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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FORMULATION OF NIOSOMES CONTAINING *CAESALPINIA SAPPAN* L. HEARTWOOD EXTRACT

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Caesalpinia sappan L. หรือฝาง ได้มีการนำมาใช้ในทางอาหาร เครื่องดื่ม สีย้อมผ้า และเครื่องสำอาง มีรายงานมากมายเกี่ยวกับฤทธิ์ทางชีวภาพของสารสกัดแก่นฝาง ได้แก่ ฤทธิ์ต้านออกซิเดชัน ฤทธิ์ยับยั้งกระบวนการสร้างเม็ดสี ฤทธิ์สมานแผล ฤทธิ์ต้านเชื้อแบคทีเรีย และฤทธิ์ต้านการอักเสบ ในงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของวิธีการสกัดแบบกึ่งบริสุทธิ์ของสารสกัดแก่นฝางต่อคุณสมบัติของสารสกัดที่เตรียมได้ และเพื่อพัฒนาระบบนิโอโซมที่บรรจุสารสกัดแก่นฝาง โดยเตรียมสารสกัดหยาบจากแก่นฝางด้วยเอทานอล และนำมาทำให้บริสุทธิ์ขึ้นด้วยวิธีการสกัดแบบแยกส่วนและการใช้ไอออนเอ็กซ์เชนจ์โครมาโตกราฟี การสกัดแบบแยกส่วนจะได้สารสกัดส่วนน้ำ ส่วนไดคลอโรมีเทน และส่วนเอทิลอะซิเตท สารสกัดส่วน ไดอะไอออน® เอชพี-20 ได้จากไอออนเอ็กซ์เชนจ์โครมาโตกราฟี สารสกัดทั้งหมดจะนำมาประเมินลายพิมพ์นิ้วมือทีเอลซี ปริมาณบราซิลิน ปริมาณสารกลุ่มฟีนอลิก และฤทธิ์ต้านอนุมูลอิสระดีพีพีเอช สารสกัดแก่นฝางที่มีปริมาณสารสำคัญและฤทธิ์ต้านออกซิเดชันสูงที่สุดจะนำมาเตรียมเป็นนิโอโซมด้วยวิธีการใช้คลื่นเสียงความถี่สูง และประเมินสัณฐานวิทยาและขนาดอนุภาคของนิโอโซม จากผลการทดลอง ลายพิมพ์นิ้วมือทีเอลซีชี้ให้เห็นว่าบราซิลินเป็นสารสำคัญหลักในสารสกัดแก่นฝาง สารสกัดส่วนไดอะไอออน® เอชพี-20 มีปริมาณบราซิลิน (13.24 ± 0.25 เปอร์เซนต์โดยน้ำหนักต่อน้ำหนัก) และสารกลุ่มฟีนอลิก (604.82 ± 2.45 มิลลิกรัมสมมูลของกรดแกลลิคต่อปริมาณสารสกัด 1 กรัม) มากที่สุด สารสกัดน้ำให้ฤทธิ์ต้านอนุมูลอิสระดีพีพีเอชดีที่สุด (ค่าความเข้มข้นที่ยับยั้งได้ 50 เปอร์เซนต์ = 1.19 ± 0.02 ไมโครกรัมต่อมิลลิลิตร) และดีกว่าวิตามินซี จากผลการศึกษา สารสกัดไดอะไอออน® เอชพี-20 จึงถูกเลือกเพื่อนำมาเตรียมระบบนิโอโซม โดยเตรียมเป็นสารละลายอิ่มตัวของสารสกัดในอะซิเตทบัฟเฟอร์ พีเอช 4.0 ซึ่งพบว่าสารละลายมีความคงตัวดีเมื่อเก็บไว้ในตู้เย็นเป็นเวลาอย่างน้อย 30 วัน เมื่อนำสารสกัดมาบรรจุในระบบนิโอโซม พบว่า ไม่สามารถเกิดนิโอโซมที่คงตัวได้ เนื่องจากเกิดการเกาะกลุ่มและการแยกชั้นของเวสซิเคิล ซึ่งผลการทดลองที่ได้นี้อาจเป็นผลมาจากการเกิดผลึกของสารประกอบในสารสกัดในสูตรตำรับหรือเกิดอันตรกิริยาระหว่างสารประกอบในสารสกัดกับโครงสร้างของนิโอโซม ดังนั้น งานวิจัยที่ควรทำการศึกษาต่อไป ได้แก่ การศึกษาผลขององค์ประกอบของนิโอโซมต่อคุณสมบัติการเกิดนิโอโซมที่บรรจุสารสกัดแก่นฝาง การพัฒนาความบริสุทธิ์ของสารสกัดแก่นฝางให้มากขึ้น และการพัฒนาระบบอื่น ๆ สำหรับนำส่งสารสกัดแก่นฝางเข้าสู่ผิวหนัง

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต .....
สาขาวิชา	วิทยาศาสตร์เครื่องสำอาง	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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SAKAN WARINHOMHUAN: FORMULATION OF NIOSOMES CONTAINING *CAESALPINIA SAPPAN* L. HEARTWOOD EXTRACT. ADVISOR: DUSADEE CHARNVANICH, Ph.D., CO-ADVISOR: ASST. PROF. VIPAPORN PANAPISAL, Ph.D., ASSOC. PROF. BOONCHOO SRITULARAK, Ph.D., 110 pp.

Caesalpinia sappan L. or commonly known sappanwood is used in food, beverage, fabrics and cosmetics. Biological activities of sappanwood extract such as antioxidant, anti-melanogenesis, wound healing, antibacterial and anti-inflammation have been considerably reported. The purpose of this study aimed to investigate the effect of semi-purifying methods of the sappanwood extract on properties of the extract obtained and to develop niosomes containing the sappanwood extract. Ethanolic crude extract from *C. sappan* heartwood was semi-purified with partition method and ion-exchange chromatography. The water, dichloromethane and ethyl acetate fractions of sappanwood extract were obtained from the partition method. The Diaion® HP-20 fraction was prepared from ion-exchange chromatography. These extracts were evaluated for TLC fingerprint, brazilin content, total phenolic content and DPPH free radical scavenging activity. The sappanwood extract showing the highest active content and antioxidant activity was used to prepare niosomes. Niosomes was prepared with sonication method and evaluated for morphology and particle size. As the results of evaluation, TLC fingerprint indicated brazilin as a major compound of all sappanwood extracts. The Diaion® HP-20 fraction had the highest brazilin content (13.24 ± 0.25 %w/w) and total phenolic content (604.82 ± 2.45 mg GAE/g extract). The water fraction showed the greatest antioxidant activity (IC_{50} = 1.19 ± 0.02 µg/ml) and more than ascorbic acid. As a result, the Diaion® HP-20 fraction of sappanwood extract was selected for preparation of niosomes. The saturated solution of this extract in acetate buffer pH 4.0 showed good stability when kept in a refrigerator for at least 30 days. When the extract solution was loaded into niosomes, it was found that all formulations could not form stable niosomes because of aggregation and separation of vesicles. This finding may result from the occurrence of crystal of extract compounds in the formulations or the interaction between the extract compounds and niosome structure. Consequently, further research studies such as effect of niosome compositions on formation of niosomes containing sappanwood extract, development of more purified extract and other skin delivery systems of the sappanwood extract should be performed.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
		Advisor's Signature	.....
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LIST OF ABBREVIATIONS

μg	microgram (s)
μl	microliter (s)
μm	micrometer (s)
Acetone-d_6	deuterated acetone
br	board singlet (for NMR spectra)
°C	degree celsius
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
CHO	cholesterol
^{13}C NMR	carbon-13 nuclear magnetic resonance
d	doublet (for NMR spectra)
dd	doublet of doublets (for NMR spectra)
DEPT	distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	ethyl acetate
EC_{50}	half maximal effective concentration
EE	entrapment efficiency
g	gram (s)
GAE	gallic acid equivalent
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography

HR-ESIMS	high resolution electrospray ionization mass spectroscopy
Hz	hertz
IC_{50}	half maximal inhibitory concentration
MBC	minimum bactericidal concentration
MeOH	methanol
mg	milligram (s)
MIC	minimum inhibitory concentration
min	minute (s)
mL	milliliter (s)
mM	millimolar (s)
MMP	matrix metalloproteinase
MW	molecular weight
NMR	nuclear magnetic resonance
PdI	polydispersity index
pH	the negative logarithm of hydrogen ion concentration
R^2	coefficient of determination
Rf	retention factor
rpm	revolution per minute
SD	standard deviation
T_c	phase transition temperature
TEAC	trolox equivalent antioxidant capacity
UV	ultraviolet
VCEAC	vitamin c equivalent antioxidant capacity

v/v	volume by volume
w/w	weight by weight

CHAPTER I
INTRODUCTION

Nowadays, research trends in anti-aging skin care products are moving towards, especially products from natural substances due to their safety, low irritation and low cost. A number of herbs was found to be effective for skin repair (Sasaki et al., 2007; Mukherjee et al., 2011) such as Indian gooseberry extract, gotu kola extract and turmeric extract. Thailand has many herbs which consists of interesting substances for being the active ingredients in research and development of cosmetic products.

Caesalpinia sappan L. or sappanwood has been used for Thai traditional medicine for wound healing, diarrhea, epilepsy, blood disease and menstrual disorder etc. In addition, red color of sappanwood has been used in food colorants, dyes and cosmetics. Heartwood of *C. sappan* was found to contain homoisoflavonoid which is a subclass of flavonoid (Lin, Liu, and Ye, 2014). Brazilin was a major homoisoflavonoid compound isolated from *C. sappan* extract (8.7-22.2 %w/w) in which its content depended on sources of sappanwood (Temsiririrkkul et al., 2007). Several pharmacological activities of chemical compounds in sappanwood were reported as antioxidant (Badami et al., 2003; Wetwitayaklung, Phaechamud, and Keokitichai, 2005; Hu et al., 2008; Saenjum et al., 2010; Wongtana et al., 2013), anti-inflammatory (Sasaki et al., 2007; Washiyama et al., 2009; Wu et al., 2011; Cuong et al., 2012; Tewtrakul et al., 2015), anti-bacterial (Xu and Lee, 2004; Batubara, Mitsunaga, and Ohashi, 2009; Nirmal and Panichayupakaranant, 2014), wound healing (Nirmal, Prasad, and Keokitichai, 2014; Tewtrakul et al., 2015), type 1 and type 3 matrix metalloproteinase (MMP) inhibitors (Lee et al., 2012) and type 9 MMP inhibitor in human keratinocytes (Youn et al., 2011), and anti-melanogenesis (Chang, Chao, and Ding, 2012; Mitani et al., 2013). Regarding to all these beneficial activities, sappanwood extract has interested to be developed as an active ingredient in cosmetic product.

In the preparation of the extract from herbs, extraction method is an important factor to be considered because it can affect properties of the extract obtained. Maceration method has been widely used for plant extraction (Azwanida, 2015) and is

suitable for initial and bulk extraction (Seidel, 2012). Ethanol was the most widely used solvent for extraction of sappanwood (Han and Rhee, 2005; Batubara et al., 2009; Wang, Sun, and Zhou, 2011; Wu et al., 2011; Toegel et al., 2012; Wongtana et al., 2013; Tewtrakul et al., 2015) due to its safety with low toxicity and appropriateness for the extraction of phenolic compounds (Meireles et al., 2008; Wendakoon, Calderon, and Gagnon, 2012). However, the crude extract contained unwanted impurity compounds such as sugar, wax, lipid, cholesterol and tannin. They may affect properties and activities of the extract obtained.

The semi-purifying methods of the crude extract were processed to remove unwanted compounds. The obtained extracts' properties depended on the type of organic solvent and procedure. The partition method was a conventional and simple method for separation of compound groups in the extracts which based on two immiscible solvents and solubility of the compounds (Jones and Kinghorn, 2012; Santos-Buelga et al., 2012). Thus, the partition technique was used to semi-purify crude extract and to increase content of major compounds of the extract (Huh, Hong, and Hong, 2004; Seidel, 2012).

Furthermore, the ion-exchange chromatography could also be used to semi-purify crude extract. Chromatographic resin (Diaion® HP-20) is a synthesis absorbent for both organic and inorganic compounds. Diaion® HP-20 was used to increase content of phenolic compounds (Huh et al., 2004; Panichayupakaranant, Itsuriya, and Sirikatitham, 2009) and remove unwanted compounds. The advantages of Diaion® HP-20 included high efficiency for purification, less time required, no interaction with samples, stable at wide pH range, low cost and reusable (Dufresne, 1998; Martin, Kau, and Wrigley, 2005; Dragull and Beck, 2012). The previous studies indicated that the semi-purified extracts with Diaion® HP-20 had better antioxidant activity than the initial extract such as of ellagic acid-rich pomegranate fruit peel extract (Panichayupakaranant et al., 2009) and oligomeric proanthocyanidin-enriched grape seed extract (Huh et al., 2004). In addition, more antibacterial activity of rhinacanthins-rich *Rhinacanthus nasutus* extract (Puttarak, Charoonratana, and Panichayupakaranant, 2010), anthraquinone of *Senna alata* extract (Sakunpak, Sirikatitham, and

Panichayupakaranant, 2009) and *Sesbania Grandiflora* bark extract (Anantaworasakul and Okonogi, 2013) were observed. Properties of sappanwood extract were also improved after passing Diaion® HP-20. Diaion® HP-20 semi-purified extract showed to contain two-fold higher brazilin than the crude extract and to have the antibacterial activity against *P. acnes, S. epidermis,* and *S. aureus* more than the crude extract (Nirmal and Panichayupakaranant, 2014). In addition, brazilin-rich extract showed the free radical scavenging activity more than brazilin compound (Nirmal et al., 2014)

Stability of chemical constituents in extract was a factor to be taken into consideration for development of cosmetic products. Brazilin was previously reported that it could be oxidized to brazilein when exposed to light and air (Dong et al., 1996; de Oliveira et al., 2002; Rondao et al., 2013). Color of aqueous solution of sappanwood extract was changed depending on the pH values of the solution (Sinsawasdi, 2012). Moreover, in preliminary study brazilin was found to be stable in weak acid solution more than weak base solution. Furthermore, the physiochemical property of compounds is the key factor in developing cosmetic products. Brazilin is soluble in water and has molecular weight of 286.4 (Namikoshi et al., 1987; Nirmal et al., 2015). Delivery of hydrophilic drugs into skin was limited due to the barrier nature of stratum corneum (Andrews, Jeong, and Prausnitz, 2013). To develop efficient cosmetic product, special delivery systems should be used to increase stability and skin permeation of hydrophilic drugs.

Niosomes are a vesicular system that consists of non-ionic surfactant, cholesterol or membrane additive. It can entrap hydrophilic drug within aqueous component and hydrophobic drug in surfactant bilayer. The advantages of niosomes were low cost, easy to prepare, low toxicity and ability to entrap both hydrophilic and hydrophobic drugs (Sahin, 2007). Niosomes was widely used for transdermal drug delivery such as glycolic acid (Ohta, Ramachandran, and Weiner, 1996), caffeine (Khazaeli, Pardakhty, and Shoorabi, 2007), gallidermin (Manosroi et al., 2010), n-acetyl glucosamine (Shatalebi, Mostafavi, and Moghaddas, 2010), black tea extract (Yeh et al., 2013), luteolin (Abidin et al., 2014) and salidroside (Zhang et al., 2015). Moreover, niosomes could reduce irritation of drugs such as acetazolamide (Guinedi et al., 2005),

glycolic acid (Klinhom, 2008) and tretinoin (Patel et al., 2011). In addition, niosomes improved stability and photostability of drugs when compared with drug solution for example adriamycin (Rogerson, Cummings, and Florence, 1987), tretinoin (Manconi et al.2003), minoxidil (Kanjanapadit, 2005), β-carotene (Palozza et al., 2006) and diclofenac sodium (Ioele et al., 2015). In this study, niosomes containing sappanwood extract were developed to improve extract stability and to deliver it into the skin.

The composition of niosomes affected properties of niosomes. Non-ionic surfactants widely used in niosome formulation were sorbitan fatty acid ester (Span®), polyoxyethylene sorbitol ester (Tween®) and polyoxyethylene cetyl ether (Brij®). The alkyl chain length of non-ionic surfactants and the difference of hydrophilic-lipophilic balance (HLB) influenced to size, entrapment (Uchegbu and Vyas, 1998; Hao et al., 2002) and drug release of niosomes (Baillie et al., 1985; El-Ridy et al., 2012). Cholesterol can stabilize bilayers, decrease phase transition and prevent drug leakage. However, the higher amount of cholesterol caused a decrease in drug release (Uchegbu and Vyas, 1998; Alsarra et al., 2005; Bayindir and Yuksel, 2010; El-Ridy et al., 2012). Cholesteryl poly-24-oxyethylene ether (Solulan® C24) is a non-ionic membrane additive which has been widely used for preventing aggregation by steric stabilization of niosomes (Beugin-Deroo, Ollivon, and Lesieur, 1998; Uchegbu and Vyas, 1998).

In the present study, the ethanolic crude extract of sappanwood was prepared. Then partition method and ion-exchange chromatography were used to semi-purify the crude extract. Brazilin content, DPPH radical scavenging activity, and total phenolic content of the semi-purified extracts were compared. After that, the selected extract was loaded into niosomes. Influence of non-ionic surfactant types on properties of niosomes containing the sappanwood extract were investigated.

The objectives of this study were as follows:

1. To study the effect of the semi-purifying methods of the sappanwood crude extract including partition method and ion-exchange chromatography on properties of the obtained extracts.
2. To evaluate properties of the sappanwood extracts including brazilin content, DPPH free radical scavenging activity and total phenolic content.
3. To develop niosomes containing the sappanwood extract.
4. To study the effect of non-ionic surfactant type on properties of niosomes.

CHAPTER II
LITERATURE REVIEW

1. *Caesalpinia sappan* L.

Caesalpinia sappan L. commonly known as sappanwood is a plant in the Fabaceae family. It is widely distributed and implanted in Southeast Asia, America as well as Africa (Edwards, de Oliveira, and Nesbitt, 2003). It is a small to medium size, shrubby tree 4 to 8 meters tall, trunk up to 14 centimeters in diameter, and bark with district ridges and many prickle and grayish brown (Saenjum et al., 2010). Heartwood is the important part of sappanwood which gives natural red color (Figure 1). In Thai traditional medicine, the heartwood of sappanwood is used for wound healing, diarrhea, epilepsy, blood disease and menstrual disorder etc. Moreover, red color from heartwood is widely used in foods, beverages, fabrics and cosmetics.

Figure 1 Sappanwood tree (left) and heartwood (right)
(Available: https://commons.wikimedia.org)

2. Chemical constituents of *C. sappan* heartwood

Homoisoflavonoid is a major compound of sappanwood. It is subclass of flavonoids which contain one additional atom. These compounds are found in different parts of plants such as seeds, barks, roots, heartwood, bulbs and leaves. The homoisoflavonoids can be classified into 5 types of compounds depending on their

carbon skeleton: sappanin-type, scilliascillin-type, brazilin-type, caesalpin-type and protosappanin-type (Lin et al., 2014). The heartwood of sappanwood has various compound types. The major active compound was brazilin (Hikino et al., 1977; Dong et al., 1996; Yang et al., 2002). Other compounds were found from heartwood of sappanwood for example 3'-O-methylsappanol, 3'-O-methylepisappanol, 3'-O-methylbrazilin, sappanol, episappanol, 3'-deoxysappanol (Namikoshi, Nakata, and Saitoh, 1987), protosappanin A (Nagai et al., 1986), sappanchalcone (Saitoh et al., 1986), brazilein (Dong et al., 1996), brazilide A (Yang et al., 2002), sappanone B, 3-deoxysappanone B, 3-deoxysappanchalcone (Chen and Tu , 2008), caesappanin A and B (Shu et al., 2011), protosappanin B, protosappanin C, protosappanin D, protosappanin E (Washiyama et al., 2009), sappanone A (Chang et al., 2012), and caesalpiniaphenol A-C (Cuong et al., 2012). Moreover, some phenols, alkaloids and tannins isolated from heartwood have been reported (Senthilkumar et al., 2011). The chemical structures of some isolated compounds are shown in Figure 2.

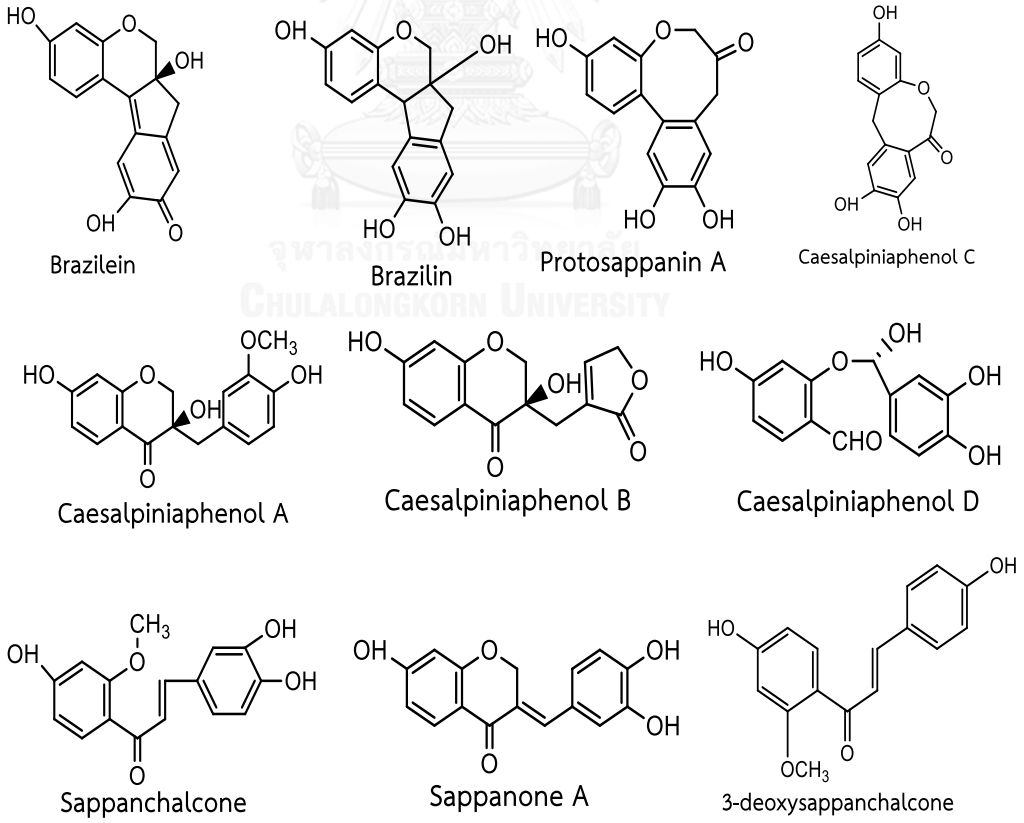


Figure 2 Structures of isolated compounds from heartwood of sappanwood

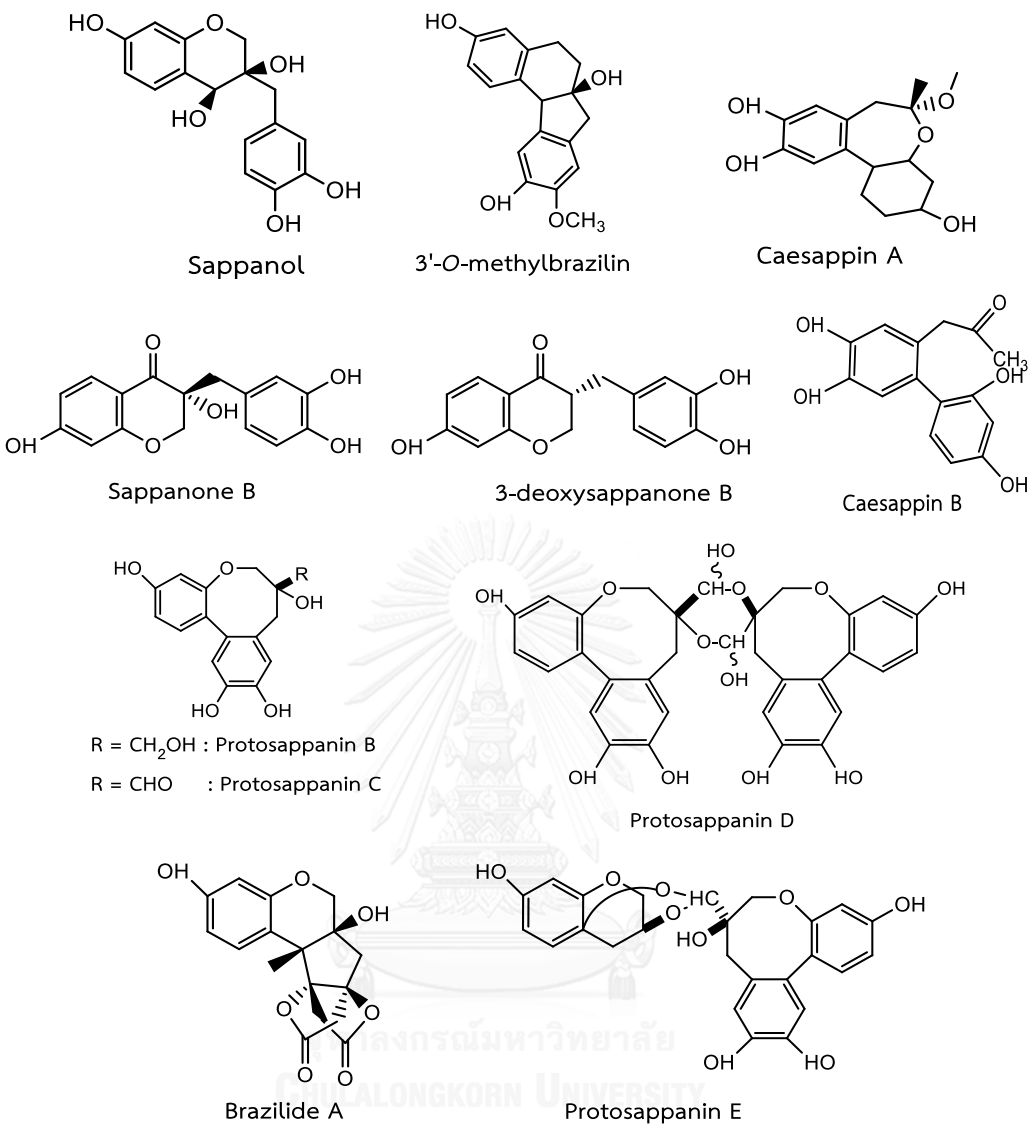


Figure 2 Structures of isolated compounds from heartwood of sappanwood (continued).

