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CELLULOSE DERIVATIVE PARTICLES AS ANTI-TUBERCULOSIS AGENT CARRIERS

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โรควัณโรคเกิดจากการติดเชื้อแบคทีเรีย *Mycobacterium tuberculosis* (*M.TB*) และมีรายงานวิจัยจำนวนมากกล่าวถึงการดื้อยาของเชื้อวัณโรค จึงมีความจำเป็นที่จะต้องพัฒนายาชนิดใหม่ โดยในงานวิจัยนี้เกี่ยวข้องกับการใช้สารสกัดจากธรรมชาติในกลุ่มของ bisbenzylisoquinoline alkaloids (BBQs) ซึ่งเป็นสารสกัดจากรากของต้นย่านาง (*Tiliacora triandra*) ที่มีฤทธิ์ต้านเชื้อวัณโรคทั้งชนิดดื้อยาและไม่ดื้อยา เนื่องจาก BBQs มีความเป็นพิษต่อเซลล์เม็ดเลือดขาว ดังนั้นในที่นี้จึงทำการกักเก็บสารในกลุ่มดังกล่าวลงในอนุภาคขนาดนาโนเมตรที่สร้างจากเอทิลเซลลูโลสโดยงานวิจัยนี้ได้ทำการกักเก็บไทมอลร่วมกับ BBQs โดยพบว่าการกักเก็บสารในกลุ่ม BBQs และไทมอลลงในอนุภาคขนาดประมาณ 140 นาโนเมตรได้อนุภาคที่กระจายตัวในน้ำได้ดี อีกทั้งอนุภาคดังกล่าวยังมีฤทธิ์ในการต้านเชื้อวัณโรคเทียบเท่าสารที่ไม่ได้กักเก็บ แต่มีความเป็นพิษต่อเม็ดเลือดขาวลดลงกว่า BBQs ที่ไม่ได้ถูกกักเก็บ อนุภาคสามารถเข้าสู่เซลล์เม็ดเลือดขาวได้อย่างมีประสิทธิภาพ

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Tuberculosis (TB) is an infectious disease that is caused by *Mycobacterium tuberculosis* (*M.TB*). Multi-drug resistant tuberculosis had been reported. Therefore, the development of new anti-tuberculosis agents is needed. This work involves the use of bisbenzylisoquinoline alkaloids (BBQs) compounds isolated from the roots of *Tiliacora triandra*, for combating with *M.TB*. Anti-mycobacterial activity against multidrug-resistant strains of both TB and *M.TB* has been reported. Since BBQs is toxic to macrophage cell, here we encapsulate BBQs and thymol into nanoparticles fabricated from ethyl cellulose (EC) and show that 140 nm particles possess similar anti-TB activity to unencapsulated BBQs, but are less toxic to macrophages. We also show that the particles can be effectively taken up into the macrophages.

Field of Study: Petrochemistry and Polymer Science Student's Signature .....
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List of Abbreviations

%	Percent
℃	Degree Celsius
g	Gram
h	Hour
mg	Milligram
ml	Milliliter
µg	Microgram
mW	Miliwatt
nm	Nanometer
DSC	Differential scanning calorimetry
SEM	Scanning electron microscope
EC	Ethyl cellulose
PBS	Phosphate buffer solution
FBS	Fetal bovine serum
RPMI 1640	Roswell Park Memorial Institute 1640
EtOH	Ethanol
MIC	Minimum inhibitory concentration
HPLC	High performance liquid chromatography
CLSM	Confocal laser scanning microscopy
%EE	Encapsulation efficiency
%loading	Loading capacity
BBQs	Bisbenzylisoquinoline alkaloids

BBQ1	Tiliacorinine
BBQ2	13'-Bromo-tiliacorinine
M.TB	*Mycobacterium tuberculosis*
TB	Tuberculosis

CHAPTER I

INTRODUCTION

Tuberculosis (TB) has become a major public health problem. About one-third of the world's population is infected by this disease. However, not everyone infected with TB becomes immediately sick. Ninety percent of the population infected with TB develops this disease only after their immune systems become weak [1]. In 2010, the World Health Organization (WHO) reported that there were 9.4 million people infected with TB and this caused the deaths of 17 million people per year [2].

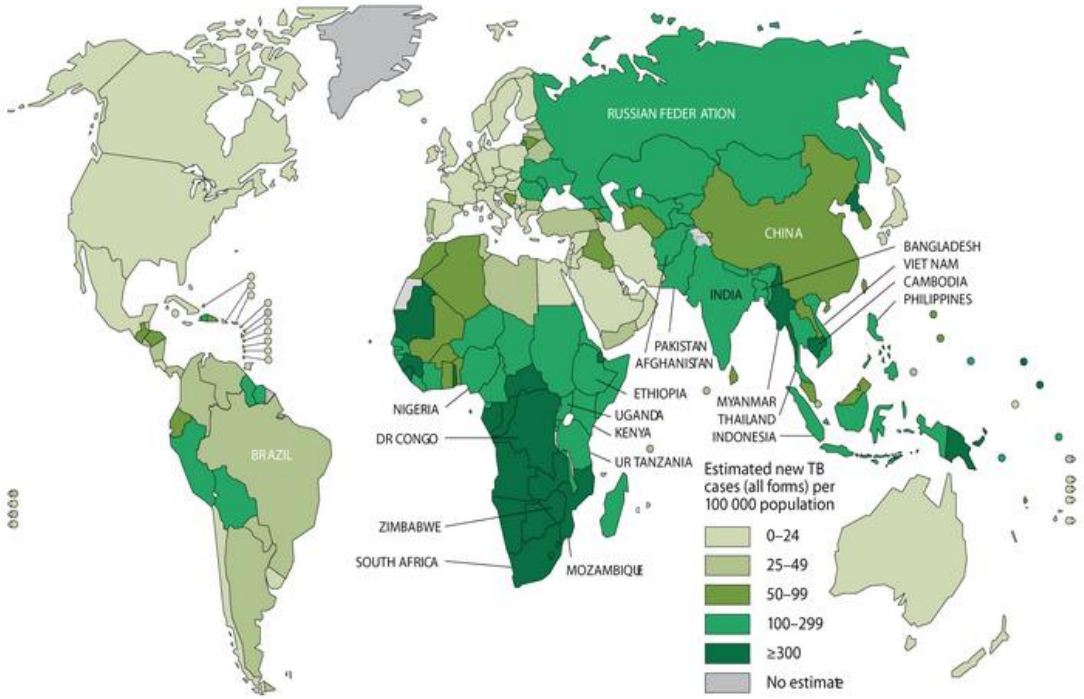


Figure 1.1 Estimated TB incidence rates 2010 [2].

TB is chronic disease caused by *Mycobacterium tuberculosis* (*M.TB*) which usually spread through the air from persons to persons. The TB bacteria are expelled into the air when a person with TB coughs, sneezes or speaks. The bacteria usually attack lungs, but they can also attack any parts of the body such as brain, bone, lymph nodes, skin and kidney [3, 4]. The TB bacteria are able to remain in the air for a long time. They are resistant to dry air, cold, heat, chemicals but not resistant to sunlight. TB is often found in people who are physically weak from other diseases such as cold, measles, pertussis, diabetes, and HIV [5-7] or people who have overloaded works, or those with malnutrition, alcoholism or people with a history of close contact with persons with latent TB infection. An increasing rate of TB spreading is due to the fact that TB is the most common opportunistic infection associated with HIV. In addition, a patient with TB has a lack of understanding of the treatment and does not follow a strict procedure of medical treatment, causing of drug resistance in *M.TB* [8].

1.1 *Mycobacterium tuberculosis* (*M.TB*)

Mycobacterium tuberculosis (M.TB) is a fairly large non-motile aerobic bacterium. The bacterium divides every 16–20 hours, which is very slow compared to other bacteria. The bacteria are rod-shaped, 0.2-0.5 μm in width, 2-5 μm in length. It can grow well in aerobic condition [9] The structure of cell wall composes of three major macromolecules which are peptidoglycan, arabinogalalactan and mycolic acids [10].

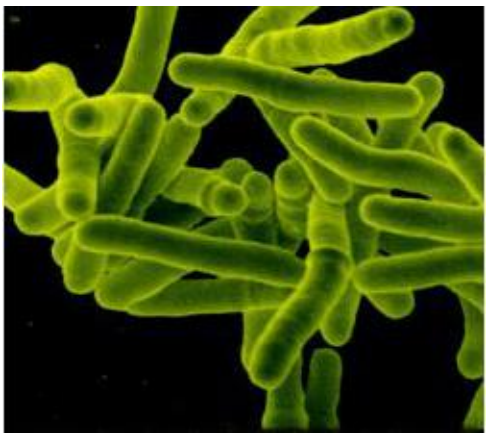


Figure 1.2 Structure of Mycobacterium tuberculosis.
(http://info.agscientific.com/blog/bid/141197/Antibiotics-Against-Mycobacteria)

1.2 Problem of drug-resistance in tuberculosis

Tuberculosis (TB) is an infectious disease that has become a major health problem as it can spread quickly due to a lack of understanding of the prophylaxis. Because of this, World Health Organization (WHO) has been alerted and has greatly expanded its awareness and prevention programs. The major factor that causes for the outbreak of tuberculosis is the spread of AIDS. Patients with AIDS have immunodeficiency and thereby result in various infection including M.TB. People with both AIDS and TB co-infection are often sick violently and their sickness are difficult to cure. Not follow drug prescription currently causes drug resistance to tuberculosis, resulting in ineffective treatment. Table 1.1 shows the drug list used to treat tuberculosis.

Table 1.1 Drug compounds with greater than 80% of growth inhibition of *M.TB*. at 5 μM [11].

i. WHO recommended drugs			
Compound	**Current Use**	**Route of delivery**	**Anti-tuberculosis MIC (μM)**
Rifampicin (Rifampin)	Antibacterial (First-line anti-tuberculosis agent)	Oral	0.02
3-formyl Rifamycin	Antibacterial (Anti-tuberculosis agent)	Oral	0.08
Isoniazid (Isonicotinic acid hydrazide)	Antibacterial (First-line anti-tuberculosis agent)	Oral	0.16
Moxifloxacin HCl	Antibiotic (Fluoroquinoloine-anti-tuberculosis agent)	Oral	0.16
Amikacin	Antibiotic (Anti-tuberculosis agent)	Intravenous	0.31
Ofloxacin	Antibiotic (Fluoroquinolone-anti-tuberculosis agent)	Oral	0.63
Clofazimine	Antibacterial (Anti-tuberculosis agent)	Oral	1.25

i. WHO recommended drugs			
Compound	**Current Use**	**Route of delivery**	**Anti-tuberculosis MIC (μM)**
Gatifloxacin	Antibiotic (Fluoroquinolone-anti-tuberculosis agent)	Oral	1.25
Levofloxacin	Antibiotic (Fluoroquinoline-anti-tuberculosis agent)	Oral	1.25
Ofloxacin	Antibiotic (Fluoroquinoline-anti-tuberculosis agent)	Oral	2.5
Protionamide	Antibacterial (Second line anti-tuberculosis agent)	Oral	>5 μM
Rifamycin sv	Antibacterial (First-line anti-tuberculosis agent)	Oral	>5 μM
Thiacetazone (Amithiozone)	Antibacterial (Second-line anti-tuberculosis agent)	Oral	>5 μM
p-Aminosalicylic acid	Antibacterial (Second-line anti-tuberculosis agent)	Oral	>5 μM

1.3 Literature reviews of drug resistant in tuberculosis

In 2001, Khan et al. [12] studied the increase of drug resistance to *M.TB* in Saudi Arabia. The treatment result of tuberculosis with patients in hospital during 1996-1998 compared with 1993-1995 indicated the increase of drug resistance as summarized in Table 1.2.

Table 1.2 Prevalence (%) of drug resistance to *M.TB.* during two different periods.

Drugs	1996-1998 (*N* = 101) (%)	1993-1995 (*N* = 101) (%)
Isoniazid	28.7	10.3
Streptomycin	22.7	7.7
Rifampicin	20.7	5.1
Ethambutol	6.9	1.3
Pyrazinamide	7.9	1.3

In 2003, Espinal et al. [13] studied the global situation of drug resistance in tuberculosis. They found that the number of infectious patients was increasing due to non-observance with the full medication, thus producing multi-drug resistant (MTB).

In 2007, Khue et al. [14] studied drug-resistance in tuberculosis from 13,283 patients in France. During 1995-2004, the trend gradually increased.

In 2010, Calver et al. [15] investigated the emergence and evolution of drug-resistant tuberculosis in a co-infected HIV in population at a South Africa from 128 patients. During January 2003-November 2005, the trend of drug-resistance dramatically increased.

In 2011, Beltran et al. [16] reported trend of drug-resistance tuberculosis, during 1997-2005 in Sinaloa, Mexico. The result show the new infectious patients, which had dramatically increase and also the TB pathogen from patients that withstand various drugs from the treatment process.

In 2014, Nasiri et al. [17] studied distribution of drug-resistant tuberculosis in Iran. They found that the rate of drug-resistance with isoniazid and streptomycin in TB, which dramatically increased.

