Effects of Phytoestrogen Extracts from *Linum usitatissimum* on the Jeg3 Human Trophoblast Tumour Cell Line

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Abstract. *Background: Phytoestrogens are a diverse group of nonsteroidal plant compounds which have similar effects to endogenous estrogens in humans and have been ascribed potential anticarcinogenic activities. We tested the effects of phytoestrogen extracts from different plant organs of flax, Linum usitatissimum, on cell proliferation in trophoblast tumour cells of the cell line Jeg3. Materials and Methods: Phytoestrogen extracts were prepared from leaves, stems and roots of L. usitatissimum using different extraction methods. The isolated phytoestrogens were identified using HPLC-MS analysis. The influence on cell proliferation (MTT test) was determined in the trophoblast tumour cells, Jeg3. Results: Cell proliferation of trophoblast tumour Jeg3 cells was significantly affected by the phytoestrogens isolated from leaves, stems and roots of L. usitatissimum. Root extracts inhibited Jeg3 cell growth significantly. Conclusion: A cell culture model system of the human trophoblast tumour cell line, Jeg3, was established to test the effect of potential phytoestrogens on cell proliferation. It was shown that the roots of L. usitatissimum contain measurable concentrations of lignans and isoflavones.*

Phytoestrogens are a diverse group of nonsteroidal compounds synthesized by plants. The major classes of phytoestrogens are the isoflavones and lignans found at high levels in legumes such as soybean, chickpea, clover and in various plant parts, including roots, stems, leaves, flowers, fruits and seeds (1-3). Of more than 1000 types of isoflavones, genistein and daidzein are the most commonly investigated isoflavones (4, 5). The mammalian lignans enterolactone and enterodiol, commonly found in human plasma and urine, are formed by the conversion of dietary precursors such as secoisolariciresinol and matairesinol by colonic microflora (6). Numerous *in vitro* cell culture studies and *in vivo* animal experiments demonstrated that phytoestrogens can inhibit tumour growth (1, 7). In comprehensive reviews on the potential of phytoestrogens to reduce tumour growth, Fournier *et al.* (8), Rickard-Bon and Thompson (1), and Westcott and Muir (9) noted that in *in vitro* cell studies and *in vivo* animal studies, the addition of soybean and flaxseed products reduced tumour incidence or cell multiplicity in tumour models of the breast, colon, prostate, liver, oesophagus and lung.

The aim of the present study was to isolate and identify potential phytoestrogens from different parts of the flax plant *L. usitatissimum* and to test their effect on cell proliferation in Jeg3 trophoblast tumour cells.

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Key Words: Flax, *Linum usitatissimum*, phytoestrogens, Jeg3, cell proliferation.

Materials and Methods

Preparation of phytoestrogen extracts from leaves, stems and roots of L. usitatissimum. The seeds from *L. usitatissimum*, cultivar Barbara, were obtained from the Agricultural Research Institution Mecklenburg-Vorpommern (LUFA), Rostock, Germany. The seeds were sown on soil and grown under field conditions. When the plants reached a height of 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 till extraction.

Different extraction methods were performed to obtain either isoflavones or lignans from the various plant organs of *L. usitatissimum*.

(i) Extraction of isoflavones. This was adapted from the method of Franz and Köhler (10) and modified by Matscheski (11). Plant material (8 g) was ground in a mortar with liquid nitrogen and extracted with methanol (160 ml). After addition of distilled water (160 ml), concomitant substances were separated by addition of petroleum ether. The aqueous-methanol solution was evaporated to dryness. Additionally, aliquots were hydrolyzed with HCl (1 M) according to Dorbritz (12).

(ii) Extraction of lignans. This was performed according to Luyengi *et al.* (13) and modified by Matscheski (11). Plant organs (8 g) were ground in a mortar with liquid nitrogen and extracted with methanol (80 ml). The extract was suspended in

2 ml of distilled water and partitioned with ethyl acetate (five times) to give, on drying, an ethyl acetate soluble residue. In addition aliquots were hydrolyzed with HCl (1 M) according to Dorbritz (12).

(iii) Extraction of cytotoxic lignans. Cytotoxic lignans such as podophyllotoxin were extracted according to Windhövel *et al.* (14 (lignan/toxin extraction)). A fine powder (4 g) of lyophilized plant material was extracted with methanol (60 ml). After addition of distilled water (180 ml) and *o*-phosphoric acid to adjust the pH to 5.0 the sample was treated with β-glucosidase (20 mg) at 35˚C for 1 h. Methanol (360 ml) was added and after centrifugation, the supernatant was evaporated to complete dryness. Additionally, aliquots were prepared without the hydrolysis step.