3. Brazilin

Brazilin or [(6aS, 11bR)-7,11 b-dihydro-6H-indenol[2,1-c] chromene-3,6a,9,10-tetrol] is the main chemical constituent of sappanwood (Namikoshi et al., 1987). It was isolated as red or amber yellow crystal (Batubara et al., 2009; Nirmal et al., 2014). Brazilin is soluble in water and has molecular weight of 286.4 (Namikoshi et al., 1987; Nirmal et al., 2015). However, it was oxidized to brazilein when exposed to light or air

as shown in Figure 3 (Dong et al., 1996; de Oliveira et al., 2002; Rondao et al., 2013). Sinsawasdi (2012) explained about two chromophores of aromatic rings (ring A and ring B) that when molecule is oxidized, quinone structure of ring B becomes conjugated with ring A and absorbs light at higher visible wavelength. The oxidized brazilin is called as brazilein.

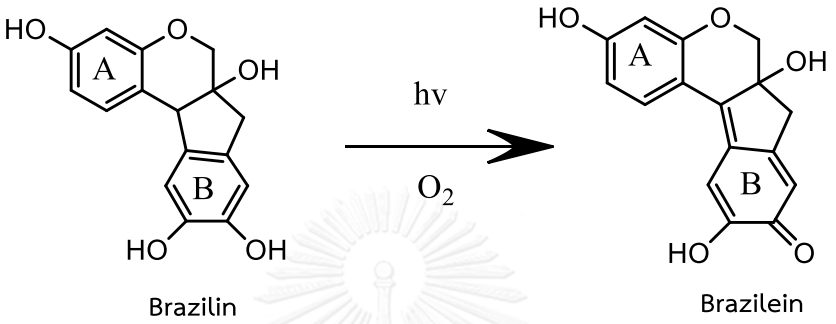


Figure 3 Oxidation of brazilin to brazilein

4. Preparation of sappanwood extract

The extraction procedures are an important step in the processing of the bioactive compounds from medical plants. The extraction method is the separation of chemical compounds from plant material using the solvent through standard procedures. It has influenced to percentage of yield and biological activities of extracts (Jones and Kinghorn, 2012). The extraction method of sappanwood have been reported in many studies.

Maceration is a widely used for initial extraction of medical plants. Solvent is a key factor for the extraction of phenolic compounds from plants. The solvents that have been frequently used for extraction are methanol, ethanol, acetone, and ethyl acetate etc. (Santos-Buelga et al., 2012). Extraction of sappanwood heartwood with various ratios of methanol-dichloromethane and various ages was found that the ratio of 1:3 gave the highest amount of brazilin (1.930) followed by 3:1 (1.782) and 1:0 (1.772) brazilin equivalent (mg) in 1 mg extract. The four years old of heartwood had brazilin content more than 2, 6, 10, and 30 years old. The results indicated that ages of

heartwood was not factor for brazilin content and antioxidant activities of sappanwood extract (Wetwitayaklung et al., 2005). Batubara et al. (2009) reported that percentage yield of sappanwood extracted with methanol was 8.63%. The different parts of sappanwood such as leaves, pods, twigs and heartwood were extracted with water, methanol, and ethyl acetate. The results showed that heartwood gave the highest flavonoid (28 mg/g), phenolic (150 mg/g) and tannin (171 mg/g) content but only 10 mg/g alkaloid (Senthilkumar et al., 2011). The sappanwood macerated with 95% ethanol showed high total phenolic content (178.56 ± 0.36 mg GAE/g) (Wongtana et al., 2013).

Soxhlet extraction is often used technique for phenolic and flavonoid extracts. Advantage of this method is using small amount of solvent because it is continuous extraction process (Santos-Buelga et al., 2012). Sappanwood was extracted by using soxhlet extractor with petroleum ether, chloroform, ethyl acetate, methanol, 50% methanol, and water, respectively. The results showed that 50% methanol had the highest percentage yield (15.9% w/w) followed by water (13.4% w/w), methanol (8.9% w/w), ethyl acetate (3.8% w/w), chloroform (1.6% w/w), and petroleum ether (1.4% w/w). Types of solvents affected yield, bioactive compounds and biological activities of the extracts obtained.

Ion-exchange chromatography (Diaion® HP-20 resin) is a non-polar polymeric adsorbent resin. The advantages of Diaion® HP-20 are high purification efficiency, less time required, no interaction with samples, stable at wide pH range, low cost, and reusable (Dufresne, 1998; Martin et al., 2005; Dragull and Beck, 2012). Diaion® HP-20 has been widely used for extraction of secondary metabolites from natural products because it could separate unwanted compounds (Yu, 2014). The previous study reported that Diaion® HP20 could increase content of active compound from crude extract (Panichayupakaranant et al., 2009; Sakunpak et al., 2009; Chan, Lim, and Tan, 2011). Sappanwood crude extract was purified to obtain brazilin-rich extract by using Diaion® HP-20 column. The results showed brazilin-rich extract contained almost two-fold (39.9 ± 0.34% w/w) brazilin content when compared with the crude extract (20.0 ± 0.26% w/w).

5. Pharmacological activities of sappanwood extract

Crude extracts and isolated compounds from *C. sappan* heartwood have been reported to have various pharmacological activities including antioxidant, anti-melanogenesis, wound healing, matrix metalloproteinase (MMP) inhibition, anti-bacterial and anti-inflammatory activities.

5.1 Antioxidant activity

Free radicals are the main cause of many diseases. There are several studies that the extract and bioactive compounds of sappanwood heartwood showed effective antioxidant activities. Types of solvent used for extraction affected antioxidant activity of the sappanwood extracts. The free radical scavenging activities of sappanwood extracts prepared with six different solvents were determined by 1, 1-Diphenyl-2-pieryl hydrazyl (DPPH) assay. The results presented that they showed strong antioxidant activities. The IC_{50} values of the sappanwood extracts were ordered from low to high as follows: methanolic (1.44 ± 0.12 µg/ml), ethyl acetate (1.71 ± 0.15 µg/ml), 50% methanol (2.31 ± 0.17 µg/ml), water (4.09 ± 0.31 µg/ml), petroleum ether and chloroform fractions (> 1000 µg/ml) (Badami et al., 2003). The first three fractions had higher activities than ascorbic acid (2.85±0.20 µg/ml). Sasaki et al. (2007) reported that IC_{50} values of brazilin (57.4 µM) and protosappanin A (62.2 µM) showed lower than vitamin E. In addition, Hu et al. (2008) found that ethanolic extract, brazilein, protosappanin A and protosappanin B showed different inhibition percentages of malondialdehyde (MDA), scavenging of hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and superoxide anions (O_2^{-}). These results may be due to different functional groups of substances affecting to the radicals. Evaluation of ABTS scavenging activity of the extracts was expressed by trolox equivalent antioxidant activity (TEAC) and vitamin C equivalent antioxidant activity (VCEAC), respectively. Saenjum et al. (2010) found that crude extract has strong ABTS scavenging activity with TEAC of 0.9159 ± 0.0055 trolox/gram extract and VCEAC of 0.5782 ± 0.0042 L-ascorbic acid/gram extract. The IC_{50} values of trolox equivalent antioxidant activity of crude extract (10.24 µg to 13.40 µg) showed

stronger than brazilin (28.79 μg). The results suggested that the potential antioxidant activity was not only brazilin but also other compounds in sappanwood (Wetwitayaklung et al., 2005).

5.2 Anti-melanogenesis

Recently, Chang et al. (2012) reported that methanolic sappanwood extract showed strong melanogenesis inhibition activity in mouse melanoma B16 cells. In addition, sappanone A isolated from the heartwood showed inhibition activities of both melanogenesis and cellular tyrosinase depending on its concentration (1.1-4.4 μM). The mechanism of sappanone A in inhibition of melanogenesis may be due to suppression of tyrosinase gene expression. The similar results were investigated by Mitani et al. (2013) reporting that the crude extract and six isolated compounds presented inhibition activity of melanin synthesis in human melanoma HMV-II cells. 3'-deoxy-4-O-methylsappnol and brazilin had potential anti-melanogenesis with EC50 values of 3.0±0.5 μM and 4.6±0.7 μM, respectively. Furthermore, brazilin at the concentration of 10 μM repressed the gene expression of mRNA for tyrosinase-related protein (TRYP) type I but did not affect TRYP type II.

5.3 Wound healing activity

The effects of ethanolic crude extract of sappanwood and brazilin on wound healing activity have been studied. Tewtrakul et al. (2015) reported that ethanolic extract at 6.25 μg/ml increased proliferation rate and cell migration of L929 fibroblast cells while brazilin did not. In addition, ethanolic extract at concentration of 6.25 μg/ml and 12.5 μg/ml showed an increase in collagen Type-I, whereas brazilin did not expanded collagen type-I. Moreover, brazilin-rich extract (BRE) from sappanwood at 250 μg/ml showed significantly higher effective enhancing human fibroblast cell line than the control (Dulbecco's Modified Eagle Medium, DMEM) (Nirmal et al., 2014).

5.4 Matrix metalloproteinase (MMP) inhibition on the skin

Lee et al. (2012) reported the effect of brazilin on suppressions of MMP-1 and MMP-3 expressions and secretions which were induced by Ultraviolet B (UVB) irradiation in Human Dermal Fibroblasts (HDFs). Where these activities depended on the concentrations of brazilin (20 μM and 50 μM). In addition, nuclear factor-κB (NF-κB) stimulated with UVB irradiation was inhibited by brazilin treatment. The results concluded that brazilin could directly inhibit of MMP-1, MMP-3 and NF-κB activations.

5.5 Anti-inflammatory activity

Sasaki et al. (2007) reported that brazilin, brazilein, sappanchalcone, and protosappanin A isolated from sappanwood exhibited potential inhibition of nitric oxide (NO) production in Murine peritoneal macrophages at IC_{50} values of 7.3, 3.6, 12.2 and 40.4 μM, respectively. The inhibition of iNOS gene expression was found that only protosappanin A showed very weak inhibition activity when compared with other compounds. Moreover, Washiyama et al. (2009) also reported that brazilin could inhibit of NO production and mRNA expression of iNOS with IC_{50} of 3.7 μM and 3.6 μM, respectively but it could not inhibit prostaglandin E_2 (PGE_2). On the other hand, sappanchalcone, protosappanin D and protosappanin E were able to inhibit the NO and PGE_2 production including mRNA expression of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2) and iNOS. These results suggested that their potential anti-inflammation activity may be due to several compounds in sappanwood which affected different mechanisms for inhibition of inflammation process. Recently, the ethanolic sappanwood extract presented inhibition of the expression and inducement of mRNA level of Interleukin-1 beta (IL-1β) in THP-1 macrophage and tumor necrosis factor-alpha (TNF-α) in chondrocytes. The activity depended on the concentration of extract. Furthermore, the crude extract showed suppression of inducible nitric oxide

synthase (iNOS) expression which induced nitric oxide (NO) synthesis in primary osteoarthritis chondrocytes (Wu et al., 2011).

5.6 Anti-bacterial activity

The brazilin, protosappanin A and sappanone B which were isolated from sappanwood extract showed anti-bacterial activity against *Propionibacterium acnes* (*P. acne*). Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of brazilin (MIC/MBC = 0.5 mg/ml) was greater anti *P. acne* activity than protosappanin A (MIC/MBC = 1 mg/ml) and sappanone B (MIC/MBC more than 2 mg/ml). Moreover, brazilin and protosappanin A showed the strongest lipase inhibition with IC_{50} values of 6 μM and 100 μM, respectively while sappanone B did not show this activity (Batubara et al., 2009). These results agreed with Nirmal et al. (2014A), who reported that brazilin exhibited the strongest inhibition against *P. acne*, *S. epidermis* and *S. aureus* with MIC of 15.6, 31.3, and 62.5 μg/ml and MBC were 31.3, 62.5 and 62.5 μg/ml, respectively followed by brazilin rich extract and crude extract.

6. Niosomes

Non-ionic surfactant based vesicles or commonly known niosomes had been developed from liposomes. Niosomes are formed by self-assembly of non-ionic surfactant in aqueous medium which results in closed bilayer. The properties of heat or physical agitation were used to achieve a close of bilayers. In the structure of bilayer, the hydrophobic head groups are in contact with the aqueous media, whereas the hydrophilic head groups are shielded from the aqueous media (Uchegbu and Vyas, 1998) as shown in Figure 4. Niosomes can entrap hydrophilic drug into aqueous compartment while hydrophobic drug into surfactant bilayers. The advantages of niosomes are great stability, low cost, simple preparation, large scale production and avoid of hazardous solvents.

Moreover, niosomes are an interesting system for skin delivery of active in cosmetic skin care. Both of chemical ingredient and natural extract were developed into niosomes for improving skin permeation such as N-acetyl glucosamine (Shatalebi et al., 2010), tretinoin (Patel et al., 2011), *Terminalia chebula* galls extract (Manosroi et al., 2011), rice bran extract (Manosroi et al., 2012) black tea extract (Yeh et al., 2013), and rosmarinic acid (Budhiraja and Dhingra, 2015). The possible mechanisms of niosomes for improving delivery of actives into skin were diffusion, fusion, modification or adhesion with stratum corneum, reformation of niosomes vesicles, and property of non-ionic surfactant as penetration enhancer (Sahin, 2007).

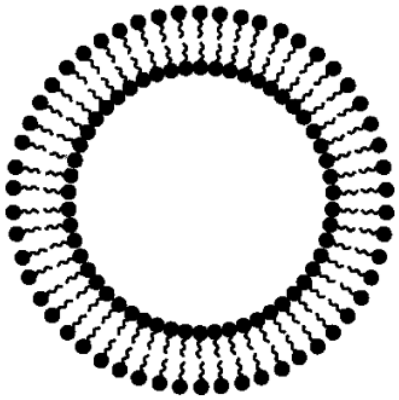


Figure 4 Structure of niosomes (Sahin, 2007)

6.1 Materials used in niosome preparation

6.1.1 Non-ionic surfactant

Non-ionic surfactants are widely used in preparing niosomes because of their advantage including an increase in drug solubility, stability, compatibility and less toxicity when compared with anionic, amphoteric and cationic surfactants (Jiao, 2008). The non-ionic surfactants with alkyl chain length from C_{12}- C_{18} were usually used to form vesicle of niosomes (Uchegbu and Vyas, 1998). Span® or sorbitan fatty acid ester is mixture of a partial ester of sorbitol and its mono- and di-anhydrides with fatty acid. It is widely used in pharmaceutical, food, and cosmetic products (Roson, 1989; Kibbe, 2000).The hydrophilic-lipophilic balance (HLB) is a good indicator for the forming ability of vesicle. The

HLB values of Span® in the range of 4 and 8 were suitable for vesicle formation (Yoshioka, Sternberg, and Florence, 1994; Uchegbu and Florence, 1995; Uchegbu and Vyas, 1998). Types of Span® have been extensively used in preparation of niosomes, including Span® 20, Span® 40, Span® 60, Span® 80, and Span® 85 (Yoshioka et al., 1994; Uchegbu and Florence, 1995; Uchegbu and Vyas, 1998; Hao et al., 2002; Manconi et al., 2002; Manconi et al., 2003; Manosroi et al., 2003; Suwakul, Ongpipattanakul, and Vardhanabhuti, 2006; Khazaeli et al., 2007; Junyaprasert, Teeranachaideekul, and Supaperm, 2008; Mokhtar et al., 2008; Shatalebi et al., 2010; Hao and Li, 2011; Sharma, Anandhakumar, and Sasidharan, 2015)

6.1.2 Cholesterol (CHO)

Cholesterol is well known as the membrane additive used in niosomes. Cholesterol abolishes the gel to liquid phase transition of liposomes and niosomes resulting in less leaky. The mixture of surfactant and CHO are commonly used to prepare niosome system. They can form stable niosomes because CHO can reduce the fluidity of membrane above the phase transition temperature, with a corresponding reduction in a permeability to aqueous solute which is an increase in niosome stability. The ratio between non-ionic surfactant and CHO was usually a molar ratio of 1:1 (Uchegbu and Vyas, 1998).

6.1.3 Solulan® C24

Poly-24-oxyethylene cholesteryl ether or Solulan® C24 is one of non-ionic stabilizers used in preparation of niosomes to prevent aggregation by steric stabilization and to improve physical stability of system (Uchegbu and Vyas, 1998; Arunothayanun, Sooksawate, and Florence, 1999). Moreover, Solulan® C24 has been used in many formulations of niosomes (Uchegbu and Florence, 1995; Arunothayanun et al., 2000; Nasseri, 2005; Suwakul et al., 2006; Junyaprasert et al., 2008; Junyaprasert et al., 2012). The concentration of Solulan® C24 at 5% by

weight was reported to be appropriate for adding in niosome formulations without toxicity (Dimitrijevic et al., 1997).

6.2 Niosome preparation with sonication method

Niosomes can be formed with various preparation methods. The niosome formation requires the input of some energy. All of preparation methods compose of the hydration of a mixture of the surfactant and lipid at elevated temperature following by optimal size reduction to obtain a colloidal dispersion. The sonication method is prepared by melting mixture of lipid phases (surfactant: CHO: membrane additive). Then preheated aqueous phase is added into the lipid phases. After that, the mixture was immediately sonicated to obtain niosomal dispersion. This method is simple for preparing vesicles by applying some energy to the system without organic solvents (Baillie et al., 1985; Suwakul et al., 2006).

6.3 Characterization of niosomes

After preparation process, the formation of vesicle is characterized to confirm that properties of niosomes formed are appropriate for their intended usage. They are evaluated in term of morphology, size and size distribution, entrapment efficiency and physical stability.

6.3.1 Morphology

Optical microscopy was used to characterize shape, aggregation and formation of niosomes (Manosroi et al., 2003; Suwakul et al., 2006; Junyaprasert et al., 2008; Junyaprasert et al., 2012). Moreover, niosome formation was determined by electron microscopy to examine lamellarity and morphology of vesicles. Moreover, transmission electron microscopy (TEM) (Manconi et al., 2002; Guinedi et al., 2005), and scanning electron microscopy (SEM) (Manconi et al., 2006; Rungphanichkul et al., 2011) were also used.

6.3.2 Size and size distribution

Size and size distribution of niosomes were determined using photon correlation spectroscopy or dynamic light scattering. The physical stability of niosomes depended on size and size distribution of vesicles. (Arunothayanun et al., 2000; Suwakul et al., 2006).

6.3.3 Entrapment efficiency

Entrapment efficiency of the active in niosomes was determined by removing unentrapped active from the vesicles before evaluating the total amount of active in the vesicles. The centrifugation and ultracentrifugation have been widely used to evaluate for entrapment efficiency. In addition, exhaustive dialysis and gel filtration were also used to evaluate entrapment efficiency of niosomes (Ucheghu and Vyas, 1998).

6.3.4 Stability

Niosomes were usually evaluated for physical stability at ambient temperature or in refrigerator including entrapment efficiency, changing in the vesicular structure, particle size and size distribution, aggregation and chemical instability of entrapped drug in niosomes (Guinedi et al., 2005; Suwakul et al., 2006; Khazaeli et al., 2007).

6.4 Factor influencing niosome characteristics

6.4.1 Effect of non-ionic surfactant on entrapment efficiency (EE)

Type of non-ionic surfactants affected entrapment efficiency of active ingredient in niosomes. Yoshioka et al. (1994) reported that phase transition temperature (T_c) of Span® had an effect on EE of active in niosomes. The results showed that Span® 40 and Span® 60 presented high EE because both of the higher T_c values. It was also found that alkyl chain length and size of the hydrophilic head

group influenced entrapment efficiency (Ucheghu and Vyas, 1998). As for Span® series, Chandraprakash et al. (1990) reported that Span® 60 gave maximum entrapment efficiency (EE) of methotrexate because of increased lipophilicity of Span® 60. Difference of alkyl chain length resulted in the different EE of colchicine and 5-fluorouracil (5-FU) in niosomes with EE of Span® 60 (C16) > Span® 40 (C14) > Span® 20 (C12) (Hao et al., 2002). Other studies supported that niosomes composed of sorbitan fatty acid ester showed various EE depending on alkyl chain length of surfactant which niosomes with Span® 60 had the highest %EE (Yoshioka et al., 1994; Ucheghu and Vyas, 1998; Manconi et al., 2002; Suwakul et al., 2006; Mokhtar et al., 2008; Hao and Li, 2011)

6.4.2 Effect of non-ionic surfactant on particle sizes

The vesicle size of niosomes depended on type of non-ionic surfactant in formulation. The chain length of surfactant had affected vesicle size. An increase in hydrocarbon chain length of surfactant monomer leaded to smaller vesicles (Barlow, Lawrence, and Timmins, 2000). Moreover, HLB value also affected the niosome size. The previous study of Yoshioka et al. (1994) was reported that size of niosomes prepared by hand shaking method relied on the HLB value of Span®. The alkyl chain length also influenced to vesicle size (Hao et al., 2002). Guinedi et al. (2005) reported that vesicle size of acetazolamide niosomes prepared with Span® 60 was higher than those of Span® 40. Tretinoin niosome with Span® 60 prepared with thin film hydration method was larger than the Span® 40 niosome (Manconi et al., 2002). Similar result was observed by Bharkatiya, Nema, and Bhatnagar (2007) who reported that metoprolol niosome prepared from Span® 80 with film hydration method had smaller vesicle size than Span® 60.

6.4.3 Effect of concentration of active ingredient on niosome formation

Recently, El-Samaligy, Afifi, and Mahmoud (2006) found that increasing concentrations of silymarin in niosomes resulted in an increase of vesicle size because the higher saturation of the media in vesicle that forced silymarin to be encapsulated into vesicles. Mokhtar et al. (2008) reported that increasing concentrations of flurbiprofen upto 100 mg showed drug crystal in niosomes or leaded to drug precipitation.

CHAPTER III
MATERIALS AND METHODS

Materials

1. 2,2 -diphenyl-1-picryhydrazyl (DPPH) (Lot no. STBD4146V, Sigma-Aldrich, USA)
2. Absolute ethanol, AR grade (Lot no. E7025-1-2501, B & J Scientific Co., Ltd., USA)
3. Acetic acid, glacial (Lot no. K41857863, Merck, Germany)
4. Acetone (Lot no. PR-25342/11184AC1, Cambridge Isotope Laboratories, Inc., England)
5. Ascorbic acid (Lot no. 9L253119M, Carlo Erba Reagenti, Italy)
6. Brazilin, purity >98% (Lot no.14010702, Chengdu Biopurify Phytochemical Ltd., China)
7. Cholesterol (Lot no. BCBF1727V, Sigma, USA)
8. Diaion® HP-20 (Lot no. 56323, Sigma-Aldrich, USA)
9. Ethanol 95% (Lot no. 3C200614, Liquor Distillery Organization Excise department, Thailand)
10. Gallic acid (Lot no.SZE61290, Sigma-Aldrich, USA)
11. Isopropanol (Lot no. 09BG1A, Burdick & Jackson, USA)
12. Methanol (Lot no. 14E-494, Cambridge Isotope Laboratories, Inc., USA)
13. Methanol, HPLC grade (Lot no. P7AG1H, Burdick & Jackson, USA)
14. Propylene glycol (Lot no. 66352266091, S. Tong Chemicals Co., Ltd, Thailand)
15. Sephadex LH-20 (Lot no.10231655, GE Healthcare Bio-Sciences, Sweden)
16. Silica gel 60 (0.063-0.200 mm) (Lot no. TA1185434, Merck, Germany)
17. Silica gel 60 (0.040-0.063 mm) (Lot no. TA843085, Merck, Germany)
18. Sodium acetate (Lot no. K41857863, Univar Inc., USA)

19. Solulan® C24 (Lot no. 23C149, Lubrizol Corporation, USA)
20. Span® 20 (Lot no. 27663, CRODA, Thailand)
21. Span® 40 (Lot no. 27679, CRODA, Thailand)
22. Span® 60 (Lot no. 28342, CRODA, Thailand)

Apparatuses

1. 96-well plate (Lot no. 11214005, Costar® assay plate, Corning Inc., USA)
2. Analytical balance (Model UMT2, Mettler Toledo, Switzerland)
3. Analytical balance (Model Ax105 DeltaRange, Mettler Toledo, Switzerland)
4. Centrifuge bottles polycarbonate with cap assemblies (lot no. P10644, Beckman Coulter, Inc., USA)
5. Dry bath incubator (Model 112001, Boekel Scientific, Japan)
6. Lyophilizer Dura-Dry II MP (Model FD2080C0000, FTS system Inc., USA)
7. High Performance Liquid Chromatography system (HPLC) (Model LC-20AP, Shimadzu, Japan)
8. High Performance Liquid Chromatography column (Halo C18, Advanced material technology, Inc., USA)
9. Hot plate stirrer (Model GEM HS-101, Harikul Science Co., Ltd., Thailand)
10. Light optical microscope (Nikon Eclipse E200, Japan)
11. Mastersizer (Malvern 2000, Malvern Instrument, UK)
12. Micropipette 100-1000 µl (Transferpipette®, Trefflab, Switzerland)
13. Micropipette 20-200 µl (Transferpipette®, Trefflab, Switzerland)
14. Micropipette 20 µl (Model PIPETMAN®, Gilson Inc., USA)
15. Microplate reader (Model Victor®, Perkin Elmer Ltd., USA)

16. Nylon membrane disc (47mm, 0.45 µm) (Lot no.N06121304008, Fortune scientific CO., LTD, Thailand)
17. Nylon syringe filter (13 mm, 0.45 µm) (Lot no. 12731000, ANPEL Laboratory Technologies, Shanghai)
18. pH meter SevenCompact™ (Model S220, Mettler Toledo, Switzerland)
19. Rotary evaporator (Model R-114, Buchi, Switzerland)
20. TLC scanner (Model CAMAG Scanner 3, CAMAG Co. Ltd, USA)
21. TLC semi-automatic samples (Model CAMAG LINOMAT 5, CAMAG Co. Ltd., USA)
22. TLC Silica gel 60 F254 (Lot no. HX391037, Merck, Germany)
23. Ultracentrifuge (Model L-80, Beckman, USA)
24. Ultrasonic bath (Model S30H Elmasonic, Elma, Germany)
25. Vortex mixer (Vortex Genie-2, Scientific Industries, USA)

Methods

1. Extraction and isolation

1.1 Extraction

Caesalpinia sappan heartwood was collected from Baan Dong Bung, Prachinburi province, Thailand in July, 2014. The heartwood was cut into small pieces, then dried in a hot air oven at 60℃ for 6 hours and grounded to coarse powder. The sappanwood powder was macerated with 95% ethanol (ratio 1:4) for 24 hour, followed by filtration (Whatman No.1). The maceration was repeated three times with the same volume of solvent. Ethanol was removed by using rotary evaporator at 40°C under vacuum to obtain the crude extract. The crude extract was weighted and calculated using equation (1). Then, the extract was kept in an amber bottle packed in a box with silica gel and placed in the refrigerator.

$$\%\text{Yield} = \frac{\text{Weight of an obtained extract}}{\text{Weight of dried sappan heartwood}} \times 100\% \qquad (1)$$

1.2 Semi-purification of the crude extract

The crude extract from section 1.1 was semi-purified with two methods as follows:

1.2.1 Partition of crude extract

Partition method was used to separate chemical compounds of sappanwood extract. The crude extract (100 g) was dissolved in a tiny amount of ethanol (20 ml), mixed with distilled water (180 ml) and partitioned with dichloromethane (200 ml) and ethyl acetate (200 ml), respectively. The fractions of water, dichloromethane and ethyl acetate were obtained by using rotary evaporator at 40°C. Each fraction was weighed and its yield was calculated using equation (2). All of the fractions were kept in amber bottles packed in a box with silica gel and placed in the refrigerator.

$$\%\text{Yield} = \frac{\text{Weight of an extract}}{\text{Weight of the crude extract}} \times 100\% \qquad (2)$$

1.2.2 Ion-exchange chromatography

The method was slightly modified from a previous study (Nirmal et al., 2014). The crude extract (20 g) was dissolved in 35% ethanol, then sample was loaded on Diaion® HP-20 column (1 g of crude extract per 60 g of resin) and eluted with 35% ethanol. The fractions were pooled and dried by using rotary evaporator at 40°C under vacuum to obtain semi-purified crude extract (Diaion® HP-20 fraction). Percentage of yield was determined using equation (2). The extract was kept in amber bottles packed in the box with silica gel and placed in refrigerator.

