CHAPTER 4
RESULTS

The main objectives of this present work were to identify the active fractions with high antioxidant activity, and develop nanoparticle systems for encapsulating *Butea monosperma* (Lam.) Taubert flowers extract.

4.1 Medicinal plant materials

The flowers of *Butea monosperma* (Lam.) Taubert used in this study were collected between January to March in 2009 from Chiang Mai province, the northern part of Thailand. The herbarium specimen of this plant (Voucher No. 002968) has been deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. (Figure 13)

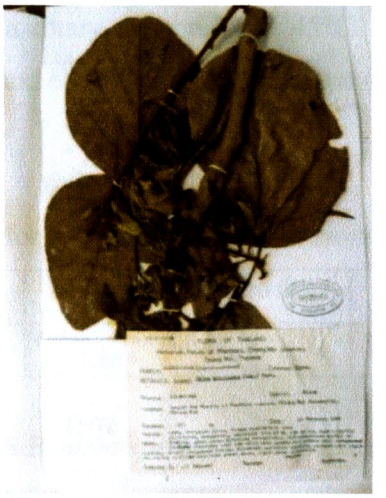


Figure 13: The herbarium specimen of *B. monosperma*

B. monosperma is a medium sized deciduous tree of up to 50 feet high. It has stunning flower clusters, and the trunk is normally crooked and twisted with irregular branches with rough, grey bark. (Figure 14)

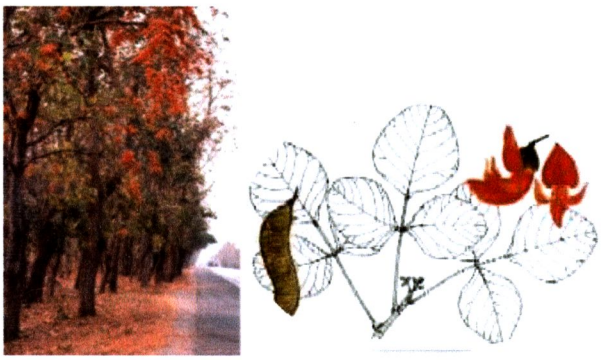


Figure 14: The characterization of *B. monosperma* (68)

4.2 Extract preparation

The method in this study used the maceration method. First, the flowers of *B. monosperma* were washed, dried in an oven at 50°C for 72 hours, and ground into powder. Second, the dried powder was divided into two groups for maceration and fractionation. All parts of extract were filtered through Whatman filter paper no.1, the filtrate was evaporated by a rotary evaporator at 45°C. The yield values of dried powder from fresh flowers and each solvent extract from dried powder were shown in Table 6 and Table 7.

Table 6: The percent yield for powder from fresh *B. monosperma.* flowers

Fresh of flowers (kg)	Powder of flowers (kg)	% Yield
36.15	4.65	12.86

Table 7: The yield values of *B. monosperma* flowers extracts.

Extracts		Yield value (%w/w)
Maceration	80 % Ethanol Ethanolic crude extract	35.25
Fractionation	Hexane	0.95
	Ethyl acetate	1.78
	80 % Ethanol	25.35

4.3 Quality control of the extract

4.3.1 Appearance of *B. monosperma* flowers extract

The ethanolic crude extract of *B. monosperma* flowers (BMF) which macerated with 80 % ethanol was studied for its antioxidant activity and compared with the extracts from sequential macerations (hexane, ethyl acetate, and 80 % ethanol). The ethanolic crude extract showed highly effective antioxidant activity, so the ethanolic crude extract was selected for further study. The obtained extract, after freeze drying, was yellow solid mass, loose texture and herbal odor as shown in Figure 15.

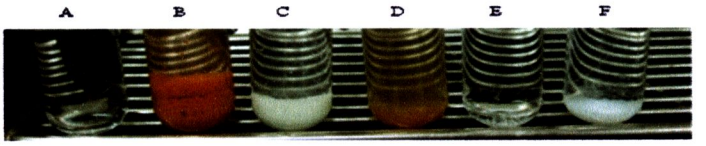
Figure 15: Appearance of *B. monosperma* flowers extract (maceration with 80% ethanol)

4.3.2 Phytochemical screening of *B. monosperma* flowers extract

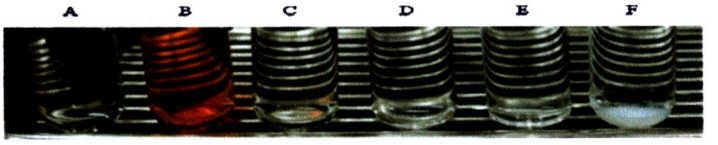
The alkaloids testing

An acidic extract solution was placed into five test tubes. The specific reagents 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 show the turbidity or precipitation with specific reagent which indicate the presenting of the alkaloid group in the extract. The results from the alkaloids testing were showed in Figure 16(A1) to 16(A6). The *B. monosperma* flowers extract did not exhibit turbid samples or precipitation by testing with the above reagents. Thus, alkaloid is not a major group in the extract.

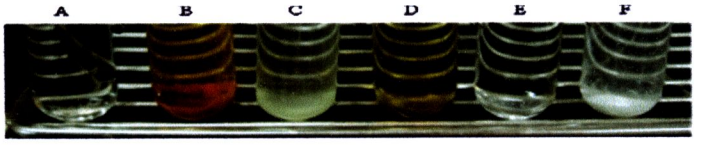
(A1) Standard strychnine (Alkaloids standard)



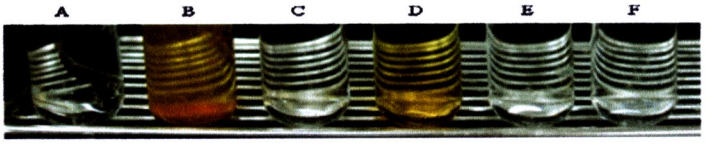
(A2) Standard atropine (Alkaloids standard)



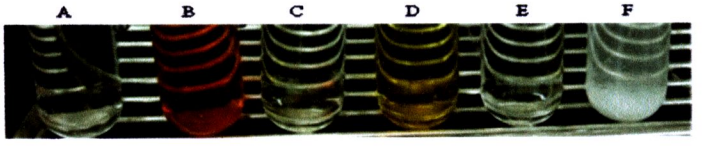
(A3) Standard scopolamine HBr (Alkaloids standard)



(A4) DI water (control)



(A5) 95 % Ethanol (control)



(A6) *B. monosperma* solution

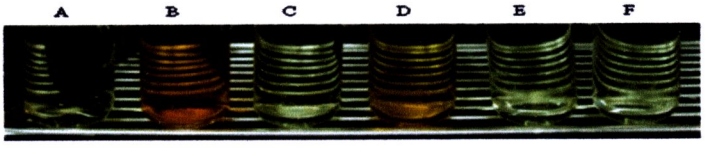


Figure 16: The results from alkaloid testing with specific reagents

Note: A = Alkaloids standard / *B. monosperma* solution / control

B = Alkaloids standard / *B. monosperma* solution + Dragendorff's reagent

C = Alkaloids standard / *B. monosperma* solution + Mayer's reagent

D = Alkaloids standard / *B. monosperma* solution + Wagner's reagent

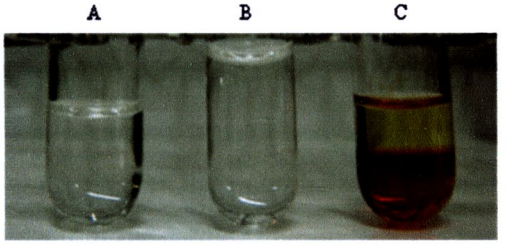
E = Alkaloids standard / *B. monosperma* solution + Marme's reagent

F = Alkaloids standard / *B. monosperma* solution + Kraut's reagent

The glycoside testing

Anthracene/ Anthraquinone glycoside testing

To determine the anthracene or anthraquinones in the extract, the Borntrager's reaction was used in this study. Twenty-five percent of ammonia solution was added into the concentrated ether layer of the extract and mixed homogeneously. The red color in an ammonia layer which was in the lower layer after mixing, indicated the emodol group in the extract. The result showed that the extract of *B. monosperma* flowers presented the anthraquinone glycoside (emodol group) by observing the red color in the ammonia layer (Figure 17).



Note: A = Ethanol
B = DI water
C = *B. monosperma* solution

Figure 17: The result from anthracene / anthraquinone glycoside test

Sterol glycoside/ Triterpene glycoside testing

The sterol glycoside and triterpene glycoside tests were observed by Lieberman Burchard's reaction. The obtained residue of ether layer was dissolved with 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 was added slowly using a dropper. The obtained color was formed between both layers. Appearance of red/reddish brown can be visualised at beginning step and then changes to violet and green/blue-green color in the final which indicates the sterol glycoside. For the triterpine glycoside, the violet color is observed at the final step difference from the sterol glycoside. As shown in Figure 18, the violet ring between the two layers was observed. This result indicated the extract of *B. monosperma* flowers consisted of triterpine glycoside compound(s).

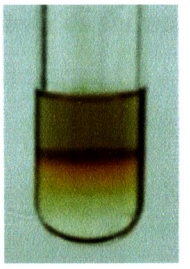


Figure 18: The result from the sterol glycoside/ triterpene glycoside test

Cardiac glycoside testing

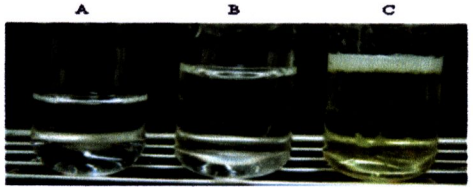
Methods of Kadde's test was applied for the cardiac glycoside testing. The ether layer was completely evaporated in an evaporating dish. The obtained residue was dissolved in methanol and 1 – 2 ml of 1 N potassium hydroxide solution in ethanol was added. The solution was then added with 1% 3, 5-dinitrobenzoic acid in ethanol and boiled on the water bath. The violet color in the final step represents as a positive result if the cardiac glycoside exists in an extract. From this study, the violet color occurred in the *B. monosperma* flowers extract indicating the present of cardiac glycoside compound(s) in the extract (Figure 19).

Figure 19: The result from cardiac glycoside test

Saponin glycoside testing

Foam test was used to identify the saponin glycoside compound in the extract. The ether layer of the extract 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 indicates the present of saponin glycoside

compounds. Froth on the topmost layer as shown in Figure 20, exhibited the existing of saponin glycoside compound in the extract .



Note: A = Ethanol
B = DI water
C = *B. monosperma* solution

Figure 20: The result from saponin glycoside test

Flavonoid glycoside testing

Flavonoid glycoside compound in the extract was tested by Shibata's reaction. The ether layer of extract was completely evaporated in an evaporating dish. The obtained residue was dissolved in 50% methanol on the water bath. Then, a small thin piece of magnesium metal was put and 5-6 drops of the concentrated hydrochloric acid (HCl) was added. The red solution and orange solution after reaction present the flavonol and flavanone compound, respectively. The ether layer of the *B. monosperma* flowers extract showed the dark yellow solution. From this data, the ethanolic crude extract could not summarize that there was the flavonoid glycoside in the extract because the extract's color interfered the assay (Figure 21).

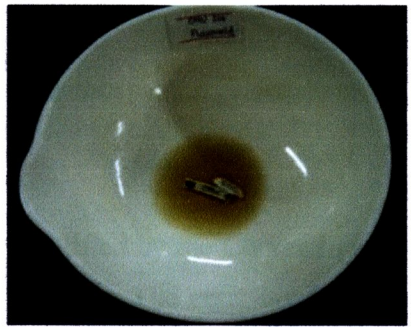
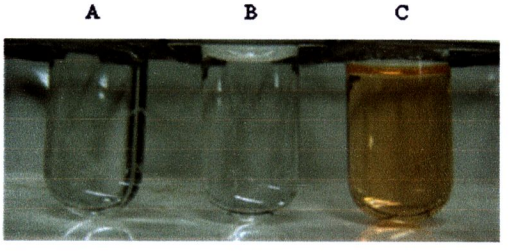


Figure 21: The result from flavonoid glycoside test

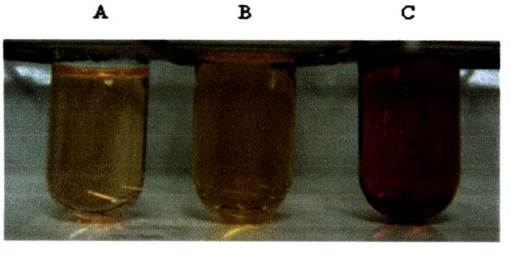
Anthocyanine testing

The anthocyanine was identified by an acidic - alkaline's test. Twenty-five percent of ammonia solution was added in an aqueous acidic solution to adjust the pH of this solution from acidic to neutral and finally to basic solution. The positive result of anthocyanine glycoside compound can be observed from the red color in an acidic solution, violet color in a neutral solution and a blue / green color in a basic solution. In this study, the *B. monosperma* flowers extract did not exhibit the color series same as the positive result indicating the absence of the anthocyanine glycoside compound in the extract (Figure 22-23).



