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Vorstand: Prof. Dr. med. Sven Mahner

Influence of the phytoestrogens of Sambuccus nigra and Petroselinum crispum on the proliferation and receptor expression in breast cancer cell lines

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Dr. med. univ. Lennard Schröder

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Berichterstatter:	Prof. Dr. rer. nat. Udo Jeschke
Mitberichterstatter:	Prof. Dr. Christian Schindlbeck
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
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1. INTRODUCTION

Nature does amazing things… We are what we eat… In the following pages the effects of phytoestrogens on the estrogen receptor (ER) and other cellular pathways will be explained. The two attached papers outline the effects of two phytoestrogen containing plants, sambucus nigra (elder flower) and petrosilium crispum (parsley root) on breast cancer cell lines.

1.1. Epidemiology Breast Cancer

Until today breast cancer remains the most abundant cancer in women. 2.1 million Women are confronted with the diagnosis breast cancer every year and it is responsible for the greatest cancer mortality in women. 627,000 women succumbed due to breast cancer in 2017 – representing more than 15% of all women dying of cancer worldwide. Although traditionally breast cancer rates were higher in developed regions of the world, today breast cancer incidence is also increasing rapidly in developed countries[1].

Nevertheless during the last three decades breast cancer therapy has evolved rapidly, it remains an incurable disease once metastasis are diagnosed. Most women will only live two to three years with the disease (median overall survival) and after five years of metastatic breast cancer only 25% of the women affected remain alive[23].

1.2. Breast cancer treatment

The basic therapeutic options of early breast cancer usually involve standardized algorithms including surgery, radiation therapy, chemotherapy and endocrine therapy in hormone receptor positive breast cancer and the use of antibody therapy in Her2 positive breast cancer[4].

Once hormone receptor positive metastatic breast cancer has been diagnosed systemic therapy with extended endocrine therapy including CDK4/6 inhibitors and aromatase inhibitors are first line therapy in patients that are not affected by visceral crisis[5]. In Her2 positive metastatic breast cancer chemotherapy with paclitaxel and the application of the antibodies trastuzumab and pertuzumab is the first line treatment of choice[4]. In any type of breast cancer patients presenting with visceral crisis, usually chemotherapy is the preferred therapeutic option[4].

Endocrine resistance and resistance to chemotherapeutic drugs are one of the greatest problems in the treatment of breast cancer[6]. The majority of breast cancer is ER positive[4]. The already mentioned CDK4/6 inhibitors are a new class of drugs that intervene in the cellular process of endocrine resistance[78]. The ER plays a central role as a ligand-dependent transcription factor for the expression of genes that lead to key mechanisms associated with carcinogenesis like tumor growth, cellular proliferation and survival[9]. Therefore one of the main targets of endocrine therapy is estrogen receptor interaction and modulation by means of selective receptor interaction or by depriving the tumor of estrogen[10]. Although effective therapies for hormone receptor positive breast cancer have been developed such as Tamoxifen and aromatase inhibitors, approximately 30-50% of breast cancer patients will present with progressive disease in the course of treatment due to the cancers ability to adapt and mutate thus developing resistance pathways[11].

Breast cancer pathogenesis is complex and besides a substantial genetic burden mostly related to BRCA mutations and factors like age of menarche and menopause, lifestyle factors such as age at first child, parity, breast feeding, contraception, nutrition, obesity, nicotine and alcohol consumption also play major roles[1213]. Therefore the question to which extend phytoestrogens influence the pathogenesis of breast cancer or the course of breast cancer is of great interest[14]. Because of the preventive properties of phytoestrogens that have emerged from epidemiological evidence and from clinical research, where substance safety and therapeutic effects were observed, in this work the effects of two phytoestrogen containing plants, sambucus nigra (elder flower) and petrosilium crispum (parsley root) on breast cancer cell lines will be assessed.

1.3. Phytoestrogens:

1.3.1. Definition, Sources, Chemical Structure, History

To withstand environmental stressors, evolution drove some plants to produce secondary metabolites with antioxidant, antifungal and antibiotic properties belonging to the large family of polyphenols[15]. Phytoestrogens are a group of polyphenols produced by plants that functionally and structurally demonstrate certain similarities with 17β-estradiol and therefore interact as agonists or antagonists with the estrogen receptor and estrogen receptor independent pathways[1617]. All phytoestrogens contain one or more aromatic rings with at least one hydroxyl group. Hydroxyl groups may be free, but usually they are engaged in another function with an ester, ether or a glycoside[18].

Phytoestrogens are found at a wide great variety in whole grains, vegetables and fruits and are a natural component of human diet[19]. Nevertheless the affinity of phytoestrogens to interact with the estrogen receptor is low compared with 17β-estradiol, the two substances compete for the receptor binding domain[20]. Phytoestrogens can only enter human metabolism by ingestion and cannot be synthesized by the endocrine system[21]. The name "phytoestrogen" is a combination of the Greek word phyto (plant) and the word estrus (sexual desire) and gene (to generate)[2223].

Natural polyphenolic compounds that are classified as phytoestrogens are the coumestans, the stilbenes, the isoflavones and the lignans[24]. Coumestans can be found in soybean sprouts, alfalfa and clover whereas resveratrol is the stilbene present in grape and red wine[19]. Isoflavones from *flavus* (yellow in Latin), are mostly found in legumes such as soybean, kalachanna, mung bean, red lentils, and red clover. On the one hand soy and its phytoestrogen compounds genistein and daidzein represent the most important isoflavones often found in Asian diets, on the other hand lignans which are components of plant cell walls are often found in traditional occidental diets such as grains, fiber-rich cereals such as flaxseed, seaweed, whole grains, oil seeds, fruits, and vegetables [2526]. Secoisolariciresinol and matairesinol are the most abundant and important lignans in human diet[27]. It was demonstrated in clinical studies that enterolignans exposure at high concentrations reduces the risk of developing breast cancer by 16%[28]. Furthermore other

studies associated a significant reduction of breast cancer mortality with elevated enterolactone levels in the blood of postmenopausal women[29][30].

Phytoestrogens first made headlines in Western Australia when sheep feeding on subterranean clover leaves demonstrated decreased fertility rates and an elevated number of miscarriages and malformations[31][32]. As a result of the studies listed, the hypothesis has been made, that plants use phytoestrogens to protect themselves from herbivore animals by controlling female fertility thus preventing overpopulation and overgrazing[22].

In the last fifty years phytoestrogens research has expanded rapidly because evidence of epidemiological studies suggest that phytoestrogen rich diets cause beneficial health effects. Associated data reports positive effects such an improvement in metabolic features like a lowered risk of obesity, metabolic syndrome, type 2 diabetes and effectively cardiovascular disease. Moreover phytoestrogen drugs as treatment options for common menopausal symptoms like osteoporosis and hot flushes are available and other data suggests an improvement in a variety of brain function disorders and a positive influence on various cancers such as bowel cancer, prostate cancer and breast cancer[33][34].

After the publication of the first results of the women's health initiative study, where alarming side effects of hormonal replacement therapy where published in 2002 many physicians ceased to prescribe and women individually stopped their hormonal replacement therapy[35]. Due to this the already existing trend towards plant derived therapeutic options emerged increasingly and the demand for phytoestrogens as a save therapeutic alternative for climacteric symptoms became apparent[36].

Besides the efficacy in hormonal replacement therapy, epidemiological observations demonstrated chemo preventive properties of soy ingestion ('Japanese Phenomenon') on breast cancer prevalence, as women originating from Asian countries, where soy is a regular part of the diet, have a three to five times lower risk of developing breast cancer than European or American women, not consuming soy products on a regular basis[37][14]. Interestingly, in the offspring of female Asian immigrants in the second or third

generation who migrated to occidental countries, breast cancer prevalence resembles the prevalence of women living in occidental countries, emphasizing the importance of lifestyle factors associated with breast cancer genesis [14]. Some Asian diets lead to a high isoflavone consumption of concentrations around 47 mg/day compared with 0.1–1.2 mg/day in Western diets. Vegetarian diets or dietary soy supplements can elevate soy blood levels to the concentrations of the Asian diets mentioned above[38394041]. Interestingly and often underestimated, soy is found besides soy milk and soy compounds such as tempeh and tofu in a broad variety of processed foods (up to 60 %)[25]. Soy textured protein, were the soy protein percentage is as high as 70 %, can be found in fast foods meats such as burger patties, hot dog sausage meat and other meat compounds such as chicken nuggets[25]. Isolated soy protein, where the soy protein percentage is up to 90% is used in many different processed foods ranging from imitation dairy products such as infant formula, cheese and ice cream to sports drinks, granola bars and cereals[25].

The general perception of soy being healthy and a rewarded popular food additive is not only due to the phytoestrogen content, but also because of attractive appealing features such as being rich in complex carbohydrates, free of lactose and unsaturated fats. Furthermore it is a cholesterol-free, vegetable protein with a relatively high fiber content[25]. Due to this in the United States school breakfast and lunch programs include textured soy protein as a part of federal assistance programs[2542].

After the US Food and Drug Administration (FDA) approval in 1999, which stated that the risk of coronary artery disease can be effectively reduced by daily soy consumption[43], prevalence of soy products and consumption increased tremendously[25]. The soy product sales data clearly reflects the soy boom in the USA; in the time between 2000 and 2007, up to 2700 new products containing soy were put on the market resulting in a boost in sales from $300 million in 1992 up to $4 billion in 2008[25]. Nowadays, soy compounds and other phytoestrogens containing food compounds can be purchased as dietary supplements with substrate concentrations excessively higher than present in traditional soy and soy based foods[4425]. To sum up, these marketing and sales data clearly demonstrate that phytoestrogen consumption is increasing tremendously. Therefore

experimental and clinical data evaluating the effect of phytoestrogens has to be critically analyzed to determine definite positive and negative health effects at a broad spectrum[25].

1.3.2. Phytoestrogen Metabolism

A common problem with phytoestrogens is that in order to achieve high serum concentrations of the substrates, proportional high quantities of the referred phytoestrogen have to be ingested[41]. After ingestion of foods, phytoestrogens are not ready to enter the blood in their bioavailable chemical structure because they are bound to metabolically passive glycoside conjugates consisting of carbohydrate or glucose moieties[45]. Thus in order to be activated they require digestion in the form of enzymatic conversion by a group of enzymes called the glucosidases[45]. These enzymes break the phytoestrogen down to their respective aglycon leading to efficient absorption[45]. Some phytoestrogens also depend on the intestinal bacterial flora for further metabolism, as it is the case with the lignans secoisolariciresinol and matairesinol [28]. Once ingested, the metabolic inactive secoisolariciresinol and matairesinol are being metabolized by intestinal aerobe and anerobe bacteria into the bioavailable metabolic active enterolignans enterodiol and enterolactone[2930].

Genistein and daidzein are another interesting example demonstrating the complexity of phytoestrogen metabolism. They are metabolized to the three substrates; equol and to *O*-desmethylangolensin (*O*-DMA) and/or *p*-ethyl phenol, depending on the individual intestinal flora[39]. Only 30% of the western population and 60% of the asian population possess gut microbiota that can metabolize daidzein into the isoflavan equol and approximately 80%-90% *0*-DMA[3846]. Therefore, it becomes clear that it is not only dependent on an individual's diet, but also on the intestinal flora to which extend phytoestrogens are being absorbed[46].

After enzymatic conversion and in some cases bacterial metabolism the phytoestrogens are absorbed from the intestine and then, in the hepatic circulation conjugated for the most part to glucuronic acid and for the lesser part to sulphuric acid [4745]. After

conjugation, the conjugated phytoestrogens are de-conjugated prior to excretion[48]. The amount of phytoestrogen consumption correlates with urinary excretion[47]. The actual bioavailable active unconjugated phytoestrogen is usually found in proportional very low levels of less than 3 % of the amount ingested[49]. Detectable concentrations are usually in the ng/ml range and sometimes even lower[49]. In most in vitro studies using cancer cell lines the concentrations demonstrating effectiveness are much higher than the concentrations that can be physiologically achieved in humans[5051].

Properly indicated therapeutic implications remain scarce although as already mentioned a large body of data in the preclinical and clinical setting exist.

1.3.3. Mechanism of action and in *in vitro* data of phytoestrogens

Phytoestrogens are able to interact within cellular processes in different ways[14]. Evidence exists, represented by a large body of research that besides ER interaction a wide variety of cellular pathways and mechanisms such as angiogenesis, cell signaling pathways, mitochondrial metabolism, oxidative reactive stress, antioxidant properties, regulation of autophagia, apoptosis, cell cycle alternation and epigenetic changes are influenced by phytoestrogens[5214]. The most studied effect of phytoestrogens is ER interaction[14]. When activated by a ligand ER-α and ER-β are considered ligand-activated transcription factors that translocate from the cytoplasm into the nucleus of the cell where they attach to estrogen response elements (ERE) at the promoter regions of the referred gene causing specific DNA transcription and mir-RNAs leading to cellular modification and action[5354].

Phytoestrogens have an increased affinity for ER-β than for ER-α [5556]. ERβ binding affinity is considered an important feature of phytoestrogens because ERβ signaling is associated with anti-proliferative and anti-carcinogenic effects, while ER-α signaling is related to carcinogenesis[1457]. As the loss of ERβ is associated with aggressive breast cancers, ERβ has been considered as a tumor suppressor gene controlling ERα-induced proliferation[58]. In a study, the phytoestrogen calycosin upregulated ERβ, thus causing many different effects in downstream cellular signaling pathways, like the stimulation of p38 MAPK and

oppression of the serine/threonine kinase (Akt), the poly(ADP-ribose) polymerase 1 (PARP-1) division to induce apoptosis and the inactivation of insulin-like growth factor 1 receptor (IGF-1R) in MCF-7 cells[1459].

Another possible ER independent mechanism of action is the binding of a phytoestrogen ligand with receptors of the cell surface, thus promoting the creation of cytoplasmic cyclic nucleotides and associated protein kinases, affecting target cells by the induction of signaling pathways[1414]. For example, the soy flavonoid genistein demonstrated the ability to induce apoptosis in MCF-7 cells by inactivating IGF-1R, downregulating phosphatidylinositol 3-kinase(PI3K)/Akt signaling pathway[606162], and decreasing the Bcl-2/Bax protein ratio[1463]. In MCF-7 cells daidzein induced apoptosis via the mitochondrial pathway by generating reactive oxygen species leading to disruption of the transmembrane potential of the mitochondria, the downregulation of Bcl-2/Bax ratio leading to a liberation of mitochondrial cytochrome C into the surrounding cytosol effectively activating caspase-7 and incaspase-9[1464]. Equol induced in ER-negative MDA-MB-453 cells an up-regulation of cytochrome c expression and an activation of the p53 pathway finally leading to significant cell cycle arrest at the G1/S transition and in the G2/M phase[65]. Also in triple negative breast cancer MDA-MB-231 cells genistein inactivated NF-kappaB via the Akt or Notch-1 signaling pathway signaling pathway by downregulating the expression of cyclin B1, Bcl-2, and Bcl-xl [1466].

Besides the mechanisms described above, phytoestrogens are able to induce epigenetic modulation by histone modification, DNA methylation and microRNA regulation[18]. The most investigated phytoestrogens in this context are genistein listing the highest amount of publications, followed by daidzein and its intestinal flora metabolite equol[67].

