

# 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; ER $\alpha$  and ER $\beta$ ). Glabrene-rich fractions displayed an estrogenic response, predominantly to the ER $\alpha$ . 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 17 $\beta$ -estradiol

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(E<sub>2</sub>). 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 ER $\alpha$  or ER $\beta$  subtype. Prolonged exposure of the yeast to the estrogenic fractions that showed superinduction did, contrary to E<sub>2</sub>, 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 ER $\alpha$ -selective antagonism, similar to the ER $\alpha$ -selective antagonist RU 58668. Whereas glabridin was able to reduce the estrogenic response of E<sub>2</sub> by approximately 80% at 6 × 10<sup>-6</sup> M, glabrene-rich fractions only exhibited agonistic responses, preferentially on ER $\alpha$ .

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

## 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  $\alpha$  (ER $\alpha$ ) [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 ER $\alpha$  [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 ER $\alpha$  and ER $\beta$  [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<sub>50</sub> values of  $5 \times 10^{-5}$  M and  $5 \times 10^{-6}$  M, respectively. These values were obtained using an MCF-7 cell line that is known to express the ER $\alpha$  type mainly (no detectable ER $\beta$  amounts on the protein level), indicating that both compounds were agonists [18, 19]. However, the specific estrogenic potencies of glabrene and glabridin towards ER $\alpha$  and ER $\beta$  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  $\mu$ L) were injected onto an Acquity UPLC BEH C18 column (2.1  $\times$  150 mm, 1.7  $\mu$ m particle size) with an Acquity UPLC BEH C18 Vanguard pre-column (2.1  $\times$  5 mm, 1.7  $\mu$ m 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  $\mu\text{L}/\text{min}$ , 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 ( $\text{MS}^n$ ) analysis was performed with a normalized collision energy of 35%. The  $\text{MS}^n$  fragmentation was always performed on the most intense daughter ion in the  $\text{MS}^{n-1}$  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  $\alpha$  ( $\text{ER}\alpha$ ) or the human estrogen receptor  $\beta$  ( $\text{ER}\beta$ ). 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  $\text{ER}\alpha$  or  $\text{ER}\beta$  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 ( $\text{ER}\alpha$ ) or histidine ( $\text{ER}\beta$ ) to an optical density (OD) value (630 nm) of  $0.05 \pm 0.01$  ( $\text{ER}\alpha$ ) and  $0.15 \pm 0.05$  ( $\text{ER}\beta$ ). For exposure, 200- $\mu\text{L}$  aliquots of these diluted yeast cultures were combined with 2  $\mu\text{L}$  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 17 $\beta$ -estradiol ( $\text{E}_2$ ) 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  $\text{ER}\alpha$  or  $\text{ER}\beta$  assay, respectively, at 30 °C and orbital shaking at 200 rpm.

Fluorescence and OD were measured at 0 and 24 h for the  $\text{ER}\alpha$  and 0 and 6–8 h for the  $\text{ER}\beta$  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  $\mu\text{g}/\text{mL}$ .  $\text{EC}_{50}$  calculations were performed in Sigma Plot (8.02, SPSS Inc.). In a number of cases, a concentration of 10  $\mu\text{g}/\text{mL}$  for the  $\text{ER}\alpha$  and 3  $\mu\text{g}/\text{mL}$  for the  $\text{ER}\beta$  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  $\text{EC}_{50}$  values in the  $\text{ER}\alpha$  bioassay were 0.86 nM and 1.73  $\mu\text{M}$  for estradiol and genistein, respectively, and 0.12 and 9.1 nM in the  $\text{ER}\beta$  bioassay, respectively. All  $\text{EC}_{50}$  values were in line with those reported previously [20].

For the determination of ER antagonism, the yeast cells were exposed to the  $\text{EC}_{70}$  ( $\text{ER}\alpha$ ) or the  $\text{EC}_{90}$  ( $\text{ER}\beta$ ) 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  $\text{EC}_{70}$  of estradiol in combination with the known  $\text{ER}\alpha$  antagonist RU 58688 [23]. For  $\text{ER}\beta$  antagonism, the yeast cells were exposed to the  $\text{EC}_{90}$  of estradiol in combination with *R,R*-THC, a known antagonist on the  $\text{ER}\beta$  [24].

