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LC-MS analysis of extracts from beech and sea buckthorn to correlate the phytoestrogen content and anti-cancer activity

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ABSTRACT

Compared with Asian countries cancer is one of the most common diseases in the Western world. Studies show that a phytoestrogen-rich diet could have a protective effect against cancer. The effect of phytoestrogens is specific to hormone-dependent diseases like breast and prostate carcinoma. The article deals with the microwave supported preparation of ethanolic extracts from different parts of local plants like the berries of the sea buckthorn (Hippophae rhamnoides) and the bark of the European beech (Fangus sylvatica). Their cell biological anticancer action on cellular metabolic features (adhesion, respiration and extracellular acidification) in the breast cancer cell line MCF-7 was investigated. The analysis of secondary plant metabolites by means of preparative and mass spectrometric methods provides information about the important substances and classes of substances which influence the proliferation inhibition on breast cancer cell lines. The intention of this study was to evaluate and discuss the structure-activity relationships of the obtained extracts and compare the herbal ingredients in connection with the cell activity tests for the whole plant extracts and their fractions. So this study gives new insights in the anticancer function of extracts and fractions of sea buckthorn berries and European beech bark.

Keywords: sea buckthorn juice (*Hippophae rhamnoides)*, beech bark *(Fangus sylvatica)*, LC-MS, anticancer activity, phytoestrogens

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INTRODUCTION

Cancer is a generic term for different diseases. These diseases can affect any part of the body and are characterized by uncontrolled growth and spread of abnormal cells. If the uncontrolled growth of cancer cells is not covered, it can result in death. On the world scale, according to the World Health Organization [1,2] cancer is a very popular disease with 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 [3]. Worldwide deaths caused by cancer are projected to rise to 13 million in 2030 [3]. With an annual estimated incidence of 1,676,300 new cases, breast cancer is the most common cancer among women worldwide [3-5]. However, recent studies showed clearly that incidences of breast cancer are not equally distributed worldwide and strong differences are found between Europe and Asia [3]. A comparison of breast cancer diseases between Europe and Asia shows that Asia has considerably fewer newly diagnosed cancer cases than Europe. According to the World Cancer Report 32.1% of approx. 7.1 million Asia women from the total female population were diagnosed with breast cancer in 2012 [3,1]. On the other hand, the number of European women is much higher, the incidence rate was estimated to be about 40% (out of a total female population of approx. 4.6 million) [3]. Some scientists assumed that this distribution can be attributed to the traditional diets [6-12]. In Asia people eat food that is mostly rich on phytoestrogens for example soya products and rice [13-16]. This phenomenon is often discussed as the reason for the lower cases of breast cancer in Asia [11]. Other studies determined that in the developing countries cancer rate will increase in the next years, due to western lifestyle which is assumed to be associated with the increasing risk of developing cancer [3]. In Germany the interest to combat and prevent breast cancer is also very high because of the 73,000 newly diagnosed cases every year. After all, the standard cancer treatment is still symptomatically treated by surgery, chemotherapy and radiation. These methods are dangerous and physically demanding for the patients. A popular new approach to fight against cancer is the hormone-supported therapy. Specifically, some studies have shown that it is assumed that

phytoestrogens are able to positively influence the growth of breast tumors [17-21]. Therefore it is possible to exert a direct influence to their own health and nutrition to reduce the risk to suffer from breast cancer [22,23].

Phytoestrogens are secondary plant metabolites, which act as phytoprotectants against predators. They can be divided into three main groups: isoflavones (e.g. genistein), lignans (e.g. secoisolariciresinol) and coumestanes (e.g. coumestrol). The main stocks for these phytoestrogens are fruits and vegetables, such as soya, beans, berries or red clover.

These phytoestrogens from plants may show activity towards mammalian estrogen receptors when supplied as food. In addition they may also modulate the effectiveness of endogenous estrogens. This biological response is based on their structural similarity to 17β-estradiol and their ability to bind to the human estrogen receptors [24].

To find a new source for possible cancer drugs we choose local plants such as European beech bark or sea buckthorn berries for the production of extracts containing phytoestrogens.

Common beech *(Fangus sylvatica*) is a deciduous tree about 40 m high and native in the most parts of Europe. With a total forest area of 14% is this one of the most common deciduous trees in Germany. With their astringent, fever reducing and antiseptic affects people used bark extracts previously against different diseases like respiratory diseases, stomatitis (infections of oral mucosa) and colds. Furthermore with a beech logging of 7 million square meters per year, *Fangus sylvatica* is one of the most important deciduous trees as lumber and industrial wood [25].

Sea buckthorn *(Hippophae rhamnoides)* is a shrub which reaches from 0.5 up to 6 meters. The plant grows naturally in sandy soil and is popular over a wide area of Europe and Asia. Sea buckthorn has been shown to have a potent antioxidant activity, mainly attributed to its flavonoids and vitamin C content [26]. Sea buckthorn berry components have potential activity against cancer. Today the product line includes different teas, oils and vinegars, spirits, juice and spreads or cosmetics. However, through more difficult harvesting conditions and a long period from 6 to 8 years for the first harvesting, sea buckthorn becomes a quite expensive resource.

There are many different methods to obtain extracts from plants e.g. the conventional methods such as soxhlet, ultra sonic, etc. [27-29]. All these methods need a lot of time and solvents. A new method is the microwave assisted solvent extraction [30,31]. It can be alternatively used for extraction of natural products, facilitating the separation and isolation of secondary metabolites such as alkaloids, saponins, tannins, flavonoids and essential oils. For the aim of this work we used this as an alternative and fast method to obtain plant extracts for the LC-MS and anticancer activity measurements. In this work we evaluate and discuss the structure-activity relationships of the examined extracts and compare the herbal ingredients in connection with the cell activity tests.

