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ORIGINAL PAPER

Agonistic and antagonistic estrogens in licorice root (*Glycyrrhiza glabra*)

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Abstract The roots of licorice (*Glycyrrhiza glabra*) are a rich source of flavonoids, in particular, prenylated flavonoids, such as the isoflavan glabridin and the isoflavene glabrene. Fractionation of an ethyl acetate extract from licorice root by centrifugal partitioning chromatography yielded 51 fractions, which were characterized by liquid chromatography–mass spectrometry and screened for activity in yeast estrogen bioassays. One third of the fractions displayed estrogenic activity towards either one or both estrogen receptors (ERs; ERα and ERβ). Glabrene-rich fractions displayed an estrogenic response, predominantly to the ERα. Surprisingly, glabridin did not exert agonistic activity to both ER subtypes. Several fractions displayed higher responses than the maximum response obtained with the reference compound, the natural hormone 17β-estradiol (E_2). The estrogenic activities of all fractions, including this so-called superinduction, were clearly ER-mediated, as the estrogenic response was inhibited by 20–60% by known ER antagonists, and no activity was found in yeast cells that did not express the ERα or ERβ subtype. Prolonged exposure of the yeast to the estrogenic fractions that showed superinduction did, contrary to E_2, not result in a decrease of the fluorescent response. Therefore, the superinduction was most likely the result of stabilization of the ER, yeast-enhanced green fluorescent protein, or a combination of both. Most fractions displaying superinduction were rich in flavonoids with single prenylation. Glabridin displayed ERα-selective antagonism, similar to the ERα-selective antagonist RU 58668. Whereas glabridin was able to reduce the estrogenic response of E_2 by approximately 80% at 6×10^{-6} M, glabrene-rich fractions only exhibited agonistic responses, preferentially on ERα.

Keywords Prenylation · Isoflavonoids · Licorice · Estrogenicity · Antagonism · Superinduction

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Introduction

Flavonoids are a broad class of phenolic compounds mainly found in plants with a wide range of bioactivities [1]. Prenylated flavonoids, in particular, are of interest with respect to bioactivity, as prenylation is considered to modulate the responses towards the estrogen receptor [1, 2]. Prenyl-substitution of the flavonoid subclasses flavones, flavanones, and flavonols has been linked to an increased affinity to the estrogen receptor α (ERα) [3–5], e.g., prenylation of the eight-position of the flavanone naringenin results in a 200–1,000 higher estrogenic activity [6]. Furthermore, the

prenylation of isoflavonoids has been suggested to induce antagonistic activity when binding to ERα [5, 7, 8].

Licorice roots (*Glycyrrhiza glabra*) are a rich source of prenylated flavonoids. They might offer opportunities for the development of new food supplements related to, e.g., the alleviation of osteoporosis and menopausal complaints [9]. Approximately 75 prenylated flavonoids have been identified, mainly belonging to isoflavans, isoflavenes, and flavanones [10, 11].

Previous studies have shown that licorice root extracts have estrogenic activity towards the ERα and ERβ [12, 13]. The key estrogenic compounds isolated from *G. glabra* were identified as glabrene and glabridin, both prenylated isoflavonoids [14, 15]. The estrogen-like activities of both compounds have been established by means of competitive ligand binding assays, in vitro cell assays, and in vivo animal models [16, 17]. It has been demonstrated that glabrene and glabridin bind to the ER with EC_{50} values of 5×10^{-5} M and 5×10^{-6} M, respectively. These values were obtained using an MCF-7 cell line that is known to express the ERα type mainly (no detectable ERβ amounts on the protein level), indicating that both compounds were agonists [18, 19]. However, the specific estrogenic potencies of glabrene and glabridin towards ERα and ERβ and their potential antagonistic activities have not yet been investigated. Such information is vital for understanding their specific estrogenic activity in the human body.

The aim of the present study was to determine the predominant estrogenic compounds of licorice roots that are active on both ER subtypes and investigate their agonistic and antagonistic potencies. To this end, fractions of a licorice root extract obtained by centrifugal partitioning chromatography were characterized by liquid chromatography-mass spectrometry (LC-MS) and subsequently screened for (anti)estrogenic activity using yeast estrogen bioassays.

Experimental section

Materials The roots of *G. glabra*, collected in Afghanistan, were provided by Frutarom US (North Bergen, NJ, USA). Estradiol was purchased from Sigma Aldrich (St. Louis, MO, USA) and glabridin from Wako Chemicals GmbH (Neuss, Germany). RU 58668 and *R*,*R*-diethyl-THC (*R*,*R*-THC) were purchased from Tocris Bioscience (Bristol, UK). Analytical reagent-grade *n*-hexane, acetone, and absolute ethanol and ultra-LC-MS grade acetonitrile were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). Dimethylsulfoxide (DMSO) and all other chemicals were purchased from Merck (Darmstadt, Germany).

Preparation of licorice extract The roots were milled with a ZM 200 Retsch Ultra Centrifugal Mill (Haan, Germany) using a 1-mm sieve. The root powder was extracted with ethyl acetate (EA) in a ratio of 1 to 25 (*w*/*w*) for 2 h at 40 °C under continuous stirring. The extract was obtained by pressing the mixture with a Fischer Maschinenbau hydraulic press type HP 5M (Gemmrigheim, Germany) under 40 bar for 1 h. The dried extract was obtained after evaporation of the EA under reduced pressure at 40 °C.

CPC fractionation of licorice extract Centrifugal partitioning chromatography (CPC) was performed using a thermostated Kromaton FCPC machine (Angers, France) connected to an Armen AP 100 (Chromtech, Boronia, VIC, Australia) plunger pump. The two-phase solvent system used consisted of *n*-hexane/acetone/water in a ratio of 5:9:1 (*v*/*v*/*v*). It was equilibrated under stirring at 22 °C for at least 1 h. Small-scale fractionations as part of the method development were done with a 200-mL rotor in ascending mode (i.e., lower phase is stationary phase) at 22 °C, a rotation speed of 1,000 rpm, and a flow rate of 10 mL/min. The volume of displaced stationary phase was approximately 83 mL. Eighty-five milligrams dried extract was dissolved in a mixture of upper and lower phase, 4 mL of each phase. The fractionation process was monitored using a Jasco UV-2075 UV detector equipped with a 1-mm preparative cell at a wavelength of 330 nm (absorbance is expressed as relative response to the highest peak).

