and play several roles for the survival of the organism.^{98,99} Fruits and flowers are coloured and scented by the flavonoids to attract pollinators for reproduction.⁹⁹ The aromatic molecules provide protection against pathogens, UV rays, and extreme temperatures.⁹⁹ The wide diversity of structures within the flavonoid class affords opportunities for targeting novel medicinal targets within inflammatory pathways.¹⁰⁰

Quercetin was found to directly inhibit inflammatory proteins (i.e. MMP-9),¹⁰¹ upregulate expression of anti-inflammatory cytokines (i.e. IL-10),⁹⁸ and reduce expression of pro-

inflammatory factors (i.e., MMP-2, MMP-9, ICAM-1, and VCAM-1) in the lacrimal gland of a mouse model.¹⁰² Catechins found in green tea downregulated pro-inflammatory cytokines IL-6 and IL-1 β , and reduced reactive oxygen species (ROS) in a human corneal epithelial cell (HCEC) model.¹⁰³ Participants with DED showed improvement in symptoms scores and tear stability after using a topical administration of green tea extract containing the aforementioned catechins.¹⁰⁴ Finally, isorhamnetin was found to induce tear fluid secretion on the ocular surface in mice models.¹⁰⁵ These examples represent the potential flavonoids have as anti-inflammatory agents.

Molecules found in the flavonoid class are generally considered safe due to the fact that many foods such as fruit, vegetables, and other plant-based food products, are rich in these polyphenols.¹⁰⁶ The dosage of flavonoids from normal daily dietary consumption is well established and deemed safe.¹⁰⁷ As with any substance, there is a maximum dosage, beyond which the substance becomes toxic. With flavonoids, the threshold of toxicity is typically above that of normal consumption. A clinical study observed the effects of 500 mg of rutin supplements for 3-months in human patients with Type 2 Diabetes and reported no adverse events or toxicity.¹⁰⁸ Another study observed the effect of citrus flavonoid extract (400-500 mg) on athletic performance and also reported no adverse events or toxicity.¹⁰⁹ Another clinical trial assessed the effect of a multi-flavonoid supplement (1000 mg) with up to 450 mg of a single flavonoid daily for 8 consecutive weeks.¹¹⁰ This study identified only mild adverse side effects after 20 days of administration. While the mentioned human studies have not reported toxicity, the number of studies is few, and more clinical trials are needed to fully establish safe levels of consumption.¹¹¹

1.4 Thesis Objectives

The purpose of this thesis was to identify flavonoids that can inhibit pro-inflammatory proteins. The approach consisted of conducting a drug discovery study with three objectives:

1. Identify candidate flavonoids and protein targets associated with DED using network analysis.
2. Predict binding conformations and stability of protein-ligand complexes with computational modelling.
3. Validate the inhibitory effect of flavonoids in vitro against protein targets and assess the biocompatibility of candidate flavonoids using an immortalized HCEC line.

Chapter 2 The Discovery of an IL-17A Inhibitor

2.1 Introduction

The treatment of moderate-to-severe DED requires anti-inflammatory therapy;^{28,82} however, the therapeutics available to alleviate inflammation may only be suitable for short-term treatment. The nature of long-term use of these treatments is not well-established (i.e. efficacy, safety, and tolerability) and prolonged use may lead to increased risk of developing adverse side effects (e.g. cataracts or increased intraocular pressure).^{83-90,92,93} Flavonoids are a plant-based subset of secondary metabolites which serve important roles in the survival of plants,⁹⁹ and may offer solutions to the limitations of anti-inflammatory treatments. The diverse structures within this chemical class allow plants perform various functions, which include attracting pollinators, resisting extreme temperatures, UV rays, and provide anti-bacterial protection.⁹⁹ This diversity could be leveraged to help discover small molecule therapeutics against multiple protein targets in human disease, such as various pro-inflammatory proteins.¹¹² Examples of known anti-inflammatory flavonoids include quercetin, catechins, and isorhamnetin; all of which have demonstrated activity against specific proteins in various biological pathways.^{98,101-105} The purpose of this thesis was to use a hybrid approach, consisting of computational modeling and in vitro validation experiments, to identify and validate novel anti-inflammatory flavonoid compounds.

2.2 Methods

2.2.1 Candidate Flavonoids and Protein Target Determination

The protein-protein interaction (PPI) network of proteins associated with DED was generated using the STRING-DISEASES database (<https://string-db.org/>) using the keywords “Dry Eye Disease” (STRING Consortium, USA).¹¹³ Proteins within confidence score >0.9 were included in the PPI network and were considered DED-associated proteins. Functional enrichment analysis tool, ToppFun, within the ToppGene Suite enrichment analysis toolkit (Cincinnati Children's Hospital Medical Center, OH, USA),¹¹⁴ was used to cross reference DED-associated proteins with several drug databases for statistically significant ($p < 0.05$) known and predicted drug interactions and associations. The drug databases accessed were Drug Bank (<https://go.drugbank.com/>),¹¹⁵ Search Tool for Interacting Chemicals (STITCH) (<http://stitch.embl.de/>),¹¹⁶ and Comparative Toxicogenomics Database (CTD) (<https://ctdbase.org/>)¹¹⁷. The collection of drugs was included in the drug-protein interaction network and encompassed all small molecules stored in the databases including non-drug small molecules and toxic compounds. Python scripts were written to select for molecules categorized as “flavone”, “flavonoid”, “flavanol”, and/or “polyphenol” by PubChem (<https://pubchem.ncbi.nlm.nih.gov/>)¹¹⁸ and CTD; and the dataset was restructured to be compatible with Cytoscape edge interaction analysis (3.1 Appendix A). The visualization and analysis of the PPI and drug-protein network were conducted using Cytoscape 3.9.1. (Cytoscape Consortium, USA).¹¹⁹ The nodes, representing either proteins or flavonoids, were ranked according to number of edge interactions, which was then used as a metric for candidate flavonoid and protein target selection.

2.2.2 Protein-Ligand Complex Computational Modelling

The initial 3-D protein structures of IL-17A (IL-17A; PDBID: 7AMA) and tumour necrosis factor α (TNF- α ; PDBID: 6X81) were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (<https://www.rcsb.org/>).¹²⁰ The PDB protein structure was prepared by removing extraneous molecules, including water and ligands. Missing chains of TNF- α were repaired with a secondary TNF- α structure as a template (PDBID: 6RMJ); polar hydrogen molecules were added to both protein structures. Initial ligand structures of flavonoids were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>): luteolin (CID: 5280445), and rutin (CID: 5280805). The ligand structures were energetically minimized using chemical informatics software, OpenBabel,¹²¹ within the molecular docking virtual screening program, PyRx (The Scripps Research Institute, CA, USA).¹²²

Molecular docking was conducted to model the protein-ligand complex of the selected protein receptors and flavonoid ligands in preparation for molecular dynamics (MD) simulations. All docking were performed using PyRx. Docking grid boxes were defined to compass the receptor binding site of the proteins (Table 1). Detailed protein-ligand interactions were identified using the molecular visualizer tool, BIOVIA Discovery Studio (Dassault Systèmes, France). A total of four complex combinations were generated: IL-17A – Luteolin, IL-17A – Rutin, TNF- α – Luteolin, and TNF- α – Rutin.

