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**„Immunomodulatory effects of anthocyanin-rich berry extracts
in tumorigenic and non-tumorigenic human colon cells
and NF- κ B pathway interactions of SN38 in THP-1 monocytes
in vitro“**

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III. Index of Abbreviations

ADI	acceptable daily intake
ATP	adenosine triphosphate
BW	bodyweight
BB	blackberry
BC	black currant
Bil	bilberry
CA	citric acid
cDNA	complementary DNA
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CRD	cysteine-rich domains
CS	citrate synthase
CSF	colony stimulating factors
C _T	threshold cycle
CTB	CellTiter-Blue® Cell Viability Assay
CTLA4	cytotoxic T-lymphocyte-associated antigen 4
CVD	cardiovascular diseases
CYP	cytochrome p450 monooxygenase
Cy-3-glc	cyanidin 3-glucoside
CXCL8	chemokine IL-8
DC	dendritic cells
DD	death domain
Del-3-glc	delphinidin 3-glucoside
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	elderberry
EFSA	European Food Safety Authority
EGC	epigallocatechin

EPIC-study	European prospective investigation into cancer and nutrition
EPO	erythropoietin
EU	european union
FA	formic acid
FBS	fetal bovine serum
FDA	Foundation US Food & Drug Administration
FWF	Austrian Science Fund (German: Fonds zur Förderung der wissenschaftlichen Forschung)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GH	growth hormone
GIT	gastro intestinal tract
GLUT	glucose-associated transporters
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
HMBS	hydroxymethylbilane synthase
HPLC	high performance liquid chromatography
HPRT1	hypoxanthine-guanine phosphoribosyltransferase
HKG	housekeeping gene
IBDs	Intestinal bowel diseases
IFN	interferon
IGDs	immunoglobulin domains
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
iNOS	inducible nitric oxide synthase
IL	interleukin
ILR	interleukin receptor
JAK	janus kinase

JNK	c-Jun N-terminal kinases
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	limit of detection
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MFI	median fluorescence intensity
MS	mass spectrometry
NHANES	National health and nutrition survey study
NF- κ B	nuclear factor kappa-B
NK	natural killer cells
Nrf2	nuclear factor erythroid 2–related factor 2
NOX	NADPH oxidase
OAA	acetyl-CoA and oxaloacetate
PBS	phosphate-buffered saline
PCR	polymerases chain reaction
PI3K	phosphoinositide 3-kinase
PD-L1	programmed cell death protein ligand 1
PenStrep	penicillin-streptomycin
POLR2A	polymerase II subunit A
qRT-PCR	quantitative real-time PCR
RelA	v-rel avian reticuloendotheliosis viral oncogene homolog A
RNA	ribonucleic acid
ROS	reactive oxygen species
SCF	Scientific Committee on Food
SD	standard deviation
STAT	signal transducer and activator of transcription
SRB	sulforhodamine B

TCA	tricarboxylic acid
T/C	test over control
Th2	T helper 2 cells
TFRC	transferrin receptor
TIR	the Toll/IL-1R
TPO	thyreoperoxidase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Treg	regulatory T cells
WST-1	water-soluble tetrazolium salt 1

1. Introduction

Anthocyanins are colouring plant substances that are subject of frequent discussion regarding their potential protection against several chronic and degenerative diseases such as cardiovascular disease, diabetes and cancer, mainly gastrointestinal cancer.¹ Research on bioavailability, mechanisms of action, effectiveness, possible toxicity and potential interactions with drugs or other bioactive compounds is needed in order to be able to utilize these promising pigments to the full capacity.

After a brief overview of anthocyanins in general, the theoretical part focuses on their prevalence, bioavailability and effects, while going into more detail on their potential effects on the immune system with special focus on inflammation and also potential interactions with the chemotherapeutic irinotecan. Therefore, the intestinal immune system is also discussed, with focus on the main immunomodulatory markers investigated in this thesis, in order to give a base to the experimentally obtained data and the discussion thereof following. Additionally, a brief overview is given on the chemotherapeutic irinotecan and citric acid regarding the effects on cancer.

1.1. Anthocyanins

The name anthocyanin derives from the Greek “anthos” meaning flower, and “kyanos” translating to blue. Anthocyanins are water-soluble vacuolar pigments in plants and belong to the large group of polyphenols. They represent a subclass of flavonoids, consisting of phenolic rings with differing side chains and are a widespread group of secondary plant metabolites. Anthocyanins are responsible for blue, purple and red colouring in plants and occur mainly in berries, flowers and fruit, although they are also present in many vegetables, legumes, roots and cereals. Anthocyanins have a strong impact on the sensory properties of food, as they are responsible for characteristic colours.^{2, 3} In the plants themselves, they play a key role for pollination and protection from cold, stress and UV ray-induced damage by absorbing light.⁴ Anthocyanins are bioactive compounds and several health benefits have been described and linked to their consumption. These health benefits include prevention from cardiovascular diseases (CVD), anti-angiogenesis, anti-obesity, anticancer and anti-diabetic effects, improved visual health, antimicrobial activity, and neuroprotection (summarized i.e. by Khoo *et al.*

2017).¹ Those beneficial effects have mainly been attributed to their free-radical scavenging capacity and to their action on a number of enzymes such as cyclooxygenase, and on inflammatory cytokine signalling.⁵ Despite these and other anthocyanin-associated health effects speculated on, they remain yet to be fully understood, because the bioavailability of anthocyanins and other polyphenols appears to be relatively limited. Described below are the chemical structure, exposure, stability, digestion and later some of the most common health benefits attributed to anthocyanins, mainly focussing on inflammation (in chapter 1.2.5).

1.1.1. Chemical structure

Anthocyanins are glycosides of anthocyanidins (aglycons), polymethoxy and polyhydroxy derivatives of 2-phenylbenzopyrylium or flavylium salts. The anthocyanidins consist of an aromatic ring [A] fused to an heterocyclic ring [C] that contains oxygen, which is also bound to a third aromatic ring [B] by a carbon–carbon bond (Figure 1).^{2, 6}

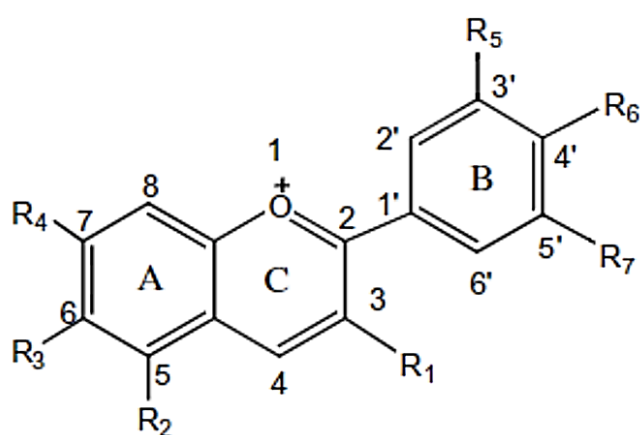


Figure 1: Structural formula of anthocyanidins (Castaneda-Ovando, et al. 2009).⁶

There is a huge variety of anthocyanins found in nature with some examples of common anthocyanins listed in Table 1. The main differences between their chemical structures are the number of hydroxyl groups, the number and nature of bound sugars to their structure, the aliphatic or aromatic carboxylates bound to the sugar and the position of these bonds.^{3, 6} According to Kong and colleagues, the most common anthocyanins in plants for human consumption are cyanidin-based with 50 %, followed by delphinidin, peonidin, pelargonidin, petunidin and malvidin.³

Table 1: Structural identification of anthocyanidins, associated abbreviations and colours. (Modified from Castaneda-Ovando et al. 2009⁶ and Kong et al. 2003³)

Name:	Abbreviations:	Substitution pattern:							Colour:
		R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	Orange
Arrabidin	Ab	H	H	OH	OH	H	OH	OMe	N.R. ^a
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Red-blue
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Red-blue
6-HydroxyCy	6OHCy	OH	OH	OH	OH	OH	OH	OH	Red
6-HydroxyDp	6OHDp	OH	OH	OH	OH	OH	OH	OH	Red-blue
6-HydroxyPg	6OHPg	OH	OH	OH	OH	H	OH	H	N.R. ^a
Luteolin	Lt	H	OH	H	OH	OH	OH	H	Orange
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Red-blue
5-MethylCy	5-MCy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	Orange
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Red-blue
Rosinidin	Rs	OH	OH	H	OMe	OMe	OH	H	Red

^a N.R: not reported.

Glycosylation of anthocyanidins to form the individual anthocyanins can occur on different hydroxyl moieties of the molecule, 3-OH being the most widespread glycosylation site in nature to produce 3-O- β -glucosides. Depicted in Figure 2 are some of the most commonly naturally occurring monomeric anthocyanins, the glycosides of cyanidin, delphinidin, malvidin and pelargonidin.⁵

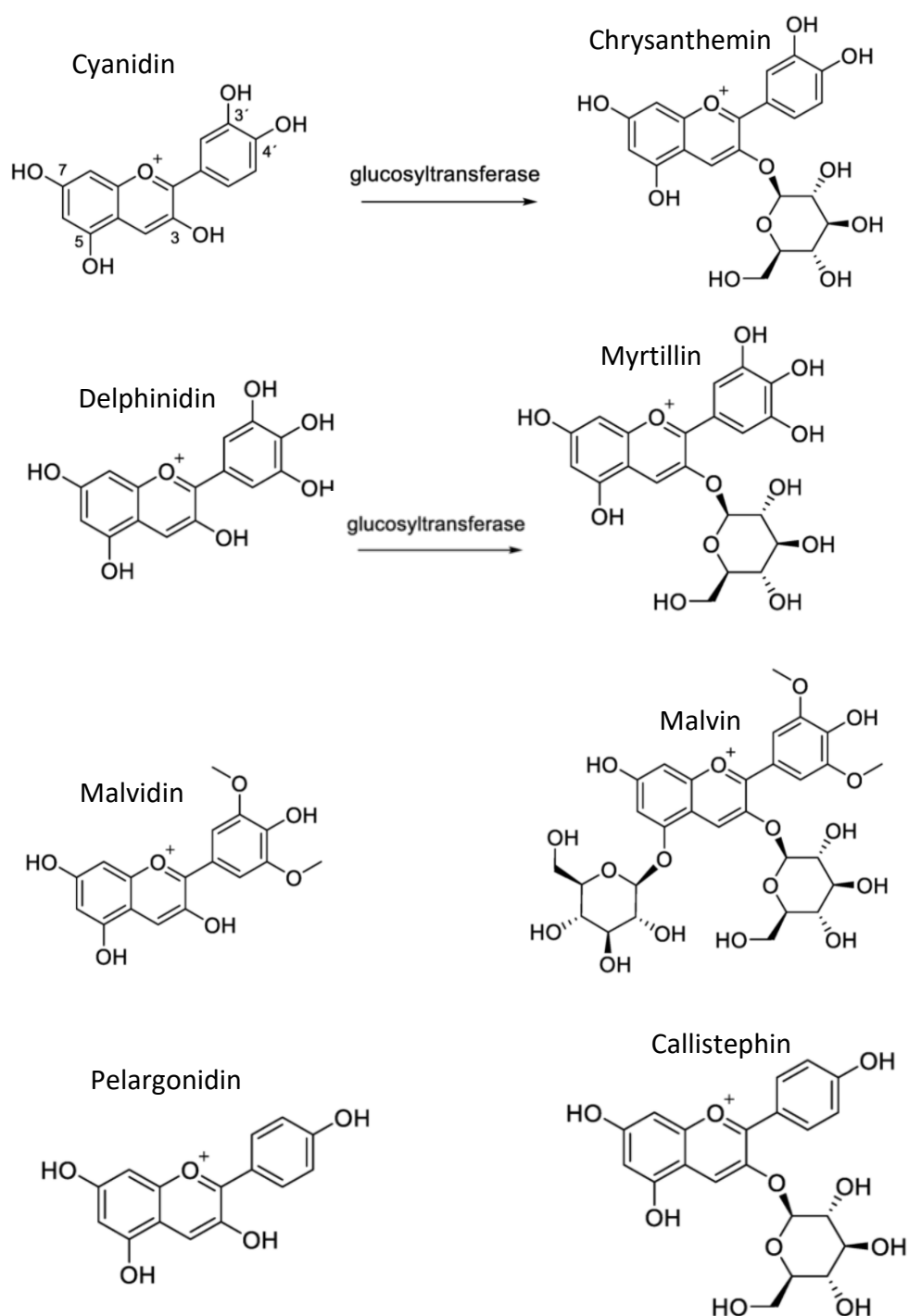


Figure 2: Chemical structure of some naturally occurring anthocyanidins (left) and corresponding mono- or di-glycosylated anthocyanins (right). (Modified from Mattioli et al. 2020)⁵

1.1.2. Exposure

Anthocyanins are not considered essential nutrients, hence there is no official recommended daily intake provided by the responsible bodies. As estimated by the EPIC-study (European prospective investigation into cancer and nutrition) and nutrition databases, the average consumption of anthocyanins in Europe ranges between 18.73 and 64.88 mg/day with a mean of 29.44 mg for men and 33.52 mg for women.⁷ In the US, consumption was estimated with

data from the National health and nutrition survey study (NHANES) to average 12.5 mg/day.⁸ Exposure to anthocyanins can occur through many sources, from natural food content and food additives to a wide variety of dietary supplements.

1.1.2.1. Food content

Anthocyanins are predominantly found in highly pigmented foods, with colours ranging from red to blue. The content of anthocyanins in dark berries can be very high and increases as the fruit ripens, for example in black currant or blackberries. Table 2 presents some examples of the anthocyanin content in common berries. Other foods, besides berries, with a considerably large anthocyanin content are aubergines (7500 mg/kg), rhubarb (2000 mg/kg) and red wine (250-300 mg/L), amongst others.⁹

Table 2: Anthocyanin content in common berry fruits (mg/kg) (from Ponder et al. 2021)¹⁰

Source	Anthocyanin content in mg/kg*
Raspberry	900
Blackberry	950
Red currant	50
Black currant	1110

*Mean values determined chromatographically (method described by Ponder and Hallmann, 2019¹¹)

1.1.2.2. Food additives

According to the International Food Information Council and Foundation US Food & Drug Administration (FDA), anthocyanins, derived from natural sources, are amongst the most utilised colorants utilised in the food industry.¹² They can be extracted from berries, grapes, red cabbage, apples, radishes, tulips, roses and orchids, to name some commonly used examples.¹³ Anthocyanins, under the E-number E 163, are authorised as food additives in the EU, and have been previously evaluated on their safety by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food (SCF) in 1975. One of the main sources of these colourants is grape skin extract, containing peonidin, malvidin, delphinidin and petunidin, and black currant extract, containing cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-rutinoside and delphinidin-3-glucoside. JECFA has established an acceptable daily intake (ADI) for anthocyanins from grape skin of 2.5 mg/kg bw/day, while the SCF has not derived an ADI for any anthocyanin. In their 2013 re-evaluation,

the European Food Safety Authority (EFSA) was also not able to set a numerical ADI due to the inadequate toxicological data available at the moment. Although, grape skin and black currant extracts were not seen as likely to be of safety concern. In most foods, the maximal permitted level of anthocyanins from fruits and vegetables as a colorant is at *quantum satis*, with no numerical value set. In the EU specifications there is no indication as to which fruits or vegetables can be used to obtain the food additive anthocyanins, and the composition and identity of anthocyanins which may be present in the food additive E163 is not stipulated.¹⁴ However, the low stability of anthocyanins limit their use as food colorants and functional food ingredients.^{15, 16, 17}

1.1.2.3. Dietary supplements

Anthocyanins are also available in a wide variety of dietary supplements, in the form of extracts, powders, gels, capsules, and purified compounds. Compared to the delivery through their natural food matrix, here, much larger doses can be ingested. A critical consideration when obtaining anthocyanins as a supplement is whether their chemical and biological activity has been maintained. Anthocyanins are, as mentioned above, relatively unstable compounds, particularly under the influence of high temperatures and light. They are most stable in acidic conditions (pH < 3) and reduced water activity, such as a freeze-dried, and for maximum shelf-life should be stored at low temperatures in appropriate packaging with little to no oxygen exposure.^{18, 19} Anthocyanin amounts in dietary supplements available for purchase can vary greatly. For example, Vlachojannis and team investigated nine different anthocyanin products in 2015, three containing elderberry preparations and six containing chokeberry preparations. Anthocyanin content was analysed by HPLC-MS using cyanidin 3-O-glucoside (cy-3-glc) as reference. The content in the elderberry preparations varied greatly between 158 and 9806 mg/L, and the same was true for the content in chokeberry preparations which varied between 60 and 1118 mg/l.²⁰

1.1.3. Stability

Most anthocyanins are highly reactive and can easily degrade or react with their environment, especially in their aglycon form. Degradation is caused by various external influences such as pH, light, oxygen, metals, enzymes, presence of other oxidants and temperature.²¹ Anthocyanins undergo degradation processes before and after consumption. These processes can be partly hampered by glycosylation, generally with a glucose at position 3, and

esterification with various organic acids (such as citric and malic acids) and phenolic acids. In addition, anthocyanins can also be stabilized by complex formation with other molecules (co-pigmentation).⁹ The stability of anthocyanidins is influenced by their structure, specifically by the B-ring in their structure and the presence of hydroxyl or methoxyl groups.⁶ These groups are known to decrease anthocyanidin stability in a solution.¹ The major stable products arising from anthocyanin degradation are phenolic acids, resulting from the B-ring, and aldehydes resulting from the A-ring.^{22, 23} The influence of pH on anthocyanins, can also lead to colour changes. At acidic pH, anthocyanins are red pigments which then shift to purple and blue with increasing pH values. The reason being; at pH 1, the flavylium cation is the primary species leading to red colours, at pH values between 2 and 4, the blue quinoidal species are predominantly present. Two colourless species can be observed at pH values between 5 and 6; a carbinol pseudo-base and a chalcone, respectively. At basic conditions, anthocyanins become unstable and can undergo different degradation pathways, leading to colour-loss to dark brown oxidized compounds.^{5, 6} After consumption, the changing pH, water content, metabolic activity and the dissolved gas composition of the gastro intestinal tract (GIT) can be expected to influence the stability and form of anthocyanins. The anthocyanin degradation already starts in the mouth; through oral microbiota, pH, high temperatures, and, to a lesser extent, binding of anthocyanin to salivary proteins, and continues through the stomach to the small and large intestine, where they become increasingly modified by biological and chemical events and movement throughout the GIT.²⁴ Figure 3 below shows the wide variety of possible degradation products emerging from anthocyanins under physiological conditions. The metabolism will be discussed later in chapter 1.1.5. Beside phenolic acids and phenylaldehydes, other degradants, such as phenylalcohols, phenylacetic acids and phenylpropenoic acids, have been identified.²⁵

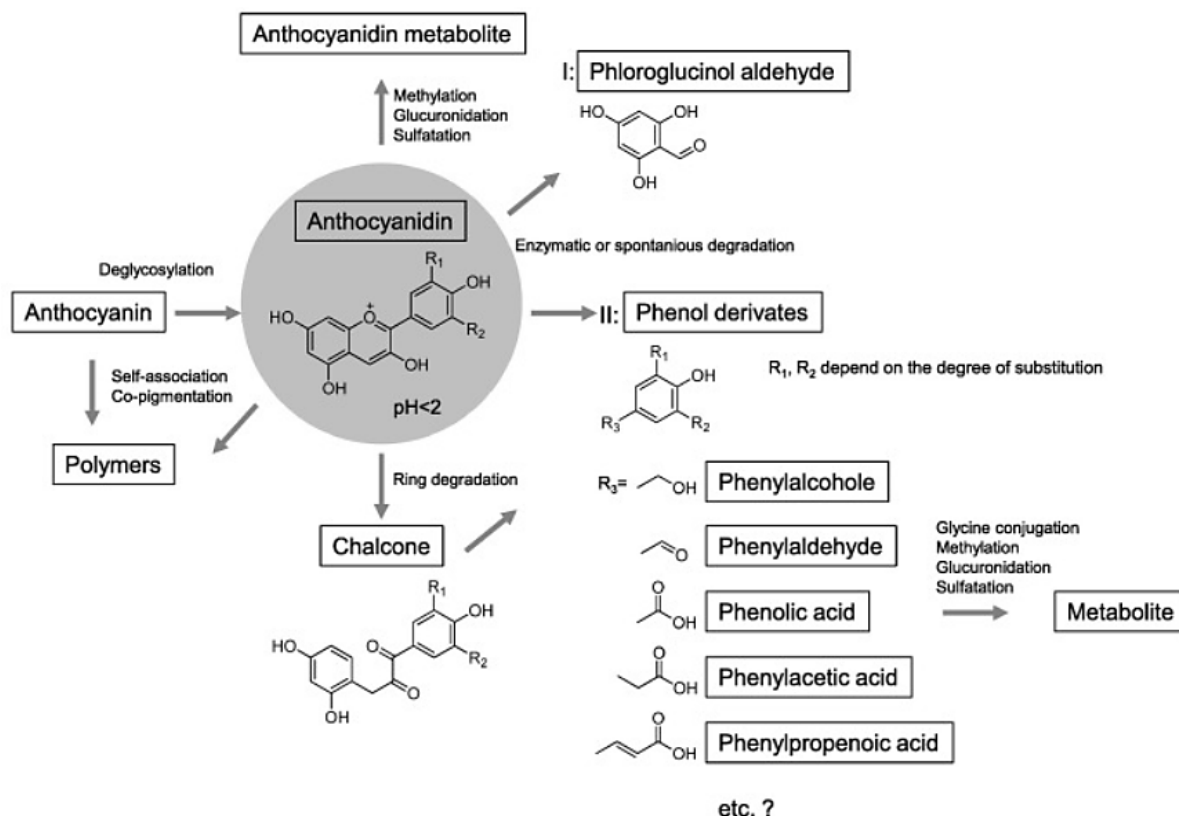


Figure 3: Potential degradation processes of anthocyanins under physiological conditions (modified from Mueller et al. 2017)²⁵

1.1.4. Bioavailability

The term bioavailability refers to the extent in which a substance reaches systemic circulation after a specific route of administration. Anthocyanins are ingested via the oral route; therefore, bioavailability is limited by their ability to pass through the epithelial surface of the gastrointestinal tract, pass the liver and appear in systemic circulation. Several works have been published over the last decade dedicated to the bioavailability of anthocyanins (summarized by Lila *et al.* 2016).²⁴ Bioavailability of the intact glycoside is dependent on a lot of factors, mainly aglycone structure and sugar moiety, but most reports indicate a bioavailability lower than 1 %. With a review by Manach *et al.* (2004) stating that peak plasma concentrations of anthocyanins, which are reported to transpire between 30 min and 2 h after consumption, are on the order of a few tens of nanomoles per liter for an intake of approximately 110–200 mg anthocyanins and urinary excretion percentages mainly lie under 0.1 % of intake.²⁶ However, the metabolites generated from anthocyanin ingestion (including aglycones, phase I and phase II metabolites, conjugated products, and microbial generated metabolites), also have to be taken into account.²⁷ There is a high inter- and intra-individual variation in the response to anthocyanin intake. An essential role in this variability may be

ascribed to intrinsic aspects such as age, sex, genetics and physiological or pathological states, but bioavailability may also be affected by the food matrix, food processing and meal composition, which can modulate their bio-accessibility. There is still research needed for better assessment of the bioavailability of anthocyanins, especially regarding important metabolites that might have been ignored so far, as well as the methods for analysis itself. Studies also need to take the different chemical forms of anthocyanins, present in equilibrium depending on the pH, into account.^{27, 24} New technologies like microencapsulation or nano-formulation are on the rise and could potentially be of help regarding the low bioavailability of anthocyanins. It has been theorized that besides providing superior stability, encapsulation may have positive effects on intestinal absorption and metabolism of anthocyanins.²⁸ Another factor to be considered in this context is the bioavailability in patients with compromised intestinal barriers. Intestinal bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis, are chronic, self-destructive inflammatory disorders of the gut. Characteristics of Crohn's disease include the formation of granuloma and chronic inflammation of the entire intestinal wall (transmural inflammation).²⁹ Impairment of the intestinal barrier during chemotherapy is also a well-known phenomenon, modulating absorption in the GI tract. This could potentially lead to a higher bioavailability in cancer patients or patients with similar impairment of the intestinal barrier.³⁰

1.1.5. Absorption, Metabolism and Excretion

After consumption, the anthocyanins are mainly absorbed in the small intestine. Enzymes present on the brush border of the enterocytes, can release the aglycone that is then able to enter the epithelial cells by passive diffusion or various suggested transport mechanisms including the sodium dependent glucose co-transporter as well as other glucose-associated transporters (GLUT). Anthocyanins can reach the colon if they are previously unabsorbed, where they are extensively modified by the colonic microbiota which may release aglycones and generate simple phenolics, that then have the potential to be absorbed by the colonic mucosa.^{5, 31} After absorption in the gut, anthocyanins travel the liver via the portal vein. Metabolism occurs mainly in the liver, but also at the renal and enterocyte level. Anthocyanins are metabolized by phase I and phase II enzymes, which generate hydroxylated, sulphated, glucuronidated and methylated molecules, giving rise to many different molecular intermediates. They can also be secreted and reabsorbed in the enterohepatic circle to restart

the pathway and create even more metabolites.²⁴ Czank and his team performed a ¹³C-tracer study to trace metabolites derived from the anthocyanin cyanidin-3-glucoside (cy-3-glc), to establish a comprehensive profile of their absorption and elimination kinetics. A total of 25 ¹³C-labelled compounds were identified, ¹³C₅-Cy-3-glc and 24 metabolites. Metabolites included phase II conjugates of cy-3-glc and cyanidin (methyl cyanidin-glucuronide, cyanidin-glucuronide and methyl cy-3-glc-glucuronide), degradants (protocatechuic acid (PCA) and phloroglucinaldehyde (PGA)), phase II conjugates of PCA (including PCA-3-sulfate, PCA-3-glucuronide and vanillic acids amongst others), phenylacetic acids (3,4-dihydroxyphenyl acetic acid and 4-hydroxyphenylacetic acid), phenylpropenoic acids (caffeic acid and ferulic acid), and hippuric acid.³² Anthocyanins are mainly excreted through urine and feces. As shown by Czank *et al.* in 2013, for the first 6 h urine was the primary route of elimination, representing 66.33 ± 13.22 % of the total ¹³C recovery, followed by that in breath (28.94 ± 6.89 %). Feces depicted the major route of elimination over the time periods 6–24- and 24–48-h. The highest concentrations were shown in 6–24-h urine samples (10.77 ± 4.52 μmol/l) and 6–24-h fecal samples (43.16 ± 18.00 μmol/l).³² Ultimately the route, time and form of elimination depends on the type of conjugates produced and their site of production, and also has a wide interindividual variability. Glucuronides formed in the intestine, for example, are likely to enter the systemic circulation directly and are not promptly available for biliary excretion unlike those newly formed in the liver and predominantly excreted into the bile.³³

1.2. Intestinal Immune System

The intestinal immune system plays an important part in human health, due to the vast mucosal surface and the constant challenge of antigens of dietary or bacterial origin and a large number of possibly toxic, mutagenic or mitogenic stimuli. This leads to a continuous condition of physiological inflammation. In this state, all types of cells - immune cells as well as nerve, mesenchymal, epithelial and endothelial cells - play a key role in orchestrating the intestinal immune response. A malfunction of any of these components can lead to a dysregulation of intercellular signalling and therefore to the induction of pathological and chronic inflammation.³⁴

1.2.1. Inflammation

Inflammation is part of the convoluted protective response of body tissues to harmful stimuli, such as pathogens, cell damage or irritation, involving immune cells, blood vessels and molecular mediators. The purpose of inflammation is to eliminate the cause of cell injury, clear out damaged tissues and necrotic cells and initiate repair mechanisms. The five principal signs of inflammation are heat, redness, swelling, loss of function and pain.^{35, 36} Chronic inflammation is a prolonged immune response that lasts for months to years. Multiple diseases are mediated by chronic inflammation, such as diabetes, cardiovascular disease, allergies and chronic obstructive pulmonary disease (COPD). Obesity, smoking and stress, amongst other factors, promote chronic inflammation.³⁷ Chronic inflammation can also contribute to the pathogenesis of GI cancers.³⁸ Under chronic inflammatory conditions, intestinal barrier function can be impaired by the loss of the mucosal epithelial layer integrity layer.³⁹ Inflammatory cell response consist of activating inflammatory signalling pathways, usually the nuclear factor kappa-B (NF- κ B), mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT), which regulate inflammatory mediator levels.⁴⁰

1.2.1.1. *NF- κ B Pathway*

The NF- κ B transcription factor plays an important role in inflammation, immune response, survival and apoptosis as it regulates the expression of various components of the immune system. These include proinflammatory cytokines, chemokines, adhesion molecules and inducible enzymes regulating the innate immune response such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Proteins that regulate the specific immune response, such as major histocompatibility complex (MHC), co-stimulatory molecules pivotal to the induction of specific immunity and cytokines such as interleukin (IL)-2, IL-12 and interferon- γ that control lymphocyte proliferation and differentiation, can also be induced. The NF- κ B family includes five related transcription factors: p50, p52, RelA (v-rel avian reticuloendotheliosis viral oncogene homolog A, also known as p65), RelB, and c-Rel (members of the Rel protein family).⁴¹ NF- κ B is activated by a vast range of stimuli, including pathogen-derived substances, intercellular inflammatory cytokines, and various enzymes. I κ B proteins present in the cytoplasm inhibit NF- κ B under physiological conditions. Pattern-recognition receptors, including the Toll-like receptors (TLRs), and inflammatory cytokines,

such as tumor necrosis factor (TNF) and IL-1, activate the pathway. They use signal transduction mechanisms to activate I κ B kinase (IKK) by phosphorylation, resulting in its degradation by the proteasome and the following release of NF- κ B for nuclear translocation and gene transcription activation.^{42, 43} The RelA/p50 complexes for example, regulate expression of inflammatory cytokines, as shown in Figure 4.⁴⁰

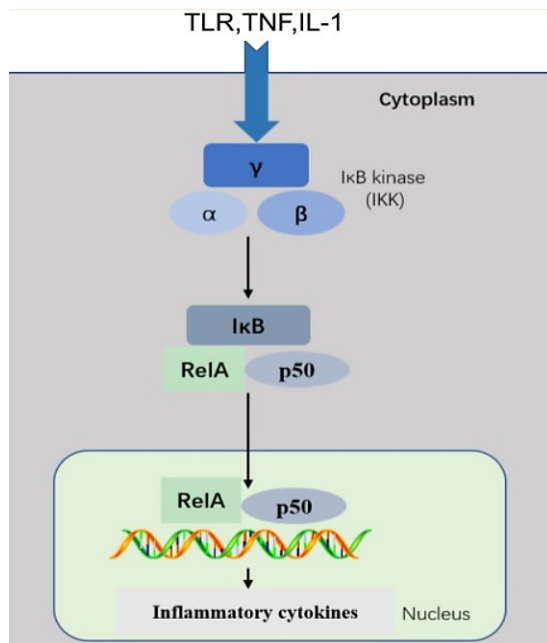


Figure 4: NF- κ B pathway (taken from Chen et al. 2018)⁴⁰

1.2.1.2. MAPK Pathway

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that mediate intracellular signalling initiated by extracellular stimuli, including osmotic stress, mitogens, heat shock and inflammatory cytokines. The mammalian MAPKs include extracellular-signal-regulated kinase ERK1/2, p38 MAPK and c-Jun N-terminal kinases (JNK). Each MAPK signalling pathway comprises at a minimum three components: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). MAP kinases are regulated by phosphorylation cascades. MAPKKKs phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs. Activated MAPKs phosphorylate a variety of proteins, including transcription factors, subsequently regulating inflammatory responses.⁴⁴

1.2.1.3. JAK-STAT Pathway

The JAK-STAT pathway is a signalling mechanism through which gene expression can be controlled by extracellular factors. It involves various cytokines, growth factors, interferons and related molecules, such as growth hormone and leptin (see Table 3). Receptor-associated

JAKs are activated by ligands and phosphorylate one another, creating docking sites for the cytoplasmic transcription factors known as STATs. Cytoplasmic STATs recruited to these sites undergo phosphorylation and subsequent dimerization and translocate to the nucleus. There, they bind to target gene promoter regions to control transcription of inflammatory genes, leading to activation of downstream cytokines (see Table 3).⁴⁵

Table 3: JAKs and STATs and their related cytokines (modulated from O'Shea et al.).⁴⁵

JAK/STAT	Important for signalling by
JAK1	IFN α/β , IFN- γ , IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, IL-6 family cytokines, IL-10 family cytokines
JAK2	IFN γ , IL-3, IL-5, GM-CSF, EPO, TPO, G-CSF, GH, leptin
JAK3	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
tyk2	IFN α/β , IL-12, IL-23
STAT1	All interferons
STAT2	Type I IFNs
STAT3	IL-6 and other gp130 cytokines
STAT4	IL-12, IL-23, type 1 interferons
STAT5a/STAT5b	IL-2, EPO, TPO, GM-CSF, GH, IL-7
STAT6	IL-4, IL-13

Abbreviations: GM-CSF; Granulocyte-macrophage colony-stimulating factor, EPO; Erythropoietin, TPO; Thyreoperoxidase, G-CSF; Granulocyte colony-stimulating factor, GH; growth hormone

1.2.2. Cytokines

Cytokines are biologically active components predominantly released from immune cells, including macrophages, monocytes and lymphocytes, but also by intestinal epithelial cells and other non-immune cells. In their role as key regulators and effectors of inflammation, cytokines often exert overlapping activities and their complex interaction networking can sometimes be seemingly contradictory. They include a vast number of interferons, chemokines, interleukins, growth factors and colony-stimulating factors, that express pro- and anti-inflammatory activities. To guarantee intestinal homeostasis and control inflammation, a complex crosstalk between the different cells and the microbiota via extracellular, soluble regulators and mediators is necessary. Excessive inflammatory cytokine production can lead

to tissue damage, organ failure, hemodynamic changes and ultimately death. Cytokines also regulate the suppression of inflammation, including the mediation of healing and repair mechanisms.²⁹ Individual cytokines perform specific roles depending on cell type and location, Table 4 shows cytokine action, defined by target cell and primary function.

Table 4: Cytokines, defined by main source, target cell and primary function. (modified from Turner et al. 2014)²⁹

Cytokine		Main sources	Target cell	Major function
Interleukins	IL-1	Macrophages, B cells, DCs	B cells, NK cells, T-cells	Pyrogenic, pro-inflammatory, proliferation and differentiation, cell proliferation
	IL-2	T cells	Activated T and B cells, NK cells	Proliferation and activation
	IL-3	T cells, NK cells	Stem cells	Hematopoietic precursor, proliferation and differentiation
	IL-4	Th-cells	B cells, T cells, macrophages	Proliferation of B and cytotoxic T cells, enhances MHC class II expression, stimulates IgG and IgE production
	IL-5	Th-cells	Eosinophils, B-cells	Proliferation and maturation, stimulates IgA and IgM production
	IL-6	Th-cells, macrophages, fibroblasts	Activated B-cells, plasma cells	Differentiation into plasma cells, IgG production
	IL-7	BM stromal cells, epithelial cells	Stem cells	B and T cell growth factor
	IL-8	Macrophages	Neutrophils	Chemotaxis, pro-inflammatory
	IL-9	T cell	T cell	Growth and proliferation
	IL-10	T cell	B cells, macrophages	Inhibits cytokine production and mononuclear cell function, anti-inflammatory
	IL-11	BM stromal cells	B cells	Differentiation, induces acute phase proteins
	IL-12	T cells	NK cells	Activates NK cells

Cytokine		Main sources	Target cell	Major function
Tumor necrosis factors	TNF- α	Macrophages	Macrophages	Phagocyte cell activation, endotoxic shock
		Monocytes	Tumor cells	Tumor cytotoxicity, cachexia
	TNF- β	T-cells	Phagocytes, tumor cells	Chemotactic, phagocytosis, oncostatic, induces other cytokines
Interferons	IFN- α	Leukocytes	Various	Anti-viral
	IFN- β	Fibroblasts	Various	Anti-viral, anti-proliferative
	IFN- γ	T-cells	Various	Anti-viral, macrophage activation, MHC-I and -II expression on cells, increases neutrophil and monocyte function
Colony stimulating factors	G-CSF	Fibroblasts, endothelium	Stem cells in BM	Granulocyte production
	GM-CSF	T cells, macrophages, fibroblasts	Stem cells	Granulocyte, monocyte, eosinophil production
	M-CSF	Fibroblast, endothelium	Stem cells	Monocyte production and activation
	EPO	Endothelium	Stem cells	Red blood cell production
Others	TGF- β	T cells and B cells	Activated T and B cells	Inhibit T and B cell proliferation, inhibit haematopoiesis, promote wound healing

Abbreviations: NKs; natural killer cells, DCs; dendritic cells, MHC; major histocompatibility complex, Ig; immunoglobulin, BM; bone marrow

The main pro-inflammatory cytokines include interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF- α), all of which signal via the type I cytokine receptors. By contrast, the critical pro-inflammatory chemokine, IL-8, signals via G protein-coupled receptors (GPCRs) (Figure 5).

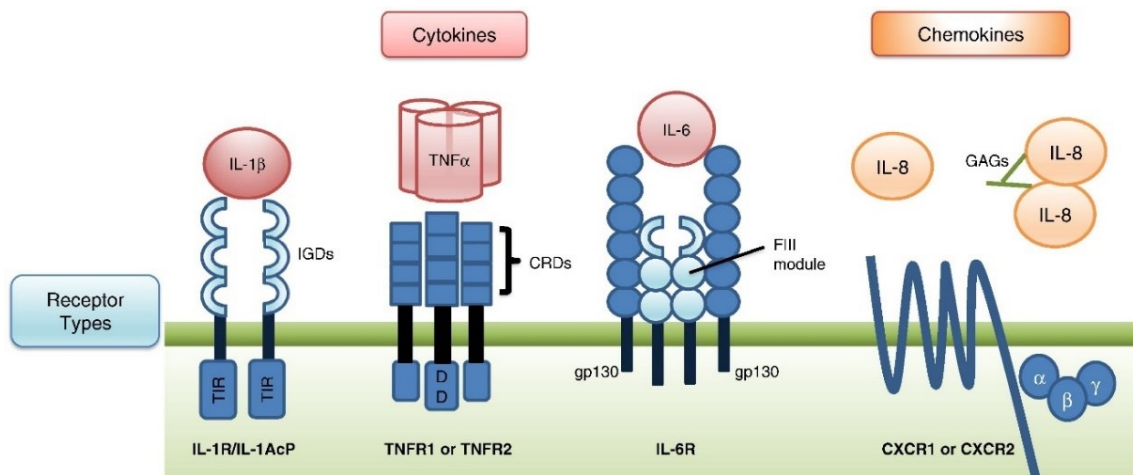


Figure 5: Cytokines (IL-1 β , TNF- α and IL-6), chemokines (IL-8) and their receptors. (Modified from Turner et al. 2014)²⁹

Cytokines signal via oligomers of single-pass, type I transmembrane receptors, with specific extracellular domains for ligand binding and intracellular domains for signal transduction. The receptor for IL-1 consists of IL-1R1 and the IL-1RAcP, which following ligand binding is formed to the extracellular immunoglobulin domains (IGDs); intracellular signalling is mediated via the Toll/IL-1R (TIR) domain. Trimeric TNF- α binds to the cysteine-rich domains (CRDs) of the TNF receptor (TNFR) and the receptor's death domain (DD) mediates the signal. Finally, the IL-6R consists of a multimeric structure with IL-6R chains complexed with gp130. The ligand-binding region is within the fibronectin III (FIII) modules of the IL-6R chains but the signal transduction is mediated by the associated gp130 molecules. Chemokine signalling is mediated via activation of the seven transmembrane GPCRs. IL-8 binds to the extracellular region of either CXCR1 or CXCR2, causing a conformational change that triggers activation of the G-proteins.²⁹ The cytokine interleukin 1 β (IL-1 β) belongs to the IL-1 family, that is generally known to for proinflammatory signalling. IL-1 β expression is induced predominantly in response to microbial or viral molecules, although it can also stimulate its own expression. The IL-1 receptor (IL1R) (shown in Figure 5) is present at the membrane of most cell types – including intestinal epithelial cells.⁴⁶ Following ligand binding the adaptor molecule, MyD88, interacts with IL-1R1 via its TIR domain. Signal transduction leads to activation of MAPKs and the transcription factor NF- κ B, consequently resulting in pro-inflammatory cytokine expression.²⁹ The monocyte-derived tumor necrosis factor TNF- α has diverse functions in cell differentiation, immunity, inflammation and apoptosis and represents the prototypic member of the TNF superfamily of type II transmembrane proteins.⁴⁷ Signal transduction of TNF- α leads to activation of several pathways finally leading to induction of mainly proinflammatory cytokine expression. As potent inflammatory mediator, it also leads to expression or activation

of adhesion molecules and growth stimulation, proliferation of normal cells, causes antiviral, inflammatory and immunoregulatory effects and exerts cytolytic or cytostatic effects against tumor cells. TNF- α has also been shown to perform a number of additional functions as well, being linked with lipid metabolism, insulin resistance, coagulation and endothelial function.²⁹ IL-6 belongs to the interleukin 6 superfamily and is classified as mainly proinflammatory. IL-6 is also expressed by an array of cells. IL-6 is involved in a number of important processes in the body, including haematopoiesis, maturation of B-cells, T cell activation, differentiation and regulation of T helper 2 cells (Th2) and regulatory T cells (Treg) phenotypes, and is also important in the secretion of acute phase proteins by the liver. Signalling of IL-6 is mediated through binding of IL-6 to the IL-6 receptor (IL-6R) (shown in Figure 5), associating with the membrane glycoprotein gp130 leading to signal transduction activating the JAK/STAT, phosphoinositide 3-kinase (PI3K) and MAPK pathways.²⁹

The chemokine IL-8 (CXCL8) is another critical mediator of inflammation. Pro-inflammatory chemokines are produced by cells primarily to recruit leukocytes to the sites of infection or injury. IL-8 signal transduction is mediated via the CXCR1 and CXCR2 receptors, that belong to a seven-transmembrane superfamily of G protein-coupled receptors (Figure 5). The G protein subunits stimulate enzymes (adenylate cyclase or phospholipase C) that can activate MAPK signalling and modify gene expression to promote cell survival, proliferation and inflammation.²⁹

IL-2 is part of the adaptive immunity, it is a T cell growth factor and is crucial for the proliferation of T cells and the generation of effector and memory cells.⁴⁸ IL-33 is a more recently added member of the IL-1 family, mostly produced by stromal cells, such as epithelial and endothelial cells. It is passively released when tissues are damaged or during cell necrosis, leading to the belief that it may function as an alarmin that alerts the immune system after cell damage. IL-33 can regulate the allergic inflammation in various allergic diseases, is either pro- or anti-inflammatory depending on the disease and the model.⁴⁹ IL-18 is part of the IL-1 family as well, exhibits pro inflammatory signalling and is also involved in activation and differentiation of various T cell populations.⁵⁰

This master thesis will focus mainly on the up- and downregulation of the cytokines IL-1 β , TNF- α , IL-8 and IL-6, as they have been reported to be excreted by HCT116 cells^{51, 52} and

HCEC-1CT cells⁵³ in culture. The cytokines IL-18, IL-33 and IL-2 are also of interest, even though the literature is uncertain about the ability of colonic epithelial cells to express them.^{54, 55, 50}