Note: A = Ethanol
B = DI water
C = *B. monosperma* solution

Figure 22: The result from anthocyanine glycoside test in an acidic solution



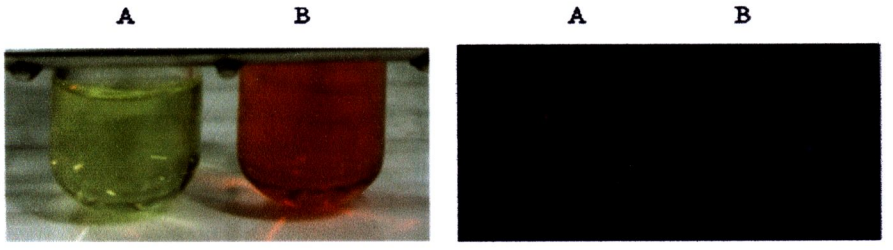
Note: A = Acidic solution
B = Neutral solution
C = Basic solution

Figure 23: The result from anthocyanine glycoside test in *B. monosperma* solution

Coumarin testing

The coumarin was identified by the Coumarin's test, which can observe from its fluorescence under a UV lamp. The ether layer of the extract was evaporated to dry powder. The dry powder was dissolved in hot DI water and divided into 2 test tubes. One test tube was added with 10% the ammonia solution. The second tube was used as a control. The final appearance of a blue or green fluorescence under the UV lamp observation reveals the conjugated coumarin ring in the structure. The *B. monosperma* flowers extract in both test tubes did not show blue or green

fluorescence under the UV lamp, referring that the *B. monosperma* flowers extract did not contain the coumarin as shown in the Figure 24.



Visible light UV 365 nm

Figure 24: The result from coumarin testing in visible light and under the UV lamp

Note: A = *B. monosperma* solution (control)
B = *B. monosperma* solution + 10 % Ammonia

Phenolic and Tannin testing

The phenolic compound was identified by ferric chloride's test, and the tannin was presented by ferric chloride, and gelatin's test. In this study, the ethanolic crude extract of the *B. monosperma* flowers was completely evaporated on a water bath. After that, 25 ml heated DI water was added and stirred until it cooled. Then, a few drops of 10 % NaCl solution was added to salting out the precipitates of other compounds, except tannin and filtered by a buchner funnel. The obtained clear solution was divided into 4 test tubes, approximately 2 ml each. In the first test tube, 1 % gelatin solution were added into the test tube. In the second tube, 1 % gelatin solution and 10 % NaCl was added into the test tube. In the third test tube, 1 % ferric chloride solution was added. The last one was as a control test tube. The results interpreted that, if the solution is not reacted with ferric chloride solution that refers to the extract is not consist of the phenolic or tannin compounds. In the case of the catechol, subgroup of tannin, find in the extract, the sample solution is a blue-green or dark green almost black when adding ferric chloride solution and precipitates with gelatin in saline solution. Whereas, the result shows that the sample is a dark blue after adding ferric chloride solution and precipitation of gelatin in saline solution, it can be summarized that the pyrogallol or gallic tannin, subgroup of tannin, was found

in the extract. Finally, if the result indicates that the sample solution reacts with ferric chloride solution occurs in a green or blue solution, it can be concluded that the other phenolic find in the extract. The *B. monosperma* flowers extract showed the only reaction with ferric chloride solution to give a green or blue solution, so it has the phenolic compound. The result displays in Figure 25.

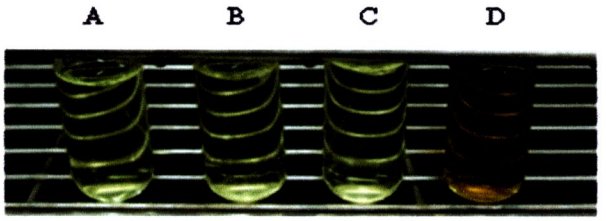


Figure 25: The result from the phenolic and tannin test

Note: A = *B. monosperma* solution (control)

B = *B. monosperma* solution + 1 % gelatin

C = *B. monosperma* solution + 1 % gelatin + 10 % NaCl

D = *B. monosperma* solution + $FeCl_3$

In summary, the primary phytochemical tests indicated that the chemical constituents found in *B. monosperma* flowers extract consisting of phenolic and glycoside such as anthraquinone glycoside, triterpene glycosidde, cardiac glycoside, and saponin glycoside. The data were shown in Table 8, 9 and 10. The phenolic was as important active compounds for biological activities (antioxidant activity) of *B. monosperma* flowers extract.

Table 8: Alkaloids screening test

	Reagents					
	Control	**Dragendorff**	**Mayer**	**Wagner**	**Marme**	**Kraut**
Preliminary test						
Standard strychnine	-	+	+	+	+	+
Standard atropine	-	-	-	-	-	+
Standard scopolamine HBr	-	+	+	-	-	+
DI water	-	+	-	-	-	-
95% ethanol	-	-	-	-	-	+
Ethanolic crude extract (BMF)	-	-	-	-	-	-

Note: + = positive

\- = negative

Table 9: Glycosides screening test

TEST		DI water	95% EtOH	Ethanolic crude extract (BMF)
Glycosides	**Anthraquinone**	-	-	+
	Sterol / Triterpene	-	-	+, triterpene
	Cardiac	-	-	+
	Saponin	-	-	+
	Flavonoid	-	-	-
	Anthocyanine			
	Acidic	-	-	-
	Neutral	-	-	-
	Basic	-	-	-
Coumarin		-	-	-

Note: + = positive

\- = negative

Table 10: Phenolic and Tannin test

	Reagents			
	Control	**1% gelatin**	**1% gelatin + 10% NaCl**	**1% $FeCl_3$**
DI water	-	-	-	-
95% ethanol	-	-	-	-
Ethanolic crude extract (BMF)	-	-	-	+

Note: + = positive

\- = negative

4.4 Antioxidant activity of *B. monosperma* flowers extract (crude extract)

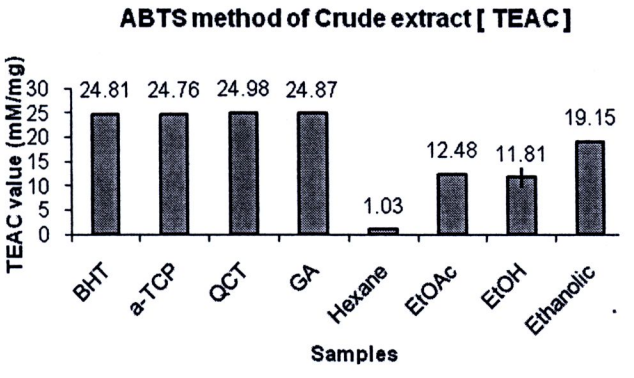
The oxidation mechanisms are divided into two main types of antioxidants. Primary antioxidants can inhibit oxidation by scavenging free radicals by donation of hydrogen atom or electrons, which converts them to more stable product. Secondary antioxidants function by many mechanisms, including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. In this experiment, the inhibition of oxidation reactions by scavenging free radicals which donated electrons have ABTS and DPPH method. And another one, FRAP method, is reducing power property. There are 2 groups for antioxidant activity test, the first is an ethanolic crude extract (single solvent maceration) and another group is fractionated crude extracts (hexane, ethyl acetate (EtOAc), and 80 % ethanol (EtOH)). All of the method was compared with butylated hydroxyl toluene (BHT), quercetin (QCT), α-Tocopherol (a-TCP) and gallic acid (GA) as positive controls.

4.4.1 ABTS method:

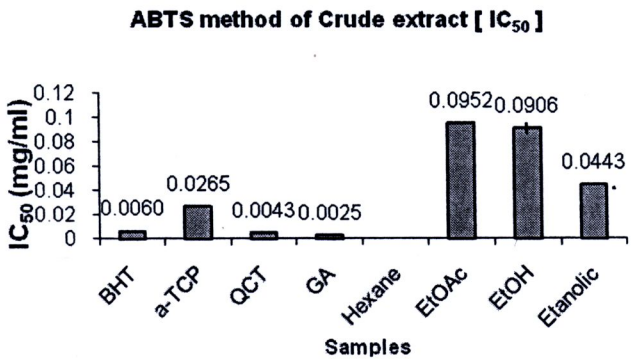
The comparative study of antioxidant activity from each fractionate part of *B. monosperma* crude extracts was carried out by a ABTS free radical-scavenging method. This is an excellent method for determining the antioxidant activity of broad diversity of substances (55). Inhibition of the $ABTS^{\cdot+}$ free radicals by different fractions of *B. monosperma* was monitored. The antioxidant activity of all crude extracts were expressed as trolox equivalent antioxidant capacity (TEAC) value and IC_{50} as shown in Figure 26.

TEAC value represents the antioxidant activity of 1 mg extract equivalent to trolox concentration in millimolar (mM). This value is calculated from a standard curve plotted from the trolox concentrations and the corresponded absorbance. IC_{50} is the inhibitory concentration of bioactive compounds which inhibit the biological process at 50%. This value is calculated from the standard curve plotted between % inhibition and concentration (mg/ml).

The TEAC values and IC_{50} value obtained from all tested extracts were shown in Figure 26. The TEAC value from EtOAc was higher than EtOH crude extracts, but these were not significantly different ($p > 0.05$). However, the IC_{50} value of EtOAc crude extract was significantly higher than EtOH crude extract ($p < 0.05$), the data were expressed in Figure 26 (A) and Figure 26 (B). Then comparison of ethanolic crude extract and EtOH extract from the fractional crude extract, showed the ethanolic extract was significantly higher than EtOH crude extract with TEAC value and IC_{50} ($p < 0.05$), were shown in Figure 26 (A) and Figure 26 (B).



(A)



(B)

Figure 26: Free radical-scavenging activity of *B. monosperma* flowers crude extracts expressed as TEAC value (A), and IC_{50} (B) by ABTS method

4.4.2 FRAP method:

The principle of the FRAP method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process. The reducing power of all *B.monosperma* crude extracts were expressed as an EC value, as shown in Figure 27.

EC value represents the concentration of antioxidant having a ferric reducing ability equivalent to 1 mM $FeSO_4$.

From the experiment, it was clearly indicated that the EtOAc crude extract had the highest reducing power with the EC value of 167.31 ± 2.66 mM/mg extract. This EC value was significantly higher than the EC values from the other crude extracts ($p < 0.05$). This was the reason for the selection of EtOAc crude extract for active fraction isolation by column chromatography, besides, the ethanolic crude extract.

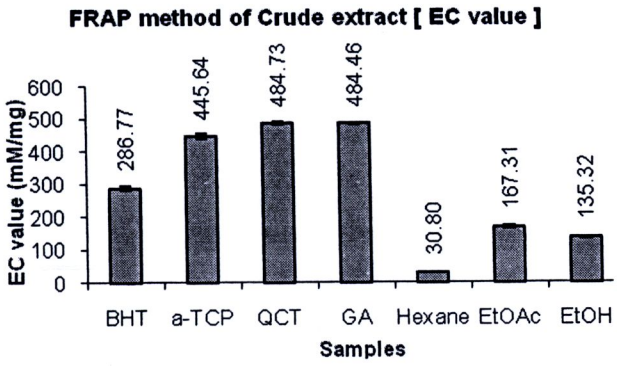


Figure 27: Reducing power of *B. monosperma* flowers extracts were expressed as EC value, crude extract

4.4.3 DPPH method:

The radical scavenging activity on DPPH was expressed as IC_{50}. This value was the concentration of the extract required to inhibition 50 % of an absorbance measured from the initial concentration of DPPH free radical. The IC_{50} values of the crude extracts of *B.monosperma* were expressed in Figure 28. The results of the antioxidant activity testing by DPPH method exhibited that the EtOAc, EtOH, and ethanolic crude extracts yielded similar IC_{50} values ($p > 0.05$). However, in this study selected only the EtOAc and ethanolic crude extracts for further isolation by column chromatography.

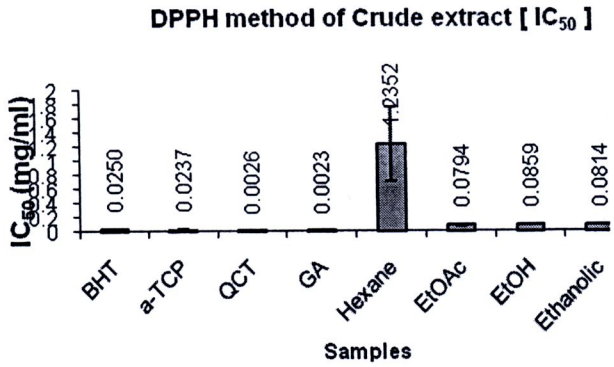
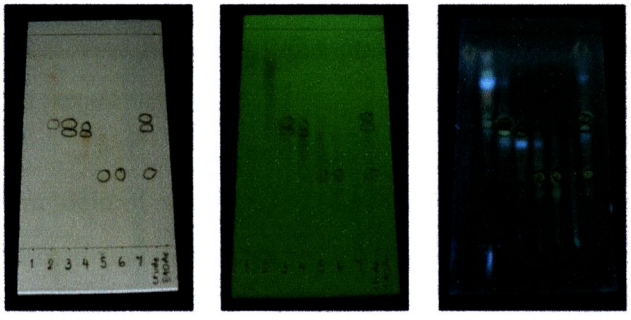


Figure 28: Free radical-scavenging activity of *B. monosperma* flowers extracts were expressed as IC_{50} value, crude extract

From the antioxidant activity test results, an ethanolic crude extract (single solvent maceration) showed the highest antioxidant activity followed by the ethyl acetate (EtOAc) fractional extract (Figure 26). In order to isolate the active marker from the *B. monosperma* flower extract, the EtOAc and ethanolic crude extracts were selected for further isolation by column chromatography.

4.5 Isolation of *B. monosperma* flowers extract

The ethyl acetate crude extract was fractionated by column chromatography. The eluates were examined by TLC under visible and UV light and 7 combined fractions (F1-F7) were obtained. The results were shown in Figure 29, and Table 11.



