

Effects of Phytoestrogen Extracts Isolated from Pumpkin Seeds on Estradiol Production and ER/PR Expression in Breast Cancer and Trophoblast Tumor Cells

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25 Phytoestrogens have a controversial effect on hormone-dependent tumours. Herein, we investigated the effect of the pumpkin seed extract (PSE) on estradiol production and estrogen receptor (ER)- α /ER- β /progesterone receptor (PR) status on MCF7, Jeg3, and BeWo cells. The PSE was prepared and analyzed by mass spectrometry. MCF7, Jeg3, and BeWo cells were incubated with various concentrations of PSE. Untreated cells served as controls. Supernatants were tested for estradiol production with an ELISA method. Furthermore, the effect of the PSE on ER- α /ER- β /PR expression was assessed by immunocytochemistry. The PSE was

found to contain both lignans and flavones. Estradiol production was elevated in MCF7, BeWo, and Jeg3 cells in a concentration-dependent manner. In MCF7 cells, a significant ER- α downregulation and a significant PR upregulation were observed. The above results after properly designed animal studies could highlight a potential role of pumpkin seed's lignans in breast cancer prevention and/or treatment. 35 40

BACKGROUND

Phytoestrogens are plant polyphenolic compounds with a molecular structure that closely resembles mammalian estrogens. It has been proven that such compounds can bind to human estrogen receptors exerting both estrogenic and antiestrogenic effects (1). According to their molecular characteristics, 45

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phytoestrogens can be classified into different groups, the most studied of which are isoflavones and lignans.

Isoflavones are found in legumes, with the most common representative being soy and its derivative products, whereas lignans are usually found in seeds and fiber-rich cereals (2). In contrast to isoflavones, which are quite abundant in Asian diet, lignans are the phytoestrogens mostly consumed in Europe (2, 3).

The role of lignans as hormonal compounds has been excessively discussed and to date it is still unclear whether their consumption may lead to beneficial results or not. To this direction, several reports have used lignans either in their isolated form or as product extracts using several *in vitro* or *in vivo* models (4), especially in the aim to study their association as well as their effect on hormone-dependent malignancies and mainly breast cancer (5). In addition, lignans are expected to play a potentially crucial role in other hormone-dependent processes in humans, such as reproduction and menopause (6).

Based on the facts mentioned above and having already presented relevant data about the effect of isoflavones and lignans on tumor trophoblast cells (well accepted as a trophoblast model) and on the estrogen receptor positive MCF-7 breast cancer cell line (7–9), we expanded our research to the effect of the pumpkin seed extract (PSE), because pumpkin is very abundant in the North European diet. We have shown that the PSE may alter the hormone receptor profile of the MCF-7 but not of the BeWo and Jeg-3 cells, while at the same time it triggers estradiol production.

MATERIALS AND METHODS

Preparation of the PSE

The PSE, from the species *Curcubita Pepo*, was made as previously described (10) and was afterwards dissolved in 100% ethanol. In the aim to verify the previously reported increased lignan concentration in pumpkin seeds (11, 12) the molecular–chemical composition of the extract was further analyzed by pyrolysis-field ionization mass spectrometry by using a LCQ-Advantage (Thermo Finnigan, USA). The peaks were identified by ion trap technology on ESI mode. The source voltage was set at 4.5 KV, while the mass detection range was 150–2000 amu.

Cell Lines

For the current work the chorion carcinoma cell lines Jeg-3 and BeWo and the breast carcinoma cell line MCF-7 were used. All cell lines were obtained by the European Collection of Cell Cultures (ECACC, UK). The cells were grown in DMEM (Biochrom AG, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH, Austria), 100 $\mu\text{g/ml}$ Penicillin/Streptomycin (Biochrom AG, Germany) and 2.5 $\mu\text{g/ml}$ Amphotericin B (Biochrom AG, Germany). Cultures were maintained in a humidified incubator, at 37°C with a 5% CO₂ atmosphere. Prior to cell culture the pres-

ence of estrogen or progesterone in the medium was excluded, by an automated Immulite (DPC Biermann, Freiburg, Germany) hormone analyzer.