1.4 Infectious tuberculosis of macrophage

Typically, when *Mycobacterium tuberculosis (M.TB)* suspended in the air enters through airways and lungs, the macrophages in the lung then responds to *M.TB*. The results of the response by the immune system are divided into 3 groups which are [18, 19]

1. Pathogen is removed.

2. Pathogen entering into body is in latent TB infection. Macrophage envelopes pathogen so the bacteria is in remission and does not cause a disease known as Latent tuberculosis. Infected people do not show any symptoms and there is no spread of infection. However, if patients get weak, white blood cell that engulfs bacteria cannot control the infection, they tend to become ill with tuberculosis disease.

3. The patients can become infected (active tuberculosis) after the pathogen exposure. These patients usually have immune system disorders.

1.5 Literature reviews of infectious tuberculosis in macrophage

In 2004, Raja [20] reported that *Mycobacterium tuberculosis*, which could pass into the macrophage cells by endocytosis pathway. The pathogen generate substance in phagosome, which cannot fuse with lysosome, resulting in an inability to eliminate the pathogen causing the rise in collection of immune cells called granulomas.

In 2008, Egan et al. [21] studied the *M.TB* infection in macrophages by immunofluorescence microscopy. They discovered granulomas which are immune cell aggregates that are the pathologic hallmarks of tuberculosis.

In 2009, Palaga [22] reported that *Mycobacterium tuberculosis* normally enters macrophages and proliferates inside them resulting in patients with low immune system. This symptom will develop into tuberculosis disease i.e. macrophages cannot uptake the pathogens and undergo the invaders elimination processes. These processes is caused by a generation of toxic substances from the pathogens which disrupt phagosome membrane fusing activity with lysosome. Then pathogens are not destroyed by macrophages and continue to grow rapidly.

1.6 Literature reviews of new drug from natural products

Multi-drug resistant TB strains have caused drug treatments to be ineffective, therefore, the discovery of new anti-tuberculosis agents is needed. The most interesting anti-tuberculosis agents can be obtained from the natural products extraction.

In 2012, Sureram et al. [23] extracted bisbenzylisoquinoline alkaloids, the natural products isolated from the roots of *Tiliacora triandra*. Natural bisbenzylisoquinoline alkaloids (substance 1-3 Figure 1.3) are confirmed to have anti-mycobacterial activity against multidrug-resistant strains of *Mycobacterium tuberculosis* (MDR-MTB). In addition, they chemically modified the structure of

bisbenzylisoquinoline alkaloids, generating derivative 4 (substance 4 Figure 1.3). This particular derivative showed increasing activity against MDR-MTB and decreasing cytotoxicity to human cells. These bisbenzylisoquinoline alkaloids and derivative showed MDR-MTB inhibition activity more than many first-line drugs used in present tuberculosis treatment.

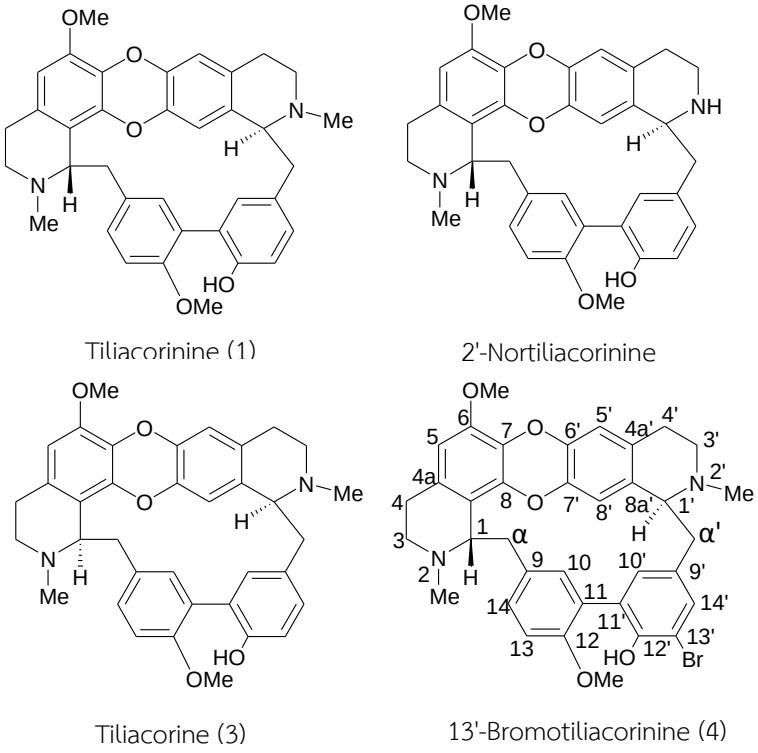
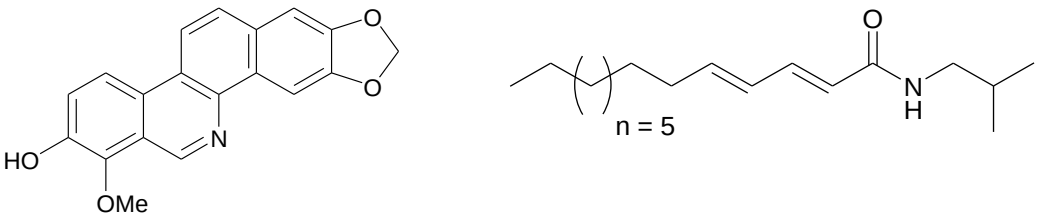


Figure 1.3 Chemical structure of bisbenzylisoquinoline alkaloids [23].

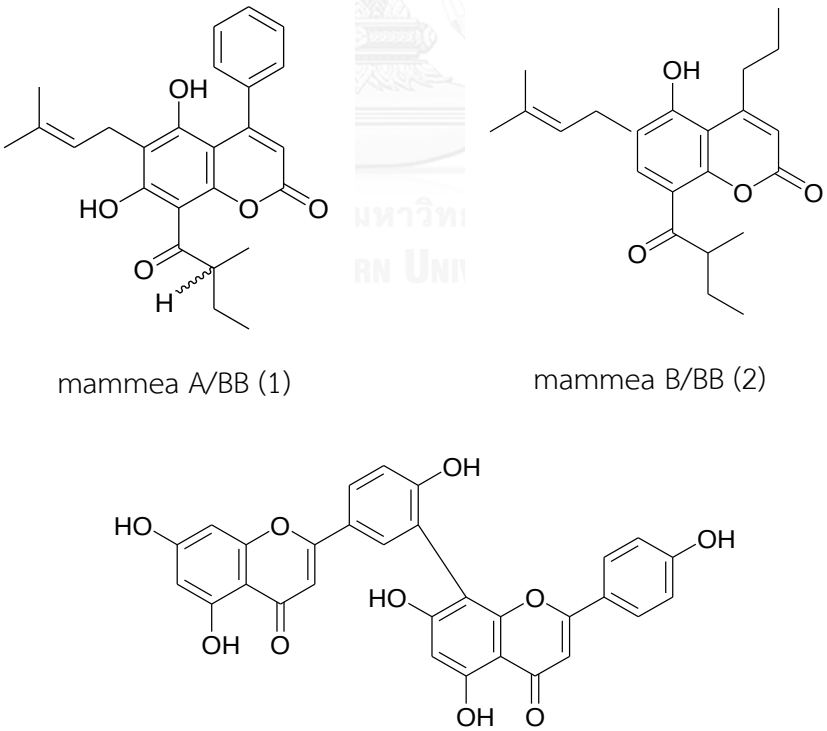
In 2013, Xuan Luo et al. [24] isolated 16 compounds from the thumbs of *Zanthoxylum capense*. Benzophenanthridine alkaloid (substance 1 Figure 1.4) and *N*-isobutylamide (substance 15 Figure 1.4) have the best anti-mycobacterial activity of all (MIC of 1.6 μg/ml)



decarine (1) N-isobutyl-(2E,4E)-2,4-tetradecadienamide

Figure 1.4 Structure of benzophenanthridine alkaloid and N-isobutylamide [24].

In 2014, Clauda et al. [25] isolated key compounds from the medicinal plant leaves of Calophyllum brasiliense. They got 3 compounds from it which are coumarins (substance 1-2 Figure 1.5) and amentoflavone (substance 3 Figure 1.5). Both types of coumarins showed activity against anti-Mycobacterium tuberculosis.



mammea A/BB (1) mammea B/BB (2)

amentoflavone (3)

Figure 1.5 Structure of Coumarins and amentoflavone [25].

In 2014, Uc-Cachón et al. [26] isolated nathoquinones 8 compounds from the stem bark of medicinal plant *Diospyros anisandra*. They isolated and tested anti-mycobacterial activity of the extracted compounds. Three compounds such as plumbagin (substance 1 Figure 1.6), maritionone (substance 4 Figure 1.6) and 3,3'-biplumbagin (substance 5 Figure 1.6), showed potent anti-mycobacterial activity (MIC of 1.56-3.33 μg/ml).

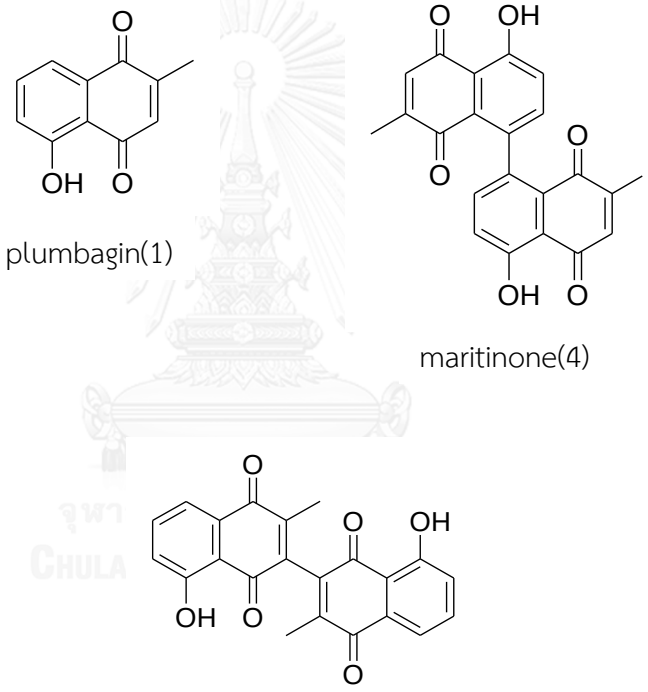


Figure 1.6 Chemical structure of napthoquinones from Diospyros anisandra [26].

In 2015, Scodro et al. [27] extracted a natural product from the leaves of *Piper diospyrifolium* by supercritical fluid method. They obtained the compound called 4-methoxy-3-[(*E*)-3-methyl-1,3-butadien-1-yl]-5-(3-methyl-2-buten-1-yl)-benzoic acid (Figure 1.7) Then the anti-mycobacterial activity test yielded the MIC value of 125 μg/ml.

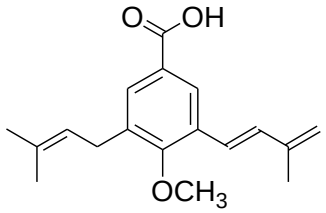
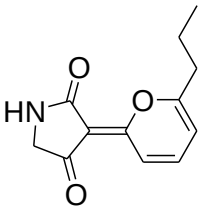


Figure 1.7 Chemical structure of 4-methoxy-3-[(E)-3-methyl-1,3-butadien-1-yl]-5-(3-methyl-2-buten-1-yl)-benzoic acid from Piper diospyrifolium [27].

In 2015, Ganihigama et al. [28] studied anti-mycobacterial activity of 27 natural products. They reported that vermelhoin (substance 19 Figure 1.8) can act as a good anti-mycobacterial agent with the MIC of 1.5-12.5 μg/ml. They also synthesized various anti-tuberculosis compounds, and found out that pyrroldiquinolines type compounds such as *5-Methylpyrrolo[1,2-a:3,4-c']diquinolin-5-ium iodide* (substance 85 Figure 1.8) and *5-Methyl-5,6-dihydropyrrolo[1,2-a:3,4-c']diquinoline* (substance 86 Figure 1.8) have better anti-mycobacterial activity than several first-line drugs used in tuberculosis treatment with low cytotoxicity on human cells (MIC of 125 μg/ml.)



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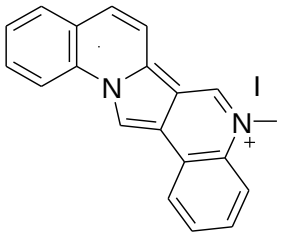

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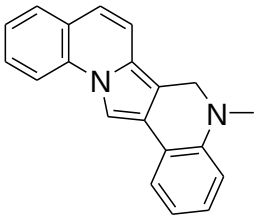

(86)

Figure 1.8 Chemical structure of vermelhotin and pyrroldiquinolines [28].

1.7 Thymol

The other name is 2-isopropyl-5-methylphenol or IPMP ($C_{10}H_{14}O$) monoterpene phenol derivative of cymene. It is an isomer of carvacrol. Thymol is found in oil of *Thymus vulgaris*, commonly called thyme. The compound has proven benefits in medical (antimicrobial, antifungal and antibacterial activity.), food and agriculture. It is a white crystalline powder with the molecular weight of 150.22 g/mol. The maximum absorption wavelength of thymol is 274 nm. Thymol is slightly soluble in water, but it is well soluble in alcohols and other organic solvents [29-32].

