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Flax-seed Extracts with Phytoestrogenic Effects on a Hormone Receptor-positive Tumour Cell Line

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Abstract. *The higher soy intake in the Asian population compared to Europeans is believed to be an essential factor for the lower incidence of hormone-dependent tumours in Asia. It has already been shown that soya beans, with their ingredients genistein and daidzein from the isoflavonoid group, have protective effects on hormone-caused diseases. Lignans are another, less investigated, group of phytoestrogens. The aim of this study was to investigate the effects of flax-seed, which is typically found in Northern European diets, on the proliferation and hormone production of an estrogen receptor (ER)-positive trophoblast tumour cell line. Materials and Methods: Trophoblast tumour cells of the cell line Jeg3 were incubated with 2 different concentrations of the isolated crude extract of flax-seed and 7 chemically partitioned extract fractions. Untreated cells were used as controls. After 48 h of stimulation, cell proliferation was measured using the BrdU method. The concentrations of hCG and progesterone produced by the trophoblast tumour cells were measured 48 h after stimulation. Extract fractions with antiproliferative effects in the BrdU- test were analysed by HPLC-MS. Results: Our study showed an inhibitory influence of some of the isolated flax-seed fractions on the Jeg3 tumour cells. Proliferation of the Jeg3 cells was decreased by flax-seed fractions I, V, VI and VII in a dose-dependent manner. Inhibition of hCG production by flax-seed extracts III, V, VI and VII was also dose-dependent. Extract fractions V and VI decreased the production of progesterone by 58% to 86%. Some extract fractions showed a stimulating effect on hormone production and cell proliferation. HPLC-MS analysis showed the presence of matairesinol and biochanin A in flax-seed fraction VI. Discussion: Flax-seed seems to have similar inhibitory effects to soya on hormone production and proliferation of hormone-sensitive tumour cells. Our results showed a dose-dependent inhibition by isolated flax-seed extracts on the Jeg3 cell line. Matairesinol and biochanin A seem to be useful candidates for extended tests on other tumour cell lines and normal tissues to evaluate the potential benefit of a lignan-containing therapy in hormone-dependent diseases.*

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Hormone-dependent tumours such as breast cancer have a higher incidence in Western than in Asian countries. This is believed to be due to the higher intake of soy products in the Asian population which contain large amounts of the phytoestrogens genistein and daidzein (1). Both are isoflavones with a close structural similarity to the estrogen molecule so that they can bind to the estrogen receptor (ER). Phytoestrogens have a higher binding affinity to the estrogen receptor β (2). This characteristic enables them to mediate estrogen-like actions on cells expressing ER but with a lesser efficacy than mammalian estrogens (3). Depending on the presence of endogenous estrogen, phytoestrogens may exert both estrogenic and antiestrogenic effects (4, 5). Their prospective anticarcinogenic potency seems to be due to: competition with estradiol for the nuclear type II estrogen- binding site; aromatase inhibition; inhibition of proteine tyrosine kinases; antioxidant effects; stimulation of SHGB synthesis (=decrease the relative amount of free estradiol) and/or inhibition of angiogenesis (1, 6).

Lignans comprise another less investigated group of phytoestrogens. They are found in grains, seeds and legumes. Flax-seed is the richest known source of lignans (7,

