

Actin cytoskeleton reconstitution in MCF-7 breast cancer cells initiated by a native flax root extract

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ABSTRACT

The intention of this systematic cell biological study was to analyze the effects of the phytoestrogen-rich, ethanolic flax root extract (*Linum usitatissimum* L.) on human MCF-7 estrogen receptor positive breast cancer cells in order to identify the main anti-tumor action by focusing on adhesion and migration related features. Cell impedance, initial adhesion capacity, cell migration ability, and actin cytoskeleton formation was determined by live cell monitoring, flow cytometry, scratch assay and confocal microscopy. Detailed expression analyses of adhesion- and actin-related proteins were performed by flow cytometry and western blotting, respectively. The effect on anchorage-independent growth of MCF-7 cells was analyzed by colony formation on soft agar. 50 µg/ml flax root extract reduced cell impedance (50%), initial adhesion capacity (18%), migration ability (72%) and colony formation (83%) in MCF-7 cells significantly. Increased stress fiber formation (9-fold higher filament number and a 12-fold elevation of the total filament length) was initiated by overexpression of profilin-1 and down regulation of arp-2, two important regulators of actin dynamics. In conclusion, the flax root extract exhibits anti-tumor potential for estrogen receptor positive breast cancer cells mainly by remodeling of the actin cytoskeleton, leading to significant reduction of migration and colony formation *in vitro*.

Keywords: Breast cancer, flax, lignans, actin cytoskeleton, profilin, migration.

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DMEM, modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ESI, electron spray ionization; FAK, focal adhesion kinase; FCS, fetal calf serum; HA, hyaluronic acid receptor; HPLC, high-performance liquid chromatography; HPR, horse radish peroxidase; IDES, Interdigitated electrodes sensor; LC-MS, liquid chromatography-mass spectrometry; LTQ, linear trap quadrupole; PDA, photodiode array; PFA, paraformaldehyde; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, scanning electron microscopy; TBS, tris-buffered saline; TCPS, tissue-culture polystyrene; ZO-1, zona occludens.

INTRODUCTION

Natural products from a variety of herbal, animal, and microbial sources contain a huge potential of pharmacologically active substances that might play a role in drug development. Interest in natural products for drug development has waxed and waned, but there is no denying their importance in oncology. Today, over half of the drugs approved to treat cancer originate from natural products or natural product prototypes. Single

compounds, such as paclitaxel, isolated from the bark of the Pacific yew tree, *Taxus brevifolia*, are standardized in chemotherapy. However, these substances often cause serious side effects, and healing from cancer is not guaranteed. Therefore, the present trend is towards cancer prevention. In this context, phytoestrogens play an essential role, as in epidemiological studies it was shown that especially women who consume soy products

since childhood, have a significantly reduced risk of developing breast cancer (Shu et al., 2001; Wu et al., 2008; Nagata, 2010). The active phytoestrogens found in soy are primarily isoflavones e.g. genistein and daidzein.

Phytoestrogens, plant-derived phytochemicals, are thought to prevent breast cancer by modulating estrogen receptor dependent mechanisms, reacting similar to the endogenous hormone 17 β -estradiol because of structural similarities (Makiewicz, 1993). Some phytoestrogens, e.g. genistein belonging to the category of isoflavones, exhibit biphasic effects in the estrogen-receptor positive breast cancer cell line MCF-7: estrogenic effects are achieved at low concentrations (<10 μ M), whereas higher concentrations (>10 μ M) show antiestrogenic activity (Hilakivi-Clarke et al., 2002; Allred et al., 2001; Zava and Duwe, 1997). The estrogenic stimulation can result in the up-regulation of proliferation and migration, while high concentrations of genistein are able to induce growth arrest and apoptosis in MCF-7 cells, most likely by inhibiting the intrinsic tyrosine kinase activities of growth factor receptors (Pagliacci et al., 1994).

This biphasic behavior can be avoided by using phytoestrogen-rich plant extracts, for example the native flax root extract (Engel et al., 2012; Abarzua et al., 2007). The native flax root extract includes various phytoestrogens like genistein, daidzein, secoisolariciresinol and matairesinol beside various other not yet analyzed compounds. This mixture of secondary plant metabolites does not produce biphasic effects in MCF-7 cells, possibly due to its synergistic mode of action (Engel et al., 2012). Regional herbal products are of particular interest, if they are already used in the agricultural science field or industry. Flax (*Linum usitatissimum*), a member of the genus *Linum*, is a food and fibre crop that is grown in humid continental, arid and dry arid climates zones, predominantly cultivated in Canada, China and Russia. Its medicinal use is established in the German speaking area for treatment of various disorders since centuries (Vogl et al., 2013). Recently flax has raised the focus on its potential for the treatment of breast and prostate cancer (Thompson et al., 2005; Azrad et al., 2013). This might be due to its high levels of lignans, a major class of secondary plant compounds belonging to the category of phytoestrogens. Especially secoisolariciresinol but also lariciresinol, matairesinol, and pinoresinol are known to reduce breast tumor growth in athypic mice (Adolphe et al., 2010).

In this study a native flax root extract was used, which includes both a variety of lignans and isoflavones (Abarzua et al., 2007). Its availability is high, as flax is an intensively used agricultural crop. In *in vitro* studies no biphasic effect could be identified on estrogen receptor positive breast cancer cells MCF-7, the extract is effective at rather low concentrations (10 to 50 μ g/ml). Also, until no negative effects on non-tumorigenic breast cells (MCF-10A, MCF-12A) could not be demonstrated *in vitro* (Engel et al., 2012). Therefore, we turned our attention to the investigation of adhesion-related

processes, which are associated with the tumorigenicity of the cell. These include the potential for migration, the expression of adhesion receptors and the organization of the actin cytoskeleton, which is substantially responsible for the motility of the cancer cell (Kallergi et al., 2003). Because increased levels of soluble actin and decreased levels of polymerized actin are correlated with tumorigenesis (Jordan and Wilson, 1998). The present study provides a systematic cell biological analysis of the effects of a phytoestrogen-rich plant extract isolated from the root of *Linum usitatissimum* on human MCF-7 breast cancer cells intending to identify its main anti-tumor actions.

MATERIALS AND METHODS

Chemicals

Methanol (HPLC gradient grade, J.T. Baker, Center Valley, PA, USA) and absolute ethanol (MERCK, Darmstadt, Germany) with the purity ACS, ISO, Reag. PhEur were used as extraction solvent. The LC-MS (liquid chromatography combined with mass spectrometry) Chromasolv[®] grade solvents, methanol with 0.1% formic acid and water with 0.1% formic acid were obtained by FLUKA (St. Louis, USA). The reference compounds of genistein, glycitein, biochanin and secoisolariciresinol were purchased from Sigma-Aldrich (St. Louis, USA) with the highest-possible purity. The standards of fisetin and daidzein were intent on FLUKA (St. Louis, USA) with the best-possible purity. All of the standard samples were dissolved to a concentration of 1 mg/ml in absolute ethanol. Phytoestrogens were identified by comparing the mass spectra and retention times with those of the reference compounds, or with published mass spectra (Ford et al., 2001; Bambagiotti-Alberti et al., 1994).

Plant material and extract preparation

Linum usitatissimum seeds were obtained from the Agriculture Research Institution (LUFA, Rostock, Mecklenburg-Vorpommern, Germany), seeded in April and were grown in pots filled with soil under field conditions. Initial, the plants were watered with 0.1% Wuxal liquid fertilizer (aglucone Fertilizers GmbH). The plants were not protected from the natural weathering or attack by pests. When plants reached a height of 1 m (~100 d), roots were harvested, cleaned manually and stored at -80°C in plastic bags until extraction. Preparation of the flax root extract was performed according to Luyengi et al. (1996) and described previously (Abarzua et al., 2007; Abarzua et al., 2010). Extract powder was dissolved in ethanol (HPLC, gradient grade, \geq 99.8%, Sigma, Germany) to give a stock solution of 100 mg/ml. The extract was aliquoted (100 μ l portions) and stored at -80°C.

