PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 259–268

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Comparison of hormonal activity (estrogen, androgen and progestin) of standardized plant extracts for large scale use in hormone replacement therapy☆

V. Beck[a], E. Unterrieder[b], L. Krenn[b], W. Kubelka[b], A. Jungbauer[a,*]

[a] *Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria*
[b] *Institute of Pharmacognosy, University of Vienna, Althanstraße 14, 1090 Vienna, Austria*

Abstract

Extracts from red clover (*Trifolium pratense*), soybean (*Glycine max.*) and black cohosh (*Cimicifuga racemosa*) are frequently used as alternative compounds for hormone replacement therapy (HRT) to treat menopausal disorders. Fifteen commercially available products made either from red clover, soybean or black cohosh were tested in in vitro assays in this study. The main polycyclic phenolic compounds of soy and red clover products were biochanin A, genistein, daidzein, formononetin, and glycitein. In red clover products glycitein was not abundant. All the compounds showed clear estrogenic activity through estrogen receptor α (ERα) and estrogen receptor β (ERβ) and affinity to progesterone receptor (PR) and androgen receptor (AR), whereas the compounds from black cohosh did not. This was corroborated by synthetic isoflavones such as biochanin A, daidzein, genistein and formononetin. They exerted affinity to PR and AR in the range of 0.39–110 mM. Statistical analysis applying principal component analysis (PCA) revealed that all red clover and soy products are grouped in different clusters. Red clover products showed a higher affinity to AR and PR than soy products, which is explained by the higher amount of isoflavones present. In vitro assays and chemical analysis showed that theoretical estrogenic activity expressed as equivalent E2 concentration is in the same range as recommended for synthetic estrogens. Broader spectrum of action and hypothesized lower side effects by action through ERβ make them suitable for alternative hormone replacement therapy.

Keywords: Phytoestrogens; *Trifolium pratense*; *Glycine max.*; *Cimicifuga racemosa*; Isoflavones

1. Introduction

Menopausal disorders caused by estrogen deficiency such as hot flushes, sweating, reduced bone density, eye dryness and anxiety are treated by hormone replacement therapy (HRT) using either synthetic or equine estrogens. An increased risk for breast and endometrial cancer by conventional HRT has been debated in the past [1–3]. Plant extracts containing polycyclic compounds with estrogenic activity, the so-called phytoestrogens have become increasingly popular as alternative HRT for treatment of menopausal disorders. Epidemiological studies suggested that women in countries with a high dietary intake of phytoestrogens have a decreased risk of breast cancer [4]. Sources of such phytoestrogens are among others soy (*Glycine max.*) and red clover (*Trifolium pratense*) [5]. Clinical studies are controversial about beneficial effects, but side effects and increased risk of breast cancer as reported for conventional HRT, have not become evident [6,7]. Antiproliferative action of red clover on endometrium has been suggested but could not be confirmed [6,8]. Plant extracts showed weak estrogenic activity in ovariectomized rats, but did not stimulate cell proliferation in mammary glands [9]. Other reports show a clear tumor promoting activity of genistein when administered to rats with implanted MCF7 breast tumor cells [10]. Studies with healthy women suggested that phytoestrogens influence not only estrogen receptor related functions but also the hypothalamo–hypophysis–gonadal axis [11,12].

Also beneficial effects of phytoestrogens on men's health have been hypothesized [13,14] supported by epidemiological data [15]. Incidence and mortality of prostate cancer are considerably lower in Asian populations than in US and European populations, yet the incidence of precancerous lesions is the same for these populations [16]. Prevention of hormone-dependent prostate cancer results either from interaction of phytoestrogens with aromatase or from regulation

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany, 17–20 May 2002.

* Corresponding author. Fax: +43-1-3697615.
E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00034-7

of the sex steroid hormone receptors and growth factor pathways. Such mechanisms also became evident from in vivo studies with mice and rats [17]. Phytoestrogens used as food supplements had no adverse effects on endocrine measurement and the phytoestrogen dose had no effect on semen quality [18]. There are no indications that such extracts of food supplements are not safe.

A big variety of plants have been screened for hormonal activity [19]. Active compounds in such plant extracts exerting estrogenic activity have been classified as isoflavones, coumestans, lignans and dihydroxychalcones [20,21]. Coumestans and isoflavones exert high estrogenic activity. The isoflavones biochanin A, genistein, daidzein and formononetin are present in high concentrations in red clover and soy products [22,23]. Thus, red clover and soy have become most popular as food supplement for HRT. Biochanin A is metabolized in the gut to genistein, formononetin to daidzein and daidzein to equol [24,25]. Metabolic pathways may depend on individual, race and gender.

Different cellular and biochemical properties have been attributed to the various isoflavones besides their affinity for the estrogen receptors α and β [26]. It has been hypothesized that the high affinity for estrogen receptor β (ERβ) makes them preferred candidates for alternative HRT, since effects such as endometrial growth and increased risk of breast cancer observed with conventional HRT can be avoided. Loss in bone mineral density, which is often observed during menopause can be prevented considerably by dietary phytoestrogens from soy or red clover. In in vitro assays daidzein down-regulated osteoclast differentiation by interfering with apoptosis via caspase 3 pathway [27]. Isoflavones and/or their metabolites also interact with 17β-hydroxysteroid oxidoreductase [28,29], aromatase [30], and sulfotransferase [31]. Isoflavone composition of soy and red clover extracts is remarkably different. In the plant the major fraction of isoflavones is present in their conjugated form. In soy they are mainly bound as β-glycosides, acetyl β-glycosides [22,32], and malonyl β-glycosides while in red clover they are present as malonyl β-glycosides [33]. The conjugated forms do not exert estrogenicity, since they do not bind to the estrogen receptor. In order to increase potency of such plant extracts, technology has been developed to process them in a way that free isoflavones are liberated.

Extracts from black cohosh are also used for alternative HRT [34]. Several clinical studies showed that side effects of menopause like hot flushes, menopausal anxiety and depression can be treated successfully with black cohosh-derived products [34,35]. Such extracts did not exert estrogenic activity in mice and rats [36] and also in in vitro tests with the estrogen-dependent breast cancer cell line MCF7 estrogen-like action could not be observed [5]. In a preliminary study we did not find affinity of *Cimicifuga racemosa* extracts to estrogen receptor α (ERα) and ERβ. It is likely that a metabolic activation of the extract is necessary to evoke an estrogenic effect.

Clinical data on prevention of prostate cancer by red clover and soy preparations as well as in vivo experiments in mice and rats [37,38] suggest interaction of isoflavones and other phytoestrogens with the androgen receptor (AR). Clear evidence has not been published so far. Progesterone-like activity was found for red clover extracts, whereas reports for soy and *C. racemosa* are not available [19].

