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Anti-androgenic activities of the triterpenoids fraction of *Ganoderma lucidum*

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Abstract

The ethanol extract of *Ganoderma lucidum* showed inhibitory activity on both isozymes (types 1 and 2) of 5α-reductase and suppression effects of ventral prostate growth induced by testosterone in castrated rat, but not induced by dihydrotestosterone. Activity-guided fractionation and TLC analysis suggested that the active principles in vivo were triterpenoids. These results indicate that the triterpenoids fraction of *G. lucidum* might be a useful ingredient in the treatment of benign prostatic hyperplasia.

Keywords: 5α-Reductase; *Ganoderma lucidum*; Anti-androgen activities; Benign prostatic hyperplasia; Triterpenoid

1. Introduction

Nowadays androgen-mediated diseases such as prostate cancer, hirsutism, acne, androgenic alopecia, and benign prostatic hyperplasia (BPH) are considered to be a serious problem (Bartsch, Rittmaster, & Klocker, 2002; Wasser & Weis, 1999). Above all, BPH is one of the most common symptoms seen in older men, and 40% of men 50–60 years of age and 90% of those 80–90 years of age are diagnosed with BPH. The principal prostatic androgen is dihydrotestosterone (DHT), which is synthesized by steroid enzyme 5α-reductase from its substrate testosterone (Russell & Wilson, 1994). Since the weight of the seminal vesicles depends on the 5α-reduced androgens, it is important to maintain adequate levels of the DHT. Two isoforms of 5α-reductase have been cloned, expressed, and characterized (types 1 and 2) that display different tissue expression patterns, enzyme kinetic parameters, and chromosomal localization (Jenkins et al., 1991). Both isozymes are overexpressed in BPH tissue (Iehle et al., 1999). Because BPH therapy can reduce DHT levels by blocking its conversion from testosterone, 5α-reductase inhibitors could be useful in the treatment (Bartsch, Rittmaster, & Klocker, 2000). A number of compounds have been identified as that, including both a steroidal and a nonsteroidal inhibitor. However, it has been reported that these inhibitors may cause adverse effects such as gynecomastia, impairment muscle growth, and severe myopathy (Uygur, Gur, Arik, Altug, & Erol, 1998). Therefore, the emergence of therapeutic materials with fewer side effects, especially edible natural products, has been considered desirable if the safety of these products can be guaranteed. For thousand of years, mushrooms have been known to be a source of medicine. They are widely sold as nutritional supplements and touted as beneficial for health. We have therefore focused on edible and medicinal mushrooms as possible sources of 5α-reductase inhibitory ingredients.

The fungi *Ganoderma lucidum* has been used for centuries in East Asia. Its fruiting body is called as "Reishi" in

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Japan and "Lingzhi" in China. In these areas, *G. lucidum* has been a popular folk or oriental medicine to cure various human diseases such as hepatitis, hypertension, hypercholesterolemia, and gastric cancer (Wasser & Weis, 1999; Yun, 1999). However, the precise mechanism and active compounds of *G. lucidum* against these biological activities have remained unclear.

In the continuing search for active principles from the ethanol extract of *G. lucidum* against the growth inhibition of the ventral prostate induced by testosterone in rat, we have found a characteristic fraction containing triterpenoids after separation by silica gel column chromatography that showed significant activity.

2. Materials and methods

2.1. Materials

The fruiting body of *G. lucidum* was obtained from Bisoken Inc. (Fukuoka, Japan). The fruiting bodies were dried and ground to a powder before use. Unless otherwise specified, chemicals were obtained from Sigma Chemical Co. (Japan). Organic solvents were purchased from Wako Pure Chemical Industries Co. (Japan). [4-^{14}C] Testosterone was obtained from Perkin–Elmer (Japan). Sprague–Dawley (SD) rat was obtained from Charles River (Japan). Ganoderol B, ganodermanontriol, ganoderic acid G and ganoderic acid C2 were provided by the Laboratory of Systematic Forest and Forest Products Sciences, Department of Forest Products, Faculty of Agriculture, Kyushu University in Japan.

2.2. Ethanol extracts of G. lucidum

Dried and chipped *G. lucidum* (15 kg) was extracted with 95% ethanol (126 l) at room temperature for 24 h using a blender. The extracts were filtered through ADVANTEC No. 2 filter paper, concentrated under vacuum, and then freeze-dried. The extracts (571.1 g) were stored at −20 °C before assay.