Table 1. Molecular Docking Grid Box Parameters

Protein	Center Coordinate			Size (Å)		
	X	Y	Z	X	Y	Z
IL-17A	-14.98	-13.66	-14.14	14.78	15.64	16.92
TNF- α	2.95	34.47	-27.42	19.47	16.82	18.46

MD simulations were conducted to further differentiate good small molecule candidates. MD simulations permit protein and flavonoid structure movement/flexibility and model their atomic interactions in a dynamic environment. For each complex combination, ligand positions that exhibited the lowest binding energy from molecular docking were selected for MD simulation preparation. Simulation trajectories were conducted at the average temperature of the ocular surface, 308K (34.85°C),¹²³ for 150 ns with AMBER22 (Assisted Model Building and Energy Refinement 2022) to carry out MD simulations.¹²⁴ The preparatory package *tleap* within AMBER22 was utilized for system preparation. All hydrogen atoms were added to the protein residues, and the complex was solvated with the optimal point charge (OPC) water model within a cubic box measuring 15 Å x 15 Å x 15 Å and neutralized with sodium ions. All residues were adjusted to protonation states at pH 7 and at a salinity of 300 mM. All systems underwent the standard minimization, heating, and equilibration step prior to production steps. The production steps of the trajectories were extended until the system has stabilized by monitoring the root-mean-square deviation (RMSD) using trajectory analysis package, *cpptraj*, within AmberTools22. RMSD is a measure of the structural deviation of the system from the initial reference structure. Free binding energy calculations were performed for snapshots between 120 ns -140 ns of the trajectories, where the RMSD was relatively consistent, using the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) method with *MMPBSA.py*.¹²⁵

2.2.3 In Vitro Validation

Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific International (Hampton, NH, USA). Luteolin and rutin were obtained from TCI America (Portland, OR, USA) and Fisher Scientific International (Hampton, NH, USA), respectively. The alamarBlue cell viability reagent was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The

live/dead viability/cytotoxicity kit, for mammalian cells; annexin V, AlexaFluor 647 stain; and 5X annexin-binding buffer were also obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

The inhibition of IL-17A and TNF- α by luteolin and rutin was quantified using inhibitor screening ELISA kits (ACRO Biosystems, DE, USA). The kits quantified the ability of the flavonoids to inhibit IL-17A or TNF- α from binding to their respective receptors (i.e., IL-17 receptor A (IL-17RA) and TNF receptor 1 (TNFR1)). The assays were conducted in technical replicates of 2 and experimental replicates of 3. Luteolin solutions were prepared at varying concentrations with 50% DMSO and rutin solutions were prepared with 61% DMSO. Briefly, coating protein (TNFR1 or IL-17A) and respective biotinylated cytokine/receptor (TNF- α or IL-17RA) was exposed to only assay buffer (assay control), 50% or 61% DMSO (vehicle control) or various concentrations (0.5 mM, 1.0 mM, 5.0 mM, 10 mM, 50 mM) of luteolin or rutin with either 50% or 61% of DMSO, respectively, for 1 hour. Streptavidin-conjugated horseradish peroxidase substrate was added for quantification. The absorbance of each well was then measured at $\lambda = 450$ nm for activity, with background subtracted at $\lambda = 630$ nm.

2.2.4 Cell Culture and Biocompatibility

HPV-immortalized human corneal epithelial cells (HCEC) (Ottawa Eye Research Institute, Ottawa, ON, Canada)¹²⁶ were cultured in Dulbecco's Minimal Essential Medium/Nutrient Mixture F12 (DMEM/F12 media); (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). HCECs were cultured at 37°C, 100% humidity and 5% CO₂. The media was replaced every two days.

HCECs were cultured in 48-well plates in DMEM/F12 media. The cells were treated with the following experimental conditions: assay control (only DMEM/F12 media), vehicle control (DMEM/F12 media and 1% DMSO), and various concentrations (0.03 mM, 0.06 mM, 0.3 mM, 0.6 mM, 3 mM, 6 mM) of rutin dissolved in DMSO (1%) and media for 24 hours. Metabolic activity was assessed using an alamarBlue assay. Briefly, 10% alamarBlue reagent in serum-free media was prepared and added to the treated cells for 4 hours after experimental condition treatment. Fluorescence was measured at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 540 \text{ nm}/590 \text{ nm}$. The assay was conducted in technical replicates of 2 and repeated 3 times for experimental replicates.

HCECs were cultured and treated with the same experimental conditions and controls as described above to image apoptotic, live, and dead HCECs. After the treatment period, the HCECs were washed with cold phosphate-buffered saline and stained for imaging. The staining solution was prepared with 5% (v/v) annexin-V, 0.05% (v/v) calcein AM, and 0.2% (v/v) ethidium homodimer-1 in 1X annexin-binding buffer. The apoptotic cell stain, annexin V ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 650 \text{ nm}/665 \text{ nm}$), binds to phosphatidylserine, which is normally located in the inner leaflet layer of the cell membrane. Phosphatidylserine is presented on the outer surface of the membrane when a cell undergoes apoptosis.¹²⁷ Live cells are stained with calcein AM ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 494 \text{ nm}/517 \text{ nm}$). Calcein AM passes through the cell membrane and is modified by intracellular esterases of living cells to become fluorescent.¹²⁸ Non-living cells lacking esterase activity would not activate calcein AM and the stain will be lost during subsequent washing steps. Ethidium homodimer-1 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 528 \text{ nm}/617 \text{ nm}$) is a nucleic acid stain that is normally impermeable to intact cell membranes. Dead cells with damaged membranes permit entry to this fluorescent stain, which binds strongly to DNA/RNA in the cell.¹²⁹ The combined stain solution was applied to the cells for 30 minutes at room temperature, and then washed with 1X annexin-V binding

buffer. This experiment was conducted in technical replicates of 2 and repeated 3 times. Fluorescent signal from each representative image was binarized and quantified using ImageJ (National Institutes of Health, Bethesda, MD). The images were split into red (annexin V), blue (ethidium homodimer-1), and green (calcein-AM) channels. Threshold values were assigned to all images as followed: (red) 96-225, (blue) 110-225, (green) auto. The thresholds for each stain were determined by visual inspection where all cells were clearly defined, and no background noise was detected (Figure 2). Individual particles were counted for each image.

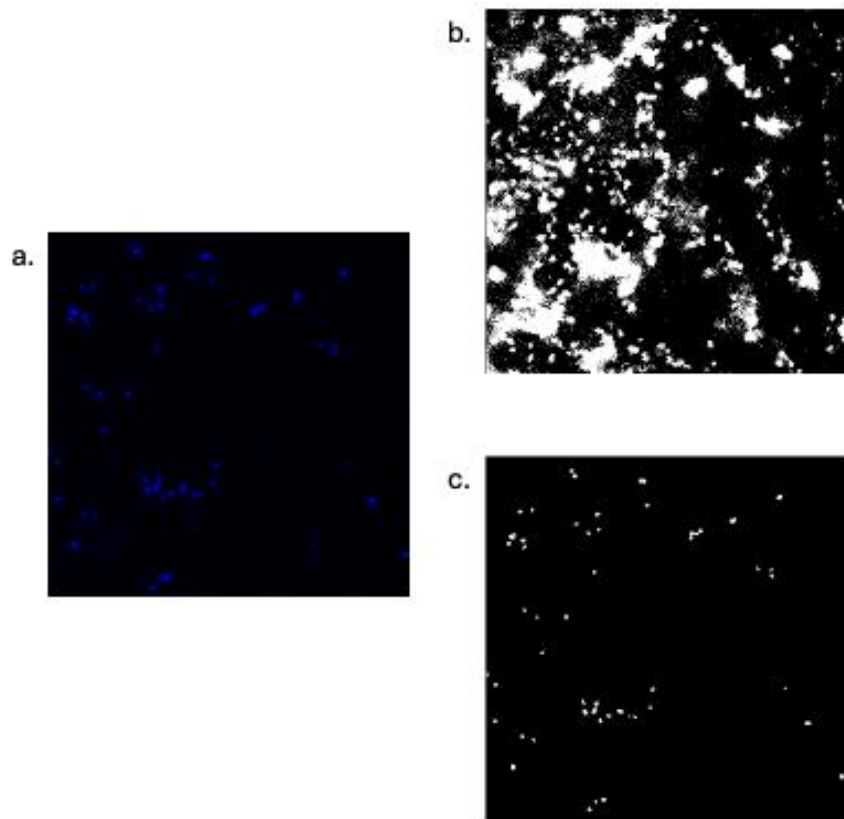


Figure 2. ImageJ threshold parameters. A) Original fluorescent microscopy image of stained dead cells (ethidium homodimer-1). B,C) Images of poor (56-255) and acceptable threshold (96-255) parameters respectively. All selected areas are coloured white, and background is represented in black. Figure 2B demonstrates poor threshold parameters where cells are not well defined, and background signal is included. Figure 2C demonstrates acceptable threshold parameters where individual cells are well defined, and background noise is minimized, to best represent the image in 1A.