It was demonstrated that genestein induces DNA methylation in exposed mice during gestation. Genestein exposed mice had lower incidence of developing obesity early in life compared to mice without gestational exposure [68]. The results of this study led to the hypothesis that the observed epigenetic modulation takes place more likely when soy exposure starts already during gestation or early in life and that later exposure is less effective[6970]. Moreover it was concluded that the effect observed is a possible reason for

the earlier described Asian phenomenon; decreased obesity and cancer prevalence in Asian countries[71].

Due to the mechanisms described above it seems clear that phytoestrogens can cause a wide variety of effects ranging from ER mediated effects to the regulation of the cell cycle, enhancement of antioxidant properties, induction of apoptosis or autophagy, modulation of cell signaling pathways to epigenetic alterations[72].

1.4. Clinical data and experience with phytoestrogens

A systematic review investigating the effects of dietary soy consumption on the prognosis of breast cancer patients published in 2013 analyzed 80 observational studies 11 uncontrolled trials, and 40 randomized controlled trials [737414]. The study resulted with the conclusion that soy consumption may decrease the risk of breast cancer mortality, recurrence and incidence and that the amount of soy ingested (2–3 meals/day, approximately 25–50 mg isoflavones) in a traditional Japanese diet is likely to decrease breast cancer recurrence and incidence. The effects and safety of high dose isoflavones (≥100 mg) need further assessment before recommended for breast cancer patients[18]. Other epidemiological studies such as meta-analysis and case–control studies demonstrated that increased genistein ingestion is associated with decreased breast cancer risk[1875]. Moreover it was pointed out that the intestinal metabolite equol has positive effects on breast cancer incidence, but can only be metabolized by 30 – 40 % of the population[767778].

Besides soy and other flavones, the lignans have demonstrated positive effects on estrogen receptor but also estrogen receptor negative breast cancer. In a Canadian study, breast cancer risk was significantly reduced by flaxseed or flax bread consumption (OR = 0.77, 95 % CI 0.67-0.89) [79]. In another study by Buck et al high enterolactone levels in the serum of postmenopausal women with breast cancer was associated with increased survival[80]. Besides having influence on breast cancer risk and survival, it was shown in a case-control study that lignan ingestion may also have an beneficial impact on tumor

characteristics; increased lignan uptake inversely correlated with the odds of developing grade 3 tumors and a decreased rate of triple negative tumors[81].

A broad variety of clinical data with phytoestrogens and breast cancer dealing in the clinical setting exists. As observed in the *in vitro* setting, data and substrate heterogeneity of phytoestrogens is great. Therefore, even the data assessing the impact of soy diet on breast cancer results in different conclusions. As phytoestrogen metabolism depends on multiple factors such as ethnicity, gut flora and the phytoestrogen ingested it becomes clear that thoroughly planned and designed clinical studies are crucial to gain further understanding and knowledge.

1.5. Overview on Results and Achievements

In both publications extracts of the plants were prepared in different concentrations and analyzed by mass spectrometry. In the parsley root publication, breast cancer MCF12A and MCF7 cells were exposed with different PCE concentrations and incubated. After incubation the effect of the different concentrations of PCE on MCF12A and MCF7 breast cancer cells was analyzed using various tests. For cytotoxicity, metabolic activity and DNA synthesis performance LDH, MTT and BrdU proliferation assays were used respectively.

In the elder flower publication, trophoblast tumor cell lines BeWo and JEG-3, as well as MCF7 breast cancer cells were exposed and then incubated at different EFE concentrations. Cells remaining untreated served as test controls. In supernatant cells estradiol production was tested using an ELISA method. Changes induced by EFE in ER/PR expression were analyzed by immunocytochemistry.

It was demonstrated that the plants investigated contain a substantial amount of phytoestrogens. Moreover, both plants demonstrated anti-cancerogenic properties on breast cancer cell lines by means of receptor expression patterns and the inhibition of cell proliferation. If the effects observed are only caused by phytoestrogen action remains unclear. Further investigations on different breast cancer cells and with the isolated phytoestrogen substances is needed before proper clinical investigations can be planned.

1.6. Contribution to Publications Included in this Thesis

Lennard Schröder analyzed the data, did statistical evaluation and wrote the papers. Udo Jeschke, Dagmar Richter and Birgit Piechulla designed and conceived the studies and supervised the experiments. Simone Hoffmann and Sandra Schulze took care of the cell culture. Mareike Chorbak and Christina Kuhn established the dye protocols and led the immunohistobiochemical laboratory work. Jens Haumann and Julian Schmedt where involved with laboratory work, Sven Mahner, Bernd Kost, Tobias Weissenbacher and Julian Koch helped with data interpretation and proofread the manuscript.

2. PUBLICATIONS INCLUDED IN THIS THESIS

2.1. Publication 1

Title:

The Effects of Petroselinum Crispum on Estrogen Receptor-positive Benign and Malignant Mammary Cells (MCF12A/MCF7).

Authors:

Schröder L, Koch J, Mahner S, Kost BP, Hofmann S, Jeschke U, Haumann J, Schmedt J, Richter DU.

Journal:

Anticancer Res. 2017 Jan;37(1):95-102.

Abstract:

BACKGROUND:

Phytoestrogens have controversial effects on hormone-dependent tumors. Herein we investigated the effects of parsley root extract (PCE) on DNA synthesis performance, metabolic activity and cytotoxicity in malignant and benign breast cells.

MATERIALS AND METHODS:

The PCE was prepared and analyzed by mass spectrometry. MCF7 and MCF12A cells were incubated with various concentrations of PCE and analyzed for DNA synthesis performance, metabolic activity and cytotoxicity by BrdU proliferation, MTT and LDH assays, respectively.

RESULTS:

PCE was found to contain a substantial ratio of lignans. At a concentration range of 0.01 μg/ml-100 μg/ml the LDH assay analysis showed no significant cytotoxicity of PCE in both cell lines. However, at 500 μg/ml PCE's cytotoxicity was well over 70% of total cell population in both cell lines. According to the BrdU proliferation assay analysis, PCE demonstrated significant DNA synthesis inhibition of up to 80% at concentrations of 10, 50, 100 and 500 μg/ml in both cell lines. Based on the MTT assay analysis, only at a concentration of 500 μg/ml, PCE demonstrated a statistically significant inhibition of cellular metabolic activity of 63% in MCF7 and 75% in MCF12A of their

respective normal capacity.

CONCLUSION:

PCE showed anti-proliferative effects in MCF7 and MCF12A cells. Further investigation is required to determine whether this effect can be solely attributed to its phytoestrogens.

ANTICANCER RESEARCH *37*: 95-102 (2017)
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The Effects of Petroselinum Crispum on Estrogen Receptor-positive Benign and Malignant Mammary Cells (MCF12A/MCF7)

LENNARD SCHRÖDER[1], JULIAN KOCH[1], SVEN MAHNER[1], BERND P. KOST[1], SIMONE HOFMANN[1], UDO JESCHKE[1], JENS HAUMANN[2], JULIAN SCHMEDT[2] and DAGMAR ULRIKE RICHTER[2]

[1]*LMU Munich, Department of Obstetrics and Gynecology, Munich, Germany;*
[2]*University of Rostock, Department of Obstetrics and Gynecology, Klinikum Sued, Rostock, Germany*

Abstract. *Background: Phytoestrogens have controversial effects on hormone-dependent tumors. Herein we investigated the effects of parsley root extract (PCE) on DNA synthesis performance, metabolic activity and cytotoxicity in malignant and benign breast cells. Materials and Methods: The PCE was prepared and analyzed by mass spectrometry. MCF7 and MCF12A cells were incubated with various concentrations of PCE and analyzed for DNA synthesis performance, metabolic activity and cytotoxicity by BrdU proliferation, MTT and LDH assays, respectively. Results: PCE was found to contain a substantial ratio of lignans. At a concentration range of 0.01 μg/ml-100 μg/ml the LDH assay analysis showed no significant cytotoxicity of PCE in both cell lines. However, at 500 μg/ml PCE's cytotoxicity was well over 70% of total cell population in both cell lines. According to the BrdU proliferation assay analysis, PCE demonstrated significant DNA synthesis inhibition of up to 80% at concentrations of 10, 50, 100 and 500 μg/ml in both cell lines. Based on the MTT assay analysis, only at a concentration of 500 μg/ml, PCE demonstrated a statistically significant inhibition of cellular metabolic activity of 63% in MCF7 and 75% in MCF12A of their respective normal capacity. Conclusion: PCE showed antiproliferative effects in MCF7 and MCF12A cells. Further investigation is required to determine whether this effect can be solely attributed to its phytoestrogens.*

Correspondence to: Professor Dr. Udo Jeschke, Department of Gynecology and Obstetrics, Campus Innenstadt, Ludwig Maximilian University Munich, Maistr. 11, D-80337 Munich, Germany. Tel: +49 89440054240, Fax: +49 89440054169, e-mail: udo.jeschke@med.uni-muenchen.de

Key Words: Petroselinum crispum, estrogen receptor, breast cancer, MCF7, MCF12A.

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Breast cancer, alongside bronchial and colorectal cancer, is one of three most commonly diagnosed cancers in women. It accounts for almost one third of all cancers diagnosed in women (1). The most prevalent type of breast cancer (65%) is estrogen (ER) and progesterone (PR) receptor-positive (2). For all patients diagnosed with hormone receptor positive breast cancer, endocrine therapy is recommended either with tamoxifen, a selective estrogen receptor antagonist (SERM) or with an aromatase inhibitor, depending on the menopausal status and comorbidities. Although endocrine therapy has led to a considerable decline in breast cancer mortality, endocrine resistance often causes disease progression, leading to metastasis with a poor 5-year relative survival outcome (23%) (3).

Phytoestrogens are a group of plant-derived polyphenolic compounds, their chemical structures resemble that of estrogen (4). Due to their molecular structures these compounds can bind and interact with human ER and PR resulting in both estrogen and anti-estrogen effects (5, 6). Because phytoestrogens are abundant in human and animal food sources, it has been suggested that they can influence hormone-dependent cancers. Besides interacting with ER and PR, phytoestrogens may contribute to low cancer risk by inhibiting aromatase enzymatic activity, decreasing *CYP19* gene expression in human tissues (7) and by other biochemical actions identified that lead to tumor suppression and inhibition of neovascularization (8, 9). Moreover, phytoestrogens have been applied as an alternative to hormone replacement therapy. In menopausal women, besides improving the cardiovascular system, the lipid and bone metabolism, they also positively affect major symptoms such as hot flushes and mood swings (10, 11).

Parsley root (*Petroselinum crispum*) is a perennial and herbaceous plant. It originated in the Mediterranean region and is cultivated as a dietary and medicinal plant. Parsley root is used in traditional and folklore medicines for digestive disorders, kidney and liver problems, menstrual irregularities

and for detoxification. It contains a substantial amount of vitamin C and constitutes as a good source of iron, potassium, calcium, manganese and folic acid (12, 13). The active compounds identified in *Petroselinum crispum* are phenolic compounds, flavonoids (particularly apigenin, apiin and 6-acetylapiin) and essential oils – mainly myristicin and apiol (14). Also notable are the photosensitizing furocoumarines bergapten and isoimperatorin in the root that increase the photosensitivity of the skin and are associated with an increased incidence of malignant melanoma (15).

Research on the presence of phytoestrogens in parsley root has attracted little attention. Only the research team led by M. Yoshikawa demonstrated that the methanolic extract from the aerial parts of *Petroselinum crispum* showed potent estrogenic activity, which was equal to that of isoflavone glycosides from soybean. In addition, they demonstrated that the methanolic extract of parsley, apiin and apigenin restored the uterus weight in ovariectomized mice when orally administered for seven consecutive days (16).

Due to the interesting characteristics of parsley root described above, the current *in vitro* study aims to identify the molecular chemical composition of parsley root extract (PCE) by mass spectrometry and to evaluate the potential phytoestrogen effects of PCE in terms of DNA synthesis performance (BrdU proliferation assay), metabolic activity (MTT assay) and cytotoxicity (LDH assay) on MCF7 breast cancer and MCF12A benign breast cell lines. Furthermore, the receptor status of MCF7 and MCF12A cells will be determined by immunohistochemical staining of ERα, ERβ and PR.

Materials and Methods

Origin of the PCE. The parsley root (*Petroselinum crispum* subsp. tuberosum) was grown, harvested and obtained commercially in 2011 in Mecklenburg-Western Pomerania, at vegetable farming Hofer Neubrandenburg.

Preparation of the PCE. The lignan-isolations were prepared as previously described (17-18). Deep frozen (–20˚C) parsley root (40 g) was cut into pieces of 5 mm. The pieces were ground in a mortar with liquid nitrogen and extracted under reflux with 360 ml methanol (methanol 99.5%; Roth, Karlsruhe, Germany) in a water bath at 75˚C. The extract was suspended in 10 ml of distilled water and partitioned with 16 ml ethyl acetate (five times) to give on drying an ethyl acetate soluble residue. After cooling the sample, the larger components were removed twice by using a folded filter (diameter: 185 mm; Schleicher und Schuell GmbH, Dassel, Germany). The solution was then evaporated in a 100 ml round bottom flask using a rotational damper (VV2011, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) under uniform reduction of pressure from 350 mbar to approximately 100 mbar. Adhering extract to the wall of the round bottomed flask was removed with 10 ml distilled water and then dissolved in an ultrasonic bath (Ultrasonics (300/H); Omnilab, Bremen, Germany) and extracted 5 times with 16 ml ethyl acetate (99.5%; Roth, Karlsruhe, Germany) in a 50 ml Falcon tube. This process leads to significant phase separation into a lower, denser phase (water phase), and an upper phase (lipid phase, containing lignans). The upper phase was carefully aspirated by a pasteur pipette and transferred to a 50 ml round bottomed flask. The sample was again evaporated in a rotational evaporator at 45˚C, under a uniform decrease in pressure from 400 mbar to about 250 mbar. The evaporated extract was dried overnight using a desiccator (with dried silica). To determine the net weight of the extract, the difference of the tare of the round bottom flask and the weight of the flask with dried extract was made (MC1 Analytic scale AC120S; Sartorius GmbH, Göttingen, Germany). The extract was stored protected from light in a refrigerator at 4˚C.

In order to verify the previously reported increased phytoestrogen concentration in PCE the molecular–chemical composition of the extract was further analyzed by pyrolysis-field ionization mass spectrometry by using a LCQ-Advantage (Thermo Finnigan's city & state). The peaks were identified by ion trap technology on ESI mode. The source voltage was set at 4.5 kV while the mass detection range was 150-2000 amu.

Creation of different PCE concentrations. After determining the weight of the extract it was dissolved with 96% ethanol (Roth, Karlsruhe, Germany) in an ultrasonic bath (Ultrasonics (300/H); Omnilab, Bremen, Germany) and the concentration of 100 mg/ml was determined (stock solution). For the following test series, 7 dilutions (excl. the stock solution) were set at the concentrations of 50,000 - 10,000 - 5,000 - 1,000 - 100 - 10 - 1 μg/ml. The extract was then further diluted, 1:100 giving the final concentrations 500 - 100 - 50 - 10 - 1 - 0.1 to 0.01 μg/ml. The dilutions were frozen at –85˚C.

