

Full Length Research Paper

Polyphenol compounds with anti-carcinogenic qualities: Effects of quercetin (flavonol), chrysin (flavon), kaempferol (flavanol), naringenin (flavanon) and hesperidin (flavanoid) on *in vitro* breast cancer

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Epidemiological, clinical or animal studies have suggested an inverse association between the consumption of polyphenol and polyphenol-rich foods or beverages and the prevention of diseases. Previously, the following effects have been shown on flavonoids from the black elderberry, *Sambucus nigra*, on cells. Quercetin: apoptosis induction, antioxidant, protein kinase inhibitor, interaction with type II oestrogen-binding site. Chrysin: aromatase inhibitor and apoptosis-induction affect PPAR alpha and synthetic analogue: dihydroxy-8-nitrochrysin (NOC) = apoptosis-induction. Kaempferol: induction of apoptosis, cell proliferation, and reduction in ER-alpha mRNA. Naringenin: cell proliferation. Hesperidin: cell proliferation. The question now arises as to whether the flavonoids, quercetin, chrysin, kaempferol, naringenin and hesperidin are candidates for primary and secondary cancer prevention. The effect of crude extracts of elderflower and elderberries and the aforementioned flavonoids at concentrations of 5, 10, 20 and 50 µg/ml on vitality parameters of the breast cancer cell lines MCF-7 (ER α +, ER β +) and BT20 (ER α-, ER β-) was demonstrated. Cell proliferation (BrdU assay), mitochondrial activity (MTT assay) and cytotoxicity (LDH assay) were examined. Elderflower crude extract inhibits cell proliferation of MCF-7 (62%) and BT20 (10%) cells. Elderberry crude extract inhibits cell proliferation in MCF-7 (50%) cells and proliferation in BT20 (20%) cells. Quercetin, chrysin, kaempferol, naringenin and hesperidin inhibit proliferation in MCF-7 (quercetin, 50 µg/ml (22%); chrysin, 50 µg/ml (30%); kaempferol, 5 µg/ml (36%); naringenin, 5 µg/ml (25%); hesperidin, 5 and 10 µg/ml (21%) and BT20 (quercetin, 5 µg/ml (18%); chrysin, 10 and 50 µg/ml (8%); kaempferol, 20 µg/ml (15%); naringenin, 5 and 50 µg/ml (20%); hesperidin, 5 and 10 µg/ml (14%)) cells, no cytotoxicity, and little effect on mitochondrial activity.

Key words: *Sambucus nigra*, polyphenols, breast cancer, LC-MS analysis.

INTRODUCTION

Breast cancer is the most often diagnosed type of cancer (23% of all cancer cases) and the main cause of the cancer death among women (14%) (Meeran et al., 2012). Breast cancer is therefore one of the most threatening chemotherapeutic options for breast cancer include drugs

such as tamoxifen and the cytotoxics paclitaxel and docetaxel, all of which show severe side effects (Marsh et al., 2007). Consequently, research is being carried out to diseases of modern time (Harum et al., 2012). Current find new therapeutic options for this type of cancer,

including the use of naturally occurring substances (Harum et al., 2012). Vegetable products are known to be able to induce apoptosis by various mechanisms (Harum et al., 2012). Epidemiological results indicate that the regular consumption of fruit and vegetables can decrease the incidence of cancer (Fleschhut, 2004; Jing et al., 2010). Natural product research has revealed a large variety of phytochemicals that have been proven successful against breast cancer in several epidemiological and *in vitro* studies. Polyphenolic compounds are found in plants, fruits, nuts and vegetables (White et al., 2012). The three main groups of polyphenols include flavanoids, stilbenoids and lignans (White et al., 2012). Epidemiological, clinical and animal studies have described the role of polyphenols in the prevention of various diseases, such as cardiovascular diseases, cancer and neuro-degenerative diseases (Milenkovic et al., 2012). To date, more than 4000 flavonoic compounds have been identified, many of which have anti-tumour activity (Thomas et al., 2012). In the plants that we have examined, the black elder *Sambucus nigra* contains primarily ethereal oils, flavanoids, triterpenes, triterpenic acid, derivatives of hydroxycinnamic acid and vitamins in its flower and berries. It has been shown that raw extracts of the blossoms and berries of *S. nigra* influence the cell proliferation of the breast cancer cells MCF-7, an estrogen receptor (ER) positive control cell line and BT20, an estrogen receptor (ER) negative control cell line. MCF-7 and BT20 cells are useful for *in vitro* breast cancer studies, because the cell lines have retained several ideal characteristics particular to the mammary epithelium. Further establishment by high performance liquid chromatography/mass spectrometry (HPLC/MS) analysis have shown that the flavanoids quercetin, kaempferol, chrysin, naringenin and hesperidin occur in these raw extracts.

The present aim has been to examine the effects of these flavonoids *in vitro* on the human breast cancer cell lines MCF-7 and BT20 by focusing on cytotoxicity, viability and influences on the cell proliferation of these cells, with a long-term view regarding the prevention of breast cancer.

