Assessment of hormone-like activities in *Ginkgo biloba, Elettaria cardamomum* and *Plantago ovata* extracts using in vitro receptor-specific bioassays

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Abstract

Medicinal plants are widely used for treatment of several diseases, as well as for developing new drugs. The present study was designed to determine the presence of hormone-like activities dependent on the activation of human estrogen receptor alpha (hERα) and/or androgen receptor (hAR) in methanol extracts prepared from three medicinal plants historically and currently used for diverse therapeutic purposes [*Ginkgo biloba* leaves (GBL), *Elettaria cardamomum* seeds (ECS) and *Plantago ovata* seeds (POS)]. After a solid-liquid extraction (SLE) step, their effects on hERα function were assessed in MCF-7 breast cancer cells using the E-Screen bioassay whereas the ability to induce hAR-mediated reporter gene expression was explored in the androgen-sensitive PALM cells, a stable prostatic cell line. Unlike POS extracts, GBL and ECS extracts showed estrogenic (71.11 and 205.26 pM E_2Eq/mg, respectively) and anti-estrogenic activity (13.22 and 20.81 nM ICI182780Eq/mg, respectively). Androgenic activity was found in extracts of ECS (300.19 pM R1881Eq/mg) whereas anti-androgenic activity was exclusively observed in POS extracts (22.30 µM ProcEq/mg). These findings provide evidence that plant extracts may interfere with the endocrine system *via* one or more hormonal receptors and that further investigation is warranted into their role as endocrine disrupters in humans.

Key words: *Ginkgo biloba* leaves (GBL); *Elettaria cardamomum* seeds (ECS); *Plantago ovate* seeds (POS); E-Screen bioassay; Human androgen receptor (hAR).

Abbreviations and definitions used in the manuscript: 17β-estradiol (E_2); 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT); benign prostatic hyperplasia (BPH); dextran-coated charcoal (DCC); dimethyl sulfoxide (DMSO); electron spray ionization-mass spectroscopy (ESI-MS); *elettaria cardamomum* seeds (ECS); endocrine disrupting chemicals (EDCs); estrogens receptors (ERs); fetal bovine serum (FBS); flame-retardant polybrominated diphenyl ether (PBDE); geneticin (G418); *ginkgo biloba* leaves (GBL); high performance liquid chromatographic (HPLC); human androgen receptor (hAR); human estrogen receptor alpha (hERα); ICI 182,780 (ICI); methyltrienolone (R1881); nuclear receptors (NRs); *plantago ovata* seeds (POS); polychlorinated biphenyls (PCBs); procymidone (Proc); proliferative effect (PE); selective estrogen receptor modulators (SERMs); solid-liquid extraction (SLE); sulforhodamine B (SRB); trichloroacetic acid (TCA); world health organization (WHO); yeast estrogen screen (YES).

Introduction

The world health organization (WHO) has estimated that about 80% of the population in developing countries depends on traditional medicine for their primary health care needs. Of the 75,000 plants used in different systems of medicine, more than 20,000 species of higher plants are used in the traditional treatment practices of indigenous cultures living around the world (Pal and Shukla, 2003). Therefore, medicinal plants play a key role in world health including the maintenance of health as well as in the introduction of new treatment. The widespread use of medicinal plants is partly due to the low toxicity attributed to these natural products (Philomena, 2011). However, medicinal plants may cause a series of toxic effects, including metabolic disorders, alterations in immune and endocrine system, hepatic toxicity and behavioral effects among others (Bush et al., 2007; Buttar and Jones, 2003; Tovar, 2009).

Herbal products are complex mixtures of organic chemicals that may come from any raw or processed part of a plant, including leaves, stems, flowers, roots, and seeds. As far as they are considered dietary supplements products manufacturers can produce, sell, and market them without first demonstrating safety and efficacy, as is required for pharmaceutical drugs (Bent, 2008).

Among herbal products phytoestrogens are plant-derived compounds with estrogenic activity found in natural dietary sources. In the last decades, these compounds have received considerable attention and many of them are now assimilated to endocrine disrupting chemicals (EDCs) (Cederroth et al., 2012). However phytoestrogens remain widely believed to provide an array of beneficial effects, including preventative or therapeutic actions in carcinogenesis, atherosclerosis, and osteoporosis (Cassidy et al., 2006; Kim, 2008). In contrast, exposure to most synthetic EDCs such as pesticides, fungicides, pharmaceutical agents, dioxins, plasticizers, plastics, polychlorinated biphenyls (PCBs), flame-retardant polybrominated diphenyl ether (PBDE), is associated with harmful effects regarding declining reproductive health, effects including interference with both the male and female reproductive systems, causing a spectrum of disorders throughout life, including sexual precocity, reproductive tract abnormalities and infertility (Arrebola et al., 2013; Diamanti-Kandarakis et al., 2009; Fernandez et al., 2007, 2012).