Cell lines. The ER-positive, malignant breast cancer cell line MCF7 and the ER-positive, benign breast cell line MCF12A were used. Both cell lines were obtained commercially from the American Type Culture Collection (ATCC) and for further use in 1.5 ml culture medium (PAA, Germany) + 10% DMSO + 20% fetal calf serum (fetal bovine serum, Biochrom, Germany) stored at –180˚C.

Cell culture. After slow thawing, the cryopreserved cells were washed by 5 ml cell type-specific medium. For MCF7 cells, DMEM, high Glucose 4.5g (PAA, Germany), for MCF12A, Ham's F12 medium (PAA Germany) were used. For this, the cell suspension was carefully pipetted into 15 ml tubes and diluted with 5 ml culture medium. After vortexing and subsequent centrifugation for 5 min at 1000 rpm (centrifuge Universal 320R Hettich Zentrifugen, Gemany), the supernatant was discarded. The cells were re-suspended in 5 ml culture medium, transferred to 25-cm^2 cell culture flasks (Biochrom, Germany) and incubated at 37˚C, 5% CO_2 for 2-3 days. At a confluence rate of 80%, the medium was changed and the cells were splitted in a larger culture flask (75 cm^2, 150 cm^2, Biochrom, Germany). The cells were rinsed with phosphate buffered saline (PbS: Dulbecco's PbS powdered buffer, without Ca & Mg, PAA, Germany) to remove the upper layer of protein on the cells which would prevent an effective engagement of the trypsin before incubating them with trypsin (10%, trypsin EDTA, PAA, Germany) for 5 min at 37˚C. The reaction was stopped by adding DMEM (DMEM High Glucose (4,5g) +L-glutamin; without phenol red, PAA, Germany). The complete detachment of the cells was monitored by light microscopy. Following centrifugation for 5 min at 1000 rpm, the supernatant was discharged and cells were re-suspended in 5 ml cell-specific medium. For MCF7,

Table I. *Salient features of the antibodies used in the present study.*

Antibody/Source	Origin	Dilution in PBS	Incubation	Temperature
Anti – ERα, (Dako, Germany)	Mouse monoclonal	1:150	1h	RT
Anti – ERβ, (Serotec, Germany)	Mouse monoclonal	1:600	O/N	4˚C
Anti – PR, (Dako, Germany)	Mouse monoclonal	1:50	1h	RT

O/N: Overnight, RT: room temperature.

10% FKS, (PAA, Germany), for MCF12A, 10% Horseserum, (SIGMA, Germany) were used. Penicillin/Streptomycin 0.2% (PAA, Germany) and Amphotericin B 0.5% (PAA, Germany) were added to both cell lines.

Cell counting. Despite the fact that cell proliferation depends on the cell concentration and that cells need contact with neighboring cells in order to grow, when cell count is elevated nutrient shortages occur that may lead to apoptosis. The optimal cell concentration, determined by previous studies was set at 5×10^5 cells/ml (19). Most cell vitality assays that allow cell count determination are based on the change in membrane permeability of dead cells. Vital cells are impermeable for dyes such as trypan blue. Therefore, only the colorless cells are counted in phase contrast microscopy. To cell count, 20 μl of the cell suspension were diluted with 340 μl of cell culture medium and then combined with 20 μl trypan blue (corresponding dilution 1:20). To avoid trypan blue staining of intact cells, prompt counting was done. For this, about 20 μl of the probe was added to both sides of a Bürker cell counting chamber. Counting was done under a phase contrast microscope at a magnification of 100×.

General and statistical considerations. In all tests, a positive control (17β-estradiol, E2) and a negative control (tamoxifen, TAM) were carried along with the extract in different concentrations levels. Preliminary tests done using the same workgroup yielded optimal concentrations for E2: 10^{-9} mol/l and TAM: 10^{-4} mol/l. All calculations of the results were performed using Microsoft Excel considering the standard deviation. Statistical analysis was performed using the t-test. Each observation with $p<0.05$ was considered as statistically significant.

Measurement of cytotoxicity by LDH assay. The LDH assay (Roche, Mannheim, Germany) was performed in accordance with the manufacturer's instructions. Best results were achieved after incubation of treated cells (5×10^5 cells/ml) for 24 h in the absence (controls: TAM, E2) or the presence of PCE at different concentrations. Maximum LDH release (high control) was determined by incubating cells with Triton X-100 at 1% final concentration (Ferak, Berlin, Germany). Untreated cells were used to determine spontaneous LDH release (low control). After incubation, cells were gently centrifuged for 5 min 1,000 rpm. Subsequently, cell-free supernatants were carefully removed and transferred into a new 96-well microplate. Supernatants were mixed 1:1 with freshly prepared reaction mixture and incubated protected from light for 30 min at room temperature. The principle of the assay is based on a LDH/diaphorase coupled reaction with creation of a red colored formazan. Absorbance of the color was measured at 490 nm (620 nm wave length was used as a reference).

Proliferation measurement: MTT assay. Cell proliferation was analyzed using an MTT-kit according to the instructions of the manufacturer (Roche, Germany). After incubation of treated cells (5×10^5 cells/ml) for 24 h in the absence (controls: TAM, E2, N2) or presence of PCE at different concentrations, MTT labelled reagent was added to each well in a final concentration of 0.5 mg/ml. Subsequently, cells were incubated under culture conditions for 4 h. During this time the metabolic active cells transformed the yellow tetrazolium salt MTT to purple colored formazan crystals. After addition of the solubilization solution the plates were incubated overnight in a humidified atmosphere at 37˚C. With a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA) the color intensity was measured at 570 nm using a reference wave length of 650 nm.

BrdU proliferation-assay. Cell proliferation was analyzed with a 5-bromo-2'-deoxy-uridine (BrdU) labelling and detection kit (Roche, Germany) according to the instructions of the manufacturer. MCF7 and MCF12A cells (5×10^5 cells/ml) were grown in 96-well tissue culture plates for 24 h in the absence (controls: TAM, E2, N2) or presence of PCE at different concentrations. After labelling with BrdU for 3 h, the cells were fixed and BrdU incorporation into DNA was measured by an ELISA technique. Cellular proliferation inhibition is expressed in relation to controls (100%)±SD.

Immunohistochemical staining for the ERα, ERβ and PR. For immuno-detection of the steroid receptors ERα, ERβ and PR, the Vectastain R Elite ABC-kit (Vector Laboratories, USA) was used according to manufacturer's protocol. The slides were first air dried, rinsed in PBS for 5 min and then incubated with the ABC normal serum for 60 min in a humidified environment. The slides were then washed again and incubated with the respective primary antibodies. Salient features of the antibodies used are presented in Table I. The slides were then incubated with the diluted biotinylated secondary antibody (30 min), followed by incubation with the ABC reagent (30 min) and the ABC substrate (15 min). A single wash (PBS, 5 min) was applied between steps. Finally, the slides were counterstained with Mayer's acidic hematoxylin (30 sec), rinsed with water and covered with Aquatex.

Results

Immunohistochemistry. Expression of ERα, ERβ and PR receptors was evaluated in MCF7 and MCF12A cells (Figure 1). A negative control measurement was carried out in which instead of the primary antibody only secondary antibodies were added to show possible non-specific fluorescence signals. In both cell lines secondary antibodies were

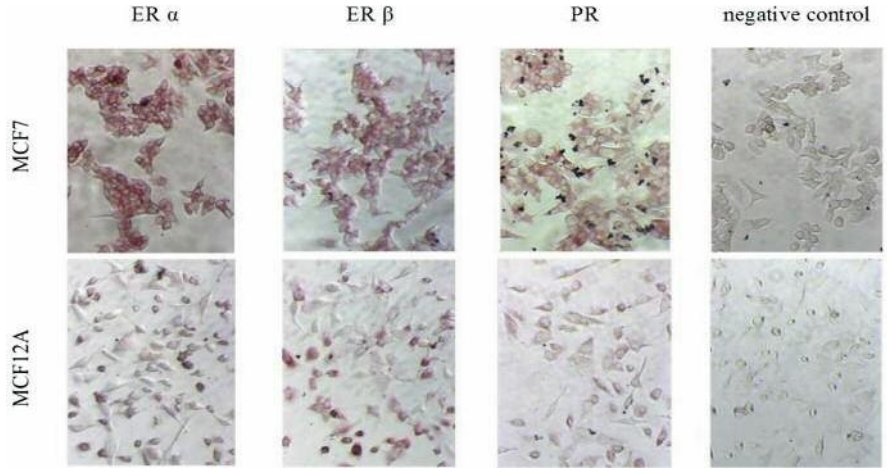

Figure 1. *Expression of hormone receptors in cell culture systems (MCF7 & MCF12A) used for the study.*

negative, ruling out non-specific fluorescence signals. ERα demonstrated the strongest expression in both cell lines. ERβ and PR were less pronounced and comparable in intensity. MCF12A cells expressed lower levels of all receptors tested compared to MCF7 cells, which can be due to the benign nature of MCF12A and its lower metabolic activity and growth rate.

LDH cytotoxicity assay. The lactate dehydrogenase assay (LDH assay) indicates non-specific cytotoxic effects of the given extract that are measurable by enzymatic reactions of LDH. LDH is a cytosolic enzyme that is released from dead cells, making its concentration suitable as a quantitative indicator of cell death. As a positive control and to establish a reference value, cells were incubated with 1% Triton X- 100, resulting in complete cell death. LDH activity in these controls was, therefore, set at 100%. Figure 2 shows that no significant cytotoxicity was observed at concentrations of 0.01 μg/ml to 100 μg/ml. Also estradiol (E2) did not cause significant cytotoxicity. However, significant cytotoxicity ($p<0.01$) was caused by PCE at the concentration of 500 μg/ml in up to 80% of the cells of both cell lines (MCF7: 74.9±36.6%, MCF12A: 81.5%±14.7%), which is approximately equivalent to the effect observed with TAM (MCF7: 69.6%±22.6%, MCF12A: 98.7%±1.7, $p<0.01$).

DNA synthesis performance: BrdU proliferation assay. The bromodeoxiuridine (BrdU) proliferation assay provides insights into the DNA synthetic capacity of cells, making it a suitable marker for cell proliferation. During the DNA synthesis phase of the mitotic cycle, cells build the added base analogue BrdU into their genome. An antibody directed against BrdU catalyzes a colour reaction whose intensity directly correlates with the synthetic capacity of the cells. As a reference value, the negative control (N2, ethanol/DMSO) was set at 100%. Figure 3 compares proliferation rates of MCF7 and MCF12A cell lines incubated with the respective extract concentrations. PCE showed less pronounced antiproliferative effects in MCF12A cultures compared to MCF7, as more significant results were obtained using MCF7 cells. At PCE concentrations of 0.01, 0.1 and 1 μg/ml, little growth inhibition of MCF12A cells (max. 15%, at PCE 0.1 μg/ml: 85.5%±5.6%, $p<0.01$) was demonstrated. At the concentrations of 50 and 100 μg/ml the antiproliferative effect of PCE in MCF12A intensified, and statistical significance was demonstrated (50 μg/ml: 56.7%±13.8%, $p<0.01$, 100μg/ml: 61.1%±14%, $p=0.01$). At 500 μg/ml extract concentration, significant inhibition of proliferation of 84.5% was noted (15.5%±11.7%, $p=0.01$) in MCF12A cells. In MCF7 cells, the PCE concentration of 0.01 μg/ml caused, compared to the concentrations of 1 and 0.1 μg/ml an increased, although not significant inhibition of cell proliferation of approximately 40% (61.77%±13.0%, $p=0.08$). Statistical significant inhibitions over 40% were demonstrated in MCF7 cells at the concentration levels of 10, 50, 100 and 500 μg/ml. The strongest inhibition of proliferation in MFC7 cells of approximately 80% was observed at a PCE concentration of 100 μg/ml (19%±2.7%, $p<0.01$).

Cellular metabolic activity: MTT cell viability assay. Metabolically-active cells have high activity of the mitochondrial enzyme succinate dehydrogenase, whose catalytic activity can be measured directly by a color reaction. High color intensities indicate increased metabolism and cell viability. As a reference value, the negative control N2 was set to 100%. In MFC7 and

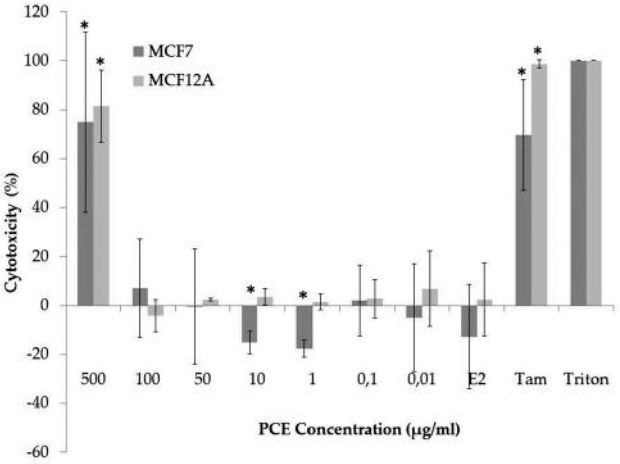

Figure 2. *LDH test of the PCE (µg/ml) on MCF7 and MCF12A cells for the assessment of cytotoxicity. Values are given as a percentage of the triton control (100%). *Marks values below the significance level of α=5%.*

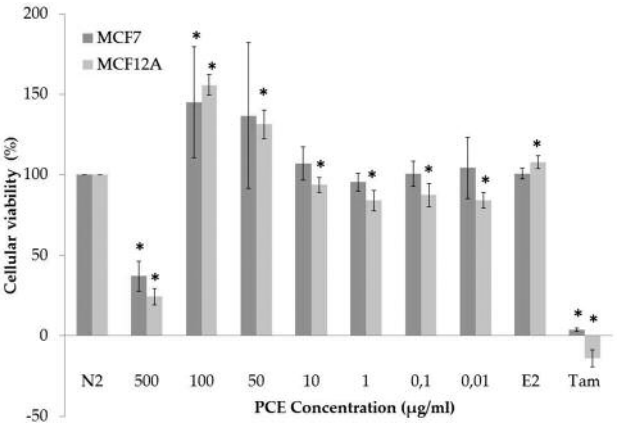

Figure 4. *MTT cell viability assay of the PCE at different concentrations (µg/ml) on MCF7 and MCF12A cellscfor the assessment of cellular metabolic activity. Values are given as a percentage of negative control 2 (N2). *Marks values below the significance level of α=5%.*

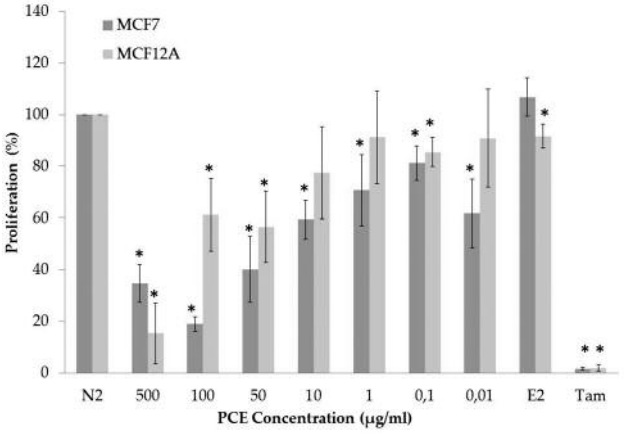

Figure 3. *BrdU proliferation test of the PCE in different concentrations (µg/ml) on MCF7 and MCF12A cells for the assessment of cell proliferation. Values are given as a percentage of negative control 2 (N2). *Marks values below the significance level of α=5%.*

