CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and instruments

Chemicals

1. Absolute ethanol (Analytical grade, Labscan, Ireland)
2. Acetone (Analytical grade, Labscan, Ireland)
3. Acetonitrile (HPLC grade, Labscan, Thailand)
4. Alginate (Analytical grade, Sigma-Aldrich, USA)
5. Butylated hydroxytoluene (BHT) (Analytical grade, Sigma, Germany)
6. Calcium chloride (Analytical grade, Merck, Germany)
7. Chitosan (CS) MW 15000 (Analytical grade, Seafresh Chitosan(Lab)Company Limited, Thailand)
8. Deionized water and pyrogen free water (Elga, England)
9. Ethanol (Commercial grade, Liquor Distillery Organization Excise Department, Thailand)
10. Ethyl acetate (Analytical grade, RCI Labscan, Thailand)
11. Ferric chloride ($FeCl_3.6H_2O$) (Analytical grade, Sigma, USA)
12. Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Analytical grade, Rankem, India)
13. Gallic acid (Analytical grade, Fluka, Spain)
14. Glacial acetic acid (Analytical grade, Labscan, Ireland)
15. Hydrochloric acid (Analytical grade, Labscan, Thailand)
16. Methanol (Analytical grade, Merck, Germany)
17. Methanol (HPLC grade, Fisher, U.K.)
18. *n*-Hexane (Analytical grade, RCI Labscan, Thailand)
19. PLGA : Poly (DL-lactide-co-glycolide) with a 50:50 lactide:glycolide ratio, acid end group, and inherent viscosity of 0.26 dL/g (LACTEL Absorbable Polymers International, USA)
20. Polyvinyl alcohol (PVA) MW 15000 (Analytical grade, Fluka AG, Germany)
21. Potassium persulfate ($K_2S_2O_8$) (Analytical grade, Rankem, India)

22. Quercetine (Analytical grade, Sigma, Switzerland)
23. Silica gel 60 (0.063-0.200 mm) (Analytical grade, Merck, Germany)
24. Sodium acetate trihydrate (Analytical grade, Merck, Germany)
25. Sodium carboxymethylcellulose (SCMC) (Analytical grade, Fluka Biochemical, Finland)
26. TLC Aluminium sheet Silica gel 60 F_{254}, layer thickness 210 µm (Merck, Germany)
27. Toluene (Analytical grade, Labscan, Thailand)
28. Vitamin E (Analytical grade, Fluka Biochemical, Switzerland)
29. 2,2'-Azino-bis(3-ethylbenzo-thiazoline-6sulfonic acid) diammonium salt (ABTS) (Analytical grade, Fluka Chemical, Switzerland)
30. 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) (Analytical grade, Fluka Chemical, Switzerland)
31. 2,4,6-tris(2-pyridyl)-s-trizine (TPTZ) (Analytical grade, Fluka, Switzerland)
32. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Analytical grade, Aldrich, Germany)

Instruments

1. Analytical balance (Sartorius AC210 S, Scientific Promotion, Germany)
2. Differential Scanning Calorimeter (DSC) (7 series/UNIX DSC7, Parkin Elmer, USA)
3. Fourier Transform Infrared Spectrophotometer (Nicolet 470 FT-IR spectrometers, USA).
4. Freeze-dryer (Advantage, Vertis, USA)
5. ^{1}H NMR spectrometer (Bruker AVANCE™ NMR spectrometer, USA)
6. High intensity ultrasonic processor (Vibra cell™, Sonics & Materials, USA)
7. High Performance Liquid Chromatrography (HPLC) (Hewlett Packard series 1100, Agilent Technologies, U.S.A)
8. Microplate UV/VIS Spectrophotometer (Model 680, BIO RAD, Japan)
9. Milli-Q water filter (Milli-Q®, U.S.A)
10. pH meter (pH Level 2, Inolab, Germany)
11. Photon Correlation Spectrophorometer (Zetasizer ZS, Malvern Instruments, UK)

12. Polytron (PT-MR 3000, Kinematica AG, Switzerland)
13. Refrigerate Micro Centrifuge (SORVALL® SUPER T21, USA)
14. Rotary vacuum evaporator (EYELA, Japan)
15. Sonicator (Elma; Transsonic T460/H, Germany)
16. Transmission Electronic Microscopy (TEM, JEM-2010, JEOL, Japan)
17. Ultraviolet-Visible Spectrophotometer (UV-2450, Shimadzu, Japan)
18. UV viewer (Model Chromato-VUE® C-70G, USA)