Figure 1. *Jeg3 cell line expression of estrogen receptor α.*

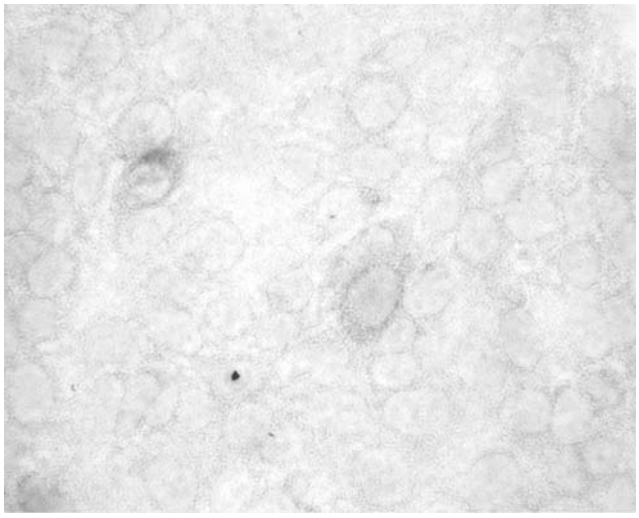

Figure 2. *Jeg3 cell line expression of estrogen receptor β.*

8). It can be cultivated in northern Europe where it is an essential part of the diet. Matairesinol (MATA) and secoisolariciresinol (SECO) are the main plant lignans. It has already been described that MATA and SECO are precursors for the mammalian lignans enterolactone (ENL) and enterodiol (END) (9). Their metabolism depends on the presence of specific human faecal bacteria (10, 11). Scientific opinion varies greatly with regard to the possible carcinogenic or anticarcinogenic role of lignans (6, 13). Some authors support the view that lignans have a stimulating effect on tumour growth (14, 15), while others describe anticarcinogenic properties (14, 16-18).

The aim of this study was to investigate the direct effects of lignan-containing extract obtained from seeds of *Linum usitatissimum* (flax-seed) on the ER-positive tumour cell line Jeg3. Thus, the proliferation and hormone production of the cells were measured in several cell culture test with reference to different concentrations and extract fractions.

Materials and Methods

Cell culture. The chorion carcinoma cell line Jeg3 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL Life Technologies, Paisley, Scotland) with 10% inactivated foetal bovine serum (FCS) (Sigma, Taufkirchen, Germany) without antibiotics and antimycotics, in a water-saturated atmosphere (95 % air; 5% CO_2) at 37˚C.

Lignan extraction from flax-seed. Seeds from *Linum usitatissimum* (15 g) were mechanically pulverized and subsequently defatted with petroleum ether (T 55˚; 56 ml) and extracted with MeOH (90 ml). The extract was suspended in 4 ml of H_2O and partitioned with EtOAc (5 x 4 ml) to give, on drying, 65.5 mg of EtOAc-soluble residue. Column chromatography (CC) fractionation of the residue, using silica gel as the stationary phase and eluting with $CHCl_3$ and MeOH mixtures of increasing polarity (0-50%), afforded 7 fractions. The crude extract and the 7 extract fractions were dried and dissolved in phosphate-buffered saline (PBS) for the cell tests.

Immunocytochemistry. For the immunocytochemical characterization of steroid hormone receptors in trophoblast tumour cells of the cell line Jeg3, the Vectastain®Elite ABC-Kit (Vector Laboratories, Burlingame, USA) was used. Mouse monoclonal antibodies used for the experiments were: mouse anti-human progesterone receptor (Dako, Hamburg, Germany) and mouse anti-human estrogen receptor (Dako). Cytospins of the cells were prepared by centrifugation of 300 µl cell suspension per slide at 1000 rpm for 1 min. The slides were air dried, briefly washed in PBS, fixed in 3.7% formalin for 10 min, washed again in PBS and then for 10 min in cold methanol. The slides were incubated with the ABC-Kit's normal serum for 30 min, followed by incubation with the described primary antibodies for 1 h. After washing with diluted biotinylated serum, a secondary antibody was added for another 30 min. Following incubation with the Reagent ABC for 30 min, H_2O_2/DAB (Dako) was added for 2 min until brown staining of "positive" cells could be observed. The slides were further counterstained with methyl blue and washed with 5% NH_3-solution and alcohol (50%-98%). After xylol treatment, the slides were covered. Positive cells showed a brownish colour and negative controls as well as unstained cells were blue.