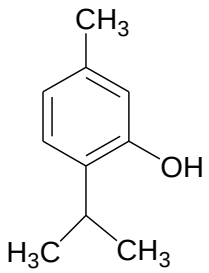

Figure 1.9 Chemical structure of thymol [32].

1.8 Literature reviews of thymol antimicrobial activity

In 2008, Dorman et al. [33] reported the essential oil from various types of plant that thymol show effective of growth inhibition of gram positive and negative bacteria, e.g., *S. typhimurium*, *E. coli*, *S. dysenteria, B. cereus* and *S. aureus.*

In 2012, Shaaban et al. [34] studied antibacterial activity of essential oils and their fragrance components, result show that thymol had effectiveness of antibacterial properties.

In 2012, Orhan et al. [35] studied antimicrobial activity of essential oils obtained from plants with various types of microbacteria e.g. *E.coli, P. aeruginosa, P. mirabilis, K. pneumonia, A. baumannii, S. aureus*, *E. faecalis*, *B. subtilis*. The thymol shows the inhibition against the microbacteria.

1.9 Drug delivery systems

Drug delivery systems are the drug preparation in variety formulations, which can control drug release and are specific to organs in the body. The right delivery system can enhance drug efficacy. Three main roles of delivery systems include:

1. To regulate releasing and amount of drug, which depends on each work.

2. To protect sensitive drug or reducing side effect in the body.

3. To deliver drug to the selected organs [36-38].

Consequently, the selected polymer as the drug carrier should be the biologically compatible, biodegradable or eliminateable non-toxic properties. For example polylactic acid (PLA), poly-lactide-co-glycolide (PLGA), poly- **ε**-caprolactone (PCL), chitosan, gelatin, poly-alkyl-cyano-acrylates (PAC) and ethyl cellulose etc [39-42].

1.10 Literature reviews of particles uptake into macrophages

In 2011, Sigh et al. [43] encapsulated isoniazid into niosomes particles by reverse phase evaporation method. The particles show the spherical shape and the amount of drug encapsulation efficiency of 61.8%. This result also demonstrates the noisome particles can uptake into mouse macrophage.

In 2013, Park et al. [44] trapped ofloxacin into glutaraldehyde-crosslinked chitosan microspheres by water-in-oil emulsification method. The particles show spherical shape and size around 1-6 μm. Moreover, the particles can hold the drug on with 27.41% of encapsulation efficiency. They studied delivery mechanism of particles into alveolar macrophage and showed that the particles could be uptaken into alveolar macrophage.

In 2014, Maretti et al. [45] prepared microparticles from solid lipid microparticles and encapsulated rifampicin into the microparticles. The microparticles were spherical. Furthermore, the particles showed high encapsulation efficiency of 91.6%. Moreover, the studying of particles uptake into the murine macrophage and monitoring by laser confocal scanning microscopy indicated that the particles could be uptaken into murine macrophage.

1.11 Ethyl cellulose (EC)

Ethyl cellulose (EC) is a derivative of cellulose. The hydrogen on hydroxyl groups of glucose until was replaced by ethyl groups. It is used for pharmaceutical industry as the coating agent, flavoring fixation, tablet binder, tablet filler, film-former, viscosity-increasing agent, and emulsifier agent in food manufacturing industry. EC is slightly solvate in water and soluble in ethanol.

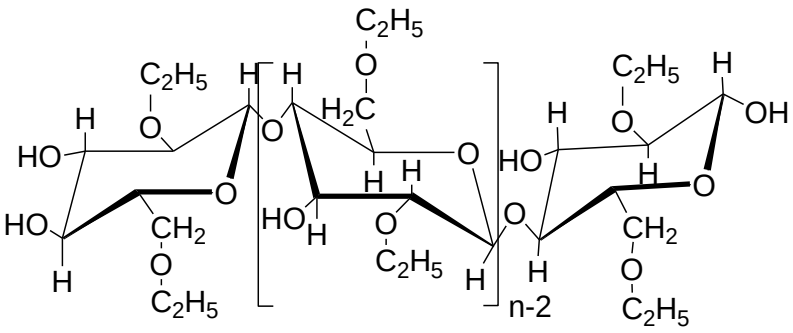

Figure 1.10 Structure of ethyl cellulose (EC).

EC is a hydrophobic material used in a variety of applications such as sustained release. Furthermore, it possesses many versatile to be used for encapsulating drug [46] e.g., 1. water insoluble but soluble in many organic solvents such as alcohol, ether, ketone and ester, 2. biocompatible and compatible with many cellulose, 3. white to light tan odorless and tasteless powder, 4. specific density range 1.07-1.18 g/cm^3 with 135-155°C heat distortion point and 330-360°C fire point, 5. stable against light, heat, oxygen and wetness and chemicals, 6. non-toxic and non-irritant.

1.12 Literature reviews of ethyl cellulose for drug carriers

In 2001, Cheu et al. [47] encapsulated acyclovir into ethyl cellulose (EC) particles. The average size of particles was around 44-74.1 μm. The process gave the encapsulation efficiency of 20 – 56.6%. After that, they studied acyclovir releasing behavior from EC particles compared with un-encapsulated acyclovir into EC particles at 25°C, 37°C and 50°C and the result showed that encapsulated acyclovir were degraded more slowly than unencapsulated acyclovir drug.

In 2009, Prasertmanakit et al. [48] prepared EC microcapsules and encapsulating folic acid through emulsion solvent evaporation method. After that, they studied folic acid releasing behavior in solution pH = 1.2. The result show, that the rate of folic acid releasing from EC microcapsules slower than free folic acid.

In 2012, Zheng et al. [49] encapsulated polyphenols, which had been extracted from bayberry into EC particles through phase separation method. They studied the stability of the encapsulated polyphenols under simulated gastric fluid at pH of 2-6. The result indicated that the encapsulated polyphenols were more stable than the unencapsulated polyphenols.

1.13 Research goals

The aim of this research is to develop the water dispersible particles that contain bisbenzylisoquinoline alkaloids (BBQs) and thymol. Then the BBQs/thymol-loaded particles will be tested for cellular toxicity in macrophage cells and multi-drug resistant *M.TB*. Uptake of the particles into macrophage cells will also be studied. The scope of this work is as follows:

1. Preparation of carriers that can deliver bisbenzylisoquinoline alkaloids and thymol into cell simultaneously.
2. Evaluation of the uptake of the obtained particles into macrophage cells
3. Evaluation of the anti-tuberculosis activity of the obtained particles.
4. Determine the cytotoxicity of the obtained particles in macrophage cells.

CHATER II

EXPERIMENTAL

2.1 Materials and Chemicals

Ethyl cellulose (EC; viscosity 300 cP; ethoxy content 48%), was purchased from Sigma-Aldrich (St. Louis, USA). Thymol was purchased from Thai-China Flavors and Fragrances Industry (Nonthaburi, Thailand). Bisbenzylisoquinoline alkaloid (BBQ) was kindly provided by Dr. Prasat Kittakoop, Chulabhorn Research Institute (Bangkok, Thailand).

2.1.1 Media culture for anti-mycobacterial test

Lowenstein-Jensen medium (LJ medium) and middlebrook 7H9GC were purchased from Becton, Dickinson and company (New Jersey, USA). Dimethyl sulfoxide (DMSO), Tween80 and Rifampin and *M. tuberculosis* (H37Ra ATCC 25177) were obtained from Sigma Aldrich (Steinheim, Germany). Alamur blue was purchased from SeroTec (Oxford, UK).

2.1.2 Media culture and chemical reagent for cytotoxic study

Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 2.05 mM L-glutamine media, sodium pyruvate, HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfuric acid) (free acid 1 M solution (238.3 g/L)) and 100 mM fetal bovine serum was obtained from Hyclone (Utah, USA). Streptomycin sulphate and

penicillin G (sodium salt) were purchased from M & H manufacturing (Samutprakarn, Thailand). 2-Mercaptoethanol was purchased from Gibco Invitrogen coporation (Massachusetts, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide or MTT was purchased from USB Corporation (Ohio, USA).

2.2 Instruments and Equipment

2.2.1 Morphology of the drug-loaded nanoparticles and EC-TAMRA nanoparticles

Morphology of drug-loaded nanoparticles and EC-TAMRA nanoparticles were characterized by scanning electron micropscopy (SEM). Drug-loaded nanoparticles were subjected to differential scanning calorimetry (DSC) analysis.

SEM images were acquired by JEM-6400 scanning electron microscope (JEOL, Tokyo, Japan). To prepare samples for SEM, a drop of aqueous product suspension was placed on a glass slide and dried in desiccator overnight. After that, the samples were coated with a gold layer under vacuum at 15 kV for 90 s.

DSC was measured by Netzsch DSC 204 Phoenix. Ten milligrams of the dried samples were weighed into aluminum cup and sealed. The analysis was carried out from 25-300 ℃. The analysis was performed under nitrogen gas with scanning rate of 10^{o}C/min.

2.2.2 Determination of loading capacity and encapsulation efficiency of drug-loaded nanoparticles

High performance liquid chromatography (HPLC) was used for analyzing all of the obtained suspension of drug-loaded nanoparticles (No.1-6). It was performed through Water 1525 binary high-performance liquid chromatograph (pump) a 100 mm × 4.6 mm column packed with Hypersil C18 (Thermo Fisher Inc., Waltham, MA) and connected with Waters 2489 UV-vis detector (Milford, MA) operating at 274 nm, 290 nm and 295 nm for thymol, BBQ1 and BBQ2, respectively. 10 μl of sample injection volume was performed. The mobile phase was an isocratic system of 20:80 (v/v) 1% triethylamine solution/MeOH. Flow rate of mobile phase was 0.5 ml/min and The UV-vis detector was operated at 290 nm for BBQ1 (tilacorinine). Then, the BBQ2 (13'-Bromo-tiliacorinine) was quantified by isocratic system of 30:70 (v/v) 1%triethylamine solution-ACN, flow rate was 0.5 ml/min, and UV-vis detector operating at 295 nm. Finally, thymol was analyzed through isocratic system of 20:80 (v/v) 1% triethylamine solution-MeOH and 30:70 (v/v) 1%triethylamine solution-ACN, respectively. The continuous flow rate was performed 0.5 ml/min, and monitor compound at 274 nm of UV-detector.

2.2.3 Cellular uptake of drug-loaded nanoparticles into Jurkat cell

Confocal laser scanning microscopy (CLSM) used for monitoring drug-loaded nanoparticles uptake into Jurkat cell was a Nikon Digital Eclipse C1-Si Confocal Microscope system (Tokyo, Japan). CLSM was equipped with Plan Apochromat VC 100×, Melles Griot Diode Laser and 85 YCA-series Laser at 405 nm, a Nikon TE2000-U microscope, a 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software).

2.3 Encapsulation and characterization of drug-loaded nanoparticles

BBQs and thymol were encapsulated into EC at various drug to polymer ratios (Table 2.1)

Table 2.1 Ratios of EC used the encapsulation of drug

Order	EC + drug					
	EC	BBQ1	BBQ2	thymol	EtOH	H_2O
1	7 mg	-	-	-	4 ml	6 ml
2	7 mg	4.5 mg	-	-	4 ml	6 ml
3	7 mg	4.5 mg	-	2.5 mg	4 ml	6 ml
4	7 mg	-	4.5 mg	-	4 ml	6 ml
5	7 mg	-	4.5 mg	2.5 mg	4 ml	6 ml
6	7 mg	-	-	2.5 mg	4 ml	6 ml

BBQs and thymol were encapsulated into EC polymer by solvent displacement method [50]. Briefly, EC was dissolved in 2 ml of ethanol with continuous stirring. At the same time, BBQs and thymol were dissolved in of ethanol (2 ml) and poured into

the EC solution, while continuous stirring. Then, drop-wise of deionized water (6 ml) was added into the mixture. The final product was characterized by SEM. To determine encapsulation efficiency (% EE) and loading capacity (% loading), 1 ml of each of the obtained suspension of drug-loaded nanoparticles (No.1-6) was filtered through filtering centrifugal tube (MWCO 100,000 (Amicon Ultra-0.5)). After that, the obtained after filtering solution was analyzed by HPLC. The encapsulation efficiency (%EE), loading capacity (%loading) were calculated using equation (1) and (2), respectively.

$$\%EE = \frac{\text{Weight of encapsulated drug in the particles} \times 100}{\text{Weight of drug initially used}} \quad (1)$$

$$\%loading = \frac{\text{Weight of encapsulated drug in the particles}}{\text{Weight of encapsulated drug + polymer}} \times 100 \quad (2)$$

2.4 Determination of minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentration (MIC) against *M. tuberculosis* was determined by microplate Alamar blue assay [25]. Briefly, stock solutions of the 11 samples (BBQ1, BBQ2, thymol, BBQ1 mixed thymol, BBQ2 mixed with thymol and samples of No.1-6 (Table 2.1) were diluted two-fold in 100 ml of Middlebrook 7H9GC in 96-well microplates. Mycobacterial suspension was prepared in 0.04% Tween 80 and diluted with sterile distilled water until a turbidity of the McFarland no. 1. The suspension was diluted 1:50 with 7H9GC, then 100 ml of this suspension was added

to wells of microplates. After that incubation at 37°C for 7 days, 12.5 ml of 20% Tween 80 and 20 ml of Almar blue were added to all wells. After that incubation at 37 ℃ for 24 h, the growth of the organisms was determined by colorimetric method of solution transfer blue to pink solution. The MIC was defined as the lowest concentration that prevents the colour changing. Rifampin was used as a standard drug.

