CHAPTER III
MATERIALS AND METHODS

1. Chemicals

For PCR process, the chemicals used were Molecular Biology agarose (Bio-Rad, Spain), 1kb DNA ladder (Promega, U.S.A.), Blue/Orange 6X loading dye (Promega, U.S.A.), primer β-actin, VEGF, KGF, TGF-β1, 5α-reductase (Type I and II), tyrosinase, TRP-1 and TRP-2 (Eurofins MWG Operon, Germany), tris base, glacial acetic acid, EDTA (Ajax/Australia), Omiscript RT Kit (QIAGEN, Germany), TopTaq MasterMix kit (QIAGEN, Germany) and Novel Juice (GeneDirex). RNA extraction kit (GE Healthcare, UK) was used to extract total RNA from the cells. α-melanocyte stimulating hormone (Sigma, USA), 3-[4, 5- dimethylthiazol-2-yl]-2,5-dyphenyl tetra-zolium bromide (Invitrogen, USA), Minoxidil (Sigma, USA), Hydroxysafflor yellow A (NICPBP, China), NADPH (Sigma, USA), NADH (Sigma, USA), DMEM (Invitrogen, UK), FBS (Invitrogen, UK), RPMI (Invitrogen, UK), Ficoll Hypaque. The HPLC standard compounds including gallic acid, chlorogenic acid, vannillin, epicatechin gallate, quercetin, caffeic acid, *p*-coumaric acid, ferulic acid were HPLC grade and other analytical grade chemicals were used.

2. Materials and apparatus

High performance liquid chromatography (HPLC) instrument equipped with UV-detector and auto-sampler system (Agilent Technologies 1200 Series), HPLC column: C18 column (Hypersil ODS, USA), Ultrasonic bath (Elma S60, Elmosonic), Two milliliters vials with caps, Autopipette (LABMATE), Rotary evaporator (ETERA, Japan), Analytical Balance (Mettler Toledo, Germany), Lyopilizer (Christ, Germany), High speed centrifuge (Kubota, Japan), Centrifuge (Mini spin, eppendorf), CO_2 incubator (JENCONS-PLS, UK), Laminar air flow (Esco, Australia), Thermo-cycler (Biometra), Side by side diffusion cell apparatus (Crown glass company, New Jersey), UV-spectrophotometer (Shimadzu, Japan), Vortex mixer (Vision, USA), Inverted microscope (Nikon Eclipse E200, Japan), Binocular inverted microscope (Nikon Eclipse TS100, Japan), Electrophoresis apparatus (Major Science, MP-250N),

Micro-plate reader (Anthos, Austria), pH meter (Mettler Toledo, Germany), Gel Documentation and system analysis machine (InGenius L).

3. Plant materials

Fifteen herbal plants were purchased from the local market in Amphoe Meuang, Khon Kaen Province, Thailand (Table 6).

4. Preparation of the plant extract

4.1 Plant preparation for the screening test

The part used of each plant was pulverized and macerated in 50% ethanol for 3 days and then filtered through thin cloth and centrifuged at 3000 g, 25 °C for 10 min. The clear supernatant was concentrated using a rotary evaporator at 45°C, and then freeze-dried. The extract was stored at -20°C for further use.

4.2 Preparation of *Carthamus tinctorious*

C.tinctorious floret was extracted in boiling water for 30 minutes, maceration in water, 50% ethanol and methanol. Each supernatant of 4 fractions was centrifuged, evaporated and finally freeze-dried. The 4 fractions of *C.tinctorius* were obtained. The schematic of extraction method was shown below (Figure 18).

