**Original Article**

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**Tectona grandis**, a potential active ingredient for hair growth promotion

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**Abstract**

This study aimed to investigate the biological activities of *Tectona grandis* L. f. related to hair loss treatment, including steroid 5α-reductase (S5AR) inhibition, effect on Human Follicle Dermal Papilla Cells (HFDPCs), anti-testosterone activity, cytotoxicity on macrophage cells and interleukin 1 beta (IL-1β) secretion inhibition. Among the crude extracts, *T. grandis* leaf-hexane and ethyl acetate (EtOAc) extracts possessed potent S5AR inhibitions, with IC₅₀ values of 31.39±3.38 µg/mL and 20.92±2.59 µg/mL, respectively. *T. grandis* leaf-hexane extract showed lower cytotoxicity on HFDPCs than EtOAc extract. Hexane extract at 25 µg/mL had similar anti-testosterone activity with a positive control, finasteride, and exhibited 70% inhibition of IL-1β secretion in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Meanwhile, EtOAc extract had lower anti-testosterone activity at 6.25 µg/mL and exhibited 52% IL-1β secretion inhibition at 1.5 µg/mL. This discovery suggests *T. grandis* leaf extracts as a new source of active ingredients for the development of hair care products.

**Keywords:** *Tectona grandis*, 5a-reductase inhibition, anti-testosterone, IL-1β secretion inhibition

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1. Introduction

Testosterone is the most abundant androgen in the serum and is synthesized by the Leydig cells of the testes under the control of hypothalamus and anterior pituitary gland (Imperato-McGinley & Zhu, 2002). Testosterone can be converted to a more potent androgenic, dihydrotestosterone (DHT), the preferred ligand for androgen receptor transactivation, by steroid 5α-reductase (5SRAR) enzyme using NADPH as a cofactor (Russell & Wilson, 1994). Both testosterone and DHT play important roles in normal male growth. However, the excessive production of androgen hormones may lead to androgen dependent disorder, including androgenic alopecia (AGA), benign prostatic hyperplasia (BPH), acne and female hirsutism (Cilotti, Danza, & Serio, 2001; Luu-The, Belanger, & Labrie, 2008; Occhiato, Guarna, Danza, & Serio, 2004; Randall, 1994).

AGA (male pattern baldness) is the common type of hair loss alongside other types of hair loss associated with SSAR type 1. At present, anti-androgen drugs either inhibiting SSAR or blocking the androgen receptor may be useful for the treatment of AGA. Finasteride (SSAR type 2 inhibitor) and minoxidil (vasodilator) are two synthetic drugs that have been approved by US FDA for the treatment of AGA. However, there are several undesirable side effects associated with these drugs, such as erythema, scaling, pruritus, dermatitis, itching or skin rash (minoxidil) (Robinson, Deluca, Drummond, & Boswell, 2003), impotence, abnormal ejaculation, gynecomastia, testicular pain, impairment of muscle growth and severe myopathy (finasteride) (Libecco & Bergfeld, 2004). Therefore, alternative anti-androgenics need to be discovered and identified from the natural products.

*Tectona grandis* L. f. or teak, a native tree from Southeast Asia, is considered high quality timber due to its natural durability, and it is widely reputed in the exterior timber industry. Aside from its economic importance, *T. grandis* also plays a role in traditional medicine. Traditionally, various parts of *T. grandis* are used to relieve fever, inflammation, cancer, skin disease, bronchitis, biliousness, hyperacidity, diabetes, leprosy, astringence, and hemintiaasis (Harborne, 1994). Some classes of bioactive compounds in *T. grandis* have been reported, such as quinones (Aguinaldo, Ocampo, Bowden, Gray, & Waterman, 1993), terpenes (Macias et al., 2010), nortignans (Lacret, Varela, Molinillo, Nogueiras, & Macias, 2012) and betulins (Pathak, Neogi, Biswas, Tripathi, & Pandey). Those bioactive compounds are spread all over the plant or located at distinct sites/tissues of *T. grandis*, including bark, wood, leaves, roots and fruit.

The seeds of *T. grandis* are traditionally acclaimed as hair tonic in the Indian system of medicine. In addition, a study of petroleum ether extract of *T. grandis* seeds in albino mice revealed that hair growth initiation time was significantly decreased to half and the treatment was also successful in bringing a greater number of hair follicles in anagenic phase than standard minoxidil (Jaybhaye et al., 2010). In this study, we evaluated the inhibitory activity of *T. grandis* extracts against 5SRAR and their bioactivities related to hair loss treatment, including effects on Human Follicle Dermal Papilla Cells (HFDPcs), and anti-testosterone and anti-inflammatory activities via interleukin 1 beta (IL-1β) secretion inhibition.

