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 mankind, 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 Specranskia tuberculate, leaf of Breyria patens, whole plant of Teucrium viscudum, whole plant of Euphorbia lunulate, whole plant of E. humifusa, root of E. kansui, seed of E. lathyrus 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

The extract of P. urinaria was further investigated suppression of androgen activity of dihydrotestosterone in LNCaP cell line.8 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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (µg/mL)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. urinaria</td>
<td>50</td>
<td>24.3**</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>64.6**</td>
</tr>
<tr>
<td>G. eriocarpum</td>
<td>50</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>25.5**</td>
</tr>
<tr>
<td>S. tuberculata</td>
<td>50</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>31.9**</td>
</tr>
<tr>
<td>B. patens</td>
<td>50</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>49.7**</td>
</tr>
<tr>
<td>T. viscudum</td>
<td>50</td>
<td>31.5**</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>57.4**</td>
</tr>
<tr>
<td>E. lunulata</td>
<td>50</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>43.7**</td>
</tr>
<tr>
<td>E. humifusa</td>
<td>50</td>
<td>23.4**</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>54.0**</td>
</tr>
<tr>
<td>E. kansui</td>
<td>50</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>21.3</td>
</tr>
<tr>
<td>E. lathyrus</td>
<td>50</td>
<td>22.5*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>34.8**</td>
</tr>
<tr>
<td>E. pekinensis</td>
<td>50</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19.6</td>
</tr>
<tr>
<td>Finasteride</td>
<td>0.093</td>
<td>72.3**</td>
</tr>
</tbody>
</table>

Significant differences at p<0.01; ** and p<0.05; * against control group (n=3).

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naria 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 petersiana, Atriplex nummularia, Thalassodendron ciliatum, Cissus javana and Ambroma augusta as a biologically active compound along with various biological cytotoxic, anti-inflammatory, anti-bacterial and antimalarial activities. Although stigmasterol glucoside was previously isolated from P. urinaria, 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. tabercccute: ST110506-2, leaf of B. patens: BP20130614, whole plant of T. viscidum: TV110506-3, whole plant of E. lunulate: 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. Type II 5αR was prepared according to the method reported with modifications. 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
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 Laboratories, Inc., Hercules, CA, U.S.A.). The crude enzyme solution was diluted to 10 mg/mL and stored at −85°C until use.

A 5αR inhibition assay was performed according to the method reported (6) with minor modifications. The sample solution of various concentrations in methanol (50 µL), 590 µL of citrate/phosphate (Mcllvaine) buffer (pH 5.0), 20 µL of testosterone solution [0.4 mM in propylene glycol-citrate/phosphate buffer pH 5.0 (1:1 v/v)], and 120 µL of the enzyme solution were mixed in a plastic tube. The reaction was initiated by the addition of 20 µL of 34 mM nicotinamide adenine dinucleotide phosphate (NADPH). The mixture was incubated at 37°C for 30 min. After addition of 1.0 mL of dichloromethane and 20 µL of p-hydroxybenzoate-α-hexylester as an internal standard (I.S.), the organic layer was obtained by centrifugation (3000 × g for 10 min) and transferred into another tube. The solvent was evaporated and the residue was dissolved in 200 µL of methanol. An aliquot of 30 µL was injected into the HPLC under these conditions: column; YMC-Pak ODS-AM302 (4.6 i.d. × 150 mm), column temperature; 40°C, mobile phase; methanol/water (65:35, v/v), flow rate; 1.0 mL/min, detection; UV at 254 nm, retention time; 7 min for testosterone and 14 min for I.S. The control-0 min tube received 1.0 mL of dichloromethane before addition of the enzyme solution, while the control-30 min tube received 50 µL of methanol instead of the test sample. A similar procedure to that described above was carried out for these control tubes. 5αR inhibitory activity was determined from the following equation using the peak-area ratios (rr = testosterone/I.S.). Finasteride (Tokyo Chemical, Tokyo, Japan), a potent 5αR inhibitor and widely used to treat prostate hyperplasia was used as a reference drug.

\[
\text{Inhibition} (%) = \frac{C_{\text{sample}}}{C_{\text{control}}} \times 100
\]

\[
C_{\text{conversion rate}} = \frac{r_{\text{testosterone}} - r_{\text{dihydrotestosterone}}}{r_{\text{dihydrotestosterone}}} \times 100
\]

\[
C_{\text{control}} (C\text{ for control group}) = r \text{ of control-0min} - r \text{ of control-30min}
\]

**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:

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

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. (7) Male C57BL/6NCrSlc mice were purchased from Shimizu Laboratory Supplies Co., Ltd. Water and pellet 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, 28, and 30 after starting application, a hair growth score was given to each mouse as referred to in the picture depicted in Fig. 2. Oxe ndolone 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**
Structural Elucidation The 50% ethanol extract of *P. urinaria* (18g) was dissolved into 900mL of ethyl acetate, partitioned with water (900mL) and subjected to solvent partitioning using ethyl acetate and water (900mL). The active ethyl acetate soluble fraction (2.3g) was subjected to silicic acid column chromatography (250g, silica gel 60, Merck Millipore) 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 (0:1), 99:1, 9:1) 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