DEVELOPMENT OF NIOSOME CONTAINING ROASTED COFFEE RESIDUE EXTRACT FOR ANTIAGING PREPARATION

BY

MR. AHMAD ZAKWAN MUSTAFA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF SCIENCE AND TECHNOLOGY
THAMMASAT UNIVERSITY
ACADEMIC YEAR 2015

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THESIS

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ENTITLED

DEVELOPMENT OF NIOSOME CONTAINING
ROASTED COFFEE RESIDUE EXTRACT FOR ANTIAGING PREPARATION

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ABSTRACT

Roasted coffee bean is claimed as the second most traded commodity in the world, it is used to brew coffee, the most popular and highly consumed beverage. Six million tons roasted coffee residue (RCR) was produced annually, worldwide, harmful to the environment as it decomposed in a year. Due to environment impact, it was reused as adsorbent, fertilizer, animal feed, industrial substitute and fuel. Coffee bean contains caffeine (CAF), caffeic acid (CA), chlorogenic acid (CGA), nicotinic acid (NA), trigonelline, melanoidins, protein, lipids, fiber and minerals. Coffee drink is abundant with antioxidant active compounds, higher than tea. Therefore, the objectives of this study were to investigate the utility of RCR as a sustainable and economical source of antioxidant compounds such as CA and CGA. The compounds were reported that can reverse premature skin aging and damage by reducing radical oxygen species (ROS), inhibits ROS formation and down-regulate ultra-violet (UV) induced skin damage. In this study, RCR was extracted via water decoction, ethanol maceration, and Soxhlet extraction methods, then the extracts were assayed with DPPH radical antioxidant, total phenolic content (TPC) assay and quantified its antioxidant compound by high performance liquid chromatography (HPLC). RCR extraction in 95% ethanol Soxhlet extraction yielded higher crude extract than maceration at 3.42 ± 0.37% and 0.51 ± 0.01% (w/w), respectively. However, it was equivalent to water decoction and residue decoction extraction at 3.98 ± 0.29%

and 3.84 ± 0.13%, (w/w), respectively. Contrarily, Soxhlet extraction yielded higher antioxidant activity and TPC due to higher CGA and CA compounds in its crude extract than water decoction. Water decoction extraction attained high CGA compound, but poorly extracts CA. Presumably, ethanol has a higher affinity towards dissolving and extracting phenolic compounds, thereby higher yields of phenolic contents in maceration and Soxhlet extraction. Ethanolic extract with the highest antioxidant compound from Soxhlet extraction was chosen as the candidate for product development. Topical delivery is the most suitable method to deliver antioxidant rich RCR extract directly onto the skin, and this study uses drug delivery system such as non-ionic surfactant vesicles. Non-ionic surfactant vesicles or niosome enhances solubility, bioavailability, stability, delivery, sustain release and reduces side effects of its loading agent. RCR extract was entrapped in niosome vesicle in various formulations. The vesicles were characterized for RCR extract entrapment efficiency, diameter size and appearance. Formulation which yields the highest entrapment and smallest size was preferred. Niosome was prepared using Span 60, Tween 20 non-ionic surfactants and cholesterol with heating and sonication method, which is simple, fast and inexpensive. Niosome formulations condition were varied by adjusting total niosome molar, cholesterol molar ratio, hydrophilic-lipophilic balance (HLB) and RCR crude extract loading agent concentration. The highest RCR extract entrapment efficiency in niosome vesicle achieved at 56.51 ± 5.21% and 4.47 ± 1.14 μm diameter size. This study proposed that an optimum formulation for high entrapment efficiency of RCR extract and micron range size vesicle can be achieved using 900 μmol total niosome material, 1:1 ratio Tween 20 and Span 60 non-ionic surfactants, 2:1 ratio non-ionic surfactant to cholesterol, 10.7 HLB, 6 mL 10 mM PBS hydration and 0.75 mg/mL RCR extract concentration. In conclusion, future improvements and enhancement of the niosome formulation preparation with the objective of higher entrapment efficiency and smaller size are possible and should be undertaken to improve RCR extract entrapment even further.

Keywords: Roasted coffee residue, Soxhlet extraction, chlorogenic acid, caffeic acid, niosome

หัวข้อวิทยานิพนธ์	การพัฒนาไนโอโซมที่มีการกักเก็บสารสกัดจากกากกาแฟเพื่อนำไปใช้ในการชะลอวัย
ชื่อผู้เขียน	นายอาหมัด ซาฟัน มัสตาฟา
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ปีการศึกษา	2558

บทคัดย่อ

เมล็ดกาแฟคั่ว เป็นวัตถุดิบที่ถูกนำมาใช้ในกระบวนการชงกาแฟซึ่งจัดเป็นเครื่องดื่มที่มีการบริโภคกันอย่างแพร่หลายและได้รับความนิยมมากที่สุด โดยขั้นตอนการทำเครื่องดื่มกาแฟนั้น จะได้กากกาแฟซึ่งเป็นสิ่งเหลือทิ้งทางอุตสาหกรรม ซึ่งแต่ละปีจะมีกากกาแฟเหลือทิ้งจากการทำเครื่องดื่มกาแฟมากกว่าหกล้านตันต่อปีซึ่งเมื่อเกิดการย่อยสลายจะส่งผลกระทบที่เป็นอันตรายต่อสภาพแวดล้อม ดังนั้นจึงได้มีการนำกากกาแฟมาใช้ประโยชน์ในด้านต่างๆ เช่นนำมาใช้เป็นตัวดูดซับปุ๋ย อาหารสัตว์ สิ่งทดแทนทางอุตสาหกรรม และเชื้อเพลิง เมล็ดกาแฟนั้นประกอบด้วยไปด้วย คาเฟอีน กรดคาเฟอิก กรดคลอโรจินิก กรดนิโคตินิก ไตรจีโนลีน เมลานอยดิน โปรตีน ไขมัน ใยอาหาร และ แร่ธาตุ และจากการศึกษาพบว่าเครื่องดื่มกาแฟมีสารต้านอนุมูลอิสระมากกว่าเครื่องดื่มชา ดังนั้น งานวิจัยนี้จึงมีวัตถุประสงค์ในการศึกษาการใช้กากกาแฟซึ่งวัตถุดิบที่มีปริมาณมากและมีราคาถูกเป็นแหล่งของสารต้านอนุมูลอิสระ ได้แก่ กรดคาเฟอิก และ กรดคลอโรจินิก โดยสารดังกล่าวมีการรายงานว่าสามารถช่วยชะลอการชราก่อนวัยและการถูกทำลายของผิว โดยจะช่วยลดการเกิดสารอนุมูลอิสระจำพวกออกซิเจน โดยยับยั้งการสร้างออกซิเจนดังกล่าว และลดอิทธิพลของแสงอัลตราไวโอเลตที่ทำลายผิว ในการศึกษานี้กากกาแฟจะถูกสกัดด้วยน้ำ สกัดด้วยการแช่ในเอธานอล และการสกัดด้วยชุดสกัดซอห์กเลต จากนั้นสารสกัดที่ได้จะถูกวิเคราะห์หาระดับการต้านอนุมูลอิสระด้วยวิธี DPPH หาปริมาณสารประกอบ ฟีนอลิก และการวิเคราะห์หาสารต้านอนุมูลอิสระด้วยเครื่องโครมาโตกราฟีชนิดของเหลวสมรรถนะสูง จากการทดลองพบว่า การสกัดกากกาแฟด้วยชุดสกัดแบบซอห์กเล็ตด้วยเอทานอล 95 เปอร์เซ็นต์ จะได้ปริมาณของสารสกัดเท่ากับ 0.51 ± 0.01 เปอร์เซ็นต์โดยน้ำหนักต่อน้ำหนัก ซึ่งพบว่ามากกว่าการสกัดด้วยการแช่ในเอธานอล การสกัดกากกาแฟที่ต้มด้วยน้ำ และการสกัดจากกากของกากกาแฟที่ต้มด้วยน้ำ ที่มีปริมาณของสารสกัดที่ใกล้เคียงกันดังนี้

3.42 ± 0.37, 0.51 ± 0.01, 3.98 ± 0.29 และ 3.84 ±0.13 เปอร์เซ็นต์ โดยน้ำหนักต่อน้ำหนักตามลำดับ แต่เมื่อทำการศึกษาหาฤทธิ์ในการต้านอนุมูลอิสระและปริมาณสารประกอบฟีนอลิกพบว่าการสกัดด้วยชุดสกัดซอห์กเลตพบว่ามีปริมาณสารต้านอนุมูลอิสระและสารประกอบฟีนอลิกสูงกว่าสารสกัดจากกากกาแฟที่ต้มด้วยน้ำ เนื่องจากมีปริมาณของกรดคลอโรจินิก กรดคาเฟอิก สูงกว่า ทั้งนี้สารสกัดที่ได้จากกากกาแฟที่ต้มด้วยน้ำพบว่ามีกรดคลอโรจินิกในปริมาณที่สูง แต่มีปริมาณของกรดคาเฟอิกต่ำ ซึ่งเป็นผลของเอทานอลที่มีต่อการละลายและการสกัดสารฟีนอลิก ดังนั้นจึงพบสารประกอบฟีนอลิกในปริมาณที่สูง เมื่อทำการสกัดด้วยการแช่ในเอทานอลและใช้ชุดสกัดแบบซอห์กเล็ต ดังนั้นผู้วิจัยจึงเลือกวิธีการสกัดที่ใช้ชุดสกัดแบบซอห์กเล็ตที่ให้ปริมาณสารต้านอนุมูลอิสระสูงที่สุดมาใช้ในการพัฒนาเป็นผลิตภัณฑ์ การนำส่งสารสกัดผ่านผิวเป็นวิธีที่เหมาะที่สุด ซึ่งจะนำสารต้านอนุมูลอิสระจากสารสกัดกากกาแฟเข้าสู่ผิวหนังได้โดยตรง ซึ่งงานวิจัยนี้ได้ใช้ระบบการนำส่งที่เป็นวัสดุที่มีลักษณะเป็นอนุภาคของสารลดแรงตึงผิวชนิดไม่มีประจุ อนุภาคของสารลดแรงตึงผิวชนิดไม่มีประจุหรือไนโอโซมนั้นจะช่วยเพิ่มความสามารถในการละลาย การออกฤทธิ์ ความคงตัว และการนำส่ง โดยรักษาระดับการปลดปล่อยและลดการเกิดผลข้างเคียงจากสารสกัดที่ใช้ ไนโอโซมที่กักเก็บสารสกัดจากกากกาแฟที่เตรียมจากสูตรต่างๆ จะถูกนำมาศึกษาประสิทธิภาพในการกักเก็บสารสกัดขนาดอนุภาค และลักษณะปรากฏ โดยสูตรที่เหมาะสมจะเป็นสูตรที่มีการกักเก็บสูงและมีอนุภาคเล็กขนาดเล็ก ไนโอโซมนั้นถูกเตรียมจากสารลดแรงตึงผิวชนิดไม่มีขั้วซึ่ง ได้แก่ สแปน 60 และ ทวีน 20 ร่วมกับคอเลสเตอรอล ด้วยวิธีการให้ความร้อนและการสั่นด้วยคลื่นเสียงความถี่สูง ซึ่งเป็นวิธีง่ายรวดเร็วและประหยัด สูตรในการเตรียมไนโอโซมจะมีการแปรผันปริมาณไนโอโซม โดยปรับสัดส่วนโมลระหว่างสารลดแรงตึงผิวชนิดไม่มีขั้วกับคอเลสเตอรอล ค่าสัดส่วนระหว่างส่วนที่ชอบน้ำกับส่วนที่ชอบน้ำมัน และ ความเข้มข้นของสารสกัดจากกากกาแฟ จากการศึกษาพบว่า ประสิทธิภาพการกักเก็บสารสกัดจากกากกาแฟสูงสุดที่เตรียมได้ คือ 57 เปอร์เซ็นต์ ซึ่งมีขนาดของอนุภาคอยู่ระหว่าง 1 ถึง 8 ไมโครเมตร จาการศึกษาพบว่าสูตรในการเตรียมไนโอโซมที่มีประสิทธิภาพในการกักเก็บสารสกัดจากกากกาแฟสูงและมีขนาดอนุภาคที่เล็ก คือ การใช้วัสดุในการเตรียมในโอโซมทั้งสิ้น 900 ไมโครโมล ดังนี้ สัดส่วนของสารลดแรงตึงผิวชนิดไม่มีขั้วและคอเลสเตอรอลเท่ากับ 2 ต่อ 1 โดยที่สัดส่วนของสารลดแรงตึงผิวชนิดไม่มีขั้ว (ทวีน 20 และ แสปน 60) เท่ากับ 1 ต่อ 1 มีค่าสัดส่วนระหว่างส่วนที่ชอบน้ำกับส่วนที่ชอบน้ำมันเท่ากับ 10.7 โดยใช้ฟอสเฟตบัฟเฟอร์ซาลีนเข้มข้น 10 มิลลิโมลาร์ ปริมาณ 6 มิลลิลิตร เป็นสารเติมน้ำในกระบวนการเตรียมไนโอโซมและใช้สารสกัดจากกากกาแฟเข้มข้น 0.75 มิลลิกรัมต่อมิลลิลิตร โดยสรุปการปรับปรุงสูตรการเตรียมไนโอโซมในอนาคต จะมุ่งเน้นที่การเตรียมไนโอโซมที่มีประสิทธิภาพในการกักเก็บสารสกัดจากกากกาแฟสูง และอนุภาคสำหรับกักเก็บมีขนาดเล็ก

คำสำคัญ: กากกาแฟ การสกัดแบบซอห์กเลต กรดคลอโรจินิก กรดคาเฟอิก ไนโอโซม

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Mr. Ahmad Zakwan Mustafa

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms/Meaning
RCR	Roasted coffee residue
CGA	Chlorogenic Acid
CAF	Caffeine
CA	Caffeic Acid
QA	Quinic Acid
HOM	Hydroxymethyl
ROS	Radical oxygen species
UV	Ultraviolet
ICO	International Coffee Organization
DDS	Drug delivery system
HLB	Hydrophilic Lipophilic Balance
TC	Critical temperature
RT	Room temperature
SPSS	Statistical Package for Social Sciences
SD	Standard deviation
ANOVA	Analysis of variance
HPLC	High performance liquid chromatography
CPP	Critical packaging factor
DLS	Dynamic light scattering
ELS	Electrophoretic light scatter
PDI	Poly-dispersity index
EE	Entrapment efficiency

ZP	Zeta potential
TEM	Transmission electron microscope
SEM	Scanning electron microscope
PEG	Poly-ethyl glycol
XG	Xanthan gum
NG	Niosome-gel
DPPH	2,2-diphenyl-1-picrylhydrazyl
GA	Gallic acid
FCR	Folin-Ciocalteu reagent
WD	Direct water decoction
RD	EM residue water decoction
EM	Ethanol maceration
nm	nanometer
LOD	Limit of detection
LD	Laser diffraction
SUV	Small unilamellar vesicles
LUV	large unilamellar vesicles

CHAPTER 1

INTRODUCTION

1.1. Problem and statement

Roasted coffee bean is one of the most traded commodity in the world, amounting to 90 billion USD industry yearly (1,2), it is claimed as the second most traded commodity after oil, which are among the highly demanded goods. Roasted coffee bean is used to brew coffee – the most popular and highly consumed beverage (3-6). Coffee industry is an industry which is huge and still actively growing year by year at an exponential rate since the last three centuries after its discovery (4). Its prospect in the future is advantageous as concern on sustainable living become more important. In sustainable living, mankind strives to utilize resource more efficiently, reduce wastage, preserve and nurture green life abundantly.

Coffee bean contains caffeine (CAF), caffeic acid (CA), chlorogenic acid (CGA), nicotinic acid (NA), trigonelline, melanoidins, protein, lipids, fiber and minerals at slightly varying concentration depending on the variety of the bean, harvesting, roasting, and brewing techniques (4). However, among all the bioactive compounds available, roasted coffee ground and green coffee bean are especially well known and rich with CAF (3). CAF is an alkaloid, it is a psychoactive drug that stimulates brain activity, enhances concentration and affects various physical and mental state of human (6). In a cup of coffee, its bitter taste and aromatic smell is the effect of CAF and volatile compounds extracted. A stronger and bitter coffee taste is observed in *Robusta*, whereas *Arabica* coffee taste is sweeter and richer (6). Besides CAF, per cup of coffee also exhibits antioxidant activity, which is higher than tea (7). Chlorogenic acid (CGA), caffeic acid (CA) and quinic acid (QA) are highly active phenolic compounds (8,9), these compounds and also hydroxymethyl shows antioxidant activity and could be useful for radical oxidization stress and other antioxidant applications (10,11).

Once roasted coffee bean is brewed, it produces coffee and leftover, the leftover is generally known as roasted coffee residue (RCR). It is estimated that six million ton RCR were generated from seven million ton of roasted coffee bean

consumed worldwide, annually (12-14). RCR has no significant value, thus it is mostly discarded as domestic waste. Its environment impact however is minimal and manageable, as it naturally decomposed within a year or so. Consequently, during that long period, the residue will amass landfills, clogs drainage, pollute environment and creates an eyesore sight. To overcome that, RCR was repurposed into adsorbent, fertilizer, animal feed, industrial substitute and even fuel source (4). Nevertheless, RCR is a material with huge potential for value added benefits in the long chain of coffee processing industry. It is also easily available ubiquitously at most coffee shops at zero cost, at any time of the year and it is potentially an alternative source of antioxidant.

As the main bioactive compounds were already extracted from roasted coffee ground during coffee brewing, other compounds such as CA and trigonelline still remains in RCR with some traces of CAF, CGA, NA and hydroxymethyl (15). Upon RCR extraction, the remaining bioactive compounds could be obtained, identified and assayed for further possible purpose. Several of the bioactive compounds in RCR extract such as CA and CGA have potential antioxidant scavenging property (9,16-18). Antioxidant is a good bioactive compound which is capable to inhibit oxidation activity by reducing free radical agent and consequently preventing it from causing cell damages, especially skin cells. Antioxidant agent mechanism of action to protect skin cells not only by reducing free radical oxygen species (ROS), but also inhibits ROS formation, down-regulate ultra-violet (UV) induced skin damage, pigmentation, wrinkles that leads to skin aging and skin cancer (19-21). The agent can be developed and added into various products from food, supplements, drugs and even also in cosmetics to improve the product and provide additional health benefits.

In view of that, this study is interested to utilize RCR as a sustainable and economical source of antioxidant active compound from coffee such as caffeic acid (CA) and CGA to add value to an otherwise RCR laid to waste. For cosmetic application, RCR extract antioxidant agent is natural and safe to be use. The advantages of this source of antioxidant is the agent is environmentally friendly as it is obtained from coffee residue, it adds value to coffee residue and freely available with infinite supply at any time of the year. Niosome is one of many nanocarrier of drug delivery system which able to entraps and enhances agent delivery by

improving agent solubility, bioavailability, stability, permeation, prolong release and reduce side effects (22). Topical application is the best approach to deliver an antioxidant agent directly onto the skin, drug delivery such as vesicles delivery system with non-ionic surfactant polymer enhances agent's solubility, bioavailability and stability, it also improves delivery by eases transdermal adsorption, sustained release and reduced side effects.

This study uses RCR obtained from Hom Krun coffee shop, a local coffee shop for extraction and identification of its antioxidant compound. The use of RCR represent the support and push for creating a sustainable and economical source of an important bioactive compound. This approach also will subsequently upgrade RCR value of an otherwise waste. RCR was extracted and compared among several extraction methods and solvent systems conditions. RCR extracts were compared for crude extract yield, antioxidant activity, 50% inhibition concentration (IC_{50}), total phenolic content, identification and quantification of CA, CAF and CGA compounds yields. CA, CAF and CGA identification and quantification were performed on modification of an established HPLC method. Then the extracts were evaluated and selected for the most optimum extraction method that yields the most bioactive compounds based on high antioxidant activity and total phenolic content.

This study aims to back information on the effects of water and ethanol solvent extraction to the active compounds yield and suggest ways to improve its yield. A modified HPLC method was designed for improved simplicity, speed and detection resolution of CA, CAF and CGA quantification. The dynamics of each CA, CGA and CAF bioactive compounds quantity towards its antioxidant activity and total phenolic content was scrutinized and elaborated. The results supported other studies on effects of water and ethanol solvent in activity and active compounds yields, which were due to higher yield of CGA and CA in water and 95% ethanol (v/v), respectively.

1.2. Objectives

The aim of this research is to extract RCR and obtain an extract with the highest antioxidant activity and total phenolic content. RCR extract is then isolated, characterized and quantified for its bioactive compounds such as CA, CGA and CAF through HPLC method. Subsequently, selected RCR extract is entrapped in various niosome preparation and evaluated for its appearance, size and entrapment efficiency. The niosome preparation was aimed for the highest entrapment efficiency and smallest size property for improved RCR amount delivered and improved bioavailability.

1.3. Scope of study

This study covered two scopes of works, in the first scope, the study performed an extraction method on the RCR samples obtain its crude extract. Then, the extracts were identified for antioxidant activity and total phenolic content, followed by separation, identification and quantification of several known bioactive compounds, such as CA, CGA and CAF. The extract with the most active antioxidant activity and total phenol content was established its relation to the quantity of its bioactive compounds identified for selection. The extracts were used as the candidate agent for the next scope.

The second scope of the study involves the preparation of niosome vesicles for entrapment of the selected RCR extract. Niosome were prepared using Tween 20 and Span 60 non-ionic surfactants and cholesterol as the main materials. It was formulated with various formulation conditions such as the effect total niosome molar, cholesterol molar, HLB ratio and loading agent concentration. Niosome preparation obtained was characterized for visual appearance, entrapment efficiency and size. Niosome entrapped RCR extract preparation with the highest entrapment efficiency and smallest size was selected as the most suitable formulation.

1.4. Benefits

This study provides an additional knowledge and information to an already know-how and widely performed RCR extraction method. A simple RCR extraction method for high antioxidant active compound was laid out for comparison with previous methods. In line with that, this study also shed some additional information on which bioactive compounds contributed the most to higher antioxidant activity, and the dynamics and effects involves in the extraction method to yields the compounds. The study also developed a modified HPLC system for the separation of RCR known bioactive compounds such as CA, CGA and CAF with simplicity, speed and accuracy. RCR, extracted with the method proposed in the study benefits from a higher yield of antioxidant active compounds, and for other applications.

Furthermore, in this research, for the first time ever, the RCR crude extract was developed for entrapment into niosome vesicles to improve its protection, delivery, bioavailability, storage, uptake and reduce possible side effects. This new niosome vesicle development formulation specifically focused for the entrapment of RCR extract provided an informative knowledge and insight to the potential of such possibility. In this study, various niosome formulation were performed and studied for its effect on its appearance, entrapment efficiency and size. The effects obtained gives a lot of information and benefitted many other researchers in the planning for further improvement of the niosome preparation. Moreover, RCR entrapped niosome obtained aided its delivery, bioavailability and stability in its application.

CHAPTER 2
REVIEW OF LITERATURE

2.1. Coffee

2.1.1. Introduction to Coffee

Historically, coffee was first found in Kaffa, Ethiopia and then cultivated in Yemen by the Arabs in the early thirtieth century. Nowadays, after more than eight centuries (4), coffee industry is still growing and becoming more popular as ever with total consumption topping seven million ton each year (12). Out of seven million of roasted coffee bean which has been consumed annually only 20 percent of roasted coffee beans were brewed into coffee drinks leaving behind six million ton of dried roasted coffee residue (RCR) as domestic waste heading to landfills everywhere in the world. Residual organic materials which still present in RCR pose a threat to the environment such as green house effect (12). This situation creates a continuous cycle of waste production and environmental impact which could became a catastrophe in the near future. However, the risk may present an advantage if handled well using biotechnological know-how to reutilize the waste into a valuable resource.

2.1.2. Coffee bean species

Coffee originates and produced from coffee berry fruit, it is highly claimed as the second most traded commodity after petroleum in the world by some economic reviewers (5,23). Under the genus of *Coffea*, there are over 500 *Coffea* species available (3,14), but only two species which are the most cultivated and produced. They are *Coffea arabica* and *Coffea robusta* at 70 and 30 percent of total world production respectively. *C. arabica* is generally more favorable due to its rich aroma, taste and value compared to *C. robusta*. They were cultivated mostly at topical climate areas (4) as it grows well in the countries which lays along the topical climates areas at 1000 meters altitude (12). Two of the largest coffee producers in

the world are Brazil and Vietnam at 32.4 and 17.9 percent market share, respectively (3,14).

Figure 1: Distributon of *Coffea robusta* and *Coffea arabica* cultivation

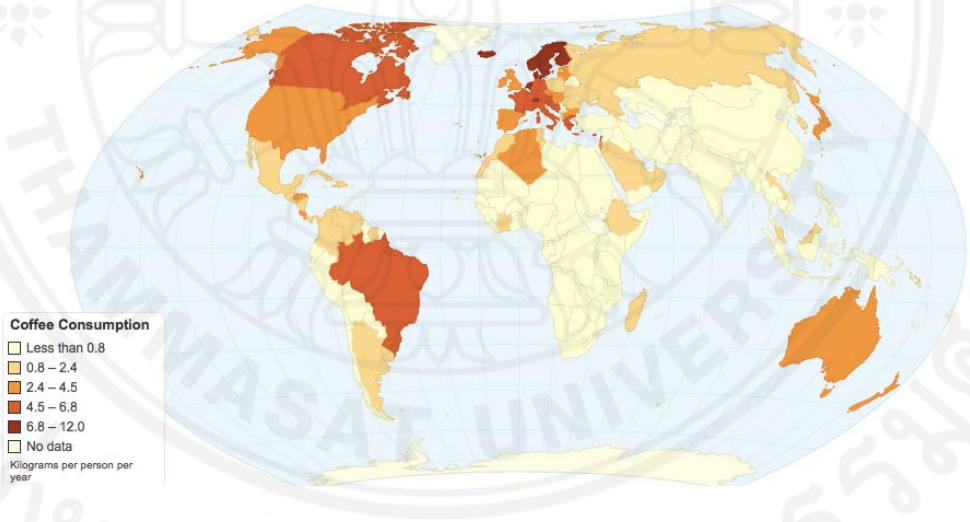


Figure 1: Distribution of coffee consumption worldwide in 2014 (24)

2.2. Coffee benefits

Coffee is preferred due to its unique aroma and taste compared to other beverages. Its aroma and taste were developed from volatile compounds and caffeine, as a result of complex coffee bean roasting process. Different brewing method, varieties of coffee mixture and blends with other flavor keep consumers taste buds afresh with unique taste and flavors. A recent report from large study associates coffee drinking to the reduced risk of premature death due to heart disease, stroke, diabetes, neurological disease and suicide, among many others (25-27). But besides of huge and wide range of such benefits, drinking coffee has been largely a favorite beverage to be enjoyed at anytime of the day and a culture (28).

2.2.1. Caffeine

The most well-known and important active compound in coffee is caffeine (CAF) (3), it is mostly sought for its stimulant property, which has paradoxical claims ranging from beneficial to harmful. CAF is a natural alkaloid served as natural plant insecticide (29), but for human, it is the most common, natural psychoactive drug consumed. Moderate dose of caffeine provides stimulant, energy, concentration and invigorate. However, its overdose consumption causes nausea, nervousness, seizure and genetic mutation (30). Also, some study suggested an *in vivo* antioxidant activity such as radical oxygen species (ROS) scavenging (12).

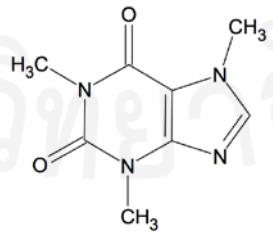


Figure 1: CAF molecule structure, 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione

2.2.2. Antioxidant

Other property in coffee drinks that attracts consumer is its claimed health benefits such as high antioxidant property compared to tea and other beverages (31). Antioxidant property of coffee drink is mostly contributed by coffee's phenolic compounds, which is mostly secondary metabolites compounds produced by higher plants for defense against insects and other metabolic functions. Bioactive phenolic compounds inside coffee drink which shows high and active antioxidant property are identified as chlorogenic acid (CGA), caffeic acid (CA), hydroxymethyl (HOM) (6,15) and melanoidins (brown pigments). Most phenolic compounds were extracted during coffee bean brewing, leaving behind trace amount of the remaining phenolic compounds in RCR. These phenolic compounds show active radical scavenging activity in antioxidant assay against standard (15).

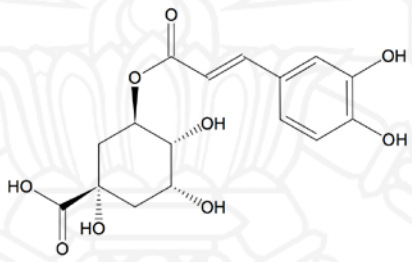


Figure 2: Chlorogenic acid (CGA) molecule structure, (1S, 3R, 4R)-3-{[(2E)-3-(3,4-dihydroxylphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxylcyclohexanecarboxylic acid.

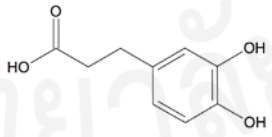


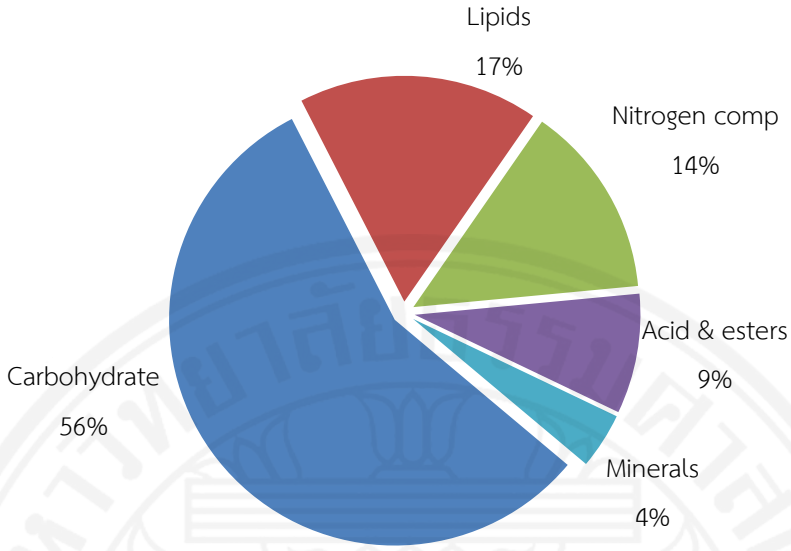
Figure 3: Caffeic acid (CA) molecule structure, 3,4-dihydroxy-cinnamic acid.

2.3. Roasted coffee residue (RCR)

Roasted coffee bean is rich with CAF (6,12,32), an alkaloid psychoactive drug that stimulates brain activity, enhances concentration, affects various physical and mental state of human (6,29). Other compounds such as caffeic acid (CA) and trigonelline remains in RCR along with traces of CAF, chlorogenic acid (CGA), nicotinic acid (NA) and hydroxymethyl (HOM) (6,11,12,15,18,33-35). RCR extraction could yield these compounds, identified and assayed for further possible purpose. HOM and phenolic compounds in RCR, such as CGA and CA contributes 65 to 70% antioxidant activity (4), potent for radical oxidization stress, free radical protection, food preservation, stabilizer, skin care and other antioxidant applications (10,11). CA reportedly reduce and inhibits ROS formation and down-regulate ultra-violet (UV) induced skin damage, pigmentation and wrinkles which all leads to skin aging and cancer. Coffee is richer in phenolic compounds than other beverages such as tea (7,31,36). Among carrot, grapes, apple and kiwi peels fruit wastes, RCR has more phenolic content (11,37)

80 percent of the roasted coffee bean turns into RCR after brewed for coffee drink. It amounts to 6 million tons of RCR dry weight produced annually in the world, which is equal to one great pyramid filling up the landfills every year (16). This poses an environmental threat due to its massive amount and unpleasant odor from degradation process. Conveniently, RCR is naturally biodegradable, its decomposition is quick and takes about a year depends on the content of polysaccharide in the residue (4). However, instead of laying the residue on the landfill, the economic benefits and potential of the residue could be maximized with the use of biotechnology methods.

Composition of 100g Roasted *Coffea arabica* (Farah, *et al.,* 2012)



2.3.1. Carbohydrate

At 56% (w/w), carbohydrate and fiber are the main component of roasted coffee bean, which is more than half of its total weight. The component consists of 41% polysaccharide, 9% sucrose, 3% lignin and 3% pectin. Plant polysaccharide, which is cellulose is a natural polymer and makes up about 35 percent of coffee dry weight. Comparatively, the composition of cellulose in cotton and wood are 90 and 45 percent, respectively (6). Cellulose is the main component in the production of materials in paper making industry, which usually uses cellulose from pulp and cotton, RCR cellulose could be used as an additional substitute to the main component. In renewable energy sector, cellulose and lignin is used as combusting material for energy production, cellulose can also be converted into biofuel using fermentation technology as substrate. Beside cellulose, coffee bean also composed of high organic matter, nitrogen and carbon/nitrogen ratio, which is an attractive property for natural fertilizer. The presence of high carbon/nitrogen ratio composition was the result of extensive roasting process of coffee bean with amino acid and sugar (6).

2.3.2. Bioactive compounds

Beside the possibility of direct usage of RCR's polysaccharide and its organic matter in its solid form, RCR also harbor some trace amount of bioactive compounds such as caffeic acid, chlorogenic acids, melanoidins and volatile compounds. These bioactive compounds are potentially to be extracted with a suitable and optimized extraction condition and solvent system. The knowledge to recover trace amount to bioactive compounds available in RCR was recently discovered, reported and potentially promising for improvement and enhancement. Besides, this provides an additional value added benefits to RCR, which is otherwise a valueless waste. The presence of bioactive compound which shows antioxidant activity is valuable and potentially to be utilize in many biotechnology applications such as in food, drink, cosmetic, medicine, supplement and materials. One of many properties of an antioxidant is its protection to free radical damage. Free radical damage is a serious problem in food industry as it is the cause of food damage, in cosmetic industry it is as the main active agent of skin health and for health it can be taken as supplement, food and beverage additives and other applications.

2.4. Roasted coffee residue (RCR) extraction

Previous study has performed a comparison of different type extraction solvent ranging from low to very polar such as hexane, water, methanol and ethanol with aim for the highest RCR crude extract yield reported by Yen *et. al.,* (15). The study found that water extract yields a suitable amount of crude extract and subsequently used for the rest of investigation to determine its antioxidant activity, total phenolic content (TPC), flavonoid content, browning index, and HPLC analysis. Among the extraction solvent compared, hexane and water extract yield the highest crude extract. However, the content of the crude hexane extract yield was mainly lipids instead of antioxidant compounds as shown by its poor performance in liposome antioxidant activity assay. Meanwhile, methanol, water and ethanol extracts shows higher antioxidant activity than hexane extract. This is because hexane is a non-polar organic solvent, and thus it is not very effective in extracting polar compounds such as phenolic compounds. Instead, hexane is good at

extraction of non polar compounds such as triglyceride, glycerol, terpene, esters, sterols, fatty acids and lipids which are known to be the compounds with the highest content in coffee bean and RCR. For the lack of reports on RCR antioxidant assay compared to roasted coffee bean is the motivation the researcher to perform the study. For this purpose, an array of antioxidant assay such as liposome oxidation, DPPH free radical scavenging, protein oxidation, reducing activity and chelating activity were performed on RCR water extract. It shows a strong radical scavenging activity, 50% protective effect in protein oxidation, but poor iron reducing ability and meager iron chelating activity. Furthermore, water extract of RCR was compared against roasted coffee bean, and its active compounds were quantified by HPLC. Phenolic and non-phenolic active compounds which has been identified are trigonelline (TG), nicotinic acid (NA), 5-(hydroxymethyl)furfuraldehyde (HOM), chlorogenic acid (CGA), caffeine (CAF) and caffeic acid (CA). Most of the compound identified in RCR shows 99 to 80 percent of reduction from roasted coffee bean, except for CA and TG. Standard compounds were assessed against RCR water extract in lipid oxidation system. The study concludes that RCR crude water extract has higher lipid oxidation activity against pure standard compounds due to the effect synergism among many bioactive compounds in the crude extract. The study identifies that HOM and CGA isolates are the compounds with the most active antioxidant activity and the main contributor to the crude extract activity in the lipid oxidation system.