1.3 Isolation of brazilin and protosappanin A

The crude extract (100 g) was initially separated by partition method as described in 1.2.1. The ethyl acetate fraction (77 g) was obtained for isolation of brazilin and protosappanin A. This material was initially separated by vacuum liquid chromatography. Silica gel (No. 7734) was used as the stationary phase and hexane-ethyl acetate (100:0 to 0:100) as the mobile phase. The eluates were collected about 500 ml per fraction and examined by thin layer chromatography (hexane-ethyl acetate 4:6), with detection under the ultraviolet light at wavelengths of 254 and 366 nm. All similar thin layer chromatography (TLC) patterns were pooled to give seven fractions (A-G). Fraction G (9.27 g) was separated by silica gel column (hexane-ethyl acetate 0:100 to 100:0) to give eight fractions (G1-G8).

Fraction G2 (120 mg) was separated by column chromatography using silica gel (No. 9385) as a stationary phase and hexane-ethyl acetate (100:0 to 0:100) was a mobile phase. Each of fraction was examined by thin layer chromatography (hexane-ethyl acetate 4:6), with detection under the ultraviolet light at wavelengths of 254 and 366 nm. All similar TLC patterns were pooled to give

seven fractions (G2.1-2.7). Fraction G2.5 (22.4 mg) was purified by Sephadex LH-20 column and eluted with acetone to give protosappanin A (4.1 mg) as colorless needles.

Fraction G3 (18 mg) was subjected to column chromatography using silica gel (No. 9385) as a stationary phase and hexane-ethyl acetate (100:0 to 0:100) as a mobile phase. Each fraction was examined by thin layer chromatography (hexane-ethyl acetate 4:6), with detection under the ultraviolet light at wavelengths of 254 and 366 nm. All similar TLC patterns were pooled to give eight fractions (G3.1-3.8). Fraction G3.4 (180 mg) was separated by silica gel (No. 9385), and eluted with hexane-ethyl acetate (0:100 to 100:0). Each fraction was examined by thin layer chromatography (hexane-ethyl acetate 4:6), with detection under the ultraviolet light at wavelengths of 254 and 366 nm. All similar chromatography patterns were pooled to give six fractions (G3.4.1-3.4.6). Brazilin was obtained from fraction G3.4.2 (3.1 mg).

The structure identifications of protosappanin A and brazilin were performed by high-resolution electrospray ionization mass spectrometry (HR-ESIMS) and nuclear magnetic resonance (NMR) in comparison with previously reported data (Batubara et al., 2009). Brazilin and protosappanin A obtained were then used as reference standards in the section 2.1.

2. Characterization of the sappanwood extracts

2.1 Thin layer chromatographic densitometric method

All extracts and standard solutions at concentration of 100 µg/ml were spotted in band length of 5.0 mm (20 µl/spot) with a Linomat V automatic sample spotter (CAMAG) under nitrogen flow. Thin layer chromatography plate was developed in chamber with mobile phase (hexane-ethyl acetate 4:6). The densitometric scanning at a wavelength of 280 nm (Nirmal et al., 2014A) was presented using TLC scanner 3 (CAMAG) with winCAT software.

2.2 Analysis of brazilin content using HPLC method

2.2.1 HPLC assay

Analysis of brazilin content in the sappanwood extracts was performed by HPLC method modified from the previous study (Nirmal et al., 2014). The HPLC conditions were set as follows:

HPLC instrument	: Shimadzu Model LC-20AP
Column	: Halo® C18 (150 mm × 4.6 mm, 5 µm)
Mobile phase	: 2.5% acetic acid: MeOH (90:10 v/v)
Run time of process	: 0.1-12 min (10% MeOH v/v)
	: 13-25 min (100% MeOH v/v)
	: 26-40 min (10% MeOH v/v)
Injection volume	: 20 µl
Flow rate	: 1 ml/min
Detector	: UV detector at 280 nm
Temperature	: 35°C

The HPLC method was partially validated in terms of system suitability, specificity and linearity.

- The system suitability test was performed by analyzing six replicates of a reference standard solution (10 µg/ml). Percentage relative standard deviations (% RSD) for retention time, peak area response, tailing factor, and theoretical plates was calculated.
- The method specificity was evaluated by comparing the chromatograms of the extracts and standard. Peak purity of the sample and standard was analyzed using photodiode array detection. In addition, chromatograms of brazilin solution (10 µg/ml), blank niosomes without brazilin, and niosomes

spiked with brazilin (10 µg/ml) were compared to ensure the absence of interfering peaks of the niosome formulations.

- The linearity of the method was determined with standard brazilin solutions at six different concentrations in range of 2.5-25 µg/ml. All the samples was evaluated in triplicate.

2.2.2 Preparation of standard and sample solutions

Standard brazilin solution (2.0 mg/ml) was prepared by dissolving in methanol. The standard solution was diluted with mobile phase to obtain stock solution of brazilin standard (0.2 mg/ml). Final concentrations of brazilin solutions were ranged between 2.5-25 µg/ml for calibration curve. For analysis of the sappanwood extract, a sample was dissolved in methanol (2.0 mg/ml). The extract solution was filtered through 0.45 µm syringe filter, then diluted with mobile phase to obtain the final extract concentration of 0.2 mg/ml. Brazilin content in sappanwood extract was determined by HPLC method and calculated with standard curve of brazilin versus area under curves. It was presented as %w/w of the extract. Each determination was repeated in triplicate.

2.3 Total phenolic content

Total phenolic content of the sappanwood extract was determined by Folin-Ciocalteau method modified from the study of Miliauskas and Venskutonis (2004). Folin-Ciocalteau reagent consists of a mixture of phosphomolydate and phosphotungstate. They will oxidize phenolates and reduce heteropoly acids to a blue Mo-W complex (Singleton and Rossi, 1965). The 20 µl of extract solution was mixed with 100 µl of ten-fold diluted Folin–Ciocalteu reagent and 80 µl of sodium carbonate (75 g/L). The mixture was incubated at room temperature for 1 hr and was measured the absorbance at 765 nm with a microplate reader. All samples were determined in triplicate. Solutions of gallic acid in the range of 4-15 µg/ml were used

to prepare a standard curve for determining total phenolic content. The results were expressed as milligram gallic acid equivalent per gram dry weight of the extract (mg GAE/g).

2.4 DPPH radical scavenging activity

Free radical scavenging activity of each sample was determined by DPPH assay (Miliauskas, Venskutonis, and van Beek, 2004). Briefly, a sample solution at concentration between 0.2-10 µg/ml (100 µl) was added to 6×10^{-5} M of DPPH solution (100 µl) in a 96-well plate. The mixture was incubated for 30 min at room temperature and protected from light. When DPPH receives hydrogen atom from an antioxidant, it can change color from violet to light yellow. Its absorbance was measured at 517 nm with a microplate reader (VICTOR® 3, Model 1420-012). Each sample was determined in triplicate. Ascorbic acid and gallic acid were used as positive references. Percentage of DPPH inhibition was calculated using equation (3). IC_{50} value (a concentration providing 50% inhibition) of each sample was determined from the graph plotted between concentration and % inhibition.

$$\%\text{Inhibition} = \left(\frac{(A-B)-(C-D)}{(A-B)}\right) \times 100\% \qquad (3)$$

Where; A is absorbance of solution of ethanol and DPPH

B is absorbance of ethanol

C is absorbance of solution of a sample and DPPH

D is absorbance of solution of a sample and ethanol

The sappanwood extract having the highest brazilin content, DPPH radical scavenging activity, and total phenolic content with suitable % yield was selected for further studies.

3. Preparation of niosomes containing the sappanwood extract

3.1 Preparation of blank niosomes

Niosomes was prepared by sonication method (Suwakul et al., 2006). The lipid phase of niosomes contains a non-ionic surfactant, cholesterol and a membrane additive. Type of non-ionic surfactant was varied to study the effect on the properties of niosomes. The non-ionic surfactants studied were Span® 20, Span® 40 and Span® 60. Cholesterol was used at various weight ratios depending on type of surfactant. Solulan® C24 was used as a membrane additive at 5% by weight to anti-aggregation by steric hindrance (Ucheghu and Vyas, 1998). The total lipid concentration was 100 mg/ml. The weight ratios of lipid phases (surfactant: cholesterol: Solulan® C24) were set at 57.5:37.5:5 for Span® 20, 67.5:27.5:5 for Span® 40, and 57.5:37.5:5 for Span® 60. Each composition was accurately weighed in a 10-ml glass test tube and melted in a dry bath incubator at 130°C. The aqueous phase was acetate buffer pH 4.0 (0.2 M) (Stoll and Blanchard, 1990). One milliliter of the aqueous phase warmed at 70°C was added to the lipid phase. The mixture was immediately sonicated at 70°C for 10 minutes using an ultrasonic bath at 40 kHz and vortexed for 1 min. The niosomal dispersion was cooled down and kept in refrigerator at 4°C for 24 hours. Then, niosomes were visually observed for sedimentation, flocculation and turbidity (Janyaprasert et al., 2008). The homogenous niosome formulations were stored in the refrigerator and evaluated for their morphology, size and size distribution as described in section 4.1 and 4.2, respectively. They were used for further preparing the extract-loaded niosomes.

3.2 Preparation of saturated solution of the sappanwood extract

The saturated solution of the selected sappanwood extract was prepared for loading into niosomes. One percent (w/v) of the extract was added into a volumetric flask, mixed with acetate buffer pH 4.0 and stirred using magnetic stirrer at 4°C for 5 hours. After that, the suspension was kept in the refrigerator for 3 days and filtered with a membrane filter (0.45 µm pore size) to remove insoluble compounds. The

obtained solution was kept in the refrigerator and evaluated for brazilin content, total phenolic content and DPPH scavenging activity as methods described in section 2.2, 2.3 and 2.4, respectively. In addition, stability of the saturated solution was also investigated for sedimentation, color change and content of brazilin remaining by HPLC method after storage in the refrigerator for 30 days.

3.3 Preparation of niosomes containing the sappanwood extract

The extract-loaded niosomes were prepared as the method in section 3.1. The saturated solution of the selected sappanwood extract prepared in section 3.2 was used as an aqueous phase. After preparation, niosomes containing the sappanwood extract were kept in the refrigerator for 24 hours and then visually observed for homogeneity and color of dispersion. The homogenous formulations were stored in the refrigerator and characterized as section 4.

4. Evaluation of niosomes containing the sappanwood extract

4.1 Morphology

4.1.1 Optical microscopy

The niosomes were examined by optical microscopy for the completeness of vesicle formation, morphology, aggregation and the absence of extract crystal. A drop of sample was diluted with acetate buffer pH 4.00. The mixture was placed on a glass slide, closed with a cover slip and viewed under an optical microscope at 400x magnification.

4.1.2 Polarized light microscopy

Polarized light microscopy was used to evaluate the lamellar structure of bilayer membrane (Manosroi et al., 2003). The polarized light photomicrographs were recorded using a digital camera.

4.2 Size and size distribution

Size and size distribution of the vesicles were investigated by laser diffraction techniques (Mastersizer 2000, Malvern Instruments, UK). The refractive index was set at 1.53 (refractive index of cholesterol). Size and size distribution were expressed as D [4, 3] and SPAN index, respectively. The experiment was done in triplicate with pooled samples.

5. Statistical analysis

All of the data were presented as mean ± standard deviation (SD). One-way analysis of variance (One-way ANOVA) with a Tukey's post-hoc test was performed to test the statistical significance at $p < 0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

1. Preparation of the sappanwood extracts and isolated compounds

1.1 Extraction and semi-purification of crude extract

In this study, the initial extraction has been done by maceration of *C. sappan* heartwood with 95% ethanol to obtain the crude extract. However, crude extract contained various compounds which affected their physical and chemical properties (Otsuka, 2005). Thus, semi-purify methods by using partition method and ion-exchange chromatography were used to separate compounds of sappanwood. The partition is the method using two immiscible solvents to separate chemical components of the crude extract based on polarities of compounds and solvents. Finally, water fraction, ethyl acetate fraction, and dichloromethane fraction were dried by solvent evaporation and further evaluated. As for ion-exchange chromatography, the resin (Diaion® HP20) could absorb and separate unnecessary compounds to obtain Diaion® HP20 fraction which was dried by freeze-drying method.

The physical appearances of various sappanwood extracts depended on drying methods and components in each extract as shown in Figure 1. The crude extract was sticky semisolid with orange-brown color. The ethyl acetate fraction, the water fraction and the dichloromethane fraction were coarse mass with reddish-brown, pasty with orange-brown color and yellow-brown color, respectively. The Diaion® HP-20 fraction was fine powder with orange-red color.

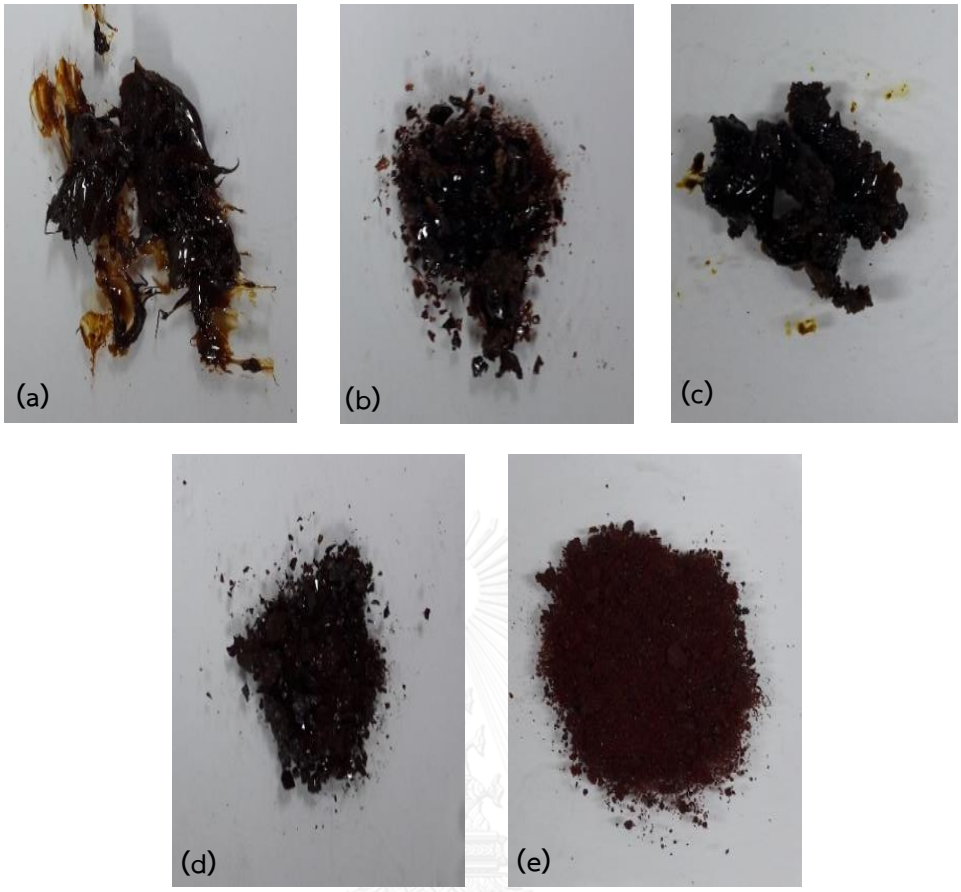


Figure 5 Appearance of various sappanwood extracts: crude extract (a), ethyl acetate fraction (b), dichloromethane fraction (c), water fraction (d), and Diaion® HP-20 fraction (e)

The percentage yields of crude extract and semi-purified extracts are shown in Table 1. For partition method, the ethyl acetate fraction gave the highest percentage yield followed by the water fraction and the dichloromethane fraction, respectively. Difference of percentage yield were a result of compound properties in each extract such as polarity and solubility. The ethyl acetate is a semi-polar solvent which was effective for extraction of phenolic compounds (Santos-Buelga et al., 2012). Yield of Ion-exchange chromatography method was similar to that of the ethyl acetate fraction because sappanwood contained rich of phenolic compounds which could be soluble in ethyl acetate and hydro alcohol.

Table 1 Percentage yields of the crude extract and semi-purified extracts from sappanwood

Method	**Extraction solvent**	**Weight of extract (g)**	**%Yield**
Maceration	Ethanol 95%	433	6.185*
Partition	Water	12.69	12.69**
	Ethyl acetate	71.05	71.05**
	Dichloromethane	5.02	5.02**
Ion-exchange chromatography	Ethanol 35%	13.91	69.55**

*Based on weight of sappanwood powder

**Based on weight of crude extract for partition method (100 g) and ion-exchange chromatography (20 g)

1.2 Isolation of brazilin and protosappanin A

Brazilin and protosappanin A were isolated from the crude extract and characterized their structure by HR-ESIMS and NMR. The ESI mass spectrum of compound **1** showed a pseudomolecular ion $[M+Na]^+$ at *m/z* 295.0582, corresponding the molecular formula of $C_{15}H_{12}O_5$ (Figure 6.1). The ESI mass spectrum of compound **2** showed a pseudomolecular ion $[M+Na]^+$ at *m/z* 309.0738, preferring the molecular formula of $C_{16}H_{14}O_5$ (Figure 6.2). The tables of ^1H and ^{13}C NMR for protosappanin A and brazilin are showed in Tables 2 and 3, respectively. The chromatograms of ESI mass spectrum, NMR and MS of protosappanin A and brazilin are shown in Appendix A. The brazilin and protosappanin A obtained were used as reference standards in Section 2.1.

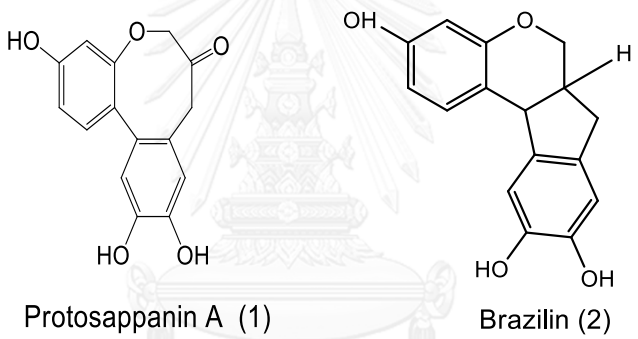


Figure 6 Structures of protosappanin A (1) and brazilin (2)

Table 2 ^{1}H and ^{13}C NMR of compound 1 (in acetone-d_6) and protosappanin A (in $CDCl_3$)

Position	Compound 1		Protosappanin A*	
	δ_H (mult., J in Hz)	**δ_c**	**δ_H (mult., J in Hz)**	**δ_c**
1	7.13 (d, J = 7.8 Hz)	131.4	7.11 (d, J = 8.2 Hz)	129.9
1a	-	126.8		124.1
2	6.75 (dd, J = 2.1,9.3 Hz)	113.0	6.67 (dd, J = 2.1,8.2 Hz)	112.2
3	-	158.8	-	158.1
4	6.74 (d, J = 2.1 Hz)	108.7	6.63 (d, J = 2.1 Hz)	108.0
4a	-	159.2	-	158.5
6	4.47 (s)	78.4	4.45 (s)	77.6
7	-	205.8	-	204.6
8	3.41 (s)	48.8	3.43 (s)	45.1
8a	-	126.8	-	126.0
9	6.77 (s)	117.2	6.69 (s)	116.5
10	-	145.2	-	144.4
11	-	145.0	-	144.2
12	6.77 (s)	117.1	6.69 (s)	116.4
12a	-	131.4	-	130.7

*Batubara et al., 2009

Table 3 ^{1}H and ^{13}C NMR of compound 2 (in CD_3OD) and brazilin (in CD_3OD)

Position	Compound 2		Brazilin*	
	δ_H (mult., J in Hz)	**δ_c**	**δ_H (mult., J in Hz)**	**δ_c**
1	7.18 (d, J = 8.1 Hz)	132.2	7.15 (d, J = 8.2 Hz)	130.9
1a	-	115.5	-	114.2
2	6.46 (dd,8.4,2.4 Hz)	109.9	6.44 (dd, J = 8.2,2.7 Hz)	108.7
3	-	155.7	-	154.4
4	6.29 (d, J = 2.4 Hz)	104.2	6.26 (d, J = 2.7 Hz)	102.9
4a	-	157.8	-	156.5
6	3.69 (d, J = 11.1Hz)	70.8	3.67 (d, J = 10.9 Hz)	69.5
6a	-	78.0	-	76.8
7	2.77 (d, J = 15.1 Hz) 3.02 (d, J = 15.3 Hz)	42.9	2.73 (d, J = 15.8 Hz) 2.97 (d, J = 15.8 Hz)	41.5
7a	-	131.3	-	130.0
8	6.70 (s)	112.8	6.68 (s)	111.6
9	-	145.6	-	144.3
10	-	145.3	-	143.9
11	6.59 (s)	112.4	6.58 (s)	111.1
11a	-	137.4	-	136.1
12	3.96 (s)	51.0	3.93 (s)	49.7

*Batubara et al., 2009

2. Characterization of the sappanwood extracts

2.1 Thin layer chromatographic densitometric method

In preliminary study, TLC-densitometric method was used to determine major compounds in the crude extract and the semi-purified extracts in comparison with the reference standards prepared from section 1.2. TLC fingerprints of brazilin, protosappanin A and various sappanwood extracts showed chromatographic bands after detecting by UV detector at 254 and 366 nm as shown in Figures 7. From the TLC fingerprints, it was found that all of extracts showed the chromatographic band of brazilin, whereas the chromatographic band of protosappanin A was found very thin when comparing with a reference standard. In addition, peak areas of reference standard and all of extracts were detected by UV detector at 280 nm as presented in Figure 8. The Rf (retention factor) values of brazilin and protosappanin A were 0.24 and 0.43, respectively. The TLC-densitometric chromatograms showed that crude extract, dichloromethane fraction, water fraction, ethyl acetate fraction and Diaion® HP-20 fraction were detected the Rf values of brazilin at 0.25, 0.25, 0.27, 0.27 and 0.27, respectively. For protosappanin A, its peak area was rarely found in all extracts. The results was consistent with the previous study reported that brazilin was a major compound of sappanwood (Temsiririrkkul et al., 2007). The peak area of protosappanin A indicated that it was a minor compound of sappanwood.

However, TLC-densitometric method was not performed standard curve for determining amount of brazilin and protosappanin A in each of the sappanwood extract. It may be because some phenolic compounds in samples were dissociated which leaded to the tailing of the spots observed on TLC plate (Jayaprakasha et al., 1998). Therefore, only qualitative analyses of brazilin and protosappanin A could be done by TLC-densitometric method.

