BIOFUNCTIONAL PROPERTIES OF BLUEBERRIES

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Dedication

I dedicate this thesis to the memory of my Father RAHEEM TAHER AL-SAEDI

And

I dedicate this thesis to the memory of my Brother ALI RAHEEM AL-SAEDI

Abstract

Epidemiological studies associate fruit and vegetable consumption with a reduced risk of degenerative diseases. Bioactive components of these foods may be responsible for their health-promoting effects. Blueberries are a rich source of candidate bioactive components such as vitamins, phenolic acids and anthocyanins. Anthocyanins are pigments that contribute to the intense colours of blueberries; they have numerous bioactive properties, such as anti-inflammatory, antioxidant and anticancer effects. Gut microflora metabolises anthocyanins to phenolic acids and aldehydes. These metabolites have higher chemical and microbial stability, and they may contribute to beneficial health effects.

This study investigated the properties of a commercial blueberry extract, malvidin-3-galacotside, as one of the most abundant anthocyanins in blueberries and metabolites (malvidin in its aglycone form and 2,4,6-trihyodroxybenzaldehyde) that can be derived from malvidin-3-galacotside during gastrointestinal transit.

Analysis of the blueberry extract confirmed it is rich in phenolic and flavonoids and exhibited antioxidant properties in vitro, including radical scavenging and metal chelating activities. High-performance liquid chromatography demonstrated that malvidin-3-galactoside was the most abundant form of malvidin-glycoside in the extract.

The effects of the blueberry-derived agent on the Caco-2 colorectal cell line were then investigated. All these agents exhibited concentration-dependent cytotoxicity and stimulated apoptosis of Caco-2 cells. At non-cytotoxic concentrations, none of the test agents provided significant antioxidant protection against oxidative stress-induced Caco-2 cells using tert-butyl hydroperoxide. Instead, when used at higher concentrations, the blueberry-derived agents stimulated the production of intracellular ROS, with the blueberry extract and the 2,4,6-trihydroxybenzaldehyde producing the most marked effects.

Finally, the effects of the blueberry-derived agents were tested when used in combination with the chemotherapeutic agent 5-fluorouracil. In all cases, the cytotoxic effects of the combination treatments were at least equivalent to the additive effects of the individual agents. However, the blueberry extract, and more marginally, the 2,4,6-trihydroxybenzaldehyde elicited synergistic effects with 5-fluorouracil.

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My lovely brothers and sisters for being there for me whenever I need their support.

Finally, I have left till last the mention of my greatest gratitude to my family: ***my kids Yousef, Maya and Meryam;*** you have been my inspiration, motivation and the reason for my happiness. ***My beloved husband Hussain*** thank you for your love and support throughout my study. What I owe my family is priceless.

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Table of Abbreviations

5FU	5-Fluorouracil
AlCl3-6H2O	Aluminum Chloride Hexahydrate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine Serum Albumin
Caco-2	Human Epithelial Colorectal Adenocarcinoma Cells
Caspases	Cysteine aspartate proteases
CM-H_2DCFDA	5-(and-6)chloromethyl-2',7' dichloro dihydrofluorescein diacetate
DMEM	Dulbecco's Modify Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EA	Ellagic Acid
EDTA	Ethylenediaminetetraacetic Acid
FBS	Foetal Bovine Serum
FTC	Ferric thiocyanate
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
IC	Inhibitory concentration
LDL	Low-density lipoprotein
LOOH	Lipid hydroperoxide

MDA	Malondialdehyde
MTT	Tetrazolium Salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide
NADPH	Nicotine Amide Adenine Dinucleotide Phosphate Reduced
NaNO2	Sodium nitrite
NaOH	Sodium Hydroxide
NEAA	Non-Essential Amino Acid
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
R•	Free radical
RH	Unsaturated fatty acid
ROO•	Peroxy radical
ROS	Reactive Oxygen Species
T-BOOH	Tert-Butyl Hydroperoxide
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TFC	Total flavonoids content
TPC	Total phenols content

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CHAPTER ONE

1. Introduction

1.1. Blueberries

Blueberries were established as food in North America before the colonies, and they have long been a part of a European traditional food habit as they have been used in baking cakes and bread (Routray and Orsat, 2011).

Blueberries are a kind of woody shrub plant that fall under the *Ericaceae*, family and the *Vaccinium*, genus (Gough *et al*., 1994). The *Vaccinium* genus covers many popular berries, including blueberries, bilberries, cranberries, huckleberries, and lingonberries. Depending on the growing season and harvesting time, various types of blueberry were presented, including highbush blueberry, lowbush blueberry, bilberry and rabbiteye blueberry (Michalska and Lysiak, 2015).

Blueberries are nutritious fruits; in fresh form, they can consist of about 80% water, 10% carbohydrates, under 1% protein and fat, as well as fibres that comprise 3-3.5% of fruit weight. One of the main nutritional interests in blueberries is for them as a rich source of vitamin C; 100g of blueberries can provide 10 mg of ascorbic acid (Michalska and Lysiak, 2015). Equally, several studies report that blueberries are a great source of other potential health-promoting compounds as they contain substantial amounts of phytochemicals, mainly phenolic derivatives and, in particular, anthocyanins. The concentration of anthocyanins in blueberries can reach up to 800 mg/100g fresh weight in highbush blueberries and around 1000 mg/100g fresh weight in lowbush blueberries (Cho *et al*., 2004). This high content of anthocyanins in blueberries contributes to their antioxidant activity (Zhou *et al*., 2020)

Anthocyanins consist of one or more hydroxylated phenolic rings, which contribute to their antioxidant activity. These compounds exhibit potentially important functionality as inhibitors or activators of many mammalian enzymes, as metal chelators and as scavengers of free oxygen radicals (Gavrilova *et al*., 2011). Anthocyanins have been investigated extensively for being associated with antioxidant, anti-inflammatory and anticarcinogenic properties, for protection

against certain types of cancer and heart disease and for the reduction of the risk of diabetes and cognitive function disorders (Prior *et al*., 2001),

To this end, it is clear that there is a growing interest in studying the biological activity and content of phytochemicals and especially anthocyanins in blueberries in response to the increase in consumer awareness of the possible relationship between these plants products and the inhibition of degenerative diseases (Castrejón *et al*., 2008).

1.2 Reactive oxygen species and oxidative stress

1.2. 1 Free radical

A free radical is defined as any molecular species that contains an unpaired electron capable of independent existence in an atomic orbital. Free radicals are typically unstable and highly reactive. They behave as oxidants or reductants by either donating an elector to, or accepting an electron from other molecules (Halliwell, 2005).

The field of free radical research in biology has been under active for many years. Free radicals are produced in our body by many endogenous processes, pathological states or exposure to different physicochemical conditions. For healthy physiological function, a balance between the free radical production and antioxidant defence is required. Reactive oxygen species (ROS) are molecules that contain an oxygen atom and are highly reactive free radicals or due to the structure of the oxygen atom within the molecule whereby there are more electrons than usual (Halliwell, 1991). Hydroxyl radical, superoxide anion radical, nitric oxide radical, peroxynitrite radical, and oxygen singlet are the main ROS species in biological systems that are capable of causing biological damage to key cellular components including DNA, proteins and lipids (Young and Woodside, 2001).

1.2.2 Free radical production

Free radicals are produced endogenously as products or by-products of essential metabolic processes including mitochondrial respiration, inflammation, peroxisomal function, phagocytosis, ischemia/reperfusion injury and exercise. Free radicals can also be produced as a result of exposure to exogenous agents such as cigarette smoke, industrial solvents, radiation, certain drugs, environmental pollutants and ozone (Halliwell, 2005).

1.2.3 Oxidative stress

An imbalance between antioxidant defences and free radical production results in a condition known as oxidative stress, which leads to increased damage production within cellular components including nucleic acids, proteins and lipids (McCord *et al*., 2000). In acute injuries, short-term oxidative stress may occur; this type of oxidative stress causes mild inflammation, which typically goes away after the immune system fights off an infection or repairs an injury. Long term inflammation link to many chronic health conditions for instance type 2 diabetes, metabolic syndrome, cardiovascular disease, cancer, rheumatoid arthritis and inflammatory bowel disease (Zhong *et al*., 2019). Recent studies have identified a link between the imbalances of the antioxidant defence system with ROS and cancer progression (Rao *et al*., 2006).

1.2.4 Lipid peroxidation

Oxidative damage to lipids comprising one or more carbon-carbon double bonds is known as lipid peroxidation. The peroxidation of lipids produces free radicals, damaging the cell membrane (Catalá, 2006).

1.2.4.1 Types of lipid oxidation

Lipid oxidation occurs when the free radicals attack the unsaturated lipids through the following different pathways:

Autoxidation: When free radicals present in the living cells interact with polyunsaturated fatty acids and monounsaturated fatty acids, the process of autoxidation is triggered and results in the production of intermediate substances such as alkyl and peroxyl radicals (Sikorski and Kolakowska, 2002).

Photoxidation: In this process, light (including ultraviolet light) works as a source of energy which raises the oxygen from the ground state (two electrons with same spin in two orbitals) to the excited energy state (two electrons with different spin in one orbital) that is referred to as singlet oxygen ($^1O_2$). This reacts with the unsaturated fatty acids to form free radicals. Photosensitisers like chlorophyll, riboflavin and myoglobin mediate the production of singlet oxygen, as explained above (Sikorski and Kolakowska, 2002).

Enzymatic oxidation: The presence of a lipoxygenase enzyme system determines the enzymatic oxidation reaction as it catalyses the reaction between the oxygen and unsaturated fatty acids in order to produce hydroperoxides (Sikorski and Kolakowska, 2002).

1.2.4.2 Mechanism of lipid peroxidation

Lipids are essential components in human and animal cell membranes. Exposure to light, oxygen, free radicals, pro-oxidants, and high temperatures can cause them to become oxidised, affecting their structural and functional properties (Frankel *et al.,* 1999). In food systems, during the storage of food containing lipids, oxidation may cause serious deterioration to the food (Sakanaka *et al.,* 2004). The oxidation of lipids produces undesirable rancid odours and decreases the sensory and nutritional quality of food. Polyunsaturated fatty acid, which can be found in cell membranes are particularly susceptible to radical chain reactions resulting in extensive lipid peroxidation. Hydroperoxides are srecognised as the primary products of lipid peroxidation, and they are unstable molecules. Hydroxyl radicals initiate the reaction by removing a hydrogen atom from polyunsaturated fatty acids, converting them into radicals. The unstable lipid radicals undergo rearrangement into conjugated dienes that, in the presence

of oxygen, can be converted into the peroxyl radical form. These lipid peroxyl radicals are highly reactive and capable of attacking other fatty acids, forming lipid hydroperoxide (LOOH) and a new lipid radical. Thus lipid peroxidation can be propagated in a chain reaction. Alkanes, malonaldehyde, and isoprostanes are a number of compounds formed due to lipid peroxidation, and these compounds have been observed at elevated concentrations in many degenerative diseases and are used as markers for lipid peroxidation (Joseph *et al*. 2009).

Lipid peroxidation steps:

Initiation:

RH (fatty acids) + initiator → R· (lipid free radical)

ROOH (lipid-hydroperoxide) + initiator → ROO· (peroxy radical)

Propagation:

R· (lipid free radical) + O2 → ROO· (peroxy radical)

ROO· (peroxy radical) + RH (fatty acids) → ROOH (lipid-hydroperoxide)

+ R· (lipid free radical)

Termination:

R· (lipid free radical) + R· (lipid free radical) → R-R (stable molecule)

ROO· (peroxy radical) + R· (lipid free radical) → ROOR (stable molecule).

These processes can be initiated by many physical or chemical factors, including temperature, ROS, and photosensitises.

1.3 Oxidative stress and chronic disease

Multiple lines of evidence indicate that oxidative stress is linked to increase risk of a range of chronic diseases. The studies described in this thesis focus upon cancer as an example, and so this is considered in detail below.

1.3.1 Human cancer

Cancer is associated with uncontrolled cell growth and other changes in cell characteristics. This unregulated growth can lead to the development of tumours. The lymphatic system and bloodstream can play a role in spreading cancer to neighbouring healthy tissues, which can lead to severe health problems (Siegel *et al*., 2012). In the human body, there are more than 200 different types of cells and cancer can be derived from any of these. Thus, many types of cancer can develop (Alberts *et al*., 2013).

The development of cancer is a multi-step process. Initiation is the first step. This occurs when chemicals, metabolic, environmental and genetic factors lead to damage at critical sites in the cellular DNA. The second step is promotion, when the cells that have passed through the initiation process start uncontrolled proliferation. Progression is the final step defined as the metastasis of the tumour cells.

Tumours can either be non-cancerous (benign tumour) or cancerous (malignant tumour). Benign tumours are comprised of cells similar to normal cells that do not spread to other parts of the body and generally grow at a comparatively slow rate. Typically, these only become a source of concern if the benign tumour develops to too large size. Malignant cancer cells usually grow at a faster rate than benign tumour cells, and they can spread beyond the initial tumour destroying neighbouring tissues or developing as secondary tumours at sites remote to the primary tumour (Alberts *et al*., 2013).

1.3.2 Cancer therapy

Several types of intervention are used to treat cancer. These include chemotherapy, radiotherapy, biological therapy, hormone therapy, stem cell transplants and surgery. These treatments may be used individually or in combination, depending on the type of cancer (Guimaraes *et al.,* 2011). One of the most widely used cancer treatments is chemotherapy and is probably the cancer treatment option of most obvious relevance to the research presented in this thesis. Chemotherapy involves the use of cytotoxic drugs with the aim of explicitly killing the cancerous cells. Most commonly, chemotherapeutic agents act to disrupt cell division of the rapidly proliferative cancer cells by inducing DNA damage (Kaufmann and Earnshaw, 2000).

Unfortunately, chemotherapy is often associated with a range of side effects. Common side-effects include nausea, vomiting, fatigue, hair loss and loss of appetite, weight loss, increased risk of infection, bleeding, anaemia, diarrhoea, constipation, sleep problems along with sexual and fertility issues (Carelle *et al*., 2002). Thus any strategy that might be used to reduce the dose of cytoxic agent given to patients without reducing its overall efficacy in terms of killing cancer cells could be of substantial benefit to patients.

1.3.2.1 Cell death Pathways

Several different cell death pathways have been identified, each leading to characteristic morphological changes (shown in figure 1.1). Cell death via apoptosis and autophagy are coordinated via specific molecular pathways and, as such, are considered programmed forms of cell death. In contrast, necrosis is classified as a non-programmed cell since it proceeds via dysregulation of normal cellular processes.

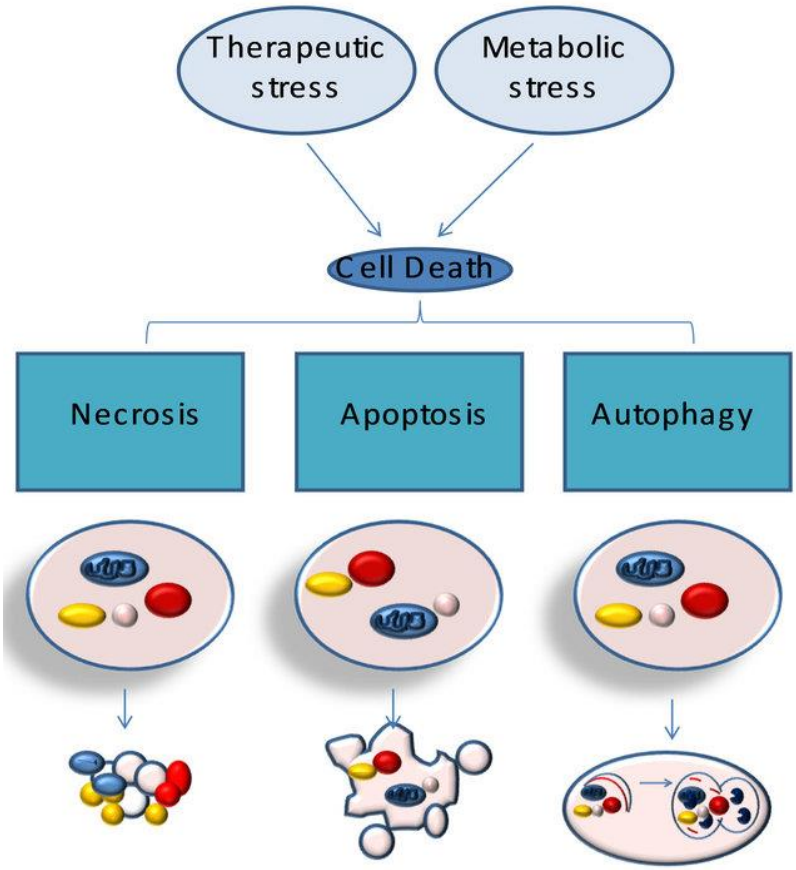


Figure 1.1 Representation of different cell death mechanism (From Zappavigna *et al*., 2013)

1.3.2.1.1 Necrosis

Necrosis is non-programmed cell death and can be characterised by an increase in cell volume, cytoplasmic swelling, mechanical plasma membrane rupture, and dilation of cytoplasmic organelles and a reasonable condensation of chromatin (Weerasinghe and Buja, 2012). External factors such as toxins and infectious agents can activate necrosis and can lead to the unregulated release of cell contents, provoking inflammation localised around the dying cell (Edinger and Thompson, 2004).

1.3.2.1.2 Apoptosis

As noted above, apoptosis is a programmed cell death pathway and can be characterised by biochemical events that lead to characteristic changes in cell morphology such as cell shrinkage, blebbing, chromatin condensation as well as chromosomal DNA fragmentation (Rowan and Fisher, 1997). Certain anticancer therapies can induce the apoptosis pathway through the induction of the intracellular caspases, which are cysteine-dependent aspartate-directed proteases (Persson *et al*., 2009). Both intrinsic and extrinsic pathways control cell apoptosis. The intrinsic pathway is achieved by the release of caspase activation protein from the mitochondria. The extrinsic pathway is controlled by trans-membrane death receptors like tumour necrosis factor (TNF-R) (Seeram *et al*., 2005).

1.3.2.1.3 Autophagy

Autophagy is a word of Greek origin first described by Christian de Duve over 40 years ago, which means 'eating of self'. In this mechanism, dysfunctional cytoplasmic components are isolated from the rest of the cell inside the autophagosome; these components are subsequently combined with lysosomes and decomposed or recycled (Edinger and Thompson, 2004).

1.4 Antioxidants

1.4.1 Mechanism of antioxidants

Both in living systems and food, it is essential to limit the occurrence of oxidation, which can cause both cellular damage and food spoilage (Yildirim *et al*., 2001). ROS capable of causing oxidative damage within cells and tissues are produced as part of the normal body process and, under certain circumstances, can be produced in excess, causing oxidative stress. Oxidation plays a crucial role in the pathogenesis of many degenerative diseases such as cancer (Valko *et al*., 2004), cardiovascular disease (Cai and Harrison, 2000) and neurodegenerative diseases

such as Alzheimer's disease (Christen, 2000). Equally, oxidation has a key role in food deterioration, shortening the shelf-life of food products (Lin and Liang, 2002). An antioxidant is defined as a stable molecule that can neutralise a free radical, hence reducing the capacity of free radicals to cause oxidative damage. Antioxidants can delay or inhibit the damaging of vital cellular components by interacting with free radicals to safely terminate oxidative chain reactions. For antioxidants, there have been two standard mechanisms of action proposed (Rice-Evans and Diplock, 1993); one mechanism is a chain-breaking mechanism when the primary antioxidant donates an electron to the free radical presented and the second involves removing the initiators of the ROS or reactive nitrogen species (RNS) production by reducing chain-initiating catalyst (Krinsky, 1992).

1.4.2 Endogenous sources of antioxidants

Antioxidants can be synthesised within the body, such as non-enzymatic antioxidants that are produced endogenously as part of normal cellular metabolism. These include glutathione (GSH), uric acid and ubiquinol (Fernández-Gajardo *et al*., 2014). Additionally, a variety of antioxidant enzymes also synthesised within cells. These include enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Krishnamurthy and Wadhwani, 2012). Some of the defence system enzymes are described below:

Superoxide dismutase (SOD) is responsible for the dismutation of the superoxide anion to hydrogen peroxide, which is less harmful, and a molecule of oxygen according to the following reaction:

$$2O^{\cdot}{}_2 + 2H^+ \rightarrow H_2O_2 + O_2$$

In humans, SOD can be found in mitochondria, cytoplasm as well as extracellular fluids. There are two different forms of SOD in humans, which can be characterised according to the type of metal(s) which acts as a cofactor to catalyse the dismutation reaction. The copper/zinc-SOD form is found in the cytoplasm and extracellular fluids while manganese-SOD form found in the mitochondria (Halliwall and Gutteridge, 1990).

Catalase (CAT) protects the cells by converting hydrogen peroxide H_2O_2 to water and oxygen. CAT is found in all eukaryotic cells in peroxisome organelles (Del Río *et al*., 2002). For the active site of catalase, two molecules of H_2O_2 are needed to convert them to H_2O (Halliwall and Gutteridge, 1990).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Glutathione Peroxidases (GPx) eliminate hydrogen peroxides using glutathione (GSH) in its reduced form as substrate, catalysing their conversion to water, alcohols and oxidised glutathione (GSSG) (Halliwall and Gutteridge, 1990).

$$H_2O_2 + 2\ GSH \rightarrow 2H_2O + GSSG$$

GSSG can be reduced back to GSH by glutathione reductase. Thus, the ratio of GSH/GSSG should be maintained (Halliwall and Gutteridge, 1990).

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

CAT and GPx both participate in the breakdown of H_2O_2 to H_2O. Thus the concentration of H_2O_2 determines the right enzyme to be activated; for high levels of H_2O_2, CAT is dominant while, at the low levels, GPx is produced (Chance *et al*., 1979).

1.4.3 Synthetic sources of antioxidants

Fatty oils in food and medicine need to be protected against oxidation; synthetic and natural antioxidants are used routinely to protect these products against oxidation. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are varieties of synthetic phenolic antioxidants compounds that have been extensively used as antioxidants in food industry, cosmetics, and for other commercial uses due to their stability which can be higher than natural antioxidants (Iverson, 1995). However, these synthetic antioxidants are a cause for concern to consumers due to their potential toxicity and carcinogenicity at high levels of intake (Vandghanooni and Forouharmehr, 2013). As such, there is a focus on the potential use of natural antioxidants (Pokorný, 2007).

1.4.4 Natural sources of antioxidants

Other important antioxidants are obtained exogenously from the diet. These include the essential nutrients vitamins E (tocopherols and tocotrienols), and C (ascorbic acid) as well as a range of food components that are strictly considered as non-essential, such as carotenoids and polyphenols, but which nonetheless may have significant health-promoting bioactivities (Al Salhen, 2014). In numerous *in vitro* studies, phenolic compounds can exhibit higher antioxidant activity compared to vitamins and carotenoids. However, this does not necessarily make them more beneficial in vivo, and while many consider natural food antioxidants to be safe and beneficial, there still remains the possibility that excessive intake could prove harmful (Ataa and Mohamed, 2017).

Competition occurs *in vivo* between oxidative and antioxidative processes that depends on many factors, including the polyunsaturated fatty acid composition of tissue lipids and the presence of various pro- and antioxidants. According to the oxidant-antioxidant balance hypothesis, to achieve optimum health, the damaging effects of ROS and lipid oxidation products must be counteracted by an adequate supply of antioxidants acting in combination

with cellular repair systems (Lobo *et al*., 2010). If antioxidant protection is insufficient, an imbalance develops, referred to as oxidative stress, with the resulting accumulation of tissue damage and increased susceptibility to disease.

1.5 Anticancer activity

The phytochemicals in blueberries such as polyphenols and specific anthocyanins are proposed be crucial to their apparent beneficial health effect through their biological activity as antioxidants and as potential anticancer agents. As discussed above, the antioxidant activity of these compounds may help to reduce the extent of cellular oxidative damage via ROS scavenging (Lima *et al*., 2006). Additionally, these compounds appear to have the capacity to inhibit cell proliferation in different cancer cell types arresting the cell cycle in a specific phase and inducing apoptosis (Seeram *et al*., 2005; Hafeez *et al*., 2008). A combination of epidemiological and experimental studies support a link between inflammation and cancer that has been confirmed by the efficacy of anti-inflammatory therapies in cancer prevention and treatment (Reuter *et al*., 2010). An emergent body of evidence has suggested that numerous phytochemicals have anti-inflammatory effects on various chronic inflammatory diseases (Yatoo *et al*., 2018). Phytochemicals can reduce inflammation thru the inhibition of prostaglandin production and nuclear factor-κB activity, enzyme inhibition, and the increase of cytokine production (Yao *et al*., 2015).

1.6 Phytochemicals

Phytochemical is a general term meaning plant (phyto) chemical. It refers to a wide range of compounds that occur naturally in plants (Guaadaoui *et al*., 2014). Based on their chemical structures and characteristics, phytochemicals can be classified into six primary categories (Figure 1.2), including carbohydrates, lipids, phenolics, terpenoids and alkaloids (Huang *et al*., 2016). Recently the term phytochemical has been used increasingly to refer to plant chemicals that are not categorised as ‘essential nutrients’ (Campos-Vega *et al*., 2013).

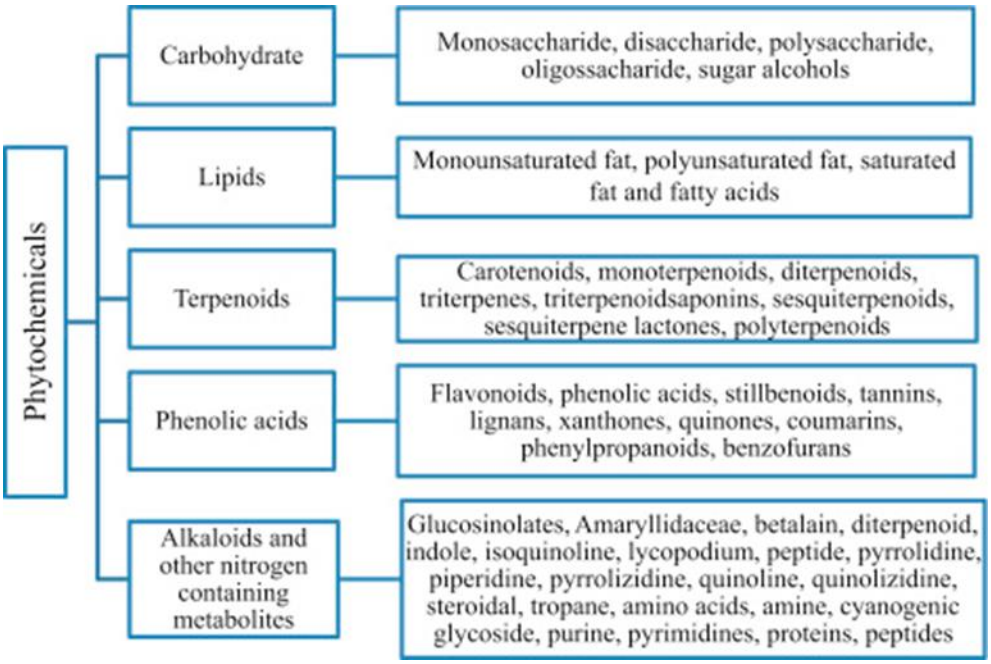


Figure 1.2 Classification of phytochemicals (From Huang *et al*., 2016)

Phenolic phytochemicals are plant secondary metabolites; metabolites that are considered to have no or minimal role in the main processes of photosynthesis, respiration and growth. They can accumulate to comparatively high concentrations in some plants, and as dietary components, these phytochemicals can be treated as non-nutrient xenobiotics and metabolised to be eliminated competently (Crozier *et al*., 2009).

1.7 Phenolic compounds

Phenolic compounds can be identified by having at least one aromatic ring with one or more hydroxyl groups attached. Phenolics are found widely across the plant kingdom, with many occur in plant-based foods. To date, more than 8000 plant-derived phenolic compounds have been characterised (Strack, 1997).

Phenolic compounds range from simple single aromatic-ring compounds, with low molecular weight, to highly complex compounds and derived polyphenols. Phenolic compounds are classified by the arrangement and number of their carbon atoms, and are commonly found in plants bound to organic acids or sugars. Phenolic compounds arising naturally in plant tissue are classified into polyphenols and simple phenols (Figure 1.3).

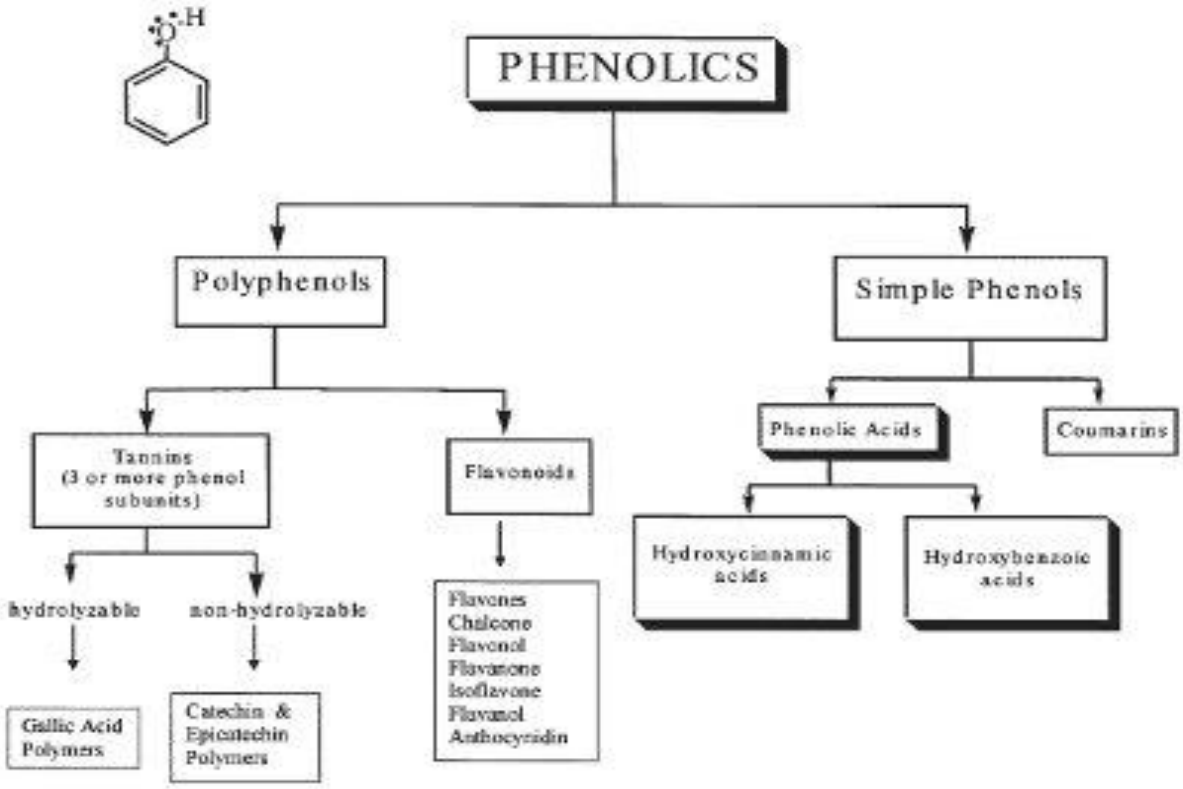


Figure 1.3 Basic structural skeleton of phenolic and polyphenolic compounds. (From Crozier *et al*., 2009)

1.7.1 Polyphenols

Dietary polyphenols are secondary metabolites usually found in food such as fruits, vegetables, tea, cocoa and other products derived from plants (Williamson and Clifford, 2010). The consumption of polyphenol-rich food can reduce the risk of degenerative diseases (Hooper and Frazier, 2012), and this association has been supported by many intervention studies (Williamson and Manach, 2005). Most of these activities can be attributed to their essential antioxidant property as from the in vitro antioxidant activity of many phenolic compounds and the role of oxidation in several disease pathways; evidence suggests that antioxidant activity explains the link between dietary polyphenols and disease prevention (Bennett *et al*., 2015).

1.7.2 Flavonoids

Flavonoids are polyphenolic compounds containing of the core structure of 15 carbons, arranged as two aromatic rings linked by a three-carbon bridge, thus C6–C3–C6 Figure 1.4. Flavanoids are the most common phenolic compounds found in plants (Crozier et al., 2009).

Flavonoids are found primarily in the skin of fruits and the epidermis of leaves. The main subclasses of dietary flavonoids (Figure 1.4) are isoflavones in which the B ring is linked in position 3 of the C ring; neoflavonoids, where we can find the B ring, is linked in position 4. While the subclass that has the B ring linked in position 2 represents the (flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins and chalcones) (Figure 1.4).

