Growth Suppression of Hamster Flank Glands by Topical Application of an Extract from "Kwao Keur" (*Pueraria mirifica*)

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Abstract

Hamster flank gland growth is androgenic dependent and a widely used model for the study of topically applied androgens and anti-androgens. A chloroform-methanol extract of "Kwao Keur" (*Pueraria mirifica*) was tested using this model. The extract was applied on one of the paired flank glands leaving other as a control. The results showed that the extract effectively suppressed flank gland growth. The local effect was also indicated by the different growth between treated site (-2.6 %) and untreated site (6.8 %). Acute dermal irritation test of the extract was also conducted according to the Test Guidelines (TG) No. 404 of OECD Guidelines for Testing of Chemicals. Very slight degree of erythema formation was observed in one out of three treated sites on the skin of rabbits. This skin reaction was fully recovered within 24 hours. Since the effect of the extract was localized with low skin irritation, it may be potentially useful for treatment of androgen-dependent skin disorders.

Keywords: Anti-androgen; *Pueraria mirifica*; Suppression hamster flank growth

Introduction

Androgenic alopecia, acne, seborrhea, hidradenitis suppurativa and hirsutism are the skin disorders that are associated with hyperactivity of androgenic hormones (Mauvais-Jarvis et al., 1969; Milne, 1969; Wilson and Walker, 1969; Birgham and Shaw, 1973; Liang et al., 1983; Labrie, 1991). While anti-androgens are accepted as therapeutic agents for prostatic diseases, the use of these compounds for the mentioned androgen-mediated cutaneous disorders is still limited. Since these skin conditions are non-life threatening, it is important for the drug candidate and its formulation to exert their effect directly at the area of application and at a minimum of any systemic side effects. Thus, in an interest of exploring and enhancing the local anti-androgenic action and reducing the potential systemic side effects, there has been considerable interest into the topical delivery of anti-androgenic substances. Paired hamster flank glands are highly sensitive to androgen stimulation (Hamilton and Montagna, 1950). and widely used for the study of topically applied androgens and anti-androgens (Matias and Orentreich, 1983; Chen et al., 1995; Liang and Liao, 1997). An advantage of hamster flank gland model is that a test substance can be topically applied to one flank area leaving another as a control. Many compounds including naturally occurring substances have been investigated and their effects have been reported, for example, unsaturated fatty acids (Liang and Liao, 1992), (-)-epigallocatechin-3-gallate (Liao and Hiipakka, 1995), and curcumin (Liao et al., 2001). Therefore, we tested an extract of "Kwao Keur", *Pueraria mirifica* Airy Shaw & Suvatab (Family: Leguminosae), for its anti-androgenic activity using the hamster flank model. "Kwao Keur" is a rejuvenating Thai folk medicine which has been reported to exert an effect on the sexual related physiological conditions. In addition, we examined the potential acute dermal irritation of the extract.

Materials and methods

Materials

"Kwao Keur" powder was a gift from Associate Professor Yuthana Smitasiri, Mae Fah Luang University, Chiang Rai, Thailand. The sample was collected from Ban-Tum District, Ampur Dok-Kom-Tai, Phayao Province, Thailand. A voucher specimen (MFLU # 307) has been deposited at the Herbarium of Mae Fah Luang University. Ethanol USP, chloroform, and methanol were purchased from Labscan Asia Co., Ltd. (Thailand). Flutamide was ordered from Sigma Chemical Company (USA). Propylene glycol was a product of K.H. Co., Ltd. (Thailand). All water used was deionized.

