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Promotion of Hair Growth by *Rosmarinus officinalis* Leaf Extract

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Topical administration of *Rosmarinus officinalis* leaf extract (RO-ext, 2 mg/day/mouse) improved hair regrowth in C57BL/6NCrSlc mice that experienced hair regrowth interruption induced by testosterone treatment. In addition, RO-ext promoted hair growth in C3H/He mice that had their dorsal areas shaved. To investigate the antiandrogenic activity mechanism of RO-ext, we focused on inhibition of testosterone 5α-reductase, which is well recognized as one of the most effective strategies for the treatment of androgenic alopecia. RO-ext showed inhibitory activity of 82.4% and 94.6% at 200 and 500 μg/mL, respectively. As an active constituent of 5α-reductase inhibition, 12-methoxycarnosic acid was identified with activity-guided fractionation. In addition, the extract of *R. officinalis* and 12-methoxycarnosic acid inhibited androgen-dependent proliferation of LNCaP cells as 64.5% and 66.7% at 5 μg/mL and 5 μM, respectively. These results suggest that they inhibit the binding of dihydrotestosterone to androgen receptors. Consequently, RO-ext is a promising crude drug for hair growth.

Keywords: Rosmarinus officinalis; testosterone 5α-reductase; 12-methoxycarnosic acid; androgenic alopecia; hair growth.

INTRODUCTION

Hair growth is induced by vigorous proliferation of hair matrix cells and differentiation during migration to the surface of the scalp (Malkinson and Keane, 1978). Dermal papilla cells, which are mesenchyme cells under hair matrix cells, play key roles in differentiation, proliferation, and hair cycle control of hair follicles (Botchkarev and Kishimoto, 2003). Recently, the number of alopecia patients has tended to increase not only in terms of middle-aged and elderly men but also in young men and women. This can be attributed to the increasing stress from changes in society and diet (Arck *et al*., 2006). Alopecia is recognized as a serious cosmetic and mental problem, and the development of an effective agent for treatment has long been anticipated.

The major alopecia recognized is androgenic alopecia (AGA), and excess testosterone in blood capillaries is known as one of the causative factors (Hamilton, 1942). In fact, testosterone-treated mice exhibit AGA-like symptoms, and the hair regrowth effect of antiandrogenic agents has been demonstrated in various reports. Among them, we have reported the hair growth effects of *Schisandra chinensis* (Hirata *et al*., 2008), *Piper nigrum* (Hirata *et al*., 2007), *Lygodium japonicum* (Matsuda *et al*., 2002), *Anemarrhena asphodeloides* (Matsuda *et al*., 2001), Ginseng Radix (Matsuda *et al*., 2003), and Pueraria Flos (Murata *et al*., 2012).

In our recent report on Pueraria Flos, the activities of crude drug extracts showing estrogen activities were screened. Among them, the leaves of *Rosmarinus officinalis* were selected as a potent agent. *R. officinalis* is classified as Lamiaceae and is an indigenous plant around the Mediterranean Sea. The plant has been reported to possess antioxidative (Aruoma *et al*., 1996), antiinflammatory (Altinier *et al*., 2007), antibacterial (Bernardes *et al*., 2010), and antitumor effects (Singletary *et al*., 1996).

In the present study, *R. officinalis* leaf showed an antiandrogenic effect and hair growth promoting activity in each of the corresponding *in vivo* assays. To clarify the mechanism of the *R. officinalis* leaf extract hair growth effect, testosterone-5α-reductase (5αR) inhibitory activity and LNCaP cell growth inhibitory activity were studied. Furthermore, an active constituent on 5αR inhibition was determined.

MATERIALS AND METHODS

Materials. Leaves of *R. officinalis* were purchased from Maechu (Nara, Japan). Voucher specimens were deposited at the Faculty of Pharmacy, Kinki University. Reagents used in this study were of analytical grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise stated.

Preparation of *R. officinalis* leaf extract. The leaves of *R. officinalis* (50 g) were extracted with 500 mL of 50% aqueous ethanol for 2 h under reflux and filtered through filter paper (No. 2, Advantec, Tokyo, Japan). The extract manipulation was repeated, and the combined filtrates were evaporated under reduced pressure to obtain RO-ext (12.8 g, 26.0% yield).