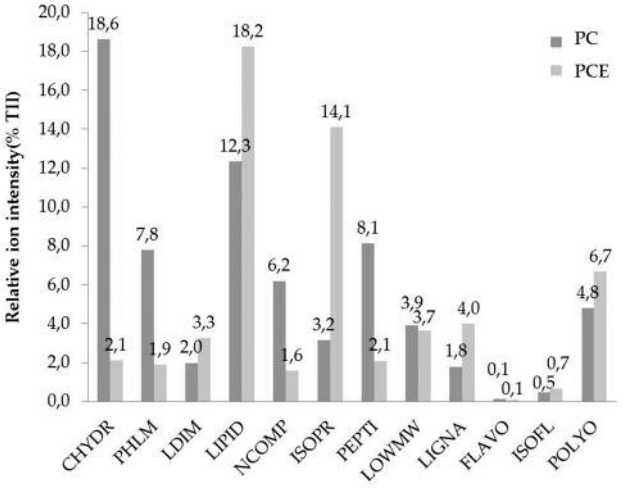

Figure 5. *Comparison of the main classes of organic extract components between untreated parsley root (PW) and a parsley root extract (PWE, 100 mg/ml, according to Luyengi et al.). The mean values are shown from 3 measurements, expressed as a percentage of the total ion intensity (TI). LIPID=Alkanes, alkenes, aldehydes, alcohols, fatty acids, n-alkyl esters, waxes, fats; NCOMP=N-containing compounds; ISOPR=isoprenoid compounds (sterols, terpenes, carotenoids); PEPTI=peptides and free amino acids; LOWMW=low molecular weight compounds m/z 15 to 56; POLYPH=other polyphenols (suberin, cutin, stilbene, tannins, etc.).*

MCF12A cell lines, both inhibitory and proliferative effects of PCE were demonstrated. At PCE concentration levels of 0.01 µg/ml to 10 µg/ml, weak effects in both cell lines were observed (Figure 4). Generally, metabolic activity was lower in MCF12A cells than in MCF7 cells, except at PCE concentration of 100 µg/ml (MCF12A: 155.7%±6.3%, $p<0.01$, MCF7: 144.9%±34.5%, $p<0.01$). Nevertheless, statistically significant results were found for all MCF12A concentrations, in contrast to MCF7 cells, were increased metabolism and significant results were achieved only at concentration levels of 100 µg/ml (proliferative effect, 144.9%±34.5%, $p<0.01$) and 500 µg/ml (inhibitory effect, 37.0%±9.3%, $p<0.01$). Strong, significant inhibition of cell

growth and metabolic activity was demonstrated in both cell lines at PCE concentration of 500 μg/ml, 37.0%±9.3% ($p<0.01$), cell viability for MCF7 cells and 24.4%±4% ($p<0.01$) for MCF12A cells. No influence on cell viability was observed by E2. However, Tamoxifen caused significant cytotoxicity and thus reduced metabolic activity in both cell lines (MCF7: 3.8%±0.9%, $p<0.01$, MCF12A: -14.0%±5.4%, $p<0.01$).

Pyrolysis/FI mass spectrometry. The results from pyrolysis mass spectrometry were compared with masses of different substance classes from public databases. In Figure 5 the main categories identified are presented with the respective percentage of the substance class from the total ion intensity. Untreated parsley root (PC) showed high carbohydrate and lipid fractions (18.6% and 12.3% respectively). Peptides and free amino acids are represented with 8.1%, as the third largest group. The extraction process caused a shift of the respective proportions of substances classes, resulting in less carbohydrates (2.1%), more lipids (18.2%) and more isoprenoids (14.1%) in PCE compared to PC. Together, the fractions monolignols, lignin dimers, lignans, flavones, isoflavones and other polyphenols (PHLM, LDIM, LIGNA, FLAVO, ISOFL, POLYO) represent the group of phytoestrogens (PC: 17%; PCE: 16.6%).

Discussion

In this study we demonstrated that PCE has cytotoxic, inhibitory and anti-proliferative effects on cultures of both the benign MCF12A and cancer-derived MCF7 cell lines. The BrdU proliferation assay was used as the main method to detect inhibitory or stimulatory effects of PCE. To verify its accuracy, a LDH assay was performed to determine cytotoxicity. In both cell lines and at all PCE concentration levels with the notable exception of 500 μg/ml, no substantial increase in LDH activity was observed. In combination with the BrdU assay, these results suggest antiproliferative effects of PCE not related to cytotoxicity.

So far, the exact cytotoxic effects of PCE remain unknown. However, according to Dorman *et al.* it is likely that at high concentrations, toxicity is not mediated by caspase 3 apoptosis pathways but by strong pro-oxidative effects (20). Other possible apoptosis pathways are intrinsic signal molecules (cytochrome *c*, p53, p21) which can be activated by isoflavones, such as genistein (21, 22), apigenin (23) and lignans (24).

The BrdU assay showed little to no effect of PCE in both cell lines at low concentrations, but at higher concentrations (≥10 μg/ml) the following strong inhibitory effects: a reduction of cell proliferation by 42% in MCF12A (at 50 μg/ml) and by 80% in MCF7 cell lines (at 100 μg/ml). In further studies the effect of PCE at the given different concentrations on ERα/ERβ/PR expression and the assessment by immunocytochemistry could clarify whether it is likely that MCF12A as a benign cell line is less sensitive to possible anti-estrogenic properties of PCE due to a decreased receptor expression compared with malignant MCF7 cells.

A stronger inhibition was demonstrated in MCF12A cells at the concentration of 500 μg/ml, attributable to increased cytotoxicity and the associated cell loss. Paradoxically, in MCF7 cells PCE demonstrated a weaker inhibition of cell proliferation at 500 μg/ml than at 100 μg/ml, despite cell death rates of up to 75%. Accordingly, the remaining intact cells (25%) would have to operate at a higher synthesis performance than, in this case, 90% of the cells at a PCE concentration of 100 μg/ml. Similar results were obtained by Moorghen *et al.* (25). They concluded that a higher proliferation rate compensates the cells under apoptosis (secondary effect). However, the low MTT assay values contradict this assumption. At a PCE concentration of 500 μg/ml, when high cytotoxicity was reported, low metabolic activity in both cell lines (max. 37%) was registered.

Interestingly, in MCF12A and MCF7 cells that demonstrated an inhibition of proliferation at the PCE concentrations of 100 μg/ml and 50 μg/ml, excessive metabolic activities were reported (max. 155% in MCF12A cells or max. 145% in MCF7 cells). A potential explanation is the already mentioned compensatory secondary effect which was also reported by Abarzua *et al.* in MCF7 cell lines with similar values (26). Moreover, in the E2 control no stimulatory effect on MCF7 cells was observed. Therefore, PCE-induced enhancement of viability at 100 and 50 μg/ml is probably not related to an ER-mediated process. Other possibilities that increase cellular viability at 100 and 50 μg/ml could be the activation of protective or resistance pathways (elimination of the active compounds by MDR transporters, metabolic detoxification, activation of alternative metabolic pathways). At low PCE concentrations (10 μg/ml-0.01 μg/ml) no eminent metabolic changes were visible, matching the weakly altered proliferation status of the MCF7 and MCF12A cells.

Only one other study investigating the phytoestrogen effects of *Petroselinum crispum* on MCF7 cell lines exists (16) where the influence of parsley leaf extracts and other isolates on MCF7 breast cancer cell lines was investigated. The methanol extract showed progesterone-like properties associated with increased proliferation rates of up to 156% (at a concentration of 10 μg/ml). However, the cell proliferation was not measured by BrdU but only with the MTT assay. It reported similar MTT values (155% at a concentration of 100 μg/ml in MCF12A cell cultures). However, it remains unclear to what extent the cellular metabolic activity is linked with the proliferation rate.

In all assays, estrogen was used as a positive control. In both cell lines it demonstrated no significant cytotoxicity and proliferation compared with the negative control. Previous works using the same cell lines demonstrated similar results

(27, 28). It is likely that both receptor subtypes (ERα/ERβ) are activated by E2, whereby ERβ intracellularly suppresses the proliferative effect of ERα. Thus the ratio of the expression of ERα/ERβ would be decisive for the different proliferation patterns of the cells. Immunohistochemistry analysis showed that both ERα and ERβ are present on cells (Figure 1). Looking at the images, it is evident that the ERβ expression is weaker but likely strong enough to inhibit ERα mediated cell proliferation.

TAM, which was used as a negative control is a SERM used for the treatment of hormone-sensitive breast cancer for already more than twenty years. It acts as a competitive inhibitor or partial agonist on the ER. On breast cancer cell lines it has an inhibitory effect (29). Correspondingly, a high cytotoxicity and a low metabolism and proliferation were observed in both cell lines treated with TAM.

Previous results demonstrated that PCE has anti- carcinogenic properties. The question of whether and to what extent phytoestrogens are involved cannot be answered precisely. For this reason, mass spectrometry was performed which provided information on the different extract components. In order to determine the efficiency of the extraction method used based on the Luyengi *et al.* study, a sample of 5 mg raw material (PC) was analyzed in addition to the extract sample.

Mass spectrometry of PCE and PC demonstrated a loss of carbohydrates, monolignols, and nitrogen-containing compounds as well as free amino acids in PCE compared to PC. Except for the monolignols these compounds are not probable constitutes of phytoestrogens. An increase in concentration was registered in the groups of lipids, isoprenoids, lignindimeres, lignans and other polyphenols. Particularly the latter three groups are known to have phytoestrogen activity. This leads to a total sum of 16.6% phytoestrogen compounds in the PCE. The isoprenoids group contains sterols such as cholesterol (animal) and ergosterol (fungi/protozoa). Other metabolites can form steroids or steroid hormones like estradiol. It seems likely that isoprenoids may have estrogenic activity. Further, non-estrogenic effects can be found in the group of lipids: free fatty acids inhibit cell proliferation through activation of peroxisome proliferator-activated receptors PPARα and PPARγ, which bind as transcription factors to the promoters of retinoid X receptors, regulating the expression of various genes (30, 31). In MCF7 breast cancer cells, PPARγ counteracts the transcription factor NFκB, inducing the promoter of *p53* to drive apoptosis (32). Also, the group the alkyl esters cytotoxic effects have been assigned: n-alkyl ester as a basic component of the (methyl) acrylates have lipophilic side groups that mediate cytotoxicity (33). Due to the overall high percentage of lipids (18.2%) in PCE it cannot be ruled out that phytoestrogens exhibit little or no influence on its overall effect. Therefore, in further studies, lipopolysaccharide could be used as an additional control substance to demonstrate the extent to which lipids are involved in the observed effects.

So far, few studies have been conducted with extracts of *Petroselinum crispum*. Differently to our study, Yoshikawa *et al.* used a methanol extract of the leaves, but not of the root (16). By means of high pressure liquid chromatography various fractions were separated and subsequently detected by electron impact mass spectrometry. Ten individual substances were identified, of which five possess proven anti-estrogenic properties: apigetrin, apiin, diosmetin, kaempferol, 6-acetylapiin (all from the group of flavone glycosides, which also include the strong growth inhibitors genistin and daidzein). Furthermore, an increase in uterine weight of ovariectomized mice fed with the parsley leave extract was observed, which suggests progesterone like growth-promoting effect.

This work proved that PCE causes anti-carcinogenic effects on MCF7/MCF12A cell lines. Growth inhibitory potency of parsley root extract (PCE) on MCF7 mammary tumor cells at non-toxic concentrations (up to 100 µg/ml) was reported. Complementary observation of a less marked inhibition on related MCF12A benign cells suggests an ER-mediated process. Whether this apparent anti-carcinogenic effect of PCE can be solely attributed to its phytoestrogens requires complementary investigations. As a possible approach to determining the role of hormone receptor mediated cell response, PCE could be tested on malignant and benign ER- negative cells (*e.g.* BT-20/MCF-10A). Additional control substances to assess the lipid signal pathway, such as lipopolysaccharide could demonstrate the extent to which lipids are involved in the effects observed. Furthermore, fractional chromatography could provide information of the individual substances and their impact on breast cancer cell lines. Cytotoxicity could be evaluated in detail by immunohistochemistry or RTQ-PCR quantification apoptosis induced markers like, p53, p21, BCL2 and Caspase 8/9. Other extraction methods besides the ones used (17) could probably create qualitatively improved extracts (*e.g.*, water extraction, resuspension of the dry extract in various solvents).

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2.2. Publication 2

Title:

Effects of Phytoestrogen Extracts Isolated from Elder Flower on Hormone Production and Receptor Expression of Trophoblast Tumor Cells JEG-3 and BeWo, as well as MCF7 Breast Cancer Cells.

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Effects of Phytoestrogen Extracts Isolated from Elder Flower on Hormone Production and Receptor Expression of Trophoblast Tumor Cells JEG-3 and BeWo, as well as MCF7 Breast Cancer Cells

Lennard Schröder [1], Dagmar Ulrike Richter [2], Birgit Piechulla [3], Mareike Chrobak [3], Christina Kuhn [1], Sandra Schulze [1], Sybille Abarzua [3], Udo Jeschke [1,*] and Tobias Weissenbacher [1]

1 Department of Obstetrics and Gynaecology, Ludwig-Maximilians-University of Munich, Munich 80337, Germany; lennard.schroeder@med.uni-muenchen.de (L.S.); Christina.kuhn@med.uni-muenchen.de (C.K.); sandra.schulze@med.uni-muenchen.de (S.S.); tobias.weissenbacher@med.uni-muenchen.de (T.W.)

2 Department of Obstetrics and Gynaecology, University of Rostock, Rostock 18059, Germany; dagmar.richter@kliniksued-rostock.de

3 Department of Biological Sciences, University of Rostock, Rostock 18059, Germany; birgit.piechulla@uni-rostock.de (B.P.); chrobak@bni-hamburg.de (M.C.); sybille.abarzua@uni-rostock.de (S.A.)

* Correspondence: udo.jeschke@med.uni-muenchen.de; Tel.: +49-89-51604240; Fax: +49-89-51604916

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Abstract: Herein we investigated the effect of elderflower extracts (EFE) and of enterolactone/enterodiol on hormone production and proliferation of trophoblast tumor cell lines JEG-3 and BeWo, as well as MCF7 breast cancer cells. The EFE was analyzed by mass spectrometry. Cells were incubated with various concentrations of EFE. Untreated cells served as controls. Supernatants were tested for estradiol production with an ELISA method. Furthermore, the effect of the EFE on ERα/ERβ/PR expression was assessed by immunocytochemistry. EFE contains a substantial amount of lignans. Estradiol production was inhibited in all cells in a concentration-dependent manner. EFE upregulated ERα in JEG-3 cell lines. In MCF7 cells, a significant ERα downregulation and PR upregulation were observed. The control substances enterolactone and enterodiol in contrast inhibited the expression of both ER and of PR in MCF7 cells. In addition, the production of estradiol was upregulated in BeWo and MCF7 cells in a concentration dependent manner. The downregulating effect of EFE on ERα expression and the upregulation of the PR expression in MFC-7 cells are promising results. Therefore, additional unknown substances might be responsible for ERα downregulation and PR upregulation. These findings suggest potential use of EFE in breast cancer prevention and/or treatment and warrant further investigation.

