CHAPTER IV
RESULTS

1. Effect of the plant extracts on the viability of dermal papilla cells (DPCs) and keratinocytes (HaCaT)

Fifteen plants traditionally used for hair growth promoting treatment were selected (Table 6 and Figure 20). After the extraction of these plants with 50% ethanol, the crude extracts were obtained with %yield of 0.7-34.4 (Figure 21)

After 24 h of incubation with various concentrations of the extracts (0.03-5.00 mg/ml), six extracts stimulated the proliferation of DPCs (Kham foi, Thong Phan Chang, Kaphao, Makrut, Anchan and Buabok) and five extracts stimulated the proliferation of HaCaT (Kham foi, Kaphrao, Thong Phan Chang, Malako and Phlu) (Table 7). However, the ethanolic extract of the floret of *C. tinctorius* L. (CTE) showed the highest hair growth promoting activity with proliferation of 166.02 ± 4.89% and 114.83 ± 6.83% on DPCs and HaCaT, respectively. Moreover, the yield of the CTE also was high.

In addition, to find the most effective fraction of *C.tinctorius* on the hair growth promotion, 4 different methods of extraction were prepared and tested on the viability of DPCs and HaCaT. The results showed that 50% ethanolic fraction with 28.30% yield have the highest potential on the proliferation of DPCs and low toxicity on HaCaT (Table 8). Therefore, the 50% ethanolic fraction of *C.tinctorius* or CTE was selected for further hair growth promoting study.

Table 6 Fifteen selected plants for hair growth promoting study

Thai name*	English name*	Scientific name*	Family*	Part used
1. Anchan	Butterfly pea	*Clitoria ternatea* L.	Papilionoideae	Flower
2. Boraphet	Boraphet	*Tinospora crispa* (L.) Miers ex Hook.f. & Thomson	Menispermaceae	Vine
3. Bua bok	Asiatic pennywort	*Centella asiatica* (L.) Urb.	Umbelliferae	Leaf and Stem
4. Cha om	Cha om	*Acacia pennata* (L.) Willd. subsp. *insuavis* (Lace) I.C.Nielsen	Mimosoideae	Leaf
5. Ka phrao	Holy basil	*Ocimun tenuiflorum* L.	Labiatae	Leaf
6. Kham foi	Safflower	*Carthamus tinctorius* L.	Compositae	Florets
7. Kluai hom	Banana	*Musa sapientum* L.	Musaceae	Fruit
8. Krachiap	Roselle	*Hibiscus sabdariffa* L.	Malvaceae	Flower
9. Makrut	Leech lime	*Citrus hystrix* DC.	Rutaceae	Fruit skin
10. Malako	Papaya	*Carica papaya* L.	Caricaceae	Fruit (ripe)
11. Maphrao	Coconut	*Cocos nucifera* L.	Palmae	Fresh fruit meat
12. Phlu	Betel pepper	*Piper betle* L.	Piperaceae	Leaf
13. Som poi	Som poi	*Acacia concinna* (Willd.) DC.	Minosoideae	Fruit
14. Thai hom mali rice	Rice	*Oryza sativa* L.	Gramineae	Grain
15. Thong phan chang	Thong phan chang	*Rhinacanthus nasutus* (L.) Kurz	Acanthaceae	Leaf and Stem

* (Smitinand, 2001)

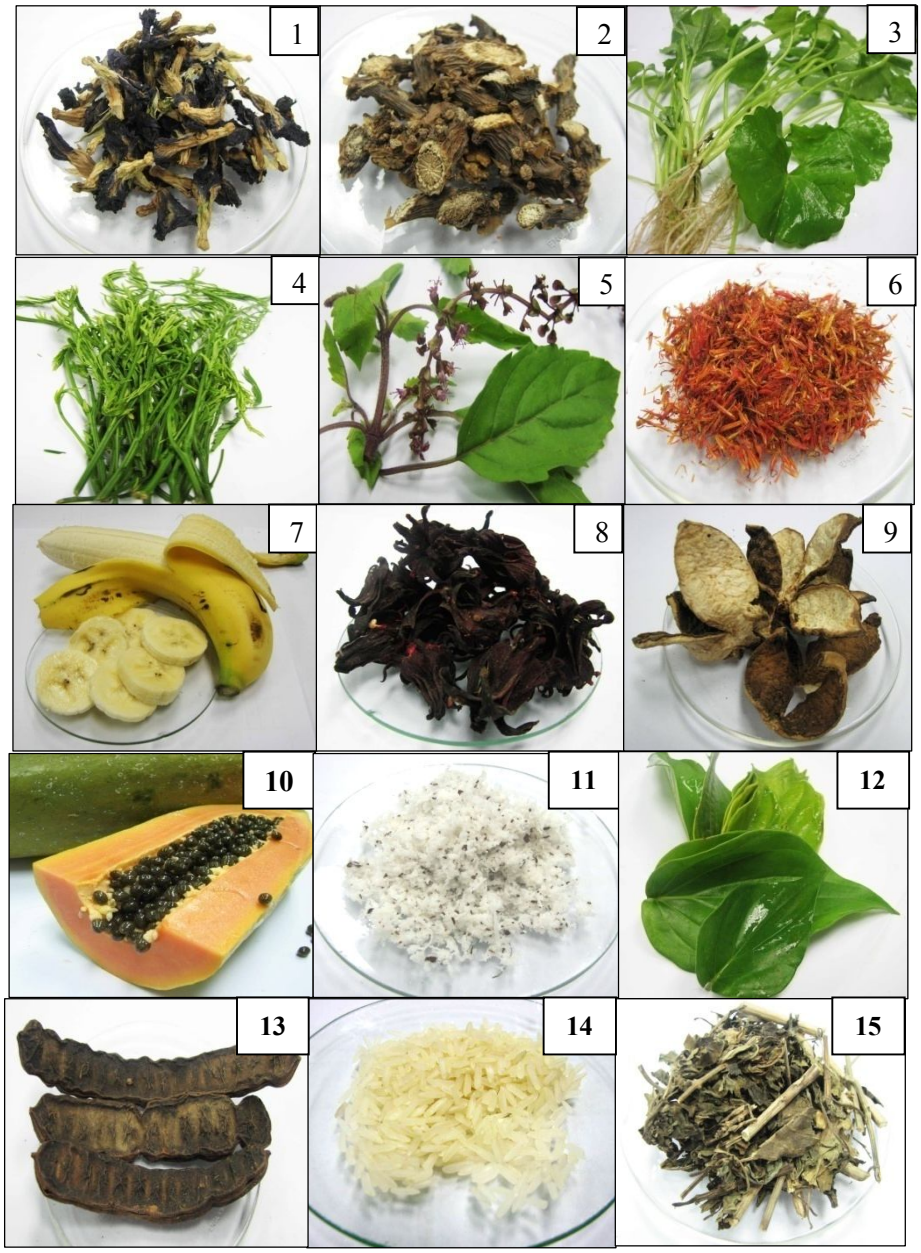

Figure 20 Fifteen plant materials (1=Anchan, 2=Boraphet, 3=Bua bok, 4=Cha om, 5=Kaphrao, 6=Kham foi, 7=Kluai hom, 8=Krachiap, 9=Makrut, 10=Malako, 11=Maphrao, 12=Phlu, 13=Som poi, 14=Thai hom mali, 15=Thong phan chang)

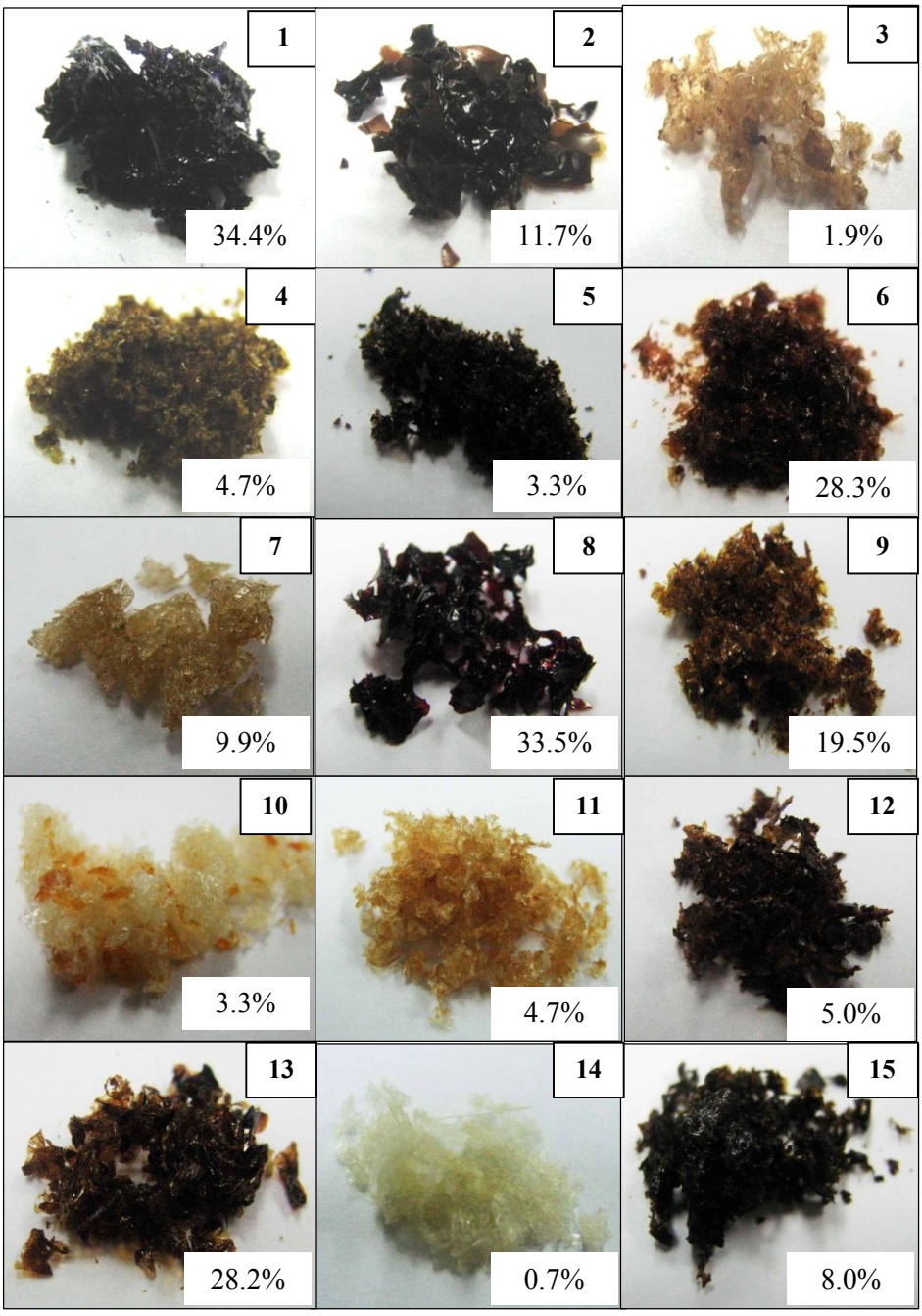

Figure 21 Crude ethanolic extracts and % yield of 15 plants (1=Anchan, 2=Boraphet, 3=Bua bok, 4=Cha om, 5=Kaphrao, 6=Kham foi, 7=Kluai hom, 8=Krachiap, 9=Makrut, 10=Malako, 11=Maphrao, 12=Phlu, 13=Som poi, 14=Thai hom mali, 15=Thong phan chang)