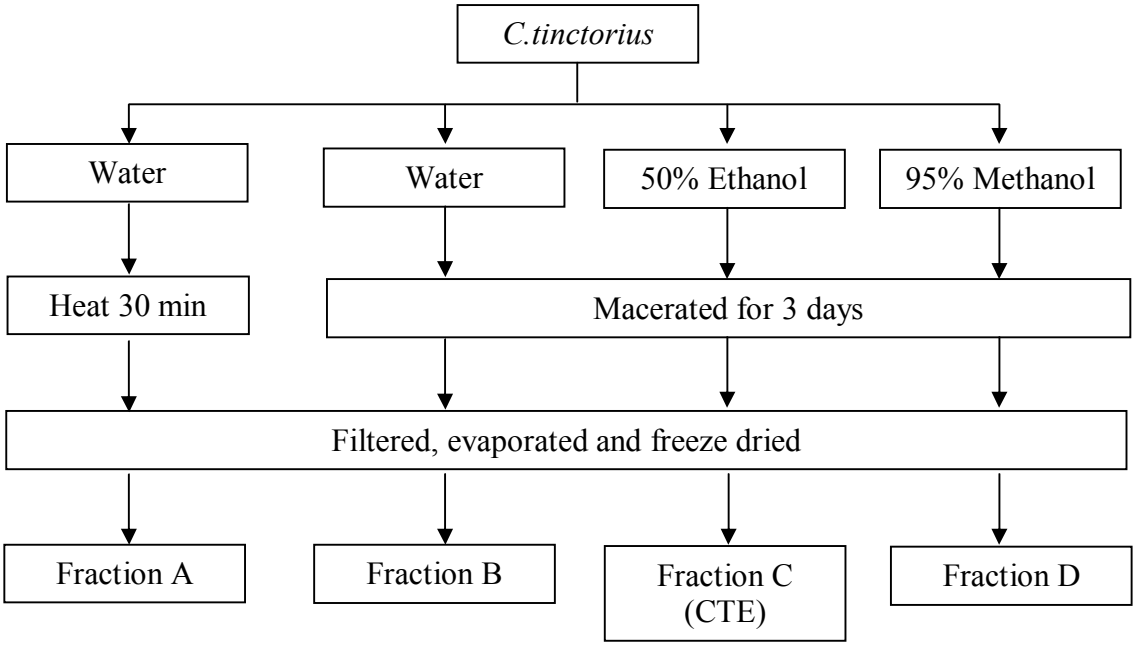

Figure 18 Extraction chart of 4 fractions of *C.tinctorius*

5. Safety evaluation

5.1 Cytotoxic effect on white blood cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood donated to Blood Bank of Khon Kaen University hospital. Fresh heparinized blood was diluted with RPMI-1640 medium (1:4) and then 10 ml of diluted blood was slowly overlaid on 3 ml of Ficol Hypaque solution (density 1.077 g/L). After centrifugation at 900×g, 20°C for 30 min, the plasma in the top layer was removed and then the PBMCs white thin layer on the surface of Ficoll Hypaque was collected. The collected cells were washed 3 times with 10 ml of RPMI-1640 medium and then centrifuged at 400×g, 20°C for 10 min. The PBMCs were resuspended in 5 mL of RPMI-1640 medium supplemented with 10% FBS and incubated at 37°C, 5% CO_2 for overnight. The cells were added with various concentrations of the plant extract and then the cells were incubated at 37°C, 5% CO_2 for 24 hours. Cell viability was analyzed by using MTT assay (Mosmann, 1983) and measured the absorbance at 570 nm. The triplicate experiments were performed and the results were calculated for % viability followed Eq.

$$\% \text{ viability} = A570_{treated} / A570_{control} \times 100$$

5.2 Mutagenicity effect evaluation

5.2.1 S-9 mixture preparation The rat liver S-9 fraction was used for the metabolic activation. The liver S-9 fraction was prepared from Sprague-dawley rats as described by Matsushima et al. (1976). The S-9 mixture (S-9 mix) contained 10%S-9, 4 mM NADPH, 4 mM NADH, 5 mM G-6-P, 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer, pH 7.4.

