Biomedical Research 2015; 26 (1): 51-54 *ISSN 0970-938X*
www.biomedres.info

Studies on the extraction process of total flavonoids in *Radix puerariae* and their hypoglycemic effect in mice.

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Abstract

***Radix Puerariae* is the dried root of *Pueraria lobata (Willd.)* Ohwi, which releases a variety of isoflavones. The objective is to investigate the extraction process of total flavonoids in *Radix Puerariae* and to examine their effects on blood glucose MDA in alloxan-diabetic mice. Solvent extraction and column chromatographic isolation were used to extract the total flavonoids in *Radix Puerariae*, and colorimetry was used to determine the content of total flavonoids. Mouse model (persistent hyperglycemia) was established by intravenous injection of alloxan to observe the effects of *Radix Puerariae* total flavonoids in these diabetic mice. MDA level was also determined. Colorimetric determination showed that the content of total flavonoids in *Radix Puerariae*, the main active constituents in the extraction product, averaged 47.65%. Serum glucose level reduced significantly in animals following the use of *Radix Puerariae* total flavonoids of high- and medium-dose.The extraction process of total flavonoids in *Radix Puerariae* and quality control methods established in this study are feasible and cost effective. *Radix Puerariae* total flavonoids have a significant hypoglycemic effect, and can reduce lipid peroxidation.**

Keywords: Total Flavonoids in *Radix Puerariae*, Extraction Process, Hypoglycemic Effect, MDA.

Accepted September 24 2014

Introduction

Radix Puerariae is the dried root of *Pueraria lobata (Willd.)* Ohwi of the family Leguminosae. It has effects of curing fever, facilitating fluid secretion and promoting eruption, and checking diarrhea. *Radix Puerariae* contains a variety of isoflavones including daidzein, daidzin, puerarin, puerarin-7-xyloside, etc. [1], of which puerarin is a special constituent that activates blood circulation, blood stasis dissipation, microcirculation and aldose reductase inhibiting effects.

Polyhydroxy phenolic compounds in *Radix Puerariae* have a hypoglycemic effect, as well as an effect in the prevention of vascular complications of diabetes [2-6]. Clinically, *Radix Puerariae* is often combined with other *TCM (Traditional Chinese Medicine)* to treat diabetes. In this paper, crude *Radix Puerariae* was extracted and purified, and the hypoglycemic effect of total flavonoids extract in *Radix Puerariae* was explored in order to lay the foundation for the clinical application of *Radix Puerariae*.

Materials

Instruments and reagents

UV-160A UV/Vis spectrophotometer, wavelength range: 200~800 nm (Shimadzu, Japan), reflux unit, chromatography nylon PX0608060 - 80 mesh. 5% $NaNO_2$ test solution, 5% Al(NO3)3 test solution, 4% NaOH test solution, all other experimental reagents used were of analytical grade.

Radix Puerarae

Crude *Radix Puerariae* was purchased from a medicinal material company by pharmaceutical department of our college, which was identified as the root of *Pueraria lobata (Willd.)* Ohwi. The National Institute provided Puerarin reference substance for the Control of Pharmaceutical and Biological Products. *Radix Puerariae* total flavonoids; metformin tablets (batch number: 20130201, Liaoning Pharmaceutical Co., Ltd.); alloxan (batch number: 112365, Sigma Company); glucose kit (batch number: 2012223698B, Shanghai Meilun Biotechnology Co., Ltd.); and malondialdehyde (MDA) test kit (provided by the Dalian Liming Bioengineering Institute, batch number: DMA212653).

Animals

Kunming mice, provided by the Laboratory Animal Center of Guangxi Medical University (animal quality certificate No.: BXSC212563). Animals were approved for use in relevant experimental studies by the Chinese Ethics Committee for Laboratory Animals.

Methods

Extraction and refinement of total flavonoids in Radix Puerariae

40g (60 mesh) of herbal powder of *Radix Puerariae* was taken in triplicate in two batches, made lipid free using petroleum ether, extracted with 75% ethanol and then the reflux extract was collected. The extract was allowed to stand overnight, filtered to remove the solvent and concentrated to give an intermediate product of dry extract. The yield was about 10% by weight of original material. The dry extract was reduced to red purple in colour in the ethanol solution by HCl-Mg reaction.

Qualitative reactions with lead acetate, aluminum trichloride, sodium carbonate and ferric trichloride, all showed positive flavonoid reactions on the filter paper. The dry extract was dissolved in hot water, passed through polyamide column chromatography, washed and purified with distilled water - diethyl ether - petroleum ether, and then eluted with 95% ethanol. After removal of ethanol by reduced pressure, it was dried to give a pale yellow flaky solid. The yield was 0.35～0.55% of original materials. The solid extract turned bright yellow-green when dissolved in methanol and its qualitative reaction was consistent with the results of the dry extract.

