Full Length Research Paper

Effects of extracts from *Linum usitatissimum* on cell vitality, proliferation and cytotoxicity in human breast cancer cell lines

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The seeds of flax (binomial name: *Linum usitatissimum L.*) are well known for their high content of phytoestrogens. In the present study, extracts from roots, leaves and stems of flax were analysed for their content of compounds, which might have phytoestrogen-like properties, by pyrolysis field ionisation mass spectrometry. All extracts were tested on the human breast cancer cell lines MCF7 (estrogen receptor positive) and BT20 (estrogen receptor negative). Specific tests were applied for cell vitality ((3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (MTT) test), proliferation (BrdU test) and cytotoxicity (lactate dehydrogenase (LDH) test). In the flax root extract, the amounts of monolignols and polyphenols were three times higher than in the stem and leaf extracts. Even at higher concentrations, the root extract also was the least cytotoxic one of all extracts in MCF7 cells, while it showed a dose-dependent and much higher cytotoxicity in BT20 cells. Furthermore, at higher concentrations (> 100 µg/ml), the root extract reduced cell vitality in MCF7 significantly less than in BT20 cells and inhibited proliferation in MCF7 by up to 85%. Since flax root extracts induce significant inhibition of cell vitality and proliferation without performing strong cytotoxicity in the human mamma carcinoma cell lines MCF7, the potential phytoestrogens in flax root extracts could have beneficial effects in hormone-dependent tumours.

Key words: Flax, *Linum usitatissimum*, phytoestrogens, MCF7, cell proliferation, breast cancer.

INTRODUCTION

Plants produce more than 100,000 different low-molecular-mass compounds, known as secondary metabolites. Amongst the most numerous are lignans, isoflavonoids and coumarins (Dixon, 2001). Being able to bind to the human estrogen receptors alpha and beta (ERα, ERβ), with higher affinity to ERβ, they were named phytoestrogens (Kuiper et al., 1997). In several publications, anti-proliferative effects of these substances on tumour (cell) growth were described (Lamartiniere et al., 1995; Zhou et al., 1999). They inhibit enzymatic activities

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involved in intracellular signal transduction, which are involved in the stimulation of cellular growth factors (Scholar and Toews, 1994). Phytoestrogens act also on the cellular metabolism by ER-independent mechanisms such as induction of apoptosis, inhibition of topoisomerase II and angiogenesis, or by anti-oxidative effects (Hostanska et al., 2004; Kulling and Watzl, 2003; Peterson and Barnes, 1991).

For several crude extracts, for example from peas, beans, pumpkin seed, red clover or soy beans, which contain phytoestrogens binding to ERs, an anti-tumour activity was demonstrated (Booth et al., 2006; Boue et al., 2003; Liu et al., 2001). A diet with flax seeds, which are very rich in lignans, significantly reduced MCF7 cell tumour size in mice (Adlercreutz and Mazur, 1997; Horn-Ross et al., 2000; Saarinen et al., 2006). Also, other parts of flax may be of interest, as it was shown that the cell vitality of the chorion carcinoma cell line Jeg3 was more strongly inhibited by flax root extracts than by flax leaf or stem extracts (Abarzua et al., 2007).

Flax is a food and fibre crop that is grown in cooler regions of the world, for example Canada, Russia and Germany. It is an annual plant, reaching a height of up to more than 200 cm. Anthesis is between July and August; the main growth phase is in May and June. The plant does not make great demands on soil and thus it can be easily cultivated.

The aim of the present study was the identification of putative phytoestrogens in methanolic extracts of leaves, stems and roots of flax and to test the effect of these extracts on cell vitality, proliferation and cytotoxicity in ER-positive and ER-negative human breast cancer cell lines *in vitro*.

MATERIALS AND METHODS

Preparation of extracts

Seeds of the flax *Linum usitatissimum* L., cultivar Barbara, provided by the Agricultural Research Institution (LUFA), Rostock, Germany, were sown on soil and grown under field conditions. The flowering plants were harvested, and leaves, stems and roots were frozen separately in liquid nitrogen and stored at -80°C until extraction. The extracts were prepared according to Luyengi et al. (1996) as modified by Matscheski et al. (2006). Plant material (20 g) was ground with liquid nitrogen in a mortar and extracted with 180 ml 100% methanol in a water bath for 15 min at 70°C by using a reflux condenser. The solution was cooled, filtered and evaporated almost to dryness. The extract was resuspensed in 8 ml of distilled water and partitioned with ethyl acetate for five times. After drying, the ethyl acetate soluble fraction was dissolved in absolute ethanol to provide a stock solution of 100 mg/ml. The extracts were stored at -20°C.

