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Contributed Paper

5α-Reductase Inhibition and Melanogenesis Induction of the Selected Thai Plant Extracts

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ABSTRACT

Five selected Thai plant extracts, including Lotus seeds, Black Sesame seeds, White Kwao Krua tubers, rice bran and Asiatic Pennywort leaves were extracted by different solvents (ethyl acetate, ethanol and water). The mixture of the rice bran extract and the White Kwao Krua extract at 1:1 weight ratio, the water and chloroform fractions of the decolorized Asiatic Pennywort extract and the rice bran extract loaded in β-cyclodextrin were prepared. The samples were investigated for 5α-reductase inhibition in DU-145 prostate carcinoma and melanogenesis in B16F10 melanoma cells. The rice bran extract not loaded and loaded in β-cyclodextrin exhibited the high linoleic acid contents of 21.13±1.22 and 21.47±0.91% w/w, respectively. The highest inhibitory effect of 5α-reductase type 1 was observed in 0.5 mg/mL of the rice bran extract at 87.43±7.15% of the control, which was higher than the standard dutasteride of about 1.60 times, followed respectively by the mixture of the rice bran extract and the White Kwao Krua extract, and the Lotus seed extract. The rice bran extract loaded in β-cyclodextrin at 1 mg/mL and the White Kwao Krua n-hexane fraction at 1 mg/mL demonstrated the highest melanogenesis induction and tyrosinase activity of 1.25 and 1.82 folds of the control, and 0.52 and 0.55 folds of the standard theophylline, respectively. This study has demonstrated the potential of the rice bran extracts on 5α-reductase inhibition and melanogenesis induction when loaded in β-cyclodextrin, which can be further developed as the anti-hair loss and hair darkening products.

Keywords: 5α-reductase inhibition, melanogenesis, Thai plant extracts, rice bran extract, White Kwao Krua extract

1. INTRODUCTION

The elderly suffer not only from baldness, but also the grey hair. They have spent annually $3 billion on hair dye and $1.5 billion on hair loss therapies [1]. Canities (grey hair) is caused by the decreasing of the natural pigment (melanin), which is present in hair. Not only the age progression takes part in the cause of canities, but also the vitamin B deficiency (B5 and B6) and psychic factors. Melanocytes locate in the basal layer of the skin and hair follicles with their major function acting as the melanin producing cells. They produce pigments in melanosomes, which will subsequently be transferred to the skin and hair shaft keratinocytes. Melanogenesis, the complicated pigment formation process occurring in melanocytes, involves the enzymatic oxidation of tyrosine to melanin. Tyrosinase, a rate-limiting melanogenic enzyme, catalyses the two substrates in the first two steps of melanogenesis, which are tyrosine to L-dihydroxy phenylalanine L-dopa and L-dopa to dopaquinone. Dopachrome tautomerase (tyrosinase-related protein-2; TRP-2) and dihydroxyindole-2-carboxylic acid (DHICA) oxidase (tyrosinase-related protein-1; TRP-1) are the other two enzymes that convert dopachrome to DHICA and DHICA to eumelanin, respectively [2]. For hair loss, dihydrotestosterone (DHT), a potent male hormone, appear to be the cause of genetic male pattern baldness or androgenic alopecia. An increase of the DHT levels, numbers of DHT receptors in the hair follicles and the 5α-reductase enzyme activity, which converts testosterone to DHT, have been reported in the balding scalp of androgenic alopecia patients. DHT is the main cause of androgenic alopecia leading to the miniaturization of hair follicle and hair shedding. The 5α-reductase enzymes, also known as 3-oxo-5α-steroid 4-dehydrogenases, are classified into 2 types; type 1 (SRD5A1) related to androgenic alopecia is expressed predominantly in skin, scalp, sebaceous gland, liver and brain, while the 5α-reductase type 2 (SRD5A2) is found predominantly in the androgen target organs, such as prostate, genital skin and seminal vesicles [3].

Chemicals containing in hair colorants and anti-hair loss drugs have been reported to have several and sustained side effects. For example, *p*-phenylenediamine, a major chemical component of hair dye, is well known to induce contact dermatitis in its unpolymerized state [4]. Dutasteride, a dual 5α-reductase inhibitor may cause a decrease of libido, erectile dysfunction, ejaculation disorder and gynecomastia [5]. Several natural extracts from many plants are alternative agents for hair darkening and hair growth promotion. Nowadays, natural products from several plants have been used for hair growth promotion. The unsaturated fatty acids, such as γ-linolenic acid, linoleic acid and oleic acid, have been proved to have anti-hair loss activity by inhibiting 5α-reductase enzyme in androgen responsive organs [6]. In fact, several edible plants contain these unsaturated fatty acids in variable amounts, such as the bran of *Oryza sativa* (rice) and seeds of *Nelumbo nucifera* (Lotus) [7-8]. The saturated fatty acids and their methyl ester, such as palmitic acid, stearic acid and palmitic acid methyl ester, have been shown to have melanogenesis induction activity in melanocyte and B16F10 melanoma cells [9-10].

This present study has investigated the potential of the Thai plant extracts (Lotus seeds, Black Sesame seeds, White Kwao Krua, rice and Asiatic Pennywort) and their formulations (the mixture of the rice bran extract and the White Kwao Krua extract, the water and chloroform fractions of the

decolorized Asiatic Pennywort extract and the rice bran extract loaded in β-cyclodextrin) on 5α-reductase type 1 inhibition in DU-145 cells and melanin induction in B16F10 melanoma cells for the further development of the androgenic alopecia and canities hair products.

2. MATERIALS AND METHODS

2.1 Materials

Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640) and penicillin/ streptomycin solution were obtained from GIBCO (Maryland, USA). Fetal bovine serum was from PAA Laboratories GmbH (Pasching, Austria). Theophylline, synthetic melanin, mushroom tyrosinase and sulforhodamine B (SRB) were from Sigma Aldrich Co. (Missouri, USA). Bovine serum albumin was from Amresco (Ohio, USA). Linoleic acid and sesamin were from Wako Pure Chemical Industrial Ltd (Osaka, Japan). Methanol, ethyl acetate, acetic acid and acetonitrile were obtained from RCI Labscan Limited (Bangkok, Thailand). β-cyclodextrin and the standard dutasteride (99.5%) were purchased from Ka-Shing Business Macau Co., Ltd. (Macau, China). Other reagents were of analytical grade.

