In vitro estrogenic activities of Chinese medicinal plants traditionally used for the management of menopausal symptoms

C.Z. Zhang a,b, S.X. Wang b, Y. Zhang a, J.P. Chen a, X.M. Liang a,*

a Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Zhongshan Road No. 161, Dalian 116011, PR China
b College of Bio and Food Technology, Dalian Institute of Light Industry, Qinggong-yuan No. 1, Ganjingzi-qu, Dalian 116034, PR China

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Abstract

The estrogenic activity of 70% EtOH extracts of 32 traditional Chinese medicinal plants, selected according to their reported efficacy for the treatment of menopausal symptoms, was assessed using a recombinant yeast system with both a human estrogen receptor expression plasmid and a reporter plasmid. Among them, 11 (34%) species proved to be active. Polygonum cuspidatum had the highest estrogenic relative potency (RP) (3.28 × 10⁻³), followed by Rheum palmatum (3.85 × 10⁻⁴), Cassia obtusifolia (3.49 × 10⁻⁴), Polygonum multiflorum (2.87 × 10⁻⁴), Epimedium brevicornum (2.30 × 10⁻⁴), Psoralea corylifolia (1.90 × 10⁻⁴), Cynomorium songaricum (1.78 × 10⁻⁴), Belamcanda chinensis (1.26 × 10⁻⁴), Scutellaria baicalensis (8.77 × 10⁻⁵), Astragalus membranaceus (8.47 × 10⁻⁵) and Pueraria lobata (6.17 × 10⁻⁵). The EC₅₀ value of 17α-E₂-galactosidase used as the positive control was 0.205 ± 0.025 ng/ml (RP = 100). This study gave support to the reported efficacy of Chinese medicines used for hormone replacement therapy.

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1. Introduction

Hormones such as estrogen and progesterone play a very important role in human growth. It is responsible in regulating the complex cellular events associated with differentiation, function and growth of female reproductive tissues. Women in the menopause have always had to suffer bone density reduction, sweating and anxieties because of a lack of hormones (Harlow and Signorello, 2000). Hormone replacement therapy (HRT) was introduced to improve the menopausal symptoms 20 years ago (Nichols et al., 1984), which quickly took effect but increased the risk of breast cancer (Beral et al., 1999). It was found that natural compounds from certain plants called phytoestrogens could be used for management of menopausal symptoms and have few side effects (Thompson, 1993; Glazier and Bowman, 2001).

Traditional Chinese medicine has been used to heal many diseases for thousands of years and is now well known as natural medicine throughout the world. Many herbal medicines such as Angelica sinensis (Oliv.) Diels, Panax notoginseng (Burk.) F. H. Chen and so on are effective for improving female function according to the oldest traditional Chinese medical book, Sheng-nong Ben-cao Jing. It has been proven that some plant extracts have estrogenic components possessing a potential human use in dietary supplements and treatment of menopausal symptoms (Liu et al., 2001).

In vivo and in vitro assays have been developed to test estrogenic substances. Although in vivo assays are widely used,
2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2) and o-nitrophenol-β-D-pyrogalactoside (oNPG) were purchased from Sigma. Yeast nitro-
gen base without amino acids was purchased from Fluka. All other reagents used in the study were of analytical
grade.

2.2. Plant materials and extraction

A total of 32 Chinese medicinal plants were purchased from Darentang drugstore in Dalian, China, originating from
different regions in China. The plants were identified by Dr. H. Sun, College of Pharmacy, Hei Longjiang University.
Voucher specimens were preserved in College of Bio and Food Technology, Dalian Institute of Light Industry, China.
The voucher numbers are shown in Table 1.

The minced plants (100 g) were extracted with 70% EtOH
and for the samples, it was less than 90 min. The result-
was kindly provided by Prof. W.Z. Wu, Institute of Hydrobi-
ology, Chinese Academy of Sciences. This strain carried the
ER expression plasmid YEPE10 and the estrogen respons-
ive reporter plasmid YRPE2 (Santiso-Mere et al., 1991).
The reporter gene (β-galactosidase) was controlled by ERE. The activity of β-galactosidase resulted in a color reaction,
which was measured absorbance at 420 nm. The absorbance
at 600 nm was selected to measure cell density and viability.
The yeast strain was grown at 30°C, 180 rpm, in selective
medium with 50 μM CuSO4 but without tryptophan and
uracil (Wu et al., 2002a).

2.4. Preparation of test samples

The plant extracts were dissolved in dimethylsulfoxide (DMSO) and used as samples for screening tests. 17β-
Estradiol was dissolved in DMSO and used as positive con-
trol.

