การศึกษาเบื้องต้นถึงความเป็นพิษทางผิวหนังของผลิตภัณฑ์พอกหน้าที่มีส่วนผสมของสารสกัดจากไพลและหม่อน

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สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย

บทคัดย่อ

"ไพล" มีชื่อทางวิทยาศาสตร์ว่า *Zingiber montanum* จัดอยู่ในวงศ์ Zingiberaceae ในตำรับยาไทยแผนโบราณมีการนำเหง้าไพลมาใช้ประโยชน์ในการรักษา เช่น การต้านอักเสบ และ แก้ปวด เป็นต้น "หม่อน" มีชื่อทางวิทยาศาสตร์ว่า *Morus alba* L. จัดอยู่ในวงศ์ Moraceae ในหลายประเทศแถบทวีปเอเชีย มีการนำส่วนที่เป็นใบของหม่อนมาใช้ประโยชน์อย่างแพร่หลาย ซึ่งมีข้อมูลทางวิทยาศาสตร์ยืนยันว่าใบหม่อนมีฤทธิ์ในการต้านอนุมูลอิสระ ช่วยยับยั้งการสร้างเม็ดสี ทำให้ผิวดูขาวขึ้น ในงานวิจัยได้มีการนำสารสกัดเอธานอลของเหง้าไพล และใบหม่อนมาใช้เป็นส่วนผสมสำคัญในผลิตภัณฑ์พอกหน้า (PM) เพื่อใช้ในการบำรุงผิวหน้า สำหรับวัตถุประสงค์ในการศึกษาครั้งนี้ คือ เพื่อประเมินผลความเป็นพิษทางผิวหนังของ PM ในสัตว์ทดลอง โดยทำการศึกษาการก่อความระคายเคืองของ PM ที่มีต่อผิวหนังในกระต่าย ศึกษาความเป็นพิษเฉียบพลันทางผิวหนังในหนูขาว และศึกษาการก่ออาการแพ้ทางผิวหนังในหนูตะเภา จากผลการทดลอง สรุปได้ว่า PM อาจก่อให้เกิดอาการระคายเคืองเพียงเล็กน้อยซึ่งเป็นแบบเฉียบพลันในผิวหนังกระต่าย แต่ไม่ก่อให้เกิดอาการแพ้ต่อผิวหนังในหนูตะเภา และมีค่า LD_{50} ทางผิวหนังในหนูขาวมากกว่า 2,000 มิลลิกรัม/กิโลกรัม น้ำหนักตัว

คำสำคัญ: *Zingiber montanum*, *Morus alba* L., การทดสอบความเป็นพิษทางผิวหนัง, สารที่ทำให้ผิวขาว, ตัวยับยั้งเอนไซม์Tyrosinase

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Preliminary studies on dermal toxicity effects of the mask product derived from the ethanolic extracts of *Zingiber montanum* (Koenig) Link ex. Dietr. and *Morus alba* L.

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ABSTRACT

According to the Thai tradition medicine, the rhizome of *Zingiber montanum* (Koenig) Link ex. Dietr. (Syn. *Z. cassumunar* Roxb; in Thai "Phlai"; Fam. Zingiberaceae) has been therapeutically used as anti-inflammatory, analgesic drugs etc. Also Mulberry (*Morus alba* L.; in Thai "Mon"; Fam. Moraceae) leaves which are widely used in an oriental medicine, has been scientically proved as antioxidant, melanocyte inhibition and whitening agent. In this research, both of the ethanolic extracts of Phlai rhizome and mulberry leaves are active ingredients for the Mask Product (PM) which is proposed to be used as skin care commodity. The objective of this study was to estimate and establish dermal toxicity effects of PM in animal models. The skin irritation, acute dermal toxicity and skin sensitization studied were conducted by following the Test Guidelines No. 404; Acute Dermal Irritation / Corrosion, No. 402; Acute Dermal Toxicity Test and No. 406; Skin Sensitization of the OECD Guidelines for testing of chemical, in the rabbits, rats and guinea pigs, respectively. From the results, we concluded that the PM might cause mild skin irritation in rabbits but did not produce skin sensitization (Buehler method) in guinea pigs. The dermal LD_{50} of the PM in Wistar rats was more than 2,000 mg/kg body weight.

