CHAPTER V
DISCUSSION

Based on the traditional uses in hair treatment products, *Carthamus tinctorius* was one of 15 selected plants in this study. The part used of these 15 plants was extracted with 50% ethanol which is a moderate polarity solvent. The plant extracts were screened on the viability of dermal papilla cells (DPCs) and keratinocytes (HaCaT). DPCs and HaCaT were used as cell models in this study because they play roles in the growth of hair (Schlabe et al., 2008). Dermal papilla cell is a major component of hair. Many growth factors produced by DPCs were found to have the function of modulating the proliferation of follicular epithelium and acted as a cytokine network controlling follicle development and hair cycling (Rushan et al., 2007). Keratinocyte is one of the cells that composed in the hair follicle and hair shaft (Yoo et al., 2009). Major protein of hair shaft called keratin is produced from keratinocyte. The cross-talks between dermal papilla cells and keratinocyte is thought to be key for successful reconstitution of hair follicles (Fujie et al., 2001). After the screening test, the results revealed that *C.tinctorius* floret extract (CTE) showed low toxicity on DPCs and HaCaT. CTE also showed high potency on hair growth promotion by increasing the proliferation of DPCs and HaCaT with proliferative value at 166.02±4.89% and 114.83±6.83%, respectively.

To find the best fraction for hair growth promotion of *C.tinctorius*, 4 different systems were used to extract this plant including fraction A (boiled water), fraction B (macerated with water), fraction C (macerated with 95%methanol) and fraction D (macerated with 50% ethanol). Fraction A showed the highest extraction yield (29.28%) whereas the extraction yield of fraction B, fraction C and fraction D was 16.30%, 16.15% and 28.30%, respectively. However, fraction D (CTE) was selected for further study due to its highest proliferative effect on DPCs and low toxicity on HaCaT whereas other fractions exhibited high toxicity on HaCaT.

The safety of the extract is an important issue that we have concerned. The safety assessment of pharmaceutical agents is a specialized process. In general, an evaluation of the safety of pharmaceutical agents can be divided into two phases: the

preclinical or non-clinical phase and the clinical phase. Bacterial and cell culture models are non-clinical phase that could answer the safety of agents before further study in animal or human. Since, there are very few scientific reports available on safety of *C.tinctorius* extract, thus, in this study, the toxicity of CTE was examined on the peripheral white blood cell and bacterial mutagenicity test. White blood cells are immune system cells involved in defending the body against both infectious disease and foreign materials. The abnormal white blood cells directly related to the diseases that affect to the body. For the mutagenicity test, this test serves as a quick and convenient assay to estimate the carcinogenic potential of a compound. The results showed that CTE at doses of 0.25-1.00 mg/ml with 24 h of incubation had no toxic on white blood cells and had no mutagenicity. The mutagenicity results confirmed the previous study of Sripanidkulchai et al. (2001). Therefore, CTE is a safe herbal extract.

DPCs and HaCaT were further used in the evaluation of CTE on mRNA level using RT-PCR. CTE at 0.25-1 mg/ml induced the expression of VEGF mRNA in DPCs and HaCaT. From these results, it can be noted that CTE promoted hair growth by stimulating the expression of VEGF mRNA as we know that VEGF is involved in the regulation of hair morphogenesis and hair growth. It plays important roles in angiogenesis, as well as in a number of inflammatory and neoplastic diseases that are associated with neovascularization (Witner et al., 2003). The vascular network is associated with the hair growth cycle. The perifollicular capillary network is coupled to the hair cycle, increasing during the anagen phase and then regressing during the catagen and telogen phase (Lee et al., 2010). The importance of VEGF in hair growth stimulation has been further supported by the study on the activity of minoxidil. Minoxidil has been used extensively over the last 30 years to treat androgenic alopecia (AGA). Several reports demonstrated the mechanism of action of minoxidil to stimulate the hair growth via the expression of VEGF (Rho et al., 2005; Han et al., 2004). In this study, it was found that CTE at 1 and 0.5 mg/ml showed the induction of VEGF expression higher than that of 0.5 mg/ml of minoxidil. These results were observed in both DPC and HaCaT. Moreover, CTE also stimulated the expression of KGF. It has been reported that KGF was one of the growth factor that stimulates hair fiber elongation and protected hair follicles from cell death (Braun et al., 2006). The

regulatory role of KGF in hair growth was well implicated in transgenic mice model in which KGF directly affected the development of hair follicles; therefore, KGF in dermal papilla is a fundamental factor of hair growth process (Roh et al., 2002). It is likely that, VEGF and KGF may closs-talk and sending signal to each other as we know that the auto- and cross-induction mechanisms of growth factor are very effective in the stimulation of keratinocyte proliferation and the growth of hair (Hashimoto, 2000).

