Screening of Euphorbiaceae Plant Extracts for Anti-5α-reductase

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In our research program to find novel agents for alopecia from natural plant resources, we screened Euphorbiaceae plant extracts using an anti-5α-reductase assay. Among the samples tested, the extract of Phyllanthus urinaria showed the most potent activity with 24.3 and 64.6% inhibition at 50 and 200 µg/mL against the enzyme, respectively. The extract also suppressed the androgen activity of dihydrotestosterone in LNCaP cell line. These results show that the extract of P. urinaria may be a multi-potent agent for androgen-derived alopecia. We tested for activity on a hair regrowth model using mice. The extract of P. urinaria showed hair regrowth activity at 5 mg/mouse/d administration. Furthermore, the active principle for anti-5α-reductase activity was determined as stigmasterol glucoside from activity-guided fractionation and the IC50 was 27.2 µM. These results suggest that extract of P. urinaria may be a promising candidate anti-alopecia agent.

Key words 5α-reductase; androgen receptor; Euphorbiaceae; Phyllanthus urinaria; stigmasterol glucoside

Hair loss is a major cosmetic problem in the prime of manhood, and may reduce QOL and lead to serious depression. Hair loss is mainly attributable to a change in testosterone level. Testosterone is a hormone in males that is dehydrolyzed to dihydrotestosterone by 5α-reductase, which is a more potent androgen than testosterone. Dihydrotestosterone strongly binds to androgen receptor and the complex migrates to the nucleus. This migration enhances the mRNA transcription of the anti-proliferative factor, transforming growth factor β (TGF-β) and suppresses the proliferation of hair follicle cells. Alopecia attributed to androgen is known as androgenetic alopecia and is recognized as a disease. Medical therapy for androgenetic alopecia is available; however, an alternative medicine for androgenetic alopecia using affordable self-care agents has long been awaited. In our research program to investigate hair growth agents from natural plant resources, we discovered that extracts from the flower of Pueraria lobata,1 the leaves of Rosmarinus officinalis2 and the rhizome of Panax ginseng3 were effective for hair growth via their inhibition of 5α-reductase.

In our research program to find effective plant materials from natural resources, we focused on Euphorbiaceae plants, which possess various active components and biological activities.4–6 In addition, Euphorbiaceae plants have been said to be effective for burns to the skin since ancient times, which indicates cell proliferation activity. Thus, if a plant material extract has both cell proliferating and anti-5α-reductase activities, it may be a suitable agent for hair growth. In these circumstances, we screened 10 plant extracts for anti-5α-reductase activity and the active principles were determined.

We selected plant samples randomly from Euphorbiaceae plants for screening as follows; whole plant of Phyllanthus urinaria, root of Glochidion eriocarpum, whole plant of Spe ranckia tubercula, leaf of Breynia patens, whole plant of Teucrum viscidum, whole plant of Euphorbia lunulata, whole plant of E. humifusa, root of E. kansui, seed of E. lathyris and root of E. pekinensis. Among the extracts tested, the extract from the whole plant of P. urinaria showed the most potent activity with 24.3 and 64.6% inhibition at 50 and 200 µg/mL, respectively (Table 1). The whole plant of P. urinaria is known as a hepatoprotective agent in ancient Chinese medicine. Scientific evidence on its hepatoprotective effects have been reported.7–9

The extract of P. urinaria was further investigated suppression of androgen activity of dihydrotestosterone in LNCaP cell line. The extract of P. urinaria suppressed androgen activity of dihydrotestosterone (10 nM) as 36.9 and 62.6% at 1 and 5 µg/mL, respectively (Fig. 1). The extract of P. urinaria was shown to possess a suppression of androgen activity of dihydrotestosterone, as well as inhibitory activity against 5α-reductase. These results suggest that the extract of P. urinaria may be a promising candidate anti-alopecia agent.

