CHAPTER 4

CONCLUSION

The results from this study can be concluded as the followings:

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

1.1 Leaves of Long Kong were prepared in six different processes including hot water, cold water, hot methanol, cold methanol, hot chloroform and cold chloroform.

1.2 The hot water crude extract was selected to prepare the semi-purified extracts because of these following reasons:

1.2.1 The crude extract prepared by the hot water process from leaves of Long Kong gave the percentage yield of 17.97%. The phytochemical constituents of this crude extract were alkaloids, flavonoids and reducing sugars including fructose and sucrose.

1.2.2 The total phenolic and flavonoid contents containing in the hot water crude extract were 55.37 ± 0.10 µg GAE/g dry extract and 403.10 ± 0.03 µg QAE/g dry extract, respectively.

1.2.3 The hot water crude extract showed *in vitro* anti-aging activities with the DPPH radical scavenging (SC_{50} value of 5.40 ± 1.23 mg/ml), lipid peroxidation inhibition (IPC_{50} value of 3.29 ± 0.30 mg/ml) and metal ion chelating (CC_{50} value of 32.31 ± 0.84 mg/ml) which were

1.2.4 lower potent than ascorbic acid, α-tocopherol and EDTA of 67.50, 4.16 and 5.39 times, respectively. The crude extract also exhibited high tyrosinase inhibition with the IC_{50} value 0f 0.49 ± 0.23 mg/ml) which was lower potent than Kojic acid of 16.33 times.

1.2.5 The hot water crude extract at 1 mg/ml gave no cytotoxicity on human skin fibroblast with the %cell viability of 80.52±15.16% which was lower than ascorbic acid of 1.42 times. 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.

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

2.1 The hot water crude extract was partitionated and obtained three semi-purified extract including water, butanol and ethyl acetate semi-purified extracts. Percentage yields of the butanol, water and ethyl acetate semi-purified extracts were 17.97, 15.90 and 10.32%, respectively.

2.2 The ethyl acetate semi-purified extract was selected for the further development according to these following reasons:

2.2.1 The ethyl acetate semi-purified extract prepared from the hot water crude extract by partition technique gave the percentage yield of

2.83% when calculated from the crude extract and 0.3% of the dry leave powder of Long Kong.

2.2.2 The phytochemical constituents of the ethyl acetate semi-purified extract were triterpenoids and flavonoids. The phenolic and flavonoid contents of this semi-purified extract were 868.90 ± 0.02µg GAE/g dry extract and 422.39 ± 0.01 µg QE/g dry extract, respectively.

2.2.3 The ethyl acetate semi-purified extract showed high *in vitro* anti-aging activities including DPPH radical scavenging activity (SC_{50} value of 0.23±0.02 mg/ml), lipid peroxidation inhibition (IPC_{50} value of 33.48±14.49 mg/ml) and metal ion chelating activity (CC_{50} value of 0.45±0.02 mg/ml) which were lower potent than ascorbic acid and α-tocopherol of 3.29 and 40.34 times, respectively, but the ethyl acetate semi-purified extract gave higher metal ion chelating activity than EDTA of 1.27 times. This semi-purified extract also showed strong tyrosinase inhibition activity (IC_{50} value of 0.19±0.16 mg/ml) which was lower potent than kojic acid of 19 times.

2.2.4 The ethyl acetate semi-purified extract at 1 mg/ml exhibited no cytotoxicity on human skin fibroblast with the cell viability of 95.94±0.55% which was lower than ascorbic acid of 1.25 times. The ethyl acetate semi-purified extract at 1 mg/ml also demonstrated the pro and active MMP-2 inhibition of 52.37±8.67

and 60.23±7.89%, respectively, which were lower than ascorbic acid of 1.78 and 1.60 times, respectively.

