ANALYSIS OF THE POTENTIAL FOR *RUBUS* FRUIT ELLAGITANNINS TO INDUCE ANTI-INFLAMMATORY EFFECTS IN *IN VITRO* MODELS

A dissertation presented in fulfilment of the requirements for the degree of

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Abstract

Nutrigenomics is the study of interactions between human diets and the genome with the aim of discerning how food components influence gene and protein expression in individuals. Previous studies have established the potential for ellagitannins (ETs) isolated from various fruits, to induce anti-inflammatory responses that may be beneficial to those afflicted with Inflammatory Bowel Disease (IBD).

The hypothesis of this thesis is that *Rubus* fruit extracts, rich in ETs, induce an anti-inflammatory effect in mammalian cells involved in inflammatory processes.

Methods were developed to extract, purify, and quantify the phytochemical composition of three selected *Rubus* fruit cultivars (HB19, Wakefield and ZZ).

Extracts (EX: phenolic extract, ETx: ET-enriched extract) were initially assessed using a secondary cell line: RAW 264.7 macrophages. Cell viability using the MTT assay, found no cytotoxic effect at polyphenolic concentrations up to 100 µg/mL. The Griess assay measured levels of nitric oxide (NO) production and found a decrease at 50 µg/mL of polyphenolic exposure for most extracts except ZZ EX. Cytokine assessment showed that the extract, Wakefield ETx, lowered production of Interleukin 6 (IL6) by macrophages. All extracts increased production of Tumour Necrosis Factor alpha (TNFα) by macrophages.

Colonic intestinal epithelial cells (colonic IEC) and bone marrow derived macrophages (BMDM), were isolated from control C57BI/6J mice and Interleukin 10 gene-deficient mice (*Il10*$^{-/-}$). Viability results suggested that the polyphenolic concentration of 50 µg/mL, was the highest tolerated concentration for NO and cytokine assays. NO production was decreased for BMDM (*Il10*$^{-/-}$) with extracts Wakefield EX and ZZ EX, and for BMDM (C57) with most extracts except HB19 EX. IL6 production by colonic IEC (*Il10*$^{-/-}$) was decreased for all extracts. The cytokine profile for BMDM (*Il10*$^{-/-}$) mice showed a decrease in IL12 and IL6 production, and no change in TNFα production in response to co-incubation with all extracts.

There are indications from research done in this thesis that the *Rubus* fruit extracts from “ZZ” and “Wakefield” cultivars reduce inflammatory immune responses or potentially having the ability to act on different pathways or induce a wound healing response.

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Abbreviations Used

Abbreviation	Full meaning
BMDM	Bone marrow derived macrophages
C57	C57BI/6J mice
CA	Cellulose acetate
CD	Crohn's Disease
CFU	Colony forming units
CIF	Conventional intestinal flora
COX2	Cyclooxygenase-2
Complete BRPMI	Complete Bone Marrow RPMI-1640
Complete IRPMI	Complete IEC RPMI-1640
DM	Dry matter
Complete DMEM	Liquid high-glucose Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
DTT HBSS	HBSS 1% Pen/Strep with 15 mM DTT
EA	Ellagic acid
EGF	Epidermal growth factor
ES	*Enterococcus* suspension
ET	Ellagitannin
ETx	*Rubus* fruit ellagitannin enriched extract
EX	*Rubus* fruit phenolic
FBS	Foetal bovine serum
FW	Fresh weight
GHS	General Health Score
GIT	Gastrointestinal tract
GWAS	Genome-wide association studies
HBSS	Hanks' Balanced Salt Solution
HETx	HB19 ellagitannin extract
HEX	HB19 phenolic extract
HHDP	Hexahydroxydiphenoyl
HPLC	High-performance liquid chromatography
IBD	Inflammatory Bowel Disease
IEC	Intestinal epithelial cells
IFNγ	Interferon γ
IL	Interleukin cytokine (family group name)
Il10$^{-/-}$	IL10 gene-deficient mice
Lam C	Lambertianin C
LCMS	Liquid chromatograph-mass spectrometry
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
M-CSF	Macrophage colony stimulating factor
MTT	MTT assay
NED	Naphthylenediamine dihydrochloride
NF-κβ	Nuclear factor-κβ
NO	Nitric Oxide
NOD2	Nucleotide oligomerisation domain 2

PBS	Phosphate buffered saline
PE	Pomegranate extract
Pen/Strep	Penicillin and Streptomycin
Plant & Food Research	The New Zealand Institute for Plant & Food Research Limited
RPMI	Liquid Roswell Park Memorial Institute medium
RT	Room temperature
SH6	Sanguiin-H6
SNP	Single nucleotide polsymorphisms
T_h 1	T helper 1
TLR	Toll-like receptor
TNFα	Tumour Necrosis Factor alpha
UC	Ulcerative Colitis
Uro	Urolithin
WETx	Wakefield ellagitannin extract
WEX	Wakefield phenolic extract
WST-1	WST-1 assay
ZETx	ZZ ellagitannin extract
ZEX	ZZ phenolic extract

Chapter 1: Literature Review

1.1 Preface

Diet can be an important contributing factor to many chronic diseases, including inflammatory conditions such as Inflammatory Bowel Disease (IBD). IBD has two main forms, Crohn's Disease (CD) and Ulcerative Colitis (UC), which differ in physical presentation and immunohistopathology [1]. To date a number of genes have been identified that contribute to IBD susceptibility and disease development, which in combination with certain external factors leads to full disease expression [2, 3]. At present there has been little investigation as to specific food components that can influence the development of IBD. Consequently, only limited general dietary advice for IBD patients is available, rather than advice tailored to an individual's dietary preference, tolerance or sensitivity, factors which may be influenced by their genetic profile.

Nutrigenomics is the study of interactions between human diets and the genome, with the aim of discerning how food components influence gene and protein expression in individuals or how their genetic makeup influences their metabolic interactions with certain food compounds. Using a nutrigenomic approach, personalised dietary advice may be able to provide individuals with an understanding of whether or not they will have any effect on the amelioration or aggravation of a given disease [4-6].

Ellagitannins (ETs) are polyphenolic compounds that are naturally occurring secondary metabolites in some plant families [7, 8]. These compounds have demonstrated anti-oxidant, anti-cancer and anti-inflammatory properties. *Rubus* fruits, such as red raspberry and Boysenberry, contain high concentrations of these compounds [7, 9, 10]. Recent research in other ET-rich fruit species, has demonstrated potential for these compounds to reduce inflammation, which may be of benefit to some IBD patients [11-18].

This thesis examines the putative anti-inflammatory effects that Boysenberry and red raspberry extracts may have in the context of IBD. Phytochemical analysis was used to identify the composition and concentration of ETs found in three selected cultivars, followed by preparation of phenolic extracts and further purification to prepare ET-enriched extracts. *In vitro* models were then used to investigate the potential anti-inflammatory effects of these extracts. The introduction section of this thesis presents general background for the key topics

of nutrigenomics, IBD, and *Rubus* phytochemistry. At the beginning of each subsequent chapter a more specific introduction has been included pertaining to the research addressed.

1.2 Nutrigenomics in Applied Research

Nutritional research has traditionally focused on dietary intervention and the development of generic healthy guidelines with preference for the ease of accessibility of information for the general public, without allowing for genetic differences between individuals [5]. These dietary guidelines are designed to aid in the maintenance of a healthy lifestyle and disease prevention, are generally based solely on the basic "food pyramid", and may include reference to nutritional recommendations that are dependent on age and sex. Such guidelines are set out in each country and differ slightly but all have the same goal of preventing disease onset [5].

Classical research has concentrated primarily on the effects of nutritional deficiencies, and the potential harm of both imbalanced macronutrients, vitamins and minerals or toxic concentrations of certain food components. The shift in focus to beneficial non-nutrient components of food and their effects on metabolism has only begun to be explored more deeply in recent years [4]. Correspondingly, the understanding behind nutrient-gene interactions and health outcomes, that are dependent on individual genotypes are still being developed. The combination of these two key aspects of research, along with recent technical advancements in molecular biology and chemistry, has led to the emergence of new fields of study based on personalised nutrition for maintenance of health, or to preven, or delay the onset of disease.

Nutritional science has two main branches that investigate the effect of genetic variation and personalised dietary response to different nutrients and food components: nutrigenomics and nutrigenetics [5]. Nutrigenetics looks at the effects of genetic variation on the interaction between diet and health, and how diet can influence an individual's predisposition to develop or suffer an illness [19]. Nutrigenomics is the study of how nutrition influences metabolic pathways and homeostasis, through the alteration of genes, proteins, and metabolites, and the resulting effect on physiology. Through insight into which genes or biochemical pathways are affected by food components, inferences can be made as to which individuals or population groups (based on genotype) are likely to respond to particular foods. The aim of both disciplines is to unravel diet-gene interactions, albeit via different approaches.

Ultimately, the amalgamation of both will be required to achieve the long term goal of optimising an individual's health through nutritional intervention [6].

The completion of the Human Genome Project in 2011 (*20*), helped launch the study of Nutrigenomics and Nutrigenetics. The publication of whole genome sequences for humans and other species combined with on-going studies to characterise genetic variation within a genome [20, 21], has allowed researchers to begin to link genotype to phenotype.

Genome wide association studies (GWAS) test for statistical associations between genotype and phenotype for hundreds of thousands of single nucleotide polymorphisms (SNP) [22], has allowed for the identification of genetic variants for many chronic diseases. In IBD research, a candidate gene study in 2001 identified Nucleotide oligomerisation domain 2 (NOD2) as an important disease gene [23, 24], followed soon after by the immune recognition gene Toll-like receptor 4 (TLR4). However, subsequent GWAS on this disease have revealed the importance of other SNPs in unsuspected genes such as Interleukin 23 receptor (IL23) [25], and the autophagy gene ATG16L1 [26]. These genes are involved with the response to environmental factors such as bacteria and diet.

Coinciding with the development of whole genome sequencing, the advent of new research tools such as the "omics" technologies and bioinformatics, has provided mechanisms to define a phenotype at a specific point or physiopathological state. By employing transcriptomics (gene expression analysis), proteomics (protein expression analysis), metabolomics (metabolite profiling), to compare healthy and diseased phenotypes, biomarkers associated with a specific phenotype can be identified [4]. The ability to link genotype to phenotype and to correlate changes in gene, protein and metabolite expression with nutritional intervention are core to the concept of nutrigenomics and the ultimate goal of personalised nutrition for favourable health outcomes.

Food composition databases are now being established that contain information on the composition, and biological effects in humans of bioactives in plant-based foods [27]. These databases, in combination with a wealth of literature on the anti-inflammatory activity of plants and plant-based extracts, provide a good basis for the selection of food types to assess for efficacy in targeted intestinal health nutritional intervention studies.

In vitro model systems, utilising murine primary or secondary macrophages and intestinal epithelial cells, are often used initially to assess the anti-inflammatory efficacy of food components or extracts prior to more in-depth and expensive *in vivo* studies. *In vivo* studies

typically use small animal models of IBD, prior to human studies, to provide a complex system, including interactions between the host, commensal microbiota, diet and environment to further assess anti-inflammatory efficacy, and allow the collection of tissue samples to investigate dietary-induced changes in gene and protein expression levels using transcriptomic and proteomic techniques.

1.3 Inflammatory Bowel Disease

IBD is a chronic inflammatory condition that affects the gastrointestinal tract (GIT) whose incidence is on the rise in the "developed" world. UC and CD are the two most common forms of IBD. Although the two forms have distinctive characteristics, they share many common symptoms and can be difficult to distinguish clinically [28].

While the exact aetiology and pathogenesis of IBD is still unclear, it appears to involve complex interactions between the immune system, the environment, microbiota, and a number of host susceptibility genes (Figure 1). Dysbiosis (irregular symbiotic relationship) to the commensal intestinal microbiota is one of the important environmental factors identified as a likely trigger in the chronic inflammatory response seen in IBD [29].

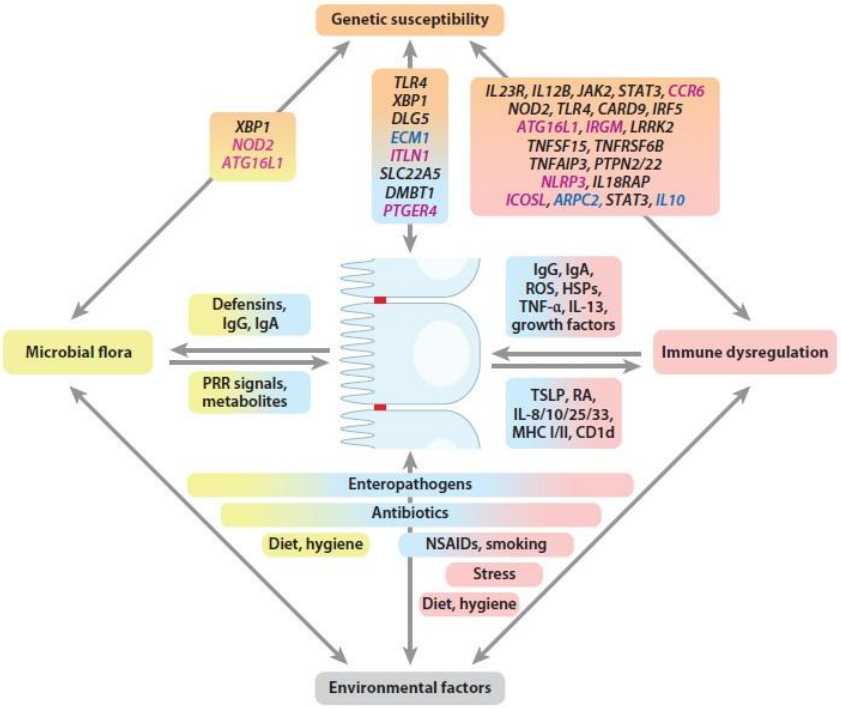

Figure 1: Demonstration of the complex interactions in the development of IBD. Colours indicate the relationships between the identified factors. From Kaser et al., 2010 [1].

Therapeutic options for the management of IBD include aminosalicylates, antibiotics, corticosteroids, antimetabolite immunomodulators and biologic therapies such as anti-TNF agents. However, clinical efficacy is limited with low rates of sustained remission and increased risks of serious infections and malignancies with some of these treatments [30]. The increased recognition of the limits of conventional medicine for the management of IBD has helped drive growing interest in complementary approaches such as nutritional intervention.

1.3.1 Characteristics and Pathology

The two main forms of IBD, UC and CD, have varied immunohistopathological presentations and are multifactorial on whether or not an individual develops the disease. The pathology of IBD is characterised by inflammation and ulceration of the GIT and presents clinically with abdominal pain and altered intestinal function, including diarrhea, constipation, improper sense of evacuation, bloating and rectal bleeding [31]. While CD and UC share many common symptoms and can be difficult to distinguish clinically, they typically differ in the nature and location of the inflammation in the GIT (Figure 2).

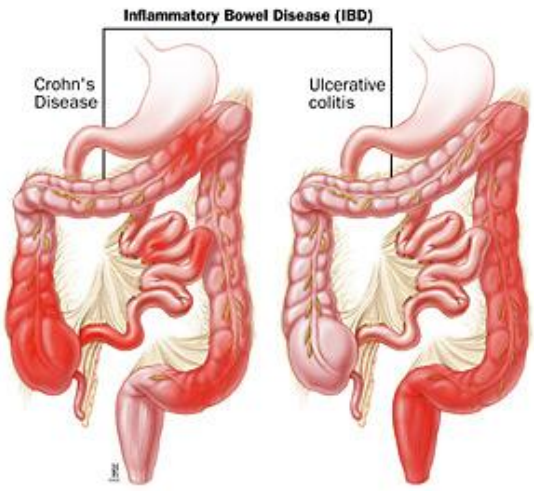

Figure 2: Anatomic distribution of CD and UC in GIT. Taken from http://hopkins-gi.org/GDL_Disease.aspx?CurrentUDV=31&GDL_Disease_ID=2A4995B2-DFA5-4954-B770-F1F5BAFED033&GDL_DC_ID=D03119D7-57A3-4890-A717-CF1E7426C8BA

In CD, the inflammation is not confined to any specific region of the GIT but is most commonly found in the ileum [28], and the inflammation presents with discontinuous patches,

predominantly with transmural involvement and granulomas. In contrast, UC is characterised by the inflammation beginning in the rectum, and spreading continuously and proximally through the GIT (Figure 2).

Disease related malnutrition, weight loss, and sub-optimal nutritional status may be present at any stage of IBD [32]. This is primarily due to poor dietary intake which occurs as a result of altered GIT state, impaired nutrient digestion and absorption [32]. Lean body mass and bone density have also been shown to be significantly reduced in IBD patients [32]. As with any chronic inflammatory disease the long-term effects associated with IBD are atrophy (wasting), fibrosis and increased rick of cancer.

It has been demonstrated that there is a change of normal cellular characteristics in both forms of IBD. There are also notable changes in cytokine production, although whether or not this is genetically or microbially based is yet to be fully quantified. Interestingly, studies examining the involvment of the nervous system have found a sensitivity of enteric nerve cells to the cytokines Interleukin 1 beta (IL1β), TNFα and Interleukin 23 (IL23), which are already known to be important in IBD as they are generally over-expressed [33]. Overstimulation of the nervous system in IBD can lead to enhanced neuronal signalling which can induce the perception of normally harmless stimuli as being painful [33], likely caused in association with the rise in cytokine levels.

1.3.2 Genetic Component

Twin and large scale population studies provide the most compelling evidence for a genetic influence in IBD [33, 34]. Concordance of the disease in monozygotic twins approaches 50% in CD, and up to 18 % for UC. These rates are higher than the concordance rates in dizygotic twins, which are no different from the expected rate in siblings of ~5 % [35]. Although genetic influences are clear, these studies also indicate important environmental inputs, particularly in UC where no concordance of disease is found in the vast majority of patients with identical genetic background (twins). The complex familial inheritance patterns seen in IBD indicate a number of gene variants working in concert to contribute to overall disease susceptibility rather than a single gene polymorphism (SNP) or mutation, interacting with environmental factors to cause disease [35].

Currently GWAS have identified 163 genetic risk loci for IBD [36], of which 28 are shared between CD and UC (Figure 3). Analyses of the genes and genetic loci implicated in IBD show

the disruption of several pathways that are crucial for the development of correct intestinal homeostasis including: barrier function, epithelial restitution, microbial defense, innate immune regulation, reactive oxygen species generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum stress and metabolic pathways that are associated with cellular homeostasis (Figure 4) [28].

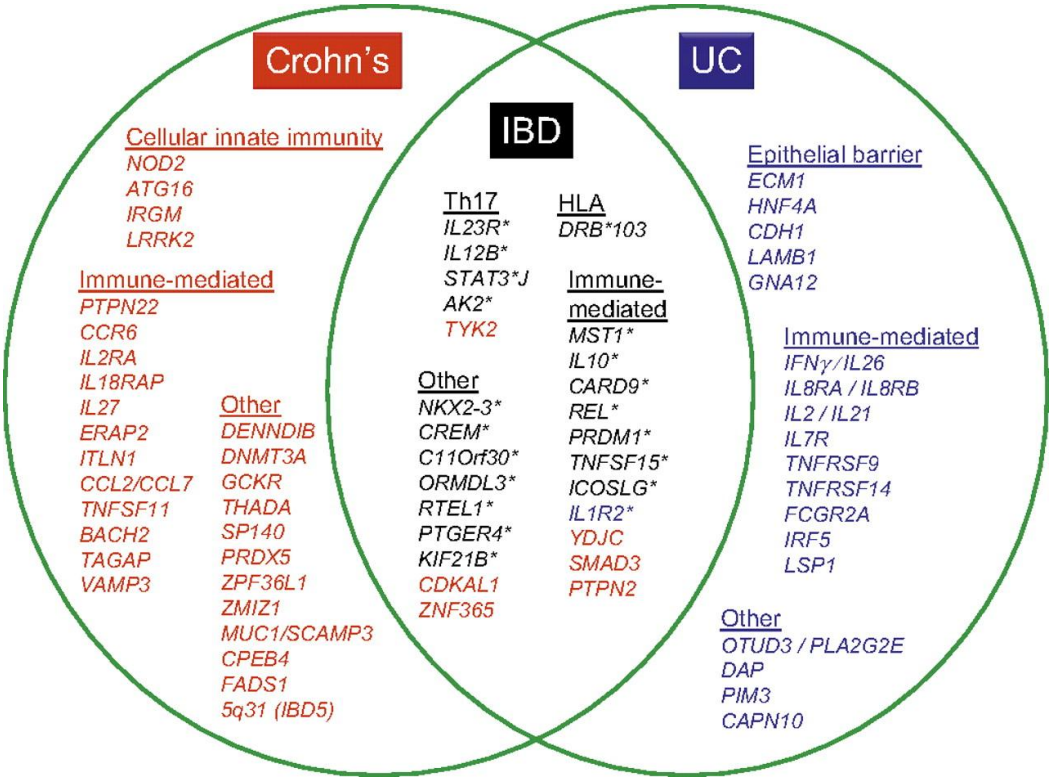

Figure 3: IBD susceptibility loci. The loci (depicted by lead gene name) attaining genome-wide significance (P < 5×10−8) are shown for CD (red), UC (blue) and IBD (black). Taken from Lees et al., (2010) [37].

Multidisease comparative analysis has revealed that more than 50% of IBD susceptibility loci have also been associated with other inflammatory and autoimmune diseases, indicating that there is a common link between many chronic diseases. Interestingly, some of the most important CD susceptibility genes identified from GWAS - NOD2, autophagy related 16-like protein 1, and Immunity-related GTPase family M protein - are not associated with UC [35] (Figure 3). These genes are important in sensing and responding to microbial antigens

suggesting an underlying difference in the significance of microbial antigens between the two main forms of IBD in their pathogenesis.

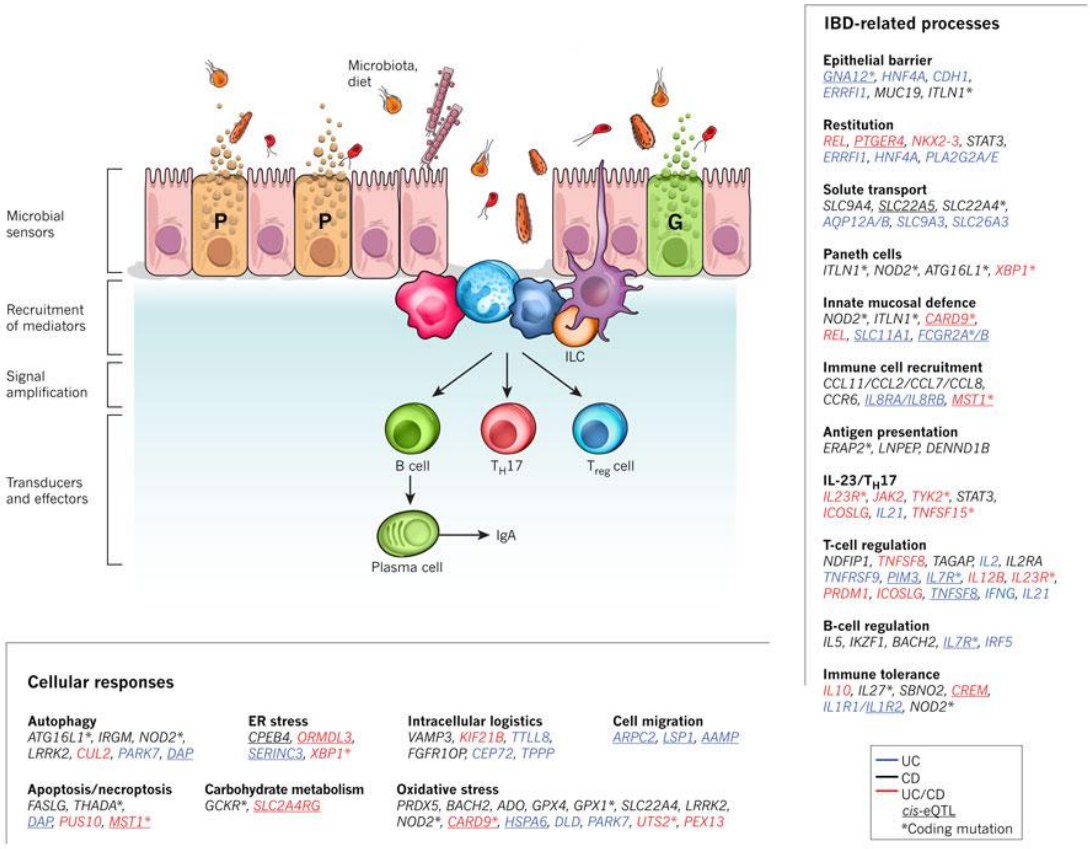

Figure 4: A model for IBD pathways based on genome-wide association studies. Take from Khor et al., (2011) [28].

To date, however, genes identified from GWAS account for only 23% and 16% of the heritability in CD and UC, respectively. These may be underestimates owing to the net effect of common variants that are individually too small to calculate accurately. Other genetic and epigenetic, as well as, interaction with non-genetic (including environmental) components are believed to be important factors [28].

1.3.3 Microbial Involvement

The intestinal microbiota are a natural community of microbes that have co-evolved with the host and provide a number of beneficial effects such as aiding in metabolism, controlling immune responses and defense against pathogens [38]. When inflammation or changes in diet disrupt the balance in this environment it can induce the onset of IBD [39]. Several studies within the context of IBD and microbial symbiosis have found that there is an increase in the pathogenic bacterial species *E.coli* and *Bacteriodes* spp., with a corresponding decrease in the healthy intestinal microbial families *Lactobacillus* and *Bifidobacterium [40]*.

The dynamic balance at the mucosal barrier between naturally occurring microbes (commensal) and the host immune defense system responses, has a pivotal role in the initiation and development of IBD [41, 42]. Commensal bacteria can increase growth of pro-inflammatory microbial species or can restrict the growth of beneficial microbial species and their protective by-products [29].

It is difficult to grasp the complexity of how the composition of the intestinal microbiota and their interaction with the host's immune system contributes to the interplay of gene-environment interactions. In the context of IBD this interaction is not fully understood, but research into this area is on the rise [28]. The importance of microbial involvment with IBD is evidenced by both noticable microbiota changes in patients, and the discovery that many of the underlying susceptibility genes linked to IBD are associated with sensing and response to microbiota [39].

In humans there is an inverse relationship between the presence of *Faecalibacterium praunitzii* in the colon microbiota and the development of IBD [43]. *F. praunitzii* belongs to the family group *Firmicutes* which is a dominant phylum within the intestinal microbiota. There has been repeated observation of the decrease in abundance and biodiversity in intestinal microbiota in CD patients which was demonstrated through metagenomic analysis [44]. In recent work, it was found that *F. praunitzii* has anti-inflammatory effects *in vitro*, as well as, *in vivo* [44]. Treatment by either *F. praunitzii* or its culture supernatant tended to counterbalance the dysbiosis that is observed in colitic control mice when compared with other bacterial types under investigation [44].

1.3.4 Environmental Influences

Environmental stimuli, such as nutrients, are one of the key factors affecting the incidence and development of disease [19]. Nutrients have the ability to affect cells by altering the expression levels of genes. This change in expression level can be brought about by modulation via transcription factors or through induction of epigenetic changes in the cell (among other factors) [5].

Understanding of genotype-diet interaction could help inform dietary changes to mitigate the risk of developing chronic disease in genetically predisposed individuals. Many studies have looked at providing definitive evidence for a specific food component that may cause IBD, but nothing conclusive has been shown, other than a linkage to certain risk factors such as smoking, low fibre, high sugar, stress, and high amounts of animal fat [32, 45].

Currently basic nutritional advice for disease looks at modulating the immune system to create a desired outcome, and does not take into consideration an individual's genetic differences. Therefore, further exploration into understanding how the susceptibility of genetic variants facilitates the development of chronic diseases, such as IBD, whilst also taking into consideration associated environmental risk factors (such as diet) is needed [46].

1.3.4.1 Nutrigenomic Assessment of Environmental Influences

There is much economic interest in the outcomes of nutrigenomic research [47, 48]. The food industry is continually developing new products that cater to consumers' specific dietary needs or preferences. A clearer understanding of the genetic basis for the potential health benefit of these foods could bring about a dramatic change in food consumerism and marketing to also take into consideration the genetic basis of responses to food [49]. At present, food is generally selected according to preference for taste, convenience, and appearance rather than potential health benefits [5].

To understand the potential health benefits, one approach is to use targeted *in vitro* investigation to first examine the effects of a selected food component or nutrient of interest, before moving forward to animal models that mimick a human disease of interest [50]. Presently human clinical trials are not commonly used in nutrigenomic research [5]. Two examples are discussed below to demonstrate different methods that can be applied to study the interaction of nutrients with specific genes in association with IBD.

Philpott et al., (2007) [51] developed two cell-based assays in order to test the ability of food components to modulate pathways associated with specific genotype variants of IBD. The first assay tested the ability of the food components to inhibit Tumour Necrosis Factor alpha (TNFα) secretion (an important cytokine in the development of inflammation and measured at high concentrations in people with IBD), via two stages of testing. The first stage utilised RAW 264.7 murine macrophages stimulated with unpurified lipopolysaccharide (LPS) to assess the decrease in TNFα [52], and found that two extracts of kiwifruit demonstrated strong inhibition. The second stage of experimentation specifically looked at these extracts' ability to inhibit responses controlled by specific ligands of NOD2, Toll-like receptor 4 (TLR4) and TRL9, all of which proved to inhibit TNFα production no matter which ligand was involved with stimulation [51].

The second assay developed by Philpott et al., was a NOD2 genotype-specific assay. This gene has been found to play an important role in the development of CD. Using directed mutagenesis, three cell lines were generated to express no NOD2, mutant NOD2, or wild-type NOD2, and all were co-transfected with a reporter plasmid. The three cell lines were incubated with or without food extracts at various concentrations, followed by further incubation with or without muramyl dipeptide. After incubation the nuclear factor-κβ (NF-κβ) activity and cell survival were assessed (NF-κβ is a protein complex that controls transcription of DNA encoding cytokine and other stress responses). A blueberry extract tested using this assay demonstrated a decrease in NF-κβ activity, in a concentration-dependent manner for the NOD2-overexpressing lines, but not in control or mutant cell lines suggesting that the extract acts purely as a general anti-inflammatory.

Larossa et al., (2010) [43] used a rat model to study the effect of pomegranate extract (PE) and a PE metabolite, Urolithin A (Uro A), on colonic inflammation. Male Fisher rats were fed the extracts for 25 days, following which dextran sodium sulfate (DSS) was used to induce colitis-like inflammation (a well known model of IBD). There was a noted attenuation of the severity of colonic injury in the group fed Uro A. Both extracts led to an increase of the favourable microbial species *Bifidobacteria* and *Lactobacilli* in feces compared with controls. Dietary supplemention with both extracts led to a noticeable decrease in the expression of cyclooxygenase-2 (COX2), inducible-nitric oxide synthase (iNOS), nitric oxide (NO) levels, and prostaglandin E_2, when compared with a DSS control group [43].

Microarray analysis was conducted to assess the changes in gene expression with the different treatments. Of the genes modified by PE, 32% were also modified by Uro A. Genes

modified of significance were tumour suppressor retinoblastoma 1 and protein p53 [43], which are improtant genes related to cancer. In combination, these results suggest that Uro A is most likely a strong anti-inflammatory compound, and its production through metabolism of the PE extract would have also contributed to the results demonstrated by PE.

1.4 Plant Polyphenols

Polyphenolic compounds are secondary metabolites synthesised by plants, both during normal development and in response to stress conditions such as infection or wounding,to protect against UV damage, microbial and insect attack [12, 53, 54]. They are often found in the outer layers of the plant or fruit, likely as a result of their function in protecting the plant from environmental stresses and pathogenic attack [15]. Concentrations can be affected by stress, cultivar, cultivation techniques, growing conditions, ripening process, as well as processing and storage [55]. Polyphenols have been reported to confer beneficial biological properties in foods that may contribute to disease risk reduction and human health promotion, including anti-oxidant, anti-inflammatory, metal chelation, antimutagenic, anticarcinogenic, antimicrobial, and clarifying agent properties [12, 56].

Polyphenols can be divided into several different classes: phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavonols, flavones, isoflavones, anthocyanins, and proanthocyanidins), lignans and polymeric tannins [15]. They show great diversity in structure, ranging from simple molecules (monomers and oligomers) to polymers. Higher-molecular-weight structures (with molecular weights of >500) are usually identified as tannins.

1.4.1 Ellagitannins (ETs)

Plant tannins may be divided into two large groups: condensed and hydrolysable. Condensed tannins are formed through the condensation of flavan-3-ols (catechins or epicatechins) and are often referred to as proanthocyanidins. Among the more than 500 hydrolysable tannins characterised to date, the ETs constitute the largest tannin group of this type [18]. ETs produce ellagic acid (EA) upon hydrolysis. The remaining group of tannins, gallotannins (galloylglucoses), produce gallic acid upon hydrolysis.

ETs are characterised by the presence of one of more hexahydroxydiphenoyl (HHDP) moieties esterified to a sugar, usually glucose. The HHDP group is biosynthetically formed through intramolecular oxidative C-C bonds between neighbouring galloyl groups in galloylglucoses. ET

compounds have enormous structural variability due to the many different possibilities for the linkage of HHDP residues with the glucose moiety, and their strong tendency to form dimeric and oligomeric derivatives [38, 57].

The HHDP-sugar ester linkage is easily hydrolysed under strongly acidic or basic conditions and the HHDP group spontaneously rearranges itself to form EA, a dimeric derivative of gallic acid. EA is present in plants in either its free form as an EA derivative, or bound as an ET. Numerous derivatives of ETs exist in plants, formed through methylation, glycosylation, and methoxylation of hydroxyl groups [12].

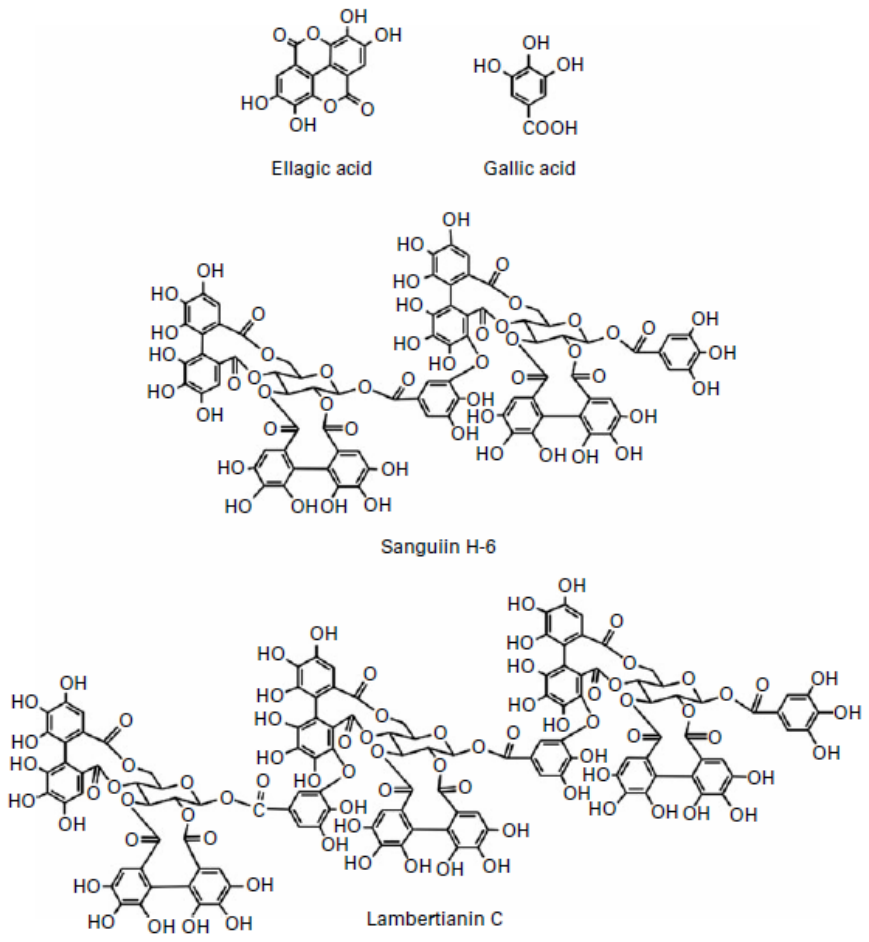

Figure 5: The chemical structure of the two main ETs found in red raspberry and Boysenberries (the focus of this thesis), as well as, EA and gallic acid. EA is the hydrolysed product of these ETs, and dimeric derivative of gallic acid. From Del Rio et al., (2010) [58].

ETs have the potential to play an important role in human nutrition and health. They are purported to have numerous biological properties such as: antioxidant [8, 10, 17, 53, 59-63], anticancer [53, 60, 63-65], anti-inflammatory [11, 43, 66-68], and antibacterial [15, 16] activities. These beneficial biological activities have led to the increased scientific interest in ETs for their potential application in the promotion of human health and in alleviation of chronic disease [69].Common dietary sources of ETs and EA are fruits, seeds and in foods or beverages based on fruit, such as juices or jams. Berries of the family Rosaceae (red raspberry, cloudberry, rose hip, strawberry, and Boysenberry) contain high levels of ETs or EA equivalents, as do pomegranate, walnut and pecan nut.

1.4.2 *In Vivo* Metabolism of Ellagitannins (ETs) and Ellagic Acid (EA)

Available experimental data indicates that intact dietary ETs are generally not detected in plasma or other biological fluids after the intake of ET-rich foods [70]. ETs have been shown by *in vitro* digestion simulation studies to be stable under the physiological acidic conditions of the stomach, and no degradation to EA or absorption was observed. Under the physiological near neutral conditions of the small intestine, however, there is a release of free EA from ETs. This hydrolysis is believed to be due to the pH conditions (neutral to mild alkaline pH, 7.0–7.3) rather than to the effect of pancreatic enzymes and bile salts [57, 71, 72]. EA released is poorly absorbed in the small intestine, and largely metabolised by unidentified bacteria in the GIT to produce dibenzopyranones or urolithins (Uro) (Figure 6). Analyses of GIT tissues show that the metabolites are absorbed preferentially as their degree of hydroxylation decreases and corresponding lipophilicity increases as they move through the GIT (Figure 7) [38].

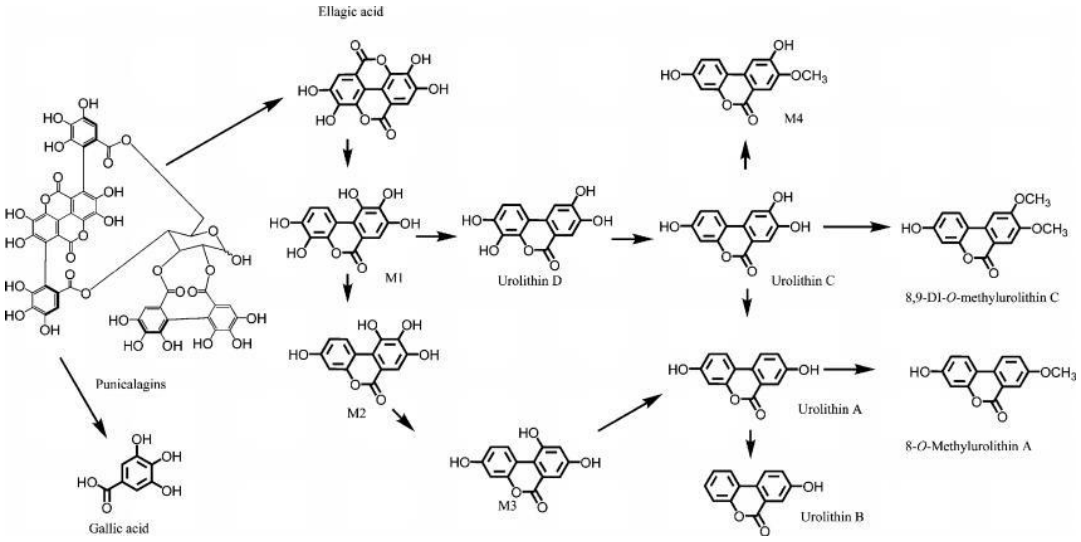

Figure 6: The proposed metabolism of EA (from ET origin: punicalagin) to Uro's by microbial action. The exact enzymes and microbes that are involved in these processes are unknown. From Bialonska et al., (2009) [73].

The absorbed metabolites of ET are conjugated by phase II metabolism enzymes with glucuronic acid, sulfates and/or are methylated. Uro A and Uro B conjugates are the main metabolites detected in plasma and urine. Uro C, Uro D and EA derivatives are not detected in the peripheral plasma, but are absorbed in the small intestine and transported to the liver. Here they are further metabolised and excreted with bile to the small intestine leading to an entero-hepatic circulation that is responsible for the long life of urolithins in plasma. Several tissue disposition studies report that urolithins are found in intestinal, colon and prostate tissues in mice, in liver and kidney of rats [74] and in human prostate [69] following consumption of ET-rich foods.

A study following the fate of EA, ETs and anthocyanins after consumption of red raspberries by either healthy human volunteers or subjects with an ileostomy showed marked variation in the Uro profiles amongst individuals. This variation was attributed to differences in the colonic microbiota responsible for ET degradation [69].

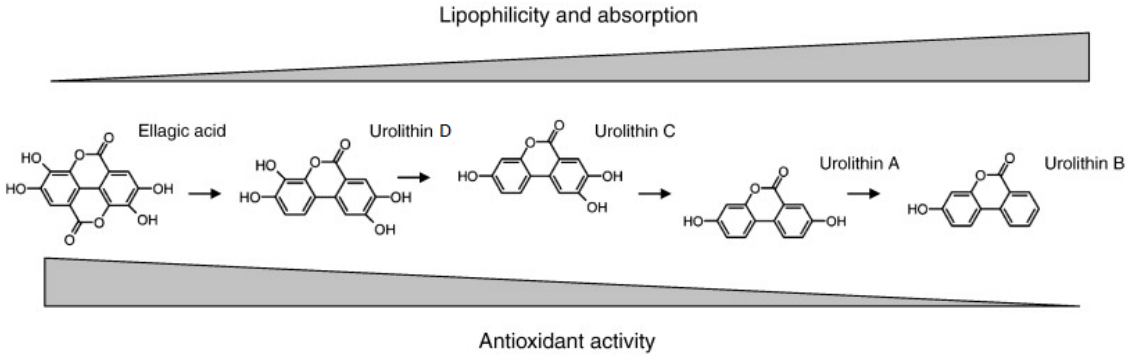

Figure 7: EA bacterial metabolites. Increasing bacterial metabolism involves decreasing anti-oxidant activity and increasing lipophilicity and intestinal absorption. From Landete (2011) [38].

1.4.3 Ellagitannins (ETs) and Ellagic Acid (EA):

Potential for Amelioration of IBD

Research indicates that intake of ET and EA-rich foods may be protective against certain chronic diseases, including those associated with chronic inflammation.

A recent rat study by Ramirez et al., (2010) [75] suggested that EA may have potential to ameliorate symptoms associated with UC. Rats fed EA both before and after 2,4,6-trinitrobenzenesulfonic acid induced UC onset, showed reduced inflammation formation, and accelerated healing through proinflammatory down-regulation-mediated mechanisms [75]. They also noted a change in intestinal microbiota, observing increased growth of protective intestinal strains. As previously mentioned, the interactions between the microbiota and polyphenolic extracts are critcal in the role that the polyphenolic extracts may have in disease alleviation and prevention.

A study by Larrosa et al., (2010) [43] investigated the anti-inflammatory properties of pomegranate in a colitis rat model to determine whether the ETs or the microbial-derived ET metabolite Uro A was the main anti-inflammatory compound. Oral administration of pomegranate extract (PE) or Uro A prior to induction of colitis resulted in the modulation of intestinal microbiota. Uro A supplementation was more effective than PE in ameliorating inflammation, although both showed a clear effect on inflammatory markers. It was also reported that pomegranate metabolism by rats with colitis was different from those without colon inflammation. The low metabolism exhibited by the altered microbiota of inflamed rats

allowed EA and even some ETs from the PE to reach the colon and exert a possible antioxidant effect. The authors concluded that Uro A is likely the main active anti-inflammatory compound related to pomegranate consumption by healthy subjects (with normal metabolism). However, in the case of inflamed colon such as in IBD, the ET and EA fraction together with some Uro A could act as a synergistic anti-inflammatory cocktail [76].

More recently, Gimenez-Bastida, et al., (2012) [77] reported that a mixture of EA, Uro A and Uro B, at concentrations representative of those found *in vivo* in the human colon after dietary intake of ET-rich foods, ameliorated cytokine induced inflammation and associated downstream molecular markers in human colon fibroblasts. Of the three metabolites tested, Uro A exhibited the most significant anti-inflammatory effects.

Furthermore, it has been suggested that ETs may change the composition of intestinal microbiota. A recent human study has shown that patients suffering from IBD, in particular CD, exhibit overall higher intestinal bacterial cell counts, a general loss of biodiversity, a preponderance of *Bacteroidetes* with a parallel decrease of *Firmicutes* phylum. The authors theorised that ETs may change the ratio of the two phyla, thus helping to correct the microbial imbalance [78].

1.4.4 Rubus fruits of New Zealand and their Polyphenols

Red raspberries, blackberries and hybrid berries (including Boysenberries, loganberries and other hybrid types) belong to the genus *Rubus*, in the family Rosaceae. *Rubus* berries, like other berryfruit (e.g. blueberry), are a rich source of dietary phenolics including flavonoids (anthocyanins and flavanols), condensed tannins, hydrolysable tannins, stilbenoids, and phenolic acids. They differ, compositionally, however, from commonly consumed berries, such as blueberries and blackcurrants, in the particular class and levels of tannin molecules. While many berry fruit varieties contain predominately condensed tannins (proanthocyanidins), *Rubus* berries are rich sources of hydrolysable ETs. The classes and specific chemical structure of tannins present in particular berry types may contribute to their unique biological properties [79]. There are four main classes of polyphenolic compounds that are found in *Rubus* fruit: flavonoids, phenolic acids, lignans, and tannins [17].

Boysenberries (Figure 8) are a thorny septaploid that produce large red-wine fruit [80]. They are derived from a cross between loganberry (*Rubus loganobaccus*) and blackberry (*Rubus baileyanus* Britt.) and were introduced to New Zealand from Knott's Berry Farm, California, in the mid-1930s. The plant produces flavoursome fruit, that is suitable for freezing, canning and jam-making. Plant & Food Research's, *Rubus* berry breeding programme has developed several new Boysenberry cultivars with varying beneficial agronomic characteristics over the last 25 years, such as pest and disease resistance, yield and chill requirements and fresh market appeal [80]. The most commonly commercially grown varieties in New Zealand include 'Mapua', 'Tasman' and 'Riwaka Choice'.

New Zealand is a significant producer in the global Boysenberry market, producing approximately 4000 ton of Boysenberry each year, up from 2500 ton in 2005 [81]. At present 95 % of the crop is sold on the international market as frozen whole fruit, processed puree or juice concentrate, with annual sales of NZD $6–10 million [82].

Red raspberries (*Rubus idaeus*) (Figure 8) have been grown in New Zealand since early European settlement; however, their commercial cultivation in New Zealand has been a story of boom and bust. At its market peak in 1985, red raspberries were New Zealand's fifth most valuable fresh export (~2600 ton/annum), with Australia being the main market. A pest infection in 1988 by raspberry bud moth (*Heterocrossa rubophaga*), which is endemic to New Zealand, led to the closure of this market and significant industry decline [83].

Figure 8: The two main *Rubus* fruit grown in New Zealand Boysenberries (right) and red raspberries (left). Picture courtesy of The New Zealand Institute for Plant & Food Research Limited.

In collaboration with Northwest Plant Company (USA), Plant & Food Research has recently bred a new red raspberry cultivar called 'Wakefield', which has a number of improved benefits over other red raspberry cultivars, such as improved disease resistance, higher crop yield, and higher phenolic levels. This cultivar is now commercially available in the USA and New Zealand (and was one of the cultivars selected for this study).

ETs are the predominant phenolic components of Boysenberry and red raspberry, comprising at least 30% of the total phenolic content for the latter [17]. Anthocyanins are the next most abundant phenolic class, with the remainder of the phenolic profile comprising minor components such as phenolic acids, flavanol monomers and flavonol–glycosides [84].

The major ETs detected in *Rubus* fruit are SanguiinH-6 (SH6) and Lambertianin C (Lam C). However, the levels of these compounds reported in the literature are closely dependent on the analytical method employed, and are not always comparable [9]. Due to the lack of commercially available standards, quantification of ETs has usually been carried out after acid hydrolysis and ET levels reported as EA equivalents. More recently, researchers have endeavoured to measure ETs in their native form by isolating and purifying major ETs, such as SH6, for use as external standards, allowing other ETs to be quantified more meaningfully as SH6 equivalents [85].

Gasperotti et al., (2010) [85] characterised the ET profile of a number of red raspberry cultivars and reported that SH6 and Lam C, represent 73–86% of the total ETs measured. Ten minor ETs and three EA conjugates were also identified, with concentrations at least an order of magnitude less than that found for SH6 and Lam C. Additional studies reporting ET profiles for red raspberries have been recently summarised by Lee et al., (2012) [84].

Less data is available for Boysenberry ET composition. Kool et al., (2010) [86], identified SH6 and an isomer of sanguiin H-10 as the major ETs in a dried Boysenberry powder extract. Two less-abundant ETs were identified as sanguiin H-2 and [galloyl-bis-HHDP-glucose]$_2$ – gallate (galloyl SH-6). More recently, Furuuchi et al., (2011) [87], quantified the ETs present in Boysenberry juice and seeds from a mixture of commercially harvested cultivars from New Zealand (Berryfruit Export, NZ). Six ETs were characterised in the juice (total ETs 116 mg/100ml) and ten in the seeds (total ETs 912 mg/100g); three of which were in common with the structures reported by Kool et al., SH6 was the predominant ET for both juice (51 mg/100 ml) and seeds (371 mg/100g). Interestingly, both Lam C and galloyl SH-6 were detected, the former at high levels in juice (38 mg/100ml).

Anthocyanins from *Rubus* fruit are unlike other fruit in that they are predominately cyanidin-based in the non-acetylated form [84]. Boysenberry has been reported to contain four major anthocyanins, although reports in the literature regarding the identification of these anthocyanins has been inconsistent [88]. These assignments have been confirmed by liquid chromatograph-mass spectrometry (LCMS) [89] and nuclear magnetic resonance (NMR) [90], as cyanidin-3-[2-(glucosyl)glucoside], cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside], cyanidin-3-glucoside and cyanidin-3-[6-(rhamnosyl)glucoside], and agree with those previously reported by Torre and Barritt, 1977 [91]. Red raspberry has a similar anthocyanin composition to Boysenberry [92]; however, the ratio of detected cyanidin-3-[2-(glucosyl)glucoside] to other anthocyanins detected was typically higher [17]. Total levels of anthocyanins reported in Boysenberry and red raspberry differ according to cultivar and geographic growing region but typically range from 62 to 213 mg/100 g FW for Boysenberry [92] and from 19 to 116 mg/100 g FW for red raspberry [17].

Processing of berry fruit for juice and puree leaves behind waste in the form of seeds and pomace, which could be utilised to expand the market if a use was identified for them [87, 93]. Testing of these waste products yielded flavanol monomers, proanthocyanidins, anthocyanins, EA, ET and flavonol glycosides, all of which have differing chemical structures and biological function [94]. The most abundant polyphenols found in the seeds were ETs [86, 87]. Therefore, with the high ET content found in the waste produce of Boysenberry, this resource has the potential to provide a unique application as a food supplement. This would be a boost for New Zealand's economy, as it is the world's largest exporter and producer of Boysenberries.

1.5 Outline of the thesis

Nutritional research explores the ability of a nutrient to induce a health effect in genetically predisposed individuals, through the tools of nutrigenomics. Of interest to this thesis is the assessment of *Rubus* fruit extracts and their potential to ameliorate inflammatory symptoms in the context of IBD. *Rubus* fruits are known to have high concentrations of ETs of which SH6 and Lam C are found in the highest concentration. Chosen for study are three cultivars previously untested for their anti-inflammatory effects. Based on the literature, which predominantly focuses on pomegranate, ETs have been shown to induce anti-inflammatory effects in both *in vitro* and *in vivo* models of inflammation, and may therefore have the ability to provide a protective effect in certain chronic inflammatory diseases (such as IBD).

The hypothesis of this thesis is that *Rubus* fruit extracts, rich in ETs, induce an anti-inflammatory effect in mammalian cell involved in inflammatory processes.