LC-MS analysis

The samples and standard solutions were identified on a Thermo Scientific HPLC-LTQ system (Thermo Scientific, Dreieich, Germany) comprising of a Surveyor Plus[™] HPLC system equipped with a three simultaneous channel PDA detector and a linear trap quadrupole mass spectrometer (LTQ) fitted with an electron spray ionization source. Data were evaluated and interpreted by Xcalibur software (Thermo Scientific, Dreieich, Germany) and a special interpretation HighChem[®] Mass Frontier[™] Software (Thermo Scientific, Dreieich, Germany). The separation was performed on a Kinetex 2.6u C18 100A (150 \times 4.6 mm, Phenomenex, Germany). The column temperature was kept at

35°C and the mobile phases consisted of solvent A (methanol with 0.1% formic acid, LC.MS Chromasolv®, Fluka, Germany) and B (water with 0.1% formic acid, LC.MS Chromasolv®, Fluka, Germany). Elution of the extracts was performed by the following solvent gradient: 40% A to 95% A (15 min), 95% A isocratic (10 min), 95% A to 80% A (10 min), 80% A to 40% A (5 min) and 40% A isocratic (20 min). The flow rate was 0.15 ml/min and the injection volume amount 2 µl. MS spectra were recorded in both positive and negative modes and in a range of m/z 90.00 to 2000.00. The compounds were identified by ion trap technology, and the mass spectrometric detection was realized with electron spray ionization (ESI).

Cell culture and treatment conditions

The human epithelial estrogen-sensitive breast cancer cell line MCF-7 was purchased from ATCC (www.atcc.org) and cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Germany) with 10% fetal bovine serum (FCS; PAN Biotech GmbH, Germany) and 1% gentamicin (Ratiopharm GmbH, Ulm, Germany) at 37°C and in a humidified atmosphere with 5% CO₂. Prior to treatment with the flax root extract (L; *Linum usitatissimum*; final concentrations: 0.1, 1, 10, 25 and 50 µg/ml) or 17β-estradiol (E2; final concentration: 1 nM; purchased from Sigma, Germany), cells were adapted to phenol-red-free DMEM (PAA Laboratories GmbH, Germany) with 10% charcoal stripped FCS (PAN Biotech GmbH, Germany) for 48 h to avoid unspecific stimulation of endogenous hormones in the serum (assay medium). Then, treatment with substances was carried out in the assay medium for 48 h. For negative controls (C) the respective vehicle (EtOH; final concentration: 0.1%) was used in the same manner.

SEM visualization of cell morphology

Cells were fixed with 2.5% glutaraldehyde (24 h, 4°C), dehydrated through a graded series of alcohol (30% 5 min, 50% 5 min, 75% 10 min, 90% 15 min, 100% twice for 10 min) and dried in a critical point dryer (K 850, EMITECH, Taunusstein, Germany). Subsequently probes were sputtered with a thin goldlayer. The cell morphology was examined with the scanning electron microscope (SEM) Zeiss DSM 960A (Carl Zeiss, Jena, Germany).

Initial adhesion measurement

Cells treated for 48 h were trypsinized with 0.05% trypsin/0.02% EDTA for 5 min, washed once with phosphate buffered saline (PBS) and resuspended in assay medium. Cell number and vitality were measured with the CASY cell counter DT (Schärfe System, Reutlingen, Germany). A definite number of 50,000 vital cells were seeded in 6-well plates. After 2 h incubation, the number of non-adherent cells in the assay medium was measured by flow cytometry (BD FACSCalibur, NJ, US). Each independent measurement was repeated at least for six times. Adherent cells were back-calculated from the non-adherent cells in the medium.

Wound healing assay

Wound healing assay was conducted on MCF-7 cells, pre-incubated in assay medium for 48 h adaption in 6-well plates (Greiner, Germany). A scratch wound was made by Ibidi culture inserts (µ-Dish 35 mm; Ibidi GmbH, Martinsried, Germany) following the instructors recommendations. When cell layers reached confluency, the culture insert was removed and cells were treated with the flax root extracts or control (vehicle). Photographs of the

gap were taken at different time points (0, 12, 24, 48, 62 h) by a bright field microscope (Axiovert 40, Carl Zeiss, Jena, Germany) equipped with the camera lcc1 (Carl Zeiss, Jena, Germany).

Expression of adhesion receptors

Cells grown in T25 culture flasks up to a confluency of 70% were either treated for 48 h with the vehicle (0.1% EtOH) or with different concentrations of the flax root extract (10, 50 µg/ml). After trypsinization, cells were washed with PBS (with 0.133 g/L CaCl₂·2H₂O and 0.1 g/L MgCl₂·2H₂O, Sigma, Germany) and then incubated with 100 µl of the primary antibodies (dilution 1:20; mouse anti human IgG₁) against the adhesion receptors: CD29 (β1-integrin); CD61 (β3-integrin); CD49b (α2-integrin); CD49c (α3-integrin); CD49d (α4-integrin); CD49e (α5-integrin); CD49f (α6-integrin); CD51 (αv-integrin); CD44 (HA; hyaluronic acid); control Mouse IgG₁; (all from Beckman Coulter, USA) for 1 h at room temperature. Thereafter, cells were washed and secondarily labeled with fluorescein isothiocyanate-conjugated anti-mouse IgG (FITC; Sigma) for 1 h at room temperature in the dark. After a final washing step with PBS, cells were diluted in 300 µl CellFix (Beckman Coulter, USA). Adhesion receptor expression levels were measured as described previously by Nebe et al. (2006). 10.000 events were recorded for each measurement. At least three replicates for each treatment were performed.

For visualization of integrins, cells were incubated on glass coverslips until a confluency of 70% was reached. After adaptation to the assay medium for 48 h, cells were treated with the flax root extract for 48 h. Prior to integrin labeling, cells were washed three times with PBS (with Ca and Mg) and then incubated with 100 µl of the primary antibody (CD29 (β1-integrin), Beckman Coulter, USA) for 1 h at room temperature. After a PBS washing step, the secondary antibody (Alexa Fluor488; Invitrogen, UK) was used for 1 h to label β1 integrins on the cell surface. Thereafter cells were fixed by treatment with 3.7% paraformaldehyde (PFA) for 10 min, washed with PBS (with Ca and Mg) and then permeabilized with 0.1% Triton X-100. Finally the nucleus of cells were counterstained with 100 µl of 300 nM DAPI (4',6-diamidino-2-phenylindole)-dihydrochloride (Invitrogen, UK) for 15 min. After washing with PBS, cells were embedded on glass slides in mounting medium and stored at +4°C in the dark. Cellular integrins were visualized with the Axio Scope.A1 fluorescence microscope (Carl Zeiss, Germany) using AxioVision Imaging Software 4.8.2.0 (Carl Zeiss, Germany).

Actin visualization and quantification

MCF-7 cells (10,000 cells/cm²) were cultured on glass coverslips for 24 h. After adaption to assay medium for 48 h, cells were treated with the flax root extract for 48 h, and finally fixed with 4% PFA for 10 min. They were washed twice with PBS and permeabilized with 0.1% Triton X-100 for 10 min. Then cells were incubated with 100 µl 6.6 µM Alexa Fluor594 phalloidin (Invitrogen, Germany) for 30 min in the dark at room temperature, washed again and embedded in mounting medium. Actin was investigated with an inverted confocal laser scanning microscope (LSM780, Carl Zeiss, Germany) equipped with a helium/neon-ion laser and a ZEISS 63x oil immersion objective. The confocal images (1024 x 1024 pixel) were used for subsequent actin quantification via mathematical image processing by the FilaQuant software (University of Rostock, Institute of Mathematics, Mathematical Optimization) (Matschegewski et al., 2012; Birkholz et al., 2010; Birkholz, 2011).

Western blotting

The general steps of the Western blot procedure have been

described previously (Engel et al., 2012). For protein detections primary antibodies (Collagen Type I (600-401-103-01, Rockland, USA); Fibronectin (sc-8422); Vinculin (sc-55465); Focal adhesion kinase (FAK, 610088, BD-Transduction; USA); Paxillin (sc-5574); E-Cadherin (3195, Cell signaling, Germany); ZO-1 (8139, Cell signaling, Germany); α -E-Catenin (3240; Cell signaling, Germany); Profilin-1 (3246, Cell signaling, Germany); Arp-2 (3128, Cell signaling, Germany) and β -Actin (sc-47778); sc: Santa Cruz, USA) were incubated overnight at 4°C followed by labeling with a horseradish peroxidase (HPR)-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1 h at room temperature. Band intensity was analyzed densitometrically with the Molecular Imager ChemiDoc XRS and Image Lab 3.0.1 software (Bio-Rad, USA). Protein detection was repeated at least three times with individually prepared cell lysates from independently passaged cells.

