CHAPTER 5
DISCUSSION

Antioxidant activity of *Butea monosperma* (*B. monosperma*) flowers was studied on their extracts by a maceration method. The dried powder of the *B. monosperma* flowers was devided into 2 groups; the first group was for a single maceration with 80 % ethanol (ethanolic crude extract) and the second was for a sequential maceration with increasing polarity solvents (*n*-Hexane, EtOAc and 80% EtOH). The additional sequential maceration and further fractionation of the selected extract were performed in order to identify the most appropriate extraction and optimal marker(s) for quality control of the extract.

According to the physicochemical study of the ethanolic crude extract from *B. monosperma* after freeze drying, the physical observation including color, appearance and odor was made. This extract was employed in the observation due to the popularity in extraction of most medicinal plants, applicability in the industry and relatively safe and inexpensive solvent when used in a commercial grade. The appearance of ethanolic crude extract was yellow solid powder with herbal odor. Chemical components found in the ethanolic crude extract were phenolic compounds and glycoside such as anthraquinone glycoside, triterpene glycoside, cardiac glycoside and saponin glycoside. The phytochemical screening could not confirm the presence of flavonoid glycoside in the ethanolic crude extract because of the interference from the color of the extract. However, the UV spectrum presented the existing of a flavonoid nucleus in the ethanolic crude extract. From the previous reports, the chemical groups found in the extract were flavonoids, saponin, triterpenes, sterols, phenolic compounds, proteins and amino acid, which were in line with previous studies (3, 5, 12-13, 15).

The antioxidant activity of the *B. monosperma* flowers extract were studied by 3 methods, (ABTS, FRAP, and DPPH method). The antioxidant activity were used to select the crude extracts for the further isolation by column chromatography. The results indicated that the ethanolic crude extract and the ethyl acetate (EtOAc) crude

extract possessed higher antioxidant activity than the other extracts. This is the reason for selecting the ethanolic crude extract and the EtOAc crude extract for the isolation. However, the isolation might be increase or decrease the antioxidant activity from crude extracts depend on the active compounds consist in each fractions. From this study, the isolation of the ethyl acetate crude extract gave 7 combined fractions (F1-F7) and the ethanolic crude extract gave 5 combined fractions (F1-F5). All the crude extracts and the combined fractions from the ethanolic crude extract and the EtOAc crude extract were compared for their antioxidant activities. The fraction which showed the highest antioxidant activity was selected to use as the marker for monitoring of the active fraction entrapment and releasing profile. In this experiment, ABTS and DPPH method were used for analysis of scavenging free radicals activity, represented value in TEAC and IC_{50} values. In addition, FRAP method was used for measuring of reducing power property, reported value in EC value. All of the methods were compared with butylated hydroxyl toluene (BHT), quercetin (QCT), α-tocopherol (a-TCP) and gallic acid (GA) as positive controls. The antioxidant activities of positive controls were significantly higher than all crude extracts from all method. The TEAC values and EC values decreased in the order of ethanolic > EtOAC > EtOH > hexane crude extract. After isolation, some fractions showed the significant higher antioxidant activity when compare to the crude extract. For EtOAc crude extract, the F6 and F4 presented the highest values in ABTS and FRAP method, respectively. For the ethanolic crude extract, the F2 showed the highest values in both methods. From DPPH method, the IC_{50} values of the ethanolic, EtOAC, EtOH crude extracts were comparable and significantly lower than the hexane crude extract. This study found that the F4, F5 and F6 fractions revealed the lowest IC_{50} values of EtOAc crude extract. And the lowest IC_{50} values of ethanolic crude extract were found in the F2 and F3 fractions. From the previous report, antioxidant activity of *B. monosperma* flower extract was studied by DPPH method which reported the percentage of reduced DPPH tested with butrin, butin (as samples) and (+)-catechin (as positive control) (2), and EC_{50} value of total methanol extract along with its ethyl acetate, butanol (as samples) and rutin (as the positive control) (3). In this study, the DPPH method represented in the IC_{50} value which differed from the previous reports (2, 3) and positive controls. The highest TEAC and EC values from the ABTS and FRAP

method indicated that the active compounds in the F2 of ethanolic crude extract were good electron donors. TLC pattern of the F2, F3 of ethanolic and F4 of EtOAC crude extract showed similar patterns and antioxidant activities. The TLC patterns and antioxidant activities presented the active compounds in these fractions. However, the F2 ethanolic crude extract was the highest potential antioxidant activity (ABTS method: TEAC value = 24.72 ± 0.02 mM/mg, and IC_{50} value = 0.0269 ± 0.0004 mg/ml, FRAP method: EC value = 240.59 ± 2.88 mM/mg, DPPH method: IC_{50} value = 0.0306 ± 0.0008 mg/ml) which conclude that the F2 ethanolic crude extract could be used as a marker of *B. monosperma* flowers extract for the further experiments in this study.