Keywords: lignans; isoflavones; elder flower; breast cancer; trophoblast tumor

1. Introduction

A growing body of data points to health benefits of phytoestrogens in diet and to possible pharmaceutical applications [1]. The two main groups of phytoestrogens, isoflavones and lignans, are polyphenolic compounds derived from plants with a molecular structure that closely resembles mammalian estrogens [2]. Due to their molecular structure, these compounds can bind and interact with human estrogen receptors (ER) resulting in both estrogen and anti-estrogen effects [3]. Thus, it is assumed that some phytoestrogens can be classified as selective estrogen receptor modulators (SERM) [4,5].

Isoflavones are mostly found in legumes, with the most common representative being soy and its derivative products [6], making them more common in Asian diets, whereas lignans, more common in occidental diets, are usually found in seeds and fiber-rich cereals [7,8]. Their role in the pathogenesis of hormone-dependent malignancies, especially breast cancer, has been investigated using chemically pure isolates or product extracts in several in vitro or in vivo models [9–11]. Their effects as hormonally-active diet components have been excessively and controversially discussed [12,13]. Isoflavone extracts and supplements are often used for the treatment of menopausal symptoms and for the prevention of age-associated conditions, such as cardiovascular diseases and osteoporosis in postmenopausal women [12].

In humans the most important lignans are secoisolariciresinol and matairesinol [14].

After oral intake they are transformed by intestinal aerobe and anerobe flora into bioavailable enterolignans enterodiol and enterolactone [15].

Clinical studies proved that a high exposition to enterolignans reduced the risk of breast cancer by 16% [16]. Moreover, increased blood concentrations of enterolactone in postmenopausal women are related with a significant reduction of breast cancer mortality [17].

With the goal of identifying potential sources of phytoestrogens and selecting those with beneficial functions, our group has tested, in prior trials, the phytoestrogen properties of pumpkin and flax seed lignan and isoflavone extracts on the proliferation of trophoblast and breast cancer cell lines [18,19]. Moreover, the effect of the phytoestrogens genistein and daidzein on human term trophoblasts and their influence on fertility was investigated [20].

Elder flower (*Sambucus nigra*) is a historically-significant herbal medicinal plant used for centuries as a cold remedy. It is used as a general nutritive tonic and due to its strong taste as a flavor enhancer in meals and beverages. Elder extracts possess significant antioxidant activity and have been shown to impair angiogenesis. The anthocyanins present in elderberries protect vascular epithelial cells against oxidative insult, and reduce low-density lipoprotein (LDL) and cholesterol, therefore, preventing vascular disease [21]. Elder extracts boost cytokine production [22]. The influenza A virus subtype H1N1 inhibition activities of the elder flavonoids compare favorably to the known anti-influenza activities of oseltamivir and amantadine [23]. The terpenes extracted from elder flower show notably strong antimicrobial effects in vitro upon methicillin-resistant *Staphylococcus aureus* [24]. Moreover elder flower could improve bone properties by inhibiting the process of bone resorption and stimulating the process of bone formation [25].

Due to the interesting characteristics of elder flower described above, this in vitro study aims to identify the distribution of lignans and isoflavones in elder flower extracts (EFE) and evaluate the potential phytoestrogen effects of EFE on tumor trophoblast BeWo and JEG-3 cells and the ER-positive MCF7 breast cancer cell lines, and compare those with the effects of enterodiol and enterolactone.

2. Materials and Methods

2.1. Preparation of the EFE

In total six EFE from the species *Sambucus nigra* were produced. Three lignan-isolations were prepared as previously described [26] and, afterwards, dissolved in 100% ethanol. In the aim to verify the previously-reported increased lignan concentration in elder flowers [27] the molecular–chemical composition of the extract was further analyzed by pyrolysis-field ionization mass spectrometry by using an LCQ-Advantage (Thermo Finnigan's, Arcade, NY, USA). The peaks were identified by ion trap technology in electrospray ionisation (ESI) mode. The source voltage was set at 4.5 kV, while the mass detection range was 150–2000 amu. For the production of the three flavonoid extracts, the method previously described by Franz and Koehler was used [28].

2.2. Cell Lines

For the current work the chorion carcinoma cell lines JEG-3 and BeWo, and the breast carcinoma cell line MCF7, were used. All cell lines were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Biochrom AG, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 100 µg/mL Penicillin/Streptomycin (Biochrom AG) and 2.5 µg/mL Amphotericin B (Biochrom AG). Cultures were maintained in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Prior to cell culture, the levels of estrogen or progesterone in the medium were measured, using an automated Immulite (DPC Biermann, Freiburg, Germany) hormone analyzer, in order to exclude their presence.

2.3. Effect of EFE on Cell Lines

For all experiments, the cells were seeded on Quadriperm tissue slides with or without added lignan and flavonoid EFE separately. In brief, cells were seeded at a concentration of 400,000 cells per slide. The cells were left to attach for 24 h. Then, the medium was replaced by medium supplemented with lignan and flavonoid EFE separately at final effective concentrations of 10, 50, and 100 µg/mL. Since the original EFE was diluted in 100% ethanol, medium supplemented with 100% ethanol at a concentration of 5 µg/mL (this being the maximum ethanol concentration achieved during these experiments) served as the internal control. In addition, enterolactone and enterodiol (Sigma-Aldrich, Taufkirchen, Germany) were added to the same cell cultures as used for EFE in concentrations of 10, 50, and 100 µg/mL, respectively. After the cells were cultured for 72 h, 1 mL from each supernatant was stored at −80 °C for estradiol analysis. The remaining supernatant was then discarded and the slides were washed in phosphate-buffered saline (PBS), fixed in acetone for 10 min, and left to dry at room temperature. Cells treated with equal concentrations of estradiol (10, 50, and 100 µg/mL) served as external controls.

2.4. Estradiol Determination in the Cell Culture Medium

For the determination of estradiol in the culture medium, a competitive enzyme immuno-assay (EIA) was applied as described previously [29]. The measurements were performed using an automated Immulite 2000 (DPC Biermann, Freiburg, Germany) hormone analyzer.

2.5. Immunocytochemistry for the ERα, ERβ, and Progesterone Receptor (PR)

For immuno-detection of the steroid receptors ERα, ERβ, and PR, the Vectastain R Elite Avidin/Biotin Complex (ABC) Kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's protocol. After being air dried, the slides were rinsed in PBS for 5 min and incubated with the ABC normal serum for 60 min in a humidified environment. The slides were then washed and incubated with the respective primary antibodies. Salient features of the antibodies used are presented in Table 1. The slides were then incubated with the diluted biotinylated secondary antibody (30 min), followed by incubation with the ABC reagent (30 min), and the ABC substrate (15 min). A PBS wash (5 min) was applied between steps. Finally, the slides were counterstained with Mayer's acidic hematoxylin (30 s), rinsed with water, and covered with Aquatex. The intensity and distribution patterns of the specific immunocytochemical staining was evaluated using a semi-quantitative method (IRS score) as previously described [30]. Briefly, the IRS score was calculated as the product of the optical staining intensity (0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong straining) multiplied by staining extent (0 = no staining; 1% ≤ 10% staining; 2 = 11%–50% staining; 3 = 51%–80% staining and 4 ≥ 80% staining). The percentage of positively-stained cells was estimated by counting approximately 100 cells.

Table 1. Antibodies used for expression analysis of steroid hormone receptors.

Salient Features of the Antibodies Used in the Present Study				
Antibody	**(Source)**	**Origin**	**Dilution in PBS**	**Temperature**
Anti-ERcr	(Dako, Germany)	Mouse monoclonal	1:150	1 h RT
Anti-ERβ	(Serotec, Germany)	Mouse monoclonal	1:600	O/N 4 °C
Anti-PR	(Dako, Germany)	Mouse monoclonal	1:50	1 h RT

ER = estrogen receptor; PR = progesterone receptor; O/N = overnight; RT = room temperature.

2.6. Statistical Analysis

The results are presented as mean ± sem of three independent experiments. Statistical analysis was performed using the Wilcoxon's signed rank tests for pairwise comparisons. Each observation with $p < 0.05$ was considered statistically significant.

3. Results

3.1. EFE Contains Phytoestrogen Compounds

Mass spectrometry was performed to identify the different substrates and to determine their proportions in EFE. The results showed that the EFE contains phytoestrogen compounds. Lignan dimers (LDIM) were found with a total intensity of 2.6%, lignans (LIGNA) with 1.3%, isoflavones (ISOFL) with 0.6%, and flavones (FLAVO) with 0.1%. Figure 1 demonstrates the distribution of the different substance classes found in EFE. With a total intensity of 18.1% the most abundant substance class in EFE were lipids, including alcanes, alcenes, fatty acids, waxes, and fats (LIPID). Monolignoles (PHLM) were found with an intensity of 13.4% and carbohydrates (CHYDR) with 11.1%. Nitrogen (NCOMP) compounds were found with a total intensity of 6%, amino acids and peptides with 5.4% (PEPTI), isoprenoid compounds (ISOPR) with 1.5%, other polyphenolic (POLYO) with 5.2%, and low molecular compounds (LOWMW) with 4.7%.

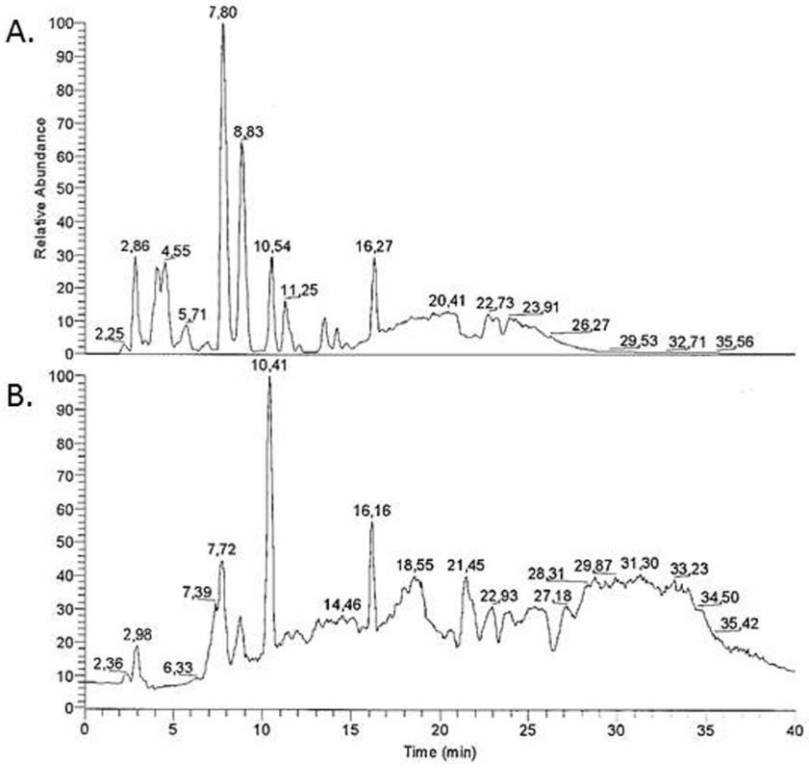

Figure 1. *Cont.*

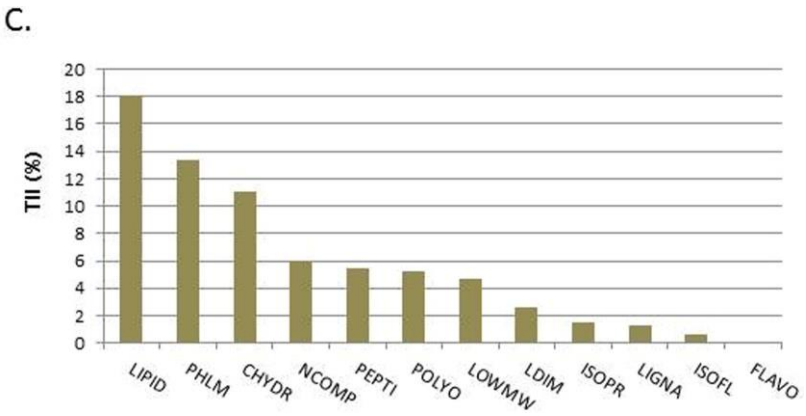

Figure 1. Characteristic diagram of mass spectrometry analysis results of the EFE using both the microwave extraction (**A**) and the extraction method modified from Luyengi et al. [26] (**B**); moreover, the different substances extracted are presented (**C**).

3.2. EFE Lignan and Flavonoid Extracts Induce the Inhibition of Estradiol Secretion in JEG-3, BeWo, and MCF7 Cells in a Dose-Response Pattern and the Inhibition of Progesterone Secretion in JEG-3 Cells

To assess the estradiol and progesterone secretion, all three cell lines were cultured for 72 h in the presence of different EFE concentrations. An automated hormone analyzer was used to determine the estradiol and progesterone concentration in the medium by applying a competitive EIA. All cell lines were incubated with elder flower flavonoid and lignan extracts. The EFE lignan and flavonoid extracts demonstrated a statistical significant inhibition in estradiol secretion in a dose-response pattern in all three cell lines (Figure 2). Only statistical significant data is demonstrated in the figures. The cell culture medium with 10% FCS did not contain any measurable amounts of estrogen and progesterone, as determined with the automated hormone analyzer Immulite (DPC Biermann, Freiburg, Germany).

In JEG-3 cells, the estradiol production was inhibited from 5634.96 ± 235.77 pg/mL in the control to 4547.48 ± 145.89 pg/mL, 1283.88 ± 29.78 pg/mL, and 1030.43 ± 24.50 pg/mL when the EFE lignan concentration was 10 µg/mL, 50 µg/mL, and 100 µg/mL, p = 0.018, respectively (Figure 2A). EFE flavonoids had a similar effect using the same concentrations, as the estradiol production was inhibited from 5634.97 ± 235.77 pg/mL in the control to 5049 ± 187.28 pg/mL, 1264.5 ± 151.26 pg/mL, and 1137 ± 138.08 pg/mL (Figure 2B).

In JEG-3 cell lines progesterone secretion was also significantly inhibited using EFE lignan extracts from 87.95 ± 1.36 pg/mL in the control to 84.88 ± 1.98 pg/mL, 66.22 ± 2.25 pg/mL, and 45.98 ± 1.92 pg/mL when the EFE concentration was 10 µg/mL, 50 µg/mL and 100 µg/mL.

The cultivation of the BeWo cell line with EFE lignan extracts resulted again in an inhibition of estradiol secretion from 245.25 ± 16.25 pg/mL in the control to 230.85 ± 8.17 pg/mL, 231.95 ± 6.1 pg/mL, and 206.81 ± 5.69 pg/mL when the EFE concentration was 10 µg/mL, 50 µg/mL, and 100 µg/mL (Figure 2C). The differences between the stimulated cells and the control were only significant at a concentration of 100 µg/mL, with p = 0.05.