Outline of the Experiments

- Plant material
- Preparation of *B. monosperma* flowers extract
- Phytochemical screening of *B. monosperma* flowers extract
- Isolation of *B. monosperma* flowers extract
    - Column chromatography (CC)
    - Thin layer chromatography (TLC)
- Identification and structure elucidation
    - UV-Visible Spectroscopy
    - High performance liquid chromatography (HPLC)
    - Mass spectrrometry (MS)
    - Proton nuclear magnetic resonance spectra (^{1}H-NMR)
- Antioxidant activity
    - ABTS method or Trolox equivalent antioxidant capacity (TEAC) assay
    - Ferric reducing antioxidant power (FRAP) method
    - DPPH method
- Determination of physicochemical properties of *B. monosperma* flowers extract using

  Differential Scanning Calorimeter (DSC)
- Development of nanoparticles protocol
    - Preparation of Chitosan nanoparticles
    - Preparation of Chitosan alginate nanoparticles
    - Preparation of PLGA nanoparticles
    - Preparation of Solid lipid nanoparticles (SLN)
    - Preparation of Nanostructured lipid carrier (NLC)
- Particle size diameter and zeta potential
- Transmission Electronic Microscopy (TEM)
- Percentage of entrapment efficiency (%EE)
- *In vitro* release of ethanolic extract loaded NLC
- Statistical analysis

Outline of the experiments

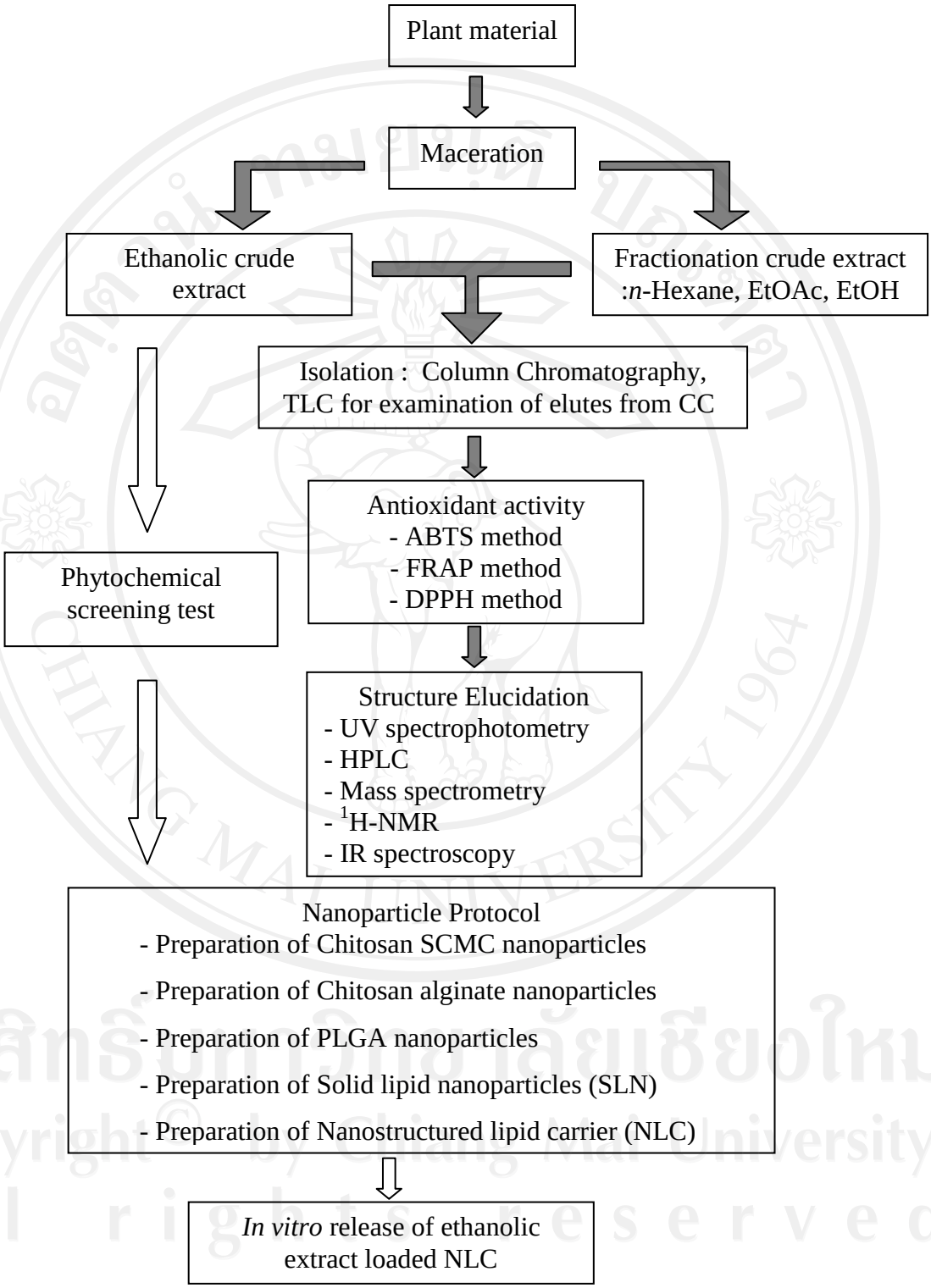

Figure 12: Flow chart of the overall experimental methods

3.2 Acquisition of bioactive compounds from B. monosperma flowers

3.2.1 Plant material

The flowers of *Butea monosperma* (Lam.)Taubert were collected between January to March in 2009 from Chiang Mai province, the northern of Thailand. The voucher specimen of this plant (No. 002968) has been deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. The flowers were washed, dried and ground into powder. The dried powder was stored at - 20 °C for further used.