The aim of our work was to compare different preparations intended for alternative HRT regarding estrogenicity and affinity for progesterone receptor (PR) and androgen receptor. As there is little evidence about the effects of isoflavones on androgen or progesterone receptor, we tried to elucidate this subject in our study. We correlated biological in vitro assays with chemical analysis in order to compare the actual isoflavone content of various products with their biological activity in yeast transactivation assays as well as in competitive radioligand binding assays. Statistical evaluation of data by means of principal component analysis (PCA) was performed to get maximum information out of all accumulated tests.

2. Materials and methods

2.1. Chemicals

Buffer reagents, dimethylsulfoxide (DMSO), dextran-coated charcoal (DCC) and *o*-nitrophenyl-β-galactopyranoside (ONPG) were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). The [17α-methyl-^{3}H]-methyltrienolone (^{3}H-R1881) and [1,2,6,7-^{3}H(*N*)]-progesterone as well as unlabeled methyltrienolone (R1881) were purchased from NEN LifeScience. For yeast media preparation, yeast nitrogen base was obtained from Difco (Franklin Lakesm, NJ), amino acids from Serva Feinbiochemica (Heidelberg, Germany) and from Sigma (St. Louis, MO). The 17β-estradiol, unlabeled progesterone and isoflavones (biochanin A, daidzein, formononetin, genistein) were obtained from Sigma (St. Louis, MO). Glass beads (0.25–0.5 mm diameter) were purchased from Merck (Darmstadt, Germany). Human recombinant histidine-tagged progesterone receptor B and recombinant rat thioredoxin-fusion androgen receptor were purchased from Panvera (Madison, WI). Samples of different preparations intended for hormone replacement therapy were provided by Melbrosin (Vienna, Austria). The solvents used were HPLC grade.

2.2. Yeast strain and growth conditions

Yeast strain 188R1 (*Saccharomyces cerevisiae*) was transformed as a two-plasmid system with a human ERα (YEpE12; CUP1 promoter) or human ERβ (YEpFERβH3, CUP1 promoter) [39] expression plasmid and a corresponding reporter plasmid (YRpE2), which contained two copies of the vitellogenin ERE and the *iso*-1-cytochrome *c* (CYC 1)

in a lacZ fusion vector [40]. The auxotrophy markers were tryptophan (trp) for the expression plasmid and uracil (ura) for the reporter plasmid. Gold medium without trp/ura was used for yeast cultivation.

2.3. Extraction of samples

The pills or capsules were pulverized in a mortar, the powder was suspended in a defined volume of DMSO and stirred at 720 rpm. After 5 h the suspension was centrifuged at 13,000 rpm for 3 min. The supernatant was collected for further analysis and stored at −80 °C.

2.4. Transactivation assay

For each test run a new overnight culture (30 °C, 180 rpm) was taken and diluted to $OD_{600} = 0.4$ before use. Amounts of 10, 30 and 50 μl of the diluted sample-extracts were added to 5 ml of the diluted yeast culture. The same amount of DMSO was added to each sample. Expression of hER was induced by addition of 10 μM $CuSO_4$. DMSO alone was used as blank. A calibration curve with 17β-estradiol was made within each test run. Determinations were carried out in duplicates. After 4 h of incubation at 30 °C the yeast cells were disintegrated.

2.5. Preparation of yeast extracts

Cells were collected by centrifugation at 2500 rpm for 5 min. The pellets were resuspended in 1 ml of lacZ buffer (100 mM sodium phosphate buffer, pH 7.0, containing 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol), transferred to test tubes and centrifuged again (5 min, 10,000 rpm, 4 °C). The supernatant was discarded and 100 μl of lacZ buffer were added to the pellet. Disintegration of the yeast cells was performed by vortexing with glass beads. The samples were vortexed three times for 30 s with a rest of 15 s on ice in the intervals. After centrifugation (10 min, 10,000 rpm, 4 °C) β-galactosidase assay and protein assay were performed.

2.6. β-Galactosidase assay and protein determination

A small amount of the clear supernatant of the samples was transferred into the wells of a 96-well microtiter plate. An amount of 250 μl of a 4 mg/ml solution of the chromogenic substrate *o*-nitrophenyl-β-galactopyranoside were added and the plate was incubated at 37 °C for about 15 min until a yellow color had developed. The color reaction was stopped by adding 100 μl of 1 M Na_2CO_3. The absorption at 405 nm was measured with a SLT EAR 400 AT plate reader (SLT, Salzburg, Austria).

Amount of total protein was quantified with the Bio-Rad protein assay reagent. A BSA-standard curve was created within each test run.

The specific enzyme activity was expressed in Miller units which take the amount of total protein into account. They are defined as follows:

$$\text{Miller units} = \left(\frac{OD_{405}}{\mu g \text{ of protein/ml}}\right)\left(\frac{1}{\Delta t}\right) \times \left(\frac{\text{sample volume protein assay } (\mu l)}{\text{sample volume } \beta\text{-galactosidase assay } (\mu l)}\right) \times 1000 \quad (1)$$

where Δt (given in minutes) is the incubation time at 37 °C.

2.7. Curve fitting

Data of the transactivation assay were fitted using a logistic dose–response model. Calculation was performed with Table Curve 2D software (Jandel Scientific). The function is described as:

$$Y = a + \frac{b}{1 + (c/x)^d} \quad (2)$$

where the parameter *a* equals the baseline, *b* is the plateau of the curve (ligand efficiency) and *c* gives the transition center of the curve (ligand potency), which is the concentration that causes 50% efficiency.

By using this calibration curve the estrogenic activity of the samples can be expressed in equivalents of 17β-estradiol.

2.8. Competitive radioligand binding experiments

Affinity of preparations to androgen and progesterone receptor was determined by competitive radioligand binding assays. Dilutions of the samples were combined with 7.98 nM [^{3}H]-R1881 or 3.21 nM [1,2,6,7-^{3}H(*N*)]-progesterone, respectively. An amount of 50 μl incubation buffer (10 mM Tris, pH 7.4, containing 1.5 mM EDTA and 10% (v/v) glycerol) and 30 μl of an appropriate dilution of the androgen or progesterone receptor, respectively, were added and incubated for 16–18 h. In parallel non-specific binding was determined by adding a 200-fold excess of unlabeled R1881 or unlabeled progesterone, respectively, which have a binding affinity to the corresponding receptors similar to the radioactively labeled ligands. As a carrier protein casein was added to the incubation buffer in a concentration of 1 mg/ml. The unbound radiolabeled ligand was removed by incubation with 300 μl dextran-coated charcoal for 15 min at 4 °C. Samples were centrifuged (6000 × *g* for 10 min at 4 °C), aliquots of the supernatant (100 μl) were added to scintillation cocktail (3 ml) and counted for 1 min. All determinations were carried out in duplicates. Counter efficiency was measured in parallel to each assay by measuring a known amount of [^{3}H]-R1881 and [1,2,6,7-^{3}H(*N*)]-progesterone, respectively.