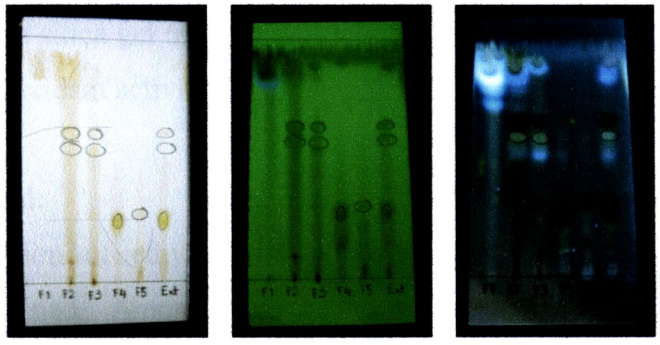
Visible light UV 245 nm UV 365 nm

Figure 29: The combined fractions of *B. monosperma* flowers EtOAc crude extracts examined by TLC

Table 11: The percent yield of combined fractions (F1-F7) from ethyl acetate crude extract

Combined fractions	Yield (% w/w)
F1 (1-4)	12.32
F2 (5-6)	9.67
F3 (7-17)	38.26
F4 (18-22)	8.63
F5 (23-28)	8.04
F6 (29-49)	21.36
F7 (50-66)	6.71

The ethanolic crude extract was fractionated by column chromatography. The eluates were examined by TLC and 5 combined fractions (F1-F5) were obtained. The results were shown in Figure 30, and Table 12.



Visible light UV 245 nm UV 365 nm

Figure 30: The combined fractions of *B. monosperma* flowers ethanolic crude extracts examined by TLC

Table 12: The percent yield of combined fractions (F1-F5) of ethanolic crude extract

Combined fractions	Yield (% w/w)
F1 (1-3)	3.20
F2 (4-12)	5.25
F3 (13-16)	4.99
F4 (17-27)	67.77
F5 (28-38)	22.10

4.6 Antioxidant activity of the isolation of ethyl acetate fraction and ethanolic crude extract from *B. monosperma* flowers

The ethyl acetate crude extract (4.0 g) was isolated by normal-phase column chromatography (Merck silica gel 60, 320 g), eluting with the different compositions of toluene and ethanol with increasing gradient polarity. The eluates were concentrated and examined by TLC. Fractions exhibited similar TLC pattern were combined. After isolation of ethyl acetate (EtOAc) crude extract, seven combined fractions were obtained

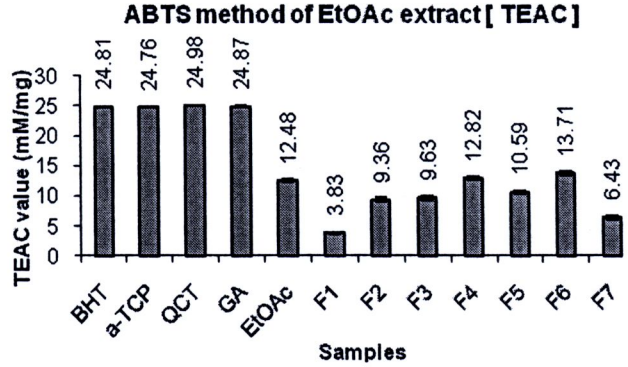
As a forementioned, the ethanolic crude extract was another crude extract with high antioxidant activity that was isolated in the same manner as the ethyl acetate crude extract. Ten grams of the ethanolic crude extract was isolated on the opened column packed with silica gel 60, 500 g. Five combined fractions were obtained and tested for their antioxidant activity.

4.6.1 ABTS method:

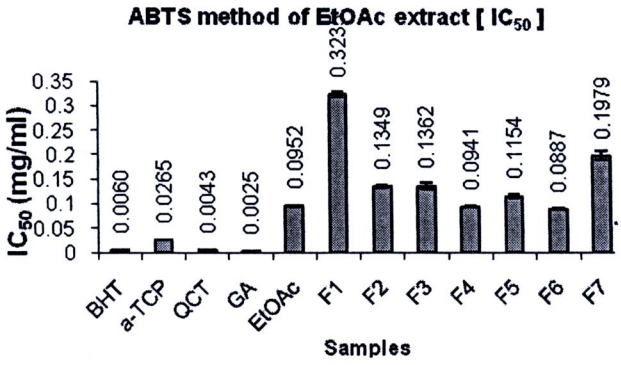
After isolation of ethyl acetate (EtOAc) crude extract, seven combined fractions were obtained. Among those fractions, F6 showed the highest antioxidant activity with a TEAC value and IC_{50} of 13.71 ± 0.45 mM trolox equivalents/mg extract, and 0.0886 ± 0.0000 mg/ml, respectively, as shown in Figure 31(A) and Figure 31(B).

As a forementioned, the ethanolic crude extract obtained 5 combined fractions. It was found that the F2 showed the highest antioxidant activity with a TEAC value, and IC_{50} was 24.72 ± 0.02 mM trolox equivalents/mg extract, and 0.0269 ± 0.0000 mg/ml, respectively, the results as shown in Figure 31(C) and Figure 31(D).

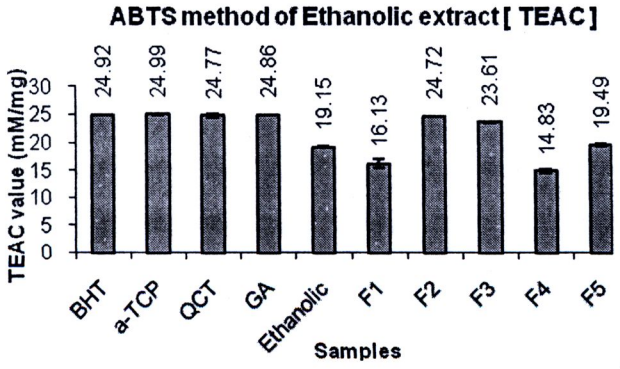
The comparison of the F6 (EtOAc extract) and the F2 (ethanolic extract) showed that the TEAC value and IC_{50} of the F2 (ethanolic extract) were significantly higher and lower than F6 (EtOAc extract), respectively ($p<0.05$). In the other words, the F2 was the best in free radical scavenging activity as investigated by ABTS method.



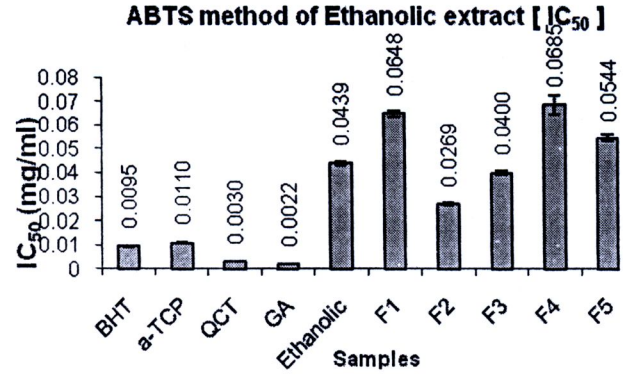
(A)



(B)



(C)



(D)

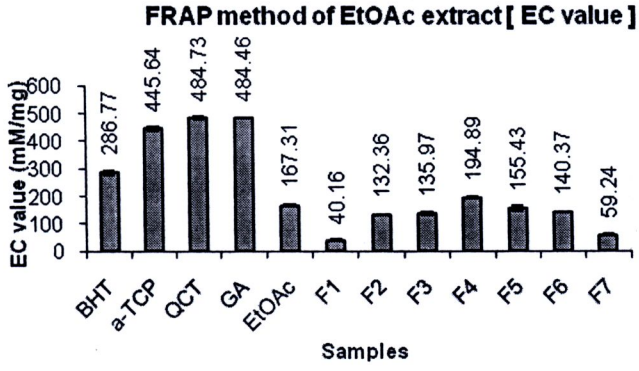
Figure 31: Free radical-scavenging activity of *B. monosperma* flowers extracts expressed as TEAC value of EtOAc extract (A), Ethanolic extract (C) and IC_{50} value of EtOAc extract (B), Ethanolic extract (D) by ABTS method

4.6.2 FRAP method:

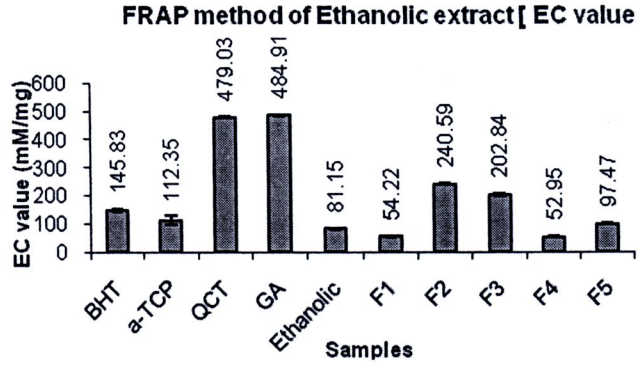
The isolation of ethyl acetate crude extract had 7 combined fractions. Comparison with 7 combined fractions of the EtOAc crude extract, the F4 showed the highest antioxidant activity with a EC value was 194.89 ± 1.61 mM/mg extract, the results as shown in Figure 32(A).

In the another crude extract, the isolation of ethanolic crude extract had 5 combined fractions. After that comparison with 5 combined fractions, it was found that the F2 showed the highest antioxidant activity with a EC value was 240.59 ± 2.88 mM/mg extract, the results as shown in Figure 32(B).

The EC value of the F2 (ethanolic extract) was expressed significantly higher than the F4 (EtOAc extract) ($p < 0.05$). According to its high EC value could be considered that compounds in the F2 of ethanolic crude extract was good electron donor(s) by reducing the oxidized intermediates into the stable form.



(A)



(B)

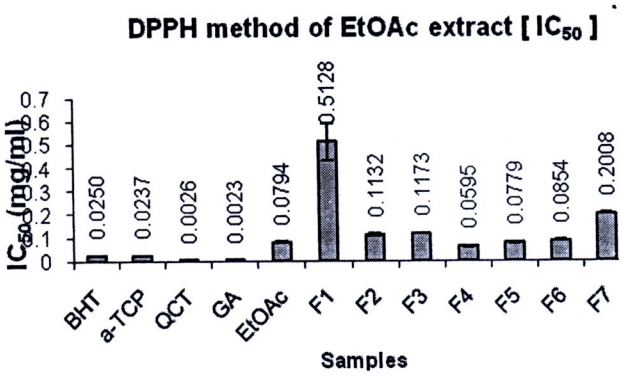
Figure 32: Reducing power of *B. monosperma* flowers extracts were expressed as EC value, EtOAc extract (A), and Ethanolic extract (B) by FRAP method

4.6.3 DPPH method:

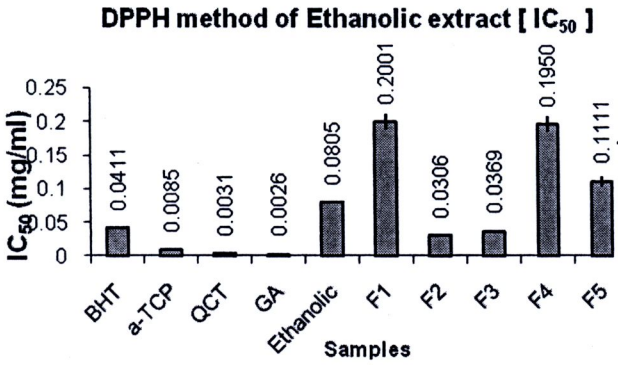
The isolation of ethyl acetate crude extract gave 7 combined fractions. Comparison among 7 combined fractions of the EtOAc crude extract, the IC_{50} of the F4, F5, and F6 were 0.0595, 0.0779, and 0.0854 mg/ml, respectively. Statistically, the IC_{50} of the F4, F5, and F6 fractions were not significantly different ($p>0.05$), as shown in Figure 33(A).

The isolation of ethanolic crude extract had 5 combined fractions. After that comparison with 5 combined fractions, it was found that the IC_{50} of the F2 and F3 from the ethanolic crude extract were 0.0306 and 0.0369 mg/ml, respectively. The statistic test showed that the IC_{50} of the F2 and F3 from the ethanolic crude extract were not significantly different ($p>0.05$), as shown in Figure 33(B).

The F4, F5, and F6 (EtOAc extract) were compared with the F2 and F3 (ethanolic extract), represented that the IC_{50} values of the F2 and F3 were significantly lower than three fractions of the EtOAc extract ($p<0.05$). In the other words, the F2 and F3 was the best of free radical scavenging activity by DPPH method



(A)

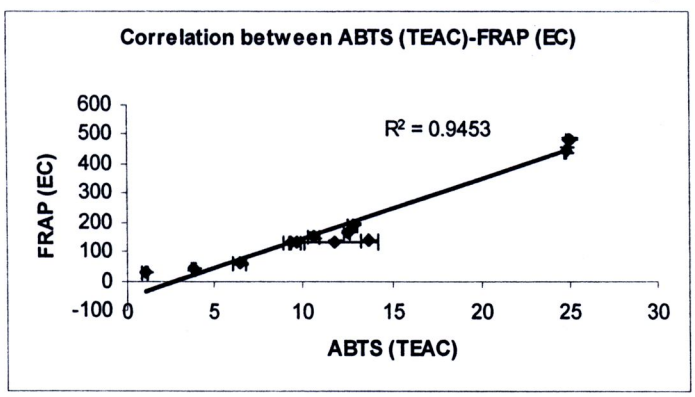


(B)

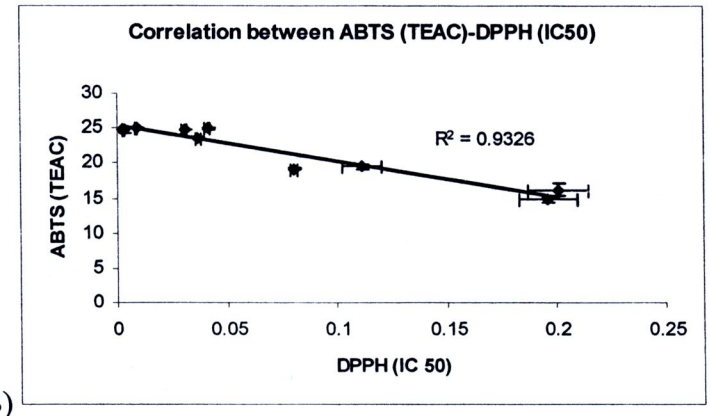
Figure 33: Free radical-scavenging activity of *B. monosperma* flowers extracts were expressed as IC_{50} value, EtOAc extract (A), and Ethanolic extract (B) by DPPH method

4.6.4 Correlation of antioxidant activities

The correlation between TEAC and EC values of all samples was shown in Figure 34(A). High correlation (R^2=0.9453), indicated that crude and combined fractions extract possessed antioxidant mechanisms in both ways; the free radical scavenging and the reducing power. Moreover, the correlation between TEAC values from ABTS method and IC_{50} from DPPH method was shown in Figure 34(B), exhibited the high correlation (R^2=0.9326), this result suggested that the crude extract and the combined fractions extract had antioxidant mechanism in free radical scavenging activity. The active compounds from *B. monosperma* flowers extract had the antioxidant activities in both mechanisms; the free radical scavenging and the reducing power.