2.2.5 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 10 (GraphPad Software, CA, USA). The cytokine/receptor inhibition signals were normalized against the assay buffer control and linear regression analysis was conducted to determine the presence of a dose-independent inhibitory effect. Next, half maximum inhibitory concentration for the protein-ligand pairs demonstrating a dose dependent inhibitory effect was quantified using nonlinear regression analysis. Metabolic activity signals were normalized against the DMEM media control. Annexin V (apoptotic) or ethidium homodimer-1 (dead) stain signals were normalized to calcein-AM (live cells) stain to generate ratios that quantified relative cell death and apoptosis. A Kruskal-Wallis test was conducted to test the difference in conditions against their respective vehicle control (1% DMSO) for both the metabolic activity and fluorescent cell imaging data. For all statistical tests, a p-value of <0.05 was considered statistically significant.

2.3 Results

2.3.1 Network Analysis

Generated from the STRING-DISEASES (11.0) database, the PPI network (Figure 3a) consisted of 64 DED-associated proteins (confidence score >0.9). All nodes in the PPI network were ranked by number of edge interactions with neighbouring nodes. The highest-ranking proteins (number of edges) were TNF- α (21), IL-6 (21), CD4 (18), IL-1 β (15), and IL-17A (14) (Table 2). Two of the five top proteins were selected strategically as the protein targets of interest for molecular docking for their roles in the innate and adaptive immune response respectively: TNF- α and IL-17A. The drug-protein network identified a total of 108 flavonoids after cross-referencing against chemical interactions databases and removal of known toxic

compounds. Nodes within the drug-protein network were also ranked by number of edge interactions. The top 5 highly connected flavonoids were luteolin, rutin, kaempferol, catechin, and naringin (Figure 3b, Table 3). Of the 5, the two highest ranking flavonoids, luteolin and rutin, were selected for docking as well.

Table 2. Highest Interacting Proteins Associated with DED

Protein Name	Number of Edge Interactions	String Database Disease Score
TNF-α	21	2.52
IL-6	21	2.56
CD4	18	2.47
IL-1β	15	2.56
IL-17A	14	2.51

Table 3. Highest Interacting Flavonoids with DED-associated Proteins

Flavonoid Name	Number of Edge Interactions	p-value
Luteolin	19	8.35×10^{-23}
Rutin	17	3.62×10^{-21}
Kaempferol	17	1.23×10^{-18}
Catechin	17	1.91×10^{-7}
Naringin	16	9.30×10^{-5}

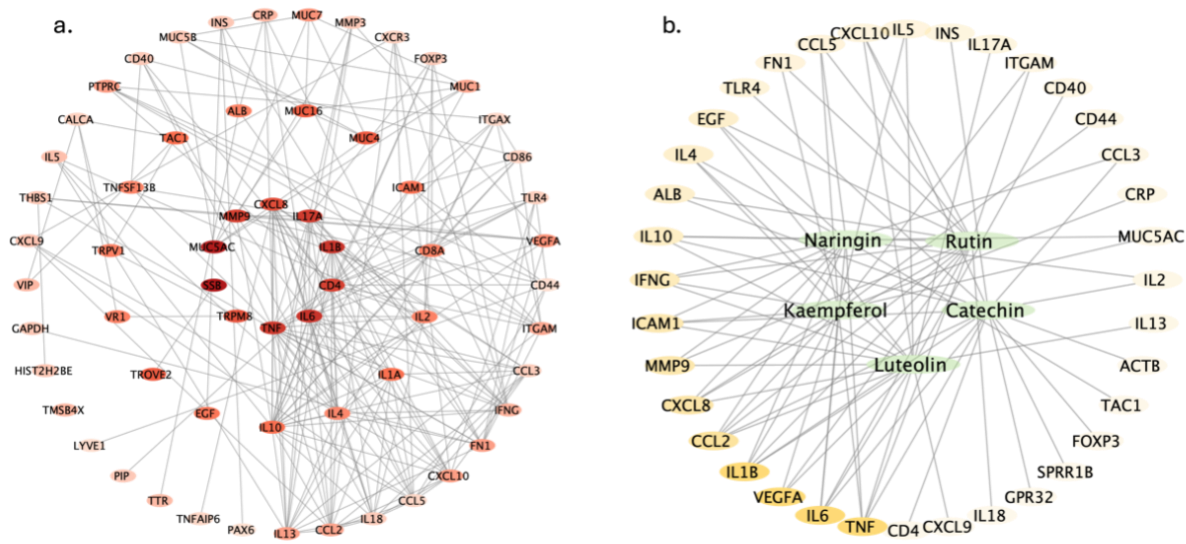


Figure 3. Visualized network results. (A) Protein-protein interaction network of dry eye disease associated proteins; nodes are organized by greatest (dark) edge interactions to least (light) edge interactions. (B) Drug-protein network showing the interaction of the top-ranking flavonoids (green) with dry eye disease associated proteins (yellow). The shade of yellow colour indicates a higher number of edge interactions.

2.3.2 Molecular Docking

Each docking instance generated 10 positions for each protein-ligand complex; but only the best docking score position was used to examine direct interactions. Of the 4 complexes (Table 4, Figure 4), TNF- α and luteolin exhibited the most favourable binding affinity of -9.90 kcal/mol with TNF- α and rutin following closely at -9.20 kcal/mol, rutin and IL-17 with -8.10 kcal/mol and the least favourable binding affinity of -6.80 kcal/mol was luteolin and IL-17A.

Table 4. Docking Scores

Flavonoid	Docking Score (kcal/mol)	Favourable Interacting Residues
IL-17A (7AMA)		
Luteolin (CID 5280445)	-6.80	Chain A: PRO63, ILE96 Chain B: GLU95, LEU97, LEU99
Rutin (CID 5280805)	-8.10	Chain A: TYR62, TRP67, VAL98, LEU112 Chain B: GLN94, GLU95
TNF-α (6X81)		
Luteolin (CID 5280445)	-9.90	Chain A: LEU57, GLN61, TYR151 Chain B: TYR119
Rutin (CID 5280805)	-9.20	Chain A: TYR59, GLY121 Chain B: SER60, LEU120 Chain C: TYR119, GLY121, GLY122

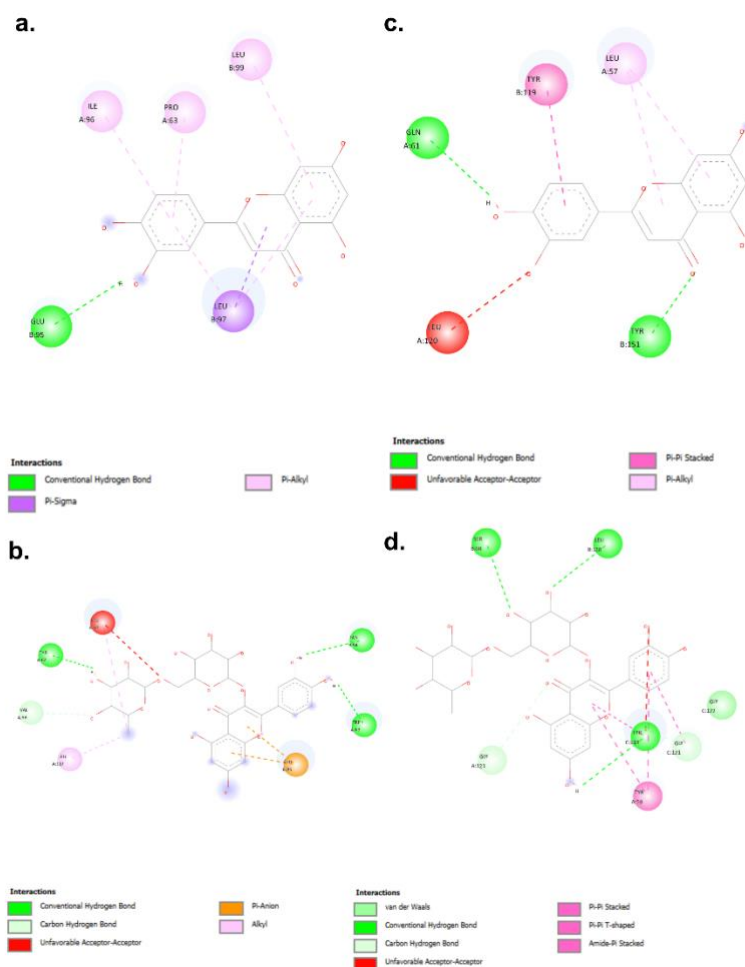


Figure 4. Ligand interaction map: Residue-ligand interaction map of (a) IL17A and luteolin; (b) TNF- α and luteolin; (c) IL17A and rutin; (d) TNF- α and rutin.

