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Original article

Interactions between plant-derived oestrogenic substances and the mycoestrogen zearalenone in a bioassay with MCF-7 cells

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Abstract

Human and animal diets may contain several non-steroidal oestrogenic compounds which originate either from plants (phytoestrogens) or from fungi that infect plants (mycoestrogens such as zearalenone (ZEN)). Phytoestrogens may compete with ZEN in binding to the oestrogen receptor β and thereby may counteract the oestrogenic activity of ZEN. Using a modified version of the E-screen assay, plant-derived oestrogenic substances were tested for their proliferative or anti-proliferative effect on oestrogen-dependent MCF-7 cells. The samples were additionally tested for their ability to influence the oestrogenic activity of ZEN (1 μM). Among the individual substances tested, 8-prenylnaringenin had the strongest effect, as cell proliferation was increased by 78% at the lowest concentration (0.23 μM), and by 167% at the highest concentration (29.4 μM). Coumestrol (5.83 μM) increased cell proliferation by 39%, and genistein (370 μM) by 61%, respectively. Xanthohumol and enterolactone did not stimulate cell proliferation significantly. In the co-incubation experiments with ZEN, none of the single substances was able to decrease the oestrogenic activity of ZEN. Only for 8-prenylnaringenin (14.7 and 29.4 μM) was a trend towards an increase in the ZEN-induced cell proliferation up to 72% observed. In conclusion, with the exception of 8-prenylnaringenin, no substantial interaction between phytoestrogens and the mycotoxin ZEN could be detected using a bioassays with MCF-7 cells.

Key words: E-screen assay, oestrogen receptor, mycoestrogen, phytoestrogens, zearalenone

Introduction

Zearalenone (ZEN) is a resorcylic acid lactone (Urry et al. 1966) produced by several *Fusarium* species and is commonly found worldwide in cereal grains intended for food and feed (Schatzmayr and Streit 2013). In farm animals, the ingestion of ZEN-contaminated feed can lead to alterations of the reproductive tract, disturbance of the oestrous cycle and decreased fertility due to malfunction of the ovary

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(Gajecki 2002, Fink-Gremmels and Malekinejad 2007, Malekinejad et al. 2007, Tiemann and Danicke 2007, Zinedine et al. 2007, Minervini and Dell'Aquila 2008, Doll and Danicke 2011). These effects ultimately lead to lower performance. Pigs are particularly sensitive to ZEN (EFSA 2011). In this species, even very low concentrations of ZEN may lead to hyperoestrogenic syndrome (Gajecka et al. 2012, Schoevers et al. 2012).

ZEN and its derivatives are the only known mycoestrogens and exhibit oestrogenic activity due to the structural resemblance of 17 β-oestradiol (E2), the main hormone produced by the mammalian ovary. ZEN competes with E2 for the specific binding sites of the oestrogen receptors (ER) α and β in mammalian target cells (Kuiper-Goodman et al. 1987). E2 binds to ERα via its aromatic ring. However, the volume of the binding cavity of the receptor is almost twice as large as E2, which could allow other compounds with a comparable 3-dimensional structure, such as ZEN and various non-steroidal phytoestrogens, to bind simultaneously (Brzozowski et al. 1997, Jungbauer and Medjakovic 2014, Leclercq and Jacquot 2014). ERα and ERβ are assumed to have different and even opposing biological functions (Lindberg et al. 2003). ERα seems to play a predominant role in cell proliferation (Pearce and Jordan 2004), while ERβ may negatively regulate cell proliferation, and has a protective role for example in normal breast cells (Hilakivi-Clarke et al. 2002). When the same cell is co-exposed to multiple agonists, ERβ inhibits ERα-mediated gene expression (Matthews and Gustafsson 2003, Paruthiyil et al. 2011).

The human breast cancer cell line MCF-7 expresses both types of oestrogen receptors with a predominance of ERα (Nadal-Serrano et al. 2013). MCF-7 cells are very sensitive to oestrogenic compounds and cell proliferation depends on the presence of oestrogen-receptor agonists. This sensitivity facilitated the development of the E-screen assay (Soto et al. 1992) which measures oestrogen-induced proliferation of MCF-7 cells. As demonstrated in transfected MCF-7 cells, E2 is able to activate both ER isomers with similar potency (Harris et al. 2005). Phytoestrogens on the other hand show differential binding to the two ER subtypes, with preference for ERβ (Leclercq and Jacquot 2014). Therefore, phytoestrogens are presumed to inhibit excessive cell proliferation, for example in hormone dependent breast cancer cells (Ramanathan and Gray 2003, Rietjens et al. 2013). The mycoestrogen ZEN induces proliferation of MCF-7 cells by binding preferentially to ERα (Wang et al. 2010). Phytoestrogens which bind preferentially to the oestrogen receptor ERβ may counteract the oestrogenic activity of ZEN. Such an effect was shown for GEN, which repressed the proliferative activity of ZEN on MCF-7 cells (Wang et al. 2010).

