Journal of Ethnopharmacology 143 (2012) 109–115

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Er Zhi Wan, an ancient herbal decoction for woman menopausal syndrome, activates the estrogenic response in cultured MCF-7 cells: An evaluation of compatibility in defining the optimized preparation method

Hong Xu [a,c,d], Zi-Ren Su [b], Wen Huang [a,b], Roy Chi-Yan Choi [a], Yu-Zhong Zheng [a], David Tai-Wai Lau [a], Tina Ting-Xia Dong [a], Zheng-Tao Wang [c,d], Karl Wah-Keung Tsim [a,*]

[a] Division of Life Science, Center for Chinese Medicine and State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China
[b] New Drug R&D Center, Guangzhou University of Chinese Medicine, 12 JiChang Road, Guangzhou 510405, China
[c] Key Laboratory of Standardization of Chinese Medicines of Education, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 CaiLun Road, Shanghai 201203, China
[d] Shanghai R&D Centre for Standardization of Chinese Medicines, 199 GuoShouJin Road, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 13 January 2012
Received in revised form
29 May 2012
Accepted 6 June 2012
Available online 16 June 2012

Keywords:
Er Zhi Wan
Ecliptae Herba
Ligustri Lucidi Fructus
Estrogenic activities
Compatibility

ABSTRACT

Ethnopharmacological relevance: Er Zhi Wan (EZW), a Chinese medicinal preparation, has been used clinically for treating menopausal syndrome for its kidney-invigorating function, which contains simply two herbs, Ecliptae Herba (EH) and Ligustri Lucidi Fructus (LLF). Although this herbal extract has been used for many years, there is no scientific basis about its effectiveness on menopausal symptom. Here, we aimed to evaluate the estrogenic activities of EZW and to study the compatibilities of two herbs including different processed-LLF in single and mixed preparation of EZW. Moreover, the weight ratio of EH to LLF in EZW was determined according to their estrogenic activities.

Materials and methods: The extractions of LLF, processed-LLF and EH were prepared separately by extracting the powders with water, 50% alcohol or 95% alcohol. Steamed-LLF and EH were extracted separately, or together, in preparing EZW extracts. A promoter-reporter construct (pERE-Luc) containing three repeats of estrogen responsive elements (ERE) was stably transfected into MCF-7 cells, and this stable breast cancer cell line was used to determine the estrogenic property. The cell proliferation was measured by MTT assay.

Results: The results showed that EZW could significantly induce the expression of luciferase driven by an estrogen responsive element in a pERE-Luc vector. The proliferation of MCF-7 cells was not altered by this herbal treatment. The best preparation of EZW was from: (i) LLF was firstly steamed over water and then dried to make steamed-LLF; and (ii) steamed-LLF and EH were extracted separately by 95% alcohol and then mixed together according to a weight ratio of 1:1.

Conclusions: Under the optimized extracting method, EZW possessed robust effect in activating the estrogenic activity, but which did not alter the proliferation of cultured MCF-7 cells. Thus, EZW is an effective and safe estrogenic herbal extract.

1. Introduction

Menopause is an unavoidable change that every woman has to experience. Women in menopause often suffer from vasomotor symptoms (e.g., hot flashes and palpitations), psychological effects (e.g., depression, anxiety, irritability, mood swing, memory problem and lack of concentration), and atrophic effects (e.g., vaginal dryness and urgency of urination). These disorders are mainly due to the deficiency of ovarian hormones, especially estrogen (Harlow and Signorello, 2000). Hormone replacement therapy (HRT) has been used to treat menopausal symptoms for over 20 years. In spite of this, the majority of people will stop taking the treatment after 2–5 years for the fear of adverse events of HRT, especially having an increased risk of breast cancer and heart disease (Beral, et al., 1999; Timins, 2004). To reduce these problematic side effects, many phytoestrogens from plants, including isoflavonoids, lignans or coumestans, as well as different preparations from traditional Chinese medicine (TCM), are being developed as a new source of estrogen: these phytoestrogens do not simply mimic the effects of human steroidal estrogen but also exhibit similar and divergent actions (Brzezinski and Debi, 1999; Glazier and Bowman, 2001; Geller and Kronenberg, 2003).