2.3.3 Molecular Dynamics Simulations

All simulations were extended to 150 ns to capture the equilibration of the system (i.e., stable RMSD) (Figure 5). The production steps of all simulations were all extended for approximately 20-30 ns once the system had plateaued to a consistent RMSD value, indicating no major conformation changes were occurring. Visual inspection of all simulations confirmed that all the complexes remained intact as none of the ligands were energetically repulsed from the binding site of the protein receptors. If protein-ligand complexes were energetically unfavourable, the visualization of the ligand leaving the binding site due to atomic forces would have been observed. Binding free energy (ΔG) was calculated using the MM-PBSA method (**Error! Reference source not found.**). The results suggested that the two most probable protein-ligand complex pairs to be rutin/TNF- α (-11.31 kcal/mol) and luteolin/IL-17A (-9.51 kcal/mol). While luteolin/TNF- α (-2.96 kcal/mol) and rutin/IL-17A (+2.19 kcal/mol) showed the least probability for binding, it should be noted these values were relative to the four complexes assessed in this thesis.

Table 5. MM-PBSA Free Energy Calculations

Protein	Ligand	ΔG Binding (kcal/mol)
IL-17A	Luteolin	-9.51
	Rutin	+2.19
TNF- α	Luteolin	-2.96
	Rutin	-11.31

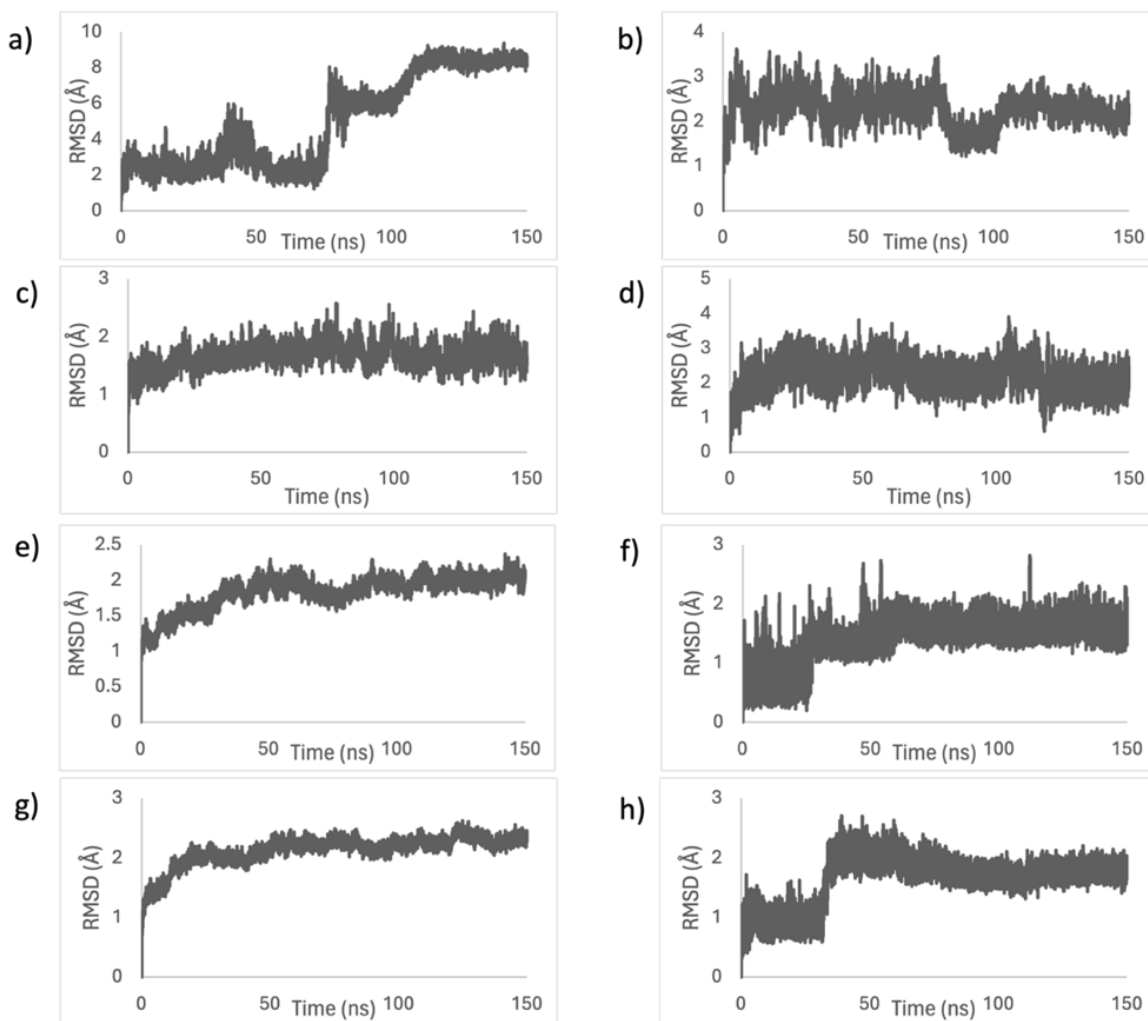


Figure 5. RMSD plot of MD simulations: IL-17A-luteolin (a) backbone, (b) luteolin; IL-17A-rutin (c) backbone, (d) rutin; TNF- α -luteolin (e) backbone, (f) luteolin; TNF- α -rutin (g) backbone, (h) rutin.

2.3.4 Inhibitor Screening Assay

Regression analysis of inhibitor screening assays detected significant inhibitory activity of IL-17A/IL-17RA activity by both luteolin (slope = -1.70 ± 0.53) and rutin (slope = -0.41 ± 0.09) (both $p < 0.01$). Neither luteolin nor rutin exhibited any significant inhibitory effect on TNF- α /TNFR1 activity (all $p \geq 0.17$). Nonlinear regression analysis detected an $IC_{50} = 8.73$ mM for rutin, compared to $IC_{50} = 16.54$ mM for luteolin against IL-17A/IL-17RA activity (Figure 7).

