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Research on antioxidant effects and estrogenic effect of formononetin from *Trifolium pratense* (*red clover*)

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Abstract

Antioxidant and estrogenic effects of formononetin on ovariectomized mice have been investigated in the present study. The adult female Kunming mice were divided into 5 groups: sham-operated group, ovariectomized group, stilbestrol replacement therapy group (0.20 mg/kg day), low-dose formononetin group (0.05 g/kg day) and high-dose formononetin group (0.5 g/kg day). The mice in the latter 4 groups were ovariectomized. The drug was given by oral administration for 6 months. Estrogenic effect was determined by the change of uterine weight, and oxidant effects were determined by the content of SOD, GSH-Px, CAT and MDA. The intake of formononetin increased the uterine weight of the mice significantly as well as the content of SOD, GSH-Px, CAT, and reduced MDA in body. Formononetin had obvious antioxidant effects and estrogenic effect, and the estrogenic effect was not dosage-related.

Keywords: Formononetin; Isoflavone; Estrogenic effect; Antioxidant effect; *Trifolium pratense* (*red clover*)

Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anions, which are produced as by-products in aerobic organisms, are implicated in oxidative damage to various cellular macromolecules (Farber, 1994). Antioxidant enzymes including superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), etc., constitute an important defense system in clearing up the detrimental ROS *in vivo*. Although almost all organisms possess antioxidant defence and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent the damage. Against this background, evaluation of the antioxidant properties of specific chemical scavengers is of particular importance for their potential use in preventing or limiting the damage induced by free radicals. Several synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are available, but are quite unsafe and their toxicity is a problem of concern (Madhavi and Salunkhe, 1995). Therefore, in recent years, considerable attention has been directed towards the identification of natural antioxidants (plant derived) that may be used for human consumption.

Red clover (*Trifolium pratense* L) is one of several botanical dietary supplements that is being marketed for use in alleviation of hot flashes and other menopausal symptoms (Coon et al., 2007). Commercial extracts of *red clover* contain high amounts of the mildly estrogenic isoflavones daidzein, genistein, formononetin and biochanin A (Rull Prous and Arias, 2004). The general premise supporting the use of *red clover* supplements in menopause is that isoflavones will act as a natural estrogen replacement in older women, and thus alleviate climacteric symptoms.

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Isoflavones are reported to have beneficial estrogenic effects in the treatment of menopausal disorders and cardiovascular diseases and may lower the risk of several cancers (Herman, 1995; Adlercreutz et al., 1992; Herman and Witold, 1997; Reinli and Block, 1996). Soy beans and soy products are known to be the richest sources of the two estrogenic active isoflavones genistein and daidzein (Takamatsu, 2001). However, recent research has shown that *red clover* extracts contain significant high amounts of all the four major estrogenic isoflavones genistein, daidzein, and their methyl ether derivatives biochanin A and formononetin, and indicate the potential application of *red clover* preparations for alternative hormone replacement therapy (HRT) as selective estrogen receptor modulators and in the management of menopause. It is well-known that polyphenolic compounds are responsible for the potential antioxidant activity and radical scavenging capacity of plant food (Kanner et al., 1994; Salah et al., 1995; Vinson and Hontz, 1995). Studies also indicate that isoflavones, especially genistein, like flavonoids posses antioxidant activity (Ruiz-Larrea et al., 1997; Wei et al., 1993). Formononetin is one of the predominant isoflavones in *Trifolium pratense* L. The content of formononetin varies in different parts, among which the highest was in the leaf and the lowest in the flower (Booth et al., 2006). Recently, formononetin extracted from *Trifolium pratense* L has been reported to have weak antioxidant activity (Yu et al., 2005; Corinna and Sabine, 2006), but most of the investigations focused on assays *in vitro*. Few reports investigate the antioxidant activity of formononetin *in vivo*. The metabolites of tritiated formononetin were studied *in vivo* and *in vitro* in sheep (Davies et al., 1989). *In vivo* most of the formononetin was metabolized to equol and 81% of the administered radioactivity was excreted in the urine within 48 h, the main component being equol (Xu et al., 2006). *In vitro* formononetin was converted to equol in rumen liquor from sheep (Davies et al., 1989). Studies showed that equol inhibited LDL oxidation, superoxide anion and H_2O_2 (Hwang et al., 2000; Desiree et al., 1999). In this paper, antioxidant activity *in vivo* and the estrogenic effect of formononetin from *Trifolium pratense* L were studied.

Materials and methods

Materials

Fifty female mature Kunming mice aged 2–3 months and weighing 25–35 g were used in our experiments (all the animals were obtained from Animal Laboratories of Xi'an Jiaotong University, China). All the mice were either ovariectomized (OVX) or falsely operated (SHAM). After surgery, the mice were divided into 5 groups of ten mice each and treated with vehicle alone (Control1: SHAM and Control2: OVX), or stilbestrol (DES: OVX, 0.20 mg/kg body weight), or formononetin (F1, F2: OVX, were administered orally with formononetin at doses of 0.05 and 0.5 g/kg body weight, respectively). Among the 5 groups, OVX, F1 and F2 were used in the assay of detection of ROS, and all the groups were used in the assay of estrogenic effect. After being sacrificed by ether, blood samples were collected and their uteri, livers, hearts and kidneys were removed immediately. All procedures were conducted in strict compliance with applicable laws and regulations as well as with the principles expressed in Guide for the Care and Use of Laboratory Animals of China for the use of experimental animals. The studies conducted on animals were approved by the Animal Care Committee of Xi'an Jiaotong University.

Preparation of formononetin from *red clover* (*Trifolium pratense* L)

Dried *red clover* (*Trifolium pratense* L) was collected from Liuyang, Hunan, China. The collected plant materials were confirmed by Dr. L. Zhou, Professor of medical school, Xi'an Jiaotong University, China. Dried *red clover* (*Trifolium pratense* L) 15 kg from PR China were soaked in 80% ethanol for 24 h and then boiled under reflux for 4 h. After cooling, the extract was filtered using filter paper. The filtrate was extracted by petroleum ether and acetic ether separately. 30 g out of 96 g acetic ether extraction was dissolved in methanol and mixed with gel silica. They were eluted in gel silica with gradient elution of chloroform and methanol (from 100:1 to 1:1). Thin-layer chromatography was used to monitor elute. All elute containing formornonetin were collected and concentrated. The condensate was purified again with silica gel with gradient elution and 4.9 g formononetin was collected after recrystallization.

Measurement of superoxide dismutase activity (SOD)

The tissue to be tested homogenate was prepared (100 mg tissue/ml of 0.9% NaCl). Samples were assayed by measuring the inhibition of NADH oxidation by mercaptoethanol in the presence of EDTA and Mn, following the methods of Paoletti and Mocali (1990). The assays were run by adding the following sequentially to the cuvette: 0.70 ml of 0.1 mM triethanolamine–diethanolamine buffer, 25 μl EDTA-Mn solution, 40 μl NADH solution and 100 μl sample. The reaction was then initiated by adding 200 μl mercaptoethanol solution. Changes in absorbance were measured at 340 nm in a UV spectrophotometer for 16 min; we chose