DEVELOPMENT OF TOPICAL FILM FORMING SPRAY CONTAINING *GARCINIA COWA* LEAF EXTRACT NANOEMULSION FOR PHARMACEUTICAL AND COSMECEUTICAL PRODUCTS

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MASTER OF SCIENCE

IN

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Thesis Title	Development of Topical Film Forming Spray Containing *Garcinia Cowa* Leaf Extract Nanoemulsion for Pharmaceutical and Cosmeceutical Products
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ABSTRACT

The aim of this study was to fabricate and characterize topical film forming spray containing *Garcinia cowa* leaf extract (GC) nanoemulsion for use as pharmaceutical and cosmeceutical products. The GC nanoemulsion was first prepared by ultrasonic method. Properties of the GC nanoemulsion, including morphology, particle size, polydispersity index (PDI), zeta potential, thermal properties, and stability were investigated. In addition, encapsulation efficiency (%EE), release study, antibacterial activity, antioxidant activity, anti-inflammatory activity, and indirect cytotoxicity were investigated. The results showed that the particle size of GC nanoemulsion ranged between 66 and 71 nm, PDI ranged between 0.17 and 0.28, and zeta potential ranged between -17 and -10 mV. The cumulative released amount of GC nanoemulsion ranged between 14 and 31%. In addition, the GC nanoemulsion exhibited the inhibition against both gram-negative and gram-positive bacteria and showed good antioxidant activity. The GC nanoemulsion was also non-toxic to NCTC clone 929 cells. Thus, the GC nanoemulsion could incorporate into the topical film forming spray for use as pharmaceutical and cosmeceutical products.

The topical film forming spray was prepared using Eudragit E100 (EuE100) as polymeric system, dibutyl phthalate (DBP) as plasticizer, GC nanoemulsion as drug, polyvinyl pyrrolidone (PVP) as binder and enhancer, and ethanol as solvent. From the results, the increase of EuE100 concentration caused the viscosity to increase but the spray pattern to decrease. All formulations (S1-S6) showed the evaporation time in the range of 3.27-7.10 min and the contact angle was low, in the range of 19.05-30.04°. Finally, the topical film forming film containing GC nanoemulsion showed good antibacterial, anti-inflammatory, and non-toxic to the cells. Thus, this topical film forming spray containing GC nanoemulsion have the potential for use as pharmaceutical and cosmeceutical products.

Keywords: *Garcinia cowa* Leaf Extract, Nanoemulsion, Topical Film Forming Spray, Pharmaceutical and Cosmeceutical Products

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ABBREVIATION AND SYMBOL

GC	*Garcinia cowa* leaf extract
EuE100	Eudragit E100
NLCs	Nanostructured lipid carriers
SLNs	Solid-lipid nanoparticles
PHB	Polyhydroxybutyrate
SPF	Sun protection factor
UV	Ultraviolet-visible light
O/W	Oil in water
W/O	Water in oil
PVP	Polyvinyl pyrrolidone
DBP	Dibutyl phthalate
SC	Stratum corneum
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CFU/ml	Colony Forming Units per milliliter
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
°C	Degree Celsius

ABBREVIATION AND SYMBOL (continued)

h	Hour
nm	Nanometer scale
mm	Millimeter scale
mL	Milliliter or unit of volume
µL	Microliter or unit of volume
pH	measure of acidity or alkalinity
g	Gram
cP	Centipoise
DMEM	Dulbecco's modified eagle medium
LPS	Lipopolysaccharide
FBS	Fetal bovine serum
SFM	Serum-free medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
rpm	Revolutions per minute
PDI	Polydispersity index
DLS	Dynamic light scattering
TEM	Transmission electron microscopy

ABBREVIATION AND SYMBOL (continued)

DSC	Differential scanning calorimetry
PBS	Phosphate buffer saline
NO	Nitric oxide
IC_{50}	The half maximal inhibitory concentration
%EE	Percent encapsulation efficiency
%AA	Percent antioxidant activity
%w/w	Percent weight per weight
%w/v	Percent weight per volume
%v/v	Percent volume per volume

CHAPTER 1

INTRODUCTION

1.1 Background and Significance of the Research Problem

Skin is the largest organ covered human body. There are three main functions of skin including protection, regulation, and sensation. The skin protects inner organs from temperature variance, bacteria or pathogen, radiation, and chemicals (Tortora et al.,1996). It consists of three main layers which are epidermis, dermis, and subcutis. The epidermis is the outermost layer of the skin and its function is protecting skin from chemicals, bacteria, and moisture loss from the skin. This layer is thick and highly flexible which keeps good skin and looks youthful (Lai-Cheong & McGrath, 2009). However, if skin is infected by bacteria and pathogens, these infections cause inflammation and tissue damage within the skin and slow the healing process. To solve this problem, pharmaceutical and cosmeceutical products are employed to prevent further injury and bacterial exposure to the skin. Since the epidermis layer mostly consists of lipid and fatty acid, thus the ingredients of products must have lipophilicity (Ansari et al., 1970). Besides, the particle size of products is a key factor for the absorption capacity of the human skin since the human skin consists of many hair follicles with pore size of around 100-200 nm. Therefore, the particles size of the products is assumed to be no larger than 200 nm in diameter (Ryman-Rasmussen et al., 2006; Rosen et al., 2015).

There are many products used in pharmaceutical and cosmeceutical products such as hydrogel, facial mask, and topical film forming spray (Sood et al., 2014; Kathe & Kathpalia, 2017). Topical film forming spray is a novel approach as a promising choice for transdermal drug delivery used in pharmaceutical and cosmeceutical products due to its many advantages compared to conventional topical preparation. In addition, it provides excellent coverage of the skin or wound, high drug penetration, lower

incidence of irritation, and accelerates wound healing through moisture control, and protects skin or wound from bacteria or pathogen (Kathe & Kathpalia, 2017). Topical film forming spray is a liquid solution and formed transdermal film after applied to the skin. The topical film forming spray has four main components which are polymer, solvent, penetration enhancers, and drug (Schroeder et al., 2007). Polymer is the main component of topical film forming spray that should be breathable film and non-occlusive, for example, methyl methacrylate (Eudragit), polyvinyl acetate, cellulose acetate, polyvinyl alcohol, and hydroxyl ethyl cellulose. The topical drug delivery system has been a favored route of drug administration since the topical drug delivery system is easy to release the drug and drug cannot be destroyed by the digestive tract and liver. However, this system also shows some limitations such as lower drug delivery through the skin because the outermost layer of epidermis called stratum corneum prevents the penetration of substances (Radhakrishnan et al., 2018).

The particle size of active substance has an effect on the delivery of an active substance to various sites within the body. Nanoencapsulation technique such as nanoemulsion, nanostructured lipid carriers, nanosuspension, solid lipid nanoparticles, nanosized liposomes, biopolymer nanoparticles, micelles are a process for coating or entrapment of active substance within other materials to perform nanoparticles (Jafari, 2017). Thus, nanoencapsulation has the potential to improve biocompatibility, protect decomposition from enzymes, temperature, or moisture, improve the controlled release, and increase bioavailability (Suganya & Anuradha, 2017; Asija et al., 2013). Therefore, many researchers have been worked on this technique for developing drug delivery since particle with nano-size can penetrate through stratum corneum (Neethirajan & Jayas, 2011; Huang et al., 2010). In addition, many bioactive compounds have been incorporated using nanoencapsulation technique such as antibiotics, lipophilic drugs, hydrophilic drug, vitamins, and polyphenolic compounds (Rashidinejad & Jafari, 2020; Ungaro et al., 2012; Barichello et al., 1999; Desai & Park, 2005; Fang & Bhandari, 2010; Munin & Edwards-Lévy, 2011). Consequently, the nanoencapsulation of the active substances plays the important role for development of the topical film forming spray.

Garcinia cowa or chamuang is the indigenous plant growing in Thailand. Many parts of the plant including leaves, flowers roots, stems, fruits and latex can be used.

Garcinia cowa leaves have received a lot of interest in pharmaceutical applications because *Garcinia cowa* leaf extract (GC) contains many bioactive compounds such as xanthones, benzophenones, flavonoids, and flavones. Thus, it has several biological activities such as antimicrobial, antioxidant, antidiabetic, and cytotoxic activities (Phukhatmuen et al., 2020; Wahyuni et al., 2015). GC was encapsulated using nanoemulsion technique to improve release profile and permeability of GC through the skin.

Thus, in this study, the topical film forming spray containing GC nanoemulsion was developed for use in pharmaceutical and cosmeceutical products. The size, zeta potentials, PDI, morphology, encapsulation efficiency, release characteristic, antioxidant activity, antibacterial activity, anti-inflammatory activity, cytotoxicity, cell internalization, and stability of GC nanoemulsion were investigated. The topical film forming spray containing GC nanoemulsion was characterized for its physicochemical properties (pH, viscosity, evaporation time, weight per spray, contact angle, and spray pattern), encapsulation efficiency, release characteristic, antibacterial activity, anti-inflammatory activity, cytotoxicity, and stability.

1.2 Research Objectives

1.2.1 To prepare and characterize *Garcinia cowa* leaf extract (GC) nanoemulsion.

1.2.2 To prepare topical film forming spray containing GC nanoemulsion.

1.2.3 To characterize the topical film forming spray containing GC nanoemulsion for use in pharmaceutical and cosmeceutical products.

1.3 Scopes of Research

1.3.1 Characterize various properties (i.e., antioxidant activity, antibacterial activity, anti-inflammatory activity, and cytotoxicity) of GC.

1.3.2 Find the optimal condition (concentration of GC) to fabricate the GC nanoemulsion.

1.3.3 Characterize various properties (i.e., size, zeta potential, PDI, morphology, encapsulation efficiency, release study, antioxidant activity, antibacterial activity, anti- inflammatory activity, cytotoxicity, cell internalization, and stability) of GC nanoemulsion

1.3.4 Find the optimal condition (concentration of EuE100) to prepare the topical film forming spray containing GC nanoemulsion.

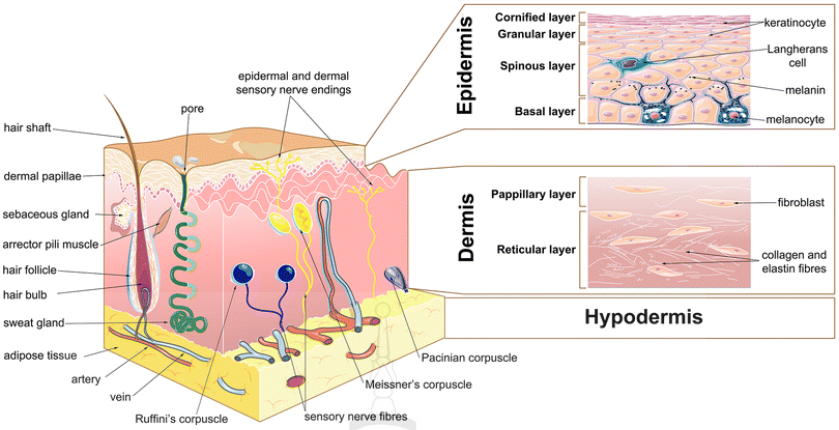
1.3.5 Characterize various properties (i.e., pH, viscosity, contact angle, spray pattern, weight per spray, evaporation time, encapsulation efficiency, release study, antibacterial activity, anti-inflammatory activity, cytotoxicity, and stability) of the topical film forming spray containing GC nanoemulsion.

CHAPTER 2

LITERATURE REVIEW

2.1 Transdermal Route and Drug Delivery by Skin

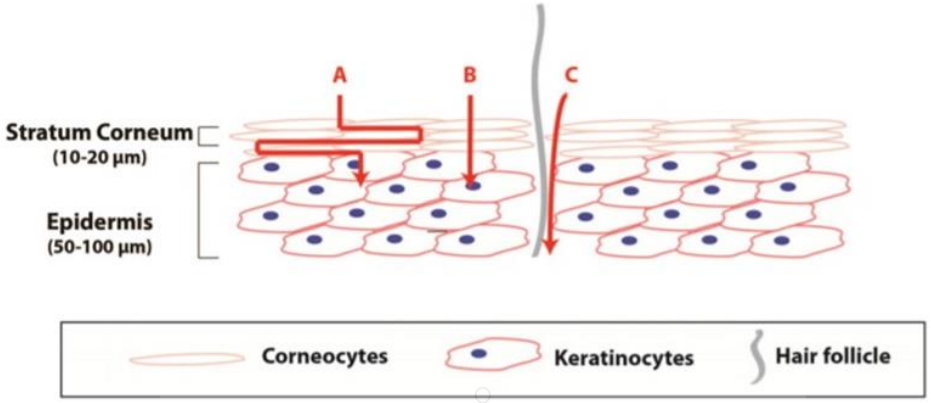
Skin is the furthest tissue of the body and the biggest organ in terms of weight and surface area. It has a region of around 16,000 cm^2 for a grown-up and it was approximately 8% of the body weight. The function of skin is to regulate body temperature, prevent the entry of bacteria, and provide a selectively permeable barrier (Boer et al., 2016). Skin contains different layers of cells including epidermis, dermis, and hypodermis (Figure 2.1). The epidermis is the first layer. Its main job is to protect the body by acting as a barrier and help control body temperature. It is made up of four types of cells such as keratinocytes (which comprised 90% of the epidermis), melanocytes, Langerhans cells, and Merkel cells. The dermal-epidermal junction offers the mechanical support for the epidermis and acts as a partial barrier against exchange of cells and large molecules. The dermis is the fatty layer of panniculus adiposus tissues. The blood and lymph vessels bring nutrients to the dermis and epidermis. The hypodermis is the layer present below the dermis which connects the skin to the underlying fascia (fibrous tissue) of the bones and muscles. It is mostly made of fat and connective tissue. It connects the skin to muscles and bones. It also saves body heat, stores energy and absorbs shock to protect the body from injury (Zerres & Stahl, 2020). Skin is a major factor for determining the permeation and absorption of drug across the dermis. The diffusional resistance of the skin is extremely dependent on its anatomy and ultrastructure. Over the past decades, the development of controlled drug delivery has become increasingly important in the pharmaceutical industry. Transdermal Drug Delivery System (TDDS) is defined as self-contained, discrete dosage forms. It is administrated by skin and drug is delivered directly into systemic circulation maintaining continuous efficacy (Tanwar & Sachdeva, 2016).



Source Pereira (2017)

Figure 2.1 Multilayer Structure of Human Skin Tissue

Pharmaceuticals are agents used to change or protect skin from the abnormal conditions. Cosmeceuticals indicate a category of products placed between nonprescription and prescription products. Cosmeceuticals may also be examined as hybrids between cosmetics and pharmaceuticals that are used to improve the health and beauty of the skin. Cosmeceuticals remain popular in the skin care marketplace (Amer & Maged, 2009). Cosmeceuticals are characterized as any substance or arrangement expected to be used on the outer parts of the human body primarily for cleansing, perfuming, beautifying, odorant, and advancing well-being without influencing body structure or capacities. Normally, active ingredients are diffused through the tortuous lipid channels and/or traverse transcellularly through corneocytes, or enter the skin through hair follicles or sweat ducts (Figure 2.2) (Zakrewsky et al., 2015; Ahsan, 2018). However, since skin has a stratum corneum as a skin barrier to drug transport and the transport of drugs through the skin is a complex process. Thus, the chemical skin permeation enhancers are used to increase the transport across the barrier by partly solubilizing or extracting the skin lipids and creating hydrophobic pores (Cevc, 1997).



