RESEARCH ARTICLES

Subchronic Exposure of *Pueraria Mirifica* in Normal- and High Cholesterol Diet-fed Rats : Influence on Lipid Profile and Toxicity

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Abstract

Pueraria mirifica Airy Shaw and Suvatabandhu, known locally as White Kwao Keur, is a plant in family Leguminosae. In this study, effects of *P.mirifica* on serum lipid profile and subchronic toxicity were investigated in male Wistar rats. Rats were randomly divided into four treatment groups as following: normal diet-fed group; normal diet-fed supplemented with *P.mirifica* group; high cholesterol diet-fed group; high cholesterol diet-fed supplemented with *P.mirifica* group. Each group comprised 10 rats. *P.mirifica* was administered orally at a dosage of 100 mg/kg/day for 90 consecutive days. During the treatment period, body weights of the animals were recorded every two weeks. At the end of the treatment, rats were anesthesized. Blood samples were collected by heart puncture and serum sample were prepared for determination of hematology and clinical blood chemistry, respectively. The results showed that body weight of rats given *P.mirifica* in either normal diet or high cholesterol diet conditions were significantly lower than their corresponding control groups. There was no significant difference of these following hematology and clinical blood chemistry: hemoglobin, hematocrit, RBC morphology, WBC count, % differential WBC, platelet count, glucose, BUN, SCr, total bilirubin, and direct bilirubin in all experimental groups. *P.mirifica* did not affect serum level of AST, ALT, and ALP in normal diet-fed condition. High cholesterol diet-fed condition caused a significant increase of AST, ALT, and ALP but *P.mirifica* attenuated these effects. *P.mirifica* significantly decreased serum total cholesterol and LDL-C in either normal diet-fed or high cholesterol diet-fed rats. Serum triglyceride was increased in normal diet-fed rats but decreased in high cholesterol diet-fed rats. *P.mirifica* caused a significant decrease of HDL-C in both normal and high cholesterol diet-fed rats whereas its improvement in the LDL-C/HDL-C ratio was shown only in high cholesterol diet-fed rats. Although, *P.mirifica* demonstrated a benefit on lipid profile and did not show any toxic effects on liver, kidney, and blood system in this study, an increment of serum triglyceride in normal rat receiving *P.mirifica*, however, is not favorable. Effects of *P.mirifica* at various doses, long term used as well as mechanism of the effects should be further investigated.

Key words : *P.mirifica*, lipid profile, subchronic toxicity, normal diet-fed rat, high cholesterol diet-fed rat.

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การได้รับกวาวเครือแบบกึ่งเรื้อรังในหนูขาวที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง : ผลต่อระดับไขมันในเลือด และความเป็นพิษ