Table 7 The highest cell viability and 50% toxicity dose (TC_{50}) when treated with 0.03-5.00 mg/ml of plant extracts on DPCs and HaCaT

Plant extract	DPCs		HaCaT	
	Cell viability (%)*	**TC_{50} (mg/ml)***	**Cell viability (%)***	**TC_{50} (mg/ml)***
1. Anchan	106.88 ± 5.87	2.62±0.15	97.45 ± 9.54	3.55 ± 0.11
2. Boraphet	78.59 ± 3.45	1.25±0.09	85.93 ± 8.24	2.53 ± 0.10
3. Bua bok	102.03 ± 6.29	2.35±0.23	98.72 ± 6.55	4.16 ± 0.15
4. Cha om	87.49 ± 9.45	1.20±0.11	94.80 ± 8.19	1.27 ± 0.10
5. Kaphrao	119.10 ± 4.58	0.49±0.02	113.59 ± 3.59	0.18 ± 0.03
6. Kham foi	166.02 ± 4.89	3.88±0.63	114.83 ± 6.83	2.19 ± 0.11
7. Kluai hom	90.56 ± 4.56	0.45±0.06	95.93 ± 9.93	0.41 ± 0.05
8. Krachiap	75.26 ± 3.11	1.08±0.09	96.64 ± 0.23	1.33 ± 0.09
9. Makrut	116.50 ± 4.65	1.66±0.22	105.82 ± 5.82	2.16 ± 0.09
10. Malako	78.49 ± 3.23	0.52±0.07	74.56 ± 0.51	0.18 ± 0.07
11. Maphrao	100.02 ± 8.47	0.51±0.03	89.05 ± 0.36	0.33 ± 0.01
12. Phlu	90.47 ± 1.29	0.67±0.05	102.20 ± 5.03	0.86 ± 0.10
13. Thai hom mali	100.12 ± 6.98	1.06±0.09	98.05 ± 0.79	1.91 ± 0.10
14. Thong phan chang	130.56 ± 3.67	1.80±0.12	112.48 ± 6.08	2.76 ± 0.09
15. Som poi	98.76 ± 3.46	0.54±0.06	79.36 ± 8.27	0.25 ± 0.01

* Results expressed as mean±SD, n=4

Table 8 % yield and effect of *C.tinctorius* fractions on viability of DPCs and HaCaT

Extraction method/ Solvent	Yield (%)	Cell viability of DPCs (%)*	Toxicity on HaCaT (TC_{50}, mg/ml)*
Boil / water	29.28	122.33±7.20	1.92±0.19
Maceration / water	16.30	120.15±6.21	2.22±0.21
Maceration / 95% Methanol	16.15	112.68±5.68	1.04±0.10
Maceration / 50% ethanol (CTE)	28.30	166.02±8.20	2.14±0.11

* Results expressed as mean±SD, n=4

2. Safety evaluation

From the screening test, CTE was selected for further study due to its strongest potency on the proliferation of DPCs and HaCaT. However, the safety evaluation of CTE has been performed, by checking for the toxicity on peripheral white blood cell and the mutagenicity in *Salmonella typhimurium*.

2.1 Effect of CTE on white blood cells

After 24 h of incubation, CTE at the doses of 0.25-1 mg/ml showed insignificantly toxic effect on human white blood cells as shown by the percent cell viability in Figure 22.

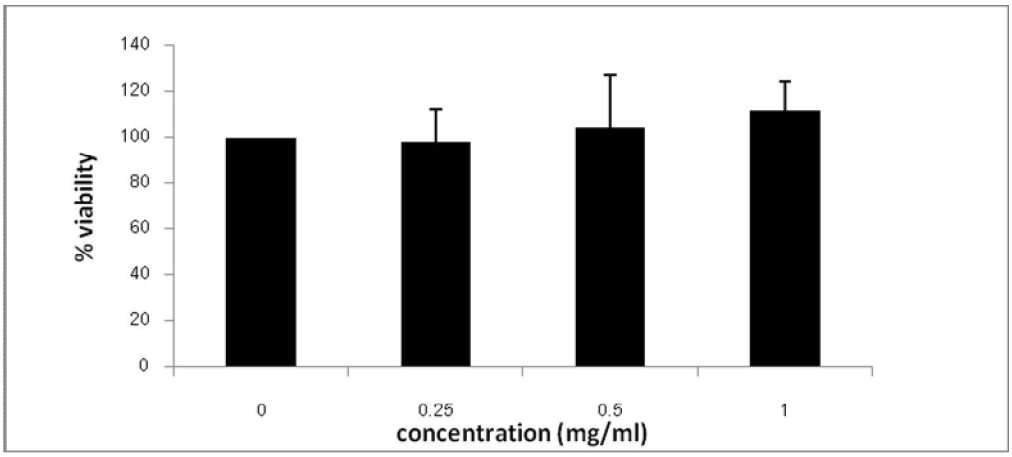

Figure 22 Cytotoxic effect of CTE on white blood cell (mean±SD, n=3)

2.2 Mutagenicity evaluation

With the criteria that the extract possesses positive mutagenicity must show the number of revertant colonies at 2 times of the background with dose-dependant manner in both TA98 and TA100. The result demonstrated that CTE at concentrations of 1-10 mg/plate had no mutagenicity against *Salmonella typhimurium* TA98 and TA100 in the presence and absence of enzymatic activation. At the low and high doses of CTE, the number of revertant colonies of both TA98 and TA100 did not increase (Figure 23).

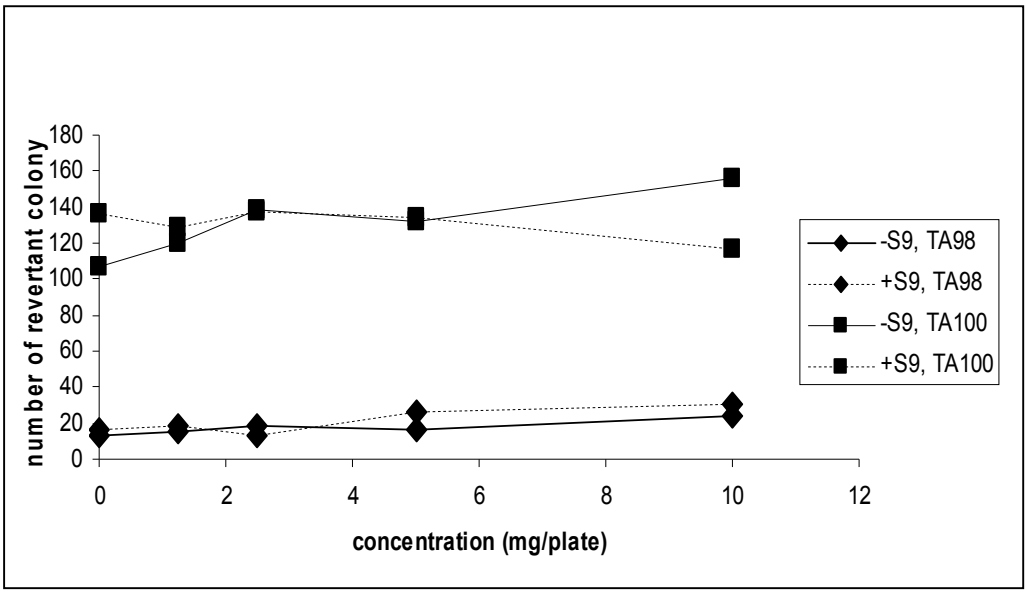

Figure 23 Effect of CTE on *Salmonella typhimurium*

3. Chemical component analysis

By scanning at the wavelength of 200-800 nm, the spectrum of hydroxysafflor yellow A and CTE were similar with λ_{max} at 413 nm (Figure 24). The result suggests that hydroxysafflor yellow A is the major compound of CTE.

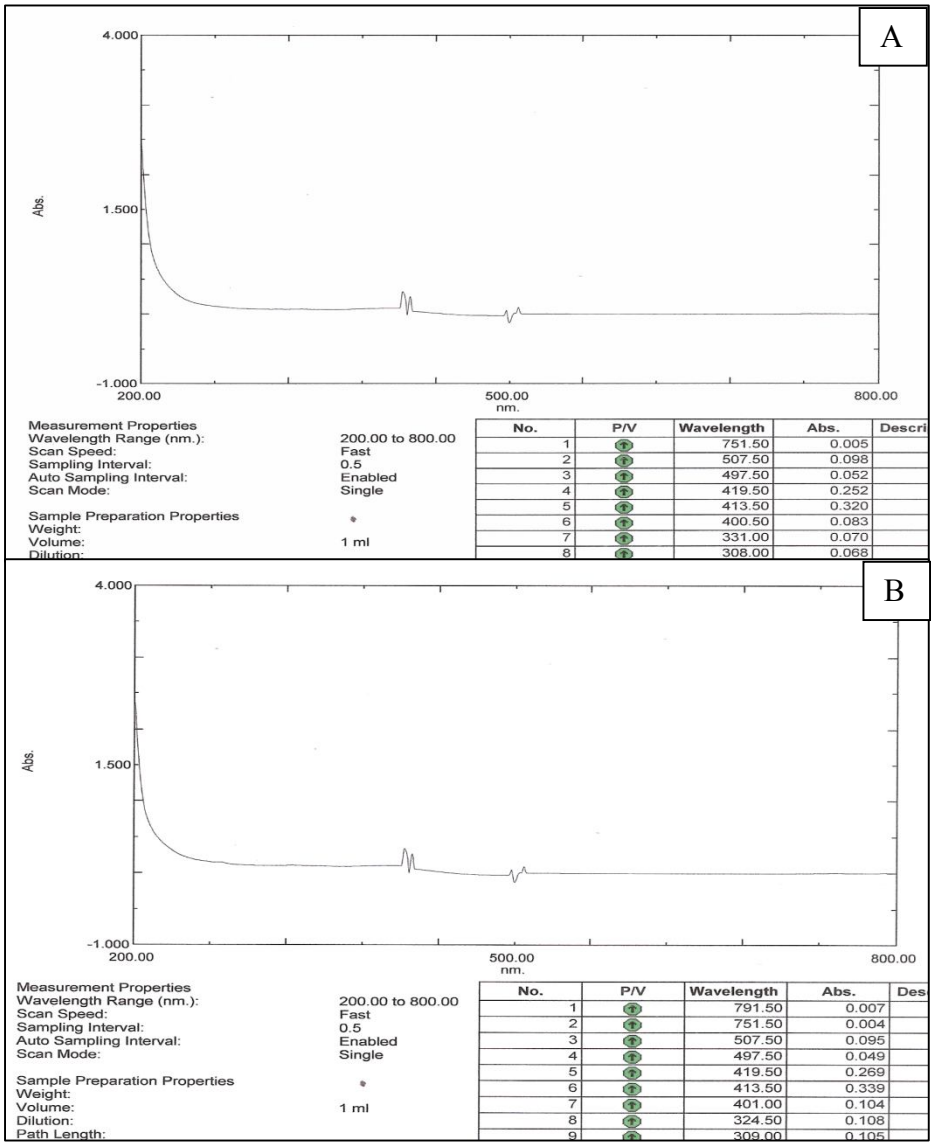

Figure 24 UV-vis spectrum of hydroxysafflor yellow A (A) and CTE (B)

HPLC technique was used to identify the chemical components of CTE with two mobile phase systems. The first system is for analysis of phenolic compounds, using 2 standard mixtures, i.e., standard mixture 1 containing gallic acid, chlorogenic acid, vanillin, epicatechin gallate and quercetin; and standard mixture 2 containing caffeic acid, *p*-coumaric acid and ferulic acid (Figure 25).