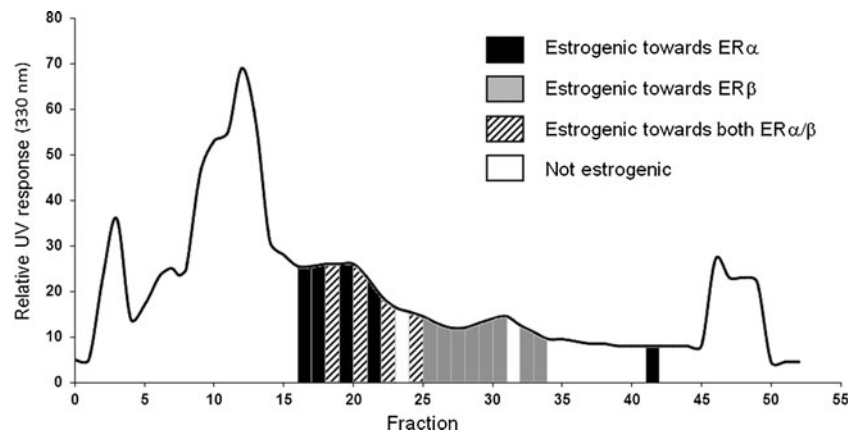
In addition to the yeasts expressing the yEGFP reporter gene in combination with either  $\text{ER}\alpha$  or  $\text{ER}\beta$ , a third yeast strain was used that only contained the reporter gene but not the vector with the ER. This yeast strain was used as a negative control.

## Results and discussion

### Estrogenic activity of CPC fractions for $\text{ER}\alpha$ and $\text{ER}\beta$

CPC fractionation of the licorice root extract resulted in 51 fractions (Fig. 1). After the 51<sup>st</sup> 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 <sub>2</sub>	100		100		F26 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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) <sup>b</sup>	F33 <sup>a</sup>	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 <sup>b</sup>	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) <sup>b</sup>	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) <sup>b</sup>	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 <sup>a</sup>	159.9 (±9.5)	186.9 (±15.0)	28.3 (±4.9)	34.9 (±13.5)
F16 <sup>a</sup>	74.8 (±4.0)	20.4 (±28.9) <sup>b</sup>	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 <sup>a</sup>	109.1 (±2.3)	49.3 (±4.4) <sup>b</sup>	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 <sup>a</sup>	105.3 (±6.2)	104.6 (±10.5) <sup>b</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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 <sup>a</sup>	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)

<sup>a</sup> Estrogenically active

<sup>b</sup> 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 ER $\alpha$  in the yeast assay was determined to be  $1.0\text{--}1.6 \times 10^{-9}$  M, which corresponds to  $2.7\text{--}4.4 \times 10^{-4}$   $\mu\text{g/mL}$ . Therefore, only CPC fractions giving a response above the  $EC_{50}$  at a dilution below 3  $\mu\text{g/mL}$  were indicated as active towards ER $\alpha$  in Fig. 1. The application of this threshold value for the ER $\alpha$  resulted in nine active fractions out of 51 (see Table 1).

