Determination of Physicochemical and Antifungal Properties of Nanoliposome Containing Rhinacanthin-C Extracted from *Rhinacanthus nasutus* Leaves

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Pharmacy in Pharmaceutical Sciences

Prince of Songkla University

2011

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ชื่อวิทยานิพนธ์	การศึกษาคุณสมบัติทางเคมีกายภาพและฤทธิ์ต้านเชื้อราของนาโนไลโป-โซมที่มีไรนาแคนติน–ซี ที่สกัดจากใบทองพันชั่ง
ผู้เขียน	นายจีรศักดิ์ เผ่าสุภาพ
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บทคัดย่อ

สามารถเตรียมนาโนไลโปโซมที่มี rhinacanthin–C ได้ด้วยวิธี modified ethanol injection method โดยมีสองสูตรที่ดีที่สุดประกอบด้วย ซอยบีนฟอสฟาติดิลโคลีน (SPC), คอเลสเตอรอล (CHL) และ ทวีน 80 (T80) ในอัตราส่วน 1:1:0 (F19) และ 1:1:0.5 (F28) โดยโมล ในแต่ละสูตรประกอบด้วยปริมาณของฟอสโฟไลปิดทั้งหมดเท่ากับ 40 μmol/mL และมีปริมาณ rhinacanthin-C เท่ากับ 4 mg ต่อปริมาณ ไลโปโซม 3 mL ไลโปโซมที่ได้นำไปศึกษาคุณสมบัติทางเคมีและกายภาพรวมถึงการศึกษาฤทธิ์ต้านเชื้อรา ไลโปโซมที่มี rhinacanthin–C มีประสิทธิภาพในการกักเก็บสารคิดเป็นร้อยละ 94.69 ± 1.20 (F19) และ 84.94 ± 1.32 (F28) โดยมีขนาดอนุภาคประมาณ 221.4 ± 13.76 (F19) และ 115.8 ± 23.33 (F28) นาโนเมตร และมีค่าศักย์ซีตา (zeta potential) วัดได้เท่ากับ -38.16 mV (F19) และ -40.98 mV (F28)

การศึกษาความคงตัวของนาโนไลโปโซมที่เตรียมได้โดยเก็บที่อุณหภูมิห้องในภาชนะป้องกันแสงเป็นระยะเวลาสามเดือนพบว่า ขนาดอนุภาคของไลโปโซมทั้งสองสูตรมีความคงตัวดีในระหว่างการเก็บรักษาสองเดือนแรก จากนั้นขนาดอนุภาคจะลดลงเล็กน้อยในเดือนที่สาม การศึกษาความคงตัวของ rhinacanthin–C ที่อยู่ในไลโปโซมในสภาวะเร่งที่ความเป็นกรด – ด่างต่างๆ ด้วยเทคนิค reversed–phase high–performance liquid chromatography เป็นเวลาหกเดือน พบว่า ที่ pH 4.0 และ 6.6 ไลโปโซม rhinacanthin–C ที่เตรียมด้วยสูตร F19 สามารถรักษาปริมาณของ rhinacanthin–C ได้มากกว่า 80 เปอร์เซ็นต์นานถึงสามเดือน ขณะที่สูตร F28 สามารถรักษาปริมาณของ rhinacanthin–C ได้มากกว่า 80 เปอร์เซ็นต์นานถึงสองเดือนและสามเดือน เมื่อเก็บที่ pH 4.0 และ 6.6 ตามลำดับ ซึ่งทั้งสองสูตรช่วยรักษาความคงตัวของ rhinacanthin–C ได้ดีกว่าที่ไม่ได้เตรียมเป็นไลโปโซม ส่วนที่ pH 10.0 ไลโปโซมทั้งสองสูตรสามารถรักษาปริมาณของ rhinacanthin–C ได้มากกว่า 80 เปอร์เซ็นต์ ได้เพียง 7 วัน โดยสูตร F28 การสลายตัวจะเร็วกว่า rhinacanthin–C ที่ไม่ได้เตรียมอยู่ในรูปของไลโปโซมและไลโปโซมที่เตรียมด้วยสูตร F19 ตามลำดับ

การศึกษาฤทธิ์ต้านเชื้อราที่ก่อโรคกลากและเกลื้อนของนาโนไลโปโซมที่มี rhinacanthin–C พบว่า ไลโปโซมที่เตรียมด้วยสูตร F28 มีฤทธิ์ยับยั้งเชื้อ *Microsporum gypseum* และ *Trichophyton rubrum* ได้ดีกว่าสูตร F19 โดยมีค่า MICs เท่ากับ 1.87, 1.87 และ 30.0, 7.5 µg/mL ตามลำดับ สูตร F19 มีฤทธิ์ยับยั้งเชื้อ *T. rubrum* ได้ดีกว่า *M. gypseum* ในขณะที่สูตร F28 มีฤทธิ์ยับยั้งเชื้อราทั้งสองได้ไม่แตกต่างกัน และเมื่อเปรียบเทียบฤทธิ์ยับยั้งเชื้อราดังกล่าวกับ rhinacanthin–C ที่ไม่ได้เตรียมอยู่ในรูปไลโปโซมพบว่า ไลโปโซมที่เตรียมด้วยสูตรF28ให้ฤทธิ์การยับยั้งที่ดีกว่า ดังนั้นจากการศึกษาแสดงให้เห็นว่า rhinacanthin–C ที่นำมาเตรียมเป็นไลโปโซมสามารถช่วยเพิ่มความคงตัวและเพิ่มประสิทธิภาพในการยับยั้งเชื้อราที่ก่อโรคกลากและเกลื้อนของ rhinacanthin–C ได้

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Academic Year	2011

ABSTRACT

Nanoliposome containing rhinacanthin-C can be prepared by using modified ethanol injection method. The two best formulations composed of soybean phosphatidylcholine (SPC), cholesterol (CHL) and Tween 80 (T80) in a molar ratio of 1:1:0 (F19) and 1:1:0.5 (F28) with the total phospholipids 40 μmol/mL containing rhinacanthin-C 4 mg per 3 mL were obtained. Their physicochemical characterization and antifungal activities were investigated and compared with pure rhinacanthin-C. The encapsulating efficiencies of rhinacanthin-C of the two formulas were 94.69 ± 1.20 % (F19) and 84.94 ± 1.32 % (F28), respectively. Their particles sizes were in the range of nanometer about 221.4 ± 13.76 and 115.8 ± 23.33 nm, with zeta potential values of -38.16 and -40.98 mV for F19 and F28, respectively.

The stability study of nanoliposome particles were performed by storing at room temperature in a container protected from light for a period of three months. The result demonstrated that the particle size of nanoliposome of the two formulations were stable during first two months and slightly decreased after storage for three months. A reversed-phase high-performance liquid chromatography was used to determined amount of rhinacanthin-C in liposome during six months storage in various conditions (buffer solutions pH 4.0, 6.6 and 10.0). The result showed that at pH 4.0 and 6.6, liposome containing rhinacanthin-C (F19) could maintain amount of rhinacanthin-C more than 80% for up to three months. Formulation number 28 could maintain amount of rhinacanthin-C more than 80% up to two and three months when storage in pH 4.0 and 6.6, respectively. Both formulations could maintain stability of rhinacanthin-C better than pure rhinacanthin-C in solutions with the same pH. At pH 10.0 the both liposome formulations could preserve amount of rhinacanthin-C more than 80% for only 7

days. Moreover, formulation number 28 showed decomposition profile of rhinacanthin-C faster than rhinacanthin-C in solution and F19 formulation.

Antifungal activities study of nanoliposome containing rhinacanthin-C demonstrated that the formulation (F28) showed better inhibition activity against *Microsporum gypseum* and *Trichophyton rubrum* than the formulation (F19). The MIC values of formulations (F28) were 1.87, 1.87 µg/mL and formulations (F19) were 30.0, 7.5 µg/mL against *M. gypseum* and *T. rubrum*, respectively. The formulations (F19) could inhibit *T. rubrum* better than *M. gypseum*, while the formulations (F28) showed no different in inhibition properties against those two fungi. The formulation (F28) also showed better antifungal activity MIC value when compared with pure rhinacanthin-C (MICs = 240, 3.75 µg/mL against *M. gypseum* and *T. rubrum* for 7 days). Therefore, nanoliposomes were able to improve stability and antifungal activity of rhinacanthin-C.

ACKNOWLEDGEMENTS

This thesis is not successfully completed if without support from many parts. Firstly, I would like to express my gratitude to my advisor Assist. Prof. Dr. Chitchamai Ovatlarnporn for giving me the opportunity performing this work, including her helpful suggestion, guidance, and encouragement for me throughout the course of this work. I would like to especially thank my co-advisor Assist. Prof. Dr. Sirirat Pinsuwan for her helpful introduces and comments to me about liposomes.

Secondly, I would like to give special thank to all financial supported by Graduate School, Prince of Songkla University; Drug Delivery System Excellence Center in Faculty of Pharmaceutical Sciences, Prince of Songkla University; scholarship from the Faculty of Pharmaceutical Sciences, Prince of Songkla University and grants supported by the Thailand Graduate Institute of Science and Technology, TGIST (No. TG-55-18-51-042M).

I also wish to thank the various internal organizations of Prince of Songkla University as well as Scientific Equipment Center, Songklanagarin Hospital and Microbiology Department, Faculty of Sciences for supporting equipment and microorganism to use in experiments.

I would like to the express my appreciation to staffs of Faculty of Pharmaceutical Sciences, Prince of Songkla University for their kindness and help. Thank you to all my friends and colleagues for their friendship and encouragement as well as stimulating intellectual environment to work and study in Faculty of Pharmaceutical Sciences, Prince of Songkla University.

Lastly, I would like to express my deepest sincere and gratitude to my family for their loves, understanding and encouragement during my study.

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LIST OF ABBREVIATIONS AND SYMBOLS

AmB	=	amphotericin B
AUC	=	area under the curve
Balb/c mice	=	an albino, laboratory-bred strain of the House Mouse
BDMCA	=	bis-demethoxy curcumin analogue
β-	=	beta-
BSV	=	bubblesome vesicle
c-AMB	=	conventional amphotericin B
^{13}C – NMR	=	carbon-13 nuclear magnetic resonance
^{14}C	=	carbon-14
$CD4^{+}$	=	cluster of differentiation antigen 4
C-DOPE	=	*N*-citraconyl- dioleoylphosphatidylethanolamine
CHEMS	=	cholesterylhemisuccinate
CHL	=	cholesterol
cm	=	centimeter
cm^2	=	square centimeter
C_{max}	=	maximum concentration
CMC	=	critical micelle concentration
CTL	=	cytotoxic T lymphocyte
DA	=	deoxycholic acid
^{3}H	=	deuterium
DDAB	=	dimethyldioctadecylammonium bromide
DDPC	=	didecanoylphosphatidylcholine
DEPC	=	dielaidoyl phosphatidylcholine
DLPC	=	dilauroylphosphatidylcholine
DMPA	=	dimyristoylphasphatidic acid
DMPC	=	dimyristoylphosphatidylcholine

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

DMPE	=	dimyristoyl phosphatidylethanolamine
DMPG	=	dimyristoyl phosphatidylglycerol
DMRIE	=	1 ,2-dimyristoyl-3-dimethyl-hydroxyethyl ammonium bromide
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
DODAC	=	*N,N*-dimethyl-*N,N*-di-9-*cis*-octadecenylammonium
DODAP	=	1,2-dioleoyl-3-dimethylammonium propanediol
DOGS	=	dioetadecylamidoglycyl spermine
DOPC	=	dioleoylphosphatidylcholine
DOPE	=	dioleoyl phosphatidylethanolamine
DOPS	=	dioleoylphosphatidylserine
DOSPA	=	2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*
DOTAP	=	1 ,2-dioleoyl-3-trimethylammonium-propane
DOTMA	=	*N*-[1-(2,3-dioleoylox-y)propyl]-*N,N,N*-trimethylammonium chloride
Dox-LCL	=	doxorubicin entrapped within long-circulating liposomes
DPOPE	=	dipalmitoleoylphosphatidylethanolamine
DPPA	=	dipalmitoyl phosphatidic acid
DPPC	=	dipalmitoylphosphatidylcholine
DPPE	=	dipalmitoylphosphatidylethanolamine
DPPE	=	dipalmitoyl phosphatidylethanolamine
DPPG	=	dipalmitoylphosphatidylglycerol
DPSG	=	dipalmitoylglycerosuccinate
DSPA	=	distearoylphosphatidic acid
DSPC	=	distearoylphosphatidylcholine
DSPE	=	distearoyl phosphatidylethanolamine

DSPG	=	disaturated phosphatidylglycerol
EC_{50}	=	50% effective concentration
EE	=	entrapment efficiency
EPC	=	egg phosphatidylcholine
EPC	=	egg yolk phosphatidylcholine
EPG	=	egg phosphatidylglycerol
5-FU	=	5-fluorouracil .
F-THP	=	free 4′-*O*-tetrahydropyranyldoxorubicin
g	=	gram
G1 phase	=	first phase within interphase of cell cycle
G2/M phase	=	a period in the cell cycle to DNA damage checkpoint
GdDTPA-BMA	=	gadodiamide diethylenetriaminepentaacetic-acid-bis-methylamide
GM1	=	monosialoganglioside
>	=	greater than
h	=	hours
HBsAg	=	hepatitis B surface antigen
HCl	=	hydrochloric acid
HPI	=	hydrogenated phosphatidyl inositol
HPLC	=	high Performance Liquid Chromatography
HSPC	=	hydrogenated soy phosphatidylcholine
i.v.	=	intravenous
IC_{50}	=	50% inhibition concentration
Ig^{+}	=	immunoglobulin-positive
IL-2	=	interleukin 2
IR	=	infrared spectroscopy

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

K^+	=	potassium ion
kg	=	kilogram
KH_2PO_4	=	potassium dihydrogen phosphate
La^{3+}	=	lanthanum
L-AMB	=	liposomal amphotericin B
$\leq$	=	less than or equal to
log K	=	partition coefficient
L-THP	=	liposomal 4′-*O*-tetrahydropyranyldoxorubicin
mg/kg/d	=	milligrams per kilogram per day
µg/mL	=	microgram per milliliter
µl	=	microliter
µm	=	micrometer
µmol/ml	=	micromole per milliliter
min	=	minute
mM	=	millimolar
mm	=	millimeter
MS	=	mass spectrometry
mV	=	millivolt
MW	=	molecular weight
MWCO	=	molecular weigh cutoff
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
ng	=	nanogram
NH_3	=	ammonia
NH_4Cl	=	ammonium chloride
nm	=	nanometer

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

nM	=	nanomolar
NMD	=	nimodipine
OA	=	oleic acid
OAlc	=	oleyl alcohol
ODN	=	oligonucleotide
PA	=	palmitic acid
%	=	percent
PBS	=	phosphate buffered saline
PDA	=	photodiode array detector
PE	=	phosphatidylethanolamines
PEG	=	polyethylene glycol
PEG-PE	=	polyethyleneglycol/phosphatidyl-ethanolamine
PG	=	phosphatidylglycerol
pH	=	potential of hydrogen ion
PHC	=	*N*-palmitoyl homocysteine
PI	=	polydispersity index
PLGA	=	poly(lactic-co-glycolic acid)
PLN	=	popliteal lymph node
POPC	=	palmitoyloleoyl phosphatidylcholine
POPG	=	palmitoyloleoyl phosphatidylglycerol
PS	=	phosphatidylserine
^{1}H – NMR	=	proton nuclear magnetic resonance
$\Delta\Psi$	=	psi
PVA	=	polyvinyl alcohol
R.S.D.	=	relative standard deviation
RES	=	reticuloendothelial system

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

Rh	=	rhinacanthin
RIF	=	rifampicin
RNA	=	ribonucleic acid
S phase	=	part of the cell cycle in which DNA is replicated (synthesis phase)
SA	=	stearylamine
SCS	=	sodium cholesterol sulfate
SD	=	standard deviation
SDA	=	sabouraud dextrose agar
SDB	=	sabouraud dextrose broth
SEM	=	scanning electron microscope
Sit-G	=	β-sitosterol β-d-glucoside
SM	=	sphingomyelin
SNARE proteins	=	soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor
SPC	=	soy phosphatidylcholine
SPC	=	3-sn-phosphatidylcholine from soybean
suc-DOPE	=	*N*-succinyldioleoylphosphatidylethanolamine
$t_{1/2}$	=	half life
T80	=	tween 80
T_c	=	critical temperature
TFU	=	N_3-*O*-toluyl-fluorouracil
THP	=	4′-*O*-tetrahydropyranyldoxorubicin
TPGS	=	D-α-tocopheryl polyethylene glycol succinate
UV	=	ultraviolet
w/w	=	weight per weight

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Rhinacanthus nasutus is a local plant widely distributed in Southeast Asian region, South China, India and Taiwan. It is often used in several skin complaints such as ringworm, tinea, eczema, and other diseases. It has been reported to have several biological activities, for example, cytotoxicity, antiviral, antiproliferative, antitumor and antibacterial activities (Gotoh et al., 2004; Kongkathip et al., 2004; Prabakaran and Pugalvendhan, 2009; Puttarak et al., 2010; Sattar et al., 2004; Siripong et al., 2006a and 2006b). However, these properties were only studied in laboratory scale except antifungal activity that used as traditional medicines. In Thailand, *R. nasutus* was selected to be one of the effective herbs and can be used safety under the advisation of public health committee. It has been utilized in treatment of skin diseases caused by fungal infection (Bunyapraphatsara and Chokchaijareun, 2541). In India, it is used in poultice for the treatment of eczema and ringworm. In Malaysia, the leaves of *R. nasutus* are ground with benzoin and sulphur, and the paste is applied to the ringworm area. In Taiwan, Philippines and Thailand, it has been used in the treatment of skin diseases caused by fungi such as *Trichophyton rubrum*, *Trichophyton mentagophytes*, *Microsporum canis*, *Microsporum gypseum*, and etc (Gotoh et al., 2004; Sattar et al., 2004). Many compounds have been isolated from different parts of *R. nasutus* for example, flavonoids, steroids, triterpenoids, anthraquinones, lignans and naphthoquinone derivatives. A number of naphthoquinone compounds extracted from *R. nasutus* have been reported such as rhinacanthin-A, -B, -C, -D, -G, -H, -I, -J, -K, -L, -M, -N, -O, -P, -Q. They were also reported to have some interesting biological activities for examples, anticancer, anti-inflammation, antibacterial, antiviral, anti-allergic and antidermatophyte activities (Gotoh et al., 2004; Panichayupakaranant et al., 2009; Puttarak et al., 2010; Sendl, et al., 1996; Tewtrakul et al., 2009a and 2009b; Wu et al., 1998). Among those naphthoquinones rhinacanthin-C are the major naphthoquinone and possesses the highest

antifungal activity.

Unfortunately, utilization of rhinacanthin-C extracted from *R. nasutus* has been limited by its chemical and physical properties. Rhinacanthin-C is hydrophobic compounds, low aqueous solubility and not stable to light, acid and base conditions (Siripong et al., 2006b; Puttarak, 2010; ชุลีกร แซ่อุ่ย และคณะ, 2548). It was easily degradable when kept in unsuitable conditions. It has been known that, improvement of solubility and stability properties of nonpolar drugs may increase treatment efficiency in the treatment. Therefore, in order to improve these properties of rhinacanthin-C including antifungal activities, liposome formulation was taken into account.

Liposomes are drugs delivery systems (DDS), widely used with a number of drugs, because of their biphasic characters and can be used as carriers for both lipophilic and hydrophilic drugs. There are several advantages of utilization of liposome in drug encapsulation such as decrease toxicity of drugs as well as improve their therapeutic efficacy. Increasing stability of the encapsulated drug and reduces side effects of encapsulated to non target tissue were also reported. For the objective of DDS, liposomes having rigid lipid bilayer usually composed of phospholipids with saturated fatty acyl chains and cholesterol (Siripong et al., 2006b). Liposomes have often been used for encapsulation hydrophobic drugs, since hydrophobic materials can be encapsulated in between the lipid bilayer of the liposomes. Liposome was reported to use for reduction of side effects and toxicities of many drugs as well as improving bioavailability of the drugs. Moreover, liposomes are favored for drugs used for cancer treatment, since most of anticancer drugs accompany severe side effects. Liposomes have long circulating characteristics and are known to accumulate in interstitial spaces of tumor tissues of which blood vessels are highly permeable (Kohno et al., 1998). Additionally, liposome was reported to encapsulate hydrophobic/hydrophilic antibiotics such as amphotericin B (Seaman et al., 1995; Juster-Reicher et al., 2000; Lagler et al., 2006; Lanternier and Lortholary, 2008), albendazole (Panwar et al., 2010), gentamicin (Abraham and Walubo, 2005), nystatin (Mehta et al., 1987), cephalexin (Shafaa et al., 2008), amikacin, tobramycin, ciprofloxacin, ofloxacin, isoniazid, rifampicin, sparfloxacin, ampicillin, meropenem, metronidazol, polymyxin B, triclosan, vancomycin (Drulis-Kawa and Dorotkiewicz-Jach, 2010) and etc. to improve antimicrobial activity, pharmacokinetics and increase the clinical efficacy with decreased toxicity. This may be due to the liposomes

protect the drugs by isolating them from degrading enzymes or promoting their diffusion across the bacterial envelope. Liposomes are easily captured by macrophages and neutrophils, and they accumulate in the organs of reticuloendothelial systems, therefore, liposome-encapsulated antibiotics is useful in treatment infections caused by intracytoplasmic pathogens (Kohno et al., 1998).

Numerous of researches base on nanotechnology in drug delivery system, especially in cancer treatment were reported. Nanotechnology is purposely engineered and constructed systems that are measured in nanometer (nm) size (10^{-9}). Usually these particles range from a few nm to several hundred nm depending on their intended use. The several applications of nanotechnology to medicine, known as nanomedicine, include numerous architectural designs such as, dendrimers, micelles, nanospheres, nanocapsules, fullerenes and nanotubes, liposomes and etc (Haley and Frenkel, 2008; Shanmugam et al., 2009).

Many advantages of nanoparticles for drug delivery have been recognized. It improves the solubility of poorly water-soluble drugs, prolongs the half-life of drug in systemic circulation by reducing immunogenicity, releases drugs at a sustained rate or in an environmentally responsive manner and thus lowers the frequency of administration. The system could delivers drugs in a target manner to minimize systemic side effects, and delivers two or more drugs simultaneously for combination therapy to generate a synergistic effect and suppress drug resistance (Zhang et al., 2008). These properties can be used to overcome some of the limitations in hydrophobic compounds.

In this study liposomes were chosen to encapsulate rhinacanthin-C, which is a hydrophobic compound and easily decomposed in order to increase antifungal property as well as stabilities. Nanoliposomes containing rhinacanthin-C were prepared and investigated for their chemical and physical properties. The suitable formulation was described from sizes and entrapment efficiencies, as well as various lipid compositions. The best formulations of nanoliposome containing rhinacanthin-C were determined for their stability and antifungal activity in comparison with free rhinacanthin-C in solution.

1.2 Objectives

The objectives of this study are:

1. To prepare of liposomes containing rhinacanthin-C.
2. To determine physico-chemical properties of the liposome containing rhinacanthin-C.
3. To investigate antifungal activity of the liposome containing rhinacanthin-C in comparison with free rhinacanthin-C.
4. To study stability of rhinacanthin-C in liposome and liposome during storage period in different conditions.

CHAPTER 2

REVIEW OF LITERATURES

2.1 Botanical descriptions of *Rhinacanthus nasutus*

Rhinacanthus nasutus (Linn.) Kurz is one of local plants in the Acanthaceae family. It is a shrub widely distributed in some parts of the sub-continent India, South China and in the region of Southeast Asia including Thailand. *R. nasutus* or locally known in Thai as Thong-pan-chung, has been used in Thai folk remedy for treatment of several types of skin diseases (Gotoh et al., 2004; Kupradinun et al., 2009; Tunsaringkarn et al., 2009; Tewtrakul et al., 2009a).

R. nasutus is a shade loving perennial shrub and is found wild in the road bushes. The plant is a small slender, erect, branched plant with hairy shrub and 1-2 m in height. The leaves (Figure 2.1) are ovate to oblong, sparsely to densely pubescent, 4-10 cm in length, base rounded to cordate, apex short acuminate. The inflorescence is a spreading and violent in color, leafy, hairy panicle with the flowers usually in clusters. The calyx is green, hairy and about 5 mm long. The corolla-tube is greenish, slender, cylindrical and about 2 cm long. The flowers (Figure 2.1) are 2-lipped; the upper lip is white, erect, oblong or lancet like, 2-toothed at the apex and about 3 mm in both lengths and width; and the lower lip is broadly obovate, 1.1-1.3 cm in both measurements, 3-lobed and white with a few small brownish dots near the base. The fruit (capsule) is club-shaped and contains 4 seeds. During rainy season the plant grows vigorously whereas in summer, the aerial part mostly dries up and the root portion remains intact. It is highly susceptible to water logging and water stagnation for period of 1-2 days cause damage to the plant (Upendra Rao et al., 2010).

Figure 2.1 The pictures of *Rhinacanthus nasutus* (Linn.) Kurz with leaves and flowers.

2.2 Utilization of *R. nasutus* in traditional medicines

R. nasutus has long been used in Thai traditional medicines to treat several skin diseases such as pruritis, tinea versicolor and ringworm. Several parts of *R. nasutus* have been used in a number of Thai traditional recipes. They have been utilized as a main component in the recipes for the treatment of skin diseases caused by microbial infection as summarized in Table 2.1 (Farnsworth and Bunyapraphatsara, 1992).

Table 2.1 Applications of *R. nasutus* in disease treatment (นันทวัน บุณยะประภัศร, 2530)

Plant parts	Applications
Roots	Recipe 1: the roots (6-7 roots) are pounded with lemon and tamarinds juices then the mixtures are applied over the infected area for use to treat dermatitis and ringworm, anti-cancer, fever, snake venom. Recipe 2: the roots (6-7 roots) are pounded with match tips and vasaline then the mixture is applied over the infected area for use to treat dermatitis caused from fungi.
Whole plants	Grind whole plant (30 g) and mix with alcohol, then the paste is applied over the infected area for use to treat dermatitis, eruption, and frambesia.
Stalks	Grind 10-15 g of dried stalk and boil in water for drink for use to use as nourishment, pneumonosis and hair loss treatment.

Table 2.1 Applications of *R. nasutus* in disease treatment (continued)

Plant parts	Applications
Leaves	Recipe 1: fresh leaves (5-8 leaves) are pounded and mixed with an alcohol then the paste is applied over infected area for use to treat prickly heat, ringworm, abscess and dermatitis. Recipe 2: a tincture is prepared by soaking fresh leaves and roots in alcohol. The mixture is then applied over the infected area for use to treat prickly heat, ringworm, abscess and dermatitis.

There are also several recipes utilizing *R. nasutus* in tradition medicines reported in other countries for diseases treatments (Sendl et al., 1996; Wu et al., 1998a) as following:

- In Malaysia, roots were ground and mixed with vinegar or alcohol, leaves were mixed with benzoin and sulfur, and applied to the area infected by ringworm.
- In Indonesia, flowers and young leaves were rubbed with vinegar and lime and applied to the skin to treat prickly heat.
- In Taiwan, indigenous medical practitioners used roots and leaves pounded with vinegar or alcohol for the treatment of hepatitis, diabetes, hypertension and skin diseases.

Many reports have focused on antiproliferative activity of rhinacanthins against cancer cells in order to develop toward anticancer drug (Wu et al., 1988; Siripong et al., 1997; Gotoh et al., 2004; Siripong et al., 2006b; Siripong et al., 2006c; Kupradinun et al., 2009). Moreover, traditional medicines containing *R. nasutus* were well known as remedy for treatment of skin diseases caused by fungi (Darah and Jain, 2001; Sattar et al., 2004; Panichayupakaranant et al., 2009).

2.3 Chemical constituents of *R. nasutus*

A number of compounds have been isolated from different parts of *R. nasutus*. This plant is well known as a rich sources of flavonoids, steroids, triterpenoids, anthraquinones, lignans and

especially naphthoquinone analoges such as, rhinacanthin-A, -B, -C, -D, -G, -H, -I, -J, -K, -L, -M, -N, -O, -P, -Q and etc (Kodama et al., 1993; Sendl et al., 1996; Kerman et al., 1997; Wu et al., 1998a). Chemical structures of some naphthoquinone derivatives which were extracted from *R. nasutus* are shown in Table 2.2.

Rhinacanthin naphthoquinone compounds have been reported to have some interesting biological activities such as, antiviral, antiproliferative, antifungal, antibacterial activities and etc (Babula et al., 2009; Kernan et al., 1997; Panichayupakaranant et al., 2009; Puttarak et al., 2010; Siriwatanametanon et al., 2010). Rhinacanthin-C is one among naphthoquinone derivatives which could be isolated in the highest amount from whole plants and it showed good antifungal activity in laboratory studied. Moreover, amount of rhinacanthin-C was found the most when compare to the other components in the extracts of *R. nasutus* (Gotoh et al., 2004). In 2006, Siripong and co-worker extracted 1.5 kg of dried root of *R. nasutus* with chloroform affording rhinacanthin-C (1.99 g), rhinacanthin-N (0.73 g) and rhinacanthin-Q (0.58 g), respectively. The identification of those three active compounds was confirmed by spectroscopic data (UV, IR, ^{1}H- and ^{13}C-NMR and MS).

Tewtrakul et al. (2009b) used 200 g dried weight of *R. nasutus* leaves and isolated rhinacanthin naphthoquinone compounds with ethyl acetate (EtOAc). The EtOAc extract was concentrated to dryness giving 9.6 g of the crude extract. Rhinacanthin-C (450 mg), rhinacanthin-N (14 mg) and rhinacanthin-D (25 mg) were obtained after chromatographed over silica gel column and further purified on Sephadex LH-20 column. Their structures were elucidated by comparing the ^{1}H and ^{13}C-NMR spectral data with those reported (Sendl et al., 1996; Wu et al., 1998a).

Table 2.2 Chemical structures of rhinacanthin naphthoquinone compounds

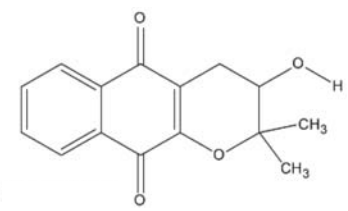
Chemicals name	**Structures**	**Plant parts (References)**
Rhinacanthin-A		Roots (Wu et al., 1988; Wu et al., 1998a; Wu et al., 1998b)

Table 2.2 Chemical structures of rhinacanthin naphthoquinone compounds (continued)

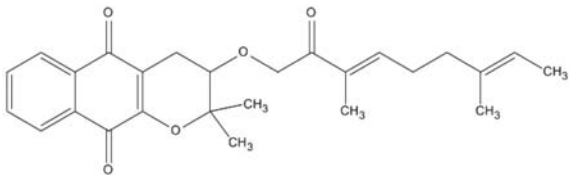
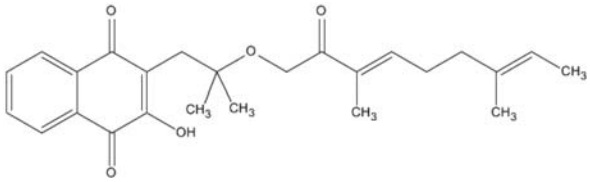
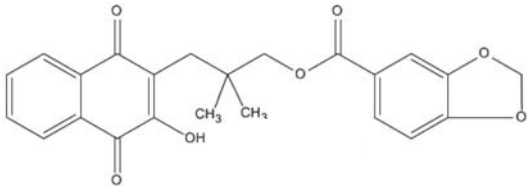
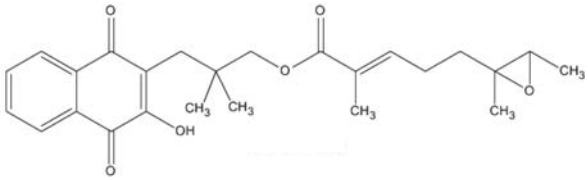
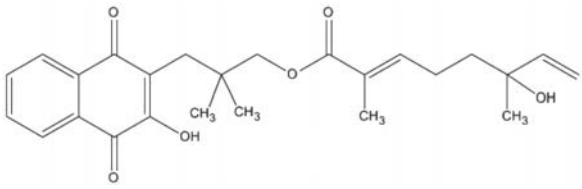
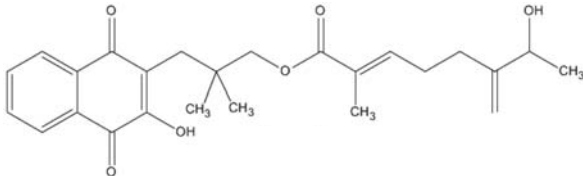
Chemicals name	Structures	Plant parts (References)
Rhinacanthin-B		Roots (Wu et al., 1988; Wu et al., 1998a; Wu et al., 1998b)
Rhinacanthin-C		Whole plants (Wu et al., 1988; Sendl et al., 1996; Siripong et al., 1997; Wu et al., 1998b)
Rhinacanthin-D		Roots (Wu et al., 1988; Sendl et al., 1996; Wu et al., 1998b)
Rhinacanthin-G or epoxyrhinacanthin-C		Roots (Wu et al., 1988; Siripong et al., 1997; Wu et al., 1998b)
Rhinacanthin-H		Roots (Wu et al., 1988; Wu et al., 1998b)
Rhinacanthin-I		Leaves and roots (Wu et al., 1988; Wu et al., 1998b)

Table 2.2 Chemical structure of rhinacanthin naphthoquinone compounds (continued)

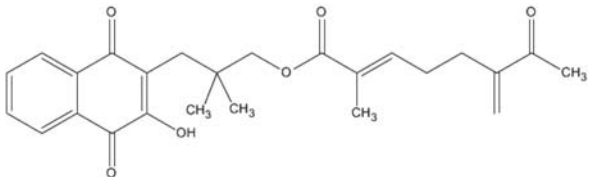
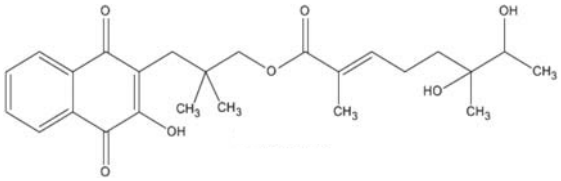
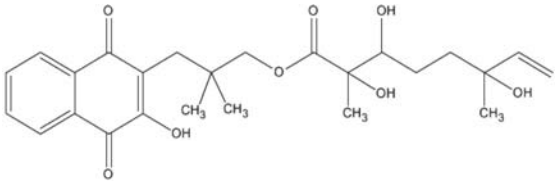
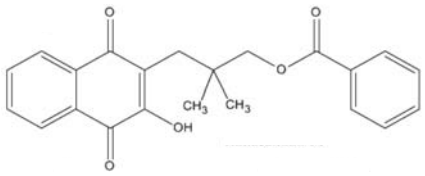
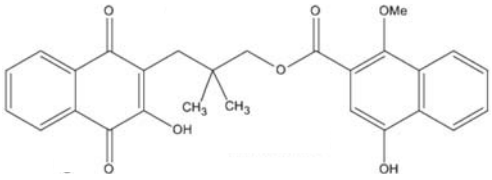
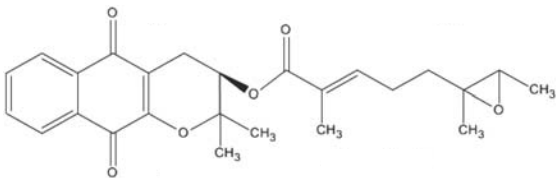
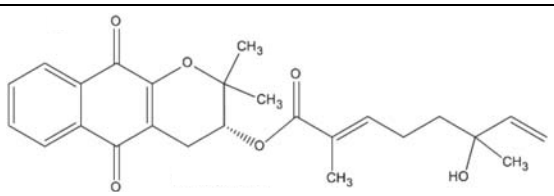
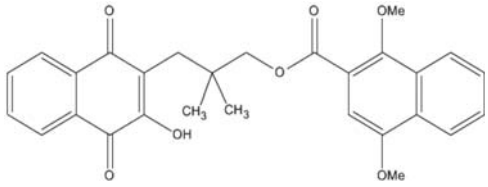
Chemicals	Structure	Plant parts (Reference)
Rhinacanthin-J		Leaves and roots (Wu et al., 1988; Wu et al., 1998b)
Rhinacanthin-K		Roots (Wu et al., 1988; Wu et al., 1998b)
Rhinacanthin-L		Roots (Wu et al., 1988; Wu et al., 1998b)
Rhinacanthin-M		Roots (Wu et al., 1988; Wu et al., 1998b)
Rhinacanthin-N		Leaves and roots (Wu et al., 1988; Wu et al., 1998b)
Rhinacanthin-O or epoxyrhinacanthin-B		Roots (Wu et al., 1988; Siripong et al., 1997; Wu et al., 1998b)
Rhinacanthin-P		Roots (Wu et al., 1988; Wu et al., 1998b)

Table 2.2 Chemical structure of rhinacanthin naphthoquinone compounds (continued)

Chemicals	Structure	Plant parts (Reference)
Rhinacanthin-Q		Roots (Wu et al., 1998b)

2.4 Biological activities of rhinacanthin-C

Rhinacanthin-C, the main naphthoquinone ester isolated from *R. nasutus* plants has been widely determined for it biological activities. In 1996, Sendl and co-worker isolated two naphthoquinones from *R. nasutus* (226 g), rhinacanthin-C (11.6 mg, 0.005%) and rhinacanthin-D (1.8 mg, 0.0007%) were found. Those two compounds exhibited inhibitory activity against cytomegalovirus (CMV), with EC_{50} values of 0.02 and 0.22 μg/mL, respectively.

Siripong and his team (2006d) extracted naphthoquinones ester from the dried roots of *R. nasutus*. (1.5 kg) with methanol using a Soxhlet apparatus. The isolated compounds were chromatographed on a silica gel column. Rhinacanthin-C (1.99 g), -D (1.5 mg), -G (7.3 mg), -O (12.8 mg), -M (2.4 mg), -N (620 mg), -Q (580 mg) and rhinacanthone (1.65 mg) were obtained. These isolated compounds were used to test antiproliferative activities against several cancer and a nontumorigenic cells and the result are summarized in Table 2.3.

Another study demonstrated that the extracts from *R. nasutus* have been found to have apoptotic activity against human cervical carcinoma (HeLaS3) tumor cells. The result showed that after the HeLaS3 cells were exposed to different concentrations of rhinacanthins-C, -N and -Q, the IC_{50} values of antiproliferative effects on HeLaS3 cells were 80, 65, 73 mM; 55, 45, 55 mM; and 1.5, 1.5, 5.0 mM for 24, 48 and 72 h after incubation period were obtained, respectively (Siripong et al., 2006c).

In 2004, Kongchai extracted rhinacanthin-C from *R. nasutus* leaf and its antifungal

activity against dermatophytes were examined. Minimum inhibitory concentration (MIC) values of rhinacanthin-C against *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum gypseum* were found to be 31.25, 31.25 and 125 µg/mL, respectively (Kongchai, 2004).