2.5 Cytotoxicity by MTT assay

Jurkat cell viability was measured using the MTT based on colorimetric assay. Cells were seeded into 96 well plate at density of 1×10^5 cells/ well.Cells were treated with varoius concentrations of stock solutions of the 11 samples (BBQ1, BBQ2, thymol, BBQ1 mixed with thymol, BBQ2 mixed with thymol and samples of No.1-6). 10 ml, concentration of BBQs = 450 µg/ml were diluted with completed RPMI 1640 media, using three fold serial dilution and incubated for 24 h at 37 ℃. After that, 10 µL of MTT (25 mM in PBS) was added to each well and incubation further for 4 h. Finally, the formazan crystals formation from the reaction were solvated by addition of 100 µl of 0.04N HCl in isopropanol to each well and mixed. The absorbance was measured at 540 nm. All the experiments were repeated in triplicate. Finally, the results were showed as percentage of cell viability with respect to the untreated wells or controls and calculated by using the following equation:

Cell viability (%) = (Abs test cells/Abs control cells) × 100

2.6 Cellular uptake of nanoparticle into macrophages

5-Carboxytetramethylrhodamine or TAMRA was grafted onto the EC using EDCI/HOBt coupling method [51]. First, TAMRA (4.3 mg) was dissolved in 10 ml of DMF under nitrogen atmosphere the reaction work on ice bath. Then, EDCI·HCl (3.88 mg in 5 ml of DMF) was added to TAMRA solution with continuous stirring for 30 min at 0 ℃. Then HOBt (3.83 mg in 5 ml of DMF) was added to the mixture and continuous stirring was performed overnight, then the product was purified by dialysis against water using cellulose membrane with 12000-14000 MW cutoff before being-dried. The product was subjected to ^{1}H NMR analyzes. ^{1}H Nuclear magnetic resonance (NMR) spectra were obtained in deuterated chloroform (CDCl3-*d1*) with tetramethylsilane (TMS) as an internal reference using ACF 200 spectrometer which operated at 400 MHz for 1H nuclei (Varian Company, Palo Alto, CA, USA).

Jurkat macrophage cells were seeded into 48 well plate at density of 5×10^{5} cells/ well. After that, cells were treated with EC nanoparticles, EC containing the fluorescence-labelled carriers (EC-TAMRA 0.43 g/ml) were diluted with completed RPMI 1640 media at the concentrations of 300 µg/ml and incubation for 4 h at 37 ℃. After that, cell was washed with 500 µl of PBS for each well. Then, 100 µl of 4% para-formaldehyde was added to each well for 30 min at room temperature. Finally, cells were cleaned up several times with PBS. The cellular uptake of nanoparticles were evaluated by confocal laser scanning microscopy (CLSM).

CHAPTER III

RESULTS AND DISCUSSION

The aim of this work is to develop the drug carrier that contains bisbenzylisoquinoline alkaloids (BBQs) and thymol and test their anti-*M.TB* activity. Therefore the work includes the preparation of BBQs/thymol-loaded nanoparticles and cytotoxicity evaluation and cellular uptake determination of the obtained particles.

3.1 Encapsulation of bisbenzylisoquinoline alkaloids (BBQs) and thymol

This study includes the preparation of tilacorinine-loaded nanoparticles, tilacorinine+thymol-loaded nanoparticles, 13'-bromo-tilacorinine-loaded nanoparticles, 13'-bromo-tilacorinine+thymol-loaded nanoparticles and thymol-loaded nanoparticles. The solvent displacement method was used to encapsulate five different drugs; (1) tilacorinine (BBQ1), (2) 13'-bromo-tilacorinine (BBQ2), (3) BBQ1+thymol, (4) BBQ2+thymol and (5) thymol into EC nanoparticles. The ethanolic solution containing EC polymers and drugs was first prepared and then added with water. The aqueous suspension of drug-loaded nanoparticles appeared as a milky yellow suspension. When ethanol was slowly displaced with water, it was likely that the EC chains self-assemble into nanoparticles. The EC chains probably orientated the hydrophilic moieties (hydroxyl groups) in contact with the water medium while the

hydrophobic moieties (ethoxyl groups) were at the inside of the forming particles away from the water medium, thus drug-loaded EC particles were formed.

The morphology of all particles were characterized by scanning electron microscopy (SEM). The SEM results showed that the EC nanoparticles, BBQ1-loaded nanoparticles, BBQ1+thymol-loaded nanoparticles, BBQ2-loaded nanoparticles, BBQ2+thymol-loaded nanoparticles, and thymol-loaded nanoparticles had spherical shape (Figure 3.1). Dried sizes of EC nanoparticles, BBQ1-loaded nanoparticles, BBQ1+thymol-loaded nanoparticles, BBQ2-loaded nanoparticles, BBQ2+thymol-loaded nanoparticles, and thymol-loaded nanoparticles could be estimated from the SEM pictures to be 137 $\pm$ 3.50, 137 $\pm$ 31.80, 137 $\pm$ 4.90, 138 $\pm$ 20.50, 137 $\pm$ 5.70 and 120 $\pm$ 14.80 nm, respectively.

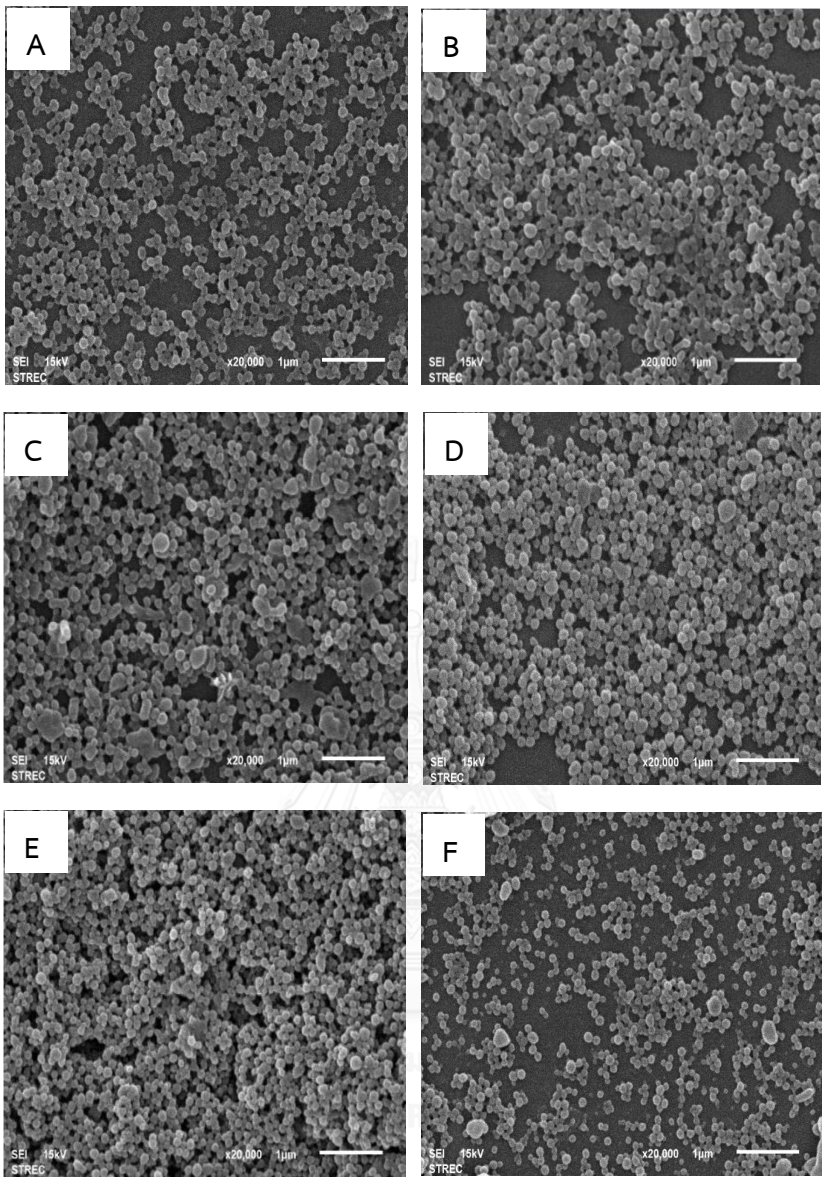

Figure 3.1. SEM photograph (A) EC nanoparticles, (B) BBQ1-loaded nanoparticles, (C) BBQ1+thymol-loaded nanoparticles, (D) BBQ2-loaded nanoparticles, (E) BBQ2-loaded nanoparticles and (F) thymol-loaded nanoparticles.

Centrifugal filtration of the particles suspension allowed separation of the unencapsulated BBQ1, BBQ2 and thymol from the particles suspension. Percentage of encapsulation efficiency (%EE) of the encapsulation process and loading capacity of the particles were analyzed by quantifying the unencapsulated BBQ1, BBQ2 and thymol (Table 3.1) by high performance liquid chromatography (HPLC). The process gave high efficiency for the encapsulation of BBQ1+thymol, BBQ2 and BBQ2+thymol and only BBQ1 loading gave less than 70% encapsulation efficiency. The obtained particles possess high loading of more than 30% for BBQ1+thymol, BBQ2 and BBQ2+thymol. Only BBQ1-loaded particles gave less than 30% loading. Thymol could be loaded into the particles with less than 50% efficiency, thus its loading in all three particles were around 10%.

Table 3.1 Encapsulation efficiency and loading capacity of BBQ1, BBQ2 and thymol in EC particles

Particles	%EE		%loading	
	BBQ	thymol	BBQ	thymol
BBQ1-loaded nanoparticles	48.05 ± 2.52	-	23.59 ± 0.95	-
BBQ1+thymol-loaded nanoparticles	74.01 ± 0.42	38.06 ± 1.63	32.24 ± 0.12	11.97 ± 0.45
BBQ2-loaded nanoparticles	81.94 ± 0.72	-	34.50 ± 0.20	-
BBQ2+thymol-loaded nanoparticles	87.88 ± 3.75	31.60 ± 0.94	36.09 ± 0.99	10.14 ± 0.27
Thymol-loaded nanoparticles	-	43.07 ± 1.50	-	13.33 ± 0.40

DSC analysis of the BBQ1-loaded nanoparticles and BBQ1+thymol-loaded nanoparticles (Figure 3.2) showed no melting peaks of the BBQ1 (110.5 ℃) and thymol (54.1 ℃). The result indicated that there was neither crystalline thymol nor crystalline BBQ1 in the particles. This implies that BBQ1 and thymol had interaction with EC polymer. The BBQ2-loaded nanoparticles and BBQ2+thymol-loaded nanoparticles (Figure 3.3) and thymol-loaded nanoparticles (Figure 3.4) also showed no melting peaks of thymol at 54.1 ℃ (Figure 3.3 and 3.4), indicating that there was no crystalline thymol in those particles. Thus thymol probably existed in the particles in the solid solution state. This was confirmed through the disappearance of all endothermic peaks of EC and appearance of new peaks, probably related to the shift of the polymer absorption isotherm due to the molecular interaction with the encapsulated molecules. Since BBQ2 itself showed no sharp melting peak (Figure 3.3), when it was encapsulated into particles, no characteristic peak related to BBQ2 could be identified. Nevertheless, the change of the endothermic peaks of EC for the particles loaded with BBQ2 indicated that the molecular interaction between BBQ2 and EC polymer did take place. Thus, we concluded that the BBQ1, BBQ2 and thymol molecules resided in the particles in the solid solution state with the EC polymer chains (Figure 3.5).

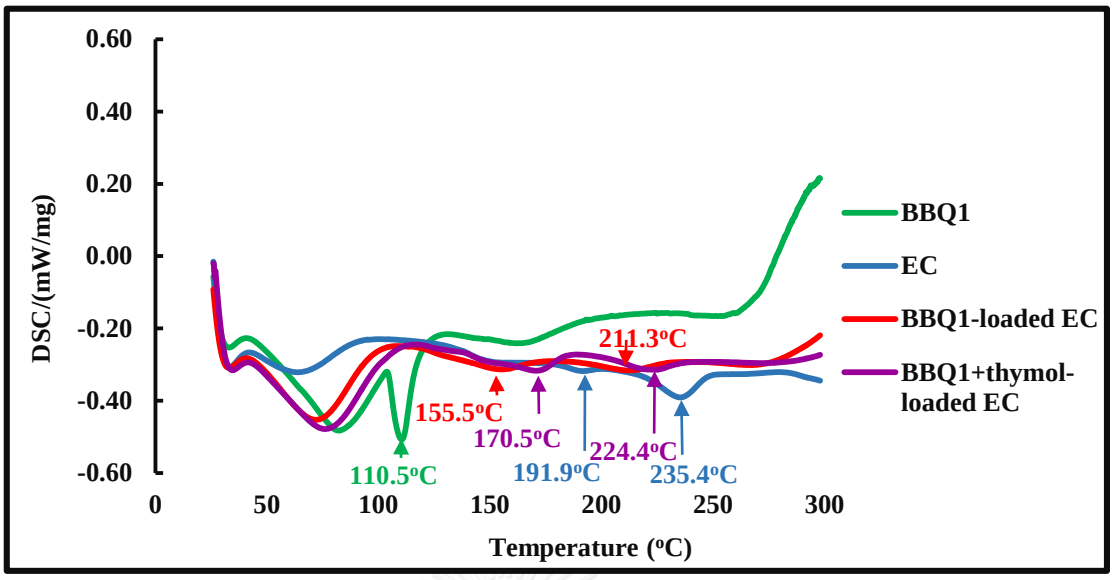

Figure 3.2 Differential scanning calorimetric thermograms of unencapsulated BBQ1 (green line), BBQ1-loaded nanoparticles (red line), BBQ1+thymol-loaded nanoparticles (brown line) and ethyl cellulose (blue line).

