RESEARCH ARTICLES

Chronic Toxicity of *Pueraria mirifica* in Rats

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Abstract

Pueraria mirifica Airy Shaw and Suvatabandhu or White Kwao Keur has been used in folk medicine as a rejuvenating agent for the elderly. To date, toxicological data of this plant are still incomplete and therefore the six-month chronic toxicity study was undertaken. Wistar rats of each sex were orally administered *Pueraria mirifica* powder (PM) at the doses of 10, 50 and 250 mg/kg/day whereas the control group received water at 10 ml/kg/day for 6 months. The significantly decreased body weights were observed in both male and female rats receiving PM at the doses of 50 and 250 mg/kg/day. Food consumptions were suppressed in PM-treated male rats at the doses of 50 and 250 mg/kg/day and also in the highest dose-treated female rats. During the experiment, some rats receiving PM had alopecia for two weeks and then recovered. Hematology revealed that PM at the dose of 250 mg/kg/day produced significant decreases of hematocrit, RBC and hemoglobin in both sexes of rats. Triglyceride levels in the female rats treated with 250 mg/kg/day of PM were significantly increased. Cholesterol levels were significantly decreased in male rats receiving PM at the doses of 50 and 250 mg/kg/day and in female rats at the highest dose. The testicular weight of male rats receiving the highest dose of PM was significantly decreased whereas the uterine weight of female rats receiving this dose was significantly increased. Histopathological examinations of visceral organs revealed no changes related to the toxicity of *P. mirifica* except that male rats receiving the highest dose of PM had significantly higher incidence of kidney tubular cysts. Results of the study indicated that prolonged administration of 10 mg/kg/day *P. mirifica* did not cause any hematological and biochemical alterations. Nor did pathology of the internal organs indicating the toxicity at this dose.

Key words : *Pueraria mirifica*, toxicity, White Kwao Keur

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พิษเรื้อรังของกวาวเครือขาวในหนูขาว

ทรงพล ชีวะพัฒน์, ปราณี ชวลิตธำรง, สดุดี รัตนจรัสโรจน์, สมเกียรติ ปัญญามัง

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บทคัดย่อ

กวาวเครือขาวเป็นสมุนไพรพื้นบ้านที่ใช้เป็นยาบำรุงสุขภาพในผู้สูงอายุ เนื่องจากข้อมูลด้านพิษวิทยาของสมุนไพรชนิดนี้ยังไม่สมบูรณ์ คณะผู้วิจัยจึงได้ทำการศึกษาพิษเรื้อรังของหัวกวาวเครือขาวในหนูขาวพันธุ์วิสตาร์ เป็นระยะเวลา 6 เดือน โดยป้อนผงกวาวเครือขาวแขวนตะกอนในน้ำแก่หนูขาวในขนาด 10, 50, และ 250 มก./กก./วันทุกวัน เปรียบเทียบกับกลุ่มควบคุมที่ได้รับน้ำ 10 มล./กก./วันเป็นเวลานาน 6 เดือน พบว่าหนูเพศผู้และเพศเมียที่ได้รับกวาวเครือขาวขนาด 50 และ 250 มก./กก./วัน มีน้ำหนักตัวต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ หนูเพศผู้ที่ได้กวาวเครือขาวขนาด 50 และ 250 มก./กก./วันและเพศเมียที่ได้รับกวาวเครือขนาด 250 มก./กก./วัน กินอาหารได้น้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ หนูที่ได้รับกวาวเครือขาวขนาด 250 มก./กก./วัน บางตัวมีอาการขนร่วงนานประมาณ 2 สัปดาห์ต่อมาจะดีขึ้นจนปกติ ผลทางโลหิตวิทยาแสดงให้เห็นว่า หนูขาวที่ได้รับกวาวเครือขนาดสูงทั้งสองเพศมีค่าฮีมาโตคริต จำนวนเม็ดเลือดแดงและปริมาณฮีโมโกลบินลดลงอย่างมีนัยสำคัญ ระดับไขมันไตรกลีเซอไรด์ของหนูเพศเมียที่ได้รับกวาวเครือขาวขนาด 250 มก./กก./วันเพิ่มขึ้นอย่างมีนัยสำคัญ กวาวเครือขาวขนาด 250 มก./กก./วัน ทำให้น้ำหนักอัณฑะของหนูเพศผู้ลดลง แต่น้ำหนักมดลูกของหนูเพศเมียเพิ่มขึ้น ผลการตรวจเนื้อเยื่ออวัยวะทางจุลพยาธิวิทยาไม่พบการเปลี่ยนแปลงของอวัยวะภายในต่าง ๆที่มีความสัมพันธ์กับความเป็นพิษของกวาวเครือขาวยกเว้นไตของหนูเพศผู้ที่ได้รับกวาวเครือขนาดสูงมีอัตราการเกิด tubular cyst เพิ่มขึ้นอย่างมีนัยสำคัญ ดังนั้นจากการศึกษาพิษเรื้อรังครั้งนี้ สรุปได้ว่าเมื่อให้กวาวเครือขาวขนาด 10 มก./กก.นานติดต่อกัน 6 เดือน ไม่ทำให้เกิดการเปลี่ยนแปลงของค่าทางโลหิตวิทยา ค่าทางชีวเคมีของซีรั่ม และพยาธิสภาพของอวัยวะภายในต่าง ๆ ที่บ่งชี้ถึงความผิดปกติอันเนื่องมาจากกวาวเครือขาว

