Effects of Phytoestrogen Extracts Isolated from Flax on Estradiol Production and ER/PR Expression in MCF7 Breast Cancer Cells

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Abstract. *Background: In this study, we tested the effects of crude extracts from flax (Linum usitatissimum) on the production of estradiol and expression of estrogen receptor (ER) and progesterone receptor (PR) in human breast cancer MCF7 cells. Materials and Methods: Isoflavone and lignan extracts from flax plant Linum usitatissimum were obtained, using different extraction methods. Breast carcinoma cells (MCF7) were incubated with various concentrations of the isolated extracts. Untreated MCF7 cells were used as controls. Supernatants were removed at designated times and tested for estradiol with an ELISA method. Furthermore, the effect of phytoestrogen extracts on the production of ERa and ERβ as well as on PR was examined. Results and Conclusion: Production of estradiol is elevated in MCF7 cells in a concentration-dependent manner after stimulation with isoflavone and lignan extracts from Linum usitatissimum. Expression of ERα is up-regulated after stimulation with lower concentrations of lignan extracts from flax plants, unchanged at median concentrations and down-regulated at high concentrations. Expression of ERβ is down-regulated in a concentration-dependent manner.*

Phytoestrogens are polyphenolic compounds that are naturally occurring in plants. Their major biological role is to prevent the plants from stress or to take part in the plant's defense mechanisms (1-6). A common attribute is their oestrogenic activity, because they are able to bind to both oestrogen receptors in humans (ERα and ERβ), but with a higher affinity to ERβ than steroidal oestrogens (7). In spite of their ability to bind to ERs, their physiological effect is much weaker than that of human oestrogen. Their activity is reduced by 10^2 to 10^5 compared with 17-β-oestradiol (8-10).

Phytoestrogens are classified into three groups: isoflavones, lignans and coumestans. Isoflavones belong to the major group of flavonoids and are often found in legumes such as soy and lens, but also in many types of clover. From more than 1000 isolated compounds, genistein and daidzein are the most commonly investigated isoflavones (11). Because of their structural diversity, lignans occur in many food types including grains, drupes and onions (12). Lignans are also present in wholemeal cereals, linseed and wild berries (13). The mammalian lignans enterolactone and enterodiol, mostly found in human plasma and urine, are formed by the conversion of dietary precursors such as secoisolariciresinol and matairesinol in the colonic microflora (14). Based on the structural similarities to estrogens and on *in vitro* studies, several health effects have been hypothesized for phytoestrogens. Many epidemiological studies correlated high dose consumption of soy isoflavones with multiple beneficial effects on breast and prostate cancer, menopausal symptoms, osteoporosis and many other hormone dependant diseases (2). Numerous *in vitro* cell culture studies and *in vivo* animal experiments have also shown that phytoestrogens are able to inhibit tumour growth (15). In comprehensive reviews on the potential of phytoestrogens to reduce tumour growth, Fournier *et al*. (16), Westcott and Muir (17) and Thompson (18) noted that in *in vitro* cell studies and *in vivo* animal studies, the addition of soybean products and flaxseed products reduced tumour incidence or cell division in tumour models of the breast,

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Key words: MCF7, flax roots, flax leaves, progesterone, ER, PR.

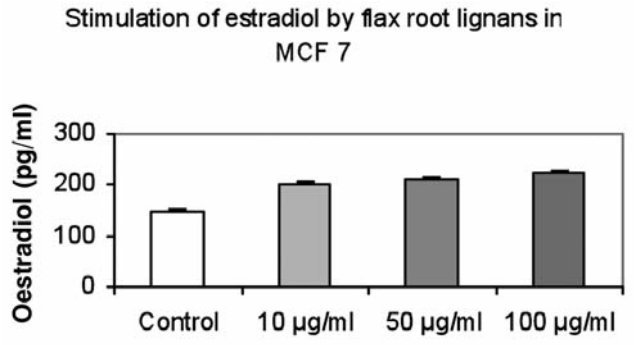

Figure 1. *Stimulation of oestradiol production by flax root lignans in MCF7 cells in vitro.*

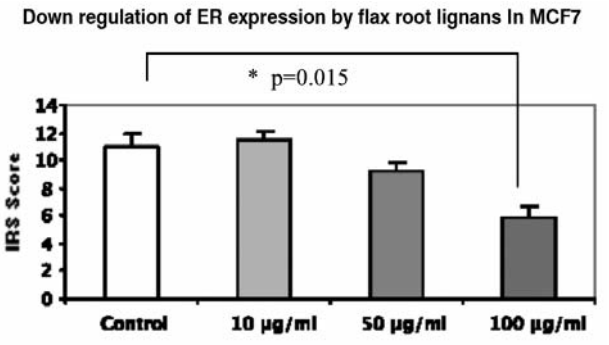

Figure 6. *Summary of staining results of ERα after stimulation with flax root lignans in MCF7 cells in vitro.*