Keywords: *Zingiber montanum*, *Morus alba* L., Dermal toxicity test, Skin whitening, Tyrosinase ihibitor

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INTRODUCTION

Zingiber montanum (Koening) Link ex. Dietr. (Syn. *Z. cassumunar* Roxb.; in Thai "Phlai" Fam. Zingiberaceae) has been used for medicinal purposes in Thailand and Southeast Asia for centuries. It is distributed in tropical and subtropical Asia which has been under cultivation in India, China and Southeast Asian countries for a long time.[1,2] It is probably a native plant of India and is now widely cultivated in tropical Asia. It has traditionally been used in these regions as spice, food flavoring and medicine.[3] As in Thai traditional medicine, it is used as deflatulance and anti-inflammatory for sprain. In many countries, it is widely used in traditional remedies as a single plant or as a component of herbal recipes. The extract from its rhizome possess anti-inflammatory action, analgesic action, anti-allergic and antioxidant activities which has been used topically for the treatment of sprains, contusions, joint inflammation, muscle pain, abscesses and similar inflammation-related disorders.[4,5] *Morus alba* L. (Mulberry; in Thai "Mon" Fam. Moraceae) is a short-lived, fast-growing, small to medium sized mulberry tree which grows to 10 – 20 meters tall. It is also known as *Tuta* in Sanskrit and *Tuti* in Marathi. It is a native plant of the northern China and is widely cultivated in the north and northeast regions of Thailand. Mulberry leaves contain many nutritional components and the extracts from them have a potent antihyperglycemic activity in diabetic mice. Many phenolic compounds have been identified from the root bark of mulberry tree and shown a skin whitening effect. The methanolic extract of dried mulberry leaves has been reported for its *in vitro* effects on melanin biosynthesis. The extract inhibited the tyrosinase activity that converted dopa to dopachrome in the biosynthetic process of melanin.[6]

There are various causes for the darkening of skin color, UV rays considered the primary source. Once skin is exposed to UV rays, melanin is synthesized in melanocytes and released to darken the skin. Melanin pigmentation in human skin is a major defense mechanism against UV light from the sun, but abnormal pigmentation such as freckles or chloasma can be a serious aesthetic problem. Tyrosinase is responsible and plays the most important role for the melanin biosynthesis. The extracts and components isolated from mulberry, commonly used as tyrosinase inhibitor.[7] An understanding of the melanin synthesis mechanism is very important to the development of cosmetic products having whitening effect. Thus, both of these plants are highly beneficial herbs due to their therapeutic indications and the reported ethnomedical uses which make them as attractive candidates for the development of cosmetics, spa products and skin whitening products. In this research, both of the ethanolic extracts of Phlai rhizome and mulberry leaves are active ingredients for the Mask Product (PM) which is proposed to be

used as skin care commodity. The assessment of dermal toxicity in animal models is needed to establish the level and reversibility of the toxicity. The results may provide dose for other studies and will be beneficial to clinical manifestations.

MATERIALS

Animals

Healthy adult albino rabbits of New Zealand white hybrid strain, weighting 2-3 kg were used in acute dermal irritation test. They were purchased from Department of Animal Science, Faculty of Agriculture, Kasetsart University. They were kept in controlled conditions at the room temperature of 22-25°C and relative humidity of 65 ±5 percent. They were housed individually in stainless steel cages, 1 animal per cage and were fed with food (Pokphan Animal Feed Co., Ltd., Thailand) and water *ad libitum*.