EXPERIMENTAL SECTION

Chemicals

Absolute ethanol obtained from MERCK, with the purity ACS, ISO, Reag. PhEur was used as extraction solvent. The LC-MS Chromasolv® grade solvents, methanol with 0.1% formic acid and water with 0.1% formic acid were obtained by Sigma-Aldrich, Switzerland. Reference compounds biochanin A, catechine, β-estradiol, hesperidin, kaempferol, quercetin, quercetin-3-β-D-glucoside, rhamnetin, rutin hydrate, rutin trihydrate, secoisolariciresinol, taxifolin and vitexin were purchased from Sigma-Aldrich. Fisetin and isorhamnetin were acquired from Sigma-Aldrich, Switzerland as well and quercetrin was from HWI ANALYTIK GmbH (Rülzheim, Germany). All of the standard samples were dissolved in a concentration of 1 mg/ml in pure ethanol. Phytoestrogens were identified by comparing the mass spectra and retention times with those of the reference compounds, or the mass spectra published in the literature. The HPLC gradient grade methanol from J.T. Baker was used as eluent for the preparative separation.

Plant Material

Sea buckthorn *(Hippophae rhamnoides)* berries juice was purchased from the Sanddorn Storchennest GmbH (Ludwigslust, Germany). The growth areas are situated in Ludwigslust (Mecklenburg-Vorpommern, Germany) with an altitude above sea level of 22 to 64 meters. From the beginning of August till the end October the little sea buckthorn berries were picked manually. The berries were frozen and stored in a deep-freeze warehouses at < -20 °C for further processing. Approximately five to six kilograms berries were used for the production of one liter sea buckthorn juice.

Beech bark was received (*Fangus sylvatica)* from the sawmill Drewes & Suderow GmbH (Malchow [Mecklenburg], Germany]. The tree has been felled in the immediate surroundings of Malchow in 2007.

The mechanical comminution of the raw-material was done with the help of a cutting mill into wood chips with a size of 0.25-1.0 mm.

Extraction procedure

Five ml of the sea buckthorn juice were clarified by centrifugation at 40,000 RPM for 10 minutes at 18 °C. The resulting clear liquid is the stock solution for preparing samples for the LC-MS and for cell activity tests.

For the beech bark extract three grams of beech bark shavings were used. The shavings were extracted with 27 ml of absolute ethanol in a CEM MARS Microwave Synthesis System. An extraction time of 30 minutes at 800 W microwave power and 60 °C temperature proved to be best.

LC-MS Analysis

The plant extracts were analyzed on a Finnigan Surveyor high pressure liquid chromatography (HPLC) system equipped with a Finnigan linear trap quadrupole (LTQ) mass spectrometer (Thermo Scientific). The chromatographic separation was performed on a Kinetex C18 column (2.6 u, 100 A) produced by Phenomenex. The temperature of the column was set at 30 °C. As mobile phase a gradient was used. 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). Elution of the extracts were performed by the following solvent gradient: 40% A to 95% A (10 min), 95% A isocratic (10 min), 95% A to 80% A (10 min), 80% A to 40% A (5 min) and 40% A isocratic (25 min). The flow rate of the mobile phase was 0.2 ml/min and the volume of injection 5 µl.

The compounds were identified by ion trap technology and the mass spectrometric detection was realized with electron spray ionization. MS spectra were recorded consecutively in one segment with two scan events in the range m/z 90.00–2000.00. In the first scan event a full scan was conducted in a positive ion mode with a skimmer induced dissociation of 35.00. In the second scan, a full scan was realized in a negative ion mode with a skimmer induced dissociation of 95.00. All Data were evaluated and interpreted with Xcalibur and Mass Frontier^{TM} Software (Thermo Scientific, Dreieich, Germany). The obtained data were compared with standards and characteristic fragmentation patters using databases for substance classification.

Preparative HPLC

The ethanolic plant extracts were fractionated on a Knauer HPLC system connected to a fraction collector Foxy Jr. FC from ISCO inc. (Lincoln NE). As mobile phase an isocratic eluent with 40% HPLC gradient grade methanol (J.T. Baker) and 60% ultrapure water was used. The chromatographic separation was performed on a Eurospher-10 C18 column (250 x 10 mm) (Knauer) with a flow rate of 3 ml/ min. The run time and UV detector setting were varied for the different extracts. For the sea buckthorn extract we used a run time of 40 minutes and 350 nm, for the European beech extract the run time was 30 minutes and the UV detector set to 280 nm.

The sea buckthorn berries juice was cut into 12 fractions with the following time slots: fraction 1 (6.7-7.6 min), fraction 2 (8.1-9.0 min), fraction 3 (9.5-10.1 min), fraction 4 (10.5-11.8 min), fraction 5 (12.1-13.5 min), fraction 6 (13.9-14.7 min), fraction 7 (15.4-16.2 min), fraction 8 (16.6-19.0 min), fraction 9 (19.4-20.3 min), fraction 10 (23.0-26.0 min), fraction 11 (31.5-34.5 min) and fraction 12 (35.3-38.0 min). For the ethanolic European beech extract the following time slots were used: fraction 1 (5.2-6.1 min), fraction 2 (8,4-9.4 min), fraction 3 (9.4-10.8 min) and fraction 4 (12,1-13.6 min). The received fractions were reduced and concentrated in an argon inert atmosphere at 60 °C. Afterwards the fractions were analyzed molecular and cellular biological.