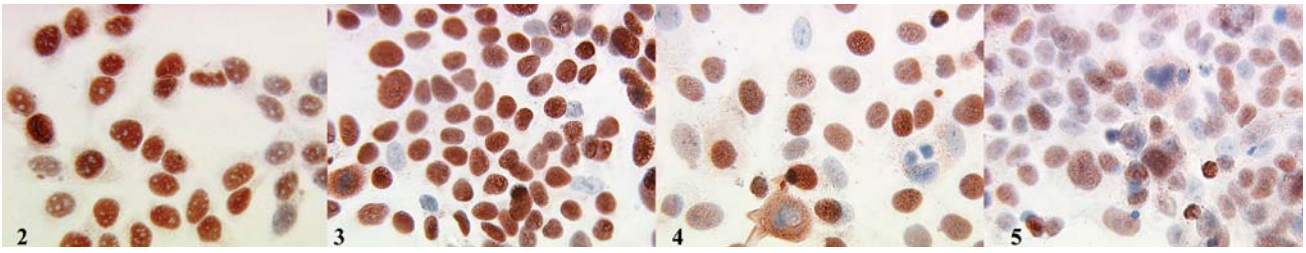

Figure 2. *Expression of ERα in MCF7 cells in vitro.* Figure 3. *Expression of ERα in MCF7 cells in vitro after stimulation with 10 µg/ml flax root lignans.* Figure 4. *Expression of ERα in MCF7 cells in vitro after stimulation with 50 µg/ml flax root lignans.* Figure 5. *Expression of ERα in MCF7 cells in vitro after stimulation with 100 µg/ml flax root lignans.*

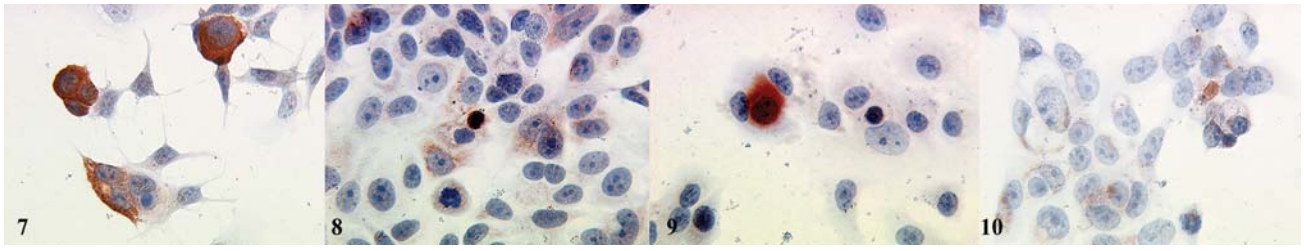

Figure 7. *Expression of ERβ in MCF7 cells in vitro.* Figure 8. *Expression of ERβ in MCF7 cells in vitro after stimulation with 10 µg/ml flax root lignans.* Figure 9. *Expression of ERβ in MCF7 cells in vitro after stimulation with 50 µg/ml flax root lignans.* Figure 10. *Expression of ERβ in MCF7 cells in vitro after stimulation with 100 µg/ml flax root lignans.*

colon, prostate, liver, oesophagus and lung. The aim of the present study was to isolate isoflavones and lignans from flax roots, stems, leaves and seeds and to test the effects of the isolated phytoestrogens on oestradiol production as well as the expression of ER and progesterone receptors (PR) in MCF7 breast cancer cells *in vitro*.

Materials and Methods

Cell culture. The human breast cancer cell line MCF7 was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL Life Technologies, Paisley, Scotland) with 10% inactivated foetal bovine serum (FCS gold) with antibiotics and antimycotics in a water saturated atmosphere (95% air, 5% CO_2) at 37°C. This cell culture medium with 10% FCS did not contain any measurable amounts of oestrogen and progesterone as determined with an automated hormone analyzer Immulite (DPC Biermann, Freiburg, Germany).