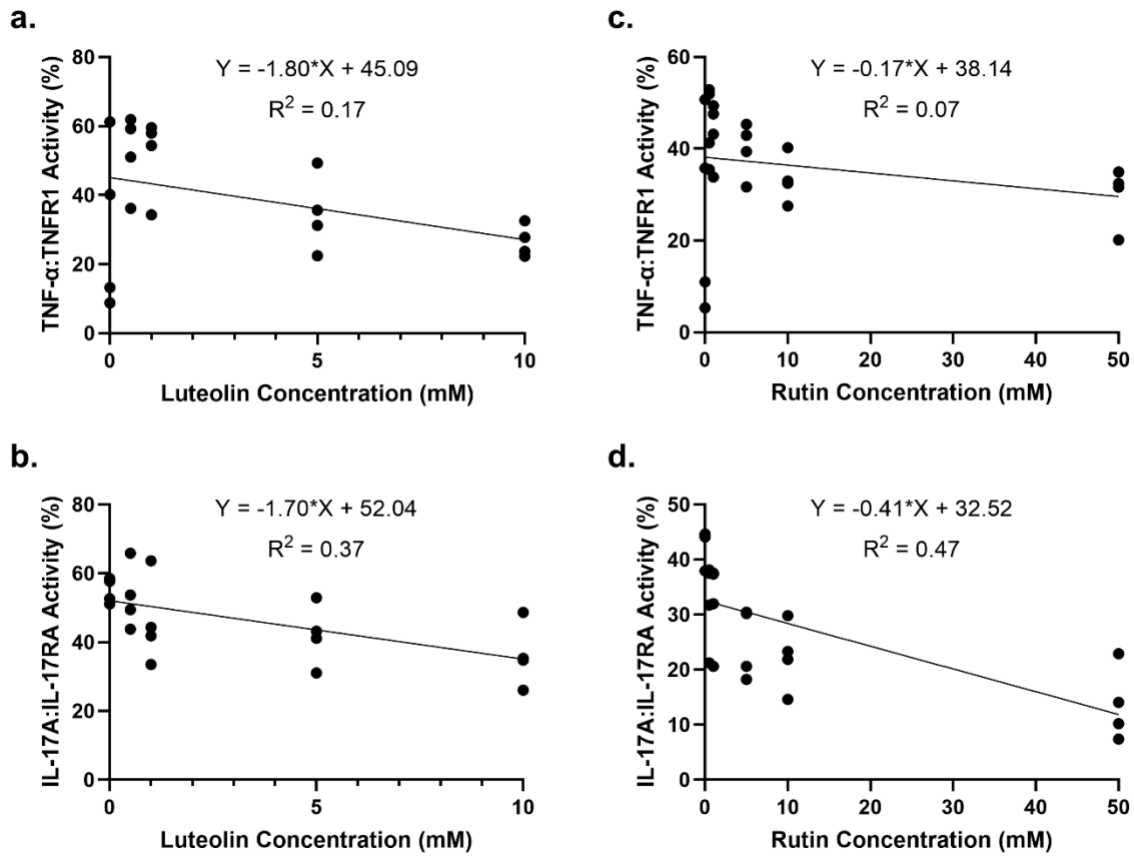


Figure 6. Activity of cytokine-receptor interaction in the presence of luteolin or rutin normalized against assay control with linear regression results. a) TNF- α /TNFR1 and luteolin; b) IL-17A/IL-17RA and luteolin; c) TNF- α /TNFR1 and rutin; d) IL-17A/IL-17RA and rutin.

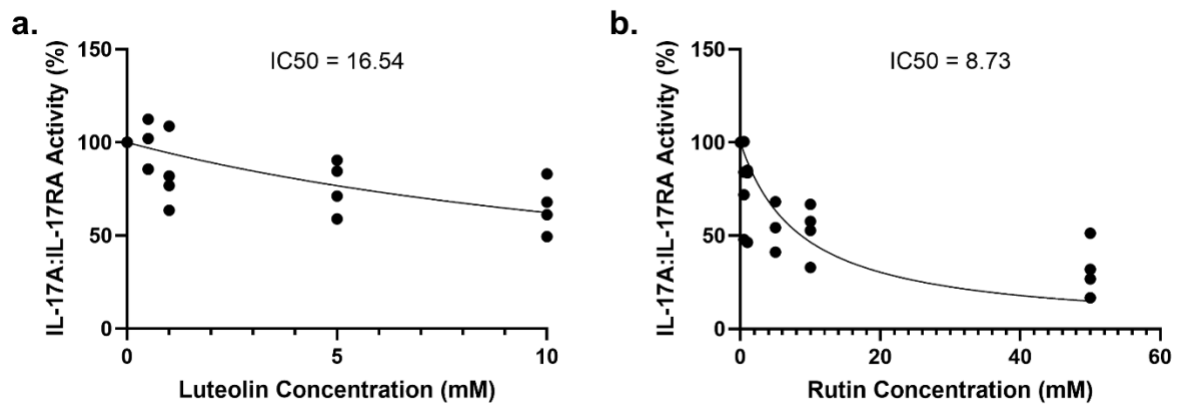


Figure 7. Activity of cytokine-receptor interaction in the presence of luteolin or rutin normalized against vehicle control with nonlinear regression analysis. a) IL-17A/IL-17RA and luteolin; b) IL-17A/IL-17RA and rutin.

Due to its ability to demonstrate greater cytokine inhibition compared to luteolin, rutin was selected to continue with biocompatibility evaluation. The metabolic activity of HCECs was assessed at varying concentrations of rutin (Figure 8). While a significant difference of metabolic activity was detected within the group ($p=0.02$), no significant differences between all concentrations compared to the vehicle control were detected in the post-hoc multiple comparisons analysis (all $p \geq 0.13$). However, it was noted that exposure to 3.0 mM rutin resulted in reduced metabolic activity (52.90%) in comparison to the vehicle control (84.21%) and all other lower concentrations of rutin (86.95% - 96.71%). It was also noted that the effect of the vehicle (1% DMSO) did not significantly affect metabolic activity compared to the assay control (DMEM media) ($p = 0.13$).

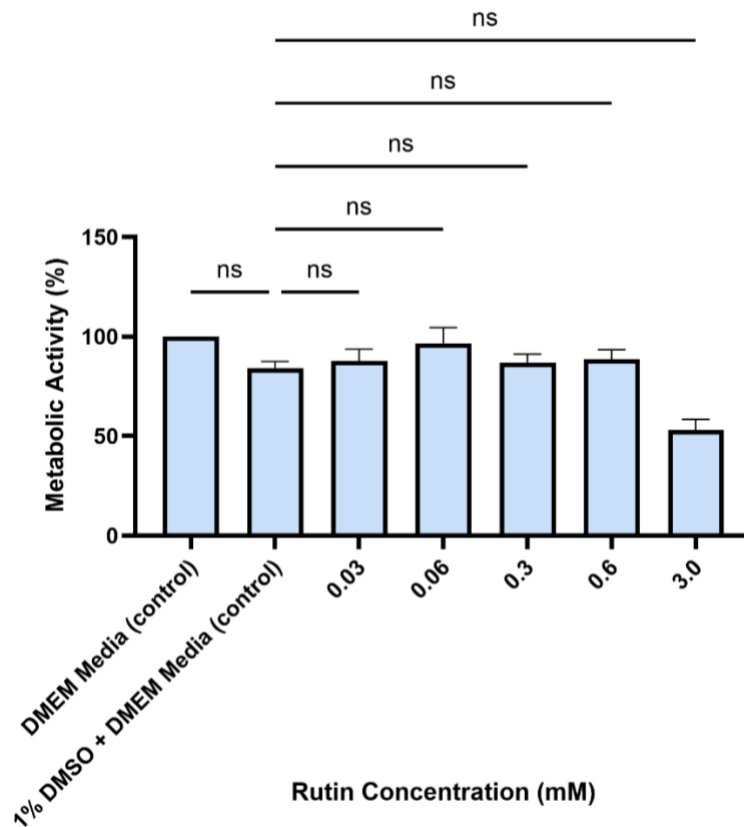


Figure 8. Metabolic activity of HCECs after various concentrations of rutin treatment.

The proportion of apoptotic and dead cells to live cells were quantified, (Figure 9) and no significant difference in proportion of apoptotic cells ($p=0.50$) nor dead cells ($p=0.06$) were detected within the groups (Figure 9). Therefore, rutin had no significant effect to apoptotic and dead cell populations compared to the media control.

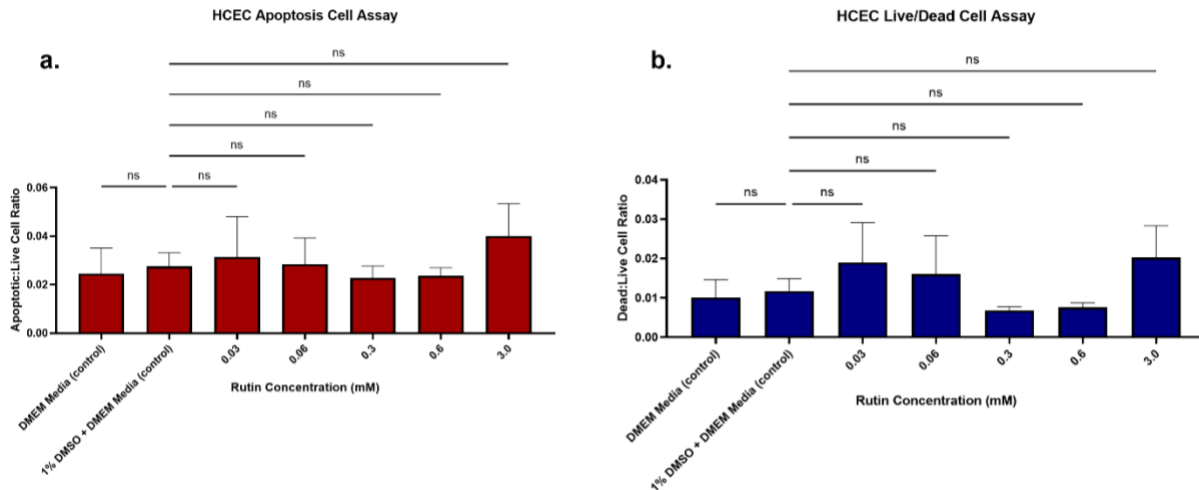


Figure 9. Apoptosis cell staining assay. a) Apoptotic/live cell ratio (annexin V/calcein AM signal) b) Dead/live cell ratio (ethidium homodimer-1/calcein AM signal).