2.3. Fractionation of the ethanol extracts of G. lucidum by silica gel column chromatography

A portion of the ethanol extracts (50 g) was fractionated into three fractions (Fr. A–C) by column chromatography on silica gel (Wakogel C-200, Wako, Osaka, Japan) (2 kg, column size; 20 cm i.d. × 150 cm) eluting with an *n*-hexane-EtOAc step-gradient [Fr. A: *n*-hexane:ethyl acetate = 9:1 (2 l), 8:2 (2 l); Fr. B: 7:3 (2 l), 6:4 (2 l), 5:5 (2 l), 4:6 (2 l), 3:7 (2 l), 2:8 (2 l); Fr. C: 1:9 (2 l), MeOH (6 l)]. [Fr. A: TLC, silica gel, I_2 detection, EtOAc/*n*-hexane, 7:3, Rf 0.97–0.48, Fr. B: Rf 0.67–0.03, Fr. C: Rf 0.04–0] (Fig. 3). These procedures were repeated 11 times and combined into each fraction [Fr. A (240 g), Fr. B (35 g), Fr. C (269 g)]. From the further separation of a part of Fr. A (1.0 g) by a silica gel column guided with a 5α-reductase inhibitory assay, the palmitic acid (9.3 mg), oleic acid (9.8 mg), and linoleic acid (12.3 mg) were isolated and identified by comparison with GC–MS data of commercially available standard samples.

2.4. Preparation of rat microsomes

Rat liver and prostate microsomes from female SD rat (7 weeks age) and male SD rat (13 weeks age) were prepared, respectively, by a method previously reported by Shimizu et al. with some modifications (Shimizu, Fukuda, Kondo, & Sakai, 2000). Two mature SD female rats were killed. The liver was removed, and minced tissue was then homogenized in a 4-tissue volume medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5). Also, three mature SD male rats were killed. The prostate was removed, and minced tissues were then homogenized in 4-tissue volume medium A. The homogenate was then centrifuged at 10,000*g* for 10 min. The resulting supernatant from the centrifugations was further centrifuged twice at 105,000*g* for 1 h twice. The washed microsomes were suspended in 1-pellet volume medium A, and the dispersion of microsomes was achieved using a syringe with 18 G, 23 G, and 26 G needles in succession. The microsome suspension was divided, diluted with medium A, and stored at −80 °C until just before use.

2.5. Measurement of 5α-reductase inhibitory activity

The complete reaction mixture included 1 mM dithiothreitol, 20 mM phosphate buffer (pH 6.5 for type 1 or pH 5.0 for type 2), 1.9 nCi [4-^{14}C] testosterone, 150 μM testosterone, 167 μM NADPH, and the enzyme preparation (1.54 mg of protein) in a final volume of 0.3 ml. The concentration of testosterone contributed by [4-^{14}C] testosterone was negligible. The incubation was carried out for 10 min at 37 °C, and was started by the addition of 10 μl microsomes to the preheated reaction solution in a tube. After 10 min, the incubation was terminated by adding 10 μl of 3 M NaOH. To extract the metabolites, 1 ml of diethyl ether was added, and the tubes were capped and shaken. The organic phase was applied to a silica plate (Kieselgel 60 F_{254}). The plate was developed in ethyl acetate–*n*-hexane (7:3) at room temperature. The radioactivity profile was determined with an imaging analyzer (FLA-5000 RF, Fuji Film Co. Ltd., Tokyo, Japan). The 5α-reductase activity was calculated from the percentage of the extent of the conversion of [4-^{14}C] testosterone to [4-^{14}C] dihydrotestosterone.

2.6. Growth suppression of the rat prostate by G. lucidum

The assay for growth suppression of the rat prostate was carried out as described by Fukuta et al. (Fukuta et al., 1999). The testes of SD rat were removed at 4 weeks of age under light anesthesia with pentobarbital. After 4 days, testosterone (100 μg/ body) or dihydrotestosterone

(300 μg/ body) was injected s.c. into the rats once daily for 7 days. The animals were freely administered CL-2 food (Oriental yeast Co., LTD). The samples suspended in 0.5% methylcellulose were orally administered at each concentration once daily. The flutamide (10 mg/kg/day) was used as the positive control and was suspended in 0.5% methylcellulose and orally administered once daily for 7 days. After 7 days, the rats were fasted for 18 h and sacrificed by pentobarbital. The prostate was then removed, and the weight was determined.

