

Anti-androgenic and Hair Growth Promoting Activities of *Lygodii Spora* (Spore of *Lygodium japonicum*) I. Active Constituents Inhibiting Testosterone 5 α -Reductase

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The aqueous ethanol extract of *Lygodii Spora* (spore of *Lygodium japonicum* (THUNB.) SW.) showed *in vitro* testosterone 5 α -reductase inhibitory activity and *in vivo* anti-androgenic activity using growth of flank organ in castrated Syrian hamsters and hair regrowth after shaving in testosterone-treated C57Black/6CrSlc mice. From the lipophilic constituents of *Lygodii Spora*, oleic, linoleic and palmitic acids were identified as the main active principles inhibiting testosterone 5 α -reductase.

Key words *Lygodium japonicum*; testosterone 5 α -reductase; anti-androgenic activity; hair regrowth

In many tissues including the prostate and skin, testosterone is metabolized intracellularly by the enzyme testosterone 5 α -reductase to an active androgen, 5 α -dihydrotestosterone, which binds to androgen receptors and shows various hormonal actions.¹⁾ Androgens have been indicated to be factors inciting common baldness, and both androgenic action and a genetic predisposition to be prerequisite to development of alopecia.²⁾ Thus a topical treatment with an anti-androgenic agent which exhibited inhibitory activity in testosterone 5 α -reductase and/or in the successive biological processes, e.g. binding between 5 α -dihydrotestosterone and androgen receptor, and protein synthesis may be useful for protection of alopecia.

During the course of our search for natural crude drugs having anti-androgenic activity, we have reported active constituents inhibiting testosterone 5 α -reductase from *Anemarrhena Rhizoma* (tubers of *Anemarrhena asphodeloides* BUNGE, Liliaceae)³⁾ and anti-androgenic activity of *Myrica Cortex* (barks of *Myrica rubra* SIEB. et ZUCC., Myricaceae).⁴⁾ In a continuing literature search on Chinese medicinal herbals, *Lygodii Spora* (spore of *Lygodium japonicum* (THUNB.) SW., Schizaeaceae) has been prescribed for the treatment of dysuria, male urodynia, nephritis, urinary calculus, and urinary tract infection.^{5,6)} Among these functional disorders, dysuria and male urodynia are nowadays said to result from benign prostatic hyperplasia. Several anti-androgens including testosterone 5 α -reductase inhibitors have recently been used for the therapeutic treatment of this hyperplasia. Thus, *Lygodii Spora* was our targeted natural crude drug and was expected to have anti-androgenic and hair growth promoting activities. We found that the aqueous ethanol extract of *Lygodii Spora* showed *in vitro* testosterone 5 α -reductase inhibitory activity and *in vivo* anti-androgenic activity using growth of the flank organ in castrated Syrian hamsters and hair regrowth after shaving in testosterone-treated C57Black/6CrSlc mice.

In this paper, we describe the *in vitro* and *in vivo* anti-androgenic activity of *Lygodii Spora* and the testosterone 5 α -reductase inhibitory activity guided fractionation of *Lygodii Spora* extract leading to obtain active fatty acids: oleic, linoleic and palmitic acids.

MATERIALS AND METHODS

Plant Materials *Lygodii Spora* produced in China was purchased from Tochimoto Tenkaido Pharmacy Co. Ltd. (Osaka, Japan).

Reagents Testosterone, 5 α -dihydrotestosterone, ethinyl-estradiol, and standard fatty acids were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and oxendolone (Prostetine[®]) was purchased from Takeda Chemical Industries Ltd. (Osaka, Japan). Other chemical reagents were reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. unless otherwise noted.

Extraction and Fractionation of *Lygodii Spora* 1) 50% Ethanol Extract: *Lygodii Spora* (50 g) was extracted with 50% ethanol (EtOH) (250 ml) at room temperature for 3 d. After filtration, the extract was concentrated under reduced pressure followed by lyophilization to give a 50% ethanol extract (3.75 g, 7.2% yield). This extract was used for *in vitro* assay of inhibition of testosterone 5 α -reductase activity and for *in vivo* anti-androgenic experiments.