In addition, a rapid microwave extraction method was performed: 5 g of the plant material was cut, dissolved in 20 ml methanol and extracted in a microwave extraction system (MES 1000) (CEM, Kamp Lintford, Deutschland) at 60˚C for 10 min. After this procedure the samples were concentrated to complete dryness.

Identification of isolated phytoestrogens with HPLC-MS. The phytoestrogen extracts were dissolved in methanol and used for analysis. Chromatographic separation of the isolated phytoestrogen fractions was performed using reversed-phase HPLC using a gradient elution program: 0.2 ml/min, 20% methanol (A), 80% water with 0.1% formic acid (B), linearly to 80% A: 20% B in 15 min, followed by a hold for 25 min to reach initial conditions for an additional 10 min. A Discovery C18 (15 cm x 2.1 mm) column produced by Supelco (Taufkirchen, Germany) was used.

For MS analysis an LCQ-Advantage (Thermo Finnigan, San Jose, USA) mass spectrometer was used. Identification of the compounds was obtained by ion trap technology, using the ESI mode and positive ion. The source voltage was 4.5 kV and a mass range of 150-2000 amu was used for detection.

Cell culture. The chorion carcinoma cell line Jeg3 was obtained from the Department of "Human and Animal Cell Cultures" Braunschweig, Germany. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% inactivated fetal calf serum and antibiotics (1% penicillin/streptomycin) and antimycotic (0.5% amphotericin) at 37˚C and 5% CO_2.

Proliferation assay (MTT test). Cell proliferation was analyzed using an MTT-kit according to the instructions of the manufacturer (Roche, Germany). For reproducible results it was important to work with the same cell number in all MTT tests. For this purpose, the optimal cell number of Jeg3 cells/ml were estimated in preliminary experiments. The following considerations were taken into account: (i) during the test phase the medium should not be consumed completely and hence should be added in excess, (ii) the cells should adhere to the plastic material and should not float, and (iii) a measurable absorbance at 570 nm to determine inhibitory effects should be reached. The test conditions were optimized in preliminary experiments and the optimal cell number was found to be $1x10^6$ Jeg3 cells/ml.

The phytoestrogen extracts were dissolved in 1% DMSO to provide a stock solution of 100 mg/ml. From this stock solution, aliquots were taken and added to 0.1 ml supplemented culture medium producing final concentrations of 0.05 mg/ml, 0.5 mg/ml, 1 mg/ml and 5 mg/ml (final concentration of DMSO: 0.05%). Jeg3 cells ($1x10^5$/0.1 ml supplemented culture medium) were grown in 96-well tissue culture plates for 48 h in the absence (controls) and presence of different phytoestrogen extracts at 37˚C and 5% CO_2. Two negative controls were prepared (i) Jeg3 cells in DMEM and (ii) Jeg3 cells in DMEM and DMSO, final concentration of DMSO: 0.05%. After incubation with MTT for 4 h at 37˚C and 5% CO_2, solubilization solution was added and the plates were incubated in a humidified atmosphere (37˚C, 5% CO_2) overnight. The spectrophotometrical absorbance of the purple formazan crystals was measured at 570 nm using a microplate ELISA reader (BioRad, Hercules, CA, USA).

Table I. *Classes, representatives and forms of phytoestrogens identified in leaf, stem and root extracts of Linum usitatissimum using HPLC-MS analysis. Phytoestrogen extracts were prepared according to Franz and Köhler (10), Luyengi et al. (13) and Windhövel et al. (14) with and without HCl or β-glucosidase hydrolysis.*

Class of phytoestrogen	Representative	Chemical form
Isoflavones	Genistein	Aglycone
	Daidzein	Glycoside
	Biochanin A	Diglycoside
		Dimer
		Glycoside dimer
		Deoxydiglycoside
Lignans	Secoisolariciresinol	
	Matairesinol	
	Pinoresinol	
	Lariciresinol	
	Isolariciresinol	
	Arctigenin	
	6-Methoxypodophyllotoxin	

Statistical analysis. Statistical analysis was performed using the Student's *t*-test for comparison of the means. $P<0.01$ was considered statistically significant.

Results

Preparation of phytoestrogen extracts from leaves, stems and roots of *L. usitatissimum*. The application of the different isoflavone, lignan and lignan/toxin extraction methods (10, 13, 14) resulted in different yields of extracts using 1 g of plant material.