2. Materials and Methods

2.1 Chemicals

Hexane, ethyl acetate (EtOAc) and ethanol (EtOH) were purchased from RCI Labscan (Thailand). All solvents were AR grade. Water was produced with a Milli-Q water purification system (Millipore, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Labscan (Dublin, Ireland). Nicotinamide adenine dinucleotide 20-phosphate reduced tetrasodium salt (NADPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human androgen-dependent LNCaP cells were purchased from CRL-1740TM, American Type Culture Collection (ATCC, VA, USA). HFDPCs were purchased from Promo Cell and RAW 264.7 cells were purchased from ATCC (Manassas, Virginia, USA). Mouse IL-1β ELISA Instant Kit was purchased from E-Bio Sciences (Bender-med systems GmbH, Vienna, Austria).

2.2 Plant materials

Plant materials from *T. grandis*, including leaves, wood, bark, roots, fruit, peels of fruit, and seeds were collected from Phitsanulok Province, Thailand, in January 2017 and identified by Assist. Prof. Dr. Prance Nangngam, Department of Biology, Faculty of Science, Naresuan University. The voucher specimen (No. 004479) was deposited at PNU Herbarium, Faculty of Science, Naresuan University. The collected plant samples were then cleaned, rinsed, cut into small pieces and eventually dried in a hot air oven at 50°C for 3 days. Afterwards, the dried plant materials were ground into powder and passed through a 60 mesh sieve.

2.3 Plant extract preparation

2.3.1 Sequential solvent extraction

The powdered plant materials were sequentially extracted using various organic solvents; hexane, EtOAc and 95% EtOH. Firstly, the powdered plant materials (100 g of leaves and peels, 50 g of roots and fruits, 45 g of woods and 25 g of barks and seeds) were macerated with hexane (250 mL) and placed on a shaker to allow agitation at room temperature for 24 hr. The filtrate and residue were separated by filtration using No.1 Whatman™ filter paper (GE Healthcare Life Sciences, Thailand) and the filtrate was concentrated using a rotary evaporator under a reduced pressure to obtain the crude hexane extract. The residue was then macerated with EtOAc and 95% EtOH in sequence, with the same method to obtain crude EtOAc and 95% EtOH extracts. All extracts were stored in –20°C until further use.

2.3.2 Water extraction

For water extract, the powdered plant materials (30 g of roots, 10 g of leaves, fruits, woods and peels, 5 g of seeds and barks) were infused in 80°C hot water (100 mL) for 15 min and then filtered through a No.1 Whatman™ filter paper. The filtrate was frozen at –80°C and lyophilized to obtain the water extract. All extracts were stored in –20°C until use.
2.4 Determination of steroid 5α-reductase inhibitory activity

2.4.1 Enzyme preparation

Human androgen-dependent LNCaP cells expressing SSAR type 1 were cultured in cell culture medium RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 100 µg/mL streptomycin (Gibco, Paisley, Scotland). The cells were cultured in 175 cm² cell culture flasks and incubated at 37°C under 5% CO₂ humidified atmosphere. Once the cells reached ≥80% confluence, the medium was removed, the cells were rinsed with Tris-HCl buffer pH 7.4 and scraped off from the flask. The collected cells were then centrifuged at 1900 g for 10 min. Lysis buffer (containing 10 mM Tris-HCl buffer pH 7.4; 50 mM KCl; 1 mM EDTA; 0.5 mM phenylmethylsulfonyl fluoride) was added to the cell pellet to obtain ≥9 x 10⁷ cells/mL. The cell pellet was homogenized on ice using a sonicator probe with 10 s pulse on, 10 s pulse off for 1 min at 40% amplitude (Sonics VibraCell™ VCX130 probe V18, Newtown, CT, USA). Glycerol (Invitrogen, Carlsbad, CA, USA) was then added to the homogenized cell pellet to 20% (v/v) prior to storing it in −80°C until use. Pierce bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) was used to examine the protein content and bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) was used as the standard. The calibration curve over BSA concentration range 10–500 µg/mL was plotted for absorbance at 595 nm.