5.2.2 Determination of mutagenic activity

For mutagenesis assay, the modified pre-incubation bacterial mutation assay (Ames et al., 1973; Sripanidkulchai et al., 2001) was carried out in both the presence and absence of S-9 mixture in order to detect indirect and direct mutagenicity of the plant extracts. Two standard test strains, *Salmonella typhimurium* TA98 and TA100 were used. The extract was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mg/ml. The mixture of plant extract (0.01-0.1 ml) with 0.5 ml of S-9 mix or 0.1 M phosphate buffer (pH 7.4) and 0.1 ml of the test strain of bacteria was incubated at 37°C for 30 min. Then this mixture was rapidly mixed with 2 ml of molten top agar containing 0.1 μmol of histidine and biotin, and poured rapidly into a minimal glucose agar plate and incubated at 37°C for 48 hr. The background or negative control was included in each experiment by using DMSO alone instead of the plant extract. All tests were duplicated. The revertant colonies were counted, and the toxic effects were determined by viewing the background lawn under a stereo microscope. The results with revertant colonies at 2x background and dose-response manner of both TA98 and TA100 were reported to be positive mutagenicity.

6. Chemical component analysis

6.1 UV-visible spectrophotometer

The spectrum of light absorption in ethanolic extract of *C.tinctorius* (CTE) and hydroxysafflor yellow A was investigated using ultraviolet-visible spectrophotometer. The solution of the compound was prepared in water to achieve the final concentration of 50 μg /ml. After baseline adjustment; the sample contained in a quartz cell was scanned from wavelength of 200-800 nm.

6.2 HPLC analysis

To identify the chemical components of the extract, two HPLC analytical systems were performed on Agilent Technologies 1200 (AG1100 Autosampler, USA) equipped with isocratic pumping system, four channel in-line vacuum degasser, an autosampler injector and using a C18 column (5 µm, 4.6 x 250 mm). For the phenolic analysis condition, the gradient mobile phase consisted of solution A (acetonitrile: H_2O: phosphoric acid = 79.7:20:0.3) and solution B (0.3% phosphoric acid) at a flow rate of 0.8 ml/min. A concave gradient was applied to the ratio of solution A: solution B from 5:95 to 50:50 in 45 min (the gradient of mobile phase as showed in Table 3). A UV-vis detector at wavelength of 210 nm was used. The temperature of analytical room and analysis column was maintained at 25°C. For the hydroxysafflor yellow A (HSYA) analysis system, mobile phase was an isocratic phase of 10% aqueous acetonitrile at a flow rate of 0.8 ml/min and detection at 403 nm.

Table 3 Gradient elution of HPLC mobile phase system

Time	Solution A (%)	Solution B (%)
0	5	95
5	10	90
10	20	80
25	30	70
35	40	60
45	50	50

6.3 Determination of phenolics content and antioxidant activity

The total phenolics content was determined by the Folin-Ciocalteu method (Singleton et al., 1999). CTE (5 mg) was dissolved with methanol up to 1 mL, and then the CTE solution (0.5 mL) was mixed with 0.25 mL of the 1N Folin-Ciocalteau reagent and 1.25 mL of 20% sodium carbonate. After mixing and standing for 40 minutes at the room temperature, the optical density was measured at 725 nm. The total phenolic contents were expressed as mg tannic acid equivalent (TAE)/g dry basis.

The DPPH free radical-scavenging activity (Oh et al., 2008) of CTE was determined. 0.1 mM solution of ethanolic DPPH solution was prepared. The various

concentrations of CTE dissolved in 50% ethanol were added to 0.2 ml of ethanolic DPPH solution. The absorbance was measured at 515 nm after incubation for 15 min at room temperature. Measurements were performed in triplicate. The percentage of inhibition was calculated using:

$$\%\text{inhibition} = [(Abs_{control}-Abs_{sample})/Abs_{control}]*100$$

IC_{50} values calculated denote the concentration of plant extract required to decrease the absorbance at 515 nm by 50%. Vit.C and Vit.E were used as the reference antioxidants.

