

Investigation of the estrogenic activity of *Pueraria candollei* variety *mirifica* extract on rats

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ABSTRACT

Background: *Pueraria candollei* variety *mirifica* (PM) has been widely used as ingredient in many rejuvenating products. In this study, we aimed to assess the estrogenic activity of PM extract grown in Vietnam.

Methods: Estrogenic activity of PM extract was estimated on immature female rats by using uterotrophic method to measure the weight of the reproductive organs. Estrogenic activity of PM extract also was investigated in mature female ovariectomized rats by evaluating the vaginal cells growth, reproductive organs weight, serum estradiol concentration.

Results: Our results showed that PM extract at doses of 100 mg/kg, 200 mg/kg had increased the reproductive organs weight in immature rats and female ovariectomized rats. In addition, PM extract had increased the serum estradiol concentration and the vaginal cells growth by increasing the percentage of keratinocytes in female ovariectomized rats.

Conclusions: Our results showed that PM extract has strong estrogenic activity in rats.

Keywords: Estrogenic activity, *Pueraria mirifica*, Uterotropic assay, Vaginal cytology assay, Ovariectomized rats

INTRODUCTION

Phytoestrogen is a plant estrogen proven effective, less unwanted effects than estrogen in complementary therapies for women with estrogen deficiency. Therefore, the finding for phytoestrogen-rich medicinal plants is a new direction for scientists. *Pueraria candollei* variety *mirifica* (Shaw and Suvat) Niyomdham (PM) is widely distributed in the highlands of northern Thailand, Myanmar and Vietnam which has been identified in a high-phytoestrogen component.¹ Some phytochemicals

compounds have been identified in PM extract such as miroestrol, deoxymiroestrol, puerarin, daidzin, genistin, daidzein, genistein and isoflavonoid.^{2,3} Miroestrol was the first phytoestrogen isolated from PM, which has the strong estrogenic potency.¹ In traditional medicine, PM has been used for a long time with the purpose of improving tonic, beauty for women. Pharmacological studies have shown that PM has anti-osteoporotic effects, anti-collagenase, anti-elastase and antioxidant and also improving estrogenic activity.⁴⁻⁷ This study was carried out to evaluating the improving estrogenic activity of the

PM extract on immature female rats and female ovariectomized rats.

METHODS

Plant material

Roots of PM planted in Bac Giang province, Vietnam. The sample was identified as *Pueraria candollei* var. *mirifica* (Shaw and Suvat) Niyomdham, Legume (*Fabaceae*). A voucher specimen has been deposited in the Department of Botanicals-Hanoi Pharmacy University, the code number of HNIP/18159/16.

Dried roots of PM were pulverized and were extracted. Water was added to boil for one hour. This step was repeated three times and combined water extract for all steps. The mixture was then filtered and dried at 60°C until it became concentrated extract.

Animals

White rats, 21-22 days old, healthy, recently weaned female wistar strain (immature), weight 60-80 g, and healthy adult wistar strain, 8 weeks old, weight 140-160 g, were provided by the Military Medical University. Animals were maintained accordingly to a protocol approved by the Hanoi Pharmacy University, Hanoi, Vietnam and following the international rules for animal research. Animals were received water ad libitum as vehicle and standard diet administration.

Effect of PM extract on immature female rats

After adaptation for five days, immature female rats were randomly divided into four groups, n=9 for each group.

- Group 1 (normal group): were given orally distilled water of 1 ml/100 g body weight (b.w.).
- Group 2 (control group): were injected with subcutaneous of estradiol at a dose of 10 µg/kg b.w.
- Group 3: were given orally PM extract at a dose of 100 mg/kg b.w.
- Group 4: were given orally PM extract at a dose of 200 mg/kg b.w.

The rats were treated during continuous for 3 days. On the 4th day, sacrificed rats were decapitated under light anesthesia, the reproductive organs were removed (uterines, ovaries, vaginal), weight, then dry at 70°C to constant weight and measure weight.