For the actual fractionation of the licorice root extract, a 1,000-mL rotor was used (22 °C; rotation speed 1,100 rpm; flow rate 25 mL/min). The volume of displaced stationary phase in the 1,000 mL rotor was approximately 625 mL. Seven hundred fifty milligrams dried extract was dissolved in 28 mL of a mixture of upper and lower phase (1:1). Seven subsequent runs were performed that resulted in 51 fractions per run; the fraction size was 50 mL. Based on the CPC UV profile, corresponding fractions were combined and evaporated in combination with lyophilization in order to remove solvents. The combined fractions were resolubilized in absolute ethanol (EtOH) and stored at −20 °C. All samples were thawed and centrifuged before analysis. Fractions collected were analyzed by ultra-high performance liquid chromatography (UHPLC)-mass spectrometry at a concentration of 1 mg/mL.

Reversed-phase UHPLC Samples were analyzed using an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with pump, autosampler, and PDA detector. Samples (1 μL) were injected onto an Acquity UPLC BEH C18 column (2.1×150 mm, 1.7 μm particle size) with an Acquity UPLC BEH C18 Vanguard pre-column (2.1×5 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Eluents were water-acidified with 0.1% (*v*/*v*) acetic acid (eluent A) and acetonitrile-acidified with 0.1% (*v*/*v*) acetic

acid (eluent B). The flow rate was 300 μL/min, and the PDA detector was set to measure at a range of 205–400 nm. The following elution profile was used at 0–18 min, linear gradient from 10–100% (*v/v*) B; 18–22 min, isocratic on 100% B; 22–23 min, linear gradient from 100–10% B; and 23–25 min, isocratic on 10% B.

Electrospray ionization mass spectrometry (ESI-MS) Mass spectrometric data were obtained by analyzing samples on an ion trap LTQ-XL (Thermo Scientific) equipped with an ESI-MS probe coupled to the reversed-phase UHPLC. Helium was used as sheath gas and nitrogen as auxiliary gas. Data were collected over an *m/z*-range of 150–1,500. Data-dependent tandem mass spectrometry (MS^n) analysis was performed with a normalized collision energy of 35%. The MS^n fragmentation was always performed on the most intense daughter ion in the MS^{n-1} spectrum. Most settings were optimized via automatic tuning using "Tune Plus" (Xcalibur 2.0.7, Thermo Scientific). To this end, the system was tuned with glabridin in both positive ionization and negative ionization mode. In both modes, the ion transfer tube temperature was 350 °C and the source voltage, 4.8 kV. Data acquisition and reprocessing were done with Xcalibur 2.0.7. Mass spectral data interpretation and peak determination were performed with Mass Frontier 5.0 (Highchem, Bratislava, Slovakia).

Determination of estrogenic activity The protocols for the yeast estrogen bioassays were adopted from Bovee et al. [20] with slight modifications. The genetically modified yeast strains have a strong constitutive expression vector stably integrated in the genome to express either the human estrogen receptor α (ERα) or the human estrogen receptor β (ERβ). The yeast genome also contains a reporter construct. This reporter construct contains an inducible yeast-enhanced green fluorescent protein (yEGFP) regulated by the activation of a minimal promoter with estrogen-responsive elements. Cultures of the yeast estrogen biosensor with either ERα or ERβ were grown overnight at 30 °C with shaking at 200 rpm. At the late log phase, the cultures of both estrogen receptors were diluted in the selective minimal medium supplemented with either leucine (ERα) or histidine (ERβ) to an optical density (OD) value (630 nm) of 0.05± 0.01 (ERα) and 0.15±0.05 (ERβ). For exposure, 200-μL aliquots of these diluted yeast cultures were combined with 2 μL of test compound or extract (in various concentrations) in a 96-well plate to test the agonistic properties of these compounds. DMSO (blank) and control samples containing 17β-estradiol (E_2) or genistein dissolved in DMSO were included in each experiment. Dilution series of each sample were prepared in DMSO, and the final concentration of DMSO in the assay did not exceed 1% (*v/v*). Each sample concentration was assayed in triplicate. Exposure was performed for 24 or 6 h for the ERα or ERβ assay, respectively, at 30 °C and orbital shaking at 200 rpm.

Fluorescence and OD were measured at 0 and 24 h for the ERα and 0 and 6–8 h for the ERβ in a Tecan Infinite F500 (Männedorf, Switzerland), using an excitation filter of 485 nm (bandwidth, 20 nm) and an emission filter of 535 nm (bandwidth, 35 nm). The fluorescence signals of the samples were corrected with the signal obtained with the diluted yeast suspension at t 0 h (background signal). In order to check the viability of the yeast in each well, the absorbance was measured at 630 nm. Each fraction was tested in a concentration series ranging from 0.1–100 μg/mL. EC_{50} calculations were performed in Sigma Plot (8.02, SPSS Inc.). In a number of cases, a concentration of 10 μg/mL for the ERα and 3 μg/mL for the ERβ resulted in decreased yeast growth during incubation of more than 50% during incubation due to cytotoxicity. This cytotoxicity could be due to the anti-microbial properties of licorice root constituents as reported before [21, 22].

A dilution series of estradiol and genistein were used as reference controls in this bioassay. The EC_{50} values in the ERα bioassay were 0.86 nM and 1.73 μM for estradiol and genistein, respectively, and 0.12 and 9.1 nM in the ERβ bioassay, respectively. All EC_{50} values were in line with those reported previously [20].