The second system is for analysis of hydroxysafflor yellow A (HSYA). By comparing the retention time, it is suggested that CTE contained quercetin, *p*-coumaric acid, vanillin, HSYA and several un-identified peaks (Figure 26). Based on the distinct and clear peak, HSYA was determined to be the major compound of CTE. As calculated from the standard curve, hydroxysafflor yellow A content was highest in the boiled water fraction, followed by the macerated 50% ethanolic, water and methanol fractions (Table 9). There were also 3-7 un-identified peaks in these fractions. The structures of four identified compounds in CTE were showed in Figure 27.

DPPH, a stable free radical with a characteristic absorption at 515 nm, was used to study the radical scavenging effects of CTE. The CTE exhibited slight anti-oxidative activity with IC_{50} of DPPH at 365.62 ± 17.80 µg/ml. The total phenolic contents from Folin-Ciocalteu method which compared from standard curve was 78.92±2.75 mg/g crude (Table 10).

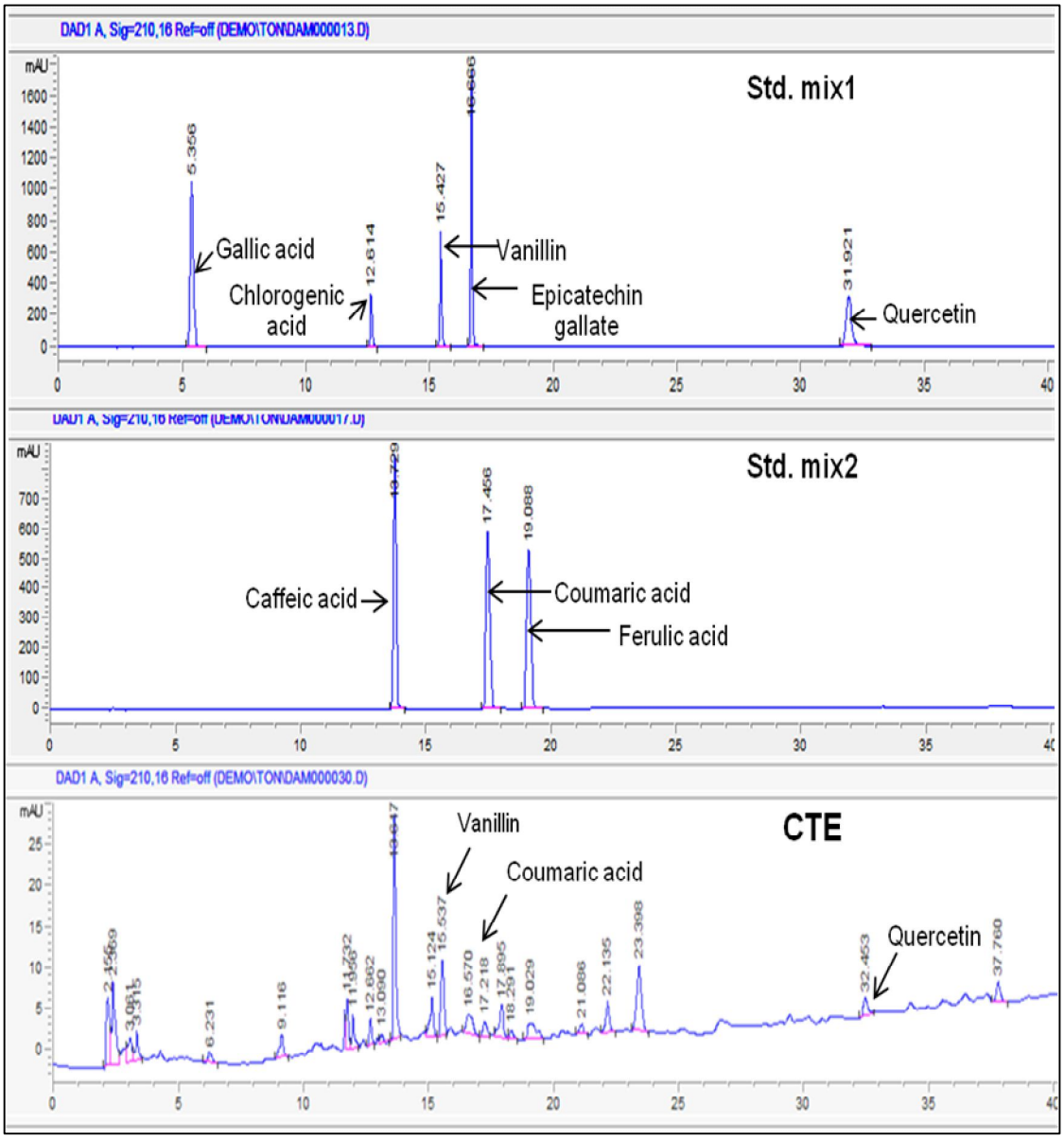

Figure 25 HPLC profiles of standard mixture (A-B) and CTE (C)

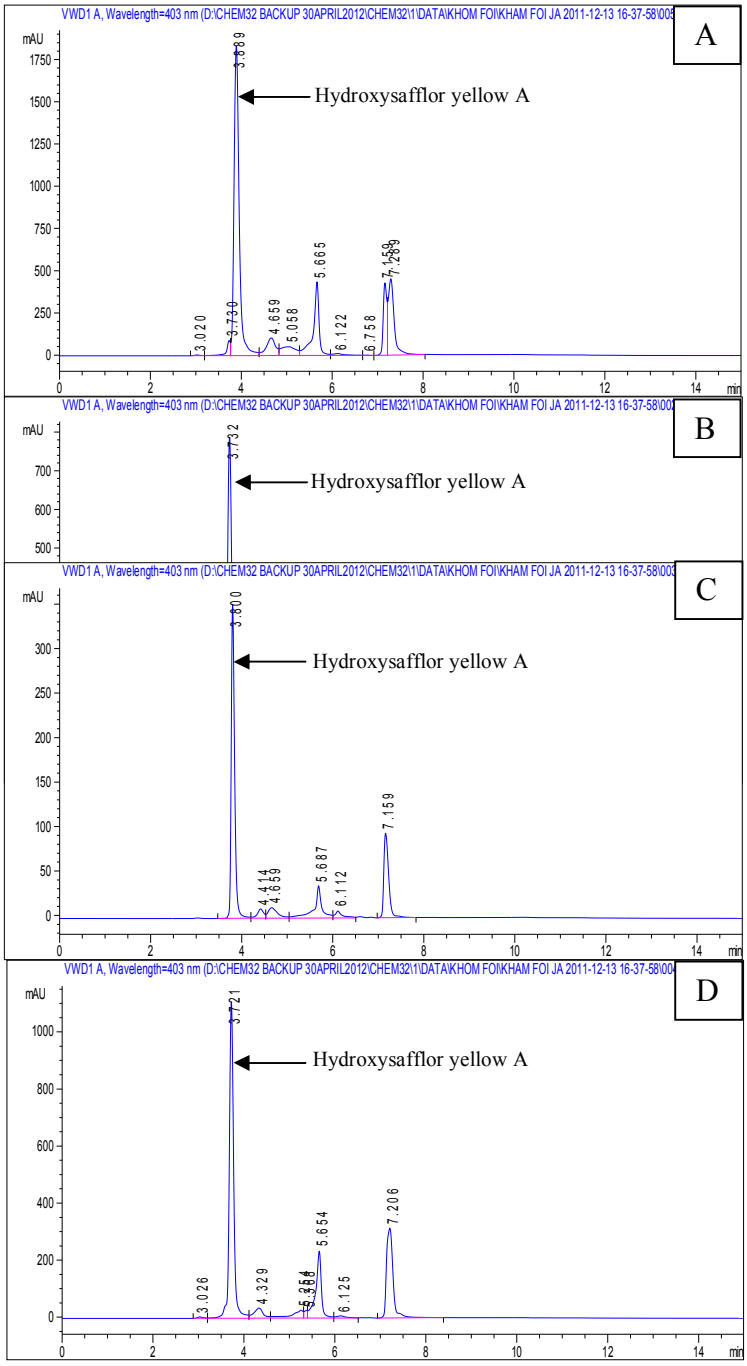

Figure 26 HPLC profile of *C.tinctorius* (2 mg/ml) (A= Boil / water, B= Maceration / water, C= Maceration / 95% Methanol, D= Maceration / 50% ethanol)

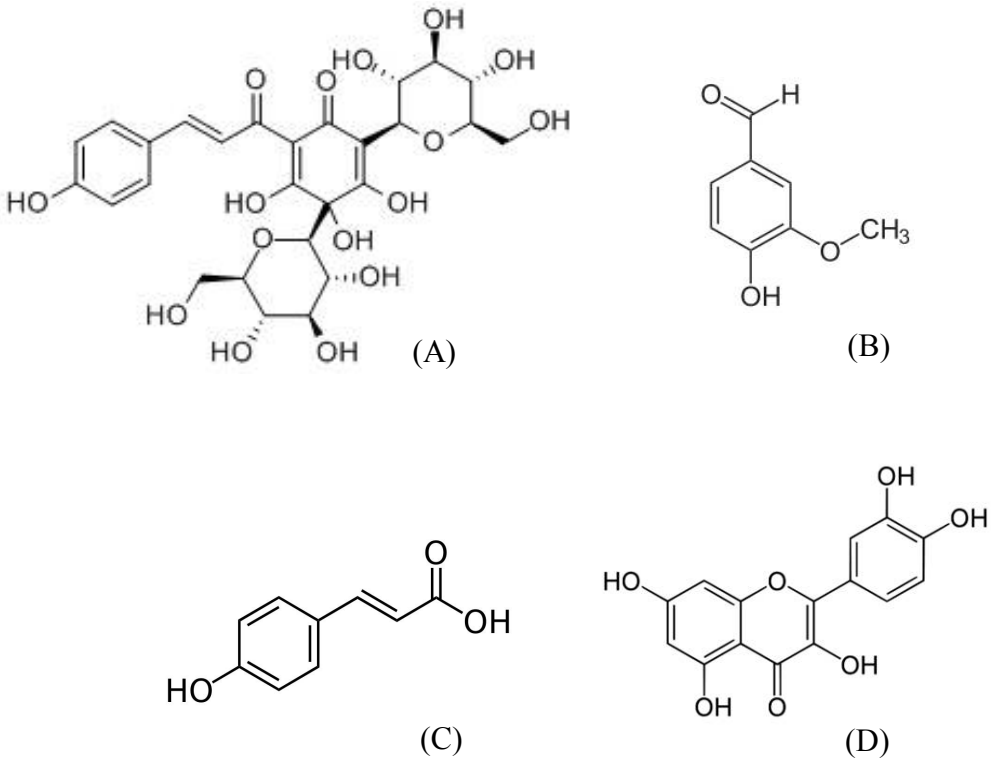

Figure 27 The structures of four identified compounds in CTE (A = HSYA, B = vanillin, C = *p*-coumaric acid, D = quercetin)

Table 9 Hydroxysafflor yellow A content in four fractions of *C.tinctorius*

Extraction method/ Solvent	**Hydroxysafflor yellow A content (mg/g crude)**
Boil / water	245.82±14.28
Maceration / water	124.44±22.22
Maceration / 95% Methanol	4.78±2.10
Maceration / 50% ethanol	212.00±17.56

* Results expressed as mean±SD, n=3

Table 10 The total phenolic compounds and anti-oxidative activity of CTE

Sample	Phenolics contents (mg eq. tannic acid/g)*	Anti-oxidative activity, IC_{50} (µg/ml)*
CTE	78.92 ± 2.75	369.62 ± 17.80
Ascobic acid, Vit.C	-	3.21± 0.12
α-tocopherol, Vit.E	-	6.51± 0.15

* Results expressed as mean±SD, n=3

4. Preliminary stability test of CTE

The CTE was kept in 4 conditions including -2°C, 4°C, room temperature (RT) and 45°C for 5 months. The result exhibited that within 5 months of storage, there are gradual changes in the hydroxysafflor yellow A content of CTE at -20°C, 4°C and RT. But at 45°C storage, there are dramatically lost of hydroxysafflor yellow A content (Figure 28). Moreover after 5 months storage, the physical appearance of CTE kept at RT and 45°C changed from powder to semi-solid form as shown in Figure 29.