คำสำคัญ : *Pueraria mirifica*, toxicity, White Kwao Keur

Introduction

Pueraria mirifica Airy Shaw and Suvatabandhu or White Kwao Keur[1] is a Thai indigenous woody climber belonging to the family Leguminosae.[2] Tuberous roots of *P. mirifica* were traditionally used by Thai people as a rejuvenating drug.[3] Phytochemical studies have shown that the tuberous root contains various compounds such as miroestrol,[4] puerarin,[5] coumestrol, daidizin, daidzein, mirificin,[6] mirificoumestan,[7] genistin[8] and kwakurin.[9] Many of these substances are classified as phytoestrogens. Recently, deoxymiroestrol has been isolated from roots of *P. mirifica* and shown to possess stronger estrogenic effect on MCF-7 human breast cancer cells than miroestrol.[10]

Several pharmacological studies of *P. mirifica* mostly concerned with its estrogenic-like activities in animals. *P. mirifica* could inhibit courtship, mating behavior and testicular development in male pigeon whereas in female it suppressed egg laying by inhibition of follicular development.[11] Experiments conducted in female rats have shown that White Kwao Keur suppresses lactation by inhibiting mammary gland growth and milk production.[12] This herb exhibited effective postcoital antifertility in rats and increased the uterine weight as well as fluid content in the uterus of immature ovariectomized rats.[13] In male rats, *P. mirifica* reduced reproductive behavior and caused weight reduction of testis, epididymis, prostate gland and seminal vesicles.[14]

Toxicity of *P. mirifica* tuberous root has been investigated in some experimental animals. In Japanese quail, this herb affected hemopoietic systems i.e. decrease hematocrit, hemoglobin and red blood cells.[15] It also caused suppurative inflammation in some parts of the body.[16] Hepatic lesions and degeneration of testicular leydig cells was reported in rats orally treated with 100 mg/kg of *P. mirifica* for 14 consecutive days.[14] Subchronic toxicity study at the doses range between 10-1,000 mg/kg for a period of ninety days in Wistar rats showed that the highest dose affected hematological values and no estrogenic effect was observed at the lowest dose.[17] Since the currently available toxicological data of this plant are still incomplete to evaluate the safety of this plant, therefore Medicinal Plant Reseach Institute, Department of Medical Science conducted chronic toxicity study of *P. mirifica* tuberous root so as to provide additional toxicological information in order to ascertain the safety of using this plant.

Materials and Methods

Preparation of *Pueraria mirifica* suspensions

Tuberous roots of *P. mirifica* were collected and identified by Associate professor Yudthana Smitasiri, Mae Fah Luang University. The roots, approximately 2 kg each, were sliced and oven-dried at 50°C. The dried roots were pulverized and passed through sieve no 100. Chemical constituents of *P. mirifica* powder (PM) were assayed by the Research and Development Institute, Government Pharmaceutical Organization. The percentage amount of isoflavones : genistin, daidzin, and puerarin were 0.0113, 0.0016 and 0.0152 respectively whereas genistein was not found. Another isoflavone, daidzein, was also qualitatively found by using HPLC/photodiode array (PDA) in our institute. Bioassay of PM using an immature rat uterine weight method was also performed by Associate Professor. Yudthana. It was found that the estrogenic potency of 1 mg of dried powder of PM indicated by uterine weight increase was equal to that of about 1.5-2.0 micrograms of conjugated estrogen (Premarin®). The powders were suspended and diluted to the desired concentrations with water for chronic toxicity study

Experimental animals

One hundred and fifty Wistar rats (75 of each sex) weighing 150 ± 10 g were purchased from the National Laboratory Animal Center, Salaya, Mahidol University.

The animals were housed in conventional hygienic laboratory animal room at the Institute of Health Science Research, Department of Medical Sciences. Temperature in the room was maintained at 25 ± 1°C with 60% relative humidity and 12 hours-light-dark-cycle. The animals were given commercially pelleted diets and clean tap water ad libitum.

Chronic toxicity study

Seventy five rats of each sex were randomly divided into 5 groups of 15 animals per sex. Group 1 (water control) received water 10 ml/kg/day and groups 2-5 were orally administered with the suspensions of PM at the doses of 10, 50, 250 and 250 mg/kg/day respectively for six months. After the six-month period of PM administration, only group 5 (250-R), high recovery group, were further raised for two weeks without PM in order to study recovery or delay effects of PM. During the period of experiment, body weight and food consumption were measured weekly and the rats were closely observed for signs of abnormality. At the end of six-month-treatment period, the animals were fasted for 18 hours and then were dissected under ether anesthesia. Blood was collected from posterior vena cava for hematological and serum biochemical determinations.