2.2.5 The ethyl acetate semi-purified extract (0.1% dissolved in water) was in pale greenish-brown color and cloudy solution with the pH value of 4.5. This semi-purified extract was soluble in propylene glycol, and sparingly soluble in methanol, ethanol, mineral oil and glycerol, but insoluble in hot (60ºC) and cold (25ºC) water. The ethyl acetate semi-purified extract was not stable in strong base and reducing agent, but stable in weak base, weak acid, strong acid, sodium salt of weak acid and oxidizing agent.

2.2.6 The HPLC fingerprint profile of the ethyl acetate semi-purified extract gave the peak of gallic acid at the retention time of 3.272 min and had the gallic acid content of 6.16%.

2.2.7 The ethyl acetate semi-purified extract was consequently selected to test for irritation on rabbit skin by the Draize test. This extract showed no irritation on rabbit skin at 0.1, 0.3 and 0.5%.

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

3.1 The color of the ethyl acetate semi-purified extract was eliminated by the partition technique. The discolored water and chloroform fractions were obtained with the percentage yields of 37.01 and 11.12%, respectively.

3.2 The discolored water fraction was selected because of the following reasons:

3.2.1 The discolored water fraction contained flavonoids, while the discolored chloroform fraction had triterpenoids and flavonoids as the phytochemical constituents. 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).

3.2.2 The discolored water fraction exhibited *in vitro* anti-aging activities including DPPH radical scavenging activity (SC_{50} values of 0.09±0.04 mg/ml), lipid peroxidation inhibition (IPC_{50} value of 26.46±6.24 mg/ml) and metal ion chelating activity (0.28±0.17 mg/ml) which were lower potent than ascorbic acid and α-tocopherol of 1.125 and 220. 25 times, respectively, but the fraction exhibited metal ion chelating activity more potent than EDTA of 1.61 times. This fraction also showed tyrosinase inhibition (IC_{50} value of 0.11±0.05 mg/ml) which was lower potent than kojic acid of 22 times and also gave no cytotoxicity on human skin fibroblast with the cell viability of 126.29 ± 0.81% which was less than ascorbic acid of 1.14 times.

3.2.3 At 1 mg/ml, the discolored water fraction indicated the pro and active MMP-2 inhibition of 56.51 ± 0.04 and 64.83 ± 0.88% which was lower than ascorbic acid of 1.65 and 1.54 times

3.2.4 The discolored water fraction (0.1% dissolved in water) showed the pH value of 3.0 which gave the pale green color and clear solution. The discolored water fraction was soluble in hot (60ºC) and cold (25ºC) water, methanol, ethanol and propylene glycol, and slightly soluble in mineral oil and glycerol. The discolored water fraction was unstable in strong base and reducing agent, but stable in weak base, weak acid, strong acid, sodium salt of weak acid and oxidizing agent.

3.2.5 The HPLC fingerprint of the discolored water fraction showed the peak of gallic acid which presented at the retention time of 3.415 min with the percentages of 0.39%.

3.3 The discolored water fraction at 0.3 and 0.5% demonstrated slightly irritation on the rabbit skin. Since the percentage yield of the discolored water fraction was very low, therefore, the ethyl acetate semi-purified extract was selected to incorporate to the cosmetic formulations.

Part 4: Development of the cosmetic base formulations

4.1 Each type of base formulations including gel, serum and cream were prepared in three different formulas. After tested for the physical stability by heating-cooling cycles (6 cycles) for 12 days, gel No.1, serum No.1 and cream No.1 showed good physical stability.

4.2 The above three cosmetic base formulations were selected to prepare the cosmetic formulations containing the ethyl acetate semi-purified extract.

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

5.1 After kept at various temperatures (4±2, 27±2 and 45±2ºC) for 3 months, cream containing the ethyl acetate semi-purified extract at 0.1 and 0.5% exhibited good physical stability at all temperatures for 3 months.

5.2 The shelf lives of the gallic acid in the cream containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 0.37 and 0.30 days at 27±2ºC, 0.19 and 0.76 days at 4±2ºC, 0.54 and 0.54 days at 45±2ºC, respectively. The half lives of the gallic acid in the cream containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were 1.87 and 2.74 days at 27±2ºC, 1.28 and 3.78 days, 2.86 and 2.86 days at 45±2ºC, respectively.