Table 2.3 Antiproliferative activity of isolated compounds from *R. nasutus* roots (Siripong et al., 2006d)

Cell culture	Antiproliferative activity (IC_{50}) of naphthoquinone compounds (µM)							
	Rh-C	**Rh-D**	**Rh-G**	**Rh-O**	**Rh-M**	**Rh-N**	**Rh-Q**	**Rhinacanthone**
KB	0.46	0.47	4.7	5.5	2.6	0.33	1.4	3.8
Hep-2	0.80	7.6	3.3	3.7	6.1	1.2	3.6	4.4
MCF-7	0.88	14.7	8.7	8.1	8.9	2.6	10.6	4.9
HepG2	0.41	1.9	1.2	6.5	3.8	0.37	3.0	4.9
HeLa	0.29	0.49	4.7	6.1	4.3	0.87	3.8	4.2
SiHa	0.49	6.6	18.8	7.4	39.1	3.9	6.1	2.9
C-32	9.8	14.7	16.4	30.7	37.0	39.1	8.4	2.1
LLC	0.98	6.1	6.1	8.2	54.4	5.4	8.3	2.9
Colon-26	0.44	2.3	1.5	6.6	5.4	1.1	1.1	3.4
P388	1.5	9.3	3.3	8.9	8.1	3.7	10.3	4.4
Vero	11.0	34.3	16.4	12.3	36.1	12.7	41.1	4.2

KB: human epidermoid carcinoma; Hep-2: human laryngeal carcinoma; MCF-7: human breast adenocacinoma; HepG2: human hepatocellular carcinoma; HeLa: human cervical carcinoma; SiHa: human squamous cervical carcinoma; C-32: haman amelanotic melanoma carcinoma; LLC: murine Lewis lung carcinoma; Colon-26: murine colon adenocarcinoma; P388: P-388 mouse lymphocytic leukemia; Vero: kidney of Afrcan green monkey; IC_{50}: 50% inhibition concentration; Rh: Rhinacanthin.

In 2007, Charoonratana found that the extracts from *R. nasutus* leaves (ethyl acetate extract) showed inhibition activities against *T. rubrum*, *T. mentagrophytes* and *M. gypseum* with MIC values are 31.2, 62.5 and 500.0 µg/mL, respectively. While pure rhinacanthin-C showed

good antidermatophyte activities than crude extract with MIC values of 15.6, 31.2 and 125.0 for *T. rubrum*, *T. mentagrophytes* and *M. gypseum* µg/mL, respectively.

In addition, rhinacanthin compounds have been reported to have other interesting biological activities such as, antibacterial (Puttarak et al., 2010) (Table 2.4), antiviral (Sendl et al., 1996; Kernan et al., 1997), immunomodulatory, antiplatelet (Wu et al., 1998a), hypotensive (วรรณดี แต้โสตถิกุล, 2528), anti-inflammatory (Tewtrakul et al., 2009b) and antiallergic activities (Tewtrakul et al., 2009a).

Table 2.4 Anti-bacterial activity of *R. nasutus* extract[a] (Sattar et al., 2004)

Concentration of extract (mg/mL)	**2.5**	**5**	**10**	**50**
Bacterial strains				
Gram-positive				
Bacillus cereus	7.9±0.5	8.0±0.4	9.0±0.4	11.9±1.8
Bacillus globigii	7.7±0.2	7.9±0.1	8.5±0.1	12.3 ±2.1
Bacillus subtilis	7.4±0.1	8.0±0.5	8.4 ±0.5	10.1±1.6
Staphylococcus aureus	--	--	7.4±0.2	9.3±0.5
Gram-negative				
Proteus morgani	--	--	--	--
Proteus mirabilis	--	--	--	--
Salmonella typhi	--	--	--	--
Pseudomonas aeruginosa	--	--	--	--
Escherichia coli	--	--	--	--

[a,] Values presented are zone of inhibition (mm) in response to different dosages of extract and are mean±SD of three independent observations.

--, No anti-bacterial activity observed.

The mechanism of antidermatophyte property was suggested by Darah and Jain (2001) that the rhinacanthins may interact with cell wall of the dermatophyte which subsequently leading to the formation of cytopathological and membrane structural degeneration and finally leading to

cell lysis and death. Moreover, rhinacanthins was found to induce suppression of cell division of cancer cell at G2/M phase. This result suggested that rhinacanthins could inhibit cell division cycle during the G2/M phase and undergoes apoptosis (Siripong et al., 2006b). The cell cycle control mechanism is the major regulatory mechanism of cell growth. Many cytotoxic agents including rhinacanthin and/or DNA damaging agents could arrest the cell cycle at the G1, S or G2/M phase and then induce apoptotic cell death (Wu et al., 2006).

2.5 Limitations of using rhinacanthin-C

Since rhinacanthin-C is a hydrophobic compound with log K about 1.73 ± 0.154. It is poor soluble in aqueous medium (Siripong et al., 2006b; Charoonratana, 2007). It is known that the solubility problems are major problems that may affect on dissolution rate and bioavailability of poorly water-soluble drugs (Serajuddin, 1999). The latter are difficult to formulate as a result of their lack of significant solubility in water. However, when a molecule needs to penetrate through biological membrane to be absorbed, the molecule generally must possesses some hydrophobic or lipophilic characteristics (Gulati et al., 1998; Merisko-Liversidge and Liversidge, 2008).

Rhinacanthin-C was known to be unstable when exposed to light, acid, base and oxidation conditions (Puttarak et al., 2010). It has been found that when storing the extract from *R. nasutus* in the presence of light at room temperature (30 ± 2 °C) for 4 months, rhinacanthin-C content of the rhinacanthins-rich *R. nasutus* (RRn) extract reduced from 65.40 ± 0.56 to 41.26 ± 0.53 %w/w. When storing the extract in acid condition (pH 5.5), rhinacanthin-C contents reduced from 64.72 ± 0.66 to 43.26 ± 0.59 %w/w. When storing in the buffer pH 7.0, rhinacanthin-C contents reduced from 64.85 ± 0.65 to 42.21 ± 0.58 %w/w and when storing in base condition (pH 8.0), rhinacanthin-C contents reduced from 64.88 ± 0.66 to 39.12 ± 0.60 %w/w.

In previous study, rhinacanthin-C was used to prepare inclusion complexes with β-cyclodextrin and found that the inclusion complexes could enhance water solubility, control release and stability of rhinacanthin-C. Moreover, the inclusion complexes gave significantly

increase in antibacterial and antioxidant activities higher than of pure rhinacanthin-C. However, its antifungal activity did not improve (Ovatlarnporn et al., 2008). Therefore, to improve antifungal activity of rhinacanthin-C was thought to be prepared in liposome formulation.

2.6 Liposomes

Liposomes are drug delivery vehicles which first described by Bangham in 1965 while studying membrane model system of biological membranes (Bangham et al., 1965a; Bangham et al., 1965b). They compose of amphiphilic phospholipids and cholesterol that self-associate into bilayers encapsulating an aqueous interior. These may be formulated into small structures (80 - 100 nm in size) that could encapsulate either hydrophilic drugs in the aqueous interior or hydrophobic drugs within the lipid membrane, Figure 2.2 (Fahmy et al., 2005; Zamboni, 2005).

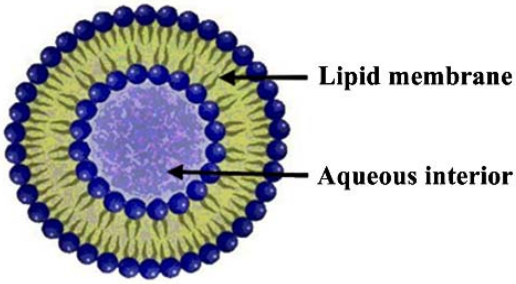


Figure 2.2 Schematic of liposome

(From: http://www.uni-magdeburg.de/imos/mea_sen/img/pictures/Lipo.jpg)

Liposomes are microscopic vesicles composed of a phospholipid bilayer that are capable of encapsulating several types of active drugs. The drug could be encapsulated in the core or in between the bilayer of the liposome, dependent on the characteristics of the drug and the encapsulation process. In general, water-soluble drugs could be encapsulated within the central aqueous core, whereas lipid-soluble drugs are incorporated directly in between the lipid membrane. The drug molecules are located differently in the liposomal environment and exhibit different entrapment and release properties, depending upon their solubility and partitioning characteristics (Figure 2.3). Liposomes can alter both the tissue distribution and the rate of clearance of the drug by making the drug take on the pharmacokinetic characteristics of the

carrier depend on the physiochemical characteristics of the liposomes, such as size, surface charge, membrane lipid packing, steric stabilization, dose, and route of administration. The tumor, liver, and spleen are the primary sites of accumulation of conventional liposomes compared with nonliposomal formulations. The development of STEALTH liposomes was based on the discovery that incorporation of polyethylene glycol (PEG) - lipids into liposomes yields preparations with superior tumor delivery compared with conventional liposomes which composed of natural phospholipids. Incorporation of PEG-lipids causes the liposome to remain in the blood circulation for extended periods of time (i.e., $t_{1/2} > 40$ hours) and distribute through an organism relatively evenly with most of the dose remaining in the central compartment (i.e., the blood) and only 10% to 15% of the dose being delivered to the liver. This is a significant improvement over conventional liposomes where typically 80% to 90% of the liposome deposits in the liver. The clearance of conventional liposomes has been proposed to occur by uptake of the liposomes by the reticulo-endothelial system (RES). The mononuclear phagocyte system uptake of liposomes results in their rapid removal from the blood and accumulation in tissues involved in the RES, such as the liver and spleen. Uptake by the RES usually results in irreversible sequestering of the encapsulated drug in the RES, where it can be degraded. In addition, the uptake of the liposomes by the RES may result in acute impairment of the mononuclear phagocyte system and toxicity. Sterically stabilized liposomes, such as STEALTH liposomes, prolong the duration of exposure of the encapsulated liposome in the systemic circulation. The presence of the PEG coating on the outside of the liposome does not prevent uptake by the reticuloendothelial system, but simply reduces the rate of uptake. The exact mechanism by which steric stabilization of liposomes decreases the rate of uptake by the reticuloendothelial system is unclear (Zamboni, 2005).

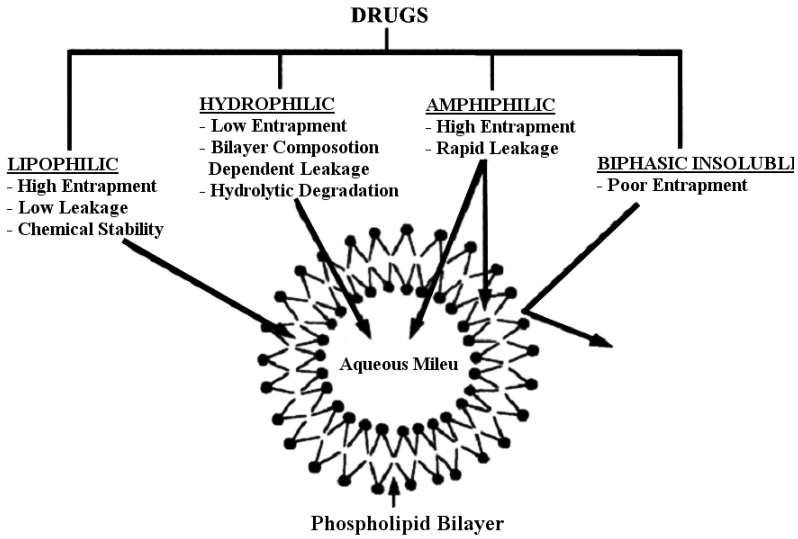


Figure 2.3 Types of drugs and site of their incorporation into liposomal vesicle (From: Gulati et al., 1998).

As their structures have some similarities to the biological cell membrane may lead to acceptance of a liposome into a cell. Liposomes are consisting of lipids and phospholipids. Each phospholipid has a polar hydrophilic "head group" and two hydrophobic "tails". When phospholipid molecules are hydrated under low-shear conditions, they spontaneously arrange themselves in sheets with their heads up and tails down. These sheets then join tails to tails and form a bilayer membrane that encloses water and if added water soluble compounds (e.g., pharmaceuticals and larger biomolecules) in the center of the sphere. If liposomes come into contact with phospholipid cell membranes, the liposome membrane fuses with the cell membrane facilitating the entry of the encapsulated drug into the interior of the cell as shown in Figure 2.4 (Siekmeier and Scheuch, 2008).

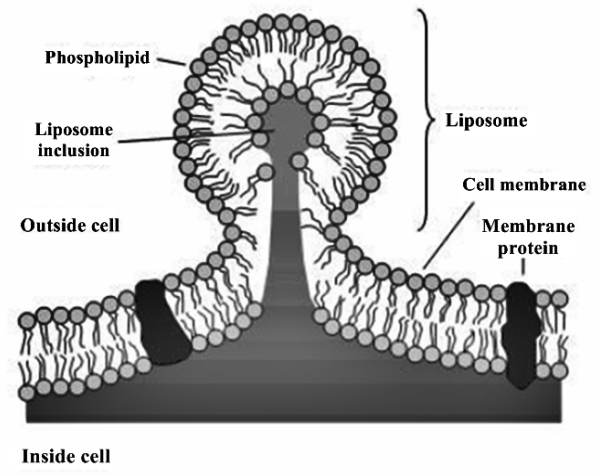


Figure 2.4 Picture showed the fusion of liposome when their contact with biological cell membrane (From: Siekmeier and Scheuch, 2008).

2.7 Structural components of liposomes

Liposomes are uncomplicated vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous media, giving rise to a population of vesicle which may range in size from nanometers to microns in diameter. During liposomes construction they could entrap quantities of materials both within their aqueous compartment and within the membrane. The value of liposomes as model membrane systems derives from the fact that liposomes can be constructed to contain bilayer structure which in a principal identical to the lipid portion of natural cell membranes. The similarity between liposome and natural membranes can be promoted by using proper chemical modification of the liposome membrane. Therefore, they can be exploited in many areas such as drug targeting or immune modulation, both *in vivo* and *in vitro*. They could be used to mimic the behavior of natural membranes, and also be degraded by the same pathways, hence, makes them a very safe and efficacious vehicle for medical applications. Alternatively, liposomes can be constructed by using entirely artificial components which were chosen for their improved chemical properties (Roger, 1989). Liposome features are strictly related to chemical properties of the phospholipids used for their preparation. Since, lipids can modify biodistribution, surface charge, permeability, and release and clearance of liposomal drug delivery (Calvagno et al., 2007). Improving chemical property of liposome is

usually for therapeutic treatment of various cancer diseases. In particular, pH-sensitive liposomes containing unsaturated fatty acids, i.e. oleic acid or linoleic acid, can be used both to obtain fusogenic vesicles at low pH values (≤ 6.5) thus improving the intracellular drug entrance and increasing the percutaneous drug passage when an anticancer topical treatment is possible. While, the presence of polyethylene glycol moieties on the surface of liposomes provides long circulating properties, improved stability, drug defence from metabolic degradation/inactivation and increased intracellular uptake (Calvagno et al., 2007). Løkling and co-worker (2004) prepared pH-sensitive liposomes which were found to be stable in blood and selectively release the encapsulated paramagnetic agent when exposed to lower pH in the target tissue. The pH-sensitive liposomes system was composed dipalmitoylphosphatidylethanolamine/palmitic acid (DPPE/PA) liposomal GdDTPA-BMA, which was shown to be unstable in blood. This formulation was modified to improve its stability by incorporation of cholesterol into the DPPE/PA liposomes and found to have significantly increased their stability in blood, but the pH sensitivity was diminished. Polyethylene glycol modified DPPE/PA liposomes were pH-insensitive in buffer, and unstable in blood. However, exchanging PA with the double-chained amphiphile dipalmitoylglycerosuccinate (DPSG) yielded liposomes with improved properties. DPPE/DPSG liposomal GdDTPA-BMA was stable in blood at physiological pH, and displayed marked pH sensitivity. The pH sensitivity was not diminished after preincubation in blood.

Since liposomes are similar for biological membranes. A fundamental feature of cell membranes is the organization of lipids into bilayers, providing permeability barriers between exterior and interior compartments. A large group of biological membrane lipids that spontaneously form bilayers in water are the phospholipids. A class of phospholipids commonly used to construct liposomes for drug delivery is phosphatidylcholine. The ability of phospholipids to form a bi-layer structure is because of their amphipathic character resulting from the presence of a polar or hydrophilic (water-attracting) head-group region and a non-polar, lipophilic (water-repellent) tail [Figure 2.5 (a)]. Normally, the hydrophilic head groups orientate toward the aqueous phase and the lipophilic tails orientate to each other in the presence of water to form bilayer membrane leaflets [Figure 2.5(b)], followed by transformation into liposomes [Figure 2.5 (c)]. A single bilayer component of liposome of typically ~5 nm thickness consist of neatly

arranged individual lipid molecules with their hydrophobic tails facing each other and their hydrophilic head groups facing toward the internal and external aqueous medium [Figure 2.5 (d)]. Apart from the structural characteristics of the liposomes, their properties and functionality are usually defined by their size and the composition of the four distinct regions which are (1) lipid-soluble material, (2) intravesicular medium, (3) extravascular medium and (4) membrane-anchored/conjugated material (Edwards and Baeumner, 2006). Various natural and synthetic lipid molecules are available for the preparation of bilayer membranes and liposomes (De Leeuw et al., 2009) such as, egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG), soy phosphatidylcholine (SPC), hydrogenated soy phosphatidylcholine (HSPC), sphingomyelin (SM) and etc. Natural lipid and synthetic lipid molecules for examples, didecanoylphosphatidylcholine (DDPC), dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), palmitoyloleoyl phosphatidylcholine (POPC) and dielaidoyl phosphatidylcholine (DEPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoylphosphatidylglycerol (DPPG), disaturated phosphatidylglycerol (DSPG) and palmitoyloleoyl phosphatidylglycerol (POPG), dimyristoylphasphatidic acid (DMPA), dipalmitoyl phosphatidic acid (DPPA) and distearoylphosphatidic acid (DSPA), dimyristoyl phosphatidylethanolamine (DMPE), dipalmitoyl phosphatidylethanolamine (DPPE), distearoyl phosphatidylethanolamine (DSPE) and dioleoyl phosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS) and etc. were also utilized in liposome formulations (http://nofamerica.net/store/index.php?target=categories&category_id=216).

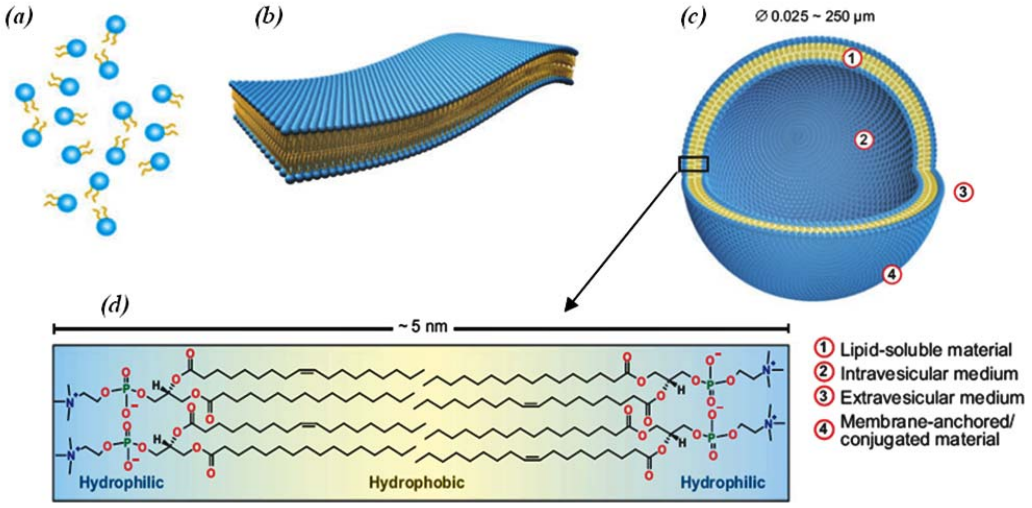


Figure 2.5 The fundamental process to form liposome from phospholipid molecules (a) phospholipid molecules (b) bilayer of phospholipid molecules (c) phospholipid vesicle (d) single bilayer of ~5 nm thickness (From: Jesorka and Orwar, 2008).

2.7.1 Chemical constituents of liposome

2.7.1.1 Phospholipids

Phospholipids are known to be the major structural components of biological membrane such as a cell membrane. The most common phospholipid used in liposome formulation is naturally occurring phospholipids, phosphatidylcholines. They are amphipatic molecules in which contain a hydrophilic polar head group, a glycerol bridge and a pair of hydrophobic acyl hydrocarbon chains (Figure 2.6). Phosphatidyl cholines, also known as 'lecithin' can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean, but less readily from bovine heart and spinal cord. They are often used as the principal lipid component in liposomes for a wide range of applications due to their low cost relative to other phospholipids, and their natural charge and chemical inertness. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while lecitin from mammalian sources contains a higher proportion of fully saturated chains (Roger, 1989; http://www.azayatherapeutics.com/faq/questions.php).

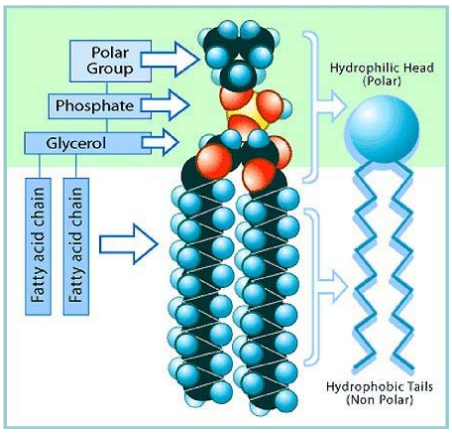


Figure 2.6 chemical structures of phospholipid molecules

(From: http://www.bioteach.ubc.ca/Bio-industry/Inex/).

A phospholipid is a unique molecule that possesses a polar head group, e.g. a charged phosphate with a polar moiety (choline) which soluble in water and a hydrophobic or nonpolar fatty acid chains that are water insoluble. Since, oil and water are immisible, therefore, the oily tails of the phospholipid align to form a layer of oil, while their polar heads align to the outside or inner core forming a shell or lipid bilayer (Figure 2.2). The shell is solvated outside by water and the inner core is lined with polar heads forming an interior aqueous core.

These small molecules are held together by non-covalent interactions; hydrophobic interactions (oil characteristics), hydrogen bonds or hydrophilic interactions (water soluble characteristics), ionic and van der waals interactions (electrostatic charges or dipole moments). These interactions form the lipid bilayer that can open and close to release the contents of the inner aqueous core to the extra cellular media (leakage of inner core material). Thus, drugs encapsulated in the inner aqueous core will rapidly escape through momentary cracks or openings in the lipid bilayer, exchanging aqueous media from the outside with the inner core where the aqueous soluble drug is encapsulated (Roger, 1989).

The shape of phospholipid molecules has also effect on the structure of phospholipid arrangement. Lasic (1998) described that a symmetry of lipid self assembly and liquid-crystalline phase formation shows strong dependence on the molecular shape of the amphiphile. Amphiphiles molecules include soaps, detergents and polar lipids (lecithins, kephalins) and in

aqueous mixtures, these molecules are able to form several different phases. At high concentrations, they form liquid-crystalline phases (characterized by long-range order) that can, upon dilution in excess water, be dispersed into relatively stable colloidal particles. The most frequently formed liquid-crystalline phases are the lamellar and, to a lesser extent, hexagonal and cubic phases. These macroscopic phases can be dispersed into colloidal particles known as liposomes, hexasomes and cubosomes, respectively; the particles retain the short-range symmetry of their original parent phase. Figure 2.7 shows the influence of molecular geometry on the symmetry of the phases formed. Cone-like molecules tend to pack into structures with high radii of curvature and inverse-cone-like molecules form structures with large negative curvatures; cylindrical molecules organize into flat bilayers. Combining cone- and inverse-cone-like molecules can result in the formation of a flat bilayer. Such a bilayer can, however, be unstable and undergo a phase transition into a normal or inverse-micellar structure.

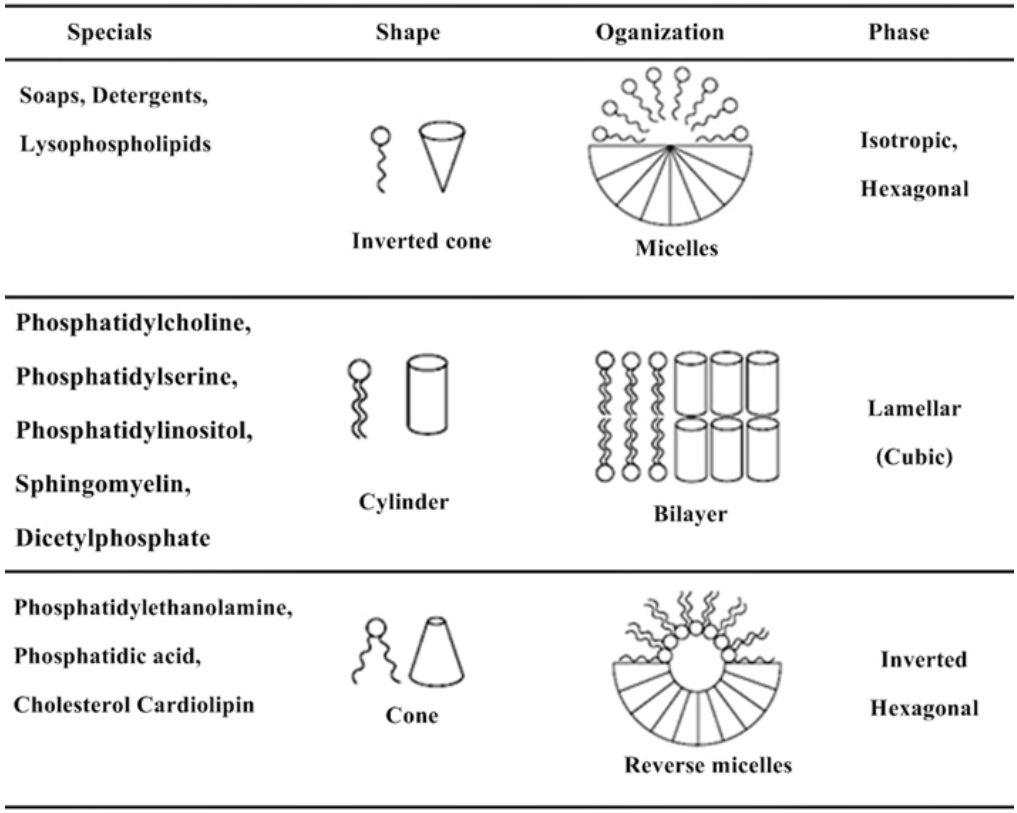


Figure 2.7 The different shapes of phospholipids molecules effected on arrangement (From: Lasic, 1998).

2.7.1.2 Cholesterol

Incorporation of sterols in liposome bilayer caused major changes in the preparation of these membranes. Cholesterol by itself does not form a bilayer structure. However, cholesterol acts as a fluidity buffer below the phase transition temperature. It makes the membrane less ordered and slightly more permeable, while above the phase transition temperature it makes the membrane more ordered and stable. It can be incorporated into phospholipid membranes at high concentration upto 1:1 or even 2:1 molar ratios of cholesterol to phosphatidylcholine [Figure 2.8 (a)].

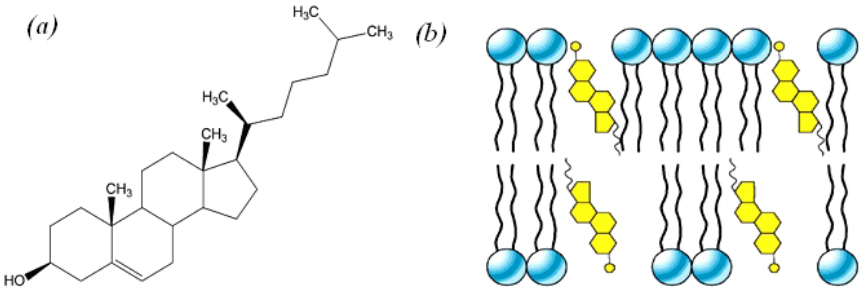


Figure 2.8 (*a*) structure of cholesterol and (*b*) Insertion of cholesterol inner lipid bilayer (From: http://www.etseq.urv.es/dinamic/catala/places/liposome_lit_review.doc).

For mechanism of cholesterol acting as a fluidity buffer, cholesterol incorporation increases the separation between the choline head groups [Figure 2.8 (b)] and eliminates the normal electrostatic and hydrogen-bonding interactions, thus pushing the phospholipids apart making the layer less ordered at lower temperatures. However, at the higher concentrations of cholesterol, the membrane area occupied by the combination of acyl chains and cholesterol is greater than (or equal to) that taken by phosphocholine head group. This difference in area retards chain tilt. Above the transition temperature, the reduction in the freedom of the acyl chains causes the membrane to remain condensed and rigidized, with a reduction in area through closer packing and resultant decrease in fluid (http://www.iqm.unicamp.br/graduacao.pdf).

2.7.1.3 Amphiphiles

Amphiphiles are chemical compounds possessing both hydrophilic and hydrophobic properties. Such a compound is called amphiphilic or amphipathic. It may have both polar and

nonpolar parts in its structure. Amphiphiles compound usually be added during liposome preparation, to enhance entrapment efficiency of active compound or drug interior liposome structure. There are several examples of molecule that present amphiphilic properties such as hydrocarbon based surfactants. Their polar region can be either ionic, or non-ionic. Some typical members of this group are sodium dodecyl sulfate (anionic), benzalkonium chloride (cationic), cocamidopropyl betaine (zwitterionic) and octanol (long chain alcohol, non-ionic). Many biological compounds are amphiphilic for example: phospholipids, cholesterol, glycolipids, fatty acids, bile acids, saponins, and etc (http://en.wikipedia.org/wiki/Amphiphile). Two groups of amphiphiles are normally utilized in liposome preparation. The amphiphile will result in positive charge on the surface region of liposome such as stearylamine. Another group is a group of amphiphiles which will give negative charge on surface region of liposome such as phosphatidylserine, dicetyl phosphate, phosphatidic acid, deoxycholic acid (อรัญญา มโนสร้อย, 2538). Manosroi and co-worker (2004) prepared topical amphotericin B (AmB) liposome formulations to investigate the stability and transdermal absorption of amphotericin B. Their liposome formulations composed of various lipids as well as hydrogenated soya phosphatidylcholine/cholesterol/charged lipid in a molar ratios of 1:1:0, 7:2:0, 7:2:1(-) and 7:2:1(+). The lipids which were used in the formulations were dicetyl phosphate gave negative charge (-) or stearylamine gave positive charge (+). The amount of AmB in each formulation was 0.05mg/mg lipid. The result shown that, the positive liposome (7:2:1(+), AmB) was displayed the slowest degradation at 30 ˚C, with shelf life of ~1 year. In comparison, the shelf lives of AmB in solution and powder were 4 and 14 days, respectively. In addition, AmB in positive liposomes seemed to demonstrate the highest flux in stratum corneum (58 ng/cm^2/h), while the highest flux in viable epidermis (23 ng/cm^2/h) was observed in negative liposomes. AmB entrapped in charged liposomes (+ or -) showed sustained skin absorption than the neutral liposome. Lian and Ho (2001) explained that the surface charge of liposomes may bear a negative, neutral, or positive charge on their surface depended on the head group composition of the lipid and pH. The nature and density of charge on the surface of the liposomes influences stability, kinetics, and extent of biodistribution, as well as interaction with and uptake of liposomes by target cells. Liposomes with a neutral surface charge have a lower tendency to be cleared by cells of the reticuloendothelial system (RES) after systemic administration and the

highest tendency to aggregate. Although negatively charged liposomes could reduce aggregation and have increased stability in suspension, their nonspecific cellular uptake is increased *in vivo*. Negatively charged liposomes containing phosphatidylserine (PS) or phosphatidylglycerol (PG) were observed to be endocytosed at a faster rate and to a greater extent than neutral liposomes. Negative surface charge is recognized by receptors found on a variety of cells, including macrophages. Inclusion of some glycolipids, such as the ganglioside GM1 or phosphotidylinositol (PI), inhibits uptake by macrophages and RES cells and results in longer circulation times. It has been suggested that a small amount of negatively charged lipids stabilize neutral liposomes against an aggregation-dependent uptake mechanism. Positively charged, cationic liposomes, often used as a DNA condensation reagent for intracellular DNA delivery in gene therapy, have a high tendency to interact with serum proteins; this interaction results in enhanced uptake by the RES and eventual clearance by the lung, liver, or spleen. This mechanism of RES clearance partly explains the low in vivo transfection efficiency. Other factors, including DNA instability, immune-mediated clearance, inflammatory response, and tissue accessibility may also contribute to low transfection efficiency in animals. In fact, high doses of positively charged liposomes have been shown to produce varying degrees of tissue inflammation. The surface of the liposome membrane can be modified to reduce aggregation and avoid recognition by RES using hydrophilic polymers. This strategy is often referred to as surface hydration or steric modification. Surface modification is often done by incorporating gangliosides, such as GM1 or lipids that are chemically conjugated to hygroscopic or hydrophilic polymers, usually poly(ethyleneglycol). Miller and co-worker (1998) prepared the large unilamellar liposomes composed of 1,2-dioleolyl-*sn*-glycero-3-phosphatidylcholine with and without the addition of either a positively charged lipid, 1,2-dioleoyl-3-dimethylammonium propanediol (DODAP), or a negatively charged lipid, 1,2-dioleolyl-*sn*-glycero-3-phosphatidylserine. The anionic PEG_{2000}-PE or a neutral PEG lipid (5 mol %) of the same molecular weight were added in some experiments. The results displayed in a human ovarian carcinoma cell line (Mura et al., 2007) were found to endocytose positively charged liposomes to a greater extent than either neutral or negatively charged liposomes. The both cationic and anionic liposomes showed greater the extent of liposome interaction with a murine derived mononuclear macrophage cell line (J774) than for neutral liposomes. The greater uptake of positively charged liposomes by HeLa cells was also

observed with sterically stabilized liposomes (PEG liposomes), whereas negatively charged PEG_{2000}-PE liposomes were hardly endocytosed by the HeLa cells. Incorporation of a neutral PEG lipid into liposomes permits the independent variation of liposome steric and electrostatic effects in a manner that may allow interactions with cells of the reticuloendothelial system to be minimized, yet permit strong interactions between liposomes and proliferating cells.

Abraham and Walubo (2005) prepared liposome encapsulated gentamicin by the hydration method and also surface charge was varied. Neutral, negative and positive liposomes were prepared by using phosphatidyl choline and cholesterol in a molar ratio of 9.7 : 6.9 for neutral liposome, while negative and positive liposomes added dicetyl phosphate (negative charge) or stearylamine (positive charge) in a molar ratio of 5 : 1 : 0.5. The liposomes were injected in intraperitoneal of rat and studied the effect of surface charge to distrbution in the rat liver, brain, lungs and kidneys. The observations in this study indicated that distribution of gentamicin with higher concentrations in the brain and liver, while concentrations were lower in the kidney. The average concentrations of gentamicin in the liver and the brain were highest with positive liposomes, while, gentamicin concentrations in the kidneys and lungs were not influenced by surface charge of the liposomes. It has been shown that encapsulation efficiency for gentamicin is better with charged than neutral lipids, indicating that surface chargemay enhance encapsulation of gentamicin. Due to charged lipids may increase the volume of entrapped material in the liposome due to repulsion between similar charges on the lipid bilayers. The results also indicated that charged liposomes were more effective drug carriers to the liver than neutral liposomes; this suggests that liposomal surface charge is a major determinant in hepatic uptake of liposomes.

2.8 Classification of liposomes

The types of liposome could be classified on the basis of their applications, compositions or physical properties as well as size, lamella and surface charge. Sharma and Sharma (1997) classified the types of liposome in two terms; depend on compositions and mechanism of intracellular delivery and size/number of bilayers (lamellae) of liposomes.

2.8.1 Liposome classified base on compositions and mechanism of intracellular delivery

Generally, liposomes compose of natural and/or synthetic lipids (phospholipids and sphingolipids). They may be also containing other bilayer constituents such as cholesterol and hydrophilic polymer conjugated lipids. The net physicochemical properties of the lipids composing the liposomes, such as membrane fluidity, charge density, steric hindrance, and permeability, determine liposomes interactions with blood components and other tissues after systemic administration. The nature and extent of liposome-cell interaction in turn determines the mode of intracellular delivery of drugs. Thus, the predominant mechanism of intracellular delivery of drugs by liposomes may mainly depend on their composition. Therefore, liposomes can be classified in terms of composition and mechanism of intracellular delivery into five subtypes as following:

2.8.1.1 Conventional liposomes

Conventional liposome (Miller et al., 1998) is always composed of neutral and/or negatively charged phospholipids and cholesterol. They have been used to coat active drug and diffused into cell by endocytosis. The contents of active drug ultimately delivered to lysosomes, drug or prodrug encapsulated in liposome was delivered into the inner cell (Sharma and Sharma, 1997). On the contrary, if they do not diffuse from endosome, it will be rapid and saturable uptaken by reticuloendothelial systems (RES) such as liver and spleen (Figure 2.9). They have short circulation half-life and dose-dependent pharmacokinetics (Lipke and Ovalle, 1998).

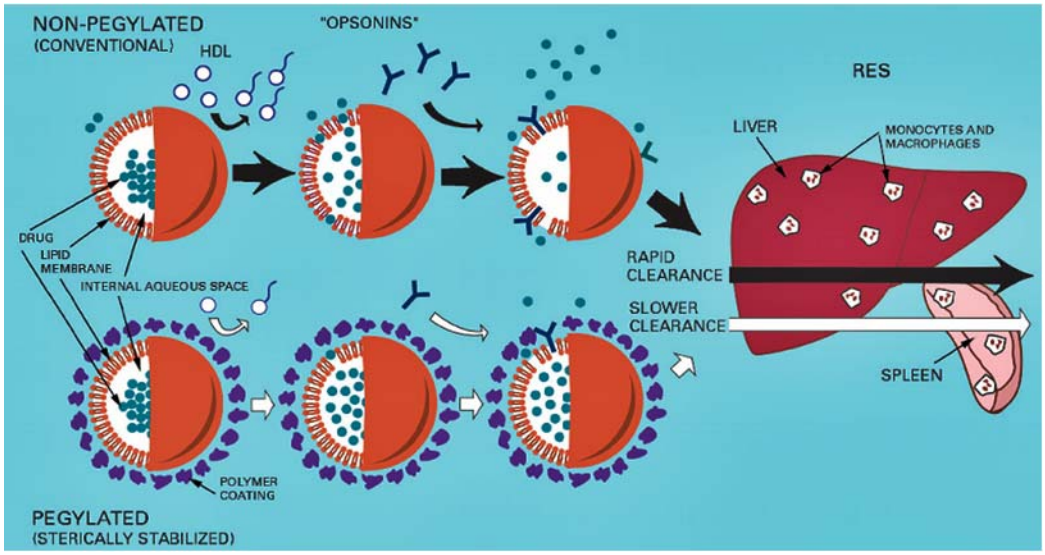


Figure 2.9 Clearance of pegylated (sterically stabilized) and nonpegylated (conventional) liposomes via the reticuloendothelial system (RES) in the liver and spleen. Nonpegylated liposomes undergo greater breakdown in blood and more rapid clearance via the RES compared with pegylated liposomes (Zamboni, 2005).