For the determination of ER antagonism, the yeast cells were exposed to the EC_{70} (ERα) or the EC_{90} (ERβ) of estradiol in combination with different dilutions of a test compound or fraction (measured in triplicates). As a positive control, the yeast cells were exposed to the EC_{70} of estradiol in combination with the known ERα antagonist RU 58688 [23]. For ERβ antagonism, the yeast cells were exposed to the EC_{90} of estradiol in combination with *R*,*R*-THC, a known antagonist on the ERβ [24].

In addition to the yeasts expressing the yEGFP reporter gene in combination with either ERα or ERβ, a third yeast strain was used that only contained the reporter gene but not the vector with the ER. This yeast strain was used as a negative control.

Results and discussion

Estrogenic activity of CPC fractions for ERα and ERβ

CPC fractionation of the licorice root extract resulted in 51 fractions (Fig. 1). After the 51st fraction, no UV response was observed anymore. Fractions F1 to 5, F6 to 21, and F22 to 51 comprised ~25, ~40, and ~35% DW of the total extraction yield, respectively (Electronic Supplementary Material Table S1). Most fractions showed some estrogenicity on both ERs, indicating the presence of phytoestrogens (Table 1).

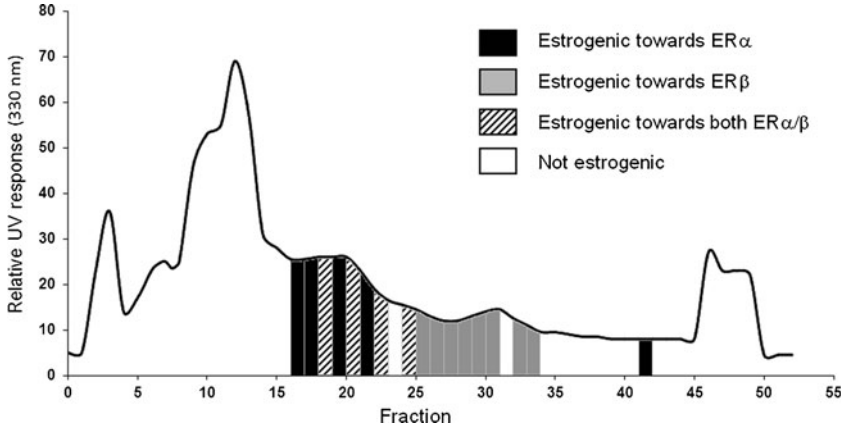

Fig. 1 UV profile of the licorice root extract fractionation by CPC. Estrogenically active fractions are indicated

Table 1 Estrogenic response of CPC fractions from the EA extract of licorice roots

	3 μg/mL	10 μg/mL	0.3 μg/mL	1.0 μg/mL		3 μg/mL	10 μg/mL	0.3 μg/mL	1.0 μg/mL
Fr	ERα	ERα	ERβ	ERβ	Fr	ERα	ERα	ERβ	ERβ
E_2	100		100		F26[a]	38.7 (±1.9)	93.7 (±6.1)	95.5 (±3.4)	151.3 (±15.1)
F1	ND	ND	0.0 (±2.0)	0.0 (±2.0)	F27[a]	36.6 (±0.3)	90.8 (±2.1)	74.6 (±5.9)	176.5 (± 11.8)
F2	ND	1.1 (±1.2)	0.0 (±6.5)	0.0 (±4.9)	F28[a]	19.4 (±1.9)	48.2 (±2.7)	68.7 (±4.0)	104.4 (± 8.3)
F3	ND	4.9 (±0.7)	1.6 (±1.5)	0.0 (±4.5)	F29[a]	19.9 (±1.3)	23.0 (±3.9)	57.2 (±12.5)	93.5 (±0.6)
F4	ND	7.8 (±0.8)	13.4 (±5.0)	22.4 (±7.7)	F30[a]	23.8 (±2.7)	22.4 (±1.8)	57.3 (±1.1)	48.2 (±2.8)
F5	3.0 (±2.3)	7.8 (±0.3)	12.0 (±5.1)	19.5 (±2.2)	F31	25.0 (±1.5)	34.2 (±1.9)	25.8 (±8.5)	40.5 (±17.8)
F6	ND	ND	ND	ND	F32[a]	26.8 (±2.3)	34.5 (±2.0)	57.4 (±4.7)	47.4 (±10.7)
F7	17.1 (±3.0)	21.0 (±2.7)	21.4 (±5.6)	12.2 (±2.2)[b]	F33[a]	32.0 (±1.4)	38.0 (±0.4)	50.2 (±9.5)	36.0 (±3.2)
F8	10.5 (±2.2)	21.8 (±6.3)	17.2 (±2.3)	58.3 (±3.4)	F34	29.6 (±5.3)	44.9 (±1.3)	27.7 (±8.8)	46.1 (±5.2)
F9	13.3 (±1.5)	7.1 (±6.4)	37.8 (±10.0)	49.1 (±21.5)	F35	30.8 (±3.1)	45.5 (±4.2)	43.6 (±7.6)	37.5 (±21.0)
F10	9.7 (±3.3)	9.3 (±2.0)	23.0 (±9.5)	57.3 (±12.9)	F36	42.8 (±2.3)	42.9 (±6.5)	19.9 (±2.3)	45.3 (±2.5)
F11	ND	ND	46.3 (±12.0)	ND [b]	F37	13.6 (±1.6)	25.3 (±2.5)	24.8 (±2.6)	35.1 (±9.9)
F12	15.6 (±2.9)	15.7 (±3.2)[b]	30.7 (±0.1)	77.2 (±7.3)	F38	14.8 (±1.7)	28.8 (±0.5)	14.2 (±6.8)	30.8 (±15.4)
F13	ND	ND	40.3 (±5.0)	2.4 (±4.9)[b]	F39	19.8 (±1.6)	32.4 (±2.6)	21.1 (±4.4)	45.6 (±14.7)
F14	4.8 (±4.7)	ND	42.7 (±6.6)	73.3 (± 4.1)	F40	25.6 (±4.3)	33.9 (±2.3)	26.5 (±5.5)	35.4 (±17.4)
F15	ND	ND	28.4 (±7.1)	77.7 (± 1.8)	F41[a]	159.9 (±9.5)	186.9 (±15.0)	28.3 (±4.9)	34.9 (±13.5)
F16[a]	74.8 (±4.0)	20.4 (±28.9)[b]	40.7 (±1.0)	62.6 (± 2.1)	F42	21.6 (±6.0)	26.2 (±2.2)	19.8 (±11.0)	53.8 (±25.5)
F17[a]	109.1 (±2.3)	49.3 (±4.4)[b]	36.7 (±9.5)	88.8 (± 8.2)	F43	26.5 (±1.8)	24.7 (±4.3)	25.4 (±1.2)	38.9 (±20.0)
F18[a]	105.3 (±6.2)	104.6 (±10.5)[b]	57.1 (±9.4)	98.8 (± 15.1)	F44	18.9 (±1.9)	24.6 (±1.5)	22.7 (±7.3)	33.8 (±12.2)
F19[a]	89.1 (±4.2)	131.2 (±16.2)	48.3 (±4.6)	123.8 (±17.1)	F45	20.9 (±1.5)	21.0 (±4.3)	19.6 (±3.4)	66.3 (±15.1)
F20[a]	60.2 (±3.1)	140.5 (±10.7)	60.5 (±14.5)	116.7 (±14.3)	F46	16.9 (±1.2)	20.5 (±3.2)	18.0 (±11.6)	73.8 (±7.3)
F21[a]	71.9 (±8.4)	125.3 (±1.2)	45.2 (±3.8)	145.9 (±25.2)	F47	21.2 (±2.9)	23.3 (±4.2)	31.0 (±5.1)	79.4 (±6.1)
F22[a]	101.4 (±11.6)	134.1 (±2.7)	87.0 (±10.3)	144.4 (±10.7)	F48	23.2 (±4.6)	21.7 (±2.4)	26.8 (±2.5)	49.1 (±17.8)
F23	16.0 (±0.9)	83.0 (±9.9)	26.3 (±8.7)	84.8 (±7.0)	F49	26.4 (±0.6)	26.7 (±0.3)	16.5 (±3.0)	82.9 (±4.9)
F24[a]	79.6 (±1.6)	156.3 (±14.4)	103.1 (±2.3)	159.3 (±23.1)	F50	18.8 (±2.7)	16.7 (±3.2)	7.3 (±9.3)	41.3 (±3.1)
F25[a]	44.2 (±5.7)	108.6 (±3.9)	97.9 (±10.6)	172.8 (±20.7)	F51	22.0 (±3.2)	15.6 (±2.5)	18.8 (±2.7)	40.5 (±18.3)