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บทคัดย่อ

กวาวเครือขาว (*Pueraria mirifica* Airy Shaw and Suvatabandhu) เป็นพืชในวงศ์ Leguminosae งานวิจัยนี้ทำการศึกษาผลของการได้รับกวาวเครือขาวแบบกึ่งเรื้อรังต่อระดับไขมันในเลือด และความเป็นพิษในหนูขาวเพศผู้พันธุ์วิสตาร์ที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง โดยแบ่งหนูขาวแบบสุ่มเป็น 4 กลุ่ม กลุ่มละ 10 ตัว ดังนี้ กลุ่มที่ได้รับอาหารปกติ, กลุ่มที่ได้รับอาหารปกติและกวาวเครือขาว, กลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง และกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูงและกวาวเครือขาว ให้กวาวเครือขาวในขนาด 100 มิลลิกรัม/กิโลกรัม/วัน โดยวิธีป้อนทางปาก เป็นเวลา 90 วัน ทำการชั่งน้ำหนักหนูขาวทุก 2 สัปดาห์ เมื่อครบระยะเวลา ทำให้หนูหมดความรู้สึก เก็บตัวอย่างเลือดจากหัวใจ เพื่อตรวจค่าโลหิตวิทยาและแยกซีรัมตรวจค่าเคมีคลินิก ผลการทดลองพบว่ากวาวเครือขาวทำให้การเพิ่มของน้ำหนักหนูขาวต่ำกว่ากลุ่มควบคุม แต่ไม่มีผลต่อค่าโลหิตวิทยาและค่าเคมีคลินิกดังต่อไปนี้ hemoglobin, hematocrit, RBC morphology, WBC count, %differential WBC, platelet count, glucose, BUN, SCr, total bilirubin และ direct bilirubin กวาวเครือขาวไม่มีผลต่อค่า AST, ALT และ ALP ในซีรัมของหนูที่ได้รับอาหารปกติ อาหารคลอเรสเตอรอลสูงมีผลทำให้ AST, ALT และ ALP สูงแต่ค่าเหล่านี้ลดลงเมื่อให้กวาวเครือขาว กวาวเครือขาวทำให้ค่า total cholesterol และ LDL-C ในซีรัมลดลงอย่างมีนัยสำคัญทั้งในกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง ในขณะที่ค่าไตรกลีเซอไรด์สูงขึ้นอย่างมีนัยสำคัญในกลุ่มที่ได้รับอาหารปกติ แต่มีค่าลดลงในกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง กวาวเครือขาวทำให้ค่า HDL-C ในซีรัมลดลงอย่างมีนัยสำคัญทั้งกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง ส่วนอัตราส่วนของ LDL-C ต่อ HDL-C มีค่าต่ำลงอย่างมีนัยสำคัญเฉพาะในกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง ถึงแม้ว่ากวาวเครือขาวจะมีผลที่เป็นประโยชน์ต่อค่าไขมันในเลือดและไม่มีผลพิษใดๆ ต่อตับ ไต และระบบเลือด ผลที่ไม่พึงปรารถนาของกวาวเครือขาวที่พบคือมีผลเพิ่มไตรกลีเซอไรด์ในหนูที่ได้รับอาหารปกติ ควรทำการศึกษาต่อไปถึงผลของกวาวเครือขาวที่ขนาดต่างๆ ผลของการใช้สารนี้ในระยะเวลานาน และกลไกที่ใช้อธิบายผลต่างๆที่เกิดขึ้นนี้

คำสำคัญ : กวาวเครือขาว, ระดับไขมันในเลือด, พิษกึ่งเรื้อรัง, หนูขาวที่ได้รับอาหารปกติ, หนูขาวที่ได้รับอาหารคลอเรสเตอรอลสูง

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Introduction

Pueraria mirifica Airy Shaw and Suvatabandhu, known locally as "White Kwao Keur", is a plant in family Leguminosae. Several indications of this plant were suggested for a traditional purpose such as using for skin enrichment, thickening and blackening hair, a relief of weakness, an increase of appetite, treatment of insomnia, and breast enlargement in women[1]. These uses of *P.mirifica* in traditional medicine may be attributed to its estrogenic properties of the constituents. Several previous studies demonstrated that this plant possessed various compounds including phytoestrogens, the compounds with estrogen-like biological activity. Phytoestrogens found in tuberous roots of *P.mirifica* include miroestrol[2], kwakhurin[3], puerarin[4], coumestrol, daidzin, daidzein, mirificin[5], genistein, genistin[6] and deoxymiroestrol[7]. Epidemiological studies showed that frequent consumption of phytoestrogen rich diet, as seen in traditional Asian food, is associated with lower risks of many diseases such as breast, prostate, and colon cancers as well as cardiovascular diseases[8,9,10]. Several studies suggested that genistein and daidzein possess cancer chemopreventive effects[11,12,13,14], of which the specific mechanisms have not been clearly identified. *In vitro* and *in vivo* studies found that genistein exhibited antiproliferative effects in human breast cancer cells[15]. It also inhibited tyrosine specific protein kinases[16], DNA topoisomerase II[17], epidermal growth factor induced phosphatidylinositol turnover[18] and angiogenesis[19]. In addition to the chemopreventive effects, isoflavone phytoestrogens, genistein and daidzein, which are found mostly in soy foods, also possess a benefit in reducing risk of cardiovascular diseases by a proposed hypothesis of decreasing of total cholesterol, LDL-C, and triglyceride but an increase of HDL-C in both normal and hypercholesterolemic conditions[8,20]. The cardioprotective effects of these compounds may be attributed to its estrogenic like-activity.

Effect of *P.mirifica* on lipid profile, particularly in hypercholesterolemic condition, which may contribute to its cardioprotective potential have never been investigated. In addition, there are few studies regarding the subchronic toxicity of *P.mirifica*[21]. Therefore, the objectives of this study were primarily to investigate subchronic effects of *P.mirifica* on rat hematology and clinical blood chemistry so as to preliminarily investigate lipid-lowering effects and subchronic toxicity of this plant in both normal and hypercholesterolemic rats.