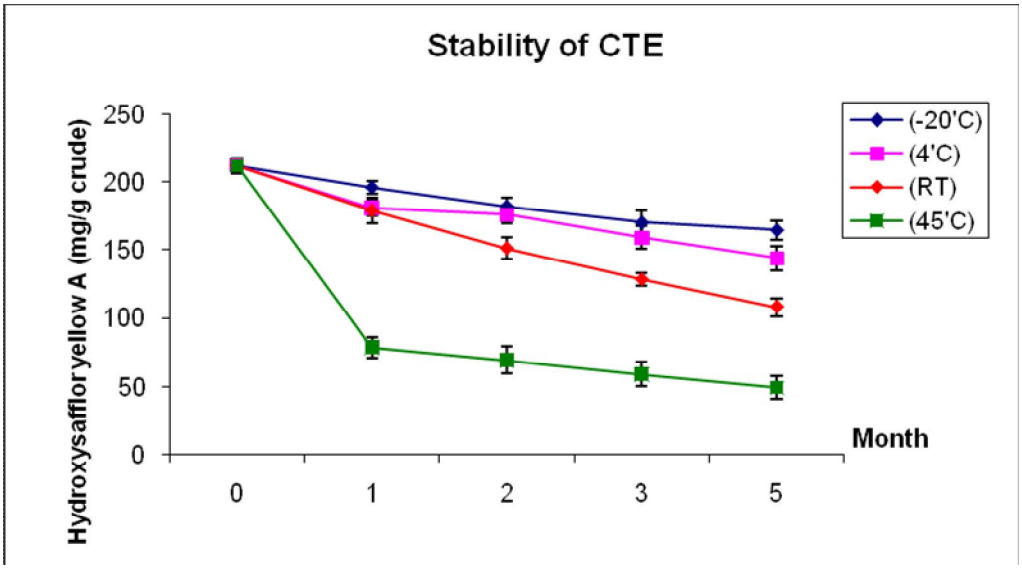

Figure 28 The stability of CTE

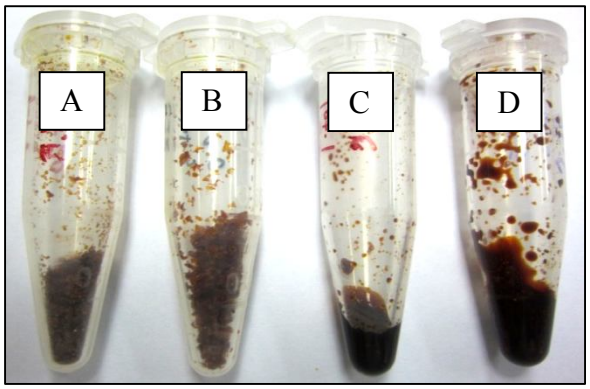

Figure 29 The physical appearance of CTE after 5 months of storage (A=-20°C, B=4°C, C=RT, D=45°C)

5. Hair growth promoting effect of CTE

5.1 *In vitro* study

5.1.1 Effect of CTE on the expression of hair growth-related genes

5.1.1.1 Effect on hair growth promoting genes

By semi-quantitative RT-PCR techniques, CTE at 0.25-1 mg/ml significantly increased the level of VEGF mRNA expression in DPCs at dose-dependent manner. Especially, CTE at dose of 1 mg/ml induced the expression of VEGF mRNA higher than minoxidil. CTE slightly increased the level of VEGF mRNA in HaCaT cells (Figure 30). CTE at these doses also significantly increased the level of KGF mRNA expression in both DPCs and HaCaT at dose-and –time dependent manners (Figure 31).

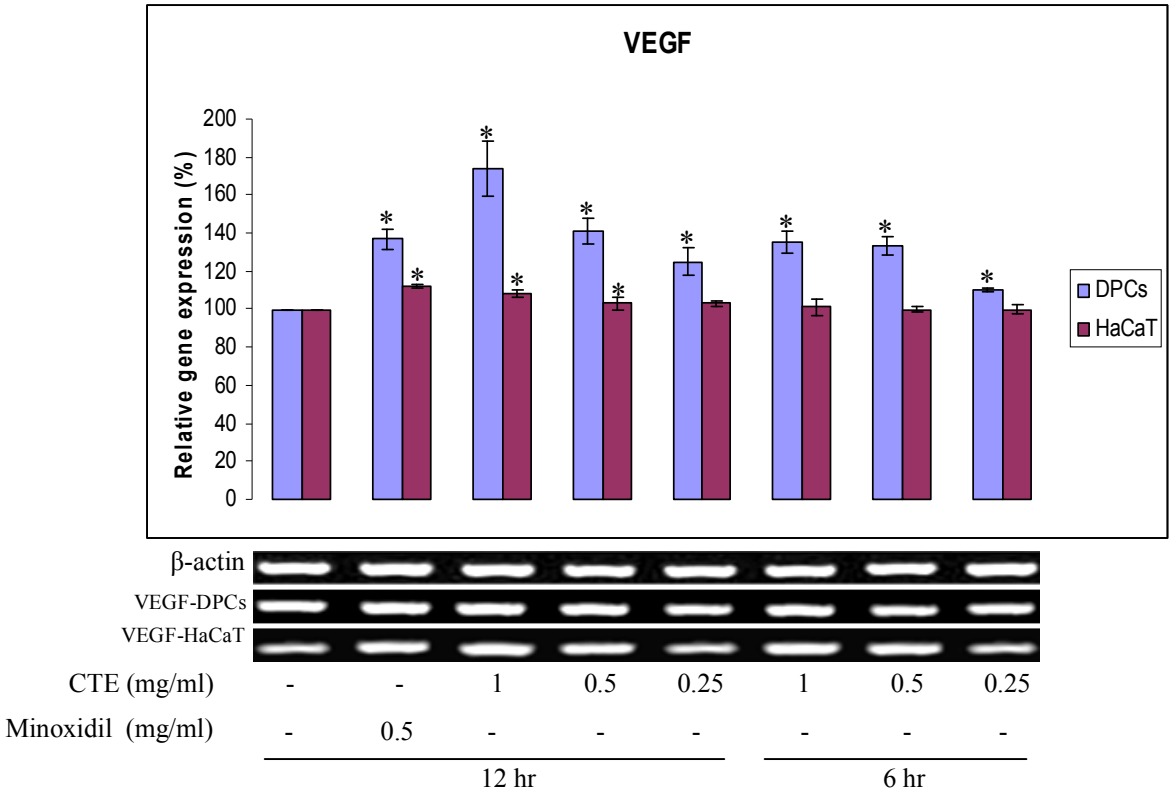

Figure 30 Effect of CTE on mRNA expression of VEGF in DPCs and HaCaT cells (results are expressed as % of control, $P<0.05$ vs. untreated group)

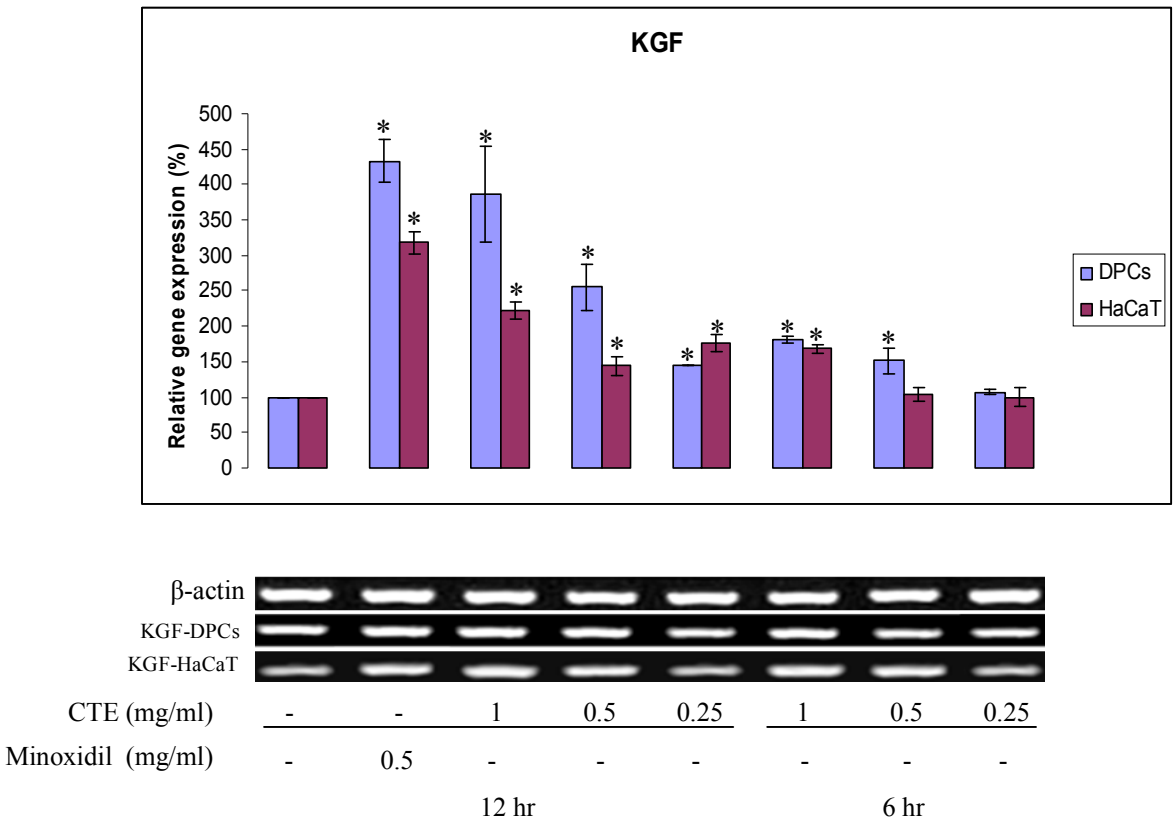

Figure 31 Effect of CTE on mRNA expression of KGF in DPCs and HaCaT cells (results are expressed as % of control, $P<0.05$ vs. untreated group)