From the viewpoint of TCM, the menopausal syndrome is primarily caused by *kidney* deficiency with aging and imbalance

Abbreviations: EZW, Er Zhi Wan; EH, Ecliptae Herba; LLF, Ligustri Lucidi Fructus; TCM, Traditional Chinese Medicine; E_2, 17β-estradiol

* Corresponding author. Tel.: +852 2358 7332; fax: +852 2358 1559.
E-mail address: botsim@ust.hk (K.W.-K. Tsim).

0378-8741/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.06.009

of *yin* and *yang*. If *yin* declines more rapidly than *yang*, the manifestations of over-activity, such as hot flushes, palpitation, insomnia, anxiety and night sweating become prominent. In contrast, depressive mood, dizziness, and fatigue are more of a concern of women having severe *yang* deficiency (Scheid, 2007). Er Zhi Wan (EZW), a TCM herbal formulation, has been developed as a restorative decoction for hundreds of years, which is a simple combination of only two herbs, Ecliptae Herba (EH; the herbs of *Eclipta prostrata* L.) and Ligustri Lucidi Fructus (LLF; the fruits of *Ligustrum lucidum* Ait.). EZW is first described in *FuShouJingFang* by *Wu MinJi* in China during Ming Dynasty (AD 1530). According to Chinese medicinal theory, EZW is used to prevent, or to treat, various *kidney* diseases for its actions of nourishing the *kidney's yin* and strengthening tendon and bone. Pharmacological results indicated that EZW could have the abilities of hepatoprotective (Zheng, et al., 2007), anti-aging (Zhou and Ding, 2008), promoting hematopoietic function (Zhong, 2006; Chen et al., 2007), increasing anti-oxidation activities (Ding et al., 2006) and stimulating immune system (Yao and He, 2006). Nowadays, EZW is commonly used for the treatment of menopausal symptoms in clinical practice (Hu, 1997; Xu, 2005): indeed this is the most frequently prescribed herbal preparation in clinical visits of Taiwan (Chen et al., 2011). However, the effectiveness and action mechanism of EZW, as well as the compatibility rationale, remain to be determined, in particular its association with HRT as a herbal decoction.

In preparing EZW, various processing methods of LLF have been reported: (i) LLF should be soaked by wine according to *FuShouJingFang*; (ii) LLF should be steamed by wine according to *YiFangJiJie* by *Wang Ang* in China during Qing Dynasty; and (iii) LLF should be steamed over the water according to China Pharmacopoeia (2010) today. Thus, this is a puzzle that none of the historical methods have been demonstrated to be biologically superior that others. Owing to the traditional functions of EZW in treating post-menopausal symptoms, the estrogenic activity was employed here to evaluate the biological efficacy of EZW. The classical estrogenic pathway involves the phosphorylation of estrogen receptors and the transcriptional activation of estrogen responsive element (ERE) of target genes. Cultured MCF-7 breast cell is well-known to contain estrogen receptors and to be responsive upon estrogen treatment (Gao et al., 2007; Zhan et al., 2011). In addition, the proliferation of MCF-7 cells could serve as another parameter in revealing the potential risk of developing breast cancer. Thus, this MCF-7-based estrogenic activity assay is a well-accepted method for evaluation of estrogenic properties by phytochemicals (Galluzzo and Marino, 2006; Wanda et al., 2006; Hu et al., 2007), plant extracts (Innocenti et al., 2007; Cherdshewasart et al., 2008; Dall'Acqua et al., 2009), animal products (Mishima et al., 2005) and Chinese medicinal decoctions (Dong et al. 2006; Gao et al., 2007). Here, we employed this in vitro model system to determine the estrogenic activity of EZW by the activation of luciferase that was derived from a luciferase-reporter tagged upstream with an estrogen response element and being stably transfected into cultured MCF-7 cells. In addition, the compatibility principal of EZW of having two distinct herbs was elucidated according to the estrogenic activities.