Preparation of phytoestrogen extracts from roots, stems, leaves and seeds of Linum usitatissimum. The flax roots and leaves were obtained from the Agricultural Research Institution Mecklenburg-Vorpommern (LUFA), Rostock, Germany. Seed growing was realized under field conditions. When the plants reached a height of

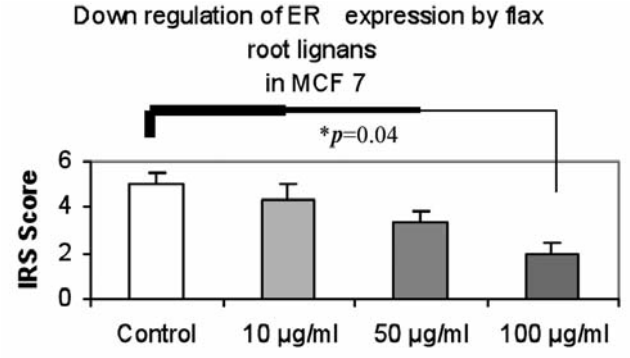

Figure 11. *Summary of staining results of ERβ after stimulation with flax root lignans in MCF7 cells in vitro.*

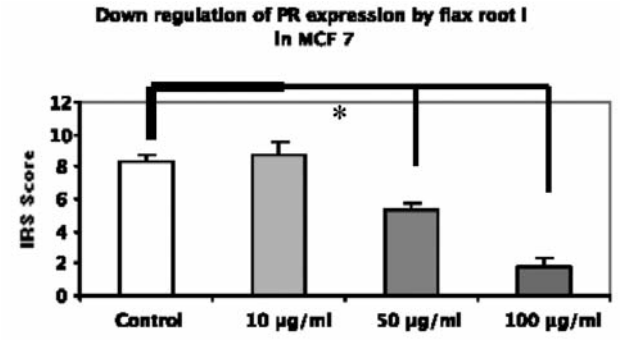

Figure 21. *Summary of staining results of PR after stimulation with flax root lignans in MCF7 cells in vitro.*

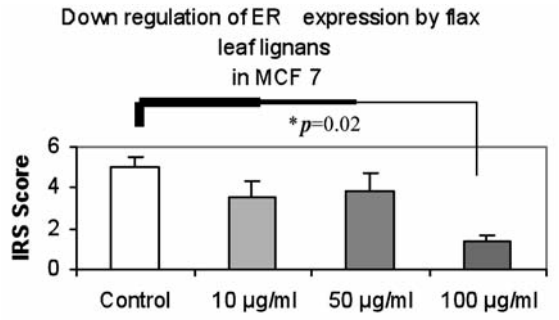

Figure 16. *Summary of staining results of ERβ after stimulation with flax leave lignans in MCF7 cells in vitro.*

about 1 m, they flowered and the leaves, stems and roots were harvested. These plant organs were frozen in liquid nitrogen and stored at –70°C until extraction. Different extraction methods were performed to obtain either isoflavones or lignans. The procedure for the extraction of isoflavones was adapted from Franz and Köhler (19) and modified by Matscheski (12). The method for the extraction of lignans was performed according to Luyengi *et al*. (20) and modified by Matscheski (12).

Determination of oestradiol. MCF7 cells ($0.4x10^6$/5 ml supplemented cell culture medium) were grown on quadriperm tissue slides for up to 96 hours in the absence (controls) and presence of the isolated phytoestrogen extracts. Extracts from flax

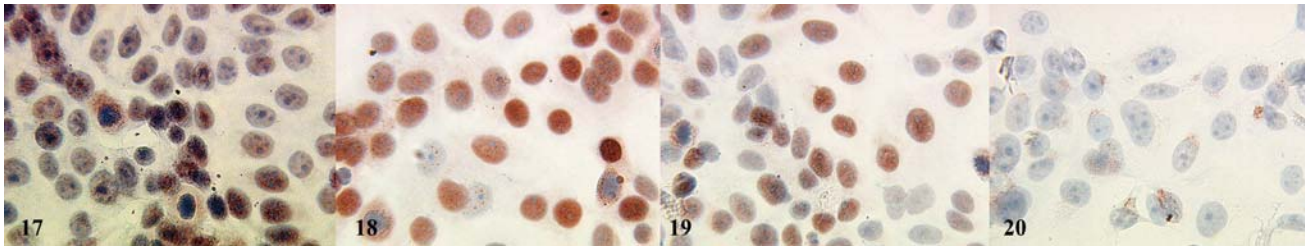

Figure 17. *Expression of PR in MCF7 cells in vitro*. Figure 18. *Expression of PR in MCF7 cells in vitro after stimulation with 10 µg/ml flax root lignans*. Figure 19. *Expression of PR in MCF7 cells in vitro after stimulation with 50 µg/ml flax root lignans*. Figure 20. *Expression of PR in MCF7 cells in vitro after stimulation with 100 µg/ml flax root lignans*.