Wetted weight of reproductive organs was calculated as following:

$$= \frac{\text{The wetted weight of reproductive organs} - \text{The weight of reproductive organs just removed (mg)}}{\text{The weight of the rat (g)}} \times 100$$

Dried weight of reproductive organs was calculated as following:

$$= \frac{\text{The dried weight of reproductive organs} - \text{The weight of reproductive organs after drying (mg)}}{\text{The weight of the rat (g)}} \times 100$$

Percentage of the increased weight of reproductive organs of treated group as compared to normal group was calculated as following:

$$= \frac{\% \text{ Increased} - \text{The weight of reproductive organs of treated group} - \text{The weight of reproductive organs of normal group}}{\text{The weight of reproductive organs of normal group}} \times 100$$

Effect of PM extract on mature female ovariectomized rats

Female rats were bilaterally ovariectomized by surgical to remove the ovaries, under light ether anesthesia. Healthy ovariectomized rats were maintained after 14 days of removing ovaries. Microscopic examination of vaginal epithelial cells assessing the presence of epithelial cells was conducted to confirm the ovarian removed successfully. Then rats were randomly divided into 4 groups, n=9 for each group:

- Group 1 (control): Ovariectomized rats were given drinking distilled water with a volume of 1 ml/100 g b.w.
- Group 2: Ovariectomized rats were subcutaneously injected estradiol at 2 mg/kg b.w.
- Group 3: Ovariectomized rats were orally administered at a dose of PM extract 100 mg/kg b.w.
- Group 4: Ovariectomized rats were orally administered at a dose of PM extract 200 mg/kg b.w.

Rats were treated continuously during 14 days at 10 am every day. Then vaginal cell growth, increasing weight of reproductive organs, blood estradiol concentration were evaluated.^{8,9}

Evaluating the-growth of the vaginal cells

From the day of treatment vaginal fluids were taken daily, and calculated the percentage of keratinocytes. Vaginal fluids were taken at 9 am every day by pipette 100 µl of distilled water into vaginal and then take 25 µl, stained within 1 minute with 0.5% methylene blue solution. Counted the number of neutrophils, epithelial cells and keratinocytes. Calculation of the percentage of keratinocytes according to the formula as

$$\% \text{ Keratinocytes} = \frac{\text{Number of keratinocytes}}{\text{Number of neutrophils} + \text{Number of epithelial cells with nucleus} + \text{Number of keratinocytes}} \times 100$$

Evaluating on the weight of reproductive organs

On the 15th day, rats were sacrificed by decapitation under light anesthesia, the reproductive organs were removed (uterines, ovaries, vagina), weighed, then dried at 70°C to constant weight and measure weight.

Wetted weight of reproductive organs was calculated as following:

$$= \frac{\text{The wetted weight of reproductive organs} - \text{The weight of reproductive organs just removed (mg)}}{\text{The weight of the rat (g)}} \times 100$$

Dried weight of reproductive organs was calculated as following:

$$= \frac{\text{The dried weight of reproductive organs} - \text{The weight of reproductive organs after drying (mg)}}{\text{The weight of the rat (g)}} \times 100$$

Percentage of increased weight of reproductive organs of treated group as compared to normal group was calculated as following:

$$\% \text{Increased} = \frac{\text{The weight of reproductive organs of treated group} - \text{The weight of reproductive organs of normal group}}{\text{The weight of reproductive organs of normal group}} \times 100$$

Estimating the blood estradiol concentration

Before taking blood, mice were allowed to starve for 12 hours. On the 15th day, before sacrificing rats, take blood and then blood was centrifuged at 3000 rpm for 10 min at 4°C. The serum was used to quantify estradiol level using Estradiol ELISA kit (Eagle Biosciences) according to the manufacturer's protocols. Estradiol level was expressed as pg/ml.

Statistical analysis

All results are expressed as mean±SEM. Serial measurements were analyzed by using one-way ANOVA with Tukey's post hoc test using SigmaStat 3.5 program and figures were performed by using SigmaPlot 10.0 program (Systat Software Inc). The critical significance level α was 0.05, and statistical significance was defined as $p < 0.05$.

RESULTS

The effect of PM extract on estrogenic activity on immature female rats

Estrogen is a female sex hormone that plays a role in developing and maintaining female characteristics, developing the uterus, ovary, vagina, mammary gland. Compounds with estrogenic activity may increase these weights. Therefore, we can use the weight of reproductive organs (uterines, ovaries, vaginal) to demonstrate the estrogenic activity.