Molecular-chemical analysis of extracts

The extracts (stock solution) were screened by pyrolysis field ionisation mass spectrometry (Py-FIMS) (Schulten and Halket, 1986). It combines a temperature-resolved volatilisation during pyrolysis and a soft ionisation of molecules at high-vacuum (Beckey, 1977; Schulten, 1996). About 5 µl of the extract were transferred to a quartz capsule that was placed in the micro-oven of a double-focusing Finnigan MAT 900 mass spectrometer (Finnigan, MAT, Bremen, Germany). The micro-oven heated the sample from 110 to 700°C in 12 min, and 91 magnetic scans were recorded for the mass range m/z 15 to 900 (single spectra). The marker signal selection was based on the concepts of Schulten et al. (1989), Hempfling et al. (1991) and Hempfling and Schulten (1991). A detailed description of the Py-FIMS method and the statistical treatment of TII and data normalisation are provided by Sorge et al. (1993).

Cell lines and cell culture

The human breast cancer cell lines MCF7 and BT20 were obtained from the Department of Human and Animal Cell Culture, Braunschweig, Germany. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich-Chemie, Germany), supplemented with 10% inactivated fetal calf serum (FCS, Biochrom, Germany), 1% antibiotics (penicillin/streptomycin, PAA, Germany) and 0.5% amphotericin B (PAA, Germany) under a humidified atmosphere (37°C and 5% CO_2). MCF7 cell line is described to be ER alpha, beta and progesterone receptor positive. BT20 cell culture does not express the ER receptors (manufacturer's protocol). We confirmed this by immunocytochemistry.

### Cell vitality, cell proliferation and cytotoxicity

Cell vitality, cell proliferation and cytotoxicity in the human breast cancer cell lines MCF7 and BT20, treated with the extracts from *L. usitatissimum* were analysed by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method, a 5-bromo-2′-desoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (colorimetric), and a cytotoxicity detection kit (LDH kit for lactate dehydrogenase) as was recommended by the manufacturer (all Roche, Germany). For MTT, BrdU and LDH test, 5×10^5 cells/ml were grown in 96-well tissue plates for 24 h at 37°C and 5% CO_2. Subsequently, extracts and controls were added and incubated for 24 h at humidified atmosphere. Aliquots from the stock solutions of root, leaf and stem extracts were dissolved in absolute ethanol and added to the supplemented culture medium to give final concentrations of 0.01, 0.1, 1, 10, 50, 100, 500 and 1000 µg/ml (final concentration of ethanol: 1%). In all assays, two negative and two positive controls were used. Negative controls: (i) cells in DMEM (control 1) and (ii) cells in DMEM and ethanol (final concentration of ethanol: 1%; control 2). Positive controls: 17ß-estradiol (estrogen, Sigma-Aldrich, Germany) and tamoxifen (anti-estrogen, Sigma-Aldrich, Germany). Both positive controls were performed to show the accuracy of the tests and the usual behaviour of the cell cultures (data not shown). MTT test: After incubation with MTT for 4 h at 37°C and 5% CO_2, the solvent was added and the plates were incubated overnight. The absorbance of the formazan crystals was measured at 570 nm (reference wavelength 670 nm) by using a microplate ELISA reader (BioRad, Hercules, CA, USA).

BrdU test: After labelling with BrdU for 3 h, the cells were fixed and BrdU incorporation into DNA was measured at 450 nm (reference wavelength 620 nm).

LDH test: The LDH activity of the supernatants was measured at 492 nm (reference wavelength 620 nm). Additionally, a Triton X-100 control was added at a final concentration of 1% for the determination of the maximum release of LDH activity (100%).