1.2.3. Inflammatory Markers

Markers for inflammation are mainly cytokines and chemokines, as discussed above. Besides, proteins and enzymes can be used as inflammation, infection and trauma biomarkers in medicine. Inflammatory proteins in the blood, such as haptoglobin, C-reactive protein, fibrinogen and alpha 1-acid glycoprotein, assist in restoring homeostasis and reducing microbial growth independently of antibodies during infection, trauma or stress.⁵⁶ Abnormal activation of certain enzymes, including NADPH oxidase (NOX), iNOS and COX-2, play important roles in the development of inflammation-related diseases, such as cardiovascular disease and cancer, as they can influence the intracellular signalling cascades involved in activation of the NF- κ B and MAPK pathways.⁵⁷ This master thesis will focus on the up- and downregulation of iNOS and COX-2, as they have been reported to be excreted by colonic cells, namely HCT116, in culture.⁵⁸ iNOS is expressed in a variety of cell types under both normal and pathological conditions, including epithelial cells. With infectious and proinflammatory stimuli, iNOS protein is highly induced to produce NO. Cyclooxygenase (PGH-2 synthase, COX) leads to the formation of a bioactive prostanoid (PEG-2), via concerted activation of PGE synthase (PGES). COX-2 protein is only moderately expressed in most mammalian tissues in response to physical, biological or chemical stimuli, including inflammation.⁵⁷

1.2.4. Immuno-Checkpoint Markers

Immune-inhibitory pathways, termed immune checkpoints, are inhibitory pathways hardwired into the immune system that are pivotal for maintaining self-tolerance and modulating the extend and duration of physiological immune responses with the purpose to minimize collateral damage in tissue. Tumors are required to develop specific immune resistance mechanisms to avoid recognition and attacks by the immune system. They co-opt certain immune-checkpoint pathways as a critical mechanism of immune resistance, especially against T cells that are distinct for tumor antigens. Those mechanisms in turn, are targets of cancer immunotherapy. Many of the immune checkpoints are facilitated by ligand–receptor interactions, meaning they can be blocked by antibodies or modulated by recombinant forms of receptors or ligands. Cytotoxic T-lymphocyte-associated antigen 4

(CTLA4) antibodies for example achieved US Food and Drug Administration (FDA) approval, as the first of this class of immunotherapeutics.^{59, 60} In this thesis the immune-checkpoint markers programmed cell death protein ligand 1 (PD-L1) and CD70 are examined, since according to literature, both are potentially expressed in colonic epithelial cells.^{61, 62} The immune-checkpoint receptor, programmed cell death protein 1 (PD1), limits T cell effector functions within tissues at the time of an inflammatory response to infection, to limit autoimmunity (Figure 6). By upregulating ligands for PD1 (PD-L1) tumor cells block antitumor immune responses in the tumor microenvironment. When engaged by one of its ligands, PD1 inhibits kinases that are involved in T cell activation through the phosphatase SHP250, although additional signalling pathways are likely. PD1 is highly expressed on regulatory T cells (TReg), where it is likely to enhance their proliferation in the presence of ligands. Since many tumors are highly infiltrated with TReg cells, that may further suppress effector immune responses, blockade of the PD1 pathway possibly enhances antitumor immune responses by decreasing the number and/or suppressive activity of intratumoral TReg cells.⁵⁹

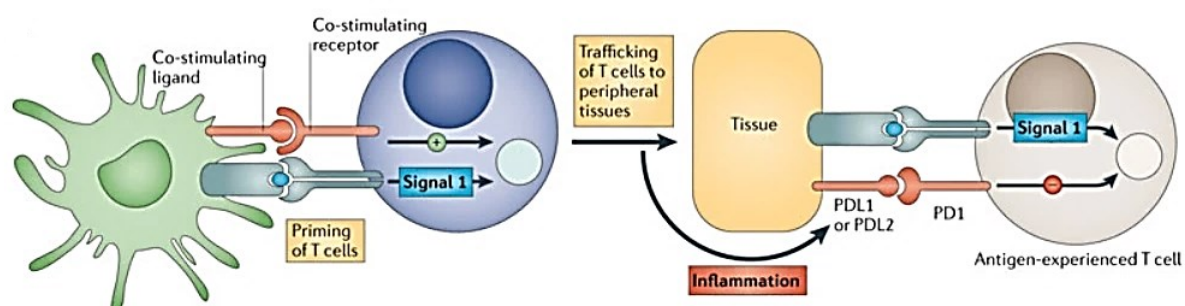


Figure 6: PD-L1 pathway. (Modified from Pardoll 2012)⁵⁹

CD70 is the TNF-like ligand of the tumor necrosis factor receptor (TNFR) family member CD27, a transmembrane receptor with a co-stimulatory role in immune cell activation. CD70 is upregulated in several tumors where it promotes cytotoxic effects on B and T lymphocytes, stimulating immune escape.⁶³

1.2.5. Anti-Inflammatory Activity of Anthocyanins

The plant pigments anthocyanins have been reported to exhibit anti-inflammatory activity. Anthocyanins have been shown to improve the intestinal barrier integrity, impaired under chronic inflammation conditions, by upregulating the expression of crucial barrier-forming proteins via upregulation of glucagon-like peptide (GLP)-2 intestinal hormone levels.³⁹ In addition, anthocyanins seem to be able to improve barrier function by regulating cell

permeability on the GIT.⁶⁴ Besides their effects on the intestinal barrier, anthocyanins have also been shown to down-regulate the expression of various pro-inflammatory biomarkers such as NF- κ B, TNF- α , multiple interleukins, interferon (IFN-) γ , inducible nitric oxide synthase (iNOS), cyclooxygenase (COX) -2 and more.^{65, 39, 66, 67, 68, 69} The transcription factor NF- κ B controls the expression of numerous genes involved in the inflammatory response and is activated by pro-inflammatory stimuli and oxidative stress. Dampening NF- κ B activation and consequentially limiting the inflammatory response have been proposed as a potential strategy to prevent chronic inflammatory diseases. *In vitro*, anthocyanins seem to be able to suppress induced NF- κ B.⁷⁰ As for inflammatory cytokines, Venancio *et al.* showed the decreased expression of NF- κ B and IL-1 β , IL-6 and TNF- α by anthocyanins in human colon cancer and non-malignant colon cells.⁶⁷ In THP-1 derived macrophages, different fractions of an anthocyanin-rich blackberry extract seem to display both anti- and pro-inflammatory properties, influencing NF- κ B activity and expression of IL-8, IL-6 and TNF- α , with the extract overall expressing anti-inflammatory activity.⁶⁸ Besides various *in vitro* models, Peng *et al.* report that the long-term consumption of anthocyanins from *Lycium ruthenicum* Murray reduces inflammation of the colon by reducing the expression of iNOS, Cox-2, TNF- α , IL-6, IL-1 β , and IFN- γ mRNAs in C57BL/6 male mice.⁶⁶ And Karlsen *et al.* (2007) reported reduced plasma concentrations of pro-inflammatory mediators in healthy adults after consumption of anthocyanins.⁷⁰

1.3. Irinotecan and Cancer

A commonly used drug in the treatment of colorectal cancer is irinotecan. Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) also known as CPT-11 (see Figure 7) is regularly used as a chemotherapeutic agent in the treatment of colorectal cancer, due to its activity as a DNA topoisomerase I inhibitor.⁷¹ It is a semi-synthetic derivative of the naturally occurring plant alkaloid camptothecin. After application, CPT-11 is mainly metabolized in the liver to its active metabolite SN38, which can then be detoxified in the liver by glucuronidation.⁷² DNA topoisomerases are ubiquitously expressed enzymes involved in the maintenance of DNA topology. They are involved in numerous nuclear processes such as replication, transcription, recombination and chromosome dynamics.⁷³ Anti-cancer drugs often target topoisomerase, including derivatives of camptothecin (irinotecan and topotecan) targeting topoisomerase I and epipodophyllotoxin (etoposide and teniposide) targeting

topoisomerase II. These so-called topoisomerase poisons stabilize covalent topoisomerase/DNA intermediates by forming a ternary complex, thereby converting the endogenous enzyme to a cellular toxin. An increase in cleavable complexes ultimately triggers cell death by generating permanent strand breaks in the genetic material.^{74, 75} Chemotherapeutics treating colorectal cancer, including irinotecan, often have severe side effects, which include neutropenia and diarrhea, making the treatment difficult for the patients.^{76, 77} In the case of irinotecan, those side effects seem to be mainly caused by intestinal exposure to SN38, since the detoxified SN38 glucuronide can be reconverted to SN38 with the help of bacterial β -glucuronidases in the intestinal tract and can thus undergo enterohepatic circulation.⁷⁸

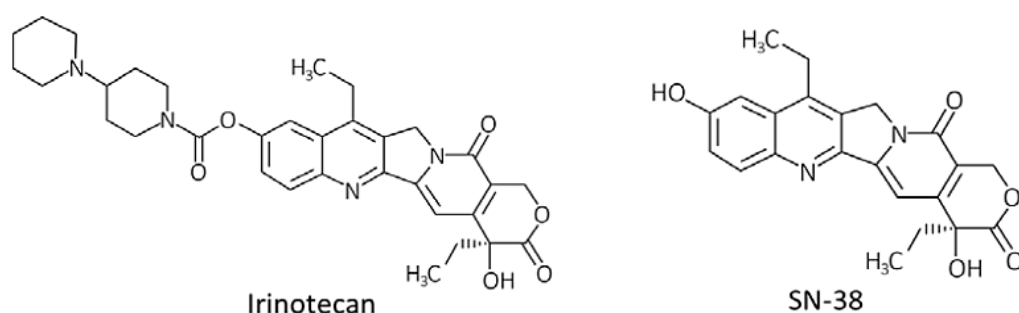


Figure 7: Structures of irinotecan (CPT-11) and its activated metabolite SN-38

1.3.1. Effects on Oxidative Stress and Inflammation

As mentioned before, the main dose-limiting toxicities of chemotherapeutics like irinotecan, used for the treatment of colorectal cancer, are neutropenia and diarrhea, especially late-onset diarrhea.^{76, 79} The cytotoxic effects of irinotecan, and other chemotherapeutics, are potentiated by the fact that the drug reportedly upregulates TNF production and activates the NF- κ B pathway.⁸⁰ Chemotherapeutics like irinotecan are proposed to activate the NF- κ B pathway, leading to the release of pro-inflammatory cytokines and affecting tumor cell growth via pro-apoptotic pathways.⁸⁰ NF- κ B activation is one mechanism by which tumors become resistant to apoptosis.⁸¹ In fact, multiple chemotherapeutics (camptothecin with the active metabolite SN38 amongst others) have already been listed as known NF- κ B inducers in 1999.⁸² To help to understand the relationship between chemotherapeutics and inflammation, specifically NF- κ B, multiple studies have been conducted. Numerous *in vitro* publications showed that irinotecan or SN38 activates NF- κ B in different cell lines. Bottero *et al.* (2001) found that, in HeLa cells, SN38 activates NF- κ B using a preexisting pathway that requires a

functional I κ B-specific kinase complex, I κ B-specific kinase activation, I κ B- α phosphorylation and degradation.⁸³ Camp *et al.* (2003) studied NCI-N87 and AGS human gastric cancer cells and concluded a significant induction of NF- κ B by SN38.⁸⁴ Kokura *et al.* (2005) reported that, *in vitro*, SN38 induced activation of NF- κ B, activation of caspase-3, production of intracellular reactive oxygen species and apoptosis in colon26 cells.⁸¹ Whereas Li *et al.* (2015)⁸⁵ concluded in their *in vitro* study, that SN38 only influences the activation of NF- κ B by LPS (lipopolysaccharide) and Wong *et al.* (2019)⁸⁶ observed no activation of NF- κ B by SN38. Wong *et al.* even describe an anti-inflammatory effect of SN38 by targeting TLR4 receptor and downregulating NF- κ B activity, induced by LPS. It is also proposed that irinotecan and SN38 induce the generation of reactive oxygen species (ROS) which activate the inflammasome and caspase 1 leading to the production of cytokines like IL-1 β , IL-18 and IL-33 in mouse models.^{87,88}

1.3.2. Interactions between Anthocyanins and Chemotherapy

Anthocyanins have been proposed to interact with chemotherapeutic agents such as irinotecan. This phenomenon has already been documented *in vivo* in 2013, where anthocyanins have been shown to suppress the cleavable complex formation by irinotecan and decrease its DNA-strand-breaking activity in the colon of Wistar rats.⁸⁹ Anthocyanins have been shown to act as topoisomerase inhibitors, potentially inhibiting the catalytic activity of topoisomerases without stabilizing the covalent enzyme/DNA intermediate. They are able to modulate the effect of topoisomerase poisons *in vitro*, decreasing their DNA damaging properties.^{90, 91} In the aforementioned *in vivo* study from the year 2013, the question was addressed whether anthocyanins interfere with the topoisomerase I poison irinotecan (Figure 7). To detect irinotecan-induced stabilization of topoisomerase I/ DNA complexes *in vivo* complexes-of-enzyme-to-DNA-bioassay was used and single cell gel electrophoresis was used to determine DNA-strand-break induction in the colon of male Wistar rats. Anthocyanins were found to significantly reduce irinotecan-mediated topoisomerase I/DNA cleavable complex formation.⁸⁹ Cellular targets of anthocyanins also include the antioxidant defence (by activating Nrf2-regulated enzymes) and anti-inflammatory cytokines, as discussed in chapter 1.2.6.^{92, 91} Hence, potential interference with targets of irinotecan and other chemotherapeutics are possible. Since anthocyanins have been shown to suppress induced NF- κ B⁷⁰, they could be proposed to enhance the anti-tumor effects of chemotherapeutics by increasing apoptosis via inhibition of NF- κ B.

1.4. Citric Acid and Cancer

Citric acid (CA) (Figure 8) is a naturally occurring organic acid presented primarily in fruits and vegetables, especially in citrus fruits.⁹³

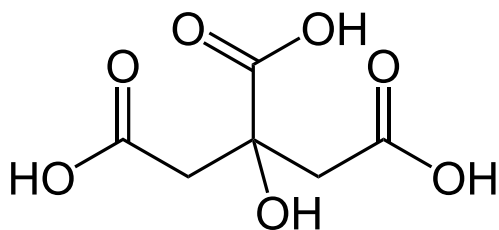


Figure 8: Structure of citric acid

CA, under the E-number E330, is widely used as a food additive and preservative in many processed foods and beverages and as an ingredient in cosmetic products, cleaning agents and more. It functions as an acidifier and as a flavouring and chelating agent. Citric acid anhydrous and monohydrate are both authorised as food additives in Commission Regulation (EU) No 231/2012 and categorized as “additives other than colours and sweeteners” with a maximum level at *quantum satis*.⁹⁴ In the biochemical process, citric acid is an intermediate in the citrate cycle, which occurs in the metabolism of all aerobic organisms and is involved in the chemical conversion of carbohydrates, fats and proteins into carbon dioxide and water to generate energy. Citrate is synthesized in mitochondria from acetyl-CoA and oxaloacetate (OAA) by citrate synthase (CS) before entering the tricarboxylic acid (TCA) cycle. Besides the production of energy in the TCA cycle, citrate can be transported into the cytoplasm and catalysed to create acetyl-CoA, providing a major source for lipid biosynthesis.⁹⁵ Besides directly influencing the TCA cycle activity, citrate is also an important metabolic regulator involved in various pathophysiological processes, including inflammation and cancer.^{96, 97} Cancer cells synthesize, through various pathways, great quantities of macromolecules to proliferate and build new cells, while requiring to continuously produce ATP and cofactors in order to sustain synthetic pathways. They therefore consume large amount of glucose and amino acids (in particular glutamine derived from muscle proteolysis). In cancer cells glucose metabolism, even in the presence of oxygen, predominantly results in the formation of lactic acid. This phenomenon is often referred to as “aerobic glycolysis” or the “Warburg effect”. CA is considered an effective agent in the suppression of glycolytic enzyme activities in cancer cells. Intracellular citrate levels are both an essential metabolic intermediary and an important regulator of energy production. Usually, when citrate, being the first product of the TCA cycle,

is sufficiently produced, it exerts a negative feedback on glycolysis and the TCA cycle itself, slowing down or arresting these pathways. Simultaneously it stimulates gluconeogenesis and ATP-consuming lipid synthesis. This knowledge leads to the theory that large amounts of citrate may fool the energy level of the cell and inhibit ATP production pathways (glycolysis, TCA cycle and β -oxidation), whereas it could stimulate synthesis routes which consume ATP (gluconeogenesis and lipid synthesis). These mechanisms may then lead to cell growth arrest and cell death following severe energy depletion inside cells.⁹⁶ In preclinical cancer models, the administration of citrate in high doses shows various anti-cancer effects, such as the inhibition of glycolysis, the promotion of cytotoxic drugs sensibility, the neutralization of extracellular acidity and the inhibition of tumor growth and of key signalling pathways (in particular, the IGF-1R/AKT pathway).⁹⁸ *In vitro* for example; Chen *et al.* (2017) investigated the effect of citric acid on the growth of human esophageal carcinoma cells EC109 and saw a decrease in cell proliferation when the cells were treated with citric acid.⁹⁹ Similar effects were observed for MSTO-211H human mesothelioma cells¹⁰⁰, human ovarian carcinoma cells¹⁰¹ or gastric carcinoma cells¹⁰². Ren *et al.* (2017) found that citrate suppresses tumor growth in multiple models through inhibition of glycolysis, the tricarboxylic acid cycle and the IGF-1R pathway.¹⁰³ Zhao *et al.* (2022) came to the conclusion that citric acid treatments significantly inhibited tumor cell proliferation and growth in various tumor types and identified that citrate-induced tumor suppression was caused by the promotion of excessive lipid biosynthesis and induction of cell senescence in tumor cells. In addition, Zhao *et al.* determined that citrate administration not only inhibited tumor growth and progression *in vivo*, but also displayed synergistic antitumor effects when combined with the conventional chemotherapy drugs *in vivo* in colon cancer xenograft models. To be more specific, they investigated the effect of citric acid in cancerous and non-cancerous cell lines and reported a dose dependent inhibition of cell growth and proliferation in cancer cells, including breast cancer MCF7 and MDA-MB-231, colon cancer HCT116 and HT29, lung cancer H1299 and A549 cell lines, starting from 10 mM (0.2 %) but little to no effect in human fibroblasts (HFF and WI-38) and a normal breast cell line (MCF10A). This indicates that the anti-proliferative effect is specific to cancer cells.⁹⁵ These results indicate that targeting citrate can be a promising therapeutic strategy for tumor treatment.

2. Aim of the Thesis

This thesis is part of the project “Protective Properties of Anthocyanins During Chemotherapy” funded by the Austrian Science Fund (German: Fonds zur Förderung der wissenschaftlichen Forschung, FWF) which revolves around interactions between anthocyanins and chemotherapeutics and investigates, if they might be able to avert side effects of chemotherapeutic drugs in the colon. This thesis focuses primarily on the immunomodulatory effects of anthocyanins contained in multiple berry extracts in HCT116 human colon carcinoma cells and HCEC-1CT immortalized human colonic epithelial cells and on the effects of SN38 on NF- κ B in THP-1 monocytes. As a potential point of interaction between chemotherapeutics and anthocyanins, the interaction of SN38 with the NF- κ B pathway is investigated. The NF- κ B transcription factor plays an important role in inflammation, immune response and apoptosis as it regulates the expression of numerous components of the immune system including proinflammatory cytokines. The NF- κ B activation potential of SN38 is evaluated on human THP-1 monocytes, specifically THP-1 Lucia™ cells, with and without an NF- κ B activating stimulus (LPS). The four chosen berry extracts, blackberry, bilberry, elderberry and black currant, are studied regarding their immunomodulatory effects. They each have unique anthocyanin profiles. As discussed in 1.2.5, anthocyanins have been shown to interfere with the immune system on multiple levels, specifically in the expression of various inflammatory biomarkers. The immunomodulatory markers of interest are chosen based on their expression and measurability in the cell lines according to the available literature. The aim of this thesis is to investigate the immunomodulatory effects of each berry extract, by characterizing their effects on inflammation associated (TNF- α , IL-2, IL-6, IL-8, IL-18, IL-33, IL-1 β , COX-2, iNOS) and immuno-checkpoint (PD-L1, CD70) marker levels. In this context the question is addressed, whether there is a difference in the immunomodulatory effects of the berry extracts between cancerogenic (HCT116) and non-cancerogenic (HCEC-1CT) cells. On a transcript level the target RNA will be measured by a custom Invitrogen™ QuantiGene™ Plex Gene Expression Assay (Thermo Fisher Scientific) with Luminex® xMAP technology and the additional use of qRT-PCR.

Also, the effect of citric acid, used as a solvent in the first *in vivo* study of the project, will be assessed *in vitro* as an additional experiment. The cytotoxic effects of citric acid, alone and in combination with the berry extracts, are investigated via WST-1 and SRB assay in HCT116,

HCEC-1CT and murine CT26 cells. The aim is to investigate and compare the effects of citric acid in tumorigenic (HCT116) and non-tumorigenic (HCEC-1CT), as well as murine tumorigenic (CT26) cell lines. Additionally, to investigate the bioavailability of the anthocyanins in the berry extracts, data of the *in vivo* interim studies II (only feces) and III of the project is evaluated as well. This includes the sample preparation of urine, plasma and feces samples provided by the group of Univ.-Prof. Dr. Walter Berger at the Medical University of Vienna, who performed the *in vivo* experiments. The subsequent sample measurement is conducted via LC-MS/MS. Other additional experiments include cytotoxicity testing of four polyphenols included in the berry extracts (namely (+)-rutin, (-)-epicatechin, (-)-epigallocatechin and catechol)

3. Results:

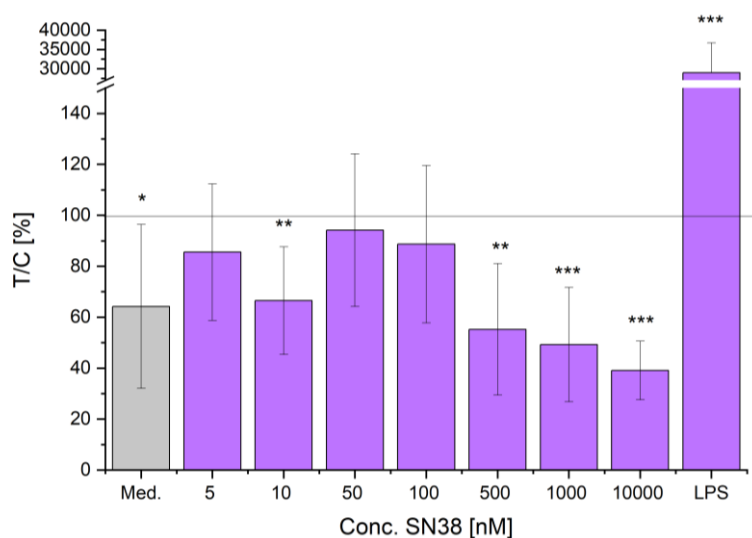
3.3. NF- κ B Activation by SN38

Chemotherapeutics, such as irinotecan, are proposed to activate the NF- κ B pathway, leading to the release of pro-inflammatory cytokines, thereby affecting tumor cell growth via pro-apoptotic pathways. There is no clear consistency in literature regarding the questions; whether irinotecan or SN38 can activate the pathway themselves or if they modulate already activated NF- κ B. Based on the available literature, discussed in chapter 1.3.1, two experiments were performed evolving around the NF- κ B activation by SN38, one with the addition of LPS to activate the pathway and one without, to investigate whether SN38 can modulate the activated pathway or activate it by itself, respectively. Simultaneously, CellTiter-Blue® Cell Viability Assay (CTB) was performed to determine the toxicity of SN38. The NF- κ B activation was determined with the use of THP1-Lucia™ NF- κ B cells (by InvivoGen), which are derived from the human THP-1 monocyte cell line by stable integration of an NF- κ B-inducible luciferase reporter construct. Consequently, the cells allow the monitoring of NF- κ B activation by determining the activity of secreted luciferase (Lucia). The levels of NF- κ B-induced Lucia in the cell culture supernatant are readily assessed with QUANTI-Luc™, a luciferase detection reagent.¹⁰⁴ To detect the cytotoxicity the CTB was performed immediately after the NF- κ B assay with the same plate. For the incubation 100k cells/well were seeded in 96 well plates and SN38 was added in the concentrations 5, 10, 50, 100, 500, 1000 and 10000 nM. The maximum DMSO concentration in all incubation conditions was 0.05 %. As positive control 10 ng/ml LPS was used in the NF- κ B assay and 0.1 % triton X in the CTB assay. The incubation time was 20 h. For the co-incubation experiments with LPS, 10 ng/ml LPS was added after 2 h of incubation to co-incubate for the 18 remaining hours.

As shown in Figure 9 the addition of LPS, serving as a positive control for NF- κ B stimulation, significantly differed from the solvent control (DMSO) and all used SN38 concentrations ($p < 0.001$). In the experiments without any additional LPS, SN38 showed no NF- κ B induction in the used concentrations (see Figure 9 A), ranging from 5-10000 nM SN38. The concentrations 5, 50 and 100 showed no significant deviation from the solvent control. There was a significant reduction of NF- κ B activation compared to the control with added concentrations of 10, 500 ($p < 0.01$), 1000 and 10000 ($p < 0.001$) nM SN38. No SN38 concentrations significantly different from each other ($p < 0.05$). However, SN38 was found to be cytotoxic in the highest

concentrations by the CTB-assay, 1000 and 10000 nM SN38, with cell viability going down to 20 %, as shown in Figure 9 B. The SN38 concentrations 5-500 nM showed no significant cytotoxicity compared to the control, but the toxicity looked to be concentration dependent with slight downregulations starting from 500 nM. The downregulation of the NF- κ B activation in the higher concentrations, mainly 1000 and 10000 nM, shown in the NF- κ B assay, are most probably caused by the concentration dependent cytotoxicity of SN38, as demonstrated by the CTB assay.

(A)



(B)

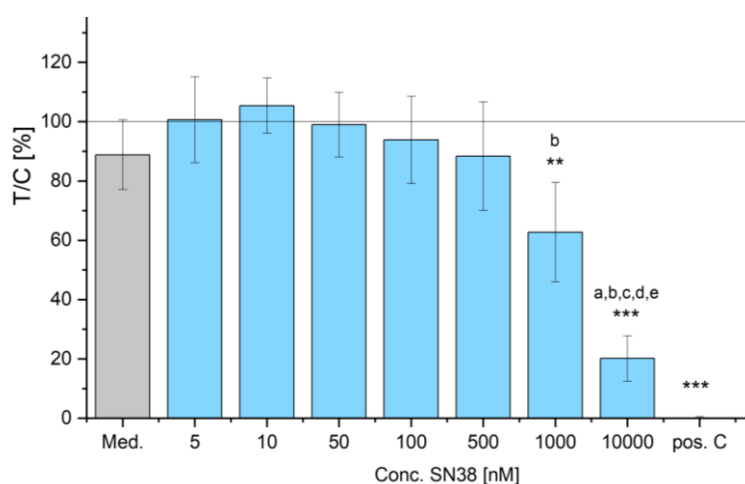
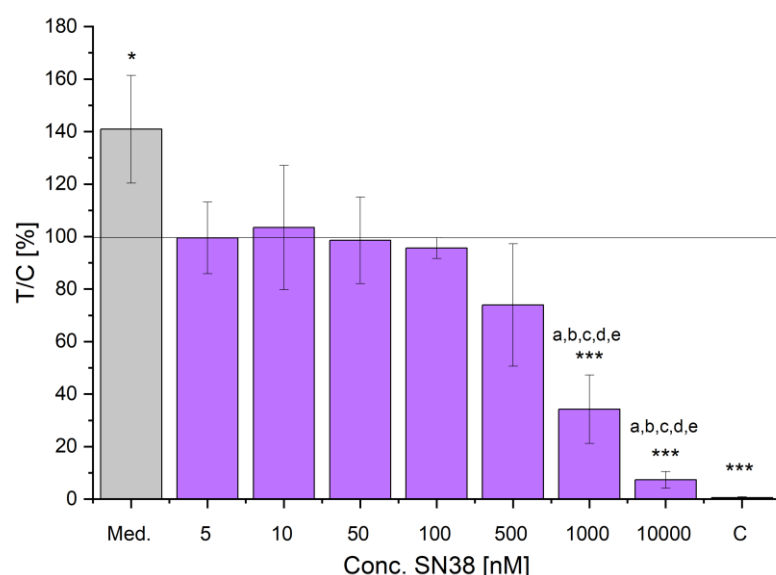


Figure 9: Effects of SN38, in a range from 5-10000 nM, on the NF- κ B activation (A) and cytotoxicity (assessed with the CellTiter-Blue® Cell Viability Assay) (B) in THP1-Lucia™ NF- κ B cells (seeded 100000 cells/well) after incubation time of 20 h. Medium (med.) alone. Results are expressed as means $\pm$ SD of 5-7 independent experiments, normalized to the solvent control (Ctrl., 0.05 % DMSO, γ =100). Statistical significances were determined by one-sample Student's t-test to determine differences between samples and the solvent control (* p <0.05, ** p <0.01 and *** p <0.001) and bonferroni ANOVA (p <0.05) for differences between test concentrations. As a positive control 10 ng/mL LPS was used for the NF κ B assay and 0.1 % triton X for the CTB assay.

In the second experiment, the same SN38 concentrations were co-incubated with 10 ng/ml LPS which serves as a stimulus for NF- κ B activation in THP1 monocytes. The solvent control (C) without LPS was significantly different from any incubation that included added LPS ($p < 0.001$), meaning LPS activated the NF- κ B pathway potently. For statistical analysis 10 ng/ml LPS was used as a control to determine potential significant differences of SN38. As shown in the figures below, in the co-incubation of SN38 and LPS, SN38 did not upregulate the NF- κ B induction by 10 ng/ml LPS in any of the used concentrations, ranging from 5 to 10000 nM SN38 (Figure 10 A). The concentrations 5-500 did not significantly differ from the LPS control. The highest concentrations 1000 and 10000 nM SN38 downregulated the LPS induced NF- κ B activity significantly ($p < 0.001$). They also significantly differ from the other SN38 concentrations ($p < 0.05$). The medium without DMSO addition differ significantly ($p < 0.05$) from the LPS control as it showed higher NF- κ B activity. As shown in Figure 10 (B), SN38 was again cytotoxic in the higher concentrations in a dose dependent manner, with significant downregulations starting from 100 and 500 nM SN38 ($p < 0.05$) with approximately 80 % cell viability, 1000 nM ($p < 0.01$) with 50 % to 10000 nM ($p < 0.001$) with 20 % cell viability. The CTB assay showed no significant difference between the control and the concentrations of 5, 10 and 500 nM SN38, cytotoxicity is therefore unlikely.

(A)



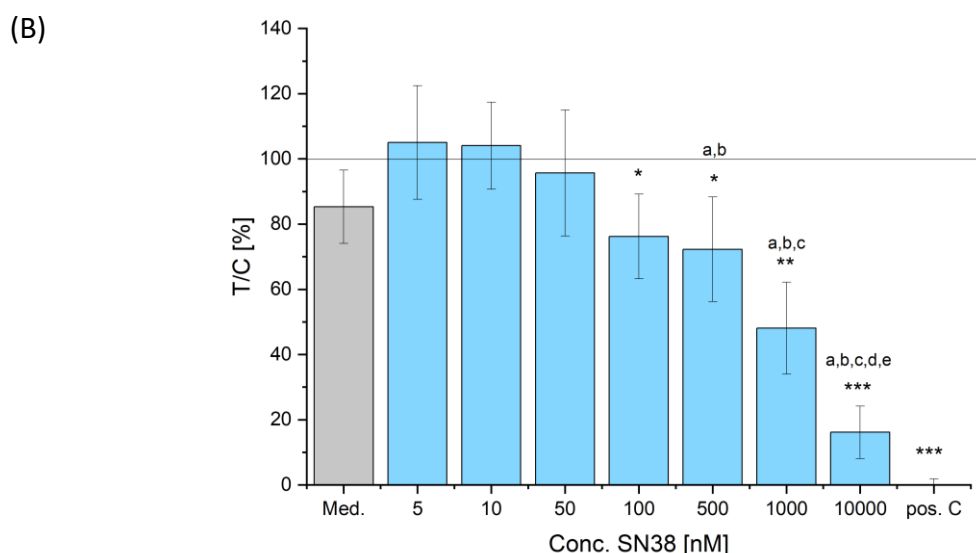


Figure 10: Effects of SN38, in a range from 5-10000 nM on the NF κ B activation (induced by 10 ng/ml LPS) (A) and cytotoxicity (assessed with the CellTiter-Blue® Cell Viability Assay) (B) in THP1-Lucia™ NF- κ B cells (seeded 100000 cells/well) after incubation time of 20 h. Medium (med.) alone. Results are expressed as means $\pm$ SD of 5-7 independent experiments, normalized to the control (10 ng/ml LPS $y=100$). Statistical significances were determined by one-sample Student's t-test for differences between samples and the control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and bonferroni ANOVA ($p<0.05$) between test concentrations. The NF κ B assay contains a solvent control without added LPS (C) and the positive control for the CTB assay was 0.1 % triton X.

The stimulation of THP1-Lucia™ cells with SN38 in concentrations between 5 and 100000 nM, does not seem to activate the NF- κ B pathway by itself nor upregulate already activated NF- κ B by 10 ng/ml LPS. The NF- κ B activity was downregulated by SN38 in higher doses, corresponding to the cytotoxicity observed with the CTB assay.

3.4. Immunomodulatory Effects of Berry Extracts

In the present thesis, immunomodulatory effects of berry extracts were investigated in the cancerous colon cell line HCT116 and the non-cancerous colon epithelial HCEC-1CT cells after incubation of 5 hours with concentrations 1, 10 or 100 μ g/ml of either blackberry, bilberry, elderberry or black currant extract. Additionally, after 2 hours 25 ng/ml IL-1 β was added as an inflammatory stimulus. On a transcript level the target RNA was measured by a custom Invitrogen™ QuantiGene™ Plex Gene Expression Assay (Thermo Fisher Scientific) based on Luminex® xMAP technology, quantitative real-time PCR (qRT-PCR) was employed additionally. The Invitrogen™ QuantiGene™ Plex Assay Kit allows the multiplexed measurement of gene expression by combining branched DNA (bDNA) signal amplification with Luminex® xMAP™ multi-analyte profiling technology. The initial targets were determined by literature research and included the following: IL-1 β , IL-2, IL-6, IL-8, IL-18, IL-33, TNF- α , iNOS, COX-2, CD70 and

PD-L1. Four housekeeping (HKG) genes recommended by the manufacturer for expected low expression were used; TFRC (transferrin receptor), POLR2A (polymerase II subunit A), HPRT1 (hypoxanthine-guanine phosphoribosyltransferase) and HMBS (hydroxymethylbilane synthase), which are expressed in constant rates and are used to normalize mRNA expression. Besides the different berry concentrations, solvent control without stimulation (DMSO 0.5 %), IL-1 β control from three biological replicates and three blanks were added and measured. All samples were measured as duplicates. The solvent control contained 0.5 % DMSO and was not stimulated with IL-1 β . IL-8 was expressed in very high concentrations in both cell lines and could not be quantified due to signal saturation. Subsequently, qRT-PCR was employed for quantification of IL-8 and other selected targets. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a widely-used method to elucidate and quantify changes of gene expression patterns. The combined advantages of high throughput, practical simplicity, reliable specificity and its sensitivity make it the most commonly used method for quantification of RNA in research applications.^{105, 106} In this case, it is applied to analyse minimal quantities of gene transcripts to monitor biological responses to diverse stimuli. The HK used for qRT-PCR experiments were HPRT1 and GAPDH and all the samples were measured as duplicates as well.

HCT116 cells:

After a pilot test to determine the optimal RNA concentration needed to detect the targets, the first measurement was performed with the HCT116 cells. The pilot test included 150, 300 and 500 ng RNA per well (20 μ l) and afterwards, according to the observed gene expression, it was decided on a concentration of 500 ng RNA per well. IL-8 was expressed so highly that the signal was saturated in the measurement and had to be excluded. Unfortunately, besides the saturated IL-8, IL-2, IL-33, IL-6, CD70 and iNOS also had to be taken out of the experiment due to low expression in HCT116 cells. The detected signals of these genes laid generally under 20 median fluorescence intensity (MFI) and could not be significantly differed from the blank and controls. The remaining genes, IL-18, IL-1 β , TNF α , PD-L1 and COX-2, were normalized to the mean of all four housekeepers after blank deduction and set in relation to the IL-1 β control. Table 5 shows the results of the signal relation to the IL-1 β control, after blank deduction, as a mean of the 3 replicates with the corresponding standard deviation. The raw data of the measurement is listed in the appendix (Table 25).

Table 5: Relative gene expression of IL-1 β , TNF α , PD-L1, IL-18 and COX-2 in HCT116 cells treated with 1, 10 or 100 μ g/ml of blackberry (BB), bilberry (Bil), elderberry (EB) or black currant (BC) extract for 5 hours, 3 of which with 25 ng/ml IL-1 β as a stimulus (n=3). The DMSO control was not stimulated. The relative gene expression was calculated with cells only treated with 25 ng/ml IL-1 β as calibrator.

	IL-1 β	SD	TNF α	SD	PD-L1	SD	IL-18	SD	COX2	SD
DMSO	0.85	0.54	0.07	0.04	0.26	0.07	0.73	0.09	0.28	0.12
1BB	1.22	0.66	0.95	0.74	0.97	0.14	1.01	0.17	1.07	0.30
10BB	1.58	1.18	0.85	0.02	1.07	0.10	1.07	0.15	1.26	0.37
100BB	1.71	1.22	0.93	0.22	1.12	0.12	1.09	0.06	1.19	0.35
1Bil	1.58	1.06	1.10	0.08	1.05	0.10	1.03	0.07	0.98	0.20
10Bil	1.47	0.69	1.06	0.27	1.00	0.12	1.05	0.08	1.21	0.22
100Bil	1.34	0.63	1.13	0.38	1.08	0.11	1.12	0.13	1.36	0.36
1EB	1.06	0.69	1.26	0.27	1.06	0.19	1.02	0.11	1.09	0.51
10EB	1.27	0.99	0.95	0.08	1.02	0.20	1.00	0.11	1.22	0.05
100EB	1.34	0.70	1.01	0.30	1.09	0.31	1.16	0.06	1.53	0.31
1BC	1.33	1.19	0.73	0.14	1.14	0.23	1.05	0.08	1.26	0.50
10BC	1.16	0.48	0.87	0.37	1.06	0.25	1.06	0.02	1.37	0.50
100BC	1.21	0.28	1.12	0.18	1.00	0.11	1.35	0.15	1.44	0.25

The graphs below in Figure 11 show the relative gene expression of HCT116 cells treated with the different extracts, blackberry, bilberry, elderberry and black currant in concentrations of 1, 10 and 100 μ g/ml for 5 h and stimulated with 25 ng/ml IL-1 β for 3 h, as a mean of 3 biological replicates with the standard deviations.

In the HCT116 cells the solvent controls of TNF α , PD-L1, IL-18 and COX-2 were significantly lower than the control stimulated with IL-1 β during incubation (*p<0.5, **p<0.01, ***p<0.001), indicating that those targets are stimulated by IL-1 β . IL-1 β itself did not significantly differ between the two controls or berry extract. The standard deviations were particularly high for IL-1 β and it was expressed relatively poor (raw data in appendix Table 25).

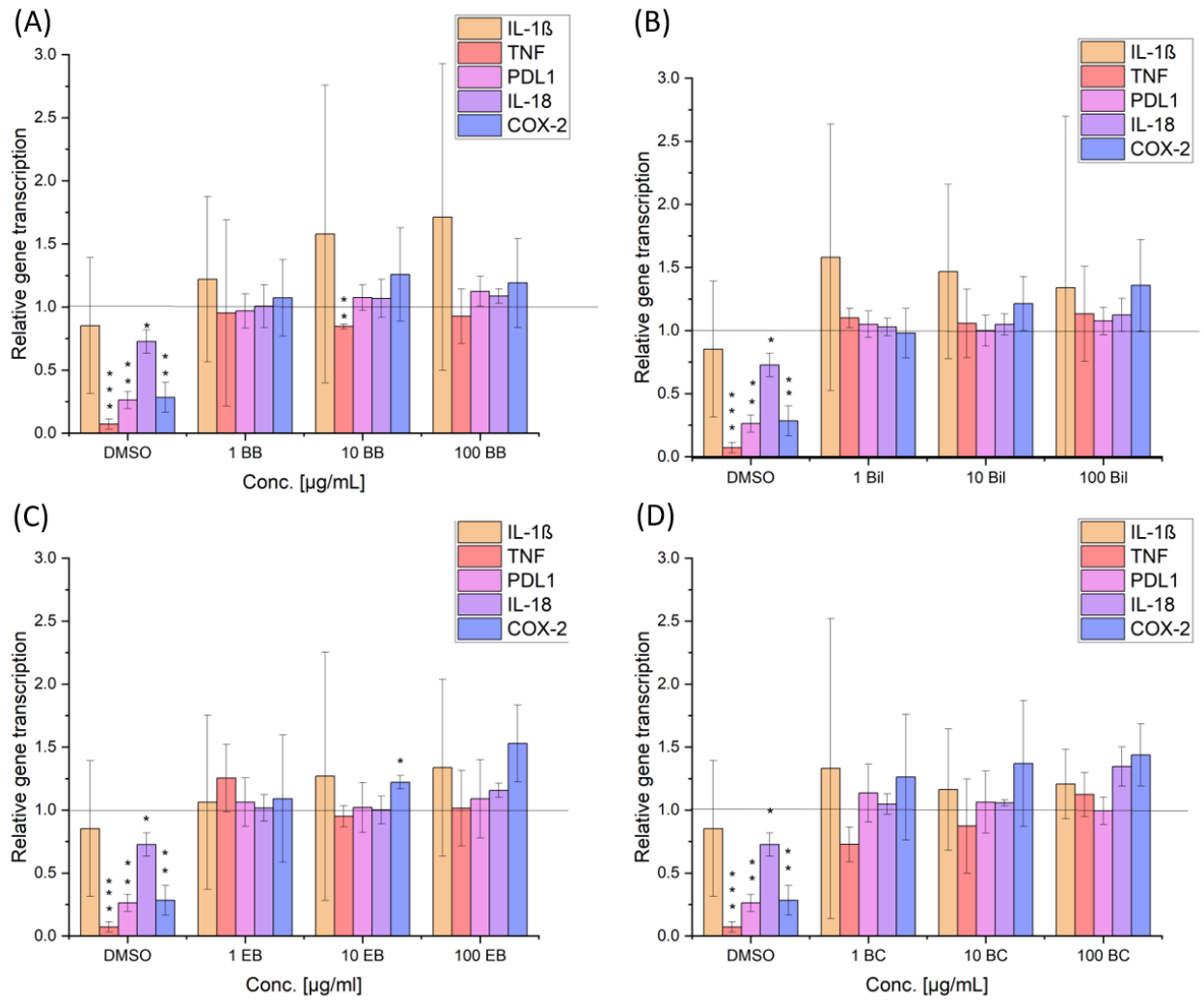


Figure 11: Relative gene transcription of IL-1 β , TNF α , PD-L1, IL-18 and COX-2 in HCT116 cells after 5 h exposure to berry extract; Blackberry (BB)(A), bilberry (Bil) (B), elderberry (EB) (C) and black currant (BC) (D) and stimulation with 25 ng/ml IL-1 β for 3 h. The DMSO control was not stimulated with IL-1 β . Results are expressed as relative transcription levels normalized to the HKGs TFRC, POLR2A, HPRT1 and HMBS compared to the IL-1 β control (calibrator, $y=1$). Data is shown as means $\pm$ SD of 3 independent experiments measured in technical duplicates with Luminox[®] xMAP technology. Statistical significances were determined by one-sample Student's *t*-test for significances between the extracts and the IL-1 β control. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

HCT116 cells treated with blackberry, bilberry, elderberry and black currant extract in doses of 1, 10 and 100 μ g/ml for 5 hours, 3 of which stimulated with 25 ng/ml IL-1 β , did not show a significant down or upregulation in relative gene transcription of either IL-1 β , PD-L1 or IL-18. No significant concentration dependent trends could be observed for COX-2 and TNF α either. However, 10 μ g/ml of elderberry extract showed a slight but significant upregulation of COX-2 gene expression (Figure 11 C) and 10 μ g/ml of blackberry extract showed a slight but significant downregulation of the TNF α expression (Figure 11 A). There might be a tendency

of COX-2 expression being upregulated in treatment with elderberry and black currant extract but, partly due to the large standard deviations, it is hard to draw conclusions. There were multiple problems with the Luminex® instrument during measurement of the plate and the measurement had to be stopped multiple times. In the end, the measurement took over a week to complete. This is a possible reason for the large standard deviations and makes it difficult to interpret the data. Taking the data as it is, the experiment showed; HCT116 cells do not express multiple of our selected targets in significant concentrations even when stimulated with IL-1 β and using the highest RNA concentration recommended (500 ng/well). This led to IL-2, IL-33, IL-6, CD70 and iNOS having to be removed from the evaluation. In the targets which could be used for the evaluation, IL-1 β , TNF α , PD-L1, IL-18 and COX-2, standard deviations were very high and no significant trends could be observed after treatment with either blackberry, bilberry, elderberry nor black currant extract in concentrations between 1 and 100 μ g/ml. Since the IL-8 expression in the HCT116 cells was so high after stimulation with IL-1 β that the signal was saturated in the Luminex® instrument, qRT-PCR was performed additionally. After evaluating the data of the Luminex® experiment and due to the problems during measurement, IL-1 β and TNF α were also added to the qRT-PCR experiment to verify the data.

qRT-PCR was used to quantify changes of gene expression patterns in the experiment at hand. It is the most commonly used method for quantification of RNA in research applications.^{105, 106} The HKGs used in this experiment were HPRT1 and GAPDH and all the samples were measured as duplicates. Table 6 shows the results expressed as means $\pm$ SD of 3 independent experiments. Results are expressed as relative transcription levels ($2^{-\Delta\Delta C_T}$) normalized to the HKGs HPRT1 and GAPDH, compared to the solvent control (calibrator, $y=1$). The C_T (threshold cycle) is the quantitative endpoint of PCR experiments, where the fluorescent signal is in the exponential phase of cycling and reaches a discretionary placed threshold.

$$\Delta C_T = C_T(\text{target}) - C_T(\text{HKGs})$$

$$\Delta\Delta C_T = \Delta C_T(\text{target}) - \Delta C_T(\text{calibrator})$$

$2^{-\Delta\Delta C_T}$ values express gene transcription as n-fold change relative to the IL-1 β control (calibrator).