Cell culture conditions

The estrogen-positive breast cancer cell line MCF-7 (ATCC no. HTB-22) was cultivated in Dulbecco's modified Eagle's medium (Invitrogen, Germany) with 10% fetal bovine serum (PAN Biotech GmbH, Germany) and 1% gentamycin (Ratiopharm, Germany) and maintained at 37 °C and in a 5% CO_2 atmosphere. Medium was changed every two days. Confluent cells were passaged by treating them with 0.05% trypsin – 0.02% EDTA.

Live cell monitoring of adhesion, acidification and respiration

Online monitoring of the cellular processes was performed with the Bionas® 2500 analyzing system [32] with the metabolic chip SC 1000 (Bionas GmbH, Rostock, Germany) and the measurement software Bionas15002 CS1.47. Prior to experiments, chips were cleaned with 70% ethanol for 10 minutes, washed with phosphate buffered saline (PBS) and were adapted to the measurement medium for 5 minutes. Measurement medium was composed of Dulbecco's modified Eagle's medium without $NaHCO_3$ (Invitrogen, Germany), 0.1% charcoal stripped fetal bovine serum (PAN Biotech GmbH, Germany) and 1% gentamycin (Ratiopharm, Germany). The pH value was set to 7.4 and afterwards the medium was sterile filtered. On each chip 2 x 10^6 cells were seeded with normal cell culture medium and were let to adhere overnight so that approximately 80% confluence on the sensor chips was reached. Bionas measurement of cell adhesion, acidification and respiration was carried out with a pump rate of 56 µl/min for 24 hours [32,33]. Within the first four hours cells could adapt to the new measurement medium. Thereafter cells

were treated with the plant extracts (final dilution 1:1000) with the solvent Ethanol (control) for 20 hours. Every measurement was repeated three times, obtained data were evaluated and normalized with the software Bionas1500[2] Data analyzerV1.07.

RESULTS AND DISCUSSION

Identification and structure elucidation of compounds in the sea buckthorn juice and beech bark

LC-MS measurements were carried out with samples of sea buckthorn juice and ethanolic beech bark extracts to compare the chemical fingerprints of the solved ingredients. The chromatograms figure 1 show the different samples scanned with different modes (figure 1, negative scan mode A and C; positive scan mode B and D) to detect the positive and negative ion current in the MS.

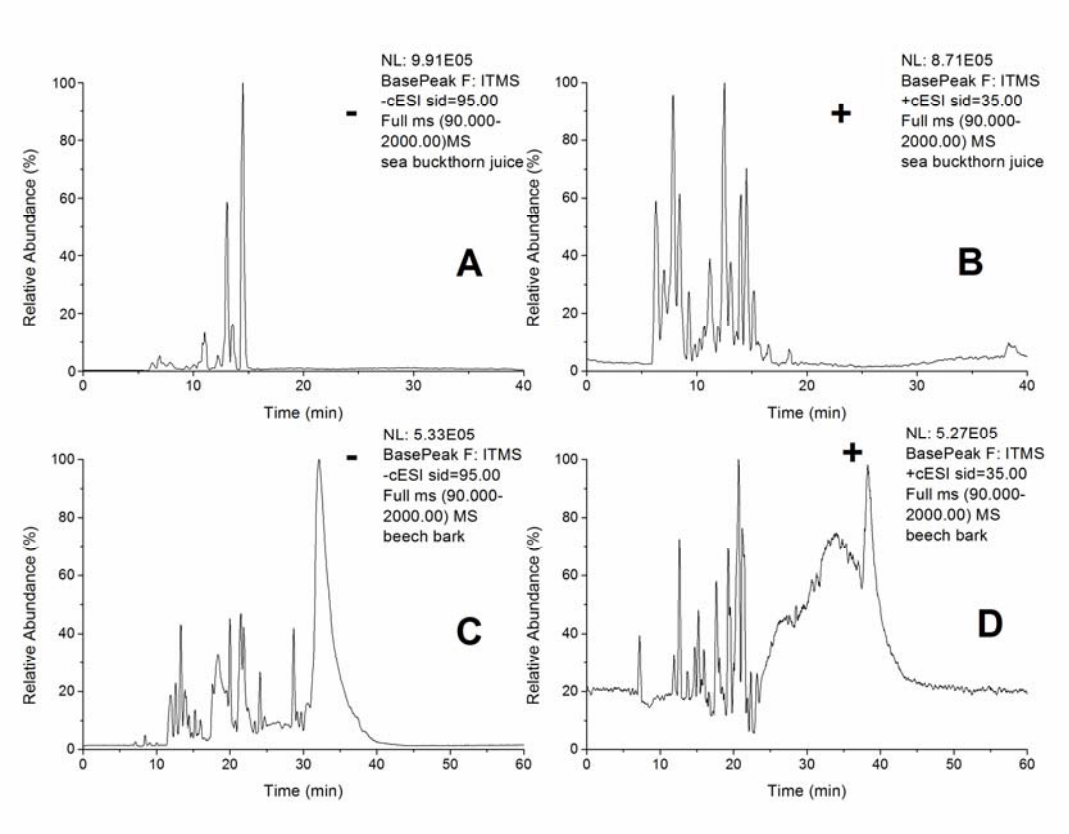


Figure 1: LC-MS chromatograms of the different scan modes. A: negative scan mode of the sea buckthorn juice, NL: 9.91E05 BasePeak F: ITMS- cESI sid=95.00 Full ms (90.000-2000.00)MS B: positive scan mode of the sea buckthorn juice, NL: 8.71E05 BasePeak F: ITMS+ cESI sid=35.00 Full ms (90.000-2000.00)MS C: negative scan mode of the beech bark extract, NL: 5.33E05 BasePeak F: ITMS- cESI sid=95.00 Full ms (90.000-2000.00)MS D: positive scan mode of the beech bark extract, NL: 5.27E05 BasePeak F: ITMS+ cESI sid=35.00 Full ms (90.000-2000.00)MS