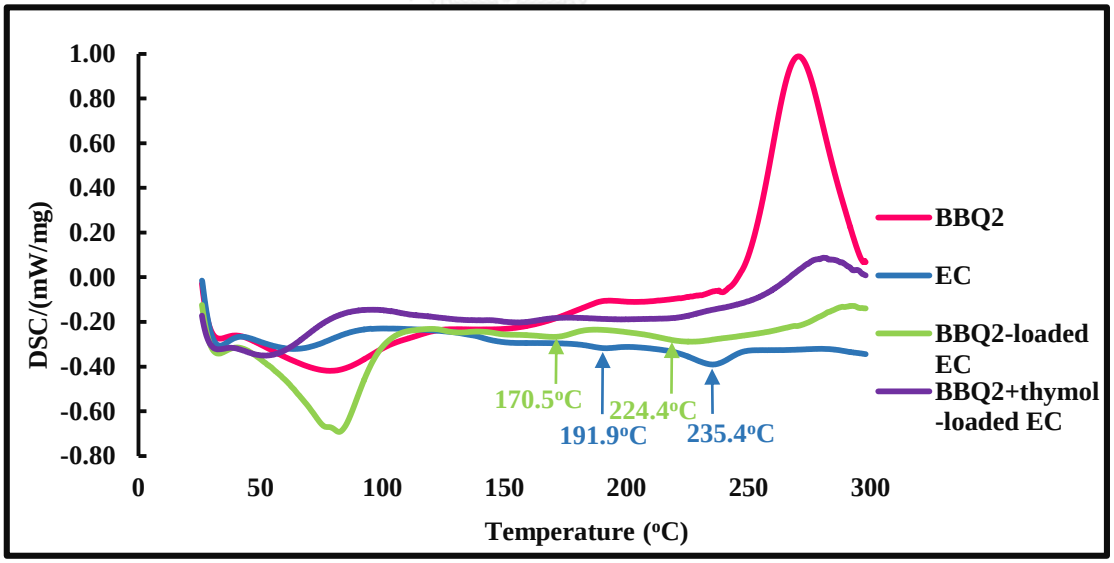

Figure 3.3 Differential scanning calorimetric thermograms of unencapsulated BBQ2 (magenta line), BBQ2-loaded nanoparticles (green line), BBQ2+thymol-loaded nanoparticles (purple line) and ethyl cellulose (blue line).

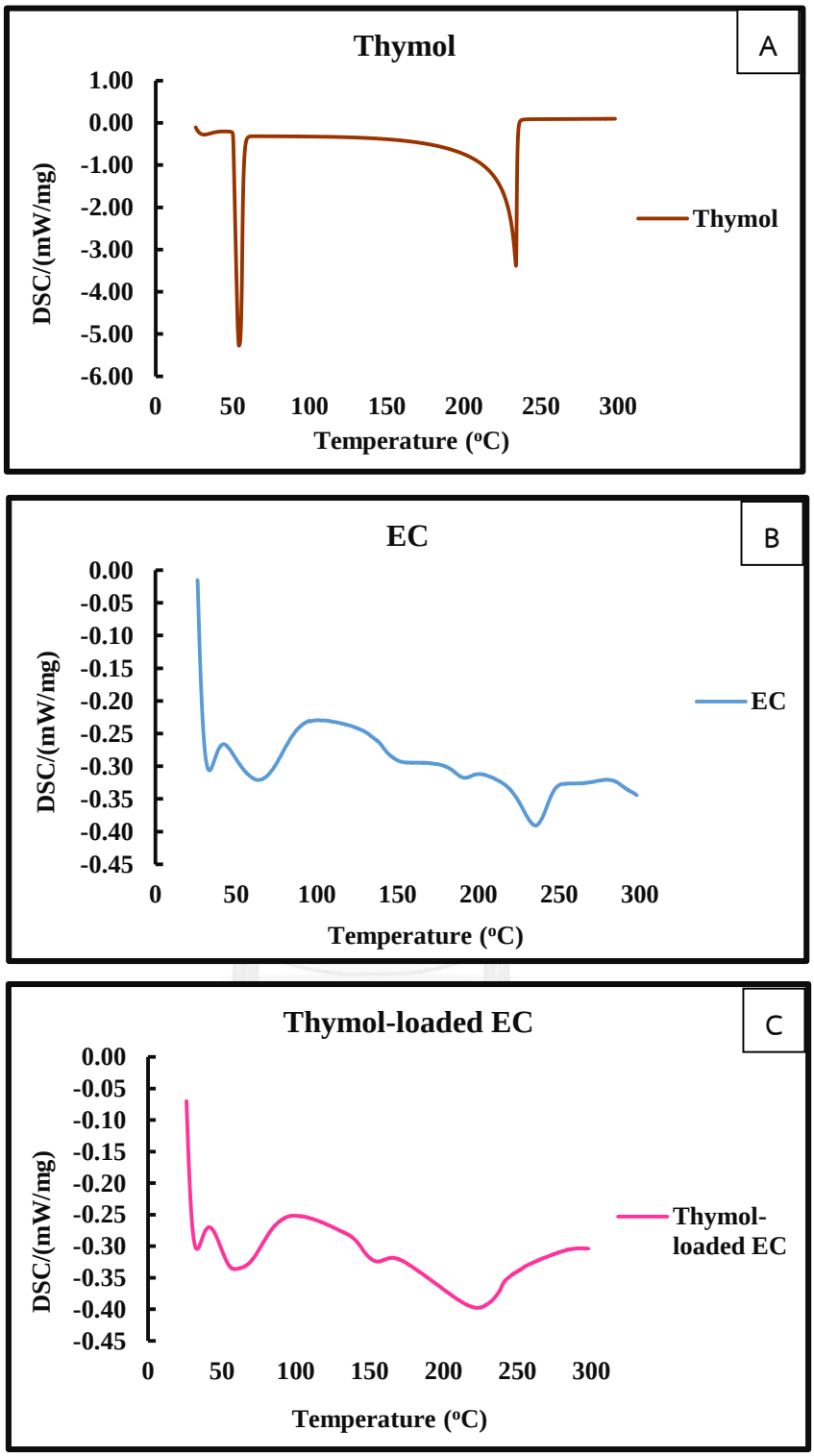

Figure 3.4 Differential scanning calorimetric thermograms of unencapsulated (A) thymol, (B) ethyl cellulose (EC) and (C) thymol-loaded nanoparticles.

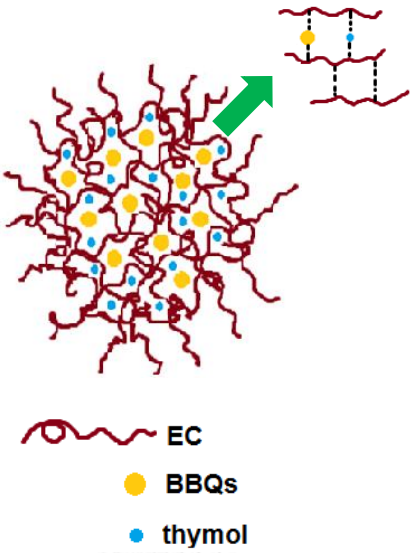

Figure 3.5 The model of drugs-loaded nanoparticles.

3.2 In vitro anti-mycobacterial activity

BBQ1 and BBQ2 have been shown to possess anti-mycobacterial activity against H37Ra (non-virulent strain), H37Rv, H37Rv-ETA-R ATCC35830 (ethionamide resistant), H37Rv-PAS-R ATCC35821 (*p*-aminosalicylic acid resistant), and H37Rv-PZA-R ATCC 35828 (pyrazinamide resistant) [23]. Nevertheless, they are toxic against MRC-5 cells line (human fetal lung fibroblast cells line) [23]. For reducing the toxic of BBQ1 and BBQ2, we proposed to make BBQ1- and BBQ2-loaded particles that could be effectively taken up into the macrophages. This is because the *M.TB* resides in the macrophage. If the particles could get into the macrophage more effectively than into lung fibroblast, then we had a chance to attack the *M.TB* without harming the good normal lung cells. In this work, we also co-encapsulate thymol with the two drugs with the rationale that it might help enhance the anti-mycobacterium activity of the drug-

loaded particles. Thymol has been shown to possess antimicrobial activity against various bacteria including *Bacillus subtilis*, *Escherichia coli*, *Klebsiella peneumoniae* and *Staphylococcus aureus* [33]. Thus, BBQ1, BBQ2, BBQ1+thymol, BBQ2+thymol and thymol were also tested for their *in vitro* anti-mycobacterial activity against *M.TB* (H37Ra).

As shown in Table 3.2, the best anti-mycobacterial activity observed from rifampicin (MIC = 0.01 µg/ml) was not surprising, because it is one of commonly used drug in the treatment of tuberculosis. The MIC values of BBQ1 and BBQ2 were 3.50 µg/ml while the MIC value of thymol was 7.80 µg/ml. Interestingly, all of the encapsulated samples showed a similar anti-*M.TB* activity to their free compound. Therefore, the result indicates that anti-*M.TB* activity of these materials was still preserved after encapsulation into a polymeric nanoparticles. It is likely that the drug-loaded particles interacts well with the *M.TB* and thus are toxic to them.

Furthermore, the anti-*M.TB* activity of BBQ1 and BBQ2 were also investigated when they were mixed with thymol. The results indicates that thymol possesses less anti-*M.TB* activity than the BBQ1 and BBQ2. Since in the BBQ-thymol mixtures, the percentage of thymol was only 2.50%, the mixture showed similar anti-*M.TB* activity to the activity of unmixed BBQ1 and BBQ2.

Table 3.2 Anti-mycobacterial activity (as MIC values)

Sample	**MIC (μg/ml)**
free BBQ1	3.50
free BBQ2	3.50
free BBQ1+thymol	3.50
free BBQ2+thymol	3.50
free thymol	7.80
BBQ1-loaded EC	3.50
BBQ2-loaded EC	3.50
BBQ1+thymol-loaded EC	3.50
BBQ2+thymol-loaded EC	3.50
Thymol-loaded EC	7.80
Rifampicin	0.01

3.3 Cytotoxicity test

The cytotoxicity of BBQ1, BBQ2, BBQ1+thymol, BBQ2+thymol and thymol in free form and in nanoparticles, were determined by MTT assay in Jurkat cells line (T-cell line). MTT assay is designed to quantify cell viability. The various concentrations of BBQ1 and BBQ2 (0.1-10 μg/ml) were incubated Jurkat cells at 1×10^5 cells per well for 24 h. The cytotoxicity values of BBQ1 and BBQ1+thymol groups are shown in Figure 3.6 and those of BBQ2 and BBQ2+thymol groups are shown in the Figure 3.7. At the concentration of 0.1-3 μg/ml, all samples presented only a little cytotoxicity because cell viability were more than 70%. When the concentration increased to 10 μg/ml, the unencapsulated BBQ1, BBQ1+thymol, BBQ2 and BBQ2+thymol showed a high cytotoxicity because cell viabilities were less than 40%. In contrast to unencapsulated drugs, cells viabilities of encapsulation of BBQ1, BBQ1+thymol, BBQ2 and BBQ2+thymol were more than 60% at the concentration of 10 μg/ml. Interestingly, the encapsulation of BBQ1, BBQ1+thymol, BBQ2 and BBQ2+thymol into EC nanoparticles exhibited lower cytotoxic levels compared to their free form at all of tested concentrations, especially at the concentration of 10 μg/ml. These results could be concluded that encapsulation of BBQ1 and BBQ2 could reduce cytotoxicity of BBQ1 and BBQ2. It should be pointed out here that the cytotoxicity level to the *M.TB* of the BBQ-loaded particles are similar to those of the free drugs. This implied that the particles interacted well with the *M.TB* and thus could effectively harm the bacteria.