Table 6: Relative quantification of IL-1 β , TNF α and IL-8 mRNA upon 5 h exposure to 1, 10, 100 μ g/ml blackberry (BB), bilberry (Bil), elderberry (EB) and black currant (BC) extract and 3 h exposure to 25 ng/ml IL-1 β in HCT116 cells. Results are expressed as relative transcription levels ($2^{-\Delta\Delta C_T}$) normalized to the HKGs HPRT1 and GAPDH, compared to the IL-1 β control (calibrator, $y=1$).

	IL-1 β	SD	TNF α	SD	IL-8	SD
IL-β	1.01	0.01	1.01	0.01	1.01	0.01
DMSO	0.00	0.00	0.01	0.01	0.00	0.00
BB 1	0.81	0.20	0.92	0.29	0.90	0.15
BB 10	0.89	0.24	0.80	0.29	0.81	0.15
BB 100	0.94	0.32	0.92	0.43	0.90	0.27
Bil 1	0.95	0.33	0.98	0.38	0.93	0.26
Bil 10	1.04	0.14	1.29	0.51	1.13	0.18
Bil 100	1.71	0.27	1.76	0.65	1.11	0.11
EB 1	1.34	0.55	1.90	0.42	1.59	0.36
EB 10	1.17	0.52	2.49	1.56	1.58	0.32
EB 100	1.24	0.65	1.97	0.76	1.66	0.23
BC 1	1.29	0.30	1.73	1.02	1.38	0.38
BC 10	1.41	0.38	2.17	0.80	1.39	0.23
BC 100	1.31	0.21	2.20	0.92	1.12	0.22

The graphs below in

Figure 12, show the relative gene expression of HCT116 cells treated with the different extracts, corresponding to the data shown in Table 6. Overall, the results of the qRT-PCR experiment align with the data from the Luminex[®] experiment. Treatment of HCT116 cells with 1, 10 or 100 μ g/ml of blackberry, bilberry, elderberry or black currant extract for 5 h, 3 of which with 25 ng/ml IL-1 β stimulation, shows no significant deviations in gene expression regarding IL-1 β , TNF α and IL-8. The upregulation of those 3 genes when comparing stimulated and non-stimulated samples is shown more clearly in the qRT-PCR than the Luminex[®] experiment, with a relative gene expression of almost zero for the non-stimulated cells compared to the IL-1 β control. Here IL-1 β expression differs significantly between the two controls ($p<0.001$), in contrast to the Luminex experiment. The extracts, however, show no

significant differences to the IL-1 β control. Tendencies could be observed for TNF α being upregulated in a dose dependent manner by BC and Bil extract and IL-1 β being upregulated by Bil extract in a dose dependent manner as well, but neither was significant.

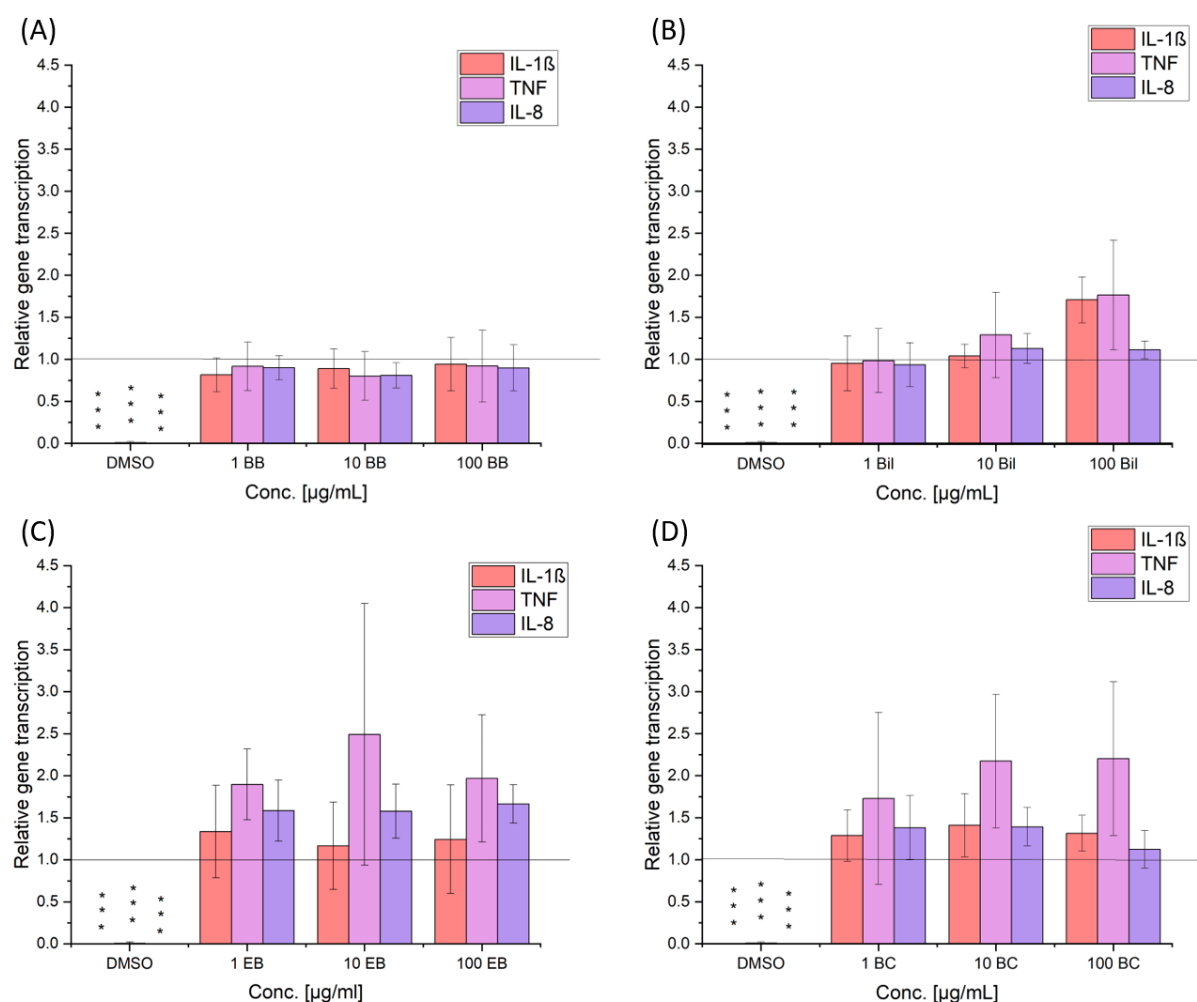


Figure 12: Relative gene transcription of IL-1 β , TNF α and IL-8 in HCT116 cells after 5 h exposure to berry extracts, 3 h of which with 25 ng/ml IL-1 β stimulation. Results are expressed as relative transcription levels ($2^{-\Delta\Delta CT}$) normalized to the HKGs HPRT1 and GAPDH, compared to the IL-1 β control (calibrator, $y=1$). Data is shown as means $\pm$ SD of 3 independent experiments measured in technical duplicates with qPCR. Statistical significances were determined by bonferroni ANOVA ($p<0.05$) for test concentrations and by one-sample Student's t-test for significances between the IL-1 β stimulus and the solvent control or the different concentrations, 1, 10, 100 μ g/ml of blackberry (BB) (A), bilberry (Bil) (B), elderberry (EB) (C) and black currant (BC) (D) extract. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Overall, no significant immunomodulatory effects by berry extracts, blackberry, bilberry, elderberry and black currant, could be observed in HCT116 cells.

HCEC-1CT:

The HCEC-1CT samples were also measured with the Luminex[®] instrument at first and later qRT-PCR was added additionally. According to the test sample already measured with the HCT116 samples, also 500 ng RNA per well were used in this plate. Based on this test sample, it was also possible to eliminate the targets IL-8 and IL-18 from the plate. IL-8 was expressed very potently and the signal was saturated, thus it had to be measured by qRT-PCR as well. IL-18 showed to be expressed very poorly and was excluded due to low expression.

Although the test samples looked promising, ultimately three targets also had to be eliminated from this evaluation due to poor expression and thereby low signals. The signals of IL-2, IL-33 and iNOS were on average under 20 MFI and were thus cut from the evaluation (raw data in appendix). Unfortunately, it was due to problems with the Luminex[®] instrument again not possible to measure the plate immediately and the 24 h storage period recommended by the manufacturer was exceeded. The plate was measured in one sitting after a storage period of around 48 h. There were some abnormalities when evaluating the data of the HCEC-1CT cells; the values for the housekeeping genes showed fluctuations in both the Luminex[®] and PCR experiment. Because of this, the results have to be treated with caution. Shown in Table 7 is the relative gene expressions of the remaining targets, IL-1 β , TNF α , IL-6, PD-L1, CD70 and COX-2, as a mean of 3 biological replicates with the standard deviations, in HCEC-1CT cells treated with blackberry, bilberry, elderberry and black currant extract in concentrations of 1, 10 and 100 μ g/ml for 5 h and stimulated with 25 ng/ml IL-1 β for 3 h. The graphs below in Figure 13 show the relative gene expression of HCEC-1CT cells treated with the different extracts, blackberry (A), bilberry (B), elderberry (C) and black currant (D) in concentrations of 1, 10 and 100 μ g/ml for 5 h and stimulated with 25 ng/ml IL-1 β for 3 h, as a mean of 3 biological replicates with the standard deviations, corresponding to the data in Table 7.

Table 7: Relative gene expression of IL-1 β , TNF α , IL-6, PD-L1, CD70 and COX-2 in HCEC-1CT cells treated with 1, 10 or 100 μ g/ml of blackberry (BB), bilberry (Bil), elderberry (EB) or black currant (BC) extract for 5 hours, 3 of which with 25 ng/ml IL-1 β as a stimulus (n=3). The DMSO control was not stimulated. The relative gene expression was calculated from cells only treated with 25 ng/ml IL-1 β .

	IL-1 β	SD	TNF α	SD	IL-6	SD	PD-L1	SD	CD70	SD	COX-2	SD
DMSO	0.01	0.01	0.06	0.04	0.00	0.00	0.50	0.16	0.04	0.03	0.02	0.00
IL1β	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
BB1	1.01	0.02	1.08	0.50	0.95	0.10	0.93	0.22	0.96	0.07	1.07	0.06
BB10	0.99	0.07	1.09	0.02	0.90	0.19	0.88	0.14	1.01	0.19	1.23	0.19
BB100	0.69	0.60	0.48	0.42	0.60	0.56	0.66	0.58	0.47	0.46	0.75	0.68
Bil1	1.03	0.29	0.53	0.11	1.18	0.16	0.95	0.25	0.59	0.09	1.18	0.20
Bil10	1.12	0.08	0.69	0.43	1.33	0.19	0.97	0.34	0.70	0.19	1.15	0.06
Bil100	1.19	0.13	0.93	0.35	1.17	0.12	1.06	0.28	0.88	0.24	1.26	0.04
EB1	1.03	0.18	0.97	0.40	1.07	0.07	0.91	0.05	0.81	0.32	1.08	0.06
EB10	1.06	0.15	1.12	0.33	1.04	0.11	1.10	0.10	0.75	0.20	1.15	0.23
EB100	1.39	0.19	1.00	0.30	1.19	0.05	0.94	0.23	0.68	0.06	1.62	0.20
BC1	1.00	0.20	0.82	0.18	0.98	0.15	1.06	0.24	0.84	0.14	1.02	0.19
BC10	1.03	0.10	1.20	0.11	0.84	0.11	0.91	0.12	1.09	0.11	1.06	0.18
BC100	1.11	0.10	1.36	0.61	0.83	0.33	1.05	0.26	1.17	0.25	1.29	0.28

In HCEC-1CT cells, the relative gene expression of IL-1 β , TNF α , IL-6, COX-2, PD-L1 and CD70 differed significantly (*p<0.5, **p<0.01, ***p<0.001), between the solvent control (0.5 % DMSO) and the control stimulated with IL-1 β during incubation, indicating that those targets are stimulated by 25 ng/ml IL-1 β . The BB extract (Figure 13 A) showed a slight upregulation of TNF α with the concentration of 10 μ g/ml, but to a very small degree (1.09 fold). No other target showed significant up or downregulation in any concentration of BB extract. Unfortunately, for the concentration of 100 μ g/ml BB only two biological samples were available for evaluation, thus no statistical analysis could be performed, making data interpretation difficult. However, the means of these two replicates would point towards a downregulation of TNF α and CD70 by 100 μ g/ml BB, while for the other target genes the SDs are unfortunately so high that no interpretation can be drawn. The Bil extract showed some significant changes compared to the control (Figure 13 (B)); 1 μ g/ml Bil significantly

downregulated TNF α and CD70, 10 μ g/ml Bil significantly upregulated IL-6 and COX-2 and 100 μ g/ml Bil also significantly upregulated COX-2. Next to the significant downregulation of TNF α at 1 μ g/ml, tendencies could be observed for a concentration dependent increase of TNF α gene transcription. The downregulation of CD70 seems to reverse in higher concentrations but the expression is still low (not significant) with 10 μ g/ml Bil. EB (Figure 13 (C)) also showed a downregulation of CD70 at the highest concentration of 100 μ g/ml. At this concentration the EB extract showed an upregulation of COX-2 as well. IL-1 β showed slight, non-significant tendencies for upregulation when treated with 100 μ g/ml EB. No other significant changes could be observed in HCEC-1CT cells treated with the EB extract and no significant changes could be observed with the BC extract in any concentrations. All extracts showed slight non-significant tendencies for COX-2 upregulation in a concentration dependent matter. No other trends were observable throughout the extracts.

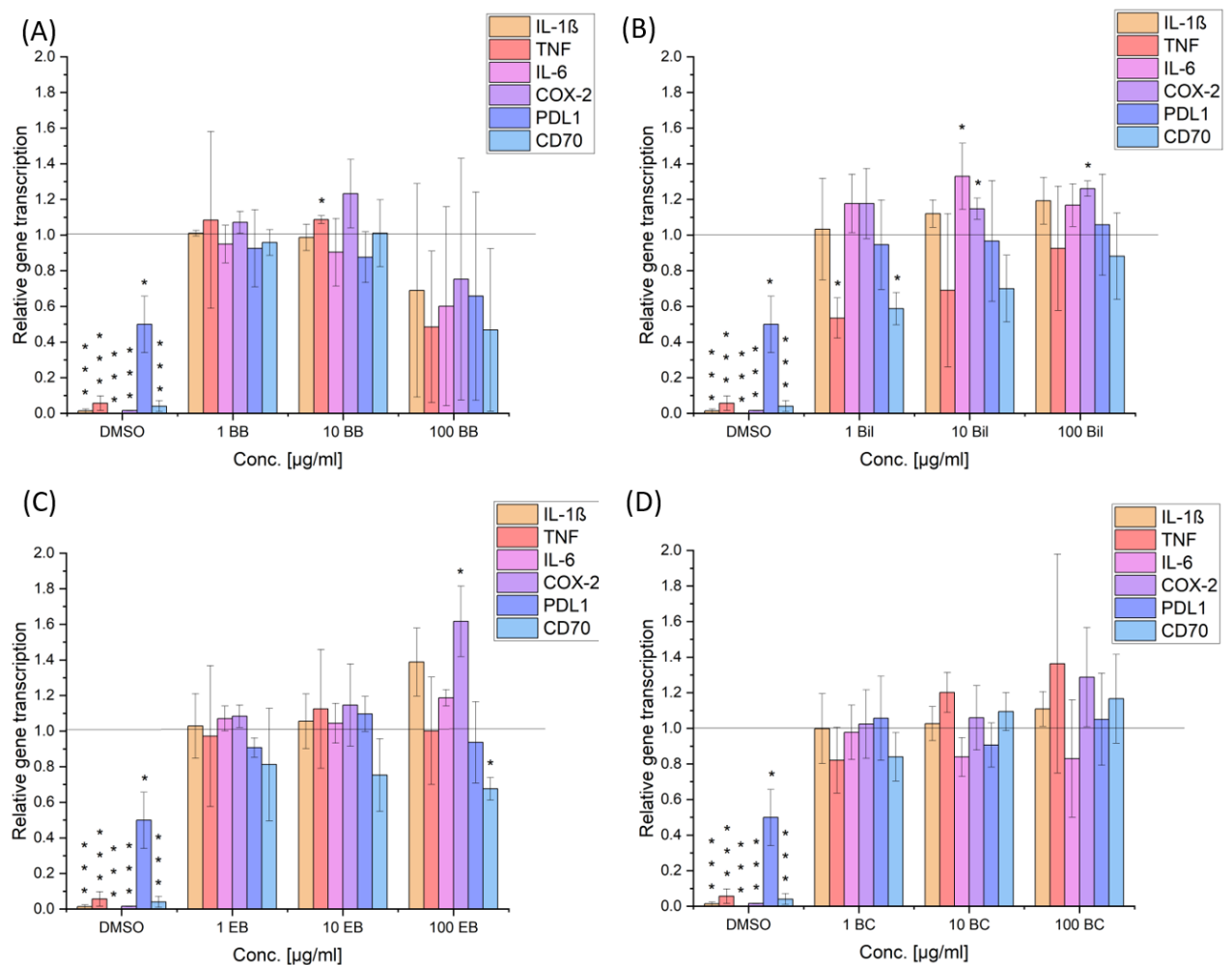


Figure 13: Relative gene transcription of IL-1 β , TNF α , IL-6, COX-2, PD-L1 and CD70 in HCEC-1CT cells after 5 h exposure to berry extract; Blackberry (BB) (A), bilberry (Bil)(B), elderberry (EB) (C) and black currant (BC) (D) and stimulation with 25 ng/ml IL-

18 for 3 h. The DMSO control was not stimulated with IL-18. Results are expressed as relative transcription levels normalized to the HKGs TFRC, POLR2A, HPRT1 and HMBS compared to the IL-18 control (calibrator, $y=1$). Data is shown as means $\pm$ SD of 3 independent experiments measured in technical duplicates with Luminex® xMAP technology. Statistical significances were determined by one-sample Student's *t*-test for significances between the extracts and the IL-18 control. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. No statistical analysis could be performed for 100 $\mu\text{g/ml}$ BB since there were only 2 replicates.

As mentioned above, IL-8 was excluded from the Luminex® experiment due to signal saturation in the test wells and thus was evaluated via qRT-PCR. To again verify the Luminex® data two other targets were added to the PCR experiment; IL-1 β and COX-2. The same qRT-PCR method was applied as with the HCT116 cells. Table 8 shows the relative gene expression measured by qRT-PCR expressed as means $\pm$ SD of 3 independent experiments.

Table 8: Relative quantification of IL-18, IL-8 and COX-2 mRNA upon 5 h exposure to 1, 10, 100 $\mu\text{g/ml}$ blackberry (BB), bilberry (Bil), elderberry (EB) and black currant (BC) extract and 3 h exposure to 25 ng/ml IL-18 in HCEC cells. Results are expressed as relative transcription levels ($2^{-\Delta\Delta C_T}$) normalized to the HKGs HPRT1 and GAPDH, compared to the IL-18 control (calibrator, $y=1$).

	IL1 β	SD	IL-8	SD	COX-2	SD
IL1β	1.04	0.03	1.03	0.03	1.03	0.03
DMSO	0.00	0.00	0.00	0.00	0.02	0.00
BB 1	1.01	0.47	0.64	0.13	0.68	0.12
BB 10	1.25	0.26	1.06	0.17	1.38	0.43
BB 100	1.37	0.21	1.70	0.96	1.63	0.16
Bil 1	1.36	0.29	1.30	0.27	2.02	1.06
Bil 10	1.32	0.34	1.52	0.54	2.20	1.21
Bil 100	1.23	0.50	2.01	1.20	2.34	1.29
EB 1	1.17	0.21	0.91	0.18	0.70	0.27
EB 10	1.39	0.52	1.40	0.67	0.96	0.34
EB 100	1.24	0.49	1.11	0.28	0.81	0.36
BC 1	1.23	0.39	1.00	0.21	1.21	0.51
BC 10	1.48	0.53	0.77	0.16	1.07	0.56
BC 100	0.90	0.08	0.65	0.20	1.30	0.37

The graphs below in Figure 14 show the relative gene expression of HCEC-1CT cells treated with the different extracts, blackberry (A), bilberry (B), elderberry (C) and black currant (D),

corresponding to the data shown in Table 8. Overall, the results of the qRT-PCR experiment somewhat but not completely align with the data from the Luminex® experiment. Treatment with 1, 10 or 100 µg/ml of blackberry, bilberry, elderberry or black currant extract for 5 h, 3 of which with 25 ng/ml IL-1β stimulation, showed no significant deviations in gene expression regarding IL-1β, and IL-8 and slight upregulations of COX-2. The qRT-PCR showed, like the Luminex® experiment, that the three genes are significantly (*p<0.05, **p<0.01, ***p<0.001) induced by the stimulation with 25 ng/ml IL-1β. The extracts, however, mainly show no significant differences to the IL-1β control. IL-1β showed no significant deviations from the control in either extract concentration. IL-8 also showed no effects of the extracts in any concentration although a slight tendency towards upregulation in the higher concentrations of BB and Bil might be present. Tendencies could also be observed for COX-2 being upregulated in a dose dependent manner, in the BB extract the upregulation was significant in the highest concentration of 100 µg/ml. Bil showed COX-2 upregulation in a dose dependent manner in all concentrations, although this was not significant due to the relatively large standard deviations. EB and BC did not show upregulations of COX-2. BC showed a slight trend for concentration dependent downregulation of IL-8, this was also not significant. Overall, immunomodulatory effects of the berry extracts in HCEC-1CT cells were, similarly to the HCT116, rather rare. IL-1β, IL-8 and PD-L1 showed no influence by either extract, concentration or test system. In the Luminex® experiment IL-6 showed one irregularity, being upregulated by 10 µg/ml Bil extract. TNFα showed a significant upregulation with the BB extract and a significant downregulation with the Bil extract. CD70 was downregulated with concentrations of Bil and EB. COX-2 showed tendencies for upregulation in both test systems but partly in different extracts. The Luminex® showed upregulations with Bil and EB, whereas the PCR showed a significant upregulation with BB and no effects with Bil and EB.

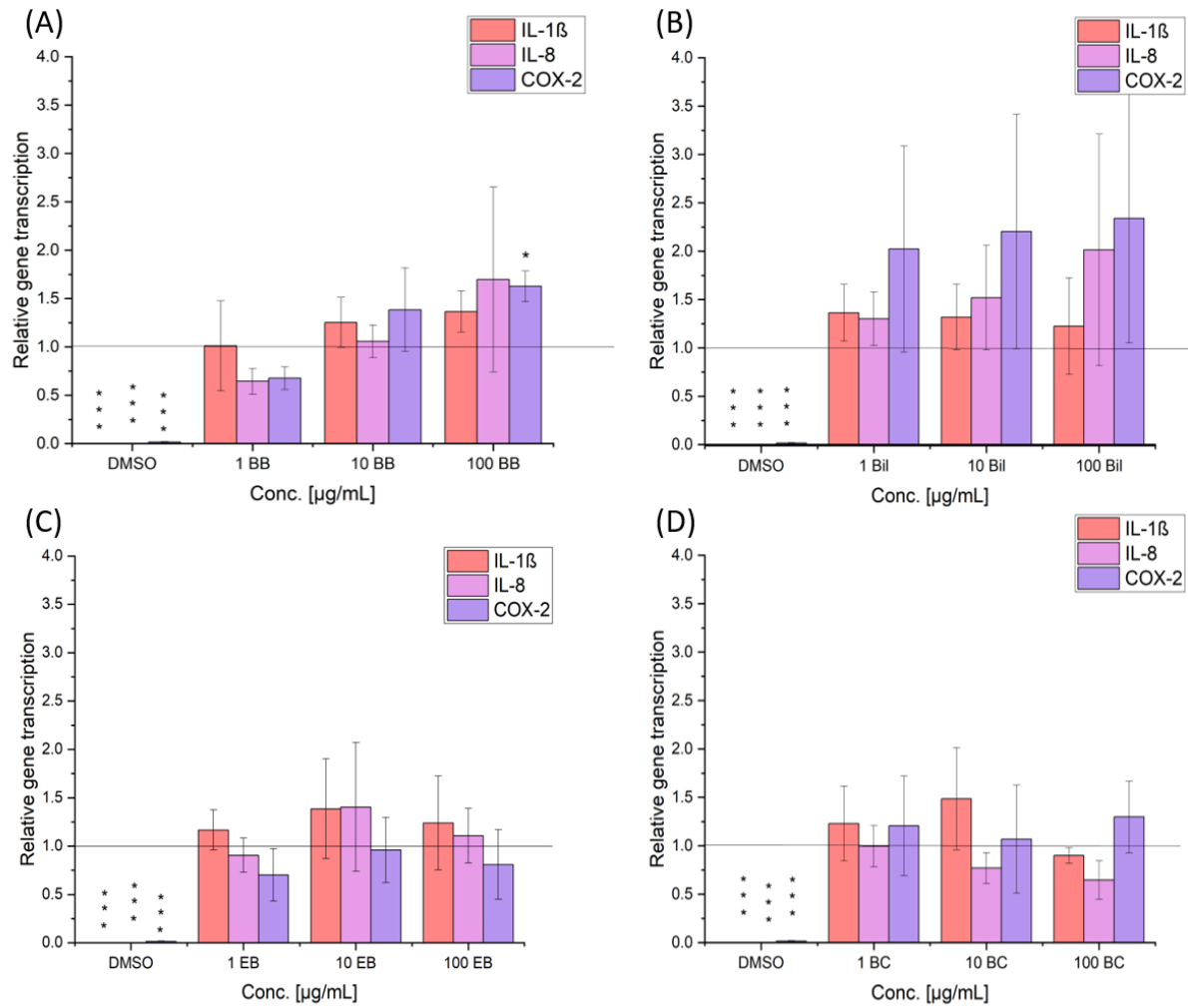


Figure 14: Relative gene transcription of IL-1 β , IL-8 and COX-2 in HCEC-1CT cells after 5 h exposure to berry extracts, 3 h of which with 25 ng/ml IL-1 β stimulation. Results are expressed as relative transcription levels ($2^{-\Delta\Delta C_T}$) normalized to the HKGs HPRT1 and GAPDH, compared to the IL-1 β control (calibrator, $y=1$). Data is shown as means $\pm$ SD of 3 independent experiments measured in technical duplicates with qPCR. Statistical significances were determined by bonferroni ANOVA ($p<0.05$) for test concentrations and by one-sample Student's t -test for significances between the IL-1 β stimulus and the solvent control or the different concentrations, 1, 10, 100 μ g/ml of blackberry (BB) (A), bilberry (Bil) (B), elderberry (EC) (C) and black currant (BC) (D) extract. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

4. Discussion

Anthocyanins are colouring plant substances that are subject of frequent discussion regarding their potential protection against a variety of chronic and degenerative diseases such as cancer, mainly gastrointestinal cancer, cardiovascular disease and diabetes.¹ Multiple immunomodulatory and anti-inflammatory effects have been reported. Anthocyanins have been shown to down-regulate the expression of various pro-inflammatory biomarkers such as NF- κ B, multiple cytokines and interleukins, IFN- γ , iNOS, COX -2 and more.^{65, 39, 66, 67, 68} NF- κ B controls the expression of numerous genes involved in the inflammatory response and *in vitro* anthocyanins seem to be able to suppress induced NF- κ B.⁷⁰ Dampening NF- κ B activation and thereby limiting the inflammatory response have been suggested as a potential strategy to prevent chronic inflammatory diseases and protect against the side effects of chemotherapy caused by inflammation. This thesis, therefore, aimed to shed light on immunomodulatory properties of anthocyanin-rich extracts and SN38. Firstly, possible activation of the NF- κ B pathway by the chemotherapeutic SN38 was investigated in THP-1 monocytes, as a possible point of interaction between chemotherapeutics and anthocyanins. Then immunomodulatory effects of four anthocyanin rich berry extracts, blackberry, bilberry, elderberry and black currant, were evaluated on tumorigenic (HCT116) and non-tumorigenic (HCEC-1CT) colon cells.

Specifically designed THP-1 monocytes, THP1-Lucia™ NF- κ B Cells (by InvivoGen), were incubated with concentrations ranging from 5 to 10000 nM SN38 with and without the additional stimulus of LPS (10 ng/ml) for 20 h and the NF- κ B activity was measured with the help of a luciferase detection reagent. According to the results obtained, SN38 does not seem to affect the NF- κ B pathway in human THP-1 monocytes at all; surprisingly no activation could be observed with sole SN38 treatment and also in combination with LPS, SN38 did not upregulate the expression. This is in contrast to most available literature. As discussed in chapter 1.3.1. it is assumed that chemotherapeutics, such as irinotecan and SN38, activate the NF- κ B pathway. This was observed in HeLa cells⁸³, NCI-N87 and AGS human gastric cancer cells⁸⁴ and colon26 cells⁸¹, amongst others. However, some studies also came to different results. Li *et al.* concluded in their *in vitro* study, that CPT-11 with its active metabolite SN38, only influences already activated NF- κ B by LPS.⁸⁵ The cell line and incubation times were similar to the ones used in this experiment but the NF- κ B activation was determined differently. Li *et*

al. also used THP-1 cells which were primed by LPS for 3 h, then treated with CPT-11 for 24 h. CPT-11 treatment significantly upregulated phosphorylation levels of JNK, STAT1 and NF- κ B in a dose-dependent manner. They then used an NF- κ B inhibitor to disturb the pathway and the accumulation of IL-1 β /IL-18 in the supernatant induced by CPT-11 decreased, leading to the conclusion that NF- κ B plays an important role in the inflammasome activity of CPT-11.⁸⁵ Wong *et al.* (2019) investigated RAW 264.7.Luc murine macrophages that stably bear the luciferase reporter gene controlled by the NF- κ B sensitive promoter, and concluded a downregulation of LPS induced NF- κ B, and thereby an anti-inflammatory effect of SN38. Cells were incubated for 6 h with 0.2, 2 and 20 μ M SN38 and an addition of 100 ng/ml LPS after 0.5 h. All three SN38 concentrations attenuated LPS-induced luciferase activity.⁸⁶ In the performed experiment, 10 ng/ml LPS were used for activation of NF- κ B, the SN38 concentration ranged from 5 to 10000 nM and the incubation time was 20 h. In the model used, a dose dependent downregulation of NF- κ B induced by LPS was also observed at concentrations of 1 and 10 μ M but this can be attributed very clearly to the dose dependent cytotoxicity observed by the CTB in those concentrations (see Figure 10). It is possible that results vary between cell lines, incubation duration and detection methods. The results obtained however indicate that SN38 does not activate the NF- κ B pathway directly, and thus, should not lead to the induction of proinflammatory cytokines, chemokines, adhesion molecules, inducible enzymes (COX2 and iNOS), proteins that regulate the specific immune response (major histocompatibility complex), co-stimulatory molecules crucial to the induction phase of specific immunity, and cytokines (IL-2, IL-12 and interferon- γ) that control lymphocyte proliferation and differentiation, through this pathway. However, it is also possible that the NF- κ B pathway gets activated indirectly in a way that is not reproducible in this single cell line, or simply in the cell line at hand. Further research needs to be done regarding the effects of chemotherapeutics on the NF- κ B pathway and its targets, since NF- κ B activation seems to be a mechanism by which tumors become resistant to apoptosis.⁸¹ Therefore, clarification is needed in regard to whether and how chemotherapeutics influence the pathway to better understand the relationship between chemotherapeutics and inflammation. The NF- κ B pathway would be potential point of interaction between chemotherapeutics and anthocyanins since *in vitro* they are able to suppress induced NF- κ B and could thus be proposed to enhance the anti-tumor effects of chemotherapeutics by increasing apoptosis via inhibition of NF- κ B.^{68, 70} Cen *et al.* (2021) showed LPS-boosted activity of NF- κ B of differentiated THP-1 reporter gene cells

was marginally inhibited by cy-3-glc, the main anthocyanin in the blackberry extract.⁶⁸ Since SN38 however, does not seem to activate the NF- κ B pathway, it was ruled out as a possible point of interaction and not included in the experiments regarding the immunomodulatory effects of anthocyanins. Still, inflammation plays a major role in chemotherapeutic side effects.⁷⁹ Hence, potential interference between anthocyanins and targets of irinotecan and other chemotherapeutics in this area are still likely.

The immunomodulatory effects of anthocyanin-rich berry extracts were investigated in HCT116 and HCEC-1CT cells. The cells were incubated for 5 h, 3 of which with the additional stimulation with IL-1 β , with 1, 10 or 100 μ g/ml of the berry extracts and the extracted RNA was measured using a custom Invitrogen™ QuantiGene™ Plex Gene Expression Assay (Thermo Fisher Scientific) based on Luminex® xMAP technology and qRT-PCR.

According to the results obtained, the colon cancer HCT116 cells seem to have very low expression of the targeted gens in general, even when stimulated. Only IL-8, IL-18, IL-1 β , TNF- α , PD-L1 and COX-2 were expressed in measurable quantities. Despite initial literature research showing otherwise; Salla and Zhang *et al.* additionally detected IL-6 and iNOS^{51, 58}, Dehghan *et al.* detected IL-33⁵⁴ and Wang *et al.* detected CD70⁶², in HCT116. Multiple targets had to be excluded from the experiment. The excluded genes were IL-8, IL-2, IL-33, IL-6, CD70 and iNOS. In the case of IL-8, it was excluded due to signal saturation, because the expression was too high in the used 500 ng RNA/well. IL-8 was then measured additionally by qRT-PCR. The genes that could be measured via Luminex®, IL-18, IL-1 β , TNF- α , PD-L1 and COX-2, exhibited relatively large standard deviations between the three biological replicates. This can be attributed to the fact that the measurement had to be stopped multiple times due to malfunction of the reader. Ultimately, the plate had to be measured over the course of a week instead of in one read. The plate was stored in the fridge with a plate seal and aluminium foil for light protection during this time, according to manufacturer recommendations, but loss of target RNA or capture beads is still possible, if not likely. Another possibility for this outcome could be, that the genes are simply expressed too poorly in this cell line and thus making it more difficult to measure them correctly. IL-1 β had an extremely large standard deviation and the solvent control did not significantly differ from the control stimulated with IL-1 β during incubation. Because of the problems during measurement and the high standard deviations, the results should be handled carefully. To verify the results from the Luminex® experiment,

some targets were additionally measured via qRT-PCR. IL-8, due to signal saturation, and also IL-1 β and TNF α were measured in the three biological replicates in duplicates. In the qRT-PCR, clearer distinctions could be drawn between the un-stimulated and the stimulated control. Here it was very clear that the IL-1 β stimulation stimulated IL-1 β expression in the HCT116 cells ($p < 0.001$). Ultimately both, the Luminex[®] and the PCR measurement, came to the same results in regards to the question at hand; the berry extracts did not impact the expression of the immunomodulatory markers targeted in a significant way. There was no significant difference between the IL-1 β stimulated control and samples treated with 1, 10 or 100 $\mu\text{g/ml}$ blackberry, bilberry, elderberry or black currant extract regarding the expression of IL-1 β , IL-8, IL-18, TNF α , PD-L1 and COX-2, apart from two exceptions. In the Luminex[®] measurement, 10 $\mu\text{g/ml}$ of blackberry extract showed a slight but significant downregulation of the TNF α expression. This could not be verified by the qRT-PCR measurement which showed no influence of the extracts on TNF α expression. Since no concentration dependent trends could be observed in either method and the Luminex[®] measurement had some problems in general, this is most likely not relevant. 10 $\mu\text{g/ml}$ EB extract showed a slight but significant upregulation of COX-2 expression in the Luminex[®] measurement. This is accompanied by a non-significant slight trend of COX-2 upregulation after treatment with EB and BC extract.

As opposed to the HCT116, the HCEC-1CT cells did express more of the selected targets. IL-8 was saturated in the HCEC-1CT cells as well, with the RNA amount (500 ng/well) used. Having gained this knowledge from adding a HCEC-1CT sample to the first HCT116 Luminex[®] plate, IL-18 and IL-8 were excluded from the QuantiGene plex assay[™] when measuring the HCEC-1CT samples. The HCEC-1CT cells expressed TNF α , IL-1 β , IL-6, IL-8, COX-2, CD70 and PD-L1 in measurable quantities and their expression of was induced by the inflammatory stimulus (IL-1 β) significantly. The expression of some of those genes has previously been shown in HCEC-1CT cells, but not all of them. Woelflingseder *et al.* showed in multiple publications revolving around the immunomodulatory effects of mycotoxins, the ability of HCEC-1CT cells to express TNF α , IL-1 β , IL-6 and IL-8.^{107, 53} Besides those cytokines, their expression of PD-L1 has also been stated in literature.¹⁰⁸ There was no literature found in regards to the remaining targets. Interestingly, the immune-checkpoint markers PD-L1 and CD70, that should be upregulated in tumorigenic cells^{59, 63}, were also expressed more prominently in the HCEC-1CT cells than the HCT116 cells. CD70 did not reach measurable signal intensity in the HCT116 cells measured by Luminex[®], but in the HCEC-1CT concentrations could be measured.

Before discussing the results of the measurements in HCEC-1CT cells, the aforementioned fluctuations of the housekeeping signals have to be addressed. In both the Luminex® and PCR measurements the signals of the housekeeping genes, and target genes, showed significant fluctuations between samples. After eliminating all other possibilities, this was attributed most likely to loss of RNA. After noticing the irregularities, the RNA quantities in the samples were measured again (via Nanodrop). The time between initial extraction immediately followed by measurement and the Luminex®/PCR was about 3-4 months. In this time an RNA loss occurred in some, but not all, of the samples up to 30 ng/μl. No RNA loss was observable in the samples from the HCT116 cells, leading to the belief a RNase contamination might have been the cause for the RNA loss in the HCEC-1CT cells. The results have to be handled with caution.

Regarding the immunomodulatory effects of the berry extracts in the HCEC-1CT cells, the results showed some hints and tendencies for immunomodulation but they were overall rather rare and sometimes inconsistent. IL-1β, IL-8 and PD-L1 expression was not influenced by either extract, concentration or test system. IL-6 was upregulated with 10 μg/ml of Bil extract but showed no other trends. TNFα showed some irregularities, being significantly upregulated with the BB extract and significantly downregulated with Bil (each only in one concentration). CD70 was significantly downregulated with concentrations of Bil and EB. The main trend was like in the HCT116 cells, an upregulation of COX-2. In the HCEC-1CT cells COX-2 showed tendencies for upregulation in both test systems but with some differences. The Luminex® showed significant upregulations with Bil (10 and 100 μg/ml) and EB (100 μg/ml) concentrations, whereas the PCR showed a significant upregulation with BB (100 μg/ml) and no effects with Bil and EB extracts. Due to the differences between the test systems the results regarding COX-2 upregulation need to be taken with caution.

Overall, these results suggest that the berry extracts at hand do not exhibit major immunomodulatory properties in HCT116 colon cancer cells and show only some irregular trends in the HCEC-1CT cells, most of which were upregulations of inflammation markers. This stands in contrast to multiple publications postulating anti-inflammatory properties of anthocyanins. Regarding the available literature, immune cells are often the *in vitro* system of choice when investigating immunomodulation, but studies are also performed on cells of the gastrointestinal tract, as they are the first point of contact. Immune cells have a higher

expression of immunomodulatory markers in nature, which makes it easier to investigate modulation. Cenk *et al.* (2021) for example, investigating the effect of highly purified anthocyanin fractions of blackberry juice in LPS-challenged THP-1-derived macrophages, which were found to display both anti- and pro-inflammatory properties at plasma-relevant concentrations depending on their structure and substitution pattern. With some fractions up- or downregulating, for example, NF- κ B activity or the cytokines TNF α , IL-6, and IL-8 as well as IL-10. The BB extract itself, only managed to downregulate TNF α significantly at the highest concentration of 5 μ g/ml.⁶⁸ As it is the model investigated in this work, the main focus of discussion is in regards to studies on colon cell systems. The literature available does point to immunomodulatory effects in colon cells, with at least downregulation of TNF α observed multiple times. Venancio *et al.* for instance, investigated the effects of an anthocyanin rich fruit (Cocoplum) in CCD-18Co non-malignant colonic fibroblasts and HT-29 colorectal adenocarcinoma cells. In HT-29 cells they observed increased intracellular ROS production as well as decreased TNF- α , IL-1 β , IL-6, and NF- κ B RNA levels, accompanied by suppressed cell proliferation. On a protein level only TNF α was significantly downregulated by the anthocyanins extracted from the fruit. Within the same concentration range (1 to 20 μ g/ml gallic acid equivalents (GAE)), there was no cytotoxic effect in CCD-18Co cells and TNF α -induced intracellular ROS-production was decreased by the treatment. Only NF- κ B levels were decreased on an RNA level by anthocyanin treatment in TNF α -treated CCD-18Co cells. Protein expression of IL-1 β , IL-6 and TNF- α was also reduced in TNF α -treated CCD-18Co cells by the anthocyanins. According to this study, HT-29 cells did not need an inflammatory stimulus, like addition of IL-1 β , to excrete those targets. The fibroblast cells, which are known immune regulators, were stimulated and excreted the inflammation related targets as a response. The concentrations applied by Venancio *et al.* that showed immunomodulatory effects, where however, in contrast to the concentrations applied here, cytotoxic in the HT-29 cells with 20 μ g/ml GAE reducing the cell proliferation to 50 %.⁶⁷ Triebel *et al.* also investigated the immunomodulatory properties of berry extracts. They showed that bilberry extract significantly and dose-dependently inhibited expression of TNF α in IFN- γ /IL-1 β /TNF α stimulated human colon epithelial T84 cells, IL-8 was slightly but not significantly inhibited. They observed that the effects of selected single anthocyanins on the expressions of TNF α , IL-8, and IP-10 genes in the stimulated T84 cells depended on both their aglycone and sugar moieties, and can severely differ.⁶⁹ Cancer preventive potentials of several food

phytochemicals targeting the induction of iNOS and COX-2 have previously been described in literature. COX-2 has a central role in cancer and inflammation in general and is induced by pro-inflammatory mediators and mitogenic stimuli such as cytokines.⁵⁷ Publications revolving around anthocyanins often speak of a reduced COX-2 expression in context of anti-inflammatory properties that decrease NF- κ B expression. For example, delphinidin inhibited IL-1 β -induced expression of COX-2 and production of PGE2 in human chondrocytes¹⁰⁹ and anthocyanin extract derived from sweet potato decreased the expression of COX-2 and iNOS in DMN-intoxicated rats.¹¹⁰ In the experiment at hand, no downregulation of COX-2 could be observed instead there were tendencies for COX-2 upregulation in some models indicating an inflammatory stimulus. However, because of the fluctuation between test systems in the HCEC-1CT cells it is difficult to interpret them as such.