3.2.2 Preparation of *B. monosperma* flowers extract (55)

A) Ethanolic crude extract: The dried powder of *B. monosperma* flowers (500 g) was macerated in 2000 ml of 80 % ethanol with continuous shaking in 30 °C water-bath for two days. The extracted solvent was decanted and the residues were repeatedly macerated 5 times. The extracted macerates from 5 times extraction were pooled andfiltered through Whatman No.1 filter paper and evaporated under reduced pressure at 40 °C by using a rotary evaporator until dryness. Then the concentrated extract was freeze-dried at 0.05 mbar pressure and –60 °C for 4 days. The obtained crude extract was stored at -20 °C for the further study.

B) Fractionation of extract: The dried powder of *B. monosperma* flowers (300 g) was macerated in 1500 ml of *n*-hexane with continuous shaking 30 °C for two days. The extracted solvent was collected and the total maceration was processed in triplicates. After the third filtration, the residue was dried at room temperature for 3 hours to ensure that *n*-hexane was completely evaporated. This dried residue was further macerated in the same manner when the solvent was changed to ethyl acetate (EtOAc), and 80 % ethanol (EtOH), respectively. Differently, maceration cycles were increased to five cycles. The filtrates of the same solvent were pooled together. And the solvent was then evaporated under reduced pressure at 30 - 40 °C by using a rotary evaporator. Then the concentrated extract was freeze-dried at 0.05 mbar pressure and – 60 °C for 4 days. The obtained crude extracts from each

solvent was stored at - 20 °C for the further study. The fractionation extracts were named hexane, EtOAc, and EtOH crude extract, respectively.

3.3 Phytochemical screening of *B. monosperma* flowers extract (56-57)

The ethanolic crude extract was evaluated for the primary phytochemicals related to chemical constituents including alkaloids, glycosides, phenolics and tannins. The methods of phytochemical screening are as following.

A) Alkaloids testing

Alkaloids were identified by the methods of Dragendorff using the potassium bismuth iodide solution, Mayer using potassiomercuric iodide solution, Wagner using solution of iodine in potassium iodide, Marme using the solution of potassium iodide and cadmium iodide, and Kraut using a modified Dragendorff's reagent.

Firstly, *B. monosperma* flowers extract which is the ethanolic crude extract from single solvent maceration (80 % ethanol) was placed in an evaporating dish. After that 0.5 ml of 2 N HCl was added and heated with continuous stirring for 10 min. Then, 0.5 mg NaCl was added and stirred until it was completely dissolved. An acidic extract solution was filtered for the primary testing.

Primary testing

Methods of Dragendorff, Mayer, Wagner, Marme and Kraut were applied for the primary testing. An acidic extract solution at volume of 0.5 ml was placed into five test tubes. The specific reagent such as Dragendorff's reagent, Mayer's reagent, Wagner's reagent, Marme's reagent and Kraut's reagent, were dropped into each test tubes, respectively. The positive results which indicated the present of alkaloids in the extract, exhibit the present of turbidity and precipitation. If the positive results found, the confirm testing will be continued.

Confirm testing

Ten milliliters of the acidic extract solution was placed in a separating funnel. Then 10 ml of ether was added. Slow swirling was performed. The mixture was then sat at room temperature for appropriate separation. The ether layer was removed. An aqueous layer was added with NH_3 solution until the color of litmus paper changed from red to blue. Then, 10 ml of the ether was added and gently swirled to extract unionized base. The unionized base would be dissolved in the ether layer. After that

the extract was partitioned with ether twice. The ether layers were pooled and evaporated to dryness in an evaporating dish. For alkaloid testing, 2.5 ml of 2 N HCl was added and stirred until the extract was completely dissolved. The acidic extract solution was filtered and filled into the test tubes for testing with Dragendorff's, Mayer's, Wagner's, Marme's, and Kraut's reagent, respectively. If the precipitation occurred, it could be concluded that the extract had the primary, secondary or tertiary alkaloids as the chemical constituents. For the basic activity test, one drop of 2 N HCl gradually added until the color of litmus paper changed from blue to red and filtered to obtain the clear solution. The clear solution was placed into 2 tubes and then Wagner's and Mayer's reagents were added seperately as specific reagent. If the precipitation occurred, it could be concluded that the extract contained the quaternary amine or amine oxide as a chemical constituents.