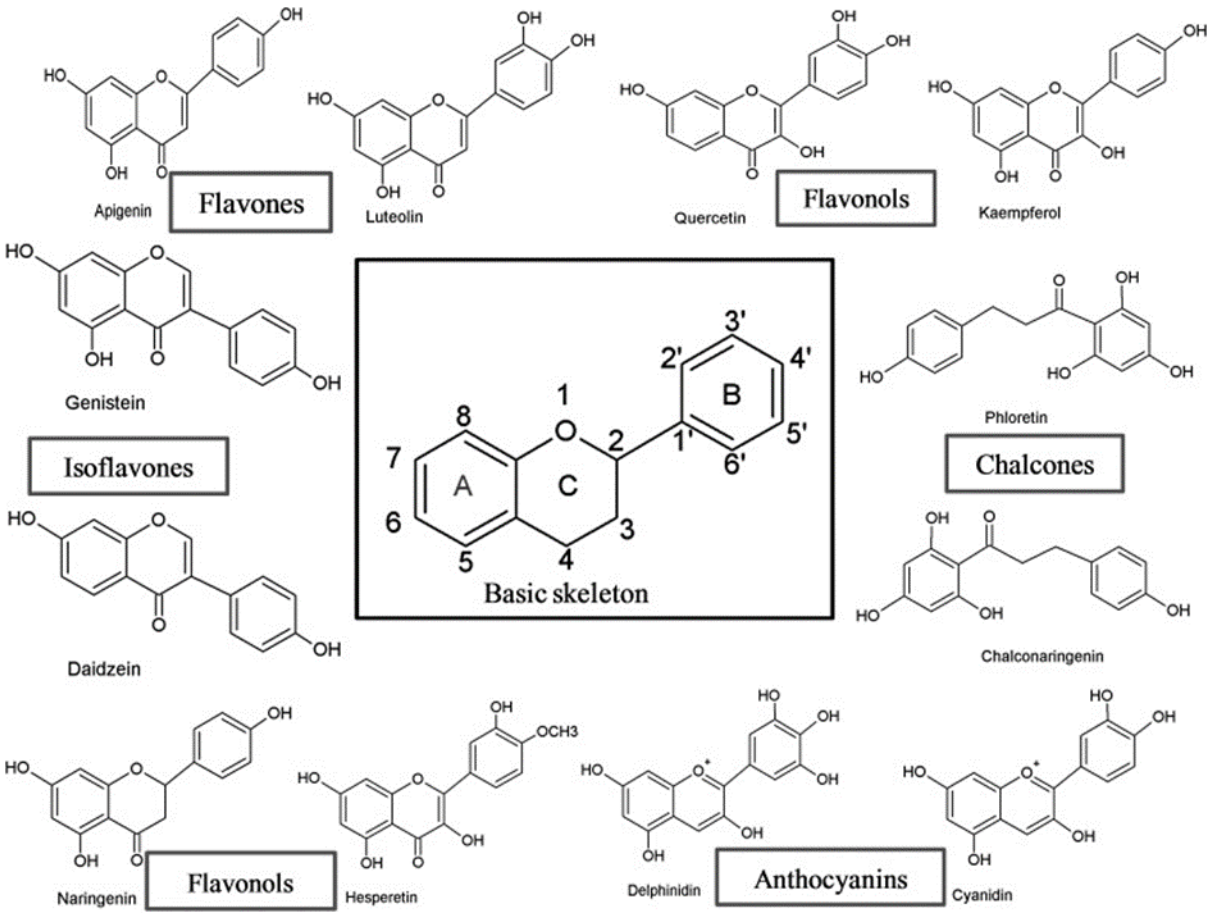
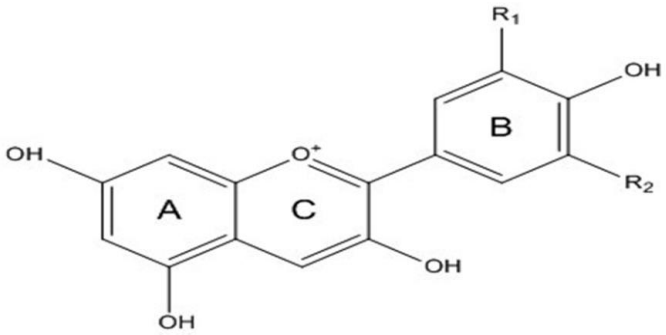


Figure 1.4 The basic flavonoid skeleton structure and their classes. (From Panche *et al*., 2016).

1.7.3 Anthocyanins

Anthocyanins in plant tissues are phenolic secondary metabolites, and they fall under the flavonoid group. The core components of anthocyanins are the anthocyanidins, based on the flavylium cation (Figure 1.5), which consist of a phenolic ring attached to a pyran connected at the 2 positions to an additional phenolic ring. This structure can be bonded to a sugar moiety to form an anthocyanin (Barnes *et al*., 2009). Anthocyanins are responsible for, or contribute

to, the red, blue and purple colours in plant organs like fruits, flowers and leaves (Strack and Wray, 1989). Members of the anthocyanin family vary in their structure according to the number and position of hydroxyl groups, the degree of methylation of these hydroxyl groups, the number and nature of the attached sugar moieties beside the position of their attachment in addition to the nature and number of aliphatic or aromatic acids attached to the sugars (Monagas and Bartolomé, 2009).

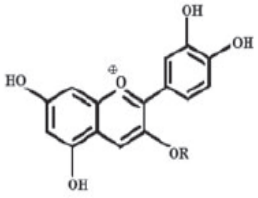
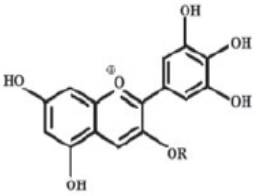
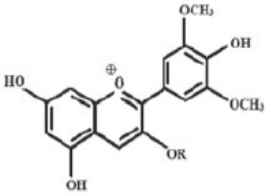
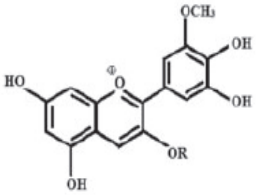
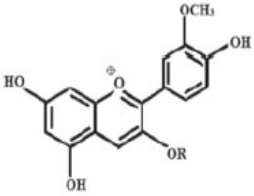


Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1.5 the chemical structure of anthocyanidins

While there are approximately 17 anthocyanins found in nature, only six were commonly distributed: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Table 1)

Table 1. Most common Anthocyanins, their structures, sugar moieties, and colour. From Routray and Orsat, 2011.

Anthocyanin	Basic structure	R (Sugar moiety)	Colour
Cyanidin		Galactose, Glucose, Arabinose	Orange-red
Delphinidin		Galactose, Glucose, Arabinose	Blue-red
Malvidin		Galactose, Glucose, Arabinose	Blue-red
Petunidin		Galactose, Glucose, Arabinose	Blue-red
Peonidin		Galactose, Glucose, Arabinose	Orange-red

The stability and water solubility of anthocyanidins increase by glycosylation; also the acylation of the sugar with cinnamic acid or an aliphatic acid can further enhance anthocyanins stability (Mazza *et al*., 2004). In the presence of oxygen, enzymes, light and high-temperature

anthocyanins can be very reactive compounds; though, pH has the most remarkable effect on anthocyanins stability (Mazza *et al*., 2004). In an aqueous solution, anthocyanins will exist as four molecular interconvertible forms (Figure 1.6) (McGhie and Walton, 2007); the most stable structure is known as the red flavylium cation, and it prevails in the stomach when the pH is < 2. In the small intestine, as the pH increases, the rapid loss of a proton produces the blue quinonoidal structure. Slower hydration of the flavylium cation occurs at the same time, generating the colourless hemiketal form, which then undergoes the tautomerisation phase through the opening of the C- ring same to produce the chalcone form. (McGhie and Walton, 2007).

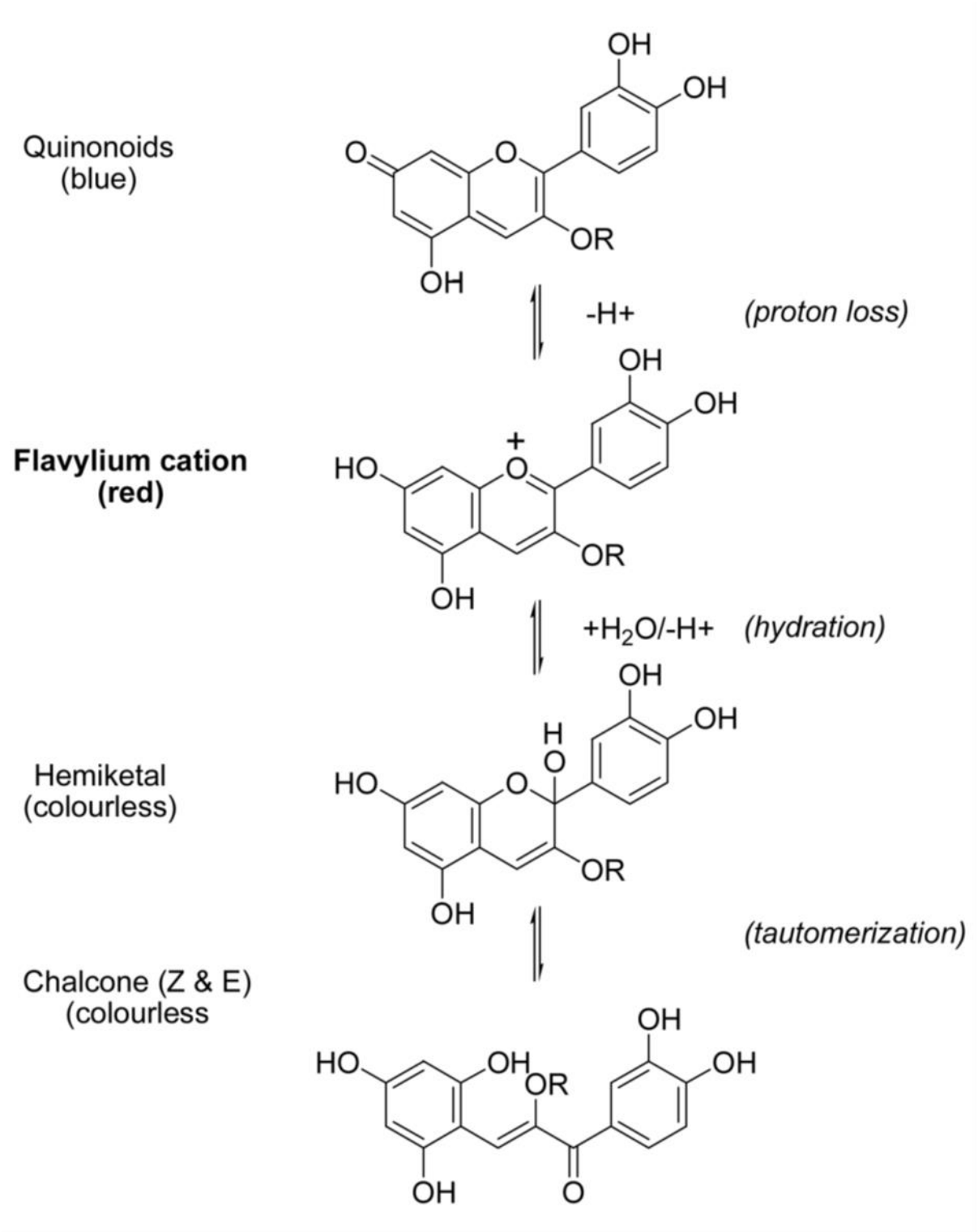


Figure 1.6 The various anthocyanin molecular structures that are known to be generated under different pH conditions. From McGhie TK and Walton MC, (2007)

Anthocyanidins, the aglycone form of anthocyanins, are hydrophobic and can be absorbed through passive diffusion. In contrast, anthocyanins are water-soluble molecules, and this feature limits their absorption by passive diffusion (Hollman, 2004). Thus, anthocyanins either need to be hydrolysed to the aglycone form in the small intestine through the action of β-glucuronidase, β-glucosidase and α-rhamnosidase to be absorbed or a particular active transport mechanism is required to allow the transport of the intact anthocyanins across enterocyte membranes, for instance, via the sodium-glucose cotransporter SGLT1 (Kay, 2006).

Some studies have suggested that anthocyanins can be absorbed in the stomach through a possible mechanism involving the organic anion membrane carrier, bilitranslocase which is located in the stomach (Passamonti, 2005). For example, some animal model studies have indicated that anthocyanins can be rapidly absorbed, appearing in the bloodstream within a few minutes (6 to 20 min) of consumption, reaching maximum blood levels after 15 to 60 min (McGhie and Walton, 2007). However, other studies suggest that anthocyanins are absorbed in the small intestine and that the extent of absorption is limited (He *et al*., 2009).

Further studies indicate that the anthocyanins not absorbed in the upper part of the gastrointestinal tract can be extensively modified by gut microflora (McGhie and Walton, 2007). There is a possibility that a significant amounts of the carbinol pseudo base might pass into the large intestine where they are metabolised to poorly characterised products by the gut microflora via glycosylation and ring fission of the C-ring to produce phenolic acids and aldehydes, such as Gallic acid, 3-O-methylgallic acid and 2,4,6-trihydroxybenzaldehyde (Keppler and Humpf, 2005). Hence not only the anthocyanins but also metabolites derived from them may be found in urine and serum (Garcia-Alonso *et al*., 2009). For example, in a study where human participants were given a large oral dose of cyanidin-3-glycoside, only 32.7% of the absorbed material was detected as intact cyanidin-3-glycoside in serum while 67.3% was identified as metabolites (Kay *et al*., 2005). Moreover, the percentage of the total anthocyanins in both native forms and metabolites absorbed and excreted in the urine is low compared to the ingested dose which indicates the bioavailability of anthocyanins poor (Pojer *et al*., 2013).

There could be numerous aspects responsible the apparent low bioavailability of anthocyanin. For instance, at normal pH values, the carbinol and chalcone forms of anthocyanins can escape detection or can be chemically bound to other components. Also, low concentrations of the metabolites may be difficult to detect (Pojer *et al*., 2013), and that may be why the biological

activities of the decomposition products and/or metabolites have been less extensively investigated than those of the parent compounds.

The human epithelial cell line Caco-2 has remained extensively used to model the intestinal epithelial barrier and absorption characteristics. The Caco-2 cell line is derived initially from colon carcinoma. One of its most valuable properties is its aptitude to instinctively differentiate into a monolayer of cells with many properties distinctive of absorptive enterocytes thru the brush border layer, for instance, originating in the small intestine (Verhoeckx *et al*., 2015).

1.8. Hypotheses

1. The apparent health-promoting effects of blueberries are due to a combination of the polyphenols they contain rather than a single component

2. Some metabolites derived from the action of the colonic microbiota on blueberry polyphenols have biological effects as important as, or more important than, the parent compounds

3. As well as potentially reducing the risk of colorectal cancer, blueberry polyphenols could find use as adjunctive therapeutic agents for the treatment of colorectal cancer through chemotherapy

1.9. Conclusion

The potential bioactive properties of phytochemicals, and in particular the anthocyanins, found in abundance in berries provide a basis for scientific interest in their potential as dietary agents that may help prevent against the development of chronic disease or even as therapeutic agents to help in the treatment of chronic diseases. The aim of the research detailed in this thesis was to investigate the biological effects of blueberry extract consisting of a complex mix of phytochemicals present in the fruit, as well as a selected purified candidate bioactive component of blueberries (malvidin-3-galactoside) and common metabolites of this

anthocyanin (malvidin and 2,4,6-trihydroxybenzaldehyde) in an *in-vitro* cell model of colon cancer. To achieve this aim, the following objectives were considered:

1. Preparation and characterisation of blueberry extracts.
2. Analysis of the radical scavenging and antioxidant activity of blueberry extract.
3. Investigation of the effects of blueberry extract, a major blueberry anthocyanin, and its metabolites on the human colon adenocarcinoma Caco-2 cell line viability.
4. Investigation of the effects of blueberry extract, a major blueberry anthocyanin, and its metabolites in the presence and absence of induced oxidative stress on the Caco-2 cell line.
5. Investigation of the effects of blueberry extract, a major blueberry anthocyanin, and its metabolites on cell death pathways in the Caco-2 cell line.
6. Evaluation of the synergistic effects of blueberry extract, a major blueberry anthocyanin, and its metabolites in combination with a cytotoxic chemotherapy agent (5-fluoruracil) in the Caco-2 cancer cell line.

CHAPTER TWO

CHARACTERISATION, SCAVENGING, AND ANTIOXIDANT ACTIVITY OF A BLUEBERRY EXTRACT

Free radicals form in all living organisms as a result of redox reactions; they are molecules with unpaired electrons that are highly reactive in normal metabolic pathways. The production of free radicals increases under certain circumstances such as wounding, pathogen attack, and environmental stress and can cause damage to the living organisms. Free radical-induced damage development of degenerative diseases (Wolfe *et al.*, 2008).

Antioxidant compounds obtained from our diet can help to neutralise free radicals, reducing cellular and tissue damage and, thereby, potentially helping to reduce the risk of chronic disease development (Briviba and Sies, 1994). Lipid oxidation can also be delayed or prevented by antioxidants through delaying the initiation or propagation of oxidising chain reactions (Bakowska-Barczak and Kolodziejczyk, 2011).

The increasing focus on the impact on health promoting antioxidative compounds in fruit and vegetables was a motivation for numerous studies to investigate the bioactive components in these fruits and vegetables. Berries for instant comprising anthocyanins, phenolics acids, flavonols and tannins, which can possess many health-promoting properties, such as, anticancer, antidiabetic, anti-inflammatory, antimicrobial, and antioxidant. A wide variation in antioxidant capacity, anthocyanin content, phenolic contents, sugar and organic acid values among various blueberry genotypes and that these values vary significantly between different growing seasons (YI et al., 2005; Wang et al., 2012; Baby et al., 2018). Castrejón and colleges publicised that ripening processes, maturation besides climate conditions can have additional distinct effect on flavonoid biosynthesis and phenolic composition in comparison to genetic variances of highbush blueberry varieties (Castrejón et al,. 2008). Consumption of sufficient berries, such as blueberries, in diets may help decrease the risk of many kinds of chronic disease (Wang and Ballington, 2007). The complex anthocyanin composition of blueberries, containing over 25 different anthocyanins, is distinct from all other berries (Wu and Prior

2005). Thus, the consumption of blueberries is proposed to lessen the risk associated with developing certain diseases, including cancer, heart disease, and stroke (Neto *et al*., 2008). Alternatively, blueberry antioxidants could be used to stabilise food products to increase their shelf life (Gulcin, 2012).

In view of current interest in the inverse association between consumption of berries and degenerative diseases, there is a clear need to identify the components responsible and quantify them. Blueberries represent a promising model food to evaluate in this context due to their rich and complex anthocyanin content (Cho *et al*., 2005). The work described here focused on analysing blueberries for total phenolic content, total flavonoid content, and evaluation of the antioxidant activity. As many factors affect the antioxidant activity, particularly in biological systems, different assays are required to gain a clear overview. I used assays based on the ability of antioxidant compounds to scavenge free radicals, chelate metal ions, and their ability to act as a reducing agent.

Initially, I aimed to prepare blueberry extracts from the whole fruit sample collected from different cultivars for subsequent detailed analysis of the biological properties of blueberries in vitro and in vivo. However, this proved problematic due to the nature of the physical properties of the fruit. Consequently, it was decided to investigate commercial blueberry extracts for the subsequent cell-based experiments.

The hypothesis of this chapter is that the commercial blueberry extract rich in phenolics and flavonoids besides exhibiting antioxidant properties and potent antioxidant activity, in addition malvidin-3-galactoside is the most abundant form of malvidin-glycoside in the extract.

The objectives of the studies described in this chapter were:

- To determine the total phenolic and flavonoid content in blueberry extracts.
- To determine the finding of malvidin-3-glucoside and malvidin-3-galacotside in blueberry extract using the HPLC analysis.
- To determine the scavenging activity of the blueberry extract via,

    1. Analysis of the concentration-dependent reducing power of Commercial blueberry extracts.

2. Analysis of the concentration-dependent capacity of the Commercial blueberry extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.
3. Analysis of the concentration-dependent capacity of blueberry extracts to scavenge hydrogen peroxide.
4. Analysis of the concentration-dependent capacity of blueberry extracts to chelate metal ions.

- To determine the antioxidant activity of blueberry extracts using two methods:

1. The measurement of peroxide formation using the ferric thiocyanate method (FTC).
2. The measurements of oxidation using the thiobarbituric acid method (TBA).

2.1 Materials and Methods

2.1.1 Materials

Commercial blueberry extract Life Extension Blueberry Extract Capsules (Wild blueberry extract of whole fruit, maltodextrin, vegetable cellulose capsule, rice concentrate and silica). It was obtained from Amazon (Manufactured for Quality supplement and Vitamins, Inc. Ft. Lauderdale, FL 33309. LifeExtension.com). Folin-Ciocalteu reagent, catechin, gallic acid, linoleic acid, thiobarbituric acid (TBA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ferrozine ascorbate, potassium ferric cyanide, phosphate buffer, ferric chloride, DPPH, hydrogen peroxide (H_2O_2), ferrous chloride and ethylenediamine tetra-acetic acid (EDTA) were all obtained from Sigma-Aldrich. All reagents and solvents used for experiments were of analytical grade.

A UVIK 860 spectrophotometer (Zurich,Switzerland) was used in all experiments described in this chapter to measure the absorption and the specific wavelength required for the different methods.

2.1.2 Methods

2.1.2.1 Characterisation of blueberry antioxidant content and properties

2.1.2.1.1 Total phenolic content (TPC)

A sample of the commercial blueberry extract (0.4 g) was extracted with 20 ml methanol, then filtered through a 13 mm syringe filter with a 0.2 µm PTFE membrane. The Folin Ciocalteu assay, as described by Kim *et al*., (2003), was used to determine the TPC content of the blueberry extract. Briefly, the methanol blueberry extract (0.2 ml) was mixed with 1ml Folin Ciocalteu reagent and 10 ml of deionised water, and the mixture was incubated for 4 min at room temperature. The reaction mix was then neutralised with 1ml of 20% w/v sodium carbonate. The mixture was incubated in the dark at room temperature for two hours, after which the absorbance was measured at 765 nm. A calibration curve with a series of Gallic acid standards at various concentrations was prepared (0.0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 mg/ml). All absorbance values were corrected for background using a blank in which the extracted sample or Gallic acid standard was replaced with deionised water. All analyses were performed in triplicate, and results were presented as mg of Gallic acid equivalents/mg of sample (dry weight). The average value of absorbance was used to plot the calibration curve to determine the level of phenolics in the extracts.

2.1.2.1.2 Total flavonoid content (TFC)

A sample of the blueberry extract (0.5 g) was extracted with 100 ml methanol at room temperature. The extract was filtered through a 13 mm syringe filter with a 0.2 µm PTFE membrane. The TFC of the extract was determined by using a colourimetric method, according to the method of Park *et al*. (2008). Briefly, an aliquot (0.3 ml) of the methanol solubilised extract was mixed with 3.4 ml of 30% v/v methanol, 0.15 ml of 0.3 M $AlCl_3$, and 0.15 ml of

0.5 M $NaNO_2$ in a (10 ml) test tube. Then 1 ml of 1 M NaOH was added. The absorption of the mixture was measured at 506 nm. A calibration curve was prepared using catechin as the standard (0.0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 mg/ml). TFC was calculated as mg of catechin equivalents per mg of sample (dry weight).

2.1.2.2 HPLC analysis of malvidin-3-glucoside and malvidin-3-galacotside in blueberry extract

Malvidin-3-glucoside (oenin) and malvidin-3-galactoside (primulin) were purchased from Sigma-Aldrich. An Accucore RP-MS 150 x 3mm 2.6µm HPLC column was purchased from Thermo Scientific. HPLC was performed with the column maintained at 30°C in a column oven and with a constant flow rate of 0.5ml/min. Separation was achieved using a gradient system with a combination of two mobile phases: phase A was 0.1% trifluoroacetic acid in water; phase B was 100% methanol. The system was programmed to start each run at 100%. The percentage of phase B was changed in a linear fashion as follows: 0-7% during minutes 1-5, from 7-20% during minutes 5-15, 20-25% during minutes 15-20, 25-30% during minutes 20-25, 30-60% during minutes 25-45, 60-0% during minutes 47-47 and finally maintained at 0% during minutes 47-60. The eluate was monitored at 520 and 280nm (HPLC analysis was completed by Anuska Mann).

The blueberry extract was prepared in two ways for analysis. It was either dissolved in 100% DMSO or 70% methanol to a final concentration of 10mg/ml. The malvidin-3-glucoside and malvidin-3-galacotside content of the extract was estimated by interpolation based on linear regression analysis of the peak areas for serial dilutions of the standards.

2.1.2.3 Scavenging activities of blueberry

2.1.2.3.1 Preparation of extract

A sample of the commercial blueberry extract (100 mg) was dissolved in 10 ml methanol at room temperature and then filtered through a 13 mm syringe filter with a 0.2 nm PTFE membrane.

2.1.2.3.2 Reducing power

The method of Oyaiz (1986) was used to determine the reducing power of the extract. This method is based on determining the ability of the test material to reduce ferricyanides to the ferrous form. Briefly, 2.5 ml of extract was diluted in methanol to various concentrations (0.0125, 0.025, 0.075, 0.100, 0.300, 0.500 mg/ml in) and mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% w/v potassium ferricyanide. Then the mixture was incubated at 50°C for 20 min in a water bath. Next, 2.5 ml of 10% w/v trichloroacetic acid was added, and the mixture was centrifuged at 200 x g for 10 min. Finally, 5 ml of the supernatant was mixed with 5ml of deionised water and 1 ml of 0.1% ferric chloride. The absorbance was read at 700 nm and corrected for background using a blank prepared in the same way except that the sample was replaced with deionised water. BHT was used as the positive control.

2.1.2.3.3 Scavenging effect on 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) radicals

The radical scavenging activity of the commercial blueberry extract was determined according to the method of Liu *et al*. (2010). A volume of 1 ml of extract diluted in methanol to various concentrations (0,1, 6, 8, 10, 20, 30, 40, or 50 mg/ml) was mixed with 1 ml of 1 mM DPPH in methanol. The reagents were carefully mixed and left to stand in the dark at room temperature for 60 min. By using a spectrophotometer, the absorbance was measured at 517 nm. BHA

(0.1mg/ml in deionised water) was used as a positive control. The scavenging activity, or % inhibition, was measured using the following formula:

Radical scavenging activity (%) = $[(AC_{DPPH} - AC_{DPPH}) / AC_{DPPH}] \times 100$

Where: AC_{DPPH} represents the absorbance of the control, which contains DPPH, and AS_{DPPH} refers to the absorbance of blueberry extract in the presence of DPPH.

2.1.2.3.4 Scavenging effect on hydrogen peroxide

The ability of blueberry to scavenge hydrogen peroxide was determined by the method of Ruch *et al*., (1989). Briefly, 0.2 ml of the extract diluted in methanol to various concentrations (0,1, 6, 8, 10, 20, 30, 40, 50 mg/ml) was mixed with 2.8 ml of 4 mM hydrogen peroxide solution. The mixture then was incubated for 10 min at room temperature, and the absorption was measured at 532 nm. The absorbance of the positive control BHA (at a concentration of 0.1 mg/ml) was also read. The scavenging activity or % inhibition was calculated using the following formula:

H_2O_2 scavenging activity (%) = $[(AC_{H_2O_2} - AS_{H_2O_2}) / AC_{H_2O_2}] \times 100$

Where AC_{H2O2} is the absorbance of the control with H_2O_2 and AS_{H2O2} is the absorbance of the testing sample in the presence of H_2O_2.

2.1.2.3.5 Metal chelating activity

The metal chelating activity of the commercial blueberry extract was determined using a modified version of the method of Dinis *et al*. (1994). This assay measures the ability of the test material to chelate ferrous ions, thereby inhibiting the subsequent formation of a ferrous-ferrozine complex. Deionised water (1.6 ml) was mixed with 0.5 ml of blueberry extract

diluted in methanol to various concentrations (0, 1, 6, 8, 10, 20, 30, 40, 50 mg/ml). Then 0.05 ml of 2mM ferrous chloride was added to the mixture and vortexed. After 30 seconds, 0.1 ml of 5mM ferrozine was added, the solution was then incubated for 10 min at room temperature. A (0.01% w/v) EDTA solution was used as a positive control. The absorbance of the sample/positive control mixtures was measured at 562 nm. The ferrous ion chelating activity was calculated according to the following formula:

Chelating rate (%) = $[(A_0 – A_1) / A_0] \times 100$

Where A_0 is the absorbance of the control that contains $FeCl_2$ and ferrozine complex and

A_1 is the treated sample in the presence of $FeCl_2$ and ferrozine complex.

2.1.2.4 Antioxidant activity determined by measuring the oxidation of linoleic acid

The inhibition of lipid peroxidation by the commercial blueberry extract was measured in an oxidising linoleic acid model system (Osawa and Namiki, 1985). The formation of peroxides as primary products from lipid oxidation was measured using the FTC method. In contrast, the production of carbonyl compounds as secondary lipid oxidation products was estimated by using the TBARS method (Ohkawa *et al*., 1979).

2.1.2.4.1 Preparation of reaction mixture for the lipid oxidation assays

Briefly, 100 mg of the commercial blueberry extract was dissolved in 2.5ml absolute methanol, 2.5 ml of 50 mM phosphate buffer (pH 7.0), and 0.13 ml linoleic acid, and the final volume was adjusted to 6.25 ml with distilled water in glass tubes. The samples were then homogenised using a sonicator (Labsonic M, B. Braun Biotech International, Germany). The tubes were sealed tightly with silicone rubber caps and placed in an oven at 60°C to accelerate oxidation. Aliquots for FTC and TBARS analysis were taken from these tubes daily to measure the antioxidant activity over 7 days. All analyses were performed in triplicate. BHT was used as a positive control and distilled water as negative control Sample of both were prepared in the same manner.

2.1.2.4.2 Measurement of peroxide formation using the ferric thiocyanate method (FTC)

The FTC method is a valuable method of evaluating antioxidant activity in the early stages of lipid oxidation using mild conditions (Antolovich *et al*., 2002). The peroxides produced from linoleic acid oxidation oxidise ferrous iron to ferric iron that reacts with thiocyanate ions to form a red complex. The absorbance of the red complex, which is determined using a spectrophotometer at 500 nm, quantifies the degree of linoleic acid oxidation (Nugraheni *et al*., 2011). Once every 24 hours, a 0.1ml aliquot was drawn from each of the reaction mixtures and placed in a test tube that contained 4.7 ml of 75% v/v ethanol, 0.1 ml of 30% w/v ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride solution in 3.5% hydrochloric acid. Three minutes after the addition of ferrous chloride to the reaction mixture, the concentration of thiocyanate was measured by reading the absorbance at 500 nm on a spectrophotometer (Uvikon 860, Kontron instrument, UK) (Rezaeizadeh *et al*., 2011).

The percentage (%) of lipid oxidation inhibition was calculated according to the following formation:

Lipid oxidation inhibition (%) = $[A_{control} - A_{sample} / A_{control}] \times 100$

Where $A_{control}$= absorbance of negative control, A_{sample}= absorbance of the sample.

2.1.2.4.3 Measurements of the oxidation using the thiobarbituric acid (TBA) method

This is a widely used method in which the reaction of malondialdehyde and thiobarbituric acid (TBA) forms a red complex pigment that can be detected spectrophotometrically at 532 nm (Antolovich *et al*., 2002). The TBA test was conducted according to the method of Yoshikawa *et al*. (1993) with some modifications. At 24 hour intervals, 0.05 ml aliquots were drawn from each of the reaction mixtures and added to test tubes containing 0.8 ml of distilled water, 0.2 ml of 8.1% (w/v) sodium dodecyl sulphate, 1.5 ml of 20% (w/v) acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) (TBA solution in water. The mixtures were placed in a water bath at 100°C for 60 minutes. After cooling, the mixtures were centrifuged at 4300 x g for 10 minutes. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Uvikon 860, Kontron instrument, UK). The antioxidant activity of the sample was determined based on the absorbance on the final day of the FTC method. The percentage inhibition (%) of lipid oxidation was calculated according to the following formula:

$$\% \text{ inhibition} = [A_{control} - A_{sample} / A_{control}] \times 100$$

Where $A_{control}$= absorbance of negative control, A_{sample}= absorbance of sample.

2.1.3 Statistical Analysis

All assays were conducted with three replicates. The data were analysed and presented using Graph pad prism. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test where appropriate. Statistical significance was assumed at $P<0.05$. Data are presented as means ± standard deviation.

2.2 Results and Discussion

2.2.1 Total phenolic content of the blueberry extract

The standard curve produced using the Folin Ciocalteu method for serial dilutions of Gallic acid, as shown in Fig 2.1, was used to estimate the total phenolic content of the commercial blueberry extract. Were the total phenols were expressed as mg/g Gallic acid equivalent using the standard curve equation.

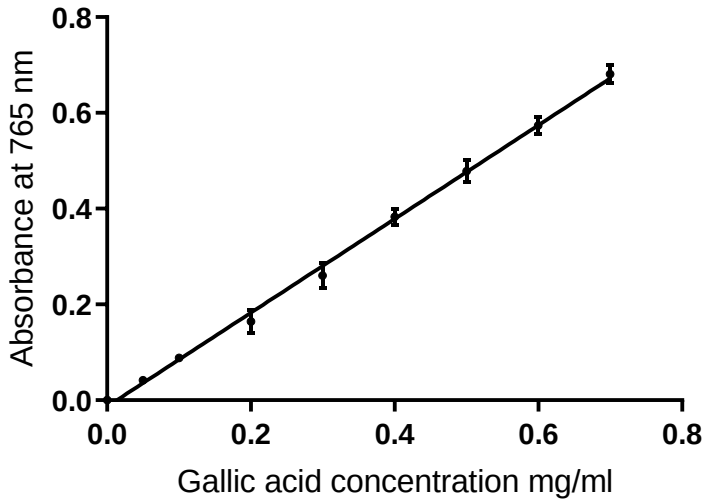


Figure 2.1. Gallic acid Standard curve. The standard curve represents as mean ± SD of Gallic acid (0-0.7mg/ml). The standard curve equation Y = 0.9791*X - 0.01328 was used to determine the total phenolic content, were Y = Absorbance of your test sample and X = concentration from the calibration curve. To calculate total phenolic content the formula: C = c V/m was used, where C = total phenolic content mg GAE/g dry extract, c = concentration of Gallic acid obtained from calibration curve in mg/mL, V = volume of extract used, m = mass of extract used.

The quantitative determination of the total phenolic content was expressed in mg of Gallic acid corresponding to 0.4g dry weight of the blueberry extract. Phenolic compounds were found in a considerable quantity for (TPC) 7.64±0.14 (mg Gallic acid/g DW)

2.2.2 Total flavonoid content of the blueberry extract

The standard curve produced using serial dilutions of catechin, as depicted in Figure 2.2, was used to evaluate the total flavonoid content in the commercial blueberry extract. The total flavonoids were expressed as mg/g catechin using the standard curve equation.