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Animal experiments. Improvement in hair regrowth on testosterone-treated C57BL/6 mice was investigated according to the method reported previously (Yokoyama, 1999). Male C57BL/6NCrSlc mice were purchased from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan). Water and pellet chows were freely available. After 1 week of acclimatization, the dorsal hairs of ten male mice (7 weeks of age) for each administration group were shaved. After 30 min from the topical application of testosterone solution [0.07% in 50% ethanol (w/v)] to the shaved skin area, sample solutions of 100 μL in 80% ethanol were applied daily for 30 days. 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 described in Figure 1.

The hair growth effect on C3H/He mice was evaluated according to the method previously reported (Ogawa and Hattori, 1983). Male C3H/He mice were purchased from Shimizu Laboratory Supplies Co. Ltd. After 1 week of acclimatization, the dorsal hairs of ten male mice (7 weeks of age) for each administration group were shaved. From the next day, sample solutions in 100 μL of 80% ethanol were applied to the shaved skin areas daily for 30 days. 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 (Fig. 1).

All animal experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Inhibitory assay for 5α-reductase. Type II 5αR was prepared according to the method reported with modifications (Imai, 1965). Rats (Wistar, 9 weeks) were purchased from Shimizu Laboratory Supplies Co. Ltd. and kept at constant temperature (25°C) and humidity with 12 h light and dark cycles for 11 days. Water and pellet chow (Labo MR stock, Nosan Corporation, Tokyo Japan) were freely available. The epididymis was taken from 100 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, USA). The crude enzyme solution was diluted to 10 mg/mL and stored at −85°C until use.

A 5αR inhibition assay was performed using the method reported (Ibata, 1988) with minor modifications. The sample solution of various concentrations in methanol (50 μL), 590 μL of citrate/phosphate (McIlvaine) 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 the addition of 1.0 mL of dichloromethane and 20 μL of *p*-hydroxybenzoate *n*-hexyl ester as an internal standard (IS), the organic layer was obtained by centrifugation (3000 × g for 10 min) and transferred into another tube. The solvent was evaporated, the residue was dissolved in 200 μL of methanol, and 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, t_R; testosterone, 7 min, t_R; IS, 14 min. The control-0-min tube received 1.0 mL of dichloromethane before addition of the enzyme solution, whereas the control-30-min tube received 50 μL of methanol instead of the test sample. A similar procedure to that described previously was carried out for these control tubes. 5αR inhibitory activity was determined from the following equation using the peak-area ratios (r = testosterone/IS). Finasteride (Tokyo Chemical, Tokyo, Japan), a potent 5αR inhibitor and widely used to treat prostate hyperplasia, was used as a reference drug.

$$\text{Inhibition } (\%) = 100 \times C_{\text{sample}}/C_{\text{control}}$$

C; conversion rate (%) of testosterone to DHT

C_{sample} (C for sample groups) = r of test sample − r of control − 30 min

C_{control} (C for control groups) = r of control − 0 min − r of control − 30 min

Isolation and structural elucidation of the active constituent from RO-ext. RO-ext (3.0 g) was submitted to column chromatography over 50 g silica gel (4.0 i.d. × 13 cm, Merck, Darmstadt, Germany). Elution was performed with hexane and ethyl acetate (EtOAc) in increasing proportions to give hexane/EtOAc (1:1, v/v), EtOAc and MeOH fractions were obtained. Each hexane/EtOAc (1:1) fraction was submitted again to column chromatography over 40 g silica gel (1.2 i.d. × 10 cm). Elution with chloroform ($CHCl_3$) monitored with thin layer chromatography (TLC; Merck, silica gel

