CHAPTER 3

RESULTS AND DISCUSSION

The results and discussion of this study is divided into 6 parts as the followings:

Part 1 : Preparation and *in vitro* anti-aging activities of the crude extracts from leaves of Long Kong

Part 2 : Preparation and *in vitro* anti-aging activities of the semi-purified extracts

Part 3 : Discoloration and *in vitro* anti-aging activities of the selected semi-purified extracts

Part 4 : Development of the cosmetic base formulations

Part 5 : Development of the cosmetic formulations containing the extract from leaves of Long Kong

Part 6 : *In vivo* anti-aging activities on human volunteers of the cosmetic formulations containing the extract from leaves of Long Kong

Part 1 : Preparation and *in vitro* anti-aging activities of the crude extracts from leaves of Long Kong

1.1 Preparation of the crude extracts

1.1.1 Percentage yields of the crude extracts

The percentage yields of the crude extracts from leaves of Long Kong were presented in **Table 31**. The methanolic crude extract by the hot process exhibited the highest percentage yield at 23.67% (w/w), while the lowest percentage yield was from the chloroform crude extract by the cold process [7.47% (w/w)]. As

known, a solute will dissolve well in a solvent with similar chemical structure. The leaves of Long Kong may contain both the polar and non-polar compounds which can be better extracted by methanol than by water and chloroform. The extracts from the hot processes also showed significant higher percentage yields than those from the cold processes ($p < 0.05$). In fact, the heated solvents have been reported to be able to release the cell wall bioactives by breaking down the cellular constituents and resulting in high percentage yields (Tachibana et al., 2001).

Table 31 Percentages yield of the crude extracts from leaves of Long Kong prepared by six different processes

Samples	%yield (w/w) of the dried leaf powder
Hot chloroform extract	17.95
Cold chloroform extract	7.47
Hot methanol extract	23.67
Cold methanol extract	15.90
Hot water extract	17.97
Cold water extract	10.32

Note: Percentage yields of the crude extract were from 100 g of the dried Long Kong leaf powder

1.2 Characteristics of the crude extracts

1.2.1 Phytochemical constituents of the crude extracts

Table 32 showed the phytochemical constituents existing in the crude extracts. In the same solvent systems, both hot and cold crude extracts gave the same phytochemical constituents. However, the different polarity of the extracted solvent contributed to different phytochemical compositions of the crude extracts. Methanol which was a medium polar solvent, gave the most abundant phytochemicals of triterpenoids, reducing sugars, and flavonoids. Triterpenoids were available both in

the methanol and chloroform crude extracts, but not found in the water crude extract, while alkaloid was only presented in the water crude extracts.

Table 32 Phytochemical constituents of the crude extracts from leaves of Long Kong prepared by six different processes

Processes	Triterpenoids	Alkaloids	Anthraquinones	Reducing sugars			Flavonoids	Carotenoid	Tannins
				F	G	S			
Hot water	-	+	-	+	-	+	+	-	-
Hot methanol	+	-	-	+	-	+	+	-	-
Hot chloroform	+	-	-	-	-	-	-	-	-
Cold water	-	+	-	+	-	+	+	-	-
Cold methanol	+	-	-	+	-	+	+	-	-
Cold chloroform	+	-	-	-	-	-	-	-	-

Note: "+" represented presence in the extract. "–" represented absence in the extract. F represented for fructose. G represented for glucose. S represented for sucrose

1.2.2 Total phenolic and flavonoid contents of the crude extracts

The total phenolic and flavonoid contents of the six crude extracts were demonstrated in **Figure 38.** The mean total phenolic contents of the crude extracts were decreased in the order of methanol > chloroform > water. The methanolic extract by the cold process contained the highest phenolic content (107.63±0.05 µg GAE/g dry extract). The similar previous finding has been reported that the lesser amounts of the phenolic compounds were extracted by chloroform due to their polar nature, and the low molecular weight phenolic compounds were readily extracted by methanol (Sun et al., 2006). The mean total flavonoid contents of the crude extracts were decreased in the order of chloroform > methanol > water. The hot

chloroform extract exhibited the highest flavonoid content (582.70±0.04 µg QE/g dry extract). As known, flavonoids are usually existing in the form of lipophillic aglycone (without the sugar moiety such as quercetin, apigenin and luteolin) and hydrophyllic glycoside (with the sugar moiety such as rutin, naringin and hesperidin). The lipophillic flavonoid may be presented in the Long Kong leaves more than the hydrophillic flavonoid which can be extracted more by chloroform than methanol and water. In addition, temperatures did not affect the phenolic and flavonoid contents in the crude extracts.

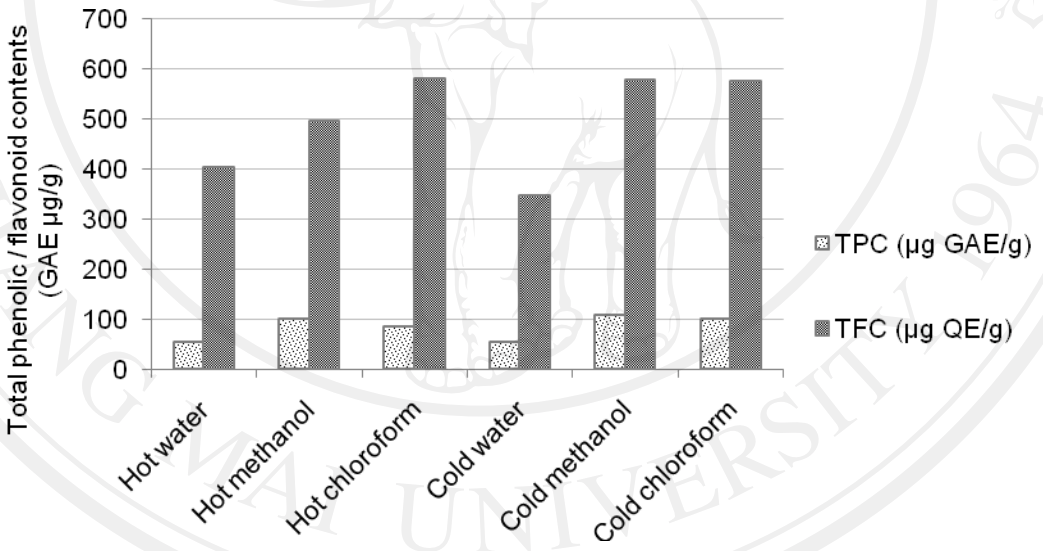


Figure 38 Comparison of the total phenolic and flavonoid contents of the crude extracts from leaves of Long Kong prepared by six different processes

1.2.3 *In vitro* anti-aging activities of the crude extracts

The antioxidant activities including DPPH radical scavenging, lipid peroxidation inhibition and metal ion chelating activity of the crude extracts were shown in **Table 33**.

Table 33 Comparison of the antioxidant activities of the crude extract from leaves of Long Kong prepared by six different processes

Crude extracts	DPPH radical scavenging	Metal chelating	Lipid peroxidation inhibition
	SC_{50} (mg/ml)	CC_{50} (mg/ml)	IPC_{50} (mg/ml)
Cold chloroform extract	6.06±0.27	19.21±1.46	-
Hot chloroform extract	6.15±0.78	12.34±0.35	18.77±1.78
Cold methanol extract	7.59±0.02	12.38±0.16	6.22±0.06
Hot methanol extract	8.72±0.54	12.65±0.50	4.61±0.65
Cold water extract	8.45±0.47	-	-
Hot water extract	5.40±1.23	32.31±0.84	3.29±0.30
Ascorbic acid	0.08±0.02	ND	ND
α-tocopherol	ND	1.00±0.68	ND
EDTA	ND	ND	0.61±0.24

Note: Values represent mean±S.D. (n = 3).
SC_{50} = scavenging concentration at 50% activity (mg/ml)
IPC_{50} = inhibition peroxidation concentration at 50% activity (mg/ml)
CC_{50} = chelating concentration at 50% activity (mg/ml)
ND = not done
- = no activity

1.2.3.1 DPPH radical scavenging activity of the crude extracts

The water crude extract by the hot process exhibited the highest DPPH radical scavenging activity (SC_{50} values of 5.40±1.23 mg/ml), but lower than ascorbic acid (SC_{50} values of 0.08±0.02 mg/ml) of 63 times. The ability to scavenge DPPH radicals of the hot water crude extract might be not only from the phenolic contents, but also other polar antioxidants such as ascorbic acid (Lim et al., 2007), maleic acid and malic acid (Chairgulprasert et al., 2006).

1.2.3.2 Lipid peroxidation inhibition activity of the crude extracts

The water crude extracts by the hot process gave the highest lipid peroxidation inhibition at the IPC_{50} values of 3.29±0.30 mg/ml, but lower than α-tocopherol (0.79±0.08 mg/ml) of 4.16 times. The sugar moiety in the form of glycosides containing in the hot water crude extract might prevent the lipid peroxidation by forming the hydrogen bonds between the hydroxyl groups and hydroperoxides (Judis et al., 2010).

1.2.3.3 Metal ion chelating assay of the crude extracts

The hot chloroform and cold methanol crude extracts exhibited similar metal chelating activity with the CC_{50} value of 12.34±0.35 and 12.38±0.16 mg/ml, respectively, but lower than EDTA (CC_{50} value of 0.72±0.04 mg/ml) of 17.14 and 17.19 times, respectively.

1.2.4 Tyrosinase inhibition activity of the crude extracts

The hot water crude extract showed the highest tyrosinase inhibition activity (IC_{50} value of 0.49±0.23 mg/ml), but lower than kojic acid (IC_{50} value of 0.03±0.01 mg/ml) of 16.33 times (**Table 34**). Tyrosinase inhibition might be from the hydroxyl groups of the phenolic compounds in the crude extract that could form a hydrogen bond to the active site of the tyrosinase enzyme, leading to the low enzymatic activity. Some tyrosinase inhibitors act through the binding of their hydroxyl groups to the active site of the enzyme, resulting in the steric hindrance or conformation change. Also, antioxidant activity may be one of the important mechanisms for tyrosinase inhibitory activity (Alam et al., 2011). Several studies have reported the tyrosinase inhibitory effect of Long Kong, but did not clearly confirm this inhibition mechanism. Tilaar et al. and Arung et al. have reported that

the Long Kong extract significantly decreased the *in vivo* skin melanin index in human volunteers and strongly inhibited the melanin production of $B_{16}F_{10}$ melanoma cells (Arung et al., 2009; Martha et al., 2007).

Table 34 Comparison of the *in vitro* tyrosinase inhibition of the six crude extracts from the leaves of Long Kong

Samples	Tyrosinase inhibition IC_{50} (mg/ml)
Hot water extract	0.49±0.23
Hot methanol extract	2.30±1.72
Hot chloroform extract	4.32±1.98
Cold water extract	3.44±0.76
Cold methanol extract	7.39±1.15
Cold chloroform extract	4.42±3.40
Kojic acid	0.03±0.01

Note: Values represent mean±S.D. (n = 3).
IC_{50} = tyrosinase inhibition concentration at 50% activity (mg/ml)
- = no activity

1.2.5 Cytotoxicity on human skin fibroblast of the crude extracts

Table 35 showed the percentages of cell viability of the crude extracts at 1 mg/ml. The concentration-dependent manner was observed. The chloroform crude extracts by the hot and cold processes tended to be toxic with the cell viability of 41.22±8.66 and 24.22±7.59%, respectively, while both the hot and cold processes of the water and methanolic crude extracts gave no cytotoxicity with the cell viability of more than 75% (Martins et al., 2008). The cold methanolic extract exhibited higher cell viability (%cell viability of 118.70±29.53%) than ascorbic acid (%cell

viability of 114.06±17.53%) of 1.04 times. The non-polar compounds existing in the chloroform crude extract might cause cell death via the activation of apoptotic pathway following the significant membrane damage (Ricci et al., 2009), and the disruption of the cytoskeleton and cellular membrane as well as the disturbance of the energy metabolism and gene expression associated pathways, and DNA damage accompanied by cell cycle arrest (Ma et al., 2011). In addition, the resulting toxicity depends on a number of factors such as size, concentration, solubility, chemical and biological properties, and stability (Singh et al., 2007; Zhao et al., 2007).

Table 35 Comparison of the percentages cell viability on human skin fibroblast of the crude extracts from leaves of Long Kong

Samples	% cell viability
Hot water extract	80.52 ± 15.16
Hot methanol extract	87.13 ± 16.36
Hot chloroform extract	41.22 ± 8.66
Cold water extract	75.16 ± 21.97
Cold methanol extract	118.70 ± 29.53
Cold chloroform extract	24.22 ± 7.59
Ascorbic acid	114.06 ± 17.53

Note: Values represent mean±S.D. (n = 3).
Percentages of cell viability at the sample concentration 1 mg/ml

1.2.6 Gelatinolytic activity on MMP-2 inhibition activity of the crude extracts

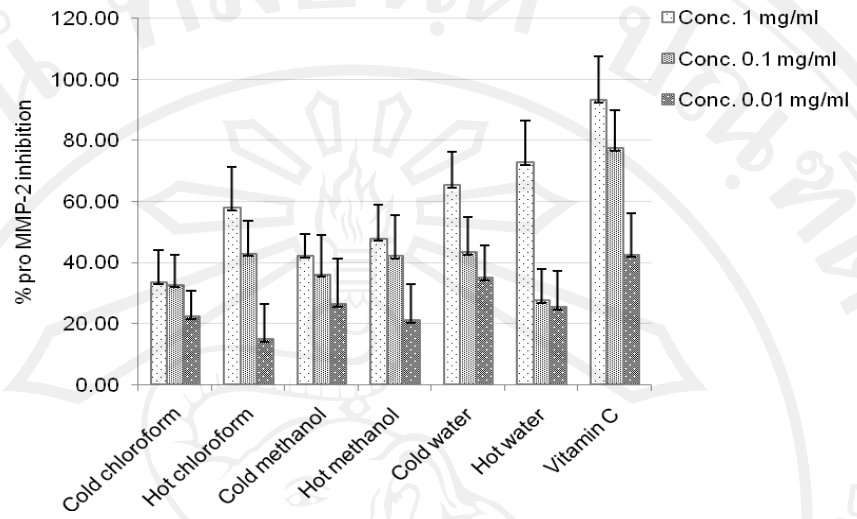
Figures 39 presented the gelatinolytic activity on MMP-2 inhibition of the crude extracts at various concentrations. The hot water crude extracts at 1 mg/ml indicated the highest pro and active MMP-2 activity with the %MMP-2 inhibition of 72.96±13.44 and 85.77±13.77%, but lower than ascorbic acid (%MMP-2 inhibition of

93.46±14.09 and 96.22±13.22%, respectively) of 1.28 and 1.12 times, respectively. The inhibition of MMP-2 expression of the hot water crude extract related to its DPPH radical scavenging activity. In fact, it has been reported that the exogenous hydrogen peroxide and endogenous ROS can induce the MMP expression in endothelial cells, cardiac fibroblasts, macrophages and breast cancer cells (Zhang et al., 2002). Actually, the pro MMP-2 activation does not only depend on ROS, but the membrane type-1-MMP (MT-1 MMP) and tissue inhibitor of metalloproteinase (TIMP)-2 also play a critical role to activate the pro MMP-2. In addition, ROS suppression is a bypassed way to inhibit pro MMP-2 activation (Kim et al., 2007). The zymograms of pro and active MMP-2 inhibition of the semi-purified extract at various concentrations (0.01, 0.1 and 1 mg/ml) were shown in **Figure 40**. The intensity of bands of pro and active MMP-2 were decreased with increasing concentrations of the samples, while the area of the bands of pro and active MMP-2 were increased with decreasing concentrations of the samples.