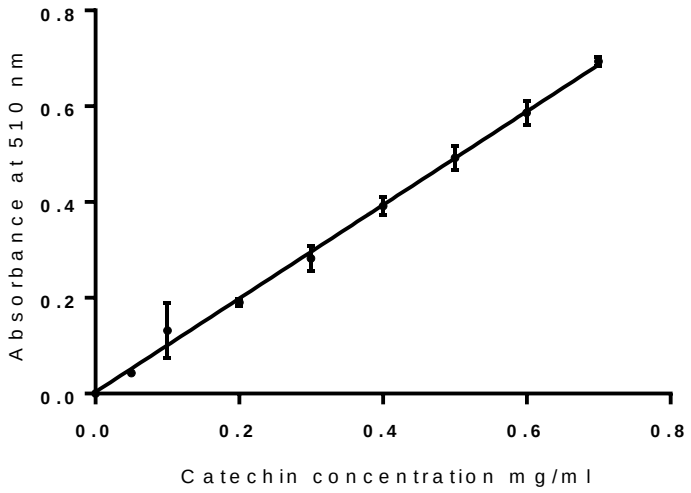


Figure 2.2. Catechin Standard curve. The standard curve represents as mean ± SD of catechin (0-0.7mg/ml). The standard curve equation Y = 0.9762*X + 0.003385 was used to determine the total flavonoids content were Y = Absorbance of your test sample and X = concentration from the calibration curve. To calculate total flavonoids content the formula: C = c V/m was used where, C = total flavonoids content mg catechin /g dry extract, c = concentration of catechin obtained from calibration curve in mg/mL, V = volume of extract used, m = mass of extract used.

The quantiteve determination of the total flavonoid content was expressed in mg of catechin corresponding to 0.5g dry weight of the blueberry extract. Flavonoids compounds were found in a considerable quantity for (TFC) 14.18±0.16 TFC (mg catechin /g DW)

2.2.3 HPLC analysis of malvidin-3-glucoside and malvidin-3-galacotside in blueberry extract

A typical chromatogram obtained using the blueberry extract is shown in figure 2.3 A. The analysis revealed the presence of multiple components with a notably large peak of an unidentified component eluting after 27.4 minutes. The extract was spiked with malvidin-3-galactoside or malvidin-3-glucoside (figures 2.3 B and C, respectively) to confirm the retention times of these two compounds. There were determined at 30.9 and 31.6 minutes for the galactoside and glucoside, respectively. The concentrations of malvidin-3-galactoside or malvidin-3-glucoside in the extract were determined by interpolation of the corresponding peak areas against serial dilutions of the pure compounds. The apparent concentrations of both anthocyanins were slightly (~30-40%) higher when 70% methanol was used as the solvent for the extract compared with DMSO (Table 2.3). Regardless of the solvent used, malvidin-3-galactoside was found to be present at 2.5 to 2.6-fold higher concentrations (on a weight basis) than mavidin-3-glucoside.

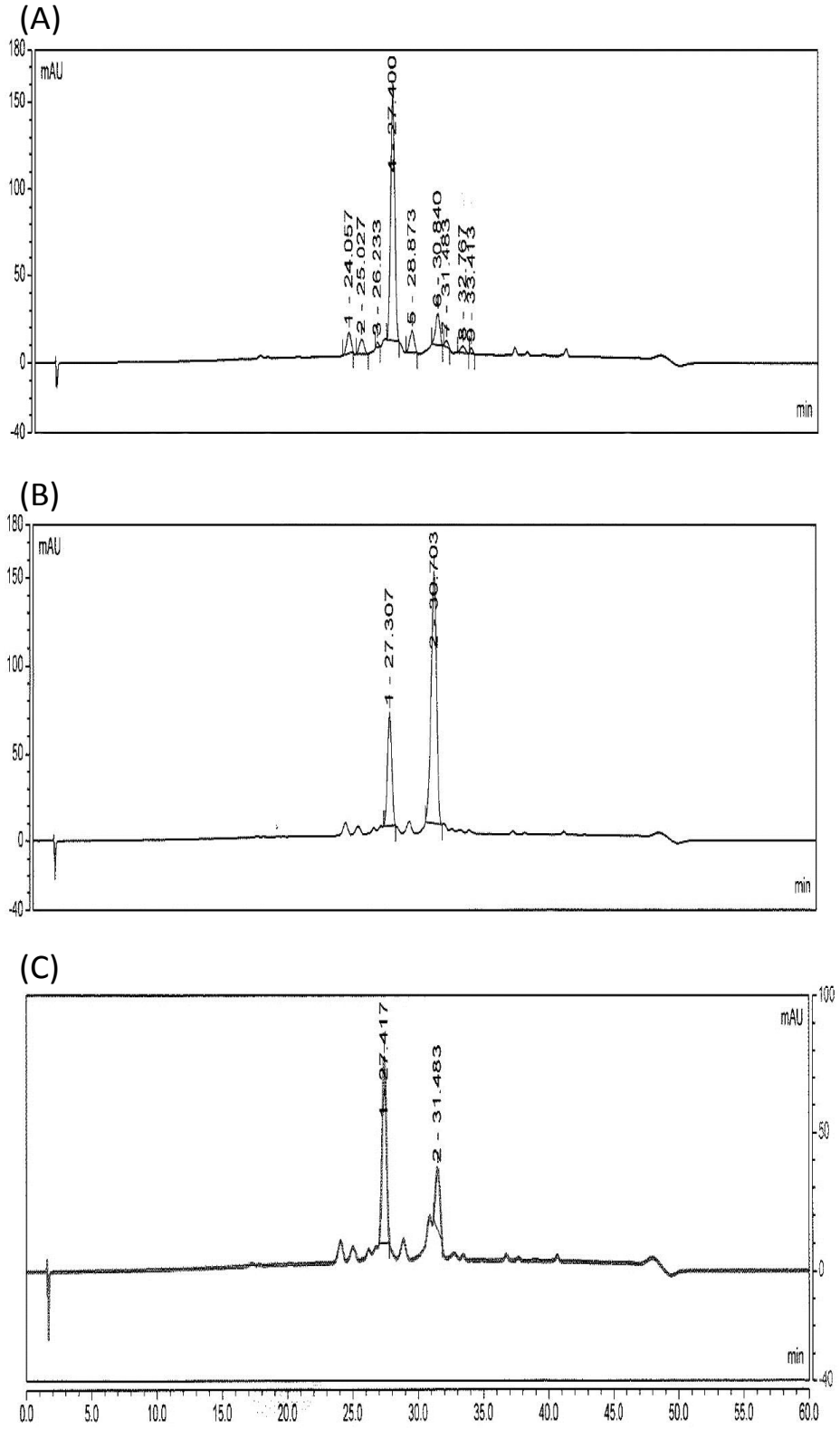


Figure 2.3. HPLC chromatograms of blueberry extract. (A) Blueberry extract alone, (B) blueberry extract spiked with malvidin-3-galacotside and (C) blueberry extract spiked with malvidin-3-glucoside.

Table 2.3 Malvidin-3-glucoside and malvidin-3-galacotside content of blueberry extract determined by HPLC analysis

Sample solvent	Concentration (mg/g total extract)	
	Malvidin-3-glucoside	**Malvidin-3-galactoside**
DMSO	0.48±0.02	1.25±0.02
70% methanol	0.67±0.13	1.67±0.21

2.2.4 Reducing power

Figure 2.4 shows the concentration-dependent reducing power of blueberry extract. It was found that the reducing power activity of the blueberry extract increased with the increasing concentrations and was similar to the reducing power of the BHA positive control.

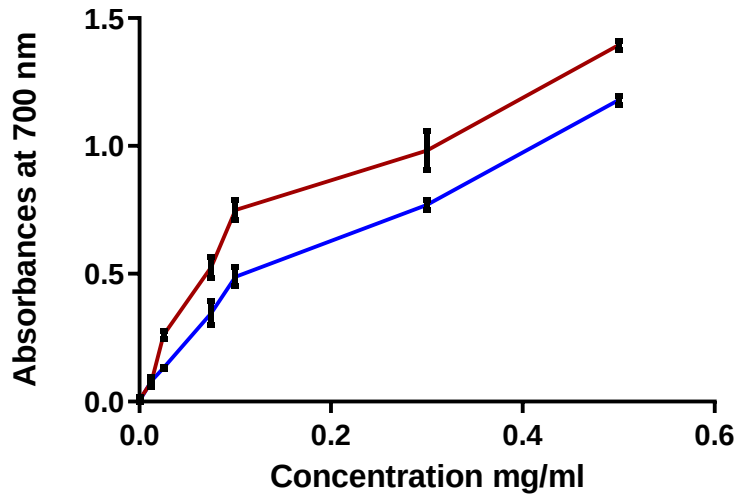


Figure 2.4. Concentration-dependent reducing power of blueberry extract and butylated hydroxyanisole. Reducing power was measured over an extract concentration range of (0.0125, 0.025, 0.075, 0.100, 0.300, 0.500 mg/ml). The activity was measured at 700 nm. The data for the blueberry extract is shown in blue, and the data for the BHA in red. Data are presented as mean±S.D (n=3).

2.2.5 DPPH radical-scavenging activity

The radical-scavenging activity of the blueberry extract was concentration-dependent, as shown in Figure 2.5.

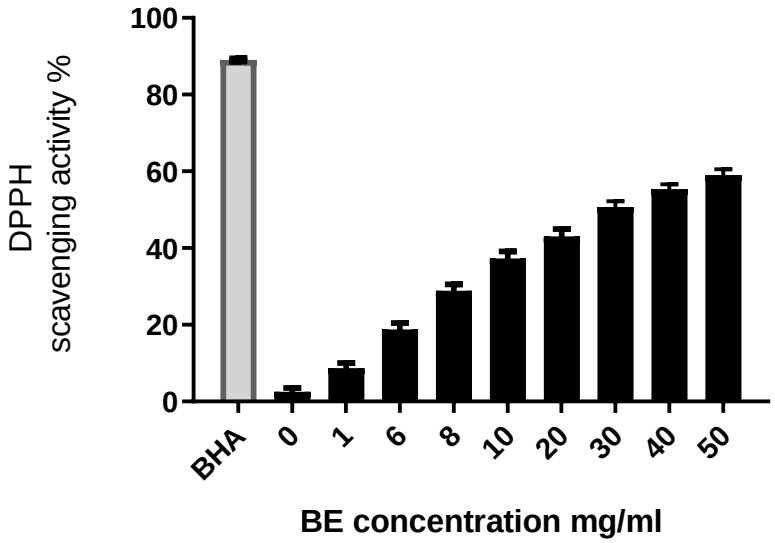


Figure 2.5. Concentration-dependent DPPH scavenging activity of the blueberry extract. The scavenging activity was measured over a concentration range of blueberry extract (BE) (0,1, 6, 8, 10, 20, 30, 40 and 50 mg/ml) using DPPH scavenging radical. The activity was determined by spectrophotometric analysis at 517 nm and was compared to butylated hydroxyanisole (BHA) (0.1 mg/ml) as a positive control. The data for the scavenging activity of the blueberry extract is presented in black. Data for the positive control (BHA) is shown in grey. Data points and error bars indicate the mean ± S.D. of triplicate analyses.

2.2.6 Hydrogen peroxide scavenging

Although H_2O_2 is not itself highly reactive, it can react with redox-active agents within biological systems, such as free iron, to generate hydroxyl radicals that can initiate lipid peroxidation chain reactions, leading to the accumulation of cellular damage. Thus, the capacity to remove H_2O_2 is critical throughout biological systems. Figure 2.6 shows the concentration-dependent H_2O_2 scavenging activity of the blueberry extract. The scavenging activity of the extract increased moderately up to a maximum of 30mg/ml but then decreased at concentrations higher than this. This suggests that the extract acted as an antioxidant in the context of hydrogen peroxide at the lower concentrations tested but that this antioxidant effect started to be lost at higher concentration.

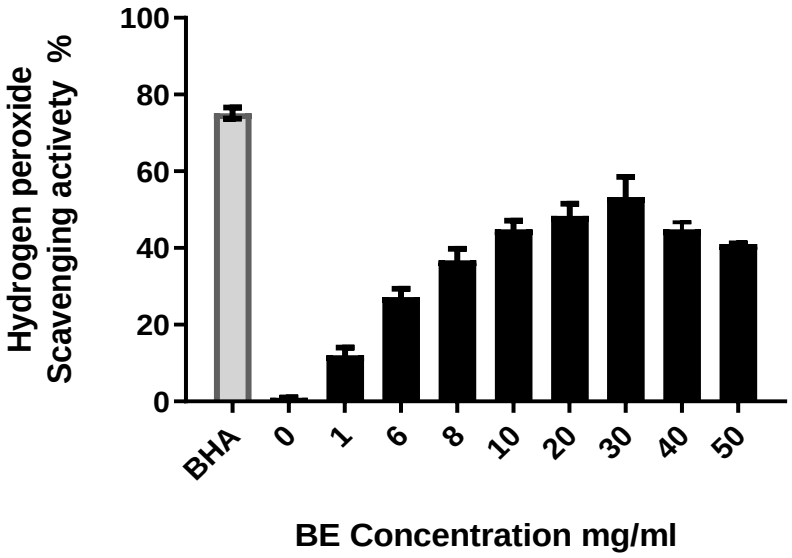


Figure 2.6. Concentration-dependent hydrogen peroxide scavenging activity of the blueberry extract. Scavenging activity was measured over a concentration range of blueberry extract (BE) (0,1, 6, 8, 10, 20, 30, 40 and 50mg/ml). The activity was determined by spectrophotometric analysis at 532 nm and compared to BHA (0.1 mg/ml) as a positive control. Data for the blueberry extract are depicted in black and for the BHA positive control in grey. Data points and error bars indicate the mean ± S.D. of triplicate analyses.

2.2.7 Metal chelating activity

Metal ions have the capability of generating free radicals from peroxides and may contribute to the development of non-communicable diseases such as cardiovascular disease (Gutteridge and Halliwell, 2000). For example, the production of oxyradicals and lipid peroxidation can be caused in biological systems by Fe^{2}, and the best way to protect against this type of oxidative damage is by minimising the concentration of free Fe^{2+} (Ebrahimzadeh *et al*., 2008). Some chelating agents help to make Fe^{2+} unavailable to participate in the redox reaction. The assay used to quantify the metal chelating capacity of the blueberry extract is based on the capacity of chelating agents in the test material to interfere with the formation of a Fe^{2+}-ferrozine complex. The Fe^{2+}-ferrozine complex is red in colour. Therefore, iron chelating activity in the test material interferes with the development of the red colour and can be quantified spectrophotometrically.

Using this method, the metal chelating activity of the blueberry extract was found to increase in a concentration-dependent manner over the entire range of concentrations tested (0 – 50) mg/ml (Figure 2.7.).

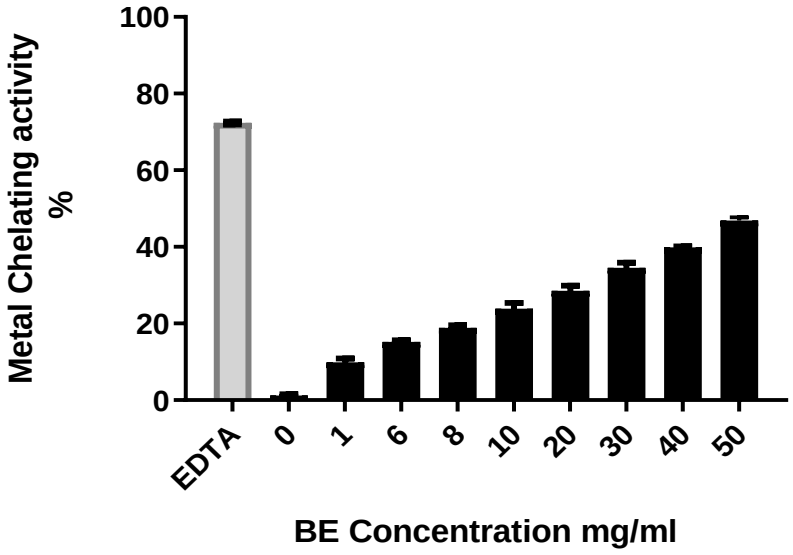


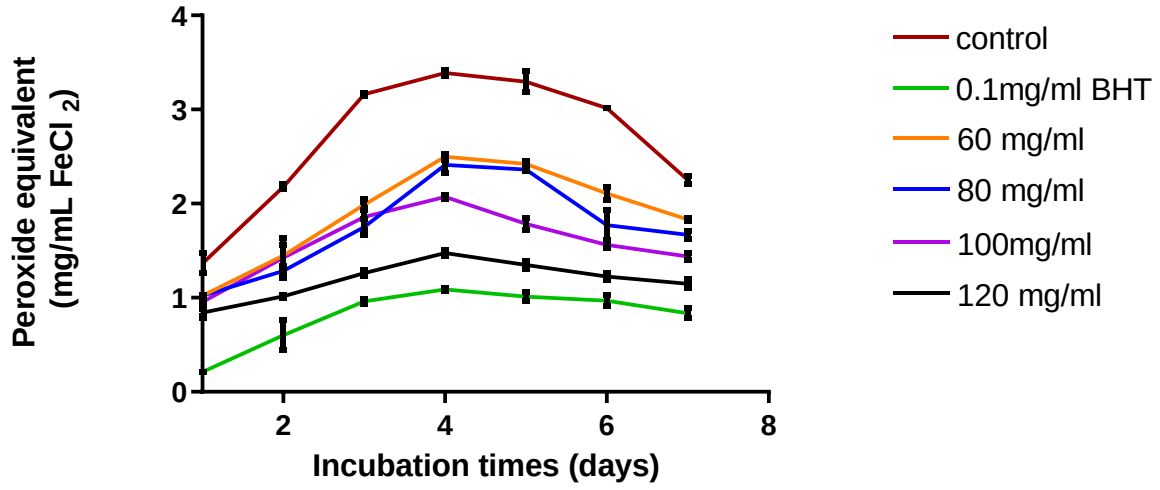
Figure 2.7. Concentration-dependent metal chelating activity of the blueberry extract. The chelating activity was measured over a concentration range of blueberry extract (BE) (0,1, 6, 8, 10, 20, 30, 40 and 50mg/ml). Using Fe^{2+} chelating activity assay. The activity was measured at 562 nm, and results were compared with EDTA (0.1 mg/ml) as a positive control. Data for the blueberry extract are depicted in black and for the EDTA positive control in grey. Data points and error bars indicate the mean+S.D. of triplicate analyses

2.2.8 Antioxidant activity

A wide range of methods has been used to evaluate the total antioxidant capacity of food and dietary supplements. In this study, the antioxidant activity of blueberries was tested using a combination of ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. The FTC assay monitors the amount of peroxide formed at the initial stage of linoleic acid peroxidation. In contrast, the TBARS method measures the carbonyl compounds formed at a later stage of lipid oxidation. For both methods, the apparent antioxidant activities of the blueberry extract were compared with those of the positive control BHT.

Using the FTC method, the individual activity of samples was measured. Samples were incubated with linoleic acid in the model system at 40 °C, and peroxide formation was monitored for seven days as shown in Figure (2.8.A). Peak peroxide concentration was detected on day 4 in all samples; thus, the percentage of lipid oxidation was estimated at this time point. Blueberry extract significantly reduced the absorbance values compared with the negative control between days three and four; the results were then compared on day four as a general trend, as shown in figure (2.8. B).

A



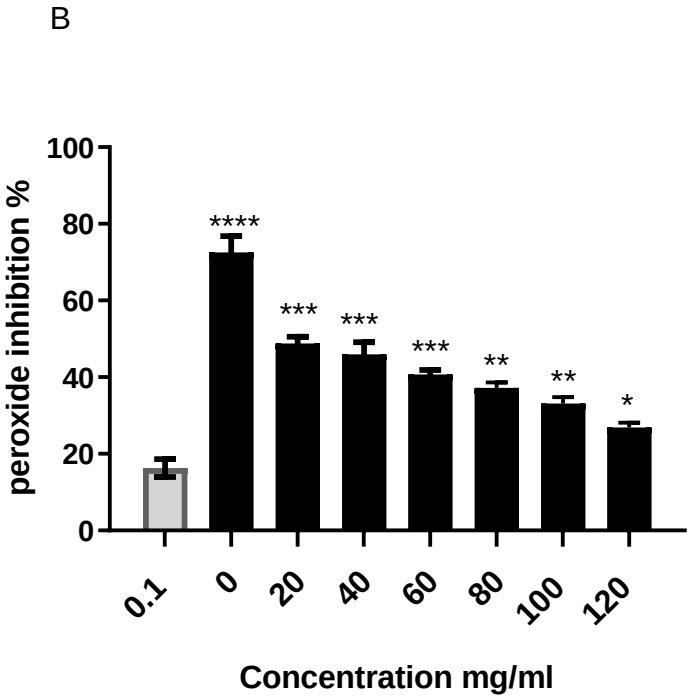
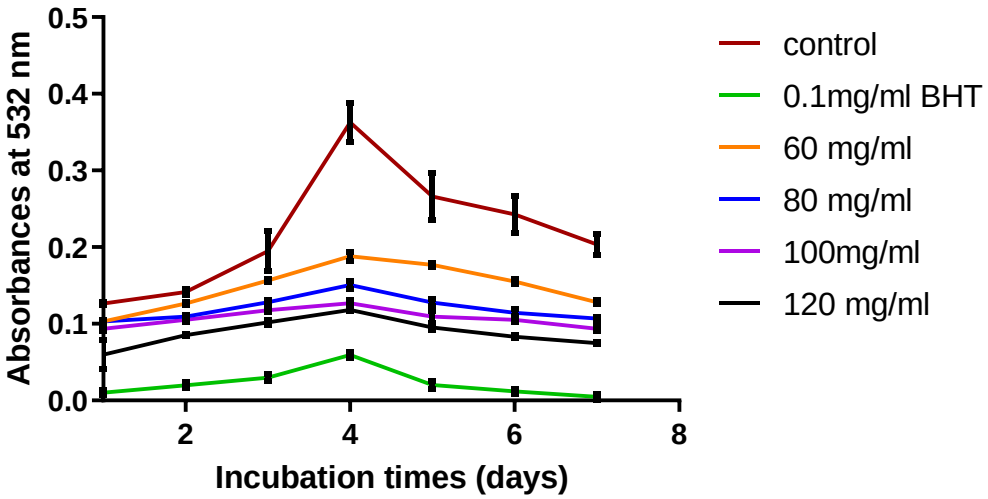


Figure 2.8. Effect of blueberry extract on lipid oxidation analysed by the ferric thiocyanate (FTC) method. Lipid oxidation was measured in a linoleic acid model system using the FTC method. A) Peroxide concentration was determined based on absorption at 500nm every 24 hours for seven days. B) On day four, the percentage of lipid oxidation was measured, and concentration-dependent inhibition of lipid peroxidation by the blueberry extract was determined. BHT (0.1 mg/ml) was used as a positive control, while milli-Q water was used as a negative control. Data for the blueberry powder are depicted in black and for the BHT positive control in grey. Data points and error bars indicate the mean ± S.D of triplicate analyses. ANOVA was performed in Graphpad prism, followed by Dunnett's multiple comparisons test. The result was considered statistically significant (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with positive control)

With the TBARS assay, there were no detectable differences between absorbance values for the samples and the negative control on day one. The absorbance value for the negative control increased gradually until it reached a peak on day 4. After that point, the absorbance began to decline. The absorbance values of all test samples were significantly below the negative control curve between days three and seven, indicating lower oxidation and better protection due to the presence of the blueberry extract (Figure 2.9.A). Based on these observations, the antioxidant activity of samples for the TBARS method was determined using the data from day 4. At this point, all test samples were significantly different from the

negative control. The antioxidant activity of the blueberry extract was similar to the positive controls (as shown in Figure (2.9.B).

A



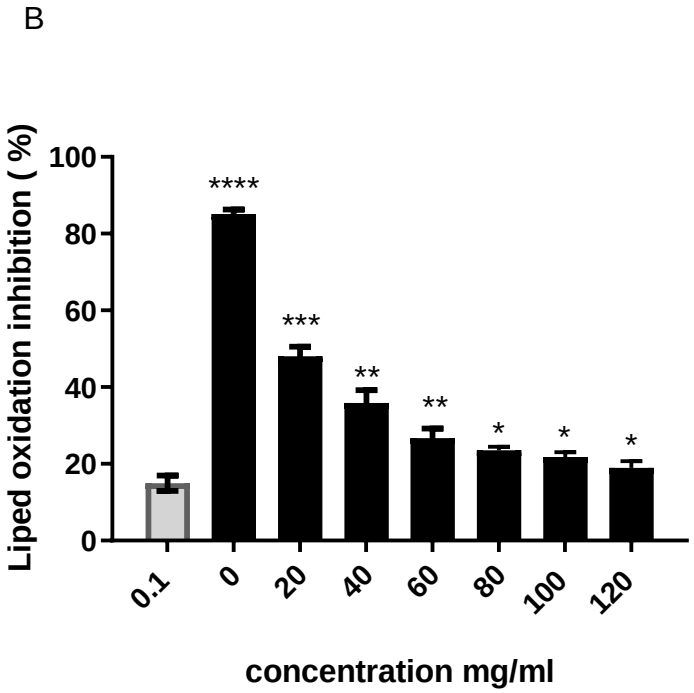


Figure 2.9. Concentration-dependent inhibition of lipid peroxidation inhibition activity was measured using the TBARS assay. A) Malondialdehyde concentration was measured every 24 hours for seven days at 532 nm. B) After four days of incubation at 40◦C percentage of lipid oxidation was measured. BHT (0.1 mg/ml) was used as a positive control, whereas milli-Q water was used in the negative control as an alternative sample. Data for the blueberry extract are depicted in black and for the BHT positive control in grey. Data points and error bars indicate the mean ± S.D. of triplicate analyses. ANOVA was performed in Graphpad prism, followed by Dunnett's multiple comparisons test. The result was considered statistically significant (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with positive control)

2.3 Discussion

The objectives of the work presented in this chapter were to evaluate the content of phenolics: total phenolic content (TPC), total flavonoids content (TFC), as well as antioxidant activity and radical scavenging potential of the commercial blueberry extract. Phenolic compounds were found in a considerable quantity for (TPC) 7.64±0.14 (mg Gallic acid/g DW) and (TFC) 14.18±0.16 TFC (mg catechin /g DW), close to the finding of (Huang et al., 2012) where the (TPC) for blackberry was 5.58±0.18 and for the strawberry was 2.72±0.18 (mg Gallic acid/g DW) while the (TFC) for blackberry was 11.83±0.24 and for the strawberry 7.04±0.59 (mg Rutin/g DW). Moreover, a positive correlation has been previously found between antioxidant capacity and phenolic and anthocyanin content (Connor *et al*., 2002), indicating that phenolic compounds are significant contributors to the antioxidant properties of blueberry (Stoner & Seeram, 2011).

The ability of the blueberry extract to reduce Fe3+/ferricyanide complex to the ferrous form was measured using the reducing power assay, where the reducing power reflects the electron-donating capacity of bioactive compounds, a mechanism also known as antioxidant activity (Gulcin *et al*., 2011). The reducing power of blueberry extract increased in a concentration-dependent manner.

The DPPH assay was selected due to the stability of the DPPH radical. The results obtained here indicate that blueberry extract can scavenge DPPH free radicals in a concentration-dependent manner due to its hydrogen donating ability. Similar results were conducted by Gulcin et al., 2010 when tannic acid concentrations between (15-45 µg/ml) produced significant inhibition of DPPH.

In biological systems, when abundant, the hydrogen peroxide reacts with biological molecules, which may contribute to the development of cancer and numerous degenerative diseases (Valko *et al*., 2004). The blueberry extract had the ability to scavenge hydrogen peroxide (H_2O_2) and convert it to H_2O by donating an electron to H_2O_2. In this research, blueberry showed a moderate concentration-dependent effect up to a maximum at 30mg/ml but then decreased at higher concentrations. This suggests higher concentrations may start to lose some of their antioxidant capacity.

In this study, the ferrous ion chelating activity for blueberry extract was measured. Metals such as iron, copper, manganese, nickel, and cobalt can directly initiate lipid oxidation thru the

development of electron transfer and lipid alkyl radical formation (Wong and Kitts, 2001). The blueberry extract had the ability to chelate Fe^{2+} ions, decreasing the appearance of the water-soluble ferrozine complex in a concentration-dependent manner.

The blueberry extract showed an excellent capacity to inhibit linoleic acid peroxidation when the FTC and TBARS assays were performed. The results suggest that the blueberry extract had a more potent antioxidant activity in the TBARS assay than in the FTC assay. This indicates that the components of the blueberry extract may be better at preventing malondialdehyde production than at inhibiting peroxide formation.

2.4 Conclusions

The tested blueberry extract showed a reasonable content of phenolic compounds (TPC) 7.64±0.14 (mg Gallic acid/g DW) and (TFC) 14.18±0.16 TFC (mg catechin /g DW) and (TFC). The extract also demonstrated the ability to act as an antioxidant free radical scavenger with comparable activity to synthetic antioxidants BHA and EDTA in DPPH, reducing power, hydrogen peroxide, and chelating assays. Additionally, the blueberry extract exhibited good antioxidant capacity based on the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods and compared with that of BHT. Based on these observations, it was concluded that the commercial blueberry extract would potentially be suitable for further investigations of the biofunctional properties of blueberries in cell systems.

CHAPTER THREE

EFFECTS OF EXTRACT AND PHYTOCHEMICALS ON HUMAN COLON ADENOCARCINOMA CACO-2 VIABILITY

3.1 Introduction

Cancer is one of the leading causes of morbidity and mortality globally (Surh, 2003). Epidemiological studies have consistently identified an inverse relationship between the risk of several types of cancer and diets rich in fresh fruit and vegetables (Ito *et al*., 2006; Williams and Hord, 2005; Vainio and Weiderpass, 2006). Phytochemicals present in such foods (e.g., flavonoids, phenolics, and carotenoids) are prime candidates for reducing cancer risk as they are proposed to have the capacity to play a critical role in defeating early and late stages of carcinogenesis (Nishino *et al*., 2007). Small berry fruits are of interest and an excellent source of natural antioxidants (Vendrame *et al*, 2016). The biological activities of berries are proposed to be partially due to their unique content of a various range of phytochemicals, for instance, flavonoids (anthocyanins, flavonols, and flavanols), tannins, stilbenoids, phenolic acids, and lignans (Seeram, 2008). Considerable attention has remained dedicated to laboratory studies that showed blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts could inhibit the growth of human oral, breast, colon, and prostate cancer cell lines in a dose-dependent manner (Seeram, 2008). Amongst these, blueberries (the focus of the research described here) contain substantial amounts of anthocyanin, especially malvidin-3-glucoside and malvidin-3-galactoside (Li *et al.*, 2013). Anthocyanins have potent free radical scavenging and antioxidant activities as well as the ability to inhibit the growth of some cancer cells (Meyers *et al*., 2003). There is also evidence that anthocyanins can inhibit the promotion and progression of tumour cells. A study suggested that anthocyanins such as delphinidin and malvidin from blueberry extract could block the proliferation of DLD-1 and COLO205 human colorectal carcinoma by inducing apoptosis (Zu *et al*., 2010).

As a result of public concerns about general health and chronic disease prevention, these observations have led to an increase in consumer interest in the potential health benefits of

phytonutrients (Guhr and Lachance, 1997). Colon cancer is particularly relevant in this context because it is a common form of cancer that appears to be highly influenced by diet.

Colorectal cancer is the third most common cancer in the world and the fourth most common type of cancer death globally (Parkin, 2001). In the United Kingdom, colorectal cancer mortalities were 47th highest for males and 48th highest for females out of 184 countries worldwide, according to the International Agency for Research on Cancer (2010) (Jemal *et al.*, 2011).

Colon cancer can be defined as any malignant neoplasm starting at the inner lining of the colonic epithelium, and colon cancer incidence demonstrates clear associations with dietary habits. For example, low intake of vegetables and fruits and high intake of salted, smoked and nitrated foods are all associated with increased risk (Kelley and Duggan, 2003; Bastide *et al.*, 2016). Carcinogenesis is a multistep process resulting in the generation of abnormal cells with abnormal molecular signal cascades (Manson *et al.*, 2005). Convincing evidence has been provided by epidemiological studies showing that dietary factors can modify the process of carcinogenesis, including initiation, promotion and progression of several types of human cancer and, in particular, colon cancer (Ray, 2005). In addition to diet, the incidence of colon cancer is associated with both age and gender: both genders have a similar risk for colon cancer until the age of 50, while 90% of the cases occur in people over 50 years, with a higher incidence in men than women (Vălean *et al.*, 2008).

Cell death is a normal feature of cell turnover in the human body; in healthy individuals, there is an appropriate balance between cell division and cell death (Khan *et al.*, 2021). Apoptosis is one distinct type of cell death, also known as a programmed cell death that usually eliminates damaged cells through the activation of caspase enzymes (Chang *et al.*, 2009). This contrasts with necrotic (unprogrammed) cell death, where injury or insult leads to cell rupture and release of products of cell death, which trigger an inflammatory response in the surrounding tissue. Many anticancer drugs and cancer chemopreventive agents act, at least in part, by inducing apoptosis, thereby helping to prevent tumour promotion, progression, and the occurrence of cellular inflammatory responses (Brown and Attardi, 2005). Some flavonoids can act as apoptosis-inducing agents in addition to inhibiting cancer cell growth via activating the pre-existing apoptosis machinery. By way of, treatment with flavonoids can stimulate the induction of caspase-3 activity and degradation of PARP [poly-(ADP- ribose) polymerase] that can lead to the initiation of apoptosis. (Wang *et al.*, 1996).

The work described in this chapter compared the potential anticancer effects of blueberry extract (containing a complex mix of anthocyanins as described in chapter 2), malvidin-3-galactoside (the most abundant anthocyanin in the extract), malvidin in its aglycone form and a major metabolite of malvidin produced by the colonic microflora (2,4,6-trihydroxybenzaldehyde) (Aura *et al*., 2005). Their effects on cell proliferation, cytotoxicity, and cell death pathways were evaluated in Caco-2 cells, a human colon cancer line selected as a suitable model for this work.

The hypothesis of this chapter presents Blueberry polyphenols and their metabolites act as chemopreventative and/or chemotherapeutic agents.

