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The Effect of Wild Blueberry Anthocyanins and Phenolic Acids on Innate Immunity: The Compliment System

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THE EFFECT OF WILD BLUEBERRY ANTHOCYANINS AND PHENOLIC ACIDS ON INNATE IMMUNITY: THE COMPLEMENT SYSTEM

By

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An Abstract of the Thesis Presented

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Ingestion of berries containing polyphenols is associated with lower risk of inflammatory, metabolic, cardiovascular and degenerative diseases. Diet has been shown to modulate the activation of the complement system, a set of over 50 proteins present in the circulation and tissues that reacts in response to damage or microbial encounter and is critical for the maintenance of homeostasis. Imbalanced activation is tightly correlated with inflammation and various pathologies. Wild blueberries are a rich source of anthocyanins and phenolic acids, which can be found in plasma shortly after consumption. Given the involvement of both complement and polyphenols in the modulation of inflammation, we investigated whether wild blueberries modulate the activation of the complement system. Phenolics (Phen) and Anthocyanins (ACNs)

were extracted from freeze-dried wild blueberry powder, characterized by liquid chromatography and used in in-vitro complement inhibition assays. We documented that Phen and ACNs inhibit the activation of the complement classical pathway in a dose-dependent manner with IC_{50} of 325.6μg/ml and 605.6μg/ml respectively. The activation of the alternative pathway was not impacted by the bioactives. Phenolic metabolites syringic, protocatechuric, gallic, chlorogenic, and hippuric acids also showed inhibitory activity with an IC_{50} of approximately, 1mM. Mechanistically, we determined that polyphenols impact specifically the complement classical pathway by targeting the activation of complement protein C4 through the C1s enzyme. This study presents novel data on the inhibition of the complement classical pathway by phenolic compounds extracted from wild blueberries, shedding new light on the anti-inflammatory properties and potential health benefits of berry consumption.

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CHAPTER 1

INTRODUCTION

Inflammation is a non-specific and complex immune response which despite being essential in its early stages, can be detrimental in later studies. Prolonged and unresolved inflammation is associated with a plethora of chronic diseases and complications (Makki et al. 2013, Samad et al. 2013).

Complement is a sophisticated system, an important part of innate immunity and an orchestrator of immunity in general. Complement functions are not only limited to host defense against microbial invaders, but expand to closely collaborate with other biological systems (Leslie 2012, Ricklin et al. 2010). This system acts immediately and effectively protecting the host organism especially during the early stages of inflammation. However, unbalanced activity of the complement has been linked to many pathologies (Ricklin & Lambris 2013). Therefore, proper complement function ensures homeostasis and health. Another of its functions is the association with chronic inflammation, metabolic-related diseases such as fatty liver, obesity, diabetes, and cardiometabolic diseases (Phieler et al. 2013, Hertle et al. 2014).

Lowbush, wild blueberries (*Vaccinium ang*.) are among the fruits and vegetables with the highest antioxidant activity and the highest concentration of anthocyanins (ACNs) (Prior et al. 1998, Hosseinian & Beta 2007). Anthocyanins and other polyphenols in these species, have been documented to be anti-inflammatory, and have antioxidant and cardioprotective properties (Tavernitti et al. 2014, Del bo' et al. 2016, Kristo et al. 2010, Kalea et al. 2006, Vendrame et al. 2015).

Recent evidence supports that diet can affect complement activity (Doerner et al. 2016, Phieler et al. 2013) however, there is paucity in studies which relate polyphenols to complement activity,

with a few studies documenting a negative correlation between polyphenols and complement activation (Ho et al. 2014, Shahat et al. 1996, Kosasi et al. 1989).

Thus, the goal of this study is to evaluate the effect of Anthocyanins (ACNs) and Phenolic acids (Phen) extracted from wild blueberries on the complement system and its related pathways (Classical and Alternative), as well as their potential mechanism of action.

The objectives are:

1. Whether ANCs and Phen activate or inhibit the Classical and Alternative pathways of complement system and
2. Explore the mechanism of their action on complement proteins (C4) and/or proteases (C1s).

This is the first study to examine polyphenols found in wild blueberries and their metabolites on the complement system of innate immunity and is novel in the field of nutrition. Wild blueberries have been extensively studied the past two decades in relation to chronic diseases and efforts have been made to unravel their effect on immunity, chronic inflammation and diseases related to inflammation. Thus, understanding their role on an important system as the complement, can enhance our understanding towards their anti-inflammatory effects. Additionally, understanding their mechanism(s) of action could help us use them not only for the prevention but also for the modulation of complement-related pathologies. Since they are natural compounds, their moderate consumption may be accompanied by beneficial effects without the adverse effects that usually go hand-in-hand with pharmacotherapy. Moreover, this study is not only beneficial to the scientific community, but to Maine wild blueberry commodity groups, in that Maine is the major producer of wild blueberries worldwide.

CHAPTER 2

LITERATURE REVIEW

2.1 Innate and Adaptive Immunity

Innate immunity constitutes the first line of defense against pathogen intruders, being activated immediately to defend all multicellular organisms. In contrast, the adaptive immune system (or acquired) responds days after an invasion, and antigen-specific lymphocytes such as T and B cells are responsible for the stimulation and expression of this type of immune response (Wherry and Masopust 2016). Lymphocytes are generated in the bone marrow by stem cells (Wherry and Masopust 2016). Two distinctive categories of adaptive immune responses exist, with lymphocytes being the protagonists in both. In humoral immunity, B cells secrete antibodies (immunoglobins) that bind to the antigen of viruses, neutralizing them. During cell-mediated responses, T cells spot and directly kill virus-infected host cells (Alberts et al. 2002). Immunological memory is a distinct characteristic of adaptive immunity along with diversity and antigen-specific responses (Alberts et al. 2002). Despite the fact that vertebrates rely on innate immunity for rapid responses, this system does not generate lasting protective immunity and has limited antigen-specificity. In particular, the innate response includes the immediate recognition and attack of various classes of pathogen-associated immunostimulants which are non-specific and common molecules that appear in a repeated pattern on invading pathogens (Alberts et al. 2002). Innate immunity is mainly composed of phagocytic leukocytes, dendritic cells, natural killer cells (NK), epithelial barriers and plasma proteins. Such structures that behave as immunostimulants are the CpG motif and various lipopolysaccharides (LPS) (Hajishengallis and Lambris 2010). Innate immunity has a crucial role defending the organism the first days of an infection, due to the delayed activation of the adaptive immune system. Eventually, the innate responses along with the pathogen intruders

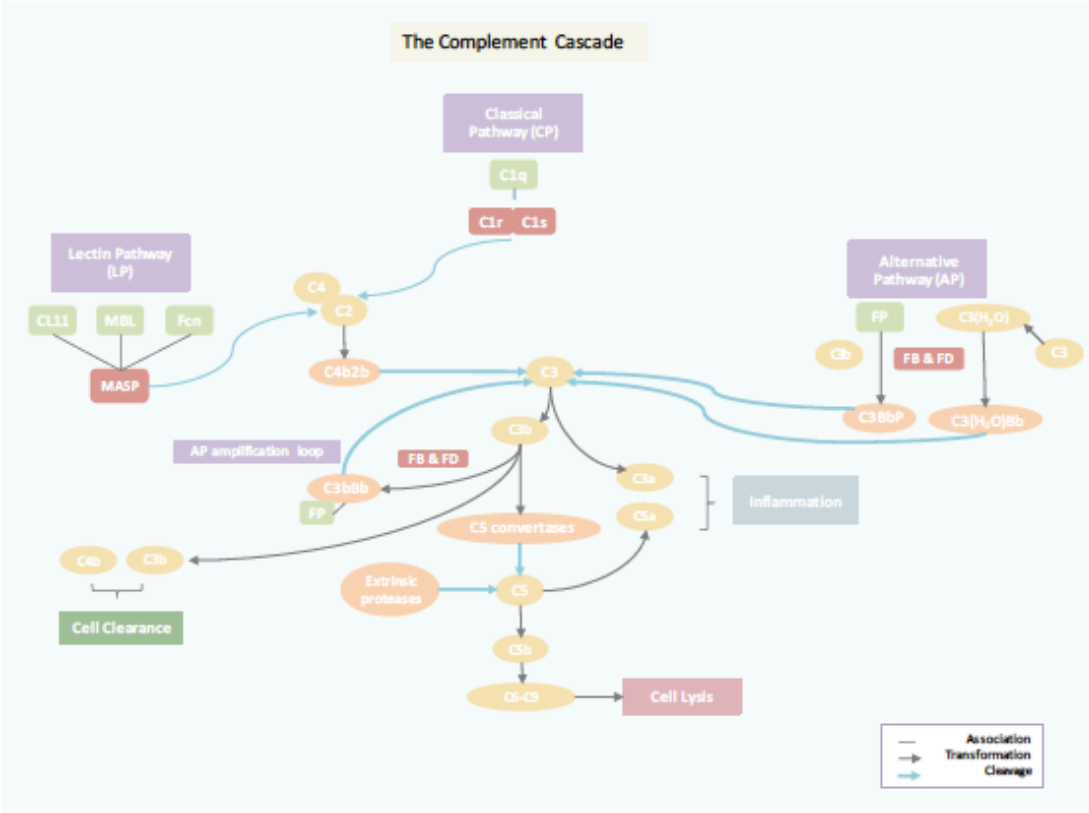
lead to the stimulation of adaptive immunity which in turn enhances defense against infections (Alberts et al. 2002). Recent evidence affirms that there is a significant crosstalk between the two arms of the immune system and that innate immunity plays a significant role instructing the adaptive immune responses (Phieler et al. 2013). An example of collaboration between the two immune systems, is the ability of B cells to mark specific pathogens, leading to their consumption by phagocytic cells (Alberts et al. 2002). Therefore, innate immunity may affect the development of long term specific immunity and autoimmune disease (Alberts et al. 2002).

2.2 The Complement System: Role, Pathways, Activation

The complement system is composed of at least thirty proteins, soluble or membrane-bound receptors and regulators which are mainly produced by hepatocytes in the liver and are found in the circulation, extracellular fluid and cell surfaces. They act as a cascade and ensure protection of the host against microbial intruders (Mastellos et al. 2003). Contrary to previous beliefs, plethora of evidence suggests that the role of the complement system is not limited to being the first line of defense against invading microbes. In fact, complement controls and partakes in a multitude of biological processes including but not limited to tissue regeneration, lipid metabolism, coagulation, modulation of cell responses, bacterial opsonization, clearance of cell debris and initiation of inflammation. It bridges innate and acquired immunity and crosstalks with other physiological systems (Ricklin et al. 2010). Thus, complement is fully working through constant collaboration with other biological systems and is not limited to just fighting microbial intruders (Ricklin et al. 2016). The activation of complement's proteolytic cascade is achieved through three distinct pathways: the classical (CP), alternative (AP) and lectin (LP) pathways. The cascade is regulated tightly and includes controlled proteolytic cleavage of the complement proteins (Markiewski & Lambris 2007, Ricklin et al. 2016).

As Figure 1 shows, the classical pathway (CP) involves complement component C1q. This subcomponent of C1 complex has the ability to recognize and bind to antigen-antibody complexes and activate the serine proteases C1r and C1s which in turn cleave complement proteins C2 and C4. C1s (Complement serine protease 1) is an enzyme associated with C1q and C1r. Literature suggest C4 is a complement protein (200 kD) which consists of a thioester bond and three peptide chains (α. β, γ) connected by disulfide bonds. Complement component 4 (C4) is eventually cleaved (by serine protease C1s) into its fragments C4a (9 kD) and C4b (195 kD). C4a is cleaved off the N-terminal of α-chain generating C4b. Then, C4b along with C2a participate in the formation convertase C4b2b (C3 convertase). When C4 is not cleaved, the α-chain is intact, while when it gets cleaved, α-chain gets activated (activated form: α'-chain) (Gigli et al. 1997, He and Lin 1998, Schifferli JA and Paccaud J 1989, Ricklin et al. 2016).

Figure 1: **The complement cascade**



(Modified version from Ricklin et.al, Nat Rev Nephrol. 2016 Jul;12(7):383-401. doi: 10.1038/nrneph.2016.70)

Regarding the lectin pathway (LP) (Figure 1), mannose-binding lectin (MBL) is another pattern recognition molecule structurally similar to C1q. Ficolin (FCN) and and collectin (CL-11) are pattern-recognition molecules (PRMs) as well, and along with MBL can identify high density mannose on pathogen surfaces and activate MBL-associate serine proteases (MASPs). Upon activation, MASPs cleave C2 and C4 (similar to classical pathway) which leads to the creation of C3 convertase (C4b2b) and the activation of C3 (Phieler et al. 2013, Ricklin et al. 2016, Markiewski and Lambris 2007).

