Toxicity Tests of a Phytoestrogen-Rich Herb; *Pueraria mirifica*

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A study to determine the toxicity of the powder and extract derived from the phytoestrogen-rich herb; *Pueraria mirifica* cultivar Wichai-III was carried out using a selection of animals and some human volunteers. The powder was used to determine the acute toxicity in mice with a 0.5 dilution criterion. It was found that the LD_{50} of the oral consumption of the powder was out of the range of 2,000 mg / kg b.w. A skin irritation test was performed on rabbits with 1 ml / head epidermal application. The rabbits showed no signs of irritation symptoms. A Draize test was performed on rabbits along with an eye irritation test. No irritation was observed up to the end of the 7^{th} day of the test period. A skin irritation test was conducted on guinea pigs by applying GPMT to assess contact allergic response. It was scored as a no allergic response. The extract-treated skin showed no allergic response after UV-A irradiation during the photo-toxicity test on guinea pigs. The primary human skin test using an applied Draize test revealed no meaningful allergic response. From all established toxicity tests, it may be concluded that *P. mirifica* cultivar Wichai-III should be outside any significance acute toxicology range if orally consumed or should not cause any significance skin sensitization if topically applied.

Key words: *Pueraria mirifica*, phytoestrogen and toxicity test.

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พิษวิทยาของสมุนไพรที่มีสารไฟโตเอสโตรเจนสูง ; *Pueraria mirifica*

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การศึกษาพิษวิทยาของผงและสารสกัดจากสมุนไพรที่มีสารไฟโตเอสโตรเจนสูง ; *Pueraria mirifica* สายพันธุ์วิชัย –3 ในการศึกษาพิษเฉียบพลันในหนูถีบจักรด้วยวิธีการเพิ่มความเข้มข้น 0.5 เท่าผลการทดสอบโดยการป้อนผงทางปากมีค่า LD_{50} มากกว่า 2,000 มก / น้ำหนักตัว 1 กก. การทดสอบผิวหนังกระต่ายด้วยสารสกัดในปริมาณ 1 มล / ตัวไม่พบอาการระคายเคือง การทดสอบผิวหนังโดยวิธีแดรซควบคู่กับการทดสอบเยื่อตาเป็นเวลา 7 วัน ไม่พบอาการอักเสบ การทดสอบการแพ้ต่อแสงของผิวหนังในหนูตะเภาโดยใช้วิธี GPMT พบว่าผิวหนังไม่แสดงอาการอักเสบภายหลังจากการได้รับแสงยูวี–เอ การทดสอบผิวหนังในอาสาสมัครโดยวิธีแดรซประยุกต์ไม่พบอาการระคายเคือง จากผลการทดสอบพิษวิทยาทั้งหมดสรุปได้ว่า *Pueraria mirifica* สายพันธุ์วิชัย –3 ทั้งในรูปผงและสารสกัดไม่ก่อให้เกิดพิษอย่างมีนัยสำคัญ

คำสำคัญ กวาวเครือขาว พิษวิทยา ไฟโตเอสโตรเจน

INTRODUCTION

Phytoestrogen consumption is becoming of great interest in the nutrition and public health sectors due to the rapid increasing awareness of the benefits to human health after long-term consumption, most frequently from legumes and beans [1, 2, 3] even though it was first noticed to induce sterility in animals.[4] Children who were baby-fed with soybean showed lower evidence of breast cancer when they became mature.[5] The maternal consumption of phytoestrogens was passed to the neonatal before birth.[6] Long-term consumption of phytoestrogen-rich diets in females also showed lower evidence of breast cancer development as well as colon cancer, artherosclerosis, menopausal symptoms and postmenopausal osteoporosis.[7, 8]

The key phytoestrogens in soy are isoflavones especially daidzin, daidzein, genistin and genistein. Daidzin was found to exhibit bone loss protection activity.[9] Daidzein could act as an immune enhancer,[10] and inhibitor of specific mutagenesis.[11] Genistin showed inhibitory effects to myoblast[12] as well as prostate cell line proliferation.[13] Genistein was seen as the most important ingredient as it could exhibit anti-breast cancer activity.[14-16]