7. Preliminary stability test

CTE was kept in four conditions including -20°C, 4°C, room temperature (RT) and 45°C for 5 months. At various time points (0, 1, 2, 3 and 5 month), hydroxysafflor yellow A which is a chemical contents of the CTE was measured by HPLC method.

8. The study on hair growth promoting effect

8.1 *In vitro* study

8.1.1 Cell cultures

These cultured hair growth related-cells were used in this study:

1) Human keratinocytes (HaCaT) was purchased from CLS-cell lines service (Germany) and carefully grown and maintained in complete media containing Dulbecco's Modified Eagle's Medium (DMEM), 10%FBS, 2mM L-glutamine, 4.5 g/L glucose, 100 IU penicillin and 100µg streptomycin. The cells were cultured at 37°C, 5%CO_2.

2) Human follicle dermal papilla cells (DPCs) was obtained from PromoCell and cultured in Basal medium supplemented with SupplementMix which contains 0.04 ml/ml fetal calf serum, 0.004 ml/ml bovine pituitary extract, 1 ng/ml basic fibroblast growth factor and 5 µg/ml insulin. The cells were cultured at 37°C, 5%CO_2.

3) B16F10 murine melanoma cells (B16F10) was purchased from ATCC (JR scientific U.S.A.). B16F10 cells were cultured in DMEM containing 10%

heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured at 37°C, 5%CO_2.

8.1.2 Cell proliferation

The cells were seeded into 96-well plates ($1x10^4$ cells per well) and incubated at 37°C, 5% CO_2 for 24 h. The cells were added with various concentrations of the plant extract and then the cells were incubated at 37°C, 5% CO_2 for 24 h. The cell viability was determined by using MTT assay (Mosmann, 1983). Briefly, the treated cells were added with 50 µl of MTT (0.5 mg/ml) and incubated for 2 h at 37°C, 5% CO_2. The supernatant was then removed, and 150 µl of DMSO was added to dissolve formazan products. Absorbance was determined spectrophotometrically at 570 nm using micro-plate reader. The absorbance values were calculated and expressed as % proliferation.

8.1.3 The expression of hair growth-related gene

Semi-quantitative-reverse transcription-polymerase chain reaction (RT-PCR) was used to study the effect of CTE on hair growth promoting related genes including, VEGF and KGF and hair growth inhibiting related genes including TGF-β1, Type I-5α Reductase and Type II-5α Reductase.

DPCs or HaCaT were separately seeded at a density of 10^6 cells / well in 12-well tissue culture plate and incubated for overnight. The cells was treated with samples and incubated at 37°C in 5%CO_2 for 24 h. Then, the cells were harvested. Total RNA was isolated by RNA isolation kit according to the manufacturer's instructions and treated with DNase. RNA purity was confirmed with the spectrophotometric absorbance ratio at 260/280 and RNA quantity was determined by the absorbance at 260 nm. cDNA was synthesized from 40 ng of total RNA with two step RT-PCR kit using a thermal cycler. Specific oligonucleotides based on the published sequences as shown in Table 4 were used. cDNA was amplified by using thermo cycler machine as specific conditions (Table 5). There were 30 cycles of denaturation for VEGF, KGF and TGF-β1, 33 cycles for 5α-R, 27 cycles for β-actin, tyrosinase, TRP-1 and TRP-2. The PCR products were then analyzed on 2%agarose gel, visualized by Novel Juice staining and RT-PCR product density was measured by Gel Documentation and system analysis machine. The hair growth related genes expression was expressed as the relative mRNA expression level with β-actin.

