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Effects of Natural Products on the Inhibition of 5α-Reductase Type 2 for the Development of Chemopreventive Agents in LNCaP Cells

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Abstract – The enzyme steroid 5α-reductase is responsible for the conversion of testosterone into the most potent androgen dihydrotestosterone (DHT). In man, this steroid acts on a variety of androgen-responsive target tissues to mediate such diverse endocrine processes as male sexual differentiation in the fetus and prostatic growth in men. Androgen levels in the prostate may influence carcinogenesis in this organ. The use of a 5α-reductase inhibitor, finasteride, in the chemoprevention of prostate cancer is being evaluated in a clinical trial and have been used successfully for treatment of benign prostatic hyperplasia. Therefore, for the discovery of 5α-reductase type 2 inhibitors, we have evaluated the inhibitory effects of solvent fractionated extracts of natural products on 5α-reductase type 2 activity. We have tested approximately 80 kinds of natural products after partition into n-hexane, ethyl acetate and aqueous layers from 100% methanol extracts of plants. The ethyl acetate fractions of *Perilla sikokiana* (seed, IC_{50} : 6.2 ug/ml), *Sophora flavescens* (root, IC_{50} : 8.9 ug/ml), and *Angelica tenuissima* (root, IC_{50} : 11.7 ug/ml) revealed inhibitory effects on 5α-reductase 2 activity in LNCaP cells. The effective ethyl acetate fractions of *Perilla sikokiana*, *Sophora flavescens*, *Hydnocarpus anthelmintica*, and *Angelica tenuissima* were subfractionated by column chromatography and tested. The subfractions F4 (IC_{50} : 1.1 ug/ml), F5 (IC_{50} : 2.0 ug/ml), and F6 (IC_{50} : 5.8 ug/ml) of the ethyl acetate fraction of *Perilla sikokiana* and the subfraction F8 (IC_{50} : 5.3 ug/ml) of the ethyl acetate fraction of *Sophora flavescens* displayed greater inhibition of 5α-reductase type 2 than did finasteride in LNCaP cells. These active fractions are under the process of further sequential fractionation to find the effective pure compounds against 5α-reductase 2 activity.

Key words – 5α-reductase type 2, Chemoprevention, Prostate cancer, Benign prostatic hyperplasia, LNCaP cells.

Introduction

Androgens are required for the normal development and function of the prostate gland. Prostate cancer and benign prostatic hyperplasia (BPH) are common in men and develop in an environment of continuous androgen exposure. In most western countries, prostate cancer is one of the leading causes of cancer-related death in men (Dijkman and Debruyne, 1996). Androgens play an important role in carcinogenesis of the prostate. Exogenous androgen supplementation is required in most animal prostate carcinogenesis models (Pollard *et al.*, 1993; Shirai *et al.*, 1995). The higher level of circulation testosterone may explain the higher incidence of prostate cancer in a human population (Ross *et al.*, 1986; Ellis and Nyborg, 1992). Clinical prostate cancers often respond to androgen-deprivation therapy. Thus, reduction in androgen levels should affect carcinogenesis processes, although systemic androgen ablation is not acceptable as a preventive measure because of associated male sexual dysfunctions.

In the prostate, testosterne is rapidly and irreversibly converted to 5α-dihydrotestosterone (Bruchovsky and Wilson, 1968; Anderson and Liao, 1968). This metabolite plays a much more important role than testosterone in the organogenesis and homeostasis of the prostate (Cheng *et al.*, 1993; Wilson, 1996;

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Perterson *et al.*, 1977). The administration of 5α-reductase inhibitors results in a substantial decrease in prostatic secretion of the normal gland and a substantial increase in cell death in normal and transformed prostatic cells (Kadohama *et al.*, 1984; Lamb *et al.*, 1992). The racial populations with a higher incidence of prostate cancer were shown to have a higher activity of 5α-reductase (Wu *et al.*, 1995; Lookingbill *et al.*, 1991). Also, men with prostatic cancer have an increased conversion rate of testosterone to its potent 5α-reduced metabolites (Meikle *et al.*, 1987).