Proliferation assay. The effects of the crude extract and the 7 extract fractions on the cell proliferation of Jeg3 cells were tested by using the 5-bromo-2'-deoxy-uridine Labelling and Detection Kit III (Roche). This test is based on the cell ELISA principle. Cells (5x

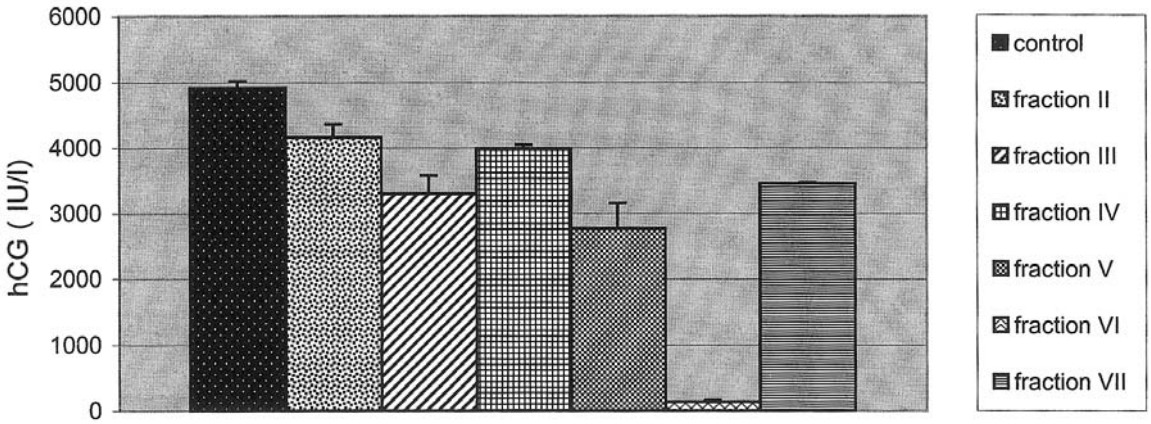

Figure 3. *HCG production of Jeg3 cells after 48 h of stimulation with 300 μg/ml of flax-seed extracts.*

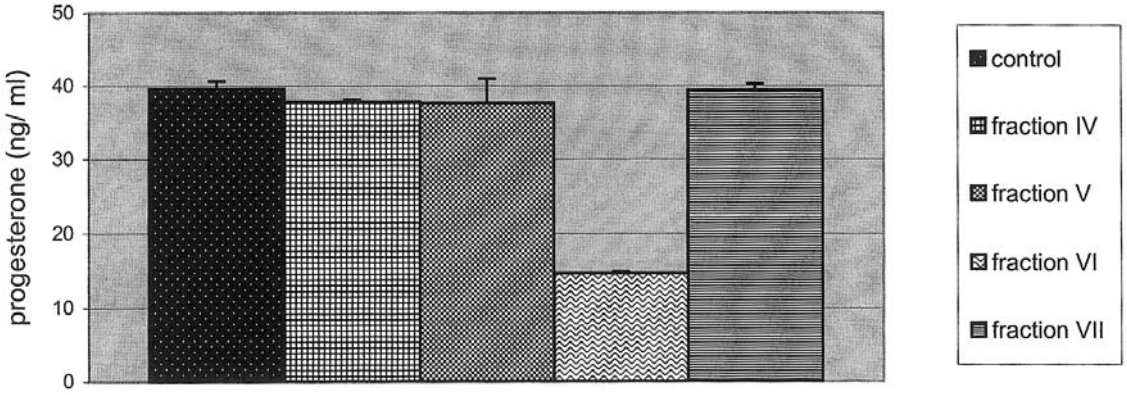

Figure 4. *Progesterone production of Jeg3 cells after 48 h of stimulation with 300 μg/ml of flax-seed extracts.*