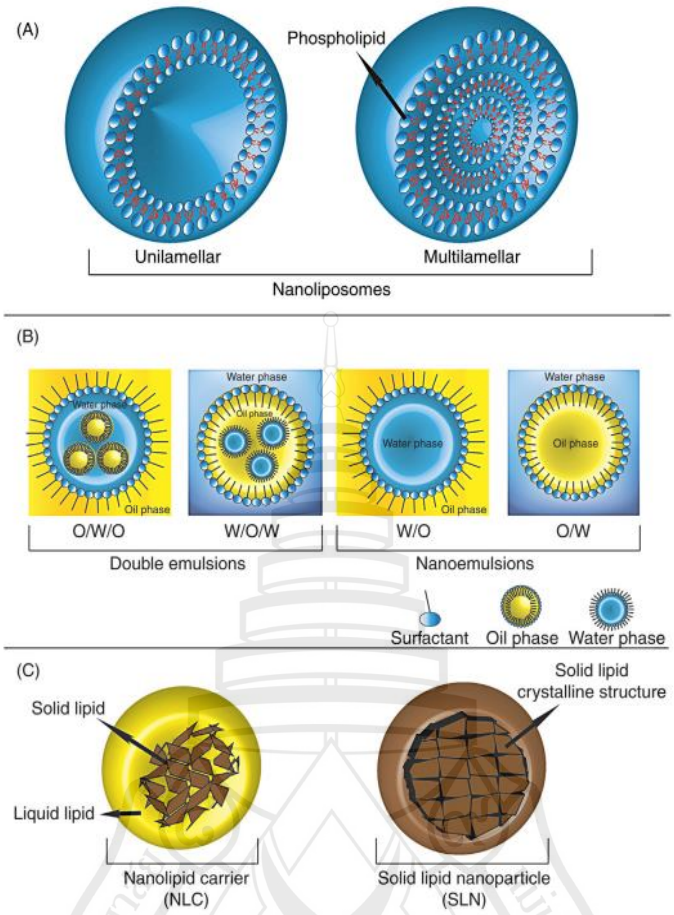
Note A: Intercellular Pathway through Lipid Bilayers. B: Transcellular Pathway through Keratin-Rich Corneocytes. C: Shunt Pathway through Hair Follicles and Sweat Ducts

Source Ahsan (2018)

Figure 2.2 Transport Pathways into the Skin

2.2 Nanoencapsulation

Nanoencapsulation is a process for encapsulating drugs or active ingredients within a wall material to form capsules at the nanoscale range. The small particles easily penetrate the skin and protect them from adverse environments for the controlled release at targeted sites (Apsara et al., 2020). The nanoparticles have diameters between 0 and 1,000 nm (in the colloidal range) and can be found as nanocapsules or nanospheres (Pisoschi et al., 2018). Nanoencapsulation can enhance bioavailability, improve controlled release, and enable precision targeting of the bioactive compounds greater than microencapsulation. Most of the bioactive compounds, including hydrophobic vitamins, fatty acids, flavonoids, aromas, preservatives, etc. have hydrophobic characteristics. For encapsulating these compounds, the addition of emulsifiers (typically, oil in water) are often required. There are various types of nanoencapsulation including nanoliposomes, nanostructured lipid carriers (nanolipid carrier and solid lipid nanoparticle), and nanoemulsions (Figure 2.3) (Demir, 2014; Jafari, 2017).



Note (A) Nanoliposomes, (B) Nanoemulsions, and (C) Nanostructured Lipid Carriers (NLCs)

Source Jafari (2017)

Figure 2.3 Schematic Illustration of Nanostructured (Lipid-Based) Encapsulation Formulations

2.2.1 Nanoliposomes

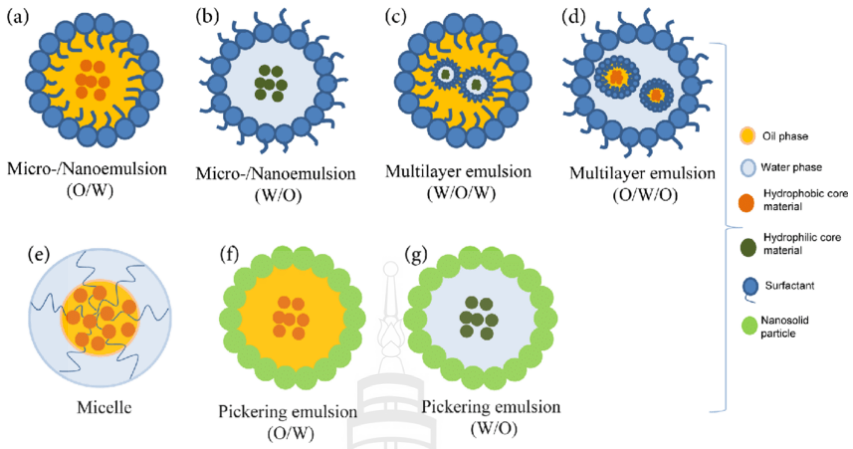
Nanoliposomes are membrane-based vesicles commonly used as delivery systems of drugs. It is sphere-shaped, consisting of one or more phospholipid bilayers that can dissolve both water-soluble and lipid-soluble compounds. In 2020, Pavelkova et al. prepared a new UV-protecting system based on liposomes/polyhydroxybutyrate (PHB) with encapsulated coffee extracts. The result showed that adding PHB into liposome particles increased UV-protective effect and increased colloid stability and SPF value during long-term storage and non-toxicity (Pavelkova et al., 2020). The disadvantages of this technique are decreased stability and slow release of encapsulated molecules during long-term storage (Akbarzadeh et al., 2013).

2.2.2 Nanostructured Lipid Carriers (NLCs)

Solid-lipid nanoparticles (SLNs) were first conceptualized by Professor R.H. Müller (Germany) and Professor M. Gasco (Italy). SLNs are aqueous colloidal dispersions that the matrix of which comprises solid biodegradable lipids. The advantages of SLNs are improved bioavailability of poorly water-soluble, non-toxicity, and better stability compared to liposomes. Karamchedu et al. encapsulated morin hydrate into a SLNs to develop nanomedicines. The results showed that the nanoscale size of particles with good steric stability was obtained. In addition, the encapsulation of morin hydrate showed higher cytotoxicity with cancer cells than pure morin hydrate. However, the disadvantage of this technique is poor drug loading capacity (Garud et al., 2012; Karamchedu et al., 2020). Nanostructured lipid carriers (NLCs) are the second generation of lipid nanoparticles. They are developed to overcome the shortcomings of first generation is SLNs. The NLC or oil-loaded SLN contain lipid droplets that are partially crystallized and have a less-ordered crystalline structure or an amorphous solid structure. The composition of inner phase of the NLCs can offer higher encapsulation efficiency, higher loading volume, and higher bioavailability of nanoencapsulated ingredients in comparison to the SLNs (Tamjidi et al., 2013; Chauhan et al., 2020).

2.2.3 Nanoemulsions

Nanoemulsions are the simplest form composed of a hydrophilic phase and a hydrophobic phase where one is dispersed through the others. Nanoemulsions are the colloidal particulate systems in the range of submicron size. They act as carriers of drug molecules and can be used in personal care and cosmetics as well as in health care. Since they have reduction in the creaming or sedimentation, prevent flocculation and coalescence, perform efficient delivery of active ingredients through the skin, and enhance penetration of actives (Maruno & Rocha-Filho, 2009). Nanoemulsions are divided into several types including oil in water (O/W), water in oil (W/O), water in oil in water, oil in water in oil, micelles, and pickering emulsion (O/W or W/O) as shown in Figure 2.4 (Katouzian & Jafari, 2016). The diameter of the droplet from the nanoemulsions technique reaches approximately 20–500 nm. The major ingredients of nanoemulsions are oil, emulsifying agents, and aqueous phases. The main application of nanoemulsions in the biomedical fields is the delivery of poorly soluble drugs to the specific site. Shakeel and Ramadan reported that the nanoemulsion had permeation of the drug better than the aqueous solution (Shakeel & Ramadan, 2010). In 2017, Mazzarino et al. developed and characterized nanoemulsions containing jaboticaba extract (Plinia peruviana) for pharmaceutical and cosmetic applications. Nanoemulsions were prepared by high-pressure homogenization method. The result showed that the particles size of colloidal around 200 nm and the stability of nanoemulsions was stable over 120 days of storage at room temperature. Therefore, nanoemulsions technique could reduce particle size and improve stability (Mazzarino et al., 2017). Zainuddin et al. improved the properties of *Parthenium hysterophorus* L. crude extract (PHCE) by nanoemulsion technique. The results showed that *P. hysterophorus* nanoemulsion showed the better inhibitory effect on *D. ocimifolia* seed germination than *P. hysterophorus* without encapsulation (Zainuddin et al., 2019). The nanoencapsulation of drug improved the efficiency of the drug delivery system. Therefore, it is commonly used in topical drug delivery products.



Source Yang (2020)

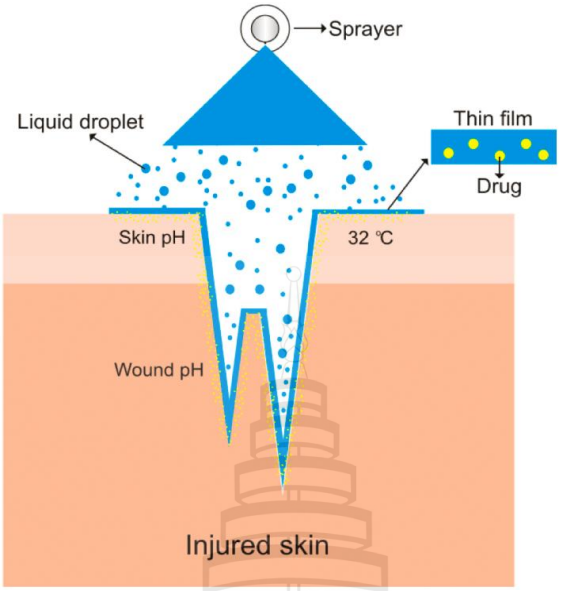
Figure 2.4 Types of Nanoemulsion Delivery System

2.3 Topical Film Forming Spray

Topical drug delivery system is an advanced technology in pharmaceutical application. Topical routes of drug delivery aim to avoid the hepatic first-pass metabolism, gastric pH variations, and fluctuations in plasma levels that are frequently found when a drug is administered through the oral route. The advantages of topical drug delivery system are improved drug bioavailability, ease and convenience, and painless and noninvasive technique. There are various forms of topical drug delivery system such as patches, creams, and film forming sprays (Singh et al., 2016; Umar et al., 2020). The disadvantages of patches are skin irritation, difficulty in applying on curved surfaces, and pain while peeling off. Ointments and creams have low adhesive leading to easily being wiped off by clothes. Therefore, there is a need for the development of a dosage form. The topical film forming spray was developed to permit less frequent dosing by maintaining close contact with the skin for a prolonged time period (Kathe & Kathpalia, 2017; Dhiman et al., 2011; Allen & Ansel, 2013).

Topical film forming spray is a novel approach as a promising choice for transdermal drug delivery used in pharmaceutical and cosmeceutical products due to its many advantages compared to conventional topical preparation. Moreover, the advantages of topical film forming spray are easily to apply, provide uniform drug distribution and dose, increase bioavailability, decrease incidence of irritation, provide continuous drug release, and accelerate wound healing through moisture control (Kathe & Kathpalia, 2017; Saingam et al., 2018). After the application of the spray solution to the skin, the film was formed due to the loss of the volatile components of the vehicle. After the sprayed solution contacts the target therapeutic site, film formation was formed by utilizing the polymer as a matrix. After that, drug will be released in a sustained fashion (Figure 2.5) (Umar et al., 2020). Sneha et al. reported that the topical film forming sprays have the ability to provide an accurate, long lasting and patient compliant delivery of drugs on the skin as compared to conventional gels (Ranade et al., 2017). Tehreem et al. developed novel VRC-loaded polymeric nanoparticles to improve the topical delivery in the form of film

forming spray. The result showed that the film forming spray was non-irritant as confirmed by histological study on the rabbit's skin (Mumtaz et al., 2022). Vaskuri et al. prepared topical delivery film forming topical dermal spray for augmenting inflammation and pain. The results showed that the revealed marked diminution in inflammation because the potential of FFTDS in alleviating pain and inflammation (Jyothi et al., 2022). The topical film forming spray has four mains components: polymer, solvent systems (volatile or nonvolatile vehicle), penetration enhancers, and drug (Schroeder et al., 2007).



Source Umar (2020)

Figure 2.5 Mechanism of Film Forming Spray

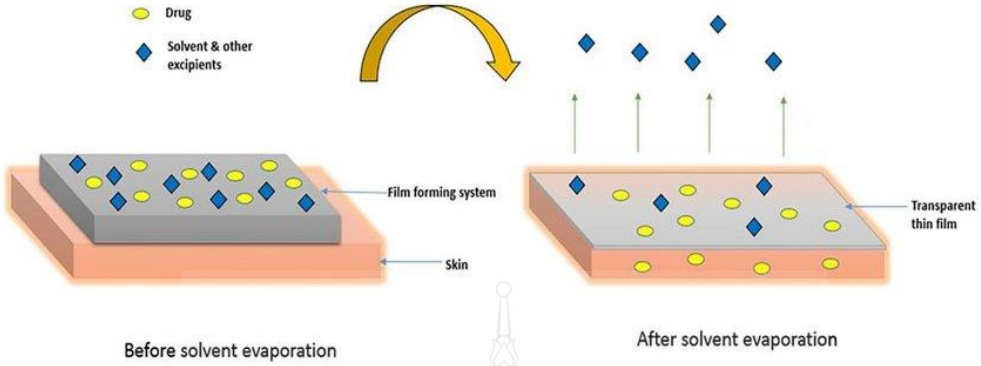
2.3.1 Polymer

Polymer acts as the film-forming base. Polymer agent used in film-forming spray must function as anti-nucleating agent and crystallization inhibitors to prevent crystallization of drug even after solvent evaporation (Oh et al., 2017). Polymer used in topical film forming spray can be natural or synthetic polymers such as chitosan, ethyl cellulose, hydroxypropyl methylcellulose, Carbopol 940, and Eudragit (EPO, E100, RSPO, RLPO) (Umar et al., 2020; Zhuang et al., 2018; Patra et al., 2017). Eudragit is available in various types with different purposes in topical film forming spray. Generally, Eudragit is synthetic polymers containing ratios ranging from two to three methacrylate monomers. It has been suitable for a wide variety of pharmaceutical applications because it has many advantages such as increased drug permeation in the skin, good sprayability, adhesiveness, and flexibility (Patra et al., 2017).

Eudragit® E100 is an adhesive cationic copolymer that is the matrix for controlled drug release. It possesses defined swelling capacity and permeability for water and dissolved drug, respectively. Eudragit® E100 hold considerable promise in targeted, site-specific administration of active pharmaceuticals where they can prove valuable for loading of sensitive drugs (Alasino et al., 2012). Eudragit® E100 has been reported to produce transparent and shiny films, while Eudragit® RSPO and RLPO can not produce transparent and shiny films. Eudragit RS100 can form flexible and dermal-adhesive film (Gohel et al., 2009; Ranade et al., 2017). In addition, Eudragit® L100 films have been reported to be unable to prevent the crystallization of testosterone (Leichtnam et al., 2007). In 2015, Vijaya et al. reported that the transdermal film using Eudragit® E100 and polyvinyl pyrrolidone (PVP) could provide an extended release and effective permeation of drug due to the addition of a hydrophilic substance of PVP (Vijaya et al., 2015). While medical polymers are gaining more attention in pharmaceutical production. Sritharadol et al. (2017) developed mupirocin topical spray using Eudragit® E100 as a film forming agent to treat wound healing (Sritharadol et al., 2017).

