



# *Health effects of bioactive compounds present in vegetables and fruits*

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*Master thesis:*

**Blueberry extracts**

**and**

**their protection against cancer**

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2009-2010

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## Abbreviations

WCRF, World Cancer Research Fund

MMP, matrix metalloproteinase

TIMP, tissue inhibitors of matrix metalloproteinases

NO, nitric oxide

GSH, glutathione

NADPH, nicotinamide adeninedinucleotide phosphate reduced

8-oxo-dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine

RT-PCR, reverse transcriptase polymerase chain reaction

ESR, electron spin resonance

BPDE, benzo(a)pyrene diolepoxide

FACS, fluorescence activated cell sorting

ORAC, oxygen radical absorbance capacity

HBSS, Hank's buffered salt solution

EDTA, ethylenediaminetetraacetic acid

DMSO, dimethyl sulfoxide

PBS, phosphate buffered saline

FPG, formamidopyrimidine DNA glycosylase

TBST, TRIS buffered saline/ Tween-20

FITC, fluorescein isothiocyanate

PI, Propidium Iodide

BSA, bovine serum albumin

DMPO, 5,5-dimethyl-1-pyrroline-N-oxide

TEAC, Trolox equivalent antioxidant capacity

ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

## **Acknowledgements**

During the 30 weeks of my senior internship I experienced what it is like to do research. I learned that it is impossible to get results without motivation, perseverance and patience. During my internship, I had to deal with numerous problems and the results of my experiments were not always how I hoped. Despite of all the problems and frustrations I persisted and managed to complete my master thesis. However, it was impossible to finish my internship and to write this thesis without the professional advice and the absolute support of a lot of people. First of all I would like to thank my supervisor, dr. Simone van Breda. You were always ready to help me when there was a problem, and you always encouraged me to continue. Dr. Theo de Kok and dr. Jacco Briedé, thank you for your opinions during our regular meetings, I learned a lot from it. I also would like to thank my second examiner prof. Dr. Johan Heemskerk for making time to examine my thesis. In addition, I would like to say thank you to Alexander and Sandra for helping me with my experiments. You were always ready for me and I really appreciate that. I also would like to thank the people that coloured my workday by having a little chat with me or by inviting me for a coffee break. Astrid, Daniëlle, Kristien and Leen, thank you. In addition, I am very grateful to have friends who are always willing to make time for me. Our dates always give me a boost of positive energy. Stéphanie, Fna, Linde, Myrthe, Annelies, Laura, Katrien and Natalie, I love you guys. Lien, my little sister, I love you too. Thank you for making time for me and accompanying me to the gym. You know how important that is for me. Finally, I want to thank my parents for their unconditional support. Thank you, mam and dad, for the chances you gave me and for being there for me!

## **Abstract**

Recent research has shown that several bioactive compounds present in vegetables and fruit may contribute to a lower risk of numerous chronic diseases including cancer. An important group of bioactive compounds are the antioxidants. Antioxidants protect the body against free radicals that are able to react with DNA, thereby inducing DNA damage which, in turn, can lead to cancer. The body is constantly exposed to endogenous and exogenous sources of free radicals. Therefore, the body contains several antioxidant mechanisms to encounter these radicals. However, sometimes this endogenous protection is not enough to maintain a healthy pro-oxidant-antioxidant balance. It is thus also important to increase the antioxidant intake by means of diet. Many studies indicated that blueberries are an important source of antioxidants that possess anticarcinogenic activity. To reveal the protective effects of four different blueberry varieties at the level of the gut, the present study hypothesised that blueberry extracts and their individual antioxidants protect caco-2 colon cancer cells from oxidative DNA damage, and that the analysis of cellular and genetic responses will identify the molecular mechanisms involved in this protective effect, as well as potential synergistic effects of combinations of individual antioxidants. To test this hypothesis, caco-2 cells were pre-incubated with different concentrations of blueberry extract and subsequently challenged with menadione. Menadione is a synthetic oxidant that was selected to be used because of its ability to induce radicals intracellularly. The results on the levels of DNA damage and ROS production, measured using the alkaline comet assay and ESR spectroscopy respectively, do not show a blueberry-induced protective effect. However, probably the conditions in these experiments were wrong. On the contrary, the FACS experiment showed that the two extracts that were tested did show significant pro-apoptotic activity in cells that were damaged by menadione without affecting cell cycle of the surviving cells. These findings suggest that blueberries possess anticarcinogenic effects by inducing apoptosis. However, more research is needed to reveal other blueberry-induced protective mechanisms.



## Samenvatting

Recent onderzoek heeft aangetoond dat verscheidene bioactieve componenten in groenten en fruit zouden kunnen bijdragen aan een verlaagd risico op een aantal chronische ziekten waaronder kanker. Een belangrijke groep bioactieve stoffen zijn de antioxidanten. Antioxidanten beschermen het lichaam tegen vrije radicalen die kunnen reageren met DNA waarbij ze DNA schade kunnen veroorzaken die vervolgens kan leiden tot kanker. Het lichaam wordt continu blootgesteld aan vrije radicalen en bevat dan ook verschillende antioxidant beschermingsmechanismen. - Soms is deze endogene bescherming echter niet voldoende om een gezonde pro-oxidant-antioxidant balans te behouden. Daarom is het zeer belangrijk om de antioxidant inname te verhogen via de voeding. Vele studies hebben aangetoond dat blauwe bessen een belangrijke bron zijn van antioxidanten en dat deze bessen anticarcinogene activiteit bezitten. Om de beschermende effecten van vier verschillende blauwe bes rassen te onderzoeken op het niveau van de darm, stelt deze studie dat blauwe bes extracten en hun individuele antioxidanten caco-2 colon kankercellen beschermt tegen oxidatieve DNA schade en dat de analyse van cellulaire en genetische responsen de moleculaire mechanismen die betrokken zijn bij dit beschermend effect zullen identificeren, net als de mogelijke synergetische effecten van combinaties van individuele antioxidanten. Om deze hypothese te testen werden caco-2 cellen gepre-incubeerd met verschillende concentraties blauwe bes extract waarna menadione werd toegevoegd. Menadione is een synthetische oxidant die in deze studie gebruikt wordt omdat hij de eigenschap bezit om intracellulair radicalen te induceren. De resultaten op de niveaus van DNA schade en ROS productie, respectievelijk gemeten met de alkaline comet assay en met ESR spectroscopie, vertonen geen beschermend effect van de blauwe bes extracten. Echter, de experimentele condities voor deze experimenten waren mogelijk verkeerd. De FACS experimenten daarentegen toonden aan dat de twee extracten die gemeten werden een significante verhoging van apoptose veroorzaakten in cellen die beschadigd waren door menadione, zonder invloed te hebben op de celcyclus van de overlevende cellen. Deze bevindingen suggereren dat blauwe bessen anticarcinogene effecten bezitten door het induceren van apoptose. Meer onderzoek is echter nodig om de andere beschermende mechanismen van de blauwe bes te ontdekken.

## **Introduction**

### **Diet and cancer**

Cancer is a major health problem in which environmental factors such as diet play an important role. It is suggested by the World Cancer Research Fund (WCRF) report of 1997 that physical activity, maintenance of appropriate body weight and a healthy diet, rich in vegetables and fruit, can reduce cancer risk by at least 30-40% [1]. Vegetables and fruit contain many protective elements such as selenium, folic acid, vitamin B-12, vitamin D, chlorophyll and antioxidants. Increased consumption of vegetables and fruit will reduce cancer risk by 60-70% for breast, colorectal and prostate cancers, and by 40-50% for lung cancer and cancers at other sites [2]. Several epidemiological studies support this suggestion [2]. However, according to the update of the WCRF report (2007), the evidence that vegetables and fruit protect against cancer is less compelling than in 1997 [3]. One of the reasons for this is that it is difficult for prospective cohort studies to measure dietary intake precisely and that the dietary variability in each cohort is limited [4]. In addition, genetic differences within the study population can influence cancer risk because of differences in taste preference, food tolerance, phytochemical absorption and metabolism [5]. However, there still are vegetables and fruit that “probably” protect against several cancers. For example fruits and non-starchy vegetables probably protect against cancers of the mouth, pharynx, larynx, oesophagus and stomach. Fruits also probably protect against lung cancer [3]. However, in order to make the scientific evidence stronger, more experimental studies using sensitive biomarkers have to be performed in addition to the epidemiological studies. In these experimental studies, the effects of specific bioactive compounds on specific endpoints such as DNA damage or gene expression can be investigated. As well as the effects of combinations of specific compounds.

### **Blueberries**

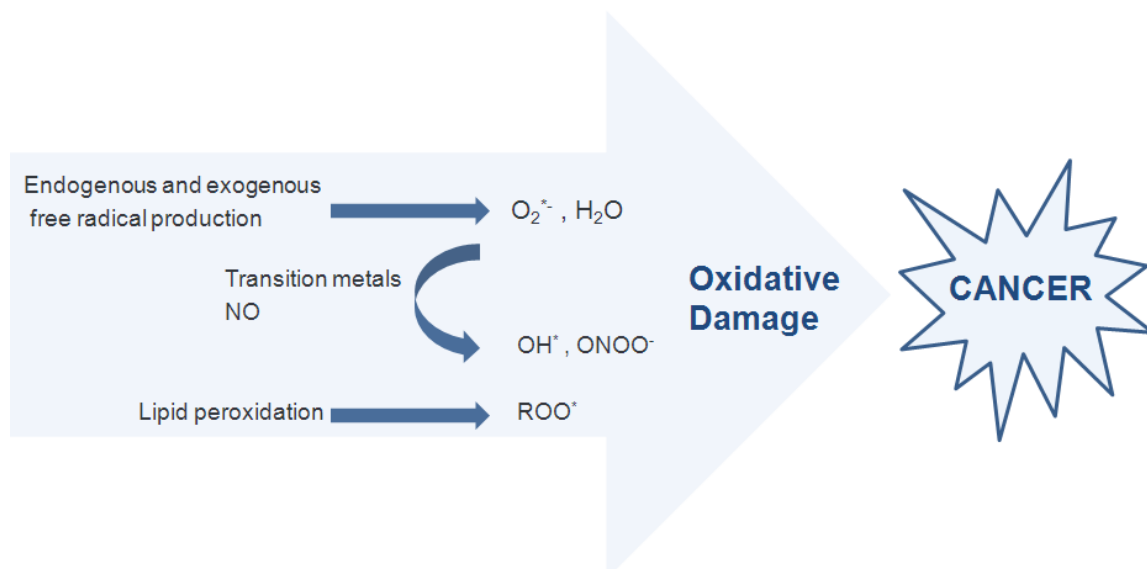
To improve the evidence for the cancer-protective role of vegetables and fruit the study described in this thesis will focus on blueberries. Blueberries originate from North America and they belong to the *Vaccinium* genus, the genus which also includes cranberries. There are three main blueberry species. Firstly, the lowbush or