Table 4 Nucleotide sequence of the primers and PCR product size

Cell Type	Gene	Primer	Nucleotide sequence	Size	Ref.
DPCs/ HaCaT	VEGF	Forward	CTACCTCCACCATGCCAAGT	536	1
		Reverse	GCGAGTCTGTGTTTTTGCAG		
	KGF7	Forward	GACATGGATCCTGCCAACTT	552	1
		Reverse	GGAAGAAAGTGGGCTGTTTTT		
	TGF-β1	Forward	GTGGAAACCCACAACGAAAT	579	1
		Reverse	CTACCTCCACCATGCCAAGT		
	Type1 5α-R	Forward	GGTTTTGGCTTGTGGTTAACA	142	2
		Reverse	CAAAATAGTTGGCTGCAGTTAC		
	Type2 5α-R	Forward	TACACAGACATACGGTTTAGC	127	2
		Reverse	CTTGTGGAATCCTGTAGCTGA		
	β-actin	Forward	GGCACCACACCTTCTACAATGAG	661	3
		Reverse	CGTCATACTCCTGCTTGCTGATC		
B16	Tyrosinase	Forward	ACAGAGGAGAACATCTGCCAGCTT	368	4
		Reverse	TGGTGACTCAACAGGTGTGAAGGT		
	TRP-1	Forward	ATACTGGGACCACATGGCAACACA	428	4
		Reverse	ATTGGTCCACCCTCAGTGCTGTTA		
	TRP-2	Forward	AGACTACGTGATCACCACGCAACA	366	4
		Reverse	TTCCGACTAATCAGCGTTGGGTCA		
	β-actin	Forward	TCATGAAGTGTGACGTTGACATCCGT	285	5
		Reverse	CCTAGAAGCATTTGCGGTGCACGATG		

1= Rho et al., 2005; 2= Iehle et al., 1999; 3= Lovkvist et al., 2008; 4= Sato et al., 2011; 5= Won et al., 2006

Table 5 PCR conditions

Cell	Gene	Denaturation / RT Inactivation	For Cycles			Final Extension
			Denaturation	Annealing	Extension	
DPCs/ HaCaT	VEGF	95˚C, 2 min	94˚C, 1 min	58˚C, 1 min	72˚C, 1 min	72 ˚C, 10 min
	KGF7	95˚C, 2 min	94˚C, 1 min	58˚C, 1 min	72˚C, 1 min	
	TGF-β1	95˚C, 2 min	94˚C, 1 min	58˚C, 1 min	72˚C, 1 min	
	Type1 5α-R	95˚C, 5 min	94˚C, 1 min	57˚C, 1 min	72˚C, 1.2 min	
	Type2 5α-R	95˚C, 5 min	94˚C, 1 min	57˚C, 1 min	72˚C, 1.2 min	
	B-actin	95˚C, 2 min	94˚C, 1 min	60˚C, 1 min	72˚C, 1 min	
B16	Tyrosinase	95˚C, 2 min	95˚C, 30 sec	60˚C, 30 sec	72˚C, 1 min	
	TRP-1	95˚C, 2 min	95˚C, 30 sec	60˚C, 30 sec	72˚C, 1 min	
	TRP-2	95˚C, 2 min	95˚C, 30 sec	60˚C, 30 sec	72˚C, 1 min	
	B-actin	95˚C, 2 min	94˚C, 1 min	60˚C, 1 min	72˚C, 1 min	

8.1.4 Hair growth activity

Neonate of ICR mice were obtained from Animal Laboratory, Faculty of Medicine (Khon Kaen University, Thailand). The mice were immediately terminated and proceed to the experiment. The research method was under supervision of the Animal Ethics Committee of Khon Kaen University (record number AEKKU 21/2554 and reference number 0514.1.12.2/38).