Feasibility of extracting phenolic compounds from RCR was explored by Zuorro and Lavecchia using RCR obtained from coffee bars and coffee capsules (11). The study utilized organic solvent due to its environmental friendly and cost-effective benefits in the extraction process, total phenolic content (TPC) from coffee capsules RCR and coffee bars RCR collected across Rome was quantified. Additionally, the study also performs an energy potential assessment on the RCR samples before and after its extraction process with aim to identify its potential to be recycled into other innovative purposes. TPC quantification of RCR extraction shows up to 90 percent of phenolic compounds was able to be extracted in both ethanol extract. Optimal extraction condition at 50°C heating, 120-minute extraction, 40 mL/grams solvent to sample ratio yielded the highest TPC of coffee bar and

coffee capsules at 17.09 and 19.98 mg GAE/g RCR sample respectively. Furthermore, the study found that RCR extraction residue has the potential to be utilized as heating purpose due to its significantly high colorific value determination.

Panusa *et. al*., have also prepared an extraction of RCR acquired across bars in Rome and coffee capsules. RCR form that two sources were extracted in pure water and 60% ethanol, and their extract was characterized and compared for TPC, AO, flavonoid and active compounds such as CGA and CAF to observe the effect of different extraction method and source of RCR. The study also developed an ultra high performance liquid chromatography-photodiode array-time-of-flight-mass spectrometer (ULHPLC-PDA-TOF-MS) characterization protocol for CGA and CAF in RCR extract. CAF compound was possessed in prominent quantity in both extract followed by CGA and lastly CA in the least quantity. Appallingly, CA content was very low which its quantity that it was found below the level of quantification (12).

Succeeding the study above, Zourro and Lavecchi embarked on a systematic investigation to establish the optimum process parameters which influence the yield of phenolic compounds in RCR extract (37). The study focused on the parameters of ethanol solvent composition, extraction temperature, extraction time and RCR sample to solvent volume ratio. At predefined 40°C, 105-minute and 30 mL/g extraction temperature, time and sample to solvent ratio respectively, ethanol solvent composition was determined at 60% as the most optimum composition which yield the highest RCR crude extract. Subsequently, the study proceeds to determine the most optimum extraction temperature, extraction time and sample to solvent ration using 3^3 full factorial experimental design. The optimum extraction conditions were determined at 60°C, 180-minutes and 50mL/g extraction temperature, time and sample to solvent ratio respectively which yields the highest percentage of extracted phenolic from the initial amount of phenolic in the RCR. At 18.75 mg GAE per gram sample, the yield obtained was in the ranges of other study and higher than other agro-industrial wastages such as grape pulp, carrot, kiwi and apple peels.

Comparison of extraction solvent found that hexane, a non-polar solvent ideal for non polar compounds extraction, lipids. Meanwhile, polar solvent extracts

phenolic compounds better. Water extract show strong radical scavenging activity, 50% protective effect in protein oxidation, but meager iron chelating and poor iron reducing ability. Up to 99% phenolic and non-phenolic compounds were lost in coffee brews, except CA and trigonelline. Crude water extract has better activity than pure compounds due to synergy among compounds (15). At 50°C, 120 minute, 40 mL/g solvent to sample ratio, high total phenol content were able to be extracted from RCR coffee bars and capsules, yielding 17.09 and 19.98 mg GAE/g total phenolic content of coffee bar and coffee capsules RCR, respectively (11). Water and 60% ethanol (v/v) extracts were reportedly shows equal quantity of non-phenolic compound CAF and phenolic compound CGA. CAF dominates the composition followed by CGA and CA at below the limits of ultra high performance liquid chromatography (UHPLC) quantification (12). Optimized RCR extraction was reported at 40°C, 105-minute, 30 mL/g RCR and 60% ethanol for the highest crude extract. High total phenolic content was achieved by increasing temperature, time and sample ratio (37).

Table 1: Review of previous RCR extraction conditions best conditions (12,15,37-39)

Author	Extraction method	Yields
Yen, 2005	1:10 g/mL, water decoction, 5 min	Crude extract; 5.78 % w/w Antioxidant, 0.2 mg/mL TPC; 2.04, 2.16 mg GAE/g
Bravo, 2013	24 g in 400 mL, water decoction	TPC; 13.94 mg GAE/g Antioxidant 82.40 µmolTrolox/g
Mussatto,, 2011	1:40 g/mL, 60% methanol, 90 min, solid-liquid extraction	TPC; 16 mg GAE/g Antioxidant: 0.1 mM Fe(II)/g
Panusa, 2013	2g in 100 mL 60% ethanol, 30 min 60°C heat macerate,	TPC; 28.62 mg/GAE/g Antioxidant, EC_{50}; 1.47 % v/v
Zuorro, 2013	1g in 50 mL, 180 min 60°C heat macerate	TPC; 18.75 mg GAE/g Antioxidant, EC_{50}; 0.86 % v/v

2.5. RCR extract bioactive compound identification

Every 100 grams roasted coffee bean contains 56% carbohydrate, 17 percent lipids, 14% nitrogenous compound 9 percent acid and esters and 4 percent minerals(6). In a cup of coffee beverage, some of important bioactive compounds were extracted in brewing process such as CAF, CGA, CA and NA. Then, traces amount of these bioactive compounds can be further extracted and identified in RCR extract with a suitable extraction system. For example, a RCR extracted with hexane yields mostly lipids and oil compounds (15). Meanwhile, polar organic solvent such as methanol, ethanol and water yields mostly polar compounds such as phenol, nitrogenous compound and traces of oil (5,12,39). The compound of interest in this study are bioactive compounds such antioxidant and phenolic compound. Thus, these compounds can be determined by antioxidant radical scavenging and total phenolic content (TPC) assay.

2.5.1. Antioxidant assay

The compound of interest in RCR in this study are the valuable bioactive compounds such as antioxidant and phenolic compound. Other study has highlighted that it contains the highest phenolic contents than other fruit wastes (11,37). Antioxidant assay is the most common assay to identify antioxidant active compounds. Several antioxidant assays based on the principle of free radical, lipid oxidation, protein oxidation, reducing ability, chelating activity, electron spinning and more are available to be utilized (3,15,31,40-46).

Antioxidant assay assess the capability of the biologically active compound to reduce or scavenge free artificial radicals into its normal state. Free radicals are dangerous as it can weaken, injure and damage healthy cells and also it’s DNA causing systematic degradation of its vigor and active state. Antioxidant activity can be assessed using several standard antioxidant activity assay such as radical scavenging method with 2,2-diphenyl-1- picrylhydrazyl (DPPH) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), or via oxygen radical absorbance capacity (ORAC) or ferric reducing ability of plasma (FRAP) methods.

Antioxidant activity assay with DPPH method is based on the determination of free radical scavenger concentration, DPPH free radical after its reaction with antioxidant agent in RCR extract DPPH was selected because of its sensitivity, stability, economical and suitability for assaying an extract's active agent. DPPH is sensitive as its radical and stable forms are detectable with visible light spectrophotometry at 320 and 517 nm wavelengths respectively. DPPH is a popular and most widely used method to determine natural extracts antioxidant activity in more than 800 studies since 1969 (40,45-49). Selection of a suitable and sensitive antioxidant assay is importance to ensure the credibility and accuracy of the antioxidant assay obtained.

2.5.2. Total phenolic content (TPC) assay

Phenolic compound is found in most vegetables, fruits, plants and also including coffee berry. It is claimed as an antioxidant potent compound due to presence of a hydroxyl group which has reducing ability. Phenolic compounds are found in varied classes and structure, but they are characteristically similar in the polyphenol rings which is an aromatic benzene and hydroxyl group. The ring can be found in many complex and bigger compounds and classified as phenolic compound. Phenolic compound can be found mostly as flavonoids and phenol with ester linkages. Chlorogenic acids, chicoric acids and its derivatives are phenolic compounds with ester linkages, it is found in coffee beans, blueberries and tomato (10).

The most common, simple, easy and standard phenolic compound quantification assay is based on the colorimetric method such as Folin-Ciocalteu reaction (50). However, the assay reaction is not specific to phenolic compounds as it may react with any reducing substances, thus may reducing the sensitivity of the assay to detect potential phenolic compounds in RCR.

2.5.3. RCR extract high performance liquid chromatography (HPLC) analysis

HPLC method is an analytical method to separate, identify and quantify the available compounds in RCR extract. HPLC separation and analysis of RCR extract was based on the reverse phase-HPLC screening method for flavonoids and phenolic compounds commonly used in plant extracts. Earlier separation and analysis of coffee berry uses a more orthodox method such as thin-layer chromatography (TLC), which envisage the study by several researchers to establish a HPLC method for coffee separation and analysis of its many compounds. HPLC analysis for berries reported by Hakkinen (1998) and adopted in other studies for coffee bean summarized (Table 2).

Table 2: Overview of HPLC condition for RCR separation and analysis (15,23,51)

Author	**Yen, 2005**	**Madhava, 2008**	**Stalmach, 2015**
Purpose	Flavonoid and phenol identification	CGA identification	CGA lactones identification
Instrument	HITACHI, HPLC	Waters HPLC	Surveyor gradient HPLC
Stationary phase	Lischosorb RP 18 (10 x 4 mm, 5µm)	Hypersil C18 (250 x 4.6 mm, 5µm)	Polar RP 80A (250 x 4.6 mm, 4µm)
Mobile phase	A: 50mM ammonium dihydrogen phosphate, pH 2.6 B: 0.2mM 0-phosphoric acid, pH 1.5 C: 20% A in 80% acetonitrile	A: 5% acetonitrile in 0.04% trifluoroacetic acid B: 80% acetonitrile in 0.03% trifuoroacetic acid	A: 1% formic acid in acetonitrile
Elution type	Gradient	Gradient	Gradient
Temp. (°C)	25	30	40
Flow rate (mL/min)	1.0	1.0	1.0
Detection	UV at 280nm	UV at 325nm	UV at 325nm
Retention time (min)	30	20	60

2.6. Introduction to niosome

Niosome was invented due to several biological limitations infested by the earlier vesicular system, which is liposome. Liposome is a good vesicle delivery system as it is a natural polymer thus biodegradable, biocompatible and non-toxic. It has been used to entrap various agents such as antioxidant agents, proteins and others for drug deliveries such as oral and dermal with considerable success (52-55). However, the weaknesses of liposome or phospholipid is that it is highly unstable because of its low physical and chemical stability in aqueous suspension leading to limited shelf life and prone to rancidity (56). On the contrary, non-ionic surfactant is more stable than phospholipids because the surfactant is charge free thus more stable than phospholipid, niosome also formed by additional low heat and shaking to induce the forming of self-assembly vesicle. Cost-wise, non-ionic surfactant is cheaper than phospholipid, because of its wide availability as it is produced in large amount for domestic detergents and cleaners (57-62).

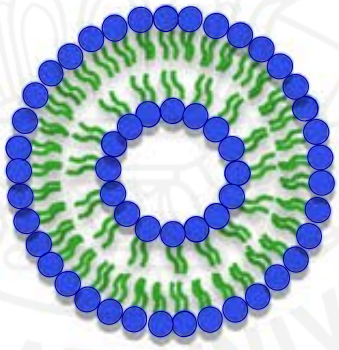


Figure 4: Niosome structure

Table 3: Overview of some selected niosome preparation for agent improvement (67,69,71,78)

Author	**Coviello, 2015**	**Budhiraja, 2014**	**Vyas, 2011**	**Mahosroi, 2011**
Agent	MonoammoniumGlycyrihizinate	Rosemarinic acid	Benzoyl Peroxide	Gallic acid
Surfactant	Tween 20, Tween 85	Span 85	Span 80	Tween 61
Method	Thin film hydration	Reverse phase evaporation	Thin film hydration	Thin film hydration
Entrapment	< 50%	65%	83%	55%
Size	105 – 110 nm	814 nm	4.6 nm	115 nm
Releasing	100% after 24 hours	50% after 24 hour	32% after 12 hour	41% after 12 hour
Shelf life	1 year	-	-	60% after 3 month
Improvement	Prolonged release	Prolonged release	Prolonged release High entrapment	Prolong release Storage stability

In its earlier application, niosome was intended to be use for the improvement of topical cosmeticeutical products such as in cream, lotion and gel formulations (63-72). Within time, niosome many advantages have found its way into medicinal application via pharmaceuticals development of therapeutic drugs delivery (59,73-76). This development arises due to the challenges of conventional therapeutic drug delivery such as low bioavailability, side effects, poor uptake and short acting. By applying niosome drug delivery system (DDS), the drug agent delivery is improved physically and biologically. Niosome DDS, depends on the type of drug agent, improve drug physical property such as solubility and stability for delivery. Its biological property is improved by niosome DDS by increasing the chances of bioavailability, permeation and release for prolonged effect (58,59,61,62,77).

2.7. Niosome building blocks

Niosome building block materials is consisted of non-ionic surfactants as the main materials and also extras such as additives, stabilizers, surface identifier, surface charge modifiers and surface targeting agent materials. Since the last 50 years,

surfactant production industry has been growing and getting bigger (79). Nowadays, surfactant can be synthetically prepared and produced in tons for various household, industry and scientific applications such as detergents, foaming agent, wetting agent, emulsifiers and also niosomes.

Non-ionic surfactant is an amphiphilic structure, which is derivative of lipid which consists of a long chain alcohol tail (hydrophobic) and non-charged head (hydrophilic) instead of charged heads and long chain hydrocarbon tail. The functional groups attached on the long alcohol chain determine the type of non-ionic surfactant, and some of the most well known and commonly used Spans are polyoxyethylene glycol alkyl ethers, polyoxyethylene glycol sorbital alkyl ethers and Sorbitan alkyl esters which also commercially known by their brand names as Brij, Tween and Span respectively. Span and tween are mild non-ionic surfactants, usually used in food and pharmaceuticals industries because of it is more stable, flexible for various formulation and has a wide compatibility with other compounds.

2.7.1. Span

Span is produced by dehydration of sorbitol and esterification with fatty acids forming sugar alcohol or polynol. Span or sorbitan esters is an emulsifying agent and stabilizer used in cosmeticeutical products. It is classified according to its hydrocarbon tail length, as the longer its tail is, the higher Span number is given reflecting higher lipophilicity of the tail. For example, Span 20 has a nine-carbons chain tail, followed by thirteen-carbons and fifteen-carbons tail chain is Span 40 and Span 60 respectively. Conversely, higher Span number with longer tail is more lipophilic, thus lower HLB values. Span 60, which has 4.7 HLB is mostly used as it is more stable, flexible for various formulations and has a wide range of compatibility with the agent loading. Span is also environmentally safe as it is safe to be handled, non-toxic and readily biodegradable in nature like other hydrocarbon. Like other long chain alcohol, with increasing carbon chain its critical temperature (TC) increases such as that Span 20 are liquid at room temperature (RT), Span 40 TC is 46 – 47°C and Span 60 is 56 – 58°C (80).

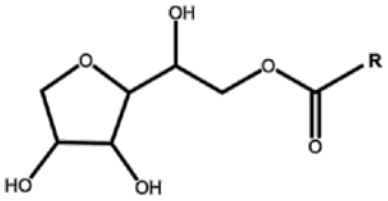
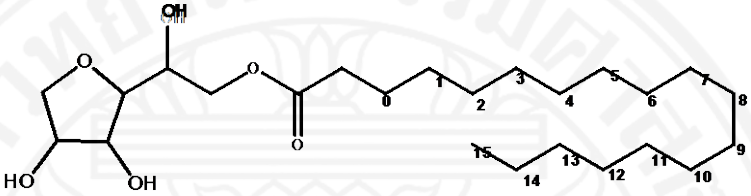


Figure 2: Basic structure of Span (sorbitan monoester)



Span 60 (R = C15)

Figure 3: Sorbitan monostearate

Table 4: Span classifications

Span classification	Hydrophilic-Lipophilic balance (HLB) value	Carbon chain
Span 20	8.6, water insoluble	C9
Span 40	6.7, partially water soluble	C13
Span 60	4.7, partially water soluble	C15
Span 80	4.3, partially water soluble	C18
Span 65	2.1, oil soluble	C15
Span 85	1.8, oil soluble, very hydrophobic, lipophilic	C11

Span 60 has been reported for in niosome preparation formulation to entrap drugs such as beclometasonedipropionate, morin hydrate, diclofenac sodium, ellagic acid, diallyl disulfide, hydroxycamptothecin, 5,6-carboxylflurescein, minoxidil, nimesulide, tenoxicam, valsartan, naltrexone, acetazolamide and many more for various route of administrations (81). The possibility to utilize Span 60 is enormous due to its high phase transition temperature, low HLB value and optimum critical

packaging factor (CPP), increasing the possible for forming a stable, flexible inverted micelle vesicle structure with uniform sizes and shapes (82).

2.7.2. Tween

Table 5: Tween classification

Tween classification	Hydrophilic-Lipophilic balance (HLB) value	Carbon chain	R
Tween 20	16.7 partial water insoluble	(w + x + y + z =C20)	Lauric acid (C12)
Tween 40	15.6, water soluble	(w + x + y = C20) + (z = R)	Palmitic acid (C16)
Tween 80	15.0, water soluble	(w + x + y = C20) + (z = $C_{17}H_{33}$) + R	Oleic acid (C18)
Tween 60	14.9, partially water soluble	(w + x + y = C20) + $C_{17}H_{35}$	Oleic acid (C18)
Tween 85	11.0,	(w = C20) + (x = 18) + (y = C18) + (z = R)	Oleic acid (C18)

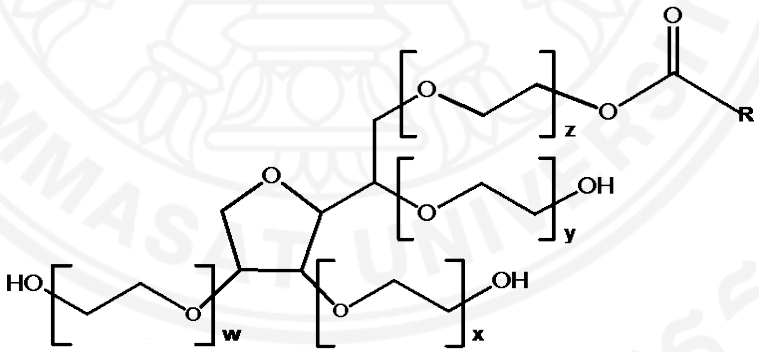


Figure 4: Basic structure of Tween (polyethoxylated monoester)

Tween, a derivatives of Span is one of the non-ionic surfactant used in the niosome preparation, it is usually added with Span to modify the combined niosome non-ionic surfactant material HLB value for better emulsification, stability and entrapment efficiency. Tween differs to Span in its function groups, and Tween is the derivative of span upon ethoxylation of sorbitol esters into polysorbate. Due to longer and more hydrophilic nature of the tail of Tween molecule, thus its

hydrophilic lipophilic balance (HLB) is higher than Span, from 10.5 to 16.7, which is Tween 65 and Tween 20 respectively.

2.7.3. Additive - cholesterol

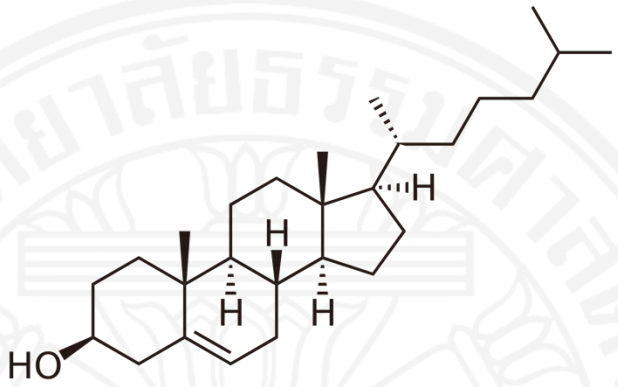


Figure 5: Cholesterol

Cholesterol is the most commonly used niosome vesicle forming additives in the preparation. It is recommended to be added especially in the niosomes prepared using less stable, hydrophilic and non-ionic surfactants with hydrophilic-lypophilic balance (HLB) value below 6. Cholesterol encourages the formation of niosome bilayer and also provided additional benefits to the niosome vesicle such as improved rigidity, strength, containment and entrapment efficiency. In a some of niosome formulation, increasing the cholesterol concentration ratio may increase the niosome entrapment efficiency and reduce drug leakage due to higher vesicle membrane rigidity. Physically, cholesterol molecules will position itself together among with the non-ionic surfactant on niosome vesicle membrane and improve its property.

Occasionally, other kind of additives are also used to improve the characteristics of niosome. They were dicetyl phosphate (DCP), phosphatidic acid (PA), stearylamine (SA) and cetylpyridinium chloride (CPC). These additives add charges on the surface of the niosome vesicles to improve its stability and prevent the formation of niosome aggregates. Particularly, DCP and PA additives functions by

adding negative charge, meanwhile SA and CPC additives adds positive charge on the surface of the niosome. The effects of the added additives are mixed and depends on many factors such as drug properties, preparation method and type of non-ionic surfactant used.

2.8. Hydrophilic-lyphophilic balance (HLB)

Table 6: HLB values and its general characteristic (79)

HLB value	Hydrophobicity-Lipophilicity characteristic	Application
20	Hydrophilic (water soluble)	Solubilizing agent
18		Detergent
15		Oil in water emulsifier
9	Dispersible in water	Wetting agent
6		Water in oil emulsifier
3		Antifoaming agent
0	Hydrophobic (oil soluble)	-

HLB value was introduced by William Griffin in the 40s to determine the characteristic nature of any given surfactant as it interacts in water or oil phase system. The value, designated in the range from 0 to 20 represents the hydrophilicity or lipophilicity of a surfactant. In theory of HLB, surfactants with HLB of 0 to 6 are oil soluble (lipophilic), whereas surfactants with HLB of 9 to 20 are water soluble (hydrophilic). Meanwhile, surfactants with HLB between 6 to 9 are water dispersible and suitable as wetting and spreading agents. These HLB value could be the rule of thumb in preparing a suitable emulsifier, however, in most cases surfactants react strangely and unpredictable (79).

HLB value and characteristic of an unknown surfactant can be easily determined experimentally or theoretically or using a simple mathematical formula proposed by William Griffin based on the composition of surfactant's saponification and acid number (79). Equation 1 as described below determines the HLB value using ester saponification number (S), and acid number (A). Meanwhile, for

surfactants with poor saponification, HLB can be alternatively calculated from oxyethelene content weight percent (E), and polyhydric alcohol content weight percent (P) (Equation 2).

$$HLB = 20\left(1 - \frac{S}{A}\right)$$

Equation 1: HLB determination formula based on saponification and acid number (79)

$$\mathrm{H}LB = \frac{E+P}{5}$$

Equation 2: Alternative HLB determination formula based on oxyethylene and polyhydric alcohol content (79,83).

(a) $$HLB = \frac{xHLB_a + (1-x)HLB_b}{100}$$

(b) $$\%A = \frac{100(x - HLB_a)}{(HLB_a - HLB_b)}$$

(c) $$\%B = 100 - \%A$$

Equation 3: Calculation of HLB value of a combined surfactants (79,83). (a) Total HLB of surfactant A and B, (b) determination of the required surfactant A% and (c) surfactant B%.

Therefore, HLB value can be adjusted to fit into the required HLB value for a stable emulsion to occur, oil in water (hydrophobic) or water in oil emulsion (hydrophilic). The optimum combined HLB value of the surfactant for the required HLB can be determined by calculating the percent ratio and HLB value of each surfactant added, using Equation 3.

2.9. Niosome preparation

Niosome vesicles have been prepared in various methods, such as using thin film hydration, ether injection, sonication, reverse phase evaporation, micro fluidization, and bubbling methods. These preparation methods are slightly varied but in principle it involves two basic steps, which is (i) the dispersion of non-ionic surfactant in its solvent and (ii) hydration of the molecules in a suitable buffer with suitable introduction of energy for forming niosome vesicles (56). Niosome is spontaneously formed under the optimized condition of molecule ratio, buffer and applied suitable amount of energy for the desired disassembly and melting the surfactants molecules into a homogenous mixture. Each preparation method has its feasibility, advantages and disadvantages associated, and those methods were reviewed here in brief.

2.9.1. Heating and sonication

Heating and sonication method is a simple, quick and safe 1-step method which involves no dangerous solvent, gas and complex procedure (56). This method mainly involves extensive heating process, coupled with stirring or sonicating. The heating step was applied to the aqueous niosome materials in the buffer to dissolve the materials and promote self-assembly of the materials when the temperature was reduced (61).

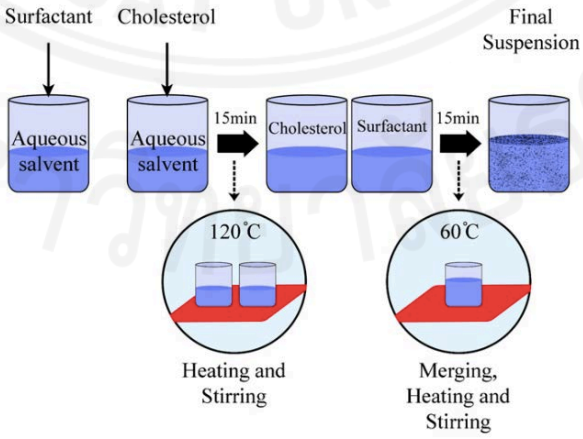


Figure 2: Thin film hydration preparation method (81)

2.9.2. Thin layer hydration method

Thin film hydration method is the most preferred and commonly used, it has been mostly reported thus far. This method is simple, easy to prepare, and economical for small or large-scale production. However, the concern about this method is its use of large amount of solvent to dissolve the surfactant and consequently requires long period of evaporation to dry and obtain thin film layer of surfactant. Meanwhile, other methods suffer from complicated steps, use of toxic solvent, use of nitrogen gas and special equipment to be carried out.

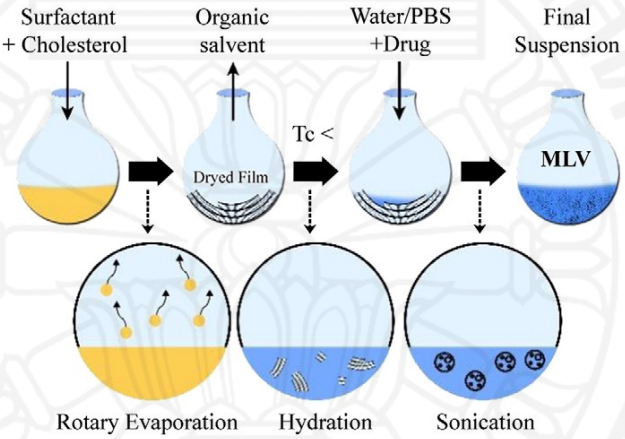


Figure 3: Thin film hydration preparation method (81)

2.9.3. Bubbling of nitrogen

Niosome vesicles prepared with nitrogen bubbling method requires no use of solvent, instead it uses an inert nitrogen gas. The homogenized niosome materials mixture were prepared in a three neck glass reactor and bubbled with an inert nitrogen gas. The method is however complex and laborious (84).

2.9.4. Ether injection

Ether injection niosome preparation method is simple, easy and yields large unilamellar (LUV) niosomes with high entrapment of drug. However, this method requires the use of diethyl ether solvent and high temperature. The preparation, in

which niosome materials in the solvent is injected slowly into an aqueous solution. Additionally, the aqueous solution must be heated above the boiling point of the solvent used to evaporate the solvent and obtain niosome in aqueous solution (84).

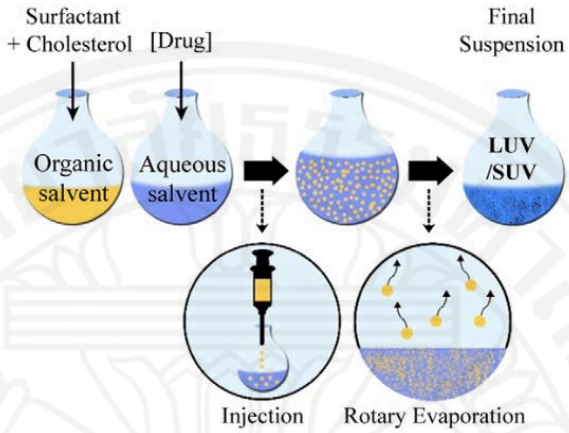


Figure 4: Ether injection method (81)

2.9.5. Reverse phase evaporation

Reverse phase evaporation method is similar with ether injection, but without the injection of diethyl ether – instead, the solvent is directly added into the aqueous solution and then later evaporated in entirely. It yields similar benefits and disadvantages as ether injection method. However, the most critical part of this method is in the step of solvent evaporation, which requires a long period of time for complete removal.

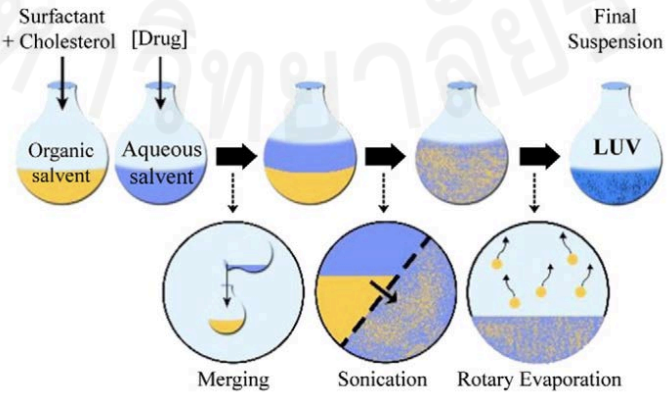


Figure 5: Reverse phase evaporation method (81)

2.9.6. Microfluidization

Microfluidization is a conventional niosome preparation method which requires no harmful organic solvent, but the process is complex and not suitable for heat sensitive drug. The preparation involves pumping niosome materials with loading agents in high pressure into a chamber at very high rate, producing small unilamellar vesicles (SUV) (84).

2.10. Niosome characterization

Niosome is characterized to obtain information about its physical, chemical properties and entrapment efficiency for selection of the best preparation method and formulation, control the quality of niosome, understand its biological behavior and has influence in its intended application performance. The main parameters of concerns when characterizing niosome preparation is size, polydispersity index (PDI), entrapment efficiency (EE), zeta potential (ZP), stability, lamellar and pH value (61).

2.10.1. Size

Niosome size characterization process involves complicated measurement and using special equipment to sense, count and measure the particles at a very small size. Dynamic light scattering (DLS) and laser diffraction (LD) are some of the methods utilized by particle sizing analyzer to measure the size and polydispersity index (PDI) of the particles. It uses Brownian motion theory and random light scattering in an aqueous medium to measure each particle size property. DLS provides particle size estimation based on the intensity of the particle to scatter light, thus concerns about turbidity, dust and aggregation may affect the measurement accuracy.

DLS analyzer size measurement has limits of detection (LOD) for particles sizes between 0.3 to 8000 nm, meanwhile LD analyzer size measurement LOD is at 10 – 50,000 nm (Figure 6). Thus, DLS particle analyzer is mostly suitable for nano-

suspension and nano-emulsion particle measurement. In addition, it also can measure zeta potential and molecular weight. On the other hand, LD analyzer are specialized for particles with sizes above nanometer ranges, such as powder, suspension and emulsions. However, it lacks zeta potential and molecular weight capabilities (85).

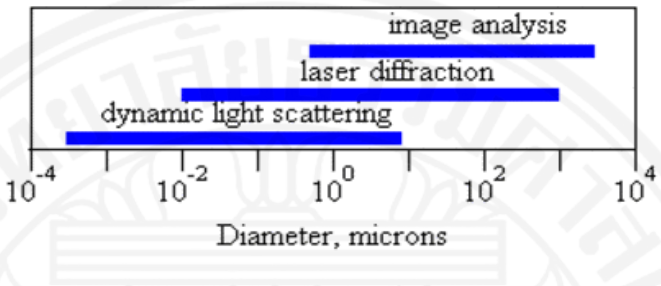


Figure 6: Limits of detection of several particle size analysis methods (85)

2.10.2. Mean, median, mode size value

Particle size measurement were reported in mode, median and mean values diameter size values. Among those values, the recommended and relevant value to be reported is in median as it gives a more accurate representation of the niosome suspension than mode or mean value. Principally, mode value reports the niosome vesicle size which are the highest among the population size. Meanwhile, mean value is the result of calculated population average value. Thus, median size value is more preferred as the value is the value of the middle of the population, reflecting the population size (85).

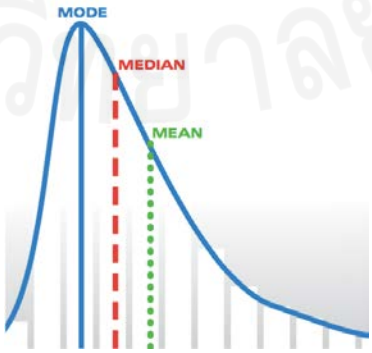


Figure 7: Histogram visualizing the differences of mode, median and mean

2.10.3. Polydispersity index (PDI)

Polydispersity index (PDI) obtained from particle size measurement describes the widths of the normal distribution graph at 2 standard deviations (±2SD). The width representing the 95% of the niosome vesicle population. High PDI values indicates the niosome vesicle size population are spread quite large between small and large vesicles, while low PDI value niosomes indicates a more uniform size and less size variation in the population which is more preferred characteristics of the niosome suspension.

2.10.4. Zeta potential

Beside size, DLS particle analyzer is also capable in measuring particle surface potential (mV), or also known as zeta potential. Zeta potential reports the electrical potential in mV of a particle surface, measured at the edge of particle surface outer layer or slipping plane. Using additional equipment to move particle based on its charge, the charge property of the particle can be measured and reported as zeta potential (mV). Particle with higher zeta potential, which has more positive value is more stable, less likely to aggregates and disperse well in an ionic suspension compared to particle with lower zeta potential value.

2.10.5. Morphology

Niosome size can also be estimated using an electron microscope such transmission electron microscope (TEM), scanning electron microscope (SEM) and others. Beside providing size, electron microscope can also provide valuable morphology characteristic information of the particle such as lamellarity, aggregation and distribution of the particle. However, electron microscopy technology is very limited and expensive to be used. Overall, information about the niosome size, polydispersity index (PDI), zeta potential, morphology and lamellarity provide better understanding of the niosome physical properties for improved application.

2.10.6. Entrapment efficiency (EE)

$$EE\% = \frac{T}{I} \times 100$$

Equation 4: Entrapment efficiency (EE) calculation (direct method)

$$EE\% = \frac{I - F}{I} \times 100$$

Equation 5: Entrapment efficiency (EE) calculation (indirect method)

EE is the characteristics of niosome vesicle measured to quantify the amount of a specific loading agent intended has been trapped inside the niosome. EE is reported as percentage by dividing the amount of agent freed or entrapped to the total amount of agent and times 100 percentage. Thus, a high EE value nearing 100 percent, indicates that higher amounts of the loading agents were entrapped and vice versa. EE percentage can be measured directly (Equation 4) or indirectly (Equation 5) (86), by estimating the initial concentration or amount of the agent used for entrapment (I), then the concentration or amount of the agent after entrapment either from the remaining agent freed from the niosome (F) or by quantifying the agent inside the niosome (T), depends on the ease and feasibility of procedure. The separation of the agent freed and not entrapped in niosome preparation (F) can be performed by dialysis (2.10.6.1), gel filtration or centrifugation methods. (61,62,69).

2.10.6.1. Dialysis

Niosomes vesicles separation from free loading agents performed via dialysis method is based on the principle of diffusion and osmosis. Due to the smaller pores of dialysis bag, niosomes vesicles are trapped inside the bag, while the free loading agent molecules can diffuse across the dialysis membrane bag from the niosome suspension into the buffer solution. Usually the buffer solutions used are normal saline, phosphate buffer, glucose or distilled water, depends on the suitability and solubility of the loading agent. Dialysis may took about 9 – 12 hours and up to an

overnight until all the amount of agents in the buffer reaches constant plateau indicating an equilibrium (77). The agents, free from niosome vesicles entrapment and diffused across the dialysis bag can be determined in the buffer solution. Meanwhile loading agents entrapped inside the niosome vesicles can be determined by lysing the vesicle with propane-1-ol or methanol. Dialysis separation has more advantage than gel filtration and centrifugation as it is better at separating smaller niosome vesicle which unable to be separated by low speed centrifugation, cheaper, simpler and easier to be performed. However, dialysis separation took longer time than centrifugation.