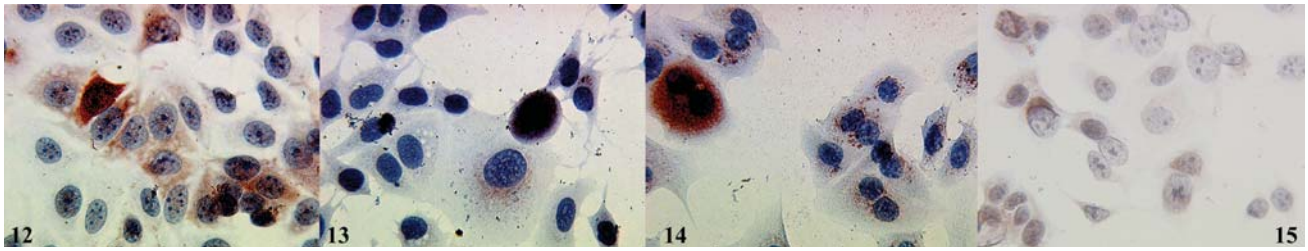

Figure 12. *Expression of ERβ in MCF7 cells in vitro*. Figure 13. *Expression of ERβ in MCF7 cells in vitro after stimulation with 10 µg/ml flax leave lignans*. Figure 14. *Expression of ERβ in MCF7 cells in vitro after stimulation with 50 µg/ml flax leave lignans*. Figure 15. *Expression of ERβ in MCF7 cells in vitro after stimulation with 100 µg/ml flax leave lignans*.

were resuspended in PBS and DMSO or ethanol before being added to 5 ml of the cells giving final concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml, respectively. In addition, oestradiol or phytoestrogen extracts (dextran encapsulated; Sigma, Deisenhofen, Germany) were added to the cells giving final concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml. After 96 hours, a 1 ml cell culture supernatant was removed and all solutions were analysed for production of oestradiol. Secretion of oestradiol was determined by automated hormone analyzer Immulite 2000 from DPC Biermann GmbH, Freiburg, Germany. Oestradiol was analysed by competitive enzyme-immuno assay (EIA). Specific antibodies were labelled with fluorescein and after binding to magnetic particles the separation was performed. Colour intensity was measured at three different wavelengths. Samples with oestradiol were diluted 1:3 with hormone-free diluents, due to the intense colour of the culture medium.

Immunocytochemistry. MCF7 cells were grown on quadriperm tissue slides for up to 96 hours in the absence (controls) and presence of the isolated phytoestrogen extracts. Extracts from flax were re-suspended in PBS, DMSO or ethanol before being added to 5 ml of the cells yielding final concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml, respectively. In addition, oestradiol (dextran encapsulated; Sigma) was added to the cells giving final concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml.

For the immunocytochemical characterization of steroid hormone receptors in human MCF7 breast cancer cells, the Vectastain® Elite ABC-Kit (Mouse IgG)(Vector Laboratories, Burlingame, USA) was used. The mouse monoclonal antibodies used for these experiments were: mouse human PR (Dako, Hamburg, Germany), mouse anti-human ERα (Dako) and mouse anti-human ERβ (Serotec, Düsseldorf, Germany). The slides were briefly washed in PBS, fixed in acetone for 10 min and air dried. Then the slides were washed in PBS (pH 7.2 – 7.6) for five minutes and incubated with the ABC-Kit's normal serum for 60 min in a water saturated chamber, followed by incubation with the described primary antibodies for one hour. After washing in PBS/Brij for 5 min the slides were incubated again with diluted biotinylated secondary antibody for another 30 min. After washing in PBS/Brij for 5 min the slides were incubated with the Reagent ABC for 30 min, washed in PBS for 5 min and AEC substrate was added for 15 min until brown staining of positive cells could be observed. The slides were counterstained with Mayer's acidic hematoxylin for 30 s and rinsed with water. Afterwards the slides were covered with Aquatex. Positive cells showed a brownish colour and negative controls as well as unstained cells were blue.