The aims of the work described in this chapter were:

1. To determine and compare the relative cytotoxicity of blueberry extract, malvidin-3-galactoside, malvidin (aglycone), and 2,4,6-trihydroxybenzaldehyde on Caco-2 cells.

2. To study and compare the morphological changes in the Caco-2 cell line induced by blueberry extract, malvidin-3-galactoside, malvidin, and 2,4,6-trihydroxybenzaldehyde.

3. To determine and compare the extent and types of cell death type induced by blueberry extract, malvidin-3-galactoside, malvidin, and 2,4,6-trihydroxybenzaldehyde in Caco-2 cells.

4. To determine and compare the effects of blueberry extract, malvidin-3-galactoside, malvidin, and 2,4,6-trihydroxybenzaldehyde on specific apoptotic pathways by analysing caspase activation in Caco-2 cells.

3.2 Materials and Methods

3.2.1 Materials

The Caco-2 cell line was obtained from the European Collection of Cell cultures (ECACC) Salisbury, UK. Phosphate buffered saline (PBS), penicillin, 3-(4,5-Dimethylthiazol-2-diphenyl)-2,5-diphenyltetrazolium bromide (MTT dye), dimethyl sulphoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), non-essential amino acids (NEAA), trypsin-EDTA solution, staurosporine and hydrogen peroxide were obtained from Sigma-Aldrich Chemical Co, Poole, UK. The Annexin V-FITC apoptosis detection kit was purchased from Merck Chemicals Ltd. The Caspase-GloR 3/7 assay system kit was purchased from Promega (Southampton, UK).

3.2.2 Methods

3.2.2.1 Solutions and Reagents for Tissue Culture

DMEM **c**omplete medium for the routine culture of Caco-2 cells was prepared by supplementing 450ml DMEM with 10% v/v 50 ml of FBS (10% v/v), 1% v/v (5 ml) non-essential amino acids (NEAA) and 1% v/v (5 ml) penicillin/streptomycin antibiotic solution.

The freezing medium was prepared by mixing 10% v/v DMSO with 90% v/v FBS. Cells were resuspended in the freezing media so that the concentration was no more than 1-2 x 10^6 cells/ml of freezing media. Aliquots (1 ml) of cell suspension were transferred to labelled cryovials and maintained on ice for approximately 30 minutes. The vials then were transferred to a NALGENE Cryo 1 °C freezing container pre-filled with isopropanol, and this was placed in a –80°C freezer for 24 hrs to freeze slowly. After transfer 24 hours in the -80°C freezer, the cryovials were transferred to liquid nitrogen for long-term storage.

The MTT reagent was dissolved in PBS to a final concentration of 5mg/ml and sterilized by passing through 0.2 µM filter.

3.2.2.2 Cell Culture Conditions

Caco-2 cells were seeded in DMEM complete medium; 3×10^6 cells were added to a 75cm^2 tissue culture flasks (seeding density of 40,000 cells/cm^2) and were incubated at 37 °C in a 5% CO_2, humidified incubator. The medium was removed from cells when 70% - 80% confluent and cells were washed with PBS, the PBS was aspirated, and 3 ml trypsin was added to each 75 cm^2 flask. The cells were incubated for 3 minutes in the incubator and then were detached from the flask surface by tapping. Complete DMEM medium (10 ml) was then added to inactivate the trypsin. Cells then were harvested, pelleted by centrifugation, and the supernatant was aspirated. Complete media (10 ml) was added, and cells then were ready for counting and sub-culturing or freezing.

3.2.2.3 Cell viability (MTT assay)

3.2.2.3.1 Principle

The MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium) assay is associated with the conversion of the soluble, yellow MTT dye to the insoluble purple formazan by the mitochondrial enzyme succinate dehydrogenase. This condition occurs in metabolically active cells. Succinate dehydrogenase cleaves the tetrazolium ring in the MTT structure and converts it to formazan. Water-insoluble formazan accumulates within healthy cells due to being impervious primarily to cell membranes (Jouvet *et al*., 2000). Formazan crystals can be solubilised with DMSO and quantified by spectrophotometry at a wavelength of 540nm (Roehm *et al*., 1991).

3.2.2.3.2 Determination of the Cytotoxicity of blueberry extract, malvidin-3-galactoside malvidin and 2,4,6-trihydroxybenzaldehyde

The cytotoxic effects of different concentrations of blueberry extract (0-500 µg/ml) and derivatives of the extract (0-500 µM) were determined using Caco-2 cells. A stock solution of the treatment (blueberry extract or defined compound) was prepared in DMSO. Caco-2 cells were cultured in 96-well tissue culture plates by adding 10^4 cells into each well in a volume of 200 µl (i.e., 30,300 cells/cm^2 seeding density). After 24 hours, the culture medium was replaced with 200µl of fresh medium containing the different concentrations of blueberry extract or individual compounds derived from those in the extract. The cells were treated with these for 24 hours. At the end of the treatment period, 20 µl of 5 mg/ml MTT dye was added to each well and incubated for 3 hours at 37 °C. At the end of the incubation, the medium was aspirated, and 200µl of DMSO was added to each well to solubilize the formazan crystals. The absorbance of the solution in each well was determined at 540 nm using an automated plate reader (Omega Fluostar microplate reader (BMG Labtech, Aylesbury, UK) with the MARS Data Analysis Software. Each experimental condition was tested in triplicate within each experiment, and the experiments were repeated three times to ensure reproducibility. Wells containing untreated cells were used as controls.

3.2.2.4 Cell morphology

A total of 1.5 x 10^6 Caco-2 cells [were] seeded in 25 cm^2 cell culture flasks (seeding density of 60,000 cells/cm^2). After 24 hours, when the cells reached approximately 50- 60% confluence, they were treated with blueberry extract or derivatives of the anthocyanin malvidin individually at a concentration of 120 µg/ml blueberry extract or 120 µM for the single compounds. Changes in cellular morphology were determined by microscopy after 24 hours. Pictures of the cells were taken using a phase-contrast microscope (Zeiss Telaval inverted microscope) equipped with a camera (Nikon, Japan) at 100 × magnification.

3.2.2.5 Apoptosis assay by Annexin V-FITC

3.2.2.5.1 Principle of the Annexin V-FITC Assay

A well-known method for evaluating the occurrence of apoptosis is to stain cells with annexin V labelled with fluorescein isothiocyanate (annexin V-FITC). In healthy viable cells, phosphatidylserine (PS) is located in the inner layer of the plasma membrane bilayer. However, the plasma membrane loses its integrity when apoptosis is stimulated, allowing PS to relocate on the outer membrane layer. Annexin V binds to PS but is cell impermeable. Thus, only cells with PS on the out surface of the plasma membrane stains positively with annexin V-FITC. Propidium iodide (PI) is a cell impermeable dye that will fluoresce strongly when it binds to DNA. PI is usually used to distinguish apoptotic from dead cells. In the early stages of apoptosis, cells stain positively with Annexin V-FITC though at this stage, PI staining would be negative as the plasma membrane is not sufficiently compromised to permit ingress of the PI. As cells progress towards death, the plasma membrane integrity becomes sufficiently compromised that they stain positive for both annexin V-FITC and PI. In necrosis, PI staining only is typically observed. Healthy cells with intact cell membranes do not stain with either PI or annexin V-FITC (Koopman *et al.*, 1994).

3.2.2.5.2 Determination of Apoptosis by Annexin V-FITC

Caco-2 cells were cultured in 25 cm^2 cell culture flasks at 1.5 x 10^6 cells (seeding density of 60,000 cells/cm^2) and incubated in 5% CO_2 at 37 °C. After allowing the cells to settle, adhere and start to grow for 24 hours, different concentrations of the test compounds (0-500µg/ml for the blueberry extract or 0-500µM for the individual compounds) were added and incubated for 24 hours. At the end of the treatment, the medium was collected into Falcon tubes from each flask prior to trypsinisation; this medium contained any floating (dead cells) cells that may have been produced during the treatments. The adherent cells remaining in the flasks were trypsinised for 1 min before the trypsin was inhibited by adding medium. Then all cells from

each flask (floating and adherent) were combined and centrifuged for 3 min at 150 x *g*. The liquid was aspirated, and 500 µl of ice-cold binding buffer (from the Annexin V-FITC Apoptosis Detection Kit) was added to each tube. Next, 5 µl of the Annexin V-FITC conjugate solution and 5 µl of the PI solution were added and gently mixed. Then the cell suspensions were incubated for 15 minutes in the dark at room temperature. At the end of incubation time, cells were kept on ice until analysed by flow cytometry using a Beckman Coulter Epics XL.

Flow cytometer set up: forward and side scatters gatings were set to exclude cell debris and analysed in FL-1 for Annexin V-FITC (520 nm emission) and FL-3 for PI (620 nm emission). At least 10,000 events were acquired in list mode for each run. Staurosporine at 1 µM was used as a positive control for apoptosis. Hydrogen peroxide treatment (3mM) was used as a positive control for cell death (most likely via a combination of apoptosis and necrosis). Three independent experiments were performed for each treatment to ensure reproducibility.

3.2.2.6 The Caspase-Glo 3/7 assay

3.2.2.6.1 Principle of Caspase-Glo 3/7 assay

The Caspase-Glo 3/7 is a luminescent assay used to measure the activities of caspase-3 and caspase-7. The caspase family (cysteine aspartic acid-specific proteases) are known to play the key effector roles in apoptosis (programmed cell death) in mammalian cells (Manabu and Sally, 2009). The assay uses a luminogenic caspase-3/7 substrate, which consists of tetrapeptide with the amino acid sequence DEVD linked to aminoluciferin. Adding the Caspase-GloR 3/7 reagent to cultured cells leads to cell lysis allowing active caspase 3 and 7 to cleave the substrate. The aminoluciferin released following caspase cleavage is a substrate for luciferase also present in the reagent mix leading to a luminescent signal. The luminescence generated is proportional to the amount of caspase activity present in the sample.

3.2.2.6.2 The Caspase Glo 3/7 assay procedure

The assay was performed in white 96-well tissue culture plates. Caco-2 cells were seeded into the wells at 1×10^4 cells/ well in 100 µl of DMEM complete medium. The cells were cultured for 24 hours; then, the cultured medium was replaced with 100 µl fresh medium/well-containing different concentrations of blueberry extract (30 – 250µg/ml) or malvidin-3-galactoside, malvidin or 2,4,6-trihydroxybenzaldehyde at (30 – 250µM) and incubated for 24 hours. At the end of the treatment, 100 µl of Caspase-Glo 3/7 reagent was added and incubated with the cells for 30 minutes. Luminescence was measured using the Omega Fluostar microplate reader (BMG Labtech, Aylesbury, UK).

3.2.3 Statistical analyses

For MTT and annexin V-FITC/PI analyses, three independent experiments were performed for each treatment condition and for the MTT assay, each treatment was also performed in triplicate within each independent experiment. All data were analysed using a one-way analysis of variance (ANOVA). Statistical significance was accepted for $p<0.05$. Where statistically significant differences were identified by ANOVA, post hoc multiple comparisons were performed using Dunnett's test. Graphpad Prism software was used to perform the statistical analyses.

3.3 Results and Discussion

3.3.1 Cytotoxicity of blueberry extract, malvidin-3-galactoside, malvidin and 2,4,6-trihydroxybenzaldehyde on Caco-2 cells determined using the MTT assay

The concentration-dependent cytotoxicity of blueberry extract, the major anthocyanin present in the extract (malvidin-3-galacotside) and its metabolites (malvidin and 2,4,6-trihydroxybenzaldehyde) in Caco-2 cells was evaluated first using the MTT assay. Following a 24-hour exposure, all four treatments were observed to lead to concentration-dependent decreases in Caco-2 cell viably (Figure 3.1). For the blueberry extract, the reductions in Caco-2 cell viability were not statistically significant using concentrations in the range of 3-30 µg/ml but were at higher concentrations (60, 120, 250 and 500 µg/ml). Both the malvidin-3-galactoside and malvidin in its aglycone form (malvidin chloride) appeared to be less cytotoxic, eliciting significant decreases in Caco-2 cell viability only at the higher concentrations tested (250 and 500 µM) compared to the control. For the 2,4,6-trihydroxybenzaldehyde substantial reductions in viability were identified at concentrations of 60, 120, 250 and 500µM compared with the control.

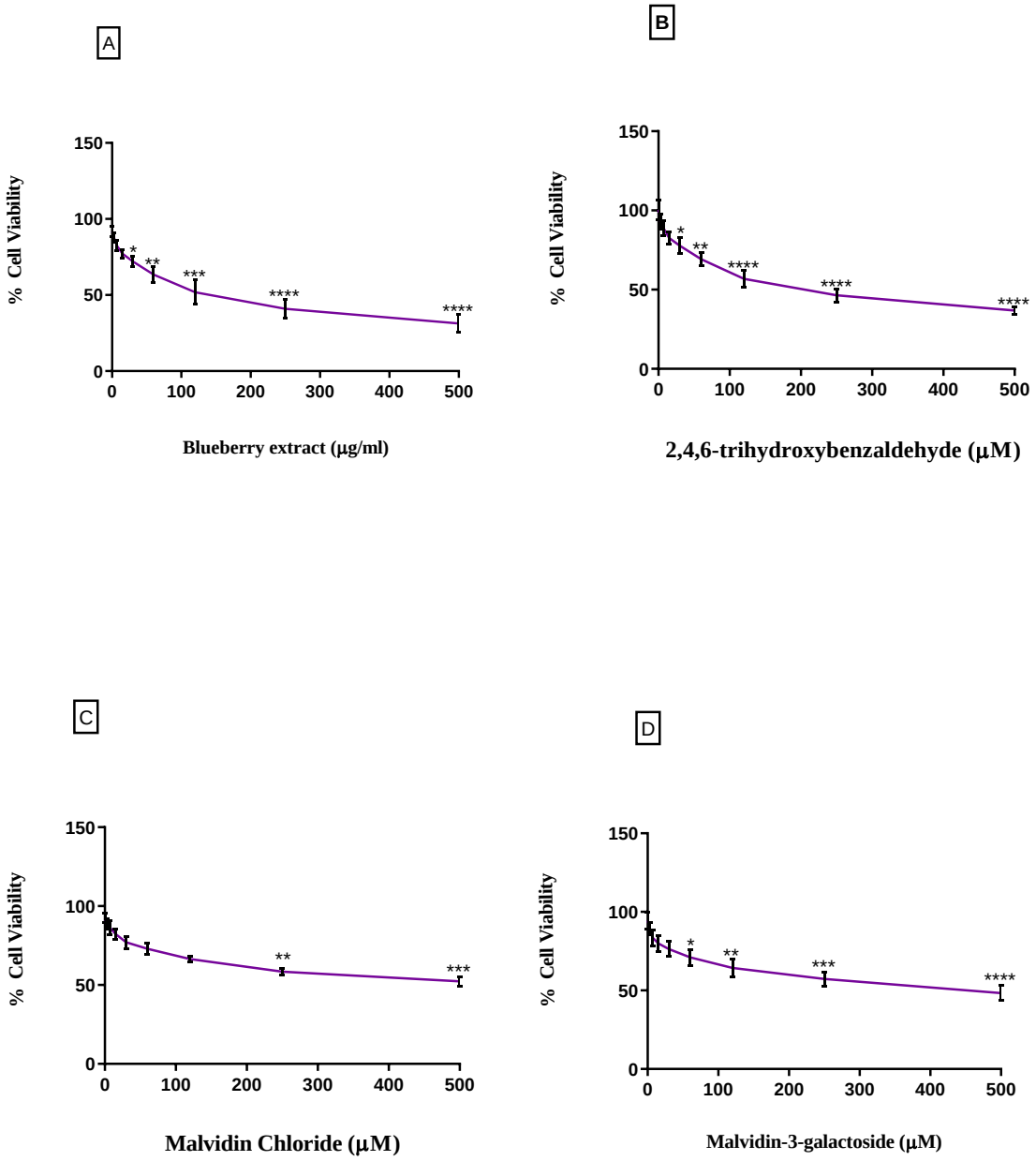


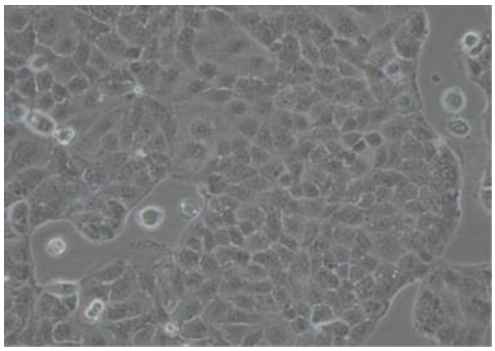
Figure 3.1 Cell viability of Caco-2 cells treated with A) blueberry extract, B) 2,4,6-trihydroxybenzaldehyde, C) malvidin chloride, and D) malvidin-3-galactoside. Caco-2 cells were cultured and treated with blueberry extract and derivatives individually over a concentration range of 3-500 µg/ml for the blueberry extract or 3-500 µM for the derivatives. The MTT assay evaluated cell viability after 24 hours of treatment. IC50 estimated for blueberry extract 200 µg/ml, 2,4,6-trihydroxybenzaldehyde 250 µM, malvidin chloride 500 µM and malvidin-3-galactoside 500 µM. The absorbance of solubilised formazan crystals was measured at 540nm. These data represent the mean ± SD of at least three independent experiments. Data were analysed by 1-way ANOVA followed by

Dunnett's test comparing different treatment conditions with the untreated controls (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$).

3.3.2 Morphological changes of caco-2 cells induced by blueberry extract, malvidin chloride (aglycon), malvidin-3-galactoside (glycoside) and 2,4,6-trihydroxybenzaldehyde (metabolite)

The effects of blueberry extract, the most abundant anthocyanin in the extract and two of its metabolites on the morphology of Caco-2 cells were examined following 24 hours of exposure. Cells were treated with (120 µg/ml) blueberry extract or (120 µM) malvidin-3-galactoside, malvidin, or 2,4,6 trihydroxybenzaldehyde individually. Morphology changes were analysed qualitatively by light microscopy. Figure 3.2 illustrates the observation that fewer cells were evident after treatment with 120 µg/ml of the blueberry extract or 120 µM 2,4,6-trihydroxybenzaldehyde and that there were more bright rounded cells, reminiscent of apoptosis, in the flasks (Figure 3.2 B, C) subject to these treatments than in the untreated controls (Figure 3.2 A). On the other hand, the effects of 120 µM malvidin-3-galactoside or malvidin in its aglycone form appeared a little less marked compared to control, at least in terms of cell numbers (Figure 3.2 D, E). These observations were generally in line with the results obtained from the MTT cell viability assay.

A



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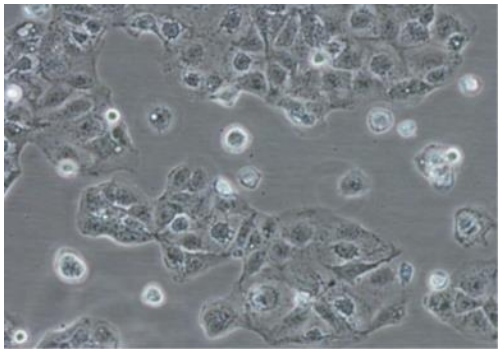

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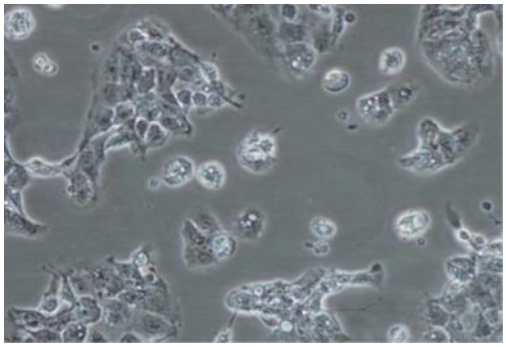

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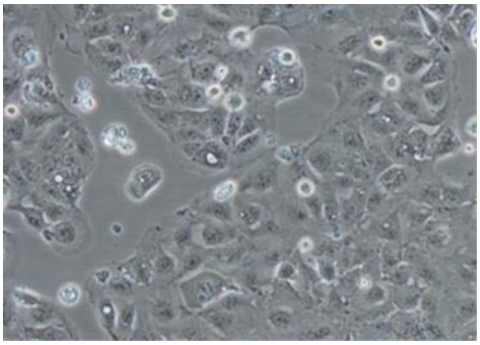

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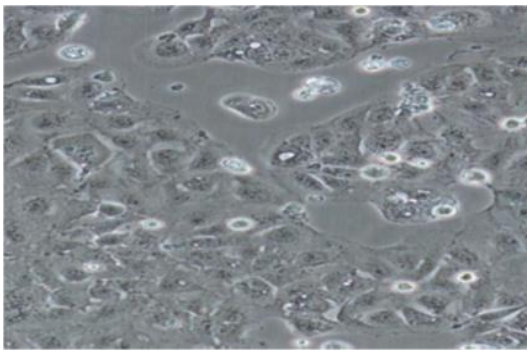


Figure 3.2 Morphological features of Caco-2 cell line after 24 hours of exposure to 120 µg/ml of blueberry extract (B) or 120 µM of 2,4,6 trihydroxybenzaldehyde (C) or 120 µM malvidin (D) or malvidin-3-galactoside (E) compared with untreated control (A). Images were captured using light microscopy at 100x magnification.

3.3.3 Effect of blueberry extract, malvidin-3-galactoside, malvidin and 2,4,6-trihydroxybenzaldehyde on cell death pathways

A combination of annexin V-FITC and PI staining, combined with flow cytometric analysis, was used to distinguish living cells from apoptotic and dead cells the following exposure to blueberry extract, malvidn-3-galactoside or its metabolites. Figure 3.3 depicts a typical scatter plot obtained from the flow cytometry. The cells captured by this analysis are categorised variously as live (low annexin V-FITC and low PI staining in quartile 1 (Q1), early apoptotic (high annexin V-FITC staining with low PI staining in quartile 2 (Q2), late apoptotic (high annexin V-FITC staining and high PI staining in quartile 3 (Q3), or dead cells (low annexin V-FITC staining with high PI staining in quartile 4 (Q4).

Caco-2 cells were used as a model of colon cancer cells to detect the effects of blueberry extract at concentrations of (30, 60, 120 or 250 µg/ml) or of malvidin-3-galacoside or its metabolites **(**malvidin chloride and 2,4,6-trihydroxybenzaldehyde) at concentrations of (30, 60, 120 or 250 µM). Compared with negative control (untreated cells) and positive controls (staurosporine and hydrogen peroxide treated cells) after 24 hours of incubation using flow cytometry. The data generated are summarised in Figures 3.4, 3.5, 3.6, 3.7 and 3.8.

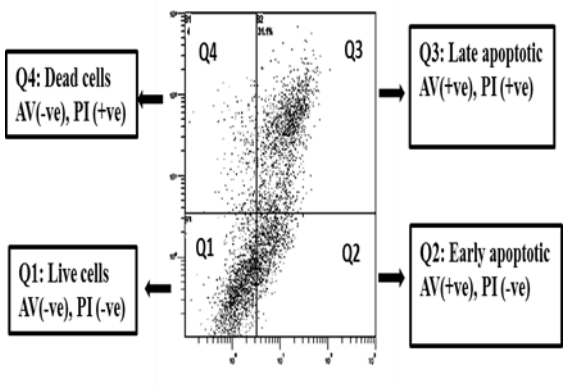


Figure 3.3 An example scatter plot obtained by flow cytometric analysis of cells stained with annexin V-FITC and PI indicating the classification of cells according to the quadrant they are located in. Q1 = healthy cells; Q2 = cells staining strongly for annexin V-FITC only; Q3 = cells staining intensely for annexin V-FITC and PI; Q4 = cells staining intensely only for PI.

Throughout these experiments, the majority of the untreated cells were located in Q1 on the scatter plot with much smaller proportions in Q2, Q3, and Q4 (Figures 3.4 to 3.8), suggesting that, in the absence of any additional experimental exposures, most cells were viable in the standard culture conditions. As expected, in all experiments, the staurosporine treatment led to a marked increase in the proportion of cells in Q2 (early apoptosis), whereas hydrogen peroxide treatment led to a moderate increase in cells in Q3 (late apoptosis) and a marked increase in cells in Q4 (dead) (Figure 3.4).

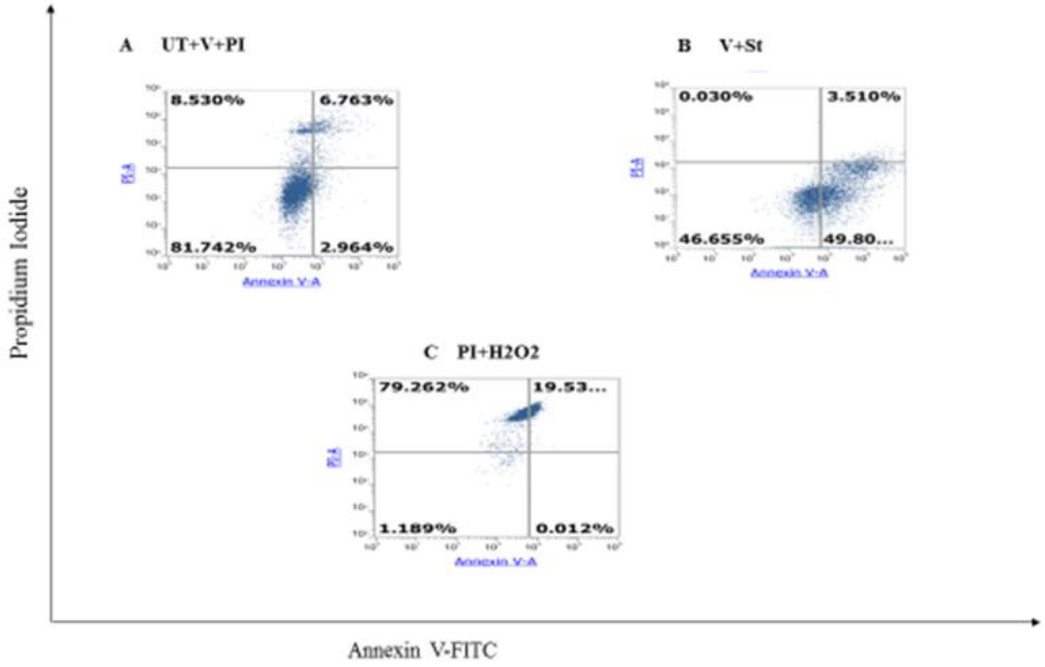
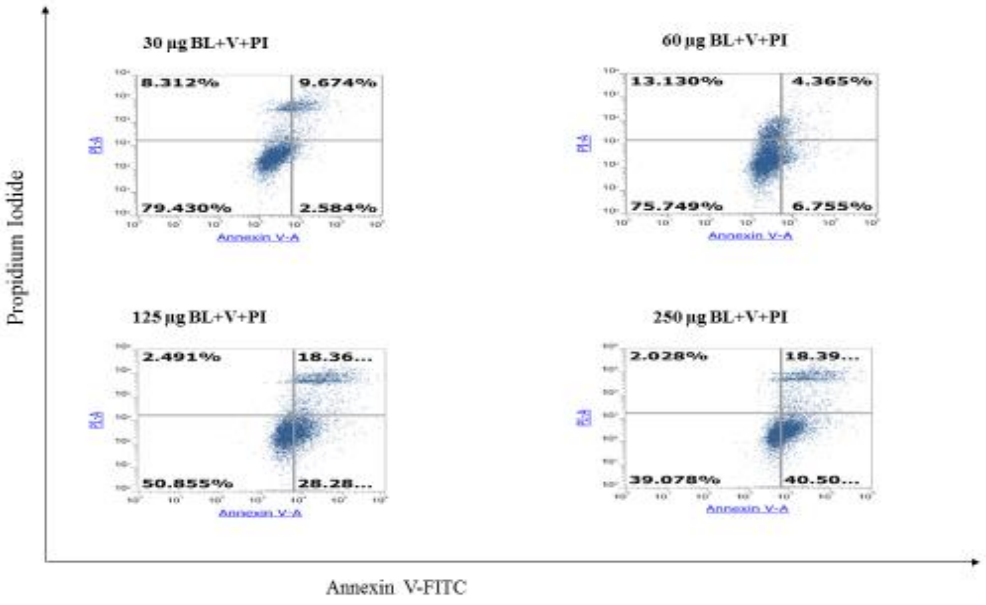


Figure 3.4 Flow cytometric analysis of cell death processes. Example scatter plots derived from flow cytometric analysis of Caco-2 cells (A) Untreated, (B) treated with staurosporine, (C) treated with hydrogen peroxide for 24 hours. At the end of the treatment, cells were trypsinised, stained with annexin V-FITC and PI, and analysed by flow cytometry. A total of 10,000 events were counted for each treatment. Cells were categorised into quartiles according to the extent of annexin V-FITC staining (x-axis) and propidium iodide staining (y-axis).

The data for experiments in which cells were treated with the blueberry extract are shown in figure 3.5. The blueberry extract led to a concentration-dependent decrease in the proportion of cells in Q1 and concentration-dependent increases in the proportions in Q2 and Q3 (figure 3.5B), with the effects achieving statistical significance at concentrations of 120 and 250 µg/ml ($P<0.0001$). These observations are consistent with the extract stimulating cell death primarily via apoptosis.

A



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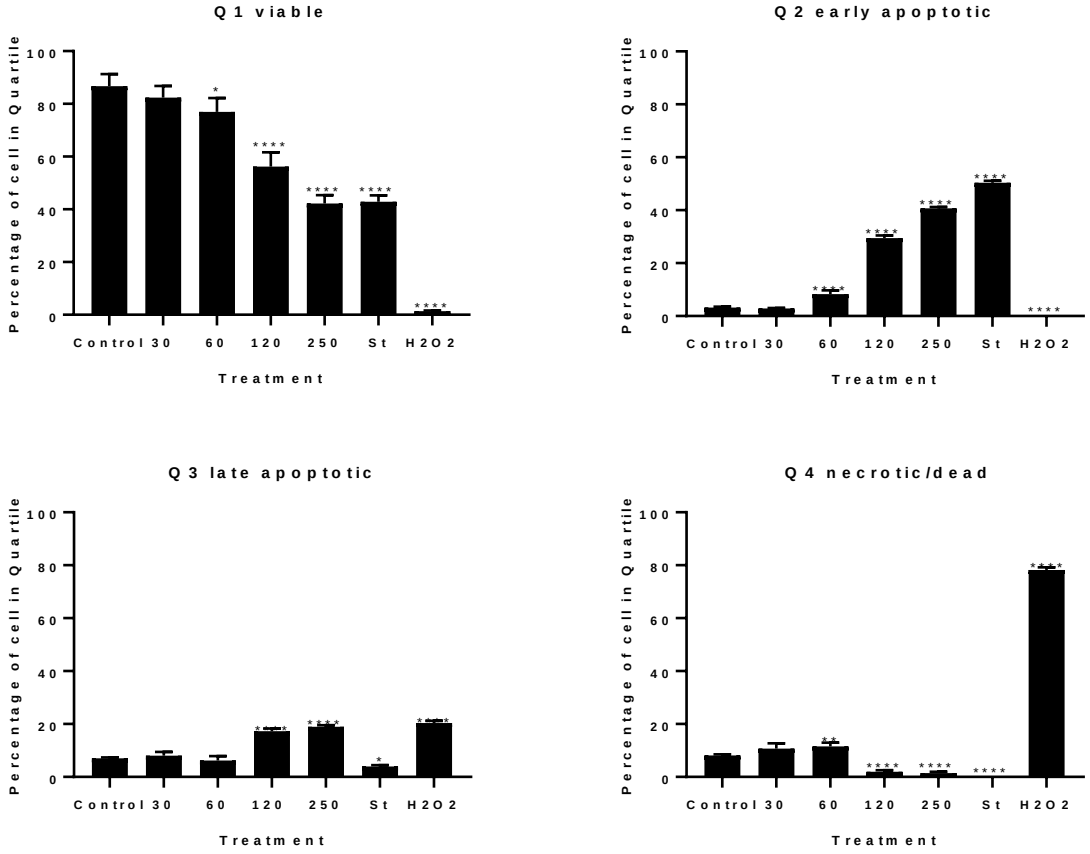

Figure 3.5 Flow cytometric analysis of cell death pathways triggered by blueberry extract based on phosphatidylserine externalisation and membrane permeability. (A) Example scatter plots derived from flow cytometric analysis of Caco-2 cells treated with different concentrations (30-250 µg/ml) of blueberry for 24 hours. At the end of the treatment, cells were trypsinised, stained with annexin V-FITC , and PI , and analysed by flow cytometry. A total of 10,000 events were counted for each treatment. Cells were categorised into quartiles according to the extent of annexin V-FITC staining (x-axis) and propidium iodide staining (y-axis). (B) Proportions of cells in each quartile following the different treatments. Q1 represents healthy cells; Q2 represents cells in early apoptosis; Q3 represents cells in late apoptosis; Q4 represents dead cells. Values are the mean+SEM for three independent experiments. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

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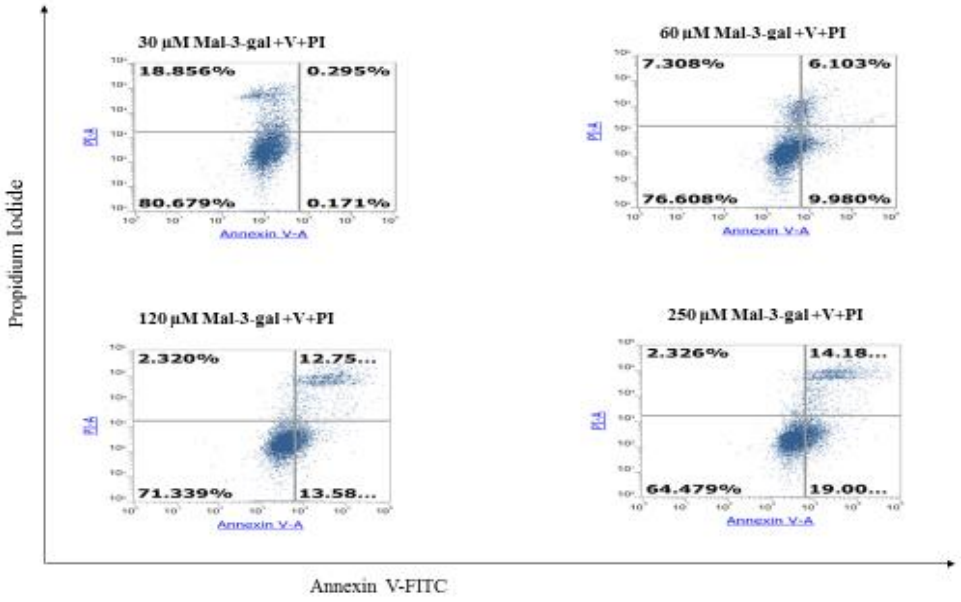
The mavidin-3-galacostide also appeared to stimulate apoptosis in the Caco-2 cells but to a lesser extent than blueberry extract (Figure 3.6). The decrease in the proportion of cells in Q1 was significant at 120 and 250µM ($p<0.0001$), but the magnitude of the reductions was smaller. In line with this observation, the proportion of early apoptotic cells (Q2) increased but to a lesser extent than observed with equivalent concentrations of the blueberry extract.

