CHAPTER III

MATERIALS AND METHODS

Mushroom Tyrosinase Inhibition Assay

This assay is used to screen the extract samples for the inhibitory effect of enzyme tyrosinase. Its principle is based on the catalytic activity of enzyme which is a rate-limiting enzyme in melanogenic pathway. In this reaction, tyrosinase catalyzed the conversion of L-tyrosine to L-dopaquinone which can be measured by spectrophotometer.[76] Briefly, 1.0 ml of PBS (pH 6.8), 1 ml of distilled water containing 0.3 mg/ml of L-tyrosine (Sigma) and 0.9 ml of sample solution (herbal extracts or kojic acid (Sigma) dissolved in 10% dimethylsulfoxide (DMSO) were mixed thoroughly in test tube and preincubated at 30°c for 10 min. Then, 0.1 ml of 480 units/ml of tyrosinase (Sigma) was added into the solution mixture at 30°c for 30 min. Finally, 0.1 ml of 1 M sodium azide was added to stop the reaction, and the absorbance was measured at 475 nm by microtiter plate reader. A control reaction a 10% DMSO solution (in the absence of crude extracts or kojic acid). Then, the inhibitory activity was calculated according to the following formula: Inhibitory activity (%) = (C-(S-B)) / C x 100 where C is the absorbance of the control at 475 nm; S is the absorbance of the sample at the same wavelength; and B is the absorbance of reaction mixture without tyrosinase enzyme.

Cultures and treatments of melanocytes

To elucidate the effect of ethanolic cruded extract from *Mallotus spodocarpus* and *Excoecaria bicolor* on the mRNA expression of tyrosinase and microphthalmia-associated transcription factor, I have selected the melanocyte cells (Catalog No.CRL-1676 purchased from ATCC), which is the adherent cell line that derived from skin female human melanoma cell, as an *in vitro* model for these studies.

Both extracts were obtained from Dr. Saroj's Research Lab Co., Ltd. CRL-1676 cells were plated at density $1x10^4$ cells/well in 24-well plates for RT-PCR or 96-well plates for MTT assay (see Appendix B for trypan blue dye exclusion method) and grown in 10 % FBS supplemented Dulbecco's modified Eagle's medium (DMEM), containing 2 mM L-glutamine, and 1% penicillin-streptomycin and let they grown in a humidified, 5 % CO_2 incubator at 37^0c. All were purchased from HyClone. When cells reached 80% confluence, cells were then allowed to grow in 2% FBS DMEM for 24 h before the experiments. To begin the treatments, the medium in each well was completely removed. Cells in each well were added with freshly prepared 2% FBS DMEM containing Crude extract from *Mallotus spodocarpus*, *Excoecaria bicolor* and kojic acid in various doses. Cells in serum-free DMEM served as the untreated control. Times for treatments were indicated in each experiment (see below).

RNA Isolation

After 24 h treatments, total RNA from each condition was isolated using Trizol reagent (Invitrogen). Briefly, the medium in each well was completely removed. The cells were washed once with phosphate-buffered saline (PBS), pH 7.4. Then, Trizol reagent (300 µl) was added into each well. The homogenates were placed on a low speed rotator for 5 min at room temperature. This is to allow the complete dissociation of nucleoprotein complexes. Next, the homogenates were transferred into steriled 1.5 ml tubes. Then, chloroform (60 µl) was added into each tube followed by vortexing vigorously for 15 seconds. The mixture was allowed to stay at room temperature for 5 min before centrifugation at 12,000 g for 15 min at 4°c. RNA in the colorless upper aqueous phase was carefully transferred to a fresh tube. Then, isopropanol (150 µl) was added into each tube. Each sample was stored at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4°c. RNA will precipitate and form a white pellet at the bottom of the tube. The supernatant was

carefully removed before adding iced-cold 75% ethanol (300 µl) into each tube to wash RNA pellet for 5 min. Subsequently, the pellet was centrifuged at 10,000 g at 4°c for 5 min. At the end of procedure, the RNA pellets were allowed to air dry briefly. It is important not to let the RNA pellet dry completely as it will greatly decrease its solubility. Dissolve the RNA pellets in RNase free water. The amount of RNA obtained by spectrophotometer was calculated using the formular below.

RNA (µg) = Absorbance at 260 nm x 40 x dilution factor

The solution containing RNA sample can be stored at -80°c until use.