5.1.1.2 Effect on hair growth inhibitory related-genes

CTE at doses of 0.25-1 mg/ml significantly suppressed the level of TGF-β1 expression in DPCs and HaCaT at dose- and time dependent manners (Figure 32). CTE showed similar and significant suppression of 5α-reductase (Type I and Type II) in DPCs and HaCaT with dose- and time-dependent manner, it is interesting to observe that this suppression is higher than the effect of minoxidil (0.5 mg/ml) (Figure 33, 34)

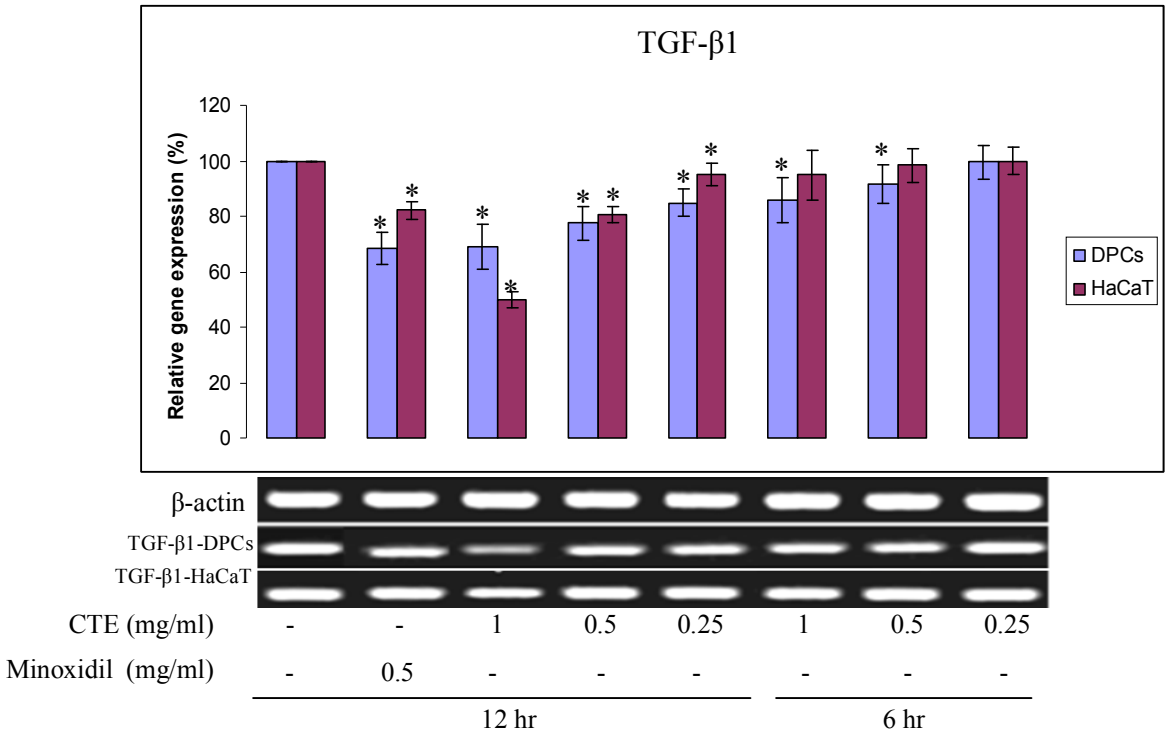

Figure 32 Effect of CTE on mRNA expression of TGF-β1 in DPCs and HaCaT cells (results are expressed as % of control, $P<0.05$ vs. untreated group)

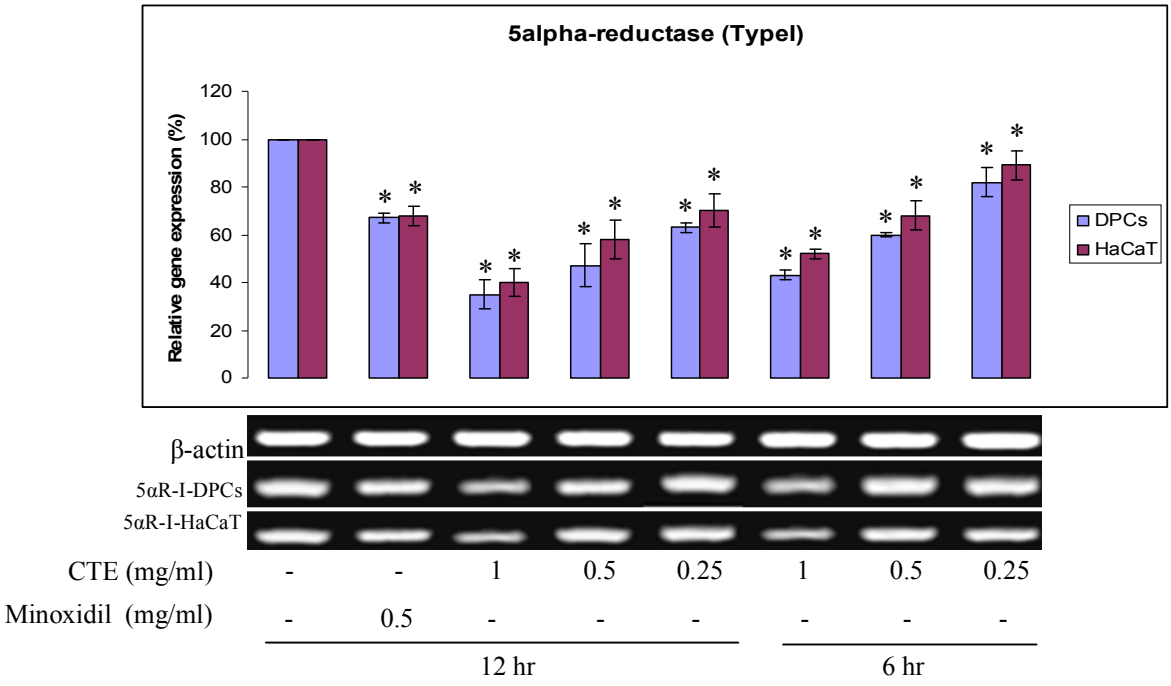

Figure 33 Effect of CTE on mRNA expression of 5α-reductase (Type I) in DPCs and HaCaT cells (results are expressed as % of control, $P<0.05$ vs. untreated group)

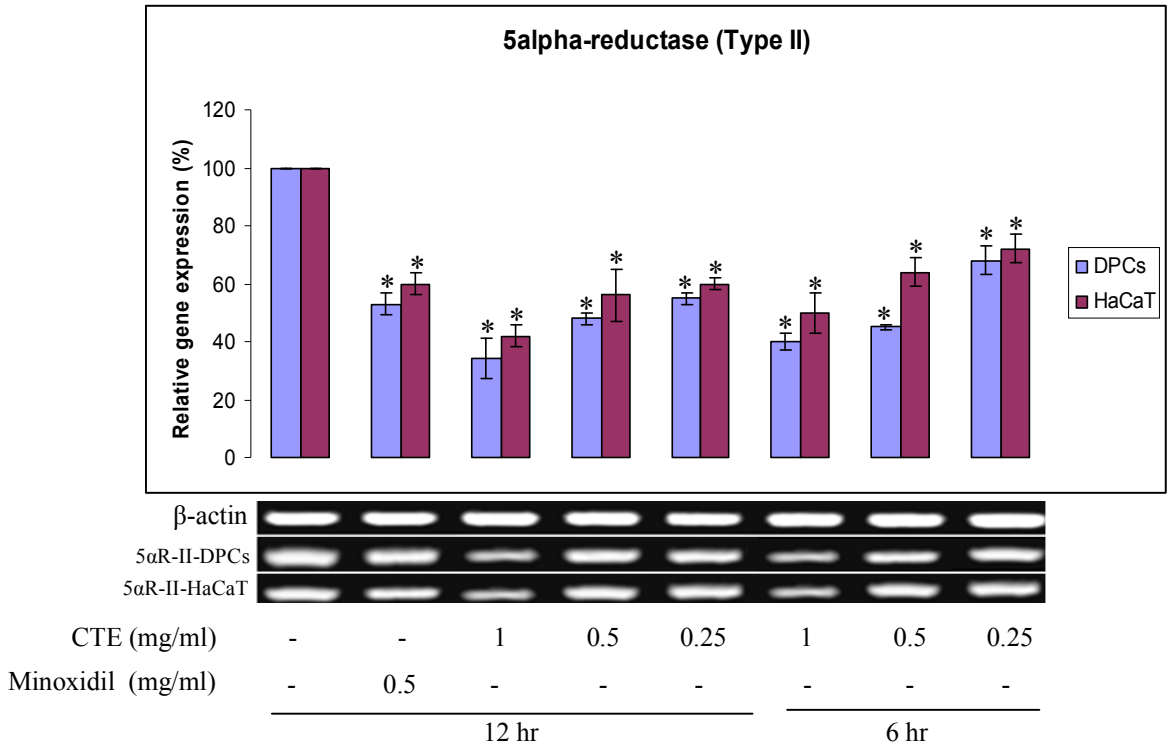

Figure 34 Effect of CTE on mRNA expression of 5α-reductase (Type II) in DPCs and HaCaT cells (results are expressed as % of control, $P<0.05$ vs. untreated group)

5.1.1.3 Effect on hair melanogenesis related-genes

After treated various concentrations of CTE (0.02-5.00 mg/ml) for 48 h with melanocyte cell, the IC_{50} was at 3.06 mg/ml (Figure 35). Based on these results, we further evaluated the effect of CTE on melanogenesis at doses lower than 3.06 mg/ml.

As shown in Figure 36, the mRNA expression of tyrosinase, TRP-1 and TRP-2 of CTE-treated cells increased in dose-dependent manner. CTE at 25 µg/ml increased the expression of tyrosinase mRNA level more than α-MSH (3.13 µg/ml).