wild blueberry (*Vaccinium angustifolium* Aiton). Secondly, the highbush or cultivated blueberry (*Vaccinium corymbosum* L. Ericaceae) which is grown in the more temperate climates. Finally, the rabbiteye blueberry (*Vaccinium ashei*) which is common in the southern US [6]. Blueberries are an important source of antioxidants. They rank very highly in antioxidant quality and quantity [6]. Several studies have been performed that show blueberry-induced chemopreventive effects. For example, Olsson et al. showed the anti-proliferative effects of ten extracts of fruits and berries on both colon cancer cells HT29 and breast cancer cells MCF-7. These extracts differed greatly in antioxidant content [7]. Baghi et al. evaluated several combinations of edible berry extracts and developed a synergistic formula, namely OptiBerry IH141. This extract significantly inhibited both H<sub>2</sub>O<sub>2</sub>- and TNF $\alpha$ -induced VEGF expression in human keratinocytes, indicating its anti-angiogenic role. In addition, pretreatment of endothelioma cells with OptiBerry resulted in a decreased ability to form hemangioma and a decreased tumor growth by more than 50%, indicating the anticarcinogenic potential of this berry extract [8]. Matchett et al. used three flavonoid-enriched fractions from lowbush blueberry to investigate the mechanisms by which this berry down-regulates matrix metalloproteinase (MMP) activity in DU145 human prostate cancer cells. The results indicated that blueberry flavonoids may use multiple mechanisms to down-regulate MMP activity. These mechanisms include an increased TIMP-1 (tissue inhibitors of matrix metalloproteinases) and TIMP-2 activity. In addition, it was also observed that protein kinase C and mitogen-activated protein kinase pathways are involved in the flavonoid-induced decrease in MMP activity [9].

### **Blueberry antioxidants**

In this section, the function of antioxidants – the primary bioactive compounds present in blueberries – will be explained in more detail. Antioxidants protect the body from free radical-induced damage. Free radicals are molecular species that contain at least one unpaired electron. Therefore, they are unstable and capable of reacting with DNA, thereby inducing DNA damage which, in turn, can lead to cancer. The most important free radicals in human health and disease are oxygen derivatives such as superoxide radical anion (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Interaction of these oxygen derivatives with transition metals such as iron (Fe) will lead to the formation of the highly reactive hydroxyl radical (HO<sup>•</sup>) [10]. Though, not all oxygen derivatives are that harmful. The nitric oxide (NO) radical for example, has a lot of biological

functions including modulation of vascular tone, memory formation and inflammation [11]. However, when NO interacts with oxygen, a cytotoxic species will be generated, namely peroxynitrite ( $\text{ONOO}^-$ ). In addition, during lipid peroxidation, other radicals such as the lipid peroxy radical ( $\text{ROO}^*$ ) will be generated (figure 1) [12]. These free radicals can all be formed endogenously in the body during normal physiological processes such as food digestion, respiratory burst and mitochondrial leak. Additionally, the body is also continuously exposed to several exogenous sources of free radicals, such as cigarette smoke, pollution and radiation [10]. To protect against these free radicals, the body contains several endogenous antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidases and glutathione reductase [10]. However, to prevent cancer, it is very important to maintain a healthy pro-oxidant-antioxidant balance by consuming antioxidant-rich vegetables and fruit. For colorectal cancer in particular, knowing that apart from luminal nutrients, the gut is constantly exposed to diet-derived oxidants, mutagens and carcinogens [13]. In general, there are four pathways in which antioxidants can act to protect the body against oxidative damage induced by free radicals. Firstly, they can prevent the formation of active oxidants. Secondly, they can scavenge, quench and remove the active oxidants. Thirdly, they can repair the oxidative damage and excrete the toxic oxidation products. Fourthly, they can induce adaptive responses [12].



**Figure 1:** Schematic overview of the production and actions of the major free radicals in the human body.

Several animal studies revealed the protective effects of antioxidants, present in vegetables and fruit such as blueberries. For example, studies in mice have shown the anticarcinogenic effect of several antioxidants including  $\alpha$ -carotene, lutein and polyphenols [14]. Khanduja et al. performed a study in which the mechanisms behind the chemopreventive effects of quercetin and ellagic acid - both present in small fruits including blueberries - were investigated. They showed that these polyphenols prevented N-Nitrosodiethylamine-induced lung tumorigenesis in mice by increasing the glutathione (GSH) content and by decreasing nicotinamide adeninedinucleotide phosphate reduced (NADPH)- and ascorbate-dependent lipid peroxidation [15]. There are also many *in vitro* studies which indicate the health-promoting role of vegetables and fruit [16]. Liu et al. showed that raspberry extracts, rich in antioxidants, inhibited the proliferation of HepG<sub>2</sub> human liver cancer cells in a dose-dependent manner [16]. Another study in which researchers tried to find mechanisms behind the blueberry-induced chemopreventive action is the study performed by Yi et al. They showed that anthocyanin, an antioxidant present in blueberries, can inhibit colon cancer cell proliferation by inducing apoptosis in HT-29 colon cancer cells and in caco-2 cells [17]. Mertens-Talcott et al. performed a study to demonstrate synergy between quercetin and ellagic acid. They investigated the effect of both antioxidants on cell death and proliferation using leukemia-derived MOLT-4 cells. The results showed that the combination of quercetin and ellagic acid enhanced the anticarcinogenic effect, which did not result solely from the additive effect, confirming the suggested synergy [18].

### **Preliminary data**

The previously mentioned *in vitro* studies already revealed a few mechanisms by which antioxidants achieve their chemopreventive effects. These studies also indicated the effects of some specific blueberry antioxidants. Yet, the results are still very limited. Therefore, our department - the department of health risk analysis and toxicology – performed several studies to gain more insight into these pathways. For example, Wilms et al. showed that pre-treatment with quercetin induced a clear dose-dependent decrease in H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in human lymphocytes [19]. Subsequently, they examined the radical scavenging capacity of quercetin in human lymphocytes, in which hydroxyl or superoxide radicals were generated, using electron spin resonance (ESR) spectroscopy. The results indicated that quercetin is a very

potent inhibitor of the hydroxyl radical rather than a scavenger of superoxide radicals [19]. In a following study, Wilms et al. showed that quercetin pre-treatment of human lymphocytes, which were subsequently exposed to benzo(a)pyrene, resulted in a decrease in benzo(a)pyrene diolepoxide (BPDE)-DNA adduct levels [20]. These *in vitro* studies show that quercetin accomplishes its chemopreventive effects by protecting human lymphocytes from oxidative DNA damage and BPDE-DNA adducts. However, apart from *in vitro* studies, it is also very important to study the blueberry-induced chemopreventive action *in vivo*. In this regard, our department performed a human dietary intervention study in which 168 healthy volunteers had to drink one litre blueberry-apple juice - containing high amounts of quercetin and vitamin C - per day for four weeks. This resulted in an increased plasma antioxidant capacity, indicating that the body takes up these compounds. Apart from increasing plasma antioxidant capacity, intervention with blueberry-apple juice also protected lymphocytes against oxidative DNA damage, in line with the results of the *in vitro* studies which were also performed by our department [20].

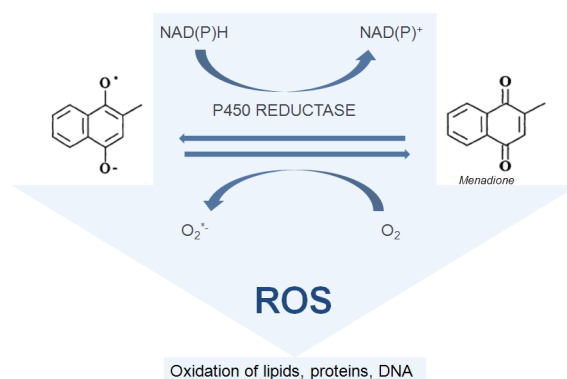
### **Current project**

Several studies have shown the chemopreventive effects of blueberry extracts and their antioxidants, both *in vitro* and *in vivo*. However, the molecular pathways involved in this blueberry-induced chemopreventive action are not entirely clear yet. It is also not clear which antioxidants apart from quercetin are responsible for this action, and which combinations of individual antioxidants act synergistically. Therefore, it is hypothesized that blueberry extracts and their individual antioxidants protect caco-2 cells from oxidative DNA damage, and that the analysis of cellular and genetic responses will identify the molecular mechanisms involved in this protective effect, as well as potential synergistic effects of combinations of individual antioxidants. Human colon adenocarcinoma-derived caco-2 cells will be used because these cells were reported to exhibit morphological, immunocytochemical and biochemical differentiation patterns characteristic for mature enterocytes [21]. To test the hypothesis, several objectives have been formulated. These objectives are to show the capacity of blueberry extracts, individual antioxidants and combinations of individual antioxidants to:

- lower the number of intracellular free radicals in caco-2 cells, using electron spin resonance (ESR)

- protect caco-2 cells from oxidative DNA damage by assessing DNA strand breaks and 8-oxo-dG (8-oxo-7,8-dihydro-2'-deoxyguanosine) levels (this oxidized derivative of deoxyguanosine is one of the major products of DNA oxidation [22]), using the alkaline comet assay and fluorescence activated cell sorting (FACS) respectively
- stimulate apoptosis and/or cell cycle arrest of caco-2 cells, using FACS
- change expression of genes that are important in detoxification, antioxidative mechanisms and DNA repair in caco-2 cells, using reverse transcriptase polymerase chain reaction (RT-PCR)

These objectives will be reached by pre-treating the caco-2 cells with a concentration range of blueberry extracts, individual antioxidants or combinations of individual antioxidants for two hours. Subsequently, some pre-treated caco-2 cells will be challenged for one hour with the oxidative agent menadione (2-methyl-1,4-naphthoquinone), and some cells will not be challenged. This experimental setup was selected according to preliminary results from our department. Menadione was chosen as oxidative agent for this project because this oxidant induces the physiological most important free radical, namely superoxide radical, intracellularly by redox cycling with the semiquinone radical. [23]. This reaction is mediated by cytochrome P450/P450 reductase. In the presence of hydrogen peroxide and transition metals, the induced superoxide radicals can lead to hydroxyl radicals and subsequent cytotoxicity [24]. Therefore, menadione provides a good model for intracellular free radical production, and to test if blueberry extracts and their antioxidants are able to decrease intracellular free radicals and free radical-induced DNA damage.