Reverse transcription (cDNA synthesis)

A volume of 2 µg total RNA from each sample was added into the reaction mixture containing 10x reaction buffer, 25mM $MgCl_2$, dNTPs, random primer, ribonuclease inhibitor, AMV reverse transcriptase and RNase free water. All reagents were purchased from Promega. Each sample was kept at room temperature for 10 min incubated at 42°c for 60 min followed by inactivation at 99°c for 5 min. Then, distilled water was added into each sample to a final volume of 100 µl. At this point, each sample can be kept at -20°c until use. See appendix C for preparing the reaction mix.

Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 25 µl. A 5 µl of cDNA (as described above) was used as templates for subsequent PCR reaction in the Perkin Elmer Thermocycle. PCR reaction was composed of distilled water, PCR buffer, dNTP mix; Taq DNA polymerase (Promega) and primer pairs (see Table 2). To ensure there was no genomic DNA contamination in the RNA samples, the PCR reaction was performed without reverse transcription. As a control reaction, the RNase free water

was replaced the RNA samples in the PCR protocol. Subsequently, amplified PCR products were visualized by gel electrophoresis. Briefly, a 10 μl of each PCR product was mixed with 2 μl of loading buffer, vortexed, spined, and then loaded on to 1.5 % agarose gel containing ethidium bromide. The gel was run at 95 volts until front dye reaches the end of gel. Estimate size of PCR products was compared to the DNA marker (100 bp ladder, Fermentas). No PCR products were observed in any control reactions (data not shown). See appendix C for preparing the reaction mix.

Table 2. Showing specific primer for human MITF, tyrosinase and beta-actin.

Gene	Primer sequences (5′ ⟶ 3′)	PCR Protocols
Tyrosinase	CTG TGG CCA GCT TTC AGG CAG A	1
	AGA TCC CCC AAG CAG TGC ATC CA	
MITF	TCC TGT CCA GCC AAC CTT CCC AA	2
	TGG TGC CAT CCG TGA GAT CGA GA	
Beta-Actin	CAC CCA CAC TGT GCC CAT C	3
	CTA GAA GCA TTT GCG GTG GAC	

Protocol 1 Pre denature at 94°c for 4 min followed by 30 cycles of 94°c for 1 min, 62°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 2 Pre denature at 94°c for 4 min followed by 30 cycles of 94°c for 1 min, 62°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 3 Pre denature at 94°c for 4 min followed by 30 cycles of 94°c for 1 min, 62°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

MTT Cell Proliferation Assay

MTT assay is the measurement of cell cytotoxic or proliferation. This method is based on the reduction of yellow tetrazolium salts MTT (3-(4, 5-dimethylthiazoly-2)-2, 5-diphenyltetrazolium bromide) by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple of formazan can be solubilized and quantified by spectrophotometric means. [77] CRL-1676 were plated at a density of $1x10^4$ cells/well in 96-well plates in 100 µl Cells was incubated at 5 % CO_2, 37^0c under treated-conditions for 24 h. Then, 10 µl of 5 mg/ml MTT reagent was added to each well, including control (DMEM without cells), then plate was returned to the incubator for 2 to 4 h. When the purple precipitate was clearly visible inside the cells under the microscope. Then, 100 µl of DMSO was added to each well, including controls. Plate was swirled gently and covered in the dark for 2 to 4 h. Absorbance in each well was measured, including the blanks, at 570 nm using a microtiter plate reader.

Western blot analysis

Immunoblot analysis was included in this study to demonstrate that the herbal extract added into the cell culture medium could induce tyrosinase signalibg cascades in melanocytes. Protein expression of total form of ERK (p44/42 MAP-Kinase) and phosphorylated form of ERK (at Thr202/Tyr204) were examined using the polyclonal antibodies from Cell Signaling. Briefly, following 24 h treatments, total cells from each sample were collected and lysed in lysis buffer containing 1% Triton-X 100. Total protein content in each sample was quantified using the Bio-Rad protein determination assay. Then, equal amounts (100 µg) of total protein from each sample was mixed with sample buffer containing beta-mercaptoethanol, boiled for 10 min, and separated on 10% SDS-PAGE. Then, proteins were transferred to PVDF membranes,

blocked in 5% dry milk for 1 h at room temperature, rinsed with TBS, and incubated overnight at 4°c in the primary antibody (1: 1000). Subsequently, the membranes were washed with TBS containing 1% Tween 20 and incubated in the secondary antibody (1 : 2000) goat anti-rabbit IgG peroxidase conjugate (Sigma) for 1 h at room temperature. Bound antibodies were visualized with the supersignal solutions (Pierce) and detected band with film explosion. See appendix D for buffer preparations.

Statistical analysis

All data were presented as means and standard error of means (mean ± SEM). One way analysis of variance (one-way ANOVA) followed by LSD was used to compare the significance between the control and the treatment groups. The p-value of less than 0.05 was set for the significant difference.