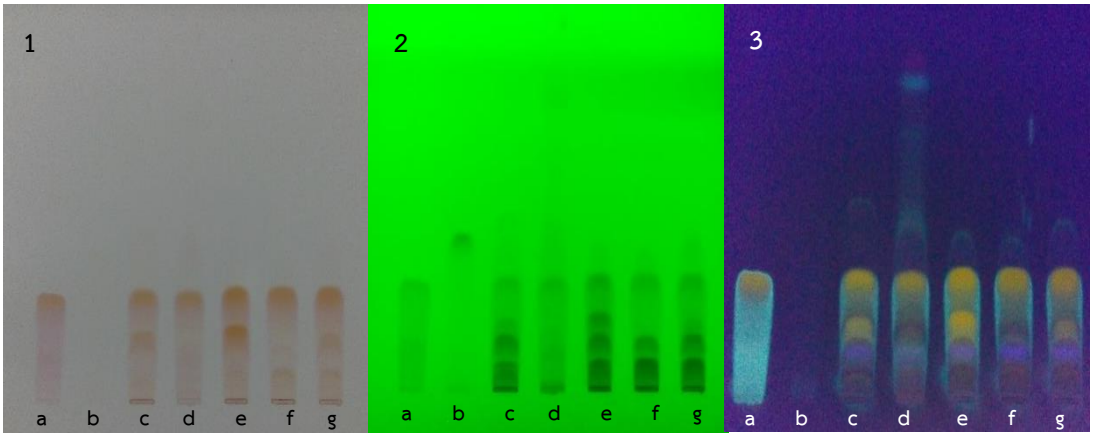


Figure 7 TLC fingerprints of standard brazilin (a), standard protosappanin A (b), crude extract (c), dichloromethane fraction (d), water fraction (e), ethyl acetate fraction (f), and Diaion® HP-20 fraction (g); Stationary phase: silica gel GF_{245}, Solvent system: ethyl acetate-hexane (6:4). Detection: (1) natural light, (2) UV at 254 nm, (3) UV at 366 nm

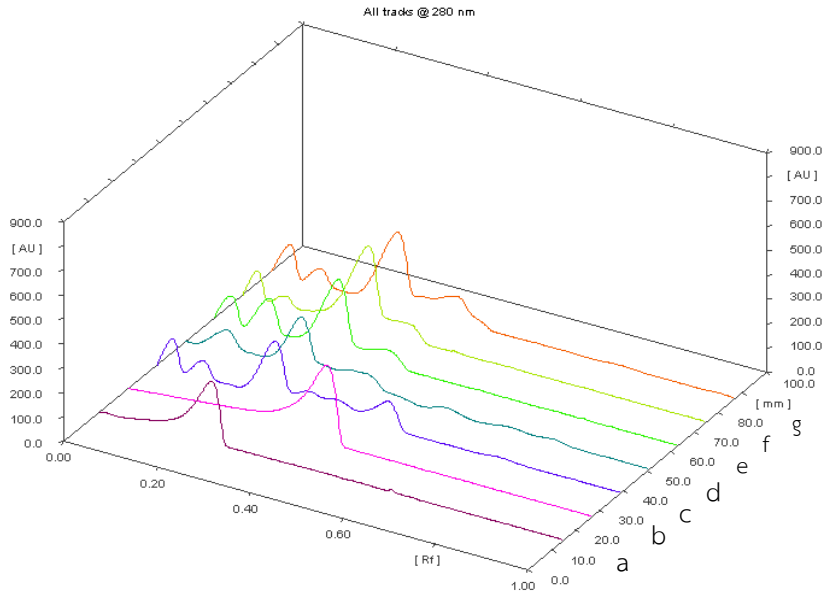


Figure 8 3D TLC densitograms of standard brazilin (a), standard protosappanin A (b), crude extract (c), dichloromethane fraction (d), water fraction (e), ethyl acetate fraction (f), and Diaion® HP-20 fraction (g)

2.1 Analysis of brazilin content by HPLC method

Brazilin contents of the sappanwood extracts were determined by HPLC method modified from the method of Nirmal et al. (2015). The example of full HPLC chromatogram of the sappanwood extract (Diaion® HP20 fraction) detected for 40 min is shown in Figure 9. The chromatogram showed a lot of peaks not only brazilin (retention time of 6.080) but also other impurity compounds in the extract. It was assumed that some compounds could be not separated with the semi-purified method. However, this study was focused on brazilin as a major compound of sappanwood extracts. Thus, other compounds were removed from the column with 100% v/v methanol at time of 13-25 min. The use of 100% methanol at the end of the chromatographic run was to elute very hydrophobic compounds from the column. This process could therefore avoid clogging of the column. Then the mobile phase was adjusted to 10% v/v methanol to equilibrate the column prior to the next injection for 15 min (at time of 26-40 min). Peaks of area were reduced to baseline when adjusted to 10% methanol at time of 26 min. Then peak of area was not found after about 33 min. Thus, it was concluded that the HPLC condition for analysis of brazilin could remove other impurity compounds and be used to detect brazilin in the sappanwood extract. The results of partial validation of the HPLC method were presented as follows:

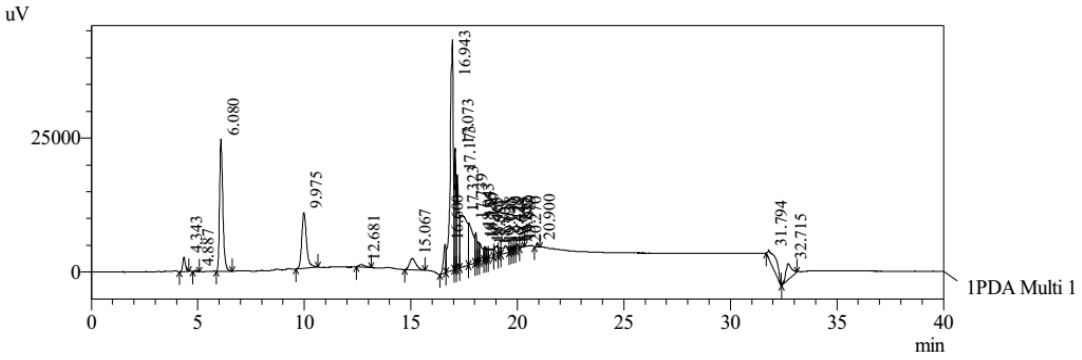


Figure 9 Full HPLC chromatogram of the sappanwood extract (Diaion® HP-20 fraction)

2.2.1 System suitability

System suitability of HPLC method was determined for checking the performance of the analysis system and the analytical instrument. The 10 μg/ml of brazilin standard solution was measured in six successive replicates. The percentage of relative standard deviation (%RSD) should be less than 2% of retention time, peak area, theoretical plate, and tailing factor as shown in Table 4. The results showed that %RSD values of all parameters were lower than 2% which revealed the system suitability of this method. The theoretical plate more than 1500 showed good efficiency of column. The tailing factor of brazilin was 1.320 which indicated symmetry of brazilin peak.

Table 4 System suitability test of HPLC method for analysis of brazilin (n = 6)

Sample no.	Retention time (min)	Peak of area (μV)	Theoretical plate	Tailing factor
1	6.107	227022	47235.604	1.318
2	6.101	227291	47377.497	1.320
3	6.105	227886	47369.069	1.320
4	6.104	228136	47422.541	1.319
5	6.108	228011	47371.652	1.321
6	6.106	228516	47200.501	1.319
Mean	6.105	227810	47329.477	1.320
SD	0.002	555.110	89.174	0.001
%RSD	0.037	0.240	0.19	0.078

2.2.2 Specificity

The specificity describes the ability of HPLC method to analyze the active ingredient in the presence of other components in the sample. The chromatograms of the sappanwood extracts were compared with the brazilin standard. In addition, the specificity was investigated by comparing the chromatograms of brazilin standard, blank niosomes without brazilin and niosomes spiked with brazilin. The chromatogram of brazilin standard (10 µg/ml) presented a peak at the retention time about 6.1 min. The chromatograms of the various sappanwood extracts exhibited major peak of brazilin with retention time detected about 6 min (Figure 10) which was comparable with the reference standard. Values of peak purity index were in range of 0.99761 to 0.9979.

However, brazilin was previously reported that it could be oxidized to brazilein when exposure to light and air (Dong et al., 1996; de Oliveira et al., 2002; Rondao et al., 2013). Thus, the preliminary stability of brazilin in the extract for photostability and pH sensitivity was evaluated. The results showed that peak area of brazilin in the extract solution at pH 4.5 and 5.5 was decreased after exposure to UV light. However, the peak purity index values of brazilin before and after exposure to UV light at pH 4.5 for 0.9987, 0.9981 and pH 5.5 for 0.9988, 0.9984, respectively. In addition, the UV spectrum using HPLC UV detector at wavelength 190-800 nm was not detected interference from other peaks even through peak area of brazilin was decreased. The results may be because pH values and UV light influenced deprotonation of brazilin to oxidized form of brazilein, which had absorbance around 520 nm. Moreover, the color change of extract solution investigated by photostability and pH sensitivity may be because brazilein was increased degradation involving the breakdown of the carbon skeleton and decrease in absorption in the visible region (Rondao et al., 2013). The results indicated that degradation products of brazilin did not interfere analysis of brazilin with HPLC method.

For niosomes containing brazilin, their chromatograms showed a peak and the retention times as the brazilin standard which were at 6.095 min, 6.094 min and

6.102 min for niosomes with Span® 20, Span® 40 and Span® 60, respectively (Figure 11). All of blank niosomes were not detected peaks in the chromatograms (Figure 12). The result showed that these components did not interfere the analysis of brazilin and also revealed specificity of the method. Therefore, this HPLC condition was appropriate for analysis of brazilin in the sappanwood extracts and the niosome formulations.

2.2.3 Linearity

The relationship of the peak area and brazilin concentration was plotted to show as a calibration curve. Brazilin standard solutions were prepared at six different concentrations. Each of brazilin concentration was analyzed in triplicate. The linearity of the calibration cure was measured by calculating coefficient of correlation (r) and coefficient of determination (r^2). The calibration graph was found to be linear over the concentration range of 2.5-25 g/ml with r of 0.9996 and r^2 of 0.9993 which indicated good linearity of the method (Table 5 and Figure 13). The %RSD of the slope and of the intercept of the three lines were 0.51% and 1.40%, respectively which showed low deviation from linearity.

The results of partial validation studies indicated that the HPLC method showed system suitability, specificity, linearity and no interference from the excipients. Therefore, this HPLC method could be applied for quantitative analysis of brazilin in the sappanwood extracts and the niosome formulations under the experimental conditions.

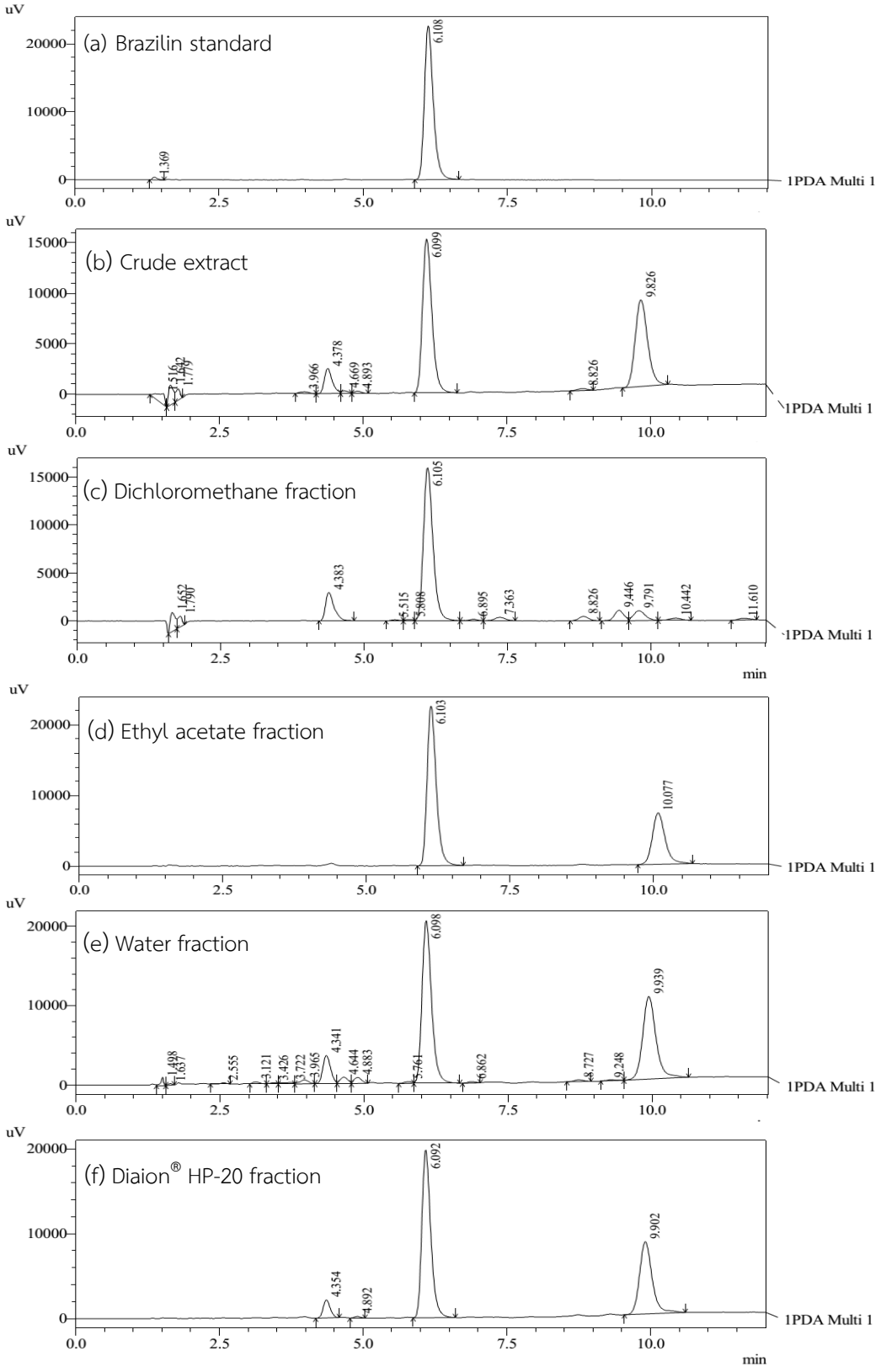
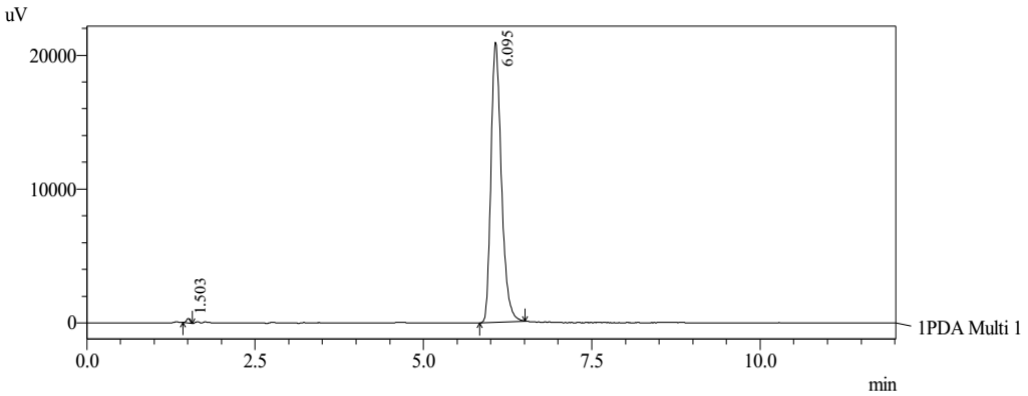
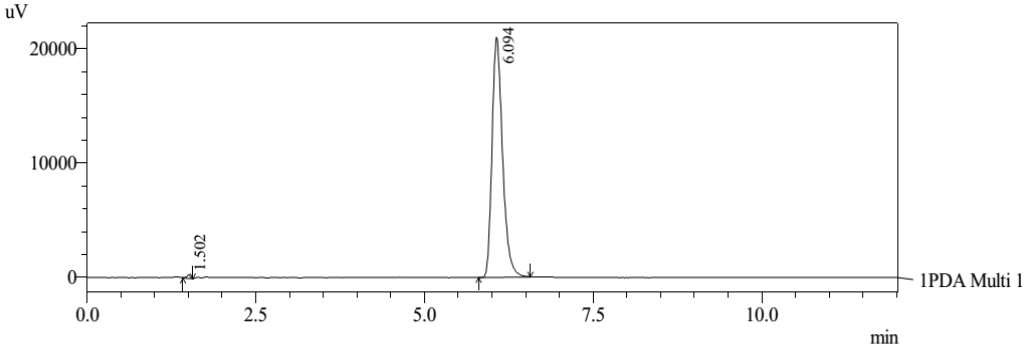


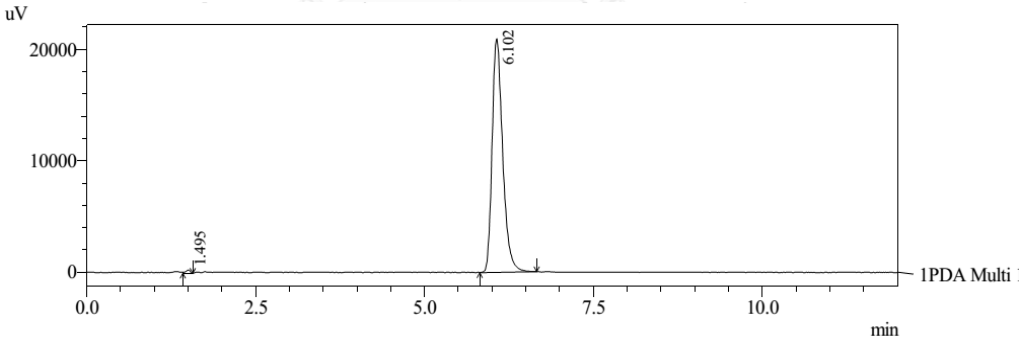
Figure 10 HPLC chromatograms of brazilin standard and various sappanwood extracts



(a) Span® 20/CHO/Solulan® C24

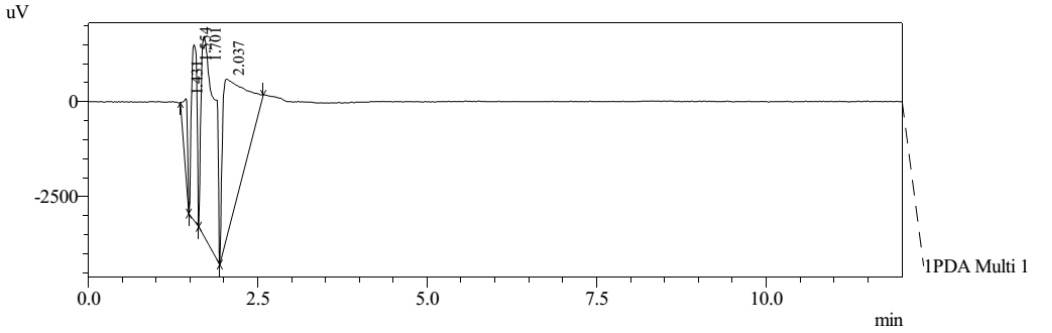


(b) Span® 40/CHO/Solulan® C24

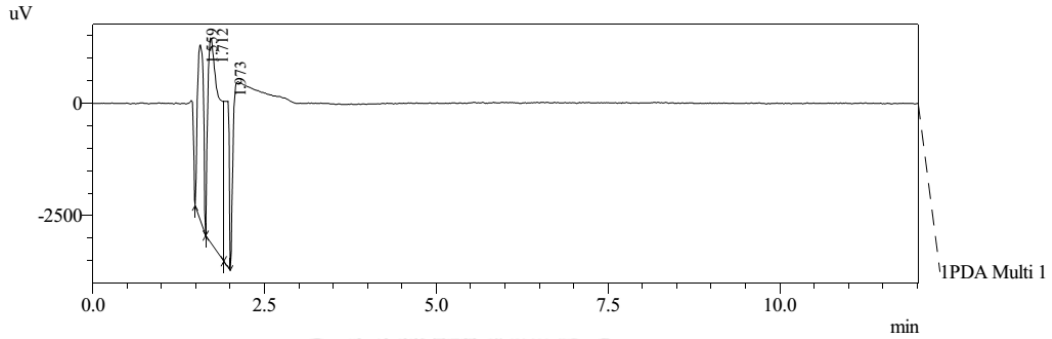


(c) Span® 60/CHO/Solulan® C24

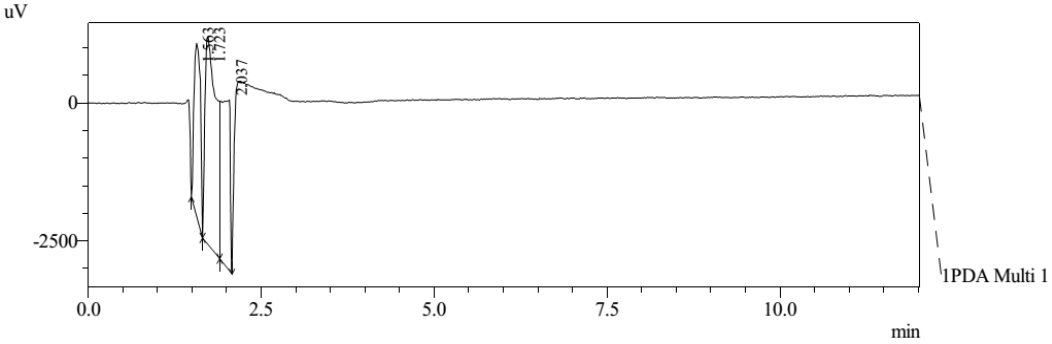
Figure 11 HPLC chromatograms of blank niosome formulations spiked with brazilin standard (10 μg/ml)



(a) Span® 20/CHO/Solulan® C24



(b) Span® 40/CHO/Solulan® C24



(c) Span® 60/CHO/Solulan® C24

Figure 12 HPLC chromatograms of blank niosome formulations

Table 5 Data of calibration curve of brazilin standard by HPLC method (n = 3)

Concentration (µg/ml)	Peak area (mV)			Mean	SD	%RSD
	Set 1	Set 2	Set 3			
2.5	58756	58565	58616	58646	99.1	0.17
5	123859	122292	123753	123301	875.7	0.71
10	228391	227659	227420	227823	506.25	0.22
15	327164	326130	326891	326728	437.30	0.13
20	426982	427381	428279	427547	664.12	0.16
25	534502	531435	538130	534689	3351.42	0.63
R^2	0.9992	0.9993	0.9993	0.9993	-	-
Slope	20794	20741	20945	20827	105.9	0.51
Y-intercept	14692	14335	14349	14459	202.2	1.40

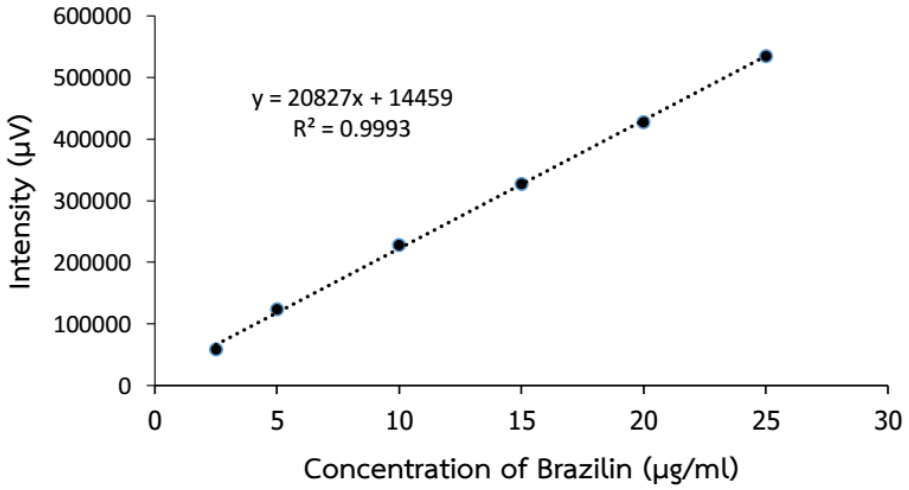


Figure 13 The representative linearity curve for brazilin by HPLC method (n =3)

Table 6 presents brazilin contents in various sappanwood extracts determined by the HPLC method. The results showed that Diaion® HP-20 fraction gave the significantly highest brazilin content ($p < 0.05$) followed by ethyl acetate fraction, water fraction, dichloromethane fraction and crude extract, respectively. In this study, the brazilin content in crude extract was less than that reported by Temsiririrkkul et al. (2007) that the percentage brazilin content of sappanwood extract in Thailand was ranged from 8.7-22 %w/w. The difference of brazilin content in sappanwood extracts may be because of different sources of sappanwood.

Table 6 Brazilin contents in various sappanwood extracts (n = 3, mean ± SD)

Sappanwood extracts	Brazilin content (%w/w)
Crude extract	7.70 ± 0.21
Water fraction	11.14 ± 0.27
Ethyl acetate fraction	11.38 ± 0.15
Dichloromethane fraction	7.98 ± 0.17
Diaion® HP-20 fraction	13.24 ± 0.25

Both of semi-purifying methods were proved that they could significantly increase content of brazilin ($p < 0.05$) in the extracts except the dichloromethane fraction when compared with the crude extract. However, brazilin contents of the ethyl acetate fraction and the water fraction were not significantly different ($p > 0.05$) because brazilin could dissolve in ethyl acetate and water (Hikino et al., 1977; Nirmal et al., 2015). The dichloromethane fraction had the lowest content of brazilin for the partition method but not significantly different from the crude extract ($p > 0.05$). Dichloromethane is less polar solvent (Otsuka, 2005) which could dissolve less brazilin. The ion-exchange chromatography using Diaion® HP-20 could increase brazilin content about two-fold when compared with the crude

extract. The result was in accordance with Nirmal et al. (2014) who reported the increased brazilin content of brazilin-rich extract prepared with Diaion® HP-20 which was about two-fold (39.9 ± 0.34% w/w) higher than crude extract (20.0 ± 0.26% w/w). Similar study was reported by Nirmal et al. (2014) who found that Diaion® HP-20 could increase brazilin content up to 49% w/w more than column chromatography by silica gel and Sephadex® LH-20 column (5.7% w/w). In this study, however, Diaion® HP-20 fraction had less brazilin content than the previous studies which may be due to different sources of sappanwood. This results could be implied that large pore size of Diaion® HP-20 could adsorb unwanted compounds and had superior adsorption or desorption ability (Sung Phil, 2014).

Diaion® HP-20 is a synthesis adsorbent for both organic and inorganic compounds. It is a non-polar polymeric adsorbent resin. Diaion® HP-20 had spherical particle and was considered as the most widely used adsorbent to extract secondary metabolites from plants and to separate unwanted or impurity compounds (Korda, Wróbel, and Gwardiak, 2006). Many studies were reported that Diaion® HP-20 could increase content of major active compounds in plant materials. Kao, Lu, and Chen (2005) presented that Diaion® HP-20 column was capable of separating five groups of compounds including flavones, malonylglucosides, glucosides, acetylglucosides and aglycones from soybran cake. Panichayupakaranant et al. (2010) reported that Diaion® HP-20 increased almost two-fold ellagic acid content of pomegranate fruit peel extract. The study of Puttarak et al. (2010) showed that the rhinacanthins content of rhinacanthins-rich *Rhinacanthus nasutus* was increased to 82.59 %w/w when compared with the crude extract. Chan et al. (2011) also showed that chlorogenic acid (CGA) and caffeoylqunic acid (CQA) contents from leaves of *Etlingera elatior* were increased four-fold of CGA and three-fold of CQA when compared with crude extract.

2.3 Total phenolic content

Total phenolic contents (TPC) of the sappanwood extracts measured using the Folin-Ciocalteu method are shown in Table 7. The Folin-Ciocalteu reagent was reduced by phenolic compounds in the extract with a concomitant formation of blue complex (Singleton and Rossi, 1965). As for the crude extract, it was prepared using maceration method of the *C. sappan* heartwood with 95% ethanol. Its phenolic content was higher than that of the previous study reported about the sappanwood extract with the same extraction method. Wongtana et al. (2013) reported that the ethanolic extract had TPC of 178.56 ± 0.36 mg GAE/g extract. However, Han and Rhee (2005) reported that the ethanolic sappanwood extract showed high TPC of 791 mg GAE/g extract. In addition, the extraction of sappanwood by using Soxhelt apparatus gave the extract with high TPC of 544.0 ± 1.92 mg GAE/g extract (Saenjum et al., 2010). These results indicated that plant material of sappanwood and extraction method had influenced to their total phenolic content.