Materials and Methods

Animals

Adult male Wistar rats of body weight between 200-250 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakompathom. Rats were housed two per cage at the Faculty of Medicine, Srinakharinwirot University and acclimatized for at least seven days prior to the experimentation. They were maintained at 25 °C on a 12-hour light/dark cycle and had free access to the diet and water throughout the study. High cholesterol rats had high cholesterol diet containing 1% cholesterol plus 2% sodium choleate. All diets were purchased from C.P.company.

P.mirifica

Dried fine powder of *P.mirifica* tuberous root was obtained from Dr. Amphawan Apisariyakul at the department of Pharmacology, Faculty of Medicine, Chiang Mai University, Thailand. *P.mirifica* used in this study was cultivated at Aumpor Ban Tak and Mae Sod, Tak Province and the tuberous roots were collected during March and April, 2000.

The powder was identified for its estrogenic activity by dissolving in water, filtered and analyzed by immunoassay. The result showed that the solution of *P.mirifica* used in this study possessed estrogenic activity in a concentration-related manner (unpublished data).

P.mirifica for animal administration was prepared freshly by dissolving 6 g of the powder with 100 ml of double distilled water, mixed well, filtered out any remaining fiber with cloth filter. During the time of drawing the suspension into the gavage tube, the suspension was thoroughly mixing by magnetic stirrer

Experimental model

Rats were randomly divided into four treatment groups as following: normal diet-fed group; normal diet-fed supplemented with *P.mirifica* group; high cholesterol diet-fed group; high cholesterol diet-fed supplemented with *P.mirifica* group. Each group comprised 10 rats. *P.mirifica* was administered orally at a dosage of 100 mg/kg/day for 90 consecutive days. During the treatment period, body weights of the animals were recorded every two weeks. At the end of the treatment, rats were anesthesized. Blood samples were collected by heart puncture and serum sample were prepared for determination of hematology and clinical blood chemistry, respectively.

Whole blood samples were determined for complete blood count (CBC), white blood cell (WBC) count, %differential WBC, platelet count and red blood cell (RBC) morphology. Serum samples were determined for various blood clinical biochemistry parameters using commercial test kit of bioMerieux company (France) as following: glucose, blood urea nitrogen (BUN), serum creatinine (SCr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride, and cholesterol. The assays mentioned above were performed by Faculty of Allied Health Sciences, Chulalongkorn University.

Determination of total bilirubin and direct bilirubin (using commercial test kit of Merieux Vitex, France), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) (using commercial test kit of Roche company, Germany) in serum were performed by Professional Laboratory Management, Bangkok.

Statistics

All quantitative data were presented as mean ± SEM. An independent *t*-test was used for statistical comparisons between two groups (Normal diet-fed group vs Normal diet-fed supplemented with *P.mirifica* group; High cholesterol diet-fed group vs High cholesterol diet-fed supplemented with *P.mirifica* group) at significant level of $P<0.05$.

Results

1. General effects of P.mirifica

During the experimental period, five rats (accounted for 50% of the total rats in the group) from normal diet-fed supplemented with *P.mirifica* group and four rats (account for 40% of the total rats in the group) from high cholesterol diet-fed supplemented with *P.mirifica* group had hair loss. No rats died at the end of the study.

Body weight gain of rats receiving *P.mirifica* fed with either normal diet or high cholesterol diet was significantly lower than their corresponding control groups (Figure 1). High cholesterol diet caused no change of body weight gain as compared to the normal diet condition.

2. Effect of *P.mirifica* on clinical blood chemistry and hematology

In both normal diet-fed and high cholesterol diet-fed conditions, *P.mirifica* exhibited no deteriorated effects indicated by these following hematological and clinical blood chemistry parameters: hemoglobin, hematocrit, WBC count, %differential WBC, RBC morphology, platelet count, glucose, BUN, SCr, total bilirubin, direct bilirubin AST, ALT, and ALP. Interestingly, *P.mirifica* even helped attenuating the liver injury-induced by hypercholesterolemic condition as shown by a significant decrease of AST, ALT and ALP in high cholesterol diet-fed supplemented with *P.mirifica* rats as compared to the corresponding high cholesterol diet-fed rats (Table 1).

Comparing to normal diet-fed rats, high cholesterol diet fed rats demonstrated a significant increase of AST (170.60±10.63 vs. 278.30±24.66; $P<0.05$), ALT (36.10± 1.58 vs.198.9±39.15; $P<0.05$), ALP (63.40± 3.41 vs. 97.10±6.50; $P<0.05$).