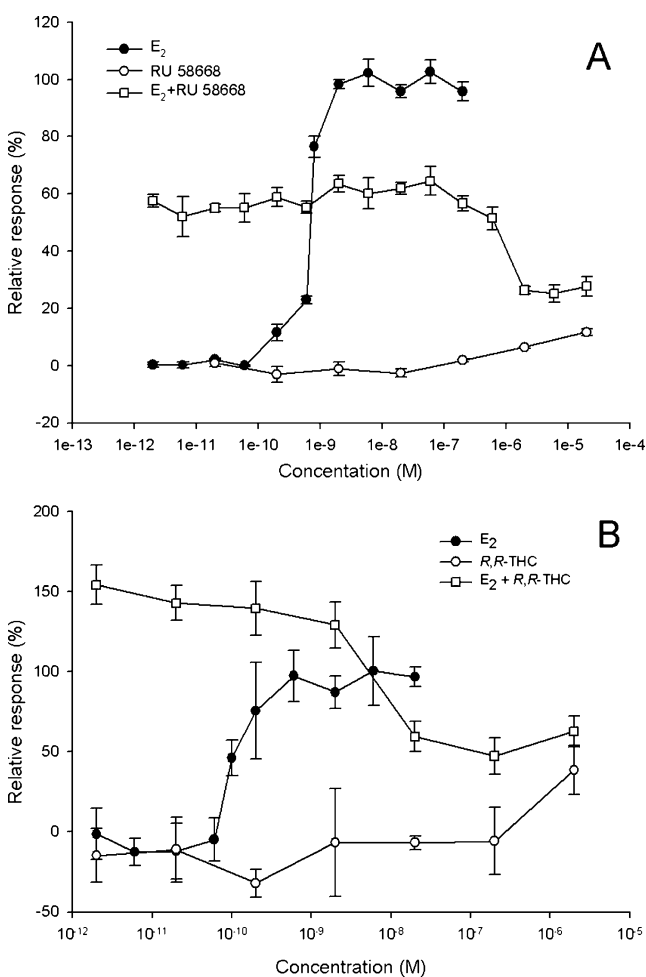
The  $EC_{50}$  value of  $E_2$  towards the ER $\beta$  ranged from  $1.1 \times 10^{-10}$  to  $2.1 \times 10^{-10}$  M, corresponding to an  $EC_{50}$  of  $3.2\text{--}5.9 \times 10^{-5}$   $\mu\text{g/mL}$ . Therefore, only CPC fractions giving a response above the  $EC_{50}$  at a dilution below 0.3  $\mu\text{g/mL}$  were indicated as active towards ER $\beta$  in Fig. 1. The

application of this threshold value for the ER $\beta$  resulted in 12 active fractions out of 51 (Table 1).

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 ER $\beta$ . 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 (ER $\alpha$ -selective) [27] and *R,R*-THC (ER $\beta$ -selective) [24] were tested in the yeast estrogen bioassays to confirm their antagonistic properties. Co-incubation of  $E_2$  and RU 58668 showed that  $6 \times 10^{-6}$  M RU 58668 was able to decrease the  $E_2$ -induced response of ER $\alpha$  by  $\sim 60\%$  (Fig. 2A). RU 58668 itself showed a weak agonistic activity of  $\sim 12\%$  in concentrations above  $1 \times 10^{-5}$



**Fig. 2** Transcription activation by ER $\alpha$  (A) and ER $\beta$  (B) in response to  $E_2$  and subtype-selective antagonists RU58668 (ER $\alpha$ ) and *R,R*-THC (ER $\beta$ ). 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 ER $\alpha$  and ER $\beta$ , respectively. Both graphs were normalized to  $E_2$ . The response of *R,R*-THC on ER $\beta$  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 $\pm$ SD ( $n=3$ )

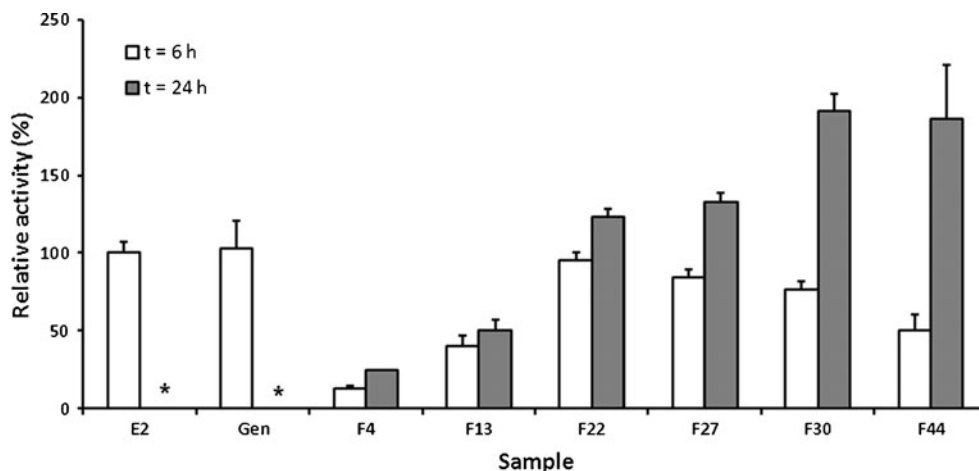
**Table 2** Inhibition of estrogenic response of representative CPC fractions by addition of a subtype-specific antagonist