The alternative pathway (AP) differs from the classical and lectin pathways and is considered as the pathway with the biggest effect on complement activity (approximately 80%) (Ricklin et al. 2016). In fact there are different ways of activation. The cascade is stimulated by rapid hydrolysis (tick-over) of the thioester bond of the complement protein C3 into C3(H2O) and the binding of hydrolyzed C3 to factor B (FB). Upon this binding, factor D (FD) activates FBC3(H2O). This results in the formation of the C3(H2O)Bb complex which leads to the initial cleavage protease of C3 (Ricklin et al. 2010, Ricklin et al. 2016, Markiewski & Lambris 2007). Complement protein C3 continues to become activated by C3(H2O)Bb leading to more C3b production, initiating an amplification loop (characteristic of the AP). Properdin or factor P (FP) is a pattern recognition molecule, which can bind to several host cells and microorganisms, enhancing deposition of C3b and C3bBb assembly (Cortes et al. 2012). It also tailors AP and promotes stability of C3 convertase, feeding the amplification loop (Ricklin et al. 2016, Alcorlo et al. 2013). Factor P has been categorized as a positive complement modulator (Ricklin et al. 2016).

As seen in Figure 1, all three pathways lead to the cleavage of the central protein of the complement system C3 into C3a and C3b (Ricklin et al. 2010, Markiewski and Lambris 2007). C3a is an anaphylatoxin along with other complement effectors (C5a, C4a). C3b is an opsonin which

deposits on cell surface and forms the C5 convertase that activates complement protein C5, a central molecule and player of the complement cascade. C5 fragment, C5b interacts with complement proteins C6, C7, C8, C9 which bind to cell membranes forming the Membrane Attack Complex (MAC). MAC enters the membrane and can lead to cell lysis (Ricklin et al. 2016).

Cp40, is a fourteen amino-acid synthetic cyclic peptide which belongs to the group of compstatin peptides which are C3 complement protein inhibitors. (Mastellos et al. 2015). This compstatin analogue, inhibits complement activation in human and non human primates acting on complement protein C3, inhibiting its cleavage into C3a and C3b (Mastellos et al. 2015). In April 2017, the first-in-human clinical trial began in healthy male volunteers, testing Cp40 (AMY-101, sponsored by AMYNDAS pharmaceuticals) (NIH 2018).

Complement system activation is not limited only to the ways described above. Frequently, extrinsic proteases such as thrombin can act directly on C3 and C5 activating them (Ricklin et al. 2016). Imbalance of the mechanisms of activation and modulation of the complement system has dramatic effects. Insufficient activation is associated with pathogenesis of diseases, while excess activation may lead to host injury and initiation of inflammation (Ricklin & Lambris 2013).

2.3 Complement System and Chronic Diseases

Adipose tissue, liver and vasculature are key contributors of chronic diseases and systemic complement levels. Excessive caloric intake, unhealthy diet and lifestyle along with genetics can induce inflammation, insulin resistance and deregulation of metabolism in adipose tissue leading to central obesity. Insulin resistance, deregulated metabolism, inflammation and injury are noticed in liver tissue as well (which is the central source of complement) (Hertle et al. 2014).

Additionally, diet can act on vasculature inducing inflammation, endothelial dysfunction, hypertension and atherosclerosis. All the above lead to the state of metabolic syndrome (MetS)

(Hertle et al. 2014). These phenomena affect the complement system by either activating or inhibiting its pathways. There is local complement production in adipose tissue, liver and vascular tissue. Specifically, in vasculature, variation in levels of complement, may lead to endothelial activation, coagulation, attraction and assembly of immune cells or debris removal. On the adipose tissue, complement dysregulation can lead to (or worsen) inflammation which is associated with insulin resistance, hypoxia and adipose necrosis. Finally, in the liver, complement proteins and receptors may have both beneficial or adverse effects, modulating insulin resistance and inflammation which may lead to hepatic injury (Hertle et al. 2014).

All these document that complement cross talks with several organs and metabolic sites interfering with complex metabolic processes. The immune response in several tissues such as the adipose, endothelial and liver promote the establishment of chronic inflammation and related pathologies. Additionally, the above conditions illustrate that diet and lifestyle have a pivotal effect on complement regulation and immune homeostasis. (Hertle et al. 2014, Phieler et al. 2013, Lafontan 2014).

In general, lifestyle such as good quality diet and exercise may influence complement activity, promoting a healthier and more balanced anti-inflammatory profile (Hertle et al. 2014). Recent evidence links diet to chronic inflammation and specifically the formation of intestinal polyps. Specifically, corn oil (mainly consisted of polyunsaturated fatty acids – 61.5%) and coconut oil (consisted of 99.1% saturated fatty acids) were found to induce intestinal neoplasia in genetically susceptible male mice through the activation of C5a complement anaphylatoxin, other inflammatory mediators (neutrophil infiltration, NF-κB, IL-1B, IL-6, MCP-1, TNF-a, etc) and expression of pro- oncogenes which together create a pro-inflammatory environment (Doerner et al. 2016). High fat diets which consist of fatty acids other than monounsaturated have the ability

to cause tumor genesis before the onset of obesity. In contrary, high fat diet based on olive oil (which is composed mainly of monounsaturated fatty acids) did not lead to the above condition (Doerner et al. 2016).

Recent evidence documents that the upregulation of the chemoattractant C5a in the hepatocytes following a four-week HFD diet results in stimulation of neutrophils along with increased MPC-1 expression, and the accumulation of leukocytes in the femoral artery in wild type mice. This evidence supports the role of C5a in the development of atherosclerosis. (Osaka et al. 2016).

2.4 Relationship of the Complement with Acute and Chronic Inflammation

Inflammation is a pivotal part of our body's immune system and the hallmark of several diseases. Despite the fact that acute inflammation is an essential mechanism because it defends the host against microbial intruders, identifies debris and apoptotic cells and augments injury repair, unresolved and chronic inflammation may have detrimental effects on the organism. In particular, low grade systemic inflammation is tightly associated to several chronic pathological conditions such as the metabolic syndrome (MetS), cardiovascular disease (CVD), diabetes mellitus, insulin resistance and cancer (Manach et al. 2002, Makki et al. 2013, Samad and Ruf 2013).

The complement and the inflammatory cascades work hand in hand. Once the immune system becomes triggered by endogenous or exogenous stimuli, complement acts immediately and effectively (Janeway et al. 2005). The fast response of the complement whenever there is cell injury and immune stimulation is attributed not only to the antigen-antibody complex but to specific pattern recognition receptors as well (Kohl 2006). During acute inflammation, tissue injury stimulates innate immunity and the complement and thus initiates the expression of a series of inflammatory mediators. Meanwhile, complement effectors C3a and C5a operate as anaphylatoxins (Complement Activation Fragments). They actively participate in the

inflammatory process inducing and enhancing vascular changes. In particular, they lead to increased vascular flow and permeability along with leukocyte extravasation and chemotaxis. C5a is a particularly effective chemoattractant and macrophage regulator (Marder et al. 1985, Dreyer et al. 1991). Complement activation fragment C5a promotes inflammation and is linked to a variety of pathologies such as degenerative diseases, sepsis and arthritis (Ricklin 2013).

The anaphylatoxins C5a and C3a interact with and bind to their mutual receptors C5aR and C3aR which are expressed by inflammatory and endothelial cells. This results in additional release of inflammatory mediators such as eicosanoids and cytokines which induce changes in the microvasculature (increase of permeability, vasodilation leukocyte extravasation, chemoattraction). Moreover, the binding of the anaphylatoxins to their receptors in mast cells, induce the release of proteases and histamine which enhances vascular permeability with implications in LDL (low density lipoprotein) binding and transmigration to the endothelium (Markiewski and Lambris 2007).

Complement participates in all parts of inflammatory sequel. Specifically, its role is protective during the early stages of inflammation or infection (Tichaczek-Goska 2012). However, if the homeostasis of complement regulation becomes disturbed by either excessive or deficient activation, it is the reason for immunodeficiency, autoimmune and inflammatory disorders and diseases, as well as infections (Ricklin and Lambris 2013, Ricklin et al. 2016). These lines of evidence help us understand how the involvement of complement in inflammation can lead to several chronic pathological conditions.

As mentioned earlier, complement crosstalks with several biological systems. It has the ability to identify host versus intruder cells, debris and apoptotic cells, responding each time according to perceived danger. It harmonically communicates with other lines of immunity to minimize threats,

supervising the immune system (Ricklin et al. 2010, Leslie 2012). Thus, we understand that it plays an important role in the development of pathological conditions and the inflammatory sequel. The complement system has been linked to several chronic diseases derived from chronic low-grade inflammation such as; metabolic syndrome, obesity, cardiovascular disease, diabetes and fatty liver. Leukocyte accumulation is the hallmark of vascular inflammation and each of the three pathways of complement system has unique role on chronic inflammation and the development of cardiovascular disease (CVD). Classical and Alternative pathway activation is associated to CVD, while Lectin Pathway activation is linked to anti-atherogenic effects (due to low MBL plasma levels or genotypes) (Hertle et al. 2014). Furthermore, complement does have dual role; protecting against endothelial debris assembly but triggering the recruitment of white blood cells on the site of injury as well (Hertle et al. 2014, Speidl et al. 2011).

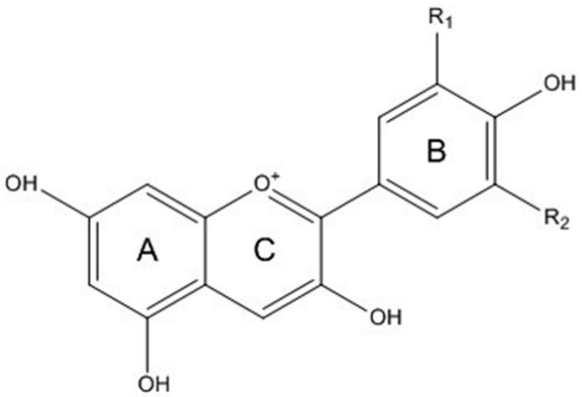
2.5 Wild Blueberry Bioactives (*Vaccinium angustifolium)*

Wild blueberries (lowbush) are Maine's native berry. They grow mainly on Northeastern US and Canada where the soil is acidic. This species does not require special treatment and can flourish on its own (USDA, 2013). The leaves are green, the flower is white and the fruit small dark blue/purple (Hokanson and Hancock 1993). Polyphenols are secondary plant metabolites and are classified as flavonoids and non-flavonoids. They are widely distributed in the plant kingdom. All phenolic compounds consist of one or more hydroxyl (-OH) group(s) attached to a benzene aromatic ring (Dai and Mumper 2010, Bravo 1998). ACNs are a group of flavonoids found in abundance in the *Vaccinium* species (Upton 2001) which include among others, blueberries, cranberries, bilberries, pomegranates. They are water soluble pigments and the most well-known are delphinidin, pelargonidin, petunidin, malvidin, peonidin and cyanidin (Andersen and Jordheim

2006). It has been documented that wild blueberries are the most abundant in polyphenols and in particular anthocyanins (Hakkinen & Torronen 1999, Hosseinian & Beta 2007).

As Figure 2 shows, aromatic ring A has two hydroxyl groups and is attached to heterocyclic C which has both a hydroxyl group and oxygen atom. In turn, ring C is connected thru a C-C bond to the third aromatic ring B. This ring consists of a hydroxyl group and two side chains (R1,2). Depending on whether R1 and R2 are hydrogens, hydroxyls, methoxyls or a combination of them, different anthocyanidins occur.

Figure 2: **Structure of ACNs**



Anthocyanidin	R_1	R_2
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Malvidin (Mv)	OCH_3	OCH_3
Pelargonidin (Pg)	H	H
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OH	OCH_3

(Kamiloglu et.al Int. J. Mol. Sci. 2015, 16(9), 21555-21574; doi:10.3390/ijms160921555)

Previous analyses of wild blueberry powder has revealed a range of ACN concentrations: 1.56+0.10mg/ml (Tavernitti et al. 2014), 29.9+5.2mg/ml (Del Bo et al. 2016) and phenols 0.71+0.02mg/ml (Tavernitti et al. 2014). The individual anthocyanins that are predominant on each HPLC analysis may vary due to changes in growing conditions or cultivars. Del Bo et.al found in one study Mv-3gal and Pn-3glc (Del Bo et al. 2010) to be predominant and in another Mv-glc was the most abundant followed by Dp-glc and Cy-glc (Del Bo et al. 2016).

Tavernitti et.al 2014, recorded higher amounts of Mv-glc, Dp-glc and Cy-glc. Several parameters such as pH, enzymes, oxygen, temperature and light fluctuations can potentially alternate the stability and consistency of anthocyanins (Fernandes et al. 2014).

Storage of freeze-dried wild blueberries at 25°C seems to be the least damaging, preserving the content and the quality of anthocyanins (Fracassetti et al. 2013). It is estimated that the average daily consumption of anthocyanins ranges from 180 to 215 mg (McGhie and Walton 2007). The pH of the stomach, the activity of digestive enzymes and gut microbiota, lead to immediate biotransformation of anthocyanins upon consumption (De Ferras et all. 2014, Faria et al. 2014). The glycoside forms of ACNs do not get absorbed in the stomach and instead continue their journey on the gastrointestinal track (GI) until they reach the small intestine where they are being absorbed (Talavera et al. 2004). Moreover, ACNs undergo constant enterohepatic recycling suggesting that they are more bioavailable than previously thought (Lila et al. 2016).