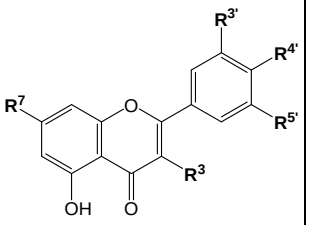
The fingerprints of the two samples indicate a number of different ingredients. Simultaneously recording of both ion currents allow a lossless analysis and registration of all ions (figure 1). Depending on the size and weight of the different plant-based ingredients electrospray ionization will produce simple charged ions such as $[M+H]^+$ and $[M-H]^-$.

The structure of the detected compounds were identified by HPLC-ESI-MS investigations either by comparison with standards or identifying substances by their characteristic fragmentation pattern using databases. MS-MS and MS^n experiments were performed by isolation and fragmentation of the most abundant pseudo molecular ions. A typical HPLC chromatogram of sea buckthorn juice and European beech bark is shown in figure 1. Most of the interesting compounds elute in the first 30 minutes. The LC-MS analysis in ESI positive and negative mode of these compounds revealed the presence of many glycosides flavonoids in both extracts.

It is well known and has been described in the literature that sea buckthorn berries contain many flavonoids [26,34-37]. They are structured with two aromatic rings, which are based on the backbone of 2-phenylchromen-4-one. By the different substitution patterns on both aromatic rings arise a large number of different compounds like isoflavones, flavanones, flavones and anthocyanins [38]. Among other we identified

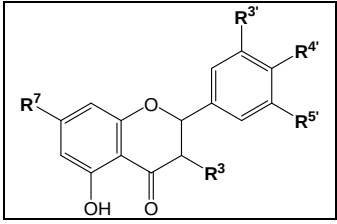
quercetin-3-β-D-glucoside, rutin, hesperidin isorhamnetin-deoxydiglycoside or isomers of xanthorhamnin and scopoletin, bond to glucoside or rutinoside (table 1, table 2).

Table 1: List A/ Structures of some identified plant ingredients of the sea buckthorn berries and European beech bark, A: identified in the sea buckthorn berries juice, B: identified in the beech bark



Substance	R^7	R^3	$R^{3'}$	$R^{4'}$	$R^{5'}$
quercetin-O-pentose[B]	OH	O-pentose	OH	H	H
isoquercetin[A]	OH	O-glucoside	OH	H	H
isorhamnetin[A]	OH	OH	OCH_3	H	H
isorhamnetin-3-glucoside[A]	OH	O-glucoside	OCH_3	H	H
isorhamnetin-3-glucoside-7-rhamnoside[A]	O-rhamnoside	O-glucoside	OCH_3	H	H
isorhamnetin-3-rutinoside[A]	OH	O-rutinoside	OCH	H	H
isorhamnetin-3-sophoroside-7-rhamnoside[A]	O-rhamnoside	H	O-sorphoside	H	OCH_3
kaempferol-rutinoside[A]	OH	O-rutinoside	H	H	H
quercetin-3-glucoside-7-rhamnoside[A]	O-rhamnoside	O-glucoside	OH	H	H
rutin[A]	OH	O-rutinoside	H	H	OH
xanthorhamnin[A]	OCH_3	O-gal-rha-rha	OH	OH	H

Table 2: List B/ Structures of some identified plant ingredients of the sea buckthorn and European beech bark, A identified in sea buckthorn berries juice, B: identified in the beech bark



substance	R^7	R^3	$R^{3'}$	$R^{4'}$	$R^{5'}$
hesperidin[A]	O-rutinoside	H	OH	OCH_3	H
taxifolin[B]	OH	OH	H	OH	OH
taxifolin-*O*-pentose[B]	OH	O-pentose	H	OH	OH
glucodistylin[B]	OH	O-glucoside	H	OH	OH

We also found flavonoids linked to a trisaccharide. These flavonoids bind mainly the sugar molecules over their C_3 -, C_5 - or C_7 - hydroxyl group standing atom.

Rutin is responsible for bright orange color of the sea buckthorn berries. The other ingredients like hesperidin protect the plant against predators or infections caused by fungi [39]. The glycosidic bonds influence the polarity of the flavonoids and their solubility, so it is easier for the plant to store these compounds into their vacuoles [40,38]. The occurrence of many triglycosidic bound flavonoids is another interesting aspect of the sea buckthorn berries. For example in xanthorhamnin the trisaccharide is bound over the C_3 - atom to the simple aglycone rhamnetin. This can be clearly seen in the fragmentation pattern of the mass spectrum. In the ESI negative scan we found $[M-H]^-$ at m/z 769. At m/z 623 $[M-H-C_6H_{10}O_4]^-$ there is the loss of the first sugar fragment a rhamnose. The second sugar fragment also rhamnose was split off with a mass difference of 163 amu and the third sugar fragment with 147 amu standing for a galactose. This is visible in the mass spectrum by the mass peaks at m/z 460 $[M-H-C_{12}H_{21}O_9]^-$ and at m/z 313 $[M-H-C_{18}H_{31}O_{13}]^-$. At m/z 313 there is the simple aglycone rhamnetin.