Conventional liposomes tend to be trapped by RES and liposomes having specific ligands also have a chance to be trapped by reticuroendothelial systems before encountering the target (Oku, 1999). For example, Immordino and co-workers (2003) compared formulation, stability, biodistribution and pharmacokinetics of docetaxel in conventional and PEGylated liposomes. Both formulations of docetaxel in conventional liposomes and PEGylated liposomes were composed of egg yolk phosphatidylcholine (EPC), phosphatidylglycerol (PG) and cholesterol (CHL) in a molar ratio of 9 : 1 : 2, and EPC/PG/DSPE-PEG_{2000}/CHOL (DSPE; distearoylphosphatidylethanolamine and PEG_{2000}; polyethylene glycol of molecular weight 2,000) in a molar ratio of 9 : 1 : 2 : 0.7, respectively and containing 6 mol% of docetaxel. Both conventional and PEGylated docetaxel liposomes were stable at 4 °C after 15 days, whereas in the presence of serum at 37 °C they were less stable. This may be due to the vesicles were in contact with serum at body temperature, docetaxel rapidly partitions between lipids and serum proteins, to which it is usually strongly bound. Pharmacokinetics and biodistribution were evaluated in Balb/c mice (an albino mice strain useful in laboratory-bred) after i.v. injection of docetaxel labeled with ^{14}C, formulated in Tween 80 or in conventional liposome labeled with ^{3}H or PEGylated liposomes. The half-life of docetaxel rose from 52.3 min to 260 min for conventional

docetaxel liposomes and to 665 min for PEGylated docetaxel liposomes. Their cytotoxicity against HT-29 and Igrov1 cell lines were evaluated and the results are summarized in Table 2.5. The lower cytotoxicity of PEGylated than conventional liposomes was probably related to the steric effect of PEG chains, which may delay internalisation of the drug into cells by masking the negative liposomal charge: negatively charged liposomes generally exhibit stronger binding than do neutral ones because of the existence of a membrane receptor recognizing negatively charged particles.

Prentice et al (1997) compared the safety of liposomal amphotericin B (L-AMB) with conventional amphotericin B (c-AMB) for the treatment of pyrexia of unknown origin in neutropenic patients. The result indicated that liposome formulation containing 1 or 3 mg/kg/d of amphotericin B were significantly safer than c-AMB in children and adults. In both the paediatric and adult populations, L-AMB treated patients had a 2 – 6 fold decrease in the incidence of test-drug-related side-effects, compared to c-AMB. The result was concluded that renal toxicity (Nephrotoxicity) caused by c-AMB could be reversed in the majority of cases when patients were switched to L-AMB.

Table 2.5 The IC_{50} values of docetaxel liposomes, evaluated on HT-29 and Igrov1 human tumor cell lines, measured as the inhibition of [^{3}H]leucine incorporation (Immordino et al., 2003).

Cell line	**Exposure time (h)**	**IC_{50} (nM)**		
		Docetaxel	**Conventional docetaxel liposomes**	**PEGylated docetaxel liposomes**
HT-29	2	13±1	31±2	40±2
Igrov1	2	63±3	151±5	187±5
HT-29	48	0.8±0.1	1.7±0.3	3.5±0.4
Igrov1	48	4.5±0.5	7.5±0.3	11±0.5

2.8.1.2 pH-sensitive liposomes

pH-Sensitive liposomes compose of mild acidic amphiphiles lipid (Figure 2.10) such as (a) *N*-palmitoylhomocysteine, (b) oleic acid (OA), (c) cholesterylhemisuccinate (CHEMS), (d) *N*-

citraconyl- dioleoylphosphatidylethanolamine (C-DOPE), (e) phosphatidylethanolamines (PE), (f) dioleoylphosphatidylethanolamine (DOPE), (g) oleyl alcohol (OAlc), (h) ***N***-succinyldioleoylphosphatidylethanolamine (suc-DOPE) and (i) *N*-palmitoyl homocysteine (PHC) (Sudimack et al., 2002) to form stable liposomes at neutral pH.

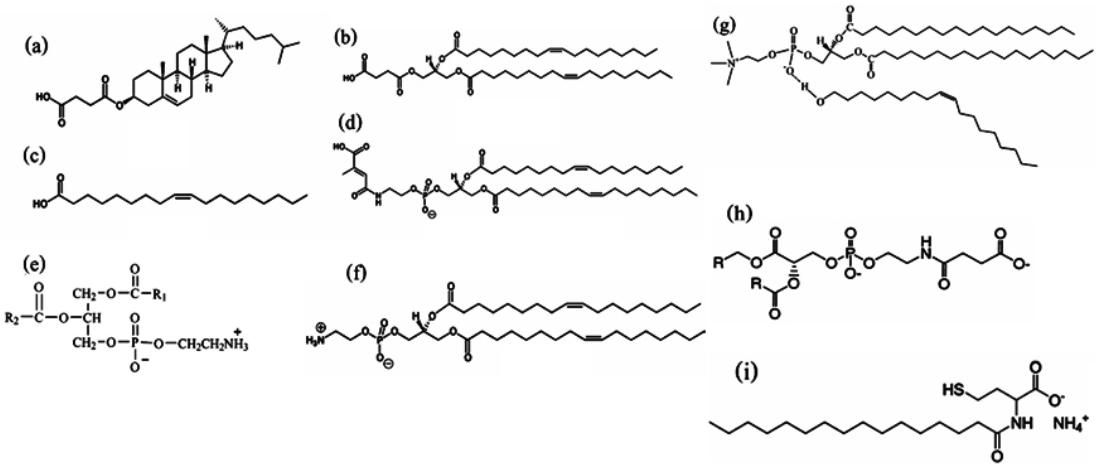


Figure 2.10 Chemical structures of several mildly acidic lipids used to stabilize pH-sensitive liposomes. (a) *N*-palmitoylhomocysteine, (b) oleic acid (OA), (c) cholesterylhemisuccinate (CHEMS), (d) *N*-(succinyl) dioleoylphosphatidylethanolamine, (e) phosphatidylethanolamines (PE), (f) dioleoylphosphatidylethanolamine (DOPE), (g) oleyl alcohol (OAlc), (h) ***N***-succinyldioleoylphosphatidylethanolamine (suc-DOPE) and (i) *N*-palmitoyl homocysteine (PHC) (From: Drummond et al., 2000; Sudimack et al., 2002) .

They can be destabilized when the external pH is changed; usually from a neutral or slightly alkaline pH to an acidic pH. They were designed to circumvent delivery of liposome contents to the lysosomes of cells following internalization of the vesicle via the endocytic pathway as shown in Figure 2.11 (Torchilin et al., 1993; Chu and Szoka, 1994).

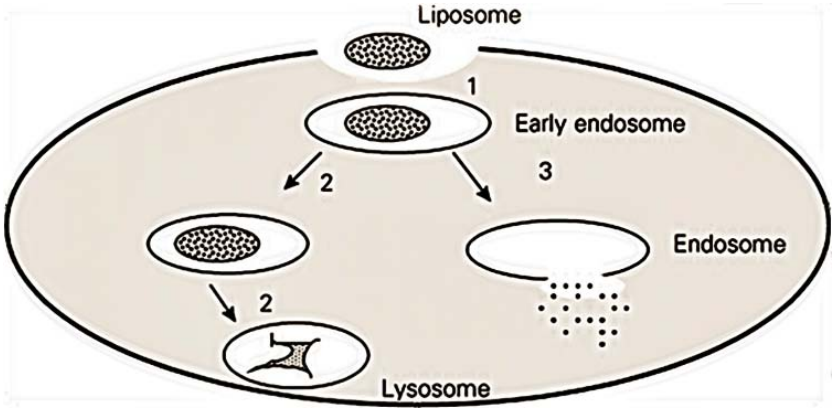


Figure 2.11 Uptake and delivery by pH-sensitive and non-pH-sensitive liposomes. (1) Endocytosis process: liposome in an early endosome. (2) Degradation of a non-pH-sensitive liposome and its content in a lysosome. (3) Destabilization of a pH-sensitive liposome and liberation of its content into the cytosol (From: Ropert, 1999).

Since pH-sensitive liposomes are usually stable under neutral conditions, but become destabilized under acidic pH dependent on the lipid composition and the pH of the incubation medium. For example, Connor and co-worker (1984) reported that liposomes containing PE and PHC fuse rapidly when the medium pH is lowered from 7 to 5, while pure suc-DOPE vesicles released their encapsulated contents at pH 7.4 but not at pH 4.0 (Nayar and Schroit, 1985).

Thus, after liposomes enter cells via endocytosis, the acidic pH inside the endosomes causes pH-sensitive liposomes destabilized the vesicles resulting in releasing their contents into the cytoplasm or into the endosomal compartment, most likely by destabilizing the endosome membrane (Torchilin et al., 1993; Slepushkin et al., 1997).

The liposomes could be applied in drug delivery especially antitumor agent to enhance cytotoxic effect and target specific carrier for antitumor drugs (Connor and Huang, 1986). They could delay the onset of tumorogenesis and reduce the cumulative numbers and sizes of tumor papillomas (Khan et al., 2007). They were used in deliver soluble proteins (antigen) to activated immune response. It was demonstrated that a potent primary CTL (cytotoxic T lymphocyte) response against a soluble protein can be achieved by delivering antigen in pH-sensitive liposomes to dendritic cells (Mozafari et al., 2009) both in vivo and in vitro.

Since the pH-sensitive liposome delivery system has results in a fraction of the material gaining access to the nonendosomal processing pathway necessary for CTL induction and recognition. The liposome bound antigen may be delivered to macrophages and that such cells may preprocess the material and pass on appropriate antigen fragments to the DC (Nair et al., 1992). Moreover, many publications reported in application of pH-sensitive liposomes in various fields such as enhancing immunogenicity (Vyas et al., 2008), specific targeting hepatocyte (Wen et al., 2004) or delivery of foreign substances into living cells (Sánchez et al., 2011).

2.8.1.3 Cationic liposomes

The liposomes composed of cationic lipid such as dimethyldioctadecylammonium bromide (DDAB), dioetadecylamidoglycyl spermine (DOGS), 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N* (DOSPA), 1 ,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1 ,2-dimyristoyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE), *N*-[1-(2,3-dioleoylox-y)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and etc. The chemical structures of some molecules are shown in Figure 2.12.

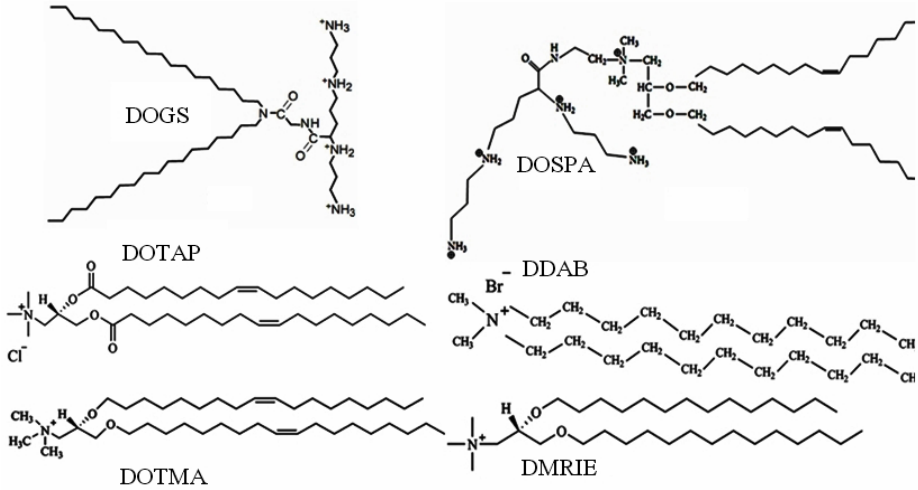


Figure 2.12 The chemical structure of cationic lipids

Their delivery mechanism is possibly due to cell or endosome membrane fusion. They are suitable for delivery of negatively charged macromolecules such as DNA, RNA, oligos and etc. Bailey and Cullis (1997) prepared cationic liposome to determine the role the lipid composition of “target” membranes which may promote delivery. They examined the ability of

large unilamellar vesicles composed of l,2-dioleoyl-sn-phosphatidylethanolamine (DOPE) and *N,N*-dimethyl-*N,N*-di-9-*cis*-octadecenylammonium chloride (DODAC) (1:1) in membrane fusion property. The result demonstrated that membrane fusion was promoted by increased negative surface charge and by increasing acyl chain unsaturation in target liposomes. However, the presence of disaturated phospholipids promoted fusion below the gel to liquid crystalline transition temperature, an effect which was eliminated by the addition of cholesterol. It was also shown that DOPE/DODAC (1:1) LUVs fused with erythrocyte ghosts and this fusion was blocked by the presence of serum.

2.8.1.4 Immunoliposomes

Immunoliposomes have similarly compositions of conventional liposome with attached to antibody or recognition sequence (antibody-coated liposome). They bind specifically bind with target cells expressing surface antigen (Huang et al., 1987; Sharma and Sharma, 1997). The liposomes were also used as a marker in immunoassays by conjugate with antigens or antibodies on liposomal surface to increasing sensitivity and specificity in analytical methods as described by Rongen and co-workers (1997). They can be used in delivery of cytotoxic drugs for tumour therapy by incorporate with tumour-specific ligands onto the liposome surface allows active targeting to tumour cells. Tumor specific ligands such as antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers were reported (Forssen and Willis, 1998; Mastrobattista et al., 1999). However, antibodies or antibody fragments were the type of targeting ligand to actively cross target liposomes mostly used (Phillips and Dahman, 1995; Völkel et al., 2004; Brignole et al., 2005; Manjappa et al., 2011).

Huwyler and co-workers (1997) studied pharmacokinetics and tissue distribution of daunomycin delivered to different tissues in the rat using immunoliposomes. Three different types of nanoliposomes were used to compare tissue distribution (Figure 2.13): 1) conventional liposomes, 2) liposomes sterically stabilized with 2000 Dalton polyethylene glycol and 3) immunoliposomes prepared by coupling a control IgG_{2a} or monoclonal antibody to the distal end of the polyethylene glycol spacer. The antibody used was the OX26 monoclonal antibody to therat transferrin receptor.

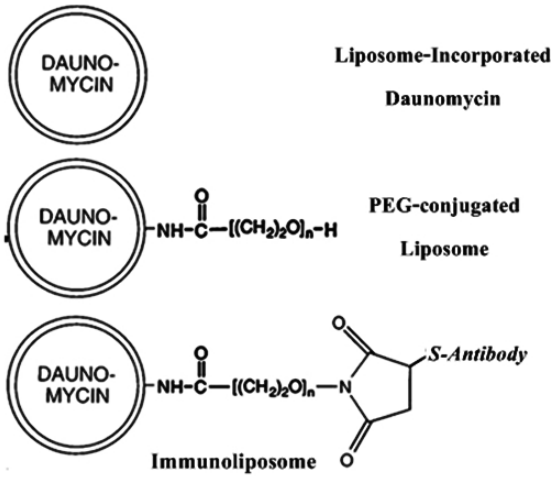


Figure 2.13 Schematic representations of the different types of liposomes used in pharmacokinetic and tissue distribution study. Immunoliposomes were prepared by attachment of a thiolated monoclonal antibody to the terminal end of a maleimide-PEG conjugated linker lipid (From: Huwyler et al., 1997).

Immunoliposomes composed of DSPC (5.2 μmol), cholesterol (4.5 μmol), and DSPE (0.3 μmol). Daunomycin and liposomes were administered by i.v. injection to the rat. The result showed that daunomycin and daunomycin in conventional liposomes were rapidly cleared from the plasma compartment. In comparison to the free drug, daunomycin in conventional liposomes did accumulate to higher levels in liver and spleen and to lower levels in heart, lung and liver. In contrast, daunomycin in liposomes sterically stabilized with polyethylene glycol could not be detected in heart, lung, kidney, liver and spleen. Using nonspecific IgG_{2a} isotype immunoliposomes, tissue concentrations of immunoliposomes were reduced by at least a factor of two. Attachment of more than 29 OX26 monoclonal antibodies per liposome did not increase tissue levels in heart, kidney or lung. Tissue levels of OX26 immunoliposomes were reduced in all organs by coinjection of unbound OX26.

In 1993, Vingerhoeds and his teams used immunoliposomes bearing enzymes (immuno-enzymosomes) for site-specific activation of prodrugs for application in cancer therapy. Their strategy is displayed in Figure 2.14, the immuno-enzymosomes were first allowed to bind to the target cells. Then a prodrug is activated by the immuno-enzymosomes in close proximity of the target cell. The active drug can subsequently kill the cell.

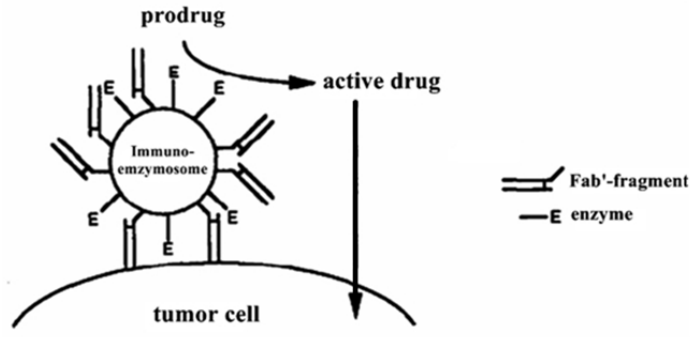


Figure 2.14 Schematic representation of the concept of antibody-directed enzyme prodrug therapy with immuno-enzymosomes (From: Vingerhoeds et al., 1993).

The composition of the bilayer of the liposomes was eggPC : eggPG : CHOL : MPB-PE at a molar ratio of 38.5 : 4 : 16 : 1.5. The result showed that the enzyme ß-glucuronidase, capable of activating the prodrug epirubicin-glucuronide (epi-glu), was coupled to the external surface of immunoliposomes directed towards ovarian cancer cells. A significant increase in cytotoxicity of the prodrug epi-glu was observed when the in vitro cultured cancer cells were pretreated with these immuno-enzymosomes.

2.8.1.5 Long-circulating liposomes (LCL)

Long-circulating liposomes were always composed of neutral charged and high T_c lipids, cholesterol and might be mixed with 5 - 10% of PEG-DSPE, GM1 or HPI (Sharma and Sharma, 1997). The PEG was added to produce a surface of the liposomes gives the liposomes a hydrophilic "sterically stabilized" surface. This property contributes to a lower affinity of macrophages of the mononuclear phagocyte system (MPS) for the circulating liposome particles and consequently to a prolonged blood circulation. One disadvantage of conventional liposome or referred short-circulating liposomes is toxicity of drug trapped in vesicle may occur in other organs due to cleared rapidly from the circulation and predominantly taken up by macrophages in the liver (Daemen et al., 1995). The toxicity in the liver and, more specifically, in the liver macrophages may be due to release of the entrapped drug within the cell (Daemen et al., 1997). Daemen and co-workers (1997) prepared doxorubicin entrapped within long-circulating liposomes (Dox-LCL) and studied the effect of phagocytic capacity and bacterial blood clearance capacity of rat liver macrophages. Dox-LCL (125 nm in diameter) composed of egg

phosphatidylcholine (PC), cholesterol (CH) and polyethyleneglycol distearoylphosphatidylethanolamine (PEG-PE) (55 : 45 : 5 molar ratio; MW PEG 1900. The doxorubicin/lipid ratio was 0.36 : 1 (mol /mol). The result showed that reducing of phagocytic activity and bacterial blood clearance was significantly reduced as compared to clearance in control rats and in rats injected twice with doxorubicin combined with placebo liposomes. Dox-LCL appears less toxic than Dox-L (short-circulating liposomes) for the liver macrophage population following i.v. administration both with respect to specific phagocytic activity and cell numbers. This due to the delay in onset of toxic effects and the faster recovery from Dox-LCL treatment as compared to Dox-L treatment. It is conceivable that therapeutic protocols can be designed with Dox-LCL that circumvents long-term impairment of the liver macrophage population. The faster recovery from Dox-LCL treatment as compared to Dox-L treatment might be explained by the prolonged circulation time of LCL and the lower accumulation of Dox-LCL in the liver. Doxorubicin in short-circulating liposomes was taken up very rapidly by the liver macrophages, resulting in an almost instantaneous blockade of the liver macrophage population. On the other hand, Dox-LCL was taken up relatively slowly. While some of the liver macrophages containing sufficient amount of Dox-LCL were damaged. Other macrophages which were not damaged might be able to initiate the process of renewal of the population by secreting cytokines that induce influx of immature monocytes/macrophages.

2.8.2 Liposome classification base on the basis of size and lamellae

In many circumstance types of liposome can be distinguished according to their particle size as a range from small (0.025 μm) to large (2.5 μm) vesicles. They were also classified by single or multiple of membranes bilayer (lamellae) as shown in Figure 2.15. It is known that the vesicle size is a critical parameter in determining circulation half-life of liposomes. Both size and number of bilayers were also have influence the extent of drug encapsulation in the liposomes.

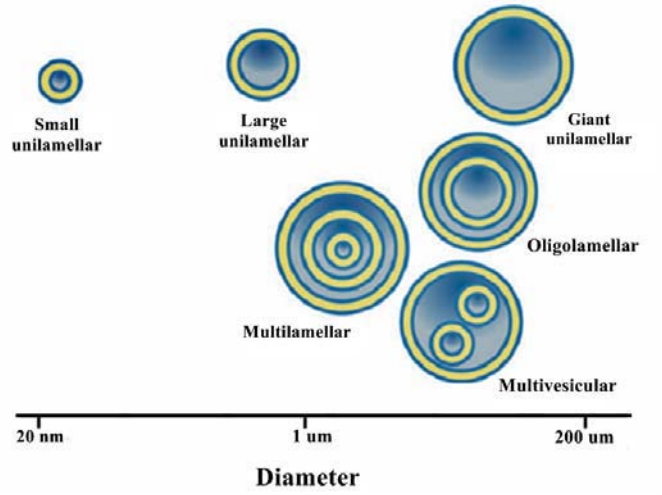


Figure 2.15 The liposomal classification according to size and lamellae (From: Jesorka and Orwar, 2008).

Small unilamellar vesicles (~0.02 μm to ~0.2 μm), large unilamellar vesicles (~0.2 μm to ~1 μm), and giant unilamellar vesicles (>1 μm) are the three most important groups for pharmaceutical applications (Sharma and Sharma, 1997; Jesorka and Orwar, 2008). Each liposomal type was described as below:

2.8.2.1 Multilamellar vesicles (MLV)

This type of liposomes has more than one membranes bilayer (lamellae). Their size are in a range of 1 – 50 μm or >0.1 μm (Riaz, 1996; Sharma and Sharma, 1997; Groth et al., 2003). Desjardins and co-worker (1995) found that when injection of MLV liposomes (size about 0.5-12.0 μm) containing distearoyl phosphatidylcholine (DSPC) and dipalmityl phosphatidylcholine (DPPC) resulted in a marked popliteal lymph node (PLN) reaction resulting in increasing numbers of $CD4^+$, CDS^+, Ig^+ subsets and proportions of large/activated EAM^+ ($CD69^+$) and $CD2.5^+$ (IL-2 receptor$^+$) cells. In contrast, when injection MLV liposomes which containing lipids of low T_c, such as egg phosphatidylcholine (egg PC) and dimyristoyl phosphatidylcholine (DMPC), did not show any immunoactivation. Large liposomes were shown to be unable to enter blood circulation because they were taken up by macrophages either at the site of injection or as they moved down the draining node. In contrast, the small size of some liposomes (~110 nm) was shown to be critical to their ability to move into the circulation. Two distinct biological phenomena could be implicated in non-drug-containing liposome

adjuvanticity: (1) macrophage activation following phagocytosis of large sized MLV liposomes; and (2) alterations of plasma membrane fluidity by artificial entrapment of liposome phospholipids into membranes of lymphoid and/or macrophage cells. Thus, depending on high Tc of phospholipids such as DSPC and DPPC, the liposome uptake could affect macrophage-lymphocyte membrane fluidity and subsequently activate the cells.

2.8.2.2 Large unilamellar vesicles (LUV)

These liposomes have single bilayer walls encapsulating a single aqueous core (known as small or large unilamellar vesicles (SUVs or LUVs) (Jones, 1996). The vesicle size may have diameters of > 0.1 μm (Sharma and Sharma, 1997). Bally and co-workers (1985) have demonstrated an ability of LUV systems to rapidly and efficiently accumulate certain species of lipophilic cations (commonly employed as membrane-potential probes) when a membrane potential ($\Delta\Psi$, inside negative) is generated across the vesicle membrane. Preliminary investigations suggested that clinically important drugs with lipophilic and cationic properties can also be accumulated and retained by a similar mechanism. They characterized the ability of LUV systems exhibiting a membrane potential to actively accumulate and retain the cationic anticancer drugs, adriamycin and vinblastine. The ability of LUV systems can entrap drugs which do not have lipophilic or cationic characteristics such as methotrexate, cytosine arabinoside were also investigated. The result showed that the presence of K^+ diffusion potential can result in the rapid accumulation of adriamycin or vinblastine into LUV systems with entrapment efficiencies approaching 100%. In 1985, Mayer and co-workers reported that the anticancer drugs, adriamycin and vinblastine, can be rapidly accumulated into egg phosphatidylcholine large unilamellar vesicles in response to a valinomycin-dependent K^+ diffusion potential ($\Delta\Psi$) to achieve high effective interior concentrations. Trapping efficiencies approaching 100% was reported. The influence of lipid composition and the requirement for valinomycin have been examined for adriamycin. Equimolar of cholesterol levels inhibited the uptake process at 20°C. However, incubation at higher temperature resulted in enhanced uptake. Similarly, the presence of egg phosphatidylserine or incubation at elevated temperatures results in significant adriamycin uptake in the absence of valinomycin.

2.8.2.3 Small unilamellar vesicles (SUV)

Lipid vesicles have single bilayer and their size about of ≤ 0.1 μm (Sharma and Sharma, 1997). Pitcher and Huestis (2002) prepared and analyzed of small unilamellar phospholipid vesicles of a uniform size. The result showed that addition of carbonmonoxyhemoglobin to dimyristoylphosphatidyl choline : dimyristoylphosphatidyl serine small unilamellar vesicles resulted in an increase of average vesicle size from 17.4 to 32.0 nm. Addition of heme to vesicles produced a smaller size increase, from 17.4 to 21.0 nm. They also reported a method for preparing small unilamellar lipid vesicles of a uniform size that can be characterized by NMR spectroscopy.

Sulfatide (cerebroside sulfate) has been used in SUV preparation and their hydration, stability properties and pH sensitivity were investigated. These vesicles were found to be pH-sensitive and became leaky at pH 6.0 or when there was a pH-gradient across the membrane bilayer. Under such conditions, the amount of calcein released after 24 h incubation at 37 °C was increased by one-fold compared to that found at pH 7.4. The results indicated that the hydration and partial dehydration of the headgroup of sulfatide upon changing pH played an essential role in determining the pH sensitivity of DOPE/sulfatide vesicles, while the importance of the condensing effect of the glycolipid on membrane bilayer is less significant (Wu et al., 1996).

2.8.2.4 Giant unilamellar vesicles (GUVs)

Giant unilamellar vesicles (GUVs) are spherical bilayer vesicles composed of a single lipid bilayer. Their sizes could be upto 5-200 μm in diameter (Menger and Keiper, 1998; Peetla et al., 2009; Wesołowska et al., 2009). GUVs have been subjected to physical and biological investigations, such as elastic properties of phospholipid membranes, shape change of vesicles, interaction of cytoskeleton proteins with the membranes, membrane fusion, hydrodynamic interactions of water-soluble amphiphiles and active membranes, and reconstitution of artificial cells (Döbereiner, 2000; Tamba et al., 2004). Since their structure and size are very close to those of cells and to their ease of observation by light microscopy techniques (Carvalho et al., 2008). For examples, Tanaka and Yamazaki (2004) used GUVs to investigate membrane fusion of vesicles of biomembranes which played various important roles in cells, such as, in membrane

fusion induced by SNARE proteins (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor). The mechanism of the association between vesicles and cell membranes has been revealed, but the mechanism of their membrane fusion is still not clear. They were found that 30 μM to1 mM La^{3+} induced membrane fusions of two giant unilamellar vesicles (GUVs) composed of a mixture of dioleoylphosphatidylcholine (DOPC) and dipalmitoleoylphosphatidylethanolamine (DPOPE). The process of membrane fusion in detail was observed. First, two GUVs became strongly associated, with a partition membrane between them composed of two bilayers, one from each GUV. Then, the partition membrane was suddenly broken at one site on its edge. The area of this breakage site gradually spread, until it was completely separated from the GUV to complete the membrane fusion. This proposes was a novel model (i.e., the partition breakage model) for the mechanism of La^{3+} induced membrane fusion of GUVs.

2.8.2.5 Multivesicular vesicles (MVV)

Multivesicular vesicles or multivesicular liposomes (MVL) composed of several small vesicles which have 100 nm – 20 μm or usually large >1 μm in diameter (Storm and Crommelin, 1998; Wiedmer et al., 2004). They have hundreds of bilayer-enclosed, includes water-filled compartments and is designed to be a depot to localize drug delivery to affected areas, while avoiding uptake by the reticuloendothelial system (Spector et al., 1996). MVLs were used to depot delivery system for controlled systemic delivery of acyclovir sodium. Due to acyclovir sodium has limitations of conventional therapies and to investigate its in vivo effectiveness for sustained delivery. MVLs of acyclovir were prepared by the reverse phase evaporation method. The loading efficiency of the MVLs (45% - 82%) was found to be 3 to 6 times higher than conventional multilamellar vesicles (MLVs). The in vitro release of acyclovir from MVL formulations was found to be in a sustained manner and only 70% of drug was released in 96 hours, whereas conventional MLVs released 80% of drug in 16 hours. The MVL formulations showed effective plasma concentration for 48 hours compared with MLVs and free drug solution (12 - 16 hours) when intradermal administration to Wistar rats. C_{max} (maximum plasma concentration) values of MVL formulations were significantly less (8.6 - 11.4 mg/mL) than MLV and free drug solution (12.5 mg/mL). The AUC_{0-48} (area under the drug plasma concentration-

time curve) of the MVL formulations was 1.5- and 3-fold higher compared with conventional liposomes and free drug solution, respectively. The increase in AUC and decrease in C_{max} reflects that the MVL formulations could reduce the toxic complications and limitations of conventional IV and oral therapies (Jain et al., 2005).

2.9 Liposomes preparation methods

Nowadays, many methods were used for preparation of liposomes. Numerous of review literatures have been reported for techniques of liposome preparation including lab-scale and large-scale. Several techniques gave different vesicle types of liposome. The procedures can be categorized as summarized in Table 2.6 (Wagner and Vorauer-Uhl, 2010).

Table 2.6 Methods of liposome preparation and vesicle types from each preparation technique (Wagner and Vorauer-Uhl, 2010).

Methods	Vesicle types
Mechanical methods	
Vortex or hand shaking of phospholipid dispersions	MLV
Extrusion through polycarbonate filters at low or medium pressure	OLV, LUV
Extrusion through a French press cell "Microfluidizer" technique	Mainly SUV
High-pressure homogenization	Mainly SUV
Ultrasonic irritation	SUV of minimal size
Bubbling of gas	BSV
Methods based on replacement of organic solvent(s) by aqueous media	
Removal of organic solvent(s)	MLV, OLV, SUV
Use of water-immiscible solvents: ether and petroleum	MLV, OLV, LUV
Ethanol injection method	LUV
Ether infusion (solvent vaporization)	LUV, OLV, MLV
Reverse-phase evaporation	LUV

Table 2.6 Methods of liposome preparation and the resulting product (continued).

Methods	Vesicle types
Methods based on detergent removal	
Gel exclusion chromatography	SUV
"Slow" dialysis	LUV, OLV, MLV
Fast dilution	LUV, OLV
Other related techniques	MLV, OLV, LUV, SUV

Furthermore, the physical properties of liposome could be preliminary categorized from preparation procedures, such as their size distribution, lamellar or trapping efficiency. Mayer and his team (1986b) compiled a review literature of preparation techniques for liposome formulations including their several physical properties as shown in Table 2.7. However, these results were depended on liposomal composition, lipid concentration and aqueous trapped volumes inherent in the specific liposome preparation procedures. Ability of prepare to be accurately reproduced or replicated by someone else working independently (reproducibility).

Table 2.7 Physical properties of liposome formulation (modified from: Mayer et al., 1986b)

Vesicle type	Preparation methods	Vesicle diameter (μm)	Entrapped agent	% Trapping efficiency
SUV	Sonication	0.020 - 0.040	Cytosine arabinoside, methotrexate, carboxyfluorescein	1 - 5
SUV	French press	0.020 - 0.050	Carboxyfluoresceln, inulin, trypsin, BSA	5 - 25
SUV	Detergent removal	0.036 - 0.050	Carboxyfluorescein, inulin	12
LUV	Detergent removal	0.1 - 10.0	Inulin, cytochrome C, carboxyfluorescein	12 - 42

Table 2.7 Physical properties of liposome formulation (continued)

Vesicle type	Preparation methods	Vesicle diameter (μm)	Entrapped agent	% Trapping efficiency
LUV	Reverse phase evaporation	0.2 - 1.0	Carboxyfluorescein, cytosine, arabinoside	28 - 45
LUV	Solvent vaporization	0.1 - 0.5	Chromate, glucose, soy bean trypsin inhibitor,	2 - 45
LUV	Extrusion	0.056 - 0.2	Inulin, methotrexate	15 - 60
MLV	Mechanical mixing	0.4 - 3.5	Glucose albumin, DNA	1 - 8.5
MLV	Sonicate-freeze-thaw	0.17 - 0.26	Asparaginase	50 - 56
MLV	Freeze-thaw	0.5 - 5.0	Inulin	35 - 88
MLV	Sonicate-dehydrate-rehydrate	0.3 - 2.0	Sucrose, vincristine, melphalan	27 - 54
MLV	Solvent evaporation-sonication	0.3 - 2.0	streptomycin sulfate, oxytetracycline sulfamerazine	6.3 - 38

From the table 2.7 showed that several methods in liposome preparation have influence to vesicle structure, efficiency of drug entrapment and size diameter of the liposomes. Significant differences in trapping efficiencies were reported depending on liposome preparation procedures.

2.9.1 Mechanical methods

2.9.1.1 Hand-shaken method: In order to produce liposomes, lipid molecules must be introduced into an aqueous environment. When dry lipid film is hydrated the lamellae swell and grow into myelin figures (thin lipid tubules). Only mechanical agitation provided by

vortexing, shaking, swirling or pipetting causes myelin figures to break and reseal the exposed hydrophobic edges resulting in the formation of liposomes. Large multilamellar liposomes can be made by hand-shaken method (Shaheen et al., 2006).

Karki et al (2009) used this technique to formulate liposome co-encapsulated which rifampicin and isoniazid (Antitubercular drugs). The rifampicin and isoniazid co-encapsulated in liposome formulation showed good stable when kept for long term at 5 ± 3 °C for one year (Table 2.8). *In vivo* studies showed that co-encapsulation liposome retard the release of drug from circulation compared to free drug due to slow drug release into systemic. Increasing in the bioavailability of drug from these systems compared to free drug. This formulation also reduces the accumulation of drug in the liver kidney and lungs. The result from this study revealed that liposomes could be promising delivery systems for rifampicin and isoniazid with prolonged drug release profiles and reasonably good stability characteristics.

Table 2.8 Stability studies of rifampicin and isoniazid from co-encapsulated of liposomal formulation at 5 ± 3 °C (modified from: Karki et al., 2009).

Times (Months)	Rifampicin		Isoniazid	
	Drug contents (%)	**Morphology**	**Drug contents (%)**	**Morphology**
0	100.00 ± 0.0	-	100.00 ± 0.0	-
1	99.98 ± 0.1	-	99.97 ± 0.1	-
2	99.80 ± 0.1	-	99.80 ± 0.1	-
4	99.79 ± 0.1	-	99.42 ± 0.1	-
6	98.72 ± 0.1	-	98.62 ± 0.1	-
8	97.98 ± 0.1	-	97.83 ± 0.1	-
10	97.89 ± 0.2	-	97.52 ± 0.1	-
12	97.53 ± 0.2	+	97.32 ± 0.1	+

(-) is no change in morphology of the formulation and (+) is mild change in morphology formulation characteristics (aggregation/discoloration/increased vesicle diameter).

2.9.1.2 Sonication method: This method is probably the most widely used method for the preparation of SUVs. The method involves the subsequent sonication of MLVs prepared by the convectional method either with a bath type or a probe type sonicator under an inert atmosphere, usually nitrogen or argon. The principle of sonication can be performed by using pulsed, high frequency sound waves (sonic energy) to agitate a suspension of the MLVs. Such disruption of the MLVs produces SUVs with diameter in the range of 15–50 nm. The purpose of sonication, therefore, is to produce a homogenous dispersion of small vesicles with a potential for greater tissue penetration (Uhumwangho and Okor, 2005). Two types of sonication technique that were the most used in liposome preparation.

(a) Probe sonication

The tip of sonicator is directly immersed into the liposome dispersion. The energy input into lipid dispersion is very high. The dissipation of energy at the tip results in local overheating and therefore the vessel must be immersed into an ice/water bath. However, during the sonication up to one hour more than 5% of the lipids can be de-esterify. Moreover, titanium which is the probe composition can be slough off and contaminate the solution (Shaheen et al., 2006).

(b) Bath sonication

During the liposome dispersion, tube should be placed into a bath sonicator. Controlling the temperature of the lipid dispersion is easier in this method compare to sonication the dispersion directly using the tip. Material being sonicated can be kept in a sterile container, unlike the probe units, or under an inert atmosphere. The lipid bilayer of the liposome can fuse with other bilayer (e.g., cell membranes), thus delivering the liposome contents such as DNA or drugs (which would normally be delivered past the lipid bilayer) can be delivered past the lipid bilayer (Shaheen et al., 2006).

Comparatively, sonication with a probe results in faster breakdown of the MLVs to smaller structures than by a bath sonication. The reduction in size of the liposomes, however, also decreases the amount of interior aqueous space, thereby limiting the amount of water-soluble

drugs that can be entrapped. However, degradation of lipids, metal particle shedding from the probe tip (titanium particles) and generation of aerosols from solutions can be achieved. Bath sonication is a closed system allowing for temperature control to minimise thermo degradation of the lipid and entrapped substance. The position of the tube and water level in the bath is also regulated for maximum efficiency (Uhumwangho and Okor, 2005).

Bacalum and Radu (2007) used sonication technique for preparing liposome to study the proper functioning of the membrane proteins. The result of this study found interaction of proteins with the lipid bilayer in artificial lipid membranes (liposomes) by using a lipid bilayer fluorescence marker. This method was used to study the membrane insertion of a bacterial outer membrane porin (OmpF). The OmpF is a major porin transporting ions and small molecules (up to 600 Daltons) across the outer membrane of gram negative bacteria, as *Escherichia coli*.