Numerous herbal products have been historically and currently used for diverse therapeutic uses, but only a few have been thoroughly characterized for their hormone-like activity. In this context, we focused our investigations on three medicinal plants widely employed: *Ginkgo biloba* (GB), *Elettaria cardamomum* (EC) and *Plantago ovata* (PO). Extracts of GB leaves (GBL) in various forms can be purchased in many countries as a dietary supplement and is one of the most widely sold products in herbal supplement sales in the United States (Lindstrom et al., 2013) as well as in Europe (Vargas-Murga et al., 2011). GBL extracts contain numerous chemical constituents, for example, flavonol glycosides and terpene lactones that are the two major class components associated with the reported therapeutic effects (van Beek and Montoro, 2009). So far, some studies have detected sex hormone-like activities in commercially available extracts from GBL. For instance, GBL extracts has demonstrated *in vitro* estrogenic and anti-estrogenic activity in MCF-7 human breast cancer cells (Oh and Chung, 2004) and in a recombinant MCF-7 cell line (Oh and Chung, 2006). However, these studies have focused only on the agonistic/antagonistic activity of GBL extracts *via* estrogen receptors (ERs) with no mention to other nuclear receptors (NRs) mediated activities, *e.g*. hAR.

EC is a perennial herb, known as “queen of spices”, produced in India, Guatemala, Tanzania, Papua New Guinea, Costa Rica, Sri Lanka, El Salvador, Vietnam, Laos and Cambodia. In addition to its wide use for culinary purpose, EC seeds (ECS) are used as carminative, stomachic, diuretic, abortive, antibacterial, antiviral, antifungal treatments and is considered useful in treatment of constipation, colic, diarrhea, dyspepsia, vomiting, headache, epilepsy and cardiovascular diseases (al-Zuhair et al., 1996; Gilani et al., 2008). Finally, PO is an annual herb, native to Iran, India and Middle East countries that belongs to the *plantaginaceae* family, containing 10-30% mucilage. PO seeds (POS) are widely used as laxative and cholesterol-lowering properties (Fernandez-Banares et al., 1999; Romero et al., 2002).

Except for GBL, no information is available about sex hormone-like activities of the above mentioned medicinal plants. Therefore, the present study was designed to determine the presence of sex hormone-like activities dependent on the activation of human estrogen receptor alpha (hERα) and/or androgen receptor (hAR) in extracts prepared from GBL, ECS and POS using receptor-specific bioassays after a solid-liquid extraction (SLE) step. The experimental approach focused on EDCs that are active *via* hERα binding and subsequent MCF-7 cell proliferation using the E-screen bioassay and

on those active *via* hAR binding in PALM cells, a stable prostatic cell line, determining the consequent gene expression.

Materials and methods

Chemicals, plant material, and instrumentation

All reagents were analytical grade unless otherwise specified. Reference standards 17β-estradiol (E_2), methyltrienolone (R1881), procymidone (Proc) and ICI 182780 (henceforth, ICI), puromycin, geneticin (G418), luciferin (sodium salt), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), sulforhodamine B (SRB), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich Inc. (St Louis, MO). Stock solutions (10 mM) of E_2 (≥ 98% purity), R1881 (≥ 98% purity), Proc (> 99.7% purity), and ICI (≥ 99% purity) were prepared in ethanol, and successive dilutions were performed in culture medium. Stock solutions were kept at -20 °C, and dilution series were freshly prepared before each experiment. HPLC-grade solvent (methanol) was from Merck (Darmstadt, Germany) and dimethyl sulfoxide (DMSO) from Panreac (Barcelona, Spain). Culture medium and fetal bovine serum (FBS) came from Gibco (Invitrogen, Barcelona, Spain), and all cell culture plastics were supplied by Falcon (VWR International Eurolab, Barcelona, Spain).

For cell proliferation assays, the absorbance was read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm, while an infinite M200 luminometer (Tecan, Barcelona, Spain) was used to detect luciferase activity in intact cells.

All three plant samples [(*Ginkgo biloba* leaves (GBL), *Elettaria cardamomum* seeds (ECS) and *Plantago ovata* seeds (POS)] were purchased from different local herbalists in Granada (Southern Spain). To avoid anthropogenic influence, samples were purchased from ecological production. Extracts from these plants were kindly provided by Ingredientis Biotech, SLU. Granada, Spain.

Plant treatment: solid-liquid extraction (SLE)

Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. Here, we performed a solid-liquid extraction (SLE) using methanol as solvent in order to obtain the bioactive compounds from GBL, ECS and POS. In brief, all three plant samples

(GBL, ECS and POS) were dried and pulverized before extraction. Next, 20 g of each dry sample powder was placed in a 1000-ml round-bottom flask and macerated with 500 ml of methanol. Extraction was performed at room temperature for 24 h using an electric shaker (shaking intensity 120 rpm). After extraction, the residues were separated from the extracts by filtering through a filter paper. The methanol extract obtained was then concentrated under reduced pressure at 45 ºC in a rotary evaporator. Finally, the residues obtained (0.96, 1.24 and 0.76 g for GBL, ECS and POS, respectively) were placed in glass vials stored at 4 °C until analysis in the different bioassays. An initial stock solution for each extract was prepared as follows: 20 mg were first dissolved in DMSO (100 µl) and next diluted in test culture medium to a concentration of 2 mg/ml (1% final solvent concentration).