The structure elucidation of the active compounds from *B. monosperma* flowers extract was done by UV spectroscopy, FT-IR, mass spectroscopy, and ^{1}H-NMR. The UV-Visible spectrum of the ethanolic crude extract and the F2 ethanolic crude extract revealed the maximum UV absorption wavelengths (λmax) at 374.00 and 371.80 nm, respectively. The UV spectra of the crude extract and the combined fractions especially the ethanolic (Band I: 374.00nm, Band II: 268.00 nm) and F2 ethanolic crude extract (Band I: 371.80nm, Band II: 2685.60 nm) represented the flavonoid group in the extract which was reported the two band of UV spectrum of flavonoid nucleus showed two bands spectrum (Band I: 300-400 nm, Band II: 240-285 nm.) (69). The wavelength at 375 nm was selected for recording the HPLC fingerprint and active components. The analysis by HPLC was developed under both gradient and isocratic conditions. The F2 ethanolic crude extract was used as the marker for quality control of the *B. monosperma* flowers extract. The isocratic reversed phase chromatographic condition of the Liquid Chromatography Coupled with Atmospheric Pressure Ionization Mass Spectrometry (LC/API-MS) (ACN : DI water = 17 : 83, reversed phase column, Zorbax SB-C18 (150 mm x 4.6 mm i.d., 5 μm, and flow rate 1.0 ml/min) was used for identifying the major compound in the F2 ethanolic crude extract. From ^{1}H-NMR and MS spectra of the F2 ethanolic crude extract which isolated from *B. monosperma* flowers extract revealed 4 possible compounds: butin (m/z = 274.1 $[M+2H]^+$), isomonospermoside (m/z = 436.2 $[M+2H]^+$), butein (m/z = 273.2 $[M+H]^+$) and monospermoside (m/z = 435.2 $[M+H]^+$), chemical shifts and mass to charge ratios were compared with the previous report (4).

From the literature reviews, *B. monosperma* flowers extract found to contain the flavonoids; butin, butein, butrin, isobutrin, palasitrin, coreopsin, isocoreopsin, sulphuresin, monospermoside, isomonospermoside, 7,3',4'-tri-hydroxyflavone, chalcones, and aurones (2-4, 15)

The nanotechnology is an innovation sciences and develops for making particles size in a range of 10-1000 nanometers (17). The nanotechnology has advantages in many fields, especially medical and pharmaceutical fields. In this study, types of the nanoparticle systems used for entrapment of active principles were polymeric nanoparticles (chitosan-SCMC, chitosan alginate, and PLGA nanoparticles) and lipid nanoparticles (SLN and NLC). The ethanolic crude extract was the sample used in preparation of the extract in nanoparticle form. The measurement of particle diameter size, polydispersity index (PDI) and zeta potential of the obtained nanoparticles was performed by photon correlation spectrophotometer (PCS) and the percentage of entrapment efficiency of *B. monosperma* flowers extract analyzed by Ultraviolet-Visible spectrophotometer.

Chitosan (CS) is a polymer which is widely used in pharmaceutical and medical fields. CS had various biological properties such as biodegradability, biocompatibility and low toxicity. Therefore, CS was chosen to be a core polymer for nanoparticles preparation. The CS nanoparticles could be prepared from various methods (70). The ionotropic complexation is one of the simple and mild technique to prepare nanoparticles, in which the opposite charge polymers or molecules can form complex through electrostatic interaction without the assistance from the chemically cross-linking agents (70). The CS carries positive charges on amino groups at pH lower than pKa which are available for the interaction with negatively charged moieties. Moreover, sodium carboxymethylcellulose (SCMC) was used in this study. The SCMC is also the natural and safe polymer, which contained negative charges to form nanoparticles with positive charge of CS. In this study, the CS-SCMC nanoparticles were prepared from 0.1% CS and 1% SCMC. The effect of mixing ratio between CS : SCMC were studied, including the ratio of 1 : 1, 2 : 1, and 1 : 2 (by weight), respectively. The interesting concentrations of ethanolic crude extract were 0.125, 0.250, and 0.500 %. In comparison for the results between the CS : SCMC mixing ratio of 1 : 1 and 2 : 1, the average particle sizes of nanoparticles

in the mixing ratio at 1 : 1 was smaller than that of 2 : 1. In the experiment, ethanolic crude extracts, 0.125, 0.250, and 0.500 % were calculated in 6.25, 12.50, and 25.00 % ethanolic extract per polymer (CS : SCMC = 1 : 1), respectively, and 4.18, 8.33, and 16.67 % ethanolic extract per polymer (CS : SCMC = 2 : 1), respectively. The increasing of the CS polymer increased the particles size and the percentage of drug entrapment efficiency. However, when the mixing ratio of CS : SCMC was changed from 1 : 1 to 1 : 2, the aggregation was observed as white fibers because of charge influence nanoparticles. The excess of the polyanion complex structure could form an electrostatically stabilizing shell on the surface of neutralized complex core, leading to the increase of nanoparticles diameter (71). The nanoparticles prepared from CS : SCMC (1 : 1) nanoparticles exhibited the particles size in the range of 148.5 to 156.8 nm, PDI were between 0.155 to 0.206, zeta potential were 42.05 to 46.08 mV and percentage of flavonoid entrapment was 3.04 – 6.19 %. And the other ratio of CS : SCMC (2 : 1) nanoparticles showed the particles size in the range of 166.7 to 178.4 nm, PDI were between 0.120 to 0.179, zeta potential were 46.00 to 48.70 mV and percentage of flavonoid entrapment was 4.05 – 6.97 %.

