Antipyretic, Analgesic and Muscle Relaxant Activities of Pueraria Isoflavonoids and Their Metabolites From *Pueraria lobata* Ohwi —a Traditional Chinese Drug

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We evaluated the antipyretic, analgesic, and muscle relaxant activities of Pueraria isoflavonoids and their metabolites in mice. The glycosides daidzin and genistin significantly reduced fever induced by lipopolysaccharide (LPS). Their metabolites, daidzein and *p*-ethylphenol, also significantly reduced fever induced by LPS. In addition, daidzin, daidzein, dihydrodaidzein, and *p*-ethylphenol showed analgesic activity as assessed by the acetic acid-induced writhing test. Furthermore, equol and *p*-ethylphenol showed muscle relaxant activity in the rotarod and horizontal wire test. These results suggest that these compounds play a major role in the therapeutic activity of Pueraria isoflavonoids.

Key words Isoflavonoid; *Pueraria lobata*; antipyretic activity; analgesic activity; muscle relaxant activity; metabolite

Pueraria Root, consisting of the root of *Pueraria lobata* Ohwi (Leguminosae), has been clinically used as an antipyretic and spasmolytic agent in traditional Chinese medicine. Many Chinese herbal formulas contain Pueraria root as their major ingredient; most well-known being "Kakkon-To" (in Japanese), which is indicated for fever and chills with stiffness or rigidity of the neck and upper back. The constituents of Pueraria root have been studied extensively with various isoflavonoids, such as puerarin, daidzin, daidzein, genistin, and genistein, having been identified.[1] The isoflavone *O*-glycoside daidzin inhibits cyclic AMP phosphodiesterase[2] and induces differentiation in murine erythroleukemia cells.[3] The *C*-glycoside puerarin exhibits hypoglycemic activity[4] and increases coronary artery blood flow.[5] The spasmolytic activity of daidzein, the aglycone of daidzin, has been demonstrated by a Magnus experiment using excised murine small intestine.[6] On the other hand, genistein had estrogenic activity,[7] a tyrosine kinase inhibitory action,[8] and a histidine kinase inhibitory action.[9] However, the active compounds in Pueraria root that produce antipyretic activity have not been identified. We previously showed that orally administered glycoside daidzin and genistin were hydrolyzed to the aglycone daidzein and genistein *in vivo*, respectively.[10,11] Furthermore, daidzin or genistein were metabolized to dihydrodaidzein, equol or dihydrogenistein, *p*-ethylphenol through hydrogenation, reduction or ring-fission *in vivo*, respectively.[12,13] Therefore, we investigated the pharmacological relationship between the antipyretic, analgesic, and muscle relaxant activities and chemical components of Pueraria root to identify the active compounds and to elucidate the empirical use.

MATERIALS AND METHODS

Chemicals Daidzin, daidzein, puerarin, genistin, genistein, *p*-ethylphenol and aminopyrine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tween 80 and dantrolene sodium salt were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.). Lipopolysaccharide (bacterial endotoxin) from *Salmonella typhimurium* and methocarbamol were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents used were of analytical grade.

Animals Male ddY mice weighing 22—26 g were obtained from Japan SLC Inc. (Hamamatsu, Japan), maintained in an environmentally controlled room (22±2 °C, 55±10% relative humidity, and 12-h light dark cycle), and given free access to feed and water. They were deprived of feed, but had free access to water, 2 h prior to the experiments.

Preparation of Dihydrodaidzein, Dihydrogenistein and Equol Dihydrodaidzein: The reaction mixture from the hydrogenation of daidzein in EtOH and $Pd/BaSO_4$ under a hydrogen atmosphere was purified by Sephadex LH-20 column chromatography and recrystallized from H_2O–MeOH to give dihydrodaidzein (yield 64%).

Dihydrogenistein: The reaction mixture from the hydrogenation of genistein in MeOH and $Pd/BaSO_4$ under a hydrogen atmosphere was purified by Sephadex LH-20 column chromatography and recrystallized from H_2O–MeOH to give dihydrogenistein (yield 62%).