B) Glycoside testing

The *B. monosperma* flowers extract, which is the ethanolic crude extract, was dissolved in absolute ethanol at a concentration of 1 mg/ml. Twenty five milliliters of *B. monosperma* flowers extract solution was added with 15.0 ml of 10% HCl and then refluxed for 30 min. The obtained solution was cooled down at an ambient temperature (27-30 °C) and filled in a separating funnel. Then 10-20 ml of ether was added and partitioned for 3 times. The ether layers were pooled and added with anhydrous Na_2SO_4 for absorbing the excess water from the ether layer. The obtained ether layer and aqueous acidic layer were observed for specific types of glycosidic group as the following methods.

Anthracene/ Anthraquinone glycoside testing

The anthracene or anthraquinones were identified by the method of Borntrager's reaction. Four milliliters of the ether layer from the partitioning was evaporated until the volume was reduced to approximately 2 ml. Then 1-2 ml of 25% ammonia solution was added and shaken. The red color in an ammonia layer represented to a positive result, indicating the emodol group presented in the extract.

Sterol glycoside/ Triterpene glycoside testing

The sterol glycoside/ triterpene glycoside testing were done by Lieberman Burchard's reaction. Briefly, 10 ml of the ether layer from the partitioning was completely evaporated. The obtained residue was dissolved in 0.5 ml of acetic anhydride and 0.5 ml of $CHCl_3$. This solution was poured into the dry and clean tube. The concentrated sulfuric acid, 1-2 ml, was added slowly by using a dropper. The color of obtained ring which formed between both layers was observed at 5, 15 and 30 min. Appearance of red/reddish brown at beginning and then changing to violet and finally to green/blue-green color indicated the presences of sterol glycoside. The result of triterpine glycoside showed that the color was at first red/reddish brown and then changed to violet and stood continually.

Cardiac glycoside testing

Methods of Kadde's test was applied for the cardiac glycoside testing. Four milliliters of the ether layer was completely evaporated in an evaporating dish. The obtained residue was dissolved in 1-2 ml of methanol and added 1-2 ml of 1 N potassium hydroxide solution in ethanol. And then, the solution was added 3-4 drops of 1% 3, 5-dinitrobenzoic acid in ethanol and boiled on the water bath. The violet color was as a positive result, showing the extract had cardiac glycoside.

Saponin glycoside testing

The saponin glycoside was identified by a foam test. Four milliliters of the ether layer was completely evaporated in an evaporating dish. The obtained residue was dissolved in DI water and then transferred into a test tube. Appearance of froth after shaking indicated the presence of saponin glycoside.

Flavonoid glycoside testing

Flavonoid glycoside testing was tested by Shibata's reaction. Five milliliters of the ether layer was completely evaporated in an evaporating dish. The obtained residue was dissolved in 1-2 ml of 50% methanol on the water bath. Then, one small thin piece of magnesium metal was put and added 5-6 drops of the concentrated HCl.

The color of solution showed that the red solution related to flavonol compounds. But if the color of solution was orange that referred to flavanone compounds.

Anthocyanine testing

The anthocyanine was identified by an acidic - alkaline's test. Twenty-five percent ammonia solution was added to an aqueous acidic solution to adjust pH to be a neutral and finally to basic solution. If the test solution was red in an acidic solution and changed to violet in a neutral solution and finally to green/blue in a basic solution, the positive result was indicated. It could be concluded that the extract contained the anthocyanine compounds.

C) Coumarin testing

The coumarin was identified by the Coumarin's test. Five milliliters of the ether layer was evaporated to dry powder. The dry powder was dissolved in 1-2 ml of hot DI water and divided into 2 test tubes. One test tube was added with 2.5 ml of 10% the ammonia solution. Other test tube was used as a control. The final appearance of a blue or green fluorescence detected under the UV lamp due to the conjugated coumarin ring indicated the presence of coumarins.

D) Phenolic and Tannin testing

The phenolic was identified by ferric chloride's test, and the tannin was identified by ferric chloride, and gelatin's test.