A



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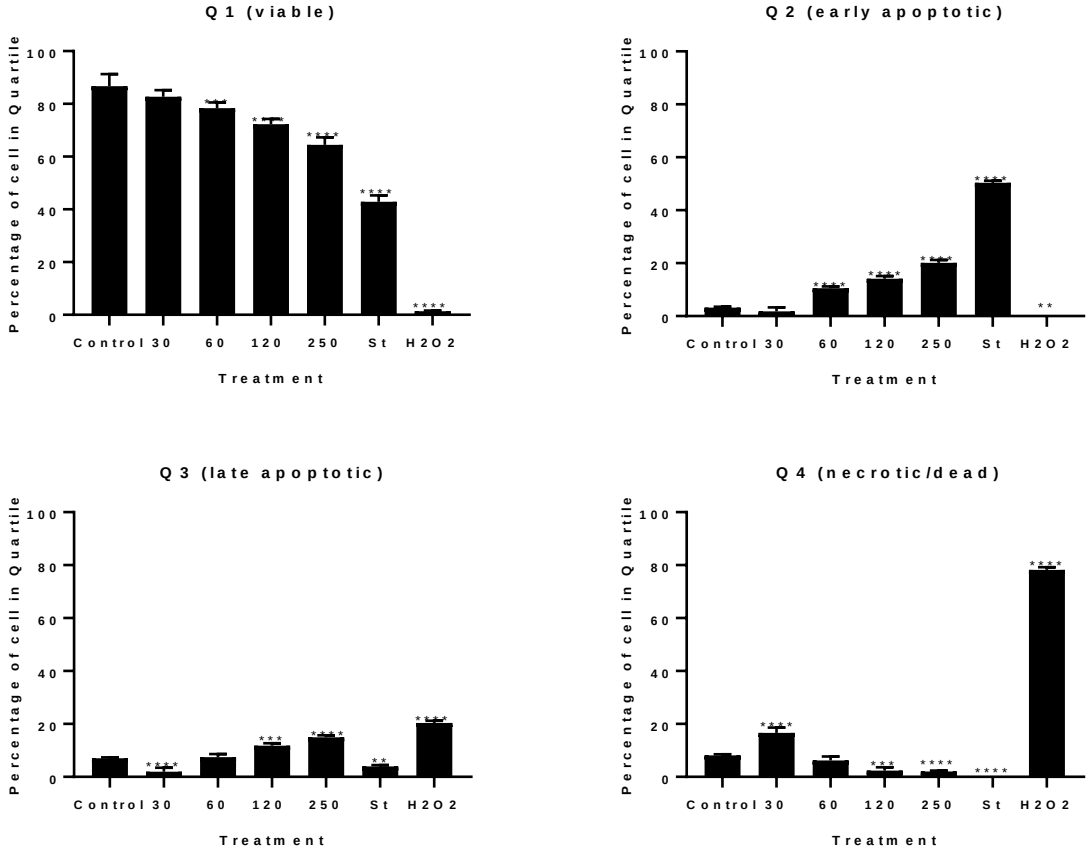

Figure 3.6 Flow cytometric analysis of cell death triggered by malvidin-3-galactoside pathways based on phosphatidylserine externalisation and membrane permeability. (A) Example scatter plots derived from flow cytometric analysis of Caco-2 cells treated with different concentrations (30-250 µM) of malvidin-3-galactoside for 24 hours. At the end of the treatment, cells were trypsinised, stained with annexin V-FITC , and Propidium iodide , and analysed by flow cytometry. A total of 10,000 events were counted for each treatment. Cells were categorised into quartiles according to the extent of annexin V-FITC staining (x-axis) and propidium iodide staining (y-axis). (B) Proportions of cells in each quartile following the different treatments. Q1 represents healthy cells; Q2 represents cells in early apoptosis; Q3 represents cells in late apoptosis; Q4 represents dead cells. Values are the mean+SEM for three independent experiments. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

The concentration-dependent effects of the aglycone form of malvidin (Figure 3.7) were almost identical to those of the mavidin-3-galacostide. The proportion of cells in Q1 was significantly decreased by malvidin treatment at concentrations of 60µM and above ($p<0.001$ at 60µM and $p<0.0001$ at 120 and 250µM), and this was coordinated with an increase in the proportion of early apoptotic cells in Q2 at malvidin concentrations of 120 and 250µM ($p<0.0001$ in both cases). The proportion of cells in Q3 was also moderately but significantly increased by malvidin treatments at 60, 120 and 250µM ($p<0.0001$ in all cases, while the proportion in Q4 was significantly decreased at concentrations of 120 and 250µM ($p<0.0001$ in both cases).

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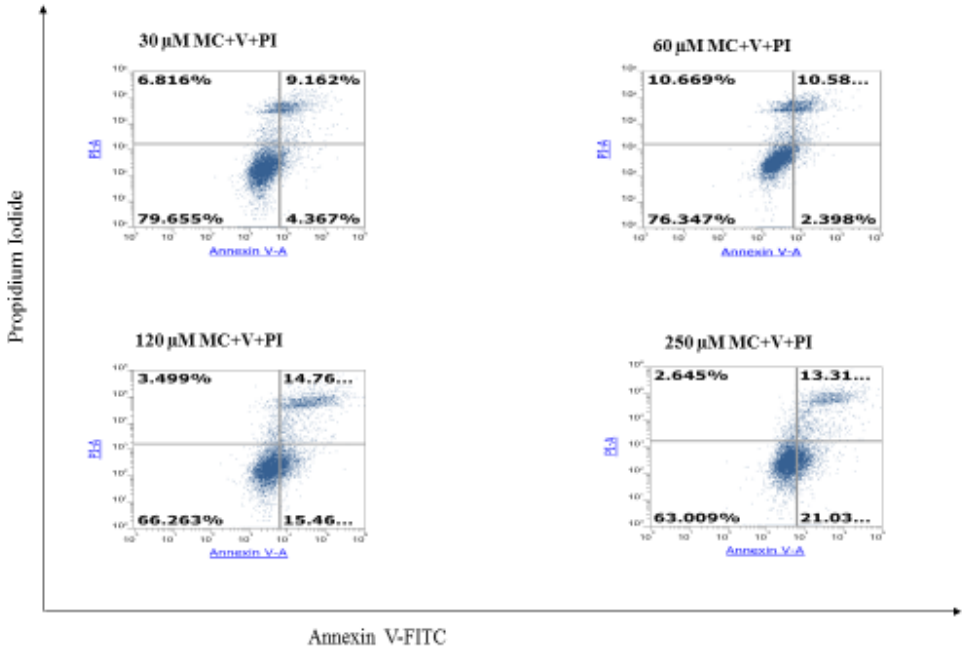

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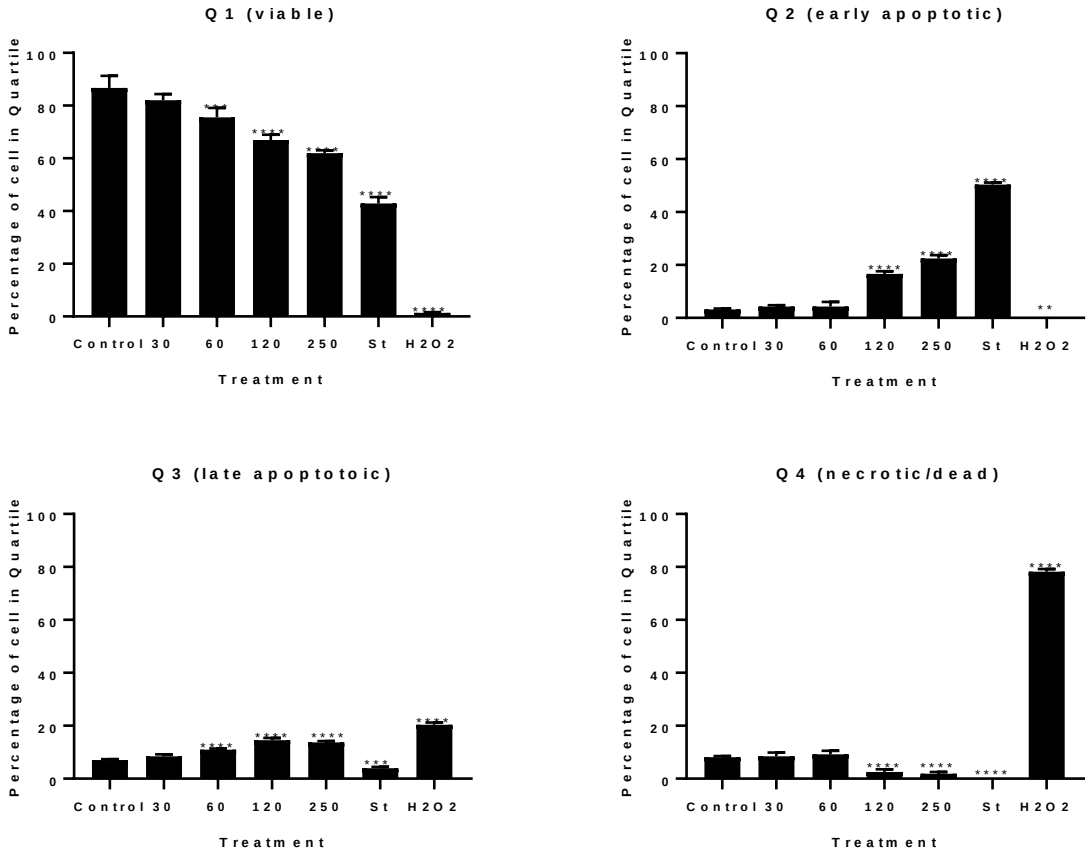

Figure 3.7 Flow cytometric analysis of cell death triggered by malvidin chlorid pathways based on phosphatidylserine externalisation and membrane permeability. (A) Example scatter plots derived from flow cytometric analysis of Caco-2 cells treated with different concentrations (30-250 µM) of malvidin chlorid for 24 hours. At the end of the treatment, cells were trypsinised, stained with annexin V-FITC , and Propidium iodide , and analysed by flow cytometry. A total of 10,000 events were counted for each treatment. Cells were categorised into quartiles according to the extent of annexin V-FITC staining (x axis and propidium iodide staining (y-axis). (B) Proportions of cells in each quartile following the different treatments. Q1 represents healthy cells; Q2 represents cells in early apoptosis; Q3 represents cells in late apoptosis; Q4 represents dead cells. Values are the mean+SEM for three independent experiments. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

Treatment of Caco-2 cells with 2,4,6 trihyodroxybenzaldehyde elicited a very simliar pattern of effects (Figure 3.8) to that described for the blueberry extract. The proportion of cells in Q1 decreased in a concentration depedent manner at concentrations of 60µM and above ($p<0.0001$ in all cases). The proportion of cells in Q2 and Q3 significantly increased at all concentrations tested ($p<0.0001$ in all cases). The proportion of cells in Q3 was also moderately but significantly increased by malvidin at all concentrations tested ($p<0.00010$ while the proportion in Q4 was decreased at all concentrations tested ($p<0.0001$).

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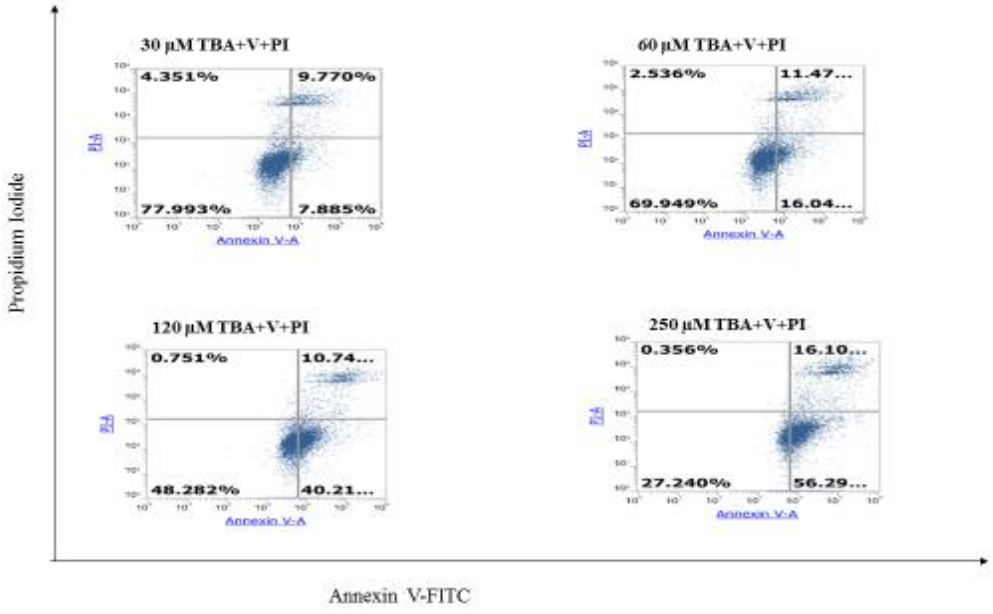

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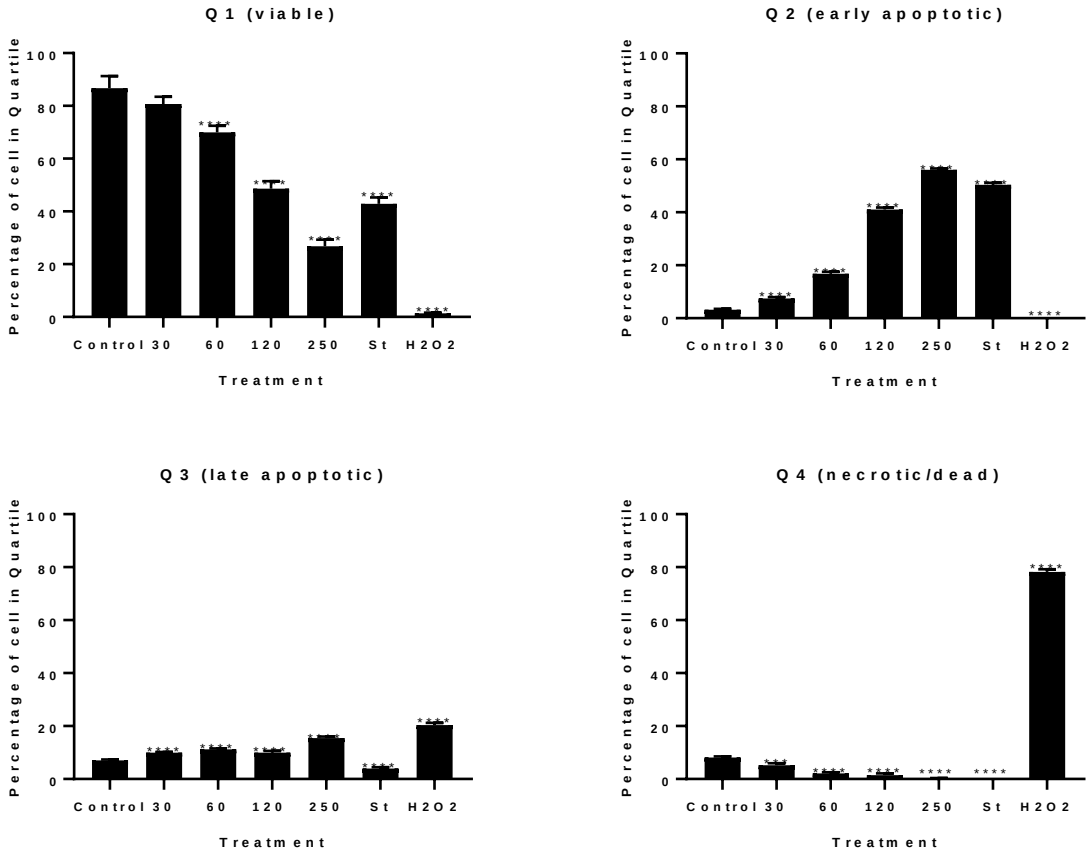

Figure 3.8 Flow cytometric analysis of cell death triggered by 2,4,6-trihydroxybenzaldehyde pathways based on phosphatidylserine externalisation and membrane permeability. (A) Example scatter plots derived from flow cytometric analysis of Caco-2 cells treated with different concentrations (30-250 µM) of 2,4,6-trihydroxybenzaldehyde for 24 hours. At the end of the treatment, cells were trypsinised, stained with annexin V-FITC , and Propidium iodide , and analysed by flow cytometry. A total of 10,000 events were counted for each treatment. Cells were categorised into quartiles according to the extent of annexin V-FITC staining (x axis) and propidium iodide staining (y-axis). (B) Proportions of cells in each quartile following the different treatments. Q1 represents healthy cells; Q2 represents cells in early apoptosis; Q3 represents cells in late apoptosis; Q4 represents dead cells. Values are the mean+SEM for three independent experiments. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

3.3.4 The Effects of blueberry extract, malvidin-3-galactoside malvidin and 2,4,6-trihydroxybenzaldehyde on caspase 3/7 activity in Caco-2 cells.

The results of the caspase 3/7 activity analysis are presented in figure 3.9. Treatment of the cells with staurosporine led to a significant increase in signal (figure 3.9A) confirming that activation of caspase 3/7 was observed in response to stimulation of apoptosis. Treatment of Caco-2 cells with blueberry extract and 2,4,6 trihydroxybenzaldehyde also both led to concentration-dependent increases in caspase 3/7 activity. The caspase activation was statistically significant compared to the untreated cells at concentrations of 125 and 250 µg/ml for the blueberry extract and 125 and 250 µM for the 2,4,6 trihydroxybenzaldehyde, compared to the negative control (untreated cells) and positive control (staurosporine). Neither the malvidin-3-galactoside nor the malvidin in its aglycone form caused any significant activation of caspase 3/7 at any of the concentrations tests (30-250 µM).

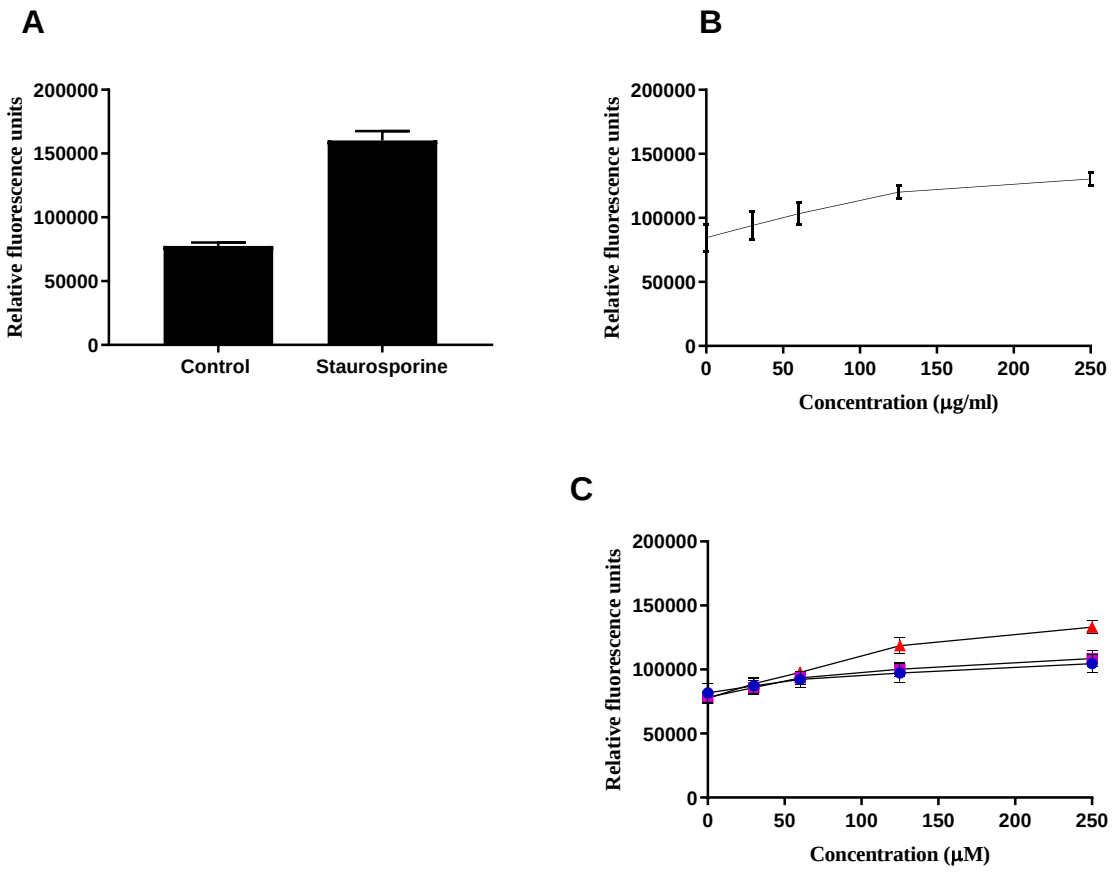

Figure 3.9 Effects of blueberry extract, malvidin-3-galacotside and two of its metabolites on caspase 3/7 activity in Caco-2 Cells. (A) comparsion of caspase 3/7 activity in untreated Caco-2 cells (negative control) and Caco-2 cells treated with staurosporine (positive control). (B) Caspase activity in Caco-2 cells treated with blueberry extract at concentrations of 30-250 µg/ml. (C) Caspase activity in Caco-2 cells treated with varying concentrations of, □ malvidin-3-galactoside, ● malvidin chloride or Δ 2,4,6-trihydroxybenzaldehyde for 24 hours. At the end of the treatment 100 µl/well Caspase-Glo 3/7 reagent was added, cells were incubated again for 30 minutes and caspase 3/7 activity was measured using a luminometer. The data represent the mean ± SD of 3 replicates.

3.4 Discussion

Numerous studies have shown that berry extracts and their metabolites can inhibit cell proliferation and stimulate apoptosis in cancer cells with little or no cytotoxic effects on healthy cells (Seeram *et al*., 2005). The current work aimed to study the impact of blueberry extract, the major anthocyanin in the extract and two of its metabolites on several properties related to cancer cell viability, using Caco-2 cells as a suitable model. The experimental conditions were chosen so that the Caco-2 cells were still actively dividing, rather than at confluence when Caco-2 cell growth is inhibited and the cells start to differentiate into monolayers structurally similar to the small intestinal epithelium (Buzza *et al*., 2010).

The first analyses were performed using the MTT assay. This is a widely used colorimetric assay based on the reduction of a tetrazolium compound to a formazan product by metabolism in live cells. The colour development is dependent on a combination of cell numbers and their metabolic activity. As presented in figure 3.1, blueberry extract and metabolites have the capacity to decrease either Caco-2 cell numbers or their viability (or a combination of the two), leading to a concentration-dependent decrease in the signal obtained. The blueberry extract and the 2,4,6 trihydroxybenzaldehyde were more potent than the mavidin-3-galacotisde or the malvidin in its aglycone form.

The morphological observations depicted in figure 3.3, support the observations from the MTT assay, suggesting that blueberry extract at 120 µg/ml or 2,4,6,-trihydroxybenzaldehyde at 120 µM caused a reduction in cell numbers with some evidence of increased numers of apoptotic cells, while the effects of malvidin-3-galactoside and malvidin in its aglycone form were less apparent.

The annexin-V FITC/PI cell staining strategy was employed to investigate the mechanisms responsible for the effects observed with the MTT assay. This assay has been used widely (for examples, see Seeram *et al*., (2005) and Zhao *et al*., 2013).

This approach was used to determine the type of cell death induced by polyphenols and anthocyanins. The present study results indicate that Caco-2 cell death following exposure to blueberry extract, malvidin-3-galactoside, and two of its metabolites occur primarily via stimulation of apoptosis.

The mechanism of action was further analysed by explicitly examining the activity of caspase 3 and 7 (Figure 3.10). Caspases are a family of cysteine proteases activated in sequence during apoptosis and hence used to analyse apoptosis induction. One of the most studied mammalian caspases is caspase-3, due to it being an effector which has the ability to progress caspase-2, -6, -7, and -9 proenzymes besides shearing a sequence recognition with caspase-7 (Miret *et al*., 2006). The stabilized luciferase and proprietary buffer system improve caspase glow 3/7 assay performance and, as a result, the assay is less likely to be affected by compound interference in addition to the advantage that it is designed for use with multi well plate formats. This approach revealed significant caspase 3/7 only with the blueberry extract and the 2,4,6-trihydroxybenzaldehyde, and only at the higher concentrations tested.

3.5 Conclusion

Based on the results presented in this chapter, blueberry extract (at 120 -250 µg/ml) and 2,4,6-trihydroxybenzaldehyde (at 120-250 µM) elicit increased Caco-2 cell apoptosis. It is not clear from these data whether these is the sole mechanisms of action other than an anti-proliferative effect on Caco-2. Malvidin-3-galactoside and malvidin in its aglycone form have less pronounced effects and may not act through exactly the same pathways as there was no clear evidence they activated caspase 3/7.

CHAPTER FOUR

EFFECTS OF BLUEBERRY EXTRACT AND BLUEBERRY COMPONENTS ON CELLULAR ROS

The imbalance between the production of free radicals and reactive oxygen species (ROS), and the elimination of these products by protective antioxidant mechanisms is known as oxidative stress. This imbalance can lead to the accumulation of cellular damage with a possible impact on the whole organism that could contribute to the development of various pathophysiological conditions, including cancer, cardiovascular disease, and arthritis (Durackova, 2014). The mitochondrial respiratory chain is typically the source of the majority of ROS generated within cells (Poyton *et al*., 2009). ROS such as superoxide anion ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and organic peroxides are natural produces of the biological reduction of molecular oxygen formed by aerobic cells during endogenous metabolic reactions (Fridovich *et al*., 1978). In response, aerobic cells have evolved a wide range of enzymatic and non-enzymatic mechanisms that, in healthy conditions, limit ROS accumulation. For example, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase are cellular antioxidative enzymes that can protect cell membranes and cellular content by scavenging ROS (Cimen, 2008). In addition to these endogenous cellular antioxidants, exogenous antioxidants also play an essential role. For instance, ascorbic, lipoic acid, polyphenols, and carotenoids are all antioxidants derived from dietary sources.

The association between the consumption of fresh fruits and vegetables with the prevention or delay of chronic degenerative diseases, including cancer, has been investigated in numerous studies (Heber *et al.,* 2004). These foods are rich sources of dietary antioxidants. Hence, there is a long-standing interest in evaluating the role of such food-derived antioxidants in protecting cells from free radical-mediated oxidative stress. For instance, the consumption of blueberry polyphenols can enhance protection against free radicals and oxidative stress within red blood cells *in vitro* and *in vivo* (Youdim *et al.,* 2000). Also, black raspberries exhibit significant antioxidant protection in the gut epithelium of weanling pigs in line with their high content of anthocyanins (Wu *et al.,* 2006). Anthocyanins have been identified as potentially important food-derived antioxidants that are potent inhibitors of lipid peroxidation compared with many

other common antioxidants (Prior, 2003; Karakaya, *et al.*, 2004). Anthocyanin glycosides can be rapidly absorbed from the stomach after ingestion. Anthocyanin glycosides that are not absorbed from the stomach move into the small intestine, similar to flavonols, where the glycosides may be removed by various hydrolases and the phenolic aglycone may be absorbed (Keppler and Humpf, 2005). Anthocyanins that reach the colon are exposed to a substantial microbial population and may be degraded to sugar and phenolic components (McGhie and Walton, 2007).

Based on this, the Caco-2 cell line model of human epithelium was selected as a model for the studies described here due to the similarity in physiological and morphological characteristics compared to the intestinal epithelial cells under standard culture conditions, (Meunier *et al.*, 1995). The organic hydroperoxide, tert-butyl hydroperoxide (t-BHP) was used to produce oxidative stress in Caco-2 cells (Lapshina *et al.*, 2005) as a model system for examining the antioxidant properties of the commercial blueberry extract (detailed in chapters 2 and 3) and selected individual components or metabolites of the blueberry extract (as set out in chapter 3).

The hypothesis of this chapter is that Blueberry polyphenols and their metabolites act as an antioxidant agents

The aims of this studies described in this chapter were:

1. To determine the protective effects of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde on Caco-2 cells exposed to oxidative stress using the MTT assay.
2. To evaluate the antioxidant effects of non-cytotoxic concentrations of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde as ROS scavengers in the Caco-2 cell system.
3. To determine the effects of cytotoxic concentrations of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde on the level of intracellular ROS in Caco-2 cells.

4.1 Materials and Methods

4.1.1 Materials

The human colon epithelial carcinoma cell line (Caco-2) was purchased from the European collection of cell cultures (ECACC) Salisbury, UK. Phosphate buffered saline (PBS), penicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), dimethyl sulphoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), non-essential amino acids (NEAA), trypsin-EDTA solution, staurosporine, 2´,7´-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) and t-BHP were obtained from Sigma-Aldrich Chemical Co, Poole, UK.

4.1.2 Methods

4.1.2.1 Cell culture and treatment

Caco-2 cells were routinely cultured in DMEM cell containing 20% v/v FBS, 1% v/v L-glutamine, 1% v/v NEAA and 1% v/v penicillin. Cells were seeded in 25cm^2 tissue culture flasks, starting with a concentration of 1x 10^6 cells/ml (60,000 cell/cm^2), and were incubated at 37°C in a 5% CO_2 humidified incubator. Cells were sub-cultured on reaching approximately 80% confluence during routine culture. The cells were subcultured by removing the medium, washing with 3ml PBS, and then detached by incubating at 37°C 1ml trypsin/EDTA. Once detached, the trypsin was inhibited by the addition of 1ml of the full medium.

4.1.2.2 Determination of the IC_{50} for Tert-butylhydroxide

The effects of a range of concentrations of t-BHP on Caco-2 cells were determined using the MTT assay. Caco-2 cells were seeded into 96-well tissue culture plates at $1x10^4$ cells per well in 200 µl complete media. After 24 hours, the culture medium was replaced with 200µl fresh complete medium containing t-BHP at concentrations ranging from 0 to 40mM, After 2 hours exposure, the MTT assay was used to determined cell viability as described in section 3.1.2.3.

4.1.2.3 Evaluation of the protective effect of blueberry extract, malvidin-3-galactoside malvidin chlorideand 2,4,6-trihydroxybenzaldehyde on Caco-2 cells oxidatively challenged with 3mM t-BHP

The MTT assay was used to evaluate the effect of blueberry extract, malvidin-3-galactoside, malvidin chloride, and 2,4,6-trihydroxybenzaldehyde on Caco-2 cells subjected to oxidative stress by exposure to 3mM t-BHP. Caco-2 cells were seeded in 96-well plates at a density of $1x10^4$ cells per well in a 200µl complete medium. After 24 hours, the culture medium was replaced with fresh medium containing different concentrations of blueberry extract (0-15µg/ml) or malvidin-3-galactoside, malvidin chloride or 2,4,6-trihydroxybenzaldehyde (each at concentrations of 0-15µM). Trolox, at a concentration of 0.01% w/v was used as a positive control. After 24 hours of incubation, t-BHP (3mM) was added to the pretreated cells and incubated for 2 hours at 37°C. The MTT assay was then use to determine cell viability.

4.1.2.4 Determination of the antioxidant effect of blueberry extract, malvidin-3-galactoside, malvidin chloride, and 2,4,6-trihydroxybenzaldehyde as ROS scavengers

The antioxidant activity of the blueberry extract, malvidin-3-galactoside, malvidin chloride, and 2,4,6-trihydroxybenzaldehyde as ROS scavengers, was determent by using the dichlorofluorescin diacetate (CM-H_2DCFDA) cell permeable fluorogenic dye.

The non-fluorescent CM-H_2DCFDA dye has the capability of diffusing through the cell membranes. In the cytoplasm, this dye is rapidly converted to the membrane impermeable fluorescin dye due to the removal of the ester groups by intracellular esterases. This reagent can then be oxidised by cellular ROS to produce highly fluorescent dichlorofluorescein. Fluorescence intensity is then determined (by flow cytometry in the experiments described here), providing a marker of intracellular ROS production (Osseni *et al*., 1999).

Caco-2 cells were seeded in 25cm^2 flasks at 60,000 cells/cm^2. After 24 hours, the medium was aspirated and fresh medium containing blueberry extract (0-15µg/ml), malvidin-3-galactoside, malvidin chloride or 2,4,6-trihydroxybenzaldehyde (each a 0-15µM), or 0.01% w/v Trolox (positive control) was added and the cells incubated at 37°C with 5% CO_2 for 24 hours. At the end of the incubation, 3mM t-BHP was added to the treated cells and incubated for a further 2 hours. At the end of incubation, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of the medium. The cell suspensions were centrifuged for 3 minutes at 150 x g. The medium was aspirated and each cell pellet was resuspended in 500µl PBS. CM-H_2DCFDA (prepared freshly in DMSO) was added to a final concentration of 5µM, and the cells were incubated for 30 minutes at 37°C with 5% CO_2. At the end of incubation time, cells were kept on ice in the dark to reduce the photo-oxidation of dye via light. Fluorescence was measured at 520nm using a Beckman Coulter Epics XL flow cytometer. At least 10,000 events were acquired for each sample in the gated regions. The data from the flow cytometer were initially gated to exclude cell debris (gated regions designated R1 for cell debris and R2 for intact cells). The cells within R2 were then divided into two sub-populations based on the fluorescein fluorescence intensity (designated R3 for high fluorescence and R4 for low fluorescence regions, respectively). The boundary between R3 and R4 was set to best discriminate between the low fluorescence cells observed in the control treatments and the high fluorescence sub-population created as a result of the tBHP treatment. Both cell counts and

average fluorescence intensities were determined for the R3 and R4 sub-populations under all treatment conditions.