2.2 Plant Extract Preparation

Plants with scientific evidences used for hair cares, including *Nelumbo nucifera* Gaertn. (Lotus) [8], *Sesamum indicum* Linn. (Black Sesame) [11], *Pueraria candollei* Graham ex Benth. var mirifica (White Kwao Krua) [12], *Oryza sativa* Linn. (rice) [7] and *Centella asiatica* (Linn.) Urban (Asiatic Pennywort) [13] were selected for this study. The plant specimens were collected in Chiang Mai Province, Thailand and their authentic specimens were identified by a botanist and kept at Manose Health and Beauty Research Center (www.manose.co). The extraction process used for each plant was according to our previous studies and from the solubility of the active compounds existing in the plant. Lotus seeds, Black Sesame seeds and White Kwao Krua tubers were extracted with ethyl acetate. The crude ethyl acetate of White Kwao Krua extract was dispersed in distilled water and further performed by partition extraction with solvents sequentially of methanol and n-hexane. For the rice bran, it was extracted with absolute ethanol. The Asiatic Pennywort leaves were extracted with water and then decolorized by partitioning with the mixture of water and chloroform. The solvents were evaporated and the final dried extracts or fractions were obtained. The percentage yields of all crude extracts and fractions were obtained from the weight basis of the initial dried plants and the crude extracts, respectively.

2.3 Linoleic Acid Contents Determination

Linoleic acid contents in the extracts were determined by HPLC (Gemini-NX 5 µm C18 250 mm × 4.60 mm Phenomenex Column [Phenomenex Inc., Torrance, CA, USA], LC1200UV/VIS Detector and LC1100HPLC pump) using the mobile phase of 95% v/v acetonitrile mixed with 5% v/v of 0.1% v/v glacial acetic acid, injection volume at 20 µL, flow rate of 1 mL/min and the UV detector at 205 nm. The samples were weighed, dissolved in the mobile phase (1 mg/mL) and filtered through 0.45 µm membrane filter. Linoleic acid contents in the samples were determined from the HPLC chromatogram in comparing to the standard linoleic acid (0.02-1.96 mg/ mL).

2.4 *In vitro* Cytotoxicity by SRB Assay

2.4.1 Cell cultures

The human prostate carcinoma cell line (DU-145) was provided by Prof. Dr. Toshihiro Akihisa at the College of Science and Technology, Nihon University in Tokyo, Japan. B16F10 melanoma cell line was obtained from ATCC (Virginia, USA). Both DU-145 and B16F10 melanoma cells were cultured in RPMI 1640 and DMEM media, respectively, supplemented with 10% v/v fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were incubated in a temperature-controlled and humidified incubator (Shel Lab, model 2123TC, USA) with 5% CO_2 at 37°C. Cells were grown to 70-75% confluence and harvested by 0.25% trypsin/0.06 mM EDTA solution. Cells were resuspended in culture medium and the cell numbers were then counted by a hemacytometer. All experiments were performed in triplicate.

2.4.2 Sample preparation

Nine plant extracts including Lotus seed extract, Black Sesame seed extract, White Kwao Krua n-hexane fraction, rice bran extract, the mixture of the rice bran extract and the White Kwao Krua extract (1:1 w/w), rice bran extract loaded in β-cyclodextrin, Asiatic Pennywort extract, water and chloroform fractions of the decolorized Asiatic Pennywort extract, and dutasteride (a positive control for 5α-reductase inhibitory activity), were dissolved in absolute ethanol and added to the cell cultures with the final ethanol concentration of 1% or less (v/v). Theophylline, a standard melanogenesis stimulator, was dissolved in culture medium and sterilized by filtering through a 0.2 μm membrane filter.

2.4.3 Cytotoxicity by SRB assay

The extract samples and the positive control were tested for cytotoxicity in DU-145 and B16F10 melanoma cells by SRB assay as previously described [14]. DU-145 and B16F10 melanoma cells were plated at the density of 1.0×10^5 and 1×10^4 cells/well, respectively, in 96-well plates and left overnight for cell attachment on the plate. DU-145 cells were then exposed to serial concentrations of the extracts (0.0005-50 mg/mL) and 0.5 mg/mL of dutasteride, a standard reference, and further incubated for 24 h. For B16F10 melanoma cells, 0.01-10 mg/mL of the extracts and 0.05 mg/mL of theophylline were added and incubated for 72 h. After incubation, the adherent cells were fixed *in situ*, washed and dyed with SRB. The bound dye was solubilized and the absorbance was measured at 540 nm by a microplate reader (Bio-Rad, model 680, Japan). The experiments were performed in triplicate. The percentages of cell viability were calculated according to the following equation: $\%\ Cell\ viability = (A/B) \times 100$, where A was the absorbance of the sample and B was the absorbance of the control. The concentrations of the samples which gave % cell viability of more than 90 and 80% were selected to perform the 5α-reductase inhibition and melanogenesis assays, respectively.

2.5 Effects of the Extracts on 5α-Reductase Activity

2.5.1 Cultivation of cells

The pellets of DU-145 cells were plated in 6-well plates separately at the density of 8.0×10^5 cells/well and incubated for cell adhesion. Cells were then exposed to the extracts and the standard dutasteride at 0.5 mg/mL for 24 h. The medium were removed and the cells were washed with PBS, trypsinized with 0.25% trypsin/0.06 mM EDTA solution for 2 min and suspended in PBS.

2.5.2 Total RNA extraction

The total RNA from the cell pellets was extracted by the RNA extraction kit (NucleoSpin®, Macherey-Nagel, CA, USA) according to the instructions of the manufacturer. The concentration of the total RNA was quantified by Qubit Fluorometer and Quant-iT™ RNA BR assay kit (Invitrogen, CA, USA). The total RNA solution was kept at -20°C until used.