The *B. monosperma* flowers extract which ethanolic extract part was completely evaporated on the water bath. After that, 25 ml of heated DI water was added and stirred until it cooled. Then, a few drops of 10 % NaCl solution was added to salting out the precipitate of other compounds, except tannin and filtered by using a buchner funnel with whatman no. 1 (filter paper). The obtained clear solution was divided into 4 test tubes, approximately 2 ml each. In the first test tube, approximately 4 – 5 drops of 1 % gelatin solution were added into one of the test tube. In the second tube, approximately 4 - 5 drops of 1 % gelatin solution and 10 % NaCl was added into the test tube. Approximately 3 – 4 drops of 1 % ferric chloride solution was added into the third tube. The last one was untreated and used as a

control test tube. The results interpreted that, if the solution was not reacted with ferric chloride solution that referred to the extract did not contain the phenolic compounds or tannin. In case, the result found that the sample solution was a blue-green or dark green almost black when adding ferric chloride solution and precipitated with gelatin in saline solutio. It could be inferred that the catechol, subgroup of tannin, was found in the extract. Whereas, the result showed that the sample was dark blue after adding ferric chloride solution and collaborated with precipitation of gelatin in a saline solution. It could be summarized that the pyrogallol or gallic tannin, subgroup of tannin, was found in the extract. Finally, the result indicated that the sample solution reacted with ferric chloride solution to give a green or blue solution. It could be concluded that the other phenolic compounds were found in the extract.

3.4 Isolation of *B. monosperma* flowers extract

The *B. monosperma* flowers extract from fractionated ethyl acetate and single maceration 80 % ethanol (ethanolic crude extract) were selected for the antioxidant test-guide isolation to isolate the active compounds.

3.4.1 Column Chromatography (CC)

Column chromatography was used for isolating the active compounds from *B. monosperma* flowers extract. The condition of CC was shown as following.

Column size: Diameter 13 cm

Adsorbent: Silica gel 60

Packing: Adsorbent was wet-packed after suspending in the eluent overnight. The slurry of adsorbent was poured into a column. The column was then equilibrated by slowly flowing with the eluent 2-3 column volumn.

Sample loading: The sample was dissolved in methanol and mixed with silica gel. The mixture was dried and put into the top of column.

Examination of elutes: Fractions were examined by TLC under visible light and ultraviolet light at the wavelengths of 254 and 365 nm.

The ethyl acetate (4.0 g) and ethanolic (10.0 g) crude extracts were fractionated by normal-phase column chromatography (Merck silica gel 60, 320 g, 500 g respectively), eluting with the mixture of toluene and ethanol in a gradient composition with increasing polarity. The gradient compositions were shown in Table 2. The eluates were concentrated and examined by TLC. Fractions exhibited similar TLC pattern were combined and stored at - 20 °C for further study.

Table 2: The mixture of toluene and ethanol in the gradient composition

Ratio of the mixture		Volume (ml)	
Toluene	Ethanol	EtOAc extract	Ethanolic extract
70	30	2000	3000
60	40	1500	1500
30	70	1500	1500
0	100	1500	2500

3.4.2 Thin Layer Chromatography (TLC)

TLC technique was used to indicate the differences between each fraction from column chromatography. After that all fractions which exhibited the same TLC pattern was combined together. The TLC condition was shown below.

Adsorbent: Silica gel 60 F_{254} aluminium sheet

Developing solvent: Ethyl acetate : Methanol : Water = 2.5 : 1 :1.5

Layer thickness: 210 µm.

Technique: One way, ascending

Distance: 5.5 cm.

Temperature: Room temperature 25-30 °C

Detection: 1) Visual detection under daylight

2) Ultraviolet light at the wavelengths of 254 and 365 nm

3.5 Antioxidant activity

3.5.1 ABTS method or TEAC assay (55, 58-59): This method measures the scavenging capacity of the stable cation radical $ABTS^{\bullet+}$ compared to trolox, a water soluble analogue of vitamin E. Briefly, the pre-formed radical monocation of ABTS was generated by the reaction of 7 mM ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 16-18 hours in dark at room temperature. One milliliter of the solution was diluted with 20 ml ethanol to obtain the absorbance of 0.7 ± 0.2, at 750 nm. The standard trolox was prepared for a trolox calibration curve in a concentration range of 10 – 50 µM. The plant extracts were separately dissolved in ethanol to different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml). An aliquot of 20 µl of each the plant extract solution was added to 180 µl of $ABTS^{\bullet+}$ free radical cation solution. The absorbance was monitored by spectrophotometry at 750 nm by using a microtitre plate reader. The reaction was incubated for 5 min in the dark at room temperature. All measurements were performed in triplicate. The antioxidant activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC) which is defined as the millimolar concentration of a trolox solution whose antioxidant capacity is equivalent to 1.0 mg of the extract. And the expression for $ABTS^{\bullet+}$ was IC_{50}, which was a concentration causing 50 % inhibition. Butylated hydroxytoluene (BHT), quercetin (QCT), α-tocopherol (a-TCP) and gallic acid (GA) were used as positive controls.