Table 1. Inhibitory Activities of the Extracts from Euphorbiaceae Plants against 5α-Reductase

| Samples             | Concentration (µg/mL) | Inhibition (%) |
|---------------------|-----------------------|----------------|
| P. urinaria         | 50                    | 24.3**         |
|                     | 200                   | 64.6**         |
| G. eriocarpum       | 50                    | 8.1            |
|                     | 200                   | 25.5**         |
| S. tuberculata      | 50                    | 18.1           |
|                     | 200                   | 31.9**         |
| B. patens           | 50                    | 19.7           |
|                     | 200                   | 49.7**         |
| T. viscidum         | 50                    | 31.5**         |
|                     | 200                   | 57.4**         |
| E. lunulata         | 50                    | 15.3           |
|                     | 200                   | 43.7**         |
| E. humifusa         | 50                    | 23.4**         |
|                     | 200                   | 54.0**         |
| E. kansui           | 50                    | 16.2           |
|                     | 200                   | 21.3           |
| E. lathyris         | 50                    | 22.5*          |
|                     | 200                   | 34.8**         |
| E. pekinensis       | 50                    | 11.6           |
|                     | 200                   | 19.6           |
| Finasteride         | 0.093                 | 72.3**         |

Significant differences at p<0.01; ** and p<0.05; * against control group (n=3).
P. urinaria may demonstrate hair growth activity in vivo.

In order to demonstrate the hair growth effect of the P. urinaria extract in vivo, we carried out a hair re-growth assay using a testosterone-treated C57BL/6 mouse model. Successful administration of the P. urinaria extract at 5 mg/d showed hair re-growth in 30 d compared to a testosterone-treated (50 µg/d) group (Fig. 2).

Although the contribution of 5α-reductase inhibition to hair growth may be smaller than that of suppression of androgen activity of dihydrotestosterone, the active principle of 5α-reductase inhibition was investigated. Activity-guided fractionation led to the isolation of stigmasterol glucoside (Fig. 3). The compound possessed inhibitory activity against 5α-reductase at 27.2 µM (IC50) and may inhibit 5α-reductase in a competitive mode against testosterone as they share a similar steroidal skeleton. However, the functional groups of testosterone and stigmasterol glucoside at the C-3 position differ as ene-ketone and glucoside, respectively. Glucosylation may improve the solubility of the compound into water and express a relatively high potency. Stigmasterol glucoside was isolated from various natural resources, such as Cassia peteriana,\textsuperscript{9} Atriplex nummularia,\textsuperscript{10} Thalassodendron ciliatum,\textsuperscript{11} Cissus javana\textsuperscript{9} and Ambroma augusta as a biologically active compound,\textsuperscript{13} along with various biological cytotoxic, anti-inflammatory, anti-bacterial and antimalarial activities. Although stigmasterol glucoside was previously isolated from P. urinaria,\textsuperscript{14} this is the first report to clarify its anti-5α-reductase activity. Further investigation of the active principle is underway in our laboratory.

MATERIALS AND METHODS

Materials All reagents used were analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise stated. All plant samples were a generous gift from Maechu Co., Ltd. (Nara, Japan). Voucher specimens were deposited in the Faculty of Pharmacy, Kindai University and voucher numbers are shown as follows, whole plant of P. urinaria: PU20130614, root of G. eriocarpum: GE20130614, whole plant of S. taberculata: ST110506-2, leaf of B. patens: BP20130614, whole plant of T. viscidum: TV110506-3, whole plant of E. lunulata: EL20130614, whole plant of E. humifusa: EH11Q072, root of E. kansui: EK20130614, seed of E. lathyris: EL11Q071 and root of E. pekinensis: EP20130614.

Preparation of Extracts Each plant sample was pulverized to powder and 10 v/w of 50% ethanol was added. The suspension was extracted by reflux for 2 h. The suspension was then filtered and the filtrate obtained. The extraction was repeated and the two filtrates were combined and evaporated to remove the ethanol. Water was removed by lyophilization to obtain extracts. The extraction yields are shown in Table 2.

Assay for 5α-Reductase Inhibition The assay was performed according to the method reported previously\textsuperscript{15} Type II 5αR was prepared according to the method reported with modifications\textsuperscript{15} Rats (Wistar, 9 weeks) were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and kept at a constant temperature (25°C) and humidity with 12 h light and dark cycles for 11 d. Water and pellet chow (Labo MR stock, Nosan Corporation, Tokyo Japan) were freely

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**Fig. 1.** Suppression of Androgen Activity of Dihydrotestosterone by P. urinaria Extract Using LNCaP Cells Numbers Indicate Suppression Percentage of Cell Proliferation

Significant differences detected at $p<0.01$; ** against control group ($n=3$).