2.4.2 Enzymic inhibition

Inhibitory activity against the conversion of testosterone into DHT by SSAR enzyme was determined in vitro using the method from Srivilai et al. (2016). Briefly, the sample was dissolved in DMSO to give an assay final concentration of 100 µg/mL. Final volume of the enzymic reaction mixture was 200 µL and the assay was performed in a deep 96-well plate (Agilent Technologies, Santa Clara, CA, USA) covered with well-cap mats (Thermo Scientific, Waltham, MA, USA), where to each well, 10 µL of extract solution was added, followed by 20 µL of Tris-buffer pH 7.4, 20 µL of 34.7 µM testosterone, 50 µL of 1 mM NADPH in Tris buffer pH 7.4 and 100 µL of LNCaP cell homogenate enzyme (75 µg total protein). This enzymatic reaction mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 300 µL of hydroxylamine (10 mg/mL in 80% (v/v) EtOH) and incubated for another 60 min at 60°C to allow derivatization. Afterwards, the reaction mixture was centrifuged for 5 min at 8500 rpm and the supernatant was collected for LC-MS analysis.

2.4.3 LC-MS analysis

The control group was prepared as a complete reaction mixture but lacking the tested sample, in place of which DMSO was used. The C₀ was the control group that was terminated before the first incubation (0 min) and represented 100% enzymatic inhibition, whereas Cₙₙ was the control group that was terminated after 60 min incubation and represented 0% enzymatic inhibition. The inhibition of the tested sample was analyzed by measuring the area under curve (AUC) of extracted ion chromatogram (EIC) of derivatization DHT (m/z [M+H]+; 306.2426) and was calculated as follows:

\[
\%\text{SSAR inhibitory activity} = \left(1 - \frac{\text{AUC of } C_0 - \text{AUC of } C_n}{\text{AUC of } C_0 - \text{AUC of } C_{\text{sample}}}\right) \times 100
\]

For LC-MS analysis, Agilent 1260 Infinity Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) connected to Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA) equipped with a dual electrospray ionization (ESI) in positive mode and m/z range 100-1200 was used. The analytical reversed phase column Phenomenex Luna® C18 (2) (150 mm x 4.6 mm, 5 µm) was used as the stationary phase. The mobile phase was the gradient elution of 0.1% (v/v) formic acid in purified water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (LC-MS grade, ACI Labscan, Bangkok, Thailand) as solvent B. The initial mobile phase system was 60% solvent B, then solvent B was linearly increased up to 80% after 8 min and held constant for 4 min and followed by 2 min post-run. The flow rate was 0.5 mL/min, the injection volume was 20 µL and the column temperature was maintained at 35°C.

2.5 Effect of T. grandis extracts on HFDPCs

2.5.1 Cytotoxicity of T. grandis extracts on HFDPCs

Cytotoxicity of T. grandis leaf extracts on androgen target mesenchymal HFDPCs was examined using the MTT assay. Ten thousand cells (cultured in follicle dermal papilla cell growth medium from PromoCell, GmbH) were seeded into a 96-well plate and incubated for 24 hr at 37°C under 5% CO₂ humidified atmosphere. The medium was then removed and 100 µL of the tested sample in DMSO with the various concentrations ranging from 0.195 to 100 µg/mL were added and the cells were re-incubated for another 24 hr. Afterwards, 50 µL of 1 mg/mL of MTT in PBS buffer (Sigma-Aldrich, St. Louis, MO, USA) was added into each well and incubated for 3 hr. The medium was removed and the formed formazan crystals of the viable cells were dissolved in 100 µL of DMSO. The absorbance was determined at 595 nm using a microplate reader and cell viability was calculated by comparing with the control group (cells incubated in the medium only). The tested samples that had maintained cell viability were used for further study.

2.5.2 Anti-androgenic activity analysis

Ten thousand of HFDPCs were seeded into a 96-well plate and incubated for 24 hr at 37°C under 5% CO₂ humidified atmosphere. The cells were then treated with 200 µM testosterone, 25 µg/mL of hexane extract and 6.25 µg/mL of EtOAc extract of T. grandis leaf and incubated for four days. On day 4, cell viability was determined by the MTT assay. Anti-androgenic activity was evaluated by comparing with the control group. Finasteride at 75 nM was used as the positive control.
2.6 Determination of IL-1β secretion inhibition

2.6.1 Cytotoxicity of \textit{T. grandis} extracts on macrophage cells

The effects of \textit{T. grandis} leaf extracts on macrophage cells were examined using the MTT assay similarly as described in section 2.5.2, except that RAW 264.7 cells were used instead of HDFPCs. The concentrations of the extracts that maintained cell viability of at least 80% were used for IL-1β secretion inhibition analysis.