Multiple factors make literature comparisons difficult in regard to immunomodulatory effects of berry extracts. In general, there are a lot of different models investigating immunomodulatory effects of anthocyanins. Firstly, this is due to the fact that diverse extracts can be used. The extracts can have different concentrations, sources, components and preparations, leading to different anthocyanin compositions. The berry extract quantities applied in this work should represent realistic amounts, achievable through diet. With regular consumption of fruits and berries, an intake of 100 mg per day is easily reachable.¹⁰ With high consumption of food additives and food supplements not even taken into account.^{14, 18} Hence, it can be postulated that high local concentrations of biologically active components may occur in the GIT in direct contact with epithelial cells. However, in healthy individuals a comparably low systemic bioavailability is assumed and low levels are detected in plasma after ingestion.²⁴ Besides concentration; extracts are individual samples. Other substances may also be included, such as other polyphenols for example. Furthermore, the obvious differences in the source itself, a variety of external factors, such as climate, influence the amounts of anthocyanins contained. This leads to complex and individual composition patterns. The stability of anthocyanins also presenting a source of varieties between studies, as it also depends on various variables as discussed in chapter 1.1.3. As Cenk *et al.* and Venancoi *et al.* both demonstrate, even different fractions of one extract can have different immunomodulatory properties.^{68, 67} It is therefore possible that the anthocyanins in one extract express immunomodulatory properties. Those properties however can differ and

possibly cancel each other out when the extract itself is applied. The different anthocyanin composition of the extracts can lead to markers being upregulated by one extract and downregulated by another, as shown in the HCEC-1CT cells for TNF α .

Then, there are also different models of investigation, just regarding *in vitro* models there are multiple options to choose from; from immune cells to colon cells, cancerogenic to non-cancerogenic cells. As mentioned above, immune cells are often the model of choice when investigating immunomodulatory properties. Here, two colon cell lines were chosen, one cancer cell line (HCT116) and one non-cancerous epithelial cell line (HCEC-1CT). Unfortunately, the chosen cancer colon HCT116 cells did not express measurable levels of multiple desired targets, even with an inflammatory stimulus, namely CD70, IL-6, IL-2, IL-33 and iNOS. This could be specific to HCT116 cells or to colon cancer cells in general, although at least IL-6 seems to be expressed adequately in other colon cancer cell lines (HT-29).⁶⁷ In an inflammatory state provoked by IL-1 β , the HCEC-1CT cells however additionally showed expression of CD70 and IL-6 but did not express IL-18 in measurable concentrations. IL-2, IL-33 and iNOS had to be excluded in both cell lines due to poor expression. Hence, no immunomodulation by the extracts could be observed in those targets. Not only immune cells but all types of cells - nerve, mesenchymal, epithelial and endothelial cells - play a key role in orchestrating the intestinal immune response.³⁴ It is therefore possible, that the extracts, the anthocyanins included or a mixture of multiple constituents exhibit more prominent immunomodulatory and anti-inflammatory effects in other *in vitro* or *in vivo* models.

Next is the mode of stimulation. In the present thesis IL-1 β was used as an inflammatory stimulus to induce an immune-response from the treated cells. There are various different compounds used for this; LPS or other cytokines such as TNF α or a mixture of multiple stimuli (IFN- γ /IL-1 β /TNF α). Different stimulations could lead to different activation patterns in cell lines. It is possible that the HCT116 cells might produce measurable target concentrations when stimulated differently. However, the stimulus is there to mimic intestinal inflammation and IL-1 β is a pro-inflammatory stimulus that is reported to be upregulated during inflammation and IBDs. IL-1 β is also suggested to activate both the transcription factor NF- κ B and MAPKs.²⁹ Importantly, different stimuli might affect which markers are measurable but not the influence of the extracts on those markers.

And lastly, the methods of detection can differ between studies, with the method of choice being RT-PCR since it is still considered “gold standard” while the QuantiGene Plex Assay[™]

utilizing xMAP Luminex® beads only being described in fewer more recent works. Additionally, qRT-PCR also exhibited a higher sensitivity in this case.¹¹¹ In the qRT-PCR 40 cycles (annealing – elongation – denaturation) are employed which exponentially double the DNA, leading to signal amplification up to 2^{40} .¹¹² In the QuantiGene Plex Assay™ each amplification unit provides a 400x signal amplification and there are six amplification units per target RNA copy, resulting in a 2400x signal amplification per copy RNA.¹¹³ The clear advantage of the QuantiGene plex assay™ measured by Luminex® technology is that it allows the measurement of up to 80 genes of interest in a single well. It uses branched signal amplification rather than template amplification. There is no need for RNA extraction or amplification, which can eliminate bias and sources of error.¹¹⁴ Different multiplexed gene assays like the QuantiGene Plex Assay™ are on the rise since the last decade in *in vivo* and *in vitro* models, also being proposed for clinical use to detect inflammation markers^{115, 116}, since they allow detection of multiple targets at once. Only one *in vivo* study investigating immunomodulatory effects of anthocyanins could be found utilizing this specific QuantiGene Plex Assay™; Pfohl *et al.* investigated in 2021 the anti-inflammatory effects of polyphenol-enriched extracts and their gut microbial metabolites in a mouse model with pomegranate extract supplementation in high fat diet-induced obese C57BL/6 mice leading to significant reduction in the gene expression of IL-1 α , IL-1 β , IL-7, IL-11, TNF α and others.¹¹⁷ Other multiplexed gene assays have been used in multiple publications also investigating immunomodulation by anthocyanins *in vitro*, for example in human aortic endothelial cells¹¹⁸, but not specifically in colon cells. It is possible that the assay lacks the sensitivity to detected smaller amounts of immunomodulation markers in colon cells such as the ones used here.

Not only literature comparisons, but also comparisons between the two cell lines were challenging. Unfortunately, due to the lack of ability to express some of the target genes in HCT116, it was not possible to fully draw comparisons regarding the immunomodulatory effects between non-cancerous (HCEC-1CT) and cancerous (HCT116) cells in this thesis. CD70 and IL-6 were only detected in the HCEC-1CT cells and there both exhibited some hints of immunomodulatory effects with CD70 being downregulated with concentrations of both Bil and EB extract. Only the markers expressed by both cell lines could be compared. The markers IL-8, IL-1 β and PD-L1 showed no significant up- or downregulation in HCT116 cells upon incubation of the cells with the different berry extracts, TNF α downregulation by BB extract

was only significant in the Luminex experiment and not the PCR and slight tendencies of COX-2 upregulation, significant for one EB concentration, could be observed with all extracts but mainly EB and BC. The HCEC-1CT cells showed no effects on IL-1 β , PD-L1 and IL-8 as well but showed some effects in the expression of TNF α , being slightly upregulated with BB and downregulated with Bil. COX-2 showed a trend for upregulation in this cell line as well but the tendencies were fluctuant depending on the test system and thus most likely not relevant. The accuracy of the obtained results in general can be called into question. There were instances in both cell lines where the results of the Luminex[®] were not reflected in the PCR, in the HCT116 cells it was the significant downregulation of TNF α by 10 μ g/ml BB extract and in the HCEC-1CT cells the aforementioned upregulations of COX-2. Since not every target was also investigated via PCR, it can not be excluded that the results would differ for those targets as well. Although no major immunomodulatory effects could be observed overall, both cell lines showed anti-inflammatory and pro-inflammatory effects of the berry extracts.

To above mentioned problems and the relatively large standard deviations make definitive statements difficult. For a better understanding more replicates would need to be measured so that statistical outliers can be identified and excluding. Due to the differences between the applied methods, it would be optimal to investigate all targets with both Luminex[®] and qRT-PCR. Unfortunately, the at the moment still very costly Luminex[®] technology make this challenging.

5. Conclusion

Firstly, this thesis aimed to investigate the potential influence of SN38 on the NF- κ B pathway, as a target causing the rise of inflammation-markers during treatment and as a potential point of interaction between SN38 and anthocyanins. Anthocyanins are known to downregulate NF- κ B activity.⁶⁸ Surprisingly, SN38 did not exhibit any influence on NF- κ B activity in the THP1-Lucia™ NF- κ B cells, whether by itself nor in combination with LPS as an NF- κ B stimulus. In higher doses, concomitantly with reduction of NF- κ B activity an increased cytotoxicity of SN38 was detected with the CellTiter-Blue® Cell Viability Assay. This opposes the general belief, that chemotherapeutics such as SN38 activate the NF- κ B pathway⁸² and speaks against the NF- κ B pathway being a point of interaction between SN38 and anthocyanins, which might downregulate its induced activation.

Secondly, this thesis aimed to enlighten possible immunomodulatory effects of different anthocyanin rich berry extracts in an *in vitro* model with cancerogenic and non-cancerogenic human colon epithelial cells. After short-term incubation (5 h) of HCT116 and HCEC-1CT cells with blackberry, bilberry, elderberry or black currant extract, in doses of 1, 10 or 100 μ g/ml, combined with an inflammatory stimulus for 3 h (25ng/ml IL-1 β), selected immunomodulatory markers were measured on a transcription level using the Invitrogen™ QuantiGene Plex Assay™ Kit with Luminex® xMAP technology and qRT-PCR. The selected targets consisted of the inflammation markers IL-1 β , IL-2, IL-6, IL-8, IL-18, IL-33, TNF α , iNOS, COX-2 and the immune-checkpoint markers CD70 and PD-L1. The HCT116 cells did not express multiple of the selected targets in measurable amounts, namely IL-2, IL-6, IL-33, iNOS and CD70. The remaining markers were significantly induced by the inflammatory stimulus (IL-1 β) but the extracts had few effects on the expression levels, with only tendencies for COX-2 upregulation being observed. None of the four berry extracts investigated, in none of the applied concentrations, effected the expression of IL-1 β , IL-18, IL-8 or PD-L1 and the results for TNF α varied between test systems, with Luminex® showing a small downregulation with 10 μ g/ml BB extract not visible in the PCR experiments. In non-cancerous HCEC-1CT colon epithelial cells the expression of IL-1 β , IL-6, IL-8, TNF α , COX-2, CD70 and PD-L1 was induced by the inflammatory stimulus significantly. However, extracts of blackberry, bilberry, elderberry and black currant did not influence the expression of IL-1 β , IL-8 and PD-L1 at all. Hints of TNF α and IL-6 modulation in individual concentrations and tendencies regarding downregulation of

CD70 (with 1 µg/ml Bil and 100 µg/ml EB extract) and upregulation of COX-2 were observed. COX-2 was upregulated significantly with the Bil and EB extract in the Luminex® experiment and only with the BB extract in the qRT-PCR. Due to the fluctuations between test systems the COX-2 upregulation is unlikely to be relevant.

The anthocyanin-rich berry extracts did not exhibit anti-inflammatory effects, often described in literature, in either colon cell line. Hints of both pro- and anti-inflammatory effects could be observed. In previous works, anthocyanins have been shown to down-regulate the expression of various inflammatory biomarkers such as NF-κB, TNFα, IL-1β, IL-6, IL-8, iNOS, COX-2 and more.^{65, 39, 66, 67, 68, 69} But in this thesis no such conclusions can be drawn. The berry extract quantities applied in this work should represent realistic amounts, achievable through diet. With regular consumption of fruits and berries, an intake of 100 mg per day is easily reachable.¹⁰ Even higher doses of anthocyanins can be reached by regular consumption of food additives and food supplementes.^{14, 18} Hence, it can be postulated that high local concentrations of biologically active components may occur in the GIT in direct contact with epithelial cells. However, in healthy individuals a comparably low systemic bioavailability is assumed and low levels are detected in plasma after ingestion.²⁴

There are multiple cell types present in the intestinal immune system that can exhibit different reactions to the various constituents of berry extracts. Immune response includes various cell types that activate and stimulate each other and themselves. Hence, it is difficult to reproduce a realistic immune reaction *in vitro*, especially using non-immune cells. However, taking the obtained results as a whole, no anti-inflammatory properties of anthocyanin-rich berry extracts in colon cells could be observed.

6. Additional Experiments

Apart from the main experiments, detecting immunomodulatory markers in the colon cell cultures incubated with berry extracts and investigating the effect of SN38 on the NF- κ B pathway, other additional experiments around the project were performed.

6.1. Cytotoxicity of Citric Acid and Berry Extracts

Findings from the main *in vivo* study I, suggested an inhibition of tumor growth by citric acid (unpublished data), which was used as a solvent for the berry extracts. The main study I also suggested that BB extract inhibited the toxic effects of citric acid on the tumor cells. The findings of Zhao *et al.*⁹⁵, Chen *et al.*⁹⁹ and Ren *et al.*¹⁰³ also suggest that citric acid can inhibit tumor cell proliferation and tumor growth in multiple tumor lines *in vivo* and *in vitro*. Based on these findings *in vitro* cytotoxicity testing was carried out, combining citric acid with the different berry extracts. The cytotoxicity was determined via WST-1 and SRB assay after an incubation time of 72 h in HCT116, HCEC-1CT and CT26 cells. First, a range of citric acid concentrations was tested to determine the optimal concentration for the experiment going forward. For this, cells were seeded in 96 well plates and after allowing the cells to grow for 24 hours, citric acid was weighed, diluted with medium (with catalase) and added in different concentrations to the cells. Going forward, a citric acid concentration was used, at which a visible cytotoxicity at a medium range was present to test the effect different concentrations of all four anthocyanin-rich extracts, elderberry, blackberry, bilberry and black currant, have on the cytotoxicity of CA.

Cytotoxicity range of citric acid in different cell lines:

Figure 15 below are the graphs depicting the cytotoxicity of citric acid, assed by WST-1 and SRB assay, in HCT116 cells. The cytotoxicity is concentration dependent, starting from 1.5 mg/ml citric acid according to the WST-1 assay. The SRB assay shows similar results, in that CA exerts concentration dependent cytotoxicity, although here already starting from 1 mg/ml. At 1 mg/ml the cell viability is still around 100 % in the WST-1 but already around 80 % in the SRB assay, whereas at 1.5 mg/ml it decreases significantly to around 70 % in the WST-1 and 30 % in the SRB assay.

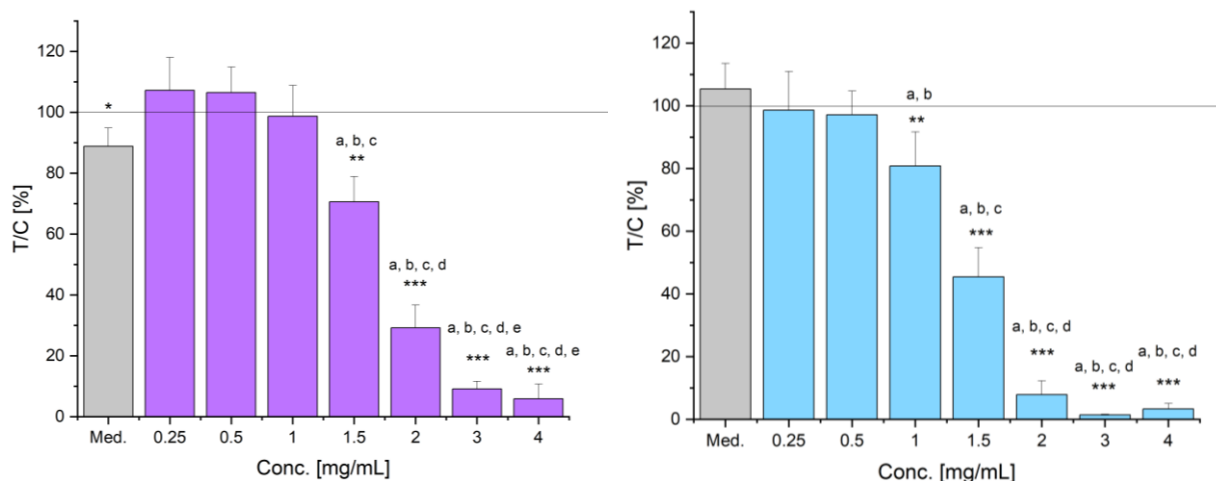


Figure 15: Cytotoxic effects of citric acid (CA) in a concentration range between 0.25 and 4 mg/ml on HCT116 cells after 72 h of incubation determined by WST-1 (left) and SRB (right) assay. Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5 % DMSO, $y=100$). Statistical significances were determined by one-sample Student's t-test for significances between the concentrations and the solvent control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and one-way ANOVA with post-hoc bonferroni ($p<0.05$) for significances among test concentrations.

Figure 16 below shows the graphs depicting the cytotoxicity of citric acid, assed by WST-1 and SRB assay, in the non-cancerogenic HCEC-1CT cells. The cytotoxicity here is concentration dependent as well and also starts at 1 -1.5 mg/ml citric acid. A significant downregulation of cell viability is already observable at 1 mg/ml, but it is still above 80 % in the WST-1 (left) assay. In the SRB assay (right) the cytotoxicity of 1 and 1.5 mg/ml is very similar, around 60-70 %.

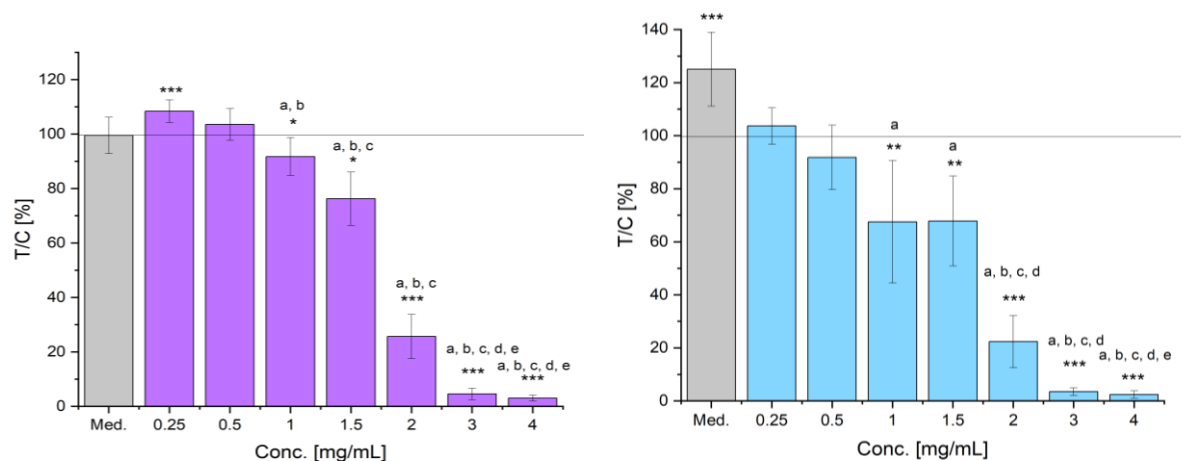


Figure 16: Cytotoxic effects of citric acid (CA) in a concentration range between 0.25 and 4 mg/ml on HCEC-1CT cells after 72 h of incubation determined by WST-1 (left) and SRB (right). Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5% DMSO, $y=100$). Statistical significances were determined by one-sample Student's t-test for significances between the concentrations and the solvent control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and one-way ANOVA with post-hoc bonferroni ($p<0.05$) for significances among test concentrations.

In Figure 17 are the graphs depicting the cytotoxicity of citric acid, assed by WST-1 and SRB assay, in murine CT26 cells. The cytotoxicity in CT26 cells is concentration dependent and is already significant at a concentration of 0.5 mg/ml in the WST-1 assay (left). The SRB assay (right) even shows a significant downregulation of cell viability at 0.25 mg/ml although, with a percentage of over 90 %, the observed reduction is still marginal. At a concentration of 0.5 mg/ml the viability lies at 70-80 % and at 0.75 mg/ml at 20-40 % in both assays.

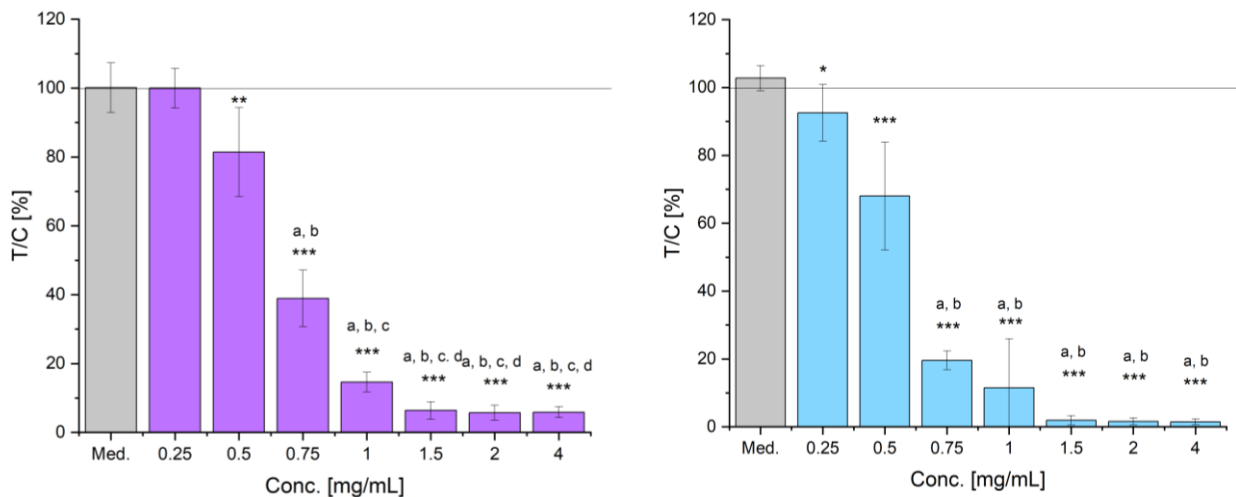
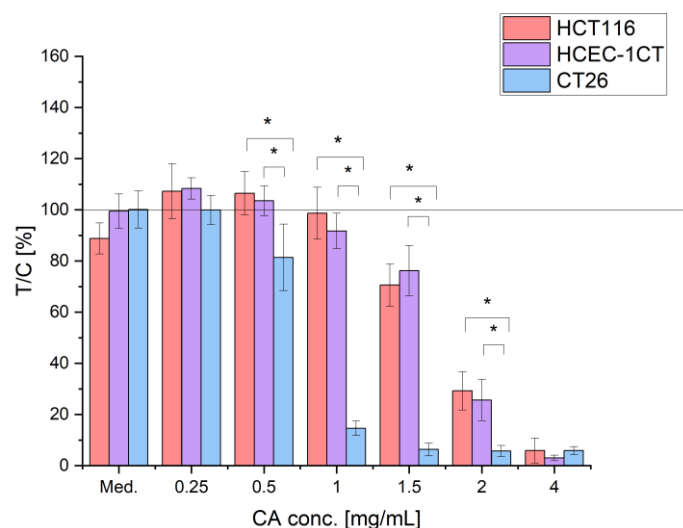


Figure 17: Cytotoxic effects of citric acid (CA) in a concentration range between 0.25 and 4 mg/ml on CT26 cells after 72 h of incubation determined by WST-1 (left) and SRB (right). Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5% DMSO, $\gamma=100$). Statistical significances were determined by one-sample Student's *t*-test for significances between the concentrations and the solvent control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and one-way ANOVA with post-hoc bonferroni ($p<0.05$) for significances among test concentrations.

These findings suggest significant differences in cell lines reacting to citric acid. The murine CT26 cells seem to be way more sensitive to the exposition to citric acid. As shown in Figure 18 below, CA already has a cytotoxic effect at 0.5 mg/ml whereas the HCT116 and HCEC-1CT cells only start reacting at higher concentrations of 1 mg/ml. There are no significant differences between the human colon cancer cells HCT116 and normal human colon cells HCEC-1CT cells in any concentration or test system but they both differ significantly from the CT26 cells regarding their cell viability when treated with 1, 1.5 and 2 mg/ml CA in the WST-1 assay. In the SRB assay, at 0.5 mg/ml CA the HCT116 cell viability differs significantly from the CT26 cells, at 1 and 1.5 mg/ml CA both human cell lines have a significantly higher cell viability than the CT26 cells. At 2 mg/ml only the HCEC-1CT cells differ significantly from the CT26 cells regarding the cytotoxicity of CA. Overall, CT26 cells express a significantly higher cytotoxicity than the HCT116 and HCEC-1CT cells upon CA treatment.

(A)



(B)

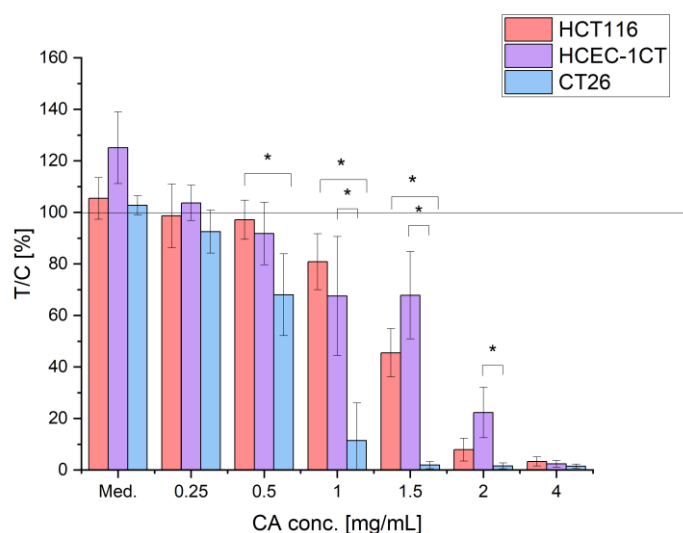


Figure 18: Cytotoxicity of citric acid (CA) in the concentrations; 0.25, 0.5, 1, 1.5, 2 and 4 mg/ml, determined by WST-1 assay (A) and SRB assay (B) in the cell lines HCT116, HCEC-1CT and CT26. Differences between the cell lines when treated with specific CA concentrations, were determined by bonferroni ANOVA ($p < 0.001$)

Co-incubation of CA with berry extracts:

Based on the findings above, a citric acid concentration was chosen for each cell line to be used for further testing. Going forward, a concentration was used, at which a visible cytotoxicity at a medium range was present to test what effect different concentrations of all four anthocyanin-rich extracts, elderberry, blackberry, bilberry and black currant, would have on the cytotoxicity. For HCT116 and HCEC-1CT cells a concentration of 1.5 mg/ml and for CT26 cells a concentration of 0.75 mg/ml was opted for. These citric acid concentrations where then co-incubated with a range of concentrations of the four extracts. The used berry extract concentrations where; 6.25, 12.5, 25, 50, 100 and 200 mg/ml. In the Figure 19 below are the

results for the WST-1 assay (A) and the SRB assay (B) in the HCT116 cells with CA and berry extract co-incubation. The DMSO control had no citric acid.

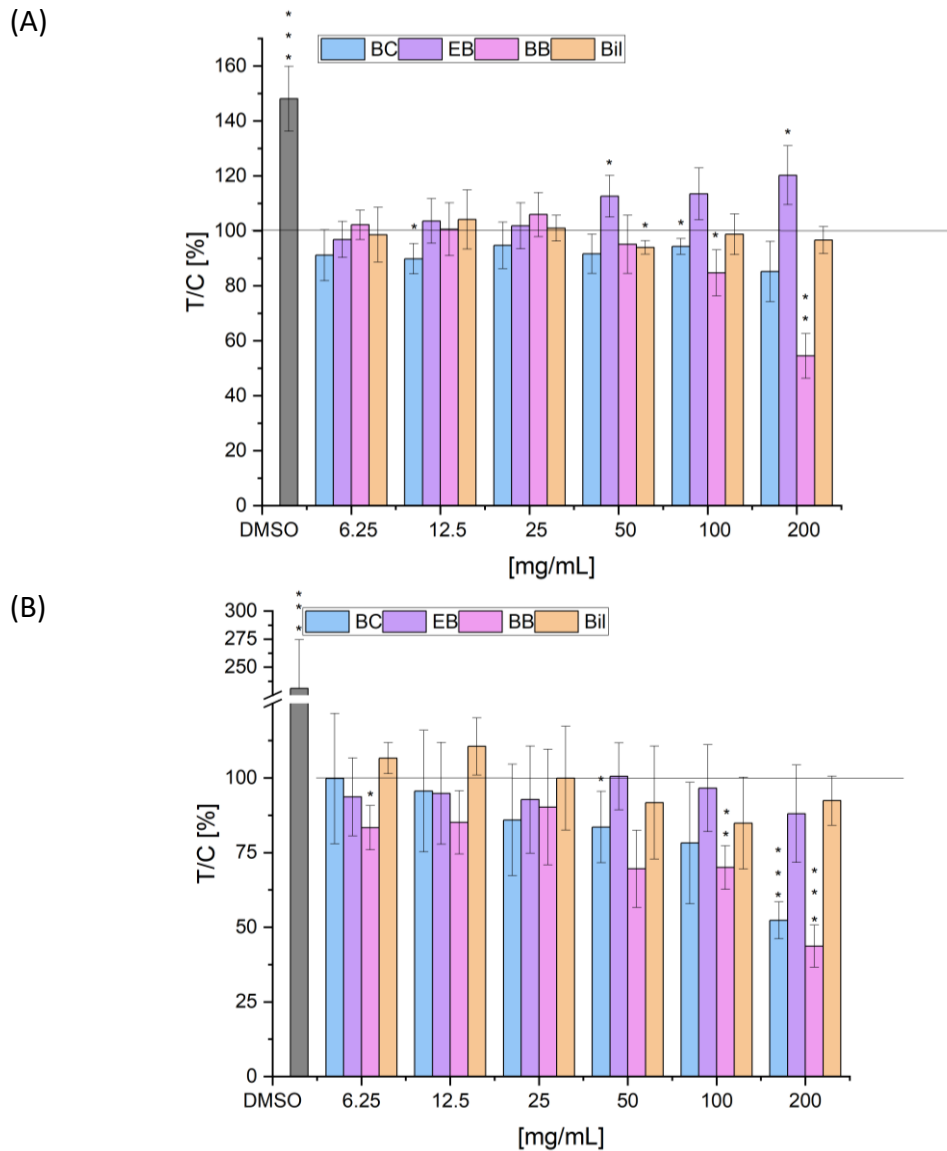


Figure 19: Cytotoxic effects of 1.5 mg/ml citric acid (CA) and berry extracts (black currant, elderberry, blackberry and bilberry) in a concentration range between 6.25 and 200 mg/ml on HCT116 cells after 72 h of incubation determined by WST-1 (A) and SRB (B) assay. The DMSO control was not treated with CA. Results are expressed as means $\pm$ SD of 4 independent experiments, normalized to the citric acid control (1.5 mg/ml CA, 0.5 % DMSO, $y=100$). Statistical significances were determined by one-sample Student's *t*-test for significances between the concentrations and the citric acid control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

The WST-1 assay shows that at a concentration of 6.25 mg/ml none of the extracts have an effect on the cytotoxicity of CA (1.5 mg/ml) in the HCT116 cells. Black currant extract seems to slightly further downregulate the cell viability (compared to the downregulation already caused by CA) in the concentrations 12.5 and 100 mg/ml. Blackberry extract also

downregulates the cell viability at 100 and 200 mg/ml in a concentration dependent matter. No effect on the cell viability is shown by the bilberry extract in all concentrations. Elderberry extract slightly but significantly upregulates the cell viability in the concentrations 50 and 200 mg/ml. There is also a slight upregulation at 100 mg/ml but it is not significant. The SRB assay in the HCT116 cells shows no upregulations in cell viability caused by the extracts, meaning none of the extracts counteract the cytotoxicity caused by the 1.5 mg/ml CA. The cell viability seems to be downregulated in a dose dependent manner by blackberry extract in the concentrations 50, 100 and 200 µg/ml. 6.25 µg/ml of BB extract also seems to cause a slight downregulation in cell viability. At 200 µg/ml the BC extract also downregulates the cell viability significantly down to approximately 50 % from the damage already caused by CA. Overall the SRB assay shows stronger downregulations of cell viability in the HCT116 cells then the WST-1 but with the same tendencies for cytotoxicity by the blackberry and black currant extracts. The slight upregulation of cell viability caused by elderberry shown in the WST-1 assay could show signs of a protective effect against the cytotoxicity of CA, but the SRB assay does not reflect that.

Figure 20 shows the results for the WST-1 assay (A) and the SRB assay (B) in the HCEC-1CT cells with CA and berry extract co-incubation. The WST-1 shows a significant downregulation of cell viability in the HCEC-1CT cells after treatment with CA (1.5 mg/ml) and 200 µg/ml of BB, Bil, BC and EB extract. BB had the strongest toxic effect, downregulating the cell viability significantly starting from 25 mg/ml and going down to 20 % after treatment with 200 µg/ml of the extract. BC and Bil downregulated the cell viability similarly, starting from 25-50 µg/ml and going down to around 50 % in the highest concentrations. EB only showed toxic effects in the highest concentration (200 µg/ml), downregulating cell viability to 80 % when compared to CA treatment alone. The SRB assay showed similar concentration dependent toxicity of the BB, BC and Bil extracts starting from around 50 µg/ml. All three extracts showed strong cytotoxic effects, downregulating cell viability to 10-15 % in the highest concentration. The EB extract also led to slight downregulated cell viability, although the downregulation is not significant. None of the extracts exhibited protective effects against the toxicity of 1.5 mg/ml CA in the HCEC-1CT cells.

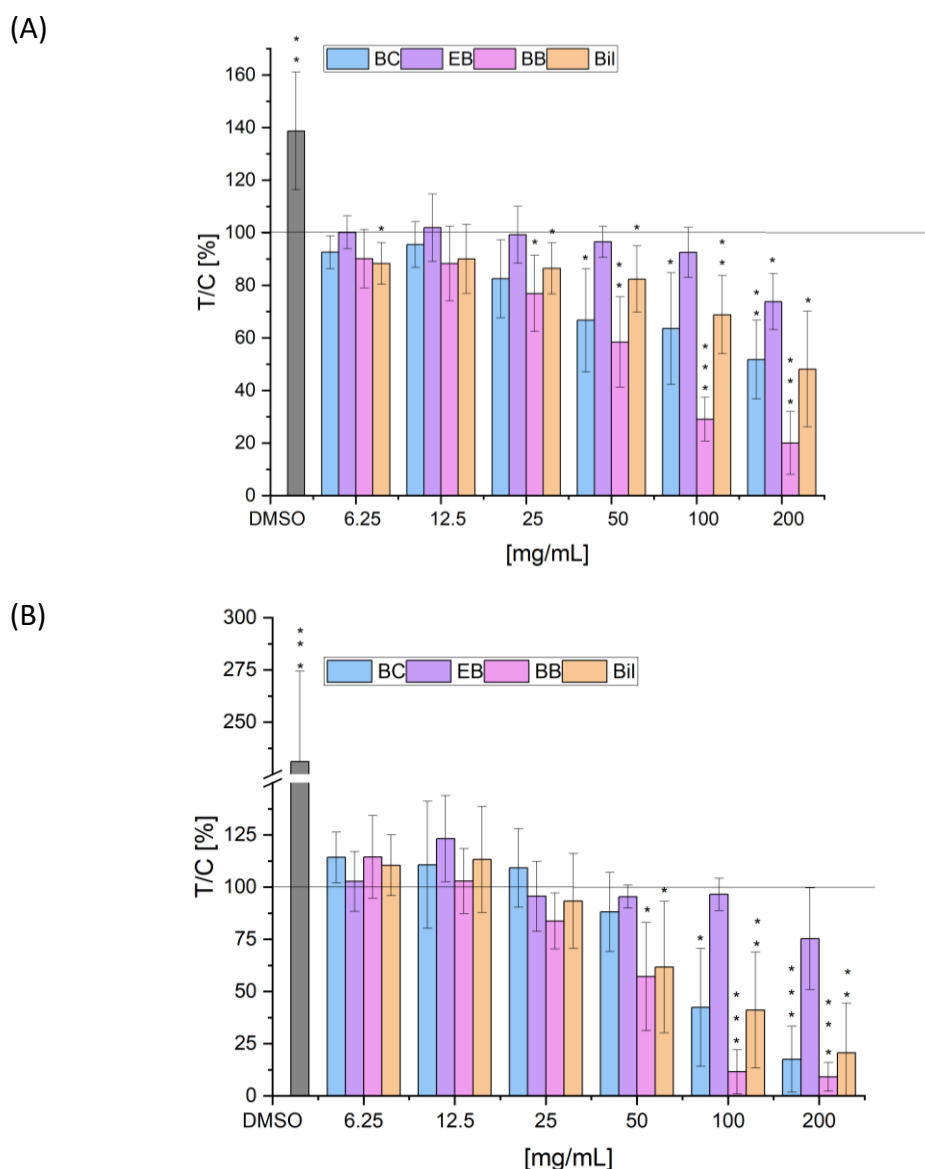


Figure 20: Cytotoxic effects of 1.5 mg/ml citric acid (CA) and berry extracts (black currant, elderberry, blackberry and bilberry) in a concentration range between 6.25 and 200 mg/ml on HCEC-1CT cells after 72h of incubation determined by WST-1 (A) and SRB (B). The DMSO control was not treated with CA. Results are expressed as means $\pm$ SD of 4 independent experiments, normalized to the citric acid control (1.5 mg/ml CA, 0.5% DMSO, $y=100$). Statistical significances were determined by one-sample Student's t-test for significances between the concentrations and the citric acid control. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Figure 21 shows the results for the WST-1 assay (A) and the SRB assay (B) in the CT26 cells with CA and berry extract co-incubation. The WST-1 assay shows that at a concentration of 6.25 mg/ml none of the extracts have an effect on the cytotoxicity of CA (0.75 mg/ml) in the CT26 cells. At a concentration of 12.5 mg/ml bilberry shows a slight downregulation of cell viability, at all other concentrations bilberry seems to have no effect. Black currant extract downregulates cell viability at a concentration of 200 mg/ml and blackberry, comparable to

the other cell lines, downregulates the viability concentration dependently at 100 and 200 mg/ml. The SRB assay in the CT26 cells shows the same picture as in the other cell lines: a more prevalent downregulation in cell viability. At the concentration 200 µg/ml all the extracts show further downregulated cell viability. Elderberry, bilberry and blackberry extract already show a significant downregulation in cell viability at a concentration of 100 µg/ml with blackberry having the strongest effect. None of the extracts are seen to have a protective effect against the cytotoxicity of 0.75 mg/ml CA in the CT26 cells in neither of the test systems. Overall, signals in the SRB assay were seen to be lower than the equivalent ones observed in the WST-1 assay.

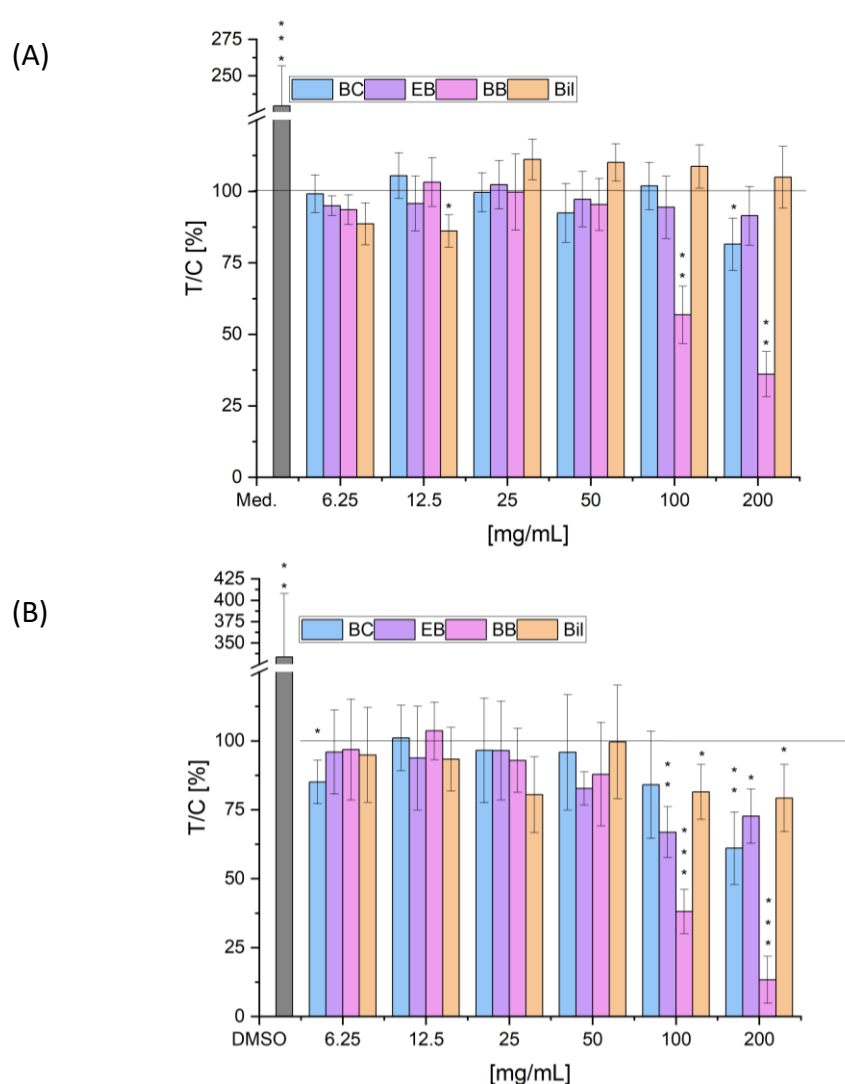


Figure 21: Cytotoxic effects of 1.5 mg/ml citric acid (CA) and berry extracts (black currant, elderberry, blackberry and bilberry) in a concentration range between 6.25 and 200 mg/ml on CT26 cells after 72 h of incubation determined by WST-1 (A) and SRB (B). The DMSO control was not treated with CA. Results are expressed as means $\pm$ SD of 4 independent experiments, normalized to the citric acid control (0.75 mg/ml CA, 0.5% DMSO, $y=100$). Statistical significances were determined by one-sample Student's *t*-test for significances between the concentrations and the citric acid control. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Combinatory effects:

To evaluate the combinatory effects of CA and the berry extracts, the mathematical method of Independent Joint Action was employed. This model can be applied for substances with different modes of action to determine whether their combined effects are simply additive or if there are synergistic or antagonistic combinatory effects present.¹¹⁹ The WST-1 assay performed with the HCEC-1CT cells was evaluated with this method.