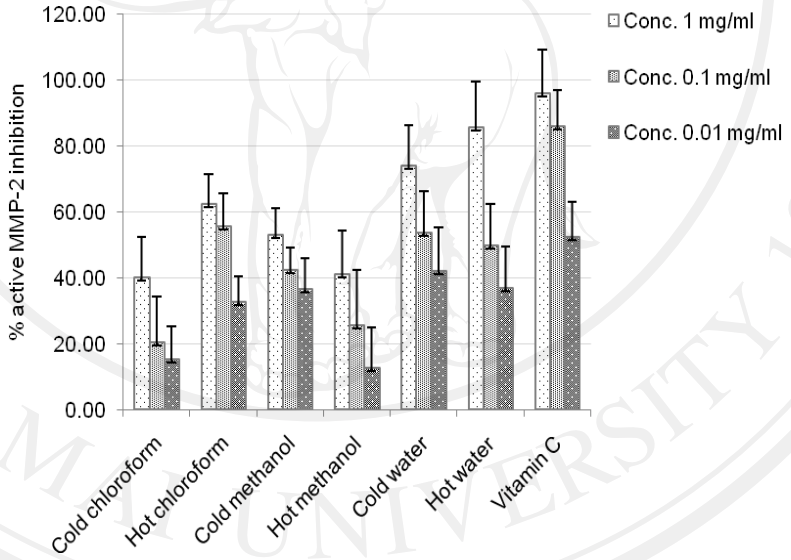
1.3 Selection of the crude extract

Among the six crude extracts, the hot water crude extract was subsequently selected to prepare the semi-purified extracts because of its high DPPH radical scavenging, lipid peroxidation inhibition and tyrosinase inhibition, moderate metal ion chelation as well as high MMP-2 inhibition activity in comparing to other crude extracts. Although this hot water crude extract contained low total phenolic and flavonoid contents, it prepared from water which is the non-toxic and inexpensive solvent in comparing to methanol and chloroform.

(A)

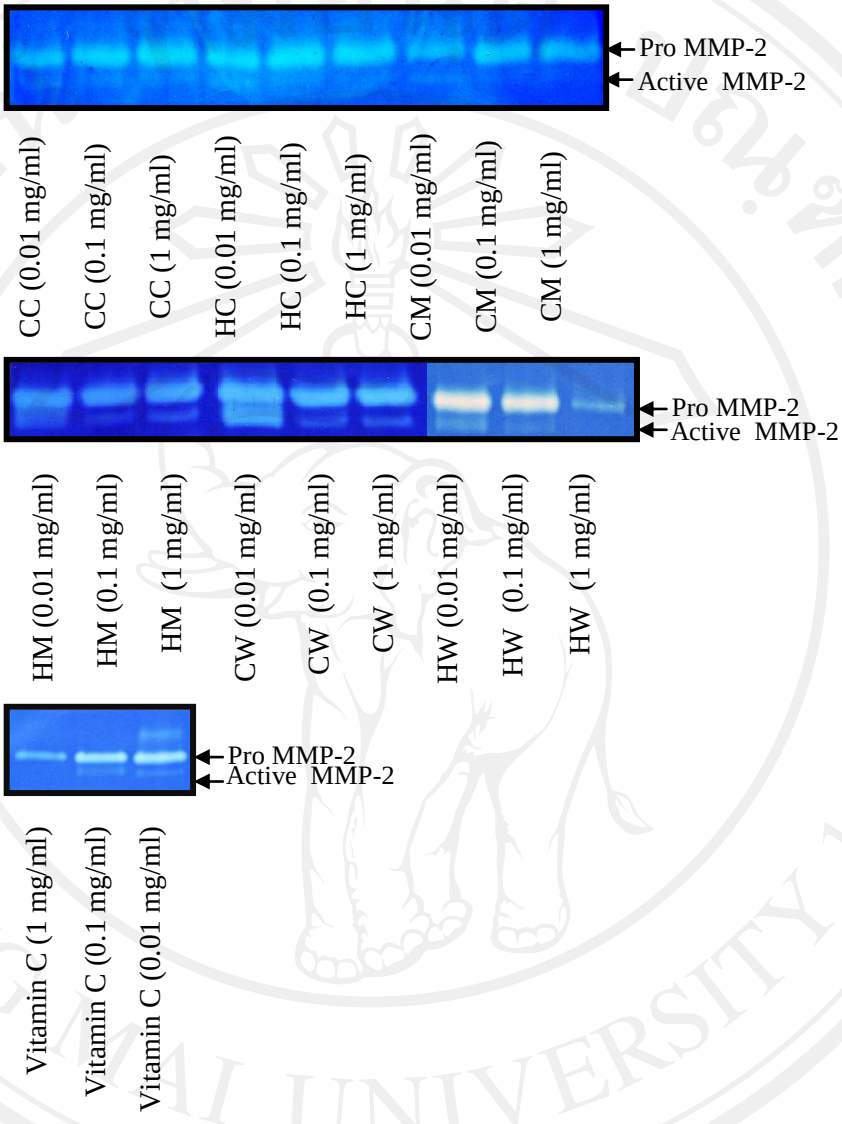


(B)



Note: MMP-2 inhibition (%) =100 − [(*MMP − 2 content of the sample* / *MMP − 2 content of the control*) × 100]

Figure 39 The gelatinolytic activity of pro (A) and active (B) MMP-2 inhibition (% of the control) of the six crude extracts prepared by the six different processes of Long Kong leaves and ascorbic acid at 0.01, 0.1 and 1 mg/ml



Note: CC: cold chloroform process, HC: hot chloroform process, CM: cold methanol process, HM: hot methanol process, CW: cold water process and HW: hot water process

Figure 40 Zymograms of pro and active MMP-2 inhibition of the six crude extracts prepared by the six different processes from leaves of Long Kong and ascorbic acid at 0.01, 0.1 and 1 mg/ml

Part 2: Preparation and *in vitro* anti-aging activities of the semi-purified extracts

2.1 Percentage yields of the semi-purified extracts prepared from the selected crude extract

Among the three semi-purified extracts of the hot water crude extract, water semi-purified extract gave the highest % yield (47.61 % (w/w)), followed by butanol (9.59 % (w/w) and ethyl acetate soluble fractions (2.83 % (w/w)), respectively (**Table 36**). The different solubility of the solute in different polarity solvent is expected (Zhang et al., 2007). Water and butanol are polar protic solvents with the dielectric constants of 80 and 18 respectively, while ethyl acetate is a polar aprotic solvent with the dielectric constant of 6. Thus, the high polar constituents in the extract should prefer to dissolve in water and butanol than in ethyl acetate. Likewise, moderate or non-polar compounds in the extract such as cycloartanoid triterpene should be found in the ethyl acetate semi-purified extract (Nishizawa et al., 1989).

Table 36 Percentages yield of the semi-purified extracts prepared from the hot water crude extract from leaves of Long Kong

Semi-purified extracts	%yields (w/w) of the dried leaf powder	%yields (w/w) of the hot water crude extract
Water semi-purified extract	8.56	47.61
Butanol semi-purified extract	1.72	9.59
Ethyl acetate semi-purified extract	0.51	2.83

2.2 Characteristics of the semi-purified extracts

2.2.1 Phytochemical analysis of the semi-purified extracts

The phytochemicals of the semi-purified extracts were different according to the distinct polarity of the partitioning solvents. However, all semi-

purified extracts contained flavonoids including glycone and aglycone groups. The less polar flavonoids and aglycones (isoflavones, flavanones, methylated flavones, and flavonols) may be partitionated by ethyl acetate, while flavonoid glycosides and the more polar aglycones (flavan-3-ols, dihydroflavonol and amentoflavone) were probable extracted by water and butanol. Alkaloids, glycosides and flavonoids which were high polar constituents were extracted both in the water and butanol semi-purified extracts. Triterpenoids which were moderate polar compounds were found only in the ethyl acetate soluble extract (**Table 37**).

Table 37 Phytochemical constituents of the semi-purified extract prepared from the hot water crude extract of leaves from Long Kong

Samples	Triterpenoids	Alkaloids	Anthraquinones	Reducing sugars			Flavonoids	Carotenoid	Tannins
				F	G	S			
Water semi-purified extract	-	+	-	+	-	+	+	-	-
Butanol semi-purified extract	-	+	-	+	-	+	+	-	-
Ethyl acetate semi-purified extract	+	-	-	-	-	-	+	-	-

Note: "+" represented presence in the extract. "–" represented absence in the extract. F represented for fructose. G represented for glucose. S represented for sucrose.

2.2.2 Total phenolic and flavonoid contents of the semi-purified extracts

For the semi-purified extracts, the ethyl acetate semi-purified extract gave the highest total phenolic and flavonoid contents (868.90±0.02µg GAE / g dry extract and 422.39±0.01 µg QE/g dry extract, respectively), suggesting that the

existing flavonoids were the free flavonoids or aglycone flavonoids which were lipophillic components (**Figure 41**).

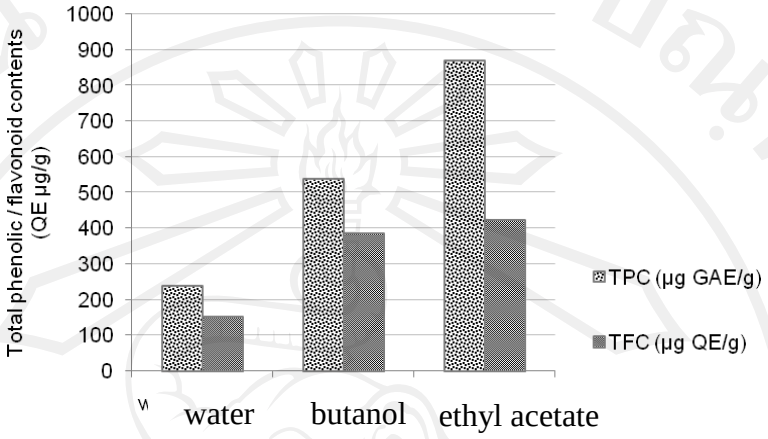


Figure 41 Comparison of the phenolic and flavonoid contents of the three semi-purified extracts prepared from the hot water crude extract of leaves from Long Kong

2.2.3 *In vitro* anti-aging activities of the semi-purified extracts

The antioxidant activities including DPPH radical scavenging, lipid peroxidation inhibition and metal ion chelating activity of the semi-purified extracts were shown in **Table 38**.

2.2.3.1 DPPH radical scavenging activity

The ethyl acetate semi-purified extract showed the highest SC_{50} values of 0.23 ± 0.02 mg/ml which was higher than the crude extract of 23.48 times, but lower than ascorbic acid of 2.88 times. The high phenolic and flavonoid compounds containing in the extract may be responsible for this scavenging ability. These bioactive compounds can interrupt with the free-radical chain of oxidation and donate the hydrogen or electron from the hydroxyl group (Singh et al., 2010), thereby

forming the stable free radicals, which do not initiate or propagate to the further oxidation reaction (Sherwin, 1978).

Table 38 Comparison of the *in vitro* antioxidant activities (DPPH radical scavenging, metal ion chelating and lipid peroxidation inhibition) of the three semi-purified extracts prepared from the hot water crude extract of leaves from Long Kong

Samples	DPPH radical scavenging SC_{50} (mg/ml)	Metal chelating CC_{50} (mg/ml)	Lipid peroxidation inhibition IPC_{50} (mg/ml)
Water semi-purified extract	2.90±0.30	132.52±72.85	2.70±1.50
Butanol semi-purified extract	0.45±0.02	32.14±0.34	16.69±7.35
Ethyl acetate semi-purified extract	0.23±0.02	0.45±0.02	33.48±14.49
Ascorbic acid	0.07±0.01	ND	ND
EDTA	ND	0.57±0.04	ND
α-Tocopherol	ND	ND	0.83±0.05

Note: Values represent mean±S.D. (n = 3).
SC_{50} = scavenging concentration at 50% activity (mg/ml)
IPC_{50} = inhibition peroxidation concentration at 50% activity (mg/ml)
CC_{50} = chelating concentration at 50% activity (mg/ml)
ND = not done

2.2.3.2 Lipid peroxidation inhibition activity

The water semi-purified extract indicated the highest activity with the IPC_{50} value of 2.70 ± 1.50 mg/ml which was higher than the crude extract of 1.22 times, but lower than α-tocopherol of 3.42 times. The sugar moiety in the form of glycosides containing in the water semi-purified extract might prevent the lipid peroxidation inhibition by forming the hydrogen bonds between the hydroxyl groups and hydroperoxides (Judis et al., 2010). This agreed with the report of Özgen et al.

that have demonstrated the more lipid peroxidation inhibition of luteolin derivatives containing sugar group than the luteolin itself (aglycone) (Özgen et al., 2011). However, the ethyl acetate semi-purified extract which showed the highest free radical scavenging gave the lowest lipid peroxidation inhibition activity. Thus, free radical scavenging may not be the only factor to suppress lipid peroxidation.

2.2.3.3 Metal ion chelating assay of the semi-purified extracts

The ethyl acetate semi-purified extract of the hot water crude extract gave the highest metal chelating activity with the CC_{50} value of 0.45 ± 0.02 mg/ml, more than EDTA and its crude extract (CC_{50} value of 32.31± 0.84 mg/ml) of 1.60 and 71.80 times, respectively. The high metal chelating activity of the ethyl acetate semi-purified extract might be from its tetranortriterpenoid contents which are one of the major bioactive compounds found in the plants of Meliaceae family including Long Kong (Nishizawa et al., 1989). After partition of the hot water crude extract, the phenolic content was found in the ethyl acetate semi-purified extract more than in the crude extract which could render to serve as a chelator, by chelating with Fe^{2+} instead of ferrozine to prevent the formation of the ferrozine complex. However, it remains unclear that which specific components in this plant are responsible for this activity.

2.2.4 Tyrosinase inhibition activity of the semi-purified extracts

The ethyl acetate semi-purified extract gave the highest tyrosinase inhibition activity (IC_{50} value of 0.19±0.16 mg/ml), but less than kojic acid and the crude extract of 3.80 and 2.60 times, respectively. Tyrosinase inhibition might be from the hydroxyl groups of the phenolic compounds in the semi-purified extract that could form a hydrogen bond to the active site of the tyrosinase enzyme, leading to the

low enzymatic activity. Some tyrosinase inhibitors act through the binding of their hydroxyl groups to the active site of the enzyme, resulting in the steric hindrance or conformation change. Also, antioxidant activity may be one of the important mechanisms for tyrosinase inhibitory activity (Alam et al., 2011). Several studies have reported the tyrosinase inhibitory effect of Long Kong, but did not clearly confirm this inhibition mechanism. Tyrosinase inhibition (IC_{50}) of the ethyl acetate, butanol and water semi-purified extracts prepared from the hot water crude extract from leaves of Long Kong were shown in **Table 39**.

Table 39 Comparison of the *in vitro* tyrosinase inhibition of the three semi-purified extracts prepared from the hot water crude extract of leaves from Long Kong

Samples	Tyrosinase inhibition IC_{50} (mg/ml)
Water semi-purified extract	-
Butanol semi-purified extract	2.14 ± 0.32
Ethyl acetate semi-purified extract	0.19 ± 0.16
Kojic acid	0.01 ± 0.00

Note: Values represent mean±S.D. (n = 3).
IC_{50} = tyrosinase inhibition concentration at 50% activity (mg/ml)
- = no activity

2.2.5 Cytotoxicity on human skin fibroblast of the semi-purified extracts

The percentages of cell viability of the ethyl acetate, butanol and water semi-purified extracts (cell viability of 95.94 ± 0.55, 88.97 ± 0.23 and 81.51 ± 0.41%, respectively) were higher than the crude extract of 1.19, 1.10 and 1.01 times, respectively (**Table 40**). It is possible that after partition the purity of the crude extract increased and some toxic agents may be removed, thereby increasing cell viability.