2.7. Statistics

Results were expressed as means ± SD. Statistical significance was determined by ANOVA and Bonferroni-type multiple *t*-test.

3. Results and discussion

In our previous screening of 19 edible and medicinal mushrooms, we discovered that the ethanol extract of the fruiting body of *G. lucidum* showed the strongest 5α-reductase inhibitory activity (Fujita et al., 2005). In addition, the treatment of the ethanol extract prepared from *G. lucidum* at 1.5 and 15 mg/kg/day significantly inhibited the growth of the ventral prostate induced by testosterone in rat (Fujita et al., 2005). These results led us to further investigation of the ethanol extract of *G. lucidum*. The inhibition of the 5α-reductase prepared from rat liver by the ethanol extract of *G. lucidum* was concentration-dependent, as shown in Fig. 1. As the concentrations of ethanol extract increased, the residual enzyme activity rapidly decreased. The inhibitory concentration leading to 50% activity loss (IC_{50}) was estimated to be 93.6 μg/ml. It should be noted that finasteride (Liang, Cascieri, Cheung, Reynolds, & Rasmusson, 1985), which is known as a potent steroidal inhibitor, showed an IC_{50} of 0.73 μM in our assay system.

To clarify the active principles of the ethanol extract of *G. lucidum*, 5α-reductase inhibitory activity-guided fractionation was carried out. The ethanol extracts were roughly separated into three fractions (Fr. A, B, C), which were analyzed by TLC as illustrated in Fig. 2. Fr. A and Fr. B showed 5α-reductase inhibitory activity of more than 90% at 200 μg/ml, while Fr. C did not show the 5α-reductase inhibitory activity (less than 25% inhibition at 200 μg/ml) (data not shown). Most of constituents of Fr. A were clarified to be fatty acids by GC–MS analysis (data not shown). A portion of Fr. A was submitted to further separation by silica gel CC, which led to the isolation of palmitic acid (IC_{50} = 870 μM), oleic acid (IC_{50} = 42.4 μM), and linoleic acid (IC_{50} = 190 μM) as 5α-reductase inhibitory components.

Some fatty acids have been reported as 5α-reductase inhibitory materials (Liang & Liao, 1992). Also, amongst the numerous phytotherapeutic compounds aimed at treating lower urinary tract symptoms (LUTS) secondary to BPH, the most popular and widely used is the extract of the dried ripe fruit of *Serenoa repens* (also named, *Sabal serrulata*) (Lowe & Fagelman, 1999). The lipid–sterolic extract of *S. repens* (LSESr, Permixon®), obtained by hexane extraction, is essentially composed of fatty acids (a mean from 15 different batches of 94 g/100 g of extract), of which free fatty acids account for a mean of 83.5 g/100 g of extract. The mean percentage values in the free fatty acid fractions are 35%, 30%, 11%, and 5% for oleic, lauric, myristic, and linoleic acids, respectively (Niederpruem, Schweikert, & Zaenker, 1994; Weisser, Tunn, Behnke, & Krieg, 1996).

In light of these previous reports indicating that *S. repens* has high levels of fatty acids, fatty acids such as oleic acid, linoleic acid, and palmitic acid isolated from Fr. A were expected to be involved in the growth suppression of prostate caused by the ethanol extract of *G. lucidum*. However, the treatment of Fr. A and oleic acid which