Hematological analysis was performed using an automatic hematological analyzer Cell-Dyn®3500. These following hematological parameters were measured: % hematocrit, hemoglobin, red blood cell (RBC), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cells (WBC), % neutrophil, % eosinophil, % lymphocyte, % monocyte, % basophil and platelets number. Biochemical values were assayed by using automatic chemistry analyzer Hitachi®912. These following parameters were measured: alkaline phosphatase (ALP), alanine aminotranferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, glucose, uric acid, triglyceride, total cholesterol, sodium, potassium and chloride ion.

Necropsy was then performed to observe gross pathological changes of various visceral organs. Brain, heart, lung, stomach, liver, kidney, spleen, bladder, testis in male rats, ovary and uterus in female rats were weighed and then calculated in term of % relative organ weight. The visceral organs were preserved in 10% buffered formalin solution and were subsequently subjected to histological preparing process for tissue slides stained with hematoxylin and eosin (H&E) for histopathological examinations.

Data analysis

Body weight, organ weight, food consumption, hematological and biochemical values were statistically analyzed by SPSS program. One way ANOVA was performed and the data was tested for homogeneity of variance by Levene test. Bonferroni test was used in case of equal variance wheras Tamhane test was applied for unequal variance in multiple comparison. Histopathological results were evaluated by Fisher's Exact test and statistical significance of all data was set at $P<0.05$.

Results

Effects of *P. mirifica* on body weight, food consumption and physical appearance

Average body weight of male rats treated with *P. mirifica* powder (PM) at the doses of 50 and 250 mg/kg/day were significantly lower than that of the control group ($P<0.05$) since week 2 and 3 through the end of the experiment, respectively. Female rats receiving PM at the doses of 50 and 250 mg/kg/day had significantly lower average body weight than their control group ($P<0.05$) since week 3 and 4, respectively until the end of the experiment (Fig.1).

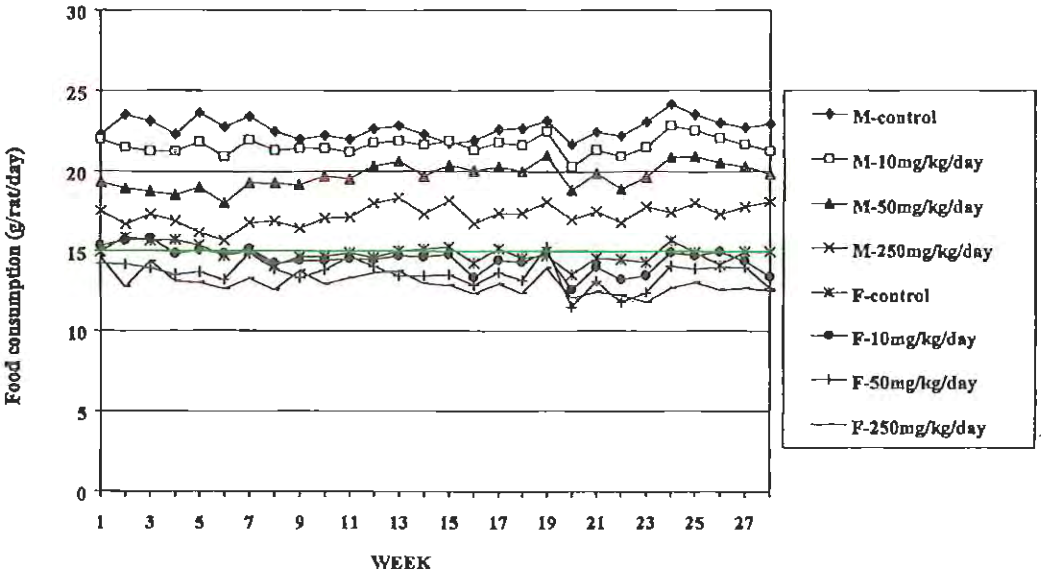

Figure 1 Average body weight of male(M) and female (F) rats treated with *P. mirifica* for 6 months

Food consumptions of male rats receiving 50 and 250 mg/kg of PM were significantly decreased when compared with that of the control group ($P<0.05$). Male rats receiving 10 mg/kg of PM had significantly lower food consumption than the control group for several weeks i.e. week 2 to week 8 of the study and after that there was no difference of food consumption until the end of the study (Fig.2). Female rats receiving the highest dose of PM had significantly lower food consumption than the control group ($P<0.05$) at each week as shown in Fig. 2. During the experiment, some animals receiving PM had alopecia areata from left or right shoulder to thoracic skin. The lesions were present for two weeks and then recovered. The number of rats showing alopecia was summarized in Table 1.