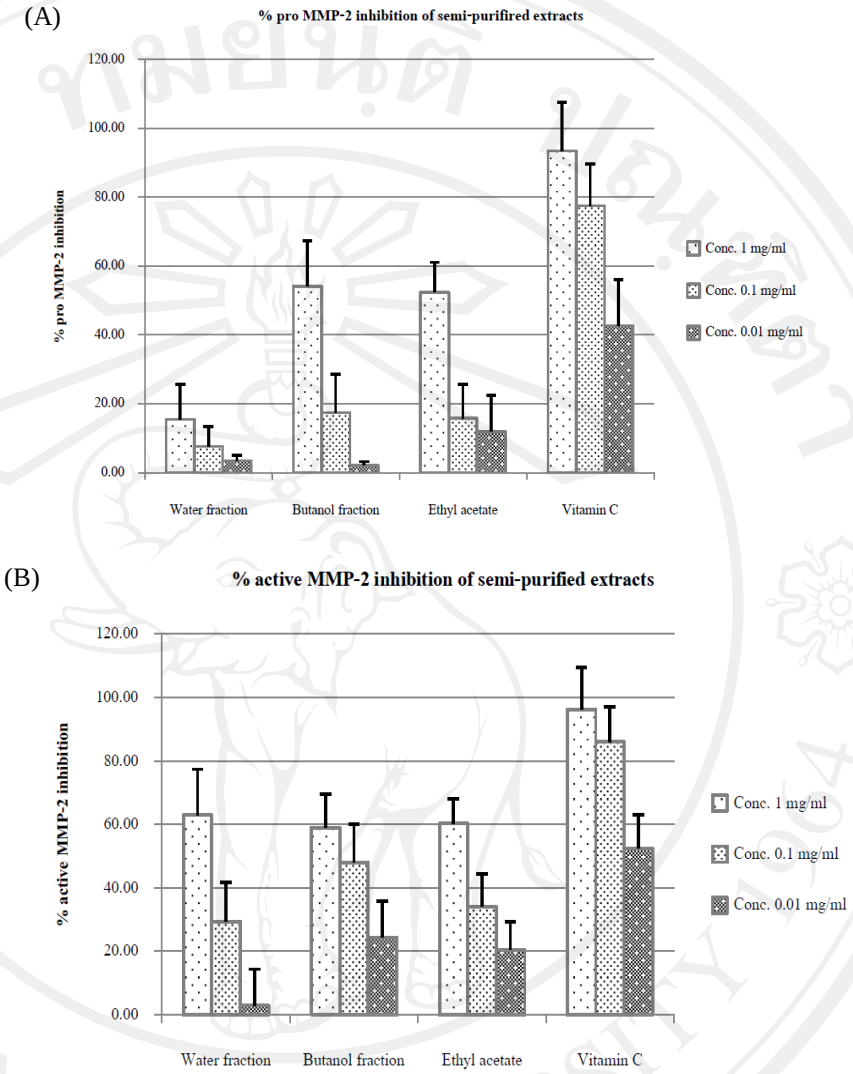
Table 40 Comparison of the percentages cell viability on human skin fibroblasts of the semi-purified extract prepared from the hot water crude extract of leaves from Long Kong

Samples	%cell viability
Water semi-purified extract	81.51 ± 0.41
Butanol semi-purified extract	88.97 ± 0.23
Ethyl acetate semi-purified extract	95.94 ± 0.55
Ascorbic acid	119.91 ± 9.78

Note: Values represent mean±S.D. (n = 3) Percentages of cell viability at the sample concentration 1 mg/ml

2.2.6 Gelatinolytic activity on MMP-2 inhibition activity of the semi-purified extracts

All semi-purified extracts at 0.01-1 mg/ml showed the inhibitory effect against pro and active MMP-2 expressions in the dose-dependent manner (**Figure 42**). The water semi-purified extract (1 mg/ml) indicated the highest active MMP-2 inhibition of 62.99±14.35%, but not significant different in comparing to the ethyl acetate (60.23±7.89%) and butanol (58.95±10.52%) semi-purified extracts. The water semi-purified extract inhibited significantly ($p < 0.05$) the active MMP-2 lower than ascorbic acid (96.22 ± 11.04%) and the crude extract (72.96 ± 13.44%) of 1.53 and 1.16 times, respectively. The butanol, ethyl acetate and water semi-purified extracts exhibited the pro MMP-2 inhibition of 54.11 ± 13.15 and 52.37 ± 8.67%, respectively, which were lower than ascorbic acid (93.46 ± 14.09%) of 1.73 and 1.78 times and the crude extract of 1.35 and 1.39 times, respectively. Thus, all semi-purified extracts showed more active MMP-2 inhibition than pro MMP-2. As known, the pro MMP-2 can be activated not only from the ROS production, but also other factors such as MT-1-MMP and TIMP-2. The high free radical scavenging abilities



Note: MMP-2 inhibition (%) =100 − [(*MMP − 2 content of the sample* / *MMP − 2 content of the control*) × 100]

Figure 42 The gelatinolytic activity of pro (A) and active (B) MMP-2 inhibition (% of the control) from the three semi-purified extracts prepared from the hot water crude extract of leaves of Long Kong and ascorbic acid at 0.01, 0.1 and 1 mg/ml.

of the semi-purified extracts may lead to the inhibition of the expression levels of MMP-2. Moreover, the phenolic compounds have also been reported to inhibit MMP-2 (Weng et al., 2012). The crude extract showed higher activity than the semi-purified extracts, owing to its synergistic effect with the phenolic compounds of many other compounds existing in the crude extract such as ascorbic acid (Lim et al., 2007), maleic acid and malic acid (Chairgulprasert et al., 2006). The zymograms of gelatinolytic activity on MMP-2 inhibition of the semi-purified extracts were shown in **Figure 43**.

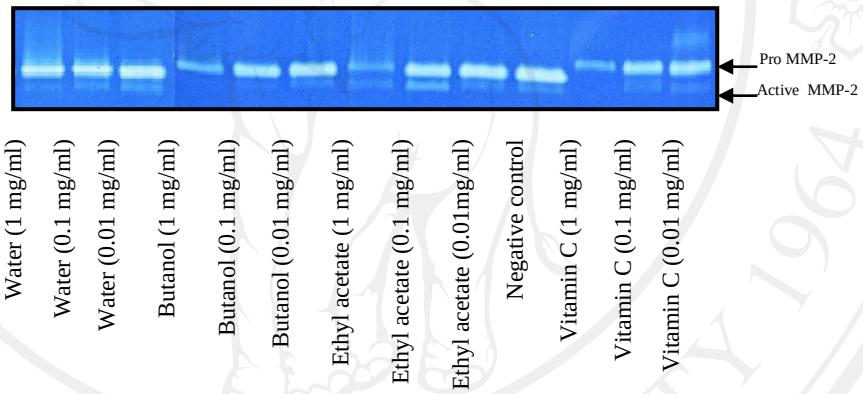


Figure 43 Zymograms of pro and active MMP-2 inhibition of the semi-purified fractions partitioned from the hot water crude extract from leaves of Long Kong and vitamin C at 0.01, 0.1 and 1 mg/ml and the negative control (5% DMSO)

2.2.7 Specification of the semi-purified extract

2.2.7.1 Solubility test

The physical appearances of the three semi-purified extracts at 0.1% dissolved in distilled water were shown in **Table 41**. The pH value of the water and butanol semi-purified extracts were acidic with the pH value of 6. The pH value

Table 41 Physical appearances and pH values of the semi-purified extracts (0.1% dissolved in distilled water)

Samples	pH	Physical appearances of the solution
Water soluble extract	6.0	Pale brown and clear solution
Butanol soluble extract	6.0	Pale brown and clear solution
Ethyl acetate soluble extract	4.5	Pale greenish brown and clear solution

of the ethyl acetate semi-purified extract was more acidic than the water and butanol semi-purified extract with the pH value of 4.5. The physical appearances of the water and butanol semi-purified extracts were in pale brown color and clear solution, while the ethyl acetate semi-purified extract was in pale greenish-brown color and clear solution. For the solubility, the water semi-purified extract prefer dissolved in polar solvents including hot (60ºC) and cold water (25ºC), ethanol and propylene glycol, but sparingly soluble in methanol and glycerol. The butanol and ethyl acetate semi-purified extracts can dissolve in propylene glycol which is a medium polar solvent.

However, the butanol semi-purified extract was slightly dissolved in water, but the ethyl acetate semi-purified extract was insoluble in hot (60ºC) and cold (25ºC) water. The solubility properties of the three semi-purified extracts were indicated in **Table 42**.

2.2.7.2 Chemical stability test

The chemical stability properties of the three semi-purified extracts were exhibited in **Table 43**. All semi-purified extracts were unstable in the strong basic solution (10% sodium hydroxide) and reducing solution (10% ferric chloride). As known, the semi-purified extract with the specific pH value of 4.5 – 6.0 was acidic. Therefore, the major chemical constituents in these semi-purified extracts

Table 42 Solubility of the three semi-purified extracts prepared from the hot water crude extract of leaves from Long Kong

Samples	hot water	cold water	methanol	ethanol	mineral oil	propylene glycol	glycerol
Water semi-purified extract	Soluble	Soluble	Sparingly soluble	Soluble	Sparingly soluble	Soluble	Sparingly soluble
Butanol semi-purified extract	Sparingly soluble	Sparingly soluble	Sparingly soluble	Very slightly soluble	Sparingly soluble	Soluble	Sparingly soluble
Ethyl acetate semi-purified extract	Insoluble	Insoluble	Sparingly soluble	Sparingly soluble	Sparingly soluble	Soluble	Sparingly soluble

Note: Temperatures of the hot and cold water were 60ºC and 25ºC, respectively

Table 43 Chemical stability of the three semi-purified extracts prepared from the hot water crude extract of leaves from Long Kong

Samples	10% NH_4OH	10% CH_3COOH	10% HCl	10% CH_3COONa	10% H_2O_2	10% NaOH	10% $FeCl_3$
	weak base	weak acid	strong acid	sodium salt of weak acid	oxidizing agent	strong base	reducing agent
Water semi-purified extract	-	-	-	-	-	+	+
Butanol semi-purified extract	-	-	-	-	-	+	+
Ethyl acetate semi-purified extract	-	-	-	-	-	+	+

Note: "-" represents stable and "+" represents unstable

might susceptible to higher pH value. In addition, the chlorophyll existing in the semi-purified extracts could be a factor affecting the unstability in the basic condition. According to the cationic aromatic acyl group (positive charge) of chlorophyll, the acid such as hydrochloric acid could hydrolyse or gave a proton to the native chemical structure of the chlorophyll and contributed to their stability in acidic condition. As

the pH raise, a rapid loss of proton or anion (negative charge) of the chlorophyll will tend to be unstable which become deeper colors (Torskangerpoll et al., 2005). The results of this study agreed with the previous study that catechins derivatives and gallic acid from tea leaves, were chemically unstable in alkaline environment (Aucamp et al., 2000).

2.2.7.3 HPLC fingerprint profile

The peak of the standard gallic acid was at the retention time of 3.235 min. The gallic acid peak presented in the chromatogram of the hot water crude extract of leaves from Long Kong (20 mg/ml) was at the retention time of 3.188 min, whereas the gallic acid peak presented in the chromatograms of the water, butanol and ethyl acetate semi-purified extracts (20 mg/ml) were at the retention time 3.012, 3.147 and 3.272 min, respectively. The percentages of the gallic acid contents containing in the crude extract and its semi-purified extracts were determined as shown in **Table 44**.

Table 44 Percentages of the gallic acid contents in the hot water crude extract and its semi-purified extracts from leaves of Long Kong

Samples	Retention time (min)	Peak area	Percentages of gallic acid content (g/g)
gallic acid	3.235	28220570	100.0
hot water crude extract	3.188	13383356	2.37
water semi-purified extract	3.012	6038084	1.07
butanol semi-purified extract	3.147	25705832	4.55
ethyl acetate semi-purified extract	3.272	34756720	6.16

The percentages of gallic acid contents presented in the ethyl acetate and butanol semi-purified extracts were higher than the crude extract of 2.60 and 1.92

times, respectively. The percentages of the gallic acid contents in the water semi-purified extract was lower than the crude extract of 2.21 times. However, the total phenolic contents existing in the semi-purified extract may contain other phenolic compounds which had the higher content in the semi-purified extract than the crude extract. The chromatographic fingerprint profile using HPLC of the three semi-purified extracts were demonstrated in **Figures 44 - 45**.

The percentages of the gallic acid contents presented in the three semi-purified extracts were decreased in the order of ethyl acetate, butanol and water semi-purified extracts, respectively. The percentages of the gallic acid contents in the three semi-purified extracts related to the total phenolic and flavonoid contents. In fact, gallic acid was found naturally both in free form and a part of tannins (Soong et al., 2006). However, gallic acid was produced during the hydrolysis reaction that had the influence to reduce the pH of the black tea (Aucamp et al., 2000). Therefore, natural gallic acid existing in the crude extract might be more hydrolyzed to free gallic acid in ethyl acetate which was a hydrolysis reacting solvent than water and butanol (Dutia, 2004).

2.3 Selection of the semi-purified extract

The ethyl acetate semi-purified extract was selected for the further development according to its high DPPH radical scavenging, metal ion chelation and tyrosinase inhibition, moderate lipid peroxidation inhibition as well as high MMP-2-inhibition. This semi-purified extract also showed no cytotoxicity on human skin fibroblasts and gave the highest gallic acid content as bioactive compounds in comparing to other semi-purified extracts.

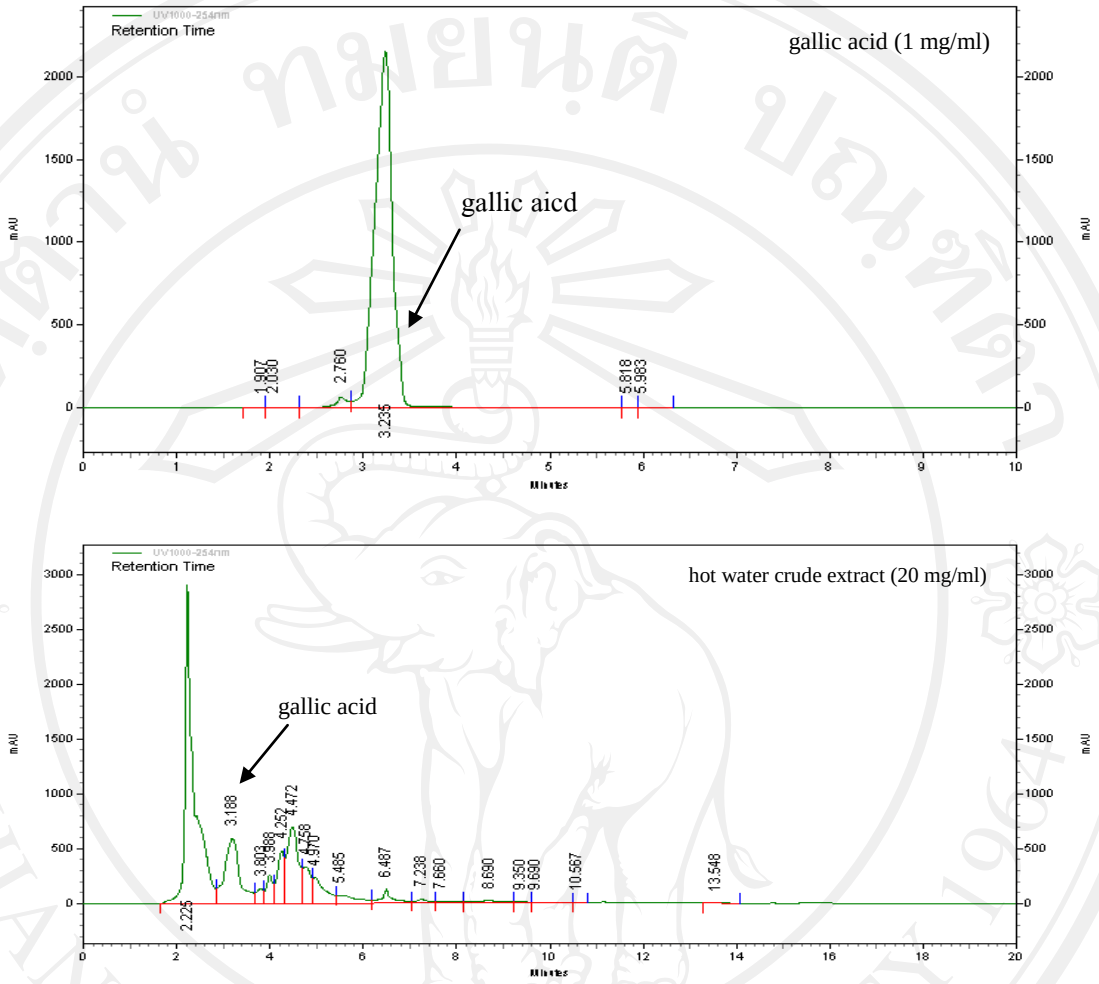


Figure 44 Chromatograms of the gallic acid (1 mg/ml) at the retention time of 3.235 min and the hot water crude extract of leaves from Long Kong (20 mg/ml) which gave the gallic acid peak at the retention time of 3.188 min