Chapter 2 aimed to develop a method to optimise the extraction of ETs from *Rubus* fruit and to purify the resultant phenolic extracts for preparing ET-enriched extracts. The ET and anthocyanin concentrations of the extracts were determined to verify composition and enable structure-activity bioactivity correlations to be made in *in vitro* assays conducted in chapters 3 and 4.

In chapter 3 the anti-inflammatory potential of the extracts prepared in chapter 2 were evaluated with a RAW 264.7 murine secondary cell line. Initial testing of the extracts assessed potential cytotoxic effects on the cells using the thiazolyl blue tetrazolium bromide (MTT) assay. Two assays were used to evaluate the anti-inflammatory potential of the extracts; measurement of nitrite production by the cells using the Griess assay, and measurement by ELISA of four key cytokines associated with the development and progression of IBD.

In chapter 4, the anti-inflammatory potential of the prepared extracts were evaluated with two primary cell types isolated from mice, macrophages and colon cells. A preliminary study was conducted to develop and refine the methods required for isolation and culturing of primary cells. The extracts were then evaluated with primary cells isolated from two genetically distinct strains of mice, C57 mice and *Il10*$^{-/-}$ mice. The C57 mouse is a commonly used control mouse strain, while the *Il10*$^{-/-}$ mouse bred on a C57 background is a well-established model of IBD known to produce consistent colonic inflammation. The same assays described in Chapter 3 for the secondary cell cultures (with the exception that water soluble tetrazolium -1 (WST-1) assay replaced MTT for cytotoxicity testing) were used to evaluate the anti-inflammatory potential of the extracts against the primary cell cultures.

Chapter 5 provides an overview and general discussion summarising the experimental work in this thesis and provides suggestions for future work to more fully evaluate the anti-inflammatory potential of *Rubus* extracts.

Chapter 2: Chemical Preparation and Analysis of Rubus Fruit Extracts

2.1 Introduction

Polyphenols have been reported to confer multiple beneficial biological properties in foods that may contribute to disease risk reduction and human health promotion. As discussed in chapter 1, polyphenolic compounds can be categorised into several different classes [15]. Of interest to this thesis are ETs, a class of higher-molecular-weight structures (>500 dalton) of hydrolysable tannins, that produce EA upon hydrolysis [18]. They have considerable structural variability due to the many different possibilities for the linkage of HHDP residues with the glucose moiety, and their strong tendency to form dimeric and oligomeric derivatives [38, 57].

ETs may play an important role in human health and are purported to have numerous biological properties such as: anti-oxidant [8, 10, 17, 53, 59-63], anti-cancer [53, 60, 63-65], anti-inflammatory [11, 43, 66-68], and anti-microbial [12, 15, 16, 56] activities. These beneficial biological activities have led to the increased scientific interest in ETs for their potential application in the promotion of human health and in reducing risks of chronic disease.

2.1.1 Polyphenolic Composition of Rubus Fruits

Rubus berries are a rich source of dietary phenolics including flavonoids, condensed tannins, ETs, stilbenoids, and phenolic acids. ETs are consumed in the diet through consumption of whole berry fruits, berry-containing foods or beverages. ETs are the predominant phenolic compounds found in Boysenberry and red raspberries [17], and are also found in pomegranate, walnut and pecan nut. The major ETs detected in *Rubus* fruit are SH6 and Lam C (see Figure 5). However, the levels of these compounds reported in the literature are dependent on the analytical method employed in extraction and quantification, and are not always comparable (measurement of native form or as EA equivalents post acid hydrolysis). Anthocyanins are the next most abundant phenolic class, with the remainder of the phenolic profile comprising of trace elements of minor phenolic groups [84].

Knowing the exact chemical composition and structure of the polyphenolics found in food or food extracts is critical for the prediction of their potential biological activities and corresponding health effects. The stability, absorption or bioavailability of these compounds is primarily determined by their chemical structure. For example, absorption and bioavailability can be influenced by a variety of factors such as glycosylation or hydroxylation [38], while molecular size and degree of polymerisation can affect solubility and as a consequence absorption. Polyphenolic stability can also be affected by pH, storage, temperature, concentration, light, oxygen and the presence of enzymes [95-98].

For example, phytochemicals, such as ETs and anthocyanins, are known to be stable in acidic conditions but anthocyanins in particular are sensitive to pH and can change conformation in response to different conditions [99]. The methods for *in vitro* assays used in this study to evaluate the anti-inflammatory potential of the polyphenolic extracts require the extracts to be co-incubated with cells in the presence of cell culture media in a solution that has a pH of 7.4–7.6. These factors need to be considered when using assays to determine structure-activity bioactivity correlations.

2.1.2 Variation in Ellagitannins (ETs) in Rubus Fruits

Multiple factors can lead to variation in the amounts and types of polyphenolics present in fruit, which in turn will impact their biological activity.

Genetic and environmental influences affect polyphenolic composition and concentration. Variation is observed amongst different genus, species and cultivars, and is influenced by environmental factors, such as fruit maturity, plant age, growing season, geographic location, solar radiation, temperature, virus exposure, and other biological stresses [84, 85, 100].

Currently there is no standard method for gathering, storing, extracting, purifying, analysing or reporting the key polyphenolic components of berries. Upon reviewing the literature to determine the best published method to adopt for a particular sample type, there are issues in comparability between different methods (see Table 1). Therefore, determination of the optimal analytical method for a particular sample needs to be verified independently.

To date, the most widely used method for quantification of ETs or EA has been high-performance liquid chromatography (HPLC) analysis of free EA after acid hydrolysis [85]. This method gives variable results depending on the conditions used for extraction and acid

hydrolysis. Hydrolysis to EA also means key information about the original ET composition is lost (e.g. amount of SH6 vs Lam C). The example in Table 1 from Kahkonen, et al., (2001) [8] demonstrates the difference in the reported polyphenolic content (8%) yielded from the same fruit type through the use of different extraction solvent.

Table 1: Comparison between different extraction methods for *Rubus* fruits and the effect on total polyphenolic content recorded.

Paper	Extraction Method	Fruit	Polyphenolic content
Ascota-Montoya et al., (2010)	Acetone 70% with 2% Formic acid	blackberry	77 (µmol/g DM[1])
Kahkonen et al., (2001)	Methanol 60%	raspberry	2488 (mg/100 g FW[2])
	Acetone 70%	raspberry	2702 (mg/100 g FW)
Jeong et al., (2010)	Ethanol 60%	black raspberry	8.25 (mg/g)
Nohynek et al., (2006)	Acetone 70%	raspberry	530 (mg/g DM)

2.1.3 Aims

The aim of this study was to optimise the methods used for the extraction of ETs from *Rubus* fruits. The extracts were later used to examine the anti-inflammatory effects of ETs found in *Rubus* fruit (Boysenberry and red raspberry) present in either a phenolic extract or ET-enriched extract using *in vitro* assays (see Chapters 3 and 4).

The first step was to compare the efficacy of solvents commonly used for extraction of polyphenols from fruit, in order to determine which solvent combination maximised the yield of the key phenolic compounds found in *Rubus* fruit. Once this was established, a method was developed to enable purification of ET-enriched extracts. The optimised extraction and purification method were employed to prepare chemically characterised phenolic and ET-enriched extracts from three selected *Rubus* cultivars.

[1] DM – Dry matter
[2] FW – Fresh weight

The anti-inflammatory potential of the prepared extracts were evaluated on primary and secondary cell models (see Chapters 3 and 4), using changes in inflammatory indicators such as nitrite concentration and cytokine analysis. These assays were used to assess whether it is the ETs action alone that has the effect or if there is a need for the whole phenolic complement to be present to induce a biological effect. In preparation for the *in vitro* studies, the prepared extracts were assessed for their stability at conditions mimicking those required for cell culture assays.

2.2 Methods

2.2.1 Rubus Fruit Cultivar Preparation

Rubus fruit cultivars were sourced from Plant & Food Research, Motueka, by Jo Stephens and staff. Three different *Rubus* cultivars with different ratios of the two predominant ETs found in *Rubus* fruit, SH6 and Lam C, were selected for the preparation of extracts:

- 'HB19'. A Plant & Food Research Boysenberry cultivar known to be high in Lam C.
- 'ZZ'. A developing Plant & Food Research Boysenberry cultivar expected to have similar levels of SH6 and Lam C.
- 'Wakefield'. A Plant & Food Research red raspberry cultivar known to be high in SH6.

Berries were harvested three times during the season (early, middle and late) in 2010/11, and placed into a clamshell container and frozen at -20°C. Berries were sent to Plant & Food Research Ruakura, as frozen whole berries, where they were stored at -80°C until extraction.

2.2.2 Ellagitannin (ET) Extraction Method Optimisation

Previous research using *Rubus* fruit has employed a variety of methods to extract and isolate ETs [8, 101]. The initial experiments were designed to optimise an extraction method to maximise the yield of ETs from *Rubus* fruit.

2.2.2.1 Sample Preparation

Frozen Boysenberries were sourced from a local supermarket in Hamilton, New Zealand, in October 2011: a 1 kg packet of Fruzio brand Boysenberries (Fruzio, Fruit Solution, 19 Packham Cres, Stoke, Nelson, New Zealand).

DM for the Fruzio Boysenberry sample was determined by drying a representative sample (45 g) in an oven at a constant temperature of 40–45°C and recording the weight daily until no further change in weight was noted.

Frozen Boysenberries (25 g) were ground using a Waring blender (Model 36BL29) to a coarse purée. This purée was used as a representative bulk sample for the extraction method optimisation.

2.2.2.2 Solvent Extraction Efficiency

Eight different extraction solvents were tested: 70:30 acetone:water, 60:40 methanol:water, 40:60 methanol:water, 50:50 ethanol:water, 70:30 acetone:water + 1% formic acid, 60:40 methanol:water + 1% formic acid, 40:60 methanol:water + 1% formic acid, 50:50 ethanol:water + 1% formic acid. These solvents were selected to determine which solvent combination would maximise the extraction yield of ETs from the *Rubus* fruit sample.

For each of the eight extraction solvents tested, a 50 mL aliquot was added to a 25 g subsample of the Boysenberry purée, in a 200 mL Schott bottle, and then blended using a Waring Blender to give a homogeneous sample equivalent to 0.5 g FW/ml; there were three replicates per extraction solvent. Each sample was shaken at RT overnight, then transferred into two 50 mL falcon tubes and centrifuged for 15 min at 3000 rpm. An aliquot comprising 0.028 g DM equivalent of the supernatant was subsampled, transferred to an autosampler vial, and evaporated to dryness under nitrogen. This subsample was then resuspended in 1 mL of 5:95 acetonitrile:water with 0.1% formic acid for HPLC analysis.

Using the optimal extraction solvent determined by the experiment described above (70:30 acetone:water) a time course study was set up to establish the extraction time required to maximise ET yield. As in the previous experiment, 50 mL of solvent was added to 25 g Boysenberry purée and blended until homogeneous to give a suspension of 0.5 g FW/mL. An initial subsample of 2 mL was taken at t = 0 min, followed by further subsamples at 1, 2, 4 and 24 h. Each subsample was aliquoted into an Eppendorf tube and centrifuged at 10,000 rpm for

1 min. An aliquot comprising 0.028 g DM equivalent was taken for each sample, evaporated to dryness with nitrogen, and resuspended in 1 mL of 5:95 acetonitrile:water with 0.1% formic acid for HPLC analysis.

Two hours extraction time was used for the later preparation of *Rubus* phenolic extracts in Section 2.2.3.1.

2.2.2.3 Ellagitannin (ET) Purification Method Optimisation

ETs were purified from anthocyanins, the other major phenolic component of *Rubus* fruit, using a modified method from Gasperotti et al., (2010) [85]. An aliquot containing the equivalent of 7 g DM Fruzio extract, extracted using 70:30 acetone:water, was taken to dryness using a rotary evaporator, resuspended in 6 mL 70:30 acetone:water and applied to a Sephadex LH-20 resin column (3 cm x 4 cm) that had been preconditioned with 30:70 methanol:water. The anthocyanins were eluted from the column with four washes of 100 mL 30:70 methanol:water (these fractions were collected and evaporated for HPLC analysis). The remaining ET fraction was eluted from the column with 80 mL 70:30 acetone:water. From each fraction, an appropriate subsample was evaporated under nitrogen and resuspended in 1 mL of 5:95 acetonitrile:water with 0.1% formic acid to give a sample comprising 0.028 g DM equivalent for HPLC analysis to confirm the composition and thereby the purification efficacy.

To simplify the method for future purifications, the four separate wash steps were combined into one wash of 400 mL 30:70 methanol:water.

2.2.3 Preparation of Extracts from Selected Rubus Cultivars

The DM for the *Rubus* fruit cultivars was determined using the same method as described in Section 2.2.2.1, using 30 g of each cultivar as a representative sample.

Figure 9 briefly summarises the methods employed to prepare extracts for future use in *in vitro* work.

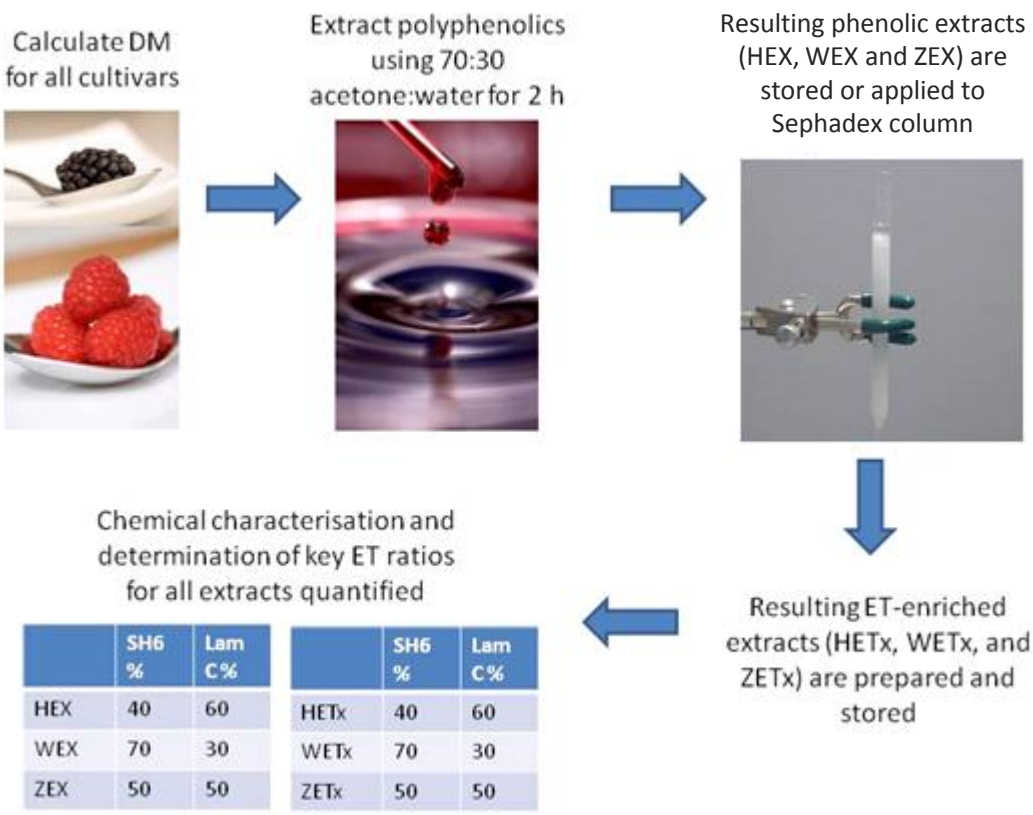

Figure 9: Schematic demonstrating the work flow used to prepare extracts for *in vitro* study from three *Rubus* cultivars.

2.2.3.1 Preparation of Phenolic Extracts

The optimised extraction method was used to prepare phenolic extracts from the three selected *Rubus* cultivars, each having different concentrations of the two predominant ETs: SH6 and Lam C. Briefly, each cultivar was extracted with 70:30 acetone:water (ratio 0.5 g FW/mL) for 2 h with shaking, followed by centrifuging at 3000 rpm for 15 min. The supernatant was decanted and retained with the pellet discarded. The resulting phenolic extracts were designated as follows:

- 'HB19' Extract – HEX
- 'Wakefield' Extract – WEX
- 'ZZ' Extract – ZEX

Each extract was divided into two portions; one portion was used for the preparation of the purified ET-enriched extracts (see Section 2.2.2.3) and the other portion was stored at -80°C for use in *in vitro* cell assays (see Chapter 3 and 4).

2.2.3.2 Preparation of Ellagitannin (ET) -enriched Extracts

A 7 g DM equivalent of each of the prepared phenolic extracts (HEX, WEX, ZEX), was purified as described in Section 2.2.2.3, to give an equivalent ET-enriched extract (HETx, WETx, ZETx). A simplified protocol was used whereby anthocyanins were eluted from the Sephadex column with one 400 mL wash step with 30:70 methanol:water. ETs were eluted with 80 mL 70:30 acetone:water. The protocol was repeated 3 times for each cultivar to prepare sufficient purified ET-enriched extracts for *in vitro* work.

The resulting ET-enriched extracts were designated as follows:

- 'HB19' ET extract – HETx
- 'Wakefield' ET extract – WETx
- 'ZZ' ET extract – ZETx

2.2.4 HPLC and LCMS Compositional Analysis of Extracts

Each extract was analysed by HPLC and LCMS to determine the phenolic profile and quantify ET and anthocyanin levels. Both phenolic extracts (EX) and ET-enriched extracts (ETx) were evaporated under nitrogen and reconstituted in 5:95 acetonitrile:H_2O with 0.1% formic acid to give a sample comprising 0.028 g DM equivalent for HPLC analysis.

ETs were quantified by HPLC (UV at 280 nm) using a standard of SH6 that had been isolated previously (> 95% purity by LCMS); and all results for individual and total ETs are expressed as SH6 equivalents. Identification of individual ETs was confirmed by LCMS.

Anthocyanins were quantified by LCMS in order to calculate the overall polyphenolic content in the extracts, using a pure standard of cyanidin 3-*O*-glucoside and all results for individual and total anthocyanins are expressed as cyanidin 3-*O*-glucoside equivalents.

2.2.4.1 Ellagitannins (ETs)

ETs were quantified by HPLC using a Shimadzu 10 Avp gradient system (Shimadzu Scientific Instruments Rydalmere, NSW, Australia) with PDA detection. Extracts were dissolved in 5:95 acetonitrile/water + 0.1% formic acid and the injection volume was 10 µL. Compound separation was achieved using a Gemini 5 µ C18 110A (Phenomenex, Torrance, CA USA) 250 × 4.6 mm column maintained at 35^{o}C. Solvents were (A) 5:95 acetonitrile/water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid and the flow rate was 1 mL/min. The initial mobile phase, 100% A, was ramped linearly to 5% B at 5 min, held for 5 min, and then ramped linearly to 15% B at 25 min, 19% B at 30 min, 95% B between 33 and 36 min before resetting to the original conditions. The detection wavelength was 280 nm.

ET analysis by HPLC was completed by Wendy Smith, Plant & Food Research Ruakura, with assistance by Alana Srubar-Vernon.

ET identification was confirmed by LCMS using an LTQ linear ion trap MS fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA, USA) coupled to an Ettan^{TM} MDLC (GE Healthcare Bio-Sciences) and a Surveyor^{TM} PDA detector. Compound separation was achieved using a Gemini 3 µm C18 110 Å (Phenomenex) 150 x 2 mm analytical column maintained at 35^{o}C. Solvents were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid and the flow rate was 0.2 mL/min. The initial mobile phase, 95% A/ 5% B, was held for 5 min then ramped linearly to 10% B at 10 min, 17% B at 25 min, 23% B at 30 min, 30% B at 40 min, 97% B between 48 and 53 min before resetting to the original conditions. Sample injection volume was 10 µL. UV-vis detection was by absorbance at 200-600 nm. MS data were acquired in negative ion mode using a data-dependent LCMS^{3} method. The ESI voltage, capillary temperature, sheath gas flow, auxiliary gas flow and sweep gas flow were set at -42 V, 400^{o}C, 40, 20, and 5, respectively.

ET analysis by LCMS was completed by Tania Trower, Plant & Food Research Ruakura.

2.2.4.2 Anthocyanins

Anthocyanins were quantified by LCMS using a 5500 QTrap triple quadrupole/linear ion trap (QqLIT) MS equipped with a TurboIon-Spray^{TM} interface (AB Sciex, Concord, ON, Canada) coupled to an Ultimate 3000 UHPLC (Dionex, Sunnyvale, CA, USA). Compound separation was achieved on a Poroshell 120 SB-C18 2.1 x 100 mm ID 2.7-micron column (Agilent Technologies, Santa Clara, CA, USA) maintained at 70°C. Solvents were (A) 5:3:92 acetonitrile:formic acid:water v/v/v and (B) 99.9:0.1 acetonitrile:formic acid v/v and the flow rate was 600 µL/min. The initial mobile phase, 100% A was held isocratically for 0.5 min, then ramped linearly to 7% B at 10 min, followed by another linear ramp to 100% B at 10.1 min and held for 1.9 min before resetting to the original conditions. Sample injection volume was 5 µL. MS data were acquired in the positive mode using a multiple reaction monitoring method. The turbo spray voltage, temperature, gas one, gas two and curtain gas pressure was set at 2500 V, 700°C, 60 psi, 90 psi and 45 psi, respectively.

Anthocyanin analysis by LCMS was completed by Dwayne Jensen, Plant & Food Research Ruakura.

2.2.5 Determination of Extract Stability

The methods for *in vitro* assays used in this study require the extracts to be co-incubated with cells in cell culture media which has a pH of 7.4–7.6. Preliminary stability experiments were conducted to assess the stability of the key compounds of interest over time at a range of pHs, and in the presence of cell culture media.

2.2.5.1 Stability of Test Extract at Different pH Conditions

A solution of phosphate buffered saline was prepared and the pH adjusted using hydrochloric acid or sodium hydroxide to prepare buffer solutions at pHs 5, 6, 7, 8, and 9. ET-enriched Fruzio extract aliquots (0.028 g DM/mL) were evaporated to dryness under nitrogen and dissolved in the appropriate pH buffer solution.

HPLC analysis (see Section 2.2.4.1) was used to assess the compositional change of the Fruzio extract for each pH buffer solution. Sampling occurred at time zero, followed by further sampling at 4, 12, 16 and 31 h, for each of the buffer solutions (apart from pH buffer solution pH 9, where sampling ceased after 12 h).

2.2.5.2 Stability of Extracts in Cell Culture Medium

Each cultivar extract prepared in Section 2.2.3 (HEX, HETx, WEX, WETx, ZEX, and ZETx) was analysed to assess stability of ET components in cell culture medium ahead of application in *in vitro* work.

A subsample containing 0.028 g DM/mL equivalent of each extract was dried under nitrogen, resuspended in complete DMEM cell culture medium (see Chapter 3 for composition), and transferred to a T25 cell culture flask. Sample solutions for each extract were placed in an incubator at 37°C, with 5% CO_2, to mimic cell culturing conditions. Subsamples of 1 mL were taken at t = 0, 5, 10, and 24 h, transferred to an HPLC vial and immediately analysed by HPLC to determine the resultant polyphenolic composition.

2.2.6 Statistical Analysis

All data analysis was done by Alana Srubar-Vernon, with assistance from Mark Wohlers (Biometrician, Mt Albert, Plant & Food Research), using a one-way Analysis of Variance (ANOVA), followed by post-hoc testing using Fishers Unprotected LSD, with a *P* value of 0.05. All statistical analysis was performed using GenStat (14^{th} edition, VSN International, Hemel Hempstead, United Kingdom 2011). Error bars shown represent the standard deviation from mean.

2.3 Results

2.3.1 Extraction Method Optimisation

2.3.1.1 Characterisation of the Major Ellagitannins in Rubus Fruit

The prepared *Rubus* fruit extracts were analysed by HPLC and LCMS as described in Section 2.2.4, to determine the phenolic profile and quantify ET levels. A representative HPLC phenolic UV profile at 280 nm for the phenolic *Rubus* extracts prepared in this study is shown in Figure 10. This profile was designed to separate the ETs from the anthocyanins, and resolve the main ET components Lam C and SH6. The order of chromatographic elution and structural identity of the main ETs was confirmed by LCMS (Figure 11).

HPLC was used to monitor ET levels to determine the optimal extraction method for maximising the yield of ETs from *Rubus* fruit.

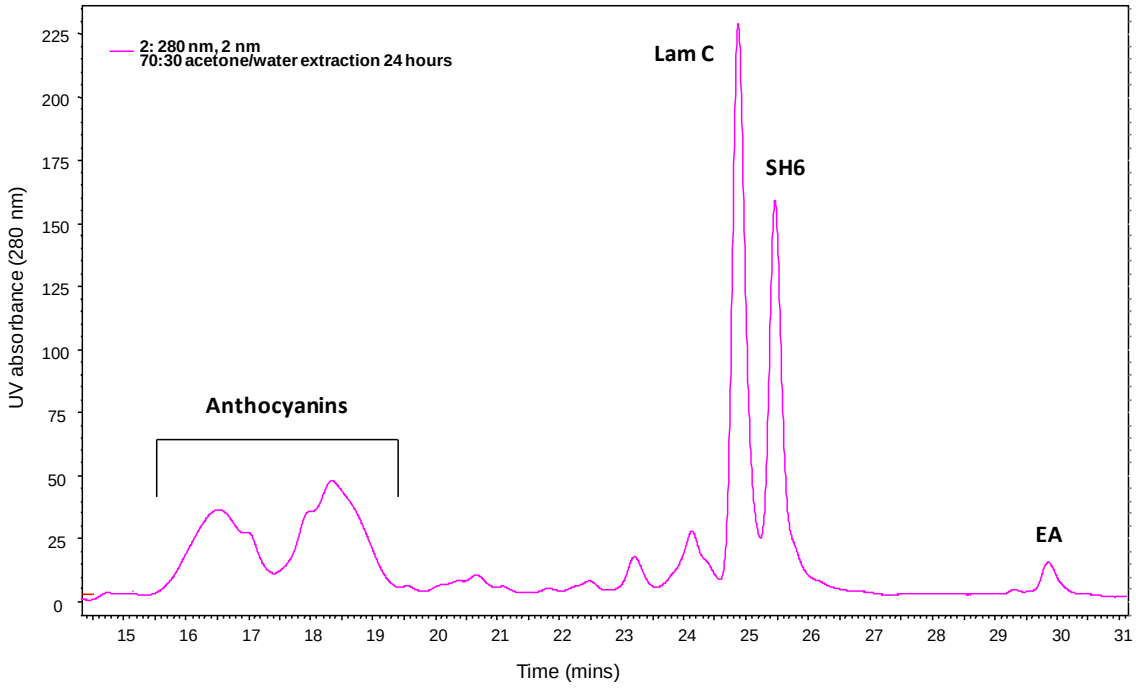

Figure 10: Representative Boysenberry phenolic HPLC UV profile (280 nm) showing the separation of the key polyphenolic compounds found in the test *Rubus* fruit extract.

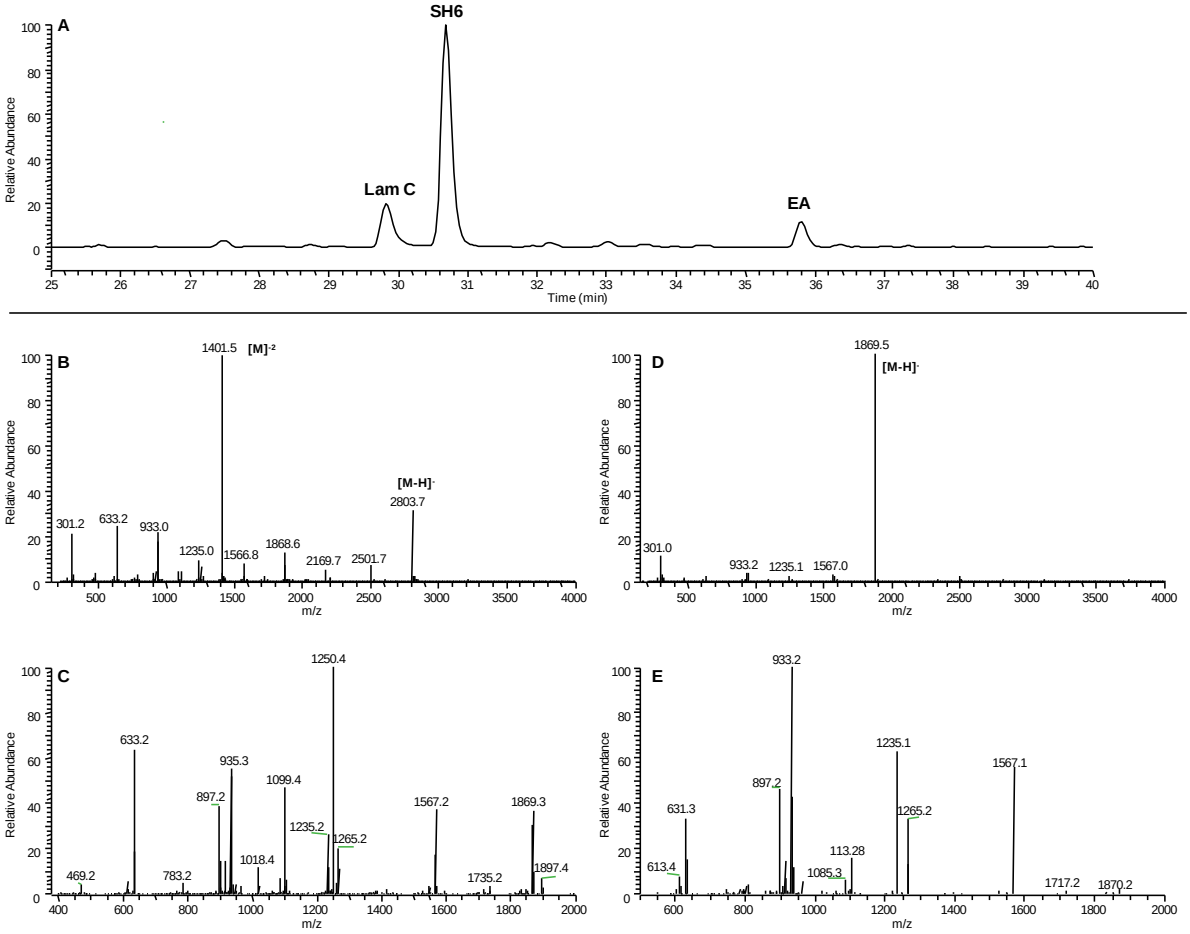

Figure 11: LCMS confirmation of ET identity: A) base peak plot in negative mode for ET extract, B) full scan data for Lam C, C) MS^2 data for Lam C [MS^2 1401.5], D) full scan data for SH6, and E) MS^2 data for SH6 [MS^2 1869.5].

2.3.1.2 Solvent Extraction Efficiency

Of the eight extraction solvents tested, 70:30 acetone:water with and without formic acid, gave the highest ET yields from the test bulk Boysenberry sample (see Figure 12), with the other 6 solvents tested proving less effective.

Pairwise testing revealed that there was a significant difference between the 70:30 acetone:water, with and without formic acid, compared with 50:50 ethanol:water, 60:40 methanol:water and 40:60 methanol:water with formic acid (*P* = <0.05). Pairwise testing also demonstrated that 40:60 methanol:water was significantly lower and different to all solvents tested (*P* = <0.05).

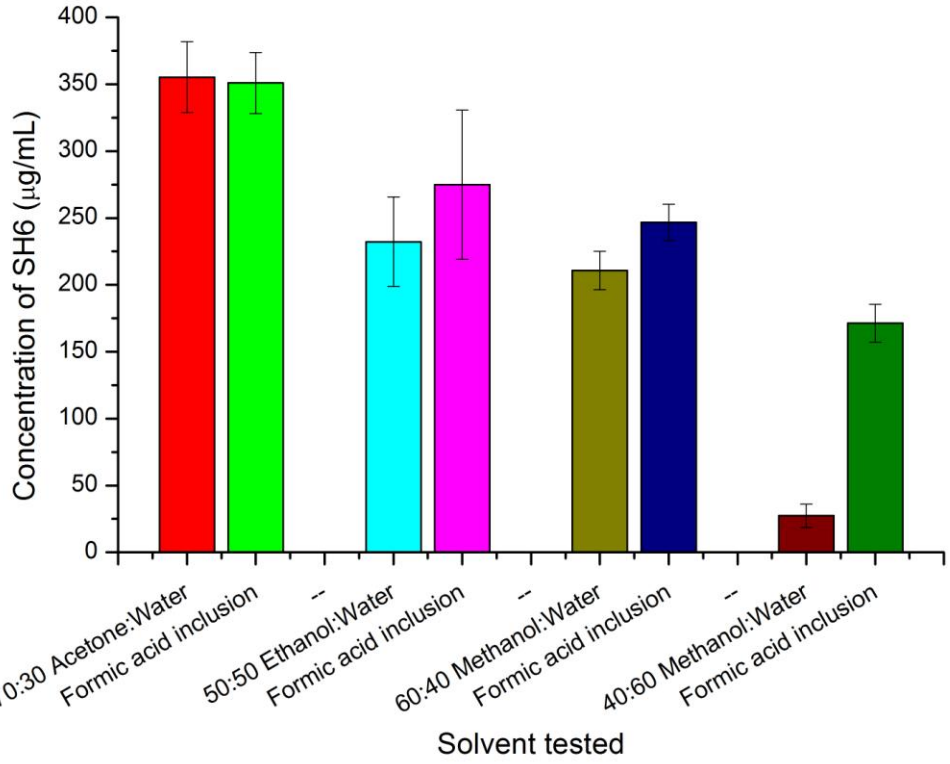

Figure 12: Effect of extraction solvent on yield of SH6 (µg/ml) from the representative Fruzio sample. Each bar represents the standard deviation of three technical replicates.

By monitoring the concentration of SH6 at time points over 0–4 h, and then again overnight (~24 h), no significant change in the yield was observed by increasing the extraction time for longer than 2 h (Figure 13). Two hours was determined to be the most practical extraction time for efficiently extracting high yield of ETs.

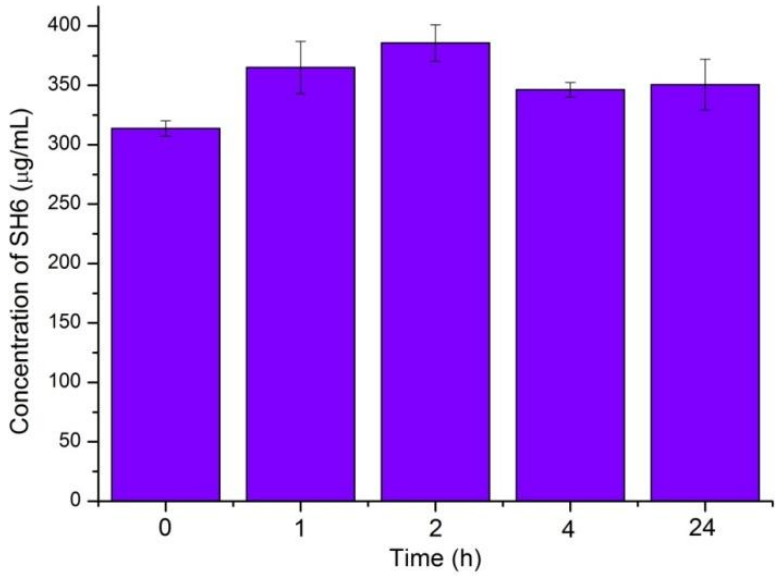

Figure 13: Effect of time on yield of SH6 from Fruzio extract. 0 point represents an aliquot being taken as soon as solvent as added to extract roughly <1 min. Each bar represents the standard deviation of three technical replicates.

2.3.1.3 Ellagitannin (ET) Purification Method Optimisation

A Sephadex column was used to successfully purify the ETs of interest from the phenolic extract, without significantly decreasing the yield relative to the initial amount of ETs extracted. Quantification of the polyphenolic components was performed as described in Section 2.2.4, to confirm the composition and thereby purification efficacy. Figure 14 illustrates the effectiveness of the method by comparing the relative percentages of polyphenolic composition for the three *Rubus* cultivars used in this study.

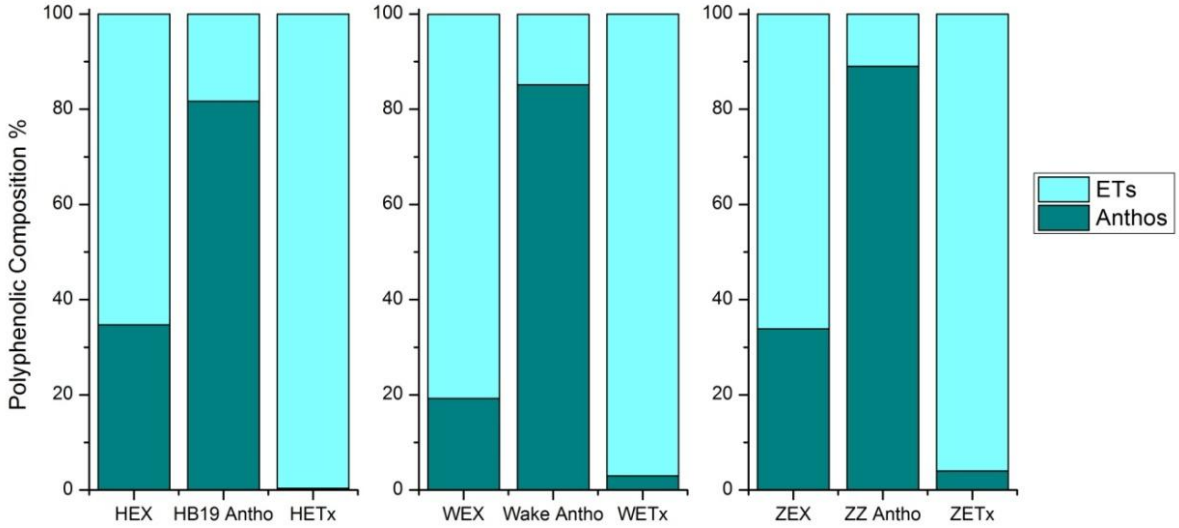

Figure 14: Percentage polyphenolic composition for *Rubus* cultivar extracts demonstrating the effectiveness of the Sephadex column for separating ETs from the other major polyphenolic component, anthocyanins. ETs denotes the ET component and Anthos denotes the anthocyanin component of each extract.

2.3.2 Preparation of Extracts from Selected Rubus Cultivars

Three *Rubus* cultivars, each with different ratios of SH6 and Lam C, were selected for the preparation of extracts for future assessment of their anti-inflammatory properties in *in vitro* studies. DM data for each cultivar are (the figure represents percentage): 'HB19'; 13.64, 'Wakefield'; 18.56, 'ZZ'; 13.81.

The phenolic extracts (HEX, WEX and ZEX) and their ET-enriched equivalent extracts (HETx, WETx, ZETx) were prepared as previously described. Each extract was analysed by HPLC and LCMS to determine the polyphenolic profile, and quantify the levels of ETs and anthocyanins. The absolute polyphenolic levels are shown in Table 2, and these results were used for calculating the total polyphenolic exposure when preparing extracts for assessment *in vitro* assays. The relative percentages of the polyphenolic composition for each extract are shown in Figure 15. After the completion of experimental work the ET-composition of the extracts was re-assessed to determine the long-term extract compositional stability when stored at -80°C. This experimental work was completed by Wendy Smith at Plant & Food Research

Ruakura. Percentage changes in ET concentration, compared to the original extract composition are shown in Table 2 (in brackets) for each extract.

Table 2: Polyphenolic composition of extracts prepared in this study. Lam C is reported as SH6 equivalents. Anthocyanins are reported as equivalents of cyanidin 3-*O*-glucoside. Figures in brackets indicate the percentage change in concentration of the ETs compared to the original composition after storage for 18 months at -80°C.

Sample	**Amount of SH6 (µg/gFW)**	**Equivalent amount of Lam C (µg/ gFW)**	**Total Amount of Anthocyanins (µg/ gFW)**
HEX	1746 (+1%)	2654 (-39%)	2317
HETx	2446 (-31%)	3775 (-62%)	46
WEX	2474 (+14%)	1191(-3%)	315
WETx	2572 (-2%)	1123 (-14%)	58
ZEX	1305(+13%)	1103 (+13%)	447
ZETx	1231 (+1%)	1388 (-21%)	75

The extracts show changes in ET composition after storage for ~ 18 months at -80°C. The increased concentration of SH6 and decreased concentration of Lam C observed for many of the extracts is likely due to the degradation of Lam C into smaller subunits such as SH6.

Figure 15 displays the chemical composition of the extracts used in this study. The polyphenolic profiles of the two Boysenberry cultivar phenolic extracts (HEX and ZEX) were comparable, with ~35% being composed of various anthocyanins and the remainder comprising the two ETs of interest. While the composition of the minor anthocyanins differed between the two cultivars, the major anthocyanin for both cultivars is cyanidin-3-*O*-glucoside. The raspberry extract (WEX) had a lower total anthocyanin concentration of 20% compared with the two Boysenberry cultivars (~35%) and therefore has a greater relative proportion of the key ETs, with a high concentration of SH6 in particular.

The ET-enriched extracts (HETx, WETx, and ZETx) have minimal anthocyanin component of < 5%. They have different levels of the key ETs of interest which relates to the choice of these extracts for study.

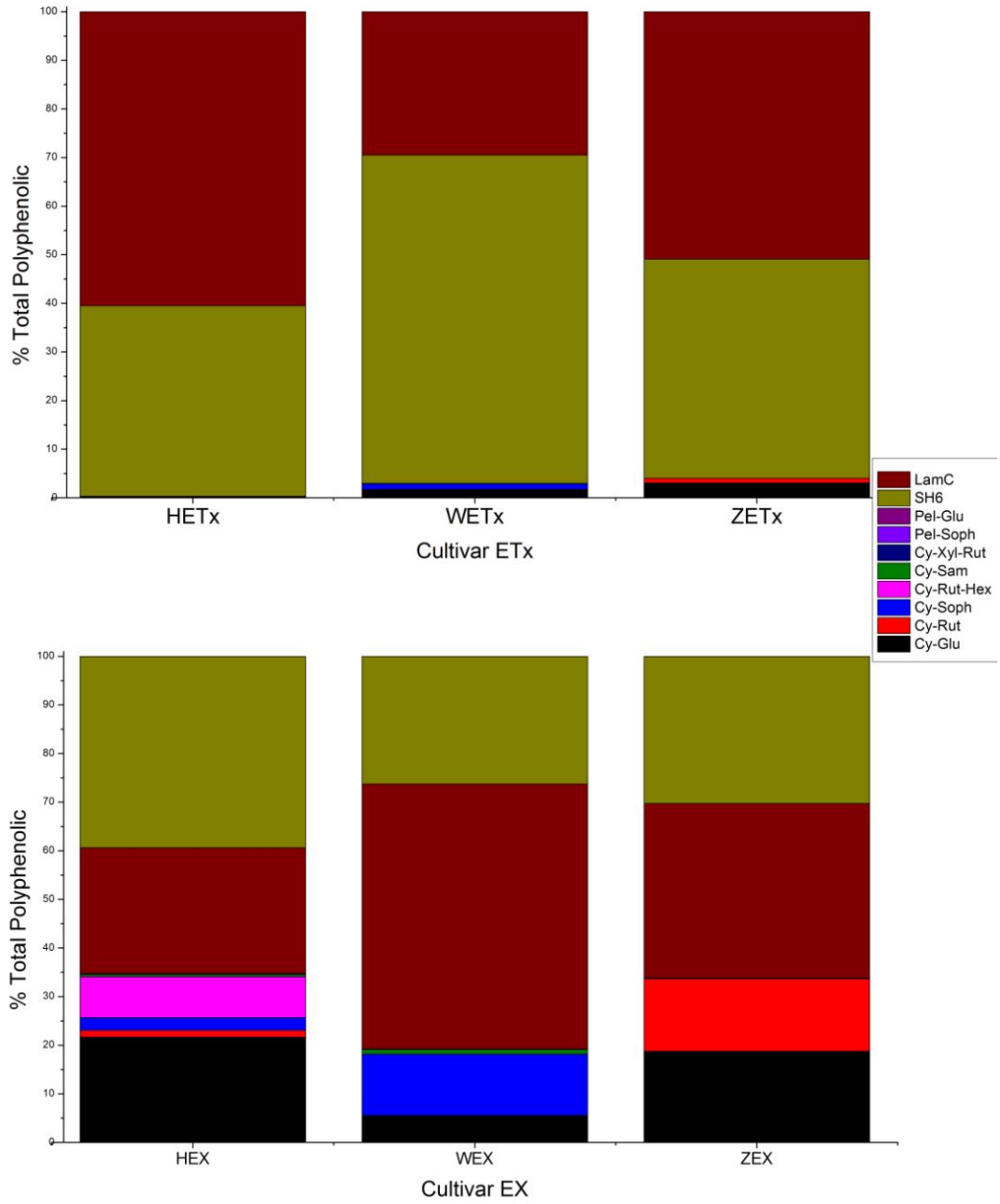

Figure 15: Polyphenolic profiles of the cultivar extracts prepared in this study. Polyphenolic compounds are presented as the relative percentage composition calculated to be present in 0.028 g DM as determined by HPLC and LCMS analysis: Lam C; Lambertianin C, Sanguiin H6; SH6, Pel-glu; Pelargonidin-3-*O*-glucoside, Pel-soph; Pelargonidin-3-*O*-sophoroside, Cy-Xyl-Rut; Cyanidin-3-xylosylrutinoside, Cy-Sam; Cyanidin-3-*O*-sambubioside, Cy-Rut-Hex; Cyanidin-3-rutinoside-hexose , Cy-Soph; Cyanidin-3-*O*-sophoroside, Cy-Rut; Cyanidin-3-*O*-rutinoside, Cy-Glu; Cyanidin-3-*O*-glucoside.

2.3.3 Determination of Extract Stability

The first experiment investigated the ET stability of the ET-enriched Fruzio Boysenberry extract over a range of pH conditions. The results demonstrated that over pH 6 there was a significant decrease in the stability of SH6 over time.

The second experiment investigated the levels of SH6 in the extracts over time, with incubation at cell culture conditions. The levels of SH6 in the phenolic extracts HEX, WEX, and ZEX were relatively stable over the assessment period of 24 h, in cell culture media (pH 7.4–7.6) as shown in Figure 17A. In comparison their ET-enriched extracts, HETx, WETx, and ZETx, showed rapid and significant degradation within 5 h exposure to cell culture media (Figure 17B), in a similar manner to the preliminary pH stability assessment at this pH.

Typically, cell culture experiments measure a physiological cell response after 24 h co-incubation with extracts. These results indicated there was unlikely to be significant benefit in exposing the cells to the extract for longer than 10 h. However, as a 10 h assay would present logistical challenges, 20 h was chosen for practicality and convenience, allowing sufficient time for the cells to metabolise the extracts and still maintain a small concentration (~2-5% dependent on ETx) of the ETs within the media for the pH sensitive ETx.

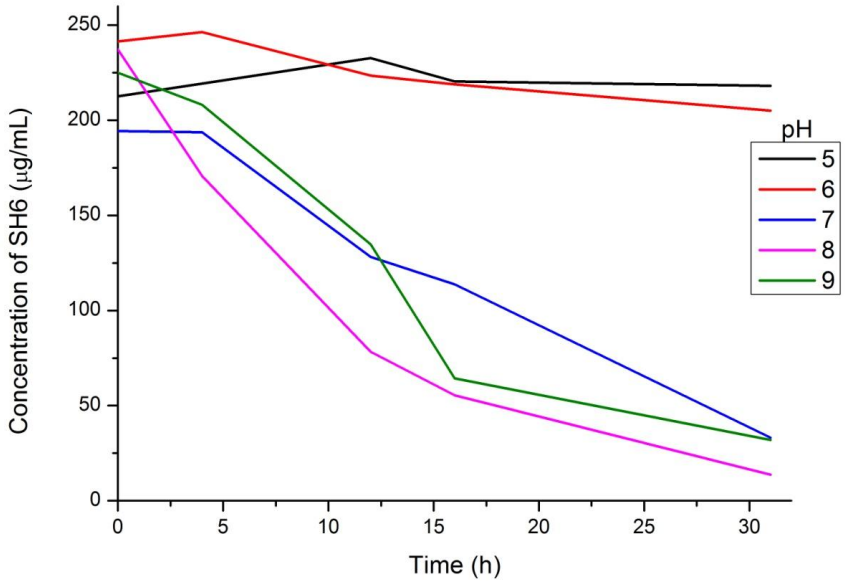

Figure 16: pH sensitivity of Fruzio extract analysed by HPLC (n = 1 technical replicate), over time.

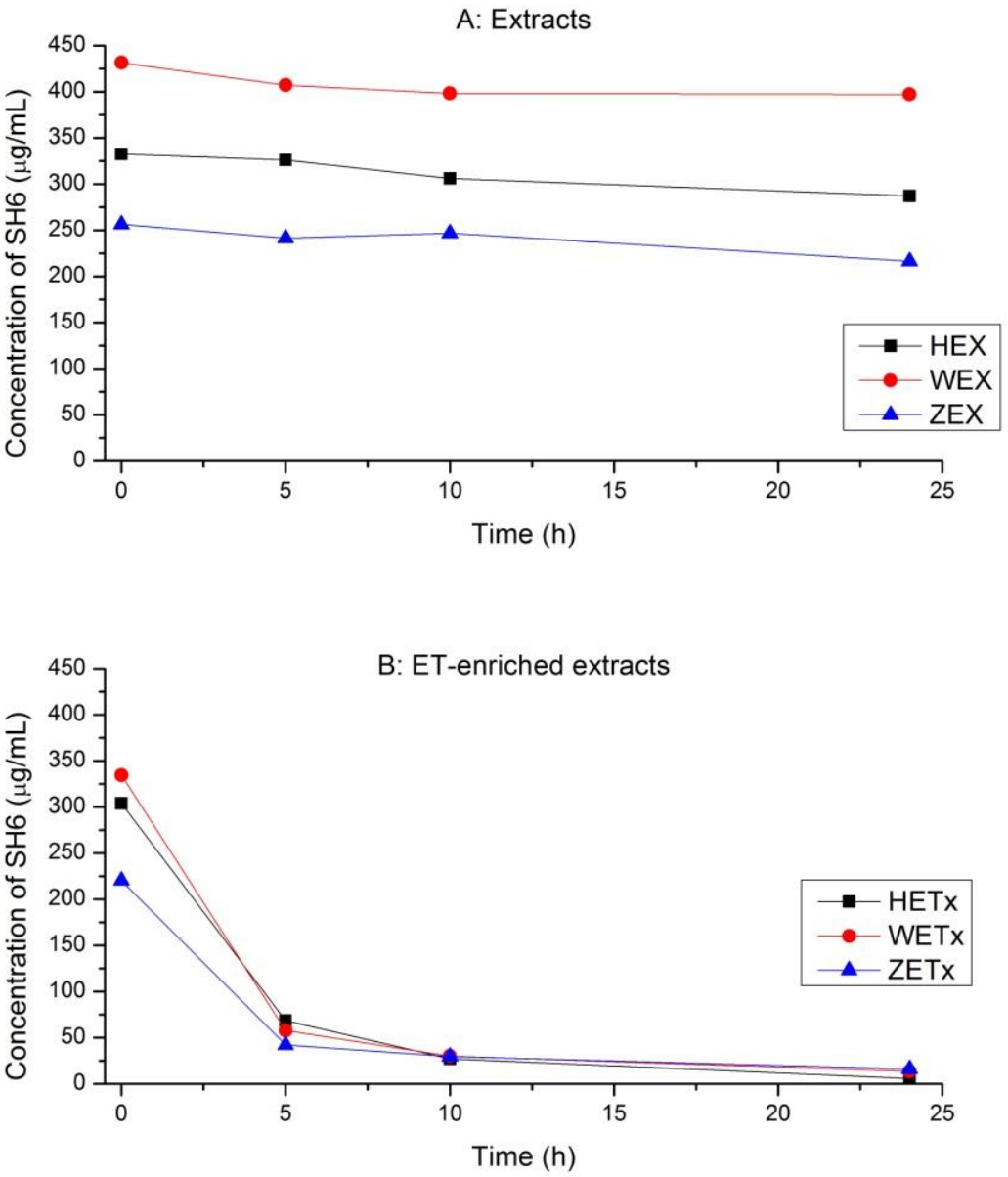

Figure 17: Stability of SH6 in cell culture media for all cultivar extracts over time. A: Extracts (EX) for each cultivar. B: ET-enriched (ETx) extracts for each cultivar (n = 1 technical replicate).

2.4 Discussion and Conclusions

2.4.1 Extraction Method Optimisation

The results of the method optimisation are consistent with findings from previous work [8], and confirm that the choice of extraction solvent significantly affects the overall yield of the key berry fruit polyphenolic compounds extracted.

