Testosterone 5 α -Reductase Inhibitory Active Constituents of *Piper nigrum* Leaf

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Previously we reported that *Piper nigrum* leaf extract showed a potent stimulation effect on melanogenesis and that (–)-cubebin (**1**) and (–)-3,4-dimethoxy-3,4-desmethylenedioxycubebin (**2**) were isolated as active constituents. As a part of our continuous studies on *Piper* species for the development of cosmetic hair-care agents, testosterone 5 α -reductase inhibitory activity of aqueous ethanolic extracts obtained from several different parts of six *Piper* species, namely *Piper nigrum*, *P. methysticum*, *P. betle*, *P. kadsura*, *P. longum*, and *P. cubeba*, were examined. Among them, the extracts of *P. nigrum* leaf, *P. nigrum* fruit and *P. cubeba* fruit showed potent inhibitory activity. Activity-guided fractionation of *P. nigrum* leaf extract led to the isolation of **1** and **2**. Fruits of *P. cubeba* contain **1** as a major lignan, thus inhibitory activity of the fruit may be attributable to **1**. As a result of further assay on other known constituents of the cited *Piper* species, it was found that piperine, a major alkaloid amide of *P. nigrum* fruit, showed potent inhibitory activity, thus a part of the inhibitory activity of *P. nigrum* fruit may depend on piperine. The 5 α -reductase inhibitory activities of **1** and piperine were found for the first time. In addition, the *P. nigrum* leaf extract showed *in vivo* anti-androgenic activity using the hair regrowth assay in testosterone sensitive male C57Black/6CrSlc strain mice.

Key words *Piper nigrum*; testosterone 5 α -reductase; cubebin; piperine; anti-androgenic activity; hair regrowth

Because of the increase of the elderly population, a larger fraction of people are now afflicted with alopecia and/or gray hair. Thus the market for hair growth agents, hair-dye, and anti-gray hair agents is growing. Testosterone 5 α -reductase catalyzes the conversion of testosterone to an active androgen, dihydrotestosterone, which binds to androgen receptors and shows various hormonal actions. An excessive accumulation of dihydrotestosterone is recognized as leading to male pattern baldness and benign prostatic hyperplasia.^{1,2)} Treatment with a testosterone 5 α -reductase inhibitor would be expected to lead to a decrease of dihydrotestosterone concentration in many tissues, and may be useful for protection against alopecia. On the other hand, gray hair is caused by aging, decrement of melanocytes by environmental stress, and decrement of the melanogenesis.^{3,4)} Therefore, it was expected that agents which exhibit both melanogenesis stimulation activity and testosterone 5 α -reductase inhibitory activity may be desirable ingredients of hair-care cosmetic products for prevention of gray hair and alopecia. During the course of our screening program using cultured murine B16 melanoma cells for the development of gray hair prevention agents from natural resources, we found that extracts of some *Piper* species (Piperaceae) showed a potent stimulation effect on melanogenesis without any effects on cell proliferation, and two lignans, (–)-cubebin (**1**) and (–)-3,4-dimethoxy-3,4-desmethylenedioxycubebin (**2**), were isolated as active constituents from the leaves of *Piper nigrum* L.^{5–7)} With the expectation that we might find new desirable agents from *Piper* species to prevent gray hair and alopecia as described above, the testosterone 5 α -reductase inhibitory activity of 50% ethanolic extracts obtained from several different parts of six *Piper* species (*P. nigrum* L., *P. methysticum* FORST., *P. betle* L., *P. kadsura* (CHOISY) OHWI, *P. longum* L., and *P. cubeba* L.) was examined. This paper deals with the screening results of *in vitro* testosterone 5 α -reductase inhibitory activity of *Piper*

species, the activity-guided fractionation of *P. nigrum* leaf extract led to the isolation of active lignans, **1** and **2**, and the *in vivo* anti-androgenic activity of the leaf extract.