Flow cytometry

MCF7 cells (5×10^5/ml) were grown for 24 h in 6-well-plates. After
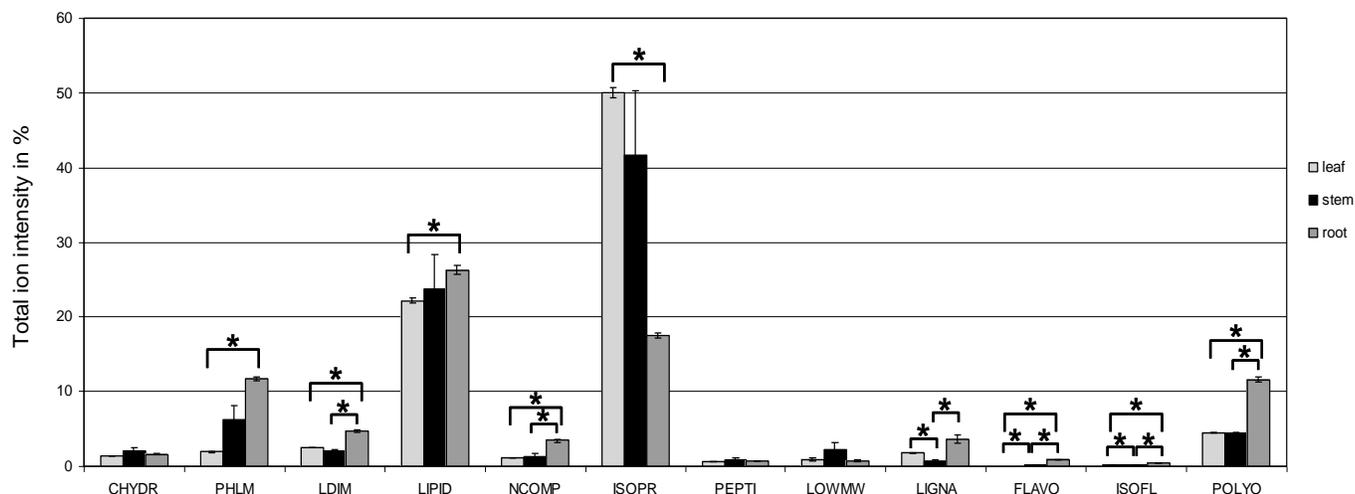
Figure 1. Pyrolysis Field Ionisation Mass Spectrometry: Data (mean ± SE) (standard error) represent the relative abundance of the total ion intensity (% TII) of 12 compound classes in leaf, stem and root extracts from flax (*Linum usitatissimum).* Asterisks (*) indicate significant differences between the extracts of the plant samples. CHYDR: carbohydrates; PHLM: monolignols; LDIM: lignin dimers; LIPID: alkanes, alkenes, aldehydes, alcohols, fatty acids, n-alkyl ester, waxes, fats; NCOMP: N-containing compounds (mainly heterocyclic); ISOPR: isoprenoid compounds (sterols, terpenes, carotinoids); PEPTI: peptides and free amino acids; LOWMW: low molecular compounds; LIGNA: lignans; FLAVO: flavones; ISOFL: isoflavones; POLYO: other polyphenols (for example, suberins, cutins, stilbenes).

the exchange of the medium, only root extract (0.01, 50 and 500 µg/ml) or tamoxifen (50 µg/ml) were added and incubated for 24 h at 37°C and 5% CO_2. Cells were washed with phosphate-buffered saline (PBS), treated with trypsin, centrifuged and washed again. They were treated with 1 mg/ml RNase (Sigma-Aldrich, Germany) at 37°C for 20 min and incubated with propidium iodide (50 µg/ml, Sigma-Aldrich, Germany) for 3 h on ice. Flow cytometry was performed with BD FACSCalibur, equipped with an argon-ion laser of wavelength 488 nm (BD Bioscience). For data acquisition, CellQuest Pro 4.0.1 (BD Bioscience) was used.

Statistical analysis

Statistical analysis was performed by using the Student´s *t*-test for a comparison of the means. Data are presented as mean ± standard deviation (SD). $P < 0.01$ was considered as being statistically significant and is denoted by an asterisk.

RESULTS

**Pyrolysis field ionisation mass spectrometry**

The molecular-chemical screening of leaf, stem and root ethanolic extracts from *L. usitatissimum* by Py-FIMS revealed a broad mass signal pattern with 429, 374 and 442 *m/z* signals (data not shown). The signals were higher than 0.01% of total ion intensity (TII) for leaf, stem and root extract from the flax plant, respectively. The bulk data of summed and averaged mass spectra was condensed to 12 compound classes. The relative abundance of TII is visualised in Figure 1, where obvious differences can be observed between the various plant extracts. Thus, significantly higher percentages of TII

were found in root extracts of *L. usitatissimum* compared with those of the leaf and stem for the 8 compound classes monolignols (PHLM), lignin dimers (LDIM), alkanes (LIPID etc.), N-containing compounds (NCOMP), lignans (LIGNA), flavones (FLAVO), isoflavones (ISOFL) and other polyphenolics (POLYO). These compound classes, with the exception of LIPID, showed up to three times higher relative abundances in the root extract compared with those of the leaf or stem extract (Figure 1).