Cells and culture conditions

Cell lines used in this study were cultured as previously described (Molina-Molina et al., 2013, 2014). In brief, human breast cancer MCF-7 cells were cultured for routine maintenance in DMEM with phenol red supplemented with 10% FBS, while PALM cells, a human androgen-dependent stable transfected cell line (Terouanne et al., 2000), were cultured in Ham's F12 supplemented with 10% FBS, 1 mg/ml G418, and 1 µg/ml puromycin. Because of the hormonal activity of phenol red and FBS, experiments were performed in a test culture medium, *i.e.*, phenol red-free DMEM supplemented with 10% dextran-coated charcoal-FBS (10% DCC-FBS) for MCF-7 cells or Ham's F12 supplemented with 6% DCC-FBS and 1% antibiotic for PALM cells, in a 5% CO_2 humidified atmosphere at 37 °C.

E-Screen bioassay

Assay procedure and data analysis were conducted as previously described (Molina-Molina et al., 2013, 2014) with some modifications for the testing of plant extracts. Briefly, MCF-7 cells were trypsinized and plated in 96-well culture plates at initial concentrations of 4 x 10^3 cells per well. On the second day, the seeding medium was removed and replaced with 150 µl test culture medium. Sample extracts (2 mg/ml in test culture medium) were serially diluted (10x) in the same medium to concentrations of 2-0.002 mg/ml and 50 µl were added per well, resulting in final concentrations of 500 to 0.5 µg/ml. A dose-response curve (0.1 pM-1000 pM) for E_2 and negative control (cell

treated only with hormone-free medium) and solvent controls (blank and solvent) were included in each experiment. The bioassay was ended on day 6 (late exponential phase) by removing the media from the wells, fixing the cells, and staining them with SRB. Finally, bound dye was solubilized and the absorbance was read at 492 nm.

Agonistic assays were performed with increasing concentrations (0.5, 5, 50 or 500 μg/ml) of sample extracts, resulting in final solvent concentrations of 0.0012 to 0.25% (v/v) in the well. The ratio between the cell yield obtained and the proliferation of hormone-free control cells (negative control) was calculated for each concentration. Tests were done in triplicate and results were expressed as proliferative effect (PE) [MCF-7 cell proliferation (-fold over control)]. The antagonistic activities of extracts were determined by co-incubation with the agonist E_2 at 100 pM. Finally, the PE of each sample extract was referred to the maximal PE obtained with E_2 or ICI and transformed into E_2 or ICI equivalent units (E_2Eq or ICI Eq) by reading from the dose-response curves for these compounds. For each sample extract, E_2Eq or ICIEq were calculated using the concentration obtaining the greatest induction or inhibition of cell proliferation, respectively. Next, derived E_2Eq and ICIEq values were corrected for the dilution factor and reported as E_2Eq or ICIEq per mg of sample extract and per g of dry matter plant.

PALM cell luciferase assay

Luciferase assays were conducted by using the stably transfected luciferase reporter PALM cell line according to previously described protocols (Molina-Molina et al., 2006, 2008, 2013, 2014). Cells were seeded at a density of 5×10^4 cells per well in 96-well white opaque tissue culture plates in 150 μL test culture medium. Sample extracts were serially diluted (as described above for the E-Screen bioassay) and 50 μL were added per well 8 h after seeding. On each plate, alongside the test samples, serial dilutions of the agonist R1881 (1 pM-10,000 pM) were included as positive and negative controls (test culture medium alone or with solvent). PALM cells were incubated for 40 h at 37 °C, and the medium containing the sample extracts was then removed and replaced by test culture medium containing 0.3 mM luciferin. Next, the 96-well plate was introduced into a luminometer for 2 s to measure luminescence from intact living cells.

hAR-agonistic activities were tested with increasing concentrations of sample extracts, performing tests in triplicate for each concentration. Maximal luciferase activity (100%) was obtained in the presence of 10 nM R1881. The antagonistic activity of extracts was determined by coincubation with R1881 agonist (0.3 nM). At this concentration, activity reaches approximately 80% of maximal luciferase activity. Results were expressed as percentage of maximal luciferase activity. Finally, the luciferase activity in each sample extract (calculated as a percentage of the maximal luciferase activity obtained with R1881 or Proc) was transformed into R1881 or Proc equivalent units (R1881Eq or ProcEq, respectively) by reading from dose-response curves of R1881 or Proc (standard serial dilutions) included on each plate. R1881Eq or ProcEq were calculated from the concentration obtaining the greatest induction or inhibition of luciferase activity, respectively. Derived R1881Eq or ProcEq were corrected for the dilution factor and reported as R1881Eq or ProcEq per mg of sample extract and per g of dry matter plant.

MTT assay for evaluating cell toxicity

The effect of sample extracts on cell viability was assessed with the MTT test, using Denizot and Lang's modified technique (1986). Briefly, cell lines (MCF-7 and PALM) were seeded at a density of 5 x 10^4 cells per well in 96-well culture plates for 8 h, followed by treatment with different concentrations (0.5 to 500 µg/ml) of each extract for a further 40 or 144 h (for PALM and MCF-7 cells, respectively). Cells were washed with PBS, and 100 µl of MTT solution (0.5 mg/ml) were then added to each well. After incubation (2 h), viable cells cleaved the MTT tetrazolium ring into a dark blue formazan reaction product, whereas dead cells remained colorless. The MTT-containing medium was gently removed, and 150 µl DMSO were added to each well. Cell viability of treated cells was calculated in reference to the untreated control cells using the following formula: viability (%) = 100 x (Sample Abs)/(Control Abs), where Abs is the absorbance value at 540 nm. Medium alone with no cells served as an additional control. Three independent experiments run in triplicate were performed, and data were expressed as the mean of three wells.