A series of 5α-reductase inhibitors have been synthesized (Lamb *et al.*, 1992; Brooks *et al.*, 1981; Liang *et al.*, 1984; Hirosumi *et al.*, 1995a, b). Fortunately, 5α-reductase inhibitors, such as finasteride, while lowering intraprostatic concentration of dihydrotestosterone, do not affect adversely serum testosterone level or sexual functions (Gormley *et al.*, 1992b). These observations provide the rationale for a chemoprevention trial using a 5α-reductase inhibitor as the preventive medicine, and such a study involving 18,000 men randomly assigned to receive finasteride or placebo has been started in the United States (Brawer and Ellis, 1995; Gormley, 1992a). Besides, these inhibitors of 5α-reductase are of considerable interest for the treatment of the diseases that are at least partially dependent on DHT such as BPH, acne, and male pattern baldness (Wilson, 1980; Mooradian *et al.*, 1987; Cunha *et al.*, 1987; Metcalf *et al.*, 1987).

In man, two isozymes of 5α-reductase, designated types 1 and 2, have been reported (Anderson and Russell, 1990; Anderson *et al.*, 1991). 5α-Reductase type 1 enzyme is expressed at low levels in prostate and has been found mainly in skin and liver. The enzyme has a neutral to basic pH optimum and is insensitive to finasteride inhibition (Anderson and Russell, 1990). 5α-Reductase type 2 enzyme, often referred to as the prostatic 5α-reductase, is expressed at high levels in prostate and has been found in seminal vesicles, liver, and epididymis also (Thigpen *et al.*, 1993). The enzyme has an acidic pH optium and is sensitive to finasteride (Anderson *et al.*, 1991). Since both isozymes contribute to the plasma level of dihydrotestosterone, it is necessary to design potent inhibitors of both the isozymes.

Previous studies that described subjects with 5α-reductase deficiency suggested that 5α-reductase inhibitors could be used safely in adult men because low DHT levels throughout life did not compromise normal muscular development or health (Imperato-McGinley *et al.*, 1974, Walsh *et al.*, 1974).

Therefore, as a strategy for the development of chemopreventive agents of prostate cancer and BPH treatment agents, we evaluated natural products to find inhibitors of the type 2 isozyme of human 5α-reductase with higher potency and efficacy than finasteride.

Experimental

Chemicals and reagents – [1,2,6,7,-^{3}H] Testosterone (97Ci/mmol) was purchased from Dupont - New England Nuclear (Boston, MA, USA). Cell culture media, reagents and finasteride were obtained from Sigma (St. Louis, MO, USA) and Gibco/BRL(Grand Island, NY, USA). The serum was heat-inactivated and stripped of endogenous steroids by incubation with dextran-coated charcoal before use.

Plant materials – Medicinal plants were purchased from herb markets in Seoul, Korea and voucher specimens have been deposited at Herbarium of Natural Products Research Institute, Seoul National University, Seoul, Korea. Each of the dreid herbs was sliced, and extracted 3 times with 100% methanol at room temperature. The methanol extracts were concentrated under reduced pressure below 40°C, and then the concentrated methanol extracts were partitioned into *n*-hexane, ethyl acetate and then water.

Cells and cell culture – Human prostatic carcinoma cell line LNCaP was purchased from American Type Culture Collection (Rockville, MD, USA). They were maintained in RPMI 1640 medium (Sigma Chem. Co. USA) supplemented with 5% fetal bovine serum (Gibco BRL, USA) at 37°C in a humidified atmosphere(5% CO_2).

5α-Reductase assay – The enzyme assay is based on the previous method with some modification (Bologna *et al.*, 1995). Confluent cells were washed with phosphate-buffered saline (PBS, pH 7.4) and then digested wtih trypsin-EDTA for 10 min. The trypsinized LNCaP cells have been plated at a density of 5×10^4 cells/well in 24-well culture plate and allowed to grow until they reached 80% confluence. Test compounds dissolved in dimethylsulfoxide (DMSO) and ethanol solutions containing [1,2,6,7,-^{3}H] testosterone (100,000 dpm) were added to the wells in a final volume of 1 ml of medium (pH 7.4).

Two wells (background) containing medium and substrate but no cells were also included to account

for the nonenzymatic conversion of substrate. After addition of the compound and substrate, the cells were incubated at 37 for 12 h. At the end of the incubation, cytotoxic effects of plant extracts were observed with a microscope. The plant extracts showed cytotoxic effects were diluted until no cytotoxicity and then tested. The medium was collected and transferred to an extraction tube containing 2 ml of ethyl acetate to which was added 40 ug each of unlabeled carrier steroids (estradiol, testosterone, DHT). The samples were mixed for 30 seconds and then centrifuged for 10 min at 500×g. The organic phase was collected, taken to dryness, brought up in 25 ul of ethyl acetate, and analyzed on silica gel thin-layer chromatography (TLC) plates (0.2 mm thick, Merck, Germany). The 5α-Reductase inhibitory effects of treated samples were determined by with the negative control group (final 0.5% DMSO) using a imaging analyzer BAS-1500 (Fuji Photo Film Co., Japan).