2) Fractionation: *Lygodii Spora* (200 g) was successively extracted with acetone (2 l, at room temperature for 24 h, 3 times), EtOH (2 l, under reflux for 1 h, 3 times), and 70% EtOH (2 l, under reflux for 1 h, 3 times) as shown in Chart 1. The combined acetone, EtOH, and 70% EtOH extracts were evaporated to dryness to give acetone fraction (fr.), EtOH fr., and 70% EtOH fr., respectively. The EtOH fr. was treated with hexane (100 ml, at room temperature, 3 times) to afford a hexane soluble fraction (EtOH–hexane soluble fr.) and an insoluble residue (EtOH–hexane insoluble residue). The insoluble residue was extracted with AcOEt (200 ml, at room temperature, 2 times) to give an AcOEt extract. Resulting AcOEt insoluble residue was partitioned by an AcOEt–H₂O (1 : 4) mixture (500 ml). The aqueous layer was lyophilized to give an EtOH–H₂O fr. The AcOEt layer was combined with the AcOEt extract and evaporated under reduced pressure to give an EtOH–AcOEt fr. Yield of each fraction is shown in Chart 1. Each fraction was used for *in vitro* assay of inhibition of testosterone 5 α -reductase activity.

The EtOH–hexane soluble fr. (15.35 g) which showed a remarkable inhibitory activity in testosterone 5 α -reductase assay was chromatographed over silica gel (Merck, No.

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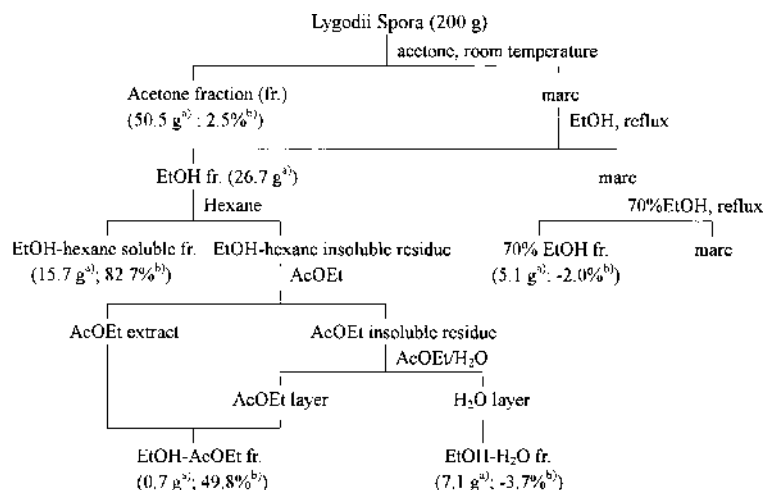


Chart 1. Fractionation of *Lygodii Spora* and Testosterone 5α -Reductase Inhibitory Activity of Each Fraction

a) Yield. b) Inhibitory percentage against testosterone 5α -reductase activity at 1.0 mg/ml.

1,07734) eluted with a gradient of hexane and AcOEt to give seven fractions; fr. A [hexane–AcOEt (100:0 to 95:5, v/v), yield; 0.014 g, inhibition %; not determined], fr. B [hexane–AcOEt (95:5), yield, 5.882 g, inhibition %; 1.9% at 0.5 mg/ml], fr. C [hexane–AcOEt (95:5), yield, 0.350 g, inhibition %; 38.8% at 0.5 mg/ml], fr. D [hexane–AcOEt (95:5), yield, 1.976 g, inhibition %; 77.6% at 0.5 mg/ml], fr. E [hexane–AcOEt (9:1), yield, 2.663 g, inhibition %; 65.3% at 0.5 mg/ml], fr. F [hexane–AcOEt (9:1 to 4:1), yield, 1.817 g, inhibition %; 8.8% at 0.5 mg/ml], and fr. G [hexane–AcOEt (4:1 to 7:3), yield, 1.694 g, inhibition %; 57.4% at 0.5 mg/ml]. Repeated chromatography of the most active fr. D [hexane–AcOEt (95:5) elute, 1.74 g] over silica gel eluted with hexane–AcOEt (95:5, v/v) afforded three fractions; fr. D-1 (yield, 0.833 g, inhibition %; 75.4% at 0.25 mg/ml), fr. D-2 (yield, 0.747 g, colorless oil, inhibition %; 73.0% at 0.25 mg/ml), and fr. D-3 (yield, 0.013 g, inhibition %; not determined). Fraction D-2 showed a single spot on TLC [Merck No. 1.05715 silica gel 60F₂₅₄, solvent system; hexane–AcOEt–AcOH (10:4:0.1 v/v), detection; 10% H₂SO₄, *R_f*-value 0.6]. By gas chromatographic analysis using a J&W DB-23 capillary column on a Shimadzu GC-1700 after methylation, the active fr. D-2 was found to be comprised 53.0% of oleic acid, 29.6% of linoleic acid, 11.8% of palmitic acid, 3.0% of stearic acid and 1.8% of asclepic acid [(*Z*)-11-octadecenoic acid]. The ¹H- and ¹³C-NMR spectral data and *R_f*-value of fr. D-2 were in good accordance with those of these authentic fatty acids. A part of the inactive fr. B [hexane–AcOEt (95:5) elute, 3.1 g] was purified by rechromatography on silica gel to give oily triglyceride (2.5 g) which showed a single spot on TLC [Merck No. 1.05715 silica gel 60F₂₅₄, solvent system; hexane–AcOEt (10:1 v/v), detection; 10% H₂SO₄, *R_f*-value, 0.5]. Comparison of its ¹H- and ¹³C-NMR spectral data with those of authentic samples showed that the triglyceride was a mixture, and that acyl parts of triglyceride were comprised of several fatty acids such as oleic, linoleic, and palmitic acids.