In MCF7 cell lines the EFE flavonoid concentrations of 10 µg/mL and 50 µg/mL first provoked a transient increased secretion of estradiol from 146.37 ± 9.91 pg/mL in the control to 185.44 ± 4.28 pg/mL at 10 µg/mL and 164.07 ± 3.16 pg/mL at 50 µg/mL (Figure 2D). Then, at 100 µg/mL, the estradiol secretion was inhibited to 140.21 ± 2.22 pg/mL, p = 0.08 respectively.

Using the same concentrations with EFE flavonoid-extracts, progesterone secretion was also significantly inhibited in JEG-3 cells (Figure 2E) from 104.83 ± 5.13 pg/mL in the control to 77.94 ± 1.32 pg/mL, 56.18 ± 1.7 pg/mL, and 47.76 ± 1.56 pg/mL (p = 0.043).

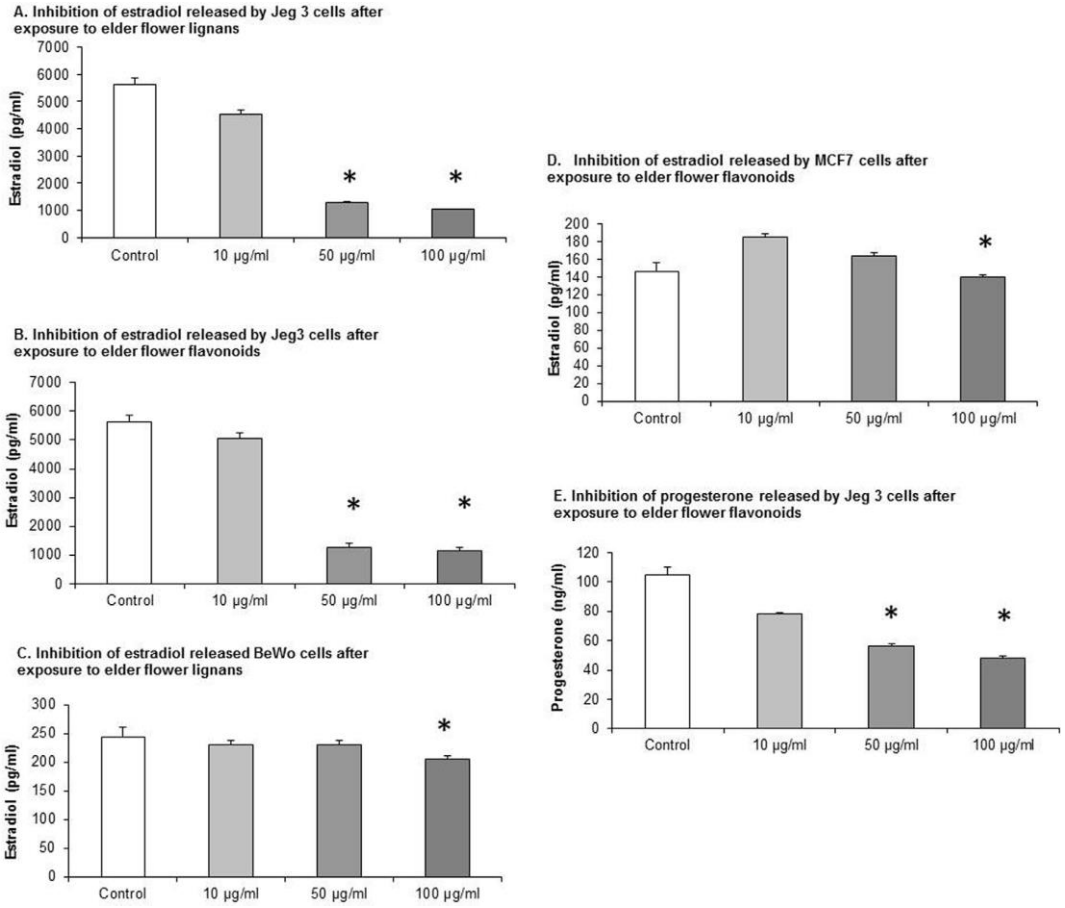

Figure 2. Estradiol and progesterone concentration in the tissue culture medium of JEG-3, BeWo, and MCF7 cells in the absence or presence of EFE. The effective EFE concentrations were 10 µg/mL, 50 µg/mL, and 100 µg/mL. Significantly different observations are highlighted with an asterisk.

3.3. EFE Flavonoid Extracts up Regulates ERα in JEG-3 Cells

JEG-3 cell lines that were cultivated with EFE flavonoid in the concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL an upregulation of ERα was demonstrated. The IRS score of ERα was increased from 1 ± 0 in the control to 1.33 ± 0.23, 1.67 ± 0.54, and 2.167 ± 0.44. At 100 µg/mL statistical significance was demonstrated, $p = 0.015$, respectively (Figure 3A).

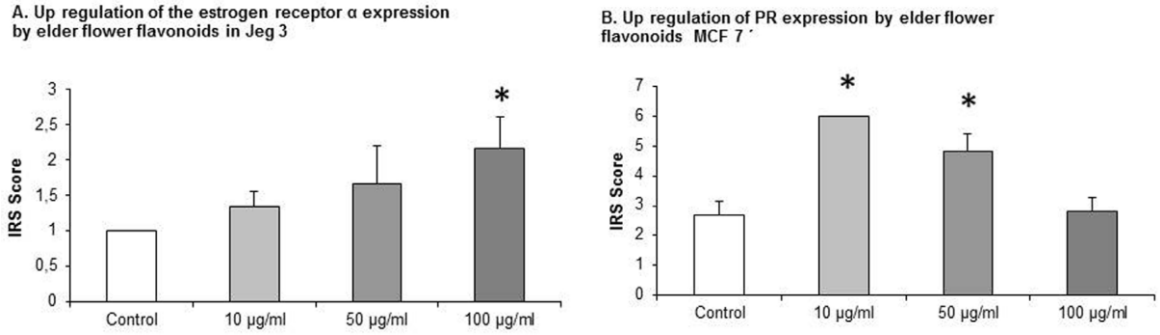

Figure 3. Upregulation of ER α and progesterone receptor by elder flower flavonoids in JEG-3 and MCF7 cells. The effective EFE concentrations were 10 µg/mL, 50 µg/mL, and 100 µg/mL. Significantly different observations are highlighted with an asterisk.

3.4. EFE Flavonoids Downregulate ER α and EFE Lignans and Flavonoids Upregulate the PR in a Dose-Response Pattern Predominantly in Lower Concentrations in MCF7 Cells

MCF7 cells that were exposed to EFE flavonoids with the concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL responded significantly with a downregulation of ER α at the concentrations of 10 µg/mL (3.5 ± 0.55) and 50 µg/mL (6.3 ± 0.88) compared to the control (11.33 ± 0.73, p = 0.002 and 0.004), (Figure 4A).

MCF7 cells that were exposed to EFE lignan and flavonoid extracts with the concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL responded significantly in an upregulation of the PR in a dose-response pattern (Figure 4B). The upregulation of the progesterone IRS score significantly reached a peak at the EFE lignan concentration of 10 µg/mL (8 ± 0.98) compared to the control (3.3 ± 0.36, p = 0.002). As the EFE concentration increased, the IRS score decreased at 50 µg/mL to 7.66 ± 1.04, and at 100 µg/mL to 5.83 (Figure 4B).

The same phenomenon was observed using EFE flavonoids where the IRS score increased from 2.66 ± 0.46 in the control to 6 ± 0 at 10 µg/mL (p = 0.002), and then decreased to 4.83 ± 0.59 (p = 0.026) at 50 µg/mL, and to 2.83 ± 0.44 at 100 µg/mL (Figure 3B).

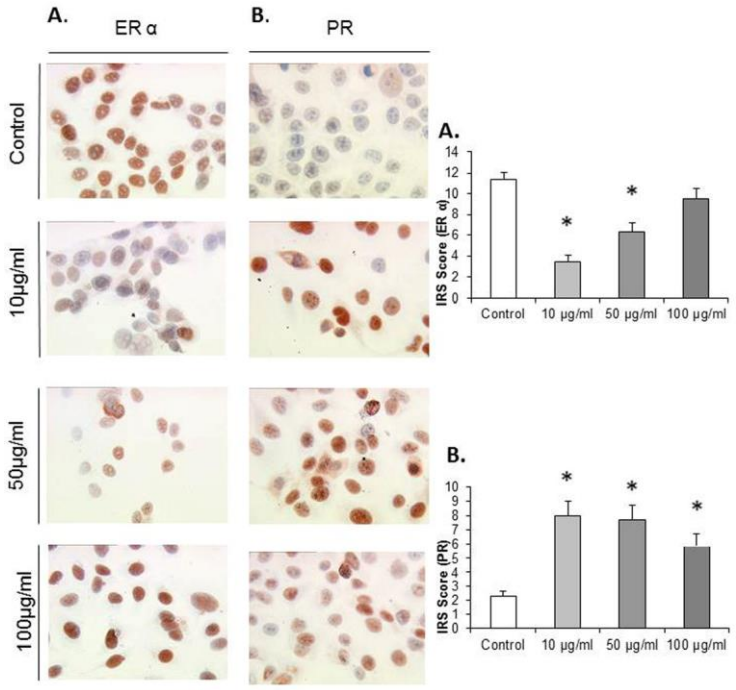

Figure 4. Representative microphotographs of MCF7 cells grown in the absence or presence of elder flower extract (at effective EFE concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL), after immuno-detection of ER-α (**A**) and PR (**B**); and presentation of the immunocytochemistry results by the semi-quantitative immunoreactivity score (IRS). Significantly different observations are highlighted with an asterisk.

3.5. Enterolactone Downregulates Expression of ERα and PR in a Dose-Response Pattern in MCF7 Cells

MCF7 cells that were exposed to enterolactone at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL responded significantly with a downregulation of ER α at concentrations of 50 µg/mL (IRS score 2.5) and 100 µg/mL (IRS score 0) compared to the control (IRS score 5, p = 0.027 and 0.024) (see Figure 5). MCF7 cells that were exposed to enterolactone at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL responded with a dose-response-related downregulation of the PR. The downregulation of the PR was significant at enterolactone concentrations of 50 µg/mL (IRS score 4) and 100 µg/mL downregulation (IRS score 2) compared to the control (IRS score 9, p = 0.028 for both concentrations).

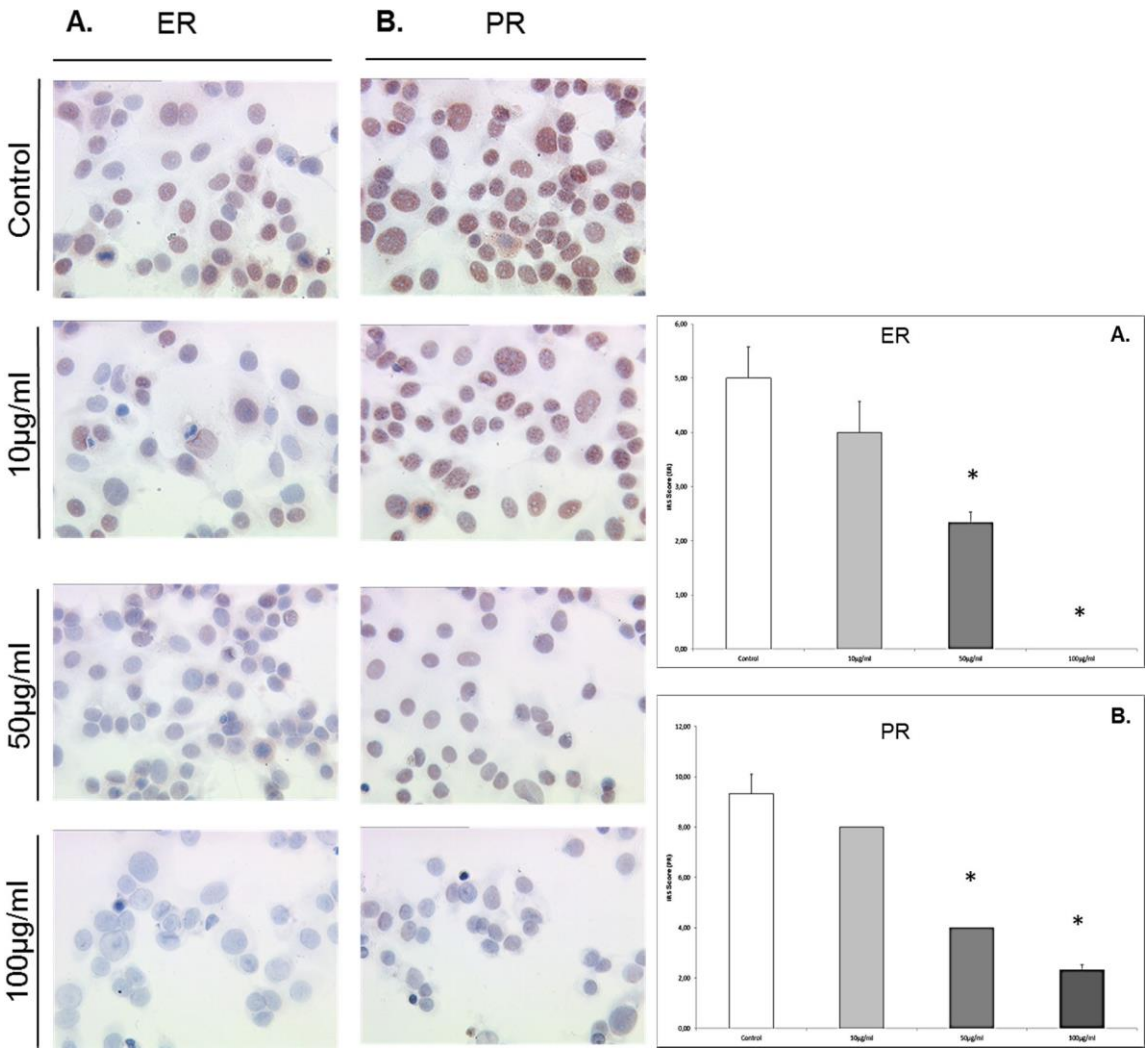

Figure 5. Representative microphotographs of MCF7 cells grown in the absence or presence of enterolactone at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL), after immuno-detection of ER-α (**A**) and PR (**B**); and presentation of the immunocytochemistry results by the semi-quantitative immunoreactivity score (IRS). Significantly different observations are highlighted with an asterisk.

3.6. Enterodiol Downregulates Expression of ERα and PR Only at High Concentrations in MCF7 Cells

MCF7 cells that were exposed to enterodiol at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL responded with a significant downregulation of ERα only at 100 µg/mL (IRS score 0) compared to the control (IRS score 5, $p = 0.023$) (Figure 6). MCF7 cells that were exposed to enterodiol at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL responded with a significant downregulation of the PR at 100 µg/mL (IRS score 0) compared to the control (IRS score 5.5, $p = 0.023$).