2.9. Determination of isoflavones by HPLC

The HPLC analyses of the isoflavone content of the samples were performed according to Krenn et al. [41]. Briefly, the tablets or the content of the capsules were homogenized. For red clover preparations, 50 mg of the homogenous material were dissolved by sonication with 5.00 ml dimethylsulfoxide/water (3:1). After filtration, to 500 μl of the filtrate 40 μl of internal standard (15 mg 6-methoxyflavanon/ml) were added and 10 μl of the mixture were analyzed.

The extraction of the soy products was performed according to Jones (slightly modified) [42]. An amount of 200 mg of the homogenized material were extracted under reflux with 9 ml methanol and 4 ml of concentrated HCl for 3 h. The hydrolysate was washed with 65% methanol and brought to pH 5.5–6 with sodium hydroxide solution. The solution was washed three times with 20 ml hexane. The aqueous layer was then extracted with 20 ml and three times 5 ml diethyl ether. After evaporation of the combined extracts the residue was dissolved in 2 ml DMSO. An amount of 500 μl of the solution were treated as given earlier.

HPLC was performed on a 250 mm × 4 mm i.d. Hypersil BDS-C_{18} 5 μm column (Shandon, Runcorn, UK). The eluent consisted of water, adjusted with sulfuric acid to pH 2.7 (A) and acetonitrile (B). The gradient profile was: 0–35 min, 20–37% B; 35–45 min, 37–100% B; 45–50 min, 100% B; 50–51 min, 100–20% B; 51–61 min, 20% B. Flow rate: 1 ml/min. Wavelength of detection: 254 nm.

2.10. Calculation of theoretical estrogenic activity from HPLC-results

Relative potency (pot_{rel}) of the individual compounds was calculated by dividing the potency of each individual compound (pot_{iso}) by the potency of E2 (pot_{E2}) (Eq. (3)):

$$pot_{rel} = \frac{pot_{iso}}{pot_{E2}} \tag{3}$$

The theoretical equivalent concentration of 17β-estradiol ($E2_{theor}$) is the sum of the individual theoretical E2 concentrations of the standard red clover phytoestrogens, which are calculated by dividing the content of the individual isoflavone (c_{iso}) by its relative potency pot_{rel} (Eq. (4)):

$$E2_{theor} = \sum_{i=1}^{n} \frac{c_{iso,i}}{pot_{rel}} \tag{4}$$

2.11. Calculation of affinity constant

Affinity constants of plant extracts to androgen and progesterone receptor were calculated from the radioligand competition data by using an equation described by Cheng–Prusoff [43] (Eq. (5)):

$$K_H = \frac{[H_{0.5}]}{1 + ([L]/K_L)} \tag{5}$$

where K_H is the affinity constant of the individual ligand $[H_{0.5}]$ is the potency of the individual ligand, [L] is the concentration of the free radioactive ligand in the displacement experiment and K_L is the affinity constant of the radioactive ligand to the receptor. K_L was assumed to be in the range of 1 nM.

2.12. Statistical analysis

Principal component analysis was performed with accumulated data from biological in vitro assays and from chemical HPLC analysis. Data were autoscaled and PCA was performed with Matlab software (Mathworks, Natick, MA) using singular value decomposition.

3. Results

The effect of various standardized plant extracts intended for hormone replacement therapy on the different steroid hormone receptors was subject of this study. The impact of phytoestrogens extracted from red clover or soybean on human steroid hormone receptors has not been fully investigated yet. In this study the potential of a preparation to transactivate estrogen receptor α and estrogen receptor β was tested in a yeast estrogen screen (YES), whereas binding to androgen receptor and progesterone receptor was determined in a competitive radioligand binding assay. Isoflavone content of the samples was determined by reversed phase HPLC. The results of the accumulated data were analyzed in a principal component analysis. A list of the tested preparations including the source from which they originate is shown in Table 1.

Yeast estrogen screens of all preparations with ERα and ERβ were performed in different dilutions. Data shown here are averages of at least two determinations at various concentrations. The equivalent estrogenic activities were calculated using an E2-standard curve performed within each test run (Table 2). Except Rimostil all samples originating from soy or red clover showed higher activity in the yeast transactivation assay using ERβ than using ERα. The potency of Liviella, a hormone preparation containing the synthetic compound Tibolon, to activate ERβ was very weak, while it showed significant activity on ERα. Soy-derived products had very little estrogenic activity on ERα. Usually their equivalent E2 concentration was lower than that of red clover-derived preparations also in ERβ. Remifemin, the only sample that contained isoflavones from black cohosh, showed no activity in any of the assays performed in this study.

Standard compounds of red clover were assayed for activation of ERα and ERβ. The logistic dose–response curves are shown in Figs. 1 and 2. The potency, which is the concentration of ligand at half maximal response, and efficiency, which is the maximum response, were calculated from the fitted function according to Eq. (2). The values

Table 1
List of the samples (drugs) tested in this study

#	Sample	Supplier	Source	Botanical nomenclature	Recommended daily dose (mg isoflavones)
1	Promensil	Novogen	Red clover	*T. pratense*	40
2	Rimostil	Novogen	Red clover	*T. pratense*	57
3	Trinovin	Novogen	Red clover	*T. pratense*	40
4	Rotklee Activ tablets	HWS OTC-Service GmbH	Red clover	*T. pratense*	Not available
5	Rotklee tablets	MeRoSan GmbH	Red clover	*T. pratense*	Not available
6	Red clover	Novogen	Red clover	*T. pratense*	40
7	Isoflavones	Boots	Red clover	*T. pratense*	40
8	Menoflavon	Melbrosin	Red clover	*T. pratense*	40–80
9	Soy Plus capsules	Ökopharm GmbH	Soybean	*G. max.*	80
10	Orthomol femin	Orthomol GmbH	Soybean	*G. max.*	40
11	Isoflavones from soybean extract	Sanamed	Soybean	*G. max.*	40
12	Aria	Lichtwer Pharma	Soybean	*G. max.*	50
13	Remifemin	Schaper & Brümmer	Black cohosh	*C. racemosa*	–[a]
14	Life Extension	LifeScience	Soybean, black cohosh	*G. max., C. racemosa*	110
15	Tibolon Liviella	Organon	Synthetic	Not applicable	Not applicable

[a] Isopropanolic extract (40% (v/v)) equivalent to 40 mg of the drug.