Phytoestrogens from legumes have been analyzed and ranked for isoflavone content. It was found that *Pueraria lobata* or Kudzu which is abundance in Japan, Korea and China, contained daidzin and daidzein with many profound pharmacological actions including antidipsotropic activity,[17] was classified as the top ranking for daidzein content.[18] Even though there has been no direct comparison between that plant and the Thai herb; *Pueraria mirifica* (Airy Shaw et Suvatabandhu) or otherwise known as White Kwao Krua, it would be of great interest to introduce *P. mirifica* into the world of phytoestrogens as its consumption in Thailand had been long-recorded with a purpose similar to ERT at the present time.

P. mirifica is widely distributed in Thailand, and it has been domestically consumed as a rejuvenating drug for both male and female for a long time, especially by mature people. It was possible to prove the presence of the active ingredients which affect rejuvenation.[19] It was brought to medicinal attention as a potent phytoestrogen source, even though this terminology was not established at that moment. The study in animals showed the strong estrogenic effect of its key active ingredient, namely miroestrol, in both immature female mice as well as ovariectomized rats,[20,21] which was also confirmed in clinical trials in Great Britain.[22] Recently deoxymiroestrol was found to be the real key active ingredient with its strong estrogenic effects[23] as well as isoflavonoids when tested with the ER α-harboring human breast cancer cells.[24] Other active ingredients have also been found, including some of similar to those contained in soy such as daidzin, daidzein, genistin and genistein and different chemicals such as puerarin, kwakhurin, and coumesterol.[25-28]

As a proven phytoestrogen-rich Thai herb with a comparable strength for estrogen, the toxicity studies are not clear especially for the powder and extract derived from the plant. The powder derived from *P. mirifica*, especially cultivar Wichai III was recently developed into traditional medicines and dietary supplement products whereas the extract is widely used in various cosmetic products. Three patents were applies for based on product development from this plant cultivar. We therefore set up a range of toxicity tests in various animals to clarify *P. mirifica* as it is becoming a novel raw material for dietary supplements, and traditional medicines as well as cosmetics and topically applied pharmaceutical products.

MATERIALS AND METHODS

Plant materials

The fresh tuber of *P. mirifica* cultivar Wichai-III was collected from a field-grown plant, cleaned, peeled, sliced into pieces, dried in a hot air oven until nearly completely dried, ground into fine powder of 100 Mesh size and used as powder for the acute toxicity test as well as a serial dosage test. For the extract preparation, the fresh tuber was ground and mixed with the equal volume of extraction solution containing propylene glycol and sterile de-ionized water. The extract was kept for all skin tests.

Animals

The mice (ICR) used in the acute toxicity test, the New Zealand white rabbits used in the primary skin irritation test and the primary eye irritation test, Hartley guinea pigs used in the skin sensitization test as well as the photo-toxicity test, were inbred.

Animal husbandry

The animals were housed in polypropylene cages; balanced pellet animal feed and community tap water was provided, *ad libitum.* A standard laboratory environment of 25^{o}C, 12 hrs dark-light period was maintained.

Human subjects

Thirty healthy woman volunteers from the ages of 20-30 years old were selected with standard exclusion criteria for the skin sensitization test. The following were excluded from the tests. These including any volunteers undergoing medical treatment, pregnant women, those with spots or scars or any skin diseases, those with a history of Psoriasis, Eczema, atopic dermatitis or that of family members' disease history, as well as any volunteer involved in another on-going test or in a test last finished within a certain time lapse. They were informed that the test material was the extract derived from *P. mirifica* and signed a form of consent before testing.

Acute toxicity test

Five mature male (weight 30-35 g) and female (weight 28-32 g) mice (ICR) per group and per cage was set up as a 5 case study group-dilution with a common ration of 0.5 as 250, 500, 1,000 and 2,000 mg / kg b.w. as well as one control group. *P. mirifica* powder was mixed with 2,000 mg animal feed per mouse. The animals were kept under observation for the maximum period of 14 days whereas the group of 5 mice were chosen hourly, at the end of 1, 2, 3, 4, 5, 6 and 12 hrs and daily from day 1-14 for histopathology and gross findings of necropsy including brain, kidney, heart, lung, spleen, liver, stomach, intestine, pancreas, adrenal gland, pituitary gland, testis and ovary.