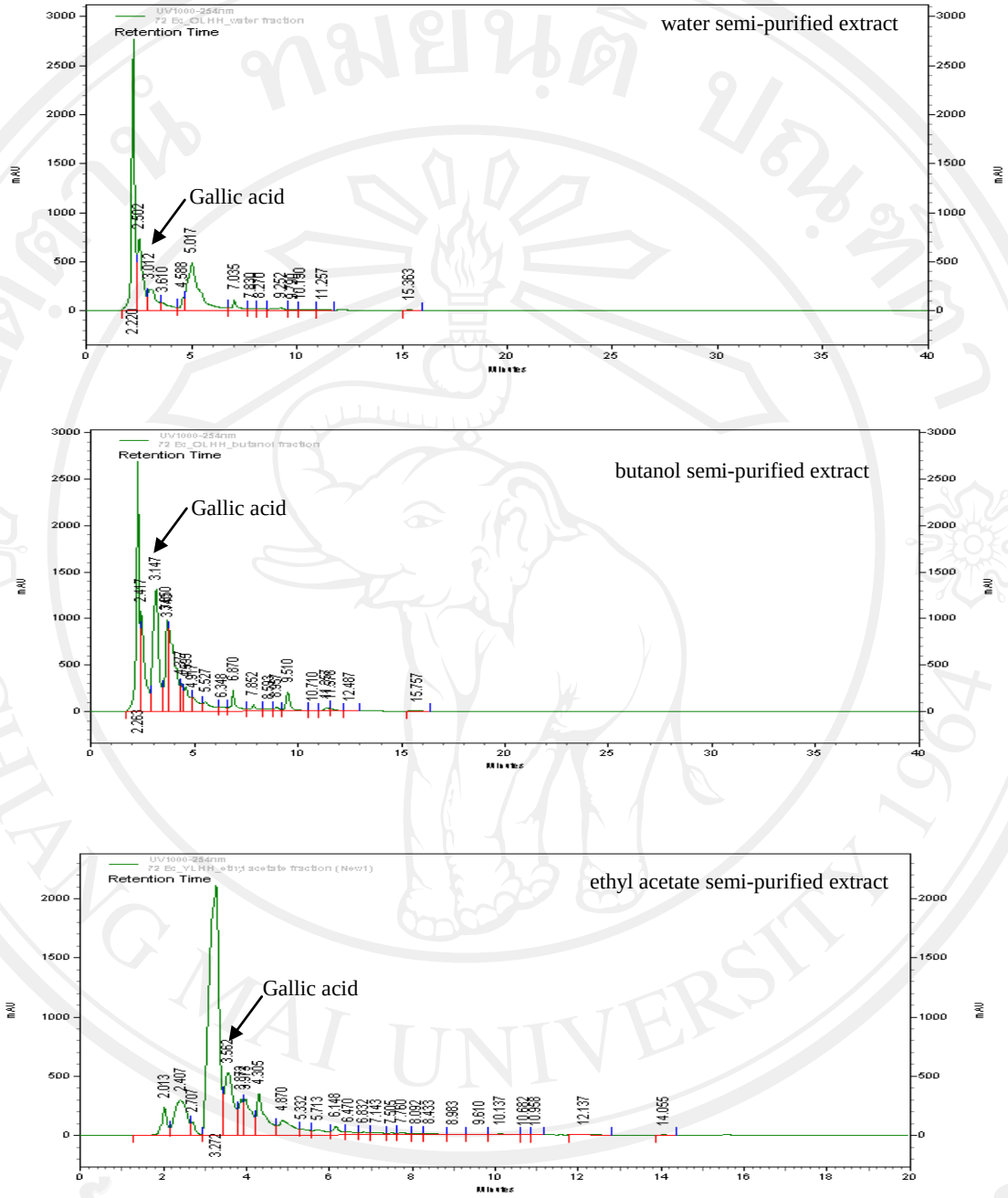


Figure 45 Chromatograms of the water, butanol and ethyl acetate semi-purified extracts prepared from the hot water crude extract of the leaves from Long Kong showed the gallic acid peak at the retention time of 3.012, 3.147 and 3.272 min, respectively.

Part 3 : Discoloration and *in vitro* anti-aging activities of the semi-purified extract

3.1 Percentages yield of the discolored fractions

After discoloration of the ethyl acetate semi-purified extract, the water and chloroform fractions were obtained. The discolored water fraction was faded to paler green than the discolored chloroform fraction. The result indicated that the chlorophyll from leaves of Long Kong might be eliminated by the non-polar solvent (chloroform). The other bioactive compounds such as phenolic and flavonoid were expected to be in the polar solvent (water). The discolored water fraction (37.01%) gave higher percentage yield than the discolored chloroform fraction (11.12%) of 3.34 times (**Table 45**).

Table 45 Percentages yield of the discolored water and chloroform fractions

Samples	%yields (w/w) of the dried leaf powder	%yields (w/w) of the hot water crude extract	%yields (w/w) of the ethyl acetate semi-purified extract
Discolored water fraction	0.19	1.05	37.01
Discolored chloroform fraction	0.06	0.31	11.12

3.2 Characteristics of the discolored fractions

3.2.1 Phytochemical tests of the discolored fractions

The phytochemicals of the discolored fractions were different depending on the distinct polarity of the extracting solvents. The phytochemical constituents of the discolored water and chloroform fractions were related to the ethyl acetate semi-purified extract. All discolored fractions contained flavonoids which could be divided to glycone and aglycone. The low polar flavonoids and aglycones

(i.e. isoflavones, flavanones, methylated flavones, and flavonols) may be partitionated by chloroform, while the flavonoid glycosides and the more polar aglycones (i.e. flavan-3-ols, dihydroflavonol and amentoflavone) were probable extracted by water. Triterpenoids which were moderate polar compounds were found only in the discolored chloroform fraction (**Table 46**).

Table 46 Phytochemical constituents of the discolored fractions prepared from the ethyl acetate semi-purified extract

Samples	Triterpenoids	Alkaloids	Anthraquinones	Reducing sugars			Flavonoids	Carotenoid	Tannins
				F	G	S			
Discolored water fraction	-	-	-	-	-	-	+	-	-
Discolored chloroform fraction	+	-	-	-	-	-	+	-	-

Note: "+" represented presence in the extract. "−" represented absence in the extract. F represented for fructose. G represented for glucose. S represented for sucrose

3.2.2 Determination of the total phenolic and flavonoid contents of the discolored fractions

The discolored water fraction gave higher phenolic (750.12±0.12 µg GAE/g extract) and flavonoid contents (515.30 ± 0.64 µg QE/g extract) than the discolored chloroform fraction (543.21 ± 0.24 µg GAE/g extract and 445.23 ± 0.21 QE/g extract). However, the total phenolic content containing in the discolored water and chloroform fractions was lower than the ethyl acetate semi-purified extract (868.90±0.02 µg GAE/g extract) of 1.16 and 1.60 times, respectively, while the discolored water and chloroform fractions exhibited higher flavonoid contents than

the ethyl acetate semi-purified extract (422.39±0.01 µg QE/g extract) of 1.22 and 1.05 times, respectively. This indicated that phenolic compounds containing in the ethyl acetate semi-purified extract might not be only the flavonoid compounds, but also other phenolic compounds which might be loss during the discolored fraction preparation process. As known, phenolic compouds are the large and diverse group of molecules, which includes many different families of aromatic secondary metabolites in plants. These phenolics can be classified into non-soluble compounds such as condensed tannins, lignins, cell wall bound hydroxycinammic acids, and the soluble compounds such as phenolic acids, phenylpropanoids, flavonoids and quinines (Harborne et al., 2000). The total phenolic and flavonoid contents of the discolored water and chloroform fractions were shown in **Figure 46**.

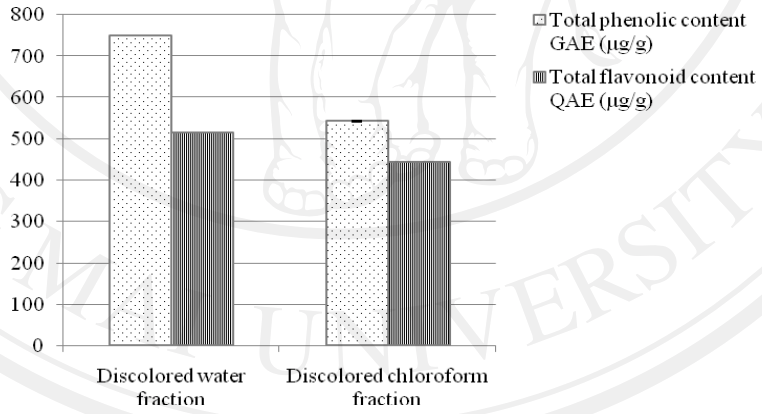


Figure 46 Total phenolic and flavonoid contents of the discolored fractions prepared from the ethyl acetate semi-purified extract

3.2.3 *In vitro* anti-aging activities of the discolored fractions determination

The antioxidant activities including DPPH radical scavenging, lipid peroxidation and metal ion chelating were demonstrated in **Table 47**.

Table 47 Antioxidant activity of the discolored fractions prepared from the ethyl acetate semi-purified extract

Discolored fractions	DPPH radical scavenging	Metal chelating	Lipid peroxidation inhibtion
	SC_{50} (mg/ml)	CC_{50} (mg/ml)	IPC_{50} (mg/ml)
Discolored water fraction	0.09 ± 0.04	0.28 ± 0.17	26.46 ± 6.24
Discolored chloroform fraction	0.18 ± 0.16	113.87 ± 36.98	28.36 ± 3.02
Ascorbic acid	0.08 ± 0.01	ND	ND
EDTA	ND	0.45 ± 0.06	ND
α-Tocopherol	ND	ND	0.12 ± 0.05

Note: Values represent mean±S.D. (n = 3).
SC_{50} = scavenging concentration at 50% activity (mg/ml)
IPC_{50} = inhibition peroxidation concentration at 50% activity (mg/ml)
CC_{50} = chelating concentration at 50% activity (mg/ml)
ND = not done

3.2.3.1 DPPH radical scavenging activity of the discolored fractions

The discolored water fraction showed the highest DPPH radical scavenging activity with the SC_{50} values of 0.09±0.04 mg/ml which was higher than the ethyl acetate semi-purified extract of 2.56 times, but lower than ascorbic acid of 1.125 times. According to the semi-purified extracts prepared from the leaves, it may be brought to the impurities composition such as chlorophyll which was a hindrance for scavenging ability. Therefore, after pigment elimination, the scavenging ability was higher. In fact, the pigment including chlorophyll and riboflavins initiating photosensitized oxidation in foods via photochemically and can activate free radicals initiation (Bradley et al., 1992).

3.2.3.2 Lipid peroxidation inhibition activity of the discolored fractions

The discolored water fraction gave slightly higher lipid peroxidation inhibition with the IPC_{50} value of 26.46 ± 6.24 mg/ml than the discolored chloroform fraction (28.36 ± 3.02 mg/ml) of 1.07 times, and also higher lipid peroxidation

inhibition than the ethyl acetate semi-purified extract (33.48 ± 14.49 mg/ml) of 1.27 times. However, the discolored water fraction indicated lower lipid peroxidation inhibition than α-tocopherol (0.12 ± 0.05 mg/ml) of 220.50 times. The lipid peroxidation inhibition of the discolored fractions which was higher than the ethyl acetate semi-purified extract might be from the pigment removing that caused the generation of the primary hydroperoxides (Wanasundara et al., 2005).

3.2.3.3 Metal ion chelating assay of the discolored fractions

The discolored water fraction gave the highest metal ion chelating activity with the CC_{50} value of 0.28 ± 0.17 mg/ml which was higher than the ethyl acetate semi-purified extract (0.45 ± 0.02 mg/ml), and slightly more than EDTA of 1.61 times. The increasing metal chelaing ability of the discolored water fraction might be due to the polar chelating agents containing in this plant such as citric acid, malic acid and maleic acid (Chairgulprasert et al., 2006).

3.2.4 Tyrosinase inhibition activity of the discolored fractions

The discolored water fraction gave the highest tyrosinase inhibition with the IC_{50} value of 0.11 ± 0.05 mg/ml, which was slightly higher than the discolored chloroform fraction (IC_{50} value of 0.14 ± 0.03 mg/ml) and the ethyl acetate semi-purified extract (0.19 ± 0.16 mg/ml) of 1.27 and 1.73 times, respectively (**Table 48**). However, the discolored water fraction exhibited lower tyrosinase inhibition than kojic acid of 22 times. The tyrosinase inhibition of the discolored fractions did not demonstrate the significantly difference from the ethyl acetate semi-purified extract. This indicated that the natural tyrosinase inhibitor compounds might be not eliminated during the discoloration and also presented slightly higher inhibitory effect

Table 48 Tyrosinase inhibition activity of the discolored fractions prepared from the ethyl acetate semi-purified extract

Samples	**IC_{50} (mg/ml)**
Discolored water fraction	0.11 ± 0.05
Discolored chloroform fraction	0.14 ± 0.03
Kojic acid	0.005 ± 0.00

Note: Values represent mean±S.D. (n = 3).
IC_{50} = tyrosinase inhibition concentration at 50% activity (mg/ml)

against tyrosinase enzyme. The tyrosinase inhibitor containing in the discolored water and chloroform fractions might have different polarities from using the different extracting solvents. Indeed, flavonoids can be used as tyrosinase inhibitors due to their formation of the copper-flavonoid complexes. The tyrosinase enzyme is primarily quenched by the hydroxyl groups in the rings of the flavonoids. The numbers of hydroxyl group of these flavonoids refers to their polarities which could be partitioned by the extracting solvents (Donghyun et al., 2006).

3.2.5 Cytotoxicity on human skin fibroblasts of the discolored fractions

At 1 mg/ml, the discolored water and chloroform fractions showed no cytotoxicity against human skin fibroblasts with the percentages of cell viability at 126.29 ± 0.81 and 96.86 ± 0.86%, respectively (**Table 49**). The discolored water fraction also gave higher cell viability than the ethyl acetate semi-purified extract (95.94 ± 0.55%) of 1.32 times, but lower than the ascorbic acid of 1.14 and 1.48 times, respectively. The high cell viability of the discolored fractions might be due to the removal of chlorophyll. The formation of a semiquinone free radical which damaged the living cell derived from chlorophyll in the reaction of photoreduction has been reported (Krasnovsky, 1953). However, the discolored fractions were not the

pure compounds but in complex mixture of the naturally occurring phytochemicals that might be toxic to human skin fibroblasts.