Statistical analysis. Statistical analysis was performed using the Wilcoxon's signed rank tests for comparison of the means. $P<0.05$ was considered statistically significant. The intensity and distribution patterns of the specific immunocytochemical staining was evaluated using a semi-quantitative method (IRS score) as previously described (21). The IRS score was calculated as follows: IRS = SI x PP, where SI is the optical staining intensity (graded as 0, no staining; 1, weak staining; 2, moderate staining and 3, strong straining) and PP the percentage of positively stained cells. The PP was estimated by counting approx. 100 cells and it was defined as 0, no staining; 1, <10% staining; 2, 11-50% staining; 3, 51-80% staining and 4, >80% staining. The Mann-Whitney rank-sum test was used to compare the means of the different IRS scores (22).

Results

Determination of oestradiol. The cell line MCF7 does produce estradiol. Oestradiol concentration in phytoestrogen stimulated cells and unstimulated controls was measured. In this cell line only the crude lignan extracts from flax root cultured at concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml stimulated oestradiol production in comparison to unstimulated cells (Figure 1). Differences in oestradiol production between stimulated and unstimulated cells were statistically significant in all cases (p=0.012). None of the other crude extracts from flax had any significant stimulation or inhibition of the production of oestradiol.

Immunocytochemistry. MCF7 cells were analysed for expression of ERα, ERβ and PR after stimulation with several concentrations of phytoestrogens, oestradiol and in unstimulated controls.

Figure 2 shows that MCF7 cells strongly expressed ERα. Expression of ERα was inhibited by addition of flax root lignans (Figures 3-5). A significant inhibition was obtained with 100 μg/ml flax root lignans (p=0.015). A summary of staining results is presented in Figure 6.

In contrast to ERα, MCF7 cells showed median expression of ERβ (Figure 7). Expression of ERβ was inhibited by the addition of flax root lignans (Figures 8-10). A significant inhibition was obtained with 100 μg/ml flax root lignans (p=0.04). A summary of staining results is presented in figure 11. Also flax leave lignan extracts inhibited ERβ expression. Unstimulated MCF7 cells showed again median expression of ERβ (Figure 12). Expression of ERβ was inhibited by addition of flax root lignans (Figures 13-15). Significant inhibition was obtained with 100 μg/ml flax root lignans (p=0.02). A summary of staining results is presented in Figure 16.

MCF7 cells also showed median expression of PR (Figure 17). There was a non-significant rise of PR expression with 10 μg/ml flax root lignans (Figure 18), higher concentrations of flax root lignans (50 μg/ml, Figure 19) and 100 μg/ml (Figure 20) showed significant inhibition of PR expression (p=0.004). A summary of these staining results is presented in Figure 21.

Discussion

The results obtained in this study showed that lignan extracts from flax *Linum usitatissimum* can stimulate the production of estrogen production in MCF7 cells. In addition we also observed a down-regulation of ERβ receptor expression and down-regulation of PR expression in MCF7 cells after treatment. The expression of ERα was also significantly altered in MCF7 cells after stimulation with extracts from *Linum usitatissimum,* and down-regulation. A down-regulation of ERα expression was also observed.

In a former study we evaluated the interaction between phytoestrogens (genistein and daidzein) and chorion carcinoma cell lines (BeWo and Jeg3). These tumour cell lines express ERα and ERβ and in addition produce a variety of specific hormones such as progesterone and hCG (11). We were able to show that high doses of the phytoestrogens genistein and daidzein (1-2 μmol/ml) reduced the cell proliferation and the production of the steroid hormone progesterone. In addition, high doses of daidzein reduced the production of hCG in both cell types tested. High doses of genistein had no influence on the hCG production in BeWo and Jeg3 cells. Surprisingly low doses of daidzein and genistein induced both cell types to produce high amounts of hCG. Therefore, we assumed that in parallel measurements of progesterone but not hCG and in addition cell proliferation give comparable results in dose-dependent effects of phytoestrogens in Jeg3 and BeWo cell cultures. Our group has previously shown that crude flax seed extracts containing the lignans matairesinol and biochanin A decreased the proliferation and production of progesterone and hCG in Jeg3 cells (23). We also showed that these cells express both estrogen receptors (ERα and ERβ). In another study the distribution patterns of steroid hormone receptors (ER, PR) and glycodelin A (GdA) expression of proliferative endometrial glandular cells after stimulation with phytoestrogens genistein and daidzein were evaluated. ER expression showed a significant decline with genistein, whereas PR expression increased significantly with genistein. GdA did not show any significant expression under genistein stimulation. Stimulation with daidzein resulted in no statistically significant alterations in ER expression, whereas the PR and GdA expression significantly increased (24).