There was an increase in the yield of the two major *Rubus* ETs from the test *Rubus* fruit using a 70:30 acetone:water extraction solution. The other solvent combinations evaluated, 40:60 methanol:water, 60:40 methanol:water, and 50:50 ethanol:water, were all chosen from other examples in the literature [8, 61, 85-87, 93, 102, 103]. There was a considerable difference in extraction efficiency noted between 40:60 methanol:water and 60:40 methanol:water, with approximately 10x more SH6 extracted (Figure 12) for the latter solvent combination. However, this increase was negated by the addition of formic acid to 40:60 methanol:water.

For solutions with less than 50% water there was no difference in extraction efficiency observed by the addition of formic acid. As the greatest yield was achieved with 70:30 acetone:water and no difference observed with the addition of formic acid, it was omitted from further experimentation and method development. However, in future experimental work, the addition of formic acid to solvent would likely be beneficial for extract stability, particularly for long-term storage, as this work and other studies have established that the main polyphenolics found in *Rubus* fruits have increased stability under acidic conditions [71, 104]. This is further supported by the finding that at the end of this study there was evidence of ET-degradation occurring (Table 2) in the test extracts which had been stored at -80°C for approximately 18 months.

Many methods for fruit extraction commonly use an overnight period to extract polyphenolic components for use in experimental work. However, results from this study demonstrated that there was no benefit of yield with time for extraction periods longer than 2 h using the optimal evaluated solvent combination, 70:30 acetone:water. Therefore, a 2 h standard extraction period was adopted for this work for convenience and time efficiency.

Purification using a Sephadex column was used to successfully isolate the ETs of interest from the crude phenolic extracts, without decreasing the initial amount of ETs extracted. Compositional analysis of the ETx extracts post-column confirmed only ETs predominantly remaining (< 5% anthocyanin component). The effectiveness of the purification method is evidenced in Figure 15 by comparing the polyphenolic composition as a relative percentage of

ETs compared with anthocyanins for the extracts prepared from each of the 3 *Rubus* cultivars used in this study.

A review of the ET chemical composition of the extracts prepared in this study revealed a discrepancy in the amount of ET isolated between extracts HEX and HETx. As the HETx extract was prepared by purification of the HEX extract and all solutions for chemical analysis were prepared using a standard DM equivalent/mL calculated from the original extract, the concentration of key ETs should be similar, or less for HETx if any losses arose during purification. The observed apparent increase in the amount of ETs in HETx is not able to be explained from experimental records. The numbers reported in this table were used throughout the study and the discrepancy was only noted upon review of the thesis. For future study, it would be advantageous to compile multiple HPLC results for any extract after each purification step, and prior to every biological assay in order to remove potential errors that can be carried through experimental work, and to monitor and compensate for any degradative extract compositional changes over time.

2.4.2 pH Stability of Phenolic Compounds

In this study it was found that under the cell culture assay conditions tested, while the ET-composition of the phenolic extracts (EX) did not change significantly, their ET-enriched ETx counterparts showed extensive ET-degradation, evidenced after even just 5 h (or less). The presence of anthocyanins (and other trace phenolic compounds) in the extract may provide some form of stability for the key ETs and total phenolic composition. The ET-enriched ETx extracts were very sensitive to the pHs commonly used for cell culture (see Figure 17). This susceptibility of the ETx extracts to higher pH is a likely result of the removal of anthocyanins from the extract.

An interesting study to test the critical amount of anthocyanins (and other trace phenolics) required to confer ET stability at higher pH conditions, would be to add the removed phenolics back into the extract at known concentrations, and monitor ET degradation.

After the completion of this chapters work, a study by Kosinska et al., (2012) [104] assessed the stability of phenolic compounds found in strawberries for their application in cell culture assays. They showed a slight decrease in the level of SH6 after 2 h and complete degradation after 24 h (at pH 7.4), which concurs with the results of this study [104]. The main conclusion of Kosinska's study replicates the results of this thesis, that pure phenolic compound extracts

are sensitive to pH and likely undergo oxidation, and that co-occurring phenolic substances have a protective effect against degradation [104].

Another study found that a number of phenolic compounds, including gallic acid (of which EA is the dimeric equivalent) were very pH sensitive [105]. The authors concluded that susceptibility to pH was predominately determined by phenolic structure, and rationalised the results in terms of relative resonance stability of phenoxide ions and quinone oxidation intermediates [105].

As a result of the findings that the key ETs of interest in this thesis were sensitive to the pH conditions required for the planned cell culture assays, the decision was made to decrease the period of time the extracts were co-incubated with the cells in the assay from 24 h to 20 h. Although the results suggested there may be little benefit in applying the ET-enriched extracts for longer than 10 h, as evidenced by the rapid decrease in SH6 concentration by this time point, for convenience and logistics a 20 h standard assay time was chosen. The measured levels of SH6 at 24 h suggested there should still be detectable ET component in solution at 20 h (although minimal) and this period of time should also allow the cells to metabolise and respond to the applied extracts.

This aspect of the research provided some interesting results about the stability of ETs, using SH6 as a model, at different pHs. Cell culture assays are a useful tool for their ability to investigate the potential metabolic effects food components may have in *in vivo* studies. While there is considerable interest in the application of polyphenolics in the study of gut health, and many examples in the literature where extracts are applied to cell assays for assessment, few studies consider the solubility or stability of the extract components under the assay conditions, or eventual *in vivo* conditions. Cell culture methods generally require neutral to mildly alkaline pH conditions which can have oxidising effects upon phenolic components [104]. Optimisation of experimental work to take account of extract component stability should be an important consideration when using *in vitro* assays for evaluation of extract efficacy. For nutritional research the stability and bioavailability of extract components under the physiological conditions experienced *in vivo* after dietary intake should also be carefully considered.

Chapter 3: *Rubus* fruit extracts and their effects on RAW 264.7 macrophages

3.1 Introduction

Recent studies have shown that ET-rich pomegranate fruit are anti-inflammatory [11, 66, 73, 106], and suggest that fruit with high concentrations of ETs may prove to be beneficial to patients with IBD in reducing inflammation. In research, experimental work involving animal or human models involves considerable expense, difficulty and ethical cost. Therefore, *in vitro* models are often employed in initial testing to screen different food components to identify promising leads for further *in vivo* tests, and to eliminate components that may show negative side effects.

Numerous cellular models using a variety of different cell types, such as RAW 264.7 macrophages, HT-29 human intestinal cells, human aortic endothelial cells, or human breast cancer cells MCF-7, have been used to assess the anti-inflammatory effect of polyphenolic components from fruit [11, 72, 77, 107-112].

In vitro models only provide an overview or simplified version of part of a biological process rather than the entire process. Therefore, it is important to choose a relevant model that allows investigation of the changes in biological responses related to the disease or process of interest [113].

One of the key attributes of IBD is an altered immune state; therefore, cells isolated from an immune origin are of interest to examine the effects of an extract, for the potential of ameliorating IBD symptoms. Macrophages are one such cell type, and play an important role in antigen sensing and immune regulation. RAW 264.7 macrophages are a genetically transformed cell line of murine origin that are widely used due to their ability to produce homogenous cultures that can proliferate indefinitely [114]. This cell line was used in this thesis as a preliminary screen to assess the potential effects of the *Rubus* fruit extracts before application on more in-depth and expensive primary cell models.

3.1.1 Macrophages

Macrophages are activated, differentiated monocytes present in almost every tissue. They are important for host defence and adaptive immune response activation. Macrophages are initially derived from myeloid-specific progenitor cells and have the ability to differentiate into a variety of leukocytes, such as neutrophils and macrophage-precursor monocytes [115]. These monocytes migrate to specific tissues in response to chemical signalling, where they are activated to differentiate into macrophages. This process is initiated by macrophage-specific growth factors such as macrophage colony stimulating factor (M-CSF) [115].

Macrophages carry out a number of important functions in regulating immune response such as: antigen presentation, phagocytosis, production of reactive oxygen and nitrogen radicals, and cytokine production. All these components of the immune response have the goal of eradicating invading organisms and causing wider spread inflammation in order to further clear any infection. Eventually the biochemical responses also act to regulate the immune response and dampen inflammation once the invader has been cleared.

Circulating monocytes, from which macrophages are derived, have the ability to differentiate into distinct phenotypes through differential activation as a result of microenvironments [115, 116]. In their differentiated form they have the ability to induce changes in gene and protein expression in target tissues [115]. The process of activation classically occurs in response to stimulation by a pathogen associated molecular pattern such as lipopolysaccharide (LPS) or by interferon γ (IFNγ) [117]. Classically activated macrophages are important immune effector cells for both normal physiology [116] and in IBD. In IBD, chronic inflammation is initiated with macrophages releasing chemokines and cytokines, which activates a T helper 1 (T_h 1) or T helper 2 response in the gut mucosa (associated with CD, but not conclusively with UC) [118], which in turn leads to the activation of further naïve macrophages, causing the formation of a positive feedback loop for inflammation.

3.1.1.1 Secondary Murine RAW 264.7 Macrophages

Secondary cell lines are sourced from cancer cells or cells genetically transformed to become immortal, and are able to be stored and proliferate indefinitely. They generally produce homogeneous cultures [119], although over time genetic differences can arise after multiple subcultures.

The murine macrophage-like cell line RAW 264.7 was derived from a tumour developed in a BAB/14 mouse through exposure to Abelson murine leukaemia virus [114]. Cells were collected from ascites in the peritoneal cavity. The cell line is widely used as a model for the investigation of cell signalling in inflammation, metabolic and apoptosis studies [120]. It has the ability to be easily propagated yet retains the capacity for further genetic manipulation [114], making it a useful and easy to work with model.

The RAW 264.7 cell line was chosen for this study as it can be compared with the primary cell culture work in Chapter 4, which uses cells isolated from murine origin, in particular bone marrow derived macrophages. The RAW 264.7 cell line is also a relatively straightforward cell model to use and is well established.

3.1.1.2 Inflammatory Biomarkers

Measurement of inflammatory biomarkers provides information about two areas: detection of acute inflammation that might indicate specific diseases, and to give a biomarker of treatment response. In this study, nitric oxide (NO) and cytokine production levels were monitored in LPS stimulated RAW 264.7 macrophages after co-incubation with *Rubus* fruit extracts to determine if the *Rubus* fruit extracts induce a potential anti-inflammatory effect.

NO is a highly reactive free radical molecule that has an important functional role as a second messenger in relation to inflammatory events [121]. In inflammatory diseases, such as CD, there is substantial evidence that the expression of the gene encoding the enzyme, inducible nitric oxide synthase, and also NO level, are upregulated from normal cellular responses [121]. This upregulation results in excessive production of NO by cells such as macrophages, promoting an inflammatory disease state [110, 121], which has been shown to exacerbate the histopathological expression of IBD [122].

To assess the level of NO, nitrite can be used as an indirect measure [123]. Nitrite is the stable degradation product of NO and can be measured using the Griess reaction [123]. In this reaction, nitrite reacts under acidic conditions with sulfanilic acid to form a diazonium cation, which then couples with N-1-napthylethylenediamine to produce an azo dye (Figure 18). The resultant azo dye can be measured colorimetrically [124].

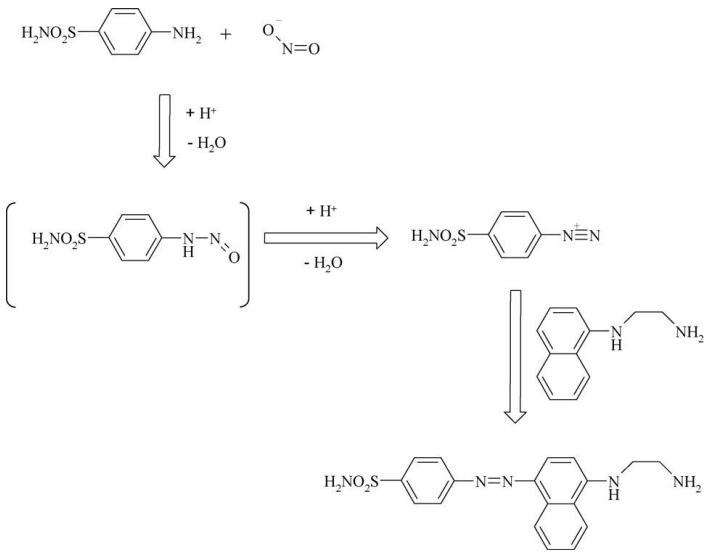

Figure 18: Schematic diagram demonstrating the Griess reaction. Taken from Tsikas, D., (2007) [124].

As well as, producing NO upon exposure to LPS, macrophages release a large number of immunoregulatory cytokines. This release of cytokines is aimed to control the immune response and fight infection [117]. By assessing the changes in cytokine production by macrophages as a result of cell exposure to a food component or extract, we can gain insight into the potential metabolic pathways and changes in immune functionality that may be altered *in vivo* after dietary intake.

In this study four cytokines of interest were chosen for assessment: TNFα, IL1β, IL10 and IL6. These cytokines were selected as they are known to be important mediators of inflammation in IBD, and their production by RAW 264.7 macrophages has been demonstrated previously [109, 125-128].

IL6 is produced by various cell types and has been found to be elevated in numerous inflammatory states, including CD, UC, fever, and rheumatoid arthritis [129]. IL6 has an important role in differentiation and growth of blood and immune cells under non-inflammatory conditions, but when examined in the context of an inflamed GIT state, levels were increased compared with controls and there was a correlation noted between IL6 levels and disease severity [129]. Generally IL6 levels are lower in UC than CD.

TNFα is a critical immune communicator that has become a target for treating IBD through anti-TNF drugs [130]. IBD is associated with increased levels of TNFα, which is implicated in

helping to perpetuate the sustained inflammatory response [52]. TNFα has a physiological role in stimulating the proliferation and differentiation of immune effector cells (like T_h 1) and apoptosis, in response to activation by microbes (in particular LPS) [131]. It is important for regulating acute inflammation and is produced by activated macrophages.

Like TNFα, IL1β has a raised profile in IBD and can influence the severity and course of the disease by contributing to the disbalance of inflammatory responses [132]. Studies to date show an association of higher IL1β expression, which is more strongly linked to CD than UC [52, 133]. IL1β is involved with the regulation of pro-inflammatory responses in normal physiology and has a role in the induction of fibroblast activity [132].

IL10 is an anti-inflammatory cytokine that has an important role in immunomodulation [134]. Although the regulatory mechanisms are not fully understood, it is believed to act through the dampening of T_h 1 and 2 cells (and their corresponding effect) and antigen presentation [135, 136]. IL10 helps to inhibit the production of pro-inflammatory cytokines commonly produced by macrophages: TNFα, IL1β, IL12 and IL6 [137]. Associated with IBD there appears to be a compensatory increase in the levels of IL10 (and other anti-inflammatory cytokines), but overall the levels are insufficient, especially in UC, to negate the imbalanced immune activation [136].

3.1.3 Aims

In this chapter we used a secondary murine RAW 264.7 macrophage cell line as an *in vitro* model to study the effects that phenolic and ET-enriched extracts from three selected *Rubus* fruit cultivars (two Boysenberry and one red raspberry) have on two key indicators of inflammation, NO and cytokine production.

Initial work focused on assessing the effect of the *Rubus* fruit extracts on cell proliferation and viability using the MTT assay. The results from this assay were used to assess the suitability of the samples for use in *in vitro* work, and to determine the maximum concentration of the *Rubus* fruit extracts able to be tested in the NO and cytokine assays without giving rise to cytotoxic effects .

Measurement of nitrite, as an indicator of inflammatory cellular NO production, was conducted using the Griess assay. RAW 264.7 macrophages were co-incubated with six *Rubus* fruit extracts and stimulated with LPS to induce an inflammatory state. The effect of the

extracts on NO production was determined to evaluate their anti-inflammatory potential, and to establish the optimum extract concentration to be used for cytokine assay evaluation.

Finally, the effect the *Rubus* cultivar extracts have on cytokine production was evaluated using an ELISA assay. By assessing the changes in cytokine production, inferences can be established as to the impact on viability and potential immune effect the compounds of interest in the extracts have on the model, and their potential mechanism for the amelioration of inflammation associated with IBD.

3.2 Methods

3.2.1 Cell Culture Materials

The following cell culture materials were purchased from Sigma Aldrich (Auckland, New Zealand): dimethyl sulfoxide (DMSO; D2650), trypan blue (T0887), LPS from *Escherichia coli* serotype 0111:B4 (L4391), dithiothreitol (DTT; D0632), sulfanilamide (S9251), N-1-naphthylethylenediamine (70720) and MTT (M5655). The Griess Assay kit (G2930) was purchased from Promega, Australia.

Liquid high-glucose Dulbecco's Modified Eagle Medium (DMEM; Life Technologies 11995) and heat-inactivated foetal bovine serum (FBS; 10437028) were purchased from Life Technologies, New Zealand. All non-sterile solutions were filtered immediately prior to use with cellulose acetate (CA) 0.22 µm syringe filters (13 mm diameter: SFCA013022S) purchased from Membrane Solutions, Ohio, USA.

3.2.2 Cell Counting

To count cells, 15 µL of cell suspension and 15 µL trypan blue dye (0.4% w/v solution) were mixed together in a 1.5 mL Eppendorf tube and this mixture was loaded onto both sides of a Neubauer haemocytometer. At least one square (1 mm x 1 mm) from each side was counted and a minimum of 100 viable cells were counted over 2–4 squares. Only cells that did not absorb the dye were deemed viable, as they were impervious to the dye evidencing a complete cell membrane. The following formulae were used for seeding plates.

Viable count:

Number of cells in n squares x 10^4 (number of field volumes/mL) x 1/n x 2 (dilution factor)

n = number of squares counted

The viable count was then used in the equation below to calculate the final volume needed for plating.

$C_1V_1 = C_2V_2$

$V_1 = C_2V_2/C_1$

Where V_2 is the number of wells, C_2 is the concentration of cells wanted per well (10,000 or 40,000), $C_1$is the viable cell count and V_1 is the final volume of cell suspension.

3.2.3 Culture of Macrophage-Like RAW 264.7 Cell Line

The RAW 264.7 murine macrophage cell line (European Collection of Cell Cultures, Cat. no. 91062702) was grown in liquid high-glucose Dulbecco's Modified Eagle Medium with 10% FBS (complete DMEM) at 37°C, with 10% CO_2 in a humidified incubator.

Cells were prepared previously by Dr. Jeffrey Greenwood at passage number 6 (passage number indicates the number of times confluent cells have been removed and seeded into new vessels at a lower density in order to continue proliferation) and stored in liquid nitrogen until use. A 1.5 mL cryovial of prepared frozen cells was removed from liquid nitrogen storage and quickly thawed, swirling in warm water at 37°C. The cell suspension was removed and re-suspended in 7 mL of complete DMEM medium (DMEM with 10% FBS) in a T25 cell culture flask and mixed gently before being left to grow at 37°C, with 10% CO_2 for 2 days. Cells were then gently removed from the flask with a sterile cell scraper and the cell suspension transferred into a 5 mL falcon tube and centrifuged at 125 x*g* for 5 min. The supernatant was discarded and the pellet was resuspended in 15 mL complete DMEM and transferred into a T75 flask. A further two passages were carried out before cells were used for experimental work. Cells were maintained and passaged (with a split ratio of 1/8 - 1/2 dependent on confluence) every 3–4 days using complete DMEM at 37°C, with 10% CO_2, in a T75 filter-cap flask. For consistency in experimental work, cells from passages 10–30 were used, as cell lines at high passage numbers have been reported to show differences in responses and morphology compared with lower passage numbers [138].

Assays involved growing cells at 37°C, with 10% CO_2, in a 96 well culture plate (Greiner 655180), at a density of 10,000 or 40,000 cells per well with 100 µL/well culture medium. Cell suspensions for assay work were prepared by aspirating spent medium and replacing it with 10 mL complete DMEM and then gently detaching the cells using a sterile cell scraper. The suspension was collected in a 50 mL falcon tube, prepared at the correct density by counting and diluting the required cell suspension out in fresh media and plated out using an automated multichannel pipette.

3.2.4 In Vitro Assays

All RAW 264.7 assays were repeated with a minimum of three technical replicates per experiment. Each assay consisted of a different frozen cell stock aliquot and variable cell passage number (10–30). The same LPS stock and *Rubus* fruit extraction preparations were used throughout experimental work at the Mt Albert Plant & Food Research facility.

3.2.4.1 Rubus Fruit Extract Solution for Cell Culture Testing

The amount of phenolic extract used for assay work was chosen based on a literature review pertaining to berry extracts and/or ET-containing extracts in *in vitro* work [11, 38, 68, 72, 103, 111, 126, 139, 140]. For cytotoxicity testing the initial amount chosen for inclusion in cell culture work was up to 200 µg/mL of overall polyphenolic exposure to the cells. The required volume of each extract (using the polyphenolic composition results calculated from HPLC and LCMS analysis), was placed into a 1.5 mL Eppendorf tube and evaporated to dryness using a Scanvac vacuum centrifuge, for 5–8 h, at +4°C (1700 rpm).

After determination of the highest non-toxic concentration of phenolic extracts, concentrations up to 50 µg/mL were used in subsequent experiments.

3.2.5 Cytotoxicity of Rubus Fruit Extracts for RAW 264.7 Macrophages

The first step when assessing the effect of a food component in any cell model is to establish the cells' ability to tolerate the food component. In this study preliminary cytotoxicity testing used the alamarBlue® assay (based on the resazurin reaction: where resazurin is reduced to resorufin, a pink fluorescent product) to quantify viable cells [141, 142]. However, background

fluorescence from the extracts, most likely due to the presence of anthocyanins, interfered with the fluorescence readings.

Cell growth and cytotoxicity data for all extracts were therefore determined using the thiazolyl blue tetrazolium bromide (MTT) assay [143]. The MTT assay allowed for removal of the medium and sample prior to cell assessment, thereby removing the interference. The live cells convert the MTT into an insoluble purple formazan dye through a potential mitochondrial mechanism, where the concentration of the dye directly correlates with the number of metabolically active cells in the culture [143, 144].

Cell growth and viability was quantified using the MTT assay in order to determine the highest non-cytotoxic concentration of EX and ETx for each cultivar tested. A series of cell growth and viability experiments were performed using the RAW 264.7 cell line.

All assay work was conducted with cells seeded into 96 well plates (Greiner 655180) at 10,000 cells/well in 100 µL medium. This sub-confluent cell plating density was used to ensure that cells were actively proliferating and therefore would be responsive to testing [145] with the *Rubus* extracts throughout the assay period. Plates were initially incubated for 24 h at 37°C, 10% CO_2 before extract addition.

Rubus fruit extracts were prepared at three times the final concentration to allow for dilution. Dried extracts were resuspended and dissolved in 1 mL of medium and immediately passed through a 0.22 µm CA filter. A series of two-fold dilutions were prepared to test the following polyphenolic concentrations for all extracts (µg/mL): 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78. A multichannel pipettor was used to dispense 50 µL of extract solutions or culture medium to the appropriate wells. Control and blank wells had additional medium added only.

After 18 h of co-incubation of extracts with the cells, 15 µL of MTT at the concentration of 5 mg/mL (or 10% of total well volume) were added to each well, and incubated for a further 2 h to allow for formazan crystal formation. At this point the medium, MTT and extracts were aspirated out of the wells, and 100 µL of DMSO was added to solubilise the purple crystalline precipitate. After 2.5–3 h incubation in the dark at RT, the absorbance at 570 nm and 700 nm (700 nm was for background correction) was measured using a BMG FLUOstar Omega plate reader. All measurements were conducted in quadruplicate. Visual assessment of viability and proliferation was carried out using light microscopy (subjective assessment of confluence by operator).

3.2.6 Cell Activation with LPS

The addition of LPS into the medium is critical for macrophage activation or differentiation into an activated phenotype. It is only after a macrophage has been activated (such as with stimulation by LPS) that it will produce significant amounts of nitric oxide (NO), which also correlates with an increase in certain pro-inflammatory cytokines. Therefore determining the amount of LPS to add to the *in vitro* model to ensure that activation of the cells has occurred, yet not to over-stimulate the assay, is essential.

Preliminary experiments were conducted to assess the concentration of LPS required for stimulation of NO production by RAW 264.7 cells. The assay was conducted with cells seeded in 96-well plates, at 40,000 cells/well in 100 µL complete DMEM medium, and grown overnight at 37°C, 10% CO_2.

LPS dilutions were prepared in culture medium at three times the final concentration to allow for subsequent dilution on addition to cells. Half-log dilutions of LPS were prepared to yield final concentrations of 3.16, 1, 0.316, 0.1, 0.0316, 0.01, 0.00316, and 0.001 µg/mL. Fifty microlitres of each dilution was added to appropriate wells and co-incubated for a further 20 h.

At the end of the incubation, cells were visually assessed by light microscopy for proliferation and morphological changes in response to LPS stimulation. Culture supernatant was collected and assayed for nitrite using the Griess assay (as in Section 3.2.4.4), in order to determine the optimum LPS concentration for macrophage stimulation.

3.2.7 Measuring Nitric Oxide (NO) Production

Production of NO by RAW 264.7 macrophages stimulated with LPS was used as an *in vitro* inflammatory assay for observing any anti-inflammatory effects of co-incubation with the *Rubus* fruit extracts. Nitrite concentration was used as an indirect measure of NO production and was determined using the Griess assay.

Cells were seeded in a 96-well culture plate at a density of 40,000 cells per well in 100 µL complete DMEM medium per well, and were cultured for 24 h at 37°C, 10% CO_2. A multichannel pipettor was used to dispense 50 µL of culture medium with or without *Rubus* extracts. After incubation for 1 h, 3 µL of LPS solution in culture medium (or culture medium

only for control wells) was added to wells to a final LPS concentration of 0.05 µg/mL, and the plate was incubated for a further 19 h.

Measurement of nitrite in culture media was carried out using the Griess Reagent System (Promega), following the manufacturer's instructions. A 100 µM nitrite solution was prepared by diluting with the culture medium the 0.1 M nitrite standard provided in the kit. Two-fold serial dilutions were prepared to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM), and 50 µL of each dilution was added to the appropriate triplicate wells of a 96-well assay plate. In the 0 µM nitrite wells 50 µL of culture medium was added. Fifty microlitres of supernatant from each of the wells of the culture plate was added to the assay plate before adding the Griess assay reagents.

One percent sulfanilamide in 5% phosphoric acid and 0.1% naphthylenediamine dihydrochloride (NED) in water were allowed to equilibrate to RT. Once at RT 50 µL of sulfanilamide solution was dispensed to all wells of the assay plate. The plate was then incubated at RT for 7 min, protected from light. Fifty microlitres of the NED solution was then added to all the wells, and the plate was allowed to incubate at RT, protected from light, for a further 7 min before the absorbance was measured at 535 nm using a BMG FLUOstar Omega plate reader.

3.2.7.1 Measuring Interference by Anthocyanins

In acidic media preparations, such as those used in the Griess assay, anthocyanins have an intense absorbance in the same wavelength range as the azo dye produced in the assay [146]. Therefore, a method developed from Wang and Mazza, (2002) [146], was followed to allow the accurate quantification of nitrite in the presence of anthocyanins found in the *Rubus* fruit extracts.

Using the same supernatant analysed in Section 3.2.7, 50 µL of supernatant was added into wells on a separate 96-well plate. To each well 100 µL of 2.5% phosphoric acid was added (instead of Griess assay reagents). The plate was left to incubate for 20 min in the dark, after which the absorbance was read at 535 nm using a BMG FLUOstar Omega plate reader. This absorbance due to anthocyanins was then subtracted from the corresponding Griess assay absorbance, from Section 3.2.4.4, to give a net absorbance corrected for anthocyanin interference.

3.2.8 Measuring Cytokine Production for RAW 264.7 Macrophages

Cytokine production (IL6, IL10, IL1β, and TNFα) was measured using ELISA analysis. RAW 264.7 macrophages were grown and stimulated with 0.05 µg/mL of LPS in order to induce an immune response, and were co-incubated with 50 µg/mL of each of the *Rubus* fruit extracts.

Cells were seeded in a 96-well culture plate at a density of 40,000 cells per well in 100 µL complete DMEM medium per well, and were cultured for 24 h at 37°C, 10% CO_2. A multichannel pipettor was used to dispense 50 µL of culture medium with or without *Rubus* extracts at a final concentration of 50 µg/mL extract. After incubation for 1 h, 3 µL of LPS solution in culture medium (or culture medium only for control wells) was added to wells to a final LPS concentration of 0.05 µg/mL, and the plate was incubated for a further 19 h.

At the end of the incubation period supernatant from replicate wells was pooled into a 1.5 mL Eppendorf tube and cell debris removed by centrifuging for 10 min, at RT (RT), at 10,000 x*g*. After centrifugation, supernatants were transferred into fresh 1.5 mL Eppendorf tubes and frozen at -80°C to await analysis.

Four cytokines were measured concurrently from each supernatant sample: IL1β, IL6, IL10 and TNFα. Concentrations of these cytokines were analysed using custom designed Multiplex ELISArray plates (SABioscience through Qiagen: QIA336111) with plate layout demonstrated in Figure 19. Each plate consisted of 12 columns that had 8 wells pre-coated with capture antibodies for the selected cytokines.

IL6	IL10	TNFα	IL1β	IL6	IL10	TNFα	IL1β	IL6	IL10	TNFα	IL1β
←	Negative Control		→	←	Negative Control		→	←	Negative Control		→
←	Sample 1		→	←	Sample 1		→	←	Sample 1		→
←	Sample 2		→	←	Sample 2		→	←	Sample 2		→
←	Sample 3		→	←	Sample 3		→	←	Sample 3		→
←	Sample 4		→	←	Sample 4		→	←	Sample 4		→
←	Sample 5		→	←	Sample 5		→	←	Sample 5		→
←	Sample 6		→	←	Sample 6		→	←	Sample 6		→
←	Positive Control		→	←	Positive Control		→	←	Positive Control		→

Figure 19: Layout of custom designed Multiplex ELISArray for RAW 264.7 macrophages. Plate consists of 12 strips running vertically, with pre-coated capture antibodies, allowing triplicate cytokine representation per plate.

Two biological replicates were performed on each plate and triple technical replicates were comassessed from repeat experiments, following the manufacturer's protocol. All incubations were carried out at RT and in the dark.

Fifty microlitres of assay buffer were loaded into each well. Into the appropriate wells, 50 µL of the sample of interest were loaded. The negative control wells had 50 µL of sample dilution buffer added, and in the positive control wells, 50 µL of the antigen standard cocktail were loaded. The plate was then incubated for 2 h. After incubation the samples were removed and the plate was washed (with prepared wash buffer) three times by hand using an automated pipette. One hundred microlitres of the appropriate detection antibody solutions (supplied with the kit) were added to each well (as per Figure 19) and incubated for 1 h. The antibody solution was then removed, and the plate was washed by hand three times. To the plate, 100 µL of diluted Avidin-HRP was added to all wells and the plate was incubated for a further 30 min. This solution was then removed and the plate was washed by hand four times. One hundred microlitres of development solution (supplied with the kit) was then added to each well and incubated for 13 min, after which 100 µL of stop solution (supplied with the kit) was added (in the same order). The plate was then read within 30 min at 450 nm with a background wavelength reading of 570 nm, using a BMG FLUOstar Omega plate reader.

The range of this assay was confirmed by performing a reference plate analysis and substituting the sample columns with diluted positive control solutions (500, 250, 100, 50, 25, and 10 pg/mL) using the antigen standard cocktail. Test control samples were assayed to ensure that samples were diluted to optimal concentrations to be within the linear range of the assay.

3.2.9 Statistical Analysis

All data analysis was done by Alana Srubar-Vernon, with assistance from Mark Wohlers (Biometrician, Mt Albert, Plant and Food Research), using a one-way Analysis of Variance (ANOVA), followed by post-hoc testing using Fishers Unprotected LSD, with a *P* value of 0.05. All statistical analysis was performed using GenStat (14th edition, VSN International, Hemel Hempstead, United Kingdom 2011). Error bars shown represent the standard deviation from mean.

3.3 Results

3.3.1 Cytotoxicity of Cultivar Extracts on RAW264.7 Cells

The RAW 264.7 macrophages were co-incubated with the *Rubus* fruit extracts to determine if the phenolic extracts had any cytotoxic effects on the cells, and at what phenolic concentration any cytotoxicity was significant. Cytotoxicity was assessed using the MTT assay as described in Section 3.2.5. Figure 20 (below) shows that for polyphenolic concentrations of < 100 µg/mL there were no indication of cytotoxic effects for any of the extracts tested.

There was no significant difference in viability observed between HEX and HETx, and no significant change in viability with concentrations of either extract. For WEX and WETx there was no significant difference in relative cell viability observed between the extracts and a phenolic concentration of 200 µg/mL of both extracts were significantly different from the control (P = < 0.05). There was a significant difference observed between the ZEX and ZETx responses ($F_{1,57}$ = 10.70, P = 0.002), and 200 µg/mL was significantly different from the control (P = < 0.05).

Throughout these experiments cell viability was visually assessed using light microscopy and any growth changes observed were noted at the time. The results from the MTT assay were consistent with the visual assessments made at the time of the assay, with significant decreases in cell density observed at the highest phenolic concentrations for each extract (100 µg/mL = ~10%, 200 µg/mL = ~20%).

Based on these results phenolic extract concentrations up to 50 µg/mL was used for all further assays to preclude cell cytotoxicity.

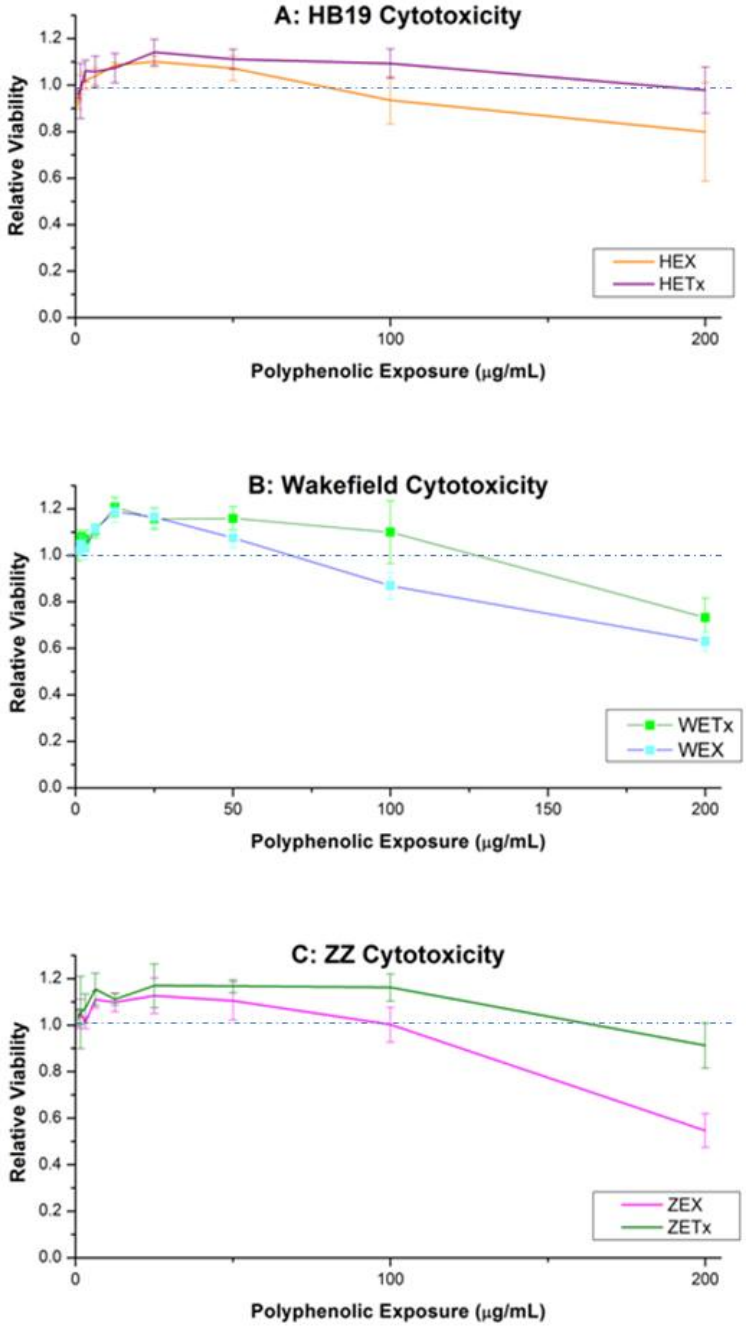

Figure 20: MTT cytotoxicity results for RAW 264.7 macrophages co-incubated with *Rubus* fruit extracts. Cells were incubated for 20 h with *Rubus* fruit extracts prior to MTT assay. Results are displayed as relative viability of extract-treated cells compared with controls exposed to cell culture medium only, plotted against polyphenolic concentration (µg/mL). A) HEX and HETx response for HB19 cultivar, B) WEX and WETx response for Wakefield cultivar, C) ZEX and ZETx response for ZZ cultivar. The horizontal dotted line represents viability of control RAW 264.7 macrophages. Each bar represents the standard deviation for four technical replicates

3.3.2 Nitric Oxide Production (NO) by RAW 264.7 Cells

Measurement of nitrite, as an indicator of inflammatory cellular NO production, was conducted using the Griess assay. RAW 264.7 macrophages were stimulated with LPS to induce an inflammatory state, and the effect of Rubus fruit extracts on NO production was determined to gain insight into the anti-inflammatory potential of these extracts.

3.3.2.1 Optimisation of LPS Stimulation

The addition of 0.001–3.16 µg/mL of LPS into the cell medium using a half-log dilution method gave a regular concentration-dependent response (see Figure 21). There was a relationship observed with increasing LPS concentrations, resulting in increasing levels of nitrite. The LPS concentration of 0.05 µg/mL was used in further experimentation, as this concentration yielded a significant increase in nitrite compared with naive cells and is in the middle of the observed stimulation response, therefore enabling more sensitive measurement of potential increases and/or decreases to be made.

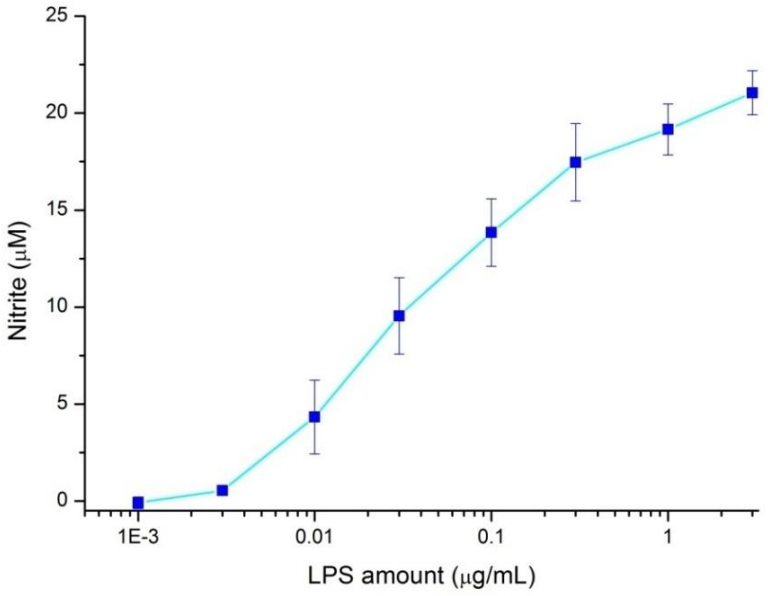

Figure 21: Optimisation of LPS concentration for stimulating NO response in RAW 264.7 macrophages. The cells were grown for 24 h LPS free, and then co-incubated with varying concentrations of LPS, after which the concentration of NO was determined using the Griess assay. Each bar represents the standard deviation for three technical replicates

3.3.2.2 Effect of Rubus Fruit Extracts on Nitric Oxide (NO) Production

After establishing the optimal concentration of LPS needed for stimulation of the RAW 264.7 macrophages, the *Rubus* fruit extracts were co-incubated with the cells at phenolic concentrations up to 50 μg/mL. The culture supernatants collected were assessed using the Griess assay.

Figure 22 graphically represents the relative production (to control) of NO by the cells co-incubated with the *Rubus* extracts. The results show that there is no significant change in NO production for extracts at phenolic concentrations < 25 μg/mL. At phenolic concentrations of 25 and 50 μg/mL several extracts were able to induce a small but significant reduction in NO production relative to untreated control cells (LPS stimulated but no phenolic treatment), implying an anti-inflammatory effect for these extracts.

At 25 and 50 μg/mL the 'HB19' Boysenberry extracts HETx and HEX both significantly reduced NO production relative to the LPS stimulated control ($P < 0.05$). At these concentrations there was no significant difference between the HEX and HETx extracts in their effect on NO levels.

For Boysenberry cultivar 'ZZ' extracts the ET-enriched extract ZETx at 50 μg/mL showed a small but significant reduction in NO production compared to the LPS stimulated control ($P < 0.05$). At the other phenolic concentrations tested there was no significant difference in NO production observed between ZEX and ZETx, and no significant difference in NO production relative to controls.

For the red raspberry 'Wakefield' extracts there was a significant reduction in NO production in comparison to the LPS stimulated control for WETx at 25 and 50 μg/mL, and at 50 μg/mL for WEX ($P < 0.05$). At other concentrations there was no significant change in comparison to the control for either extracts. At 25 and 50 μg/mL there was a significant difference between the extracts WEX and WETx ($P < 0.007$), in their ability to reduce NO production.

As all cultivars showed a significant reduction in NO production at polyphenolic concentrations of 50 μg/mL relative to untreated control cells, this concentration was used in the cytokine assay experimental work in Chapters 3 and 4.

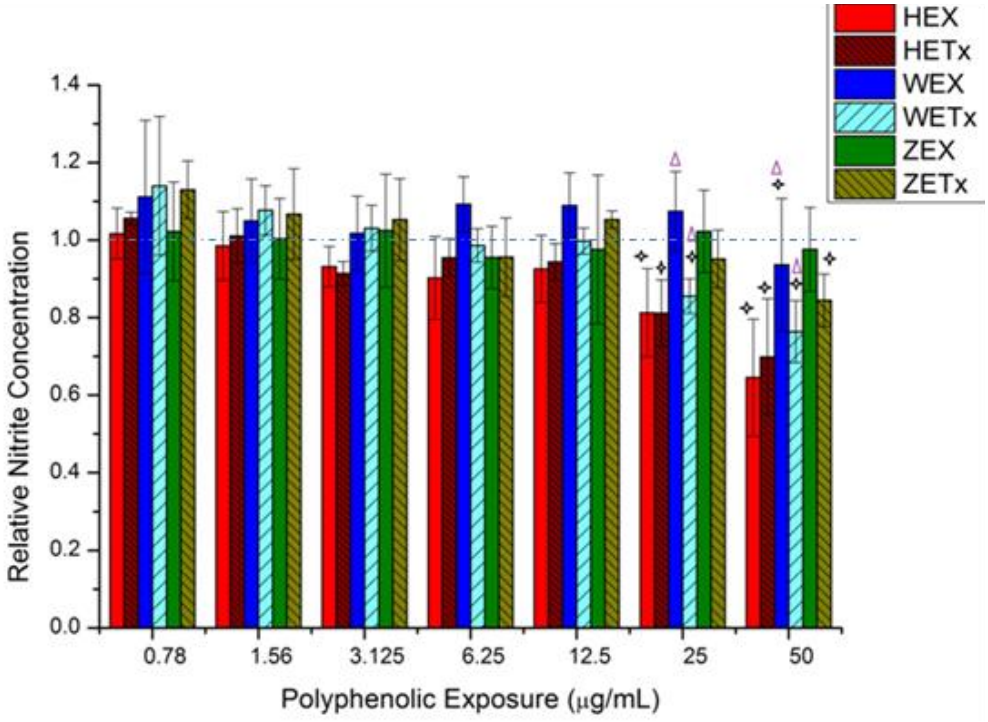

Figure 22: Effect of *Rubus* fruit extracts on nitrite concentration, as an indicator of NO production, by LPS stimulated RAW 264.7 macrophages. The cells were grown for 24 h LPS free, then co-incubated with increasing polyphenolic concentrations of the *Rubus* extracts with 0.05 μg/mL LPS. At the end of the assay the concentration of nitrite was determined as a measure of cellular NO production using the Griess assay. Results are displayed as relative NO production of extract-treated cells compared with controls exposed to cell culture medium only. ✧ statistically significant compared to controls. Δ statistically significant difference between paired extracts (EX vs ETx). The horizontal dotted line represents NO production of control RAW 264.7 macrophages. Each bar represents the standard deviation for three technical replicates

3.3.3 Effect of Rubus Fruit Extracts on Cytokine Production

Of the four cytokines assayed for in this study, IL6, TNFα, IL1β and IL10, only IL6 and TNFα were produced by the RAW 264.7 macrophages at detectable levels under LPS-stimulation. Results for IL6 and TNFα production in RAW 264.7 cells treated with *Rubus* fruit extracts relative to the untreated positive control (LPS stimulated but no phenolic treatment), are presented in Figure 23. The negative control (no LPS stimulation) did not produce detectable levels of any cytokine.

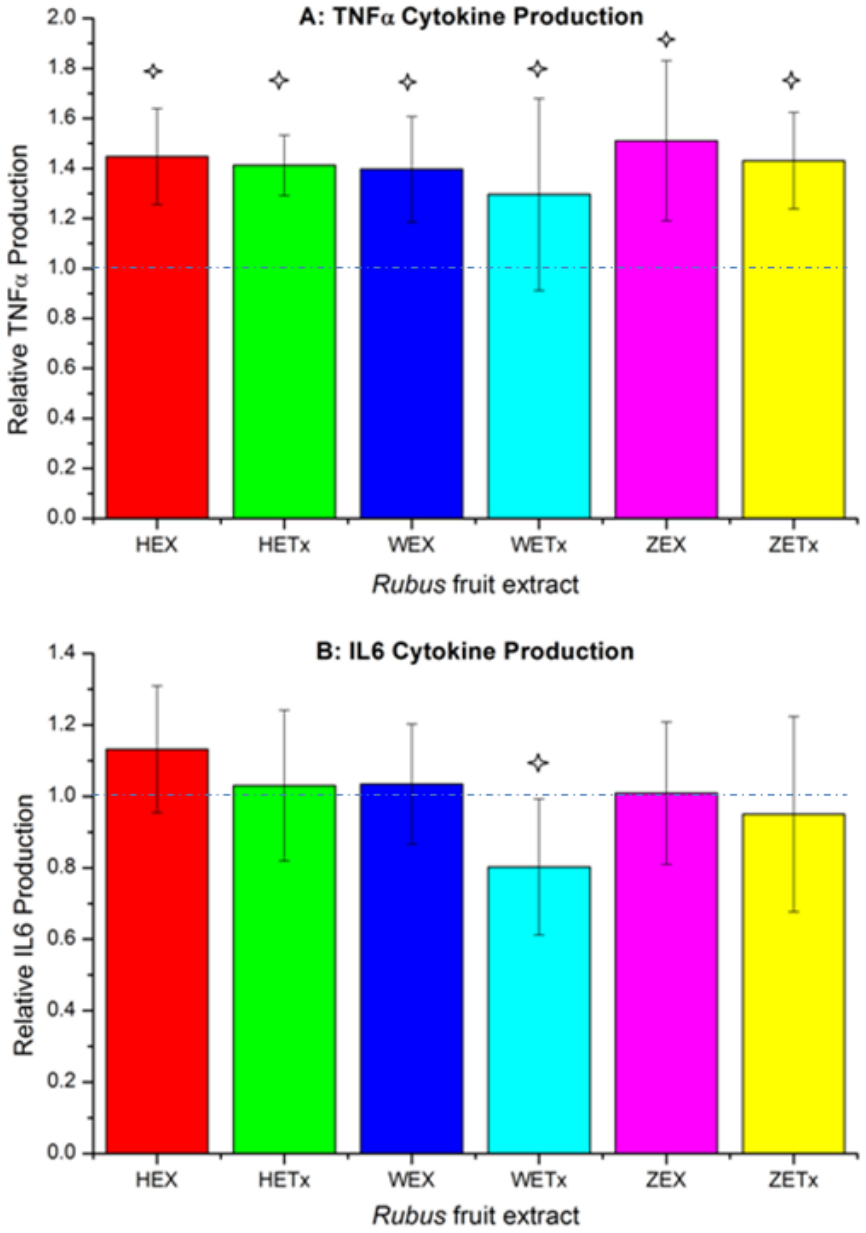

Figure 23: Cytokine production in LPS-stimulated RAW264.7 macrophages as a result of co-incubation with *Rubus* extracts. The cells were grown for 24 h LPS free, then co-incubated with 50 µg/mL of the *Rubus* fruit extracts and stimulated with the addition of 0.05 µg/mL LPS. At the end of the assay the supernatant was collected and cytokine concentration determined through custom ELISA assay plates. Results are displayed as relative cytokine production of extract-treated cells compared with controls exposed to cell culture medium only. ✧ statistically significant compared to controls. The horizontal dotted line represents cytokine production of control RAW 264.7 macrophages. Each bar represents the standard deviation for three technical replicates

All the *Rubus* fruit extracts tested significantly increased TNFα production in LPS stimulated RAW 264.7 macrophages relative to the LPS only stimulated control ($P < 0.001$), demonstrating a treatment wide effect. In contrast WETx was the only extract that significantly modulated IL6 production, a small but significant decrease observed ($P < 0.05$).

3.4 Discussion and Conclusions

In this chapter murine RAW 264.7 cells were used as a model to assess the potential anti-inflammatory effect of the six *Rubus* fruit extracts.

3.4.1 Cytotoxicity

The first step in this study was complicated by technical issues with the alamarBlue® cytotoxicity assay. Background fluorescence from the extracts, most likely due to the presence of anthocyanins, interfered with the assay fluorescence readings. Cell growth and cytotoxicity data for all extracts were therefore determined using the MTT assay. The MTT assay is an older method in which live cells convert the yellow tetrazolium salt into an insoluble purple formazan dye where the concentration of the dye directly correlates to the number of metabolically active cells in culture. The key difference for this method compared with the alamarBlue® assay was the removal of media and extracts prior to measurement, effectively reducing interference by the extracts.

The results from the MTT assay were consistent with the visual assessments made at the time of the assay, with ~10-20% decreases in cell density observed at the highest phenolic concentrations (100-200 µg/mL) for each extract. The MTT assay revealed that for polyphenolic concentrations of < 100 µg/mL there was no statistically significant indication of cytotoxic effects for any of the extracts tested. Based on these findings *Rubus* fruit extracts were applied to cells at overall polyphenolic concentrations of up to 50 µg/mL for all further assays to preclude cell cytotoxicity.

Although the MTT assay was used to evaluate extract cytotoxicity, there are alternative methods that could have been used such as such as ATP analysis or measurement of protease components [147]. These methods assess cellular density through different means than absorbance or fluorescence, and may be more suited to working with these extracts. Another assay that uses similar parameters to MTT is the WST-1 assay. This assay also uses tetrazolium

salts (as with MTT), but is a more recent development for this form of cytotoxicity testing and has logistical advantages over the older MTT method.

3.4.2 Nitrite

There was no change in NO production (inflammatory marker) by RAW 264.7 cells when exposed to *Rubus* fruit extracts at phenolic concentrations <25 µg/mL. At phenolic concentrations of 50 µg/mL all extracts, with the exception of the Boysenberry 'ZZ' extract ZEX, were able to induce at least a 10% reduction in NO production relative to untreated control cells (LPS stimulated but no phenolic treatment), suggesting an anti-inflammatory effect for these extracts. The 'HB19' Boysenberry extracts HEX and HETx and red raspberry 'Wakefield' extract WETx were also able to reduce NO production at a phenolic concentration of 25 µg/mL (Figure 22). There was no difference in efficacy between phenolic extracts (EX) and their ET-enriched equivalents (ETx) with the exception of the 'Wakefield' extracts WETx compared to WEX, where WETx was able to induce a greater decrease in relative NO production.

Further examination is needed to explore how the extracts might have an anti-inflammatory effect relating to NO production, especially with the observed increase in TNFα. One alternative test would be to examine a transduction enzyme, like mitogen-activated protein kinases, which have a role in regulating nitrite production and is known to be expressed in RAW 264.7 macrophages [148]. This enzyme has the ability to influence several target genes that are involved with immunity, such as the expression of pro-inflammatory cytokines [149]. The measurement of its activity through Western Blot analysis, could clarify how the extracts induce the anti-inflammatory effect [148].