Following Rösch and Llorach there are also indications for flavonoid systems with hydroxycinnamic acid derivatives in the ethanolic sea buckthorn extract [26,41]. The main mass fragments $[M-H]^-$ in the ESI negative scan mode at m/z 991 and at m/z 961 could be quercetindimethylether-3-hydroxferulylglucosyl-glucoside-7-rhamnoside and quercetin-dimethyl-ether-3-cafferoylglucosyl-glucoside-7-rhamnoside.

The dried bark of the European beech mainly consists of lignin (40.4%) and different carbohydrates (35.2%) such as glucose (18.5%), xylose (10.5%), arabinose (2.9%), rhamnose (0.8%) and mannose (0.5%) [42]. These compounds are a part of the cellulose and hemicelluloses and form together with lignin the structural and support plant substances [43,44]. Even though the high share of the woody material it was possible to extract secondary plant substances by the use of microwave extraction. A typical LC-MS chromatogram of the European beech bark extract is represented in figure 1. Once again it is obvious that many phenolic compounds mainly ionize negative via ESI (figure 1 C and D). There are also many glycosides flavonids and some aglycones. The special feature was the occurrence of many molecule fragments similar to lignans. This group of substances consists of phenylpropanoid molecules (dimeric C_6C_3 structure) which are linked to the middle β-C atom [45]. The different structure variants are the results of the different configuration and combination of the C_3-side chain [46,47]. In comparison to the sea buckthorn berry extract the beech bark extract is rich in lignans or molecule fragments similar to lignans which are not completely identified yet. Furthermore, it was possible to isolate an oligometric proanthocyanidin (OPC) at a

retention time of 8.47 min it consists of catechine dimers or trimers. In the mass spectrum this substance is proven by the following fragment patterns (figure 2).

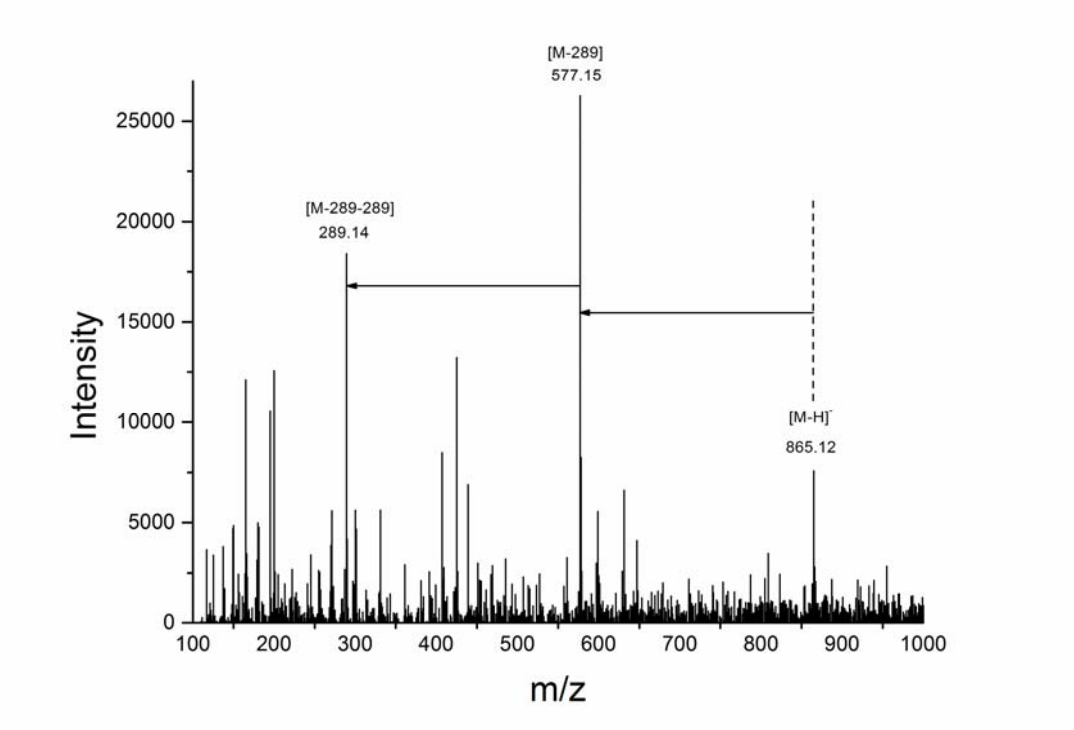


Figure 2: Mass spectrum of OPC in beech bark extract F: ITMS : - c ESI MS sid=95.00 Full ms 90.00-2000.00

Through the negative ionization by ESI a base peak $[M-H]^-$ with a value of 865 amu for the OPC is found. After splitting-off of one catechine molecule a value of 577 amu is to be found for the remaining fragment [M-288]. The separation of the second catechine molecule leads to the mass fragment with a value of 289 amu for a single catechine molecule. This compound is important for the protection against predators, UV radiation and to altered climatic conditions. This substance is found in large quantities in the shells and cores of the fruits and vegetables like apples, strawberries, onions, peanuts and oranges [47]. Studies have shown that the OPC has a dose-dependent growth inhibition on colon cancer cells [48]. Furthermore, the OPC's are able to increase the effect of vitamins [49,50]. Further analysis showed that other structures similar to flavonoids are also components of the beech bark extract. Special for the beech bark is that many flavonoids are structurally based on taxifolin. The bioflavonoid taxifolin, also known as dihydroquercetin, has a molecular structure similar to that of quercetin without the double bond in the C-ring. This missing double bond has a big influence on the properties of the taxifolin. So the double bond from the quercetin is the reason for its antioxidant activity [51], but may also cause mutagenicity and toxicity [52]. Taxifolin is less mutagenic and toxic than quercetin but has also an antioxidant efficacy, but this effect is 50% lower than the effect of quercetin [51]. These two components can be correlated to the plant metabolism. Taxifolin and quercetin are metabolites of the herbal flavonoid biosynthesis [53-57]. In this case, taxifolin is one of the possible precursors of quercetin, which will be formed by the enzyme flavonol synthase belonging to the group of oxidoreductases.