The estrogenicity toward the ERα and the ERβ was measured at two concentrations. The activity was standardized to the maximum induced response of 2 μM estradiol (100%). Values are the mean±SD (n=3). Estrogenic fractions are marked

Fr fraction, *ND* not detected (estrogenicity values were zero or slightly negative)

[a] Estrogenically active

[b] Inhibited yeast growth due to cytotoxicity

A compound is considered a phytoestrogen when it activates the ER at concentrations $\leq 10^4$ times than that of estradiol (E_2) [25]. The EC_{50} value of E_2 towards the ERα in the yeast assay was determined to be $1.0–1.6\times10^{-9}$ M, which corresponds to $2.7–4.4\times10^{-4}$ μg/mL. Therefore, only CPC fractions giving a response above the EC_{50} at a dilution below 3 μg/mL were indicated as active towards ERα in Fig. 1. The application of this threshold value for the ERα resulted in nine active fractions out of 51 (see Table 1).

The EC_{50} value of E_2 towards the ERβ ranged from 1.1×10^{-10} to 2.1×10^{-10} M, corresponding to an EC_{50} of $3.2–5.9\times10^{-5}$ μg/mL. Therefore, only CPC fractions giving a response above the EC_{50} at a dilution below 0.3 μg/mL were indicated as active towards ERβ in Fig. 1. The application of this threshold value for the ERβ resulted in 12 active fractions out of 51 (Table 1).

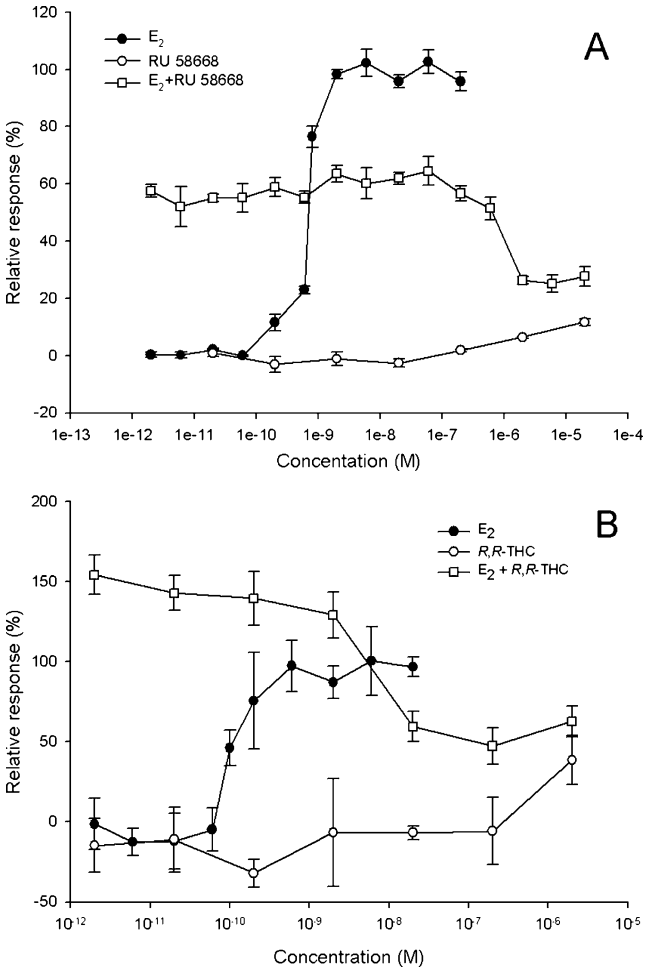

Fig. 2 Transcription activation by ERα (**A**) and ERβ (**B**) in response to E_2 and subtype-selective antagonists RU58668 (ERα) and *R*,*R*-THC (ERβ). The antagonistic activity of both receptor-specific antagonists were assayed in the presence of the EC_{70} (0.8 nM) and EC_{90} (0.2 nM) E_2 for ERα and ERβ, respectively. Both graphs were normalized to E_2. The response of *R*,*R*-THC on ERβ for every concentration was lower than the minimum response of E_2. This was corrected by normalizing the lowest concentration of *R*,*R*-THC to 0%. Values are the mean±SD (n=3)