For the LIPID class, including alkanes, alkenes, n-alkyl ester, aldehydes, alcohols, fatty acids and waxes, the difference of the percentage of TII between the root, the leaf and the stem extract was negligible. The largest difference of TII was determined for the PHLM: about 12% for the root extract versus 2 and 6% for the leaf and stem extract, respectively. POLYO was also twice as high in the root versus the leave and stem extracts. For isoprenoids (sterols, terpenes, carotinoids), the TIIs were ranked in the reverse order. This compound class dominated in leaf (50%) and stem (41%) extracts, but was low in root extract (17%). The compound classes of carbohydrates, peptides and low molecular compounds were identified both with low TII and without significant differences between root, leaf and stem extracts.

Influence of flax extracts on cell vitality

In MCF7 cell lines, low concentrations of leaf, stem and root extracts (0.01 to 100 µg/ml) did not affect cell vitality. However, high extract concentrations (500 and 1000 µg/ml) activated (leaf extract) or inhibited (root extract)

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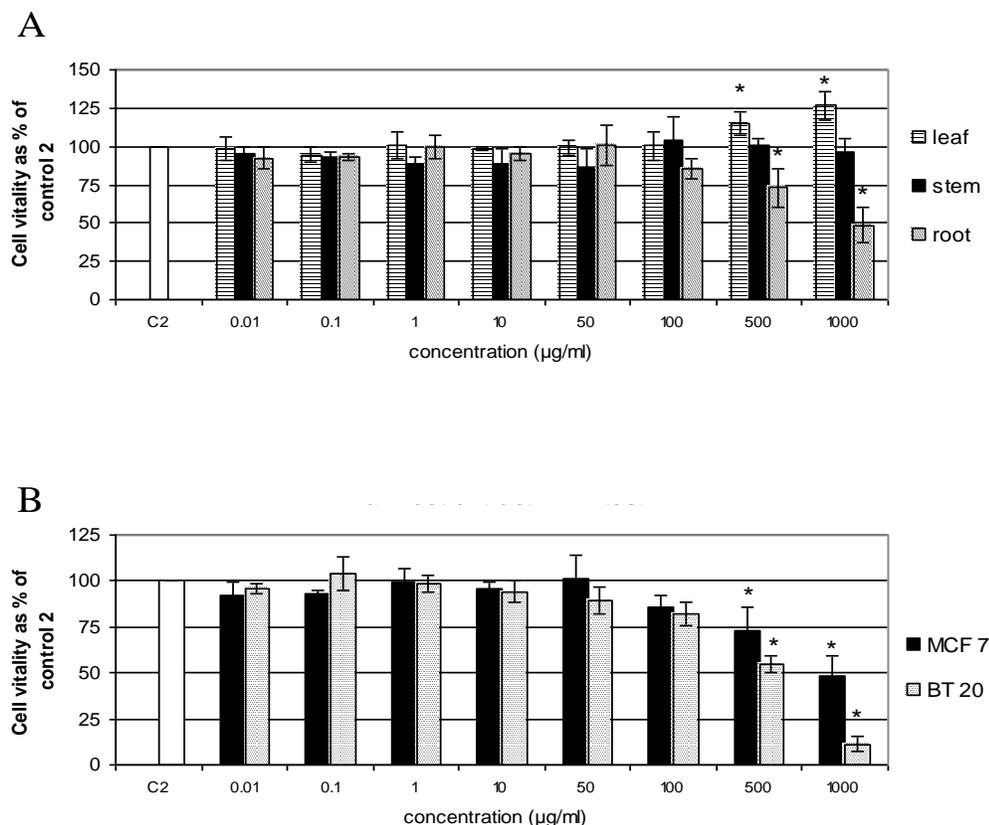
Figure 2. Effect of various concentrations of leaf, stem and root extracts from flax (*Linum usitatissimum)* on the cell vitality (MTT test) of the MCF7 cell line (A) and the effect of various concentrations of flax root extracts on the cell vitality of the MCF7 and BT20 cell lines (B). Data (mean ± SD) represent the relative formation of formazan from MTT in % in comparison with negative control 2 (100%) as obtained in at least 3 experiments. Asterisks (*) indicate significant differences between treated cells and the negative control 2 (*P*<0.01). C2: negative control 2, cells treated with ethanol (final concentration 1%).