Content determination

Based on the characteristics of flavonoids contained in the *Radix Puerariae*, the content of flavonoids was determined by colorimetry [7].

Preparation of reference solutions: Appropriate amount (0.1007 g) of puerarin reference substance was precisely weighed, placed in a 100 ml volumetric flask, and ultrasonically dissolved with appropriate amount of methanol, cooled at room temperature, methanol was added to make the volume constant, and shaken uniformly to prepare the reference solution containing 1.007 mg/ml puerarin. 10.0 ml of the above solution was taken, placed in a 100 ml volumetric flask; methanol was added to make the volume constant, and shaken uniformly to prepare the reference solution containing 0.1007-mg/ml puerarin.

Preparation of standard curves

0.1 mg/ml methanol solution was precisely prepared with puerarin as the standard reference, 0.00, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 ml of the solution (each containing 0, 50, 100, 200, 300, 400 and 500 μg) were separately drawn in 10 ml volumetric flasks, diluted to 5.00 ml with 60% ethanol, added with 0.3 ml of 5% NaNO2 test solution, shaken uniformly, allowed to stand for 6 min, then added with 0.3 ml of 5% Al(NO3)3 test solution, shaken uniformly, allowed to stand for 6 min, then added with 4.00 ml of 4% NaOH test solution, diluted to the mark with 60% ethanol, allowed to stand for 12 min, then absorbance was measured at 510 nm with the first tube as a blank control, solution concentration C was regressed by absorbance A, linearity was good within the puerarin concentration range of 5.00~50 μg/ml, which obeyed Beer's law, the regression equation of absorbance and concentration was A = 0.8356C-0.07256, r = 0.999 (n = 6).

Determination of total flavonoids in the sample: About 150 mg of the pale yellow flaky solid sample refined and extracted under section 2.1 was precisely weighed, placed in a 100 ml volumetric flask, diluted to the mark with 60% ethanol to give sample solution. 1.00 ml of the sample solution was precisely pipetted, placed in a 10 ml volumetric flask, and operated according to the procedures in section “Preparation of standard curves” to measure the absorbance.

Blood glucose level in alloxan-induced hyperglycemic mice [8-10]

Kunming male mice, weighing 18-22 g, were injected (iv) with 80 mg/kg/bw alloxan to induce hyperglycemia. On the 4th day, orbital blood sample was collected, centrifuged to obtain serum to measure the blood glucose. Mice with blood glucose greater than 11 mmol/L were divided into 5 groups (n=10 each), namely the model group, metformin-treated group (0.2 g/kg) group, *Radix Puerariae* total flavonoids of high-, medium- and low- dose (4.0g, 2.0g and 1.0g extract/kg, respectively) - treated groups, and the animals of control group. Animals in each group were gavaged once a day for 10 consecutive days. One hour after the last dose, orbital blood sample was collected, centrifuged to obtain serum to measure blood glucose.

Determination of MDA

MDA level was determined by thiobarbituric acid assay according to the kit instructions.

Statistics

Data were processed using SPSS 11.0 statistical software and comparison among groups was performed by one-way ANOVA. P<0.05 was considered statistically significant.

Results

Results for the determination of total flavonoids content in sample

According to the above method, two copies total Puerariae flavones are prepared and measured three times respectively. The value of the total flavonoids in the first and second mean are 48.78 and 46.56. Total content mean (%) was 47.67; RSD (%) was 1.4326. The results are shown in Table. 1. The experiment results show that the content of total flavonoids prepared in *Radix puerariae* gets promotion and the yield is stable and reproducible.

Table 1. *Results showing total flavonoids content in samples (n = 6)*

No.	1	2	3	4	5	6
Total flavonoids in sample	48.56	49.23	48.56	46.32	47.01	46.35
Mean	48.78			46.56		
RSD	0.3004			0.7826		

Total content mean (%) was 47.67; RSD (%) was 1.4326

Table 2. *Effect of Radix Puerariae total flavonoids on blood glucose level in alloxan-induced hyperglycemic mice (x ±s, n=10)*

Group	Dose	Blood glucose level
Control group	Equivalent volume of distilled water	7.2±1.2**
Model group	Equivalent volume of distilled water	23.5±5.6
Metformin group	0.2g/kg	11.0±2.3**
Radix Puerariae total flavonoids groups	4.0g/kg	13±3.6**
	2.0g/kg	15±1.3*
	1.0g/kg	19±1.6