(A)



(B)

Figure 34: Correlation between free radical scavenging activity versus reducing power (A), free radical scavenging activities in different method, ABTS versus DPPH, between TEAC-IC_{50} (B) of the crude extract and the combined fractions of *B. monosperma* flowers extract

4.7 The structure elucidation of the active compounds from *B. monosperma* flowers extract

The structure elucidation of the active compound(s) used many instruments such as UV spectrophotometer, HPLC, LC-MS, FT-IR and ^{1}H-NMR. The F2 of ethanolic crude extract which was the highest antioxidant activity, used as a marker for this study

4.7.1 UV-Visible Spectroscopy

The UV spectrophotometer used for identification and analysis, it had been used for studying flavonoid glycoside, it showed that the flavonoid group had two bands spectrum. The result could be to consider that the crude extract and the combined fractions of *B. monosperma* was the flavonoid (Band I: 300-400 nm., Band II: 240-285 nm.) (16, 69).

The UV spectrum showed the wavelength of the crude extract and the combined fractions, which had the antioxidant activity, were shown the spectral data in Figure 35, and Table 13, and the maximum wavelength (λmax) was used in the further study.

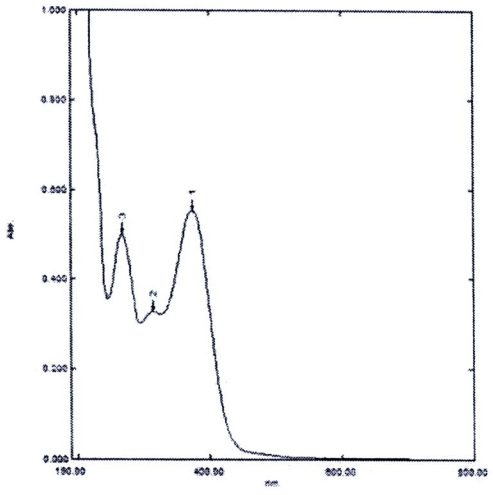


Figure 35: The UV Spectrum of *B. monosperma* flowers extract (Ethanolic extract)

Table 13: Wavelengths (λ) of the crude extract and the combined fractions of *B. monosperma*, which had the antioxidant activity

Samples	Wavelength (λ), nm.	
	Band I *	**Band II**
EtOAC crude extract	374.60	266.20
F4_EtOAc crude extract	374.60	268.40
F6_EtOAc crude extract	375.60	267.40
Ethanolic extract	374.00	268.00
F2_ Ethanolic extract	371.80	265.60

* The maximum wavelength (λmax), nm.

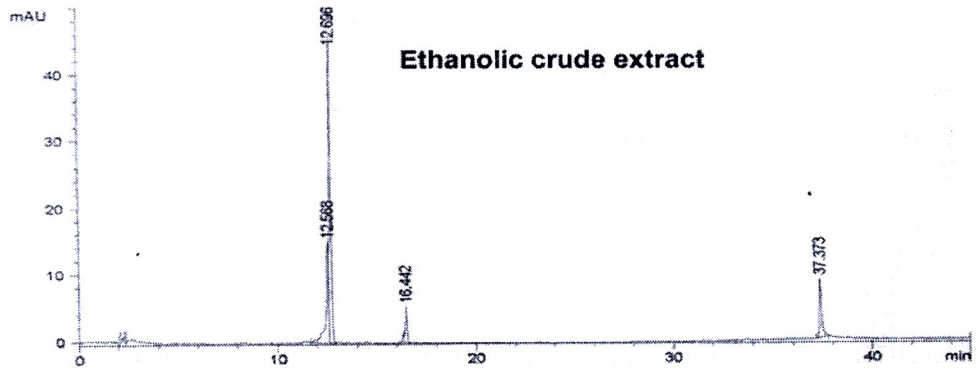
4.7.2 High performance liquid chromatography (HPLC) *B. monosperma* flowers extract chromatogram

From the UV spectrum used the maximum wavelength (λmax) in UV detector of HPLC. According to the significantly high antioxidant activity of the F2 fraction from the ethanolic crude extract, F2 fraction was selected as a marker fraction for quality control of the *B. monosperma* flowers extract and further active compound

identification. The analysis by HPLC was developed under gradient and isocratic conditions. The chromatograms of the markers in *B. monosperma* flowers extract were shown in Figure 36, and Figure 37. In a gradient condition, the condition as shown in Table 14, retention time (t_R) of *B. monosperma* flowers extract had two peaks at range of 12-13 and 16-17 min., and the F2 ethanolic crude extract was found at range of 16 – 17 min. In isocratic condition, the F2 ethanolic crude extract was found the peaks at 1.655 and 7.695 min. The isocratic condition (ACN : DI water = 17.5 : 82.5) of the F2 ethanolic crude extract was the condition used to investigate of major compound(s) by liquid chromatography-mass spectrometry (LC-MS).

Table 14: The gradient condition for isolation *B. monosperma* flowers extract using HPLC

Time (min)	% ACN	% DI water
0	0	100
25	50	50
35	100	0
45	100	0



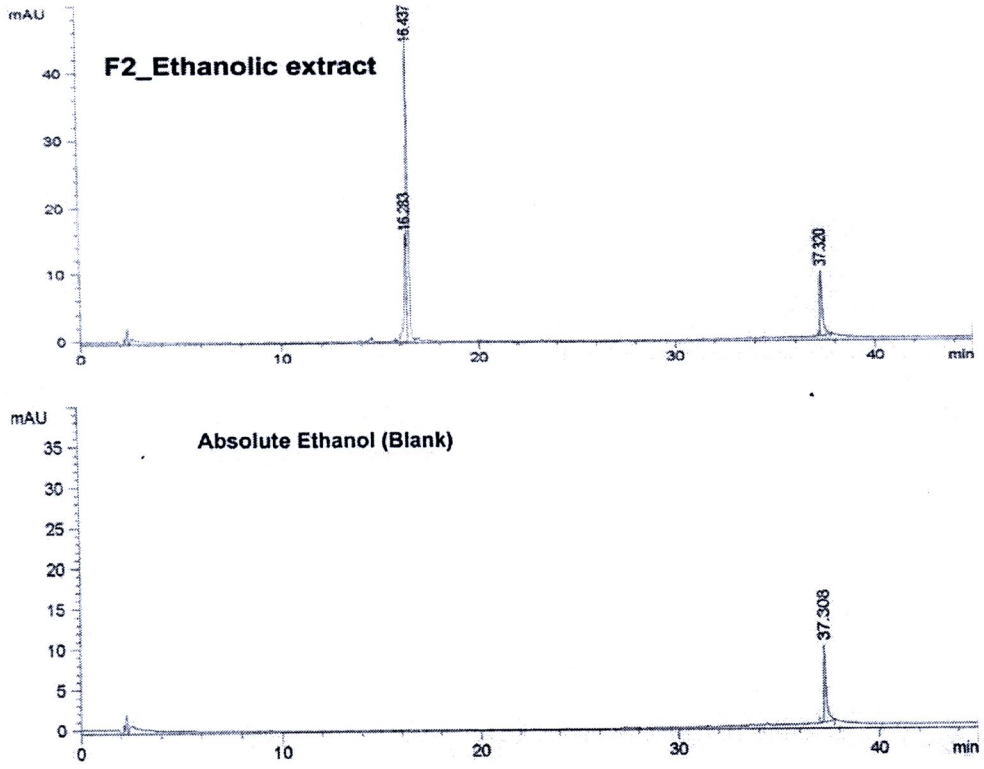
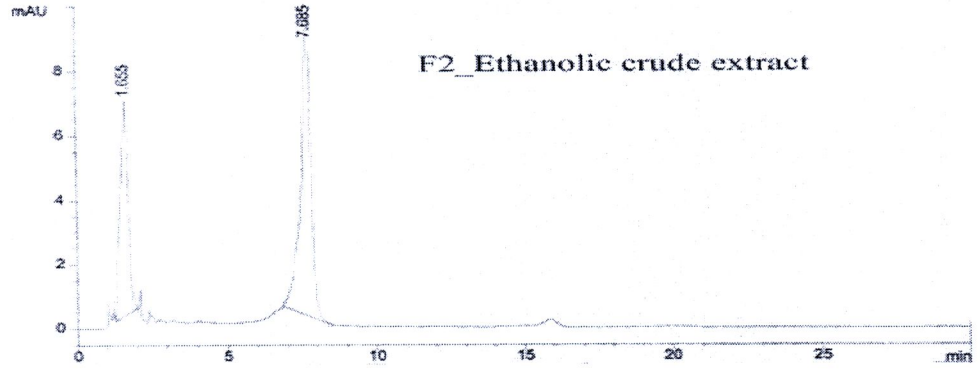


Figure 36: HPLC chromatograms were developed under gradient condition

Isocratic Condition

ACN 17.5 % + DI water 82.5 %



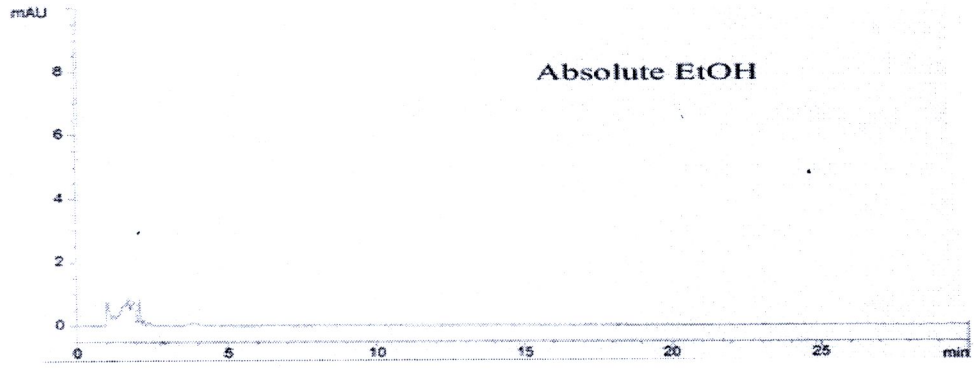
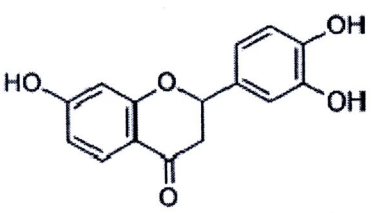


Figure 37: HPLC chromatograms were developed under isocratic condition

4.7.3 Liquid chromatography - mass spectrometry (LC-MS)

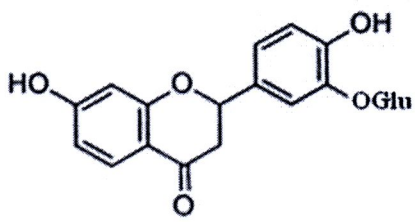
The LC-MS condition for isolation of the F2 ethanolic crude extract was developed from the HPLC-UV condition with the gradient and isocratic conditions. The mass spectra showed the molecular weights of bioactive compounds that isolated from *B. monosperma* flowers extract. F2 fraction from ethanolic crude extract, contained 4 major 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 $[M+H]^+$). The structures of butin, isomonospermoside, butein and monospermoside were shown in Figure 38(A) to 38(D), respectively. The mobile phase with an isocratic condition (ACN : DI water = 17 : 83) was run through a reversed phase column with the flow rate of 1.0 ml/min before flowing into the UV detector and subsequently mass spectrometric detector. In Figure 39, the F2 ethanolic crude extract HPLC chromatogram obtained from the UV detector exhibited 2 major peaks at 9.994, and 44.371 min, the corresponding mass spectra of these peaks were shown in Figure 40 and Figure 41, respectively.