Microbial metabolism in colon is also crucial, affecting significantly the bioavailability of polyphenols (Czank et al. 2013, Cardona et al. 2013). Approximately 90-95% of consumed polyphenols remain unabsorbed) and accumulate in high concentrations in the intestinal tissues where they undergo extensive transformation leading to the polyphenol metabolites (Cardona et al. 2013, Bowey et al. 2003, Romier et al. 2009). In particular, unabsorbed anthocyanin glycosides are transformed by the intestinal environment and the colonic bacteria into aglycones and phenolic acids (Fernandez 2014). A common (for Phen and ACNs) metabolite is the hippuric acid, which was found to be predominant in urine of animals and humans suggesting that it may be a metabolic product of processes in the hepatic tissue, therefore, organs such as the liver may contribute to the metabolism and the biotransformation of these bioactives (Del'Bo et al. 2010).

Anthocyanins have been found in organs such as the stomach, jejunum, liver, kidney, eye and brain either in native or methylated and conjugated forms (Felgines et al. 2009, Passamonti et al. 2005, Talavera et al. 2005). Cyanidin-3-glucoside has been detected in adipose tissues (Felgines 2009) and protocatechuic acid (PCA) is its most significant metabolite (70% of total absorbed

anthocyanins in the intestines) (Vitaglione et al. 2007). Contrary to the traditional notion that anthocyanins have low bioavailability (Manach et al. 2005), recent evidence suggests the opposite (Czank et al. 2013, De Ferras et al. 2014, Lila et al. 2016). Moreover, it has been shown that ACNs are found in tissues or circulation in their modified (methylated, sulfated or glucuronidated) forms (Kay et al. 2009, Del Bo et al. 2016).

The detected concentration of metabolites in plasma ranges from 0.01 to 2 μM (De Ferras et al. 2014). Increased cellular uptake, metabolism and excretion may be the reason why only 1% of ingested ACNs has been detected in plasma. (Fernandes et al. 2014, Manach et al. 2005).

For the current study, gallic, protocatechuic and syringic acids were utilized because, they are metabolites of single anthocyanins Dp (delphinidin), Cy (cyanidin) and Mv-3-O-glucosides (malvidin) respectively and have been studied in the past *in-vitro* in HUVECs (Human Umbilical Vein Endothelial Cells) and instestinal epithelial caco-2 cells (Del Bo et al. 2016, Taverniti et al. 2014). Chlorogenic acid was chosen is the most predominant phenolic acid based on previous HPLC studies, and hippuric acid was used because it is a common metabolite of phenolic acids and anthocyanins (Del Bo et.al 2016, Taverniti et.al 2014, De Ferras et.al 2014). Chlorogenic acid is an ester of caffeic and quinic acid (Olthof et al. 2001). In this study, protocatechuic acid was preferably used because its parent metabolite cyanidin, has been extensively studied in pharmacokinetic studies (Pace et al. 2018, Warner et al. 2017, De Ferras et al. 2014, Czank et al. 2013).

2.6 Wild Blueberries (Vaccinium angostifolium) and Chronic Inflammation

A series of in vivo and in vitro studies assert that phenols are effectors of the inflammatory status shifting it towards an improved and more balanced state. In particular, they can modulate the expression of various adhesion molecules and anti-inflammatory cytokines (Vendrame et al.

2015). Research in our laboratory has documented that consumption of wild blueberries (8% w/w) for eight weeks can have an anti-inflammatory effect through the downregulation of nuclear factor kappa beta (NFkB), asserting a protective effect against the Metabolic Syndrome (MetS) (Vendrame et al. 2013). These bioactives act also on mitogen-activated protein kinases (MAPK) inhibiting their pathway of action (Vendrame et al. 2015). Additionally, it has been found that they can protect lymphocytes against DNA damage (after eight weeks consumption), displaying antioxidant properties (Del Bo et al. 2010).

Additionally, our laboratory has documented their ability to beneficially influence the contractile machinery and the structural properties of the aortic smooth muscle cells in both hypertensive and Sprague –Dawley rat models by preserving (Nitric Oxide) NO bioavailability (Norton et al. 2005, Kalea et al. 2006, Kristo et al. 2010).

Protein complex NF-kB bridges inflammation and oxidative stress. In particular, its activation induces the expression of plethora of pro-inflammatory and pro-oxidative factors, inhibiting at the same time the expression of anti-inflammatory molecules such as adiponectin. Anthocyanins inhibit the above cascade affecting the NF-kB translocation and activation, and they can also act inhibiting nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) expression. This beneficial activity of the bioactive compounds found on wild blueberries attenuates the Nf-kB signaling. (Vendrame et al. 2013, Vendrame et al. 2015).

In-vivo studies support the *in-vitro* findings regarding the way of action of anthocyanins. anthocyanins target NF-κB and MAPKs pathways and inhibit overexpression of pro-oxidative and inflammatory mediators such as COX-2 (inhibiting production of PGE2), iNOs (inhibiting production of NOs) (Hwang et al. 2011, Min et al. 2010, Kwon et al. 2009, Vendrame et al. 2015).

2.7 Phytochemicals and the Complement System

There is a limited number of studies which examine the effect of ACNs and Phen on the complement system. Ho et al. 2014, examined polyphenols extracted from *Aronia Melanocarpa* on classical and alternative pathways. Specifically, they found that cyanidin aglycone, procyanidins B1, B5 and C1 along with proanthocyanidin fractions extracted from *Aronia Melanocarca* inhibit complement activation (Ho et al. 2014). This study raises some issues of ambiguity as far as data interpretation is concerned, in that there is lack of complement-specific controls in the hemolytic assays. However, the results and graphs presented might be indicative of complement inhibitory activity in the Aronia's extracts.

Another study conducted in 1996 also documented that procyanidin C1 and proanthocyanin A1 extracted from Crataegus sinaica were more active inhibiting the Classical pathway and less active inhibiting the Alternative pathway (Shahat et al. 1996).

Kosasi et al., in 1989 reported the inhibitory activity of *Jatropha Multifida Latex* proanthocyanidin extracts. The extract was more active inhibiting the Classical versus the Alternative pathway. Catechin was the major constituent of the extract and its anti-complement effect was attributed to Ca+2 chelation (Kosasi et al. 1989). The above studies reveal the potential of bioactive compounds found on the different berries on the complement system and its activation. Since there is paucity of research in this area and no studies published on the possible effect of wild blueberry bioactive compounds on the complement system, the goal of this project is to evaluate the effect of ACN and/or Phen extracts found in wild blueberries on complement system and its known pathways.

This study is significant because it is first time that the relation between bioactives in wild blueberries and their possible effect on the complement system is investigated.

CHAPTER 3

MATERIALS/METHODS

3.1 Wild Blueberries

3.1.1 Wild Blueberry powder

Wild Blueberries were provided as a composite from Wyman's (Cherryfield, ME), freeze-dried and vacuum-packed by FutureChemicals (Momence, IL) and stored in the dark at -20 °C until use.

3.1.2 Phen- and ACN-rich fractions extraction

For the extraction, 100mg of lyophilized wild blueberry powder (stored in -80°C) and 10ml water was vortexed and centrifuged at 5000rpm for 10 minutes at 4°C. The supernatant was removed and then was added to a Strata cartridge (initially equilibrated with 5ml methanol and 5ml water) C18-E. It then eluted and the eluate was discarded. Two ml of HCl (0.01M) was added to the column to elute the water soluble fraction. The addition of 2ml ethyl acetate resulted in the Phen-rich fraction and the further addition of 2ml acidified ethanol (0.1% HCl) resulted in the ACN-rich fraction. The fractions underwent lyophilization under nitrogen steam and were stored at -20°C until use. When ready to use, they were reconstituted with mQ water. This protocol is a modified version described by Wrolstad (Wrolstad et al. 2015).

3.1.3 Phen- and ACN-rich fraction analysis

The specific ACN and Phen profile of the extracts from the wild blueberries were analyzed by high performance liquid chromatography (Alliance mod, 2695, Water, Milford, MA) at the University of Milan (Italy) according to the protocol described in Del Bo' et al. (2010).

3.1.4 ACN metabolites

Individual ACNs (Mv-glc and Cy-glc), their metabolites, syringic, protocatechuic, gallic and hippuric acids and the Phen metabolite chlorogenic acid were purchased from *Sigma Aldrich* (Missouri, USA).

The complement peptidic inhibitor Cp40 - dTyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-His-Arg-Cys]- mIle-NH2; 1.7 kDa) was used was synthesized at Dr.Lambris laboratory (University of Pennsylvania). It inhibits the cleavage of C3 complement protein into C3a and C3b and in this study it was used as a control (Mastellos et al. 2015).

3.1.5 Donor blood samples

For the experiments, human plasma was used from healthy donors. Specifically, whole blood was collected with routine phlebotomy. Plasma was centrifuged for 15min. at 2200-2500 rpm and was transferred into new aliquots. Only plasma from donors whose complement can be activated upon a challenge was used.

3.2 Immunoassays

ELISAs were performed to detect the activity of:

- ACN and Phen extracts on Classical (CP), and Alternative (AP) pathways.
- Their respective metabolites gallic, protocatechuic and syringic acids along with chlorogenic acid (the primary compound of the Phen extract) and hippuric acid (common metabolite, found in high concentrations in plasma (~2uM) (De Ferras et al. 2014).

3.2.1 ELISA

3.2.1.1 Classical Pathway (antigen/antibody complex-initiated complement activation)

Plate was coated with 50ul/well 1% Ovalbumin in PBS 1x, pH 7.4 and was incubated overnight at 4C. 50ul/wll of anti-Ovalbumin antibody UP17969 (1:1000 in PBS/BSA) was added following

incubation for 1 hour at room temperature. Serial dilutions of Cp40, Phenols and ACNs in VBS (x2 the desired final volume) were performed on ice and 30ul/well were transferred on the ELISA plate. Added 30ul/well of lepirudin plasma on the ELISA plate and incubated for 15 minutes at room temperature. 50ul/well of anti C3-HRP (1:1000 dilution in PBS/BSA) and incubated for 1 hours at room temperature were added and developed with 50ul/well of the developing buffer with ABTS substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) added. The plate was read at 405nm using photometer.

3.2.1.2 Alternative Pathway (LPS (lipopolysaccharide from E.coli) initiated complement activation)

Plate was coated with LPS 50ug/ml in PBS, pH 7.4, 50ul/well, and incubated overnight at 4C. Blocking with 200ul/ml PBS/1%BSA and 1 hour incubation at room temperature followed. Serial dilutions of inhibitors (2x the desired final concentration) in VBS were performed and transferred to ELISA plate, 30ul/well. Next, 50ul/well of the polyclonal ani-human C3 conjugated with HRP (dilution 1:1000) in PBS/BSA were added followed by 1 hour incubation at room temperature. Finally, developed with 50ul/well of the developing buffer with ABTS substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was added and the plate was read at 405nm using a photometer. The basis of these assays are also described in Schmidt et.al (Schmidt et al. 2013).

The peptidic inhibitor Cp40 was used as a positive control, with maximum concentration (for both ELISA assays) of 17.8 uM. This concentration was serially diluted to get: 8.9, 4.45, 2.22, 1.1, 0.5, 0.27, 0.13, 0.06, 0.03, and 0.01 uM.

For both Classical and Alternative Pathways, maximum concentration of:

ACN extract was 5363ug/ml (with serial dilutions of: 2681, 1340, 670, 335, 167, 83, 41, 20, 10, 5.2 ug/ml) while for Phenolic acid extract was 3944ug/ml (with serial dilutions of: 1972, 986, 493, 246.5, 123.2, 61.6, 30.8, 15.4, 7.7, 3.85 μg/ml).

Individual metabolites had a maximum concentration of 1000ug/ml each (with serial dilutions of: 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95, 0.97 μg/ml).

3.2.2 Western Blot

Effect of Protocatechuic acid (PCA) on the activation of the complement C4 protein

3.2.2.1 Test of PCA inhibition of complement protein C4 through detection of C4 alpha prime (PVDF membrane (Bio-Rad)).

Protocol was identical to that of Classical Pathway up to plasma incubation. After that, EDTA was added (2.5ul/well) and samples were collected. 7.5% polyacrylamide gel was used. Samples for Western blot combined 16ul of bioactive compounds (taken from ELISA) and 4ul of 5x running buffer (with 5ul of beta-metcaptoethanol). The samples upon the addition of running buffer were denatured at 90°C for 8min. and were then run on a 7.5% polyacrylamide gel at 100V for approximately 2 hours under denaturing conditions at room temperature. The samples were transferred on a PVDF membrane using FierceG2-Fast Blotter. The membrane was blocked with TBSTween/4% Casein followed by binding of anti-serum human C4 (dilution 1:1000 in TBST/4%Casein) overnight at 4C. Goat anti-rabbit (dilution 1:5000 in TBST/4%Casein) was bound (1 hour at 4C) followed by the addition of Western Chemiluminescent HRP substrate (Immobilon, Millipore) for development with Western Chemiluminescent HRP substrate (Immobilon, Milipore) following manufacturer's instructions. The concentration for PCA will be 1mM and for Cp40 0.3uM.