Table 7 Total phenolic contents of various sappanwood extracts (n = 3, mean ± SD)

Sappanwood extracts	mg GAE/g extract
Crude extract	499.55 ± 2.31
Water fraction	542.84 ± 2.04
Ethyl acetate fraction	594.64 ± 1.67
Dichloromethane fraction	327.04 ± 1.39
Diaion® HP-20 fraction	604.82 ± 2.45

For the semi-purified extracts, all fractions except the dichloromethane fraction had significantly higher total phenolic contents than the crude extract ($p < 0.05$). Diaion® HP-20 fraction had significantly the highest TPC followed by ethyl acetate

fraction, water fraction and dichloromethane fraction, respectively. The difference of total phenolic contents in various sappanwood extracts was influenced by solubilities of phenolic compounds in each extracting solvent. Polarity and type of extracting solvent affected contents of extracted phenolic compounds (Naczk and Shahidi, 2006).

The highest TPC of Diaion® HP-20 fraction may be due to using the solvent mixture of ethanol and water, which could dissolve a wide range of phenols (Alothman, Bhat, and Karim, 2009). Furthermore, it was assumed that ion-exchange chromatography could remove unwanted phenolic and non-phenolic substances such as waxes, terpenes, fats or chlorophylls (Naczk and Shahidi, 2006). This result was consistent with the previous study of Chan et al. (2011) who reported that the extracts obtained from *Etlingera elatior* leaves using Diaion® HP-20 showed higher TPC (706 ± 12 mg GAE/g extract) than the crude extract (444 ± 20 mg GAE/g extract). In addition, total phenolic content of the ethyl acetate fraction was significantly higher than that of the water fraction in this study. Ethyl acetate is semi-polar solvent that can dissolve both low molecular weight phenolic compounds and high molecular weight polyphenol (Yadav, Yadav, and Yadav, 2014). Therefore, ethyl acetate could be used to increase amount of phenolic compounds in the extracts (Khalili et al., 2013). The dichloromethane fraction showed the lowest TPC because it was less polar solvent which would dissolve less phenolic compounds (Alothman et al., 2009)

2.4 DPPH radical scavenging activity

The free radical scavenging activities of all sappanwood extracts were determined by DPPH assay. DPPH is a stable free radical which changes color from violet to yellow by hydrogen or electron donation of extracts (Alam, Bristi and Rafiquzzaman, 2013). The IC_{50} values of the extracts and positive controls (IC_{50}) are shown in Table 8. Brazilin and protosappanin A standard obtained from section 1.2 were also evaluated for this activity to compare with the sappanwood extracts. The

results showed that DPPH inhibition effect of various sappanwood extracts were corresponding with increasing concentrations. The order of DPPH scavenging activity was similar to the results of TPC in section 2.3 except for the water fraction. The scavenging effect of extracts was ordered from high to low as follows: water fraction > brazilin ≅ protosappanin A > Diaion® HP-20 fraction ≅ ethyl acetate fraction > crude extract > dichloromethane fraction, respectively.

Difference of their DPPH scavenging activities was resulted from the presence of different active compounds in the extracts which were obtained from various extracting solvents and extraction methods. The antioxidant activity of water fraction was the highest when compared with other extracts studied. Moreover, the water fraction had better activity than reference standards and ascorbic acid but lower than gallic acid. *C. sappan* heartwood had tannins of 150 mg/g extract, phenolic compounds of 171 mg/g extract and other compounds (Senthilkumar et al., 2011). Tannins can be soluble in water (Scalbert, 1991; Chung et al., 1998). Therefore, it was explained that potential antioxidant activity of the water fraction may be occurred from water-soluble tannins and phenolic compounds as well as brazilin.

Brazilin and protosappanin A had similar DPPH scavenging activities. They showed higher antioxidant activities than all sappanwood extracts except for the water fraction. This result was in accordance with previous studies that brazilin presented powerful DPPH radical scavenging activity (Sasaki et al., 2007; Chen et al., 2014; Nirmal et al., 2014b). The possible activity mechanism of brazilin was its hydroxyl group structure which had potential for inhibition of free radical (Chen et al., 2014). This result agreed with Sasaki et al. (2007) who showed that IC_{50} value of protosappanin A in DPPH radical scavenging activity was 62.2 μM higher than vitamin E as positive control.

Table 8 DPPH radical scavenging activity of various sappanwood extracts, isolated compounds and positive controls (n = 3, mean ± S.D.)

Samples	IC_{50} (μg/ml)
Crude extract	2.72 ± 0.03
Water fraction	1.19 ± 0.02
Ethyl acetate fraction	2.08 ± 0.02
Dichloromethane fraction	3.95 ± 0.05
Diaion® HP-20 fraction	2.03 ± 0.04
Brazilin	1.34 ± 0.01
Protosappanin A	1.35 ± 0.01
Ascorbic acid	1.58 ± 0.02
Gallic acid	0.63 ± 0.01

DPPH scavenging activities of the Diaion® HP-20 fraction and the ethyl acetate fraction were not significantly different ($p > 0.05$) and were better than the crude extract but less than brazilin standard and ascorbic acid. Huh et al. (2004) found that semi- purification of oligomeric proanthocyanidin (OPC) from wild grape seeds using Diaion® HP-20 showed higher DPPH scavenging activity (IC_{50} = 31.60 μg/ml) than crude extract (IC_{50} = 40.70 μg/ml) because of impurities in crude extract were removed by Diaion® HP-20. Although brazilin content and TPC of the Diaion® HP-20 fraction were more than the ethyl acetate fraction, their DPPH scavenging activities were similar. This result may be because other components in the ethyl acetate fraction also exhibited this antioxidant activity.

The dichloromethane fraction had the lowest antioxidant activity which was consistent with the lowest content of brazilin and total phenolic content. However, its antioxidant activity was not only from brazilin but also various less polar compounds of sappanwood extract.

The results indicated that both partition method and ion-exchange chromatography with Diaion® HP-20 could be used to semi-purify the crude extract. They could improve the brazilin content and DPPH scavenging activity of the sappanwood extracts. The ethyl acetate fraction and Diaion® HP-20 fraction had similarity of extract yield and DPPH scavenging activities. However, Diaion® HP-20 fraction had significantly higher brazilin content than ethyl acetate fraction. Moreover, Diaion® HP-20 fraction was fine powder which was easier to use than ethyl acetate fraction which was coarse mass. Therefore, Diaion® HP-20 fraction was selected for further development of niosome formulations.

3. Preparation and characterization of niosomes containing the sappanwood extract

3.1 Preparation of saturated solution of the sappanwood extract

The sappanwood extract of Diaion® HP-20 fraction was selected for development of niosomes. From the preliminary study, brazilin would degrade in buffer pH above 5.0. Therefore, acetate buffer pH 4.0 was used as solvent for preparation the solution of the extract. This pH value can be safely used for skin care products (Lambers et al., 2006). However, it was found that the extract was practically insoluble in this solvent (1 mg/ 100 ml) because the extract was composed of various compounds (both polar and nonpolar). Thus, the extract solution was prepared at starting concentration of 1% w/v in acetate buffer pH 4.0 and stored in a refrigerator for 3 days. Then, the extract suspension was filtered through a membrane filter (0.45 μm pore size) to obtain a clear saturated solution. The saturated solution obtained would give content of brazilin which was enough for development of cosmetic products and could be detected for *in vitro* skin permeation study by HPLC method. The appearance of the saturated solution of the sappanwood extract was yellow clear solution as shown in Figure 14. No detectable crystallization of the extract in the solution was observed by optical microscope at various magnifications. The saturated solution obtained was evaluated for brazilin content, total phenolic content and DPPH scavenging activity

after fresh preparation and storage in the refrigerator for 30 days. The content and activity were calculated based on the weight of Diaion® HP-20 fraction used in preparing the saturated solution (1% w/v) and compared with properties of the extract before preparation of solution.

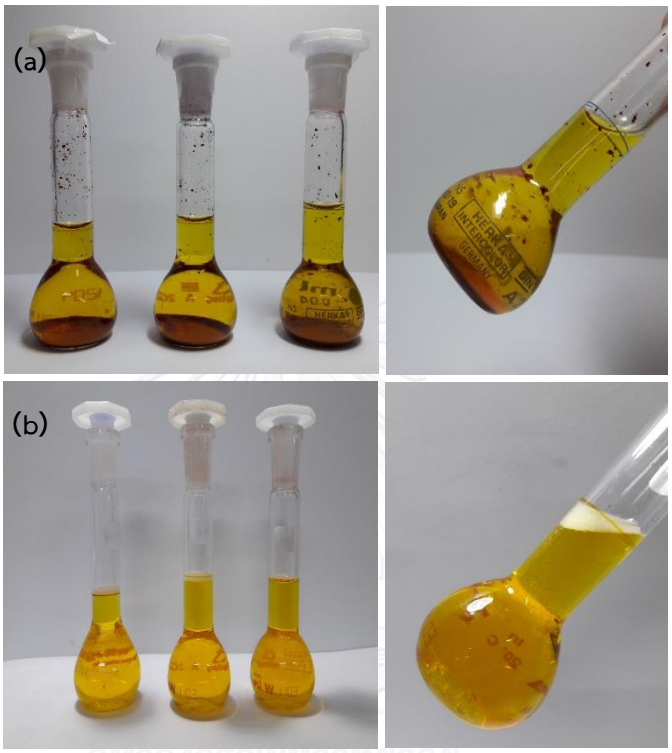


Figure 14 Appearance of the saturated solutions of Diaion® HP-20 fraction before filtration (a) and after filtration with 0.45 µm filter membrane (b)

Table 9 shows brazilin content, DPPH scavenging activity and pH of the saturated solutions of the extract after fresh preparation and storage in the refrigerator for 30 days. When compared the properties of Diaion® HP-20 extract between the saturated solution after fresh preparation and the dried extract, it was found that brazilin content, total phenolic content and DPPH scavenging activity of the saturated solution were less than those of the dried extract (Tables 6, 7, 8 and 9). The reason may be because the saturated solution was prepared from filtration of 1% w/v Diaion® HP-20 fraction suspension in acetate buffer pH 4.0. Some

phenolic compounds as well as brazilin in the extract could not be dissolved in acetate buffer pH 4.0 and were filtered out of the solution. As a result, the mentioned properties and activity were less than the dried extract. However, the saturated solution of Diaion® HP-20 extract still showed high brazilin content, total phenolic content and antioxidant activity.

Table 9 Properties of the saturated solution of Diaion® HP-20 fraction in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days (n = 3, mean ± SD)

Properties	Fresh preparation	After storage for 30 days
Brazilin content (%w/w)	11.53 ± 0.16	11.31 ± 0.17
TPC (mg GAE/g extract)	574.83 ± 2.45	523.70 ± 2.24*
DPPH IC_{50} (µg/ml)	3.07 ± 0.03	3.22 ± 0.08
pH	4.02 ± 0.02	4.03 ± 0.01

*Significantly different from fresh preparation at $p < 0.05$

After storage for 30 days, brazilin content and DPPH scavenging activity of Diaion® HP-20 extract in the saturated solution was insignificantly less than those of fresh preparation except for total phenolic content which was significantly lessen ($p < 0.05$). Its physical and chemical stabilities depended on buffer pH of the solution and type of the phenolic compounds in the extracts (Friedman and Jürgens, 2000). The decreasing of TPC after 30 days was assumed that some of phenolic compounds in the saturated solution was significantly degraded. However, a decrease in TPC after 30 days did not significantly affect DPPH radical scavenging activity. It may be assumed that brazilin was a main compound showing DPPH radical scavenging activity.

The physical appearance of the saturated solution of the extract was not found sedimentation and color change within 30 days. This result indicated that the saturated solution of the Diaion® HP-20 fraction in acetate buffer pH 4.0 was physically and chemically stable of brazilin after storage in the refrigerator for at least 30 days. Consequently, this saturated solution was further used in preparation of niosomes.

3.2 Preparation and characterization of blank niosomes

In this study, niosomes were prepared from non-ionic surfactant, cholesterol and Solulan® C24 by using sonication method. Types of non-ionic surfactant studied were Span® 20, Span® 40 and Span® 60. The ratios of Span®: CHO: Solulan® C24 depended on type of Span® and was selected from the previous study of Suwakul et al. (2006). Type and ratio of composition studied could be used to prepare niosomes of propylthiouracil in water and phosphate buffer pH 4.0 (Kanjanapadit, 2005; Suwakul et al., 2006) and niosomes of glycolic acid in acetate buffer pH 4.0 (Klinhom, 2008). The total of lipid was 100 mg/ml. The lipid phase was hydrated with acetate buffer pH 4.0 for blank niosomes.

All of blank niosomes in acetate buffer pH 4.0 were homogenously opalescent dispersion and completely formed vesicles. The crystal of lipid phase was not found in all formulations as shown in Figures 15-17. The niosomes could be prepared in acid buffer because Solulan® C24 prevented acid hydrolysis of ester bonds in Span® at pH 4.0. Thus, niosomes would form stable vesicles from steric barrier at the surface layer (Klinhom, 2008). The sizes and size distributions as SPAN index of all blank niosomes are showed in Table 10 and Figure 18. The trend of average sizes of niosomes prepared in acetate buffer pH 4.0 was Span® 20 < Span® 40 < Span® 60. The small values of SPAN index were in accordance with narrow size distribution of vesicles. The result was consistent with the previous study of Klinhom (2008). The longer alkyl chain length of surfactants resulted in larger vesicle size (Ucheghu and Duncan, 1997; Manosroi et al., 2003; Guinedi et al., 2005; Klinhom, 2008). The

structure of Span® has the same head group and different alkyl chain. The alkyl chain of Span® 60 was the longest (C_{16}) followed by Span® 40 (C_{14}) and Span® 20 (C_{12}), respectively (Hao et al., 2002) Moreover, the HLB values of Span® series also influenced the size of niosomes (Yoshioka et al., 1994). The lower HLB values of non-ionic surfactant gave larger vesicle size (Guinedi et al., 2005). HLB value of Span® 60 (4.7) is the lowest followed by Span® 40 (6.7) and Span® 20 (8.6), respectively.

Table 10 Size and size distribution of blank niosomes with various formulation (n = 3, Mean ± SD)

Formulations	Code	Size (μm)	SPAN index
Span® 20/CHO/Solulan® C24	S20	3.80 ± 0.04	1.19 ± 0.01
Span® 40/CHO/Solulan® C24	S40	7.52 ± 0.09	1.90 ± 0.01
Span® 60/CHO/Solulan® C24	S60	9.52.± 0.15	1.51 ± 0.05

3.3 Preparation and characterization of sappanwood niosomes

The saturated solution of the Diaion® HP-20 fraction in acetate buffer pH 4.0 obtained from section 3.1 was loaded into niosomes. Appearance of all niosome formulations containing the sappanwood extract was yellow opaque suspension (Figures 19). The physical appearance of formulations S20 and S60 showed immediately sedimentation and aggregation of vesicles after fresh preparation, whereas formulation S40 was homogenous dispersion. However, separation of formulation S40 was observed after kept in the refrigerator for 24 hours as shown in Figure 20. The appearance of niosomes was examined under optical microscopy for shape, aggregation of vesicles and crystal formation. The polarized light microscopy was used for detection of liquid crystal because it shows birefringence, which is typical black and white textures (Müller-Goymann, 2004).

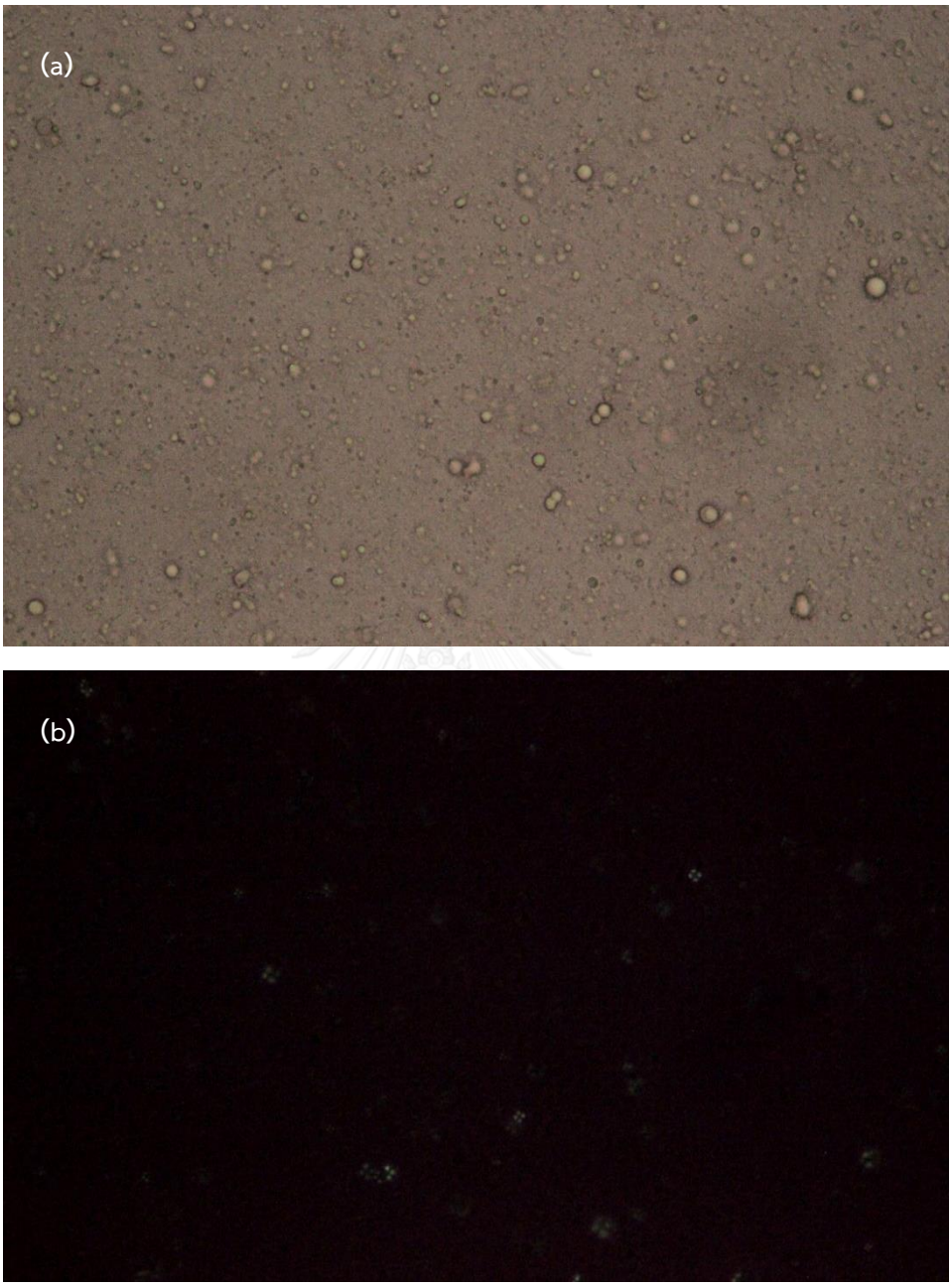


Figure 15 Appearance of blank niosomes of Span® 20/CHO/Solulan® C24 under optical microscopy (a) and polarized-light microscopy (b) (400X)

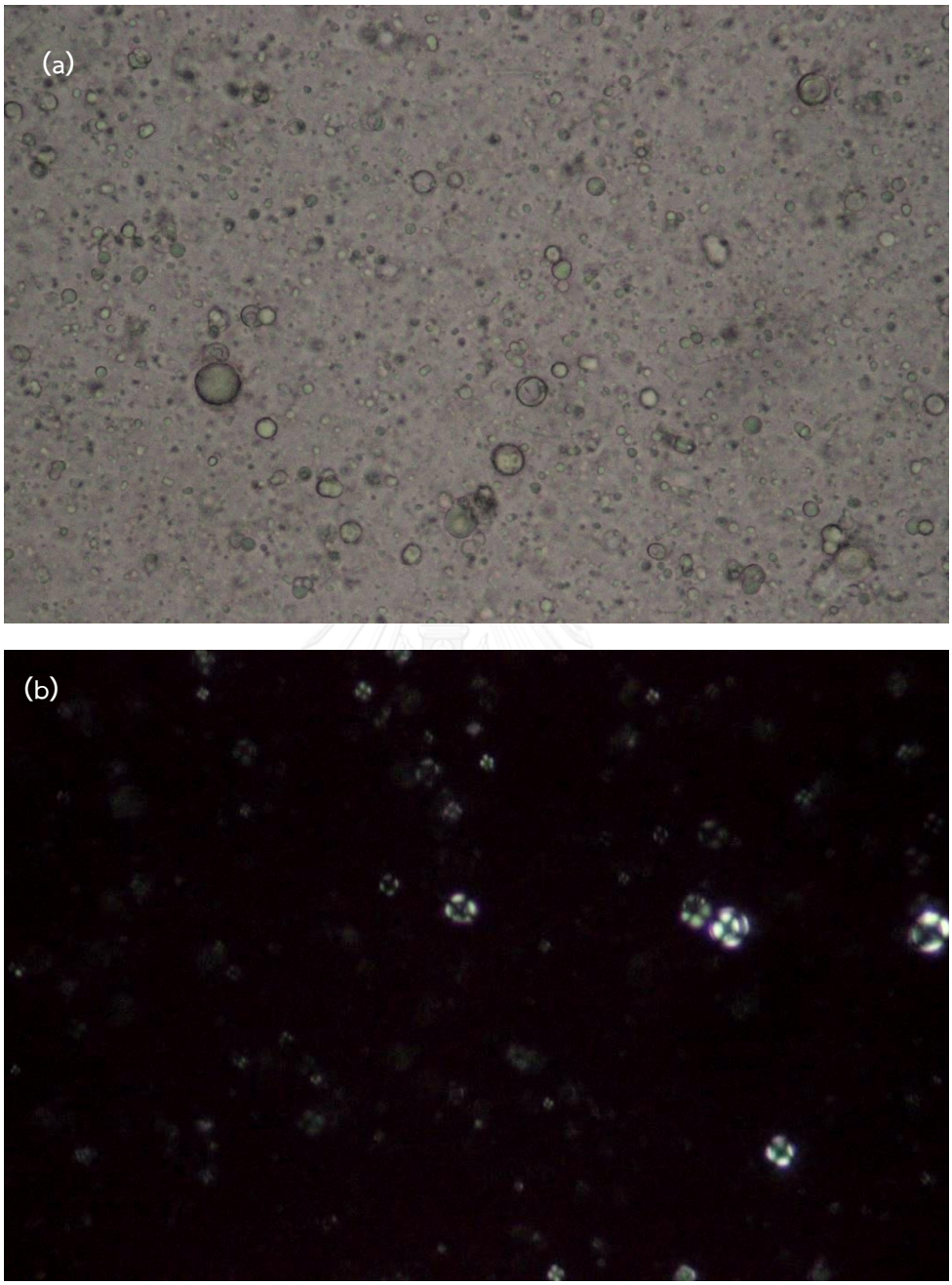


Figure 16 Appearance of blank niosomes of Span® 40/CHO/Solulan® C24 under optical microscopy (a) and polarized-light microscopy (b) (400X)

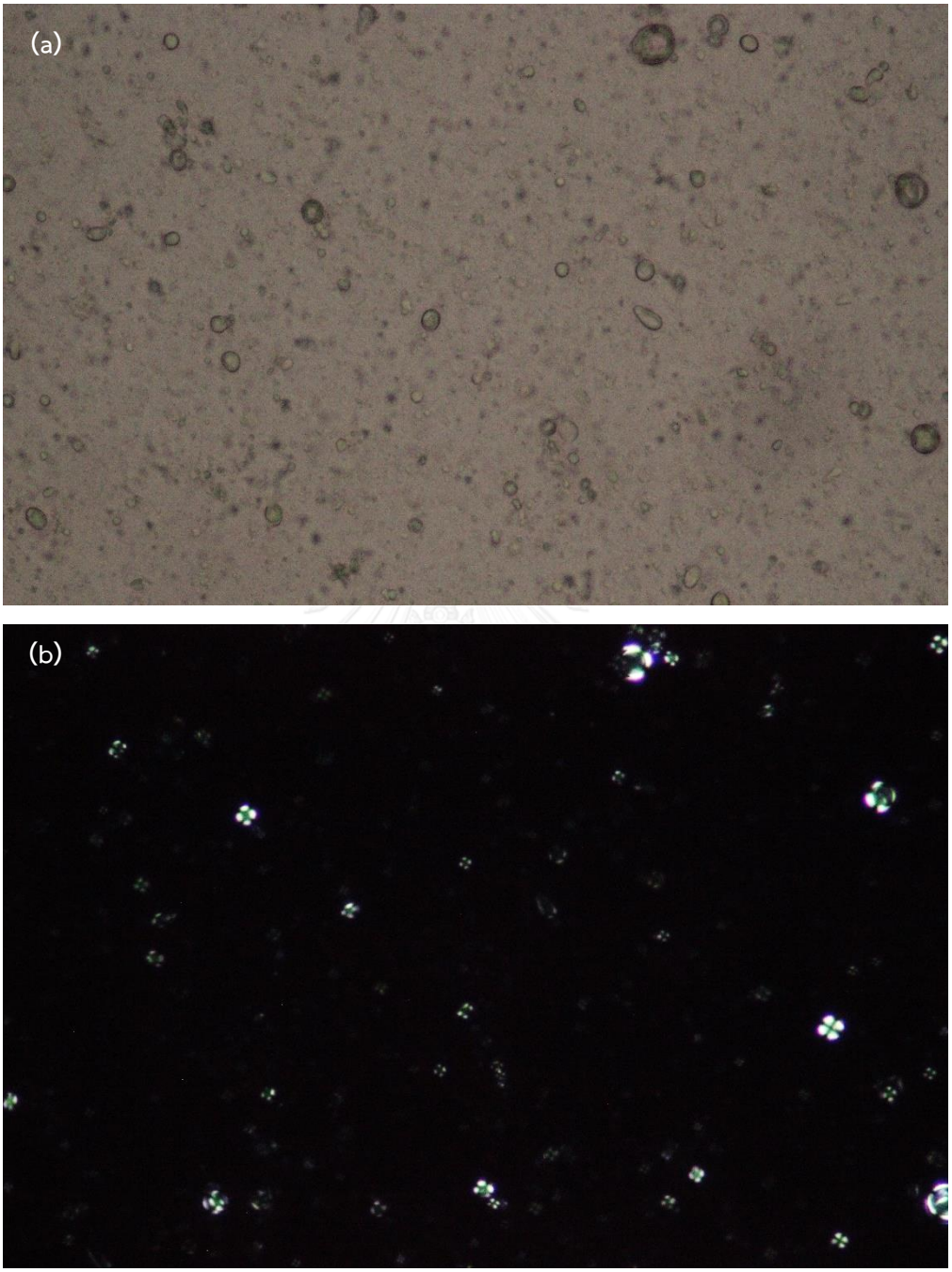
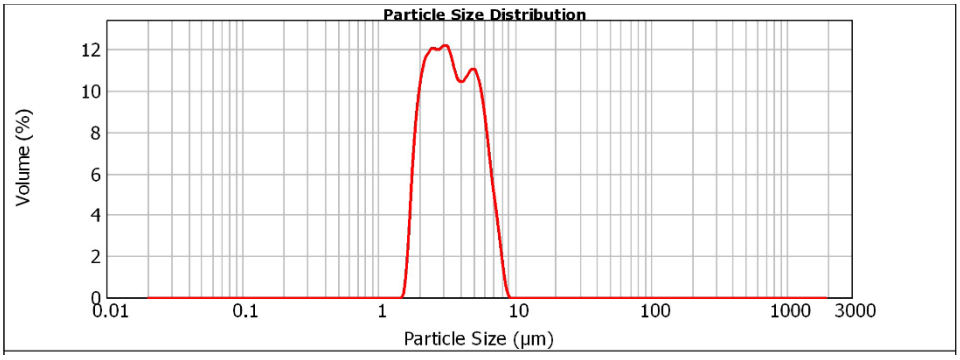
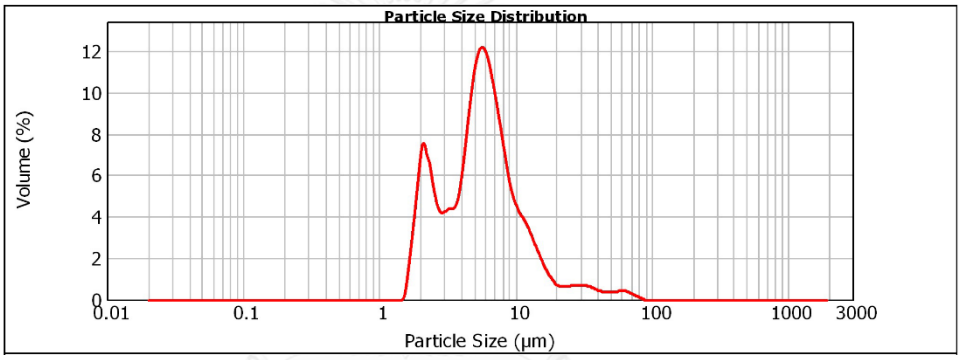