Healthy adult male and female Wistar rats, *Rattus norvegicus*, 5-7 weeks old were used in acute dermal toxicity test. They were supplied by the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon pathom. They were kept in controlled conditions at the room temperature of 22-25°C and relative humidity of 65 ±5 percent. They were housed in stainless steel cages, 5 animals with the same sex per cage and were fed with food (Pokphan Animal Feed Co., Ltd., Thailand) and water *ad libitum*.

Healthy adult male and female albino guinea pigs of Dunkin Hartley strain, weighting 250-300 g were used in skin sensitization test. They were supplied by the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon pathom. They were kept in controlled conditions at the room temperature of 22-25°C and relative humidity of 65 ±5 percent. They were housed in stainless steel cages, 5 animals with the same sex per cage and were fed with food (Pokphan Animal Feed Co., Ltd., Thailand) and water *ad libitum*.

Test material

The Mask Product (PM) was prepared by mixed compounds which used the ethanolic extracts of Phlai rhizome and mulberry leaves as active ingredients. It was a greenish cream with characteristic odor.

METHODS

1. Acute Dermal Irritation Test

The test of Acute Dermal Irritation / Corrosion was conducted according to the Test Guideline (TG) No. 404 of the OECD Guidelines for Testing of Chemicals (2002).[8] Three rabbits were employed and acclimatized to the laboratory environment for one week. One day before experimentation, an area of skin approximately 10 cm × 10 cm on the dorso-lumbar region of each rabbit was clipped free of hairs. Two areas of the shaven skin approximately 2.5 cm × 2.5 cm were selected. 0.5 ml of the PM was introduced onto a 2.5 cm × 2.5 cm gauze patch, which was served as a treated patch while 0.5 ml of base cream on

another patch was served as a control patch. The entire trunk of the rabbit was wrapped with elastic cloth to avoid dislocation of the patches for 4 hrs. At the end of the exposure period, all patches were removed and gently wiped the treated skin with moistened cotton wool to remove any residual test material. The rabbits were assessed for the degree of erythema and oedema evidence on each site at 1 hr, 24, 48 and 72 hrs after removal of the patches. Further observation would be needed, as necessary, to establish the reversibility if the irritation sign(s) still existed, but would not exceed 14 days after application. The skin reactions were independently scored by two inspectors using the numerical scoring system as follows.

Erythema and eschar formation:	**Score**
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Oedema formation:	**Score**
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well- defined by definite raising)	2
Moderate oedema (raised approximately 1 mm.)	3
Severe oedema (raised more than 1 mm. and extending beyond the area of exposure)	4

2. Acute Dermal Toxicity Test

The acute dermal toxicity test (Limit test) was carried out following the Guideline No 402 of the OECD Guideline for Testing of Chemicals (1987).[9] Twenty Wistar rats (ten of each sex) were divided into two groups defined as the control and treatment groups. Each group consisted of both sexes at equal number. They were acclimatized to the laboratory environment for one week prior to experimentation. The rats were randomly chosen using the simple random sampling method and identified numbers by tail labeling.

At 24 hrs before the test, an area of skin approximately 5 cm × 5 cm on the dorso-lumbar region of each rat was clipped free of hairs. Care was taken to avoid abrading on the skin. The PM at the dose of 2,000 mg/kg body weight was introduced onto the 3.5 cm × 3.5 cm gauze patch, applied on the shaven skin of the rats of the treatment group. In the same manner, base cream at equivolume to the treatment group was used in place of the test sample and applied to the rat's skin of the control group. The patches were then secured onto the skin by transpore

adhesive tape. The entire trunk of each rat was wrapped with elastic cloth to avoid dislocation of the patches for an exposure period of 24 hrs. Each rat was returned to its designated cage and was not restrained. At the end of the exposure period, the dressing was removed and the skin was gently wiped with distilled water moistened cotton wool to remove any residue test material.