cell activity significantly; a mean inhibition of 50% was measured with 1000 µg/ml (Figure 2A). While flax root extract concentrations between 0.01 and 100 µg/ml did not affect the vitality of BT20 cells, 500 and 1000 µg/ml were very effective. Compared to MCF7 cells, BT20 cells were much stronger inhibited (up to 90% at 1000 µg/ml) (Figure 2B). The values of the two negative controls of both cell types did not differ, indicating that 1% ethanol did not inhibit the growth of MCF7 and BT20 cells (data not shown).
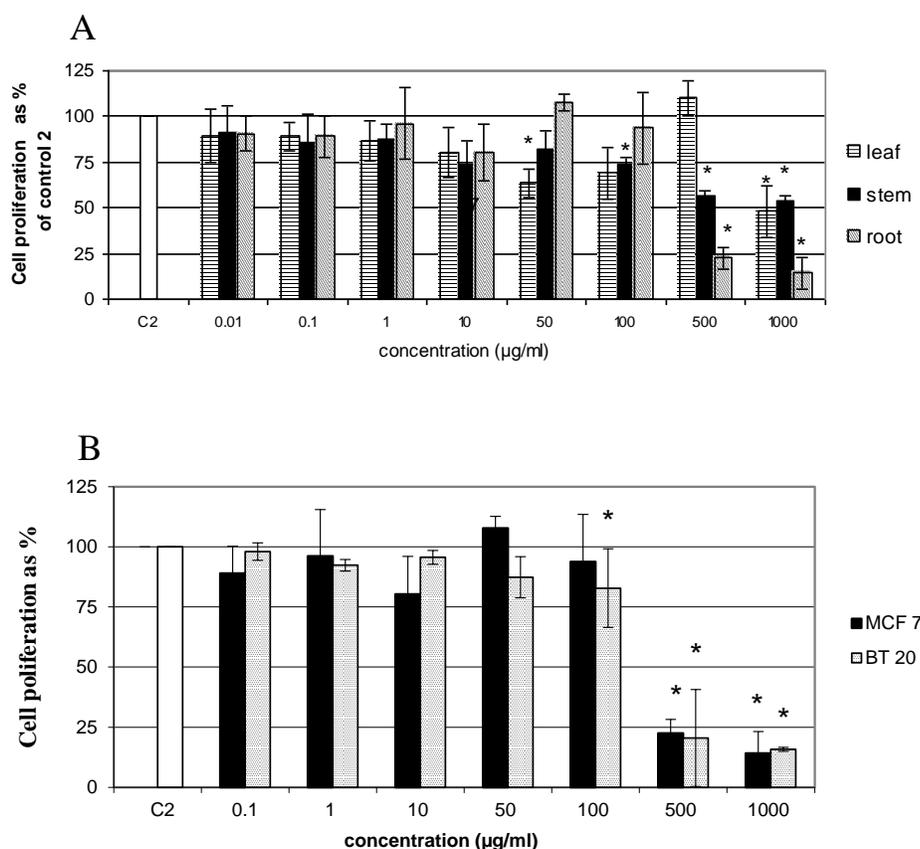
Influence of flax extracts on cell proliferation

Leaf, stem and root extracts did not affect MCF7 cell proliferation (BrdU test) at low concentrations (0.01 to 10 µg/ml) (Figure 3A). At 50 to 500 µg/ml, the leaf extract induced the activation or inhibition of cell proliferation, at 1000 µg/ml inhibition was about 50%. The stem extract was weakly inhibitory at 100 µg/ml, concentrations of 500 and 1000 µg/ml inhibited cell proliferation by 25 to 40%.

At concentrations of 500 and 1000 µg/ml, root extract showed the strongest inhibitory action (up to 85%) of all extracts. Flax root extract concentrations between 0.01 and 50 µg/ml did not influence the growth of BT20 cells, but 100, 500 and 1000 µg/ml inhibited cell proliferation up to 85% (Figure 3B).

Effect of flax extracts on cytotoxicity in MCF7 and BT20 cell lines

Low concentrations (0.01 to 10 µg/ml) of flax leaf, stem and root extracts did not induce cytotoxic effects in MCF7 cell lines (LDH test) (Figure 4A). Higher concentrations of the leaf and stem extracts (> 50 µg/ml) were dose-dependently cytotoxic. In contrast, the whole range of flax root extract concentrations produced only low cytotoxic effects on MCF7 cells (Figure 4B). However, in BT20 cell lines, the lethality reached 60 to 100% after the addition of high concentrations of flax root extract (100 to 1000 µg/ml) (Figure 4B).