The screening for estrogenicity of the CPC fractions on both receptor subtypes showed that the estrogenic response of several fractions substantially exceeded the maximum response of E_2 (Table 1). This phenomenon has been referred to as superinduction [26]. In our study, this superinduction was observed with both receptors and appeared more pronounced for ERβ. The mechanism that leads to superinduction is not well understood but sometimes occurs with colored extracts. Such colored extracts can disturb the fluorescent measurement, as, due to a decrease of the pH during the exposure period, the color can change as well.

To determine whether fractions gave an increased fluorescent response as a result of acidification (change of pH 5.0 to pH 2.9) of the culture medium due to yeast growth, six representative fractions (F4, F13, F22, F27, F30, and F44), with no, moderate, or high estrogenic activity, were measured at different pH values in the absence of yeast. No altered fluorescent signals were observed compared with the blank, showing that the observed superinduction was not related to altered fluorescent signals due to a drop in pH.

In a next series of experiments, two subtype-selective antagonists were used to determine whether the observed estrogenic activities, including the superinduction, were ER-mediated. First, RU 58668 (ERα-selective) [27] and *R*,*R*-THC (ERβ-selective) [24] were tested in the yeast estrogen bioassays to confirm their antagonistic properties. Co-incubation of E_2 and RU 58668 showed that 6×10^{-6} M RU 58668 was able to decrease the E_2-induced response of ERα by ~60% (Fig. 2A). RU 58668 itself showed a weak agonistic activity of ~12% in concentrations above 1×10^{-5}

Table 2 Inhibition of estrogenic response of representative CPC fractions by addition of a subtype-specific antagonist

	Estrogenic activity ERα	Inhibition by RU 58668	Estrogenic activity ERβ	Inhibition by *R*,*R*-THC
E_2	100	60%	100	55%
F4	ND	–	13.4 (±5.0)	70%
F13	ND	–	40.3 (±5.0)	37%
F22	101.4 (±11.6)	52%	87.0 (±10.3)	34%
F27	36.6 (±0.3)	21%	74.6 (±5.9)	61%
F30	23.8 (±2.7)	21%	57.3 (±1.1)	48%
F44	18.9 (±1.9)	30%	22.7 (±7.3)	70%

Inhibition of the estrogenic response of six representative CPC fractions were determined by co-incubation at 1 μg/mL with 6×10^{-6} M RU 58668 for the ERα and 1 μg/mL with 2×10^{-8} M *R*,*R*-THC for the ERβ. Values are the mean±SD (n=3)

ND not detected

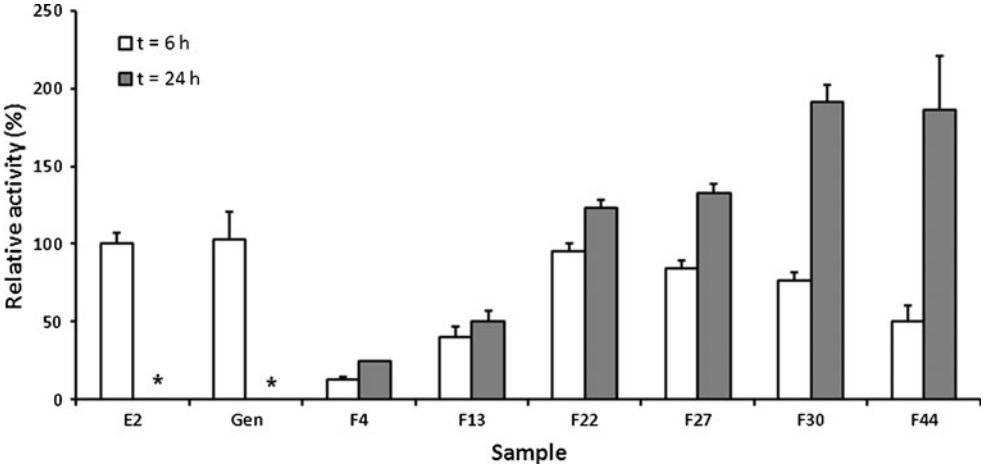

Fig. 3 Stabilizing effect of E2, genistein, and several fractions obtained from the licorice root extract on the relative activity measured after 6 and 24 h in the ERβ assay. *E_2*, 2×10^{-10} M estradiol; *Gen*, 2×10^{-7} M genistein; *F4* to *F44*, licorice root fractions obtained by CPC measured at 0.3 μg/mL. *Asterisk*, negative response

M. RU 58668 is known as a pure ERα antagonist, but its weak agonistic activity in the yeast estrogen bioassay with ERα is not expected, as several other 11β-analogues of E_2 were shown to be selective estrogen receptor modulators (SERM) with both agonistic and antagonistic activity on the ERα in the same yeast estrogen bioassay [23].

Co-incubation of E_2 and *R*,*R*-THC showed that 2×10^{-7} M *R*,*R*-THC was able to decrease the E_2-induced response of ERβ by ~55% (Fig. 2B). Meyers and co-workers reported an inhibition of ~100% at similar concentrations while using mammalian cell-based assays [24].