**Figure 2:** Redox cycling of menadione inducing oxidative damage [24].



Extracts from four different blueberry varieties will be used to pre-incubate the caco-2 cells. Two varieties with low oxygen radical absorbance capacity (ORAC) values (variety B and D) and two with high ORAC values (variety A and E). Subsequently, individual antioxidants, present in these berries, will be selected according to the results from the Plant Research Institute (Wageningen). These individual antioxidants, alone or in combination, will also be used to pre-incubate the caco-2 cells in order to identify the molecular mechanisms behind the blueberry-induced protective effect and to identify possible synergistic effects. The individual antioxidants that will be used are vitamin C, quercetin and cyanidin.

### **Expected outcome**

As previously mentioned, to prevent cancer, it is very important to maintain a healthy pro-oxidant-antioxidant balance. Increasing the vegetable and fruit consumption is therefore essential. Although everybody knows that a healthy diet is very important, many people still don't eat enough antioxidant-containing vegetables and fruit. However, if the results of this project show the molecular pathways behind the protective effects of blueberry antioxidants, people will be more convinced and motivated to eat healthy and antioxidant-containing food. In addition, the new insights can be used for the prevention and treatment of cancer.

## **Materials and methods**

### **Cell culture**

Caco-2 cells from three different cultures were used. These cells were cultured in a humidified incubator with an atmosphere of 5% CO<sub>2</sub> and 95% air (v/v) at 37°C in Dulbecco's medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 50U/ml Penicillin, 50µg/ml Streptomycin and 1% sodium-pyruvate. When 80-90% confluency was reached in the T75 flasks, cells were harvested by trypsin treatment and diluted 1:8 in supplemented growth medium in new T75 flasks. The first culture had passage numbers ranging from 32 till 45. The second cultures ranged from passage number 29 till 36, and the third caco-2 cell culture ranged from 20 till 29. HT-29 cells (passages between 9 and 11) were cultured in the same growth



medium with exception for sodium-pyruvate. Culturing occurred similar to the caco-2 cells.

### **Preparation of the blueberry extract**

Blueberries from four different blueberry varieties (A and E with high ORAC value, and B and D with low ORAC value) were frozen using nitrogen. Subsequently, the frozen berries were crunched resulting in blueberry powders which were dissolved in a 70% methanol/ 0.1% formic acid solution. However, because blueberries consist for 85% of water, the percentage of methanol/ formic acid had to be adapted to 76.5% methanol/ 0.109% formic acid. Subsequently, 0.5 gram blueberry powder was dissolved in 4.575ml 76.5% methanol/ 0.109% formic acid solution. This solution was mixed on a vortex for one minute at room temperature in a Greiner tube. Next, this mixture was centrifuged for 5 minutes at 3000 rpm. A 1cm large pellet was formed. The supernatant containing the blueberry extract was transferred into a new tube. This extract was concentrated 10 times by evaporating the methanol solution using nitrogen gas resulting in the maximal concentration of 1g/ml. The maximal concentration of the solvent 70% methanol/ 0.1% formic acid in the medium was always 0.5%. In addition, a test extract was also used. This extract was made from the blueberry-apple juice that was used in the human dietary intervention study of Wilms et. al. at our department [20]. It was also dissolved in a 70% methanol/ 0.1% formic acid solution and concentrated 10 times using nitrogen gas.

### **Pre-incubation with the blueberry extracts**

The maximal concentration of the extract to which the caco-2 cells were exposed was determined by the maximal tolerable concentration of the solvent 70% methanol/ 0.1% formic acid in the growth medium, which was always 0.5%. In addition to this maximal concentration of blueberry extract, caco-2 cells were exposed to half of this maximal concentration ( $\frac{1}{2}$  maximal), to a fourth ( $\frac{1}{4}$  maximal), and to one eighth of the maximal concentration ( $\frac{1}{8}$  maximal). In order to maintain equal levels of methanol/ formic acid to which the cells would be exposed, the extract was diluted with 70% methanol/ 0.1% formic acid. As a control 0.5 % methanol/ formic acid final concentration in growth medium was used to detect the effect of the solvent, and as a blanc, growth medium was used. Pre-incubations with the extracts were performed in

6 wellsplates with confluency between 80-90%. Cells were pre-incubated for 2 hours with the different doses of the extracts.

### **Cytotoxicity test**

The cytotoxicity of the blueberry extracts were tested using the Trypan blue exclusion assay. Caco-2 cells growing in 6 wellsplates with 80-90% confluency were used. After incubation of these cells with the different doses (max, 1/2max, 1/4max and 1/8max) and time points (2 and 24 hours) of the blueberry extracts, medium was removed and cells were washed with HBSS and harvested by trypsin treatment. All cell material including medium and HBSS was collected in greiner tubes on ice, and subsequently centrifuged (5 minutes at 400 rzb at 4°C). The supernatant was removed until 1 ml above the pellet in which the pellet was subsequently resuspended. The viability was assessed using a Bürker counting chamber. Therefore, 15 µl celsuspension was mixed with 15 µl Trypan blue and added to the Bürker counting chamber. The number of viable and death cells were counted four times in 16 squares, representing 0.1 µl celsuspension. Cells excluding Trypan blue were considered viable; the blue cells however were death cells. Viability was calculated using equation 1.

$$Viability = 100 - \left[ \left( \frac{blue}{blue + white} \right) \times 100 \right] \quad (1)$$

### **Challenge with menadione**

Menadione sodium bisulfite was purchased from Sigma (95%, FW 276.2). Challenge of caco-2 cells with menadione in growth medium was performed in 6 wellsplates with confluency between 80-90%.

### **Comet assay**

DNA single and double strand breaks in caco-2 cells induced by menadione were determined by the alkaline comet assay. Microscopic slides were degreased overnight in 100% ethanol, subsequently dried and pre-coated with 1.5% normal melting point agarose solution. They were stored in black storage boxes at 4°C until use. Caco-2 cells were harvested and resuspended in 250 µl 1xPBS at 4°C, resulting in a concentration of approximately 2\*10E6 cells/ml. Cytotoxicity was evaluated using the Trypan blue exclusion assay. Subsequently, 25 µl cell suspension and 75 µl

0.065% low melting point agarose was pipetted on the pre-coated slides, covered with a coverslip and placed on a wooden plate for 45 minutes at 4°C. After solidification, the coverslips were removed and the slides were placed in slide holders which were filled with Lyses buffer (2,5M NaCl, 100mM EDTA, 10mM Tris, 250mM NaOH, pH 10, 10% DMSO, 1% Triton X-100). The slide holders were wrapped with aluminium foil, closed with parafilm and placed at 4°C overnight or for minimum 1 hour. The next day, electrophoresis of the slides was performed. First, the slides were washed in 1xPBS for 15 minutes. Then they were placed in the electrophoresis tank which was filled with electrophoresis buffer (300mM NaOH, 1mM EDTA, pH min.12, 4°C). During 20 minutes, the DNA was unwind and denatured. Next, the tank was connected to the power source (300 mA, 25V) for 20 minutes, in order to facilitate migration of the damaged DNA strands to the positive pole. After electrophoresis, the slides were placed in the slide holders, washed with PBS for 5 minutes and subsequently dipped in 100% ethanol. Finally, the slides were dried for a few hours and stored in black storage boxes at 4°C until scoring. In order to compare different electrophoreses to each other, an internal control was used. Internal control cells were challenged with menadione (concentration to be determined), isolated, and freezed in growth medium with 10% DMSO. During every comet experiment, three comet slides were made using these internal control cells, similar to the other cells. At least 30 minutes before scoring, slides were stained with Ethidium Bromide in order to visualize the comets. Therefore, 50 µl Ethidium Bromide was pipetted at the non frosted end of the slide and than covered with a coverslip. The UV lamp was turned on 30 minutes before scoring. Fifty comets per slide were scored using the program Comet Assay III. The amount of oxidative DNA damage was defined as tail moment  $[(\text{tail intensity}/\text{total Comet intensity}) \times \text{distance from tail centre of distribution to head centre}]$ . Median values were used for statistical analyses and data presentation, because tail moments per slide are not normally distributed, and to exclude the effect of outliers.

### **Comet assay with FPG (Formamidopyrimidine DNA glycosylase)**

The protocol is similar to that of the normal comet assay. For the protocol with FPG, an additional step is added before electrophoresis of the comet slides in order to make the technique more sensitive. FPG is a base excision repair enzyme which recognizes and removes oxidized purines from correspondingly damaged DNA

resulting in a higher signal in the comet assay. After lysing the slides for 1 hour, the slides were rinsed 10 minutes in buffer F (40mM Hepes, 0.1M KCl, 0.5mM EDTA, 0.2mg/ml BSA). After this, slides were placed on a heat plate at 37°C and were incubated with 25µl FPG enzyme solution (diluted 30 times with buffer F) and covered with a coverslip. After 20 minutes, the slides were transferred to a cold plate. Control slides were incubated with buffer F alone. Subsequently, the coverslips were removed and electrophoresis occurred according the normal comet assay protocol.

### **FACS analysis of 8-oxo-dG**

To determine if blueberry extracts inhibit menadione-induced DNA oxidation, FACS analyses were performed. Cells, treated with extract alone or with extract and menadione, were harvested, resuspended in 100µl 1x PBS, and fixed with ice cold 100% methanol. Tubes were stored at -20°C until further analyses. Debris and methanol were removed by centrifuging samples at 400g for 5 minutes at 4°C and by removing the supernatant. Subsequently, cells were washed with TRIS buffered saline/ Tween-20 (TBST) (Biotrin). After removing the supernatant again, 100µl binding protein-FITC conjugate (Biotrin) was added. After incubation with the antibody for 1 hour at room temperature, cells were washed two times with TBST. Next, 500µl PBS/PI/RNase (PI, Fluka analytical; RNase, Sigma) solution was added to the cells. This suspension was put in FACS tubes and subsequently analyses on a FACScan flow cytometer (Becton Dickinson). The excitation wavelength ranged between 450 and 500 nm, the emission wavelength between 500 and 550 nm.