2.10.7. Gel filtration

Gel filtration is also a popular and versatile method to perform separation of loading agent from niosome vesicle. This method is effective in obtaining a high yield of drug separation. However, the drawback is, it is expensive as it uses commercial beads such as Sephadex-G50, G75 or G25 column for separation on the specific sizes of agents in the range of 80 – 1 kDa molecules. In the separation method, niosome suspension is eluted in the gel filtration column with buffer or normal saline. Loading agent is eluted out entirely, while niosomes vesicles trapped in the beads are recovered by flushing the column.

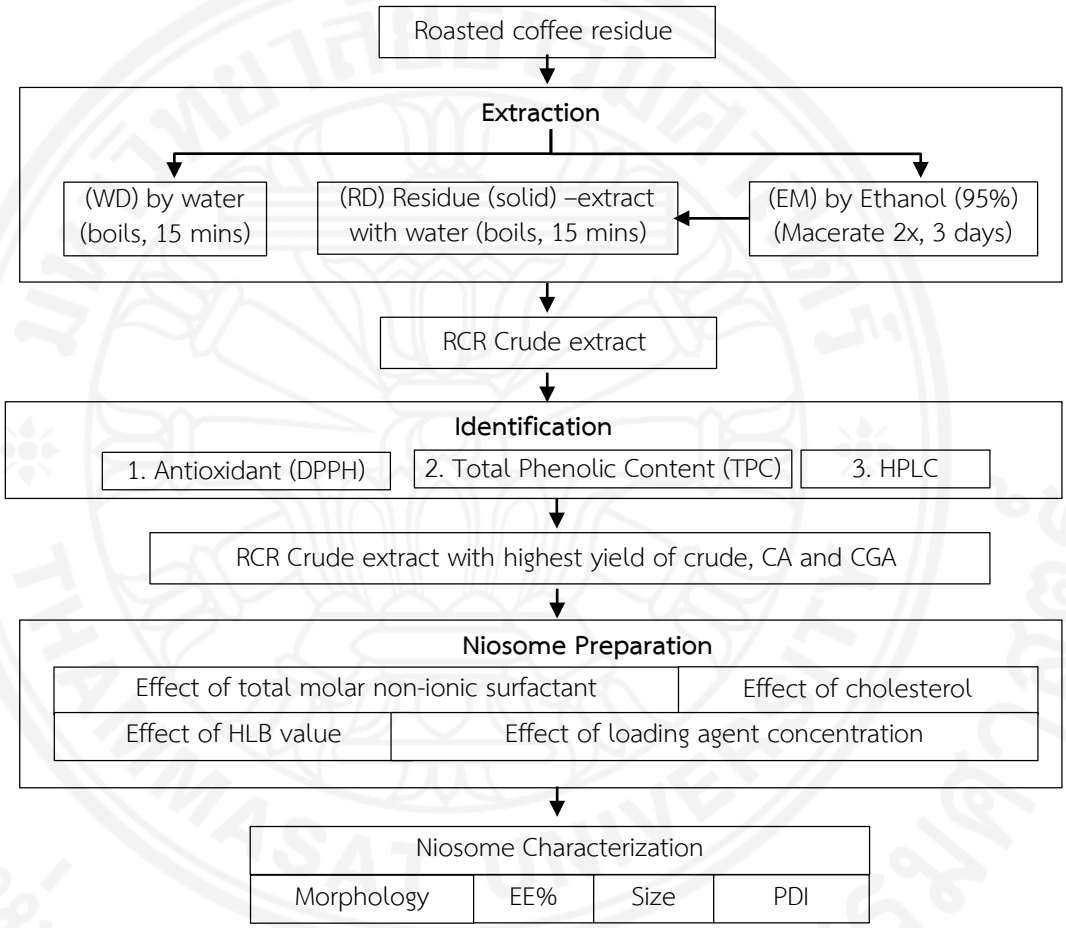
2.10.8. Centrifugation / ultra-centrifugation

Ultra-speed centrifugation separates niosome vesicles according to its sizes, smaller niosome vesicle sizes requires higher centrifugation speed and prolonged time. The separation efficiency of the niosome suspension from the loading agent can be determined by Stoke's law of sedimentation. Smaller niosome vesicles which is in sub-nano meter ranges may requires combination of ultra-filtration centrifugation system. Centrifugation separates niosome into sedimentation pallets and supernatant of free loading agent. The advantage of centrifugation separation is speed; it just took a couple of hours of ultra speed centrifugation than overnight dialysis or longer hours of gel separation.

CHAPTER 3

RESEARCH METHODOLOGY

3.1. Overview



3.2. RCR extraction

3.2.1. Source and variety of RCR

RCR from the residue of a commercial coffee bean which was obtained and used in this study was donated from Hom Krun Coffee shop at Learning Centre 4 (LC 4), Faculty of Science & Technology, Thammasat University, Pathumthani, Thailand. The roasted coffee bean of the residue was a medium roasted coffee bean of *Coffea arabica* variety cultivated in Chiang Mai, a northern province of Thailand, mixed with an imported variety of the same species and roast.

3.2.2. Drying RCR

RCR was spread evenly on a tray and then set inside a hot air oven (MEMMERT UE500, Germany) at 40°C until fully dried. Afterwards, dried RCR was sieved and packed in a sealable clear plastic bag and stored in a desiccator jar at room temperature until further use.

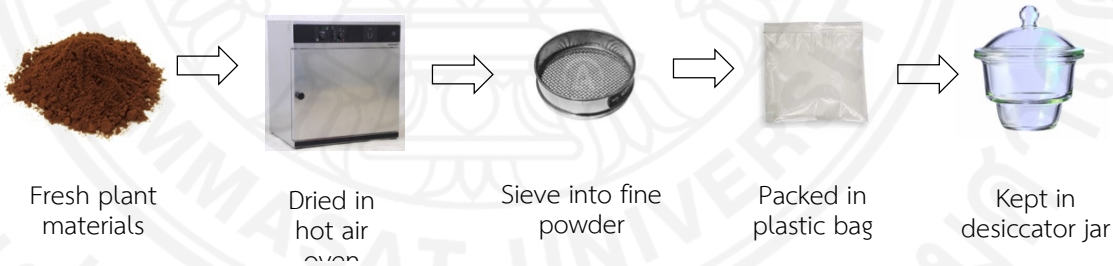


Figure 1: RCR pre-extraction preparation

3.2.3. Extraction

3.2.3.1. Extraction strategy

RCR extractions were varied in this study on the basis of extraction methods such as; (i) water decoction (WD), (ii) ethanol maceration (EM), (iii) water decoction of ethanol maceration residue (RD) and also lastly, (iv) Soxhlet extraction (SE). 40 grams RCR in 400 mL solvent was used in each extraction, solvent of the extract obtained

was filtered via filter paper (Whatman No.1) and evaporated in hot air oven at 40°C until all solvent removed and dried extract obtained. Dried RCR extract was weighted to obtain its crude yield and then kept in 4°C, fridge.

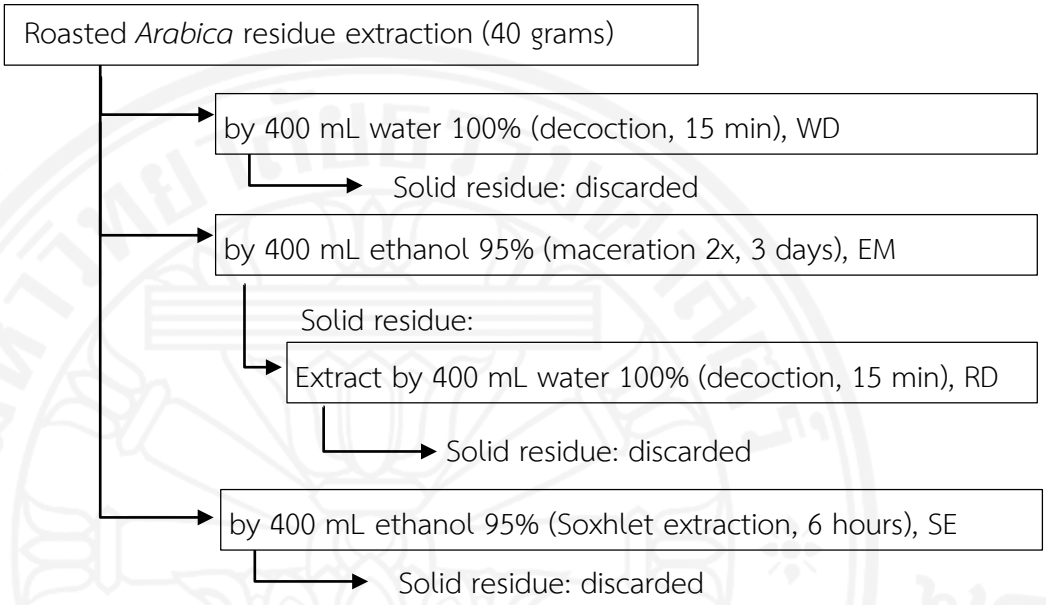


Figure 2: Overview of RCR extraction process

Table 7: RCR extraction conditions

Code	RCR (g)	Solvent, volume (mL)	Method	Duration
WD	40	Water, 400	Decoction	15 minutes
EM	40	95% Ethanol, 400	Maceration	3 days
RD	40	Water, 400	Decoction	15 minutes
SE	40	95% Ethanol, 400	Soxhlet	6 hours

3.2.3.2. Water decoction (WD)

WD was performed by soaking 40 grams RCR in 400 mL water and boiled at 100°C for 15 minutes, the residue was separated from the water extract by filtration via coffee filter, followed by Whatman No.1 filter paper. Filtrated residue was re-decocted again and the water extract was combined and dried in hot air oven at 45°C to evaporate the water.

3.2.3.3. Ethanol maceration (EM)

Maceration or also known as solid-liquid solvent extraction was performed by soaking 40 grams RCR in 400 mL 95% ethanol (ACI Labscan, Thailand) in 500 mL Erlenmeyer flask (Germany) for 3 days, ethanol extract was filtered and filtrate residue was macerated again, then the extract was combined and dried in hot air oven until all ethanol removed.

3.2.3.4. Water decoction of EM residue (RD)

RD was performed on the RCR from the EM filtrate residue, using the method similar as WD (0 ด้านบน). All filtrates were filtered using coffee filter to remove solid residue and followed by Whatman No. 1 filter paper. The water extract was evaporated in hot air oven until dryness.

3.2.3.5. Soxhlet extraction (SE)

RCR was also extracted using Soxhlet extractor to observe the effect of Soxhlet extraction versus maceration extraction in the same 95% ethanol solvent. 40 grams RCR was fitted in Soxhlet extractor chamber and washed continuously with 400 mL solvent. The solvent was percolated from the boiling flask into the RCR sample chamber and siphoned back into the flask completing one cycle. The extract solvent was filtered with Whatman No. 1 filter paper and evaporated in hot air oven until dried.

3.2.3.6. Espresso extraction (E)

Espresso was bought from Hom Krun coffee shop, filtered with Whatman No. 1 filter paper and evaporated hot air oven to obtain crude extract. The espresso was brewed from 18 grams *Coffea arabica* in 30 mL boiling water in hot air pressure of espresso maker.

3.3. Crude extract yield and calculation

Dried RCR crude extract was scrapped from the drying plate and transferred into a clean glass bottle, weighted, calculated its yield, wrapped in aluminum foil and stored at 4°C until further use. All extractions were performed in triplicate. RCR extract crude yield was calculated from its crude extract weight divided with the weight of RCR sample used in the extraction. RCR crude extract yield was reported as percentage weight per weight (%, w/w) calculated as follows:

$$Yield\ (\%, w/w) = \frac{RCR\ crude\ extract\ obtained}{RCR\ sample\ used} \times 100$$

Equation 6: Crude extract yield calculation formula

3.4. Identification of RCR extract bioactive compounds

RCR extract bioactive compounds was identified using standard biological assays. RCR is known contains phenolic and antioxidant compounds such as CA and CGA. CA is a phenol and an acrylic group compound, whereas CGA is an esterified CA, phenol with quinic acid (QA) which is a cyclitol. Thus, CA and CGA compounds both has a phenol group, phenolic compounds are rich in plants and naturally exhibits antioxidant activity from hydroxyl in the phenol group.

3.4.1. Antioxidant activity assay

3.4.1.1. DPHH free radical scavenging antioxidant activity assay

RCR extract was assayed for antioxidant activity using modified DPPH free radical scavenging assay in 96 well micro-plate method (40,44,45,49).

3.4.1.2. Antioxidant standard (gallic acid), RCR extract, CA, CGA and CAF antioxidant activity assay

Fresh 0.2 mM DPPH (Sigma Aldrich, USA) stock in ethanol with absorbance at 520 nm was prepared and stored away from light exposure. RCR extracts, Gallic acid (GA), CA, CGA and CAF (Sigma Aldrich, USA) were weighted and diluted with its suitable solvent into 1.0 mg/mL concentration of RCR extracts and standards stock respectively. 40 µL of each RCR extracts and standard stock was added in the 1st row of the 96 well micro-titer plate (Nunclon™ Delta Surface, Denmark) and diluted 5 times with 160 µL suitable solvent into 200 µL total volume and 200 µg/mL, initial diluted concentration. Then, all sample in the 1st well were serially diluted 2 times by pipetting out 100 µL sample in the 1st well to the 2nd well and repeated until the 10th well to obtain 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8 and 0.4 µg/mL serial diluted initial concentrations. The 11th and 12th wells were both filled with 100 µL solvent and then added with 100 µL, 0.2 mM DPPH stock and 100 µL of the same solvent as control and blank respectively. The experiment were conducted in dim light room and each reaction were replicated thrice. Absorbance value at 520 nm measured from a spectrophotometer micro-plate reader (BioTek, PowerWave XS2, USA) and calculated to obtain the percentage of radical scavenging activity using Equation 7.

Then, 100 µL 0.2 mM DPPH stock was added in all 100 µL sample and standards micro-plate well subsequently dilutes each standards and samples into 2 times of its initial concentration, into the final concentrations of 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 and 0.2 µg/mL. The mixture was incubated 30 minute in the dark for antioxidant's radical scavenging reaction to progress. After incubation, the reaction's absorbance was measured at 520 nm visible light wavelength with UV-vis spectrophotometer micro-plate reader against dilution solvent as blank on micro-plate reader (BioTek, PowerWave XS2, USA). Each sample was replicated thrice for reproducibility.

3.4.1.3. Antioxidant standard (gallic acid) and RCR extract fit curve plot

DPPH free radical antioxidant scavenging activity assay of the sample was reported as free radical scavenging activity percentage against the sample concentration. The serially diluted sample concentration against the percentage of

radical scavenging activity can be better visualized and comprehends in a fit curve graph. Using sample concentration as x-axis and free radical scavenging activity percentage as y-axis, each free radical scavenging percentage value are plotted against its dilution concentration. Then, the linear regression trend line and the equation of the fit curve was generated from the excel sheet format trend line menu option.

The percentage of DPPH radical scavenging activity, (D) was calculated from the formulae as follows (Equation 7). Sample absorbance, (S) subtracted from control absorbance, (C), divided by control absorbance, C and then multiplied with 100. Fit curve of DPPH radical scavenging activity was attained by plotting the percentage scavenging activity on y-axis and against its serially diluted sample concentrations on x-axis. The curve was used to determine the concentration of the standard or sample and each extract required to reduce DPPH radical into half of its original concentration (IC_{50}).

$$D = \frac{(C - S)}{C} \times 100$$

Equation 7. Free radical scavenging calculation

3.4.1.4. Antioxidant half inhibition concentration from fit curve plot

Antioxidant activity effectiveness was determined by its inhibition activity, measured by its ability to inhibits half of the free radical concentration (IC_{50}), it was measured at half concentration for economical and practicality reasons. IC_{50} value was obtained from the fit curve plot of percent radical scavenging versus sample or standard's concentration directly or calculated from its linear regression equation, at 50 percent free radical scavenging y-axis, its associated concentration is obtained from the horizontal line, x-axis is referred directly on the graph plot or calculated from the linear regression equation. Similarly, the radical scavenging and concentration can also be computed to SigmaPlot 13 (Systat Software, San Jose, CA)

graphing and statistical analysis software to plot the fitted curve and obtain its IC_{50} value accurately.

3.4.2. Determination of total phenolic content (TPC)

Cumulative amount of polyphenol contents in RCR extract, CA, CGA and CAF were quantified using colorimetric *in vitro* assay method based on phenolic compounds reaction with Folin–Ciocalteau reagent (FCR) and sodium carbonate (Na_2CO_3) (Singleton & Rossi, 1965), on modified Folin-Ciocalteu method (44,87) for micro-plate well.

3.4.2.1. Phenolic standard (gallic acid) TPC assay

FCR (Merck KGaA, Germany) and Na_2CO_3 (Ajax Finechem, Australia) were diluted into 10% and 7.5% (w/w) stock solution respectively in ddH_2O before use. Gallic acid (GA) was used as TPC assay standard which is equivalent to the total phenolic compound. 30 µL standard and RCR extraction stock were pipetted into the 1st row well, and diluted with 120 µL suitable solvent and then serially diluted 2 times by pipetting 30 µL sample from the 1st well into the 2nd well and repeated until the 10th well. 2 times serial dilution yielded 100, 50, 25, 12.5, 6.3, 3.1, 1.6 and 0.8 µg/m of initial standard and sample concentrations respectively. The 11th and 12th well were filled with solvent in TPC reagents and solvent as control and blank respectively. 150 µL, 10x FC reagent well and then after 5 minute followed by 120 µL, 7.5% Na_2CO_3 were added in each micro-plate well containing sample and control before incubated 30 minute in dark at room temperature. The reaction was prepared in triplicate and each mixture absorbance was measured at 765 nm wavelength on a micro-plate reader (BioTek, PowerWave XS2, USA).

3.4.2.2. TPC-GA standard curve plot

GA absorbance value at 765 nm wavelength (mAU) obtained was plotted against its serial diluted concentration (µg/mL) to obtain a TPC-GA standard curve, plotted in a Microsoft Office Excel application sheet. A straight linear dots were

visible and connected to form a linear regression lines representing the standard curve plot slope where its linear regression equation (y=mx+c) and regression coefficient (r^2) were generated from the Excel sheet. TPC-GA standard curve slope equation was used to obtain the concentration of TPC, equivalent to GA of RCR extract, CA, CGA and CAF standards. TPC was expressed in milligram GA equivalence per gram sample (mg GAE/g RCR). TPC concentration was calculated using the equation below (Equation 8). T represent total phenolic content (TPC), G is GAE concentration of RCR extract (mg/mL) established from TPC-GA standard curve and R is concentration of RCR extract (g/mL) used in the assay

$$T = \frac{G}{R}$$

Equation 8. Formula to determine GAE TPC concentration (88)

3.4.2.3. RCR extract, CA, CGA and CAF TPC assay

RCR extract, CA, CGA and CAF standards were diluted 10x in distilled water and then 0.1 mL of the samples were assayed as per 3.4.2.1 procedure. The concentration of TPC in RCR extract, CA, CGA and CAF were estimated based on the GA equivalence (GAE) by referring its absorbance value obtained at 765 nm wavelength to the GAE concentration in the TPC-GA standard curve plot obtained in procedure 3.4.2.2 above. TPC concentration in RCR, CA, CGA and CAF were expressed as mg GAE per gram RCE (mg GA/g RCR), calculated from Equation 8.

3.4.3. RCR extract bioactive compounds quantification

The main bioactive compounds of interest known to be present in coffee are CA, CGA and CAF, which contributed to significant benefits and function of coffee such as antioxidant activity, phenolic content and stimulant. In RCR extract, these

bioactive compounds presence are identified and quantified to determine the best separation method and yield.

3.4.3.1. HPLC quantification of RCR extract bioactive compounds

HPLC is an advanced analytical method to precisely separate and quantify complicated and complex compounds under suitable mobile and stationary phase system. It can be used to separate, identify and quantify the main bioactive compound in RCR extract such as CA, CGA and CAF. HPLC system used was modified from a previous study using HPLC system to identify the main compounds of phenolic and non-phenolic compounds in berries (15,39,89).

3.4.3.2. HPLC system

1200 Series Agilent HPLC system (Agilent Technologies, USA), courtesy of Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani, Thailand was used. The system was equipped with quaternary pump, standard auto-sampler, multiple wavelength photodiode array detector (PDA) and 150 mm x 4.6 mm, 5 µm i.d particle size, HPLC column (Luna C18, Phenomenex, USA). The HPLC mobile phase system were consisted of gradient mixture of (A) 50 mM potassium dihydrogen phosphate buffer (Ajax Finechem, Australia), adjusted to pH 2.6 with phosphate acid (ACl Labscan, Thailand) and (B) HPLC grade acetonitrile (ACI Labscan, Thailand), running for 50 minutes per injection. Standards, and RCR extract samples were dissolved in a suitable solvent, diluted with buffer A, filtered with 13 mm, 0.45 µm pore syringe nylon filter and 1 mL was filled into HPLC vial. At the beginning of the HPLC system start-up, the system solvent system was purged and then equilibrated with 100% buffer B, acetonitrile, 50% buffer B, acetonitrile and 50% water, 100% water, 50% water and 50% buffer and finally 100% buffer B, acetonitrile for 15 minute each. 20 µL sample was injected by auto-sampler at 1.0 mL/min flow rate in HPLC column heated at 30°C and equilibrated with 100% buffer A for 10 minute.

HPLC system mobile phase gradient begin with 100% (v/v) buffer A eluted for 10-minute at 0.1 mL/min flow rate, then buffer A was gradually reduced to 90% (v/v), compensated with 10% (v/v) acetonitrile for the next 10 minutes. This buffer ratio was maintained at the 20th to 30th minute before the solvent gradient system was immediately switched to 30% (v/v) buffer A, with the rest of the solvent was 70% (v/v) buffer B for another 10-minute beginning at the mark of 1 second past 30-minute time. The HPLC column elution for one injection ends at the mark of 50th minute, the system was re-equilibrated with 100% (v/v) buffer A (Table 8). Eluted HPLC fractions were analyzed with integrated HPLC spectrophotometry UV detector set at **λ** max 280 nm wavelength for CAF compound spectrum detection and 321 nm wavelength for CGA and CA compound spectrum detection.

Table 8: HPLC mobile phase gradient elution system, consist of (A) 50 mM potassium dihydrogen phosphate buffer, pH 2.6 and (B) acetonitrile

Time (min:sec)	A (%, v/v)	B (%, v/v)
00:00	100	0
10:00	100	0
20:00	90	10
30:00	90	10
30:01	30	70
40:00	30	70
40:00	100	0
45:00	100	0

3.4.3.3. Preparation of CA, CGA and CAF standard curve plot

Five dilution concentration of CA, CGA and CAF standard compounds (Sigma Aldrich, USA) at 25, 12.5, 6.25, 3.12 and 1.56 µg/mL were prepared by 2-fold serial dilution for HPLC standard curve preparation. The standard compound concentration range used was estimated with proximity to the detectable range of identified CA, CGA and CAF compounds in RCR extract. All three standards compounds mixtures

were prepared in triplicate, mixed together into 1mL total volume in each HPLC vial and loaded into HPLC analyzer auto sampler tray (Table 9). 20 µL of the standard was injected in the HPLC system in duplicate for analysis as per 3.4.3.1 procedure.

Table 9: CA, CGA and CAF standards dilution concentration used in HPLC

Dilutions \ Standards	CA (µg/mL)	CAF (µg/mL)	CGA (µg/mL)
1	25.0	25.0	25.0
2	12.5	12.5	12.5
3	6.25	6.25	6.25
4	3.12	3.12	3.12
5	1.56	1.56	1.56

HPLC chromatograms of CA, CGA and CAF standards compounds were generated from ChemStation LC 3D systems (Agilent Technologies, USA) and obtained at the end of the HPLC elution. The retention time of CA and CGA standards compound at 321 nm and CAF standard compound at 280 nm detection wavelength HPLC chromatogram peaks were identified as well as the peak total area (mAU) and total height (mAU). The standard compounds peak total area (mAU) and dilution concentration (µg/mL) were used to plot CA, CGA and CAF standard curve. Y-axis, represents peak area (mAU) was plotted against CA, CGA and CAF dilution concentration (µg/mL) as x-axis of the standard curve in Microsoft Office Excel application sheet. The standard curve linear regression line was aligned to intercept y-axis at zero and its linear regression equation (y=mx+c) and regression coefficient (r^2) were generated from the Excel sheet. The linear regression equation was used to estimate the peak area (mAU) in RCR extract which representing CA, CGA and CAF compounds concentration equivalent to each standard concentration per gram RCR extract injected.

3.4.3.4. Separation and quantification of RCR extract by HPLC

1 mL, 1.0 mg/mL RCR extract prepared in a suitable solvent was filtered through a 0.45 µm filter, injected into a HPLC vial and placed on the auto sampler, the sample was aspirated into the HPLC system at 1.0 mL/min flow rate. Each RCR extract samples were prepared in triplicate and injected twice to confirm peak reproducibility and accuracy. RCR extract samples were eluted in HPLC system according to procedure 3.4.3.2 ด้านบน. The chromatogram data and spectrum response of RCR extract were collected and analyzed by ChemStation for LC 3D systems (Agilent Technologies, USA). HPLC chromatogram of RCR extract was screened and identified for CA, CGA and CAF compound peak by comparing the similarity of the peak retention time and spectrum profile to CA, CGA and CAF standard chromatogram as reference.

The peak area (mAU) of confirmed CA, CGA and CAF compound peaks in RCR extract HPLC chromatogram were compared to CA, CGA and CAF standard curve plot to obtain the equivalent concentration of the compound in RCR extract. Using linear regression equation of CA, CGA and CAF standard curve obtained in procedure 3.4.3.3 ด้านบน, CA, CGA and CAF equivalent concentration (µg/mL) of RCR extract identified CA, CGA and CAF peak area (mAU) were obtained. CA, CGA and CAF peak area (mUA) equivalence of CA, CGA and CAF standard curve concentration were expressed as milligram equivalent of CA, CGA and CAF per gram RCR extract (mg CAE/g RCR, mg CGAE/g RCR and mg CAFÉ/g RCR) calculated from Equation 9 below. The denominator C is the total CA, CGA or CAF contents (mg/g RCR) in the CA, CGA or CAF equivalent concentration, S divided by the gram weight of RCR extract (R).

$$Total\ C = \frac{S}{R}$$

Equation 9: Total CA, CGA or CAF equivalent concentration estimation

3.5. Niosome preparation

3.5.1. Heating and sonication method

Niosomes were prepared using the selected modified hydration and sonication method (2.9.1 ด้านบน) based on the established method in a previous study. In this method, non-ionic surfactants of choice (Tween 20 and Span 60®) (Sigma Aldrich, USA) and additive (cholesterol) (Sigma Aldrich, Japan) were weighed at the specific molar ratios and combined in a 12 mL glass vial (WHEATON, New Jersey, USA). The mixture was hydrated with 5 mL 10 mM PBS buffer pH 7.4 (Sigma Aldrich, USA) as the medium for the niosome forming. The niosome suspension was subjected to heating at 56 – 58°C, the phase transition temperature (Tc) of Span 60 (Kumar, 2011) with sonication at 50 –60 Hz in a bath sonicator (Tru-Sweep™, Ultrasonic Cleaner, New York, USA) for 45 minutes. Afterwards, the hydrated mixture was cooled to RT for an hour before incubated in 4 – 5°C water bath overnight, for the formation and annealing of the non-ionic surfactant and additive into niosome vesicles.

3.5.2. Agent loaded niosome

Niosomes were also prepared loaded with different loading agents such as GA, CA and RCR extract. Each of the agents were dissolved in its suitable solvent and then diluted in 10 mM PBS with 20% EtOH into 1 mg/mL concentration. PBS prepared with the loading agent was used to hydrate the non-ionic surfactant and cholesterol mixtures prepared. The hydrated mixtures was heated and sonicated similar to the procedure 3.5.1 ด้านบน.

3.5.3. Non-ionic surfactant and cholesterol ratios

3.5.3.1. Effect of span 60 and cholesterol total molar and loading agent

Table 10: Niosome formulation 1 (effect of Span 60: cholesterol 2:1 total molar ratio)

Code	Span 60 (mg): Cholesterol (mg)	Molar ratio (µmol)	PBS Hydration volume (mL)	Niosome Concentration (mM)
1.1	64.6 : 58.0	1:1 (150:150)	6	0.50
1.2	86.1 : 38.7	2:1 (200:100)	6	0.50
1.3	129.2 : 58.0	2:1 (300:150)	6	0.75

Initially, in the study the niosomes were prepared using only Span 60 as non-ionic surfactant and cholesterol as additives. At 2:1 molar ratio of Span 60 and cholesterol, the total molar ratios were varied at three levels of 300, 450 and 600 µmol. The niosome formulations were hydrated with 6 mL 10 mM PBS containing 1 mg/mL GA, CA and RCR extract and blank for control. The hydrated mixtures were proceeded as per procedure 3.5.1 ด้านบน.

3.5.3.2. Effect of span 60 and cholesterol ratio and hydration volume

The formulation was varied on the effect of Span 60 to cholesterol ratio at 2:1, 1:1 and 1:2 with the total molar fixed at 600 µmol. Additionally, the 10 mM PBS hydration volume was varied at 6, 9 and 12 mL for the effect of higher volume of buffer to the niosome formation and loading agent entrapment. The hydrated mixtures were proceeded as per 3.5.1 ด้านบน.

Table 11: Niosome formulation 2 (effect of Span 60:cholesterol ratio and hydration volume)

Code	Span 60 (mg): Cholesterol (mg)	Molar ratio (µmol)	PBS Hydration volume (mL)	Niosome Concentration (mM)
2.1	86.1 : 154.7	1:2 (200:400)	6	1.00
2.2	86.1 : 154.7	1:2 (200:400)	9	0.67
2.3	86.1 : 154.7	1:2 (200:400)	12	0.50
2.4	129.2 : 116.0	1:1 (300:300)	6	1.00
2.5	129.2 : 116.0	1:1 (300:300)	9	0.67
2.6	129.2 : 116.0	1:1 (300:300)	12	0.50
2.7	172.2 : 77.3	2:1 (400:200)	6	1.00
2.8	172.2 : 77.3	2:1 (400:200)	9	0.67
2.9	172.2 : 77.3	2:1 (400:200)	12	0.50

3.5.3.3. Effect of tween 20 in span 60 and cholesterol

Combination of non-ionic surfactants, Tween 20, 16.7 HLB and Span 60, 4.7 HLB modifies the HLB value mixture of non-ionic surfactants forming niosome. At 1:1 Tween 20 and Span 60 ratios, produces 10.7 HLB value. Increasing Tween 20 ratio increases HLB, while increasing Span 60 reduces the combined HLB value.

3.5.3.3.1. Effect of tween 20 in span 60 and cholesterol total molar

Niosomes were prepared in 1:1 molar ratio of Tween 20 and Span 60 as non-ionic surfactants with combined HLB of 10.7 and added with cholesterol as additives at 1:1 molar ratios of non-ionic surfactant to cholesterol. The total molar ratios of the niosome materials were varied at 150, 200, 450, 600 and 900 µmol. 6 mL 10 mM PBS with 1 mg/mL RCR extract and GA loading agent were used to hydrate the niosome material mixtures. The hydrated mixtures were proceeded as per procedure 3.5.1 ด้านบน to obtain niosome suspension.

Table 12: Niosome formulation 3 (effect of total niosome material molar)

Code	Tween 20 (uL): Span 60 (mg): Cholesterol (mg)	Molar ratio (µmol)	PBS Hydration volume (mL)	Niosome Concentration (mM)
3.1	368 : 129.2 : 116	300 : 300 : 300 (900)	6	1.50
3.2	246 : 86.1 : 77.3	200 : 200 : 200 (600)	6	1.00
3.3	184 : 64.6 : 58	200 : 200 : 200 (600)	6	0.75
3.4	123 : 43.1 : 38.7	150 : 150 : 150 (450)	6	0.50
3.5	61 : 21.5 : 19.3	100 : 100 : 100 (300)	6	0.25

3.5.3.3.2. Effect of cholesterol molar ratio

At the suitable total molar of niosome materials (Tween 20, Span 60 and cholesterol), the effect of cholesterol additive ratio in the niosome preparation entrapment efficiency was observed by varying the ratio of cholesterol in the total niosome materials preparation. Total non- ionic surfactant (1;1, Tween 20 and Span 60) to cholesterol molar ratios to were varied from 1;1, 2:1, 5:1 and 11:1. Meanwhile, total molar niosome material, PBS hydration volume and loading agent concentration were fixed at 600 µmol, 6 mL and 1.0 mg/mL respectively.

Table 13: Niosome formulation 4 (effect of cholesterol molar ratio)

Code	Tween 20 (uL): Span 60 (mg): Cholesterol (mg)	HLB	Molar ratio (µmol)	PBS Hydration volume (mL)	Niosome Concentration (mM)
4.1	184 : 64.6 : 116	10.7	150 : 150 : 300 (1:1)	6	1.00
4.2	246 : 86.1 : 77.3	10.7	200 : 200 : 200 (2:1)	6	1.00
4.3	307 : 107.7: 38.7	10.7	250 : 250 : 100 (5:1)	6	1.00
4.4	338 : 118.4 : 19.3	10.7	275 : 275 : 50 (11:1)	6	1.00

3.5.3.3.3. Effect of non-ionic surfactant molar ratio and HLB value

At fixed 600 µmol total molar of niosome materials, 6 mL 10 mM PBS hydration volume, 1 mg/mL loading agent (RCR and GA) concentration and 100 µmol cholesterol molar, non-ionic surfactant (Tween 20 and Span 60) ratios were varied from 1:1 to 1:1.5, 1:0.8 and 1:0.6, subsequently modified the combined HLB into 8.7, 11.3 and 12.7 respectively.

Table 14: Niosome formulation 5 (effect of non-ionic surfactant molar ratio)

Code	Tween 20 (uL): Span 60 (mg): Cholesterol (mg)	HLB	Molar ratio (600 µmol)	PBS Hydration volume (mL)	Niosome Concentration (mM)
5.1	246 : 129.2 : 38.7	8.7	200 : 300 (1:1.5) : 100	6	1.00
4.3	307 : 107.7 : 38.7	10.7	250 : 250 (1:1.0) : 100	6	1.00
5.2	368 : 86.1 : 38.7	12.7	300 : 200 (1:0.8) : 100	6	1.00
5.3	368 : 107.7 : 19.3	11.3	300 : 250 (1:0.6): 50	6	1.00

3.5.3.3.4. Effect of high total niosome molar at low cholesterol ratio

Total molar niosome materials was increased to 900, 1200 and 1500 µmol at fixed 8.3% (mol/mol) cholesterol concentration and 1:1 Tween 20 and Span 60 ratio, respective to 10.7 HLB. The 10 mM PBS hydration volume and loading agent concentration were fixed at 6 mL and 1 mg/mL respectively.

Table 15: Niosome formulation 6 (effect total niosome molar at low cholesterol molar)

Code	Tween 20 (uL): Span 60 (mg): Cholesterol (mg)	HLB	Molar ratio (600 µmol)	PBS Hydration volume (mL)	Niosome Concentration (mM)
6.1	460 : 177.6 : 29.0	10.7	413 : 413 : 75 (900)	6	1.50
6.2	675 : 236.8 : 38.7	10.7	550 : 550 : 100 (1200)	6	2.00
6.3	767 : 296.0 : 48.3	10.7	688 : 688 : 125 (1500)	6	2.50

3.5.3.3.5. Effect of loading agent concentration

RCR loading agent concentration added in PBS hydration buffer was decreased to 1.25, 0.75, 0.50 and 0.25 mg/mL. Other parameters were fixed such as total niosome materials at 900 µmol, non-ionic surfactant to cholesterol ratio at 2:1, Tween 20 to Span 60 ratios at 1:1 correspond to 10.7 HLB and 6 mL PBS hydration buffer.