Equol: The reaction mixture from the hydrogenation of daidzein in AcOH and Pd/C under a hydrogen atmosphere was purified by Sephadex LH-20 column chromatography and recrystallized from H_2O–MeOH to give equol (yield 53%). Identification of these prepared compounds was made by comparing their EI-MS and NMR spectral data with published values.[12,13]

Antipyretic Activity Test Mice with a higher body temperature than normal mice (normothermic) were used 12 h after the subcutaneous injection of LPS (50 mg/kg). Samples were injected i.p., and rectal temperatures were measured hourly. Test samples were suspended in saline with 10% Tween 80. Control mice received only the vehicle, positive controls were treated with aminopyrine, and the experimental groups received 50 and 100 mg/kg of the test compounds. Aminopyrine was used as positive control at a dose of 50 mg/kg (i.p.).

Analgesic Activity Test Test compounds were given i.p. 15 min prior to an i.p. injection of 0.7% acetic acid (0.1 ml/10 g body weight of mouse). The number of squirms was counted in each mouse for 15 min beginning 5 min after

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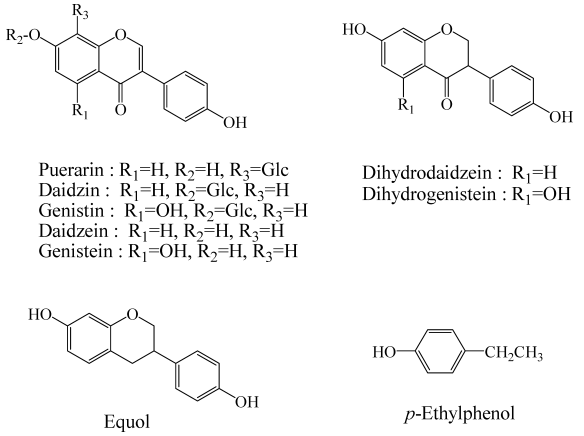

Fig. 1. Chemical Structures of Pueraria Isoflavonoids and Their Metabolites *in Vivo*

Pueraria isoflavonoids: puerarin, daidzin, genistin, daidzein, genistein. Metabolites: dihydrodaidzein, dihydrogenistein, equol, *p*-ethylphenol.

the last injection. Aminopyrine was used as a positive control at a dose of 25 mg/kg (i.p.).

Muscle Relaxant Activity Test Rotarod Test: Mice were placed on a horizontal wooden rod (diameter 3 cm) that was 23 cm above the bench and rotating at a rate of 15 rev min^{-1}. After a preliminary run of naive animals, those that did not remain on the rod for 2 consecutive minutes within a period of 5 min were discarded. Immediately before giving test samples the mice were tested once more and those that did not stay on the rod for 1 min were discarded. The mice were placed on the rotarod at 0, 15, 30, 45, 60 and 75 min after injection. The time taken for each mouse to fall off the rotarod was recorded as the endurance time. If a mouse remained on the rod for more than 10 min, then its endurance time was recorded as 10 min. Total endurance time was calculated during 75 min after test sample administration.

Horizontal Wire Test: Mice were lifted by their tails and allowed to grasp a horizontally strung wire (20 cm high, 1 mm diameter, 15 cm long) with their forepaws and then released. The trials were executed two times at 30 min after injection. The number of animals from a total of ten per treatment group that did not grasp the wire with the forepaws or actively grasp the wire with at least one hind paw within 3 s was determined. In the vehicle treatment group, this number was consistently zero. Methocarbamol and dantrolene sodium salt were used as positive controls at doses of 200 mg/kg (i.p.) and 80 mg/kg (i.p.), respectively.

Statistical Analysis Values are expressed as the mean±S.E.M. except the horizontal wire test. The results were analyzed by analysis of variance (ANOVA), supplemented by Student's unpaired *t*-test for the antipyretic and rotarod tests, Dunnett's test for the horizontal wire test, and LSD-test for the analgesic test. Values with $p<0.05$ were considered significant.

RESULTS

Antipyretic Activity Test The results of the antipyretic effects are summarized in Figs. 2 and 3. Hyperthermia was observed 14 h after LPS injection and continued through out the test. LPS (50 mg/kg, s.c.) significantly ($p<0.01$) increased the rectal temperature compared to the control