### **FACS analysis of apoptosis/ cell cycle**

To determine if blueberry extracts induce apoptosis, FACS analyses were performed. Cells, treated with extract alone or with extract and menadione, were harvested, resuspended in 100µl 1x PBS, and fixed with ice cold 100% methanol. Tubes were stored at -20°C until further analyses. Debris and methanol were removed by centrifuging samples at 400g for 5 minutes at 4°C and by removing the supernatant. Subsequently, cells were washed with PBS/BSA. After removing the supernatant again, 100µl M30 CytoDeath (Peviva) antibody was added. After incubation with the primary antibody for 1 hour at room temperature, cells were washed two times with PBS/BSA. Then, 100µl FITC conjugated anti-mouse Ig (DAKO F313) was added. After 1 hour incubation at room temperature, cells were washed again two times with

PBS/BSA. Next, 500µl PBS/PI/RNase (PI, Fluka analytical; RNase, Sigma) solution was added to the cells. This suspension was put in FACS tubes (BD Falcon) and subsequently analyses on a FACScan flow cytometer (Becton Dickinson). The excitation wavelength ranged between 450 and 500 nm, the emission wavelength between 500 and 550 nm.

### **Electron spin resonance (ESR) measurements**

Reactive oxygen species and the reduction of their production by blueberry extracts were measured using electron spin resonance (ESR) spectroscopy. For ESR experiments, cells were seeded in petri dishes (9,6cm<sup>2</sup>). After treatment, the caco-2 cells were harvested by scraping the cells. As a spin trap 50mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used and was added to the cells 30 minutes before the addition of menadione. First, cells were pre-incubated with 70% methanol/ 0.1% formic acid in medium or HBSS. In case of pre-incubating in medium, the medium-solution was removed after 2 hours, then cells were washed 2 times with HBSS, subsequently DMPO was added. In case of pre-incubating in HBSS, cells were washed 2 times before adding the HBSS-solution, after 90 minutes DMPO was added. After 30 minutes incubation with menadione, the cells were harvested by scraping with a cell scraper (Greiner Bio-one). The cellsuspension was resuspended and collected in a glass capillary (100µl, Brand AG Wertheim, Germany). Subsequently, the ESR spectrum was recorded at room temperature on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field, 3490G; scan range, 60G; modulation amplitude, 1G; receiver gain, 1x10<sup>5</sup>; microwave frequency, 9.85GHz; power, 50mW; time constant, 40.96ms; scan time, 20.97s; number of scans, 35. Spectra were quantified by peak surface measurements using WIN-EPR spectrum manipulation program (Bruker, Germany).

### **Statistical Analyses**

Means and standard deviations of means were calculated for all the experiments. The significance of differences between the control condition and the different concentrations of the blueberry extracts was determined by a T-test. To specify

significance, p-values of 0.01 and/or 0.05 were used. All analyses were performed by Excel.

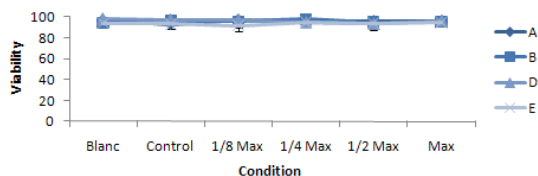
## Results & Discussion

### Part I: define the optimal experimental conditions

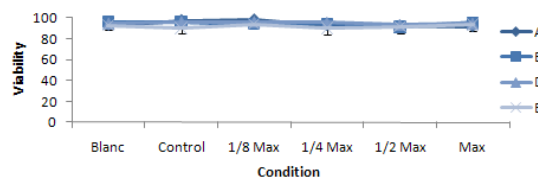
#### Assessing cytotoxicity of the blueberry extracts

To assess potential cytotoxic effects of the four different blueberry extracts (A and E with high ORAC values, and B and D with low ORAC values), caco-2 cells were incubated with several concentrations of these extracts for 2 and 24 hours. The maximal concentration, a half (1/2 Max), a fourth (1/4 Max) and one eighth (1/8 Max) of this maximal concentration were used. Figure 2 shows that the viability for all conditions and for all time points was above 90%, suggesting that the blueberry extracts were not harmful for the caco-2 cells. The test extract was also not cytotoxic (data not shown).

A



B

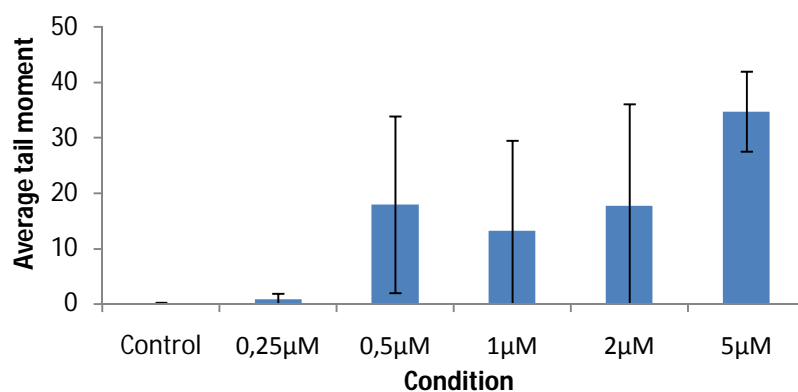


**Figure 3:** Viability of caco-2 cells exposed to increasing concentrations of four blueberry extracts (A, B, D and E) for (A) 2 hours and (B) 24 hours.

#### Selection of an optimal internal control

Internal control cells were used to be able to adjust for possible differences in electrophoresis. These cells were challenged with menadione, isolated, and frozen at  $-80^{\circ}\text{C}$  until use. During every comet experiment, three comet slides were made

containing these internal control cells. However, first an optimal menadione concentration for the internal control had to be selected. Therefore, several concentrations of menadione were tested. Figure 4 shows that menadione did induce DNA damage after 1 hour, however, there was a very large variation indicating that the results were not reliable. Consequently, an optimal menadione concentration for the internal control was selected using scientific literature. Burdette et al. showed that exposure of S30 breast cancer cells to 2 $\mu$ M menadione for 30 minutes resulted in the largest detectable difference between samples treated with menadione alone or with menadione and FPG [24]. According to Burdette et al. and our own findings, the conditions for the internal control were selected, namely 2 $\mu$ M menadione for 1 hour.



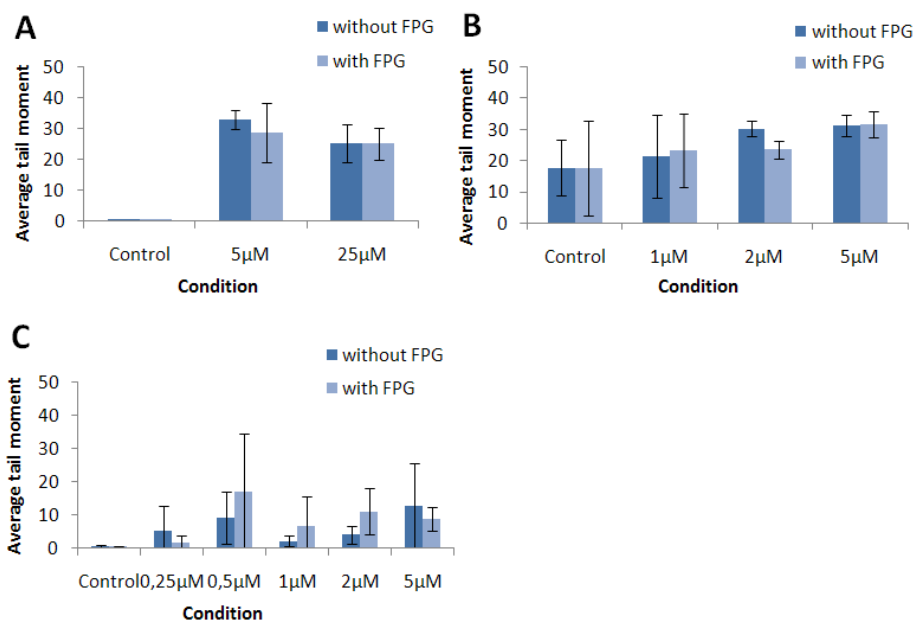
**Figure 4:** Comet Assay: Challenge of caco-2 cells with different menadione concentrations (0.25, 0.5, 1, 2 and 5 $\mu$ M) for 1 hour.

### FPG experiments

The base excision repair enzyme, formamidopyrimidine DNA glycosylase (FPG), recognizes and removes a wide range of oxidized purines from correspondingly damaged DNA. To assess if intervention with FPG could make the technique more sensitive by increasing the menadione-induced DNA damage, FPG was added to the comet assay protocol. If intervention with FPG could increase tail moments, a lower menadione concentration could be used to challenge the caco-2 cells. This lower menadione concentration would still be high enough to detect dose dependent decreases of DNA damage by the blueberry extracts, however, the lower menadione concentration would physiologically be more relevant. Figure 5A demonstrates that high menadione concentrations (5 and 25 $\mu$ M) did not show an addition in DNA damage due to FPG. Subsequently, the experiment was repeated with lower menadione concentrations (1, 2 and 5 $\mu$ M). This experiment showed large standard deviations for the control condition and the exposure with 1 $\mu$ M. In addition, the



resulting DNA damage for the control condition was unusually high, indicating that something went wrong (Figure 5B). Another experiment with low menadione concentrations (0.25, 0.5, 1, 2 and 5 $\mu$ M) showed a normal control, however, the other conditions showed very large standard deviations (Figure 5C). In addition, this signal was much lower than the signal in previous experiment when comparing concentrations 1, 2 and 5 $\mu$ M (Figure 5A and B). Besides this, in all three cases, FPG did not have a clear additional effect on DNA damage. From these results, the conclusion was made that the extra FPG step in the comet assay protocol was unnecessary in our experiments. The extra step might influence the large standard deviations, and therefore, in future experiments, FPG was not used anymore.