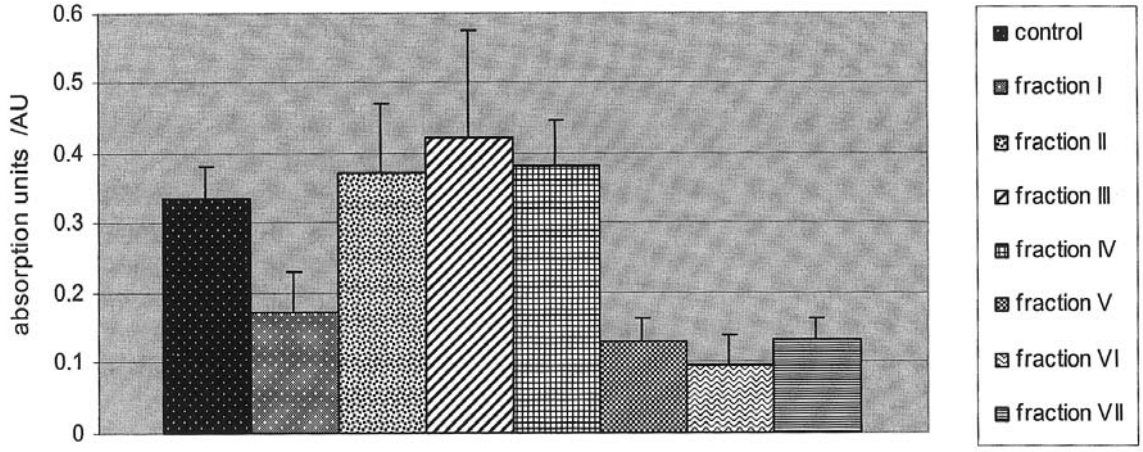

Figure 5. *BrdU incorporation into Jeg3 cells after 48 h of stimulation with 300 μg/ml of flax-seed extracts.*

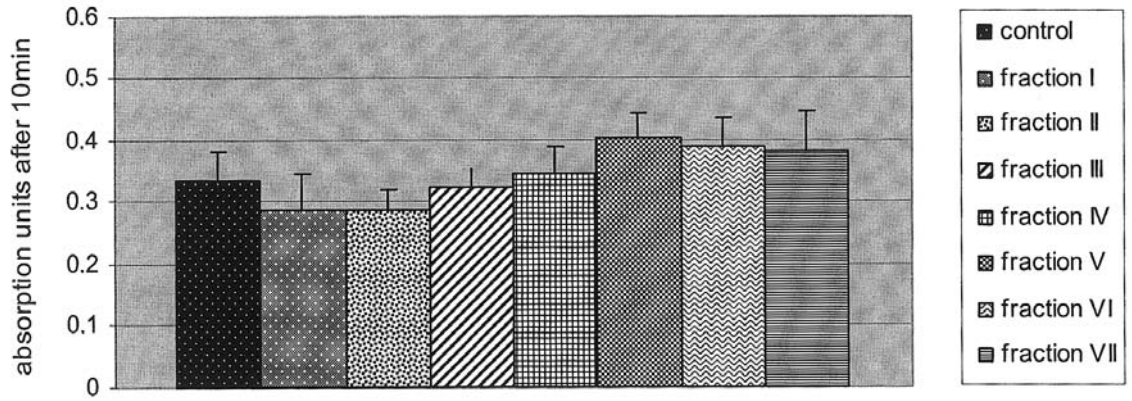

Figure 6. *BrdU incorporation into Jeg3 cells after 48 h of stimulation with 9 μg/ml of flax-seed extracts.*

10^3 / 100 μl) were incubated with 9 mg/ml and 300 μg/ml of the substances in triplicates for each concentration and were cultivated in 96-well plates for 48 h. BrdU labelling reagent was added and the cells were cultured for a further 2 h at 37˚C. The following procedure was practiced according to the manufacturer's protocol.

Determination of progesterone and hCG. Cells ($1x10^6$/ml) were incubated with the crude extract and 7 extract fractions in concentrations of 9 μg/ml and 300 μg/ml in triplicates and cultivated for 48 h. The cell culture supernatant was then removed and stored at –20˚C. At the end of the tests, all solutions were analysed for production of progesterone and hCG. Secretion of hCG and progesterone was determined by the automated hormone analyser SR1 from BioChemImmunoSystems GmbH. Progesterone was analysed by competitive enzyme-immuno assay (EIA) and hCG by immuno-enzymometric assay (RIA), respectively. Specific antibodies were labelled with fluorescein and, after binding to magnetic particles, the separation was performed. Colour intensity was measured at 3 different wavelengths. Sensitivity and the intra-assay coefficient of variation were: progesterone EIA <6.4 nmol/l and 6.8% (at a medium value of 11.5 nmol/l; 5.4% at 65.6 nmol/l, respectively) and hCG immuno-enzymometric assay (IEMA) (1 IU/l, related to 1 IRP 75/537, and 6.4% at 11.1 IU/l, 4.2% at 104 IU/l, respectively).