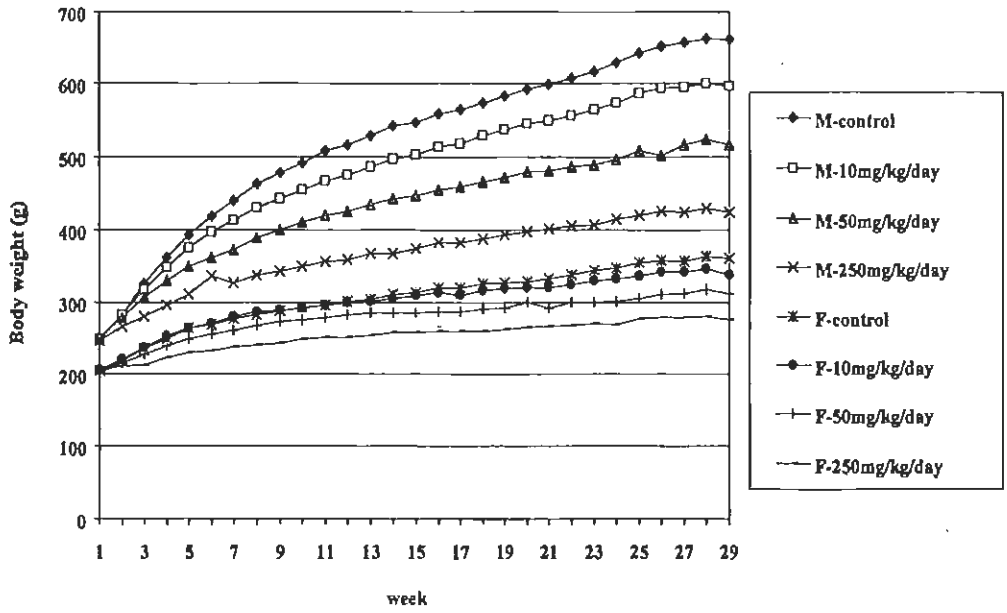

Figure 2 Food consumption of male (M) and female (F) rats treated with *P. mirifica* for 6 months

Table 1 The number of rats developed alopecia during the chronic toxicity study of *P.mirifica* (n=15/group/sex)

Dose of *Pueraria mirifica* (mg/kg/day)	No. of rats with alopecia male	female	Duration of exposure (day)
0	0	0	-
10	0	1(6.7%)	87
50	1 (6.7 %)	1 (6.7%)	60 - 64
250	4 (26.67)	2 (13.33%)	61-156
250-R	4 (26.67%)	3 (20%)	23 - 54

250-R= high dose recovery group

Effects of P. mirifica on hematological values

Significant decreases of hematocrit, RBC and hemoglobin were observed in male and female rats treated with 250 mg/kg/day of PM as compared with those of their corresponding control groups ($P<0.05$). Male rats receiving PM at the dose of 50 mg/kg/day and higher as well as female rats receiving PM at 250 mg/kg/day had significantly decreased eosinophil (%) when compared with the corresponding control groups ($P<0.05$). The percentage of lymphocyte in female rats receiving PM at the dose of 250 mg/kg/day was significantly higher than that of the control group ($P<0.05$). Hematological values of male and female rats were shown in Table 2 and 3.

Effects of P. mirifica on clinical chemistry values

Male rats receiving PM at the dose of 50 and 250 mg/kg/day had significantly lower AST and cholesterol levels than the control group ($P<0.05$). Albumin level was significantly lower in the highest dose of PM-treated group than that of the control group ($P<0.05$). Bilirubin and chloride levels of high dose recovery group were significantly increased as compared to the corresponding control group (Table 4).

Female rats receiving PM at the highest dose had significantly lower levels of AST and cholesterol than the control group ($P<0.05$) whereas triglyceride level was significantly increased ($P<0.05$). The levels of total protein were significantly increased in female rats treated with PM at the doses of 50 and 250 mg/kg/day as well as in the 250-R group (Table 5).

Effects of *P. mirifica* on organ weight

At necropsy, no remarkable gross pathological lesions of the internal organs of all PM-treated and control groups were observed. Male rats treated with PM at the highest dose had significantly decreased weight of brain, lung, liver, testicles, and adrenal gland as compared to the control group ($P<0.05$). The significant increases of cardiac weight were observed in male rats receiving PM at any doses (Table 6). Female rats treated with the highest dose of PM had significantly decreased cardiac weight while uterine and adrenal weight was significantly increased ($P<0.05$) (Table 7).

Effects of *P. mirifica* on histopathology of internal organs.

It was demonstrated that the incidence of fatty degeneration in liver and adrenal gland in all groups of PM-treated male rats was significantly decreased as compared to their control group ($P<0.05$). The incidence of kidney tubular cyst was significantly increased in male rats receiving the highest dose of PM and in the high dose recovery group. Additionally, male rats treated with PM at the dose of 10 mg/kg/day onward had significantly higher incidence of tubular cast than their control group (Table 8).