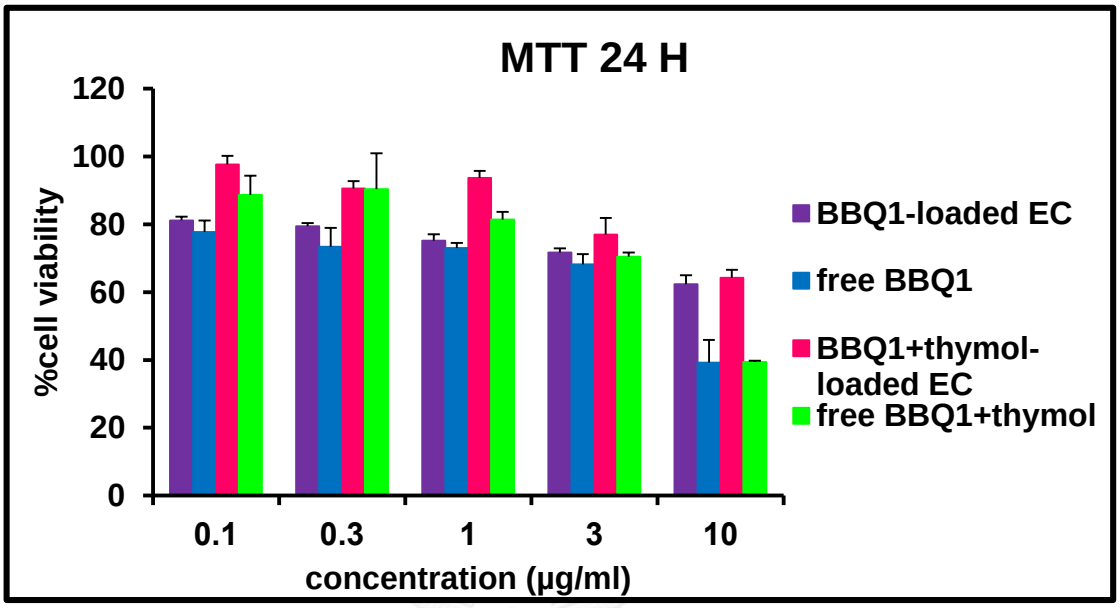

Figure 3.6 Cytotoxicity of BBQ1-loaded nanoparticles and BBQ1+thymol loaded nanoparticles.

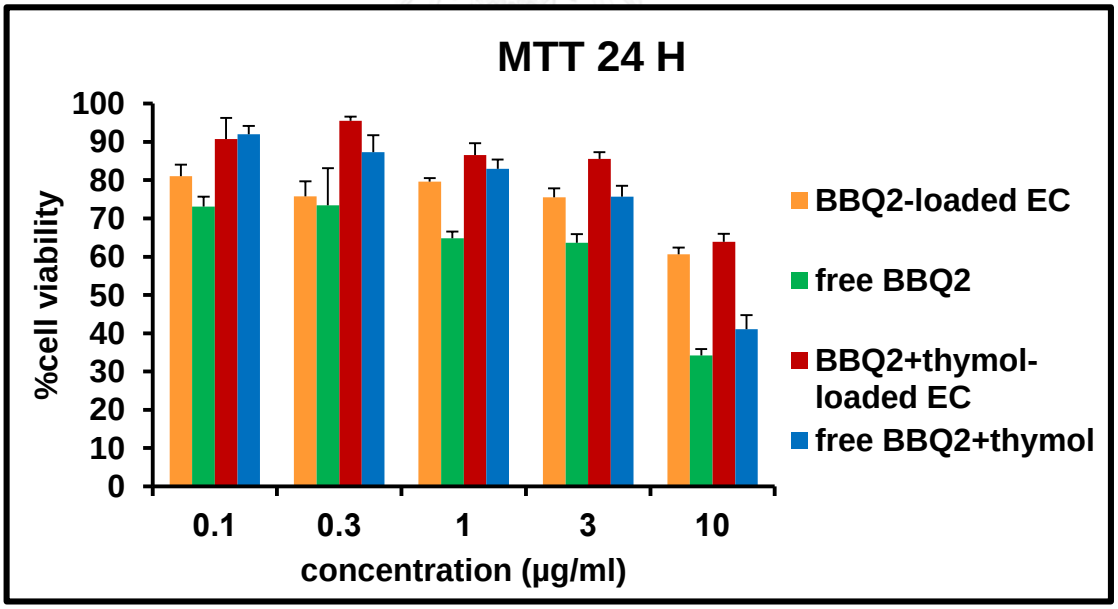

Figure 3.7 Cytotoxicity of BBQ2-loaded nanoparticles and BBQ2+thymol loaded nanoparticles.

As mentioned earlier that the rationale here is that nanoparticles loaded with BBQ and thymol might be able to get into macrophage and then kill the *M.TB* inside the macrophage. Thus here we verified that the particles could effectively get into macrophage.

3.4 Cellular uptake of nanoparticles

3.4.1 Preparation of the nanoparticles

3.4.1.1 Synthesis of fluorescence polymer

The determination of the cellular uptake of nanoparticles were performed by monitoring the nanoparticles uptake into macrophage cells. However, it is difficult to determine nanoparticles in the cells. Thus, one strategy to solve this problem is to attach fluorophore groups on the nanoparticles.

Grafting of fluorescence dye onto the particles was carried out by first grafting the dye onto EC chains and then used this dye-grafted EC to make particles. Figure 3.8 showed the coupling reaction for grafting TAMRA on EC chain. 1-Ethyl-3-(3-dimethylaminoqropyl) carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt) allowed ester to occur more easily under mild condition, through the formation of active ester (Figure 3.8).

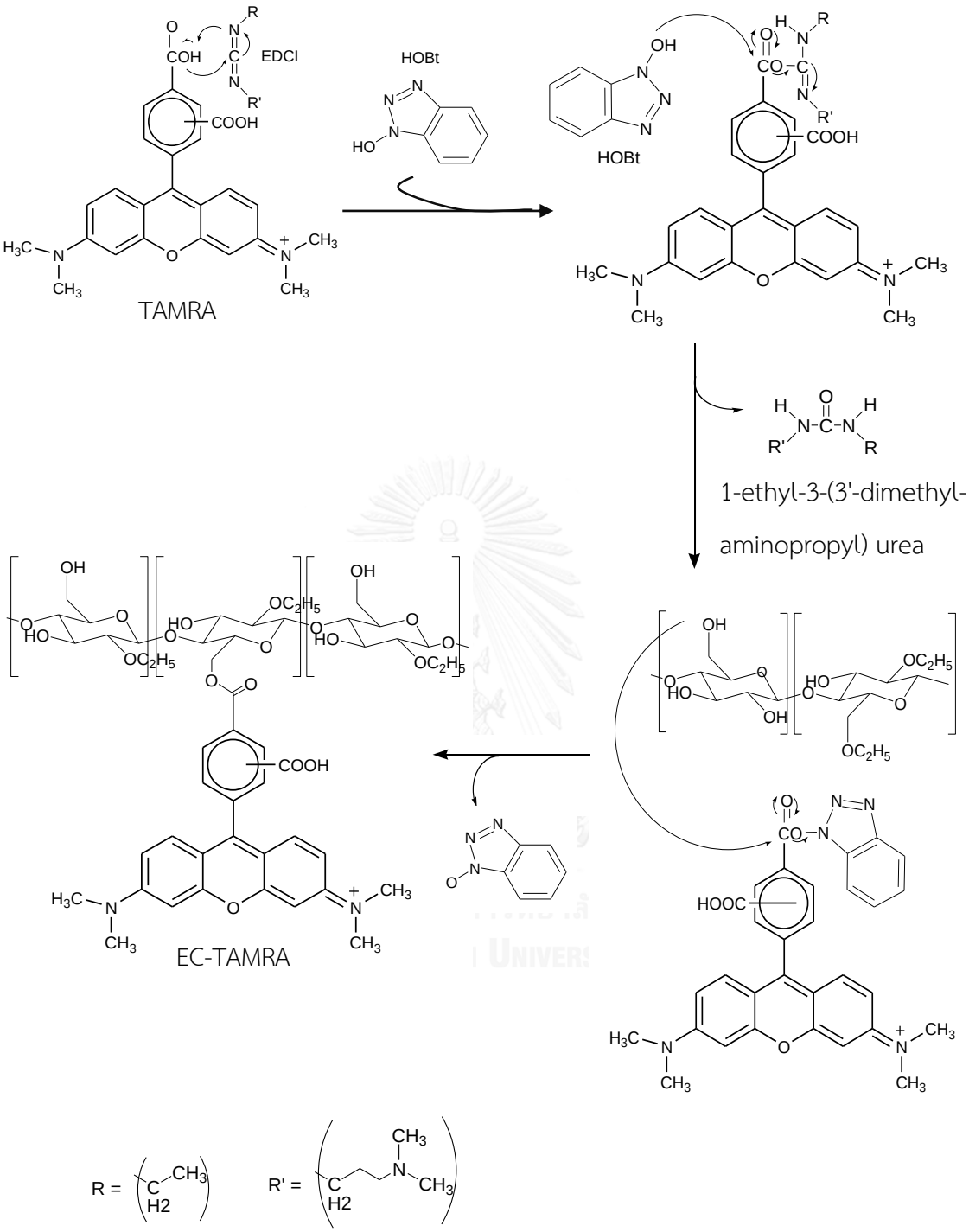

Figure 3.8 Synthesis of EC-TAMRA by coupling reaction.

3.4.1.2 Preparation of unloaded nanoparticles

Solvent displacement method was used to prepare the nanoparticles. The ethanolic solution containing polymers and drugs was slowly added with water so that the polymeric chains of EC could self-assemble into particles. The aqueous suspension of the product appeared as a milky pink suspension. The EC-TAMRA was characterized by nuclear magnetic resonance spectroscopy (^{1}H NMR). The morphology of particles was characterized by scanning electron microscopy (SEM). The fluorescence property of TAMRA-grafted-EC (EC-TAMRA) was also confirmed by confocal laser fluorescence scanning microscopy (CLSM).

The ^{1}H NMR spectrum (Figure 3.9) showed the resonance peak of aromatic protons of TAMRA on EC-TAMRA at 7.35 ppm.

The SEM results showed that the EC-TAMRA nanoparticles had spherical shape (Figure 3.10A). Dried sizes of EC-TAMRA nanoparticles could be estimated from the SEM pictures to be 154 **±** 31.11 nm.

The CLSM results showed the fluorescence image of EC-TAMRA (Figure 3.10B). The ^{1}H NMR and CLSM results confirmed that TAMRA could be grafted on the polymer chain.

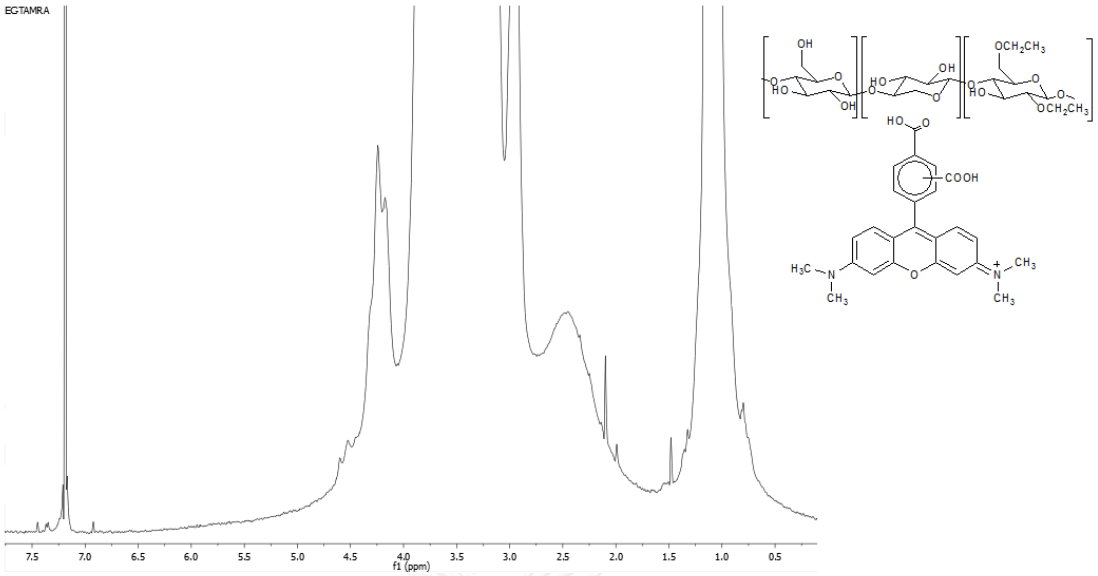

Figure 3.9 ^{1}H NMR spectrum of EC-TAMRA in $CDCl_3$.

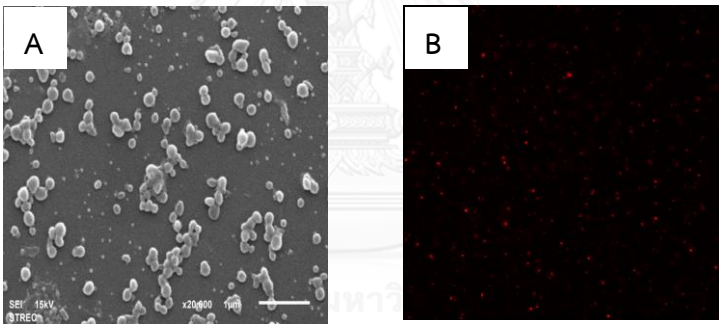

Figure 3.10 SEM and CLSM photographs (A) and (B) EC-TAMRA nanoparticles.