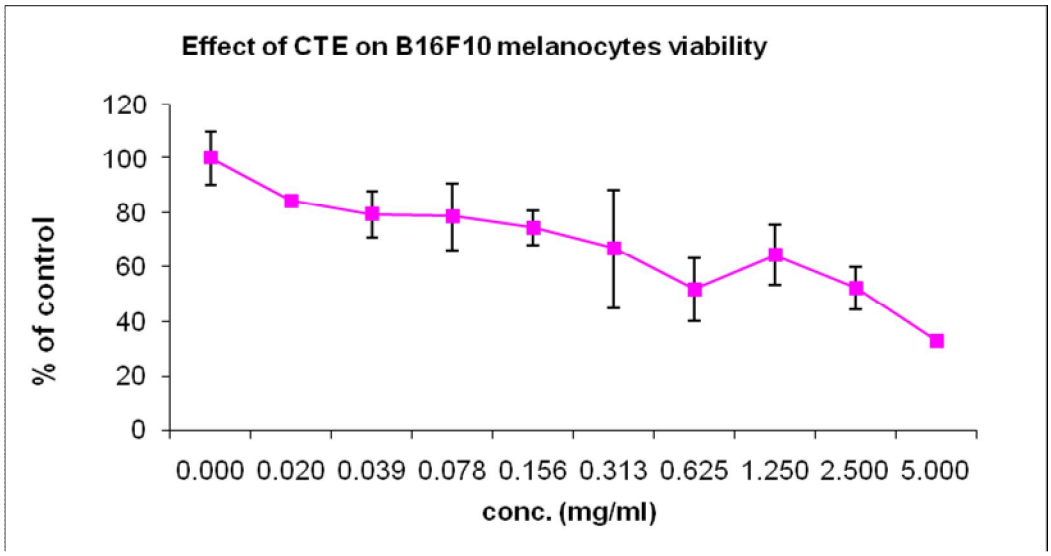

Figure 35 Effect of CTE on B16F10 melanocytes viability (each value is a mean percentage ± SD compared to control, n=3)

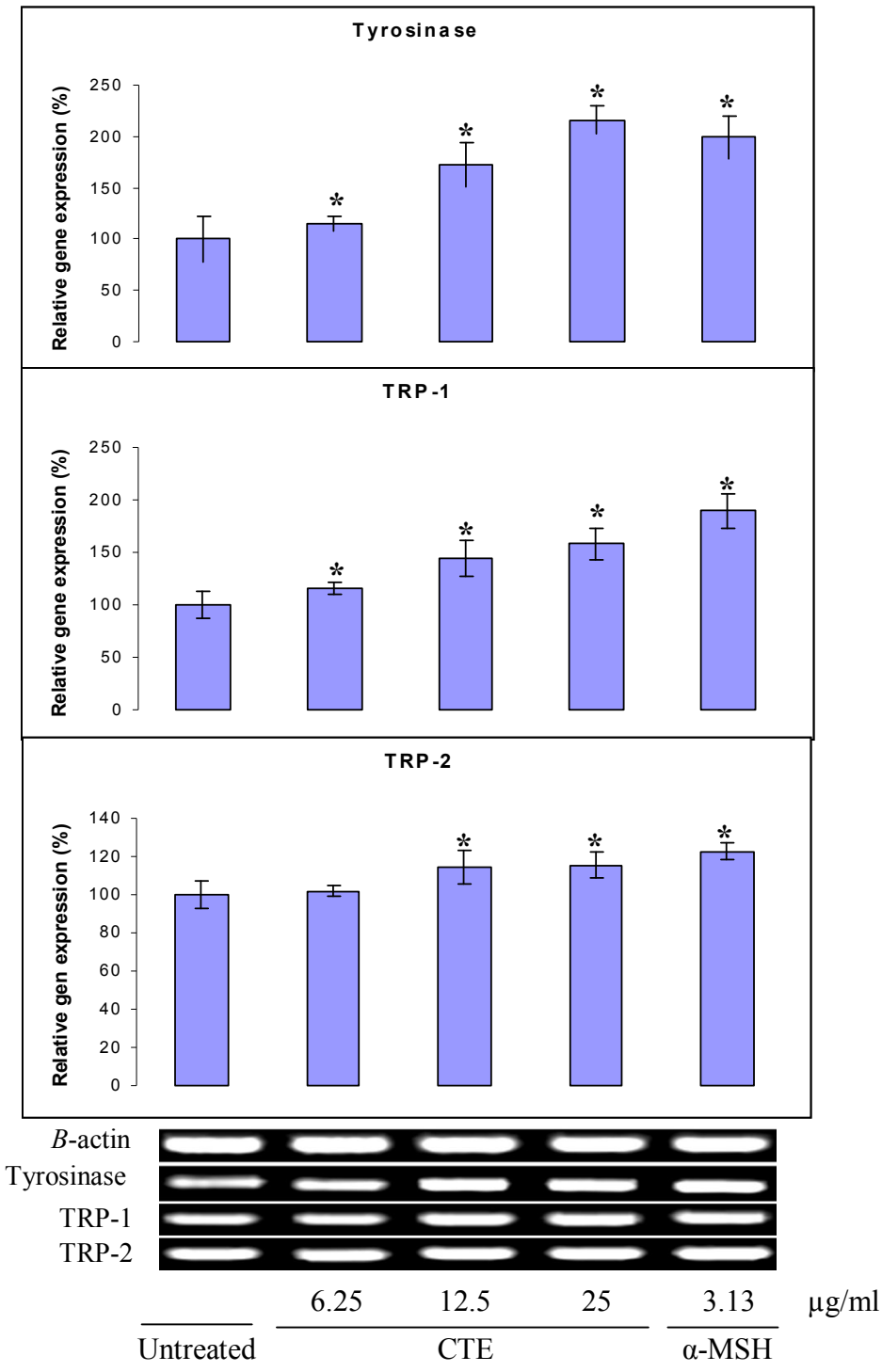

Figure 36 Effect of CTE on mRNA expression of tyrosinase, TRP-1 and TRP-2 in B16F10 cells. The level of RT-PCR product for each gene is corrected according to the level of β-actin, and the results are expressed as % of control. (*$P < 0.05$ vs. control)

5.1.2 Melanin content

The 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 (Figure 37). CTE at doses of 6.25, 25 and 100 µg/ml increased melanin content by 8.67, 19.46 and 30.57%, respectively (Figure 38a). Moreover, CTE at a dose of 100 µg/ml was slightly toxic to B16F10 cells as observed that cell viability were decreased to 83.33% (Figure 38b)

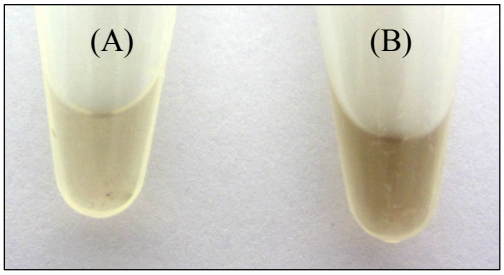

Figure 37 Photograph of darken B16F10 cells, after incubated for 48 h without any substances (A) and with CTE 25 µg/ml (B)

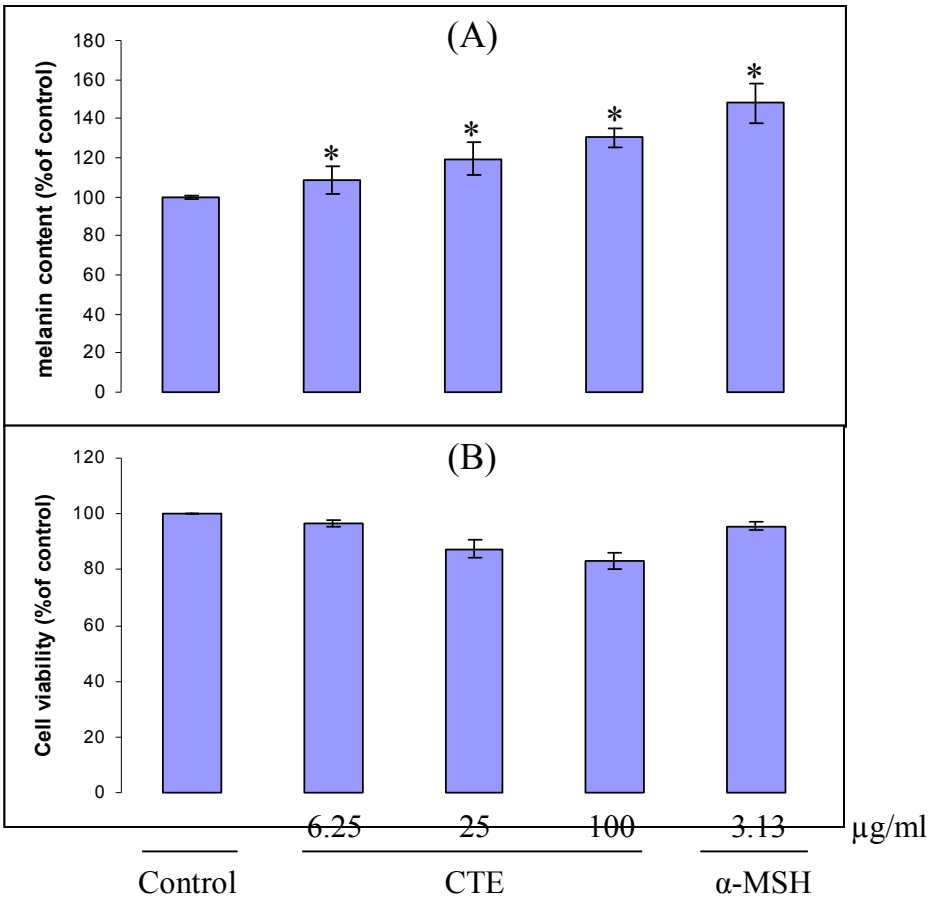

Figure 38 Melanin content (A) and viability (B) of B16F10 cells treated with different concentrations of CTE for 48 h. Data are expressed as percentage of control and calculated as the mean ± SD of three independent experiments. (*$P < 0.05$ vs. control)

5.1.3 Effect of CTE on the growth of mouse neonate hair follicle

The CTE showed a significant increase in length of cultured mouse neonate hair follicles at dose and time-dependent manner, as shown in Table 11 and Figure 39.

Table 11 Effect of CTE on the cultured hair follicle growth length

Compound	Increase in hair follicle length (mm)		
	24 h	48 h	72 h
CTE 200 µg/ml	0.51±0.05*	0.66±0.07*	0.68±0.07*
CTE 100 µg/ml	0.42±0.06*	0.58±0.08*	0.61±0.06*
CTE 50 µg/ml	0.39±0.04*	0.56±0.08*	0.57±0.07*
Minoxidil 50 µg/ml	0.39±0.02*	0.57±0.08*	0.63±0.05*
Control	0.32±0.05	0.46±0.08	0.49±0.06

* $P < 0.05$, when compared to control values (n=30 hair follicles)
* mean ± SD (n=30)

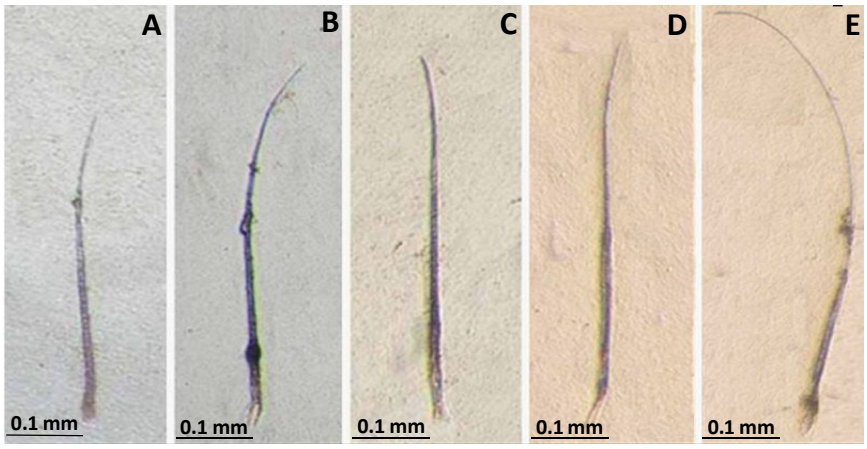

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Figure 39 Light microscope picture of mouse neonate hair follicles treated with CTE and minoxidil. The intact morphology was observed at 72 h (A=control, B=minoxidil 50 µg/ml, C=CTE 50 µg/ml, D=CTE 100 µg/ml, E=CTE 200 µg/ml)

5.2 *In vivo* study

Hair growth was observed from the hair shaved-area of the mouse skin at day 15. In comparison to the control, the whole hair shaved-area was covered with darken skin and showed the growth of hair in dose-dependent manner in the CTE-treated groups (Figure 40A-40C). Minoxidil-treated group also stimulated the hair growth but with some parts of the pink skin which was in the telogen phase (Figure 40D). In untreated-group, the mouse skin was pink with resting hair follicles (Figure 40E). In all groups, there was no considerable change in hair texture. Therefore, CTE possessed potent promoting effect on hair growth in mice.