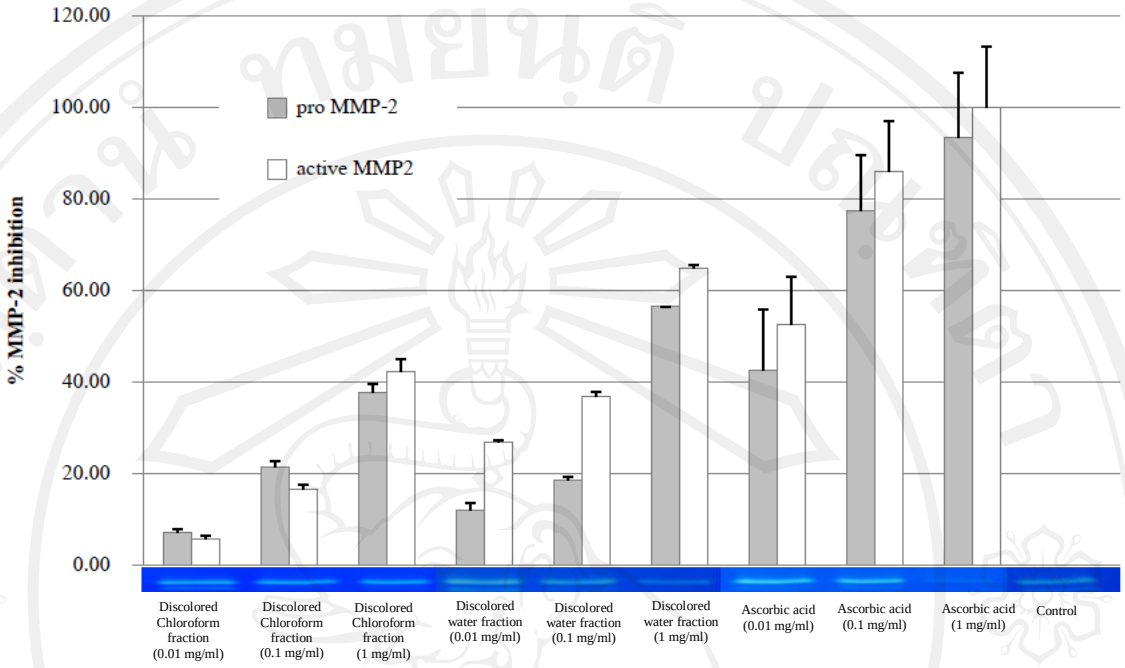
Table 49 Cytotoxicity on the human skin fibroblasts of the discolored fractions prepared from the ethyl acetate semi-purified extract

Samples	%cell viability
Discolored water fraction	126.29 ± 0.81
Discolored chloroform fraction	96.86 ± 0.86
Ascorbic acid	143.77 ± 0.30

Note: Values represent mean±S.D. (n = 3).
Percentages of cell viability at the sample concentration 1 mg/ml

3.2.6 Gelatinolytic activity on MMP-2 inhibition activity of the discolored fractions

All discolored fractions at 0.01-1 mg/ml showed the inhibitory effect against pro and active MMP-2 expressions in the dose-dependent manner. At 1 mg/ml, the discolored water fraction indicated the active MMP-2 inhibition of 64.83 ± 0.88%, which was not significant different from the discolored chloroform fraction (60.23±7.89%) and the ethyl acetate semi-purified extract (60.23 ± 7.89%). However, the discolored water and chloroform fraction inhibited significantly ($p < 0.05$) the active MMP-2 lower than ascorbic acid (100.00±13.22%) of 1.54 times. The discolored water fraction exhibited the pro MMP-2 inhibition of 56.51 ± 0.04% which was not significant different from the ethyl acetate semi-purified extract (52.37 ± 8.67 %), but lower than ascorbic acid (93.46±14.09%) of 1.65 times. The inhibitory effect of pro and active MMP-2 of the discolored water and chloroform fractions and ascorbic acid at 0.01-1 mg/ml were illustrated in **Figure 47**.



Note: MMP-2 inhibition (%) =100 − [(*MMP − 2 content of the sample / MMP − 2 content of the control*) × 100]

Figure 47 Comparison of the percentages of pro and active MMP-2 inhibition of the discolored water and chloroform fractions prepared from the ethyl acetate semi-purified extract of leaves from Long Kong

3.2.7 Specification of the selected discolored fractions

3.2.7.1 Solubility test

After color elimination of the ethyl acetate semi-purified extract, the pH values of the discolored water fraction was slightly lower than the discolored chloroform fraction and the ethyl acetate semi-purified extract, while the pH value of the discolored chloroform fraction was similar to the ethyl acetate semi-purified extract. This might be due to the pH levels of the chlorophyll (which were in the pH range of 5.5 – 7.5 that may interfere with the ethyl acetate semi-purified extract) was removed (Ryan-Stoneham et al., 2000). In addition, the leaves of Long

Kong might contain acidic compounds such as malic acid, gallic acid ascorbic acid which could be better dissolved in the polar than the non-polar solvent (Chairgulprasert et al., 2006). Physical appearances and pH values of the 0.1% discolored fractions dissolved in distilled water were shown in **Table 50**.

Table 50 Physical appearances and pH values of the 0.1% discolored fractions dissolved in distilled water

Samples	pH	Physical appearances of solutions
Discolored water fraction	3.0	Pale green and clear solution
Discolored chloroform fraction	4.5	Pale greenish-brown and clouded solution

For the solubility, the discolored water and chloroform fractions prepared from the ethyl acetate semi-purified extract could be dissolved in different polarity solvents. The discolored water fraction which was expected to be the polar extract could be sparingly soluble in hot (60ºC) and cold (25ºC) water, but insoluble in methanol, ethanol, mineral oil, propylene glycol and glycerol. The discolored chloroform which expected to be non-polar extract could be sparingly soluble in methanol and ethanol, and slightly soluble in propylene glycol, but insoluble in hot (60ºC) and cold (25ºC) water, mineral oil and glycerol. These results were slightly different from the ethyl acetate semi-purified extract. The ethyl acetate semi-purified extract could be soluble in the medium of the polar solvents in comparing to others including methanol, ethanol, mineral oil and glycerol. However, propylene glycol could be miscible with both water and chloroform. Therefore, both discolored fractions could be soluble in propylene glycol. The solubility of the discolored water

and chloroform fractions prepared from the ethyl acetate semi-purified extract of leaves from Long Kong were presented in **Table 51**.

3.2.7.2 Chemical stability test

All discolored fractions exhibited unstability in the strong basic solution (10% sodium hydroxide) and reducing solution (10% ferric chloride). The chemical stabilities of these discolored fractions were similar to the ethyl acetate semi-purified extract. As known, natural concomitant compounds in the plant extract can affect the physical and chemical stability of the plant extract (Eder et al., 1998).

Table 51 Solubility of the discolored water and chloroform fractions prepared from the ethyl acetate semi-purified extract of leaves from Long Kong

Samples	Hot water	Cold water	Methanol	Ethanol	Mineral oil	Propylene Glycol	Glycerol
Discolored water soluble fraction	Soluble	Soluble	Soluble	Soluble	Slightly soluble	Soluble	Slightly soluble
Discolored chloroform soluble fraction	Insoluble	Insoluble	Sparingly soluble	Sparingly soluble	Insoluble	Slightly soluble	Insoluble

Therefore, the stability trend of the discolored fractions which was similar to the ethyl acetate semi-purified extract might be from the same chemical compositions. The chemical stability of the discolored water and chloroform fractions prepared from the ethyl acetate semi-purified extract against various chemicals were demonstrated in **Table 52**.

Table 52 Chemical stability of the discolored fractions prepared from the ethyl acetate semi-purified extract from leaves of Long Kong

Samples	10% sodium hydroxide	10% acetic acid	10% hydrochloric acid	10% sodium acetate	10%ammonium hydroxide	10%sodium hydroxide	10%feric chloride
Discolored water fraction	-	-	-	-	-	+	+
Discolored chloroform fraction	-	-	-	-	-	+	+

Note: "-" represents stable in the chemical and "+" represents unstable in the chemical

3.2.7.3 HPLC fingerprint profile

The chromatographic fingerprint profile using HPLC of the discolored water fraction was shown in **Figure 48**.

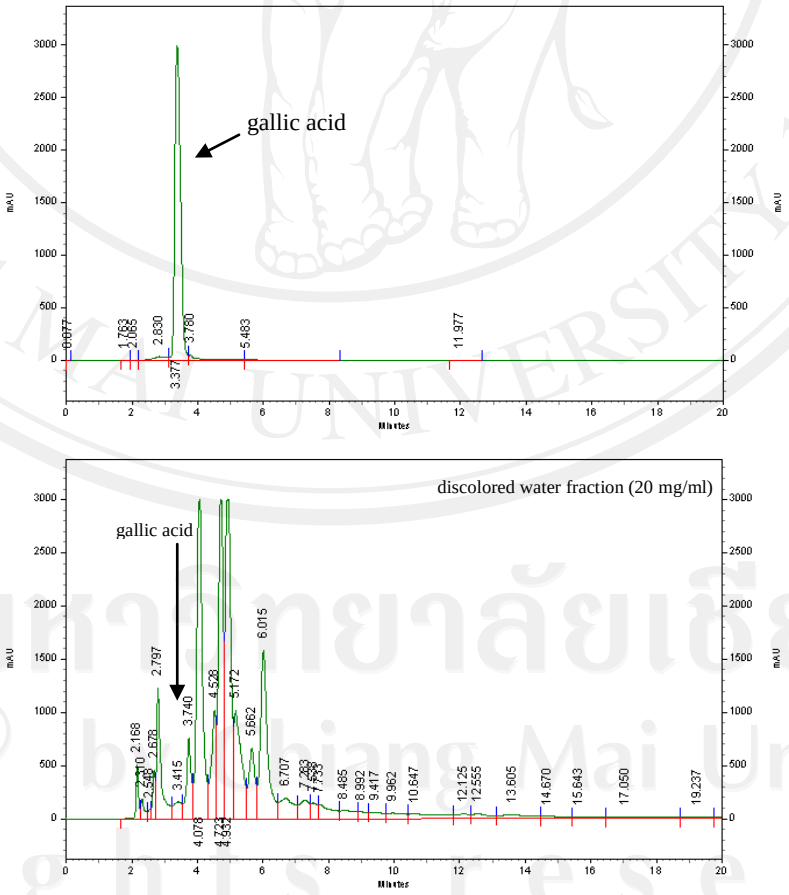


Figure 48 Chromatograms of the gallic acid and the discolored water fraction

The gallic acid peak was indicating the gallic acid peak at the retention time of 3.377 and 3.415 min, respectively. The gallic acid peak in the chromatogram of the discolored water fraction was observed at the retention time of 3.415 min, while the gallic acid peak was absent in the chromatogram of the discolored chloroform fraction.

The percentages of gallic acid contents in the discolored water fraction (0.36%) was lower than those in the ethyl acetate semi-purified extract (6.16%) of 17.11 times. The percentages of the gallic acid contents of the discolored water fraction were shown in **Table 53**. After solvent-solvent partition of the ethyl acetate semi-purified extract, gallic acid might be lost during the process, therefore, the quantity of gallic acid was reduced.

Table 53 Percentages of the gallic acid contents of the discolored water fraction prepared from the ethyl acetate semi-purified extract

Samples	**Concentration** (mg/ml)	**Retention time** (min)	**Peak area**	**Percentages of gallic acid content (g/g)**
gallic acid	1	3.377	36046532	100.00
discolored water fraction	20	3.415	2809305	0.39

3.3 Selection of the discolored semi-purified fraction

The discolored water fraction was clearly better biological activity than the discolored chloroform extract including DPPH radical scavenging, lipid peroxidation inhibition, metal ion chelation, tyrosinase inhibition and no cytotoxicity on human skin fibroblasts. However, the percentage yields and gave the gallic acid contents in the discolored water fraction were significant lower than the ethyl acetate semi-

purified extract. The comparison of *in vitro* the anti-aging activities of the ethyl acetate semi-purified extract and the discolored water fraction was shown in **Table 54**. In addition, the pH values of the discolored water extract were lower than the ethyl acetate semi-purified extract that when incorporated in the cosmetic formulations might affect the physicochemical stability. The optimal pH values of the developed topical formulations should be 4.5-5.5, since the skin's pH value is approximately at 5.5 (Boron et al., 2003). Therefore, the ethyl acetate semi-purified extract and the discolored water fraction were consequently selected for the *in vivo* rabbit skin irritation by the Draize test.

Table 54 Comparison of the *in vitro* anti-aging activities of the ethyl acetate semi-purified extract and the discolored water fraction prepared from leaves of Long Kong

Samples	%yield (w/w)	GA content (%)	DPPH (SC_{50}) (mg/ml)	Lipid IPC_{50} (mg/ml)	Metal CC_{50} (mg/ml)	Tyrosinase IC_{50} (mg/ml)	Cytotoxicity (1 mg/ml)	MMP-2 inhibition (%) (1 mg/ml)	
								Pro MMP-2	Active MMP-2
hot water crude extract	17.97	-	5.40±1.23	3.29±0.30	32.31±0.84	0.49±0.23	80.52±15.16	72.96±13.44	85.77±13.77
ehthyl acetate semi-purified extract	0.51	6.16	0.23±0.02	33.48±14.49	0.45±0.02	0.19±0.16	95.94±0.55	52.37±8.67	60.23±7.89
discolored water fraction	0.19	0.39	0.09±0.04	26.46±6.24	0.28±0.17	0.11±0.05	126.29±0.81	56.51±0.04	64.83±0.88

Note: Percentage yields of the hot water crude extract, ethyl acetate semi-purified extract and discolored water fraction were from 100 g of dried Long Kong leaf powder.

3.4 *In vivo* rabbit skin irritation by the Draize test of the ethyl acetate semi-purified extract and the discolored water fraction

The ethyl acetate semi-purified extract and the discolored water fraction were dissolved in the propylene glycol at 0.1, 0.3 and 0.5%, separately, and evaluated for the *in vivo* rabbit skin irritation by Draize test. The primary irritation index (PII) at 24, 48 and 72 h of the ethyl acetate and the discolored water fraction were shown in **Table 55**. The discolored water fraction gave no irritation on rabbit skin at 0.1% (PII = 0.2), but showed slightly irritation on rabbit skin at 0.3 and 0.5% (PII = 0.5 and 0.7,

respectively). Whereas, the ethyl acetate semi-purified extract demonstrated no irritation on rabbit skin at 0.1, 0.3 and 0.5% (PII = 0.1, 0.3 and 0.4, respectively). The 20% sodium lauryl sulfate which was used as a positive control exhibited moderate irritation on the rabbit skin (PII = 2.9). The discolored water fraction increased irritation on the rabbit skin with the dose-dependent manner, while the irritation on rabbit skin decreased with the time-dependent manner. The irritation on the rabbit skin of the discolored water fraction might be due to the acidic condition with the pH value of 3. These results demonstrated that the ethyl acetate semi-purified extract indicated a good safety profile on rabbit skin and appeared to be suitable for further development as the topical cosmetic formulations.

Table 55 Primary irritation index (PII) and category of irritation based on PII of the ethyl acetate semi-purified extract and the discolored water fraction prepared from leaves of Long Kong

Samples	Primary irritation index (PII)				Category of irritation based on PII
	24 h	**48 h**	**72 h**	**Average**	
0.1% ethyl acetate semi-purified extract	0.2	0.1	0.1	0.1	Negligible
0.3% ethyl acetate semi-purified extract	0.3	0.3	0.2	0.3	Negligible
0.5% ethyl acetate semi-purified extract	0.5	0.4	0.4	0.4	Negligible
0.1% discolored water extract	0.3	0.2	0.2	0.2	Negligible
0.3% discolored water extract	0.6	0.5	0.4	0.5	Slightly irritation
0.5% discolored water extract	0.8	0.6	0.6	0.7	Slightly irritation
20% sodium lauryl sulfate (positive control)	3.0	3.0	2.7	2.9	Moderate irritation
propylene glycol (negative control)	0.0	0.0	0.0	0.0	Negligible
Untreated area (control)	0.0	0.0	0.0	0.0	Negligible

Note: Grading scale for skin irritation effect following OECD Test Guideline 404
Primary Irritation Index (PII) = [(∑ *erythema grade at 24/48/72 h* + ∑ *edema grade at 24/48/72 h)/ 3* × *Number of animals*]

Part 4 : Development of cosmetic base formulations

4.1 Preparation of the cosmetic base formulations

The cosmetic base formulations were prepared in three different types including gel, serum and cream. Each type of the base formulation was prepared in three different formulas. The prepared gel base formulations were shiny, homogenous, transparent and odorless with the pH value of 5.5, while the prepared serum base formulations were glossy, had slippery texture and transparent appearance with the pH value of 5.0. Whereas, the prepared cream base formulations were homogenous, had smooth texture with white appearance and the pH value of 4.5.