3.4.3 Cytokines

Four cytokines were chosen to assess the anti-inflammatory potential of the Rubus fruit extracts based on their importance to IBD: TNFα, IL1β, IL6 and IL10 (143). However, in this study only IL6 and TNFα were expressed by the RAW 264.7 cells at detectable levels by the assay. It is unclear why IL10 and IL1β were not detected, as previous studies using this cell line have detected these cytokines [109, 150]. There is a possibility that the batch of cells used for this experimental work did not produce these cytokines, however, without further experimental work using multiple cell batches, it is impossible to deduce why the cytokines

were not produced. Low passage numbers for cells, 10-30, were used which was consistent with the literature, however differences in number for replication may also have influenced the cells reaction to LPS stimulation (137). There is also the potential that the LPS stock or dose used may have been ineffective in inducing the activation of necessary pathways in these cells. Studies by Gakwaed, et al., (2010) have shown that the dosage and differentiation state of macrophages is critical in determining the pattern of cytokine secretion (144).

There was an increase in TNFα production by the RAW 264.7 cells relative to the control for all extracts. There was no change in IL6 production by the cells for most extracts; WETx extract did decrease IL6 production relative to the control. This result was unexpected as in most examples in the literature, if there is a rise or decrease in TNFα levels, there is also a concomitant rise or decrease in IL6 levels (107, 151-154). Examination of cytokines with LPS stimulation was not conducted, so there is remote possibility that the cytokines could have been produced at maximum saturation and no increase or decrease could be affected (although with the rise of TNFα relative to the control this is not likely).

The immune response of the RAW 264.7 macrophages induced by WETx in the presence of LPS, show a trend of decreased immune activation relative to the other extracts. WETx produced the least rise in TNFα relative to the control and was the only extract to decrease in IL6 relative to the control. These results suggest that the response produced by this ET-enriched extract could be related to its higher proportion of SH6 relative to Lam C (see Figure 15), compared with the other ETx extracts. However, the uncertainty around some of the ET composition data for the extracts, as discussed in Chapter 2, means that it is not possible to firmly correlate SH6 and Lam C levels with anti-inflammatory activity. The effectiveness of WETx relative to its phenolic counterpart, WEX, is however supported by the NO assay results, and does indicate that the ETs alone have the ability to induce an anti-inflammatory response, without the presence of other phenolic compounds such as anthocyanins.

The anti-inflammatory effect observed for WETx could be further investigated via evaluation of additional inflammatory markers or measurement of key transcription factors to elucidate modulated signalling pathways. For example prostaglandin E2 (PGE2) is increasingly attracting attention as a marker and potential therapeutic target in several inflammatory conditions (155). The formation of PGE2 has been shown to be decreased by other polyphenols (156-158) and levels are easily assayed through ELISA. Two transcription factors implicated in the pathogenesis of IBD are STAT3 and NF-κB. IL6-mediated activation of STAT3 is a target of therapeutic treatment of IBD and has been shown to be decreased by polyphenols (159-161).

NF-κB is a transcription factor that plays a key role in regulating the inflammatory response through control of the expression of genes encoding inflammatory cytokines and control of other immune responses (128). Both STAT3 and NF-κB levels can be assayed using commercially available kits (e.g. Cignal STAT3 Reporter luc Kit: CCS-9028L, SABiosciences, USA and NF-κB (p65) Transcription Factor Assay Kit: 10007889, Cayman, USA). Using these assays in addition to the cytokines evaluated in this study, may help to understand the observed anti-inflammatory effect and would complement the work undertaken in this thesis.

Chapter 4: Effects of *Rubus* Fruit Extracts on Murine Isolated Primary Cell Cultures of Bone Marrow Derived Macrophages and Colon Cells

4.1 Introduction

Previous research carried out by Edmunds et al., (2011) (162), developed methods to test kiwifruit extracts, rich in polyphenols , for potential anti-inflammatory effects on an IBD model utilising murine primary cells. The cells isolated were bone marrow derived macrophages (BMDM) and intestinal epithelial cells (IEC), from C57BI/6J (C57) (healthy) and interleukin 10 gene deficient mice (*Il10*$^{-/-}$). *Il10*$^{-/-}$ mice develop colitis-like symptoms through an interaction between the GIT mucosa and innate GIT microbiota (163). The disease presentation is similar to human CD with a loss of tolerance to commensal bacteria, infiltration of activated macrophages and alterations of normal immune function (164).

Primary cells have the same genetic background as the whole animal from which they are derived, and provide a more in depth physiologically relevant assessment of the potential response to treatments when compared with secondary cell lines, such as the RAW 264.7 cells. RAW 264.7 cells have undergone genetic mutations in order to be immortalised (119), and were used in the preliminary *In vitro* screening done in Chapter 3. Secondary cell lines are commonly used as models for applied gut research but frequently show major structural or functional alterations as compared with their *in vivo* counterparts (168, 169). Primary cells on the other hand closely reflect the *in vivo* situation [151]. The disadvantage of primary cells is that they are more difficult to purify and culture, and are limited in their ability to proliferate (165, 166).

BMDM cells allow *in vitro* assessment of immunological responses to food components (125). BMDM are differentiated *in vitro* into mature macrophages which have not been exposed to any immune stimulus in *in vivo* conditions. The unstimulated macrophages are then able to be classically activated (such as through exposure to LPS), mimicking the natural inflammatory responses observed with activated macrophages found in patients with IBD (162). Using

unstimulated macrophages allows a clearer picture of the immunological impact the food components could induce, without confounding factors that may be present in pre-activated macrophages (162). IEC have a role in nutrient absorption, inflammatory regulation through initiation of immune responses in the intestinal mucosa, and form a physical barrier to protect against pathogens (167). The lumenal contents of the GIT, whether from microbial or food sources have the ability to directly affect the immune response, as recognition of microbial antigens occurs at the luminal face. IEC are able to be cultured *in vitro* and display normal physiology.

Using BMDM and IEC primary cells, Edmunds et al., (2011) (162), showed that kiwifruit extracts reduced inflammatory activation after LPS-stimulation, causing a reduction in NO and cytokine production in cells isolated from C57 and *Il10*$^{-/-}$ mice. This study demonstrated the successful application of a polyphenolic rich extract on primary cells isolated from murine sources, and provided an appropriate model with which to investigate the potential anti-inflammatory effects of *Rubus* fruit extracts.

4.1.2 Aims

The aim of this research was to evaluate the anti-inflammatory effect the *Rubus* fruit extracts may have in primary cell cultures of IEC and BMDM as a model of cells involved in immune responses during IBD.

A preliminary study was conducted to optimise the small intestinal and colonic IEC, and BMDM culture and assays for use of these primary cells with *Rubus* extracts using eight C57 mice (in two groups of four). The preliminary study used the same tests as detailed in Chapter 3 for the RAW 264.7 cells; cell viability, NO production and cytokine production. The cytokines examined in Chapter 3 (IL10, IL6, IL1β and TNFα), were assessed to optimise methodology for this model system.

In the main study, two genotypes of mice were used to isolate colonic IEC and BMDM: C57 and *Il10*$^{-/-}$. The effects of the Rubus fruit extracts on cell viability, NO production and cytokine production by the small intestinal and colonic IEC, and BMDM were investigated using the parameters optimised in the preliminary study. IEC were harvested only from the colon for logistical reasons. IL12 production was analysed for *Il10*$^{-/-}$ mice, instead of IL10, as *Il10*$^{-/-}$ mice do not produce IL10.

4.2 Methods

4.2.1 Materials

The following cell culture materials were purchased from Sigma Aldrich (Auckland, New Zealand): Liquid Roswell Park Memorial Institute medium (RPMI-1640; R8758), Hanks' Balanced Salt Solution (HBSS; H6648), dimethyl sulfoxide (DMSO; D2650), trypan blue (T8154), LPS from *Escherichia coli* serotype 0111:B4 (L4391), dithiothreitol (DTT; D0632), collagenase from *Clostridium histolyticum* (C9697), D-sorbitol (S1876), sulfanilamide (S9251), N-1-naphthylethylenediamine (70720), epidermal growth factor (EGF; E4127) and thiazolyl blue tetrazolium bromide (MTT; M5655).

Heat-inactivated foetal bovine serum (FBS; 10437028), phosphate buffered saline (PBS; 70011044), and penicillin-streptomycin (Pen/Strep: 10,000 U/mL penicillin and 10,000 µg/mL streptomycin) were purchased from Life Technologies (Auckland, New Zealand). Dispase II from *Bacillus polymyxa* (04942078001) was purchased from Roche (Auckland, New Zealand). Recombinant murine macrophage-colony stimulating factor (M-CSF; EBI-14-8983-80) and ELISA Ready-Set-Go! IL6 Cytokine plates by eBioscience (88-7064), were purchased from Huntingtree (Auckland, New Zealand). Mix-N-Match ELISArray plates (QIA336111) were purchased from SABiosciences through Qiagen (Auckland, New Zealand). CellStripper non-enzymatic cell dissociation solution by cellgro (25-056-01), was purchased from DKSH (Palmerston North, New Zealand). Pre-mixed WST-1 (630118), by Clontech was purchased from Norrie Biotech (Auckland, New Zealand). All non-sterile extract solutions were filtered immediately prior to use with cellulose acetate (CA) 0.22 µm syringe filters (13 mm diameter: SFCA013022S) purchased from Membrane Solutions (North Bend, Ohio, USA).

4.2.2 Preliminary Study

Experimentation was approved by the Grasslands Animal Ethics Committee, Palmerston North, New Zealand (AE Tissue Collection 99 Application), and was performed in accordance with the New Zealand Animal Welfare Act 1999. Eight C57 mice were transported from AgResearch Ruakura facility to AgResearch Palmerston North Ulyatt-Reid Facility. The mice had a seven day stand down period for acclimation to the new environment during which they had *ad libitum* access to standard chow and water. Animals were housed in a temperature and humidity controlled room with standard housing cages and environmental enrichment

with a 12 h light/dark cycle. After seven days the animals were humanely euthanised using CO_2 and cervical dislocation, in two groups of four (a day apart) for use in cell culture.

Three primary cell types (small intestinal and colonic IEC, and BMDM) were isolated from C57 mice (in two groups of 4) using aseptic methods and cultured using methods developed from Edmunds et al., (2011) [126]. The methods were optimised for BMDM and IEC culture conditions with *Rubus* fruit extracts (see Figure 24). BMDM were isolated from hind leg femurs and tibiae, and IEC isolated from the small intestine and colon. BMDM and IEC were each pooled from 4 mice to provide sufficient yield for the assays using these cell types.

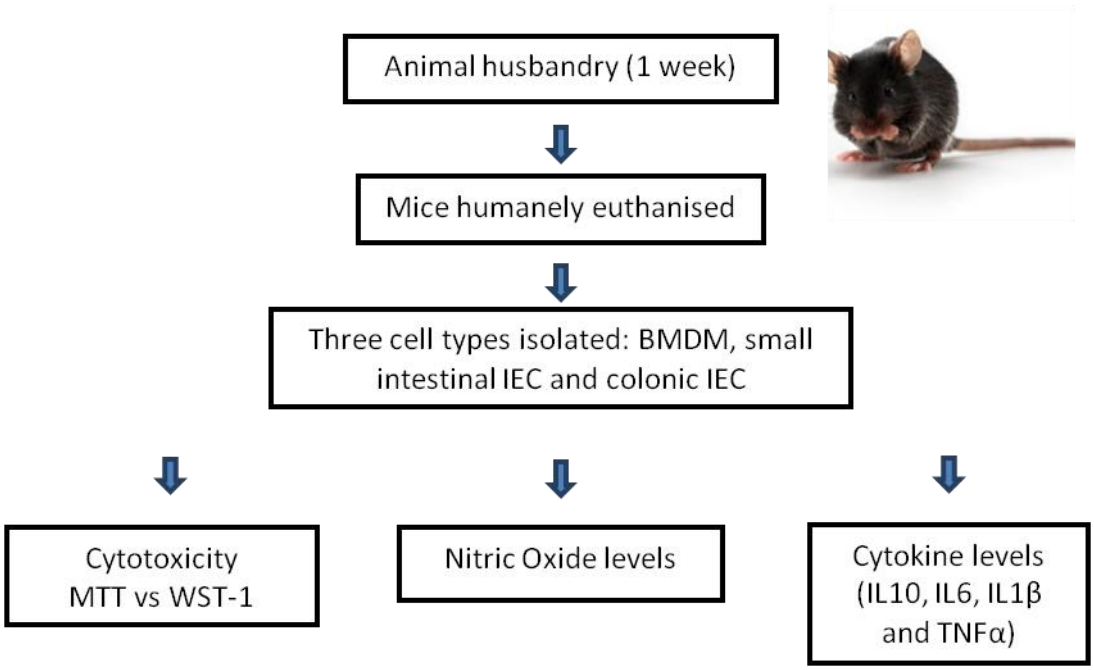

Figure 24: Schematic summary showing experimentation for preliminary study.

4.2.2.1 Bone Marrow Derived Macrophages Isolation

Isolated BMDM are relatively uniform in population characteristics and are likely to have a homogenous response to external stimuli for biochemical analysis [116]. Isolated BMDM were cultured with M-CSF to differentiate macrophage progenitor cells from the bone marrow into mature macrophages [116].

Both hind legs from mice were removed and the femur and tibiae isolated and placed in HBSS containing 5% FBS and 1% Pen/Strep. The bone tissue was then cleaned using scissors (to remove flesh) and sterile gauze (for fine cleaning of flesh). The bone cavity of each was exposed and then flushed with serum-free RPMI-1640 using a 5 mL syringe and 22 gauge needle. Once the passage ran clear the cell clumps were disrupted using an 18 gauge blunt end needle and a 3 mL syringe. The resulting cell suspension was passed through a 70 µm cell strainer to remove unwanted cellular debris and remove tissue contamination. The cell suspension was then centrifuged at 300 *xg* for 5 min at RT to remove excess media, and was resuspended in Complete Bone Marrow RPMI-1640 (Complete BRPMI) (see A.1 Additional Media Preparations) to give a final cell count of 2-4 x 10^6 cells/mL. Of the resulting suspension, 10 mL was plated out onto 10 cm cell culture treated Petri dishes (to promote cell adhesion) and placed in a humidified incubator at 37°C and 5% CO_2.

After three days 5 mL of Complete BRPMI were added to each Petri dish and cells were checked for growth and attachment. Seven days after plating, cell medium was removed and dishes were washed with sterile PBS to remove non-mature cells (unattached cells). Cells were harvested by adding 10 mL of CellStripper to the plates, which were incubated for 75 min in a humidified incubator at 37°C and 5% CO_2. After incubation cells were removed using a cell scraper and collected into a conical tube and washed twice in 10 mL Complete BRPMI. The cell suspensions were centrifuged at 300 *xg* for 4 min at RT after each wash. The cell count was then performed using a Countess automated cell counter (Life Technologies) on the resulting cell suspension. To count cells, 15 µL of cell suspension and 15 µL trypan blue dye were mixed together in a 1.5 mL Eppendorf tube. This mixture was loaded onto both sides of the disposable cell counting chamber slides allowing two readings of the same sample to be performed. Only the average viable count calculated was used for seeding plates, as detailed in Chapter 3.

4.2.2.2 Intestinal Epithelial Cells Isolation

The whole GIT was removed from the mice and caecum discarded in preparation of isolating IEC (Table 3). The tissues of the small intestine and colon were isolated and then separately placed in HBSS with 1% Pen/Strep. Both tissues were flushed with ice cold HBSS 1% Pen/Strep and opened longitudinally with any remaining mesenteric tissue removed. The tissues were then cut into small sections and minced with scissors. Afterwards the tissue was pipetted vigorously to further break up the tissues (resultant fragments were less than 1 mm) and both were transferred to conical bottom tubes and centrifuged at 300 x*g* for 5 min.

Medium was removed and 15 mL of HBSS 1% Pen/Strep with 15 mM dithiothreitol (DTT) (DTT HBSS) were added to the colon tissue to remove mucus. After shaking incubation for 15 min at 37°C, colon tissue was centrifuged at 300 x*g* for 5 min to remove DTT HBSS. Both the small intestinal and colonic sedimented cells and tissues, were resuspended in 20 mL of prepared Low Collagenase/Dispase Enzyme Solution (see A.1 Additional Media Preparations) which was filtered just prior to use. Both tubes were incubated in a shaker at 25°C for 45 min at 150 rpm.

After this incubation the small intestinal cell suspension was removed and the temperature increased for the colon tissue, to 30°C for a further 45 min. The small intestinal cell suspension was then centrifuged at 300 x*g* for 5 min to remove the enzyme mix and resuspended in Complete IEC RPMI (Complete IRPMI), (see A.1 Additional Media Preparations) with 10% D-Sorbitol by vigorous pipetting. Cells were separated under gravity and the supernatant (containing IEC) was collected and passed through a 70 µm cell strainer. The passing of supernatant through the strainer was repeated a total of three times.

This process of straining cells was then applied to the colon cell suspension at the end of the remaining incubation. The filtered suspensions were then centrifuged and the media replaced with Complete IRPMI. The cell count was carried out on the resulting cell suspension as described earlier for BMDM and the cells were plated out. IEC cells were cultured with EGF (at 10 ng/mL) to improve cell growth and differentiation [152].

Table 3: Summary flow chart describing experimental procedures for isolating IEC.

Small intestinal IEC	Colonic IEC
Whole GIT isolated from mouse.	
Tissue isolated and placed in HBSS with 1% Pen/Strep.	Tissue isolated and placed in HBSS with 1% Pen/Strep.
Tissues processed and centrifuged.	Tissues processed and centrifuged.
Resuspended in Low Collagenase/Diapase Enzyme Solution. 45 min shaking incubation at 25°C.	Resuspended in DTT HBSS. 15 min shaking incubation at 37°C.
Centrifuged and resuspended in Complete IRPMI, with 10% D-Sorbitol.	Centrifuged and resuspended in Low Collagenase/Diapase Enzyme Solution. 45 min shaking incubation at 25°C.
Cells settled under gravity and supernatant passed 3x through 70 µm cell strainer.	Further 45 min shaking incubation at 30°C.
Cells counted and plated.	Centrifuged and resuspended in Complete IRPMI, with 10% D-Sorbitol.
	Cells settled under gravity and supernatant passed 3x through 70 µm cell strainer.
	Cells counted and plated.

4.2.2.3 Cytotoxicity Assay of *Rubus* Fruit Extracts

Cell growth and viability were quantified using MTT or WST-1 in order to determine if there was any cytotoxic effect of the *Rubus* fruit extracts on primary cell cultures of BMDM and small intestinal and colonic IEC. The MTT cytotoxicity assay used in Chapter 3 was compared to the WST-1 cytotoxicity assay. The WST-1 assay is similar to the MTT assay in that it is a colorimetric method that assesses the cytotoxic effect of a sample of interest by measuring the number of metabolically active cells in a culture [144]. This assay works under the same principle as MTT, by the reduction of a tetrazolium salt by metabolic activity, resulting in the production of a dark red formazan dye that is able to be quantified through absorbance [144]. However, this assay differs from MTT in that it is non-lethal to the cells meaning it can therefore additionally be used for time course assessment of cytotoxicity, and the resultant formazan dye produced is water soluble.

The IEC were seeded into 96 well plates at a concentration of 10,000 cells per well with 100 µL loaded into appropriate wells. The plates were incubated for 24 h at 37°C, 5% CO_2. To decrease the serum concentration, 50% of the media was gently extracted and replaced with fresh Assay IRPMI (see A.1 Additional Media Preparations). In primary cell cultures it is important to decrease serum concentrations (through media replacement not containing FBS), as it has been shown that, over time, higher concentrations of serum inhibit long term epithelial growth [153]. After media replacement plates were returned to the incubator for a further 24 h period.

Rubus fruit extracts were prepared and resuspended in 1 mL of Assay IRPMI and immediately passed through a 0.22 µm CA filter. Two-fold dilutions were prepared in Assay IRPMI to test the following final polyphenolic concentrations for all *Rubus* extracts (µg/mL): 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78. Fifty microlitres of *Rubus* extract solutions or culture medium were added to the appropriate wells and co-incubated for 18 h. To each well, 15 µL of MTT at the concentration of 5 mg/mL was added, and co-incubated for a further 2 h. At this point the medium, MTT and extracts were aspirated from the wells, and 100 µL of DMSO was added to solubilise the purple crystalline precipitate. After 2.5–3 h incubation at RT in the dark, the absorbance at 570 nm (and 700 nm for background correction) was measured using a FlexStation® 3 Benchtop Multi-Mode Microplate Reader. Separately, 15 µL of pre-mixed WST-1 was added to each well containing IEC isolated from group 1 C57 mice. The plates were then incubated for a further 2 h, after which the absorbance at 450 nm (and 650 nm for

background correction) was measured using a FlexStation® 3 Benchtop Multi-Mode Microplate Reader.

Due to low BMDM cell isolation cytotoxicity was assessed using BMDM pooled from group 1 C57 mice and inflammatory marker assays were assessed using BMDM pooled from group 2 C57 mice. Cells were seeded into 96 well plates at a concentration of 10,000 cells per well, with 100 µL loaded into appropriate wells. Plates were incubated for 24 h at 37°C, 5% CO_2. *Rubus* fruit extracts were prepared and added to the growth plate in the same manner as described for IEC but using Complete BRPMI medium. After 18 h of co-incubation of extracts with the cells, the MTT assay was completed as described for IEC cellular work.

4.2.2.4 Nitric Oxide Assay

IEC were grown overnight at 37°C, with 5% CO_2, in a 96-well culture plate at a density of 40,000 cells per well with 100 µL cell suspension in Complete IRPMI medium per well. Fifty percent of the medium was gently replaced (to minimise cell disturbance) and then plates were returned to the incubator for a further 24 h period.

Fifty microlitres of culture medium with or without *Rubus* fruit extracts (150 µg/mL) was added to give a final extract concentration of at 50 µg/mL (or control equilvalent), and the plates incubated for 1 h. Three microlitres of LPS solution in culture medium at a final concentration of 0.05 µg/mL, or cell culture medium (to control wells), was added and the plates were incubated for a further 19 h. As previously described for RAW 264.7 macrophages in Section 3.1.1.2, the production of NO by cells stimulated with LPS was determined as nitrite using the Griess assay.

BMDM were grown at a lower density of 30,000 (compared with the density of 40,000 for IEC) cells per well due to low cell isolation yields, with 100 µL cell suspension in Complete BRPMI per well. The same preparation of extracts and LPS solutions were applied as described for NO assay of IEC.

4.2.2.5 Cytokine Assay

Pro- and anti-inflammatory cytokines (IL10, IL1β, TNFα, and IL6) were measured to assess immunological changes induced by co-incubation with the extracts. Small intestinal and colonic IEC were seeded on 96-well plates at a density of 40,000 cells/well, with the BMDM

seeded at a density of 30,000 cells/well. The supernatant was collected from the same plates as seeded in Section 4.2.2.4. The supernatant from three wells was pooled into a 1.5 mL Eppendorf tube and cell debris removed by centrifugation at 10,000 x*g* for 10 min, at RT. Supernatants were then transferred into fresh Eppendorf tubes and frozen at -80°C until analysis.

On the day of analysis the samples were loaded onto the custom designed plates which had the same layout as Figure 19 and assayed using ELISA following the same protocol as outlined in Section 3.2.8. Only supernatant isolated from group 2 mice, for all cell types, was used due to the low yield of cells isolated from group 1 for BMDM and experimental error in processing for IEC from group 1.

4.2.3 Chemical Interference by Extracts and Optimisation of LPS-Stimulation for Bone Marrow Derived Macrophages

Two additional studies were carried out after the preliminary study and prior to the main study to further optimise methods.

Firstly, experiments were conducted to assess if there was any interaction between the chemical components of the medium and/or the *Rubus* fruit extracts with the viability detection reagents. This study used the same methods as previously described in Section 4.2.2.3 for both the MTT and WST-1 assays with the exception that no cells were used.

Low yields of BMDM in the preliminary study meant that determining the optimal amount of LPS needed for adequate BMDM stimulation was not assessed. Using C57 mice from an on-going study (Ethics Approval Number: AEC 12579), the assay was conducted with cells isolated as described in Section 4.2.2.1, and grown at 37°C, with 7% CO_2. Cells were seeded in a 96-well culture plate at a density of 40,000 cells per well with 100 µL cell suspension in Complete BRPMI per well. The change in CO_2 level was a result of a change in laboratory room for experimental work. This change in level is within normal primary cell culturing techniques as levels can vary from 5-10%, however it can slightly decrease the pH of the media.

Fifty microlitres of fresh Complete BRPMI was added after 24 h incubation. LPS dilutions were prepared in Complete BRPMI at final concentrations of 1, 0.5, 0.25, 0.1, 0.05 and 0.01 µg/mL and 3 µL of each (or fresh media for control) was added after 1 h and co-incubated for a further 19 h.

At the end of the incubation, cells were subjectively assessed by light microscopy for visual changes of proliferation in response to co-incubation with the extracts and morphology in response to LPS-stimulation. Changes from controls were noted, and are reported in results. Changes from controls were noted, and are discussed if relevant in results. Supernatant was collected and assessed for NO production by the cells using the Griess assay as described in Section 4.2.2.4.

4.2.4 Main Study

Colon IEC and BMDM were isolated from two genetically distinct types of mice - C57 (control mice; n = 10) and *Il10*$^{-/-}$ (IBD model mice; n = 10), in order to assess the anti-inflammatory properties of *Rubus* fruit extracts using *in vitro* conditions (see summary Figure 25). Experimentation was approved by the Grasslands Animal Ethics Committee, Palmerston North, New Zealand (AEC 12826 Application) and performed in accordance with the New Zealand Animal Welfare Act 1999. Mice aged 5-5.5 weeks arrived in two groups of 10 (with five of each genotype) with a two week interval between groups to allow for logistics of cell culturing work. Isolation and culture methods were adapted according to the results and modifications from the preliminary study.

Upon arrival at Grasslands, the mice were housed and taken care of as described in Section 4.2.2. After the seven day acclimation period mice were switched to the AIN diet and fed three times a week, and given *ad libitum* access to water. Monitoring of animal health, food intake, and weight was conducted during feeding times to decrease undue handling and stress to animals. After 1 week of the AIN diet, all animals received an inoculation with intestinal bacteria (see Section 4.2.4.1) to ensure colon inflammation was induced in the *Il10*$^{-/-}$ mice and for comparison to C57 (or control) mice [154]. At 11–11.5 weeks of age mice were euthanised using humane methods (CO_2 asphyxiation followed by cervical dislocation) and primary cells isolated as described in Section 4.2.4.2 and Section 4.2.4.3.

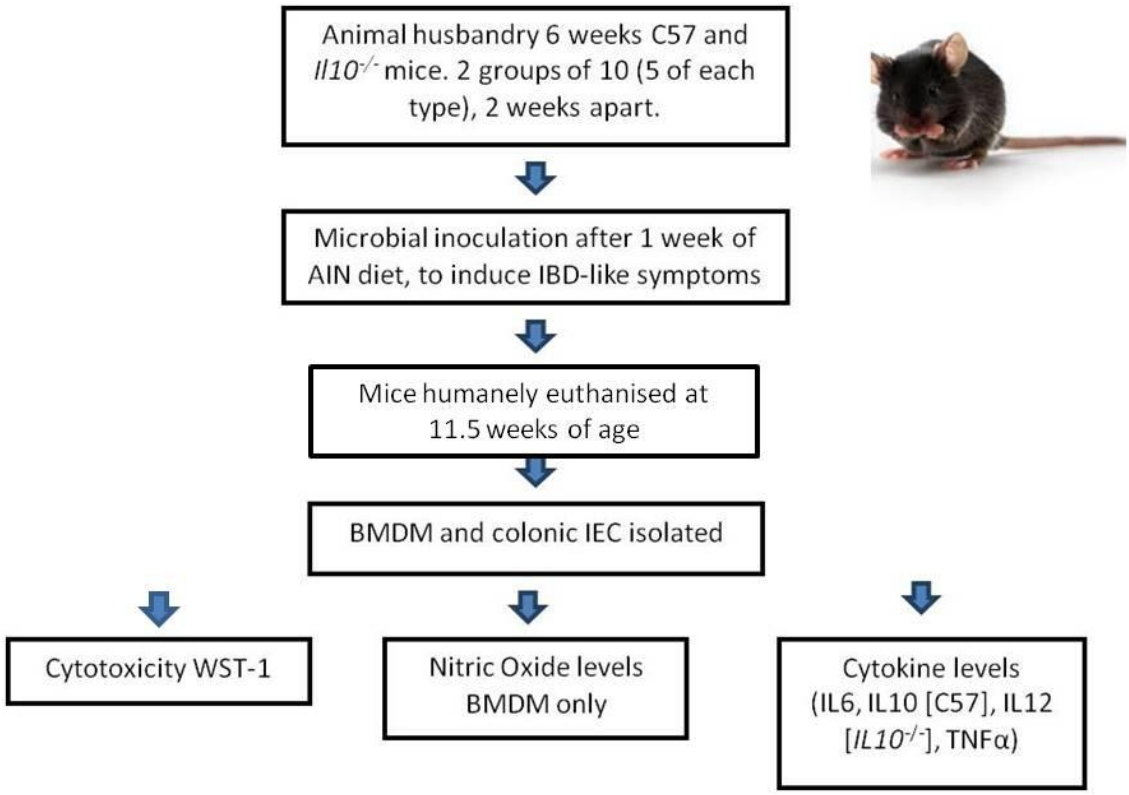

Figure 25: Summary schematic for main study focusing on BMDM and colon cells.

4.2.4.1 Intestinal Bacteria Inoculation

A fresh inoculation was prepared for each group of mice, with the same methods used for each inoculation preparation [154]. Twelve strains of *Enterococcus* isolated from calf and poultry sources, were grown on Slanetz & Bartley medium (see A.1 Additional Media Preparations) Petri dishes for 48 h at 37°C (in between preparations, *Enterococcus* strains were frozen at -80°C). After incubation, one colony from each agar plate was transferred into separate sterile polypropylene tubes, each containing 5 mL of Todd Hewitt Broth (see A.1 Additional Media Preparations). The tubes were then capped and incubated for 24 h at 37°C. Three cultures were then randomly pooled into 4 large falcon tubes (total of 15 mL culture broth per tube) and centrifuged at 3000 x*g* for 10 min to pellet the bacteria. The supernatant was removed and each bacterial pellet resuspended in 15 mL of sterile phosphate buffered saline (PBS). Then resuspensions were pooled in a sterile 100 mL Schott bottle, resulting in the final *Enterococcus* suspension (ES).

Leigh Ryan (AgResearch Research Technician) prepared the conventional intestinal flora (CIF) on the same day the ES was prepared. For each inoculum, one C57 mouse, sourced from

AgResearch, Ruakura, was humanely euthanised and the GIT removed. The lumenal contents were flushed out by running PBS through the GIT, and splitting the caecum open and washing the contents. The slurry produced was collected and diluted out with PBS to a final volume of 10 mL and this suspension was used in inoculum preparation.

To generate the final microbiota inoculation (CIF-ES) 5 mL of the fresh ES and 5 mL of the CIF were mixed in a sterile 50 mL Falcon tube. Each mouse was inoculated orally with 200 µL of the final suspension (apart from mouse 18 which only had 100 µL due to poor health at the time of inoculation).

To determine the colony forming units (CFU) given to each group of mice, the ES was ten-fold serially diluted down to 10^{-4}. Three 20 µL drops of each of the four lowest concentrations were plated out on Slanetz & Bartley plates and incubated at 37°C for 48 h. The final colony count was then used to calculate the CFU that the mice were inoculated with.

4.2.4.2 Bone Marrow Derived Macrophages Isolation

The methods used to isolate BMDM from C57 mice and *Il10*$^{-/-}$ mice were as described in Section 4.2.2.1, with a number of minor modifications to increase yield of isolated cells. After the tissue was cleaned off the bone, the bones were dipped (1-2 seconds) in 70% ethanol followed by a wash in sterile PBS. Seven days after plating, cell medium was removed and dishes were washed with sterile PBS to remove non-mature cells. BMDM were harvested by incubation with 10 mL of CellStripper for 75 min at 37°C, 7% CO_2, followed by vigorous pipetting to dislodge loosely attached cells. The suspension was extracted from the plates and collected into a conical tube. To the plates, 5 mL of Complete BRPMI was then added and remaining BMDM were removed by using a cell scraper and collected into the same conical tube.

4.2.4.3 Colon Cells Isolation

The methods used to isolate colon cells from C57 mice and *Il10*$^{-/-}$ mice were as described in Section 4.2.2.2, with a number of minor modifications to increase expected yield of isolated cells. After the removal of mucus the colon cell suspension was resuspended in 20 mL of prepared High Collagenase/Dispase Enzyme Solution (see A.1 Additional Media Preparations)

which was filtered using a 0.22 µm CA filter just prior to use. The colon suspension was incubated in a shaker at 25°C for 45 min at 150 rpm and at 30°C for a further 45 min.

The colon suspension was then centrifuged at 300 x*g* for 5 min to remove the enzyme solution. The pellet was resuspended in Complete IRPMI with 10% D-Sorbitol by vigorous pipetting and allowed to settle under gravity (repeated three times) prior to separation.

4.2.4.4 Cytotoxicity of *Rubus* Fruit Extracts

Cell growth and viability was quantified using the WST-1 assay in order to determine the highest non-cytotoxic dose of EX and ETx for each *Rubus* fruit cultivar tested, for both BMDM and colon cells. The same extract preparations were used and all assay work was conducted with cells seeded into 96-well plates with 10,000 cells/well for BMDM assay work, and 20,000 cells/well for colonic IEC assay work, in order to ensure that cells were actively proliferating through the assay period.

Methods for *Rubus* extract cytotoxicity testing on colon cells were as described in Section 4.2.2.3, with the following modification. After 20 h of co-incubation of extracts with the colon cells, the medium was removed and replaced with fresh medium (with the same ratio of 2:1 Assay and Complete IRPMI) and 15 µL of pre-mixed WST-1 was added to each well. The plates were then incubated for a further 4 h.

BMDM cytotoxicity measurement was as described previously with the modification that after 20 h of co-incubation of extracts with the BMDM, the medium was removed and replaced with fresh Complete BRPMI and 15 µL of pre-mixed WST-1 was added to each well. The plates were then incubated for a further 2 h.

For both cell types the absorbance at 450 and 650 nm was recorded using a FlexStation® 3 Benchtop Multi-Mode Microplate Reader. Visual assessment of viability and proliferation was carried out using light microscopy before each step of the above procedure.

4.2.4.5 Measuring Nitric Oxide Production in Bone Marrow Derived Macrophages

The methods used for the measurement of NO were as described in Section 4.2.2.4, with the only modification being an increase in the LPS concentration in culture medium to 0.5 µg/mL for BMDM. The NO levels of colonic IEC were not measured, due to evidence from a previous study by Edmunds, 2011 *[126]* using similar methods, which showed a lack of production of NO for this cell type and which was verified from results of the preliminary study.

4.2.4.6 Measuring Cytokine Production in Bone Marrow Derived Macrophages

BMDM isolated from *Il10*$^{-/-}$ mice do not produce the IL10 cytokine, and therefore levels of another cytokine, IL12, were monitored for this cell type instead. IL12 is a pro-inflammatory cytokine produced by activated macrophages, and is upregulated in both CD and UC [155]. It has been previously reported to be produced by BMDM using similar primary cell culturing methods [126].

The methods for measuring cytokine production by BMDM were as described in Section 4.2.2.5, other than an increase in the LPS concentration to 0.5 µg/mL. The layout of the custom designed Multiplex ELISArray plate (SABioscience through Qiagen: QIA336111) as shown in Table 4. Cytokine concentrations were analysed with only one freeze-thaw cycle prior to analysis. Three cytokines were measured concurrently from each supernatant sample. For C57 derived supernatant IL6, IL10 and TNFα were analysed. For *Il10*$^{-/-}$ derived supernatant IL6, IL12 and TNFα were measured.

BMDM isolated from mice from both genotypes and groups were analysed with a randomised plate design. All assays were performed following the manufacturers protocol as described in Section 3.2.8

Table 4: Layout of custom designed Multiplex ELISArray for cytokine analysis of supernatants collected from C57 or *Il10*$^{-/-}$ mice. Plate consisted of 12 strips running vertically, with pre-coated capture antibodies (C57 mice: IL6, IL10, and TNFα, *Il10*$^{-/-}$ mice: IL6, IL12, and TNFα), allowing quadruplicate cytokine representation per plate.

IL6	IL10 /IL12	TNFα	IL6	IL10 /IL12	TNFα	IL6	IL10 /IL12	TNFα	IL6	IL10 /IL12	TNFα
				Negative	Control	Row					
					Samples						
				Positive	Control	Row					

4.2.4.7 Measuring Cytokine Production from Colon Cells

The results of the preliminary study indicated that the colon cells did not produce cytokines other than IL6 at detectable levels. A more sensitive product called Ready-Set-GO! Pre-coated mouse IL6 ELISA plates were used to measure IL6 production.

Each plate layout was randomised to avoid potential bias, and to reflect the uneven replication between groups in sample sets isolated. Plate layout design was done by Alana Srubar-Vernon with assistance from Siva Ganesh (Biostatistican, Grasslands, AgResearch, Palmerston North).

The IL6 ELISA assays were carried out following the manufacturer's instructions. Cytokine concentrations were analysed with only one freeze-thaw cycle prior to analysis. Prior to thawing of the prepared supernatants, plates were prepared with an overnight incubation with 100 µL of capture antibody prepared in coating buffer. The next day the plates were washed three times (using an automated plate washer) with wash buffer, and 200 µL of Assay Diluent was loaded into each well and incubated for 1 h at RT. After one wash to remove

Assay Diluent, 100 µL of standards (with appropriate dilutions for reference curve production) and thawed supernatants were loaded appropriately onto the plate and incubated at RT for 2 h. After 2 h the plate was then washed four times with wash buffer and 100 µL of prepared detection antibody was loaded into each well and incubated for 1 h. The plate was then washed four times and 100 µL of prepared Avidin-HRP was loaded into each well and incubated for 30 min. Six washes were then carried out and 100 µL of substrate solution was loaded into each well and incubated for 15 min in the dark at RT. Fifty microlitres of stop solution (1M H_3PO_4) was then loaded into each well and the absorbance read at 450 and 570 nm using a BMG FLUOstar Omega plate reader.

4.2.5 Statistical Analysis

Statistical design of experiments and plate layouts for the final study was done with assistance from Siva Ganesh (Biostatistican, Grasslands, AgResearch, Palmerston North). All data analysis was done by Alana Srubar-Vernon, with assistance from Mark Wohlers (Biometrician, Plant and Food Research, Mt Albert), using a one-way Analysis of Variance (ANOVA), followed by post-hoc testing using Fishers Unprotected LSD, with a *P* value of 0.05. All statistical analysis was performed using GenStat (14^{th} edition, VSN International, Hemel Hempstead, United Kingdom 2011). Where replication in samples was possible, error bars shown on graphs represent standard deviation from technical replicates.

4.3 Results

4.3.1 Preliminary Study

4.3.1.1 Cytotoxicity

Cytotoxicity assays (MTT and WST-1) were conducted to assess the effects of the *Rubus* fruit extracts on both BMDM and IEC cell viability and growth changes. Eight C57 mice from two groups (4 in each) were used to isolate pooled IEC or BMDM (with technical replicates where possible). A comparison of MTT and WST-1 assays was made to assess suitability of the WST-1 assay for application in the main study.

Figure 26 shows the relative viability of *Rubus* extract-treated small intestinal IEC compared to that of IEC exposed to control medium only (control IEC) using the WST-1 assay (group 1 mice)

and the MTT assay (group 2 mice). WST-1 assay results were more consistent between extracts (HETx an outlier) than the MTT assay results (Figure 26). For WST-1, with the exception of HETx, small intestinal IEC displayed up to 5-10 fold cell density compared to control IEC, at a polyphenolic concentration of 200 µg/mL, relative to control IEC. Small intestinal IEC exposed to the *Rubus* fruit extract WEX, showed an increase in growth 400 times that of the control IEC using the MTT assay (data not shown in Figure 26 B). The results obtained for *Rubus* extracts with both the MTT and WST-1 assays did not agree with the visual assessment of small intestinal IEC growth, the latter consistent with estimated 1.5 fold increase compared to the control IEC (data not shown).

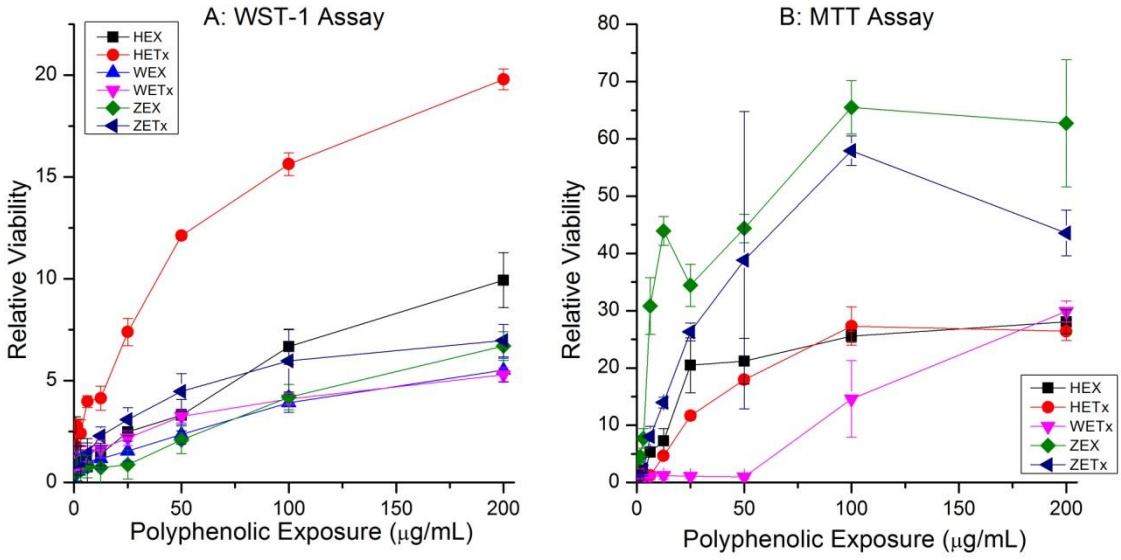

Figure 26: Relative viability of *Rubus* fruit extract-treated small intestinal IEC compared with control IEC (IEC exposed to control medium only) plotted against polyphenolic concentration (µg/mL) for A: WST-1 with cells isolated from group 1 mice, and B: MTT with cells isolated from group 2 mice. One experiment is shown for each assay consisting of pooled cells from four mice. Each bar represents standard deviation from two technical replicates.

Figure 27 shows the relative viability of *Rubus* extract-treated colonic IEC compared to that of control IEC, using the WST-1 assay (group 1 mice) and the MTT assay (group 2 mice). A low yield of colonic IEC was obtained from group 1 mice and only one viability point was generated using the WST-1 assay. This was likely due to processing steps not being completed

in optimal time, which was refined for group 2 mice for the MTT assay. Results from both methods were not reflective of the visual assessment of colonic IEC growth, which was increased 1.5-2 fold when IEC were co-incubated with the *Rubus* fruit extracts relative to the control IEC.

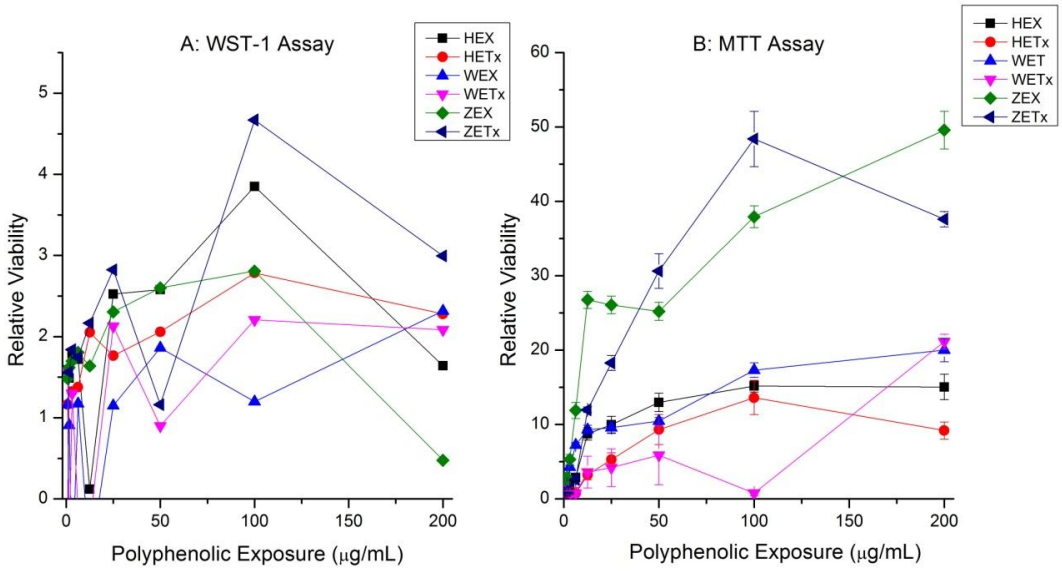

Figure 27: Relative viability of *Rubus* fruit extract-treated colonic IEC compared with control IEC (IEC exposed to control medium only) plotted against polyphenolic concentration (µg/mL) for A: WST-1 with cells isolated from group 1 mice and B: MTT with cells isolated from group 2 mice. There were not enough cells isolated from group 1 mice to allow technical replication for the WST-1 assay. One experiment is shown for each assay consisting of pooled cells from four mice. Each bar represents the standard deviation from three technical replicates for group 2 mice using the MTT assay.

Figure 28 shows the relative viability of *Rubus* extract-treated BMDM isolated from group 1 mice compared to that of BMDM exposed to control medium (control BMDM). The yield of BMDM was insufficient and only one experiment was carried out using the MTT assay (group 1 mice). The results indicate an increase of BMDM growth (1.5-5 fold) when cells were co-incubated with the *Rubus* extracts. These results did not concur with the visual assessment; with an estimated 1.5 fold increase associated with the extracts at the highest polyphenolic concentration.

The results of *Rubus* extract-treated IEC and BMDM suggest that further experimentation is needed to address the inconsistencies between the cell viability obtained from both the WST-1 and MTT assays and the visual observations of cell density. These inconsistencies may be due to interference between the *Rubus* extracts and cell culture medium.

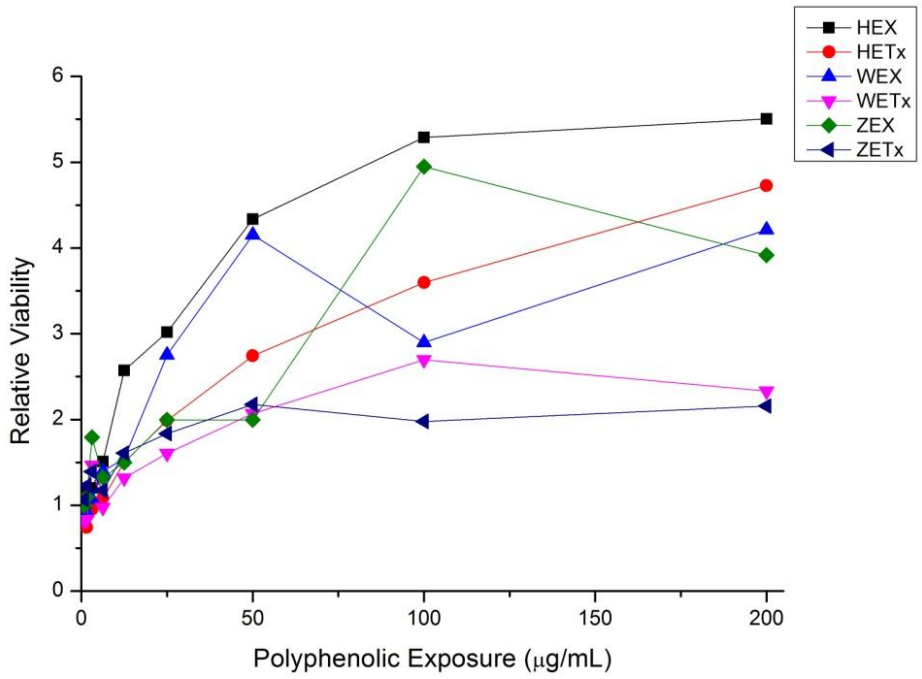

Figure 28: Relative viability of *Rubus* fruit extract-treated BMDM compared with control BMDM (BMDM exposed to control medium only) plotted against polyphenolic concentration (μg/mL) for the MTT assay, isolated from group 1 mice. One experiment consisting of pooled BMDM from four mice is shown. Due to the low number of BMDM isolated no technical replication or comparison to WST-1 assay was possible for this cell type.

4.3.1.2 Nitric Oxide Production

The Griess assay was used to determine the nitrite concentration as an indirect measure of NO production (indicator of inflammation) by IEC and BMDM treated with *Rubus* extracts followed by stimulation with LPS.

Small intestinal and colonic IEC from group 1 and 2 mice did not produce detectable levels of NO (data not shown). Similarly, the BMDM isolated from group 2 mice did not produce detectable levels of NO (data not shown), which could be due to the low cell density obtained or insufficient LPS-stimulation.

4.3.1.3 Cytokine Production

Assessment of IL6, IL10, IL1β and TNFα production by LPS-stimulated small intestinal and colonic IEC, and BMDM after co-incubation with the *Rubus* fruit extracts was carried out using pooled cells from group 2 mice. Overall, the low yield of all cell types: small intestinal and colonic IEC and BMDM, and experimental issues, meant that for each cell type only one data point per treatment for cytokine production was obtained. The lack of replication means that the results should not be over-interpreted and can only be used as an indication of the potential effects of *Rubus* fruit extracts on cytokine production by colonic IEC and BMDM.

Due to experimental error, supernatant for analysis was not collected from group 1 small intestinal and colonic IEC. The small intestinal IEC isolated from group 2 mice did not produce IL6, IL10, IL1β and TNFα at detectable levels (data not shown). Results displayed in Figure 29 and Figure 30 show the absolute levels of cytokines produced by the colonic IEC and BMDM co-incubated with the extracts rather than the levels of cytokines produced in response to exposure to the extracts relative to their LPS positive or negative controls.

Colonic IEC (group 2 mice) with and without LPS-stimulation produced low levels of IL6 (Figure 29), however, no IL10, IL1β or TNFα were detected (data not shown). Within the limits of the replication, the addition of LPS raised the level of IL6 produced for positive LPS control (Con +) and *Rubus* extract-treated colonic IEC (apart from HEX), compared with no LPS-stimulation. The *Rubus* extracts with LPS (+) or without LPS (-) stimulation decreased IL6 production compared to their relative control (apart from HETx -) (Figure 29). Co-incubation of colonic IEC with the extracts WETx, ZEX and ZETx without LPS-stimulation (-) did not produce detectable levels of the IL6 cytokine.

There were not enough BMDM isolated from group 1 mice to be used for cytokine assessment. Therefore, only BMDM isolated from group 2 mice were used, with only enough cells isolated for one growth plate at a lower cell density of 30,000 cells/well (compared with 40,000 cells/well for small intestinal and colonic IEC). BMDM were examined for changes in cytokine production as a result of co-incubation with the *Rubus* fruit extracts. Within the limits

of the replication, BMDM co-incubated with medium alone (Con -) did not produce IL6 but did produce TNFα and IL10. There was a decrease in the amount of IL6 and IL10 produced by BMDM for all *Rubus* extracts compared to the positive control BMDM (Con +). An increase in production of TNFα was observed compared with the positive control BMDM for all extracts apart from WEX. IL1β was not detected for any extracts tested (data not shown).