Besides the growth factors, there are other factors which involved in the growth and loss of hair. As we know that AGA is the most common type of hair loss which affects both men and women (Sinclair, 2004). AGA is caused by the increase in the activity of steroid 5α-reductase enzyme in hair follicles (Ornelas et al., 2005). Changing of testosterone to dihidrotestoseterone (DHT) which is considered to be more potent in triggering hair loss by the action of 5α-reductase (Rho et al., 2005; Faragalla et al., 2003). Thus, 5α-reductase enzyme is considered to be one of the most important targets for developing drugs for treatment of hair loss. In this study, CTE suppressed the RNA expression of both type I and type II 5α-reductase enzyme in DPCs and HaCaT. These results supported the report of Kumar et al. (2012), which demonstrated the inhibitory effect of ethanolic extract of *C.tinctorius* on the activity of 5α-reductase enzyme by using *in vitro* enzymatic test. CTE may be potential as anti-hair loss *via* the inhibition of 5α-reductase enzyme. At present, there are some medicines used to treat AGA such as finasteride and dutasteride. Finasteride is a competitive inhibitor of type II 5 α-reductase while dutasteride is a dual inhibitor of both types I and type II 5α-reductase enzymes. Dutasteride is approximately 3 times as potent as finasterides at inhibiting type II 5α-reductase and more than 100 times as potent at inhibiting the type I enzyme (Olsen et al., 2006). CTE also suppressed the expression of TGF-β1 which is one of hair loss stimulating factors. TGF-β1 is a part of cytokine super-family in mammals and regulates the apoptosis in many cell types. TGF-β1 has a central role in the inflammation that is associated with hair follicle miniaturization, fibrosis and its eventual loss. DHT stimulates the expression of TGF-β1 in dermal papilla cells resulting in the epithelial cell growth inhibition (Rho et al., 2005).

In addition, CTE significantly increased mouse neonate hair follicle length within 72 h of cultivation. The follicle length of CTE-treated group was significantly higher than that of the control group. However, after 72 h incubation, the rate of increase in hair length was reduced and subsequently ended due to the follicles enter into catagen phase. Despite the length of hair follicle increased in culture, the rate of hair growth is markedly less than *in vivo*. Several reports tried to develop and culture hair follicles from rodents and also from human. It has been showed that the hair follicles could be cultured *in vitro* cultured approximately 9 days for human and 48 h for rat (Adhirajan et al., 2003). In this study, the increasing in hair follicle length in the first 72 h may be associated with the production of a keratinized hair shaft after that the rate of hair growth is markedly decreased. Since the culture conditions could not completely mimic the complicated *in vivo* environment. Furthermore, when dermal papilla cells are cultured *in vitro*, they gradually lose their innate hair follicle-inducing ability after about 3-4 passages, resulted in the decreasing of the length of cultured hair follicles (Reynolds and Jahoda, 1996). However, this method is very useful *in vitro* for testing of hair growth promoting activity and it is the important model of hair follicle multiplication for hair transplantation (Yoo et al., 2010a; Qiao et al., 2008).

In vivo study, CTE at doses of 0.1-2.0 mg/ml stimulated the re-growth of hair both in C57BL/6 mice and Sprague-dawley rats. Since we know that, the active phase of hair follicle cycling is accompanied by increase in size and number of follicles resulting in increase in thickness of subcutis layer between dermis and panniculus carnosus. Numerous studies on morphological changes in the follicle showed that the hair bulb extends into the deep subcutis during the anagen and catagen (Datta et al., 2009). The histological study proved that there was the difference in the number of hair follicles, the anagen phase and the skin thickness between the CTE-treated and control groups. Uno (1991) reported the quantitative evaluation of hair growth potential of minoxidil in macaque monkey and fuzzy rat by determining the percentage transformation of hair follicles from telogen to anagen. The study revealed that the topical application of minoxidil produced the conversion of telogen into the anagen follicles. CTE showed the similar effect on hair related-growth factors as minoxidil did. It may act by induction the proliferation of epithelial cells near the base

of the hair follicle and may induce the vasodilation of scalp blood vessels (Savin and Atton, 1993).