Extraction

One and a half kg dry powder from a root part of "Kwao Keur" was extracted with 3.5 l ethanol USP at 50-60^{o}C for 24 hrs by Soxhlet extraction method. The ethanolic extract was collected and evaporated at 50^{o}C under vacuum for 12 hrs. The concentrated viscous liquid was then mixed with the small amount of silica gel 60. The mixture was packed in a pre-packed (6 cm height silica gel 60) column of 13 cm diameter and 10 cm height. The column was eluted with $CHCl_3$:Ethyl acetate (1:1) until the elucidate was clear and colorless. The column was further eluted with $CHCl_3$:MeOH (1:1) until the elucidate was clear and colorless. The elucidate of $CHCl_3$:MeOH was then evaporated to dryness at 50 ^{o}C under vacuum. The extract was kept in a refrigerator at 4^{o}C until use. Fingerprint of extracts were detected using HPLC equipped with a LC-10AT pump, a Rheodye injector with 20 µl loop, and a SPD-M10A photodiode array detector (Shimadzu, Japan). Separation of the compositions in an extract was achieved with a flow rate of 1 ml/min. on a 4.60 x 250 mm, 5 µm Phenomenex Luna C18 column with C18 guard column. The mobile phase consisted of water:MeOH (9:1) pH 3.2. Compositions of an extract were detected by UV absorption at 225 nm.

Formulations

Test formulation (GX):

"Kwao Keur" extract 1 mg/20 µl consisting of propylene glycol 40% and ethanol USP 60%.

Positive control (F):

Flutamide 300 g/20 µl consisting of propylene glycol 40% and ethanol USP 60%.

Negative control (C):

Propylene glycol 40% and ethanol USP 60%.

In vivo anti-androgenic test

Young adult male golden Syrian hamsters of 8 weeks old were used. All animal handling and testing were conducted in accordance with the US guidelines (NIH publication #85-23, revised in 1985) for laboratory animal use and care. On day 0, the animals were anesthetized by intraperitoneal injection (IP) of 50-70 mg/kg sodium pentobarbital. Hairs on both sides of the flank areas were removed with an electric clipper and the area was measured with a caliper. Twenty microlitrs of test formulation were administered on only one side of hamster flank gland per animal once daily from Monday to Friday for 4 weeks. The other flank gland of each animal served as a control for systemic action. The animals were kept under anesthesia for at least 2 hrs and then put into custom-made jackets to prevent the possibility of oral intake of the formulation by the hamsters from grooming. Minimum of six animals were treated with each formulation. On day 29, the animals were euthanized by a lethal dose (200 mg/kg) injection of sodium pentobarbital. The area of flank glands from both treated and untreated sides were compared in the product of the

long axis and the short axis of the pigmented macule. The skin containing the flank glands was excised, fixed in 10 % buffer formalin and sectioned along the long axis of the gland. The tissue sections were stained with hematoxylin and eosin for microscopic examinations with microscope.

Acute Dermal Irritation Test

Acute dermal irritation test was conducted according to the Test Guidelines (TG) No. 404 of the OECD Guidelines for Testing of Chemicals (1993). Three healthy adult White New Zealand rabbits (weight 2 - 3 kg) were used. The rabbits were housed and acclimatized to the laboratory environment for one week. One day before experimentation, an area of skin approximately 10 cm x 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 x 2.5 cm were selected and marked. Half militirs of the test formulation (GX) was applied onto 2.5 cm x 2.5 cm gauze patch while 0.5 ml distilled water on another patch was served as control. Both gauze patches were applied to the selected skin sites on each rabbit. The patches were then secured to the skin by transpore adhesive tape. The entire trunk of the rabbit was wrapped with an elastic cloth to avoid dislocation of the patches for 4 hrs. At the end of the exposure period, all patches were removed and the treated skin was gently wiped with moistened cotton wool to remove any residual test formulation. The animals were assessed for the degree of erythema and oedema evidence on each site at 1, 24, 48 and 72 hrs after removal of the patches. Further observations would be needed to establish reversibility if the irritation sign(s) still existed (not exceeding 14 days after application). In addition to the observation of irritation, any lesions and other toxic effects were recorded. The skin reactions were scored by two inspectors independently by the following numerical scoring system.