Primary skin irritation test [29, 30]

Six healthy male New Zealand white rabbits weight 4-5 kg with abraded skin at the back position were kept individually per cage, each assigned as 4 test sites, 2 of which were for tests by applying 0.5 ml of *P. mirifica* extract with the aid of the patch, the other two sites were for control by applying 0.5 ml of distilled water with the aid of the patch. The animals were kept for 24, 48 and 72 hrs observation.

Primary eye irritation test

Nine healthy male New Zealand white rabbits weight 4-5 kg were kept individuaaly per cage, each received eye-drop on the mucous membrane, 3 non-rinsed, and 6 with rinsed, 0.1 ml *P. mirifica* extract on one eye without any treatment on the other eye as a control to evaluate the damage to the cornea as described before as Draize test (29, 30). The animals were kept for 1, 2, 3, 4, and 7 days observation.

Skin sensitization test

Thirty mature male guinea pigs (Hartley) weight 1,000 – 1,150 g were divided into 3 group, 10 of each, and submitted to the GPMT. The first group was a test group receiving 0.1ml *P. mirifica* extract. The second group was a positive control group receiving 0.1 ml of 0.1% DNCB. The third group was a negative control group receiving 0.1 ml of 0.1% saline solution. At the primary sensitization stage, 0.1 ml of each test material was applied, 0.1 ml of FCA, 0.1 ml of test material and FCA was injected into both sides of a hairless guinea pig's stomach skin. While the primary skin sensitization test on *P. mirifica* extract was carried out to determine the secondary sensitization concentration. After obtaining the result that no positive response was found on the site applied with 100 % of test material when applying *P. mirifica* extract on the back of the guinea pig by various concentrations, the secondary sensitization induced concentration was determined as 100 % pure solution. After one week of the primary sensitization, the hair which grew on the site was cut and mild irritation was induced by 10% SDS (petrolatum) and test material was applied with the dosage of 1 ml to each animal on the 2 x 4 cm^2 of Whatman No. 3 filter paper and the paper was attached to the animal, enclosed with non-permeable plastic tape, rolled with pressure-dressing then close-patched for 48 hrs. After 2 weeks, the hair of the animal's waist was cut (5 x 5 cm^2) and as the previous case, 1 ml of test material was uniformly applied on Whatman No. 3 filter paper at a size of 2 x 4 cm^2 and the paper was covered with non-permeable plastic tape to keep it on and rolled with a pressure-dressing, and then close-patched for 24 hrs. After 24 hrs, the residual test material on the test site was cleaned and the occurrence of erythema and edema was checked. The next day, the test site was observed by the Magnusson and Klingman test method.[31]

Photo-toxicity test

Twenty mature male guinea pigs (Hartley), weight 1,000 – 1,150 g were divided into 4 groups, the same number for the *P. mirifica* extract-treated group, the CP-treated group, the 8-MOP treated group and the saline solution-treated group. The hair at the back part of the guinea pigs was cut and 3 sites of 2 x 2 cm^2 area was drawn on both sides, which totalled 6 sites. The right sites were specified as light-intercepted control ones and the left sites as light-radiated test ones. The treatment was carried out as followings. In the *P. mirifica* extract-treated group, a test material was prepared in 100% extract, 50% and 25% in saline solution. In the CP and 8-MOP treated group, test materials were prepared in 10 %, 1%, and 0.1% in DMSO solution and each solution was uniformly applied with 50 µl on each site. In the case of the saline solution group, 50 µl of saline solution was applied on all sites.

In all treated groups except for the saline solution-treated group, each test material was prepared as 10, 1 and 0.1% in DMSO solution and uniformly applied with 50 µl on the sites. In the saline-solution-treated group, 50 µl of saline solution was uniformly applied on all the sites. Thirty minutes after the application, the right site of the animal was light-intercepted with aluminum foil and UV-A (300-380 nm) was irradiated at 10 cm distance for 2 hrs by a photo-toxicity-generating system (Ultra irradiation system, BIOTRONIC, VILBER LAURMAT, France). The final energy of the system was set to be 15 J/cm. The intensity of the irradiated light was measured by photo-radiometer (Vilber). After 24, 48, 72 hrs of irradiation of UV-A, photo-toxicity was evaluated by observing the guinea pig's skin response.