A

B

Figure 3. Effect of various concentrations of leaf, stem and root extracts from flax (*Linum usitatissimum)* on the cell proliferation (BrdU test) of the MCF7 cell line (A) and the effect of various concentrations of flax root extracts on the cell proliferation of the MCF7 and BT20 cell lines (B). Data (mean ± SD) represent relative BrdU uptake in % in comparison with negative control 2 (100%) as obtained in at least 3 experiments. Asterisks (*) indicate significant differences between treated cells and the negative control 2 ($P < 0.01$). C2: negative control 2, cells treated with ethanol (final concentration 1%).

Induction of apoptosis

Induction of apoptosis was tested in MCF7 cells. The apoptotic potency of flax root extracts in MCF7 cells was analysed by flow cytometry (Figure 5). Only the high concentration (500 µg/ml extract) resulted in a significant increase of apoptosis (about 25%). For comparison, tamoxifen at 50 µg/ml resulted in 35% apoptosis of the cells.

DISCUSSION

The major classes of phytoestrogens, the isoflavones and lignans, are found at high levels in soybean, flax seed and in various other parts of plants (Kulling and Watzl, 2003; Rickard and Thompson, 1997). Inhibitory effects of isolated isoflavones and lignans on breast and colon cancer were reported in several studies (Rickard-Bon and

Thompson, 2003; Zaizen et al., 2000). However, in this context the different roles of receptor-dependent and independent mechanisms of phytoestrogens are still only poorly investigated. We have therefore embarked on a systematic investigation to test the influence of extracts from three parts of *L. usitatissimum* on human mamma carcinoma cell lines *in vitro*. We have exposed human ER-positive (MCF7) and ER-negative (BT20) breast cancer cell lines to flax extracts. Different responses indirectly could provide information on the involvement of ERs and of phytoestrogens.

High-pressure liquid chromatography (HPLC)-MS analysis has shown by using various extraction methods that root, stem and leaf extracts of *L. usitatissimum* contain more representatives from the lignan domain (for example, secoisolariciresinol, matairesinol, arctigenin) than isoflavones (for example, genistein, daidzein) (Abarzua et al., 2007). In the present study, Py-FIMS has provided evidence that flax root extracts are especially