3.5.2 FRAP method (55, 60): The reducing power of antioxidants was determined based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form. Briefly, the FRAP reagent contained 2 ml of 10 mM TPTZ solution in 40 mM HCl plus 2 ml of 20 mM $FeCl_3$ and 20 ml of 0.3 M acetate buffer, pH 3.6, was freshly prepared. The plant extracts were separately dissolved in ethanol to yield a concentration of 1 mg/ml. An aliquot of 20 µl of each ethanol solution was added to 180 µl of FRAP reagent. The absorbance of the reaction mixture was measured at 595 nm after left for 20 min in the dark at room temperature by using a microtitre plate reader. Ethanol solutions of known Fe(II) concentration, in the range of 10-200 µM ($FeSO_4$), were used as calibration curve. The reducing power was expressed as equivalent concentration (EC value). This parameter was defined as the concentration

of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$. BHT, QCT, a-TCP and GA were used as positive controls.

3.5.3 DPPH method (58, 61): This method examines the free radical scavenging activity of the extracts, based on the scavenging activity of the stable 2,2-diphenyl-1- picrylhydrazyl (DPPH). Briefly, DPPH was dissolved in ethanol and adjusted to a final DPPH concentration of 100 µM. The plant extracts were separately dissolved in ethanol to different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml). An aliquot of 20 µl of each ethanol solution was added to 180 µl of DPPH reagent. The absorbance of the reaction mixture was measured at 540 nm by using a microtitre plate reader. The reaction was incubated for 30 min in the dark at room temperature. All measurements were performed in triplicate. The radical scavenging activity was expressed as the IC_{50} which was concentration causing 50 % inhibition. BHT, QCT, a-TCP and GA were used as positive controls.

3.6 Structure Elucidation

3.6.1 UV-Visible Spectroscopy

The crude extracts, and the combined fractions which showed an antioxidant activities were scan in a spectrum mode at the range of 800 – 200 nm. The spectrum peaks which expressed the maximum wavelength (λ max) was used for further study.

3.6.2 High performance liquid chromatography (HPLC)

The crude extracts, and the combined fractions, which showed the antioxidant activities were selected for HPLC analysis. HPLC analysis was performed using an HP1100 system with a thermostatically controlled column oven and a UV detector set at 375 nm (Hewlett-Packard, USA). Samples and mobile phases were filtrated through a 0.45 µm millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. The HPLC condition was shown in Table 3.

3.6.3 Mass spectrometry (MS)

Liquid Chromatography Coupled with Atmospheric Pressure Ionization Mass Spectrometry (LC/API-MS) for isolated compounds were obtained on Agilent, USA (The Chiang mai Laboratory of the Central Laboratory (Thailand) Co., Ltd.). The

condition for mass spectrometry which developed from HPLC condition in gradient and isocratic condition, as shown in Table 4.

Table 3: HPLC conditions for quality control of *B. monosperma* flowers extract

Operating parameter	**Conditions**
HPLC	
Stationary phase (column)	A reversed phase column Zorbax SB-C18 (150 mm x 4.6 mm i.d., 5 µm, Agilent, USA)
Guard column	Zorbax SB-C18 guard column (25 mm x 4.6 mm i.d., 5 µm, Algilent, USA)
Mobile phase	Acetonitrile (ACN) and DI water
Detector	UV-Visible 375 nm
Flow rate	1.0 ml/min
Injection volume	20 µl

Table 4: MS conditions for *B. monosperma* flowers extract (F2 Ethanolic crude extract)

Operating parameter	**Conditions**
MS	
Stationary phase (column)	A reversed phase column Zorbax SB-C18 (150 mm x 4.6 mm i.d., 5 µm, Agilent, USA)
Mobile phase	Acetonitrile (ACN) and DI water
Detector	UV-Visible 375 nm
Flow rate	1.0 ml/min
HPLC analysis	HP1100 system Agilent, Germany
MSD	Agilent, USA MSD SL
ESI	Mode scan 100 – 800 m/z (+4000 V, - 3000 V)
Drying gas	N_2 10 L/min
Temperature	300 °C

3.6.4 Infrared (IR) absorption spectrometry

The IR spectra were obtained by using a Nicolet 470 FT-IR spectrophotometer (Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang mai university). The F2 ethanolic crude extract was mashed with KBr (IR grade). The mixing powder was compressed to KBr disc before measured.

3.6.5 Proton nuclear magnetic resonance spectrometry (^{1}H-NMR)

The NMR spectra were measured with a Bruker A VANCE^{TM} NMR spectrometer; 400 MHz for ^{1}H-NMR (Department of Chemistry, Faculty of Science, Chiang mai university). The F2 ethanolic crude extract was dissolved in MeOH-d_4 (NMR grade) before measured.

3.6.6 Differential Scanning Calorimetry (DSC)

The thermal behavior of *B. monosperma* flowers ethanolic extract was determined by DSC. The condition was shown in Table 5. The thermal behavior could indicate the melting point, and purity of the compound.