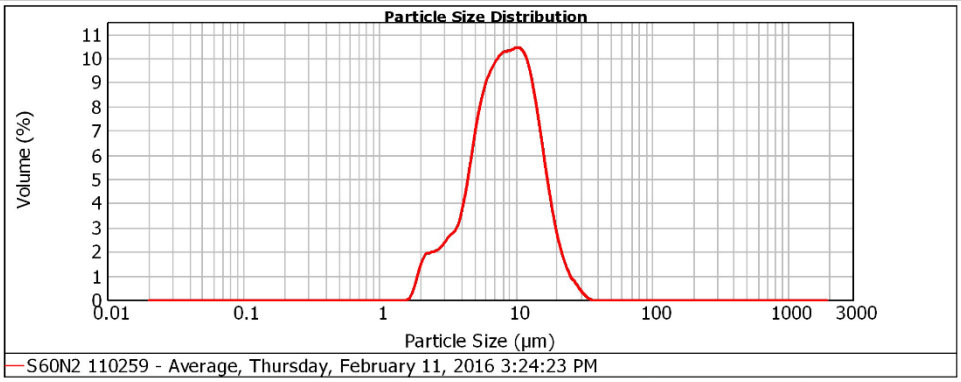
Figure 17 Appearance of blank niosomes of Span® 60/CHO/Solulan® C24 under optical microscopy (a) and polarized-light microscopy (b) (400X)



(a) Span® 20/CHO/Solulan® C24



(b) Span® 40/CHO/Solulan® C24



(c) Span® 60/ CHO/ Solulan® C24

Figure 18 Size and size distributions of blank niosomes with various formulations

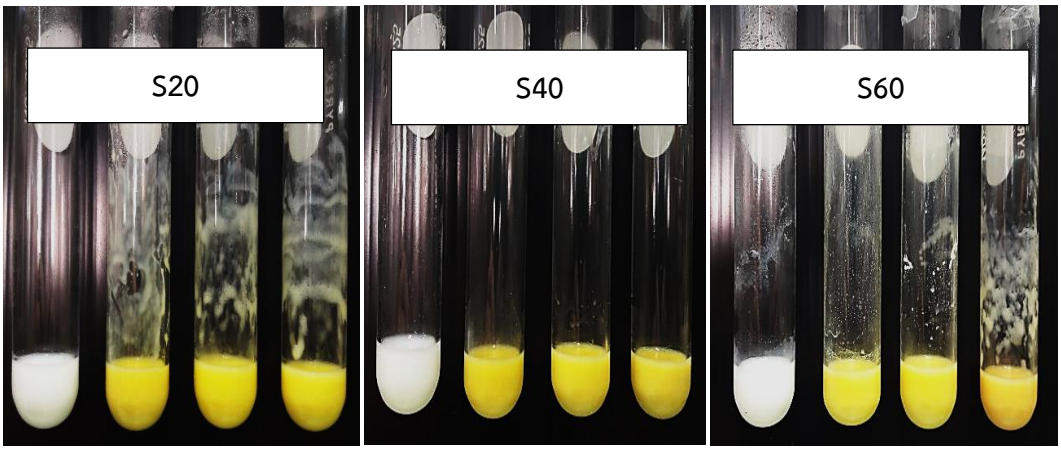


Figure 19 Physical appearance of blank niosomes (left) and niosomes containing the extract at initial concentration (1% w/w) prepared with various surfactants after fresh preparation (n = 3)

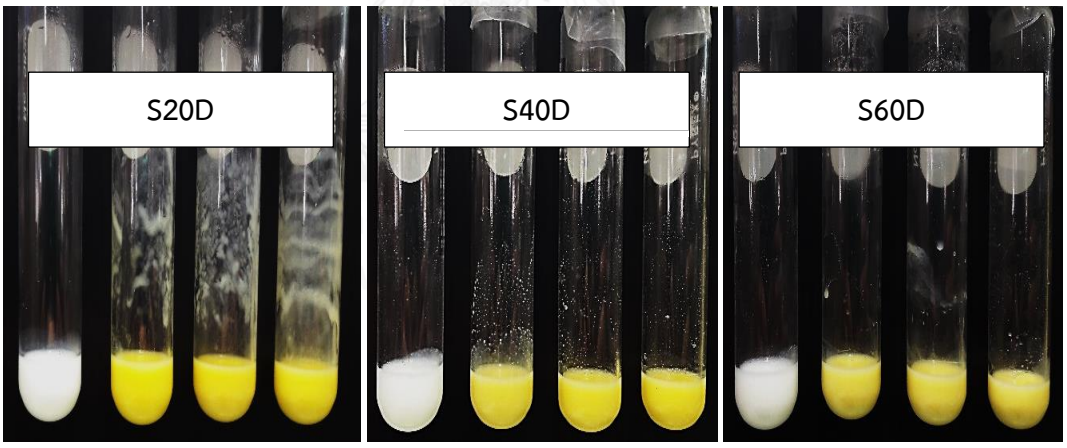


Figure 20 Physical appearance of blank niosomes (left) and niosomes containing the extract at two-fold diluted concentration prepared with various surfactants after fresh preparation (n = 3)

Under the optical microscope, all of niosomes containing the extract showed both spherical and tubular shapes and aggregation of vesicles as shown in Table 11 and Figure 21-23. In addition, the sizes of Span® 60 niosomes with the extract were larger than those of blank niosomes due to rupture of Span® 60 niosomes.

Moreover, red crystals of the extract were found in aqueous phase of all niosome formulations under optical microscope, while these crystals were not seen before with the saturated solution of the extract.

Table 11 Morphology of niosomes containing the extract at the initial concentration (1% w/v) and two-fold diluted concentration prepared with various surfactants under optical microscope and polarized-light microscope

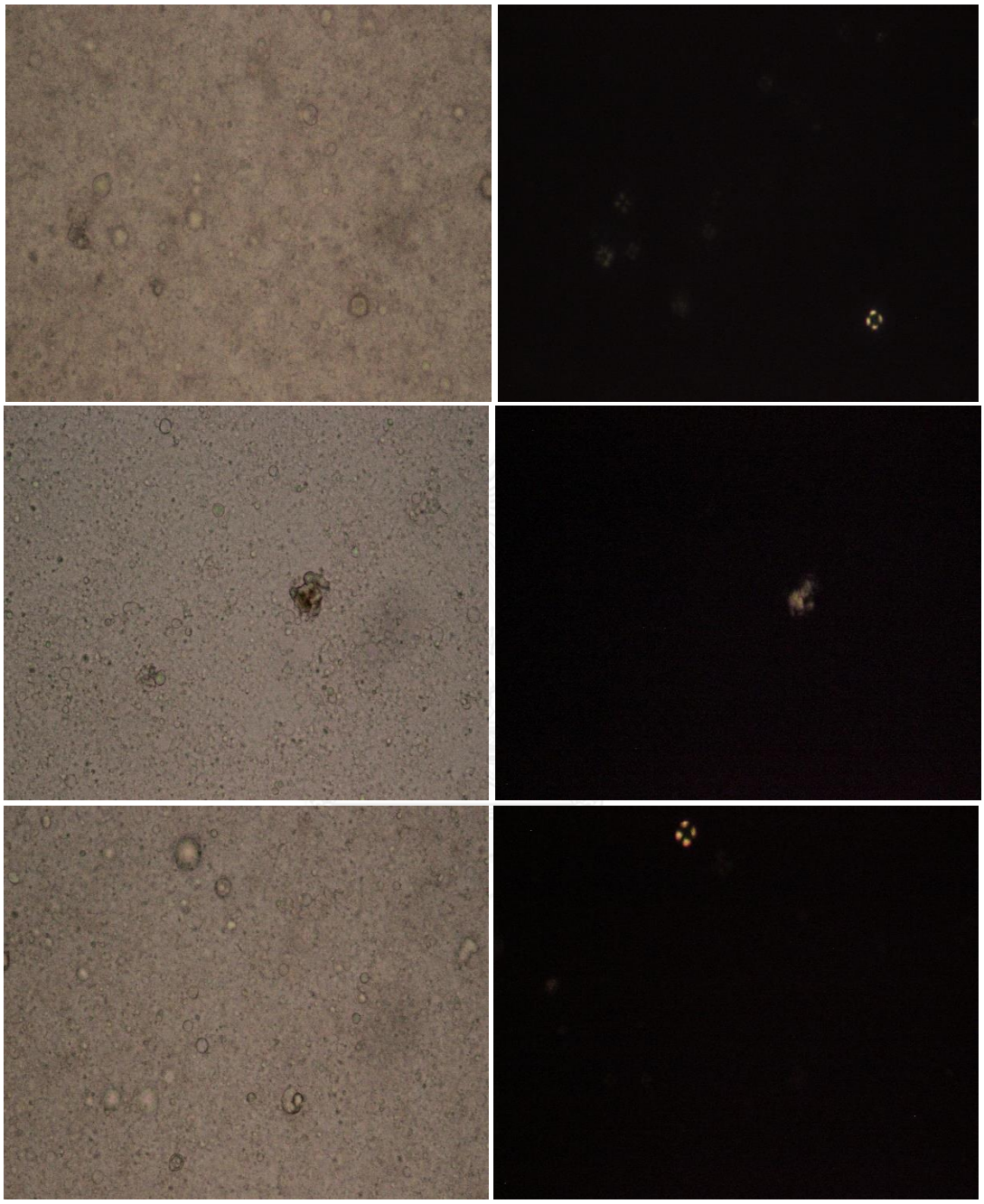
Code of formulations	Shape of vesicles	Aggregation	Crystal formation	Rupture of vesicles
Initial solution				
S20	S,T	+	+	-
S40	S,T	+	+	-
S60	S,T	+	+	+
Diluted solution				
S20D	S,T	+	+	-
S40D	S,T	+	+	-
S60D	S,T	+	+	+

S = Spherical vesicles, T = Tubular vesicles, + = Detected, - = Not detected

To study the effect of the extract concentration on property of vesicles, the two-fold diluted solution of the extract was used for preparation of niosomes. The saturated solution of the 1% w/v initial concentration (section 3.1) was diluted with acetate buffer pH 4.0. Physical appearance of all niosome formulations obtained was light yellow opaque dispersion (Figure 20). Their color intensity was depended on the loading concentration of the extract. Aggregation and sedimentation of vesicles were still immediately found in all formulations after fresh preparation but

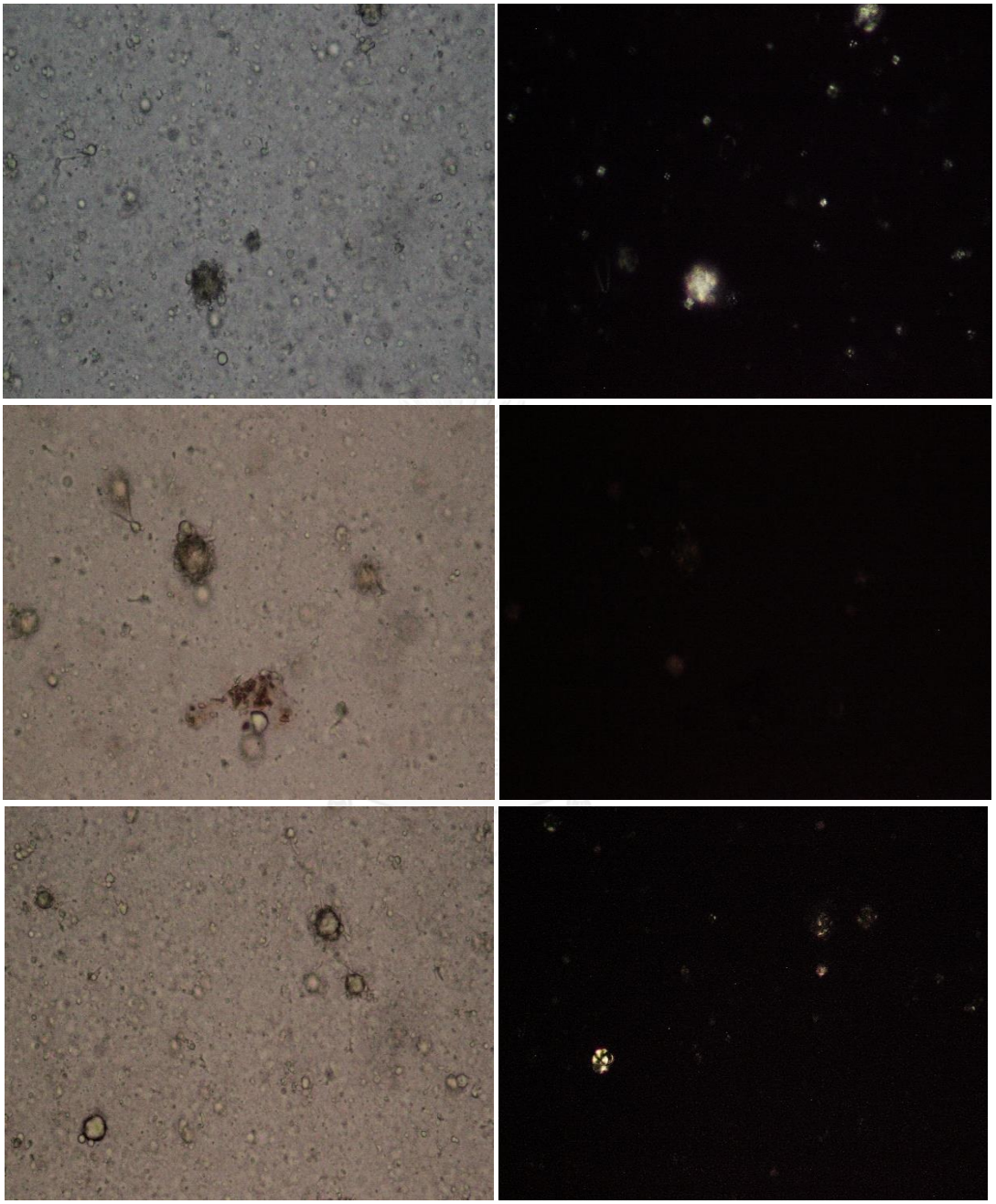
less than niosome preparation at 1% w/w as shown in Figure 20. The formulation with Span® 60 also showed rupture of vesicles. The observation under optical microscope and polarized light microscope indicated aggregation of vesicles and the presence of red crystals as shown in Table 11 and Figure 24 -26.

From the preliminary study, stability test of the extract solution in acetate buffer pH 4.0 after passing the same condition as the niosome preparation showed good stability of brazilin and no crystals in the solution. Thus, the presence of red crystals in niosome formulations may be explained that compositions of niosomes caused crystallization of compounds in the extract. Consequently, aggregation of vesicles may be attributed to the presence of crystals in formulations which resulted in disturbance of bilayer membrane (Mokhtar et al., 2008). Another possible reason for the aggregation may be due to the occurrence of interaction between various compounds in the sappanwood extract and structure of niosomes leading to unstable form of vesicles. Chemical compounds of sappanwood were reported to be phenolics, flavonoids, tannins and alkaloids (Senthilkumar et al., 2011). These results indicated that the sappanwood extract of Diaion® HP-20 fraction in the concentration studied could not be prepared as niosomes of all formulations investigated in this study.



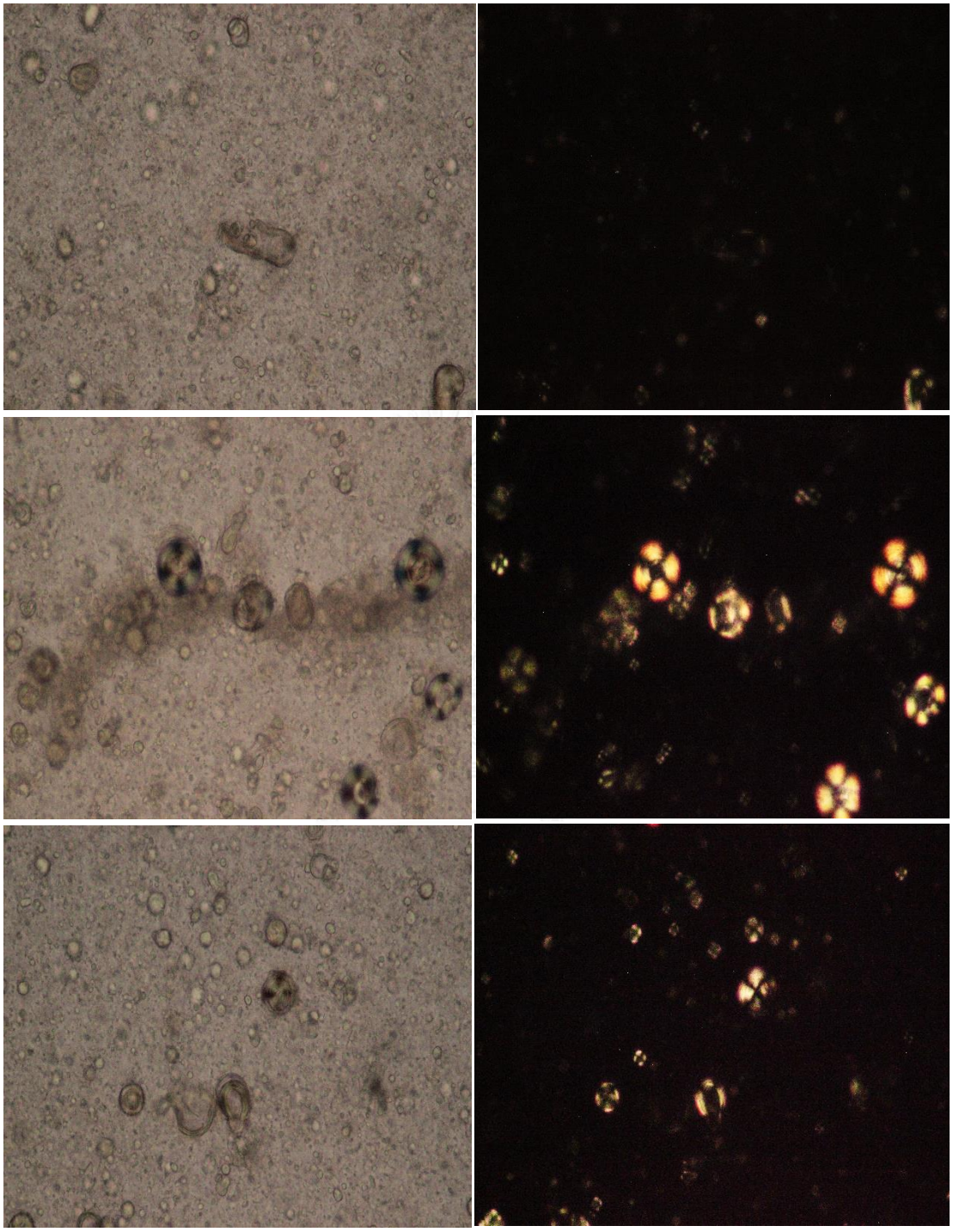
(a) (b)

Figure 21 Appearance of niosomes of Span® 20/CHO/Solulan® C24 containing the extract at initial concentration (1% w/w) under optical microscopy (a) and polarized light microscopy (b) (400X)



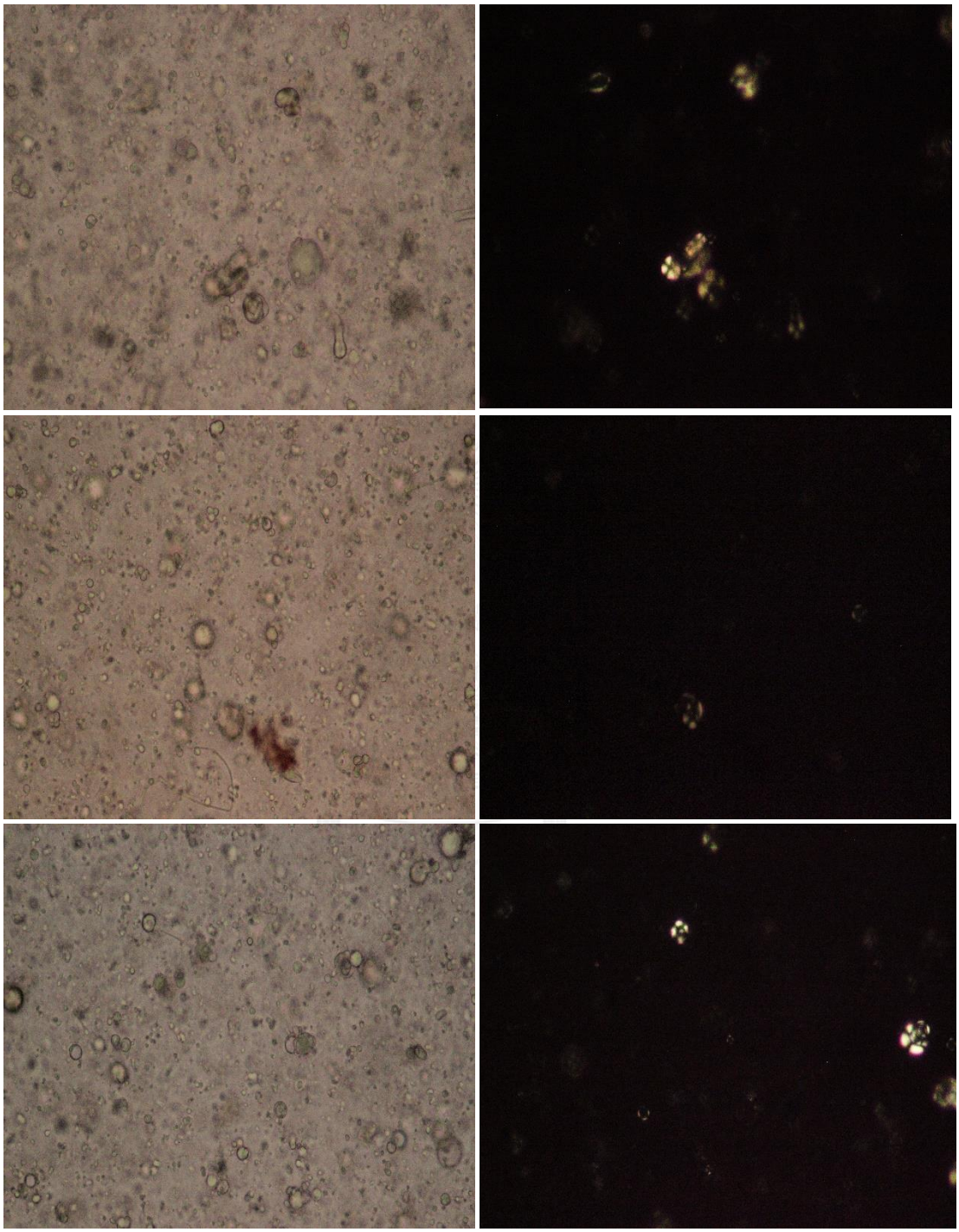
(a) **(b)**

Figure 22 Appearance of niosomes of Span® 40/CHO/Solulan® C24 containing the extract at initial concentration (1% w/w) under optical microscopy (a) and polarized light microscopy (b) (400X)