Table 2
Equivalent E2-activity of the preparations tested in the yeast estrogen screen using ERα and ERβ

#	Sample	ERα		ERβ	
		Equivalent E2 concentration (nmol/g)	S.D. (nmol/g)	Equivalent E2 concentration (nmol/g)	S.D. (nmol/g)
1	Promensil	80.6	31.9	168.3	2.8
2	Rimostil	167.2	2.8	94.2	7.7
3	Trinovin	58.5	4.1	160.9	41.9
4	Rotklee Activ tablets	67.0	5.7	165.2	51.5
5	Rotklee tablets	71.5	7.4	167.2	9.6
6	Red clover	53.5	20.4	101.4	7.4
7	Isoflavones	63.7	26.2	114.8	7.0
8	Menoflavon	99.6	27.9	192.7	22.7
9	Soy Plus capsules	1.7	0.3	43.7	15.5
10	Orthomol femin	0.8	0.0	23.9	2.4
11	Isoflavones from soybean extract	8.5	0.5	332.0	101.4
12	Aria	1.8	0.5	33.7	0.2
13	Remifemin	No activity	–	No activity	–
14	Life Extension	3.7	0.9	36.8	9.7
15	Tibolon Liviella	49.5	3.1	6.1	1.1

S.D.: standard deviation.

for potency and efficiency of the standard isoflavones are shown in Table 3. In the transactivation assay using ERα or ERβ potencies of the standard isoflavones were about two to five orders of magnitude lower than the potency of the standard compound 17β-estradiol. The potency of genistein was about 160-fold higher in the assay using ERβ than in the assay with ERα. Also the relative response of genistein in respect of 17β-estradiol was higher in ERβ than in ERα.

Table 3
Transactivation induced by phytoestrogens on human ERα and human ERβ measured by yeast estrogen screen

Ligand	Potency (EC_{50}) (mol/l)		Efficiency[a] (%)	
	ERα	ERβ	ERα	ERβ
17β-Estradiol	6.8E−10	8.9E−11	100.0	100.0
Biochanin A	1.1E−06	4.3E−07	58.9	103.0
Daidzein	1.3E−05	5.3E−07	30.7	94.5
Formononetin	1.4E−06	1.1E−06	82.9	84.0
Genistein	8.8E−07	5.2E−09	110.1	229.0

[a] Fold induction, 17β-estradiol: 100%.

Due to the structural similarity of steroid hormone receptors it is likely that phytoestrogens show activity not only on the estrogen receptors α and β but also on other steroid hormone receptors. Competitive radioligand binding assays of different dilutions of the isoflavone preparations resulted in a binding to androgen and progesterone receptor in a dose-dependent manner. Binding curves of the products to PR and AR have been fitted with a logistic dose–response model. IC_{50} values of the tested substances are shown in Table 4. The affinity of the isoflavone preparations tested in this study usually was higher to AR than to PR whereas the synthetic product Tibolon bound more strongly to PR.

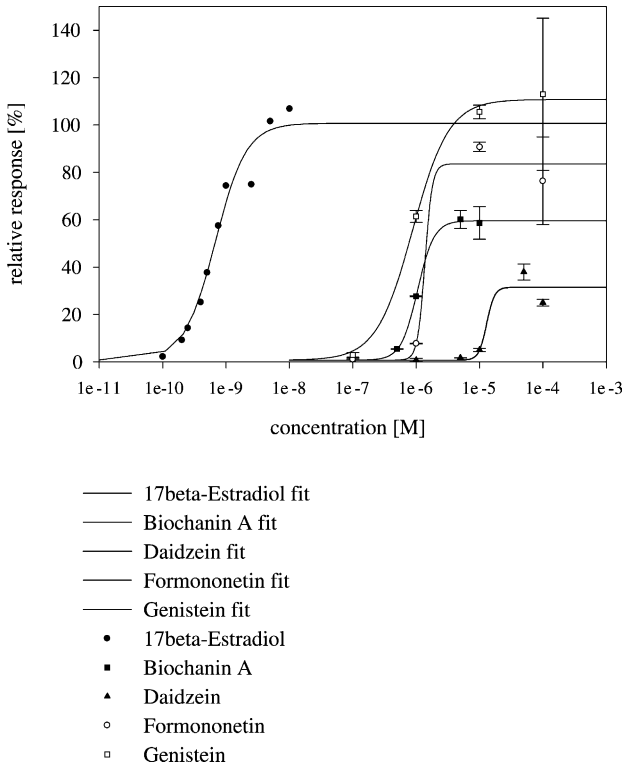

Fig. 1. Logistic dose–response curves of 17β-estradiol and isoflavone standards determined in a yeast assay using ERα. The relative response has been calculated in respect to the response toward 17β-estradiol (100%). Concentrations are denoted in mol/l.

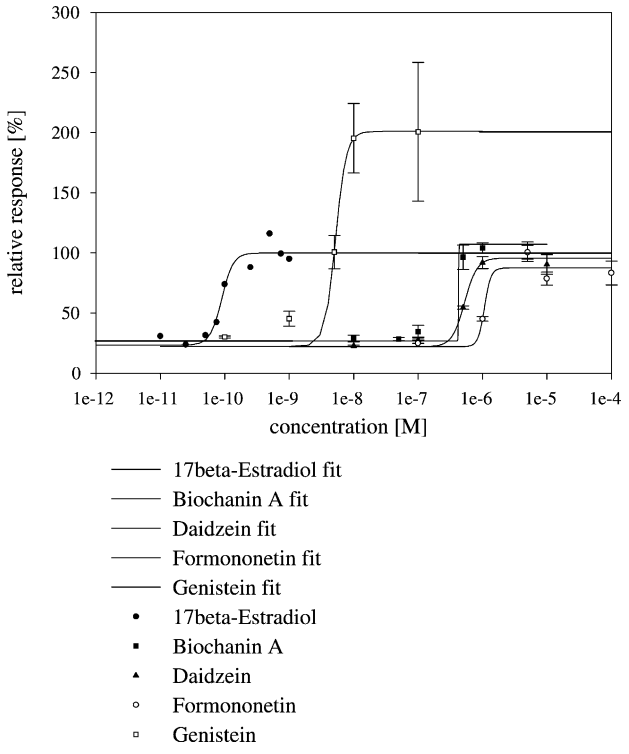

Fig. 2. Logistic dose–response curves of 17β-estradiol and isoflavone standards determined in a yeast assay using ERβ. The relative response has been calculated in respect to the response toward 17β-estradiol (100%). Concentrations are denoted in mol/l.

Remifemin, which is derived from black cohosh showed no binding to either of the steroid hormone receptors.

Binding studies of standard isoflavones derived from red clover showed that these compounds could compete with methyltrienolone and progesterone in binding to their respective receptors. The potencies of binding of these substances to both receptors are shown in Table 5. Formononetin had the lowest affinity to both PR and AR, whereas biochanin A was the most potent isoflavone extracted from red clover, regarding binding to AR and to PR. Affinity constants of the standard isoflavones were calculated using Eq. (5). Dissociation constants for the receptor–ligand complexes were assumed to be in the range of 1 nM.