Colony formation in soft agar

Anchorage-independent growth was determined by assaying colony formation in soft agar. MCF-7 cells were adapted to assay medium for 48 h. Thereafter, cells were treated with the flax extract (1, 25 and 50 μ g/ml) and the vehicle (C; 0.1% ethanol) for further 48 h. To form the base layer 1 ml of 0.5% agar (Gibco, USA) in assay medium was added to each well of a 12-well plate (Greiner, Germany) and allowed to polymerize on the tissue culture plastic until the agar reached room temperature. The top layer was made by 0.35% agar in assay medium. After cooling down to about 40°C, 5×10^3 cells were mixed with 1 ml top layer agar and plated over the base layer. Cells were cultivated with assay medium which was changed every 3rd day. The growth of the cells in soft agar was monitored daily by a light microscope (Axiovert 40, Carl Zeiss, Jena, Germany) equipped with the camera lcc1 (Carl Zeiss, Jena, Germany). Images of the colonies were taken at day 16.

Statistical analysis

All experiments were replicated at least three times with individually passaged cells, and data sets were expressed as means \pm standard deviations (SD). Statistical significance was determined by the unpaired student's *t*-test (***P* < 0.001; ***P* < 0.01; **P* < 0.05).

RESULTS

Chemical composition of the flax root extract

To identify the major classes of active substances in the ethanolic flax root extract, LC separation and MS analysis were performed. For qualitative measurements, the negative and positive ion currents via Xcalibur software were analyzed because all examined analytes could form deprotonated molecules [M-H]⁻ or protonated molecules [M+H]⁺ as major precursor ions (Figure 1). The investigations revealed that the plant ingredients mainly tend to negative ionization.

The root extract is a mixture of many different complex ingredients the presence of the lignan secoisolariciresinol with the main mass fragment [M-H]⁻ at *m/z* = 361 at a retention time of 10.48 min was verified in the negative ion current. Furthermore, matairesinol, pinoresinol, lariciresinol, anhydrosecoisolariciresinol diglycoside and arctigenin were identified (Table 1). All compounds are

characterized by their dimer phenylpropanoid, molecules which are linked to a middle β -C atom (Larkin, 2000). These properties are characteristic for lignans.

Besides the lignans, also some flavonoids were identified, e.g. fisetin (3, 3', 4', 7 - tetrahydroxyflavon) which was found in the negative ion current. Fisetin eluted with a medium intensity and a retention time of 16.25 min. Other flavonoids such as daizein, glycitin and biochanin, were also detected by MS. A summary of the identified plant ingredients is given in table 1. The peak with the highest intensity (NL: 7.20E6) in the positive ion current at a retention time of 13.98 min (Figure 1) has a *m/z* ratio of 701 amu. Comparison with databases, standards and various publications did not provide any clear structure. After *ms*ⁿ investigation with syringe pump we could identify the lignan basic structure of a secoisolariciresinol, which is linked double glycosidic. One of the sugar parts contained a methoxy-group. Thus, in the *ms* profile of this structure there is clear evidence that based on the main mass fragment [M+H]⁺ with a *m/z* value of 701 amu, a direct sugar separation [M-162]⁺ followed by an α,β -hydrogen rearrangement with a *m/z* value of 539 amu is discernible. The second separation of a sugar molecule and some other hydrogen and charge rearrangements induce the basic lignan molecule secoisolariciresinol with a *m/z* of 361 amu. In accordance to the literature, the presence of podophyllotoxin could not be demonstrated by MS (Abarzua et al., 2007).

Cell impedance, adhesion and migration ability

We analyzed the influence of the flax root extract on the cell impedance with the Bionas® 2500 analyzing system combined with the metabolic chip Bionas DiscoveryTM SC1000 equipped with an IDES sensor (Interdigitated Electrodes), which are solid state electrochemical devices able to detect dielectric properties of a sample. After an adaption phase of 3 to 4 h (highlighted in grey) to the new culture conditions, cell impedance of MCF-7 cells was measured continuously for 20 h (Figure 2). In comparison to the control treatment with 0.1% ethanol, which was set to 100%, a concentration of 10 μ g/ml flax extract only slightly lowered the MCF-7 cell impedance. The highest concentration of 50 μ g/ml reduced the impedance rate by up to 50% of the initial value.

To determine the initial adhesion capacity, suspended MCF-7 cells were let to adhere on tissue culture polystyrene (TCPS) for 2 h. Non-adherent cell were counted via flow cytometry. Treatment with 50 μ g/ml flax root extract significantly reduced initial adhesion of MCF-7 cells compared to the control (18% reduction; *p* < 0.0009; Figure 3A). Lower concentrations of the flax extract did not change initial adhesion properties. The positive control 17 β -estradiol (1 nM) stimulated the initial adhesion capacity significantly (*p* < 0.049).

A further important marker for the tumorigenicity of cancer cells is the migration ability. Figure 3B clearly

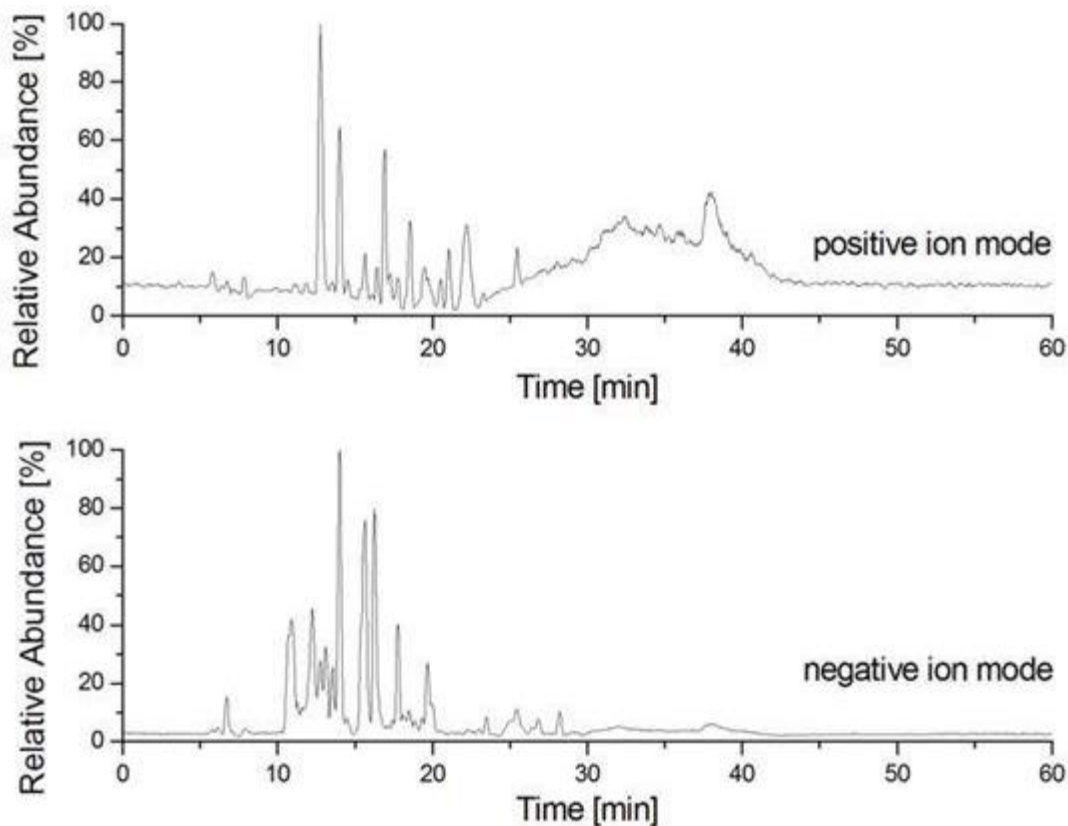


Figure 1. Chromatograms of the positive and negative ion mode of the ethanolic *Linum usitatissimum* root extract. Positive: NL: 7.20E6 BasePeak F: ITMS + c ESI sid = 35.00 Full ms [90.00-2000.00] MS. Negative: NL: 1.06E6 BasePeak F: ITMS - c ESI sid = 95.00 Full ms [90.00-2000.00] MS. Note that the detected secondary plant compounds appear at retention times between 10 and 20 min.