Table 2 Hematology values of male rats treated with *P. mirifica* for 6 months

Parameters	Dose of Pueraraia mirifica (mg/kg/day)					Normal value
	Control n=15	10 n=15	50 n=13	250 n=15	250-R n=14	
Hematocrit (%)	46.65±0.52	46.78±0.68	45.58±0.65	42.88±0.88*	46.48±0.64	42.5-49.4
RBC(x 10^6/μL)	9.13±0.09	9.09±0.12	8.85±0.14	8.05±0.18*	8.64±0.14	7.2-9.6
Hb (g/dl)	15.72±0.12	15.77±0.16	15.46±0.12	14.70±0.18*	16.51±0.15*	12-17.5
MCV (fl/red cell)	51.15±0.64	51.51±0.78	51.56±0.52	53.25±0.44	53.83±0.58	57-65
MCH (pg/red cell)	17.24±0.21	17.38±0.18	17.58±0.30	18.38±0.36	19.19±0.31*	14.6-21.3
MCHC (g/dl RBC)	33.76±0.33	33.80±0.35	34.08±0.50	34.61±0.79	35.64±0.39*	26-38
WBC (K/μL)	5.38±0.38	6.37±0.37	5.25±0.32	4.95±0.51	4.31±0.25	5-8.96
Neutrophil (%)	14.68±1.54	13.78±0.93	13.72±1.14	14.62±1.33	16.05±1.17	9-34
Eosinophil (%)	1.54±0.14	1.45±0.10	1.00±0.72*	0.98±0.61*	1.59±0.14	0-2.5
Lymphocyte (%)	81.29±1.70	82.53±1.09	83.72±1.06	82.67±1.37	80.24±1.28	65-84.5
Monocyte (%)	1.10±0.30	1.39±0.35	0.94±0.29	1.07±0.25	1.14±0.36	0-5
Basophil (%)	1.19±0.19	0.78±0.09	0.66±0.06	0.61±0.07	0.97±0.10	0-1.5
Platelet (K/μL)	877.13±21.06	829.00±31.75	844.38±23.15	844.57±39.40	882.79±37.02	662.0-992.0

250-R=high dose recovery group
The values are expressed as mean ± SEM
*significantly different from the control group at $P< 0.05$

Table 3 Hematology values of female rats treated with *P. mirifica* for 6 months

Parameters	Dose of *Pueraria mirifica* (mg/kg/day)					Normal value
	Control n=15	10 n=15	50 n=14	250 n=15	250-R n=15	
Hematocrit (%)	45.30±0.91	45.23±0.76	45.95±0.36	41.12±0.65*	43.81±0.68	42.5-49.4
RBC(x 10^6/μL)	8.36±0.17	8.23±0.12	8.43±0.10	7.58±0.11*	8.02±0.12	7.2-9.6
Hb (g/dl)	15.52±0.27	15.33±0.18	15.34±0.16	14.03±0.17*	15.86±0.12	12-17.5
MCV (fl/red cell)	54.31±0.38	54.95±0.49	54.52±0.48	54.21±0.34	54.61±0.48	57-65
MCH (pg/red cell)	18.63±0.19	18.65±0.17	18.20±0.19	18.54±0.16	19.82±0.26*	14.6-21.3
MCHC (g/dl RBC)	35.32±0.34	33.97±0.35	33.38±0.18	34.20±0.31	36.31±0.46*	26-38
WBC (K/μL)	2.92±0.23	2.56±0.28	2.69±0.27	2.68±0.17	2.43±0.19	5-8.96
Neutrophil (%)	23.44±3.10	20.77±2.47	21.23±2.93	21.37±1.57	21.20±1.92	9-34
Eosinophil (%)	1.93±0.16	2.16±0.16	1.52±0.12	1.21±0.17*	1.86±0.14	0-2.5
Lymphocyte (%)	70.19±3.22	73.81±2.71	73.84±2.92	81.02±2.01*	73.23±2.27	65-84.5
Monocyte (%)	3.64±0.86	2.62±0.74	2.75±0.71	2.14±0.59	2.35±0.56	0-5
Basophil (%)	0.80±0.10	0.64±0.08	0.66±0.12	0.79±0.11	1.37±0.16*	0-1.5
Platelet (K/μL)	847.03±25.03	859.63±21.93	923.42±29.42	826.65±33.01	871.39±27.01	403.0-979.0

250-R=high dose recovery group
The values are expressed as mean ± SEM
*significantly different from the control group at $P< 0.05$

Table 4 Clinical chemistry values of male rats treated with *P. mirifica* for 6 months