4.2 Physical stability determination of the cosmetic base formulations by the heating and cooling cycle

The cosmetic base formulations, which were gel No.1, serum No. 1 and cream No.1, gave good physical stability with no phase separation and no change of the pH and had the slight viscosity change at the heating and cooling cycles (45±2 and 4±2^{o}C) for 12 days. The gel No.1 was shiny, had smooth texture and transparent appearance with the pH value of 5.5 (**Table 56**).

After stored under the heating and cooling cycles, the viscosity of gel No.1 slightly changed with no significant difference from at initial. The serum No.1 was glossy, had slippery texture, transparent appearance and odorless with the pH value of 5.0, and also gave no significant difference viscosity between at the initial and after the heating cooling cycles. The cream No.1 was homogenous, had smooth texture, white color and odorless. After the heating cooling cycles, the pH value of cream No.1 was slightly decreased, while its viscosity was not significantly different from at the initial (**Figure 49**).

Table 56 Physical appearances and characteristics (color, odor, texture and pH value) of the nine cosmetic base formulations kept at the heating and cooling cycle (4±2 and 45±2°C) for 6 cycles (12 days)

Formulations		Physical appearances	Color	Odor	pH	viscosity (cP)
Gel No.1	Before	Shiny and smooth texture	transparence	odorless	5.5	15210
	After	Shiny and smooth texture	transparence	odorless	5.5	15530
Gel No.2	Before	Shiny and smooth texture	transparence	odorless	5.5	8660
	After	Gloss and rough texture	transparence	odorless	5.5	8010
Gel No.3	Before	Shiny and smooth texture	transparence	odorless	5.5	7020
	After	Gloss and sedimentation	transparence	odorless	5.5	9510
Serum No.1	Before	Gloss and slippery texture	transparence	odorless	5.0	100
	After	Gloss and slippery texture	transparence	odorless	5.0	120
Serum No.2	Before	Gloss and slippery texture	transparence	odorless	5.0	260
	After	Bubble and rough texture	transparence	odorless	5.5	250
Serum No.3	Before	Gloss and slippery texture	transparence	odorless	5.5	50
	After	Gloss and rough texture	transparence	odorless	5.5	110
Cream No.1	Before	Uniformity and smooth texture	white	odorless	5.5	30940
	After	Uniformity and smooth texture	white	odorless	5.0	31430
Cream No.2	Before	Uniformity and smooth texture	white	odorless	5.5	13640
	After	Phase separation	white	odorless	5.0	19460
Cream No.3	Before	Uniformity and smooth texture	white	odorless	5.0	15080
	After	Phase separation	white	odorless	5.0	19050

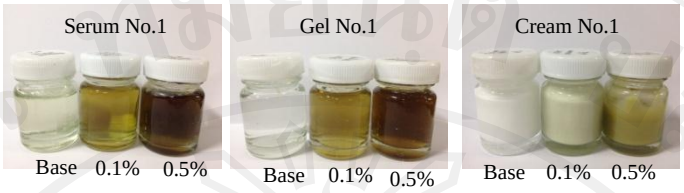


Figure 49 Physical appearance of serum No.1, gel No.1 and cream No.1

4.3 *In vivo* rabbit skin irritation by Draize test of the cosmetic base formulations

The best three cosmetic base formulations including gel No.1, serum No. 1 and cream No.1 which gave good physical stability were determined for irritation on the rabbit skin by the Draize test. **Table 5**7 presented the primary irritation index (PII) and the category of the irritation based on PII of various cosmetic base formulations. All cosmetic base formulations demonstrated no irritation on the rabbit skin after treated with the base formulations at 24, 48 and 72 h.

Table 57 Primary irritation index (PII) and category of the irritation based on PII of various cosmetic base formulations

Samples	Primary irritation index (PII)				Category of the irritation base on PII
	24 h	48 h	72 h	Average	
Gel No.1	0.0	0.4	0.2	0.2	Negligible
Serum No.1	0.0	0.2	0.3	0.3	Negligible
Cream No.1	0.0	0.4	0.4	0.4	Negligible
20% sodium lauryl sulfate (positive control)	3.0	3.0	2.7	2.9	Moderate irritation
Distilled water (negative control)	0.0	0.0	0.0	0.0	Negligible
Untreated area (control)	0.0	0.0	0.0	0.0	Negligible

4.4 Selection of the cosmetic base formulations

The best three cosmetic base formulations which were gel No.1, serum No.1 and cream No.1 were selected to prepare the topical formulations containing various concentrations (0.1 and 0.5%) of the semi-purified extract from the Long Kong leaves because of their good physical stability and no irritation on the rabbit skin.

Part 5 : Development of the cosmetic formulations containing the selected semi-purified extract prepared from the hot water crude extract from leaves of Long Kong

5.1 Preparation of the cosmetic formulations containing the semi-purified extract at various concentrations

Figure 50 presented the prepared ethyl acetate semi-purified extract dissolved in propylene glycol, propylene glycol base, cream formulations containing the ethyl acetate semi-purified extract at various concentrations, cream base and the commercial product used for *in vivo* anti-aging activity on human volunteers. The ethyl acetate semi-purified extract at 0.1 and 0.5% were incorporated in the selected cosmetic base formulations including gel, serum and cream. The physical appearance of the gel formulations containing the ethyl acetate semi-purified extract were in green color, transparent and sticky with the pH value of 5.0, while the serum formulations were in green color, transparent, slippery and low viscosity with the pH value of 5.0. The cream formulations containing the ethyl acetate semi-purified extract were in light green color, moderate viscosity and smooth texture with the pH value of 5.0. The ethyl acetate semi-purified

extract at 0.1 and 0.5% dissolved in propylene glycol gave green color, shiny and transparent with the pH value of 5.5. The ethyl acetate semi-purified extract which was in green color with the pH value of 4.5 might affect the color, odor and the pH value of each base formulation.

5.2 Physico-chemical stability of the cosmetic formulations containing the semi-purified extract at various concentrations

The physical stability of various cosmetic formulations containing the ethyl acetate semi-purified extract at 0.1 and 0.5% when kept at 4±2, 27±2 and 45±2^{o}C at the initial and after stored for 3 months was shown in **Table 58**.

Figure 50 Propylene glycol base (A), the ethyl acetate semi-purified extract at 0.1 (B), 0.3 (C) and 0.5% (D) dissolved in propylene glycol, the commercial product (E), cream base (F) and cream formulations containing the ethyl acetate semi-purified extract at 0.1 (G), 0.3 (H) and 0.5% (I)

All formulations gave good physical stability with no sedimentation, no layer separation and no color change at all temperatures (4±2, 27±2 and 45±2^{o}C) for 3 months, except the gel containing the ethyl acetate semi-purified extract at 0.1 and 0.5% which

Table 58 The physical stability of various cosmetic formulations containing the ethyl acetate semi-purified at 0.1 and 0.5% kept at 4±2, 27±2 and 45±2°C at initial and after stored for 3 months

Formulations	At initial	3 months		
		4±2°C	27±2°C	45±2°C
Viscosity (cP)				
Gel base	15340	16570	13220	10240
Gel containing 0.1% of the semi-purified extract	14250	18460	12040	9260
Gel containing 0.5 % of the semi-purified extract	13310	15320	11050	8410
Serum base	110	140	100	100
Serum containing 0.1% of the semi-purified extract	120	130	100	110
Serum containing 0.5% of the semi-purified extract	120	130	100	110
Cream base	31720	32140	29460	29120
Cream containing 0.1% of the semi-purified extract	30870	31610	29310	26730
Cream containing 0.5% of the semi-purified extract	30130	31220	29450	24200
pH value				
Propylene glycol base	6.0	6.0	6.0	6.0
0.1% of the semi-purified extract in propylene glycol	5.5	5.5	5.5	5.5
0.5% of the semi-purified extract in propylene glycol	5.5	5.5	5.5	5.5
Gel base	5.0	5.0	5.0	5.0
Gel containing 0.1% of the semi-purified extract	5.0	5.0	4.5	4.5
Gel containing 0.5 % of the semi-purified extract	5.0	5.0	4.5	4.5
Serum base	5.0	5.0	5.0	5.0
Serum containing 0.1% of the semi-purified extract	5.0	5.0	5.0	5.0
Serum containing 0.5% of the semi-purified extract	5.0	5.0	5.0	5.0
Cream base	5.0	5.0	5.0	5.0
Cream containing 0.1% of the semi-purified extract	5.0	5.0	5.0	5.0
Cream containing 0.5% of the semi-purified extract	5.0	5.0	5.0	5.0

Note : The viscosity of cream and gel formulations was measured using spindle L4 with speed at 10 rpm, while that of the serum formulations was measured using spindle TL7 with speed at 100 rpm.

exhibited significantly the decrease viscosity, pH value and color change at high temperature in comparing to low temperature. This might be due to the thermal degradation of the gel formulations. It has been reported that the viscosity of the Carbopol gel was decreased with the increase temperatures (Barry et al., 1979). Heat energy may also cause the breaking down of the polymer structure. The viscosity of the serum and cream were slightly decreased at the increasing temperatures according to the low amount of Carbopol which was used as a gelling agent.

The percentages remaining of gallic acid in various cosmetic formulations at various storage temperatures (4±2, 27±2 and 45±2°C) for 3 months were shown in **Figure 51**. The order of reactions, half lives and shelf lives of various cosmetic formulations containing the semi-purified extract at 0.1 and 0.5% when stored at various temperatures (27±2, 4±2 and 45±2ºC) were shown in **Table 59**.

The ethyl acetate semi-purified extract at 0.1 and 0.5% dissolved in propylene glycol showed the most correlation between $[A_0]$ and times of the zero order kinetic equation of r^2 = 0.9933 and 0.9726, respectively. This indicated that the ethyl acetate semi-purified extract dissolved in propylene glycol was thermally decomposed and the concentrations of the extract did not affect the rate of the degradation reactions. The shelf lives of gallic acid in the solution at 0.1 and 0.5% were 0.41 and 0.55 days at 4±2ºC, 0.34 and 0.54 days at 27±2ºC, 0.16 and 0.58 days at 45±2ºC, respectively. The half lives of these semi-purified extract solutions at 0.1 and 0.5% were 2.03 and 2.76 days at 4±2ºC, 1.69 and 2.71 at 27±2ºC and 1.44 and 2.90 at 45±2ºC, respectively (**Table 59**).

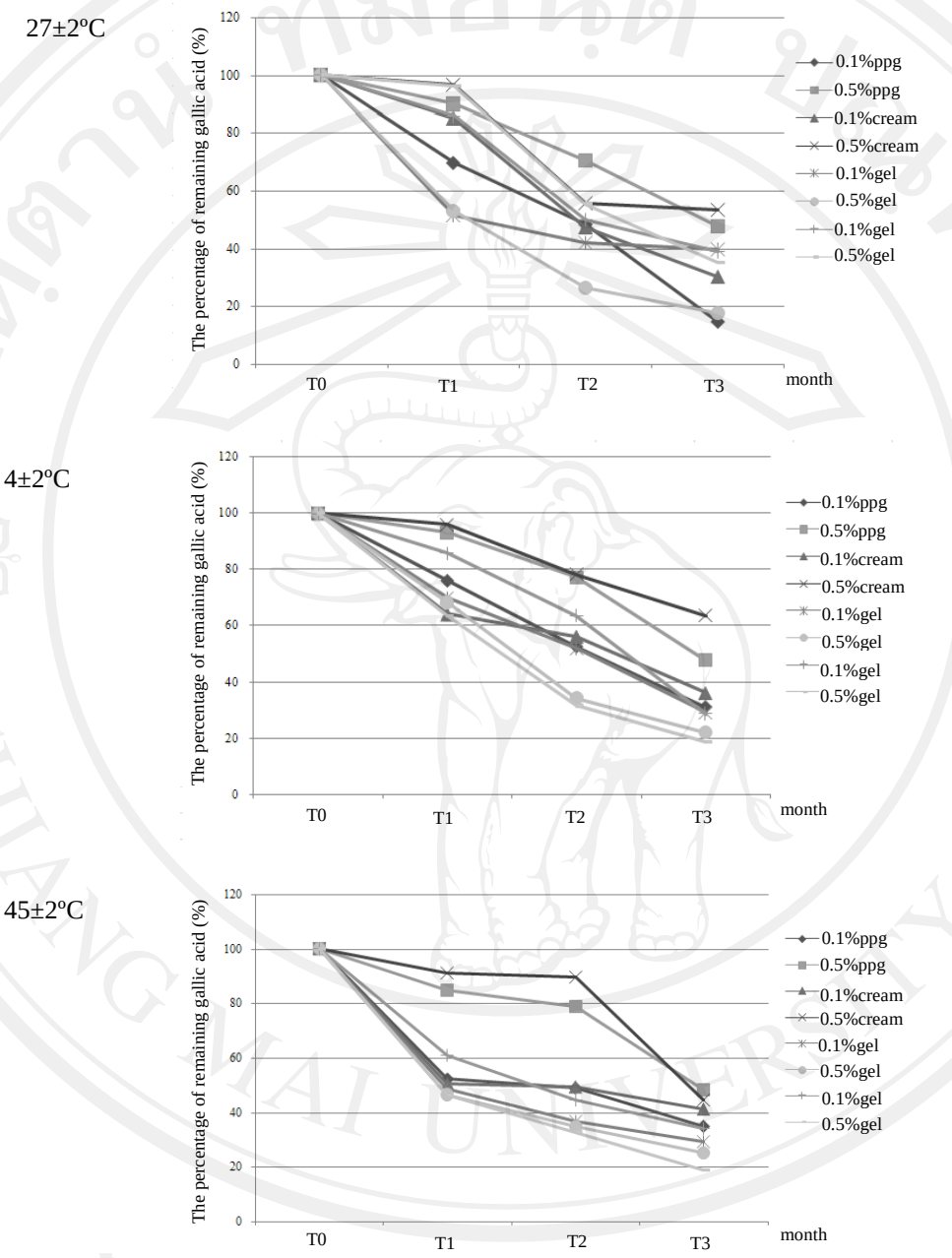


Figure 51 The percentages of gallic acid remaining in various cosmetic formulations at different storage temperatures (27±2, 4±2 and 45±2°C) for 3 months

Table 59 The order of reactions, half lives and shelf lives of various cosmetic formulations stored at various temperatures (27±2, 4±2 and 45±2ºC)

Samples	Stored temperature	Order of reaction	Half lives (days)	Shelf lives (days)
0.1% ppg	27±2ºC	zero order (r^2 = 0.9933)	1.69	0.34
	4±2ºC	zero order (r^2 = 0.9994)	2.03	0.41
	45±2ºC	zero order (r^2 = 0.8189)	1.44	0.16
0.5% ppg	27±2ºC	zero order (r^2 = 0.9726)	2.71	0.54
	4±2ºC	zero order (r^2 = 0.9209)	2.76	0.55
	45±2ºC	zero order (r^2 = 0.7459)	2.90	0.58
0.1% cream	27±2ºC	zero order (r^2 = 0.9696)	1.87	0.37
	4±2ºC	first order (r^2 = 0.9686)	1.28	0.19
	45±2ºC	first order (r^2 = 0.8722)	2.86	0.57
0.5% cream	27±2ºC	second order (r^2 = 0.8484)	2.74	0.30
	4±2ºC	zero order (r^2 = 0.9512)	3.78	0.76
	45±2ºC	zero order (r^2 = 0.7546)	2.86	0.57
0.1% gel	27±2ºC	second order (r^2 = 0.9066)	1.59	0.17
	4±2ºC	zero order (r^2 = 0.9896)	1.98	0.40
	45±2ºC	second order (r^2 = 0.8224)	0.90	0.10
0.5% gel	27±2ºC	first order (r^2 = 0.9888)	2.08	0.42
	4±2ºC	zero order (r^2 = 0.9966)	2.03	0.41
	45±2ºC	second order (r^2 = 0.9907)	0.84	0.09
0.1% serum	27±2ºC	zero order (r^2 = 0.9551)	2.08	0.42
	4±2ºC	zero order (r^2 = 0.9620)	1.90	0.38
	45±2ºC	second order (r^2 = 0.9901)	1.09	0.12
0.5% serum	27±2ºC	zero order (r^2 = 0.9224)	2.03	0.41
	4±2ºC	first order (r^2 = 0.9923)	0.03	0.01
	45±2ºC	first order (r^2 = 0.9691)	0.03	0.01

Note: 0.1% and 0.5% represented the concentrations of the semi-purified extract in the formulations and "ppg" substituted for propylene glycol.