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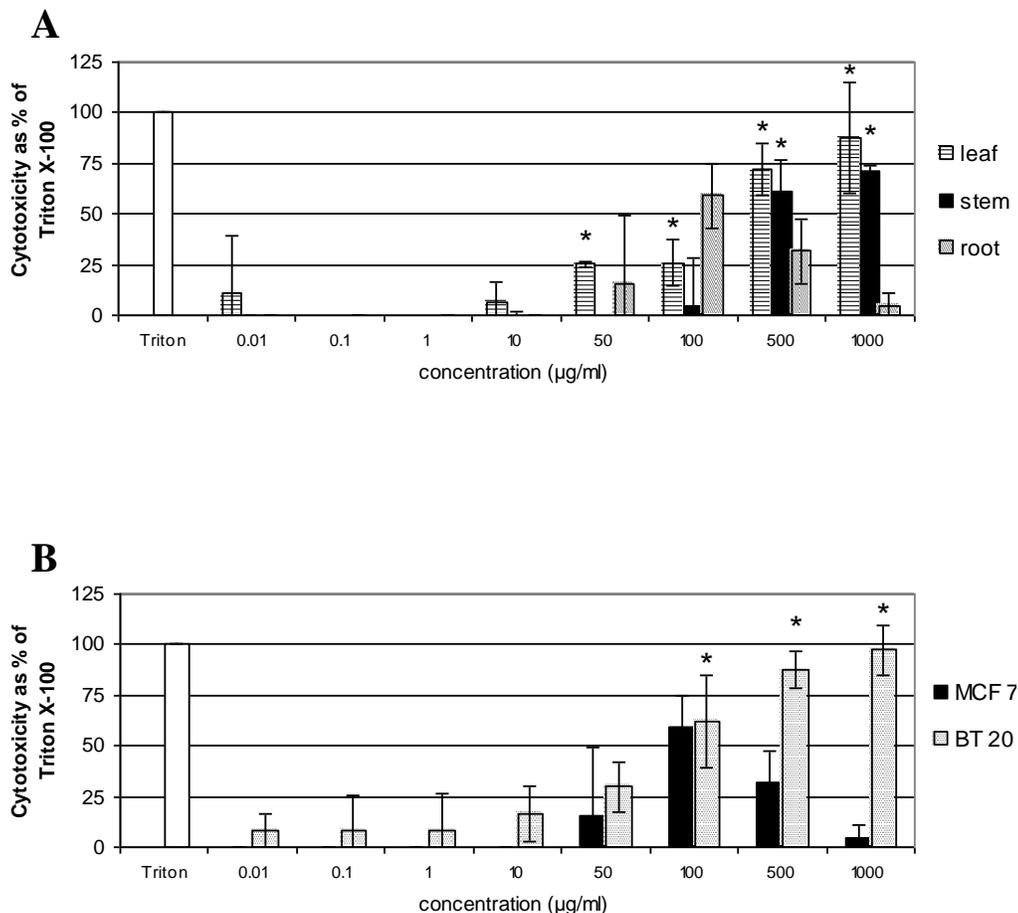
Figure 4. Effect of various concentrations of leaf, stem and root extracts from flax (*Linum usitatissimum)* on cytotoxicity (LDH test) in the MCF7 cell lines (A) and the effect of various concentrations of flax root extracts on cytotoxicity in the MCF7 and BT20 cell lines (B). Data (mean ± SD) represent relative cytotoxicity in % in comparison with negative control 2 and Triton X-100 control (100 %) obtained in at least 3 experiments. Asterisks (*) indicate significant differences between treated cells and the negative control 2 (*P*<0.01). Triton: Triton X-100 control.
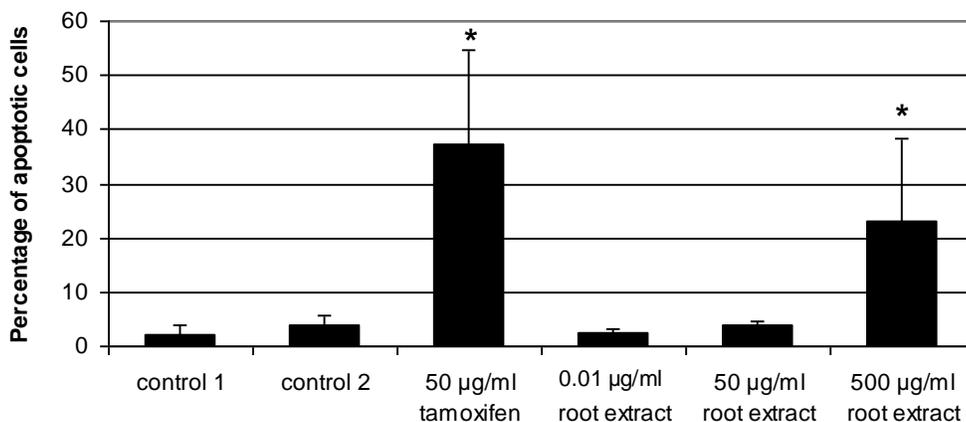
Figure 5. Effect of various concentrations of root extracts from flax (*Linum usitatissimum)* (0.01, 50, 500 µg/ml) and of 50 µg/ml tamoxifen on the percentage of apoptotic MCF7 cells. Data (mean ± SD) represent relative apoptosis in % obtained in at least 3 experiments. Asterisks (*) indicate significant differences between treated cells and the negative control 2 (P<0.01); control 1: untreated cells, control 2: cells treated with ethanol (final concentration 1%).

rich in polyphenols and monolignols (Figure 1), that is contain large amounts (21%) of polyphenols, including lignin dimers, flavones, isoflavones, lignans and related compounds such as suberins, cutins and stilbenes, whereas in leaf and stem extracts they represent less than 9% of all compounds.

Plant cell walls containing high amounts of polyphenols such as suberins and lignins have been suggested as the most likely dietary components being protective against cancer (dietary fiber hypothesis) (Ferguson et al., 2001). Several studies have documented the anti-proliferative and cytotoxic effects of isolated lignans (Awale et al., 2006; Moritani et al., 1996; Singh et al., 2007). As in the present study the flax root extract induced stronger inhibitory effects on cell vitality and proliferation of human breast cancer cells in vitro than leaf and stem extracts (Figures 2A and 3), we suppose that phenolic compounds, for example isoflavones, lignans and monolignols, play an important role in the inhibitory effects. Isoflavones (for example, genistein, daidzein, equol) can alter the expression of genes that are important for cell survival (Moiseeva et al., 2007) and cell cycle (Touny and Banerjee, 2006). They suppress NFκB (Li and Sarkar, 2002) and induce caspase-mediated apoptosis (Casp 7, 9) (Charalambous et al., 2013). For genistein, an increased expression of tumor suppressor genes p21 and p16 and a decreased expression of the tumor promoting genes BMI1 and c-MYC is described (Li et al., 2013).