*Note: Compared with the model group, * P<0.05, ** P<0.01.*

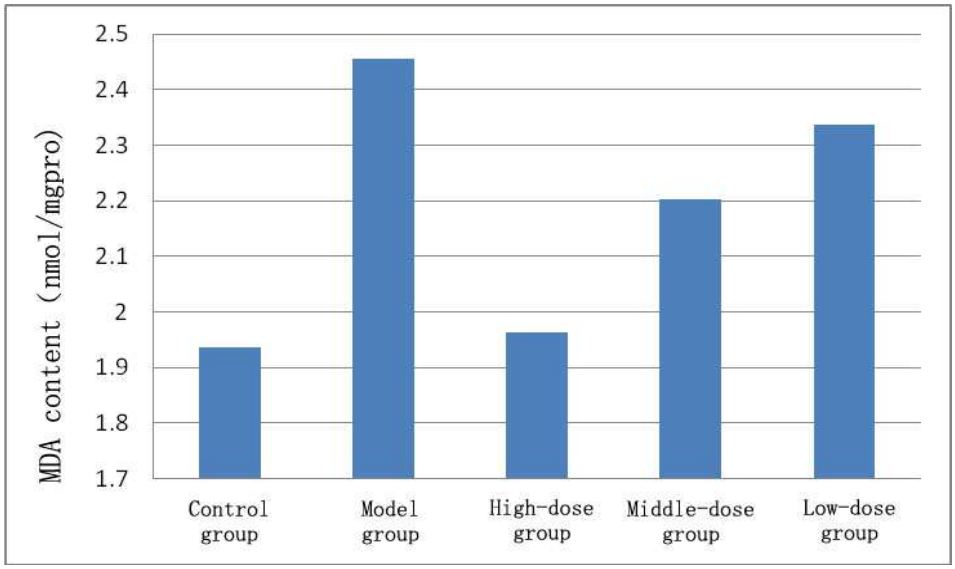

Figure 1. *Effect of Radix Puerariae total flavonoids on blood MDA level.*

Blood glucose level in alloxan-induced hyperglycemic mice

Blood glucose level in the model group was significantly higher than the animals in control group. Compared with the animals in model group, the blood glucose levels were significantly inhibited in animals treated with the *Radix Puerariae* total flavonoids of high- and medium-dose (4.0 and 2.0 g/kg) (P<0.05, P<0.01), Table 2.

Results for determination of MDA*(Fig. 1)*

After gavaging different doses of *Radix Puerariae* total flavonoids for 1 week, blood MDA levels of the treated animals showed a decreasing trend compared with the animals of control group, of which only the difference between the animals treated with high-dose and the controls was shown to be statistically significant (P<0.05).

Discussion

Relatively high purity *Radix Puerariae* total flavonoids were obtained by chemical means. Their content was determined by colorimetry. Total flavonoids had a good linearity within the range of 5.00~50 μg/ml. Content of total flavonoids in the extracted flaky solid reached 47.65%. The non-flavonoid fraction was identified as phenols and saccharides by qualitative reaction, which could hardly be isolated from flavonoids. However, for the mass production, the presences of these constituents are not harmful to the preparations, which can help increase the water solubility of flavonoids as well.

Radix Puerariae has a variety of biological effects; the pharmacodynamic relationship between its action and

polyhydroxy phenolic compounds is worthy of concern. With respect to the treatment of diabetes, Western medicine has a strong and fast-acting hypoglycemic effect, but lacks overall coordination, and has significant side effects [11-12]. The common adverse reactions include hypoglycemia, gastrointestinal symptoms, allergies, and metabolic and nutritional disorders, which are not conducive to long-term use by diabetic patients. Numerous studies of Traditional Chinese Medicine have shown that the treatment of diabetes by Traditional Chinese Medicine is characterized by multi-channel, multi-target and multi-link, which are the reflection of comprehensive therapeutic effects. Most of Traditional Chinese Medicine achieves the therapeutic effects by regulating NO-ET system, significantly improving vascular endothelial function; controlling *ad neuronal* degeneration, inhibiting *Bax expression*, maintaining normal expression of *Bcl-2*; reducing serum *5-HT* level, elevating *β-EP* level, and controlling *5-HT* level. It is thus clear that the prevention and treatment of diabetes by *TCM* have unique characteristics and corresponding mechanism to act [13-15].

Alloxan can cause impaired mRNA function, lead to cell apoptosis, results in decreased blood insulin level and high blood glucose, and form insulin-dependent diabetes (type 1 diabetes). Our experimental results showed that the *Radix Puerariae* total flavonoids of high-dose (4.0g/kg) could significantly reduce the blood glucose in alloxan-induced hyperglycemic animals. This suggests that the preventive and therapeutic effect of *Radix Puerariae* total flavonoids on diabetes is mainly associated with the Produce of new islet β cells. As an end product of lipid peroxidation chain reaction, MDA can reflect the state of lipid peroxidation in the body [16]. The level of MDA can indirectly reflect the severity of free radical attack to cells in the body.

Acknowledgement

The present study was supported by Science and Technology Project of Higher Education of Shandong Province (NO: J12LK05).

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