Effect of PSE on the Cell Lines

For all the experiments the cells were seeded on Quadriperm tissue slides at the presence or absence of PSE. In brief, cells were seeded at a concentration of 400,000 cells per slide. The cells were left to attach for 24 h. Then, the medium was replaced by medium supplemented with PSE at final effective concentrations of 10, 50, and 100 $\mu\text{g/ml}$. Because the original PSE was diluted in 100% ethanol, medium supplemented with 100% ethanol at a concentration of 5 $\mu\text{l/ml}$ (this being the maximum ethanol concentration achieved during these experiments) served as internal control. After the cells were cultured for 72 h, 1 ml from each supernatant was stored at –80°C for estradiol analysis. The remaining supernatant was then discarded and the slides were washed in PBS, fixed in acetone for 10 min, and let to dry in room temperature. Cells treated with equal concentrations of estradiol (10, 50, and 100 $\mu\text{g/ml}$) served as external controls.

Estradiol Determination in the Cell Culture Medium

For the determination of estradiol in the culture medium, a competitive enzyme immuno-assay (EIA) was applied as described previously (13). Determination was performed by an automated Immulite 2000 (DPC Biermann, Freiburg, Germany) hormone analyzer.

Immunocytochemistry for ER- α , ER- β , and PR

For immuno-detection of the steroid receptors ER- α , ER- β , and PR, the Vectastain[®] Elite ABC-kit (Vector Laboratories, USA) was used according to manufacturer's protocol. After been air dried, the slides were rinsed in PBS for 5 min and incubated with the ABC normal serum for 60 min in a humidified environment. The slides were then washed and incubated with the respective primary antibodies. Salient features of the antibodies used are presented in Table 1. The slides were then incubated with the diluted biotinylated secondary antibody (30 min), followed by incubation with the ABC reagent (30 min), and the ABC substrate (15 min). A PBS wash (5 min) was applied between steps. Finally, the slides were counterstained with Mayer's acidic hematoxylin (30 sec), rinsed with water and covered with Aquatex.

The intensity and distribution patterns of the specific immunocytochemical staining was evaluated using a semiquantitative method (IRS score) as previously described (14). Briefly, the IRS score was calculated as the product of the optical staining intensity (0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining) multiplied by the graded staining extent (0 = no staining; 1 = <10% staining; 2 = 11–50% staining; 3 = 51–80% staining and 4 = >80% staining). The percentage of positively stained cells was estimated by counting approximately 100 cells.

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TABLE 1
Salient features of the antibodies used in the present study

Antibody (source)	Origin	Dilution in PBS	Incubation	Temperature
Anti-ER α (Dako, Germany)	Mouse monoclonal	1:150	1h	RT
Anti-ER β (Serotec, Germany)	Mouse monoclonal	1:600	O/N	4°C
Anti-PR (Dako, Germany)	Mouse monoclonal	1:50	1h	RT

ER = estrogen receptor; PR = progesterone receptor; nO/N = overnight; RT = room temperature.

150 MTT Assay

To quantify the effect of the PSE on the MCF-7 cell population, the MTT assay was performed. Briefly, 10⁵ cells/well were allowed to attach on Quadriperm tissue culture slides and then were treated with 10 μ g/ml, 50 μ g/ml, or 100 μ g/ml of PSE for 72 h. Controls were set as described above. The cells were then treated according to the MTT assay protocol, as previously described (15).

Statistical Analysis

All experiments were performed in triplicates. Statistical analysis was performed using the Wilcoxon's signed rank tests for pair wise comparisons. Each observation with $P < 0.05$ was considered statistically significant.