3.5.4. Niosome vesicle evaluation and characterization

3.5.4.1. Entrapment efficiency (EE)

The entrapment efficiency percentage (EE) % of loading agents in niosome vesicles formulation preparation was performed via dialysis and estimated by indirect method. The initial amount of the loading agent in the PBS buffer added into the niosome preparation was the total loading agent available, and free loading agent, which remains in the suspension after niosome vesicles formed, was quantified using spectrophotometry (Spectrophotometer UV-vis, Biochrom Libra S22, U.K.) for its absorbance.

3.5.4.2. Dialysis method

Un-entrapped loading agent was separated from the niosome suspension by dialysis method (64,90). Priori, cellulose dialysis bag (CelluSep T4, Texas, USA)was prepared by soaking it in a PBS (Phosphate Buffer Saline, pH 7.4, Sigma Aldrich, USA) buffer for 20 minutes. Then, 4 cm long cellulose dialysis bag was prepared with its bottom end tied up. Aliquot 1mL niosome suspension prepared earlier into the dialysis bag and tied up its top end. The dialysis bag was placed in a 15 mL Falcon tube filled with 6 mL PBS buffer. The niosome suspension on dialysis was applied with gentle shake with vortex mixer to promote the diffusion of loading agent from niosome suspension inside the dialysis bad into the buffer environment and left overnight for complete dialysis.

After an overnight dialysis, the absorbance of the PBS buffer was measured with a spectrophotometer at the specific lambda max wavelength of loading agent. RCR extract, GA, and CA spectrum wavelength lambda max were determined by scanning the agents in buffer at 200 to 800 nm wavelength for 0.5 second with a UV-vis spectrophotometer to obtain its spectrum profile. Lambda max of each agent was used as the wavelength to determine the absorbance of the loading agent which reflect its amount in the buffer.

Both the loading agent in buffer and niosome suspension were dialyzed, and its absorbance were obtained. Absorbance of the prepared loading agent in the buffer represent the initial or total amount of drug used for entrapment (I), meanwhile the absorbance of loading agent in the buffer of niosome suspension dialysis represent un-entrapped loading agent (F). The amount of loading agent trapped inside the niosome vesicles were assumed as I – F. Thus, the indirect estimation of entrapment efficiency percentage was calculated from equation 10 as follows;

$$EE\ \% = \frac{I - F}{I} \times 100$$

Equation 10: Entrapment efficiency calculation (61,62,69)

3.5.4.3. Size and zeta potential

Niosomes prepared earlier were also characterized for its vesicle size, polydispersity index (PDI) and zeta potential (ZP) characteristics. These characteristics were essential for selection of a preferred and suitable niosome for further application. For sizing of the niosomes, vesicles with sub 1000 nm sizes can be measured with dynamic light scattering (DLS) and electrophoretic light scattering (ELS) analysis using Zetasizer. Meanwhile, due to the upper limit of detection (LOD) in DLS method of up to 8000 nm, thus niosomes vesicles with sizes larger than 8000 nm or 8 microns up to 50,000 microns are recommended to be measured with laser diffraction (LD) particle analyzer. HORIBA LA-950 LD particle size analyzer can only measure size and incapable of zeta potential estimation. However, new generations of LD particle size analyzer have been improved with zeta potential analysis features.

3.5.4.4. Morphology

The prepared niosome vesicles were observed for its morphological structure, visual size estimation and number of niosome using optical microscope equipped with a camera.

3.5.4.5. Visual appearance

Additionally, niosome suspension was also observed and compared visually for color, cloudy, phase separation, aggregation and sedimentation appearance (91).

3.6. Statistical analysis

Extracts, samples and standards were prepared in triplicate (n = 3) or at least in duplicates (n = 2) and summed for reporting reproducible average population data with standard deviation value. International Business Machine (IBM Corp., USA) Statistical Package for Social Science (SPSS) ® version 22.0.0 software was used to compile and compute the data from between group comparison and full factorial results for analysis of variance (ANOVA). ANOVA and Tukey HSD post hoc analysis level of significant were set at 95% or less than 0.05 probability value. Tukey HSD post-hoc analysis was performed after ANOVA to determine and group variables according to its group mean difference, significant at probability value less than 0.05.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. RCR extraction

RCR used in this study were the residues of brewed ground coffee bean, from a mixture of *Coffea arabica*, locally produced in Chiang Mai, Thailand with an imported bean of the same variety. RCR was dried thoroughly for a few days and extracted by using water and 95% ethanol solvent system with decoction and maceration process, respectively. The extractions were varied with aim to obtain high crude extract yield, antioxidant activity, total phenolic compounds, caffeic acid, chlorogenic acid and caffeine. Previous study has reported various extraction conditions such as non-continuous and continuous extraction method using decoction, percolation, filter coffee maker, maceration and Soxhlet extraction. Additionally, various solvent system such as non-polar to very polar has been utilized such as water, ethanol, methanol, hexane isopropanol and mixture of those solvents (15,23,38). After careful evaluation and consideration on the advantages, disadvantages and suitability of previous study's RCR extraction condition, this study has selected RCR extraction via decoction, maceration and Soxhlet extraction method in pure water and 95% ethanol solvent system.

4.1.1. Drying

Drying is one of the standard and most widely used post-harvest practice to preserve any sample material and its active compounds for further application. It is also the preliminary step in bioprocess technology to remove impurities and concentrate volume. Drying removes remaining moisture from damp RCR as a result of coffee brewing process. Moisture in the RCR provides a suitable condition for mold and microbial growth which leads to natural and slow RCR decomposition process (92,93).

4.1.2. Extraction yields

Initially, RCR was extracted in three extraction conditions such as water decoction (WD), ethanol 95% maceration (EM) and water decoction of ethanol maceration residue (RD). The ratio of RCR material and solvent volume was fixed at 1:10 (gram:milliliter) ratio. Overview of RCR extraction strategy is summarized in the Figure 3 as follows. Extraction of RCR via WD, EM and RD extraction methods have yielded 3.98 ± 0.29%, 0.51 ± 0.01% and 3.89 ± 0.29% RCR extract solid fractions respectively. Water solvent with decoction extraction method (WD and RD) obviously yielded the highest crude extract yield, at almost 8x higher than RCR crude extract yield of 95% ethanol solvent with maceration extraction method (EM).

However, using Soxhlet extraction (SE) with 95% ethanol, the same solvent system as EM, able to increases the yield of RCR crude extract by almost 7x bringing the yield in proximity with WD and RD RCR crude extract yields (Figure 4). Meanwhile, roasted coffee bean brewed into espresso shot obtained from coffee shop yields 16.04 ± 1.22% (w/w) crude extract. The summary of all RCR extraction and its crude yield are presented in Table 16.

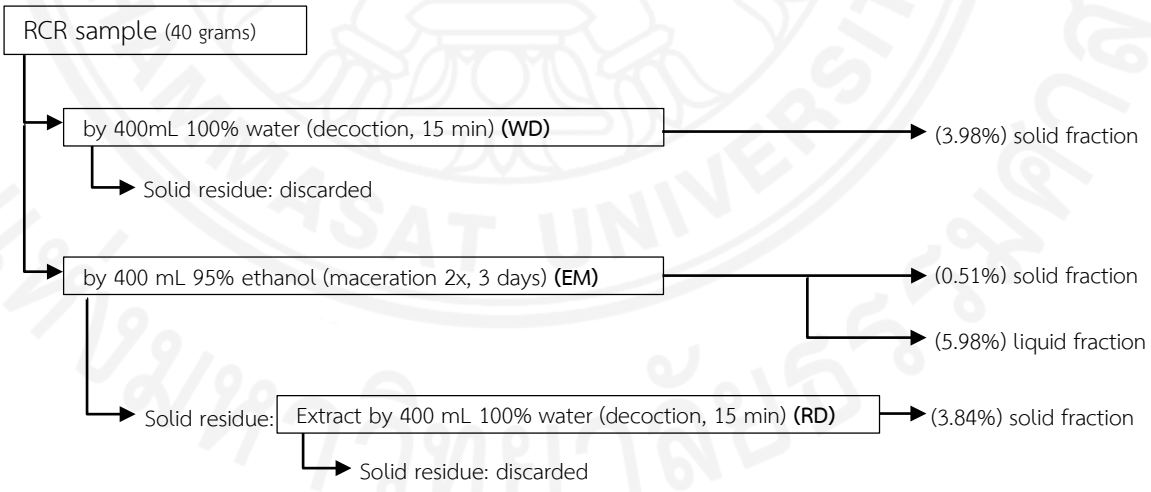


Figure 3: Overview of initial RCR extraction in decoction (WD, RD) and maceration (EM)

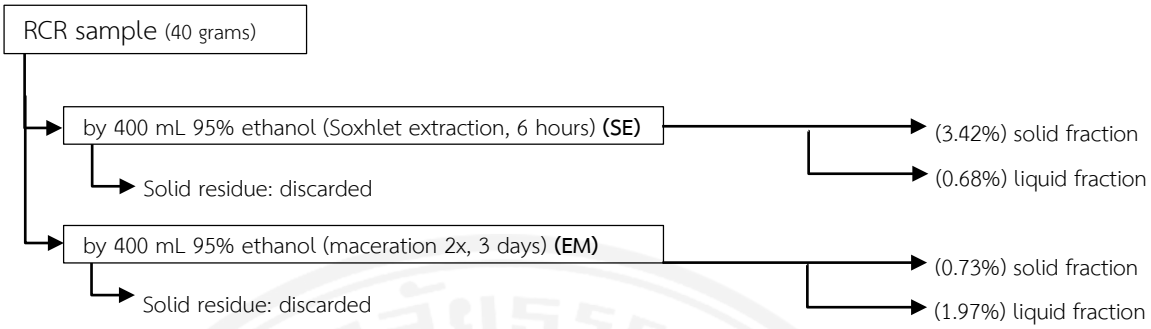


Figure 4: Overview of RCR extraction in 95% ethanol by maceration (EM) and Soxhlet (SE)

Table 16: Overview of RCR extraction yields in WD, RD, EM, SE and Espresso

	Extraction condition				Solid fraction of RCR crude
Code	**RCR (g)**	**Solvent, volume (mL)**	**Method**	**Duration**	**extract yield (%, w/w)**
WD	40	Water, 400	Decoction	15 minutes	3.98 ± 0.29[a]
EM	40	95% Ethanol, 400	Maceration	3 days	0.51 ± 0.01[b]
RD	40	Water, 400	Decoction	15 minutes	3.84 ± 0.13[a]
SE	40	95% Ethanol 400	Soxhlet	8 hours	3.42 ± 0.37[a]
E	18	Water, 30	Espresso	-	16.04 ± 1.22

[a, b] ANOVA Tukey HSD mean significant difference at 0.637 and 1.00 respectively, *p*-value 0.05.

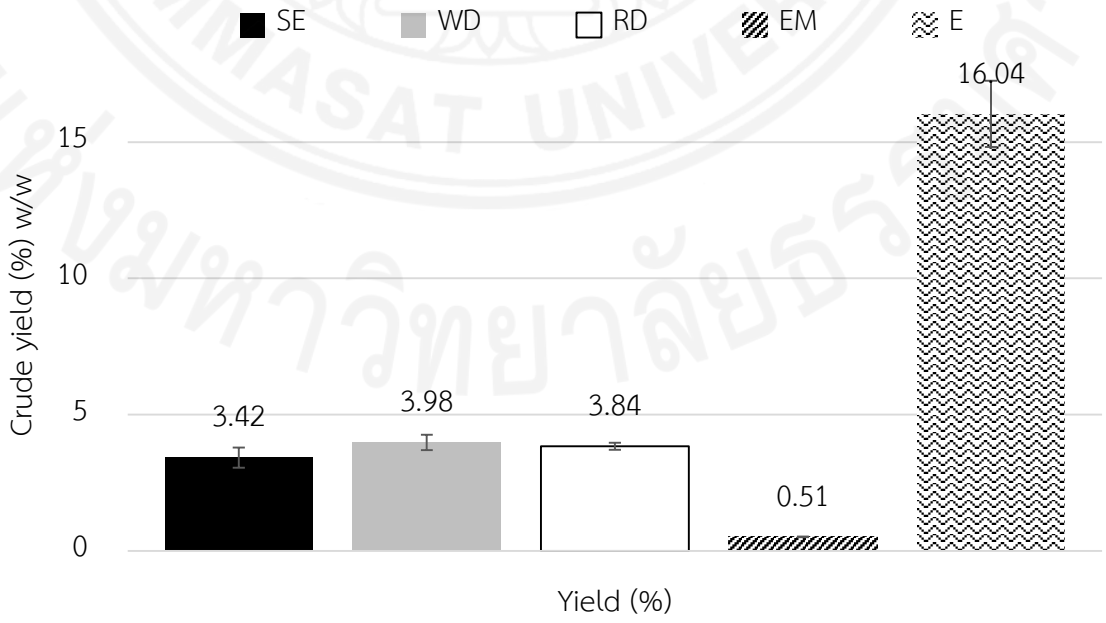


Figure 5: RCR extraction crude yield, ranked highest from Espresso, E > WD > RD > SE > EM

4.1.2.1. Effect of extraction method

Selection of a suitable and optimized extraction method and solvent system are a crucial step in biotechnology process. Water decoction is a simple, inexpensive and commonly used plant extraction process (94), it has evolved into other variations of decoction methods. A traditional technique – maceration, involves a slow process of solvent softening and absorption into the soaked material, and it may takes several days of soaking to be completed (95,96). Hexane, water, ethanol and methanol solvents has been utilized as an extraction solvent to extract RCR, and hexane yield the highest crude extract, followed by water, ethanol and the least in methanol (15,38,39).

In this study, WD, EM, RD RCR extraction has yielded 3.98 ± 0.29, 0.51 ± 0.01 and 3.84 ± 0.13 (%, w/w) solid crude extract, respectively. WD and RD water decoction extraction method yields 8 times higher crude extract than EM, 95% ethanol maceration extraction method. Water extraction method also reported high crude extract yields in other previous studies, such as water decoction (15), (38,39). EM crude extract yielded two phase fractions, which was separated into oil and solid fraction respectively. 95% ethanol maceration solid fraction yields lower than water decoction, consistent with previous reported study. Water has polarity index of 9.0 is higher than ethanol at 5.2, contributes to its capacity to harness more polar compounds from RCR (97).

4.1.2.2. Effect of water and 95% ethanol extraction solvent

Selecting a suitable extraction solvent is an important consideration to be taken in order to extract RCR active compound more efficiently. Due to different polarity and solubility nature of active compounds, polar organic solvents are better suited to exploit active compounds with high polarity and solubility and vice versa (96). RCR active compound extraction mostly were performed and reportedly yields better in water or ethanol solvent (15,38,39) than other solvents such as hexane, methanol. The authors found that RCR active compounds which are mostly phenolic

compounds preferred highly polar solvent such as water and ethanol mixture for efficient extraction interface. The mixture ratio at 40:60, 80:20 and 100% water to ethanol or methanol have been reported to yield the highest total phenolic compounds, ABTS and DPPH radical scavenging respectively (38,39).

In this study, 95% ethanol and pure water was chosen and compared to observe the effect and efficiency of each solvents in extracting RCR active compounds. Methanol was not considered due to its safety concerns and also reports of its lower crude yield than water and ethanol solvents, and then even though hexane reported higher yield, but it shows no antioxidant and total phenolic activity, thus both solvents were excluded. As for water and ethanol solvents, they are generally safe, environmental friendly, non-toxic and inexpensive to be utilized for large extraction process. These are important solvent selection criteria as RCR crude extract and its active compounds are intended for application in cosmeceutical formulation, thus the extraction solvent was considered for health and safety criteria prior to selection. Besides, water and ethanol were reported could provide a protective effect to RCR extract antioxidant compounds (15).

In the results of RCR extraction, it appears that water decoction is the most efficient extraction solvent and method which yielded higher crude extract than extraction via 95% ethanol maceration. Water's polarity index is 9.0, which is higher than ethanol at 5.2, this explains its higher ability to extract more polar compounds from RCR. However, ethanol is also a well known solvent with high affinity to extract phenolic compounds, especially from plant materials. Other very polar solvents such as acetic acid, acetonitrile, dimethyl formamide and dimethylsulfoxide with 6.2, 5.8, 6.4 and 7.2 polarity index (97) respectively are highly volatile and environmentally harmful to be utilized as RCR extraction solvent. In comparison with other study, the extraction of RCR in boiling water for 5 minute yielded higher crude extract versus overnight ethanol maceration at 6.58 and 1.38 percent respectively (15). Additionally, in another report, water extraction via filter coffee maker also shows higher yield than ethanol or methanol solvent (38). Evidently, water is a good extraction solvent to obtain higher yield of RCR crude extract.

4.1.2.3. Effect of ethanol maceration (EM) on water decoction extraction (RD)

Ethanol is the most preferred and commonly used solvent for extraction of active compounds from herbal and plant materials. Its polarity index at 5.2, which is considered as just the right polarity and affinity towards phenolic compounds for these compound's efficient extraction condition. In this study, one of the extraction condition applied was EM. In this extraction condition, 95% ethanol solvent was used and there are two observations which were evaluated. The first was to compare the efficiency of EM against RD extraction in term of crude extract and active compounds yield. And in the second condition, EM extraction was observed for its effect towards RD crude extract yield and active compounds.

In the first condition, EM yielded 0.51 ± 0.01% (w/w) solid crude extract, which was significantly lower than WD at 3.98 ± 0.29% (w/w) with (*p value*> 0.05). It was coherent that EM has lower crude extract yield than RD, probably due to its polarity, which is lower than water thus limits ethanol ability to extract more polar compounds in its crude extract. However, in other report, other solvent such as hexane which has lower polarity than water or ethanol at 0 polarity index were able to obtain higher crude extract yield of RCR crude extract at 8.4%. Hexane extract crude yield was higher than both water and ethanol solvent extract at 6.58 and 1.38% respectively. However, upon further identification, hexane extract compounds were primarily lipids, shown by lack of neither antioxidant activity nor total phenolic compound (15).

Meanwhile, in the second observation of EM extraction, it was found that EM had not effected RD extract crude yield totally. RD crude extract yield was which obtained after EM was 3.84 ± 0.13% even after 0.51 ± 0.01% was already extracted by EM earlier. These yields, if were accumulated, then both EM and RD would amount to 4.35% of total crude extracts, outright higher than WD extract yield. Comparing WD and RD extract crude yield which were 3.84 ± 0.13 and 3.98 ± 0.29% shows not even a slight evidence of significant difference at all with *p-value* of 0.862. Thus, it is assumed that EM and WD extract a different set of active compounds from

the same RCR materials. Further investigation of the extracts active compounds was performed later on in the study.

4.1.2.4. Effect of maceration (EM) and Soxhlet extraction (SE) method

As observed earlier in the initial RCR extraction (Figure 3), it has been shown that 95% ethanol solvent in EM performs poorly than water (WD and RD) in extracting RCR crude extract. In the extraction process, 95% ethanol was used with maceration extraction method and water was with decoction method. Thus, under different extraction method, will 95% ethanol performs similarly or better than maceration or otherwise? Another extraction method, Soxhlet extraction was performed for comparison (Figure 4).

Soxhlet extraction (SE) involves repeated cycle of evaporation, percolation and soaking 95% ethanol solvent on RCR sample until the appearance of the solvent soaking the sample is clear – a continuous process. Compared to maceration extraction, which is a non-continuous process, it involves RCR sample material mildly soak in its solvent for a period of time. During soaking, any compounds which has polarity or affinity towards the solvent will diffused from sample material into the solvent. The diffusion occurs until it reaches an equilibrium rate in a few days, which the solvent becomes saturated with the extract compounds and unable to diffuse more compounds from the material.

In maceration extraction, an extract diffuse mildly from sample material into the solvent until it reaches equilibrates once the solvent become saturated with the extract. At equilibration point, it is highly recommended that the saturated solvent is replaced with a new fresh solvent to repeat the process and diffuse any remaining extract from the sample material again. Clearly, Soxhlet extraction is more efficient in terms of extracting the extract from sample material to obtain higher yield, plus the advantage is that the method reduces considerable amount of time and volume of solvent used in the expense of high consumption of heat to evaporate the solvent continuously.

Then, 95% ethanol solvent was compared between maceration and Soxhlet extraction methods. Interestingly, RCR extraction with 95% ethanol solvent yielded much higher RCR crude extract with Soxhlet than maceration extraction at 3.42 ± 0.37% and 0.73 ± 0.09% respectively, which is about 5x higher yield.

4.2. Antioxidant activity

4.2.1. DPPH free radical antioxidant scavenging activity

Antioxidant agent is a compound that inhibit oxidation process of free radical by neutralizing free radical into its reduced form (40). DPPH free radical compound is a stable organic chemical compound radical, which has strong violet appearance and adsorb 325 nm wavelength, it turns yellowish and absorbs 517 nm wavelength in its stable forms (41,45,48,49). DPPH radical scavenging is a simple, sensitive and inexpensive direct free radical assay for quantification antioxidant capacity. RCR crude extract antioxidant agent was assayed to assess its ability to scavenge and reduced free DPPH free radical into its stable form.

Comparison between GA, CA, CGA and CAF standards DPPH free radical scavenging activity are shows in the graph below (Figure 6). In DPPH free radical antioxidant assay system, the result clearly indicate that GA is the most active antioxidant agent in scavenging DPPH free radicals, followed by CA and CGA. Meanwhile, CAF shows no DPPH free radical scavenging activity. At high concentration all standard compounds, GA, CA and CGA able to fully scavenge DPPH free radical, except CAF. However, the most sensitive compound, GA reaches plateau DPPH free radical scavenging at 1.56 µg/mL the lowest concentration, while CA requires 1x GA concentration at 3.13 µg/mL and CGA only fully scavenge the DPPH free radicals at 12.5 µg/mL, 6x higher concentration than GA.

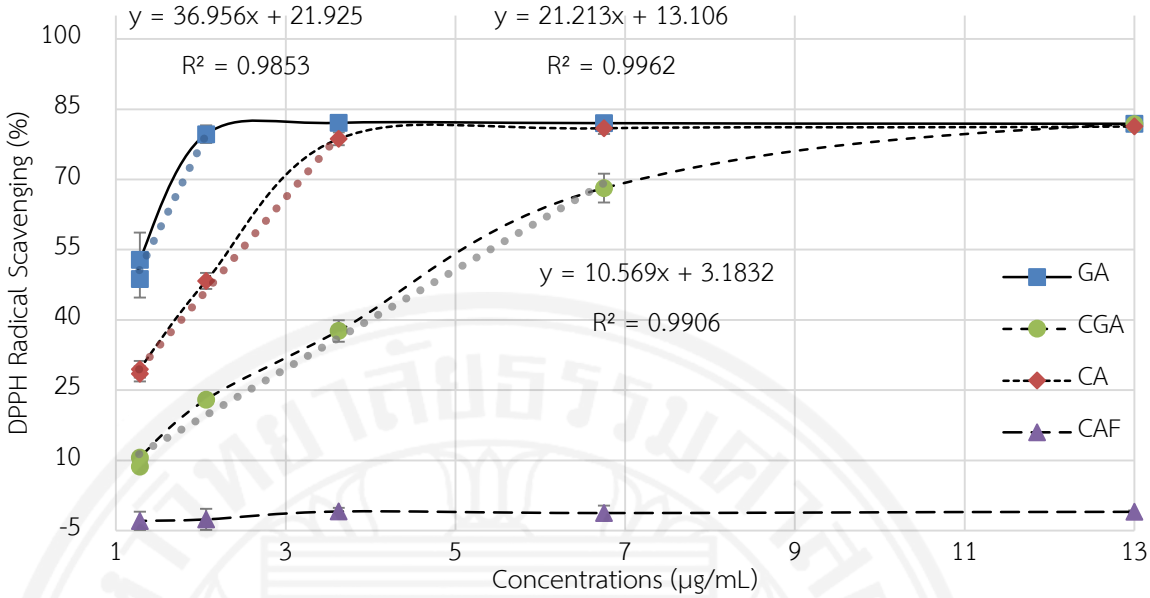


Figure 6: GA, CA, CGA and CAF DPPH free radical scavenging activity profile

Table 17: Summary of WD, RD and EM RCR extract DPPH free radical scavenging activity at 0.1 mg/mL and 50% antioxidant activity inhibition concentration (IC_{50}) alongside CA, CGA and CAF pure standards and GA as control.

Extraction method	(D) DPPH radical scavenging at 0.1mg/mL (%)	(I) IC_{50} (µg/mL)
WD	52.69 ± 15.91^{c}	86.03 ± 15.00^{e}
RD	62.54 ± 15.07^{c}	62.92 ± 12.35^{ef}
EM	78.54 ± 1.94^{d}	28.62 ± 1.89^{fg}
SE	77.06 ± 5.56^{d}	22.17 ± 3.51^{g}
E	55.83 ± 6.68^{c}	83.79 ± 7.96^{e}
GA	85.13 ± 0.68^{d}	0.70 ± 0.14^{g}
CA	84.69 ± 0.11^{d}	1.79 ± 0.07^{g}
CGA	85.99 ± 0.55^{d}	4.43 ± 0.18^{g}
CAF	0	195.44 ± 100.84^{e}

$^{c, d}$ ANOVA Tukey HSD mean significant difference at 0.758, and 0.066 respectively, *p*-value 0.05.

$^{e, f, g}$ ANOVA Tukey HSD mean significant difference at 0.511, 0.123 and 0.298 respectively, *p*-value 0.05.

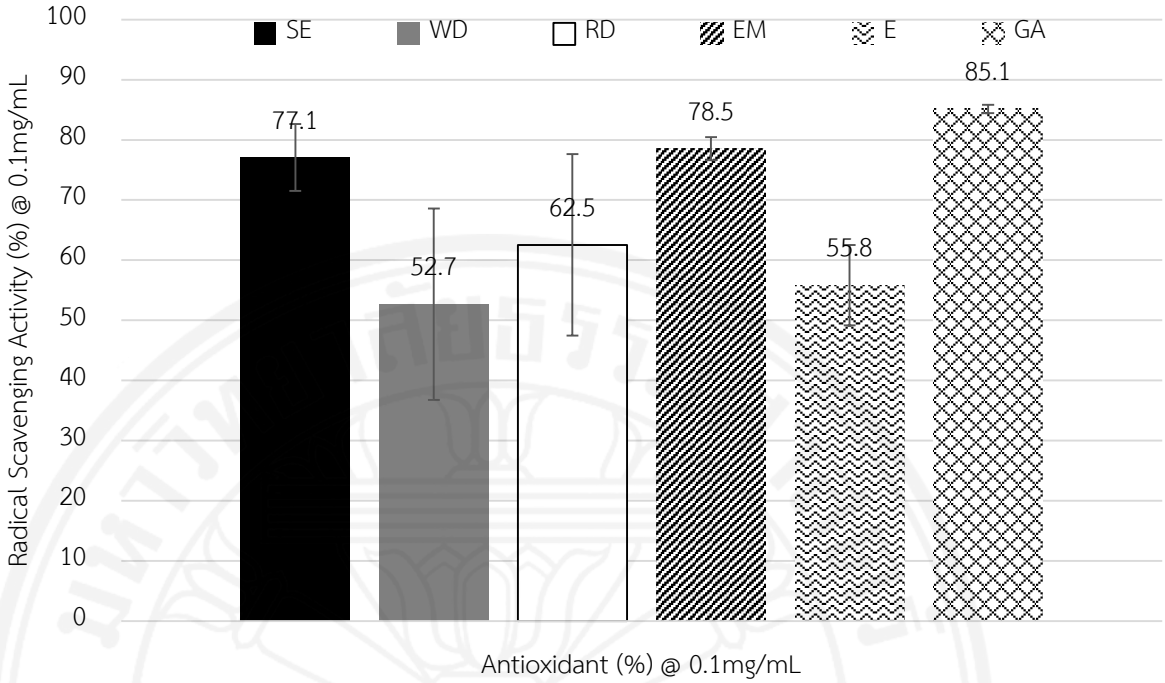


Figure 7: WD, RD, EM, SE RCR extract and Espresso DPPH free radical scavenging antioxidant activity (%) against GA standard at 0.1 mg/mL concentration

At 0.1 mg/mL, EM extract had the most active radical scavenging respond to free DPPH free radicals at 78.54 ± 1.94% scavenging activity, followed by RD and WD at 62.54 ± 15.1% and 52.69 ± 17.8% respectively, against GA standard at 85.12 ± 0.70. 95% ethanol solvent used in maceration extraction was better than water decoction at extracting polar compounds with high antioxidant activity from RCR. It also was reported as the most antioxidant active solvent extract for herbal or other plants materials extraction (33,98). Tukey HSD post-hoc analysis shows EM antioxidant activity at 0.1 mg/mL was significantly differed than WD and RD, additionally it was placed in high group indicating it is significantly equal to GA control. At 84.76 ± 0.11% and 85.99 ± 0.55% DPPH free radical antioxidant scavenging activity, CA and CGA standards respectively were comparable to GA, meanwhile CAF standards shows no activity.

4.2.2. Antioxidant activity half inhibition concentration (IC_{50})

IC_{50} is a parameter used to determine the concentration of the antioxidant sample required to reduce half (50%) of the total free radical amount in the system (99). A sensitive or active compound or extract shows lower IC_{50} value than less sensitive compound. In DPPH free radical scavenging system, GA is one of the antioxidant standard compound used. GA shows very sensitive IC_{50} at 3.9 ± 0.32 µg/mL, followed by EM, RD and WD at 28.6 ± 3.79, 62.9 ± 24.72 and 86.0 ± 30.0 µg/mL IC_{50} values respectively. Meanwhile, IC_{50} of CA and CGA standards were achieved at 1.79 ± 0.07 and 4.43 ± 0.19 µg/mL respectively. Moreover, CAF show no free radical scavenging activity even at its concentration as high as 0.1 mg/mL, other study also confirms its poor radical scavenging performance (15). CA DPPH free radical antioxidant scavenging and IC_{50} was highly active and perform very closely to GA, meanwhile CGA perform half the efficiency of CA and GA in free radical model. However, in lipid oxidation mode, CGA was reportedly performed better than CA, while CA shows half of CGA performance (15).

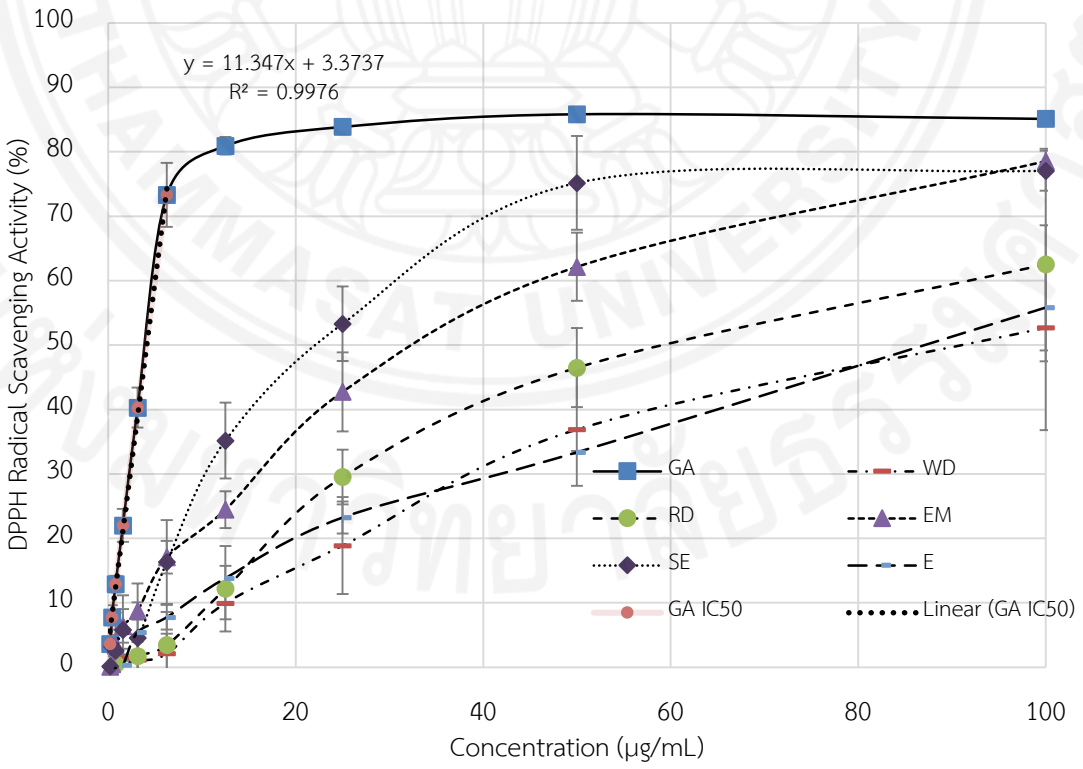


Figure 8: WD, RD, EM, SE RCR extract response to DPPH radical scavenging activity in serially diluted concentrations

4.3. Total phenolic content (TPC) quantification

4.3.1. Comparison of GA, CA, CGA and CAF standard curve

TPC was performed according to modified Folin-Ciocalteu method (87,94). Absorbance of GA TPC reaction at 765 nm was plotted against its serial dilution concentration for standard curve and shows a high regression linearity of y = 9.229 x + 0.0722 with 0.99864 regression coefficient (r^2) value, it was used as GA equivalence (GAE) concentration (mg GAE / g RCR) standard reference. CA also shows a closely equal quality of regression linearity at y = 9.8802 x + 1.1519 with 0.98417 regression coefficient. Trailing behind is CGA at y = 4.5501 + 0.1151 and 0.97829 regression coefficient. CAF did not show any content of TPC at all CAF concentration prepared. GA-TPC linear regression was used as the standard curve for TPC determination because its phenolic compounds are more sensitive to react in TPC assay.

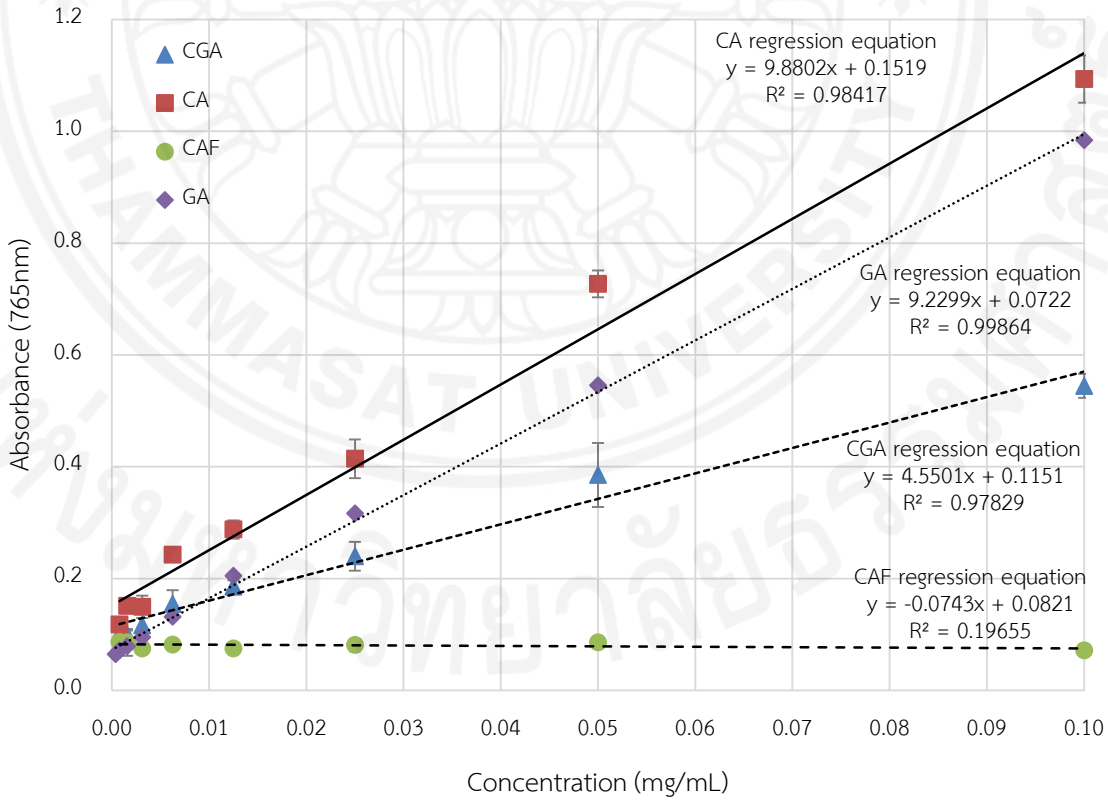


Figure 9: Comparison of GA, CA, CGA and CAF TPC standard curve

4.3.2. TPC of RCR extract in WD, RD, EM, SE and espresso

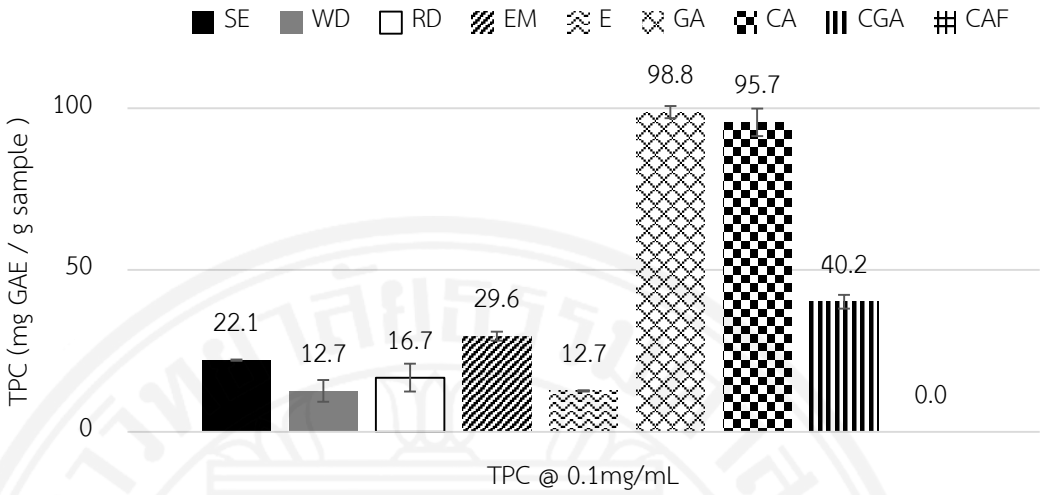


Figure 10: TPC yields in WD, RD, EM, SE RCR extract and expresso in comparison to GA, CA, CGA and CAF standards at 0.1 mg/mL of each sample concentration

RCR extract shows high quantity TPC was observed in EM at 29.6 ± 1.35 mg GAE/g RCR followed by RD and WD, at 16.7 ± 4.32 and 12.7 ± 3.36 mg GAE/g RCR respectively, revealing notable equivalent with its RCR extract DPPH free radical antioxidant scavenging activity.