**Fig. 2.** Hair Regrowth Assay for P. urinaria Extract

$\text{}$; 2 mg/mouse/d, $\text{}$; 5 mg/mouse/d. Significant differences detected at $p<0.01$; ** against control (testosterone-treated) group and ## against control (testosterone-untreated) group ($n=10$).
available. The epididymis was taken from one hundred rats and homogenized with a blender in cooled physiological saline containing 0.25 M sucrose, 1 mM dithiothreitol and a protease inhibitor cocktail. The homogenate was filtered through gauze and centrifuged at 3000 × g for 10 min. The supernatant was centrifuged again under the same conditions to obtain a supernatant as a crude enzyme solution. The protein concentration was determined using Protein Assay methodology (Bio-Rad, Tokyo, Japan), a potent 5αR inhibitor and widely used to treat prostate hyperplasia was used as a reference drug.

\[
\text{Inhibition} \% = \left( \frac{(A-B) - (C-D)}{(A-B)} \right) \times 100
\]

where \(A\) is with dihydrotestosterone, but without the sample, \(B\) is without dihydrotestosterone and the sample, \(C\) is with dihydrotestosterone and the sample, \(D\) is with the sample but without dihydrotestosterone.

Bicalutamide, an anti-androgen agent was used as a reference drug.

**Animal Experiments** Improvement in hair re-growth on testosterone-treated C57BL/6 mice was investigated according to the method reported previously.\(^{17}\) Male C57BL/6NCrSlc mice were purchased from Shimizu Laboratory Supplies Co., Ltd. Water and pelleted chow were freely available. After 1 week of acclimatization, the dorsal hairs of 10 male mice (7 weeks of age) for each administration group were shaved. After 30 min from the topical application of the testosterone solution (0.07% in 50% ethanol) to the shaved skin area, sample solutions of 100 μL in 80% ethanol were applied daily for 30 d. On days 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 30 after starting application, a hair growth score was given for each mouse as referred to in the picture depicted in Fig. 2. Oxendolone was used as a reference drug. The animal experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Kindai University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996).

**Activity-Guided Fractionation of Active Principle and**

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**Table 2. Names of the Plants, Parts and Extraction Yields Used in This Study**

| Name                  | Part          | Yield (%) |
|-----------------------|---------------|-----------|
| Phyllanthus urinaria  | Whole plant   | 18.7      |
| Glochidion eriocarpum | Root          | 14.3      |
| Speranska tuberculata | Whole plant   | 10.0      |
| Brevnia patens        | Leaf          | 17.2      |
| Tecuroid viscidula    | Whole plant   | 28.4      |
| Euphorbia hamifusa    | Whole plant   | 21.1      |
| Euphorbia kansui      | Root          | 12.8      |
| Euphorbia lathyris    | Seed          | 8.8       |
| Euphorbia pekinensis  | Root          | 39.1      |

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**Cell Culture and Inhibitory Assay for LNCaP Cell Growth**

The test was performed according to the method previously described with minor modifications.\(^{8}\) Human prostatic cancer LNCaP cells were purchased from Riken BRC Cell Bank (Tsukuba, Japan). LNCaP cells were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 100 unit/mL penicillin–streptomycin at 37°C in an incubator at an atmosphere of 95% air and 5% CO₂. Confluent cells were seeded into 96-well collagen-coated plates (2000 cells/well/50 μL) and incubated for 24 h. To each well, 150 μL of serum-free medium (0.3% dimethylsulfoxide) with dihydrotestosterone (0 or 10 nM) and a sample (0–10 μM) were added. After 96 h of incubation, the medium was replaced with 150 μL of 10% WST-8 in serum-free medium and incubated for 4 h. The resulting amount of tetrazolium salt was estimated by measuring the optical density at 450 nm with a microplate reader (Tecan, Kawasaki, Japan). The inhibitory percentage of cell growth was calculated as follows:

\[
\text{Inhibition} \% = \left( \frac{(A-B) - (C-D)}{(A-B)} \right) \times 100
\]
Structural Elucidation The 50% ethanol extract of *P. urinaria* (18 g) was dissolved into 900 mL of ethyl acetate, partitioned with water (900 mL) and subjected to solvent partitioning using ethyl acetate and water (900 mL). The active ethyl acetate soluble fraction (2.3 g) was subjected to silicic acid column chromatography (250 g, silica gel 60, Merck Milipore) using chloroform/methanol (1:0), (99:1), (9:1) and (0:1) as eluents. According to TLC analysis, 8 fractions were obtained (frs. 1–8). The active fr. 6 (chloroform/methanol (9:1), 91.6 mg) was subjected to preparative HPLC under the following conditions: column; YMC-Pack ODS AM-323 (9: i.d.×250 mm), column temperature; 40°C, mobile phase; water/methanol (9:41), flow rate; 5.0 mL/min, detection; UV 254, retention time; 12 min to obtain stigmasterol glucoside. The chemical structure was elucidated by analysis of NMR and MS spectra data compared with data reported previously.13)

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Murata K, Noguchi K, Kondo M, Onishi M, Watanabe N, Okamura K, Matsuda H. Inhibitory activities of *Pueraiae Flos* against testosterone 5α-reductase and its hair growth promotion activities. *J. Nat. Med.*, 66, 158–165 (2012).