2.5. Design of the experiments

The experiments were designed according to Wang
et al. (2003) with some modification. For all experi-
ments, overnight cultures were diluted to OD600 = 1.0
prior to the induction of hER expression and addition of
17β-estradiol (positive control), test samples or DMSO
(negative control). All the samples at concentrations of
0.1–1000 mg/ml (dried extract/ml) and 17β-estradiol at con-
centrations of 0.001–10 ng/ml were prepared in DMSO.
The final concentration of DMSO in the assays was less
than 1.0%. In this test, 5 μl of samples, 17β-estradiol or
DMSO were added to 995 μl of yeast culture containing
50 μM CuSO4, which induced the expression of estrogen re-
ceptor (ER). After incubation at 30°C for 2 h with shaking
(150–180 rpm), the yeast cells were used for β-galactosidase
assays.

2.6. β-Galactosidase assays

For the β-galactosidase assays, 100 μl cell suspensions
were added to the wells of a 96-well microplate. The
cells were permeabilized by addition of 100 μl assay buffer
(60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM
MgSO4, 2 mg/ml oNPG, 38 mM β-mercaptoethanol, 0.01% Triton X-100, 15U/ml lyticase). The microplate was incu-
bated at 30°C until the color became yellow, which resulted
from β-galactosidase cleavage of oNPG. Then, 100 μl of
1 M Na2CO3 was added to stop the reaction. In our test
system, for 17β-estradiol, the incubation time was 50 min,
and for the samples, it was less than 90 min. The result-
ing absorption was measured at 420 nm with a plate reader
(TECAN, Austria). Each test sample and E2 was assayed
in triplicate. E2 as a positive control and DMSO as a
negative control for activity were performed in each test
run.
Table 1
Selected Chinese medicinal plants, extraction yields and estrogenic activity of 70% EtOH extracts assessed by the recombinant yeast bioassay

<table>
<thead>
<tr>
<th>Plant family and species</th>
<th>Chinese name</th>
<th>Plant part</th>
<th>Collection place and voucher number</th>
<th>Extraction yield (w/w)</th>
<th>EC50 (H9262/µg/ml)</th>
<th>RIE</th>
<th>RP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaranthaceae</td>
<td><em>Achyranthes bidentata</em> Bl.</td>
<td>Niuxi Root</td>
<td>Sichuan, DL-A AB318 6</td>
<td>6.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Asteraceae</td>
<td><em>Atractylodes macrocephala</em> Koidz.</td>
<td>Baishu Rhizome</td>
<td>Jiangsu, DL-AAM617 4</td>
<td>4.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Berberidaceae</td>
<td><em>Epimedium brevicornum</em> Maxim.</td>
<td>Yinyanghuo Leaf</td>
<td>Shandong, DL-BEB414 4</td>
<td>3.8</td>
<td>100.0</td>
<td>31.5</td>
<td>2.30 × 10^{-4}</td>
</tr>
<tr>
<td>Cynomorium warneckianum</td>
<td><em>Sanqi</em></td>
<td>Root</td>
<td>Jilin, DL-APG423 6</td>
<td>6.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Convolvulaceae</td>
<td><em>Cuscuta chinensis</em> Lam.</td>
<td>Tusizi Seed</td>
<td>Guangdong, DL-CCC527 0</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dipsacaceae</td>
<td><em>Dipsacus asperoides</em> C. Y. Cheng et T. M. Ai</td>
<td>Xuduan Root</td>
<td>Yunnan, DL-DDA519 2</td>
<td>2.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Eucommiaceae</td>
<td><em>Eucommia ulmoides</em> Oliv.</td>
<td>Duzhong Bark</td>
<td>Jiangsu, DL-EUO624 3</td>
<td>5.4</td>
<td>112.2</td>
<td>37.1</td>
<td>1.78 × 10^{-3}</td>
</tr>
<tr>
<td>Leguminosae</td>
<td><em>Astragalus membranaceus</em> (Fisch.) Bge.</td>
<td>Huangqi Root</td>
<td>Gansu, DL-LAM518 9</td>
<td>9.5</td>
<td>236.1</td>
<td>17.2</td>
<td>8.47 × 10^{-5}</td>
</tr>
<tr>
<td>Liliaceae</td>
<td><em>Polygonatum odoratum</em> (Mill.) Druce</td>
<td>Yuzhu Rhizome</td>
<td>Heilongjiang, DL-LPL515 7.8</td>
<td>120.6</td>
<td>66.5</td>
<td>1.90 × 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>Magnoliaceae</td>
<td><em>Schisandra chinensis</em> (Turcz.) Baill.</td>
<td>Chuanmutong Stem</td>
<td>Sichuan, DL-MRC421 4</td>
<td>3.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Orobanchaceae</td>
<td><em>Cistanche deserticola</em> Y. C. Ma</td>
<td>Roucongrong Stem</td>
<td>Neimenggu, DL-OCD320 15.6</td>
<td>15.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Polygonaceae</td>
<td><em>Polygonatum sibiricum</em> Red.</td>
<td>Huangjing Rhizome</td>
<td>Jiangsu, DL-LPS811 8</td>
<td>8.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rubiaceae</td>
<td><em>Morinda officinalis</em> How</td>
<td>Bajitian Fruit</td>
<td>Guangdong, DL-MRD515 7.8</td>
<td>8.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Scrophulariaceae</td>
<td><em>Rehmannia glutinosa</em> Libosch.</td>
<td>Dihuang Root</td>
<td>Henan, DL-SRG043 8.9</td>
<td>8.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
2.7. Calculation of \( \beta \)-galactosidase activity