Table 1. Identified mass traces of the secondary plant compounds of the ethanolic flax root extract detected by LC-MS analysis in accordance to their retention time.

No.	Retention time (min)	Compounds	ESI (pos. mode), m/z	ESI (neg. mode), m/z
1	10.48	Secoisolariciresinol	-	361
2		Lariciresinol	361	359
3	12.03	Matairesinol	-	357
4	12.60	Pinoresinol	359	357
5	13.98	"not yet characterized"	701	722
6	14.34	Arctigenin	-	371
7	15.86	Biochanin a	285	283
8	16.25	Fisetin	287	285
9	16.42	Daidzein	255	253
10	16.90	Glyciten	285	-
11	18.48	Anhydrosecoisolariciresinol diglycosid	345	343

demonstrates a decrease of the migration rate of MCF-7 cells after treatment with 10 and 50 $\mu\text{g/ml}$ flax extract in standard scratch assays. While after 48 h control cells were able to close the gap as a sign of high migratory capacity, exposure of the flax extract inhibited MCF-7 cell

motility substantially. The highest concentration of the extract reduced cell movement significantly, very few cells were able to migrate into the gap (72% reduction). These results clearly show that the flax extract exerts a significant influence on the adhesion and migration

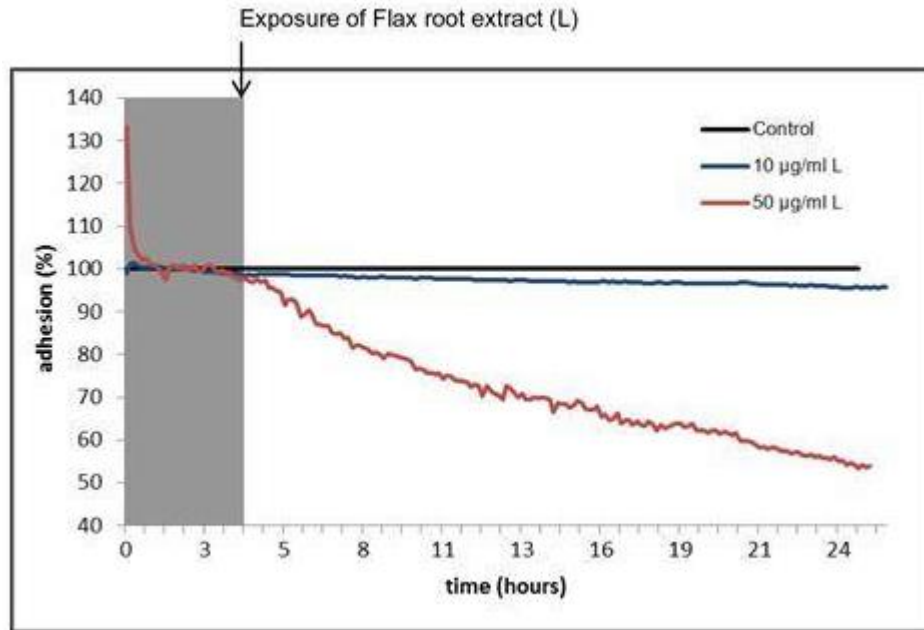


Figure 2. Online monitoring of living cell impedance of MCF-7 cells after exposure with 10 and 50 µg/ml flax root extract in comparison to the control (0.1% ethanol) with the Bionas Discovery™ 2500 system using the metabolic chip SC1000 under dynamic flow conditions (pump rate 56 µl/min). Displayed were the percentages of the standardized rates of cell adhesion (impedance) in a period of 24 h. Grey shadowed area marks the adaption phase (4 h) of the cells. Arrow: start of the addition of the flax root extract. Shown is the mean of 3 independent experiments.

processes in the cell line MCF-7.

Adhesion receptor expression

Integrins are key players in the mediation of attachment between a cell and its surroundings, e.g. other cells or the extracellular matrix. Therefore alteration of integrin expression or distribution might be involved in the significant changes of initial adhesion capacity and migration properties after application of the flax extract. The integrin expression on the cell surface was measured by flow cytometry (Figure 4A). Compared to the control treatment no significant alteration in the integrin receptor expression was detectable.

To confirm these results, integrin expression and distribution was monitored by fluorescence microscopy. In Figure 4B the distribution of $\beta 1$ integrin (green fluorescence) is exemplarily shown. Under control conditions, $\beta 1$ integrins is weakly present on the cell surface, but shows a marked increase of expression at the cell-cell contacts, indicating for strong cell cohesion. After treatment with 10 µg/ml flax extract the green fluorescence decreased, and the cells seem to slide apart. This phenomenon is even more pronounced after treatment with 50 µg/ml of the flax extract: Only a few cell-cell contacts can be recognized, and the $\beta 1$ integrin

expression is spread evenly over the whole cell surfaces.

Morphological alterations

In order to confirm that the flax root extract is able to break up the cell-cell contacts, morphological analyses were performed. Scanning electron microscopy allows deriving indications for membrane stability, induction of apoptosis (so-called "membrane-blebbing") or contact with the extracellular matrix. Figure 5 demonstrates the cell morphology of the MCF-7 cells after a 48 h exposure with the flax extract. Under control conditions, MCF-7 cells are clustered in domes, which are typical for the tumorigenic growth of these cells. The cells are well spread on the glass surface, and the cell-cell contacts are very close, therefore a visual differentiation of the cells borders hardly can be observed. After treatment with 1 µg/ml flax root extract there was no detectable change of cell morphology. At a concentration of 10 µg/ml the contacts between the cells started to dissolve. At a concentration of 50 µg/ml flax extract nearly all MCF-7 cells are separated from each other: In the 1000-fold magnification the MCF-7 cells do not show any cell-cell contacts. The contact with the glass surface, however, is not affected - the cells do not detach from the substrate. These high-resolution images confirm the suspicions that