Aukunuru et al (2009) prepared the small unilamellar vesicles (SUVs) incorporating bis-demethoxy curcumin analogue (BDMCA) by using sonication method. The size of SUVs obtained was 327 ± 15.72 nm. The result showed that the release was sustained for 10 days *in vitro* and could be described as diffusion-controlled. The liposomal formulations were able to sustain the release of drug *in vivo* also. Liposomal formulations showed better hepatoprotective activity to the drug compared to its solution form.

2.9.1.3 Preparation by Film Methods: Wagner and co-worker (2010) explained that the properties of lipid formulations can vary depending on the composition (cationic, anionic, and neutral lipid species). However, the same preparation method can be used for all lipid vesicles regardless of composition. The general steps of the procedure are preparation of the lipids for hydration, hydration with agitation, and sizing to a homogeneous distribution of vesicles. Since then, many different variations of this method have been developed differing in the organic solvents used for lipid solubilization, the way of lipid drying, and the way of film rehydration. Despite the various modifications, all these methods have in common that heterogeneous populations of multilamellar liposomes are produced. However, vesicle size is influenced by the lipid charge. Charged lipids form smaller liposomes with less lamella. Other

influencing parameters are the nature of the aqueous phase as well as energy and power input of agitation. The film method has several advantages. It can be used for all different kinds of lipid mixtures. In addition, the method is easy to perform, and high encapsulation rates of lipid as well as aqueous soluble substances can be achieved because high lipid concentrations can be used. One major drawback of this method is the difficulty of scaling up to several tens of liters. Furthermore, the process becomes more time and cost intensive because additional processing is recommended for a defined liposome suspension, whereby product losses are generated. Several downsizing techniques have been established in order to make the heterogeneous vesicles more uniform. The first published downsizing method by sonication. A very high energy input based on cavitations is applied to the liposomal dispersion either directly with a tip or indirectly in a bath sonicator. Other methods also aiming at breaking down the large MLVs are homogenization techniques, either by shear or pressure forces. This can be done by microfluidization, high-pressure homogenization, and shear force-induced homogenization techniques. The most defined method for downsizing is the extrusion technique whereby liposomes are forced through filters with well defined pores.

Changsan and co-worker (2008) used the chloroform film method to produce liposome encapsulated rifampicin (RIF). The lipid vesicles were obtained a mixture of unilamellar and multilamellar vesicles with a size of 200 - 300 nm when determined by Cryo-transmission electron microscopy. This result correlated with those obtained from dynamic light scattering. The cholesterol oriented between the phospholipid molecules and affects the fluidity of the bilayer membrane. The physical stability of liposomes increasing when increased cholesterol content. The result was also indicated that RIF was located between the methylene chains of the phospholipid bilayer, in association with cholesterol molecules.

Zimmer and his team (1999) prepared positively charged liposomes by using film method. Oligonucleotides (ODN) were efficiently adsorbed onto the cationic liposomes membrane. At an ODN/liposome ratio of 1 : 5 (10 : 50 μg/mL) 84.2 ± 5.4% of the ODNs were bound to the liposomal membrane. Within the range of 1 : 40 and 1 : 100 charge neutralization occurred and the liposome dispersion showed an increase in particle size due to aggregation.

Below or above this range of charge neutralization the ODN loaded liposome preparation was physically stable, no sedimentation, and increase of vesicle size or vesicle aggregation occurred.

2.9.1.4 Homogenization techniques: Similar to the ultrasound methods, homogenization techniques have been used in biology and microbiology for breaking up the cells. Therefore, many scientists have used them for reducing the size and number of lamellae of multilamellar liposomes. Lipids as well as proteins or other sensitive compounds can be degraded during the sonication procedure. This system is normally used in the volume of 1 to 40 mL and therefore is not suitable for large-scale production. However, a scale-up-based strategy on this technique was established as the microfluidization. This continuous and scaleable enforces downsizing of liposomes by collision of larger vesicles at high pressure in the interaction chamber of the microfluidizer. Starting volumes from 50 mL upwards are applicable, and high pressures were used for disruption of multilamellar systems. The system works in a pressure range of 0–200 bar and is equipped with heating and cooling systems to control sample temperature during processing. The liposome suspension passes the exchangeable orifices several times (up to thousands of passes). Liposomes are formed in the size range from 50 to 100 nm by this process. This technique is suitable for large-scale production and sterile liposome preparation. In contrast to the microfluidizer, where the fluid stream is split and mixed by collision in a mixing chamber, homogenizers work on a different principle. In a homogenizer, the fluid beam is pressed with high pressure through an orifice, and this beam collides with a stainless steel wall. The liposome suspension is continuously pumped through the homogenizer system, where high pressures are generated to downsize lipid vesicles. The extrusion through polycarbonate is the most prominent scalable downsizing method. Olson et al were first published this method in 1979 (Olson et al., 1979). Size reduction was managed under mild and more reproducible conditions compared to those discussed above. In this method vesicles were forced through defined membranes by a much lower pressure. All the above methods have in common that the reproducibility of downsizing is extremely high. Systems with a heating device can either be used with saturated and unsaturated lipids.

The main disadvantage of this method is the long-lasting preparation starting with preformed liposomes, eventually an additional freeze-thaw step, and finally the extrusion. In

these entire procedures, high product losses may be generated, especially if clogging of the extrusion membranes occurs, which may cause technical limitations with large-scale production of high priced goods.

In 2008, Sun and co-worker used homogenization technique to prepare liposome encapsulated 5-fluorouracil (5-Fu) and N_3-*O*-toluyl-fluorouracil (TFu) which are antitumor lipophilic prodrug. The formulated liposomes were found to be relatively uniform in size (400.5 ± 9.6 nm) with a negative zeta potential (-6.4 ± 0.8 mV). The drug entrapment efficiency and loading were (88.87 ± 3.25%) and (8.89 ± 0.19%), respectively. The physical stability experiments results indicated that lyophilized TFu-loaded liposomes were stable for at least 9 months at 4˚C. In vitro drug release profile of TFu-loaded liposomes followed the bi-exponential equation. The results of the pharmacokinetic studies in mice indicated that the bioavailability of TFu-loaded liposomes was higher than the suspension after oral administration, and was bioequivalent comparing with TFu 50% alcohol solution after intravenous (i.v.) administration. These results indicated that TFu-loaded liposomes were valued to develop as a practical preparation for oral or i.v. administration (Sun et al., 2008).

2.9.1.5 Freeze-dried rehydration method: Freeze-dried liposomes are formed from preformed liposomes. Very high encapsulation efficiencies even for macromolecules can be achieved using freeze-dried hydration method. During the hydration the lipids bilayers and the material to be encapsulated into the liposomes are brought into close contact. Upon reswelling the chances of encapsulation of the adhered molecules are much higher. The rehydration is a very important step and in should be added in very carefully. The aqueous phase should be added in very small portions with a micropipette to the dried material. After each addition the tube should be vortexed thoroughly. As a general rule, the total volume used for rehydration must be smaller than the starting volume of the liposome dispersion (Shaheen et al., 2006).

Kawano and co-worker (2003) used freeze-dried rehydration method to prepare liposomes entrapped pirarubicin (4′-*O*-tetrahydropyranyldoxorubicin; THP). After producing small unilamellar vesicles (SUVs) which composed of egg lecithin, cholesterol (CHL), β-

sitosterol β-d-glucoside (Sit-G) and oleic acid (OA). The SUVs obtained were freeze-dried with THP and sugars, rehydration of the lyophilized powders led to form the larger vesicles entrapping drugs, but the proper amount of sugars and OA to lipids (sucrose/lipid = 8 w/w) maintained the small particle size (about 340 nm) with high entrapment (80.7%) of THP. After intravenous injection of liposomal THP (L-THP), the accumulations of THP in the liver and heart were approximately 4-fold higher and half lower, respectively, than those of free THP (F-THP). L-THP had superior antitumor effect in 10 mg/kg intravenous administration without significant body weight loss. L-THP is a potential drug dosage form of liver cancer treatment since the liposomes carry THP to the liver (Kawano et al., 2003). The process of liposome preparation by using freeze-dried rehydration method was displayed in Figure 2.16, which described preparation of the dried liposomes containing hepatitis B surface antigen (HBsAg) (Kim and Jeong, 1995).

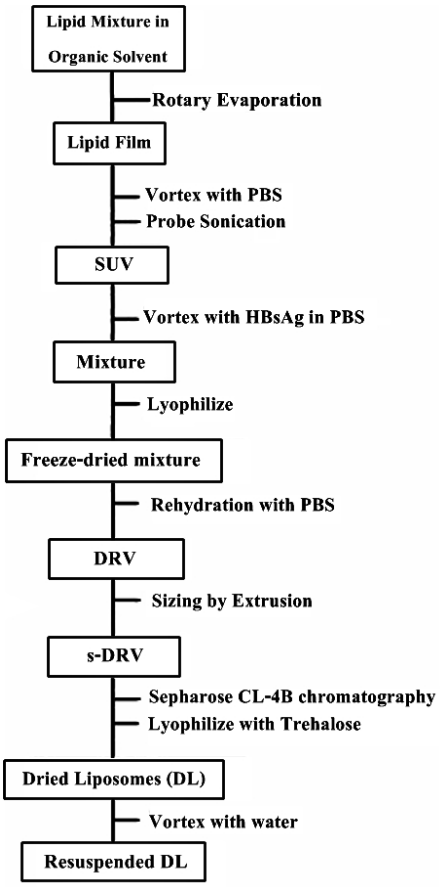


Figure 2.16 Preparation of the dried liposomes containing HBsAg (From: Kim and Jeong, 1995)

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2.9.2 Methods based on replacement of organic solvents by aqueous media

The liposome preparation methods described in this section have in common that organic solvents, either water miscible or immiscible, are replaced by an aqueous solution. This replacement is either performed by injection of the lipid carrying organic solution into the aqueous phase. The injection methods or by stepwise addition of aqueous phase to the organic phase, in particular ethanol was used in the proliposome preparation. In addition, the emulsification methods usually the reverse-phase evaporation method and the double emulsion technique were used that are based on the replacement of a water-immiscible solvent by an aqueous phase, thus forming liposomes with high encapsulation rates of hydrophilic as well as lipid phase soluble substances.

2.9.2.1 The ethanol injection method: This technique was first reported by Batzri and Korn (1973) as one of the first alternatives for the preparation of SUVs without sonication. By the immediate dilution of the ethanol in the aqueous phase, the lipid molecules precipitate and form bilayer planar fragments which themselves form into liposomal systems, thereby encapsulating aqueous phase. Batzri and Korn performed their experiments with a very low lipid concentration resulting in small liposomes and poor encapsulation efficiency. The preparation parameters influencing liposome size, size distribution, and drug encapsulation efficiency were investigated in more detail by Kremer et al in 1977. They determined the lipid concentration in ethanol as the only liposome formation influencing parameter. Neither stirring rate of the aqueous phase nor injection velocity had a significant influence on liposome size and size distribution. Another modified ethanol injection method was developed by Maitani et al (2001) which were more or less a combination of the ethanol injection method, the proliposome method, and the reverse-phase evaporation technique. This method has many advantages as the technique is easy to scale up, and ethanol is a harmless solvent. Stano et al (2004) emphasized the advantage of preparing monomodal distributed liposomes in the size range of about 100 nm and furthermore point out the suitability of the entrapment of lipophilic substances. An additional advance is the improvement of product shelf life due to the absence of mechanical forces which might lead to drug and/or lipid degradation.

Further development was initiated by several groups. In 1996, Naeff published the development of a liposome production technique in industrial scale based on the ethanol injection technique. Their production system was used for the liposomal encapsulation of econazole, an imidazole derivative for the topical treatment of dermatomycosis, and combined the principles of the ethanol injection system and high shear homogenization. Additional production technology patents from several companies were filed dealing with liposome production systems based on the ethanol injection technique. Wagner et al (2002) have also extensively worked in this field, leading to the development of the cross-flow injection system. Based on the ethanol injection technique, they developed a scalable and sterile production technique leading from the conventional batch process to a continuous procedure.

Herein, the principal item is the cross-flow injection module, especially designed for this purpose. This specially conceived unit has the benefit of defined and characterized injection streams and permits liposome manufacture regardless of production scale because scale is determined only by free disposable vessel volumes. By this, process development is performed in lab scale at a volume of about 20 mL. Once the parameters are defined, an easy scale-up can be performed by changing the process vessels only. In addition, these process vessels can be sterilized, either by steam or autoclavation. All raw materials such as buffer solutions, lipid ethanol solution, and even N_2 for applying the injection pressure are transferred into the sanitized and sterilized system via 0.2 µm filters to guarantee an aseptic production. Liposome size can be controlled by the local lipid concentration at the injection point which is defined by the lipid concentration in ethanol, the injection whole diameter, the injection pressure, and the flow rate of the aqueous phase. By varying these parameters, different liposome sizes suitable for the intended purpose can be prepared. These defined process parameters are furthermore responsible for highly reproducible results with respect to vesicle diameters and encapsulation rates. Tangential flow filtration is the next process step to remove ethanol as well as not entrapped drug. Another important advantage of this method is the suitability of the entrapment of many different drug substances such as large hydrophilic proteins by passive encapsulation, small amphiphilic drugs by a one-step remote loading technique, or membrane association of antigens for vaccines (Wagner and Vorauer-Uhl, 2010).

2.9.2.2 Proliposome-liposome method: Wagner and Vorauer-Uhl (2010) described the proliposome-liposome method based on the conversion of the initial proliposome preparation into liposome dispersion by dilution with an aqueous phase. This method is suitable for the encapsulation of a wide range of drugs with varying solubility in water and alcohol and has extremely high encapsulation efficiencies when compared with other methods based on passive entrapment. Turánek and co-workers (2003) developed a sterile liposome production procedure based on this method. Reproducible liposome preparation is feasible in a controlled manner by exact controlling of the dilution rate and process temperature. Additionally, the authors claimed their method as being easy to scale up, which makes this method an alternative approach for the production of liposomes for clinical application.

2.9.2.3 Reverse-phase evaporation method: Historically, this method provided a breakthrough in liposome technology, since it allowed for the first time the preparation of liposomes with a high aqueous space to lipid ratio and able to entrap a large percentage of the aqueous material presented. Reverse-phase evaporation is base on the formation of inverted micelles. These inverted micelles are formed upon sonication of a mixture of a buffered aqueous phase, which contains the water soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. The slow removal of the organic solvent leads to transformation of these inverted micelles into a gel-like and viscous state. At a critical point in this procedure, the gel state collapses and some of the inverted micelles disintegrate. The excess of phospholipids in the environment contributes to the formation of a complete bilayer around the remaining micelles, which results in formation of liposomes. Liposomes made reverse-phase evaporation method can be made from various lipid formulations and have aqueous volume to lipid ratios that are four times higher than multilamllar liposomes or hand shaken liposomes (Shaheen et al., 2006).

Similar method was described by Wagner and Vorauer-Uhl (2010), lipid is hydrated via solubilization in an organic phase followed by introduction into an aqueous phase. The organic phase should be immiscible with the aqueous phase, thus an oil/water emulsion is created, which is diluted with further aqueous phase for liposome formation. The advantage of

this preparation technique is a very high encapsulation rate up to 50%. One variation of the microemulsion technique, the double emulsion technique, further improves the encapsulation rates and results in unilamellar liposomes. A possible drawback of this efficient method is the remaining solvent or the proof of their absence especially for using them for pharmaceutical purposes. The other important issue is large-scale production which might be feasible if appropriate shear mixing devices for the creation of the microemulsion and pumps for the dilution step are available.

2.9.3 Methods based on detergent removal

This liposome preparation procedure, detergents, such as bile salts or alkylglycosides, are used for the solubilization of lipids in micellar systems. In contrast to lipids, detergents are highly soluble in both aqueous and organic media. There is equilibrium between the detergent molecules in the aqueous phase and the lipid environment of the micelle. Equilibrium of the mixed micelles in the aqueous phase takes quite some time and the equilibrium does not happen during a short period of time. The size and shape of the resulting vesicles are depending on the chemical nature of the detergent, their concentration, and the lipids used. The use of different detergent results in faster depletion rates produces smaller size liposome, different ratios of large unilamrllar vesicles/oligolamellar vesicles and multilamellar vesicles. To date, the most frequently applied method for membrane protein reconstitution involves the cosolubilization of membrane proteins. Common procedures of detergent removal from the mixed micelles are dilution, gel chromatography and dialysis through hollow fibers or through membrane filters. Additionally, detergents can also be removed by adsorption to hydrophobic resins or cyclodextrins (Wagner and Vorauer-Uhl, 2010).

2.9.3.1 Detergent depletion method: Shaheen et al (2006) described this method for preparation of a variety of liposome formulations. Detergent can be depleted from a mixed detergent-lipid micelles by various techniques which leads to the formation of very homogeneous liposomes. Phase transition temperature of lipids must be considered for this method. However, not all detergents are suitable for this method and only few detergents can be used for detergent

depletion method. The most popular detergents are sodium cholate, alkyl (thio) glucoside and alkyloxypolyethylenes. Mixed micelles are prepared by adding the concentrated detergent solution to multilamellar liposomes. The final concentration of detergent should be well above the critical micelle concentration (CMC) of the detergent. Detergent depletion is achieved by of four techniques as following approaches:

Dialysis: The dialysis can be preformed in dialysis bags which were immersed in large detergent free buffers or by using continuous flow cells, diafiltration and cross filtration.

Gel filtration: This method, the detergent is depleted by size exclusive chromatography. Sephadex G-50, Sephadex G-100, Sepharose 2B-6B and Sephacryl S200-S1000 can be used for gel filtration. The liposomes do not penetrate into the pores of the beads packed in a column. They percolate through the inter-bead spaces. At slow flow rates the separation of liposome from detergent monomers is very good. The swollen polysaccharide beads adsorb substantial amounts of amphiphilic lipids, therefore pre-treatment is necessary. The pre-treatment is done by pre-saturation of the gel filtration column by lipids using empty liposome suspensions.

Adsorption: Detergent absorption is achieved by shaking of mixed micelle solution with organic polystyrene adsorbers beads such as XAD-2 beads (styrene-divinylbenzene polymer) and Bio-beads SM2. The great advantage of the using detergent adsorbers is that they can remove detergents with a very low critical micelle concentration (CMC) which are not completely depleted.

Dilution: Upon dilution of aqueous mixed micellar solution of detergent and phospholipids with buffer the micellar size and the polydispersity increases dramatically and as the system is diluted beyond the mixed micellar phase boundary, a spontaneous transition from polydisperse micelles to monodisperse vesicles occurs.

Beside classification of liposome preparation method was described above, Meure et al (2008) have been categorized techniques for the production of liposomes base on conventional techniques and recent advanced techniques developed for liposome formation. Traditional techniques, for example, Bangham (Hand shaken or Thin film hydration method), detergent depletion, ether/ethanol injection, reverse-phase evaporation and emulsion methods

were described previously for some method. Since, a manufacturing method is desirable to formulate liposomes with high entrapment efficiency, narrow size distribution, long-term stability and protective properties. Liposomes for human applications are low stability, high cost for large scale production, toxicity of organic residue, and low entrapment efficiency. Pharmaceutical companies have required developing a technique to address the above issues. The new methods have been designed to alleviate the current issues for liposome formulation. Advantages and disadvantages of conventional methods were compared and summarized as shown in Table 2.9.

Table 2.9 Advantages and disadvantages for large scale production using the major conventional liposome formation methods (Meure et al., 2008)

Conventional methods	Advantages	Disadvantages
Bangham	Simple process	Large amount of organic solvent, requires vigorous agitation, large vesicles with no control on particle size, time consuming, sterilisation issue
Detergent depletion	Simple process, homogenous product, control of particle size	Large amount of organic solvent, detergent residue in the product, time consuming, poor entrapment efficiency, low yield, sterilisation issue
Ethanol/ether injection	Simple process	Organic solvent residue, nozzle blockage in ether system due to pre-evaporation, time consuming, sterilisation issue
Reverse phase evaporation	Simple design, reasonable encapsulation efficiency	Not suitable for encapsulation of fragile molecule due to large quantity of organic solvent use, time consuming, sterilisation issue

Table 2.9 Advantages and disadvantages for large scale production using the major conventional liposome formation methods (continued).

Conventional methods	Advantages	Disadvantages
Emulsion	Simple, potential to fabricate multivesicular liposome for delivery of multiple compounds that are not stable in combination	Large amount of organic solvent, requires vigorous agitation, sterilisation issue
Inkjet injection	Simple process, control on particle size and homogeneity, high potential for scale-up and encapsulation efficiency, low energy process	Organic solvent residue, low drug stability, sterilisation issue

2.10 Rationales of antifungal activity of drug containing liposomes

In order to under stand the antifungal mechanism of the liposomes prepared in this study, the general information of fungal need to be clarified.

2.10.1 The properties of fungal cell wall

The cell wall of fungal is a complex structure composed of mannoprotein, glucan, chitin, and other polymers extensive cross-linking between these components. An anionic surface and a reliance on β1,4- and β1,3-linked polysaccharides as major fibrous components (Lipke and Ovalle, 1998). Their wall structure is highly dynamic in changing constantly during cell division, growth and morphogenesis. Hydrolytic enzymes were closely associated with the cell wall, have been implicated in the maintenance of wall plasticity and may have roles during branching and

cross-linking of polymers (Adams, 2004). Furthermore, its rigid structure is useful as a force for the penetration of insoluble substrates that it colonizes or invades (Latge, 2007).

The fungal cell wall is a structure essential for the fungus that covers on the surface, and shape of the cell and protects the protoplast against adverse effects of the environment. They contain components that are not present in other cell types such as polysaccharides, chains of (1,3) β-glucan and (1,3) α-glucan, mannan and manoproteins (Figure 2.17) consequently presents an attractive target for new antifungal agents (Georgopapadakou and Tkacz, 1995; Balkovec et al., 1997; Farkaš, 2003). The cell wall maintains the shape of the cell, giving it tensile strength providing protection from extra-cellular stress and ultraviolet radiation. The wall is semi-permeable, retaining water while protecting the cell from high osmotic water pressure. The wall acts as a filter, keeping out large, dangerous molecules. Fungal cell walls enclose the cell plasma membrane and consist of three separate layers (Figure 2.17). The lower and middle glucans layers and outer glycoprotein layers are interwoven fibrillar polymers held together by covalent bonds (http://www.ehow.com/facts_6976258_structure-fungal-cell-wall.html). Chitin polymer chains are present throughout the cell wall, helping to maintain its structural morphology (Latge, 2007). Although the fungal cell wall is a rigid structure, it must be dynamic in order to allow budding, growth and adaptation to environmental stress (Pinto et al., 2008). The complexity of the cell wall means that its biogenesis demands a significant number of cellular activities that have to act in concert with the essential functions controlling cell growth and morphogenesis (Durán and Nombela, 2004).

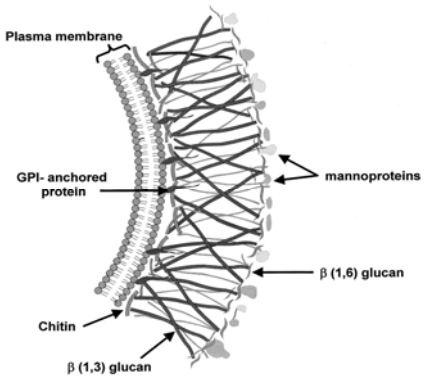


Figure 2.17 Illustration of the cell wall of fungi

(From: http://www.glogster.com/media/2/4/11/57/4115737.jpg)

De Souza Pereira and Geibel (1999) described that fungi obtain their food by digesting organic matters in their environment with enzymatic cocktails that produce small molecules that are then absorbed. Consequently, fungal cells must have efficient mechanisms for the transport and export of cellular products required for nutrient acquisition. Fungi use vesicular transport to deliver substances across their cell walls. Fungal vesicles are similar to mammalian exosomes and could originate from cytoplasmic multivesicular bodies. Vesicular transport enables the export of large molecules across the cell wall, and vesicles contain lipids, proteins and polysaccharides, many of which are associated with virulence. An alternative to trans-cell wall diffusion was suggested with the atomic force microscopy observation that *Saccharomyces cerevisiae* cell wall had pores of around 200 nm that could increase to 400 nm in stress conditions (De Souza Pereira and Geibel, 1999). The fungal cell wall was permeable to much larger molecules. However, such putative pores remain poorly characterized and their physiological function remains poorly understood. Additional evidence for the existence of pores on fungal cell walls comes from cryoporometry studies on acid-resistant melanized cell walls of *Cryptococcus neoformans* by cryoporometry, which revealed a population of pore sizes ranging from 1 – 4 nm to 30 nm (Casadevall et al., 2009). Moreover, it was also reported that contains undulations (rugosities) and diameter of pores (from 50 – 250 nm in diameter) present on the cell walls surface of *S. cerevisiae* strains (De Souza Pereira and Geibel, 1999).

2.10.2 Mechanism of cell transportation of liposome

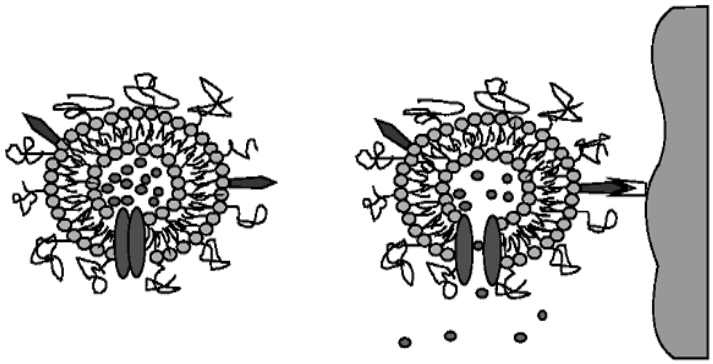
The liposome vesicle can transport active agent and interact with cells by four different mechanisms as bellow:

(1) Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils (Shaheen et al., 2006). Endocytosis delivers the liposome and its contents into the cytoplasm indirectly via a lysosomal vacuole in which low pH and enzymes may inactivate the encapsulated agent (Lasic, 1998).

(2) Adsorption to the cell surface either by nonspecific which occurs when the attractive forces (electrostatic, electrodynamics, van der waals hydrophobic insertion, hydrogen bonding, specific 'lock-and-key' etc.) exceed the repulsive forces (electrostatic, steric, hydration, ondulation, protrusion etc.). Specific interaction with cell surface components depends on the surface characteristics of liposomes (Lasic, 1998; Shaheen et al., 2006).

(3) Fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal content into the cytoplasm. The fusion of lipid membrane occurred due to change in free energy of each transition (Zimmerberg and Chernomordik, 1999) to neutralize charged of lipid species. Its was promoted by the incorporation of lipids bearing unsaturated fatty acyl, or by lipids with small uncharged head groups (Bailey and Cullis, 1997).

(4) Transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents as shown in a Figure 2.18.



Circulating closed liposome Targeted open liposome

Figure 2.18 Mechanism of transportation through liposomes (From: Shaheen et al., 2006).

In addition, liposome encapsulated antimicrobial agent can be used to enhance efficacy or safety for two reasons. First, they protect the entrapped drug against enzymatic degradation or unstable when exposed to light, acid or base conditions. For instance, the penicillins and cephalosporins are sensitive to the degradative action of β-lactamase, which is produced by certain microorganisms. Secondly, the lipid nature of the vesicles promotes enhanced cellular uptake of the antibiotics into the microorganisms, thus reducing the effective dose and the

incidence of toxicity as exemplified by the liposomal formulation of amphotericin-B (Uhumwangho and Okor, 2005).

2.11 Applications of nanoliposomes for drug delivery system

Nowadays, nanotechnologies have been widely used in drug delivery system (Haley and Frenkel, 2008). Many types of material were applied to produce nanostructures in drug delivery such as polymer, phospholipids, ceramic, metallic and carbon (Ochekpe et al., 2009). Among of these materials, nanostructures were constructed from phospholipids to give liposomes for drug delivery. Since the beginning was first found in 1965 by Bangham et al (Bangham et al., 1965a) until presently over the last 40 years have expanded considerably for liposome research.

Due to liposomes have many advantages for example, biocompatibility, biodegradability, can encapsulate of all hydrophilic or hydrophobic drugs, nontoxicity, reducing adversed effect of drug, cell specific targeting and control drug release. It is therefore appropriate system to use in delivering drugs to the body. However, limitation of liposome have been reported include low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components and poor storage stability (Ochekpe et al., 2009). Moreover, small liposomes (≤ 0.1 μm) are opsonized less rapidly and to a lower extent compared to large liposomes (> 0.1 μm) and therefore, the rate of liposome uptake by RES increases with the size of the vesicles (Sharma and Sharma, 1997). Therefore, if size of liposomal could not be well controlled, liposome encapsulated active drug may be uptaken by other cell and displayed cytotoxicity. Yin Win and Feng (2005) observed that nanoparticles of biodegradable polymers of small size and with appropriate surface coating may have great potential to be applied for oral delivery of anticancer drugs as well as other therapeutic agents (Figure 2.19).

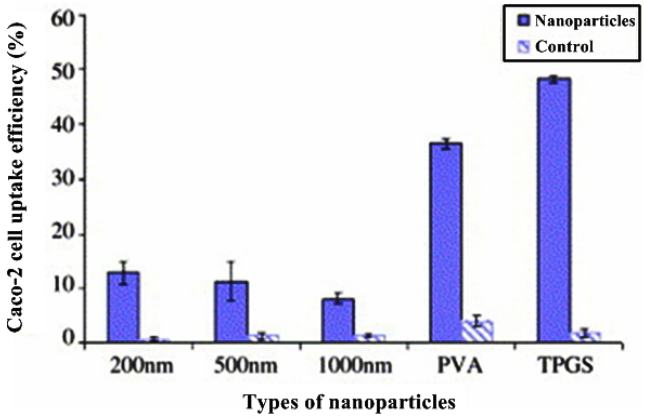


Figure 2.19 Cellular uptake efficiency of standard fluorescent polystyrene nanoparticles of 200, 500, 1000 nm diameter and poly(lactic-co-glycolic acid) (PLGA) nanoparticles coated with polyvinyl alcohol (PVA) or Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate), respectively, which is measured after 2 h incubation with Caco-2 cells at 37 °C. The control is the cellular uptake of coumarin-6 released from the nanoparticles under in vitro conditions and incubated with Caco-2 cells (From: Yin Win and Feng, 2005).

Nagayasu and co-worker (1999) described an important of the size of liposomes in delivery of an antitumor agent to a tumor. A liposome diameter of about 100 nm is likely to be an optimal size, not only for the more effective blood-to-tumor transfer of liposomes, but also for their longer retention in tumor tissue. However, it has been suggested that the pore size of tumor vessels varies depending on the type of tumor, the site of the tumor growth, and the degrees of tumor growth and regression. A liposome size of 100 nm thus is not always the most suitable for effective blood-to-tumor transfer, but this size may be a pivotal size of liposomes for tumor targeting, because it is also a pivotal size for prolonging the circulation of liposomes in blood (Nagayasu et al., 1999).

Nanoliposome could be used to improve the solubility of poorly water soluble drugs prolongs the half-life of drug in systemic circulation by reducing immunogenicity. It could release drugs at a sustained rate or in an environmentally responsive manner and thus lowers the frequency of administration. Moreover, it could deliver drugs in a target manner to minimize systemic side effects, and could deliver two or more drugs simultaneously for combination

therapy to generate a synergistic effect and suppress drug resistance (Zhang et al., 2008; Ochekpe et al., 2009).

Zhang and co-worker (2010) described two types of antimicrobial mechanism for liposome nanoparticle containing antimicrobial drug (Figure 2.20). (a) Liposome nanoparticles fuse with microbial cell wall or membrane and release the carried drugs within the cell wall or membrane; (b) liposome nanoparticles bind to cell wall and serve as a drug depot to continuously release drug molecules, which will diffuse into the interior of the microorganisms. Furthermore, in strategy of cancer therapy by nanosystem drug delivery have unique advantages are (1) they can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged; (2) they can penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord, and lymph (Liu et al., 2007). Liposomes particles especially in a range of nanosize have ability to target cancer cells. The endothelial cells of blood vessel bound in such a way to form tight junctions. These tight junctions inhibit the leakage of large particle (present in the blood) from the blood vessel. Liposomes having size less than 400 nm may target the cancer cells in the body because such a small size allows penetrating through the blood vessel (Kumar et al., 2011).

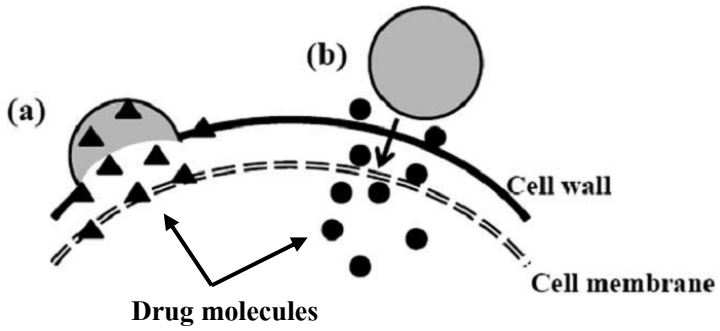


Figure 2.20 Mechanisms of liposome nanoparticles encapsulated antimicrobial drug delivery into microorganisms (modified frome: Zhang et al., 2008).

Guan and co-worker (2011) prepared nimodipine (NMD)-loaded nanoliposomes for injection. The results showed that nimodipine-loaded nanoliposomes with a 20 : 1 of egg yolk lecithin PL-100M vs. sodium cholesterol sulfate (SCS) ratio as the regulator and surfactant

instead of cholesterol. Nanoliposomes loaded nimodipine had a particle size of 86.8 ± 42.007 nm, a zeta potential of -13.94 mV and entrapment efficiency (EE) of 94.34% and could be stored for 12 days at 25˚C. SCS was superior to cholesterol because of its bulky sulfate group, which helped prevent liposomes fusing and allowed the preparation of much smaller liposomes. Lyophilized NMD-nanoliposomes were investigated by adding different cryoprotectants. Due to the bulking effect of mannitol and the membrane-protecting effect of trehalose, they were used together. Satisfactory freeze-dried products were obtained by using a mixture of 10% mannitol and 20% trehalose. Using differential scanning calorimetry, X-ray diffraction and scanning electron microscopy, it was found that trehalose could partially prevent mannitol forming crystals. The drug release from nanoliposomes before and after lyophilization in pH 7.4 PBS containing 30% ethanol was tested. Both release profiles fitted the Viswanathan equation, which showed that the drug release may be controlled by the pore diffusion resistance (Guan et al., 2011).

The solubility of hydrophobic drugs can be also improved using nanostructured delivery technologies where drug molecules are held within nanometre-width pores of porous microparticles. The drug is released through the pores as the matrix biodegrades. Solubility is increased owing to the huge surface area created by the nanostructuring process (Ogden and Parry-Billings, 2005). Presently, various nanotechnologies already have been commercialized to help deliver poorly water-soluble drugs into the body for example, Dissocubes (a patented technology currently owned by SkyePharma Plc), Nanocrystal technology (Elan Corporation, Dublin, Ireland), Nanomorph technology (Soliqs Abbott GmbH & Co. Kg, Ludwigshafen, Germany), Nanoedge technology (Baxter Healthcare Corporation, Deerfield, IL), Nanopure technology (PharmaSol GmbH, Germany), Crititech technology (CritiTech, Inc., Lawrence, KS), Nanocochleate technology (also known as bioral technology) and Controlled-flow cavitation (CFC technology (Five Star Technologies, Cleveland, OH) (Kharb et al., 2006).

2.12 Information of dermatophytosis

Fungal infection diseases have been of interest for centuries but diagnostic and causal including treatment of fungal infections remained unclear. However, a numerous publications

reported in the middle of 1800 centuries about this issue (Negroni, 2010). The historical aspects of dermatomycosis are summarized in Table 2.10. Dermatophytosis or dermatomycosis are skin diseases which caused by different genera of fungal infections, a group of related filamentous fungi also known as ringworm fungi. The infection of fungal could be found out horny layer of the skin and the appendages, such as the nails, hair, and surface of the mucous membranes (Rinaldi, 2000; Negroni, 2010). Since, they live in keratin layers and can used keratin as a nutrient source. These organisms invade keratinized tissue (skin, hair, and nails) and cause dermatophytosis or ringworm (Donnelly et al., 2000). The main fungi responsible for dermatophytosis which attack the keratinized tissue of the living host are classified into three genera: *Epidermophyton*, *Microsporum*, and *Trichophyton* (Rinaldi, 2000). These organisms can be further grouped into those that infect humans (anthropophilic). They are unable to colonize on other animals and have no other environmental sources. Those that infect animals (zoophilic) are primarily parasitic on animals, and infections may be transmitted to humans after contact with the animal host. Fungi that grow in soil (geophilic), normally inhabit the soil where it is believed they primarily decompose keratinaceous debris. However, some geophilic species may cause infections in animals and man after contact with soil.

Table 2.10 Historical aspects of dermatomycoses (Negroni, 2010).

Years (A.D.)	Occurrence
1835	Agostino Bassi discovered a fungus from the silk worm.
1837	Remak found the fungal nature of favus
1841	Berg reported oral candidosis
1846	Eichstedt described tinea versicolor clinically
1849	Wilkinson described vaginal candidosis
1853	etiologic agent of tinea versicolor was identified
1856	Beigel reported white piedra
1890	Sabouraud began the systematic study of dermatophytes
1891	Cerqueira reported tinea nigra
1910	Sabouraud published the book *Les Tiegnes*
1911	Horta described black piedra infection

Table 2.10 Historical aspects of dermatomycoses (continued)

Years (A.D.)	Occurrence
1927	Nannizzi reported the description of the sexual state of *Microsporum gypseum*
1934	Emmons published classification of dermatophytes
1952	Lodder and Kreger-van Rij described the taxonomy of yeast fungi
1958	Gentles succeed in treatment of tinea capitis with griseofulvin by using thallium
1981	Ketoconazole was introduced as the first azole derivative with systemic antifungal action.

Herein, we will be focused information of the major dermatophytes involved in human disease. Dermatophytes are fungi that infect skin, hair and nails due to their ability to utilize keratin. The organisms colonize the keratin tissue. Colonization is usually restricted to the nonliving, cornified layer of the epidermis because of the inability of dermatophytes to penetrate viable tissue of an immunocompetent host. However, infection does elicit a host response ranging from mild to severe that is related to the species and strain of fungus. Inflammation is then caused by host response to metabolic by products (Donnelly et al., 2000). The dermatophytes consist of three genera:

2.12.1 Epidermophyton spp. The maccroconidia are broadly clavate with typically smooth, thin to moderately thick walls and one to nine septa, with 20 – 60 × 13 μm in size. They are usually abundant and borne singly or in clustures. Microconidia are absent. This genus has only two known species to date and only *E. floccosum* is pathogenic.