2.5.3 Reverse transcription-polymerase chain reaction (RT-PCR)

The 5α-reductase type 1 genes were amplified from the extracted RNA by SuperScript® One-Step RT-PCR with Platinum® Taq kit (Invitrogen, CA, USA) according to the manufacturer's protocol. Briefly, five micrograms of the total RNAs were reverse transcribed with RT/ Platinum Taq® mix and subjected to PCR cycles with the primers for human 5α-reductase type 1 (SRD5A1) as follows: 94°C for 15 s, 55°C for 30 s, 72°C for 45 sec for 35 cycles. The human 5α-reductase type 1 primers were designed based on GenBank accession no. NM_001047.2 and NM_000348, respectively, with a forward (5'- CCA TGT TCC TCG TCC ACT AC-3') and reverse (5'- TTC AAC CTC CAT TTC AGC GT -3'), produced 707 bp amplicon [15]. The RT-PCR products were then loaded on 1% agarose gel in the 1X tris-acetate-EDTA (TAE) buffer chamber at 100 V for 30 min. The human 5α-reductase type 1 dsDNA samples were quantified by the Qubit fluorometer and Quant-iT™ dsDNA assay kit (Invitrogen, CA, USA).

2.6 Effects of the Extracts on Melanogenesis Activity

2.6.1 Melanin content

The melanin content was measured according to the previously described method with some modification [9]. Briefly, B16F10 melanoma cells at the density of 1×10^5cells/well were plated in 6-well plates and incubated overnight for cell adhesion. The samples were then added and incubated for 72 h. The cells were washed with PBS and dissolved in 500 µl of 2 N NaOH at 60°C for 1 h. The absorbance was measured at 450 nm using a microplate reader and the melanin amount was determined in comparing to the standard melanin. The total protein content was measured by the Bradford dye-binding method using bovine serum albumin as a standard [16]. For determination of the actual melanin formation from the same cell numbers, the melanin content of each treatment was divided by the total protein content. The percentages of the relative ratio of melanin content were calculated as the following: *% relative ratio of the melanin content* = (*Mt*/*Mc*) × 100, where Mt was the melanin content of the sample divided by the total protein content of the sample and Mc was the melanin content of the control divided by the total protein content of the control.

2.6.2 Tyrosinase activity

Tyrosinase activity was analyzed by the method described previously with some modification [10]. Briefly, the cells treated with different samples were washed with ice-cold PBS and then lysed by incubation with the lysis reagent (Fermentas, EU) containing protease inhibitors (Roche, Germany) at 4°C for 30 min. The lysates were collected and centrifuged at 15,000 rpm for 10 min. The obtained supernatants were then collected, mixed with the mixture containing 50 mM sodium phosphate buffer (pH 6.8) and 0.05% dopa, and incubated at 37°C for 2 h. After incubation, the dopachrome formation was measured at 490 nm using a microplate reader. The

enzyme activity was calculated in compared to the standard mushroom tyrosinase. The total protein content of the sample was also evaluated. Enzyme activity of the sample was compared to the control and calculated as the percentages of the relative ratio of the tyrosinase activity according to the following equation: *% relative ratio of tyrosinase activity* = $(Tt/Tc) \times 100$, where Tt was the tyrosinase activity of the sample divided by the total protein content of the sample and Tc was the tyrosinase activity of the control divided by the total protein content of the control.

2.6.3 TRP-2 activity

TRP-2 activity was performed by the method previously described with some modification [10]. Briefly, the supernatant obtained from the lysis of the treated cells was mixed with the mixture consisting of 1 mM phenylthiourea, 2 mM EDTA and 10 mM sodium phosphate buffer (pH 6.8). Dopachrome solution, separately prepared by mixing an equal volume of 1 mM dopa and 2 mM $NaIO_4$, was added into the cell lysate mixture and incubated at 37°C for 2 h. The decreased absorbance based on the consumption of the dopachrome was measured at 490 nm. The reaction mixture with bovine serum albumin instead of the cell lysate was used as a negative control. The total protein content of the treated cells was also evaluated. TRP-2 activity of the sample was compared with the control and calculated as the percentages of the relative ratio of TRP-2 activity according to the following equation: *% relative ratio of TRP-2 activity* = $(Dt/Dc) \times 100$, where Dt was the TRP-2 activity of the sample divided by the total protein content of the sample and Dc was the TRP-2 activity of the control divided by the total protein content of the control.

2.7 Statistical Analysis

Data were expressed as the mean ±S.E. of three independent experiments. One way analysis of variance (ANOVA) and LSD tests were used for the analysis of the test results at the significant level of p-value <0.05.

3. RESULTS AND DISCUSSION

3.1 Percentage Yields of the Plant Extracts

Table 1 showed the percentage yields of the plant extracts that were calculated based on the plant dry weight. Different extraction solvents for each plant used in the study were based on the preliminary previous studies. The seeds of Lotus and Black Sesame were extracted with ethyl acetate based on the contents of the active compounds, such as fatty acids and tocopherol [7]. The complicated extraction process of the White Kwao Krua was taken from our previous studies on the melanogenesis activity. The n-hexane fraction of the extract demonstrated higher melanin induction and tyrosinase activity than the fractions from water and methanol, and the crude extract [17]. The extraction of rice bran with absolute ethanol was from the previous study, since it was convenient and gave the high unsaturated fatty acid content [7]. The extraction of Asiatic Pennywort was from the traditional use and our preliminary study [17]. Due to the brown-greenish color of the Asiatic Pennywort crude extract which was not appropriate to be incorporated in the formulation, the decolorization of the Asiatic Pennywort crude extract was performed. Two fractions of the decolorized Asiatic Pennywort extract, which were the water and chloroform fractions, were obtained.

The Black Sesame seed extract gave the highest percentage yield (42.76%), while the lowest percentage yield was the Lotus seed extract (1.5%). Also, the mixture of the rice bran extract and the White Kwao Krua extract was prepared in order to evaluate the synergistic effect of these two extracts according to the traditional herbal recipes. In addition, the rice bran loaded in β-cyclodextrin was prepared for the enhancement of the extract stability and biological activity [18]. The complex formation of the rice bran extract and β-cyclodextrin was determined by the size measurement using the dynamic light scattering (Zetaziser Nano Series Nano-S, Malvern instrument Ltd.m Malvern, UK). The observed larger size of the rice bran extract loaded in β-cyclodextrin (500.30±4.93 nm) than the free extract (329.20±1.74 nm) indicated the possibility of complex formation.