Data analysis

For all assays, each sample extract was tested at various concentrations in at least three independent experiments, and data were expressed as means ± SD. Individual dose-

response curves for E_2, ICI, R1881, and Proc were fitted using the sigmoid dose-response function of a graphics and statistics software package (Graph-Pad Prism, version 4.0, 2003, Graph-Pad Software Inc., San Diego, CA), expressing the results as EC_{50} and IC_{50} values. Data were analyzed for significant differences using one-way ANOVA followed by Dunnett's post-comparison test (*vs.* control). Differences were considered statistically significant at $p < 0.05$.

Results

Estrogenic effects of plant extracts in the E-Screen bioassay

The estrogenic potential of GBL, ECS and POS extracts was characterized by using the E-Screen bioassay to investigate their ability to stimulate cell proliferation in MCF-7 cells. As depicted in Figure 1A the natural estrogen E_2 strongly induces significant proliferation in a dose-dependent manner in this cell line, with an EC_{50} value of 0.018 nM. When individual extracts were applied to MCF-7 cells, GBL and ECS extracts (both at 0.5 mg/ml) exhibited a manifest estrogenic activity, and a 2.1- and 4.2-fold increase in cell number, respectively, *versus* control-treated cells (hormone-free medium). In terms of relative proliferative effects (RPE) estrogenicity ranged from 33.5% (GBL) to 64.6% (ECS) of the maximal effect induced by E_2. By contrast, POS extracts did not stimulate MCF-7 cell proliferation in the concentration range of 0.5-500 mg/ml (Fig. 1B). Then, it was then investigated whether the estrogenic effects of GBL and ECS extracts were hER-mediated. As expected, co-incubation with pure anti-estrogen ICI (10 nM) abolished the estrogen response in these estrogen-positive samples, confirming that the cell proliferation observed by E-Screen was mediated by hER (data not shown). As shown in table 1, the estrogenic potency of plant extracts, expressed in E_2Eq/mg of extract, ranged from 0.071 nM (3.41 nM in E_2Eq/g of leaves) for GBL to 0.20 nM for ECS (12.42 nM in E_2Eq/g of seeds).

As recently reported (Real et al., 2015), the synthetic anti-estrogen ICI exhibited a marked hER antagonistic activity in MCF-7 cells, with an IC_{50} value (concentration required for 50% of maximal inhibition of E_2-induced proliferation) of 0.97 nM (Fig. 1C). Interestingly, significant hERα antagonistic activity was also detected in extracts from GBL and ECS, as they inhibited the proliferation induced by 100 pM of E_2, whereas POS extracts showed no significant antagonistic activity (Fig. 1D). The anti-

estrogenic activity of the tested extracts expressed in ICIEq/mg of extract, ranged from 0.013 μM for GBL (0.63 μM in E_2Eq/g of leaves) to 0.020 μM for ECS (1.29 μM in E_2Eq/g of seeds).

Effect of plant extracts on transcriptional activation through hAR

In line with previous reports (Molina-Molina et al., 2013, 2014), the synthetic androgen R1881 exhibited a marked hAR agonistic activity in PALM cells with an EC_{50} value of 0.1 nM (Fig. 2A). When GBL, ECS and POS extracts were tested in PALM cells, hAR was significantly activated only by ECS extracts (Fig. 2B), with a luciferase activation value of 21.4%, indicating the presence of androgen-like chemicals in the extract. However, GBL and POS extracts were unable to activate luciferase expression in this cell line after 40 h of exposure at concentrations up to 500 μg/ml. The calculated androgenic potency of ECS extracts, expressed in R1881Eq/mg of extract was 0.30 nM (18.61 nM in R1881Eq/g of seeds).

As expected, in PALM cells the well-known anti-androgen Proc exhibited a marked anti-androgenic activity in this cell line, with an IC_{50} value of 1.98 μM (Fig. 2C). When the anti-androgenic activities of these three extracts were examined, antagonistic activity was only detected in sample extracts from POS which moderately inhibits the luciferase activity induced by 0.3 nM of R1881 (Fig. 2D). In order to further characterize the antagonistic properties of POS extracts, competitive reversal assays were performed with PALM cells to determine whether the anti-androgenic activity observed in transactivation assays reflect the ability of POS extracts for binding to hAR. The antagonistic activity of these extracts was completely reversed by co-incubation with excess (1000 times the EC_{50} value) of the synthetic androgen R1881 (100 nM), demonstrating the specificity of the response (data not shown). The anti-androgenic activity of POS extracts (expressed in ProcEq/mg) was 22.30 μM (847.4 μM in ProcEq/g of seeds) (Table 1).

Cell viability

The MTT test was used to assess the cytotoxicity of GBL, ECS and POS extracts in a concentration range of 0.5-500 μg/ml for the two cell lines studied. When the viability of MCF-7 cells was analyzed after incubation with GBL or ECS extracts, the cell proliferation and therefore viability increased due to the presence of the estrogenic

compounds in these extracts, indicating that the extracts were not cytotoxic. POS extracts were also devoid of any cytotoxicity (cell survival ranging from 95 to 100%) in the 0.5-500 μg/ml range. Moreover, studies employing a saturating concentration of E_2 (10 nM) indicated that MCF-7 cell viability or survival ranged from 96 to 100% with reference to the control cells. Finally, PALM cells showed no significant changes in cell viability after incubation in the absence or presence of saturating concentrations (10 nM) of the agonist R1881, finding no cytotoxicity at any concentration (data not shown).