2. Materials and methods

2.1. Materials

The herbs of *E. prostrate* (EH) and the fruits of *L. lucidum* (LLF) were collected from Qing-Ping Medicinal Market of Guangzhou and identified by Dr. Tina T.X. Dong of the Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China. The corresponding voucher specimens, voucher LLF-S001 for LLF and voucher EH-S002 for EH, were deposited in the Center for Chinese Medicine.

17β-Estradiol (E_2), fetal bovine serum (FBS), 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), G418 and luciferase substrate A or B were purchased from Sigma (St. Louis, MO); modified Eagle's medium (MEM) were obtained from Invitrogen Technologies (Carlsbad, CA); and other cell culture supplements were obtained from Invitrogen or Sigma; the luciferase assay system was obtained from Applied Biosystems (Foster City, CA). Analytical- and HPLC-grade reagents were from Merck (Darmstadt, Germany).

2.2. Processing and chemical analysis of Ligustri Lucidi Fructus

The raw materials of LLF and EH were separately grounded into powders. The processed products of LLF using different methods as follows: (1) Steamed-LLF: the original drug was steamed over boiling water for 4 h, then dried and grounded into powders; (2) Wine-steamed-LLF: the original drug was mixed with rice wine, then which was steamed over boiling water for 4 h, dried and grounded into powders; and (3) Wine-soaked-LLF: the original drug was mixed with rice wine and left overnight, then dried and grounded into powders.

Nuezhenide was from Shanghai R&D Centre for Standardization of Chinese Medicines (Shanghai, China): its purity, confirmed by high-performance liquid chromatograph (HPLC), was higher than 98.0%. Chemical analyses of LLF and processed-LLF were carried out by HPLC method according to reference (Huang et al., 2009): this developed method was validated in the determination of nuezhenide. The HPLC system was an Aglient Technologies Series 1260 consisting of a quaternary pump, continuous vacuum degasser, thermostated autosampler, and column compartment coupled to a variable wave-length diode-array detector. The samples were separated by a Shiseido C18 column (4.6 mm × 250 mm, 5 μm) with acetonitrile (A)- water (B) as mobile phase (0 to 40 min,15% A→40% A), the flow rate was 1.0 ml/min, and the detection wavelength was set at 230 nm.

2.3. Preparation of the herbal extracts

The extractions of LLF, processed-LLF and EH were prepared separately by extracting the powders with water, 50% alcohol or 95% alcohol. For water extraction, 30 g of herb was soaked in 240 ml of water for 2 h, and then which was boiled for another 2 h. For alcohol extraction, 30 g of herb was soaked in 240 ml of alcohol, 50% or 95%, for 2 h, then which was under the reflux for 2 h. The extracts were dried by lyophilization and stored at −4 °C.

Steamed-LLF and EH were extracted separately for single preparation of EZW and extracted together for mixed preparation of EZW. For single preparation of EZW, 30 g of EH and steamed-LLF were separately extracted with 8-volume of 95% alcohol. The two herbal extracts were dried by lyophilization and then mixed well according to different weight ratios of EH to steamed-LLF. For mixed preparation of EZW extracts, EH and steamed-LLF were first mixed well according to the weight ratio of EH to steamed-LLF (total weight is 60 g), then which was extracted with 8-volume of 95% alcohol to obtain the mixed EZW extracts. In order to standardize the herbal extract, the percentage of extractive for each herb was calculated (Supplementary Table). The standardized extracts were stored at −20 °C for all biochemical experiments. In preparing the stock solution before the application to cultures, the water and 50% alcohol extracts were dissolved in PBS, while 95% alcohol extracts was in 0.2% DMSO. The herbal extraction was done on 4 independent batches of herbs, and they were used subsequently for all cell culture studies.