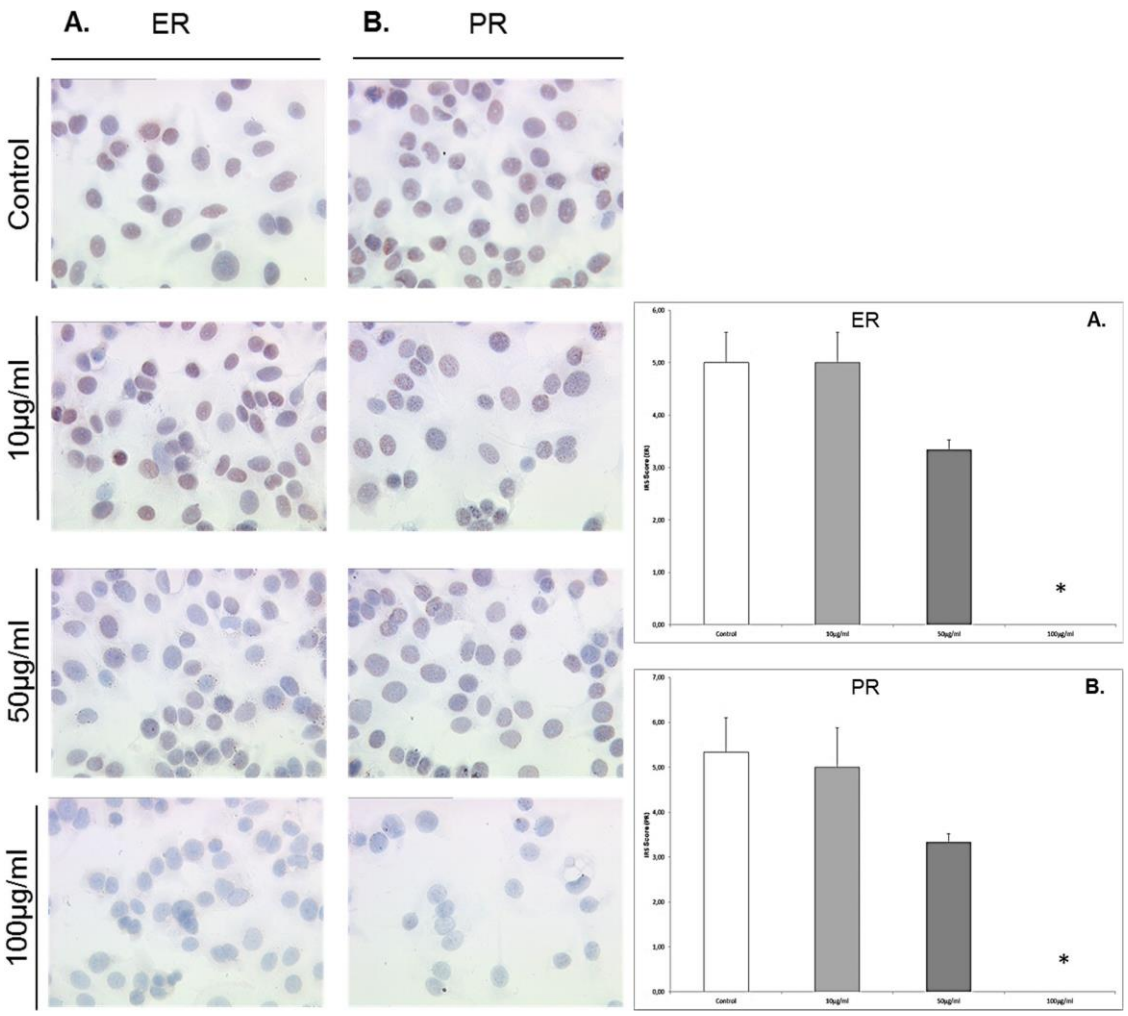

Figure 6. Representative microphotographs of MCF7 cells grown in the absence or presence of enterodiol at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL), after immuno-detection of ER-α (**A**) and PR (**B**) ; and presentation of the immunocytochemistry results by the semi-quantitative immunoreactivity score (IRS). Significantly observations are highlighted with an asterisk.

3.7. Enterolactone Inhibits Estradiol Secretion in JEG-3 Cells and Induce Estradiol Secretion in BeWo and MCF7 Cells in a Dose-Response Pattern

In JEG-3 cells, the estradiol production was inhibited from 211.8 ± 8.88 pg/mL in the control to 190.9 ± 7.9 pg/mL, and 149.59 ± 7 pg/mL at enterolactone concentrations of 10 µg/mL and 50 µg/mL, $p = 0.028$, respectively (Figure 7).

The cultivation of the BeWo cell line with enterolactone resulted again in an upregulation of estradiol secretion from 75.07 ± 2.33 pg/mL in the control to 94.66 ± 6.39 pg/mL, 137.66 ± 10.04 pg/mL, and 173.53 ±9.56 pg/mL when the enterolactone concentration was 10 µg/mL, 50 µg/mL, and 100 µg/mL. The differences between the stimulated cells and the control were significant at all concentration levels of enterolactone, $p = 0.028$, respectively.

In MCF7 cells the concentrations of 10 µg/mL, 50 µg/mL and 100 µg/mL provoked an increased secretion of estradiol from 52.65 ± 7.90 pg/mL in the control to 75.22 ± 2.11 pg/mL at 10 µg/mL, 123.93 ± 3.93 pg/mL at 50 µg/mL, and 172.12 ± 10.05 pg/mL at 100 µg/mL, $p = 0.028$, respectively.

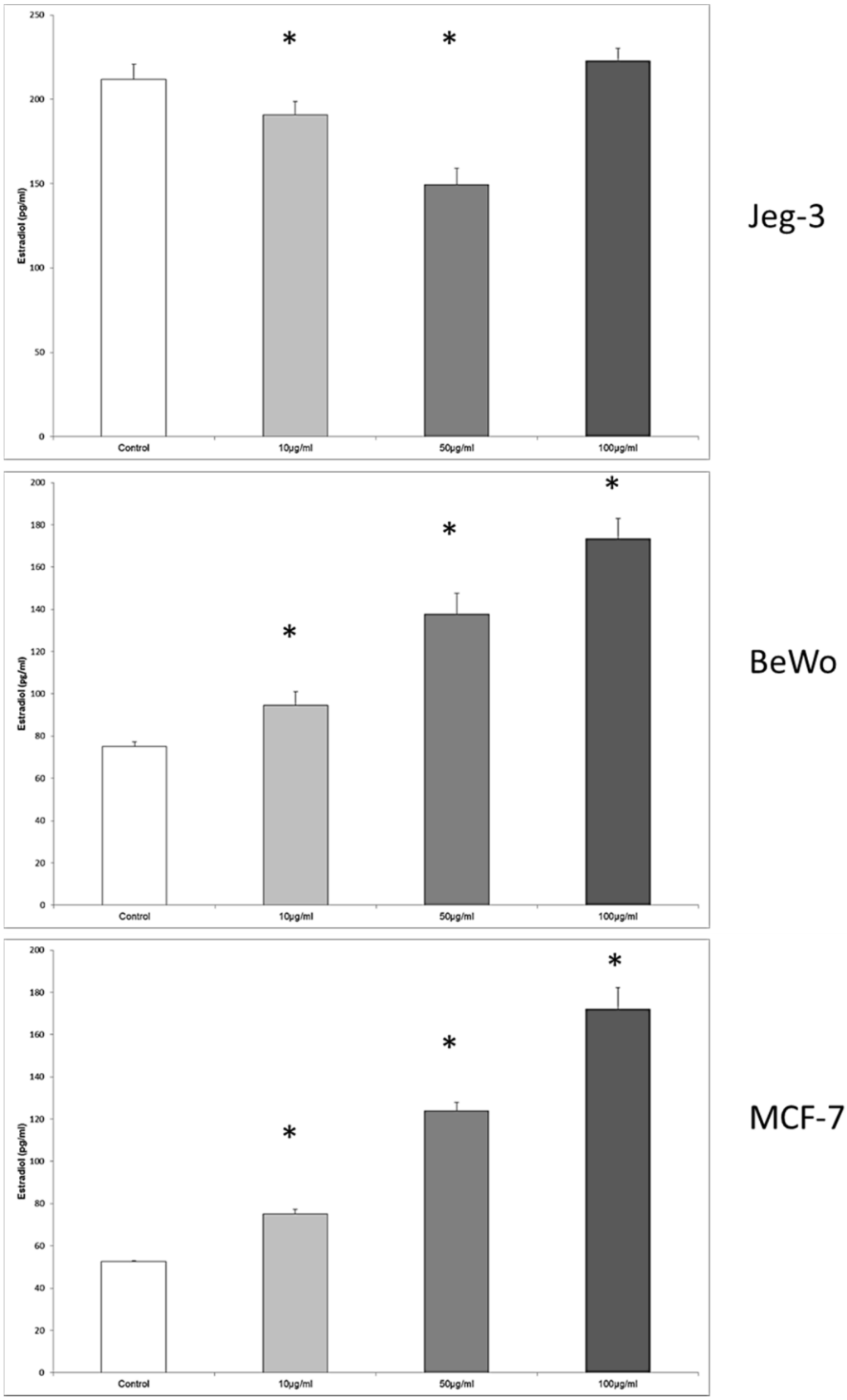

Figure 7. Estradiol concentration in the tissue culture medium of JEG-3, BeWo and MCF7 cells in the absence or presence of enterolactone. The effective enterolactone concentrations were 10 µg/mL, 50 µg/mL, and 100 µg/mL. Significantly different observation are highlighted with an asterisk.

3.8. Enterodiol Induces Estradiol Secretion in JEG-3, BeWo, and MCF7 Cells at Distinct Concentrations

In JEG-3 cells, the estradiol secretion was significantly enhanced from 79.85 ± 1.14 pg/mL in the control to 86.37 ± 1.07 pg/mL, when the concentration was 50 µg/mL enterodiol, p = 0.028 (Figure 8). The cultivation of the BeWo cell line with enterodiol resulted again in a significant upregulation of estradiol secretion from 63.71 ± 0.68 pg/mL in the control to 72.71 ± 0.79 pg/mL, and 84.37 ± 4.63 pg/mL at the enterodiol concentrations of 50 µg/mL and 100 µg/mL, respectively. The differences between the stimulated cells and the control were significant at both concentration of enterodiol, p = 0.028, respectively.

In MCF7 cells the concentrations of 50 µg/mL and 100 µg/mL provoked an increased secretion of estradiol from 35.64 ± 1.32 pg/mL in the control to 53.28 ± 0.39 pg/mL at 50 µg/mL, and 56.94 ± 2.54 pg/mL at 100 µg/mL, p = 0.028, respectively.

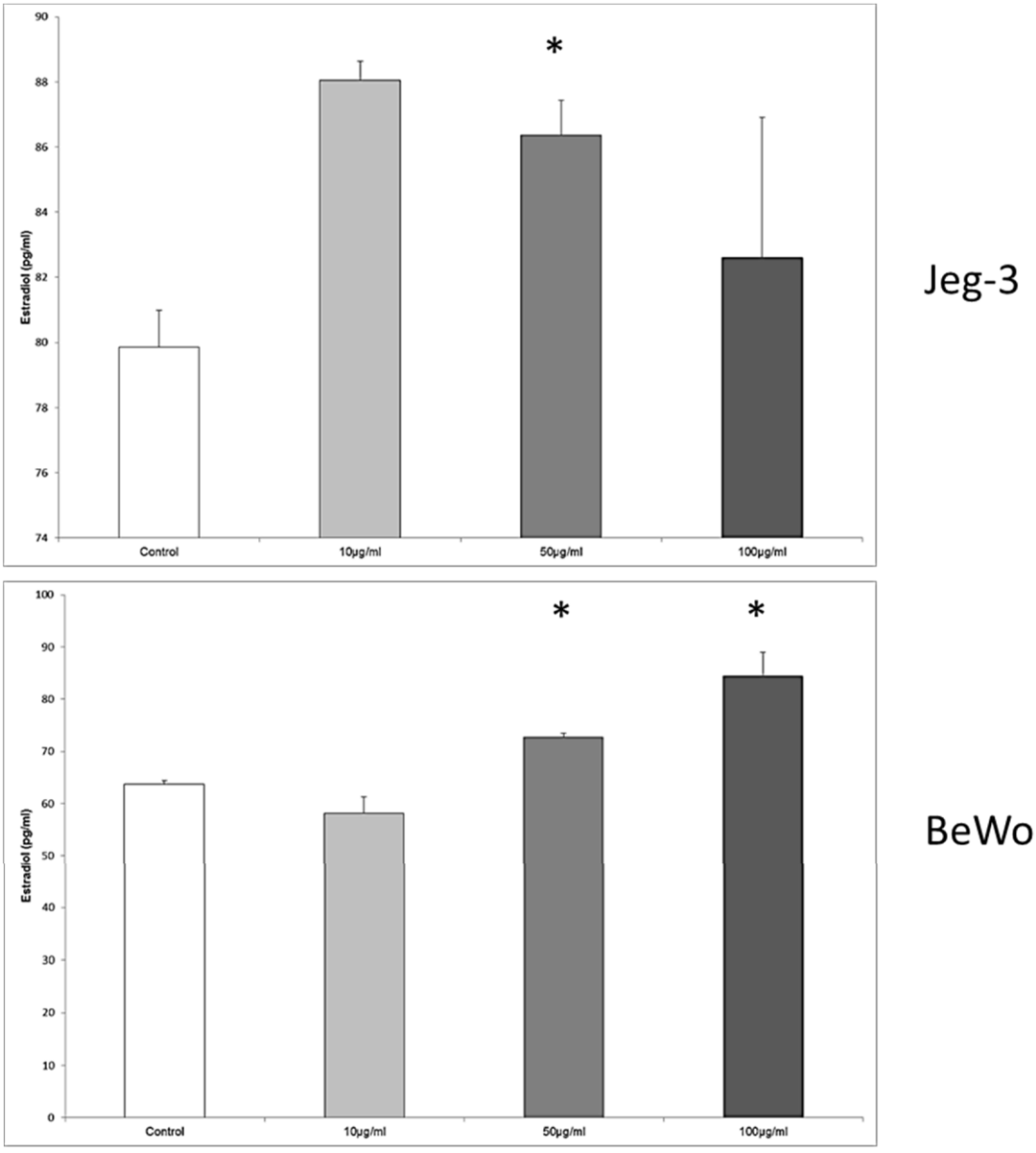

Figure 8. *Cont.*

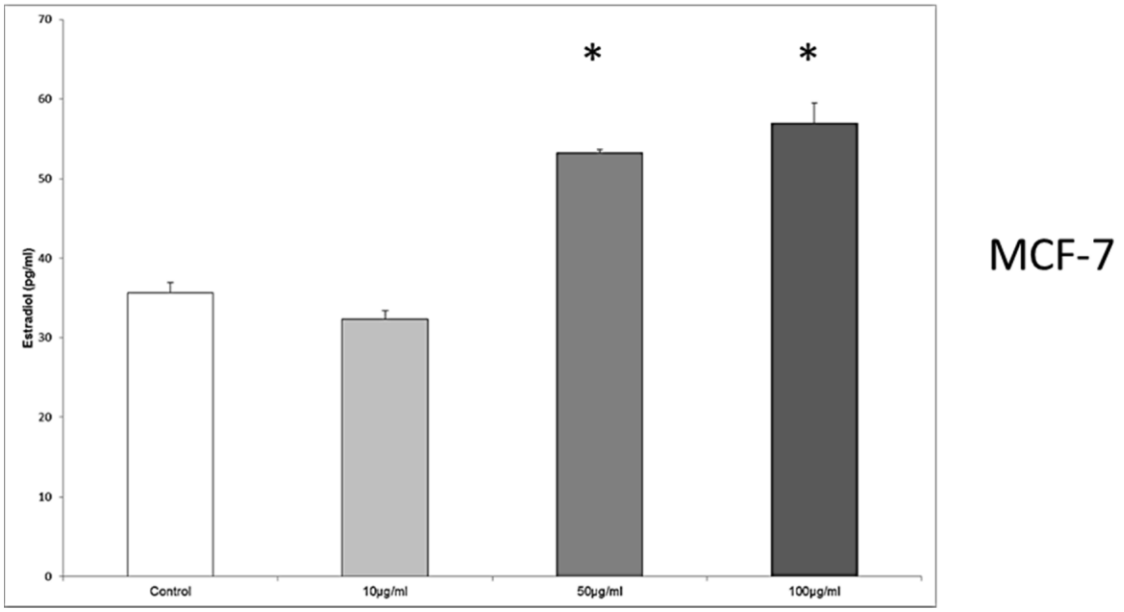

Figure 8. Estradiol concentration in the tissue culture medium of JEG-3, BeWo and MCF7 cells in the absence or presence of enterodiol. The effective enterodiol concentrations were 50 µg/mL and 100 µg/mL. Significantly different observation are highlighted with an asterisk.