Human and animal diets may contain several non-steroidal phytoestrogens which can be classified into: (1) flavonoids such as genistein (GEN), 8-prenylnaringenin (8-PN) or xanthohumol (XAN), (2) coumestans such as coumestrol (COU), and (3) lignans such as precursors for enterolactone (ENT), or cubebin (Nikov et al. 2000). GEN is a natural compound of soybeans (*Glycine max* L.) or red clover (*Trifolium pratense* L.). It has been reported that GEN has oestrogenic, anti-oxidative, and anti-inflammatory effects (Record et al. 1995, Hsieh et al. 1998, Danciu et al. 2012, Russo et al. 2016). GEN binds to ERα and ERβ, but has a stronger affinity to ERβ (Kuiper et al. 1998, Mueller et al. 2004). The flavonoids 8-PN and XAN occur in hop plants (*Humulus lupulus* L.). Its constituent 8-PN is considered the most potent phytoestrogen isolated to date and binds to ERα and ERβ (Milligan et al. 2002, Blanquer-Rossello et al. 2013, Busch et al. 2015). XAN is less potent than 8-PN, and is known to exhibit anti-carcinogenic properties and to prevent initiation and progression of cancer (Gerhauser et al. 2002, Deeb et al. 2010, Yoshimaru et al. 2014). XAN seems to inhibit the proliferation of ERα-positive breast cancer cells through suppression of the E2-signalling pathways (Blanquer-Rossello et al. 2013, Yoshimaru et al. 2014). COU is a representative of the coumestan group which naturally occurs in broccoli (*Brassica oleracea* L.), red clover sprouts, alfalfa sprouts (*Medicago sativa* L.), and soy beans (Adams 1995, Harris et al. 2005). Like GEN, COU binds to ERα and ERβ, but has a stronger affinity to ERβ (Kuiper et al. 1998, Mueller et al. 2004). Oestrogenic effects of COU were shown in different assays in MCF-7 cells (Matsumura et al. 2005). ENT is a member of the lignan group, present in plant foods and human biological fluids. The conversion of plant lignans (secoisolariciresinol and matairesinol) to mammalian lignans (enterodial and ENT) occurs in the gastrointestinal tract as a result of bacterial action (Kurzer and Xu 1997). Oilseeds, such as flaxseed (*Linum usitatissimum* L.) and unhulled soybeans, are the richest plant sources of lignans. Anti-oestrogenic effects of flaxseed and its lignans were shown by Bergman Jungestrom (2007).

In the present study, the effect of plant-derived oestrogenic substances on the proliferation of MCF-7 cells and the interaction of the samples with ZEN were tested using a modified version of the E-screen assay. The aim was to assess if these samples are able to alter the oestrogenic effect of ZEN.

Table 1. Information concerning plant-derived substances tested for their effect on proliferation of oestrogen-sensitive MCF-7 cells.

(Phyto)estrogen	Abbreviation	Group	Stock [mM]
8-prenylnaringenin	8-PN	Flavonoid	29.4
Xanthohumol	XAN	Flavonoid	282
Genistein	GEN	Flavonoid	370
Coumestrol	COU	Coumestan	373
Enterolactone	ENT	Lignan	168

Materials and Methods

Preparation of samples tested for their effect on MCF-7 cell proliferation

The samples that were tested in this study are summarised in Table 1. The phytoestrogens and enterolactone were purchased from Sigma-Aldrich (Vienna, Austria). Stocks of all substances were prepared in dimethyl sulfoxide (DMSO; cell culture tested; Sigma-Aldrich) as indicated in Table 1 and finally diluted by a factor of at least 1000 with assay medium (composition is defined in the "Proliferation assay" section). A 63 mM stock of ZEN (Romer Labs®, Tulln, Austria) was also prepared in DMSO and diluted with assay medium to the different working concentrations described below. The maximum concentration of DMSO was 0.1% in all assays.