Animals Male SD strain rats (6 weeks of age), male Syrian strain golden hamsters (4 weeks of age), and male C57Black/6CrSlc strain mice (7 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). They were main-

tained in an air-conditioned room with lighting from 7 a.m. to 7 p.m. The room temperature (about 23 °C) and humidity (about 60%) were controlled automatically. Laboratory pellet chows (Labo MR Stock and Labo R stock, Nihon Nosan Kogyo Co. Ltd., Tokyo, Japan) and water were freely available.

Assay for Inhibition of Testosterone 5α -Reductase Activity Testosterone 5α -reductase was prepared from the liver of rats (6 weeks of age) according to the method of Imai.⁷⁾ Inhibition assay of 5α -reductase was performed according to the method described by Iбата⁸⁾ as in the case of our previous papers.^{3,4)} IC₅₀ values were graphically calculated from the inhibition percent values at several concentrations.

Growth Suppression of Hamster Flank Organs An anti-androgenic assay using hamster flank organ growth measured by the increase in the area of pigmented macule was performed according to the method described by Liang and Liao⁹⁾ with minor modification. Pre-pubertal hamsters were castrated at 3 weeks by excision of the testicles; intact hamsters were not castrated. After 1 week, hamsters were divided into 6–8 animals per group in order to have similar weight in each group. Hair on the lower back of each animal was shaved with electric hair clippers weekly to expose flank organ. Five microliters of testosterone or 5α -dihydrotestosterone solution (0.01% in EtOH) was applied topically to the right flank organ once a day for 21 d with a pipette (GILSON, France) and a polypropylene disposable tip. After 30 min of testosterone or 5α -dihydrotestosterone treatment, 5 μ l of one of the following sample solutions was applied topically to the right flank organ once a day for 21 d in a similar way. Sample solutions: (1) 70% EtOH as control, (2) 2% oxendolone in 70% EtOH as positive control, (3) 50% EtOH extract of *Lygodii Spora* in an appropriate concentration in 70% EtOH. As vehicle, 5 μ l of EtOH was applied topically to the left flank organ. In the intact group, 5 μ l of EtOH was applied to both flank organs, and after 30 min both organs were again applied with 5 μ l of 70% EtOH. The surface of the flank organ was wiped with an ethanol pad before each treatment. On the day after the last treatment, animals were sacrificed by an intraperitoneal injection of pentobarbital.

Growth of both flank organs, the treated (right side) and vehicle (left side) was determined by measuring the length of the long axis and the short axis of the pigmented spot (pigmented macule) with calipers. The surface area (mm²) of the spot was calculated by multiplication of the length of the two axes.

Hair Regrowth after Shaving in Testosterone-Treated C57Black/6CrSlc Mice According to the method described by Yokoyama¹⁰ with minor modification, the dorsal hair of mice (7 weeks of age, one group using 8–10 mice) was shaved with electric hair clippers. Beginning the next day, 100 μ l of testosterone solution (0.05% in EtOH) was applied topically to the shaved dorsum once a day for 24 d with a pipette and a polypropylene disposable tip. After 30 min of testosterone treatment, 100 μ l of one of the following sample solutions was applied topically to the shaved dorsum once a day for 24 d in a similar way. Sample solutions: (1) 50% EtOH as control, (2) 2% solution of 50% ethanol extract of *Lygodii Spora* in 50% EtOH.