2.6.2 IL-1β secretion analysis

One hundred thousands RAW 264.7 cells were seeded into each well of a 24-well plate and incubated for 24 hr at 37°C under 5% CO₂ humidified atmosphere. The cells were then treated with 5 µg/mL of LPS (Sigma-Aldrich, St. Louis, MO, USA) and \textit{T. grandis} leaf extracts. Five micrometers per milliliter of hydrocortisone was the positive control. The treated cells were incubated for 24 hr. Supernatants were collected for determination of IL-1β secretion using Mouse IL-1β ELISA Instant Kit and the procedure followed manufacturer’s instructions. Briefly, 150 µL of distilled water was added into the sample wells, followed by 150 µL of each supernatant, in duplicate, to the designated wells, and the contents were mixed. The plate was covered with an adhesive film and incubated at room temperature (25°C) for 3 h and agitated using shaker at 400 rpm. After incubation, the micro-wells were washed 6 times with approximately 400 µL of washing buffer per well, allowing the buffer to stay in the wells for about 10–15 sec before aspiration. After the last wash, each micro-well was tapped with absorbent paper tissue to remove excess wash buffer. One hundred microliters of TMB solution was transferred into each well, including the blank wells. The micro-wells were incubated at room temperature (25°C) for 10 min, avoiding direct exposure to intense light. The enzyme reaction was stopped by quickly pipetting 100 µL of stop solution into each well, including the blank wells. The absorbance was determined at 450 nm by a microplate reader. The IL-1β content was calculated by using the IL-1β calibration curve.

3. Results and discussion

3.1 \textit{T. grandis} extracts

Each part of \textit{T. grandis} was extracted individually by a sequential solvent maceration using hexane, followed by EtOAc, 95% EtOH as well as infusion in hot water. The yields of all extracts are shown in Table 1. Among the extracts, the three water extracts of leaves (38.84%), wood (42.12%) and fruit (31.91%) as well as the hexane extract of seeds (29.05%) gave high yields.

3.2 Steroid 5 alpha reductase inhibitory activity of \textit{T. grandis}

Androgenic alopecia is induced by an over-production of androgen hormones by the 5αS5AR enzyme. In human hair follicles, either testosterone or DHT produced after S5AR conversion binds to androgen receptor and the resulting complex migrates to nucleus causing gene expression (Takeyasu, Wakimoto, Itami, & Sano, 1980). Moreover, increased levels of S5AR have been detected in hair follicles in males with male pattern baldness (Sawaya & Price, 1997). Therefore, enzyme inhibition could decrease such effects. In our study of cell-free S5AR inhibitory activity assay using extracts from different parts of \textit{T. grandis}, it was observed that some extracts at the final assay concentration of 100 µg/mL inhibited S5AR by over 80%, including hexane and EtOAc extracts of leaves, roots, and peels, as well as EtOAc extract of fruit (Table 2). Two known S5AR inhibitors, finasteride and dutasteride, were used as positive controls at the final concentrations of 0.001 M and 0.1 M, respectively. Considering the availability and accessibility of the plant material, hexane and EtOAc extracts of the leaves – with IC50 values of 31.39 ± 3.38 µg/mL and 20.92 ± 2.59 µg/mL, respectively – were chosen for further study.

3.3 Effect of \textit{T. grandis} leaf extracts on HDFPCs

3.3.1 Cytotoxicity of \textit{T. grandis} extracts on HDFPCs

Androgen hormones control the proliferation of human hair (Thornton, Messenger, Elliot, & Randall, 1991). However, the over-uptake of androgens to HDFPCs is considered a cause of HDFPCs apoptosis and the decrease of anagen phase of the hair cycle (Randall et al., 2000). Cytotoxicity of \textit{T. grandis} leaf extracts was measured using the MTT assay. One percent DMSO, the solvent to dissolve the extracts in the assay, showed no cytotoxicity toward the cells. The concentrations of extracts assayed were in the range from 0.195 to 100 µg/mL (the concentration was limited by the solubility of extracts in DMSO). The results revealed that hexane extract of \textit{T. grandis} leaves showed a lower cytotoxic effect on HDFPCs than that of the EtOAc extract. The cell viability remained above 80% when treated with hexane extract even at the highest assayed concentration. Meanwhile, cell viability declined when the cells were treated with EtOAc extract and the safe concentrations of EtOAc extract were 0.195–6.25 µg/mL. The concentrations of extracts that showed no cytotoxic effects in MTT analysis are shown in Figure 1.
3.3.2 Anti-testosterone activity