Discussion

We report quantitative data on the sex hormone-like activities of extracts from medicinal plants GB, EC and PO using two in vitro receptor specific bioassays. Extracts from leaves of GB and seeds of EC showed both estrogenic and anti-estrogenic activity on MCF-7 cells. Androgenic activity was observed in extracts from seeds of EC whereas anti-androgenic activity was present in extracts from seeds of PO. Overall, all the three extracts tested evidenced at least one of the four hormonal activities measured, suggesting that the consumption of their respective plants may contribute to human exposure to EDCs.

While work on phytoestrogens is fairly advanced and widely known, the concept of phytoandrogens, meaning plant-derived compounds with androgenic and/or anti-androgenic activity, is relatively new. Nevertheless, *in vitro* and *in vivo* studies have demonstrated the (anti-)androgenic potential of some natural products (Schleich et al., 2006) and several compounds or extracts derived from dietary plant material have been identified (Edouard et al., 2014; Iguchi et al., 2012). Prompted by these reports, we explored the (anti-)androgenic potential of methanol extracts from GBL, ECS, and POS using the androgen-sensitive human prostate PALM cell line. We show here, for the first time, that extracts from ECS are hAR activators (0.30 nM R1881Eq/mg of extract corresponding to 18.61 nM R1881Eq/g of seeds), suggesting the presence of androgen-like chemicals in these extracts. Moreover, POS extracts exhibited a marked anti-androgenic activity in PALM cells (22.30 μM ProcEq/mg of extract corresponding to 847.4 μM ProcEq/g of seeds) which might indicate the presence of compounds that effectively compete with the synthetic androgen R1881 for binding to the hAR. By

contrast, GBL extract showed no agonistic or antagonistic activity towards hAR after 40 h of exposure at concentrations up to 500 μg/ml.

Despite its toxicological relevance, publications dealing with the androgenic and/or anti-androgenic burden in herbal products are scarce. Based on our own data, the androgenic activity reported in this study for a recommended daily dose of 1.5 g of ECS (approximately 27.9 nM R1881Eq), is several orders of magnitude higher than the mean androgenic activity (0.033 pM R1881Eq/l) that we recently measured per liter in commercial bottled waters marketed in Southern Spain (Real et al., 2015). In the same way, the calculated anti-androgenic activity for a recommended daily dose of 3 g of POS (approximately 2,545 μM ProcEq) is also much higher than the mean anti-androgenic activity (1.61 nM ProcEq/l) that we measured in these commercial bottled waters.

To search for chemicals responsible of the (anti-)androgenicity associated to extracts of seeds of EC and PO it is far from the objectives of this study, but phytosterols, phenolic acids, and triterpenoids may count as putative candidates. For instance, it was reported that extracts of the tree bark (cortex) of the Gutta-Percha tree *Eucommia ulmoides* (used medicinally in herbal pharmacopoeias to hasten recovery from fatigue) exhibited a moderate *in vitro* androgenic activity using a reporter gene bioassay (Ong and Tan, 2007) and the purification of the androgenic extract using chromatographic and electron spray ionization-mass spectroscopy (ESI-MS) techniques revealed that this phytoandrogenic activity was being mediated by triterpenoids.

Interestingly, screening experiments to find novel anti-androgens from natural products or extracts derived from plants (Ozten-Kandaş and Bosland, 2011) found androgen antagonists which inhibit the transcriptional activity of the hAR induced by androgens and 5α-reductase inhibitors and are currently being used as potential treatment agents in the fight against prostate cancer (Syede et al., 2008). The reported anti-androgenic effects of these extracts derived from plants have been attributed to several compounds, among others some phytosterols such as β-sitosterol (Schleich et al., 2006), fatty acids (Liu et al., 2009) and phenolic acids (Sanderson et al., 2013).

On the contrary to the scarce information on androgenicity, plant-derived xenoestrogens have received much more attention, in part due to the use of selective estrogen receptor modulators (SERMs) as possible new alternative in postmenopausal therapy (Delmas et al., 1997). In fact, diets supplemented with phytoestrogens are considered an alternative to drug based hormone replacement therapy (HRT). In this regard, GB extracts obtained

from leaves represent a complex mixture of phytoestrogens with approximately 300 different compounds (Smith et al., 1996). For instance, GBL extracts contain 24% of flavone glycosides, primarily composed of quercetin, kaempferol, and isorhamnetin glycosides, and 6% terpene lactones, including ginkgolides and bilobalide (Kleijnen and Knipschild, 1992). It was previously reported that GB extracts and its major components (quercetin, kaempferol and isorhamnetin) have a double action on the ERs in competitive binding assays, with a higher binding affinity for the ERβ compared with ERα (Oh and Chung, 2004).