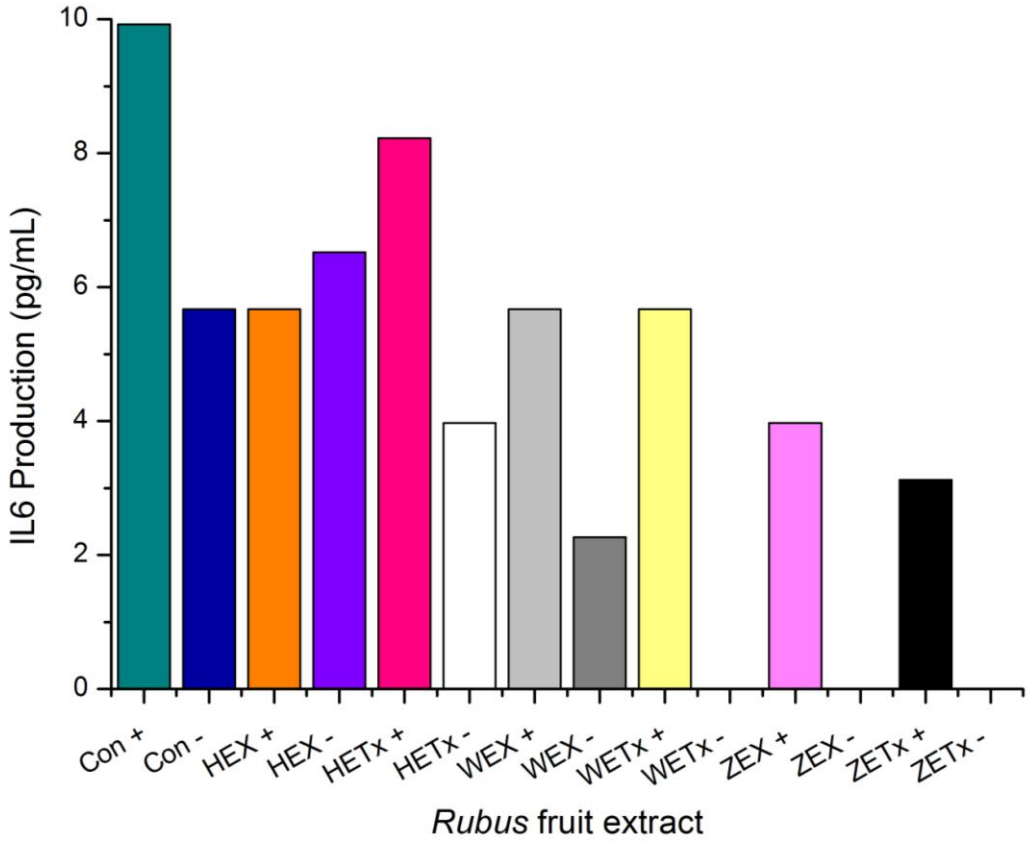

Figure 29: The effect of 50 µg/mL *Rubus* fruit extracts, after 19 h co-incubation, on the production of IL6 by colonic IEC isolated from group 2 mice (n = 1 technical replicates), compared to control IEC (IEC exposed to control medium only), with medium with (+) or without (-) 0.05 µg/mL LPS co-incubation. One experiment consisting of pooled cells from four mice is shown.

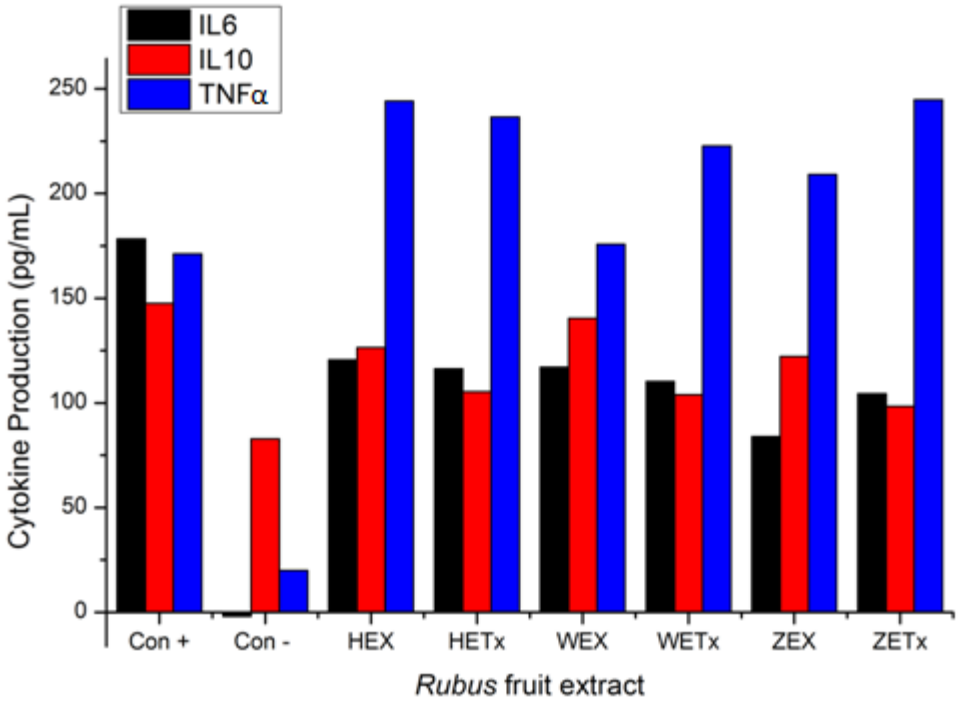

Figure 30: The effect of 50 µg/mL *Rubus* fruit extracts on the production of IL6, IL10 and TNFα by BMDM isolated from group 2 mice (n = 1 technical replicate), compared to the control BMDM (BMDM exposed to control medium only), with (+) or without (-) 0.05 µg/mL LPS-stimulation. One experiment consisting of pooled cells from four mice is shown.

4.3.2 Chemical Interference by Extracts

Tests were conducted to determine the cause of the inconsistencies observed for the viability results using the WST-1 and MTT assays in the preliminary study. The results suggested that there might be interference between the *Rubus* extracts and the medium used for the culture of IEC and BMDM. Tests were carried out in one experiment.

Figure 31 shows interference in the absorbance readings observed for both assays when increasing concentrations of extracts were co-incubated in medium without IEC or BMDM. The zero point for the extracts represents the background absorbance (WST-1: 0.15, MTT: 0.015) without the inclusion of any *Rubus* extract (i.e. control). For the MTT and WST-1 assays there was a positive association between the extract concentration and absorbance. For the MTT assay, the *Rubus* ETx extracts had the most marked increase in absorbance.

The results for the WST-1 assay were more consistent across the *Rubus* extracts than the MTT assay results. The WST-1 method was then optimised for the main study, by removing the medium containing the *Rubus* extracts and replacing it with fresh medium prior to reagent addition and the final incubation step.

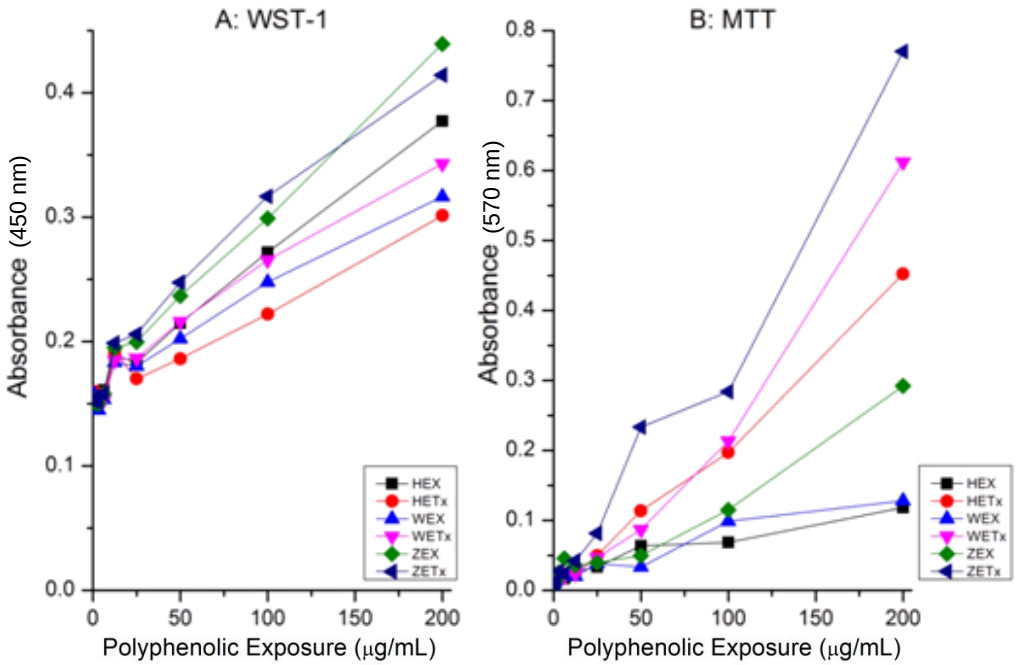

Figure 31: Effects of *Rubus* fruit extracts and medium on absorbance for the A: WST-1 and B: MTT assay methods. The extracts were co-incubated in medium without cells to determine any interference or interaction between the reagents and extracts with the medium (n = 1 technical replicate). Zero points shown represent the background absorbance without addition of extract (i.e. only reagent and medium), WST-1: 0.15, MTT: 0.015.

4.3.3 LPS Stimulation of BMDM

The lack of NO production reported in Section 4.3.1.2 indicated that a LPS dose-response experiment was required to determine the optimal LPS concentration needed to stimulate the BMDM. This dose-response was carried out in one experiment with pooled cells isolated from 2 mice gifted from another study.

From the results shown in Figure 32, a concentration of 0.5 µg/mL LPS (previous LPS concentration was 0.05 µg/mL), was chosen to ensure that the stimulation was adequate yet allowed the potential to measure increases and/or decreases in response. In the absence of LPS there was no detectable production of NO by BMDM.

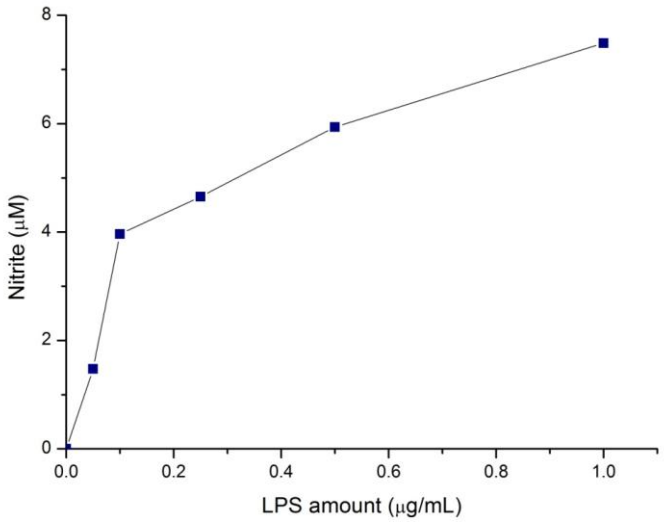

Figure 32: Optimisation of LPS concentration for stimulating NO response in BMDM cells. The cells were grown for 24 h LPS free, and then co-incubated with increasing concentrations of LPS, after which the concentration of NO was determined using the Griess assay (n = 1 technical replicate). One experiment consisting of pooled cells from two mice is shown.

4.3.4 Main Study

Ten C57 and ten *Il10*$^{-/-}$ mice from two groups (5 of each type) were used to assess the effect of *Rubus* fruit extracts on two cell types that are involved in inflammation, BMDM and colonic IEC. Data on cell viability, NO production and cytokine production were obtained from two independent experiments, each consisting of pooled cells from five mice from each genotype with three technical replicates, unless otherwise stated.

4.3.4.1 Animal Performance

Food intake, weight and health were monitored for mice throughout the study. Average daily food intake was 2.7 g/day for C57 and *Il10*$^{-/-}$ mice (see A.2 Additional Mouse Methods). Group 1 had no mice showing abnormal growth (Figure 33). In group 2, mouse 18 lost body weight at the beginning of the study, and mouse 17 showed a decline in body weight towards the end of the study (Figure 33). The average growth rate was similar between C57 and *Il10*$^{-/-}$ mice, regardless of their groups (Figure 34).

The *Il10*$^{-/-}$ and C57 in each group were inoculated with freshly prepared CIF-ES inoculum to induce IBD like symptoms (Group 1: 3.76 x 10^{10} CFU; Group 2: 9.1 x 10^{7} CFU). The unexpected variation in CFU between the inoculums was unable to be explained from experimental records, with the only noted difference being the larger sizes of colonies used in group 1.

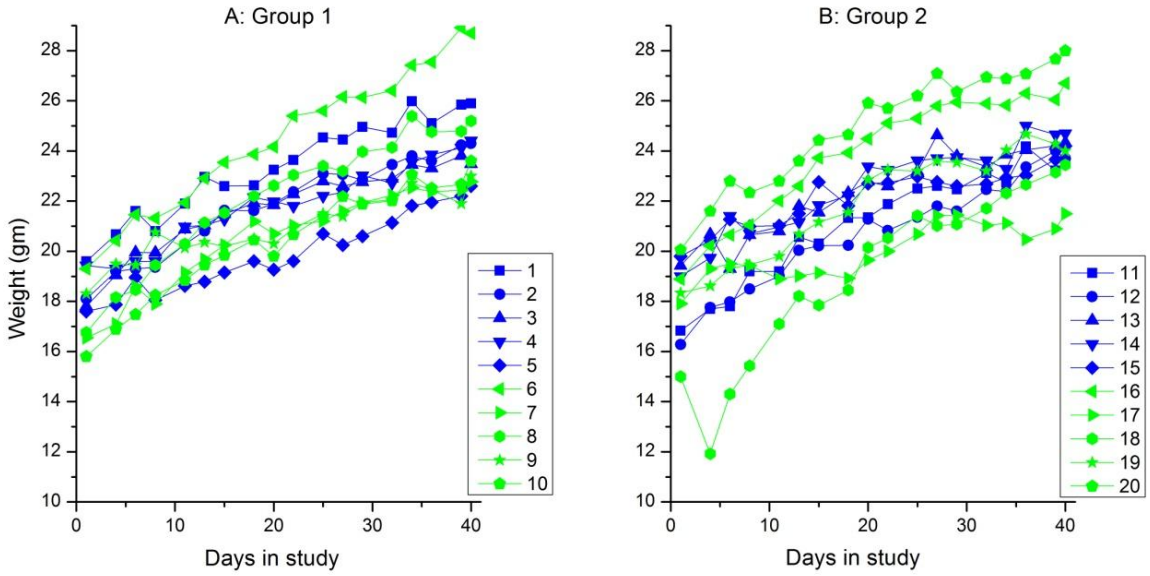

Figure 33: Individual mouse body weights throughout the study, A: Group 1 mice (10 mice) and B: Group 2 mice (10 mice). Data points for C57 mice are coloured blue and data points for *Il10*$^{-/-}$ mice are coloured green. In group 1: 1–5 are C57 mice, 6–10 are *Il10*$^{-/-}$ mice. In group 2: 11–15 are C57 mice, 16–20 are *Il10*$^{-/-}$ mice.

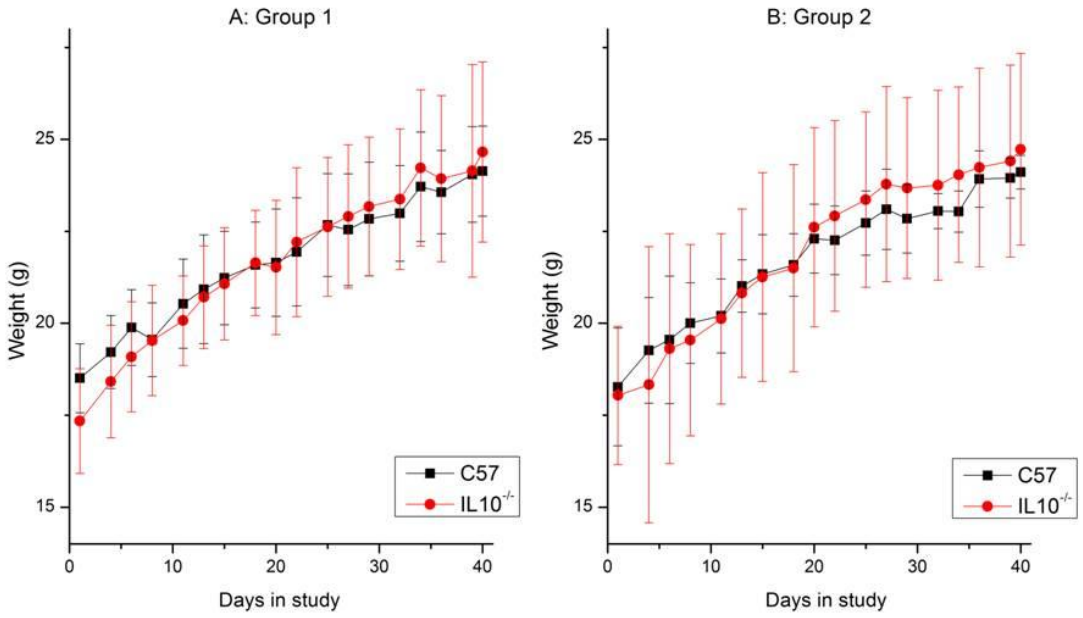

Figure 34: Average weight of *Il10*$^{-/-}$ and C57 mice in group 1 and group 2 throughout the study. Data There was no significant difference between the two groups or by genotype. The data points represent the mean body weight of five mice for each genotype. Each bar represents the standard deviation for the five mice in body weight.

Group 1 mice all maintained a General Health Score (GHS) of 5 (1- non-responsive to stimulus and 5- interested in stimulus; see A.2 Additional Mouse Methods) throughout the study. Group 2 mice had variation in GHS. Mouse 18 initially suffered growth issues due to malformed teeth (which were trimmed), and its GHS was lowered to a 4 for a period of 8 days after which it returned to a GHS of 5. Towards the end of the study mouse 17 was exhibiting IBD-like symptoms with profuse bleeding from the anus, which lowered its score to 3.5. Apart from these two mice (which were kept in the study), all other mice in group 2 maintained a normal GHS of 5.

4.3.4.2 Cytotoxicity Assay

Cytotoxicity assays were conducted to assess the effects of the *Rubus* fruit extracts "HB19", "Wakefield" and "ZZ" (EX or ETx) on the viability of colonic IEC and BMDM using the WST-1 assay (and the new procedure which removed extracts from the medium before reagent addition). Viability results for colonic IEC and BMDM are displayed for each extract as relative viability at nine increasing polyphenolic concentrations (0.76-200 μg/mL) compared to cells exposed to control medium only (control IEC or control BMDM).

The results in Figure 35 for group 1 *Il10*$^{-/-}$ mice indicate that there was no change in viability of colonic IEC relative to the control IEC, at any extract concentration. This result matches the visual assessment of colonic IEC growth (data not shown). In contrast, Figure 36 shows a decrease ($P < 0.05$) in viability of colonic IEC (relative to control IEC) with increasing extract concentration for group 2 *Il10*$^{-/-}$ mice, which did not match visual assessment (data not shown).

Group 1 C57 colonic IEC had two-fold increase in viability when co-incubated with any of the *Rubus* extracts in comparison with the control IEC (Figure 37), which was in agreement with the visual assessment of colonic IEC growth (data not shown). Some data points for group 1 C57 mice were missing due to incorrect pipetting of reagent during the WST-1 assay. As there were only sufficient colonic IEC isolated for one technical replicate these data points could not be generated. Therefore, the viability results need to be interpreted with caution. Figure 38 shows a decrease ($P < 0.05$) in viability of colonic IEC (isolated from group 2 C57 mice) relative to control IEC, when treated with any of the extracts at polyphenolic concentrations greater than 25 μg/mL. This did not match the visual observations for colonic IEC growth, with no observed change relative to the control IEC for any extract (data not shown).

The results for group 1 *Il10*$^{-/-}$ mice indicate a decline in BMDM ($P < 0.05$) at a polyphenolic concentration of 200 μg/mL for all extracts, and for HEX, WEX and ZEX at a polyphenolic concentration of 100 μg/mL (Figure 39). Group 2 *Il10*$^{-/-}$ mice results also indicate a decrease in BMDM viability at polyphenolic concentrations greater than 50 μg/mL for all *Rubus* extracts, apart from HEX which showed decline at polyphenolic concentrations greater than 100 μg/mL (Figure 40). However, due to lack of technical replication statistical confirmation of this result was not possible. Visual assessment of BMDM growth did not show any difference at any

polyphenolic concentration of *Rubus* extracts in comparison to control BMDM for either group (data not shown).

A decline in the viability of BMDM ($P < 0.05$) isolated from group 1 C57 mice was observed for all *Rubus* extracts with polyphenolic concentrations of greater than 25 µg/mL relative to control BMDM (Figure 41). Figure 42 results indicate (not enough technical replication for statistics to be done) that for group 2 C57 mice there was no change observed in BMDM viability (relative to control BMDM) for all *Rubus* extracts until BMDM were exposed to a polyphenolic concentration of 200 µg/mL. The viability results for group 2 mice most closely match the visual results observed for BMDM cell growth (data not shown).

Table 5: Summary of effect of *Rubus* fruit extracts on cell viability at polyphenolic concentration 50 µg/mL. This concentration was used in the inflammatory marker assays.

Cell Type	Group	Genotype	Result	Figure
Colonic IEC	1	*Il10*$^{-/-}$	No change (all extracts)	35
Colonic IEC	2	*Il10*$^{-/-}$	Decrease in viability (all extracts)	36
Colonic IEC	1	C57	~Two-fold increase in viability (all extracts)	37
Colonic IEC	2	C57	Decrease in viability (all extracts)	38
BMDM	1	*Il10*$^{-/-}$	No change (most extracts: decrease ZEX)	39
BMDM	2	*Il10*$^{-/-}$	Decrease in viability (most extracts: not HEX)	40
BMDM	1	C57	Decrease in viability (all extracts)	41
BMDM	2	C57	No change (all extracts)	42

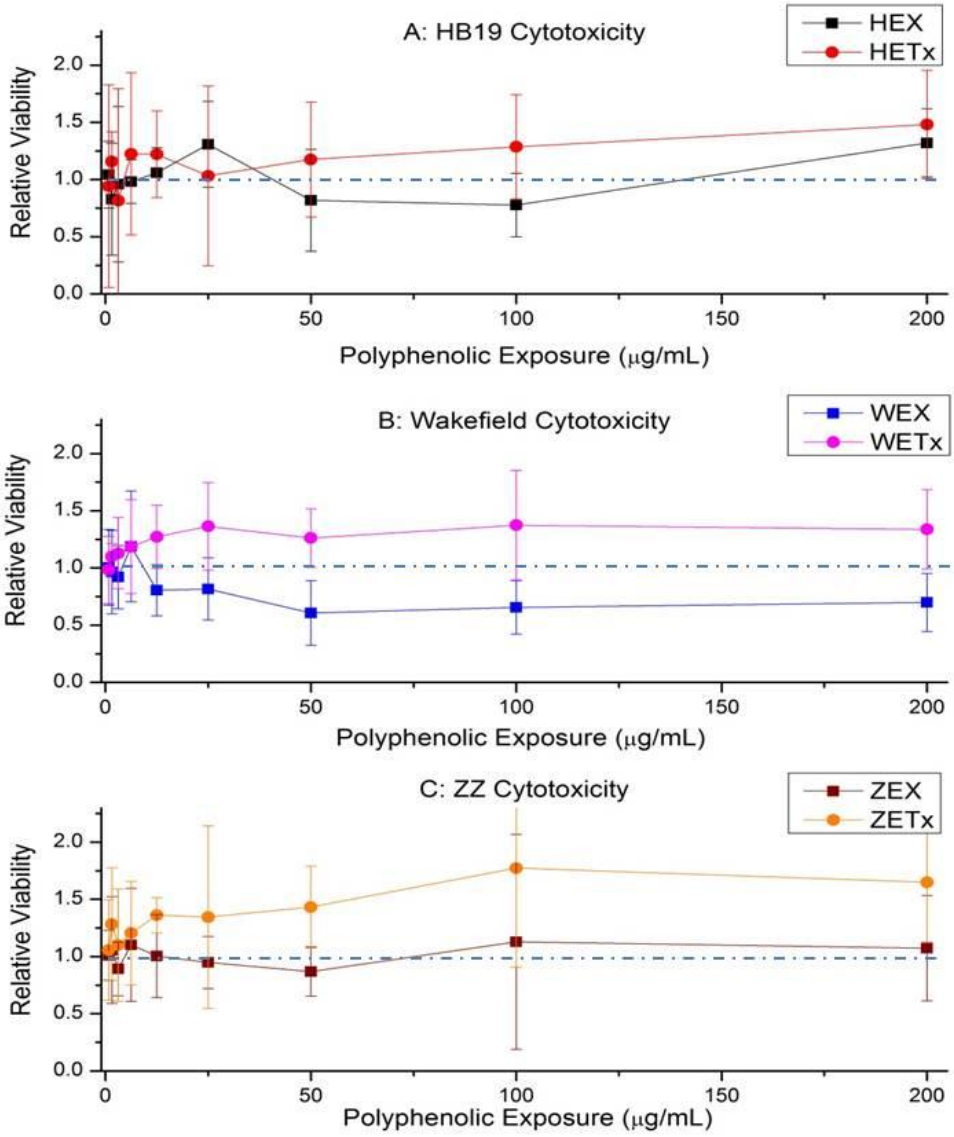

Figure 35: Relative viability of extract-treated colonic IEC compared with control IEC (IEC exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 1 *Il10*$^{-/-}$ mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice with four technical replicates. Each bar represents the standard deviation for four technical replicates. The horizontal dotted line represents viability of control IEC.

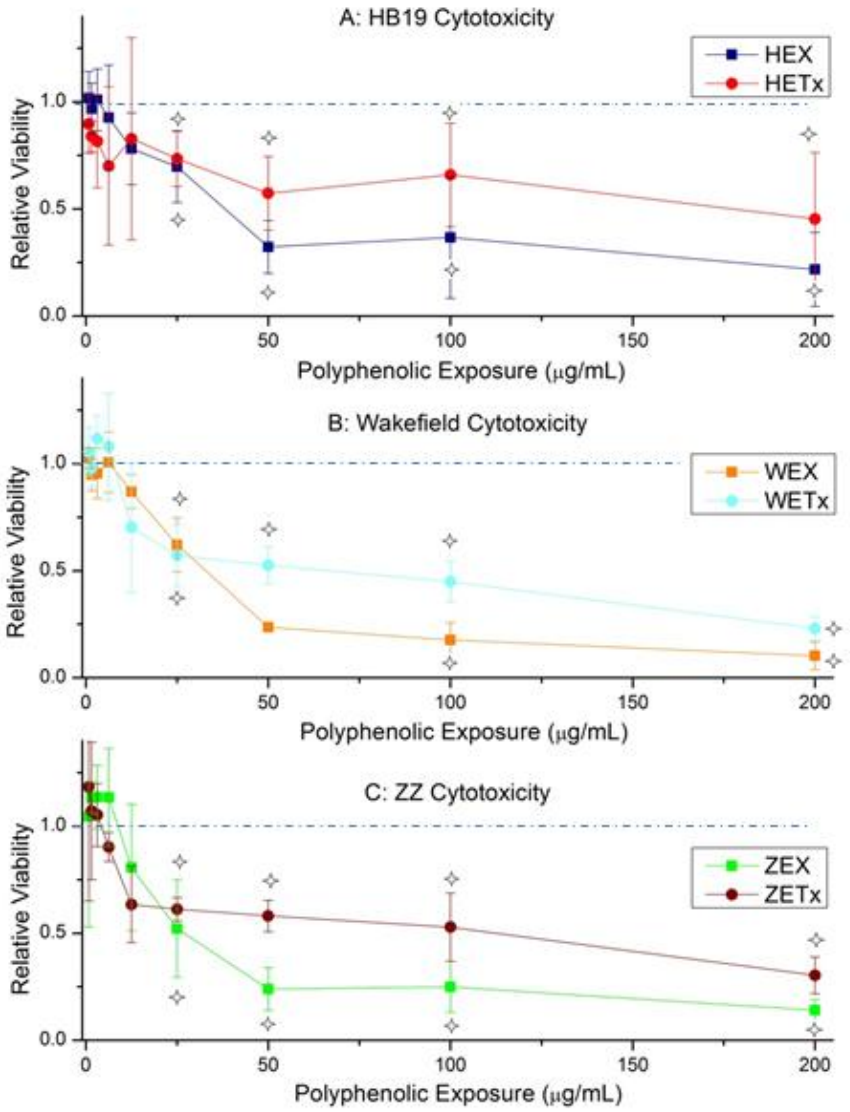

Figure 36: Relative viability of extract-treated colonic IEC compared with control IEC (IEC exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 2 *Il10*$^{-/-}$ mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice with four technical replicates. Each bar represents the standard deviation for four technical replicates. The horizontal dotted line represents viability of control IEC. ✧ indicates a significant change in viability of colonic IEC relative to control ($P < 0.05$).

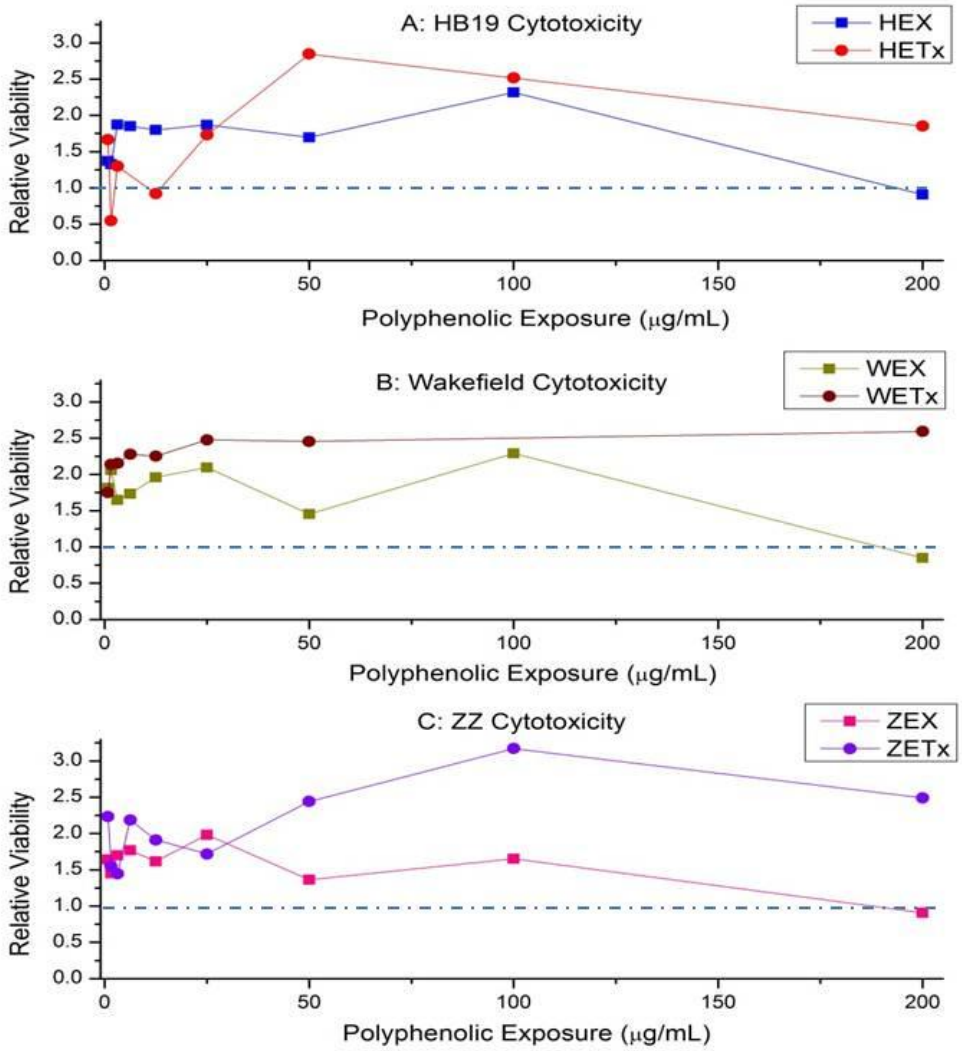

Figure 37: Relative viability of extract-treated colonic IEC compared with control IEC (IEC exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 1 C57 mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five C57 mice with one technical replicate; therefore statistical analysis was not able to be performed. The horizontal dotted line represents viability of control IEC.

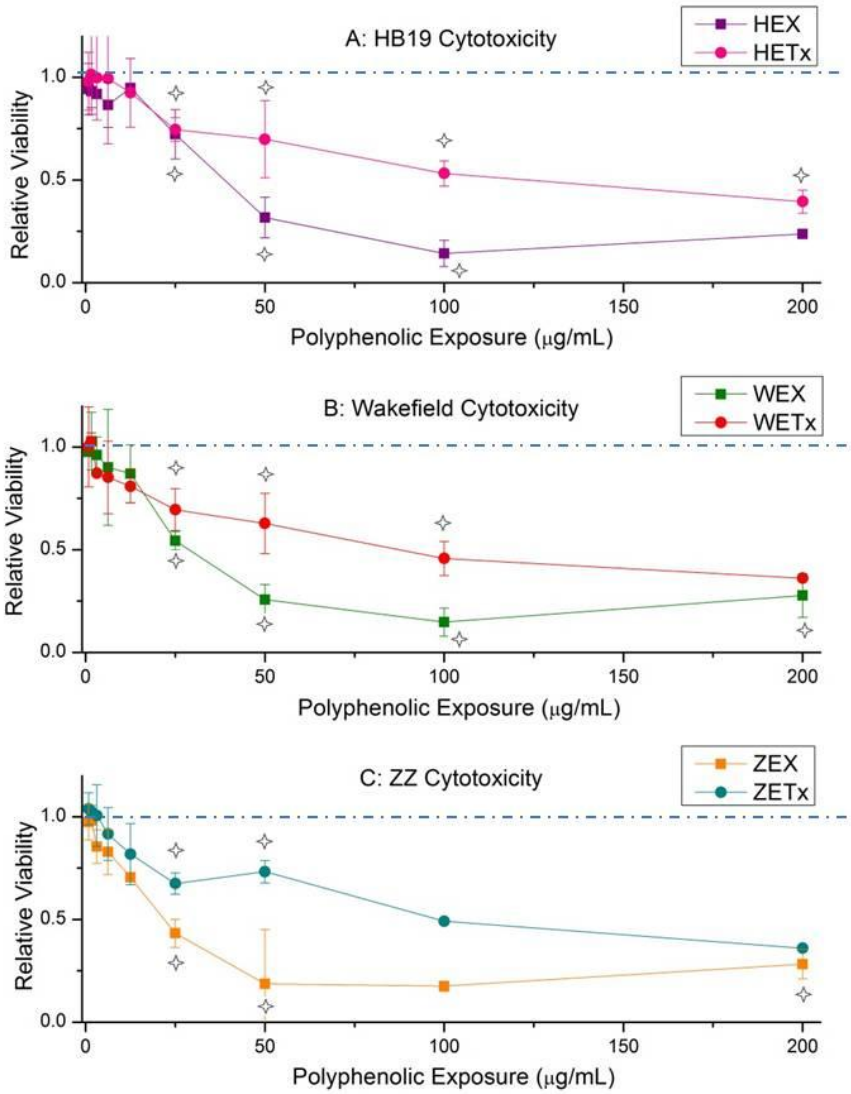

Figure 38: Relative viability of extract-treated colonic IEC compared with control IEC (IEC exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 2 C57 mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five C57 mice with two technical replicates. Each bar represents the standard deviation for two technical replicates (data points without bar show only one technical replicate due to pipetting error). The horizontal dotted line represents viability of control BMDM. ✧ indicates a significant change in viability of colonic IEC relative to control ($P < 0.05$).

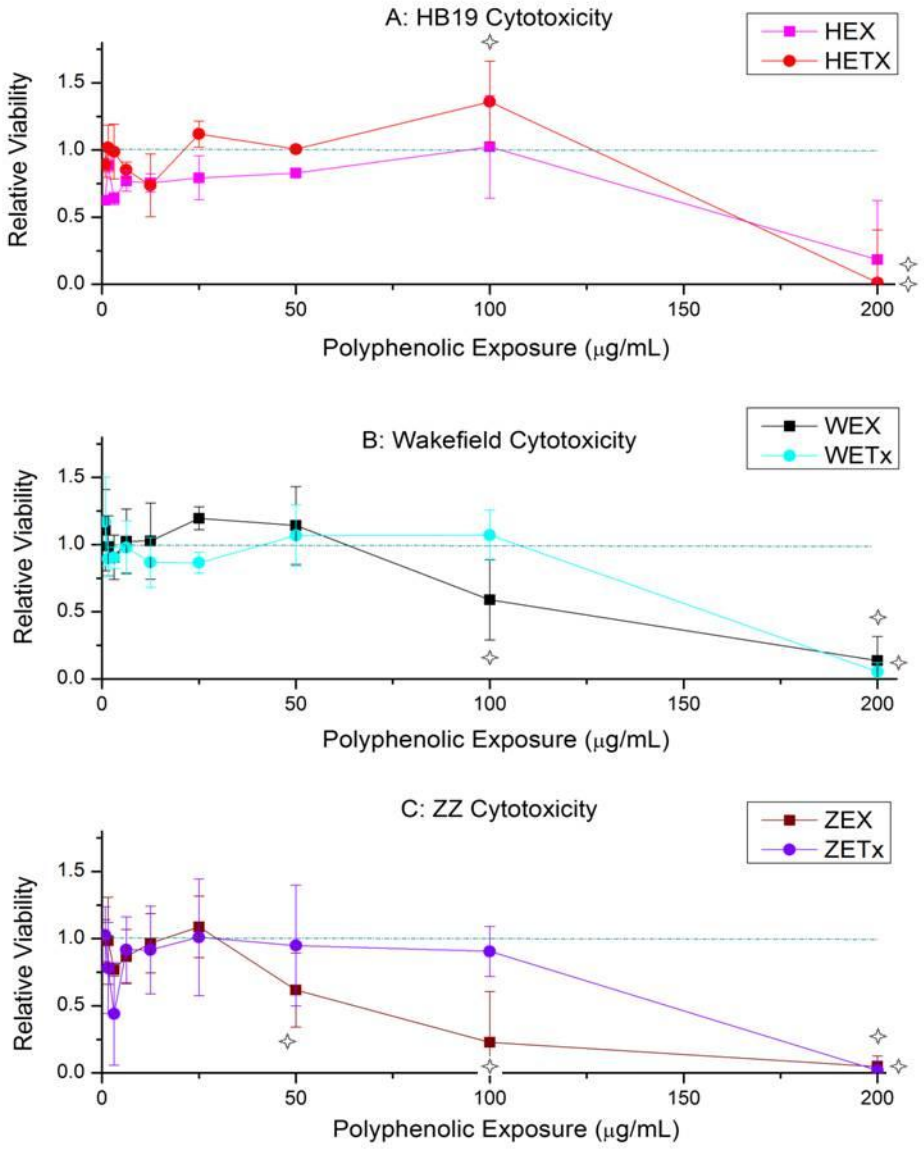

Figure 39: Relative viability of extract-treated BMDM compared with control BMDM (BMDM exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 1 *Il10*$^{-/-}$ mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice with two technical replicates. Each bar represents the standard deviation for two technical replicates. The horizontal dotted line represents viability of control BMDM. ✧ indicates a significant change in viability of BMDM relative to control ($P < 0.05$).

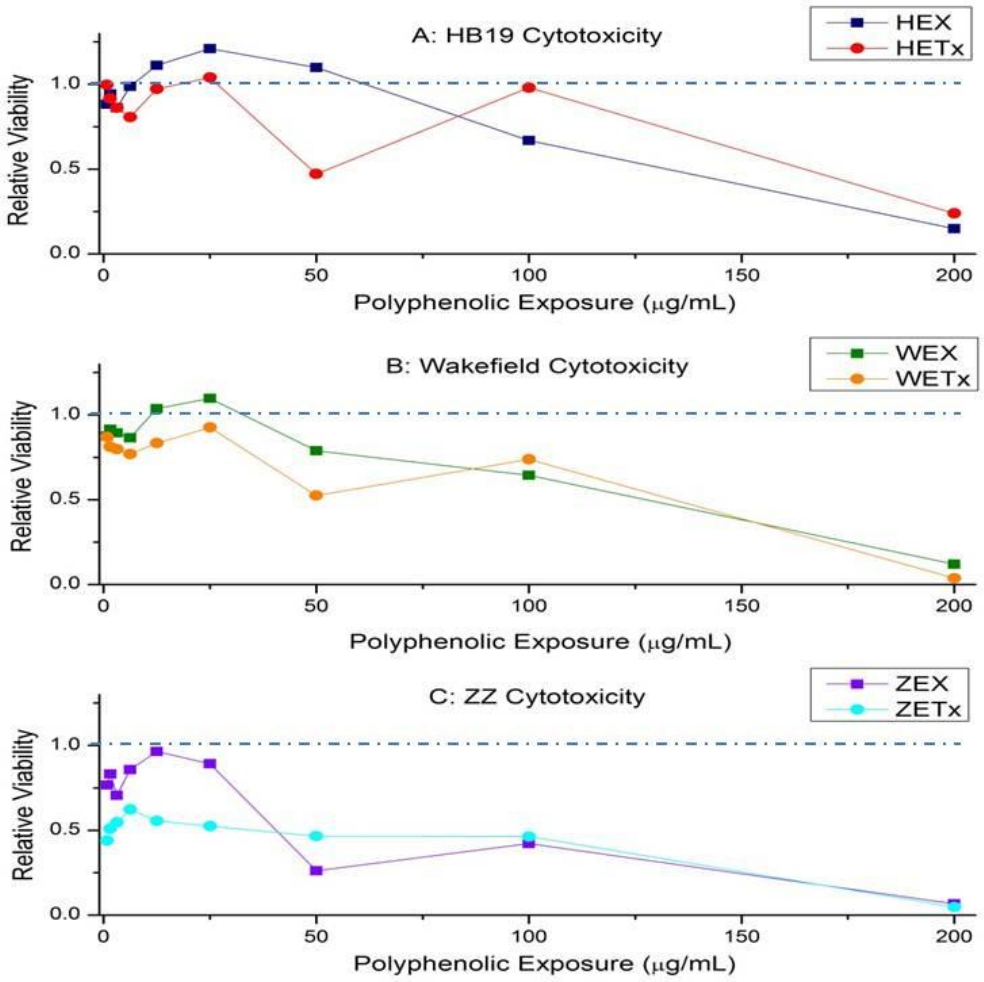

Figure 40: Relative viability of extract-treated BMDM compared with control BMDM (BMDM exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 2 *Il10*$^{-/-}$ mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice with one technical replicate; therefore statistical analysis was not able to be performed. The horizontal dotted line represents viability of control BMDM.

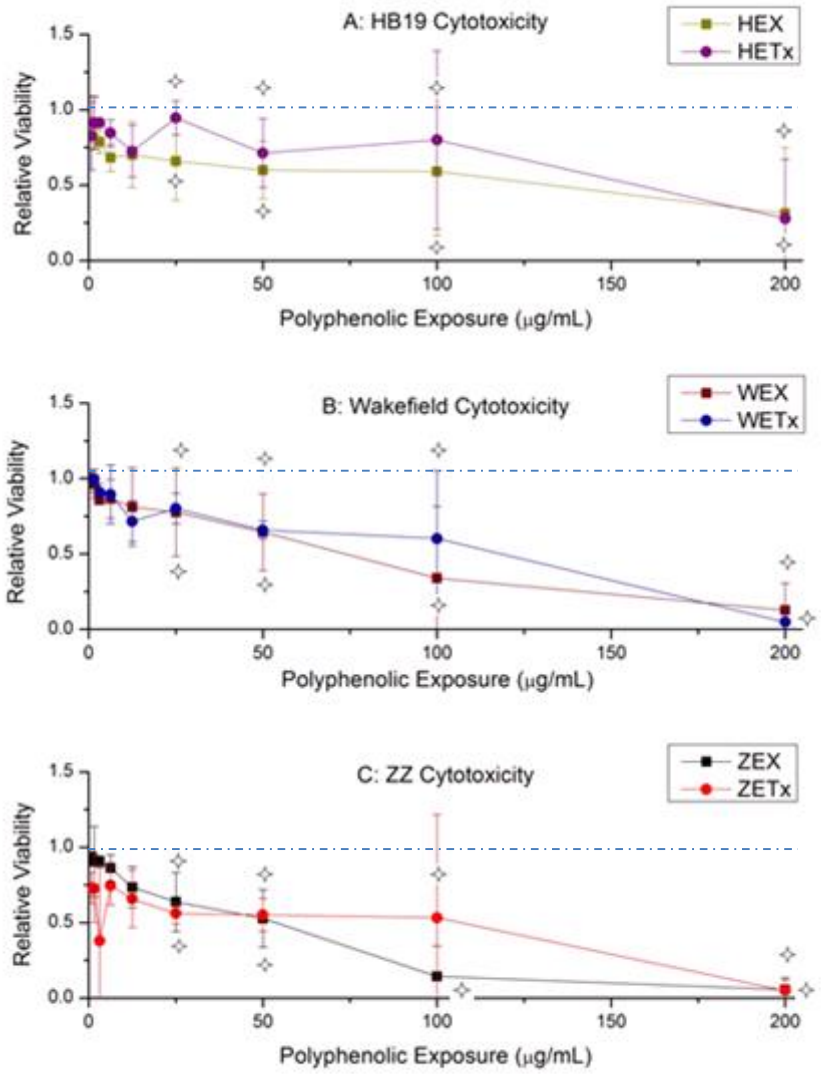

Figure 41: Relative viability of extract-treated BMDM compared with control BMDM (BMDM exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 1 C57 mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five C57 mice with two technical replicates. Each bar represents the standard deviation for two technical replicates. The horizontal dotted line represents viability of control BMDM. ✧ indicates a significant change in viability of BMDM relative to control ($P < 0.05$).

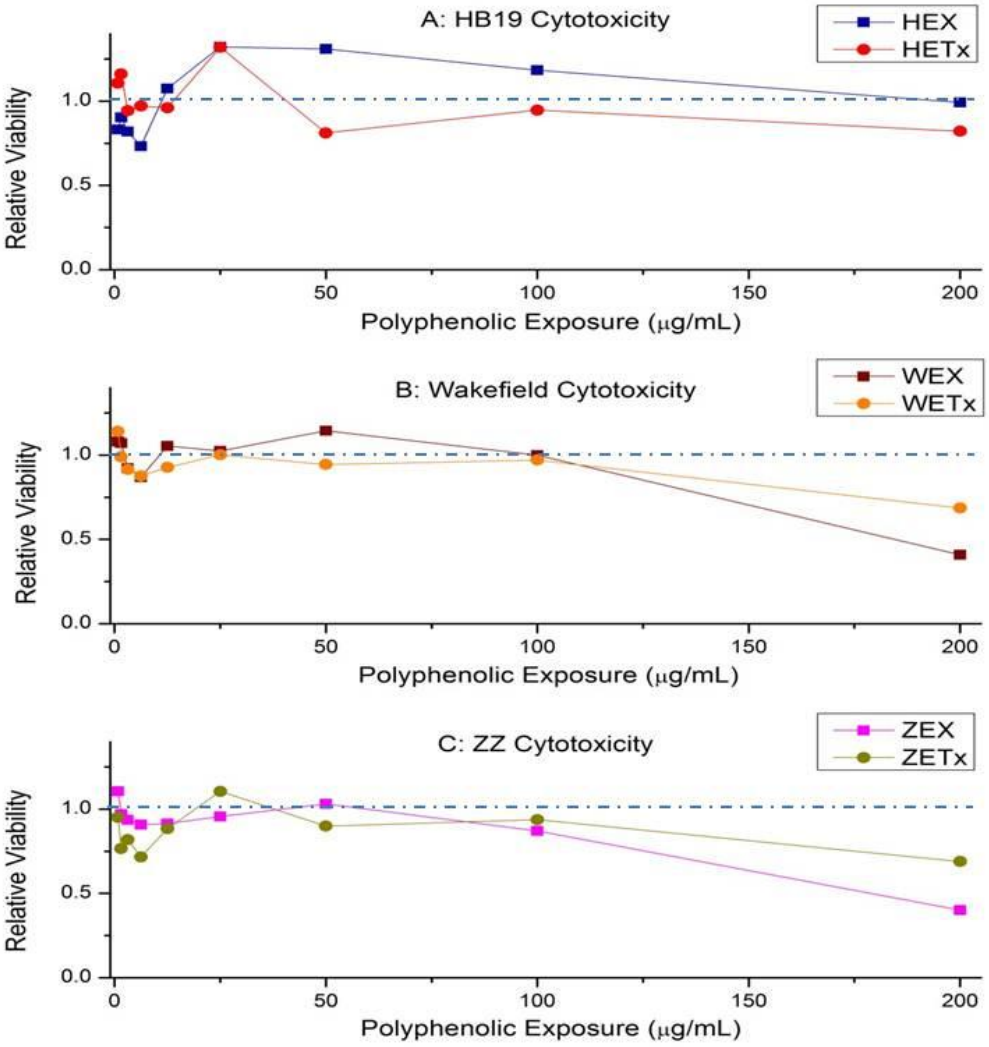

Figure 42: Relative viability of extract-treated BMDM compared with control BMDM (BMDM exposed to control medium) plotted against polyphenolic concentration (μg/mL) for group 2 C57 mice. A: 'HB19', B: 'Wakefield', C: 'ZZ'. The experiment consisted of pooled cells from five C57 mice with one technical replicate; therefore statistical analysis was not able to be preformed. The horizontal dotted line represents viability of control BMDM.

4.3.4.3 Nitrite Production

Nitrite concentration was used as an indirect measure of NO production and was determined using the Griess assay with 0.5 µg/mL LPS-stimulation. The production of NO by BMDM exposed to extracts relative to BMDM exposed to control medium (control BMDM) was consistent between groups 1 and group 2 mice. For C57 mice from both groups, there was a decrease in the NO production by BMDM for most extracts (apart from HEX), relative to control BMDM (see Figure 43 and Figure 44). ZEX was the most effective with an average decrease of approximately 35% in NO production by BMDM. These results are limited in interpretation due to lack of technical replication, however, the concordance shown between the two groups increases the likelihood of the results being reflective of the response induced by the *Rubus* extracts.

There was no significant difference in the responses between groups for C57 mice ($P = 0.25$) or between groups for *Il10*$^{-/-}$ mice ($P = 0.14$), however there is low statistical power in this analysis due to lack of replicates. For both groups of *Il10*$^{-/-}$ mice, WEX and ZEX extracts had significantly reduced ($P < 0.05$) NO production for BMDM. For group 1 *Il10*$^{-/-}$ mice, HEX, HETx, and WETx also acted to decrease NO production, with a rise observed for ZETx, however, the results cannot be conclusively interpreted due to lack of replication. For group 2 *Il10*$^{-/-}$ mice, there was no significant change for other *Rubus* fruit extracts in NO production.

Overall, there were indications of a difference between the EX and ETx extracts for the cultivars ‘ZZ’ (ZEX vs ZETx) and ‘Wakefield’ (WEX vs WETx) in their effectiveness in reducing NO production by BMDM. The EX extracts appear to be more effective in reducing the NO concentration in both C57 and *Il10*$^{-/-}$ mice compared to their ET-enriched (ETx) counterparts. However, due to the lack of technical replication for all group 1 results and the group 2 C57 result this would need to be confirmed.

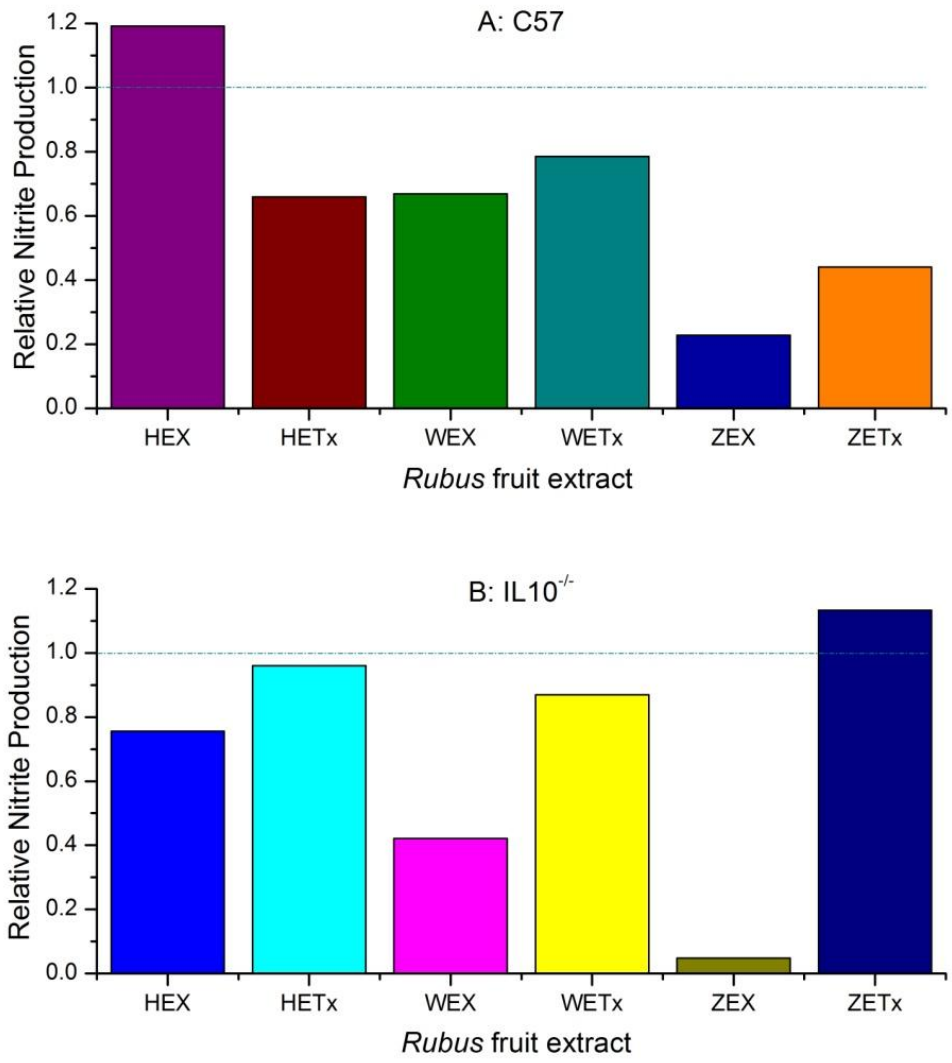

Figure 43: Effect of *Rubus* fruit extracts on NO production by LPS-stimulated BMDM relative to control BMDM, from group 1 mice. A: C57 and B: *Il10*$^{-/-}$. The experiment consisted of pooled cells from five C57 and *Il10*$^{-/-}$ mice with one technical replicate; therefore statistical analysis was not able to be performed. The horizontal dotted line represents the production of NO by the control BMDM.