RESULTS

Identification of Phytoestrogens in the PSE

The mass spectrometry results showed that the PSE contains phytoestrogens. Indeed it was found that the PSE contained 1) lignans (secoisolarisiresinol, matairesinol, and arctigenin) and 2) flavons (Genistin, Daidzin, Formononetin, and Quercetin). There was a trend for lignans to be more abundant in the PSE, compared to isoflavones. In addition, the PSE contained other phenolic compounds, as well as lipids and isoprenoids like sterols, terpenes, and carotenoids (Table 2). Despite the established knowledge that pumpkin seeds contain mainly lignans and especially secoisolarisiresinol (11, 12, 16), and the initial trend for higher lignan concentrations compared to isoflavones, quantification was technically impossible due to the presence of extremely high amounts of unsaturated and saturated fatty acids and di-/triglycerides (80% w/w). With such high concentrations of these compounds, a safe evaluation of the phytoestrogenic content is rather not feasible. The lack of quantification of PSE compounds did not affect the study design, the initial aim of which was to evaluate the effect of the crude extract and not of each of its ingredients separately.

185 PSE Induces Estradiol Secretion by Jeg-3, BeWo, and MCF-7 Cells in a Dose-Response Pattern

All 3 cell lines were cultured for 72 h in the presence of different concentrations of PSE. The estradiol concentration in the medium was determined by an automated hormone analyzer by applying a competitive EIA. As shown in Fig. 1, estradiol pro-

duction is significantly induced in a dose-response pattern. In 190 Jeg-3 cells, the estradiol production was increased from 1065.01 \pm 22.22 pg/ml in the control to 1674.48 \pm 30.34 pg/ml, 5213.62 \pm 82.35 pg/ml, and 7275.89 \pm 439.48 pg/ml when the PSE concentration was 10, 50, and 100 μ g/ml, respectively ($P < 0.05$). To the same direction, in BeWo cells, the estradiol produc- 195 tion was increased from 245.25 \pm 16.25 pg/ml in the control to 315.75 \pm 4.27 pg/ml, 752.37 \pm 25.03 pg/ml, and 3586.93 \pm 134.02 pg/ml when the PSE concentration was 10, 50, and 100 μ g/ml, respectively ($P < 0.05$). Finally, in MCF-7 cells, the estradiol production was increased from 131.42 \pm 2.21 pg/ml 200 in the control to 174.24 \pm 3.30 pg/ml, 734.86 \pm 20.61 pg/ml, and 1487.47 \pm 45.32 pg/ml when the PSE concentration was 10 μ g/ml, 50 μ g/ml, and 100 μ g/ml, respectively ($P < 0.05$).

TABLE 2
Presentation of the compounds found in the pumpkin seed extract

Pumkin seed extract composition (qualitative analysis)
Phytoestrogens
Lignans
- Secoisolariciresinol
- Matairesinol
- Arctigenin
Flavonoids
- Genistin
- Daidzin
- Formononetin
- Quercetin
Carbohydrates
Phenole and lignin monomers
Lignin dimers
Lipids, alkenes, alkanes, fatty acids, n-alkylesters
Alkyl aromatic compounds
N-containing compounds
Steroles, terpenes, carotenoids
Peptides and aminoacids
Suberin
Free saturated or unsaturated fatty acids (n-C ₁₆ to n-C ₃₄)
Low molecular weight compounds (MW 15–56)
Di- and Triglycerids