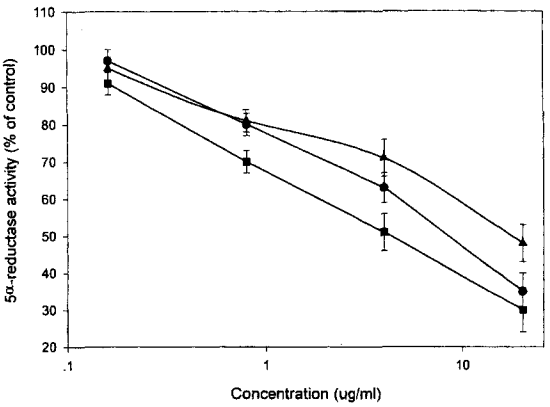

Fig. 1. Inhibitory effects of the ethyl acetate fraction of the root of *Sophora flavescens* (●), its subfration F8 (■), and finasteride (▲) against 5α-reductase activity in LNCaP cells. Data are expressed as mean ± S.D. from triplicate determinations at each concentration. Inhibition of 5α-reductase activity was determined in terms of the *in vitro* conversion of [^{3}H] testosterone to [^{3}H] DHT in LNCaP cells.

Results and Discussion

An *in vitro* system for assessing the inhibitory effects of 5α-reductase type 2 activity was used for the evaluation of plant extracts by analyzing the amount of DHT from LNCaP cell culture medium. About 80 kinds of natural products were compared for *in vitro* 5α-reductase-inhibiting potency, as judged by the abifity to attenuate the conversion of [^{3}H] testosterone to [^{3}H] DHT. The amount of extracts required for 50% inhibition at a single concentration of [^{3}H] testosterone substrate was determined to yield an IC_{50} value (Table 1). We identified 11 fractions that have the inhibitory effects on 5α-reductase 2 activity. These were the ethyl acetate fractions of *Perilla sikokiana* (seed, IC_{50} : 6.2 ug/ml), *Sophora flavescens* (root, IC_{50} : 8.9 ug/ml), *Angelica tenuissima* (root, IC_{50} : 11.7 ug/ml), *Scopolia japonica* (root, IC_{50} : 13.1 ug/ml), *Alisma orientale* (bark, IC_{50} : 13.9 ug/ml), *Zingiber officinale* (root, IC_{50} : 19.4 ug/ml), *Fritillaia verticillata* (rhizome, IC_{50} : 20.0 ug/ml), *Lilium bulbus* (fruit, IC_{50} : 20.0 ug/ml), and *Pueraria thunbergiana* (root, IC_{50} : 20.0 ug/ml), and the hexane fractions of *Amomum cardamomum* (seed, IC_{50} : 18.7 ug/ml), *Fritillaria verticillata*(rhizome, IC_{50} : 20.0 ug/ml). The ethyl acetate fractions of *Perilla sikokiana*(seed, IC_{50} : 6.2 ug/ml) and *Sophora flavescens* (Root, IC_{50} : 8.9 ug/ml) exhibited the most potent inhibitory effect as compared to that of finasteride, which was used as a positive control in this assay. A recent report by Lee *et al.* (1997) revealed that the ethyl acetate fractions of *Perilla sikokiana* also exhibited dose dependent inhibition of constitutive cyclooxygenase I in human erythroleukemia cell line (HEL).