4.1.2.5 Analysis of intracellular reactive oxygen species in Caco-2 cells following exposure to blueberry extract, malvidin-3-galactoside, malvidin chlorideand 2,4,6 trihydroxybenzaldehyde

The intracellular ROS levels within Caco-2 cells following treatment with blueberry extract, malvidin-3-galanctoside, malvidin chlorideor 2,4,6 trihydroxybenzaldehyde were determined using CM H_2DCFDA and flow cytometry as described in section 4.1.2.4. Cell seeding and treatments steps were as described above except that the t-BHP treatment step was omitted and cells were exposed to high concentrations of blueberry extract (30-250µg/ml) or to malvidin-3-galactoside, malvidin chlorideor 2,4,6-trihydroxybenzaldehyde (30-250µM) or Trolox at 0.01% w/v.

4.1.3 Statistical analysis

Each experiment was repeated three times on separate occasions. The data were analysed (using GraphPad Prim software) by one-way analysis of variance (ANOVA). Followed by Dunnett's test post hoc. Statstical significance was assumes at $p<0.05$.

4.2 Results

4.2.1 Determination of IC_{50} for t-BHP

In the experiments described below, t-BHP was used to induce oxidative stress in Caco-2 cells. Thus, as a preliminary to that work, the IC_{50} for t-BHP was determined. Caco-2 cells were exposed to t-BHP (0-40mM) for 2 hours and then analysed using the MTT assay. This demonstrated concentration-dependent toxicity caused by the t-BHP treatment (Figure 4.1), with the IC_{50} estimated at approximately 3mM. Accordingly, this concentration was used to induce oxidative stress in Caco-2 cells in the subsequent experiments evaluating the protective effect of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6 trihydroxybenzaldehyde.

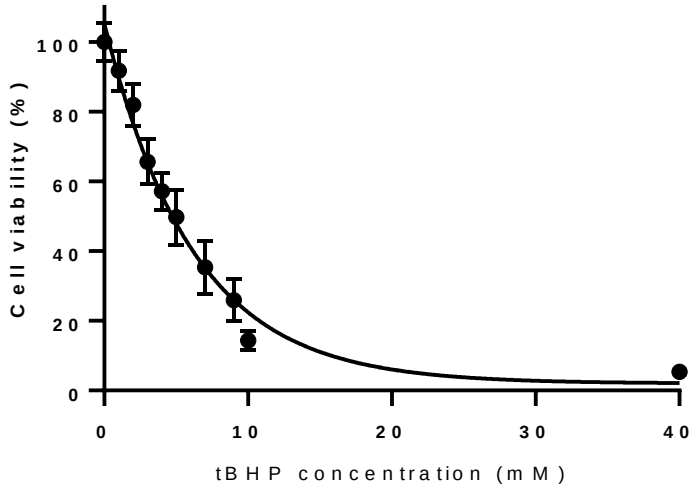

Figure 4.1 Effect of t-BHP on Caco-2 cell viability. Cultured cells were incubated with a range of t-BHP concentrations (0-40 mM) for 2 hours. The MTT assay was used to evaluate cell viability. IC50 estimated at approximately 3mM. The data represent the means ± SD of three independent experiments.

4.2.2 Protective effects of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde on Caco-2 cells exposed to oxidative stress

The effects of blueberry extract, malvidin-3-galactoside, malvidin chloride, and 2,4,6-trihydroxybenzaldehyde on the viability of Caco-2 cells subjected to oxidative stressed with 3mM t-BHP are depicted in Figure 4.2. In the absence of t-BHP treatment, none of the agents tested had any significant effect on cell viability at any of the concentrations tested. Pre-treatment with the antioxidant. Trolox (at 0.01% w/v), which was used as a positive control, partially protected the cells, significantly reducing the effects of t-BHP treatment on Caco-2 cell viability ($p<0.0001$). In contrast, neither the blueberry extract nor any of the purified compounds tested (malvidin-3-galactoside, malvidin chloride or 2,4,6-trihydroxybenzaldehyde afforded the cells any significant protection from the t-BHP at any of the concentrations tested.

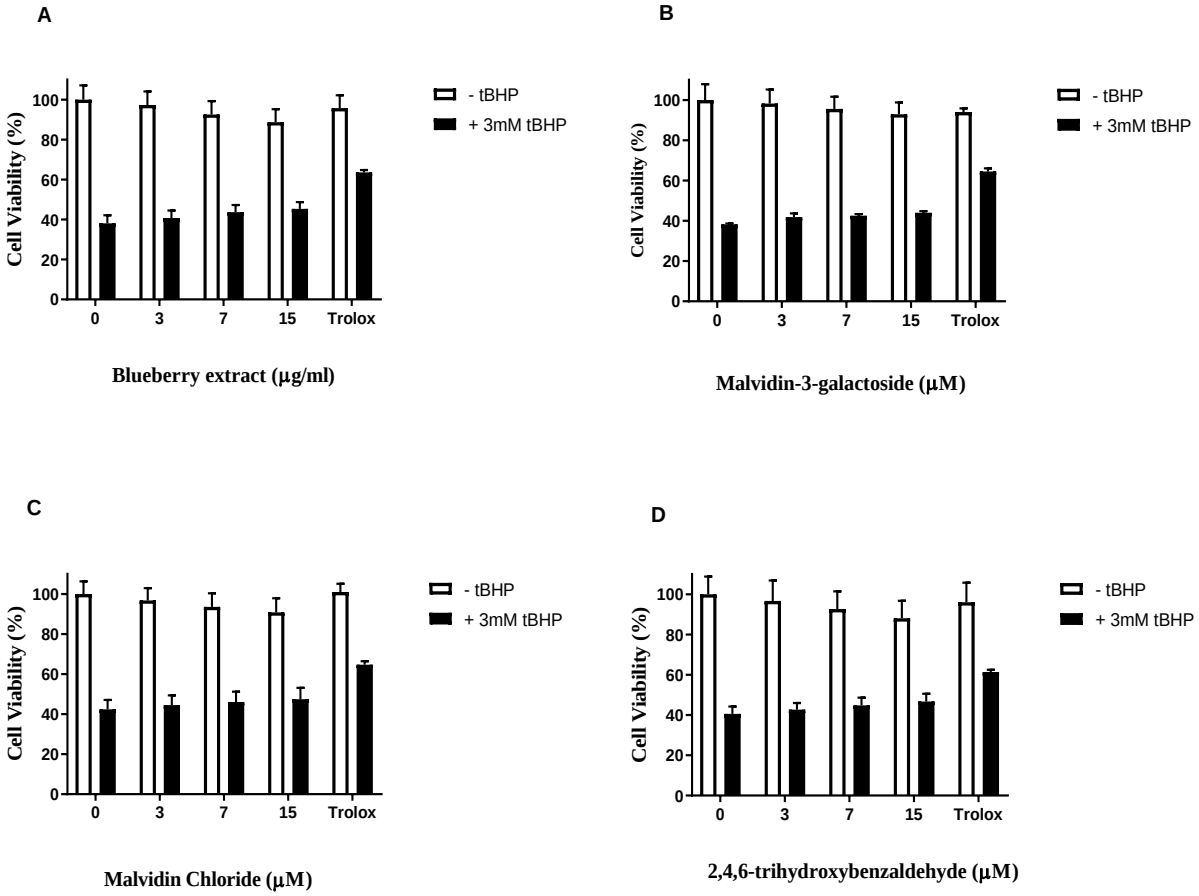

Figure 4.2 Viability of Caco-2 cells treated with different concentrations of blueberry extract (0, 3, 7 and 15 µg), malvidin-3-galactoside, malvidin chloride or 2, 4, 6-trihydroxybenzaldehyde, (0, 3, 7 and 15 µM) or Trolox (0.01% w/v) used as a positive control, with and without subsequent exposure to 3mM t-BHP for 2 hours. Cell viability of Caco-2 cells was assessed using the MTT assay. Data represent the mean ± SD of 3 independent experiments.

4.2.3 Evaluation of the antioxidant effect of non-cytotoxic concentrations of blueberry extract, malvidin chloride, malvidin-3-galactoside and 2,4,6-trihydroxybenzaldehyde as ROS scavengers

Flow cytometry with the fluorogenic dye CM-H_2DCFDA was used to assess the ROS scavenging effects of the crude blueberry extract and the three purified test compounds in

Caco-2 cells. Example data for untreated control cells and cells treated with tBHP are shown in figure 4.3. In the absence of the tBHP, most cells exhibitedlow fluorescence with a smaller subpopulation exhibitiedng much higher fluorecence (figure 4.3A). Treatment with tBHP treatment led to an increase in the high flurescence sub-population (figure 4.3B) confirming that this treatment was effective in creating intracellular ROS detectable with the dye.

(A) (B)

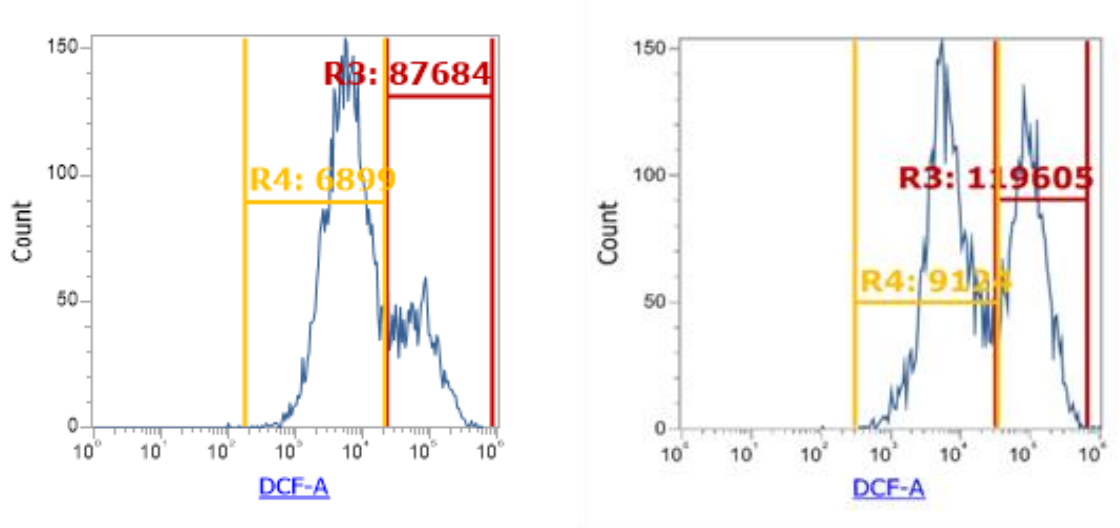

Figure 4.3 Example histograms of fluorescein staining in untreated (A) and tBHP-treated (B) Caco-2 cells. The region highlighted with red lines and text indicates the high fluorescence intensity sub-population (designated R3) and the region highlighted with orange lines and text indicates the low fluorescence intensity subpopulation (designated R4).

Trolox (0.01% w/v) was used as a positive control antioxidant in all experiments. Therefore, control data (cells with no pre-treatment or pre-treated with Trolox +/- subsequent exposure to tBHP) were pooled from all 12 independent experiments (3 each for the blueberry extract, malvidin-3-galacostide, malvidin chloride and 2,4,6-trihydroxybenzaldehyde) to examine the effects of Trolox in detail. Results are shown in figure 4.4

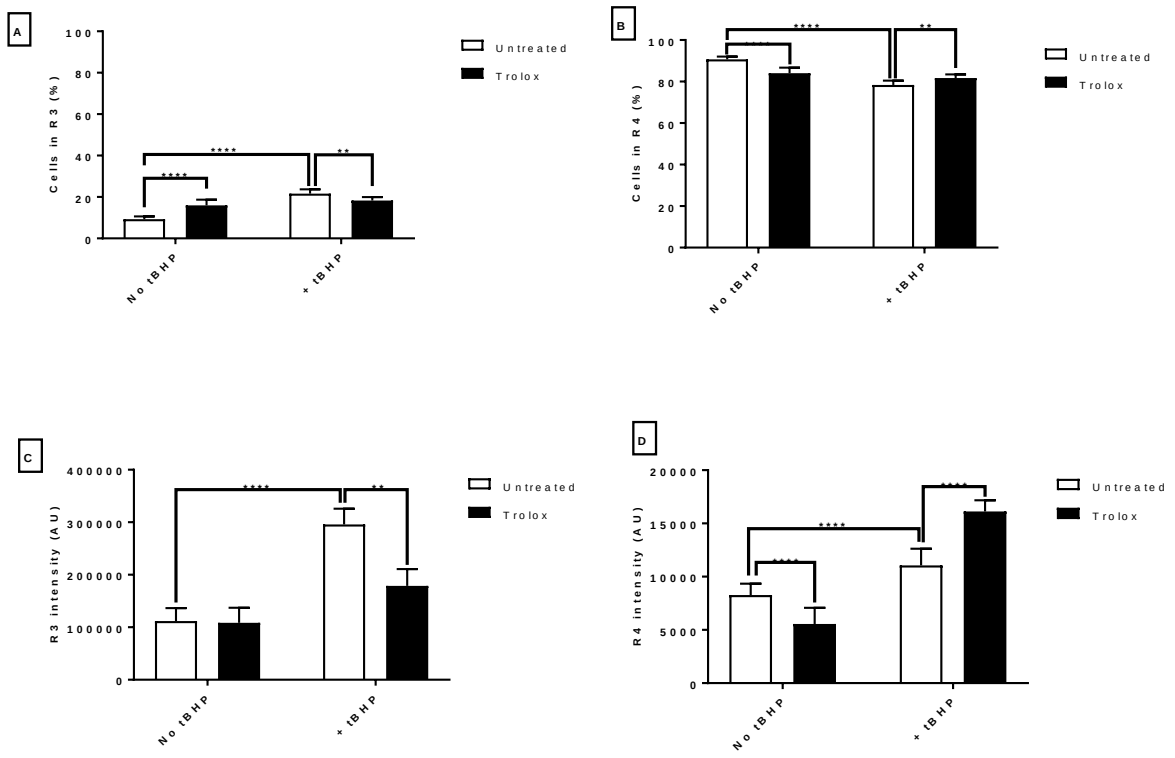

Figure 4.4 Effects of pre=treatment with Trolox on intracellular ROS in the absence or presence of subsequent exposure to 3mM tBHP. Graphs A and B depict the percentage of high fluorescence cells (defined as being within the R3 gated region by flow cytometric analysis) and low fluorescence cell (defined as being within the R4 gated region by flow cytometric analysis), respectively. Graphs C and D depict the mean fluorescence intensity of cells within R3 and R4, respectively. Bars and error bars indicate mean + S.D. for 12 independent experiments. Data were analysed by 2-way ANOVA with Tukey's test used post hoc. Significant differences are denoted by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and *** $p<0.0001$, respectively.

Analyses of these data confirm that tBHP treatment led to a significant increase in the proportion of cells within the high fluorescence intensity region R3 with a corresponding decrease in the proportion of low fluorescence intensity cells in R4 ($p<0.0001$).

tBHP treatment also led to a substantial increase in the mean fluorescence intensity of the cells in R3 ($p<0.0001$) and a smaller but still significant increase in the mean fluorescence intensity of the cells remaining in R4 ($p<0.0001$)

The antioxidant effect of Trolox treatment was evident from the significantly smaller increase in the proportion of cells in R3, and substantially smaller increase in mean fluorescence

intensity of the cells in R3, following exposure to tBHP ($p<0.0001$ in both cases). However, pre-treatment with Trolox also led to a significant increase ($p<0.0001$) in the mean fluorescence intensity of cells in R4 following subsequent exposure to tBHP.

In the absence of tBHP treatment the effects of Trolox appeared even more complex, leading to a small but significant increase in the proportion of cells in R3 ($p<0.0001$) without any change in mean fluorescence intensity of the cells in R3. The Trolox alone also led to a corresponding small reduction in the proportion of cells in the low fluorescence intensity R4 region and significantly reduced the mean fluorescence intensity of the cells within this region.

Overall, these findings suggest that Trolox provides a degree of protection in cells suffering oxidative stress as a result to exposure to tBHP but that in the absence of tBHP treatment the Trolox may cause a certain amount of low-level oxidative stress.

The effects of low, non-cytotoxic concentrations of blueberry extract are shown in Figure 4.5.

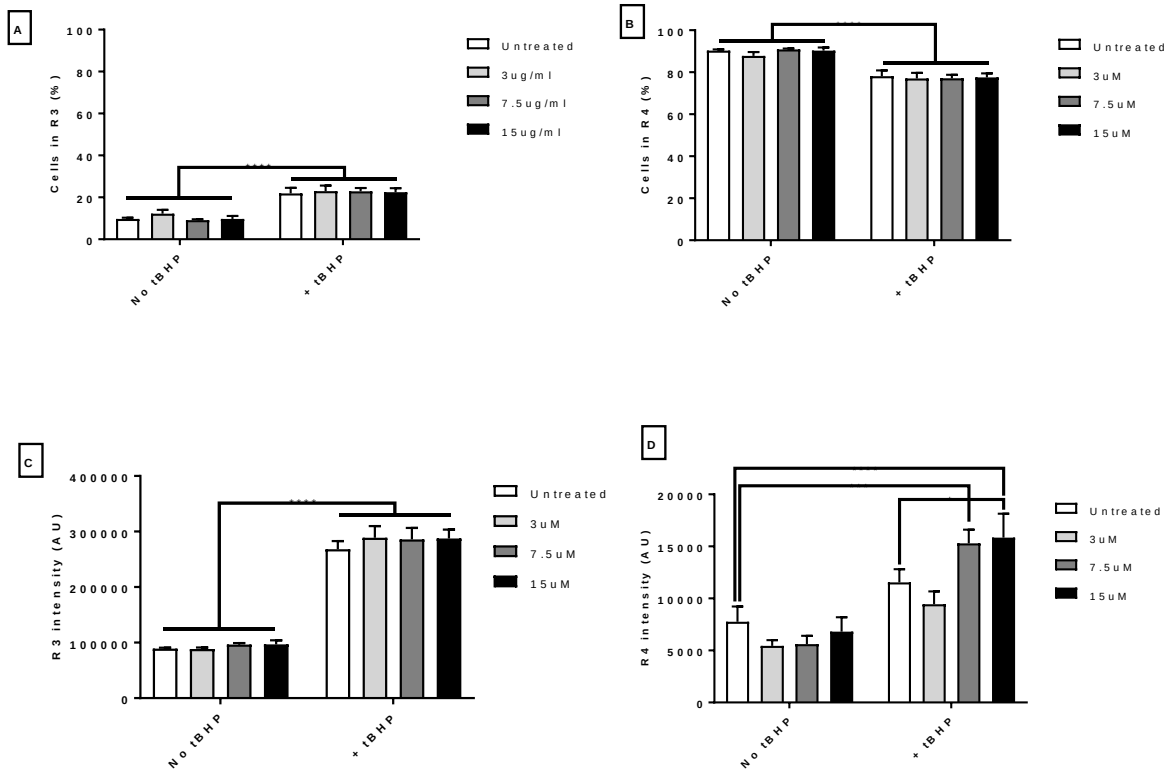

Figure 4.5 Antioxidant effects of blueberry extract as ROS scavenger with/without 3mM tBHP. The potential protective effects of low, non-cytotoxic concentrations of blueberry extract on tBHP-induced ROS production in Caco-2 cells were measured by flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration 60,000 cells/cm^2. After 24 hours the medium was aspirated, and fresh medium containing blueberry extract (at 0-15 µg/ml) was added and incubated with the cells for 24 hours. At the end of this incubation, 3mM t-BHP was added to some of the flasks and incubated for 2 hours. At the end of this incubation, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured by flow cytometry. Cells were classified into high (R3) fluorescence and low fluorescence (R4) intensities and the proportion of cells in R3 (A), or R4 (B) were determined. The mean fluorescence intensities of the cells in R3 (C) and R4 (D) were also determined. Bars indicate mean + S.D. for 3 independent experiments. Data were analysed by 2-way ANOVA with Tukey's test used for post hoc comparisons. Significant different are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

In the experiments investigating the effects of the blueberry extract, tBHP treatment led to significant increases in the proportion of cells in R3 (with corresponding decreases in the proportion of cells in R4) and in the mean fluorescence intensity of the cells in R3. Pre-treatment with blueberry extract did not have any significant effect on any of these parameters either in the absence or presence of subsequent exposure to tBHP. However, subtle effects of the blueberry extract were more apparent when the mean fluorescence intensities of the cells in R4 were evaluated. At the lowest concentration tested (3µg/ml) the blueberry extract appeared to moderately reduce R4 fluorescence intensity in the absence of tBHP treatment, although the difference did not achieve statistical significance. If this apparent effect is genuine then it appears to be lost at the higher concentrations tested under conditions where tBHP is not used. Moreover, pre-treatment with the highest concentration of blueberry extract (15µg/ml) led to a significantly larger increase in mean fluorescence intensity of the cells in R4 following tBHP treatment than was seen in cells treated with tBHP alone ($p<0.01$).

The effects of low, non-cytotoxic concentrations of malvidin-3-galactoside are shown in Figure 4.6

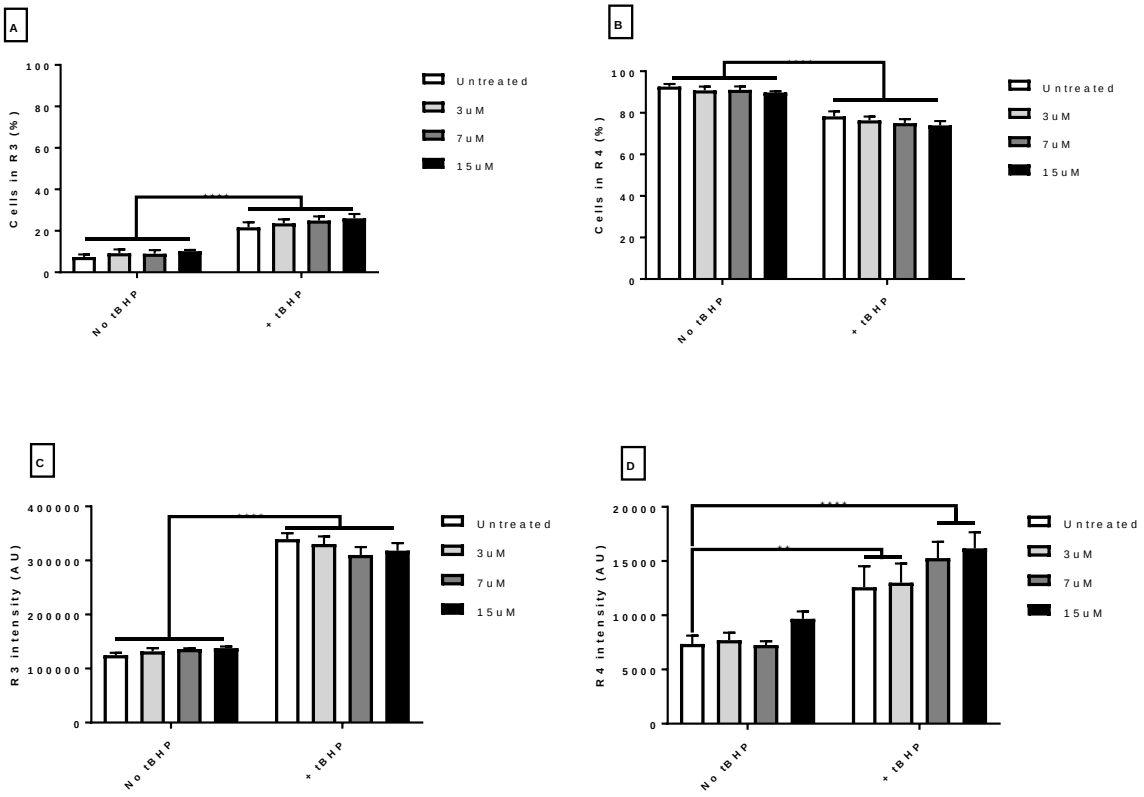

Figure 4.6 Antioxidant effects of malvidin-3-galactoside as ROS scavenger with/without 3mM tBHP. The potential protective effects of low, non-cytotoxic concentrations of malvidin-3-galactoside on tBHP-induced ROS production in Caco-2 cells were measured by flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration 60,000 cells/cm^2. After 24 hours the medium was aspirated, and fresh medium containing malvidin-3-galactoside (at 0-15 µM) was added and incubated with the cells for 24 hours. At the end of this incubation, 3mM t-BHP was added to some of the flasks and incubated for 2 hours. At the end of this incubation, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured by flow cytometry. Cells were classified into high (R3) fluorescence and low fluorescence (R4) intensities and the proportion of cells in R3 (A), or R4 (B) were determined. The mean fluorescence intensities of the cells in R3 (C) and R4 (D) were also determined. Bars indicate mean + S.D. for 3 independent experiments. Data were analysed by 2-way ANOVA with Tukey's test used for post hoc comparisons. Significant different are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

As in the previous experiments, tBHP treatment led to significant increases in the proportion of cells in R3 (with corresponding decreases in the proportion of cells in R4) and an increase in the mean fluorescence of cells in R3. Pre-treatment with malvidin-3-galactoside did not have any significant effect on any of these parameters either in the absence or presence of subsequent exposure to tBHP. However, as with the blueberry extract, more subtle effects of malvidin-3-galactoside were apparent when the mean fluorescence intensities of the cells in

R4 were evaluated. No significant effects were apparent in the absence of tBHP treatment but at the two highest concentration of malvidin-3-galactoside (7.5 and 15µM) there were larger increases in mean fluorescence intensity of the cells in R4 following tBHP treatment than were seen in cells treated with tBHP alone.

The effects of low, non-cytotoxic concentrations of malvidin chloride are shown in Figure 4.7.

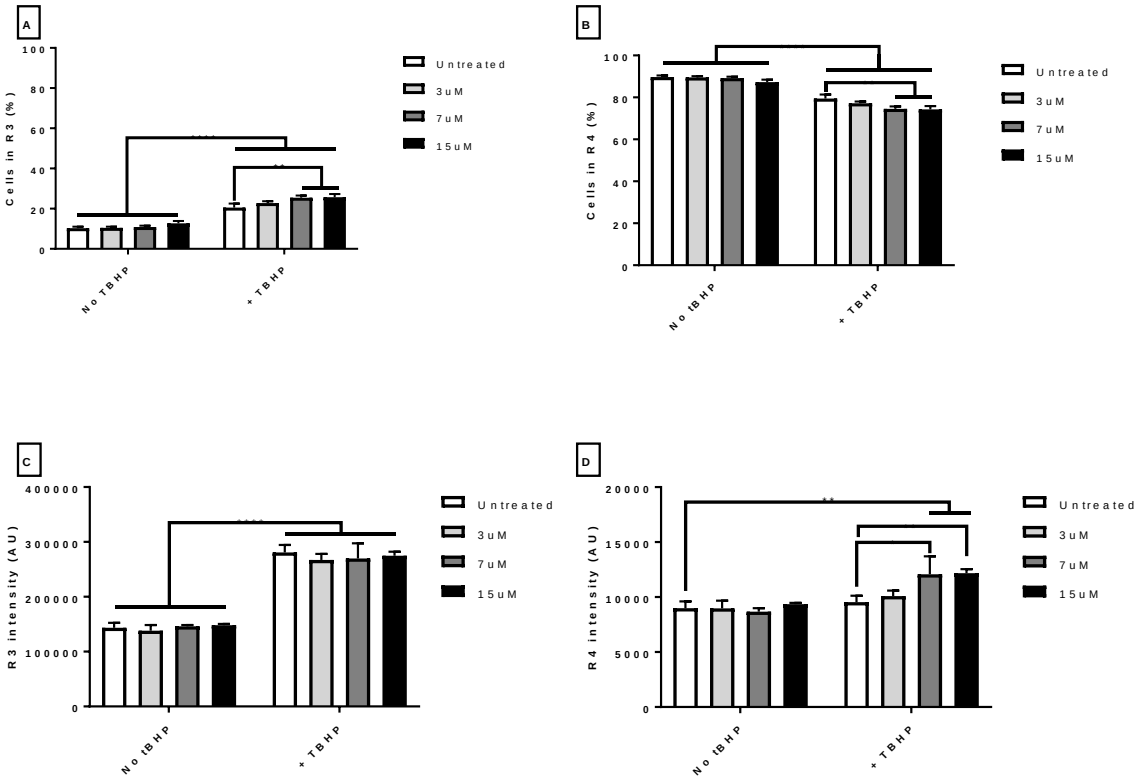

Figure 4.7 Antioxidant effects of malvidin chloride as ROS scavenger with/without 3mM tBHP. The potential protective effects of low, non-cytotoxic concentrations of malvidin chloride on tBHP-induced ROS production in Caco-2 cells were measured by flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration 60,000 cells/cm^2. After 24 hours the medium was aspirated, and fresh medium containing malvidin chloride (at 0-15 µM) was added and incubated with the cells for 24 hours. At the end of this incubation, 3mM t-BHP was added to some of the flasks and incubated for 2 hours. At the end of this incubation, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured by flow cytometry. Cells were classified into high (R3) fluorescence and low fluorescence (R4) intensities and the proportion of cells in R3 (A), or R4 (B) were determined. The mean fluorescence intensities of the cells in R3 (C) and R4 (D) were also determined. Bars indicate mean + S.D. for 3

independent experiments. Data were analysed by 2-way ANOVA with Tukey's test used for post hoc comparisons. Significant different are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Again, as expected, tBHP treatment led to significant increases in the proportion of cells in R3 (with corresponding decreases in the proportion of cells in R4) and an increase in the mean fluorescence of cells in R3. Pre-treatment with malvidin-3-galactoside did not have any significant effect on any of these parameters in the absence of subsequent exposure to tBHP. However, following exposure to tBHP, cells that had been pre-treated with 7.5 or 15µM malvidin chloride exhibited a moderately but significantly higher proportion of cells in R3 (and corresponding lower proportion of cells in R4) than cells exposed only to tBHP ($p<0.01$ in both cases). Moreover, while the mean fluorescence intensities of the cells in R3 were not affected by any of malvidin chloride pre-treatments either in the absence or presence of subsequent tBHP treatment, the mean fluorescence intensities of the cells in R4 following tBHP treatment were also significantly elevated by pre-treatment with 7.5 or 15µM malvidin chloride ($p<0.05$ for 7.5µM and <0.01 for 15µM).

The effects of low, non-cytotoxic concentrations of 2,4,6-trihydroxybenzaldehyde are shown in Figure 4.8.

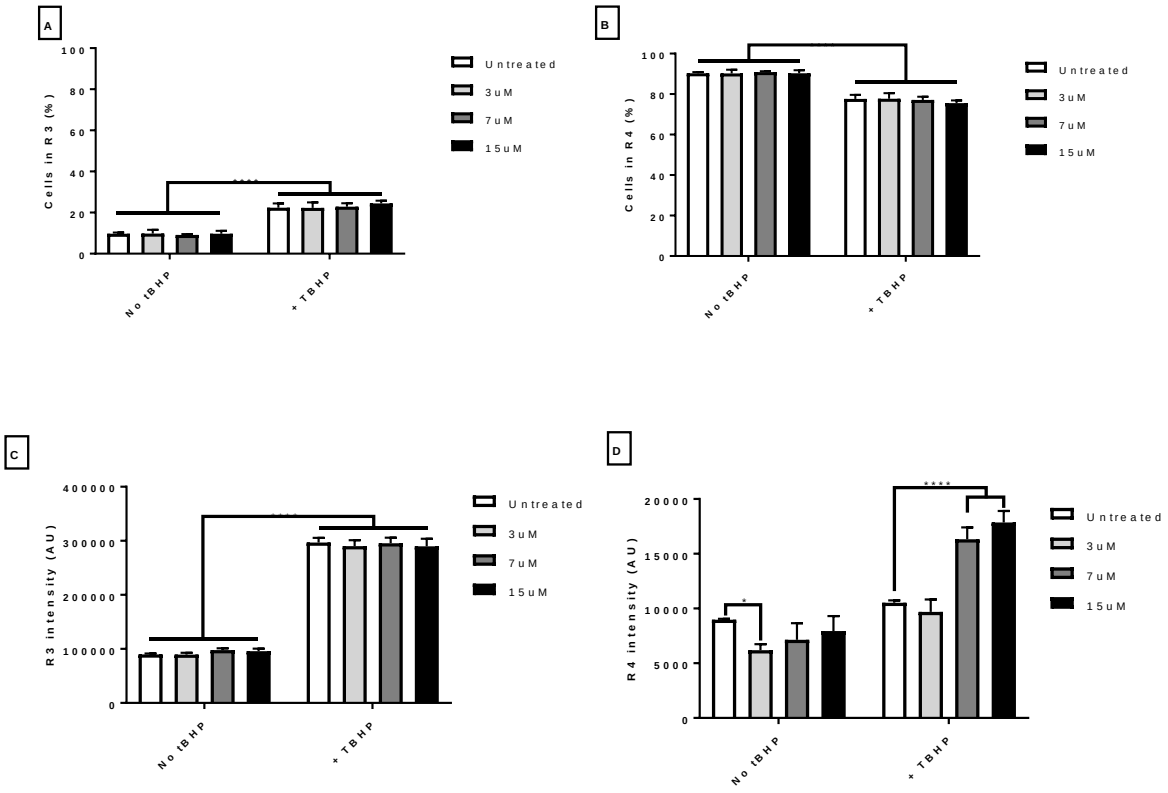

Figure 4.8 Antioxidant effects of 2,4,6-trihydroxybenzaldehyde as ROS scavenger with/without 3mM tBHP. The potential protective effects of low, non-cytotoxic concentrations of 2,4,6-trihydroxybenzaldehyde on tBHP-induced ROS production in Caco-2 cells were measured by flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration 60,000 cells/cm^2. After 24 hours the medium was aspirated, and fresh medium containing 2,4,6-trihydroxybenzaldehyde (at 0-15 µM) was added and incubated with the cells for 24 hours. At the end of this incubation, 3mM t-BHP was added to some of the flasks and incubated for 2 hours. At the end of this incubation, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured by flow cytometry. Cells were classified into high (R3) fluorescence and low fluorescence (R4) intensities and the proportion of cells in R3 (A), or R4 (B) were determined. The mean fluorescence intensities of the cells in R3 (C) and R4 (D) were also determined. Bars indicate mean + S.D. for 3 independent experiments. Data were analysed by 2-way ANOVA with Tukey's test used for post hoc comparisons. Significant different are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

As in all the previous experiments, tBHP treatment led to significant increases in the proportion of cells in R3 (with corresponding decreases in the proportion of cells in R4) and an increase

in the mean fluorescence of cells in R3. Pre-treatment with 2,4,6-trihydroxylbenzaldehyde did not have any significant effect on any of these parameters either in the absence or presence of subsequent exposure to tBHP. However, as with the blueberry extract, more subtle effects of 2,4,6-trihydorxylbenzaldehyde were apparent when the mean fluorescence intensities of the cells in R4 were evaluated. In the absence of subsequent tBHP treatment, exposure to the lowest concentration of 2,4,6-trihydroxylbenzaldehyde led to a significant decrease in mean fluorescence intensity of the cells in R4. This effect was lost at the higher concentrations tested. Moreover, pre-treatment with the higher concentrations of 2,4,6-trihydroxylbenzaldehyde, (7.5 and 15µM) led to increased fluorescence intensity of the cells in R4 compared with cells exposed only to tBHP.