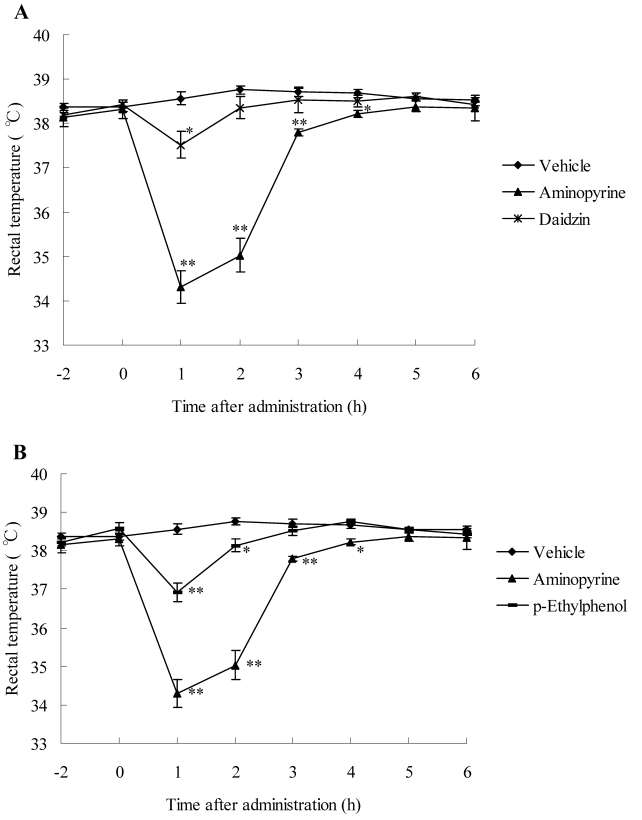

Fig. 2. Antipyretic Effects of Pueraria Isoflavonoid Glycosides (A) and Their Metabolites (B) (50 mg/kg, i.p.) in LPS-Treated Mice

Control means vehicle-treated (10% Tween 80) mice. Each point and vertical bar represent the mean and S.E.M. ($n=6$—8). * $p<0.05$ and ** $p<0.01$ as compared with LPS-treated group. Aminopyrine was used as a positive control at a dose of 50 mg/kg (i.p.).

groups over a 6 h period during the test. Aminopyrine (50 mg/kg, i.p.) reduced rectal temperature after LPS, and the antipyretic effect continued over 4 h with the maximum reduction 1 h ($p<0.001$) after administration. Low doses (50 mg/kg i.p.) of daidzin and *p*-ethylphenol significantly reduced fever induced by LPS at 1 h and 1—2 after administration, respectively (Fig. 2). However, other test compounds had no effect on hyperthermia. Aminopyrine also reduced temperature at high dose (100 mg/kg, i.p.) and the antipyretic effect continued over a 6 h period with the maximum reduction 1 h after administration (Fig. 3). In addition, a high dose (100 mg/kg, i.p.) of both glycoside daidzin and genistin significantly reduced rectal temperature 1 h after administration. Daidzein (100 mg/kg, i.p.), the aglycone and/or hydrolytic metabolite of daidzin, also significantly reduced fever at 1 h and 2 h after administration (Fig. 3). The reduction by *p*-ethylphenol (100 mg/kg, i.p.) was the strongest among the test compounds. The antipyretic effect lasted 4 h, the maximum reduction at 1 h after injection, and its potency was similar to aminopyrine. In contrast, the other test compounds had no antipyretic activity, even at the high dose (data not shown).

Analgesic Activity Test Daidzin, daidzein, dihydrodaidzein, and *p*-ethylphenol significantly inhibited in a dose dependent fashion the writhing induced by 0.7% acetic acid (Fig. 4). Aminopyrine also showed analgesic activity at 25 mg/kg (i.p.). On the other hand, other test compounds were ineffective in the same test.