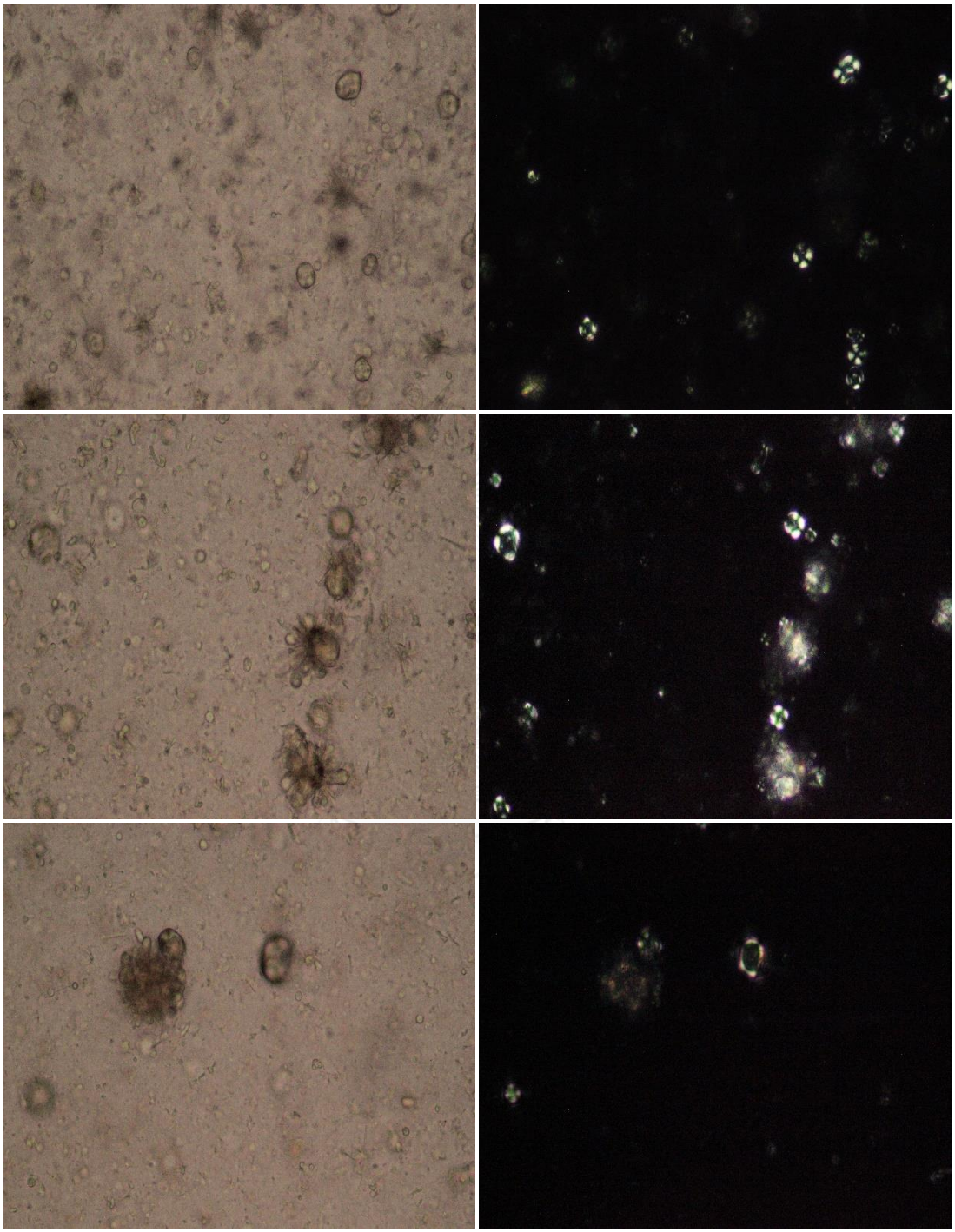
(a) (b)

Figure 23 Appearance of niosomes of Span® 60/CHO/Solulan® C24 containing the extract at initial concentration (1% w/w) under optical microscopy (a) and polarized light microscopy (b) (400X)



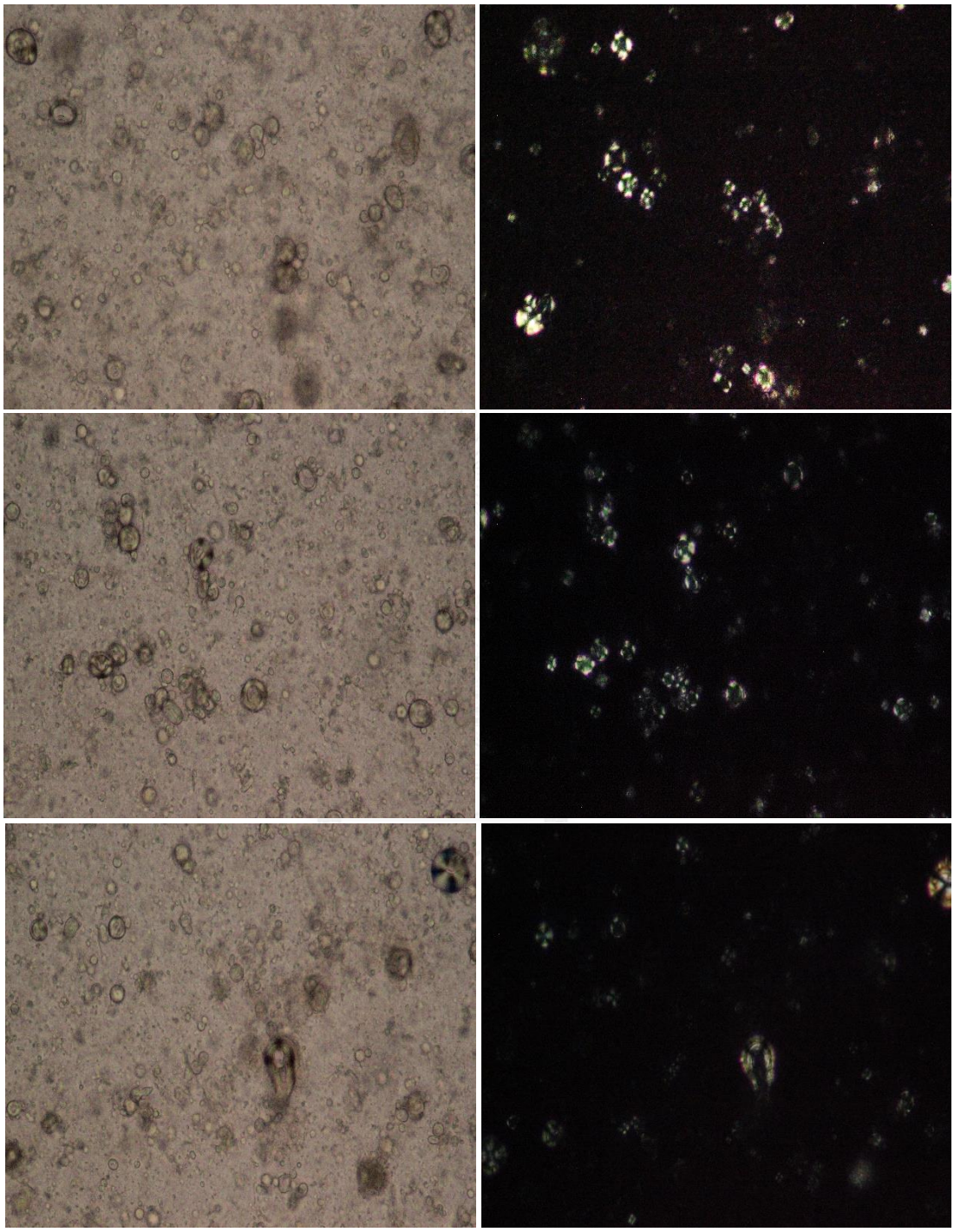
(a) **(b)**

Figure 24 Appearance of niosomes of Span® 20/CHO/Solulan® C24 containing the extract at two-fold diluted concentration under optical microscopy (a) and polarized light microscopy (b) (400X)



(a) **(b)**

Figure 25 Appearance of niosomes of Span® 40/CHO/Solulan® C24 containing the extract at two-fold diluted concentration under optical microscopy (a) and polarized light microscopy (b) (400X)



(a) **(b)**

Figure 26 Appearance of niosomes of Span® 60/CHO/Solulan® C24 containing the extract at two-fold diluted concentration under optical microscopy (a) and polarized light microscopy (b) (400X)

CHAPTER V
CONCLUSION

The present study aimed to develop niosomes containing *Caesalpinia sappan* L. (Sappanwood) extract. Partition method and ion-exchange chromatography (Diaion® HP-20) were used to semi-purify the ethanolic crude extract from *C. sappan* heartwood. Brazilin content, total phenolic content and DPPH free radical scavenging activity of various fractions were evaluated for finding the most suitable extract in development of niosomes.

The results obtained in this study can be concluded as follows:

1. The ethyl acetate fraction from partition method and the Diaion® HP-20 fraction showed similar high yields of 71.05% and 69.55%, respectively.
2. TLC densitometric method showed that brazilin was a major active compound in the sappanwood extracts. However, it could not be used for quantitative analysis of brazilin because it was tailing of spots in TLC plate.
3. Analysis of brazilin content by HPLC method showed that the Diaion® HP-20 fraction gave the highest brazilin content (13.24 ± 0.25% w/w).
4. The Diaion® HP-20 fraction presented the highest total phenolic content (604.82 ± 2.45 mg GAE/g extract).
5. The water fraction exhibited the greatest DPPH free scavenging activity with IC_{50} value of 1.19 ± 0.02 µg/ml and significantly higher than ascorbic acid, but lower than gallic acid.
6. The Diaion® HP-20 fraction was chosen for formulation of niosomes. The saturated solution of this extract which was prepared by filtering the 1% w/v extract suspension in acetate buffer pH 4.0 showed the highest brazilin content and was used for preparation of niosomes. The stability testing of in the saturated extract solution stored at 4°C for one month showed good stability

in terms of brazilin content, DPPH scavenging activity and pH except for total phenolic content.

7. Blank niosomes formulation with Span® 20/CHO/Solulan® C24, Span® 40/CHO/Solulan® C24 and Span® 60/CHO/Solulan® C24 could be prepared with sonication method. Niosomes with Span® 60 showed the largest size followed by Span® 40 and Span® 20, respectively.
8. Sappanwood extract loaded niosomes were unsuccessfully prepared due to the occurrences of vesicle aggregation and crystal formation. In addition, vesicle rupture could be observed with Span® 60 niosomes after extract loading.

From the results of the present study, the Diaion® HP-20 fraction of sappanwood extract was suitable for using as an active ingredient in cosmetic products. The saturated solution of this extract in acetate buffer pH 4.0 could be used for development of other skin delivery systems. For development of niosomes containing the sappanwood extract, other compositions of niosomes should be studied their effect on properties of niosomes. In addition, development of more purified extracts should be performed to increase more amounts of active compounds. Other delivery systems such as nanoemulsions and microemulsions should be conducted to improve skin delivery of the sappanwood extract.

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APPENDICES

APPENDIX A

^{1}H and ^{13}C NMR spectrum and high-resolution electrospray ionization mass spectrometry (HR-ESIMS) of protosappanin A and brazilin

Figure A-1 ^{13}C spectrum of protosappanin A (300 MHz in acetone-d_6)

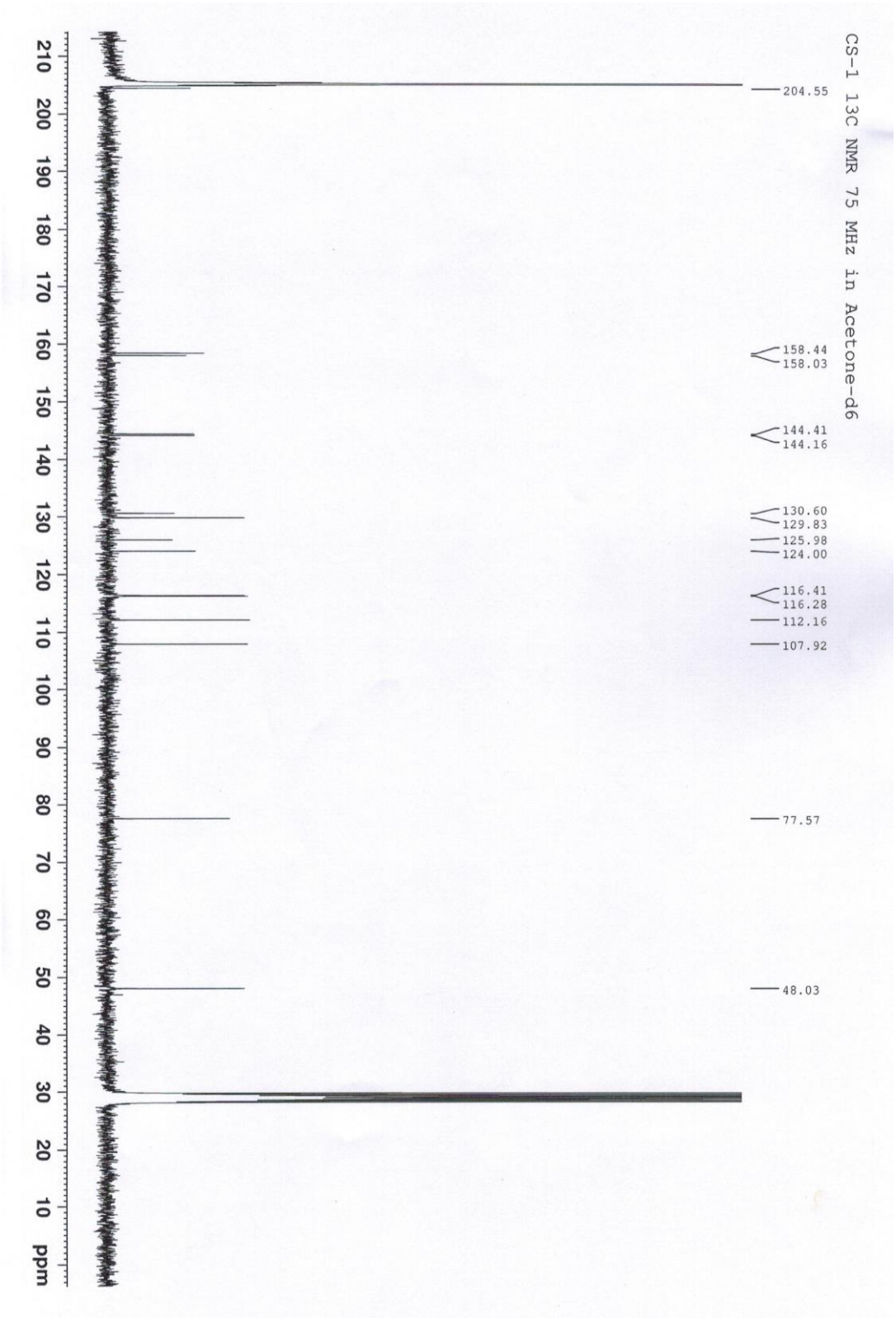


Figure A-2 DEPT 135 spectrum of protosappanin A (300 MHz in acetone-d_6)

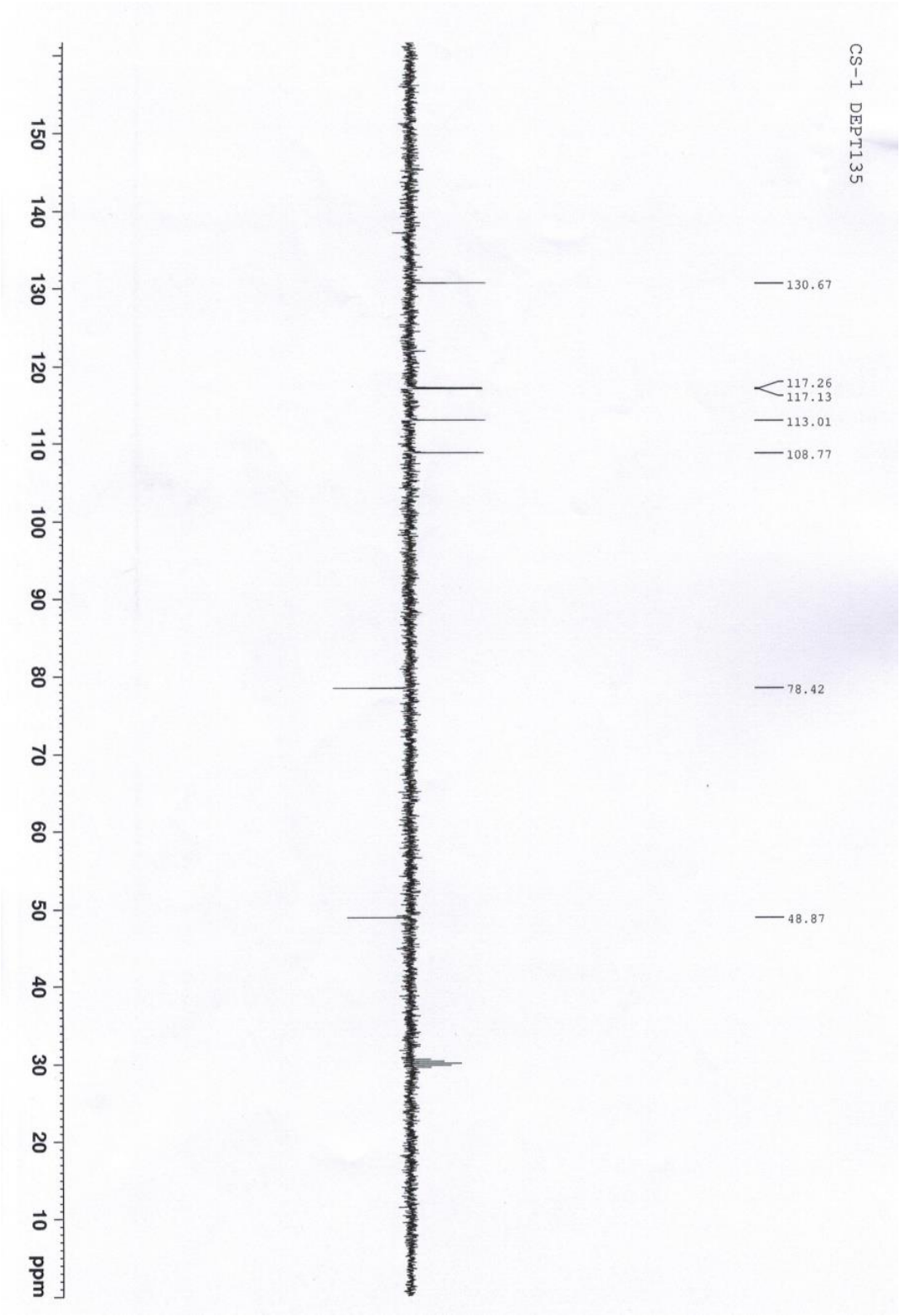


Figure A-3 ^{1}H spectrum of protosappanin A (300 MHz in acetone-d_6)

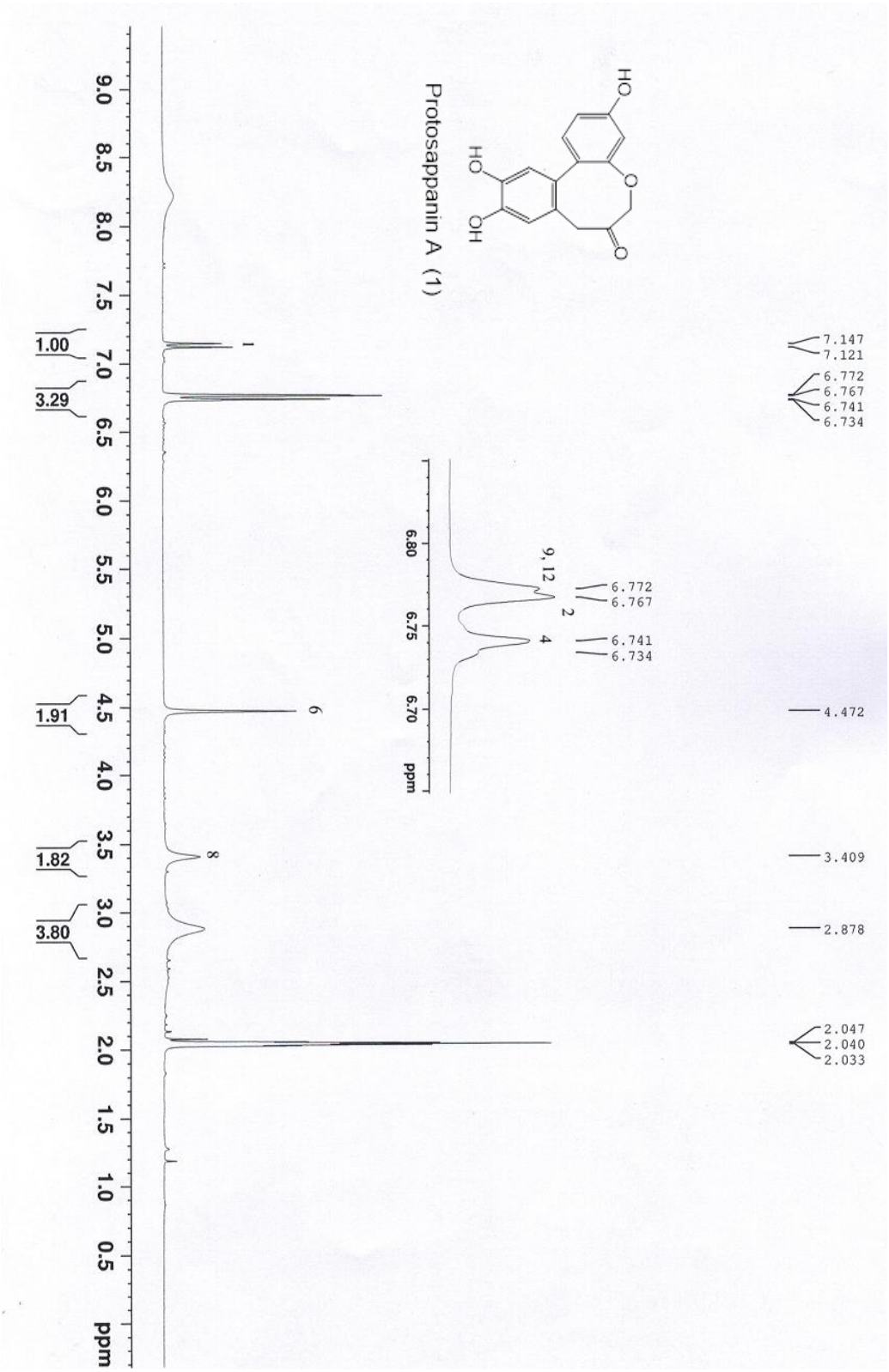


Figure A-4 HR-ESIMS of protosappanin A

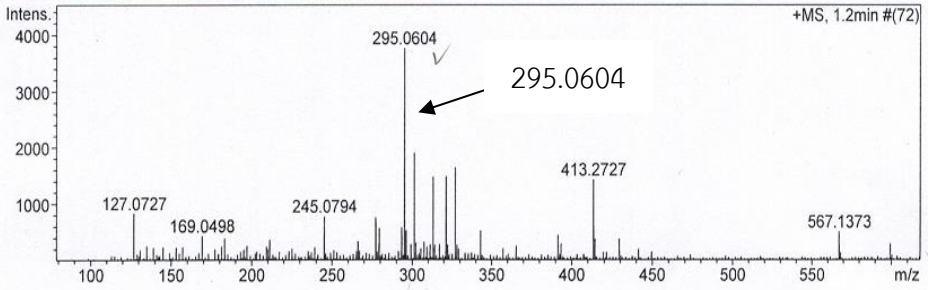
Mass Spectrum List Report

Analysis Info

Analysis Name	OSCUBS571223003.d	Acquisition Date	12/23/2014 12:38:06 PM
Method	MKE_tune_low_positive_20130204.m	Operator	Administrator
Sample Name	CS-1	Instrument	micrOTOF 72
	CS-1		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	79 V
Scan Range	n/a	Capillary Exit	110.0 V	Set Pulsar Pull	406 V
Scan Begin	50 m/z	Hexapole RF	90.0 V	Set Pulsar Push	388 V
Scan End	3000 m/z	Skimmer 1	45.5 V	Set Reflector	1300 V
		Hexapole 1	25.0 V	Set Flight Tube	9000 V
				Set Detector TOF	1910 V



#	m/z	I	I %	S/N	FWHM	Res.
1	127.0727	837	22.3	277.8	0.0203	6258
2	169.0498	438	11.6	122.8	0.0266	6362
3	183.0654	398	10.6	102.8	0.0260	7032
4	211.0974	375	10.0	83.7	0.0390	5410
5	245.0794	780	20.7	149.7	0.0326	7517
6	266.1761	348	9.3	61.3	0.0359	7414
7	277.1620	767	20.4	129.9	0.0394	7036
8	279.2288	574	15.3	96.4	0.0371	7533
9	293.2597	505	13.4	80.7	0.0786	3729
10	295.0604	3761	100.0	598.4	0.0356	8286
11	296.0636	537	14.3	85.0	0.0356	8320
12	301.1435	1905	50.7	296.8	0.0402	7489
13	302.1479	322	8.6	49.8	0.0380	7953
14	307.2565	333	8.9	50.6	0.0388	7926
15	311.0767	286	7.6	42.9	0.0495	6288
16	313.1786	1480	39.4	221.5	0.0369	8494
17	314.1823	279	7.4	41.4	0.0373	8426
18	317.1243	296	7.9	43.6	0.0445	7126
19	321.2777	1485	39.5	216.6	0.0413	7775
20	322.2795	284	7.6	41.1	0.0416	7749
21	327.0879	1640	43.6	234.9	0.0430	7606
22	328.0908	286	7.6	40.6	0.0414	7920
23	343.0650	530	14.1	72.7	0.0446	7686
24	391.3572	450	12.0	62.1	0.0533	7340
25	393.3093	301	8.0	41.4	0.0515	7640
26	413.2727	1427	37.9	198.4	0.0513	8052
27	414.2752	385	10.2	53.3	0.0495	8365
28	429.2462	384	10.2	53.2	0.0563	7623
29	567.1373	506	13.5	71.9	0.0712	7971
30	599.1651	301	8.0	42.9	0.0792	7562

Figure A-5 ^{13}C spectrum of brazilin (300 MHz in CD_3OD)