For this model the effects of the single compounds and combinations were calculated by relating the results to the DMSO control as described by following formula:

$$\% \text{ mitochondrial activity of DMSO} = (1 - \text{effect})100$$

Then, the expected values were calculated by the following equation:

$$\text{Expected values} = \text{effect substance 1} + \text{effect substance 2} - (\text{effect substance 1} * \text{effect substance 2})$$

Expected additive values were compared to actually measured values in order to determine deviations from additivity. If the calculated value of % mitochondrial activity is higher than the measured value synergistic toxicity is at play, if it is lower than the measured values the effects are antagonistic.¹²⁰

In Figure 22 are the results of the WST-1 assay performed with HCEC-1CT cells treated with 1.5 mg/ml CA and different concentrations of berry extracts (BB, Bil, BC and EB) in colour and the expected values, calculated by the mathematical model of Independent Joint Action, in grey. According to the applied model, there were no antagonistic effects on the toxicity between CA and the berry extracts. Synergistic toxicity could only be observed for one BB extract concentration. The applied CA concentration of 1.5 mg/ml and the BB concentration 100 µg/ml showed significant synergistic toxicity, with the observed cell viability being lower than the calculated value. No other significant synergistic effects could be observed. Slight differences between experimental and calculated values could also be observed for other BB concentrations and in the highest concentrations of BC and Bil hinting at possible synergism, but this was not significant.

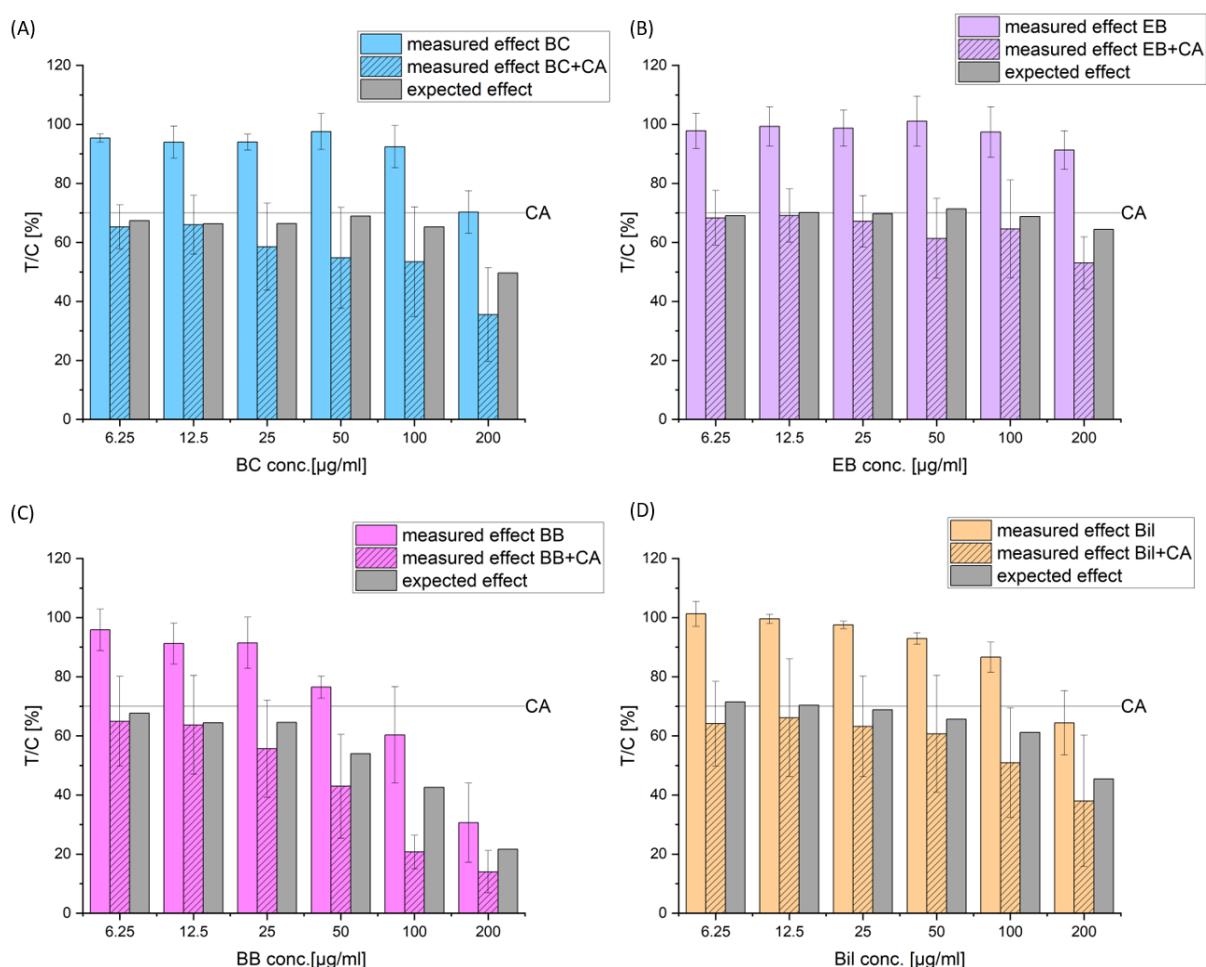


Figure 22: Cytotoxic effects of BC (A), EB (B), BB (C) and Bil (D) extracts in a concentration range between 6.25 and 200 mg/ml alone (unpublished data) and combined with 1.5 mg/ml citric acid (CA) and on HCEC-1CT cells after 72 h of incubation determined by WST-1. Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5 % DMSO, $y=100$). The grey bars represent the expected effects of binary combinations calculated on basis of Independent Joint Action. Straight line represents the mean toxicity of 1.5 mg/ml CA. Statistical significances were evaluated between expected and measured effects by unpaired Student's t-test (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Discussion:

Firstly, it was discussed in chapter 1.4, that it is hypothesised that citric acid exhibits anti-cancer effects in multiple cancer models. Cancerous cell lines investigated in this regard are; breast cancer MCF7 and MDA-MB-231, colon cancer HCT116 and HT29, lung cancer H1299 and A549 cell lines¹⁰³, human esophageal carcinoma cells EC109⁹⁹, MSTO-211H human mesothelioma cells¹⁰⁰, human ovarian carcinoma cells¹⁰¹ and gastric carcinoma cells¹⁰², amongst others. In the experiment performed, CA significantly downregulated the cell viability concentration dependently in the cancer cell lines HCT116 and CT26. In the HCT116 cells the toxicity observed was significant from 1 mg/ml upwards and in the CT26 cells already at 0.5 mg/ml, hinting at a higher sensitivity of the murine cell line. In the cancer cell lines tested

by Zhao *et al.* (including HCT116), IC₅₀ was achieved between 5-10 mM (1-2 mg/ml), which is in accordance to most other literature. The toxicity expressed by CA in the HCT116 in the experiment performed is very similar to the literature, but CT26 seem to be especially sensitive since toxicity is already observed at a relatively low concentration of 0.5 mg/ml (≈ 2.5 mM). Here, also no conclusions could be drawn in regards to the mechanisms responsible for the anti-proliferative effect, since only the toxicity itself was tested using the WST-1 and SRB assay. Furthermore, it is postulated that this anti-proliferative effect is specific to cancer cells. Zhao *et al.* investigated the effect of citric acid in cancerous and non-cancerous cell lines and reported a dose dependent inhibition of cell growth and proliferation in cancer cells, including breast cancer MCF7 and MDA-MB-231, colon cancer HCT116 and HT29, lung cancer H1299 and A549 cell lines, but little to no effect in human fibroblasts (HFF and WI-38) and a normal breast cell line (MCF10A).⁹⁵ According to the results presented in this thesis, there is no difference between cancerous (HCT116) and non-cancerous (HCEC-1CT) human colon cells, regarding the toxicity of citric acid, in any dose or test system. These results differ from Zhao *et al.* (2022) and refute the theory that CA is more toxic in cancerous than non-cancerous cells. Although they investigated multiple different cell lines, none of them being a non-cancerous colon cell line, whereas here only two human colon cell lines were compared. However, the fact remains, that the toxicity of CA did not differ between the colon cancer cell line HCT116 and then non-cancerous colon cell line HCEC-1CT.

It also has to be noted that the SRB assay seems to be more sensitive and describes a lower cell viability than the WST-1 in some cases when investigating the toxicity of CA. Differences in the sensitivity of SRB and WST-1 assays are possible, as they utilize different labelling systems to assess cell proliferation and toxicity (as described in chapter 7.3.1). The WST-1 assay represents a measure for mitochondrial enzyme activity, more specifically on the metabolic reduction of the salt to a highly coloured formazan end-product, which may be stimulated by stress, resulting in apparent higher cell viability values. Whether there is actually an increased proliferation or an initial stress response of the cells cannot be specified using this assay. The evaluation of cell viability in the SRB assay functions via cellular protein content, providing a different endpoint for a more detailed assessment. The quantity of dye extracted from stained cells is directly proportional to the total protein mass and therefore correlated to cell number. Nevertheless, the standard deviations were generally higher in the

SRB assay. The higher deviations might be due to the several washing steps conducted in the SRB assay, providing possibilities for cell loss. It is known that different toxicity assays have different sensitivities depending on cell line and treatment.^{121, 122} Both assays generally showed similar concentration dependent toxicity results and trends, but the SRB detected a lower cell viability in some cases. However, the factors stated above need to be taken into consideration when comparing results from these different assays.

One point, which a lot of the literature on this subject overlook, is the influence of the pH when applying CA in cell culture models. CA is acidic and *in vitro* cell models are kept at a very specific pH and the media used has buffer capabilities. A lowered pH could also lead to a lower cell viability in the model applied. How much of the toxicity is caused by the acidic nature of CA and how much is caused by CA specifically through the different pathways discussed in literature, remains open. Also, not all cell lines have the same pH optimum and not all cell culture media include the same buffers. If some media buffers the acidity better than others, this could also be the cause of the differences between cell lines. The pH was measured in the media with 1.5 mg/ml added CA in HCT116 and HCEC-1CT medium and 0.75 mg/ml in the CT26 medium, since those were the concentrations used in the berry extract combination experiment. The pH measurements were only performed twice with relatively high fluctuations and are thus not significant, but they can give an incentive for discussion. The pH of the HCT116 media with 1.5 mg/ml CA was around 7.05 ± 0.11 , the pH of HCEC-1CT media with added 1.5 mg/ml CA around 6.75 ± 0.18 and CT26 media with added 0.75 mg/ml CA around 6.75 ± 0.14 . According to the pH measurements the media of CT26 cells seems to be more sensitive to the CA and the pH is with 0.75 mg/ml CA already lower than the pH of the media of HCT116 and HCEC-1CT cells with 1.5 mg/ml CA. This certainly could impact the toxicity and explain why the CT26 cells show a higher toxicity of CA than the other two cell lines. HCT116 and HCEC-1CT media, though similar CA toxicity was observed in the cells, show differences in pH with the HCEC-1CT media having an almost similarly low pH than CT26. This could potentially indicate that the non-cancerous HCEC-1CT cells are more resistant to CA after all, although this was not directly reflected in the cytotoxicity assays and could only be interpreted this way after pH measurements were conducted.

Regarding the toxicity of CA in combination with the berry extracts, all three cell lines were co-incubated for 72 h with a toxic concentration of CA (HCT116 and HCEC-1CT: 1.5 mg/ml, CT26: 0.75 mg/ml) and extract concentrations of 6.25, 12.5, 25, 50, 100 or 200 µg/ml. The

effects observed do not seem to indicate an interaction between CA and the extracts. The observed downregulations lie for the most part in accordance with the cytotoxicity tests of the extracts themselves. Franziska Steiger performed cytotoxicity testing with the four extracts on the HCT116 cells in her master thesis in 2021.¹²³ Her data showed a concentration-dependent downregulation in cell viability by the blackberry extract in the WST-1 assay and a slight, mostly concentration-dependent downregulation of viability by all extracts in the SRB assay. The WST-1 assay even showed slight upregulations in cell viability for the other three extracts. The cell viability still stayed over 80 % for the most part in the SRB assay, but in the highest concentration, 200 µg/ml, viability fell slightly below 80 % and below 40 % with the BB extract. This is reflected in the results above, with BB downregulating the cell viability even further from the damage caused by CA, and the other extracts mainly staying around 100 %, EB even upregulating the viability in the WST-1 assay. There were slight downregulations for higher concentrations of BC and Bil, in the SRB the viability fell to around 60 % when treated with 200 µg/ml of BC. This would be a downregulation from the sole treatment with the extracts, possibly hinting at an increased toxicity when combining CA and BC.¹²³ Comparisons with other literature are difficult since the berry extracts are very individual in their compositions. Katsube *et al.* investigated the toxicity of ten berry ethanol extracts including blackberry, bilberry and black currant in HCT116 cells. The bilberry extract was found to exert the strongest cytotoxic effects in HCT116 cells, applied in concentrations of 2 and 4 mg/ml, whereas in this work the blackberry extract displayed the strongest cytotoxicity at 200 µg/ml (Figure 19). Comparisons are furthermore difficult as the concentrations deployed in the comparison study were significantly higher (factor 10), the incubation period (48 h) was shorter, the solvent was different (ethanol) and cell viability was determined by count. Nevertheless, all berry extracts were able to diminish growth of HCT116 cells.¹²⁴ As for HCEC-1CT, there seems to be concentration dependent cytotoxicity for all four extracts (unpublished data), which is in line with the data observed here. HCEC-1CT cells, treated with the extracts in the same manner as in this thesis except for the CA addition, show in the WST-1 assay downregulated cell viability; starting in the blackberry extract from 50 µg/ml at 80 % cell viability going down to 30 % at 200 µg/ml, the bilberry extract shows slight but significant toxicity starting from 25 µg/ml going down to 70 % at 200 µg/ml, black currant shows only significant toxicity at 200 µg/ml and elderberry shows slight but no significant toxicity. In the

SRB assay HCEC-1CT show similarly stronger concentration dependent toxicity; in the BB starting from 25 µg/ml, Bil and BC at 50 µg/ml and the EB extract shows significant toxicity at 200 µg/ml with the cell viability going down to around 70 % (unpublished data). The data observed in this thesis is similar, with the exception of the WST-1 assay potentially showing slightly higher toxicity here, BC and Bil downregulating cell viability to under 60 %. To investigate combinatory effects between CA and berry extracts in this cell line, the mathematical model of independent joint action was utilised. Upon comparing the experimental and expected values, only one BB concentration showed significant differences. 1.5 mg/ml CA and 100 µg/ml BB extract showed synergistic toxic effects in the HCEC-1CT cells. The extracts BB, BC and Bil showed tendencies for synergism in the highest concentrations, hinting toward possible combinatory effects, but this was not significant. Unpublished data regarding CT26 cell line, shows similar patterns as observed here in regards to the toxicity of the berry extracts used. In the WST-1 assay; blackberry extract shows cytotoxicity in almost every concentration, going down to cell viability of 30 % at 200 µg/ml. The bilberry, black currant and elderberry extracts have no significant impact on CT26 cell viability in concentrations applied, between 6.25 and 200 µg/ml (unpublished data). The SRB assay again shows stronger toxicity, with every extract causing down regulation of cell viability to 80 % at 25 µg/ml with Bil, BC and EB staying around 80 % in all concentrations and BB going down concentration dependently to 10 % cell viability at 200 µg/ml (unpublished data). Results from this thesis show a more or less steady cell viability at round 100 % in the WST-1 except for the BB extract which leads to a downregulation to around 40 % with 200 µg/ml. In the SRB assay the viability is lightly lower but still not significantly down regulated from 100 % at concentrations up to 100 µg/ml, there and at 200 µg/ml the viability is downregulated to 70-80 % with the Bil, BC and EB extract and to around 20 % with BB.

All three cell lines, HCT116, HCEC-1CT and CT26, seem to react in a similar manner to the extracts, whether the control is just solvent or cytotoxic CA. The toxicity from the CA was overall only worsened in concentrations where the extracts are cytotoxic themselves. Possible synergistic effects were observed for CA and BB extract, when applying the mathematical model of independent joint action in HCEC-1CT cells. No significant upregulations of viability could be observed in any cell line. The extracts seem therefore to have no inhibitory effects on the toxicity of citric acid *in vitro*, as the *in vivo* main study I suggested in case of the blackberry extract (unpublished data).

6.2. LC-MS/MS Evaluation of Anthocyanins *in vivo*

The animal studies were performed on balb/c mice and were carried out by the group of Univ.-Prof. Dr. Walter Berger at the Medical University of Vienna. The naming of the individual studies as well as the numbering of the mice was done internally by the Department of Food Chemistry and Toxicology at the University of Vienna and was independent of the Medical University of Vienna. Thus, they might carry different names. After the sacrifice of the mice, urine, feces and plasma samples were collected and anthocyanin concentrations were measured via LC-MS/MS. For this thesis, samples of the interim studies II (only feces) and III were analysed. For quantitative measurements of the anthocyanins, the software Skyline (version 21.1; MacCossLab, University of Washington, Seattle, Washington, USA) was used, calibration curves were created for each analyte using standards, by plotting the measured peak area against the analyte concentration in µg/l, and the limit of detection (LOD) and limit of quantification (LOQ) were determined by using the signal to noise ratio, which should be above 3 and 10, respectively. Values higher than the LOD but below the LOQ are included in the graphs but not in the tables.

6.2.1. Interim Study II

After findings from the main study I, that indicated an influence of citric acid on the study outcome, interim studies were carried out using 0.9 % NaCl instead of 0.1 % citric acid as a solvent for the berry extracts. The findings of Zhao *et al.*⁹⁵, Chen *et al.*⁹⁹ and Ren *et al.*¹⁰³, suggest that citric acid can inhibit tumor cell proliferation and tumor growth in multiple tumor lines *in vivo* and *in vitro*. Interim study II was conducted in tumor bearing mice.

In this study, there was a control group and one that received 200 mg/kg body weight (bw) blackberry extract (BB) every second day via oral gavage. The treatment was intended to last for fourteen days equalling 8 doses of BB extract but toxicity of the extract (visible through weight loss of the mice) allowed for only 6 doses. The next day, mice were sacrificed after receiving a final seventh oral gavage of BB extract. The BB extract was not dissolved in 0.1 % citric acid, as in previous studies, but was dissolved in a 0.9 % saline solution. The control group received only the vehicle (0.9 % NaCl) via oral gavage. For this study, female balb/c mice were used that were injected with CT26 cells 24 hours before the experiment started. Which mice were assigned to which group, sample collection and their times of sacrifice are shown in Table 9. Urine and plasma of the interim study II were evaluated by Anna-Lena Mayr in her master

thesis earlier this year. The times of sacrifice were chosen based on the conducted pilot study (pilot II), to ensure optimal concentrations for measurements in all matrices.¹²⁵

Table 9: General parameters of interim study II -female balb/c mice, injected with CT26 cells 24 hours before the experiment started, received 200 mg/kg BB extract dissolved in NaCl 0.9 % via oral gavage every second day (total 6 doses).

Mouse	Treatment	Analytics			Time	Sacrificed after BB gavage
No.		Plasma [µl]	Urine	Feces	Sacrificed	h:min
#56	Ctrl.	~200	↓	x	13:52	02:22
#57	Ctrl.	~115	↓	x	13:41	02:11
#58	Ctrl.	~105	x	x	14:00	02:30
#59	Ctrl.	~150	x	x	13:35	02:05
#60	BB	~80	↓ 1 µl	x	14:20	02:50
#61	BB	~50	x	x	15:07	03:37
#62	BB	~140	↓ 1 µl	x	14:55	03:25
#63	BB	~110	x	x	14:38	03:08

x sample taken

↓ small amount

Since anthocyanins have a rather low bioavailability, as discussed in chapter 1.1.4., lower concentrations are expected in urine and plasma, while higher ones are expected in feces. Standards of cyanidin-3-glucoside (cy-3-glc), delphinidin-3-glucoside (del-3-glc), cyanidin-3-sambubioside (cy-3-sam), cyanidin-3-rutinoside (cy-3-rut), cyanidin-3-sambubioside-5-glucoside (cy-3-sam-5-glc) and cyanidin-3,5-diglucoside (cy-3,5-diglc) were utilised to identify and quantify the anthocyanins in the samples. The anthocyanins cy-3-glc and cy-3-rut are two of the five anthocyanins (see Table 20) present in the blackberry extract, of which standards were available. They are also the anthocyanins present in the highest concentrations, with cy-3-glc making up the largest fraction with roughly 90 %.

In Table 10 the results for the feces evaluation of the interim study II are depicted. All peaks of cy-3-sam-5-glc and del-3-glc fell under the limit of detection (LOD), as well as multiple signals for cy-3,5-diglc.

Table 10: Results of interim study II: anthocyanin concentrations ($\mu\text{g}/\text{kg}$) in feces samples:

Group	Mouse	Conc. $[\mu\text{g}/\text{l}]$					
		Cy-3-glc	Del-3-glc	Cy-3-sam	Cy-3-Rut	Cy-3-sam-5-glc	Cy-3,5-diglc
Ctrl.	56	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD
Ctrl.	57	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD
Ctrl.	58	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD
Ctrl.	59	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD
BB	60	11800	<LOD	300	4580	<LOD	487
BB	61	238	<LOD	<LOQ	94.07	<LOD	<LOQ
BB	62	45500	<LOD	1690	23500	<LOD	3210
BB	63	40.4	<LOD	<LOQ	<LOQ	<LOD	<LOD

LOD $\approx 0.80 \mu\text{g}/\text{kg}$, LOQ $\approx 12.0 \mu\text{g}/\text{kg}$

Figure 23 shows the concentrations of cy3-glc, cy-3-rut, cy-3-sam and cy-3,5-diglc in $\mu\text{g}/\text{kg}$ in feces found approximately after 2 to 3 hours after administration of the extract. Small concentrations of supposed anthocyanins were measurable in the control group but not quantifiable. There is a clear distinction between control mice and mice given the blackberry extract but the concentrations in the group vary greatly between animals. Concentrations of cy-3-glc range from 40.4 to 45500 $\mu\text{g}/\text{kg}$ feces and cy-3-rut concentrations range from 94.1 to 23500 $\mu\text{g}/\text{kg}$ feces in the mice administered blackberry extract. Concentrations of cy-3-sam and cy-3,5-diglc were also measurable in the samples but considering they are not contained in the blackberry extract, they were probably falsely identified as such and are metabolites of the anthocyanins contained with similar structures.

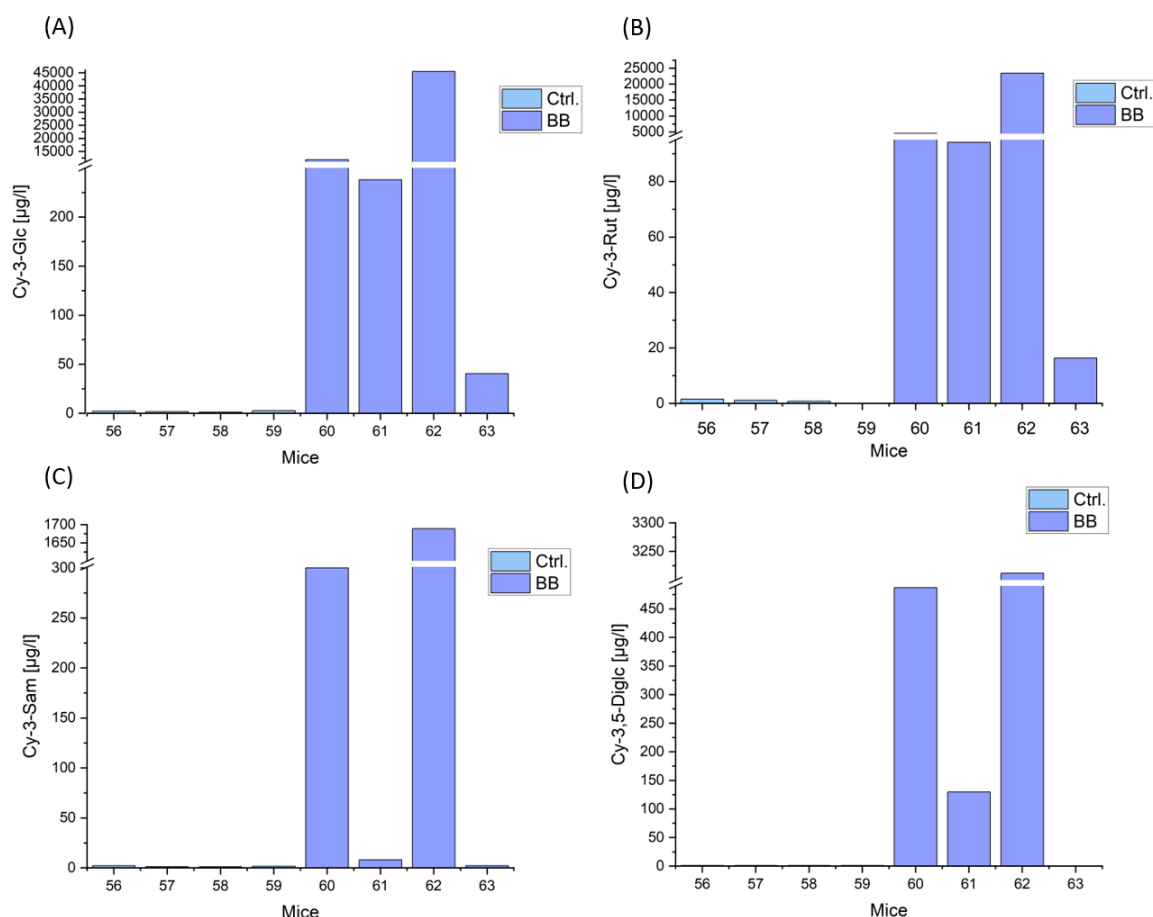


Figure 23: Concentrations of (A) cy-3-glc, (B) cy-3-rut, (C) cy-3-sam and (D) cy-3,5-diglc are shown in the feces of tumor bearing mice that received no extract (ctrl.) or 200 mg/kg body weight blackberry extract in 0.9 % saline solution every second day for 12 days via oral gavage. The concentration scales vary between graphs. Concentrations in the control groups were not quantifiable but lie over the LOD.

The differences between the four mice, given the same amount of BB extract, are very high. Thus, means were not calculated for the group. Since only the fecal samples were evaluated for the interim study II, the results will be discussed with the results from the master thesis of Ana-Lena Mayr, as she evaluated the plasma and urine samples of this study with the help of 3 standards; cy-3-glc, cy-3-rut and cyanidin-3-xyloside (cy-3-xyl). No urine could be collected from mice #60 and #62, leaving only 2 samples in the BB group. According to Mayr, the highest concentration in urine stemmed from cy-3-glc at around 10400 ± 1200 µg/l, but both cy-3-xyl and cy-3-rut were also found in the urine samples, at around 400 and 200 µg/l, respectively. Out of the 3 standards used, only cy-3-glc, and cy-3-rut could be found in the plasma at concentrations above the LOD. Concentrations of cy-3-glc ranged between 0.5 (#61) and 20 (#60) µg/l and cy-3-rut concentrations fell below the LOQ for two mice (#61 and #63), leaving mouse #60 with 1.8 µg/l and mouse #62 with 1 µg/l.¹²⁵ Mouse #63 had slightly higher

anthocyanin concentration in the urine than mouse #61, which could partly explain the very low concentrations in feces but only to a degree since the low concentrations in feces is quite striking by comparison. After sacrifice of mouse #63, the extracted tumor was described as very dark and bloody and the feces samples also had a dark colour. This would suggest that the feces should have relatively high anthocyanin concentrations which the measurements did not confirm. It is possible that the dark colour in the feces was caused by blood not anthocyanins. Significantly higher concentrations of anthocyanins were detected in the feces than in urine and especially plasma. This is in accordance to the relatively low bioavailability of anthocyanins, discussed in literature, since anthocyanins excreted through the feces are not absorbed in the intestine and do not reach the bloodstream.²⁴

6.2.2. Interim Study III

For this study, there were a control group and one group per berry extract, that received 200 mg/kg extract every second day via oral gavage totalling 6 doses. Blackberry (BB), elderberry (EB) and bilberry (Bil) extracts were investigated regarding the tolerability of the mice and the bioavailability of the contained anthocyanins. The extracts did not contain 0.1 % citric acid but were dissolved in a 0.9 % NaCl solution. For this study non tumor bearing balb/c mice were used. The mice received the extract treatments at 7:00 (Ctrl, BB), 8:00 (Bil) or 9:10 (EB). Upon sacrifice and sample collection, urine and plasma samples were acidulated with ~1.5 % FA. Which mice were assigned to which group, sample collection and their times of sacrifice are shown in Table 11.

Mouse #64 had no urine sample available and mouse #68 was sacrificed after the second BB gavage since it lost a lot of weight. Due to the observed toxicity within the BB extract group, it was decided to pause the oral administration of the BB extract. As a result, mice of the BB treated group only received three doses in total: two at the beginning of the study and one last dose at the day of sacrifice to investigate bioavailability. To obtain comparable data for bioavailability studies of the different extracts, mice were treated in a time-delayed manner resulting in a time of sacrifice for each extract group of 3-4 h after treatment.

Table 11: General parameters of interim study III – no tumor bearing balb/c mice received 200 mg/kg extract (BB, EB or Bil) dissolved in 0.9 % NaCl every second day via oral gavage (total of 6 doses).

Mouse	Treatment	Analytics			Time	Sacrifice after
No.		Plasma [μl]	Urine [μl]	Feces	Sacrificed	h:min
#64	Ctrl.	120	○	x	09:40	02:40
#65	Ctrl.	260	↓ 15	x	09:15	02:15
#66	Ctrl.	110	120	x	09:55	02:55
#67	Ctrl.	140	120	x	09:27	02:27
#68	BB	○	○	○	○	○
#69	BB	180	120	x	10:10	03:10
#70	BB	110	80	x	10:30	03:30
#71	BB	120	200	x	10:50	03:50
#72	EB	210	180	x	12:10	03:00
#73	EB	>200	↓ 20	x	12:27	03:17
#74	EB	130	>200	x	12:36	03:26
#75	EB	100	50	x	12:50	03:40
#76	Bil	200	180	x	11:00	03:00
#77	Bil	130	80	x	11:20	03:20
#78	Bil	130	200	x	11:37	03:37
#79	Bil	140	250	x	11:57	03:57

x sample taken

○ no sample

↓ small amount

Plasma:

Table 12 shows the detected concentrations of cy-3-glc, del-3-glc, cy-3-sam, cy-3-rut, cy-3,5-diglc and cy-3-gal in the plasma of mice treated with blackberry, elderberry or bilberry extract. The concentrations detected in the plasma were overall very low and a considerable amount fell below the limit of detection. The other detected concentrations often fell under the limit of quantification (LOQ) and/or did not differ significantly from the control group and are thereby not relevant. Namely, cy-3-rut, cy-3,5-diglc and del-3-glc were not detected in the plasma in relevant concentrations. Detected cy-3-rut concentrations were always under the LOQ. Cy-3-glc concentrations were very low but still over the LOD and LOQ, generally revolving around 0.20 μg/l. Cy-3-sam and cy-3-sam-5-glc were detected in the extract groups in higher concentrations than in the control group, with concentrations up to 1 μg/l. Both were detected in all extract groups, which indicates that metabolites of other anthocyanins are detected as cy-3-sam or cy-3-sam-5-glc here, since they are not part of the main anthocyanin composition of the extracts besides EB.

Table 12: Results of interim study III: anthocyanin concentrations (µg/l) in plasma samples:

Group	Mouse	Conc. [µg/l]					
		Cy-3-glc	Del-3-glc	Cy-3-sam	Cy-3-rut	Cy-3-sam-5-glc	Cy-3,5-diglc
Ctrl.	64	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
Ctrl.	65	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
Ctrl.	66	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
Ctrl.	67	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD
BB	69	0.25	<LOQ	0.90	<LOQ	0.49	<LOQ
BB	70	0.13	<LOD	0.81	<LOQ	1.89	<LOQ
BB	71	0.25	<LOD	0.69	<LOQ	1.62	<LOD
EB	72	0.19	<LOD	0.71	<LOQ	1.47	<LOD
EB	73	0.20	<LOD	0.55	<LOQ	1.43	<LOD
EB	74	0.23	<LOD	0.83	<LOQ	2.80	<LOD
EB	75	0.17	<LOD	0.32	<LOQ	<LOQ	<LOD
Bil	76	0.16	<LOD	0.27	<LOQ	<LOQ	<LOD
Bil	77	0.17	<LOD	0.24	<LOQ	<LOQ	<LOD
Bil	78	0.16	<LOD	0.26	<LOQ	<LOQ	<LOD
Bil	79	0.15	<LOD	0.26	<LOQ	<LOQ	<LOD

LOD≈ 0.06 µg/l, LOQ ≈ 0.13 µg/l

Urine:

Table 13 and Figure 24 show the concentrations of cy-3-glc, del-3-glc, cy-3-sam, cy-3-rut, cy-3,5-diglc and cy-3-gal measured in urine samples.

Table 13: Results from interim study III: anthocyanin concentrations (µg/l) in urine samples

Group	Mouse	Conc. [µg/l]						
		Cy-3-glc	Del-3-glc	Cy-3-sam	Cy-3-rut	Cy-3-sam-5-glc	Cy-3,5-diglc	Cy-3-gal*
Ctrl.	65	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	/
Ctrl.	66	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	/
Ctrl.	67	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	/
BB	69	652	<LOD	7.73	30.0	<LOD	<LOD	/
BB	70	502	<LOD	<LOQ	25.7	<LOQ	<LOD	/
BB	71	539	<LOD	<LOQ	18.70	<LOD	<LOD	/
EB	72	115	<LOD	645	5.87	590	95.2	/
EB	73	12.6	<LOD	103	<LOQ	37.6	<LOD	/
EB	74	2420	<LOD	8220	44.7	10300	1540	/
EB	75	36.1	<LOD	386	<LOQ	131	<LOQ	/
Bil	76	52.9	104	<LOQ	<LOQ	<LOD	<LOD	59.9
Bil	77	46.7	70.0	<LOQ	<LOQ	<LOD	<LOD	65.1
Bil	78	147	296	<LOQ	<LOQ	<LOD	<LOD	159.4
Bil	79	48.1	84.1	<LOQ	<LOQ	<LOD	<LOQ	62.1

*double-peak identified as cy-3-glc and cy-3-gal only in Bil

LOD≈ 2.00 µg/l, LOQ≈ 7.00 µg/l

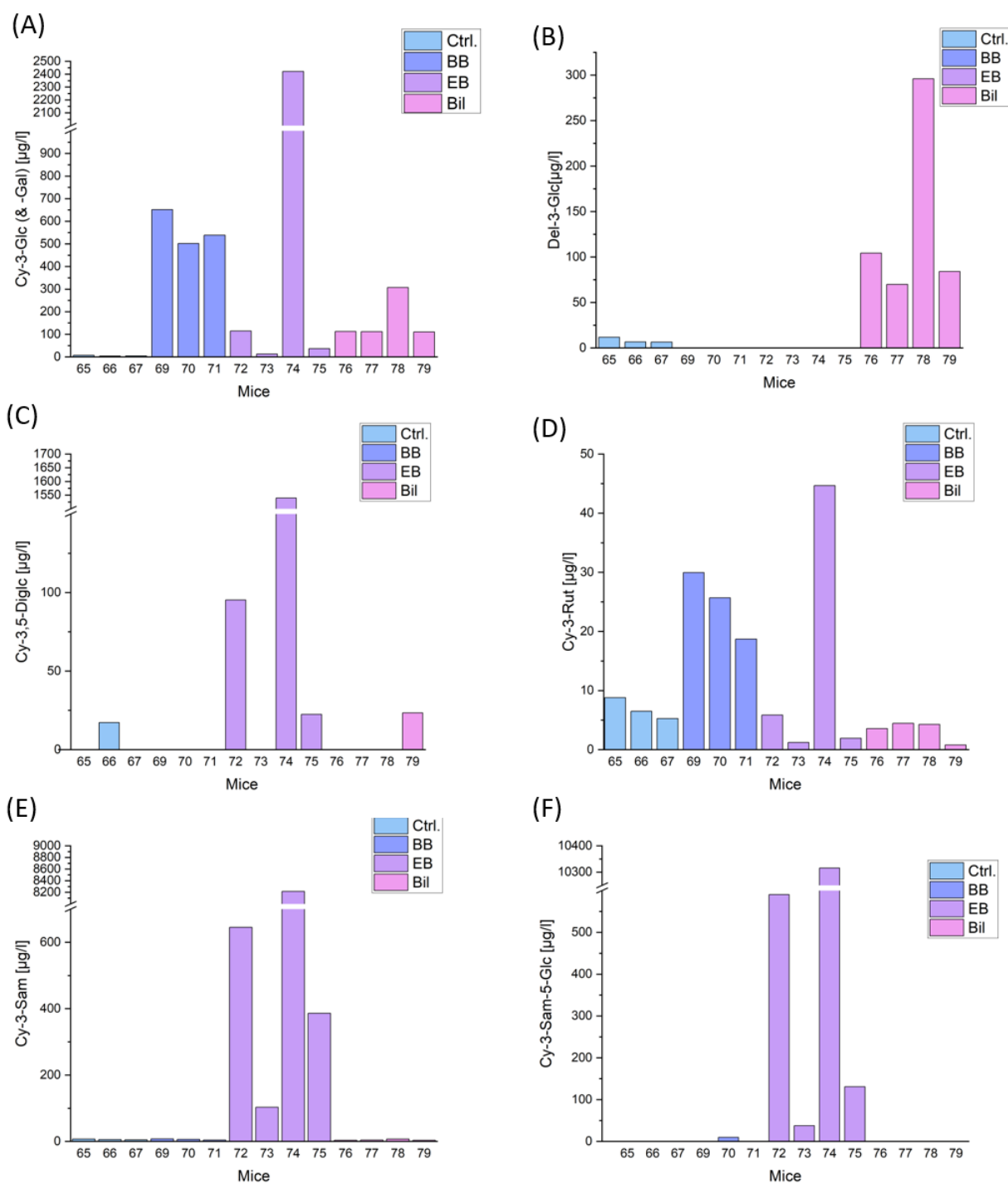


Figure 24: Concentrations of (A) cy-3-glc and cy-3-gal, (B) del-3-glc, (C) cy-3,5-diglc, (D) cy-3-rut, (E) cy-3-sam-5-glc and (F) cy-3-sam are shown in the urine of non-tumor bearing mice that received no extract (ctrl.) or 200 mg/kg body weight blackberry extract (blue), elderberry extract (violet) or bilberry extract (pink) in 0.9% saline solution every second day via oral gavage. The concentration scales vary between graphs. Concentrations in the control groups were not quantifiable.

The bilberry extract contains a variety of anthocyanins (Table 20) the main ones being del-3-glc, cy-3-glc and cyanidin-3-galactoside (cy-3-gal) and also delphinidin-3-arabino- side, delphinidin-3-galactoside and malvidin-3-glucoside for which there were no standards available. The urine samples of the bilberry group (#76, 77, 78, 79), and also feces samples,

showed a double peak for cy-3-glc (identified by standard) and the second peak derives most likely from cy-3-gal (explementary Figure 25). Since there was no standard available for cy-3-gal the concentrations were determined on the basis of the cy-3-glc standard and added to Table 13. In the graph for cy-3-glc, cy-3-glc and cy-3-gal were summed up for the bilberry samples. Other than that, del-3-glc was also detected in the bilberry mice (70.0-296 µg/l), which is in accordance with the extract composition. Delphinidin-3-galactoside is also part of the anthocyanin composition of the bilberry extract, but in this method, there is no separate peak recognizable in the chromatogram and the peak for del-3-glc probably contains also del-3-gal. The measured concentrations in the groups vary greatly between animals. Mouse #74 for example, had extremely high contents of various anthocyanins in the urine which was also represented in the sample itself, which was very dark and red. However, considerable differences in concentrations were observed in the other samples and groups as well.

Feces:

Table 14 shows the detected concentrations of cy-3-glc, del-3-glc, cy-3-sam, cy-3-rut, cy-3,5-diglc and cy-3-gal in feces of mice treated with blackberry, elderberry or bilberry extract.

Table 14: Results from interim study III: anthocyanin concentrations (µg/kg) in feces samples

Group	Mouse	Conc. [µg/kg]						
		Cy-3-glc	Del-3-glc	Cy-3-sam	Cy-3-rut	Cy-3-sam-5-glc	Cy-3,5-diglc	Cy-3-gal*
Ctrl.	64	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOQ	/
Ctrl.	65	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD	/
Ctrl.	66	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD	/
Ctrl.	67	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOD	/
BB	69	<LOQ	<LOD	<LOQ	1.23	<LOD	<LOD	/
BB	70	2950	<LOQ	59.6	344	<LOD	45.7	/
BB	71	2510	<LOQ	57.2	250	<LOD	27.6	/
EB	72	380	<LOD	13400	43.3	3460	234	/
EB	73	1720	<LOD	55277.10	96.0	5770	527	/
EB	74	<LOQ	<LOD	41.81	<LOQ	8.55	<LOD	/
EB	75	2820	<LOD	97800	170	12300	1270	/
Bil	76	1600	12900	599	19.7	91.6	<LOD	1460
Bil	77	368	1690	85.8	11.2	10.5	<LOD	79.0
Bil	78	54.2	170	12.5	<LOQ	<LOQ	<LOD	39.9
Bil	79	46.4	292	8.19	<LOQ	<LOQ	<LOD	39.1

*double-peak identified as cy-3-glc and cy-3-gal only in Bil

LOD≈ 0.80 µg/kg, LOQ≈ 9.60 µg/kg

Similar to the urine samples of the bilberry group (#76, 77, 78, 79), the feces samples also showed a double peak for cy-3-glc (identified by standard) and most likely cy-3-gal. According to the retention times of the standard, the first peak was identified as cy-3-glc and the second one is assumed to be cy-3-gal based on literature data. In Figure 25 an example of the double peak for cy-3-glc in the feces sample of the mouse #76 is shown.