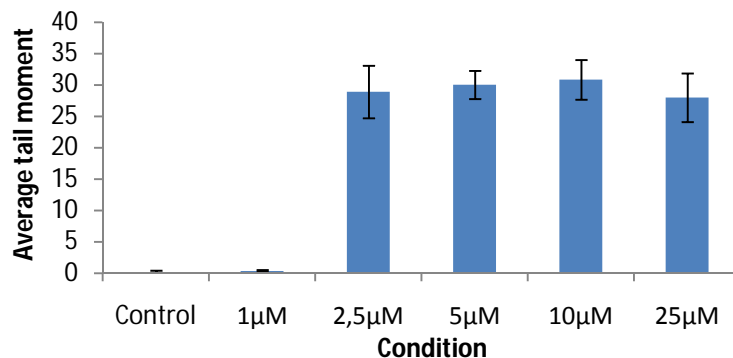
**Figure 5:** Comet assay: challenge of caco-2 cells with different concentrations of menadione for 1 hour; with or without FPG. (A) 5 $\mu$ M and 25 $\mu$ M menadione (B) 1 $\mu$ M, 2 $\mu$ M and 5 $\mu$ M menadione (C) 0.25 $\mu$ M, 0.5 $\mu$ M, 1 $\mu$ M, 2 $\mu$ M and 5 $\mu$ M menadione.

### Selection of an optimal menadione concentration

Preliminary data did already indicate that 10 $\mu$ M was an optimal menadione concentration because exposure of caco-2 cells to this concentration resulted in a cell viability around 99%. In addition, according to the comet assay, this concentration induced an optimal level of DNA damage. However, before using 10 $\mu$ M menadione in future experiments, a new comet assay had to be performed to confirm previous findings, and this concentration had to be tested in an ESR experiment to investigate if it indeed did induce ROS formation. Figure 6 demonstrates that DNA damage after exposure with a menadione concentration of 2.5 $\mu$ M or more already

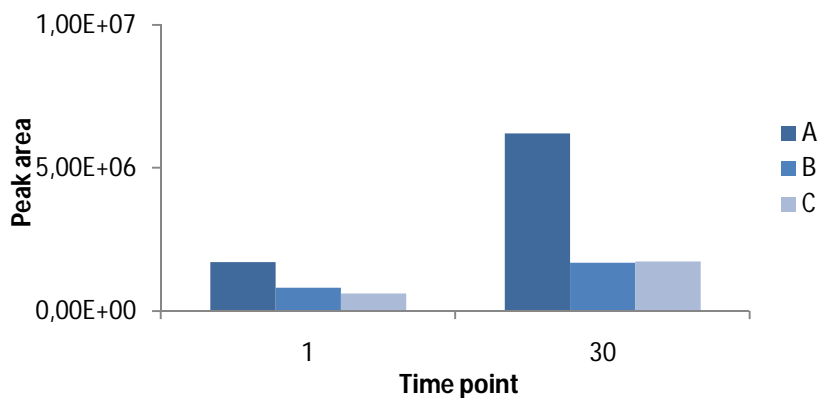


reached a maximum value. Concentrations above 5 $\mu$ M did not add extra information about DNA damage.

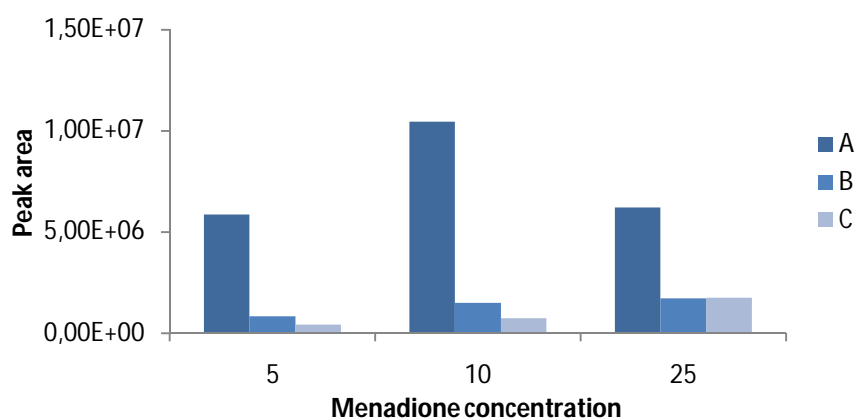


**Figure 6:** Comet assay: Challenge of caco-2 cells with different menadione concentrations (1, 2.5, 5, 10 and 25 $\mu$ M) for 1 hour.

ESR experiments were performed to measure menadione-induced ROS production in caco-2 cells and the possible protective effect of blueberry extracts. In the comet assay, menadione-induced DNA damage was measured after exposure for 1 hour. To be able to correlate the menadione-induced ROS production to menadione-induced DNA damage, ROS production had to be measured during the first hour of menadione exposure. To achieve this, an optimal experimental setup had to be developed. First, several menadione concentrations and time points had to be measured. Figure 7 and 8 show the results of these measurements. To be able to measure a significant dose dependent decrease in ROS production due to intervention with the different blueberry extracts, the menadione signal should be high enough. Measuring menadione-induced ROS production at 30 minutes after exposure was much higher than measuring this immediately after addition of menadione (approximately after 1 minute), especially for the exposure with 25 $\mu$ M menadione (Figure 7). According to the dose response experiment with menadione, shown in Figure 8, cells from experiment A did respond different than those from the other experiments (B and C). This could be explained by the fact that cells from experiments B and C were derived from a new batch. Cells of this new batch seemed to produce less ROS than cells of experiment A.

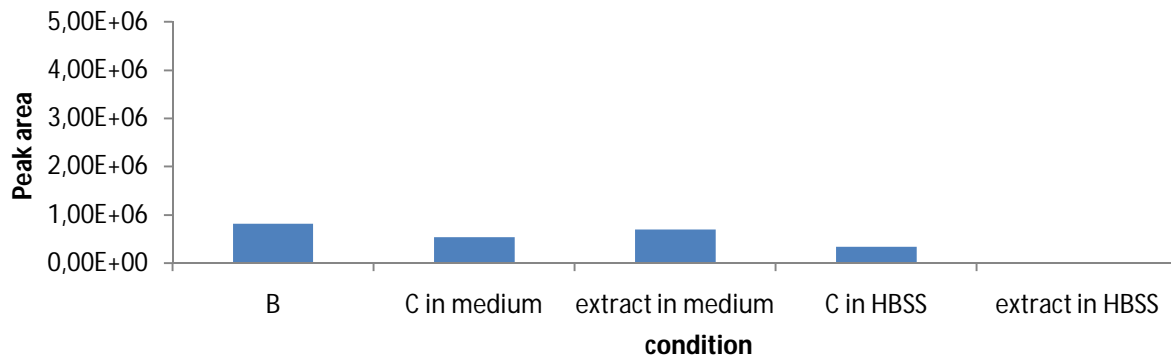


**Figure 7:** ESR: Challenge of caco-2 cells with different menadione concentrations, measured at 1 and 30 minutes after exposure. (A) 25 $\mu$ M menadione (B) and (C) 10 $\mu$ M menadione.



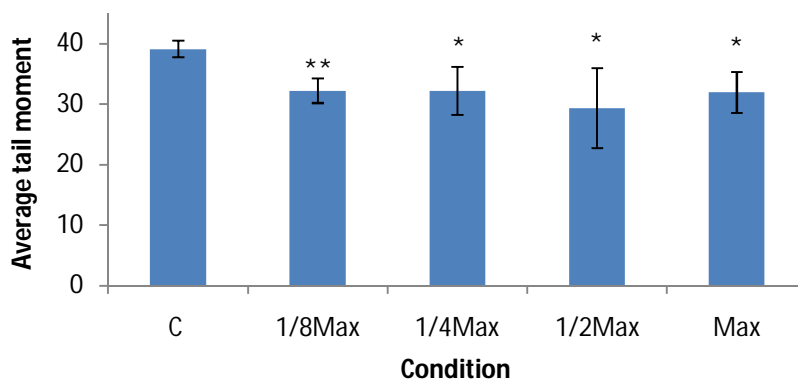
**Figure 8:** ESR: Challenge of caco-2 cells with 5, 10 or 25 $\mu$ M menadione, measured at 30 minutes after exposure. A, B and C indicate different experiments.

To investigate the effect of pre-incubating the caco-2 cells before challenging them with menadione, cells were pre-incubated for 2 hours with 70% methanol/ 0.1% formic acid (control) or with the maximal concentration of the test extract. Cells were pre-incubated in medium or in HBSS to assess the optimal experimental setup. According to Figure 9, pre-incubating in HBSS was the optimal setup, since the extract decreased ROS production till zero. Consequently, pre-incubating the test extract in medium seemed to increase ROS production (Figure 9). However, because of the low menadione signal it was difficult to assess significant decreases in ROS production induced by the extracts.



**Figure 9:** ESR: Challenge of *caco-2* cells with 10 $\mu$ M menadione after pre-incubation with 0.5% of 70% methanol/ 0.1% formic acid (C) or extract for 2 hours in medium or in HBSS. Cells were measured at 30 minutes after menadione exposure. B (blanc) is 10 $\mu$ M menadione alone. C (control) is pre-incubation with 0.5% of 70% methanol/ 0.1% formic acid.

Subsequently, a comet experiment was performed in which *caco-2* cells were pre-incubated with different concentrations of the test extract for 2 hours, followed by a challenge with 10 $\mu$ M menadione for 1 hour. Figure 10 shows that pre-incubation with the test extract did induce a little decrease in DNA damage, however, the damage was still high. Previous interventions with this test extract did have larger effects on DNA damage (data not shown).



**Figure 10:** Comet assay: Challenge of *caco-2* cells with 10 $\mu$ M menadione for 1 hour after a pre-incubation with different concentrations of the extract (Max, 1/2max, 1/4max, 1/8max) for 2 hours. C (control) is pre-incubation with 0.5% of 70% methanol/ 0.1% formic acid for 2 hours, followed by challenge with 10 $\mu$ M menadione for 1 hour. \*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone,  $P < 0.05$ . \*\*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone,  $P < 0.01$ .