2.3.2 Solvent System

Solvent system is important in topical film forming spray. Solvent system comprises volatile and non-volatile solvents. There are various types of solvent used in topical film forming spray including glycols (*i.e.*, propylene glycol and polyethylene glycols), alcohol (*i.e.*, ethanol, butanol, isopropanol, and benzyl alcohol), and other solvents (Williams & Walters, 2007). Volatile solvents showed a short period of time evaporation, causing an increase in the thermodynamic activity of the drug through the stratum corneum layer. The selected solvent should facilitate the transfer of the drug and have a high drug loading capacity (Mandal et a., 2016). After solvent evaporation, the transparent film was formed as shown in Figure 2.6 (Kathe & Kathpalia, 2017).



Source Kathe and Kathpalia (2017)

Figure 2.6 Mechanism of Film Formation

2.3.3 Penetration Enhancer

Penetration enhancer is used for improving transdermal drug delivery into the skin by decreasing the barrier resistance (Williams & Barry, 2007). It is the agent that can increase the drug diffusivity through the skin by reducing the barrier resistance of the stratum corneum (SC) without damaging viable cells (Pathan & Setty, 2009). SC serves as a rate-limiting lipophilic barrier against the uptake of chemical and biological toxins as well as transepidermal water loss. The possible pathways for epidermal penetration of active compounds include transcellular (intracellular) permeation across the corneocytes of SC, penetration through the intercellular spaces of the SC, and appendage penetration through the hair follicle, sebaceous, and sweat glands (Herman & Herman, 2014). Frequently penetration enhancers used in formulations include pyrrolidones (2-pyrrolidone, 2P), alcohols (ethanol), alkanols (decanol), sulfoxides (DMSO), glycols (propylene glycol, PG), azones (laurocapram), surfactants, and terpenes (Vyas et al., 2011; Kar et al., 2019).

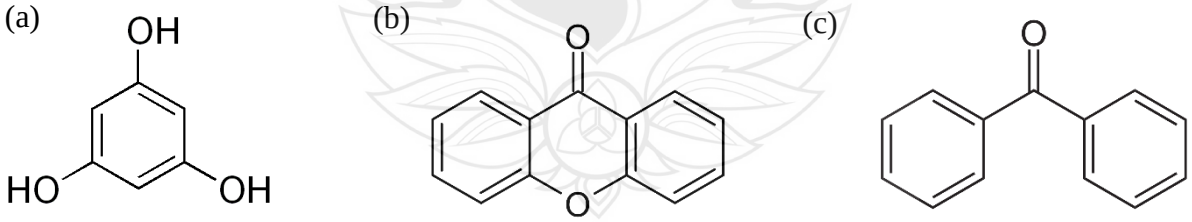
2.3.4 Drug

Drug applied to topical film forming spray should have suitable properties independent of the dosage form. It should be dissolved in the film forming polymers and after solvent evaporation, the drug saturation should remain on the skin surface (Tran & Tran, 2019). In recent research, drug solubility in the polymer has been suggested to be an important factor in controlling drugs crossing the skin. Edwards et al. reported that methylphenidate was the free base and had a lower solubility in Eudragit RS than in Eudragit E (Edwards et al., 2017). Currently, there are two forms of drugs classified as herbal extracts and commercial drugs. Herbal extracts commonly known as medicinal plant can be administered as the whole plant by extraction process. The advantages of herbal extracts are low side effect, readily available, and cheaper than modern medicines. However, the herbal extract is unstable depending on climate change and habitat destruction. In 2018, Saingam et al. prepared topical film forming spray containing *Piper nigrum* L. or Prik Thai Dam. Prik Thai Dam has many bioactivities such as anti-inflammatory, antioxidant, and analgesic activities. The results showed that the film forming spray formulations were clear, smooth and flexible in physical appearance (Saingam et al., 2018).

2.4 *Garcinia cowa* Leaf Extract

In Thai, *Garcinia cowa* commonly known as Chamuang was distributed in Malaysia, Thailand, and Myanmar. It has received much interest in pharmaceutical application due to its bioactive compounds. *Garcinia cowa* is a medium-sized tree. The fruits and young leaves are edible with a sour taste. The bark is dark brown with a yellow latex. The leaves are glossy, deep green, oblong, up to 6-15 cm in length, and 2.5-6.0 cm in width. *Garcinia cowa* has a rich source of secondary metabolites, especially benzophenone, phloroglucinols, and xanthones (Figure 2.7) (Ritthiwigrom et al. 2013). Thus, it has various pharmacological activities, including antimicrobial, antioxidant, anticancer, and anti-inflammatory activities (Panthong et al., 2006; Panthong et al., 2009; Likhitwitayawuid et al., 1998; Tian et al., 2008). Joseph et al.

reported that fruit rinds of *Garcinia cowa* extracted by hexane and chloroform showed significant antiaflatoxigenic and antioxidant activities. So, *Garcinia cowa* could controlfungal growth, prevent aflatoxin contamination, and increase the shelf life (Joseph et al., 2005). Negi et al. reported that the fruit rinds of *Garcinia cowa* extracted using hexane and chloroform exhibited antioxidant and antimutagenic activities (Negi et al., 2010). Kedare et al. reported that the leaves of *Garcinia cowa* extracted by ethanol showed antioxidant activity performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical scavenging assay (Kedare et al., 2011). In 2015, Wahyuni et al. reported that the isolated compound of the leaves of *Garcinia cowa* extracted by methanol showed cytotoxic properties against cancer cell-line (Wahyuni et al., 2015). Munira et al. investigated the antibacterial activity from the leaves and barks of *Garcinia cowa* extracted using methanol. The results showed that the crude extracts from the leaves and barks of *Garcinia cowa* possessed mild and moderate antibacterial activity (Munira & Chowdhury, 2015). In 2020, phukhatmuen et al. discovered and identified bioactive compounds from the ethyl acetate extract of the leaves of *Garcinia cowa*. The results showed that the isolation compound from fresh *Garcinia cowa* had antidiabetic activity (Phukhatmuen et al., 2020). Consequently, *Garcinia cowa* is suitable for medical applications as it contains various biological activities.



Note Phloroglucinol (a), Benzophenone (b), and Xanthone (c)

Figure 2.7 Isolated Compounds from Leaf, Fruit, Twig, and Stem of *Garcinia cowa* Containing

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials

Polyoxyethylene (20) sorbitan monolaurate (Tween 20)	PC Intertrade, Thailand
Polyoxyethylene (80) sorbitan monolaurate (Tween 80)	PC Intertrade, Thailand
Transcutol CG	PC Intertrade, Thailand
Poloxamer 188	Sigma-Aldrich, USA
Glycerol	Sigma-Aldrich, USA
2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Sigma-Aldrich, USA
Griess reagent	Sigma-Aldrich, USA
Lipopolysaccharide (LPS) (From *Escherichia coli* O55:B5)	Sigma-Aldrich, USA
Polyvinylpyrrolidone (PVP)	Sigma-Aldrich, USA
Dibutyl phthalate (DBP)	Sigma-Aldrich, USA
Dialysis bag (10,000 MWCO)	Thermo Scientific, USA
Ethanol	RCI LabScan Limited, Thailand

Chloroform	RCI LabScan Limited, Thailand
Dimethyl sulfoxide (DMSO)	RCI LabScan Limited, Thailand
Eudragit® E100 (EuE100)	Evonik industries, Germany
Nutrient Broth	Himedia, Thailand
Biomax® 500 kDa ultrafiltration disc	Merck, USA
Dulbecco's Modified Eagle Medium (DMEM)	GIBCO, USA
Fetal bovine serum (FBS)	GIBCO, USA
Phosphate buffered saline (PBS)	GIBCO, USA

3.2 Methodology

3.2.1 Antioxidant Activity of GC

Antioxidant activity of GC was investigated by 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay. First, the $ABTS^{\bullet+}$ stock solution mixture was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate solution in equal quantities and kept in dark box for 16-18 h. The $ABTS^{\bullet+}$ radical solution was diluted with distilled water to achieve a solution with absorbance of 0.7 at 731 nm. The control was prepared by mixing 100 µL of $ABTS^{\bullet+}$ solution with 100 µL of distilled water. The sample was prepared by dissolving GC in ethanol and then diluted with distilled water to make various concentrations (0.5, 0.25, 0.125, 0.063, 0.031, and 0.016 mg/mL) solution. Then, 100 µl of each sample solution was mixed with 100 µL of $ABTS^{\bullet+}$ solution. After that, the reaction mixture was kept in the dark condition at room temperature for 15 min.

The antioxidant activity of GC was determined by microplate reader at 731 nm. The percentage antioxidant activity (%AA) was calculated by the following equation (1):

$$AA\ (\%) = \frac{A_{control} - (A_{sample} - A_{control\ of\ sample})}{A_{control}} \times 100 \quad (1)$$

Where $A_{control\ of\ sample}$ is the absorbance values of GC solution. $A_{control}$ is the absorbance values of $ABTS^{\bullet+}$ solution and A_{sample} is absorbance values of the testing solution $ABTS^{\bullet+}$ with GC solution.

3.2.2 Antibacterial Activity of GC

Staphylococcus aureus, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* were used to estimate the antibacterial activity of GC. One loopful of the microorganisms was inoculated in the test tube and then shaken in an air-bath shaker at 37 °C for 24 h. The cultures of these strains containing approximately 10^5 Colony Forming Units per milliliter (CFU/mL) were prepared and used for the evaluation of antibacterial activity, and then conducted as the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) method. MIC method was defined as the lowest concentration is able to inhibit any visible bacteria growth. GC was dissolved in nutrient broth solution to prepare the different concentrations of sample (5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 mg/mL). 50 µL of the bacterial suspension was then added to all wells containing GC and to the control wells. The 96-well plates were incubated at 37 °C for 24 h. After that, the MIC of GC was measured at 625 nm using microplate reader (BioTek Instruments, USA) to observe the precipitate from the growth of bacteria. The lowest concentration at which precipitate occurred was taken as the MIC value.

The MBC is supplementary to the MIC; whereas the MIC test indicates the lowest level of antibacterial agent that greatly inhibits growth, the MBC represents the lowest level of antibacterial agent resulting in microbial death. A sample solution from

MIC test was dropped on the nutrient agar plate and incubated at 37°C for 24 h. Finally,if no growth of bacteria was observed, the original concentration contained no living bacteria and it was considered to kill bacteria at the concentration.

3.2.3 Anti-inflammatory Activity of GC

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS) at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2. GC was dissolved in 5% v/v ethanol in serum-free medium (SFM) and produce various concentrations with SFM (i.e., 5, 2.50, 1.25, 0.62, 0.31, 0.16 and 0.08 mg/mL). This assay was performed in four replicate wells in 96-well plates. First, the plates were seeded with cell suspension at 1×10^5 cells/well and incubated in humidified incubator with 5% CO_2 at 37 °C for 24 h to allow cell attachment. After that, the cells were treated with GC at various concentrations for 1 h. Then, the supernatant was discarded and the cells were stimulated with 0.1 mg/mL of lipopolysaccharide (LPS) for 24 h. After 24 h treatment, 50 µL of supernatant was transferred to a new plate and Griess reagent was added. The well plate was then incubated at room temperature for 10 min. Finally, the anti-inflammatory activity of GC was measured at 540 nm using microplate reader (BioTek Instruments, USA).

3.2.4 Cytotoxicity of GC

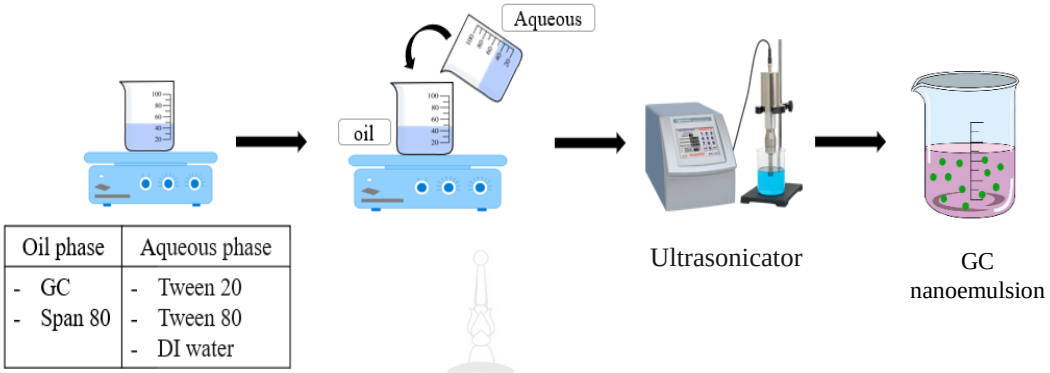
NCTC clone 929 cells were cultured in DMEM, containing 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. GC was dissolved in 5% v/v ethanol in SFM and produce various concentrations with SFM (i.e., 2, 1, 0.5, 0.25, 0.125, 0.625, and 0.313 mg/mL). This assay was operated in four replicate wells in 96-well plate. First, plates were seeded with cell suspension at 8,000 cells/well into 96-well plate and incubated in humidified incubator with 5% CO_2 at 37 ℃ for 24 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with GC and then cells were re-incubated for 24 h. After incubation period, the cells viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with the viability of the cells cultured by fresh SFM being used as control and solvent system as positive control.

3.2.5 Preparation of GC Nanoemulsion

GC nanoemulsion was prepared under oil-in-water (O/W) emulsion system using ultrasonic technique. For GC1, 1 g of GC was first dissolved in 5 g of ethanol, while for GC2 and GC3, 1 g of GC was dissolved in 3 g of transcutol CG. After that, GC nanoemulsion was prepared by mixing 2 phases of oil and aqueous phases. Compositions of GC nanoemulsion are shown in Table 3.1. After that, each phase was stirred with rpm of 300 at 60±5 °C. The aqueous phase was then added into the oil phase and mixed entirely by stirring with rpm of 300 at 60±5 °C for 5 min. Finally, the mixture solution was sonicated at 30% amplitude for 3 min using an ultrasonic processor (VC750, Sonic & Materials, INC., USA). A schematic representation of the preparation of GC nanoemulsion was shown in Figure 3.1.

Table 3.1 Compositions of GC Nanoemulsion

Formulation	**Ingredient (%w/w)**						
	Oil phase			**Aqueous phase**			
	GC	**Span 80**	**Glycerol**	**Tween 20**	**Tween 80**	**Poloxamer**	**Distilled water**
GC1	1	1	1	-	2	2	93
GC2	1	2	-	3	2	-	92
GC3	2	2	-	3	2	-	91
Blank	-	2	-	3	2	-	93



Oil phase	Aqueous phase
- GC - Span 80	- Tween 20 - Tween 80 - DI water

Figure 3.1 Schematic Preparation of GC Nanoemulsion

3.2.6 Stability Test of GC Nanoemulsion

Stability test of GC nanoemulsion was studied to observe physical properties of GC nanoemulsion under 2 conditions (room temperature and 45 °C). The GC nanoemulsions were stored for 0, 1, 2, and 3 months. After that, GC nanoemulsion was measured for its average size, PDI, and zeta potential by dynamic light scattering (DLS) (Zeta sizer, Nano series, Malvern, UK).

3.2.7 Size, Zeta potential, and Polydispersity Index (PDI) of GC Nanoemulsion

The average size, polydispersity index (PDI), and zeta potential of the GC nanoemulsion were determined by DLS (Zeta sizer, Nano series, Malvern, UK). Each sample was dispersed in distilled water and measured at 25 °C using disposable cuvettes and folded capillary cells with electrodes for size and zeta potential, respectively.