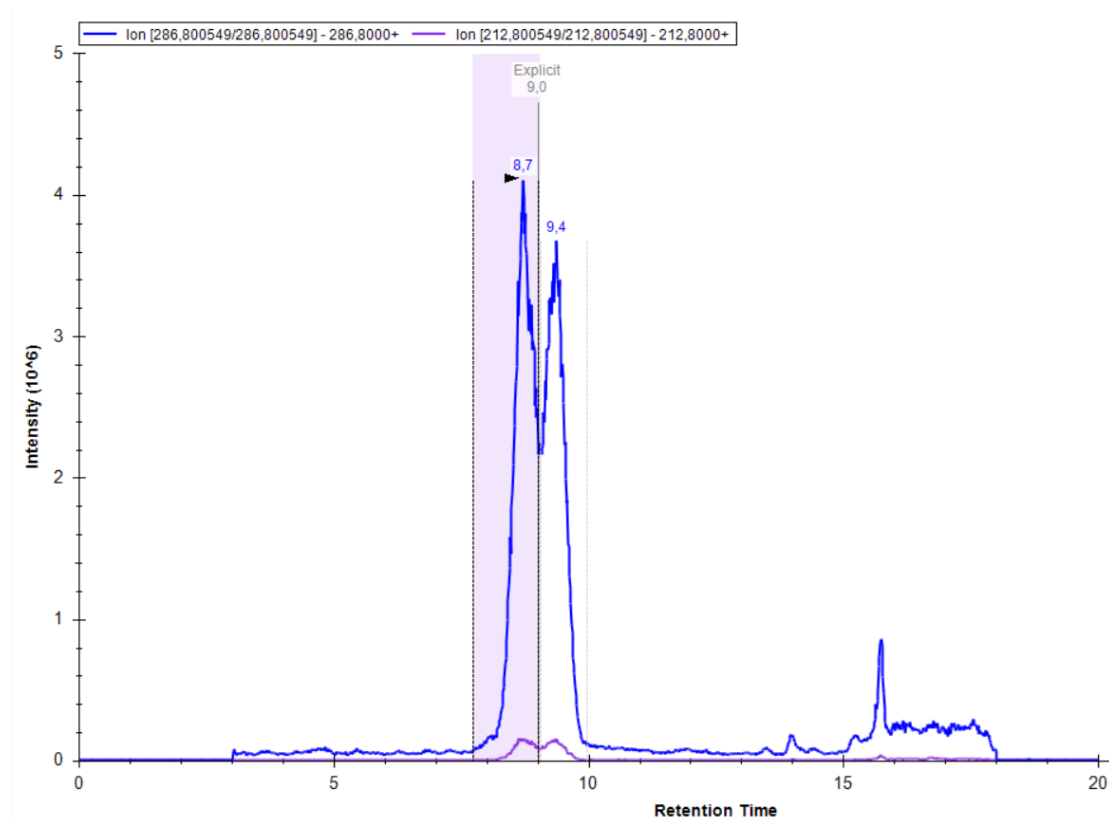


Figure 25: Chromatogram of cy-3-glc in the feces sample #64 taken with the skyline software and identified by cy-3-glc standards. The graph depicts as example the split peak seen for cy-3-glc in the mice that received the bilberry extract. The second peak was most probably identified as cy-3-gal in line with the concentrations found in the extract.

Since there was no standard available for cy-3-gal the concentrations were determined on the basis of the cy-3-glc standard and added to Table 14. In the graph for cy-3-glc, cy-3-glc and cy-3-gal were summed up for the bilberry samples. Shown below in Figure 26 are the graphs depicting concentrations of anthocyanins found in feces samples of interim study III with (A) cy-3-glc and cy-3-gal, (B) del-3-glc, (C) cy-3,5-diglc, (D) cy-3-rut, (E) cy-3-sam-5-glc and (F) cy-3-sam.

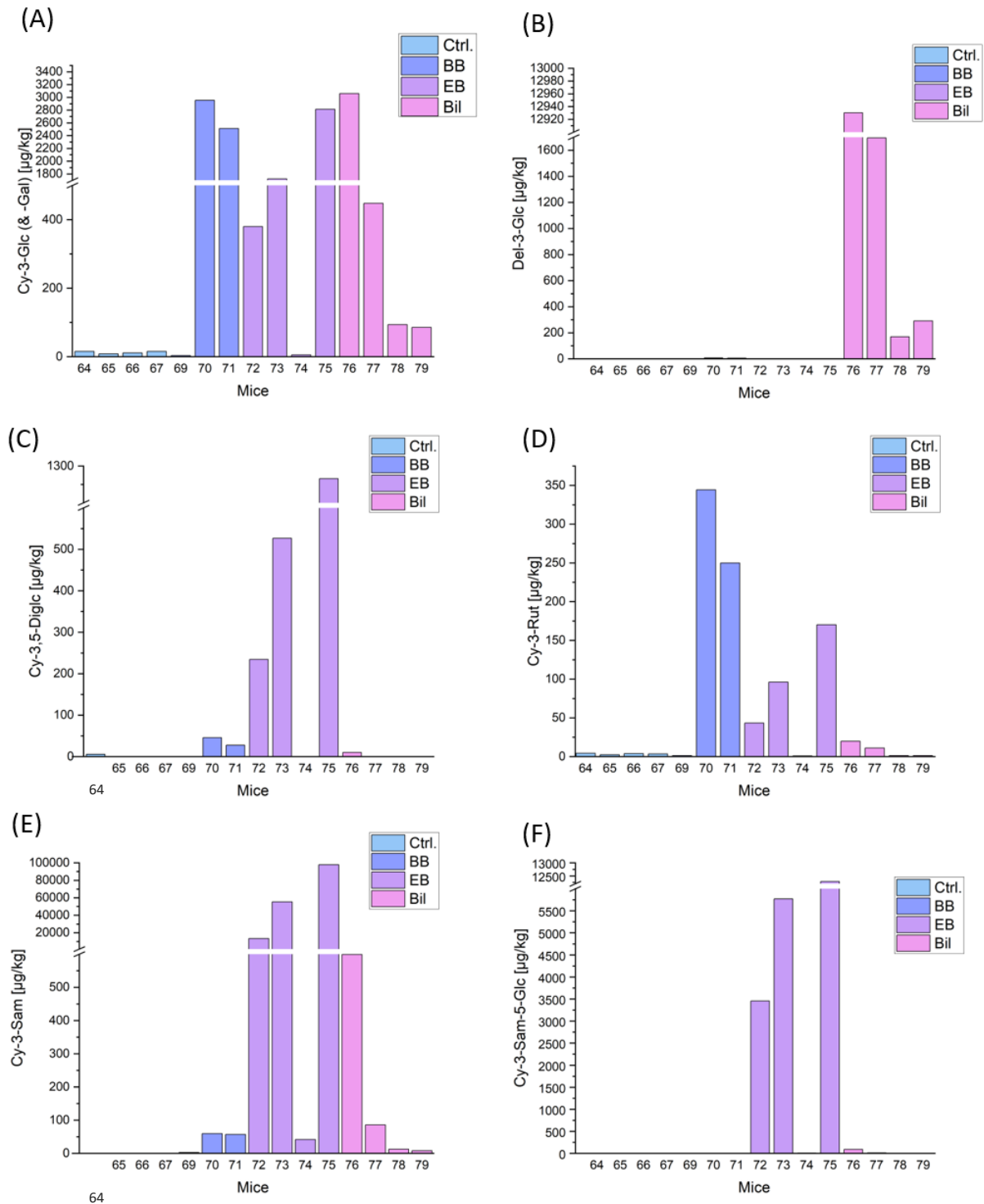


Figure 26: Concentrations (µg/kg) of (A) cy-3-glc and cy-3-gal, (B) del-3-glc, (C) cy-3,5-diglc, (D) cy-3-rut, (E) cy-3-sam-5-glc and (F) cy-3-sam are shown in feces of non-tumor bearing mice that received no extract (ctrl.) or 200 mg/kg body weight blackberry extract (blue), elderberry extract (violet) or bilberry extract (pink) in 0.9% saline solution every second day via oral gavage. The concentration scales vary between graphs. Concentrations in the control groups were not quantifiable.

In the fecal samples, the concentrations vary between mice in one group as well. In the blackberry group, mouse #69 had no anthocyanin signals distinguishable from the control

group. The other two mice in the group (#70, 71) had expectedly high concentrations of cy-3-glc, 300 and 2500 µg/kg respectively, and cy-3-rut, 344 and 250 µg/kg respectively. Smaller concentrations of cy-3-sam, 59.6 and 57.2 µg/kg, and cy-3,5-diglc, 45.7 and 27.6 µg/kg, were also detected. Those two are not in the anthocyanin composition of the blackberry extract (Table 20) and are most likely metabolites of cy-3-glc or cy-3-rut that were falsely identified as cy-3-sam and cy-3,5-diglc. In the elderberry group (#73, 74, 75, 76), concentrations of all anthocyanins investigated, except del-3-glc, were detected. Mouse #74 is an exception. As visible in all the graphs here, mouse #74 has almost no anthocyanins in the fecal sample, leading to the belief that the anthocyanins did not reach the colon and stayed in the small intestine due to slow digestion. In contrast to the findings in feces this mouse had the highest concentration in the urine. Upon sacrifice and sample collection this was also visible with the urine being dark brown and after addition of FA bright red and all of extract seemed to still be in the small intestine as it was coloured a lot darker than the small intestines of the other mice. According to the extract composition, only the sambubiosides, here also detected in the highest concentrations of up to 97800 µg/kg cy-3-sam and up to 12300 µg/kg cy-3-sam-5-glc, stem from the extract. Lower concentrations identified as cy-3-glc, cy-3-rut and cy-3,5-diglc were also detected. Cy-3-glc and cy-3,5-diglc were not identified in the extracts via HPLC method according to Table 20, but do seem to be contained in elderberry extract according to literature when characterized by LC-UV/MS.¹²⁶ The low concentration of cy-3-rut is most likely caused by metabolites. In the bilberry group, concentrations of cy-3-glc (and cy-3-gal), del-3-glc (with del-3-gal) were detected in relevant concentrations; cy-3-glc and cy-3-gal each between about 50 and 1500 µg/l and del-3-glc between about 200 and 13000 µg/l. The highest concentrations detected in feces overall stem from the elderberry extract with a maximum of 97800 µg/kg of cy-3-sam.

Discussion:

According to the extract compositions in Table 20, cy-3-glc and cy-3-rut are the main anthocyanins in the blackberry extract, cy-3-glc making up the largest fraction with roughly 90 %. Plasma concentrations in mice given the BB extract were generally low, with only cy-3-glc, cy-3-sam and cy-3-sam-5-glc in concentrations over the LOQ. Cy-3-glc concentrations were the lowest, lying between 0.13 and 0.25 µg/l. Higher concentrations were found of cy-3-sam and cy-3-sam-5-glc, with the later measuring concentrations between 0.49 and 1.89 µg/l. They

are however, not part of the extract composition, which indicates that metabolites of other anthocyanins are detected as cy-3-sam or cy-3-sam-5-glc in this case. Mice given the blackberry extract had predominantly cy-3-glc in their urine, in concentrations between 501 and 652 µg/l, with lower concentrations of cy-3-rut. This is mainly in accordance with the extract composition. In the feces samples one mouse had no anthocyanin signals distinguishable from the control group whereas the other two mice in the group also had high concentrations of cy-3-glc (up to 2950 µg/kg) and cy-3-rut (up to 344 µg/kg). Smaller concentrations of cy-3-sam and cy-3,5-diglc were detected as well, which are most likely metabolites of cy-3-glc or cy-3-rut.

The elderberry extract contains cy-3-sam and cy-3-sam-5-glc (Table 20), which is reflected in the plasma. Plasma concentrations of cy-3-sam ranged between 0.55 and 0.83 µg/l and concentrations of cy-3-sam-5-glc between 1.43 and 1.62 µg/l. The urine analysis also lies in accordance with the extract composition, with cy-3-sam ranging between 103 and 8220 µg/l and cy-3-sam-5-glc between 37.6 and 10300 µg/l in the administered group. Some of the samples from the elderberry group also contained cy-3-glc and cy-3,5-diglc in considerable concentrations. In the feces of the elderberry group, concentrations of all anthocyanins investigated, except del-3-glc, were detected. Mouse #74 is an exception since it has almost no anthocyanins in the feces sample, leading to the belief that the anthocyanins did not reach the colon and stayed in the small intestine and are responsible for the high concentrations in plasma and urine. Sambubiosides were the predominant anthocyanins detected in feces with a maximum of 97800 µg/kg cy-3-sam-5-glc. The lower concentrations identified as cy-3-glc and cy-3,5-diglc are also in accordance with the extract composition.¹²⁶

The bilberry extract contains a variety of anthocyanins (Table 20); the main ones being del-3-glc, cy-3-glc and cyanidin-3-glactoside (cy-3-gal). The plasma concentrations in this group are especially low, with only cy-3-glc (0.15-0.17 µg/l) and cy-3-sam (0.24-0.27 µg/l) being detected in quantifiable concentrations. The measured cy-3-sam concentration might stem from metabolites, since cy-3-sam is not part of the extract composition. In the urine samples, the bilberry mice showed concentrations of cy-3-glc, cy-3-gal and del-3-glc. Del-3-glc was found in the highest concentrations ranging from 70.0 to 296 µg/l. In the feces of the bilberry group, concentrations of cy-3-glc, cy-3-gal and del-3-glc were detected in relevant concentrations;

with cy-3-glc and cy-3-gal in concentrations between 39.1 and 1600 µg/kg and del-3-glc between 170 and 12900 µg/kg.

The anthocyanins identified and quantified were selected because they represent the main anthocyanins in the berry extracts (see Table 20); cy-3-glc mainly in blackberry, gly-3-sam and cy-3-sam-5-glc in elderberry, del-3-glc in bilberry, and also cy-3-rut and cy-3,5-diglc. However, relevant plasma concentrations were only detected for cy-3-sam and cy-3-sam-5-glc, with concentrations up to 1 µg/l. Both were detected in all extract groups, which indicates that metabolites of other anthocyanins are detected as cy-3-sam or cy-3-sam-5-glc here, since they are not part of the main anthocyanin composition of the extracts besides elderberry. The samples were collected approximately 3 h after the last extracts administration (200 mg/kg bw). Regarding peak plasma concentrations of anthocyanins, this is in accordance with the literature more on the later side. According to Lila *et al.*, the glucuronidated anthocyanins are predominate in human plasma and peak approximately 1 to 3 h after ingestion.²⁴ Manach *et al.* stated peak plasma concentrations of anthocyanins between 30 min and 2 h after consumption.²⁶ According to a radiolabelled cyanidin 3-O-glucoside study in mice, the maximum concentration of cy-3-glc in plasma was detected 15-30 minutes after administration of a ¹⁴C-labelled cy-3-glc compound.¹²⁷ Hence, it is possible that higher plasma concentrations would have been found earlier. Though these relatively small plasma concentrations lie in accordance with the literature since most reports indicate a very low bioavailability, for example in humans approximately a few tens of nanomoles per liter for an intake of ≈110–200 mg anthocyanins according to Manach *et al.*²⁶ The concentrations given to the mice in the present study were 200 mg/kg bw of extract, which did lead to plasma concentration up to 1 µg/l. When eight humans were fed ¹³C-labelled cy-3-glc (by Czank *et al.* 2013), approximately 30 times more cy-3-glc was recovered in urine than in plasma (0.18% in plasma and 5.37% in urine).³² Here, the concentrations found in the urine were also significantly higher than the concentrations found in plasma. Considering that, for the substances to be excreted via the urine, they needed to be absorbed in the intestine, considerable concentrations of anthocyanins seemed to be made bioavailable in the study. The concentrations of cy-3-glc, del-3-glc and cy-3-rut were high in urine samples of the mice groups taken into consideration that they were not detectable on relevant levels in the plasma. Since the anthocyanin has to be in the bloodstream before it can be excreted via the

urine, it seems that the samples might have been taken at a moment where they had already passed the bloodstream for the most part.

There were some smaller amounts of anthocyanins measured in extracts where they should not have been detected, leading to them being identified as metabolites of anthocyanins present in the extract given. Anthocyanins are metabolized by phase I and phase II enzymes, which create glucuronidated, hydroxylated, sulfated and methylated molecules, giving rise to many different molecular intermediates. In the ^{13}C -tracer study Czank and his team performed to trace metabolites derived from the anthocyanin cy-3-glc, a total of 25 ^{13}C -labelled compounds were identified. Metabolites included phase II conjugates of cy-3-glc and cyanidin, degradants, phase II conjugates of PCA, phenylacetic acids, phenylpropenoic acids and hippuric acid. Czank *et al.* also showed that urine was the primary route of elimination over the first 6 h and feces represented the major route of elimination over the 6–24- and 24–48-h time periods.³² Some of those metabolites of anthocyanins might have been present here as well, albeit not detected by the applied method. In addition, Felgines *et al.* showed that all of the metabolites, except for the native glycoside, were very unstable and thoroughly degraded when acidified urine samples were frozen for storage. This could have led to possible losses in the study at hand.¹²⁷

As expected, most anthocyanins were excreted via feces. This is in accordance with the in literature discussed relatively low bioavailability of anthocyanins, since anthocyanins excreted through the feces are not absorbed in the intestine and do not reach the bloodstream.²⁴ With the results at hand, it is difficult to calculate the bioavailability since it seemed to vary greatly between animals. Taking the BB mice, approximately 500 $\mu\text{g/l}$ anthocyanins were excreted through urine and 3000 $\mu\text{g/kg}$ through feces, meaning a quite high amount of the BB anthocyanins were bioavailable ($\approx 15\%$ of excreted anthocyanins). The interindividual differences however, are so high, that conclusions in this regard are almost impossible; in the EB group there are mice where 50000 $\mu\text{g/kg}$ anthocyanins were excreted through feces and almost none (100 $\mu\text{g/l}$) through urine (#73) and other mice where the amount in urine was up to 10000 $\mu\text{g/l}$ (#74). For a better evaluation the groups in each category would have to be larger so that a representative mean can be calculated. However, the tendencies show the favourable route of elimination is feces, meaning most of the anthocyanins are not made bioavailable. There are also considerable amounts of anthocyanins found in urine, for which

they have to be absorbed first. The plasma concentrations were very low, possibly due to the timing of sacrifice being too late for the anthocyanins to still be in the bloodstream. Overall, the large differences between the mice and the inability to account for metabolites and degradation products make it difficult to draw conclusions regarding bioavailability of anthocyanins in berry extracts from this data.

6.3. Cytotoxicity Testing of Polyphenols

In addition to the anthocyanins (listed in Table 20), other polyphenols were detected in the four berry extracts used for the experiments (unpublished data). They were detected via LC-MS/MS (according to the method by Oesterle et al. 2022¹²⁸) in the extracts and selected polyphenols with a relatively high abundance in the extracts also underwent cytotoxicity testing to investigate whether their involvement is relevant for the extracts' effects. Four polyphenols were evaluated in the course of this thesis regarding their cytotoxicity in the relevant doses, in which they were found in the extracts (listed in Table 21). To determine the cytotoxicity, the WST-1 and the SRB assay were performed on the colon cancer HCT116 cell line and the non-cancerogenic HCEC-1CT cell line with an incubation time of 72 h.

6.3.1. (+)-Rutin

The concentrations (+)-rutin (further referred to as rutin) was tested in were determined by their concentration in the berry extracts at 200 µg/ml, shown in Table 15.

Table 15: Incubation concentrations of rutin and approximate corresponding occurrence in 200 µg/ml of the extracts.

Conc. µg/ml	Extract
1. 8.8	EB
2. 4.4	
3. 2.2	
4. 1.1	BC
5. 0.55	
6. 0.275	BB
	Bil ↓

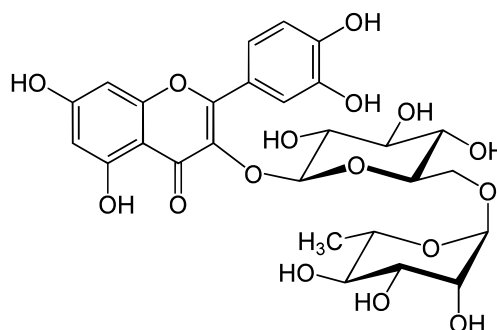


Figure 27: Structure of rutin

Graphs in Figure 28 depicting the cell viability, T/C (%), determined by WST-1 and SRB assay for HCEC-1CT and HCT116 cells. Cytotoxic effects of rutin in a concentration range between 0.275 and 8.8 µg/ml after 72 h of incubation determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT) are depicted. No cytotoxicity was observed in the concentrations tested between 8.8 and 0.275 µg/ml. All rutin concentrations show no significant downregulation of cell viability compared to the solvent control (DMSO) in either cell line.

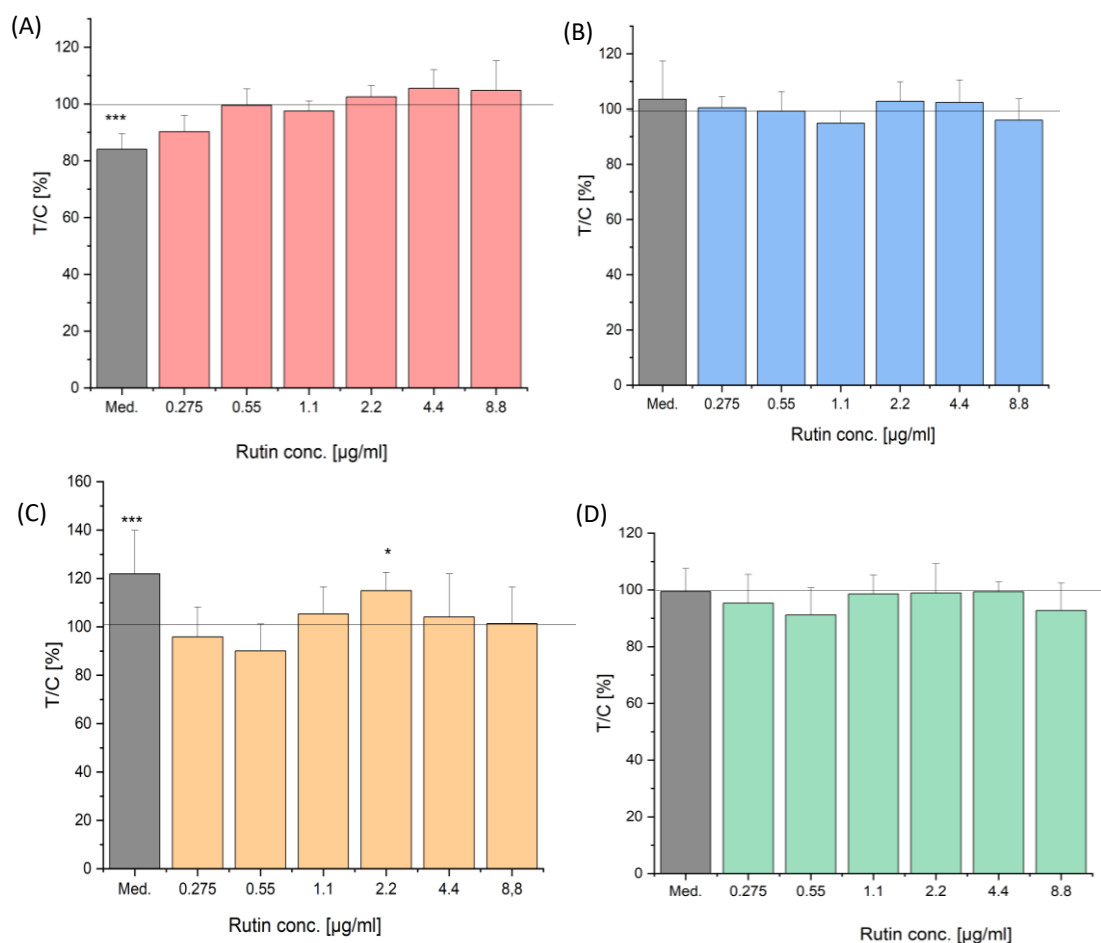


Figure 28: Cytotoxic effects of rutin in a concentration range between 0.275 and 8.8 µg/ml on HCT116 and HCEC-1CT cells after 72 h of incubation determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5 % DMSO, $y=100$). Statistical significances were determined by one-sample Student's t-test for significances between the concentrations of rutin and the solvent control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and bonferroni ANOVA for test concentrations ($p<0.05$).

6.3.2. (-)-Epicatechin

(-)-Epicatechin (further referred to as epicatechin) was tested in the concentrations shown in Table 16, corresponding to the concentrations found in the berry extracts at 200 µg/ml.

Table 16: Incubation concentrations of epicatechin and approximate corresponding occurrence in 200 µg/ml of the extracts.

	Conc. µg/ml	Extract
1.	1.4	
2.	0.7	BB
3.	0.35	Bil
4.	0.175	BC
5.	0.088	
6.	0.044	EB

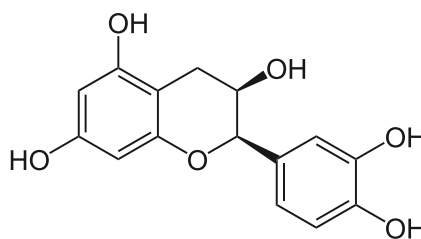


Figure 29: Sturcture of epicatechin

Graphs in Figure 30 depicting the cell viability, T/C (%) after 72h incubation with epicatechin in a concentration range between 0.044 and 1.4 $\mu\text{g/ml}$ determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). As depicted in Figure 30, there was no major cytotoxicity observed in the HCT116 and HCEC-1CT cell line with either the WST-1 or the SRB assay. Although there were some inconsistencies with a significantly lowered cell viability in different individual concentrations in the assays and cell lines (conc. 0.044 and 0.175 $\mu\text{g/ml}$ in WST1 HCT116 (A), 0.088 $\mu\text{g/ml}$ in SRB HCEC-1CT (D) and 0.175 $\mu\text{g/ml}$ in SRB HCT116 (B)), the cell viability was still above 85 % and the downregulation was not concentration dependent.

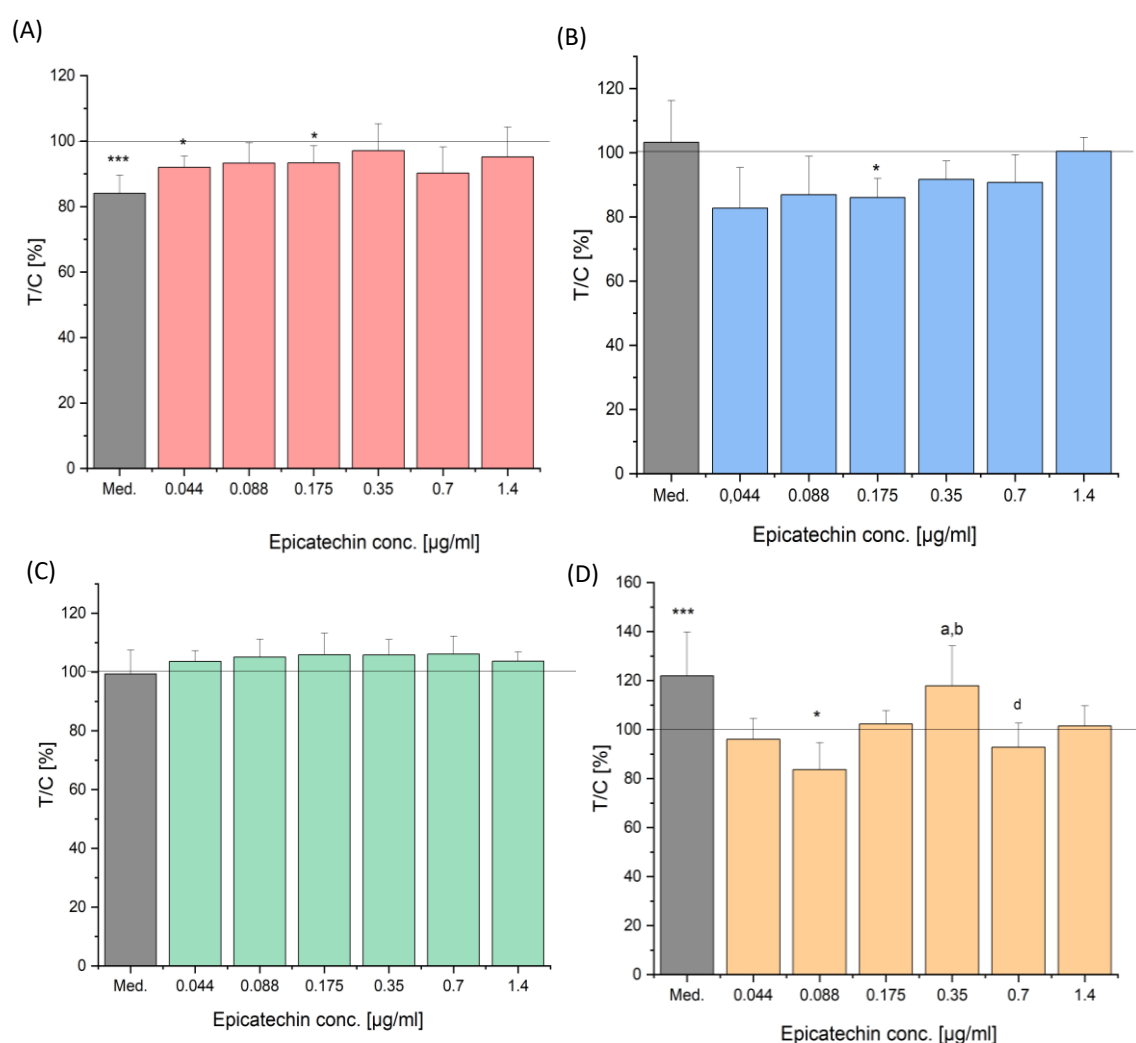


Figure 30: Cytotoxic effects of epicatechin in a concentration range between 0.044 and 1.4 $\mu\text{g/ml}$ on HCT116 and HCEC-1CT cells after 72 h of incubation determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5% DMSO, $y=100$). Statistical significances were determined by one sample Student's *t* test for significances between the concentrations of epicatechin and the solvent control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and bonferroni ANOVA for test concentrations ($p<0.05$).

6.3.3. (-)-Epigallocatechin

(-)-Epigallocatechin (EGC) was tested in the concentrations shown in Table 17, corresponding to the concentrations found in the berry extracts at 200 µg/ml.

Table 17: Incubation concentrations of EGC and approximate corresponding occurrence in 200 µg/ml of the extracts.

Conc. µg/ml		Extract
1.	0.6	BC
2.	0.3	
3.	0.15	Bil
4.	0.075	
5.	0.038	
6.	0.019	
		BB↓

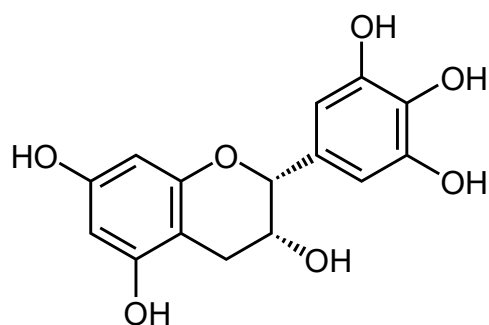


Figure 31: Structure of epigallocatechin

Graphs in Figure 32 depicting the cell viability, T/C (%) after 72h incubation with epigallocatechin in a concentration range between 0.019 and 0.6 µg/ml determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). There was no cytotoxicity observed in the HCT116 and HCEC-1CT cell line with either the WST1 or the SRB assay after incubation with epigallocatechin (0.019- 0.6 µg/ml).

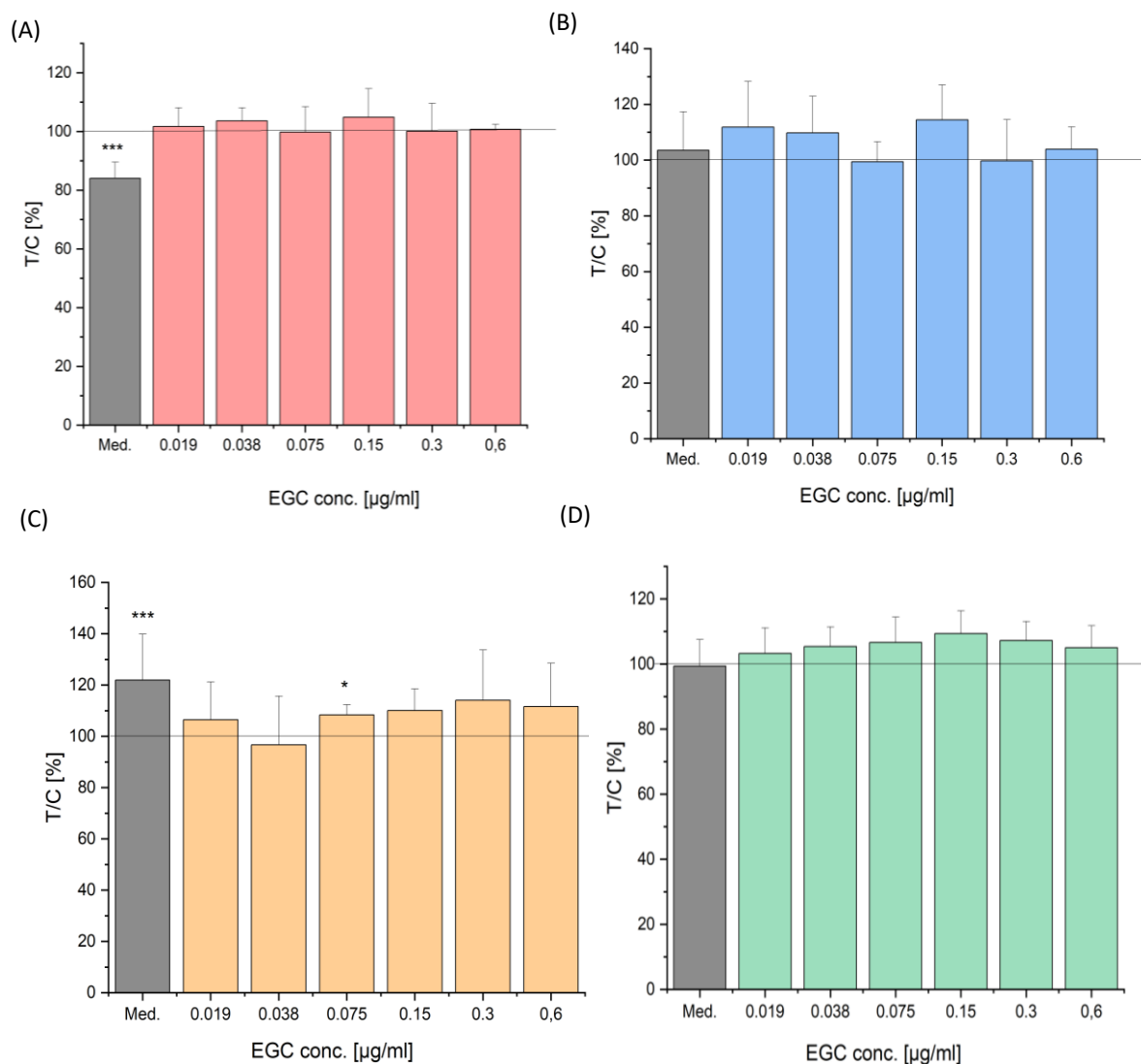


Figure 32: Cytotoxic effects of epigallocatechin in a concentration range between 0.019 and 0.6 µg/ml on HCT116 and HCEC-1CT cells after 72 h of incubation determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). Results are expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5% DMSO, $y=100$). Statistical significances were determined by one sample Student's *t* test for significances between the concentrations of epigallocatechin and the solvent control (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) and bonferroni ANOVA for test concentrations ($p<0.05$).

6.3.4. Catechol

Catechol was tested in the concentrations shown in Table 18, corresponding to the concentrations found in the berry extracts at 200 µg/ml.

Table 18: Incubation concentrations of catechol and approximate corresponding occurrence in 200 µg/ml of the extracts.

	Conc. µg/ml	Extract
1.	1	Bil, EB
2.	0.5	BB
3.	0.25	
4.	0.125	BC
5.	0.063	
6.	0.031	

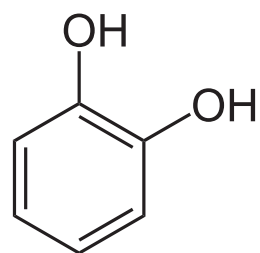


Figure 33: Structure of catechol

The graphs in Figure 34 below depicting the cell viability, T/C (%) after 72 h incubation with catechol in a concentration range between 0.031 and 1 µg/ml determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). There was no major cytotoxicity observed in the HCT116 and HCEC-1CT cell line with either the WST1 or the SRB assay.

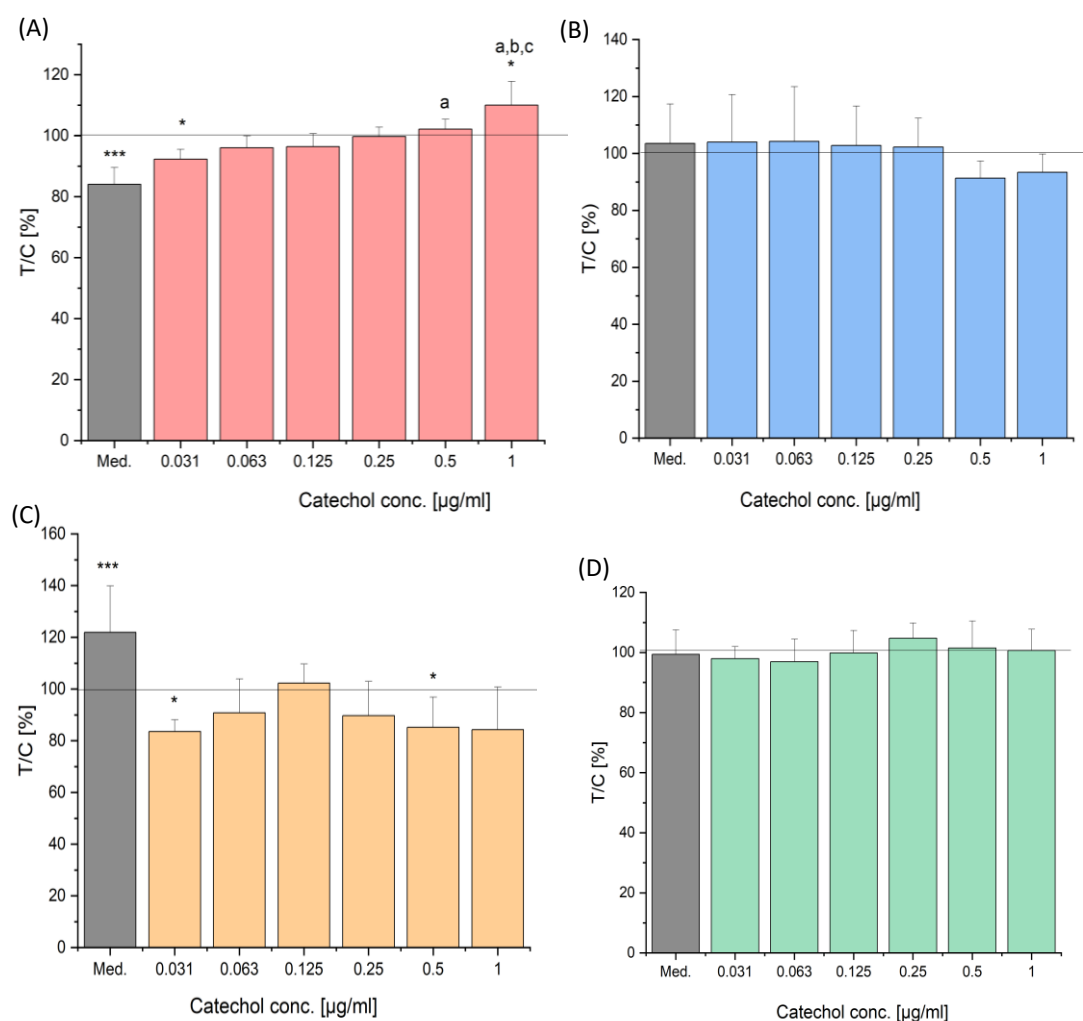


Figure 34: Cytotoxic effects of catechol in a concentration range between 0.031 and 1 µg/ml on HCT116 and HCEC-1CT cells after 72 h of incubation determined by WST-1 (A; HCT116, C; HCEC-1CT) and SRB (B; HCT116, D; HCEC-1CT). Results are

expressed as means $\pm$ SD of 4-5 independent experiments, normalized to the solvent control (Ctrl., 0.5% DMSO, y=100). Statistical significances were determined by one-sample Student's t test for significances between the concentrations of catechol and the solvent control (p <0.05, ** p <0.01 and *** p <0.001) and bonferroni ANOVA for test concentrations (p <0.05).*

Although there were some inconsistencies with a significantly lowered cell viability in different individual concentrations in the assays and cell lines (conc. 0.031 in WST1 HCT116 (A), 0.031 and 0.5 μ g/ml in WST-1 HCEC-1CT (C)), but the cell viability was still above 85 % and the downregulation was not concentration dependent. Additionally, the SRB assay did not reflect these slight downregulations in either cell line.

6.3.5. Conclusion

In summary, (-)-epicatechin, (-)-epigallocatechin, (+)-rutin and catechol showed no major cytotoxicity in the tested concentrations and cell lines HCT116 and HCEC-1CT. They are unlikely to be responsible for any potential cytotoxicity expressed by the berry extracts in the concentrations those were tested in, however they might contribute to mixture effects since the extracts contain a range of different polyphenols.

7. Materials and Methods

7.1. Berry extracts

To investigate the effects of anthocyanins, four different berry extracts were used for practical research. They include extracts of bilberry, black currant, blackberry and elderberry. The extracts were produced and dried between the 21.10. and the 15.11.2019 in the University of Geisenheim (DE) and shipped to the Department of Food Chemistry and Toxicology, University of Vienna on the 15.11.2019. Table 19 shows source and production of the berry extracts.

Table 19: Source and production of the anthocyanin-rich berry extracts used (information provided by the Hochschule Geisenheim).

Fruit	Source	Production	Storage	Column	Resin	Drying
Bilberry	Bayernwald, organic juice	readily packed	bag in box	big	SP70 (Mitsubishi)	Freeze dried
Blackberry	Trautner, frozen	thawing, mash enzyme, rack&frame press, juice enzyme, filtration	fresh	small	ADS5000 (Chemra)	Freeze dried
Black currant	Trautner, frozen	thawing, mash enzyme, Vaculiq extraction, juice enzyme, filtration	pasteurised, KEG	small	ADS5000	Freeze dried
Elderberry	Trautner, frozen	like blackberry	fresh	small	ADS5000	Freeze dried

The berry extracts have different anthocyanin compositions, as shown in Table 20.

Table 20: anthocyanin-content in extracts of bilberry, black currant, blackberry and elderberry (information provided by the Hochschule Geisenheim).

Anthocyanins	Bilberry		Black currant		Blackberry		Elderberry	
	[g/kg]	%	[g/kg]	%	[g/kg]	%	[g/kg]	%
cyanidin-3O-glucoside	13.8	13.5	2.4	5.3	67.3	88.5	-	-
cyanidin-3O-arabinoside	7.5	7.3	-	-	-	-	-	-
cyanidin-3O-galactoside	9.1	8.9	-	-	-	-	-	-
cyanidin-3O-rutinoside	-	-	18.2	39.4	2.3	3.0	-	-
cyanidin-3O-sambubioside	-	-	-	-	-	-	56.6	72.9

cyanidin-3O-sambubioside-5-glccoside	-	-	-	-	-	-	21.0	27.1
cyanidin-3O-xyloside	-	-	-	-	1.5	1.9	-	-
cyanidin-3O-(6"-O-malonyl)-glccoside	-	-	-	-	1.5	2.0	-	-
cyanidin-3O-(6"-O-dioxalyl)-glccoside	-	-	-	-	3.5	4.6	-	-
delphinidin-3O-glccoside	17.1	16.7	5.1	11.0	-	-	-	-
delphinidin-3O-arabinoside	10.5	10.2	-	-	-	-	-	-
delphinidin-3O-galactoside	12.6	12.3	-	-	-	-	-	-
delphinidin-3O-rutinoside	-	-	19.1	41.3	-	-	-	-
malvidin-3O-glccoside	7.8	7.6	-	-	-	-	-	-
malvidin-3O-arabinoside	1.2	1.1	-	-	-	-	-	-
malvidin-3O-galactoside	2.6	2.5	-	-	-	-	-	-
peonidin-3O-glucoside	4.2	4.1	-	-	-	-	-	-
peonidin-3O-galactoside	0.6	0.6	-	-	-	-	-	-
peonidin-3O-rutinoside	-	-	0.5	1.1	-	-	-	-
petunidin-3O-glccoside	9.5	9.3	-	-	-	-	-	-
petunidin-3O-arabinoside	2.0	2.0	-	-	-	-	-	-
petunidin-3O-galactoside	3.8	3.7	-	-	-	-	-	-
petunidin-3O-rutinoside	-	-	0.9	1.9	-	-	-	-
Total	102.5	100	46.3	100	76.0	100	77.6	100

Besides the anthocyanins, different polyphenols could also be found in the extracts, by utilising the LC-MS/MS method from Oesterle et al. 2022.¹²⁸ Table 21 shows the average concentrations (µg/l) of four Polyphenols measured in the extracts.