Cell line and cell culture conditions

The human breast adenocarcinoma cell line MCF-7 (Number ACC 115) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were routinely grown in a culture medium containing RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 200 mM L-glutamine, 100 mM sodium pyruvate, and 1% of the growth supplement ITS (insulin/transferrin/selenium). The cells were maintained under standard conditions at 37°C in an atmosphere of 5% CO_2 and saturating humidity. The cells were grown to 90% confluence and were sub-cultured every 2-3 days using a solution of 0.25% trypsin and 0.5 mM EDTA (Sigma-Aldrich) for detachment. Unless otherwise stated, all medium components were obtained from PAA Laboratories GmbH (Pasching, Austria).

Proliferation assay

The proliferation assay was performed according to the E-screen assay described by Soto et al. (1992) with minor modifications. Initially, the proliferative effect of ZEN was tested in a concentration range between 15.6-2000 nM in the MCF-7 cells to determine the working concentration for the main experiments. To this end, MCF-7 cells were seeded in 96-well plates at an initial concentration of $1x10^4$ cells per well in 200 µL of culture medium. Cells were allowed to attach for 24 h. The culture medium was thereafter replaced by assay medium, which was composed of culture medium without phenol red, supplemented with 10% double charcoal-stripped FBS, to exclude any oestrogenic substances in the culture medium. The cells were further cultivated for 2 h under these conditions, before the medium was discarded and replaced with 200 µL of medium containing the different concentrations of ZEN. Cells in assay medium without ZEN served as control. After 6 days of incubation, the medium was discarded and cell proliferation was determined by addition of 100 µL of 10% water soluble tetrazolium-1 (WST-1; Roche Diagnostics, Vienna, Austria) in assay medium. WST-1 is cleaved enzymatically to formazan by cellular mitochondrial dehydrogenase present in all viable cells. The absorbance of the formazan dye produced can be measured at 450 nm (A_{450nm}), and is directly correlated to the number of viable cells. The first measurement was done immediately after the addition of the WST-1 dye using a GENios microplate reader from Tecan (Grödig, Austria). The cells were then incubated with WST-1 at 37°C in an atmosphere of 5% CO_2 and saturating humidity. The absorbance was measured at regular intervals. The incubation was terminated as soon as the control cells reached an absorbance of ≥ 1.

For the main experiments, the effect of selected samples (Table 1) on MCF-7 cells was assessed. In the assays, MCF-7 cells were either incubated with the test sample alone or with the test sample and 1 µM of ZEN. Test samples (200 µL) diluted to different concentrations were added after the incubation step with assay medium to remove natural oestrogens. After 1 h of pre-incubation with the test samples, 100 µL ZEN working solution was added to the appropriate wells resulting in a final concentration of 1 µM. Cells in blanc assay medium served as cell control. Cells incubated with 1 µM of ZEN were defined as ZEN control for the co-incubation experiments.

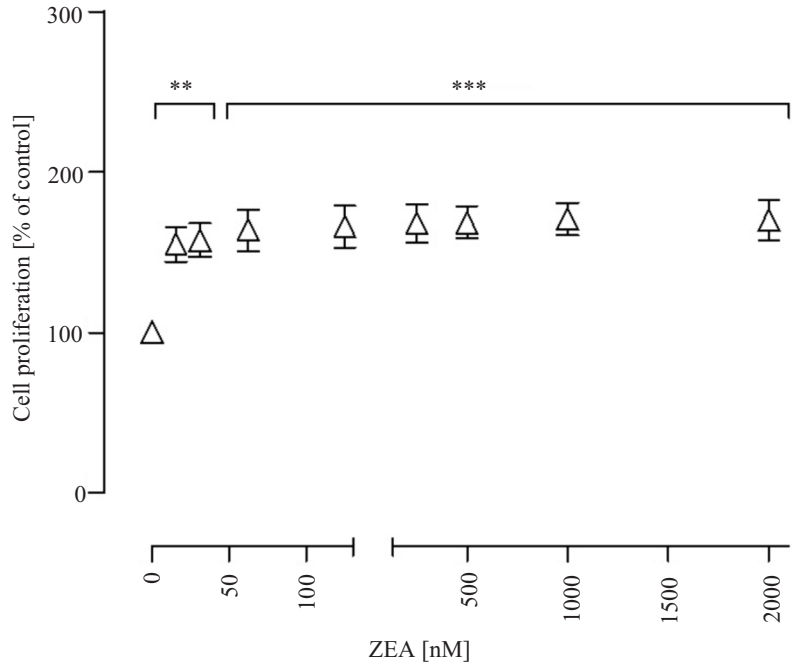

Fig. 1. Proliferation of MCF-7 cells incubated with zearalenone (ZEN; n=6). Data represent means ± standard deviation. ** p≤0.01, *** p≤0.001 vs. cell control.