The rats were observed at 1/2, 1 and 3 hrs after patch removal and once daily for 14 days thereafter for signs of gross toxicity and mortality. Body weight of rats were recorded on day 1, day 8 and day 15 (at termination) or after death. Gross necropsies were performed on all decedents. On day 15, all survival rats were euthanasia by CO_2 asphyxiation and gross pathology was performed. The mean of body weight gain of treated rats was calculated in comparison to that of the control rats using Student's t-Test ($p \leq 0.05$).

3. Skin Sensitization Test

Skin sensitization test of the PM was conducted according to the Test Guideline (TG) No. 406: Skin Sensitization (Buehler Method) of the OECD Guidelines for Testing of Chemicals (1992). Forty guinea pigs (20 of each sex) were employed and acclimatized to the laboratory environment Skin sensitization test of the PM was conducted according to the Test Guideline (TG) No. 406: Skin Sensitization (Buehler Method) of the OECD Guidelines for Testing of Chemicals (1992).[10] Forty guinea pigs (20 of each sex) were employed and acclimatized to the laboratory environment for one week. One day before experimentation, left flank of each guinea pig was clipped free of hairs on area approximately 2.5 cm x 2.5 cm The guinea pigs were randomly assigned to meet the numbers of the animals in 3 groups as follow:

(1) Treatment group (PM; 10 of each sex).

(2) Positive control group (0.075% (w/v) DNCB in absolute alcohol; 5 of each sex).

(3) Negative control group (base cream; 5 of each sex).

3.1 Induction: Topical application

Day 0 – Treatment group

0.5 ml of PM was introduced onto a 2.5 cm. x 2.5 cm. occlusive gauze patch and placed on the shaven skin and secured to the skin of the test animal by transpore adhesive tape. The entire trunk of the animal, each was wrapped with elastic cloth to avoid dislocation of the patch for 6 hrs exposure period. At the end of the exposure period, all patches were removed and gently wiped the treated skin with a water moistened cotton wool to remove any residual of test sample.

Day 0 – Positive control group

The same manners as those in Day 0 – Treatment group were conducted. But 0.5 ml of 0.075 % (w/v) dinitrochlorobenzene (DNCB) was used in place of the test sample.

Day 0 – Negative Control group

The same manners as those in Day 0 – Treatment group were conducted. But 0.5 ml of base cream was used in place of the test sample.

Days 6-8 and 13-15-Treatment group, Positive control group and Control group

The same applications as on Day 0 of each group were repeatedly carried out on the same test area (clipped newly grown of hairs, if necessary) of the same flank on Days 6-8, and again on Days 13-15.

3.2 Challenge application

Day 27-Treatment group, Positive control group and Negative control group

The untreated flank (right flank) of treated and control animals were clipped free of hair (closely-clipped). An occlusive patch, separately containing the appropriate amount of test substance and positive control substance were applied to the untreated flanks previously mentioned. The same manner as those in Day 0 - treatment group and Day 0 – positive control group were performed. Skin reactions were evaluated and recorded at 24 and 48 hrs after the patch application.

Observation of skin reaction

After an application of the challenging patch, skin reactions were evaluated and recorded at 24 and 48 hrs according to the following Magnusson and Kligman grading score system:

0 = No visible change

1 = Discrete or patchy erythema

2 = Moderate and confluent erythema

3 = Intense erythema and swelling

RESULTS

1. Acute Dermal Irritation Test

After removal of the patches, the treated skin was assessed for the degree of erythema and oedema evidence at 1 hour, 24, 48 and 72 hrs. Two out of three treated rabbits exhibited slight erythema of skin observed at 1 hr and the recovery of skin reaction was found within 24 hrs of the observation period. It indicated that the PM might cause mild skin irritation in acute. The scores of skin reactions of the rabbits treated with base cream (control area) and the PM (treated area) were shown in Tables 1 and 2, respectively.

2. Acute Dermal Toxicity Test

After removal of the PM, all rat appeared normal and survived to the termination (Day 15). The mean of body weight gain of treated rats with the PM at the dose of 2,000 mg/kg body weight was not statistically difference from those of the control rats (Table 3). No gross pathological lesions in brain, heart, lung, liver, kidney and genitals were observed on necropsy. The result of this study provided the dermal LD_{50} of the PM in Wistar rats which was more than 2,000 mg/kg body weight.