MATERIALS AND METHODS

Plant Materials Several different parts of the six *Piper* species named above were the same samples described in the previous paper.⁷⁾ Fifty percent ethanolic extracts of each air-dried and powdered material of *Piper* plant were also the same samples described that paper.⁷⁾ Methanolic extracts of *P. nigrum* leaf were obtained according to the previous paper.⁵⁾

Reagents Testosterone and ethinylestradiol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Oxendolone (Prostetone[®]) was purchased from Takeda Chemical Industries Ltd. (Osaka, Japan). Piperine was purchased from Sigma (St. Louis, MO, U.S.A.). Other chemical reagents were reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. unless otherwise noted.

Animals Male SD strain rats (6 weeks of age) and male C57Black/6CrSlc strain mice (7 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). They were maintained 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 5 α -Reductase Activity Testosterone 5 α -reductase was prepared from the liver of rats (6 weeks of age) according to the method of Imai.⁸⁾ Inhibition assay of testosterone 5 α -reductase was performed according to the method described by Ibata⁹⁾ as in

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our previous papers.^{10–12} IC₅₀ values were graphically calculated from the inhibition percent values at several concentrations.

Hair Regrowth after Shaving in Testosterone-Treated C57Black/6CrSlc Mice According to the method described by Yokoyama¹³ 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 ethanol) was applied topically to the shaved dorsum once a day for 28 d except the control group with a pipette and a polypropylene disposable tip. After 30 min of testosterone treatment, 100 μ l of the following sample solutions were applied topically to the shaved dorsum once a day for 28 d in a similar way. Sample solutions in the experiment shown in Fig. 2A: (1) 80% ethanol as control, (2) 2% oxendolone solution in 80% ethanol. Sample solutions in the experiment shown in Fig. 2B: (3) ethanol as control, (4) 2% and 5% solution of methanolic extract of *P. nigrum* leaf in ethanol.

The hair regrowth at 13, 16, 19, 22, 25 and 28 d after beginning of topical application was calculated using the following 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

Fig. 1. Chemical Structures of Lignans 1 and 2

Fig. 2. Effect of Methanolic Extract of *Piper nigrum* Leaf and Oxendolone on Hair Regrowth after Shaving in Testosterone-Treated C57BL/6CrSlc Mice

A: Control (---), testosterone-treated control (—) and 2% oxendolone solution (○). B: Control (---) and testosterone-treated control (—). 2% Solution (▲) and 5% solution (●) of methanolic extract of *P. nigrum* leaf. The regrowth after the start of topical application was calculated by scoring. Each point represents the mean $\pm$ S.E. of 10 to 12 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.

for statistical significance using Bonferroni/Dunn's multiple range test method.

