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ScienceDirect
Journal of Ethnopharmacology 107 (2006) 354–360
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Using vaginal cytology to assess the estrogenic activity of phytoestrogen-rich herb

Suchinda Malaivijitnond [a,*], Kullakanya Chansri [a], Pisamai Kijkuokul [b], Nontakorn Urasopon [c], Wichai Cherdshewasart [a]

[a] *Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Phyathai Road, Patumwan, Bangkok 10330, Thailand*
[b] *Basic Science Department, Faculty of Science, Payap University, Chiangmai 50000, Thailand*
[c] *Biological Science Ph.D. Program, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*

Received 9 June 2005; received in revised form 21 February 2006; accepted 23 March 2006
Available online 6 April 2006

Abstract

To assess the estrogenic activities of synthetic estrogen, synthetic phytoestrogen, *Pueraria lobata* and three distinct cultivars of *Pueraria mirifica*, a phytoestrogen-rich herb, a vaginal cytology assay in ovariectomized rats were used. Rats were ovariectomized and treated with DW, estradiol valerate (1 mg/kg BW), genistein (0.25–2.5 mg/kg BW), *Pueraria lobata* and *Pueraria mirifica* (10–1000 mg/kg BW) for 14 days. The vaginal cytology was checked daily and the uteri were dissected and weighed at the end of treatment or post-treatment periods. The treatments of DW, genistein and *Pueraria lobata* did not influence the vaginal epithelium, but the injection of estradiol valerate induced a vaginal cornification from day-3 of treatment to day-14 of post-treatment period. The occurrence of vaginal cornification after treatment and the recovery after the cessation was dependent on dosages and cultivars of *Pueraria mirifica*. The increments of uterus weight in all rats agreed with the cornification of vaginal epithelium. Although both uterotropic and vaginal cytology assays can be used to assess the estrogenic activity of phytoestrogen-rich herb, however, using vaginal cytology assay has two advantages: (1) we do not need to kill the animals and (2) we can follow up the recovery after the cessation of treatment.

Keywords: *Pueraria mirifica*; *Pueraria lobata*; Phytoestrogens; Vaginal cytology assay; Ovariectomized rats

1. Introduction

Nowadays, it has become popular to use phytoestrogens as an alternative choice for estrogen replacement therapy. The biological potencies of phytoestrogens vary greatly and have a stronger binding affinity to ER-β than ER-α. The majority of the compounds are nonsteroidal structures and vastly less potent than synthetic estrogens (10^{-2} to 10^{-5}) (Kuiper et al., 1997, 1998). Phytoestrogens can be found in various species of plants especially from soy and leguminous herb. The *Pueraria* spp. is one among other genera of an indigenous herb that contained phytoestrogens. The *Pueraria lobata* (Leguminosae), also known as Kadzu, is a leguminous plant and possesses a high content of isoflavonoids such as daidzein and genistein (Wang et al., 2003). As regards to the isoflavonoids contents, *Pueraria lobata* slightly increased the uterus weight in ovariectomized (OVX) rats (Wang et al., 2003, 2004) and remarkably increased the bone mineral density in OVX mice (Wang et al., 2003). However, it had neither proliferation nor anti-proliferation effect on growth of MCF-7 in vitro (Cherdshewasart et al., 2004). It is noteworthy that no reports of its estrogenic activity on the vaginal cytology have been found. Therefore, the estrogenic activity of *Pueraria lobata* was assessed using a vaginal cytology assay in comparison to that of synthetic estrogen and synthetic phytoestrogen in the present study.

The *Pueraria mirifica* (Airy Shaw et. Suvatabandhu (Leguminosae)) is a Thai vine herbal plant, known in Thai as "white kwao krua". It was firstly discovered and identified by Vatna in 1939 as "red kwao krua" (*Butea superba* Roxb.) because of their superficial resemblance. Later, in 1952, it was recognized as a new species and reidentified as *Pueraria mirifica* (Kasemsanta et al., 1952). Its tuberous root and the chemical

* Corresponding author. Tel.: +66 2 2185275; fax: +66 2 2185256.
E-mail address: Suchinda.m@chula.ac.th (S. Malaivijitnond).

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doi:10.1016/j.jep.2006.03.026

constituents were analyzed by HPLC technique and at least 13 known phytoestrogens were characterized (Chansakaow et al., 2000a,b). As regards to Thai traditional medicine, the consumption of *Pueraria mirifica* has been believed to improve the human physical appearances such as re-growing hair, promoting black hair, improving flexibility of the body and sexual performance, enlarging breast, recovering smooth skin and prolonging life (Kasemsanta et al., 1952). By this view, *Pueraria mirifica* has recently become popular in Thailand, Korea and Japan as age-rejuvenation and anti-menopausal symptom drugs and a breast enlargement cream. Many researches studied the estrogenic activity of *Pueraria mirifica* on reproductive functions and cell growths were conducted afterwards. The *Pueraria mirifica* stimulated the proliferation of vaginal and uterus epithelium in female rats and women (Sukavattana, 1940; Pope et al., 1958; Malaivijitnond et al., 2004). Feeding a suspension of *Pueraria mirifica* suppressed serum gonadotropin levels in gonadectomized rats (Malaivijitnond et al., 2004) and adult female monkeys (Trisomboon et al., 2004a, 2005). The clinical trial of *Pueraria mirifica* consumption in Thai menopausal women showed a decrease in menopausal symptoms such as hot flushes, frustration, sleep disorder and skin dryness (Muangman and Cherdshewasart, 2001). The plant also decreased serum parathyroid hormone and calcium levels in aged menopausal monkeys (Trisomboon et al., 2004b). *Pueraria mirifica* had a dual effect on the growth of MCF-7 human mammary adenocarcinoma cells, stimulated by low doses and suppressed by high doses (Cherdshewasart et al., 2004).