MATERIALS AND METHODS

Chemicals

Absolute ethanol was purchased from MERCK, with the purity ACS, ISO, Reag. PhEur was used as an extraction solvent. The liquid chromatography–mass spectrometry (LC-MS) Chromasolv® grade solvents, namely methanol with 0.1% formic acid and water with 0.1% formic acid, were obtained from FLUKA. Reference compounds, namely quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one), chrysin (5,7-dihydroxyflavone), kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one), naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one) and hesperidin (hesperetin-7-*o*-rutinoside), were purchased from Sigma-Aldrich. All of the standard samples were dissolved in

absolute ethanol to give a concentration of 1 mg/ml in absolute ethanol. Flavonoids were identified by comparing the mass spectra and retention times with those of the reference compounds or with the mass spectra published in the literature.

Plants

Elder flowers and berries were harvested from the black elder, *Sambucus nigra* (type: Haschberg) at the State Research Institute of Mecklenburg/West Pomerania (Gültzow, Germany). The growth area in Gültzow lies at a height of 12 to 17 m above sea level. Open elder flowers were harvested, during the afternoon, at the beginning of June 2009. Elderberries were harvested by hand during the middle of September 2009. Elder flowers and berries were stored at -24°C at the University of Rostock in the Department of Technical Chemistry.

Microwave extraction

Microwave energy is a non-ionizing radiation that causes molecular motion by migration of ions and rotation of dipoles (Chen et al., 2008; Gu et al., 2008). Many reports have been published on the application of microwave-assisted extraction (MAE) of secondary metabolites from plants (Chattip et al., 2008; Guo et al., 2001; Chen et al., 2007). The main advantages of MAE are the considerable reduction in time and solvent as compared to conventional techniques. Samples of 10 g plant material (blossoms and berries) from *S. nigra* were extracted with 20 ml ethanol (absolute) in a microwave oven (CEM Discover). Extraction took place under the following conditions, time: 10 min, temperature: 60°C, energy level: 800 W.

Pyrolysis-field ionization mass spectrometry (Screening)

An analysis of the substance classes contained by the elder flowers and of the elderberry extracts obtained microwave extraction was carried out by pyrolysis-field ionization mass spectrometry. For field ionization, an emitter was used, which was activated by a pyrolytic process within a benzonitrile atmosphere, with a partial pressure of approx 80 Pa and under high voltage (4.8-kV FI Emitter), via a wolfram filament heated to white heat, leading to the formation of bristle-like carbon needles. The assay material was weighed in a quartz crucible, which was introduced, via a rod, into the ionic source, and the sample was ionized. The ions were detected by a Mass Spectrometry MAT 900 S, which had a double-focus sector-field analyser.

LC/MS analysis

Crude extracts of elder flowers and of elderberries were examined by liquid chromatography coupled with mass spectrometry (LC/MS, HPLC/MS) at the University of Rostock in the Department of Technical Chemistry. Chromatographical separation was performed on a Discovery® HS-C18 column (15 × 2.1 cm, 3 µm) (Supelco). The temperature of the column was maintained at 35°C. The mobile phase consisted of an increasing liquid gradient. Solvent A was methanol with 0.1% formic acid (LC-MS Chromasolv®, Fluka) and solvent B was water with 0.1% formic acid (LC-MS Chromasolv®, Fluka). The solvents were solubilised with a gradient solvent programme, as presented in Table 1. The flow of the movable phase was 0.15 ml/min and the volume of the injection was 5 µl. Compounds were identified by ion-trap technology and by MS via electron spray ionization. MS spectra were taken successively in a segment with two scan events in the row m/z 90.00 to 2000.00.

During the first scan event, a full scan was obtained in a positive ionic form with skimmer-induced dissociation (SID): unspecific shock activation 35.00. In the second scan, a full scan was obtained in a negative ionic form with SID: unspecific shock activation 60.00. All data were evaluated and interpreted with XCalibur and FrontierTM measure software (Thermo Scientific, Dreieich, Germany). The recorded data were compared with standards and typical fragments from data banks giving substance classifications.

Cell culture

MCF-7 and BT20 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The breast cancer cell lines MCF-7 (ER α +, ER β +) and BT20 (ER α -, ER β -) were cultivated in the University Gynaecological Clinic at Rostock. The MCF-7 cells were cultivated in phenol-red-free DME-medium (PAA, with 10% fetal calf serum (FCS), Amphotericin B (250 μ g/ml) PAA, Penicillin/Streptomycin (100x), PAA). The BT20 cells were grown in RPMI medium (PAA, with 10% fetal calf serum (FCS), Amphotericin B (250 μ g/ml) PAA, Penicillin/Streptomycin (100x), PAA). Cells were cultivated at 37°C under 5% CO₂ in an incubator. Cells were passaged every 2 to 3 days using trypsin ethylenediaminetetraacetic acid (EDTA). Triple-negative (BT20) and triple positive (MCF-7) breast cancers cells are defined by the

status of estrogen receptor (ER), progesterone receptor (PR) and HER-2 expression. The receptor status of the BT20 and MCF-7 cells was identified by immunohistochemistry.