3.2.8 Morphological Investigation of GC Nanoemulsion

The morphology of GC nanoemulsions was observed using transmission electron microscopy (TEM) (JEM1010 microscope, JEOL, Tokyo, Japan) with an accelerating voltage of 200 kV. The GC nanoemulsion was dissolved in Milli-Q® water (1:20) and then the sample was dropped on a copper grid with a parafilm. The excess droplets were instantly removed using a filter paper. The TEM images of the GC nanoemulsion were obtained.

3.2.9 Encapsulation Efficiency of GC Nanoemulsion

The UV-vis spectrophotometer was used for scanning the absorbance to detect the unique wavelength maxima of GC. It was found the unique wavelength maxima of GC was 250 and 413 nm. These wavelength was used for encapsulation efficiency and release study. The encapsulation efficiency was measured to determine GC that was loaded in the GC nanoemulsion. First, the sample was added into a centrifuge tube containing chloroform at the ratio of 0.2:2 mL (sample:chloroform). The solution was then sonicated using ultrasonic bath at power level of 9 at room temperature for 30 min. After that, each solution was placed in the fridge to accelerate the stratification. Finally, the GC content in the sample was analyzed by UV-vis spectrophotometer at the wavelength of 250 nm. The encapsulation efficiency (%EE) was calculated as shown in following the equation (2)

$$\text{EE (\%)} = \frac{\text{Weight of loaded GC}}{\text{Weight of initial GC}} \qquad (2)$$

3.2.10 Release Study of GC Nanoemulsion

In vitro release of GC from GC nanoemulsion was determined by the total immersion method at 32.5 ºC. The acceptor medium was 20 mL of 94 % v/v PBS (pH 5.5), 5% v/v ethanol, and 1 % v/v Tween 80. First, 5 mL of GC nanoemulsion was loaded in a dialysis bag (SnakeSkinTM dialysis tubing, 10,000 MWCO). Then the dialysis bag was soaked in 20 mL of acceptor medium. The sample solution was withdrawn at different time points (*i.e.*, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, and 48 h). After a specific time, 1 mL of the sample. After a specific time, 1 mL of the sample solution was removed and fresh buffer solution was replaced in equal volume. Then, the released amounts of GC were measured using UV-vis spectrophotometer (Perkin-Elmer, USA) at the wavelength of 250 nm and calculated using a calibration curve of GC.

3.2.11 Antioxidant Activity of GC Nanoemulsion

Antioxidant activity of GC nanoemulsion was investigated by ABTS radical scavenging assay with some modifications. First, the $ABTS^{\bullet+}$ stock solution mixture was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate solution in equal quantities and kept in dark condition for 16-18 h. The $ABTS^{\bullet+}$ radical solution was diluted with distilled water to achieve a solution with absorbance of 0.7 at 731 nm. The control was prepared by mixing 100 µL of $ABTS^{\bullet+}$ solution with 100 µL of distilled water. The sample was prepared by dissolving GC nanoemulsion in distilled water produce various concentrations (100, 50, 25, 12.5, 6.25, and 3.125% v/v) of solution. Then, 100 µL of each sample solution was mixed with 100 µL of $ABTS^{\bullet+}$ solution. After that, the reaction mixture was in the dark condition at room temperature for 15 min. The antioxidant activity of GC nanoemulsion was determined by microplate reader at 731 nm. The percentage antioxidant activity (%AA) was calculated by the following equation (1).

3.2.12 Antibacterial Activity of GC Nanoemulsion

Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, and Pseudomonas aeruginosa were used to evaluate the antibacterial activity of GC nanoemulsion. One loopful of the microorganisms was inoculated in the test tube and

then shaken in an air-bath shaker at 37 °C for 24 h. The cultures of these strains containing approximately 10^5 Colony Forming Units per milliliter (CFU/mL) were prepared and used for the evaluation of antibacterial activity, and then conducted as the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) method. MIC method was defined as the lowest concentration able to inhibit any visible bacteria growth. GC nanoemulsion was dissolved in nutrient broth solution to prepare various concentrations of the sample (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.19 %v/v). 50 µL of the bacterial suspension was then added to all wells containing sample and to the control wells. The plates were incubated at 37 °C for 24 h. After that, the MIC of GC nanoemulsion was measured at 625 nm using microplate reader (BioTek Instruments, USA) to observe the precipitate from the growth of bacteria. The lowest concentration at which the precipitate occurred was taken as the MIC value.

The minimum bactericidal concentration (MBC) method was defined as the lowest concentration of GC nanoemulsion that kills 99.9% of bacteria. A sample solution from MIC test was dropped on the nutrient agar plate and incubated at 37 °C for 24 h. Finally, if no growth of bacteria was observed, the original concentration contained no living bacteria and it was considered to kill bacteria at the concentration.

3.2.13 Anti-inflammatory Activity of GC Nanoemulsion

The mouse macrophage-like cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. GC nanoemulsion was dissolved in DMEM to produce various concentrations of GC nanoemulsion (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.19 %v/v). This assay was performed in four replicate wells in 96-well plate. First, plates were seeded with cell suspension at 1×10^5 cells/well into 96-well plate and incubated in humidified incubator with 5% CO_2 at 37 °C for 24 h to allow cell attachment. After that the cells were treated with samples at various concentrations for 1 h. Then, the cells were stimulated with 0.1 mg/mL of lipopolysaccharide (LPS) for 24 h. After 24 h treatment, 50 µL of supernatant liquid was transferred to a new plate and Griess reagent was added. The well plate was then

incubated at room temperature for 10 min. Finally, the anti-inflammatory activity of GC nanoemulsion was measured at 570 nm using microplate reader (BioTek Instruments, USA).

3.2.14 Cytotoxicity of GC Nanoemulsion

NCTC clone 929 cells were cultured DMEM, containing 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. GC nanoemulsion was dissolved in SFM to produce various concentrations (1.56, 0.78, 0.39, and 0.19% v/v). This assay was performed in four replicate wells in 96-well plate. First, plates were seeded with cell suspension at 8,000 cells/well into 96-well plate and incubated in humidified incubator with 5% CO_2 at 37 °C for 24 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with GC nanoemulsion solution and determined by MTT assay, with the viability of the cells cultured by fresh SFM being used as control and solution system without GC nanoemulsion being used as positive control.

3.2.15 Cell Internalization

Cell internalization of GC nanoemulsion was conducted with fibroblast cell line (CRL-2522) and determined by confocal laser scanning microscope (FV10i, Olympus) and qualified by fluorescence measurement. Briefly, GC nanoemulsion was incubated on the cells for 24 h and removed from the apical surface of the cell layer. After that, the cells were washed with PBS. To track the membrane-associated nanoemulsion, the cells were labeled with Calcein-AM and rhodamine (Live/Dead Cell viability assay, Thermo Fisher Scientific) for 15 min before the measurement of internalization of nanoemulsion under confocal microscope.

3.2.16 Preparation of Topical Film Forming Spray Containing GC Nanoemulsion

First, Eudragit E100 (EuE100) was dissolved in ethanol to prepare various concentrations of solution (8, 10, 12%w/v). Next, 16 % v/v of GC nanoemulsion (based on volume of EuE100 solution) was added into EuE100 solution. The mixture was stirred until the homogenous solution was obtained. After that, PVP was added into the

mixture at weight ratio of 7:3 (EuE100:PVP) and continuously stirred. Then, 2% w/v of DBP was added and stirred until the homogenous solution was obtained. Compositions of topical film forming spray containing GC nanoemulsion are shown in Table 3.2. Finally, the topical film forming spray containing GC nanoemulsion was kept in the close container. A schematic representation of the preparation of topical film forming spray containing GC nanoemulsion is shown in Figure 3.2.

Table 3.2 Compositions of Topical Film Forming Spray Containing GC Nanoemulsion

Formulation	**EuE100 (%w/v)**	**PVP (Weight ratio of EuE100:PVP)**	**DBP (%w/v)**	**GC2 (%v/v)**	**GC3 (%v/v)**
B1	8	7:3	2	-	-
B2	10	7:3	2	-	-
B3	12	7:3	2	-	-
S1	8	7:3	2	16	-
S2	10	7:3	2	16	-
S3	12	7:3	2	16	-
S4	8	7:3	2	-	16
S5	10	7:3	2	-	16
S6	12	7:3	2	-	16

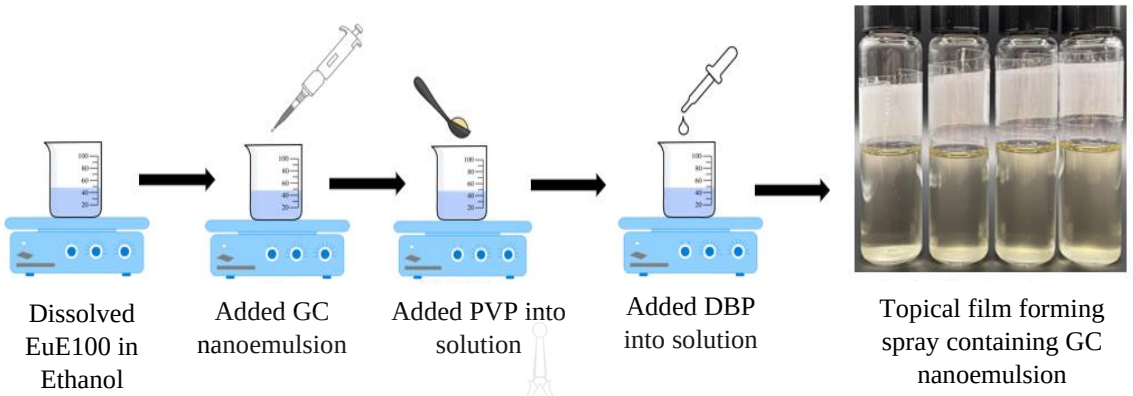


Figure 3.2 Schematic Preparation of Topical Film Forming Spray Containing GC Nanoemulsion

3.2.17 Stability Test of Topical Film Forming Spray Containing GC Nanoemulsion

Stability test of topical film forming spray containing GC nanoemulsion was studied to observe the physical properties of topical film-forming spray containing GC nanoemulsion under 3 conditions (4 °C, room temperature, and 45 °C). The solutions were stored for 0, 1, 2, and 3 months.

3.2.18 pH Study of Topical Film Forming Spray Containing GC Nanoemulsion

pH of the topical film forming formulations was characterized by pH meter. Prior to measure the pH of formulations, the pH meter was calibrated using standard solution of pH 4, 7, and 10.

3.2.19 Viscosity Study of Topical Film Forming Spray Containing GC Nanoemulsion

The viscosity of spray formulations was measured using a Brookfield viscometer with spindle rotational speed of 200 rpm at room temperature.

3.2.20 Contact Angle of Topical Film Forming Spray Containing GC Nanoemulsion

Contact angle of the topical film forming formulations was characterized by a contact angle and surface tension analyzer (KINO, SL200KS). The contact angle was investigated in pendant drop mode. The droplet was captured and analyzed by the software. The sessile drop mode was used for contact angle examination. The system was carried out at 25 °C. The sample was dropped on glass slide. After that, the software was captured and analyzed the data.

3.2.21 Spray Pattern Study of Topical Film Forming Spray Containing GC Nanoemulsion

Spray pattern was determined by actuation of the topical film forming spray formulations in the horizontal direction to white paper. First, all formulations were colorized by addition of methylene blue and then the mixture was sprayed in the vertical direction on white paper at distance of 5.0 cm. After that, the spay pattern was evaluated by measuring diameter of blue spot. The measured diameter was repeated in 4 diameters per spot.

3.2.22 Weight Per Spray Study of Topical Film Forming Spray Containing GC Nanoemulsion

Weight per spray is quantitative test that was used to determine the weight of formulations per actuation. First, topical film forming spray containing GC nanoemulsion was filled into the spray container. The container was weighed both before and after spraying. The weight per spray was calculated by equation (3)

$$\text{Weight per spray} = \frac{\text{Initial weight-final weight}}{\text{number of actions}} \qquad (3)$$

3.2.23 Evaporation Time of Topical Film Forming Spray Containing GC Nanoemulsion

Evaporation time was measured to determine the time that the solution forms the film. Each formulation was sprayed on polystyrene plate by fixing area with diameter of 3.5 cm and volume of spray solution was 130 µL. After that, the evaporation time was recorded. The system was carried out at room temperature.

3.2.24 Encapsulation Efficiency of Topical Film Forming Spray Containing GC Nanoemulsion

The encapsulation efficiency was measured to determine GC that was loaded in the topical film forming spray containing GC nanoemulsion. First, the sample was added into a centrifuge tube containing chloroform at the ratio of 0.2:2 mL (sample:chloroform). The solution was then sonicated using ultrasonic bath at power level of 9 at room temperature for 30 min. After that, each solution was placed in the fridge to accelerate the stratification. Finally, the GC content in the sample was analyzed by UV-vis spectrophotometer at the wavelength of 413 nm. The encapsulation efficiency (%EE) was calculated following the equation (2).

3.2.25 Drug Release Study of Topical Film Forming Spray GC Nanoemulsion

In vitro release of GC from topical film forming spray containing GC nanoemulsion was determined by Franz diffusion cell method. First, 130 µL of topical film forming spray containing GC nanoemulsion was dropped on glass slide (diameter 3 cm). Then, each sample was placed on polyethersulfone membrane which was placed on a modified Franz diffusion cell containing acceptor medium of 94 % v/v PBS (pH 7.4) and 1 % v/v Tween 80 at 32.5 °C. The sample solution was withdrawn at different time points (0.5, 1, 2, 3, 4, 6, 8, 10, 24, and 48 h). After a specific time, 1 mL of the sample solution was removed and fresh buffer solution was replaced in equal volume. Then, the released amounts of GC were measured using UV-vis spectrophotometer (Perkin-Elmer, USA) at the wavelength of 413 nm and calculated using a calibration curve of GC.

3.2.26 Antibacterial activity of Topical Film Forming Spray Containing GC Nanoemulsion

S.aureus, S.epidermidis, E.coli, and *P.aeruginosa* were used to evaluate the antibacterial activity of topical film forming spray containing GC nanoemulsion. One loopful of the microorganisms was inoculated in the test tube and then shaken in an air-bath shaker at 37 °C for 24 h. The cultures of these strains containing approximately 10^5 CFU/mL were prepared and used for the estimation of antibacterial activity. First, 130 µL of topical film forming spray containing GC nanoemulsion was dropped on 6-well plate and then dried in fume hood. The film sample was immersed in 1 mL of medium (PBS (pH 5.5), 1% v/v Tween 80, and 5% v/v ethanol) for 4 h to produce the extraction medium. 50 µL of the bacterial suspension was added to wells containing 50 µL of extraction medium. The plates were then incubated at 37 °C for 24 h. After that, the mixture solution was measured at 625 nm using microplate reader (BioTek Instruments, USA) to observe the precipitate from the growth of bacteria.

The bacterial killing is defined as the extraction medium from topical film forming spray containing GC nanoemulsion that kills 99.9% of bacteria. A drop from each well plate was dropped on the nutrient agar plate and incubated at 37 °C for 24 h. Finally, if no growth of bacteria was observed, the extraction medium containing no living bacteria was considered to kill bacteria.