5.3 The cream formulations containing the ethyl acetate semi-purified extract at 0.1 and 0.5% were selected for *in vivo* anti-aging performance test in human volunteers.

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

6.1 The cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% showed no irritation on rabbit skin by Draized test with the PIIs in the range of 0.0–0.2.

6.2 The cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% significantly ($p<0.05$, Student's paired T-test) decreased the skin elastic extension with the parameter changes of -35.79, -43.22 and -44.71%, respectively. The cream formulations containing the ethyl acetate semi-purified extract at 0.3 and 0.5% significantly increased the skin elastic recovery with the parameter changes of +15.46 and +23.55%, respectively.

6.3 The cream containing the semi-purified extract at 0.1, 0.3 and 0.5% after 4 weeks of application significantly decreased the skin roughness in comparing to before application ($p<0.05$, Student's paired T-test) with the parameter change of -16.19, -18.73 and -24.82%, respectively.

6.4 The cream containing the ethyl acetate semi-purified extract at 0.5% significantly increased skin hydration with the %parameter changes of 42.41%.

6.5 The skin erythema index of all developed formulations after 1, 2, 3 and 4 weeks of application was not significant different ($p>0.05$, Student's paired T-test) in comparing to before application and the untreated area ($p>0.05$, One-way ANOVA). The cream formulations containing the ethyl acetate semi-purified extract at 0.5% after 4 weeks of application significantly decreased the skin melanin in comparing to before application with the parameter change of 34.45%.

6.6 The satisfaction scales of the cream formulations containing the ethyl acetate semi-purified extract at 0.1, 0.3 and 0.5% evaluated by the volunteers using the questionnaires were in 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.

In summary, the ethyl acetate semi-purified extract prepared from the hot water crude extract from leaves of Long Kong contained the bioactive compounds including phenolic and flavonoid compounds. This semi-purified extract was effective *in vitro* anti-aging activities against DPPH radical scavenging, lipid peroxidation inhibition and metal ion chelating, and high whitening activity by the tyrosinase inhibition with no cytotoxicity on human skin fibroblast. Furthermore, the semi-purified extract also gave potent gelatinolytic activity of MMP-2 inhibition on human skin fibroblast. The cream formulations containing the ethyl acetate semi-purified extract which gave good physico-chemical stability and no irritation on rabbit skin demonstrated effective topical skin aging treatment on human volunteers, and also most volunteers satisfied on the physical appearances of the formulations. Therefore, the cream formulations containing the extract from leaves of Long Kong can be applied as a novel topical product due to their superior *in vitro* and *in vivo* anti-aging activities.

For further research study, the ethyl acetate semi-purified extract from leaves of Long Kong containing gallic acid as biomarker should be entrapped in the vesicle such as liposome and niosome, in order to improve its physico-chemical stability in cosmetic

formulations. The pure compounds obtaining from the elucidation of the ethyl acetate semi-purified extract from leaves of Long Kong might have higher biological activity and could be developed as an efficient anti-wrinkle cosmeceuticals. The other *in vitro* and *in vivo* antioxidant assay may confirm anti-wrinkle efficiency of the extract from leaves of Long Kong. The mechanisms of ethyl acetate semi-purified extract from leaves of Long Kong on MMP-2 inhibition might be thus valuable to be investigated. Phytochemical constituents such as phenolic and flavonoid compounds may inhibit the MMP-2 synthesis and secretion. However, one possible mechanism of the extract from leaves of Long Kong on MMP-2 inhibition may be from the decreasing of the activation processes by converting the latent form of MMP-2 (pro MMP-2) to an active form (active MMP-2). In fact, the effective of the anti-aging activity in human depends on several factors including sex, age and genetics of the volunteers as well as the aging level and skin location. Also, the developed cream containing the ethyl acetate semi-purified extract prepared from the hot water crude extract from leaves of Long Kong should be further investigated for *in vitro* transdermal penetration through rat skin.