Epidermophyton floccosum

E. floccosum was the first microorganism identified in tinea pedis. This anthropophilic organism is also a common cause of tinea cruris, tinea corporis, and onychomycosis. *E. floccosum* is the third most common agent associated with dermatophytosis, accounting for approximately 1 in 20 infections. Colonies of this organism are wrinkled or folded, usually

yellow to olive green in color, and have a very fine, fuzzy texture. Under the microscope, *E. floccosum* is identified by numerous, club-shaped conidia, smooth and thick cell walls, and no microconidia (Rinaldi, 2000).

2.12.2 Microsporum spp. Macroconidia is characterized by the presence of rough walls which may be asperulate, echinulate or verrucose (Weitzman and Summerbell, 1995). There are 19 described species but only 9 (*Microsporum audouinii, Microsporum canis, Microsporum cooeki, Microsporum ferrugineum, Microsporum gallinae, Microsporum gypseum, Microsporum nanum* and *Microsporum persicolo*r) are involved in human or animal infections. Some species of these genera were described as follow:

Microsporum canis

It is a zoophilic organism that occurs worldwide. *M. canis* is the major animal-associated fungus that can cause dermatophytosis of hair and skin in humans. The main reservoirs for this organism are cats and dogs. In urban areas, tinea capitis with animal origin is almost exclusively caused by *M. canis*. Although less common in humans than Trichophyton infections, lesions caused by *M. canis* appear to be more severe and are often accompanied by erythema. The *M. canis* colony is fluffy and white, with a bright yellow color sometimes seen below the mycelium. Microscopically, it has spindle-shaped macroconidia and a rough, thick outer wall (Rinaldi, 2000).

Microsporum gypseum

Infections caused by *M. gypseum*, a geophilic organism that has been isolated in almost every soil in the world, occur at a low incidence. *M. gypseum* causes ectothrix infections and has been identified in dermatophytosis of skin and hair, especially tinea barbae. As with *M. canis*, lesions caused by *M. gypseum* appear to be more severe than those involving *Trichophyton* organisms. The *M. gypseum* colony is flat, light tan to medium brown or cinnamon brown, and powdery to velvet in texture. Microscopic inspection of the organism reveals numerous spindle-

shaped macroconidia with 4 to 6 septa (Rinaldi, 2000).

2.12.3 Trichophyton spp. Macrocomidia, which have smooth, usually thin walls and one to 12 septa, are borne singly or in clusters. They may be elongate and pencil shaped, clavate, fusiform or cylindrical. Their size is in the range of 8 – 86 × 4 – 14 μm. Microconidia, usually more abundant than macroconidia, may be globose, pyriform or clavate, or sessile or stalked, and borne singly along the sides of the hyphae or in grape-like clustures (Weitzman and Summerbell, 1995). There are 20 species (*Trichophyton ajelloi, Trichophyton concentricum, Trichophyton equinum, Trichophyton erinacei, Trichophyton flavescens, Trichophyton gloriae, Trichophyton interdigitale, Trichophyton megnini, Trichophyton mentagrophytes, Trichophyton phaseoliforme, Trichophyton rubrum, Trichophyton schoenleinii, Trichophyton simii, Trichophyton soudanense, Trichophyton terrestre, Trichophyton tonsurans, Trichophyton vanbreuseghemii, Trichophyton verrucosum, Trichophyton violaceum* and *Trichophyton yaoundei*) causing infections in humans or animals. Some species of these genera were described as follow:

Trichophyton rubrum

Primarily an anthropophilic organism, *T. rubrum* is the most common agent of pedal and crural infections worldwide and is also often implicated in tinea corporis and tinea unguium. Identification of *T. rubrum* can be frustrating because cultures of this species are quite variable. The “downy” form, which is most commonly isolated in chronic tinea pedis and tinea corporis, is fluffy and white and has numerous aerial hyphae. The “granular” form manifests as a white colony that is flat, lacks aerial hyphae, and looks like sprinkles of sugar. The undersurface of both forms is usually red. Some colonies of *T. rubrum* may appear indistinguishable from *T. mentagrophytes* (Rinaldi, 2000).

Trichophyton mentagrophytes

In addition to being a common organism in tinea pedis throughout the world, *T. mentagrophytes* can cause tinea capitis, tinea corporis, tinea cruris, and tinea barbae. There are

various forms of *T. mentagrophytes* been reported. One major form, which is usually anthropophilic, has a white, fluffy, "downy" appearance. The other major form has a flat, "granular" appearance and few aerial hyphae; this form is typically zoophilic in origin. The underside of all forms of *T. mentagrophytes* is usually pale yellow. Sometimes the mycelium of *T. mentagrophytes* is red to yellow-brown, making it difficult to differentiate from *T. rubrum*. Even microscopically, the two organisms can appear indistinguishable (Rinaldi, 2000).

Trichophyton tonsurans

Also occurring worldwide, *T. tonsurans* is an anthropophilic organism that most commonly causes tinea capitis and is also seen in tinea corporis, tinea pedis, and occasionally onychomycosis. *T. tonsurans* appears to be increasing in North America, and in some laboratories it is the second most commonly isolated dermatophyte. Colonies of *T. tonsurans* have short, aerial hyphae with several wrinkles and folds, giving the appearance of suede, and the underside is a rich red to brown color. Microscopically, *T. tonsurans* is characterized by numerous microconidia that are usually clubshaped (Rinaldi, 2000).

Trichophyton verrucosum

Native to cattle and horses, *T. verrucosum* accounts for a very small percentage of dermatophytes isolated in the United States. Although it can be a significant cause of human dermatophytosis in rural areas. A vaccine for animals is available to counteract the infectious spread of *T. verrucosum*, which can remain viable on doors, fences, wood pens, and similar places for years. In humans, *T. verrucosum* can cause tinea barbae, tinea capitis, tinea corporis, and occasionally fingernail onychomycosis. The surface of the colony is dull white or gray to yellowish tan. *T. verrucosum* can be identified microscopically by its characteristic chains of chlamydoconidia (Rinaldi, 2000).

Trichophyton soudanense

As a geographically restricted anthropophilic dermatophyte, *T. soudanense* is native to the tropical lowlands and semi-dried areas of middle Africa, including Somalia, Zaire, and Angola. It can cause chronic and often disfiguring endothrix tinea capitis in the trans-Sudan belt, infecting primarily blacks and rarely seen in whites. Although not indigenous to the United States or Europe, *T. soudanense* has been isolated from African immigrants, and in the carrier stage, it may possibly exist for generations. Infections caused by *T. soudanense* are still uncommon in the United States, but are increasing as the number of immigrants from middle Africa rises. The *T. soudanense* colony looks identical to the colony of a powdery variety of *T. soudanense*. Under the microscope, however, *T. soudanense* contains a mixture of comedoconidia, with distinctive long pedicles that sit beneath the microconidia and are unique to this organism (Rinaldi, 2000).

It is known that host defence mechanisms influence the manifestation and severity of fungal infections, such that the clinical forms of the disease depend on a patient's immune response (Pinto et al., 2008). Glycoproteins, glycosphingolipids and polysaccharides exposed at the most external layers of the wall are involved in several types of interactions of fungal cells with the exocellular environment. These molecules are fundamental building blocks of organisms, contributing to the structure, integrity, cell growth, differentiation and signaling. Several of them are immunologically active compounds with potential as regulators of pathogenesis and the immune response of the host. Some of these structures can be specifically recognized by antibodies from patients' sera, suggesting that they can be also useful in the diagnosis of fungal infections (Pinto et al., 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals for used to prepare and determine properties of liposome containing rhinacanthin-C

- Egg yolk phosphatidylcholine (Fluka®, Germany)
- 3-sn-phosphatidylcholine from soybean (Fluka®, Germany)
- Cholesterol from lanolin (Fluka®, USA)
- Deoxycholic acid (Fluka®, USA)
- Sabouraud dextrose agar (Merck®, Germany)
- Sabouraud dextrose broth (Merck®, Germany)
- Crystal violet (Sigma-Aldrich, USA)
- Tween 80 (Srichand Co., Ltd., Thailand)
- Ammonium chloride (NH_4Cl) (Sigma-Aldrich, USA)
- Sodium acetate ($C_2H_3O_2Na$) (Sigma-Aldrich, USA)
- Ammoniums oxalate ($NH_4OOCCOONH_4$) (Fluka®, USA)
- Potassium iodide (KI) (Sigma-Aldrich, USA)
- Safranin O ($C_{20}H_{19}N_4Cl$) (Fluka®, USA)
- Potassium dihydrogen phosphate (KH_2PO_4) (Merck®, Germany)
- Ammonia (NH_3) solutions (Loba Chemie Pvt. Ltd., India)
- 95% ethanol (EtOH), commercial grad (High Science Ltd. Songkhla, Thailand)
- Dimethyl sulfoxide (DMSO), analytical grade (Labscan Ltd., Bangkok, Thailand)
- Sodium hydroxide (NaOH) (Labscan Ltd., Bangkok, Thailand)
- Sodium chloride (NaCl) (Labscan Ltd., Bangkok, Thailand)
- Hydrochloric acid (HCl), analytical grade (Labscan Ltd., Bangkok, Thailand)
- Ketoconazole (raw material), commercial grade (High Science Ltd. Songkhla,

Thailand)

3.1.2 Microorganisms

The tested microorganisms used for biological eavaluation consisted of *Microsporum gypseum* (SH-MU-4) obtained from the Department of Microbiology, Faculty of Science, Prince of Songkla University. *Trichophyton rubrum* (SK-10-09) obtained from Songklanagarind hospital.

3.1.3 Instruments and equipments

- Micropipette 1000 and 200 µl (Biorad®, USA)
- Tips size 10 and 200 µl (Axygen Scientific®, PIPET TIPS T-200-Y, Lot no. 070718-077, USA)
- Tips size 1000 µl (ISOLAB® Laborgeräte GmbH, Germany)
- Petri dish 90×15 mm (purchased from High Science Ltd. Songkhla, Thailand)
- Test tube 16×125 mm and 25×150 mm (PYREX®, Mexico)
- Funnel Ø 50 mm (ISOLAB® Laborgeräte GmbH, Germany)
- Cotton swabs (Ambulance®, Bangplee cotton industries co.,ltd., Thailand)
- Cuvettes (PLASTIBRAND®, semi-micro 1.5 mL, Germany)
- Vernier Caliper (Mitutoyo, Japan)
- Hot plate and magnetic stirrer (Cemarec®2 Thermolyne SP46920-33, USA)
- Hot air oven (Memmert UNV-500, Germany)
- Autoclave (HV 110, Hirayama, Japan)
- Incubator (Memmert INP 700, Germany)
- Rotary evaporator (BÜCHI, Switzerland)
- Votex-2 GINIE Scientific Industries (Model G-560E, USA)
- UV-visible spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer, USA)
- pH meter (Seven Easy S-20, USA)

- Particle size analyzer (Malvern instruments, UK)
- Zeta potential analyzer (Model Zeta PALS, Brookhaven Instrument, USA)
- High performance liquid chromatography (Thermo Fisher Scientific, USA)
- Scanning electron microscope (Model JSM-5800LV, JEOL, Tokyo, Japan)

3.2 Methods

3.2.1 Extractions and identifications of rhinacanthin-C

3.2.1.1 Plant material

Leaves of *R. nasutus* were collected from Jana district, Songkhla province, Thailand in May 2006. They were authenticated at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, where herbarium specimen (Voucher No. 001 18 14) is kept.

3.2.1.2 Chemicals and reagents for used to extract and identify of rhinacanthin-C

- Methanol (MeOH), HPLC grade (Labscan Asia co., Thailand)
- Methanol (MeOH), commercial grade (Labscan Asia co., Thailand)
- Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA)
- Acetic acid (CH_3COOH), glacial AR grade (Labscan Asia co., Thailand)
- Ethyl acetate (EtOAc) commercial grade (Labscan Asia co., Thailand)
- Amberlite® IRA-67 (SIGMA, USA)
- Hexane (C_6H_{14}), AR grade (Labscan Asia co., Thailand)
- Dichloromethane (CH_2Cl_2), AR grade (Labscan Asia co., Thailand)
- Petroleum ether, AR grade (Labscan Asia co., Thailand)
- Silica gel 60 F_{254} precoated plated (Merck®, Germany)
- Standard rhinacanthin-C was previously purified by Assoc. Prof. Dr. Pharkphoom

Panichayupakaranant (Kongchai and Panichayupakaranant, 2002) and was identified by CHNO-elemental analysis. The CHNO-elemental analysis has been performed by CHNS-O analyzer.

3.2.1.3 Extraction procedure

The leaves of *R. nasutus* were dried in hot air oven at 50-60°C for 48 hour. The dried samples were ground to the fine powder and passed through the sieve number 45. The dried leaf powder of *R. nasutus* (500 g) was extracted three times with ethyl acetate (2 L each time) by maceration for 3 days. The pooled extracts were dried *in vacuo* (Kongchai and Panichayupakaranant, 2002).

3.2.1.4 Rhinacanthin-C purification method

3.2.1.4.1 Purification using anion exchange resin

An adequate volume of methanol (250 mL) was added into 500 g anion exchange resin (Amberlite® IRA-67) and gently stirred. After allowed to stand for 15 min, the methanol was decanted and the slurry was washed twice with distilled water (2 × 500 mL), and then allowed to stand in methanol for a further 5 - 10 min. The treated resin was poured into a glass column (5×35 cm) and the excess methanol was drained. A portion of methanol (200 mL) was then added to settle the resin. The leaf extract of *R. nasutus* (5 g) was dissolved in methanol (200 mL) and filtered. The filtrate was then loaded on the anion exchange column and allowed the solution to pass through the column with a flow rate 1.5 mL/min until finish. The column was then washed with methanol until the green color in the column was disappeared. Rhinacanthins were then eluted with 10% acetic acid in methanol with a flow rate 2 mL/min. The elutent was evaporated to dryness *in vaccuo* (Kongchai and Panichayupakaranant, 2002).

3.2.1.4.2 Purification using silica gel column chromatography

The further purification was performed by using silica gel column chromatography. The silica gel column was prepared by weighing about 50 g of silica gel and wet with mobile phase [a mixture of dichloromethane:hexane (4 : 1)] 300 mL. The slurry of silica gel in mobile phase was packed into a glass column (5 × 35 cm) and the excess mobile phase was drained. The silica gel column was saturated with mobile phase for 30 min before using. The extracts (1 g) were dissolved with mobile phase 10 mL. The solution was loaded on silica gel column and allowed the solution to pass through the silica gel column with a flow rate of 1.0 mL/min.

Fractions of 50 mL each were collected and checked for the presence of rhinacanthin-C by thin layer chromatography (TLC). Fractions containing only rhinacanthin-C were pooled and evaporated to dryness *in vaccuo*. The pure rhinacanthin-C was kept in a glass bottle and stored in a refrigerator protect from light until needed.

3.2.1.5 Thin layer chromatographic system

Thin layer chromatography (TLC) was used for rhinacanthin-C identification in both *R. nasutus* extract and in final product after purification. Siliga gel $60F_{254}$ on aluminium sheet was used as stationary phase and mixtures of dichloromethane : hexane (4 : 1); ethyl acetate : hexane (3 : 7) and dichloromethane: petroleum ether (4 :1) were used as mobile phases. Standard rhinacanthin-C (Kongchai and Panichayupakaranant, 2002) was used as the reference marker. The standard rhinacanthin-C was accurately weighed about 10 mg and transferred to a 10 mL volumetric flask. Methanol was added and adjusted to volume and then mix the standard and test solutions were spotted on the TLC plate. The TLC plate was then developed in a TLC tank, using dichloromethane:hexane (4 : 1) as developing solvent. After that the plate was dried, solvent was removed, and detected the chromatogram under UV at a wavelength of 254 nm.

3.2.2 Preparation of liposomes containing rhinacanthin-C

3.2.2.1 Optimization for suitable compositions for liposome formulations

Initially, formulations for preparation of liposomes were optimized to evaluate the suitable compositions to construct liposomal membrane that will be used for rhinacanthin-C entrapment. Three types of phospholipidlipids were chosen to use in this study 3-sn-phosphatidylcholine from soybean (SPC), egg yolk phosphatidylcholine (EPC) and cholesterol (CHL). Other additive substances such as Tween80 (T80) and deoxycholic acid (DA) were also used to determine the affect on the obtained liposome formulations. Amount of rhinacanthin-C and lipid compositions used in liposome formulation are summarized in Table 3.1. The best formulations from this observation were further studied the effect of additives in liposome formulations as shown in Table 3.2.

Table 3.1 Various liposome formulations to study the effect of phospholipid compositions on physicochemical properties of liposomes containing rhinacanthin-C.

Formulation No.	Composition of lipids	Total lipid (μmol/mL)	Molar ratio	Amount of rhinacanthin-C (mg/3mL liposome)
1, 2, 3	EPC : CHL	20	1:1, 1:2, 1:3	15
4, 5, 6	EPC : CHL	40	1:1, 1:2, 1:3	15
7, 8, 9	EPC : CHL	60	1:1, 1:2, 1:3	15
10, 11, 12	SPC : CHL	20	1:1, 1:2, 1:3	15
13, 14, 15	SPC : CHL	40	1:1, 1:2, 1:3	15
16, 17, 18	SPC : CHL	60	1:1, 1:2, 1:3	15

3.2.2.1.1 Preparation of stock solution of rhinacanthin-C

Rhinacanthin-C was accurately weighed about 250 mg, and dissolved in ethanol and adjusted to a final concentration of rhinacanthin-C 25 mg/mL (Figure 3.1).

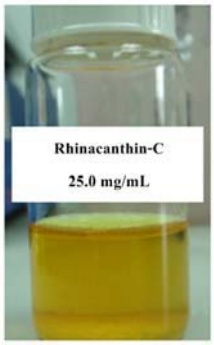


Figure 3.1 stock solution of rhinacanthin-C in ethanol

Liposomes containing rhinacanthin-C were prepared by a modified ethanol injection method (Maitani et al., 2001b) as following procedure. All lipids were dissolved in about 3 mL of 95% ethanol. Rhinacanthin-C stock solutions in ethanol (600 µL) were added to the lipids mixture (oil phase). Then oil phase (3 mL) and water (3 mL) were separately stirred in water bath at 60°C (BÜCHI, Switzerland) for 5 min. The water was then added to oil phase while stirring and continued stirring at 60°C until mixed well. The ethanol was then removed with evaporation under reduced pressure by a rotary evaporator (BÜCHI, Switzerland). Liposomes formed spontaneously after evaporation of the ethanol. Distilled water was added to the residue and volume was adjusted to 10 mL. The resulting liposomes were transferred to a vial and stored at 4°C protected from light for further determination for their physicochemical properties and antifungal activities.

The physical appearance of all liposomes containing rhinacanthin-C made of EPC gave dark yellow turbid solutions and lipids aggregation was observed. Their viscosity increased when increasing amount of total lipid from 20, 40 and 60 µmol/mL, respectively. Therefore, EPC was not chosen to be used in the further study. The physical appearances of the liposome formulations which composed of SPC displayed better appearance of yellowish turbid colloidal solutions. Lipid aggregation and sedimentation were not observed. The formula containing total lipid of 60 µmol/mL were shown to have higher viscosity and bigger particle size more than the formulations using 20 and 40 µmol/mL of total lipids. However, all formula of 20 µmol/mL of total lipid was shown to have lower entrapment efficiencies than all formula containing 40 and 60 µmol/mL of total lipid. Moreover, it was found that 15 mg of rhinacanthin-

C added in liposomal formulations were too much excess resulting in interference of drug release from membrane during dialysis method as shown in Figure 4.5. Since rhinacanthin-C is a lipophilic compound, it is poorly soluble in aqueous medium, an excess of rhinacanthin-C will present on the surface of dialysis membranes, this phenomenon also affected on determination of entrapment efficiency process.

Therefore, in order to further investigate the optimal formulation of liposome containing rhinacanthin-C, the liposomes were prepared by lowering concentration of rhinacanthin-C to 4 mg in all formulations. The total lipid of 40 μmol/mL was used in all formulations. Compositions of lipids which were used to study the effect of additives on liposome formulations are summarized in Table 3.2.

Table 3.2 Compositions of liposome formulations to investigate the effect of additives.

Formulation No.	Composition of lipids	Molar ratio	Total lipid (μmol/mL)	Amount of rhinacanthin-C (mg/3mL liposome)
19	SPC : CHL	1:1	40	4
24	SPC : CHL : DA	1:1:1	40	4
25	SPC : CHL : DA	1:1:0.5	40	4
26	SPC : CHL : DA	1:1:0.25	40	4
27	SPC : CHL : Tween 80	1:1:1	40	4
28	SPC : CHL : Tween 80	1:1:0.5	40	4
29	SPC : CHL : Tween 80	1:1:0.25	40	4

SPC = Soybean phosphatidyl choline, CHL = Cholesterol, DA = Deoxycholic acid

3.2.2.1.2 Preparation of rhinacanthin-C working solution and liposome containing rhinacanthin-C

Working solution of rhinacanthin-C was prepared by transferring the stock solution (25 mg/mL) 800 μL to 10 mL volumetric flask. Then volume was adjusted to 10 mL with ethanol to give a solution of rhinacanthin-C having concentration of 2 mg/mL.

Liposomes containing rhinacanthin-C were prepared by a modified ethanol injection method (Maitani et al., 2001b). Oil (3 mL) and aqueous phases (3 mL) were prepared separately as summarized in Table 3.3.

Table 3.3 The composition of oil phase and aqueous phase which were used in liposome preparations for investigate the effect of additives.

Formulation No.	Oil phase dissolved in 95% ethanol (3 mL)	Aqueous phase (3 mL)
20	SPC : CHL : DA (1:1:1) 0.0942 g:0.0464 g:0.0471 g + rhinacanthin-C 4 mg	distilled water only
21	SPC : CHL : DA (1:1:0.5) 0.0942 g:0.0464 g:0.0235 g + rhinacanthin-C 4 mg	distilled water only
22	SPC : CHL : DA (1:1:0.25) 0.0942 g:0.0464 g:0.0118 g + rhinacanthin-C 4 mg	distilled water only
23	SPC : CHL : Tween 80 (1:1:1) 0.0942 g:0.0464 g:0.1572 g + rhinacanthin-C 4 mg	distilled water only
24	SPC : CHL : Tween 80 (1:1:0.5) 0.0942 g:0.0464 g:0.0786 g + rhinacanthin-C 4 mg	distilled water only
25	SPC : CHL : Tween 80 (1:1:0.25) 0.0942 g:0.0464 g:0.0393 g + rhinacanthin-C 4 mg	distilled water only

Briefly, all lipids mixture of each formula were accurately weighed (as shown in table 3.3) and placed into a 15 mL glass vial, 2 mL of working solution of rhinacanthin-C was added and finally 1 mL of 95% ethanol was added to give oil phase. The total volume of oil phase was 3 mL. Next, an oil phase (3 mL) and aqueous phase (3 mL) were separately stirred in water bath at 60°C (BÜCHI, Switzerland) for 5 min. The aqueous phase was added to oil phase while stirring and continued stirring at 60°C until mixed well. The ethanol was then removed with evaporation under reduced pressure by a rotary evaporator (BÜCHI, Switzerland). Liposomes formed spontaneously after evaporation of the ethanol. Distilled water was added to the obtained liposome to adjust volume to 10 mL. The resulting liposomes were transferred to a

vial and stored at 4°C protected from light for further determination for their physicochemical properties and antifungal activities.

3.2.3 Physicochemical characterization of liposome containing rhinacanthin-C

3.2.3.1 Liposomal size measurement

The particle size distribution of liposomes was determined using the photon correlation spectroscopy (Malvern, Worcestershire, UK, Figure 3.2). The mean particle sizes and polydispersity index (PI) of samples were analyzed at 25°C with a fixed angle of 90 degrees, in triplicate for each sample. The liposome suspensions were diluted with Milli-Q water to obtain the samples until a suitable rate meter reading (100,000 counts/s). Size distribution can be monomodal (one population) or plurimodal (several populations), and monodisperse (narrow distribution) or polydisperse (broad distribution) as shown in Figure 3.3 (Gaumet et al., 2007). Usually, size distribution of particles was considered by using the polydispersity index (PI) values. The calculation of PI takes into account the particle mean size, the refractive index of the solvent, the measurement angle and the variance of the distribution. Lower values of PI (< 0.2) are indicated homogeneous vesicle population, while a higher value of PI (> 0.3) indicates heterogeneity (Song and Kim, 2006).

Figure 3.2 The particle size analyzer (Malvern instruments, UK)

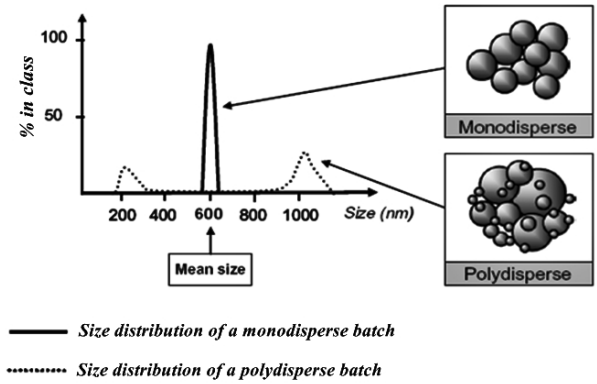


Figure 3.3 Schematic representations of two particle batches corresponding to a monodisperse population and to a bimodal polydisperse population. The graph represents the curves obtained for each batch after analysis by light scattering (From: Gaumet et al., 2007).

3.2.3.2 Microscopic investigations of liposome containing rhinacanthin-C

The morphological characteristics of liposome containing rhinacanthin-C were observed by Scanning Electron Microscope (Kremer et al., 1977) (JEOL®, Model JSM-5800LV, Japan). The particle image obtained by SEM could be used to assure the result of particle size measurement by particle size analysis. The sample preparations for SEM analysis were performed using modified Gram staining techniques.

3.2.3.2.1 Gram staining technique

Gram staining technique is technique which is usually used for identification of gram positive and gram negative bacteria. Mechanism of gram staining reaction could be explained by positively charged of crystal violet pass through the cell wall and cell membrane and binds to negatively charged components inside the cell. Addition of negatively charged iodide (in the mordant) binds to the positively charged dye and forms a large dye-iodide complex within the cell. Crystal violet (Hexamethylpararosaniline chloride) interacts with aqueous KI-I_2 via a simple anion exchange to produce a chemical precipitate. The small chloride anion is replaced by the bulkier iodide, and the complex thus formed becomes insoluble in water. During decolorization by alcohol, the lipid present in the outer membrane of gram negative bacteria is dissolved and the

dye-iodine complex out of the cell is leached. The dye-iodine complexes are washed from the gram negative cell along with the outer membrane. After decolorization, the counter stain which is usually positively charged safranin is applied and binds to negatively charged components inside the plasma membrane of negative bacteria (Figure 3.4). Hence gram negative cells readily get a pink or red color. On the other hand gram positive cells become dehydrated from the ethanol treatment, closing the pores as the cell wall shrinks during dehydration. The dye-iodine complex gets trapped inside the thick peptidoglycan layer and does not get decolorized (Figure 3.3) (Medical Chemical Corporation, 2005; Sridhar, 2006).

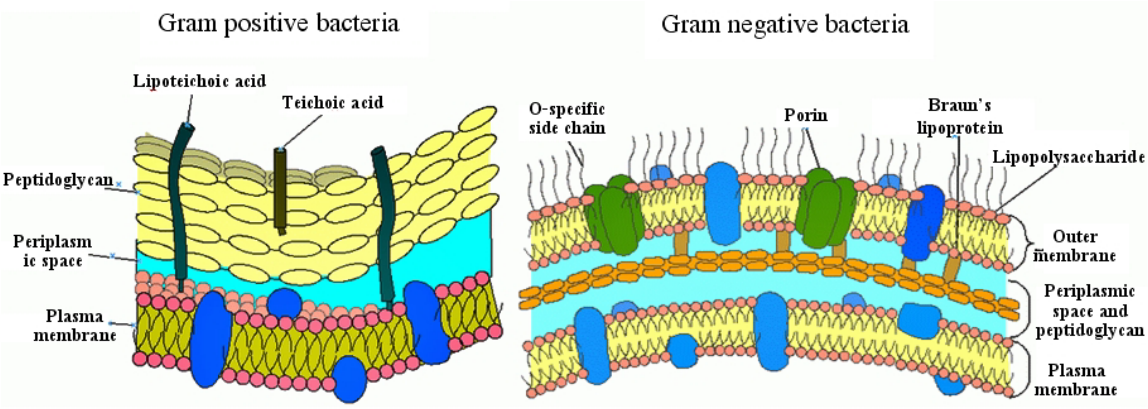


Figure 3.4 The main difference between gram negative or positive bacteria is the structure of their cell wall. Cell wall of gram negative bacteria has more layers than gram positive bacteria, these are used to identify bacteria by gram stain technique (From: http://variety.teenee.com/world/1848.html).

3.2.3.2.2 Modifications of gram staining technique for examine physical morphology of liposome particles by SEM technique

According to the main structure of liposomes composed of phospholipids bilayer similar to the cell membrane of bacteria. Therefore, using gram staining technique to investigate the physical morphology of liposome could be possible. Since in a step of sample preparation for SEM analysis need chemical fixation using glutaraldehyde or formaldehyde to preserve and stabilize structure of specimens. If the liposome was fixed by this method, it will cause liposome collapse and shrinkage. In addition, the fixation reagent caused lipid membrane decomposition. Therefore, fixation technique in preparation of liposome sample for SEM analysis should be avoided. In this study the liposome samples were fixed on a glass slide by simple film drying

technique. Two types of staining techniques were utilized to investigate the effectiveness on morphology study, staining with crystal violet and staining with safranin O. Decolorization step was skipped, since the walls of liposomes were needed to preserve for morphology determination.

Preparation of reagents for the liposomal staining

1. Crystal violet solutions:
   Solution A: crystal violet 2 g in 95%ethanol 20 mL.
   Solution B: 0.8 g ammonium oxalate in 80 mL distilled water.
   The solutions A and B were mixed and stored at room temperature for 24 hours before use.
2. Gram's iodide solution: 2.0 g of potassium iodide (KI) was weighed and dissolved in 100 mL of distilled water.
3. Safranin solution: Safranin O powder (0.25 g) was dissolved in 10 mL of ethanol and diluted to 100 mL with distilled water and mixed well before use.

Preparation of the slide samples containing liposomes for SEM

(a) Liposome staining by crystal violet solution

Liposome samples (100 µl) were diluted with Milli-Q water (200 µl) and mixed well. A drop of liposome solution was transferred to the surface of a glass cover slip (size 1.0×1.0 cm), and smeared over a small area. The film was allowed to air dry and fixed by a dryer. Crystal violet solution (2-3 drops) was dropped on the dry film and left standing for one minute and washed off briefly with water. Gram's Iodide solution (2-3 drops) was added on the film and allows standing at room temperature for one minute. An excess solution was removed by soft tissue paper.

(b) Liposome staining by safranin solution

Liposome samples (100 µl) were diluted with Milli-Q water (200 µl) and mixed well. A drop of liposome solution was transferred to the surface of a glass cover slip (size 1.0×1.0 cm),

and smeared over a small area. The film was allowed to air dry and fixed by a dryer. Safranin solution (2-3 drops) was added on the dry film and left standing for 30 seconds and washed off briefly with water. An excess solution was removed by soft tissue paper.

The slides containing liposomes samples were investigated for their physical morphology of liposome particle by scanning electron microscope (Model JSM-5800LV, JEOL, Tokyo, Japan). The slide was placed on a brass stub and then coated with gold by sputtering technique using fine coating machine (JFC-1200, JEOL, Tokyo, Japan) for 2 min. The sample was then observed under SEM at magnification ranging from 10,000 to 45,000.

3.2.3.3 Encapsulation efficiencies determinations

Optimization of dialysis techniques:

Rhinacanthin-C solution (25 mg/mL) was transferred 500 µL (12.5 mg) and filled into dialysis membrane (MW cut off: 3,500 g/mole) which wetted with phosphate buffer pH 7.4 solutions for 30 minutes. The dialysis bag was then sealed to ensure no leakage of the content inside the bag. The bag was then transferred to 400 mL of buffer pH 7.4 containing 20% ethanol as receiver medium. The receiver medium was stirred with a magnetic stirrer at room temperature until amount of rhinacanthin-C in receiver medium was 31.25 µg/mL. Ten milliliters of dialysis medium was withdrawn for measuring the amount of rhinacanthin-C releasing at different time. The amount of rhinacanthin-C was determined by using a method based on detection by UV absorbance at a wavelength of 278 nm and calculated by using calibration curve of rhinacanthin-C (Conc^{n} 1 - 50 µg/mL).

Encapsulation efficiencies of liposome containing rhinacanthin-C:

Liposome encapsulation efficiency was determined by using modified dialysis technique to separate non-entrapped or free rhinacanthin-C from liposomes (Mura et al., 2007). Cellulose membranes (MW cut off: 3,500 g/mole) were kept into phosphate buffer pH 7.4 solution for 30 minutes before use to ensure the complete membrane wetting. Ten milliliters of liposome solution containing rhinacanthin-C were placed into the dialysis bag. The dialysis bag was then

sealed to ensure no leakage of the content inside the bag. The bag was then transferred to 1000 mL of buffer pH 7.4 containing 20% ethanol as receiver medium. The receiver medium was stirred with a magnetic stirrer for 24 h at room temperature to let free rhinacanthin-C which was not entrapped into liposomes released to dialysis medium. After that, an aliquot of 10 mL of dialysis medium was withdrawn for measuring the amount of free rhinacanthin-C. The amount of free rhinacanthin-C was determined by using a method based on detection by UV absorbance at a wavelength of 278 nm (Hewlett Packard 8452A, Diode Array Spectrophotometer) (Liu et al., 2006). The concentrations of rhinacanthin-C in dialysis medium were calculated using calibration curve of rhinacanthin-C ($Conc^{n}$ 0.5-5 μg/mL). The percentage of encapsulation efficiency was then calculated according to the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{[\text{total rhinacanthin - C}] - [\text{free rhinacanthin - C}]}{[\text{total rhinacanthin - C}]} \times 100$$

Where total rhinacanthin-C is all rhinacanthin-C added in each liposome formulations, and free rhinacanthin-C is amount of the total rhinacanthin-C released to dialysis medium after stirring for 24 h. The optimized method of an UV-Visible spectrophotometric was validated and to describe in detail in section 3.2.3.5.

3.2.3.4 Stability determination of liposomes containing rhinacanthin-C

Stability of the liposome formulations was measured in terms of the size evolution and the ability of the liposome to remain rhinacanthin-C in the liposomes during the storage period (0, 7, 14, 21, 30, 60 and 90 days) at room temperature, in the neutral, acid and base conditions. Determination of amount of rhinacanthin-C remained in the vesicles was analyzed by HPLC (Figure 3.5). Size evolution was determined by Zeta potential analyzer (Model Zeta PALS, Brookhaven Instrument, USA) (Figure 3.6) during the storage period (0, 30, 60 and 90 days) at room temperature. Sample preparation method for stability determination of size particle was described in section 3.2.3.1.

Figure 3.5 High performance liquid chromatography (HPLC) systems used for determination of rhinacanthin-C stability in liposome (Themo-isocretic pump, USA).

Figure 3.6 Zeta potential analyzer (Model Zeta PALS, Brookhaven Instrument, USA) used size stability determination of liposome during storage.

Since, rhinacanthins were known to have poorly stable when stored in acid-base conditions (Charoonratana, 2007). Therefore, in this study, we determined stability of rhinacanthin-C in liposome during storage period in difference conditions [acid (pH 4.0), neutral (pH 6.6) and base (pH 10.0)] for six months.

3.2.3.4.1 Preparation of buffer solutions for liposome storage

(a) Preparation of Ammonia-ammonium chloride buffer pH 10.0 (base conditions)

Ammonium chloride (67.5 g) was dissolved with concentrate ammonia solution 570 mL and diluted with distilled water to adjust volume to 1000 mL.

(b) Preparation of acetate buffer pH 4.0 (acid conditions)

To prepare 1000 mL of the acetate buffer solution, 150 g of sodium acetate was dissolved in 250 mL of distilled water. Exactly 15 mL of glacial acetic acid was then added very slowly into the sodium acetate aqueous solution. Finally, distilled water was added into the solution to fill the volume.

(c) For neutral conditions, distilled water (pH 6.6) was used in the study.

3.2.3.4.2 Preparation of stock solution of liposome for stability study of rhinacanthin-C in liposome

Liposomes containing rhinacanthin-C (4 mg) were diluted to 10 mL with buffer solution (pH 4.0. 6.6 and 10.0) to give final samples having concentration of 400 µg/mL. Freshly prepared liposome samples were used in this study. The samples were prepared in triplicate.

Two milliliters of working solution of rhinacanthin-C (2 mg/mL, from section 3.2.2.1.2) were transferred to another 10 mL volumetric flask and then volume was adjusted to 10 mL with ethanol to give a concentration of 400 µg/mL. Next, 1 mL of rhinacanthin-C solutions (400 µg/mL) was transferred to another 10 mL volumetric flask and then volume was diluted to 10 mL with buffer solution (pH 4.0. 6.6 and 10.0) to give a concentration of 40 µg/mL for using as control samples. Samples for each condition were prepared in triplicate.

All samples (liposomes samples and control rhinacanthin-C solutions) prepared in different conditions (pH 4.0. 6.6 and 10.0) were kept at room temperature protect from light for six months. Samples were taken at the selected times during storage to determine the remaining of rhinacanthin-C and size of liposome.

3.2.3.4.3 Preparation of sample for HPLC determination

(a) Sample preparation:

Liposome samples (500 µl) which were stored at each condition (pH 4.0, 6.6 and 10.0) was separately transferred into a 10 mL volumetric flask and adjusted volume with methanol to

obtained sample having concentration of 20 µg/mL. Then, 1 mL of the resulting solution was transferred to a 10 mL volumetric flask and adjusted the volume with methanol to yield the test sample having concentration of 2.0 µg/mL. A final concentration (2.0 µg/mL) was injected into the HPLC system.

(b) Control sample preparation:

Rhinacanthin-C solutions (500 µl) which were kept at each condition (pH 4.0, 6.6 and 10.0) were separately transferred into 10 mL volumetric flask and adjusted volume with methanol. Final solutions having of 2.0 µg/mL were injected into the HPLC system.

(c) Preparation standard solutions of rhinacanthin-C:

A standard curve of rhinacanthin-C was constructed using varied concentrations (0.5, 1.0, 2.0 and 3.0 µg/mL) of rhinacanthin-C in methanol. The standard curve was obtained by plotting between concentrations versus peak area of rhinacanthin-C from each concentration from HPLC chromatogram. Standard curve of rhinacanthin-C was performed in each time of analysis.

(d) HPLC conditions:

Mobile phase: The mobile phase was a mixture of methanol and 5% acetic acid (80: 20). It was filtered through a 0.45 µm membrane filter, and degassed by sonication for 30 min before use.