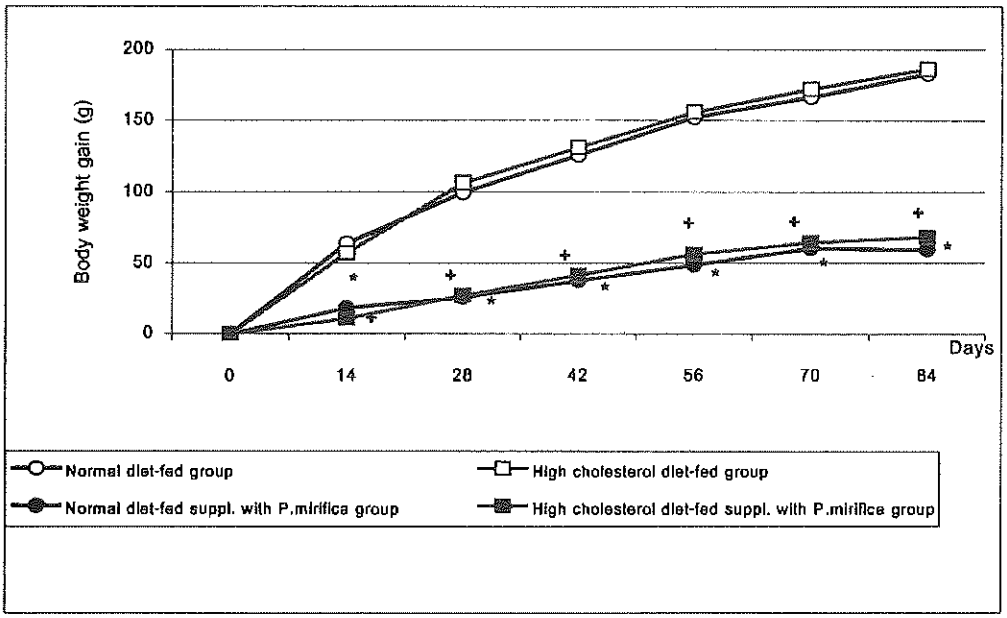

Figure 1 Effect of *P.mirifica* on body weigh gain

Data shown were mean
*P<0.05; Normal diet-fed supplemented with *P.mirifica* group vs Normal diet-fed group
+P<0.05; High cholesterol diet-fed supplemented with *P.mirifica* group vs High cholesterol diet-fed group

Table 1 Effect of *P.mirifica* on hematology and clinical blood chemistry

Hematology	**Normal diet-fed group**	**Normal diet-fed supplemented with *P.mirifica* group**	**High cholesterol diet-fed group**	**High cholesterol diet-fed supplemented with *P.mirifica* group**
Hemoglogbin (g/dl)	14.64±0.36	14.10±0.30	13.73±0.27	13.94±0.22
Hematocrit (%)	44.00±1.09	42.38±0.93	41.25±0.82	41.89±0.66
WBC count ($x10^9$/l)	1.81±0.36	1.39±0.10	2.05±0.32	1.34±0.19
Neutrophil (%)	27.57±1.78	25.13±3.24	25.75±4.20	22.56±2.59
Lymphocyte (%)	69.71±1.82	71.00±3.51	70.88±4.10	74.67±2.30
Monocyte (%)	2.14±0.51	3.00±1.04	3.00±0.58	2.00±0.33
Eosinophil (%)	0.57±0.43	0.86±0.23	0.75±0.25	0.78±0.32
Basophil (%)	0	0	0	0
RBC morphology	Normal	Normal	Normal	Normal
Platelet ($x10^3$/ul)	339.29±44.61	303.13±34.54	334.38±19.44	322.22±41.76
Clinical blood chemistry				
Glucose (mg/dl)	129.9±8.16	137.4±11.67	147.9±17.10	138.7±20.48
BUN (mg/dl)	22.05±1.27	20.08±0.60	21.27±1.19	21.81±1.47
SCr (mg/dl)	0.71±0.03	0.67±0.03	0.72±0.03	0.70±0.03
Total Bilirubin (mg/dl)	0.11±0.01	0.10±0.00	0.13±0.02	0.10±0.00
Direct bilirubin (mg/dl)	0.018±0.008	0.029±0.005	0.026±0.006	0.027±0.005
AST (U/l)	170.60±10.63	156.30±19.40	278.30±24.66	174.20±22.12 +
ALT (U/l)	36.10±1.58	29.00±2.85	198.9±39.15	57.1±17.30 +
ALP (U/l)	63.40±3.41	71.40±7.99	97.10±6.50	74.30±3.15 +