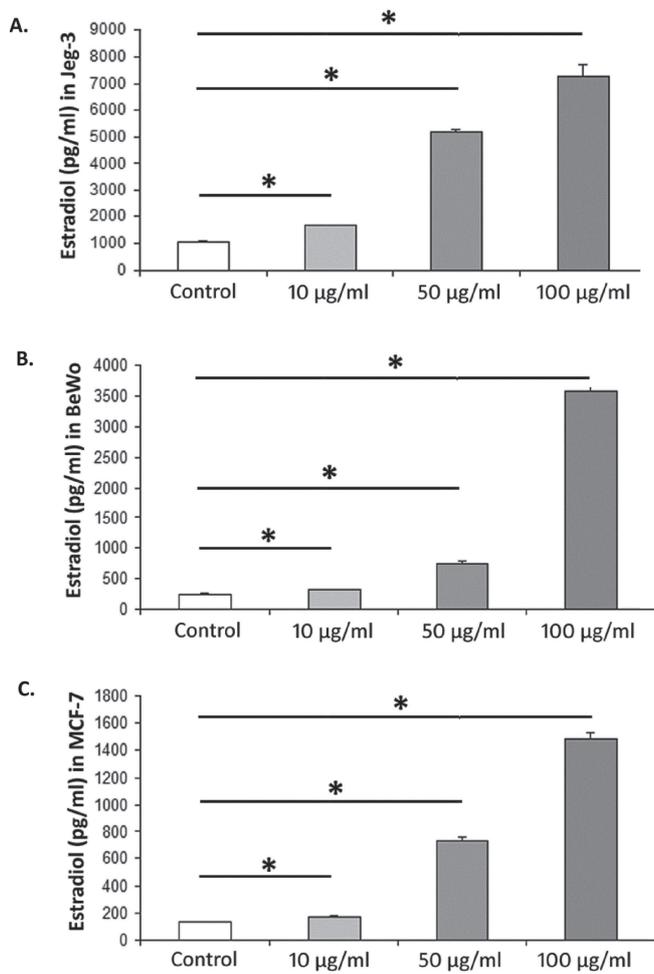


FIG. 1. Estradiol concentration in the tissue culture medium of Jeg3, BeWo, and MCF7 cells in the absence or presence of pumpkin seed extract. The effective pumpkin seed extract concentrations were 10, 50, and 100 µg/ml. Significant observations are highlighted with an asterisk.

PSE Downregulates ER-α and Upregulates PR in the MCF-7 Breast Cancer Cell Line

Jeg-3, BeWo, and MCF-7 cell lines were immunocytochemically assessed for ER-α, ER-β, and PR reactivity, after a 72-h culture in the presence of different concentrations of the PSE.

As shown in Fig. 2A and 2B, there was a significant decrease in ER-α expression in the MCF-7 cells, in a dose-response pattern, with an IRS score being reduced from 10 ± 0.98 in the controls to 0.5 ± 0.24, 0.16 ± 0.18, and 0 ± 0 when the PSE concentration was 10, 50, and 100 µg/ml, respectively (P < 0.05). On the contrary, there was a significant upregulation in the PR expression in the MCF-7 cells with an IRS score being increased from 2.33 ± 0.36 in the control to 8.83 ± 0.71, 8.66 ± 0.73, and 6.5 ± 0.54, when the PSE concentration was 10, 50, and 100 µg/ml, respectively (P < 0.05). However the PR upregulation did not present a dose-response pattern. No statistical difference was observed in MCF-7 cells regarding

ER-β expression when cultured in the presence or absence of PSE (data not shown).

MCF-7 cells treated with equal concentrations of estradiol (10, 50, and 100 µg/ml) revealed the same pattern of ER and PR expression; ERα expression was significantly downregulated, ERβ expression remained unaltered, and PR expression was upregulated (Fig. 3).

The ER-α, ER-β, and PR expression levels in Jeg-3 and BeWo cells did not differ significantly in the presence or absence of PSE (data not shown).

Only Low or Moderate (10 µg/ml or 50 µg/ml) PSE Concentrations Upregulate MCF-7 Proliferation

The effect of PSE on MCF-7 breast cancer cell proliferation was biphasic. When treated with 10 µg/ml or 50 µg/ml PSE, the MCF-7 proliferation rate was significantly increased (P < 0.05). On the contrary, increased concentrations of PSE (100 µg/ml) did not seem to affect cell proliferation (P > 0.05) (Fig. 3).