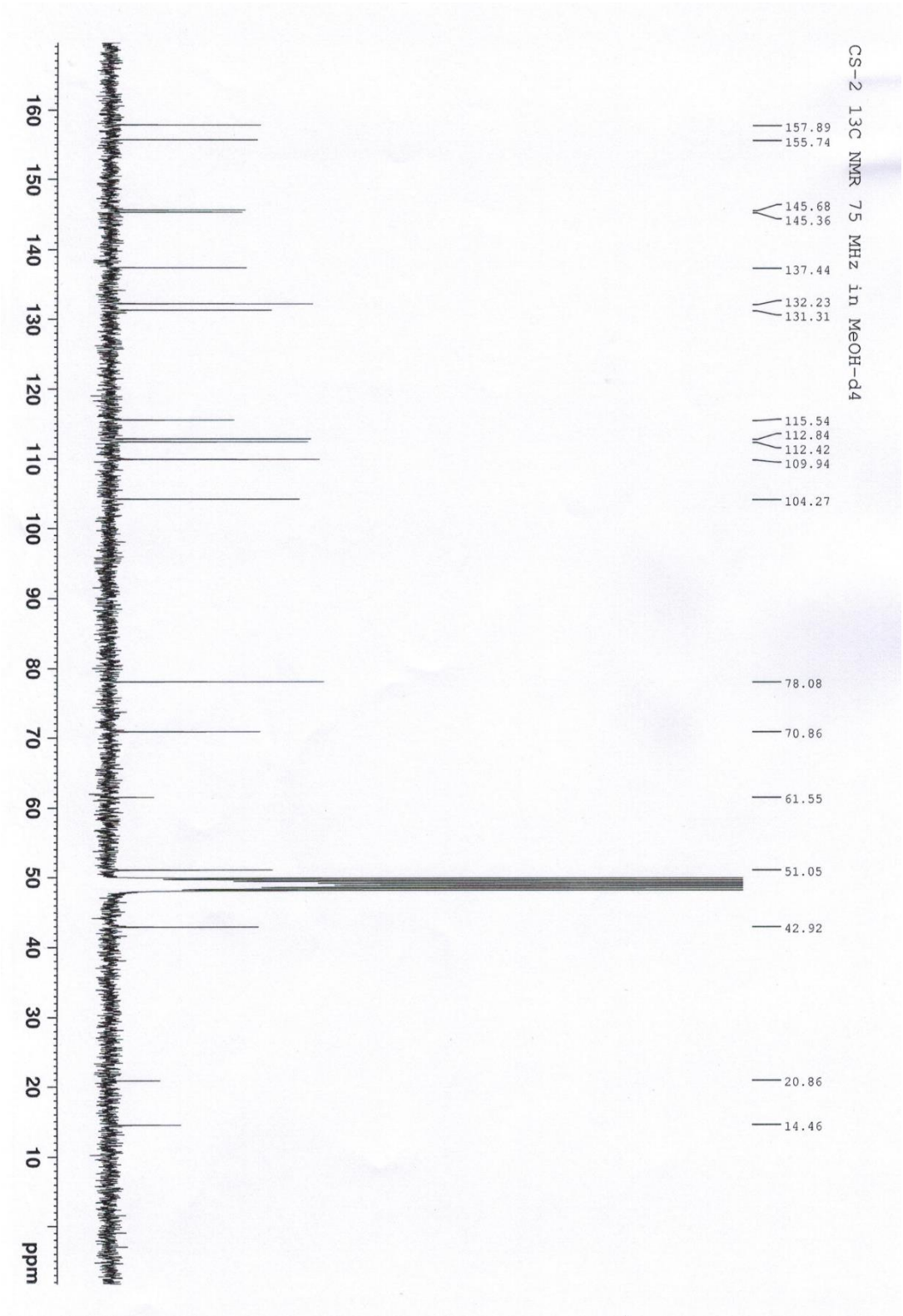


Figure A-6 DEPT 135 spectrum of brazilin (300 MHz in CD_3OD)

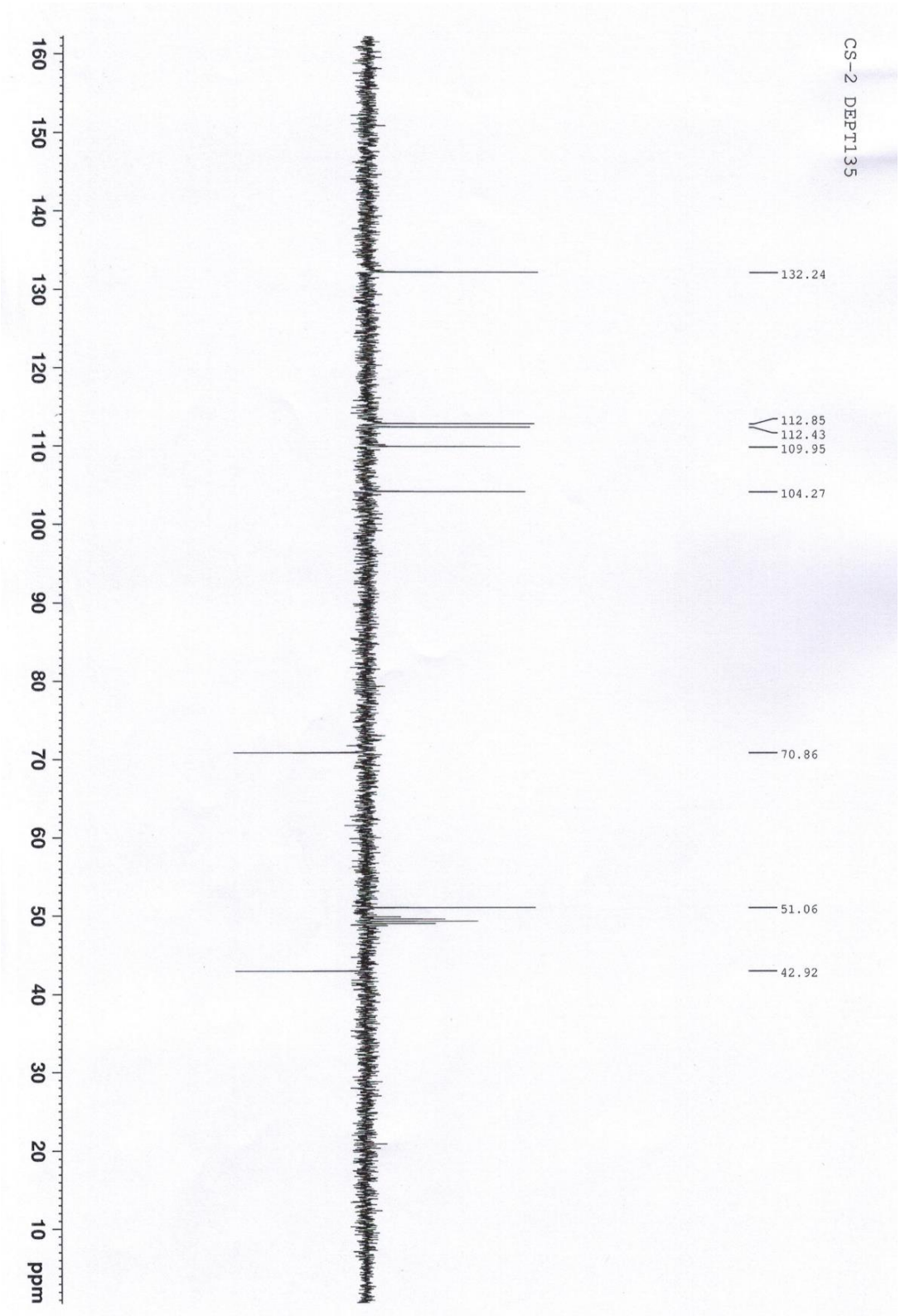


Figure A-7 ^{1}H spectrum of brazilin (300 MHz in CD_3OD)

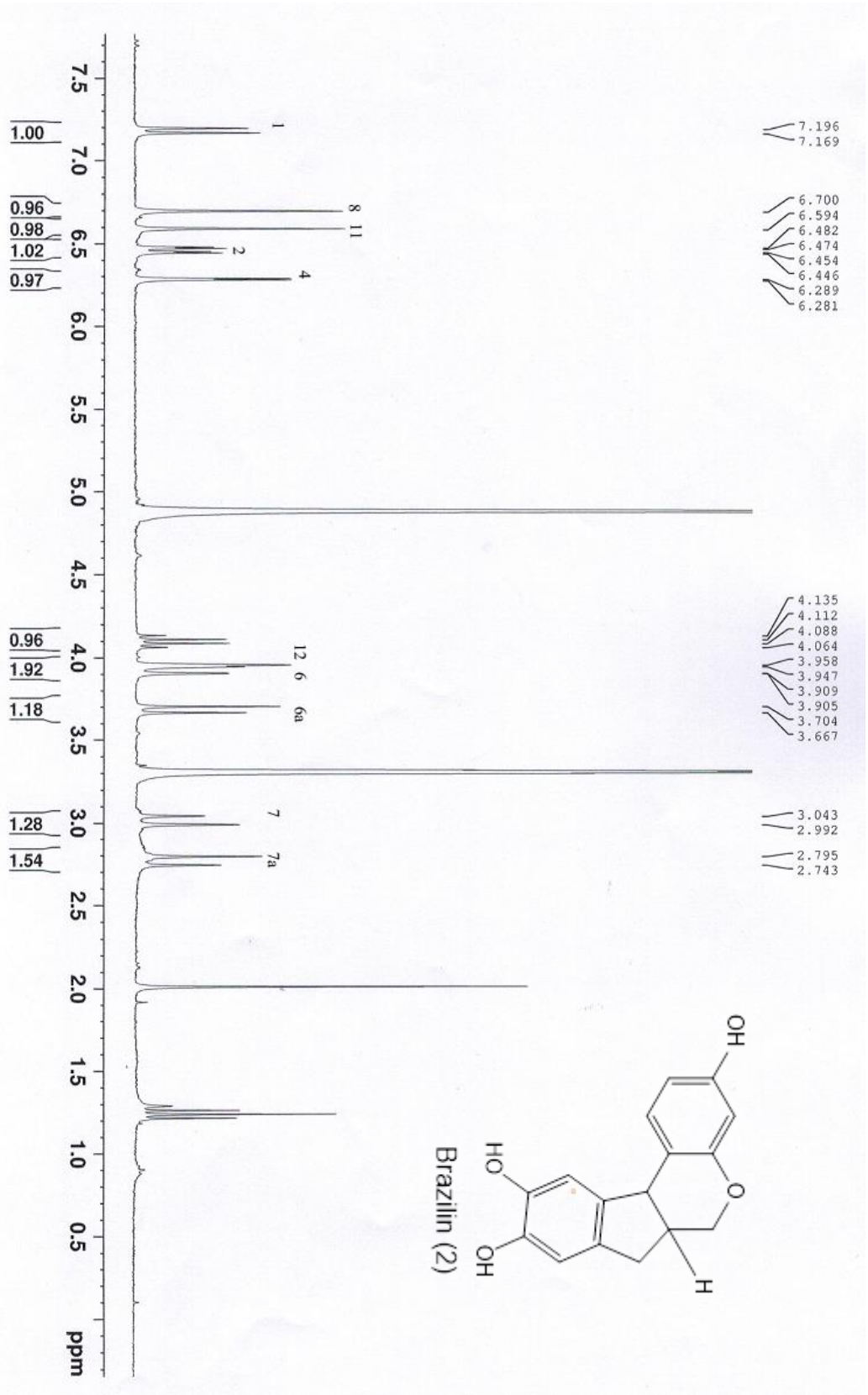


Figure A-8 HR-ESIMS of Brazilin

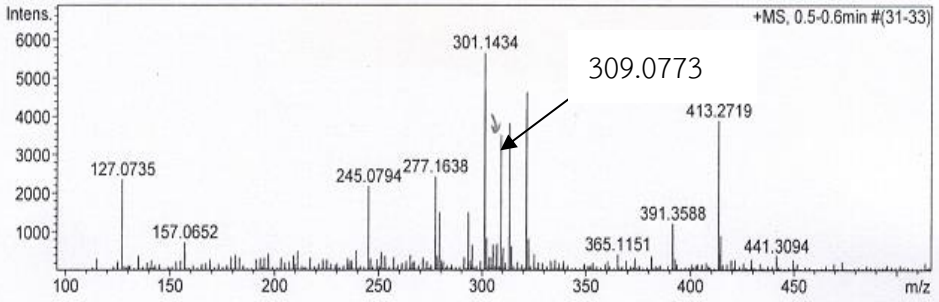
Mass Spectrum List Report

Analysis Info

Analysis Name	OSCUBS571223004.d	Acquisition Date	12/23/2014 12:43:02 PM
Method	MKE_tune_low_positive_20130204.m	Operator	Administrator
Sample Name	CS-2	Instrument	micrOTOF 72
	CS-2		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	79 V
Scan Range	n/a	Capillary Exit	150.0 V	Set Pulsar Pull	406 V
Scan Begin	50 m/z	Hexapole RF	90.0 V	Set Pulsar Push	388 V
Scan End	3000 m/z	Skimmer 1	45.5 V	Set Reflector	1300 V
		Hexapole 1	25.0 V	Set Flight Tube	9000 V
				Set Detector TOF	1910 V



#	m/z	I	I %	S/N	FWHM	Res.
1	127.0735	2355	41.7	785.9	0.0201	6335
2	157.0652	732	13.0	212.0	0.0354	4439
3	181.0846	411	7.3	96.6	0.0280	6475
4	197.0845	447	7.9	93.2	0.0307	6419
5	211.0975	505	8.9	95.9	0.0341	6193
6	239.1415	524	9.3	84.5	0.0515	4640
7	245.0794	2181	38.6	341.2	0.0331	7395
8	251.1917	491	8.7	74.3	0.0377	6662
9	265.2083	413	7.3	58.4	0.0393	6755
10	277.1638	2430	43.0	325.5	0.0370	7491
11	279.2287	1515	26.8	201.0	0.0354	7896
12	293.2565	1457	25.8	181.9	0.0669	4386
13	295.0624	671	11.9	83.0	0.0358	8242
14	301.1434	5648	100.0	683.5	0.0384	7846
15	302.1482	839	14.9	101.0	0.0368	8209
16	305.2469	674	11.9	80.1	0.0435	7021
17	307.0624	652	11.5	76.9	0.0376	8160
18	307.2599	708	12.5	83.4	0.0383	8018
19	309.0773	3526	62.4	413.3	0.0391	7896
20	310.0807	594	10.5	69.2	0.0376	8254
21	313.1796	3835	67.9	442.4	0.0380	8236
22	314.1832	645	11.4	73.9	0.0391	8028
23	321.2792	4637	82.1	518.9	0.0390	8228
24	322.2829	820	14.5	91.2	0.0390	8257
25	325.0673	432	7.7	47.5	0.0467	6957
26	365.1151	426	7.5	44.8	0.0527	6934
27	391.3588	1219	21.6	130.9	0.0514	7617
28	413.2719	3898	69.0	424.7	0.0465	8881
29	414.2749	914	16.2	99.4	0.0461	8982
30	703.4683	649	11.5	77.9	0.0839	8386

APPENDIX B

Brazilin content of the sappanwood extracts measured by HPLC method

Table B-1 Brazilin content of various sappanwood extracts

Sappanwood extracts	Brazilin content (%w/w)			Mean	SD
	N1	N2	N3		
Crude extract	7.54	7.74	7.81	7.70	0.21
Ethyl acetate fraction	11.26	11.57	11.30	11.38	0.15
Water fraction	11.36	11.29	10.78	11.14	0.27
Dichloromethane fraction	8.11	8.00	7.84	7.98	0.17
Diaion® HP-20 fraction	13.02	13.22	13.54	13.24	0.25

Table B-2 Brazilin content of Diaion® HP-20 fraction in the saturated solution in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days

Diaion® HP-20 fraction	Brazilin content (%w/w)			Mean	S.D.
	N1	N2	N3		
Fresh preparation	11.70	11.53	11.36	11.53	0.16
After storage for 30 days	11.18	11.15	11.23	11.31	0.17

Table B-3 Paired t-test of brazilin content of Diaion® HP-20 fraction in the saturated solution in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	BrazilinD1	115.3500	3	1.67502	.96707
	BrazilinD30	113.1400	3	1.77643	1.02562

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	BrazilinD1 & BrazilinD30	3	-.149	.905

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	BrazilinD1 - BrazilinD30	2.21000	2.61651	1.51064	-4.28976	8.70976	1.463	2	.281

APPENDIX C

Total phenolic content of the sappanwood extracts

Table C-1 Total phenolic content of various sappanwood extracts

Sappanwood extracts	mg GAE/ g extract			Mean	SD
	N1	N2	N3		
Crude extract	500.89	496.87	500.89	499.55	2.31
Water fraction	542.40	541.06	545.08	542.84	2.04
Ethyl acetate fraction	593.03	596.25	594.64	594.64	1.60
Dichloromethane fraction	327.85	325.44	327.85	327.04	1.39
Diaion® HP-20 fraction	607.49	602.67	605.89	604.82	2.45

Table C-2 Total phenolic content of Diaion® HP-20 fraction in the saturated solution in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days

Diaion® HP-20 fraction	mg GAE/ g extract			Mean	SD
	N1	N2	N3		
Fresh preparation	572.14	575.36	576.96	574.83	2.45
After storage for 30 days	524.19	521.26	525.66	523.79	2.24

Table C-3 One-way analysis of variance of total phenolic content of various sappanwood extracts

ANOVA

Samples

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	152523.439	4	38130.860	9482.249	.000
Within Groups	40.213	10	4.021		
Total	152563.652	14			

Table C-4 Multiple comparison of total phenolic content of various sappanwood extracts by Tukey HSD method

Multiple Comparisons

Dependent Variable: Samples

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude	Water	-43.29533*	1.63733	.000	-48.6839	-37.9067
	EtOAc	-95.09000*	1.63733	.000	-100.4786	-89.7014
	CH2Cl2	172.50533*	1.63733	.000	167.1167	177.8939
	Diaion	-105.80233*	1.63733	.000	-111.1909	-100.4137
Water	Crude	43.29533*	1.63733	.000	37.9067	48.6839
	EtOAc	-51.79467*	1.63733	.000	-57.1833	-46.4061
	CH2Cl2	215.80067*	1.63733	.000	210.4121	221.1893
	Diaion	-62.50700*	1.63733	.000	-67.8956	-57.1184
EtOAc	Crude	95.09000*	1.63733	.000	89.7014	100.4786
	Water	51.79467*	1.63733	.000	46.4061	57.1833
	CH2Cl2	267.59533*	1.63733	.000	262.2067	272.9839
	Diaion	-10.71233*	1.63733	.000	-16.1009	-5.3237
CH2Cl2	Crude	-172.50533*	1.63733	.000	-177.8939	-167.1167
	Water	-215.80067*	1.63733	.000	-221.1893	-210.4121
	EtOAc	-267.59533*	1.63733	.000	-272.9839	-262.2067
	Diaion	-278.30767*	1.63733	.000	-283.6963	-272.9191
Diaion	Crude	105.80233*	1.63733	.000	100.4137	111.1909
	Water	62.50700*	1.63733	.000	57.1184	67.8956
	EtOAc	10.71233*	1.63733	.000	5.3237	16.1009
	CH2Cl2	278.30767*	1.63733	.000	272.9191	283.6963

*. The mean difference is significant at the 0.05 level.

Table C-5 Paired t-test of total phenolic content of Diaion® HP-20 fraction in the saturated solution in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	TPC1DAY	574.8260	3	2.45430	1.41699
	TPC30DAY	523.7050	3	2.23990	1.29321

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	TPC1DAY & TPC30DAY	3	.143	.909

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	TPC1DAY - TPC30DAY	51.12100	3.07776	1.77695	43.47541	58.76659	28.769	2	.001

APPENDIX D

DPPH radical scavenging activity of the sappanwood extracts

Table D-1 DPPH IC_{50} values of various sappanwood extract, isolated compound and positive controls

Sappanwood extracts/ Standards	IC_{50} (µg/ml)			Mean	SD
	N1	N2	N3		
Crude extract	2.70	2.76	2.71	2.72	0.06
Water fraction	1.21	1.18	1.18	1.19	0.02
Ethyl acetate fraction	2.08	2.09	2.08	2.08	0.02
Dichloromethane fraction	3.68	3.76	3.73	3.72	0.04
Diaion® HP-20 fraction	1.98	2.03	2.06	2.03	0.04
Brazilin	1.34	1.32	1.36	1.34	0.01
Protosappanin A	1.36	1.33	1.35	1.35	0.01
Gallic acid	0.63	0.63	0.02	0.63	0.007
Ascorbic acid	1.57	1.56	1.58	1.57	0.01

Table D-2 DPPH IC_{50} values of Diaion® HP-20 fraction in the saturated solution in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days

Diaion® HP-20 fraction	IC_{50} (µg/ml)			Mean	SD
	N1	N2	N3		
Fresh preparation	3.10	3.07	3.03	3.07	0.03
After storage for 30 days	3.16	3.31	3.20	3.22	0.08

Table D-3 One-way analysis of variance of DPPH IC_{50} values of various sappanwood extracts, isolated compounds and positive controls

ANOVA

Group

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	180.000	24	7.500	.	.
Within Groups	.000	2	.000		
Total	180.000	26			

Table D-4 Multiple comparison of DPPH IC_{50} values of various sappanwood extracts, isolated compounds and positive controls by Tukey HSD method

Multiple Comparisons

Dependent Variable: IC50
Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude	Water	1.53333*	.02461	.000	1.4471	1.6196
	EtOAc	.64367*	.02461	.000	.5574	.7299
	CH2Cl2	-.96667*	.02461	.000	-1.0529	-.8804
	Diaion	.70000*	.02461	.000	.6138	.7862
	Ascorbic	1.15333*	.02461	.000	1.0671	1.2396
	Gallic	2.09667*	.02461	.000	2.0104	2.1829
	Brazilin	1.38033*	.02461	.000	1.2941	1.4666
	Protosappanin	1.37033*	.02461	.000	1.2841	1.4566
Water	Crude	-1.53333*	.02461	.000	-1.6196	-1.4471
	EtOAc	-.88967*	.02461	.000	-.9759	-.8034
	CH2Cl2	-2.50000*	.02461	.000	-2.5862	-2.4138
	Diaion	-.83333*	.02461	.000	-.9196	-.7471
	Ascorbic	-.38000*	.02461	.000	-.4662	-.2938
	Gallic	.56333*	.02461	.000	.4771	.6496
	Brazilin	-.15300*	.02461	.000	-.2392	-.0668
	Protosappanin	-.16300*	.02461	.000	-.2492	-.0768
EtOAc	Crude	-.64367*	.02461	.000	-.7299	-.5574
	Water	.88967*	.02461	.000	.8034	.9759
	CH2Cl2	-1.61033*	.02461	.000	-1.6966	-1.5241
	Diaion	.05633	.02461	.396	-.0299	.1426
	Ascorbic	.50967*	.02461	.000	.4234	.5959
	Gallic	1.45300*	.02461	.000	1.3668	1.5392
	Brazilin	.73667*	.02461	.000	.6504	.8229
	Protosappanin	.72667*	.02461	.000	.6404	.8129
CH2Cl2	Crude	.96667*	.02461	.000	.8804	1.0529
	Water	2.50000*	.02461	.000	2.4138	2.5862
	EtOAc	1.61033*	.02461	.000	1.5241	1.6966
	Diaion	1.66667*	.02461	.000	1.5804	1.7529
	Ascorbic	2.12000*	.02461	.000	2.0338	2.2062
	Gallic	3.06333*	.02461	.000	2.9771	3.1496
	Brazilin	2.34700*	.02461	.000	2.2608	2.4332
	Protosappanin	2.33700*	.02461	.000	2.2508	2.4232
Diaion	Crude	-.70000*	.02461	.000	-.7862	-.6138
	Water	.83333*	.02461	.000	.7471	.9196
	EtOAc	-.05633	.02461	.396	-.1426	.0299
	CH2Cl2	-1.66667*	.02461	.000	-1.7529	-1.5804
	Ascorbic	.45333*	.02461	.000	.3671	.5396
	Gallic	1.39667*	.02461	.000	1.3104	1.4829
	Brazilin	.68033*	.02461	.000	.5941	.7666
	Protosappanin	.67033*	.02461	.000	.5841	.7566
Ascorbic	Crude	-1.15333*	.02461	.000	-1.2396	-1.0671
	Water	.38000*	.02461	.000	.2938	.4662
	EtOAc	-.50967*	.02461	.000	-.5959	-.4234
	CH2Cl2	-2.12000*	.02461	.000	-2.2062	-2.0338
	Diaion	-.45333*	.02461	.000	-.5396	-.3671
	Gallic	.94333*	.02461	.000	.8571	1.0296
	Brazilin	.22700*	.02461	.000	.1408	.3132
	Protosappanin	.21700*	.02461	.000	.1308	.3032
Gallic	Crude	-2.09667*	.02461	.000	-2.1829	-2.0104
	Water	-.56333*	.02461	.000	-.6496	-.4771
	EtOAc	-1.45300*	.02461	.000	-1.5392	-1.3668
	CH2Cl2	-3.06333*	.02461	.000	-3.1496	-2.9771
	Diaion	-1.39667*	.02461	.000	-1.4829	-1.3104
	Ascorbic	-.94333*	.02461	.000	-1.0296	-.8571
	Brazilin	-.71633*	.02461	.000	-.8026	-.6301
	Protosappanin	-.72633*	.02461	.000	-.8126	-.6401
Brazilin	Crude	-1.38033*	.02461	.000	-1.4666	-1.2941
	Water	.15300*	.02461	.000	.0668	.2392
	EtOAc	-.73667*	.02461	.000	-.8229	-.6504
	CH2Cl2	-2.34700*	.02461	.000	-2.4332	-2.2608
	Diaion	-.68033*	.02461	.000	-.7666	-.5941
	Ascorbic	-.22700*	.02461	.000	-.3132	-.1408
	Gallic	.71633*	.02461	.000	.6301	.8026
	Protosappanin	-.01000	.02461	1.000	-.0962	.0762
Protosappanin	Crude	-1.37033*	.02461	.000	-1.4566	-1.2841
	Water	.16300*	.02461	.000	.0768	.2492
	EtOAc	-.72667*	.02461	.000	-.8129	-.6404
	CH2Cl2	-2.33700*	.02461	.000	-2.4232	-2.2508
	Diaion	-.67033*	.02461	.000	-.7566	-.5841
	Ascorbic	-.21700*	.02461	.000	-.3032	-.1308
	Gallic	.72633*	.02461	.000	.6401	.8126
	Brazilin	.01000	.02461	1.000	-.0762	.0962

*. The mean difference is significant at the 0.05 level.

Table D-5 Paired t-test of DPPH IC_{50} values of Diaion® HP-20 fraction in the saturated solution in acetate buffer pH 4.0 after fresh preparation and storage in the refrigerator for 30 days

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	DPPHD1 & DPPHD30	3	-.172	.890

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	DPPHD1 & DPPHD30	3	-.172	.890

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	DPPHD1 - DPPHD30	-.15367	.08954	.05170	-.37610	.06876	-2.973	2	.097

APPENDIX E

Properties of some selected materials used in niosomes

Table E-1 Properties of some selected materials

Material	INIC name	Formula	Property
Cholesterol	Cholesterol	$C_{27}H_{46}O$	MW: 386.67 MP : 174-150 °C BP : 360°C
Span® 20	Sorbitan Laurate	$C_{18}H_{34}O_6$	MW: 346 MP : 5.1 °C HLB: 8.6
Span® 40	Sorbitan Palmitate	$C_{22}H_{42}O_6$	MW: 403 MP : 44-48 °C HLB: 6.7
Span® 60	Sorbitan Stearate	$C_{24}H_{46}O_6$	MW: 431 MP : 53-57 °C HLB: 4.7
Solulan® C24	Choleth-24 and Ceteth-24	-	MW: 1,443 Cloud point : 88-95 °C HLB: 8-9

VITA

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