Reversed phase HPLC analysis of the samples showed that red clover preparations consisted mainly of biochanin A and formononetin (Fig. 3). These compounds were not present in soy preparations. The amount of genistein and daidzein was small in red clover products compared to soy preparations. The total isoflavone content determined for all red clover

Table 4
IC_{50} values and standard errors of logistic dose–response curves obtained by binding of samples to AR and PR

#	Sample	IC_{50} (mg/ml)	
		AR	PR
1	Promensil	1.1 ± 1.4	5.0 ± 14.5
2	Rimostil	4.3 ± 0.9	22.9 ± 82.6
3	Trinovin	3.5 ± 1.8	6.2 ± 5.2
4	Rotklee Activ tablets	8.0 ± 9.1	5.1 ± 0.6
5	Rotklee tablets	2.4 ± 1.0	4.5 ± 1.0
6	Red clover	3.8 ± 1.6	3.5 ± 1.8
7	Isoflavones	1.8 ± 0.5	3.2 ± 0.6
8	Menoflavon	1.9 ± 0.2	5.3 ± 0.8
9	Soy Plus capsules	11.5 ± 2.6	35.6 ± 30.1
10	Orthomol femin	5.0 ± 0.2	33.6 ± 2.4
11	Isoflavones from soybean extract	5.9 ± 0.7	17.2 ± 4.6
12	Aria	4.8 ± 165.2	23.3 ± 6.6
13	Remifemin	No activity	No activity
14	Life Extension	7.0 ± 1.4	50.2 ± 43.1
15	Tibolon Liviella	0.9 ± 0.1	0.03 ± 0.0

Table 5
Potencies of standard phytoestrogens and affinities to the respective receptor determined by competitive radioligand binding experiments to AR and PR

Ligand	PR		AR	
	IC_{50} (mg/ml)	Affinity constant (mM)	IC_{50} (mg/ml)	Affinity constant (mM)
Biochanin A	1.1	0.9	1.0	0.4
Daidzein	13.4	12.5	9.6	4.2
Formononetin	54.2	48.0	265.1	110.0
Genistein	10.4	9.2	1.0	0.4

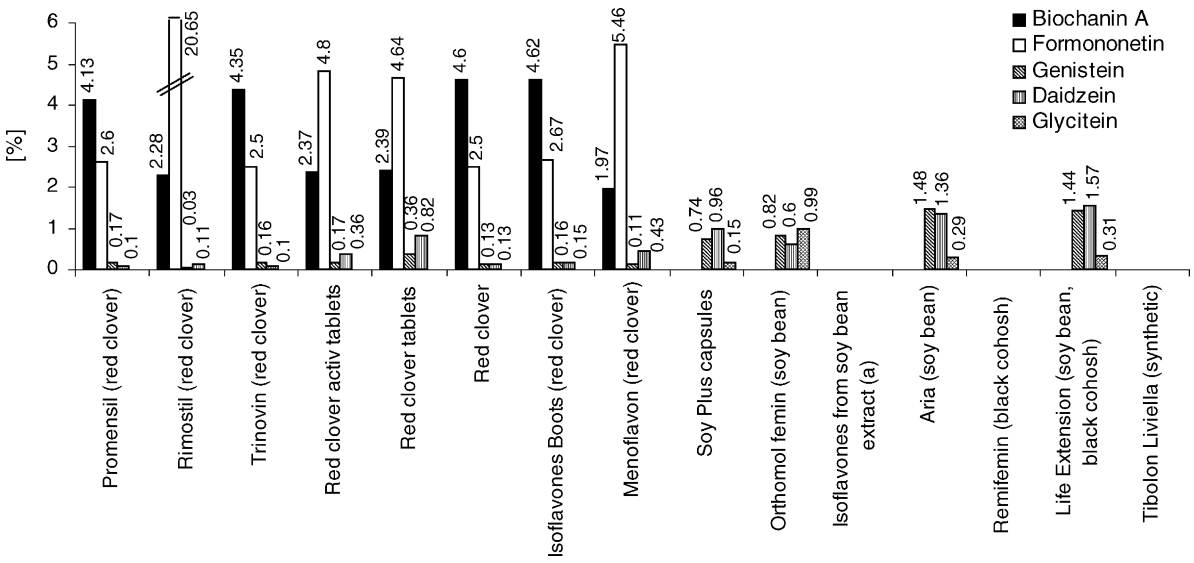

Fig. 3. Isoflavone content analyzed by reversed phase HPLC given as percent of weight; (a) implies data not available.

products corresponded to the declaration, while it was lower in soy preparations. Although the extraction of these samples was performed according to a method published, it was not clear whether the lower amounts were due to unsuitable extraction for the release of the isoflavones from the protein binding or to an actual lower amount of isoflavones in soy preparations.

A comparison of theoretical equivalent estrogenic activity calculated from HPLC data and data obtained from yeast transactivation assay showed that in most cases theoretical estrogenicity was higher than estrogenicity determined by yeast estrogen screen (Table 6). The difference between these two determinations was wider for soy products than for red clover preparations. The theoretical equivalent estrogenic activity of preparations derived from soy was higher in ERβ than in ERα. This was due to the high potency of the soy-derived isoflavone genistein in the ERβ-yeast screen.

Table 6
Equivalent estrogenic activity calculated from HPLC analysis and YES potency data of the single compounds

#	Sample	ERα (nmol E2/g)		ERβ (nmol E2/g)	
		Theoretical	YES	Theoretical	YES
1	Promensil	147.0	80.6	146.4	168.3
2	Rimostil	431.7	167.2	100.5	94.2
3	Trinovin	149.9	58.5	141.4	160.9
4	Rotklee Activ tablets	153.2	67.0	260.5	165.2
5	Rotklee tablets	152.1	71.5	264.5	167.2
6	Red clover	154.7	53.5	124.6	101.4
7	Isoflavones	159.2	63.7	144.3	114.8
8	Menoflavon	149.2	99.6	103.7	192.7
9	Soy Plus capsules	24.4	1.7	472.9	43.7
10	Orthomol femin	26.1	0.8	521.0	23.9
12	Aria	47.7	1.8	942.1	33.7
14	Life Extension	47.0	3.7	918.3	36.8

Accumulated data of all analyses performed within this study were treated statistically in a principal component analysis. Red clover preparations were clearly separated from the rest. High values of features 1 (equivalent E2 concentration in YES using ERα), 2 (equivalent E2 concentration in YES using ERβ), 5 (biochanin A content) and 6 (formononetin content) were characteristic for red clover preparations, high values of features 7 (genistein content) and 8 (daidzein content) for soy preparations and low values of features 3 (affinity to AR) and 4 (affinity to PR) for the synthetic product Tibolon. The variance of the first component was 48.2% and the variance of the second component was 30.3% (Fig. 4).

4. Discussion

Only limited information is available on standardized plant extracts intended for estrogen replacement therapy. Soy products and red clover products have been compared by chemical analysis [22,23]. The compounds responsible for estrogenic activity in products made from soy or red clover are isoflavones. Red clover has a high content of biochanin A and formononetin, which are metabolized to genistein and daidzein, respectively. The isoflavones contained in soy are mainly genistein, daidzein and glycitein. Assuming a quantitative conversion of biochanin A to genistein and of formononetin to daidzein, red clover preparations have a much higher theoretical estrogenicity compared to soy products. These plant extracts contain the same or more active estrogenic compounds compared to synthetic estrogens or Premarin, a natural product consisting of a lot of conjugated equine steroids.