Analytical column: Waters, Symmetry® C_{18} 5 µm, 3.9 × 150 mm

Detector wavelength: 278 nm

Flow rate: 1.25 mL/min

Injection volume: 10 µL

3.2.3.5 UV-Visible spectrophotometric method validation

The ability of liposome to remain rhinacanthin-C in the liposomes (Encapsulation efficiency) was analyzed by UV-Vis spectrophotometric method. This technique was utilized in this study due to its simple, rapid and suitable for quantitation of rhinacanthin-C of several

liposomal samples. Spectrophotometric method is also economical in the terms of cost and time.

3.2.3.6 Instrumental and analytical conditions for UV-Visible spectrophotometric method

UV-Visible spectrophotometric analyses were carried out on a Hewlett-Packard 8452A Diode Array Spectrophotometer. The UV-Visible spectrum of rhinacanthin-C solutions were recorded in the range of 200 to 800 nm. In the spectral analysis, the wavelength 278 nm was defined for the quantitation of rhinacanthin-C.

3.2.3.7 Preparation of solutions for spectrophotometric analysis

Rhinacanthin-C standard solutions: approximately about 250 mg of pure rhinacanthin-C compound were accurately weighed and transferred to a 10 mL volumetric flask. Absolute ethanol was added and mixed well. The solution volume was adjusted to 10 mL with absolute ethanol to obtain a solution having concentration of 25 mg/mL. An aliquot (800 µl) of the resulting solution was transferred to a volumetric flask (10 mL) and volume was adjusted with 95% ethanol to obtain a solution with a concentration of 2.0 mg/mL. This solution was used to study for the validation of spectrophotometric method.

3.2.3.8 Validation of the assay

3.2.3.8.1 Linearity and calibration curve

Rhinacanthin-C standard solutions of 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL were prepared in buffer pH 7.4 containing 20% v/v ethanol. Three replicate analyses of each solution were performed in a day (3 injections × 5 concentrations × 3 replicates). Three determinations were carried out for each solution (n = 3). Linearity was obtained by plotting the absorbance value against the concentrations of the standard solutions and analyzed using the linear least-squares regression equation. Linearity was expressed as a coefficient of determination (r^2) which should be more than 0.9990.

3.2.3.8.2 Precision

The measurements of intra-day and inter-day variability were utilized to determine the precision of the method. For intra-day precision, five concentration levels of rhinacanthin-C standard solutions at 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL in the calibration range were prepared with methanol in triplicate (n = 3). Three determinations were carried out for each solution (3 injections × 5 concentrations × 3 replicates). For inter-day precision of rhinacanthin-C standard solutions was assessed by repeating the intra-day precision on three different days. Concentrations of rhinacanthin-C standard solutions from the experiments were calculated with a linear equation of the standard curve. Precision was calculated and expressed as percentage relative standard deviation (%RSD).

3.2.3.8.3 Accuracy

The accuracy of the method was performed by recovery study by using the method of standard additions. The recovery was determined by recovery of known amounts of rhinacanthin-C reference standard used at three concentration levels of 2.0, 3.0 and 4.0 µg/mL. Ten milliliters of each rhinacanthin-C concentration were added in dialysis bag. The dialysis bag was then sealed to ensure no leakage of the content inside the bag. The bag was then transferred to 1000 mL of buffer pH 7.4 containing 20% v/v ethanol as receiver medium. The receiver medium was stirred with a magnetic stirrer for 24 h at room temperature. All solutions were prepared in triplicate and assayed (3 injections × 3 concentrations × 3 replicates). After that, an aliquot of 10 mL of dialysis medium was withdrawn for measuring the amount of rhinacanthin-C and calculating to percentage recovery. The percentage recovery of rhinacanthin-C standard was calculated with a linear equation of the standard curve.

3.2.3.8.4 Specificity

The specificity is a method producing a response for only a single analyte accurately and specifically in the present of components in the sample matrix. The specificity of the analytical method was confirmed by analysis of 4 µg/mL of rhinacanthin-C standard solution. The sample solution (4 µg/mL) was prepared in 10 mL of buffer pH 7.4 containing 20% v/v ethanol as use a dialysis medium. The UV-Visible spectrum of rhinacanthin-C was recorded as

follow previously mentioned. The identification was performed by assessing the presence of only rhinacanthin-C spectrum at 278 nm without other spectrum.

3.2.3.8.5 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection is the lowest concentration of analyte that is detectable at the most sensitive instrument settings, but not necessarily quantitated, under the stated experimental conditions. Limit of quantification is the lowest concentration of analyte that can be determined with acceptable precision and accuracy, under the stated experimental conditions. Standard solutions of rhinacanthin-C were analyzed in the range of 1, 2, 3, 4 and 5 µg/mL. LOD and LOQ were determined on the basis of response and slope of the regression equation from the calibration curve (Bruce et al., 1998; Mendez et al., 2003; Savić et al., 2008; Dey et al., 2010).

3.2.3.9 HPLC method validation

Validation of analytical method for rhinacanthin-C entrapped in liposome formulations were performed as follow; linearity and range, selectivity and accuracy. The optimized method was validated according to ICH guidelines for the validation of analytical methods (ICH, 1996).

3.2.3.10 Instrumental and analytical conditions for HPLC method

The HPLC analyses were carried out on Thermo-isocratic pump, photodiode array detector and ChromQuest sorfware. Chromatography was carried out using a reversed phase Waters Symmetry® C_{18}, 5 µm, 3.9 x 150 mm HPLC column (Symmetry Columns, Water, Ireland). The mobile phase consisted of methanol : 5% acetic acid (80 : 20 by volume), at a flow-rate 1.25 mL/min, Detection of the analytes was performed by UV absorption at 278 nm and injection volume was 10 µL.

3.2.3.11 Preparation of standard solutions

A stock solution of rhinacanthin-C was prepared by dissolving 10 mg of rhinacanthin-C reference standard in methanol in 10 mL volumetric flask, resulting in a 1 mg/mL solution of rhinacanthin-C and stored at 4 °C until used. This solution was then serially diluted with methanol to provide calibration standard solutions of 10, 20 and 30 µg/mL.

3.2.3.12 Validation of the assay

3.2.3.12.1 Accuracy

The accuracy is the closeness of an individual test result to the true value. The method was performed by recovery study by using the method of standard additions. The recovery was evaluated by analyzing the mixture prepared by adding of the rhinacanthin-C standard solutions at three concentration levels of 0.016, 0.020 and 0.024 mg/mL (QC samples) in the calibration range into the blank liposome solution containing know amount of the analyte. The three injections for each concentration were done per day (3 injections × 3 concentrations × 3 replicates). The percentage recovery was calculated by subtracting the value obtained for the control matrix (blank) preparation from those samples, divided by the amount added and multiplied by 100. The percentage recovery should be in the range of 90 to 100%.

3.2.3.12.2 Linearity and range

Linearity is the ability of the method to produce test results that are proportional, either directly or by a well-defined mathematical transformation, to the concentration of analyte in samples within a given range. Range is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with the required precision, accuracy and linearity. Rhinacanthin-C standard solutions of 2, 6 and 10 µg/mL were injected into the HPLC system. Three replicate analyses of each solution were performed in a day (3 injections × 3 concentrations × 3 replicates). Three determination were carried out for each solution (n = 3). Linearity was obtained by plotting the peak areas against concentration of

the standard solution and analyzed using the linear least-squares regression equation. Linearity was expressed as a coefficient of the determination (r^2) which should be more than 0.9990.

3.2.3.12.3 Selectivity

Selectivity is the ability of a method to quantify accurately and specifically the analytes in the presence of other compounds. The selectivity of the analytical method was confirmed by analysis of a solution containing 2.0 µg/mL of rhinacanthin-C standard and empty liposome. The methanol was used as control. A volume of 20 µL was individually injected in to the HPLC system that was previously mentioned. The ability to separate all compounds in the sample was demonstrated by assessing the resolution between the peaks corresponding to various substances. The identification was performed by comparing the retention time of rhinacanthin-C in the chromatogram of the extract solution with those in the chromatogram of the standard solutions.

3.2.4 Antifungal activity evaluation of liposome containing rhinacanthin-C

The best formulations of liposome containing rhinacanthin-C were determined for antifungal activity against *Microsporum gypseum* (SH-MU-4) and *Trichophyton rubrum* (SK-10-09). The method for assess the in vitro antifungal potential was followed the reported procedure with some modification (Lorian, 1996).

3.2.4.1 Preparation of culture media

The fungal pathogens were grown on sabouraud dextrose agar (SDA). SDA was prepared by dissolve 65 g in 1000 mL of distilled water and boiled. Melted SDA were filled in each tube for slant agar and for agar well diffusion assay, respectively. 5 and 25 mL All SDA tubes were sterilized by autoclave (HV 110, Hiruyama, Japane) at 121 °C, 15 PA for 15 min. After that, tubes with 5 mL SDA were cooled down to obtain slant agar and stored at 4 °C until use. The tubes with 25 mL SDA were collected in water bath at 55 °C for agar well-diffusion assay according to section 3.2.4.4.

3.2.4.2 Tested fungi-dermatophytes preparation

M. gypseum and *T. rubrum* cultures were grown aerobically on slant SDA and incubated in an incubator (Memmert INP 700, Germany) at 37 °C for 10 days before use (Figure 3.7). These fungi were used for the preparation of inocula.

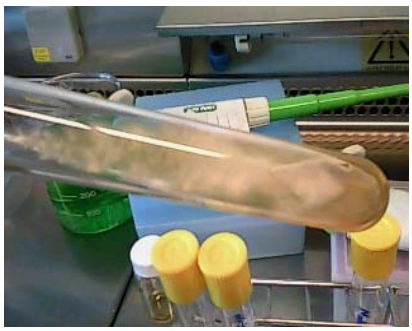


Figure 3.7 Growing of fungal on slant SDA after culture for 10 days at 37 °C.

3.2.4.3 Preparation of the spore suspension

The dermatophytes were grown slant SDA for 10 days, after which time, spores were harvested from sporulating colonies and suspended in normal saline solution sterile (0.85% NaCl w/v). The normal saline solution was transferred funnel cotton filter to only collected spore suspension (Figure 3.8). The concentration of spores in suspension was determined by transferring spore suspensions (1 mL) to a cuvette (1.5 mL) and measured turbidity by using a spectrophotometer at wave-length 625 nm. The turbidity was adjusted to give values in a range of 0.12 - 0.14 to match a final concentration that of a 0.5 McFarland standard suspensions (1.0×10^6 spores/mL) for each fungal pathogen (Kuhajek et al., 2003; Bajpai et al., 2009).

Figure 3.8 The spore suspension for antifungal activities study.

3.2.4.4 Antifungal activity (Agar well-diffusion assay)

The liposomes containing rhinacanthin-C sample was used to screen antifungal activities by using the antagonism agar well-diffusion method (Magaldi et al., 2004; Defer et al., 2009; Vaca Ruiz et al., 2009). One milliliter of spore suspension (0.5 McFarland) was mixed with 25 mL melted (55 °C) SDA in 50 mL test tube. The mixed medium was poured into Petri dishes (90 mm × 15 mm) by Pour plate technique and allowed to cool down on a leveled surface. Once the medium had solidified, four wells each 8.0 mm in diameter, were removed from the agar, and then 100 µL (contain 80 µg of rhinacanthin-C), 150 µL (contain 120 µg of rhinacanthin-C) of liposome containing rhinacanthin-C and 150 µL of rhinacanthin-C in DMSO solutions (contain 120 µg of rhinacanthin-C) were placed into each well (Figure 3.9). All plates were incubated at 37 °C and measured inhibition zone diameters (mm) by using vernier caliper (Figure 3.10 A and B). Each experiment was performed in triplicate.

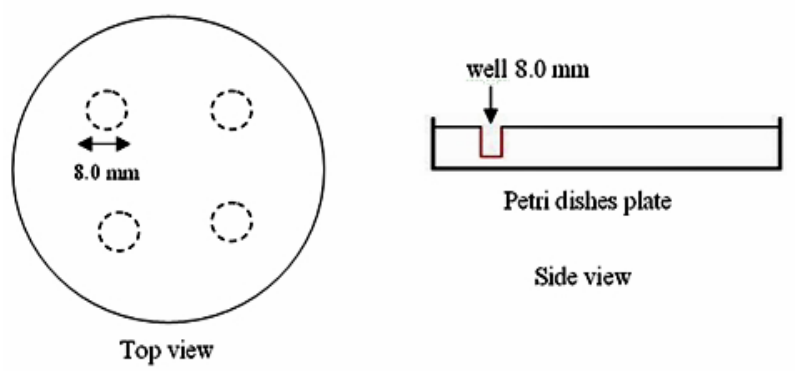


Figure 3.9 The agar-well diffusion method was used for assessing antifungal activities of liposome containing rhinacanthin-C.

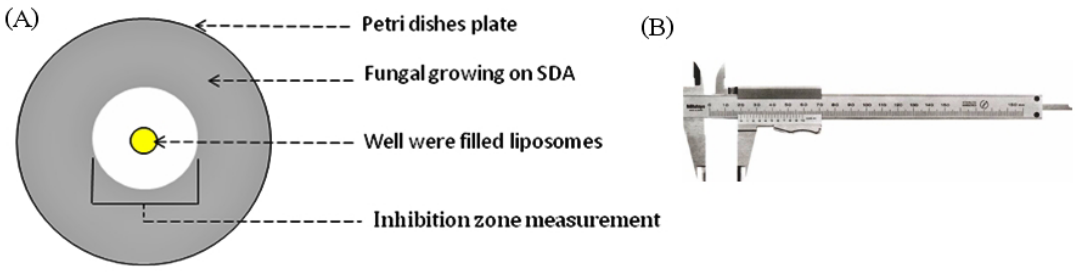


Figure 3.10 The zone of inhibited (A) was measured by Vernier Caliper (B).

3.2.4.5 Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) value was determined using micro agar dilution assay. The antifungal activity of liposome containing rhinacanthin-C, rhinacanthin-C extracted and positive drug-control (ketoconazole) were performed by the reported methods with some modification (Lorian, 1996; Bajpai et al., 2009; Kuete et al., 2009) which enabled to determine the minimum inhibitory concentration (MIC). A sequential two-fold dilution method was used in MIC test.

The MIC values of liposome containing rhinacanthin-C, pure rhinacanthin-C and ketoconazole for reference antibiotics were determined by using micro agar dilution assay as following. The stock solution of liposome containing rhinacanthin-C (800 µg/mL) was initially diluted with sterile distilled water to produce a 2-fold serial dilution of ranging from 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 400 µg of rhinacanthin-C/mL. Then, each sample of liposome containing rhinacanthin-C was further diluted with melted (55 °C) SDA in a ratio of 3 : 7 (liposome sample 300 µL : SDA 700 µL) to have final concentrations of 0.12, 0.23, 0.47, 0.94, 1.87, 3.75, 7.5, 15, 30, 60, 120, and 240 µg/mL for antifungal activity testing (Figure 3.11).

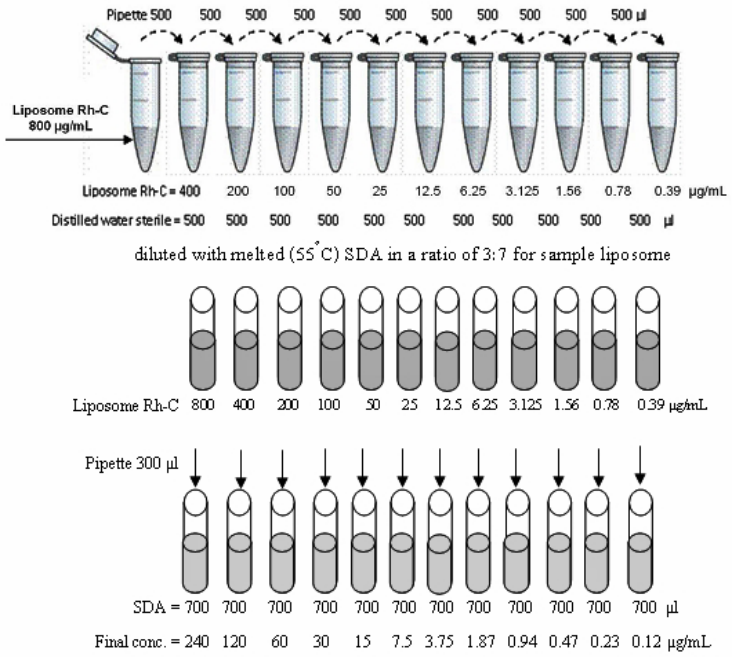


Figure 3.11 Illustration of liposome solutions prepared for testing MIC values.

The stock solution of pure rhinacanthin-C (24000 µg/mL) and ketoconazole (24000 µg/mL) in DMSO were diluted with DMSO to produce a 2-fold serial dilution having concentration of 11.72, 23.44, 46.87, 93.75, 187.5, 375, 750, 1500, 3000, 6000 and 12000 µg/mL. Then, each sample concentrations of pure rhinacanthin-C or ketoconazole were further diluted with melted (55 °C) SDA in a ratio of 1 : 100 (pure rhinacanthin-C or ketoconazole : SDA) to have final concentrations of 0.12, 0.23, 0.47, 0.94, 1.87, 3.75, 7.5, 15, 30, 60, 120, and 240 µg/mL for antifungal activity testing (Figure 3.12)

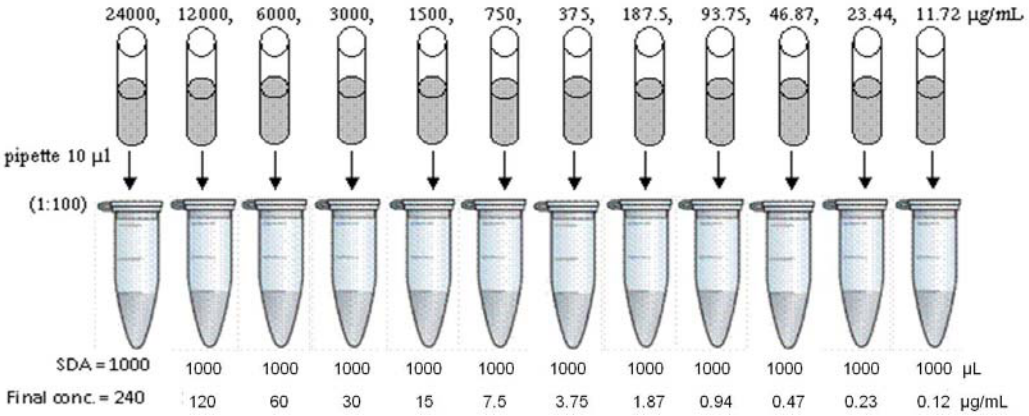


Figure 3.12 Illustration of samples preparation were diluted with DMSO.

Next, 150 µL of each concentration was added to each well of 96-well plates. Then, 2 µL of inoculate tested fungus which was adjusted to 0.5 McFarland turbidity and diluted to 1:10 with SDB was added to each well. The cultured microplates were sealed with parafilm and incubated at 37 °C for 10 days. The lowest concentration of sample that shows no growth was taken as its minimal inhibitory concentration MIC (Al-Bayati, 2009; Jung et al., 2009; Korukluoglu et al., 2009; Panáček et al., 2009).

3.3 Statistical analysis

All experimental data are expressed as mean ± standard deviation (SD). All experiments were performed in triplicate. The mean and standard deviation of at least three experiments were determined. A One-way ANOVA was used to compare the particle size of liposome in each formulation. Correlations among data obtained were calculated using regression analysis. The

differences were considered statistically significant when $p < 0.05$. The analysis was performed by using the Statistical Package for the Social Sciences (SPSS) 16.0 software package.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Extractions and identifications of rhinacanthin-C

Rhinacanthus nasutus (0.5 kg) was extracted with ethyl acetate to obtain crude extract (41.24 g). The crude extract (5 - 10 g) was subjected to purify by ion exchange chromatography and gave 3.13 g of the product. The latter product was further purified by silica gel column chromatography [dichloromethane : hexane (4 : 1) as developing solvent], and the fractions were collected in test tubes. The fractions containing only rhinacanthin-C which were identified by using UV-Visible spectrophotometry were pooled together. Only one spot were observed on thin layer chromatography (TLC) chromatograms from those developing solvents (Figure 4.2). The resulting product was collected in a round bottle flask (Figure 4.1), remove solvent by rotary evaporator. TLC was used to check purity of rhinacanthin-C by comparison with standard rhinacanthin-C (Kongchai and Panichayupakaranant, 2002) as the reference marker. Three developing solvent systems including dichloromethane : hexane (4 : 1); ethyl acetate : hexane (3 : 7) and dichloromethane: petroleum ether (4 :1) were used to confirm the purity of rhinacanthin-C (Figure 4.2). Isolated pure rhinacanthin-C was stored in a well closed bottle, kept in a refrigerator protect from light for future using in liposome preparation. The pure rhinacanthin-C was characterized by ^{1}H-NMR and FT-IR to confirm the purification. The ^{1}H-NMR spectrum of rhinacanthin-C (Figure 4.3) showed signals at δ (ppm): 8.12 – 8.108 (d, *J* = 7.77 Hz, 2H, CH-Ar); 8.093 – 8.077 (d, *J* = 7.78 Hz, 2H, CH–Ar); 7.776 – 7.744 (ts, *J* = 7.32, 7.77 Hz, *JJ* = 0.92, 1.37, 0.92 Hz, 2H, CH–Ar); 7.707 – 7.765 (ts, *J* = 7.31, 7.78 Hz, *JJ* = 1.37, 1.38, 0.91 Hz, 2H, CH–Ar); 6.710 – 6.678 (ts, *J* = 7.31, 7.32 Hz, *JJ* = 1.37, 1.38, 1.37, 1H, – CH=C– CO–); 5.226 – 5.187 (q, *J* = 5.49, 6.40, 6.86, 1H, HC=C–); 3.906 (s, 2H, –O–CH_2–CO–); 2.704 (s, 2H, CH_2–Ar); 2.193 – 2.148 (q, *J* = 7.78, 7.31, 7.32 Hz, 2H, C–CH_2–C=C–); 2.032 – 2.001 (t, *J* = 7.32, 7.77 Hz, 2H, C–CH_2–C=C–); 1.790 (s, 3H, C=C(CH_3)–CO); 1.589 (s, 3H, CH_3–C=C–); 1.568 – 1.556 (d, *J* = 5.95 Hz, 3H, CH_3–C=C–); 1.020 (s, 6H, –C–CH_3). The IR spectrum contained absorption bands of hydroxyl group and aromatic ring at 3,300 cm^{-1}, carbonyl group ester at 1,708 cm^{-1} and aromatic

ketone at 1,667 cm^{-1} (Figure 4.4). The results of ^{1}H-NMR and IR spectrum were correlated previously reported (Sendl et al., 1996).

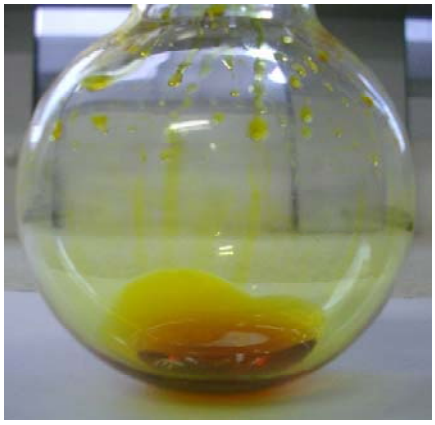


Figure 4.1 pure rhinacanthin-C.

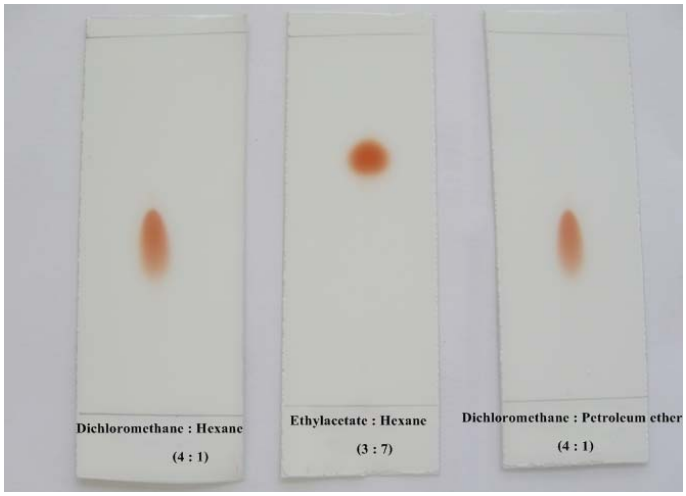


Figure 4.2 Silica gel TLC chromatograms of pure rhinacanthin-C developed by three different solvents.

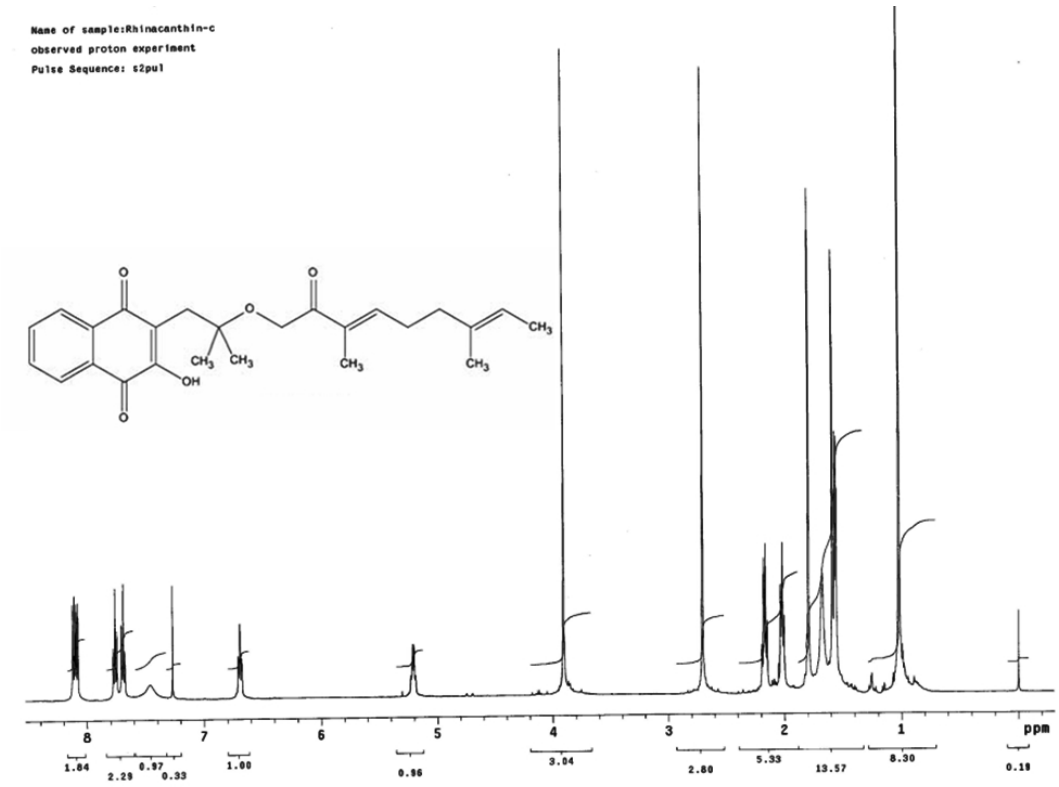


Figure 4.3 The ^{1}H-NMR spectrum of rhinacanthin-C, in $CDCl_3$ (at 500 MHz).

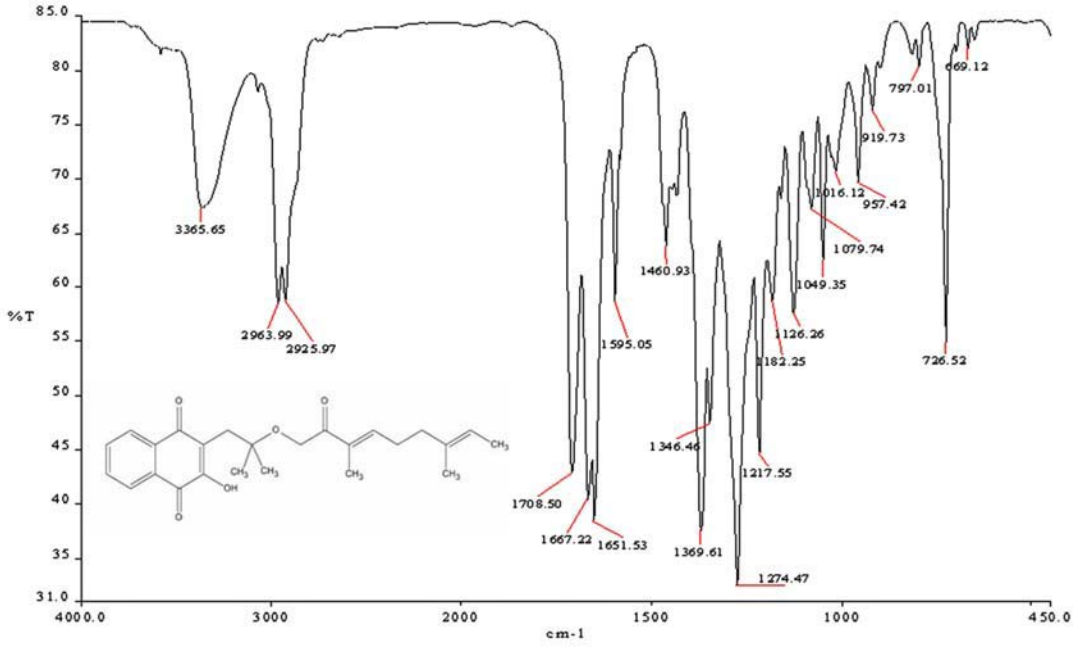


Figure 4.4 The FT-IR spectrum of rhinacanthin-C (Nujor method).

4.2 Characterization of liposome containing rhinacanthin-C

4.2.1 Physical appearances of liposome containing rhinacanthin-C

Suitable formulations of liposome containing rhinacanthin-C were preliminary chosen from physical appearances of the resulting liposome suspensions. Two types of phospholipids, egg yolk phosphatidylcholine (EPC) and soybean phosphatidylcholine (SPC) in combination with cholesterol were used in liposome formulation in this study. Firstly, each formulation of liposome were prepared by varying concentration of total phospholipids (20, 40 and 60 μmol/mL), and the ratio of phospholipids of either EPC or SPC to cholesterol (1:1, 1:2 and 1:3 molar ratio). Amount of rhinacanthin-C in each formulation was fixed at 15 mg per total volume of 3 mL (final concentration of rhinacanthin-C in liposome was 5 mg/mL) according to the result from previous report indicated that the highest MIC values of rhinacanthin-C that could inhibit the growth of dermatophytes (*Microsporum gypseum* and *Trichophyton rubrum*) were 125.0 and 15.6 μg/mL (Charoonratana, 2007); 500.0 and 3.90 μg/mL (Ovatlarnporn et al., 2008). Therefore, ten-fold of the highest concentration (0.5 mg/mL) was selected to use in liposome containing rhinacanthin-C formulation.

4.2.1.1 Physical appearance of liposome containing rhinacanthin-C composed of EPC

All liposome formulations made of EPC which contained 15 mg of rhinacanthin-C were dark yellow turbid solutions with the observation of lipids aggregation (Figure 4.5). The result was correlated to the observation of Rattanat (2008) who found that mycophenolic acid (MPA) liposomes were not formed when using EPC, the aggregation and sedimentation of lipid in the formulations after preparing were also observed. Also according to observation of Fang et al. (2005) found that liposomes composed of cholesterol and EPC were highly aggregated. Liposomes obtained from the formulation using EPC : CHL in molar ratio of 1:2 (Formulation No. 2) and 1:3 (Formulation No. 3) gave liposome having > 1000 nm in size (Table 4.1). However the formulation using EPC : CHL in molar ratio of 1:1 (Formulation No. 1), smaller size (845.33 ± 220.66) of liposomes were obtained. While blank formulation (No incorporate rhinacanthin-C) gave much smaller than the formulation having rhinacanthin-C. The result

demonstrated that the increasing amount of cholesterol in the formulation played an important role in the stability of lipid bilayers formation. The increasing of cholesterol in liposome formulation favored the aggregation process and sedimentation that may affect on the stability during storage (Lopez-Pinto et al., 2005). All formulations obtained gave negative Zeta Potential values. This due to the phospholipids introduced a negatively charge of phosphate head group onto the surface of liposome.

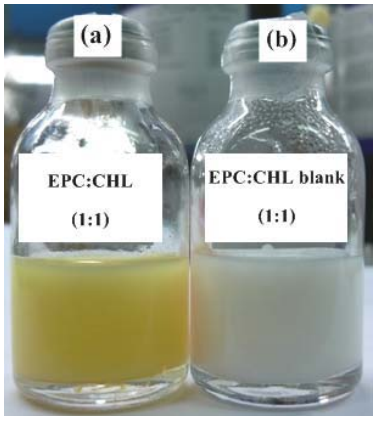


Figure 4.5 Physical appearances of liposome containing rhinacanthin-C (a) the formulation contain of EPC : CHL in 1 : 1 molar ratio containing 15 mg of rhinacanthin-C (Formulation No. 1) and (b) blank liposome of the same formulation.

Table 4.1 Size distribution and zeta potential of liposome containing rhinacanthin-C (Rh-C) prepared by using EPC.

Formulation No.	**Compositions**	**Size (nm) ± SD**	**Polydispersity Index**	**Zeta Potential (mV) ± SD**
1	EPC : CHL (1:1) 15 mg of Rh-C	845.33 ± 220.66	0.78 ± 0.15	-43.97 ± 0.57
2	EPC : CHL (1:2) 15 mg of Rh-C	1307.35 ± 404.20	0.58 ± 0.08	-53.33 ± 1.34
3	EPC : CHL (1:3) 15 mg of Rh-C	1755.4 ± 852.98	0.78 ±0.15	-56.03 ± 2.87
Liposomal blank	EPC : CHL (1:1) (No Rh-C)	239.33 ± 17.62	0.23 ± 0.00	-50.00 ± 0.44

Similar results were obtained when deoxycholic acid (0.25 mol) was added in the above formulations using EPC : CHL in molar ratio of 1:1 (Table 4.2) where the bigger liposome size was obtained when the formulation containing Rh-C than blank formulation (No incorporate rhinacanthin-C). The resulted showed that addition of deoxycholic acid did not assist in reducing the size of liposome. Bigger liposome caused by DA may be due to DA enhanced liposome agglomeration. The result was accorded with Fang and co-worker (2006), significantly increasing ($p < 0.05$) the size of the liposome vesicles when added of DA in liposome formulation. Slightly reducing of negative zeta potential values were observed after DA was added to the formulation compared to the results in Table 4.1. Observation of Fang and co-worker (2005) found that addition of DA accelerated the fusion of liposomes. This result may indicate that the negatively charged DA decreased the rigidity of the bilayers, thus facilitating vesicle aggregation.

Table 4.2 Size distribution and zeta potential of liposome containing rhinacanthin-C (Rh-C) prepared by using EPC with addition of deoxycholic acid.

Compositions	**Size (nm) ± SD**	**Polydispersity Index**	**Zeta Potential (mV) ± SD**
EPC : CHL : DA (1:1:0.25) 15 mg of Rh-C	1465.0 ± 289.91	0.46 ± 0.33	-42.90 ±0.36
EPC : CHL : DA blank (1:1:0.25) (No Rh-C)	815.33 ± 40.43	0.62 ± 0.04	-37.90 ±0.46

Moreover, their viscosities increased by visual observation when increasing amount of total lipid from 20, 40 and 60 μmol/mL, respectively. The result from this preliminary experiments demonstrated big particle sizes were obtained when using EPC. Therefore, EPC was not selected to use for further development of liposome containing rhinacanthin-C.

4.2.1.2 Physical appearance of liposome containing rhinacanthin-C composed of SPC

Liposomes which were formulated by using SPC gave better appearance than the formulation using EPC (section 4.2.1.1). Liposomes containing rhinacanthin-C gave yellow turbid solutions which resulted from the color of rhinacanthin-C (Figure 4.6, a) while blank liposome from the same formulation gave white turbid colloidal solutions (Figure 4.6, b). Phenomena of lipid aggregation and sedimentation were not observed in this formula.

Their viscosities were observed to be increased when increasing amount of total lipid from 20, 40 and 60 µmol/mL, respectively. The formula containing total lipid of 60 µmol/mL showed more viscous (Figure 4.7) and gave bigger particle size (370 - 390 nm) than the formulations using 20 µmol/mL (250 - 280 nm) and 40 µmol/mL (340 - 380 nm) (Table 4.3) of all formulation containing rhinacanthin-C 4 mg / 3 mL, 5 mg / 3 mL and 6 mg / 3 mL.

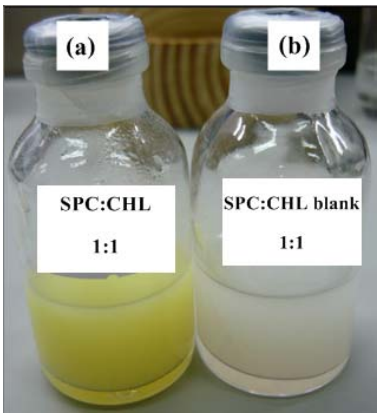


Figure 4.6 Physical appearances of liposome containing rhinacanthin-C (a) and blank liposomes (b) which were prepared from the formulation consisted of SPC : CHL in 1 : 1 molar ratio containing 15 mg of rhinacanthin-C.

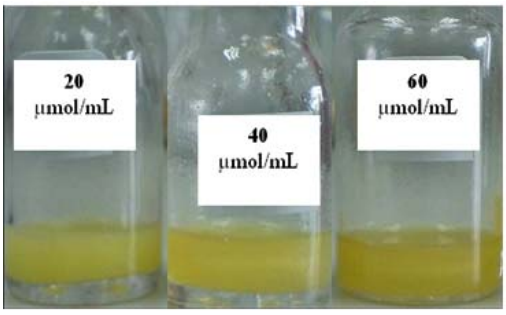


Figure 4.7 The physical appearances of liposome containing rhinacanthin-C (15 mg) composed of total phospholipid 20, 40 and 60 µmol/mL.

Table 4.3 Mean particle size of liposome containing rhinacanthin-C by using SPC : CHL (1 : 1).

Total phospholipid (μmol/mL)	Mean particle size of liposome containing different concentrations of rhinacanthin-C (nm)		
	4 mg / 3 mL	**5 mg / 3 mL**	**6 mg / 3 mL**
20	259.5 ± 7.50	258.2 ± 12.48	280.7 ± 6.92
40	343.0 ± 8.78	384.7 ± 30.12	336.0 ± 8.42
60	377.5 ± 23.95	390.0 ± 23.90	383.0 ± 10.23

At the beginning of our study, entrapment efficiency was performed by lipid precipitation method (Gabriels and Plaizier-Vercammen, 2003)]. The lipid was precipitated by additional of acetonitrile : methanol (6:1) 3 mL to the resulting liposome. Then, centrifuge at 6000 rpm for 20 minute and the supernatant were determined for amount of rhinacanthin-C by UV-Visible spectrophotometer at 278 nm. The result from this preliminary study found that the entrapment efficiencies of liposome formulations prepared by using 20 μmol/mL of total phospholipid with an increasing amount of rhinacanthin-C from 4.0, 5.0 and 6.0 mg were 1.1, 4.9 and 6.8 %, respectively (Table 4.4). In this case, trapping efficiencies were found to be increased when increasing amount of rhinacanthin-C in formulation.