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

Results and discussion

HPLC fingerprints of an ethanolic extract and an elucidate of $CHCl_3$:MeOH were shown in Figure 1. An effect of topical application of a chloroform-methanol extract of "Kwao Keur" on hamster flank gland growth is shown in Table 1. The data clearly demonstrate that the extract possessed an anti-androgenic activity. Growth suppression of the extract on the treated side is greater than that of the untreated side. Flank area of the treated side decreased by 2.6 % of original size, but that of the untreated side still increased, however, with only 6.8 % of original size. The areas of the treated (34.0 ± 1.6 mm^2) and untreated (38.9 ± 1.8 mm^2) sides after 4 weeks topical application of the extract were statistically different when compared at 95% confidence interval by

paired Student's t-test. The differences in growth suppression between treated and untreated sides are also shown in the group of hamster treated with flutamide. The reason for size reduction of untreated side is systemic absorption. The data form this study are different from that previously reported by Chen and his co-workers (Chen et al.,1995). They found no difference on the flank areas of the treated and untreated sides. The similarity in the effect of flutamide on both sides in Chen's study may be due to an oral intake of flutamide by hamster grooming. In our study, protective jackets prevented the mentioned error. However, flutamide is more potent than the extract. Flank areas of hamster treated with 300 μg flutamide decreased 13.0 % of original size while that treated with 1 mg extract decreased only by 2.6 %. The increase of the size of the flank gland of untreated side on the animals treated with the extract is, however, much lower than that of the controlled group (20.0 % and 26.7 % on the vehicle treated and untreated side, respectively). Flank areas of treated (40.1 ± 2.1 mm^2) and untreated (42.7 ± 2.9 mm^2) sides in the control group of the animals are not statistically significantly different when each animal was compared at 95 % confident interval by paired Student's t-test. Thus, the extract seems to have a local action on the hamster flank gland. Once again, the reason for the growth suppression also of the untreated side is the systemic absorption of the extract from the treated side. Once the extract get absorbed into the skin of the flank gland, blood supply underneath the skin will bring the extract to the contralateral side. However, only the slight degree of an effect implies that the systemic effect might be minimized by adjusting the dose of the extract on the treated side. The effect of the extract on the histopathology of flank glands was also examined and showed in Figure 2. No damaged tissues or physiological alteration of the flank glands were observed. There is also no indication of infiltration of any inflammatory cell.

Table 1 Effect of topical application of chloroform-methanol extract of "Kwao Keur" (*Pueraria mirifica*) and flutamide at doses of 1 mg and 300 μg respectively on the flank gland in intact young adult male golden Syrian hamsters ($n \geq 6$).

Formulations	Gland size before treatment (mm^2 ± se)		Gland size after treatment (mm^2 ± se)		% change	
	Treated side	Untreated side	Treated side	Untreated side	Treated side	Untreated side
GX	34.9 ± 1.4	36.4 ± 1.5	34.0 ± 1.6	38.9 ± 1.8	- 2.6	6.8
F	33.3 ± 1.3	36.0 ± 1.5	28.9 ± 2.5	34.0 ± 2.9	- 13.0	- 5.4
C	33.4 ± 2.3	33.7 ± 1.2	40.1 ± 2.1	42.7 ± 2.9	20.0	26.7

The scores for skin reactions on the rabbits skin treated with distilled water (control group) and test formulation (GX) are shown in Table 2. The data show that only one single treated site out of three rabbits showed a very slight degree of erythema formation 1 hr after the end of exposure time. This skin reaction recovered within 24 hrs. This indicated that the test formulation is no to very slightly irritating when topically applied onto the skin.

Table 2 Scores for skin reactions of the control and treated sites on the skin of rabbits after treated with 0.5 ml distilled water and test formulation (GX), respectively (n = 3).

Time (hrs)	Distilled water						GX					
	Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 1		Rabbit 2		Rabbit 3	
	E^a	O^b	E^a	O^b	E^a	O^b	E^a	O^b	E^a	O^b	E^a	O^b
1	0	0	0	0	0	0	0	0	1	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0	0	0	0	0