Human skin sensitization test

Thirty women were submitted to a primary skin sensitization test by applying a 8 mm Finn Chamber skin patch containing 0.1 ml *P. mirifica* extract with a control of 0.1 ml saline solution on the other patch attach to the skin of each forearm and kept for 24 hrs as a closed patch. During the test period, any kind of influence, such as baths, intensive exercises, hard work and any medications which could influence this test results were not permitted. *P. mirifica* extract was divided into 3 sets for each of the 10 volunteers and defined as A for the control section, B, C and D for the test sections B, C and D were the same material used repeatedly to check out the meaning of this test result The observation for erythema and edema was performed by the naked eye in the next 24 hrs after removal of every patch and after waiting for 2 hrs.

RESULTS

Acute toxicity test

There were no deaths due to any administered amount for the duration of 1, 2, 3, 4, 5, 6, 12 hrs and 1-14 days after treatment at the dosage of 250, 500, 1,000 as well as 2,000 mg / kg b. w. and thus the calculation for LD_{50} is out of the range of 2,000 mg/kg b.w. The body weight gain was statistically different only in the female treated mice. The gross findings of necropsy including the liver, kidney, brain, heart, lung, spleen, stomach, intestine, pancreas, adrenal gland, pituitary gland, testis and ovary were normal as compared with the control group (Table 1). The results suggest that *P. mirifica* cultivar Wichai-III powder consumption for as long as 14 days causes no acute toxicity to the mice.

Table 1. Acute toxicity test results of *P. mirifica* powder on mice.

Observation/sex	male		female	
	control	2,000 mg/kg bw	Control	2,000 mg/kg bw
Mortality	0/5*	0/5	0/5	0/5
Histopathology and gross findings	NAD/5*	NAD/5	NAD/5	NAD/5
Gain of body weight (g)	7.25 $\pm$ 2.55	6.00 $\pm$ 0.20	5.00 $\pm$ 0.10	4.50 $\pm$ 0.10

NAD = No Abnormality Detected
* n = 5
** Independent Sample Test significant at $p < 0.001$

Primary skin irritation test

There were no related changes in erythema and edema as the skin irritation response of the observed rabbit skin in both *P. mirifica* and saline solution treated groups at 24, 48 as well as 72 hrs after administration was classified as "0" (Table 2) which suggests that *P. mirifica* cultivar Wichai-III extract causes no primary skin irritation to the treated rabbits.

Primary eye irritation test

There were no abnormal clinical signs of eye irritation and any significant change in body weight after 1, 2, 3, 4, and 7 days of treatment. The pathological change of the cornea, iris and conjunctiva in the eye irritation test was classified as "0" between the *P. mirifica* extract rinsed and non-rinsed eye of the experimental rabbits (Table 2) which suggests that *P. mirifica* cultivar Wichai-III extract causes no primary eye irritation to the treated rabbits.

Table 2. Primary skin and eye irritation test results of *P. mirifica* extract on Rabbits.

Observation/test			Skin irritation	Eye irritation	
				Group I	Group II
Number of animal			6	3	6
Mortality			0/6	0/3	0/6
Clinical change			NAD	NAD	NAD
Gain of body weight (g)			46.7 $\pm$ 9.29	81 $\pm$ 16.82	97 $\pm$ 16.16
P.I.I.	Erythema and Eschar		0	-	-
	Edema		0	-	-
Tissue score	Cornea	(AxBx5)	-	0/80	0/80
	Iris	(Ax5)	-	0/10	0/10
	Conjunctiva	(A+B+C)x2	-	0/20	0/20

Group I = *P. mirifica* extract rinsed group
Group II = *P. mirifica* extract non-rinsed group
NAD = No Abnormality Detected
P.I.I. = Primary Irritation Index :
0.0~0.5 : non-irritation, 0.6~2.0 : weakly irritate, 2.1~5.0 : moderately irritate, above 5.1 : strongly irritate