Cytotoxicity assay (Cytotoxicity detection kit, Roche)

The cytotoxicity of the cells was identified with a cytotoxicity detection kit (Roche). Samples taken from the cell suspension were pipetted into 96-well plates at 100 μ l/well (5×10^5 cells/ml) for later treatment with each test substance and as controls. Cells were then incubated for 24 h at 37°C under 5% CO₂ in an incubator. The original medium (with 10% FCS) was exchanged for 198 μ l/well fresh medium (1% FCS). Some wells were left untreated (negative control 1: medium plus cells). Aliquots of 2 μ l test substance were added to the relevant wells or 2 μ l vehicles (ethanol absolute) were added to wells for the negative control 2 (medium plus cells plus ethanol absolute). As a positive (100% cell lysis) control, well reaction mixture composed of 200 μ l Triton X-100 and medium containing 1% FCS (at 1:46) was pipetted into four untreated wells. The cells were incubated for 24 h at 37°C under 5% CO₂ in the incubator. Subsequently, 100 μ l cell suspension was removed and replaced by 100 μ l well reaction mixture (at 1:46) and the reaction was stopped by the addition of 50 μ l/well 1 M N HCL. As shown in Table 2 the controls of lactate dehydrogenase (LDH)-Assay. The absorption of formazan was measured at a wavelength of 490 nm (reference 620 nm) in an ELISA reader and was evaluated with the program microplate manager.

$$\text{Cytotoxicity}(\%) = \frac{\text{Absorbance of the sample} - \text{Absorbance of the negative control 2}}{\text{Absorbance of the high control} - \text{Absorbance of the negative control 2}} \times 100$$

Viability assay (Proliferation MTT assay cell kit I, Roche)

The viability of the cells was identified with the proliferation MTT cell kit I (Roche). Cells were seeded in a 96-well plate at 100 μ l/well from the cell suspension at 5×10^5 cells/ml and incubated for 24 h at 37°C under 5% CO₂. Each test substance was added in 1 μ l to the relevant wells and the negative control 2 was treated with 1 μ l vehicle (ethanol absolute) (Table 3). Incubation of the cells with the

test substance was carried out for 24 h at 37°C under 5% CO₂ in an incubator. MTT reagent (10 μ l) was added to each well and the cells were incubated for 4 h in the incubator at 37°C under 5% CO₂. Subsequently, 100 μ l solubilising solution (10% SDS in 0.01 M HCl) was added and the cells were incubated for 24 h at 37°C under 5% CO₂. The absorption of formazan was measured by an ELISA reader at a wavelength of 570 to 655 nm and was evaluated with the computer program microplate manager as shown in Table 3, the controls of MTT-Assay.

$$\text{Viability}(\%) = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Cell proliferation (Cell proliferation BrdU assay, Roche)

Proliferating cells were revealed by the BrdU cell proliferation test (Roche). The cells were grown in a 96-well plate: 100 μ l of a cell

suspension (5×10^5 cells/ml) were pipetted into each well of a 96-well plate and incubated for 24 h at 37°C under 5% CO₂. Subsequently, 1 μ l of the test substance was added followed by 24 h incubation at 37°C under 5% CO₂. Every well, except for the background controls, received 10 μ l marker solution (1:100 dilution with sterile medium) and the cells were further incubated for 3 h at 37°C under 5% CO₂. Removal of the medium from the plates by sharply knocking the inverted plates was followed by incubation of the cells in 200 μ l FixDenat for 30 min. The FixDenat was removed by knocking the inverted plates and the cells were further incubated for 60 min with the antibody solution. The cells were then washed three times with 200 μ l washing buffer (1:10 dilution) and then incubated in 100 μ l substrate solution (tetramethylbenzidine) for 10 to 15 min. The reaction was stopped with 1 M H₂SO₄. Absorption at a wavelength of 450 nm was measured in an ELISA reader as shown in Table 4, the controls of BrdU-Assay.

$$\text{Cell proliferation}(\%) = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Statistical analysis

Statistical calculations were carried out with the Microsoft Office Excel 2007 and Statistical Package for Social Sciences (SPSS 10.0) for Windows software package. Results are expressed as the mean \pm standard error of mean (SEM) of 3 independent experiments in fourfold for each experimental point. Student's t-test was used for statistical analyses and one-way analysis of variance (ANOVA); P < 0.05 was considered to be significant.

Table 1. Program for mobile phase composition during the HPLC-MS analysis. A: methanol with 0, 1% formic acid, B: water with 0, 1% formic acid.

Parameter	Program for mobile phase composition during the HPLC-MS analysis					
Time (min)	0	10	20	30	35	40
A (v/v, %)	40	95	95	80	40	40
B (v/v, %)	60	5	5	20	60	60

Table 2. Controls of the LDH-Assay.

Parameter	LDH-Assay controls
Negative control 1	200 µl medium/well with 5×10^5 cells/ml
Negative control 2	198 µl medium/well with 5×10^5 cells/ml and 2 µl ethanol absolute
Positive control (100% cell lysis)	200 µl/well Triton x-100 + medium 1% FCS (1:46)

Table 3. Controls of the MTT-Assay.

Parameter	MTT-Assay controls
Negative control 1	100 µl medium/well with 5×10^5 cells/ml
Negative control 2	100 µl medium/well with 5×10^5 cells/ml and 1 µl ethanol absolute

Table 4. Controls of the BrdU-Assay.