DISCUSSION

Herein, the effect of the PSE on BeWo, Jeg3, and MCF7 cell lines regarding estrogen and PRs was assessed. To our knowledge this is the first time that such an effect of the PSE is investigated and thus we consider this study a pilot.

In pilot studies, the use of an extract like the PSE is considered better than investigating isolated compounds, in that case phytoestrogens. This is supported initially by the thesis that crude extracts are better simulators of the real effect, because in real life people consume foods rather than isolated compounds. To the same direction it has been shown that the processed isoflavones may trigger MCF7 growth, whereas the soy flour that served as the initial source of them did not (17, 18). The PSE used herein, was proved to contain lignans, flavones, as well as other compounds (presented in Table 2). A trend for higher lignan concentrations was found by mass spectrometry. However, because fatty acids and di-/triglycerides constituted 80% of the PSE, a safe evaluation of the phytoestrogenic content was not feasible. However based on the current evidence upon pumpkin seed phytoestrogen content (11, 16), we could speculate that the results could be mainly attributed to lignans. Further studies with isolated fractions of the PSE could clarify whether this is exclusively a single lignan or, less possibly, isoflavone effect or even a result of multiple synergies. The role of the other compounds present in the PSE needs also to be elucidated.

A matter of discussion is also the concentrations used and how these could be attributed to a possible diet intake in real life. This issue at the moment is rather difficult to clarify, because phytoestrogen intake varies significantly between individuals. However, high phytoestrogen concentrations may be achieved by constant intake of relevant foods (reported almost up to 3 µM in serum) (19). In this context, the concentrations of the PSE extract used in the present study are considered realistic.

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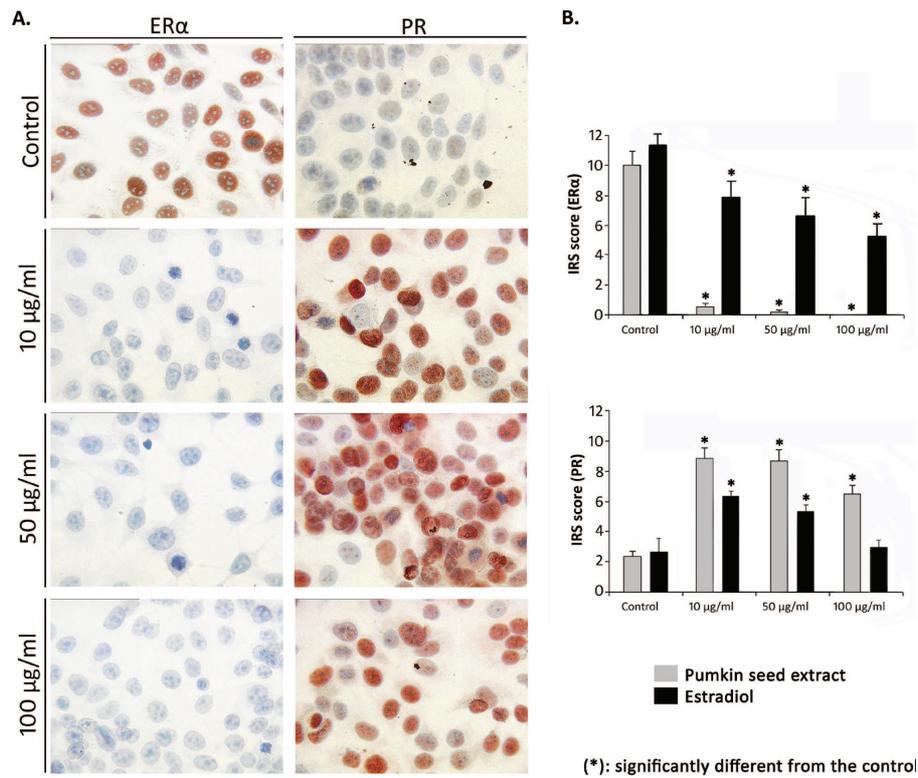


FIG. 2. A: Representative microphotographs of MCF7 cells grown in the absence or presence of pumpkin seed extract (at effective pumpkin seed extract concentrations of 10, 50, and 100 µg/ml), after immunodetection of estrogen receptor (ER)-α and progesterone receptor (PR). B: Presentation of the immunocytochemistry results by the semiquantitative immunoreactivity score (IRS). Significant observations are highlighted with an asterisk. (Color figure available online).