Pueraria mirifica is commonly found in the forests throughout Thailand and 28 cultivars are found at present. But the plants collected in different seasons induced different responses in vaginal cornification (Sukavattana, 1940). In addition, the analysis of phytoestrogen contents of *Pueraria mirifica* by thin layer chromatography (TLC) techniques showed that *Pueraria mirifica* collected in the different locations in Thailand contained the different amounts of phytoestrogens (Panriansaen, 2000). Based on these evidences, the estrogenic efficacy of drugs or cosmetics containing *Pueraria mirifica* can vary lot by lot even though it contains the same amount of plant. This has been considered as one of the problems for manufacturing *Pueraria mirifica* drugs or cosmetics in the large scale to serve the market demand. Thus, a sensitive, simple and inexpensive method to predict the estrogenic activity of *Pueraria mirifica* is needed. The present study aimed to compare the estrogenic activity of three distinct cultivars of *Pueraria mirifica* collected from different locations in Thailand using a vaginal cytology assay.

2. Materials and methods

2.1. Animals

Adult female Wistar rats, 60 and 100 days of age, with regular estrous cycles (4–5 days) for three consecutive cycles before the study period were used in this study. They were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Five animals per cage were housed in a room with controlled lighting (lights on at 06:00–20:00 h) in which the temperature was maintained at $25 \pm 1\,^{\circ}C$ at the Primate Research Unit, Chulalongkorn University, Bangkok, Thailand. The animals were fed with the rat chow diet (Pokaphan Animal Feed Co. Ltd., Thailand) and water ad libitum. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University.

2.2. Experimental design

2.2.1. Experiment I: comparison of the estrogenic activity of synthetic estrogen, synthetic phytoestrogens and a herb containing phytoestrogens

Fifty adult female rats, 60 days of age and 185–230 g of body weight, were used. When the rats showed the diestrous phase (leukocyte cells) on the fourth estrous cycle, they were ovariectomized under ether anesthesia. The treatment schedule was separated into three periods: pre-treatment, treatment and post-treatment. The duration for each period was 14 days. Five series of experiments were performed. In the first, rats were gavaged daily with 0.7 ml distilled water and kept as a negative control. In the second, rats were subcutaneously injected with 1 mg/kg BW of synthetic estrogen (estradiol valerate; Sigma Chemical Company, Merck, USA) in 200 μl of olive oil, three times a week, and kept as a positive control. In the third, rats were subcutaneously injected with 0.25 and 2.5 mg/kg BWof synthetic phytoestrogen (genistein; Sigma Chemical Company, Merck) in 200 μl of 2% dimethyl sulfoxide (DMSO; Sigma Chemical Company, Merck), three times a week. In the fourth, rats were gavaged daily with *Pueraria lobata* at doses of 10, 100 and 1000 mg/kg BW in 0.7 ml distilled water, respectively. At the end of post-treatment period in these four series, rats were euthanized, the uteri were dissected and weighed, thereafter. In the fifth, rats were treated with distilled water, 1 mg/kg BW of estradiol valerate and 1000 mg/kg BW of *Pueraria lobata* as in the first, second and fourth series, but they were euthanized at the end of treatment period, the uteri were dissected and weighed, thereafter. Vaginal smears were checked daily between 09:00 and 10:00 h in all rats throughout the experimental period. Generally, five rats were used in each group and treatments were performed at 10:00–11:00 h.

2.2.2. Experiment II: comparison of the estrogenic activity of different cultivars of the Pueraria mirifica herb

Fifty adult female rats, 100 days of age and 230–245 g of body weight, were used. When the rats showed the diestrous phase, which was determined by the presence of leukocyte cells, on the fourth estrous cycle, they were ovariectomized under ether anesthesia. The treatment schedule, duration in each period and the number of rats used in Experiment II were the same as in Experiment I. The rats were divided into three main groups and gavaged daily with *Pueraria mirifica* cultivars WichaiIII, Saraburi and Prachuab at doses of 10, 100 and 1000 mg/kg BW in 0.7 ml of distilled water, respectively. The additional negative control group of 0.7 ml of distilled water was also performed. At the end of post-treatment period, all rats were euthanized, the uteri were dissected and weighed, thereafter. Vaginal smears were