The histological profiles of mouse skin exhibited that CTE enhanced hair growing phase which could be observed as high level of growing hair follicle number and the thickness of mice skin as compare to the control treatment (Figure 41) (Table 12).

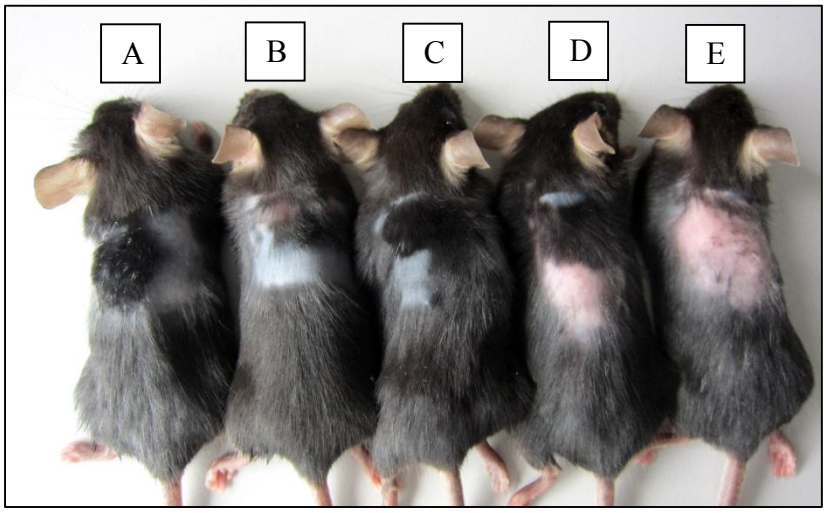

Figure 40 Effect of CTE on hair growth in C57BL/6 mice (A=0.1 mg/ml CTE, B=0.5 mg/ml CTE, C=2.0 mg/ml CTE, D=0.1 mg/ml minoxidil, E = Untreated)

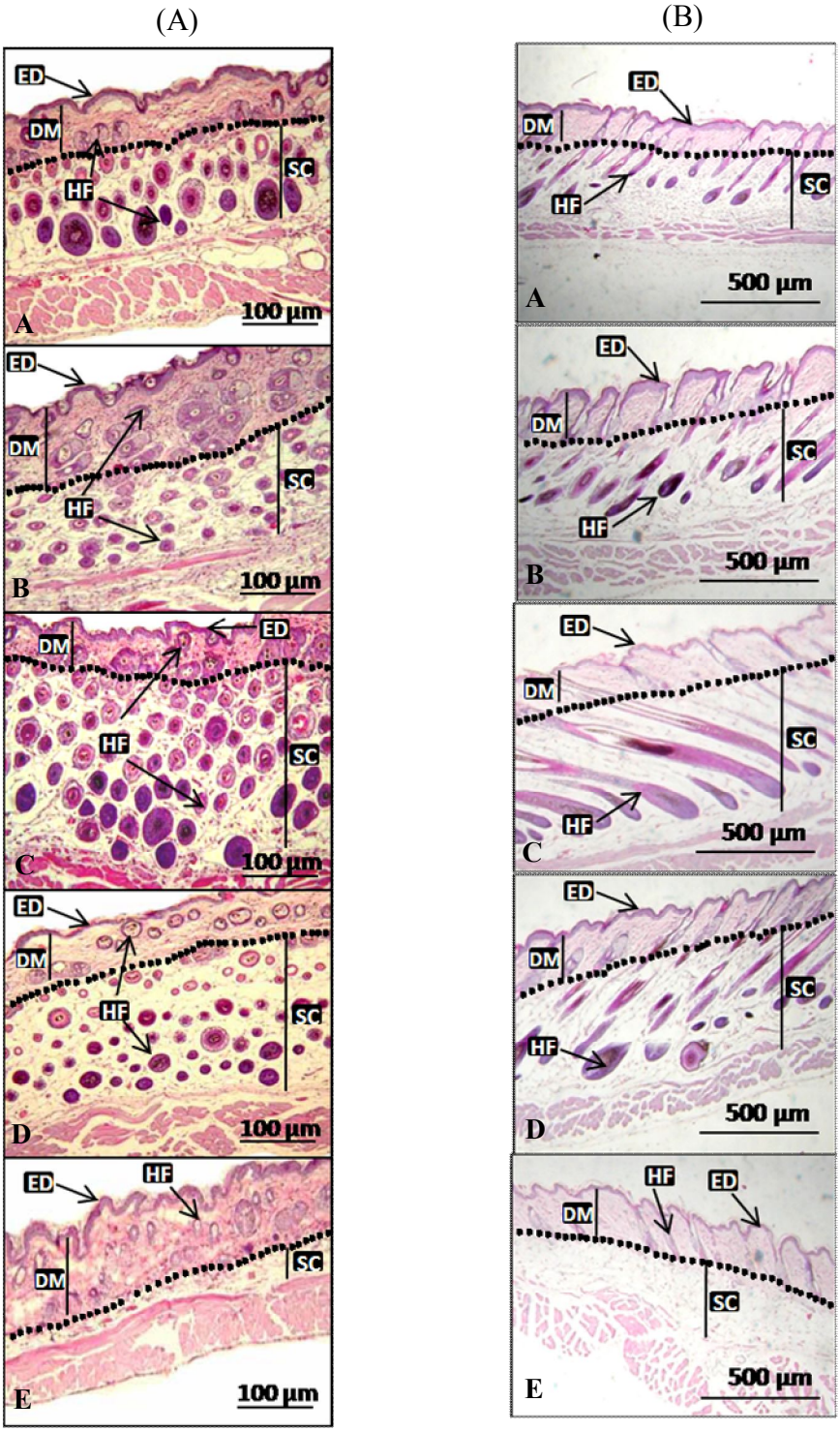

Figure 41 Representative transverse (A) and longitudinal (B) sections of C57BL/6 mice (A=0.1 mg/ml CTE, B=0.5 mg/ml CTE, C=2.0 mg/ml CTE, D=0.1 mg/ml minoxidil, E= Untreated); HF=hair follicle, SC=subcutis, DM=dermis, ED=epidermis

Table 12 Effect of CTE on anagen/telogen ratio, hair follicle number and skin thickness of mice

Group	Anagen/telogen (%)*	Hair follicle number (% of control)**	Skin thickness (μm)***
0.1 mg/ml CTE	55/45	240.7±15.8	454.5±10.8
0.5 mg/ml CTE	65/35	285.2±20.5	681.8±20.5
2 mg/ml CTE	80/20	414.8±21.8	909.1±25.2
0.1 mg/ml minoxidil	78/22	333.3±18.9	727.3±18.5
Untreated	29/71	100	363.6±16.8

* Hair follicle number in subcutis layer / hair follicle number in dermis layer, expressed as % average (n=10)

** Hair follicle number/mm^2, expressed as % of control (n=10)

*** Measured from epidermis to subcutis layer, expressed as mean±SD (n=10)

In addition, two different tested samples were applied to each shaved-area of the mouse and observed for 15 days. In comparison to the control, the hair shaved-area of the CTE-treated group was covered with darken skin and showed the growth of hair in a dose-dependent manner. In the same mouse, the results clearly exhibited the different between untreated- and CTE- treated groups (Figure 42 (A1 and A2)). At the same dose (0.1 mg/ml), minoxidil showed similar effect on hair growth to CTE (Figure 42 (B1 and B2)). In addition, there was slight hair growth effect in vehicle group as observed that was the telogen phase follicle which was the pale color of skin (Figure 42 (C1 and C2)). In all groups, there were no considerable changes in hair texture or skin irritation at the site of application. The histological profiles of mouse skin exhibited that CTE enhanced hair growing phase which increased in thickness and the presence of follicles in the subcutis layer. The % ratio of anagen to telogen phase of hair growth of animals treated with 0.5 mg/ml CTE was 65/35 whereas the untreated group showed lower number 29/71 (Table 13). In the representative sections, the anagen phase is associated with increase in follicle size and located in deep subcutis while the follicle in telogen phase lies only in the dermis (Figure 43).

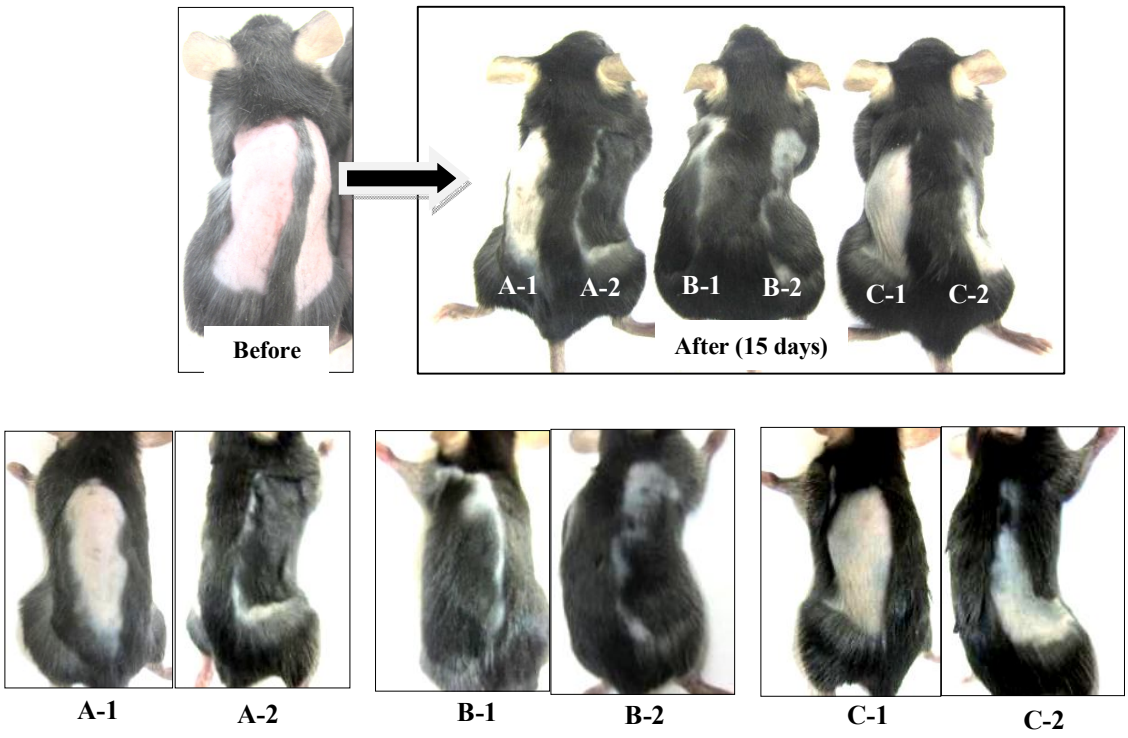

Figure 42 Hair growth-promoting effects of CTE in C57BL/6 (A-1=untreated group, A-2=CTE 0.5 mg/ml, B-1=minoxidil 0.1 mg/ml, B-2=CTE 0.1 mg/ml, C-1=vehicle, C-2= CTE 0.05 mg/ml)