4.2.4 Determination of the effects of cytotoxic concentrations of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde on intracellular reactive oxygen species in Caco-2 cells

While the experiments presented above in sections 4.2.2 and 4.2.3 examined the potential antioxidant effects of blueberry extract malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde at low (non-cytotoxic) concentration, the data presented in chapter 3 demonstrated that, at higher concentrations, all of these test compounds are cytotoxic to Caco-2 cells, Therefore, we evaluated the possible pro-oxidants effects of higher concentrations using the same CM-H_2DCFDA stain combined with flow cytometric analysis. Blueberry extract was tested at concentrations of 30-250µg/ml, and the single compounds were tested at concentrations of 30-250µM.

The effects of the higher concentrations of blueberry extract on intracellular ROS are shown in Figure 4.9. There was a clear concentration-dependent effect of the extract leading to increased intracellular ROS as evidenced both by the increasing proportion of cells within the high fluorescence gated area (R3) on the flow cytometric scatter plots and also by a significant increases of the mean fluorescence of cells within R3 (both increases being statistically significant for concentrations of 60 µg/ml and above). Equally, Figure 4.12 shows there was also a marked concentration-dependent increase in fluorescence in Caco-2 cells incubated with

high concentrations of 2,4,6-trihydroxybenzaldehyde were concentrations of 60 µM and above achieved statistical significance.

However, while concentration-dependent increases in fluorescence intensity were also seen following exposure of the cells to malvidin-3-galactoside and malvidin chloride (Figures 4.10 and 4.11), the magnitude of the changes was markedly less than that with equivalent concentrations of the 2,4,6-trihydroxybenzaldehyde. For both malvidin-3-galactoside and malvidin chloride, the effects achieved statistical significance at concentration of 125 µM and above.

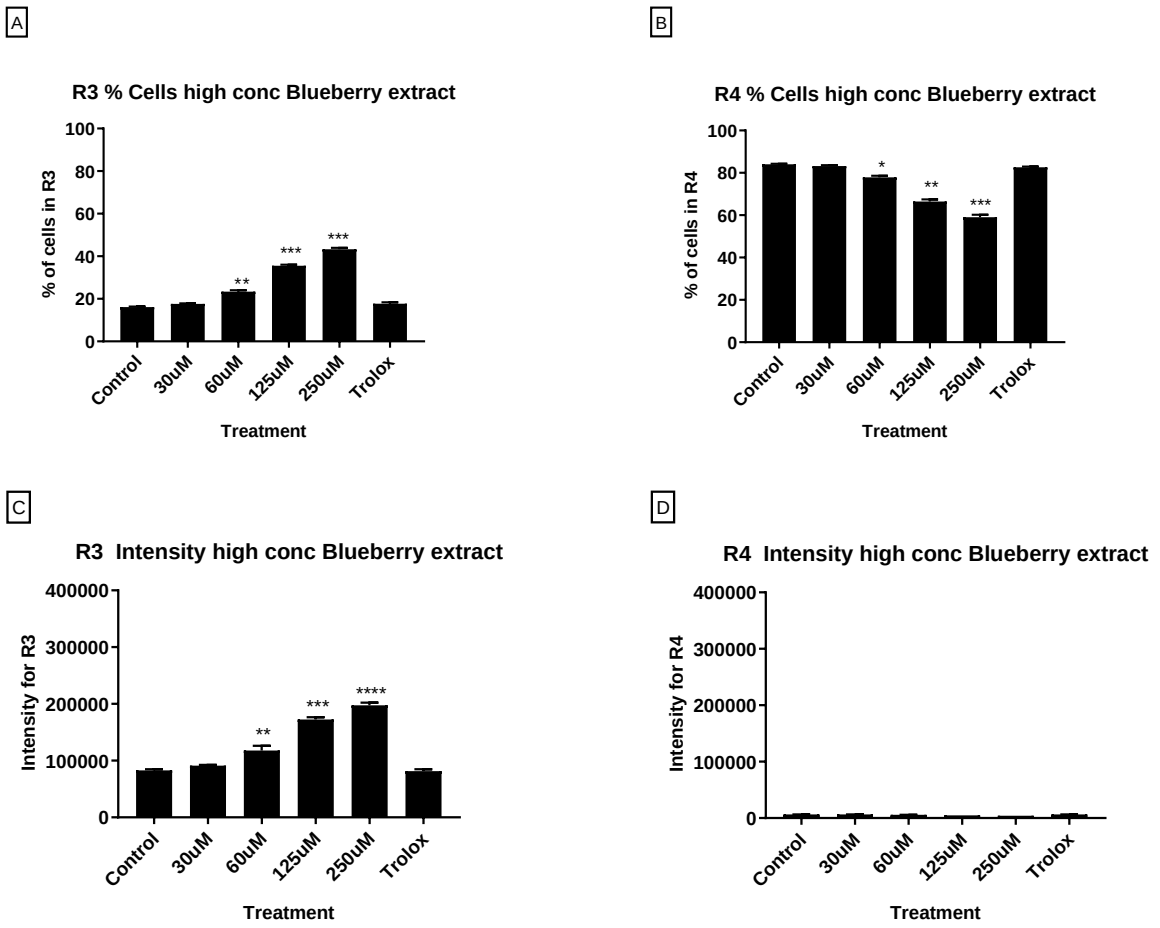

Figure 4.9 Llevels of intracellular reactive oxygen species determined using flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration 60,000 cells/cm^2. After reaching 24 hours, the medium was aspirated, and fresh medium containing blueberry extract (at 30-250 µg/ml) was added and incubated with the cells for 24 hours. At the end of incubation time, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured using a flow cytometer. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

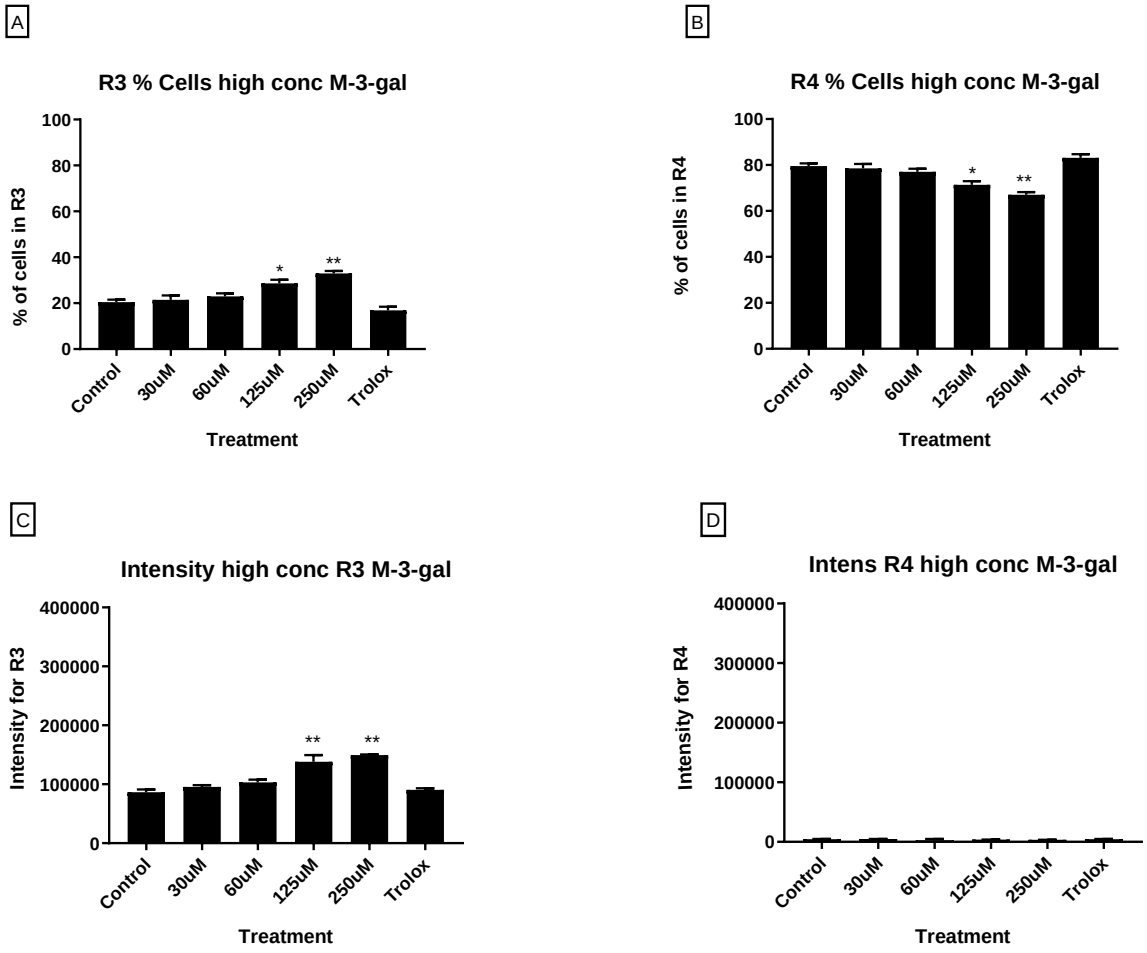

Figure 4.10 Levels of intracellular reactive oxygen species determined using flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration of 60,000 cells/cm^2. After reaching 24 hours, the medium was aspirated, and fresh medium containing Malvidin-3-galactoside (at 30-250 µM/ml) was added and incubated with the cells for 24 hours. At the end of incubation time, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured using a flow cytometer. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

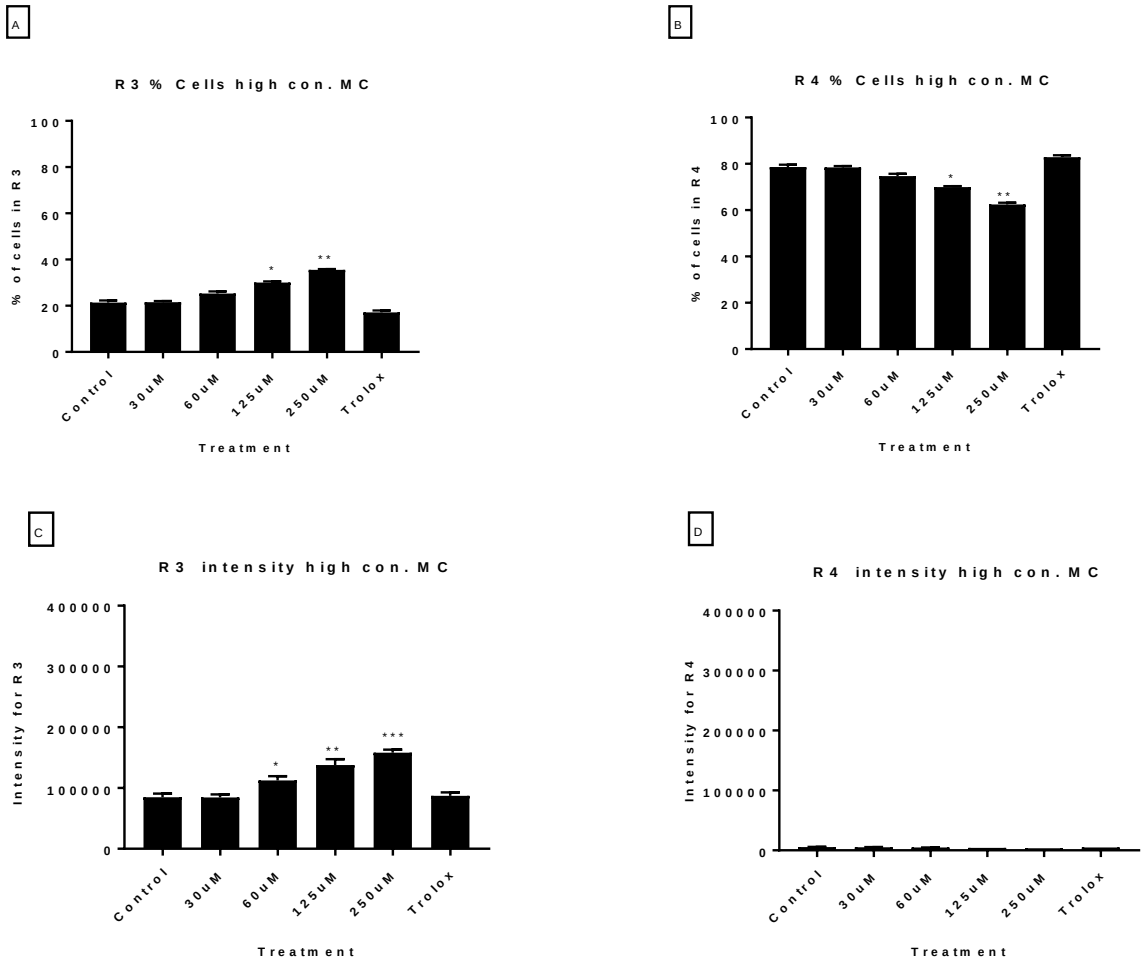

Figure 4.11 Levels of intracellular reactive oxygen species determined using flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration of 60,000 cells/cm^2. After reaching 24 hours, the medium was aspirated, and fresh medium containing Malvidin Chloride (at 30-250 µM/ml) was added and incubated with the cells for 24 hours. At the end of incubation time, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured using a flow cytometer. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

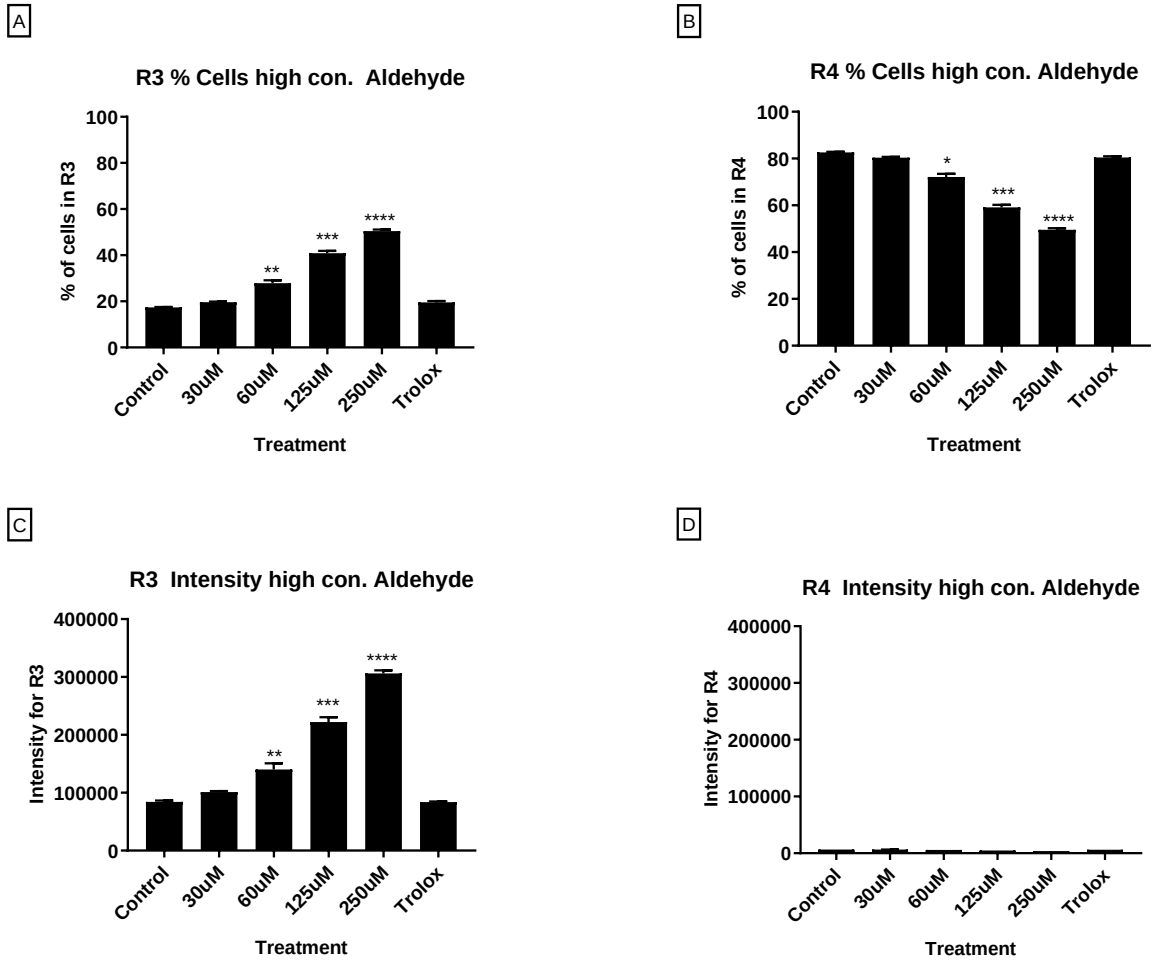

Figure 4.12 Levels of intracellular reactive oxygen species determined using flow cytometry. Caco-2 cells were seeded in 25cm^2 flasks at a concentration of 60,000 cells/cm^2. After reaching 24 hours, the medium was aspirated, and fresh medium containing 2,4,6-trihydroxybenzaldehyde (at 30-250 µM/ml) was added and incubated with the cells for 24 hours. At the end of incubation time, the medium from each flask was collected in 15ml centrifuge tubes. The cells were washed with 1ml PBS, trypsinised and the cell suspension combined with the corresponding sample of medium. DCFDA (prepared freshly in DMSO) was added to the cells at a final concentration of 5µM and incubated for 30 minutes. Fluorescence was measured using a flow cytometer. Data were analysed by one-way ANOVA followed by Dunnett's test post hoc (* $p<0.05$; ** $p<0.01$: *** $p<0.001$; **** $p<0.0001$ compared with untreated controls).

4.3 Discussion

Antioxidants are now widely accepted to help protect against chronic disease development (Palipoch *et al*., 2013), yet their precise modes of action remain to be confirmed. In this study, we focused on the effects of crude blueberry extract and selected components or metabolites of blueberries. Initial experiments were performed to test the hypothesis that the mixed components of the blueberry extract as well as a major anthocyanin component in the extract (malvidin-3-galacotside) and metabolites of this (malvidin and 2,4,6, trihydroxybenzaldehyde) would afford Caco-2 some protection from oxidative stress induced by exposure to t-BHP as evaluated using the MTT assay. The concentrations of each that were tested were selected to be in the non-cytotoxic range based on the data presented in chapter 2. At these concentrations, no evidence was found that any of the test materials provided any significant degree of protection against the cytotoxic effects of t-BHP. The validity of the model systems was confirmed using Trolox as a positive control. At 0.01% w/v, the Trolox did reduce the cytotoxic effects of the t-BHP on Caco-2 cells, albeit only moderately. As a follow-up to these initial experiments, the ROS scavenging properties of the blueberry extract and the individual compounds were assessed directly by investigating intracellular ROS production in Caco-2 cells after pre-treatment of the cells with the test materials followed by exposure to t-BHP. Flow cytometric analysis to detect oxidation of the fluorogenic dye dichlorofluorescin, suggest that 0.01% w/v Trolox can reduce ROS production in Caco-2 cells exposed to tBHP but the effects of the blueberry extract and the individual phytochemicals and phytochemical derivatives tested were more limited. There was some evidence that under basal conditions, the lowest concentrations of 2,4,6-trihydroxylbenzaldhyde and perhaps the blueberry extract may marginally reduce ROS levels in non-oxidatively stressed cells. However, any such effect was lost at the higher concentrations tested. There was no compelling evidence that the extract, purified malvidin-3 galactoside, or its metabolites were able to provide any significant protection against oxidative stress-induced by tBHP. Indeed, at the higher concentrations tested, there was some evidence that they marginally exacerbated ROS production in some cells following exposure to tBHP. In a previous study completed by Yokomizo and Moriwaki (2006), some flavonoids (kaempferol, quercetin and, luteolin) were incubated with Caco-2 cells at a concentration of 50 µM for 1 hour did provide some antioxidant protection from oxidative stress caused by treatment with 50 µM H_2O_2. However, the higher concentration of test compound used, the difference in incubations times, and the different methods for inducing

oxidative stress make comparisons between these studies difficult. Also, a study by Huang *et al*., 2016 performing the effects of malvidin and its glycosides on reactive oxygen

Species by decreasing ROS values in the endothelial cells at the concentration of 1,

5, and 10 μmol/L, respectively.

In contrast, when cells were exposed to higher concentrations of the extract or the individual compounds that had proven to have detectable cytotoxic effects based on the MTT assay (chapter 2), all were found to elicit significant increases in cellular ROS. Amongst the individual compounds, the blueberry extract and 2,4,6-trihydroxybenzaldehyde elicited more substantial increases in intracellular ROS than either the malvidin-3-galactoside or malvidin. Similar results have been reported by Mertens-Talcott *et al*. (2006), who found that red muscadine grape juice, containing the tannins ellagic acid (0.4 µmol/g) and ellagitannine (0.8 µmol/g), caused a significant increase in ROS production when applied to the Caco-2 cell line at different dilution (1:10, 1:100 and 1:1000). However, an additional study on anthocyanins, cyanidin, and delphinidin at concentrations of (25-100µM) after one hour incubation time, found to decrease ROS production in Caco- 2 cells (Cvorovic *et al*., 2010), which differs notably from the observations reported here. It could be that 24 h incubation = phase 1 metabolism, therefore less effective? Or maybe the instability of the aglycones in neutral PH values.

4.4 Conclusion

In conclusion, we found no evidence that the blueberry extract or the individual blueberry-derived components at non-cytotoxic concentrations have protective effects against oxidative damage induced by 3mM t-BHP in Caco-2 cells. In contrast, cellular ROS production was increased in Caco-2 cells treated with higher concentrations of blueberry extract or individual blueberry-derived components. These findings do not support the concept that blueberry-derived compounds provide significant antioxidant protection to gut epithelial cells but may exert other effects. However, limitations of the model system used should be acknowledged. The Caco-2 cells are a cancer cell line, and therefore their metabolism and response to the treatments may not accurately reflect the responses on healthy gut epithelial cells *in vivo*.

Moreover, the exposure of the Caco-2 cells to the compounds in culture is not under conditions that closely mirror physiological conditions in the gut *in vivo*, and these discrepancies could have a profound impact on the effects observed. Finally, these studies highlight the risk of focusing solely on the antioxidant properties of phytochemicals. They may exert health-promoting effects through completely different mechanisms, and the potential wider range of bioactivities should be considered.

CHAPTER FIVE

THE EFFECTS OF BLUEBERRY EXTRACT AND COMPONENTS IN COMBINATION WITH 5-FLUOROURACIL IN CACO-2 CELLS

Cancer death rates are still increasing, and colorectal cancer (CRC) is the fourth most common cause of cancer-related death, with 1 million new cases and 500 000 deaths worldwide each year (Bork *et al*., 2015). About 50% of patients who have undergone surgery for CRC retrogress and die from metastatic disease (Morris *et al*., 2014). Detection of colorectal tumours at an earlier stage improves prognosis as the spread of the tumour from the epithelium into the bowel wall and then into lymph nodes increases the risk of metastases (Xu W *et al*., 2020).

Despite the many side effects, chemotherapy is still the most widely used treatment for cancer (Merkow *et al*., 2013). Chemotherapy is designed to kill cancer cells via the use of cytotoxic agents, delivered either as a single drug or in combination. Chemotherapeutic agents target a range of cellular systems, for example, inhibiting cell division, damaging DNA, promoting cell differentiation, or promoting apoptosis (Shuck and Turchi, 2010; Siddik, 2002).

5-Fluorouracil, also known as 5-FU, is one of the chemotherapy drugs commonly used to treat CRC (Glimelius *et al.,* 2011). Since it was introduced about four decades ago, it has remained a central component of chemotherapeutic treatments for CRC (Moertel, 1994). The active metabolite of 5-FU, 5-fluorodeoxyuridylate binds to thymidylate synthase, forming a stable ternary complex that inhibits the enzyme (Galivan, 1977). Since thymidylate synthase provides the sole route for *de novo* synthesis of deoxythymidine monophosphate, 5-FU depletes cells of deoxythymidine monophosphate, and thereby deoxythymidine triphosphate, which is required for DNA synthesis (Klubes and Leyland-Jones 1989). However, even the best available combinations of 5-FU and other current chemotherapeutic agents for CRC produce imperfect outcomes. Hence, the search for new chemotherapeutic agents continues.

In this context, history illustrates the enormous potential that components of plants have in treating diseases. Famous examples include salicylic acid from the bark of willow, digitalis from foxglove, and morphine from the opium poppy. In particular, secondary metabolites from

plants have proven to be a rich source of commercial pharmacological compounds. Between 1983 and 1994, 41% of the newly approved drugs were derived from natural sources, including an even high proportion (>60%) of new anti-cancer and anti-infection agents. (Cragg *et al.*, 1997).

Epidemiological evidence suggests that plant-derived dietary components, such as polyphenols, may help reduce cancer incidence (Jemal *et al*., 2011). There is also some evidence that polyphenol compounds may prove helpful in reducing the side effects of chemotherapeutic agents and possibly even enhance their activity (De Kok *et al*., 2008). For example, cisplatin (cis-diamminedichloroplatinum) is a drug extensively used for treating different types of cancer that are also recognised to cause harmful side effects in humans. *In vitro* studies indicate the plant-derived phenolic compound trans-3,3',5,5'-tetrahydroxy-4'-methoxystilbene reduces cisplatin-induced DNA damage in lymphocytes and lipid peroxidation, protein carboxylation, and thiol group oxidation in blood platelets (Olas *et al*., 2006; Lewandowska *et al*., 2014). As a result of its radical scavenging antioxidative properties (Olas *et al*., 2008). It remains to be demonstrated whether these protective effects might reduce the side effects of cisplatin *in vivo* without compromising its effectiveness. Indeed, much more work remains to be done to thoroughly evaluate the potential for the use of plant bioactive as adjunct therapies for established chemotherapeutic agents.

This chapter describes an investigation of the blueberry-derived agents investigated in the preceding chapters as potential adjunct agents for use in combination with 5-FU, using Caco-2 cells as a model of CRC.

The hypothesis of this chapter presents Blueberry polyphenols and their metabolites have a synergic effect when combined with conventional chemotherapy (5-FU)

The aims of the work described in this chapter were to:

1. To determine the cytotoxicity of the 5-FU at different concentrations in Caco-2 cells.
2. To investigate the effects of blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde on 5-FU induced cytotoxicity in Caco-2 cells.

5.1 Materials and Methods

5.1.1 Materials

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). 5-Fluorouracil (5FU) and reagents for Caco-2 cell culture (as detailed in previous chapters) were obtained from Sigma-Aldrich Chemical Co, Poole, UK.

5.1.2 Methods

5.1.2.1 Cell culture and treatment

Caco-2 cells were routinely cultured as described previously (3.2.2.2).

5.1.2.2 Determination of the Cytotoxicity of 5-fluorouracil on Caco-2 cells using the MTT assay

Different concentrations of 5-FU were tested on Caco-2 cells using the MTT assay to determine its concentration-dependent cytotoxicity. Caco-2 cells were seeded into 96-well tissue culture plates at a density of $1x10^4$ cells/well in 200 µl DMEM complete media. After 24 hours, the cultured medium was replaced with a 200µl fresh medium containing different concentrations of 5-FU (0-1000 µM). After 24 hours of exposure, the MTT assay was then used to determine cell viability (as described in section 3.2.2.3.2).

5.1.2.3 Effects of blueberry extract, malvidin-3-galactoside, malvidin chloride, and 2, 4, 6-trihydroxybenzaldehyde on 5-fluorouracil induced cytotoxicity in Caco-2 cells

The cytotoxic effect of different concentrations of blueberry (0, 12.5, 25, 50µg/ml) and the individual phytochemicals (0, 12.5, 25, 50µM) in combination with 5-FU in Caco-2 cells was assessed. Caco-2 cells were seeded into 96-well tissue culture plates by adding 200 µl/well of cell suspension at a density of 1×10^4 cells/200 µl. After 24 hours, the culture medium was replaced with 200 µl fresh medium containing the various combinations of treatments. After

24 hours of treatment, the MTT assay was used to determine cell viability (as described in section 3.2.2.3.2).

5.1.3 Statistical analyses

Three independent experiments were completed with each test agent. Within each experiment, three replicate wells on the 96-well plates were used for each treatment combination. All data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's test.

5.2 Results and Discussion

5.2.1 The cytotoxicity of 5-Fluorouracil (5FU) on Caco-2 cells determined using the MTT assay

As a first step toward subsequent experiments, the concentration-dependent cytotoxicity of 5-FU on Caco-2 cells was evaluated using the MTT assay. Following a 24-hour exposure, 5-FU treatment caused concentration-dependent decreases in Caco-2 cells viably (Figure 5.1). A significant reduction was seen at all concentrations tested ($p<0.001$ for 1µM 5-FU and $p<0.0001$ for 5-1000µM). However, while the decline in viability was steep over the lower range of 5-FU concentrations, the concentration-dependent reductions slowed at the higher concentrations such that the maximum decrease in viability was approximately 50%. Based on these data, a concentration range of 0-50µM 5-FU was chosen for the subsequent experiments examining interaction with the blueberry-derived agents.

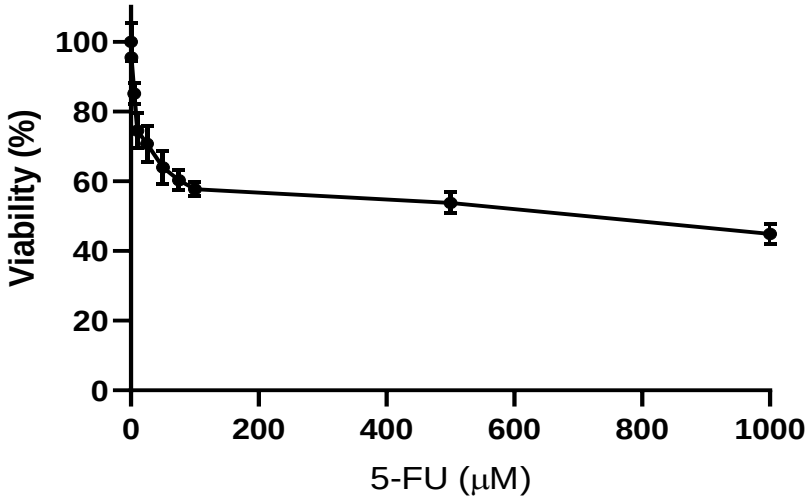

Figure 5.1 Effects of 5-Fluorouracil treatment on Caco-2 cell

Caco-2 cells were cultured and treated with 5-FU at various concentrations (0-1000 μM). The MTT assay was used to evaluate cell viability after 24 hours of exposure. These data represent the mean ±SD of three independent experiments.

5.2.2 Effects of blueberry extract, malvidin chloride, malvidin-3-galactoside and 2, 4, 6-trihydroxybenzaldehyde in combination with 5-fluorouracil on Caco-2 cell viability

The effects of different combinations of 5-FU with the blueberry extract and the blueberry-derived agents on Caco-2 cell viability were determined using the MTT assay. Caco-2 cells were incubated with blueberry extract (0, 12.5, 25 or 50μg/ml) or the individual blueberry-derived components (0, 12.5, 25 or 50μM) individually and in combination with 5-FU (0, 12.5, 25 or 50μM).

The results from combined treatment with 5-FU and blueberry extract are shown in figure 5.2. As expected, both the 5-FU and the blueberry extract exposures led to concentration-dependent increases in viability over the range of concentrations tested ($p<0.0001$ for both 5-FU and blueberry extract, based on 2-way ANOVA) with a significant interaction between the two

parameters ($p<0.0001$). In the absence of the blueberry extract, 5-FU treatment led to a significant decrease in viability at each concentration tested ($p<0.05$ for 12.5µM 5-FU and $p<0.0001$ for both 25 and 50µM 5-FU). In the absence of 5-FU, only the highest concentration of blueberry extract (50µg/ml) caused a significant decrease in viability ($p<0.001$). Moreover, as suggested by the effective interaction term from the 2-way ANOVA, the combination treatments all showed more marked effects than the individual elements of the combinations, with the most significant differentials at the highest concentrations. For example, treatment with 50µM 5-FU alone or 50µg/ml blueberry extract alone led to mean decreases of 17.4% and 8.1%, respectively, whereas the combination of the two led to a reduction of 61.3%. These data indicate the blueberry extract and 5-FU act synergistically in terms of cytotoxicity towards Caco-2 cells.