### Intermediate conclusions

According to the experiments shown above, blueberry extracts were not cytotoxic. In addition, the results of the comet assay indicated that menadione did induce DNA damage, and that FPG did not add extra information. However, these results were not reproducible and in some experiments the standard deviations were too high. Next, the ESR experiments showed that HBSS was the optimal solution to pre-incubate the caco-2 cells in. However, menadione-induced ROS production in the new batch of caco-2 cells was too low to detect a decrease after intervention with the extracts. In addition, also with the ESR experiments it was difficult to get reproducible results. To detect the problem, all variables (buffers, medium, comet slides,...) were changed, however, that did not improve the results. The only variable that still could be changed were the cells. Therefore, two new colon cancer cell lines were started up, namely HT-29 cells and new caco-2 cells (kindly provided by J. Renes, department of Human Biology, Maastricht University, the Netherlands) with a much lower passage number.

## **Part II: Determine the anticarcinogenic effects of the blueberry extracts**

### Analysis of the antioxidant content of the different blueberry extracts

The Plant Research Institute analysed the antioxidant content of the four different blueberry extracts (A, B, D and E) by means of HPLC (performed by J.P.F.G. Helsper and H.H. Jonker, Plant Research International, WUR, Wageningen). Table 1 shows the results of these analyses. Firstly, the amount of a specific antioxidant in µg per gram fresh weight (FW) was indicated. Secondly, the antioxidant capacity was indicated. This antioxidant capacity was measured using the Trolox equivalent antioxidant capacity (TEAC) assay. In this assay, the antioxidant capacity of the blueberry antioxidants was compared to the antioxidant capacity of Trolox, a vitamin E analog. To compare both antioxidant capacities, the blue 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonoc acid) (ABTS) radical cation was used. This ABTS radical was converted back to its colourless form after reaction with the antioxidants. Thirdly, the contribution of each antioxidant in the TEAC assay per gram fresh weight was calculated. The results in Table 1 show that these blueberry extracts did contain different kinds of antioxidants such as anthocyanins, which were known to be the

most important bioactive compounds present in blueberries. However, these results indicated that the antioxidant content differed strongly between the different blueberry varieties, and that the presence of a particular antioxidant did not always imply that this antioxidant had a high antioxidant capacity.

**Table 1:** Analysis of the antioxidant content of the four different blueberry extract, measured using HPLC. FW: fresh weight, (\*) not detected [25].

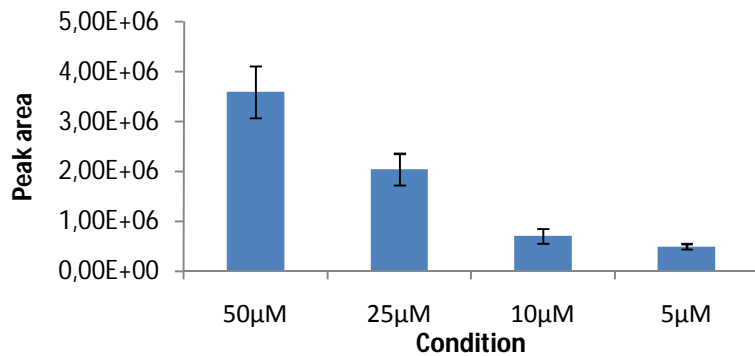
Individual antioxidants	µg/g FW				µmol Trolox eq/g FW				% Trolox eq/g FW			
	A	B	D	E	A	B	D	E	A	B	D	E
Caffeic acid derivate	8.6	4.9	13.5	(*)	0.8	0.52	0.46	0.91	3.39	3.4	2.77	2.04
anthocyanin	665	287	528	697	(*)	(*)	(*)	(*)	0	0	0	0
Delphinidin-3-glucoside	11.5	69.6	3.8	8.3	(*)	0.64	(*)	(*)	0	4.17	0	0
Delphinidin-3-rutinoside	412	138	194	560	1.34	1.35	1.85	7.39	5.63	8.83	11.15	16.62
Cyanidin-3-glucoside	304	66	149	259	0.79	0.84	1.31	3.99	3.34	5.53	7.88	8.98
Peonidin-3,5-diglucoside	47.9	73.8	29.9	69.7	0.73	0.73	0.4	1.32	3.09	4.81	2.38	2.98
Pelargonidin-3-glucoside	375	125	227	359	0.5	0.52	0.84	2.49	2.09	3.38	5.07	5.6
Peonidin-3-glucoside	2605	686	1421	1440	1.55	1.59	2.8	4.36	6.51	10.4	16.83	9.79
Malvidin-3-glucoside	29.3	158.4	16.4	13.6	(*)	(*)	(*)	(*)	0	0	0	0
Quercetin-3-glucoside	73.8	49.3	51.7	59.8	(*)	(*)	(*)	(*)	0	0	0	0
Quercetin	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	0	0	0	0

### Selection of the most favourable cell type

For both cell types, caco-2 and HT-29, several basic ESR experiments were performed to determine the effect of different menadione concentrations (10, 25 and 50µM) and the effect of pre-incubating the cells with the test extract before challenging them with menadione. Both cell types responded similar (data not shown). Therefore, the following experiments were performed with the originally preferred cell type, namely, the caco-2 cells.

### Selection of the optimal menadione concentration in the new cells

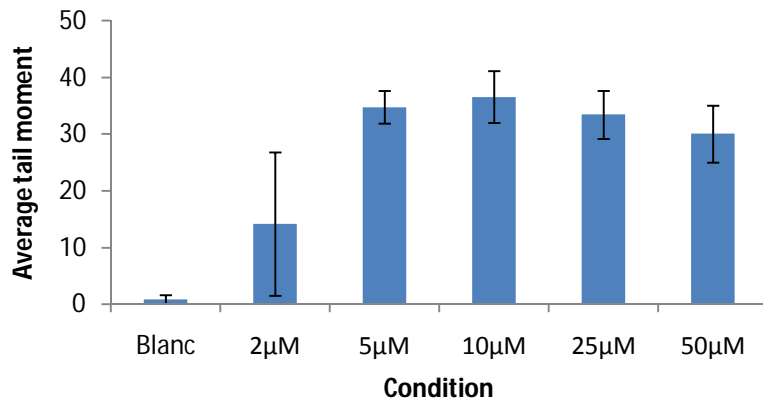
The new caco-2 cells were challenged with several menadione concentrations and were subsequently tested in the ESR as well as in the comet assay. Figure 11 shows that menadione induced ROS production in a dose-dependent manner.



**Figure 11:** ESR: Challenge of Caco-2 cells with 5, 10, 25 and 50μM menadione, measured at 30 minutes after menadione exposure.

Subsequently, Figure 12 shows the menadione-induced DNA damage in the new Caco-2 cells. Cells were challenged with 2, 5, 10, 25 and 50μM menadione for 1 hour. The result of this comet assay was similar to that of the menadione experiment from the previous section (Figure 6) and indicated that menadione-induced DNA damage happens abruptly. Low menadione concentrations (5μM and higher) did already induce high tail moments and simultaneously reach a plateau level.

In contrast with the comet assay results of Figure 12, Nutter et al. did find a dose-dependent increase in DNA damage after a challenge with 5, 10, 15, 25 and 50μM menadione [26]. However, they used other cells, namely human breast carcinoma MCF-7 cells and they analysed single- and double-strand DNA breaks using alkaline and neutral elution respectively [26]. To measure this, 24 hours before the addition of menadione, cells were radioactively labelled with [<sup>14</sup>C] thymidine or [<sup>3</sup>H] thymidine. Next, the cells were washed with PBS and lysed with proteinase K [26]. To detect single strand DNA breaks, cells were subsequently eluted using a buffer with pH 12.1 whereas double stranded DNA breaks were detected after neutral elution [26]. On the other hand, Burdette et al. showed that incubating human breast cancer S30 cells with menadione for 30 minutes induced a dose-dependent increase in DNA damage reaching a maximum at 10μM and not at 50μM [24]. However, probably different cell types and different detection techniques give different results in response to a challenge with menadione. This statement was confirmed by Janzowski et al. because they showed that different menadione concentrations were needed to generate similar levels of DNA damage, assessed using the comet assay, in two different human colon cancer cell lines, namely HT29 and Caco-2 cells [27].



**Figure 12:** Comet: Challenge of caco-2 cells with 2, 5, 10, 25 and 50µM menadione for 1 hour. Blanc is growth medium alone.

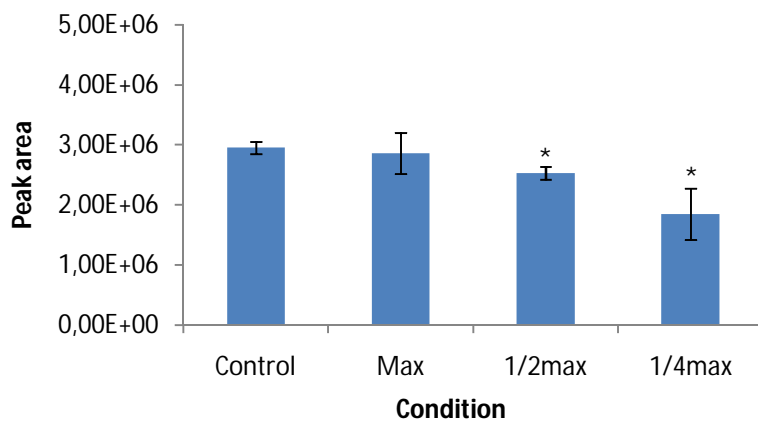
From the results of these menadione experiments, it was decided to select different menadione concentrations for the ESR and the comet experiments. For the ESR experiments it was important to select a concentration that was high enough to pick up the possible blueberry extract-induced dose-dependent decrease in ROS production. Therefore, 25µM menadione was chosen for the ESR experiments. However, if the comet experiments were also performed with this concentration (25µM), it would be likely that a possible blueberry extract-induced decrease in DNA damage would not be picked up. Therefore, a menadione concentration of 5µM was chosen to use in the comet experiments.

### The effects of blueberry extracts on ROS production

Previous ESR experiments (Figure 9) performed with the test extract indicated that pre-incubating the caco-2 cells in HBSS gave better results than pre-incubating them in medium. In case of pre-incubation in HBSS, the extract stayed on the cells during the entire experiment. The resulting effects measured with the ESR came from the blueberry antioxidants that were taken up by the cells but also from the antioxidants that were present around the cells. However, to become biologically relevant results, it was important to determine the effect of the blueberry antioxidants that were taken up by the caco-2 cells. Therefore, following ESR experiments were performed by pre-incubating the cells in medium that was removed from the cells after 2 hours of pre-incubation.