Effect of PM extract on the uterine's weight

The effect of extract PM on the uterine growth of immature female rats was shown in Table 1.

Wetted uterine's weight

The rats were treated with the PM extract at dose of 100 mg/kg and 200 mg/kg showed an increasing wet uterine's weight significantly as compared with group 1 ($p < 0.05$).

The percentage increase was 91.8%; 56.7% respectively. There were no differences between the doses of PM extract ($p > 0.05$).

Table 1: Effect of PM extract on the uterine's weight of immature female rats.

	Wetted weight uterine (mg/100 g b.w.)	% increase compared to group 1	Dried weight uterine (mg/100 g b.w.)	% increase compared to group 1
Group 1	120.9±10.8		25.2±2.6	
Group 2	297.4±31.2*	146.0	46.5±1.5*	84.5
Group 3	231.9±3.2*	91.8	38.6±3.7*	53.2
Group 4	189.4±16.5*	56.7	36.4±1.7*	44.4

*: Significantly different from group 1 ($p < 0.05$).

Table 2: Effect of PM extract on the ovarian's weight of immature female rats.

	Wetted weight ovarian (mg/100 g b.w.)	% increase compared to group 1	Dried weight ovarian (mg/100 g b.w.)	% increase compared to group 1
Group 1	48.0±4.0		10.4±1.0	
Group 2	73.5±2.6*	53.1	14.8±1.5	42.3*
Group 3	62.7±2.9*	30.6	11.3±0.6	8.6
Group 4	57.4±2.2*	19.6	12.5±1.5	20.2*

*: Significantly different from group 1 ($p < 0.05$).

Dried uterine's weight

The rats were treated with the extract of PM at doses of 100 mg/kg and 200 mg/kg also showed an increasing significantly effect on the dried uterine's weight as compared with group 1 (p<0.05). The percentage increase was 53.2%; 44.4% respectively. There were no differences between doses (100 mg/kg and 200 mg/kg) on dried uterine's weight (p>0.05).

Effect of PM extract on the ovarian's weight

The effect of the extract PM on ovarian's weight of immature female rats is shown in Table 2.

Wetted ovary's weight

The rats were treated with the extract of PM at doses of 100 mg/kg and 200 mg/kg showed an increasing wetted weight ovary significantly as compared with group 1 (p<0.05). The percentage increase was 30.6% and 19.6% respectively.

Dried ovary's weight

The results showed that estradiol and the extract of PM at dose of 200 mg/kg showed an increasing significantly effect on ovarian growth as compared with group 1 (p<0.05).

Table 3: Effect of PM extract on the vaginal's weight of immature female rats.

	Wetted weight vaginal (mg/100 g b.w.)	% increase compared to group 1	Dried weight vaginal (mg/100 g b.w.)	% increase compared to group 1
Group 1	54.9±7.0		14.7±2.6	
Group 2	195.9±5.7*	92.9	20.8±1.1*	41.5
Group 3	94.1±7.1*	71.4	17.6±1.5*	19.7
Group 4	102.9±4.6*	87.4	20.0±2.3*	36.1

*: Significantly different from group 1 (p<0.05).

Effect of PM extract on the vaginal's weight

The effect of the PM extract on the vaginal's weight in immature female rats is shown in Table 3.

Wetted vaginal's weight

The rats were treated with the extract of PM at doses of 100 mg/kg and 200 mg/kg showed an increasing wetted vaginal's weight significantly as compared with group 1 (p<0.05). The percentage increase was 71.4%; 87.4% respectively. Among doses of 100 mg/kg, 200 mg/kg, there was no difference on wetted vaginal's weight (p>0.05).

Dried vaginal's weight

The extract of PM at two doses showed a statistically significant effect on dried vaginal's weight as compared with group 1 (p<0.05).

The effect of PM extract on estrogenic activity in mature female ovariectomized rats

The removal of the ovaries leads to the function of the hypothalamus-pituitary-ovary is disrupted, and then the production of estrogen from the ovaries is inhibited, resulting in low levels of endogenous estrogen associated with the weight of secondary reproductive organs decreases. Therefore, female ovariectomized rats model is excluded the effect of endogenous estrogen on the change of the uterine and vagina. Then, the evaluation of the effect of enhancing estrogenic activity of exogenous samples is more accurately.