	Estrogenic activity ER $\alpha$	Inhibition by RU 58668	Estrogenic activity ER $\beta$	Inhibition by <i>R,R</i> -THC
$E_2$	100	60%	100	55%
F4	ND	–	13.4 ( $\pm 5.0$ )	70%
F13	ND	–	40.3 ( $\pm 5.0$ )	37%
F22	101.4 ( $\pm 11.6$ )	52%	87.0 ( $\pm 10.3$ )	34%
F27	36.6 ( $\pm 0.3$ )	21%	74.6 ( $\pm 5.9$ )	61%
F30	23.8 ( $\pm 2.7$ )	21%	57.3 ( $\pm 1.1$ )	48%
F44	18.9 ( $\pm 1.9$ )	30%	22.7 ( $\pm 7.3$ )	70%

Inhibition of the estrogenic response of six representative CPC fractions were determined by co-incubation at 1  $\mu\text{g/mL}$  with  $6 \times 10^{-6}$  M RU 58668 for the ER $\alpha$  and 1  $\mu\text{g/mL}$  with  $2 \times 10^{-8}$  M *R,R*-THC for the ER $\beta$ . Values are the mean $\pm$ SD ( $n=3$ )

ND not detected

**Fig. 3** Stabilizing effect of E<sub>2</sub>, genistein, and several fractions obtained from the licorice root extract on the relative activity measured after 6 and 24 h in the ER $\beta$  assay. E<sub>2</sub>,  $2 \times 10^{-10}$  M estradiol; Gen,  $2 \times 10^{-7}$  M genistein; F4 to F44, licorice root fractions obtained by CPC measured at 0.3  $\mu$ g/mL. Asterisk, negative response



M. RU 58668 is known as a pure ER $\alpha$  antagonist, but its weak agonistic activity in the yeast estrogen bioassay with ER $\alpha$  is not expected, as several other 11 $\beta$ -analogues of E<sub>2</sub> were shown to be selective estrogen receptor modulators (SERM) with both agonistic and antagonistic activity on the ER $\alpha$  in the same yeast estrogen bioassay [23].

Co-incubation of E<sub>2</sub> and R,R-THC showed that  $2 \times 10^{-7}$  M R,R-THC was able to decrease the E<sub>2</sub>-induced response of ER $\beta$  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

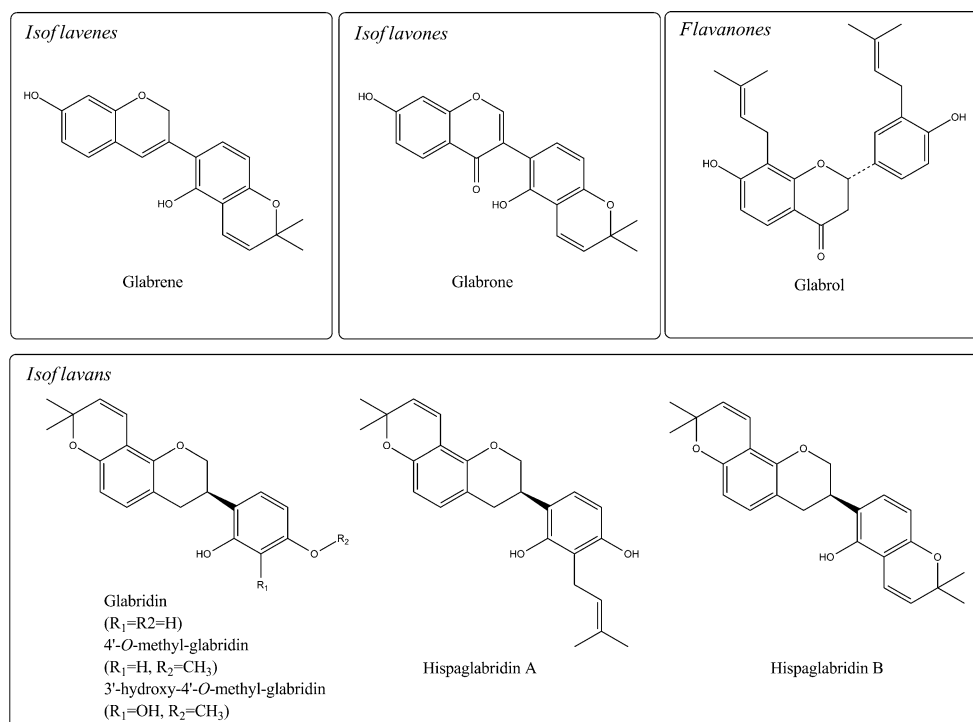
**Table 3** Fractions generated by CPC fractionation and the presence of the main flavonoids in each fraction determined by ultra-high performance LC-MS