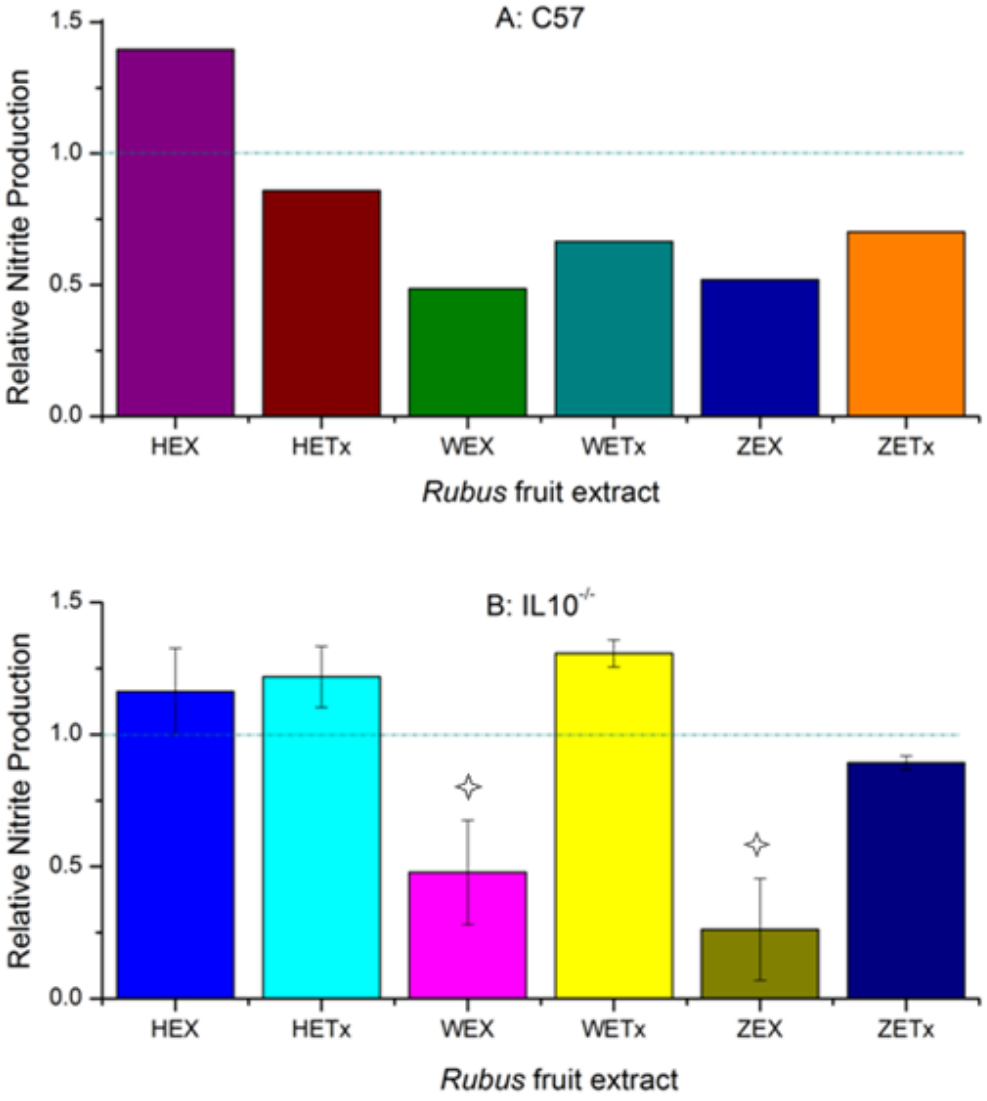

Figure 44: Effect of *Rubus* fruit extracts on NO production by LPS-stimulated BMDM relative to control BMDM, from group 2 mice. A: C57 and B: *Il10*$^{-/-}$. The experiment consisted of pooled cells from five C57 and *Il10*$^{-/-}$ mice. There was one technical replicate for C57 mice and three technical replicates for *Il10*$^{-/-}$ mice. Each bar for *Il10*$^{-/-}$ mice represents the standard deviation for three technical replicates. The horizontal dotted line represents the production of NO by the control BMDM. ✧ indicates a significant change in NO production of BMDM relative to control ($P < 0.05$).

4.3.4.4 Cytokine Production

The results presented in Figure 45 and Figure 46 are displayed on the same scale, in order to demonstrate a difference in a production of IL6 cytokine between groups. The IL6 production by colonic IEC co-incubated with *Rubus* extracts was between two to ten-fold higher for group 2 mice than for group 1 mice, for both *Il10*$^{-/-}$ and C57 mice (Figure 45 and Figure 46).

Figure 47 and Figure 48 present the results for IL6 production by colonic IEC treated with *Rubus* extracts relative to the IEC exposed to control medium only (control IEC). The results for group 1 C57 mice indicate no change in IL6 production for LPS-stimulated colonic IEC, relative to the positive control IEC for all *Rubus* extracts except ZEX+ (Figure 47). Without stimulation by LPS, IL6 production by colonic IEC from group 1 C57 was reduced by approximately 40%, when the IEC were co-incubated with *Rubus* extracts relative to control IEC. However, this is not conclusive due to lack of technical replication. There were mixed results seen for group 2 C57 mice with a significant ($P < 0.05$) increase observed *Rubus* fruit extracts for ZETx+, HEX-, HETx-, and a decrease in WETx+, ZEX+, and ZEX-, in relation to the control IEC (Figure 48).

For group 1 *Il10*$^{-/-}$ mice, there was on average a 50% decrease ($P < 0.05$) in IL6 production by LPS-stimulated colonic IEC, co-incubated with any *Rubus* extract, relative to control IEC (Figure 47). Without LPS stimulation the reduction was still significant ($P < 0.05$) and ranged between 20-25% (Figure 47). For group 2 *Il10*$^{-/-}$ mice, there was a significant decrease of IL6 production by unstimulated ($P = 0.04$) or LPS-stimulated colonic IEC ($P < 0.05$), when co-incubated with any of the *Rubus* extracts (Figure 48).

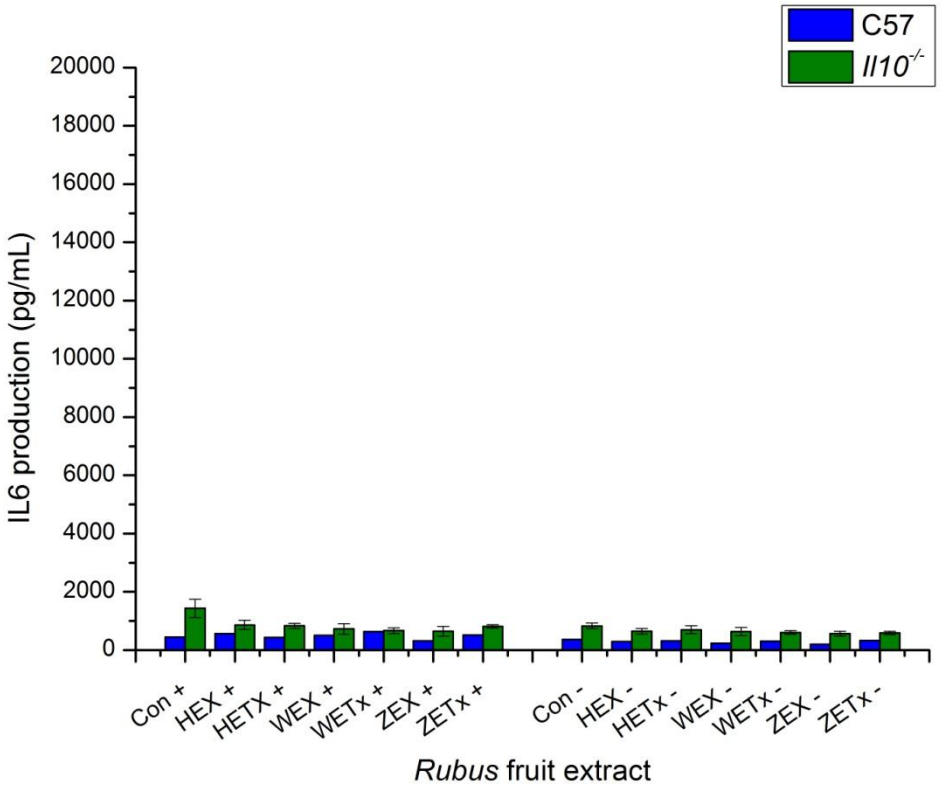

Figure 45: IL6 production for colonic IEC isolated from group 1 *Il10*$^{-/-}$ and C57 mice. Colonic IEC were co-incubated with *Rubus* fruit extracts with (+) or without (-) 0.5 µg/mL LPS-stimulation. For group 1 C57 mice only one technical replicate was carried out due to low IEC yield resulting in a lack of statistical interpretation. Triplicate technical replicates were carried out for group 1 *Il10*$^{-/-}$ mice. Each bar for *Il10*$^{-/-}$ mice represents the standard deviation for three technical replicates.

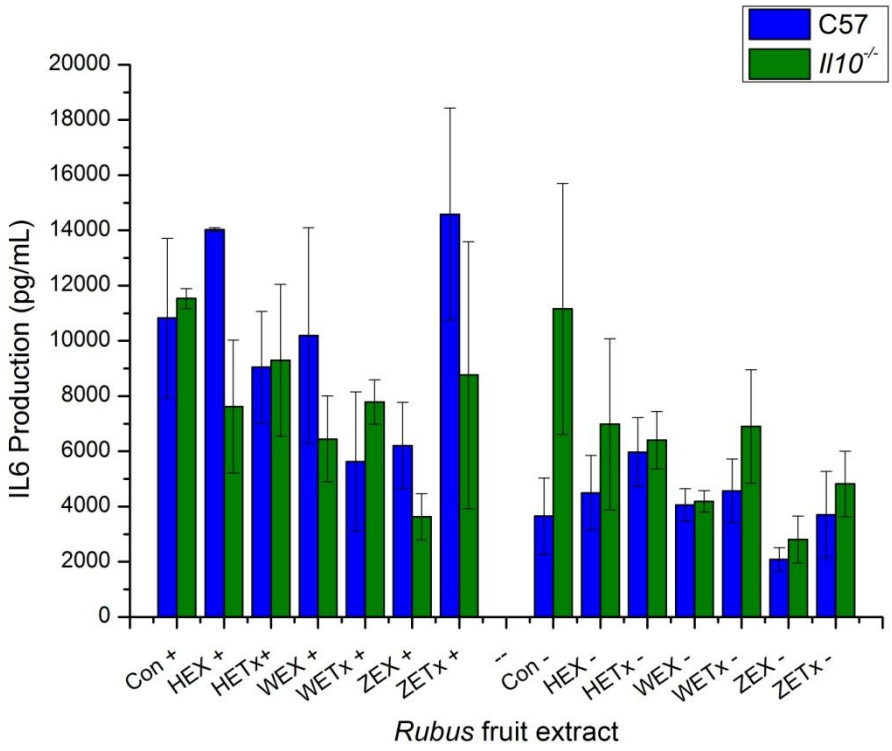

Figure 46: IL6 production for colonic IEC isolated from group 2 *Il10*$^{-/-}$ and C57 mice. Colonic IEC were co-incubated with *Rubus* fruit extracts with (+) or without (-) 0.5 µg/mL LPS-stimulation. For both genotypes triplicate technical replicates were carried out. Each bar represents the standard deviation for three technical replicates.

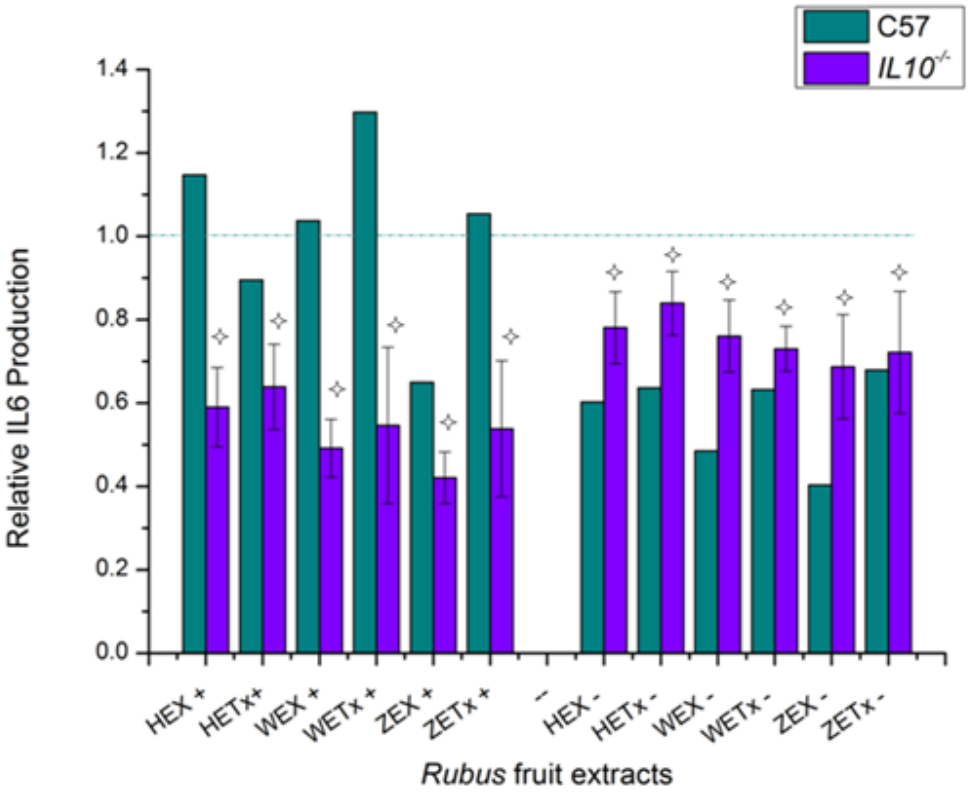

Figure 47: Relative production of IL6 by colonic IEC in comparison to control IEC. Colonic IEC were incubated with *Rubus* extracts, with (+) or without (-) 0.5 µg/mL LPS-stimulation, for group 1 *Il10*$^{-/-}$ and C57 mice. For group 1 C57 mice only one technical replicate was carried out due to low IEC yield resulting in a lack of statistical interpretation. Triplicate technical replicates were carried out for group 1 *Il10*$^{-/-}$ mice. Each bar for *Il10*$^{-/-}$ mice represents the mean standard deviation for three technical replicates. The horizontal dotted line represents the production of IL6 by the control IEC. ✧ indicates a significant change in IL6 production of colonic IEC relative to control ($P < 0.05$).

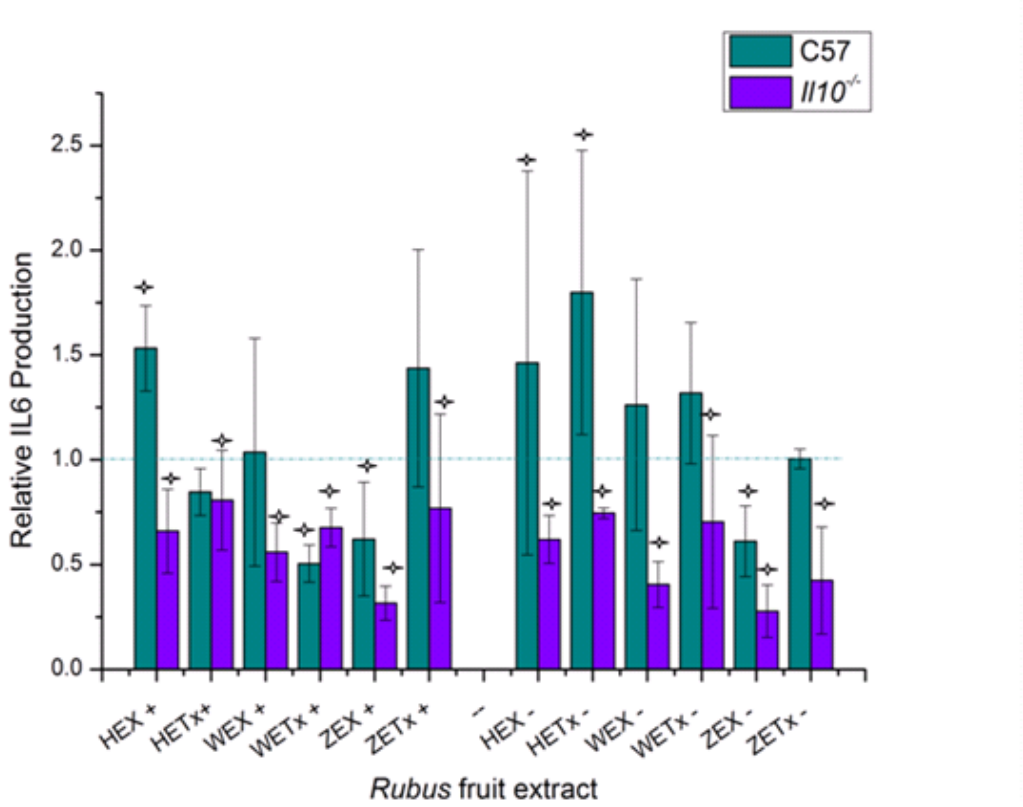

Figure 48: Relative production of IL6 by colonic IEC in comparison to control IEC. Colonic IEC were incubated with *Rubus* extracts, with (+) or without (-) 0.5 µg/mL LPS-stimulation, for group 2 *Il10*$^{-/-}$ and C57 mice. For both genotypes triplicate technical replicates were carried out. Each bar represents the mean standard deviation for three technical replicates. The horizontal dotted line represents the production of IL6 by the control IEC. ✧ indicates a significant change in IL6 production of colonic IEC relative to control ($P < 0.05$).

Figure 49 to Figure 52 present the results for the cytokine production by BMDM isolated from *Il10*$^{-/-}$ and C57 mice from groups 1 and 2. One technical replicate was carried out for C57 BMDM experiments and either two or three technical replicates were performed for *Il10*$^{-/-}$ BMDM experiments depending on the cell yield. The levels of cytokine produced were different between the two groups for both genotypes, with group 2 producing on average two-fold more cytokine as group 1 for each extract (data not shown).

TNFα production increased for extract-treated BMDM isolated from C57 mice (both groups), relative to control BMDM (Figure 49 and Figure 50). With the exception of HEX for IL6 from group 1 mice, there was decreased production of IL6 and IL10 observed for extract-treated BMDM isolated from C57 mice, relative to control BMDM (Figure 49 and Figure 50). Lack of technical replication makes these results inconclusive.

For the *Il10*$^{-/-}$ mice from groups 1 and 2, there was no significant change in TNFα production by BMDM for any extracts, relative to control BMDM (Figure 51 and Figure 52). Group 1 *Il10*$^{-/-}$ BMDM (Figure 51), showed a significant decrease ($P < 0.05$) in IL12 production for all cultivar extracts, and IL6 production was significantly reduced ($P < 0.05$), when co-incubated with extracts: HEX, HETx, WETx, ZEX and ZETx, relative to the control BMDM. In *Il10*$^{-/-}$ group 2 mice (Figure 52) all extracts induced a significant decrease ($P < 0.05$) in IL12 production. IL6 production was significantly reduced ($P < 0.05$) with ZETx or HEX-treated BMDM, relative to control BMDM.

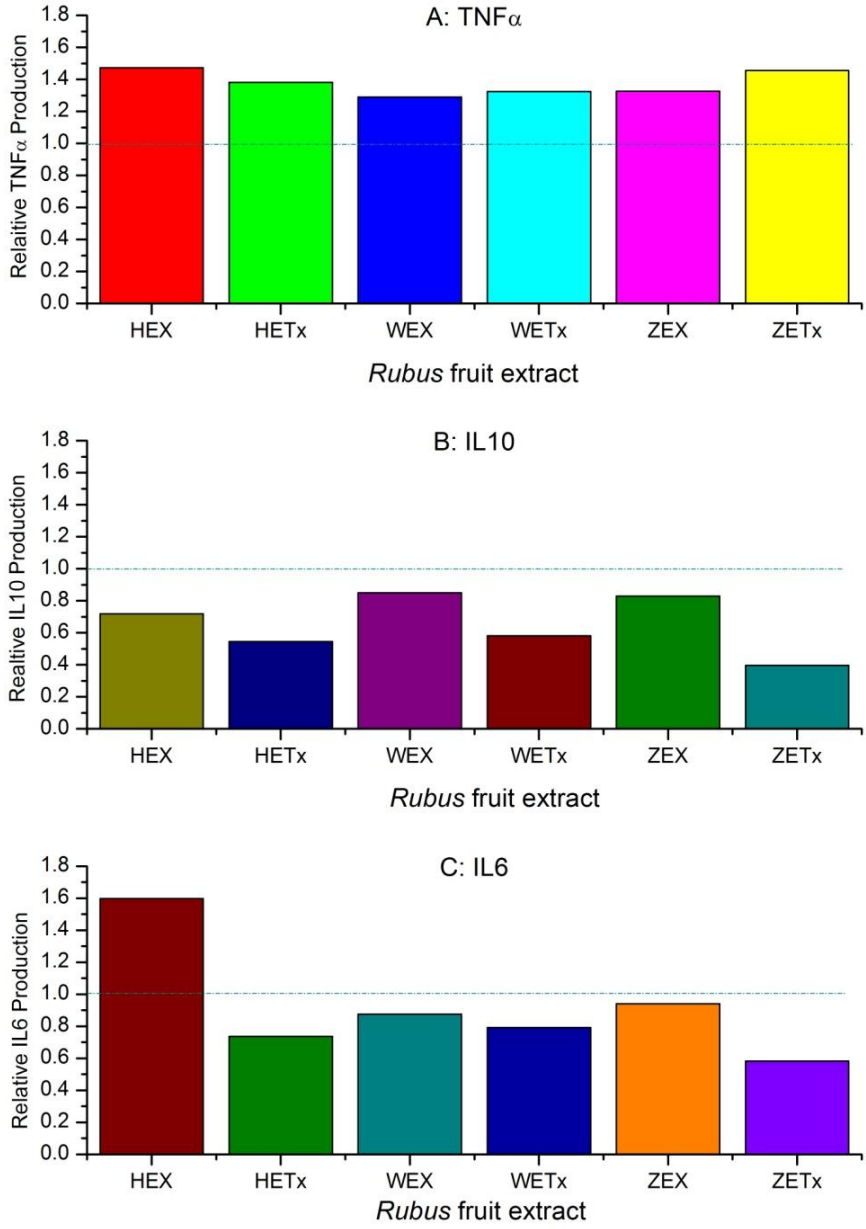

Figure 49: Effect of co-incubation with *Rubus* extracts on cytokine production relative to the control BMDM for LPS-stimulated BMDM from group 1 C57 mice. A: TNFα; B: IL10; and C: IL6 production. The experiment consisted of pooled cells from five C57 with one technical replicate; therefore statistical analysis was not able to be performed. The horizontal dotted line represents the production of cytokines by the control BMDM

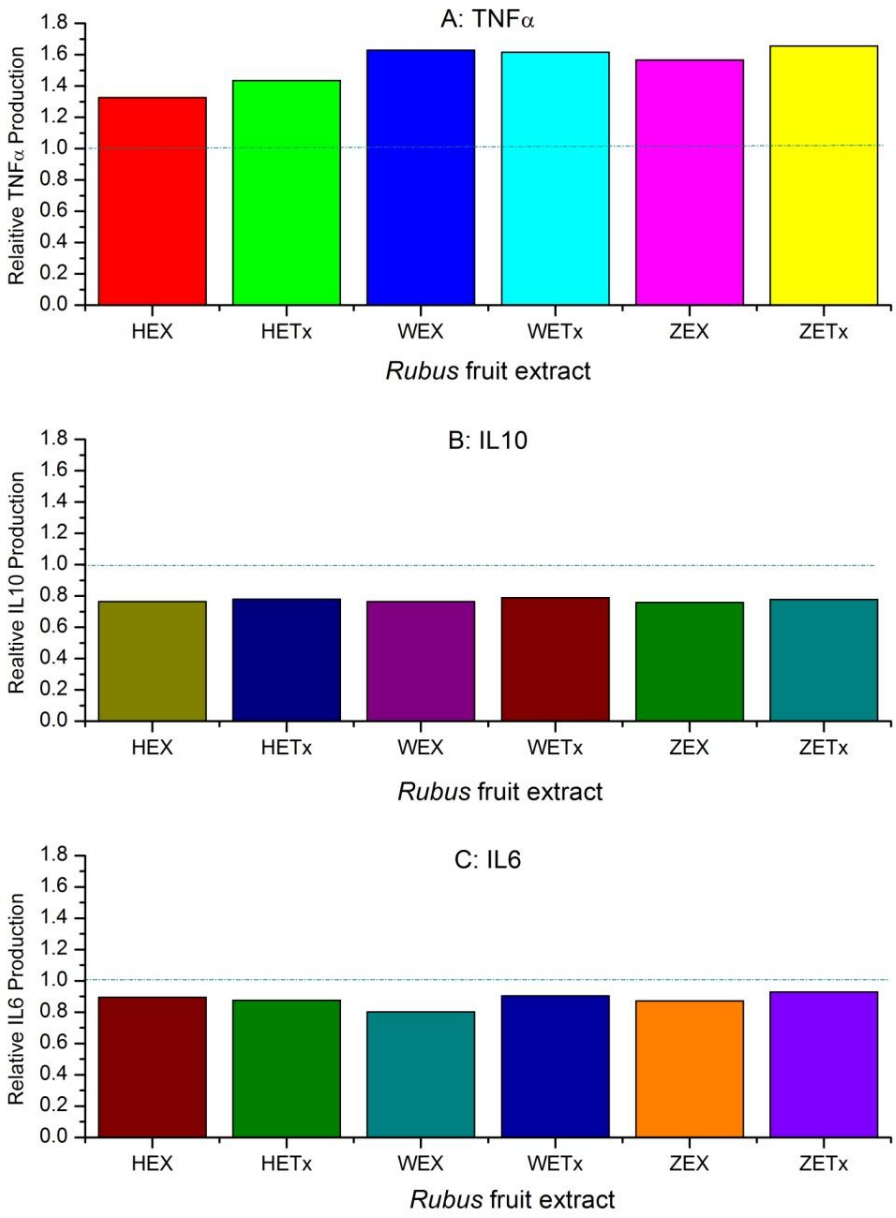

Figure 50: Effect of co-incubation with *Rubus* extracts on cytokine production relative to the control BMDM for LPS-stimulated BMDM from group 2 C57 mice. A: TNFα; B: IL10; and C: IL6 production. The experiment consisted of pooled cells from five C57 with one technical replicate; therefore statistical analysis was not able to be performed. The horizontal dotted line represents the production of cytokines by the control BMDM.

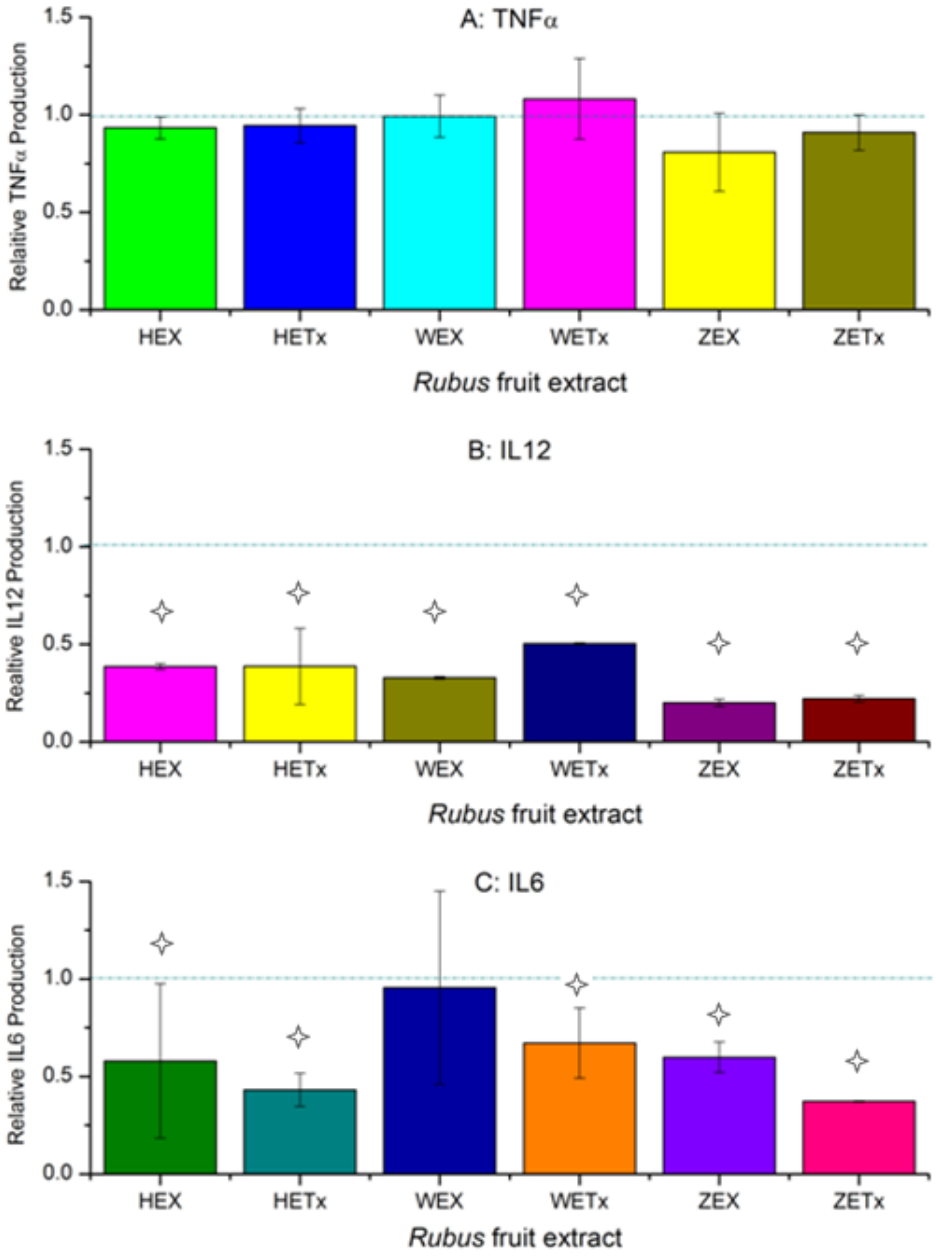

Figure 51: Effect of co-incubation with *Rubus* extracts on cytokine production relative to the control BMDM for LPS-stimulated BMDM from group 1 *Il10*$^{-/-}$ mice. A: TNFα; B: IL12; and C: IL6 production. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice with two technical replicates. Each bar represents the standard deviation for two technical replicates. The horizontal dotted line represents the production of cytokines by the control BMDM✧ indicates a significant change in cytokine production of BMDM relative to control ($P < 0.05$).

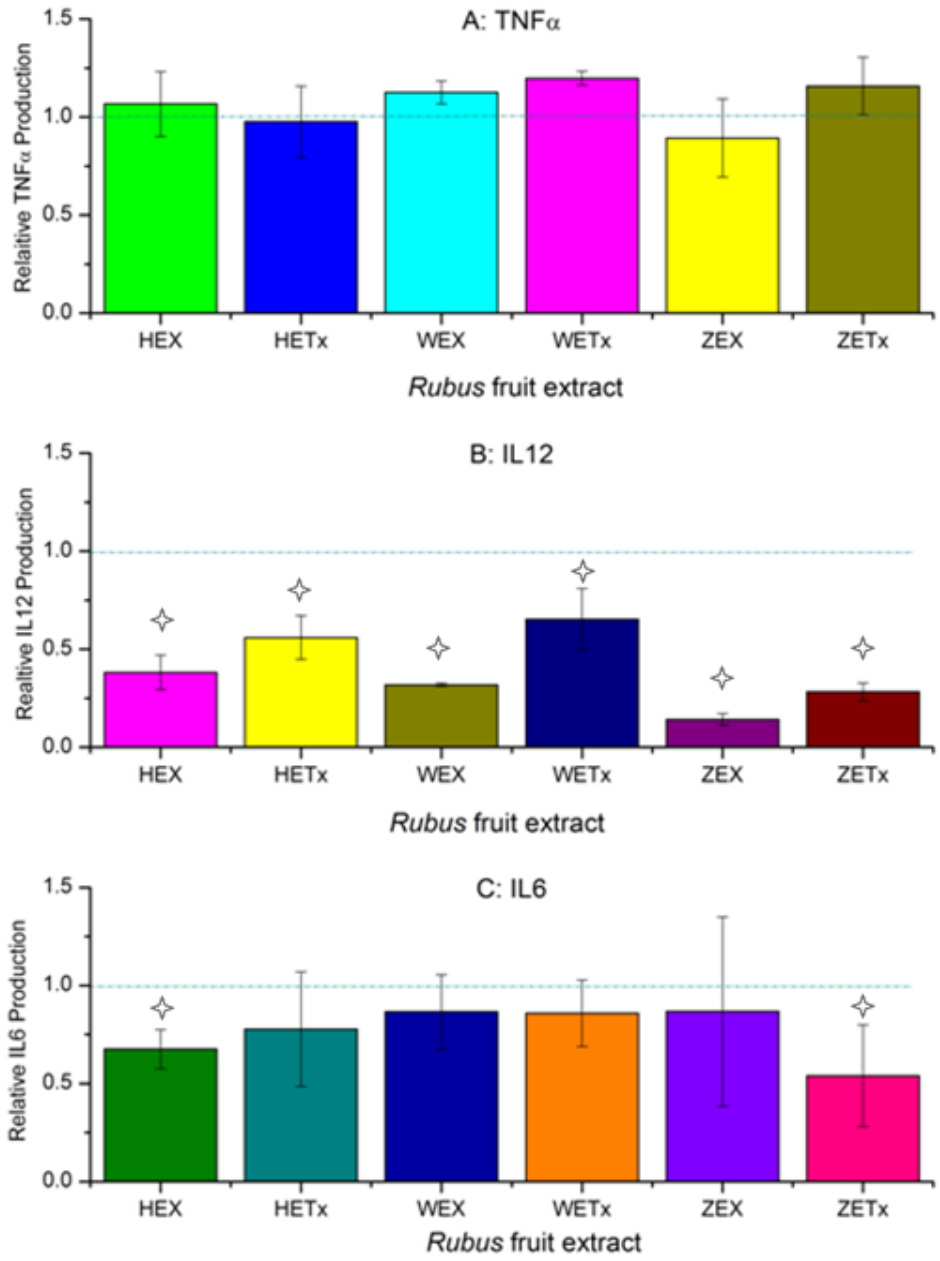

Figure 52: Effect of co-incubation with *Rubus* extracts on cytokine production relative to the control BMDM for LPS-stimulated BMDM from group 2 *Il10*$^{-/-}$ mice. A: TNFα; B: IL12; and C: IL6 production. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice with three technical replicates. Each bar represents the standard deviation for three technical replicates. The horizontal dotted line represents the production of cytokines by the control BMDM. ✧ indicates a significant change in cytokine production of BMDM relative to control ($P < 0.05$).

4.4 Discussion

4.4.1 Preliminary Study

The preliminary study using 8 C57 mice (in 2 groups of 4) aimed to optimise methods for the isolation and culture of small intestinal IEC and colonic IEC, and BMDM, and to test the effects of *Rubus* fruit extracts on cell viability, NO production and cytokine (TNFα, IL1β, IL10, and IL6) production. These methods have been used for similar experiments with kiwifruit extracts [150] or with secondary RAW 264.7 macrophages (Chapter 3), and provided guidance for this study. Initially the two groups of mice were intended to be used as biological replicates. However, due to the numerous issues with the optimisation of the methods, the mice from the two groups were solely used for that purpose.

An interesting finding from chemical interference study was the association of polyphenolic extract components and the changes in absorbance measured for the cytotoxicity assays. The use of more complex RPMI medium for primary cells instead of the DMEM medium used for secondary RAW 264.7 macrophages (Chapter 3) is the most likely reason for this interference. RPMI medium comprises a different variety and concentration of inorganic salts, amino acids and vitamins than DMEM and has growth factors EGF or M-CSF added to the medium. There were indications of a link between the *Rubus* extract polyphenolic composition and its reaction with the MTT assay and/or RPMI medium. It was noted that the ETx extracts had higher absorbance interference than EX, suggesting that the reagent and/or RPMI medium has a stronger reaction to the *Rubus* fruit extracts without the presence of anthocyanins. The results from the WST-1 assay were more consistent between extracts and there were no noted associations with *Rubus* extract polyphenolic composition.

The lack of NO production by small intestinal and colonic IEC was anticipated based on previous research [150], and the preliminary study confirmed these findings. Lack of NO production by BMDM was likely to stem from inadequate LPS-stimulation. A dose-response of LPS with BMDM showed that the optimal concentration for adequate stimulation was 0.5 µg/mL.

No TNFα, IL1β, IL10, and IL6 were detected for small intestinal IEC, and only IL6 was detected for colonic IEC. It was anticipated based on previous work [150], that only IL6 would be produced by these IEC. The lack of IL6 production from small intestinal IEC was unexpected but likely attributable to the extended wait before isolation and subsequent low IEC yield. These issues resulted in only one technical replicate for the IL6 assay for IEC. Most *Rubus*

extracts (apart from HETx-) appeared to decrease IL6 production by the colonic IEC compared to relative control IEC.

The cytokine production of BMDM was analysed at a lower cell density than used for IEC due to low cell yield, with only enough cells isolated for one technical replicate. IL6, IL10 and TNFα were produced by BMDM at detectable levels. There was a decrease in production of IL6 and IL10; but an increase in TNFα by BMDM as result of co-incubation with the *Rubus* fruit extracts. IL1β was not detected for any extracts tested and this could be related to a low level of LPS-stimulation of BMDM.

A main concern arising from the results of the preliminary study was the variable yield of primary small intestinal and colonic IEC, and BMDM, and consequently, a lack of technical replicates. As a result, to simplify the technical demands, focus was shifted to the isolation and culture of colonic IEC and BMDM only. These two cells types were most likely to provide useful insights to *Rubus* fruit extract effects as indicated by the cytokine responses by these cells. Methods from Chapter 3 and/or previous work [150], were compared and modified where needed. The WST-1 assay was used instead of MTT for cytotoxicity testing in the main study as it gave more consistent absorbance results between extracts and was simpler to use. The optimal dose for LPS-stimulation of BMDM was raised to increase their ability to produce NO. These technical changes mentioned above were implemented to simplify experimental conduction (to reduce the likelihood of experimental errors by the operator during tissue processing, isolation and assays, for the main study). Plate design had increased randomisation to decrease likelihood of experimental error resulting in loss of the same data point.

4.4.2 Main Study

The main study was designed to test the effects of *Rubus* fruit extracts on cell viability, NO production and cytokine (TNFα, IL1β, IL10, and IL6) production by colonic IEC and BMDM using methods that were modified according to the results obtained from the preliminary study.

Group 1 mice were dosed with 10^3-fold greater CFU concentration of CIF-ES inoculum compared with the dose used for group 2 mice (group 2 were exposed to normal dose). The *Il10*$^{-/-}$ mice are well characterised in their responses to this microbial inoculum and disease progression [154, 156]. While an inoculum of enteric microbiota is required to induce colitis in

Il10$^{-/-}$ mice, the same inoculum does not affect C57 mice GIT responses [154, 156]. However, it is possible that this difference in inoculum concentration could have confounded the cell viability results, NO production and cytokine responses of colonic IEC and BMDM to *Rubus* fruit extracts in this study.

The viability of colonic IEC and BMDM were tested for each *Rubus* fruit extract (0.76-200 µg/mL) using the WST-1 assay. Visual assessment of cell growth was also recorded. For the colonic IEC from C57 or *Il10*$^{-/-}$ mice, the results of viability and visual cell growth were inconclusive due to the variability in responses between group 1 and group 2, and a low number of technical replicates for group 1 C57 mice. All *Rubus* fruit extracts decreased viability of BMDM isolated from both groups of *Il10*$^{-/-}$ mice at polyphenolic concentrations greater than 50 µg/mL. The viability results for BMDM isolated from C57 mice were inconclusive between groups, most likely due to the unexpected low yield of BMDM limiting the number of technical replicates that could be carried out. Overall, this study provided inconclusive results for the C57 or *Il10*$^{-/-}$ colonic IEC and C57 BMDM, and could not be used as a guide for the selection of concentration of the *Rubus* fruit extracts to be used for NO and cytokine assay. In this study, consistent viability results were only obtained for BMDM isolated from *Il10*$^{-/-}$ mice, and the viability reduction at *Rubus* fruit extract concentrations greater than 50 µg/mL for these cells suggests that the polyphenolic concentration of 50 µg/mL was the highest concentration to use for NO and cytokine assays. The polyphenolic concentration of 50 µg/mL was used previously for RAW 264.7 cells in Chapter 3.

All *Rubus* fruit extracts, apart from HEX, decreased NO production by BMDM isolated from C57 mice from both groups. The NO production by BMDM isolated from both groups of *Il10*$^{-/-}$ mice also decreased when co-incubated with extracts WEX and ZEX. There were indications that "ZZ" cultivar may be more effective in reducing NO production, however lack of replication from group 1 *Il10*$^{-/-}$ mice precludes a conclusive statement on this matter.

The results for the cytokine production by colonic IEC from C57 mice, in response to co-incubation with *Rubus* fruit extract-treated cells were inconclusive, likely due to variability in cytokine responses between groups, and the low number of replicates carried out. The colonic IEC isolated from *Il10*$^{-/-}$ mice showed consistent decrease in IL6 production for all *Rubus* fruit cultivars with or without LPS-stimulation relative to control IEC. ZEX was the most effective in decreasing IL6 production.

BMDM isolated from *Il10*$^{-/-}$ mice produced less IL6 and IL12 but similar TNFα levels, in response to co-incubation with *Rubus* fruit extracts, relative to control BMDM. Results for the BMDM isolated from C57 mice could not be statistically analysed (due to lack of replication). Taking that into account, the cytokine response of BMDM isolated from C57 mice, to co-incubation with the extracts (increase in TNFα and decrease in IL6 and IL10) were similar to the cytokine responses observed for RAW 264.7 macrophages when exposed to the same concentration of extracts as in Chapter 3.

4.4.1 Conclusion

Despite the improved experimental design and methods, an optimal yield of cells was not isolated fully for any group. There was a major increase in the number of cells isolated for the main study, compared to the preliminary study; however it was still not enough to satisfy the requirements for statistical interpretations for all assays. This was further impacted by the need to separate the biological replicates rather than combining of results as intended. Future experimentation would ideally focus on specific extracts of interest, as determined by the main study, as the numbers of cells isolated for testing of all extracts is too low to be statistically viable.

There was an unexpected 10^3-fold difference in inoculum CFU concentration used to induce IBD-like symptoms the two groups of in *Il10*$^{-/-}$ mice (but also given to C57 mice) and this difference could have confounded the viability, NO production and cytokine responses of colonic IEC and BMDM in this study. The results for viability were inconclusive, apart from BMDM isolated from *Il10*$^{-/-}$ mice. The magnitude of the cytokine responses for colonic IEC and BMDM were different between group 1 and group 2 mice. The results for NO production by BMDM were consistent between groups. Therefore results for NO and cytokine production were used to give an indication of the effects of *Rubus* fruit extracts on their colonic IEC and BMDM responses only if there were sufficient technical replicates for statistical analysis and these responses were consistent between groups of the same genotype.

The viability results for colonic IEC isolated from C57 and *Il10*$^{-/-}$ mice, and BMDM from C57 mice were inconclusive due to lack of consistency in replication. The viability of BMDM isolated from *Il10*$^{-/-}$ mice was unaffected at polyphenolic concentrations of 50 µg/mL or less (apart from indication of ZETx for group 2 – however this is speculative). This concentration

was used for the assessment of NO production and cytokine production of colonic IEC and BMDM exposed to *Rubus* fruit extracts.

NO production by BMDM isolated from *Il10*$^{-/-}$ mice decreased with *Rubus* fruit extracts WEX and ZEX for both groups. NO production by BMDM isolated from C57 mice, showed a decrease in production for most extracts (apart from HEX). The cytokine results for BMDM isolated from *Il10*$^{-/-}$ mice showed a decrease in IL12 and IL6 production, and no change in TNFα production in response to co-incubation with some *Rubus* extracts. The IL6 produced by colonic IEC isolated from *Il10*$^{-/-}$ mice decreased for all *Rubus* fruit extracts, regardless of LPS-stimulation. The results from the analysis of inflammatory markers (NO and cytokine production) for BMDM and from cytokines for colonic IEC for the *Il10*$^{-/-}$ mice suggest that *Rubus* fruit extract ZZ, in particular ZEX, has the potential to induce an anti-inflammatory effect.

Chapter 5: Discussion and Conclusion

5.1 Summary and Discussion of Experimental Work

This thesis aimed to test the hypothesis that *Rubus* fruit extracts, rich in ellagitannins (ETs), induce an anti-inflammatory effect in mammalian cells involved in inflammatory processes. The work undertaken in this thesis encompassed multiple disciplines, including phytochemisty, cell biology and immunology.

Chapter 2 aimed to develop a method to extract high yields of polyphenolics, including ETs, from *Rubus* fruit and to purify and characterise these extracts. Chapter 3 used RAW 264.7 murine macrophages to evaluate the effects of *Rubus* fruit extracts on cell viability using the MTT assay, nitric oxide (NO) production using the Griess assay and cytokine production. Chapter 4 used intestinal epithelial cells (IEC) and bone marrow derived macrophages (BDMD) isolated from mice for method optimisation (preliminary study) and for evaluation of *Rubus* fruit extracts on the cell function mentioned above (main study).

In Chapter 2, work focused on polyphenolic chemistry using an exemplar *Rubus* fruit extract to establish methodology to isolate the key polyphenolic compounds (ETs and anthocyanins) and to purify the ET component from the extract. The results indicated that 70:30 acetone:water was the most effective solvent for extracting the highest yield of ETs, and that a Sephadex LH-20 column could be used to isolate the ETs from the other polyphenolic constituents.

The optimised extraction methods were applied to three Plant & Food Research *Rubus* fruit cultivars, which were chosen for their varying levels of key ETs; Sanguiin H6 (SH6) and Lambertianin C (Lam C). The resultant phenolic extracts (EX) were purified to prepare ET-enriched (ETx) extracts and the chemical composition of the extracts was determined. Tests were conducted in order to assess the stability of ETs in the extracts, in cell culture medium under the physiological pH conditions required (7.4-7.6) for cell maintenance. The results demonstrated a rapid decrease in the concentration of ETs (half life 3-4 h dependent on extract) over time at pH greater than 6 for ETx extracts but no such loss was observed for the EX extracts. This observed difference in stability for ETs in the EX extracts compared with the ETx extracts was hypothesised to be due to an interaction of the ETs with other polyphenolic compounds, this interaction conferring ET stability. This observation led to a decision to

decrease the period of time the extracts were co-incubated with the mammalian cells (small intestinal and colonic IEC and BMDM) from 24 h to 20 h.

In Chapter 3, the *Rubus* fruit extracts were co-incubated with a secondary cell line; RAW 264.7 macrophages, in order to generate an indication of their potential anti-inflammatory effects. The MTT assay, which measures metabolic activity as a surrogate measure of cell viability, was used to determine the maximum concentration of polyphenolic exposure to be applied to RAW 264.7 macrophages for all extracts to be 100 µg/mL. The Griess assay was used to measure, as nitrite, the levels of NO production (a marker of inflammation) by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages with concentrations up to 50 µg/mL of *Rubus* extracts. All of the *Rubus* fruit extracts apart from ZEX, reduced NO production relative to the control cells by RAW 264.7 macrophages but only at the highest concentration tested 50 µg/mL.

Cytokine (Interleukin 1 beta;IL1β, Interleukin 10; IL10, Tumor Necrosis Factor alpha; TNFα, and Interleukin 6; IL6) analysis was carried out on RAW 264.7 macrophages in order to assess their immunological responses to co-incubation with the *Rubus* fruit extracts. IL1β and IL10 were not produced at detectable levels by the LPS-stimulated RAW 264.7 macrophages but TNFα and IL6 were able to be detected. The production of TNFα, a known potent inflammatorycytokine elevated in inflammatory bowel disease (IBD), by the RAW 264.7 macrophages was raised for all extracts, whilst the production of IL6, a pleiotropic cytokine elevated in IBD [151], was lowered only by WETx. This suggests that the effect of WETx extract on cytokine production might be mediated through different pathways and demonstrates that the ET component alone has the ability to induce an anti-inflammatory response.

Evidence from the literature suggests that the cytokine response by RAW 264.7 macrophages to WETx extract could be associated with wound healing rather than inflammation. An *in vitro* study by Misha, et al., (2006), showed that a polyphenolic extract from *Rhodiola imbricata* increased IL6 and TNFα production in RAW 264.7 macrophages [157]. Furthermore, a study in rats, by Gupta et al., (2007) [158] have shown a *Rhodiola imbricata* polyphenolic rich extract accelerated wound healing through increasing cellular proliferation and collagen synthesis at the wound site. As wound healing is mediated by fibroblasts, the increase in fibroblast proliferation could be linked to increased production of cytokines TNFα and IL6. To explore whether the *Rubus* fruit extract WETx had wound healing effects, an *in vivo* study, as described by Gupta et al., (2007), could be conducted to assess their ability to alter the wound healing process.

In Chapter 4, a preliminary study was carried out to optimise the techniques needed for culturing of murine small intestinal and colonic IEC, and BMDM, and to test the effects of *Rubus* fruit extract on cell viability, NO production and cytokine (IL1β, IL10, TNFα, and IL6) production. This study was designed using guidance from similar experiments with kiwifruit extracts [150] or with secondary RAW 264.7 macrophages (Chapter 3). A main concern arising from the results of the preliminary study was the variable yield of primary small intestinal and colonic IEC, and BMDM, and consequently, a lack of technical replicates. As a result, methods from Chapter 3 and/or previous work [150], were compared and modified where needed, and focus was shifted to the isolation and culture of colonic IEC and BMDM only (to simplify technical demands of experimental work). The WST-1 assay was used instead of MTT for cytotoxicity testing in the main study as it was simpler to use and more consistent in absorbance results. The optimal dose for LPS-stimulation of BMDM was raised from 0.05 µg/mL to 0.5 µg/mL to increase the cells ability to produce NO and cytokines. Changes were implemented to improve the yield of colonic IEC and BMDM during their isolation and also to reduce the experimental errors by the operator during tissue procession, isolation and assay, for the main study.

The main study in Chapter 4, was designed to test the effects of *Rubus* fruit extracts on cell viability, NO production and cytokine (TNFα, IL1β, IL10 or IL12, and IL6) production by colonic IEC and BMDM isolated from C57BI/6J (C57) and interleukin 10 gene deficient mice (*Il10*$^{-/-}$) mice using methods that were developed from the results obtained from the preliminary study. *Il10*$^{-/-}$ mice were used as an IBD-model as they spontaneously develop colitis-like symptoms through an interaction between the gastrointestinal tract (GIT) mucosa and innate GIT microbiota. C57 mice were used as the control strain. The initial experimental plan was for two groups of C57 and *Il10*$^{-/-}$ mice to function as biological replicates, however, due to the differences in the parameter responses between groups in the assays, results were displayed separately instead of combined to give an overall effect. To enable comparison between groups, cell viability, NO production and cytokine production results were normalised and expressed as a ratio relative to that measured for control cells not exposed to the *Rubus* fruit extracts. This difference in response between groups, combined with the low number of technical replicates carried out for some assays, decreased the statistical power of the results and conclusions from the main study.