HFDPCs-based model was used to evaluate anti-testosterone activity of *T. grandis* leaf extracts. A previous study has demonstrated that testosterone and DHT in HFDPCs exhibited antiproliferative effects in time- and dose-dependent manner, and could cause programmed cell death due to the alteration of anti-apoptotic protein bcl-2 expression under physiological conditions (Winiarska et al., 2006). As the number of HFDPCs and the size of dermal papilla (DP) seem to correlate with the size and number of hair follicles, which are controlled by androgens (Elliott, Stephenson, & Messenger, 1999; Ibrahim, & Wright, 1982; Van Scott & Ekel, 1958), the appropriate amount of androgens in HDPCs has to be achieved. Our investigation of anti-testosterone activity was carried out by measuring HFDPCs viability using the MTT assay after incubation with testosterone and *T. grandis* leaf-hexane and EtOAc extracts (Figure 2). In this study, a higher cell viability corresponds to a higher anti-testosterone activity. Four days incubation with only 200 µM testosterone could decrease HFDPCs viability to below 80%. Our study unveiled that anti-testosterone activity on HFDPCs of hexane extract at 25 µg/mL was similar to that of 75 nM finasteride, which was a positive control. Surprisingly, HFDPCs viability decreased after treatment with 6.25 µg/mL of EtOAc extract (19.72%). The EtOAc extract therefore causes some safety concerns.

3.3.3 IL-1β secretion inhibition

Several cytokines are involved in the hair growth cycle. IL-1β has been found to be a key mediator of the arrest of hair growth as well as to trigger hair loss (Hoffmann, Eicheler, Huth, Wenzel, & Happle, 1996). Moreover, morphological changes within cells of the hair follicle outer root sheath and dermal papilla have been detected during...
IL-1β stimulation in vitro, and the changes were similar to those detected histologically in affected hair follicles from early alopecia areata (Phillpot, Sanders, & Kealey, 1995). In this study, anti-inflammatory activities of T. grandis leaf-hexane and EtOAc extracts were evaluated by measuring the mouse IL-1β secretion inhibition in LPS-stimulated RAW 264.7 cells. Prior to the analysis, cytotoxicity of the extracts toward the cells was evaluated. The concentrations used in the cytotoxicity evaluation were in the range 0.195–100 µg/mL. The hexane and EtOAc extracts at 0.195–25 µg/mL and 0.195-1.562 µg/mL, respectively, could maintain cell viability above 80% and were considered safe to the cells (Figure 3).

Furthermore, the highest safe concentration was selected for each extract to the analysis of IL-1β secretion inhibition (Figure 4). Hexane extract of T. grandis leaves potently inhibited IL-1β secretion (by 69.90%) at the final concentration of 25 µg/mL, slightly more than the inhibition by standard hydrocortisone. Meanwhile, the EtOAc extract exhibited moderate IL-1β inhibition (by 52.08%) at 1.5 µg/mL. Since the inhibition of IL-1β secretion is effective as treatment of androgenic alopecia, this finding suggests that T. grandis leaf extracts are a potential alternative for such treatment.

Several compounds have been found to promote hair growth. For instance, a quinone compound (avicequinone C) isolated from the heartwood of Avicennia marina (Jain, Monthakantrirat, Tengamnuay, & De-Eknamkul, 2014), fatty acids (linoleic, α-linoleic, palmitic, oleic, stearic and oleidic acids) from Boehmeria nippononivea (Shimizu et al., 2000), and sesquiterpenes isolated from the rhizome of Curcuma aeruginosa (Suphrom et al., 2012). The same group of compounds might be responsible for the bioactivities of T. grandis extracts that were investigated in this current study. However, verifying this could be pursued by first identifying relevant biomarkers, a potential topic for future studies.
4. Conclusions

The leaf extracts of *T. grandis* could serve as ingredients in alternative medicines/cosmetics for hair loss treatment. This is corroborated by their SSAR inhibitory activity, effects on HFDPCs, anti-testosterone activity, as well as anti-inflammatory activity through inhibition of IL-1β secretion.

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