The CS-alginate nanoparticles were prepared from 0.1% CS, 0.03% alginate, and 0.18 mM calcium chloride using ionotropic polyelectrolyte pregel method. The particles size of CS-alginate nanoparticles were decreased after using probe sonication. The mean particles size of CS-alginate nanoparticles was large and the flocculation was found after standing overnight. The flocculation showed the unsuitable system of CS-alginate nanoparticles for *B. monosperma* flowers extract.

The polymeric PLGA nanoparticles were prepared by the emulsion evaporation technique. The interested concentrations of ethanolic crude extract in this study were 1.88, 3.75, and 7.50 mg/ml. Ethanolic crude extracts were calculated in 4.18, 8.33, and 16.67 % per polymer (PLGA), respectively. The *B. monosperma* flowers extract entrap PLGA nanoparticles showed the small size (141.8 to 212.3 nm) and narrow size distribution (0.116 to 0.164).

The solid lipids which were used in solid lipid nanoparticles (SLN) and nanostructured lipid carrier (NLC) were selected by differential scaning calorimetry (DSC) thermogram. In this study, the solid lipid included cetyl alcohol, cetyl palmitate, glyceryl monostearate (GMS), stearyl alcohol, palmitic acid and stearic

acid. The DSC thermogram of the mixture of palmitic acid – cetyl alcohol, palmitic acid – stearyl alcohol, and stearic acid – cetyl alcohol presented the compatibility of solid lipid mixtures with the ethanolic crude extract. The palmitic acid – stearyl alcohol mixture was shown as the suitable mixture due to changing in the calorimetric peak shape demonstrating the dissolution of ethanolic extract in the solid lipid mixtures.

The solid lipid nanoparticles (SLN) for the study of SLN system, there were many factors that were studied their effect to SLN system including the solid lipid mixtures and the percent of lipid and surfactant in the formulars. The suitable formulation was the system that contained 10 % surfactant and 2.5 % lipid. The palmitic acid – stearyl alcohol mixture was selected for the solid lipid mixtures because of the DSC thermogram and the smallest particle size of SLN. The particles size of SLN which prepared from palmitic acid – cetyl alcohol and stearic acid – cetyl alcohol were large size and showed board size distribution. The percentage of entrapment efficiency was measured by an indirected method. The particles size and PDI of SLN was smallest size (46 - 49 nm) and narrow distribution (0.288 – 0.338) after probe sonication. When standing overnight, the particles size and PDI of SLN increased to large size (458 – 1562 nm) and broad distribution (0.569 – 0.918) indicating the unsuitable systems of SLN.

To formulate nanostructured lipid carrier (NLC), the palmitic acid – stearyl alcohol mixture and jojoba oil were used as the solid lipid and liquid lipid, respectively. This study varied the ratio of solid lipid and liquid lipid. The ratios of solid lipid : liquid lipid in this study were 3 : 7, 5 : 5, and 7 : 3. The mean particle size of each ratio of NLC was similar. The ratio of 7 : 3 was the most suitable ratio for NLC preparation because the ethanolic crude extract was enable entrapped when the ratio was 7 : 3. The required HLB of the lipid phase in this NLC was 12.3. This number indicated a suitable emulsifier used for preparation of emulsion. The extract with both polar and non-polar moieties could entrapped into the nanoparticular system. The particles size and PDI of NLC was smallest size (88 - 110 nm) and narrow distribution (0.354 – 0.506) after probe sonication. The interested concentrations in this study were 40.0, 80.0, and 160.0 mg/ml. Ethanolic crude extracts were calculated in 4.00, 8.00, and 16.00 % per lipid mixtures, respectively.

From the results of different nanoparticles systems, the system which showed the small size, narrow size distribution and could entrapped the ethanolic extract were NLC and PLGA nanoparticles. However, the NLC is the most optimal system for delivery the extract because there are many advantages for cosmetic production such as biocompatibility, biodegradability, low toxicity, lack of organic solvent during the production process, possibility of production on large scale for industries (48, 50-52). Moreover, NLC has the benefit for skin cosmetic include skin occlusion, increase skin hydration and elasticity (65). The chitosan nanoparticles aggregated in aqueous solution (72) and formed the film when study in cell culture (73). The irritation of acetic acid which dissolved chitosan may be occured. Then, the NLC system compared with chitosan nanoparticle presented the advantages of NLC.