3.2.27 Anti-inflammatory of Topical Film Forming Spray Containing GC Nanoemulsion

RAW 264.7 cells were cultured in DMEM containing 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. First, topical film forming spray containing GC nanoemulsion was dropped on 6-well plate and then dried in fume hood. The film sample was immersed in medium (SFM) for 4, 8, and 24 h to produce the extraction medium. After specific time, the extraction medium was removed. The RAW 264.7 cells were seeded into 96-well plate at 1×10^5 cells/well and incubated in humidified incubator with 5% CO_2 at 37 °C for 24 h to allow cell attachment. After that, the cells were treated with extraction medium for 1 h. Then, the supernatant was discarded and the cells were stimulated with 0.1 mg/mL of LPS for 24 h. After that,

50 µL of supernatant liquid was transferred to a new plate and Griess reagent was added. The well plate was then incubated at room temperature for 10 min. Finally, the anti-inflammatory activity of topical film forming spray containing GC nanoemulsion was measured at 540 nm using microplate reader (BioTek Instruments, USA).

3.2.28 Indirect Cytotoxicity Test of Topical Film Forming Spray Containing GC Nanoemulsion

NCTC clone 929 cells were cultured in DMEM containing 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. First, 130 µL of the topical film forming spray was dropped on 6-well plate and then dried in fume hood. The film sample was immersed in 1 mL of medium (SFM) for 4, and 24 h to produce the extraction medium. After specific time, the extraction medium was removed. This assay was performed in four replicate wells in 96-well plate. The cells were separately cultured in 96-well plate at 8,000 cells/well into 96-well plate and incubated in humidified incubator with 5% CO_2 at 37 °C for 24 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with extraction medium and then cells were re-incubated for 4 h. After incubation period, the cells viability was determined by MTT assay, with the viability of the cells cultured by fresh SFM being used as control.

3.2.29 Statistical Analysis

All experiment data from the studies were expressed as means ± standard deviation (SD). Analysis of variance was calculated using one-way ANOVA, followed Tukey's post hoc tests in SPSS (IBM SPSS statistics 24, USA). The statistical significance was accepted at $p<0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Antioxidant Activity of GC

The antioxidant activity of GC was investigated by ABTS assay. The results are shown in Figure 4.1. The antioxidant activity of GC increased with increasing concentration of GC. The maximum antioxidant activity of GC was 81.54±0.93% and the half-maximum inhibition concentration (IC_{50}) of GC was 0.22±0.003 mg/mL. Since, the GC contains various xanthones with phenolic functional groups. Thus, it showed good antioxidant activity (Joseph et al., 2005; Mahabusarakam et al., 2005).

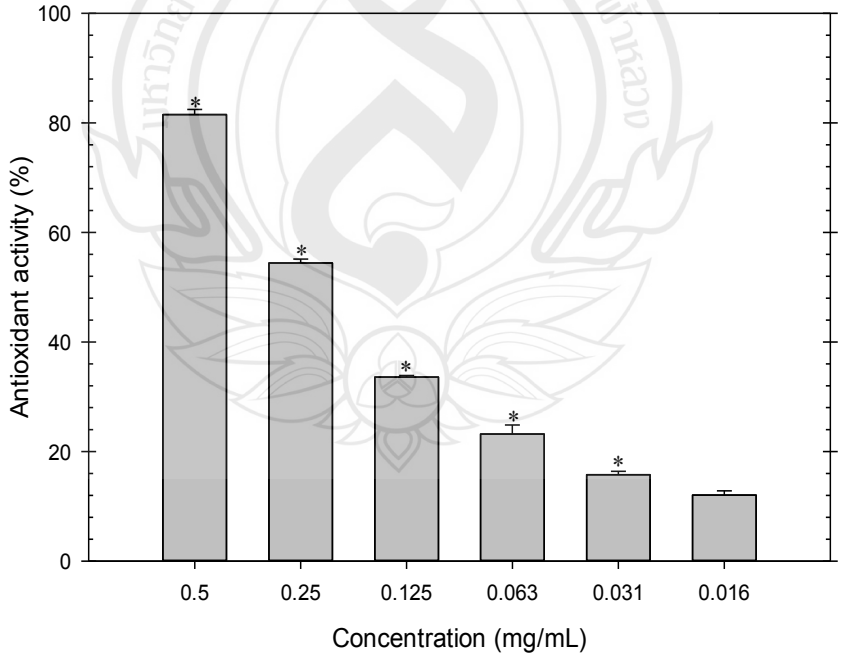


Figure 4.1 Antioxidant Activity of GC (n=3) *$p<0.05$ Compared with % Antioxidant Activity at 0.016 mg/mL

4.2 Antibacterial Activity of GC

Four bacterial candidates (Gram-negative: *E. coli* TISTR 527 and *P. aeruginosa* TISTR 1287 and Gram-positive: *S. epidermidis* DMST 15505 and *S. aureus* TISTR 746) were used to evaluate the antibacterial activity of GC. The MIC and MBC values of GC are shown in Table 4.1. The GC exhibited the strongest antibacterial activity against *P. aeruginosa* with MIC and MBC values of 0.63 and 0.63 mg/mL, respectively.

Table 4.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of GC (Mean ± Standard Deviation; n=3)

Microorganisms	MIC (mg/mL)	MBC (mg/mL)
E. coli	2.50±0.00	-
P. aeruginosa	0.63±0.00	0.63±0.0
S. epidermidis	1.04±0.00	2.50±0.0
S. aureus	2.50±0.00	-

4.3 Anti-inflammatory Activity of GC

The anti-inflammatory activity of GC was determined by nitric oxide production assay. This assay measured the accumulation of nitric oxide (NO) in a culture medium using the Griess reaction (Borges et al., 2018). The results demonstrated that the NO production inhibition with IC_{50} value of GC was 0.285±0.032 mg/mL. It has been reported that the phenylbutazone compound in GC shows strong anti-inflammatory activity. Thus, GC has anti-inflammatory activity (Panthong et al., 2009).

4.4 Cytotoxicity of GC

The MTT assay measured the cytotoxicity of GC on NCTC clone 929 cells. The results are shown as the relative cell viability referred to control. The viability of cells was determined after 24 h incubation with different concentrations of GC (Figure 4.2). The results showed that the viability of NCTC clone 929 cells cultured with GC ranged between ~76 and ~95% at GC concentration of 0.13-1.00 mg/mL. These results confirmed that GC at concentration of 0.13-1.00 mg/mL was non-toxic to the NCTC clone 929 cells.

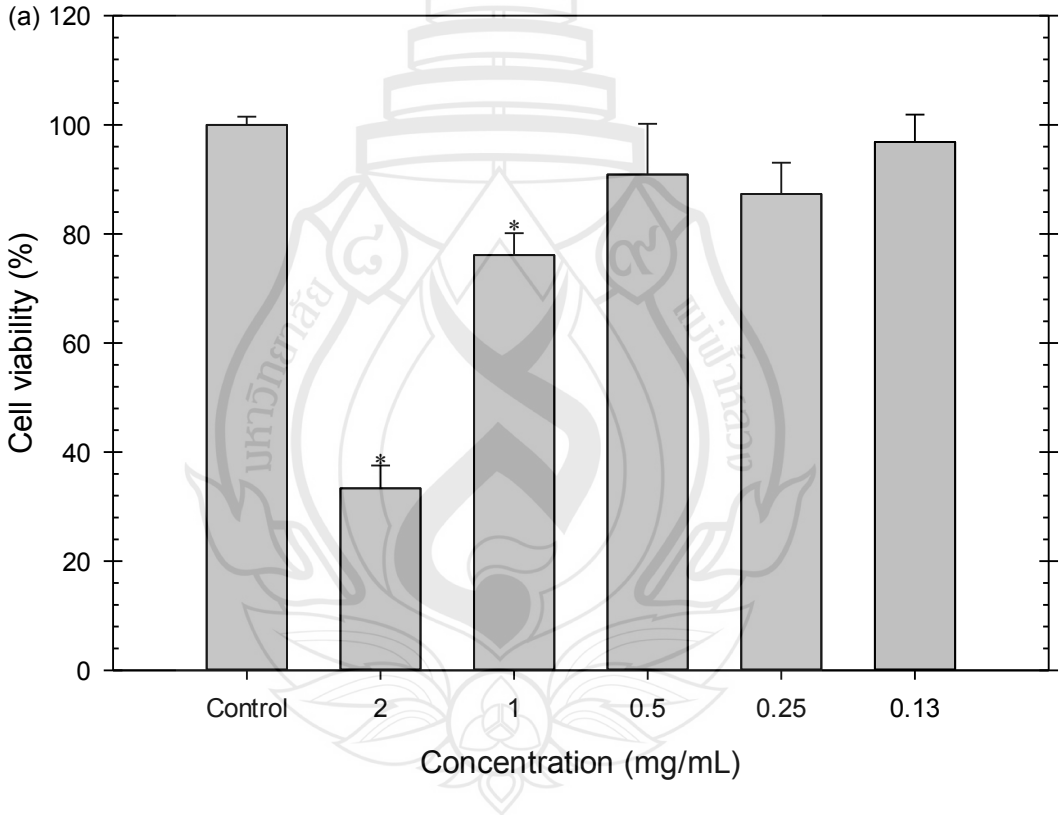
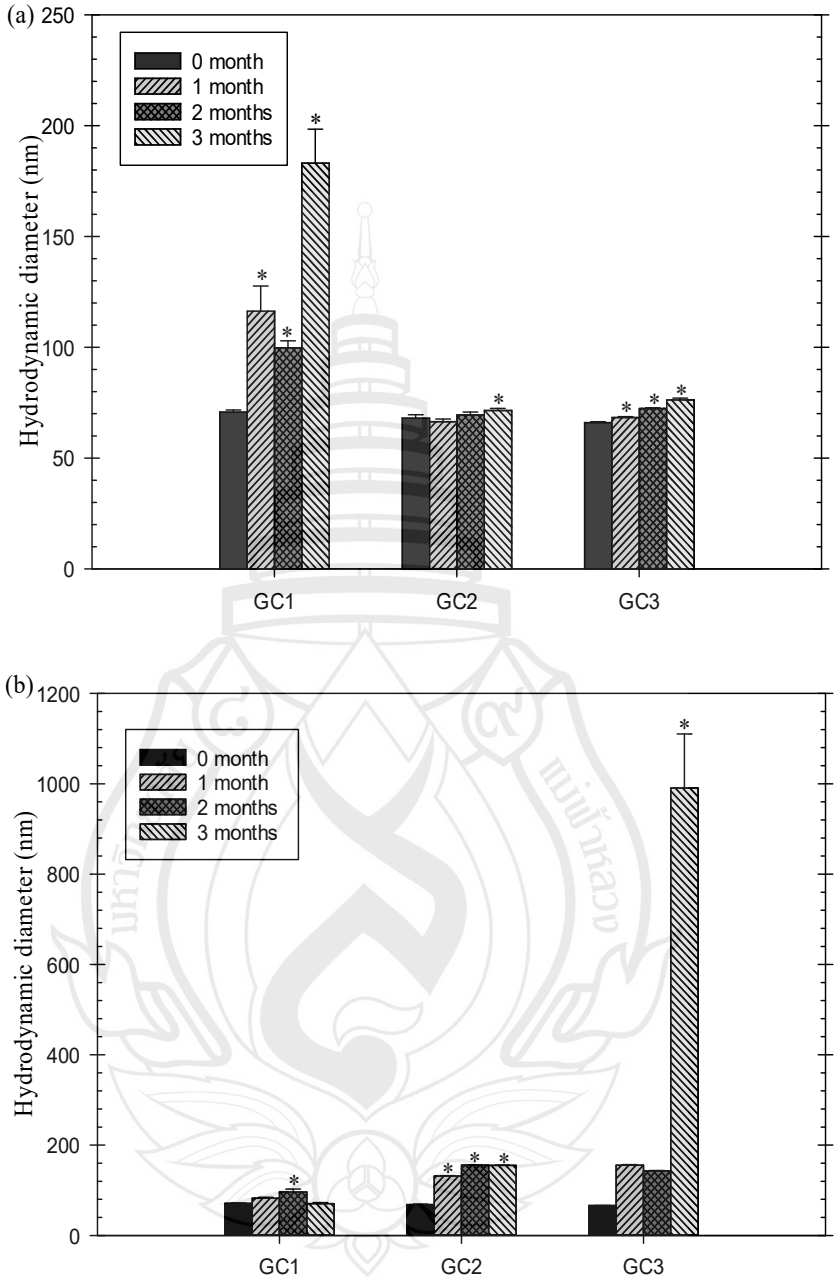


Figure 4.2 Cytotoxicity of GC on NCTC Clone 929 Cells (n=3). *$p<0.05$ Compared with Control

4.5 Formulation Development of GC Nanoemulsion

In the preliminary study, GC was tested for its solubility in ethanol and transcutol before the formulation development of GC nanoemulsion. The result showed that the solubility of GC in ethanol and transcutol CG was 10 mg/mL and 10 mg/mL, respectively. GC nanoemulsions with varying surfactant types (Tween 20, Tween 80, and poloxamer 188) were prepared to identify the optimal formulation of GC nanoemulsions. From the stability test of GC nanoemulsions under different conditions for 3 months, it was found that GC1 showed good stability without stratification. However, GC1 was not optimal formulation due to its low biological activity (See the results in Table 4.3, Figure 4.7, and Anti-inflammatory activity). This might be the amount of active ingredient added in this formulation was low concentration. Thus, the formulation was developed to identify the optimal formulation by varying concentrations of GC and changing the surfactant types. The results showed that GC2 and GC3 were clear nanoemulsion with no phase separation. The storage stability of GC nanoemulsion was determined from size particles, zeta potential and PDI of GC nanoemulsion under storage at 30 °C and 45 °C for 0, 1, 2, and 3 months (Figure 4.3). The results showed that the sizes of GC2 and GC3 under storage at 30 °C for 3 months were stable. While, GC1 and GC2 under storage at 45 °C still showed small size of particles at 3 months but GC3 showed large size of particles. However, at 30 °C that is actual usability, GC2 and GC3 were more stable than GC1. In addition, GC2 and GC3 showed good biological activities (Table 4.3, Figure 4.7, and anti-inflammatory). Therefore, GC2 and GC3 were used to formulate in the topical film forming spray.



Note (a) and 45 °C (b) for Different Time Points. *$p<0.05$ Compared with Hydrodynamic Diameter at 0 Month of Each Nanoemulsion

Figure 4.3 Hydrodynamic Diameters of GC1, GC2, GC3 After Storage at 30 °C

4.6 Size, Zeta Potential, Polydispersity Index (PDI), and Morphology of GC Nanoemulsion

The GC nanoemulsions (GC1, GC2, and GC3) were prepared by ultrasonic method. From Figure 4.4, the GC nanoemulsions exhibited the spherical shapes with dispersed particles. From the TEM images, the particle sizes of GC1, GC2, and GC3 were 59.17±4.55, 48.67±3.96, 35.25±2.75 nm, respectively. The mean particle size, polydispersity index (PDI), and zeta potential of GC nanoemulsion are shown Table 4.2. The particle sizes of GC1, GC2, and GC3 obtained from DLS were 71.06±0.62, 68.26±1.30, and 66.22±0.20 nm, respectively. These results were corresponded to the results obtained from TEM images. The low values of PDI (<0.2) of GC2 and GC3 showed the good size dispersity of nanoemulsion. The zeta potential of GC1, GC2, and GC3 was -17.4±0.95, -11.57±0.51, and -10.43±2.28 nm, respectively. The charge range of −30 to +30 mV is reported typically as high zeta potential and is desired (Long & Labute, 2010).