The ethyl acetate fractions of *Perilla sikokiana*

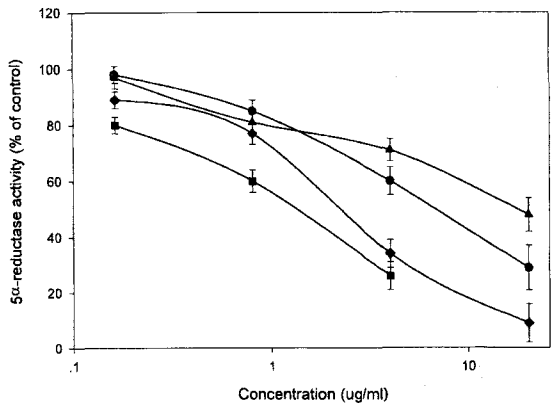

Fig. 2. Inhibitory effects of the ethyl acetate fraction of the seed of *Perilla sikokiana* (●), its subfrations F4 (■), F5 (◆), and finasteride (▲) against 5α-reductase activity in LNCaP cells. Data are expressed as mean ± S.D. from triplicate determinations at each concentration. Inhibition of 5α-reductase activity was determined in terms of the *in vitro* conversion of [^{3}H] testosterone to [^{3}H] DHT in LNCaP cells. The subfration F4 was observed to show about 30% cytotoxic effect at the concentration 20 ug/ml.

Table 1. Effects of plant extracts on the *in vitro* conversion of [^{3}H] testosterone to [^{3}H] DHT in LNCaP cells.

Scientific / Family name	Plant parts	Inhibition of 5α-reductase activity (IC_{50}, μg/ml)		
		Aqueous	Ethyl aceate	Hexane
Acohitum pseud-laeve / Ranunculaceae	Rt	>20	>20	>20
Adenophora trachelioides / Compositae	Rt	>20	>20	>20
Akebia quinata / Lardizabalaceae	Tu	>20	>20	>20
Alisma orientale / Alismataceae	Bk	>20	13.9	>20
Amomum cardamomum / Zingiberaceae	Sd	>20	>20	18.7
Angelica dahurica / Umbelliferae	Rt	>20	>20	>20
Angelica koreana / Umbelliferae	Rt	>20	>4[a]	>20
Angelica tenuissima / Umbelliferae	Rt	>20	>20	>20
Angelica tenuissima / Umbelliferae	Rt	>20	11.7	>20
Artemisia argyi / Compositae	LF	>20	>4[a]	>20
Asparagus cochinchinensis / Liliaceae	Bk	>20	>20	>20
Atractylodes japonica / Compositae	Rh	>20	>20	>20
Atractylodes japonica / Compositae	Rt	>20	>20	>20
Benicasa cerifera / Cucurbitaceae	Fr	>20	>20	>20
Boswellia carterii / Bruseraceae	Fr	>20	>20	>20
Cassia tora / Leguminosae	Sd	>20	>20	>20
Chaenomeles sinensis / Rosaceae	Fr	>20	>20	>20
Chrysanthemum siviricum / Compositae	St, Lf	>20	>20	>20
Cichorium intybus / Compositae	Rt	>20	>20	>20
Cimicifuga heracleifolia / Ranunculaceae	Rh	>20	>20	>20
Citrus Unshiu / Rutaceae	Fb	>20	>20	>20
Codonopsis pilosula / Campanulaceae	Rr	>20	>20	>20
Coptis chinensis / Ranunculaceae	Rt	>20	>20	>20
Crataegus pinnatifida / Rosaceae	Fr	>20	>20	>20
Cucumis melo var. markuwa / Cucurbitaceae	Fr	>20	>20	>20
Cynanchum wilfordii / Asclepidiaceae	WP	>20	>20	>20
Cyperus rotundus / Cyperaceae	Rt	>20	>20	>20
Dianthus chinensis / Caryophyllaceae	Wp	>20	>20	>20
Dryopteris crassirhizoma / Polypodiaceae	Rh	>20	>20	>20
Encommia ulmoides / Eucommiaceae	Bk	>20	>20	>20
Epqiseturn hyemale / Equisetaceae	Wp	>20	>20	>20
Evodia officinalis / Rutaceae	Fr	>20	>4[a]	>20
Fritillaia verticillata / Liliaceae	Rh	>20	20.0	20.0
Gardenia jasminoides / Rubiaceae	Fr	>20	>20	>20
Gastrodia elata / Orchidaceae	Rh	>20	>20	>20
Gleditschia japonica / Leguminosae	Fr	>20	>20	>20
Imperata cylindrica / Gramineae	Rh	>20	>20	>20
Juglans sinensis / Juglandaceae	Sd	>20	>20	>20
Lilium bulbus / Liliaceae	Fr	>20	20.0	>20
Lycium chinense / Solanaceae	Fr	>20	>20	>20
Malva verticillata / Malvaceae	Sd	>20	>20	>20
Mentha arvensis / Labiatae	Ap	>20	>4[a]	>20
Nelumbo nucifera / Nymphaeaceae	Fr	>20	>20	>20
Pachyma hoelen / Polyporaceae	Rt	>20	>20	>20
Paeonia japonica / Ranunculaceae	Rt	>20	>20	>20
Paeonia obovata / Ranunculaceae	Rt	>20	>20	>20
Panax ginseng / Araliaceae	Rt	>20	>20	>20
Perilla sikokiana / Labiatae	Sd	>20	6.2	>20
Pharbitis nil / Convolvulaceae	Rt	>20	>20	>20
Phlomis umbrosa / Dipsacaceae	Rt	>20	>20	>20