First, the possible radical scavenging activity of extract A was tested. Figure 13 shows that the maximal concentration of the extract did not have a significant effect on ROS production after a challenge with 25µM menadione. The lower

concentrations, however, induced a significant decrease in ROS production ( $p$ -value  $< 0.05$ ). It was known that high antioxidant concentrations could have pro-oxidant effects, and that lower concentrations could be more protective such as the results in Figure 13 indicate [28]. However, unless the maximal concentration did not induce a decrease in ROS production, it also did not induce an increase. Therefore, it was very interesting to determine the effect of the different concentrations of the blueberry extract after a challenge with a lower menadione concentration, namely  $10\mu\text{M}$  menadione.

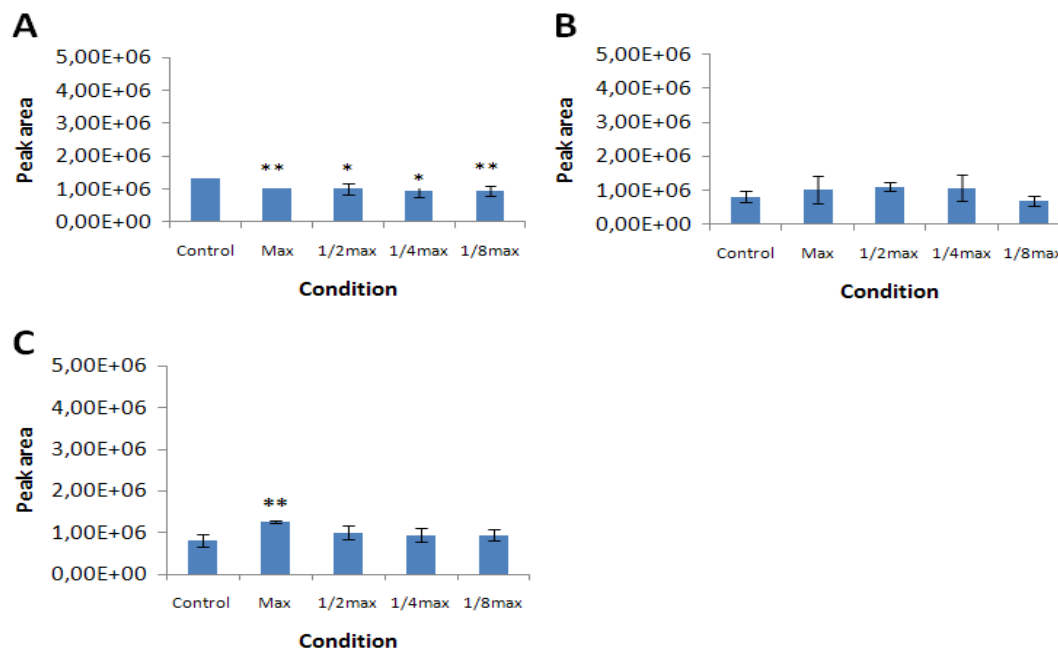


**Figure 13:** ESR: Pre-incubation of caco-2 cells with several concentrations (Max, 1/2max and 1/4max) of blueberry extract A or with 0.5% of 70% methanol/ 0.1% formic acid solution (Control) for 2 hours followed by a challenge with  $25\mu\text{M}$  menadione, measured at 30 minutes after menadione exposure. \*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone,  $P < 0.05$ .

Figure 14 shows that extract A was able to significantly decrease menadione-induced ROS production after a challenge with  $10\mu\text{M}$  menadione. However, there was only one result for the control because of a short power breakdown during the measurement of the other control condition. If there would be more results, it could be that the average control value would be lower such as the control of the experiment with extract D. Normally, the controls of both experiments should be the same. Therefore, data from experiment A were compared with the average control of experiment B (Figure 14C). This showed that pre-incubation with extract A did not decrease ROS production. On the contrary, the maximal concentration of extract A did significantly increase ROS production when compared to the control of experiment B (Figure 14C). The results of extract A taken together it was seen that after a challenge with  $25\mu\text{M}$  menadione, the maximal concentration of the extract did not show an effect on ROS production, however, after a challenge with  $10\mu\text{M}$ , the



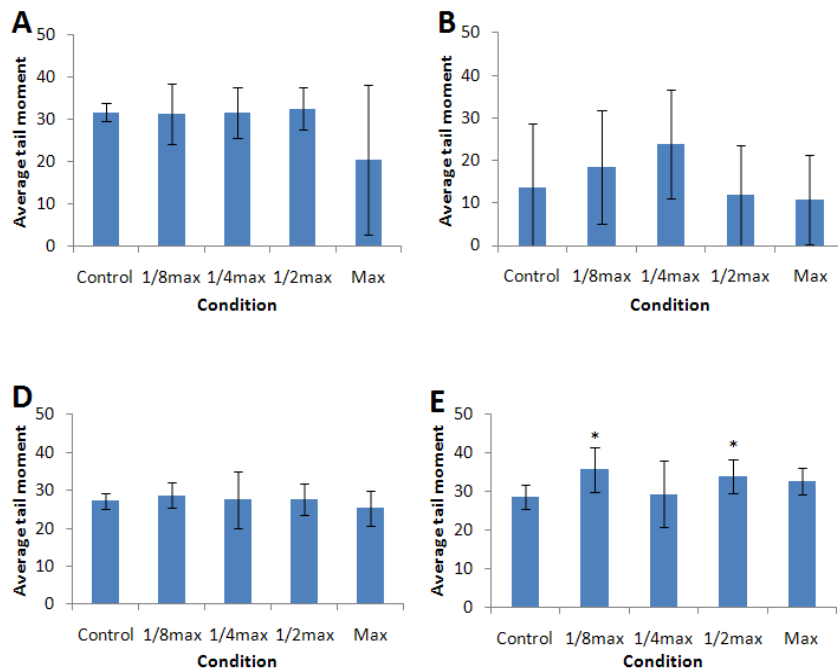
maximal concentration of the extract had a pro-oxidant effect according to the result in Figure 14C. The other conditions of both extract A and D did not induce changes in ROS production.



**Figure 14:** ESR: Pre-incubation of caco-2 cells with several concentrations (Max, 1/2max, 1/4max and 1/8max) of blueberry extract A and D or with 0.5% of 70% methanol/ 0.1% formic acid solution (Control) for 2 hours followed by a challenge with 10µM menadione, measured at 30 minutes after menadione exposure. \*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone,  $P < 0.05$ . \*\*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone,  $P < 0.01$ . (A) Blueberry extract A. (B) Blueberry extract D (C) Blueberry extract A compared to the control of the experiment with extract D.

### The effects of blueberry extracts on DNA damage

To determine if the different blueberry extracts were able to decrease DNA damage, caco-2 cells were pre-incubated for 2 hours with different concentrations (Max, 1/2max, 1/4max and 1/8max) of the blueberry extracts. Subsequently, the extract was removed from the cells and the caco-2 cells were challenged with 5µM menadione. Figure 15 shows that there was no blueberry extract able to decrease menadione-induced DNA damage. After pre-incubation with extract E, DNA damage even increased in some conditions, indicating a pro-oxidant effect. As mentioned previously, it was known that polyphenolic antioxidants could have pro-oxidative effects in some cases [28]. However, this increase in DNA damage was probably a result of the high variation that was seen in all cases.

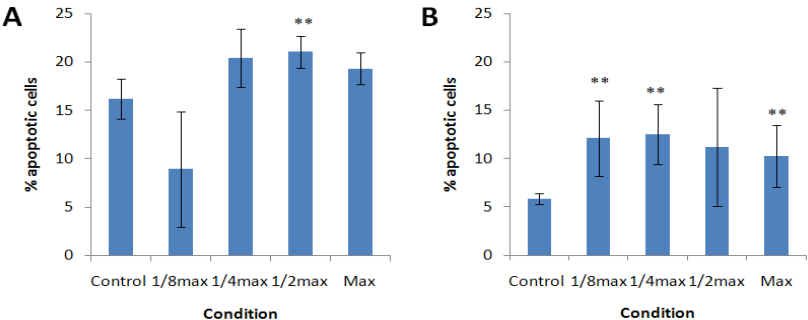


**Figure 15:** Comet assay: Challenge of caco-2 cells with 5 $\mu$ M menadione for 1 hour after a pre-incubation with different concentrations of the extract (Max, 1/2max, 1/4max, 1/8max) for 2 hours. Control is pre-incubation with 0.5% of 70% methanol/ 0.1% formic acid for 2 hours, followed by challenge with 5 $\mu$ M menadione for 1 hour. \*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone,  $P < 0.05$ . A, B, D and E indicate the four different blueberry extracts.

### The effects of blueberry extracts on apoptosis

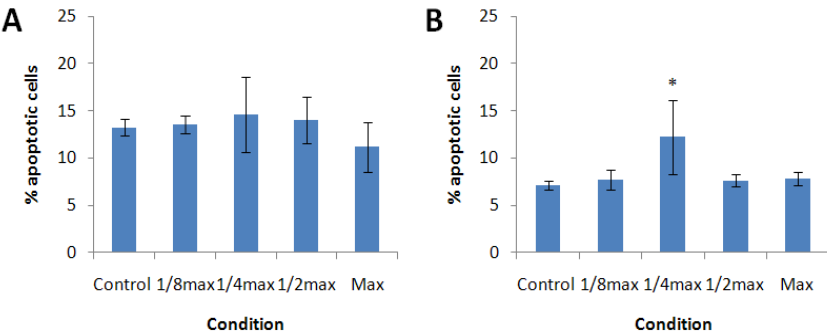
Apoptosis is one of the most important mechanisms for antitumor activity because apoptosis stops the proliferation of damaged cells. After a cell is damaged there are – next to apoptosis- two other possible reactions. Firstly, the repair system of the cell will restore the damaged cell. Secondly, the damaged cell will continue to proliferate and thereby increase the amount of damaged cells which can lead to cancer. To assess if blueberry extracts were able to induce apoptosis in cells that were damaged after a challenge with menadione, a FACS experiment was performed. Figure 16 shows that a pre-incubation for 2 hours was able to induce apoptosis in cells that were challenged with 5 $\mu$ M menadione for 1 hour. In extract A only the 1/2max concentration was able to increase apoptosis significantly. The increase in apoptosis after pre-incubating the cells with extract D was significant for the concentrations 1/8max, 1/4max and Max. These results indicated that extract D was more potent to induce apoptosis in menadione-induced damaged cells compared to extract A.