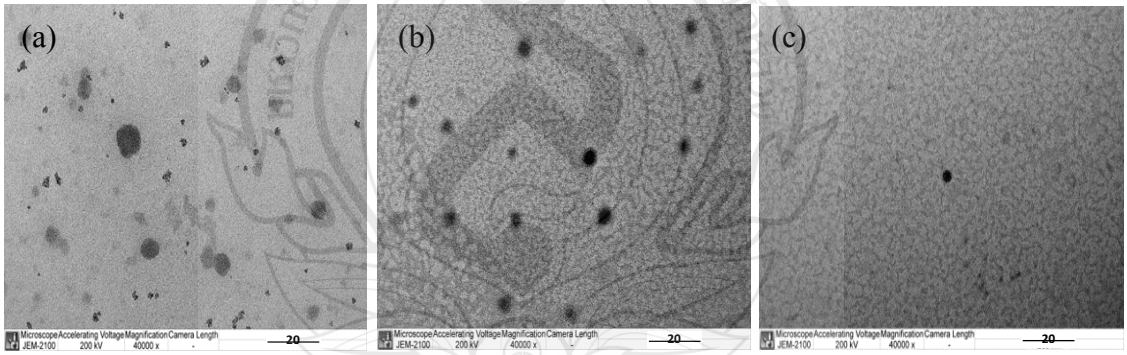


Figure 4.4 Transmission Electron Microscopy Images of (a) GC1, (b) GC2, and (c) GC3

Table 4.2 Particle Size, PDI, and Zeta Potential of GC1, GC2, and GC3 (Mean ± Standard Deviation; n=3). *$p<0.05$ Compared with GC1

Sample	Particle size (nm)	PDI	Zeta potential (mV)
GC1	71.06±0.62	0.28±0.04	-17.4±0.95
GC2	68.26±1.30*	0.17±0.01*	-11.57±0.51*
GC3	66.22±0.20*	0.19±0.01*	-10.43±2.28*

4.7 Differential Scanning Calorimetry of GC Nanoemulsion

The thermal behavior of GC nanoemulsions (GC1, GC2, and GC3), blank, and GC was investigated by DSC technique. Figure 4.5 displays the DSC thermograms of GC1, GC2, GC3, blank, and GC. The sharp endothermic peak of GC was observed at 161.17 °C. The endothermic peaks of GC1, GC2, GC3 and blank exhibited at 149.17, 145.67, 144.83, and 115.83 °C, respectively. The results showed that the encapsulation of GC in nanoemulsion decreased the melting point due to the loss of water in the nanoemulsion system (desolvation and/or dehydration process). Furthermore, the melting points of Tween20 and Tween 80 were 115.8 °C and 98.9 °C, respectively. From this information, the endothermic peak of GC nanoemulsion showed the lower temperature than that of GC (Moghaddasi et al., 2018; Ahmad et al.,2019).

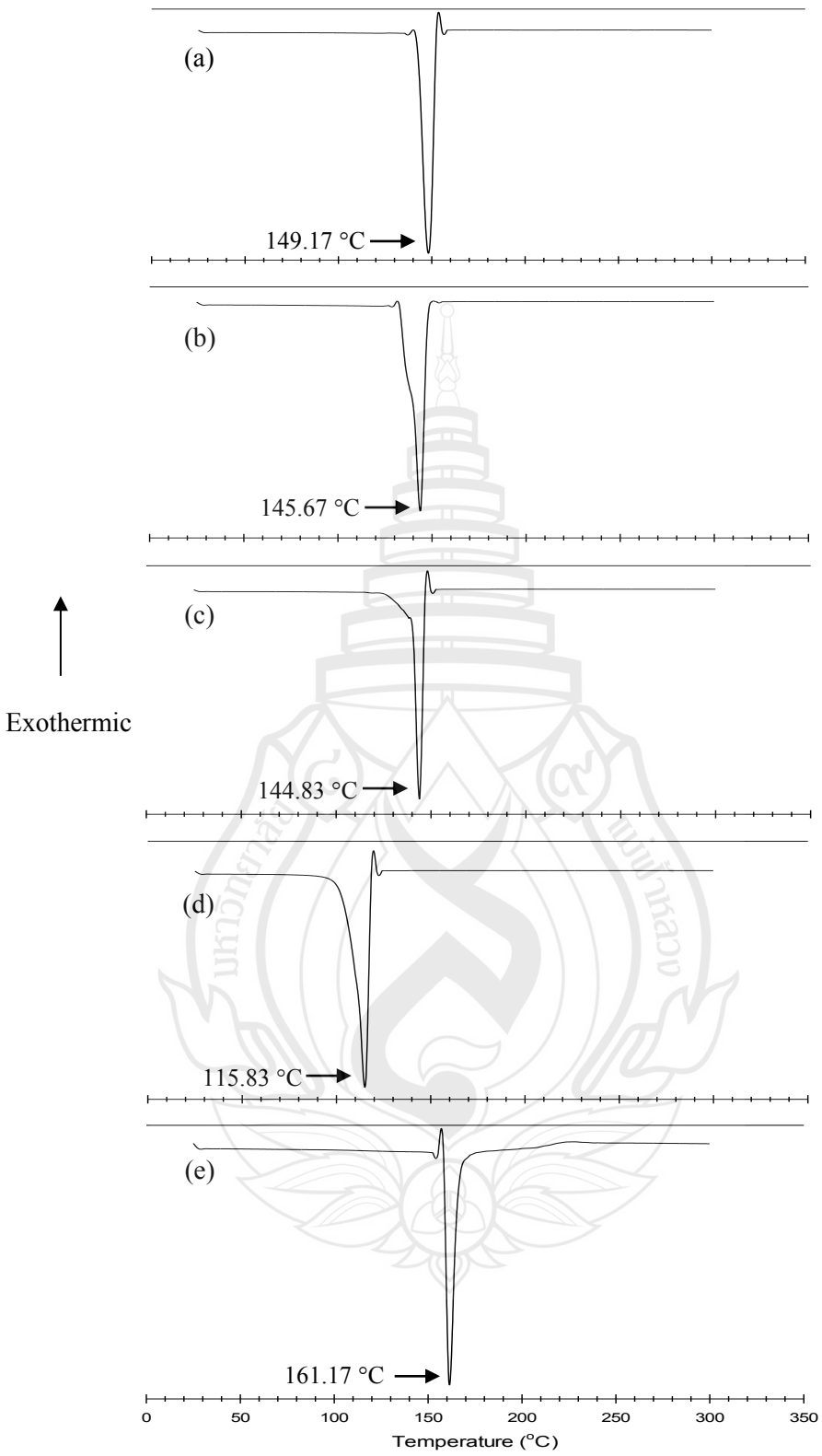


Figure 4.5 DSC Thermograms of (a) GC1, (b) GC2, (c) GC3, (d) Blank, and (e) GC

4.8 Encapsulation Efficiency of GC Nanoemulsion

The %EE is the percentage of GC that is successfully entrapped into the GC nanoemulsion. The %EE of samples was analyzed by UV-vis spectrophotometer at a wavelength of 250 nm. The %EE of GC1, GC2, and GC3 was 40.20±7.55, 42.00±8.01, and 44.78±12.81, respectively. The GC3 showed the highest value of %EE. This result might be high concentration of GC in GC nanoemulsion, while GC1 showed the lowest value of %EE due to the low concentration of GC in GC nanoemulsion.

4.9 Drug Release Study of GC Nanoemulsion

In this study, the total immersion method was employed to study the release characteristics of GC from GC nanoemulsion. The release study was carried out for 72 h in PBS containing Tween 80 (1% v/v) and ethanol (5% v/v) at 32.5 ºC. The cumulative release profiles of GC from GC nanoemulsions (GC1, GC2, and GC3) are shown in Figure 4.6. The release profiles of all samples showed similar results. All nanoemulsions exhibited a fast-released amount of GC from GC nanoemulsions between 0 – 4 h and the gradual released amount of GC from the nanoemulsion between 4 – 10 h. GC3 was released slightly more than GC1 and GC2 due to the smaller particle size of GC nanoemulsion. Thus, the smaller particle size of nanoemulsion which has high surface area showed the greater released amount of GC.

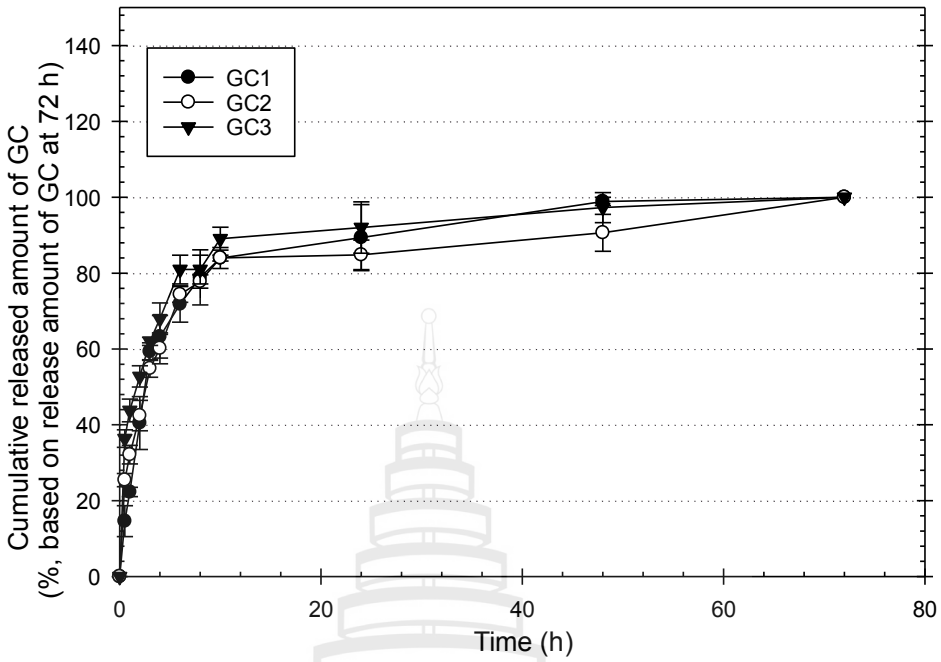


Figure 4.6 Cumulative Release Profiles of GC from GC Nanoemulsions (n=3)

4.10 Antibacterial Activity of GC Nanoemulsion

Since GC has a polyprenylated benzo-phenone which is an antibacterial active compound, the antibacterial activity of GC nanoemulsions was evaluated (Sakunpak & Panichayupakaranant, 2012; Mahabusarakam et al., 2005). Table 4.3 shows the MIC and MBC values of GC1, GC2, GC3, and blank. In MIC test, the GC2 and GC3 inhibited the growth of all bacteria, while GC1 only inhibited the growth of *P. aeruginosa* due to the low amount of GC in nanoemulsion. Furthermore, the MBC value is defined as the lowest concentration of GC nanoemulsion that kills 99.9% of bacteria. From the results, the GC3 was able to kill all bacterial stains and exhibited the lowest MIC and MBC values.

Table 4.3 Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of GC1, GC2, GC3, and Blank Against 4 Human Pathogens (Mean ± Standard Deviation; n=3)

Sample	MIC (%v/v)				MBC (%v/v)			
	SA	SE	PA	EC	SA	SE	PA	EC
GC1	-	-	50.0±0.0	-	-		50.0±0.0	-
GC2	50.0±0.0	50.0±0.0	25.0±0.0	50.0±0.0	-	50.0±0.0	50.0±0.0	-
GC3	25.0±0.0	25.0±0.0	12.5±0.0	25.0±0.0	50.0±0.0	25.0±0.0	12.5±0.0	50.0±0.0
Blank	-	-	-	-	-	-	-	-

Note SA, *S. aureus* TISTR 746; SE, *S. epidermidis* DMST 15505; PA, *P. aeruginosa* TISTR 1287; EC, *E. coli* TISTR 527.

4.11 Antioxidant Activity of GC Nanoemulsion

The antioxidant activity of GC nanoemulsions was determined by ABTS assay. From Figure 4.7, the maximum antioxidant activity of GC1, GC2, and GC3 at nanoemulsion concentration of 50 %v/v was 65.02±0.49, 82.26±1.06, and 91.73±0.12%, respectively. The half-maximum inhibitory concentration (IC_{50}) of GC1, GC2, and GC3 was 31.48±0.02, 18.09±0.13, and 9.20±0.39% v/v, respectively. The GC3 showed the highest value of antioxidant activity and the lowest IC_{50} due to the highest GC concentration. According to the literature reviews, the phenolic compounds in GC showed good antioxidant activity with excellent hydrogen or electron donor capacity (Negi et al., 2010).

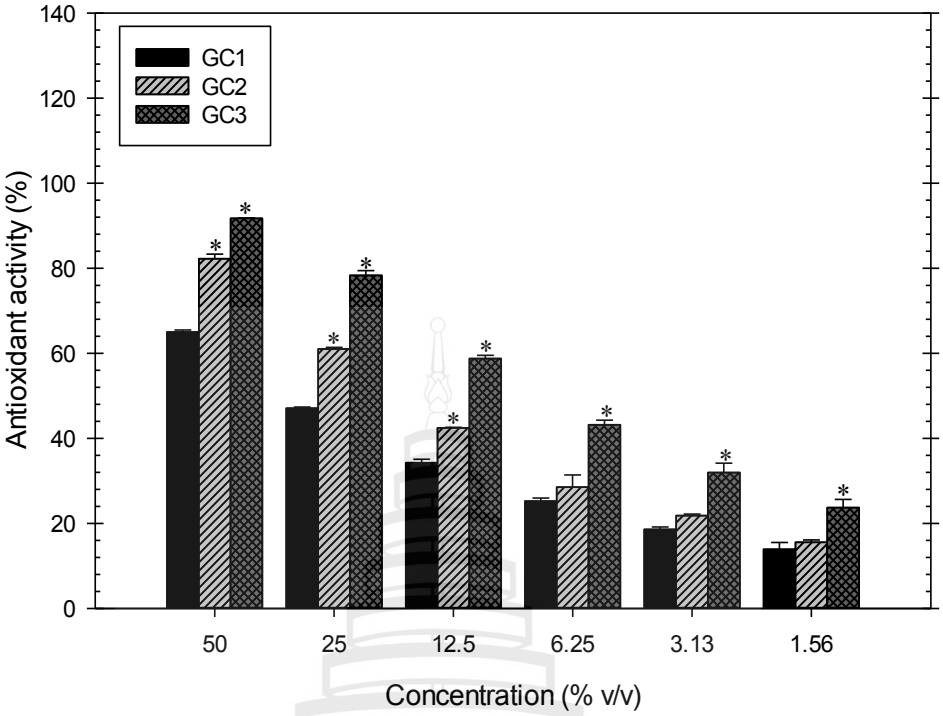


Figure 4.7 Antioxidant Activity of GC1, GC2, and GC3 (n=3). *$p<0.05$ Compared with GC1

4.12 Anti-inflammatory Activity of GC Nanoemulsion

The anti-inflammatory activity of GC nanoemulsions was determined by nitric oxide (NO) production assay. This assay measured the accumulation of NO in a culture medium using the Griess reaction (Borges t al., 2018). The results demonstrated that the NO production inhibition with IC_{50} value of GC1, GC2, GC3, and blank was 1.30±0.41, 0.26±0.01, 0.24±0.04, and 0.25±0.01 %v/v, respectively. From the result, blank showed an anti-inflammatory effect since the surfactants in the formulation of nanoemulsion affected the inflammatory reaction (Silva et al., 2013). These results might be the nano-size of emulsion greatly improves water solubility of an anti-inflammatory agent. Thus, it is possible to demonstrate that nanoemulsion could improve anti-inflammatory activity in LPS-stimulated macrophages (Han & Wang, 2012; Liao et al., 2018).