The hair follicles of neonates ICR mice were isolated and used in this study. The neonates were killed by cervical dislocation and the dorsal portion of the skin was dissected out and washed thoroughly in DMEM without FBS. The skin was cut into small pieces, approximately 0.5 cm^2 in size, and then an individual piece of

skin was placed in a petri dish containing DMEM. The skin piece was chopped thoroughly until the intact follicles come out from the skin. The separated intact hair follicles were isolated using a fine Pasteur pipette under binocular dissecting microscope. Individual, freshly isolated hair follicles were placed in 6-well plates containing 2 ml of DMEM medium supplemented with 10%FBS, 4 mM L-glutamine, 100µg/ml streptomycin, penicillin 10 units/ml and gentamycin 30 µg/ml. Finally, the test samples were added to the corresponding wells and the plates were maintained at 37°C in 5%CO_2. After incubation, the hair follicle length was measured using a binocular inverted microscope equipped with an eyepiece measuring graticule.

8.1.5 Melanin content

Melanin content measurement was developed from Sato et al. (2011). B16F10 melanoma cells were seeded in 12-well plate at a dentsity of $1x10^5$ cells per well and incubated for overnight. Then various concentrations of sample tests were added. After 48 h of incubation, the cells were collected with 0.25%trypsin and washed with DMEM media. After centrifugation, the cell pellets were solubilized in 100 µl of 1M NaOH at 60°C for 1 h. The melanin content was determined at the absorbance of 405 nm. Results were expressed as percent amount of intracellular melanin per viable cell number.

8.2 *In vivo* study

8.2.1 Sample preparation

The tested samples were dissolved in the vehicle which composed of propylene glycol: water, ethanol in a ratio of 5:3:2.

8.2.2 Animals

Two strains of animals including C57BL/6 mice and Sprague-dawley rats were used in this part. The experimental protocol was approved by the Animal Ethics Committee of Khon Kaen University (record number AEKKU 46/2554 and reference number 0514.1.12.2/65).

Five-week-old female C57BL/6 mice (18-20 g) were purchased from National Laboratory Animal Center (Mahidol University, Thailand). The mice were kept in cage and maintained on a standard laboratory diet and water *ad libitum*. They

were housed in automatically air-conditioned room (25±3°C and 65±5% humidity) with 12:12 h light and dark cycle for 7 days prior to experiment.

Seven-week-old female Sprague-dawley rats (280-310 g) were also obtained from National Laboratory Animal Center (Mahidol University, Thailand). The rats were maintained on a standard laboratory.

8.2.3 Experiment

8.2.3.1 C57BL/6 mice

C57BL/6 mice were divided into 2 experiments:

1. The mice were divided into 5 groups of each six animals (untreated, minoxidil and 3 groups of various concentrations of CTE) by one mouse was received one tested sample. About 2X3 cm^2 area of the dorsal portion of the animals, the hairs were shaved off with razor blade. All of the hair shaved-animal were synchronized in the telogen stage (Rho et al., 2005). The 100 µl of the tested solution was applied to the shaved area of the respective groups once a day and a control group was received no treatment. This treatment was continued for 15 days. Promotion of hair growth was evaluated by observing the skin color, which is indicative of the telogen-to-anagen conversion (Muller et al., 2001). The mice were killed at day 15. About 10 mm from the center of the treated skin were cut, fixed in 10%formalin buffer and then embedded in paraffin blocks to obtain both longitudinal and transverse sections into series uniform thickness of 10 µm. After that, the pieces of skin were stained with haematoxylin and eosin. From the histological characterization of the sections, the number of hair follicles per millimeter of the skin and the percentage ratio of hair follicle in anagen and telogen phases were determined using the microscope fitted with an ocular micrometer facility (Muller *et al*., 2001). Follicles were counted manually in dermis and subcutis layer by a blinded observer at a fixed size. Skin thickness from epidermis to panniculus carnosus was measured (Datta et al., 2009).