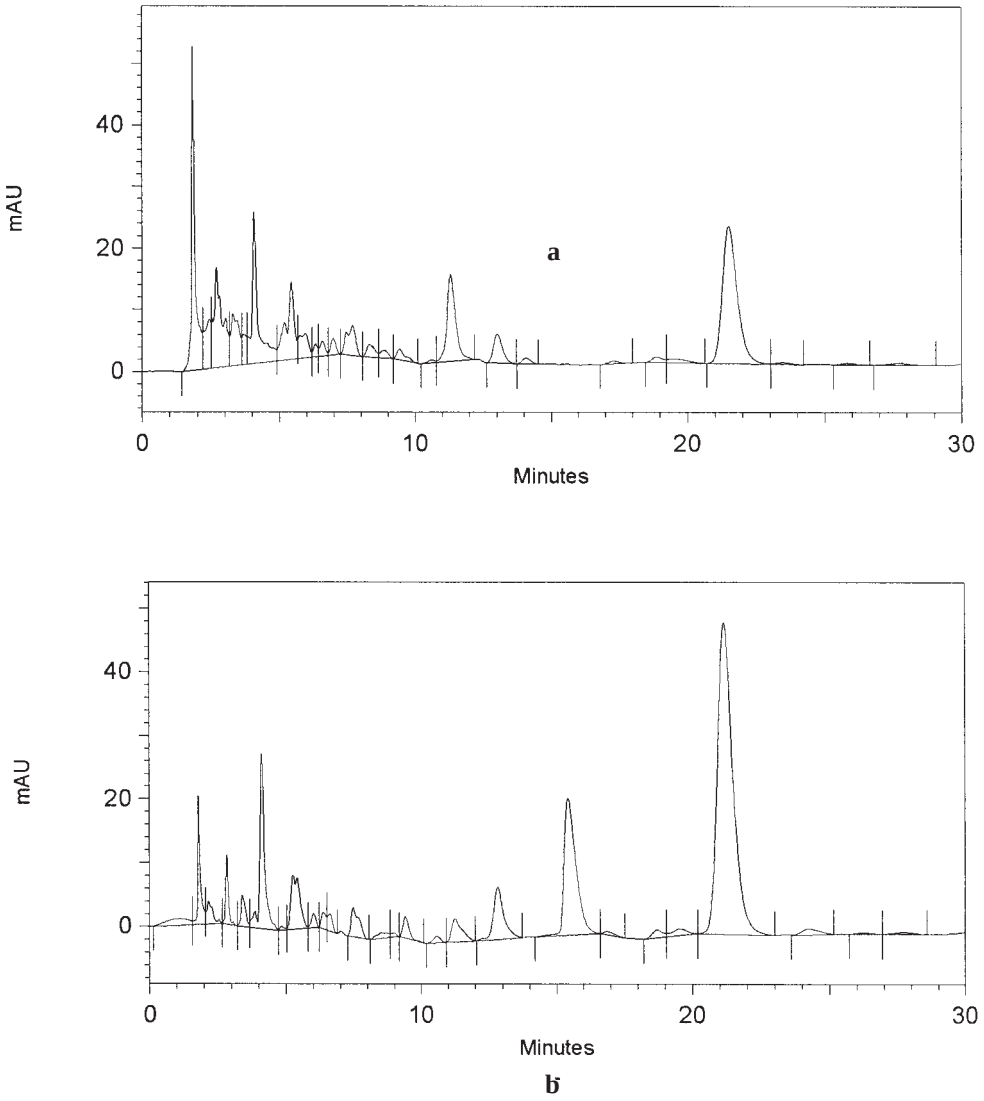

Figure 1 HPLC fingerprint of (a) an ethanolic extract of "Kwao Keur" and (b) an elucidate of $CHCl_3$:MeOH.