2.4 Discussion

Overall, this thesis was a drug discovery study that aimed to address the lack of suitable long-term anti-inflammatory treatments for DED. Current anti-inflammatory therapies for managing DED are effective short-term but have long term side effects.^{28,82} Cyclosporine and corticosteroids are commonly associated with irritation upon instillation and increased risk of developing ocular conditions (e.g. cataracts or glaucoma, respectively).^{85,89} Low-dose antibiotics are known to disrupt the gut microbiome over time⁸⁶ and long-term studies of lifitegrast and cyclosporine efficacy are insufficient.^{89,90} In the search for new anti-inflammatory drugs, the decision to focus on flavonoids was made due to their low toxicity¹⁰⁸⁻¹¹⁰ and potential anti-inflammatory activity on the ocular surface.¹⁰² Some flavonoids (e.g. quercetin, curcumin and

catechins) have already demonstrated the potential of flavonoids for treating DED.¹⁰² This thesis employed a hybrid approach to conduct a drug discovery study to identify direct flavonoid inhibitors against pro-inflammatory cytokines. The flavonoid class consists of thousands of individual molecules and therefore, a method to rapidly identify candidate flavonoids was needed. Network analysis is a common technique used to strategically select molecules from a larger subset for subsequent analyses. For example, Nogales et al.¹³⁰ used network analysis to examine protein-drug relationships to elicit novel mechanisms of drug interactions. This technique enables comprehensive visualization of all known proteins involved in the pathogenic mechanisms behind the disease. The selected protein targets and candidate flavonoid inhibitors of this thesis was then modelled computationally in their protein-ligand complexes with molecular docking. The use of these computational tools is an important part of drug discovery to understand the interactions occurring at the molecular level.¹³¹ Molecular docking calculates the strength of interaction (i.e., binding affinity) between the protein receptor and ligand using the scoring function of the docking program within PyRx, AutoDock Vina, which evaluates steric interactions, atom pairs, hydrophobic interactions, and hydrogen bonding of a static protein-ligand complex.¹³² Molecular docking uses relatively less computational resource (compared to MD simulations) to screen through a library of molecules and predict their binding conformations.¹³¹ However, in molecular docking, the protein structure is rigid in a vacuum space, which does not necessarily reflect the nature of protein-ligand interactions.¹³¹ While more computationally intensive, MD simulations simulate the complex in a customizable environment, and all molecules are free to move, directed by atomic forces.¹³¹ This method further established whether the flavonoids were binders or nonbinders by permitting the ligand to be ejected from the binding site if unfavourable conditions were encountered. Computational models serve to

inform further drug development and optimization prior to entering preclinical testing using disease models or pharmacodynamics/kinetic experiments. In this study, since both flavonoids remained within the binding sites of both cytokine targets, the subsequent experiments aimed to quantify and validate the inhibitory effect using *in vitro* techniques. Indeed, the *in vitro* experiments confirmed the predictions of the computational modeling results, confirming inhibitory interaction between the flavonoids against these TNF- α and IL-17A.

TNF- α has myriad roles, but it is well known to promote inflammation by activating the NF- κ B pathway.¹³³ Secreted as transmembrane protein pro-TNF- α , TNF- α converting enzyme (TACE) mediates and solubilizes the cytokine in its mature form, TNF- α .¹³³ Alongside with other pro-inflammatory factors, TNF- α also induces the maturation of APCs (i.e. dendritic cells, macrophages, and T cells).⁶² TNF- α is a signalling protein that upon binding to its surface receptor, will initiate the cellular inflammatory response. The two main receptors for TNF- α are TNFR1 and TNFR2.^{134,135} TNFR1 activation facilitates interaction with the TNFR1-associated death domain and forms one of three signalling complexes (I, IIa, and IIb).¹³⁶ This pathway's biological activities include induction of inflammation, tissue degradation, apoptosis, and defence against pathogens via NF- κ B signalling.^{134,135} On the other hand TNFR2 does not have an associated death domain and forms a signaling complex with a TNFR-associated factor. The main biological activities of this complex are tissue regeneration, cell proliferation and inflammation.¹³⁴ The major role of TNF- α in inflammation has made the cytokine a therapeutic target of interest for several inflammatory diseases.¹³⁴

In DED, an elevated amount of TNF- α was found in the lacrimal glands and tear film.^{137,138} Animals models that involve inducing inflammation through TNF- α exposure

demonstrated increased levels of T-cell activation transcripts and increased pro-inflammatory cytokine expression.¹³⁷ Direct inhibition of the cytokine in DED mice models have resulted in a 30-50% decrease in pro-inflammatory cytokines in tears and reduced inflammatory response.¹³⁷ Clinical trials of experimental TNF- α inhibitor, tanfanercept, demonstrated improvements in clinical assessment scores (i.e. TBUT, Schirmer's test and corneal staining).¹³⁹ However, phase III clinical trials were suspended due to logistical issues.⁵² Current FDA-approved TNF- α inhibitors are monoclonal antibodies (i.e. Infliximab, Adalimumab, Certolizumab Pegol, and Golimumab) and fusion protein therapeutic, Etanercept.¹³⁴ Both the anti-TNF- α antibodies and fusion protein therapeutic are typically administered subcutaneously and are mainly used for autoimmune diseases.^{134,141,142} While antibody therapeutics are effective in blocking TNF- α and receptor interaction, however, the typical mode of administration (i.e. injections) is invasive.¹³⁴ Antibody therapies are also expensive, costing on average \$96,731 USD per patient annually in the United States.¹⁴³ As a DED treatment, the cost and invasiveness of antibody therapies are not feasible. There are currently no direct TNF- α inhibitors indicated for DED treatment, despite the overwhelming evidence implicating this cytokine in the pathogenesis of DED.

Expressed by mainly CD4 Th17 cells, the role of IL-17A is to reinforce epithelial and neutrophil inflammatory responses.¹⁴⁴ The cytokine is a part of the IL-17 family (A to F) and bind to its receptor as a homodimer (IL-17A/A) or heterodimer (IL-17A/F).¹⁴⁵ IL-17A specifically forms a homodimer or a heterodimer with IL-17A and binds to IL-17 receptor (IL-17R) A/C, C/C, or A/D at varying levels of affinity.¹⁴⁵ Upon binding, the receptors recruit ubiquitin ligase Act1 to its respective protein domain (SEFIR) and activates the NF- κ B pathway.¹⁴⁵ IL-17A exerts bactericidal effects by recruiting neutrophils and upregulating anti-microbial proteins.¹⁴⁵ However, IL-17A may be overly expressed at the ocular surface and tear

film in DED,^{146,147} as well as in other conditions with dysregulated immune responses such as asthma, inflammatory bowel disease, and psoriasis.¹⁴⁸ As previously described, once the innate immune response is triggered, a cascade of pro-inflammatory cytokines (i.e. IL-6 and IL-23) may eventually lead to the maturation and differentiation of native CD4 T cells to effector CD4 T cells (i.e. CD4 Th17 cells) in the ocular surface tissues.^{36,60} Expression of IL-17A by Th17 cells induce more pro-inflammatory cytokines (i.e. IL-6, IL-8 and TNF- α).¹⁴⁷ Th17 cells produced in desiccating conditions remained on the ocular surface and remained resistant to regulation by Tregs in animal disease models.^{61,149} DED animal model studies showed that blockade of IL-17A with anti-IL-17A antibodies not only reduced the number of Th17 cells, but also improved corneal fluorescein staining scores and overall progression of the disease in animal disease models.^{60,61} Thus, inhibiting IL-17A as a therapeutic target could potentially halt progression of inflammation.