Parameter	BrdU-Assay controls
Negative control 1	100 µl medium/well with 5×10^5 cells/ml
Negative control 2	100 µl medium/well with 5×10^5 cells/ml and 1 µl ethanol absolute
Background control	Absent 10 µl marker solution, unspecific binding of the anti-BrdU- POD conjugate o the cells in theabsence of BrdU

RESULTS

The anti-proliferous activity of *S. nigra* extracts (BrdU-Assay)

As shown in Figure 1 the cell proliferation (%) of the MCF-7 cells and the BT20 cells was affected by crude extracts of elder flowers and elderberries. The crude extract of the elder flowers reduced the cell proliferation of the BT20 cells by about 10% and that of the MCF-7 cells by about 62%. The proliferation of the BT20 cells was promoted by the crude extract of the elderberries by about 20%. The cell proliferation of the MCF-7 cells was however lowered by approximately 50%.

Pyrolysis-field ionization mass spectrometry and identification of flavanoids by HPLC/MS

An analysis of the substance classes of the elder flowers and of the elderberry components obtained by microwave extraction was carried out by pyrolysis-field ionization mass spectrometry (Figure 2 A).

The substance class of the polyphenols to which the

flavonoids belong gave complete ion strength (%) of 4.4% in the crude extract of elderberry and 5.1% in the crude extract of the elder flower. The HPLC/MS separation and analysis of the ethanolic extractions showed that the extracts were complicated mixture of various compounds (Figure 2B). For the qualitative identification of the flavonoids, the negative ion stream with Xcalibur software was analysed, because all examined analytes were deprotonated molecules [M-H] when the major precursor ion was formed.

The recorded data were compared with the standards and typical fragments in data bases banks used for substance classification. Five flavonoids were successfully identified: hesperidin m/z 609, quercetin m/z 301, naringenin m/z 271, kaempferol m/z 285 and chrysin m/z 253 (Figure 2B).

Influence of the flavonoids on the cell proliferation (BrdU-Assay) in MCF-7 and BT20

Quercetin

Figure 3A shows the cell proliferation (%) of MCF-7 and

Table 5. The summary of the effect of the flavonoids quercetin, chrysin, kaempferol, hesperidin and naringenin on the metabolic activity and cytotoxicity of MCF-7 and BT20 cells.

Cell line	Assay	Quercetin	Chrysin	Kaempferol	Hesperidin	Naringenin
MCF-7	MTT	No effect	No effect	No effect	No effect	No effect
	LDH	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not cytotoxic
BT20	MTT	No effect	20-30% ↓, 5-50 µg/ml	No effect	No effect	No effect
	LDH	30%, 5 µg/ml	Not cytotoxic	Not cytotoxic	Not cytotoxic	30%, 5 µg/ml

of BT20 cells in the presence of quercetin (5 to 50 µg/ml, 24 h). Quercetin reduced the proliferation of MCF-7 cells by about 2 to 22% (10 to 50 µg/ml). On the BT20 cells, it had an effect dependent on concentration, with the cell proliferation being lowered by about 18% at 5 µg/ml.

Chrysin

The proliferation of MCF-7 and BT20 cells changed after incubation with chrysin (24 h). The growth of MCF-7 cells was reduced by chrysin by about 20 to 30% (10 and 50 µg/ml), whereas that of the BT20 cells was reduced by 8% at the same concentration range (Figure 3B).

Kaempferol

As shown in Figure 4A, the growth of the MCF-7 and BT20 cells was also influenced by the presence of kaempferol (5 and 50 µg/ml, 24 h). Kaempferol reduced the growth of MCF-7 cells dependent on concentration, with 5 µg/ml causing a reduction in proliferation of about 36%. At a concentration range of 10 and 50 µg/ml, growth was decreased by about 13 to 30%. The BT20 cells also exhibited lower proliferation in the presence of kaempferols, with concentrations of 5 to 50 µg/ml affecting cell growth by about 10 to 15%. No effects dependent on concentration could be identified on the proliferation of BT20 cells by kaempferol.

Naringenin

Proliferation percent of MCF-7 and BT20 cells was also affected after incubation with naringenin. Naringenin reduced the growth of MCF-7 cells dependent on concentration (5 µg/ml, 25%) (Figure 4B). The cell proliferation of cells BT20 was inhibited by about 10 to 20% (5 to 50 µg/ml, 24 h).

Hesperidin

The growth of MCF-7 and BT20 cells was reduced after incubation with hesperidin (5 to 50 µg/ml, 24 h) (Figure

4C). Hesperidin reduced the growth of MCF-7 cells by about 20% (5 to 10 µg/ml). Proliferation of the BT20 cells was decreased by hesperidin by about 8 to 14% (5 to 10 µg/ml).

Summary of effect of flavonoids on the metabolic activity (MTT-Assay) and cytotoxicity (LDH-Assay) of MCF-7 and BT20 cells

The five flavonoids showed no effect on the metabolic activity of MCF-7 cells and were not cytotoxic in the concentration range of 5 to 50 µg/ml. Quercetin and naringenin showed a cytotoxic effect of 30% at 5 µg/ml in the LDH assay of the BT20 cells. Chrysin, hesperidin and kaempferol showed no cytotoxicity with respect to BT20 cells. The flavonoids quercetin, kaempferol, hesperidin and naringenin had no effect on the metabolic activity of the BT20 cells. Chrysin reduced the metabolic activity of the BT20 cells by 20 to 30% at 5 to 50 µg/ml (Table 5).