The orders of reaction were calculated from the following equations:

Zero order equation : $[A]_t = -k_0t + [A]_0$

First order equation : $\ln[A]_t = -k_1t + \ln[A]_0$

Second order equation : $1/[A]_t = 1/[A]_0 + k_2t$

Where $[A]_t$ represents the concentration of gallic acid at a particular time, and $[A]_0$ represents the initial concentration of gallic acid and *kt* represents the slope of the resulting linear of the order rate constants.

The cream formulations containing the ethyl acetate semi-purified extract at 0.1% stored at 4±2, 27±2, and 45±2ºC indicated the chemical degradation reactions of the first order (r^2 = 0.9745), zero order (r^2 = 0.9696) and first order (r^2 = 0.8722), respectively, while the chemical degradation reactions of the cream containing the semi-purified extract at 0.5% kept at 4±2, 27±2 and 45±2ºC were zero order (r^2 = 0.9512), second order (r^2 = 0.8484) and zero order (r^2 = 0.7546), respectively. The different orders of the chemical degradation reaction may be influenced by various factors including the concentrations of the extracts and temperatures. The shelf lives of gallic acid in the cream containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 0.19 and 0.76 days at 4±2ºC, 0.37 and 0.30 days at 27±2ºC, 0.57 and 0.57 days at 45±2ºC, respectively. The half lives of gallic acid in the cream containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 1.28 and 3.78 days at 4±2ºC, 1.87 and 2.74 days at 27±2ºC, 2.86 and 2.86 days at 45±2ºC, respectively (**Table 59**).

At 4±2, 27±2 and 45±2ºC, the gel formulations containing the ethyl acetate semi-purified extract at 0.1% showed the zero order (r^2 = 0.8621), second order (r^2 = 0.9066) and second order (r^2 = 0.9976), respectively, while the concentration at 0.5% gave the zero order (r^2 = 0.9512), first order (r^2 = 0.9561) and second order (r^2 = 0.9907), respectively. The shelf lives of the gel containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 0.40 and 0.41 days at 4±2ºC, 0.17 and 0.42 days at 27±2ºC, 0.10 and 0.09 days at 45±2ºC, respectively. The half lives of the gel containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 1.98 and 2.03 days at 4±2ºC, 1.59 and 2.08 days at 27±2ºC and 0.90 and 0.84 days at 45±2ºC, respectively (**Table 59**).

The serum containing the ethyl acetate semi-purified extract at 0.1% stored at 4±2, 27±2 and 45±2ºC demonstrated the zero order (r^2 = 0.9620), zero order (r^2 = 0.9551) and second order (r^2 = 0.9901), respectively, while the serum containing the ethyl acetate semi-purified extract at 0.5% kept at 4±2, 27±2 and 45±2ºC exhibited the first order (r^2 = 0.9923), zero order (r^2 = 0.9224) and the first order (r^2 = 0.9691), respectively. The shelf lives of the serum containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 0.38 and 0.01 days at 4±2ºC, 0.42 and 0.41 days at 27±2ºC and 0.12 and 0.01 days at 45±2ºC, respectively. The half lives of the serum containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 1.90 and 0.03 days at 4±2ºC, 2.08 and 2.03 days at 27±2ºC and 1.09 and 0.03 days at 45±2ºC, respectively. The cream, gel and serum containing the semi-purified extract at 0.1 and 0.5% were less chemical stable than that of the extract in propylene glycol at various temperatures. The extract appears to be soluble in propylene glycol than other solvents such as mineral oil and glycerin. Hence, the formulations which composed of various solvents can cause the instability of the extract in the formulations. The cream and gel containing the semi-purified extract at 0.1% were more chemical stable than 0.5% at various temperatures, but the serums containing the semi-purified extract at 0.5% were more chemical stable than that of 0.1% at various temperatures. Basically, tannins such as gallic acid, tannic acid and phloroglucinol, are incompatible with alkalis, gelatin, heavy metals, iron, lime water, metallic salts, strong oxidizing agents and zinc sulfate, since they can form complexes and be precipitated in aqueous solution (Simon, 1993). The developed gel and cream at 0.1% gave lower remaining gallic acid contents than that of at 0.5%, while the developed serum composed

of water as the major ingredients at 0.5% extract indicated higher remaining gallic acid contents than that of at 0.1%. Water containing in the serum and gel formulations may hydrolyse gallic acid. During the hydrolysis, the gallic acid molecules (3,4,5-trihydroxybenzoic acid) may give carbon dioxide to form pyrogallol (1,2,3-trihydroxybenzene). In general, the shelf life of gallic acid (purity 99.0%) in the market is 2 years in a tight container at 4ºC and gallic acid has to be kept to avoid from light and reducing agents (Xiaoyuan Biotechnology Co., Ltd., Hunan, P.R.China). Furthermore, the pH value of 1% gallic acid dissolving in water is 2.8 which might be neutralized when incorporated to the cream formulations which has the pH of 5.5.

The developed cream formulations at various concentrations were accordingly selected for the *in vivo* anti-aging evaluations in human volunteers because of their good physico-chemical stability in comparing to other developed gel and serum formulations.

Part 6 : *In vivo* anti-aging activities on human volunteers of the cosmetic formulations containing the extract from leaves of Long Kong

6.1 *In vivo* rabbit skin irritation by the Draize test of the cosmetic formulations containing the semi-purified extract from leaves of Long Kong

The PIIs and the category of irritation based on PII of various samples at 24, 48 and 72 h were shown in **Table 60**. The average PIIs of cream containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% and the extract dissolved in propylene glycol at 0.1, 0.3 and 0.5% were in the range of 0.1–0.2. This demonstrated that the semi-purified extract solutions and cream formulations containing the extracts at various concentrations

gave no irritation on the rabbit skin. Therefore, the ethyl acetate semi-purified extract solutions and all cream formulations containing the extracts at various concentrations were consequently tested for *in vivo* anti-aging evaluations in human volunteers.

Table 60 Primary irritation index (PII) and category of irritation based on PII of various samples containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5%

Samples	Primary irritation index (PII)				Category of irritation base on PII
	24 h	48 h	72 h	Average	
0.1% semi-purified extract in cream	0.0	0.1	0.2	0.1	Negligible
0.3% semi-purified extract in cream	0.0	0.2	0.2	0.1	Negligible
0.5% semi-purified extract in cream	0.0	0.3	0.3	0.2	Negligible
Cream base (negative control)	0.0	0.1	0.1	0.1	Negligible
0.1% semi-purified extract in propylene glycol	0.0	0.1	0.1	0.1	Negligible
0.3% semi-purified extract in propylene glycol	0.0	0.1	0.2	0.1	Negligible
0.5% semi-purified extract in propylene glycol	0.0	0.1	0.3	0.1	Negligible
Propylene glycol base (negative control)	0.0	0.0	0.0	0.0	Negligible
20% sodium lauryl sulfate (positive control)	3.0	3.0	2.7	2.9	Moderate irritation
Untreated area (control)	0.0	0.0	0.0	0.0	Negligible

6.2 *In vivo* anti-aging activities in human volunteers of the cosmetic formulations containing the semi-purified extract from leaves of Long Kong

6.2.1 Skin elasticity

The skin elasticity measurements composed of the two parameter measurements including skin elastic extension and skin elastic recovery. The skin elastic extension is the ratio of the delayed distension (Demirezer et al.) and the immediate distension (Ue). The skin elastic recovery is the ability of the skin returning to its initial position after deformation which was the ratio of the immediate retraction (Ur) and the

final distension (Uf). The percentages of parameter change of before and after 4 weeks of applications of the formulations were shown in **Table 61**. The average values of skin elastic extension (Uv/Ue) and skin elastic recovery (Ur/Uf) parameters of various formulations before and after application in 20 volunteers measured by Cutometer® were presented in **Figure 52**.

Table 61 Percentages of the parameter changes (%) of before application and after 4 weeks of application of various formulations and the untreated area

Skin elasticity parameter	Parameter changes (%)									
	Cream base	0.1% cream	0.3% cream	0.5% cream	Ppg base	0.1% ppg	0.3% ppg	0.5% ppg	Commercial product	Untreated area
Skin elastic recovery (Ur/Uf)	+10.79	+12.47	+15.46	+23.55	+6.33	+5.95	+9.32	+18.38	+28.38	+2.61
Skin elastic extension (Ue/Uv)	-14.84	-35.79	-43.22	-44.71	-14.14	-20.80	-28.22	-30.77	-48.61	+1.74

Note : "-" represented a decrease in skin elastic extension and "+" represented an increase in skin elastic recovery or skin elastic extension

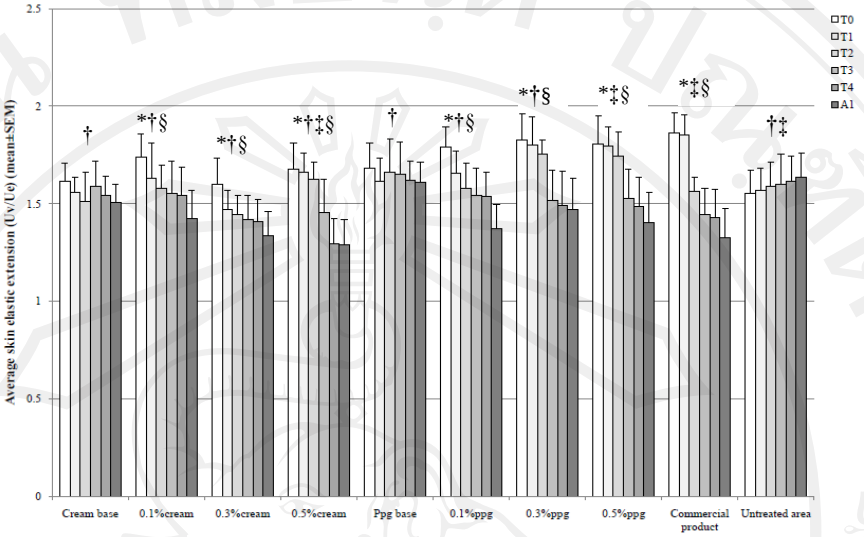
%skin elastic extension = $[(SE_1 - SE_0)/SE_0]\times100$ where SE_1 is average skin elastic extension values after application and SE_0 is an average skin elastic extension values before application

%skin elastic recovery = $[(SR_1 - SR_0)/SR_0]\times100$ where SR_1 is an average skin elastic recovery values after application and SR_0 is an average skin elastic recovery values before application

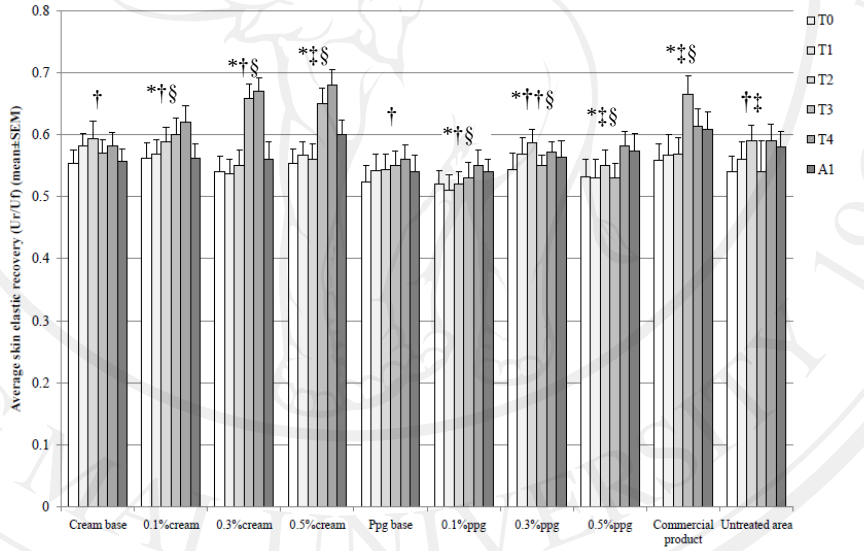
6.2.1.1 Skin elastic extension (Uv/Ue)

Figure 52 demonstrated the compartison of the average skin elastic extension (Uv/Ue) before, after application and after 1 week of the wash-out period of cream formulations containing the extract and the extract dissolved in propylene glycol at various concentrations (0.1, 0.3 and 0.5%), commercial product and the untreated area. The skin elastic extension of the cream containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% decreased with longer time and higher concentrations. After 4 weeks

(A)



(B)



Note: * represented significant different at T0 and T4 ($p<0.05$, Student's paired T-test)
‡ represented significant different at T4 and A1 ($p<0.05$, Student's paired T-test)
† represented significant different from the commercial product ($p<0.05$, One-way ANOVA)
§ represented significant different from the untreated area ($p<0.05$, One-way ANOVA)

Figure 52 Comparison of the skin elastic extension values (A) and the skin elastic recovery values (B) of the skin area treated between the cream formulations and propylene glycol solutions containing the extract at various concentrations, commercial product and the untreated area at before and after application for 1, 2,3 and 4 weeks

of application of the cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5%, they showed a significant decrease of the skin elastic extension in comparing to before application, but not significant difference from after 1 week of the wash-out period ($p<0.05$, Student's paired T-test). After 1 week of the wash-out period, the cream containing the semi-purified extract at 0.1, 0.3 and 0.5% did not exhibited the significant reduction of the skin elastic extension from after 4 weeks of applications ($p>0.05$, Student's paired T-test). The cream containing the ethyl acetate semi-purified extract gave significant difference from the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% dissolved in propylene glycol and the untreated area ($p<0.05$, One-way ANOVA), but the cream containing the ethyl acetate semi-purified extract at 0.3 and 0.5% did not show the significant difference from the commercial product ($p>0.05$, One-way ANOVA). The cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% gave the significant decrease of the skin elastic extension after 4 weeks of the percentages of the parameter change, which were determined from the variation between before and after 4 weeks of applications of the cream formulations containing the extract at 0.1, 0.3 and 0.5% gave the parameter changes of -35.79, -43.22 and -44.71%, respectively (**Table 61**). This indicated that after 4 weeks of applications of the cream formulations containing the semi-purified extract at 0.3 and 0.5% could decrease the skin elastic extension similarly to commercial product, and may maintain the skin elastic extension after 1 week of wash-out period.

6.2.1.2 Skin elastic recovery (Ur/Uf)

Figure 52 demonstrated the comparison of the average of the skin elastic recovery (Ur/Uf) before, after application and after 1 week of the wash-out period of cream formulations containing the extract and the extract dissolved in propylene glycol at various concentrations (0.1, 0.3 and 0.5%), commercial product and the untreated area. The skin elastic recovery of the cream containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% increased with longer time and higher concentrations. After 4 weeks of applications of the cream containing the ethyl acetate semi-purified extract at 0.3 and 0.5%, the significant increase of skin elastic recovery in comparing to before application ($p<0.05$, Student's paired T-test) was observed, but was not significant different from after 1 week of the wash-out period ($p>0.05$, Student's paired T-test). After 1 week of the wash-out period, the cream containing the ethyl acetate semi-purified extract at 0.3 and 0.5% gave significant different skin elastic recovery from before application ($p<0.05$, Student's paired T-test). The creams containing the ethyl acetate semi-purified extract at all concentrations were significant different skin elastic recovery from the ethyl acetate semi-purified extract dissolved in propylene glycol and the untreated area ($p<0.05$, One-way ANOVA). The cream containing the ethyl acetate semi-purified extract at 0.5% decreased the skin elastic recovery which were not significantly different from the commercial product ($p>0.05$, One-way ANOVA). The percentages of the parameter change of the skin elastic recovery before and after 4 weeks of application of the cream containing the extract at 0.1, 0.3 and 0.5% were +12.47, +15.46 and +23.55%, respectively (**Table 61**). The cream at 0.5% of the extract did not increase skin elastic

recovery the same as the commercial product after 4 weeks of application and the skin elastic recovery was maintained after 1 week of the wash-out period. The phenolic compounds, especially gallic acid, which were the constituents in the ethyl acetate semi-purified extract, may improve the skin elasticity in the dermis by scavenging the free radical and inhibiting the MMP-2 activity. This has been evidenced that some phenolic compounds, such as tannins have been reported as a direct human neutrophil elastase inhibitor. The decrease in skin elasticity, also accompanied by neutrophils, which supposed to participate in the aging process of human skin and the release of the enzymatically active human neutrophil elastase (Tsuji et al., 2001).