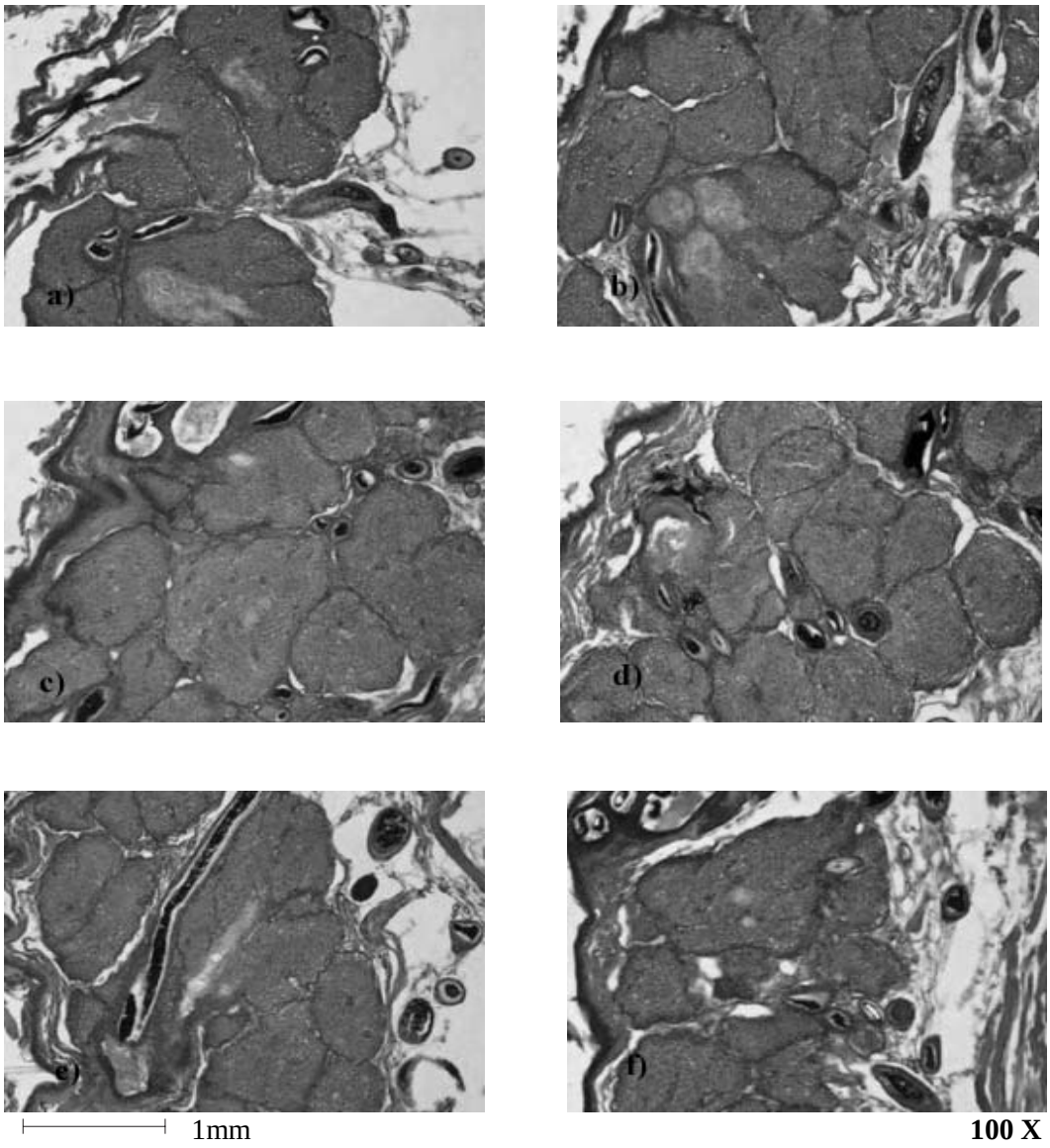

Figure 2 Light micrographs of sebaceous glands from a random cross section of flank glands of male hamsters; (a) untreated side of negative control group, (b) treated side of negative control group, (c) untreated side of "Kwao Keur" extract treated group, (d) treated side of "Kwao Keur" extract treated group, (e) untreated side of positive control group, and (f) treated side of positive control group.

Conclusions

Based on the anti-androgenic activity and very slight skin irritation, the chloroform-methanol extract of "Kwao Keur" (*Pueraria mirifica*) appears to be useful for the treatment of androgenic skin disorders. However, additional studies are needed to determine whether the findings described in this report are safe to be used in a clinical study. Anti-androgens with systemic activity may be teratogenic to embryos (Imperato-McGinley and Guatier, 1986; Russell and Wilson, 1994). Specifically, teratogenic effect of anti-androgenic substances has been found on sexual organs formation and development to male fetus.

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