3.2.2.2 Detection of C1s-dependent C4 cleavage using PCA and C1s inhibitor(INH)

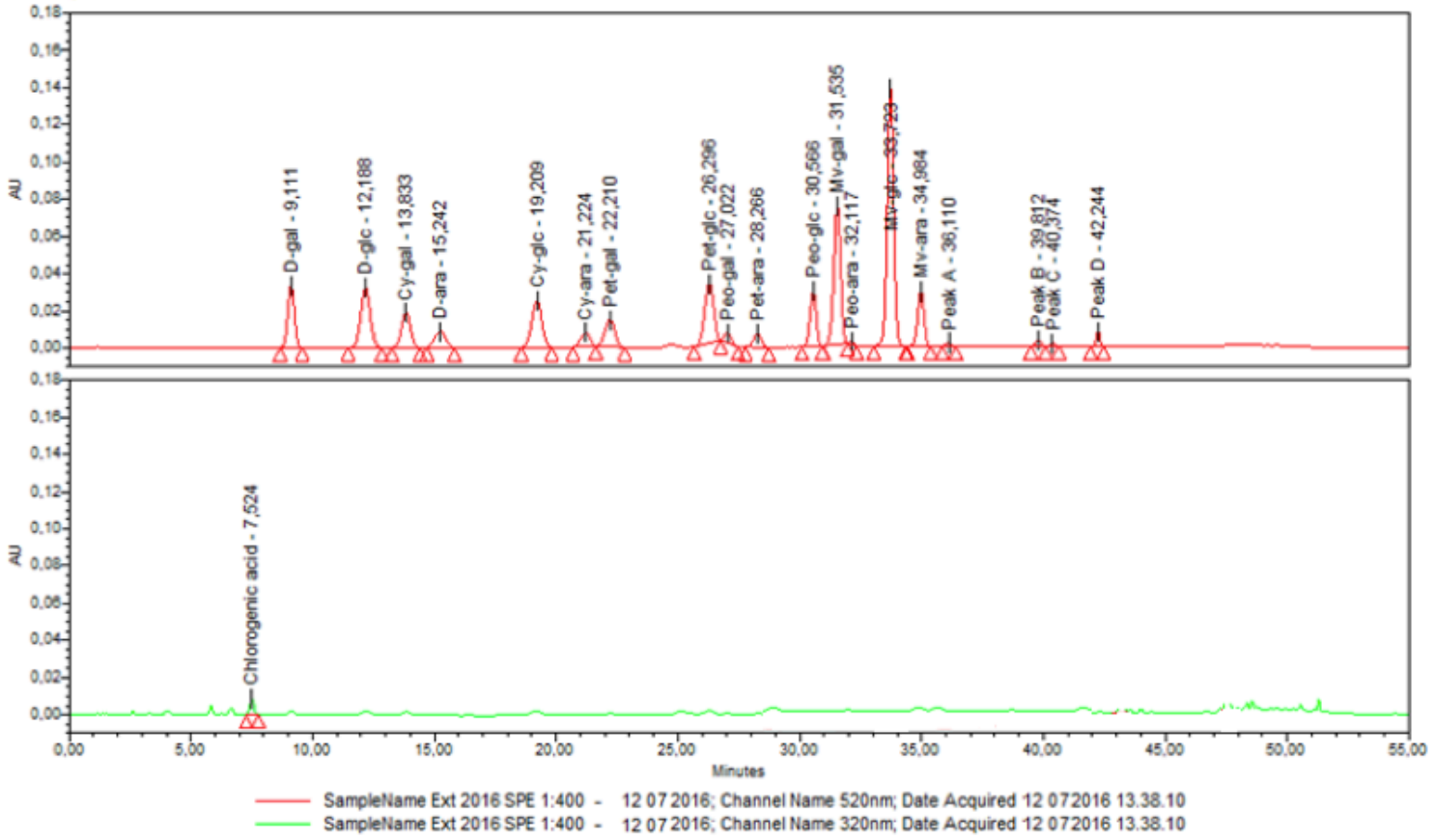
SDS-PAGE was performed as described above (i). The gel was removed and was incubated with Coomassie Blue overnight and destain buffer was added to remove excess color. It was scanned using ChemiDoc XRS imager and Imagelab software (Bio-Rad) and the presence of alpha prime (α′) of C4 complement protein was determined. The protocol for Western Blotting was optimized for the needs of these experiments as described above, but a version of them is included in Mastellos et al. (2004). PCA concentrations were 5mM, 1.5mM and 0.5mM and there was also C4, C1s and C1s-INH. Complement component C1s is a serine protease associated with C1q and C1r. Together they form the C1 complex and lead to the cleavage of complement proteins C2 and C4 (Ricklin et al. 2016).

CHAPTER 4

RESULTS

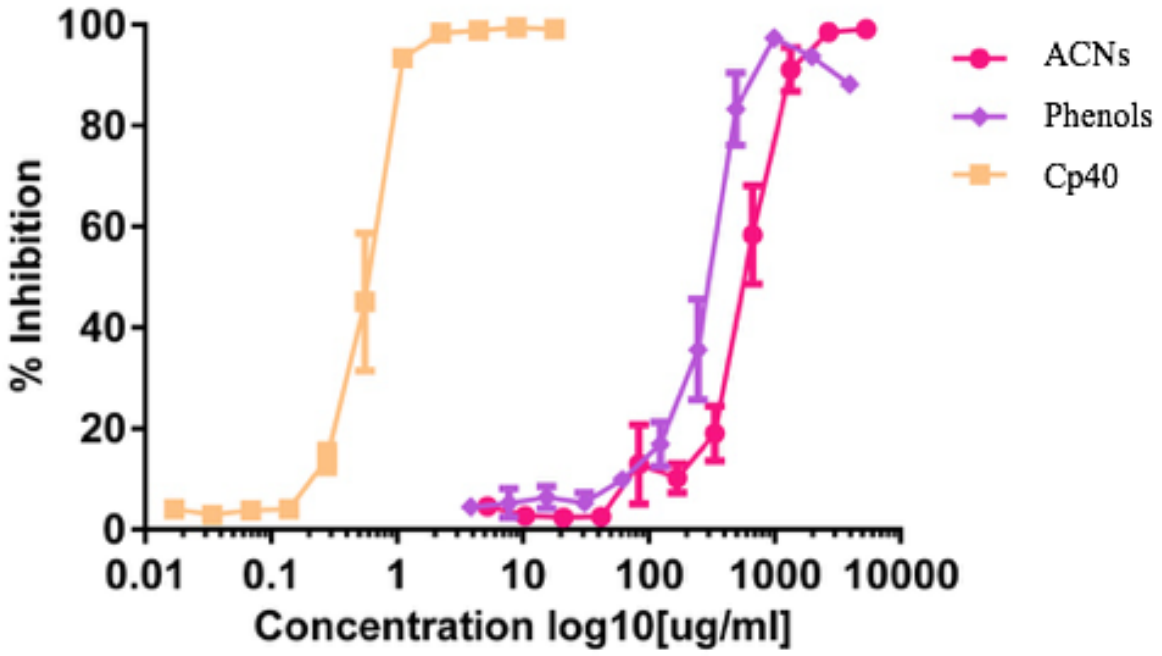
Figure 3 shows the HPLC profile of ACNs and Phenolic acid in the wild blueberry powder, at 520nm and 320nm respectively. Peak A is delphinidin-glucose-acetyl (Dp-glc-ac), peak B is cyanidin-glucose-acetyl (Cy-glc-ac), peak C is petunidin-glucose-acetyl (Pt-glc-ac) and peak D is malvidin-galactose-acetyl (My-gal-ac). The major ACNs detected were Mlv-glc and Mlv-gal, while those in the lowest concentrations were Dp-glc-ac and Pt-glc-ac. The only phenolic acid detected was the chlorogenic acid.

Figure 3: ACNs and Phen profile in the wild blueberry powder



As Figure 4 presents, both the ACN- and Phenolic-rich fractions trigger inhibition of the Classical pathway of the complement system, in a dose dependent manner, with an IC_{50} of 604.6 μg/ml and 323.6 μg/ml respectively (n=3). The peptidic inhibitor Cp40 (positive control) was found to be the most active, inhibiting activation of the Classical pathway also in a dose-depended way. The Phen fraction reached its maximum inhibitory activity at 986 μg/ml, leading to saturation at 1972 μg/ml and 3944 μg/ml. On the other hand, the ACN-rich fraction continued to have maximum inhibitory activity at concentrations of 1341.2 μg/ml, 2682.5 μg/ml and 5365 μg/ml. The bioactive fractions showed higher variation among experiments, compared to Cp40, but this may have happened due to the fact that Cp40 is a synthetic peptide, while ACN- and Phenolic-rich fractions are natural compounds.

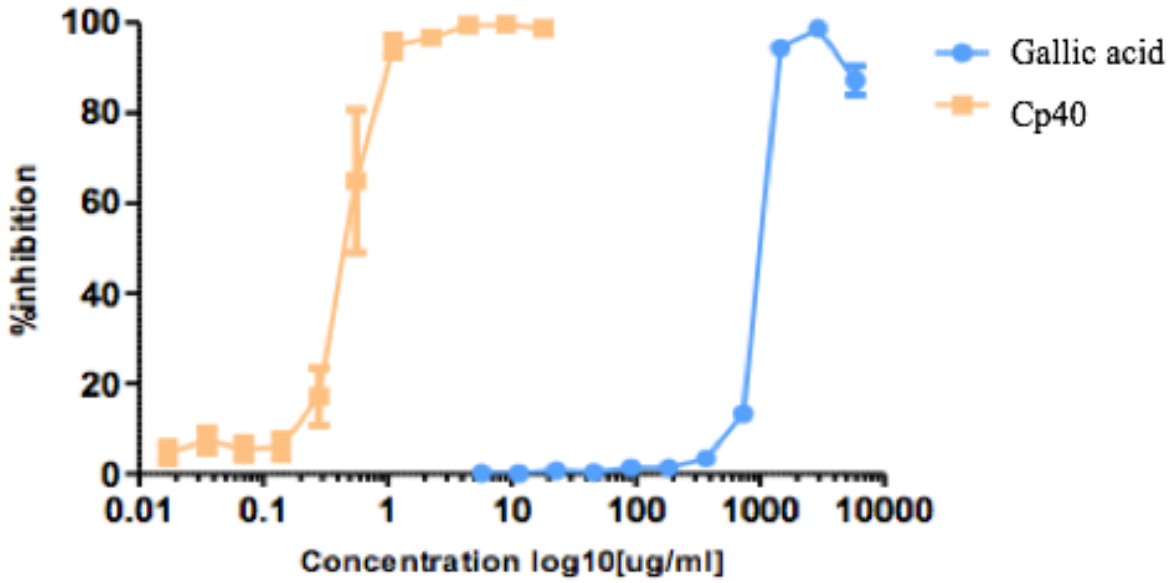
Figure 4: **Activity of ACN and Phen extracts on the activation of the Classical pathway**



(Cp40/ compstatin: control, n=3)

Gallic acid (molecular weight of 170.12 g/mol) inhibited the Classical pathway of the complement system in a dose-dependent way with an IC_{50} of 1.06 mM (Figure 5). Maximum inhibition achieved at 1.46 mM (94.3%) and 2.93 mM (98.5%) with drop at 87% at 5.84 mM. Gallic acid is less active than Cp40.

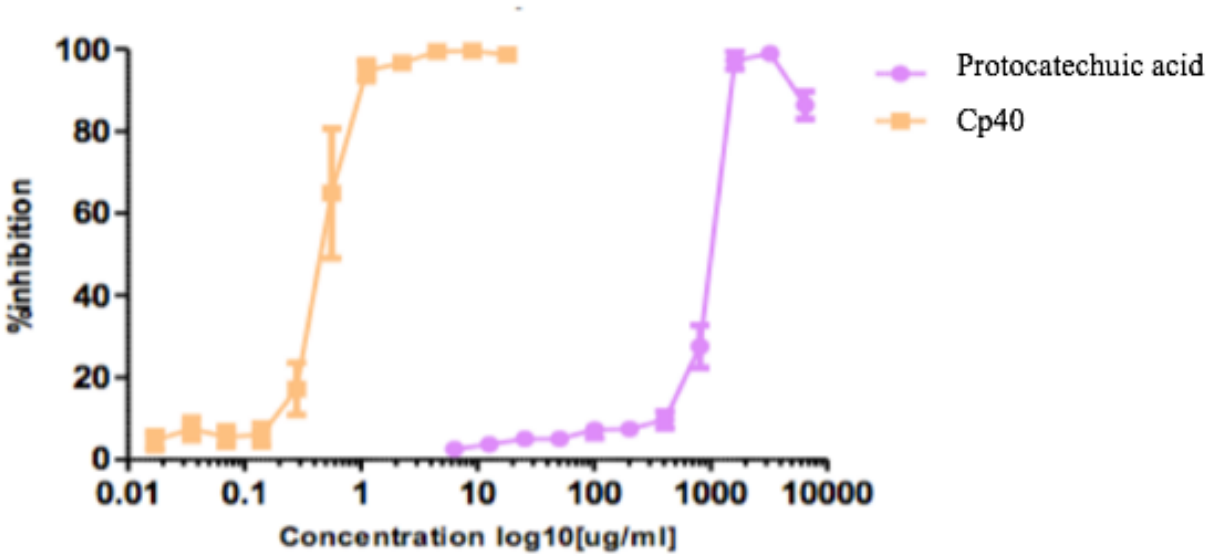
Figure 5: Activity of gallic acid (GA) (metabolite of delphinidine-3-glucoside) on the activation of the Classical pathway



(Cp40/compstatin: control, n=6)

Protocatechuic acid (molecular weight of 154.12 g/mol) triggered inhibition of the Classical pathway in a dose-depended manner with an IC_{50} of 1.06 mM. It achieved maximum inhibition at 1.62 mM (98.86%) 3.24 mM (100%) with decline at the maximum concentration used 6.48 mM (86.3%). This metabolite was less active than the control Cp40 (Figure 6).