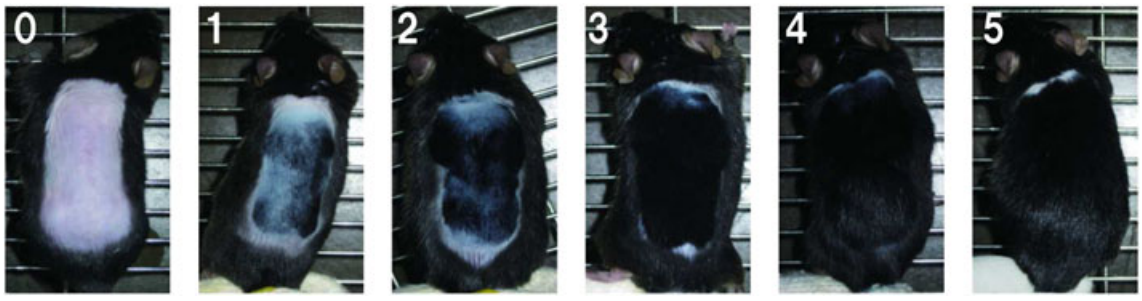

Figure 1. Scores for hair regrowth were given as follows: 0 = no growth; 1 = less than 20% growth; 2 = 20% to 40% growth; 3 = 40% to 60% growth; 4 = 60% to 80% growth; and 5 = 80% to 100% growth.

60 F254, $CHCl_3$ as the mobile phase, detection; UV and 10% sulphuric acid followed by heating) gave 204 chromatographic fractions of 15 g each, and the residue was eluted with MeOH. Collected $CHCl_3$ fractions were assembled into seven fractions as the basis for TLC analysis. The MeOH fraction was submitted to column chromatography over ODS (Lop-ODS, 1.0 i.d. × 21 cm, Nomura Chemical, Aichi, Japan). Elution was carried out with water and MeOH in increasing proportions to give 50%, 80%, and 100% MeOH fractions. The fraction of 80% MeOH was further purified with preparative HPLC utilizing these conditions: column; YMC-Pack ODS-AM323 (10 i.d. × 250 mm), column temperature; 40°C, mobile phase; methanol/water (4:1, v/v), flow rate; 4.0 mL/min, detection; UV at 254 nm, t_R; 17 min. As a result, 12-methoxycarnosic acid (12-MCA) was isolated (3.3 mg, 0.1% yield from RO-ext; Fig. 2).

The chemical structure of 12-MCA was identified by high-resolution mass spectrometry, obtained using LTQ Orbitrap Discovery (Thermo Fisher Scientific, Waltham, MA, USA) in the negative mode and nuclear magnetic resonance (NMR) spectra obtained in CD_3OD by a 700 MHz NMR spectrometer (JNM-ECA 700 with Delta 2, JEOL, Akishima, Tokyo) at 23.8°C. Chemical shifts were elucidated as delta from CD_2HOD at 3.30 ppm. High-resolution mass spectrometry of 12-MCA gave the molecular formula $C_{21}H_{28}O_4$ ($[M–H]^- = m/z$ 345.20681 observed, Δ ppm from calculated formula: −0.93). The data of ^1H and ^{13}C NMR spectra were identical to those of 12-MCA reported previously (Oluwatuyi *et al*., 2004) as shown in Table 1. The small discrepancies observed between two spectral data might be attributed from minor difference of measuring conditions.

Table 1. Nuclear magnetic resonance spectral data of ^1H (700 MHz) and ^{13}C (175 MHz) for 12-methoxycarnosic acid and isolated 12-methoxycarnosic acid in CD_3OD

	12-Methoxycarnosic acid*		Isolated 12-methoxycarnosic acid	
Position	δ (^1H) multiplicity (*J* in Hz)	δ (^{13}C)	δ (^1H) multiplicity (*J* in Hz)	δ (^{13}C)
1	3.56 dd (13, 3)	36.7	3.62 d (11)	35.6
2	1.51 m	22.1	2.20, 1.51 m	21.3
3	1.31, 1.47 m	43.6	1.49, 1.31	42.7
4		35.6		35.0
5	1.41 m	56.4	1.51, 1.28	55.7
6	1.88 d (2)	20.5	1.79 d (15)	19.8
	2.57 m		2.36 m	
7	2.75	33.9	2.79 m	33.3
8		135.7		135.3
9		129.5		128.6
10		35.6		35.0
11		151.2		149.9
12		145.2		144.4
13		140.9		140.6
14	6.42 s	118.9	6.45 s	118.4
15	3.15 sept	28.1	3.17 sept	27.5
16	1.16 dd (8, 0.5)	24.4	1.17 dd (7, 4.7)	23.9
17	1.16 dd (8, 0.5)	24.7	1.17 dd (7, 4.7)	24.1
18	0.96 s	33.9	0.98 s	33.2
19	0.92 s	22.3	0.92 s	21.0
20		180.7		NO
OMe	3.65 s	61.9	3.66 s	61.6

NO, not observed because of the extremely low sensitivity of carbon in carboxy acid.