The variation of isoflavone content in these preparations is due to varying raw material and extraction procedures [44].

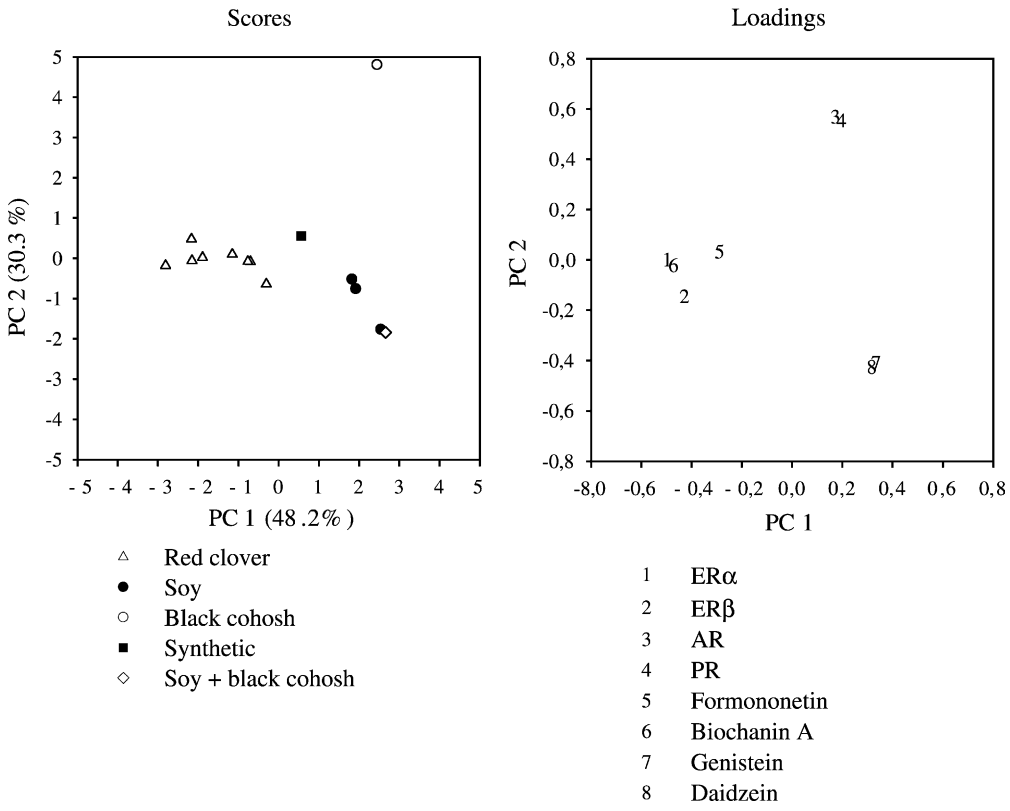

Fig. 4. Statistical treatment of accumulated data by principal component analysis.

The isoflavone content depends on the cultivar, site, soil and climatic conditions in red clover. In addition, extraction procedures influence the ratio between free and conjugated isoflavones. Conjugated isoflavones are not active, but they can be metabolized in the gut. The same properties have been observed with soy products. Both soy and red clover preparations contain sufficient estrogenic activity to be a candidate for HRT. Currently a daily dose of 40 mg isoflavones is recommended by most suppliers of red clover and soy extracts. Values stem from epidemiological studies assuming that a daily intake of at least 40 mg isoflavones through soy-rich diets have beneficial effects. This dose is often related to free isoflavones. Cleavage of conjugated isoflavones can elevate this level.

The theoretical estrogenicity of the phytoestrogen preparations expressed as equivalent E2 concentration is in the same range as recommended for synthetic estrogens. Plant extracts definitely can replace lacking estrogen and in addition they show higher affinity to ERβ than to ERα. It has been hypothesized that proliferation of endometrium and breast cells is not promoted by activation of ERβ. Thus, those compounds are considered to have a clear advantage over synthetic estrogens, since they exert higher affinity to ERβ than to ERα. Lower side effects can be expected when such substances with high affinity to ERβ are administered. Clinical data on red clover and soy preparations are rare and contradictory. More in vivo and in vitro studies are required to support the hypothesis of lower side effects.

The potencies of standard isoflavones were in good agreement with data published previously by Dornstauder et al. [45]. The same potencies and efficiencies were found for all compounds except the efficiency of formononetin, which was higher, and the efficiency of daidzein, which was lower using YES with ERβ in the present study. The accuracy of ERβ assays was always lower than the accuracy of ERα assays. Efficiency measured as the maximum level of reporter gene expression is related to the stability of the transcription complex [21]. It seems that the active transactivation complex in this artificial system using full length ERβ is not as stable as observed in transactivation assays with ERα. The more important information is potency. In this respect YES is a very robust assay [46].

Theoretical estrogenicity calculated by Eq. (4) by dividing each isoflavone content by its relative potency found in YES differed significantly from experimental values found in transactivation assays. For red clover products the variation between theoretical and experimental estrogenicity was in the same range for all products. This difference could be due to sample preparation, which was different between chemical and biological analysis. Isoflavones are present as free compounds or conjugated with sugars at the 7 and 4′ position or with malonate esters of sugars at the 7 position. Isoflavones can also be acetylated. Malonate esters are especially labile and isoflavones can be liberated during the sample preparation procedure. The complexity of such natural extracts makes rigorous standardization

extremely important. Since so many compounds with similar physicochemical properties are present, in vitro assays are a good means to complement the chromatographic methods. Chromatographic methods only quantify main compounds. Setchell et al. [22] found 78.15 mg isoflavones/g for Promensil, 73.39 mg isoflavones/g for Trinovin and 37.21 mg isoflavones/g for Soy Plus. We found 70.0 mg isoflavones/g for Promensil, 71.1 mg isoflavones/g for Trinovin and 18.5 mg isoflavones/g for Soy Plus capsules. For the latter it is not clear if we have tested the same product and comparable lots. On the other hand, as already mentioned previously, variation due to different raw material and sample preparation can also explain differences.

Glycitein, another isoflavone present in soy preparations, has been disregarded in the biological in vitro assays performed in this study. Some authors report that glycitein has an estrogenicity similar to the other soy isoflavones genistein and daidzein [47]. For some soy preparations a high glycitein content has been determined whereas in others it was low. Different extraction procedures or raw material sources may be responsible for the varying glycitein content of different products.