Interestingly, we have found that leave extracts of GB at 500 μg/ml concentration exhibited a significant but partial estrogenic activity in the E-screen bioassay (approximately 33.5% of RPE), but also and an antagonistic activity inhibiting 50% of the proliferation induced by 100 pM of E_2. The estrogenic potency of GBL extracts was 0.071 nM E_2Eq/mg of extract (corresponding to 3.41 nM E_2Eq/g of leaves) while the anti-estrogenic potency of these extracts was 0.013 μM ICI182780Eq/mg of extract (corresponding to 0.63 μM ICI182780Eq/g of leaves).

In terms of E_2Eq units and based also on our own data, the estrogenic activity calculated for a recommended daily dose of 200 mg of extract from the leaves of the GB (Chan et al., 2007), approximately 14.20 nM E_2Eq, fits well within the ranges of values (5.44 to 720 nM E_2Eq) we described per liter in liquor of vegetables packed in lacquer-coated cans (Brotons et al., 1995). However, is a little higher than the values we previously reported in baby food (Pandelova et al., 2011) where we found a maximal estrogenicity of 3.50 nM E_2Eq/ in 50 g of commercial product; and much higher in comparison with the mean estrogenic burden per liter that we measured (0.113 pM E_2Eq/l) in commercial bottled waters (Real et al., 2015).

Much difficult is to compare anti-estrogenicity levels found in this study because the scarcity of published data. Nevertheless, the anti-estrogenicity of a recommended daily dose (200 mg) of GBL extract (approximately 2.60 μM ICI182780Eq) is also much higher than the calculated anti-estrogenicity per liter (11.01 pM ICI182780Eq) in commercial bottled waters (Real et al., 2015).

Our own results confirm previous observations by Oh and Chung (2004, 2006) regarding GB hormonal activity. For instance, in MCF-7 cells, 250 μg/ml GB extracts induced a maximal cell proliferation (RPE of 24.64%) and pS2 transcription indicating that GB extracts have a moderate estrogenic activity through the estrogen response

pathway by an interaction with the hERα (Oh and Chung, 2004). Furthermore, in a bioassay based on reporter MCF-7 cells 500 μg/ml GB extracts clearly showed anti-estrogenic activity in a dose-dependent manner (Oh and Chung, 2006) by blocking the cell proliferation induced by 10 pM of E_2. Taking together these observations we can assume that leaves of GB contain active compounds, some of which are responsible for the proliferative and anti-proliferative effects observed on MCF-7 cells.

On the contrary to GB for which information is abundant, to the best of our knowledge no information is available on the endocrine properties of EC; therefore our observation on the estrogenicity of EC seed extracts is of the main interest. In fact, we have found that ECS extracts (at 500 μg/ml) exhibited a potent estrogenic activity in MCF-7 cells (0.20 nM E_2Eq/mg of seed extracts corresponding to 12.42 nM E_2Eq/g of seeds). Interestingly, we showed that ECS extracts also behaved as hERα antagonists (0.020 μM ICI182780Eq/mg of extract or 1.29 μM ICI182780Eq/g of seeds) inhibiting more than 50% the proliferation induced by 100 pM of E_2. Again, in terms of E_2Eq and ICIEq units, the calculated values of estrogenicity and anti-estrogenicity for a recommended daily dose (1.5 g) of ECS (approximately 18.6 nM E_2Eq and 1.90 μM ICI182780Eq), are much higher than the values previously reported by us in bottled waters, and comparable to the values described in cans or baby food products.

It has been reported that the seeds of EC contain mainly essential oils, sterols, phenolic acids and lipids and these seeds been prescribed in the treatment of gastrointestinal disorders (Jamal et al., 2006). In fact, clinical studies have demonstrated significant decreases in serum cholesterol concentrations after consumption of foods into which phytosterols were incorporated (Demonty et al., 2009; Laitinen et al., 2012). Early studies indicated that the main sterol components in ECS were 7-ergosterol, campesterol, γ-tocopherol, desmosterol and stigmasterol (Gopalakrishnan et al., 1990). Chemically, these are alcohols consisting of 28 or 29 carbon atoms, similar to cholesterol (Awad et al., 2000). Several *in vitro* studies have demonstrated the ability of some sterols such as 7-ergosterol, stigmasterol and its oxidation products to active hERα (Boldrin et al., 2013; Newill et al. 2007; Subbiah et al., 2003). Furthermore, in laboratory animals treated *via* parenteral with phytosterols presented adverse effects including evidence of estrogenicity (Malini et al., 1993). In contrast, oral exposure to phytosterols does not appear to cause adverse effects in rats (Waalkens-Berendsen et al., 1999). Hence, it appears that the effects of phytosterols are dependent on

bioavailability. In fact, there is agreement in many studies that the bioavailability of most phytosterols is not very high, due mainly to factors as poor absorption (Grattan, 2013). For example, in humans the absorption of phytosterols has been estimated to be 0.4-3.5%. Phytosterols have very low solubility in water (Brufau et al., 2008) and are inherently hydrophobic and tend to form stable crystals, which are not very bioavailable (Ostlund, 2007). This limited absorption is the result of the addition of a methyl/ethyl group at the C-24 position of the side chain (campesterol/β-sitosterol, respectively) increase the hydrophobicity of the molecule, thereby reducing their absorption (Heinemann et al., 1993).