4. Discussion

To our knowledge, this is the first study evaluating the phytoestrogen properties of EFE on BeWo, JEG-3, and MCF7 cells regarding the estrogen and progesterone response. Prior to this study it was uncertain if EFE contains phytoestrogen compounds. Although mass spectrometry proved that EFE contains lignans and isoflavones, the subgroups of each class were not identified and, thus, precision is lacking. EFE proved to be richer in lignans than in isoflavones (presented in Figure 1). This may explain why more significant results were found using the lignan EFE. However, further studies with isolated fractions of the subgroups of EFE lignans and isoflavones could clarify if one subgroup is more potent than the other. Therefore, it would be interesting to isolate and identify the different lignans and isoflavones in the EFE that cause phytoestrogen activity for further characterization. Before further evaluation in an animal model, in vitro evaluation of the various components' effects as single substances is required.

In a previous study of our group, the phytoestrogen properties of pumpkin seed extract were tested on the same cells, which resulted in an unexpected estrogen secretion in all cell lines [18]. As hormone-dependent tumors react with proliferation when exposed to estrogens, pumpkin seeds, thus, could provoke carcinogenic effects.

In contrast, EFE was the first of the potential phytoestrogens previously tested by our group, which had an inhibitory effect on the estradiol secretion of all three cell lines.

The effect on JEG-3 and BeWo cells was observed to be dose-dependent. Interestingly, in MCF7 cells, estrogen secretion was higher following the administration of intermediate phytoestrogen concentrations than in controls or with the highest EFE concentration tested. The degree to which the inhibition of estrogen secretion results in a decreased cell proliferation has to be tested in further investigations using EFE. In addition, it is possible that at the highest EFE concentration estrogen secretion was decreased due to cytotoxic effects of the extract itself, as other studies suggest that phytoestrogens cause cytotoxicity and decrease growth in MCF7 tumors. For example, in a study by Bergman et al. [31] ovariectomized mice were treated with continuous release of estrogen. MCF7 tumors were established and mice were fed with basal diet or 10% flaxseed, and two groups that were fed basal diet received daily injections with enterodiol or enterolactone (15 mg/kg body weight).

The regimens containing flax seeds or enterodiol or enterolactone injections resulted in decreased estrogen-induced growth and angiogenesis in solid tumors by decreasing the secretion of VEGF.

It is of interest that EFE induces not only an inhibition of estradiol secretion, but also an upregulation of the ERα in JEG-3 cells. It could be assumed that, if EFE causes an inhibition on the trophoblast estrogen secretion, the cells react by increasing ERα expression in order to obtain stimulation even in a low-estrogen environment. A recent study by Lim et al. [32] outlined that the flavonoid apigenin reduces survival of JEG-3 cells by inducing apoptosis via the PI3K/AKT and ERK1/2 MAPK pathways. Therefore, it seems likely that the phytoestrogens also found in EFE could trigger non-genomic estradiol receptor signal transduction causing apoptosis in JEG-3 cells. In contrast to the effects of EFE on JEG-3 cells, another study by our group [33] demonstrated that the two well-known phytoestrogens genistein and daidzein provoked a reduced progesterone production and a stimulation of the estrogen production in JEG-3 cells. Therefore, regarding the other extracts investigated by our group, the characteristics of EFE seem to be favorable for further research due to the properties of decreased estrogen secretion and increased ERα expression in JEG-3 cells.

MCF7 cells that were exposed to EFE extracts responded with a significant downregulation of ERα and an upregulation of PR, both predominantly in lower concentrations of EFE. Why the lower concentrations provoked a stronger effect on receptor expression remains unknown. Although it is, again, possible that higher concentrations of EFE resulted in cytotoxic effects leading to cell damage and, therefore, to decreased cellular function. Nevertheless, the fact that lower EFE concentrations resulted in a decreased expression of the ERα receptor and an increase in the progesterone receptor could be beneficial for clinical use as low blood concentrations of phytoestrogens are easier to achieve by dietary intake alone. It is important to mention that the concentrations used in this study were extremely high (non-physiological). The highest level of enterolactone that has been measured in serum/plasma in humans is 2 µmol/L (over 16 times less than the enterolactone concentration used). Furthermore, estradiol levels in adult females reach levels only as high as 300 pg/mL in the luteal phase (30,000 times less than the external control). Therefore, before realistic interpretation, our findings must be reevaluated in further studies using more physiologically relevant doses.

Our current findings partially concur with a previously-described downregulation of ERα and upregulation of PR on the MCF7 cells when treated with other potential phytoestrogen compounds such as flax and pumpkin isoflavone and lignan extracts or mixtures [19,34]. Interestingly, it has been demonstrated that estradiol has similar effects on the MCF7 ERα and on PR, as it causes a downregulation of ERα and an upregulation of PR [35,36]. Therefore, whether EFE causes MCF7 cell proliferation or inhibition has to be tested in future investigations. In a study by Stendahl et al., it was demonstrated that high progesterone receptor expression correlates with a better effect of adjuvant tamoxifen in premenopausal breast cancer patients [37]. This suggests clinical trial evaluation of elderflower as a combination partner for tamoxifen.

It is unclear whether the lignans present in the EFE require any metabolic processing prior to exerting biological effects and whether the cell culture systems used are capable of completing this conversion. For example secoisolariciresinol diglycoside (SDG) is the primary lignan in flaxseed; however, in vitro studies use bioavailabile enterodiol and enterolactone when investigating effects of flaxseed lignans. This is because in vitro systems do not have the components necessary to convert SDG to enterodiol and enterolactone. Therefore, additional in vivo studies could provide valuable information regarding EFE metabolism prior to the conduction of further in vitro studies. Nevertheless, the pattern of hormone secretion and receptor expression of enerolactone and enterodiol tested on JEG-3, BeWo, and MCF7 cells were different to those of EFE. Therefore, it is probable that the lignans in EFE are not related to the enterolignans. Enterolactone and enterodiol in contrast to EFE inhibited not only the expression of the ER but also PR in MCF7 cells. Moreover contrary to EFE, both control substances upregulated estradiol production in BeWo and MCF7 cells in a concentration-dependent manner.

5. Conclusions

Our results clearly demonstrate beneficial features of EFE in the setting of hormone receptor-positive breast cancer MCF7 cells by inhibition of estrogen secretion, downregulation of Erα, and upregulation of PR. Decreased local and circulating estrogen concentrations are certainly considered an advantage in treating breast cancer. In that view, EFE could be related to reduced tumor cell proliferation, possibly suggesting a protective effect on breast cancer. Nevertheless, the results and the conclusions made must be interpreted with caution as this is an in vitro cell culture study. In this setting, the use of plant extracts instead of chemically pure agents may be advantageous as it may more accurately reflect the effects of phytoestrogen-rich diets.

If the effects of EFE can be attributed solely to potential phytoestrogen activity remains unsolved. To which degree other non-estrogenic pathways play a role can currently not be clarified. For example, mass spectrometry demonstrated a high amount of lipids in EFE. Lipids can inhibit cell proliferation through activation of PPARα and PPARγ (peroxisome proliferator-activated receptors) which bind as transcription factors to the retinoid X receptors and, thus, regulate the expression of various genes [38,39]. In MCF7 breast cancer cells PPARγ activates p53 by stimulating the transcription factor NFkB (nuclear factor kappa-light-chain-enhancer of activated B-cells), which is a gene promoter of p53 and, thus, induces apoptosis [40]. Therefore, the following additional investigations are necessary to obtain further insight of the promising anti-carcinogenic effects of EFE: the results of hormone secretion and receptor expression of EFE should be correlated with DNA synthesis performance (BRDU proliferation assay), metabolic activity (MTT assay), and cytotoxicity (LDH assay) tests. Cytotoxicity could be evaluated in detail by immunohistochemistry or reverse transcriptase quantitative (RTQ)-PCR quantification of apoptosis-induced markers (for example, p53, p21, BCL2, Caspase 8/9). Then, as a possibility to determine the role of hormone receptor-mediated cell response, EFE could be tested on malignant ER-negative cells (e.g., BT-20). Furthermore, fractional chromatography could provide information of the individual substances and their impact on breast cancer cells. Finally, after further in vitro investigations, properly designed animal studies could highlight a potential role of EFE in trophoblast and breast cancer prevention and/or treatment.

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3. SUMMARY

Laboratory and clinical research on phytoestrogens suggests related dietary health benefits and relevant pharmaceutical implications for the treatment of breast cancer. Phytoestrogens are a group of polyphenols produced by plants that functionally and structurally resemble 17β-estradiol and can therefore interact as agonists or antagonists with the estrogen receptor and estrogen receptor independent pathways[1617]. Traditional medicinal plants like elder flower (sambuccus nigra) and parsley root (Petroselinum crispum) are known since centuries for their health benefits. Therefore, we chose them as plants for our investigations on breast cancer cells.

In both publications extracts of the plants were prepared in different concentrations and analyzed by mass spectrometry. In the parsley root publication, breast cancer MCF7 and MCF12A cells were exposed with different PCE concentrations and incubated. After incubation, the effect of the different concentrations of PCE on MCF7 and MCF12A cells was analyzed using various tests. For cytotoxicity, metabolic activity and DNA synthesis performance LDH, MTT and BrdU proliferation assays were used respectively.

In the elder flower publication, trophoblast tumor cell lines BeWo and JEG-3, as well as MCF7 breast cancer cells were exposed and then incubated at different EFE concentrations. Cells remaining untreated served as test controls. In supernatant cells estradiol production was tested with an ELISA method. Changes caused by EFE in ER/PR expression were analyzed by immunocytochemistry.

It was demonstrated that the plants investigated contain a substantial amount of phytoestrogens. Moreover, both plants demonstrated anti-cancerogenic properties on breast cancer cell lines by means of receptor expression patterns and the inhibition of cell proliferation. If the effects observed are only caused by phytoestrogen action remains unclear. Further investigations on different breast cancer cells and with the isolated phytoestrogen substances is needed before proper clinical investigations can be planned.

4. ZUSSAMENFASSUNG

Eine wachsende Anzahl von Daten weist auf die gesundheitlichen Vorteile von Phytoöstrogenen in der Ernährung und auf mögliche pharmazeutische Anwendungen hin. Phytoöstrogene sind pflanzliche Polyphenole, die strukturell und funktionell 17β-Östradiol ähneln und daher als Agonisten oder Antagonisten mit Östrogenrezeptor-abhängigen und östrogenrezeptor-unabhängigen Zellkaskaden interagieren können. Traditionelle Heilpflanzen wie Holunderblüten (Sambuccus nigra) und Petersilienwurzeln (Petroselinum crispum) sind seit Jahrhunderten für ihre gesundheitlichen Vorteile bekannt. Deshalb haben wir sie als Pflanzen für unsere Untersuchungen an Brustkrebszellen ausgewählt.

In beiden Publikationen wurden Extrakte der Pflanzen in unterschiedlichen Konzentrationen hergestellt und massenspektrometrisch analysiert. In der Petersilienwurzelveröffentlichung wurden MCF7- und MCF12A-Zellen mit verschiedenen Konzentrationen von PCE inkubiert und auf DNA-Syntheseleistung, metabolische Aktivität und Zytotoxizität durch BrdU-Proliferations-, MTT- bzw. LDH-Assays analysiert. In der Holunderblütenveröffentlichung wurden die Zellen mit verschiedenen EFE-Konzentrationen inkubiert. Als Kontrolle dienten unbehandelte Zellen. Die Überstände wurden mit einem ELISA-Verfahren auf Östradiolproduktion getestet. Darüber hinaus wurde die Wirkung des EFE auf die ER / PR-Expression durch Immunzytochemie bewertet.

In beiden Publikationen konnte gezeigt werden, dass die untersuchten Pflanzen eine erhebliche Menge an Phytoöstrogenen enthalten. Darüber hinaus zeigten beide Pflanzen durch das Rezeptorexpressionsmuster und die Hemmung der Zellproliferation anti-kanzerogene Eigenschaften in Brustkrebszelllinien. Ob dieser Effekt ausschließlich auf Phytoöstrogene zurückzuführen ist, ist unklar. Weitere Grundlagenforschung mit verschiedenen Brustkrebszellen und isolierten Phytoöstrogen sind erforderlich, bevor geeignete klinische Untersuchungen geplant werden können.

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6. EIDESSTATTLICHE VERSICHERUNG

Eidesstattliche Versicherung

Henrik Lennard Wilhelm Schröder
Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende kumulative Dissertation mit dem Thema

Influence of the phytoestrogens of Sambuccus nigra and Petroselinum crispum on the proliferation and receptor expression in breast cancer cell lines

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8. Abbreviations

Akt	serine/threonine kinase
BAX protein	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2 protein
BRCA	Breast cancer type susceptibility protein
CDK4/6 inhibitors	cyclin-dependent kinases inhibitors
DNA	deoxyribonucleic acid
EFE	elderflower extract
ERE	estrogen responsive elements
ER	estrogen receptor
Her2	human epidermal growth factor receptor 2
IGF-1R	insulin-like growth factor 1 receptor
MCF-7 cells	Michigan Cancer Foundation – 7, ER+ breast cancer cell line
MDA-MB-453 cells	androgen receptor positive, ER/PR/Her2 negative cell line
mir-RNA	small non-coding ribonucleic acid molecule
NF-kappaB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
O-DMA	*O*-desmethylangolensin
PARP-1	poly(ADP-ribose) polymerase 1
PI3K	phosphatidylinositol 3-kinase
p38 MAPK	P38 mitogen-activated protein kinases
PRE	parsley root extract
PR	progesterone receptor
ROS	reactive oxygen species
SERM	selective estrogen receptor modulator