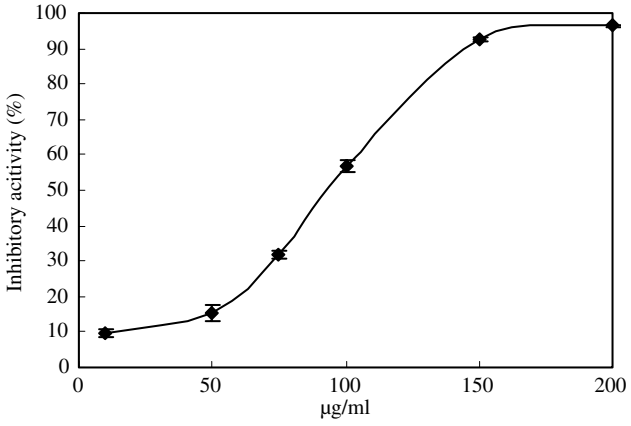
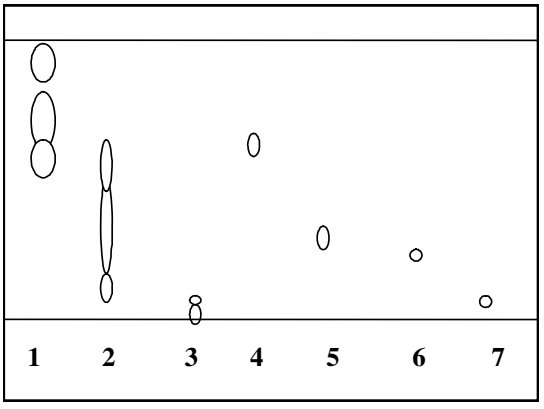


Fig. 1. The concentration dependence of the inhibitory effects of the ethanol extract of *G. lucidum* on 5α-reductase (liver microsome, pH 6.5).



1. Fr. A, **2.** Fr. B, **3.** Fr. C, **4.** ganoderol B, **5.** ganodermanontriol, **6.** ganoderic acid G, 7. **ganoderic acid C2**

Fig. 2. TLC analysis of Fr. A, B and C prepared from the ethanol extract of *G. lucidum*, and the well-known representative triterpenoids (ganoderol B, ganodermanontriol, ganoderic acid G and ganoderic acid C2) in *Ganoderma* mushroom (EtOAc:*n*-hexane = 7:3).

showed the strongest 5α-reductase inhibitory activity among fatty acids isolated from Fr. A, did not inhibit the growth of the ventral prostate induced by testosterone in rat (Fig. 3). Also, as expected, Fr. C did not show inhibitory activity in vivo. Interestingly, Fr. B showed a growth-suppression effect as similar to that of finasteride which is used as a positive control.

G. lucidum has been reported to produce many bioactive oxygenated triterpenoids. Up to now, over 120 species of triterpenoids have been isolated from *G. lucidum* and the genus *Ganoderma* (Shiao, 2003). It is likely that most of triterpenoids are present in Fr. B by the result of TLC analysis (Fig. 2) of typical triterpenoids isolated from *G. lucidum* (Shiao, 2003). For example, the Rf values of ganoderol B, ganodermanontriol, ganoderic acid G and ganoderic acid C2 which are known to be the typical triterpenoids in *Ganoderma* mushroom, developed in ethyl acetate–*n*-hexane (7:3) are 0.64, 0.30, 0.23 and 0.07, respectively (Fig. 2). Also, it has been confirmed that these triterpenoids are detected in the Fr. B by qualitative HPLC analysis, but not quantitative analysis (data not shown). Therefore, it is reasonable that we define the Fr. B as the triterpenoids fraction. Also, it should be noted that we have not examined the activities of these triterpenoids on 5α-reductase and animal test yet, because of fewer limited amount of samples. We are still trying to isolate these triterpenoids for further experiments.

Two 5α-reductase isozymes have been identified in rats and humans (Russell & Wilson, 1994). Both isozymes are overexpressed in BPH tissue (Iehle et al., 1999). Coded by two different genes (Andersson & Russell, 1990), they display a maximal activity at different pH values (6.0–8.5 for type 1 and 5.0–5.5 for type 2); they also have different biochemical characteristics (Li, Chen, Singh, & Labrie, 1995). In rats, the type 1 isozyme predominates in tissues such as liver, kidney, brain, lung, and skin, but it also exists in prostate, whereas the type 2 isozyme is more abundant in genital tissues such as prostate. Fr. A and Fr. B inhibited 5α-reductase activity derived from both isozymes prepared from prostate (Fig. 4).

The reason that Fr. A did not show a growth-suppression effect in prostate, despite its potent 5α-reductase inhibitory activity, remains unclear. However, it may not be illogical to assume that the fatty acids are not an active principle in vivo since fatty acids are easily metabolized into inactive compounds or do not reach the target organ by oral administration. Also, it has been reported that oral administration of some dietary fatty acids enhances steroid-metabolizing microsomal membrane-bound enzymes, 5α-reductase, and aromatase in rat liver (Venkatraman, Rao, Fink, & Awad, 1996).