Besides the pure taxifolin at a retention time of 13.33 min we could also identify the glycosidic linked metabolites glucodistylin at a retention time of 11.91 min and a taxifolin-*O*-pentose at 12.59 min. For the glucodistylin we found the mass trace for the whole molecule at m/z 467 in the positive ion mode and in the negative ion mode at m/z 465 and the fragments at m/z 303 $[M-C_6H_{12}O_5]^-$, m/z 285 and m/z 151. The mass trace of taxifolin-*O*-pentose and its fragments were only identified in the negative ion mode.

The comparison of both plant extracts shows that we could identify many different and complex secondary plant compounds. One special feature is that we could also identify multiple glycosidic-linked flavonoids in the sea buckthorn berry extract. In the ethanolic beech bark extract there were only known flavonoids and their simple glycosides. The occurrence of lignans or fragment patters of lignans may be not evidenced for the sea buckthorn berries. Only in the bark of the European beech we could find some fragments which indicate the occurrence of lignans.

Cell test for the extracts of the sea buckthorn berries juice and European beech bark

The most interesting ingredients are isoflavones and lignans because these substances are known to posses cancer preventing properties on breast cancer cells. The LC-MS analysis showed that sea buckthorn as well as European beech contain many of these components, so that these plant extracts may be chemopreventive and anti-tumorigenic. In order to check these properties we analyzed the metabolic effects on the breast cancer cell line MCF-7, a well-established cell line for studying hormonal responses in breast cancer. Furthermore it was shown that MCF-7 respond well to plant-derived phytochemicals and phytoestrogens and therefore are suitable for the screening of new anticancer drugs [18,58,19,59-61].

For analyzing the metabolic features of the cell line MCF-7 in response to the native plant extracts of sea buckthorn (*Hippophae rhamnoides*) and European beech (*Fagus sylvatica*) we used the Bionas® 2500 analyzing system in combination with Bionas® metabolic chip SC1000. This system allows a continuous online monitoring of adhesion, acidification and respiration in a living culture of adherent cells. Prior to treating with the plant extracts the cells were able to adapt to the measurement medium for 3 – 4 hours. After this adaption phase (highlighted in gray, figure 3) the cells should maintain a stable level of adhesion, respiration and acidification before adding the plant extracts (figure 3).

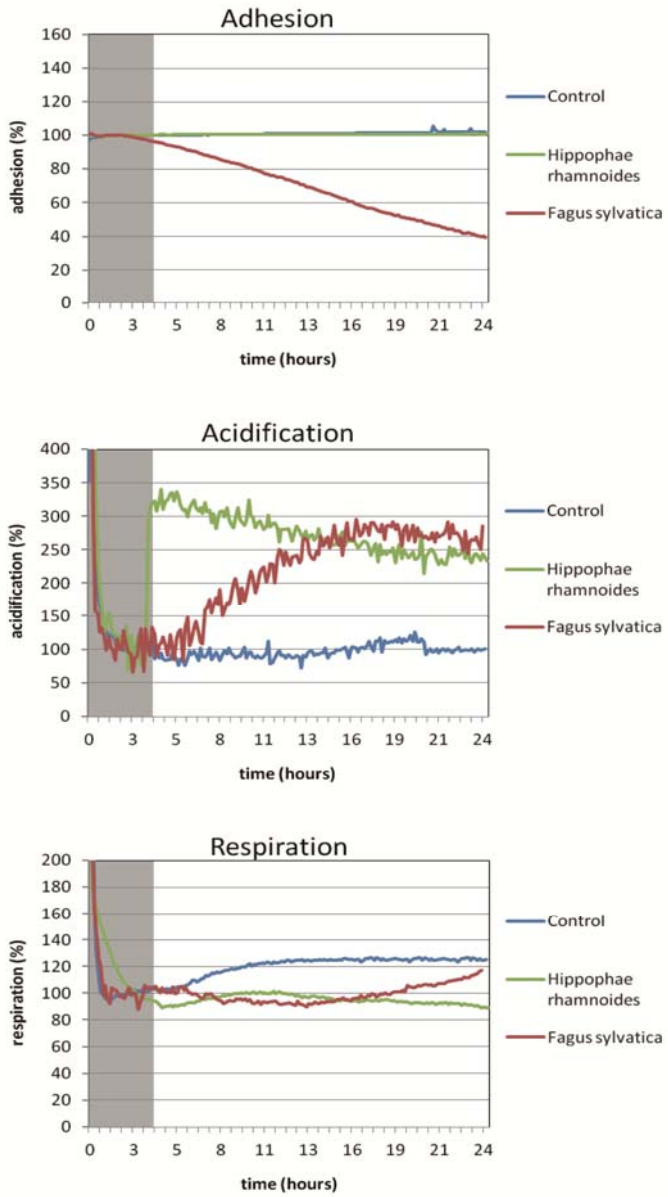


Figure 3: Continuous metabolic measurement of the human breast cancer cell line MCF-7 cells on Bionas® sensors of chip SC 1000 with the Bionas® 2500 analyzing system using the software Bionas1500[2] CS1.47. Before treatment, cells were allowed to adhere and adapt to the measurement medium for 3 - 4 h on the chips (gray area). Start point of the exposure with the plant extracts of *Hippophae rhamnoides* and *Fagus sylvatica* or with the vehicle ethanol (control) which was set to 100%. Adhesion, acidification and respiration was evaluated with the software Binos1500[2]Data AnalyzerV1.07 (n=3)