Table 21: Content of (-)-epicatechin, (-)-epigallocatechin, (+)-rutin and catechol in berry extracts measured by LC-MS/MS

	(-)-epicatechin	(-)-epigallocatechin	(+)-rutin	catechol	
Blackberry	4361.54	13.76	801.24	2856.61	µg/l
Black currant	546.19	2701.17	4131.45	790.44	µg/l
Bilberry	2137.58	573.66	47.62	4723.22	µg/l
Elderberry	276.58	N.R. ^a	N.R. ^a	4514.25	µg/l

^a N.R: not reported.

Cytotoxicity testing was performed for the extracts, as well as the anthocyanins alone, using the WST-1 und SRB assay. The polyphenols detected in the extracts were also investigated regarding their cytotoxicity. In the course of this thesis, the cytotoxicity assays WST-1 and SRB were performed with the polyphenols (-)-epicatechin, (-)epigallocatechin, catechol and (+)-rutin, in the concentrations in which they appear in the berry extracts (chapter 6.3).

7.2. Cell Culture

In the course of this thesis multiple cell lines were used for the practical experiments, namely HCT116, HCEC-1CT, CT26 and THP1-Lucia™ NF-κB cells.

7.2.1. Adherent cell lines

HCT116, HCEC-1CT and CT26 are adherent cell lines. HCT116 cells are a strain of malignant cells isolated from a male with colonic carcinoma. HCEC-1CT are immortalized epithelial cells derived from a human colon biopsy.¹²⁹ Murine CT26 cells were developed in 1975 by exposing BALB/c mice to N-nitroso-N-methylurethane (NMU), resulting in a rapid-growing grade IV carcinoma that is easily implanted and readily metastasizes.¹³⁰

7.2.1.1. Cultivation

The HCT116 cells were cultivated in 75 cm² flasks at 37 °C, 5 % CO₂ and 95 % air moisture. Dulbecco's Modified Eagle Medium (DMEM) GlutaMax was used as cell culture medium. To complete the medium, it was supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (P/S). The fetal bovine serum includes important growth factors, cytokines, hormones, binding- and transport proteins and factors for attachment and spreading of the cells. The combination of the antibiotics penicillin (5000 units/ml) and streptomycin (5000 µg/mL) was used routinely in order to reduce the risk of contamination via bacteria.¹³¹

The CT26 cells were cultivated in 75 cm² flasks at 37°C, 5 % CO₂ and 95 % air moisture. Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F2 1:1 was used as cell culture medium. To complete the medium, it was supplemented with 10 % FBS and 1 % P/S.

The HCEC-1CT cells were also cultivated in 75 cm² flasks at 37°C, 5 % CO₂ and 95 % air moisture. The complete medium used was composed of:

- DMEM (high glucose) 500 ml

- + 10 ml Medium 199 (10x)
- + 10 ml Cosmic calf serum™
- + 10 ml HEPES buffer 1M
- + 5.2 ml Insulin-transferrin-selenium (10x)
- + 0.6 ml Gentamicin (50 mg/ml)
- + 0.1 ml Recombinant human EGF (100 µg/ml)
- + 0.1 ml Hydrocortisone (water soluble) (5 mg/ml)

7.2.1.2. *Sub-cultivation*

Cells in culture have to be sub-cultivated at a regular basis. Before reaching complete confluence, the adherent cells must be loosened from the growth surface, suspended, diluted and cultivated in a new flask with fresh complete growth medium. Cells were passaged twice a week at approximately 80 % confluence using PBS (phosphate buffered saline solution) to wash away cell debris and medium rests which could hamper the effect of the trypsin used to detach the cells. Trypsin is an alkaline protease from the bovine pancreas, which detaches the cell from the bottom of the culture flask by cleaving the peptide bonds of lysine or arginine breaks the cell-matrix connections. Fresh complete growth media is then added to stop the trypsin digestion. In the HCEC-1CT cell line accutase was used instead of trypsin to detach the cells from the bottom of the flask.¹³¹

Protocol for cell passaging of adherent cells:

- Remove used growth medium with sterile Pasteur pipette without touching the cell layer
- Wash with 37 °C PBS (ca. 5 ml), remove with Pasteur pipette
- To loosen the cells add:
 - HCT116 & CT26: approx. 1 ml trypsin solution (37 °C)
 - HCEC-1CT: 1.5 ml accutase solution (37 °C)
- Solutions should be covering the cell layer, incubate for approx. 4-5 min at 37°C, 5 % CO₂ and 95 % air moisture
- Add 5 ml 37 °C medium with a sterile pipette and wash the bottom of the flask several times with cell suspension to loosen rests and disperse cell clusters
- Count the cells with Neubauer hemocytometer (see chapter 8.2.7)

- Distribute a number of cells, depending on the next passage (see Table 22 below) in 10 ml of fresh medium in a 75 cm² flask, use new flask every fourth passage

Table 22: cell numbers for passaging in 75 cm² flask

Cell line:	Passaged in 3d (cell number)	Passaged in 4d (cell number)
HCT116	500 000	400 000
HCEC-1CT	400 000	300 000/350 000
CT26	700 000	500 000

7.2.2. Suspension cell line

THP1-Lucia™ cells are distributed by InvivoGen and are specifically designed for monitoring the NF-κB signal transduction pathway in a cell line which is physiologically relevant. THP1-Lucia™ were derived from the human THP-1 monocyte cell line by stable integration of an NF-κB-inducible Luc reporter construct. Consequently, THP1-Lucia™ cells allow the monitoring of NF-κB activation by measuring the activity of secreted luciferase (Lucia). The levels of NF-κB-induced Lucia in the cell culture supernatant are readily assessed with QUANTI-Luc™, a luciferase detection reagent.¹⁰⁴

7.2.2.1. Cultivation

THP1-Lucia cells were cultivated in T-175 cm² flasks for suspension cells (green) at 37°C, 5% CO₂ and 95 % air moisture. RPMI 1640 medium (450 ml) was used as cell culture medium. To complete the medium, it was supplemented with 10 % FBS (50 ml), 1 % P/S (5 ml), 25 mM HEPES buffer (12 ml) and 100 µg/ml Normocin (1 ml).

7.2.2.2. Sub-cultivation

Cells in culture have to be sub-cultivated at a regular basis, before reaching complete confluence. Cells are suspended, diluted and cultivated in a new flask with fresh complete growth medium. The cells were passaged twice a week before they reached a cell number of 2 million cells per ml.

Protocol for cell passaging of THP-1 Lucia™:

- Pipette the cell suspension up and down several times (pipet us on the lowest speed possible)
- Pipette out of flask in tube

- For counting dilute cells 1:5 with trypan blue
- Calculate volume needed for 9 Mio cells, fill up to 30 ml with medium
- Add 30 µl of Zeocin every second passage

7.2.3. Thawing Cells

The frozen cell stocks were kept at -196 °C in liquid nitrogen. The vial was rapidly thawed in a 37°C water bath while keeping the cap above the surface to take cells into culture. The vial was decontaminated with ethanol and the cells suspended in 10 ml 37°C complete growth medium with 1 % P/S (penicillin/streptomycin) and 20 % FBS (foetal bovine serum) for HCT116, CT26 and THP1-Lucia cells, for the HCEC-1CT cells their complete medium was used without any additions. HCT116 and HCEC-1CT cell lines were immediately cultured in 75cm² flasks. Cells were initially cultured in medium with 20 % FBS for several days, if necessary. Medium was changed regularly until cells had attached to the bottom and formed islands with approx. 80 % confluency, then the cells were first passaged.

7.2.4. Determination of Cell Count with Neubauer Hemocytometer

To obtain an appropriate growth rate for subcultivation and to ensure reproducibility of experiments, cell counts must be determined correctly. To ensure this, a representative aliquot of cell suspension has to be counted with a Neubauer counting chamber, which is an optical device consisting of a thick glass slide with two etched in counting grids. Those grids usually have 9 big squares, that are again divided into smaller squares (Figure 35).¹³¹

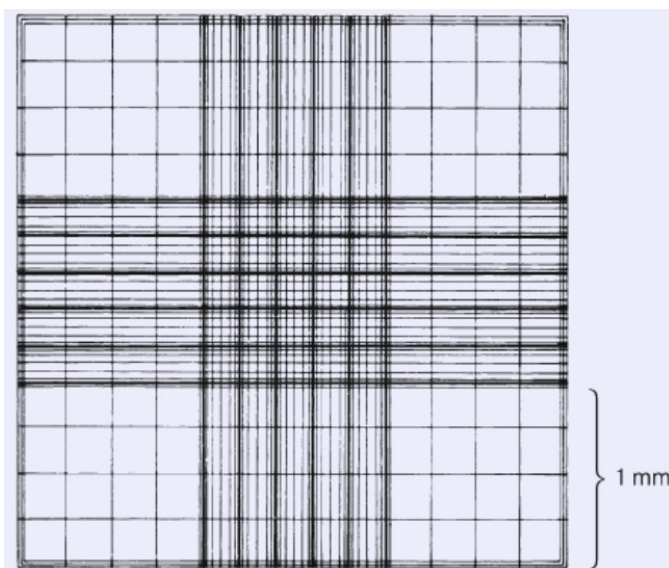


Figure 35: Counting grid of a Neubauer counting chamber (taken from Gstraunthaler & Lindl, 2021).

Each of the big squares has a volume of 0.1 mm^3 . An aliquot of cell suspension diluted in trypan blue is then pipetted between the glass slide and the cover slip and the living cells in the 4 big corner squares are counted. Calculating the mean of the cell count gives a unit of cells per 0.1 mm^3 , which is then transformed to cells per ml. To calculate the required volume of cell suspension for subcultivation or seeding the needed number of cells is divided by the counted cell concentration per ml.¹³¹

Protocol counting cells:

- Clean chamber and cover slide with 70% ethanol
- Lightly moisturize the cover slide and place in the middle of the chamber
- Add 20 μl cell suspension to 180 μl trypan blue for staining
- Vortex for approximately 5 seconds
- Add 10 μl stained cell suspension to the edge of the hemocytometer next to the cover slide, the capillary forces draw the suspension into the chamber
- Count the cells in 4x4 squares under the microscope (4 squares per chamber)
- The mean value of all squares is multiplied with the trypan blue dilution factor (10) and 10000, which results in cell concentration per ml

7.3. Methods

To investigate the cytotoxicity of (-)-Epicatechin, (-)-Epigallocatechin, (+)-Rutin and Catechol, in relevant concentrations in which they occur in the anthocyanin extracts used in the project, WST-1 and SRB assays were performed in the cell lines HCT116 and HCEC-1CT. For the investigation of the cytotoxicity of citric acid in combination with the berry extracts, WST-1 and SRB were performed in the cell lines: HCT116, CT26 and HCEC-1CT. To determine the activation of the NF- κ B pathway, NF- κ B assay was performed with THP1-Lucia™ cells which are specific design for that purpose. After the NF- κ B assay the cytotoxicity was determined in the same plate via CTB assay. To detect the markers for immunomodulation, HCT116 and HCEC-1CT cells were incubated with the anthocyanin extracts and IL-1 β . After the incubation, the collected supernatant and extracted RNA were frozen in -80°C . For the measurement of the immunomodulatory markers on a transcript level QuantiGene Plex Gene Expression Assay™ with Luminex® xMAP technology and qRT-PCR was used.

7.3.1. Cytotoxicity Testing: WST-1 and SRB

Cytotoxicity testing was performed using the coupled WST-1 and SRB assay. In short, the WST-1 assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases (see Figure 36)¹³² and in the SRB assay Sulforhodamine B binds to the protein constituents of the cells in a stoichiometric manner (see Figure 37).¹³³

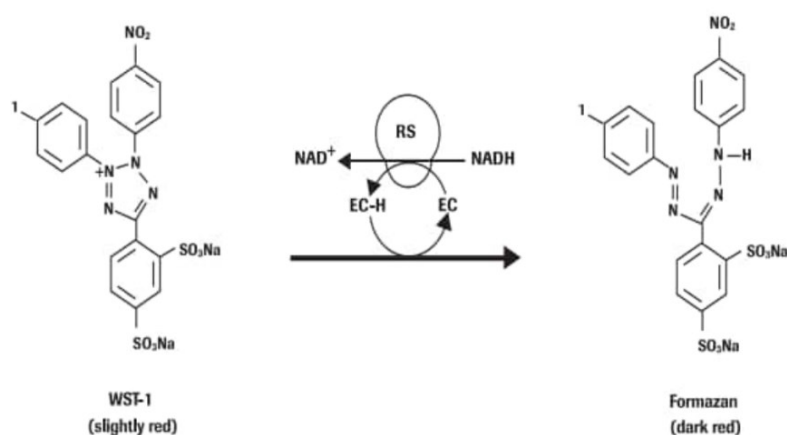


Figure 36: Cleavage of tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) to formazan. (EC = electron coupling reagent, RS = mitochondrial succinate-tetrazolium-reductase system).¹³⁴

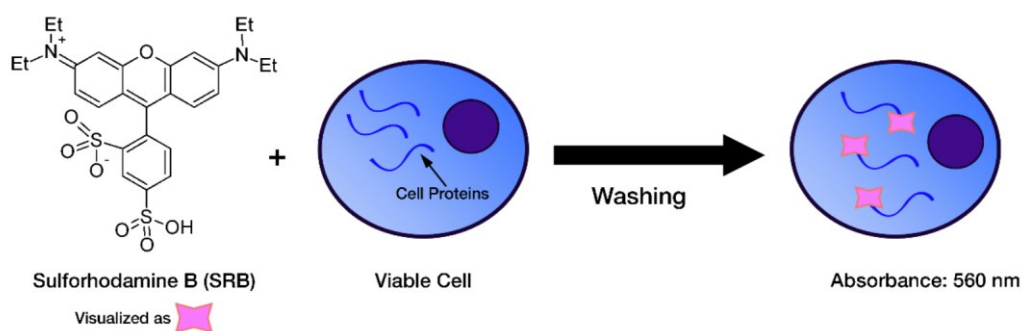


Figure 37: Sulforhodamine B (SRB) as it binds to the cell proteins¹³⁵

The assays were performed in 96 well plates with initial seeding of 4000 HCT116 cells/well, 1500 CT26 cells/well and 2000 HCEC cells/well. After 24 hours the cells were incubated with the test substances in 0.5 % DMSO for 72 hours. Medium with 0.5 % DMSO functioned as a solvent control.

Incubation:

- Weigh substances (polyphenols and berry extracts) and solve it in DMSO for stock solutions by vortexing (light sensitive, use dark tubes and turn off laminar light)
- Weigh catalase and solve in cold PBS, vortex well, dilute with medium for 100 U catalase/ml medium
- Make dilution series (0.5 % DMSO)
- Make solvent control: dilute DMSO 1:200 with medium for 0.5 % DMSO (100 U/ml catalase)
- Add 200 µl incubation medium with substance, solvent control or medium in wells (3 replicates per concentration)
- Incubate for 72h

The assays were performed according to the following protocol:

- Remove incubation media
- Incubate with 1:10 mix of WST-1 in media for 45 min; add 3 blank wells (without cells)
- Insert plate in plate reader (Synergy H1, Biotec)
- Detect absorption at 450 nm and 650 nm
- For calculation: blank deduction; 450 - 650 nm values and calculate T/C values
- Remove WST-1 solution from the wells
- Put 100 µl of PBS in each well and add 10 µl 50% TCA; incubate for 1 h at 4°C; wash with water and let dry (overnight)
- Stain the plate with 50 µl SRB reagent for 1 h at RT; wash with water and then with 1 % acetic acid until no more colour gets removed and let dry (overnight)
- Add 100 µl of Tris Base (pH 10) to each well; shake the plate in the plate reader (orbital shake for 3 min) and measure absorbance at 570 nm (if absorbance values are higher than 2, dilute each well with equal amount of Tris Base; shake and measure again)

Solutions:

- TCA (trichloro acetic acid): 50 % (w/v) in Aqua dest. → 12.5 g + 12.5 ml H₂O
- Acetic acid: 1 % (v/v) in Aqua dest. → 25 ml filled to 2.5 L
- SRB: 0.4 % (w/v) in 1 % acetic acid
- Tris-Base: 10 mM Tris in aqua dest. (pH= 10 adjusted with HCl)

7.3.2. NF- κ B and CTB assay

The NF- κ B and CTB assay were performed using the specifically for this purpose designed cell line THP1-Lucia. THP1-Lucia™ cells were specifically designed for monitoring the NF- κ B signal transduction pathway. They are derived from the human THP-1 monocyte cell line by stable integration of an NF- κ B-inducible Luc reporter construct. As a result, THP1-Lucia™ cells allow the monitoring of NF- κ B activation by measuring the activity of secreted luciferase (Lucia). The levels of NF- κ B-induced Lucia in the cell culture supernatant can be assessed with QUANTI-Luc™, a luciferase detection reagent.¹⁰⁴ The CellTiter-Blue® Cell Viability Assay (CTB) presents a homogeneous, fluorescent method to assess cell viability. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin) (see Figure 38). Nonviable cells do not generate a fluorescent signal because they lose metabolic capacity. The homogeneous assay procedure starts with adding the single reagent directly to cells cultured in serum-supplemented medium. After an incubation step, data is recorded using a plate-reading fluorometer.¹³⁶

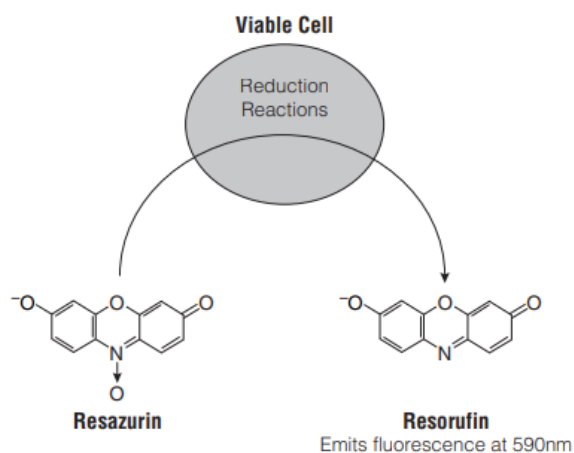


Figure 38: Conversion of resazurin to resorufin by metabolically active cells results in the generation of a fluorescent product.

Cell handling:

- 100 000 cells/well (96 well plates, for suspension cells)
- Extract cells from flask and count them
- Transfer calculated volume of cell suspension into falcon tube
- Centrifuge: 2 min, 140 rcf → pellet at the bottom

- Remove old medium above pellet and replace it with fresh medium and resuspend (calculated for 100 µl per well)

Prepare the QUANTI-Luc™ assay solution as follows:

- Pour the pouch contents into a brown 50 ml screw cap tube
- Add 25 ml of sterile water
- Swirl product gently until powder is completely dissolved
- Use QUANTI-Luc™ assay solution immediately or store until used: 1 week at 4 °C and 1 month at -20 °C

Incubation:

- Weigh substances (SN38) and solve it in DMSO for stock solution and vortex
- Make dilution series in DMSO then dilute 1:1000 in medium
- Make solvent control: dilute DMSO 1:1000 with medium (double concentration because of dilution with cell suspension)
- Add 100 µl incubation medium with substance, solvent control or medium in wells (3 replicates per concentration)
- Add 100 µl of cells suspension in wells
- After 2 h: add 10 nM LPS
- Incubate for 18 h

After 20h incubation:

- Put plate on plate shaker: 1 min; approx. 500 rpm
- Centrifuge plate for 2 min, 140 rcf
- Transfer 10 µl of each well into a white 96-well plate (do not touch cell pellet)
- For measurement of blank pipet 10 µl of medium in 3 wells

Measurement: Plate reader (Synergy H1, Biotec)

- Open program Gen 5
- Instrument Control → Prime/ Dispense
 - Insert wash plate in plate reader
 - Prime 2x with 1 ml EtOH
 - Prime 2x with 1 ml autoclaved water
 - Add tube with Quanti-Luc to syringe, prime one time (very light sensitive)
- Test Run

- Dispense → Full Plate → Clear All → Select F3 → Volume 30 µl → start dispense
- One drop should be visible on wash plate
- Measurement
 - Set the luminometer as follows: 50 µl of injection, end-point measurement with a 4 second start time and 0.1 second reading time
 - Measurement of 6 values each well, use value 5 for calculation
- Cleaning
 - Purge: the remaining Quanti-Luc in the system is transferred to tube
 - Prime: two times with water and two times with EtOH to wash the system
 - Purge: add waste tube to syringe 1, remove EtOH from system

Calculation: mean values, calculate T/C values

Protocol CellTiter-Blue® Cell Viability (CTB) Assay:

- Perform with same plate as NF-κB-assay
- Blank: three wells with only medium, add another 100 µl to get a total volume of 200 µl
- Positive control: Triton X
 - 1 % (5 µl Triton X + 495 µl medium)
 - 0.1 % (50 µl 1 % Triton X + 450 µl medium)
 - Add 20 µl of 0,1 % Triton X solution to three wells as positive control
- Add 20 µl Alamar Blue Cell Viability Reagent to every well in the dark (very light sensitive)
- Put plate on plate shaker: 1 min, 500 rpm
- Incubation: 2 hours in incubator
- Plate shaker: 1 min
- Centrifuge: 2 min, 140 rcf
- Transfer 100 µl of each well onto a black 96-well plate (do not touch cell pellet and avoid bubbles)
- Insert in plate reader (Synergy H1, Biotec)
- Measurement: fluorescent excitation at 560 nm and emission at 590 nm
- Calculation: calculate T/C values

7.3.3. LC-MS/MS

8.3.4.1. Sample Preparation

The samples used from the *in vivo* trials were urine, feces and plasma. Since *in vivo* samples have a complex matrix, samples need to be prepared in various ways. Sample preparation was executed according to the master thesis of Anna-Lena Mayr (2022)¹²⁵.

Urine:

The urine samples were diluted 1:50 with the dilution solvent, 70:30 H₂O: MeOH mixture with 1 % FA, and transferred into an HPLC vial.

Feces:

Feces were lyophilized to ensure that differences in water content in the sample did not affect the weight. Lyophilized feces were weighed and transferred into 2 ml micro tubes and 1.5 ml of extraction solvent (60:40 MeOH: H₂O mixture with 1 % FA) was added. The samples were vortexed and placed in an ultrasound bath at room temperature for 15 minutes. Afterwards they were centrifuged for 10 minutes at 1000 rcf. The supernatant was then transferred to a 15 ml tube and the extraction process was repeated two more times. The collected supernatants were placed in the vacuum concentrator till they were dry (4°C, 1.5 mbar, between 24 and 72 h). The residue was then reconstituted with 100 µl of a 30 % MeOH solution with 1 % FA on the day of the LC-MS measurement and transferred into an HPLC vial.

Plasma:

For the sample preparation of plasma, LC-MS grade acetonitrile was cooled to -20°C and equal parts of plasma and ACN were placed into a 1.5 ml Eppendorf tube. The samples were vortexed and stored at -80°C overnight. Then they were centrifuged at 4°C and 12000 rcf for 15 minutes and the clear supernatant was transferred into an HPLC vial, and the pellet was discarded.

8.3.4.2. Measurement

For the measurement the device 1290 UHPLC (Agilent Technologies) coupled to a QTrap 6500+ triplie quadrupole mass spectrometer from AB Sciex was used. Eluent A was prepared by adding 1 ml of FA to 999 ml of water. Eluent B was 100 % LC-MS grade methanol. The needle was washed with wash solution made from equal parts H₂O, ACN, MeOH and 2-propanol and 1 % FA. The injection volume was 1 or 3 µl. The method used was based on the master thesis by Anna-Lena Mayr (2022)¹²⁵.

To operate the LC MS/MS system and for identification of the anthocyanins the Analyst software (version 1.7.0; Applied Biosystems, Foster City, California, USA) was used. For quantitative measurements of the anthocyanins, the software Skyline (version 21.1; MacCosslab, University of Washington, Seattle, Washington, USA) was applied.

For the standards, the anthocyanins were weighed and dissolved in DMSO, to create 1 mg/ ml stock solutions. The anthocyanins used for the standard solutions were based on the anthocyanins contained in the berry extracts used and are shown in Table 23, together with their retention time. Then dilutions were prepared in the concentrations shown in Table 24.

Table 23: Anthocyanin standards and their retention times (RT)

	RT:
Cy-3-glc	8.94
Cy-3-sam	9.36
Cy-3-sam-5-glc	6.24
Cy-3,5-diglc	6.20
Cy-3-rut	9.27
Del-3-glc	7.46

Table 24: Concentrations of standard solutions 1 to 10 used for LC-MS/MS measurements containing: cy-3-glc, cy-3-sam, cy-3-sam-5-glc, cy-3,5-diglc, cy-3-rut and del-3-glc

	Conc. [µg/l]
Standard 10	1000
Standard 9	500
Standard 8	300
Standard 7	100
Standard 6	30
Standard 5	10
Standard 4	3
Standard 3	1
Standard 2	0.3
Standard 1	0.1

After measurement the anthocyanins present in the samples were identified by assigned peaks and retention times. For quantification, calibration curves were created for each analyte by plotting the measured peak area against the analyte concentration in µg/l. The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the signal to noise ratio, which should be over 3 and 10, respectively. The standard 10, with 1000 µg/l, was excluded from the calculations due to signal saturation. In the mice that received bilberry extract, the feces and urine samples had two peaks visible for Cy-3-glc (explementary chromatogram in Figure 25). The second peak was assumed to be the cy-3-galactoside based on literature data, and was experimentally quantified using the cy-3-glc standard, given that a standard for cy-3-gal was not available.

7.3.4. Immunomodulation Marker Analysis

7.3.4.1. *Incubation and RNA extraction*

HCEC-1CT:

For the extraction of the RNA after incubation with the anthocyanin extracts the Maxwell® RNA extraction kit was used. The HCEC cells were seeded in 12 well plates with 60000 cells/well and reached approximately 80% density after 48h.

Incubation:

- Weigh extracts and solve it in DMSO for stock solutions by vortexing (use dark tubes and turn off laminar light, anthocyanins can be light sensitive)
- Make dilution series (in DMSO) using the stock solution for the concentrations needed
- Further dilute the extract dilutions 1:200 in medium (DMSO content = 0.5%)
- Solvent controls: dilute DMSO 1:200 with medium (one without and one with IL-1β stimulation)
- Add 1.47 ml incubation medium with substance, solvent controls or medium in wells (3 replicates per concentration)
- Incubate for 2h
- Prepare IL-1β stocks:
 - add 100 µl endotoxin-free water to 10 µg of recombinant human IL-1β (provided)

- mix by pipetting, do not vortex
- prepare 100 µg/ml 2.5 µl aliquots
- freeze at -80 °C
- Add 30 µl of IL-1β (1.25 µg/ml) so that the final concentration is 25 ng/ml
- Incubate for 3h

Protocol Maxwell® simplyRNA Cells Kit:¹³⁷

- Prepare homogenization solution: 20 µl 1-Thioglycerol per ml homogenization solution
- Preparation of protein samples:
 - Take out medium with pipette and put it in tubes
 - Centrifuge: 4°C, 10.000 rpm, 15 min
 - Pipette 1 ml of supernatants into new tubes
 - Store at -80 °C until measurement (no measurements of protein samples were performed in this thesis)
- Preparation RNA samples:
 - Wash cells with PBS (0.5 ml)
 - Add 200 µl of chilled homogenization solution and pipet up and down for homogenization
 - Transfer into tubes
- RNA extraction:
 - Ensure RNase-free environment (fresh gloves, spray hands and surfaces with RNase AWAY™ Surface Decontaminant)
 - DNase I: dilute in 275 µl of nuclease free water, add 5 µl blue dye, swirl, freeze aliquots
 - Cartridge Preparation:
 - Place cartridges centred on cartridge rack (label facing away from elution tubes), snap into position and peel back seal
 - Place LEV plunger in well 8 (closest to elution tubes)
 - Put 0.5 ml elution tubes in the front, add 30 µl nuclease free water
 - Preparation of samples:
 - Add 200 µl lysis buffer to cells

- Vortex 15 sec
- Transfer cells in well 1 (last in the back)
- Add 5 µl DNase I to well 4 (yellow to green color change)
- Start program simplyRNA Maxwell (58 min)
- Measure RNA concentration with nanodrop and store samples at -80°C

HCT116:

For the extraction of the RNA after incubation with the anthocyanin extracts in the HCT116 cells the RNeasy kit from Qiagen was used, after a failed attempt to use the Maxwell kit like in the HCEC-1CT cells. Using the Maxwell® simplyRNA kit according to the protocol above, resulted in very high fluctuations in RNA concentrations with the HCT116 cells.

The incubation of the HCT116 cells was similar to the incubation of the HCEC-1CT. Except, the cells were seeded in 24 well plates with 70000 cells/well, reaching approximately 80 % density after 48 h. The amount of incubation medium per well changed to 0.98.

Protocol RNeasy Kit:

- Prepare lysis buffer (fresh or fridge 30d): RLT-lysis buffer (1% β-Mercaptoethanol, 10 µl β-ME per 1 ml)
- Preparation of protein samples:
 - Take out medium with pipette and put it in tubes
 - Centrifuge: 4°C, 10.000 rpm, 15 min
 - Pipette 1 ml of supernatants into new tubes
 - Store at -80°C
- Preparation of RNA samples:
 - Wash cells with PBS (0.5ml)
 - Add 350 µl of chilled RLT-lysis buffer and pipet up and down
 - Shear cells with one-way syringes and needles (20xG) 4x
- RNA extraction:
 - Add 340 µl 70% ethanol and homogenize
 - Transfer on spin column (close lid gently)→ 8.000 g (>10.000rpm), 15 s → discard flow through
 - Add 700 µl RW1 buffer→ 800 g, 15 s→ discard flow through
 - Add 500 µl RPE buffer→ 8 000 g, 15 s→ discard flow through

- Add 500 µl RPE buffer → 10 000 g, 2 min
- Put spin column in a new collection tube → 15.000g, 1min
- Put spin column in elution tube and add 30 µl of RNase free water directly to column membrane → 8 000 g, 1 min
- Measure RNA concentration with nanodrop and store samples at -80°C

7.3.4.2. *Detection of immunomodulation markers by Luminex® xMAP technology*

On a transcript level the target RNA was measured by a custom Invitrogen™ QuantiGene™ Plex Gene Expression Assay (Thermo Fisher Scientific) based on Luminex® xMAP technology. The Invitrogen™ QuantiGene™ Plex Assay Kit allows the multiplexed measurement of gene expression by combining branched DNA (bDNA) signal amplification with Luminex™xMAP™ multi-analyte profiling technology. The bDNA assay is a probe hybridization-based method of target-specific RNA capture and quantitation, amplifying the signal not the target. Color-coded fluorescent magnetic microspheres, called capture beads, capture specific target RNA molecules through hybridization of a custom oligonucleotide probe set, consisting of three types of probes: capture extenders, label extenders, and blocking probes. A contiguous sequence of each target RNA is hybridized by the probe set. The capture extenders segregate between different capture beads using the sequence of a capture probe conjugated to each bead, and the label extenders supply the support for the branched DNA signal amplification. Each amplification unit is constructed through sequential hybridization of bDNA oligonucleotides (preamplifier, amplifier and label probe). The label probe is biotinylated to bind Streptavidin-conjugated R-Phycoerythrin (SAPE). The resulting fluorescence signal is associated with individual capture beads by the Luminex® instrument, which combines optics, fluidics, and digital signal processing. The signal is proportional to the number of target RNA molecules present in the sample and reported as median fluorescence intensity.¹¹⁴

The shaking incubator was validated using a temperature validation kit to ensure hybridization temperatures are 54°C and 50°C.

According to the manufactures protocol:¹¹⁴

- Pre-warm lysis mixture at 37°C for 30 minutes followed by gentle swirling
- Remove RNA from freezer and thaw on ice. Vortex briefly before use. Dilute RNA using nuclease-free water so that 500 ng of RNA is present in 20 µl (per well)

- Probe Set & Blocking Reagent: Thaw and briefly vortex, centrifuge probe set briefly to collect contents at the bottom of the tube
- Capture Beads: Take out of storage right before use and protect from light
- Prepare working bead mixture per well as follows:
 - 38.74 μ l nuclease free water
 - 33.3 μ l lysis mixture
 - 2 μ l blocking reagent
 - 1 μ l capturing beads (vortex 30 seconds before adding)
 - 5 μ l probe set
- Vortex working bead mix for 10 seconds and transfer 80 μ L into each well of the hybridization plate
- Add 20 μ l of RNA sample (or 20 μ l of nuclease free water for blank)
- Seal the hybridization plate using a pressure seal: apply firm even pressure across the seal using a soft- rubber roller
- Transfere the hybridization plate in the shaking incubator and incubate at 54 °C $\pm$ 1 °C at 600 rpm for 18-22 hours
- Warm label probe solution, pre-amplifier solution and amplifier solution at 37 °C for 30 minutes to dissolve contents, mix well by inversion before use (leave at room temperature until use, solutions are viscous).
- Bring SAPE diluent to room temperature
- Prepare wash buffer: prepare 189 ml nuclease free water and add 0.6 ml wash buffer component 1 and 10 ml wash buffer component 2 (1 plate)
- Remove the hybridization plate from the shaking incubator and change temperature to 50 °C $\pm$ 1 °C
- Centrifuge hybridization plate at 240 $\times$ g for one minute at room temperature
- Completely transfer content from the hybridization plate to the magnetic separation plate and insert into the handheld magnetic plate washer
- Washing step (3x):
 - Wait 1 min to allow the magnetic beads to collect on the bottom of each well
 - Remove the solution in the wells by quickly inverting over a sink
 - Add 100 μ l of 1x wash buffer into each well
 - Wait 15 seconds to allow the magnetic beads to acollect on the bottom

- Remove the wash buffer in the wells by quickly inverting over a sink
- Pre-Amplifier Hybridization:
 - Add 100 µl of preamplifier solution to each well
 - Seal the magnetic separation plate with a day 2 plate seal
 - Shake at 800 rpm for 1 minute at room temperature
 - Place the magnetic separation plate into the shaking incubator
 - Incubate for 1 hour at 50 °C ± 1 °C with shaking at 600 rpm
- Washing step 3x
- Amplifier Hybridization:
 - Add 100 µl of amplifier solution to each well,
 - Seal the magnetic separation plate with a day 2 plate seal
 - Shake at 800 rpm for 1 minute at room temperature
 - Place the magnetic separation plate into the shaking incubator
 - Incubate for 1 hour at 50 °C ± 1 °C with shaking at 600 rpm
- Washing step 3x
- Label Probe Hybridization
 - Add 100 µl of label probe solution to each well,
 - Seal the magnetic separation plate with a day 2 plate seal
 - Shake at 800 rpm for 1 minute at room temperature
 - Place the magnetic separation plate into the shaking incubator
 - Incubate for 1 hour at 50 °C ± 1 °C with shaking at 600 rpm
- Prepare SAPE working reagent (protect from light):
 - SAPE: Briefly vortex and centrifuge to collect the contents at the bottom of the tube
 - SAPE working reagent: In a 15 ml tube, add 36 µl of SAPE to 12 ml of SAPE diluent
 - Vortex for 15 seconds to mix
- Washing step 3x
- Bind SAPE
 - Pipette 100 µl into each well
 - Seal the magnetic separation plate with a day 2 plate seal

- Wrap in aluminium foil to protect from light
- Place on a shaking platform at room temperature and shake at 800 rpm for 1 minute followed by 600 rpm for 30 minutes
- Washing step 3x using SAPE wash buffer
- Add 130 µl of SAPE wash buffer to each well
- Seal the magnetic separation plate with a day 2 plate seal
- Wrap the plate with aluminium foil to protect from light
- Place the magnetic separation plate on the microtiter plate shaker and shake at 800 rpm for 3 minutes at room temperature
- Read plate immediately on a Luminex® instrument

Use the following parameters to complete protocol:

- Sample size 100 µl
- DD Gate 5,000 - 25,000
- Timeout 45 sec
- Bead event / bead region 100

Pilot test:

To determine the optimal RNA concentration needed to detect all of the targets, a pilot test was performed with the HCT116 cells before the main experiment. The pilot test included 150, 300 and 500 ng RNA per well (20 µl) and was performed with a positive control sample (stimulated with IL-1β). According to the observed gene expression it was decided on a concentration of 500 ng RNA per well, which is the highest amount recommended by the manufacturer. This decision was based on the relatively low signals of most of the target genes in lower RNA concentrations. IL-8 was found to be measurable when using 150 and 300 ng/well but was signal saturating when using 500 ng/well. Since multiple other targets were scarcely visible when employing the lower concentrations, it was still decided upon 500 ng/well and IL-8 was measured via qRT-PCR. No pilot test was conducted with the HCEC-1CT cells, but a sample containing 500 ng RNA/well of a positive control was added to the plate when measuring the HCT116 cells. Based on the results, 500 ng/well were employed for this cell line as well.

7.3.4.3. Detection of immunomodulation markers by qRT-PCR

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a widely-used method to identify and quantify changes in gene expression. Special precaution has to

be given to avoid RNase contamination of used materials as well as in tissues, where endogenous RNases are occurring naturally. Since RNA can not be directly amplified in PCR experiments, reverse transcription to complementary DNA (cDNA) has to be done beforehand. Reverse transcriptase transcribes a RNA template to single-stranded cDNA by elongating the annealed oligo-dT or random primers at 42 °C. RNA from RNA:DNA hybrids is afterwards selectively degraded by the RNase H activity of the reverse transcriptase. The single stranded cDNA can then be applied in a conventional polymerase chain reaction, where well defined oligonucleotides (primers) attach to a specific region of their target genes (annealing). The primers are then elongated by the DNA polymerase resulting in a double-stranded DNA (dsDNA) which then denatured into two single strands and the entire process starts from the beginning and is repeated 35-40 times (annealing – elongation – denaturation). To enable quantification in real-time the reaction mix includes the fluorescent dye SYBR® Green I, that intercalates between double-stranded nucleic acids and, proportionally to the amount of dsDNA, emits green light ($\lambda_{\text{max}}=521 \text{ nm}$) upon excitation. When the fluorescent signal crosses the discretionary placed threshold in the exponential phase of cycling the so-called threshold cycle (C_T), which is also the quantitative endpoint of real-time PCR. There is an invert relation between the input of target sequence and the numerical value of the C_T .¹¹² In this master thesis relative quantification, in more detail the comparative C_T method (also referred to as $2^{-\Delta\Delta C_T}$ method) (Livak & Schmittgen, 2001), is applied. The comparative C_T method needs normalization of samples against a panel of so-called endogenous housekeeping genes (HKGs), that are presumed to maintain constant expression levels. The resulting $2^{-\Delta\Delta C_T}$ data of treated samples then represents the gene expression as fold change relative to an untreated control, the so-called calibrator, both normalized to the HKGs.¹³⁸

Protocols:

Reverse transcription of RNA to cDNA:

Purified RNA of unstimulated cells was reversely transcribed to complementary DNA (cDNA) with the QuantiTect Reverse Transcription Kit according to the manufacturer's manual. The needed volume of each sample was filled to 12 μl with RNase-free water to obtain an RNA concentration of 1000 ng/12 μl .

All steps were performed on ice.

- Samples and reverse transcriptase (RT) were thawed on ice, while gDNA Wipeout Buffer, RT Buffer and RT Primer Mix were thawed at room temperature and then stored on ice.
- The calculated volumes of each sample were transferred to 0.2 ml tubes.
- Variable volumes of RNase-free water were added to each tube to obtain a final volume of 12 µl containing 1000 ng of RNA.
- 2 µl of gDNA Wipeout Buffer were added to each tube, mixed by snapping and centrifuged.
- Samples were incubated for 2 min at 42 °C at the thermal cycler to eliminate gDNA and put back on ice afterwards.
- A master mix for x+2 samples was prepared containing 1 µl RT, 1 µl RT Primer and 4 µl RT Buffer per sample.
- 6 µl of master mix were added to each tube, mixed by snapping and centrifuged.
- Samples were then incubated at the thermal cycler for 15 min at 42 °C, followed by 3 min at 95 °C to inactivate RT.
- Samples were stored at -20 °C until further use

qRT-PCR targeting selected mRNAs:

- Template cDNA, QuantiTect SYBR® Green PCR Kit and primers were thawed on ice.
 - housekeeping genes: β-Actin and GAPDH
 - target genes: IL-8, IL-6, TNFα, IL-1β, COX-2
- Template cDNA was diluted with RNase-free water to 10 ng/µl
- A master mix per primer was prepared for x+2 samples
 - 6 µl RNase-free water
 - 2 µl primer
 - 10 µl SYBR® green
- 18 µl of master mix were pipetted in a semi-skirted 96-well plate
- 2 µl of diluted template cDNA were added in duplicates to the 96-well plate.
- For the no template controls 2 µl of RNase-free water were added to the wells.
- The plate was sealed with an adhesive film and centrifuged briefly with a plate spinner.
- The plate was put into the StepOne Plus Real time PCR instrument and the cycling program was started.

- | | | |
|---|--------|-------|
| ○ Initial activation | 15 min | 95 °C |
| 3-step cycling (40 cycles): | | |
| ○ Denaturation | 15 s | 94 °C |
| ○ Annealing | 30 s | 55 °C |
| ○ Extension | 30 s | 72 °C |
| Melt curve stage: | | |
| ▪ 15 s | | 95 °C |
| ▪ 1 min | | 60 °C |
| ▪ ~ 30 min heating in 0.5 °C steps to 94 °C | | |

Evaluation:

The generated raw data of the PCR were threshold cycle (C_T) values for each sample. At first, the mean and standard deviation (SD) of each technical duplicate was calculated and duplicates with a SD higher than 0.7 were not further evaluated. C_T values ≥ 35.5 were interpreted as if they were undetermined, since no stable or reproducible results could be obtained for those values.

- The average of the housekeeping genes (HKGs) for each replicate was needed to calculate the two ΔC_T values for each sample:

$$\Delta C_T = C_T \text{ target gene} - C_T \text{ HKGs}$$
- To calculate the $\Delta \Delta C_T$ values the average C_T value of the housekeeping genes ($|C_{T \text{ HKGs}}|$) and the residual genes ($|C_{T \text{ calibrator}}|$) of the calibrator (IL-1 β stimulated cells) were determined. The $\Delta \Delta C_T$ values for each replicate were then calculated:

$$\Delta \Delta C_T = \Delta C_T - (|C_{T \text{ calibrator}}| - |C_{T \text{ HKGs}}|)$$
- As the $\Delta \Delta C_T$ values for the calibrator should equal 0, higher $\Delta \Delta C_T$ values of samples represent a suppression of gene transcription and negative $\Delta \Delta C_T$ values indicate an induction of gene transcription. When the $2^{-\Delta \Delta C_T}$ values are calculated, the value for the calibrator should equal $1 \pm \text{SD}$ and relative to that, values for samples represent the fold change of gene expression levels.