Prostatic enlargement is dependent on tissue androgen, namely DHT, which is converted from testosterone by steroid 5α-reductase. In the present study, we investigated the effects of *G. lucidum* on steroid 5α-reductase activity and on the growth of prostate induced by testosterone in castrated rats. The Fr. B was found to inhibit both types of 5α-reductase, and this so-called dual inhibition might be advantageous in the therapy for BPH, since it has been shown that the dual inhibitor dutasteride is more powerful in reducing DHT plasma concentrations than selective type 1 or type 2 inhibitors (Graul, Silvestre, & Castaner, 1999). In addition, the treatment of ethanol extract of *G. lucidum* and Fr. B significantly inhibited the growth of the ventral prostate induced by testosterone in castrated rats. These results suggest that the suppression effects of prostatic growth by *G. lucidum* might come in part from its ability to act as a 5α-reductase inhibitory material.

There are commonly two ways to suppress prostate regrowth in animal experiments. One is inhibiting the 5α-reductase activity. The other is to act on the androgen receptors as an androgen receptor antagonist. The androgen antagonist can suppress the dihydrotestosterone-induced prostate regrowth. Therefore, blocking dihydrotestosterone from binding to the androgen receptors in the prostate grand is considered to be a possible

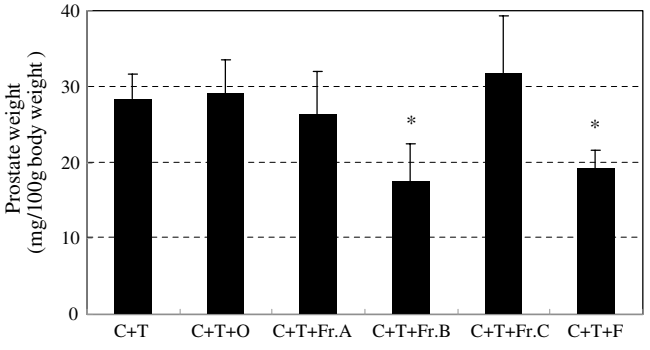


Fig. 3. Effects of the fractions prepared from ethanol extracts of *G. lucidum* on testosterone-induced regrowth of the castrated rat prostrate. Each column represents the mean ± SD, $n = 6$; C: castrated rat, T: testosterone, O: olenic acid (1 mg/kg of body weight), Fr. A, Fr. B, Fr. C (1 mg/kg of body weight), F: finasteride (1 mg/kg of body weight) $^{*}p < 0.01$ against C + T.

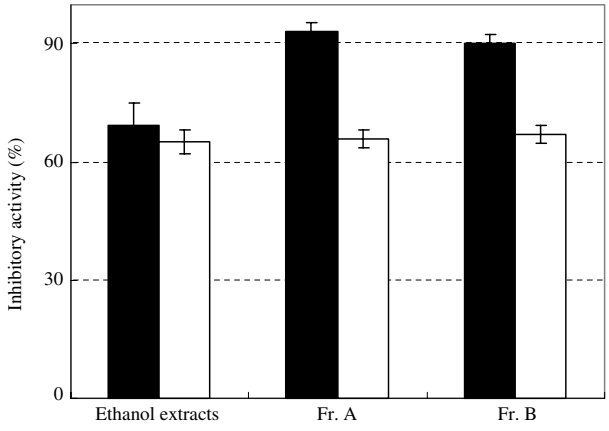


Fig. 4. The inhibitory activity of Fr. A, Fr. B and the ethanol extracts of *G. lucidum* on type 1 and type 2 5α-reductase. Each column represents the mean ± SD. Sample concentration is 200 μg/ml, $n = 3$. (■): liver microsome (pH 6.5 for type 1), (□): prostate microsome (pH 5.0 for type 2).

mechanism of action other than 5α-reductase inhibition of the extract of *G.lucidum.* To examine this possibility, the effects of ethanol extracts of *G. lucidum* on prostate growth induced by dihydrotestosterone have been investigated. In other words, if the prostate suppression is caused by only 5α-reductase inhibition, the dihydrotestosterone-induced prostatic regrowth cannot be suppressed. Four days after castration, the weights of the rat prostates were markedly reduced, and the prostate size was recovered by s.c. injections of dihydrotestosterone (300 μg/ body) (Fig. 5). Ethanol extract of *G. lucidum* had no effect on the weight of the prostate in the castrated rats that received dihydrotestosterone, whereas flutamide (Reid, Kantoff, & Oh, 1999), an androgen receptor antagonist, significantly reduced the prostate weights (Fig. 5). These results suggest that *G. lucidum* inhibited prostatic growth by the inhibition of 5α-reductase activity rather than having a direct effect on the androgen receptor, although the precise mechanism remains unclear.