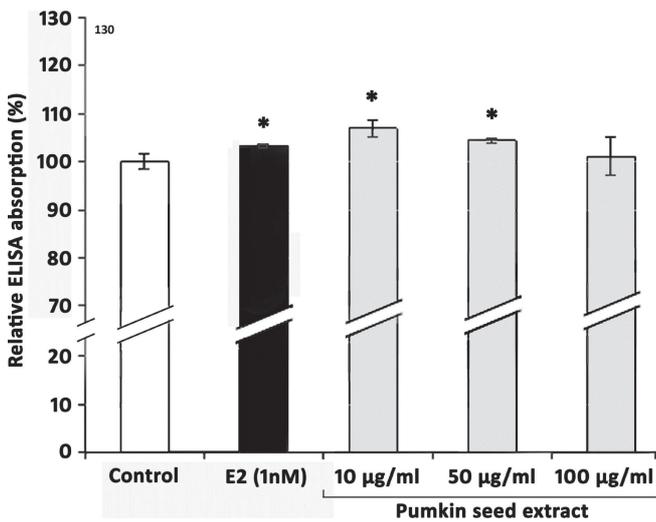


FIG. 3. MTT assay performed on MCF-7 cells. It was found that 10 and 50 µg/ml of pumpkin seed extract induce cell proliferation. On the contrary treatment of the MCF-7 with 100 µg/ml of pumpkin seed extract do not affect cell proliferation. Asterisk indicates significant differences from the control

An additional argument regarding the experimental setup of the current study is the possible use of a steroid receptor (ER/PR) negative cell line as a control. Such cell line would perhaps reveal non-estrogenic effects of the PSE; however our main aim was to clarify the possible impact of the PSE on ER/PR expression. Such clarification cannot be done by using ER/PR negative cell lines.

A previous report of our group has presented that two common isoflavones (daidzein and genistein) induce estradiol production on BeWo and Jeg3 tumour trophoblast cells (9). Our findings indicate that the PSE (containing lignans as phytoestrogens) seems to have the same effect on these cell lines regarding estradiol production. However, a deregulated estrogen production by the trophoblast may imply placenta dysfunction and thus could lead to unfavorable results regarding the ongoing gestation (20).

A significant increase of E2 production was also found when MCF7 cells were treated with the PSE. Although, it has been shown that phytoestrogens inhibit the peripheral aromatase activity, thus blocking the transformation of androgens to estrogen (4), recent evidence exist showing that in a breast cancer model at least genistein may increase aromatase activity thus enhancing a local estradiol profile (21). Because MCF-7 cells do not

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295 seem to spontaneously express aromatase (22), we hypothesize
that the increased estradiol production by the MCF7 cells could
be attributed either to an induction of aromatase expression or
by an increased 17β -HSD1 activity (reducing E1 to E2) trig-
gered by the PSE extract. Especially the sulphatase/ 17β -HSD1
300 pathway has been reported as equally important to estradiol
production (23), contributing to the high local estradiol excess
mainly seen in postmenopausal women (24). Further experi-
ments toward assessment of possible induction of aromatase
expression as well as assessment of the 17β -HSD1 activity are
305 definitely needed to clarify the exact mechanism by which PSE
up-regulates estrogen production.