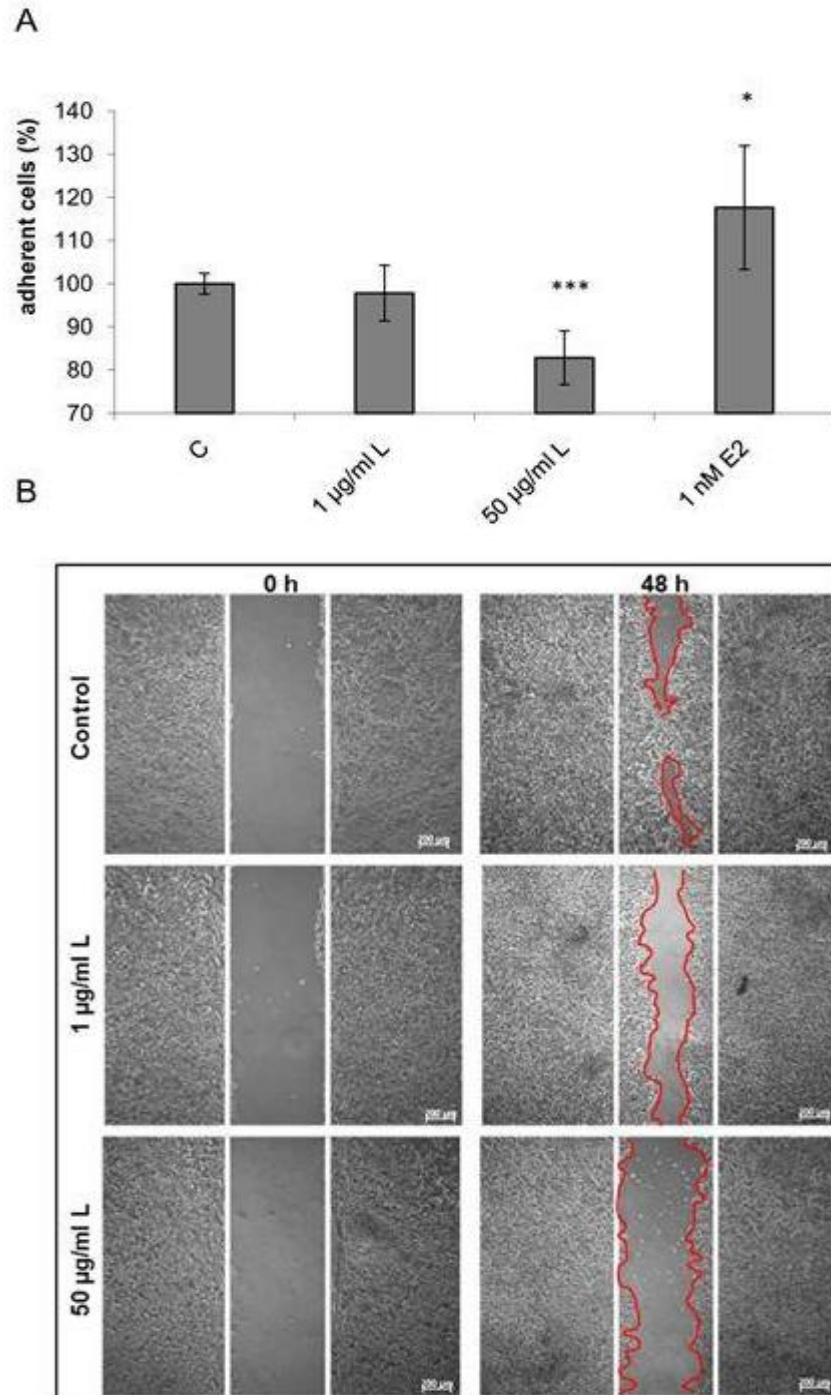


Figure 3. Influence of the flax root extract on the initial adhesion capacity (A) and the migration properties (B) of MCF-7 cells. A: Initial adhesion of suspended MCF-7 cells was measured after two hours incubation with the flax extract (L) in comparison with the vehicle control (EtOH) or the positive control of 1 nM 17 β -estradiol (E2). Significant lowered initial adhesion occurs after exposure with 50 μ g/ml flax extract. Cells were counted with the cytometer FACSCalibur. (n = 6; student's t-test; *** P < 0.001; * P < 0.05). B: Scratch assay to monitor the migration ability of MCF-7 cells after treatment with the vehicle EtOH (Control), 1 or 50 μ g/ml flax extract. Photographs were taken at the incubation start point (0 h) and after 48 h exposure with the flax extract (Camera lcc1, Carl Zeiss, Jena, Germany). Significant reduced migration potential was visible after treatment with 50 μ g/ml flax extract. Red lines surround the borderline of the cell layer. (One representative example of 5 independent experiments, bar = 200 μ m).

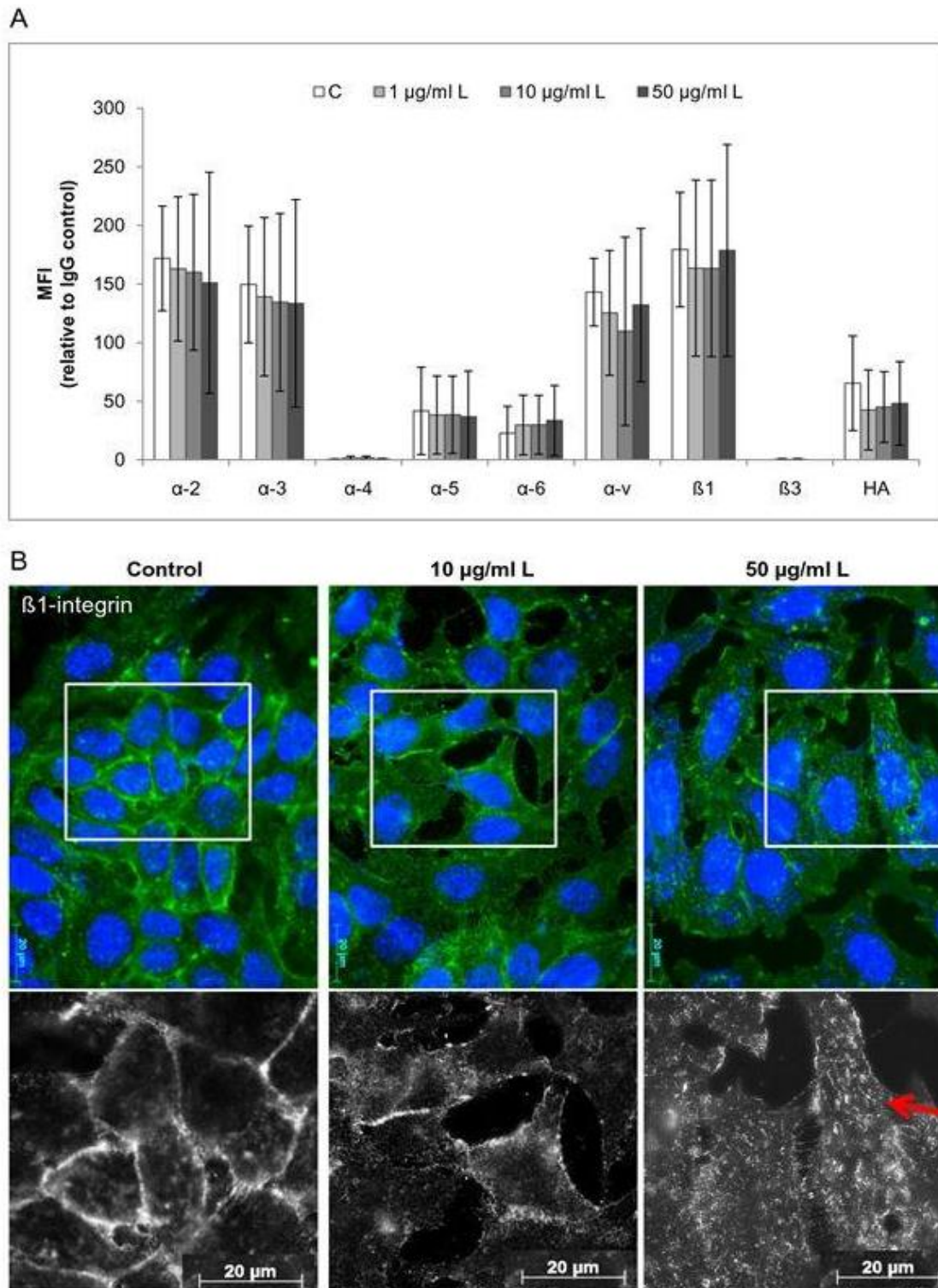


Figure 4. Expression of several integrin receptor subunits/ hyaluronic acid receptor (HA) and localization of the $\beta 1$ -integrin subunit. A: Expression of cell surface receptors was measured by flow cytometry after treatment with 1, 10 or 50 $\mu\text{g/ml}$ flax extract (L) compared to the vehicle control (C). No significant alterations in the cell surface expression of all marked integrins and HA were determined (mean \pm SD; $n=6$). B: Top row: Immunofluorescent labeling of $\beta 1$ -integrin (green) and counterstaining with Dapi to visualize the cell nucleus (blue). Bottom row: Enlarged sections of the original recordings without the representation of the cell nuclei. Notably, $\beta 1$ -integrin is strongly localized at the cell-cell contacts in control cells, whereas the exposure with 50 $\mu\text{g/ml}$ flax extract leads to a uniform distribution of $\beta 1$ -integrin on the basal side of the cells (arrow). (Axio Scope.A1 fluorescence microscope, Carl Zeiss, Germany; bar = 20 μm).