The viability results from the WST-1 assay and visual cell growth provided inconclusive results for the C57 or *Il10*$^{-/-}$ colonic IEC and C57 BMDM, and could not be used as a guide for the

selection of concentration of the *Rubus* fruit extracts to be used for NO and cytokine assays. Consistent viability results were only obtained for BMDM isolated from *Il10*$^{-/-}$ mice, and the viability reduction at *Rubus* fruit extract concentrations greater than 50 µg/mL for these cells suggested that the polyphenolic concentration of 50 µg/mL was the highest concentration to use for NO and cytokine assays. The polyphenolic concentration of 50 µg/mL was also used previously for RAW 264.7 macrophages in Chapter 3. Thus the concentration of 50 µg/mL was used for further inflammatory marker assays (NO production and cytokine profiles) of colonic IEC and BMDM for all extracts.

The main study showed that NO production for either group of isolated BMDM was similar between genotypes. Indications for BMDM from C57 mice showed a decrease of NO production with all extracts apart from HEX, and for BMDM from *Il10*$^{-/-}$ mice, a decrease in NO production for WEX and ZEX. These results are comparable with the findings reported in Chapter 3 where at 50 µg/mL there was a decrease in NO production by all *Rubus* fruit extracts apart from ZEX, in RAW 264.7 macrophages. NO is a negative free radical known to be present at higher concentrations in IBD, and the decrease in NO production by the macrophages induced by the extracts could indicate an anti-inflammatory effect.

The results for cytokine production by colonic IEC from C57 mice, in response to co-incubation with *Rubus* fruit extract-treated cells, were inconclusive likely due to variability in cytokines responses between groups, and the low number of replicates carried out. The colonic IEC isolated from *Il10*$^{-/-}$ mice showed consistent decrease in IL6 production for all *Rubus* fruit extracts with or without LPS-stimulation relative to control IEC. ZEX was the most effective in decreasing IL6 production by the colon IEC.

BMDM isolated from *Il10*$^{-/-}$ mice produced less IL6 and IL12 but similar TNFα levels, in response to co-incubation with *Rubus* fruit extracts, relative to control BMDM. Results for the BMDM isolated from C57 mice could not be statistically analysed (due to lack of replication). Taking that into account, the cytokine response of BMDM isolated from C57 mice, to co-incubation with the extracts (increase in TNFα and decrease in IL6 and IL10 levels) were similar to the cytokine responses observed for RAW 264.7 macrophages (increase in TNFα and decrease in IL6 levels) when exposed to the same extracts and concentration in Chapter 3. Cytokine assays for BMDM demonstrated both pro- and anti-inflammatory responses induced by the *Rubus* fruit extracts dependent on genotype, which could point to a difference in gene activation and metabolic pathways.

The rise in TNFα production by the RAW264.7 macrophages (Chapter 3) and BMDM isolated from C57 mice (Chapter 4), and no change in TNFα for BMDM isolated from *Il10*$^{-/-}$ mice (Chapter 4), in response to the *Rubus* fruit extracts, is unlikely to be a positive effect in the context of IBD, due to the importance of this cytokine as a critical regulator in IBD. TNFα is a pro-inflammatory cytokine that is overexpressed in IBD [159, 160], and has the ability to act upon almost every cell type within the body [161]. Many of newest drugs used in successfully controlling the inflammation in IBD are anti-TNF in mechanism [130].

The decrease in IL6 production by the RAW 264.7 macrophages, colonic IEC from both groups of mice, and indication of decrease from BMDM isolated from both mice groups, suggests a potential anti-inflammatory response induced by the *Rubus* extracts for this cytokine. This inference is in agreement with the role of IL6 in the human body; IL6 is produced by various cell types and has been found to be elevated in numerous inflammatory states, including Crohns Disease (CD), Ulcerative Colitis (UC), fever and rheumatoid arthritis [151].

The decrease in IL12 production by the BMDM isolated from *Il10*$^{-/-}$ mice might suggest an anti-inflammatory response induced by co-incubation with the *Rubus* fruit extracts. IL12 is critical for the development of T_h1 cell activation [162], and stimulates the production of interferon gamma (IFNγ) and TNFα from T and natural killer cells, and its production is strongly inhibited by IL10 [163]. The reduction of IL12 levels was also accompanied by a decrease of IL6 levels, with no change observed for TNFα levels further indicating an anti-inflammatory response. However, although IL12 production by BMDM was decreased, it could also contribute to a dysregulated inflammatory response through irregular T_h1 cell activation. Supporting this argument Chavali [164] reported an increase in TNFα and decrease in IL6 and IL10 levels, by adding sesamin and other oils to the diets of Balb/c mice. The results suggested that this response was linked to a downregulation in the immune system's ability to respond to infection [164]. It is plausible that the cytokine responses of mammalian cells might vary depending on the food component tested.

5.2 Limitations of the Methods

One limitation of this study was interference between the reagent and/or components in the cell culture media with the *Rubus* fruit extracts. Anthocyanin in samples can influence the results in colorimetric assays. Interference that can be caused by anthocyanins does not appear to be consistently reported in the literature, with some users reporting methods to correct for interference [165], and others not reporting published difficulty [62, 166]. The

issues encountered are potentially specific to the phytochemical makeup of the extract under examination and the medium used in experimentation. As a result the WST-1 assay was modified for the main study by including a media replacement step prior to incubation with the reagent, with the intent to remove the interference between reagent and cell culture medium with *Rubus* fruit extracts. However, follow up experiments to confirm the efficacy of the additional method step were not conducted due to time constraints of the main study. The results of the WST-1 assay in the main study did not show abnormal absorbance, as indicated by the preliminary study, implying the interference may have been minimised.

With the difficulties that arose with the cytotoxicity testing in the main study, assays of cell viability with *Rubus* fruit extracts other than the WST-1 assay might have been preferable. There are a variety of other tests, such as ATP analysis or measurement of protease components [147], that could be used to provide indications about cell cytotoxicity. Although which assay would be best suited to working with anthocyanin-rich fractions would first need to be examined.

Despite the optimisation of the methods for tissue processing and cell isolation in the preliminary study, the low yields of colonic IEC and BMDM isolated from the mice remained an issue for the main study. There was an unexpected lower yield of colonic IEC and BMDM isolated from C57 mice compared to that from *Il10*$^{-/-}$ mice despite using the same methods of tissue processing and cell isolation. Given the estimated yield used to design the main study was based on the same cell types isolated from C57 mice with improved methods from the preliminary study, lower yields should not have been an issue. The low yields of some cell types isolated from both groups of mice resulted in a lack of technical replication for many of the assays. This, combined with the differences in the assay parameter responses between the two groups of mice and the consequent inability to combine results across groups, decreased the statistical power of the results and conclusions from the main study.

In addition to the *Rubus* fruit extracts, colonic IEC and BMDM were also used to assess the effects of the microbially-derived ET metabolites Uro A and B (see A.3 Urolithins for experimental work and results). While a potentially important aspect of ET effects on inflammatory markers by colonic IEC and BMDM, this additional work might have been better addressed in another study allowing prioritisation of the use of the isolated cells for assessment of the effects of *Rubus* fruit extracts.

5.3 Future Perspectives

Moving forwards from *in vitro* models, the next step will be to evaluate the anti-inflammatory effects of *Rubus* fruit extract(s) that had demonstrated the greatest potential in the main study *in vivo*. However, due to inconsistent results between biological replicates and the low statistical power of the main study, further validation would be warranted.

Human cell lines could allow an improved translation of the anti-inflammatory effects of the specific *Rubus* fruit extracts in a human context based on results from immunological testing. There are a number of human secondary IEC lines that are available that could be used to repeat the assessments made in the murine models.

Human colorectal adenocarcinoma (Caco-2) cells are a human secondary IEC line that retains the ability to polarise and form a transporting epithelial monolayer. This ability makes them useful for study as they have many characteristics similar to a polarised IEC layer found *in situ* [167] and they have been previously used to examine the effects of food components, such as polyphenolic rich extracts [72, 102, 168-170]. These cells could be used to investigate the anti-inflammatory effects of *Rubus* fruit extracts using the same inflammatory marker assays (NO and cytokine production), with the addition of other cellular assays such as STAT3, PGE2 and NF-κβ assays.

Clarification regarding the changes in the production of TNFα (which was raised or unaffected in contrast to the other pro-inflammatory cytokines being lowered), induced by the *Rubus* fruit extracts could be examined by studying the changes in NF-κβ pathways. NF-κβ activation by TNFα induces an inflammatory chain reaction through the activation of multiple inflammatory mediators, one of which is forming a positive feedback loop inducing further TNFα activation [171]. Another mediator, PGE2, could be examined as some studies have shown that the regulation of PGE2 was critical for modulating TNFα [172].

Caco-2 cells could also be used to investigate ET dietary-induced changes in gene and protein expression levels using “omic” technologies, such as transcriptomics, proteomics, or metabolomics, to identify modulated metabolic pathways and key gene/protein regulatory hubs [173, 174].

There are several questions regarding the bioavailability of ETs. Uro A and B (see A.3 Urolithins), are the end metabolites of ET metabolism, and have previously been shown to have many positive biological properties such as anti-inflammatory and anti-oxidant effects [43, 73, 113, 175, 176]. Future experimental work using these compounds would be important

to validate findings about any *Rubus* fruit extracts, and potentially the focus of research could examine these compounds in greater detail.

Human intervention studies could be used to assess if Uros and/or EA are excreted in the urine and faeces or accumulate in plasma and investigate how the extracts are metabolised in the human GIT [177]. Designing this study would need to include stringent monitoring of the test subjects diet to ensure that ETs were not introduced by other means.

5.4 Conclusions

In this thesis six *Rubus* fruit extracts were prepared and characterised, in order to test their anti-inflammatory properties on RAW 264.7 macrophages, and primary cells isolated from murine sources; colonic IEC and BMDM. Future experimentation using the *Rubus* fruit extracts would need to overcome the issues found with reproducibility between mice biological replicates, cytotoxicity testing discrepancies.

There are indications that *Rubus* fruit extracts from "ZZ" and "Wakefield" cultivars can reduce immune responses (based on NO production assessment) or potentially act on different pathways or induce a wound healing response (based on cytokine assessment).

Studies like this thesis are useful for providing baseline research into understanding how a specific food component, like ETs, may influence the physiological responses in healthy or disease state. The high concentration of ETs found in the *Rubus* fruit extracts examined in this thesis, indicate the potential of inducing an anti-inflammatory response and may help those of certain genetic predispositions with ameliorating IBD. Further studies are needed to fully understand the potential of *Rubus* fruit extracts in human nutrition and health.

References:

[1] Kaser A, Zeissig S, Blumberg RS. Inflammatory Bowel Disease. Annual Review of Immunology. 2010;28:573-621.

[2] Chapman-Kiddell CA, Davies PSW, Gillen L, Radford-Smith GL. Role of diet in the development of inflammatory bowel disease. Inflammatory Bowel Diseases. 2010;16:137-51.

[3] Gassull M, Mane J, Pedross E, Cabre E. Macronutrients and Bioactive Molecules: Is There a Specific Role in the Management of Inflammatory Bowel Disease. Journal of Parenteral and Enteral Nutrition. 2005;29:179-83.

[4] Basic M, Zrnec D, Butorac A, Jurcevic I, Dikic D, Bacun-Druzina V. Sto je nutrigenomika? What is Nutrigenomics? Hrvatski Casopis za Prehrambenu Tehnologiju, Biotehnologiju i Nutricionizam. 2011;6:37-44.

[5] Fenech M, El-Sohemy A, Cahill L, Ferguson LR, French TAC, Tai ES, et al. Nutrigenetics and Nutrigenomics: Viewpoints on the Current Status and Applications in Nutrition Research and Practice. Journal of Nutrigenetics and Nutrigenomics. 2011;4:69-89.

[6] Mutch DM, Wahli W, Williamson G. Nutrigenomics and nutrigenetics: the emerging faces of nutrition. The FASEB Journal. 2005;19:1602-16.

[7] Hager TJ, Howard LR, Liyanage R, Lay JO, Prior RL. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. Journal of Agricultural and Food Chemistry. 2008;56:661-9.

[8] Kähkönen MP, Hopia AI, Heinonen M. Berry Phenolics and Their Antioxidant Activity. Journal of Agricultural and Food Chemistry. 2001;49:4076-82.

[9] Mertz C, Cheynier V, Günata Z, Brat P. Analysis of Phenolic Compounds in Two Blackberry Species (*Rubus glaucus* and *Rubus adenotrichus*) by High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ion Trap Mass Spectrometry. Journal of Agricultural and Food Chemistry. 2007;55:8616-24.

[10] Mullen W, McGinn J, MacLean MR, Gardner P, Duthie GG, Yokota T, et al. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. Journal of Agricultural and Food Chemistry. 2002;50:5191-6.

[11] Adams LS, Seeram NP, Aggarwal BB, Takada Y, Sand D, Heber D. Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. Journal of Agricultural and Food Chemistry. 2006;54:980-5.

[12] Bakkalbaşi E, Menteş Ö, Artik N. Food Ellagitannins–Occurrence, Effects of Processing and Storage. Critical Reviews in Food Science and Nutrition. 2008;49:283-98.

[13] Cerdá B, Espín JC, Parra S, Martínez P, Tomás-Barberán FA. The potent *in vitro* antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy–6H–dibenzopyran–6–one derivatives by the colonic microflora of healthy humans. European Journal of Nutrition. 2004;43:205-20.

[14] Espín JC, González-Barrio R, Cerdá B, López-Bote C, Rey AI, Tomás-Barberán FA. Iberian Pig as a Model To Clarify Obscure Points in the Bioavailability and Metabolism of Ellagitannins in Humans. Journal of Agricultural and Food Chemistry. 2007;55:10476-85.

[15] Nohynek LJ, Alakomi H-L, Kähkönen MP, Heinonen M, Helander IM, Oksman-Caldentey K-M, et al. Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens. Nutrition & Cancer. 2006;54:18-32.

[16] Puupponen-Pimiä R, Nohynek L, Hartmann-Schmidlin S, Kähkönen M, Heinonen M, Määttä-Riihinen K, et al. Berry phenolics selectively inhibit the growth of intestinal pathogens. Journal of Applied Microbiology. 2005;98:991-1000.

[17] Rao AV, Snyder DM. Raspberries and Human Health: A Review. Journal of Agricultural and Food Chemistry. 2010;58:3871-83.

[18] Yoshida T, Amakura Y, Yoshimura M. Structural Features and Biological Properties of Ellagitannins in Some Plant Families of the Order Myrtales. International Journal of Molecular Sciences. 2010;11:79-106.
[19] García-Cañas V, Simó C, León C, Cifuentes A. Advances in Nutrigenomics research: Novel and future analytical approaches to investigate the biological activity of natural compounds and food functions. Journal of Pharmaceutical and Biomedical Analysis. 2010;51:290-304.
[20] Consortium TIH. Integrating common and rare genetic variation in diverse human populations. Nature. 2010;467:52-8.
[21] The International HapMap C. A haplotype map of the human genome. Nature. 2005;437:1299-320.
[22] Begum F, Ghosh D, Tseng GC, Feingold E. Comprehensive literature review and statistical considerations for GWAS meta-analysis. Nucleic Acids Research. 2012;40:3777-84.
[23] Russell R, Drummond H, Wilson D, Anderson N, Arnott ID, Van Limbergen J, et al. Detailed assessment of NOD2/CARD15 exonic variation in inflammatory bowel disease in Scotland: implications for disease pathogenesis. Genes and Immunity. 2008;9:556-60.
[24] Hosek J, Bartosová L, Gregor P, Kolorz M, Díte P, Bátovský M, et al. Frequency of Representative Single Nucleotide Polymorphisms Associated with Inflammatory Bowel Disease in the Czech Republic and Slovak Republic. Folia Biologica. 2008;54:88-96.
[25] McGovern D, Powrie F. The IL23 axis plays a key role in the pathogenesis of IBD. Gut. 2007;56:1333-6.
[26] Van Limbergen J, Russel R, Nimmo E, Statsangi J. The Genetics of Inflammatory Bowel Disease. American Journal of Gastroenterology. 2007;102:2820-31.
[27] Gry J, Black L, Eriksen FD, Pilegaard K, Plumb J, Rhodes M, et al. EuroFIR-BASIS – a combined composition and biological activity database for bioactive compounds in plant-based foods. Trends in Food Science & Technology. 2007;18:434-44.
[28] Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature. 2011;474:307-17.
[29] Seksik P. Gut microbiota and IBD. Gastroentérologie Clinique et Biologique. 2010;34:44-51.
[30] Ali T, Shakir F, Morton J. Curcumin and inflammatory bowel disease: biological mechanisms and clinical implication. Digestion. 2012;85:249–55
[31] Kirsch RH, Riddell R. Histopathological alterations in irritable bowel syndrome. Modern Pathology. 2006;19:1638-45.
[32] O'Sullivan M, O'Morain C. Nutrition in inflammatory bowel disease. Best Practise & Research Clinical Gastroenterology. 2006;20:561-73.
[33] Taylor C, Kelly S. The autonomic nervous system and inflammatory bowel disease. Autonomic Neuroscienc: Basic and Clinical. 2007;133:104-14.
[34] Baumgart D, Carding S. Inflammatory bowel disease: cause and immunobiology. The Lancet. 2007;369:1627-40.
[35] Cooney R, Jewell D. The Genetic Basis of Inflammatory Bowel Disease. Digestive Disease. 2009;27:428-42.
[36] D'Amato M, Labbé C, Rioux J. Genetics of Ulcerative Colitis. In: D'Amato M, Rioux JD, editors. Molecular Genetics of Inflammatory Bowel Disease: Springer New York; 2013. p. 119-34.
[37] Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut. 2011;60:1739-53.
[38] Landete JM. Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. Food Research International. 2011;44:1150-60.
[39] Scaldaferri F, Gerardi V, Lopetuso LR, Del Zompo F, Mangiola F, Koski I, et al. Gut Microbial Flora, Prebiotics, and Probiotics in IBD: Their Current Usage and Utility. BioMed Research International. 2013;2013:9 pages.

[40] Barnich N, Darfeuill-Michaud A. Role of bacteria in the etiopathogenesis of inflammatory bowel disease. World Journal of Gastroenterology. 2007;42:5571-6.
[41] Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 2007;448:427-34.
[42] Chiba M, Tsuda H, Abe T, Sugawara T, Morikawa Y. Missing environmental factor in inflammatory bowel disease: Diet-associated gut microflora. Inflammatory Bowel Diseases. 2011;17:82-3.
[43] Larrosa M, Gonzalez-Sarrias A, Yanez-Gascon MJ, Selma MV, Azorin-Ortuno M, Toti S, et al. Anti-inflammatory properties of a pomegranate extract and its metabolite Urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. Journal of Nutritional Biochemistry. 2010;21:717-25.
[44] Sokol H, Watterlot L, Lakhdari O, Gratadoux J-J, Bridonneau C, Furet J-P, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:16731-6.
[45] Danese S, Sans M, Fiocchi C. Inflammatory bowel disease: the role of environmental factors. Autoimmunity reviews. 2004;3:394-400.
[46] Low Y, Tai ES. Understanding diet-gene interactions: Lessons from studying nutrigenomics and cardiovascular disease. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2007;622:7-13.
[47] Meshkin B, Chen TJ, Chen AL, Prihoda TJ, Morrisette H, Braverman ER, et al. Health economics of nutrigenomics in weight management. Gene Therapy and Molecular Biology. 2008;12:25-30.
[48] Rollin F, Kennedy J, Wills J. Consumers and new food technologies. Trends in Food Science & Technology. 2011;22:99-111.
[49] Ronteltap A, van Trijp JCM, Renes RJ, Frewer LJ. Consumer acceptance of technology-based food innovations: Lessons for the future of nutrigenomics. Appetite. 2007;49:1-17.
[50] Ferguson L, Shelling A, Lauren D. Nutrigenomics and gut health. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2007;622:1-6.
[51] Philpott M, Mackay L, Ferguson L, Forbes D, Skinner M. Cell culture models in developing nutrigenomics foods for inflammatory bowel disease. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2007;622:94-102.
[52] Dionne S, Hiscott J, D'Agata I, Duhaime A, Seidman EG. Quantitative PCR Analysis of TNF-α and IL-1β mRNA Levels in Pediatric IBD Mucosal Biopsies. Dig Dis Sci. 1997;42:1557-66.
[53] Stoner GD, Li-shu W, Casto. Laboratory and clinical studies of cancer chemoprevention by antioxidants in berries. Carcinogenesis. 2008;29:1665-74.
[54] Bidel L, Coumans M, Baissac Y, Doumas P, Jay-Allemand C. Biological Activity of Phenolics in Plant Cells. In: Santos-Buelga C, Escribano-Bailon M, Lattanzio V, editors. Recent Advances in Polyphenol Research: Blackwell Publishing Limited; 2010.
[55] Naczk M, Shahidi F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of Pharmaceutical and Biomedical Analysis. 2006;41:1523-42.
[56] El Gharras H. Polyphenols: food sources, properties and applications – a review. International Journal of Food Science & Technology. 2009;44:2512-8.
[57] Quideau S. Chemistry and biology of ellagitannins: an underestimated class of bioactive plant polyphenols: World Scientific; 2009.
[58] Del Rio D, Borges G, Crozier A. Berry flavonoids and phenolics: bioavailability and evidence of protective effects. The British Journal of Nutrition. 2010;104:67-90.
[59] Barnett LE, Broomfield AM, Hendriks WH, Hunt MB, McGhie TK. The *In Vivo* Antioxidant Action and the Reduction of Oxidative Stress by Boysenberry Extract Is Dependent on Base Diet Constituents in Rats. Journal of Medicinal Food. 2007;10:281-9.

[60] Cerda B, Tomas-Barberan FA, Espin JC. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. Journal of Agricultural and Food Chemistry. 2005;53:227-35.
[61] Ghosh D, McGhie TK, Zhang J, Adaim A, Skinner M. Effects of anthocyanins and other phenolics of boysenberry and blackcurrant as inhibitors of oxidative stress and damage to cellular DNA in SH-SY5Y and HL-60 cells. Journal of the Science of Food and Agriculture. 2006;86:678-86.
[62] Reddy M, Gupta S, Jacob M, Khan S, Ferreira D. Antioxidant, Antimalarial and Antimicrobial Activites of Tannin-rich Fractions, Ellagitannins and Phenolic Acids from *Punica granatum* L. Planta Med. 2007;73:461-7.
[63] Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary Polyphenols and the Prevention of Diseases. Critical Reviews in Food Science and Nutrition. 2005;45:287-306.
[64] Sang S, Lambert JD, Yang CS. Bioavailability and stability issues in understanding the cancer preventive effects of tea polyphenols. Journal of the Science of Food and Agriculture. 2006;86:2256-65.
[65] Umesalma S, Sudhandiran G. Ellagic acid prevents rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. European Journal of Pharmacology. 2011;660:249-58.
[66] Dell'Agli M, Galli G, Bulgari M, Basilico N, Romeo S, Bhattaharya D, et al. Ellagitannins of the fruit rind of pomegranate *(Punica granatum)* antagonize *in vitro* the host inflammatory response mechanisms involved in the onset of malaria. Malaria Journal. 2010;9.
[67] González-Sarrías A, Larrosa M, Tomás-Barberán FA, Dolara P, Espín JC. NF-[kappa]B-dependent anti-inflammatory activity of urolithins, gut microbiota ellagic acid-derived metabolites, in human colonic fibroblasts. The British Journal of Nutrition. 2010;104:503-12.
[68] Papoutsi Z, Kassi E, Chinou I, Halabalaki M, Skaltsounis LA, Moutsatsou P. Walnut extract (*Juglans regia L.*) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483. The British Journal of Nutrition. 2008;99:715-22.
[69] Gonzalez-Barrio R, Borges G, Mullen W, Crozier A. Bioavailability of Anthocyanins and Ellagitannins Following Consumption of Raspberries by Healthy Humans and Subjects with an Ileostomy. Journal of Agricultural and Food Chemistry. 2010;58:3933–9.
[70] Cerda B, Tomas-Barberan FA, Espin JC. Metabolism of Antioxidant and Chemopreventive Ellagitannins from Strawberries, Raspberries, Walnuts, and Oak-Aged Wine in Humans: Identification of Biomarkers and Individual Variability. Journal of Agricultural and Food Chemistry. 2004;53:227-35.
[71] Larrosa M, García-Conesa MT, Espín JC, Tomás-Barberán FA. Ellagitannins, ellagic acid and vascular health. Molecular Aspects of Medicine. 2010;31:513-39.
[72] Larrosa M, Tomás-Barberán FA, Espín JC. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. Journal of Nutritional Biochemistry. 2006;17:611-25.
[73] Bialonska D, Kasimsetty SG, Khan SI, Ferreira D. Urolithins, Intestinal Microbial Metabolites of Pomegranate Ellagitannins, Exhibit Potent Antioxidant Activity in a Cell-Based Assay. Journal of Agricultural and Food Chemistry. 2009;57:10181-6.
[74] Cerdá B, Llorach R, Cerón JJ, Espín JC, Tomás-Barberán FA. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. European Journal of Nutrition. 2003;42:18-28.
[75] Ramirez MAR. The effects of ellagic acid, a polyphenol dreived from pomegranate, in an experimentally acute TNBS-induced colitis model. 18. The United European gastroenterology week. Barcelona, Spain 2010.

[76] Larrosa M, González-Sarrías A, Yáñez-Gascón MJ, Selma MV, Azorín-Ortuño M, Toti S, et al. Anti-inflammatory properties of a pomegranate extract and its metabolite Urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. The Journal of Nutritional Biochemistry. 2010;21:717-25.
[77] Giménez-Bastida JA, Larrosa M, González-Sarrías A, Tomás-Barberán F, Espín JC, García-Conesa M-T. Intestinal Ellagitannin Metabolites Ameliorate Cytokine-Induced Inflammation and Associated Molecular Markers in Human Colon Fibroblasts. Journal of Agricultural and Food Chemistry. 2012;60:8866-76.
[78] Rastmanesh R. High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. Chemico-Biological Interactions. 2011;189:1-8.
[79] Seeram NP. Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact of Their Intake on Human Health, Performance, and Disease. Journal of Agricultural and Food Chemistry. 2008;56:627-9.
[80] Hall H, Langford G. The 'Boysenberry': Development of the cultivar and industries in Califonia, Oregon and New Zealand. IXth International Rubues and Ribes Symposium; 2008.
[81] Hall HK, Langford G. The 'Boysenberry': Development of the cultivar and industries in California, Oregon and New Zealand. Acta Horticulturae 2008;777:103-8.
[82] New Zealand B. History of the Boysenberry [Internet]. 2012 2012. Available from: http://www.boysenberry.co.nz/index.php?page=history-of-the-boysenberry
[83] Scarrow S. Citrus, berries, exotic fruit and nuts - Boysenberries and raspberries [Internet]. Te Ara - the Encyclopedia of New Zealand; 2009 2012]. Available from: http://www.TeAra.govt.nz/en/citrus-berries-exotic-fruit-and-nuts/5.
[84] Lee J, Dossett M, Finn CE. *Rubus* fruit phenolic research: The good, the bad, and the confusing. Food Chemistry. 2012;130:785-96.
[85] Gasperotti M, Masuero D, Vrhovsek U, Guella G, Mattivi F. Profiling and Accurate Quantification of Rubus Ellagitannins and Ellagic Acid Conjugates Using Direct UPLC-Q-TOF HDMS and HPLC-DAD Analysis. Journal of Agricultural and Food Chemistry. 2010;58:4602–16.
[86] Kool MM, Comeskey DJ, Cooney JM, McGhie TK. Structural identification of the main ellagitannins of a boysenberry (*Rubus loganbaccus × baileyanus Britt.*) extract by LC-ESI-MS/MS, MALDI-TOF-MS and NMR spectroscopy. Food Chemistry. 2010;119:1535-43.
[87] Furuuchi R, Yokoyama T, Wanatabe Y, Hirayama M. Identification and Quantification of Short Oligomeric Proanthocyanidins and Other Polyphenols in Boysenberry Seeds and Juice. Journal of Agricultural and Food Chemistry. 2011:3738-46.
[88] Wada L, Ou B. Antioxidant Activity and Phenolic Content of Oregon Caneberries. Journal of Agricultural and Food Chemistry. 2002;50:3495-500.
[89] Cooney JM, Jensen DJ, McGhie TK. LC-MS identification of anthocyanins in boysenberry extract and anthocyanin metabolites in human urine following dosing. Journal of the Science of Food and Agriculture. 2004;84:237-45.
[90] McGhie TK, Rowan DR, Edwards PJ. Structural Identification of Two Major Anthocyanin Components of Boysenberry by NMR Spectroscopy. Journal of Agricultural and Food Chemistry. 2006;54:8756-61.
[91] Torre LC, Barritt BH. Quantitative evaluation of Rubus fruit anthocyanin pigments. Journal of food science. 1977;42:488-90.
[92] McGhie TK, Hall HK, Ainge GD, Mowat AD. Breeding rubus cultivars for high anthocyanin content and high antioxidant capacity. Acta Horticulturae. 2002;585:495-500.
[93] Bushman BS, Phillips B, Isbell T, Crane JM, Knapp SJ. Chemical composition of caneberry (Rubus spp.) seeds and oils and their antioxidant potential. Journal of Agricultural and Food Chemistry. 2004;52:7982-7.
[94] Kall H, Langford G. The 'Boysenberry': Development of the cultivar and industries in Califonia, Oregon and New Zealand. IXth International Rubues and Ribes Symposium; 2008.

[95] Castañeda-Ovando A, Pacheco-Hernández MdL, Páez-Hernández ME, Rodríguez JA, Galán-Vidal CA. Chemical studies of anthocyanins: A review. Food Chemistry. 2009;113:859-71.
[96] Cordenunsi BR, Genovese MI, Lajolo FM. Effects of temperature on the chemical composition and antioxidant activity of three strawberry cultivars. Food Chemistry. 2005;91:113-21.
[97] Hager A, Howard LR, Prior RL, Brownmiller C. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed black raspberry products. Journal of Food Science. 2008;73:134-40.
[98] Hager TJ, Howard LR, Prior RL. Processing and storage effects on the ellagitannin composition of processed blackberry products. Journal of Agricultural and Food Chemistry. 2010;58:11749-54.
[99] Moldovan B, David L, Chişbora C, Cimpoiu C. Degradation Kinetics of Anthocyanins from European Cranberrybush (*Viburnum opulus L.*) Fruit Extracts. Effects of Temperature, pH and Storage Solvent. Molecules. 2012;17:11655-66.
[100] Acosta-Montoya Ó, Vaillant F, Cozzano S, Mertz C, Pérez AM, Castro MV. Phenolic content and antioxidant capacity of tropical highland blackberry (Rubus adenotrichus Schltdl.) during three edible maturity stages. Food Chemistry. 2010;119:1497-501.
[101] Vrhovsek U, Palchetti A, Reniero F, Guillou C, Masuero D, Mattivi F. Concentration and mean degree of polymerization of *Rubus* ellagitannins evaluated by optimized acid methanolysis. Journal of Agricultural and Food Chemistry. 2006;54:4469-75.
[102] Cilla A. Availability of polyphenols in fruit beverages subjected to in vitro gastrointestinal digestion and their effects on proliferation, cell-cycle and apoptosis in human colon cancer Caco-2 cells. Food Chemistry. 2009;114:813-20.
[103] Ghosh D, McGhie TK, Fisher DR, Joseph JA. Cytoprotective effects of anthocyanins and other phenolic fractions of Boysenberry and blackcurrant on dopamine and amyloid β-induced oxidative stress in transfected COS-7 cells. Journal of the Science of Food and Agriculture. 2007;87:2061-7.
[104] Kosińska A, Xie Y, Diering S, Héritier J, Andlauer W. Stability of Phenolic Compounds Isolated from Cocoa, Green Tea and Strawberries in Hank's Balanced Salt Solution under Cell Culture Conditions. Polish Journal of Food and Nutrition Sciences. 2012;62:91-6.
[105] Friedman M, Jürgens HS. Effect of pH on the Stability of Plant Phenolic Compounds. Journal of Agricultural and Food Chemistry. 2000;48:2101-10.
[106] Ismail T, Sestili P, Akhtar S. Pomegranate peel and fruit extracts: A review of potential anti-inflammatory and anti-infective effects. Journal of Ethnopharmacology. 2012;143:397–405.
[107] Jung W-J, Sung M-K. Effects of major dietary antioxidants on inflammatory markers of RAW 264.7 macrophages. BioFactors. 2004;21:113-7.
[108] Pallarès V, Calay D, Cedó L, Castell-Auví A, Raes M, Pinent M, et al. Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. Nutrition. 2012;28:447-57.
[109] Pallarès V, Calay D, Cedó L, Castell-Auví A, Raes M, Pinent M, et al. Enhanced anti-inflammatory effect of resveratrol and EPA in treated endotoxin-activated RAW 264.7 macrophages. The British Journal of Nutrition. 2012;108:1562-73.
[110] Syahida A, Israf D, Lajis N, Khozriab S, Habsah M, Permana DJ, et al. Effect of Compounds Isolated from Natural Products on IFN-ψ/LPS-Induced Nitric Oxide Production in RAW 264.7 Macrophages. Pharmaceutical Biology. 2006;44:50-9.
[111] Wang J, Mazza G. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. Journal of Agricultural and Food Chemistry. 2002;50:4183-9.

[112] Ruiz PA, Haller D. Functional Diversity of Flavonoids in the Inhibition of the Proinflammatory NF-[kappa]B, IRF, and Akt Signaling Pathways in Murine Intestinal Epithelial Cells. The Journal of Nutrition. 2006;136:664-71.
[113] Gonzalez-Sarrias A, Azorin-Ortuno M, Yanez-Gascon M-J, Tomas-Barberan FA, Garcia-Conesa M-T, Espin J-C. Dissimilar In Vitro and In Vivo Effects of Ellagic Acid and Its Microbiota-Derived Metabolites, Urolithins, on the Cytochrome P450 1A1. Journal of Agricultural and Food Chemistry. 2009;57:5623-32.
[114] Hartley JW, Evans LH, Green KY, Naghashfar Z, Macias AR, Zerfas PM, et al. Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. Retrovirology. 2008;5.
[115] Chawla A. Control of Macrophage Activation and Function by PPARs. Circulation Research. 2010;106:1559-69.
[116] Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. Current Protocals Immunology. 2008/11/20 ed2008.
[117] Hambleton J, Weinstein SL, Lem L, DeFranco AL. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. Proceedings of the National Academy of Sciences. 1996;93:2774-8.
[118] Hanauer SB. Inflammatory bowel disease: Epidemiology, pathogenesis, and therapeutic opportunities. Inflammatory Bowel Diseases. 2006;12:3-9.
[119] Freshney R. Culture of Animal Cells: A Manual of Basic Technique. 5 ed: John Wiley & Son Incorporated; 2005.
[120] Abcam. RAW 264.7 (Mouse leukaemic monocyte macrophage cell line) Whole Cell Lysate (ab7187) [Internet]. Available from: http://www.abcam.com/raw-264-7-mouse-leukaemic-monocyte-macrophage-cell-line-whole-cell-lysate-ab7187.html.
[121] Jones E, Adcock I, Ahmed B, Punchard N. Modulation of LPS stimulated NF-kappaB mediated Nitric Oxide production by PKCepsilon and JAK2 in RAW macrophages. Journal of Inflammation. 2007;4.
[122] Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. Immunology. 2004;113:427-37.
[123] Berkels R, Purol-Schnabel S, Roesen R. Measurement of nitric oxide by reconversion of nitrate/nitrite to NO. Nitric Oxide Protocols. 2 ed: Springer; 2004. p. 1-8.
[124] Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the l-arginine/nitric oxide area of research. Journal of Chromatography B. 2007;851:51-70.
[125] Berghaus LJ, Moore JN, Hurley DJ, Vandenplas ML, Fortes BP, Wolfert MA, et al. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. Comparative Immunology, Microbiology and Infectious Diseases. 2010;33:443-54.
[126] Edmunds SJ, Roy NC, Love DR, Laing WA. Kiwifruit extracts inhibit cytokine production by lipopolysaccharide-activated macrophages, and intestinal epithelial cells isolated from IL10 gene deficient mice. Cellular Immunology. 2011;270:70-9.
[127] Park E, Quinn MR, Wright CE, Schuller-Levis G. Taurine chloramine inhibits the synthesis of nitric oxide and the release of tumor necrosis factor in activated RAW 264.7 cells. Journal of Leukocyte Biology. 1993;54:119-24.
[128] Kim J-K, Oh S-m, Kwon H-S, Oh Y-S, Lim SS, Shin H-K. Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages. Biochemical and Biophysical Research Communications. 2006;345:1215-23.
[129] Mudter J, Neurath MF. Il-6 signaling in inflammatory bowel disease: Pathophysiological role and clinical relevance. Inflammatory Bowel Diseases. 2007;13:1016-23.
[130] Brown S, Mayer L. The Immune Response in Inflammatory Bowel Disease. American Journal of Gastroenterology. 2007;102:2058-69.

[131] Chatzidakis I, Mamalaki C. T cells as sources and targets of TNF: implications for immunity and autoimmunity. Current directions in autoimmunity. 2010;11:105-18.
[132] Nemetz A, Nosti-Escanilla MP, Molnar T, Kope A, Kovacs A, Feher J, et al. IL1B gene polymorphisms influence the course and severity of inflammatory bowel disease. Immunogenetics. 1999;49:527-31.
[133] Vijgen L, Van Gysel M, Rector A, Thoelen I, Esters N, Ceelen T, et al. Interleukin-1 receptor antagonist VNTR-polymorphism in inflammatory bowel disease. Genes and Immunity. 2002;3:400-6.
[134] Leach MW, Davidson NJ, Fort MM, Powrie F, Rennick DM. The role of IL-10 in inflammatory bowel disease: "of mice and men". Toxicologic pathology. 1999;27:123-33.
[135] Zhou F, Ciric B, Li H, Yan Y, Li K, Cullimore M, et al. IL-10 deficiency blocks the ability of LPS to regulate expression of tolerance-related molecules on dendritic cells. European Journal of Immunology. 2012;42:1449-58.
[136] Li MC, He SH. IL-10 and its related cytokines for treatment of inflammatory bowel disease. World Journal of Gastroenterology. 2004;10:620-5.
[137] Kucharzik T, Stoll R, Lügering N, Domschke W. Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). Clinical & Experimental Immunology. 1995;100:452-6.
[138] Briske-Anderson MJ, Finley JW, Newman SM. The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY). 1997;214:248-57.
[139] Jeong J-H, Jung H, Lee S-R, Lee H-J, Hwang KT, Kim T-Y. Anti-oxidant, anti-proliferative and anti-inflammatory activities of the extracts from black raspberry fruits and wine. Food Chemistry. 2010;123:338-44.
[140] Schmid D, Gruber M, Piskaty C, Woehs F, Renner A, Nagy Z, et al. Inhibition of NF-κB-Dependent Cytokine and Inducible Nitric Oxide Synthesis by the Macrocyclic Ellagitannin Oenothein B in TLR-Stimulated RAW 264.7 Macrophages. Journal of Natural Products. 2012;75:870-5.
[141] Al-Nasiry S, Geusens N, Hanssens M, Luyten C, Pijnenborg R. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. Human Reproduction. 2007;22:1304-9.
[142] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry. 2000;267:5421-6.
[143] Berridge MV, Tan AS. Characterization of the Cellular Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondrial Electron Transport in MTT Reduction. Archives of Biochemistry and Biophysics. 1993;303:474-82.
[144] Ngamwongsatit P, Banada PP, Panbangred W, Bhunia AK. WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic *Bacillus* species using CHO cell line. Journal of Microbiological Methods. 2008;73:211-5.
[145] Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay and drug development technologies. 2004;2:51-62.
[146] Wang J, Mazza G. Quantification of Nitrite in the Presence of Anthocyanins Using Griess and GC/MS Assays. Journal of Food Biochemistry. 2002;26:339-54.
[147] Riss TL, Moravec RA, Niles AL. Cytotoxicity testing: measuring viable cells, dead cells, and detecting mechanism of cell death. Methods in molecular biology (Clifton, NJ). 2011;740:103-14.

[148] Zhou HY, Shin EM, Guo LY, Youn UJ, Bae K, Kang SS, et al. Anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via NF-κB, JNK and p38 MAPK inactivation. European Journal of Pharmacology. 2008;586:340-9.
[149] Rosillo MA, Sánchez-Hidalgo M, Cárdeno A, Aparicio-Soto M, Sánchez-Fidalgo S, Villegas I, et al. Dietary supplementation of an ellagic acid-enriched pomegranate extract attenuates chronic colonic inflammation in rats. Pharmacological Research. 2012;66:235-42.
[150] Edmunds SJ. The effects of kiwifruit extracts on gene and protein expression levels in *in vitro* and *in vivo* mouse models of inflammatory bowel disease. Auckland, New Zealand: The University of Auckland; 2010.
[151] Schwerk J, Köster M, Hauser H, Rohde M, Fulde M, Hornef MW, et al. Generation of Mouse Small Intestinal Epithelial Cell Lines That Allow the Analysis of Specific Innate Immune Functions. PLoS ONE. 2013;8:e72700.
[152] Abud HE, Watson N, Heath JK. Growth of intestinal epithelium in organ culture is dependent on EGF signalling. Experimental Cell Research. 2005;303:252-62.
[153] Bareiss PM, Metzger M, Sohn K, Rupp S, Frick JS, Autenrieth IB, et al. Organotypical tissue cultures from adult murine colon as an in vitro model of intestinal mucosa. Histochemistry and Cell Biology. 2008;129:795-804.
[154] Barnett PGM, McNabb W, Cookson A, Zhu S, Davy M, Knoch B, et al. Changes in colon gene expression associated with increased colon inflammation in interleukin-10 gene-deficient mice inoculated with *Enterococcus* species. BMC Immunology. 2010;11.
[155] Sanchez-Muñoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. World Journal of Gastroenterology. 2008;14:4280-8.
[156] Madsen KL. Inflammatory bowel disease: lessons from the IL-10 gene-deficient mouse. Clinical and investigative medicine. 2001;24:250-7.
[157] Mishra KP, Padwad YS, Jain M, Karan D, Ganju L, Sawhney RC. Aqueous extract of *Rhodiola imbricata* rhizome stimulates proinflammatory mediators via phosphorylated IkappaB and transcription factor nuclear factor-kappaB. Immunopharmacol Immunotoxicol. 2006;28:201-12.
[158] Gupta A, Kumar R, Upadhyay NK, Pal K, Kumar R, Sawhney RC. Effects of *Rhodiola imbricata* on Dermal Wound Healing. Planta Med. 2007;73:774-7.
[159] Armstrong AM, Foulkes R, Jennings G, Gannon C, Kirk SJ, Gardiner KR. Tumour necrosis factor inhibitors reduce the acute-phase response in hapten-induced colitis. British Journal of Surgery. 2001;88:235-40.
[160] Loncar MB, Al-azzeh E-d, Sommer PSM, Marinovic M, Schmehl K, Kruschewski M, et al. Tumour necrosis factor α and nuclear factor κB inhibit transcription of human TFF3 encoding a gastrointestinal healing peptide. Gut. 2003;52:1297-303.
[161] Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A. Honey stimulates inflammatory cytokine production from monocytes. Cytokine. 2003;21:242-7.
[162] Plummeridge M, Armstrong L, Birchall M, Millar A. Reduced production of interleukin 12 by interferon γ primed alveolar macrophages from atopic asthmatic subjects. Thorax. 2000;55:842-7.
[163] Stranger BE, Stahl EA, Raj T. Progress and Promise of Genome-Wide Association Studies for Human Complex Trait Genetics. Genetics. 2011;187:367-83.
[164] Chavali SR, Weeks CE, Zhong WW, Forse RA. Increased production of TNF-α and decreased levels of dienoic eicosanoids, IL-6 and IL-10 in mice fed menhaden oil and juniper oil diets in response to an intraperitoneal lethal dose of LPS. Prostaglandins, Leukotrienes and Essential Fatty Acids. 1998;59:89-93.
[165] Schantz M, Mohn C, Baum M, Richling E. Antioxidative efficiency of an anthocyanin rich bilberry extract in the human colon tumor cell lines Caco-2 and HT-29. Journal of Berry Research. 2010;1:25-33.

[166] Gómez-Alonso S, Collins VJ, Vauzour D, Rodríguez-Mateos A, Corona G, Spencer JPE. Inhibition of colon adenocarcinoma cell proliferation by flavonols is linked to a G2/M cell cycle block and reduction in cyclin D1 expression. Food Chemistry. 2012;130:493-500.
[167] Sääf AM, Halbleib JM, Chen X, Yuen ST, Leung SY, Nelson WJ, et al. Parallels between Global Transcriptional Programs of Polarizing Caco-2 Intestinal Epithelial Cells In Vitro and Gene Expression Programs in Normal Colon and Colon Cancer. Molecular Biology of the Cell. 2007;18:4245-60.
[168] Lenaerts K, Mariman E, Bouwman F, Renes J. Differentiation stage-dependent preferred uptake of basolateral (systemic) glutamine into Caco-2 cells results in its accumulation in proteins with a role in cell–cell interaction. FEBS Journal. 2005;272:3350-64.
[169] Melillo de Magalhães P, Dupont I, Hendrickx A, Joly A, Raas T, Dessy S, et al. Anti-inflammatory effect and modulation of cytochrome P450 activities by *Artemisia annua* tea infusions in human intestinal Caco-2 cells. Food Chemistry. 2012;134:864-71.
[170] Romier B, Van De Walle J, During A, Larondelle Y, Schneider Y-J. Modulation of signalling nuclear factor-[kappa]B activation pathway by polyphenols in human intestinal Caco-2 cells. The British Journal of Nutrition. 2008;100:542-51.
[171] Kagoya Y, Yoshimi A, Kataoka K, Nakagawa M, Kumano K, Arai S, et al. Positive feedback between NF-kappaB and TNF-alpha promotes leukemia-initiating cell capacity. The Journal of Clinical Investigation. 2014;124:528-42.
[172] Chavali SR, Zhong WW, Forse RA. Dietary α-linolenic acid increases TNF-α, and decreases IL-6, IL-10 in response to LPS: effects of sesamin on the Δ-5 desaturation of ω6 and ω3 fatty acids in mice. Prostaglandins, Leukotrienes and Essential Fatty Acids. 1998;58:185-91.
[173] Pshezhetsky A, Fedjaev M, Ashmarina L, Mazur A, Budman L, Sinnett D, et al. Subcellular proteomics of cell differentiation: quantitative analysis of the plasma membrane proteome of Caco-2 cells. Proteomics. 2007;7:2201-15.
[174] Traka M, Gasper AV, Smith JA, Hawkey CJ, Bao Y, Mithen RF. Transcriptome Analysis of Human Colon Caco-2 Cells Exposed to Sulforaphane. The Journal of Nutrition. 2005;135:1865-72.
[175] Ishimoto H, Shibata M, Myojin Y, Ito H, Sugimoto Y, Tai A, et al. *In vivo* anti-inflammatory and antioxidant properties of ellagitannin metabolite Urolithin A. Bioorganic & Medicinal Chemistry Letters. 2011;21:5901-4.
[176] Larrosa M, Gonzalez-Sarrias A, Garcia-Conesa MT, Tomas-Barberan FA, Espin JC. Urolithins, ellagic acid-derived metabolites produced by human colonic microflora, exhibit estrogenic and antiestrogenic activities. Journal of Agricultural and Food Chemistry. 2006;54:1611-20.
[177] Cerdá B, Cerón JJ, Tomás-Barberán FA, Espín JC. Repeated Oral Administration of High Doses of the Pomegranate Ellagitannin Punicalagin to Rats for 37 Days Is Not Toxic. Journal of Agricultural and Food Chemistry. 2003;51:3493-501.
[178] Aiyer HS, Vadhanam MV, Stoyanova R, Caprio GD, Clapper ML, Gupta RC. Dietary berries and ellagic acid prevent oxidative DNA damage and modulate expression of DNA repair genes. International journal of molecular sciences. 2008;9:327-41.
[179] Bialonska D, Ramnani P, Kasimsetty SG, Muntha KR, Gibson GR, Ferreira D. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. International Journal of Food Microbiology. 2010;140:175-82.
[180] Crozier A, Del Rio D, Clifford MN. Bioavailability of dietary flavonoids and phenolic compounds. Molecular Aspects of Medicine. 2010;31:446-67.
[181] Gil-Izquierdo A, Fontana-Compiano LO, Morga-Egea JP, Pastor-Quirante FA. Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice. Molecular Nutrition & Food Research. 2010;54:311-22.

[182] Giménez-Bastida JA, González-Sarrías A, Larrosa M, Tomás-Barberán F, Espín JC, García-Conesa M-T. Ellagitannin metabolites, urolithin A glucuronide and its aglycone urolithin A, ameliorate TNF-α-induced inflammation and associated molecular markers in human aortic endothelial cells. Molecular Nutrition & Food Research. 2012;56:784-96.

Appendix

A.1 Additional Media Preparations

These media preparations were used in the preliminary and main study for murine primary cell culturing related to work in Chapter 4. The media preparations constituents are outlined in separate tables and if needed instructions on preparation are explained underneath.

Complete BRPMI
RPMI-1640
10% Fetal Bovine Serum
1% Penicillin/Streptomycin
10 ng/mL M-CSF

Complete IRPMI
RPMI-1640
10% Fetal Bovine Serum
1% Penicillin/Streptomycin
10 ng/mL of EGF

Assay IRPMI
RPMI-1640
1% Penicillin/Streptomycin

D-Sorbitol supplemented IRPMI
RPMI-1640
10% D-Sorbitol
1% Pen/Strep
10% FBS

Collagenase Stock Solution
25 mg Collagenase
2.5 mL HBSS

The ingredients were mixed together under sterile conditions and stored at -20°C until used.

Dispase Stock Solution
1 g Dispase II
100 mL PBS

The ingredients were mixed together under sterile conditions and stored at -20°C until used.

Low Collagenase/Dispase Enzyme Solution
15.65 mL HBSS
2 mL FBS
0.2 mL Pen/Strep
2 mL Dispase Stock Solution
0.15 mL Collagenase Stock Solution

The ingredients were mixed together under sterile conditions then sterilised with 0.22 µm single use filter units immediately before use. Used in preliminary study.

High Collagenase/Dispase Enzyme Solution
15.65 mL HBSS,
2 mL FBS
0.2 mL Pen/Strep
2 mL Dispase Stock Solution
0.3 mL Collagenase Stock Solution

The ingredients were mixed together under sterile conditions then sterilised with 0.22 µm single use filter units immediately before use. Used in main study.

Slanetz & Bartley Medium Plates

11.55 g Slanetz & Bartley medium (supplied by AgResearch sources)

275 mL dH_2O

13 sterile 100 mm diameter Petri dishes

These plates were prepared by AgResearch technician, Leigh Ryans with the following protocol: medium was microwaved, with gentle swirling every 30 seconds. The medium was removed when boiling and cooled. The medium was poured into Petri dishes, left to set within a fume hood, and then dried upside down in a 37°C incubator at least for 1 h.

Todd Hewitt Broth

1.8 g Todd Hewitt Broth (supplied by AgResearch sources)

60 mL dH_2O

12 polypropylene tubes

The powder was measured out into a 100 mL Schott bottle and water added. The suspension was then autoclaved for 15 min. The suspension was allowed to cool and a 5 mL aliquot of broth was added to each tube.

A.2 Additional Mouse Methods

Criteria for General Health Score, modified from Gill et al. (2001)

Score General Health Appearance and Clinical Signs

Score	Criteria
5	Bright eyed and alert, smooth coat with sheen, firm faeces, responds to stimulus, shows interest in its environment.
4	Fur ruffled, loss of sheen to coat, soft faeces or mild diarrhoea, mouse remains alert and active.
3	Fur noticeably ruffled, parts of coat form clumps, mouse not as alert or active, severe diarrhoea, loss of fur and damaged skin near anus, less interested in environment, signs of hyperventilating when handled.
2	Mouse hunched over and lethargic, little interest shown in environment, fur clumped.
1	Mouse non-reactive to stimulus, fur standing on end, mouse hunched over, preferring to sleep than react to environment, mouse cold to touch, paws cold to touch.

All animals in both studies maintained a GHS of 5 apart from those specifically mentioned in Chapter 4.

Animal Maintenance and Feeding

On feeding and weighing days mice were given fresh food. The cage was disassembled and mice removed for weighing, and any remaining food (left over) was weighed, recorded, and discarded. Fresh food was weighed and placed into the feeder and the cage reassembled. Food intake was estimated by the following calculation:

Food intake (g/day) = (food given - left over food) / number of days between feeding

Once a week the cages were replaced with clean ones, with new water bottles that had fresh water, new bedding material and fresh tissues (for making nests with). This process was completed by AgResearch Animal Technician Stacey Burton.

Animals were given rings to provide stimulation.