The \( \beta \)-galactosidase activity is dependant on the binding of the ligand to the estrogen receptor and was measured according to Miller (1972), using the following formula (Wang et al., 2003):

\[
u = 100\% \left[ \frac{A_{420}(\text{sample}) - A_{420}(\text{blank})}{A_{420}(\text{blank})} \right] \times \frac{S}{t}
\]

where \( u \) is the \( \beta \)-galactosidase activity, \( t \) the incubation time (min) of enzyme reaction, \( A_{420} \) the absorbance of enzyme reaction at 420 nm and \( A_{400} \) the absorbance of the cells of the sample at 600 nm.

2.8. Curve fitting and \( EC_{50} \)

Data derived from transactivation assays were fitted using a four parameters logistic model based on the Marquardt-Levenberg algorithm (SigmaPlot 4.0, SPSS Inc., Chicago, IL, USA) (Rehmann et al., 1999):

\[ Y = \frac{A - D}{1 + \left( \frac{X}{C} \right)^{B}} + D \]

where \( Y \) is the response value (\( \beta \)-galactosidase activity), \( X \) the sample concentration, \( A \) the maximum induction of \( \beta \)-galactosidase activity, \( B \) the relative slope of the middle region, \( C \) the sample concentration when half-maximal response and \( D \) the detection limit. \( EC_{50} \) value is the value of \( C \) in the equation.

2.9. Definition of estrogenic relative potency and relative inductive efficiency (RIE)

In order to compare each assay directly, the relative potency and the relative inductive efficiency are employed. The estrogenic relative potency of samples are computed by dividing the \( EC_{50} \) of 17-\( \beta \)-estradiol by the \( EC_{50} \) of the test samples and then multiplying these values by 100 (the RP value of 17-\( \beta \)-estradiol is 100 in the definition). But it is not enough to evaluate estrogenic activity using RP alone (Coldham et al., 1997). The relative inductive efficiency of \( \beta \)-galactosidase activity could be used for further evaluating estrogenic activity. The RIE is the ratio of maximal \( \beta \)-galactosidase activity of the samples to that of 17-\( \beta \)-estradiol and then multiplying these values by 100 (the RIE value of 17-\( \beta \)-estradiol is 100 in the definition).

3. Results

3.1. Standard dose–response in yeast

To the induced culture 17-\( \beta \)-estradiol was added to reach a final hormone concentration between 0.001 and 10 ng/ml and incubated for 2 h, then the \( \beta \)-galactosidase activity was assayed. The limit of detection was 0.04, the maximum estrogenic activity was 3.80 and the \( EC_{50} \) was 0.205 ± 0.025 ng/ml.

3.2. Estrogenic activities of the selected Chinese medicinal plants

The EtOH extracts of 32 Chinese medicinal plants used to treat menopausal symptoms were assayed for estrogenic activities by a recombinant yeast system. The test samples in DMSO were added to the culture reaching final concentrations between 0.1 and 1000 \( \mu \)g/ml and incubated for 2 h, and then the \( \beta \)-galactosidase activity was assayed.

Table 1 shows that 11 extracts activated the transcription of lacZ. Polygonum cuspidatum, Rheum palmatum, Cassia obtusifolia and Polygonum multiflorum had a higher estrogenic relative potency with RP ranging from 3.28 \( \times \) 10\(^{-3} \) to 2.87 \( \times \) 10\(^{-4} \). Among them, Polygonum cuspidatum had the highest estrogenic potency and was about 100,000 times less estrogenic than 17-\( \beta \)-estradiol (RP of 17-\( \beta \)-estradiol was 100). On the other hand, Belamcanda chinensis, Poriae corylifolia and Polygonum multiflorum had a higher estrogenic relative inductive efficiency with RIE ranging from 83.7 to 52.1 (RIE of 17-\( \beta \)-estradiol was 100). The results indicated their potential efficacy for the treatment of menopausal symptoms.

4. Discussion

The recombinant yeast cells, MCF-7 human breast cancer cells and a prepubertal mouse uterotrophic bioassay have been used for phytoestrogen screening and environmental estrogen assays. The recombinant yeast cell bioassay is approximately two and five orders of magnitude more sensitive to 17-\( \beta \)-estradiol than MCF-7 cells and the uterotrophic assay, respectively (Coldham et al., 1997). So it is thought to be a good method for screening potential estrogens because of its exquisite sensitivity, absence of test compound biotransformation, ease of
use and the possibility of measuring antiestrogenic activity. But the yeast system has a drawback that is the thick cell wall (Zysk et al., 1995). On the one hand, the limited permeability of small molecules might cause the error of assaying estrogenic activity. On the other hand, it blocked the permeation of small molecules might cause the error of assaying estrogenic activity. Further studies will focus on the isolation and identification of active compounds in the active species.

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