DISCUSSION

The black elder *S. nigra* was selected as a domestic plant in order to test its anti-tumour effect on breast cancer *in vitro*. No previous references in the literature are available regarding the mode of operation of the black elder, *S. nigra*, on the breast cancer *in vitro*, or for the flavonoids found in the black elder (quercetin, chrysin, kaempferol, naringenin and hesperidin). This study evaluates the cytotoxic potential, the metabolic activity and the influence on cell proliferation of crude elder extracts and the flavonoids contained therein (quercetin, chrysin, kaempferol, naringenin and hesperidin) *in vitro*. This study has been able to show that the crude extracts of elder blossom and elderberry reduce the cell proliferation of MCF-7 cells. The HPLC/MS-analysis has revealed the presence of the flavonoids quercetin, chrysin, kaempferol, naringenin and hesperidin in the crude extracts.

The anti-carcinogenic effect of flavonoids has previously been described. Various studies have shown that the flavonoid quercetin possesses anti-carcinogenic characteristics such as the inhibition of tyrosine kinase, interaction with the type II oestrogen binding site,

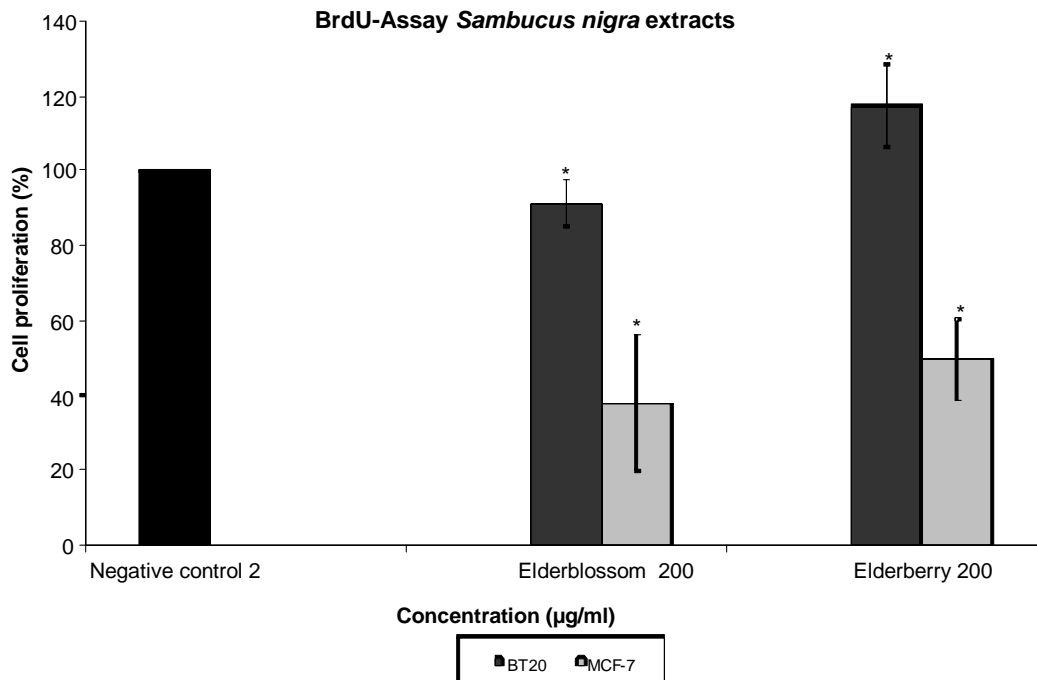


Figure 1. The BrdU-Assay show the effect of *Sambucus nigra* extracts (200 µg/ml) on cell proliferation of BT20 and MCF-7 cells. Cells were incubated for 24 h. Results are expressed as the \pm SEM of 3 independent experiments in fourfold for each experimental point. All data are reported as the percentage change in comparison with the negative control 2, which were arbitrarily assigned 100% cell proliferation. (*) $P < 0.05$, significantly different from the negative control 2.