To determine whether the observed estrogenic responses of the fractions were ER-mediated, selected fractions were co-incubated with the subtype-selective antagonists. Besides, the fractions were tested in the yeast strain that expresses no estrogen receptor and only contains the reporter construct. In all estrogen-active fractions, the responses were inhibited by either RU 58668 or *R*,*R*-THC, and the fluorescence response was reduced by up to 70%. This confirms that the estrogenic responses caused by the fractions on both receptors were ER-mediated (Table 2). Also, the controls with the yeast strain expressing no ER confirmed that the observed

Table 3 Fractions generated by CPC fractionation and the presence of the main flavonoids in each fraction determined by ultra-high performance LC-MS

Fraction	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Yield (mg)	110	141	97	136	108	126	211	345	254	156	66	176	157	115	37
Number of peaks	20	6	12	25	22	18	7	4	6	6	15	14	17	30	42
Of which are															
Non-prenylated	3	1	2	4	2	1	0	0	0	0	0	1	2	4	9
Prenylated	17	5	10	21	20	17	7	4	6	6	15	13	15	26	33
Single-prenylated	13	3	7	9	6	8	4	1	2	3	11	11	13	18	22
Chain	7	2	4	6	4	5	2	0	1	2	7	5	9	13	21
Pyran	6	1	3	3	2	3	2	1	1	1	4	6	4	5	1
Double-prenylated	4	2	3	12	14	9	3	3	4	3	4	2	2	8	11
Characterization															
Glabrene											++	++	++	++	+
Glabrone				+	+	++	+								
Glabridin						+	+	++	++	+	+	+			
Glabrol						+	++	++	+	+					
3′-OH-4′OMe-G[a]			+	+	++	++									
4′-OMe-G[b]	+	+	++	+											
Hispaglabridin A	+	++	++												
Hispaglabridin B	++	++	+												

[a] 4′-*O*-methyl-glabridin

[b] 3′-hydroxy-4′-*O*-methyl-glabridin

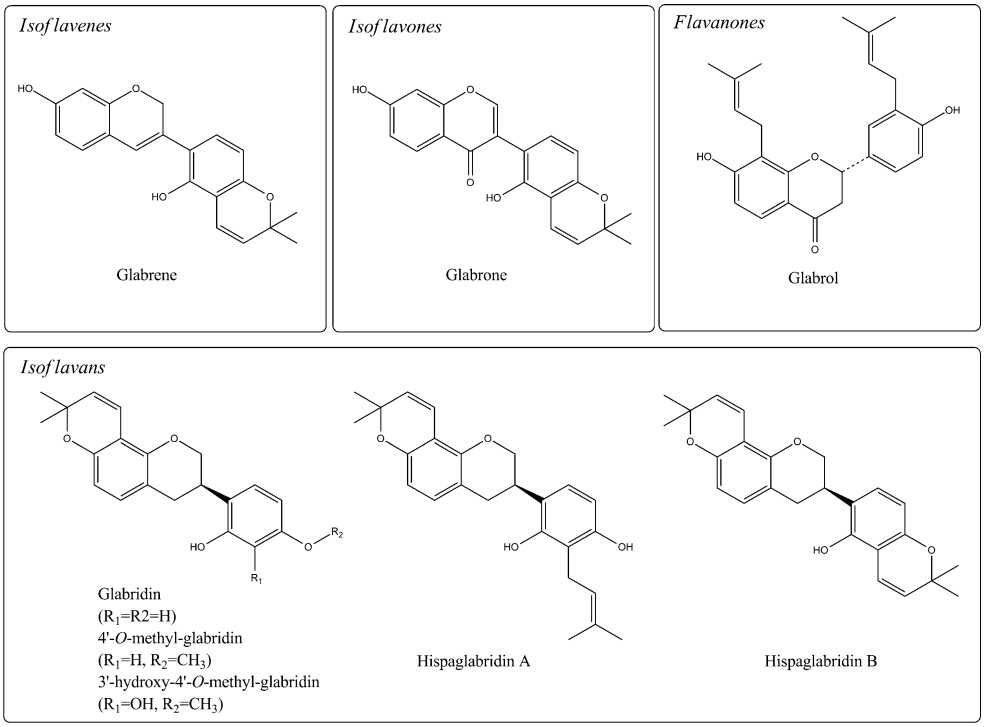

Fig. 4 Main flavonoids identified in CPC fractions 6–21 obtained from the EA extract of licorice root

responses were ER-mediated, as no fluorescent signal was observed for any of the fractions or E_2.

Superinduction by stabilization of ER-mediated response

The phenomenon of superinduction has been previously observed in several assay types and the superinduction caused by genistein in human U2OS bone cells transfected with the ERα and a luciferase reporter gene was intensively investigated [26]. It was concluded that this superinduction was caused by a post-translational stabilization of the firefly luciferase reporter enzyme by genistein and not by stabilization of the ERα. To verify the hypothesis that superinduction in the yeast was caused by the stabilization of the ER and/or the yEGFP, the yeast expressing ERβ was co-incubated with E_2, genistein, or the representative fractions (F4, F13, F22, F27, F30, and F44) mentioned before. The estrogenic responses were measured after 6 and 24 h (Fig. 3). After 6 h, both E_2 and genistein showed the maximum estrogenic response, but, as expected, the estrogenic response of E_2 completely disappeared after 24 h. Also, the response of genistein completely disappeared, whereas the estrogenic response of the fractions was similar or even higher compared with their response measured after 6 h. This strongly indicates that the responses, including the superinduction, of the fractions were stabilized. Our results do not allow speculation on whether the ER, the yEGFP, or both proteins were stabilized, but the observed estrogenic responses were without doubt ER-mediated.

LC-MS characterization of licorice fractions

Because the estrogenic responses were ER-mediated, the licorice fractions were subjected to characterization by LC-MS. Fractions F6-21 contained the main flavonoids previously annotated in the EA extract of licorice roots (Table 3, Fig. 4) [11]. These flavonoids have been annotated based on UV and MS^n spectra and, if possible, compared with spectra published in the literature. The estrogenic activity of a number of active fractions (F20-22,24-30,32-33,42) could not be traced back to individual components (Electronic Supplementary Material Table S1).