Table 1. Continued

Scientific / Family name	Plant parts	Inhibition of 5a-reductase activity (IC_{50}, μg/ml)		
		Aqueous	Ethyl aceate	Hexane
Pinellia ternata / Araceae	Tu	>20	>20	>20
Platycodon grandiflorum / Campanulaceae	Rt	>20	>20	>20
Poncirus trifoliata / Rutaceae	Fr	>20	>20	>20
Prunus humilis / Rosaceae	Sd	>20	>20	>20
Pueraria thunbergiana / Leguminosae	Rt	>20	20.0	>20
Pulsatilla koreana / Ranunculaceae	Rt	>20	>20	>20
Raphanus sativus / Cruciferase	Sd	>20	>20	>20
Rehmannia glutinosa / Scrophulariaceae	Rt	>20	>20	>20
Rheum palmatum / Polygonaceae	Rh	>20	>20	>20
Rubus coreanus / Hamamelidaceae	Fr	>20	>20	>20
Salvia miltiorrhiza / Labiatae	Rt	>20	>4[a]	>4[a]
Santallum album / Santalaceae	St	>20	>4[a]	>20
Schizandra chinensis / Magnoliaceae	Fr	>20	>20	>20
Scopolia japonica / Solanaceae	Rt	>20	13.1	>20
Seseli mairei / Umbelliferase	Rt	>20	>20	>20
Sinapis alba / Cruciferae	Sd	>20	>20	>20
Smilax glabra / Liliaceae	Rt	>20	>20	>20
Sophora flavescens / Leguminosae	Rt	>20	8.9	>20
Strychnos ignatii / Loganiaceae	Sd	>20	>20	>20
Torilis japonica / Umbelliferae	Fr	>20	>4[a]	>20
Torreya nucifera / Taxaceae	Fr	>20	>20	>20
Trichosanthes kirilowii / Cucurbitaceae	Sd	>20	>0.8[b]	>20
Xanthium strumarium / Compositae	Fr	>20	>20	>20
Zingiber officinale / Zingiberaceae	Rt	>20	19.4	>20

Abbreviations are aerial parts (Ap), bark (Bk), flower (Fl), fruits (Fr), fruits bark (Fb), leaf (Lf), root bark (Rb), rhizome (Rh), roots (Rt), ruber resin (Rr), seeds (Sd), stem (St), tuber (Tu) and whole plants (Wp).
[a]The cytotoxicity was shown at the concentration of 20 ug/ml.
[b]The cytotoxicity was shown at the concentrations of 20 ug/ml and 4 ug/ml.

(seed, IC_{50} : 6.2 ug/ml), *Sophora flavescens* (root, IC_{50} : 8.9 ug/ml), *Angelica tenuissima* (root, IC_{50} : 11.7 ug/ml), *Scopolia japonica* (root, IC_{50} : 13.1 ug/ml) were subfractionated with column chromatography and tested. Their relative potencies are listed in Table 2. The inhibitory activities of the previously described 5α-reductase inhibitor, finasteride, was included in Table 2 to serve as a standard for comparison. The subfractions F4 (IC_{50} : 1.1 ug/ml), F5 (IC_{50} : 2.0 ug/ml) and F6 (IC_{50} : 5.8 ug/ml) of the ethyl aceate fraction of *Perilla sikokiana* and the subfraction F8 (IC_{50} : 5.3 ug/ml) of the ethyl aceate fraction of *Sophora flavescens* displayed greater inhibition of 5α-reductase type 2 than did finasteride (IC_{50} : 19.4 ug/ml). The most effective fraction on the inhibition of 5α-reductase type 2 activity in LNCaP cells was the subfraction F4 of the ethyl aceate fraction of *Perilla sikokiana*, with an IC_{50} : 1.1 ug/ml (Table 2). The inhibitory effect of the subfration F4 was 15-fold more potent than that of finasteride. Finasteride has reported as a potent inhibitor in 5α-reductase type 2 activity (Smith *et al.*, 1996) but the compound showed a weak inhibitory effect in this human prostatic carcinoma cell line LNCaP culture system.