Similarly to TNF- α , the only FDA-approved drug inhibitors of IL-17 are monoclonal antibodies (i.e. secukinumab and ixekizumab), making it impractical for DED therapy due to the cost and invasive nature (i.e. injections) of the treatment.^{143,150–152} Therefore, inhibitors of IL-17A that can be administered topically are still needed. Recently, a scaffold-based drug discovery investigation identified and invented a macrocyclic small molecule IL-17A inhibitor (LY3509754).^{153,154} To date, LY3509754 is the only computer aided drug designed molecule to reach phase I of clinical trials. Unfortunately, during the trials, several participants had experienced adverse side effects and the product was terminated due to poor tolerability.¹⁵⁵

Luteolin (or 3',4',5,7-tetrahydroxyflavone) is found in a variety of plants including vegetables, fruits and herbs (Figure 10a).¹⁵⁶ The flavonoid is made up of one heterocyclic ring, two benzene rings and four hydroxyl groups. This study found that luteolin had some inhibitory

effect against IL-17A. Evidence for luteolin as a DED topical therapeutic is limited, however, Xie et al.,¹⁵⁷ found a decrease in pro-inflammatory cytokines (i.e. IL-1 β , TNF- α , IL-6 and IL-18) in serum and tears collected from depression-related DED mice models when treated with oral luteolin. Another study found that injected luteolin significantly reduced inflammatory MMP expression levels in rat models with corneal alkali burn.¹⁵⁸ A dose-dependent downregulation of IL-1 β and IL-6, and a reduction in cell apoptosis in luteolin-treated TNF- α -induced inflammatory human nucleus pulposus cell model was observed.¹⁵⁹ These studies utilized cell cultures or animal models to study the overall effect of luteolin and the majority of studies consistently found that the flavonoid exerted anti-inflammatory activity. However, the nature of TNF- α and TNFR1/2 inhibition by luteolin is not well known.

Rutin is comparatively larger than luteolin, containing a rutinoside group attached to the basic flavonoid one heterocyclic ring and two benzene ring structure alongside four hydroxyl groups (Figure 10b).¹⁶⁰ The flavonoid is found naturally-occurring in commonly consumed fruits and vegetables, including but not limited to lettuce, broccoli, watermelon, and beets.¹⁶¹ Similarly to luteolin, while this study identified rutin as a direct inhibitor against IL-17A, there are only a limited number of studies examining the use of rutin as a DED therapeutic. However, many studies have reported anti-inflammatory effects of rutin in disease models. Mice Sjögren syndrome models have exhibited a significant decrease of pro-inflammatory cytokines in salivary gland tissues when treated with oral rutin.¹⁶² Hou et al.,¹⁶³ demonstrated that rutin inhibited TLR4 signalling and subsequently downregulated pro-inflammatory cytokines in a rat liver disease model. As with luteolin, the use of rutin as an anti-inflammatory compound is not novel, however direct targets of the flavonoids are still not clear.

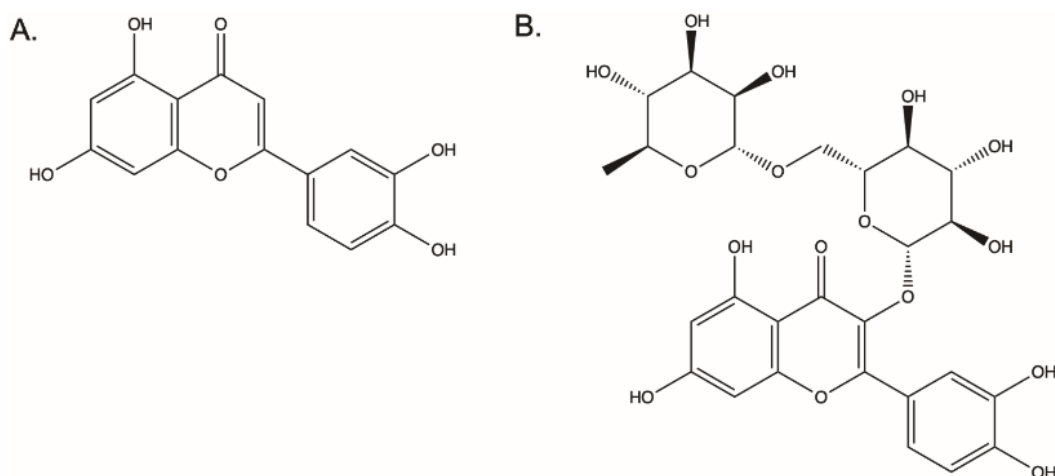


Figure 10. Chemical structures of flavonoids: luteolin (A) and rutin (B).

This study found that rutin generally appeared non-cytotoxic and was biocompatible with HCEC at concentrations < 3 mM. At 3mM, a precipitous drop-in metabolic activity and increase in apoptosis and cell death was observed. To date, no studies have observed the effect of rutin on corneal epithelial cells; however, existing studies have shown that rutin (up to 100 μ M) could maintain or increase cell viability in corneal epithelial cells that have undergone oxidative stress.^{164,165} Zhou et al.¹⁶⁵ found that rutin could reduce ROS production, lipid peroxidation, and regulated the NF- κ B signalling pathway. Emeka et al.¹⁶⁴ attributed the lack of toxicity and improvement of cell viability from rutin treatment to the upregulated expression of direct and indirect retinoic acid signaling regulators, alcohol dehydrogenase 7 and aldehyde dehydrogenase 1 family member A1, respectively. The upregulation of these enzymes reduced the production of nitric oxide by rabbit corneal epithelial cells and improved cell viability by lessened oxidative stress.¹⁶⁴

Chapter 3 Conclusions, Limitations, and Future Work

3.1 Conclusions

In conclusion, this study uncovered novel relationships between DED-associated proteins and flavonoids, having employed computational models and in vitro experiments to quantify the inhibition of IL-17A cytokine signalling by rutin. The flavonoid was also found to minimally reduce overall metabolic activity in HCEC after 24 hours of treatment and did not increase the levels of cell death at concentrations < 3 mM. These findings suggest that rutin may have a role in the development of future anti-inflammatory therapeutics against DED.

Traditional drug discovery methods are resource intensive and typically involve iterations of in vitro testing with different drugs.¹⁶⁶ Interest in using computational modelling for drug discovery began in the 1980s and has flourished recently due to increasing accessibility of tools and databases.¹⁶⁷⁻¹⁶⁹ This study aimed to leverage the strengths of bioinformatics and structural biology methods in drug discovery including the exploration of diverse molecular structures, relatively low costs, and ability to observe mechanisms at the molecular level. For example, Zhao et al.¹⁷⁰ conducted a network analysis study to compare genes associated with DED and genes targeted by the active compounds in the herbal traditional Chinese medicine drug, Qiju Dihuang. The cross-examination of active compounds led to the deduction of six compounds targeting DED, four of which were flavonoids (i.e. luteolin, kaempferol, chryseriol and diosmetin). Molecular docking was then used to predict the binding conformations of these compounds with 18 DED-associated proteins. Naddeem et al.¹⁷¹ used network analysis and functional enrichment analysis to identify common drug classes and individual drugs to interact with proteins associated with age-related macular degeneration (AMD). The list of potential

therapeutics for AMD included flavonoids such as curcumin, apigenin, luteolin, rutin and kaempferol. Several studies have demonstrated success of using computational models, leading to discovery of potential inhibitors. Wee et al.¹⁷² utilized molecular docking, MD simulations, and an inhibitor assay of several flavonoids against xanthine oxidase. Their computational models predicted kaempferol and apigenin to be strong binders to the target enzyme, which was validated with multiple in vitro experiments.¹⁷² In another hybrid study, the authors identified a potential inhibitor against a mutated signalling protein common in various cancers (BRAF).¹⁷³ They used computational techniques and in vitro validation to discover an improved inhibitor compared to one that was FDA-approved, vemurafenib.¹⁷³ These examples demonstrate the potential of bioinformatics and structural biology through the developments made in DED therapeutics, identifying potential inhibitors and their mechanisms of action. While our study did not correctly infer the flavonoid therapeutic and its direct target through computational means, the study did discover a flavonoid therapeutic (rutin) and protein target (IL-17A) amongst thousands of other molecules and many pro-inflammatory proteins through computational techniques.