The hair regrowth at 12, 15, 18, 21, and 24 d after beginning of topical application was calculated using the following hair growth score: score 0: no hair growth observed; score 1: less than 20% growth observed; score 2: 20% to less than 40% growth observed; score 3: 40% to less than 60% growth observed; score 4: 60% to less than 80% growth observed; score 5: 80% to 100% growth observed.

Statistical Analysis The experimental data were tested for statistical significance by Bonferroni/Dunn's multiple range test method.

RESULTS

In an assay for testosterone 5 α -reductase inhibitory activity, a 50% ethanol extract of *Lygodii Spora* showed a remarkable activity (inhibition %: concentration of 50% ethanol extract in 50% EtOH; 40.6%: 1.0 mg/ml, 66.7%; 2.0 mg/ml).

Summary of the activity guided fractionation of *Lygodii Spora* is shown in Chart 1. The IC₅₀ values on testosterone 5 α -reductase of five fatty acids and ethinylestradiol, a reference compound, are listed in Table 1. The values of linoleic, oleic, asclepic [(Z)-11-octadecenoic] and palmitic acids were 0.37, 0.44, 0.50 and 1.35 mm, respectively, but stearic acid was ineffective.

Results of an *in vivo* anti-androgenic activity assay using hamsters are depicted in Table 2. The pigmented macules of the intact hamsters apparently grew in response to increased production of endogenous androgen in both sides. In the castrated hamsters, the untreated left flank organ did not grow, but the testosterone or 5 α -dihydrotestosterone treated right flank organ grew noticeably. 5 α -Dihydrotestosterone was as effective as testosterone in stimulating flank organ growth.

The anti-androgenic effects of oxendolone, and 50% ethanol extract of *Lygodii Spora* on androgen stimulated growth of flank organ are shown in Table 2. Oxendolone, an anti-androgenic drug, showed a significant inhibition in both testosterone and 5 α -dihydrotestosterone treated right sides. The testosterone-stimulated growth of right flank organ was inhibited by 50% ethanol extract of *Lygodii Spora* at a 500 μ g/5 μ l concentration, while the extract showed no significant inhibition on the 5 α -dihydrotestosterone stimulated

Table 1. IC₅₀ Values of Inhibitory Effects of Five Fatty Acids and Ethinylestradiol on *in Vitro* Testosterone 5 α -Reductase Activity

Sample	IC ₅₀ (mm)
Palmitic acid	1.35 \pm 0.03
Stearic acid	Non active
Oleic acid	0.44 \pm 0.02
Asclepic acid	0.50 \pm 0.03
Linoleic acid	0.37 \pm 0.01
Ethinylestradiol	0.81 \pm 0.09

Each value represents the mean \pm S.E. of 3 experiments.

Table 2. Effects of 50% Ethanol Extract from *Lygodii Spora* (LS-ext) and Oxendolone on Testosterone (T)- or 5 α -Dihydrotestosterone (DHT)-Stimulated Growth of Pigmented Macules of Hamster Flank Organ

Treatment of right flank organ T or DHT; 0.5 μ g/5 μ l ethanol+sample solution	Pigment macule area (mm ²)		
	Left (untreated)	Right (treated)	Inhibition (%)
Non-castrated hamsters			
Ethanol+70% ethanol [Intact]	48.1 \pm 1.9	44.7 \pm 2.1	—
Castrated hamsters			
T+70% ethanol [Control]	8.1 \pm 0.9	34.2 \pm 2.5	—
T+LS-ext 50 μ g/5 μ l 70% ethanol	8.1 \pm 1.1	30.7 \pm 2.9	10.1
T+LS-ext 200 μ g	7.5 \pm 1.0	33.8 \pm 1.8	2.9
T+LS-ext 500 μ g	6.6 \pm 1.2	21.4 \pm 1.9**	37.6
T+Oxendolone 0.33 μ mol	7.3 \pm 0.8	23.7 \pm 2.7**	29.7
Castrated hamsters			
DHT+70% ethanol [Control]	4.6 \pm 0.8	40.3 \pm 2.1	—
DHT+LS-ext 50 μ g/5 μ l 70% ethanol	5.0 \pm 0.6	37.2 \pm 2.7	7.8
DHT+LS-ext 200 μ g	6.0 \pm 0.6	35.8 \pm 2.7	11.3
DHT+LS-ext 500 μ g	6.8 \pm 1.0	34.3 \pm 2.3	15.0
DHT+Oxendolone 0.33 μ mol	5.4 \pm 0.5	31.7 \pm 3.1*	21.5