Muscle Relaxant Activity Test Methocarbamol and

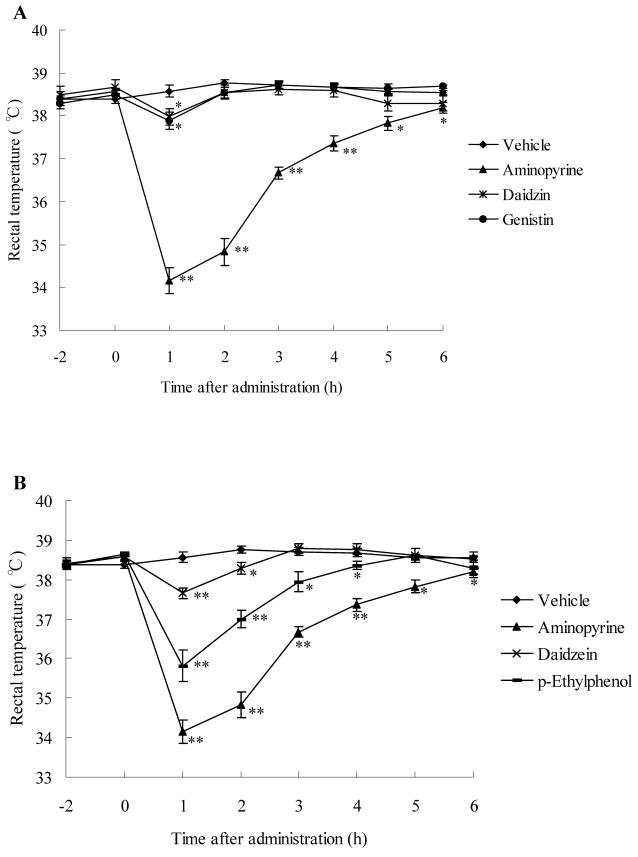

Fig. 3. Antipyretic Effects of Pueraria Isoflavonoid Glycosides (A), Aglycones and Their Metabolites (B) (100 mg/kg, i.p.) in LPS-Treated Mice

Control means vehicle-treated (10% Tween 80) mice. Each point and vertical bar represent the mean and S.E.M. (n=6—8). * p<0.05 and ** p<0.01 as compared with LPS-treated group. Aminopyrine was used as a positive control at a dose of 100 mg/kg (i.p.).

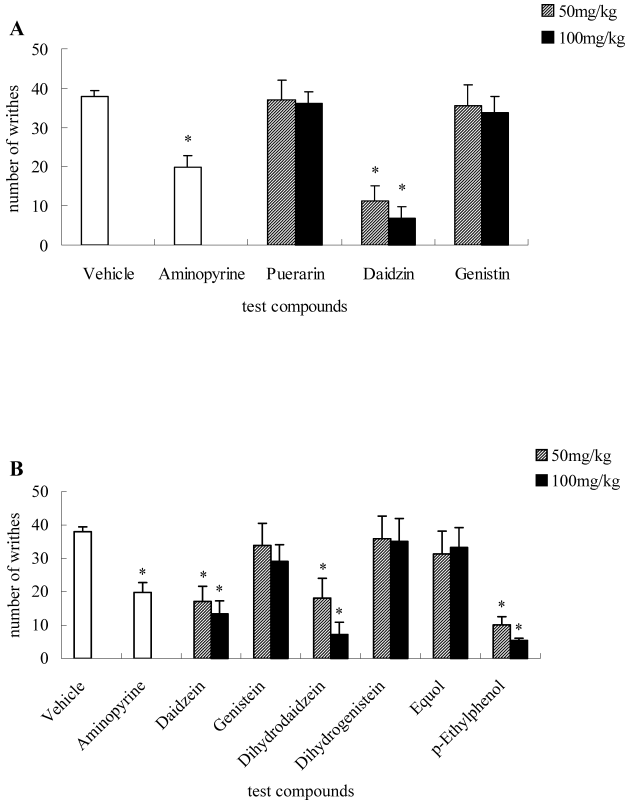

Fig. 4. Analgesic Effects of Pueraria Isoflavonoid (A) and Their Metabolites (B) (50, 100 mg/kg, i.p.) on Acetic Acid-Induced Writhing in Mice

Control means vehicle-treated (10% Tween 80) mice. Each point and vertical bar represent the mean and S.E.M. (n=8—10). * p<0.05 as compared with control group. Aminopyrine was used as a positive control at a dose of 25 mg/kg (i.p.).

dantrolene sodium, which are centrally and peripherally active skeletal muscle relaxants, were used as positive controls. In the rotarod test, the endurance times of positive control-treated mice showed a significant difference between vehicle controls at 15, 30 and 45 min for methocarbamol (200 mg/kg, i.p.) and 15, 30, 45 and 60 min for dantrolene (80 mg/kg, i.p.), respectively (Fig. 5). In addition, a low dose (50 mg/kg i.p.) of each test compound had no muscle relaxant activity (data not shown). However, a high dose (100 mg/kg, i.p.) of equol, the reductive metabolite of daidzin, daidzein and genistein, had significant muscle relaxant activity at 15, 30 and 45 min after administration, and its potency was moderate. Potent muscle relaxant activity was observed *in vivo* with *p*-ethylphenol (100 mg/kg, i.p.), the degraded metabolite of genistein. Muscle relaxant activity was present for 75 min after injection, and the potency was similar to that of the positive control. In contrast, other test compounds had no muscle relaxant activity.