Butin (m/z = 274.1 $[M+2H]^+$)



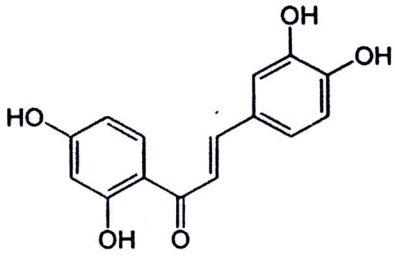
(A)

Isomonospermoside (m/z = 436.2 $[M+2H]^+$)



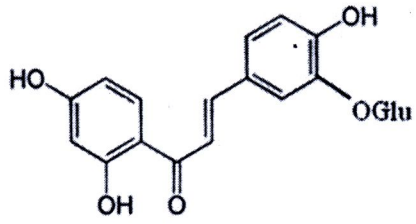
(B)

Butein (m/z = 273.2 $[M+H]^+$)



(C)

Monospermoside (m/z = 435.2 $[M+H]^+$)



(D)

Figure 38: The structures of Butin (A), Isomonospermoside (B), Butein (C), and Monospermoside (D)

The HPLC isocratic condition (ACN : DI water = 17 : 83)

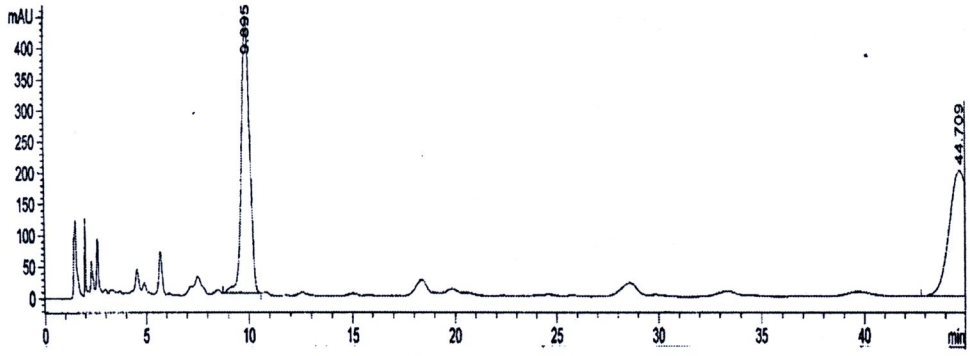


Figure 39: The HPLC chromatogram of ethanolic crude extract was developed in isocratic condition (ACN : DI water = 17 : 83)

Mass spectra

Time 9.994 min

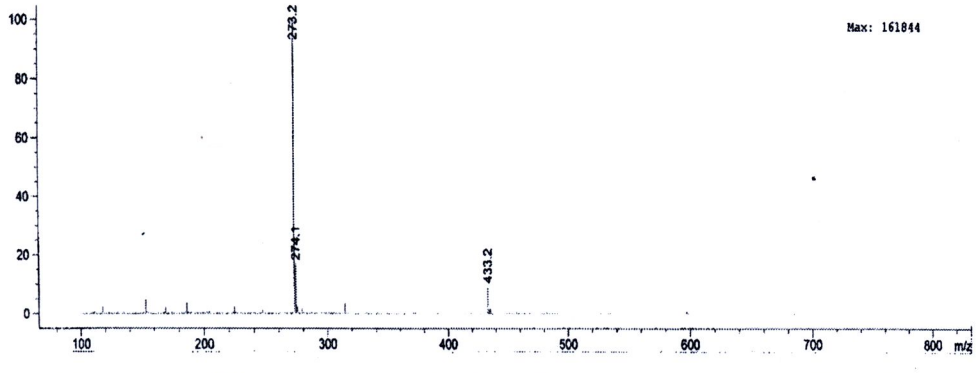


Figure 40: Mass spectrum of ethanolic crude extract at 9.994 min

Time 44.371 min

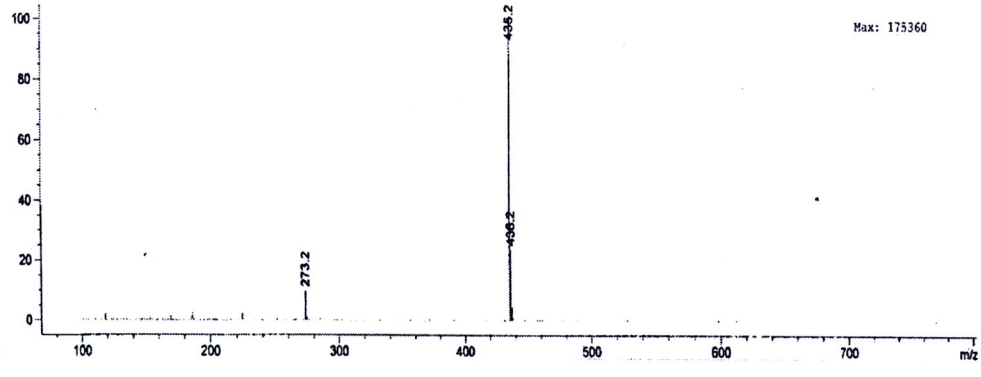


Figure 41: Mass spectrum of ethanolic crude extract at 44.371 min

4.7.4 Infrared (IR) absorption spectrometry

In this study, the FT-IR spectrogram (Figure 42) used for identify functional group in F2 ethanolic crude extract of *B. monosperma* flowers.

IR (KBr/cm^{-1}): 3400 (chelated-OH group) 2370, 1530, 700 (phenolic-OH group) 1610 cm^{-1} (C=O group).

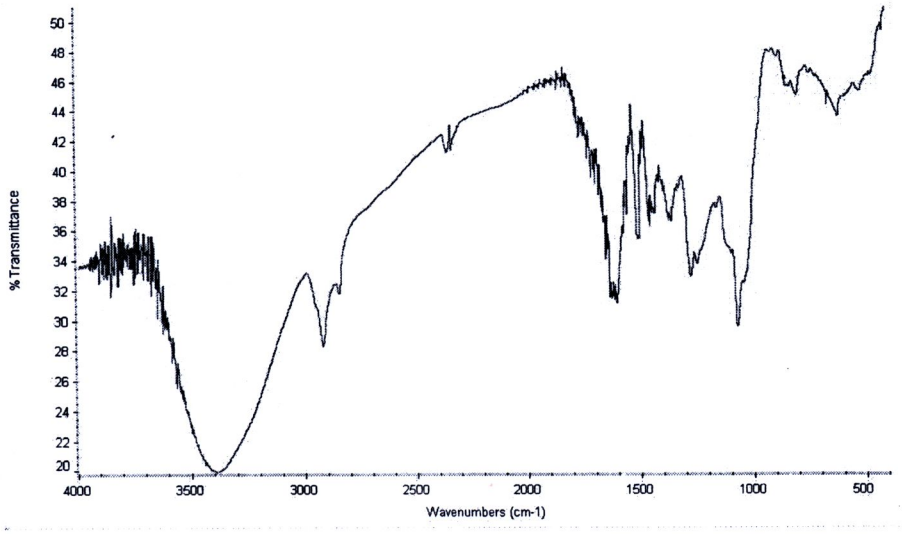
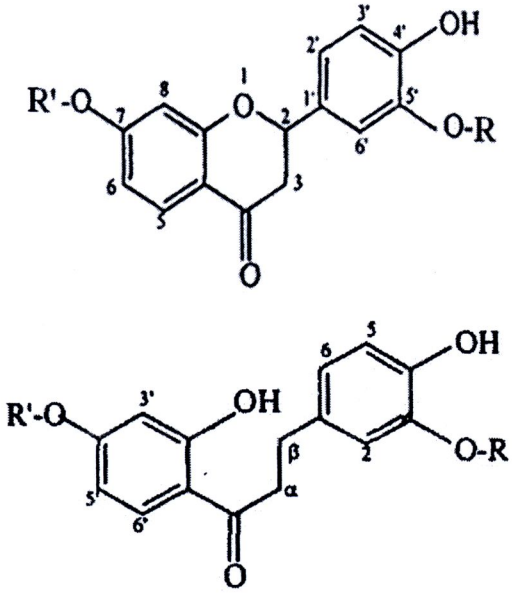


Figure 42: FT-IR spectrogram of F2 ethanolic crude extract from *B. monosperma* flowers

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

The structure of the compounds from F2 ethanolic crude extract was elucidated by spectroscopic techniques whereas those of the known compounds were identified by comparisons of spectroscopic data with those of reported compounds. The ^{1}H-NMR spectrum of F2 ethanolic crude extract exhibited ^{1}H-NMR signal at δ 2.64 to 3.89 ppm (glycosidic 6 protons), 4.83 ppm (H-1 glycosyl proton), and 6.27 to 8.10 ppm (aromatic protons). The spectroscopic data of F2 ethanolic crude extract which compared to the data of reference compounds (Figure 43) were shown in Table 15 to Table 19 (4).



Butin	R = R' = H
Isomonospermoside	R = Glu; R' = H
Butein	R = R' = H
Monospermoside	R = Glu; R' = H

Figure 43: The structure of reference compounds

Table 15: The spectroscopic data of F2 ethanolic crude extract compare with butin

F2 ethanolic crude extract : ^{1}H-NMR (400 MHz,MeOH-d_4)		Butin : ^{1}H-NMR (400 MHz, DMSO-d_6)	
Chemical shift, δ (ppm)	Description	Chemical shift, δ (ppm)	Description
13.6	s, 1H		
		9.02	brs, 2H, OH
8.10	d, *J*=8.99 Hz, 1H		
7.66	m, *J*=6.10, 8.40, 1.80, 5.29, 1.41, 7.37, 1.36 Hz, 5H	7.61	d, *J*=8.7 Hz, 1H, H-5
7.51	m, *J*= 8.48, 2.60, 8.77 Hz, 1H		
7.40	dd, *J*=1.84, 6.49, 1.82 Hz, 2H		
7.26	dd, *J*=3.99, 1.89 Hz, 2H		
7.00	d, *J*=8.48 Hz, 3H		
6.86	dd, *J*=8.23, 11.54, 8.22 Hz, 4H	6.86	s, 1H, H-2'
		6.72	s-like; 2H, H-5', H-6'
6.50	dd, *J*=2.21, 6.43, 2.23 Hz, 2H	6.47	dd, *J*=8.7, 2.1 Hz, 1H, H-6
6.41	dd, *J*=2.28, 6.55, 2.32 Hz, 2H		
6.34	t, *J*=2.33, 2.33 Hz, 2H	6.32	d, *J*=2.1 Hz, 1H, H-8
6.27	d, *J*=2.29 Hz, 1H		
		5.36	dd, *J*=12.5, 2.9 Hz, 1H, H-2
4.83	d, *J*=7.21 Hz, 4H		
4.72	t, *J*=7.84, 7.75 Hz, 6H		
4.02	d, *J*=7.79 Hz, 1H		
3.89	t, *J*=2.26, 5.58 Hz, 2H		
		3.03	dd, *J*=16.7, 12.5 Hz, 1H, H-3ax
2.64	m, *J*=2.92, 5.51, 2.98, 5.42 Hz, 3H	2.62	dd, *J*=16.7, 2.9 Hz, 1H, H-3eq
2.17	t, *J*=7.36, 7.37 Hz, 5H		
1.90	s, 2H		

Table 16: The spectroscopic data of F2 ethanolic crude extract compare with butin

F2 ethanolic crude extract : ^{1}H-NMR (400 MHz,MeOH-d_4)		Butin : ^{1}H-NMR (400 MHz, $CDCl_3$ + 5 drops CD_3OD)	
Chemical shift, δ (ppm)	Description	Chemical shift, δ (ppm)	Description
13.6	s, 1H		
8.10	d, *J*=8.99 Hz, 1H		
7.66	m, *J*=6.10, 8.40, 1.80, 5.29, 1.41, 7.37, 1.36 Hz, 5H	7.70	d, *J*=8.7 Hz, 1H, H-5
7.51	m, *J*= 8.48, 2.60, 8.77 Hz, 1H		
7.40	dd, *J*=1.84, 6.49, 1.82 Hz, 2H		
7.26	dd, *J*=3.99, 1.89 Hz, 2H		
7.00	d, *J*=8.48 Hz, 3H		
6.86	dd, *J*=8.23, 11.54, 8.22 Hz, 4H	6.87	d, *J*=1.6 Hz, 1H, H-2'
		6.78	d, *J*=8.1 Hz, 1H, H-5'
		6.73	dd, *J*=8.1, 1.6 Hz, 1H, H-6'
6.50	dd, *J*=2.21, 6.43, 2.23 Hz, 2H		
6.41	dd, *J*=2.28, 6.55, 2.32 Hz, 2H	6.44	dd, *J*=8.7, 2.1 Hz, 1H, H-6
6.34	t, *J*=2.33, 2.33 Hz, 2H	6.31	d, *J*=2.1 Hz, 1H, H-8
6.27	d, *J*=2.29 Hz, 1H		
		5.23	dd, *J*=12.9, 2.9 Hz, 1H, H-2
4.83	d, *J*=7.21 Hz, 4H		
4.72	t, *J*=7.84, 7.75 Hz, 6H		
4.02	d, *J*=7.79 Hz, 1H		
3.89	t, *J*=2.26, 5.58 Hz, 2H		
		2.93	dd, *J*=16.9, 12.9 Hz, 1H, H-3ax
2.64	m, *J*=2.92, 5.51, 2.98, 5.42 Hz, 3H	2.67	dd, *J*=16.9, 2.9 Hz, 1H, H-3eq
2.17	t, *J*=7.36, 7.37 Hz, 5H		
1.90	s, 2H		

Table 17: The spectroscopic data of F2 ethanolic crude extract compare with isomonospermoside