However, in some conditions there were high standard errors which made the results less reliable. In addition, it was strange that the values of the control conditions were not the same in both experiments.



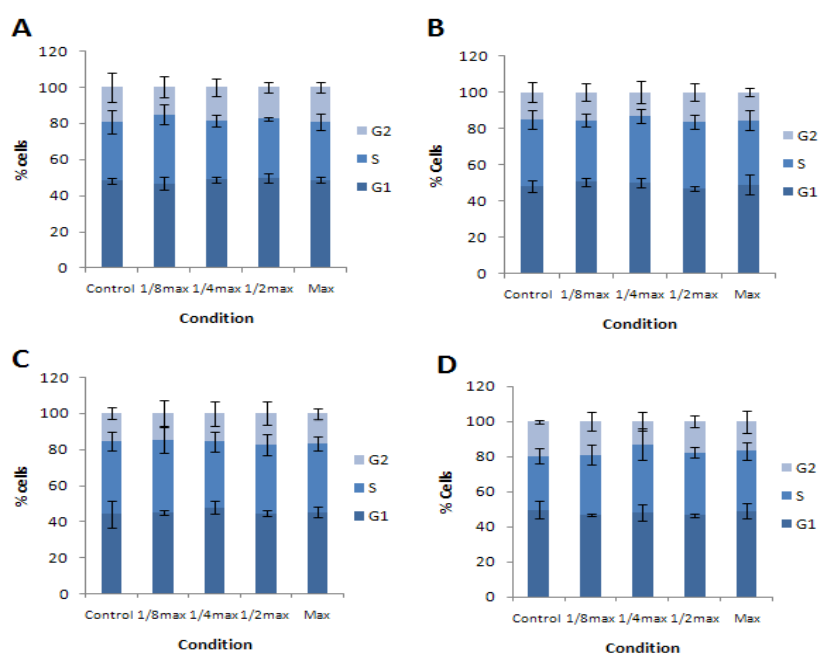
**Figure 16:** FACS: Pre-incubation of caco-2 cells with several concentrations (Max, 1/2max, 1/4max and 1/8max) of blueberry extract A and D or with 0.5% of 70% methanol/ 0.1% formic acid solution (Control) for 2 hours followed by a challenge with 5µM menadione for 1 hour. \*\*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone, P<0.01. (A) Blueberry extract A. (B) Blueberry extract D.

The conditions in which the cells were pre-incubated with different concentrations of the extracts without a challenge with menadione did not induce an increase in apoptosis except for the 1/4max concentration of extract D (Figure 17). However, the standard deviation in this condition was very large indicating that the significant increase in apoptosis was not that reliable. Therefore, to get more reliable results it was recommended to repeat this experiment. The results in Figure 16 and 17 indicated that the extracts only induced an increase in apoptosis -compared to the control- after the cells were damaged during a challenge with menadione.



**Figure 17:** FACS analysis of apoptotic cells after pre-incubation of caco-2 cells with several concentrations (Max, 1/2max, 1/4max and 1/8max) of blueberry extract A and D or with 0.5% of 70% methanol/ 0.1% formic acid solution (Control) for 2 hours. \*Significantly different from cells treated with 0.5% of the 70% methanol/ 0.1% formic acid solution alone, P<0.05. (A) Blueberry extract A. (B) Blueberry extract D.

To further investigate the effect of blueberry extracts on cell cycle, the distribution of each phase of the cell cycle was determined by flow cytometric analysis of DNA content. Figure 18 shows that pre-incubation of the cells with different concentrations of the blueberry extracts for 2 hours was not able to significantly modulate cell cycle, with and without a subsequent challenge with menadione. According to this result, it could be concluded that blueberry extracts were able to induce apoptosis in caco-2 cells that were damaged by menadione, and that these extracts were not able to induce changes in cell cycle in the surviving cells. However, the impossibility of the extracts to induce changes in cell cycle could be explained by the fact that a 2 hours pre-incubation was not sufficient to detect changes in cell cycle. Though, Lazzè et al. did also not find a significant effect on cell cycle after pre-incubating caco-2 cells with cyanidin –an anthocyanin present in blueberries- for 24 hours [29]. On the contrary, delphinidin –another anthocyanin- did have effect on cell cycle of caco-2 cells after a 24 hours pre-incubation without a subsequent challenge with an oxidant [29]. Pre-incubation with delphinidin did significantly reduce the amount of cells in the G1 phase and did increase the amount of cells in the G2/M phase [29]. In addition, some cells did have a diploid DNA content, suggesting the presence of apoptotic cells [29].



**Figure 18:** FACS cell cycle analysis: Pre-incubation of caco-2 cells with several concentrations (Max, 1/2max, 1/4max and 1/8max) of blueberry extract A and D or with 0.5% of 70% methanol/ 0.1% formic acid solution (Control) for 2 hours. (A and C) Blueberry extract A and D respectively, without menadione. (B and D) Blueberry extract A and D respectively, with 5 $\mu$ M menadione for 1 hour.

The pro-apoptotic effects of blueberry extracts were in line with the results of several other researchers, namely Seeram et al. investigated the pro-apoptotic effects of several berry extracts –rich in a diverse range of phytochemicals- on the HT-29 colon cancer cell line. They showed that black raspberries and strawberries did have a significant pro-apoptotic activity. In addition, they found that blueberries increased apoptosis compared to controls [30]. Lazzè et al. investigated the pro-apoptotic effect of several anthocyanins –present in high amounts in blueberries- in different cell types. They found that delphinidin did induce apoptosis in human cancer cells, namely in uterine carcinoma HeLaS3 cells and in colon adenocarcinoma caco-2 cells [29]. In addition, Yang et al. studied the pro-apoptotic effect of another flavonoid present in blueberries, namely quercetin. They found that quercetin was able to induce apoptosis in human lung cancer NCI-H209 cells after a pre-incubation of 48 hours [31].

In addition, the fact that the blueberry extracts did induce apoptosis in caco-2 cells after a challenge with menadione could explain the results of the previously performed ESR and comet experiments. For example, the high level of heterogeneity of the cells and therefore the high standard deviations in the comet assay could result from the fact that a lot of cells were dead because of apoptosis and that the other cells were relatively undamaged because of the blueberry extract-induced protection against menadione-induced DNA damage. To overcome this problem, it could be tried to challenge the cells with a lower menadione concentration in order to prevent that most cells become apoptotic. In addition, a longer pre-incubation period could also be tried to increase the blueberry extract-induced protective effects on the level of DNA damage. In line with this suggested approach, Janzowski et al. pre-incubated caco-2 cells with several constituents of apple juice (quercetin and phloretin) and apple juice extracts for 2 or 24 hours followed by a challenge with 3 $\mu$ M menadione for 1 hour [27]. They showed a dose-dependent modulation of DNA damage in all cases [27]. In regard to the ESR experiments, the suggested adaptation of the experimental setup could also result in a better blueberry extract-induced protection against ROS production.

## Conclusion

The aim of this study was to unravel the mechanisms behind the blueberry-induced anticarcinogenic effects in caco-2 cells, and to discover which individual antioxidants or combinations of individual antioxidants were responsible for these effects. Therefore, four different blueberry extracts were used. Two with high ORAC values and two with low ORAC values. They all contained several antioxidants such as caffeic acid and several kinds of anthocyanins in different amounts. Before their anticarcinogenic effects could be tested, an experimental setup had to be developed that could be used to discover maximal effects of these blueberry extracts. Preliminary data indicated that pre-incubation of caco-2 cells for 2 hours was sufficient to pick up protective effects of the blueberry extracts. After the pre-incubation with blueberry extracts, the cells were challenged with an oxidant, namely menadione. In the comet assay and in the FACS a 1 hour challenge with 5 $\mu$ M menadione was used, in the ESR experiments the samples were measured at 30 minutes after the addition of 10 $\mu$ M menadione.

In general it was found that pre-incubation of the caco-2 cells with different blueberry extracts did not show anticarcinogenic effects at the level of DNA damage for all four the extracts and at the level of ROS production for extract A and D. The fact that no single blueberry extract that was tested did have protective effects on the levels of DNA damage and ROS production could be explained by the fact that the menadione concentration that was used to challenge the cells was too high. Secondly, it could also be that the blueberry antioxidants did not have enough time to enter the cells. According to other studies, a pre-incubation of human cancer cells for at least 24 hours with berry extracts was needed to inhibit proliferation and to induce apoptosis [7, 16, 17]. In addition, to determine the protective effects of individual antioxidants such as quercetin, Mertens-Talcott et al. also used pre-incubation periods of at least 12 hours [18]. Despite of the fact that the comet and ESR experiments did not show protective effects of the blueberry extracts, the FACS results with extract A and D were promising. In these experiments a pre-incubation period of two hours with the blueberry extracts was sufficient to detect their pro-apoptotic effects.

For the future it is important to repeat the FACS experiments for the other extracts. In addition, the development of a new experimental setup could lead to a better understanding of the effects of the different blueberry extracts on the levels of DNA

damage and ROS production. According to the original research proposal, the effects of the different blueberry extracts on 8-oxo-dG adduct formation and on gene expression do also still have to be measured. All these future results will add a lot of interesting information that could be important to motivate people to eat antioxidant-containing food, namely vegetables and fruit. The results are promising, however, there is still a lack of *in vivo* evidence for the protective effects of a lot of bioactive compounds. In addition, there is also still less known about the bioavailability of several antioxidants such as anthocyanins. Yi et al. showed that anthocyanins from blueberries could be transported through the caco-2 cell monolayers [32]. However, McGhie et al. indicated that much information is missing about the way in which anthocyanins are absorbed and about the metabolites that are generated *in vivo*, and their specific contribution to possible health benefits [33]. Apart from the anthocyanins, there are many other antioxidants, and this group of bioactive compounds is very heterogeneous. All antioxidants vary on the basis of bioavailability, effectiveness, distribution in nature and so on. A good understanding of the numerous factors influencing bioavailability and the ability to take metabolism and bioavailability into account when distinguishing between *in vivo* and *in vitro* antioxidant activities are very important. To achieve this and to optimize the dietary advice for the population in order to improve the health benefits for the consumers of vegetables and fruit, much research is needed.

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