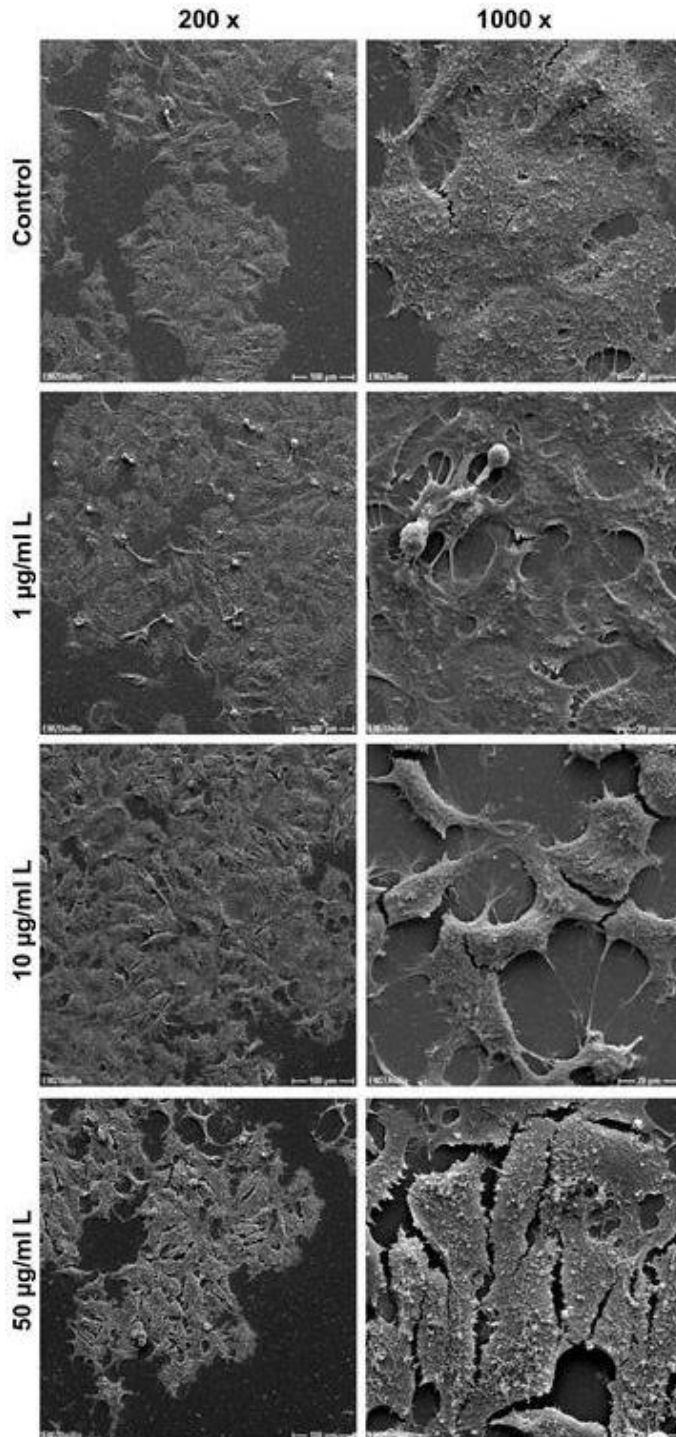


Figure 5. Scanning electron microscopic visualization of MCF-7 cell morphology after treatment with 1, 10 and 50 µg/ml flax root extract in comparison with the vehicle control. Notably, cell-cell contacts are lost after treatment with 10 and 50 µg/ml flax extract (SEM DSM 960A, Carl Zeiss, Jena, Germany; magnifications: 200x, 1000x).

have arisen by the $\beta 1$ integrin fluorescence staining: the flax extract prevents the formation or destroys the functionality of cell-cell contacts. These findings raise the

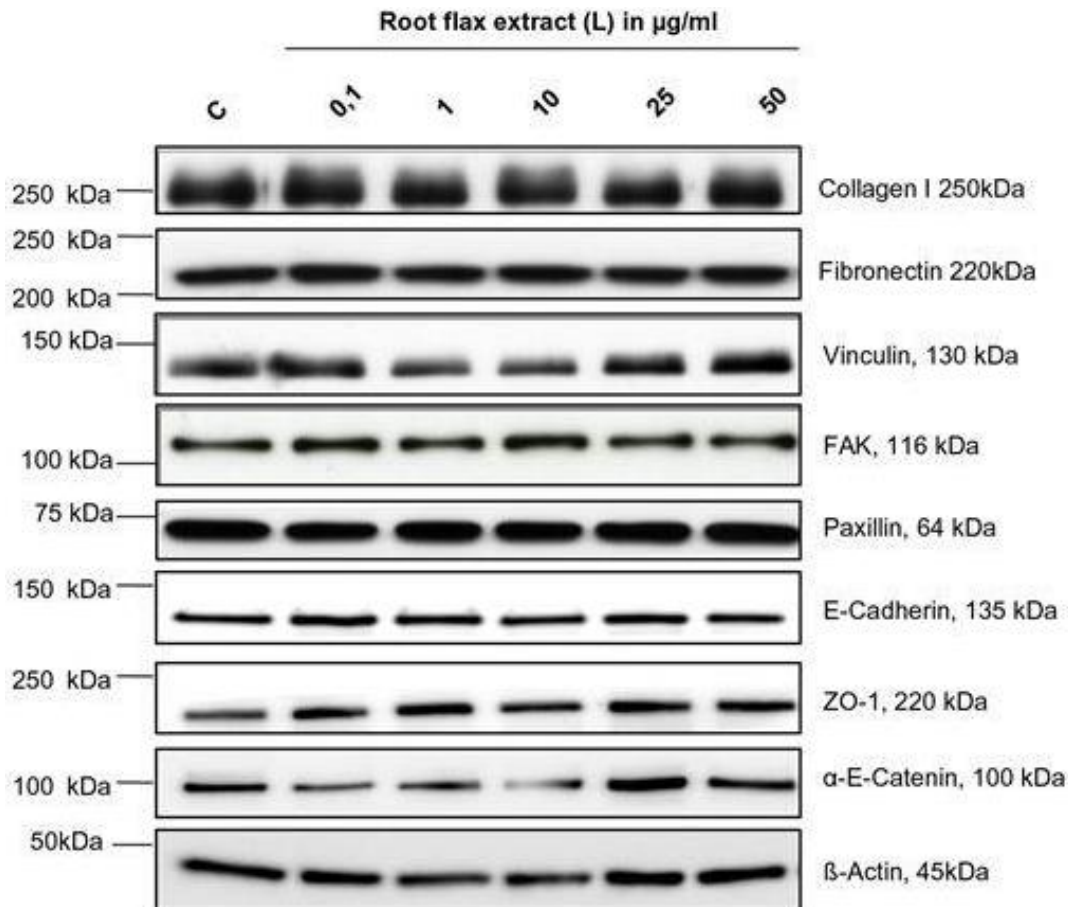
question, which of the adhesion-related proteins were affected adversely thereby resulting in breaks of the epithelial cell cluster structure.

Expression analysis of adhesion molecules

In order to explain the broken cell-cell contacts as well as the diminished adhesion, the expression of several adhesion proteins was determined on protein level (Suppl. Figure 1). We focused on calcium-dependent adhesion molecules (E-cadherin, α -E-catenin), proteins from the focal adhesion complex (focal adhesion kinase (FAK), paxillin, vinculin), and extracellular matrix proteins (collagen I, fibronectin). Furthermore, we analyzed the expression of the tight junction protein zona occludens (ZO-1), which is needed to form a continuous barrier to fluids across the epithelium. This peripheral membrane adaptor protein links junctional transmembrane proteins to the actin cytoskeleton. The western blotting results did not reveal any significant changes after treatment with the flax root extract. Neither the expression levels of collagen I, fibronectin nor paxillin, vinculin nor FAK were changed. Also E-cadherin, an important transmembrane glycoprotein mediating calcium-dependent cell-cell adhesion was not influenced in its expression. Slight variations in the expression levels were detected in the proteins ZO-1 and α -E-catenin. Also, the β -actin levels were not significantly changed after addition of the flax extract.