Extraction, Fractionation and Isolation The powdered dry leaves (500 g) of *P. nigrum* were extracted with MeOH (51 $\times$ 3 times) for 1 h under reflux. Combined extracts were evaporated under reduced pressure to give a methanolic extract (71.1 g). A part of this extract (69.6 g) was extracted with hexane (400 ml $\times$ 3), and the organic layer was evaporated to give a hexane soluble fraction (17.3 g). The hexane insoluble part was extracted with EtOAc (300 ml $\times$ 2). The EtOAc insoluble part was suspended in H₂O (500 ml) and extracted with EtOAc (300 ml $\times$ 3). The EtOAc extracts were combined and evaporated to afford an EtOAc soluble fraction (20.5 g). The aqueous layer was evaporated followed by lyophilization to give a water soluble fraction (21 g). Testosterone 5 α -reductase inhibitory activity of each fraction was assayed, and the assay results are described by inhibition % in the section Results and Discussion. The EtOAc soluble fraction (4 g) which showed a remarkable inhibitory activity in testosterone 5 α -reductase assay was chromatographed over 240 g of a silica gel column (4.5 $\times$ 30 cm). Elution with hexane, EtOAc and MeOH in increasing proportions gave 100 chromatographic fractions of 100 ml each. TLC (hexane/EtOAc, 1 : 1) analysis of the collected fractions allowed us to assemble them into 10 fractions, Fr. A to J. Testosterone 5 α -reductase inhibitory activity at a concentration of 200 μ g/ml of each fraction was assayed, and each result is indicated by inhibition %; Fr. A [hexane/EtOAc 4 : 1 to 2 : 1, fr. No. 1 to 20, yield; 0.4 g, testosterone 5 α -reductase inhibition %; 6.7%], Fr. B [2 : 1 to 1 : 1, fr. No. 21 to 36, 0.75 g, inhibition %; 57.1%], Fr. C [1 : 1, fr. No. 37 to 39, 0.11 g, inhibition %; 24.4%], Fr. D [1 : 1, fr. No. 40 to 41, 0.03 g, inhibition %; 16.5%], Fr. E [1 : 1, fr. No. 42 to 55, 0.4 g, inhibition %; 24.3%], Fr. F [1 : 1, fr. No. 56 to 58, 0.15 g, inhibition %; 27.5%], Fr. G [1 : 1 to 2 : 3, fr. No. 59 to 72, 0.3 g, inhibition %; 29.5%], Fr. H [2 : 3 to 1 : 2, fr. No. 73 to 82, 0.14 g, inhibition %; 21.1%], Fr. I [1 : 2 to MeOH/EtOAc, 1 : 1, fr. No. 83 to 87, 0.12 g, inhibition %; 3.2%], Fr. J [MeOH/EtOAc, 1 : 1 to MeOH, fr. No. 88 to 100, 1.4 g, inhibition %; 13.0%]. A mixture of active fractions, Fr. B and Fr. C, was rechromatographed over silica gel, eluted with hexane/EtOAc (1 : 1) and monitored with TLC (hexane/EtOAc, 1 : 1). Fractions showing a single spot of *R*_f-value 0.6 on TLC were collected and evaporated, and the residue was recrystallized from benzene-hexane to afford 1, mp 125–128 $^{\circ}$ C (0.17 g, isolation yield 0.18%). A mixture of other active fractions, Fr. E and Fr. F, was rechromatographed over silica gel. Fractions showing a single spot of *R*_f-value 0.4 on TLC were collected and evaporated, and a residue was recrystallized from EtOAc-hexane to give 2, mp 81–83 $^{\circ}$ C (0.15 g, 0.16%). As described in the previous paper,⁵ 1 and 2 were identified as (–)-cubebin and (–)-3,4-dimethoxy-3,4-desmethylenedioxy-cubebin, respectively.

RESULTS AND DISCUSSION

Testosterone 5 α -reductase inhibitory activities of 50% ethanolic extracts obtained from several different parts of six *Piper* species at concentrations of 1 and 2 mg/ml and 1 mM ethinylestradiol as a reference compound are shown in Table 1. Among them, the 50% ethanolic extracts obtained from *P.*

Table 1. Inhibitory Effects of 50% Ethanolic Extract from Piperaceae Plants and Ethinylestradiol on *in Vitro* Testosterone 5 α -Reductase