*Referred from Oluwatuyi *et al.*, 2004.

Cell culture and inhibitory assay for LNCaP Cell growth. The test was performed according to the method previously described with minor modifications (Katayama *et al*., 2010). 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 U/mL penicillin–streptomycin at 37°C in an incubator in an atmosphere of 95% air and 5% CO_2. 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% dimethyl sulfoxide) with 5α-dihydrotestosterone (DHT) (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:

$$\text{Inhibition } \% = [\,(A - B) - (C - D)\,]/(A - B) \times 100$$

where *A* is with DHT, but without the sample, *B* is without DHT and the sample, *C* is with DHT and the sample, and *D* is with the sample but without DHT.

Bicalutamide, an antiandrogen agent, was used as a reference drug.

Statistical analysis. Data were analysed statistically with a multiple comparison procedure using a Bonferroni/Dunn algorithm and Statcel2 (OMS Publishing, Tokyo, Japan) to detect significant differences at $p = 0.05$ and 0.01.

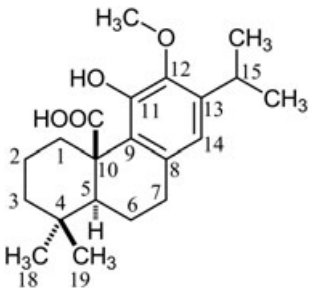

Figure 2. Chemical structure of 12-methoxycarnosic acid.

RESULTS AND DISCUSSION

Hair regrowth test in testosterone-treated C57BL/6NCrSlc Mice

The hair regrowth effect of RO-ext was assayed *in vivo* with a testosterone-sensitive male C57BL/6NCrSlc mouse model (Fig. 3). The hair regrowth of the

testosterone-treated group was delayed compared with the testosterone nontreated group. Under these conditions, topical administration of RO-ext solution (2 mg/day/mouse) improved hair regrowth in the testosterone-treated group. This data suggested that the hair growth activity of RO-ext was due to its antiandrogenic activity and therefore has potential as an effective treatment for AGA.

Hair growth effect on C3H/He mice

The hair growth effect on C3H mice that had their dorsal areas shaved was investigated. As a result, the RO-ext-treated group showed significant promotion of hair growth after 16 days of administration (Fig. 4). These data show that RO-ext possesses hair growth activity without mediating the androgenetic pathway.

Inhibitory activity of RO-ext on 5αR and identification of the active constituent

Testosterone is recognized as the key androgen for AGA. When testosterone is introduced to dermal papilla cells, it is irreversibly converted to DHT, which possesses higher androgenic activity than testosterone, by 5αR. Inhibitors of 5αR have been recognized as an effective target for treatment of AGA. Two isozymes have been characterized for 5αR, which are types I and II (Andersson *et al.*, 1991). Isozyme type I is mainly expressed in the liver and skin, whereas type II is found in prostatic glands, hair cells, and the epididymis (Thigpen *et al.*, 1993). Furthermore, isozyme type II is known to be closely associated with alopecia