The Skin sensitization test

The positive control group (DNCB treated group) induced erythema on all the tested guinea pigs which demonstrated clearly positive response. The *P. mirifica* extract treated group and the negative control group (saline solution treated group) were scored as "0" on the sensitization score after 24 and 48 hrs treatment period (Table 3) while no different changes in body weight, no mortality as well as no clinical signs to suggest that *P. mirifica* cultivar Wichai-III

extract causes skin sensitization or any adverse effect to the treated guinea pigs were observed.

Table 3. Skin sensitization test results of *P. mirifica* extract on guinea pigs.

Observation/group		*P. mirifica* extract	0.1% DNCB (in 10% PG)	Saline
Number of animal		10	10	10
Mortality		0/10	0/10	0/10
Sensitization score	24 hrs	0	II*	0
	48 hrs	0	II*	0

DNCB = Dinitrochlorobenzene
PG = propylene glycol
Sensitization score :

0 = Nonsensitization, I = Weak sensitization, II = Mild sensitization,

III = Moderate sensitization, IV = Strong sensitizatin, V = Extreme sensitization

* Erythema

The Photo-toxicity test

The *P. mirifica* extract treated test group with the dosage of 25, 50% as well as 100% of the extract concentration showed the average score of UV-A radiation response as "0" after completion of the photo-toxicity test which means non-irritating. The results demonstrated clearly no dose dependent response and no-pigmentation. In the positive control group, the UV-A radiation site applying 0.1% of 8-MOP showed average-score "1.8" which means minimally irritating, 1% of 8-MOP showed average-score "2.3" which means minimally irritating and 10% of 8-MOP showed average-scored 3.1 which means mildly irritating. Especially in 1 and 10% of 8-MOP treated site, slight erythema was formed and the irradiated skin appeared thicker, coarser and harder than the untreated site. The UV-A radiation site of 0.1% of CP average-score "1.9" which means minimally irritating, 1% of CP average score "2.5" which means minimally irritating, 10% of CP average-score "3.2" which means mildly irritating. Especially the treated site of 0.1, 1 and 10% of CP-treated skin became thicker and coarser than the untreated skin. In the case of the saline solution-treated groups with the same dosage range as the previous ones, UV-A radiation sites showed average-score "0" or non-irritating. In all treated groups, the UV-A non-radiation site showed average-score "0" or non-irritating (Table 4). The results suggest that *P. mirifica* cultivar Wichai-III extract exhibits no photo-toxicity to the skin of the treated guinea pigs.

Table 4. Evaluation of photo-toxicity test of *P. mirifica* extract on guinea pigs.

		Applied concentration (%)									
		P.mirifica extract			8-methoxypsolaren			Chloropromazine		saline
		100	50	25	0.1	1	10	0.1	1	10	0.9
Non-irritation site	Total score/ Site No.[a]	0+0/ 10	0+0/ 10	0+0/ 10	0+0/ 10	0+0/ 10	0+0/ 10	0+0 / 10	0+0/ 10	0+0/ 10	0+0 / 10
	Mean score[b]	0	0	0	0	0	0	0	0	0	0
UV-irirtation site	Total score/ Site No.	0+0/ 10	0+0/ 10	0+0/ 10	9+9/ 10	13+10/ 10	17+14/ 10	10+9/ 10	15+10/ 10	17+15/ 10	0+0 / 30
	Mean score	0	0	0	1.8	2.3	3.1	1.9	2.5	3.2	0
Evaluation of irritation		Non	Non	Non	Mild	Intensive erythema	Intensive erythema with edema	Mild	Intensive erythema	Intensive erythema with edema	Non
Number of animal		5			5			5			5
Mortality		0/5			0/5			0/5			0/5
Clinical signs		NAD			NAD			NAD			NAD
Gain of body weight (g)		7.8 $\pm$ 2.4			6.1 $\pm$ 0.9			12.6 $\pm$ 4.5			4.0 $\pm$ 1.2
Remark					Eshar formation			Pigment			

a) Total score/site No :