In addition to polyphenols, simple phenolic acids can be found in many plant species, especially derivatives of 4-hydroxybenzoic acid and 4-hydroxycinnamic acid (Rice-Evans et al., 1996). In this context, when high performance liquid chromatographic (HPLC) analysis was performed to estimate phenolic acids in ECS (Tiwari et al., 2009), gallic, vanillic, O-coumaric, ferulic, cinnamic and salicylic acids were identified. Data on the *in vitro* estrogenic effects of phenolic acids indicate that ferulic acid stimulated proliferation of human breast cancer MCF-7 cells in a concentration- and ER-dependent manner (Chang et al., 2006). Previously, Kampa et al. (2004) reported that this phenolic acid showed anti-proliferative action in the T47D breast cancer cell line. In a recent study, gallic acid was reported to show affinity for ERβ, but not for ERα (Hidalgo et al., 2012). Finally, it has been also demonstrated some essential oils show *in vitro* estrogenic and/or anti-estrogenic activity (Howes and coworkers, 2002; Tabanca et al., 2004). In this regard, α-terpenyl acetate, 1,8-cineole, linalool, α-terpineol and linalyl acetate were identified as the main components in essential oils from ECS (Marongiu et al., 2004; Sereshti et al., 2012).

In conclusion, the studied extracts either from leaves or seeds are a combination of multiple chemical compounds, among others essential oils, sterols, phenolic acids and lipids that have been poorly characterized. The observed sex hormone-like activities in these extracts could be attributed to the presence of the above mentioned compounds, but also to other naturally occurring components not identified yet. Because the significant phytoestrogens and phytoandrogens content of these extracts and the activities observed and reported here, medicinal plants must be used with caution and further studies should be performed to completely classify their active components.

Conflict of interest statement

None of the authors has a conflict of interest related to this study.

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Figure 1. Proliferative and anti-proliferative response of MCF-7 cells in the E-Screen bioassay.