Evaluation

The absorbance was corrected for the optical density of the plates measured directly after the addition of the WST-1 dye. Cell proliferation [%] in relation to the cell control was calculated using Equation 1.

$$\text{Cell proliferation [\%]} = \frac{A_{450nm} \text{ value of sample}}{A_{450nm} \text{ value of cell control}} \times 100 \quad (1)$$

The mean value of the cell proliferation was determined for each independent experiment. The results represent mean values ± standard deviation (SD) from at least three independent experiments and were plotted using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla California USA).

Statistics

Differences between the cell control and cells treated with the test substance alone (marked with *) and differences between the ZEN control and the co-incubated samples (marked with °) were determined using IBM® SPSS® Statistics statistical analysis software (Version 19; IBM Corp., USA). Data was analysed for normality using the Shapiro-Wilk test and for homogeneity using the Levene's test. Normally distributed data were assessed by one-way ANOVA and the posteriori Dunnett's test (equal variance) or the Dunnett T3 test (unequal variance). Nonparametric data were assessed using the Kruskal-Wallis test.

Results

Oestrogenic effect of ZEN on MCF-7 cell proliferation

In an initial experiment, the effect of ZEN on MCF-7 cell proliferation was studied to define the working concentration of ZEN for the co-incubation experiments. ZEN increased the cell proliferation already significantly at the lowest concentrations tested (15.6 and 31.3 nM) up to 157%. Only a minor increase was observed at concentrations above 50 nM (cell proliferation up to 170%) (Fig. 1). From these results, we selected 1000 nM (1 µM) as a working concentration for the co-incubation experiments.

Effects of plant-derived oestrogenic substances on MCF-7 cell proliferation

The flavonoid 8-PN significantly increased cell proliferation at all tested concentrations, with a maximum of 167% observed at the highest tested concen-