Total highest possible erythema score + total highest possible edema score/ No. of erythema observation site (5) + No. of edema observation site (5) = 10

b) Score of skin irritation

0 = No visible, 1 = Mild erythema, 2 = Intensive erythema, 3 = Intensive erythema with

edema, 4 = Intensive erythema with edema and vesicle

Human primary skin sensitization test

The results of the patch test for human primary skin sensitization was analyzed according to the naked eye evaluation standard. It was found that the control group A, the test group B, the test group C and the test group D showed 0.03, 0, 0.6 and 0 index of the primary irritation respectively (Table 5). Compared to the control A section among test materials mentioned above, all *P. mirifica* extracts, test section B, C and D were evaluated to have no meaningful difference regarding skin irritation.

Table 5. Naked eye evaluation of the primary skin sensitization test on human volunteers.

Group	Numbers	Index of primary reaction	Evaluation*
Control group A	30	0.03	No visible
Test group B	10	0	No visible
Test group C	10	0.06	No visible
Test group D	10	0	No visible

* score	reaction	evaluation
0	-	No visible
1	+/-	Mild erythema
2	+	Intensive erythema
3	++	Intensive erythema with edema
4	+++	Intensive erythema with edema and vesicle

Discussion

The various toxicity tests of the powder as well as the extract derived from *P. mirifica* cultivar Wichai-III were evaluated in both animals and humans. Results from acute toxicity tests with a 5 case study group-dilution with a common ration of 0.5 in the IRC mice revealed no acute toxicity and the LD_{50} was out of the range of 2,000 mg/kg b.w. A precaution was stressed only in females if a very high amount was consumed, as seen from the different in body weight gain between the male and female acute toxicity tested mice. For human consumption, it is hard to reach

such a high single dose as the miroestrol in *P. mirifica* itself could initiate a feeling of nausea.[22] A sub-chronic and chronic toxicity test should be performed to ensure the safety of long-term consumption of low amounts. Hower, because *P. mirifica* has experienced long-term consumption in Thailand in traditional medicines, any old records of endemic consumption should partially guarantee the safety of the orally consumed products.[19]

P. mirifica extract has a strong potential for development into cosmetic products for skin care and in such areas as breast or even topical pharmaceutical products as it is a phytoestrogen-rich herb, especially its tuber which is used as the raw material for such products. The skin toxicity tests should be a beneficial parameter to evaluate the great potential of the plant. The rabbit primary skin irritation test showed no related changes in erythema and edema after a topical-application of the extract. This suggests that no skin allergic reaction could be initiated by the extract. The results were confirmed in the rabbit primary eye irritation test for the 7 day observation period after the eye mucosal treatment of the extract with or without rinsing. This observation not only demonstrated the non-allergic reaction to the soft tissue such as the eye mucosa but also opens up a possibility to develop the extract into an eye drop product as one of the estrogenic effects is to refresh the cornea and eye lens. The negative results to the skin sensitization test was also confirmed in guinea pigs submitted to GPMT as well as the photo-toxicity test in the same species of test animals which would ensure the safety of the topical application of the extract even if it should be directly exposed to sun-light, an important UV-A source or even in the case of repeated topical applications. The human primary skin sensitization test revealed that the extract caused no meaningful allergic reaction to human skin. This was an important test result as it demonstrated clearly that the extract which could be present in a vast variety of cosmetics and topical pharmaceutical products, is safe for human consumption by the topical route. Taking all the toxicity tests into account, a solid conclusion could be drawn that the extract derived from *P. mirifica* cultivar Wichai-III which contains high amounts of phytoestrogens and shows effectiveness in the treatment of menopausal symptoms[32] is safe for human dermal application. This finding should be an accelerating factor in the safety and efficacy testing of various cosmetics and topically applied pharmaceutical products containing the extract from the phytoestrogen-rich herb; *P. mirifica*. As the selected cultivar had been screened from the natural existing plant, e. g. cultivar Wichai-III, the future of *P. mirifica* consumption as well as the industrial scale production in both crude powder and extract form could also take advantage from this study.

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