Table 18: Summary of WD, RD and EM RCR extract total phenolic content (TPC) at 0.1 mg/mL concentration alongside CA, CGA and CAF pure standards and GA as control.

Extraction condition	(T) TPC (mg GAE/g RCR)
WD	12.65 ± 3.36^{g}
RD	16.74 ± 4.32^{g}
EM	29.57 ± 1.35^{h}
SE	22.13 ± 0.18^{h}
E	12.65 ± 0.18^{g}
GA	98.79 ± 1.89^{j}
CA	95.67 ± 4.24^{j}
CGA	40.81 ± 2.13^{i}
CAF	0

$^{g, h, i, j}$ ANOVA Tukey HSD mean significant difference at 0.609, 1.00, 1.00 and 0.815 respectively, *p*-value 0.05.

4.4. DPPH and TPC

95% ethanol solvent in EM is the most suitable and efficient solvent system used to extract high yield of RCR extract phenolic compounds. It has been reported that organic solvent such as ethanol and methanol provides better solubility than water towards phenolic compounds (38,39,100), however it produces lower crude extract than water as solvent. Thus, a delicate balance of organic solvent and water for extraction solvent system is recommended for optimum antioxidant, phenolic compound and crude extract yield. Previous study have found and determined that the optimum balance for phenolic and antioxidant compound rich extract peaked at 60 – 70% methanol (38,39) and 20 - 80% ethanol (11,12,37,38) all claim up to 90% of phenolic compounds recovery. Contrarily other reports also shows that water extract also a good extraction solvent due to higher crude yields (15) and antioxidant activity (38).

4.4.1. Correlation between RCR extract TPC and DPPH antioxidant activity

Correlation study of RCR extract between TPC and DPP free radical antioxidant scavenging activity was performed and shown good correlation coefficient value (r^2) between both biological markers for SE, EM, RW and WD at 0.830, 0.653, 0.875 and 0.940 respectively. Conclusively, RCR extract DPPH free radical antioxidant scavenging activity was a direct response to the content of its TPC quantity.

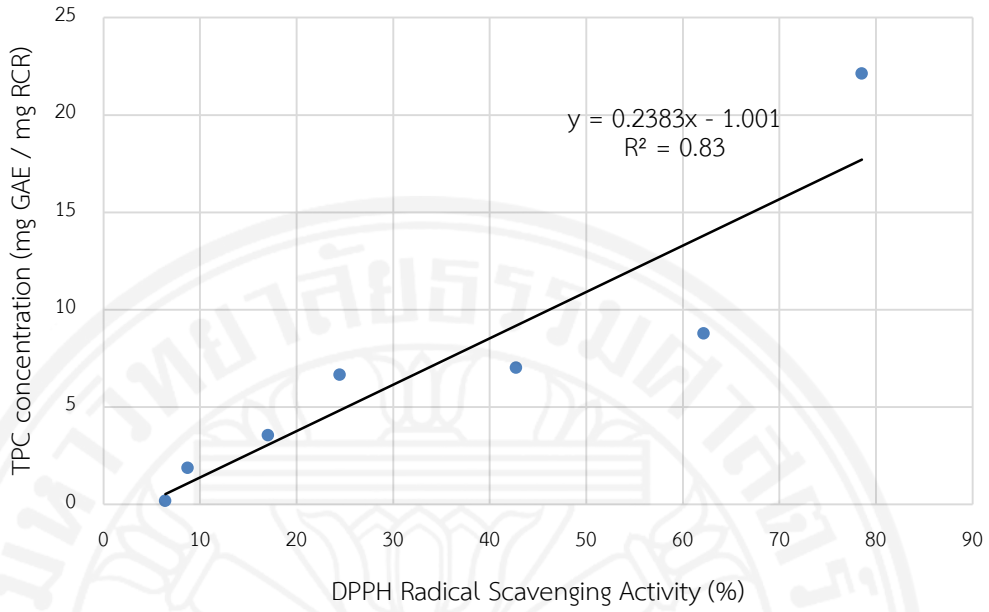


Figure 11: Correlation of SE TPC and DPPH antioxidant activity

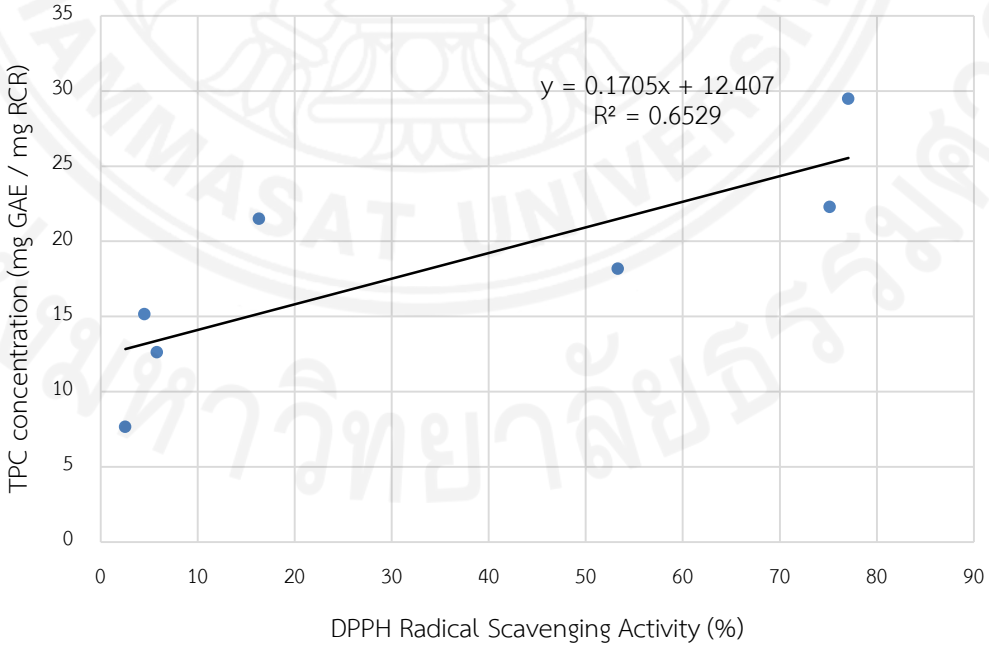


Figure 12: Correlation EM TPC and DPPH antioxidant activity

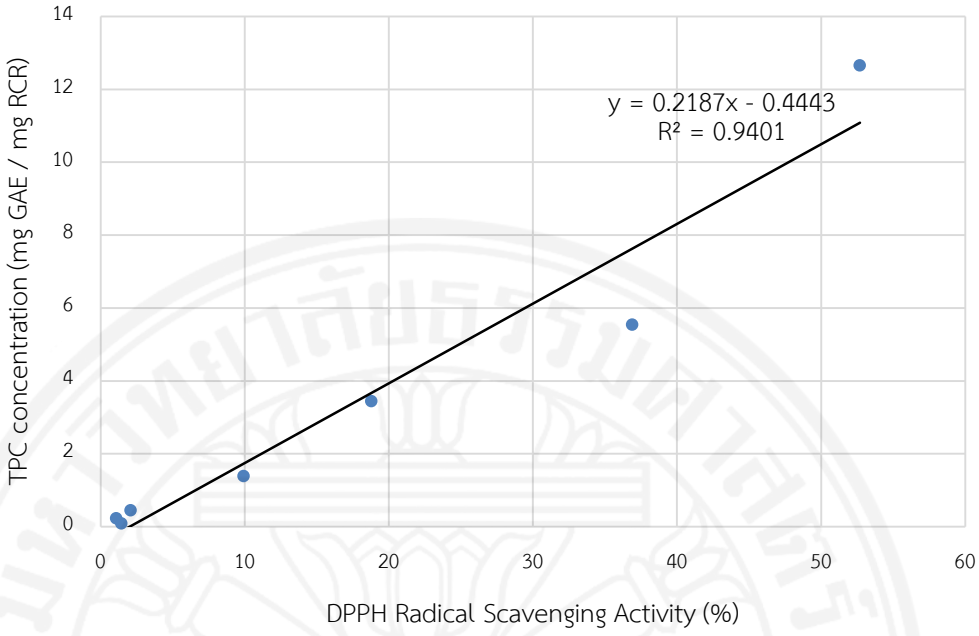


Figure 13: Correlation WD TPC and DPPH antioxidant activity

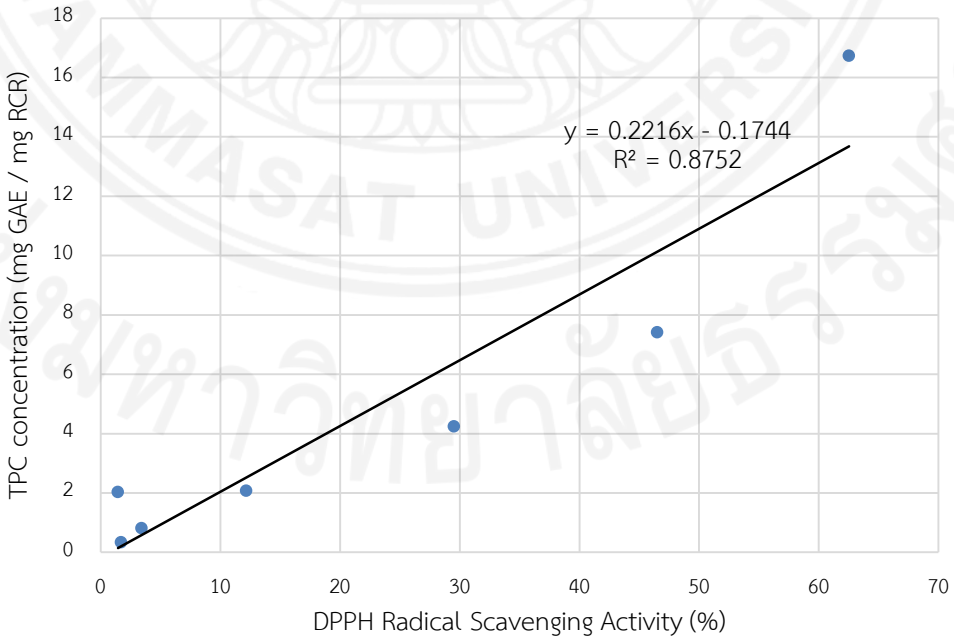


Figure 14: Correlation RD TPC and DPPH antioxidant activity

4.5. HPLC quantification of RCR extract

HPLC system was used to separate, identify and quantify RCR extract. The system used in this study was adopted and modified based on an earlier preparation proposed by Yen *et. al*. and Hakkinen*et. al.,* (15,89). The HPLC gradient elution system was designed for phenolic and flavonoid compound separation in RCR and other herbal extracts (101). The mobile phase elution was running at 1 mL/min flow rate in 45 duration for each 20 µL sample volume injection. HPLC separation for RCR extract for CA, CGA and CAF standard compounds were optimized by adjusting longer time for buffer and found good separation resolution for CA, CGA and CAF at 28 – 29 (280 and 326 nm), 25 – 26 (326 nm) and 25 – 26 (280 nm) minute, respectively.

4.5.1. CA, CGA and CAF compounds standard curve

CGA standard procured from Sigma-Aldrich (USA) was produced from ≥95% titration of 3-(3,4-Dihydroxycinnamoyl) quinic, one of the main CGA isomer. Previously, 5-(3,4-Dihydroxycinnamoyl) quinic or also known as 5-caffeoylquinic acid was the only single compound identity to represent CGA, before more than a dozen of its isomers were later discovered (102). All CGA isomers which has been identified were classified as main CQA isomers (3-0-caffeoylquinic, 4-0-caffeoylquinic, and 5-0-caffeoylquinic), FQA isomers (3-feruloylqunic, 4-0-feruloylquinic, and 5-0-feruloylquinic), di-CQA isomers (3,4-0-dicaffeoylquinic, 3,5-0-dicaffeoylquinic, and 4,5-0-dicaffeoylquini), *p*-coumaroylquinic isomers (3-pCoQA, 4-pCoQA, and 5-pCoQA) and six mixed diester isomers of caffeoylferuloy-quinic acids (3,51,103). The quantity of the each isomers varies differently across species, regions and processing method (6,18,34,51,104). Three main CQA isomers made up for almost 83 percent of total CGA identified in coffee (3), while the highest among them is 5-CQA which comprises 36 – 42 percent (18). In general, the quantity of each isomers identified in coffee were in the following decreasing order, in decreasing order, 5-CQA > 4-CQA > 3-CQA > 5-FQA > 4-FQA > 3-FQA > 3,4-diCQA > 4,5-diCQA; 3,5-diCQA (18). UV spectrum profile of all CGA isomer compound shows identical $\boldsymbol{\lambda}$ max which peaked at 220, 280 and 320 nm wavelength (12).

3-(3,4-Dihydroxycinnamoyl) quinic, the CGA standard compound was characterized via HPLC and its chromatogram peak was identified at both 280 and 320 nm wavelength spectrum detection. However, at 280 nm wavelength its **λ** max was eluted at almost the same elution time as CAF, thus CGA peak overlaps and obscured under CAF peak which was in much higher in concentration. CGA peak was only able to be separated and quantified at 320 nm wavelength, where CAF spectrum profile did not show any absorbance at 320 nm. CAF standard compound was only detected and quantified in the chromatogram at 280 nm wavelength. Since CGA is an esterified CA, thus both compounds share a significantly similar spectrum profile and **λ** max, however CA compound is de-ester of CGA, lacking quinic acid, thus it has lower molecular weight and less polar than CGA. Low molecule weight low polarity compounds were eluted later in a normal phase HPLC.

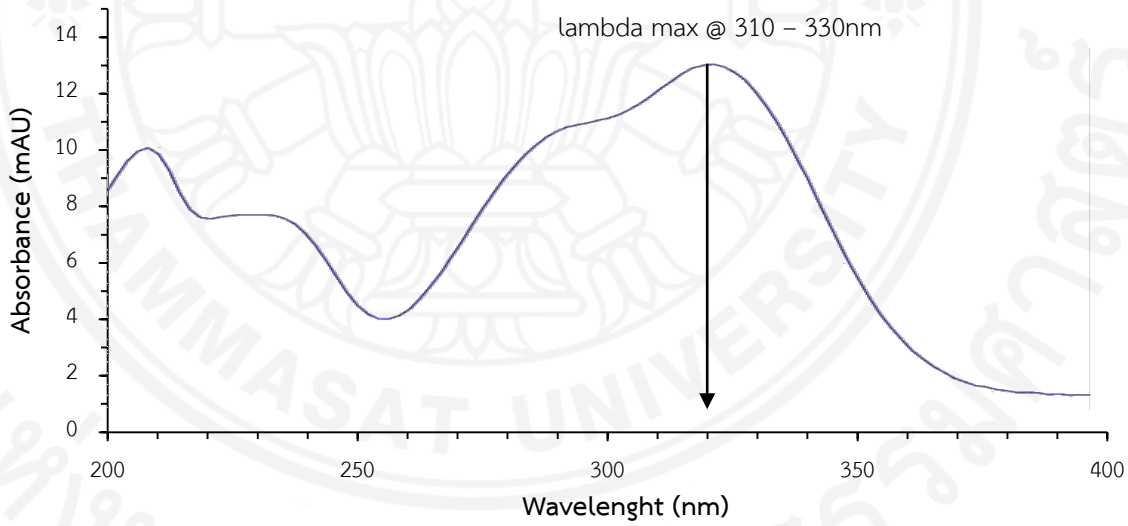


Figure 15: A typical spectrum profile of a structurally related CA and CGA compounds.

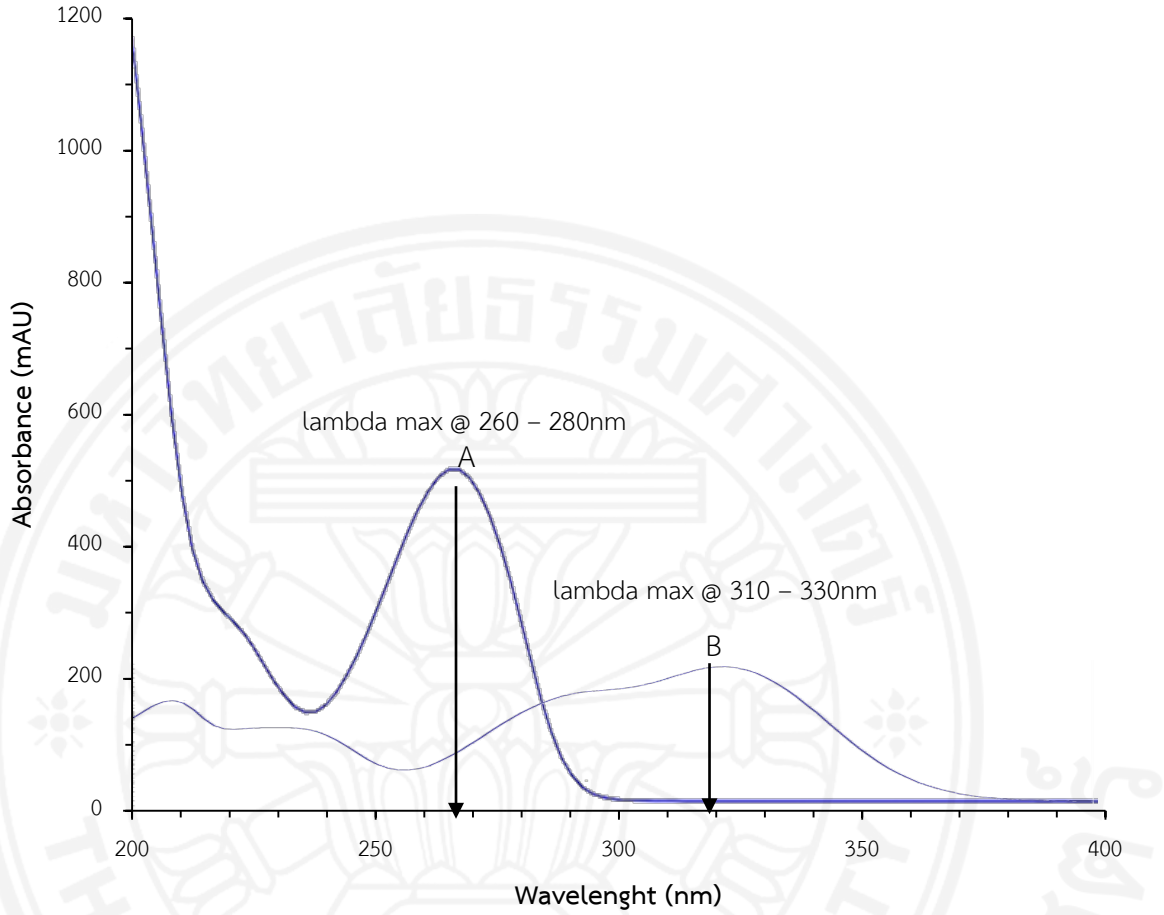


Figure 16: Spectrum profile of CAF and CGA compounds overlapped with each other showing contrasting peak profile at (A) 280 nm and (B) 320 nm lambda max, respectively.

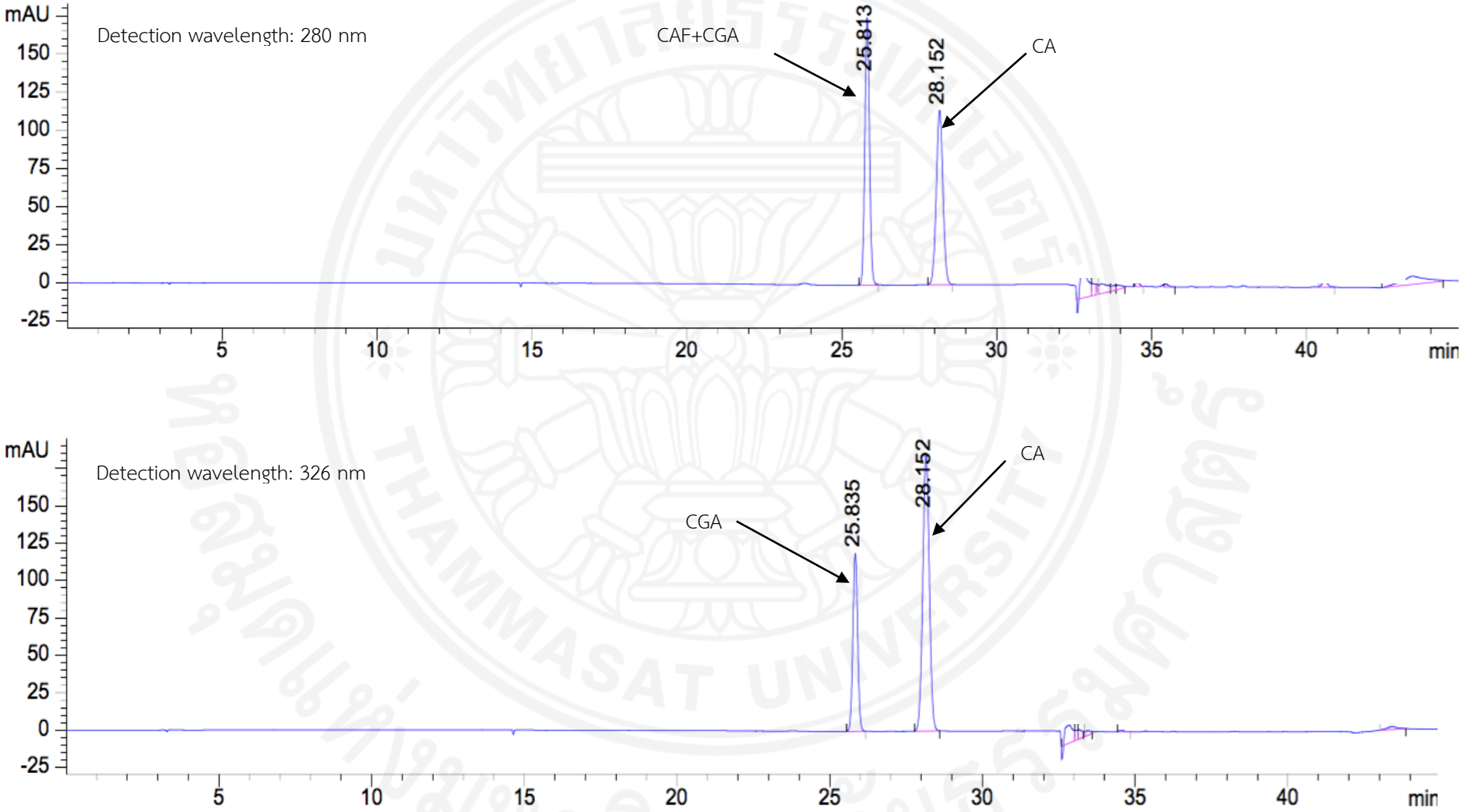


Figure 17: CA, CGA and CAF standards compounds separated and identified by HPLC chromatogram in 280 and 326 in 45 minute elution time

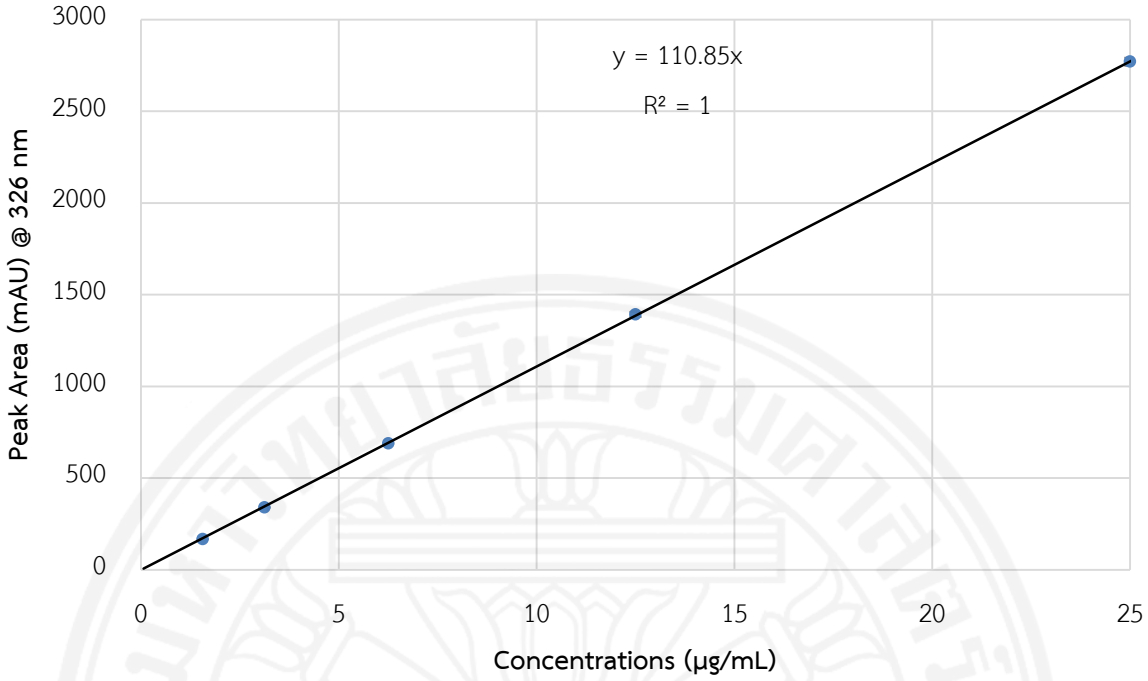


Figure 18: CA standard curve

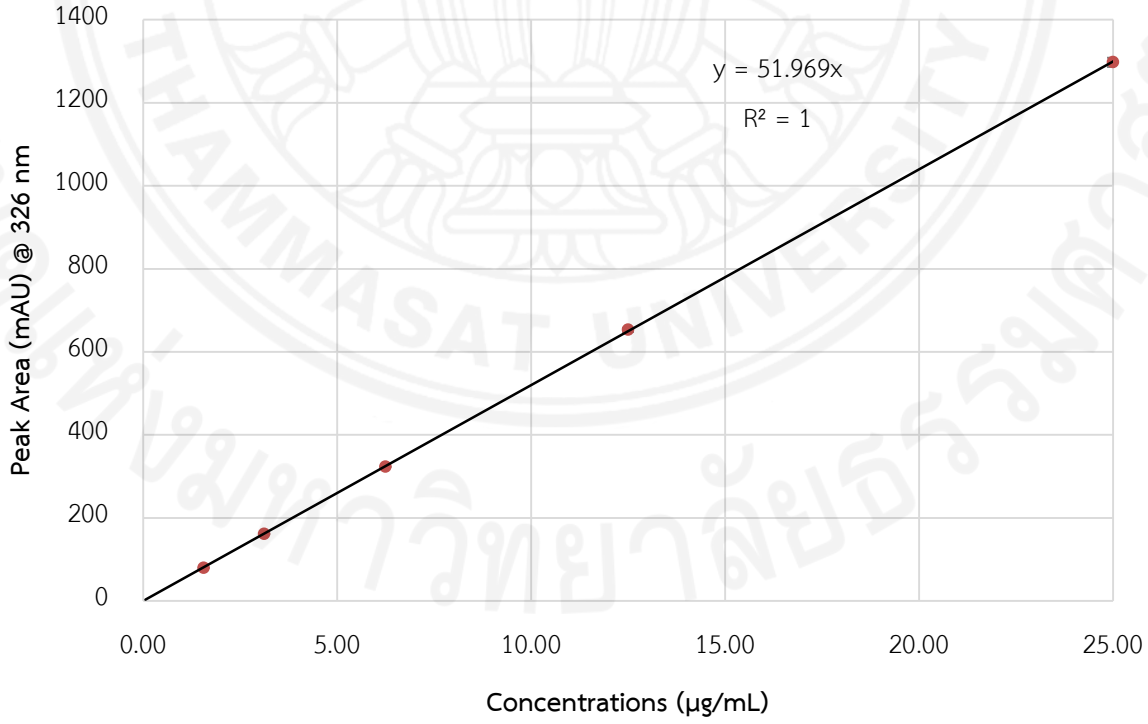


Figure 19: CGA standard curve

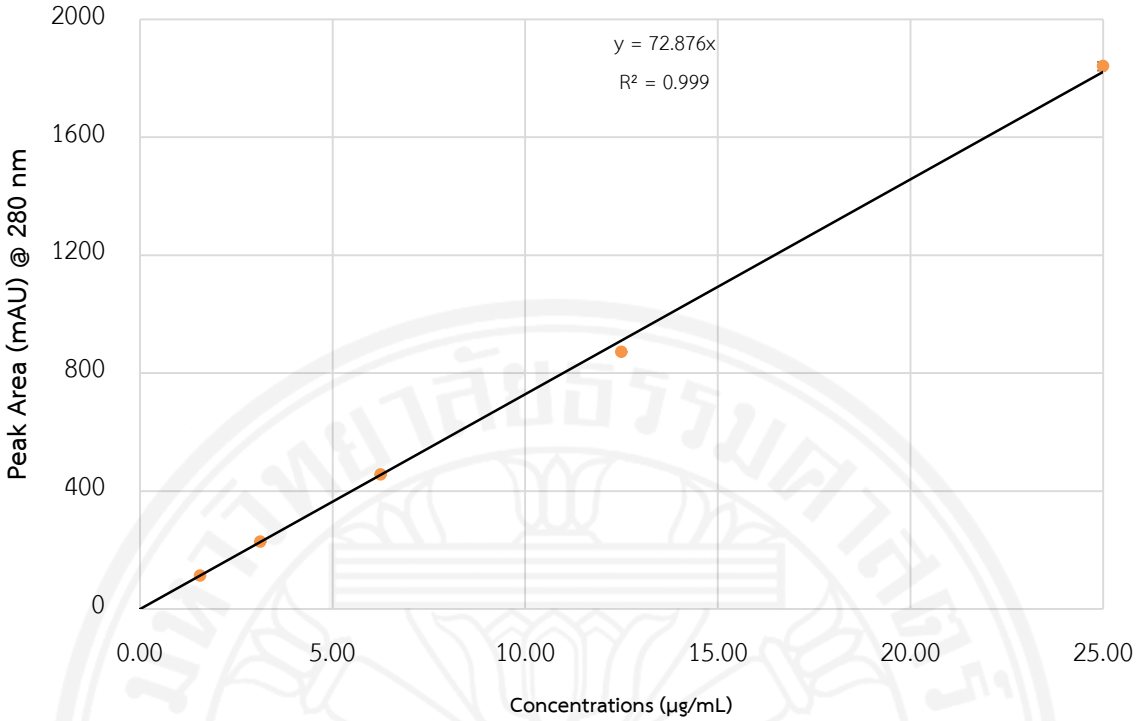


Figure 20: CAF standard curve

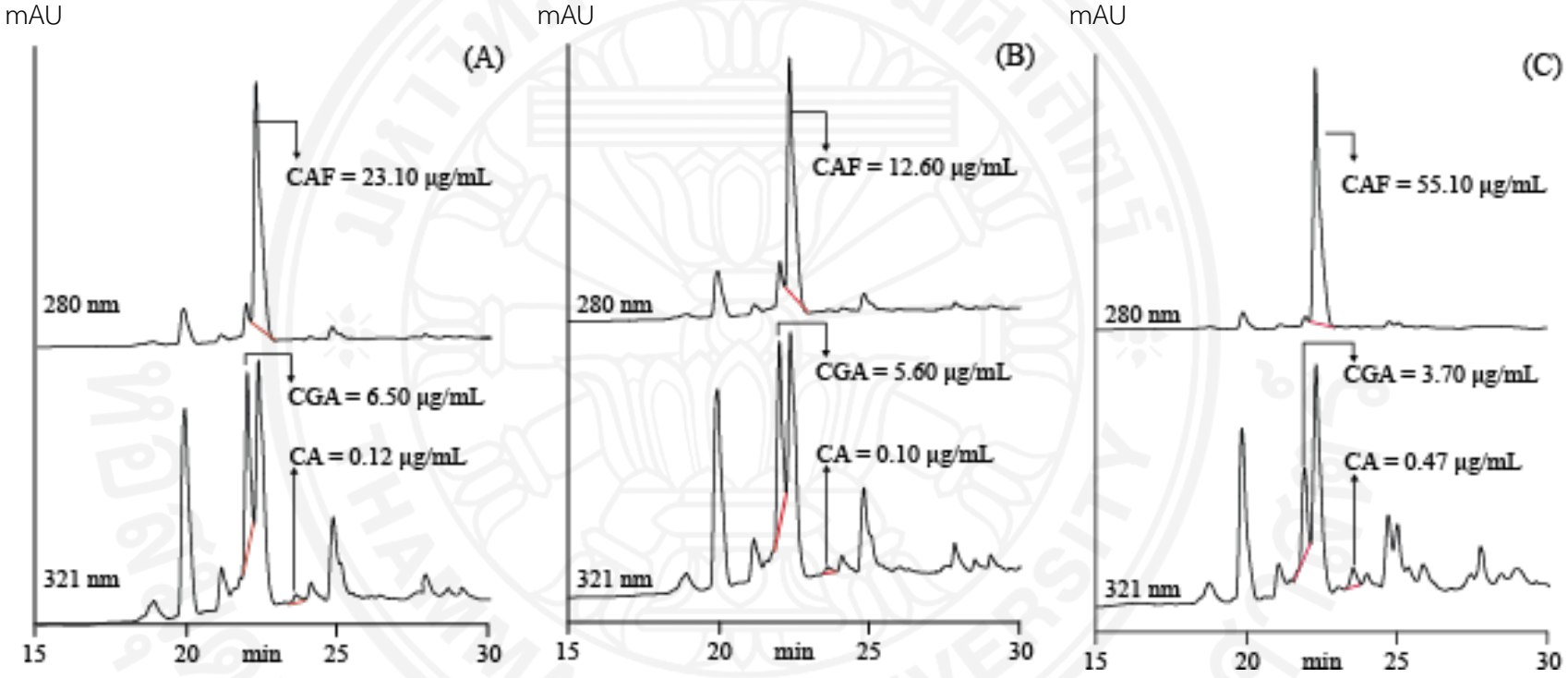


Figure 21. HPLC chromatogram profiles of water decoction, WD (A), 95% ethanol maceration residue water extraction, RD (B) and 95% ethanol extraction, EM (C) with peaks identified as CAF, CGA and CA compounds

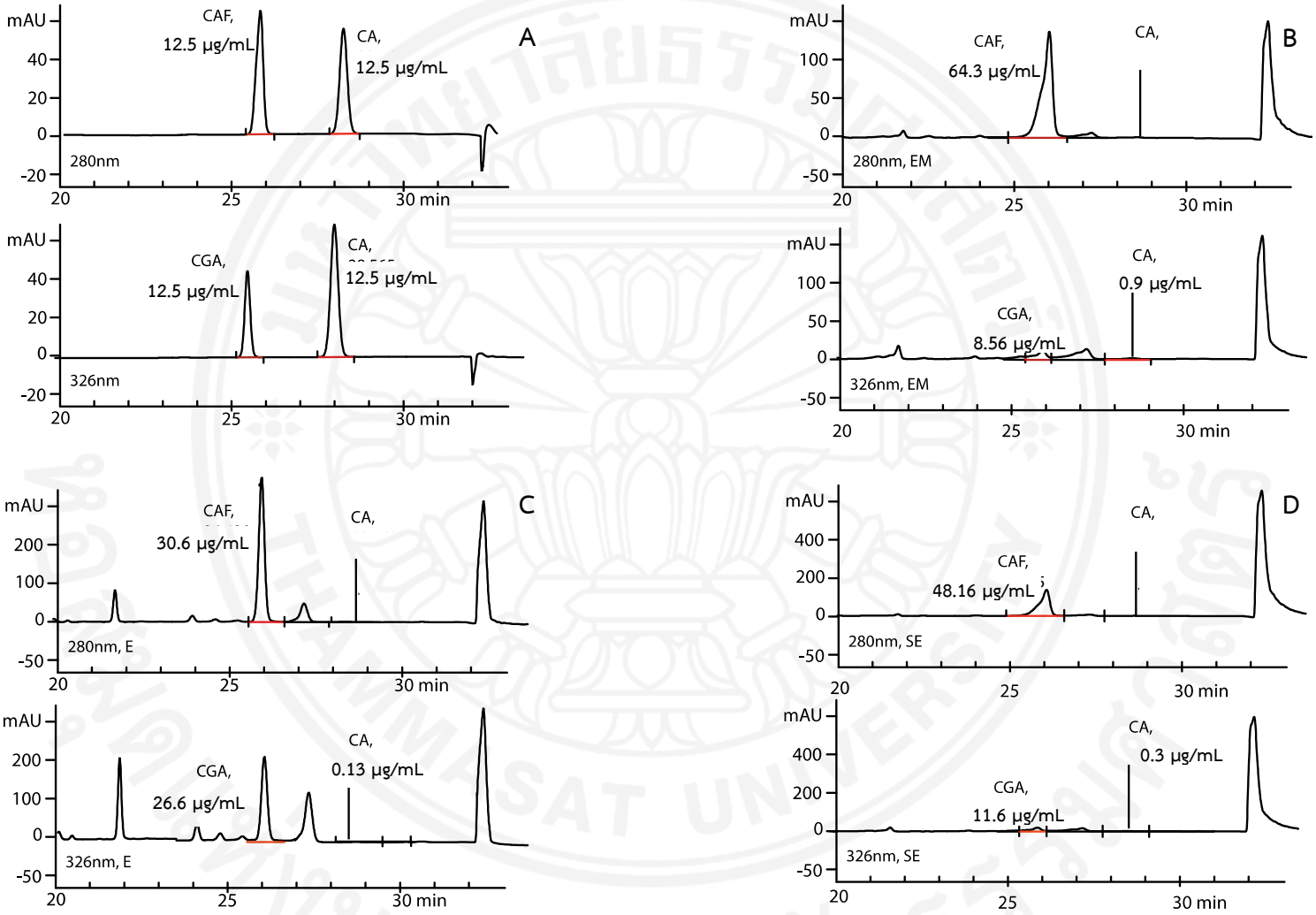


Figure 22. HPLC chromatogram profiles of CA, CGA and CAF standard at 12.5 µg/mL (A), 95% ethanol maceration, EM (B) and espresso, E (C) and 95% ethanol Soxhlet extraction, SE (D) with peaks identified as CAF, CGA and CA compounds

4.5.2. Quantification of CA, CGA and CAF in RCR extracts

CA, CGA and CAF peak area (mAU) obtained from its HPLC chromatogram were plotted against its diluted concentration (µg/mL) obtained good standard curve linearity and correlation coefficient (r^2) at 0.999 each. HPLC quantification of RCR extract shows that CAF yields are the highest among CA and CGA compounds, either in WD, RD or EM extraction method at 2.31 ± 0.09, 1.29 ± 0.11 and 5.51 ± 0.56 (%, w/w) respectively. However, CAF standard did not perform very well as an active antioxidant agent neither high with phenolic compounds. CGA and followed by CA are compounds with the lowest yield quantified from all RCR extracts. EM yields 0.047 ± 0.01 (%, w/w) CA, higher than WD and RW at 0.01 ± 0.002 and 0.012 ± 0.00 respectively. Meanwhile, WD and RW are yields higher CGA at 0.65 ± 0.02 and 0.56 ± 0.07 respectively, while EM yields 0.37 ± 0.03, half of WD and RW CGA content.