2. This experiment aimed to investigate the local effect of the tested samples thus one mouse was received two tested samples. The mice were divided into 6 groups of each 5 animals (untreated, minoxidil, vehicle (PG: water, ethanol was 5:3:2) and 3 concentrations of CTE). The hair about 1X3 cm^2 area of the

dorsal portion of both left and right sides of animals by leaving the hair line in the middle were shaved off with razor blade. The 80 µl of the tested solution was applied to the shaved-area with two different sample tests in the same animal. This treatment was continued for 15 days. Promotion of hair growth was evaluated by observing the skin color, which is indicative of the telogen-to-anagen conversion. The mice were killed at day 15. The 10 mm skin pieces were cut from the center of the treated area and fixed in 10% formalin buffer. The permanent slides of sample skin were prepared as in the first experiment. The number of hair follicles per millimeter square of the skin, the ratio of hair follicle in anagen and telogen phases and the skin thickness were determined.

8.2.3.2 Sprague-dawley rats

The rats were divided into 5 groups of each six animals (untreated, minoxidil and 3 groups of various concentrations of CTE) by one mouse was received one tested sample. About 3X3 cm^2 area of the dorsal portion of the animals, the hairs were shaved off. The 100 µl of the tested solution was applied to the shaved area of the respective groups once a day for 20 days.

9. Preliminary skin permeation study

9.1 Preparation of pig ear skin

Fresh porcine ears were obtained from a local market and cleaned with water to remove bloodstains. The epidermis was prepared by soaking the ear in water at 60°C for 45 second. The intact epidermis was subsequently teased off from dermis with forceps, washed with water and kept in the freezer at -20°C. Before using, the skin was measured the thickness by a vernier caliper and soaked in PBS (pH 7.4) for 30 minutes.

9.2 Preparation of the test sample

Two vehicle systems were used in this experiment including 1) water and 2) propylene glycol: water: ethanol (5:2:3). The CTE solutions were prepared by weighting an excess of crude extract dissolved in vehicles, mixing by vortex for 5 mins and then shaking in thermostatically controlled shaker water bath at 32°C and 100 rpm for 24 h until equilibrium was achieved.

9.3 Permeation experiment

The pig ear skins were mounted on the receptor compartment with the stratum corneum side facing towards the donor compartment in a side by side diffusion cell. The effective permeation area of the diffusion cell and receptor cell volume was 2.5 cm^2 and 3 ml, respectively. The temperature was maintained at 32°C (approximate skin temperature) with a continuous stirring of magnetic bars during the entire experiment. The saturated CTE solution was added into the donor phase whereas the receptor phase was PBS as in Figure 19. Then, a portion (1ml) of receptor solution was collected in each time points for analysis and replaced with the same volume of fresh solution to maintain sink conditions. The samples were analyzed by HPLC.

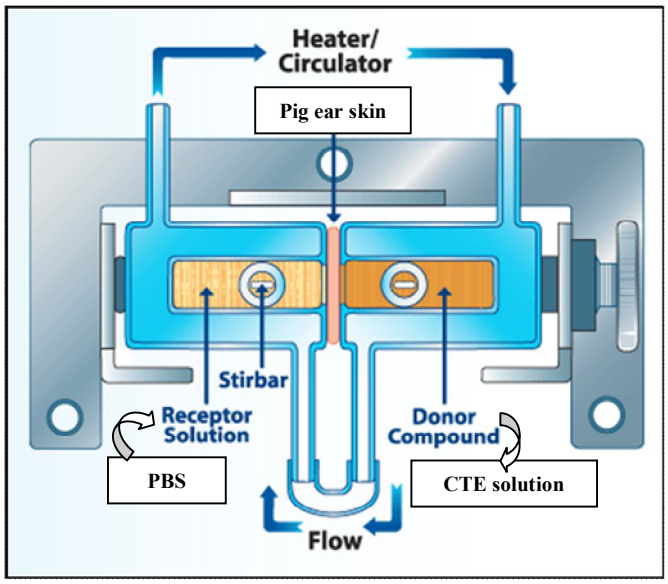

Figure 19 Side by side diffusion cell (http://www.permegear.com/sbs.htm)

10. Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean $\pm$ S.D. One-Way ANOVA and multiple comparisons were used to analyze the significant different ($P < 0.05$) by using SPSS version 19.0 software.