Pre-pubertal hamsters were castrated at 3 weeks. Intact group was not castrated. After 1 week, hair on the lower back of each animal was shaved with electric hair clippers weekly to expose flank organ. A testosterone (T) or 5 α -dihydrotestosterone (DHT) solution 5 μ l (0.01% in EtOH) was applied topically to the right flank organ once a day for 28 d with a pipette. After 30 min of T or DHT, 5 μ l of one of the sample solutions was applied topically to the right flank organ once a day for 28 d in a similar way. As vehicle, 5 μ l of EtOH was applied topically to the left flank organ. In the intact group, 5 μ l of EtOH was applied to both flank organs, and after 30 min both flank organs again applied with 5 μ l of 70%EtOH. Growth of both flank organs, the treated (right side) and vehicle (left side) was determined by measuring the length of the long axis and the short axis of the pigmented spot with calipers. The surface area (mm²) of the spot was calculated by multiplication of the length of the two axes. Each value represents the mean \pm S.E. of 6–8 hamsters. Significantly different from the control group at * p <0.05, ** p <0.01.

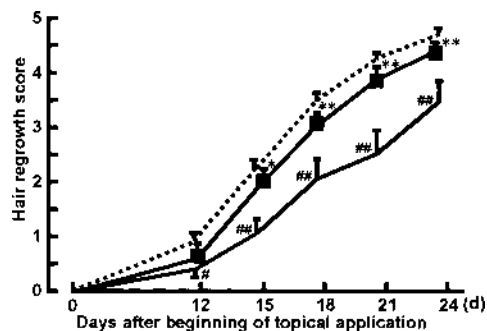


Fig. 1. Effect of 50% Ethanol Extract from *Lygodii Spora* on Hair Regrowth after Shaving in Testosterone-Treated C57Black Mice

The dorsal hair of mice was shaved. Beginning the next day, 100 μ l of testosterone solution was applied topically to the shaved dorsum once a day for 24 d. After 30 min of testosterone treatment, 100 μ l of the following sample solutions were also applied topically to the shaved dorsum once a day for 24 d. Control and testosterone-treated control were applied with 50% ethanol alone and 50% ethanol together with 0.05% testosterone, respectively. The regrowth after beginning of topical application was calculated by scoring. ---; control, —; testosterone-treated control, ■; 2% solution of 50% ethanol extract from *Lygodii Spora*. Each point represents the mean \pm S.E. of 8–10 mice. Significantly different from the control group at #: $p < 0.05$, ##: $p < 0.01$. Significantly different from the testosterone-treated control group at *: $p < 0.05$, **: $p < 0.01$.

growth of right flank organ at the same concentration.

The hair regrowth assay in testosterone sensitive mice was performed in another *in vivo* anti-androgenic assay. As shown in Fig. 1, testosterone treatment remarkably suppressed hair regrowth in these mice. Fifty percent ethanol extract of *Lygodii Spora* showed a significant anti-androgenic activity.

DISCUSSION

Lygodii Spora has been shown to contain (+)-*cis-trans*-abscisic acid and fatty acids such as palmitic, stearic, oleic, linoleic, and (+)-8-hydroxyhexadecanoic acids.^{11,12} The methyl ester of gibberellin A₇₃ was isolated in trace quantities from the culture medium of *Lygodium japonicum* prothallia.¹³ Linoleic and (+)-8-hydroxyhexadecanoic acids were identified as endogenous inhibitors for spore germination.¹¹ However, testosterone 5 α -reductase inhibitory effect of *Lygodii Spora* has not been reported. The anti-androgenic drugs which exhibited inhibitory activity in testosterone 5 α -reductase may be useful as hair growth promoting agents. Thus the present study was targeted at examining the anti-androgenic activities of the extract of *Lygodii Spora*, characterizing its active components, and then applying the extract to a hair growth promoting agent originated from a natural resource.