2) Murata K, Noguchi K, Kondo M, Onishi M, Watanabe N, Okamura K, Matsuda H. Hair growth effect of *Rosmarinus officinalis* leaf. *Phytother. Res.*, 27, 212–217 (2013).

3) Murata K, Takeshita F, Samukawa K, Tani T, Matsuda H. Effects of ginseng rhizome and ginsenoside Ro on testosterone 5α-reductase and hair re-growth in testosterone-treated mice. *Phytother. Res.*, 26, 48–53 (2012).

4) Hu Z, Lai Y, Zhang J, Wu Y, Luo Z, Yao G, Xue Y, Zhang Y. Phytochemical and chemotaxonomic studies on *Phyllanthus urinaria*. *Biochem. Syst. Ecol.*, 56, 60–64 (2014).

5) Boniface PK, Ferreira SB, Kaiser CR. Recent trends in phytochemistry, ethnobotany and pharmacological significance of *Alchornea cordifolia* (Schumach. & Thonn.) Muell. Arg. *J. Ethnopharmacol.*, 191, 216–244 (2016).

6) Ramalho SD, Pinto ME, Ferreira D, Bolzani VS. Biologically active orbitides from the Euphorbiaceae family. *Planta Med.*, 84, 558–567 (2018).

7) Chung CY, Liu CH, Burnouf T, Wang GH, Chang SP, Jassey A, Tai CJ, Tai CJ, Huang CJ, Richardson CD, Yen CC, Lin LT. Activity-based and fraction-guided analysis of *Phyllanthus urinaria* identifies lolilolide as a potent inhibitor of hepatitis C virus entry. *Antiviral Res.*, 130, 58–68 (2016).

8) Katayama H, Murashima T, Saeki Y, Nishizawa Y. The pure anti-androgen bicalutamide inhibits cyclin A expression both in androgen-dependent and -independent cell lines. *Int. J. Oncol.*, 36, 553–562 (2010).

9) Djemgou PC, Gatsing D, Kenmogne M, Nganga D, Aliyu R, Adebayo AH, Iane P, Ngadjui BT, Seguin E, Adoga GI. An anti-salmonelal agent and a new dihydroxanthracenone from *Cassia petersiana*. *Res. J. Med. Plant.*, 1, 65–71 (2007).

10) Raherim DAE. Phytochemical content and antibacterial activity of *Atriplex nummularia* extracts. *Int. J. Biol. Pharm. Allied Sci.*, 2, 1260–1269 (2013).

11) Abdelhammed RF, Ibrahim AK, Yamada K, Ahmed SA. Cytotoxic and anti-inflammatory compounds from red sea grass *Thalassodendron ciliatum*. *Med. Chem. Res.*, 27, 1238–1244 (2018).

12) Asem BD, Laitonjam WS, Oinam IS, Th J. Isolation of compounds from the aqueous methanol extract of *Cissus javana* DC leaves and determination of its trace element content through wet digestion. *Asian J. Chem.*, 26, 3820–3822 (2014).

13) Alam MS, Chopra N, Ali M, Niwa M. Oleanane and stigmasterol derivatives from *Ambroma augusta*. *Phytochemistry*, 41, 1197–1200 (1996).

14) Fang SH, Rao YK, Tseng YM. Anti-oxidant and inflammatory mediator’s growth inhibitory effects of compounds isolated from *Phyllanthus urinaria*. *J. Ethnopharmacol.*, 116, 333–340 (2008).

15) Imai Y. Preparation and subfractionation of microsomes. *Tanpakushita Kakusan Koso*, 10, 170–186 (1965).

16) Ibata Y. Phyto-ingredients and their influence on skin surface lipids-reference to ingredients of controlling testosterone-5α-reductase. *Fragrance J.*, 92, 78–83 (1988).

17) Yokoyama D. Characteristics and problems of evaluation methods for hair growing effect. *Fragrance J.*, 27, 50–56 (1999).