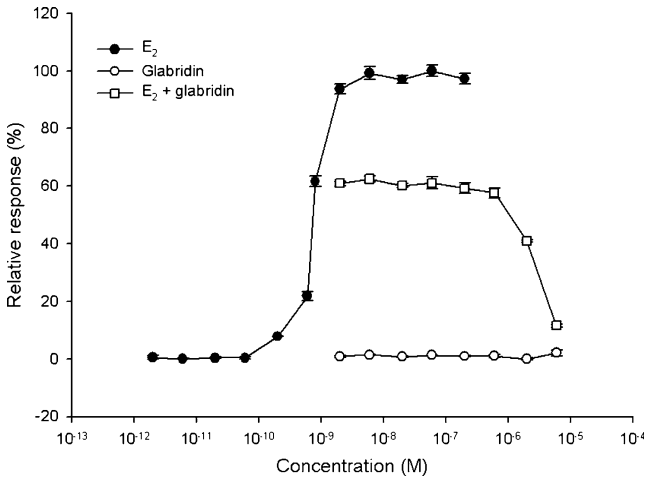

Fig. 5 Antagonistic activity of glabridin on the estrogenic response in the ERα yeast estrogen bioassay

Compositional analysis by LC-MS showed that the active fractions were complex mixtures, indicating that further purification of CPC fractions is prerequisite for the identification of the estrogenic compounds. In most cases, it was not possible to assign the identity of the predominant peaks by UV and MS^n. Furthermore, in most fractions, the majority of the compounds were prenylated, which might suggest a correlation between prenylation and superinduction (Table 3, Electronic Supplementary Material Table S1).

Fractions F16-20, rich in glabrene, showed a predominant estrogenic activity on the ERα. This is in agreement with the fact that glabrene is considered one of the principle estrogenic components of licorice root. The estrogenic potency of glabrene for both ER subtypes, however, has not yet been established. Our results indicate that the glabrene-rich fractions had a particularly high response towards ERα. Whereas phytoestrogens generally have a more pronounced affinity to the ERβ compared with the ERα, the glabrene-rich fractions showed the opposite behavior, similar to that of 8-prenyl naringenin [27].

In addition to glabrene, the estrogenic activity of licorice roots extract has been ascribed to the presence of glabridin and its derivatives. Despite the abundance of glabridin in F11-15, no significant estrogenic response on both ER subtypes was observed. EC_{50} values of 5×10^{-6} M have been reported for glabridin, using different mammalian proliferation assays [15–17]. Furthermore, several glabridin derivatives were shown to be moderately estrogenic compared with glabridin [14]. In our study, the pure reference standard of glabridin did not exert any estrogenic response in a concentration range of 1×10^{-7} to 1×10^{-4} M towards both ER subtypes (data not shown). In concentrations above 1×10^{-4} M, glabridin was toxic to the yeast cells. Because glabridin had been shown to interact with the ERs, and because it is known that different ER-based bioassays can generate different output, glabridin as well as the glabrene-rich fraction F18 (due to the lack of a glabrene reference standard) were tested for their antagonistic properties.

Antagonistic activity of glabridin and glabrene

Prenylation of isoflavonoids has been suggested to induce antagonism towards the ERα [5, 7, 8]. The glabrene-rich fraction F18 did not show antagonistic activity on both ER subtypes (no further data shown) but increased the estrogenic response upon co-exposure with E_2, confirming its agonistic character.

The reference standard of glabridin did not have antagonistic properties towards the ERβ (data not shown) but was shown to be an ERα-selective antagonist (Fig. 5). At a concentration of 6×10^{-6} M, glabridin was able to inhibit the E_2 response by ~80% without being toxic towards the yeast cells. The agonistic activity of glabridin in the MCF-7 proliferation assay, in in vivo animal models, and the ERα-selective antagonistic activity in the yeast estrogen bioassay might imply that glabridin acts as a SERM. The estrogenic activity of glabridin is similar to kievitone and phaseollin, a prenyl-chain substituted isoflavanone and a pyran-ring substituted pterocarpan, respectively. Both kievitone and phaseollin displayed agonistic activity in the MCF-7 proliferation assay and in the human HEK 293 transactivation assay but were antagonistic in the MCF-7 colony-formation assay [28]. The assay-dependent mode of action of glabridin has also been observed with other compounds. For example, both tamoxifen and 4-hydroxy-tamoxifen act as SERMs, displaying both estrogenic and anti-estrogenic activities in mammalian breast and endometrial cells, act as agonists in yeast estrogen bioassays [27].

As mentioned before, LC-MS characterization showed that fractions F6-15 were rich in glabridin derivatives. These compounds share prenyl-substitution on the A-ring with a pyran-ring (Table 3, Fig. 4). It will be worthwhile to also test the purified glabridin derivatives for antagonistic activity in the yeast estrogen bioassay.

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References

1. Barron D, Ibrahim RK (1996) Isoprenylated flavonoids—a survey. Phytochemistry 43(5):921–982
2. Botta B, Monache GD, Menendez P, Boffi A (2005) Novel prenyltransferase enzymes as a tool for flavonoid prenylation. Trends Pharmacol Sci 26(12):606–608
3. Kitaoka M, Kadokawa H, Sugano M, Ichikawa K, Taki M, Takaishi S, Iijima Y, Tsutsumi S, Boriboon M, Akiyama T (1998) Prenylflavonoids: a new class of non-steroidal phytoestrogen (part 1). Isolation of 8-isopentenylnaringenin and an initial study on its structure–activity relationship. Planta Med 64(6):511–515
4. Pinto B, Bertoli A, Noccioli C, Garritano S, Reali D, Pistelli L (2008) Estradiol-antagonistic activity of phenolic compounds from leguminous plants. Phytother Res 22(3):362–366
5. Ahn EM, Nakamura N, Akao T, Nishihara T, Hattori M (2004) Estrogenic and antiestrogenic activities of the roots of *Moghania philippinensis* and their constituents. Biol Pharm Bull 27(4):548–553
6. Roelens F, Heldring N, Dhooge W, Bengtsson M, Comhaire F, Gustafsson JÅ, Treuter E, De Keukeleire D (2006) Subtle side-chain modifications of the hop phytoestrogen 8-prenylnaringenin result in distinct agonist/antagonist activity profiles for estrogen receptors α and β. J Med Chem 49(25):7357–7365