Samples	Parts	Yield (%)	Final conc. (mg/ml)	Conversion (%)	Inhibition (%)
Control	—	—	—	83.0 $\pm$ 1.3	—
<i>P. nigrum</i>	Leaf	14.0	1	50.4 $\pm$ 3.9*	39.3
			2	39.0 $\pm$ 2.3*	53.0
	Stem	8.0	1	60.6 $\pm$ 1.7*	27.0
			2	49.1 $\pm$ 2.8*	40.8
<i>P. methysticum</i>	Fruit	4.7	1	49.9 $\pm$ 0.4*	39.9
			2	30.7 $\pm$ 1.0*	63.0
	Leaf	9.3	1	71.4 $\pm$ 0.7*	14.0
			2	59.4 $\pm$ 1.1*	28.4
<i>P. betle</i>	Stem	14.0	1	70.7 $\pm$ 1.8*	14.8
			2	64.8 $\pm$ 0.3*	21.9
	Rhizome	8.0	1	49.2 $\pm$ 1.5*	40.7
			2	47.7 $\pm$ 3.7*	42.5
<i>P. betle</i>	Whole plant	25.7	1	62.8 $\pm$ 1.8*	24.3
			2	47.9 $\pm$ 0.8*	42.3
<i>P. kadsura</i>	Leaf	25.0	1	69.1 $\pm$ 0.3*	16.7
			2	67.2 $\pm$ 3.1*	19.0
	Stem	13.0	1	69.8 $\pm$ 0.1*	15.9
			2	69.0 $\pm$ 2.7*	16.9
<i>P. longum</i>	Root	9.3	1	62.2 $\pm$ 1.3*	25.1
			2	50.5 $\pm$ 2.7*	39.2
	Rhizome	10.3	1	60.8 $\pm$ 0.6*	26.7
			2	52.7 $\pm$ 2.2*	36.5
<i>P. longum</i>	Whole plant	9.7	1	74.5 $\pm$ 1.7*	10.2
			2	72.8 $\pm$ 3.1*	12.3
<i>P. cubeba</i>	Fruit	16.8	1	62.8 $\pm$ 3.0*	24.3
			2	28.0 $\pm$ 3.2*	66.3
Ethinylestradiol			1 (mM)	30.5 $\pm$ 1.0*	63.3

The reaction solution contained 50% methanol extract solution (0.2 ml) of 50% ethanolic extract; 1.0 ml of Tris-HCl buffer (pH 7.2), 0.3 ml of testosterone (500 μ g/ml in propylene glycol-Tris-HCl buffer (1 : 1 v/v)), and 1.0 ml of the enzyme solution. The reactions were started by the addition of 0.5 ml of NADPH (0.77 mg/ml in Tris-HCl buffer). The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by addition of 5.0 ml of dichloromethane. After addition of 0.5 ml of hexyl *p*-hydroxybenzoate (0.1 mg/ml in methanol, an internal standard (I.S.) for HPLC), the tube was shaken for 10 min and centrifuged at 900 $\times$ g for 10 min. The organic layer (4 ml) was transferred to another tube and evaporated to dryness. The residue was dissolved in 5.0 ml of methanol, and an aliquot of 10 μ l was injected into the HPLC system. Each value represents from the mean $\pm$ S.E. of 3 experiments. Significantly different from the control, **p*<0.01.

nigrum leaf, *P. nigrum* fruit, and *P. cubeba* fruit showed potent activities at a concentration of 2 mg/ml. Extracts of *P. methysticum* rhizome and *P. betle* showed a moderate activity, whereas the activities of the extracts of *P. kadsura* and *P. longum* were weak.

Firstly, we targeted identification of the active principle of *P. nigrum* leaf by activity-guided fractionation of the methanolic extract of the leaves, because the activity of the methanolic extract (5 α -reductase inhibition %: 55.8% at 1 mg/ml and 91.0% at 2 mg/ml) was superior to that of 50% ethanolic extract (39.3% at 1 mg/ml and 53.0% at 2 mg/ml). As described in the Experimental section, fractionation of the extract gave three fractions, a hexane soluble fraction, an ethyl acetate soluble fraction, and a water soluble fraction. The ethyl acetate soluble fraction (5 α -reductase inhibition %: 40.8% at 0.5 mg/ml and 68.1% at 1 mg/ml) and the hexane soluble fraction (28.3% at 0.5 mg/ml and 72.1% at 1 mg/ml) showed significant activities, whereas the water soluble fraction (3.1% at 0.5 mg/ml and 6.7% at 1 mg/ml) was inactive. Activity-guided chromatography of the active ethyl acetate soluble fraction led to isolation of two active lignans, **1** and **2**.⁵⁾ The main constituent of the active hexane soluble

Table 2. IC₅₀ Values of Inhibitory Effects of Constituents from Piperaceae Plants and Ethinylestradiol on *in Vitro* Testosterone 5 α -Reductase