Table 19. Comparison of CAF, CGA and CA compound yields in SE (Soxhlet extraction), WD (water decoction), RD (residue water decoction), EM (ethanol maceration) RCR extraction and E (espresso)

Bioactive Compounds	**Bioactive compound yields (mg compound /g RCR)**				
	SE	WD	RD	EM	E
CA	0.32 ± 0.02^{b}	0.12 ± 0.03^{a}	0.10 ± 0.02^{a}	0.47 ± 0.08^{bc}	0.13 ± 0.05^{a}
CGA	11.55 ± 2.33^{e}	6.51 ± 0.25^{d}	$5.60 \pm .066^{d}$	3.70 ± 0.30^{d}	26.66 ± 7.37^{f}
CAF	48.16 ± 7.22^{i}	23.12 ± 0.92^{gh}	12.55 ± 0.11^{g}	55.08 ± 5.60^{ij}	30.57 ± 7.22^{g}

[a, b, c] Tukey HSD post hoc ANOVA difference significant at 0.13, 0.07 and 0.14, respectively.

[d, e, f] Tukey HSD post hoc ANOVA difference significant at 0.60, 0.08 and 1.00, respectively.

[g, h, I, j] Tukey HSD post hoc ANOVA difference significant at 0.95, 0.14, 0.45 and 0.60, respectively.

EM was able to extract CA compound higher than WD and RW, however perform weaker extraction towards CGA compounds than WD and RD. CA compounds has highly active antioxidant activity and high content of phenolic compounds than CGA and CAF. Its extraction yield from RCR is higher in ethanol maceration than water decoction, because ethanol is a better extraction solvent which has high affinity and dissolves phenolic compound. Contrarily, CGA are better

extracted in water decoction than ethanol maceration, coffee bean brewed with hot water are rich with CGA and CAF, meanwhile maintains it CA content into RCR (15).

Once 95% ethanol has been identified as an efficient solvent in extracting RCR extract rich with phenolic compound and antioxidant potent such as CA, 95% EM extraction method was compared against 95% ethanol Soxhlet extraction (SE). In EM, its crude extract yield is the lowest than WD and RD, as water was reported to be better than methanol or ethanol in extracting higher yield of crude extract (15) and at 95% ethanol composition (11,12,37,38). In SE of RCR extracts with 95% ethanol, its crude extract yield improves significantly than maceration at 3.24 ± 0.37 versus 0.51 ± 0.01 (%, w/w) respectively. The increase of crude extract yield may indicates that SE is more efficient in extracting more phenolic compounds than EM. Meanwhile, total phenolic content identified from SE at 2.21 ± 0.18 mg GAE/g RCR is closely comparable to EM at 2.94 ± 0.43 mg GAE/g RCR. The increase of SE yield did not increase the quantity of total phenolic content in its extract. Similarly, antioxidant activity assayed in DPPH• scavenging activity of Soxhlet and maceration extractions shows an equally active scavenging activity at 0.1 mg/mL of 77.06 ± 5.56 and 78.54 ± 1.94 percent and IC_{50} at 0.0221 ± 0.0035 and 0.0286 ± 0.0038 mg/mL, respectively.

Furthermore, HPLC analysis comparing SE and EM remarkably found that SE extract yields higher CGA but lower CA and CAF than EM. Even though in 95% ethanol solvent, its performance in extracting CGA compound is superior to WD and RD, contradicts with an earlier assumption among supporting WD and RD against 95% ethanol solvent in EM. For comparison, a cup of espresso brew of roasted coffee residue is mostly rich in CGA than CA.

4.6. Niosome

4.6.1. Niosome characterization

4.6.1.1. Appearance

Niosome prepared was visualized as milky and cloudy, sometimes masking the original color appearance of the loading agent such brown and green of RCR extract and GA, respectively. In the visual observation, it appears that niosome formulation prepared with more niosome forming materials resulting in greater cloudy and milky appearance. It is assumed that the cloudy formation was the result of niosome vesicles formed and entrapped the loading agent.

4.6.1.2. Size

In this study, the size of niosome vesicles prepared obtained was found to be in the ranges of 1 to 10 microns, the size estimates is in agreement with the recommended preparation method (56). Appropriately, the most suitable particle analyzer used was a laser diffraction (LD) particle analyzer (LA-950 Laser Scattering Particle Size Analyzer, HORIBA, USA). LD particle analyzer lacks zeta potential measurement capabilities, thus zeta potential values of the niosomes were not able to be determined. The parameters reportable by LD particle analyzer are mean, median and mode size, standard deviation or polydispersity index (PDI), and histogram of niosome size distribution. For the measurement analysis, niosome suspension was diluted 10x with 10 mM PBS 7.4 pH in 1 mL Eppendorf tube and homogenized before injected into the analyzer. The measurement analysis was performed by the scientist at Central Scientific Instrument Center (CSIC), Faculty of Science and Technology, Thammasat University, Pathumthani, Thailand, and raw data files were obtained.

4.6.1.3. Entrapment efficiency (EE)

4.6.1.3.1. Loading agent spectrum lambda max

The loading agent spectrum lambda max was determined prior to the estimation of niosome entrapment efficiency. The spectrum profile of GA, CA and RCR extract were obtained and their lambda max were 270, 280 and 275 nm, respectively (Figure 23, Figure 24, Figure 25). The lambda max values identified indicates that the compounds are phenolic compounds that contains phenolic ring structure which is identifiable around 250 – 300 nm wavelength (101).

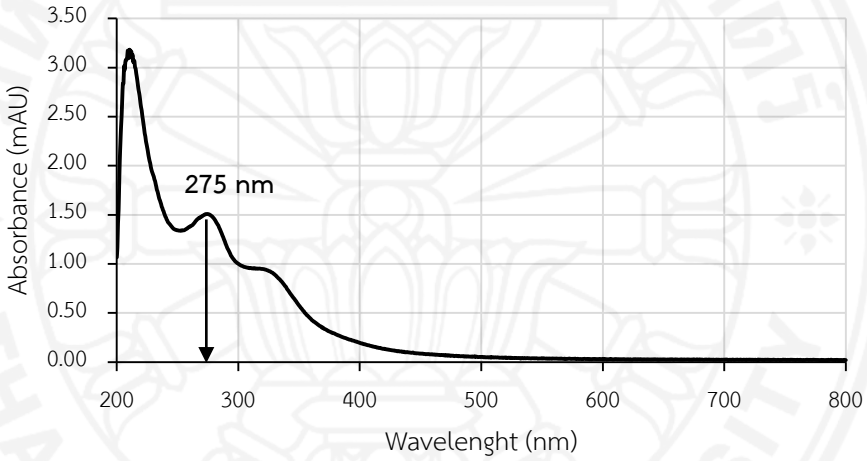


Figure 23: RCR extract spectrum profile, lambda max found at 275nm

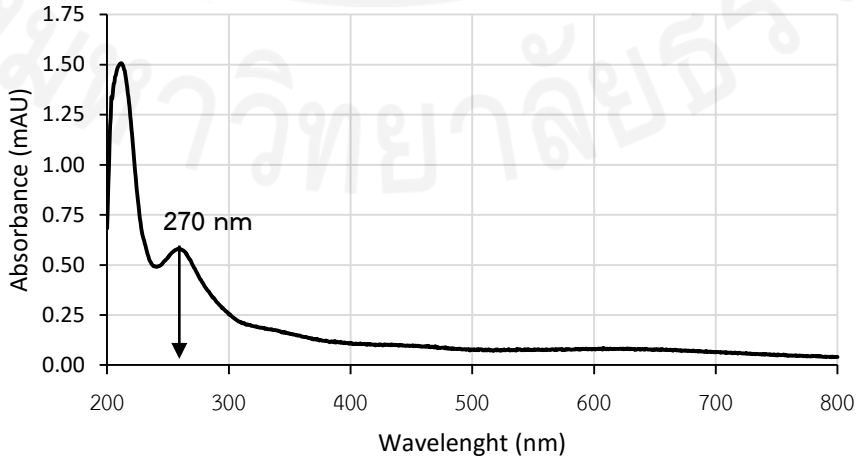


Figure 24; GA standard spectrum profile , lambda max found at 270nm

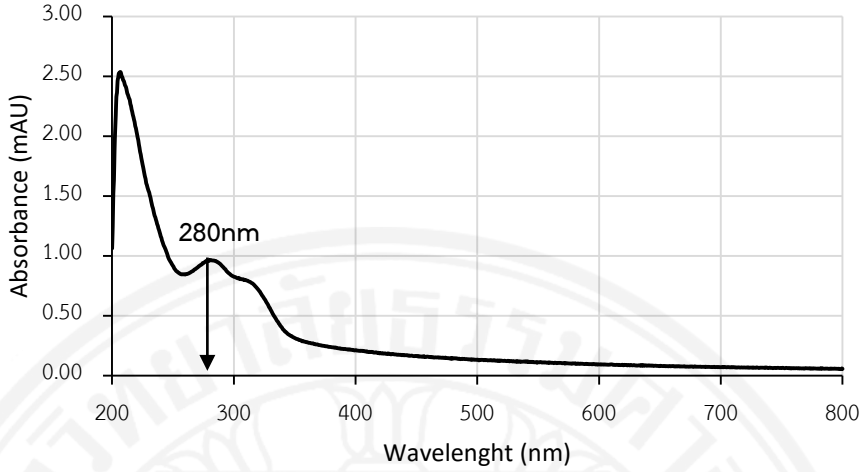


Figure 25: CA standard spectrum profile, lambda max found at 280nm

4.6.2. Effect of niosome preparation formulations

4.6.2.1. Formulation 1: effect of total niosome molar and loading agents

Table 20: Formulation 1 – effect of total molar niosome on different loading agents

Code	Molar ratio (µmol)	RCR EE (Abs)	GA EE (Abs)	CA EE (Abs)
1.1	1:1 (150:150)	15.46 ± 0.19[a]	24.11 ± 9.01[a]	39.18 ± 6.11[a]
1.2	2:1 (200:100)	16.19 ± 10.11[a]	15.29 ± 8.62[a]	-3.62 ± 0.13[b]
1.3	2:1 (300:150)	8.27 ± 1.36[a]	12.98 ± 3.85[a]	-4.73 ± 2.92[b]

[a,b]Tukey HSD shows no significant differences EE between RCR, GA and CA at 0.05. Between formulations, 1.1 significantly higher than 1.2 & 1.3 at 1.00, p = 0.05. Among loading agents, only CA shows significant EE different between 1.3, 1.2 and 1.1 at 1.00, p = 0.05.

Entrapment efficiency (EE) of GA, CA and RCR extract loading agents in niosome suspension prepared from formulation 1 are presented in Table 20. In comparison of loading agents, RCR extract and pure standard compounds (GA and CA) shows different entrapment efficiency. RCR extract obtained the highest EE of 16.19 ±

10.11% and 15.46 ± 0.19% in the formulation of 2:1 and 1:1 (300 µmol, S60:C) ratio. Meanwhile, an increase of total molar of niosome materials into 450 µmol (2:1, S60:C) reduces RCR extract EE to 8.27 ± 1.37%.

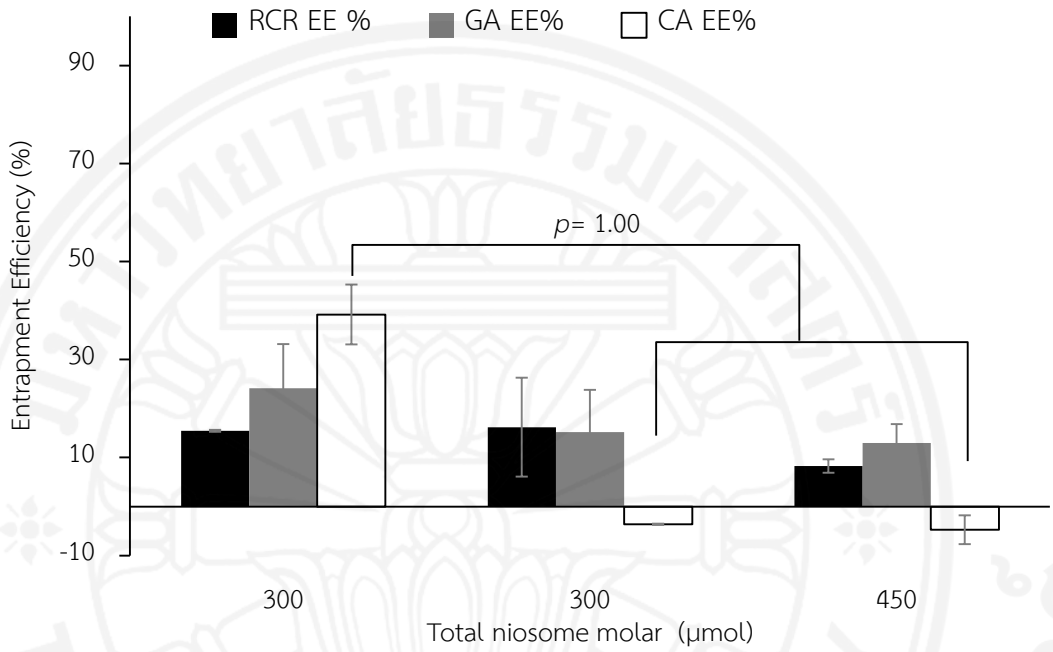


Figure 26: Formulation 1 EE of different loading agents with S60:C at 1:1 and 2:1, 300µmol total niosome materials (S60: Span 60, C: cholesterol)

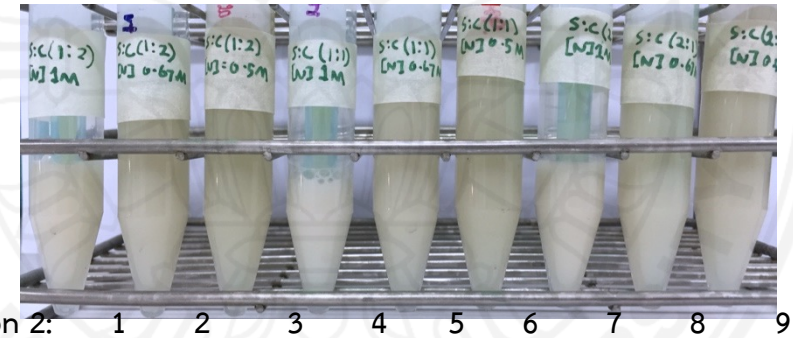
Meanwhile, GA and CA loading agent were found in its highest EE at 1:1 (150:150 µmol, S60:C) ratio niosome preparation at 24.11 ± 9.01 and 39.18 ± 6.11%, respectively. At 2:1 (S60:C) molar ratio, from 300 to 450 µmol total niosome materials, CA shows no entrapment in the niosome and GA entrapment was reduced. It was assumed that GA shows leakages and low entrapment, which is almost half of its EE compared to 1:1 (S60:C) ratio formulation. The reduction was associated with the reduction of cholesterol molar ratio from 50% (mol/mol) into 33% (mol/mol).

At 1:1 (S60:C) 300 µmol total molar niosome, CA shows the highest EE followed by GA and RCR extract. CA (180 g/mol) and GA (170 g/mol) has an almost similar structure molecule structure and molecular weight but reacts differently on

their entrapment. RCR crude extract is a partially pure extract and contains various elements and compounds which may reduce or unable to be entrapped. At 300 µmol 1:1 and 2:1 Span 60 to cholesterol ratio, RCR extract were able to be entrapped, but EE yields are below 20%, which is low.

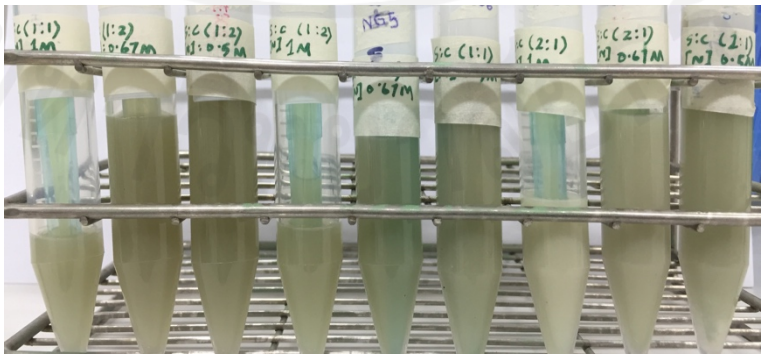
4.6.2.2. Formulation 2: effect of Span 60 and cholesterol ratio and hydration volume

The appearance of niosome suspension prepared from formulation 2, which varies the hydration volume and Span 60:cholesterol ratio is shown in Figure 27. It appears that niosome formulation prepared with 2:1 (S60:C) ratio forms a cloudier appearance than 1:2 and 1:1 ratio. Cloudier appearance may indicate the masking of the loading agents.



Formulation 2: 1 2 3 4 5 6 7 8 9

Figure 27: Formulation 2 niosome suspension appearance (RCR extract)



Formulation 2: 1 2 3 4 5 6 7 8 9

Figure 28: Formulation 2 niosome suspension appearance (GA)

Table 21: Formulation 2 – effect of Span 60, cholesterol ratio and hydration volume

Code	Molar ratio (μmol)	PBS (mL)	Niosome (mM)	GA		RCR extract	
				EE (%)	size (μm)	EE (%)	size (μm)
2.1	1:2 (200:400)	6	1.00	20.62 ± 0.45	5.245 ± 1.76	38.15 ± 0.16	2.85 ± 1.38
2.2	1:2 (200:400)	9	0.67	26.05 ± 0.29	1.874 ± 1.12	39.66 ± 0.69	5.13 ± 1.83
2.3	1:2 (200:400)	12	0.50	28.97 ± 0.28	1.912 ± 1.12	24.82 ± 0.25	1.87 ± 1.12
2.4	1:1 (300:300)	6	1.00	17.43 ± 0.54	1.912 ± 1.23	31.26 ± 0.79	2.41 ± 1.23
2.5	1:1 (300:300)	9	0.67	20.88 ± 0.23	1.912 ± 1.75	40.36 ± 0.83	5.13 ± 1.75
2.6	1:1 (300:300)	12	0.50	14.77 ±0.32	6.406 ± 2.85	42.82 ± 0.51	3.24 ± 1.38
2.7	2:1 (400:200)	6	1.00	10.96 ± 0.26	2.716 ± 1.34	49.64 ± 1.58	2.38 ± 1.19
2.8	2:1 (400:200)	9	0.67	29.02 ± 0.21	2.650 ± 1.32	41.49 ± 2.39	2.36 ± 1.17
2.9	2:1 (400:200)	12	0.50	20.98 ± 0.23	1.728 ± 1.07	40.55 ± 1.05	1.73 ± 1.08

In formulation 2, at 600 μmol total niosome and varied S60:C ratios (1:2, 1:1, 2:1), EE of GA and RCR extract are generally higher in GA than RCR extract, in the range of 25.02 – 48.52% and 10.78 – 28.88%, respectively. Overall, GA entrapment in niosomes prepared in this formulation was 2x better than RCR extracts. The highest GA EE, 48.52% is shown in formulation 2:1 (S60:C, 600 μmol) ratio hydrated with 6 mL PBS, meanwhile highest RCR extract EE, 28.88% is shown in formulation 2:1 (S60:C, 600 μmol) ratio hydrated with 9 mL PBS.

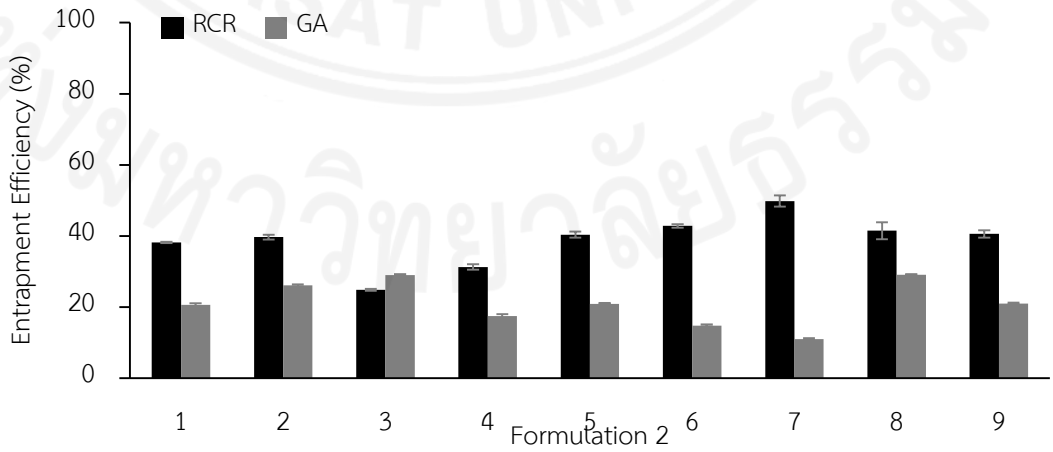


Figure 29: Formulation 2 EE of niosome prepared with RCR extract and GA loading agent

In this preparation of Span 60 with an addition of cholesterol, most of the niosome formulations with RCR extract as loading agent showed entrapment efficiency of 25 – 48%, better than GA which was around 10 – 28% EE. As for the effect of niosomes vesicle diameter sizes, all niosome preparation in this formulation was obtained in the ranges of 2 – 6 µm. GA entrapped niosome sizes obtained in the formulation 2, 3, 4, 5, 7, 8 and 9 are particularly smaller than 3 µm, except for formulation 1 and 6. Meanwhile, on average RCR crude extract loaded niosomes are smaller than 3 µm, except for the formulation 1 and 6.

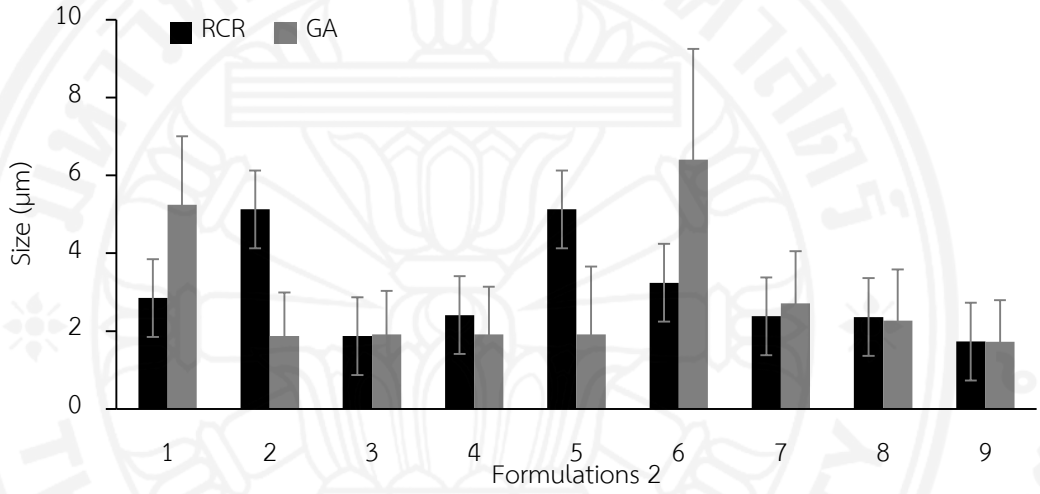


Figure 30: Formulation 2 - Size of niosome prepared with RCR extract and GA loading agents

4.6.2.3. Formulation 3: effect of tween 20, span 60 and cholesterol

Span 60 has HLB of 4.7, lipophilic which tends to forms micelle rather than a bilayer, from the arrangement of non-ionic surfactant molecules tail sticking out of the structure. Meanwhile, with the addition of 50% (w/w) Tween 20 in 50% (w/w) Span 60, the HLB of combined non-ionic surfactants increases from 4.7 to 10.7, as calculated in the Equation 11 below. At 10.7 HLB, the total molar ratios of niosome concentration were varied at 900, 600, 450, 300 and 150 µmol to observe its effect to entrapment efficiency of RCR.

$$HLB = \frac{(50 \times 4.7) + (50 \times 16.7)}{100}$$

Equation 11: Calculation of 50% (w/w) Span 60 and 50% (w/w) Tween 20 HLB value

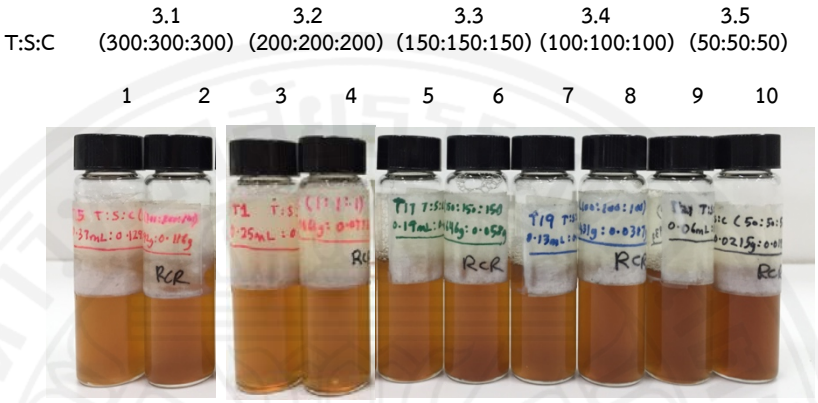


Figure 31: Formulation 3, appearance of niosome suspension after heating and sonication

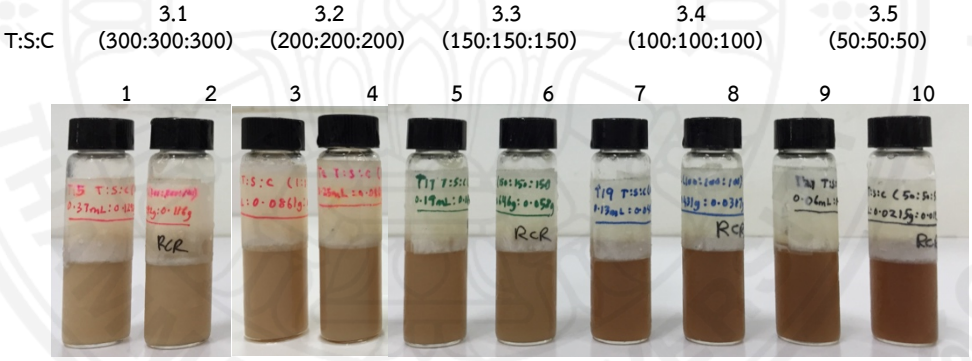


Figure 32: Formulation 3, appearance of niosome suspension after incubated in ice bath

In addition to increasing the formulation HLB value from 4.7 and fixing it at 10.7, the formulations total niosome materials molar were also adjusted by varying it from 150, 300, 450, 600 and 900 µmol to observe its effects. First of all, the appearance of niosome suspension prepared with formulation 3 shows niosome suspension increased its cloudier appearance as the total niosome materials molar used in the preparation was increased, the formulation with 900 µmol shows the cloudiest appearance.