Extracts derived from black cohosh did not interact with any of the steroid receptors (ERα, ERβ, PR and AR) in the in vitro assays performed in this study, but a positive effect on climacteric disorders in some in vivo experiments and several clinical studies has been reported [34,48]. Polycyclic compounds such as triterpene glycosides of the cycloartane type were extracted from roots of *C. racemosa* [49]. It is probable that a metabolic activation of this plant extract is necessary to evoke its estrogenic properties.

Statistical analysis using PCA revealed that there is a clear difference in the mechanisms of action of soy products and red clover products on steroid receptors. In PCA score plots, preparations extracted from the same plant were grouped together and loading plots showed that transactivation of ERα and ERβ and high formononetin and biochanin A content were characteristic for red clover preparations, whereas high genistein and daidzein content were characteristic for soy products. Tibolon, a synthetic compound with well-known properties, was only used as a reference compound.

Interestingly, soy products showed a lower affinity to PR and AR than red clover products. The affinity to PR and AR has been considered a beneficial property in Premarin, a natural equine product widely used for hormone replacement therapy. Red clover products exert a similar mode of action although totally different compounds are present.

Affinity constants of plant extracts to AR and PR are 4×10^5 to 10^8-fold lower compared to their cognate ligands. Formononetin has the lowest and biochanin A the highest affinity to both receptors. The affinities of standard isoflavones are in the same range as their affinities to ERα and ERβ. From the viewpoint of receptor binding, isoflavones from plant extracts can be considered as weak estrogens, weak progestins and weak androgens. Further studies using appropriate in vitro and in vivo models are required to assess their antagonistic and agonistic nature. The applied methodology does not allow a discrimination between agonist and antagonist.

Rimostil is the only red clover-derived product that shows a higher activity in transactivation assays using ERα than using ERβ. Beneficial effects of phytoestrogens in the treatment of menopausal disorders are often attributed to a higher affinity of those preparations to ERβ than to ERα in contrast to E2 and other synthetic estrogens.

Products considered beneficial for treating menopausal disorders may also have other activities such as inhibition or interference with aromatase, hydroxysteroid dehydrogenase, and sulfotransferase. It has been clearly demonstrated that they possess antioxidant activity [50].

The high content of phytoestrogens makes these preparations a useful alternative for conventional estrogen replacement therapy. Extracts from different plants vary in isoflavone composition and mode of action in the mammalian organism. Further investigations are necessary to fully elucidate action of phytoestrogens, not only on the estrogen, but also on the progesterone and androgen receptor. Clinical results, in vivo and in vitro data suggest red clover and soy extracts as alternative compounds for hormone replacement therapy. The spectra of action observed with red clover and soy products are thought to be different.

Phytoestrogens have also been claimed to be beneficial in protection against prostate cancer [51]. Their affinities to the steroid receptors ERα, ERβ and AR support this hypothesis.

References

[1] J. Dew, J. Eden, E. Beller, C. Magarey, P. Schwartz, P. Crea, B. Wren, A cohort study of hormone replacement therapy given to women previously treated for breast cancer, Climacteric 1 (1998) 137–142.
[2] B. Hedblad, J. Merlo, J. Manjer, G. Engstrom, G. Berglund, L. Janzon, Incidence of cardiovascular disease, cancer and death in postmenopausal women affirming use of hormone replacement therapy, Scand. J. Public Health 30 (2002) 12–19.
[3] A.H. MacLennan, B. Lawton, R.J. Baber, Hormone replacement therapy and the breast. Studies must determine the evidence, BMJ 324 (2002) 915 (discussion 915).
[4] H. Adlercreutz, Epidemiology of phytoestrogens, Baillieres Clin. Endocrinol. Metab. 12 (1998) 605–623.
[5] Z. Liu, Z. Yang, M. Zhu, J. Huo, Estrogenicity of black cohosh (*Cimicifuga racemosa*) and its effect on estrogen receptor level in human breast cancer MCF-7 cells, J. Hyg. Res. 30 (2001) 77–80.
[6] R.J. Baber, C. Templeman, T. Morton, G.E. Kelly, L. West, Randomized placebo-controlled trial of an isoflavone supplement and menopausal symptoms in women, Climacteric 2 (1999) 85–92.
[7] J. Ginsburg, G.M. Prelevic, Is there a proven place for phytoestrogens in the menopause? Climacteric 2 (1999) 75–78.
[8] G.E. Hale, C.L. Hughes, S.J. Robboy, S.K. Agarwal, M. Bievre, A double-blind randomized study on the effects of red clover isoflavones on the endometrium, Menopause 8 (2001) 338–346.
[9] J.E. Burdette, J. Liu, D. Lantvit, E. Lim, N. Booth, K.P. Bhat, S. Hedayat, R.B. Van Breemen, A.I. Constantinou, J.M. Pezzuto, N.R. Farnsworth, J.L. Bolton, *Trifolium pratense* (red clover) exhibits estrogenic effects in vivo in ovariectomized Sprague–Dawley rats, J. Nutr. 132 (2002) 27–30.