The cell vitality of ER-positive and ER-negative human breast cancer cell cultures was not affected by low concentrations of flax root extract (0.01 to 100 µg/ml). Significant inhibition in a dose-dependent manner became obvious only after the addition of high concentrations (>100 µg/ml) (Figure 2B). The inhibition of cell vitality by flax root extracts is stronger in the ER-negative cell line BT20 than in the ER-positive MCF7 (Figure 2B). These differences suggest that flax root extracts can affect the growth of the cell lines by both ER-mediated and ER-independent mechanisms of action.

However, the differences may also indicate a higher sensitivity for flax root extracts in the ER-negative BT20 cell lines compared to the ER-positive MCF7 cells. A former study demonstrated an inhibition of cell growth of ER-positive and -negative cell cultures after the application of genistein at IC_{50} values from 6.5 to 12.0 µg/ml (Peterson and Barnes, 1991). An extract from Cimicifuga racemosa reduced the cell proliferation and enhanced the rate of apoptosis in several human breast cancer cell lines, independently of their ER status (Hostanska et al., 2004).

The flax leaf and stem extracts were more cytotoxic than the root extracts (Figure 4A). Leaf extract, in particular, induced an increase of the markers of cell proliferation and vitality at concentrations of 500 and 1000 µg/ml. As only about 25% of MCF7 cells survived the addition of leaf extract, this must reflect an enormous

increase of enzymatic activity in the surviving cells. The increased viability of MCF7 cells after the addition of high concentrations of leaf extracts might be attributable to higher energy-consuming activities before death. Similar observations have been reported for soybean isoflavones (Zhou et al., 1998). Moorghen et al. (1998) have suggested that cells try to compensate for an increased apoptotic rate by an increase in cell proliferation.

With regard to the cytotoxic potential of the flax root extract, the ER status of the cells seems to be important. Whereas the cytotoxicity in BT20 cells increased with rising concentrations of flax root extract in a dose-dependent manner, in the MCF7 cell line addition of lower concentrations such as 50 and 100 µg/ml extract increased cytotoxicity, but higher concentrations decreased cytotoxicity (Figure 4B). About 90 or 98% of the BT20 cells did not survive the application of 500 or 1000 µg/ml root extract, respectively (Figure 4B). This correlates with the strong inhibition of cell vitality of the BT20 cells after the addition of 500 and 1000 µg/ml root extract (Figure 2B). An addition of root extract at 500 µg/ml caused the death of about 30% of MCF7 cells (Figure 4A). This result is supported by flow cytometric analysis, showing a percentage of 25% apoptotic cells after the application of the 500 µg/ml root extract (Figure 5).

These results confirm data of changes of cell adhesion recorded with the Bionas® 2500 analyzing system after the addition of low (0.01, 1, 50 µg/ml) and high (100, 200, 1000 µg/ml) concentrations of root extracts of L. usitatissimum (Abarzua et al., 2010). Low concentrations did not influence adhesion in MCF7 cells, whereas high concentrations resulted in dramatic morphological inhibition. Several studies have described the induction of apoptosis as a response to phytoestrogens. Jo et al. (2005) and Danbara et al. (2005) have shown that the induction of apoptosis is caused by the "human lignan" enterolactone as a result of the metabolism of plant lignans in their experiments. We therefore suggest that the apoptosis of MCF7 cells might be induced by the phenols and monolignols detected in the root extract of L. usitatissimum (Figure 1). In spite of the low cytotoxicity with regard to MCF7 cells, after the addition of 500 µg/ml of root extract significant inhibition of cell vitality and proliferation occurred in up to 25 and 75%, respectively (Figure 2A and 3).

Since the root extract of L. usitatissimum induced a significant inhibition of cell vitality and proliferation without incurring strong cytotoxicity in the human mamma carcinoma cell line MCF7, which might qualify the extract for prevention of hormone sensitive carcinomas, it seems worthwhile to further characterize the presumable phyto-estrogens in the extract and the relevant intracellular processes, in which they are involved. At the moment our investigations resulted in an extract that might possibly be used as supplement for therapy or prevention in future. For this case it is necessary to study the reactions

of normal mamma epithelial cells to addition of flax root extract in further investigations.

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