About 25-75 mg total isoflavone extract could be isolated per g plant material (2.5%-7.5%) using the isoflavone extraction method according to Franz and Köhler (10, with and without HCl hydrolysis). Lignan extract preparation according to Luyengi *et al.* ((13), with and without HCl hydrolysis) resulted in considerably smaller yields (about 1.25-4.25 mg/g fresh weight, 0.125%-0.425%). Performing the lignan/toxin extraction method with and without enzyme hydrolysis (14) the yield per g plant material was about 72.5-162.5 mg (7.25%-16.25%). The microwave extraction method using leaf, stem and root material produced 32.5-67.5 mg extract/g fresh weight (3.25%-6.75%).

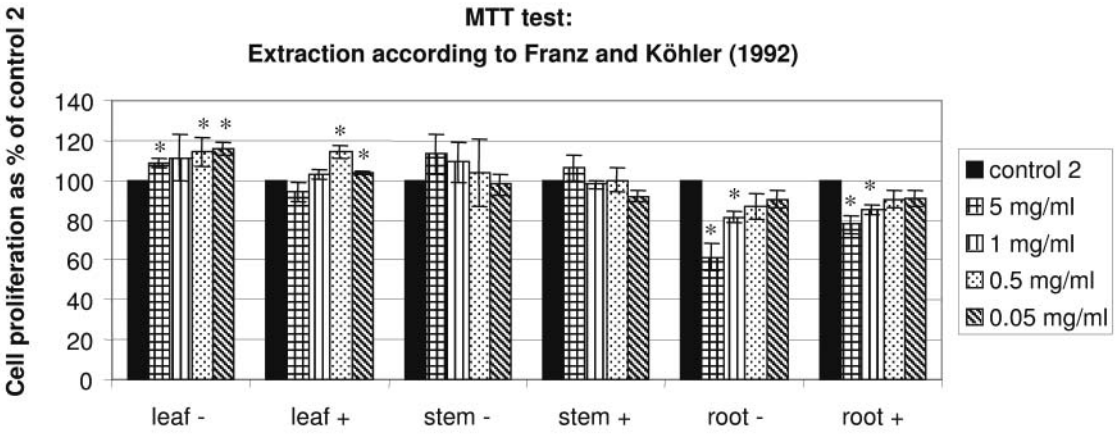

Figure 1. *Effect of different concentrations of leaf, stem and root extracts from Linum usitatissimum on the cell proliferation of Jeg3 cell lines. Extracts were prepared according to Franz and Köhler (10) with (+) and without (–) hydrolysis with 1 M HCl. Data (mean±SD (standard deviation)) represent relative formation of formazan from MTT in % in comparison to negative control 2 (100%). Asterisks (*) indicate significant differences between treated Jeg3 cell lines and the negative control 2 ($p<0.01$).*

Identification of isolated phytoestrogens with HPLC-MS. Leaf, stem and root extracts from *L. usitatissimum* were analysed using HPLC-MS and the compounds identified are given in Table I. These compounds were found in the extraction procedure as aglycones or as glycosides, independently of whether additional HCl- or enzyme hydrolysis was used or not. In the case of the special lignan/toxin extraction (14) 6-methoxypodophyllotoxin was found only in the leaf extracts (Table I).

Effects of isoflavone extracts from leaves, stems and roots of L. usitatissimum on proliferation of Jeg3 cells. Isoflavone extracts from the roots of *L. usitatissimum* obtained using the method of Franz and Köhler (10) at concentrations of 1 and 5 mg/ml significantly inhibited cell proliferation relative to the negative control 2 (100%). Negative control 1 (Jeg3 cells in DMEM) and negative control 2 (Jeg3 cells in DMEM and DMSO [final concentration 0.05%]) determinations did not differ in absorbance values, showing that DMSO at 0.05% did not produce inhibition effects on the growth of Jeg3 cells (data not shown). The root extract at a concentration of 5 mg/ml without additional HCl hydrolysis reduced the cell growth by about 40% in comparison to untreated control cultures. Application of less root extract (1 mg/ml, 0.5 mg/ml, 0.05 mg/ml) revealed less inhibition of proliferation of 19%, 15% and 10%, respectively. Addition of HCl did not induce stronger reduction of cell proliferation in comparison to those extracts obtained without hydrolysis (Figure 1), indicating that the biologically active compounds are present in an effective form in the untreated sample. It is likely that the respective compounds are not glycosilated. Leaf and stem extracts either induced Jeg3 cell proliferation (maximum 15%) or did not affect growth.