Several attempts to fractionate the 50% ethanol extract of *Lygodii Spora* failed, because any treatment of the extract caused a vigorous emulsification. In order to identify the active constituents, an alternative extraction method and bioassay-guided fractionation were examined as shown in Chart 1. Activity-guided chromatography of the EtOH–hexane soluble fraction, which showed potent inhibitory effect in the assay, afforded active oil, which showed a single spot on TLC. ¹H- and ¹³C-NMR spectral data suggested that the oil was a mixture of several fatty acids. By gas chromatography after methylation, the active oil was found to be comprised 53.0% of oleic acid, 29.6% of linoleic acid, 11.8% of palmitic acid, 3.0% of stearic acid, and 1.8% of asclepic acid

[(*Z*)-11-octadecenoic acid]. Authentic compounds of these five detected fatty acids were assayed to find out which component was responsible for the inhibition. As shown in Table 1, oleic and linoleic acids, major components of the oil, exhibited potent activity, and palmitic acid was also active. Liang and Liao¹⁴ have shown that certain aliphatic unsaturated fatty acids, e.g. γ -linolenic, α -linolenic, arachidonic, linoleic and oleic acids, are potent inhibitors of testosterone 5 α -reductase in a cell-free system. As to lipophilic constituents of the 50% ethanol extract of *Lygodii Spora*, a considerable amount of the active fatty acids described above and a trace of triglyceride were detected by TLC analysis of the extract. Thus, testosterone 5 α -reductase inhibitory activity of *Lygodii Spora* can be attributed to these three fatty acids.

It was noted that the major component of the inactive acetone fraction shown in Chart 1 was found to be triglyceride by TLC analysis. Considering the amount of inactive triglyceride mixture isolated from the EtOH–hexane soluble fraction, *Lygodii Spora* contained about 30% of triglyceride by weight.

An *in vivo* anti-androgenic assay was performed using flank organ growth measured by increase in the area of pigmented macule in castrated hamsters. As shown in Table 2, oxendolone, an anti-androgenic drug, showed a significant anti-androgenic activity on both testosterone and 5 α -dihydrotestosterone treated flank organs. The inhibitory effect of 50% ethanol extract of *Lygodii Spora* on the growth of the treated right flank organ was clear when testosterone was used but not when 5 α -dihydrotestosterone was used. These data implied that the extract specifically inhibited testosterone 5 α -reductase. This fact is consistent with the finding that the extract contains testosterone 5 α -reductase inhibiting active fatty acids: oleic, linoleic and palmitic acids. Liang and Liao⁹ reported growth suppression of hamster flank organs by certain fatty acids, e.g. γ -linolenic, α -linolenic, arachidonic, linoleic, oleic and palmitic acids. Therefore, the inhibitory effect of 50% ethanol extract of *Lygodii Spora* on the growth of pigmented macules of hamster flank organ is attributable to fatty acid inhibitors of testosterone 5 α -reductase.

Using the hair regrowth assay in testosterone sensitive male C57Black/6CrSlc strain mice, another *in vivo* anti-androgenic assay was performed. In this experimental model animal, it has been noted that testosterone caused a disorder in the stage of terogen during course of the hair growth cycle in dermal papilla cells. As shown in Fig. 1, testosterone treatment caused a remarkable suppression of hair regrowth in these mice. Fifty percent ethanol extract of *Lygodii Spora* showed a significant anti-androgenic activity as did oxendolone as described in the previous paper.⁴

Thus, it was found that 50% ethanol extract of *Lygodii Spora* showed a significant anti-androgenic activity in both these *in vivo* assays, and that *Lygodii Spora* contained the testosterone 5 α -reductase inhibitory active lipophilic constituents: oleic, linoleic and palmitic acids.

Seeds of *Sesamun indicum* L. (Pedaliaceae) which contain a considerable amount of fatty acids have been prescribed for the treatment of hair growth in Chinese medicinal herbals.⁶ This description has been confirmed by our hair regrowth experiments in mice.¹⁵ Considering the experimental results by

Liang and Liao,⁹⁾ it is expected that several fatty acids, *e.g.* palmitic, oleic, linoleic, linolenic and arachidonic acids, and a mixture of these acids show a significant anti-androgenic effect owing to their testosterone 5 α -reductase inhibitory activity.

In conclusion, *Lygodii Spora* appears to be useful for topical treatment of androgen-dependent skin disorder, and may be a candidate as a hair growth promoting agent originating from a natural resource.

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