Table 1. Percentage yields of the plant extracts.

Plants				
Common name	Scientific name	Part used of the plant	Solvent extraction/Fraction	Percentage yields* (%)
Rice (RB)	*Oryza sativa*	bran	Absolute ethanol	19.23
White Kwao Krua (KK)	*Pueraria mirifica*	tuber	Ethyl acetate and n-hexane fraction	20.99
Black Sesame	*Sesamum indicum*	seed	Ethyl acetate	42.76
Lotus	*Nelumbo nucifera*	seed	Ethyl acetate	1.50
Asiatic Pennywort	*Centella asiatica*	leaf	Water	24.87
			Water and chloroform fraction	9.45
			Water and water fraction	73.09

*The calculation of the percentage yields was based on the dry weight of the plant, whereas the fraction was based on the dry weight of the initial crude extract.

3.2 Linoleic Acid Contents in the Extracts

Figure 1 showed the chromatograms of co-injection of the sample and linoleic acid (A-C) and the standard linoleic acid (D). The retention time of the standard linoleic acid at 1.0 mg/mL was 6.432 min, while those of the plant extracts were slightly shifted and could be detect at about 7.2-7.5 min. The obtained peaks at 7.2-7.5 min were supposed to be linoleic acid, because the standard linoleic acid has been spiked in the samples and the peak of the mixture of the standard linoleic acid and linoleic acid in the sample was higher than the sample only at the retention time range (data not shown). So, this was confirmed the retention time of linoleic acid to be at 7.2-7.5 min. The contents of linoleic acid in the extracts were presented in Table 2. The highest linoleic acid content was observed in the rice bran extract loaded in β-cyclodextrin (21.47±0.91%,w/w) and rice bran extract (21.13±1.22%,w/w) with no significant difference (p <0.05), followed by the mixture of the rice bran extract and the White Kwao Krua extract (14.98±0.89%, w/w) and the White Kwao Krua n-hexane fraction (6.96±0.52%,w/w). The linoleic acid content in the rice bran extract was closely the same as the previous study, in which the rice bran extract was prepared by the supercritical carbon dioxide fluid technique [7]. The linoleic acid contents in the mixture of the rice bran extract and the White Kwao Krua extract (14.98±0.89%, w/w) were in between

the linoleic acid contents in the White Kwao Krua n-hexane fraction (6.96±0.52%,w/w) and the rice bran extract (21.13±1.22%,w/w). Variation of the linoleic acid content in various plant extracts appeared to be due to different extraction solvent and preparation method [19].

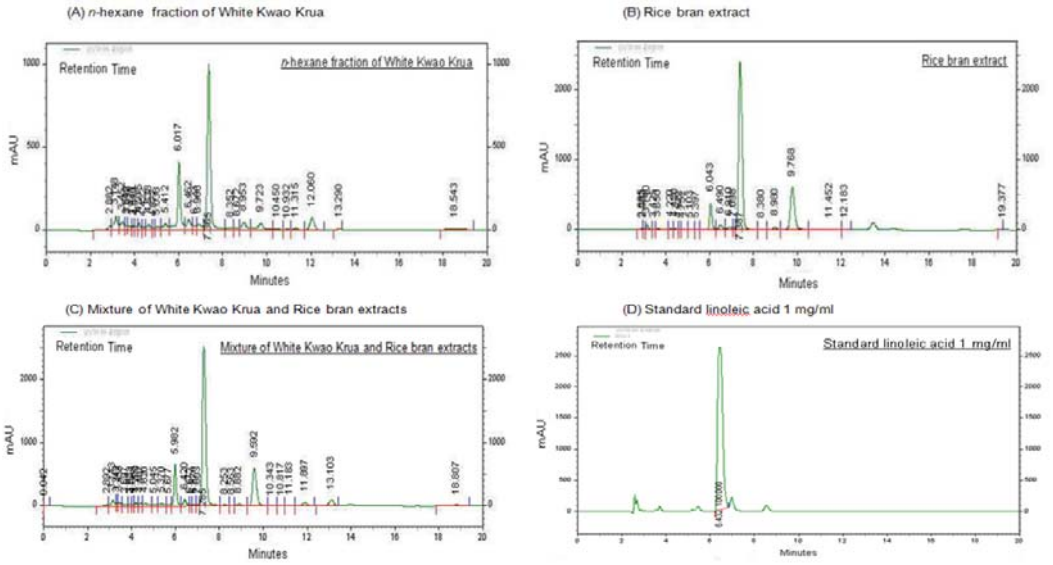

Figure 1. HPLC Chromatograms of the White Kwao Krua n-hexane fraction (A), the rice bran extract (B), the mixture of the rice bran extract and the White Kwao Krua extract (C) and the standard linoleic acid at 1 mg/mL (D).*(A)-(D) indicated the HPLC chromatograms of co-injection of the sample and the standard linoleic acid.

Table 2. Comparison of linoleic acid contents (%w/w) containing in the plant extracts by HPLC.