Substances with estrogenic activity stimulate the growth of vaginal epithelial cells; differentiate these cells into keratinocytes, leading to increased number of keratinocytes. Therefore, assessing the presence of keratinocytes helps to assess the estrogenic activity of the substances. The percentage of keratinocytes of mature female ovariectomized rats was shown in Figure 1. The vaginal fluid of rats treated with the PM extract at a dose of 100 mg/kg and 200 mg/kg at the fourth day contains mainly keratinocytes, the percentage of keratinocytes on the fourth day was 75.66% and 87.34%, respectively.

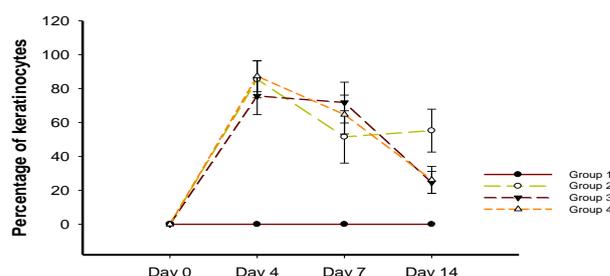


Figure 1: Effect of the PM extract on the percentage of keratinocytes of mature female ovariectomized rats.

The effect of PM extract on the growth of reproductive organs

Effect on uterine's weight

The effect of the PM extract on uterine's weight of mature ovariectomized rats was shown in Table 4 and Figure 2.

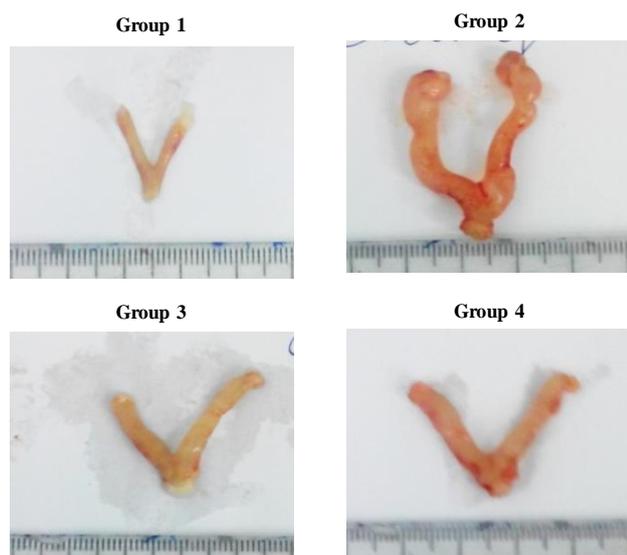


Figure 2: The effect of PM extract on uterine of mature ovariectomized rats.

Wetted uterine's weight

The rats were treated with the PM extract at both doses of 100 mg/kg and 200 mg/kg showed an increasing wetted uterine's weight, significantly as compared with group 1

($p < 0.05$). The percentage increase was 251.9% and 215.2%, respectively. There were no differences between effective doses (100 mg/kg, 200 mg/kg) on wetted uterine's weight of the studied animals ($p > 0.05$).

Dried uterine's weight

The rats were treated with the PM extract at both doses of 100 mg/kg, 200 mg/kg showed significantly increasing dried uterine's weight as compared with group 1 ($p < 0.05$). The percentage increase was 281.9% and 261.1%, respectively. There were no differences between effective doses (100 mg/kg, 200 mg/kg) on dried uterine's weight of the studied animals ($p > 0.05$).

Effect on vaginal's weight

The effect of the PM extract on vaginal's weight of mature ovariectomized rats is shown in Table 5.

Wetted vaginal's weight

The rats were treated with the PM extract at doses of 100 mg/kg and 200 mg/kg have a statistically significant increase wetted vaginal's weight as compared to the group 1 ($p < 0.01$). The percentage increase was 62.3% and 78.7%, respectively.

Table 5: Effect of PM extract on the vaginal's weight of mature female ovariectomized rats.

	Wetted weight vaginal (mg/100 g b.w.)	% increase compared to group 1	Dried weight vaginal (mg/100 g b.w.)	% increase compared to group 1
Group 1	61.0±6.0		11.9±1.3	
Group 2	107.0±12.0*	75.4	22.3±2.4*	87.4
Group 3	99.0±8.0*	62.3	29.3±7.2*	146.2
Group 4	109.0±7.0*	78.7	20.2±1.9*	69.8

*: Significantly different from group 1 ($p < 0.05$).