Table 5: DSC conditions for quality control of *B. monosperma* flowers extract

Operating parameter	**Conditions**
DSC	
Rate	10 °C/min
Temperature	30-200 °C
Pan size	40 µl
Cover pan size	0.1 µl
Weight of sample	3 - 4 mg

3.7 Development of nanoparticles protocol

In this study, the interested nanoparticle systems had 2 systems; polymeric nanoparticles (chitosan-SCMC, chitosan-alginate, and PLGA) and lipid nanoparticles (SLN and NLC). The ethanolic crude extract was entrapped into the nanoparticle systems.

3.7.1 Preparation of chitosan SCMC nanoparticles (62)

Chitosan nanoparticles were prepared using the ionotropic complexation technique which was modified from the previous report. One hundred milligrams of chitosan were dissolved in 100 ml of 1% acetic acid (0.1 % chitosan, CS) and 1 g of SCMC were dissolved in 100 ml of deionized water (1 % SCMC). Ten milliliters of 0.1 % chitosan and water was stirred at 700 rpm. The SCMC (1 %, 1 ml) was slowly dropped into the chitosan solution under stirring at 700 rpm and stand for 1 hour. The obtained nanoparticle was used as blank. While the entrapment nanoparticles contained *B. monosperma* flowers extract. The extract was dissolved in 50 % ethanol and added before the addition of SCMC. All samples were centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was determined for percentage of entrapment efficiency (%EE). Various factors affecting the nanoparticles were studied.

A) Effect of polymer ratio

The present study was interested in the ratio between chitosan solution and SCMC solution. The ratio of 2 : 1, 1 : 1, 1 : 2 was used in this study.

B) Effect of the concentration of *B. monosperma* flowers extract

The concentration of *B. monosperma* flowers extract used in this study were 1.25, 2.50, 5.00 mg/ml.

3.7.2 Preparation of chitosan alginate nanoparticles (63)

Chitosan alginate nanoparticles were prepared by pregel preparation method. One hundred milligrams of chitosan were dissolved in 100 ml of 1% acetic acid (0.1 % Chitosan), 30 mg of alginate were dissolved in 100 ml of deionized water (0.03 % alginate) and 0.18 mM Calcium chloride were dissolved in deionized water. Then 2.4 ml calcium chloride solution was added drop wise to 37.6 ml of alginate solution while stirring at 700 rpm for 60 min to form pregel preparation. Chitosan solution was added drop wise to calcium alginate pregel, while stirring at 700 rpm, 90 minutes. And then the mixed solution was placed into probe sonicator and sonicated for 10 min (work 5s, stand 5s, 40 % Amplitude). The entrapment nanoparticles contained 5.00, 2.50, 1.25 mg of *B. monosperma* flowers extract which dissolved in 50 % ethanol. The extracts were added into the alginate solution before the drop wise of calcium

chloride solution. All samples were centrifuged at 10000 rpm for 10 min 4 °C. Then the supernatant was determined for percentage of entrapment efficiency (%EE).

3.7.3 Preparation of PLGA nanoparticles (44)

PLGA nanoparticles were prepared using the emulsion evaporation technique. Forty-five milligrams of PLGA were dissolved in 3 ml of acetone. The PLGA solution was sonicated for 15 minutes. Immediately after the sonication, the solution was drop wise into 25 ml of surfactant solution of 1 % PVA. Then the emulsion was stirred for 2 hours with a magnetic stirrer at 600 rpm to evaporate away the acetone. The entrapment nanoparticles contained 1.88, 3.75, and 7.50 mg of *B. monosperma* flowers extract which dissolved in 2 ml of 50 % ethanol. The extracts were added into the PLGA solution before the drop wise into the surfactant solution. All samples were centrifuged at 10000 rpm for 10 min 4 °C. The supernatant was determined for percentage of entrapment efficiency (%EE).

Effect of the concentration of *B. monosperma* flowers extract

The concentration of *B. monosperma* flowers extract used in this study: 1.88, 3.75, and 7.50 mg/ml

3.7.4 Preparation of solid lipid nanoparticles (SLN) (64)

The ingredients of lipid and water phase of SLN were accuracy weighed. The lipid phase was melted at 70 °C when the water phase was heated at 75 °C. Then the lipid phase was poured into the water phase under continuous stirring to form pre-emulsion. Then the pre-emulsion was placed into the high speed stirrer (Polytron) and stirred at 5000 rpm for 5 min. After that the mixture was put into probe sonicator for 10 min (work 5s, stand 5s, 40 % Amplitude). The entrapment nanoparticles contained 100 mg of *B. monosperma* flowers extract which dissolved in 50% ethanol. The extract were added into the lipid phase before pouring into the water. All samples were centrifuged at 15000 rpm for 40 min 4 °C. Then the supernatant was determined for percentage of entrapment efficiency (%EE).