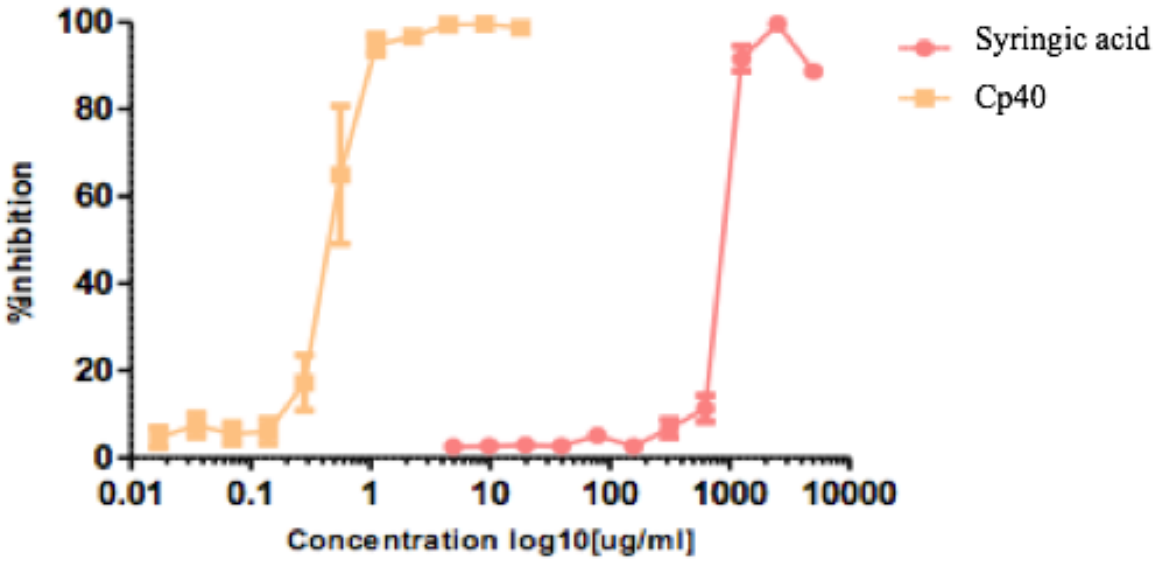
Figure 6: Activity of protocatechuic acid (PCA) (metabolite of cyanidine-3-glucoside) on the activation of the Classical pathway



(Cp40/compstatin: control, n=6)

Syringic acid (molecular weight 198.17 g/mol), according to Figure 7 inhibited the Classical pathway of the complement system in a dose-dependent way, with an IC_{50} of 0.93 mM. The concentrations that lead to maximum inhibition were 1.2 mM (91.6%) and 2.5 mM (99.6%). A higher concentration of 5 mM led to decrease in inhibitory activity (88.7%). This bioactive compound was less active than Cp40.

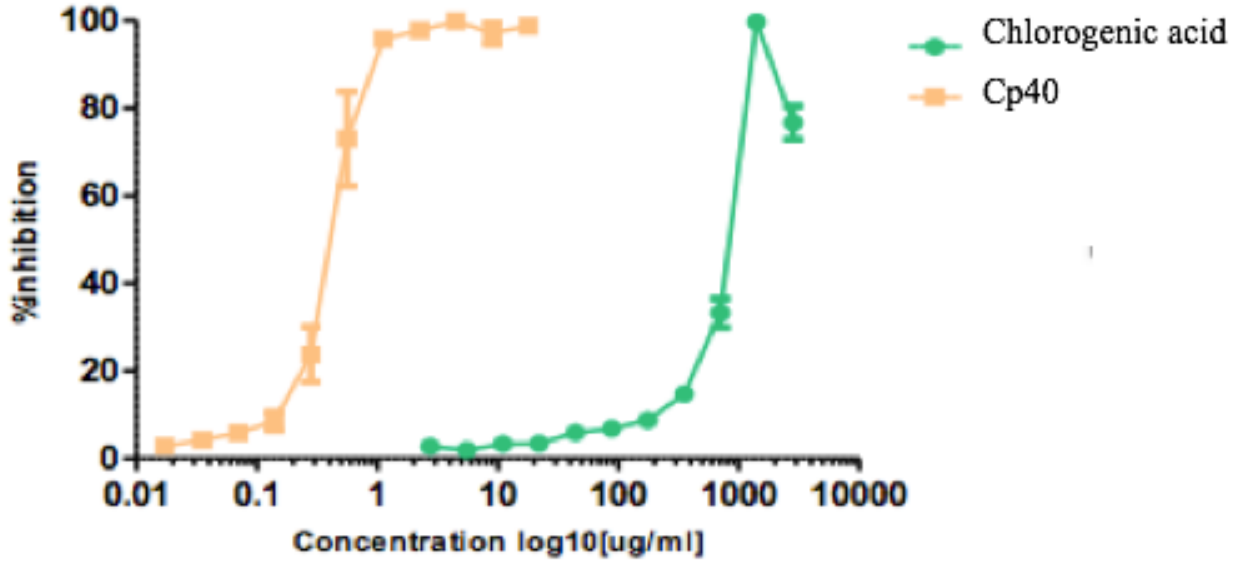
Figure 7: Activity of syringic acid (SA) (metabolite of malvidin-3-glucoside) on the activation of the Classical pathway



(Cp40/compstatin is the control, n=6)

As Figure 8 Chlorogenic acid (molecular weight 354.51 g/mol) triggered inhibition of the Classical pathway in a dose-dependent way with an IC_{50} of 0.88 mM. The most effective concentration was 1.41 mM (99.2% inhibition). Higher concentration of 2.42 mM was less effective inhibiting the Classical Pathway (76.7 % inhibition). Concentration of 0.72 mM led to 33.2 % inhibition, thus there was only a short range of concentrations that were effective. Chlorogenic acid is less effective inhibiting the complement system, compared to Cp40.

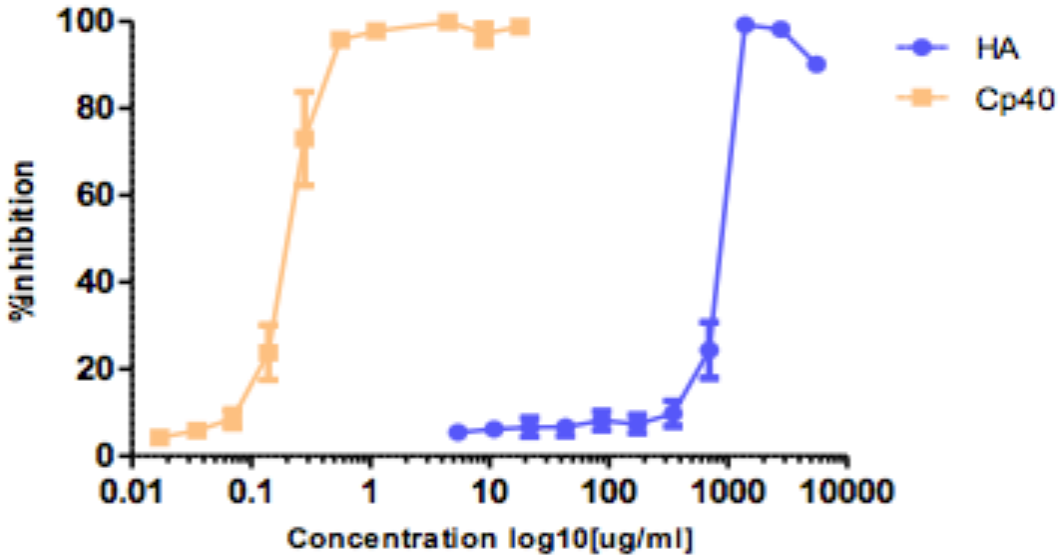
Figure 8: Activity of chlorogenic acid (predominant compound in Phen fraction) on the activation of the Classical pathway



(Cp40: compstatin is the control, n=6)

Hippuric acid (molecular weight of 179.17 g/mol) triggered inhibition of the Classical pathway of the complement system with an IC_{50} of 0.93 mM (Figure 9). The inhibitory effect was dose-depended with the most effective concentrations being 1.39 mM and 2.78 mM inhibiting the complement system by 99.2 % and 98.1 % respectively. A higher concentration of 5.58 mM inhibited the Classical pathway by 90.1 % which is high but less than the two former concentrations, meaning that anything above 5.58 mM leads to saturation.

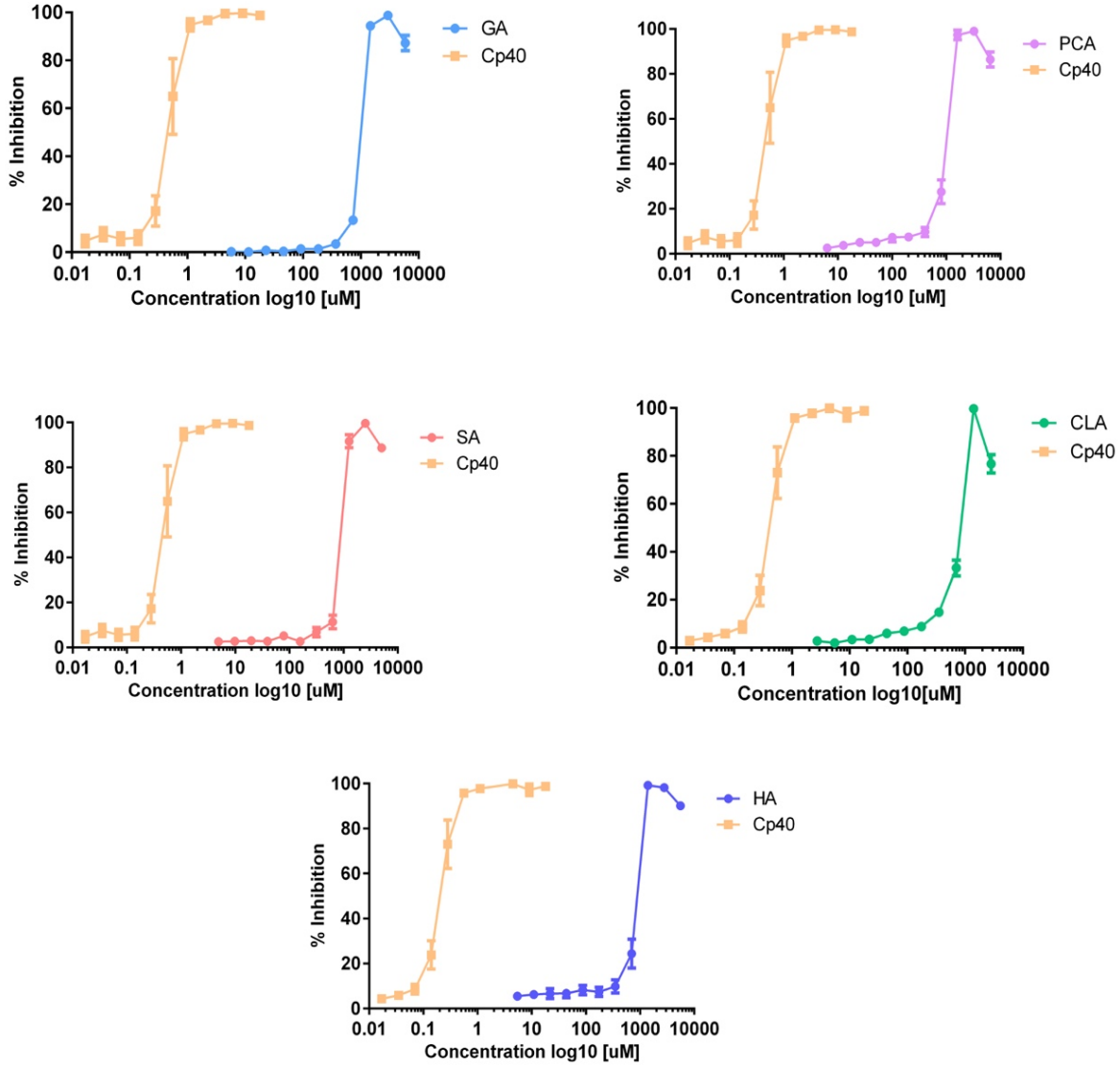
Figure 9: Activity of hippuric acid (a common metabolite of ACNs and Phen) on the activation of the Classical pathway



(Cp40/compstatin: contron, n=6)

According to Figure 10, all the metabolites tested, inhibited the Classical pathway in a dose depended way with an average IC_{50} of 1mM. These results emphasize the similarity of these bioactive compounds. Overall, they were more stable than the ACNs and Phen fractions (less variation), and more active, in that they triggered higher inhibition of the complement system in low concentrations. Specifically, ACNs and Phen fractions had an IC_{50} of 604.6 and 323.6 μg/ml respectively, while the metabolites had an average IC_{50} of 1mM. Additionally, protocatechuic (PCA), hippuric (HA) and chlorogenic (CLA) acids were the most active metabolites in low concentrations, meaning that they exhibited higher inhibitory activity in low concentrations, then the rest of the metabolites.