The Bionas measurements revealed significant alterations in the metabolic features of MCF-7 after treatment with both plant extracts. The control treatment with the extracting agent ethanol (0.1% final concentration) showed no influence on the adhesion or acidification rate of MCF-7, only the respiration value increased up to 30% indicating for stable cell culture conditions and metabolically active cells. After exposure with the extract of *Hippophae rhamnoides* MCF-7 cells revealed significant changes in the acidification rate reflecting the fact that the extract itself harbors a low pH-value. However, no influence on the adhesion was observed while the respiration rate slightly decreased (approximately 10%). In contrast, the extract of *Fagus sylvatica* displayed a drastic reduction of cell impedance resulting in nearly 50% loss of cell adhesion and a continuously rising acidification rate which is due to an elevated production of metabolic acids. Probably, it is a hint for the initiation of apoptosis because programmed cell death is often associated with cytoplasmic cell acidification and loss of adhesion [62]. The respiration rate was not affected.

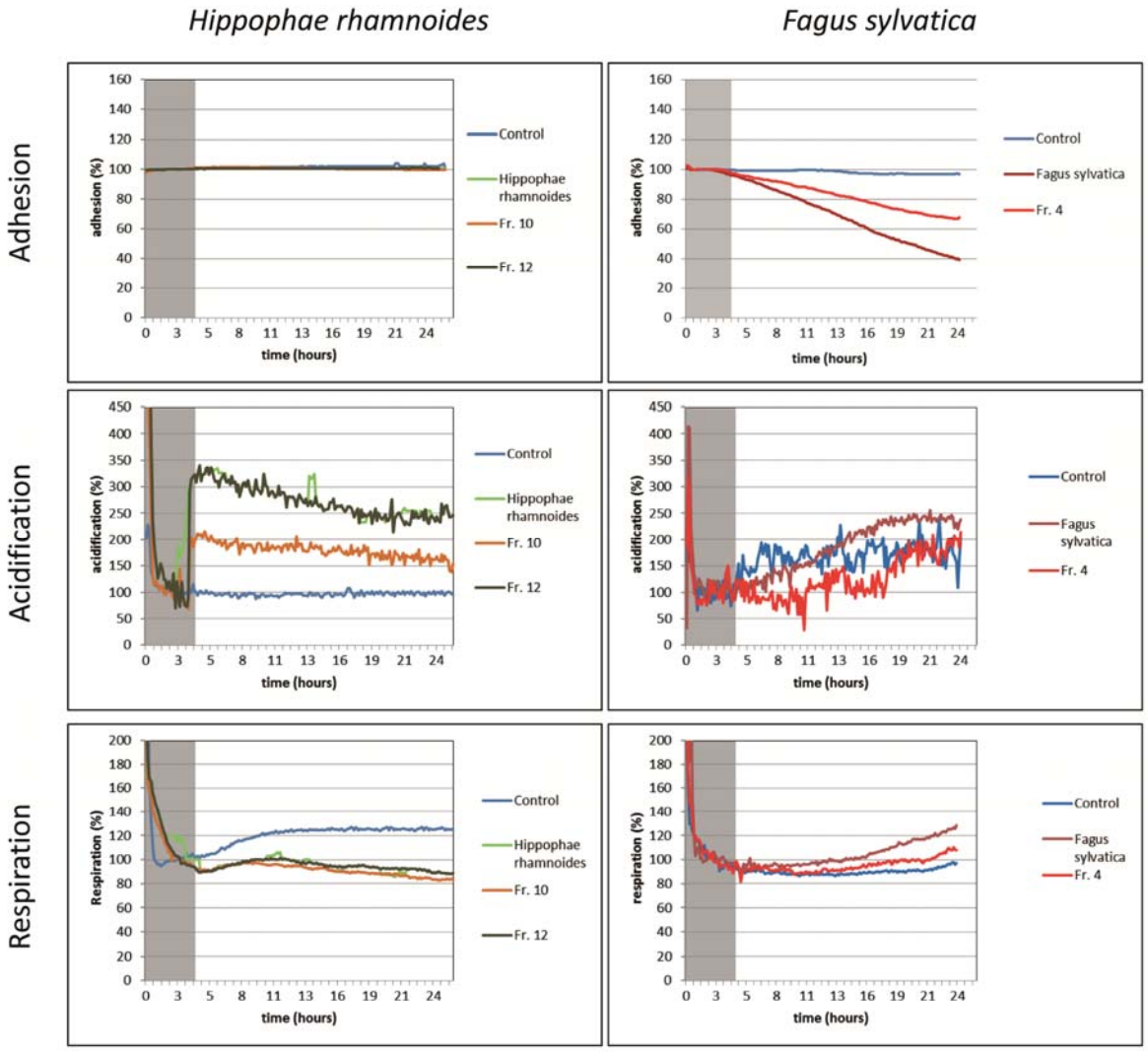


Figure 4: Online monitoring of the three metabolic features (adhesion, acidification, respiration) in the cell line MCF-7 during the treatment with the control substance, the plant extract of *Hippophae rhamnoides* and *Fagus sylvatica* in comparison with selected active fractions (Fraction 10 and 12 from *Hippophae rhamnoides*; fraction 4 from *Fagus sylvatica*). Grey shadowed: adaption phase of the cells. Adhesion, acidification and respiration was evaluated with the software Binos1500^{2}Data AnalyzerV1.07 (n=3)