Effects of lignan extracts from leaves, stems and roots of L. usitatissimum on proliferation of Jeg3 cells. All lignan extracts obtained from leaves, stems and roots of *L. usitatissimum* prepared according to Luyengi *et al.* (13) with and without HCl hydrolysis revealed significant inhibition of cell proliferation (Figure 2). Application of extracts at 1, 0.5 and 0.05 mg/ml were tested. The strongest decrease in cell proliferation was induced by treatment of Jeg3 cultures with root extracts which had not undergone HCl hydrolysis (Figure 2). Incubation of Jeg3 cells with these extracts at 1 and 0.5 mg/ml reduced cell proliferation by 93%. The addition of stem extracts at 1 mg/ml with and without prior HCl hydrolysis reduced formazan production by about 65-70%. Approximately 20% inhibition of cell proliferation was obtained with leaf and root extracts which had undergone HCl hydrolysis. Most lignan extracts exhibit concentration-dependent effects. Since extractions performed with and without HCl hydrolysis result in extracts which are effective to different extents, it can be concluded that several different compounds in the extracts are responsible for the bioactivity.

Effects of lignan/toxin extracts from leaves, stems and roots of L. usitatissimum on proliferation of Jeg3 cells. Lignan/toxin extracts obtained from the roots of *L. usitatissimum* inhibited cell proliferation significantly (Figure 3). The root extract with additional β-glucosidase hydrolysis reduced the cell growth to about 55%, most significantly at a concentration of 5 mg/ml. The extracts from the leaves and stems either induced weak activation, reduction of cell proliferation, or did not affect the growth

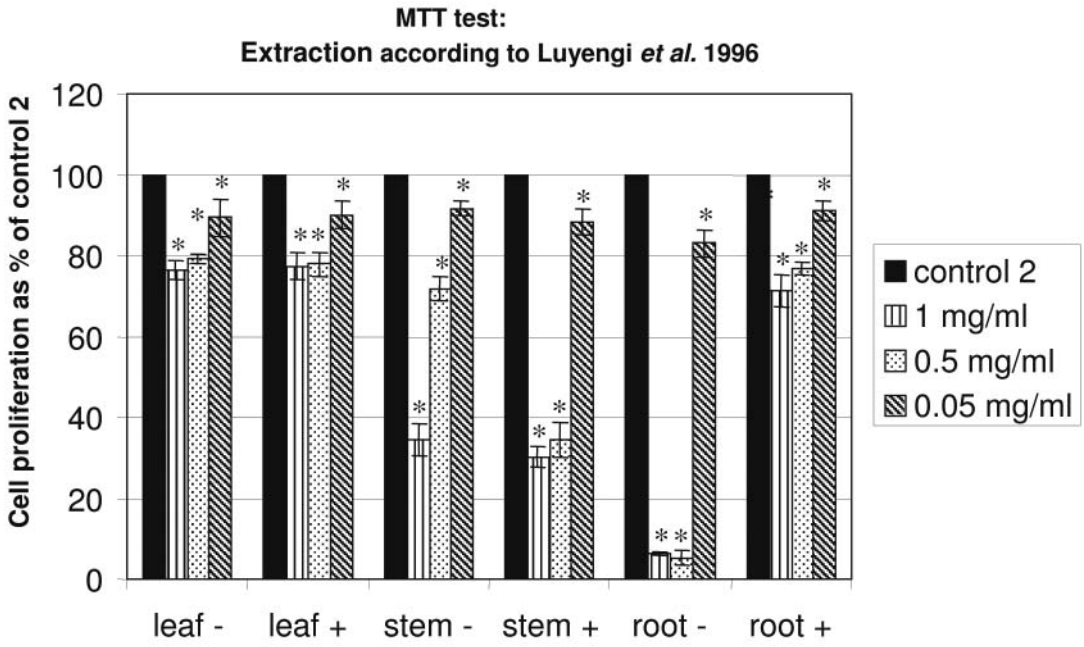

Figure 2. *Effect of different concentrations of leaf, stem and root extracts from Linum usitatissimum on the cell proliferation of Jeg3 cell lines. Extracts were prepared according to Luyengi et al. (13) with (+) and without (–) hydrolysis with 1 M HCl. Data (mean±SD) represent relative formation of formazan from MTT in % in comparison to negative control 2. Asterisks (*) indicate significant differences between treated Jeg3 cell lines and the negative control 2 ($p<0.01$).*