HPLC- MS. HPLC-MS analysis was performed using a C18 column (Discovery, Supelco) with a mobile phase flow-rate of 0.2 ml per minute and MS determination by LCQ-Advantage (Thermo, Finnegan). Samples were diluted in MeOH (500 μl). Two μl were applied to the column, separated by a gradient of MeOH to AcOH (6%) (start 20% A, 80% B; 15min 80% A, 20% B; 40 min 80% A, 20% B; 50 min20% A, 80% B) and identified by measurement of positive and negative ions within ESI-mode between 80-2000 amu.

Results

The trophoblast tumour cell line Jeg3 was used to test the new chemically isolated flax-seed extracts. The Jeg3 cells exhibited a positive immunocytochemical reaction for ER α and ER β (Figures 1 and 2). An extraction method was developed, with reference to Kinghorn *et al.* (19), for the production of a crude extract and 7 extract fractions from flax-seed (*Linum usitatissimum*). The effect of the extracts on ER-receptor-positive tumour cells was determined by measuring the production of hCG and progesterone as well as cell proliferation of Jeg3 cells, as described in previous studies (20). Flax-seed extracts showed a dose-dependent effect on hormone production. Application of 300 μg/ml of flax-seed crude extract decreased the secretion of hCG by 35% and the secretion of progesterone by 38 %. Chemical fractionation of the flax-seed crude extract by column chromatography gave 7 extract fractions. Application of 300 μg/ml of the extract fractions III, V, VI and VII to Jeg3 cells decreased the production of hCG by 30% to 97% (Figure 3). Extract fraction V decreased the progesterone production by 58%, whereas extract fraction VI decreased the production of progesterone by 86% (Figure 4). All other fractions showed either no effect or had a stimulating influence on the hormone production. The same fractions at the low concentration of 9 μg/ml did not indicate any influence on the hormone production.

The proliferation of Jeg3 cells was inhibited by the flax-seed extracts in a dose-dependent manner, as shown by BrdU ELISA. One μmol of the fractions I, V, VI and VII reduced the tumour cell proliferation significantly by 48% to 72% (Figure 5). The other fractions showed either no effect or had a proliferative influence on the cells (Figures 5 and 6).

The inhibitory effects of flax-seed fractions V and VI were shown for both proliferation and hormone production. Flax-seed fraction VI, as the most active inhibitory fraction, was analysed by HPLC-MS. The phytoestrogens matairesinol and biochanin A were detected in this fraction (VI) by typical mass ranges and their molecular weights.

Discussion

In this study, we observed an antiproliferative and a hormone-decreasing effect on the chorion carcinoma cell line Jeg3 caused by the flax-seed crude extract and some extract fractions, especially fraction VI. This fraction contains the lignan matairesinol and the isoflavone biochanin A. Thus, fraction VI seems to have a specific anticarcinogenic effect, assuming that the detected phytoestrogens have mediated this effect through the estrogen receptor.

Numerous reports on the potential tumour suppressive influence of lignans can be found in recent literature (14, 19, 20). However, they often refer to nutrition studies that discuss the effects only of the metabolized mammalian lignans ENL and END in relation to hormone-dependent cancers (9, 12, 21, 22). It has been assumed that only the mammalian lignans, produced by specific intestinal bacteria, are able to bind to the ER (23). Few studies have examined the direct effect of plant lignans on ER-positive tumour cells, like the used Jeg3 cell line (15, 24, 25).

Adlerkreutz *et al.* (14) implied that the estrogenic or antiestrogenic effect of phytoestrogens depends on the level of endogenous estrogens. Phytoestrogens may act as antagonists in pre-menopausal women and replace endogenous estrogen in the post menopause. This capability is already used in the hormone replacement therapy (HRT); herbal remedies with soy-based ingredients have become popular in recent years instead of the conventional therapy with estrogen. Soya products are considered to be gentler and to have fewer side-effects due to their natural origin, although sufficient data about dosages and long-term studies are still missing (19, 26).

Messina *et al.* (13) reported that phytoestrogens can provide a stimulating effect on breast cancer cell growth at low doses and inhibit tumour cell proliferation at higher doses. This aspect is also supported by our results with regard to the dose-dependent effect of the flax-seed extracts.

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