The results obtained in this study suggest that potential phytoestrogens isolated from flax leaves and roots can significantly stimulate estrogen production in MCF7 breast cancer cells. This study also demonstrates that a useful breast cancer cell culture model for testing new types of phytoestrogens can be established. In addition, inhibition of PR, and ERα and ERβ expression could be determined in the cells used. Measurement of oestrogen in cell culture supernatants can therefore be used as an indicator for newly isolated potential phytoestrogens in breast cancer cells. Additional experiments have to be performed in order to identify the structure of the potential phytoestrogens.

References

1 Czerwiecki L: Plants as a source of natural harmful substances. Rocz Panstw Zakl Hig *56*: 215-227, 2005.

2 Branca F and Lorenzetti S: Health effects of phytoestrogens. Forum Nutr pp. 100-111, 2005.

3 Hao HD and He LR: Mechanisms of cardiovascular protection by resveratrol. J Med Food 7: 290-298, 2004.

4 Cross HS *et al*: Phytoestrogens and vitamin D metabolism: a new concept for the prevention and therapy of colorectal, prostate, and mammary carcinomas. J Nutr *134*: 1207S-1212S, 2004.

5 Kallay E *et al:* Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. J Nutr *132*: 3490S-3493S, 2002.

6 Yu O *et al*: Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol *124*: 781-794, 2000.

7 Setchell KD and Cassidy A: Dietary isoflavones: biological effects and relevance to human health. J Nutr *129*: 758S-767S, 1999.

8 Kuiper GG *et al*: Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology *139*: 4252-4263, 1998.

9 Miksicek RJ: Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. J Steroid Biochem Mol Biol *49*: 153-160, 1994.

10 Price KR and GR Fenwick: Naturally occurring oestrogens in foods--a review. Food Addit Contam *2*: 73-106, 1985.

11 Plessow D *et al*: Effects of phytoestrogens on the trophoblast tumour cell lines BeWo and Jeg3. Anticancer Res *23*: 1081-1086, 2003.

12 Matscheski A: Untersuchungen zur Wirkung von Pflanzenextrakten aus *Linum usitatissimum, Secale cereale* und *Pisum sativum* auf die Zellproliferation und Progesteronproduktion von humanen Trophoblasttumorzellen. 2003, Universität Rostock, Fachbereich Biowissenschaften, Abteilung Biochemie.

13 Mazur W and Adlercreutz H: Overview of naturally occurring endocrine-active substances in the human diet in relation to human health. Nutrition *16*: 654-658, 2000.

14 Begum AN *et al*: Dietary lignins are precursors of mammalian lignans in rats. J Nutr *134*: 120-127, 2004.

15 Adlercreutz H: Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect *103*: 103-112, 1995.

16 Fournier DB, Erdman JWJr. and Gordon GB: Soy, its components, and cancer prevention: a review of the *in vitro*, animal, and human data. Cancer Epidemiol Biomarkers Prev *7*: 1055-1065, 1998.

17 Westcott ND and Muir AD: Flax seed lignan in disease prevention and health promotion. Phytochemistry Reviews *2*: 401-417, 2003.

18 Thompson R-BS: The role of flaxseed lignans in hormone-dependant and independant cancer, in Flax, the genus *Linum*, Westcott ND and Muir AD (eds.). London: New York Taylor & Francis Inc., 2003.

19 Franz GK, Köhler H, Allgemeine Nachweismethoden für Flavonoide in Drogen, in Drogen und Naturstoffe, F.G.a.K. H, Editor. 1992, Berlin, Springer-Verlag: pp. 120.

20 Luyengi L *et al*: A lignan and four terpenoids from *Brucea javanica* that induce differentiation with cultured HL-60 promyelocytic leukemia cells. Phytochemistry *43*: 409-412, 1996.

21 Remmele W and Stegner HE: Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe *8*: 138-140, 1987.

22 Mylonas I *et al*: Immunohistochemical analysis of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in normal human endometrium. Acta Histochem *106*: 245-252, 2004.

23 Waldschlager J *et al*: Flax-seed extracts with phytoestrogenic effects on a hormone receptor-positive tumour cell line. Anticancer Res *25*: 1817-1822, 2005.

24 Mylonas I *et al*: Immunohistochemical expression of steroid receptors and glycodelin A in isolated proliferative human endometrial glandular cells after stimulation with tamoxifen and phytoestrogens (genistein and daidzein). Anticancer Res *23*: 1119-1125, 2003.

Received August 19, 2009
Revised April 6, 2010
Accepted April 4, 2010