A) Effect of solid lipid mixture

The mixtures of solid lipid was selected by the DSC profile were palmitic acid – cetyl alcohol, palmitic acid – stearyl alcohol, and stearic acid – cetyl alcohol.

B) Effect of percentage of solid lipid and surfactant in formulation

In this study was interested in the percent of solid lipid and surfactant. The percent of solid lipid : surfactant in formulation were 10 % : 10 %, 5 % : 10 %, and 2.5 % : 10 %, respectively.

3.7.5 Preparation of nanostructured lipid carrier (NLC) (53, 64-65)

The ingredients of lipid phase and water phase of NLC were accuracy weighed. The lipid phase which composed of solid lipid, and liquid lipid (jojoba oil) was melted at 70 °C. When the water phase was heated at 75 °C. The lipid phase was poured into the water phase under stirring to form pre-emulsion. Then the pre-emulsion was placed into the high speed stirrer (Polytron) at 5000 rpm for 5 min, after that the mixture put into probe sonicator 10 min (work 5s, stand 5s, 40 % Amplitude). For the entrapment nanoparticles were containing 100 mg of *B. monosperma* flowers extract which dissolved in 50 % ethanol were added into the lipid phase before poured to the water. All samples were centrifuged at 15000 rpm for 40 min 4 °C, the supernatant was determined for percentage of entrapment efficiency (%EE).

A) Effect of solid lipid and liquid lipid ratio

In this study was interested in the ratio of solid lipid and liquid lipid. The ratio of 3 : 7, 5 : 5, and 7 : 3 were used in this study.

B) Effect of the concentration of *B. monosperma* flowers extract

The concentration of *B. monosperma* flowers extract used in this study were 40.0, 80.0, 160.0 mg/ml

3.8 Particle diameter size and zeta potential (66)

The measurement of particle diameter size, polydispersity index and zeta potential of the obtained nanoparticles was performed by photon correlation spectrophotometer (PCS). All formulations were diluted with milli Q water to appropriate concentration. The polydispersity index indicated the size distribution of the nanoparticles population. Each value reported was the average of at least three measurements.

3.9 Characterization of ethanolic crude extract loaded nanoparticles (66)

The morphology of the emptied suitable nanoparticle systems and *B. monosperma* loaded nanoparticles were determined using a transmission electron microscopy, TEM, JEM-2010, (Department of Chemistry, Faculty of Science, Chiang mai university). The nanoparticles were distributed on to sonicate 15 min. Then dropped onto copper grid and stained with uranyl acetate (UA). After that the copper grid was dried in room temperature for 30 min. The particles size was analyzed.

3.10 Assay Validation

Assay Validation was analyzed by ultraviolet-visible spectrophotometer at 375 nm according to the maximum absorption of active (flavonoids) markers.

The standard curve of *B. monosperma* flowers extract was produced for quantitative analysis. Markers solution was prepared by ethanolic crude extract of *B. monosperma* flowers. The ethanolic extract stock solution was diluted in the range of concentrations of 2.50 – 80.0 µg/ml. The standard curves were plotted the absorbance (y) against their concentration (x). The regression analysis of the data for each component gave the value for slope, along with the intercept and correlation coefficient. The accuracy and precision were determined by recovery (%) and relative standard deviation (R.S.D.), respectively.

3.11 Percentage of entrapment efficiency (%EE) (66)

The percentage of entrapment efficiency of *B. monosperma* flowers extract which was encapsulated in nanoparticles was studied using a centrifugation method and then analyzed by Ultraviolet-Visible Spectrophotometer. The supernatant was used to determine the amount of *B. monosperma* flowers extract which was not entrapped into nanoparticles. % EE was calculated as follow in equation 1

$$\%EE = \frac{\text{Mass of extract in nanoparticle}}{\text{Mass of extract used in formulation}} \times 100 \qquad \text{Equation 1}$$

3.12 *In vitro* release of ethanolic crude extract loaded nanoparticles (66)

After the complete formation of the nanoparticles, the suspension was then centrifuged and the supernatant was decanted out. The nanoparticles were subsequently re-suspended in 1 ml media prepared at acetate buffer pH 5.5. The mixture was packed into a pre-soaked dialysis bag, which its molecular weight cut off was 12,000 Da. The packed dialysis bag was suspended in a 30 ml medium at 37 °C, under agitation (600 rpm), for 7 days. At time 0 and at different time intervals, 1 ml of medium was withdrawn and the same volume of a fresh medium was replenished back. The release profiles from samples consisting of only blank nanoparticles and an ethanolic extract re-suspended in 50 % ethanol were compared with the profiles of the ethanolic extract loaded nanoparticles. The experiments were performed in triplicate.

3.13 Statistical analysis (14, 67)

Results were expressed as Means ± S.D. and all statistical comparisons were made by ANOVA and Tukey's Multiple Comparison Test, and p values less than 0.05 were considered significant.