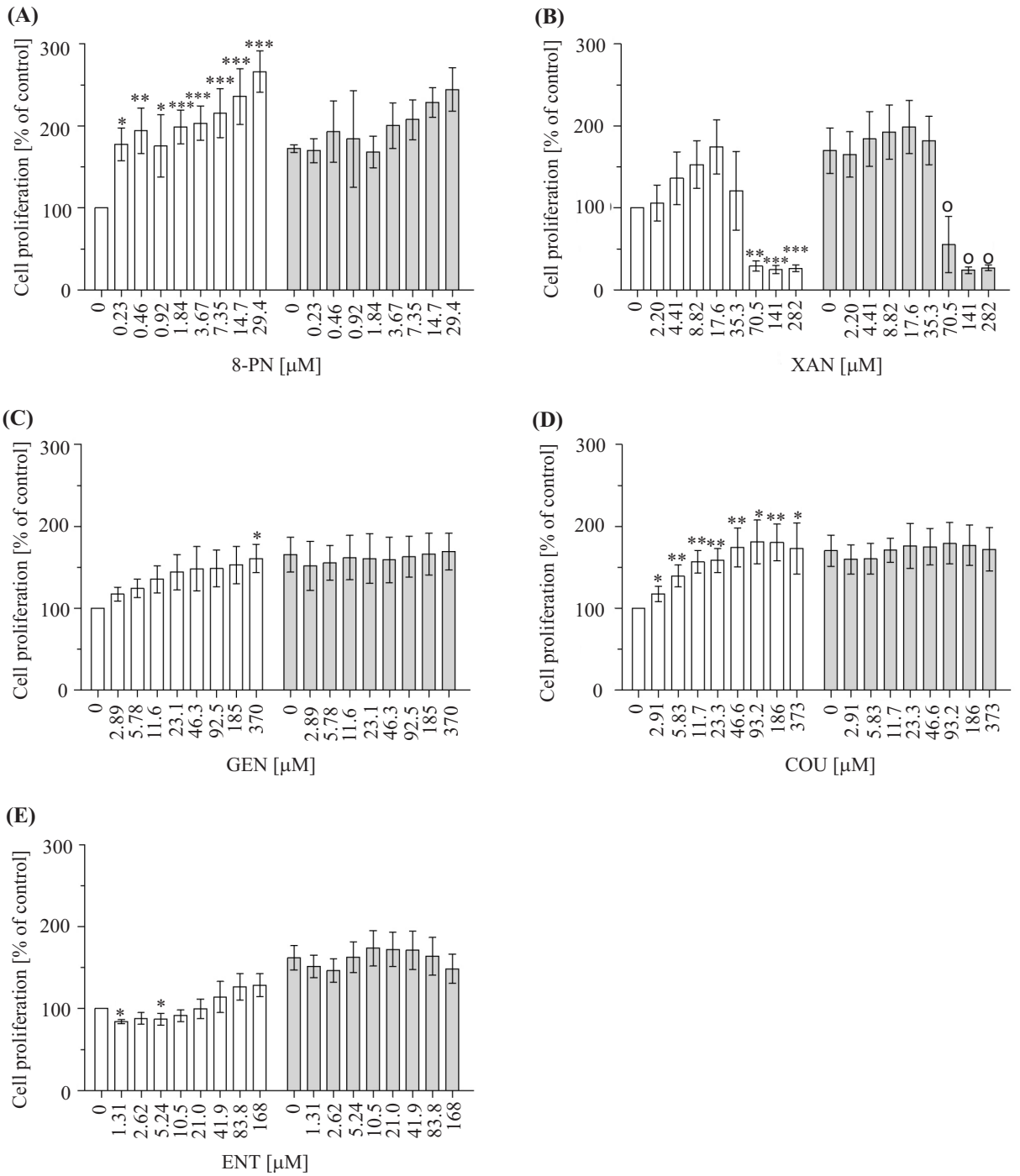

Fig. 2. Proliferation of MCF-7 cells incubated with plant-derived oestrogenic substances alone (white bars) and in co-incubation with zearalenone (ZEN; grey bars): (A) 8-prenylnaringenin (8-PN; n=3), (B) xanthohumol (XAN; n=4), (C) genistein (GEN; n=5), (D) coumestrol (COU; n=6), and (E) enterolactone (ENT; n=5). Data represent means ± standard deviation. * p≤0.05, ** p≤0.01, *** p≤0.001 vs. cell control; ^{o} p≤0.05 vs. ZEN control.

tration of 29.4 μM (Fig. 2A). When co-incubated with ZEN, the dose response curve was less steep and only an increase of up to 72% in cell proliferation rate compared to the ZEN control was observed. XAN increased cell proliferation concentration-dependently up to a concentration of 17.6 μM. At higher concentration a clear decline of cell viability was observed. The same biphasic effect was observed when cells were co-incubated with ZEN (Fig. 2B). GEN caused a concentration-dependent increase in cell proliferation (up to 64%), but the effect was only significant at the highest tested concentration (370 μM; Fig. 2C) when compared to the untreated controls. GEN had no significant effect on cell proliferation when

co-incubated with ZEN (Fig. 2C). Incubation with COU caused a significant increase (39-81%) in the cell proliferation rate at concentrations between 5.83-373 μM, but did not significantly affect proliferation of cells co-incubated with ZEN (Fig. 2D). The lignan ENT inhibited cell proliferation at 1.31 (16%) and 5.24 μM (13%), whereas higher concentrations (41.9-168 μM) increased cell proliferation by up to 29%. However, this increasing effect was not significant. Co-incubation of cells with ENT and ZEN did not significantly affect cell proliferation (Fig. 2E). Ranking the substances according to the lowest concentration showing a clear proliferative effect leads to the following order: 8-PN (0.23 μM; 78%), COU (5.83 μM; 39%), GEN (370 μM; 61%).

Discussion

Using a modified version of the common E-screen assay, plant-derived substances, previously described as exerting oestrogenic effects, were tested for their proliferative or anti-proliferative effect on MCF-7 cells. The samples were additionally tested for their ability to decrease or increase ZEN-induced cell proliferation in co-incubation experiments.

In a preliminary experiment, the working concentration of ZEN for the co-incubation experiments was established. Results show a non-linear dose-response curve. A significant increase in cell proliferation rate (up to 157%) was already achieved at the lower concentration range (15.6 and 31.3 nM), which is only slightly below the maximum proliferation rate of 170% achieved at higher concentrations. These results are in line with findings from Yu et al. (2005) describing, at concentrations of 32 and 96 nM, a significantly increased proliferation of MCF-7 cells by 154.5% and 190.4%, respectively, compared to the control. The minor differences between these studies might be explained by different assay set-ups (e.g. seeding density, incubation time, cell viability/proliferation assay). However, despite the response of low ZEN concentrations, we decided to use a concentration of 1000 nM (1 μM) for the co-incubations with phytoestrogens to ensure significant cell proliferation.