The fungi *G. lucidum* (Reishi, Mannentake, or Lingzhi) has been used for centuries in East Asia to cure various human diseases such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, and cancers (Wasser & Weis, 1999; Yun, 1999). Its dried powder was especially popular as a cancer chemotherapy agent in the Imperial Court of ancient China (Mizushina et al., 1998). Some of the triterpenes such as ganoderic and lucidic acids, recently isolated from *Ganoderma*, have demonstrated cytotoxicity against mouse sarcoma and mouse lung carcinoma cells in vitro (Min, Gao, Nakamura, & Hattori, 2000). Intraperitoneal administration of water-soluble polysaccharides isolated from *Ganoderma* has been found to inhibit the growth of sarcoma-180 solid tumors in mice (Sone & Misaki, 1985). In addition, polysaccharides from *Ganoderma* also potentiate production of cytokines, which subsequently suppress proliferation of HL-60 and U937 leukemic cell lines (Wang et al., 1997).

The use of herbal therapies in alternative medicine has been increasing, and although the number of cancer patients using herbal dietary supplements is not exactly known, there is evidence of the increasing use of dietary supplements in cancer treatment (Eisenberg et al., 1998). *G. lucidum* is one of the herbs in the herbal mixture PC-SPES, which has shown activity against hormone-refractory disease in two prostate cancer patients (de la Taille, Hayek, Burchardt, Burchardt, & Katz, 2000). Extracts of PC-SPES have demonstrated estrogenic effects (DiPaola et al., 1998) and decrease the growth of hormone-sensitive as well hormone-insensitive prostate cancer cells (de la Taille et al., 1999). Our results suggest that these effects might be related to not only the anti-cancer effects of *G. lucidum* but also anti-androgen effects. Since excessive 5α-reductase activity has been proposed to be a possible contributing factor in prostate cancer development and progression, the development and progression of prostate cancer may also be affected by diets containing inhibitors of 5α-reductase.

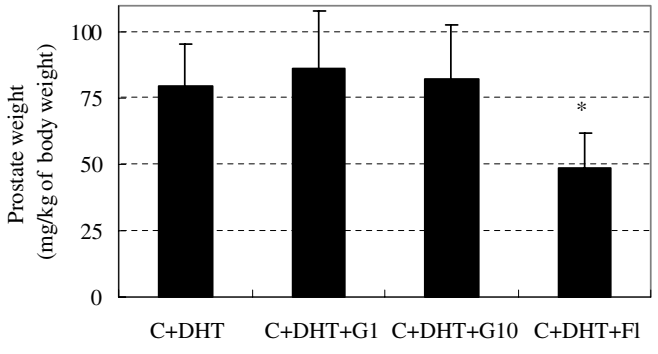


Fig. 5. Effects of ethanol extracts of *G. lucidum* on dihydrotestosterone-induced regrowth of the castrated rat prostrate. Each column represents the mean ± SD, $n = 6$; C: castrated rat, DHT: dihydrotestosterone, G1: ethanol extracts of *G. lucidum* (1.0 mg/kg of bodyweight), G10: ethanol extracts of *G. lucidum* (10 mg/kg of body weight), F: flutamide (10 mg/kg of body weight) $^{*}p < 0.01$ against C + T.

In the present study, we detected anti-androgenic activities of the ethanol extract of *G. lucidum* on in vitro 5α-reductase inhibitory activity and in vivo growth suppression of the rat prostate. Our results indicate that the active principles in vivo might be triterpenoids in Fr. B rather than fatty acids in Fr. A. In the future, herbal therapies will likely become important treatments in wide use for many diseases (Uygur et al., 1998). The anti-androgenic activity of *G. lucidum* is important biological activity that could be useful in BPH patients. At this time, the clinical implications of this activity are unknown, so further research is needed for general use to apply *G. lucidum* to the treatment of BPH.

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