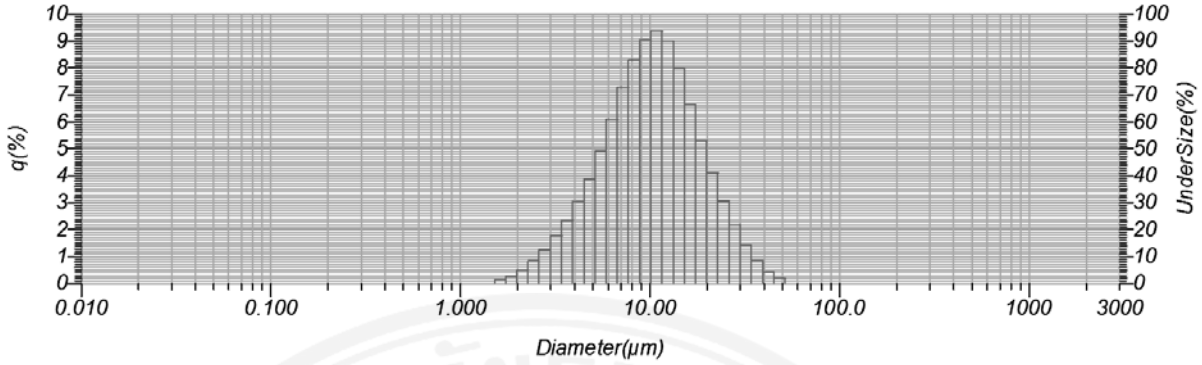


Figure 33: Niosome diameter size in formulation 3.1 at 9.09 ± 0.95 μm

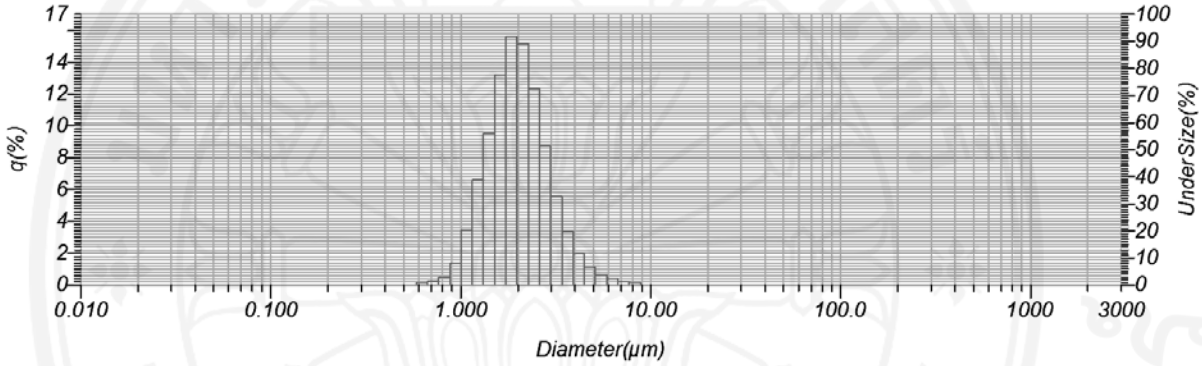


Figure 34: Niosome diameter size in formulation 3.2 at 2.27 ± 0.17 μm

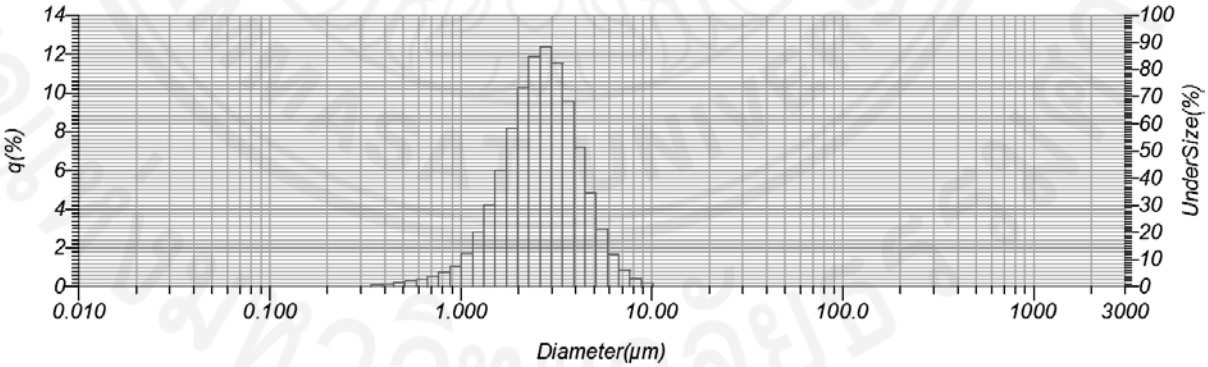


Figure 35: Niosome diameter size in formulation 3.3 at 2.92 ± 0.17 μm

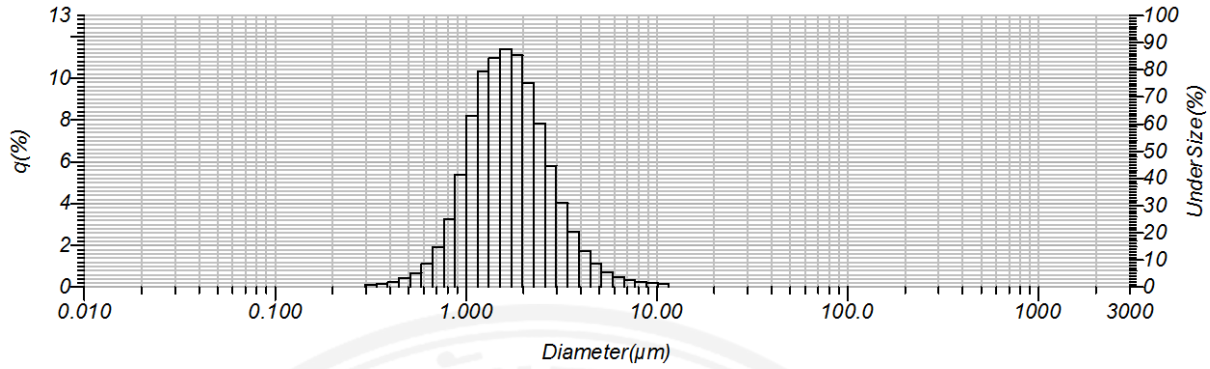


Figure 36: Niosome diameter size in formulation 3.4 at 1.91 ± 0.16 μm

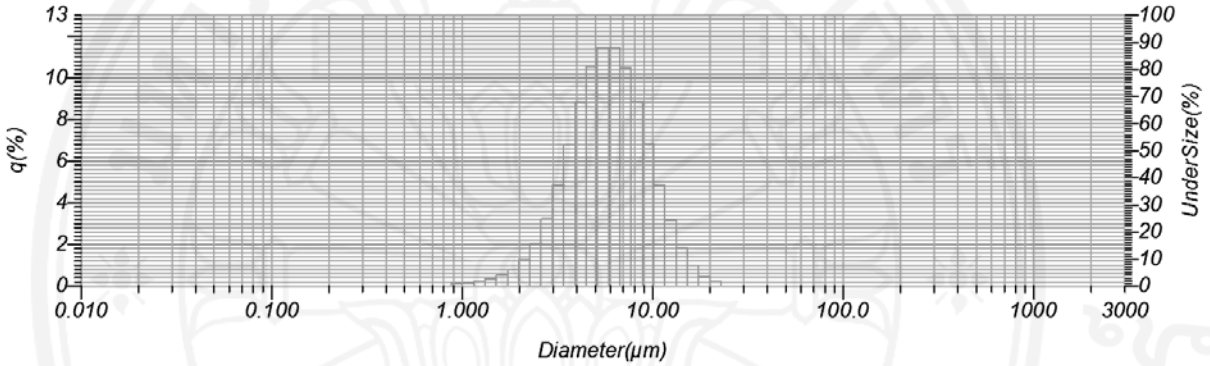


Figure 37: Niosome diameter size in formulation 3.5 at 6.40 ± 0.45 μm

Table 22: Formulation 3, (T: Tween 20 S: Span 60, C: cholesterol)

Code	T:S:C Molar Ratio, μmol	Loading agent	EE (%)	Size (μm)
3.1	300 : 300 : 300	RCR	57.22 ± 5.10[b]	9.09 ± 0.95[c]
3.2	200 : 200 : 200	RCR	28.19 ± 3.07[a]	2.27 ± 0.17[c,d]
3.3	150 : 150 : 150	RCR	40.89 ± 6.56[a,b]	2.92 ± 0.08[c,d]
3.4	100 : 100 : 100	RCR	28.19 ± 2.62[a]	1.91 ± 0.16[d]
3.5	50 : 50 : 50	RCR	24.29 ± 6.04[a]	6.40 ± 0.45[c,d]

[a,b]Tukey HSD significant different EE of 3.1 and 3.3 at 0.181 and 0.172, p = 0.05, respectively and [c,d]niosome size significantly differ in 3.1 and 3.4 at 0.054 and 0.238, p = 0.05, respectively.

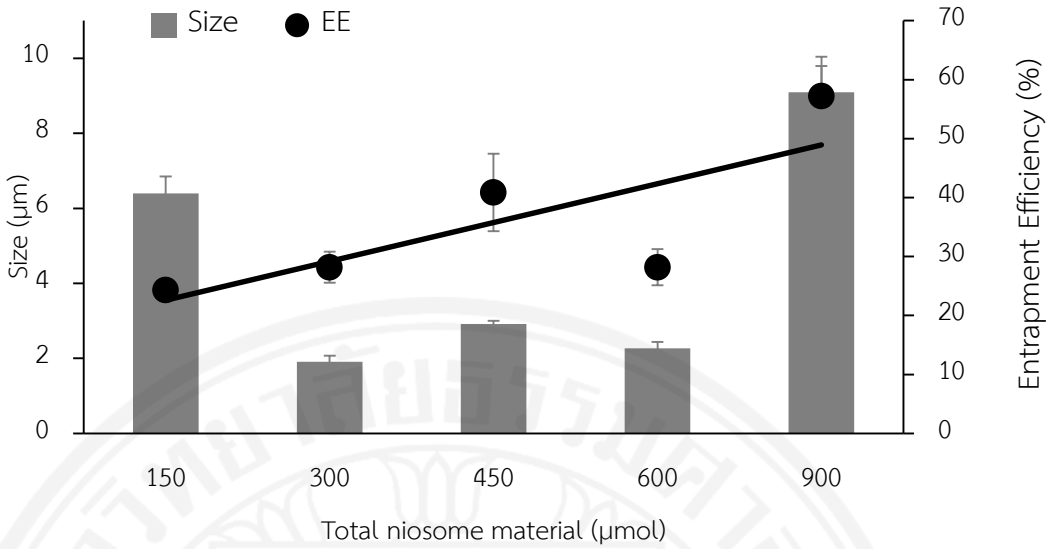


Figure 38: Formulation 3 effect on RCR extract niosome EE and size

RCR extract entrapment in niosome was highest in 900 µmol total niosome concentration, followed by 450, 600, and 150 µmol. The effect of increasing total amount of niosome materials molar further increases its entrapment efficiency. In the previous formulation at HLB 4.7 and 600 µmol total Span 60 and cholesterol, the entrapment of RCR extract ranges from 25 – 48%, this formulation has improved RCR extract entrapment up to 57% in 900 µmol total niosome molars using 1:1 ratio Tween 20 and Span 60. Adjusting the non-ionic surfactant HLB from 4.7 to 10.7 and also increasing total molar niosome has also increased RCR extract entrapment.

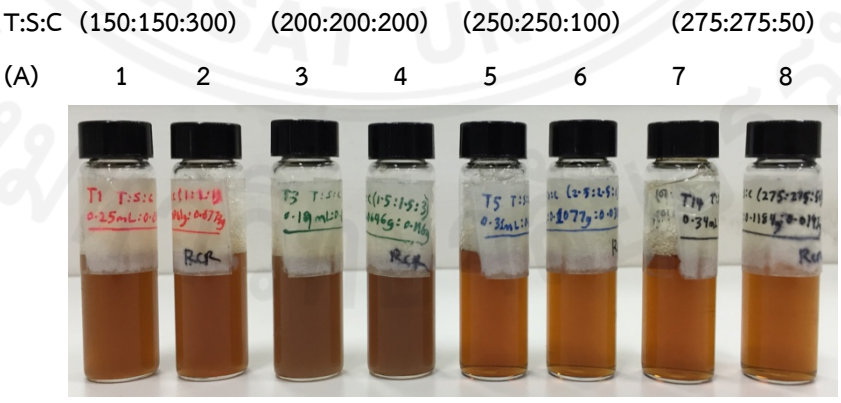
Niosome vesicle size obtained in this formulation is in the range of 2 – 9 µm, the smallest niosome vesicle was obtained at 300 µmol and the largest at 900 µmol total niosome molars with sizes of 1.91 ± 0.16 and 9.09 ± 0.95 µm, respectively. Niosome preparation which entraps more RCR extract produces larger size, compared to niosome entrapped with a lower amount of RCR extract. However, at 150 µmol total niosome molars, the amount of RCR extract was low, but it produces slightly bigger sizes niosome than its other niosomes.

At 4.7 HLB, Span 60 has low EE ability, the addition of cholesterol additive only provides more rigidity to the vesicles. Niosome EE is improved at when its HLB is adjusted at 8 – 10 HLB range, for the desired EE. Tween 20, 16.7 HLB is highly unstable to forms vesicles leading to low EE. A Mixture of high (Tween 20) and low

(Span 60) HLB non -ionic surfactant produce desired HLB in the ranges of 8 – 10 for stable niosome and higher EE. In comparison with another study, Naltrexone drug entrapment was also reported increased as its total niosome forming materials were increased from 200 to 1000 µmol, yielding EE from 5 to 25% (91).

4.6.2.4. Formulation 4: effect of cholesterol molar ratio

The effect of cholesterol as an additive was studied in the niosome preparation formulation 4. Cholesterol is recommended to be added in non-ionic surfactants with HLB lesser than 8 due to its very high hydrophobicity and high lipophilicity, thus, it has low entrapment efficiency, less stable and less lamelarity. The Addition of cholesterol was reported can increase its stability, increase its entrapment efficiency and chances to form niosome vesicles. Cholesterol and non-ionic surfactants molecules were mixed together and forms the niosome membrane bilayer structure, its reduces surface water tension and stabilize the non-ionic surfactants. In this formulation, cholesterol concentration was modified from 50, 33, 17 and 8% (mol/mol) against non-ionic surfactants of 1:1 Span 60 and Tween 20 at fixed 600 µmol total molar of niosome material, RCR extract and GA loading agents effects were studied.



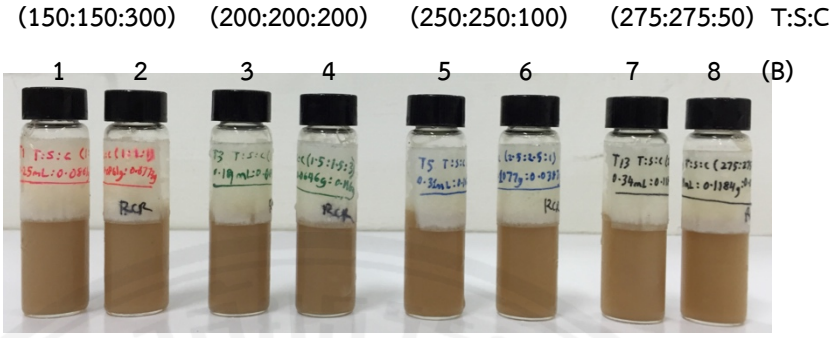
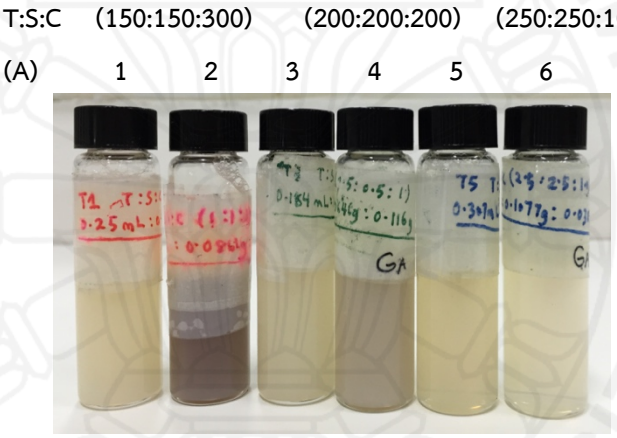


Figure 39: Formulation 4, niosome prepared with RCR extract as loading agent appearance (A) after heat and sonicate, (B) after incubate in ice bath, overnight.



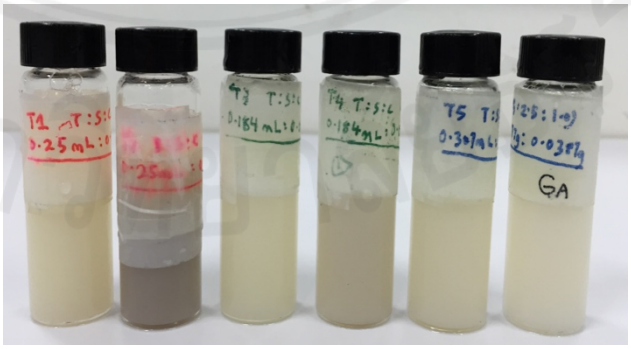


Figure 40: Formulation 4, niosome prepared with GA as loading agent appearance (A) after heat and sonicate and (B) GA after incubate in ice bath

Table 23: EE% of RCR extract and GA in niosome prepared in formulation 4

Code	T:S:C Molar Ratio, µmol	RCR extract		GA	
		EE (%)	Size (µm)	EE (%)	Size (µm)
4.1	150 : 150 : 300 (1:1)	24.57 ± 3.66[a]	9.04 ± 0.27[c]	39.06 ± 2.27[d]	6.9 ± 0.27
4.2	200 : 200 : 200 (2:1)	26.44 ± 0.08[a]	2.40 ± 0.31[c]	54.82 ± 2.12[e]	4.29 ± 0.31
4.3	250 : 250 : 100 (5:1)	52.25 ±0.04[b]	7.44 ± 1.06[c]	-0.4 ± 1.70[f]	2.05 ± 0.23
4.4	275 : 275 : 50 (11:1)	26.86 ± 3.36[a]	6.43 ± 0.74[c]	-	-

[a,b] Tukey HSD post ANOVA analysis RCR EE significant different 0.25 and 1.00 at p = 0.05, [c] RCR size no significant differ at 0.109. [d,e,f] GA EE shows significant between= 1.00,

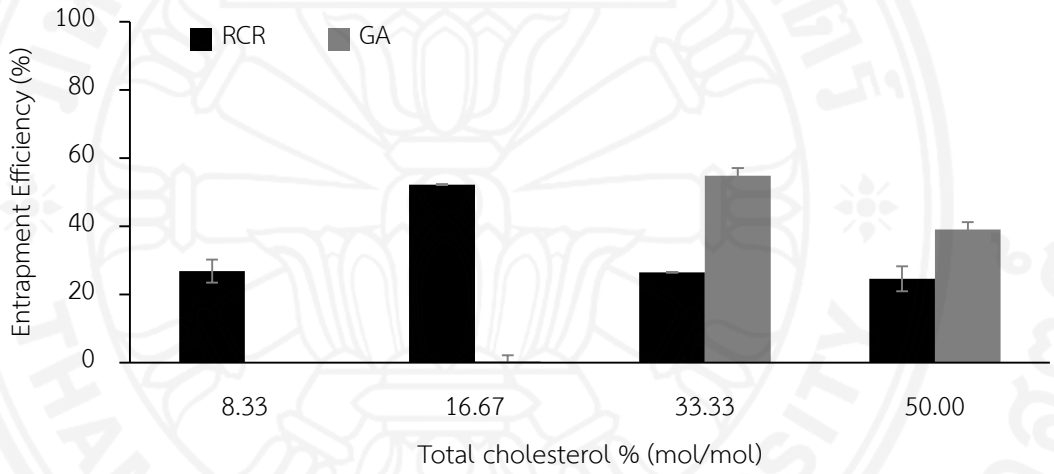


Figure 41: Formulation 4 effect on RCR extract EE % in niosome

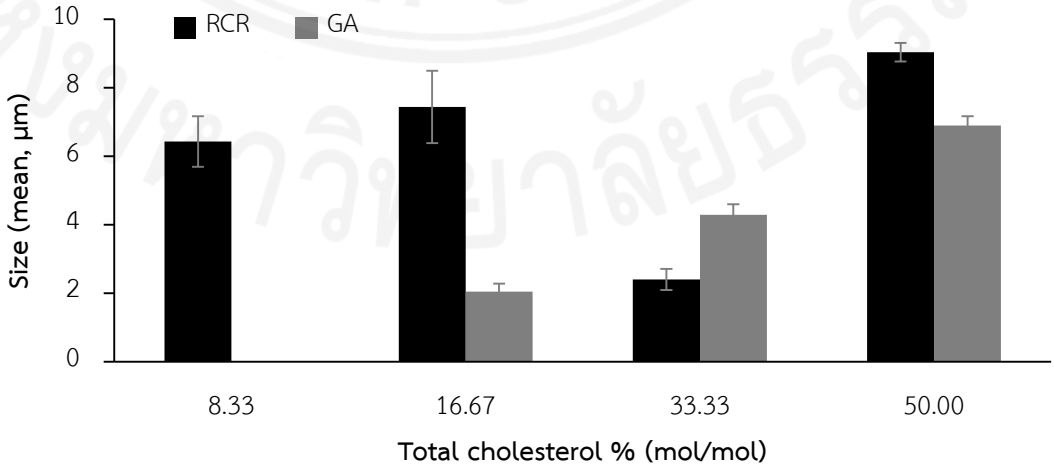


Figure 42: Formulation 4 effect on RCR extract niosome size

Niosome vesicles prepared with formulation 4 shows similarly cloudy appearance in all preparations. Meanwhile, in the effect of cholesterol concentration, the entrapment efficiency of RCR extract and GA peaked at 16.67% and 33.33% (mol/mol) cholesterol concentration, respectively. RCR extract entrapment reacts differently in the formulation. RCR extract entrapment in niosome increases as the cholesterol molar ratio was decreased from 50% to 16.67% (mol/mol), allowing more RCR extract to be entrapped in the niosome vesicles as the vesicle's non -ionic surfactant membrane rigidity decreased in as shown in 5:1 (surfactant: cholesterol) ratios yielding 52.25 ± 0.04 entrapment efficiency. Similarly, GA entrapment was increased at 33.37% (mol/mol) cholesterol at 54.82 ± 2.12%, but when cholesterol concentration was reduced further to 16.67% (mol/mol), the niosomes vesicles were leaked and unable to hold GA anymore.

Niosome size obtained in formulation 4 preparations was within the range of 2 – 8 µm for both RCR extract and GA. GA loaded niosome shows size decrease from 6.9 ± 0.27, 4.29 ± 0.31 and 2.05 ± 0.23, as the cholesterol concentration decreased 50%, 33% and 17% (mol/mol), respectively. The content of cholesterol directly affected GA loaded niosome size. On the other hand, RCR extract loaded niosome size is smallest at 33.33% (mol/mol) cholesterol concentration and at the rest of cholesterol concentration 8.3%, 16.67% and 50%% (mol/mol) its sizes were in 6.43 ± 0.74, 7.44 ± 1.06 and 9.04 ± 0.27 µm, respectively.

4.6.2.5. Formulation 5: effect of non-ionic surfactant molar ratio and HLB

Individually, Tween 20 and Span 60 has 16.7 and 4.7 HLB, respectively, which is too high and hydrophilic for the surfactant to forms niosome vesicles, also too low and hydrophobic for the surfactant to rearranged into vesicles forms and has low entrapment property. At 1:1 Tween 20 and Span 60 mixture, its combined HLB was calculated into 10.7 (Equation 11), the recommended HLB value for a suitable formation of niosome and high entrapment of loading agent. In this formulation, the

effect of HLB on niosome formation was studied. HLB value can be adjusted by changing the combined ratio of the non-ionic surfactants used. At 50% Tween 20 and Span 60 (1:1), it yields 10.7 HLB, this formulation varies the ratio of Span 60 from 1:1, 1:.1.5, 1:0.8 and 1:0.6 which represents 50% to 75%, 40%, and 30% to Tween 20 (mol/mol), producing 10, 12.7, 11.3 and 8.7 HLB values, respectively.

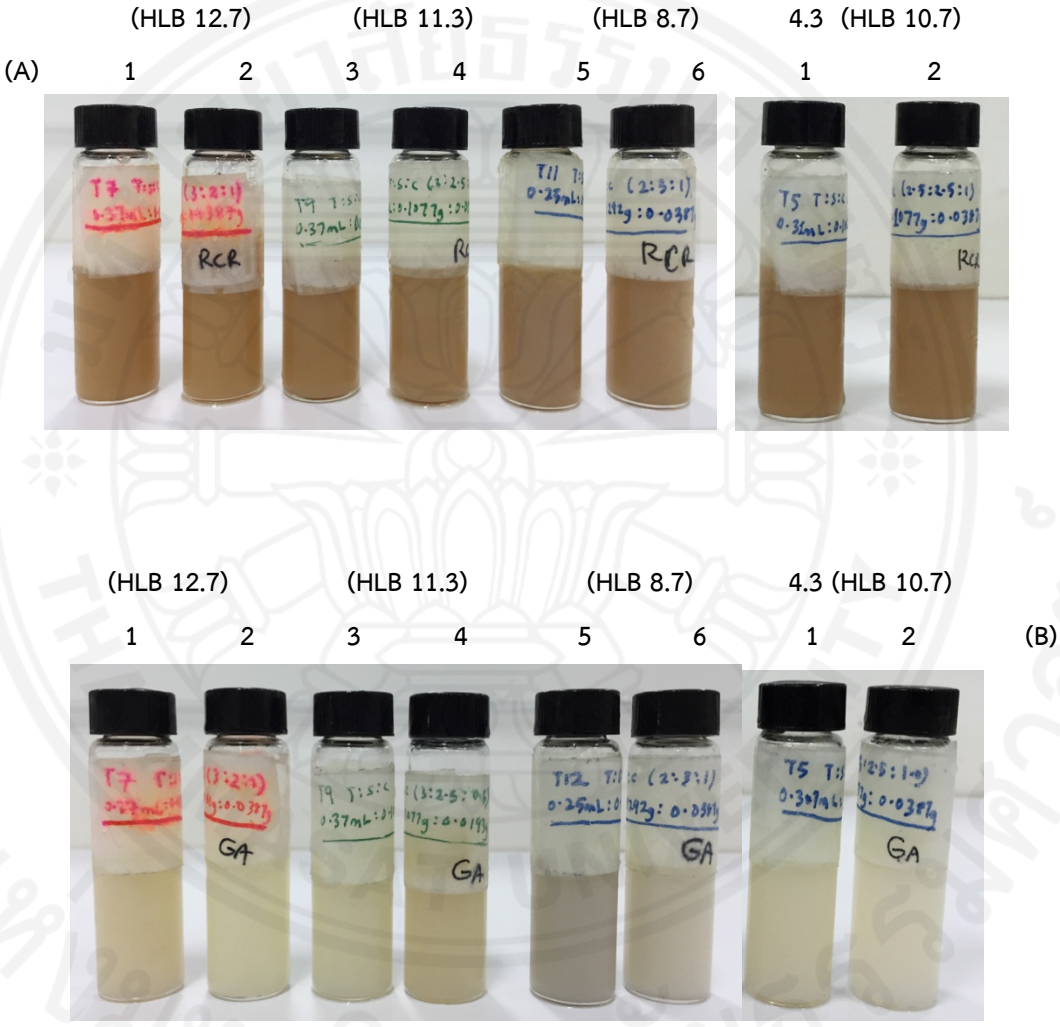


Figure 43: Formulation 5, (A) RCR niosomes suspension after incubation in ice bath, and (B) GA niosome suspension after incubate in ice bath

Table 24: EE and sizes of RCR extract and GA loaded niosomes prepared from formulation 5

Code	T:S:C Molar Ratio, μmol	HLB	RCR extract		GA	
			EE (%)	Size (μm)	EE %	Size (μm)
5.1	200 : 300 (1:1.5) : 100	8.7	31.61 ± 3.97	3.60 ± 0.94	16.28 ± 3.40[b]	5.72 ± 1.79
4.3	250 : 250 (1:1) : 100	10.7	52.25 ± 11.12	5.93 ± 1.06	-0.4 ± 1.70[a]	2.05 ± 0.28
5.2	300 : 200 (1:0.8) : 100	12.7	26.59 ± 8.82	2.57 ± 0.26	17.73 ± 6.24[b]	10.48 ± 2.12
5.3	300 : 250 (1:0.6): 50	11.3	43.12 ± 2.44	5.37 ± 0.76	37.19 ± 2.26[c]	2.27 ± 0.51

Tukey HSD no significant different among RCR niosome EE and size, 0.307 and 0.501, $p = 0.05$. [a,b,c]Tukey HSD significant different GA EE 1.00, 0.97 and 1.00, $p = 0.05$. No significant size difference.

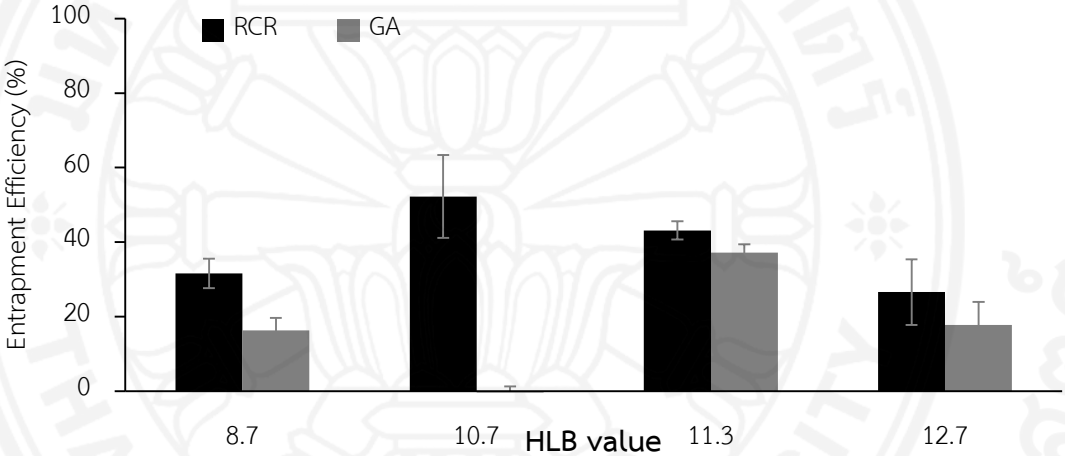


Figure 44: Formulation 5 effect on RCR extract and GA EE

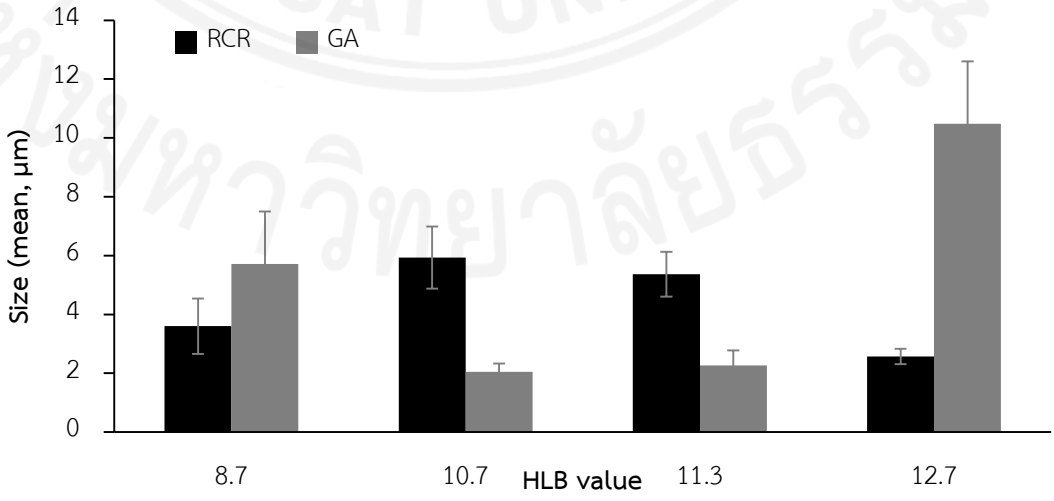


Figure 45: Formulation 5 effect on RCR extract niosome size

Entrapment of RCR extract was reduced as the HLB of the non-ionic surfactant was either increased to (11.3 and 12.7) or decreased (8.7), instead, at 10.7 HLB, 600 µmol total niosome molar, the HLB value which is recommended for good entrapment, its EE is at the highest point, 52.25 ± 11.12%. However, at that same HLB value, GA entrapment in niosome reacted differently with no entrapment. Instead, it showed better entrapment at higher or lower HLB other than 10.7 value, GA entrapment was found to be highest at 37.19 ± 2.26%, a slightly more hydrophilic HLB, 11.3. In relation to its sizes, RCR extract entrapment closely relates to its size, at high EE, its size increases and at its low EE, its size smaller, indicates that the agent inside the niosome influence the niosome structure. The same observation did not occur in GA loaded niosomes, which may be influenced by other factors such as HLB.

Niosomes vesicles obtained in this formulation were in the sizes between 2 – 10 µm, for both for RCR extract and GA loaded niosomes. The biggest niosome size 10.48 ± 2.12 µm was obtained in GA entrapped niosome prepared at 12.7 HLB, followed by 5.72 ± 1.79, 2.27 ± 0.51, 2.05 ±.0.28 µm prepared in non-ionic surfactants with 8.7, 11.3 and 10.7 HLB value. RCR extract loaded niosome vesicle sizes obtained shows contrasting effect compared to GA niosome, which at its largest of 5.93 ± 1.06 and 5.37 ± 0.76 µm, GA niosomes are smallest, and at RCR extract niosome smaller size, 3.6 ± 0.94, 2.57 ± 0.26, GA niosomes are largest. The Different response obtained towards the formulation reflects the dissimilarities among RCR extract entrapped compounds and GA. The effect of HLB has been shown in other niosome preparation with Naltrexone which increases its size significantly from 7 to 12 and 14 µm with an increase of HLB from 4 to 6.7 and 8.6, respectively (91).

4.6.2.6. Formulation 6: effect of high total niosome molar at low cholesterol ratio

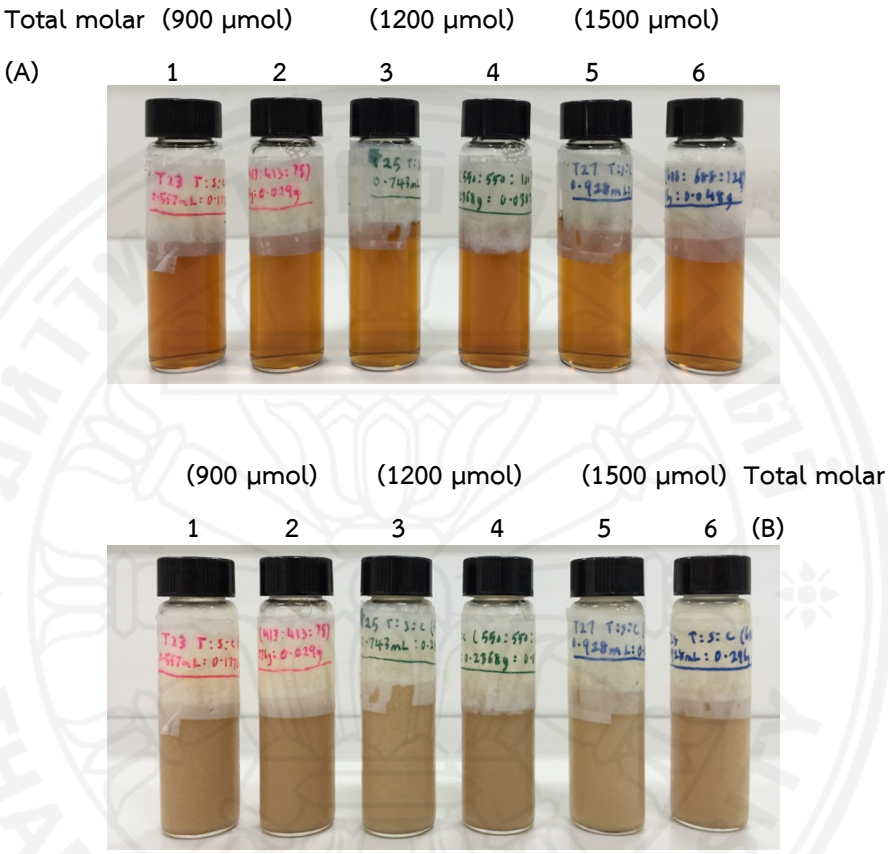


Figure 46: Formulation 6 appearance; of RCR extract entrapped niosome suspension (A) after heat and sonication and (B) after incubated in ice bath

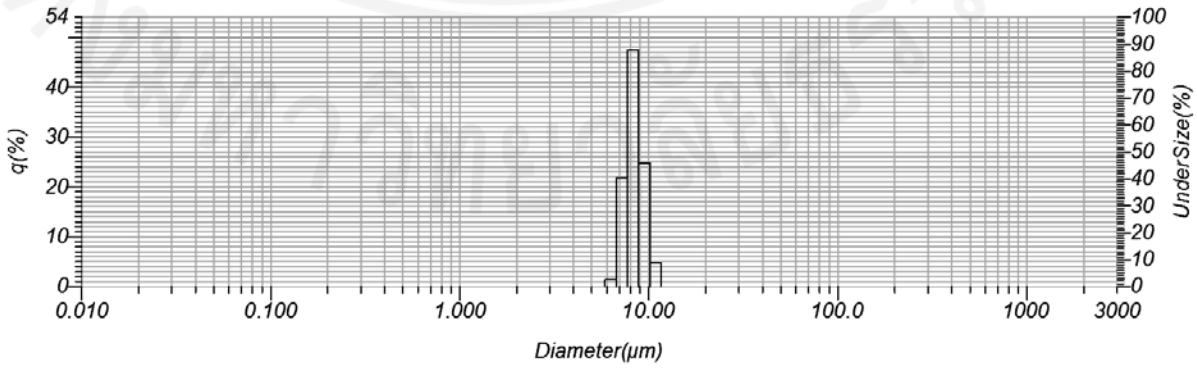


Figure 47: Niosome diameter size in formulation 6.3 at 8.27 ± 0.18 µm

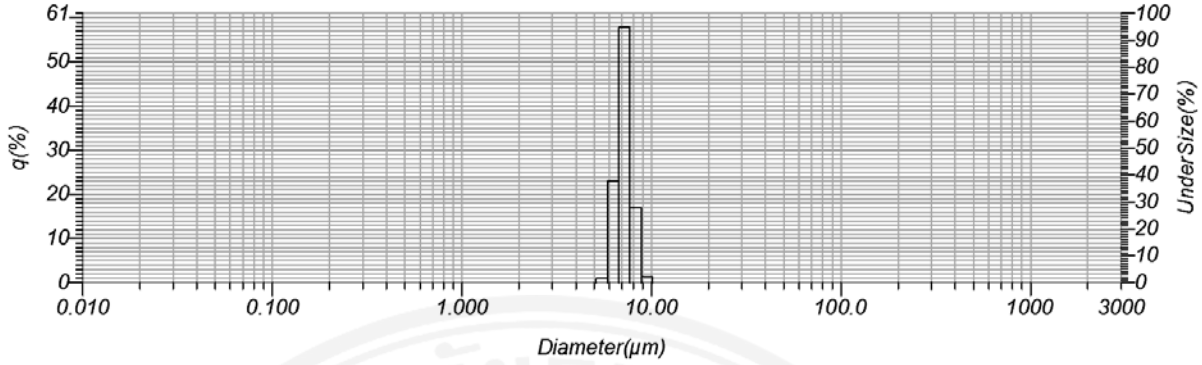


Figure 48: Niosome diameter size in formulation 6.2 at 7.41 ± 0.38 μm

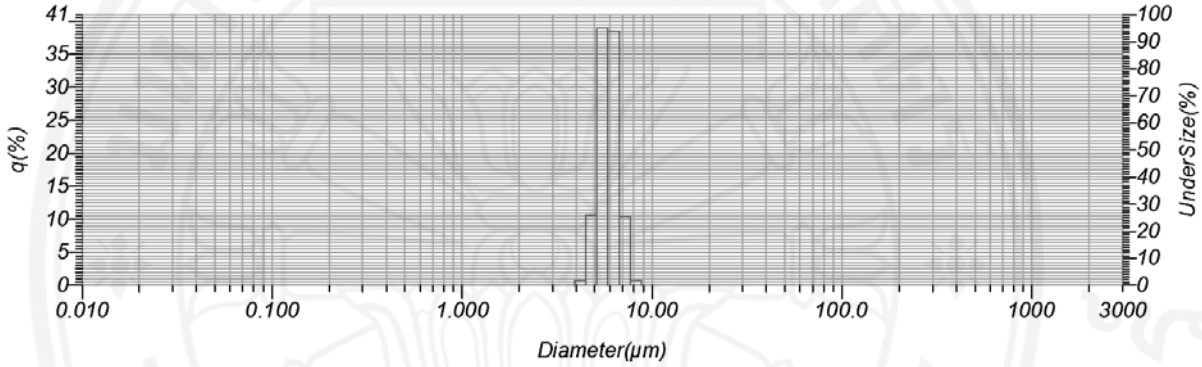


Figure 49: Niosome diameter size in formulation 4.4 at 6.43 ± 0.74 μm

Table 25: Formulation 6 – EE and sizes of RCR extract entrapped niosomes

Code	T:S:C Molar Ratio, μmol	HLB	RCR (% EE)	Size (μm)
4.4	275 : 275 : 50 (600)	10.7	26.86 ± 3.36[a]	6.43 ± 0.74[b]
6.1	413 : 413 : 75 (900)	10.7	46.35 ± 7.65[a]	7.37 ± 0.74[b]
6.2	550 : 550 : 100 (1200)	10.7	42.80 ± 4.89[a]	7.41 ± 0.34[b]
6.3	688 : 688 : 125 (1500)	10.7	48.68 ± 7.81[a]	8.27 ± 0.18[b]

[a,b]Tukey HSD RCR EE and size difference at 0.219 and 0.095, p = 0.05, not significant, respectively.