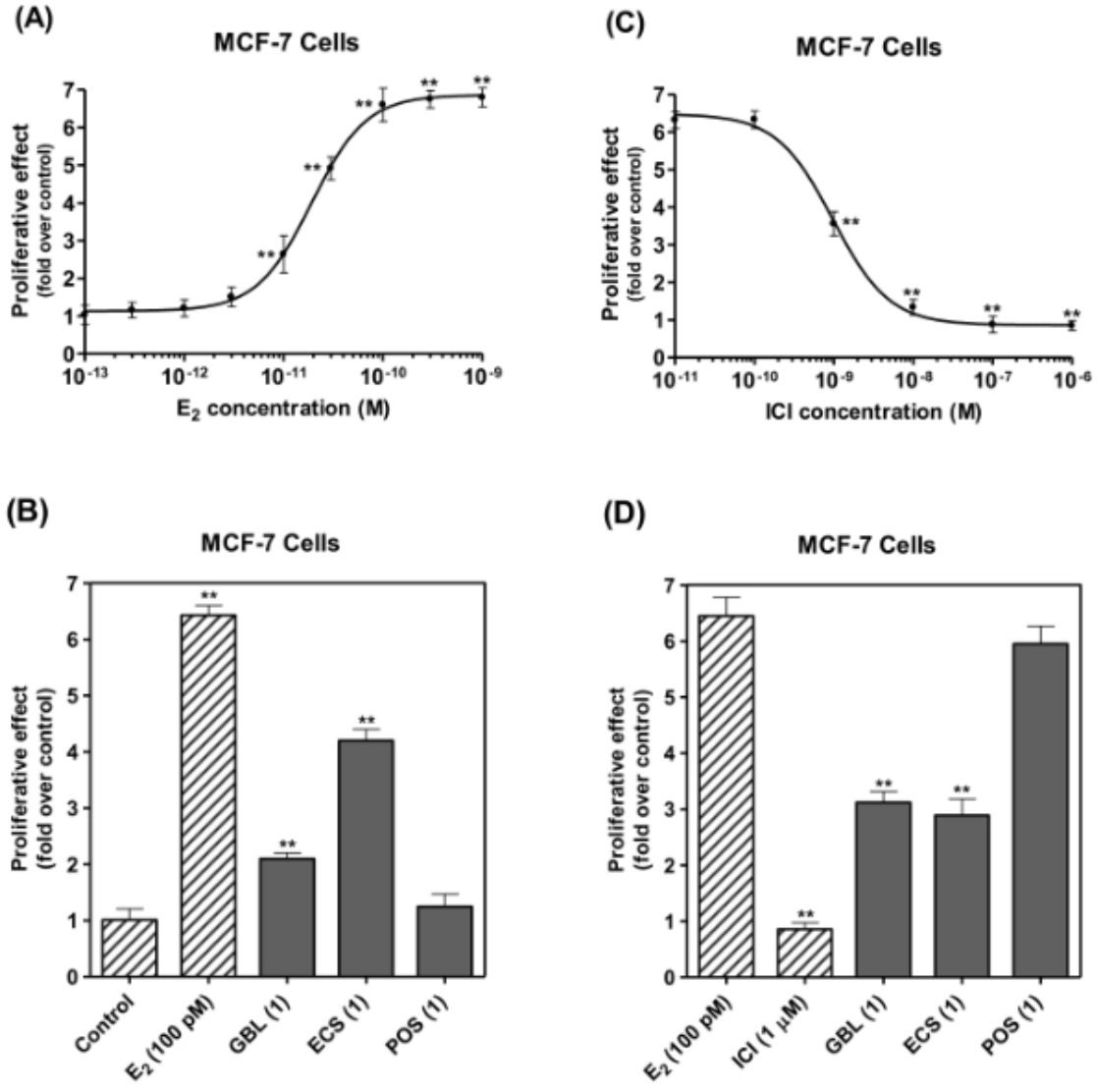

Figure 2. Gene expression modulation via hAR

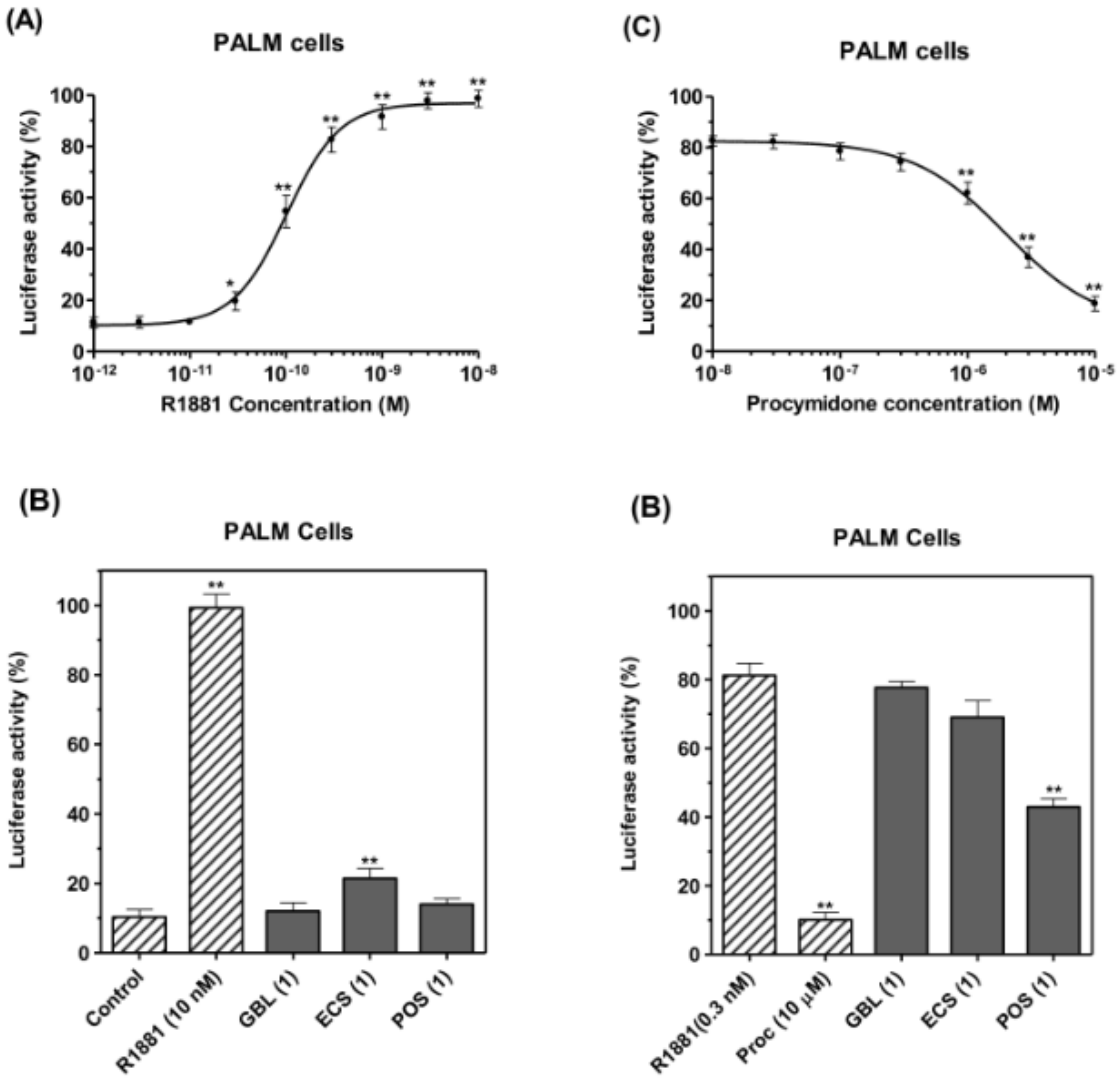

Table 1: (Anti-)estrogenic and (anti-)androgenic potencies of the 3 plant sample extracts studied determined by E-Screen or PALM luciferase assay and expressed in concentrations equivalent to E_2, ICI, R1881, or Procymidone per milligram of sample extract and per g of dry matter plant.

Extracts	E-Screen bioassay				PALM luciferase assay			
	E_2Eq/mg (nM)	E_2Eq/g (nM)	ICIEq/mg (μM)	ICIEq/g (μM)	R1881Eq/ mg (nM)	R1881Eq/g (nM)	ProcEq/ mg (μM)	ProcEq/g (μM)
GBL	0.071	3.41	0.013	0.63	-	-	-	-
ECS	0.20	12.42	0.020	1.29	0.30	18.61	-	-
POS	-	-	-	-	-	-	22.30	847.4

(-) water samples without statistically significant hormonal activity.