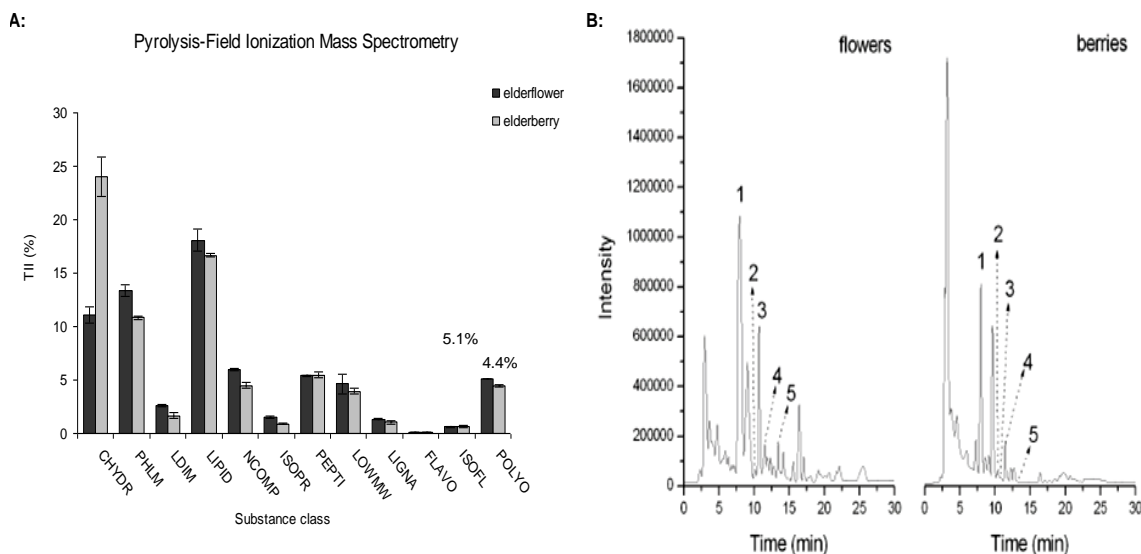


Figure 2. Pyrolysis-Field Ionization Mass Spectrometry (Screening) ITMS-cESI Full ms [90,00-2000,00] of ethanolic elder extracts. HPLC/MS analysis of ethanolic elder extracts. Fig 2A.: Results are expressed as the mean \pm SEM of 3 independent experiments in fourfold for each experimental point. A: Elder flower proportional portion of the substance class polycarboxylic acids 5.1%; Elderberry proportional portion of the substance class polycarboxylic acids 4.4%; B: HPLC/MS analysis of ethanolic elder extracts with 1: hesperidin, 2: quercetin, 3: naringenin, 4: kaempferol, and 5: chrysin. CHYDR = carbohydrates, PHLM = monolignols, LDIM = lignin dimers, LIPID = alkanes, alkenes, aldehydes, alcohols, fatty-acids, n-alkyl esters, waxes, fats, NCOMP = compounds containing nitrogen, ISOPR = isoprenoids, sterols, terpenes, carotenoids, PEPTI = peptides and free amino acids, LOWMW = low-molecular compounds m/z 15-56, LIGNA = lignans, FLAVO = flavones, ISOFL = isoflavones, POLYO = polyphenols.