F2 ethanolic crude extract : ^{1}H-NMR (400 MHz,MeOH-d_4)		(+)-Isomonospermoside : ^{1}H-NMR (400 MHz, DMSO-d_6)	
Chemical shift, δ (ppm)	Description	Chemical shift, δ (ppm)	Description
13.6	s, 1H		
8.10	d, *J*=8.99 Hz, 1H		
7.66	m, *J*=6.10, 8.40, 1.80, 5.29, 1.41, 7.37, 1.36 Hz, 5H	7.62	d, *J*=8.6 Hz, 1H, H-5
7.51	m, *J*= 8.48, 2.60, 8.77 Hz, 1H		
7.40	dd, *J*=1.84, 6.49, 1.82 Hz, 2H		
7.26	dd, *J*=3.99, 1.89 Hz, 2H	7.26	d, *J*=1.6 Hz, 1H, H-2'
7.00	d, *J*=8.48 Hz, 3H	7.01	dd, *J*=8.2, 1.6 Hz, 1H, H-6'
6.86	dd, *J*=8.23, 11.54, 8.22 Hz, 4H	6.82	d, *J*=8.2 Hz, 1H, H-5'
6.50	dd, *J*=2.21, 6.43, 2.23 Hz, 2H		
6.41	dd, *J*=2.28, 6.55, 2.32 Hz, 2H	6.48	dd, *J*=8.6, 2.0 Hz, 1H, H-6
6.34	t, *J*=2.33, 2.33 Hz,2H	6.33	d, *J*=2.0 Hz, 1H, H-8
6.27	d, *J*=2.29 Hz, 1H		
		5.40	dd, *J*=12.8, 2.6 Hz, 1H, H-2
4.83	d, *J*=7.21 Hz, 4H		
4.72	t, *J*=7.84, 7.75 Hz, 6H	4.69	d, *J*=7.2 Hz, 1H, H-1''
4.02	d, *J*=7.79 Hz, 1H		
3.89	t, *J*=2.26, 5.58 Hz,2H		
		3.68	brd, *J*=11.1 Hz, 1H, H-6''b
		3.46	m, 1H, H-6''a
		3.26-3.34	m, 3H, H-2''-H-4''
		3.13	m, 1H, H-5''
		3.10	dd, *J*=16.8, 12.8 Hz, 1H, H-3ax
2.64	m, *J*=2.92, 5.51, 2.98, 5.42 Hz, 3H	2.63	dd, *J*=16.8, 12.8 Hz, 1H, H-3eq
2.17	t, *J*=7.36, 7.37 Hz,5H		
1.90	s, 2H		

Table 18: The spectroscopic data of F2 ethanolic crude extract compare with butein

F2 ethanolic crude extract : ^{1}H-NMR (400 MHz,MeOH-d_4)		Butein : ^{1}H-NMR (400 MHz, CD_3OD)	
Chemical shift, δ (ppm)	Description	Chemical shift, δ (ppm)	Description
13.6	s, 1H		
8.10	d, *J*=8.99 Hz, 1H		
		7.92	d, *J*=8.8 Hz, 1H, H-6'
7.66	m, *J*=6.10, 8.40, 1.80, 5.29, 1.41, 7.37, 1.36 Hz, 5H	7.71	d, *J*=15.4 Hz, 1H, H-β
7.51	m, *J*= 8.48, 2.60, 8.77 Hz, 1H	7.52	d, *J*=15.4 Hz, 1H, H-α
7.40	dd, *J*=1.84, 6.49, 1.82 Hz, 2H		
7.26	dd, *J*=3.99, 1.89 Hz, 2H	7.17	d, *J*=1.6 Hz, 1H, H-6
7.00	d, *J*=8.48 Hz, 3H	7.10	dd, *J*=8.4, 1.6 Hz, 1H, H-2
6.86	dd, *J*=8.23, 11.54, 8.22 Hz, 4H	6.81	d, *J*=8.4 Hz, 1H, H-3
6.50	dd, *J*=2.21, 6.43, 2.23 Hz, 2H		
6.41	dd, *J*=2.28, 6.55, 2.32 Hz, 2H	6.40	dd, *J*=8.8, 2.4 Hz, 1H, H-5'
6.34	t, *J*=2.33, 2.33 Hz,2H		
6.27	d, *J*=2.29 Hz, 1H	6.28	d, *J*=2.4 Hz, 1H, H-3'
4.83	d, *J*=7.21 Hz, 4H		
4.72	t, *J*=7.84, 7.75 Hz, 6H		
4.02	d, *J*=7.79 Hz, 1H		
3.89	t, *J*=2.26, 5.58 Hz, 2H		
2.64	m, *J*=2.92, 5.51, 2.98, 5.42 Hz, 3H		
2.17	t, *J*=7.36, 7.37 Hz, 5H		
1.90	s, 2H		

Table 19: The spectroscopic data of F2 ethanolic crude extract compare with monospermoside

F2 ethanolic crude extract : ^{1}H-NMR (400 MHz,MeOH-d_4)		Butein 3-O-glucoside (Monospermoside) :^{1}H-NMR (400 MHz, DMSO-d_6)	
Chemical shift, δ (ppm)	Description	Chemical shift, δ (ppm)	Description
13.6	s, 1H		
8.10	d, *J*=8.99 Hz, 1H	8.10	d, *J*=8.99 Hz, 1H
		7.73	d, *J*=15.3 Hz, H-β
7.66	m, *J*=6.10, 8.40, 1.80, 5.29, 1.41, 7.37, 1.36 Hz, 5H	7.67	d, *J*= 1.5 Hz, H-2
		7.66	d, *J*=15.5 Hz, H-α
7.51	m, *J*= 8.48, 2.60, 8.77 Hz, 1H		
7.40	dd, *J*=1.84, 6.49, 1.82 Hz, 2H	7.41	dd, *J*=8.3, 1.5 Hz, H-6
7.26	dd, *J*=3.99, 1.89 Hz, 2H		
7.00	d, *J*=8.48 Hz, 3H		
6.86	dd, *J*=8.23, 11.54, 8.22 Hz, 4H	6.89	d, *J*=8.3 Hz, H-5
6.50	dd, *J*=2.21, 6.43, 2.23 Hz, 2H		
6.41	dd, *J*=2.28, 6.55, 2.32 Hz, 2H	6.40	dd, *J*=8.9, 2.2 Hz, H-5'
6.34	t, *J*=2.33, 2.33 Hz, 2H		
6.27	d, *J*=2.29 Hz, 1H		
4.83	d, *J*=7.21 Hz, 4H	4.83	d, *J*=7.1 Hz, glc H-1
4.72	t, *J*=7.84, 7.75 Hz, 6H		
4.02	d, *J*=7.79 Hz, 1H		
3.89	t, *J*=2.26, 5.58 Hz, 2H		
2.64	m, *J*=2.92, 5.51, 2.98, 5.42 Hz, 3H		
2.17	t, *J*=7.36, 7.37 Hz, 5H		
1.90	s, 2H		

4.8 Assay Validation

Standard curve of *B. monosperma* flowers extract

The standard curve of *B. monosperma* flowers extract was produced for quantitative analysis. Markers solution were prepared by *B. monosperma* flowers extract stock solution and 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 standard curve of *B. monosperma* flowers extract was found to be 0.0097 – 0.0101. The curve and data as shown in Figure 44, and Table 20, respectively.

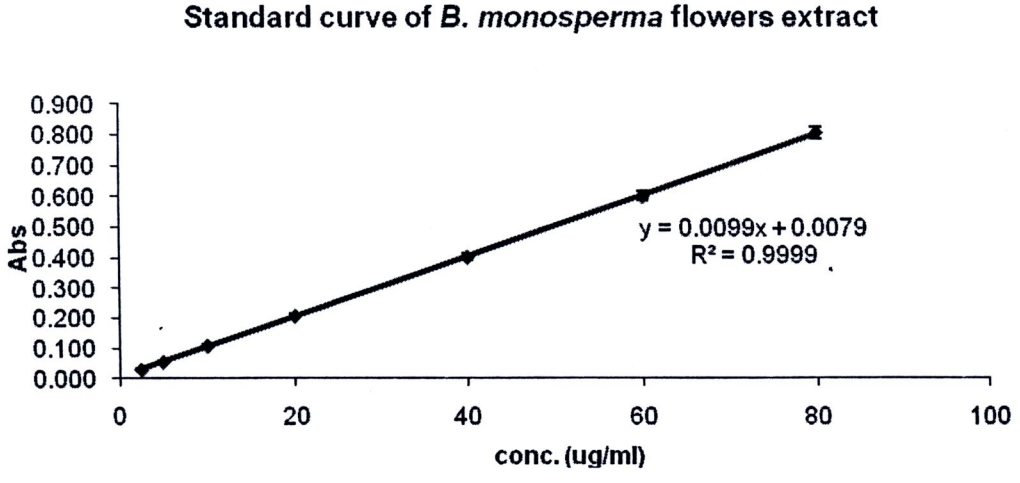


Figure 44: Standard curve of *B. monosperma* flowers extract

Table 20: The intra-day accuracy and precision of analytical method

Concentration of ethanolic crude extract (µg/ml)	Absorbance				SD	RSD	Conc. of ethanolic crude extract from standard curve (µg/ml)	% Recovery
	N1	N2	N3	Mean				
2.5	0.032	0.031	0.028	0.030	0.002	6.86	2.27	90.64
5.0	0.053	0.061	0.057	0.057	0.004	7.02	4.96	99.19
10.0	0.106	0.115	0.109	0.110	0.005	4.17	10.31	103.13
20.0	0.205	0.214	0.210	0.210	0.005	2.15	20.38	101.90
40.0	0.392	0.413	0.407	0.404	0.011	2.68	40.01	100.03
60.0	0.584	0.611	0.607	0.601	0.015	2.43	59.88	99.79
80.0	0.785	0.817	0.815	0.806	0.018	2.22	80.58	100.73
Correlation	0.9999	0.9999	0.9999	0.9999	-	-	-	-
Slope	0.0097	0.0101	0.0101	0.0099	-	-	-	-
Intercept	0.0074	0.0105	0.0058	0.0079	-	-	-	-

4.9 Particle size, size distribution and zeta potential

4.9.1 Chitosan - SCMC nanoparticles

The CS-SCMC nanoparticles were prepared from 0.1% CS and 1% SCMC with the mixing ratio by weight of 1:1, 2:1, and 1:2. The result showed that the increasing of the mixing ratio CS:SCMC from 1:1 to 2:1, the average particle sizes of the prepared nanoparticles were increased from 149.97 to 178.38 nm. When the mixing ratio of CS:SCMC was changed from 1:1 to 1:2, the aggregation was observed as white fiber (Figure 46 (C)). The results of CS-SCMC were shown in Table 21, 22 and Figure 45 (A), 45 (B), and 45 (C). The polydispersity index (PDI) of CS-SCMC nanoparticles was narrow distribution. The zeta potential of CS-SCMC was positive charge in range of 42 to 49 mV.

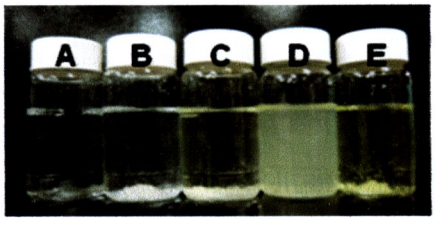
(A) The mixing ratio of 0.1% CS : 1% SCMC = 1:1 (by weight)

A = DI water
B = Chitosan + SCMC (Blank)
C = CS + SCMC + 6.25 % ethanolic extract
D = CS + SCMC + 12.50 % ethanolic extract
E = CS + SCMC + 25.00 % ethanolic extract

(B) The mixing ratio of 0.1% CS : 1% SCMC = 2:1 (by weight)

A = DI water
B = Chitosan + SCMC (Blank)
C = CS + SCMC + 4.17 % ethanolic extract
D = CS + SCMC + 8.33 % ethanolic extract
E = CS + SCMC + 16.67 % ethanolic extract

(C) The mixing ratio of 0.1% CS : 1% SCMC = 1:2 (by weight)



A = DI water
B = Chitosan + SCMC (Blank)
C = CS + SCMC + 4.17 % ethanolic extract
D = CS + SCMC + 8.33 % ethanolic extract
E = CS + SCMC + 16.67 % ethanolic extract

Figure 45: The nanoparticles were prepared from chitosan and SCMC in different ratio

Table 21: Effect of polymer ratio and concentration of *Butea monosperma* flowers extract. (CS : SCMC = 1:1)

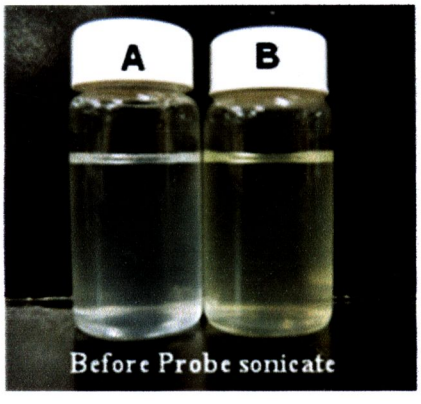
Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
Ethanolic extract	-	-	-26.68 ± 5.64
Mixing Ratio 0.1% CS : 1% SCMC = 1 : 1 (by weight)			
CS / SCMC (blank)	149.97 ± 6.24	0.155 ± 0.080	42.36 ± 4.17
CS / SCMC / 6.25% ethanolic extract	152.39 ± 13.43	0.185 ± 0.061	42.05 ± 3.38
CS / SCMC / 12.50% ethanolic extract	156.79 ± 24.97	0.206 ± 0.077	46.08 ± 4.21
CS / SCMC / 25.00% ethanolic extract	148.59 ± 7.14	0.168 ± 0.062	42.41 ± 4.34

Table 22: Effect of polymer ratio and concentration of *Butea monosperma* flowers extract. (CS : SCMC = 2:1)

Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
Ethanolic extract	-	-	-26.68 ± 5.64
Mixing Ratio 0.1% CS : 1% SCMC = 2 : 1 (by weight)			
CS / SCMC (blank)	178.38 ± 2.74	0.174 ± 0.049	46.00 ± 2.07
CS / SCMC / 4.17 % ethanolic extract	170.52 ± 3.47	0.179 ± 0.011	48.62 ± 1.51
CS / SCMC / 8.33 % ethanolic extract	169.44 ± 4.82	0.128 ± 0.060	47.60 ± 0.85
CS / SCMC / 16.67% ethanolic extract	166.76 ± 2.98	0.120 ± 0.043	47.88 ± 1.15

4.9.2 Chitosan - Alginate nanoparticles

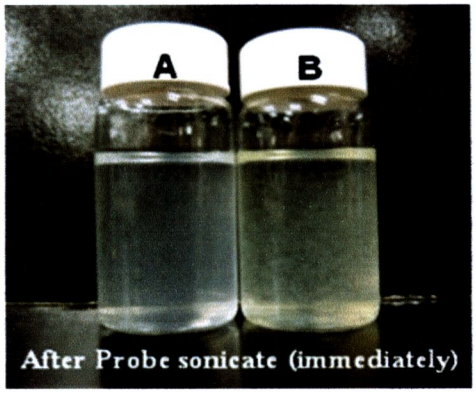
The CS-Alginate nanoparticles were prepared from 0.1% CS, 0.03% Alginate, and 0.18 mM Calcium chloride by pregel preparation method. The particles size of CS-Alginate nanoparticles was decreased after using probe sonication, and flocculated when standing overnight. The results of CS-Alginate nanoparticles were shown in Table 23 and Figure 46. The polydispersity index (PDI) of CS-SCMC nanoparticles was broad distribution. The zeta potential of CS-Alginate nanoparticles was negative charge in range of -3.57 to -25.60 mV.