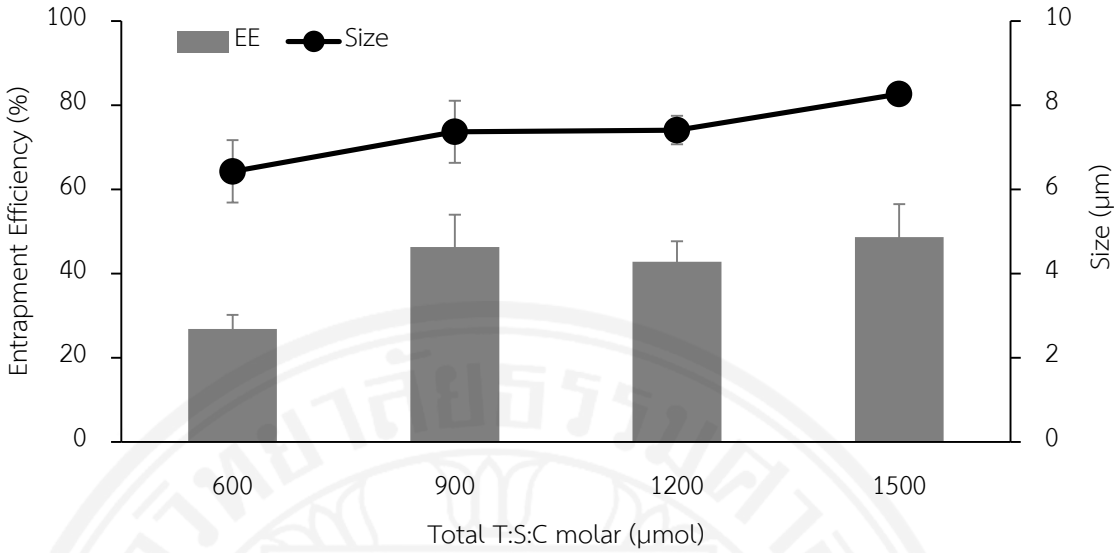


Figure 50: Formulation 6 effect on RCR extract entrapped niosome EE and size

Increasing cholesterol adds more rigidity and stability to the niosome vesicle, reducing it makes niosome vesicle less rigid, potentially leaks, poor entrapment, and poor release as the niosome becomes stiffer. However, in the formulation 4, performed earlier, reducing the cholesterol molar ratio less than 50% shows better entrapment of RCR and GA. Other studies have also reported the same, such as reducing cholesterol up to 30% has increased Naltrexone drug entrapment from 8 to 12% EE (91), increased Benzoyl Peroxide entrapment from 57 to 86% EE (67) and increased rofecoxib entrapment from 21 to 28% EE (64).

Furthermore, in continuation on the study of formulation 4 effects, this formulation 6 selected 8.33% (mol/mol) cholesterol molar ratio, and increased its total molar niosome from 600 to 900, 1200 and 1500 µmol. The niosome entrapment of RCR extract was found increased from 26.86 ± 3.36 to 46.35 ± 7.65, 42.80 ± 4.89 and 48.68 ± 7.81% EE, respectively. Similarly, its sizes also increased as the entrapment of RCR extract increased from 6.43 ± 0.74 to 7.37 ± 0.74, 7.41 ± 0.34 and 8.27 ± 0.18 µm, respectively. The finding confirms the suggestion that increasing non-ionic surfactant can also increase the yield of loading agent entrapped inside the niosome vesicles (56).

4.6.2.7. Formulation 7: effect of loading agent concentration

The concentration of RCR extract affects its PBS color appearance, a higher concentration of RCR extract produce a darker brown color of the buffer. When the buffer with RCR extract was mixed into niosome materials, the buffer brownish color was masked with the cloudy appearance of the niosome materials formulation. In this formulation, total niosome materials are fixed at 900 µmol of 1:1:1 ratios Tween 20, Span 60 and cholesterol. PBS with a low concentration of RCR extract shows cloudier appearance due to the original solution color was less brownish than PBS with a higher concentration of RCR extract, which has a darker brownish appearance. After the heated and sonicated niosome mixture was incubated overnight, its suspension shows cloudier appearance than before incubation but RCR extract with high concentration still has some brownish color. Niosome formulation added with low concentration RCR extract shows no brownish color but only milky appearance may indicate higher entrapment or because of low concentration of RCR extract.

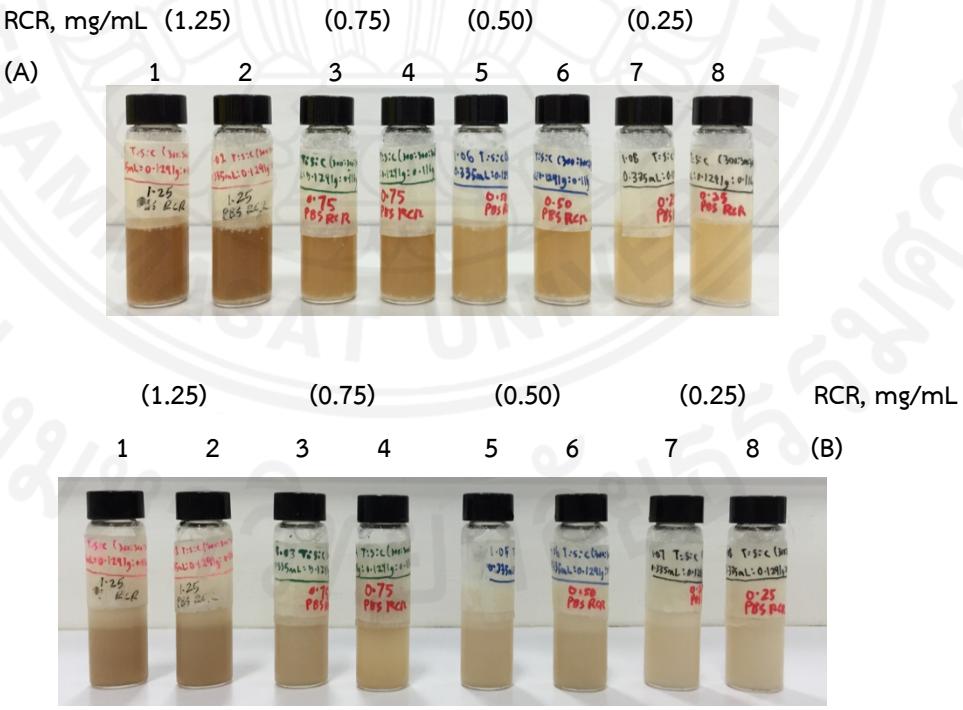


Figure 51: Formulation 7 appearance of RCR extract entrapped in niosome suspension (A) after heat and sonication and (A) after incubated in ice bath

Table 26: Formulation 7–EE and sizes of niosome prepared in vary RCR extract concentration

Code	T:S:C Molar Ratio, μmol	RCR extract (mg/mL)	RCR (EE%)	Size (μm)
7.1	300 : 300 : 300 (900)	1.25	12.21 ± 6.29[a]	3.82 ± 0.26[c]
3.1	300 : 300 : 300 (900)	1.00	57.22 ± 5.10[b]	9.09 ± 0.95[c]
7.2	300 : 300 : 300 (900)	0.75	56.51 ± 5.21[b]	4.47 ± 1.14[c]
7.3	300 : 300 : 300 (900)	0.50	29.59 ± 6.59[ab]	5.65 ± 0.12[c]
7.4	300 : 300 : 300 (900)	0.25	28.59 ± 7.67[ab]	3.88 ± 0.19[c]

[a,b]Tukey HSD RCR EE % significant difference at 0.38 and 0.09, $p = 0.05$, RCR niosome size shows no significant different in size at 0.144, $p = 0.05$.

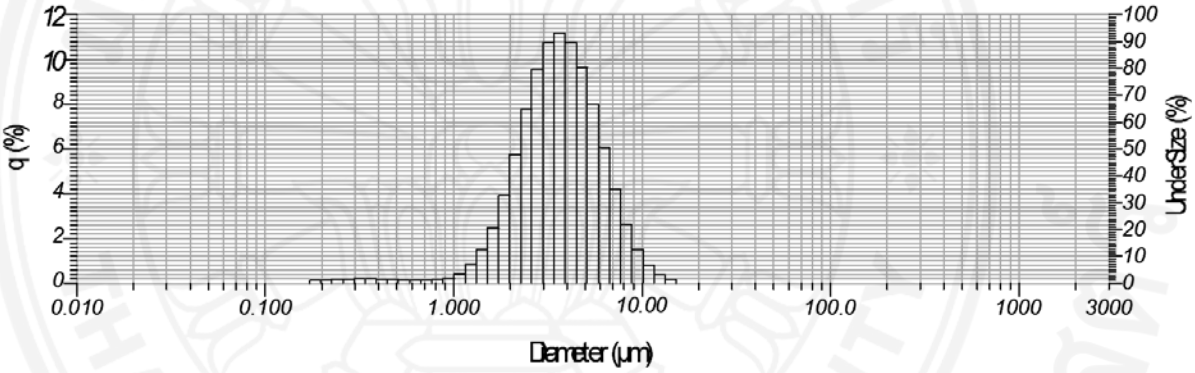


Figure 52: Niosome diameter size in formulation 7.4 at 3.88 ± 0.19 μm

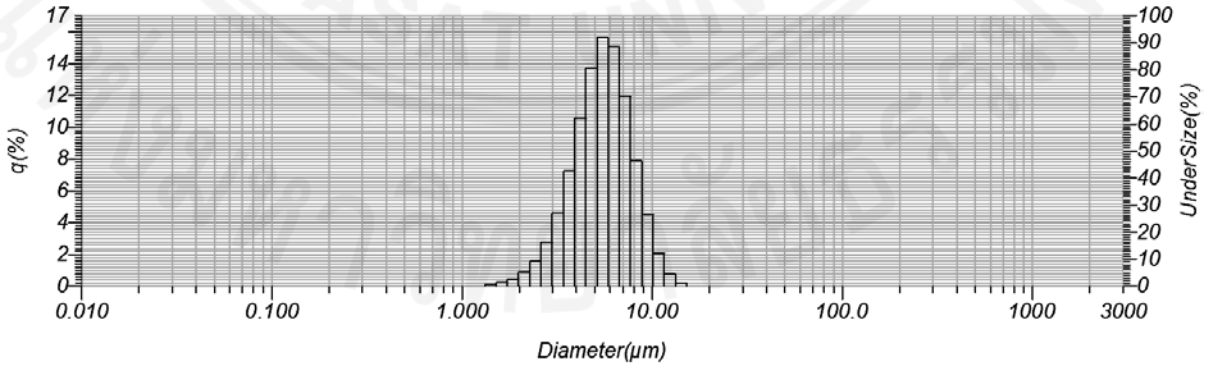


Figure 53: Niosome diameter size in formulation 7.3 at 5.65 ± 0.12 μm

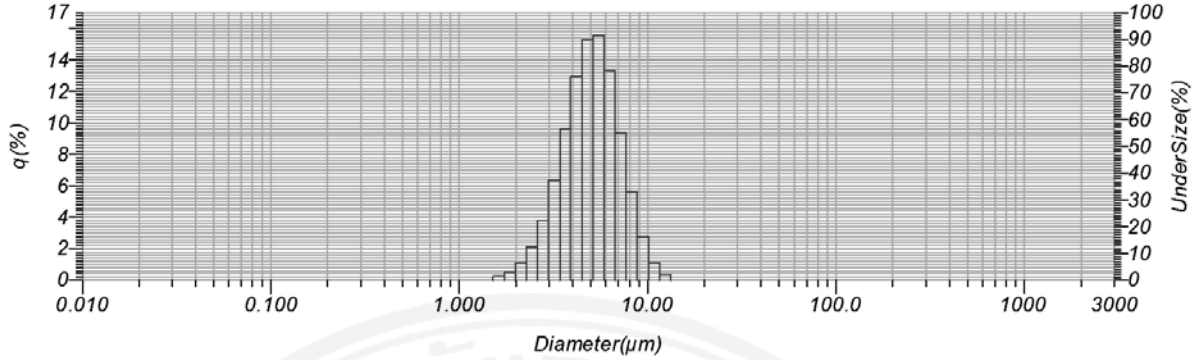


Figure 54: Niosome diameter size in formulation 7.2 at 4.47 ±1.14 μm

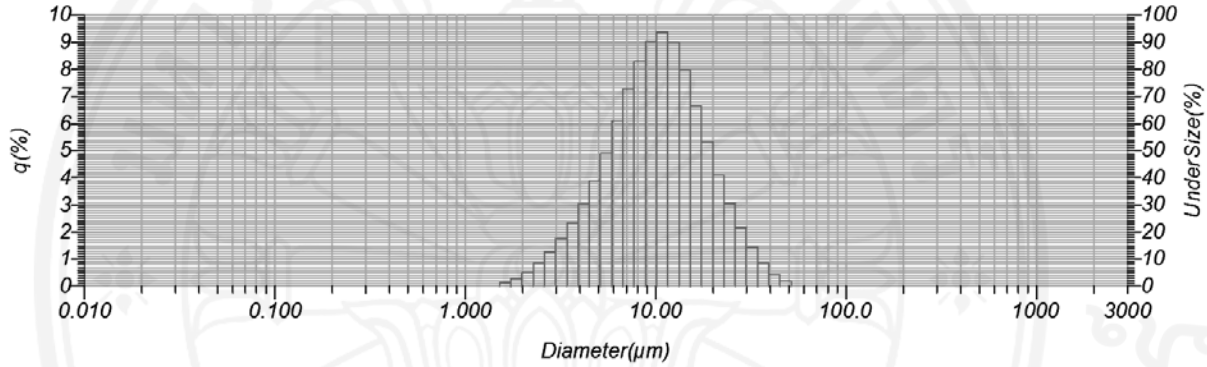


Figure 55: Niosome diameter size in formulation 3.1 at 9.09 ±0.95 μm

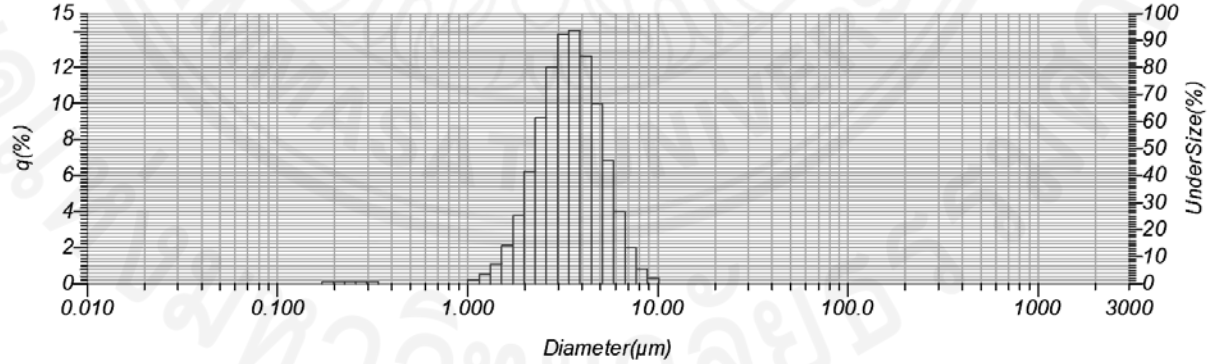


Figure 56: Niosome diameter size in formulation 7.1 at 3.82 ±0.26 μm

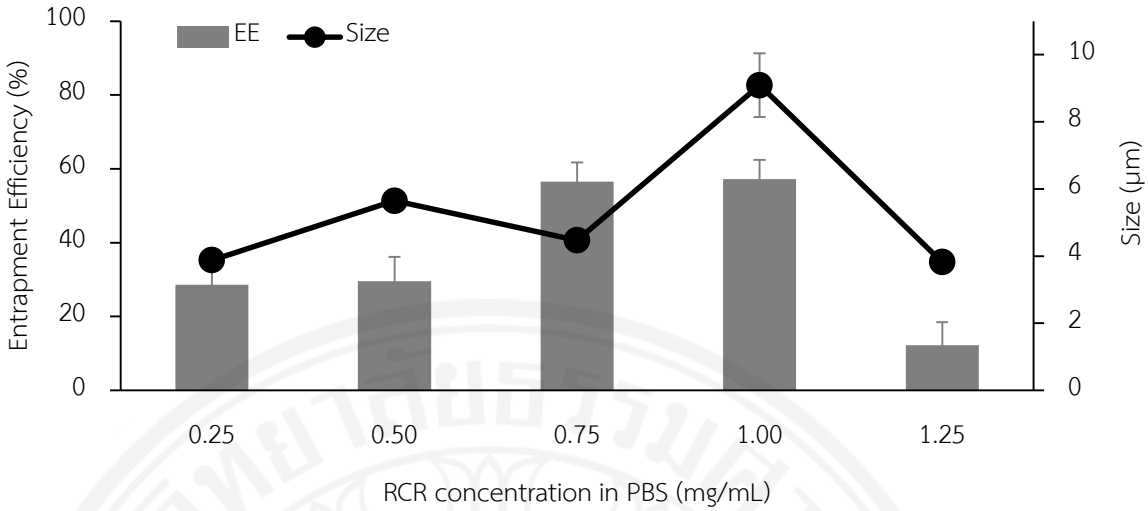


Figure 57: Summary of formulation 7 effect on RCR extract EE and size

Characterization of RCR extract entrapment in the niosome preparation shows that lower concentration of RCR extract has higher entrapment in the niosome vesicles. Reducing loading agent, RCR extract concentration from 1.25 to 0.25 mg/mL shows peaked entrapment at 1.00 and 0.75 mg/mL, at 57.22 ± 5.10 and 56.51 ± 5.21% EE, respectively. At 1.25 mg/mL RCR extract concentration, entrapment of the RCR extract is at its lowest, 12.21 ± 6.29% EE, followed by 0.25 and 0.50 mg/mL RCR concentrations at 28.59 ± 7.67 and 29.59 ± 6.59% EE, respectively. The effect of loading agent concentration on entrapment is also shared many similarities for entrapment of Naltrexone at different concentrations points. Naltrexone shows reduced entrapment from 25 to 5% as its content was increased from 5 to 15 mg (91). In an another niosome preparation, Morin hydrate drug prepared in various concentrations for entrapment shows higher % EE at 5 mg than 10 mg or 20 mg, at low total niosome molar of 95 µmol than high niosome molar 380 µmol. However, at higher niosome molar 380 µmol, the effect of high drug concentration, 20 mg entrapment is negligible compared to low niosome molar 95 µmol (105). Thus, in general, lower loading agent concentrations shows better entrapment because the niosome forming materials are available at a higher amount for entrapment of more agents, but a lower amount of loading agent may decrease its overall EE. For best result, every loading agent should be assessed for its optimum concentration for the selected niosome formulation.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1. General conclusion

5.1.1. RCR potential as antioxidant agent rich source

Phenolic compounds are a group of biological compounds which has antioxidant active activity. These compounds are available abundantly in plants in various forms of secondary metabolites Flavonoids and phenolic esters are some phenol groups available in coffee bean. Among the phenolic compounds available in RCR extract, CAF and CA are the most significant and active compounds.

CGA and CA are an important natural antioxidant agent and CAF a psychoactive stimulant agent are all brewed from roasted coffee bean into coffee drinks (32). As most of CGA and CAF are well extracted in hot water, all health benefits from these compounds are readily available to be consumed and gained from a coffee beverage. However, CA is poorly extracted in hot water and subsequently yields low quantity in a coffee beverage. Hence, it is assumed that high quantity of CA still remains in RCR alongside trace amount of CGA and CAF leftovers. In view of this opportunity, valuable CA compounds in RCR has the potential to be extracted in high yield if a suitable and efficient extraction condition were applied.

In this study, extraction of RCR obtained from a local source was compared between WD, RD, EM and also SE extraction conditions. Evidently CA was extracted better and yields 4 times higher in EM than WD and RD because ethanol is an efficient organic solvent to extract phenolic compound, such as CA. Higher CA compound in EM RCR extract was expressed into higher antioxidant activity and phenolic compounds identified. RCR extract with higher CA is definitely more potent than RCR extract with higher CGA as antioxidant agent and phenolic compound, albeit if its crude extract yield is lower. The advantages of SE against EM where it improves on the crude extract and CGA yield, but still remain actively comparable to EM. Principally, SE is faster and more efficient than EM while maintaining low -cost

production, potentially increasing its feasibility for large scale extraction of antioxidant rich RCR extract.

The extraction crude extract yields were in agreement with previous other studies, showing a consistent performance of water against ethanol. However, in active compounds identification assays, water extracts perform poorly. Meanwhile, other researchers show mixed reports due to different testing approaches and strategies. Though, in this study, the correlation between antioxidant compounds free radical scavenging activity and total phenolic content assay were performed and it shows a strong correlation, other studies did not report the relationship of the performance. Additionally, further investigation on the relationship of the activity towards active compounds involved was performed on a modified HPLC method.

This study concludes and acknowledges other prior studies that with a suitable extraction system, RCR can be redeemed for valuable antioxidant active compounds such as CGA and CA. However, even as CA is at a much lower amount than CGA, we believe that CA is a better antioxidant agent according to the evidence gathered from its pure standards, showing almost double activity than CGA (8). Thus, for this purpose, we recommend using 95% ethanol, Soxhlet extraction, in 6 hours, 80°C and 10 mL/g RCR as the suitable extraction for higher CA and CGA and lower CAF compounds. Meanwhile, the modified HPLC system coupled with spectrophotometer detection used provides a simple, fast and offers a good resolution for the separation of CAF, CGA, and CA compounds.

5.1.2. RCR extract entrapment in niosome

In niosome preparation, RCR extract and other loading agents were able to be entrapped inside niosome vesicles, in various formulations of Span 60, Tween 20 and additive, cholesterol. The niosome vesicles were prepared using heating and sonication method, and formulations were varied from different loading agents, non-ionic surfactant composition, cholesterol ratio, total molar niosome material, HLB ratio and RCR extract concentration. Among all formulation, the highest RCR extract entrapment efficiency in niosome and smallest vesicle size obtained at 56.51 ±

5.21% and 4.47 ± 1.14 μm, and there are still a lot of room for improvements. The optimized conditions were found at 1:1 Tween 20 to Span 60 non-ionic surfactant ratios, giving the niosome material HLB of 10.7, 2:1 non-ionic surfactant to cholesterol ratio, 900 μmol total niosome material, 6 mL volume of 10 mM PBS hydration with 25% ethanol, 0.75 mg/mL RCR extract concentration, 45-minute heating and sonication hydration time and overnight incubation in ice bath.

5.2. Recommendations

Vast opportunity for Improvements in the scope of niosome preparation are promising to be undertaken. For improved entrapment efficiency, several strategies can be implemented. In the study, the entrapment efficiency shows improvement as the non-ionic surfactant composition was modified by the addition of Tween 20. It provides a shift in the HLB value from hydrophobic into more hydrophilic. Similarly, the outcome from the combination of different Span and Tween non-ionic surfactants may improve its entrapment further. Besides using only two combinations of non-ionic surfactants, a combination of three or more different non-ionic surfactants may increase entrapment efficiency as the niosome membrane become more robust and diverse with different materials fusing together.

Likewise, additives such as cholesterol, dicetyl phosphate (DCP), solulan C24, sodium cholate (SC) and stearyl amine (SA) also plays a supporting role in increasing the surface charges on the niosome vesicle, and potentially increases it EE. Previous studies have shown that it improves loading agent EE greatly, but some preparations have no effects. In reality, niosome preparation using various classes of Tween and Span shows mixed effects with the addition of an additive, DCP. EE of Zidovudine drug in the various formulations sometimes shows reduced EE of the drug in some formulations and some preparations have shown small EE increases. The effect on the niosome size also mixed, such as in Tween 80 with additive its size increases but with Span 20 its size is unchanged (86). Other reports show mostly positive findings with the addition of additives, in the formulations of niosome loaded with Naltrexone, DCP shows improved EE than C24 and SC at 40%, 20%, and 10% EE, respectively (91).

As the size of the niosomes obtained in this study were in the range of reportable and expected values, it is acceptable and in general, possible for a certain application (56). A specific niosome size is particularly important for a certain type of delivery system. For example, very small niosomes size in the range of sub-nanometer is particularly desired for *in vitro* delivery of drug across cell membrane and capillaries to reach cellular target site, on the other hand, niosomes with size larger than 10 μm are recommended for ocular delivery and niosomes with size between 1 – 10 μm are suitable for intraperitoneal, intramuscular and intra cavity delivery of drug (56,91). Fortunately, niosome vesicle size has the potential and opportunity to be reduced further for a specific application using several complex and extensive methods, such as probe sonication, ultra-sonication, extrusion, high pressure homogenization, filtration and combinations of the methods (59,61,62).

5.3. Future plans

To visualize and understand the morphology of the niosome prepared from Span 60 and Twee 20 loaded with RCR extract in this study, it can be studied from the analysis of the vesicles under electron microscope such as scanning electron microscope (SEM) or transmission electron microscope (TEM). SEM and TEM analysis provides morphological information of the niosome vesicle structure surface and cross section overview of the niosome vesicle, respectively. These analysis is relevant to be performed in future study to evaluate the morphology of the niosome vesicle surface, observe the number of the niosome vesicle layer formed, and provide a visual information of the niosome vesicles prepared. From the SEM and TEM results which will be obtained, the niosome preparation can be improved to decrease size, decrease agglutination, increase lamellarity and uniformity of niosome size. However, the concern about this analysis is the high cost involved and availability of equipment.

Besides niosome surface visual morphology, its surface charge is also quite an important and relevant parameter for assessment. Surface charge value characterizes the niosome vesicle property such as its interaction behavior, stability, aggregation, entrapment efficiency and size. A charged niosome vesicle is more stable, however

an uncharged niosome vesicle tend to aggregate and fuse together, creating instability. Among charged niosome, positively charged niosome is much stable than negatively charged niosome due to the positive charge niosome is less likely to aggregates with each other. Niosome vesicles surface charge can be determined using Zetasizer analyzer which measure the electrophoretic mobility on the outer edge of the vesicle surface.

To observe the product performance and response in real world usage, RCR extract entrapped niosomes vesicles should be assessed for its strength, stability, durability and degradation in a simulated condition. International Conference on Harmonization (ICH) has provided standard guidelines for various drug and formulation stability tests such as real-time, accelerated, long term tailored for new drugs, products, and dosage. According to WHO guidelines on stability study protocols, RCR extract niosome stability testing provides valuable information on the effects of different storage conditions with variable temperature settings, humidity levels, and light intensities. The stability information provides more understanding on the limits of the product stability, to avoid damage and suggest a recommended an optimum product storage condition (106,107). Product degradation occurs by time, and RCR extract loaded niosomes also degrades over a long period of time. Degradation manifestation took a long period of time and involves a long period of research to be observed. This response is essential to be tested for new product development. In WHO guidelines, forced degradation study can be simulated in a range of degradation condition such as acid-base hydrolysis, oxidation, thermal and photolytic exposure to observe product degradation. RCR extract niosome degradation highlight the insight of the product stress threshold limits, weak spot and its byproduct produced in post-degradation. Forced degradation or stress study assist the researcher to observe and control the product destruction or after after-life condition (107-109). However, due to short and limited time allocated in this study, stability and stress study of new RCR extract entrapped niosome products formulation was impractical and thus this plan is suggested to be shelved to be undertaken in a forthcoming future study only.

RCR extract entrapped niosome is rich with antioxidant active compounds, which is potential in cosmetic application as premature skin aging or damage treatment. Skin delivery of antioxidant or other agent is recommended in gel formulations, as it is a common skin delivery system for drugs which can protect the agent, hydrate skin, spread and remains on the skin for a longer period of time. In addition, the use niosome as drug delivery system which protects and improve drug delivery, solubility, bioavailability and stability combined with gel system create a much-improved product with a multitude of protection. Other studies have shown less irritation, inflammation of drug side effects (71,110), improved permeation (67), reduced leak, heat stable, longer retention time (69) and shelf life (78).

In view of the advantages reported thus far, it is highly imperative that RCR extract entrapped in niosomes were prepared into gel formulation for its much improved topical delivery application. For future plans, niosomes loaded with antioxidant rich RCR extract were suggested to be gelled with Xanthan gum, a natural thickening agent derived from glucose or sucrose. Xanthan gum gels easily at a very low concentration of less than 2%, it also safety approved from FDA and non-toxic to human. For future development and commercialization, it is aimed that niosome gel formulation of RCR extract were compared with commercial gels to improving characteristics and property. This future endeavor is in close-knit collaboration between the experts in the field of traditional Thai medicine and cosmetics at the Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University and members of the Biopolymer Application Research Group, Faculty of Science and Technology, Thammasat University.

In closing remark, coffee is an extraordinary harvest, every part of the plant and also its by-product has tremendous potentials. This study emphasizes its potential as a potent source of an antioxidant rich extract as shown in the RCR extraction and identification. This study endeavors its first attempt to entrap RCR extract in niosome formulation with considerable success but plenty of potential for improvement. There are significant knowledge obtained in this study and it is hoped that it can contribute and spur new ideas and approaches to entraps RCR extract in niosome for improved efficiency and size.

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APPENDICES

APPENDIX A

CHEMICALS, REAGENTS, INSTRUMENTS, PLASTICS AND GLASSWARES

Table 27: List of chemicals and reagents used in this study

Item	Source
RCR, *Coffea arabica*	Hom Krun Coffee shop, LC4, Thammasat University, Thailand
Ethanol (EtOH), 95%	ACl Labscan, Thailand
Methanol (MeOH), HPLC grade	ACl Labscan, Thailand
Acetonitrile (CH_3CN), HPLC grade	ACl Labscan, Thailand
Water, HPLC grade	ACl Labscan, Thailand
Phosphoric acid	ACl Labscan, Thailand
Gallic acid	Sigma Aldrich, USA
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma Aldrich, USA
Folin Ciocalteu reagent	Merck KGaA, Germany
Sodium Carbonate	Ajax Finechem, Australia
Potassium dihydrogen orthophosphate (KH_2PO_4)	Ajax Finechem, Australia
dH_2O	Central Scientific Instrument Centre (CSIC), Faculty of Science and Technology, Thailand
3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione (Caffeine)	Sigma Aldrich, China
3,4-dihydroxy-cinnamic acid (Caffeic acid)	Sigma Aldrich, China
3-(3,4-DIhydroxycinnamoyl)quinic acid (Chlorogenic acid), >95% titration	Sigma Aldrich, USA
Sorbitan monostrearate (Span 60)	Sigma Aldrich, USA
Polyoxyethylenesorbitan monolaurate (Tween 20)	Sigma Aldrich, USA
Cholesterol	Sigma Aldrich, Japan
Phosphate buffer saline (PBS) pH 7.4 tablets	Sigma Aldrich, USA
Xanthan Gum	MySkinRecipies®, USA

Table 28: List of equipment and glassware used in this study

Item	Source
Soxhlet extractor	Thailand
Erlenmayer flask, 500 mL	Germany
Drying oven	MEMMERT, UE500, Germany
Spectrophotometer Uv-vis	Biochrom Libra S22, UK
96 well micro-plate	Nunclon™ Delta Surface, Denmark
Micro-plate reader	BioTek PowerWave XS2, USA
HPLC, 1200 series	Agilent Technologies, USA, Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Thailand.
ChemStation	LC 3D systems, Agilent Technology, USA
Luna C18 HPLC column (150 x 4.6 mm, 5µm particle size)	Phenomenex, USA
Disposable syringe	Nipro, Thailand
Glass vial, 12 mL	WHEATON, New Jersey, USA
Erlenmaer Flask, 125 mL	Germany
Magnetic stirrer hot plate	IKA® C-MAGE HS7, China
Water bath sonicator	50/60 Hz Tru-Sweep™, Ultrasonic Cleaner, NY, USA
Laser scattering particle size distributor analyzer, LA-950	HORIBA, USA
Cellulose dialysis bag	CelluSep, T4, Texas, USA

APPENDIX B

CHEMICAL REAGENTS PREPARATION

1. **Reagents for determination of antioxidant activity**
   a. **0.2 mM DPPH reagent working stock**

DPPH	15.77 mg
95% ethanol	200 mL

Freshly prepared, wrapped in foil

2. **Reagents for determination of total phenolic content**
   a. **Folin-Ciocalteu reagent 10% (w/w)**

Folin-Ciocalteu reagent diluted 10 fold with distilled water	100 mL
Distilled water (H_2O)	900 mL

Freshly prepared, wrapped in foil

   b. **Sodium carbonate 7.5% (w/w)**

Sodium carbonate (Na_2CO_3)	7.5 grams
Distilled water (H_2O)	100 mL

Freshly prepared, wrapped in foil

3. **HPLC mobile phase**
   a. **Acetonitrile, HPLC grade** **1 liter**

Filtered with vacuum filter

   b. **50 mM Potassium dihydrogenphosphate buffer, pH 2.6**

Potassium dihydrogenphosphate (KH_2PO_4)	6.80 gram
Distilled water (H_2O)	1 liter

Adjusted its pH to 2.6 with phosphoric acid, filtered with vacuum filter

4. Niosome hydration buffer

a. 10 mM Phosphate saline buffer (PBS)

PBS tablet	1 tablet
Distilled water (dH_2O)	200 mL

b. 10 mM Phosphate saline buffer (PBS), 20% ethanol

PBS tablet	1 tablet
Distilled water (dH_2O)	200 mL
95% ethanol	

c. 10 mM Phosphate saline buffer (PBS), 20% ethanol with 1 mg/mL RCR extract

10 mM PBS	33 mL
95% ethanol	12 mL
RCR extract	45 mg

d. 10 mM Phosphate saline buffer (PBS), 20% ethanol with 1 mg/mL GA

10 mM PBS	33 mL
95% ethanol	12 mL
GA	45 mg

BIOGRAPHY

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Publications

1. Mustafa, A. Z., Phonprapai, C., Itharat, A., Extraction and identification of antioxidant active agent from roasted coffee residue (RCR) for entrapment in niosome. Paper presented at the 7th Biennial Meeting of Society for Free Radical Research – Asia, Chiang Mai, Thailand.

Oral presentation

1. Mustafa, A. Z., Phonprapai, C., Itharat, A., Extraction and identification of antioxidant active agent from roasted coffee residue (RCR) for entrapment in niosome. Oral presentation presented at the 7th Biennial Meeting of Society for Free Radical Research – Asia, Chiang Mai, Thailand.