Data shown were mean ± SEM
+P<0.05; High cholesterol diet-fed supplemented with *P.mirifica* group vs.High cholesterol diet-fed group

3. Effect of *P.mirfica* on serum lipid profile

P.mirifica significantly decreased total cholesterol, LDL-C and HDL-C but significantly increased triglyceride in normal diet-fed rats. It also significantly decreased total cholesterol, triglyceride, LDL-C, HDL-C and LDL-C/HDL-C ratio in high cholesterol diet-fed rats (Table 2). High cholesterol diet-fed rats showed a significant increase of total cholesterol (64.40±3.18 vs. 85.60±9.47; P<0.05), LDL-C (8.00±0.50 vs.56.40±9.76; P<0.05) and LDL-C/HDL-C ratio (0.10±0.006 vs. 0.78±0.16; P<0.05) as compared to the normal diet-fed rats.

Table 2 Effect of *P.mirifica* on serum lipid parameters

Serum lipid parameters	Normal diet-fed group	Normal diet-fed supplemented with *P.mirifica* group	High cholesterol diet-fed group	High cholesterol diet-fed supplemented with *P.mirifica* group
Total cholesterol (mg/dl)	64.40±3.18	32.60±7.10 *	85.60±9.47	39.90±5.05 +
Triglyceride (mg/dl)	72.60±7.80	110.10±10.53 *	54.50±4.07	33.50±3.28 +
LDL-C (mg/dl)	8.00±0.50	5.00±0.67 *	56.40±9.76	17.00±2.64 +
HDL-C(mg/dl)	78.67±3.76	35.50±9.03 *	73.50±5.56	40.50±4.32 +
LDL-C/HDL-C ratio	0.10±0.006	0.18±0.04	0.78±0.16	0.45±0.06 +

Data shown were mean ± SEM
* P<0.05; Normal diet-fed supplemented with *P.mirifica* group vs Normal diet-fed group
+P<0.05; High cholesterol diet-fed supplemented with *P.mirifica* group vs High cholesterol diet-fed group

Discussion and Conclusion

This study primarily investigated subchronic toxicity and effect on serum lipid profile of *P.mirifica* in both normal and hypercholesterolemic rats. This would provide an additional information regarding subchronic effects of *P.mirifica* at the dosage of 100 mg/kg/day, the dosage which was shown to decrease serum cholesterol without any serious toxic effects in a previous study[21]. It has been well-documented that cardiovascular advantage of phytoestrogens is attributed to their lipid lowering effects. Besides studying in normal rats, this study was also performed in hypercholesterolemic rats, the model of which effect of *P.mirifica* had never been investigated.

Body weight gains of rats given *P.mirifica* and fed with either normal diet or high cholesterol diet were significantly lower than their corresponding control-diet fed groups. These were consistent to the results reported by Chivapat and collaborates[21]. In that study, they found that rats receiving *P.mirifica* orally at the doses of 100 and 1,000 mg/kg/day for 90 days had body weight gain and food consumption less than the control group. This effect was possibly dued to the effects of some phytoestrogens containing in *P.mirifica*. Miroestrol was shown to cause nausea and vomiting in human[2]. Genistein and daidzein were found to suppress food intake and body weight gain in rats[22,23]. Inhibition of 21-hydroxylase enzymes in adrenal gland cells by both genistein and daidzein resulted in a decreased synthesis of cortisol, the hormone which acted at CNS to stimulate food appetite[24]. Effects of estrogens on the growth and body weight of rodents are well documented. Both synthetic and natural estrogens decrease growth rate in rats and mice via acting centrally at the hypothalamus to decrease food consumption[25,26,27,28]. Dose-dependent growth retardation and decrease in food consumption have been reported in long-term studies with most estrogens[25,28].