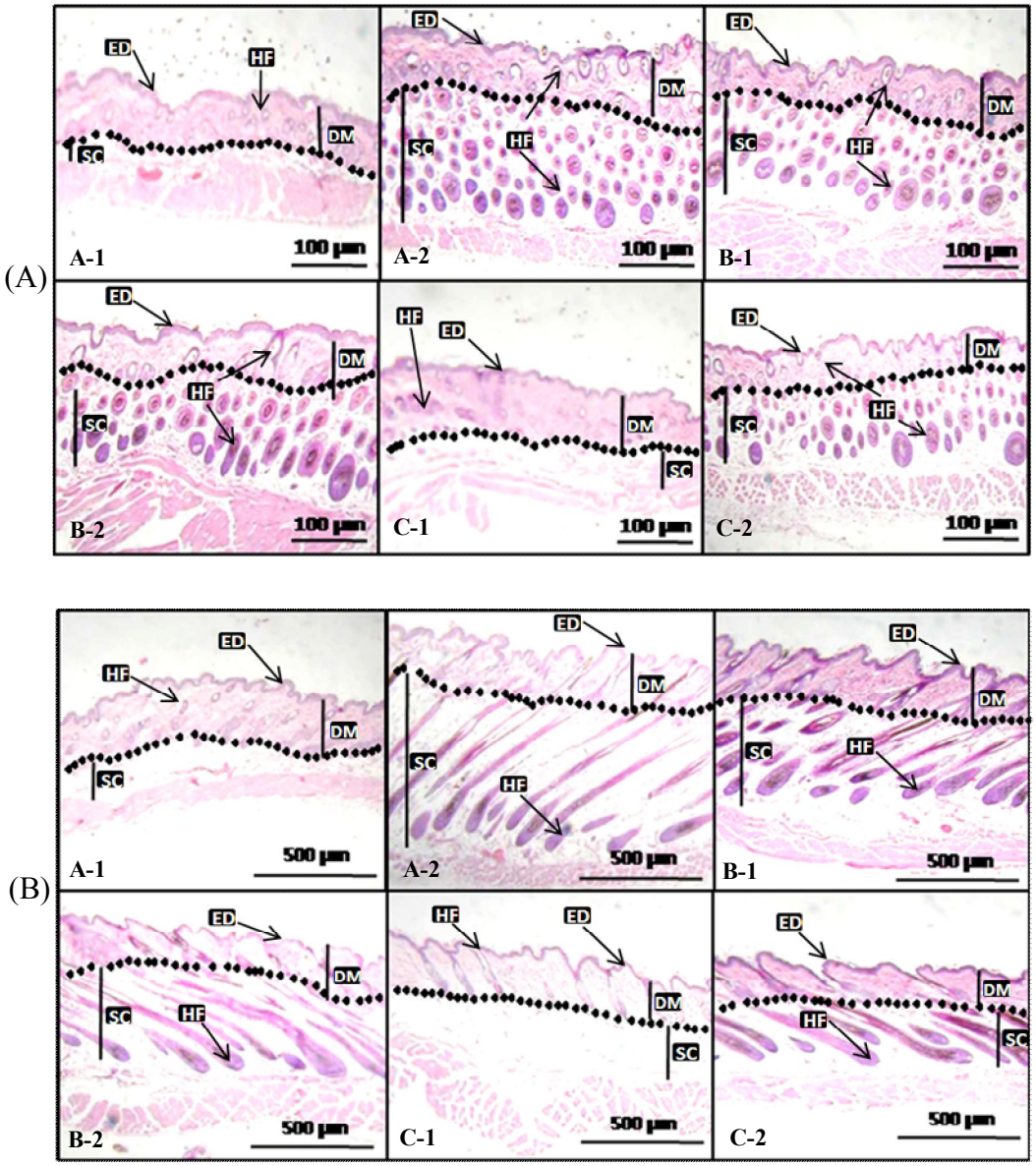

Figure 43 Representative transverse (A) and longitudinal (B) histological profiles of C57BL/6 mice (A-1=untreated group, A-2=CTE 0.5 mg/ml, B-1=minoxidil 0.1 mg/ml, B-2=CTE 0.1 mg/ml, C-1=vehicle, C-2= CTE 0.05 mg/ml); HF=hair follicle, SC=subcutis, DM=dermis, ED=epidermis

Table 13 Effect of CTE on anagen/telogen ratio, hair follicle number and skin thickness of mice

Group	Anagen/telogen (%)*	Hair follicle number (% of control)**	Skin thickness (μm)***
Untreated	30/70	100	382.2 ± 20.2
CTE 0.5 mg/ml	78/22	293.8 ± 35.5	750.6 ± 50.9
Minoxidil 0.1 mg/ml	70/30	213.5 ± 30.5	560.5 ± 26.5
CTE 0.1 mg/ml	75/25	235.8 ± 27.2	632.8 ± 40.7
Vehicle	35/65	129.5 ± 25.6	412.5 ± 35.8
CTE 0.05 mg/ml	55/45	199.5 ± 20.8	545.6 ± 30.8

* Hair follicle number in subcutis layer / hair follicle number in dermis layer, expressed as % average (n=10)

** Hair follicle number/mm^2, expressed as % of control (n=10)

*** Measured from epidermis to subcutis layer, expressed as mean±SD (n=10)

To confirm hair growth promoting effect of CTE, Sprague-dawley rats were also investigated with the same regimen for 20 days. From the observations, CTE exhibited the hair growth promoting effect in albino rat. The hair in shaved-area of CTE treated-group was markedly longer than that of the control group. CTE showed the dose-dependent manner in this study. 2 mg/ml CTE treated-group exhibited the growth of hair higher than that 0.5 and 0.1 mg/ml CTE. Moreover, the 0.5 and 2 mg/ml CTE showed the hair growth stimulating effect similar to 0.1 mg/ml minoxidil treated-group (Figure 44).

In addition, this study also confirmed the safety of CTE due to there were no irritation or any abnormalities in the animal during the application period.

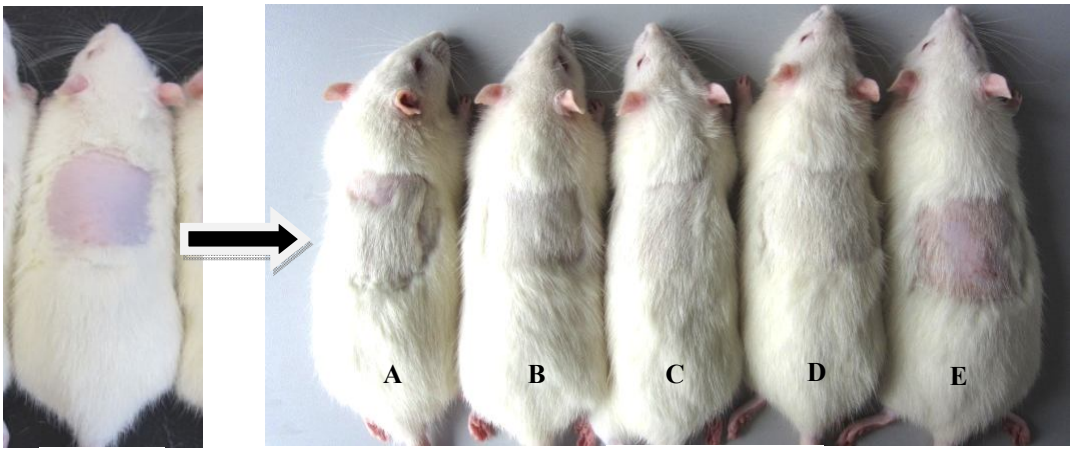

Figure 44 Hair growth-promoting effects of CTE in Sprague-dawley rat (A= CTE 0.1 mg/ml, B= CTE 0.5 mg/ml, C= CTE 2 mg/ml, D=minoxidil 0.1 mg/ml, E=untreated)

6. Skin permeation study

Under the developed HPLC system, the content of hydroxysafflor yellow A was determined from the calibration curve. The results showed that the permeation of HSYA from CTE dissolving in vehicle containing propylene glycol (PG), ethanol (EtOH) and water was higher than that of dissolving in water with the higher flux value and the shorter lag time (Figure 45 and Table 14).

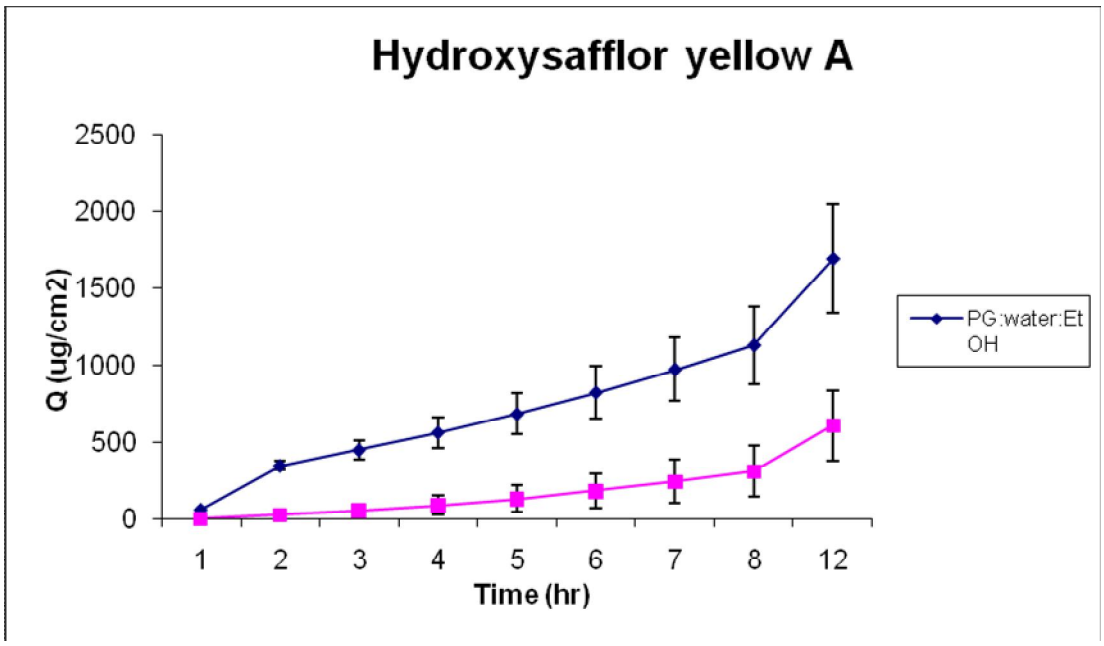

Figure 45 *In vitro* permeation profile of HSYA of CTE through porcine ear skin (results expressed as mean±SD, n=3)

Table 14 Flux and lag time of CTE permeation through pig ear skin

Vehicles	Flux (μg/cm^2/h)	Lag time (h)
Water	82.01	2.93
PG:Water:EtOH (5:2:3)	141.44	0.06