Actin stress fiber formation by alteration of profilin-1 and arp-2 expression

Actin, the major component of the cytoskeleton primarily exists as a fibrous polymer. It does not only determine the structure of the cells and serves as scaffold for signaling proteins but is also responsible for its stiffness. The breast cancer cells MCF-7 are characterized by a cortical arrangement of F-actin (Figure 6). The actin fibers show almost no stress fiber formation, and F-actin is localized submembranously, and primarily at the cell-cell contacts or close to the filopodia that extend beyond the leading edge of lamellipodia in migrating cells. After exposure to the flax root extract (50 µg/ml) the F-actin is starting reorganization, leading to the formation of long stress fibers throughout the cell. By novel software "FilaQuant", the quantification of the actin filament organization via mathematical image processing of the confocal microscopic exposures was feasible. The automatically processed images represent the actin filament formation confirming the confocal microscopic observations. They show, that total filament length after exposure to the flax extract is highly boosted (Table 2). In detail, data revealed a 9-fold higher formation of the filament number and a 12-fold increase of the total filament length after exposure to the flax extract.



Supplemental Figure 1. Western blotting experiments of adhesion and cell-cell contact relevant proteins of MCF-7 cells in a concentration dependent series (0.1 to 50 µg/ml flax root extract (L)). No expression level alterations of either one of the proteins were detected after flax extract incubation. Representative example of three western blot experiments is shown.

However, the length of the average filament is only slightly increased.

One key player in the orchestra of actin dynamic regulators is profilin-1, an actin binding protein that affects the rate of actin polymerization. A further protein relevant for actin nucleation is arp-2, which promotes branching of existing actin filaments and formation of daughter filaments by recycling existing filaments. Therefore, the expression levels of profilin-1 and arp-2 under the treatment of the flax extract were studied by western blotting analysis (Figure 7). The expression levels of profilin-1 increase dose-dependently with the extract concentration, starting from the concentration of 1 µg/ml. The expression of arp-2 decreases at all used flax extract concentrations explaining the strong formation of stress fibers in MCF-7 cells after treatment with higher concentrations (10 to 50 µg/ml) of extract. The increased profilin-1 expression initiates the polymerization of F-actin, and the reduction of arp-2 protein prevents a strong side branching of actin. The actin fibers therefore are long and not branched (Figure 6).

Effect of flax root extract on anchorage-independent growth of MCF-7 cells analyzed by colony formation on soft agar

To test if tumorigenicity of MCF-7 cells is influenced by the flax root extract we assessed the capacity for anchorage-independent growth by testing their ability to form colonies while suspended in soft agar. Control MCF-7 cells form numerous large colonies in soft agar. In contrast, flax root exposed MCF-7 cells form considerably less colonies with a reduced size in a concentration-dependent manner (Figure 8).

DISCUSSION

In previous work we could show that the flax root extract plunged a concentration-dependent decrease of the proliferation of the breast tumor cell line MCF-7 (Engel et al., 2012). In order to clarify the underlying mechanisms, a systematic study of the anti-tumorigenic properties of

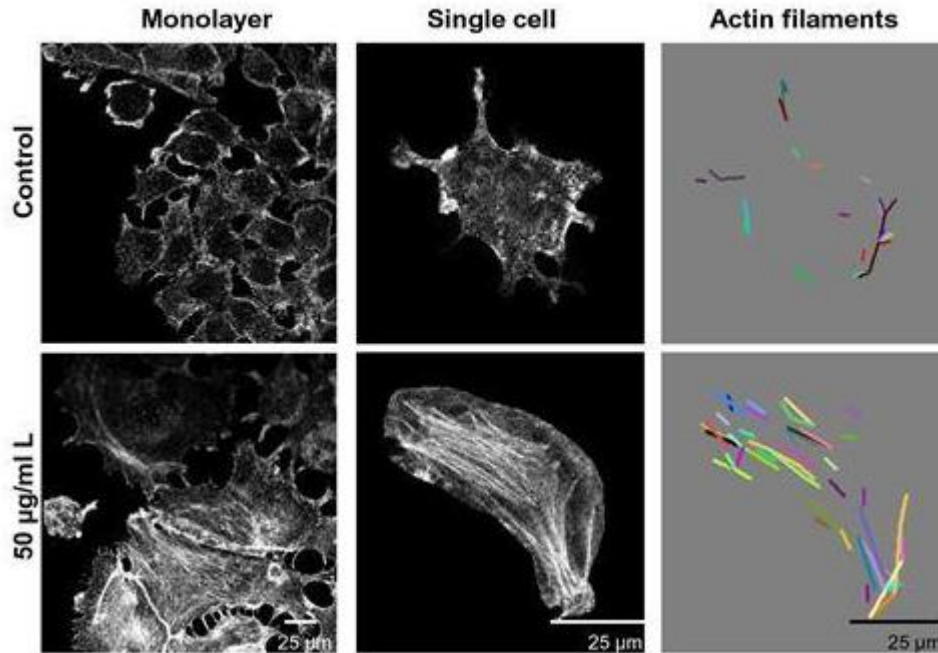


Figure 6. Visualization and quantification of actin filaments in MCF-7 cells under control conditions (vehicle 0.1% ethanol) and after treatment with 50 µg/ml flax root extract for 48 h. left column: confocal images of actin stained cells in a monolayer (LSM 780, Carl Zeiss, bar = 25 µm), middle column: single cell images, right column: automatically processed images obtained from FilaQuant software (University of Rostock, Institute of Mathematics, Mathematical Optimization). Actin filaments are shown as colored lines. Note that the actin filament length and number are impressively increased after exposure to the flax root extract.

Table 2. Quantification values of actin filament number and length calculated by the FilaQuant software. (mean ± SD, unpaired *t*-test, ****p* < 0.001; *n* = 20 cells).

Parameter	Control	50 µ/ml L
Filament number	9.24 ± 2.62	85.9 ± 6.43***
Total filament length (µm)	54.95 ± 9.18	650.46 ± 56.82***
Average filament length (µm)	5.68 ± 1.87	7.60 ± 2.01

the extract was placed in the center of this work. In addition to the proliferation as revealed earlier also adhesion and motility, important features of cancer cells, were examined. It was shown that the flax extract strongly inhibited the cell impedance, initial cell adhesion and migration ability of MCF-7 cells. By means of testing the expression and intracellular distribution of adhesion related proteins, it has been possible to evaluate that the actin cytoskeleton is remodeled by the flax root extract. This is achieved by an overexpression of profilin-1 and a reduced expression level of arp-2, which resulted in an increased formation of actin stress fibers spanning the entire cell. Normally, MCF-7 cells harbor a cortical cytoskeleton with short F-Actin fibers, which enable cells to grow, migrate and adhere much faster than normal epithelial breast cells like MCF-10A (Haynes et al., 2011). By the extension of the F-actin filaments a stabilization of

the cell is initiated - the cell has become stiff and inflexible (Kallergi et al., 2003). Among others this stiffness could lead to the disruption of cell-cell contacts (Figure 5) and a displacement of β 1-integrin (Figure 4). β 1 integrin is believed to be associated with tumor progression and metastasis-associated cell behaviors (Ivanova et al., 2013; dos Santos et al., 2012). The lack of cell-cell contacts can break down the tumorigenic epithelial cell structure, thereby interrupting cell communication. Therefore induction of apoptotic signals is likely.

The dissolution of the cell assembly is also confirmed by the impedance measurements on the Bionas device (Figure 2). The loss of 50% cell impedance can be either traced to the detachment of the cells from the metabolic chip surface or to a reduced resistance of the chip, whilst more gaps between the cells have emerged. However, a