7. Jiang Q, Payton-Stewart F, Elliott S, Driver J, Rhodes LV, Zhang Q, Zheng S, Bhatnagar D, Boue SM, Collins-Burow BM, Sridhar J, Stevens C, McLachlan JA, Wiese TE, Burow ME, Wang G (2010) Effects of 7-O substitutions on estrogenic and anti-estrogenic activities of daidzein analogues in MCF-7 breast cancer cells. J Med Chem 53(16):6153–6163
8. Okamoto Y, Suzuki A, Ueda K, Ito C, Itoigawa M, Furukawa H, Nishihara T, Kojima N (2006) Anti-estrogenic activity of prenylated isoflavones from *Millettia pachycarpa*: implications for pharmacophores and unique mechanisms. J Health Sci 52 (2):186–191
9. Boué SM, Cleveland TE, Carter-Wientjes C, Shih BY, Bhatnagar D, McLachlan JM, Burow ME (2009) Phytoalexin-enriched functional foods. J Agric Food Chem 57(7):2614–2622
10. Nomura T, Fukai T (1998) Phenolic constituents of licorice (*Glycyrrhiza species*). In: Herz W, Kirby GW, Moore RE, Steglich W, Tamm C (eds) Progress in the chemistry of organic natural products, vol 73. Springer, New York, NY, pp 1–140
11. Simons R, Vincken J-P, Bakx EJ, Verbruggen MA, Gruppen H (2009) A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts. Rapid Commun Mass Spectrom 23(19):3083–3093
12. Kohno H, Kouda K, Tokunaga R, Sonoda Y (2007) Detection of estrogenic activity in herbal teas by in vitro reporter assays. Eur Food Res Technol 225(5–6):913–920
13. Zava DT, Blen M, Duwe G (1997) Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. Environ Health Perspect 105(suppl 3):637–645
14. Tamir S, Eizenberg M, Somjen D, Izrael S, Vaya J (2001) Estrogen-like activity of glabrene and other constituents isolated from licorice root. J Steroid Biochem Mol Biol 78(3):291–298
15. Tamir S, Eizenberg M, Somjen D, Stern N, Shelach R, Kaye A, Vaya J (2000) Estrogenic and antiproliferative properties of glabridin from licorice in human breast cancer cells. Cancer Res 60(20):5704–5709
16. Somjen D, Katzburg S, Vaya J, Kaye AM, Hendel D, Posner GH, Tamir S (2004) Estrogenic activity of glabridin and glabrene from licorice roots on human osteoblasts and prepubertal rat skeletal tissues. J Steroid Biochem Mol Biol 91(4–5):241–246
17. Somjen D, Knoll E, Vaya J, Stern N, Tamir S (2004) Estrogen-like activity of licorice root constituents: glabridin and glabrene, in vascular tissues in vitro and in vivo. J Steroid Biochem Mol Biol 91(3):147–155
18. Shanmugam M, Krett NL, Maizels ET, Cutler RE Jr, Peters CA, Smith LM, O'Brien ML, Park-Sarge OK, Rosen ST, Hunzicker-Dunn M (1999) Regulation of protein kinase C δ by estrogen in the MCF-7 human breast cancer cell line. Mol Cell Endocrinol 148(1–2):109–118
19. Rachner TD, Schoppet M, Niebergall U, Hofbauer LC (2008) 17β-Estradiol inhibits osteoprotegerin production by the estrogen receptor-α-positive human breast cancer cell line MCF-7. Biochem Biophys Res Commun 368(3):736–741
20. Bovee TFH, Helsdingen RJR, Rietjens IMCM, Keijer J, Hoogenboom RLAP (2004) Rapid yeast estrogen bioassays stably expressing human estrogen receptors α and β, and green fluorescent protein: a comparison of different compounds with both receptor types. J Steroid Biochem Mol Biol 91(3):99–109
21. Nassiri Asl M, Hosseinzadeh H (2008) Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. Phytother Res 22(6):709–724
22. Nomura T, Fukai T, Akiyama T (2002) Chemistry of phenolic compounds of licorice (*Glycyrrhiza* species) and their estrogenic and cytotoxic activities. Pure Appl Chem 74(7):1199–1206
23. Bovee TFH, Thevis M, Hamers ARM, Peijnenburg AACM, Nielen MWF, Schoonen WGEJ (2010) SERMs and SARMs: detection of their activities with yeast based bioassays. J Steroid Biochem Mol Biol 118(1–2):85–92
24. Meyers MJ, Jun S, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA (1999) Estrogen receptor subtype-selective ligands: asymmetric synthesis and biological evaluation of *cis*- and *trans*-5,11-dialkyl-5,6,11,12- tetrahydrochrysenes. J Med Chem 42(13):2456–2468
25. Glazier MG, Bowman MA (2001) A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. Arch Intern Med 161(9):1161–1172
26. Sotoca AM, Bovee TFH, Brand W, Velikova N, Boeren S, Murk AJ, Vervoort J, Rietjens IMCM (2010) Superinduction of estrogen receptor mediated gene expression in luciferase based reporter gene assays is mediated by a post-transcriptional mechanism. J Steroid Biochem Mol Biol 122(4):204–211
27. Bovee TFH, Schoonen WGEJ, Hamers ARM, Bento MJ, Peijnenburg AACM (2008) Screening of synthetic and plant-derived compounds for (anti)estrogenic and (anti)androgenic activities. Anal Bioanal Chem 390(4):1111–1119
28. Boué SM, Burow ME, Wiese TE, Shih BY, Elliott S, Carter-Wientjes CH, McLachlan JA, Bhatnagar D (2011) Estrogenic and antiestrogenic activities of phytoalexins from red kidney bean (*Phaseolus vulgaris* L.). J Agric Food Chem 59(1):112–120