A.3 Urolithins

To broaden the scope of this research, the end metabolites of ET metabolism, Uro A and Uro B, were included in the study. These metabolites (as discussed in 1.4 Plant Polyphenols) have been shown to possess multiple putative effects such as anti-inflammatory and antioxidant, in multiple *in vitro* and *in vivo* models [14, 43, 58, 60, 67, 73, 113, 175, 176, 178-182]. These compounds were not in the primary aim of the thesis and were only added after research began, and they provided additional information about the potential of *Rubus* fruit extracts. Therefore the methods, results and discussion for experimental work regarding these compounds are discussed in the Appendix.

A.3.1 Urolithin Synthesis

Uro A and Uro B were synthesised by Lesley Larsen, at Plant & Food Research, Invermay, Dunedin. The compounds were sent to the Ruakura campus in powder form and stored at -20°C to await experimentation.

The amount of 10 µM was chosen as the final exposure level for all NO and cytokine assays, based on a summary of previous work using these compounds [66, 67, 73, 113, 179, 182].

A.3.2 RAW 264.7 macrophages

Uro A and B were unavailable in significant quantity at the start of experimentation and therefore only cytokine analysis of these compounds on RAW 264.7 macrophages was conducted.

The methods for growth and application to the cells were the same as described in Section 3.2.8. The preparation of the Uro samples occurred at the same time as the cultivar samples. Uro A and B were first dissolved in DMSO and then prepared in culture media at a final concentration of 10 µM with a DMSO concentration of 0.05%. The sample preparation was passed through a 0.22 µm CA filter prior to application (same as *Rubus* fruit extracts) to cells.

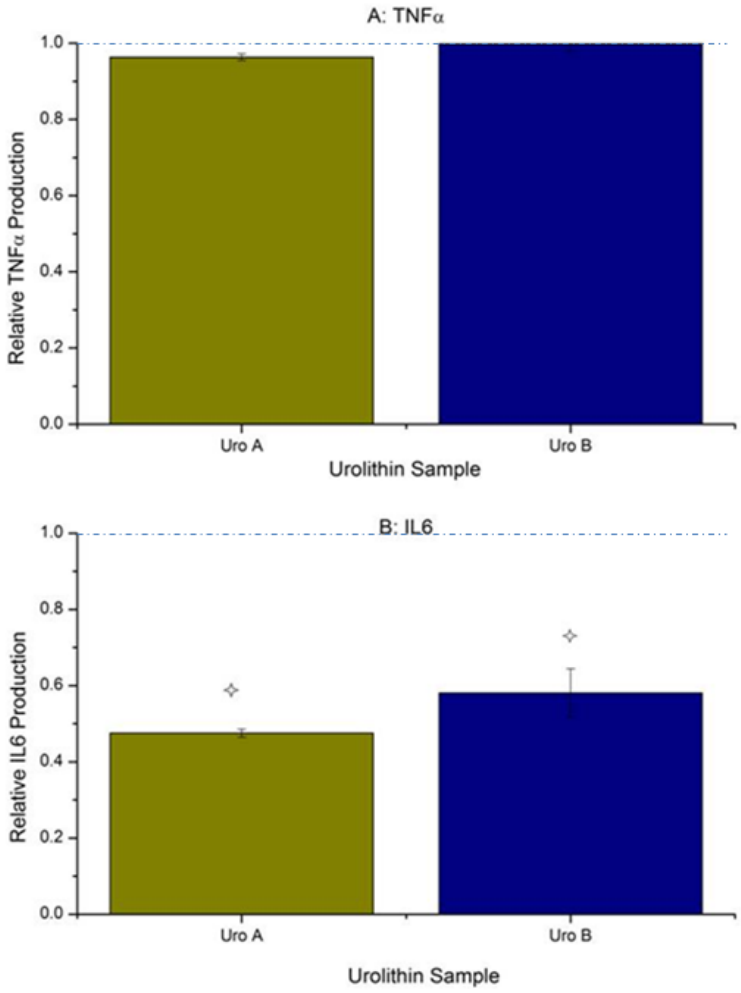

Figure 53: RAW 264.7 macrophages were co-incubated with the 10 µM Uro A and B with 0.05% DMSO, and LPS-stimulation (0.05 µg/mL). Results show relative production of cytokines by RAW 264.7 macrophages to DMSO inclusive control for Uro samples. Each bar represents the standard deviation for three technical replicates. The horizontal dotted line represents the relative cytokine production for control RAW 264.7 macrophages. ✧ indicates a significant change in viability of colonic IEC relative to control ($P < 0.05$).

The results from this assay were not congruent to those results produced by the *Rubus* fruit extracts. There was no change relative to the DMSO control for TNFα, and there was a decrease of roughly 50% in the production of IL6 observed for both Uro A and Uro B. Suggesting an anti-inflammatory effect is induced by both compounds, whereas with the *Rubus* fruit extracts only WETx had indications of inducing a decrease in IL6 production (although there was an associated rise in TNFα for this extract).

A.3.3 Primary Murine Cell Study

A greater amount of Uro A and B was made available for the main study (not tested in the preliminary study). Therefore cytotoxicity, NO and cytokine assessment was conducted at this stage of research.

As per the methods outlined in Section 4.2.4, both Uro samples were prepared and treated in the same manner but compared to a DMSO inclusive control. The concentrations tested for the cytotoxicity testing were 20, 10, 5, 2.5, and 1.25 µM. For the NO and cytokine assays the concentration tested was the same as for the RAW 264.7 macrophages of 10 µM. All samples had a final concentration of 0.05% DMSO (including controls for comparison).

A.3.3.1 Cytotoxicity

As with the *Rubus* fruit extracts, Uro A and B were prepared with the appropriate medium and filtered prior to co-incubation with the cells as per Section 4.2.4.4. The results are presented in Figure 54 – Figure 57. The results were inconclusive as there was inconsistency between biological replicates and lack of technical replication.

For colonic IEC isolated from group 1 C57 mice there was one technical replicate which did not allow for statistical interpretation (Figure 54). Group 2 colonic IEC indicated no change relative to control IEC exposed to DMSO and control medium only (control IEC) (Figure 55). Colonic IEC isolated from *Il10*$^{-/-}$ mice showed a positive association at Uro concentration greater than 10 µM for Uro A and greater than 5 µM for Uro B ($P < 0.05$) (Figure 54). There were no significant changes observed relative to control IEC, for group 2 colonic IEC (Figure 55).

For BMDM isolated from group 1 *Il10*$^{-/-}$ mice there was a decrease in viability for both Uro relative to control BMDM exposed to control medium and DMSO (control BMDM) (Figure 56). For group 2 there was only one technical replicate, which did not allow for statistical interpretation (Figure 57). BMDM isolated from group 1 C57 mice showed a decrease in viability for concentrations of Uro above 1.25 µM ($P < 0.05$) (Figure 56). BMDM isolated from group 2 C57 mice only had one technical replicate, which did not allow for statistical interpretation (Figure 57).

Results suggest that it was unlikely that Uro had a negative impact upon viability of the cells (apart from BMDM from group 1 C57 mice).

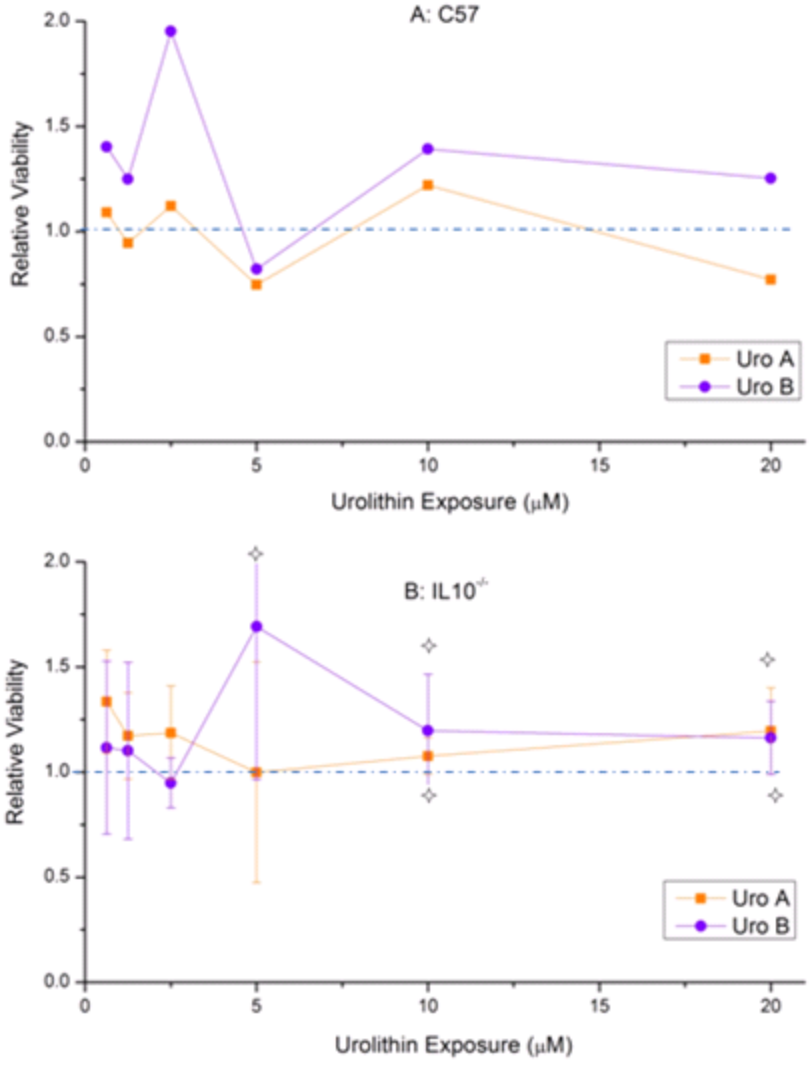

Figure 54: Relative viability results for colonic IEC co-incubated with Uro A and Uro B, from group 1 C57 and *Il10*$^{-/-}$ mice, relative to DMSO control. A: C57 and B: IL10$^{-/-}$. There was one technical replicate for C57 mice and four technical replicates for *Il10*$^{-/-}$ mice. Each bar represents the standard deviation for four technical replicates. The horizontal dotted line represents viability of control IEC. ✧ indicates a significant change in viability of colonic IEC relative to control ($P < 0.05$).

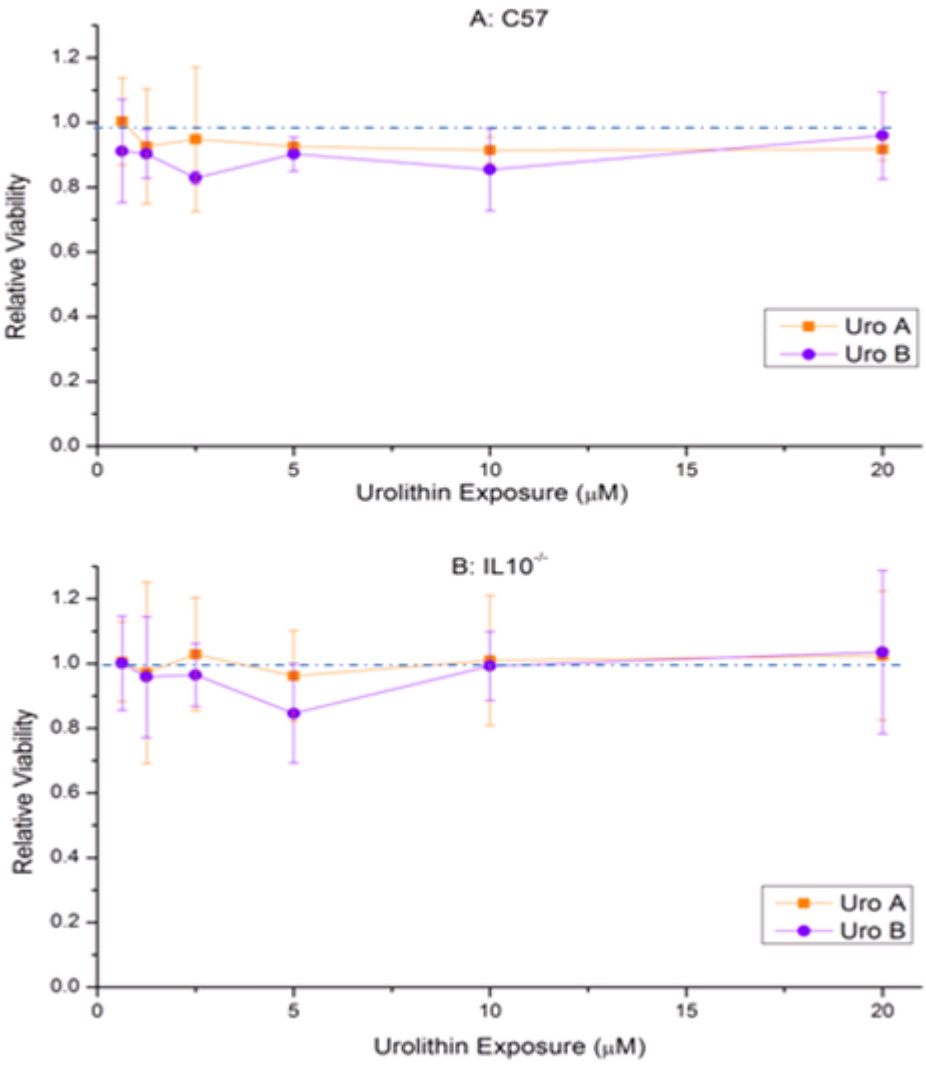

Figure 55: Relative viability results for colonic IEC co-incubated with Uro A and Uro B, for group 2 C57 and *Il10*$^{-/-}$ mice, relative to DMSO control. A: C57 and B: IL10$^{-/-}$. Two technical replicates were isolated for each genotype. Each bar represents the standard deviation of two technical replicates. The horizontal dotted line represents viability of control IEC.

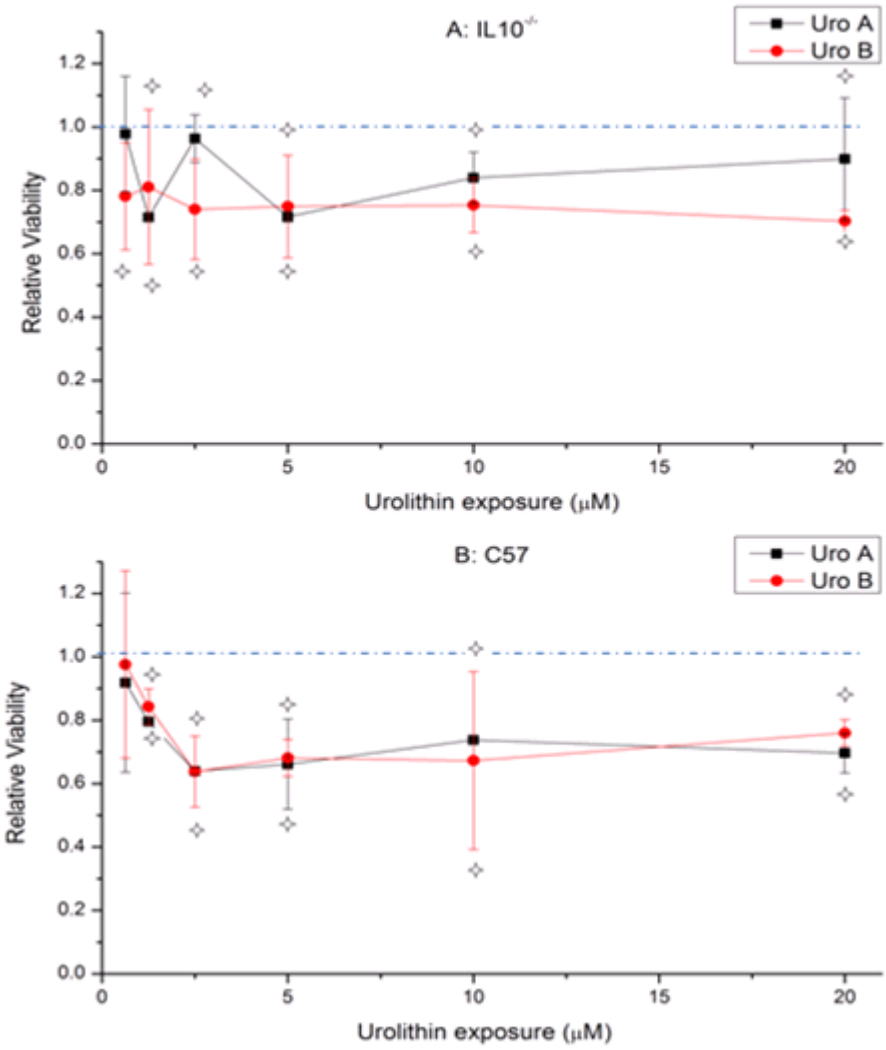

Figure 56: Relative viability results for BMDM co-incubated with Uro A and Uro B, for group 1 C57 and *Il10*$^{-/-}$ mice, relative to DMSO control. A: *Il10*$^{-/-}$ mice, B: C57 mice. There were two technical replicates for both genotypes. Each bar represents the standard deviation for two technical replicates. The horizontal dotted line represents viability of control BMDM. ✧ indicates a significant change in viability of colonic IEC relative to control ($P < 0.05$).

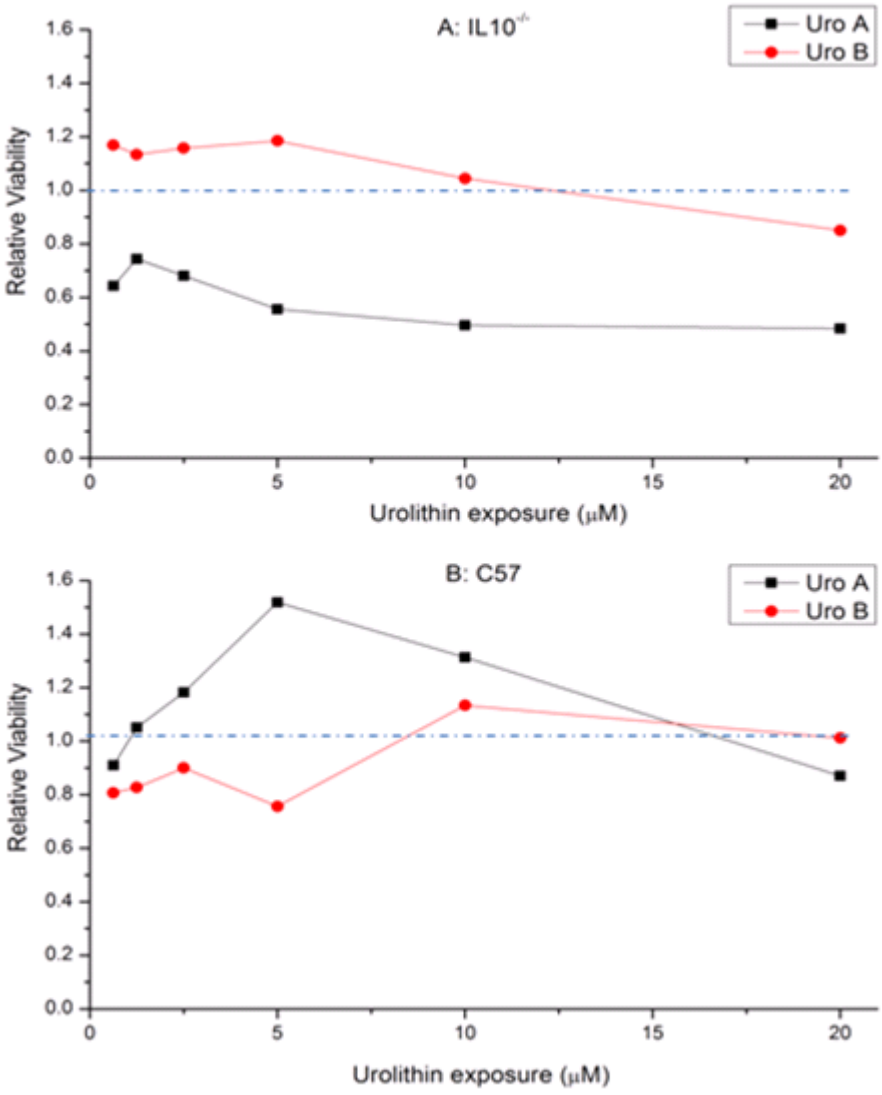

Figure 57: Relative viability results for BMDM co-incubated with Uro A and Uro B, for group 2 C57 and *Il10*$^{-/-}$ mice, relative to DMSO control. A: *Il10*$^{-/-}$ mice, B: C57 mice. There was one technical replicate for both genotypes. The horizontal dotted line represents viability of control BMDM.

A.3.3.2 Griess Assay

The Griess assay was used to measure changes in NO production as a result of co-incubation with Uro A and B. BMDM isolated from both genotypes and groups of mice were treated as per outlined in Section 4.2.4.5. Uro A and B were co-incubated with the cells at 10 µM with 0.05% DMSO final concentration and 0.5 µg/mL LPS, and compared to a 0.05% DMSO control.

The results in Figure 58 and Figure 59 indicate a difference in the responses observed between the two groups to Uro A and B (which is dissimilar to that seen with the *Rubus* fruit extracts in Chapter 4). However due to the lack of technical replicates, conclusive interpretations were not possible. There are indications that there is a decrease NO production for both Uro, with a greater decrease induced by co-incubation with Uro A.

BMDM isolated from group 1 C57 mice, indicated a decrease in NO production with Uro A but not change with Uro B. There was a lack of NO detected to be produced by C57 BMDM isolated from group 2 mice. It is unclear why this may have occurred, as there were no recorded errors with experimentation. For BMDM isolated from group 1 *Il10*$^{-/-}$ mice there were indications of a decrease in NO production for Uro A and a 10% increase for Uro B. There was no noticeable change relative to the control BMDM in the production of NO for BMDM isolated from group 2 *Il10*$^{-/-}$ mice.

Due to the lack of NO detected by group 2 C57 and lack of technical replicates, it is had to make a conclusion about the effect Uro A and B had on NO production. There are indications that Uro A had a greater impact upon decreasing NO production than Uro B. The responses observed with this assay are anti-inflammatory as a result of co-incubation with Uro.

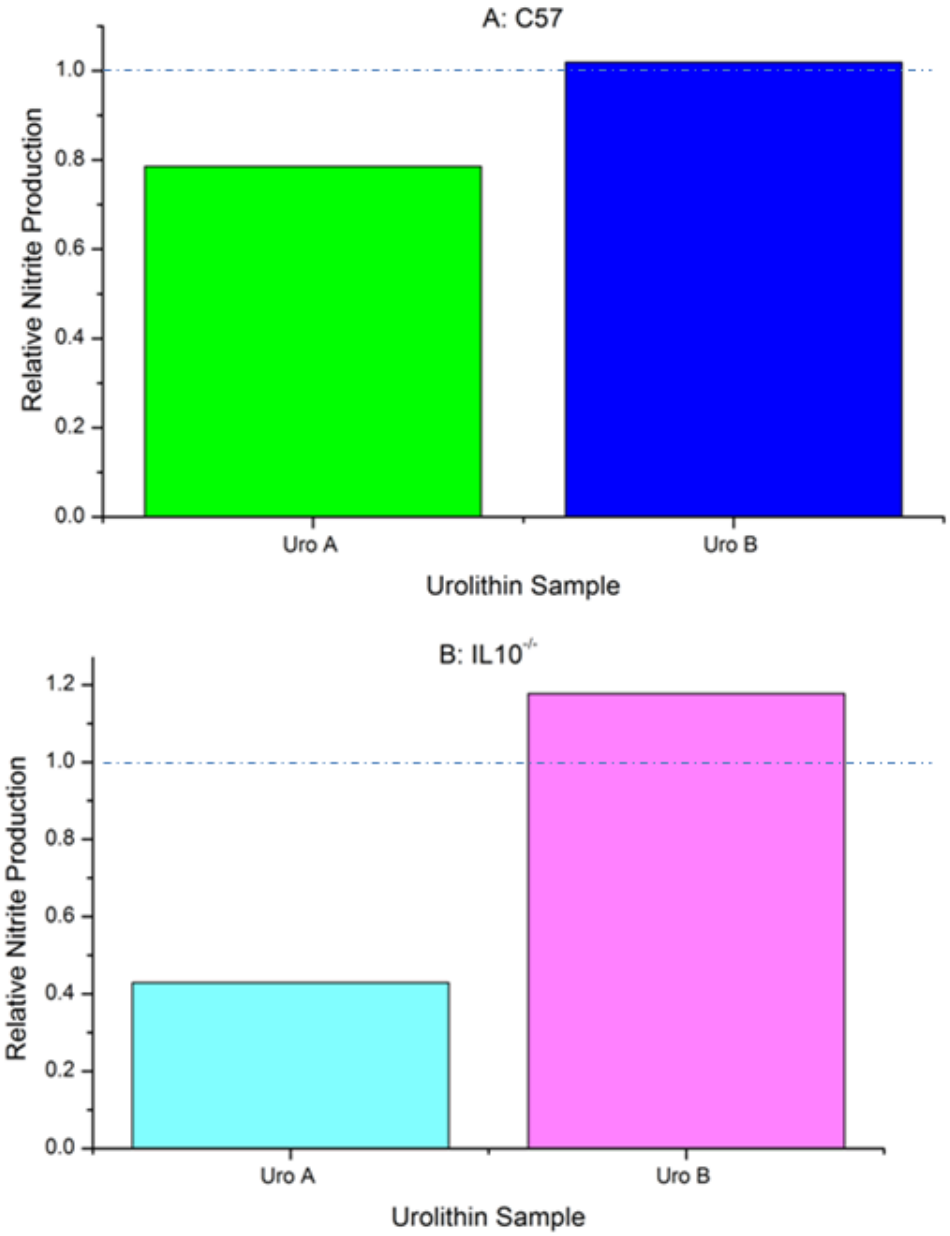

Figure 58: Relative production of NO by BMDM isolated from group 1 C57 and *Il10*$^{-/-}$ mice, relative to DMSO control. A: C57 and B: IL10$^{-/-}$. There was one technical replicate isolated for both genotypes. The horizontal dotted line represents the production of NO by control BMDM.

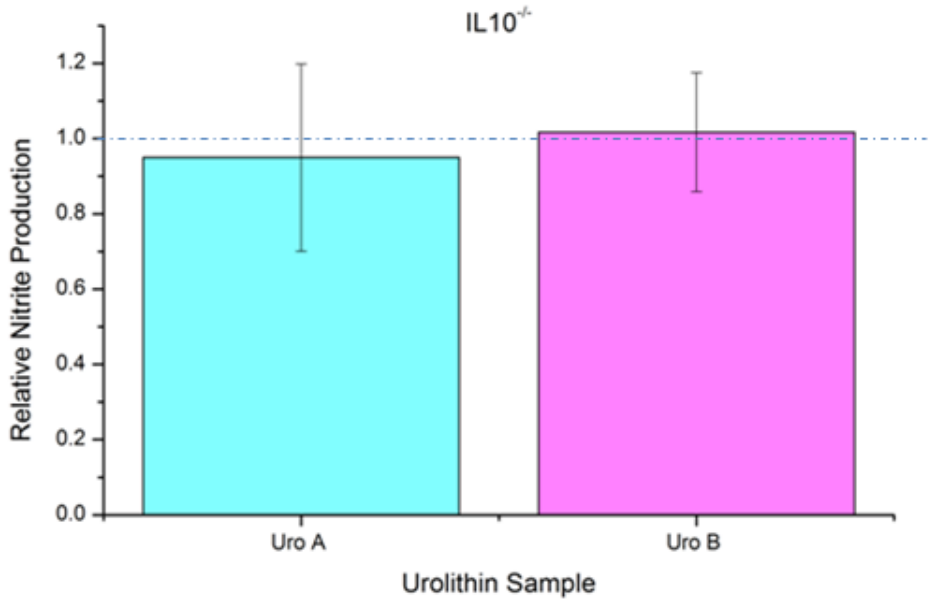

Figure 59: Relative production of NO produced by BMDM isolated from group 2 *Il10*$^{-/-}$ mice, relative to DMSO control. Each bar represents the standard deviation of two technical replicates. NO was not detected for C57 mice. The horizontal dotted line represents the production of NO by control BMDM.

A.3.3.3 Cytokine Assays and Conclusion

Cytokine assessment for colonic IEC and BMDM were conducted as outlined in the methods Section 4.2.4.6 and Section 4.2.4.7. As with the Griess assay Uro A and B were co-incubated with the cells at 10 µM 0.05% DMSO final concentration and compared to a DMSO 0.05% control. To simtulate cytokine production 0.5 µg/mL LPS was co-incubated with the cells. As with the main study, there is a lack of replication of results and therefore conclusions as to the effects induced by the Uro are speculative.

Figure 60 and Figure 61 show the actual production of IL6 by colonic IEC isolated from the two genotypes and groups of mice. As with the *Rubus* fruit extracts, there was a significant (two to ten-fold) difference between the two groups in their level of IL6 production. The reasons for this difference are unkonwn. The production levels from group 1 *Il10*$^{-/-}$ mice show no significant change in production relative to the controls (with or with LPS-stimulation). There

was only one technical replicate for group 1 C57 colonic IEC, which did not allow for statistical interpretation. IL6 production for colonic IEC from group 2 *Il10*$^{-/-}$ and C57 mice show no significant change relative to the positive control for Uro A but a significant decrease for Uro B for colonic IEC from *Il10*$^{-/-}$ mice. For both genotypes in group 2 there was a significant increase relative to the negative control ($P < 0.05$).

Cytokines co-incubated with Uro from BMDM were different to those produced by the *Rubus* fruit extracts. For C57 mice there was only one technical replicate isolated for both groups, which did not allow for statistical interpretation (Figure 62 and Figure 63). For BMDM isolated group 1 from *Il10*$^{-/-}$ mice, there was no significant change relative to control BMDM for IL16 and IL12, and a significant decrease in TNFα production (Figure 64). For group 2 BMDM from *Il10*$^{-/-}$ mice there was a significant decrease in production for all cytokines examined (Figure 65).

Results for colonic IEC are plagued by the same issues as those for *Rubus* fruit extracts and it is difficult to extrapoliate the effects the Uro are havingon the cells. The BMDM cytokine profile demonstrates an anti-inflammatory response is induced with co-incubation with Uro.

Overall conclusions are that Uro are likely to produce a decrease in inflammatory markers. Generally cytokine production was decreased for both Uro A and B, relative to controls. NO production saw a decrease with Uro A. As with the *Rubus* fruit extracts, viability testing showed dissimilar results between biological replicates and due to lack of technical replicates conclusive statements are unable to be made.

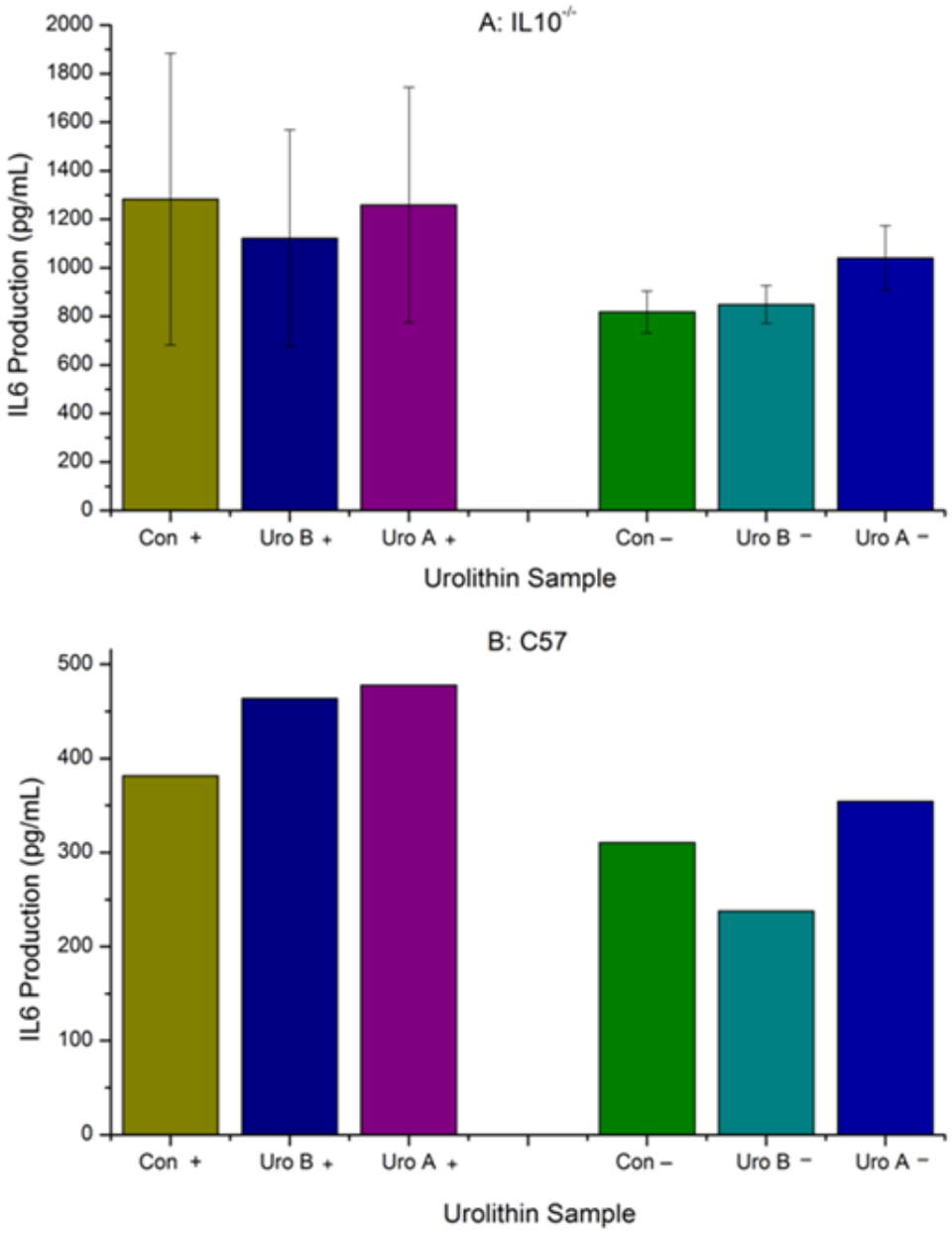

Figure 60: Production of IL6 from group 1 colonic IECs isolated from A: *Il10*$^{-/-}$ and B: C57 mice. There was one technical replicate for C57 mice and three technical replicates for *Il10*$^{-/-}$ mice. Each bar represents the standard deviation of three technical replicates.

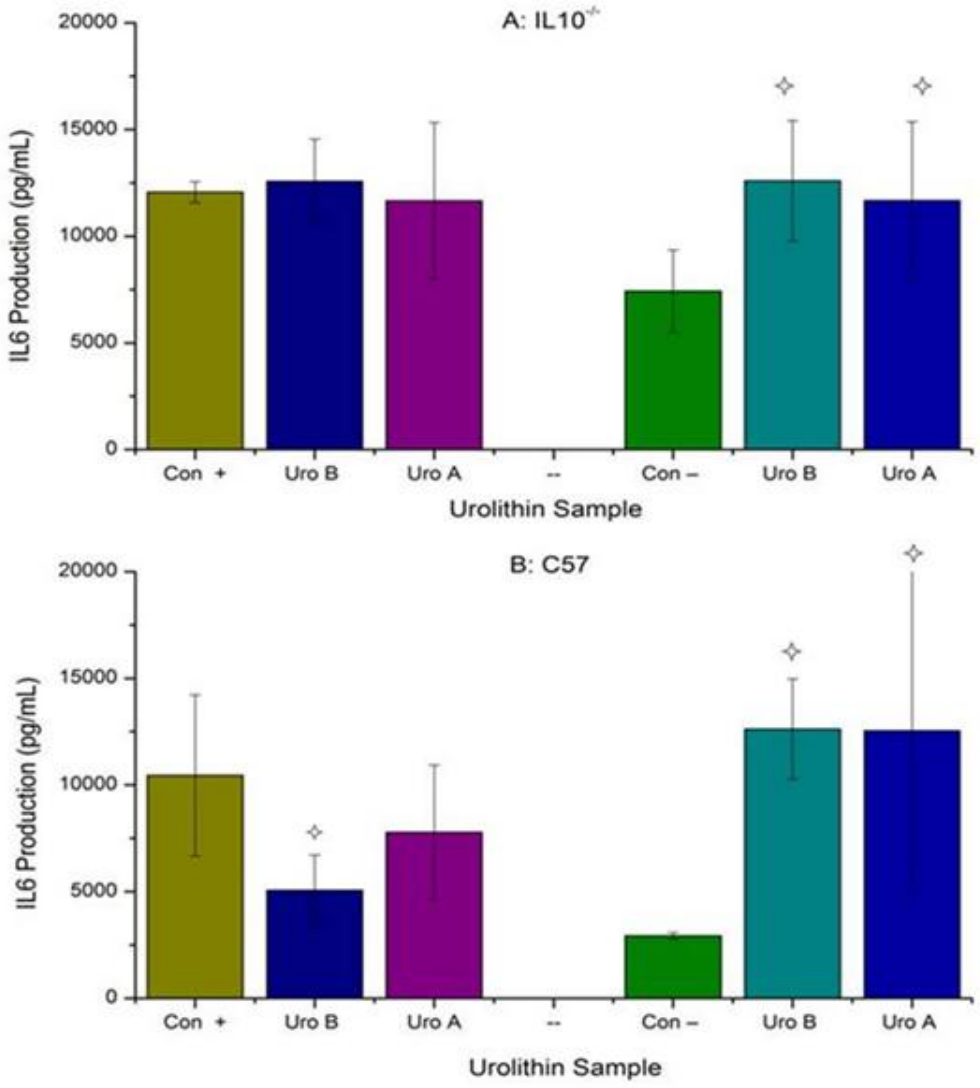

Figure 61: IL6 production from group 2 colonic IEC isolated from A: *Il10*$^{-/-}$ and B: C57 mice. There were three technical replicates for both genotypes. Each bar represents the standard deviation of three technical replicates. ✧ indicates a significant change in IL6 production of colonic IEC relative to control ($P < 0.05$).

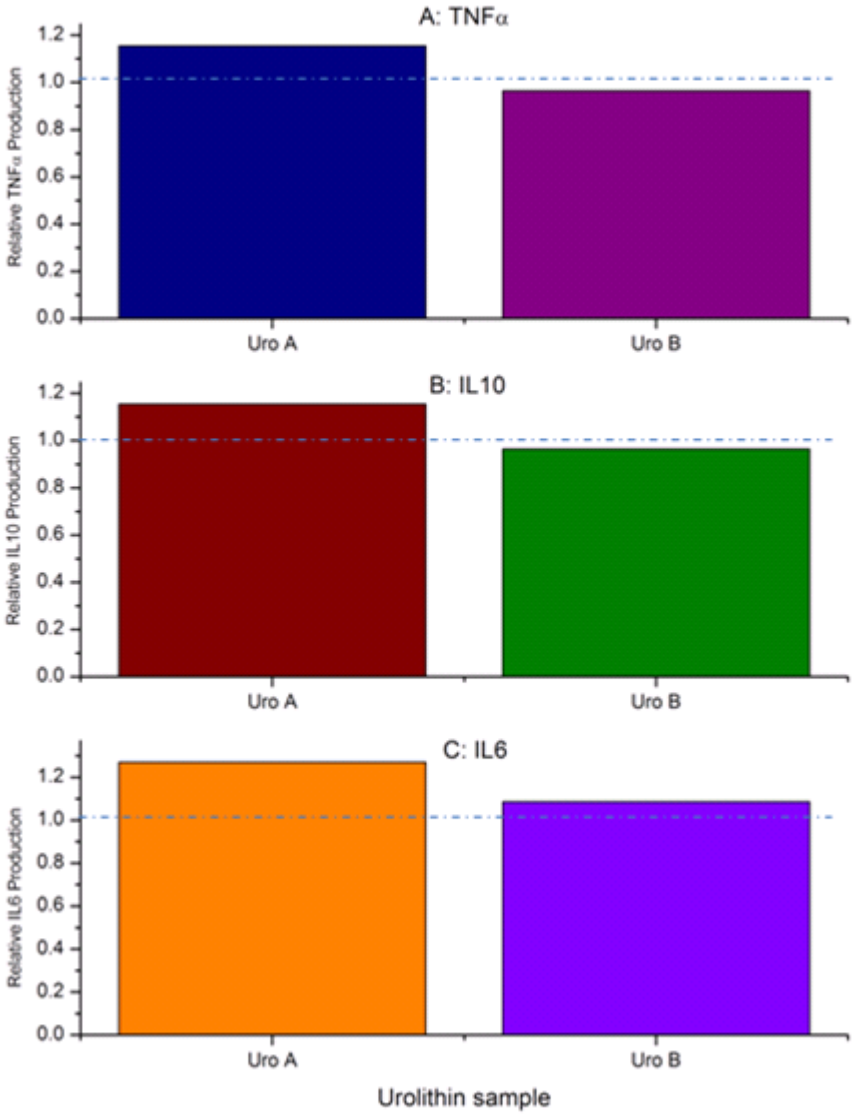

Figure 62: BMDM isolated from group 1 C57 mice, cytokine production relative to the DMSO control. A: TNFα, B: IL10 and C: IL6. The experiment consisted of pooled cells from five C57 with one technical replicate produced. The horizontal dotted line represents viability of control BMDM.

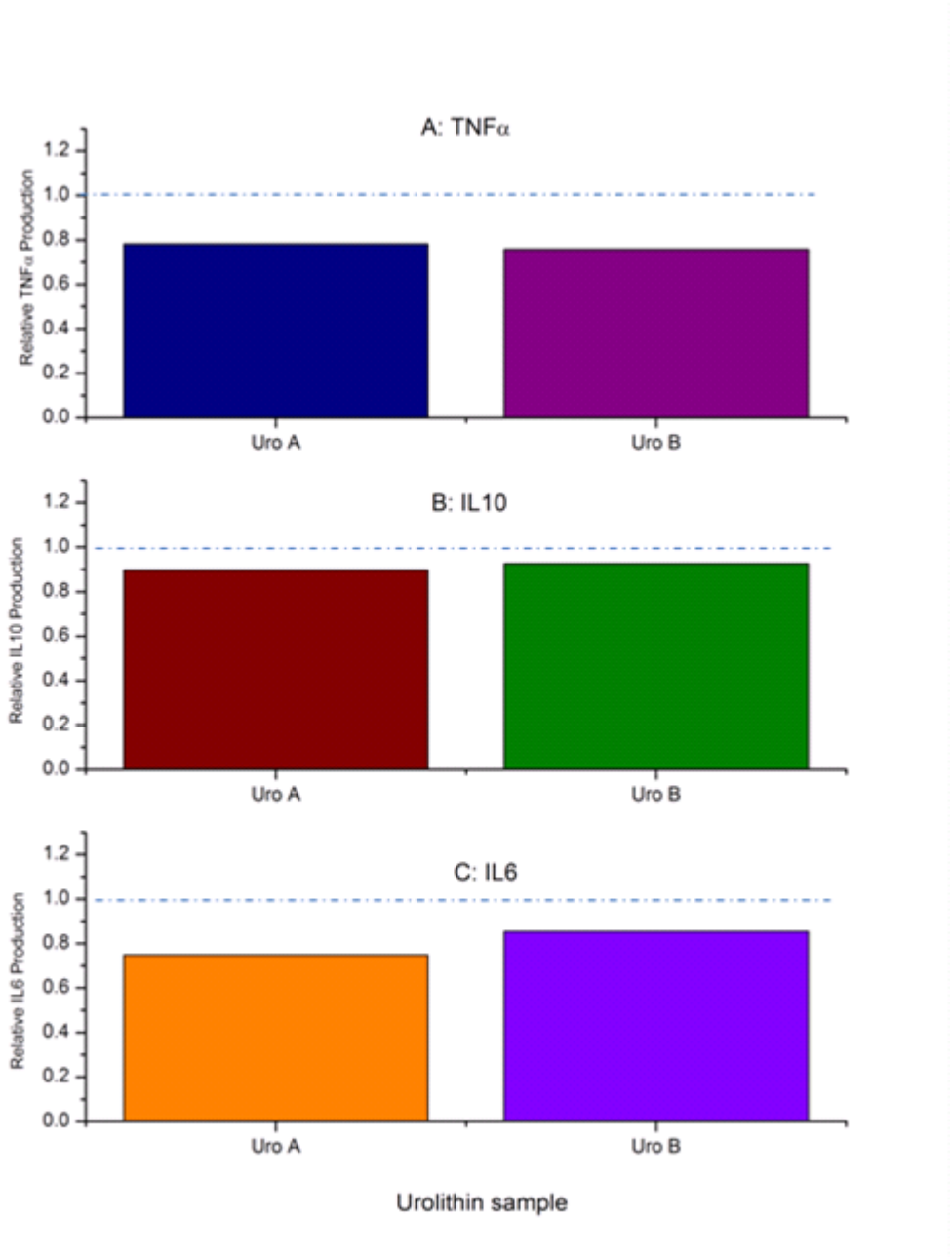

Figure 63: Group 2 results for C57 BMDM cytokine production relative to control. A: TNFα, B: IL10 and C: IL6. The experiment consisted of pooled cells from five C57 with one technical replicate produced. The horizontal dotted line represents viability of control BMDM.

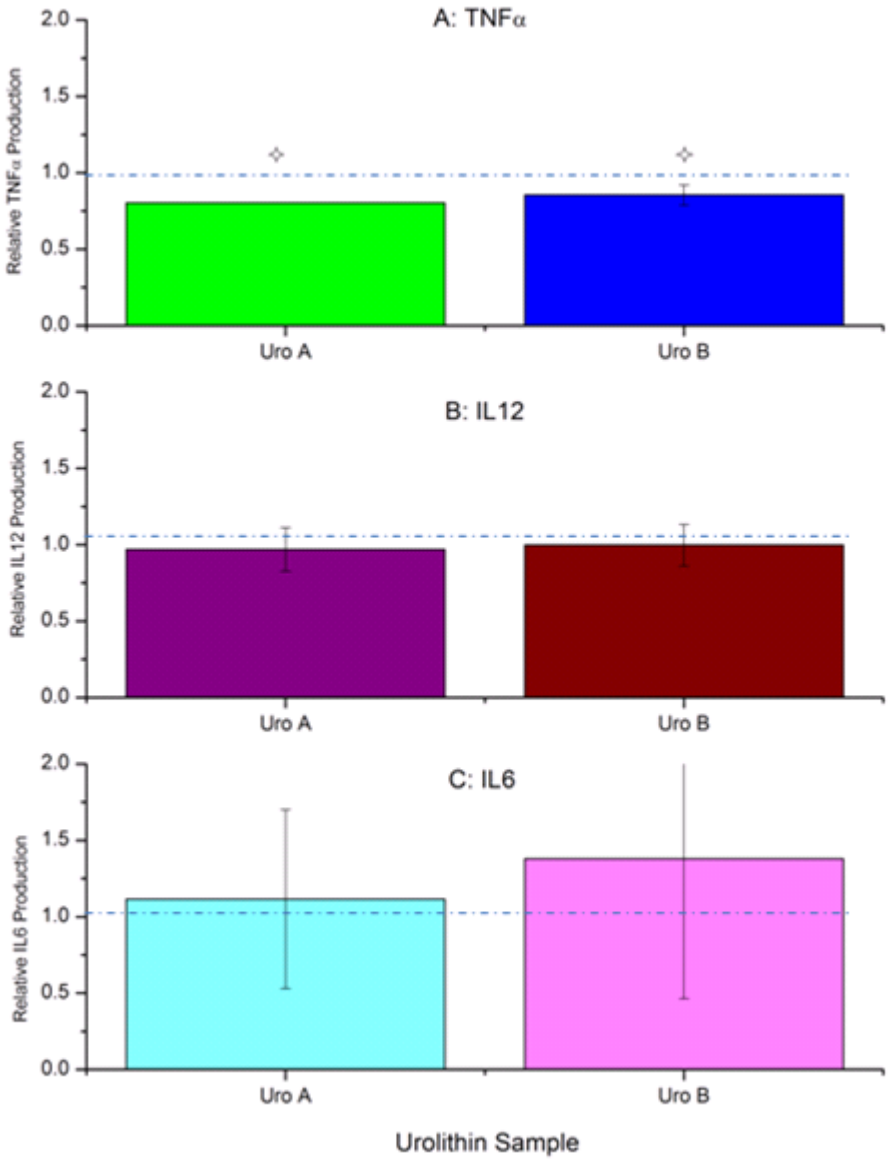

Figure 64: Cytokine production relative to control for BMDM isolated from group 1 *Il10*$^{-/-}$ mice. A: TNFα, B: IL12 and C: IL6. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice. Each bar represents the standard deviation of two technical replicates. The horizontal dotted line represents viability of control BMDM. ✧ indicates a significant change in cytokine production relative to control ($P < 0.05$).

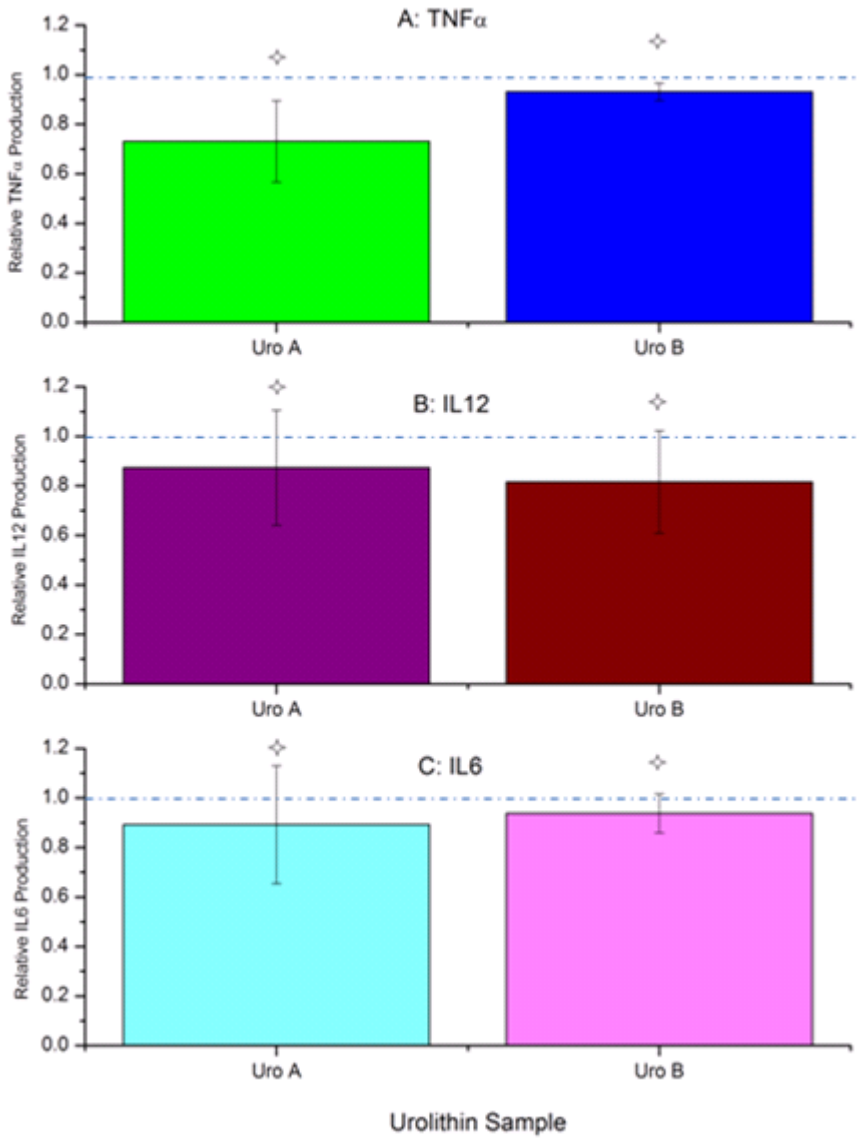

Figure 65: Cytokine production relative to control for BMDM isolated from group 2 *Il10*$^{-/-}$ mice. A: TNFα, B: IL12 and C: IL6. The experiment consisted of pooled cells from five *Il10*$^{-/-}$ mice. Each bar represents the standard deviation of two technical replicates. The horizontal dotted line represents viability of control BMDM. ✧ indicates a significant change in cytokine production relative to control ($P < 0.05$).

A.4 Declaration confirming content of digital version of thesis

I confirm that the content of the digital version of this thesis

ANALYSIS OF THE POTENTIAL FOR *RUBUS* FRUIT ELLAGITANNINS TO INDUCE ANTI-INFLAMMATORY EFFECTS IN *IN VITRO* MODELS.

is the final amended version following the examination process and is identical to this hard-bound paper copy.

Have you published articles/material from your thesis Yes / No

Alana Jocelyn Srubar-Vernon

Signature

Date 31.03.14