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 <sup>a</sup>			+	+	++	++									
4'-OMe-G <sup>b</sup>	+	+	++	+											
Hispaglabridin A	+	++	++												
Hispaglabridin B	++	++	+												

<sup>a</sup> 4'-O-methyl-glabridin

<sup>b</sup> 3'-hydroxy-4'-O-methyl-glabridin

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



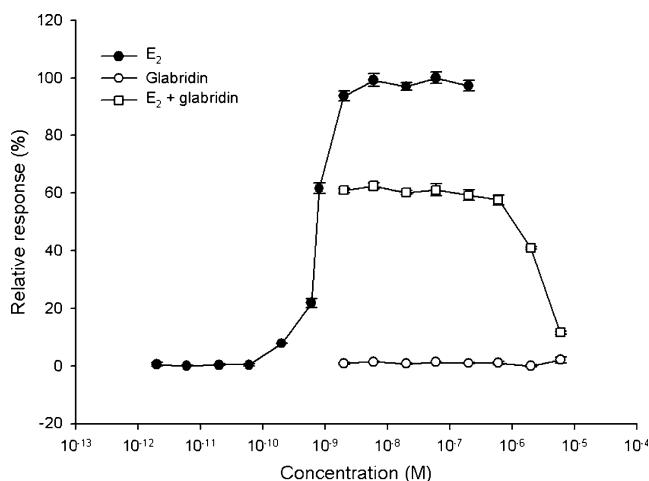
responses were ER-mediated, as no fluorescent signal was observed for any of the fractions or E<sub>2</sub>.

#### 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 ER $\alpha$  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 ER $\alpha$ . To verify the hypothesis that superinduction in the yeast was caused by the stabilization of the ER and/or the yEGFP, the yeast expressing ER $\beta$  was co-incubated with E<sub>2</sub>, 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<sub>2</sub> and genistein showed the maximum estrogenic response, but, as expected, the estrogenic response of E<sub>2</sub> 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<sup>n</sup> 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 ER $\alpha$  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<sup>n</sup>. 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 ER $\alpha$ . 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 ER $\alpha$ . Whereas phytoestrogens generally have a more pronounced affinity to the ER $\beta$  compared with the ER $\alpha$ , 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<sub>50</sub> values of  $5 \times 10^{-6}$  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  $1 \times 10^{-7}$  to  $1 \times 10^{-4}$  M towards both ER subtypes (data not shown). In concentrations above  $1 \times 10^{-4}$  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 ER $\alpha$  [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<sub>2</sub>, confirming its agonistic character.

The reference standard of glabridin did not have antagonistic properties towards the ER $\beta$  (data not shown) but was shown to be an ER $\alpha$ -selective antagonist (Fig. 5). At a concentration of  $6 \times 10^{-6}$  M, glabridin was able to inhibit the E<sub>2</sub> 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 ER $\alpha$ -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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