Plants	Preparation	Linoleic acid contents (%,w/w)
Rice	Crude extract (RB)	21.13±1.22
	Crude extract mixed with White Kwao Krua extract (RBKK)	14.98±0.89
	Crude extract loaded in β-cyclodextrin (RBC)	21.47±0.91
White Kwao Krua	n-hexane fraction (KK)	6.96±0.52
Black Sesame	Crude extract (BS)	3.19±0.62
Lotus	Crude extract (LS)	2.45±0.63
Asiatic Pennywort	Crude extract (AP)	ND*
	Chloroform fraction of crude extract (CAP)	ND*
	Water fraction of crude extract (AAP)	ND*

ND*: Not Detected

3.3 Effect on 5α-Reductase Activity

3.3.1 Cytotoxicity assay in DU-145 cells

At the final concentration of 0.5 mg/mL, all samples except the Lotus seed extract gave cell viability in DU145 cells of more than 80% (data not shown). However, at the final concentration of 0.05 mg/mL, Lotus seed extract gave no toxicity with cell viability of more than 80%. At the concentration higher than 0.05 mg/mL of the Lotus seed extract, high toxicity was observed. This may be due to the high content of saturated fatty acid and ester derivative, especially palmitic acid and its ester derivative [8-10, 20]. Previous study has reported that high amount of saturated fatty acid found in the Lotus seed extract is palmitic acid (4.2 mg/g of lipid) [20]. The Lotus seed extract at 0.05 mg/mL and others at the concentration of 0.5 mg/mL were used in the further 5α-reductase inhibition experiment.

3.3.2 The 5α-reductase type 1 inhibition assay

The 5α-reductase is the main cause of hair loss, predominated in human scalp skin especially in the dermal papilla [3]. In several studies, the DU-145 human androgen insensitive prostate adenocarcinoma cell line composing of 5α-reductase type 1 has been used for the assay of 5α-reductase type 1 inhibition [7, 21]. The rice bran extract showed the highest 5α-reductase type 1 inhibitory activity in DU-145 cells at 87.43±7.15% of the control, which was higher than the standard dutasteride (54.65±4.94%) of 1.60 times (Figure 2), followed respectively by the mixture of the rice bran extract and the White Kwao Krua extract (33.52±2.58%) and the Lotus seed extract (28.23±2.11%). The highest linoleic acid content in the rice bran crude extract (21.13% w/w) may be responsible for 5α-reductase type 1 inhibition activity. Besides linoleic acid, other bioactives existing in the rice bran extract, such as ferulic acid, vanillic acid, γ-oryzanol and phytic acid [22] may be synergistic to this activity. In addition, the 5α-reductase inhibition activity of the mixture of the rice bran extract and the White Kwao Krua extract also showed the relationship between the linoleic acid contents and the 5α-reductase inhibition activity. Nevertheless, the linoleic acid content in the rice bran extract loaded in β-cyclodextrin (21.47±0.91%) appeared not to be related to the inhibitory activity. When the rice bran extract loaded in β-cyclodextrin, it gave lower 5α-reductase inhibition activity than the free extract of about 7 times. When loaded in β-cyclodextrin, the changed physical and chemical properties of the extract may occur in combination with the tight complex forming of the extract and β-cyclodextrin, in which the extract may not easily be liberated to act with the mRNA products.

The effect of 5α-reductase activity of the White Kwao Krua n-hexane fraction may be from other bioactives in the extract, such as daidzin, genistin, daidzein and genistein, in which these compounds may act on the steroidal pathway, resulting the increased 5α-reductase activity of the extract [23]. The unsaturated fatty acid esters containing in the Lotus seed extract, such as linoleic acid methyl ester, may penetrate into the cell membrane and subsequently be hydrolyzed to give the free unsaturated fatty acids which can exhibit the synergistic effect on 5α-reductase inhibitory activity [8]. The inhibitory activity of the Black Sesame seed extract was in agreement with the previous report on the hair growth promoting effect *in vivo* model [24]. The antioxidants, such as polyphenols and flavonoids, in the Black Sesame seed extract may have the role in 5α-reductase inhibition [25]. The Asiatic Pennywort extract and the water fraction

of the decolorized extract demonstrated the 5α-reductase inhibition activity, whereas the chloroform fraction of the decolorized extract gave the stimulation activity. The different activity of extract and fraction may be from the bioactive compounds existing in the extract and fraction. For the Asiatic Pennywort extract and the water fraction, the bioactives may include alkaloids, saponins and steroids, whereas the chloroform fraction may contain only oil-soluble actives, such as steroids. The previous study has reported that saponins in the plant extracts can suppress the 5α-reductase gene expression [26]. For the chloroform fraction, the oil-soluble actives may play role in the steroidal pathway and synergistically act on the 5α-reductase activity, resulting in an increased activity of the enzyme.

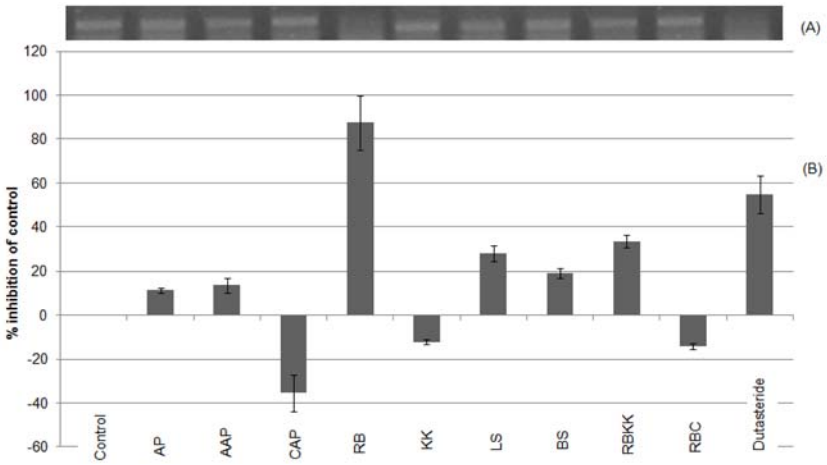

Figure 2. The 5α-reductase type 1 inhibitory activity in DU-145 cells treated with the Asiatic Pennywort extract (AP), water (AAP) and chloroform (CAP) fractions of the decolorized Asiatic Pennywort extract, the rice bran extract (RB), the White Kwao Krua n-hexane fraction (KK), the Lotus seed extract (LS), the Black Sesame seed extract (BS), the mixture of the rice bran extract and the White Kwao Krua extract (RBKK) and the rice bran extract loaded in β-cyclodextrin (RBC) in comparing to the standard dutasteride at the final concentration of 0.5 mg/mL. (A) indicated the agarose gel electrophoresis of dsDNA of 5α-reductase type 1 enzyme after treatment with the samples and (B) demonstrated the percentages of 5α-reductase type 1 inhibition which was calculated as the following equation: % inhibition = [(control-sample)/control] × 100.