Parameters	Dose of *Pueraria mirifica* (mg/kg/day)					Normal value
	control	10	50	250	250-R	
	n=15	n=15	n=15	n=13	n=15	
ALP (U/L)	63.00±2.87	62.93±2.66	67.93±4.91	68.73±4.10	72.21±6.94	56.8-128.0
ALT (U/L)	38.00±2.07	37.07±2.50	32.57±2.09	44.73±4.84	38.36±3.01	28.9-47.6
AST (U/L)	72.93±2.89	67.60±3.58	58.07±2.02*	55.80±3.02*	64.29±1.89	45.7-80.8
BUN (mg/dl)	19.25±0.49	19.98±0.75	20.84±0.77	19.56±0.91	21.76±0.94	5-29
Creatinine(mg/dl)	0.70±0.03	0.63±0.05	0.67±0.02	0.64±0.02	0.58±0.01*	0.2-0.8
Total protein (g/dl)	6.77±0.07	6.88±0.08	6.89±0.08	6.81±0.08	7.09±0.07*	4.7-8.15
Albumin (g/dl)	4.16±0.04	4.15±0.04	4.15±0.06	3.83±0.07*	4.26±0.05	2.7-5.1
Bilirubin(mg/dl)	0.07±0.01	0.08±0.02	0.13±0.03	0.08±0.01	0.34±0.01*	0.0-0.55
Glucose (mg/dl)	167.40±5.54	176.62±8.50	154.77±11.35	189.65±9.27	190.84±8.06	130.0-267.0
Uric acid (mg/dl)	1.87±0.21	2.29±0.36	1.72±0.23	2.31±0.43	1.86±0.27	1.2-7.5
Triglyceride (mg/dl)	162.27±10.92	200.63±24.33	201.15±23.44	184.80±38.26	195.05±21.25	53.0-124.8
Cholesterol (mg/dl)	84.75±4.33	96.27±7.81	47.51±4.48*	27.27±4.36*	96.71±6.28	45.0-92.0
Na^+(mmol/l)	146.73±0.54	146.20±0.35	147.14±0.36	146.67±0.35	148.43±0.45	143.0-156.0
K^+(mmol/l)	5.77±0.16	6.56±0.41	5.93±0.28	6.67±0.25	5.49±0.14	5.4-7.0
Cl^-(mmol/l)	108.47±0.54	108.53±0.40	108.43±0.40	107.60±0.49	112.57±0.58*	100.0-110.0

250-R=high dose recovery group
The values are expressed as mean ± SEM
*significantly different from the control group at $P<0.05$.

Table 5 Clinical chemistry values of female rats treated with *P. mirifica* for 6 months

Parameters	Dose of *Pueraria mirifica* (mg/kg/day)					Normal value
	control	10	50	250	250-R	
	n=15	n=15	n=15	n=13	n=15	
ALP (U/L)	27.53±2.03	26.00±2.39	25.47±1.66	23.92±1.03	25.29±0.87	56.8-128.0
ALT (U/L)	45.13±4.92	39.93±2.55	35.73±2.33	37.92±2.81	31.14±1.99*	28.9-47.6
AST (U/L)	86.67±6.87	76.40±4.06	82.73±10.59	65.08±3.41*	67.29±2.29	45.7-80.8
BUN (mg/dl)	20.27±0.96	21.05±0.77	22.09±0.60	21.90±0.55	21.58±1.02	5-29
Creatinine(mg/dl)	0.72±0.01	0.75±0.01	0.76±0.02	0.71±0.01	0.63±0.01*	0.2-0.8
Total protein (g/dl)	6.98±0.07	7.05±0.05	7.46±0.08*	7.52±0.06*	7.50±0.08*	4.7-8.15
Albumin (g/dl)	4.95±0.05	4.89±0.06	5.04±0.05	4.80±0.05	4.91±0.06	2.7-5.1
Bilirubin(mg/dl)	0.09±0.01	0.07±0.01	0.08±0.01	0.08±0.01	0.06±0.01*	0.0-0.55
Glucose (mg/dl)	149.00±4.83	139.99±5.28	165.56±7.65*	172.25±6.53*	152.81±5.14	96.0-153.0
Uric acid (mg/dl)	2.03±0.25	1.38±0.20	2.28±0.33	2.00±0.29	1.76±0.16	1.2-7.5
Triglyceride (mg/dl)	125.84±9.17	117.01±8.80	143.14±15.86	221.90±26.69*	153.00±14.76	39.1-72.8
Cholesterol (mg/dl)	71.97±4.43	69.87±5.11	67.34±6.49	39.02±3.53*	78.17±4.22	31.0-68.0
Na^+(mmol/l)	147.00±0.31	147.20±0.37	147.60±0.25	148.23±0.36*	149.57±0.25*	143.0-156.0
K^+(mmol/l)	5.74±0.32	4.93±0.26	5.29±0.31	5.44±0.31	5.48±0.16	5.4-7.0
Cl^-(mmol/l)	111.27±0.47	111.47±0.46	111.06±0.46	111.15±0.36	117.57±0.34*	100.0-110.0

250-R=high dose recovery group
The values are expressed as mean ±SDM
*significantly different from the control group at $P<0.05$.

Table 6 Organ weight of male rats treated with *P. mirifica* for 6 months

Organs	Dose of *Pueraria mirifica* (mg/kg/day)				
	Control	10	50	250	250-R
	n=15	n=15	n=14	n=15	n=15
Brain	2.15±0.02	2.12±0.02	2.12±0.02	2.04±0.02*	2.09±0.02
Heart	1.52±0.04	1.42±0.03*	1.35±0.03*	1.18±0.04*	1.24±0.03*
Lung	1.86±0.04	1.84±0.05	1.74±0.05	1.65±0.05*	1.67±0.03*
Stomach	2.16±0.06	2.40±0.10*	2.21±0.06	2.23±0.07	2.15±0.05
Liver	14.83±0.61	15.49±0.51	14.28±0.44	13.15±0.56*	13.33±0.46
Right kidney	1.42±0.06	1.43±0.02	1.42±0.06	1.39±0.07	1.33±0.05
Left kidney	1.38±0.06	1.36±0.02	1.34±0.05	1.31±0.05	1.29±0.04
Spleen	1.09±0.04	1.14±0.05	1.04±0.03	1.15±0.04	1.08±0.05
Left testis	3.19±0.08	3.10±0.06	3.02±0.06	2.84±0.14*	2.97±0.05
Right testis	3.16±0.08	3.15±0.07	3.07±0.04	2.90±0.11*	2.98±0.05
Bladder	0.15±0.01	0.13±0.01	0.14±0.01	0.13±0.01	0.13±0.01