4.13 Cytotoxicity of GC Nanoemulsion

The MTT assay measured the cytotoxicity of GC nanoemulsion on NCTC clone 929 cells and the results are shown as the relative cell viability compared to control. The viability of cells was determined after 24 h incubation with different concentrations of GC nanoemulsion (Figure 4.8). The results showed that the viability of NCTC clone 929 cells cultured with GC nanoemulsion ranged between ~72 and ~97% at GC nanoemulsion concentration of 0.19-0.39 %v/v. From the results, GC1 showed higher toxicity to the cells than GC2 and GC3 because surfactant in nanoemulsion had an effect on viability of cells (Hua et al., 2018). The percent cell viability is > 70% showing the non-cytotoxicity (Niles et al., 2008). Therefore, these GC2 and GC3 were non-toxic to NCTC clone 929 cells and these GC nanoemulsions have the potential for use as pharmaceutical and cosmeceutical products.

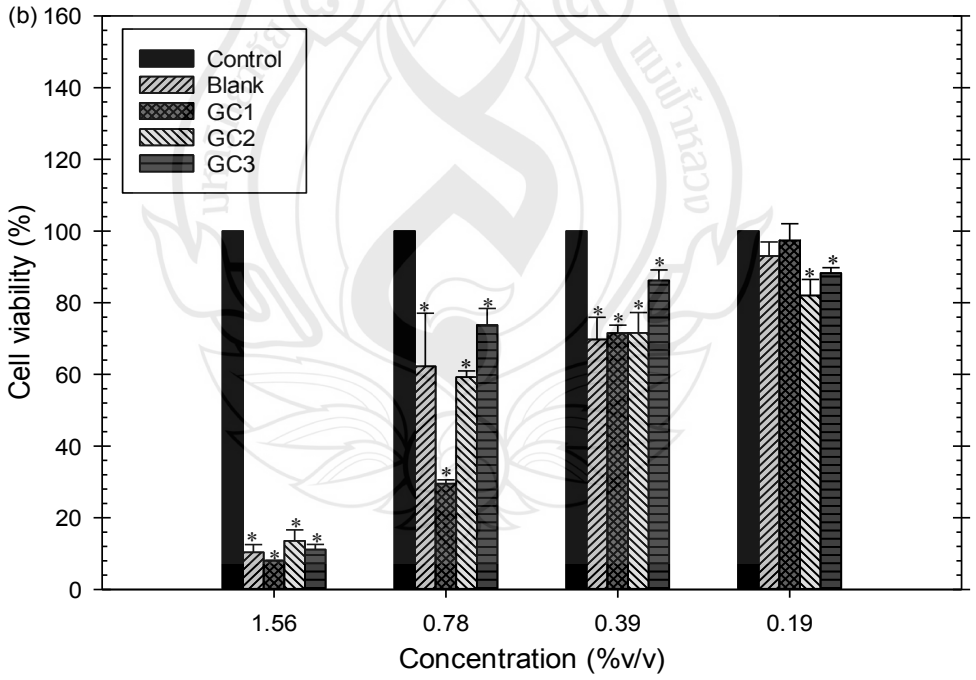


Figure 4.8 Cytotoxicity of Blank, GC1, GC2, and GC3 on NCTC Clone 929 Cells (n=3). *$p<0.05$ Compared with Control

4.14 Cell Internalization

Cellular internalization trafficking of GC nanoemulsion was visualized and quantitatively evaluated in fibroblast cells using confocal laser scanning microscope. The adherent live cells (green, stained with Calcein AM) and GC nanoemulsion internalized in cells (red, labeled with rhodamine) were quantified from Figure 4.9. From the results, GC3 showed the most internalization of the cells followed by GC2 and GC1, respectively. The results was related to the particle size of GC nanoemulsion in which GC3 represent the smallest particle size at 66.22 nm. The smaller particle size was easier to internalize and uptake into the cells (Mosquera et al., 2018). Thus, these results confirmed that the GC nanoemulsion could enhance GC into the cells and improved its efficiency for drug delivery system.

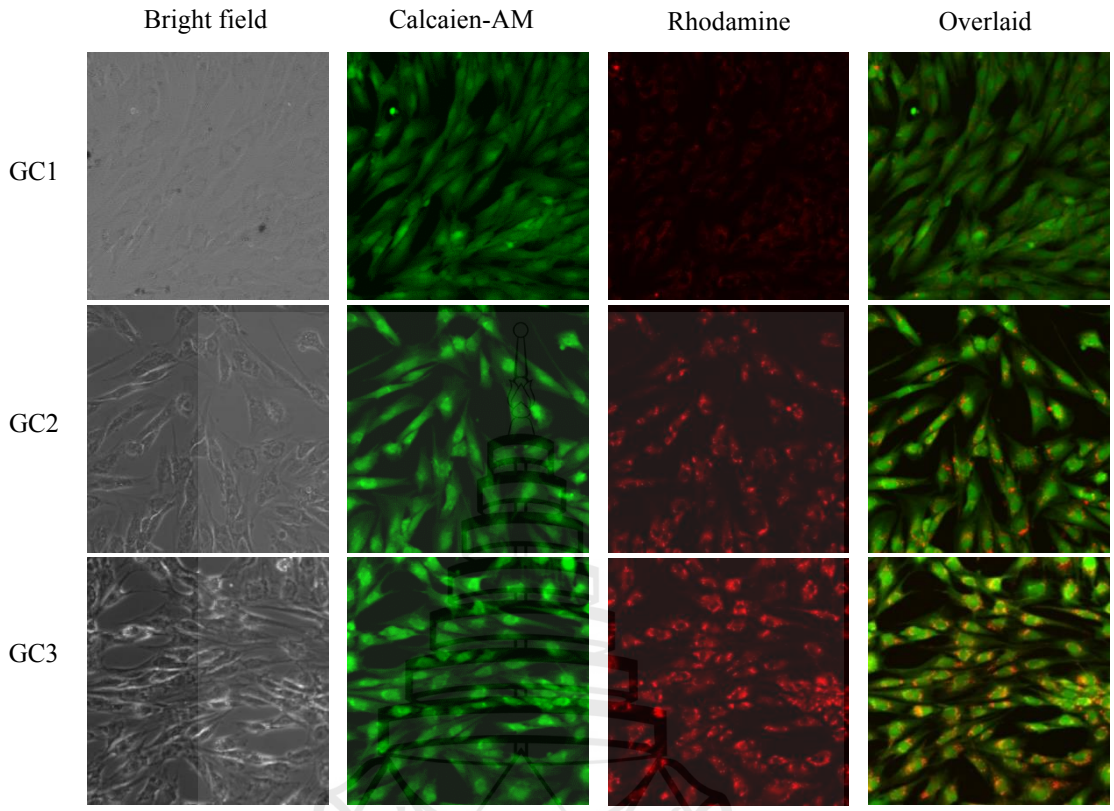


Figure 4.9 CLSM Internalization of GC1, GC2, and GC3 Cultured with Fibroblast Cell Line (CRL-2522)

4.15 Physicochemical Properties of Topical Film Forming Spray Containing GC Nanoemulsion

From the results of GC nanoemulsion, it was found that GC2 and GC3 showed clearer nanoemulsion with no phase separation and better stability than GC1 after storage at 30 °C for 3 months. In addition, GC2 and GC3 showed good biological activities (Table 4.3, Figure 4.7, and anti-inflammatory). Therefore, GC2 and GC3 was used to formulate in the topical film forming spray.

The topical film forming spray was formulated based on E100, PVP, and DBP. E100 was used for film forming and controlled drug release at proper condition. DBP as plasticizer was used to make film more pliable and softer. PVP was used to enhance the release rate. (Huang & Ru, 2010; Rashidinejad & Jafari, 2020). To determine the proper composition of EuE100 on the properties of topical film forming spray, 9 compositions were prepared and evaluated for the physicochemical properties as shown in Table 4.4. The results showed that before addition of GC nanoemulsion, the increase of concentration of EuE100 (B1, B2, and B3) caused viscosity to increase and spray pattern to decrease due to more adhesive force of EuE100. The low spray pattern showed that the film could be formed in the small area with the spherical form. Likewise, long time to evaporate of the spray was obtained when increasing concentration of EuE100.

The topical film forming spray containing GC nanoemulsion (S1-S6) showed the evaporation time in the range of 3.27-7.10 min. The pH of formulations (S1-S6) decreased when addition of GC nanoemulsion due to the acidity of GC nanoemulsion. The contact angle is the angle where a liquid interacts with a solid surface (Sritharadol et al., 2017). From the results, the contact angle of the formulations was low, in the range of 19.05-30.04°. This range suggested the good spreadability of the formulations.

From the results, S3 and S6 showed higher evaporation time and lower spray pattern. In addition, from stability testing, the formulations of S3 and S6 had white precipitates at the bottom of the bottle. Thus, the formulations of S1, S2, S4, and S5 were selected for further evaluation to study the biological activities and release profiles of the formulations.

Table 4.4 Physicochemical Properties of Topical Film Forming Spray

Formulation	pH	Viscosity (cP)	Contact angle (°)	Evaporation time (min)	Weight/spray (g)	Spray pattern (cm)	Stability
B1	8.77±0.01	4.01±0.15	28.33±1.39	3.16±0.07	N/A	3.76±0.08	N/A
B2	8.82±0.02	5.48±0.32	31.54±2.91	3.66±0.31	N/A	3.22±0.08	N/A
B3	8.92±0.00	12.89±0.18	37.10±0.41	4.03±0.05	N/A	3.05±0.02	N/A
S1	8.14±0.07	3.33±0.15	20.97±1.38	3.27±0.05	0.08±0.01	4.01±0.01	Unchanged
S2	8.53±0.02	5.25±0.20	28.50±1.44	4.31±0.12	0.09±0.02	3.23±0.09	Unchanged
S3	8.58±0.01	12.57±0.68	30.04±1.03	4.87±0.37	0.12±0.03	3.06±0.04	Precipitated
S4	8.49±0.09	4.02±0.04	19.05±0.80	4.41±0.10	0.12±0.00	3.87±0.03	Unchanged
S5	8.48±0.06	5.38±0.18	26.26±0.57	6.26±0.06	0.11±0.00	3.32±0.01	Unchanged
S6	8.49±0.00	11.08±0.37	27.68±0.95	7.10±0.02	0.10±0.04	3.07±0.04	Precipitated

4.16 Antibacterial Activity of Topical Film Forming Spray Containing GC Nanoemulsion

Four bacterial candidates (Gram-negative: *E. coli* TISTR 527 and *P. aeruginosa* TISTR 1287 and Gram-positive: *S. epidermidis* DMST 15505 and *S. aureus* TISTR 746) were used to evaluate the antibacterial activity of topical film forming spray containing GC nanoemulsion. The results of susceptibility testing using the bacteria inhibition and killing the bacteria for each formulation are reported in the Table 4.5. From the results, all of the formulations were inhibited both gram-negative and gram-positive bacteria. However, B1 and B2 showed active against the growth of all bacteria. This might be EuE100 can inhibit the growth of bacteria due to cationic polymers can be effective against gram-positive and gram-negative bacteria (Mushtaq et al., 2021).

Table 4.5 Bacterial Inhibition and Bacterial Killing of Extraction Medium from Topical Film Forming Spray Containing GC Nanoemulsion (n=3)

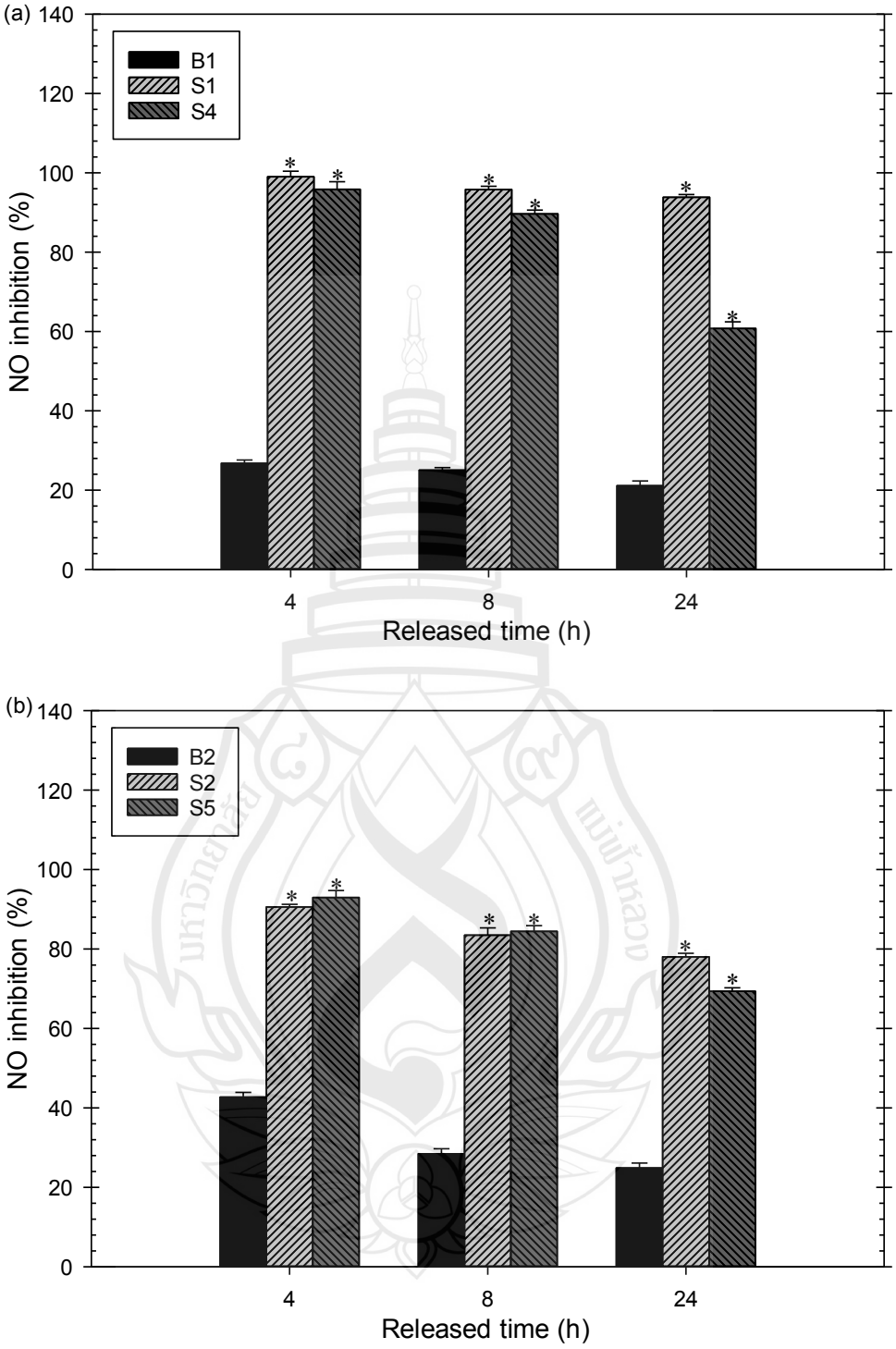
Microorganism	Bacterial inhibition						Bacterial killing					
	B1	B2	S1	S2	S4	S5	B1	B2	S1	S2	S4	S5
E. coli	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
P. aeruginosa	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
S. epidermidis	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
S. aureus	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	✓	✓

Note ✓= inhibited or killed bacteria

- = not inhibited or killed bacteria

4.17 Anti-inflammatory of Topical Film Forming Spray Containing GC Nanoemulsion

Nitric oxide (NO) assay was used to evaluate the anti-inflammatory activity of topical film forming spray containing GC nanoemulsion. From the results, S1, S2, S4, and S5 showed good NO inhibition. These results confirmed that the topical film forming spray containing GC nanoemulsion had the anti-inflammatory activity. The result demonstrated that the RAW 264.7 cells treated with the extraction medium from the topical film forming spray containing GC nanoemulsion showed higher inhibited NO than blank (topical film forming spray without GC nanoemulsion). These results might be the effect of phenylbutazone compound in GC nanoemulsion which has strong anti-inflammatory activity.