Hair loss occurred in the *P.mirifica* treated rats. This effect induced by chronic estrogen treatment has been reported[25,28]. Although less studies were performed on estrogens than on androgens, prolonged intraperitoneal, subcutaneous implant or

oral administration of estrogens has been shown to block hair growth in rats and mice[25,28,29]. Topical ICI 182 780, a pure estrogen receptor antagonist, stimulates hair regrowth in male mice[29]. Hair follicle is a complex structure that is influenced by systemic factors including androgens, glucocorticoids and estrogens. The estrogen receptor pathway within dermal papilla regulates the telogen-anagen transition of the hair follicle in CD-mice[30]. Thus, hair loss found in *P.mirifica* treated rats shown in this study was possibly attributed from estrogenic-like effect of *P.mirifica*.

Results from this study showed that *P.mirifica* given orally at the dose of 100 mg/kg/day for 90 days did not cause any toxic effects to the hematopoietic system of male rats. In addition, there were no effects of *P.mirifica* at this dose on serum glucose as well as the functions of liver and kidney. These results were consistent to the result of Chivapat and collaborates[21]. From that study, *P.mirifica* affected blood parameters only when the compound was given at 1,000 mg/kg/day. Toxic effects of estrogens on blood system have been shown in animal studies. Ninety day feeding rats with diet contained 10 and 50 ppm of 17β-estradiol demonstrated mild anemia with the mean value of hematocrit, RBC count and hematocrit lower than the control group[28]. Administration of diethy-stilbestrol, a synthetic estrogen, in the diet for two years caused a slight reduction in hemoglobin and hematocrit in both sexes of Sprague-Dawley rats[25]. A favorable effect of *P.mirifica* on the liver was interestingly demonstrated while this compound was given to high cholesterol diet rats. High cholesterol diet-fed condition caused a significant increase of serum hepatic parenchymal enzymes such as AST, ALT as well as the enzyme reflecting cholestasis such as ALP. Accompanying the unpleasant lipid profile with an increase of liver weight in high cholesterol diet rats, it is likely that an accumulation of fat in the liver might be involved in lipid-induced liver injury in this group of animals. *P.mirifica* caused an advantageous effect on serum lipid profile particularly in high cholesterol diet-fed rats as shown by an attenuation of serum hepatic enzymes which are indicators for liver injury. These findings gave a rational explanation for an attenuating effect of *P.mirifica* on lipid-induced liver injury in high cholesterol diet-fed rats.

In this study, effects of *P.mirifica* on serum lipid profile were investigated in both normal diet-fed rats and high cholesterol diet-fed rats. High cholesterol diet-fed rats in this study possessed a characteristic of type IIa hyperliproteinaemia according to Frederickson/WHO classification. This type of hyperlipoproteinaemia exhibits an elevation of LDL-C and total cholesterol in serum whereas serum triglyceride is not elevated. Type IIa hyperliproteinaemic patients possess high risk to atherosclerosis[31]. *P.mirifica* demonstrated both favorable and unfavorable effects on serum lipid profile. Advantageous effect of *P.mirifica* on serum lipid was a decrease of serum total cholesterol and LDL-C in either normal diet-fed or high cholesterol diet-fed condition. In contrast, the disadvantageous effect of this plant included an increase of serum triglyceride in normal rats and a decrease of serum HDL-C in both normal and high cholesterol diet-fed rats. This study provided an additional information to a previous study of Chivapat and collaborates[21] that found a decrease of serum total cholesterol and a slight increase of serum triglyceride in normal rats given the same dosage (100 mg/kg/day orally) of *P.mirifica* for 90 days. Eventhough excessive concentration in plasma of LDL-C is most theoretically and epidemiologically associated with atherosclerosis, hypertriglyceridaemia and reduced concentration of HDL-C are also important risk factors for this disease. Thus, precaution should be concerned for long-term administration of *P.mirifica* especially in normal person whereas the hypercholesterolemic person seem to be less affected (*P.mirifica* caused only a decrease of HDL-C, did not affect serum triglyceride, and improved the LDL-C/HDL-C ratio). Effect of *P.mirifica* on human serum lipid profile should be confirmed. Mechanism for explaining the lipid lowering effect of this plant should also be explored.

In conclusion, subchronic (90 days) exposure of *P.mirifica* given orally at 100 mg/kg/day to male Wistar rats did not show any toxic effects on blood system as well as functions of liver and kidney. *P.mirifica* even attenuated the hepatic injury induced by hypercholesterolemic condition probably due to its beneficial effects on lipid profile especially in high cholesterol diet-fed rats. Effects of *P.mirifica* at various doses, long-term uses as well as mechanism of the effects found in the study should be further investigated.

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