250-R=high dose recovery group
The values are expressed as mean ± SEM
*significantly different from the control group at $P<0.05$

Table 7 Organ weight of female rats treated with *P. mirifica* for 6 months

Organs	Dose of *Pueraria mirifica* (mg/kg/day)				
	Control	10	50	250	250-R
	n=15	n=15	n=14	n=15	n=15
Brain	1.96±0.01	1.97±0.02	1.98±0.02	1.91±0.02	1.93±0.01
Heart	0.94±0.03	0.95±0.03	0.90±0.02	0.86±0.02*	0.91±0.02
Lung	1.30±0.04	1.37±0.03	1.31±0.02	1.22±0.03	1.28±0.03
Stomach	1.62±0.03	1.73±0.05	1.68±0.06	1.72±0.05	1.58±0.04
Liver	7.52±0.19	7.52±0.29	7.46±0.27	8.01±0.18	7.66±0.14
Right kidney	0.86±0.02	0.85±0.02	0.87±0.02	0.83±0.01	0.88±0.02
Left kidney	0.82±0.02	0.82±0.02	0.81±0.02	0.77±0.02	0.82±0.02
Spleen	0.70±0.02	0.80±0.04*	0.70±0.03	0.74±0.02	0.69±0.02
Left ovary	0.08±0.00	0.07±0.00	0.07±0.00	0.07±0.00	0.06±0.00
Right ovary	0.07±0.00	0.07±0.00	0.07±0.00	0.07±0.00	0.06±0.00
Bladder	0.08±0.00	0.09±0.00	0.08±0.00	0.08±0.00	0.07±0.00
Uterus	0.69±0.04	0.69±0.03	0.86±0.06	1.05±0.08*	0.82±0.08

250-R= high dose recovery group
The values are expressed as mean ± SEM

Table 8 Histopathological results of visceral organs in Wistar rats treated with *P. mirifica* for 6 months

Organs	Microscopic findings	Dose of *P.mirifica* (mg/kg/day)									
		Male (n =15/group)					Female (n=15/group)				
		0	10	50	250	250-R	0	10	50	250	250-R
Lung	Lymphoid proliferated peribronchioles	4/15	11/15*	7/15	7/15	10/15*	8/15	9/15	0/15*	8/15	9/15
Heart	Focal myocardiosis	1/15	1/15	1/15	1/15	0/15	0/15	1/15	0/15	0/15	0/15
Liver	Fatty degeneration	7/15	2/15*	0/15	0/15	0/15	0/15	1/15	0/15	0/15	0/15
	Lymphoid aggregated periportal areas	1/15	1/15	0/15	0/15	0/15	1/15	1/15	0/15	0/15	0/15
Kidney	Tubular cast	0/15	9/15*	9/15*	7/15*	2/15	4/15	4/15	4/15	5/15	4/15
	Tubular cyst	2/15	1/15	4/15	10/15*	7/15*	0/15	0/15	0/15	0/15	0/15
Intestine	GALT hyperplasia	1/15	1/15	0/15	0/15	2/15	2/15	0/15	3/15	1/15	0/15
Testis	Atrophy	0/15	1/15	0/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15
Uterus	Subendometrial gland hyperplasia						2/15	1/15	0/15	0/15	0/15
Mammary gland	Glandular hyperplasia						3/15	1/15	1/15	1/15	0/15
Adrenal gland	Cortical fatty degeneration	11/15	4/15*	0/15*	0/15*	0/15*	0/15	0/15	0/15	0/15	0/15
	Focal hemorrhage						1/15	0/15	0/15	1/15	0/15

250-R=high-dose recovery group
The results were expressed as the number of rats with pathological findings per total number of rats treated
*significantly different from the control group at $P<0.05$

Discussion

In this six-month chronic toxicity study, the doses of PM were changed to be 10, 50 and 250 mg/kg/day. Since our previous ninety-day subchronic toxicity study revealed that PM at the doses of 100 and 1,000 mg/kg produced some adverse effects in the animals.[17] PM at the doses ranged from 50 to 250 mg/kg/day affected the body weight of the animals. The significantly lower body weight may be due to the decreasing of food intake in the PM-treated groups. It was demonstrated that an estrogen derivative, 17-beta-estradiol, can decrease food consumption and food efficiency in rats.[18] Some phytoestrogens such as miroestrol was reported to produce headache and nausea,[3] genistin and daidzein have been shown to decrease the synthesis of cortisol[19] which has an activity on stimulating appetite. Taken together, the decrease of food consumption may be contributed by the estrogenic-like effects of PM. Alopecia found in some rats receiving PM at the given doses may also be resulted from the estrogenic-like activity of PM since there was a study demonstrating that ethynylestradiol, a synthetic estrogen, produced alopecia in the albino rats.[20] However, alopecia in PM-treated rats existed for about two weeks and then it recovered.