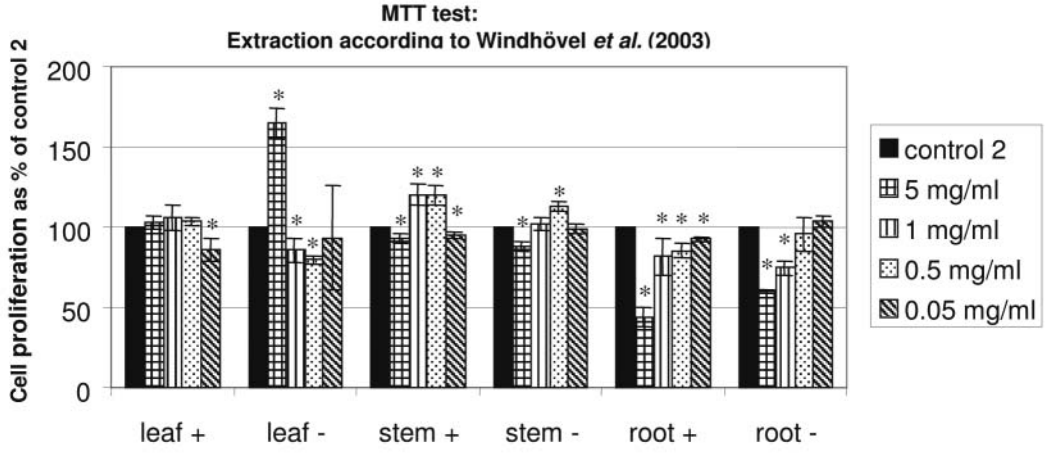

Figure 3. *Effect of different concentrations of leaf, stem and root extracts from Linum usitatissimum on the cell proliferation of Jeg3 cell lines. Extracts were prepared according to Windhövel et al. (14) with (+) and without (–) hydrolysis with β-glucosidase. Data (mean±SD) represent relative formation of formazan from MTT in % in comparison to negative control 2. Asterisks (*) indicate significant differences between treated Jeg3 cell lines and the negative control 2 ($p<0.01$).*

of Jeg3 cells. The leaf extract at a concentration of 5 mg/ml performed without enzyme hydrolysis induced an activation of proliferation of about 60% (Figure 3). The extracts with additional β-glucosidase hydrolysis did not induce stronger activation or reduction of cell proliferation in comparison to those extracts without hydrolysis (Figure 3).

Effects of isoflavone and lignan extracts from leaves, stems and roots of L. usitatissimum on proliferation of Jeg3 cells (microwave extraction). Using the microwave extraction method, extracts from leaves and stems did not induce significant effects on cell proliferation, with one exception: the leaf extracts at a concentration of 5 mg/ml induced activation of cell proliferation by approximately 40% (Figure

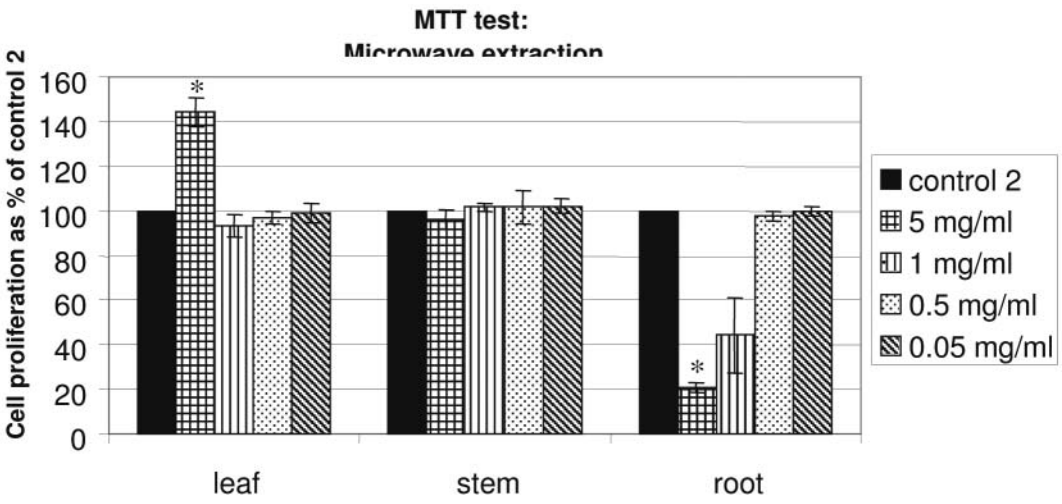

Figure 4. *Effect of different concentrations of leaf, stem and root extracts from Linum usitatissimum on the cell proliferation of Jeg3 cell lines. Extracts were prepared by microwave extraction. Data (mean±SD) represent relative formation of formazan from MTT in % in comparison to negative control 2. Asterisks (*) indicate significant differences between treated Jeg3 cell lines and the negative control 2 ($p<0.01$).*

4). Root extracts of *L. usitatissimum* at a concentration of 5 mg/ml and 1 mg/ml reduced the cell proliferation to 80% and 55%, respectively (Figure 4). The inhibition of cell proliferation was dose-dependent for the root extracts.