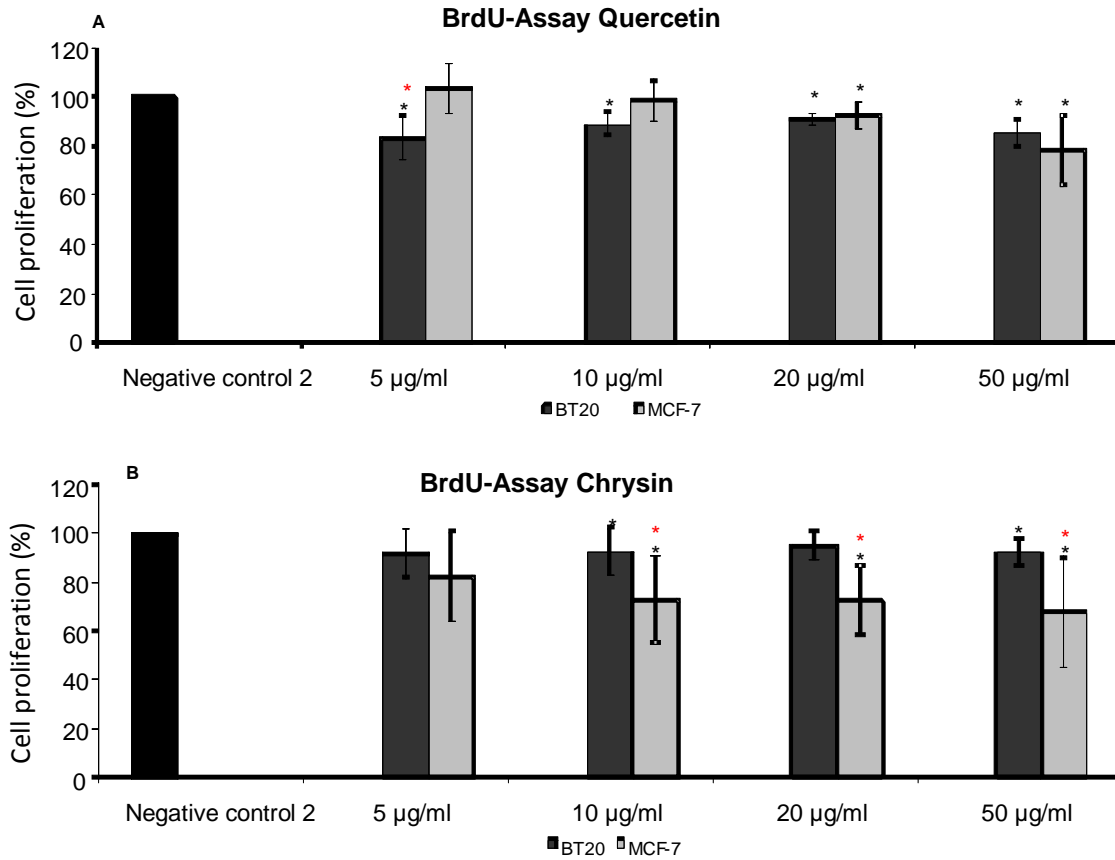


Figure 3. The BrdU-Assay show the effect of flavonoids on cell proliferation (%) of MCF-7 and BT20 cells. Cells were incubated for 24 h with A: quercetin (5-50 µg/ml) and B: chrysin (5-50 µg/ml). Results are expressed as the mean \pm SEM of 3 independent experiments in fourfold for each experimental point. Analysis of t-test (*) and one-way Anova (*) was used to compare the cell proliferation of MCF-7 and BT20 cells in different concentrations of flavonoids to control. $p < 0,05$ was regarded as statistically significant.

antioxidant characteristics and the induction of apoptosis in MCF-7 cells (Gibellini et al., 2011, 2000; Yang et al., 2010). Our results confirm an anti-tumour effect of quercetin both in MCF-7 cells and in BT20 cells by the inhibition of cell proliferation. In addition Yang et al. (2010) have demonstrated, under other test conditions and test procedures, that quercetin induces apoptosis in MCF-7 cells through the direct activation of the caspase cascade via the mitochondrial pathway (Yang et al., 2010). At the same time, the levels of Bcl-2 proteins and DeltaPSI monoclonal antibodies rises leading to an increased activation of caspase-6, 8 and 9 (Yang et al., 2010). Moreover, quercetin increases the quantity of the apoptosis-inducing factor (AIF) proteins, which pass from the mitochondria to the cell nucleus, and the quantity of GADD153 protein, which moves from the endoplasmic reticulum to the cell nucleus (Yang et al., 2010). The flavonoid chrysin exhibits a concentration-dependent effect (high concentration), in our investigation, on the inhibition of proliferation in MCF-7 cells. According to van Meeuwen et al. (2007) and Almstrup et al. (2006), chrysin is able to inhibit aromatase in MCF-7. Chrysin has been

proposed as an adjuvant for the TNF-related apoptosis-inducing ligand (TRAIL)-mediated anti-cancer therapy of Ding et al. (2012), as it initiates in various human cancer cell lines, including the breast cancer cell line MDA-MB-231, TRAIL-mediated apoptosis. In addition to chrysin, its analogue 5, 7-dihydroxy-8-nitrochrysin (NOC) has been examined for its induction of apoptosis in various types of breast cancer cells. Apoptosis caused by NOC in the breast cancer cell lines MDA-MB-453 (ER negative, HER2 over-expressed) and MCF-7 (ER positive, HER2 low), but not in the benign HBL-100 cells (ER positively, HER2 low) and is therefore a suitable candidate for breast cancer therapy (Zhao et al., 2010). In the range of concentrations and incubation times that have been selected, only a slight influence can be observed by chrysin on the cell proliferation of the receptor-negative cell line BT20, however, the results of other studies suggest that chrysin also influences the growth of receptor-negative cells. Hence, Hong et al. (2010) has found that chrysin inhibits the growth of MDA-MB-231 breast cancer cell by cytoplasmic lipid accumulation in the cells and an increased expression of the peroxisome