3.4 Effects of the Extracts on Melanogenesis

3.4.1 Cytotoxicity assay in B16F10

The cytotoxicity assay of the plant extracts was performed in B16F10 melanoma cells in order to evaluate the appropriate concentration for the melanogenesis assay. Figure 3 demonstrated the cytotoxic effects of the extracts at 0.01-10 mg/mL. At 10 mg/mL, the rice bran extract, the mixture of the rice bran extract and the White Kwao Krua extract, and the Black Sesame seed extract demonstrated cell viability of 84.64±8.33, 85.33±6.99 and 96.01±1.76%, respectively. At 1 mg/mL, the rice bran extract loaded in β-cyclodextrin, the White Kwao Krua n-hexane fraction, the Lotus seed extract and the chloroform fraction of the decolorized

Asiatic Pennywort extract showed cell viability of 98.73±2.34, 85.57±1.37, 110.93±1.97 and 80.11±4.93%, respectively. At 0.1 mg/mL, the Asiatic Pennywort extract and the water fraction of the decolorized Asiatic Pennywort extract exhibited cell viability of 89.33±3.31 and 89.7±2.08%, respectively. The appropriate concentration of the plant extracts that gave cell viability of more than 80% was selected for the melanogenesis assay.

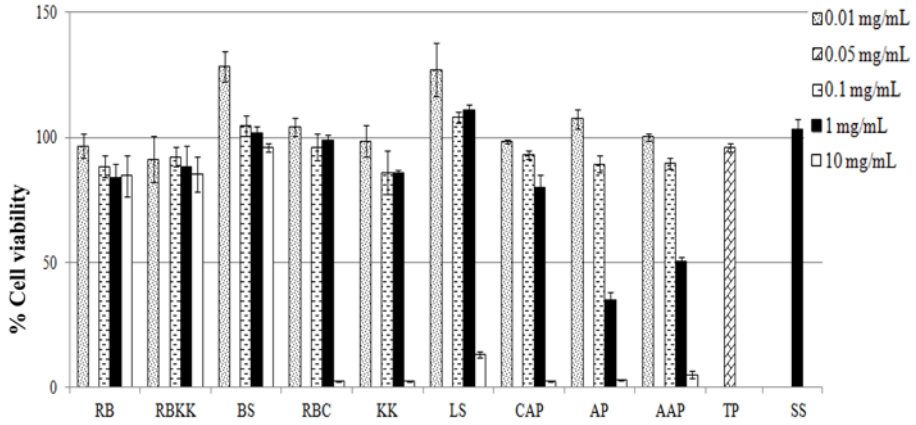

Figure 3. Cytotoxicity assay of B16F10 melanoma cells treated with the rice bran extract (RB), the mixture of the rice bran extract and the White Kwao Krua extract (RBKK), the Black Sesame seed extract (BS), the rice bran extract loaded in β-cyclodextrin (RBC), the White Kwao Krua n-hexane fraction (KK), the Lotus seed extract (LS), the Asiatic Pennywort extract (AP), chloroform (CAP) and water (AAP) fractions of the decolorized Asiatic Pennywort extract, theophylline (TP) and sesamin (SS) at 0.01-10 mg/mL.

3.4.2 Melanogenesis assay

Melanogenesis assay of all samples was performed in comparing to the positive control (theophylline) at 0.05 mg/mL in B16F10 melanoma cells. Theophylline significantly demonstrated melanin stimulation, tyrosinase and TRP-2 activities higher than the control of 2.38, 3.34 and 1.04 folds, respectively. This positive reference mediates the pigment biosynthesis through the cAMP pathway as well as increases the gamma-glutamyl transpeptidase- and tyrosinase-reactive cells, resulting in the induction of melanin production with the increased level of tyrosinase activity [10].

Melanogenesis of all samples were shown in Figures 4-6. The rice bran extract, the mixture of the rice bran extract and the White Kwao Krua extract, the rice bran extract loaded in β-cyclodextrin and the White Kwao Krua n-hexane fraction gave melanin stimulation of 1.06, 1.07, 1.25 and 1.06 folds of the control, respectively. The Black Sesame seed extract, Lotus seed extract, Asiatic Pennywort extract, chloroform and water fractions of the decolorized Asiatic Pennywort extract demonstrated melanin inhibition of 0.19, 0.24, 0.01, 0.15 and 0.10 folds of the control, respectively. Sesamin, a major lignan consisting in the sesame extract, was evaluated for the effect on melanogenesis in comparing to the Black Sesame seed extract. The standard sesamin at 1 mg/mL slightly increased the melanin content and tyrosinase activity of 1.03 and 1.10 folds of the control, respectively, while no activity change on TRP-2 was observed (98.57±1.21%).

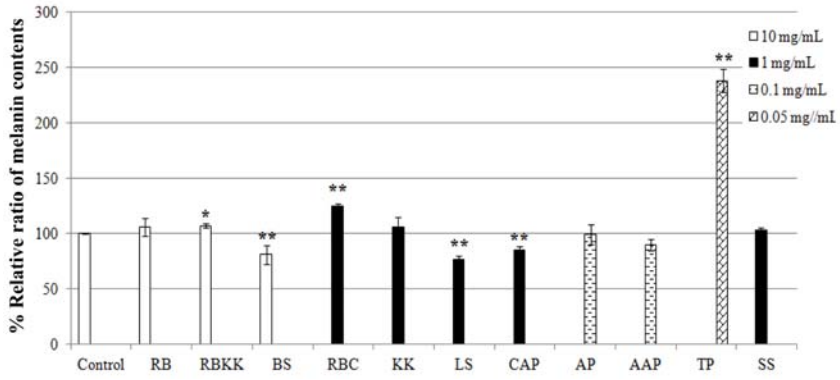

Figure 4. The % relative ratio of melanin contents in B16F10 melanoma cells treated with the rice bran extract (RB), the mixture of the rice bran extract and the White Kwao Krua extract (RBKK), the Black Sesame seed extract (BS), the rice bran extract loaded in β-cyclodextrin (RBC), the White Kwao Krua n-hexane fraction (KK), the Lotus seed extract (LS), the Asiatic Pennywort extract (AP), chloroform (CAP) and water (AAP) fractions of the decolorized Asiatic Pennywort extract, theophylline (TP) and sesamin (SS) at 0.05-10 mg/mL. *Significant difference from the control (*p<0.05,**p<0.01).