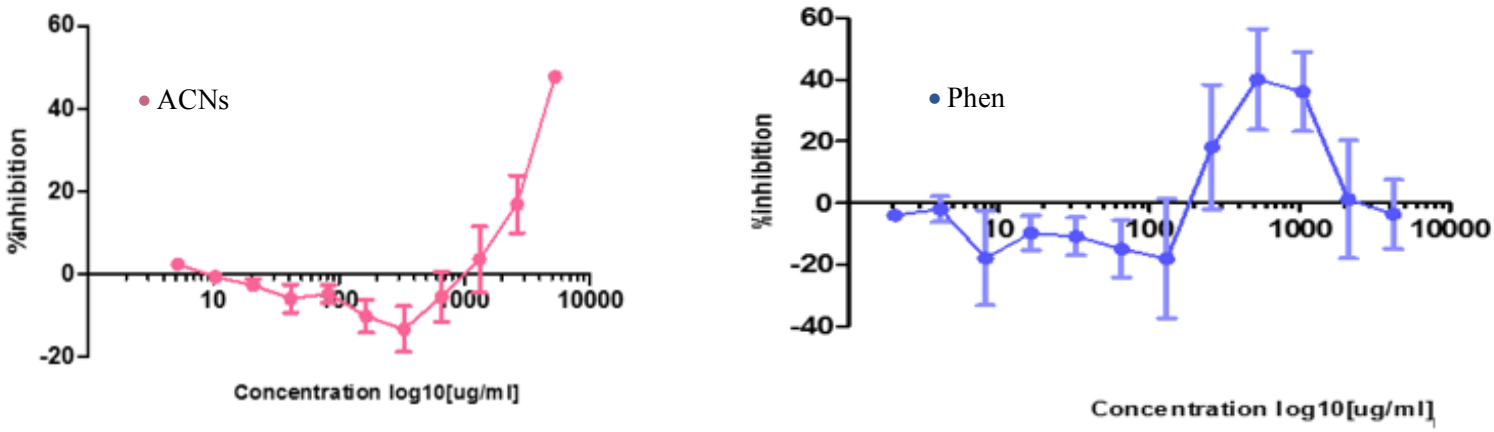
Figure 10: Activity of Polyphenol metabolites gallic (GA), Syringic (SA), protocatechuic (PCA), chlorogenic (CLA) and hippuric (HA) acids on the Classical pathway



(Cp40/compstatin: control, n=6)

As Figure 11 shows, both ACN and Phen fractions did not have any effect on the Alternative pathway of the complement system. Neither of these compounds inhibited completely the system. The maximum concentration used for the ACN fraction was 5280 μg/ml while for the Phen fraction was 4207.1 μg/ml. Only ACNs show some inhibitory effect close to 60% but in the highest concentration used. Phen exhibited large variability from experiment to experiment making the results unreliable. That could be attributed to the unstable nature of the Phen fraction. Specifically, for the Phen fraction, the OD was different in all four different experiments (1.481(A), 1.004(A), 0.757(A) and 0.717(A). This resulted in high variability of inhibition among experiments.

Figure 11: Activity of ACN and Phen extracts on the activation of the Alternative pathway

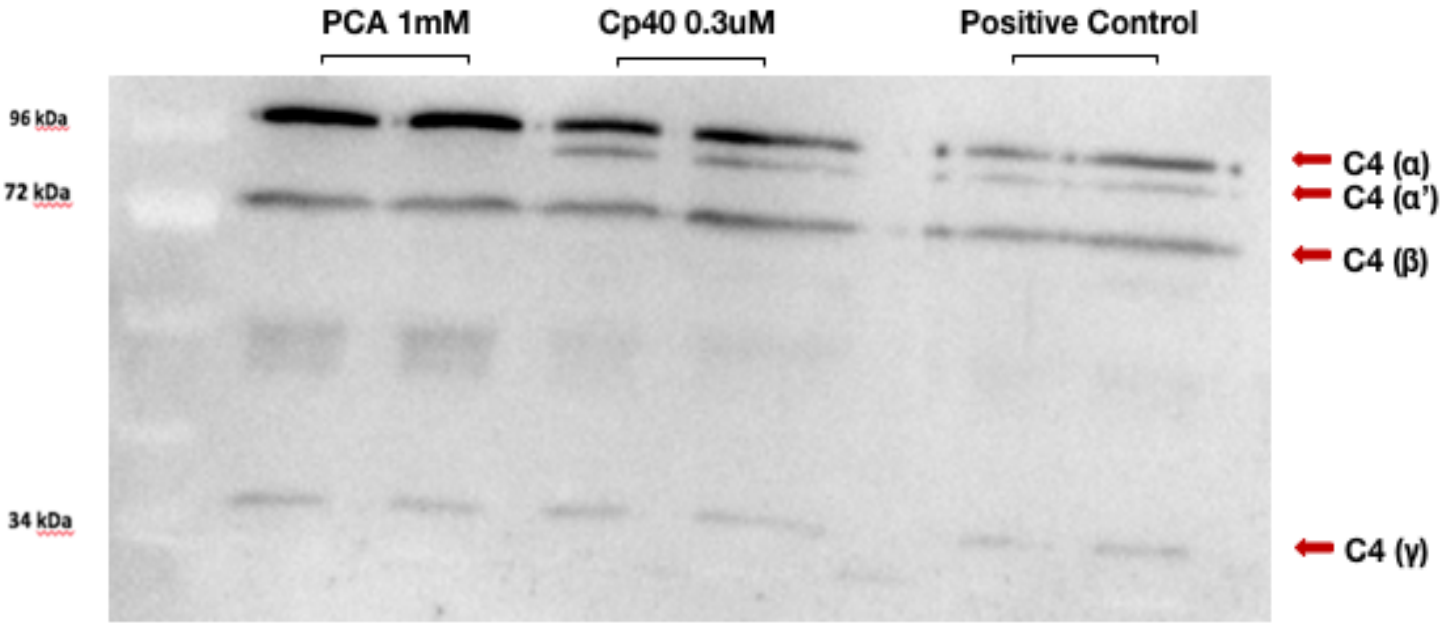


(LPS initiated complement activation, n=3 for ACNs and n=4 for Phen).

Complement protein C4 is not cleaved into its components C4a and C4b, as evidenced by the absence of C4 (α') (Figure 12). When C4 is cleaved, C4 α-chain gets activated, thus C4 α'-chain (C4 (α΄)) is detected. C4 is a complement protein (200 kD) which consists a thioester bond and three peptide chains (α. β, γ) connected by disulfide bonds. Complement component 4 (C4) is eventually cleaved (by serine protease 1/C1s) into its fragments C4a (9 kD) and C4b (195 kD). C4a is cleaved off the N-terminal of α-chain generating C4b. Then, C4b along with C2a and

participate in the formation convertase C4b2b (C3 convertase). When C4 is not cleaved, the α-chain is intact, while when it gets cleaved, α-chain becomes activated (activated form: α'-chain) (Gigli et al. 1997, He and Lin 1998, Schifferli JA and Paccaud J 1989, Ricklin et al. 2016). Cyclic peptide Cp40 (control) acts on complement protein C3 (Mastellos et al. 2015), thus we expected to detect C4 (α'). C4 α'-chain appeared on positive control, as expected. C4 (α') was not detected for Protocatechuic acid of 1 mM (the average IC_{50} for the anthocyanin metabolites).

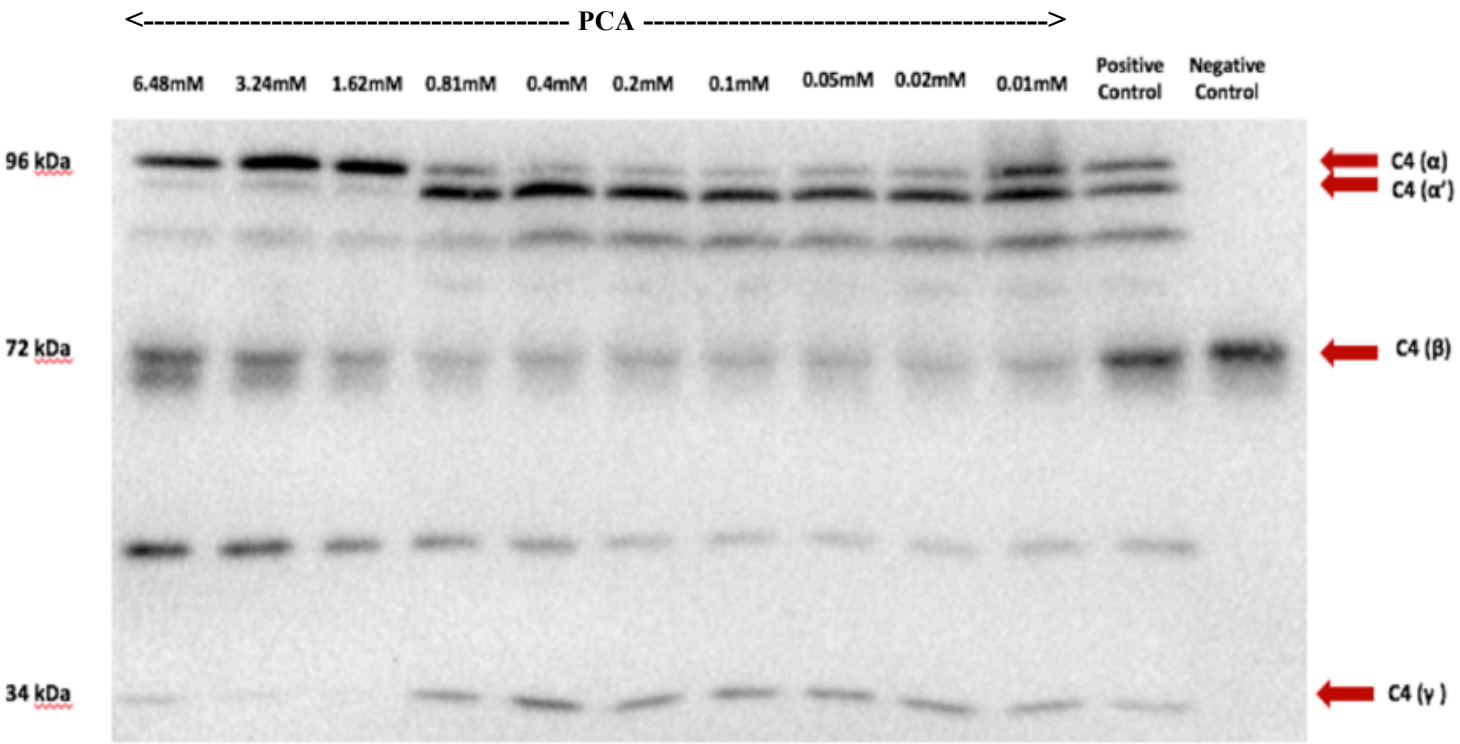
Figure 12: Protocatechic acid (PCA) inhibits cleavage of complement protein C4 (n=2)



(Positive control: only plasma from healthy donors and no inhibitors (i.e PCA or Cp40), n=2).

As shown in Figure 13 protocatechuic acid (PCA) inhibited complement C4 at higher concentrations (1.62, 3.24 and 6.48 mM). This is supported by the absence of C4 (α'). Concentrations of 0.81 mM or less did not inhibit the cleavage of C4, in that C4 (α') was present.