3.4.2 Cellular uptake of nanoparticles into Jurkat cell

The cellular uptake of 5,6-Carboxytetramethylrhodamine-grafted-EC (EC-TAMRA) into Jurkat cells line was evaluated using confocal laser scanning microscopy (CLSM) technique. The cellular uptake of EC-TAMRA was determined by detection of the fluorescence intensity at excitation wavelength of 561 nm and emission wavelength of 595 nm. The EC-TAMRA particles were incubated with Jurkat cells density of 5×10^5 cells per well for 4 h at the final EC-TAMRA concentration of 300 µg/ml.

As shown in Figure 3.11, the CLSM image of control cells showed no fluorescence (Figure 3.11A) while the CLSM image of EC-TAMRA nanoparticles showed the fluorescence signal (red colour) likely to be from EC-TAMRA nanoparticles inside the Jurkat cell (Figure 3.11D). This result confirmed that the EC-TAMRA particles could be taken up into Jurkat cell.

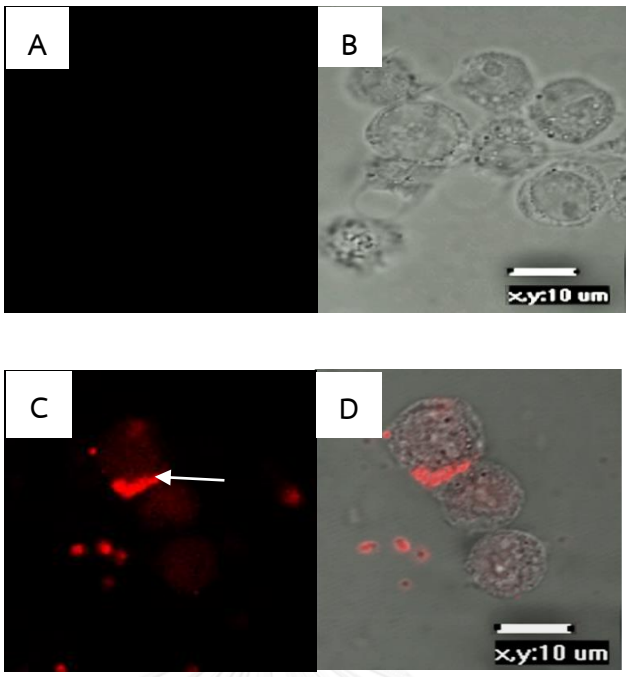

Figure 3.11 The CLSM images of the Jurkat cells after being incubated with medium control (A and B), TAMRA labeled EC (C and D).

CHAPTER IV

CONCLUSION AND SUGGESTIONS

The BBQ1 and BBQ2 were successfully loaded into EC nanoparticles. The encapsulation efficiency of BBQ1+thymol, BBQ2 and BBQ2+thymol were more than 70% and only BBQ1 gave less than 70% encapsulation efficiency. The loading capacity of BBQ1+thymol, BBQ2 and BBQ2+thymol were more than 30% and only BBQ1 gave less than 30% loading. The obtained nanoparticles showed spherical morphology and dispersed well in water. The BBQ1- and BBQ2-loaded nanoparticles exhibited anti-*M.TB* activity to their free BBQ1 and BBQ2 (MIC = 3.50 μg/ml). Thymol was added to increase anti-*M.TB* activity of BBQ1 and BBQ2. However, the results showed that thymol could not give synergistic effect with BBQ1 and BBQ2. Interestingly, the encapsulation of BBQ1 and BBQ2 into EC nanoparticles could decrease toxicity of BBQ1 and BBQ2. The cellular uptake study of EC-TAMRA nanoparticles showed that the EC-TAMRA particles could be taken up into Jurkat cells effectively. Thus with the ability of the BBQ-loaded particles to kill *M.TB*, their low toxicity towards the macrophage comparing to the *M.TB* and their ability to be taken up into macrophage cells, it is possible that these nanoparticles could help clearing the *M.TB* infection in the macrophages. In addition, although the co-encapsulated thymol no significant help for the clearing of *M.TB*, the co-encapsulation might help making the *M.TB* mutate more difficultly, Future work, thus, should involve the test of *M.TB* clearing inside the

macrophage using this BBQ-loaded particles. Long term study on the effect of the co-encapsulated thymol on bacterial resistant to BBQ is also interesting.

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APPENDIX

APPENDIX A

1. Calculation of % encapsulation efficiency and loading content of drug encapsulated into EC nanoparticles

Calibration curve of BBQ1

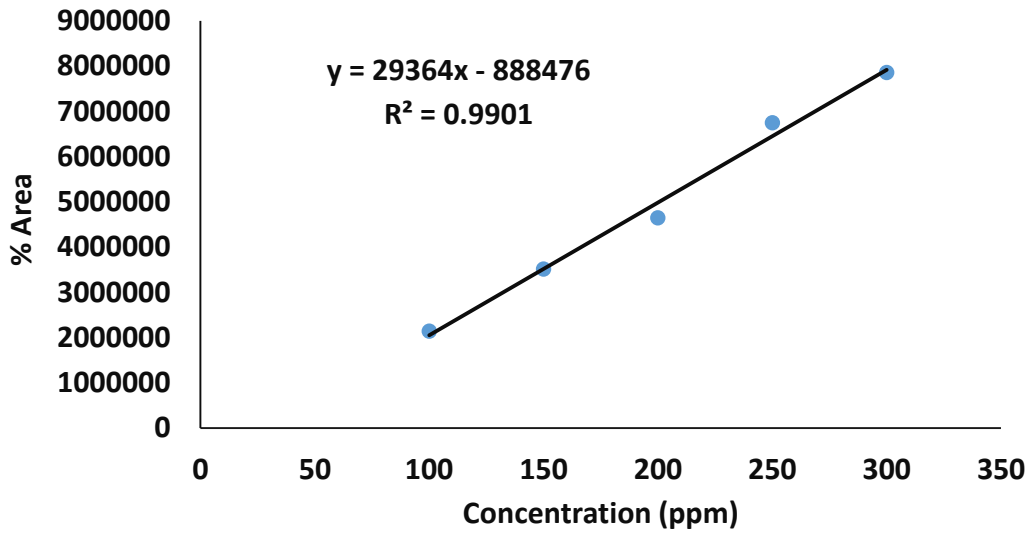

Figure A-1 Calibration curve of BBQ1 at 290 nm

By plotting a graph between percent %area and concentration of BBQ1 solution, a linear relationship was obtained and for calculation of concentrations of BBQ1.

From the equation of calibration curve;

$$Y = 29364X - 888476, R^2 = 0.9901 \quad (1)$$

The amount of BBQ1 at the outside of particles was calculated by equation (1);

5696824 = 29364X - 888476

X = 224.26 ppm = 224.26 mg/l

In final volume of 10 ml had BBQ1 of (224.26 x 10)/1000 = 2.24 mg

Weight of employed BBQ1 and EC were 4.50 mg and 7.0 mg

Weight of encapsulated BBQ1 = 4.50 – 2.24 = 2.26 mg

$$\% \text{ EE} = \frac{\text{Weight of encapsulated BBQ1}}{\text{Weight of BBQ1 initially used}} \times 100$$

= (2.26/4.50) x 100

= 50.22%

$$\% \text{ loading} = \frac{\text{Weight of encapsulated BBQ1}}{\text{Weight of encapsulated BBQ1 + polymer}} \times 100$$

= (2.26/ (4.50+7.0)) x 100

= 19.65%

Calibration curve of BBQ2

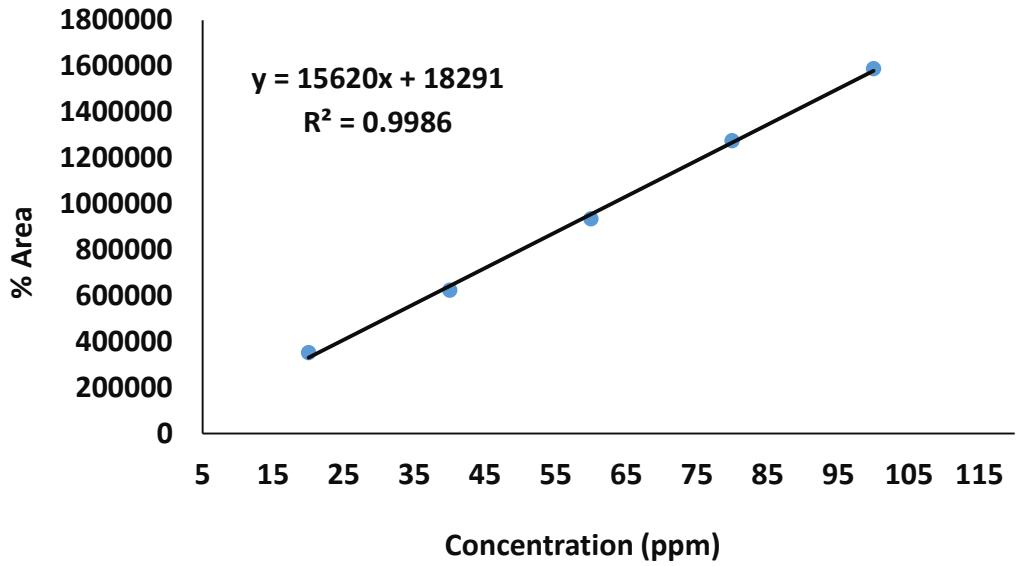

Figure A-2 Calibration curve of BBQ2 at 295 nm

By plotting a graph between percent %area and concentration of BBQ2 solution, a linear relationship was obtained and for calculation of concentrations of BBQ2.

From the equation of calibration curve;

$$Y = 15620X + 18291, R^2 = 0.9986 \qquad (1)$$

The amount of BBQ2 at the outside of particles was calculated by equation (1);

$$1229996 = 15620X + 18291$$

$$X = 77.57 \text{ ppm} = 77.57 \text{ mg/l}$$

In final volume of 10 ml had BBQ2 of (77.57 x 10)/1000 = 0.78 mg

Weight of employed BBQ2 and EC were 4.50 mg and 7.0 mg

Weight of encapsulated BBQ2 = 4.50 – 0.78 = 3.72 mg

$$\% \text{ EE} = \frac{\text{Weight of encapsulated BBQ2}}{\text{Weight of BBQ2 initially used}} \times 100$$

$= (3.72/4.50) \times 100$

$= 82.67\%$

$$\% \text{ loading} = \frac{\text{Weight of encapsulated BBQ2}}{\text{Weight of encapsulated BBQ2 + polymer}} \times 100$$

$= (3.72/ (4.50+7.0)) \times 100$

$= 32.35\%$

Calibration curve of thymol

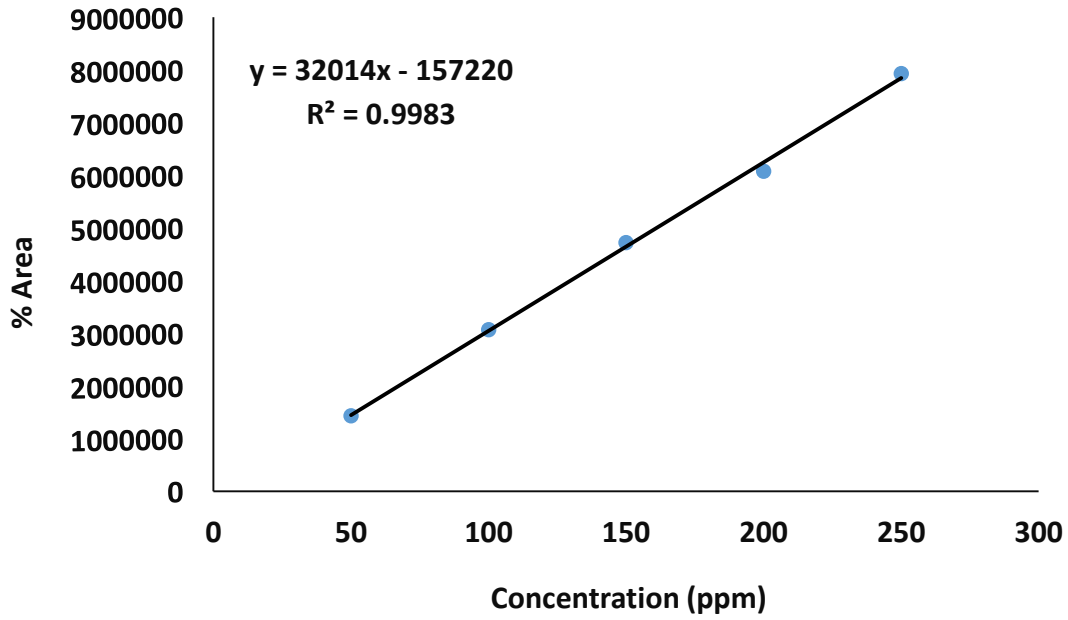

Figure A-3 Calibration curve of thymol at 274 nm

By plotting a graph between percent %area and concentration of thymol solution, a linear relationship was obtained and for calculation of concentrations of thymol.