Note:

A = Chitosan + Alginate (Blank)

B = Chitosan + Alginate + ethanolic extract



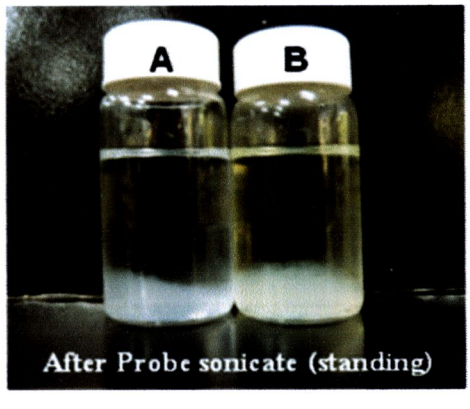


Figure 46: The nanoparticles were prepared from chitosan and alginate

Table 23: Effect of the concentration of *Butea monosperma* flowers extract (chitosan-alginate).

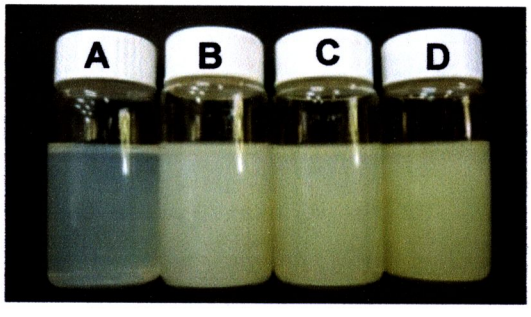
Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
Ethanolic extract	-	-	-26.68 ± 5.64
Before Probe sonicate			
CS / Alginate (blank)	2.120×10^4 ± 9406	0.545 ± 0.266	- 3.57 ± 0.68
CS / Alginate / 25.0% ethanolic extract	2.798×10^4 ± 7520	0.671 ± 0.302	-10.10 ± 2.34
CS / Alginate / 50.0% ethanolic extract	2.372×10^4 ± 6867	0.688 ± 0.311	-16.50 ± 5.05
CS / Alginate / 100.0% ethanolic extract	1.775×10^4 ± 3565	0.460 ± 0.337	-18.20 ± 3.75
After Probe sonicate			
CS / Alginate (blank)	1261 ± 121.5	0.429 ± 0.056	-25.60 ± 0.73
CS / Alginate / 25.0% ethanolic extract	1211 ± 118.3	0.532 ± 0.083	-23.20 ± 1.01
CS / Alginate / 50.0% ethanolic extract	1441 ± 72.01	0.589 ± 0.105	-19.40 ± 1.32
CS / Alginate / 100.0% ethanolic extract	1055 ± 57.99	0.469 ± 0.100	-22.40 ± 2.96

4.9.3 PLGA nanoparticles

The PLGA nanoparticles were prepared from PLGA diluted in acetone and 1% PVA. This study was interested in the concentration of *B. monosperma* flowers extract (1.88, 3.75, and 7.50 mg). The average particle sizes of the PLGA nanoparticles were 140 to 213 nm. The polydispersity index (PDI) of PLGA nanoparticles were narrow distribution. The zeta potential of PLGA was negative charge in range of -12 to -15 mV. The results of PLGA nanoparticles were shown in Table 24 and Figure 47.

Table 24: Effect of the concentration of *Butea monosperma* flowers extract (PLGA).

Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
Ethanolic extract	-	-	-45.96 ± 1.03
PLGA + 1% PVA			
PLGA_blank	141.8 ± 1.9	0.116 ± 0.036	-14.3 ± 1.83
PLGA_ ethanolic extract 4.17 %	210.1 ± 10.6	0.153 ± 0.033	-14.0 ± 1.66
PLGA_ ethanolic extract 8.33 %	198.3 ± 4.4	0.138 ± 0.035	-13.5 ± 1.23
PLGA_ ethanolic extract 16.67 %	212.3 ± 3.4	0.164 ± 0.059	-12.6 ± 3.08



Note:

A = PLGA (Blank)

B = PLGA + ethanolic extract 4.17 %

C = PLGA + ethanolic extract 8.33 %

D = PLGA + ethanolic extract 16.67 %

Figure 47: The PLGA nanoparticles were prepared from PLGA and 1% PVA in different concentration of ethanolic crude extract

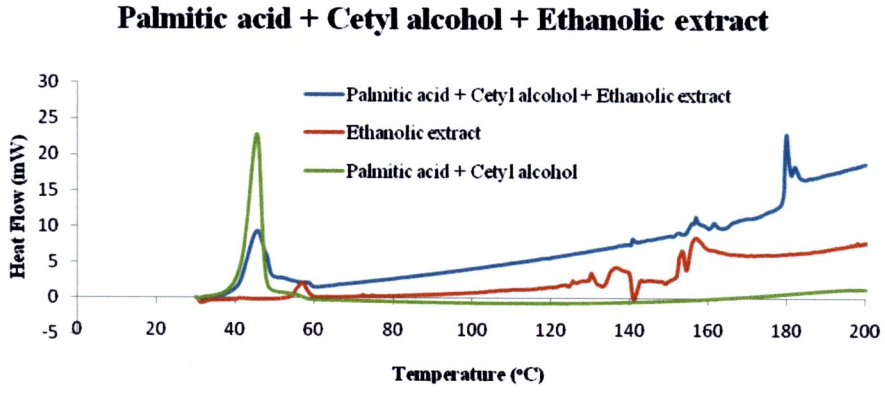
Lipid nanoparticles

In this study, solid lipid mixtures were palmitic acid – cetyl alcohol, palmitic acid – stearyl alcohol and stearic acid – cetyl alcohol. The solid lipid mixtures for SLN and NLC were selected by differential scanning calorimetry (DSC) thermogram.

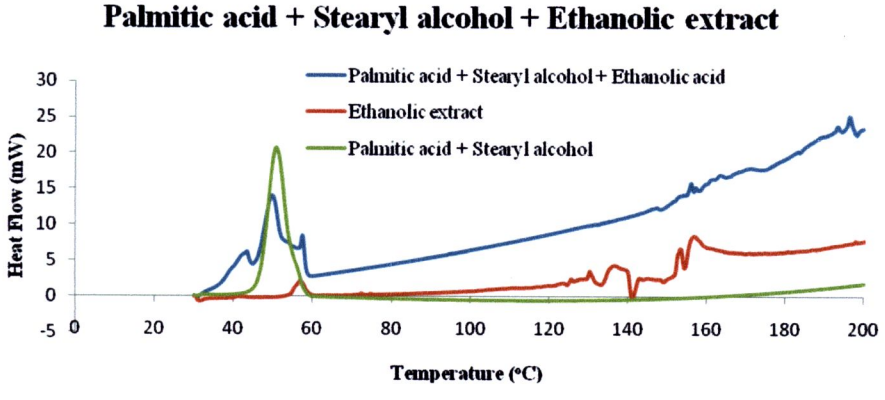
4.9.4 Solid lipid nanoparticles (SLN)

Differential Scanning Calorimetry (DSC)

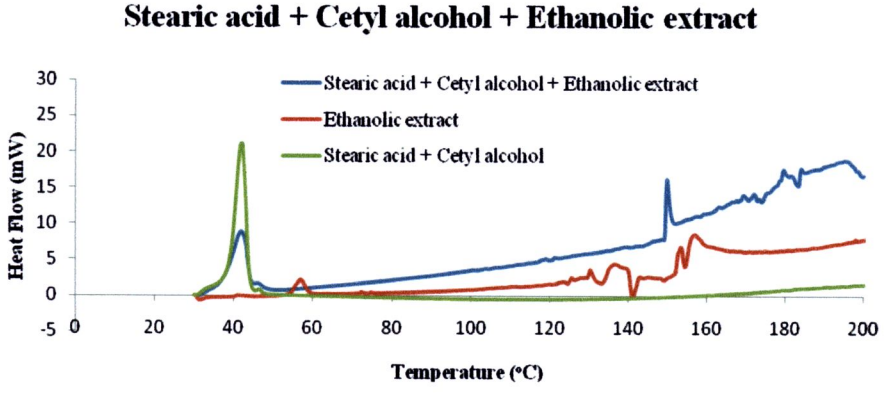
In this study, the thermal behavior of ethanolic part of *B. monosperma* flowers extract, solid lipid mixtures, and solid lipid mixture with ethanolic extract were investigated by DSC. The DSC thermograms (Figure 48(A) to Figure 48(C)) show changing in calorimetric peak shape of *B. monosperma* flowers extract and the mixtures of lipid. This change in the calorimetric peak shape demonstrated the dissolution of ethanolic extract in the solid lipid mixtures. The result indicated that the suitable solid lipid mixtures for SLN and NLC with entrapped the *B. monosperma* flowers extract was palmitic acid – stearyl alcohol. The DSC thermogram of palmitic acid – stearyl alcohol presented the preferential dissolution of ethanolic extract in the palmitic acid – stearyl alcohol.



(A)



(B)



(C)

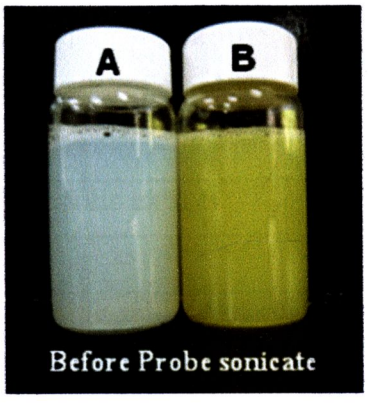
Figure 48: The DSC thermogram of *B. monosperma* flowers extract and the mixtures of lipid

The solid lipid nanoparticles (SLN) were prepared from palmitic acid and stearyl alcohol which chose from DSC. The suitable of the percent of solid lipid and surfactant in formulation was 2.5 % : 10 %. The particles size of SLN was decreased after using polytron and probe sonication, but the particles size of SLN was increased when standing overnight. The results of SLN were shown in Table 25 and Figure 49. The polydispersity index (PDI) of SLN was narrow distribution after using probe sonication immediately. However the PDI was broad distribution when standing overnight. The zeta potential of SLN was negative charge in range of -30 to -40 mV.

Table 25: Effect of the concentration of *Butea monosperma* flowers extract (SLN).

Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
Ethanolic extract	-	-	-26.68 ± 5.64
Palmitic acid + Stearyl alcohol			
After Probe Sonicate (immediately)			
SLN Blank	46.5 ± 1.1	0.288 ± 0.059	-31.8 ± 1.23
SLN+ ethanolic extract 16.00 %	48.9 ± 1.5	0.338 ± 0.055	-33.0 ± 1.21
After Probe Sonicate (overnight)			
SLN Blank	458.5 ± 40.1	0.569 ± 0.109	-39.3 ±7.86
SLN+ ethanolic extract 16.00 %	1561.7 ± 434.8	0.918 ± 0.145	-40.1 ± 5.54

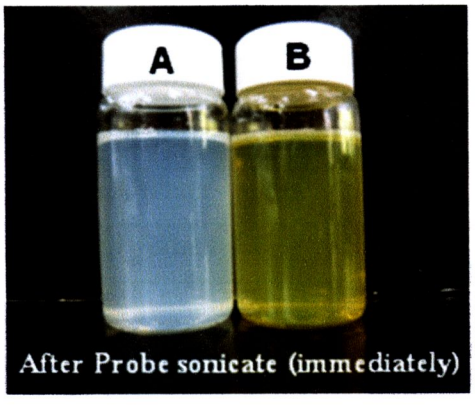
SLN : Palmitic acid + Stearyl alcohol



Note:

A = Solid Lipid Nanoparticle (Blank)

B = SLN + ethanolic extract 16.00 %



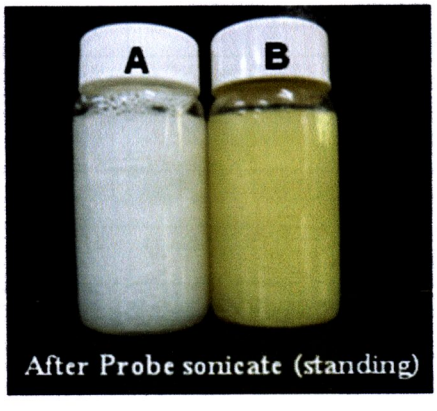


Figure 49: The solid lipid nanoparticle was prepared from palmitic acid and stearyl alcohol

4.9.5 Nanostructured lipid carrier (NLC)

The nanostructured lipid carrier (NLC) was prepared from palmitic acid and stearyl alcohol for solid lipid and jojoba oil for liquid lipid. The particles size of NLC was decreased after using polytron and probe sonication. This experiment studied the ratio of solid lipid and liquid lipid (3:7, 5:5, and 7:3), and the concentration of *B. monosperma* flowers extract (40, 80, and 160 mg). The results of NLC were shown in Table 26, 27 and Figure 50. The polydispersity index (PDI) of NLC was narrow distribution. The zeta potential of SLN was negative charge in range of -33 to -46 mV.