7.4. Statistical Analysis

All experiments during this thesis were performed in at least three independent biological and two technical replicates. The entire data is shown as mean $\pm$ SD and complete statistical analysis was performed with Origin Pro 9. With the Shapiro-Wilk normality test a normal distribution was determined. Statistical significances among the test concentrations and the control were determined using parametric statistical tests such as one-way ANOVA and one-sample Student's *t*-test. P-values <0.05 were declared significant according to the statistical gold standard.¹³⁹

7.5. Index of Materials and Devices

Materials:

Manufacturer:

Cell lines:

- | | |
|---------------------------|--|
| • HCEC-1CT cells | Prof. Jerry W. Shay, UT Southwestern
Medical Center, Dallas, Texas, USA |
| • HCT-116 cells | ATCC, Manassas, Virginia, USA |
| • CT-26 | ATCC, Manassas, Virginia, USA |
| • THP1-Lucia™ NF-kB cells | InvivoGen, San Diego, USA |

Cell culture reagents:

- | | |
|--|---|
| • Dulbecco's Modified Eagle Medium (DMEM) | Thermo Fisher Scientific Inc., Waltham, USA |
| • Fetal Calf Serum (FCS) | Thermo Fisher Scientific Inc., Waltham, USA |
| • DMEM (high glucose) | Thermo Fisher Scientific Inc., Waltham, USA |
| • Medium 199 (10x) | Thermo Fisher Scientific Inc., Waltham, USA |
| • HEPES buffer ≥ 99.5 %, PUFFERAN®
CELLPURE® | Carl Roth GmbH + Co. KG, Karlsruhe, GER |

- Insulin-Transferrin-Selenium (100X) Thermo Fisher Scientific Inc., Waltham, USA
- HyClone™ Cosmic Calf™ serum GE Healthcare Life Sciences HyClone Laboratories, South Logan, USA
- Gentamicin solution (50 mg/mL) Sigma Aldrich GmbH, St. Louis, USA
- Recombinant human EGF (100µg/ml) Szabo Scandic Handelsgmbh CoKG, Vienna, AT
- Hydrocortisone-water soluble Merck KGaA, Darmstadt, GER
- PBS (Phosphate Buffered Saline) Prepared:

1710 mM	NaCl	(100 g/l)
100 mM	Na ₂ HPO ₄	(14.2 g/l)
34 mM	KCl	(2.5 g/l)
18 mM	KH ₂ PO ₄	(2.4 g/l)

10x PBS: Solve in H₂O_{bidest}; set pH to 7.4;
Fill up to 1 l with H₂O_{bidest}; dilute to 1x PBS with H₂O_{bidest}; autoclave, store at 4°C
- Penicillin - Streptomycin (PenStrep); 5000 U/ml Pen and 5000 µg/ml Strep Thermo Fisher Scientific Inc., Waltham, USA
- Sodium pyruvate (100 mM) Thermo Fisher Scientific Inc., Waltham, USA
- Zeocin® InvivoGen, San Diego, USA
- Normocin™ InvivoGen, San Diego, USA
- RPMI 1640 medium Thermo Fisher Scientific Inc., Waltham, USA
- Dulbecco's Modified Eagle's Medium/ Ham's Nutrient Mixture D12 Sigma-Aldrich Corporation, St. Louis, USA

Consumable materials:

- Biosphere Filter tips (2.5 µl-1000 µl), Sarstedt AG & Co, Nümbrecht, GER

sterile

- | | |
|---|---|
| • Cover glasses 0.4 mm for counting chambers | Paul Marienfeld GmbH & Co. KG, Lauda-Königshofer, GER |
| • DURAN® Laborflaschen | DURAN Group GmbH, Wertheim/Main, GER |
| • Fine inject® injection needles, sterile | Henke-Sass Wolf GmbH, Tuttlingen, GER |
| • Hemocytometer | Paul Marienfeld GmbH & Co. KG, Lauda-Königshofer, GER |
| • Light-Duty Tissue Wipers | VWR International, Radnor, USA |
| • Light Protection Tubes, brown, PP, sterile | Carl Roth GmbH + Co. KG, Karlsruhe, GER |
| • Micro tubes, non-sterile | Sarstedt AG & Co, Nümbrecht, GER |
| • Nitrile Gloves, powder free | Kimberly-Clark GmbH, Koblenz-Rheinhafen, GER |
| • Pasteur pipette (230 mm), non-sterile | Carl Roth GmbH + Co. KG, Karlsruhe, GER |
| • Pipette tips (10 µl, 200 µl, 1000 µl and 5000 µl), non-sterile | Sarstedt AG & Co, Nümbrecht, GER |
| • Serological pipettes (5 ml, 10 ml, 25 ml), sterile | Sarstedt AG & Co, Nümbrecht, GER |
| • TC flask (T25, T75, T125), sterile, yellow, red and green cap→ standard growth surface for adherent cells (yellow, red), ventilation cap (yellow) | Sarstedt AG & Co, Nümbrecht, GER |
| • TC plates (12-, 24-, 96-well), sterile, red, yellow and green | Sarstedt AG & Co, Nümbrecht, GER |
| • Tube (15 ml, 50 ml), transparent, sterile | Sarstedt AG & Co, Nümbrecht, GER |
| • 96-well ELISA plate black, High Binding | Sarstedt AG & Co, Nümbrecht, GER |
| • 96-well plate, white | Sarstedt AG & Co, Nümbrecht, GER |

Chemicals:

- | | |
|---------------|-----------------------------------|
| • Accutase | Corning, Manassas, Virginia, USA |
| • Acetic acid | Fisher Chemical, Loughborough, UK |

• Acetonitrile LC-MS grade	Honeywell-Riedel-de-Haën, Seelze, GER
• Bio-plex® Sheath Fluid, 20 l	Bio-Rad Laboratories Inc., Hercules, USA
• CellTiter-Blue® Reagent	Promega, Corporation, Wisconsin, USA
• Dexamethasone ≥97 %, powder	Sigma-Aldrich Corporation, St. Louis, USA
• Dimethyl sulfoxide (DMSO)	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Di-Sodium hydrogen phosphate (Na ₂ HPO ₄), ≥99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Ethanol absolute 99.8 %	Fisher Chemical, Loughborough, UK
• Formic acid LC-MS grade	Promochem, LGC Standards Ltd., Teddington, UK
• Magnesium chloride (MgCl ₂) anhydrous ≥98 %	Sigma-Aldrich Corporation, St. Louis, USA
• Methanol LC-MS grade	Honeywell-Riedel-de-Haën, Seelze, GER
• Potassium chloride (KCl) ≥99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Potassium di-hydrogen phosphate (KH ₂ PO ₄) ≥99 %	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• QUANTI-Luc™	InvivoGen, San Diego, USA
• Recombinant human interleukin 1β	InvivoGen, San Diego, USA
• RNase AWAY™ Surface Decontaminant	Thermo Fisher Scientific MBP, Waltham, USA
• RNase free water	Qiagen N.V., Venlo, NLD
• Sodium chloride (NaCl) ≥99.8 %	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Sodium hydroxide (NaOH) ≥98 %, pearls	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Sulforhodamine B reagent	Alfa Aesar, Ward Hill, USA
• Tris Hydrochloride PUFFERAN® ≥99 %	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Trypan blue solution (0.4 %), sterile filtered	Sigma-Aldrich Corporation, St. Louis, USA
• Trypsin (1:250), powder	Thermo Fisher Scientific Inc., Waltham, USA
• Trichloroacetic acid (TCA)	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Triton X	Carl Roth GmbH + Co. KG, Karlsruhe, GER
• Water LC-MS grade	VWR International, Pennsylvania, USA

- | | |
|---------------------------|---------------------------------------|
| • WST1 (tetrazonium salt) | Abcam PLC, Cambridge, UK |
| • 2-Propanol LC-MS grade | Honeywell-Riedel-de-Haën, Seelze, GER |

Test substances:

- | | |
|---|--|
| • Bilberry extract | Hochschule Geisenheim University,
Geisenheim, GER |
| • Blackberry extract | Hochschule Geisenheim University,
Geisenheim, GER |
| • Black currant extract | Hochschule Geisenheim University,
Geisenheim, GER |
| • Catechol | Extrasynthese, Genay, FRA |
| • Citric acid | Local pharmacy |
| • Cyanidin-3-glucoside | Extrasynthese, Genay, FRA |
| • Cyanidin-3-rutinoside | Extrasynthese, Genay, FRA |
| • Cyanidin-3-sambubiosid | Extrasynthese, Genay, FRA |
| • Cyanidin-3-sambubiosid-5-glc | Extrasynthese, Genay, FRA |
| • Cyanidin-3,5-diglc | Sigma-Aldrich Corporation, St. Louis, USA |
| • Delphinidin-3-glc | Extrasynthese, Genay, FRA |
| • (-)-Epicatechin | Extrasynthese, Genay, FRA |
| • (-)-Epigallocatechin | Extrasynthese, Genay, FRA |
| • Elderberry extract | Hochschule Geisenheim University,
Geisenheim, GER |
| • (+)-Rutin | Extrasynthese, Genay, FRA |
| • 7-ethyl-10-hydroxycamptothecin (SN38) | Tocris, Bristol, UK |

Kits:

- | | |
|--|--|
| • Bio-plex® Validation Kit 4.0 | Bio-Rad Laboratories Inc., Hercules, USA |
| • Maxwell® RSC simplyRNA Cells Kit | Promega Corporation, Wisconsin, USA |
| • RNeasy Mini Kit | Qiagen N.V., Venlo, NDL |
| • QuantiTect SYBR® Green PCR Kit | Qiagen N.V., Venlo, NLD |
| • QuantiTect Reverse Transcription Kit | Qiagen N.V., Venlo, NLD |

- QuantiGene Plex Gene Expression Assay Thermo Fisher Scientific Inc.,
Waltham, USA

Primers:

All primers were purchased at Qiagen N.V., Venlo, NED.

Technical equipment and instruments:

- Acquity UPLC column,
1.8 µm particle size,
2.1 mm diameter, 100 mm length Waters Corporation, Massachusetts, USA
- Autoclave: Systec DX-150 Systec GmbH, Weitenberg, GER
- Bio-Plex 200 System –
powered by Luminex
xMAP™ Technology Bio-Rad Laboratories Inc., Hercules, USA
- CentriVap Benchtop
Vaccum Concentrator Labconco, Kansas City, Missouri, USA
- CentriVap Coldtrap Labconco, Kansas City, Missouri, USA
- Centrifuges
Galaxy MiniStar (0.2 ml or 2.0 ml) VWR International, Radnor, USA
Mikro 220 A. Hettich GmbH & Co.KG, Tuttlingen, GER
Mikro 220R A. Hettich GmbH & Co.KG, Tuttlingen, GER
Perfect Spin P PCR plate spinner VWR International, Radnor, USA
Rotina 420R A. Hettich GmbH & Co.KG, Tuttlingen, GER
Hermle Z326K Hermle, Gosheim, GER
- Crimper, Decapper 11 mm Supelco, Munich, Germany
- Fridge (-4 °C) Liebherr Medline Liebherr, Bulle, CHE
- Freezer (-20 °C) Liebherr Profi Line Liebherr, Bulle, CHE
- Freezer (-80 °C) VIPseries VIPplus Panasonic, Kadoma, JPN
- HPLC vials (glass) and caps:
32x11.6 mm, wide opening Brucker Analysentechnik, Linz, AT
- Incubator Heracell 240 i CO2 Thermo Fisher Scientific Inc., Waltham,

	USA
• Fume hood	Prutscher Laboratory Systems GmbH, Neudörfel, AUT
• Laboratory freeze dryer, Alpha 2-4 LD plus	Christ, Osterode am Harz, GER
• Laminar flow hood HeraSafe™ KS 18	Thermo Fisher Scientific Inc., Waltham, USA
• Manual Eppendorf research pipettes with adjustable volumes: 0.5-10 µL, 1-10 µL, 10-100 µL, 20-200 µL, 50-1000 µL, 500-5000 µL	Eppendorf, Hamburg, GER
• Maxwell® 16 MDx	Promega, Corporation, Wisconsin, USA
• Micro inserts (clear glass), 0.2 ml conical and 0.2 ml flat bottom (31x6 mm)	Brucker Analysetechnik, Linz, AT
• Microplate reader Cytation™ 3 Cell Imaging Multi-Mode Reader	BioTek Instruments Incorporated, Winooski, USA
• Mettler Toledo Micro Balance (XP26, Delta range)	Mettler-Toledo, Columbus, Ohio, USA
• Microscopy: Inverted microscope Axiovert 40C	Carl Zeiss Microscopy GmbH, Jena, GER
• Nano Drop 2000c Spectrophotometer	VWR International, Radnor, USA
• pH meter S20 SevenEasy™	Mettler-Toledo, Columbus, USA
• pH electrode InLab 408/120	Mettler-Toledo, Columbus, USA
• Plate shaker IKA® MS3 basic	IKA®-Werke GmbH + Co. KG, Staufen, GER
• Labnet VorTemp™ 56 Shaking Incubator	Thermo Fisher Scientific Inc., Waltham, USA
• Pipetting device: Pipetus® Akku	Hirschmann Laborgeräte, Eberstadt, GER
• RP Luna C18(2) column, 3 µm particle size, 2 mm diameter, 150 mm length	Phenomenex, Torrance, California, USA

• Synergy H1 Plate reader	BioTek, Winooski, Vermont, USA
• Scotsman MF46 Ice Flaker	Scotsman Industries Inc., Vernon Hills, USA
• StepOne Plus Real Time PCR System	Thermo Fisher Scientific Inc., Waltham, USA
• TSQ Vantage	Thermo Fisher Scientific Inc., Waltham, USA
• Ultrasonic cleaner, USC-T VWR	Radnor, Pennsylvania, USA
• Vortex Genie 2 VWR	Radnor, Pennsylvania, USA
• Water bath GD 100	Grant Instruments, Cambridge, UK
• Q-Trap 6500+ Sciex	Framingham, Massachusetts, USA
• 1290 UHPL-System	Agilent Technologies, Waldbronn, GER

8. Index of Literature

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9. Appendix

Table 25: Raw data of HCT116 treated with berry extracts, target RNA measured by Invitrogen™ QuantiGene™ Plex Gene Expression Assay.

Well	Type	IL2 (12)	CD70 (13)	IL33 (15)	IL1beta (20)	TFRC (HK) (25)	IL6 (27)	POLR2A (HK) (28)	TNF (36)	CD274 - PD-L1 (39)	IL18 (42)	PTGS2 - COX2 (44)	HPRT1 (HK) (54)	NOS2 - iNOS (56)	IL8 (57)	HMBS (HK) (61)
A1	Blank	0	0	1	0	0	2	1	0.5	0	0	0	0	0	0	0
B1	Blank*	2	0	1	1	9	0	3	1	0	0	0	1	0	50	1
C1	Blank*	1	1	0	0	23.5	0	28	0	2	3.5	0	3	0	189	0
D1	Blank*	0	0	0	1	34.5	0	33	0	0	14	1	3	0	228	2
E1	Blank	1	0	0	1	1	1	1	0	0	1	0	2	1	1	1.5
F1	(1)C1	2	7	1	11	8661.5	0	8595	1	120	2059	11	3092	3	33	2158.5
G1	(1)C2	1	9.5	2	14	8643	2	7727	25	327.5	2524	25	2971	3.5	24580	1962
H1	(1)B81	0	4	1	9	8958	2	10258	13	309	2879.5	24	3289	7	2478	2355
A2	Blank	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0
B2	Blank*	1	1	1	1	15	0	5	1	1	6	0	4.5	1	125	2
C2	Blank*	0	0	1	1	26.5	1	30.5	2	2	4	1	8	1	191	2.5
D2	Blank*	0.5	0	0.5	1	45	1	28	0	2	7.5	1	18	0	311	6.5
E2	Blank	4	1	1	1	2	2	0	4	1	3	2	5	1	2	1
F2	(1)C1	5	17	2	32	10246.5	1	10328.5	4	96	2423	15	3506	7	40	2649
G2	(1)C2	2	16	6	21	10350	15	9357	45.5	354	3133.5	52.5	3590	11	25049	2405
H2	(1)B81	6.5	10.5	3	68	10183	2	11826.5	22	330	3506	40	3620	10	25099	2781
A3	(1)B810	5	8.5	7	24	10452	3	9784	31	352	352	45	4005.5	15	25228	2446.5
B3	(1)B8100	5	11	2	55	10391	2.5	10315	35	377	3532	37	3327	3	25487	2294
C3	(1)B81	3	14.5	2	54	10203.5	1	10137	44	393	3392	29	3489	7	25381.5	2492.5
D3	(1)B810	6	9	2	26	10535	3	9758	30	339	3453.5	42.5	3659	15	25820	2653
E3	(1)B8100	8	21.5	2	42	10076	9	9231	40	349	3775	36	3520	8	25582	2340
F3	(1)EB1	3	6	2	33	9698.5	2.5	10315.5	35.5	314	3398	23	3461	27	25815	2311
G3	(1)EB10	2	28.5	5	67.5	10987.5	10	11698	46	354	3683	58.5	3644.5	9	25639	2426
H3	(1)EB100	10.5	16	3	40	10650	1	11275	63	365.5	4284.5	50	3454	12	26254.5	2498
A4	(1)B810	4.5	5.5	3	81	10417.5	6	10243	37	349	3732	39	3896.5	6	26288.5	2591
B4	(1)B8100	6	3	6	65	10556	2	10915.5	29.5	407	3799	39	3638	6	25365.5	2352
C4	(1)B81	8	14	4	50	10169	3	10100	34	337	3228	36	3381.5	7	26237.5	2318.5
D4	(1)B810	5	13	4	41	11663	2	10250	32	351	3572.5	43	3875	28	27079	2703
E4	(1)B8100	2.5	7	6	34	11042	2	10262	35	397	3723	48	3522	5	27948	2520
F4	(1)EB1	6.5	17.5	10	37	10431	2	11893	56	348	3552	21.5	3321	5	29536	2376
G4	(1)EB10	5.5	16	7	33	11078	9	12101	42.5	406	4082	50.5	4094.5	6	30821	2684.5
H4	(1)EB100	12	20	3	47	11373.5	3	12044	46	430.5	4132.5	66	3722	13	31004	2680.5
A5	(1)BC1	5	11.5	4	51	9241	2.5	8861	18	306	3206	25	3145	8	24769	2074
B5	(1)BC10	1.5	10	3	31	9729.5	2	9578	32	328.5	3315	42.5	3258	5	25819	1971
C5	(1)BC100	3.5	7.5	3	36	9374	2	7768	40.5	331	3999	62.5	3347.5	2	25950	2180
D5	(2)C1	3	14	3	25	10192.5	3	7901.5	1.5	78.5	2673	15	3856	2.5	61.5	2809
E5	(2)C2	12.5	5	2	37	10519.5	2	8404.5	31	312	3054	34	3028.5	4	29779.5	2462
F5	(2)B81	2	24	1	30.5	9863	2	8330	48	291	3838.5	38	3481	6	29529.5	2658
G5	(2)B810	7	7	1	29	9596	2	7909	32	308	3210	27	3730	10	27530	2436
H5	(2)B8100	1.5	14	2	10	8919	1	8363	13	271	3302	38.5	3473	3	26774	1951.5
A6	(1)BC1	0.5	7	1	38.5	8993	2	8483	22	313	2740.5	28	3293	6	24323.5	2062
B6	(1)BC10	2.5	8.5	3	27	8821	1.5	9326	27	232	2838	19	2624	1	23860	1876
C6	(1)BC100	6	6	3	10	8205.5	2	6316.5	31.5	231	3480.5	18	2568	8.5	21981	1965
D6	(2)C1	0	3	0	34	8190	1	6935.5	4.5	86	2068	4	3139	3	21	2332
E6	(2)C2	2.5	16	1	14	7845.5	3	5882.5	17	214	2490	11	2809.5	19.5	23081.5	1874
F6	(2)B81	0	11	3	27	8399.5	2	6826	37	244.5	3014.5	14.5	2948	2.5	22814	1907
G6	(2)B810	4	2	1	16	7994	2.5	7081	12	264	2740	42	2533	3.5	22968	1858
H6	(2)B8100	4	6.5	1	32	8319	0.5	7136	39.5	314	2836.5	28	2811	6.5	22834.5	1716.5

Well	Type	IL2 (12)	CD70 (13)	IL33 (15)	IL1beta (20)	TRFC (HK) (25)	IL6 (27)	POLR2A (HK) (28)	TNF (36)	CD274 - PDL1 (39)	IL18 (42)	PTGS2 - COX2 (44)	HPRT1 (HK) (54)	NOS2 - iNOS (56)	IL8 (57)	HMBS (HK) (61)
A7	(2) B11	13	11	1	24.5	7437	6	5409	23	215	2447	13.5	2449	4	20490	1646
B7	(2) B110	8	7.5	2	41	7555	1	5752	34	241.5	2585	23.5	2699.5	2	22833	1864
C7	(2) B1100	5	5	3	32	8673.5	2	6748	24	280	3175	25	2881	9	22575	1873
D7	(2) EB1	2	12	3	17	7051	2.5	6299	24	237	2284.5	30	2137	2	23191.5	1542
E7	(2) EB10	1	4.5	2	21	9277	3.5	6944.5	16	283	2913	34	2975	2.5	24237	2097
F7	(2) EB100	9	21.5	0.5	31	9623	1	7807	25	241.5	3227	41	3100	8.5	24683.5	1980.5
G7	(2) BC1	0	2	2	9.5	8684.5	1	7353	23	282	2705	25	2696	1.5	22207.5	1779
H7	(2) BC10	5	2	3	5.5	6932	0	5144.5	2	215	2249	41	2440	2.5	22359	1561.5
A8	(2) B11	2.5	5	1	23	7449	4	5798.5	21	214	2372	21	2264	3.5	22960	1741.5
B8	(2) B110	11	5	1	37	6873	1	5052.5	15	196	2411	20.5	2598	1.5	21580	1720
C8	(2) B1100	11	21	2	21	7402	4	6519	39	271.5	2821.5	38.5	2370	11	21512	1674
D8	(2) EB1	1	2	3	0.5	7675.5	2	7751	39	311	2949	17	2824.5	81.5	25071	1796
E8	(2) EB10	2.5	1	0.5	21	10544	0	8439.5	37.5	265.5	3691.5	26	7	9	28723.5	2530
F8	(2) EB100	2	***	16	***	***	2.5	7337.5	81	152	4174	0	***	2	29815.5	2678.5
G8	(2) BC1	0.5	0	2.5	16.5	7311	1	6782	11.5	245.5	2937	39	2673	6	25376	1874.5
H8	(2) BC10	0	0	0	26.5	7881	0.5	6541	21	244	2460.5	15.5	2754	6.5	26552.5	1768
A9	(2) BC100	2	1	2.5	29	7040	2.5	5033	17	164.5	2911.5	18	2201	0	21954	1571
B9	(3) C1	0	1.5	1	5.5	6732	1	7264	12.5	77.5	2050	2	2503	2	21	1877
C9	(3) C2	10	71	6	44.5	8404.5	1.5	7589	70	374	3914	37	2070	11	25363	2073
D9	(3) B81	6	11.5	5	41	9034	1	8549.5	63	501.5	3805	89	3675	16	24814	2409
E9	(3) B810	20	20.5	0	43	9800.5	2	9482	76	489	4245	85	3570	15.5	25468.5	2455
F9	(3) B8100	13	10	2	57.5	9931	2	9029	81	712	4690.5	90	3877	10	25624	2215.5
G9	(3) B11	9	25	2	40	10221	1	9222.5	90.5	632	4361.5	88.5	3535.5	11.5	26550	2657.5
H9	(3) B110	2	16	2	52.5	8958.5	1	9137	96	550	4089	101	3335	8	29430	2421
A10	(2) BC100	3	15	2	20	7817	3.5	6359	19	241	3201.5	37	2746	3	25015	1944.5
B10	(3) C1	1	4	1.5	19	8111	2	9058	6	76	2769	18	2903	3	32	2277.5
C10	(3) C2	11	9.5	2	54	8173	3	7578	80	472.5	3875.5	80	3104	11	29661	2285.5
D10	(3) B81	8.5	13	2	28.5	9464	2	9348.5	50	578	3838.5	90	3373.5	15.5	31184.5	2330
E10	(3) B810	0	31	2.5	17	9805	1	10391	90	614	3843.5	79	3389	26	31189	2565.5
F10	(3) B8100	15	16	5	70	8443	18	9031	57	503	4500	61	2832.5	21	30154	2289
G10	(3) B11	3	11	1	40.5	5674	4	9723	90.5	458.5	3775	61	2503	3	29802.5	2086
H10	(3) B110	4.5	4	3	21	7175	1.5	6235	75	394.5	3492	63.5	2973	8.5	26165.5	1929
A11	(3) B1100	10.5	7	2	37	8003	1.5	7730	51	416	3718	77	2418	9.5	26185.5	1961
B11	(3) EB1	18	23	3.5	68.5	9222	2	9278	103.5	584	4043	95	3391	8.5	28703	2417.5
C11	(3) EB10	8	11	1	31	9859	2	9679.5	71	666	4235.5	62.5	3655	9	28096.5	2328
D11	(3) EB100	9	20	3	42	9363	0.5	9856.5	65	743	5467	103.5	3385	12	29379.5	2505
E11	(3) BC1	6	28	2	55	9706	2	9822	91	688	4835	84.5	3635	9	31302	2462
F11	(3) BC10	3.5	13	3	61.5	11566	1	11690	148	777.5	5985	167.5	4197	16	32118.5	2819
G11	(3) BC100	10	25	2	47	10538.5	2.5	10713	146	663	6477	155.5	3761	14	32163	2567
H11	HCFC (2) C2	104.5	213	57.5	6681	7489	6459	9820	223.5	545	122	2715	4567	60.5	32744	2698.5
A12	(3) B1100	7	14.5	2	46	9483	2.5	8850	89	550	4384	91.5	3247	8	31147	2441.5
B12	(3) EB1	11	21	3	44.5	9644	2	9874	85	579	4416.5	107	3510	9	31716	2610.5
C12	(3) EB10	9	11	1.5	49	9592.5	2	9757	91	644	4305	114	3952	6.5	31648	2474.5
D12	(3) EB100	8	15	4	64	9703.5	3	10298	85.5	670.5	4983	82	3569	10	30498	2533
E12	(3) BC1	2.5	8	2	33	9748	2	10660	67.5	792	4624.5	120	3612.5	13.5	32109	2743
F12	(3) BC10	4.5	3.5	2	81.5	10452	4	10879	117.5	574	5574	96	3946	5	32056	2692
G12	(3) BC100	2.5	15	2	68.5	7559.5	2	9860	102	573	5608	86	3496	5	31864.5	2638
H12	HCFC (2) C2	64	248	61	6315	7559.5	5540	9321	206	553	154.5	2991	4447	117	32470	2602

Blank *: trial of second use; well already used for RNA concentration testing, washed and refilled

Table 26: Raw data of HCT116 treated with berry extracts, target RNA measured by Invitrogen™ QuantiGene™ Plex Gene Expression Assay.

Well	Type	IL2 (12)	CD70 (13)	IL33 (15)	IL1beta (20)	TRFC (HK) (25)	IL6 (27)	POLR2A (HK) (28)	TNF (36)	CD274 - PDL1 (39)	PTGS2 - COX2 (44)	HPRT1 (HK) (54)	NOS2 - iNOS (56)	HMBS (HK) (61)
A1	B	3	2.5	2	2.5	3	1	7	2	3	3	2	5	2
B1	B	2	2	2	2	2	2	8	2	3	3	2	2	3
C1	(1) C1	2.5	3	2	27	4771	8	6771.5	6	171	47	3025	6	2096
D1	(1) C2	5.5	49.5	13	2774	3676	3353.5	4423	22.5	222.5	1366	2296.5	4	1295.5
E1	(1) B81	23	130	50	4494	6259	4926	7361	54	365	2358	3552.5	31.5	2191
F1	(1) B810	21	123	35	4026	5369	3975.5	6613	60	283	2197.5	3209	14	2001
G1	(1) B8100	9.5	139.5	14	4440.5	5722	3555	6804	35	296	2011	3306.5	13.5	2064
H1	(1) B11	6	49.5	25	3730	3981	4439.5	4849	22	228	1852.5	2253	4	1506.5
A2	B	1	2	1	2	2	3	4.5	2	2	2	1	3	2
B2	B	1	1	2	2	2	2	6	2	2	1	0.5	2.5	1
C2	(1) C1	1	1	2	3	763.5	3.5	1085.5	2.5	28.5	5.5	577	2	292
D2	(1) C2	2.5	37	8.5	1257	1774	1425.5	1944	17	54	536.5	820.5	10	521
E2	(1) B81	2	26.5	5.5	1373.5	1778	1307	1997	13	83.5	632	1019	3	524
F2	(1) B810	2	13	3	593	690	549	816	6	33	246	446	2	256
G2	(1) B8100	3	6.5	3	542	693	393	743	4.5	34	193.5	333	4	201.5
H2	(1) B11	5	24	23	2411	2570	2761	2939	14	137	1140	1542.5	6	933.5
A3	(1) B110	12	74	36	4338	4998.5	5236	5967	59.5	368.5	2285.5	3056	29	1658
B3	(1) B1100	16.5	87	30	4766	4597	5257	6295	43.5	283.5	2486	2915	14.5	1863.5
C3	(1) EB1	2	75	17	4050.5	5323	5205	6488	35	241	2279	3017	7	1419
D3	(1) EB10	7	87	39	4727	5079	5269	5992	67.5	292	2506.5	2889	5.5	1790.5
E3	(1) EB100	10	89.5	29	5753.5	5333	5726.5	6336	54.5	301	3629	2876	20	1926
F3	(1) BC1	16	92.5	28	4149	4625.5	4607.5	5128	30	323	1904	2551.5	5	1641
G3	(1) BC10	26	129	64	5040	5467.5	4768	6495	54	284	2306	3352	20	2001
H3	(1) BC100	12	83	22	2884	3524	2033	3550	58.5	196	1948.5	1880	10.5	1278.5
A4	(1) B110	4.5	16	20	1055.5	1182	1136	1373	9	60	444	764	5	340
B4	(1) B1100	4	24	6	1148	1277	1351	1371	4	69	463	570	2	372
C4	(1) EB1	2	38	3	1254	1569	1447	1725	9	46	515.5	632.5	3	500
D4	(1) EB10	4	12	3.5	507	707	652	699	5.5	34.5	309	378	5	203
E4	(1) EB100	3	13	6.5	1659.5	1520	1452.5	1506	8	69.5	762	635	3.5	410
F4	(1) BC1	5	36	13.5	2335	3013	2648	2792	19	127	1157	1608	5	804.5
G4	(1) BC10	4	36	8	1414.5	2115	1493	2105	14.5	69	697.5	995.5	5	569.5
H4	(1) BC100	7	70	29.5	2271.5	3227.5	1899	2921	38	181.5	1484.5	1704	4	1007
A5	(2) C1	7	2.5	1	47	3476.5	11	5235	2	134	32	2508	2.5	1533.5
B5	(2) C2	36	127	41	3550	4353	3516	5070	142	367	1856	2673	60	1605
C5	(2) B81	7	79.5	7	3720	4801	3251.5	5184.5	62.5	281	2291	2688	19	1581.5
D5	(2) B810	21	125	6.5	3546	4983	2796	5132.5	109.5	277	2565	2637	51	1920
E5	(2) B8100	2	2	2	3	2	1.5	3.5	1	1	1	3	6	2
F5	(2) B11	62.5	117	40.5	4294	6609.5	5176	6983	111.5	417	2947.5	3432	65.5	2456.5
G5	(2) B110	21	95	24	5372	5410	7095.5	7492	79	386	3161.5	3796.5	15.5	2402

H5	(2) B1100	28	114	21	4673.5	5347.5	6198	6355	77.5	327	2659	2861	46.5	1969
A6	(2) C1	3	12	4	27	3268	9	4515	4	93	21.5	2001	0	1555.5
B6	(2) C2	2.5	21	4	997.5	1476	988	1395	24	64.5	534	579	3	400
C6	(2) BB1	6.5	71	4	2533	3578	2320	3468.5	60	131	1475.5	1805	5.5	1089
D6	(2) BB10	13	49	9.5	1390	2414	1202	1884	61	96	910	1042	17.5	814
E6	(2) BB100	4.5	3	2	3	2	3	5	3	3.5	2	1	8	2
F6	(2) B11	14.5	53	7	2063.5	5031	3454.5	5047.5	20	272	1543	2348.5	12.5	1732.5
G6	(2) B110	16	105	34.5	4737	3986	5871	5929	33	241	2240.5	2858	24	1893
H6	(2) B1100	26	57	18	3809.5	3844	4920.5	5150	28	171	2153	2524.5	8	1502.5
A7	(2) EB1	9	40.5	10	1764.5	2977	1836	2835.5	41.5	144	979	1163	7	748.5
B7	(2) EB10	13	69.5	12	3049.5	4730	3358.5	4722.5	100	366	1982	2454	29	1734
C7	(2) EB100	20	43.5	15	1769	3043	2531	3094	78	185	2019	1613	5	1095
D7	(2) BC1	5.5	47.5	6	1769	3113.5	1704.5	2790	39	167.5	999	1320	5.5	942.5
E7	(2) BC10	38	163	36.5	5489	6785	3919	7635	272	496	3908	3822	81.5	2548
F7	(3) C1	5.5	8	3.5	89	4129	7.5	7117.5	9.5	110	36.5	2990	3	1944
G7	(3) C2	17	77.5	10	3006	3415	2907	4000.5	57	209	1271	1844	8	1051
H7	(3) BB1	30.5	121	16	4081	4170	4409	6053	110	291	1769	2566	23	1379
A8	(2) EB1	0	16	2	1180	1989	1639	2177	40	133	991	1129.5	4.5	658
B8	(2) EB10	5	15	2	1111	1750.5	1146	1548	24.5	100.5	649	931	4	565
C8	(2) EB100	7	35.5	9	1981	2412	2105	2401	20	111.5	1368	1118	6	771
D8	(2) BC1	3	30	4.5	1339	2340.5	1524	2324	37.5	154.5	884	1224.5	10	774
E8	(2) BC10	13	57	5	2538	3257	1630	3210	56	171	1225	1491	11.5	964
F8	(3) C1	2	4.5	2	74	2072	4	3567	3	87	16	1542	2	849
G8	(3) C2	8.5	83.5	2	2923.5	3322	3045	4445	37	264	1403	1951.5	3	1104
H8	(3) BB1	5	51	8.5	2324	2729	2347.5	3058	52	140	1041	1432	9.5	834
A9	(3) BB10	13	71	38	3624	4103	4070.5	5241.5	62	217	2345.5	2241	4	1352.5
B9	(3) BB100	33	116	35	5344.5	6424	6617	8038.5	91	377	3424	3955	15	2626
C9	(3) B11	8	24	6	2802	2620.5	2951.5	3227.5	28	164	1550	1469	6	688
D9	(3) B110	15	33	6	2864	3244	3679.5	3968	32	135	1452	1785	22	1055
E9	(3) B1100	22	113	11	5361	5144.5	5278	6984.5	114	339	2593	3094	35.5	1927
F9	(3) EB1	15	112	16	4496	4400	4461	5787	70	307	1855	2582	7	1508.5
G9	(3) EB10	10	81.5	7.5	3743	4041.5	3559	5173	74	278	1571	2196	12	1338
H9	(3) EB100	12	61	7	5731.5	4270.5	4585	5833	72.5	319.5	2668.5	2710.5	31	1549
A10	(3) BB10	10	61.5	22	2711.5	3080	2890	3590.5	46	172.5	1696	1609	9	925
B10	(3) BB100	5	13	4.5	1767	2206	1789	2144	17	118.5	992	959	2	535
C10	(3) B11	1	31	9.5	1925	1810	2344	2752	9.5	70	827	1153	3	659
D10	(3) B110	3	31.5	5	2099	2305	2814	2783	11.5	129.5	922	1252	16	784.5
E10	(2) BC100	12	55	20	3107.5	3081	3069	3963	52.5	193	1564.5	1832	27	1185
F10	(3) EB1	5	74	9.5	3506	3341	3533	4922.5	72	219	1485	2051	17	1108
G10	(3) EB10	4.5	53	12	3533.5	3127	3002	4123.5	64	229	1318.5	1765	11.5	1121.5
H10	(3) EB100	10	50	5.5	3928	3146	3239	4024.5	64	113	1751	1696	29.5	947
A11	(3) BC1	5	112	8.5	4195	4285.5	3824.5	6191.5	66	250	1794	2737	5	1675
B11	(3) BC10	41	154	17	4894.5	5547.5	4521	7377.5	152	370.5	2131	3298	38	2072
C11	(3) BC100	17	89	35	5086	5406	2432	6786	125	328.5	3093	3676	31	1840
A12	(3) BC1	3	42.5	5	2082	2360	2035	3037	33	160.5	802	1386	4	772
B12	(3) BC10	5.5	54.5	5	1650	2279.5	1560	2667	22.5	93	657	1047	2	633.5
C12	(3) BC100	11	55.5	6	1216.5	1489	781.5	1563.5	18	92	503	880	7	398

10. Abstract

Anthocyanins are subject of frequent discussion regarding their potential protection against chronic and degenerative diseases and their interactions with topoisomerase poisons used in chemotherapeutics such as SN38. Multiple immunomodulatory and anti-inflammatory effects have previously been reported, with anthocyanins seemingly down-regulating the expression of various pro-inflammatory cytokines and other biomarkers. Dampening NF- κ B activation and thereby limiting the inflammatory response has been suggested as a potential strategy to prevent chronic inflammation and protect against side effects of chemotherapy caused by inflammation. This thesis, as part of the project “protective properties of anthocyanins during chemotherapy” funded by the Austrian Science Fund (FWF), aimed to shed light on immunomodulatory properties of anthocyanin-rich berry extracts. Firstly, possible activation of the NF- κ B pathway by the chemotherapeutic SN38 was investigated in THP-1 monocytes, as a potential point of interaction between chemotherapeutics and anthocyanins. Secondly, immunomodulatory effects of four anthocyanin-rich berry extracts; blackberry (BB), bilberry (Bil), elderberry (EB) and black currant (BC), were evaluated on cancerous (HCT116) and non-cancerous (HCEC-1CT) colon cells. The THP-1 cells were incubated with up to 10 μ M SN38 with and without the additional stimulus of LPS (10 ng/ml) for 20 h and the NF- κ B activity was measured with the help of a luciferase detection reagent. SN38 did not exhibit any influence on NF- κ B activity, whether by itself nor in combination with LPS as a NF- κ B stimulus. In higher doses (>1 μ M) expected cytotoxicity of SN38 was detected with the CellTiter-Blue[®] Cell Viability Assay, at which doses also the NF- κ B activity was reduced. After short-term incubation (5 h) of HCT116 and HCEC-1CT cells with berry extracts, in doses of 1, 10 or 100 μ g/ml, combined with an inflammatory stimulus for 3 h (25 ng/ml IL-1 β), selected immunomodulatory markers, IL-1 β , IL-2, IL-6, IL-8, IL-18, IL-33, TNF α , iNOS, COX-2, CD70 and PD-L1, were measured on a transcription level using the Invitrogen[™] QuantiGene[™] Plex Assay Kit with Luminex[®] xMAP technology and qRT-PCR. The HCT116 cells did not express measurable levels of IL-2, IL-6, IL-33, CD70 and iNOS. Of the remaining targets, only COX-2 was upregulated significantly after treatment with 10 μ g/ml EB. No other effects were observed. The HCEC-1CT cells additionally expressed CD70 and IL-6, but IL-18 could not be evaluated in this cell line. The expression of IL-1 β , IL-8 and PD-L1 was not affected by the treatment with berry extracts. TNF α was upregulated by 10 μ g/ml BB and downregulated by 1 μ g/ml Bil. CD70 showed downregulation with 1 μ g/ml Bil and 100 μ g/ml EB and COX-2 showed tendencies for upregulation but the results differed between the test systems. Both pro- and anti-

inflammatory tendencies were observed in both cell lines. On the whole, the obtained results show no anti-inflammatory effect of the investigated anthocyanin-rich berry extracts in colon cells and argue against NF- κ B as a potential interaction point of SN38 and anthocyanins since SN38 does not seem to influence its activation.

11. Kurzfassung

Anthocyane werden häufig diskutiert in Bezug auf Krankheitsprävention und Wechselwirkungen mit Topoisomerasegiften, welche in Chemotherapeutika zum Einsatz kommen. Es wurde bereits über zahlreiche immunmodulatorische und entzündungshemmende Wirkungen berichtet, wie die Hemmung verschiedener proinflammatorischer Zytokine und anderer Biomarker. Die Dämpfung der NF- κ B-Aktivierung zur Begrenzung einer Entzündungsreaktion wurden als mögliche Strategie zur Verhinderung chronischer Entzündungen und zum Schutz vor entzündungsbedingten Nebenwirkungen von Chemotherapie vorgeschlagen. Diese Masterarbeit, die Teil des vom Österreichischen Wissenschaftsfonds (FWF) geförderten Projekts "Schützende Eigenschaften von Anthocyanen während der Chemotherapie" ist, hat zum Ziel, die immunmodulatorischen Eigenschaften von Anthocyan-reichen Extrakten zu beleuchten. Zunächst wurde eine mögliche Aktivierung des NF κ B-Signalwegs durch das Chemotherapeutikum SN38 in THP-1-Monozyten, als möglicher Punkt der Interaktion zwischen Chemotherapeutika und Anthocyanen, untersucht. Anschließend wurden die immunmodulatorischen Wirkungen von vier Anthocyan-reichen Beerenextrakten – Brombeere (BB), Heidelbeere (Bil), Holunderbeere (EB) und schwarze Johannisbeere (BC) - auf kanzerösen (HCT116) und nicht kanzerösen (HCEC-1CT) Kolonzellen untersucht. Die THP-1-Zellen wurden für 20 Stunden mit bis zu 10 μ M SN38, mit und ohne den zusätzlichen Stimulus von LPS (10 ng/ml), inkubiert und die NF- κ B-Aktivität wurde mit Hilfe eines Luciferase-Reportergen-Assays gemessen. SN38 zeigte keinen Einfluss auf die NF- κ B Aktivität, weder allein noch in Kombination mit LPS als NF- κ B-Stimulus. In höheren Dosen ($>1 \mu$ M) wurde Zytotoxizität mittels CellTiter-Blue® Zytotoxizitäts Assay nachgewiesen, wobei bei diesen Dosen auch die NF κ B-Aktivität zurückging. Nach kurzzeitiger Inkubation (5 h) von HCT116- und HCEC-1CT-Zellen mit Beerenextrakten in Dosen von 1, 10 oder 100 μ g/ml, kombiniert mit einem Entzündungsreiz für 3 h (25 ng/ml IL-1 β) wurden ausgewählte immunmodulatorische Marker, IL-1 β , IL-2, IL-6, IL-8, IL-18, IL-33, TNF α , iNOS, COX-2, CD70 und PD-L1, auf Transkriptionsebene mit dem Invitrogen™ QuantiGene™ Plex Assay Kit mit Luminex® xMAP-Technologie und qRT-PCR gemessen. Die HCT116-Zellen exprimierten keine messbaren Mengen von IL-2, IL-6, IL-33, CD70 und iNOS, und die übrigen Zielgene wurden durch die Behandlung mit Beerenextrakten nicht beeinflusst abgesehen von einer schwachen Tendenz zu verstärkter COX-2 Expression. Die HCEC-1CT-Zellen exprimierten zusätzlich CD70 und IL-6, aber IL-18 konnte in dieser Zelllinie nicht bestimmt werden. Die Expression von IL-1 β , IL-8 und PD-L1 wurde durch die Behandlung mit Beerenextrakten nicht beeinflusst. TNF α

wurde durch 10 µg/ml BB hochreguliert und durch 1 µg/ml Bil herunterreguliert. CD70 wurde durch 1 µg/ml Bil und 100 µg/ml EB herunterreguliert und COX-2 wurde tendenziell hochreguliert, aber die Ergebnisse unterschieden sich zwischen den Testsystemen. Sowohl pro- als auch antiinflammatorische Tendenzen wurden in beiden Zelllinien observiert. Insgesamt zeigen die erzielten Ergebnisse keine entzündungshemmende Wirkung der untersuchten Anthocyan-reichen Beerenextrakte in Dickdarmzellen und sprechen gegen NF-κB als möglichen Interaktionspunkt von SN38 und Anthocyanen, da SN38 dessen Aktivierung nicht zu beeinflussen scheint.