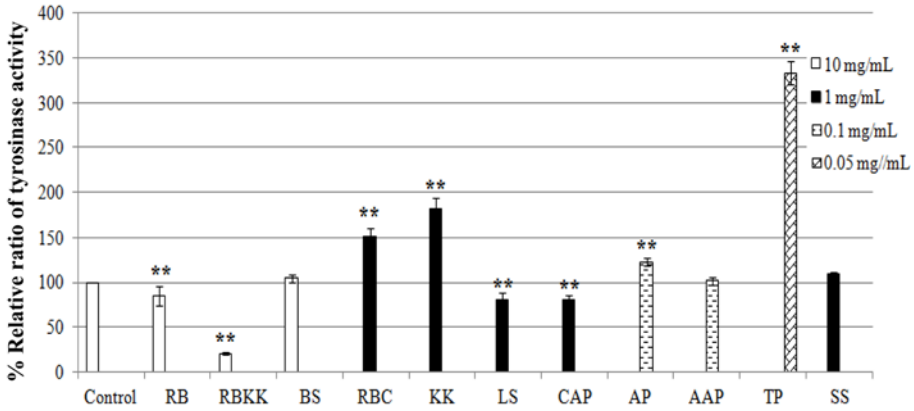

Figure 5. The % relative ratio of tyrosinase activity in B16F10 melanoma cells treated with the rice bran extract (RB), the mixture of the rice bran extract and the White Kwao Krua extract (RBKK), the Black Sesame seed extract (BS), the rice bran extract loaded in β-cyclodextrin (RBC), the White Kwao Krua n-hexane fraction (KK), the Lotus seed extract (LS), the Asiatic Pennywort extract (AP), chloroform (CAP) and water (AAP) fractions of the decolorized Asiatic Pennywort extract, theophylline (TP) and sesamin (SS) at 0.05-10 mg/mL. *Significant difference from the control (*p<0.05,**p<0.01).

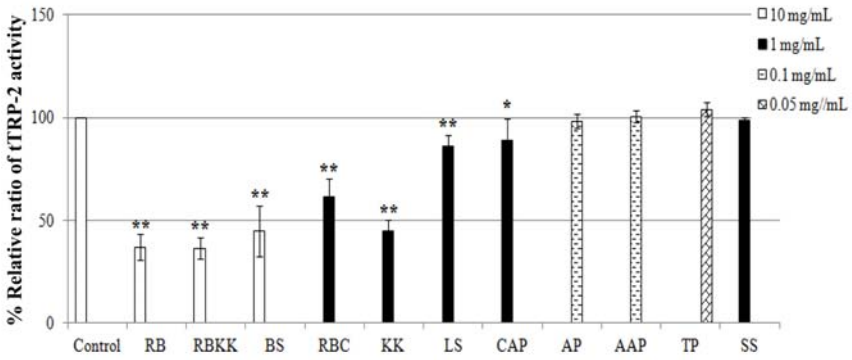

Figure 6. The % relative ratio of TRP-2 activity in B16F10 melanoma cells treated with the rice bran extract (RB), the mixture of the rice bran extract and the White Kwao Krua extract (RBKK), the Black Sesame seed extract (BS), the rice bran extract loaded in β-cyclodextrin (RBC), the White Kwao Krua n-hexane fraction (KK), the Lotus seed extract (LS), the Asiatic Pennywort extract (AP), chloroform (CAP) and water (AAP) fractions of the decolorized Asiatic Pennywort extract, theophylline (TP) and sesamin (SS) at 0.05-10 mg/mL.*Significant difference from the control (*p<0.05,**p<0.01).

For tyrosinase activity, the Black Sesame seed extract, the rice bran extract loaded in β-cyclodextrin, the White Kwao Krua n-hexane fraction, Asiatic Pennywort extract and the water fraction of the decolorized Asiatic Pennywort extract increased the tyrosinase activity of 1.04, 1.51, 1.82, 1.23 and 1.02 folds of the control, respectively. However, an increased tyrosinase activity of the samples appeared to be less than theophylline, the positive control. Tyrosinase activities of the Black Sesame seed extract, the rice bran extract loaded in β-cyclodextrin, the White Kwao Krua n-hexane fraction, the Asiatic Pennywort extract and the water fraction of the decolorized Asiatic Pennywort extract were 0.31, 0.45, 0.55, 0.37 and 0.31 folds of the positive control. In contrast, the rice bran extract, the mixture of the rice bran extract and the White Kwao Krua extract, the Lotus seed extract and the chloroform fraction of the decolorized Asiatic Pennywort extract demonstrated tyrosinase activity inhibition of 0.15, 0.80, 0.20 and 0.20 folds of the control, respectively. The rice bran extract loaded in β-cyclodextrin showed the melanin content and tyrosinase activity higher than the rice bran extract not loaded in β-cyclodextrin. This may be due to the higher stability of the extract when loaded in β-cyclodextrin. The effects of the rice bran extract β-cyclodextrin complex in 5α-reductase inhibition were different from in the melanogenesis assays, owning to the different activity of the active compounds in the extract. Previous study has also reported that reaction with the mixture of the saturated and unsaturated fatty acids, β-cyclodextrin prefers the unsaturated fatty acids (which are the active compounds for 5α-reductase inhibition) over the saturated fatty acids (which are the active compounds of melanogenesis) [27]. Therefore, the saturated fatty acids can easily be liberated from the β-cyclodextrin cavity to give the higher melanin content and tyrosinase activity, than the fatty acids not loaded in β-cyclodextrin. For the activity of the TRP-2 enzyme, the conversion of dopachrome to DHICA after converting of L-dopa to dopachrome by tyrosinase may occur. To solve this conversion, the amount of dopachrome

both in tyrosinase and TRP-2 activity assays at the same wavelength was also measured. If the conversion happens, the decreased dopachrome formation can be observed from both assays. Only Asiatic Pennywort extract and water fraction of the decolorized extract did not change the enzyme activity in comparing to the control. Other extracts including the n-hexane fraction of White Kwao Krua, chloroform fraction of the decolorized Asiatic Pennywort extract and the rice bran extract loaded in β-cyclodextrin reduced the activity of TRP-2.

