CHAPTER 4

CONCLUSION

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

4.1 The gel containing the novel Tween 61 mixed with cholesterol at 1:1 molar ratio and 25% ethanol elastic niosomes loaded with diclofenac diethyammonium (DCFD) did not only show physical and chemical stability for 3 months, but also high fluxes through rat skin and high anti-inflammatory activity in rat ear edema assay. This novel elastic niosomal formulation composed of cholesterol and Tween 61 at 3:7 molar ratio with 25% ethanolic solution prepared by chloroform film method with sonication was selected to load with the semi-purified extract of the *T. chebula* galls.

4.2 The *in vitro* anti-aging activities of *T. chebula* gall were compared with the 14 Thai Lanna medicinal plants (*Acorus gramineus*, *Cassia fistula*, *Cyperus rotundus, Dregea volubilis*, *Eclipta prostrate*, *Myristica fragrans*, *Nigella sativa*, *Plumbago indica*, *Piper nigrum*, *Pellacalyx parkinsonii*, *Piper sarmentosum*, *Plumbago zeylanica*, *Tinospora crispa* and *Zingiber officinale*) with the indication for longevity selected from the database of Thai Lanna medicinal plant recipes “Manosroi II”. The biological activities which are related to anti-aging including antioxidative, tyrosinase inhibition and proliferative stimulation as well as the MMP-2 expression inhibition on human skin fibroblasts were used to evaluate the 60 extracts prepared by aqueous and methanol with hot and cold processes.

4.2.1 For all 15 plants including *T. chebula* gall, an aqueous hot extraction process (HW) gave higher yields than the aqueous cold process (CW), whereas the methanol cold process (CM) gave better yield than the methanol hot process (HM). The highest percentage yields were from *T. chebula* gall by CM, CW and HW at 59.02, 49.85 and 60.00% respectively. For HM, *P. parkinsonii* flower gave the highest percentage yield at 29.25% while *T. chebula* gall gave 23.28%.

4.2.2 The three Thai Lanna medicinal plants, including *T. chebula* gall, *C. rotundus* root and *C. fistula* fruit gave the highest DPPH radical scavenging (IC_{50} value of 0.016 ± 0.001 mg/ml), chelating (IC_{50} value of 0.094 ± 0.001 mg/ml) and tyrosinase inhibition (IC_{50} value of 0.076 ± 0.001 mg/ml) activity, respectively. At 0.1 mg/ml, the CW extract of *T. chebula* gall exhibited the highest DPPH radical scavenging activity with the % scavenging at 84.64 ± 2.22%, whereas the HM and CM extracts of *C. rotundus* root and *C. fistula* fruit exhibited the highest chelating and tyrosinase inhibition activity with the % activity at 79.49 ± 1.10% and 63.55 ± 0.16% in comparing to the control, ascorbic acid (96.50 ± 0.10%), EDTA (87.74 ± 0.10%) and kojic acid (88.63 ± 0.10%), respectively.

4.2.3 The CW extracts of *T. chebula* gall indicated the highest stimulation index (SI) on normal human fibroblasts proliferation at 1.441 which was more active than ascorbic acid (SI 1.210), while *C. fistula* fruit and *C. rotundus* root gave almost the same effect as ascorbic acid.

4.2.4 The inhibition of MMP-2 expression determined by zymography of *T.chebula* gall CW extract was 1.37 times more potent than ascorbic acid.

4.2.5 The cold aqueous extract of *T. chebula* gall demonstrated potent *in vitro* anti-aging effects (DPPH radical scavenging activity, proliferation stimulation

activity on normal human fibroblasts and inhibition of MMP-2 expression) and was selected for isolation and purification.

4.3 The cold aqueous extract of *T. chebula* gall was fractionated on Diaion and refractionated on octadecyl silica (ODS) column. Six phenolic compounds were isolated from the aqueous extract of *T. chebula* galls and identified using NMR and MS data. Gallic acid, punicalagin, isoterchebulin, 1,3,6-tri-*O*-galloyl-β-D-glucopyranose, chebulagic acid and chebulinic acid at 1.84, 1.86, 2.20, 0.77, 1.80 and 4.93%, respectively, were found.

4.3.1 All isolated phenolic compounds showed higher DPPH radical scavenging activity than ascorbic acid, α-tocopherol and BHT which gave the SC_{50} value of 24.41, 11.86 and 17.83 μM, respectively. Chebulinic acid exhibited the highest DPPH radical scavenging activity (SC_{50} = 0.94 μM). Moreover, all isolated compounds showed less melanin content on B16 murine melanoma cells with slight less percentages cell viability than kojic acid (91.5 ± 1.99%) and arbutin (90.5 ± 3.61%). Punicalagin showed the highest inhibition acitivity of α-MSH induced melanin production on B16 murine melanoma with the melanin content of 58.9 ± 4.65%.