The subfractions of the ethyl aceate fractions of *Angelica tenuissima* (root, IC_{50} : 11.7 ug/ml) and *Scopolia japonica* (root, IC_{50} : 13.1 ug/ml) did not exhibit potencies on the inhibition of the enzyme activity. The subfractions F4 (IC_{50} : 1.1 ug/ml) and F5 (IC_{50} : 2.0 ug/ml) of the ethyl aceate fraction of *Perilla sikokiana* and the subfraction F8 (IC_{50} : 5.3 ug/ml) of the ethyl acetate fraction of *Sophora flavescens* showed dose-dependent inhibition of 5α-reductase type 2 activity (Figs. 1, 2, 3). The subfractions F4 of the ethyl aceate fraction of *Perilla sikokiana* was observed about 30% cytotoxic effect at the concentration of 20 ug/ml and the concentration was excluded from the calculation of IC_{50} value (Fig. 2).

Table 2. Effects of subfractions of *Perilla sikokiana* and *Sophora flavescens*[a] on the *in vitro* conversion of [^{3}H] testosterone to [^{3}H] DHT in LNCaP cells.

Scientific name and subfraction	IC_{50} (μg/ml)
Ethyl aceate fraction of *Perilla sikokiana*	6.2
Subfraction F01	7.1
Subfraction F02	7.3
Subfraction F03	8.7
Subfraction F04	1.1
Subfraction F05	2.0
Subfraction F06	5.8
Subfraction F07	>20
Subfraction F08	>20
Subfraction F09	>20
Subfraction F10	>20
Ethyl aceate fraction of *Sophora flavescens*	8.9
Subfraction F01	>20
Subfraction F02	>20
Subfraction F03	>20
Subfraction F04	>4[a]
Subfraction F05	>4[a]
Subfraction F06	>4[a]
Subfraction F07	>4[a]
Subfraction F08	5.3
Subfraction F09	>20
Subfraction F10	19.4
Subfraction F11	>20
Subfraction F12	>20
Subfraction F13	>20
Subfraction F14	>20
Finasteride (control)	19.4

[a]The cytotoxicity was shown at the concentration of 20 ug/ml.

These natural products displayed activity aganist 5α-reductase are considerable interest for chemopreventive agents the treatment of the diseases that are at least partially dependent on DHT such as BPH, acne, and male pattern baldness (Wilson, 1980; Mooradian *et al.*, 1987; Cunha *et al.*, 1987; Metcalf *et al.*, 1987) as well as chemoprevention of prostatic cancer (Gormley, 1992a). Currently, these active fractions and subfractions are under investigation by using activity-guided fractionation method with column chromatography to find effective pure compounds against 5α-reductase type 2 activity.

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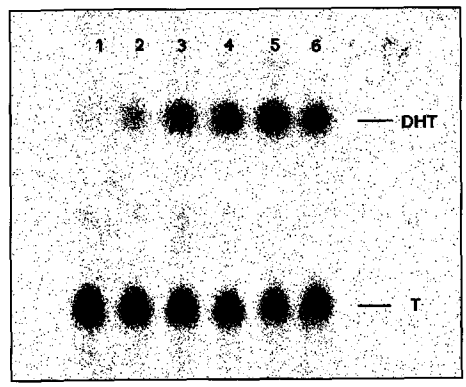

Fig. 3. Autoradiographed TLC-chromatogram of inhibitory effects of the subfration F4 of the ethyl acetate fraction of *Perilla sikokiana* (seed) against 5α-reductase activity in LNCaP cells. For the detection of the in vitro conversion of [^{3}H] testosterone (T) to [^{3}H] DHT in LNCaP cells, autoradiography was performed by TLC plate in contact with Fuji BAS-1500 imaging plate (IP) for 48 hr and the IP was scanned by BAS-1500 image analyzer. Lane 1, 20 ug/ml; lane 2, 4 ug/ml; lane 3, 0.8 ug/ml; lane 4, 0.16 ug/ml, lane 5, DMSO (control); lane 6, finasteride (20 ug/ml).

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