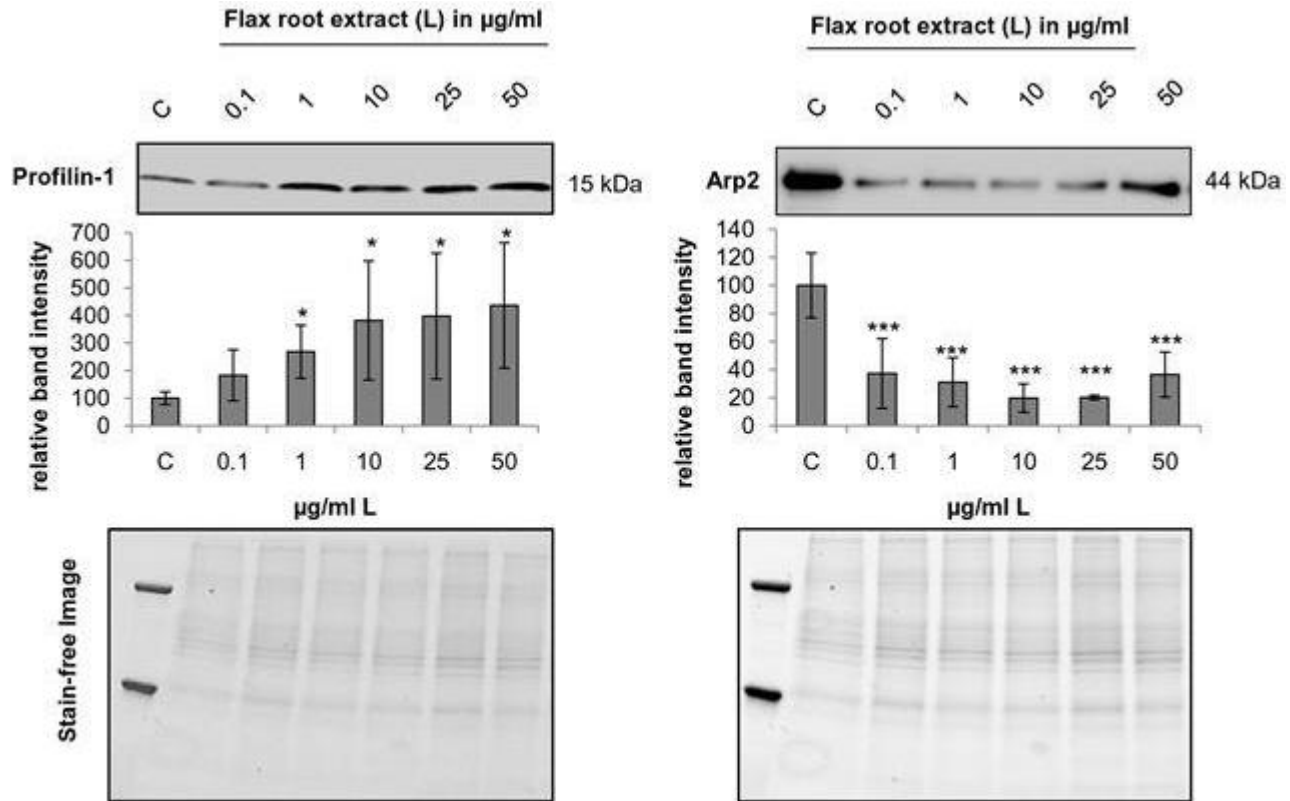


Figure 7. Expression analysis of profilin-1 and arp-2 proteins in MCF-7 cells detected by western blotting in a concentration depended series after treatment with 0.1 – 100 µg/ml flax root extract for 48 h. Representative western blots were displayed on top of the graphs. Quantification of western blotting results was carried out with individual passaged cells for at least three times (Densitometric analysis). Loading controls were guaranteed by stain-free imaging of the SDS-PAGEs prior blotting procedure. Note that profilin-1 expression lowered at all concentrations used. Representative example of three western blot experiments is shown. Unpaired t-test, * $p < 0.05$; $n = 3$.

detachment of the cells from the substrate was excluded by the micrographs (Figures 3, 4 and 5). As a consequence of the increased stiffness of the MCF-7 cells, rapid locomotion and initial adhesion capacity of suspended cells are prevented (Figure 3).

That the elasticity of a cell is sufficient for their tumorigenic potential was already published (Plodinec et al., 2012; Xu et al., 2012). But this work describes for the first time that a native plant extract directly can modulate stiffness of a breast cancer cell by the reconstitution of normal cytoskeleton features. In future studies the *in vivo* potential of the flax root extract will be tested, in order to establish a perspective view of this extract in the prevention and/or treatment of estrogen receptor positive breast tumors.

However, these cell biological alterations induced by the flax extract are probably due to the secondary plant ingredients. Beside lignans like secoisolariciresinol, lariciresinol, matairesinol, and pinoresinol, also isoflavones e.g., daidzein, fisetin, and biochanin a could be detected by LC-MS based techniques. Many studies and articles exist, describing the effectiveness of singular phytoestrogens and phytoestrogens-enriched plant

extracts *in vitro* and *in vivo*. For example, two studies have shown that dietary supplementation with flaxseeds (rich in lignans) was associated with reduced tumor biologic markers (e.g., Ki-67 labeling index) and an increased apoptosis and induced cell death in postmenopausal breast cancer patients (Saarinen et al., 2006; Thompson et al., 2005). Nevertheless, it can be assumed that the combination of a variety of lignans and isoflavones generates the biological activity of the flax root extract. It should be emphasized that it was shown in this study for the first time that the combination of phytoestrogens within a natural extract not only influenced the proliferation or apoptosis of hormone-dependent tumor cells, but also the cell architecture which decisively determined the invasive potential of a tumor cell.

CONCLUSION

For the first time, this study evaluated that a mixture of phytoestrogens within the natural root flax extract posses potential anti-tumor actin by remodeling of the actin

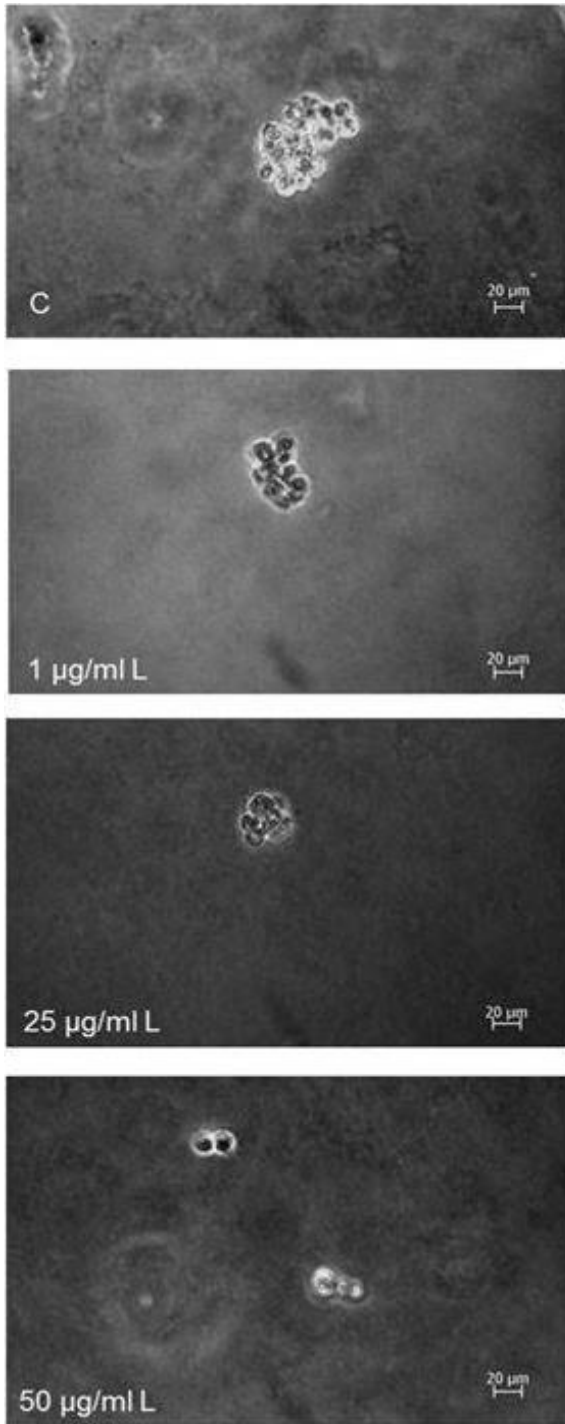


Figure 8. Representative photographs of colonies growing in soft agar by bright field imaging. MCF-7 cells treated with flax root extract (L) for two days and grown in soft agar for 16 days. Note that flax root exposed MCF-7 cells form considerably less colonies with a reduced size. (Camera Icc1; Carl Zeiss, Jena, Germany; 32x magnification, bar = 20 µm).

cytoskeleton leading to stabilization of the cells followed by significantly decreased adhesion, cell impedance,

migration and colony formation *in vitro*. This illustrates that the use of natural plant extracts in cancer therapy is a reasonable alternative to singular agents such as genistein.

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