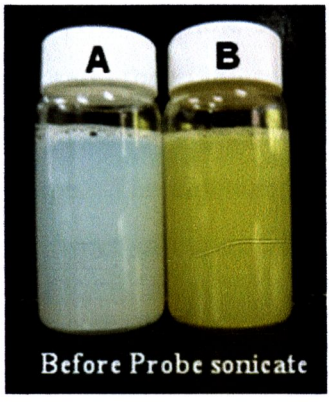
Table 26: Effect of the solid lipid and liquid lipid ratio

Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
Ethanolic extract	-	-	-45.96 ± 1.03
Palmitic acid + Stearyl alcohol			
NLC_3:7_blank	137.7 ± 1.7	0.227 ± 0.021	-43.6 ± 1.33
NLC_3:7_ ethanolic extract 160 mg	141.4 ± 2.9	0.248 ± 0.028	-42.9 ± 3.15
NLC_5:5_blank	90.8 ± 1.68	0.262 ± 0.015	-33.6 ± 2.79
NLC_5:5_ ethanolic extract 160 mg	92.4 ± 1.04	0.182 ± 0.035	-39.7 ± 2.40
NLC_7:3_blank	109.0 ± 2.50	0.512 ± 0.020	-42.6 ± 3.32
NLC_7:3_ ethanolic extract 160 mg	97.4 ± 0.94	0.444 ± 0.072	-38.0 ± 2.25

Table 27: Effect of the concentration of *Butea monosperma* flowers extract

Sample Name	Size		ZP
	Z-Ave	PDI	
	d.nm		mV
CMU-216	-	-	-45.96 ± 1.03
Palmitic acid + Stearyl alcohol			
NLC_7:3_blank	88.6 ± 9.7	0.506 ± 0.028	-36.9 ± 1.65
NLC_7:3_CMU 40 mg	92.2 ± 4.2	0.447 ± 0.026	-39.3 ± 2.12
NLC_7:3_CMU 80 mg	96.7 ± 5.2	0.393 ± 0.052	-40.2 ± 1.58
NLC_7:3_CMU 160 mg	110.1 ± 6.8	0.354 ± 0.066	-34.8 ± 1.79

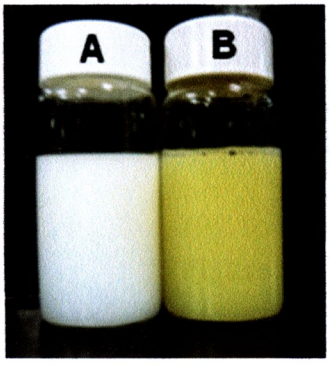
NLC: Palmitic acid + Stearyl alcohol



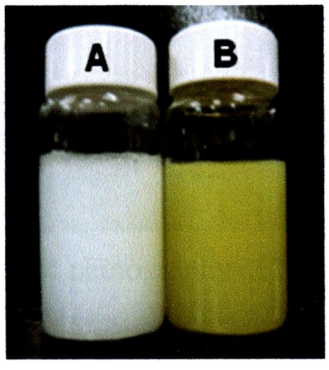
Note:

A = Nanostructured Lipid Carrier (Blank)

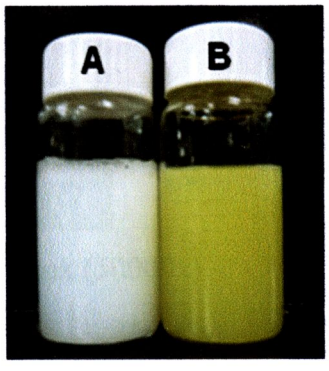
B = NLC + ethanolic extract 16.00 %



solid lipid : liquid lipid = 3 : 7



solid lipid : liquid lipid = 5 : 5



solid lipid : liquid lipid = 7 : 3

The ratio of solid lipid : liquid lipid = 7 : 3

A = Nanostructured Lipid Carrier (Blank)

B = NLC + ethanolic extract 4.00 %

C = NLC + ethanolic extract 8.00 %

D = NLC + ethanolic extract 16.00 %

Figure 50: The nanostructured lipid carrier was prepared from palmitic acid and stearyl alcohol for solid lipid and jojoba oil for liquid lipid in different ratio between solid lipid and liquid lipid, and different concentration

4.10 Characterization of ethanolic crude extract loaded nanoparticles

After particle size and zeta potential measurement, the morphology of NLC blank and ethanolic crude extract loaded NLC were determined using a transmission electron microscopy (TEM). The characteristics of the nanoparticles were spherical in shape. The characteristics of NLC blank and ethanolic crude extract loaded NLC were shown in Figure 51 and Figure 52, respectively.

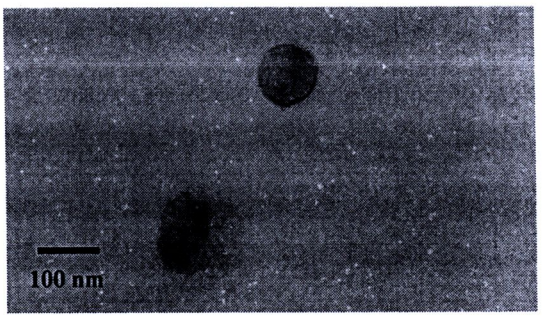


Figure 51: TEM photography of NLC blank (50000x)

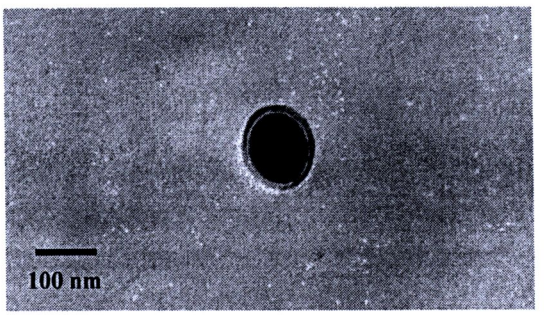


Figure 52: TEM photography of ethanolic crude extract loaded NLC (50000x)

4.11 Percentage of entrapment efficiency (%EE)

The percentages of entrapment efficiency of *B. monosperma* flowers extract in each nanoparticular system were determined by an indirect method. Approximately 4 – 25 % (w/w) ethanolic extract per polymer were added in each preparation. Detection of the untrapped extracts was performed. Nanoparticles were centrifuged and the supernatant was analyzed by ultraviolet-visible spectrophotometer at 375 nm according to the maximum absorption of active (flavonoids) markers as demonstrated in section 3.10

4.11.1 Chitosan - SCMC nanoparticles

This experiment interested in the percentage of entrapment efficiency of *B. monosperma* flowers extract loading in nanoparticles with mixing ratio of 0.1% CS and 1% SCMC by weight of 1:1, 2:1. Various concentrations of *B. monosperma* flowers extract loaded in nanoparticles with 0.125, 0.25, and 0.500 mg. The results of percentage of entrapment efficiency of *B. monosperma* flowers extract nanoparticles were shown in Table 28 which mixing ratio of 0.1% CS and 1% SCMC by weight of 1:1, and 2:1.

Table 28: The percentage of entrapment efficiency (%EE) of chitosan : SCMC : ethanolic extract

Chitosan:SCMC: ethanolic extract	**Chitosan : SCMC 1 : 1**		**Chitosan : SCMC 2 : 1**	
	% (w/w) ethanolic extract per polymer	**% EE**	**% (w/w) ethanolic extract per polymer**	**% EE**
ethanolic extract 0.125 mg	6.25	6.19 ± 0.23	4.17	6.97 ± 0.30
ethanolic extract 0.250 mg	12.50	5.01 ± 0.22	8.33	6.00 ± 0.18
ethanolic extract 0.500 mg	25.00	3.04 ± 0.17	16.67	4.05 ± 0.07

4.11.2 PLGA nanoparticles

This experiment interested in the percentage of entrapment efficiency of *B. monosperma* flowers extract loading in PLGA nanoparticles. Various the composition of *B. monosperma* flowers extract loaded in PLGA nanoparticles were 1.88, 3.75, and 7.50 mg. The results of percentage of entrapment efficiency of *B. monosperma* flowers extract in PLGA nanoparticles were shown in Table 29.

Table 29: The percentage of entrapment efficiency of PLGA nanoparticles with ethanolic crude extract

Samples	% (w/w) ethanolic extract per polymer	% EE
PLGA + ethanolic extract 1.88 mg	4.17	37.74 ± 4.21
PLGA + ethanolic extract 3.75 mg	8.33	26.30 ± 0.48
PLGA + ethanolic extract 7.50 mg	16.67	21.84 ± 0.92

4.11.3 Nanostructured lipid carrier (NLC)

In this experiment interested in the percentage of entrapment efficiency of *B. monosperma* flowers extract loading in nanostuctured lipid carrier in different ratio between solid lipid and liquid lipid, 3 : 7, 5 : 5, and 7 : 3. The percentage of entrapment efficiency of *B. monosperma* flowers extract in NLC which the ratio of solid lipid : liquid lipid = 7:3 was the best, the results were shown in Table 30. Various the composition of *B. monosperma* flowers extract loaded in NLC (solid lipid : liquid lipid = 7:3) with 40, 80, and 160 mg. The results of percentage of entrapment efficiency of NLC of *B. monosperma* flowers extract were shown in Table 31.

Table 30: The percentage of entrapment efficiency which the ratio of solid lipid : liquid lipid = 3:7, 5:5, and 7:3, respectively

Samples	% EE
NLC_3:7 + ethanolic extract 16.00 %	n.a.
NLC_5:5 + ethanolic extract 16.00 %	1.35
NLC_7:3 + ethanolic extract 16.00 %	23.80

Note: n.a. = not assay

Table 31: The percentage of entrapment efficiency which the ratio of solid lipid : liquid lipid = 7:3

Samples	% (w/w) ethanolic extract per lipid	% EE
NLC_7:3 + ethanolic extract 40 mg	4.00	24.80 ± 0.41
NLC_7:3 + ethanolic extract 80 mg	8.00	30.01 ± 0.20
NLC_7:3 + ethanolic extract 160 mg	16.00	23.82 ± 0.20

4.12 *In vitro* release of ethanolic crude extract loaded nanoparticles

The quantitative analysis of active markers in the B. monosperma flowers extract was performed after validation of UV spectrophotometric method. Percentages of active markers released from the NLC nanoparticles were monitored for 7 days. Release profile was shown a sustained release of ethanolic extract loaded NLC (Figure 53).

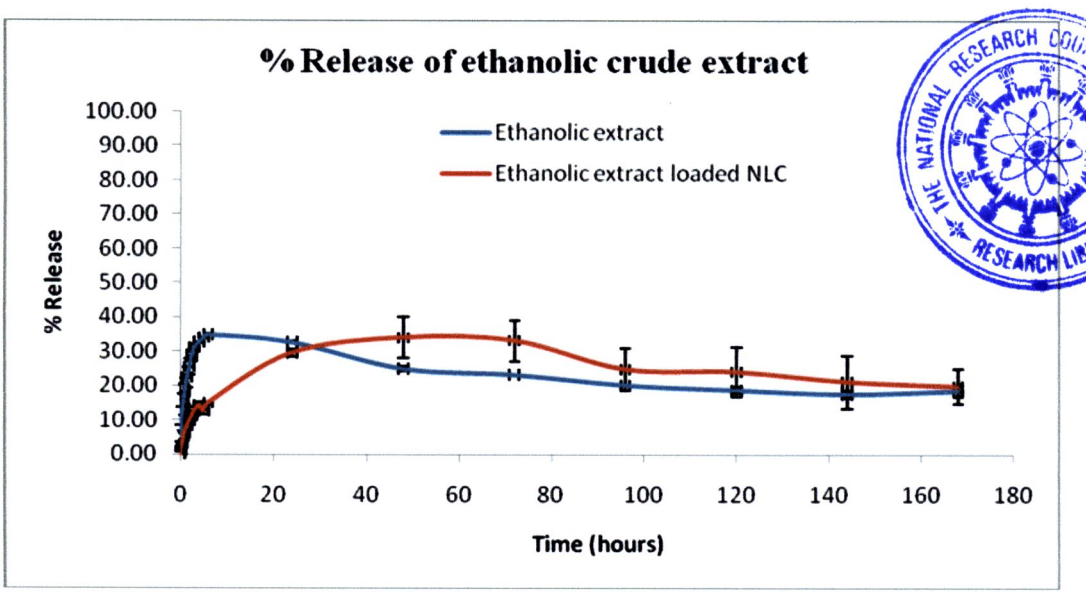


Figure 53: Release profile of ethanolic extract and ethanolic extract loaded NLC