6.2.2 Skin roughness measurement

Percentage of parameter changes of the skin roughness, skin hydration and skin melain at before and after applications of various cream formulations containing the extract at 0.1, 0.3 and 0.5% was shown in **Table 62**. The average skin roughness of various formulations before, after applications and after 1 week of wash-out period were shown in **Figure 53**. The skin roughness of the cream containing the ethyl acetate semi-purified extract decreased with longer time and higher concentrations. The cream containing the semi-purified extract at 0.1, 0.3 and 0.5% after 4 weeks of application exhibited the significant decrease of the skin roughness in comparing to before application and after 1 week of applications ($p<0.05$, Student's paired T-test). The skin roughness after 1 week of the wash-out period of the cream were significantly decreased

Table 62 Percentages changes (%) of the skin roughness, skin hydration and skin melanin of before and after 4 weeks of application of various formulations and the untreated area

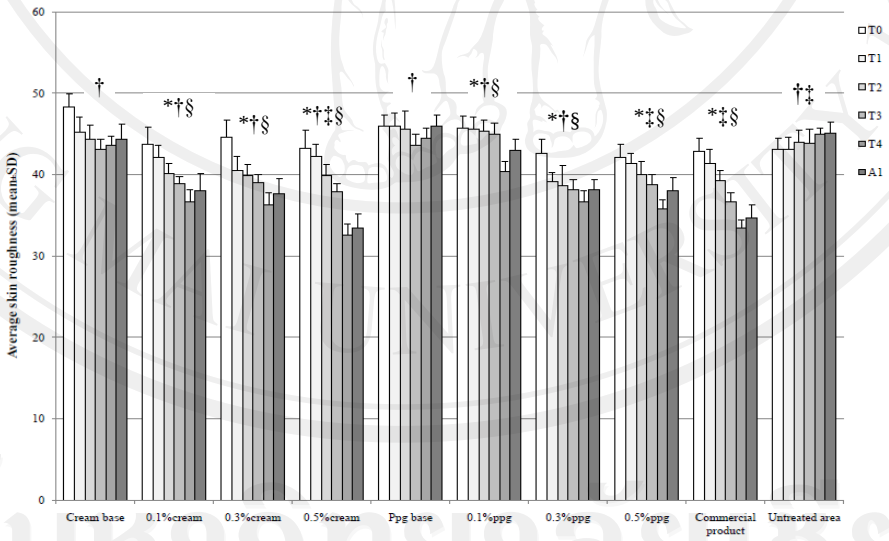
Parameters	Parameter changes (%)									
	Cream base	**0.1% cream**	**0.3% cream**	**0.5% cream**	**Ppg base**	**0.1% ppg**	**0.3% ppg**	**0.5% ppg**	**Commercial product**	**Untreated area**
Skin roughness	+9.62	-16.19	-18.73	-24.82	+3.16	-11.85	-13.98	-15.25	-22.06	+4.36
Skin hydration	+19.74	+23.14	+28.82	+42.41	+17.35	+18.57	+21.12	+28.79	+35.73	-11.87
Skin melanin	+3.02	-12.72	-16.41	-34.45	+3.58	-5.51	-9.53	-16.65	-35.04	+6.22

Note : "-" represented a decrease in skin roughness/hydration/melanin and "+" represented an increase in skin roughness/hydration/melanin

%skin roughness= $[(R_1 - R_0)/R_0]\times100$

%skin hydration= $[(H_1 - H_0)/H_0]\times100$

%skin melanin= $[(M_1 - M_0)/M_0]\times100$



Note: * represented significant different at T0 and T4 ($p<0.05$, Student's paired T-test)
‡ represented significant different at T0 and A1 ($p<0.05$, Student's paired T-test)
† represented significant different the commercial product ($p<0.05$, One-way ANOVA)
§ represented significant different from the untreated area ($p<0.05$, One-way ANOVA)

Figure 53 Comparison of the skin roughness of skin area treated with the various formulations at before, after applications and after 1 week of the wash-out period

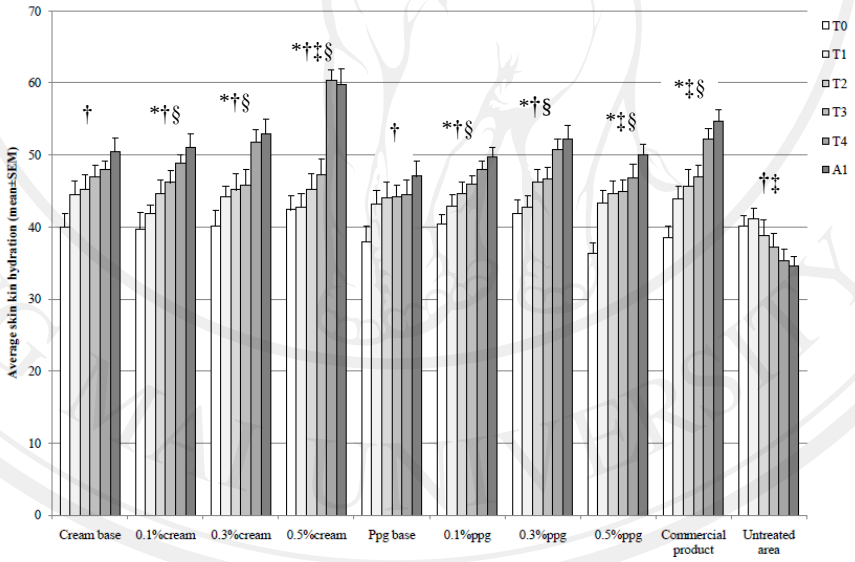
from before application ($p<0.05$, Student's paired T-test). The skin roughness of the cream containing the ethyl acetate semi-purified extract at 0.3 and 0.5% were significant different from the cream base, propylene glycol base and the ethyl acetate semi-purified extract solutions dissolved in propylene glycol at 0.1, 0.3 and 0.5% ($p<0.05$, One-way ANOVA). But the skin roughness of the cream containing the ethyl acetate semi-purified extract at all concentration did not show significant difference from the commercial product ($p>0.05$, One-way ANOVA). The results indicated that the cream containing the ethyl acetate semi-purified extract at 0.5% improved the skin roughness similar to the commercial product after 4 weeks of applications. The percentages of the parameter change of the skin roughness before and after 4 weeks of application of the cream formulations containing the ethyl acetate semi-purified extracts at 0.1, 0.3 and 0.5% were -16.19, -18.73 and -24.82%, respectively, while the commercial product gave the parameter change of skin roughness were -22.06%. This result demonstrated that the cream containing the ethyl acetate semi-purified extract at 0.5% significantly decreased the skin roughness after 4 week of application and may reduce after 1 week of the wash-out period similar to the commercial product.

6.2.3 Skin hydration

The average skin hydration of various topical formulations before application, after applications and after 1 week of wash-out period were shown in **Figure 54**. The skin hydration of cream containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% and semi-purified extract dissolved in propylene glycol at 0.1, 0.3 and 0.5% increased with longer time and higher concentrations. After 4 weeks of applications, the

cream containing the ethyl acetate semi-purified extract at all concentrations significantly increase skin hydration in comparing to before application ($p<0.05$, Student's paired T-test). After 1 week of the wash-out period, the cream containing the ethyl acetate semi-purified extract at all concentrations indicated the not significant decrease of the skin

The skin hydration of all formulations including cream base, the cream containing the ethyl acetate semi-purified extract, propylene glycol base, the ethyl acetate semi-purified extract solutions and 0.5% and the commercial product were significant different from the untreated area ($p<0.05$,One-way ANOVA).



Note: * represented significant different at T0 and T4 ($p<0.05$, Student's paired T-test)
‡ represented significant different at T0 and A1 ($p<0.05$, Student's paired T-test)
† represented significant different the commercial product ($p<0.05$, One-way ANOVA)
§ represented significant different from the untreated area ($p<0.05$, One-way ANOVA)

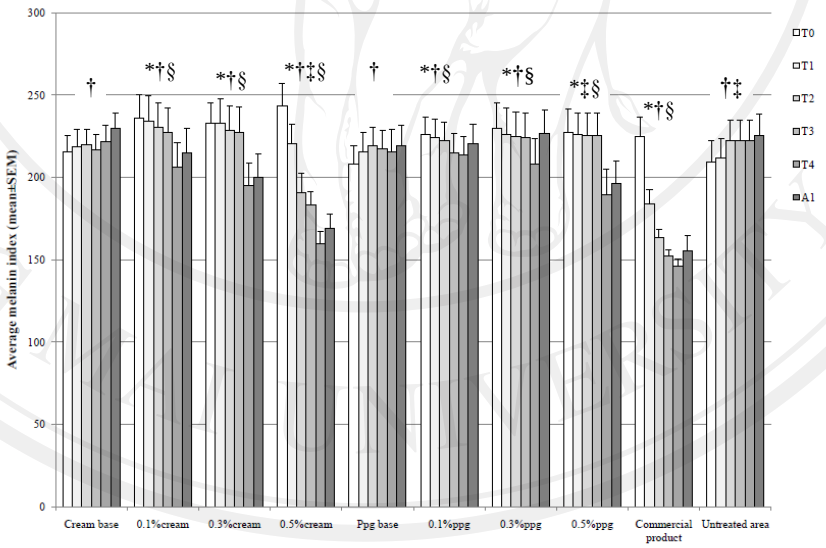
Figure 54 Comparison of the skin hydration of the skin area treated with various formulations before application, after application and after 1 week of the wash-out period hydration in comparing to after 4 weeks of applications ($p>0.05$, Student's paired T-test).

The cream containing the ethyl acetate semi-purified extract showed the decrease skin hydration which was not significant different from the ethyl acetate semi-purified extract dissolved in propylene glycol and the commercial product ($p>0.05$, One-way ANOVA). The percentages of the parameter change before application of the cream containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% and the commercial product after 4 weeks of applications were +23.14, +28.82, +42.41 and +35.73%, respectively. This indicated that the cream containing the ethyl acetate semi-purified extract improved the skin hydration significantly after 4 weeks of application and remained increased after 1 week of the wash-out period similar to the commercial product. It has been reported that the lotion containing the hydroethanol extract from the flesh fruits of *L. domesticum* significantly increased the skin moisture contents performed on a panel of 30 female volunteers aged 32-52 years old during the 4 weeks of applications measured by Corneometer® CM 820 (Martha et al., 2007). The leaves and flesh fruits of Long Kong may contain some phytochemicals that can increase the skin moisture contents in the human volunteers.

6.2.4 Skin erythema and melanin

The skin erythema index of all developed formulations performed after 1, 2, 3 and 4 weeks of applications revealed no significant differences ($p>0.05$, Student's paired T-test) in comparing to before application and the untreated area ($p>0.05$, One-way ANOVA). Therefore, all cream formulations, semi-purified extract solutions and the commercial product did not induce any irritation on the human skin.

The average skin melanin of various formulations before application, after application and after 1 week of the wash-out period were shown in **Figure 55**. The cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% decreased with longer time and higher concentrations. After 4 weeks of applications, the cream containing the ethyl acetate semi-purified extract at 0.5% showed the significantly decrease skin melanin in comparing to before application and after 1 week of the wash-out period ($p<0.05$, Student's paired T-test). After 1 week of the wash-out period, the cream containing the ethyl acetate semi-purified extract showed the significant decrease skin melanin in comparing to before application ($p<0.05$, Student's paired T-test).



Note: * represented significant different at T0 and T4 ($p<0.05$, Student's paired T-test)
‡ represented significant different at T0 and A1 ($p<0.05$, Student's paired T-test)
† represented significant different the commercial product ($p<0.05$, One-way ANOVA)
§ represented significant different from the untreated area ($p<0.05$, One-way ANOVA)

Figure 55 Comparison of the skin melanin index of the various formulations before application, after applications and after 1 week of the wash-out period

The cream containing the ethyl acetate semi-purified extract at 0.5% gave the significantly decrease skin melanin in comparing to the untreated area cream base, the ethyl acetate semi-purified extract dissolved in propylene glycol at various extract concentrations and the propylene glycol ($p<0.05$, One-way ANOVA), but was not significant different from the commercial product ($p>0.05$, One-way ANOVA). The percentages of the parameter change of the skin melanin before and after 4 weeks of application of the cream formulations containing the semi-purified extract at 0.1, 0.3 and 0.5% were -12.72, -16.41 and -34.45%, respectively, while the commercial product showed the decrease of the parameter change at +35.73%. The semi-purified extract dissolved in propylene glycol at 0.1, 0.3 and 0.5% gave the parameter changes of -5.51, -9.53 and -16.65%, respectively. After application of various cream formulations, they indicated better skin melanin than the solutions. This may be due to not only the dependence on the skin penetration of the cream formulations, but also the covering effect of other ingredients existing in the cream formulations. It has been reported that the hydroethanolic extract of the flesh fruits of Long Kong showed the inhibitory effect on tyrosinase enzyme and also the lightening effect which decreased the skin melanin index in human volunteers measured by Mexameter® MX 16. The extract from various parts including the leaves of Long Kong may contain some bioactive compounds that can inhibit tyrosinase enzyme and improve the dull skin.

6.2.5 Satisfaction evaluation on physical appearances of the cosmetic formulations by human volunteers (20 persons) using the questionnaires

The percentages of satisfaction of the volunteers on the cream formulations containing the semi-purified extract at various concentrations (0.1, 0.3 and 0.5%) were shown in **Table 63**.

Table 63 Percentages of the satisfaction parameter scale of the human volunteers on the cream formulations containing the semi-purified extract at 0.1, 0.3 and 0.5%

Parameters	0.1% cream		0.3% cream		0.5% cream	
	Mean of rating scale	Satisfaction (%)	Mean of rating scale	Satisfaction (%)	Mean of rating scale	Satisfaction (%)
Physical appearance						
Color	4.33	86.50	4.13	82.50	3.58	71.50
Odor	3.93	78.50	3.83	76.50	3.45	69.00
Consistency	3.78	75.50	3.75	75.00	3.58	71.50
Glossiness	3.80	76.00	3.90	78.00	3.85	77.00
Organoleptic evaluation						
Sticky	3.68	73.50	3.93	78.50	3.58	71.50
Absorption	3.85	77.00	3.80	76.00	3.75	75.00
Smoothness	4.05	81.00	4.23	84.50	4.03	80.50
Creamy residue	4.00	80.00	3.88	77.50	3.78	75.50

Note: Mean of the score = (median of the rating scale × percentage of the score)/Number of the samples
%satisfaction = Mean of the score × Number of the samples
The rating scale were classified into 5 levels including 1.00 – 1.49 = not satisfied, 1.50 – 2.49 = less satisfied, 2.50 – 3.49 = fairly satisfied, 3.50 – 4.49 = satisfied and 4.50 – 5.00 = very satisfied.

The satisfaction scale of the cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% were satisfied with the average score of 3.93 (78.50%), 3.93 (78.56%) and 3.70 (73.94%), respectively. This indicated that most volunteers satisfied on the physical appearances of all developed cream formulations.