From the equation of calibration curve;

$$Y = 32014X - 157220,\ R^2 = 0.9983 \qquad (1)$$

The amount of thymol at the outside of particles was calculated by equation (1);

$$4283696 = 32014X - 157220$$

$$X = 138.72 \text{ ppm} = 138.72 \text{ mg/l}$$

In final volume of 10 ml had thymol of (138.72 x 10)/1000 = 1.39 mg

Weight of employed thymol and EC were 2.50 mg and 7.0 mg

Weight of encapsulated thymol = 2.50 – 1.39 = 1.11 mg

$$\% \text{ EE} = \frac{\text{Weight of encapsulated thymol}}{\text{Weight of thymol initially used}} \times 100$$

$$= (1.11/2.50) \times 100$$

$$= 44.40\%$$

$$\% \text{ loading} = \frac{\text{Weight of encapsulated thymol}}{\text{Weight of encapsulated thymol + polymer}} \times 100$$

$$= (1.11/ (2.50+7.0)) \times 100$$

$$= 11.68\%$$

APPENDIX B

1. Chemical and culture medium preparation

1.1 Isotonic Phosphate Buffer Saline pH 7.4

NaCl	8.00 g
KCl	0.20 g
KH_2PO_4	0.20 g
Na_2HPO_4	1.44 g

Dissolve NaCl, KCl, KH_2PO_4 and Na_2HPO_4 with distilled water then adjust volume to 1000 ml with distilled water and the pH is measured with pH meter.

1.2 Preparation of MTT solution

Prepare a 12 mM stock solution by 5 mg/ml of MTT reagent was dissolved in 10 ml of PBS. The solution was stirred until completely dissolved and mixed together. The obtained stock solution was sterilized through a 0.5 μm filter and transferred into aliquot tubes. The stock solution was stored at 4 ℃ until used.

APPENDIX C

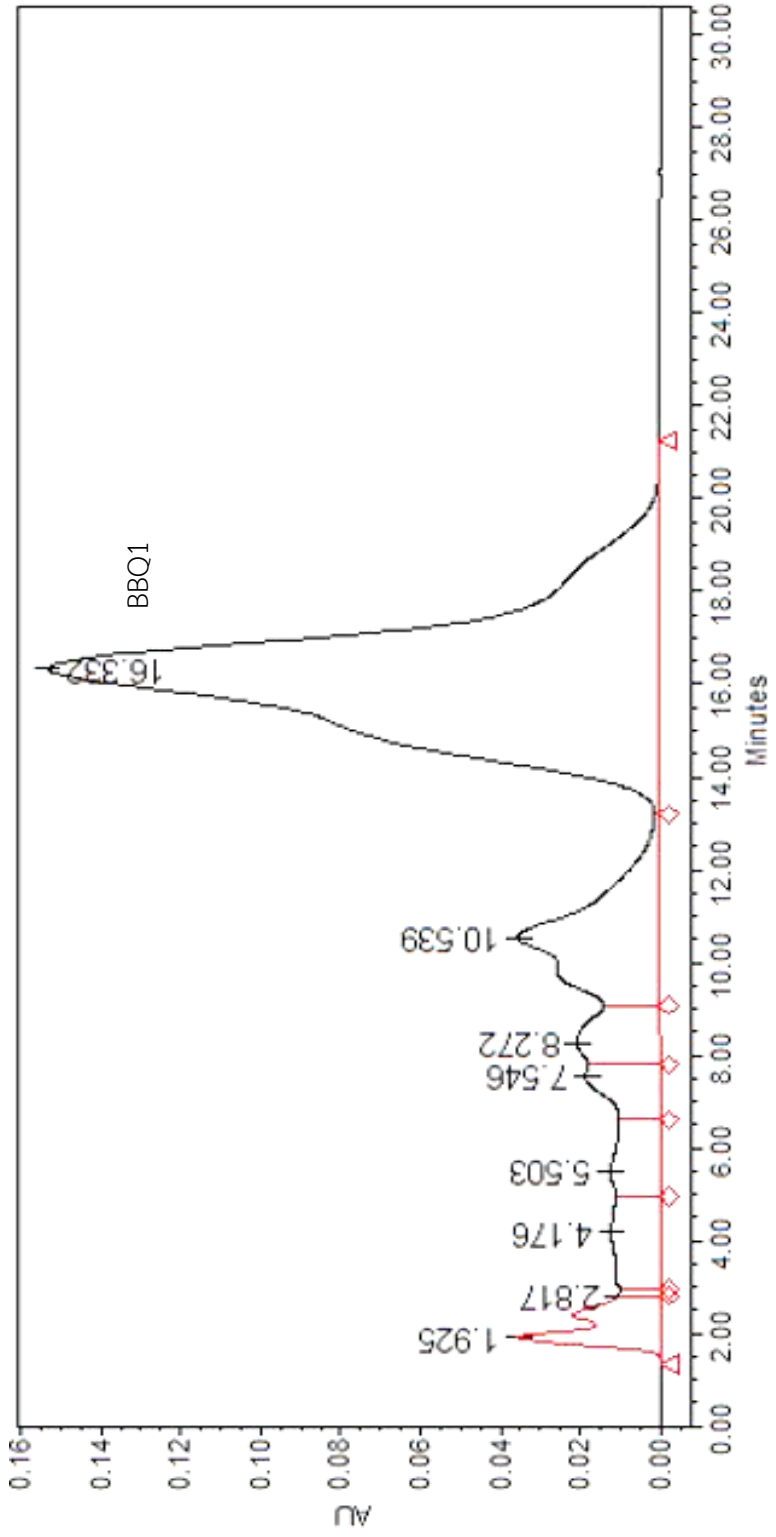

Figure C-1 HPLC chromatogram of BBQ1

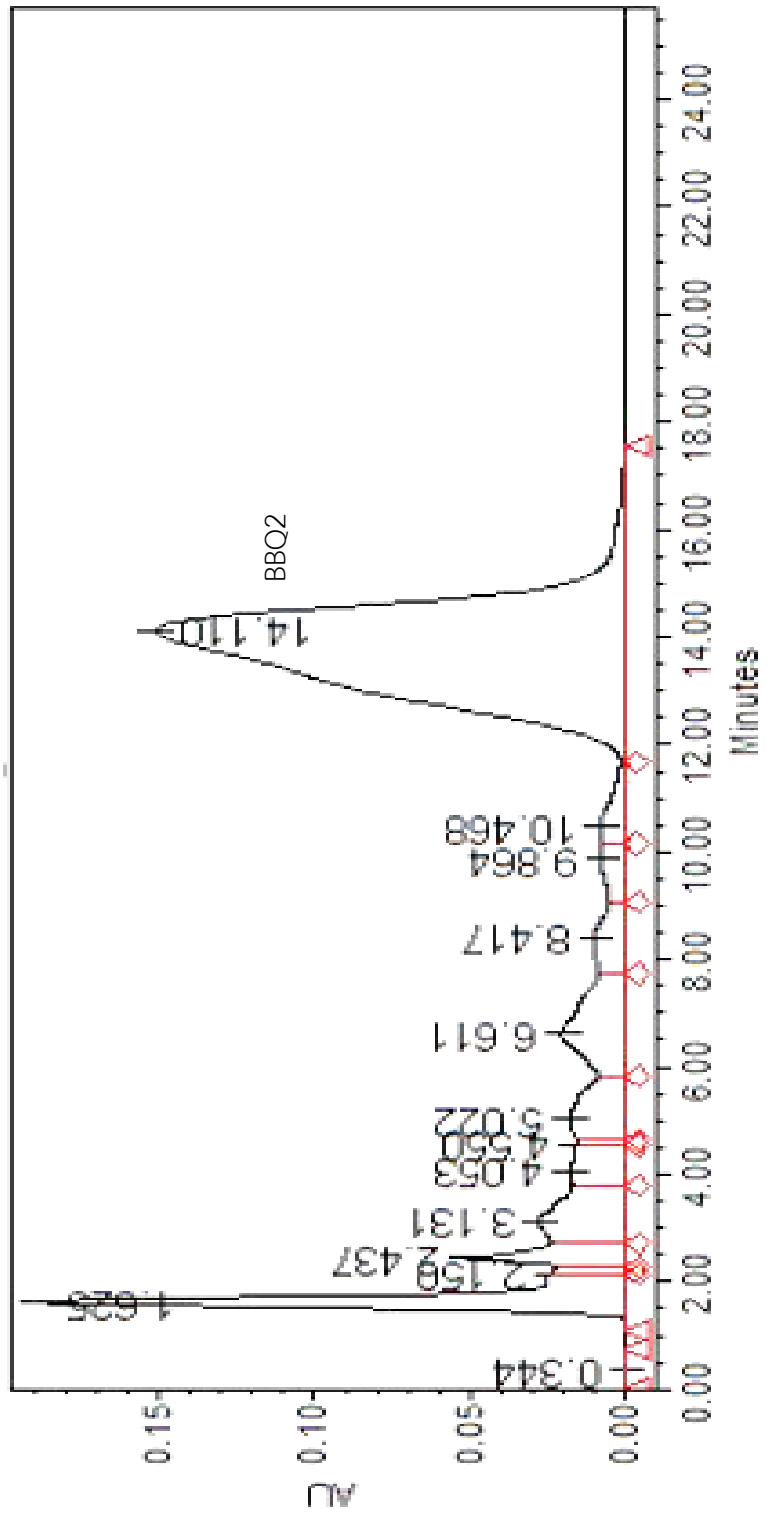

Figure C-2 HPLC chromatogram of BBQ2

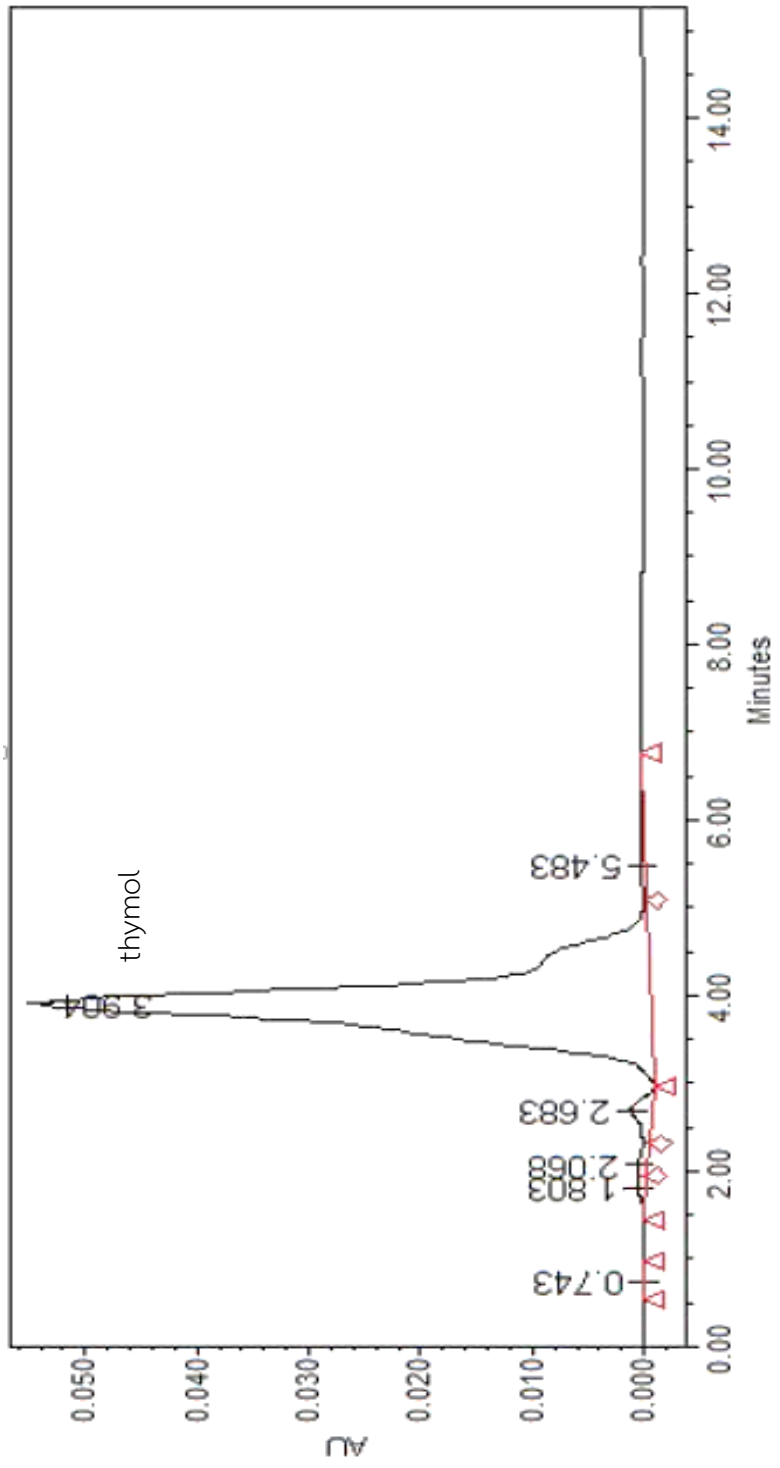

Figure C-3 HPLC chromatogram of thymol

VITA

Mister Thanawat Winairuk was born on November 14, 1988 in Bangkok, Thailand. He received a Bachelor's Degree of Science in Chemistry from Silpakorn University in 2010. Then he started his graduate study on Master's degree in the Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University. During master study, he had the great opportunity to present him work in poster session in topic "Encapsulation of Bisbenzylisoquinoline alkaloids from Tiliacora triandra" at the 2014 IUPAC World Polymer Congress (MACRO 2014), Chiang Mai, Thailand.

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