In the case of using 40 μmol/mL of total phospholipid in the preparation, the trapping efficiency was 16.8% when using rhinacanthin-C 4.0 mg. The trapping efficiency was increased to 23.12 % when increase rhinacanthin-C to 5.0 mg. However, the trapping efficiency reduced to 20.4 % when increasing rhinacanthin-C to 6.0 mg (Table 4.4). It may be indicated that a fixed amount of lipid concentration produce a constant number of liposome in the system. Those liposomes have a limited trapping capacity which leads to the decrease of trapping efficiency when concentration of rhinacanthin-C was too high (Fan et al., 2008).

Table 4.4 Trapping efficiency of liposome containing rhinacanthin-C prepared by using 20 and 40 μmol/mL of total phospholipids determined by lipid precipitation method.

Amount of rhinacanthin-C	Trapping efficiency (%)	
	20 μmol/mL	40 μmol/mL
4.0 mg/3 mL	1.10 ± 0.21	16.80 ± 0.32
5.0 mg/3 mL	4.89 ± 0.53	23.12 ± 0.46
6.0 mg/3 mL	6.77 ± 0.14	20.40 ± 0.47

When liposomes were prepared by fixing amount of rhinacanthin-C at 5.0 mg/3 mL but varied the amount of total phospholipids from 20, 40, 60 and 80 μmol/mL (Table 4.5). Trapping efficiencies of rhinacanthin-C showed increasing when increasing amount of the lipid concentration from 20 (4.89 %) to 40 μmol/mL (23.12 %). However, when the amount of lipid concentration was increased to 60 and 80 μmol/mL, the trapping efficiencies were found to be reduced to 7.3 and 6.2 %, respectively.

Table 4.5 Trapping efficiencies (determined by lipid precipitation method) of liposome containing rhinacanthin-C (5mg/3mL) of 20, 40, 60, 80 μmol/mL total phospholipids.

Total phospholipids (μmol/mL)	Trapping efficiency (%)
20	4.89 ± 0.53
40	23.12 ± 0.46
60	7.30 ± 0.76
80	6.20 ± 0.28

The result indicated that, higher or lower lipid concentration than 40 μmol/mL, the trapping efficiency was decreased. This may be due to the ultimate lipid concentration in liposomal dispersion depended on hydration volume during the preparation. A too high hydration volume made lower lipid phase which could lead to the difficulty in trapping rhinacanthin-C (Fan et al., 2008). Thus, an increasing amount of total phospholipids concentrations were not always effect to higher trapping efficiency.

Since we found that after lipid precipitation was performed, the sediments resulting from centrifugation still have yellow color. That may caused by Rh-C was also co-precipitate with all lipids used in the formulation. That may resulting in low entrapment efficiencies were obtained. Therefore dialysis method was used instead to assess entrapment efficiency of liposome containing rhinacanthin-C. This method has been utilized in many research papers in liposome preparation (Imura et al., 2003; Maestrelli et al., 2005; Mura et al., 2007; Fan et al., 2008). The result showed that 15 mg of rhinacanthin-C added in liposomal formulations was too much excess. That can be observed from the oil droplets of excess rhinacanthin-C accumulated at the surface of dialysis membrane during dialysis method as shown in Figure 4.8. Since rhinacanthin-C is a lipophilic compound and poorly soluble in aqueous medium. Therefore, the free rhinacanthin-C will present on the surface of dialysis membranes. This phenomenon has an effect on the process of the determination entrapment efficiency.

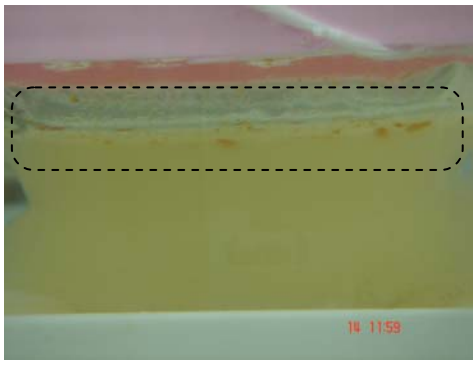


Figure 4.8 Picture of dialysis bag during removing excess of rhinacanthin-C from liposome formulation using 15 mg of rhinacanthin-C. In circle showed oily droplets of excess rhinacanthin-C presented on the surface of dialysis bag.

Therefore, in order to further development of liposome containing rhinacanthin-C and not too much excess of free rhinacanthin-C was used in the formulations, the liposomes formulations were prepared by lowering concentration of rhinacanthin-C to 4 mg/3 mL and a total phospholipid of 40 μmol/mL were used in all formulations.

4.2.2 Effect of variation of SPC content on entrapment efficiency and particle size of liposome containing rhinacanthin-C

The effect of SPC content on particle size of the resulting liposomes was determined and the results are shown in Figure 4.9. The results were analyzed by One-way ANOVA with a significant difference was considered at the level of $p < 0.05$. The results demonstrated that increasing amount of SPC from 1:1, 2:1, 3:1 and 5:1 (SPC : CHL) in liposome formulations showed no significantly difference in particle size, accepted in formulation of 4:1 (SPC : CHL) gave the largest particle size.

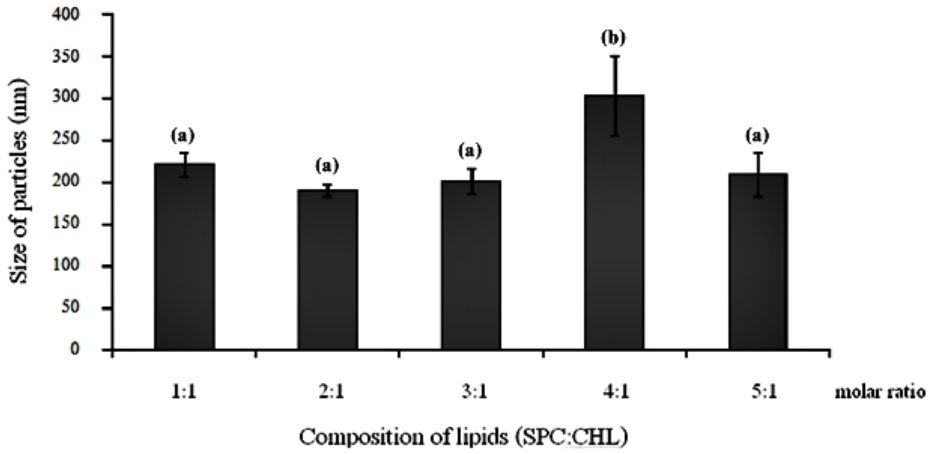


Figure 4.9 The effect of SPC content on particle size of liposome containing rhinacanthin-C which were prepared by formulation using different ratios of SPC:CHL.

(a) No significantly difference ($p > 0.05$).

(b) Significantly difference ($p < 0.05$).

The effects of SPC content on entrapment efficiency of liposome containing rhinacanthin-C are shown in Table 4.6. The formulations with high entrapment efficiency of rhinacanthin-C in liposome would be selected to use in further study. All formulations showed rather highly entrapment efficiency (> 70 %). The best formulation displayed the highest entrapment efficiency was the formulation composed of SPC : CHL in 1:1 molar ratio (Formulation No. 19). Its entrapment efficiency was 94.69 ± 1.20 %. However, an excess amount of SPC in formulations may induce physical instability of the obtained liposome such as

sedimentation or fusion of lipid membrane. The mechanism that believed to be involved in membrane fusion is schematically presented in Figure 4.10. During the process membranes of nearby liposomes must be first approach and then combine into a single bilayer, which is referred to as "hemifusion". Then, the close apposition of two lipid bilayers will lead to vesicle aggregation. It this phenomenon occurred a large increase in the size of liposomes would be obtained (Elferink et al., 1997; Cevc and Richardsen, 1999; Zimmerberg and Chernomordik, 1999; Jahn and Grubmüller, 2002; Jahn et al., 2003).

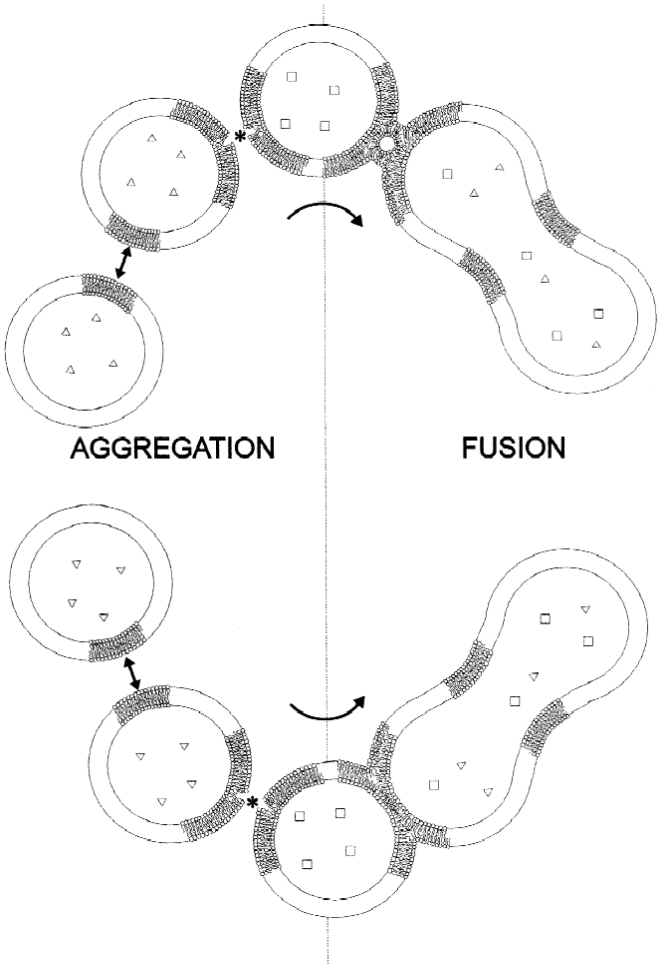


Figure 4.10 Basic sequences of steps leading to membrane unification, which all involve initial membrane approach. Inverted phases may appear at the sites of membranes contact, in the form of small inverted micellar bodies or elongated non-lamellar contact structures (upper part), both result in subsequent membrane merging. Membrane restructuring and merging can also rely on local membrane disordering and defect formation, due to the hydrophobic interaction (lower part) (Cevc and Richardsen, 1999).

In our observation found that high entrapment efficiency of the liposome formulations (more than 70 – 90 %) could be due to the strong interaction between liposome membranes with rhinacanthin-C molecules. This can be explained by the result from the preliminary experiment which demonstrated that the liposome expresses a spontaneous encapsulation capacity by themself. Blank liposome could entrap a certain amount of pure rhinacanthin-C. After pure rhinacanthin-C was added to blank liposome followed by dialysis against buffer pH 7.4 containing 20% ethanol for 24 hours simultaneously, the amount of free rhinacanthin-C which was released from dialysis bag was 0.45 μg/mL (about 11.46 %) from all pure rhinacanthin-C was added (3.96 μg/mL). Therefore strong interaction between the surfaces electrically like charges of liposomal membrane and rhinacanthin-C molecules which resulting to high entrapment efficiency.

Table 4.6 The effect of SPC content on entrapment efficiency of liposome containing rhinacanthin-C.

Formulation No.	Molar ratio of SPC : CHL	Entrapment efficiency (%) ± SD
19	1:1 molar ratio	94.69 ± 1.20
20	2:1 molar ratio	77.02 ± 3.66
21	3:1 molar ratio	87.16 ± 3.61
22	4:1 molar ratio	85.30 ± 1.90
23	5:1 molar ratio	89.68 ± 3.08

In this study, the suitable formulation of liposome (Formulation No. 19) containing rhinacanthin-C was selected for further study. The formulation composed of SPC : CHL in a molar ratio of 1 : 1. The amounts of rhinacanthin-C used in liposome formulation were 4 mg/3 mL using a 40 μmol/mL of total phospholipid.

4.3 Effective of additives in liposome formulations

4.3.1 Physical appearance of liposomes added deoxycholic acid or Tween 80

Two types of additives material were used to construct and optimize a resulting formulation of liposome suspension. Deoxycholic acid (DA) and Tween 80 were commonly chosen to add in liposome formulations. Additional of DA will give negatively charged molecules, and additional of Tween 80, non-ionic surfactants to provide the steric barrier to vesicle membrane, which prevents vesicle aggregation and leads to enhance of physical stability of liposome. Deoxycholic acid (DA) and Tween 80 were added in liposome preparation by varied the molar ratio. Liposome containing rhinacanthin-C was prepared by using SPC, CHL and DA or Tween 80 with varied a molar ratio of 1:1:1, 1:1:0.5, 1:1:0.25, respectively. The total phospholipid which was used in liposome preparation was 40 μmol/mL with 4 mg of rhinacanthin-C in 3 mL of final volume of liposome suspension. The physical appearances of the obtained liposoms were shown in Figure 4.11.

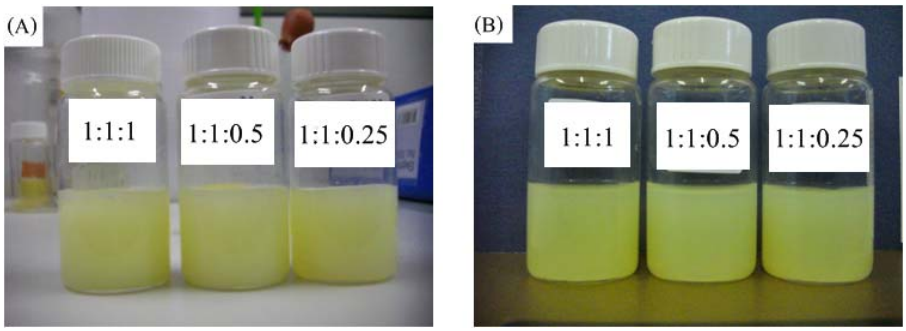


Figure 4.11 Physical appearance of liposome containing rhinacanthin-C with variation of compositions of (A) SPC : CHL : DA and (B) SPC : CHL : Tween 80 in a molar ratio of 1:1:1, 1:1:0.5 and 1:1:0.25.

The physical appearance of all liposome formulations with addition of DA and Tween 80 had a likely colloidal solution. The liposome formulations which were added DA (Figure 4.8A) showed light yellow milky and turbid solution than Tween 80 formula (Figure 4.8B). However, the formulas with additional of DA or Tween 80 in a molar ratio of 1:1:1 were appeared to have

lipids precipitation after storing at room temperature for 24 hours. The liposome formulations of SPC : CHL : DA in a molar ratio of 1:1:0.5 and 1:1:0.25 were also found to have a sediment of lipids after storing at room temperature for 2-3 days but less than SPC : CHL : DA in molar ratio of 1:1:1. However, the formulations of SPC : CHL : Tween 80 in a molar ratio of 1:1:0.5 and 1:1:0.25 no lipid sediment were observed during storage period of more than three days. Any formulations that showed sedimentation displayed a poorly stable of liposome particles and they will not be selected for further study. The incorporation of DA-containing or Tween 80-containing liposomes was reported to enhance a dramatic increasing in anionic or cationic compounds encapsulation (Fang et al., 2006). Tween 80 exposed from the outer leaflet of the bilayer membrane would increase the liposome particle curvature, whereas Tween 80 exposed to the inner leaflet did the opposite. Therefore, adding Tween 80 could reduce the initial liposomal size since more Tween 80 existed in the outer leaflet than in the inner leaflet of the liposomal bilayer membrane (Fan et al., 2008). Drug leakage from liposomes is often involved in the aggregation or fusion of liposomal membranes. The high surface potential of liposomes (DA or Tween 80 containing liposomes) tends to increase the interbilayer distance owing to electrostatic repulsive forces (Fang et al., 2006). Elsabee and co-workers (2009) explained that surfactants will form micelles in solution in order to decrease the free energy when the concentration is higher than the critical micelle concentration (CMC). Analogously, aggregates should also be formed for amphiphilic polymers such as block polymers in aqueous media. The transition concentration is consequently denoted as the critical aggregation concentration (CAC) rather than the (CMC) customary for surfactants and amphiphilic block copolymers (Elsabee et al., 2009).

4.4 Physicochemical characterization of nanoliposome containing rhinacanthin-C

4.4.1 Liposomal size measurement

The particle size of the obtained liposome was examined by the particle size analyzer using the photon correlation spectroscopy. The results from the effect of DA content on the particle size of liposome containing rhinacanthin-C are shown in Table 4.7. The obvious changes in particle size and distribution of particle sizes of liposomes were found. With increasing

amount of DA in the formulations (SPC : CHL : DA) from a molar ratio of 1:1:0.25 (Formulation No. 26) to 1:1:0.5 (Formulation No. 25) and 1:1:1 (Formulation No. 24) the particle size of liposomes containing rhinacanthin-C increased from 172.6 ± 40.49 to 241.8 ± 34.99 and 303.7 ± 37.71 nm, respectively. The addition of DA accelerated the fusion of liposomes, thus facilitating vesicle aggregation (Fang et al., 2005). Increasing DA content in liposome formulations were also displayed broad polydispersity index of the particle diameter distribution of liposome containing rhinacanthin-C (Table 4.7). Therefore increasing amount of DA in the formulation, a heterogeneity vesicle population in diameter were obtained. In preliminary study liposome containing rhinacanthin-C was prepared by using only SPC : CHL (1:1) (Formulation No. 19) without DA in formulations, the particle sizes were obtained in a range of 200 - 300 nm. Therefore, additional of DA in liposome formulations did not significantly affect on particle size. However, it may have an effect on surface charge and entrapment efficiency.

Table 4.7 The average size of liposomes containing rhinacanthin-C with additional of deoxycholic acid (DA) in formulations.

SPC : CHL : DA (Formulation No.)	**1:1:1 (24)**	**PI**	**1:1:0.5 (25)**	**PI**	**1:1:0.25 (26)**	**PI**
	333.8	1.000	211.3	0.757	187.1	1.000
	341.6	0.677	214.8	1.000	122.5	0.687
	313.7	1.000	219.2	1.000	121.6	0.916
	274.5	1.000	298.1	0.353	213.9	0.677
	315.6	1.000	238.1	0.840	186.2	0.643
	243.1	1.000	269.5	1.000	204.0	0.476
average size ± SD	303.7 ± 37.71	0.946	241.8 ± 34.99	0.825	172.6 ± 40.49	0.733

PI = polydispersity index

When liposome formulations were prepared by adding Tween 80 in formulation (SPC : CHL : Tween 80), particle size of 210.2 ± 5.84 and 320.4 ± 108.37 nm for a molar ratio of 1:1:0.25 (Formulation No. 29) and 1:1:1 (Formulation No. 27), were obtained respectively (Table 4.8). Their particle sizes were obtained insignificantly different when compare with the

formulations prepared by using only SPC : CHL (1:1) (Formulation No. 19) but without Tween 80 (section 4.2.1.2). However, when Tween 80 was added to the formulation in a molar ratio of 1:1:0.5 (Formulation No. 28), the smallest particle sizes (115.8 ± 23.33 nm) was obtained compared to the former results.

Table 4.8 The average size of liposome containing rhinacanthin-C with additional of Tween 80 (T80) in formulations.

SPC : CHL : T80 (Formulation No.)	**1:1:1 (27)**	**PI**	**1:1:0.5 (28)**	**PI**	**1:1:0.25 (29)**	**PI**
	447.2	1.000	128.0	0.351	216.9	0.614
	305.8	1.000	98.4	0.913	201.4	0.237
	169.3	1.000	157.5	1.000	213.2	0.705
	313.9	1.000	111.0	0.302	209.4	0.265
	439.3	1.000	102.5	0.523	205.6	0.635
	246.8	1.000	97.6	0.364	214.4	0.695
average size ± SD	320.4 ± 108.37	1.000	115.8 ± 23.33	0.576	210.2 ± 5.84	0.525

PI = polydispersity index

Therefore, in the case of Tween 80 incorporated in formulations indicated that an increasing amount of Tween 80 led to decreasing a particle size of liposome. But the Tween 80 beyond a certain level (for example, the molar ratio 1:1:1 of SPC : CHL : T80) (Formulation No. 27), the liposomal with the largest particles (320.4 ± 108.37 nm) was achieved. Hence, the appropriate proportions between Tween 80 and lipid were important for optimizing liposomal formulations to obtain a suitable particle sizes.

Consequently, the effects of additive materials (DA and Tween 80) content on the particle size of liposome containing rhinacanthin-C are summarized in Figure 4.12. The results indicated that an increase in DA amounts in formulations led to an increase in liposomal particle sizes from ~170 to 300 nm. Also, including an increase a sedimentation properties of liposome suspensions. While an increasing of Tween 80 did not always result in decreasing of liposomal particle sizes. Therefore, the formulations composed of SPC : CHL : T80 in a molar ratio of

1:1:0.5 (Formulation No. 28) was the suitable formulation for using in further study. However, their entrapment efficiency, stability and antifungal properties were also evaluated and the results will be discussed in the following sections.

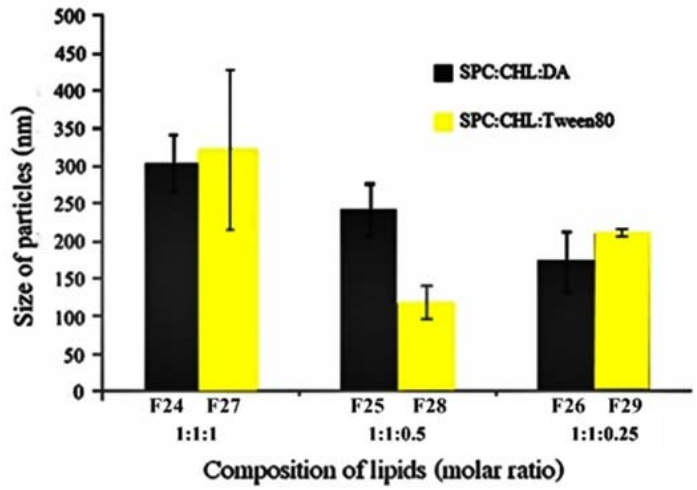


Figure 4.12 Comparable a result of particle sizes of liposome containing rhinacanthin-C between varied a molar ratio of deoxycholic acid (DA) and Tween 80 in lipid composition.

4.4.2 Entrapment efficiencies determinations

4.4.2.1 Optimization of dialysis technique for entrapment efficiencies determination

Since the dialysis technique is used for rhinacanthin-C entrapment efficiencies determination of the liposomes. Therefore, the releasing of free rhinacanthin-C from dialysis bag was optimized. A total of rhinacanthin-C of 12.5 mg was dissolved in 1 mL of absolute ethanol and placed into a dialysis bag. The dialysis bag was submerged into 400 mL dialysis medium (buffer pH 7.4 containing 20% ethanol) and stirred at room temperature. The dialysis medium (10 mL) was sampled and replaced with the same volume every hour for 23 hrs (Table 4.9). The collected solution was determined for the released amount of rhinacanthin-C by calculated from calibration curve of pure rhinacanthin-C (Figure 4.13). The result demonstrated that the free rhinacanthin-C was slowly released from dialysis bag to dialysis medium when increasing the dialysis time from the first hour to 23 hours. A total of 30.29 μg/mL of rhinacanthin-C (96.94%) was released to the dialysis medium after 23 hours of dialysis (Table 4.9). Therefore, in this

study the entrapment efficiencies of rhinacanthin-C in liposome were performed by dialysis technique. The prepared liposome was placed into the dialysis bag and dialysis against buffer pH 7.4 containing 20% ethanol (1000 mL) for 24 hours.

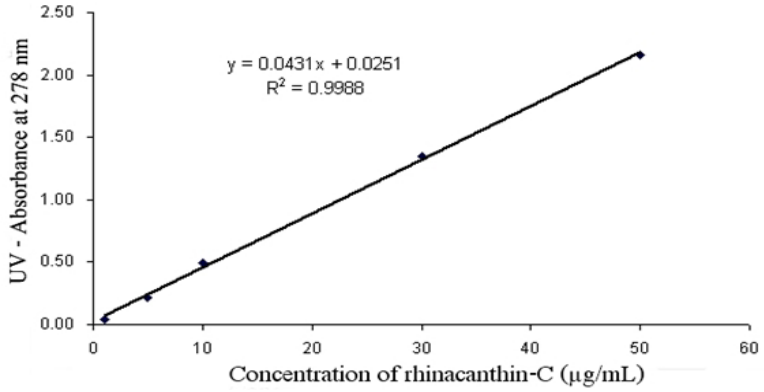


Figure 4.13 Calibration curve between concentrations (1 – 50 µg/mL) and UV absorbance values of rhinacanthin-C at 278 nm for optimization of entrapment efficiencies determination technique.

Table 4.9 Amount of rhinacanthin-C released from dialysis membrane for 23 hours.

Times (hr)	Accumulate amount of rhinacanthin-C (µg/mL)	% Release
1	1.91	6.13
2	2.29	7.34
3	2.54	8.14
4	2.94	9.43
6	3.22	10.30
8	2.80	8.96
10	2.91	9.32
12	3.00	9.60
14	3.75	12.01
20	3.67	11.74
23	3.79	12.14
Total	**30.29**	**96.94**

4.4.2.2 Quantitation of rhinacanthin-C by UV-Visible spectrophotometer

The quantitation of rhinacanthin-C entrapped into liposome was performed. The calibration curve of standard rhinacanthin-C solutions (Conc. 2.0 – 10.0 µg/ml) was obtained and shown in Figure 4.14. The equation from the linear regression line and r^2 are shown as following:

$y = 0.0455x + 0.0157$, $r^2 = 0.9994$,

where y = UV- absorbance value of rhinacanthin-C at 278 nm

x = concentration of rhinacanthin-C solutions (µg/ml) in 95% ethanol

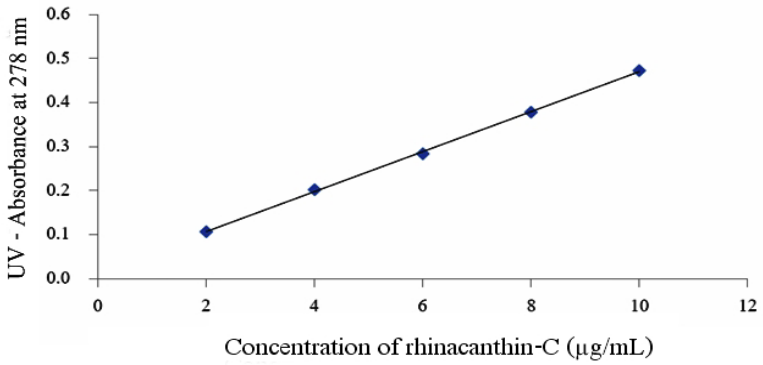


Figure 4.14 Calibration curve between concentrations and UV absorbance values of rhinacanthin-C for entrapment efficiencies determination of liposomes.

Increasing entrapment efficiencies of liposome with increasing amount of DA in formulations was observed (Table 4.10). Entrapment efficiencies of rhinacanthin-C were varies from 68.93 ± 0.71, 83.04 ± 0.50 and 88.32 ± 1.58 % when increasing amount of DA as 1:1:0.25 (F26), 1:1:0.5 (F25) and 1:1:1 (F24) molar ratio, respectively (Table 4.10). However, from previous observation, formulations (SPC : CHL in 1:1 molar ratio, F19) gave entrapment efficiency about of 94.69%. Therefore, the liposome formulations with additional of DA were not selected for further study.

Table 4.10 Effect of DA on entrapment efficiencies of liposome containing rhinacanthin-C determined by dialysis method.

Formulation No.	Compositions (molar ratio)	Entrapment efficiencies (%)			
	SPC : CHL : DA	1#	2#	3#	average ± SD
24	1:1:1	89.81	86.66	88.49	88.32 ± 1.58
25	1:1:0.5	83.16	82.48	83.47	83.04 ± 0.50
26	1:1:0.25	68.11	69.38	69.30	68.93 ± 0.71

Same situation was observed with the liposome formulations containing Tween 80. Increasing entrapment efficiencies was found when increase amount of Tween 80 in formulations. Amount of rhinacanthin-C was highly entrapped from 69.31 ± 0.20, 84.94 ± 1.32 and 92.69 ± 0.26% when varied amount of Tween 80 as 1:1:0.25 (F29), 1:1:0.5 (F28) and 1:1:1 (F27) molar ratio, respectively (Table 4.11). The highest entrapment efficiency (92.69 ± 0.26%) was found in the formulation No. 27. However, when particle size (320.4 ± 108.37 nm) was taken into an account (as described in a section 4.3.1), this formula was not selected for further study. The suitable formulation which was selected for further study was a formulation composed of SPC : CHL : Tween 80 in a molar ratio of 1:1:0.5 (F28). It's trapping efficiency and particle size were 84.94 ± 1.32% and 115.8 ± 23.33 nm, respectively.

Table 4.11 Effect of Tween 80 on entrapment efficiencies of liposome containing rhinacanthin-C determined by dialysis method.

Formulation No.	Compositions (molar ratio)	Entrapment efficiencies (%)			
	SPC : CHL : Tween 80	1#	2#	3#	average ± SD
27	1:1:1	92.75	92.42	92.92	92.69 ± 0.26
28	1:1:0.5	86.28	83.64	84.89	84.94 ± 1.32
29	1:1:0.25	69.24	69.15	69.53	69.31 ± 0.20

Figure 4.15 showed comparable result of trapping efficiencies of liposomes containing rhinacanthin-C formulations with variation molar ratio of DA and Tween 80 in preparations. The trapping efficiencies did not show significantly different between types of additive material at the

same molar ratio. Therefore, type of additive material showed no effect on trapping efficiency. However, they may have an effect on other results such as, surface charge, releasing, stability and permeability of liposome.

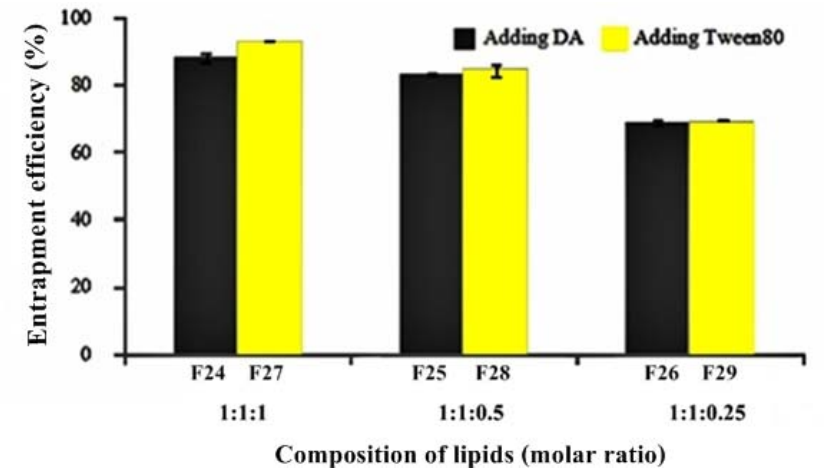


Figure 4.15 Comparable a result of trapping efficiencies of liposome containing rhinacanthin-C between varied a molar ratio of deoxycholic acid (DA) and Tween 80 (T80) in lipid composition.

4.4.3 Microscopic investigations of liposome containing rhinacanthin-C

The prepared liposome containing rhinacanthin-C was obtained in a range of nano-size. Since, particle size and particle size distribution may be crucial parameters for uptaken mechanism into the cell membrane of fungal. Morphology of the obtained liposomes was determined by visual observation and photon correlation spectroscopy. The particle size distributions of liposomes prepared in this study are shown in Figures 4.16 - 4.18. The liposomes were obtained in fine spherical shape with a relatively monodispersed size distribution and assuring a result of the previous size distribution measurement by particle size analyzer. However, some aggregation was observed (Figures 4.15) in some tested samples. This may lead to sedimentation and unstable property of liposome particles during storage period (section 4.6).

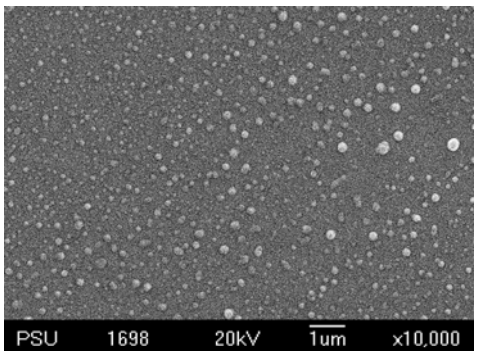


Figure 4.16 SEM image of freshly prepared of liposome containing rhinacanthin-C formulation composed of SPC : CHL in 1:1 molar ratio (Formulation No. 19) which showed size distributions of liposomal particle in resolution of x10000.

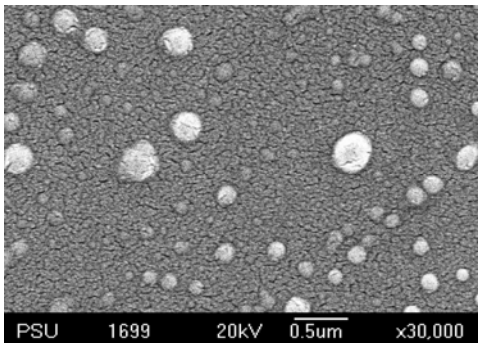


Figure 4.17 SEM image of freshly prepared of liposome containing rhinacanthin-C formulation composed of SPC : CHL in 1:1 molar ratio (Formulation No. 19) which showed size distributions of liposomal particle in resolution of x30000.

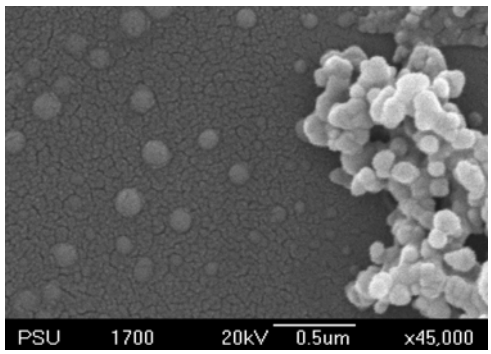


Figure 4.18 SEM imaging of aggregation of freshly prepared of liposome containing rhinacanthin-C formulation composed of SPC : CHL in 1:1 molar ratio (Formulation No. 19) in resolution of x45000.

4.5 Method validation

Method validation is a process of establishing that the performance characteristics of the analytical method are suitable for the intended application. The methods validation process for analytical procedures begins with the planned and systemic collection by the applicant of the validation data to support analytical procedures (FDA, 2000). The methods were validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures (ICH, 1996). The aim of this work was developed and validated of a HPLC method and UV methods for the estimation of rhinacanthin-C in liposome formulations. The stability of rhinacanthin-C in liposome formulation was studied by using the HPLC method. Since, we expected that a rhinacanthin-C was trapped in liposome maybe decomposed in during storage period. Therefore, HPLC was a method that is able to quantify the remaining amounts of rhinacanthin-C entrapped in the liposome without any interference of other compounds. While a UV method is commonly used in analytical laboratories for qualitative and quantitative analyses.

4.5.1 Validation of the UV-Visible spectrophotometric method

The UV-Visible spectrum recorded at 278 nm, which is a maximum absorption wavelength of rhinacanthin-C. The results from the method validation are as followed:

4.5.1.1 Linearity

The calibration curve for standard rhinacanthin-C was obtained by plotting the absorbance values versus concentrations of standard solutions of rhinacanthin-C in 20% ethanol in buffer pH 7.4. Linearity was found to be in the range of 1.0 - 5.0 µg/mL (Figure 4.19) with significantly high value of correlation coefficient r^2 = 0.9999; the representative equation was y = 0.0400x + 0.0028. The quantitative parameters for determination of rhinacanthin-C are listed in Table 4.12. The low R.S.D value (<5%) indicated the high precision of the calibration curve and accepted for further analysis method. It should be noted that at a time of the determination, a new standard curve is constructed for each experiment interpretation.

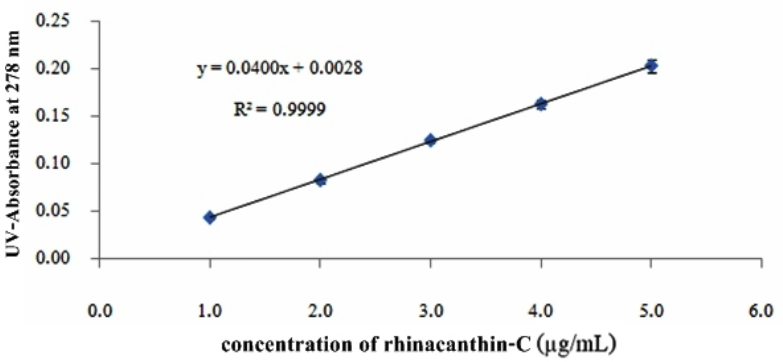


Figure 4.19 Calibration curves of rhinacanthin-C standard solutions (1.0 - 5.0 µg/mL) in buffer solution pH 7.4 containing 20% ethanol recorded at 278 nm.

Table 4.12 Quantitative parameters for rhinacanthin-C determination (solvent = buffer solution pH 7.4 containing 20% ethanol).

Parameters	Regression analysis results
λ max (nm)	278
Regression coefficient	0.9999
Slope ± standard deviation	0.0400 ± 0.0017
Intercept ± standard deviation	0.0034 ± 0.0028
Relative standard deviation (%)	2.75
Concentration range (µg/mL)	1.0 - 5.0
Number of points	5

4.5.1.2 Precision

The precision analyses result of intra-day (n = 6) and inter-day (n = 9) of various concentrations of rhinacanthin-C were obtained and reported as R.S.D. (%) values. The values of intra-day and inter-day precision were 1.61 - 3.34% (Table 4.13) and 1.98 – 2.66% (Table 4.14), respectively which lower than 5%. R.S.D. values indicated that the developed method gave a trust precision value. It is therefore, the method was suitable for using in determination the entrapping efficiencies of rhinacanthin-C in liposome formulations (Figure 4.20 and 4.21).

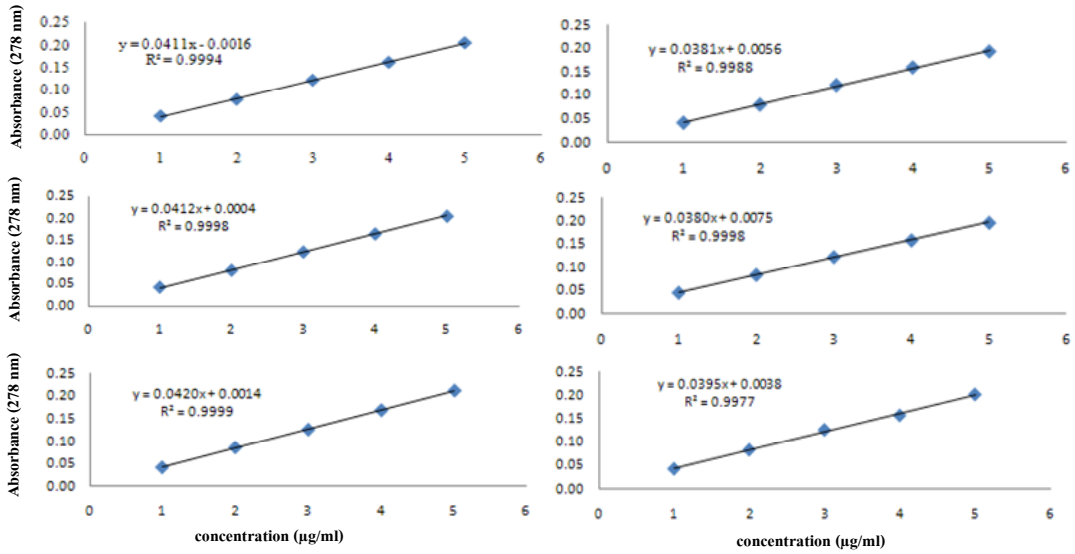


Figure 4.20 Intra-day precision of five different concentrations (n = 6); (average R.S.D. = 2.75 %).