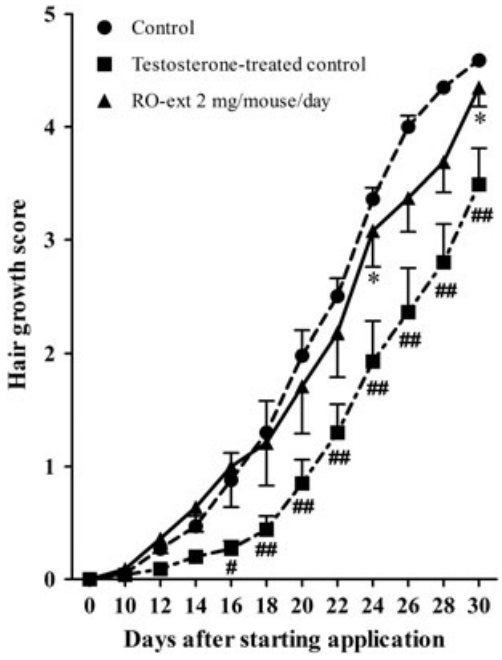

Figure 3. Effect of RO-ext on hair regrowth in testosterone-treated C57BL/6CrSlc mice. Control (no testosterone treatment, ●), testosterone-treated control (■), 2 mg/mouse/day of RO-ext (2% solution, ▲). The regrowth after beginning topical application was calculated by scoring. Each value represents the mean ± SE of $n = 10$. Significantly different from the control group at $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and from the testosterone-treated group at $^{*}p < 0.05$.

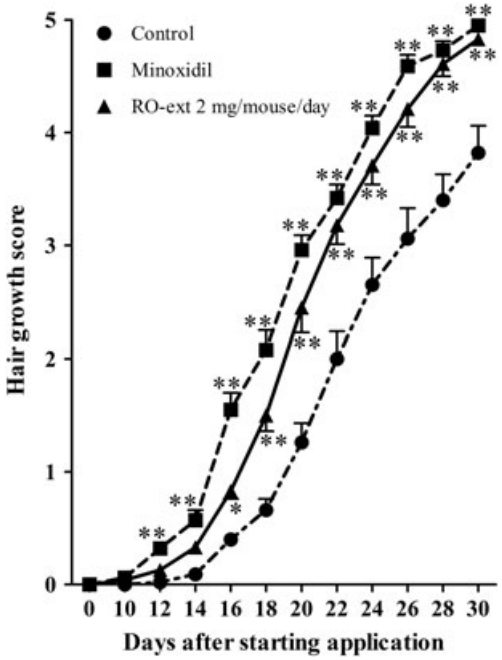

Figure 4. Effect of RO-ext on hair growth in C3H/He mice. Control (●), 1 mg/mouse/day (1% solution, ■) of minoxidil and 2 mg/mouse/day of RO-ext (2% solution, ▲). The growth after starting topical application was calculated by scoring. Each value represents the mean ± SE of $n = 10$. Significantly different from the control group at $^{*}p < 0.05$, $^{**}p < 0.01$.

(Imai, 1997; Tajima, 2001). From these facts, finasteride, a specific inhibitor of type II 5αR (Kawashima *et al.*, 2004), has been clinically applied as a hair-growth drug. Thus, 5αR inhibitors are potential hair growth agents.

The inhibitory activity on 5αR was determined, and the results are shown in Table 2. RO-ext showed 82.4% inhibition at 200 μg/mL. Successive liquid chromatography of RO-ext resulted in identifying 12-MCA as an active constituent for 5αR inhibition. The IC_{50} value of 12-MCA on the 5αR inhibitory assay was 61.7 μM. Although 12-MCA has been reported to have antioxidant activity (Oluwatuyi *et al.*, 2004), this is the first report, to the best of our knowledge, to present its inhibitory activity on the 5αR edge.

Rosmarinic acid and ursolic acid (Almela *et al.*, 2006; Ramírez *et al.*, 2006; Okamura *et al.*, 1993), which are known as major constituents in *R. officinalis* from previous reports, were tested for their inhibitory activities on 5αR and showed extremely low activities of 14.2% and 2.5% inhibition at 200 μM, respectively. Carnosic acid, which is also a known compound in *R. officinalis*, was

Table 2. Inhibitory effects of RO-ext on 5αR

Samples	Concentration (μg/mL)	Conversion rate (%)	Inhibition (%)
Control	–	37.9 ± 0.6	–
RO-ext	50	28.9 ± 0.9**	23.6
	200	6.7 ± 1.3**	82.4
	500	2.0 ± 2.6**	94.6
Finasteride	250 (nM)	6.8 ± 0.8**	81.9

Each value represents the mean ± SE of triplicates.
Significantly different from the control group at
$^{**}p < 0.01$.