In the horizontal wire test, both positive control drugs significantly reduced the number of mice grasping the horizontal wire (Fig. 6). As expected, equol (100 mg/kg, i.p.) and *p*-ethylphenol (100 mg/kg, i.p.) also significantly decreased the number of mice grasping the horizontal wire. On the other hand, other test compounds were ineffective in this test.

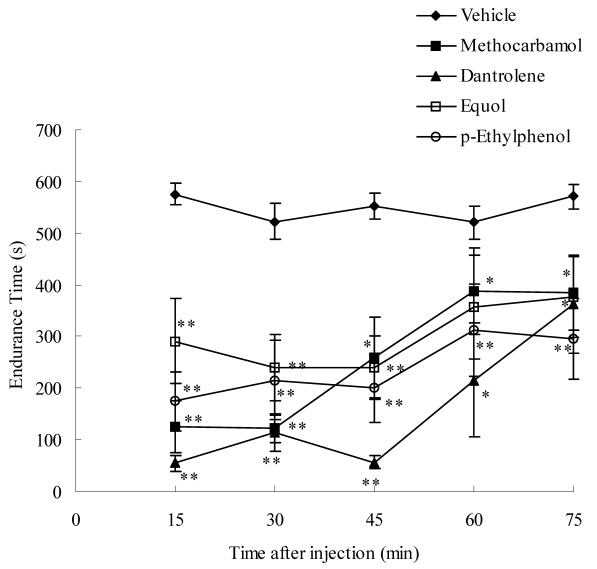

Fig. 5. Time-Course Effects of Muscle Relaxant Activities of Pueraria Metabolites at a Dose of 100 mg/kg i.p. Injection in Rotarod Test in Mice

Muscle relaxation was recorded at 15-min intervals. Each point is the mean±S.E.M. (n=6—9). * p<0.05 and ** p<0.01 were considered significant.

DISCUSSION

Pueraria root is a component of Kakkon-To, which is a traditional Chinese herbal remedy used for treating patients with the common cold or neck and shoulder stiffness/rigidity. Studies have been conducted to elucidate the pharmacologi-

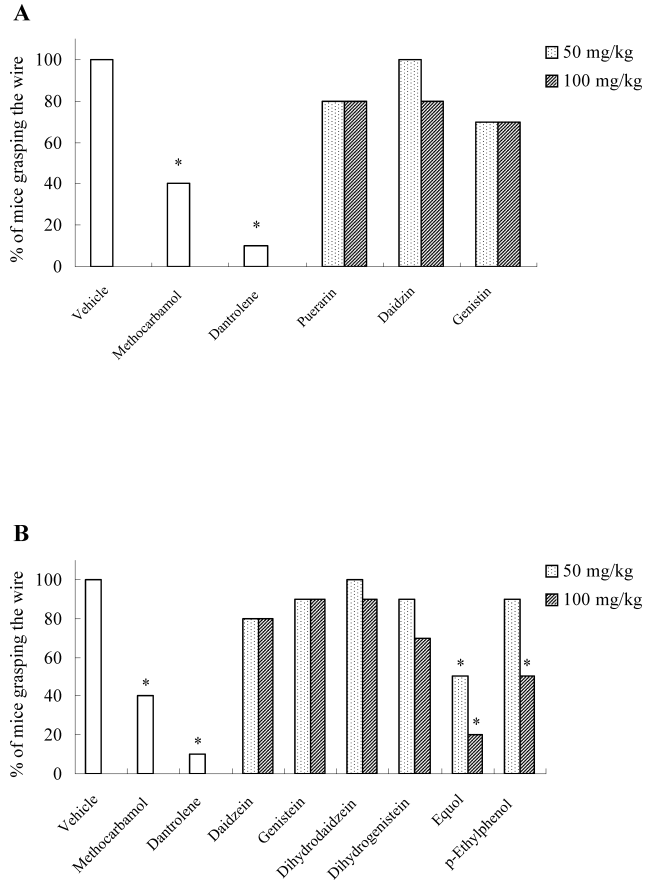

Fig. 6. Performance of Mice in the Horizontal Wire Test 30 min after an i.p. Injection with Pueraria Isoflavonoids and their Metabolites