Table 4.13 Intra-day precision of rhinacanthin-C solutions of 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL.

Conc. of rhinacanthin-C (µg/mL)	mean ± SD (µg/mL) (n = 6)	% R.S.D.	Average % R.S.D.
1	0.9983 ± 0.0012	2.84	
2	1.9860 ± 0.0027	3.34	
3	3.0307 ± 0.0020	1.61	2.75
4	3.9843 ± 0.0044	2.73	
5	4.9956 ± 0.0066	3.24	

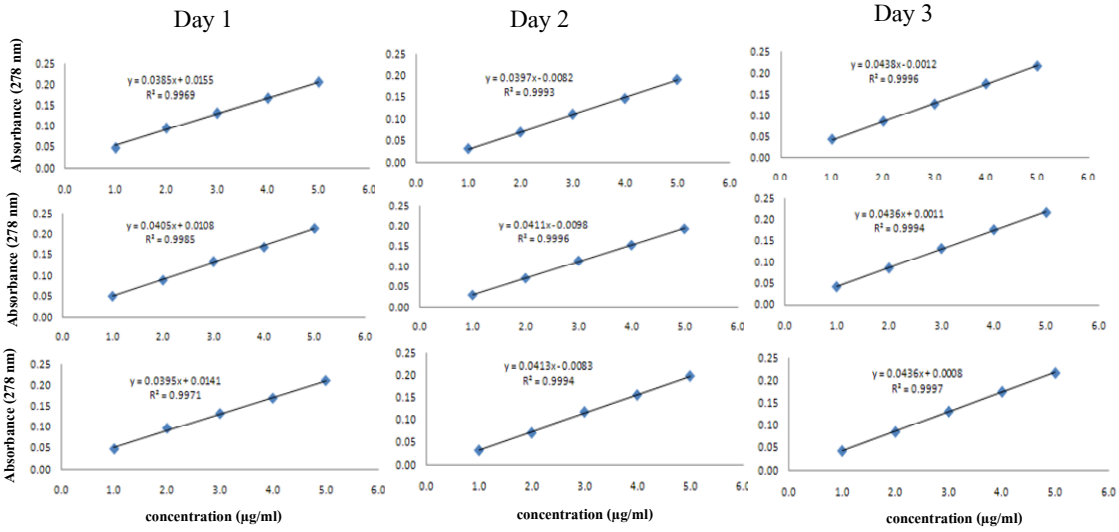


Figure 4.21 Inter-day precision of five different concentrations (n = 9); (average R.S.D. = 1.84 %).

Table 4.14 Inter-day precision of rhinacanthin-C solutions of 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL.

Conc. of rhinacanthin-C (µg/mL)	mean ± SD (µg/mL) (*n* = 9)	% R.S.D.	Average % R.S.D.
1	0.954 ± 0.0037	2.66	
2	2.064 ± 0.0053	2.04	
3	2.971 ± 0.0091	1.98	1.84
4	3.968 ± 0.0107	1.24	
5	5.020 ± 0.0103	1.25	

4.5.1.3 Accuracy

It was investigated by means of a standard rhinacanthin-C addition experiment, at three concentration levels (2.0, 3.0 and 4.0 µg/mL) in triplicate. The mean recoveries of each concentration are shown in Table 4.15, which assured the method accuracy.

Table 4.15 Recovery (%) of rhinacanthin-C solutions of 2.0, 3.0 and 4.0 µg/mL.

Concentration of added rhinacanthin-C (µg/mL)	Amount of rhinacanthin-C found (µg/mL)			Mean ± SD*	Recovery (%)*
	I	II	III		
2.0	1.66	2.01	2.25	1.97 ± 0.30	98.67 ± 13.27
3.0	2.39	2.87	2.27	2.51 ± 0.32	83.66 ± 9.47
4.0	4.10	3.94	3.70	3.91 ± 0.20	97.79 ± 4.50

* Significant differences ($P<0.05$)

4.5.1.4 Specificity

No other absorption band was found interference at 278 nm in the UV-Visible spectra of sample solution prepared from blank liposome. Only absorbance peak of rhinacanthin-C was found in the UV-Visible spectra of sample solution prepared from liposome containing rhinacanthin-C (Figure 4.22). Therefore, the determination of rhinacanthin-C was performed at 278 nm to evaluate the presence of absorption band of sample solution at 278 nm and indicative of rhinacanthin-C in complex formation.

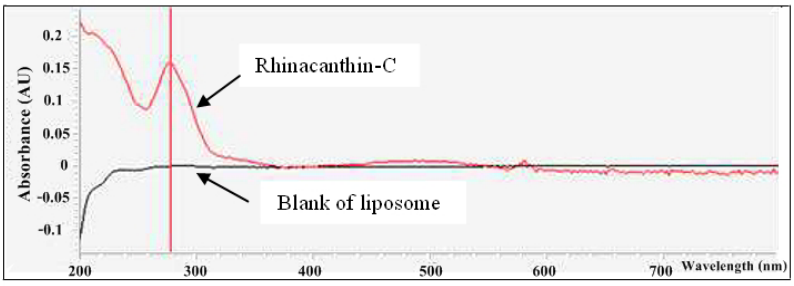


Figure 4.22 UV-Visible spectra of rhinacanthin-C and sample preparation from blank liposomal in buffer solution pH 7.4 containing 20% ethanol.

4.5.1.5 Detection and quantitation limits

In the UV-Visible spectrophotometric analysis was optimized method of diluted solutions of rhinacanthin-C in a range 1.0 - 5.0 µg/mL. The limit of detection (LOD) calculated using parameters of the calibration curve was 0.17 µg/mL. It was possible to identify the absorption band of rhinacanthin-C at 278 nm. The limit of quantification (LOQ) determined based on the parameters of the calibration curve was 0.57 µg/mL and showed good regression coefficient (r^2 = 0.9968).

4.5.2 Validation of the chromatographic method (HPLC) for quantitative analysis

The standard calibration curves for rhinacanthin-C were plotted between peak areas versus concentration of rhinacanthin-C. The standard solutions of rhinacanthin-C were varied to provide serial solutions as 0.01, 0.02 and 0.03 mg/mL for using in constructing standard curve of analysis. The standard curve of rhinacanthin-C is exhibited in Figure 4.23.

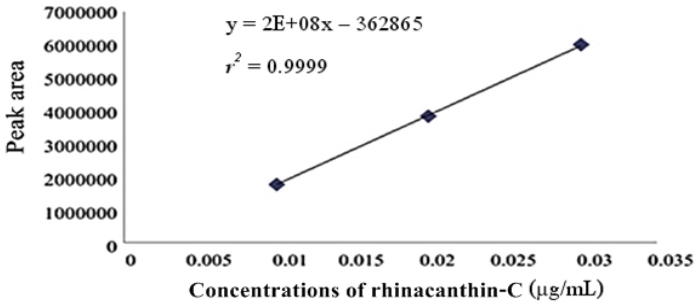


Figure 4.23 A standard calibration curves of rhinacanthin-C standard solutions determination by HPLC method.

4.5.2.1 Accuracy and precision

The recovery of the method was performed by recovery study using standard addition method. Standard solutions of rhinacanthin-C at three different concentration levels (0.016, 0.020 and 0.024 mg/mL), were added with blank liposome and the final volume was adjusted to ten milliliters. All samples were prepared in triplicate and the percentage recovery was determined. The recovery (%) results of each concentration are shown in Table 4.16. The percent recovery of the method was found to be in a range of 92.08 – 101.25 % with the percent relative standard deviations (% R.S.D.) were 6.30 – 0.71 %. It demonstrated that the analytical method showed good accuracy.

Table 4.16 Recovery (%) of rhinacanthin-C after added to blank liposome determination by HPLC method.

Concentration of added rhinacanthin-C (mg/mL)	**Recovery (%)**			**Mean recovery (%) ± SD**	**% R.S.D**
0.016	100.85	102.08	100.81	101.25 ± 0.72	0.71
0.020	99.08	101.57	99.35	100.00 ± 1.37	1.37
0.024	95.61	95.24	85.39	92.08 ± 5.80	6.30

The precision of the method was determined by intra-day and inter-day precision studies. The results are shown in Table 4.17 – 4.18, the intra-day and inter-day precision of the analyzed samples as determined by % R.S.D. were in a range of 0.58 to 1.57 % and 2.08 to 7.47 %, respectively. The precision is very much dependent on the sample matrix, the concentration of the analyte and on the analysis technique. It can vary between 2% and more than 20% (Yodhnu, 2008). These results indicated that the method for determination of rhinacanthin-C from blank liposome has good precision.

Table 4.17 Intra-day variability for determination of the rhinacanthin-C during analytical method.

Concentration of added rhinacanthin-C (mg/mL)	Recovery (%)			Average recovery (%) Mean ± SD	% R.S.D
0.016	101.25	101.25	102.50	101.67 ± 0.65	0.58
0.020	100.00	99.00	101.50	100.17 ± 1.13	1.03
0.024	92.08	95.42	95.00	94.17 ± 1.62	1.57

* R.S.D. (%) = (SD/mean) × 100; [a] All values were obtained from triplicate analyses (*n*=3).

Table 4.18 Inter-day variability for determination of the rhinacanthin-C during analytical method.

Concentration of added rhinacanthin-C (µg/mL)	Amount found (µg/mL)			[a]Mean ± SD	[*]R.S.D. (%)	% Recovery [a]Mean ± SD
	Day1	Day2	Day3			
0.5	0.49	0.51	0.49	0.50 ± 0.01	2.08	99.22 ± 2.31
1.0	0.95	1.07	1.12	1.05 ± 0.08	7.47	104.56 ± 7.74
2.0	1.99	1.89	1.88	1.92 ± 0.05	2.83	95.93 ± 2.74
3.0	2.97	3.13	3.02	3.04 ± 0.07	2.41	101.32 ± 2.53

* R.S.D. (%) = (SD/mean) × 100; [a] All values were obtained from triplicate analyses (*n*=3).

4.5.2.2 Linearity and range

The solutions of rhinacanthin-C were prepared in a range of 2 - 10 µg/mL and utilized in the linearity testing. The analytical method was performed as previously described. The peak areas obtained from each run were plotted against the concentrations. At each concentration was prepared in triplicate and relative standard deviation (%) was determined. The linear fitted equation (Y = aX + b) and correlation coefficient (r^2) were summarized in Table 4.19. The result demonstrated that rhinacanthin-C solutions displayed linear response correlated to concentration range (Figure 4.24).

Table 4.19 Linear range and correlation coefficients of the calibration curves.

Concentration (µg/mL)	Mean of peak area ± SD	R.S.D. (%)	Y = aX + b	r^2	LOD (µg/mL)	LOQ (µg/mL)
0.5	8581 ± 249.8	2.91	Y=21593x + 2130.5	0.9972	0.063	0.209
1.0	20446 ± 1670.4	8.17				
2.0	39298 ± 1181.5	3.01				
3.0	63505 ± 1637.2	2.58				

Y = peak area, X = condition (µg/mL); Limit of detection, (LOD); Limitation of quantitation, (LOQ)

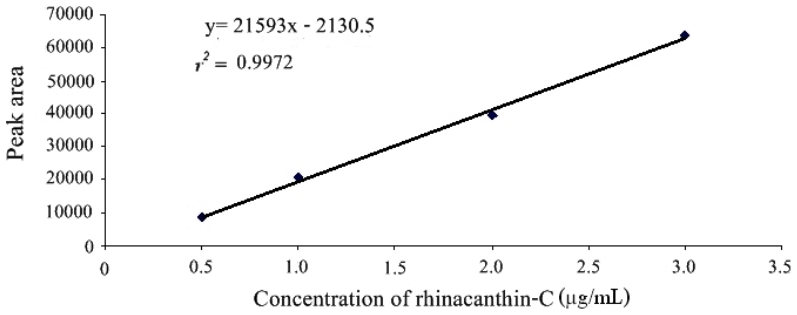


Figure 4.24 Calibration curve of rhinacanthin-C solutions (mean ± SD; *n* = 3) determination by HPLC method.

4.5.2.3 Selectivity

The selectivity of the developed method is acceptable since no interfering peak was observed at the retention time (Rt) rhinacanthin-C (Rt 7.64 min). HPLC chromatogram of rhinacanthin-C showed quite separated in mobile phase conditions for using HPLC analysis. Typical chromatograms of rhinacanthin-C were added blank liposome showed no different of complete separated and retention times (Rt 7.74 min) (Figure 4.25). All chromatogram are displayed selectivity of rhinacanthin-C in solvent or lipid matrix and confirmed the method conditions can be used for quantitative analysis of the rhinacanthin-C containing liposome formulations.

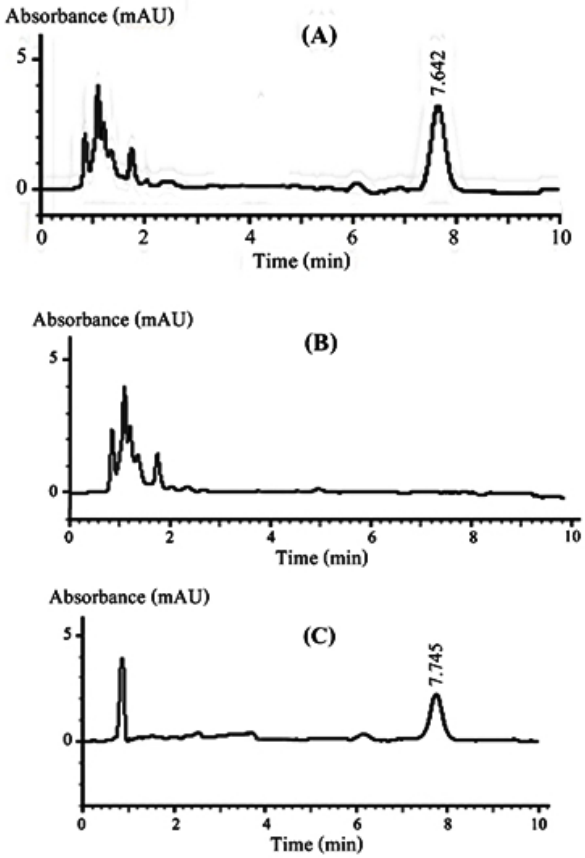


Figure 4.25 Typical HPLC chromatograms of rhinacanthin-C solutions (3.0 µg/mL) in methanol (A), Methanol solvents were mixed blank liposome (B) and rhinacanthin-C solutions (2.0 µg/mL) were spiked with the blank liposome (C).

4.6 Stability determination of nanoliposome containing rhinacanthin-C

The two best formulations composed of SPC : CHL in a molar ratio of 1:1 (F19) and SPC : CHL : T80 in a molar ratio of 1:1:0.5 (F28) were utilized in stability study. The stability of liposome was investigated in two terms. Firstly, the physical appearances of liposome formulations with changes in average particle size of liposome dispersions were monitored in the period of three months storage. The resulting of size distributions during stability study are shown in Figure 4.26, which demonstrated that the both liposome formulations (F19 and F28) had good size stability. No changing in particle size at the initial storage time (60 days) was observed for the formulations of F19, SPC : CHL (blank) and F28 but their sizes were found decreasing after storage for 90 days. This phenomenon could be explained that diffusion of particles of

freshly prepared was used for size measurement. However, when stored at a period of time the particle may have excluding behavior to systematize for suitable stability in colloid suspensions, caused smaller particle sizes. Moreover, it could be due to unstable of self-liposome particle in the term of lipid degradation by decreased gradually of lipid bilayers by the loss of outer bilayers during storage. Generally, instable of particle was described frequently in the even of particle aggregation and to be large particle by formation sediment as shown in blank formulation of F28. However, it can be observed that the entrapped substances could enhance particle size stability of liposomes. As can be seen that formulation No. 16 (with rhinacanthin-C) showed better particle size stability than the same formulation without rhinacanthin-C. That could because of the strong charge interaction between the entrapped molecules with the lipid bilayer.

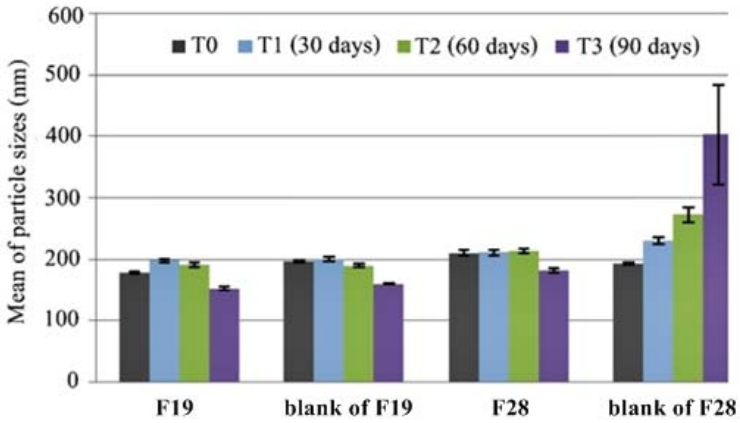


Figure 4.26 Size distribution of liposomes formulations i.e. Formulation No. 19 (with rh-C), blank of F19, Formulation No. 28 (with rh-C) and blank of F28 on stability study of liposome preparations, after storage at room temperature for three months.

In the case of blank of F28 (without rhinacanthin-C) larger particle size was observed after storage for three months. This may be due to Tween 80 exposed from the outer leaflet of the bilayer membrane would increase the liposome particle curvature. Therefore, adding Tween 80 increase the liposomal size during storage since more Tween 80 existed in the outer leaflet than in the inner leaflet of the liposomal bilayer membranes (Fan et al., 2008). In addition, it was believed that the enhanced stability of nano-liposomes was due to the surface attached polymer, which represented a steric barrier to the interacting macromolecules. To sustain the slow

clearance and sequestration of nano-liposomes in vivo circulation, sterically stabilized nano-liposomes (commercially they were referred to as Stealth) have been prepared by using polymers such as polysorbate surfactant (Tween), polyethylene glycol and polyvinyl alcohol. And when storage for long time, Tween 80 may be disrupt the rigidity of bilayers, causing instability of liposomes and thus facilitating vesicle aggregation. While the formulation No. 28 (with rhinacanthin-C), rhinacanthin-C may play important role similarly to cholesterol that made a rigid structure of liposomal membranes. Inclusion of rhinacanthin-C and Tween 80 in appropriate proportions may be resulted sterically stabilized nano-liposomes. This result may an increase in the surface potential of liposomes which led to increase the interbilayer distance owing to electrostatic repulsive forces and thus disrupt the fusion or aggregation of liposome vesicles. Therefore, the appropriate proportions of rhinacanthin-C, cholesterol, Tween 80 and lipid were important for maximizing the encapsulating efficiency for keeping the nano-liposomes' stability.

Secondly, stability of rhinacanthin-C in the formulations was determined from ability of the liposomes to protect rhinacanthin-C in liposomes during storage period at room temperature for three months. Liposome sample were prepared and kept in different conditions (buffer pH 4.0, 6.6 and 10.0) at room temperature protected from light for three months. Since these conditions, the rhinacanthin-C extracts are poor stable (Charoonratana, 2007). The remaining amount of rhinacanthin-C entrapped in liposomes was compared with rhinacanthin-C solutions storage in the same conditions. The stability of rhinacanthin-C in liposomes during 6 months storage period is shown in Figure 4.27.

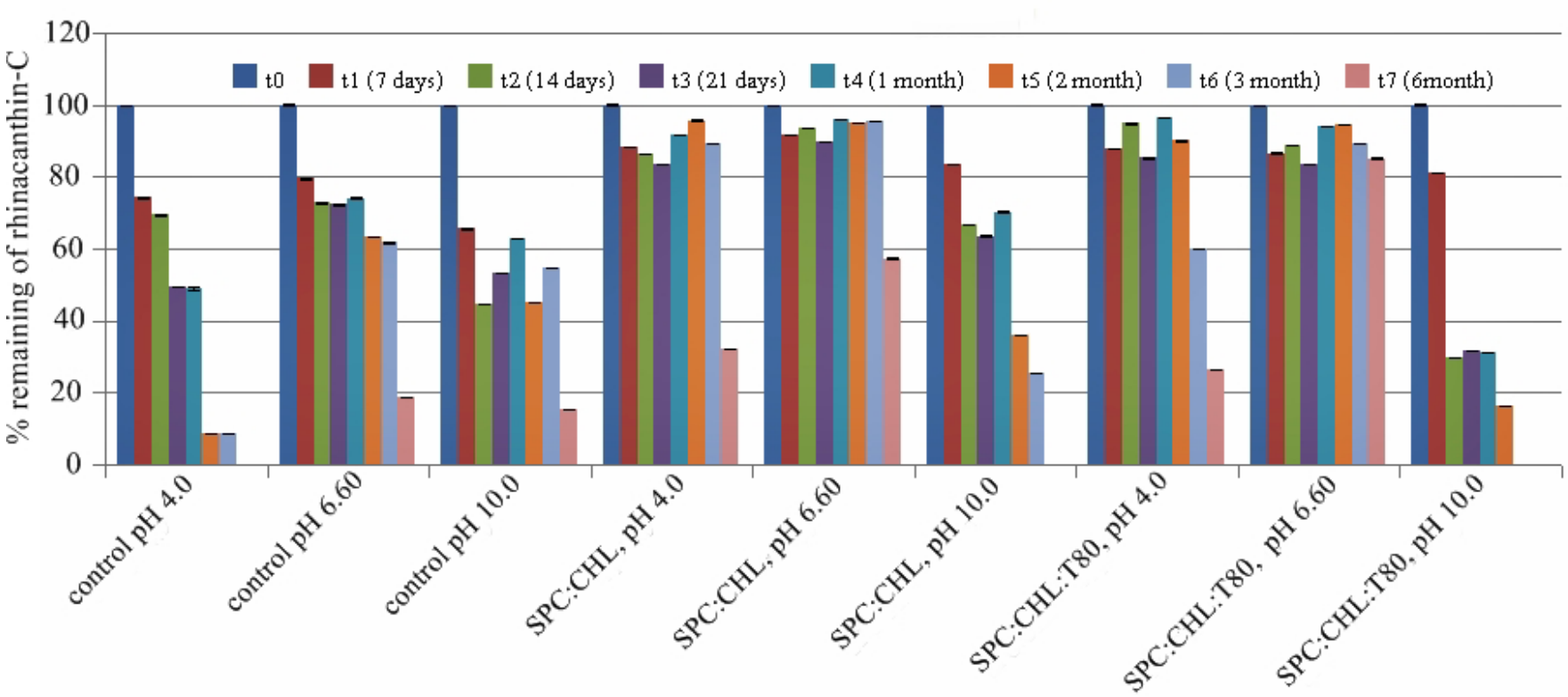


Figure 4.27 The stability study of entrapped rhinacanthin-C in liposomes, which were stored in buffer solutions having different pH values (pH 4.0, 6.6, 10.0) at room temperature for three months (t0 - t7) under light protection. Controls are solutions of rhinacanthin-C in different buffers.

The result of this study found that, rhinacanthin-C entrapped in the both liposome formulations (F19 and F28) gave a good stable better than rhinacanthin-C in solutions (control) when stored in buffer pH 4.0 and 6.6 for six months. Amount of rhinacanthin-C remaining in liposome at 3 months was analyzed by HPLC technique were 89.32% and 95.63% for formulation No. 19 in pH 4.0 and 6.6, respectively. While amount of rhinacanthin-C in formulations of F28 at 3 months were 60.05% and 89.15% in pH 4.0 and 6.6, respectively. In the case of buffer pH 10.0, rhinacanthin-C in liposome formulations (No. 19) were slightly stable than liposome formulations (No. 28) but rhinacanthin-C in the both formulations were less stable than rhinacanthin-C solutions in basic medium (pH 10.0). This probably due to base affecting to inflexible of liposomal bilayers which caused to leakage of the encapsulated rhinacanthin-C. From study of Sulkowski and his team in 2005, found that the influence of pH on the properties of liposome membranes can be the result of phosphate group protonation in acid environment and of the formation of hydrogen bonds between DPPC molecules (Sulkowski et al., 2005). It was found that liposomes prepared at low pH (1.9 or 5.0) are more rigid below the transition phase temperature (Pitcher Iii and Huestis, 2002) than those prepared at high pH (8.0 or 8.4). As described two degradation pathways might have been limit shelf-life of phospholipids in aqueous liposomal dispersions: the oxidative and hydrolytic degradation pathways (Grit and Crommelin, 1993; Samuni et al., 2000). Furthermore, rhinacanthin-C is not stable to base conditions and incident the oxidative reaction of lipids affected to rush decompose of rhinacanthin-C in liposome formulations than rhinacanthin-C solutions. Rhinacanthin-C in liposome formulation No. 28 showed the remaining of rhinacanthin-C was more than 80 % and the formulation No. 19 was more than 55 % at pH 6.6, while pure rhinacanthin-C showed that rhinacanthin-C was remained less than 20 % when kept them for six months in same conditions.

4.7 Antifungal potential determination of nanoliposome containing rhinacanthin-C

4.7.1 Determination of antifungal activities by using agar well-diffusion method

To screening activities of liposome containing rhinacanthin-C, agar well-diffusion assay (Magaldi et al., 2004) was chosen to use in preliminary screening of antifungal activities. The results are summarized in Table 4.20 and 4.21. Two concentrations per well of rhinacanthin-C in

nano-liposomes, 80 and 120 µg were used in antifungal activity determination. Control sample was a rhinacanthin-C solution of 120 µg/well. The different of the amount of rhinacanthin-C per well did not give significantly different in inhibition zone diameters against *M. gypseum* (Figure 4.28). Moreover, two different formulations (F19 and F28) gave similar results. Their inhibition zone was in a range of 12.60 - 13.45 mm. Rhinacanthin-C solutions displayed inhibition zone larger than (19.00 - 20.34 mm) liposome rhinacanthin-C formulations. Similarly results were obtained when the samples were tested against *T. rubrum* (Figure 4.29). The result showed no different in inhibition zone diameters for variation amount of rhinacanthin-C per well of the same formulations. However, for this microorganism, the formulation containing (F28) showed larger inhibition zone (25.70 - 29.50 mm) than formulation No. 19 (16.17 - 17.72 mm). It is worth to note that formulation No. 28 gave larger inhibition zone than rhinacanthin-C solutions (20.95 - 23.20 mm) against *T. rubrum*. This may be due to influence of Tween 80 in formulations on sterically stabilized liposomal membrane, the flexible or softness of lipid bilayer affecting drug release. These include approach of liposomal to fungal cell wall. While cholesterol would enhance stability and rigidity of the liposomal membrane and permeability imparted to cell (Lopez-Pinto et al., 2005; Ma et al., 2007; Xia et al., 2007; Fan et al., 2008; Dragicevic-Curic et al., 2010). Therefore, in formulation No. 28 showed better antifungal activities than formulation No. 19 (Table 4.21).

Table 4.20 Inhibition zone diameters of the both liposome formulations against *M. gypseum*.

Samples	**Amount of rhinacanthin-C (µg/well)**	**Inhibition zone (mm)**			**Average ± SD (mm)**
Liposome formulations (F19)	80	13.80	13.50	13.05	13.45 ± 0.38
	120	12.80	12.30	12.70	12.60 ± 0.26
rhinacanthin-C solution (control)	120	19.45	18.00	19.55	19.00 ± 0.87
Liposome formulations (F28)	80	12.90	14.15	11.75	12.93 ± 1.20
	120	15.10	12.95	13.65	13.90 ± 1.10
rhinacanthin-C solution (control)	120	20.08	19.45	21.50	20.34 ± 1.05

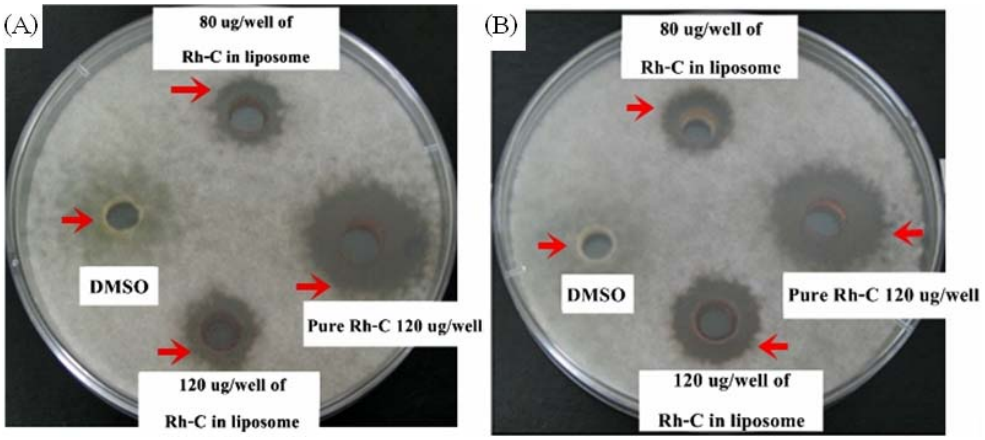


Figure 4.28 Images show antifungal activity against *M. gypseum* of liposome containing rhinacanthin-C composed of (A) formulation No. F19 and (B) formulation No. F28 by agar well-diffusion method.

Table 4.21 Inhibition zone diameters of the both liposome formulations against *T. rubrum*.

Samples	**Amount of rhinacanthin-C (µg/well)**	**Inhibition zone (mm)**			**Average ± SD (mm)**
Liposome formulations (F19)	80	17.15	16.65	14.70	16.17 ± 1.29
	120	15.90	19.30	17.95	17.72 ± 1.71
rhinacanthin-C solution (control)	120	21.50	23.80	24.00	23.10 ± 1.39
Liposome formulations (F28)	80	27.10	22.25	27.75	25.70 ± 3.01
	120	29.70	28.90	29.90	29.50 ± 0.53
rhinacanthin-C solution (control)	120	20.00	21.70	21.15	20.95 ± 0.87

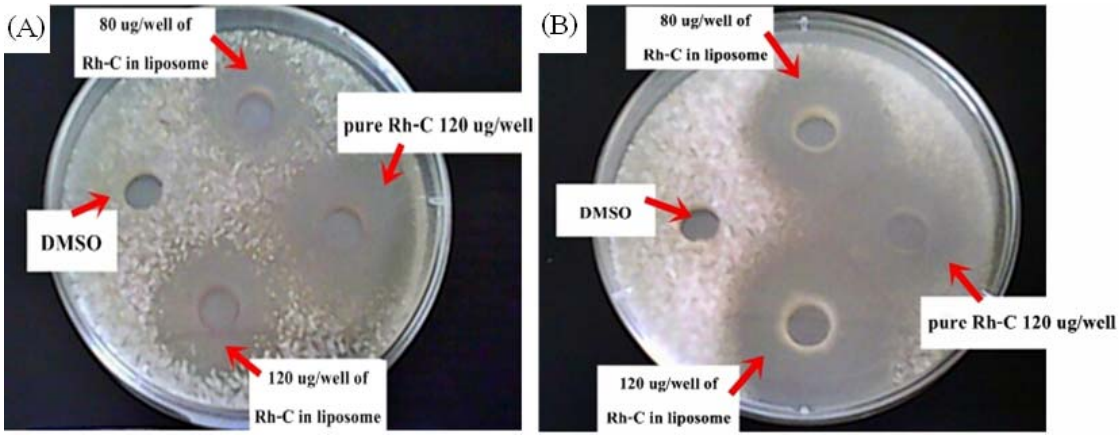


Figure 4.29 Images show antifungal activity against *T. rubrum* of liposome containing rhinacanthin-C composed of (A) formulation No. 19 and (B) formulation No. 28 by agar well-diffusion method.

The result also demonstrated that liposome containing rhinacanthin-C formulations (F28) was obtained show inhibition zone against *T. rubrum* better than *M. gypseum* (Table 4.19 and 4.20), this maybe due to *Trichophyton* sp. had smooth and thin walled of macroconidia than *Microsporum* sp. The Macroconidia is a large spore (conidia) produced in a fungus through vegetative reproduction. De Souza Pereira and Geibel found undulations (rugosities) and pores (50 – 250 nm in diameter) present on the cell walls surface of *Saccharomyces cerevisiae* strains. The pore on the surface of the cell walls is possible to transport genetic information (plasmids or genes) to the inside of microbes, using a technique called electroporation, which involves increasing the cell wall permeability via electric pulses. It is believed that pore formations generated in this manner is reversible and induce reactive oxygen species formation and, consequently, lipoperoxidation in biological membranes (oxidative stress condition), leading to the death of a considerable number of cells (De Souza Pereira and Geibel, 1999). The cell walls are the one main barrier of somatic antigens and often play an important role in pathogenesis. They contain components that are not present in other cell type which makes them potential targets for selective antifungals. Also, rigidity of cell walls is barrier impotent in antifungal drugs to uptake or cannot penetrate inside cell walls for disrupted a variety of mechanisms action into fungal cell (Chamilos and Kontoyiannis, 2005); (Shigeru Kohno et al., 1998); (Latge, 2007); (Farkaš, 2003); (Ruiz-Herrera et al., 2006); (Perez and Ribas, 2004); (Adams, 2004). Therefore incorporation of antifungal drugs into liposomes may enhance cell wall penetration of the drug.

4.7.2 Determination of minimum inhibitory concentration (MIC)

The MIC is defined as the lowest concentrations of rhinacanthin-C entrapped in liposome that have the *in vitro* antifungal activity and is expressed as MIC indicating minimal inhibitory concentration of complete growth inhibition of the tested fungi. The results from MIC determination are summarized in Table 4.22. Rhinacanthin-C entrapping in liposome formulation No. 28 showed higher antifungal activity than formulations No. 19 both after 4 and 7 days of incubation periods. This formulation also gave lower MIC values than pure rhinacnthin-C and standard antifungal agent, ketoconazole formulations both after 4 and 7 days of incubation periods. MIC values of liposome formulation No. 28 were 1.87 µg/ml both for *M. gypseum* and *T. rubrum* (Table 4.22). Even though from preliminary screening test, the formulations composed of F28 show a superior activity against *T. rubrum* compared to formulations No. 19. However, the results from MIC determination found that, rhinacanthin-C entrapping in liposome formulation No. 28 showed excellent activity against *M. gypseum* (MIC values were 1.87 and 1.87 µg/ml for 4 and 7 days, respectively.

These results suggested that the obtained nanoliposome could enhance the antifungal activities of rhinacanthin-C against dermatophytes. According to the previous results related to the stability improvement of rhinacanthin-C in liposome formulation that could affect the antifungal activity. The amount of retained rhinacanthin-C in liposome formulations during exposure and accumulated at the target site were high enough to kill the microorganisms. A number of publications reported that liposome nanoparticles could be enhanced the drug uptake, distribution, absorption, stability to cell target more than a larger size molecule (Manosroi et al., 2004); (Lian and Ho, 2001); (Zamboni, 2005); (Zrinka Abramović et al., 2008).

Table 4.22 Determination of minimum inhibitory concentration (MIC) of liposome containing rhinacanthin-C formulations against *M. gypseum* and *T. rubrum* after inoculated for 4 and 7 days.

Samples	MIC values (µg/mL) against *M. gypseum*		MIC values (µg/mL) against *T. rubrum*	
	4 days	7 days	4 days	7 days
Liposome formulations (F19)	15	30	3.75	7.50
Liposome formulations (F28)	1.87	1.87	1.87	1.87
Rhinacanthin-C solutions	60	240	1.87	3.75
Ketoconazole	7.5	15	7.50	7.50

Furthermore, the result in this study found that improved antifungal activity of rhinacanthin-C in liposome formulations in aqueous and showed quite potent/equal fungal inhibited compared with rhinacanthin-C dissolved in DMSO (Table 4.22). Therefore, the obtained nano-liposomes containing rhinacanthin-C could be improved and developed antifungal activities. All this depends on the physiochemical characteristics of the liposomes which were prepared. The result of antifungal activities of liposome containing rhinacanthin-C indicated that a smaller size of liposome of formulation No. 28 (115.8 ± 23.33 nm) could be able to pass through to cell membrane of fungal easier than a large size of liposome formulation No. 19 (221.4 ± 13.76 nm). The fungal cell wall composed of a polysaccharide based three-dimensional network. They have a rigid and insoluble structures that useful as a force for protect the penetration of insoluble substrates that it colonizes or invades (Latge, 2007). Therefore, it possible a large of particle size or insoluble substances is confined in cell wall and can not pass through to cell membrane of fungi.

CHARPTER 5

CONCLUSIONS

In conclusion, rhinacanthin-C extracted from leaves of *R. nasutus* could be used to prepare in liposome formulation. The suitable formulations of liposome containing rhinacanthin-C were composed of SPC : CHL and SPC : CHL : T80 in a molar ratio of 1 : 1 : 0 (F19) and 1 :1 : 0.5 (F28), respectively. They showed better stable and antifungal properties when investigated comparison with pure rhinacanthin-C.

The particle size of liposome containing rhinacanthin-C formulations were obtained in the range of 100 - 300 nm and their zeta potential values were -38.16 ± 3.02 (F19) and -40.98 ± 2.02 mV (F28), respectively. High trapping efficiency of rhinacanthin-C in liposome formulations (> 80%) was also achieved.

Rhinacanthin-C entrapped in liposomes (F19 and F28) showed more stable than the free rhinacanthin-C in solution, when stored in buffer pH 4.0 and 6.6 at room temperature and under protected from light for three months. However, rhinacanthin-C entrapped in the both formulations was less stable than rhinacanthin-C solutions in basic medium (pH 10.0). Tween 80 added in formulation No. 28 was demonstrated reducing stability of lipid membrane compared to the amount of rhinacanthin-C when stored in acid medium (pH 4.0) for three months with compared formulations without Tween 80 (F19). However, Tween 80 not influence on stable of liposomal size after storage for three months, excepted in formulations without rhinacanthin-C (blank of F28) was measured large particle size.

Liposome containing rhinacanthin-C composed of SPC : CHL : T80 (F28) displayed greater inhibition zone than the formulations of SPC : CHL (F19) against *T. rubrum* but showed no different against *M. gypseum*. The MIC result of formulation No. 28 showed better inhibition activity against *M. gypseum* when compared with rhinacanthin-C in solutions. These MIC data also showed a slightly stronger on inhibited *T. rubrum* inhibition activity when compared with

rhinacanthin-C in solutions. Therefore, rhinacanthin-C entrapped in liposome formulations could improve their stabilities and antifungal activities, which maybe useful for further development in the pharmaceutical dosage forms, such as topical and parenteral products.

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List of Publications and Proceedings

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