Table 6: Effect of PM extract on the serum estradiol concentration of mature female ovariectomized rats.

	Estradiol (pg/ml)	% increase compared to group 1
Group 1	27.54±11.19	
Group 2	8852.33±199.44*	320.43
Group 3	84.57±17.73*	207.08
Group 4	92.41±31.87*	235.54

*: Significantly different from group 1 ($p < 0.05$).

Dried vaginal's weight

The rats were treated with the PM extract at doses of 100 mg/kg and 200 mg/kg showed strong effect on increasing dried vaginal's weight as compared to group 1 ($p < 0.01$). The percentage increase was 146.2% and 69.8%, respectively.

The effect of PM extract on serum estradiol concentration

The PM extract at both doses of 100 mg/kg and 200 mg/kg increased the concentration of estradiol as

compared to group 1 ($p < 0.05$) with the percentage increase was 207.08% and 235.54% respectively (Table 6).

DISCUSSION

Estrogen is a hormone that stimulates cell division and growth of some tissues in female mammals such as the uterus, cervix and vagina.¹⁰ Therefore, in order to evaluate the effect of PM on initial estrogenic activity, we evaluated on immature rats through the growth of reproductive organs including uterines, ovaries, vagina

and mature female ovariectomized rats.¹¹ Our data showed that PM extract increased wetted and dried uterine weight of immature rats at doses of 100 and 200 mg/kg b.w. This increase may be due to PM increases fluid retention and electrolytes and stimulates the growth of uterine tissue. We also showed that PM extract increased wetted and dried vaginal's weight in immature rats. This shows that PM extract also enhances the development of vaginal muscle tissue, which is the characteristic of estrogenic activity.

The removing ovary cause to loss the function of the hypothalamus-pituitary-ovary, inhibit to produce estrogen from the ovaries, lead to the reduction of endogenous estrogen concentration circulating in the blood and the weight of reproductive organs.¹² Therefore, changes in weight of the uterine and vagina due to the effect of endogenous estrogens are excluded in the mature female ovariectomized rat's model. Using mature female ovariectomized rats is accurately method to evaluate the estrogenic activity of exogenous substances.¹³ The changes on the reproductive organs are mainly influenced by experimental substances. In this study, we evaluated the estrogenic effect of PM extract on mature female ovariectomized rats at two doses of 100 and 200 mg/kg b.w. on vaginal epithelial cell growth, the weight of reproductive organs including uterines, vagina and serum estradiol concentration. Our data showed that PM extract could induce the appearance of keratinocytes about 2-3 days after treatment. By day fourth, mainly keratinocytes presented in vaginal fluids. This results confirmed that PM extract had estrogenic activity through the effect of the vaginal epithelial cells converted into keratinocytes.^{6,14} We also showed that PM extract at doses of 100 mg/kg and 200 mg/kg increased both wetted and dried weight of reproductive organs including uterines and vagina significantly as compared with control group. Our results are agreed with previous studied such as Malaivijitnond and colleagues.¹⁴ These authors have assessed the estrogenic activities of three distinct cultivars of PM. Their data have shown that PM could increase the uterine weight and cornification of vaginal epithelium in manner dose-dependent. Similar, Cherdshewasart and colleagues also showed the estrogenic activity of PM depends on seasonal changes and plant cultivars.¹⁵ They also showed the PM at 1 µg/ml exhibited significant breast cancer cell line MCF-7 proliferation similar to 17 beta-estradiol.¹⁶ Furthermore, we showed that PM at dose of 100 and 200 mg/kg increased serum estradiol concentration in mature female ovariectomized rats as compared to the control group ($p < 0.05$).

CONCLUSION

In this study we demonstrated that the PM extract had estrogenic activity in immature female rats by increasing reproductive organs weight such as uterine, ovarian and vaginal. Furthermore, the PM extract also increased the reproductive organs weight in mature female

ovariectomized rats, increasing vaginal epithelial cells growth and increasing serum estradiol levels. Our study showed that PM could be useful as an alternative for hormone replacement therapy.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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