3. Skin Sensitization Test

The treatment of the PM did not show skin reactions including erythema and swelling which were evaluated at 24 and 48 hrs after challenge application. It indicated that the PM did not produce skin sensitization in guinea pigs. The summary of skin sensitization data of the control and treatment groups were shown in Table 4

Table 1. The skin reaction scores of the control area on the rabbits' skin treated with base cream.

Rabbit No.	Scoring time (hr)							
	1		24		48		72	
	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0

Table 2. The skin reaction scores of the treated area on the rabbits' skin treated with the PM.

Rabbit No.	Scoring time (hr)							
	1		24		48		72	
	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
1	0	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0	0

Table 3. The summary of skin sensitization data of the control and treatment groups.

Sex	Treatment/Dose	*Mean of body weight gain (g)	
		Day 8	Day 15
Male	**Control group** Base cream equivolume to the treatment group	15.80 ± 2.33	24.60 ± 2.85
	Treatment group "PM" 2,000 mg/kg body weight	13.80 ± 1.39	24.00 ± 1.56
Female	**Control group** Base cream equivolume to the treatment group	19.60 ± 1.16	29.80 ± 1.28
	Treatment group "PM" 2,000 mg/kg body weight	11.60 ± 0.24	24.60 ± 4.15

*Data are mean ± SEM

Table 4. The summary of skin sensitization data of the control and treatment groups.

Experimental group/ Test sample	*Response Score				Incidence of sensitization	Percentage of sensitization
	0	1	2	3		
(1). Treatment group (PM)						
Challenge - 24 hrs	20	0	0	0	0/20	0%
Challenge - 48 hrs	20	0	0	0	0/20	0%
(2). Positive control group						
Challenge - 24 hrs	0	7	3	0	10/10	100%
Challenge - 48 hrs	0	7	3	0	10/10	100%
(3). Negative control group						
Challenge - 24 hrs	10	0	0	0	0/10	0%
Challenge - 48 hrs	10	0	0	0	0/10	0%

* Number of guinea pigs exhibiting skin reactions (skin was scored based on the Magnusson and Kligman grading).

DISCUSSION

In the present study, the results showed that the PM could produce mild skin irritation in rabbits but could not produce skin sensitization in guinea pigs. The dermal LD_{50} in Wistar rats was more than 2,000 mg/kg body weight. The acute dermal toxicity test is initially conducted with the aim to establish a dosage regimen in subchronic and other studies, and may provide initial formation on the mode of the toxic action of test substance. Dosing animals with test substance at the dose of 2,000 mg/kg body weight is generally considered high enough and if no mortality or significant toxic signs are seen in animals receiving this dose, no higher doses are required for further study.[9] In the previous studies, the toxicity of the ethanolic extract of Phlai rhizome has been reported. The oral LD_{50} of Phlai rhizome extract in rats was 20 g/kg body weight and there was no toxic effect when intraperitoneally (i.p.) injection of this extract at the dose of 10 g/kg body weight to the rats.[11]

Also mulberry leaf extract has been reported as safe for use. Following a single i.p. dose, the LD_{50} of mulberry leaf extract in the mice and Wistar rats were approximately 4 and 5 g/kg body weight, respectively. This extract was administered orally dose as high as 5 g/kg body weight did not cause any significant toxic effect.[12]

CONCLUSION

In conclusion, our results demonstrated that the PM which used the ethanolic extracts of Phlai rhizome and mulberry leaves as active ingredients was rather safe for use as skin care product. All results did not show any dermal toxicity signs to the animals. The acute dermal toxicity test proved the LD_{50} dose in Wistar rats which was more than 2,000 mg/kg body weight. These toxicity studies might provide enough evidences to consider further development for cosmetics, spa products and skin whitening products.

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