4.3.2 Gallic acid exhibited inhibitory activity against nitric oxide production in lipopolysaccharide-activated macrophages with the IC_{50} value of 14.0 μM which was higher than the standards, N^G-monomethyl-L-arginine (L-NMMA) (IC_{50} value 32.1 μM). However, all phenolic compounds exhibited less activity than the standards in mushroom tyrosinase inhibition and human tumor cytotoxicity assays.

4.3.3 The three phenolic compounds including gallic acid (15.3%), 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (17.8%) and punicalagin (38.4%) from the

selected semi-purified fraction (F1, MeOH/H_2O/AcOH = 2:8:0.1) were isolated from the *T. chebula* gall cold aqueous crude extract using Diaion and ODS column chromatography.

4.4 The elastic (25% ethanol) and non-elastic niosomes composed of Tween 61 mixed with cholesterol at 1:1 molar ratio loaded with pure gallic acid or the semi-purified fraction containing gallic acid in 5 mM phosphate buffer (pH 7) exhibited the mixture of unilamellar and multilamellar vesicular structures with negative zeta potential values and in the particle size range of 200–400 nm. For vesicular deformability, the elastic niosomes loaded with pure gallic acid (10.98 ± 2.75) or semi-purified fraction (10.75 ± 3.47) showed similar deformability index to the unloaded (blank) elastic niosomes (11.36 ± 1.55), while higher than the non-elastic niosomes loaded with gallic acid (2.50 ± 0.78) and the semi-purified fraction (2.04 ± 1.13) of about 4.39 and 5.27 times, respectively.

4.5 Both elastic and non-elastic niosomes loaded with gallic acid or the semi-purified fraction containing gallic acid showed physical stability with no layer separation and gave chemical stability with more than 70, 60 and 40% of the remaining gallic acid while only more than 60, 40 and 30% of the remaining gallic acid containing in the gallic acid solution when kept at 4, 27 and 45°C, respectively for 3 months.

4.6 The gel formulations incorporated with the elastic and non-elastic niosomes loaded with gallic acid or the semi-purified fraction from *T. chebula* galls extract gave good physical stability with no sedimentation, no layer separation and no color change at all temperatures (4, 27 and 45°C) for 3 months.

4.7 For rat skin transdermal absorption by Franz diffusion cells, elastic niosomes retarded the permeation of the loaded pure gallic acid indicating of no risk of systemic effect which will be beneficial for topical application. However, elastic niosomes enhanced the permeation of the loaded gallic acid containing in the semi-purified fraction.

4.8 For the *in vivo* tests, three male rabbits were used for rabbit skin irritation test by the closed patch test and a total of 31 Thai volunteers were enrolled in the human skin anti-aging evaluation study.

4.8.1 The calculated PIIs of all gel formulations in rabbits skin irritation by the closed patch test at 72 h were in the range of 0.00–0.33 except the gallic acid gel (PII = 0.44–0.56, slight irritation), the gel base (PII = 0.11, negligible) and the positive control (5% SLS, PII = 0.78–1.22, slight irritant). The human volunteer erythema measurements of all gel samples except the gel containing pure gallic acid revealed no statistically significant differences ($p > 0.05$, Student's paired *t*-test) in comparing to the initial values on day 0 and 8 weeks after application.

4.8.2 The gel containing gallic acid and semi-purified fraction loaded in elastic (GE and SE) or non-elastic (GN and SN) niosomes significantly demonstrated higher improvement of skin elasticity and roughness more than the gel containing the unloaded semi-purified fraction. The % parameter changes of skin elastic recovery (Ur/Uf) and skin elastic extension (Uv/Ue) when applied with SN and SE gels were +28.73 and +32.57; −21.25 and −22.63%, respectively. SN and SE gel also showed a significant decrease of the maximum roughness (Rm) and average roughness (Ra) values with the parameter changes of −29.43 and −32.38; −39.47 and −35.28%, respectively. However, there was no significant difference of these effects

between the gel containing gallic acid or semi-purified fraction loaded in elastic and non-elastic niosomes.

4.9 The developed gel containing the semi-purified fraction of *T. chebula* gall extract can be applied as a novel topical product due to their superior *in vitro* and *in vivo* anti-aging activities.

The results from this study have suggested a potential of the selected plants with significant biological activities applying with nanotechnology for the further development as new cosmeceutical products. For the difficulties and problem of this research work, *T. chebula* galls are not available in the market and ethanol composed in elastic niosomal formulations may cause drying or irritation to the skin.

For the suggestion of the further study, there are the followings:

- *T. chebula* galls may replace with plants which have high *in vitro* and *in vivo* anti-aging activities.

- Sodium cholate, Span 80, Tween 80, oleic acid, and dipotassium glycyrrhizinate which are edge activators may be used the substitution of ethanol to prepare elastic niosomes.

- The process for the production of *T. chebula* gall cosmeceutical products should be further developed and scaling up in order to evaluate for possible commercialization.

- The performance tests of the satisfaction evaluation by the volunteers of the developed gel containing the semi-purified fraction of *T. chebula* gall extract should be performed and more targeted volunteers (more than 30) for the *in vivo* human skin anti-aging evaluation should be used in order to confirm the clinical anti-aging activity of the products.