Hematologic results demonstrated that PM at the dose of 250 mg/kg significantly decreased hematocrit, RBC, and hemoglobin in both male and female rats, however these alterations were within normal range[21] and recoverable as shown in 250-R group. The decrease of these parameters may be attributable to the estrogenic-like effect of PM since it was reported that dietary administration of 17 beta-estradiol produced anemia in rats.[18] The significant changes of eosinophil(%) in PM-treated male rats at the doses of 50 and 250 mg/kg/day and in female rats receiving the highest dose of PM was within normal range[21] i.e., 0-2.5%. In addition, the increase of lymphocyte in female rats receiving the highest dose of PM was also within normal range.[21]

The significant decreases of AST levels in PM-treated male rats at the dose of 50 and 250 mg/kg/day and in female rats receiving the highest dose were within normal range. However, this alteration did not indicate any damage of the concerning vital organs such as liver and heart. Our findings that triglyceride levels increased in a dose-dependent manner in all groups of female rats receiving PM and the significance effect was observed in the highest dose-treated group, suggest that this phenomenon may be PM related. Moreover, this findings are consistent with our previous subchronic toxicity study showing that high dose of PM (1,000 mg/kg/day) can cause the elevation of triglyceride level in female rats.[17] Cholesterol levels were significantly decreased in PM-treated male rats at the doses of 50 and 250 mg/kg/day as well as in female rats receiving the highest dose of PM, suggesting the hypocholesterolemic effect of PM which was consistent with our previous study[17]. There was also study indicated that phyto- estrogens from soy bean, a plant in the same family as *P. mirifica* (Leguminosae) were capable in decreasing LDL and increase HDL cholesterol levels in monkey.[22]

In this study we found that the body weights in the groups receiving PM at 50 and 250 mg/kg were significantly decreased and this might result in the significant increase of relative weight of many organs. Therefore we present actual organs weight which reveal more actual alterations than relative organ weights. The decreases of some organs weights i.e., heart in PM-treated male rat at each dose and in the highest dose-treated female rats, lung in male receiving highest dose of PM were observed. However histopathology of these organs did not indicate any abnormality The decrease of left and right testicular weight in male rats receiving the highest dose of PM and the increase of uterine weight in female rats at this dose suggest the estrogenic effects of PM as previously reported in some studies.[13,14,17]

Histopathology results showed the alterations in some visceral organs of the

male rats receiving PM. The incidence of hepatocyte fatty degeneration was significantly decreased at the doses ranging from 10 to 250 mg/kg of PM. This phenomenon may be estrogenic effect of PM, since there were some investigations showing that estrogens were able to increase apolipoprotein B (apo-B) production in human hepatocytes[23] and in mice hepatocyte.[24] Hepatocytes assemble triglyceride into VLDL-lipoproteins particles on the backbone of apo B-100 and secrete into plasma.[25] This may explain why the incidence of hepatocyte fatty degeneration in the liver decreased. The significantly higher incidence of tubular cyst in the kidneys of male rats receiving PM at the dose of 250 mg/kg/day and in those of the high dose recovery group suggests that prolonged administration of PM at this dose may cause tubular cells damage. It was demonstrated that metabolites of daidzin and daidzein were excreted in urine and bile[26]; therefore the excretion of these metabolites via kidney may affect the tubular cells. The findings of crystal-like tubular cast in the kidney of PM-treated male rats suggest that there may be the disturbance of some electrolytes reabsorption or transportation of renal epithelium. Some flavones, such as genistein and daidzein activated Cl-channels, genistein and apigenin were reported to possess a stimulatory effect on sodium, potassium and chloride ion-cotransporters in a renal epithelial cell line[27]. In addition, equol, a metabolic compound of daidezin by intestinal bacteria, is also a potent inhibitor of Na-K-Cl cotransporter.[28] However, the incidence of tubular cast decreased after PM discontinuation. The incidence of adrenocortical fatty degeneration of male rats receiving PM at each dose decreased in a dose-response relationship, suggesting the depletion of lipid accumulation. Previously, there was a study showed that daidzein, a phytoestrogen, can suppress cortisol synthesis in the adrenal gland[29]. Therefore, the reduction of cortisol levels might result in the stimulation of ACTH production. Under conditions of acute or prolonged ACTH stimulation, the lipid stores in the adrenal cortical cells might be used for corticosteroid synthesis[30]. The incidences of other histopathologic findings were not dose dependent; therefore they could not contribute to PM.

In conclusion, the chronic toxicity toxicity study of PM at the doses ranging from 10 to 250 mg/kg/day indicated that PM exerted its estrogenic-like activities in many aspects i.e., at the dose of 250 mg/kg/day affect hematology values, testicular and uterine weight. In addition, the incidence of kidney tubular cyst was significantly increased at this dose. Therefore, prolonged use and overdose of PM dose should be avoided.

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