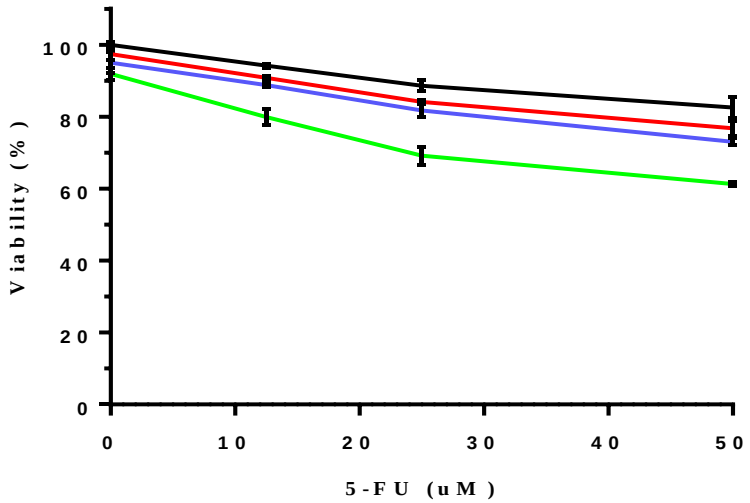

Figure 5.2. Effects of 5-flourouracil (5-FU) in combination with blueberry extract on Caco-2 cell viability. Caco-2 cells were exposed to combinations of different concentrations of 5-FU and blueberry extract for 24 hours and, cell viability was determined using the MTT assay. Black line indicates data for exposure to 5-FU only. Red, blue and green lines indicate data for cells exposed to different 5-FU concentrations in combination with 12.5, 25 or 50 µg/ml blueberry extract, respectively. Data points and error bars indicate mean ± S.D. of 3 independent experiments.

The effects of the most abundant malvidin-containing anthocyanin present in the extract (malvidin-3-galactoside) in combination with 5-FU are shown in figure 5.3. The 2-way ANOVA indicated that both the 5-FU and the malvidin-3-galactoside independently caused reductions in viability ($p<0.0001$ in both cases). However, there was no clear evidence of synergy between the action of the malvidin-3-galactoside and 5-FU on Caco-2 cell viability (interaction term in 2-way ANOVA non-significant).

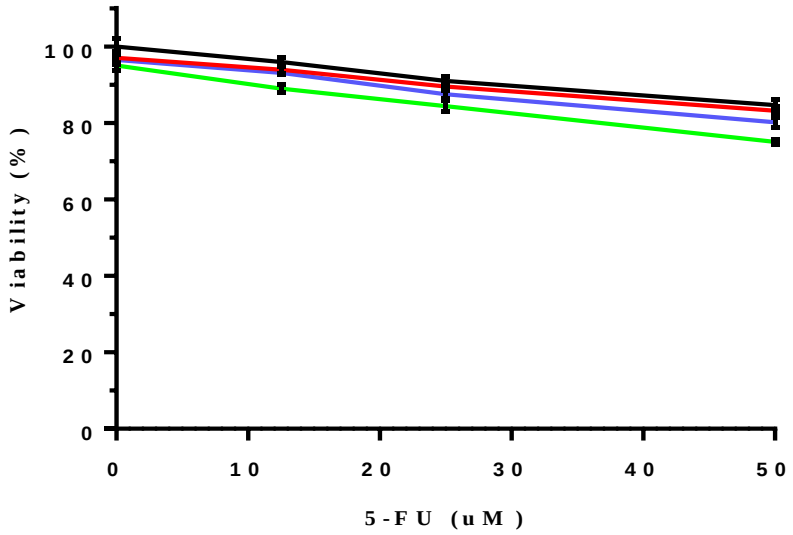

Figure 5.3. Effects of 5-fluorouracil (5-FU) in combination with malvidin-3-galactoside on Caco-2 cell viability. Caco-2 cells were exposed to combinations of different concentrations of 5-FU and malvidin-3-galactoside for 24 hours, and cell viability was determined using the MTT assay. The black line indicates data for exposure to 5-FU only. Red, blue and green lines indicate data for cells exposed to different 5-FU concentrations in combination with 12.5, 25 or 50 µM malvidin-3-galactoside, respectively. Data points and error bars indicate the mean ± S.D. of 3 independent experiments.

The results of the experiments performed using combinations of 5-FU and malvidin chloride are shown in figure 5.4. The 2-way ANOVA identified significant overall effects of the individual treatments ($p<0.0001$ in both cases). The effects of the aglycone were even more

modest than those of the galactoside form and, there was no significant interaction evidence between the malvidin and the 5-FU treatments.

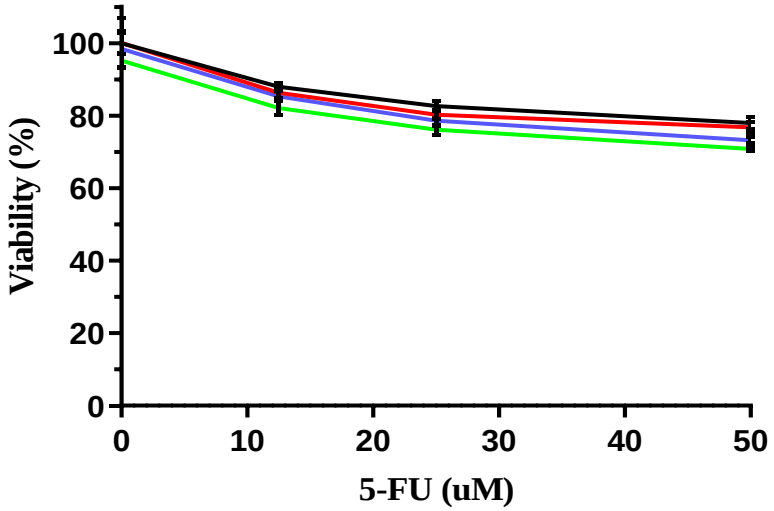

Figure 5.4. Effects of 5-flourouracil (5-FU) in combination with malvidin chloride on Caco-2 cell viability. Caco-2 cells were exposed to combinations of different concentrations of 5-FU and malvidin chloride for 24 hours and cell viability was determined using the MTT assay. Black line indicates data for exposure to 5-FU only. Red, blue and green lines indicate data for cells exposed to different 5-FU concentrations in combination with 12.5, 25 or 50 µM malvidin chloride, respectively. Data points and error bars indicate mean ± S.D. of 3 independent experiments.

Finally, results for the metabolite 2,4,6-trihydroxybenzaldehyde are shown in figure 5.5. Again, both the 5-FU and the 2,4,6-trihydroxybenzaldehyde treatments independently led to significant reductions in Caco-2 cell viability as assessed by the MTT assay ($p<0.0001$ in both cases based on 2-way ANOVA) and, there was evidence of significant interaction between the two treatments ($p<0.05$ from 2-way ANOVA) although this was substantially less marked than that between the blueberry extract and 5-FU. As in the previous set of experiments, 5-FU

treatment alone led to significant reductions in viability at all concentrations tested ($p<0.05$ at 12.5µM and $p<0.0001$ at both 25 and 50µM) while 2,4,6-trihydroxybenzaldehyde, on its own, only led to a significant reduction in viability at the highest concentration of 50µM ($p<0.01$). For the combination treatments using 2,4,6-trihydroxybenzaldehyde at 12.5 and 25µM, the mean reductions in cell viability were similar to the sum of the reductions observed with equivalent concentrations of 5-FU or 2,4,6-trihydroxybenzaldehyde individually. Only at the highest concentration (50µM) of 2,4,6-trihydroxybenzaldehyde was there any plausible evidence of synergistic interactions with the mean reductions in viability 2.0- 4.6% greater for the combined treatments compared with the sum of the effects of the individual treatments.

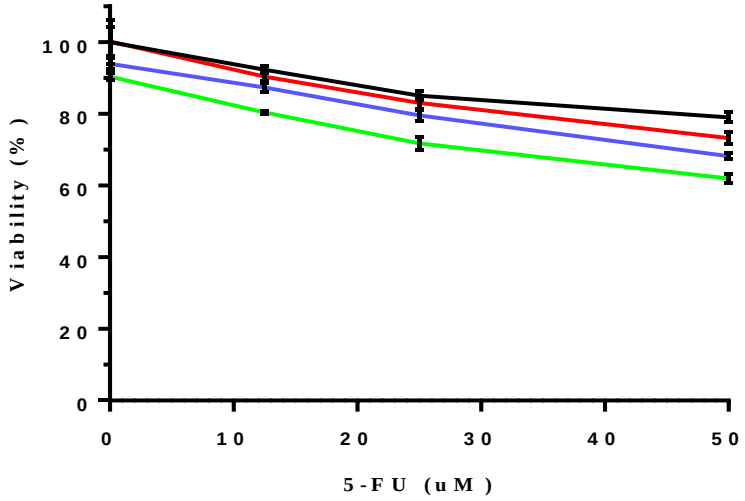

Figure 5.5. Effects of 5--fluorouracil (5-FU) in combination with 2,4,6 trihydroxybenzaldehyde on Caco-2 cell viability. Caco-2 cells were exposed to combinations of different concentrations of 5-FU and 2,4,6 trihydroxybenzaldehyde for 24 hours, and cell viability was determined using the MTT assay. The black line indicates data for exposure to 5-FU only. Red, blue and green lines indicate data for cells exposed to different 5-FU concentrations in combination with 12.5, 25 or 50 µM 2,4,6 trihydroxybenzaldehyde, respectively. Data points and error bars indicate the mean ± S.D. of 3 independent experiments.

Overall, the results indicate that the combined treatment with blueberry extract or each of the individual phytochemicals tested with 5-FU can enhance the chemotherapeutic potential of 5-FU. However, there was a clear synergistic interaction between the blueberry extract and the 5-FU. There was also a significant synergistic interaction between the 2,4,6, trihydroxybenzaldehyde, and the 5-FU, but weaker than that seen with the blueberry extract. Neither the malvidin-3-galactoside nor the malvidin chloride exhibited any clear evidence of synergy with the 5-FU, with the effects of the two treatments being simply additive.

5.3 Discussion

Conventional therapies, such as chemotherapy, are a standard component of many cancer treatment strategies. However, tumour cells often develop resistance to these therapies, leading to decreases in the effectiveness of chemotherapeutic agents over the course of the treatment regimen. Further refinements to current treatment strategies are required to improve their efficacy, overcome problems with tumour resistance and reduce side effects.

A variety of dietary compounds have been recognized as potential cancer chemopreventive agents due to their anticarcinogenic activity (Surh *et al.,* 2003). Since 2005, the number of publications concerning the combination treatment of standard cancer therapies and chemopreventive agents has increased. These studies propose that combination treatment can improve the efficacy of chemotherapeutic agents (Sarkar and Li, 2006).

The present study investigated the effects of conventional chemotherapy (5-FU) combined with blueberry extract, malvidin-3-galactoside, malvidin chloride and 2,4,6-trihydroxybenzaldehyde**,** as potentially beneficial agents in the prevention and treatment of cancer. Indeed, it has been suggested that the polyphenols, in particular, may find use in combination with conventional cancer therapies to help overcome drug resistance and reduce the side effects of standard anti-cancer treatments (Fantini *et al*., 2015).

In the initial study performed to determine suitable concentrations of 5-FU to use in the subsequent combination study, a striking observation was that low concentrations of 5-FU led to steep declines in signal from the MTT assay but, at higher concentrations, the declines

levelled out at a point consistent with maximum cytotoxicity of approximately 50% (Figure 5.1). This contrasts with the effect of tBHP, for example, which at higher concentrations effectively killed all the cells (see figure 4.1). This is potentially in line with the known mechanism of action of 5-FU, which will lead to the death of actively proliferating cells but may also stall cell cycling without causing cell death.

While all the blueberry-derived agents exerted at least additive effects in terms of reducing cell number and/or viability of Caco-2 cells when used in conjunction with 5-FU (Figure 5.2, 5.3, 5.4, 5.5). Only the blueberry extract, and more marginally the 2,4,6-trihydroxybenzaldehyde, showed evidence of synergistic effects. These data suggest that either other components in the extract that exert synergistic effects with 5-FU or that a combination of chemicals is required to obtain the synergy. Our findings are in line with previous synergic studies; for instance, Hwang and co-workers (2005) found that a combination of genistein and 5-FU could act synergistically to induce apoptosis in chemoresistant HT-29 colon cancer cell. Tolba and Abdel-Rahman (2015) found that the combined treatment with PT/5-FU [PT (pterostilbene), a class of naturally occurring phenolic compounds] improved 5-FU- mediated suppression of estrogen receptor- β by 8.9%. Equally, curcumin, a phenolic compound found in turmeric, was also found to increase the efficacy of chemotherapeutic treatments in chemoresistant colon cancer cells (Shakibaei *et al*., 2014).

Indeed, in association with classical chemotherapeutic agents, polyphenols represent a promising class as anti-cancer drugs, to target cancer cells, with a minimal toxic effect on normal cells (Russo *et al*., 2010).

5.4 Conclusion

The present study identified evidence of a synergic effect between conventional chemotherapy (5-FU) combined with blueberry extract at high concentrations of extract. There was weaker evidence of synergy when one of the major metabolites produced within the colon was

evaluated. The major anthocyanidin in the extract was used on its own, and no evidence of synergy with it or the aglycone form. Thus the synergistic effects observed with the crude extract may be due, in part, to the anthocyanins malvidin-3-galactoside, but other components or perhaps the complex mix appear to enhance the effects. The observation of synergy between the complex blueberry extract and the 5-FU, merits further investigation to identify the active agent or agents responsible.

CHAPTER SIX

GENERAL DISCUSSION

6.1 Background

In recent years there has been increased attention on phenolic compounds and flavonoids due to evidence from several research groups that consumption of fruits and vegetables high in bioactive phytochemicals is associated with the reduction of chronic diseases (Liu, 2003; Etherton *et al*., 2002; Borges *et al*., 2010). A high daily intake of fruits and vegetables is expected to provide up to 1 g of phenolics (Kuhnau, 1976). However, consumption of berry fruits can provide phenolics compounds, such as flavonols, at estimated intakes of 20–25 mg/day in different regions of the world, such as the United States, Denmark, and The Netherlands (Hertog *et al*., 1993; Sampson *et al*., 2002). Flavonol consumption may be even higher in some counties, such as Italy, where the average intake is estimated at 35 mg/day (Pietta *et al*., 1996). In Finland, where large amounts of berry fruits are consumed, anthocyanin intake may exceed 200 mg/day (Heinonen, 2001). In particular, blueberries have been recognised as a rich source of phytochemicals, specifically anthocyanins, which work to counteract oxidative damage and inflammation and have antioxidant and scavenging effects on carcinogens (Ehlenfeldt and Prior, 2001; Sellappan *et al*., 2002).

Anthocyanins are pigments that contribute to the intense colours of blueberries. The most common anthocyanidin aglycones are peonidin, pelargonidin, malvidin, delphinidin, cyanidin and petunidin; these then combine with organic acids and sugars to generate various anthocyanins (Prior *et al*., 2010). Anthocyanins have been demonstrated to have numerous bioactive properties, such as anti-inflammatory, antioxidant and anti-cancer activities (Faria *et al*., 2010; Cutler *et al*., 2016). The exact anthocyanin contents of berries depend on the species, fruit size, ripening stage, addition to pre-harvest environmental conditions and storage (Muller *et al*., 2012). After consuming food rich in anthocyanins, anthocyanins can be exposed to different PH environments during their passage through the gastrointestinal tract; thus, they might exist in various forms (Norberto et al., 2013).

Anthocyanin glycosides can be rapidly absorbed from the stomach after ingestion by a process in which bilitranslocase (an organic anion membrane carrier expressed at the sinusoidal domain of the liver plasma membrane and in epithelial cells of the gastric mucosa) is implicated (Passamonti *et al*., 2002). Sugar moiety can play a significant role in the transport pathway for anthocyanins. At the same time, absorption of aglycones can be improved by conjugation with glucose, in probability, by absorption through the sodium-dependent glucose transport system (SLGT1) (Hollman & Katan, 1999). Anthocyanins that are not absorbed further up the gastrointestinal tract and reach the colon will be exposed to a microbial population and may be degraded to sugar and phenolic components; phenolic components can undergo further degradation to produce phenolic acids and aldehydes (Aura *et al*., 2005).

The apparent health-promoting effect of phenolic compounds, such as anthocyanins, may be attributed to the whole fruit, intact glucosides or their metabolites. For example, Tsuda and colleagues (Tsuda *et al*., 1999) found that the plasma concentration of protocatechuic acids was eight times greater than that of cyanidin-3-glucoside. Protocatechuic acid is the potential degradation product of cyanidin-based anthocyanins; given that the post-prandial plasma concentrations of the degradation product were much higher than the concentration of the parent compound. The potential bioactivity of the metabolite may be more critical. Equally, fruits such as blueberries typically contain a complex mix of phenolic compounds, and these different compounds may act together, additively or synergistically, to produce chemopreventive effects (Johnson and Arjmandi 2013).

In the work presented in this thesis, we investigated the biological effects of blueberry extract consisting of a complex mix of phytochemicals present in the fruit, as well as a selected purified candidate bioactive component of blueberries (malvidin-3-galactoside) and common metabolites of this anthocyanin (malvidin and 2,4,6-trihydroxybenzaldehyde) in an *in-vitro* cell model of colon cancer (Caco-2).

6.2 Analysis and characterisation of blueberry extracts

An initial aim of the work was to prepare extracts from different varieties of blueberries to compare the biological effects on Caco-2 cells. Attempts to prepare such extracts yielded material that was gelatinous and sticky even after drying. It seems likely that this was a result of the high sugar content of the fruit, and it made the extract challenging to work with.

As an alternative, commercial blueberry extracts were investigated. The first tested one contained particulate matter that was largely insoluble in organic solvents such as DMSO. It seems likely that this extract was prepared from the skin and pulp of the fruit leftover after juicing. However, regardless of how that extract has actually been prepared, it was unsuitable for the planned cell culture work and not analysed further.

In contrast, the second commercial blueberry extract evaluated dissolved readily in DMSO to yield an intensely purple solution. Analysis of the properties of this product, presented in chapter 2, confirmed that it has potent antioxidant properties and is anthocyanin-rich.

The fact that this extract was readily and wholly dissolved in DMSO suggests it was likely to have been prepared via an organic extraction of blueberry fruit, pulp or skin. As such, it will not contain all components of the fruit. Indeed, the very different properties of the extract prepared in-house and the two different commercial blueberry supplements highlight that the process selected for preparing extracts or supplements is likely to produce very different overall compositions. For example, the fibrous cell wall components will not be present in the DMSO-soluble extract selected for the experiments described in this thesis. However, these were clearly a major component of the other commercial blueberry supplement. These fibrous cell wall components may have significant health-promoting effects of their own.

Moreover, organic extraction processes may not work equally well for all the polyphenolic components of the fruit. Consequently, the polyphenol profile of different extracts, and their biological effects, are likely to differ not only due to the different composition of the original fruits but due to the extraction process selected. Based on the findings from the research reported here, it would be interesting to return to one of the original aims of the project to prepare blueberry extracts from different varieties of the fruit using other extraction protocols and to compare the effects of these on Caco-2 cells.

The total phenolic content analysis suggested the blueberry extract contains approximately 7.6mg Gallic acid equivalent/g dry weight, and the total flavonoids content of the blueberry extract was estimated at 14.1mg catechin equivalent /g dry weight. The blueberry extract exhibited concentration-dependent reducing activity based on the determination of its capacity to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron. Initially I have planned to look only at blueberry extracts but that technical challenges led to a change in direction and the work with individual compounds. With the benefit of hindsight, it might have been appropriate to match the amount

of extract used with individual compounds/metabolites which were matched for either GAE or catechin equivalents.

To assess the free radical scavenging capacity of the blueberry extract, a widely used method based on scavenging of the stable DPPH radical was used (Ebrahimzadeh *et al*., 2008). This method has been used previously to measure the free radical scavenging effect of different polyphenols (Murcia *et al*., 2001; Julián-Loaeza *et al*., 2011). DPPH forms a stable nitrogen-centred free radical that has a violet colour in ethanolic solutions, and its reduction leads to loss of colour. Compounds that contribute to this reaction are known as radical scavengers (Dehpour *et al*., 2009). In the present. Analysis of the blueberry extract with this assays study demonstrated its radical scavenging capacity, which increased with increasing concentration over the range tested (1-50 mg/ml).

The hydrogen peroxide scavenging activity for blueberry extract was examined. Hydrogen peroxide is produced in biological systems, and while it is not a particularly reactive chemical itself, it can act as the source of hydroxyl radicals, which are highly reactive and can initiate lipid peroxidation (Halliwell and Gutteridge, 1984). The scavenging activity increased moderately up to a maximum at 30mg/ml but then decreased at concentrations higher than this, suggesting that the extract acted as an antioxidant in the context of hydrogen peroxide at the lower concentrations tested but that this antioxidant effect started to be lost at higher concentrations. This pattern of effects appeared to differ from those observed with the other antioxidant activity assays discussed above and below.

Ferrous metal (Fe^{2+}) ions are reactive and can induce free radical formation, which can lead to the initiation of lipid peroxidation (Aitken *et al*., 1993). In the presence of blueberry extract at concentrations (1-50 mg/ml), the amount of Fe^{2+} ferrozine complex was significantly reduced in a concentration-dependent manner.

Lipid peroxidation is a free radical chain reaction; in the first stage of lipid oxidation (initiation), the primary radicals are formed, such as peroxides and hydroperoxides. At the second stage of oxidation (propagation), secondary oxidation products occur, for instance, carbonyl compounds.

The antioxidant activity of the blueberry extract was measured using an oxidising linoleic acid model system, with the oxidation process being monitored for seven days. Two methods were

used to evaluate the oxidation inhibition activity: primary oxidation products were determined using the ferric thiocyanate method (FTC), and the thiobarbituric reactive substances (TBARS) test was used to monitor the secondary oxidation products. The results suggested that the blueberry extract had a more potent antioxidant activity in the TBARS assay than in the FTC assay. This indicates that the components of the blueberry extract may be better at preventing malondialdehyde production than at inhibiting peroxide formation.

Overall, the findings from this selection of different analytical methods suggested that the blueberry extract possesses a broad spectrum of inherent antioxidant properties.

Further analysis of this blueberry extract using high-performance liquid chromatography (HPLC), which is a susceptible analytical method for the separation and identification of phenolic compounds and anthocyanins (Gil *et al.*, 2000; Fischer *et al.*, 2011), revealed the presence of multiple components. The concentrations of malvidin-3-glucoside and malvidin-3-galactoside in the extract were estimated based on the use of pure standards for these compounds. Unfortunately, at the time of the analysis, we did not have access to pure standards for the other major anthocyanins in blueberries (such as delphinidin and petunidin) and so were only able to estimate the malvidin glycoside content of the extract. Nonetheless, malvidin glycosides are typically the most abundant or occasionally second most abundant anthocyanins in blueberries (Feng et al., 2013).

Depending on the organic solvent used for extraction, the HPLC analysis suggested the extract contained malvidin-3-galactoside at 1.2-1.7mg/g total extract and malvidin-3-glucoside at 0.5-0.7mg/g total extract.

DMSO was used as the solvent throughout the cell culture studies. So the calculations below use the apparent concentrations of the anthocyanins as determined above when DMSO was used as the solvent. The table below shows the actual concentrations of malvidin-3-glucoside and malvidin-3-galactoside the Caco-2 cells would have been exposed to when treated with blueberry extract solutions at concentrations of 100-500ug/ml. When adding extract at, for example, 100ug/ml to the cells, the cells were exposed to a mixture of compounds amongst which the final concentration of malvidin-3-galactoside was only 0.24uM (and the concentration of malvidin-3-glucoside was 0.1uM). This highlights that the biological effects of the complex extract were observed at much lower malvidin concentrations than when pure malvidin-3-galactoside was used alone. This suggests that the extract either contains other

components with much more potent bioactivity or that there is substantial synergy between the components such that the overall bioactivity of the mix is much greater than simply the sum of bioactivity of the individual components.

Table 6.1 Concentrations of malvidin glycosides in blueberry extract over the range of concentrations used in cell culture studies

Concentration of extract (ug/ml)	Concentration of M-3-gluc (ug/ml)	Concentration of M-3-gluc (uM)	Concentration of M-3-gal (ug/ml)	Concentration of M-3-gal (uM)
100	0.048	0.10	0.125	0.24
200	0.096	0.19	0.25	0.47
300	0.144	0.29	0.375	0.71
400	0.192	0.39	0.5	0.95
500	0.24	0.49	0.625	1.18

6.3 Investigation of the effects of blueberry extract, a major blueberry anthocyanin, and its metabolites on the human colon adenocarcinoma Caco-2 cell line viability.

Several studies on polyphenols have indicated that they can inhibit the proliferation of cancer cell lines obtained from various tissues such as the lung (Khan *et al*., 2007) and cervix (McDougall *et al*., 2008) and colon (Seeram *et al*., 2005). The current work aimed to study the effect of blueberry extract, one of, if not the most abundant anthocyanins in the extract and two of its metabolites on several properties related to cancer cell viability, using Caco-2 cells as a suitable model. The Caco-2 cell model is very well characterised (Gutmann et al., 1999; Sambuy et al., 2005). It has been widely commonly used as a model of small intestinal absorption but is, in fact, derived from a colorectal adenoma and so is also useful as a model of colorectal cancer.

The MTT assay was used as the first method to determine if blueberry extract and metabolites that can be derived from it have the capacity to decrease either Caco-2 cell numbers or their viability (or a combination of the two), leading to a concentration-dependent decrease in the signal obtained. The blueberry extract and the 2,4,6 trihydroxybenzaldehyde were more potent than the mavidin-3-galacotisde or the malvidin in its aglycone form. When considering these data, it is crucial to bear in mind that the MTT assay does not explicitly measure cell viability, although it is routinely used as a viability assay. Instead, it determines the total mitochondrial metabolic activity of the cells in the well. This is the product of the viable cell number and the average metabolic activity. Under control conditions, the Caco-2 cells continue to proliferate during the 24-hour treatment period. Consequently, the MTT assay reading could be reduced as a result of a treatment causing increased cell death, reduced proliferation or a combination of both.

To investigate the mechanisms responsible for the effects observed with the MTT assay, the Annexin-V FITC/PI cell staining strategy was employed. Annexin-V FITC/PI is a rapid and sensitive assay that uses a quantitative method to determine the type of cell death and can be measured by flow cytometry. The present study results indicated that Caco-2 cell death following exposure to blueberry extract, malvidin-3-galactoside, and two of its metabolites occurred primarily via stimulation of apoptosis. Numerous previous studies have found that polyphenols can trigger apoptosis in cancer (Zhang *et al*., 2008; Finco *et al*., 2016). Thus, the work described here is consistent with those findings.

Caspases are a family of cysteine proteases activated in sequence during apoptosis and hence used to analyse apoptosis induction. According to previous reports, caspase-3 has been reported to initiate DNA fragmentation by proteolytically inactivating ICAD (inhibitor of caspase-activated deoxyribonuclease), which releases the active endonuclease CAD (caspase-activated deoxyribonuclease), allowing CAD to enter the nucleus and degrade chromosomal DNA (Sakahira *et al*., 1998).Caspase- 3 can act as an effector that has the ability to progress caspase-2, -6, -7, and -9 proenzymes, also sharing a sequence recognition with caspase-7. Our results show significant caspase 3/7 activation only with the blueberry extract and the 2,4,6-trihydroxybenzaldehyde, and only at the higher concentrations tested. This contrasts with the findings from the flow cytometric analysis of annexin-V FITC/PI cell staining strategy, which indicated that all the different treatments were capable of stimulating apoptosis. While it may be that the annexin-V FITC/PI cell staining method is more sensitive than the caspase 3/7

activity assay or that the time points selected for analysis may have been more appropriate for one assay than the other, this apparent discrepancy merits further investigation.

6.4 Effects of blueberry extract and blueberry components on cellular ROS

Reactive oxygen species (ROS) are produced in living cells as a result of normal cell metabolism and xenobiotic detoxification. Antioxidants are compounds that have the ability to scavenge ROS; an imbalance where cellular ROS exposure exceeds the antioxidant capacity is known as oxidative stress. Under conditions of oxidative stress, the excess ROS can contribute to the accumulation of cellular damage by reacting with lipid membranes, proteins and DNA. Conversely, the damaging effects of ROS can be prevented or limited by antioxidants (Gomes *et al*., 2006).

In the studies presented here, Caco-2 cells were used as an epithelial cell model to investigate the antioxidant performance of blueberry extract and blueberry components. The protective effect of blueberry extract and blueberry components (at concentrations in the non-cytotoxic range) in Caco-2 cells were analysed under conditions of oxidative stress induced by treatment with tBHP and analysed first using the MTT assay. No evidence was found that any of the test materials provided any significant degree of protection against the cytotoxic effects of t-BHP. The validity of the model systems was confirmed using Trolox, a synthetic analogue of vitamin E, as a positive control. At 0.01% w/v, the Trolox did reduce the cytotoxic effects of the t-BHP on Caco-2 cells. This finding was confirmed by using the Flow cytometric analysis to detect oxidation of the fluorogenic dye dichlorofluorescein. These findings suggest that blueberry extract and blueberry components may have limited activity as ROS scavengers in cellular systems when used at concentrations in the non-cytotoxic range. Previous similar studies on polyphenols have found different effects. For example, Alia *et al*. (2006) established that polyphenols could decrease the level of ROS in a liver cell line.

However, the exact conditions selected may well influence the findings. For example, in the case of the studies reported here, the concentration of t-BHP was used to induce oxidative stress with 3mM. The highest concentration of any of the individual compounds used in the experiments investigating their antioxidant properties in cells was only 15µM. Thus, it could be that any oxidant effect of the t-BHP might simply have been swamped by the ROS production from the t-BHP. In this regard, the Trolox positive control was used at 0.01% w/v, which equates to a concentration of 0.48mM. While lower than the t-BHP concentration.

However, the use of the test compounds at higher concentrations would have elicited cytotoxic effects. Indeed, subsequent experiments performed with a higher cytotoxic concentration of the extract and the individual compounds demonstrated they could cause intracellular ROS production. These experiments. Amongst the different blueberry-derived materials, the blueberry extract and 2,4,6-trihydroxybenzaldehyde elicited more substantial increases in intracellular ROS than either the malvidin-3-galactoside or malvidin.

6.5 The effects of blueberry extract and components in combination with 5-fluorouracil in Caco-2 cells

Natural products, for instance, plant-derived dietary components, such as polyphenols, may help reduce the incidence of cancer (Soobrattee *et al*., 2016). They may have a low number of side effects and low toxicity. There is some evidence that polyphenol compounds may prove helpful in reducing the side effects of chemotherapeutic agents and possibly even in enhancing their activity (De Kok *et al*., 2008). In contrast, conventional chemotherapy for cancer treatment often has serious side effects such as hair loss, injury to the gastrointestinal tract, infection, nausea and vomiting, etc.

Alexander and Friedl (2012) raised the possibility of discovering alternative approaches for treatment strategies with the intention of achieving clinical success due to the increase in cancer multidrug resistance, decreased efficacy and the increasing treatment failure of the modern drugs. The present study investigated the effects of blueberry extract and blueberry-derived compounds when used in combination with 5-fluorouracil in Caco-2 cells**.** The key finding from this work was that while all the blueberry-derived agents exerted at least additive effects in terms of reducing cell number and/or viability of Caco-2 cells when used in conjunction with 5-FU (Figure 5.2, 5.3, 5.4, 5.5), only the blueberry extract, and more marginally the 2,4,6-trihydroxybenzaldehyde, showed evidence of synergistic effects. Our findings are in line with the conclusion of Tolba and Abdel-Rahman (2015). They found that the combined treatment with PT/5-FU [PT (pterostilbene) is a class of naturally occurring phenolic compounds] improved 5-FU- mediated suppression of estrogen receptor-β. It was notable in these experiments that while 5-FU alone exhibited concentration-dependent cytotoxic effects on Caco-2 cells, the maximal drug maximally appeared to reduce cell viability or number by only approximately 50%. There are at least two potential explanations for this observation: either a proportion of the Caco-2 cells are resistant to the effects of 5-FU by way of Caco-2 were

isolated from a patient who had undergone chemotherapy so may well have some resistance. Or the 5-FU reduces cell proliferation during the treatment period without inducing substantial cell death. Since 5-FU acts to inhibit thymidylate synthase, it should deplete the cells of deoxythymidine triphosphate. Therefore, it would be expected to slow or inhibit DNA replication and, thereby, slow or inhibit cell proliferation. It can also cause replicative stress triggering apoptosis. Cell viability analysis (e.g. via the annexin V, propidium iodide protocol) and cell cycle analysis by flow cytometry would provide more insight into the underlying mechanisms. Such analyses would also shed light on the mechanisms of interaction of the blueberry extract and the blueberry-derived compounds.

Overall, the data presented confirm that the blueberry extract analysed was rich in components with inherent antioxidant activity and that the extract exhibits significant cytotoxic effects on colorectal cancer cells. The individual components and metabolites analysed appeared to be much less potent in these effects than the complex mix of agents in the extract. This suggests that there are either other components that exhibit more potent bioactivities or that interaction between components enhances the bioactivity.

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6.6 Future Work

1. Further evaluation of extraction techniques to obtain blueberry extracts from different cultivars that would be suitable for cell culture studies.

2. In-death analysis of the composition of the extracts, perhaps using metabolomics technology platforms to look beyond just the anthocyanins.

3. More work would need to be done to characterise the key components of the extract responsible for the effect and the importance of potential interactions between them.

4. The synergistic effects observed between 5-FU and the blueberry extract appears to hold some promise. For this to be of use clinically, the effect would have to be specific to cancer cells. So the cytotoxicity of the extract should ideally be tested in normal colorectal cells. If such work identified specificity towards cytotoxicity in cancer cells.

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