Naturally found in plant-based foods (i.e. fruits and vegetables),¹⁶¹ rutin is well-recognized for its anti-inflammatory activity.¹⁷⁴ However, the only mechanism that has been confirmed is the inhibition of μ -calpain.¹⁷⁵ This thesis contributes to existing literature by discovering another novel anti-inflammatory mechanism of action of rutin, which is through the direct inhibition of IL-17A. Rutin supports other physiologic effects in addition to anti-inflammation. These other direct targets of rutin inhibition include histone deacetylase 6, which prevents deacetylation of α -tubulin to improve axonal transport,¹⁷⁶ and both acetylcholinesterase and butyrylcholinesterase to prevent development of pathogenic amyloid plaques in dementia

and Alzheimer's disease.¹⁷⁷ Lastly, rutin was also reported to prevent blood clot formation by directly inhibiting protein disulfide isomerase.¹⁷⁸ More studies on the mechanisms of action of rutin is needed to fully understand its effect on the body. This thesis studied the cytotoxicity of rutin in normal HCECs and determined that concentrations < 3 mM were likely to not be cytotoxic, consistent with previous studies.^{164,165} While many safety studies focused on flavonoid consumption, these results indicate rutin may be safe for topical application on the ocular surface.

A challenge that was encountered in this experiment was that luteolin and rutin were largely insoluble and required up to 61% DMSO to fully solubilize in an aqueous solution. While DMSO is still a commonly used solvent,^{179,180} future work will aim to implement alternative methods for solvating flavonoids. For example, encapsulating flavonoids within liposomes have been demonstrated to enhance their stability and bioavailability in solution.^{181,182} This study also found that DMSO demonstrated an intrinsic ability to inhibit cytokine-receptor interactions. DMSO is a commonly used solvent to solubilize nonpolar molecules in aqueous solutions,^{179,180} but its ability to bind and inhibit enzymatic reactions may confound study results.^{183,184} To address this issue in the future, DMSO-perturbing assays described by Tomohara et al.¹⁸⁵ could be used to identify whether DMSO is competing with the inhibitor to further understand the mechanism in the presence of solvent. It is also possible that aggregation of the flavonoid may contribute to reduced inhibitor activity, which could potentially be mitigated using a small amount of detergent in solution preparation.¹⁸⁵ Despite the ability of DMSO to inhibit receptor interaction, this work found that rutin provided significant inhibition in addition to that contributed by DMSO.

While the long-term goal is to formulate rutin into an ophthalmic drop for treating DED, this work is only foundational and preliminary. An important next step toward this goal includes testing the effect of rutin in an IL-17A-induced inflammation cell culture model. Gaining a better understanding of rutin and IL-17A receptor interaction and its ability to mitigate inflammation in cell culture models would be an important prerequisite for eventual animal studies. Concurrently, improved solvation and delivery of rutin must be considered. Current DED anti-inflammatory drugs are not optimal solutions for long-term treatment with adverse side effects and lack of long-term clinical studies.^{41,94-96,186} This thesis aimed to fill this gap by using in silico/in vitro drug discovery techniques. Overall, the results of this thesis suggest that rutin, a common flavonoid,¹⁶¹ could inhibit IL-17A, an important hub protein in the pathogenesis of DED. However, the long-term effects of rutin are still needed to be studied. Thus, this thesis has provided a foundation for developing an ophthalmic drop that may break the vicious circle of inflammation associated with DED.

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Appendix A

A.1 Supplementary Code for Compound Filtering

The follow code is the Python script utilized to filter the final enrichment analysis export file from ToppFun functional enrichment analysis tool for search results exported from PubChem and CTD.

```
1 #input ToppFun export file
2 filename = 'ToppGene_input.csv'
3
4 #define filter function
5 def filter(filename):
6
7     #databases:
8     #from <http://ctdbase.org/tools/batchQuery.go>
9     CTD_file = 'CTD.csv'
10    #list of CTD IDs
11    CTD_ID = []
12
13    #from <https://pubchem.ncbi.nlm.nih.gov/>
14    CID_file1 = 'CID_compound_polyphenol.csv'
15    CID_file2 = 'CID_compound_flavonol.csv'
16    CID_file3 = 'CID_compound_flavonoid.csv'
17    CID_file4 = 'CID_compound_flavone.csv'
18    #list of CID IDs
19    CID_ID1 = []
20    CID_ID2 = []
21    CID_ID3 = []
22    CID_ID4 = []
23    CID_ID = []
24
25    import csv
26    #process CTD files
27    with open(CTD_file) as f:
28        reader = csv.reader(f)
29        for row in reader:
30            if row[2] not in CTD_ID:
31                CTD_ID += [row[2]]
32
33    CTD_ID = CTD_ID[1:]
34    print(CTD_ID)
35
36    #process PubChem files
37    #CID1
38    with open(CID_file1) as f:
39        reader = csv.reader(f)
40        for row in reader:
41            if row[0] not in CID_ID1:
42                CID_ID1 += [row[0]]
43
44    CID_ID1 = CID_ID1[1:]
45    CID_ID += CID_ID1
46
```

```

47     #CID2
48     with open(CID_file2) as f:
49         reader = csv.reader(f)
50         for row in reader:
51             if row[0] not in CID_ID2:
52                 CID_ID2 += [row[0]]
53
54     CID_ID2 = CID_ID2[1:]
55     CID_ID += CID_ID2
56
57     #CID3
58     CID_ID3 = CID_ID3[1:]
59     #CID3
60     with open(CID_file3) as f:
61         reader = csv.reader(f)
62         for row in reader:
63             if row[0] not in CID_ID3:
64                 CID_ID3 += [row[0]]
65
66     CID_ID3 = CID_ID3[1:]
67     CID_ID += CID_ID3
68
69     #CID4
70     with open(CID_file4) as f:
71         reader = csv.reader(f)
72         for row in reader:
73             if row[0] not in CID_ID4:
74                 CID_ID4 += [row[0]]
75
76     CID_ID4 = CID_ID4[1:]
77     CID_ID += CID_ID4
78
79     seen = set()
80     uniq = []
81     for x in CID_ID:
82         if x not in seen:
83             uniq.append(x)
84             seen.add(x)
85
86     outfile = open("filtered.csv", "w", newline='')
87     obj = csv.writer(outfile)
88
89     with open(filename, encoding="utf-8-sig") as f:
90         reader = csv.reader(f)
91
92         for row in reader:
93             if row[0] == 'Category':
94                 obj.writerow(row)
95             elif row[2].find('ctd') == 0:
96                 ID = row[2][4:]
97                 if ID in CTD_ID:
98                     obj.writerow(row)
99             elif row[2].find('CID') == 0:
100                ID = row[2][4:]
101
102                if ID in CID_ID:
103                    obj.writerow(row)

```

```

104
105     outfile.close()
106
107 filter(filename)

```

A.2 Supplementary Code for Cytoscape Data Restructuring

The supplementary Python code provided was utilized to reformat the ToppFun export file data structure for the data structure of edge interactions in Cytoscape v 3.9.1..

```

1  def edge(filename):
2
3      import csv
4      f = open(filename)
5      read = csv.reader(f)
6      fout = open("edge_interactions.csv","w", newline = '')
7      obj = csv.writer(fout,delimiter='\\t')
8
9      ref = []
10
11     for row in read:
12         if row[0] == 'Category':
13             header = row[1:4]
14             ref = row
15             header.extend(["Type", "Interaction","Protein","Type","p-Value"])
16             obj.writerow(header)
17         else:
18             num = 5 # starting protein
19             max_num = len(row) #length of list
20
21             while num < max_num:
22                 if row[num] == "1":
23                     edge = row[1:4]
24                     edge.extend(["compound"])
25                     edge.extend(["cp"])
26                     edge.extend([ref[num]])
27                     edge.extend(["Protein"])
28                     edge.extend([row[4]])
29                     obj.writerow(edge)
30                     num += 1
31                 else:
32                     num += 1
33
34     fout.close()
35     print(header)
36
37     edge("filtered.csv")

```