Note (a) B1, S1, and S2 (*p<0.05 Compared with B1) and (b) B2, S4, and S5 (*p<0.05 Compared with B2)

Figure 4.10 Anti-inflammatory Activity

4.18 Indirect Cytotoxicity of Topical Film Forming Spray Containing GC Nanoemulsion

MTT assay was used to measure the indirect cytotoxicity of topical film forming spray containing GC nanoemulsion with NCTC clone 929 cells and the results are shown as the relative cell viability referred to control. The viability of cells was determined after 4 h of incubation with different extraction media of topical film forming spray containing GC nanoemulsion (Figure 4.11). The results showed that the viability of NCTC clone 929 cells cultured with extraction medium released from topical film forming spray containing GC nanoemulsion at 4 h ranged between ~80 and ~93%. While, the viability of NCTC clone 929 cells cultured with extraction medium released from topical film forming spray containing GC nanoemulsion at 24 h was ranging between ~59 and ~75%. From the results, S1 showed higher viability of NCTC clone 929 cells than S2, S4, and S5.

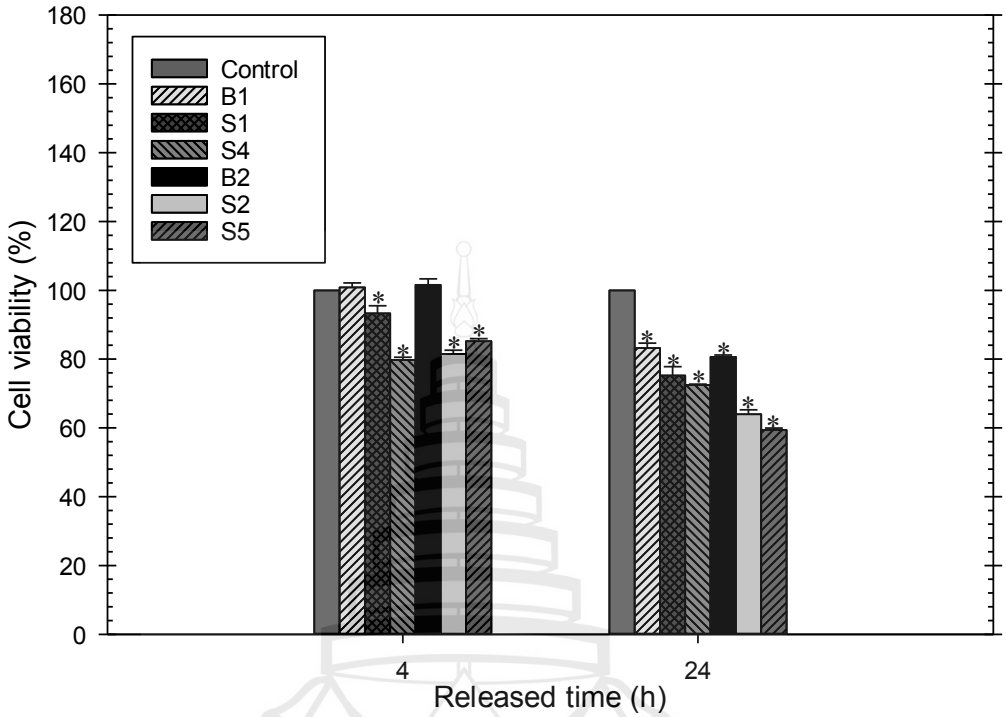


Figure 4.11 Indirect Cytotoxicity of Extraction Medium Released from B1, S1, S2, B2, S4, and S5 at 4 h Cultured with NCTC Clone 929 Cells. **p<0.05* Compared with Control

4.19 Encapsulation Efficiency of Topical Film Forming Spray Containing GC Nanoemulsion

The %EE is the percentage of GC that was successfully entrapped into the topical film forming spray containing GC nanoemulsion. The %EE of samples was analyzed by UV-vis spectrophotometer at a wavelength of 413 nm. The %EE of S1, S2, S4, and S5 was 71.81±3.56, 65.66±1.71, 60.01±0.03, and 62.25±1.27, respectively. From the results, the value of %EE of S1 and S2 was higher than that of S4 and S5. These results might be the larger particle size of GC2 that loaded into S1 and S2 increased the ability to entrap GC in higher amount (Lecaroz et al., 2006).

4.20 Drug Release Study of Topical Film Forming Spray Containing GC Nanoemulsion

The release study was carried out for 48 h in PBS containing Tween 80 (1% v/v) at 32.5 ºC. The release profiles of topical film forming spray containing GC nanoemulsion are shown in Figure 4.12. The release profiles of all samples showed similar results. All samples exhibited a gradual release of GC released from topical film forming spray containing GC nanoemulsion in the first submersion time and higher release after 10 h. After that, GC released from topical film forming spray containing GC nanoemulsion reached a plateau value at the longest submersion time 24 h. For the S2 and S5, the release amount of GC from these topical film forming spray containing GC nanoemulsion was lower than that from S1 and S4 due to higher concentration of polymer. The higher concentration of EuE100 might hinder the release of GC from the topical film forming spray.

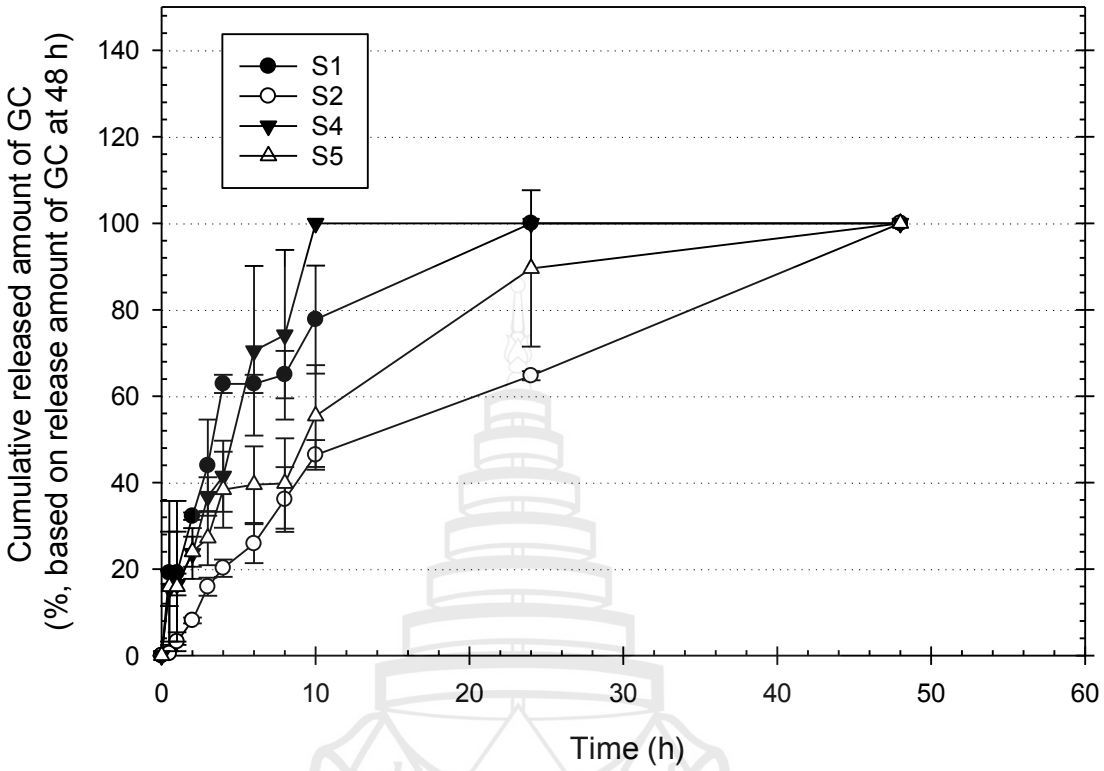


Figure 4.12 Cumulative Release Profiles of GC from the Topical Film Forming Spray Containing GC Nanoemulsions (n=3)

CHAPTER 5

CONCLUSION

In this study, the GC nanoemulsions were successfully prepared by ultrasonic method. The GC nanoemulsions showed the spherical shape. The particle size of GC nanoemulsions ranged between 66 and 71 nm, PDI ranged between 0.17 and 0.28, and zeta potential ranged between -17 and -10 mV. The GC2 showed good stability under storage at 30 ºC and 45 ºC for 3 months. The encapsulation of GC in the nanoemulsion caused the melting point of GC to decrease. %EE of GC nanoemulsion increased with increasing the GC loaded in the nanoemulsion. In addition, the increased concentration of GC in GC nanoemulsion showed the lower cumulative released amount of GC. Moreover, GC3 showed stronger growth inhibition against both gram-negative and gram-positive bacteria. For the antioxidant activity, GC3 showed the lowest value of IC_{50}. In addition, these GC nanoemulsions were non-toxic to NCTC clone 929 cells, enhanced GC into the cells, and improved the efficiency for drug delivery system. Therefore, the GC2 and GC3 were used for loading in the topical film forming spray.

The topical film forming spray containing GC nanoemulsion was fabricated for use as pharmaceutical and cosmeceutical products. GC nanoemulsions (GC2 and GC3) were added into various concentrations of EuE100 (8%, 10%, and 12% w/v). From physicochemical result, the increase of concentration of EuE100 caused the viscosity to increase and the spray pattern to decrease. The evaporation time of topical film forming spray containing GC nanoemulsion was in the range of 3.27-7.10 min. The contact angle of the formulations was low, in the range of 19.05-30.04 °. In addition, S1, S2, S4, and S5 were good stability. Moreover, topical film forming spray containing GC nanoemulsion exhibited the antibacterial activity against both gram-negative and gram-positive bacteria. Moreover, the release profiles of all topical

film forming sprays containing GC nanoemulsion showed similar profiles and a sustained release at longer immersion time points. S1 showed the highest % NO inhibition at different release timepoints. Finally, S1 showed the highest viability of NCTC clone 929 cells cultured with extraction medium from the topical film forming spray containing GC nanoemulsion at 4 and 24 h. Thus, S1 has the potential for use as pharmaceutical and cosmeceutical products.

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APPENDIX

APPENDIX

DEVELOPMENT OF TOPICAL FILM FORMING SPRAY CONTAINING *GARCINIA COWA* LEAF EXTRACT NANOEMULSION FOR PHARMACEUTICAL AND COSMECEUTICAL PRODUCTS

(a)

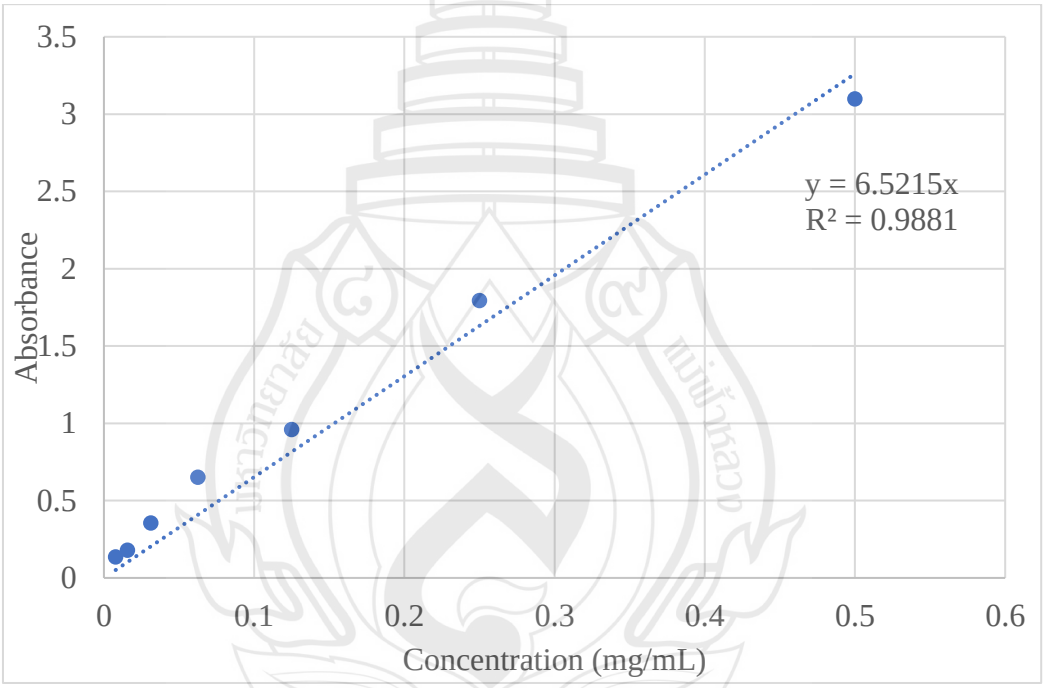
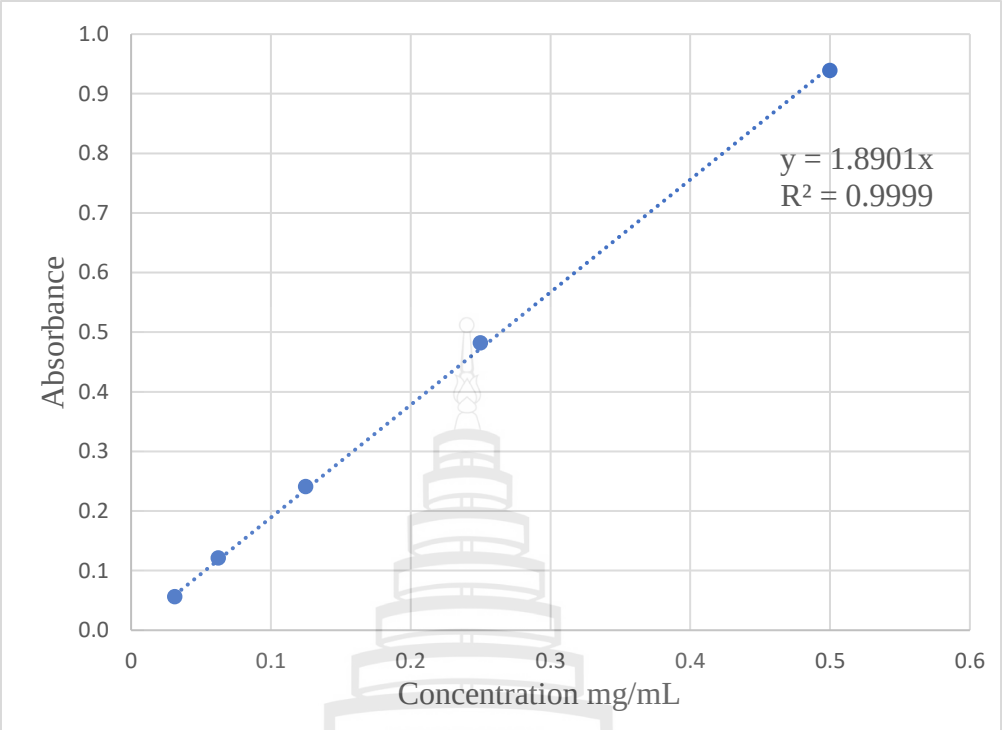


Figure A1 Standard Curve of GC

(b)



Note (a) λ_{max} = 250 nm (b) and λ_{max} = 413 nm

Figure A1 (continued)

Table A1 Visual Observation of GC nanoemulsion after storage at 4 °C, 30 °C, and 45 °C

Month	GC2			GC3		
	4 °C	**30 °C**	**45 °C**	**4 °C**	**30 °C**	**45 °C**
1						
2						
3