It was also shown that PSE induced a significant downreg-
ulation of the ER- α in the MCF7 breast cancer cell line, while
at the same time ER- β expression was unaffected. This alter-
ation, if still holds in humans, seems to be rather protective in
the view of breast cancer cell growth; it has been reported that
ER- β is associated with breast cell growth inhibition, both by
independent ER- β actions and by negative regulation of the ER-
 α pathways (25, 26). Our current findings partially concur with
315 a previously described downregulation of ER- α on the MCF7
cells, when treated with flax isoflavone and lignan extracts or
mixtures (27, 28). However, in the case of flax extracts, such a
downregulation was achieved only with high concentrations of
phytoestrogens that are unlikely to be achieved by an increased
320 phytoestrogen intake, whereas PSE seems to achieve the same
effect at more feasible concentrations. Interestingly, isolated
secoisolariciresinol—being present in the PSE as well—was
found among others to decrease ER- α expression, using a model
of MCF7 cell tumors in ovariectomized athymic mice (29).

325 On the other hand, it was shown that PSE induces a sig-
nificant increase in progesterone receptors in the MCF7 breast
cancer cell line. Such an increase though cannot be consid-
ered clearly as either beneficial or not against breast cancer cell
growth, as the role of progestins' actions in the breast is con-
troversial. Recently, by using an animal model it was described
330 that soy protein isolate may increase PR-A expression in MCF7
cell tumors and decrease apoptosis of normal ductal epithelium,
leading to more aggressive tumors (30). Further in vitro experi-
ments revealed that progestins protect T47D breast cancer cells
from serum deprivation- as well as from doxorubicin- and 5FU-
induced apoptosis (31). On the contrary, while progestins re-
duced the apoptotic rate of normal MCF10A breast cells in
vitro, at the same time progestins partially reversed the initial
estrogen-induced low apoptotic rate in breast cancer cells (32).

340 The protective role of lignans against breast cancer devel-
opment is well accepted, especially in case of high lignan in-
take (5). Flaxseed lignans were also reported to decrease cell
proliferation and c-erbB2 expression and increase apoptosis in
breast cancer patients receiving lignans for the period between
345 diagnosis (biopsy) and surgical treatment (33). Finally isolated
secoisolariciresinol is accounted for reduced MCF7 tumor cell
growth both in vitro (cell culture) and in vivo (ovariectomized
mouse model) (15, 29).

By taking into account the effects of phytoestrogens on breast
cancer cells, as presented herein, the protective role of phytoe- 350
strogens in breast cancer could be questioned. Our results show
an increased production of estrogen by MCF7 cells, although
a downregulation of ER α was noted. Increased local and cir-
culating estrogen concentrations are certainly considered as a
disadvantage in treating breast cancer. This effect in case of 355
phytoestrogen administration is counterbalanced by the pro-
found downregulation of ER α . Thus, this excess of estrogen is
rather unable to trigger ER α -mediated tumor cell proliferation.
On the contrary, because we showed that ER β expression re-
mains unaffected by phytoestrogen, it could be hypothesized 360
that both phytoestrogens and the increased estrogenic concen-
trations triggered by them, may exert ER β -mediated effects. In
that view phytoestrogens could be related to a reduced tumor
cell proliferation possibly explaining their suggested protective
effect on breast cancer. 365

Because PSE presented as having a dual role on MCF7 cells,
the results and the conclusions made must be taken into consid-
eration with caution, especially nowadays that phytoestrogens
are gaining constantly ground in the market. After all, this is a
pilot study on cell lines and thus further experiments should be 370
made: 1) isolation of fractions from PSE containing lignans to
verify whether the initial result can still be attributed to lignans,
2) study of the effect of the PSE on breast cancer cells' aro-
matase and 17β -HSD1 activity, and 3) study of the effect of the
PSE on animal models, to clarify better an overall inhibitory—or 375
not—role for the PSE in breast cancer.

We believe that in that view the PSE is a good candidate for
further research with a potential role in breast cancer prevention
and/or treatment, after properly designed studies.

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