Compound	IC ₅₀ (mM)
1	0.44
2	1.03
Piperine	0.48
Yangonin	>10
(+)-Methysticin	>10
Ethinylestradiol	0.81

fraction was identified as **1** as in the previous report.⁵⁾ As shown in Table 2, the 5 α -reductase inhibitory activities of **1** (IC₅₀, 0.44 mM) and **2** (IC₅₀, 1.03 mM) were superior to that of ethinylestradiol (IC₅₀, 0.81 mM). Anti-inflammatory,¹⁴⁾ analgesic,¹⁵⁾ and trypanocidal¹⁶⁾ activities of **1** and its analogues have been known, however, the testosterone 5 α -reductase inhibitory activity of **1** has not been reported hitherto. Both leaf and stem extracts of *P. nigrum* showed similar TLC patterns in which the spots of **1** and **2** were obviously detected. Therefore, the inhibitory activity of stem extract could be attributable to **1** and analogous lignans. Since **1** is a major lignan of the fruits of *P. cubeba*,¹⁷⁾ the inhibitory activity of *P. cubeba* fruit extract may be attributable to **1**.

Secondly, we studied *in vitro* testosterone 5 α -reductase inhibitory activities of other known constituents of the cited *Piper* species. Fruits of *P. nigrum* contain piperine as a major alkaloid amide¹⁸⁾ and a considerable amount of fatty acids such as linoleic, oleic and palmitic acids.¹⁹⁾ Piperine showed a potent testosterone 5 α -reductase inhibitory activity (IC₅₀, 0.48 mM, Table 2). Gastroprotective,²⁰⁾ monoamine oxidase inhibitory,²¹⁾ anticonvulsive,²²⁾ and other many activities of piperine have been previously reported, but the testosterone 5 α -reductase inhibitory activity of piperine was found here for the first time. We have reported that several fatty acids showed testosterone 5 α -reductase inhibitory activity, and that IC₅₀ values of oleic, linoleic and palmitic acids were 0.44, 0.37, and 1.35 mM, respectively.¹²⁾ Thus, it is suggested that the inhibitory activity of *P. nigrum* fruit extract may be attributable to its major constituents, piperine and fatty acids such as linoleic, oleic and palmitic acids. As to *P. methysticum* rhizome (Kava), we have isolated five kavalactones, yangonin, (+)-methysticin, (+)-kawain, 5,6-dehydrokawain, and 7,8-epoxyyangonin, from Kava extract.⁷⁾ None of these kavalactones showed significant 5 α -reductase inhibitory activity. The IC₅₀ value of yangonin and (+)-methysticin, major kavalactones of *P. methysticum* rhizome, was more than 10 mM. It is considered that the moderate inhibitory activity of *P. methysticum* rhizome extract depends not on the cited kavalactones, but on other unidentified constituents.

Thirdly, we examined *in vivo* anti-androgenic activity of the methanolic extract of *P. nigrum* leaf by using the hair regrowth assay in testosterone sensitive male C57Black/6CrSlc strain mice. In this experimental model animal, it has been noted that testosterone caused a disorder in the stage of terogen during the course of the hair growth cycle in dermal papilla cells.¹⁾ As shown in Fig. 2, testosterone treatment caused a remarkable suppression of hair regrowth in mice. Topical application of 5% solution of the methanolic extract of *P. nigrum* leaf showed a significant anti-androgenic activity (Fig. 2B) as did oxendolone (Fig. 2A), a positive refer-

ence drug, as described in the previous paper.¹²⁾ Thus, it was revealed that the *P. nigrum* leaf extract showed a significant anti-androgenic activity in the *in vivo* hair regrowth assay using C57Black/6CrSlc strain mice.

In conclusion, these findings led to an interesting fact that the extract of *P. nigrum* leaf and its two lignans, **1** and **2**, exhibited both testosterone 5 α -reductase inhibitory activity and melanogenesis stimulation activity. It is suggested that the extract of *P. nigrum* leaf is a desirable hair-care cosmetic material for prevention of gray hair and alopecia.

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