The effects on melanogenesis induction and tyrosinase activity of the samples were analyzed and presented as the percentage ratio in comparing to the control. The rice bran extract, the mixture of the rice bran extract and the White Kwao Krua extract, the rice bran extract loaded in β-cyclodextrin demonstrated the melanin induction. Although linoleic acid and other unsaturated fatty acids have been reported as the active compounds in the rice bran extract with melanin inhibition activity [9], this study has indicated the melanin stimulation activity of the rice bran extract and its mixture with the White Kwao Krua extract. However, the increased melanin content from the rice bran extract did not show any significant different in comparing to the control (p-value > 0.05). In addition, the mixture of the rice bran extract and the White Kwao Krua extract significantly exhibited melanin induction (p-value=0.021). The melanegenesis induction of the rice bran extract may be from other components in the extract, such as palmitic acid, stearic acid and sterols [7-10]. Also, compounds in the White Kwao Krua including daidzin, genistin, daidzein and genistein may also have a synergistic effect on their activity [23]. These compounds have been demonstrated to involve in melanin stimulation. Also, the rice bran extract loaded in β-cyclodextrin showed the melanin content and tyrosinase activity higher than the free rice bran extract. The different effect of the rice bran extract loaded in β-cyclodextrin on 5α-reductase inhibition and melanogenesis may be from the different active compounds existing in the extract. In fact, an enhanced penetration effect of the extract facilitated by the β-cyclodextrin can result in an increased melanogenesis induction activity. Nevertheless, the rice bran extract loaded in β-cyclodextrin showed lower 5α-reductase inhibition activity than the free extract. Due to complex formation of the extract with β-cyclodextrin, the physical and chemical properties of the extract may be changed and affect the liberation of the extract to react with the mRNA products, leading to the decreased inhibition activity of 5α-reductase enzyme. The tyrosinase and TRP-2 activities of the rice bran extract alone, the rice bran extract loaded in β-cyckodextrin and the mixture of the rice bran extract and the White Kwao Krua extract were not related to their melanin induction effects. Various constituents in the extracts such as saturated and unsaturated fatty acids may play different roles on melanogenesis.

Pueraria mirifica or White Kwao Krua, a medicinal plant in Leguminosae family, has been widely used as a rejuvenating plant in the traditional recipes. The White Kwao Krua n-hexane fraction demonstrated slight melanin stimulation with the significantly increased tyrosinase activity, but not the TRP-2 activity. The melanin induction activity of the White Kwao Krua n-hexane fraction may be mediated through the tyrosinase more than the TRP-2. Besides the fatty acids, the phytoestrogenic compounds, such as daidzin, genistin, daidzein and genistein in the White Kwao Krua extract may be responsible for this melanin stimulation activity [23].

The Black Sesame seed extract gave the decreased melanogenesis, while the standard sesamin slightly increased the melanin content and tyrosinase activity. Melanogenesis stimulation of the standard sesamin was in agreement with the previous study [28]. However, the Black Sesame seed extract in this study was the crude extract containing various chemical constituents, including unsaturated and saturated fatty acids, polyphenols, flavonoids, sesamol, sesamolin and sesamin [11]. These compounds together with linoleic acid and other unsaturated fatty acids, may have the synergistic effect of melanogenesis inhibition.

Lotus seeds have been intensely investigated and used for various medicinal applications, such as diuretic, cooling agent, antiemetic and tissue inflammation. In this study, the Lotus seed extract demonstrated the melanogenesis inhibition. In fact, compositions in the Lotus extract, including myricetin, quercetin, kaempferol and isorhammetin, have been reported to inhibit melanogenesis in B16 melanoma cells [29].

Asiatic Pennywort has been widely cultivated and used in many countries for the treatment of various conditions, such as skin diseases, rheumatism, inflammation, dehydration and diarrhea [30]. The Asiatic Pennywort was extracted and decolorized to obtain the chloroform and water fractions. The two decolorized fractions showed melanogeneis inhibition, but did not have any effect on melanin production. Also, the chloroform fraction of the decolorized Asiatic Pennywort extract significantly inhibited the activity of tyrosinase enzyme, the key enzyme in melanogenesis process. Thus, this potent inhibition effect of the chloroform fraction of the decolorized Asiatic Pennywort extract may be potentially developed as a depigmentation agent for whitening cosmetics. The active compounds found in the Asiatic Pennywort extract, including flavones, quercetin and kaemferol, may be the responsible compounds for this melanogenesis inhibition [30].

4. CONCLUSIONS

Various plant extracts including the Lotus seed extract, the Black Sesame seed extract, the White Kwao Krua n-hexane fraction, the rice bran extract, the mixture of the rice bran extract and the White Kwao Krua extract, the rice bran extract loaded in β-cyclodextrin, the Asiatic Pennywort extract, the water and chloroform fractions of the decolorized Asiatic Pennywort extract were investigated for 5α-reductase inhibition and melanogenesis activities on DU-145 and B16F10 melanoma cells, respectively. The rice bran extract showed the highest 5α-reductase type 1 inhibitory activity which was higher than the standard dutasteride of about 1.60 times. The rice bran extract loaded in β-cyclodextrin at 1 mg/mL and the White Kwao Krua n-hexane fraction at 1 mg/mL demonstrated the highest melanogenesis induction and tyrosinase activity of 1.25 and 1.82 folds of the control. The enhanced melanogenesis activity may be from various active components in the extracts as well as the enhanced cellular penetration from the extract loaded in β-cyclodextrin. This present study has indicated the potential 5α-reductase inhibition and melanogenesis induction of various Thai plants which can be further developed as anti-hair loss and hair darkening products, respectively.

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