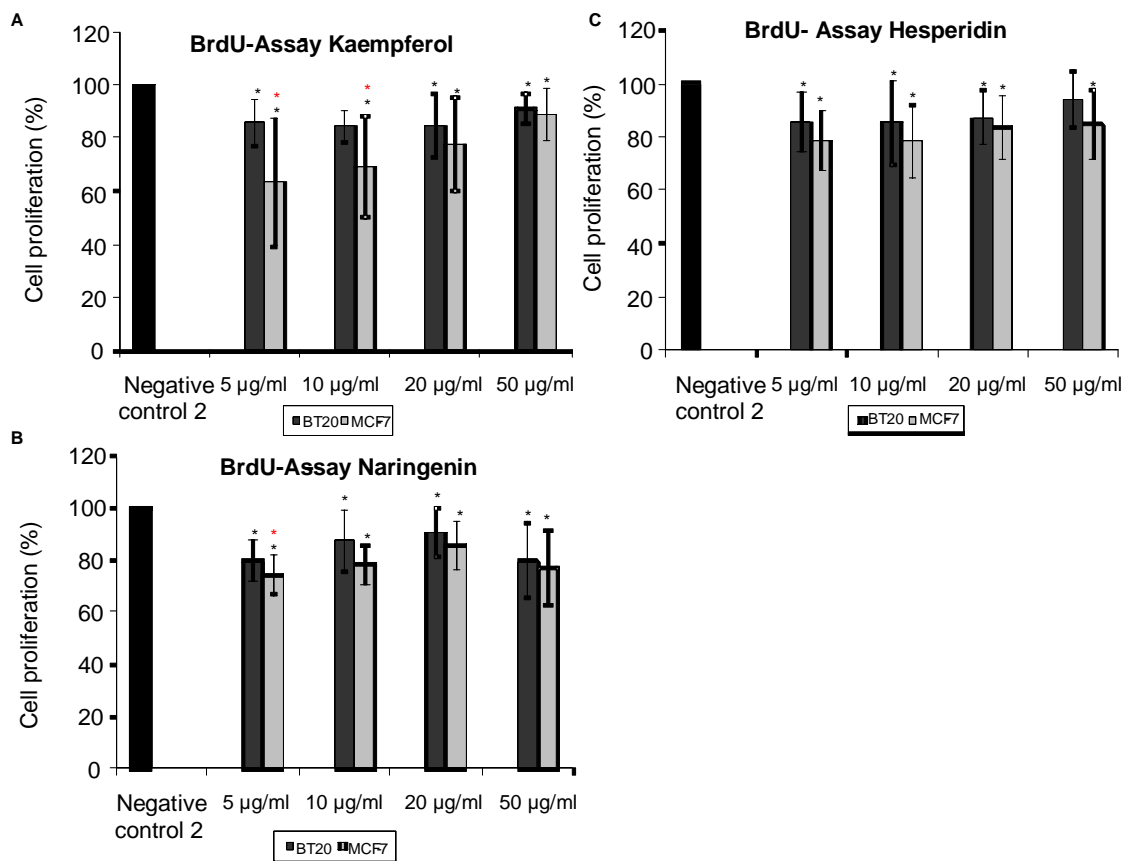


Figure 4. The BrdU-Assay show the effect of flavonoids on cell proliferation (%) of MCF-7 and BT20 cells. Cells were incubated for 24 h with A: kaempferol (5-50 µg/ml), B: naringenin (5-50 µg/ml) and C: hesperidin (5-50 µg/ml). Results are expressed as the mean \pm SEM of 3 independent experiments in fourfold for each experimental point. Analysis of t-test (*) and one-way Anova (*) was used to compare the cell proliferation of MCF-7 and BT20 cells in different concentrations of flavonoids to control. $P < 0.05$ was regarded as statistically significant.

proliferator-activated receptor (PPAR) alpha mRNA; however, the exact mechanism of chrysin on MDA-MB-231 cells remains unclear. The flavonoid kaempferol is one of the most commonly found dietary phytoestrogen. Kaempferol has both estrogenic and antiestrogenic activities, which are biphasic response on estrogen receptor (Oh et al., 2006). Therefore, it is possible that the estrogenic and antiestrogenic effects of kaempferol depend on estrogen and estrogen receptors (Oh et al., 2006). On these grounds, the flavonoid kaempferol is able to diminish proliferation of MCF-7 cells in a concentration-dependent manner (at low concentrations); however, it exhibits only a slight influence in our test on the proliferation of the receptor-negative BT20 cells. Furthermore, kaempferol is well known to cause apoptosis in MCF-7 cells through the introduction of the intrinsic caspase cascade and through the down-regulation of the Polo-like-kinase 1 (PLK-1) (Gum-Yong et al., 2009; Kim et al., 2008). Eun et al. (2008) showed that kaempferol reduces the proliferation of oestrogen-

receptor-negative MDA-MB-453 cells, interferes with the G2/M cell cycle and stimulates apoptosis by phosphorylation by P53. Furthermore, Huynh et al. (2004) demonstrated growth inhibition in receptor-positive and receptor-negative breast cancer cells and have observed a reduction of the ER alphas mRNA in MCF-7 cells treated with kaempferol. This study has shown that the naringenin can diminish the cell proliferation of both MCF-7 and of BT20 cells. Moreover, naringenin reduces the proliferation of MCF-7 cells by decreasing their glucose uptake from the culture medium (Harmon et al., 2004). According to van Meeuwen et al. (2007), naringenin inhibits the aromatase in MCF-7 cells). The flavonoid hesperidin is able to reduce the proliferation of both cell types, as has also been observed in MCF-7-GFP-Tubulin cells (Lee et al., 2010).

This investigation has shown that both the berries and blossom of the domestic plant *S. nigra* have predominantly an anticarcinogenic effect on the breast cancer cell line MCF-7. This study further presents five

flavonoids that occur in *S. nigra* and that have an anticarcinogenic potential. These substances cause, to different extents, the inhibition of proliferation in both MCF-7 and BT20 cells. However, the effect of the crude extract has a much stronger effect on the proliferation of MCF-7 cells than the single flavonoids at the concentration ranges used. Moreover, the concentration of the single flavonoids has not determined in the crude extracts and therefore no direct comparison can be performed with regard to the effects of the single substances. Nevertheless, both the crude extracts of the black elder *S. nigra* and its flavonoids are worthy of further investigation with regard to the chemoprevention of breast cancer. Further studies are necessary to elucidate the mechanisms that are responsible for the observed effects of the flavonoids in cells and to determine the optimal dose for therapeutic use. Recent reports have revealed that flavonoids can potentiate the effectiveness of existing drugs in cancer therapy (Lee et al., 2008; Choi et al., 2009; Singh et al., 2011).

The flavonoids that demonstrably occur in the flowers and berries of *S. nigra* include quercetin, chrysin, kaempferol, naringenin and hesperidin. These compounds have various anti-tumour activities under *in vitro* conditions in breast cancer cell lines MCF-7 and BT20 and thus are promising candidates for cancer prevention, possibly as adjuvants.

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