[10] C.Y. Hsieh, R.C. Santell, S.Z. Haslam, W.G. Helferich, Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo, Cancer Res. 58 (1998) 3833–3838.
[11] A. Cassidy, S. Bingham, K. Setchell, Biological effects of isoflavones in young women: importance of the chemical composition of soyabean products, Br. J. Nutr. 74 (1995) 587–601.
[12] S. Watanabe, K. Terashima, Y. Sato, S. Arai, A. Eboshida, Effects of isoflavone supplement on healthy women, Biofactors 12 (2000) 233–241.
[13] C. Nagata, N. Takatsuka, H. Shimizu, H. Hayashi, T. Akamatsu, K. Murase, Effect of soymilk consumption on serum estrogen and androgen concentrations in Japanese men, Cancer Epidemiol. Biomarkers Prev. 10 (2001) 179–184.
[14] M.S. Kurzer, Hormonal effects of soy in premenopausal women and men, J. Nutr. 132 (2002) 570S–573S.
[15] H. Adlercreutz, Phytoestrogens: epidemiology and a possible role in cancer protection, Environ. Health Perspect. 103 (1995) 103–112.
[16] L.W. Chung, Implications of stromal–epithelial interaction in human prostate cancer growth, progression and differentiation, Semin. Cancer Biol. 4 (1993) 183–192.
[17] M. Pollard, W. Wolter, L. Sun, Diet and the duration of testosterone-dependent prostate cancer in Lobund–Wistar rats, Cancer Lett. 173 (2001) 127–131.
[18] J.H. Mitchell, E. Cawood, D. Kinniburgh, A. Provan, A.R. Collins, D.S. Irvine, Effect of a phytoestrogen food supplement on reproductive health in normal males, Clin. Sci. 100 (2001) 613–618.
[19] D.T. Zava, C.M. Dollbaum, M. Blen, Estrogen and progestin bioactivity of foods, herbs, and spices, Proc. Soc. Exp. Biol. Med. 217 (1998) 369–378.
[20] R.J. Miksicek, Commonly occurring plant flavonoids have estrogenic activity, Mol. Pharmacol. 44 (1993) 37–43.
[21] A. Breithofer, K. Graumann, M.S. Scicchitano, S.K. Karathanasis, A. Jungbauer, T.R. Butt, Regulation of human estrogen receptor by phytoestrogens in yeast and human cells, J. Steroid Biochem. Mol. Biol. 67 (1998) 421–429.
[22] K.D. Setchell, N.M. Brown, P. Desai, L. Zimmer-Nechemias, B.E. Wolfe, W.T. Brashear, A.S. Kirschner, A. Cassidy, J.E. Heubi, Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements, J. Nutr. 131 (2001) 1362S–1375S.
[23] J.B. Howes, L.G. Howes, Content of isoflavone-containing preparations, Med. J. Aust. 176 (2002) 135–136.
[24] X. Xu, K.S. Harris, H.J. Wang, P.A. Murphy, S. Hendrich, Bioavailability of soybean isoflavones depends upon gut microflora in women, J. Nutr. 125 (1995) 2307–2315.
[25] D.C. Knight, J.A. Eden, A review of the clinical effects of phytoestrogens, Obstet. Gynecol. 87 (1996) 897–904.
[26] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (1998) 4252–4263.
[27] C.M. Rassi, M. Lieberherr, G. Chaumaz, A. Pointillart, G. Cournot, Down-regulation of osteoclast differentiation by daidzein via caspase 3, J. Bone Miner. Res. 17 (2002) 630–638.
[28] S. Makela, M. Poutanen, J. Lehtimaki, M.L. Kostian, R. Santti, R. Vihko, Estrogen-specific 17beta-hydroxysteroid oxidoreductase type 1 (EC 1.1.1.62) as a possible target for the action of phytoestrogens, Proc. Soc. Exp. Biol. Med. 208 (1995) 51–59.
[29] A. Krazeisen, R. Breitling, G. Moller, J. Adamski, Phytoestrogens inhibit human 17beta-hydroxysteroid dehydrogenase type 5, Mol. Cell. Endocrinol. 171 (2001) 151–162.
[30] H. Adlercreutz, C. Bannwart, K. Wahala, T. Makela, G. Brunow, T. Hase, P.J. Arosemena, J.T. Kellis Jr., L.E. Vickery, Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens, J. Steroid Biochem. Mol. Biol. 44 (1993) 147–153.
[31] C.J. Kirk, R.M. Harris, D.M. Wood, R.H. Waring, P.J. Hughes, Do dietary phytoestrogens influence susceptibility to hormone-dependent cancer by disrupting the metabolism of endogenous oestrogens? Biochem. Soc. Trans. 29 (2001) 209–216.
[32] G. Schultz, Isoflavone glucoside formononetin-7-glucoside and biochanin A-7-glucoside in *Trifolium pratense* L., Naturwissenschaften 52 (1965) 517.
[33] L.Z. Lin, X.G. He, M. Lindenmaier, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordell, LC–ESI–MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*), J. Agric. Food Chem. 48 (2000) 354–365.
[34] S. Lieberman, A review of the effectiveness of *Cimicifuga racemosa* (black cohosh) for the symptoms of menopause, J. Women's Health 7 (1998) 525–529.
[35] D.J. McKenna, K. Jones, S. Humphrey, K. Hughes, Black cohosh: efficacy, safety, and use in clinical and preclinical applications, Altern. Ther. Health Med. 7 (2001) 93–100.
[36] N. Einer-Jensen, J. Zhao, K.P. Andersen, K. Kristoffersen, *Cimicifuga* and *Melbrosia* lack oestrogenic effects in mice and rats, Maturitas 25 (1996) 149–153.
[37] R. Mentor-Marcel, C.A. Lamartiniere, I.E. Eltoum, N.M. Greenberg, A. Elgavish, Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP), Cancer Res. 61 (2001) 6777–6782.
[38] C.A. Lamartiniere, M.S. Cotroneo, W.A. Fritz, J. Wang, R. Mentor-Marcel, A. Elgavish, Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate, J. Nutr. 132 (2002) 552S–558S.
[39] E. Jisa, E. Dornstauder, S. Ogawa, S. Inoue, M. Muramatsu, A. Jungbauer, Transcriptional activities of estrogen receptor alpha and beta in yeast properties of raloxifene, Biochem. Pharmacol. 62 (2001) 953–961.
[40] C.R. Lyttle, P. Damian-Matsumura, H. Juul, T.R. Butt, Human estrogen receptor regulation in a yeast model system and studies on receptor agonists and antagonists, J. Steroid Biochem. Mol. Biol. 42 (1992) 677–685.
[41] L. Krenn, I. Unterrieder, R. Ruprechter, Quantification of isoflavones in red clover by HPLC, J. Chromatogr. B 777 (2002) 123–128.
[42] A.E. Jones, K.R. Price, R.G. Fenwick, Development and application of a high-performance liquid chromatography method for the analysis of phytoestrogens, J. Sci. Food Agric. 46 (1989) 357–364.
[43] A. Levitzki, in: T.E. Creighton (Ed.), Ligand Binding, second ed., IRL Press, Oxford, 1997, pp. 101–129.
[44] K. Kallela, I. Saastamoinen, E. Huokuna, Variations in the content of plant oestrogens in red clover–timothy-grass during the growing season, Acta Vet. Scand. 28 (1987) 255–262.
[45] E. Dornstauder, E. Jisa, I. Unterrieder, L. Krenn, W. Kubelka, A. Jungbauer, Estrogenic activity of two standardized red clover extracts (Menoflavon(R)) intended for large scale use in hormone replacement therapy, J. Steroid Biochem. Mol. Biol. 78 (2001) 67–75.
[46] A. Jungbauer, V. Beck, Yeast reporter system for rapid determination of estrogenic activity, J. Chromatogr. B 777 (2002) 167–178.
[47] T.T. Song, S. Hendrich, P.A. Murphy, Y. Zhang, G.J. Wang, J.E. Cunnick, Estrogenic activity of glycitein, a soy isoflavone, J. Agric. Food Chem. 47 (1999) 1607–1610.
[48] W. Wuttke, Phytoestrogens for Hormone Replacement Therapy, Pergamon Press, München, 2002.
[49] S.O. Kruse, A. Lohning, G.F. Pauli, H. Winterhoff, A. Nahrstedt, Fukiic and piscidic acid esters from the rhizome of *Cimicifuga racemosa* and the in vitro estrogenic activity of fukinolic acid, Planta Med. 65 (1999) 763–764.
[50] A. Harper, D.J. Kerr, A. Gescher, J.K. Chipman, Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation, Free Radic. Res. 31 (1999) 149–160.
[51] K. Griffiths, L. Denis, A. Turkes, M.S. Morton, Phytoestrogens and diseases of the prostate gland, Baillieres Clin. Endocrinol. Metab. 12 (1998) 625–647.