The comparative analysis of the influence of both plant extracts on the breast cancer cell line MCF-7 resulted in different cell responses. While a native extract of the berries of *Hippophae rhamnoides* showed relative little effects on the metabolism of MCF-7, the extract of the bark of *Fagus sylvatica* caused a great decrease in cell impedance. Both plant extracts were able to reduce the viability of MCF-7: *Hippophae rhamnoides* decreased the rate of oxygen consumption (respiration) and *Fagus sylvatica* the cell impedance (adhesion). However, the extract of *Fagus sylvatica* seems to have more potential as an anti-tumorigenic mixture because the significant reduction of adhesion and increase in acidification let to the conclusion that MCF-7 is getting apoptotic. It was already proven that the loss of extracellular matrix results in apoptosis, termed anoikis and cytoplasmic acidification is sufficient for killing cells by the inhibition of sodium/hydrogen exchange [62,63].

Fractionation of sea buckthorn berries juice and European beech bark extract and their metabolic anti-tumor activity

Testing the complete extract in cell assays indicated strong effects on the used cancer cell lines. Therefore the extract was fractionated depending on the chromatograms with UV detection of the different plant compounds. The fractions were used for the in vitro studies. The most active extracts were investigated in detail with LC-MS.

The influence of the fractions on MCF-7 cells was verified again by the metabolic measurement of the three cellular parameters: adhesion, extracellular acidification and respiration (figure 4).

Fractions of the sea buckthorn berries extract: Fraction numbers 10 and 12 of the sea buckthorn berries juice caused significant alterations in the extracellular acidification and respiration rate in comparison with the control treatment. Fraction 10 resulted in a doubling of the acidification rate. The O_2 consumption rate was reduced to the same level as with the full extract. Detailed studies revealed that fraction 10 consists of a mixture of mono- and triglycosides the following substances could be identified: isorhamnetin-3-rutinoside, isorhamnetin-4-glucoside, quercetintriglucoside (glu-rha-rha) and xanthorhamnin.

The isolated fraction number 12 showed a comparable effect on the metabolic effects as the full extract. A 2.5 to 3-fold increase in acidification and reduction of the respiration of about 20% were observed. The LC-MS analysis revealed that this fraction contains quercetindimethylether-3-cafferoylglucosyl-glucoside-7-hamnoside and also traces of xanthorhamnin. The cell impedance as an indicator for the cell adhesion was neither affected by the full extract nor the fractions.

Fractions of the beech bark extract: In contrast to the sea buckthorn extract and its fractions the full extract and fraction 4 of the European beech bark reduce the adhesion of MCF-7 cells significantly. Treatment with the full extract and fraction 4 lead to a continuously decrease in cell impedance while the full extract displayed the strongest effect with a 60% reduction after 24 hour exposure. Fraction 4 contains not only flavonoids such as taxifolin-*O*-hexoside or taxifolin-*O*-pentoside and catechines but also many typical fragments of lignans. For example, different resinols and a secondary metabolite with a structure like secoisolariciresinol was found. This mix of compounds reduced the MCF-7 cell impedance up to 40% of the control value. The acidification rate was not significantly influenced but the respiration rate increased slightly under the influence of fraction 4 as well as the full extract.

The comparison shows that the active sea buckthorn fractions contain mixtures of different glycosylated flavonoids. In the beech bark fraction besides the glycosylated flavonoids also lignans are found. Despite the differences in the content all isolated fractions altered the metabolism of the breast cancer cell line MCF-7. Their effect is less pronounced than for the full plant extracts. The components identified act at different metabolic pathways. The sea buckthorn berries fractions primarily acts on the acidification and respiration rate whereas the extract of the European beech changes the cell impedance. These results clearly illustrate that not only lignans – as it is often suggested – have a good inhibition of growth rate of mamma carcinoma. Also flavonoids with different glycosylation pattern generate a similar effect on the cell growth of mamma carcinoma. Whereas the adhesion rate is only influenced by the compounds identified, the cell respiration rate was reduced by 20%.

CONCLUSION

The extract samples of bark and berries from sea buckthorn (*Hippophae rhamnoides*) and European beech (*Fagus sylvatica*) were analyzed by cell biological and analytical methods. The analytical results show that the extracts consist of many different secondary plant compounds like flavonoids, lignans and other phytoestrogens. In the beech bark extract and fractions we found mainly lignans where as in the sea buckthorn berries extract and fractions glycosylated flavonoids were found. The cell biological results also indicated that the plant extracts have an possible anticancer effect based on the effect against the breast cancer cell line MCF-7. Full extracts of sea buckthorn berries did not influence the adhesion rate. But the fractions of sea buckthorn berries extract who contain double or triple glycosylated flavonoids were also increase the adhesion rates as well as the full beech bark extracts who mainly include lignans. Both extracts were able to alter the cell metabolism of the breast cancer cell line MCF-7. Whereas the extract of the sea buckthorn primarily addresses the acidification and respiration rate, the extract of the European beech revealed a significant influence on the cell impedance.

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