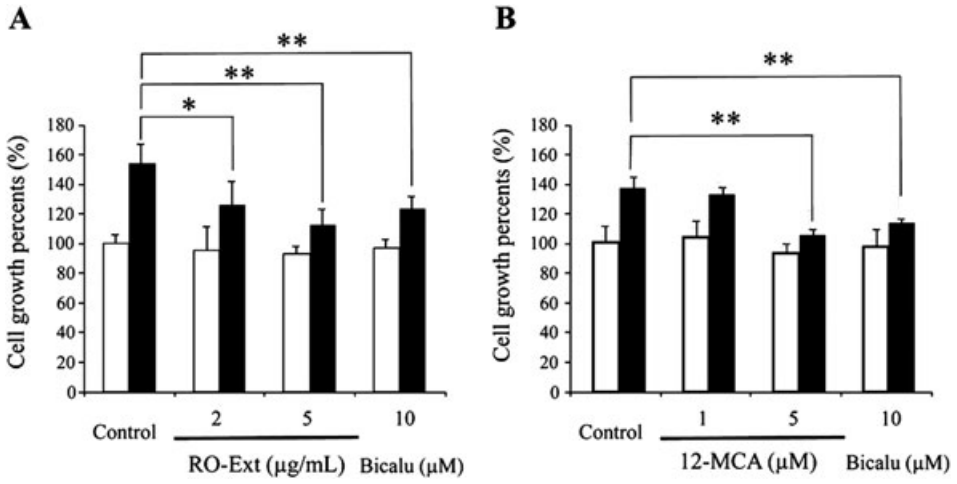

Figure 5. Inhibitory effects of RO-ext and 12-methoxycarnosic acid (12-MCA) on LNCaP cell growth induced by androgen. LNCaP cells were seeded into 96-well plates and incubated with or without 5α-dihydrotestosterone and various concentrations of RO-ext (A) or 12-MCA (B) for 3 days. Then, cell proliferation was determined to measure cell viability. Bicalutamide (Bicalu) was used as a positive control. Each value represents the mean ± SE of $n = 6$. Significantly different from the DHT-treated control at $*p < 0.05$, $**p < 0.01$.

not found at significant levels in the RO-ext used in this study from TLC analysis.

Inhibitory assay for LNCaP cell growth

5α-Dihydrotestosterone binds to androgen receptor (AR) on the nuclear envelope, and the binding complex is transported into the nucleus (Randall, 1994). The DHT/AR complex stimulates the transcription of TGF-β1 (Millar, 2002), TGF-β2 (Inui *et al*., 2002), and DKK-1 (Hibino and Nishiyama, 2004), which are known to miniaturize hair follicles (Kwack *et al*., 2008) and shorten the anagen phase in the hair cycle.

Inhibition of DHT/AR complex formation leads to suppression of negative growth regulatory factor expression for the hair follicle. Consequently, inhibition of DHT binding to the AR is recognized as an important target, as well as inhibition of 5αR, in the treatment of AGA. Inhibitory activity of binding to AR was evaluated with the cell proliferation of the prostate cancer cell line LNCaP. Because the proliferation of LNCaP can be accelerated by DHT binding to AR (Goldmann *et al*., 2001), the binding activity of DHT can be estimated using a cell proliferative assay. From our results, the proliferation was accelerated 1.4-fold compared with the control group with a 10 nM application (Fig. 3), whereas the applications of RO-ext (Fig. 5A) or 12-MCA (Fig. 5B) were suppressed in a dose-dependent manner. Inhibitory activities of RO-ext at 2 and 5 μg/mL were 43.5% and 64.5%, respectively, whereas those of 12-MCA at 1 and 5 μM were 20.0% and 66.7%, respectively. Neither sample showed significant cell toxicity at the concentration tested. Consequently, it was hypothesized that RO-ext and 12-MCA have inhibitory activity against the binding of DHT to AR.

CONCLUSION

In this report, we obtained the following results for RO-ext: (1) RO-ext showed improvement of hair growth in an AGA model mouse and a hair growth activating model; (2) RO-ext showed potent inhibitory activity of 5αR and on binding of DHT to AR; and (3) an investigation to find the active constituent of 5αR and DHT-binding inhibition revealed 12-MCA.

From these results, RO-ext is a promising candidate for treatment of AGA and/or non-AGA. The mechanisms of the hair growth effect on C3H mice and the active principle should be further studied elsewhere.

Acknowledgements

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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