Discussion

Over the last 20 years, there has been a growing interest in the role of phytoestrogens in health and disease. The major classes of phytoestrogens that have been examined are the isoflavones and lignans, found at high levels in soybean, flaxseed and in various plant parts, including roots, stems, leaves, flowers, fruits and seeds, respectively (1, 2, 15). Soybean and flaxseed based foods are reportedly potentially beneficial in treating cancer, osteoporosis and coronary heart diseases (1, 9, 16-18). Isoflavones and lignans have been shown to inhibit the *in vitro* activity of enzymes involved in estrogen metabolism, such as inhibition of aromatases and 17β-oxidoreduction of estrogens. Several studies directly examined the effects of isolated isoflavones and lignans on breast cancer, colon cancer and other cancers (1, 19), however chorion carcinomas have so far only been poorly investigated (20). We therefore started a systematic investigation to test the influence of phytoestrogens on Jeg3 chorion carcinoma cells.

Based on the results of HPLC-MS-analysis it was shown that the leaf, stem and root extracts from the flax species, *L. usitatissimum*, contain measurable concentrations of isoflavones such as genistein, daidzein and biochanin A, and lignans such as secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, isolariciresinol and arctigenin. It was shown that all extracts from *L. usitatissimum* contain more representatives of lignans compared to isoflavones, as has been shown for other *Linum* species (21).

The aryltetralin lignan, 6-methoxypodophyllotoxin, was additionally found in the leaf extracts of *L. usitatissimum*. It was previously shown that aryltetralin-type lignans (podophyllotoxin and peltatin derivatives) occur in plant parts of many *Linum* species (21) and seem to be a characteristic feature of the genus *Linum* (22). It was shown that 6-methoxypodophyllotoxin induces high cytotoxicity (21). The lignan podophyllotoxin is of special interest, since its derivatives such as Etopophos are presently used in anticancer therapy (23). Our investigations showed that leaf extracts negatively influenced cell proliferation only by a maximum of 20%, when 1 mg/ml or 0.5 mg/ml were applied to Jeg3 chorion cancer cells (Figure 2). One of the future tasks will be to determine the quantity of 6-methoxypodophyllotoxin and other lignans and isoflavones in the extracts.

It was demonstrated that of all isolation methods tested, the most effective extraction procedure was the lignan extraction method according to Luyengi *et al.* (13) (Figures 1-4) for which 20-90% inhibition of cell proliferation was detected. Comparing the effects of leaf, stem and root extracts from *L. usitatissimum* on cell proliferation of Jeg3 cell lines shows that the root extracts induced significant inhibition of cell proliferation in all experiments. The reduction of cell proliferation after the addition of root extracts was realized in a dose-dependent manner. Leaf and stem extracts induced different effects depending on the extraction method used. In the case of lignan preparation according to Luyengi *et al.* (13), approximately 20-70% of cell proliferation was observed (Figure 2). When isoflavone and lignan/toxin extraction methods were

used (10, 14), the growth of Jeg3 cell lines was promoted or not affected (Figures 1, 3).

It is known from other studies that isoflavones and lignans occur in glycosilated forms in plant material and are therefore often biologically inactive (1, 21, 24). To improve the bioavailability *in vitro*, nonspecific HCl hydrolysis and specific β-glucosidase hydrolysis were used to make the aglycons available (1, 24). However, most of the hydrolysis treatments were without effect: therefore it can be concluded that isoflavones and lignans were present in the flax extracts prior to hydrolysis as aglycons and glycoside derivates.

Conclusion

We demonstrated that the roots of *L. usitatissimum* contain measurable concentrations of lignans and isoflavones. In addition we established a cell culture model to test the effect of different doses of potential phytoestrogens on the cell proliferation of chorion tumour cell lines.

Acknowledgements

The authors thank Mrs. C. Bauer for technical assistance.

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Received February 15, 2006
Revised January 24, 2007
Accepted January 29, 2007