The trials were executed two times at 30 min after injection ($n=10$). * $p<0.05$, significantly different from controls. Methocarbamol and dantrolene sodium salt were used as positive controls at doses of 200 mg/kg (i.p.) and 80 mg/kg (i.p.), respectively.

cal effects of Pueraria root.[2—6] Recently, Choo *et al.* reported that daidzein, a metabolite of puerarin and daidzin produced by human intestinal microflora, had antithrombotic and antiallergic activities.[14] The antipyretic activity of an extract of Pueraria root was confirmed in LPS-induced hyperthermia rabbits.[15] The spasmolytic activity of daidzein was confirmed by a Magnus experiment using excised small intestine of mice.[6] However, the active components having antipyretic, analgesic, and muscle relaxant activities contained in Pueraria root have not yet been identified.

In previous studies, we identified six metabolites in the urine of rats orally administered Pueraria isoflavonoids.[10—13] These metabolites were daidzein, genistein, dihydrodaidzein, dihydrogenistein, equol and *p*-ethylphenol. We also reported that the glycosides daidzin and genistin were mainly hydrolyzed to the aglycone daidzein or genistein *in vivo*, although the glycosides were major ingredients of Pueraria root.[10,11] Therefore, we expected these metabolites to play an important roles in the antipyretic, analgesic, and muscle relaxant activities of Pueraria root.

In the present study, we demonstrated that daidzin, genistin, daidzein and *p*-ethylphenol reduced fever induced by LPS. In addition, these compounds along with dihydrodaidzein showed analgesic activities. Interestingly, the results also demonstrated that the most potent reduction in hyperthermia was produced by *p*-ethylphenol, the degraded metabolite *in vivo* of genistein and/or genistin. Furthermore, we confirmed that *p*-ethylphenol (50, 100 mg/kg, i.p.) showed a hypothermic effect on basal rectal temperature in normothermic mice, as did aminopyrine (data not shown). According to Kluger[16] and Roth and Zeisberger,[17] LPS produced fever in guinea-pigs and rabbits by stimulating the production of endogenous TNF-α. However, *p*-ethylphenol may not affect TNF-α production but rather inhibit cyclooxygenase to attenuate LPS-induced pyrexia from our experimental data. Our studies indicated that *p*-ethylphenol was the main active component of Pueraria root, and it contributed to the antipyretic effect through metabolism *in vivo*. To the best of our knowledge, this is the first report on the antipyretic effect of *p*-ethylphenol in mice. Previously, *p*-ethylphenol was reported to inhibited prostaglandin E_2 release from mouse peritoneal macrophages *in vitro*;[18] however, no antipyretic or analgesic effects have been reported in mice.

The levels of *p*-ethylphenol in plasma and brain tissue of a premenopausal woman and rats, respectively, following administration of genistein were reported.[19] Other studies,[20] using highly specific and sensitive Tandem mass spectrometry, identified glucuronide and sulfate conjugates of *p*-ethylphenol in the urine of rats after feeding isoflavones. Accordingly, it remains to be determined if the mechanism of action for *p*-ethylphenol at the central level is also involved in the antipyretic effect of this compound.

We also demonstrated that equol and *p*-ethylphenol showed muscle relaxant activity in two animal models (rotarod and horizontal wire tests) that are predictive of muscle relaxant activity in humans. Fukuda *et al*. pointed out that it is preferable to detection the effect of muscle relaxant activity using these two methods.[21]

The most important finding of the present study was that only metabolites showed muscle relaxant activity, but not the original ingredients in Pueraria root, such as daidzin, puerarin and genistin and their aglycones. Accordingly, our studies indicated that equol and *p*-ethylphenol were the main active compounds, and contributed to the muscle relaxant activity of Pueraria root after metabolism. Our study is the first report on the muscle relaxant activity of equol and *p*-ethylphenol in mice, and it remains to be determined if they act centrally or peripherally. Further studies are necessary to elucidate the mechanisms of the muscle relaxant activities of *p*-ethylphenol and equol.

Various traditional herbal medicine formulations are regularly used in clinical practice in Japan and China. The study of the pharmacological activity of a drug will help us better understand its mechanism of action, efficacy and safety. This information provides a scientific explanation for the efficacy of the herbal medicine composite formulations that have been used based only on empirical findings. While it might be difficult to directly correlate these observations with the antipyretic activity of Pueraria root, our results are consistent with the well-known properties of this drug in traditional Chinese medicine that are based on empirical observations.

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