Various species of animals such as mice, rats, sheeps and monkeys have been used, and the mouse model is most widely reported for hair growth promotion studies. The black hair C57BL/6 mice used in this study are a suitable *in vivo* model for evaluation of the hair growth promoting effect. Since C57BL/6 mice had no melanocytes on the skin, the melanogenesis in this mouse performs only in the hair follicles. Melanogenesis of C57BL/6 mice are strongly related to the growth stage of the hair cycle (Slominski et al., 1994). Therefore, the blackening of the hair-shaved dorsal area indicated that the tested sample could encourage the anagen phase of hair growth cycle in this mice. In our study, CTE showed the hair growth promoting effect both in pigmented mice and albino rats. The animals were killed at day 15 and day 20 for mice and rats, respectively because the increasing of the hair growth rate in CTE-treated group almost reaches to the maximum rate. In this period of time, the difference of hair growth between control and test samples treated group can be observed.

Moreover, CTE showed also a good candidate for gray hair treatment. Our results exhibited that CTE had the ability to stimulate melanogenesis in B16F10 cells in a dose-dependent manner. The darken B16F10 cells was observed after 48 h incubation with CTE. CTE at doses of 6.25, 25 and 100 μg/ml increased melanin content by 8.67, 19.46 and 30.57%, respectively. Moreover, CTE at a dose of 25 μg/ml increased tyrosinase level higher than that of α-MSH (3.13 μg/ml). The expressions of TRP-1 and TRP-2 mRNAs were similarly increased after treatment with CTE. Some compounds containing in CTE may play role as previously observed that quercetin had shown potent melanogenic activities in B16F10 murine melanoma cells (Nagata et al., 2004). It could be noted that CTE could promote hair growth due to its potent melanogenesis stimulating effect since we know that melanogenesis of follicular melanocytes is the process that strictly couple to the growth stage of the hair cycle and switch off at the earliest of catagen, and absent throughout telogen (Slominski et al., 1994).

More than 200 compounds were isolated from *C.tinctorius* including flavonoids, phenylethanoid glycosides, coumarins, fatty acids, steroids and safflower

polysaccharides (Jiang et al., 2008; Sagiroglu et al., 2009). In this study, the results from the spectrum of light absorption at 403 nm and HPLC analysis revealed that hydroxysafflor yellow A (HSYA) was the major compound of CTE which confirmed the study of Fan et al., (2009). Although, CTE contained several phenolics compounds such as quercetin, *p*-coumaric acid and vanillin, but they are present in low amount (total phenolic contents was 7.89%) and commonly found in other plants. Therefore, HSYA was used as a chemical marker for the quality control of CTE. However, the hair growth promoting effect of CTE comes from either HSYA or its synergistic interaction with other phytochemicals needs to be further investigated.

There may be many large molecules contained in CTE such as HSYA which was 612.534 daltons. Therefore, the preliminary skin permeability of CTE was performed using the side by side diffusion cell method. The vehicle (propylene glycol: ethanol: water, 5:2:3) used in this study was an appropriate for skin permeation enhancement. Although CTE showed high solubility in water, their skin permeability was rather low in water. The result from skin permeation study of CTE showed that the vehicle containing propylene glycol and ethanol promoted higher flux value and shorter lag time than that of the water. Moreover, this vehicle had no toxicity or any irritation to the skin throughout the animal study. In addition, these enhancers were used in the minoxidil formulation. PG is commonly used as an enhancer in topical drug formulations, it can enhance partitioning and solubility in stratum corneum and can solvate the keratin of stratum corneum (Ren et al., 2009). EtOH acts as a penetration enhancer by increasing the permeation of hydrophilic compounds and the number of free sulphydryl groups of keratin in the stratum corneum proteins (Sinha and Kaur, 2000).

HSYA also was measured in the preliminary stability study. Stability study of CTE revealed that CTE was more stable at the cool place than at room or higher temperature. Hydroxysafflor yellow A content of CTE was gradual decreased at -20°C and 4°C but it is dramatically lost at 45°C.

These findings suggest that CTE is a safe extract which could promote the growth of hair both *in vitro* and *in vivo*. Therefore, CTE should be encouraged as the ingredient in hair growth promoting products. Moreover, the preliminary of stability

and permeability studied of CTE provided the basic knowledge of this extract for further drug development.