In the main set of experiments, the plant-derived oestrogenic substances 8-PN, XAN, GEN, COU, and ENT were tested alone or in combination with ZEN. The hop flavonoid 8-PN caused the highest increase in MCF-7 cell proliferation at all tested concentrations. Comparable effects of 8-PN have been described in previous studies (Lust et al. 2005, Vanhoecke et al. 2005, Zanoli and Zavatti 2008). However, Brunelli et al. (2009) reported that this compound also exerts cytotoxic effects on MCF-7 cells when used at concentrations above 1 μM, which differs from our results. The different results might be explained by differing methods (transfected MCF-7 cells, incubation time, and viability tests). In the co-incubation experiments with ZEN, the combined dose-response curve was much less steep, pointing towards an interference of ZEN with the 8-PN induced proliferation. Despite the high concentration of ZEN, cell proliferation was increased when cells were co-incubated with 8-PN at the highest concentrations (14.7 and 29.4 μM) compared to the ZEN control. Another hop flavonoid, XAN, was shown to have anti-oestrogenic activity in Ishikawa cells, a human endometrial adenocarcinoma cell line (Gerhauser et al. 2002). In the present study, XAN inhibited cell proliferation at higher concentrations, indicating a loss of viability. No major differences between incubations with XAN alone or co-incubations with ZEN were observed. Cytotoxic effects have already been reported for 50 and 100 μM XAN, which is in line with the present observations (Monteiro et al. 2008). Other studies have shown that the flavonoid GEN can have a biphasic effect on the growth of MCF-7 cells: concentrations as low as 10 nM increased the proliferation rate of MCF-7 cells, but high concentrations (above 10-20 μM) inhibited cell proliferation (Wang et al. 1996, Hsieh et al. 1998). In a more recent study, it was shown that GEN (16 and 32 μM) reduced the cell viability of MCF-7 cells induced by 10 and 20 nM ZEN (Wang et al. 2010). In the current experiments, the cell proliferation was not increased significantly in the co-incubation experiments with GEN and ZEN. GEN alone increased the cell proliferation only in the highest tested concentration of 370 μM significantly. Our study showed no significant decrease of MCF-7 cell proliferation in the co-incubation experiments. This might be due to the higher ZEN concentration that we used (1 μM). COU is considered to be one of the most potent phytoestrogens. It exhibits strong oestrogenic activity on ERα, but an up to 100-fold stronger activation of ERβ (Kuiper et al. 1998, Schmitt and Stopper 2001, Harris et al. 2005). In the present study, COU showed a more pronounced proliferative effect on MCF-7 cells than GEN, which was also reported by Sakomoto et al. (2010). ENT at 1.31 and 5.24 μM showed a slight inhibitory effect at low concentrations, both in incubations with GEN alone and in the co-incubation experiments with ZEN, which remains unexplained. In previous studies, ENT increased MCF-7 cell proliferation at concentrations between 1-10 μM, and inhibited cell proliferation above 10 μM (Mousavi and Adlercreutz 1992). Another study has shown that concentrations of 0.5 and 1 μM ENT significantly decreased MCF-7 cell viability (Abarzua et al. 2012). Mousavi and Adlercreutz (1992) showed that ENT

inhibited the proliferative effect of E2 on MCF-7 cells. In contrast, ENT was not able to significantly reduce the ZEN-induced proliferation of MCF-7 cells in the present study.

The plant-derived substances were selected based on their ability to bind to ERα and/or ERβ (8-PN, GEN, COU) or to inhibit the proliferation of cancer cells (XAN, ENT). Despite this, there was no evidence that any of the tested compounds were able to significantly reduce the ZEN-induced proliferation and hence the pro-oestrogenic effect of ZEN. However, as it has been shown previously that at least some of the tested compounds inhibit the oestrogen-induced growth of cancer cells, it cannot be excluded that the selected concentration of ZEN (1 μM) was too high to detect an anti-oestrogenic effect in the current assay. Further studies devoted to the potential interactions between myco- and phytoestrogens should include a broader concentration range of ZEN and also its metabolites, as some of these have an even higher oestrogenic activity (EFSA 2016).

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