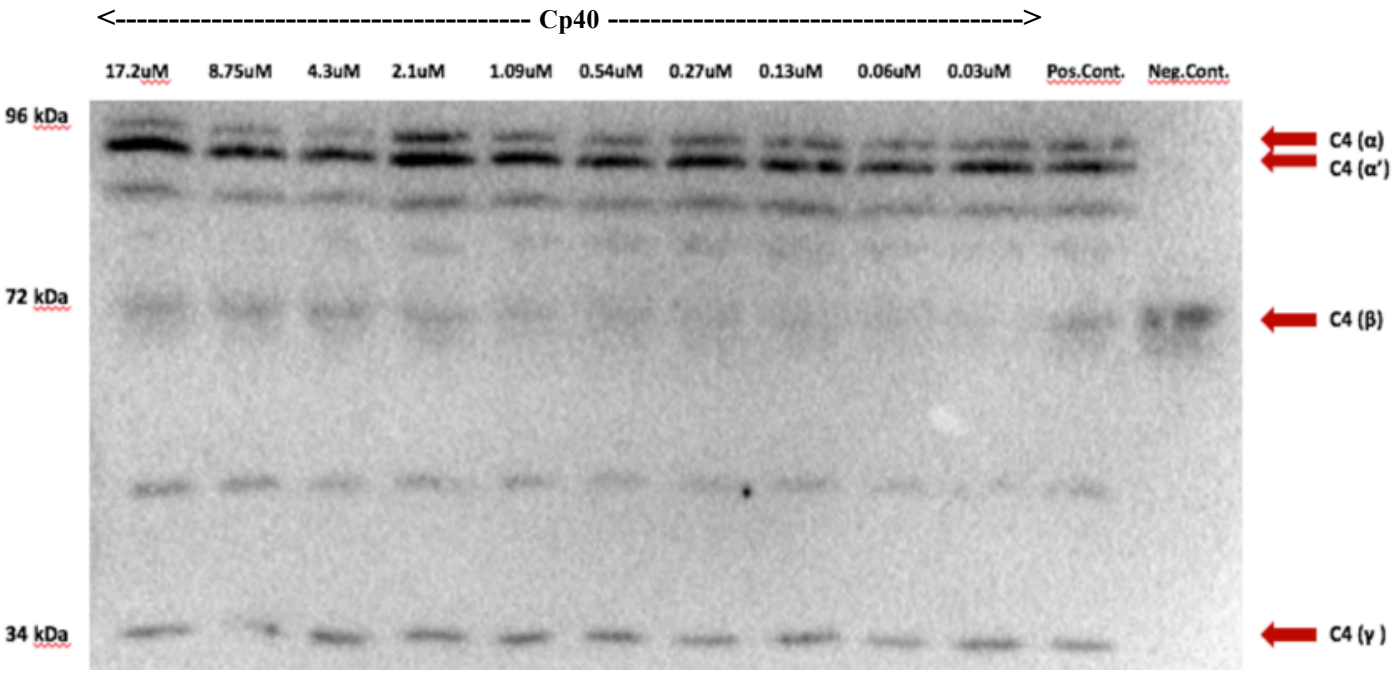
Figure 13: Effect of protocatechuic acid (PCA) concentrations on cleavage of complement protein C4 (n=2)



(Positive control: only human plasma, negative control: absence of human plasma and PCA, n=2)

Cp40 (compstatin), as shown in Figure 14 acts on complement protein C3 inhibiting its cleavage. It was expected not to affect complement protein C4. Indeed, cyclic peptide Cp40 did not inhibit the cleavage of protein C4, in that C4 (α') was detected in all concentrations that were used. That confirms that i) the experiment worked properly and ii) PCA/metabolites have an effect on C4 of the classical pathway.

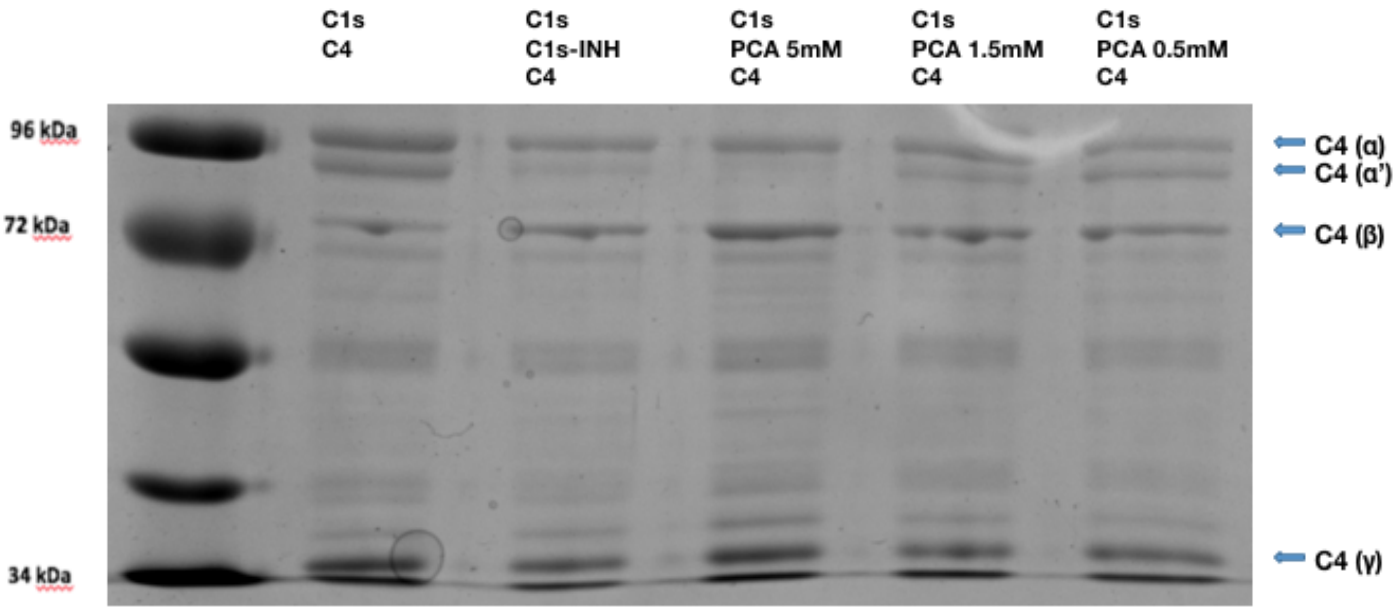
Figure 14: Cp40 (Compstatin) effect on complement C4 cleavage (n=2)



(Positive control: only human plasma, negative control: absence of human plasma and Cp40, n=2)

Figure 15 shows that protocatechuic acid completely inhibited the cleavage of complement protein C4 by the C1s (serine protease 1), mimicking the behavior of the C1s inhibitor (C1s-INH). C1s-INH is a synthetic inhibitor that prevents cleavage of C4 by binding C1s, thus no C4 α-chain remains intact and C4 (α') is expected to be observed. All columns contain human plasma from healthy donors. Only C1s and C4 were added in the second column (positive control) in order to illustrate the cleavage of complement C4 and the detection of C4 α'-chain. The third column included C1s, C4 and C1s-INH, to block C4 cleavage and the consequent appearance of C4 (α'). The column with the presence of PCA (5 mM) did not show the C4 α'-chain, showing that protocatechuic acid inhibited C4 cleavage by C1s. Lower concentrations (1.5 mM and 0.5 mM) of PCA added in the last two columns did not affect C4.

Figure 15: Protocatechuic acid (PCA) inhibits C1s mediated C4 cleavage (n=1)



(C1s-INH: compound inhibiting C1s serine protease, n=1)

Overall, the results showed that both ACN and Phen fractions inhibit the Classical pathway of the complement system in a dose-depended way. On the other hand, the Alternative pathway was not impacted by the presence of the bioactive compounds.

Metabolites of major anthocyanins and phenolic acids found in the wild blueberry extract, also triggered complete inhibition of the Classical pathway, achieving this effect in lower concentrations than the ACNs and Phen fractions. Additionally, the metabolites tested behaved similarly with an average IC_{50} of 1 mM.

Mechanistically, it was determined that polyphenols impact specifically the Classical pathway by targeting the activation of the complement protein C4 through the C1s enzyme.

CHAPTER 5

DISCUSSION

5.1 ACNs and Phen profile in wild blueberry powder

Previous researchers who have studied wild blueberries have documented the bioactive compounds in abundance in the "batch" of blueberry powder that was used for each respective work (Del Bo et al. 2016, Taverniti et al. 2014, Del Bo et al. 2010).

Two studies have documented Mv-glc, Dp-glc and Cy-glc to be predominant (Del Bo et al. 2016, Taverniti et al. 2014), while a third found Mv-3glc and Pn-3glc anthocyanins in abundance (Del Bo et al. 2010). They revealed after HPLC analysis that Mv-glc and Mv-gal were the major ACNs present. In all the above studies, along with this present one, chlorogenic acid was the major phenolic acid detected. Several factors such as temperature, light, pH, oxygen, contribute in the distribution of anthocyanins and phenolic acids in each batch of wild blueberry powder, thus it is essential to view the profile of each powder used for experimental purposes. Additionally, different soils, growing conditions and cultivar rank may affect the anthocyanin and phenolic acid content of wild blueberries (Connor et al. 2002).

5.2 The effect of wild blueberry ACN and Phen fractions on the Complement system of innate immunity

5.2.1 Classical Pathway

This research study shows for the first time that ACN and Phen fractions of wild blueberries inhibit the Classical pathway of the Complement system in a dose-depended way. In this study, it was impossible to use molar concentration for the ACN and Phen fractions, in that they are composed of multiple compounds. The IC50 for ACN and Phen fractions were 604.6 μg/ml and 323.6 μg/ml

respectively. These concentrations are very high. Regarding ACNs from wild blueberries, literature has shown that in *in-vitro* assays a concentration range of 5 to 100 μg/ml does not negatively affect the cell viability of Caco-2 cells (Taverniti et. al 2014). Several studies using ACN fractions from red raspberries, determined that concentration of 100, 150 and 200 μg/ml were not cytotoxic on Mouse RAW264.7 macrophage cells (Li et al. 2014). In most cell-culture studies, concentrations of less than 50ug/ml are used. However, Safaeian et. al (2015) in examining the effects of ACN fraction derived from the petals of *Echium amoenum*, reported that concentrations of up to 1000 μg/ml were cytoprotective in human endothelial cells (HUVECs) and not cytotoxic between 25-1000 μg/ml. In a recent study from our lab Tsakiroglou et. al (2017) showed that *in-vitro*, ACNs extracted from wild blueberries were cytotoxic on HUVECs at 1000 μg/ml, while Phen fractions were not toxic even at the highest concentration (500 μg/ml).

In the present study we tested the ACNs and Phen in human plasma from healthy donors, using ELISA immunoassays, and the most effective concentrations leading to complete complement inhibition were very high. Additionally, this is the first time these extracts from wild blueberries have been tested on the complement system using these particular assays, thus, the goal was to observe their behavior and compare them to the control Cp40. Cp40 is a synthetic cyclic peptide which belongs to the group of compstatin peptides which are C3 complement protein inhibitors (Mastellos et al. 2015). To be able to detect their effect on the complement system, we had to find and use the concentrations that would show that they act on a dose-dependent way, similar to that of Cp40. Thus, the effective concentrations for complete complement inhibition, although very high are those that help compare the natural bioactive compounds to Cp40. Inhibitor Cp40 was effective inhibiting the Classical pathway with an IC50 of 17.8 uM, thus it is more effective than

ACN and Phen fractions. This happens due to the fact that Cp40 is a synthetic peptide, thus it is synthesized to be effective in low concentrations (Mastellos et al. 2015, Lambris et. al 2015).

Future studies testing wild blueberry ACN and Phen fractions on the complement system, should incorporate experiments using cell cultures in order to establish a more accurate and realistic concentration range.

There is lack of reliable studies examining the effect of ACNs and Phen on the Complement cascade. In fact, there are no studies on the effect of wild blueberry polyphenols. Ho et. al (2014), examined the effects of on classical and alternative pathways of flavonoids extracted from *Aronia Melanocarpa in-vitro* using hemolytic complement-fixing assays and found that cyanidin, procyanidins B2, B5 and C1 along with proanthocyanidin-rich fractions inhibit the Classical pathway of the complement system. The study showed that the activity of these compounds was dose-dependent with compound C1 (trimer) being more effective than B2 and B5. It was also shown that differences in the structures of the procyanidins can affect their complement-fixing activity. Although the study of Ho et. al (2014) carries some ambiguity about data interpretation and the selection of controls for the complement hemolytic assays, it clearly showed a trend of anthocyanins and their derivatives to inhibit the Classical pathway. Specifically, the inhibitory effect of procyanidins was confirmed, with trimeric C1, being more active than dimerics B1 and B5 or monomers however, there were vague areas in methodology and analysis of results (Ho et al. 2014).

In a recent study Ho et. al (2017), also confirmed the inhibitory effect of polyphenols found in berries. Specifically, it was shown that crude extracts from elderberry and elderflower had high anti-complement activity inhibiting the complement pathway. However, in that study the crude extracts demonstrated higher inhibition of hemolysis of sensitized sheep red blood cells. Their

respective anthocyanins and procyanidins along with flavonoids failed to completely inhibit the classical pathway (Ho et. al 2017). Specifically, metabolites such as vanillic, ferulic, hippuric, cafeic, coumaric, benzoic acids demonstrated weak complement activity at 0.1-200 mM.

Both studies conducted by Ho et al. (2014 and 2017) were similar, but their methodology is not clear. Additionally, the results of Ho et al. (2017) contradict the findings of our research which showed that metabolites of ACN and Phen extracts of wild blueberries, are more effective than their parent compounds inhibiting the classical pathway with an average IC50 of 1mM. A reason for these different findings may be the fact that each plant, has different concentration of polyphenols and other bioactive compounds which may act synergistically or antagonistically with each other, determining their overall effect on immunity.

There is ambivalence in literature on whether anthocyanins are more or less effective inhibiting the complement cascade compared to their aglycones. In general, most researchers have shown that polyphenols with their sugar moieties are more active than their respective aglycones (Min et al. 2003, Jung et al. 2008, Qin et al. 2016). Therefore, sugars may have a crucial role determining the extend to which these bioactive compounds can affect the complement system.

5.2.2 Alternative Pathway

In this current study, both ACN and Phen fractions did not have any effect on the Alternative pathway of the complement system. Neither of these compounds inhibited completely the system. The maximum concentration used for the ACN fraction was 5280 μg/ml while for the Phen fraction was 4207.1 μg/ml. Only ACNs showed some inhibitory effect close to 60% but in the highest concentration used. The Phen fraction had a big variability from experiment to experiment making the results unreliable. That could be attributed to the unstable nature of the Phen (Fracassetti et al.

2013). Additionally, the Alternative pathway ELISA assay is more sensitive than the assay for the Classical Pathway. This resulted in high variability of inhibition among experiments.

The protocol for the alternative pathway may need to be modified and adjusted accordingly to match the sensitivity of these natural compounds. As of now, due to the problems in the protocol, it cannot be concluded whether these results are representative or not of the effect of these compounds on the Alternative pathway.

A study reported inhibition of the alternative pathway from extracts of *Viola tianshanica plant.* Specifically, some flavonol glycosides were reported to inhibit the alternative pathway with CH50 and AP50 of 0.113-0.536 mg/mL and 0.237-1.579 mg/mL respectively. However, those findings are slightly ambivalent as the same activity was recorded for both the classical and the alternative pathways without any further classification (Qin et al. 2016).

5.3 The effect of wild blueberry metabolites on the Complement system of innate immunity

We chose gallic, protocatechuic and syringic acids because, they are metabolites of single anthocyanins Dp, Cy and Mv-3-O-glucosides respectively and have been studied in the past *in-vitro* (Del Bo et al 2016). The latter anthocyanins have been reported to be found in human plasma upon consumption of a portion (300mg and 500mg) of blueberries (Del Bo' et al. 2012, De Ferrass et al. 2014). Anthocyanins tend to be absorbed in urine and plasma either on their intact form (as glucosides) or their derivatives (upon rapid metabolism) (Fornasaro et al. 2016, Del Rio et al. 2010). We selected chlorogenic acid as it is the most predominant phenolic acid based on our HPLC findings, and hippuric acid because it is a common metabolite of phenolic acids and anthocyanins (De Ferras et al. 2014).

In this study, all the metabolites demonstrated almost identical activity inhibiting the classical pathway with an average IC_{50} of 1mM. They were also more active than the ACN and Phen extracts

which had an IC_{50} of 604.6 μg/ml and 323.6 μg/ml respectively. This contradicts another study from Ho et al. (2017) who showed that crude extracts from elderberry and elderflower demonstrated higher inhibition of hemolysis of sensitized sheep red blood cells. Their respective anthocyanins and procyanidins along with flavonoids failed to completely inhibit the classical pathway (Ho et al. 2017). Specifically, metabolites such as vanillic, ferulic, hippuric, cafeic, coumaric and benzoic acids demonstrated weak complement activity at 0.1-200 μM. However, in that study the methodology as discussed above, was not very clear. A reason for the different findings may be the fact that each plant, has different concentration of polyphenols and other bioactive compounds which may act synergistically or antagonistically with each other, determining their overall effect on the immune system. Peake et. al (1991) later reported that the same metabolite (rosmarinic acid), inhibited the classical pathway at 2.6 mmoles of optimal concentration, in a dose-dependent way.

There is lack of studies examining the effects of the metabolites (gallic, syringic, protocatechuic, chlorogenic and hippuric acids) on the complement system. This was the first time the effect of these metabolites on the classical pathway of the complement system was explored and documented.

Moreover, there is ambivalence in literature on whether anthocyanins are more or less effective inhibiting the complement cascade compared to their aglycones. In general, most studies show that polyphenols with their sugar moieties are more active than their respective aglycones (Min et al. 2003, Jung et al. 2008, Qin et al. 2016). Therefore, sugars may have a crucial role determining the extent to which these bioactive compounds can affect the complement system.

In-vitro research on the exact same metabolites tested in our study, shows that their effect on different systems (i.e immune, cardiovascular, endocrine) is dependent on each individual

bioactive metabolite (Del Bo' et al. 2016). Moreover, different metabolites may work synergistically or antagonistically when found in plasma or tissues affecting complement system in different ways than when acting alone. This is something worth exploring, in that single phenolic derivatives are never found alone in plasma, urine or tissues. The distribution of ACNs and their metabolites in plasma and tissues varies, with most of them being found in liver, kidneys and brain, either in intact form, or in the form of derivatives (Pojer et al. 2013, Fornasaro et al. 2016). Thus, specific metabolites, may exist not only along with other metabolites, but also with parent anthocyanins (i.e malvidin, cyanidinidin, delphinidine, petunidin glucosides). Chlorogenic acid, which is found in phenol-rich extract, is hydrolyzed by the intestinal microflora into several metabolites (Gonthier et al. 2003).

In the past, researchers studying pharmacokinetics in humans have reported that phenolic derivatives have been found in trace levels in plasma, urine and tissues. For example, in one study by Kay et. al (2002), only 0.002-0.003% of 1.2 gm ingested blueberries anthocyanins were found in serum 1-4 hours upon consumption, while in two other studies, just 0.02% (of 439 mg) and 0.04% (of 690 mg) of consumed anthocyanins extracts were detected in urine (Mazza et. al 2001, Wu et. al 2002). Recently though, it has been documented that contrary to previous beliefs, polyphenol absorption is adequate (Kay et al. 2017, Lila et al. 2016, De Ferras et al. 2014, Czank et al. 2013). Fernandez et al. (2014), reported that approximately 5-10% of native forms of ACNs and their metabolites are absorbed immediately upon ingestion reaching the bloodstream. In our study, an IC_{50} of 1mM may sound unrealistic and biologically irrelevant, however, it needs to be taken into consideration that i) these are natural bioactives that have not been modified in any way, ii) they have been tested in *in-vitro* immunoassays and iii) they have been compared with a synthetic compound (Cp40) and iv) the goal of this testing was to evaluate their ability to inhibit

the classical pathway. If the protocols of the immunoassays get optimized for these compounds, it is possible that the IC50s may change. Moreover, these compounds could be possibly modified to be effective in lower concentrations. Additionally, their effect on the complement system when these compounds become accumulated in kidney, liver and brain is worth exploring. However, from a nutritional point of view, a partial complement inhibition through consumption of wild blueberries on a daily basis, would be beneficial. Balanced complement function ensures homeostasis and health. Unbalanced activity is associated with chronic inflammation metabolic related diseases such as fatty liver, obesity, diabetes, obesity and cardiometabolic diseases (Phieler et al. 2013, Hertle et al. 2014). Evidence of this study shows that these compounds are effective inhibiting only the classical pathway of the complement system.

In our study, all the metabolites had almost identical behavior inhibiting the classical pathway with an average IC50 of 1mM. Chlorogenic acid, a phenolic acid found in the phenol-rich extract, belongs to the group of hydroxycinnaminic acids. The metabolites gallic, syringic, protocatechuic and hippuric acids are found in the form of hydrobenzoic acids, thus they are benzoic acid derivatives (Rice-Evans et. al 1996, Egawa et. al 2017). Thus, the benzoic acid is their common structural characteristic which may be responsible for their similar behavior inhibiting the classical pathway. Regarding chlorogenic acid, it is an ester of caffeic and quinic acid and it may be the caffeic part of the molecule that responsible for its effect (Olthof et al. 2001). Evidence shows that chlorogenic acid gets metabolized rapidly into caffeic and quinic acid by gut microflora (Gonthier et. al 2003). Further research is required, to show why these polyphenol metabolites behave as such and if benzoic acid is related to their similar action.

5.4 Mechanism of action

In this study, we showed that protocatechuic acid (PCA) acts on the classical pathway of the complement system through C1s mediated inhibition of complement protein C4. Protocatechuic acid is a major cyanidin-3-glucoside metabolite. Cyanidin-3-glucoside has been studied extensively in pharmacokinetic studies (Pace et al. 2018, Warner et al. 2017, De Ferras et al. 2014, Czank et al. 2013).

This is the first study to show that a metabolite of wild blueberries inhibits the classical pathway with this mechanism of action. Cyclic peptide Cp40 (compstatin) acts on complement protein C3 (Mastellos 2015), while protocatechuic acid acts on an enzyme (C1s). It is possible that the other metabolites tested in this study have a similar mechanism of action, given the fact that they all have benzoic acid as part of their structure.

There are a few older studies that explore the mechanism of action of flavonoids. Engleberger et al. (1988), found that rosmarinic acid of *Melissa* and *Rosmarinus officinalis* inhbited the classical pathway acting on C3-convertase. This enzyme binds C3 complement protein, breaking it down into its C3a and C3b fragments (Ricklin et al. 2010, Markiewski and Lambris 2007). Another study reported that oleanolic acid of *Luffa cylindrica* inhibited the classical pathway in-vitro, acting on C3-convertase with optimal concentration of 100µg (Kapil and Sharma 1994). Kapil and Moza (1992) documented that boswellic acids extracted from *Boswellia serrata* also inhibited the classical pathway through inhibition of C3-convertase with threshold concentration of 100µg. Peake et al, in 1991 examined rosmarinic acid of *Melissa officinalis* and concluded that convertase C5 was responsible for the inhibitory effects of that metabolite on the classical pathway.

In this study concentrations above 0.81mM of protocatechuic acid were effective inhibiting complement protein C4. This was revealed through Western Blot. On the other hand, SDS-Page

(n=1), showed that concentration as high as 5mM of this metabolite inhibited C4 through C1s enzyme. The latter assay was only performed once, and although the results are clear in the SDS-Page image, the protocol may need to be optimized and the assay to be repeated. Both Western Blot and SDS-Page showed that C4 is inhibited but it is not clear why on the former assay 0.81 mM, while on the latter concentration as high as 5mM was needed to see an effect.

Literature suggests that C4 is a complement protein (200 kD) which consists of a thioester bond and three peptide chains (α. β, γ) connected by disulfide bonds. Complement component 4 (C4) is eventually cleaved (by serine protease C1s) into its fragments C4a (9 kD) and C4b (195 kD). C4a is cleaved off the N-terminal of α chain generating C4b. Then, C4b along with C2a and participate in the formation convertase C4b2b (C3 convertase) (Gigli et al. 1997, Schifferli JA and Paccaud J 1989, Ricklin et al. 2016).

Additional assays that could show the mechanism of action of this metabolite would be helpful as they would provide evidence which would strengthen the findings of this study. Moreover, the way protocatechuic acid acts on C1s needs to be investigated.

Uncontrolled activation of C4 protein is related to a plethora of degenerative diseases such as Alzheimer's, as well as cancer and ulcerative colitis. On the other hand, C4 deficiency is tightly associated with malnutrition, systemic lupus erythematous (SLE), hepatitis, kidney transplant rejection, liver disease, genetic complement deficiencies (NIH 2018, Macedo 2016, Crehan et. al 2012). Therefore, a partial inhibition of C4 complement protein through daily natural ingestion of wild blueberries (evidenced by low inhibition in biological relevant concentrations used in our study) could be beneficial balancing the classical pathway and possibly controlling excessive or deficient activation of the system. On the other hand, structural change of protocatechuic acid could possibly make this compound more effective inhibiting completely the classical pathway at

lower concentrations, targeting specific complement-related diseases. Therefore, the effect of protocatechuic acid, could be controlled and used based on specific goals and needs.

In general, this study presents novel data on the effect of polyphenols (anthocyanins and phenolic acids) and their metabolites on the classical pathway of the complement system. We managed to explore the mechanism of action upstream on the classical pathway cascade. The action of wild blueberry metabolites on C1s enzyme could possibly mean that other complement enzymes may be affected by these bioactive compounds. More research is required to confirm these findings and clarify the concentrations these compounds are effective *in-vivo*, as well as unravel all these new questions that have arisen.

5.5 Significance

This study is significant because it is the first time that the relation between bioactives found in wild blueberries and the complement system has been investigated. This research is also novel because wild blueberries and their bioactives are natural compounds and not pharmaceuticals. Diet has the potential to prevent and reverse several pathological conditions promoting health. At the same time, wild blueberries have been linked to prevention of several pathologies such as CVD and MetS. Wild blueberries have gained a lot of commercial interest in the past years as they are among the berries with the highest concentrations of antioxidants. Additionally, they contain the highest amounts of anthocyanins, therefore they have great potential for food manufacturers, supplement and pharmaceutical industries. Bioactives found in wild blueberries have immunomodulatory effects protecting against oxidation and inflammation which are associated with pathologies such as diabetes, obesity, CVD, MetS and cancer. Complement system is a very complex and sophisticated system which collaborates closely with other biological systems and organs in the body. It is the orchestrator and overseer of the immune system and research is

attempting to find and improve ways to coordinate and balance the activation of complement. Sparse research suggests that diet can modulate systemic complement activation which in turn affects the homeostasis of liver, adipose tissue, and the endothelium. Currently, there is paucity of studies linking bioactives and specifically anthocyanins to complement activity, and their mechanism of action remains vague. Our study generates interest in the research of berries and other fruits on the complement system. Moreover, wild blueberries as natural compounds could be goodcandidates in fighting complement dysregulation in conditions such as renal disease, degenerative and cardiovascular diseases and helping control several pathologies which result from unbalanced complement activity. Wild blueberries have a significant economic impact on the state of Maine. The ongoing research on their effects on health and disease has resulted in high consumer demand for these berries, which is growing in both the US and abroad.

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