Microencapsulation of black cumin seed oil with hydrocolloids via spray drying

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การหุ้มสารน้ำมันเมล็ดเทียนดำด้วยไฮโดรคอลลอยด์ผ่านวิธีทำแห้งแบบพ่นฝอย

น.ส.นัจวา ยานยา สันติวรกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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นัจวา ยานยา สันติวรกุล : การหุ้มสารน้ำมันเมล็ดเทียนดำด้วยไฮโดรคอลลอยด์ผ่านวิธีทำแห้งแบบพ่นฝอย. (Microencapsulation of black cumin seed oil with hydrocolloids via spray drying) อ.ที่ปรึกษาหลัก : ผศ. ดร.ทิพยเนตร อริยปิติพันธ์, อ.ที่ปรึกษาร่วม : รศ. ดร.วินัย ดะห์ลัน

เทียนดำหนึ่งเป็นในสมุนไพรที่มีประโยชน์ต่อสุขภาพที่ใช้มาแต่โบราณในหลายยุคสมัยและอารยธรรม น้ำมันเทียนดำมีกรดไขมัน ไทโมควีโนนและสารพฤกษเคมีที่มีคุณค่าทางโภชนาการ ด้วยกลิ่นที่ฉุนและรสชาติที่ขมจึงมีข้อจำกัดในการประยุกต์ใช้ในผลิตภัณฑ์อาหาร นอกจากนี้น้ำมันเมล็ดเทียนดำละลายน้ำไม่ดีและไวต่อการเสื่อมสภาพ การหุ้มน้ำมันเมล็ดเทียนดำด้วยสารห่อหุ้มที่เหมาะสมโดยผ่านวิธีทำแห้งแบบพ่นฝอยจึงเป็นกรรมวิธีที่เหมาะสมในการเพิ่มการละลายน้ำและลดกลิ่นฉุนและรสขม การศึกษานี้ออกแบบไมโครแคปซูลผสมสองชนิดในอัตราส่วนร้อยละ 50:50 จากไฮโดรคอลลอยด์ 8 ชนิด ที่สามารถละลายน้ำได้ โดยมีกัมอารบิก (GA) เป็นสารห่อหุ้มหลัก จากการศึกษาพบว่า สูตรไมโครแคปซูลที่เหมาะสมสำหรับการทำให้แห้งโดยวิธีทำแห้งแบบพ่นฝอยมีทั้งหมด 4 สูตร ได้แก่ สูตร GA กัมอารบิกชนิดเดียวและสูตรผสมของกัมอารบิกกับอินูลิน (GI) มัลโตแดกสตริน (GM) หรือเวย์โปรตีน (GW) ประสิทธิภาพในการห่อหุ้มสารของไมโครแคปซูลอยู่ในช่วงระหว่างร้อยละ 75 - 88 โดยสูตร GI มีประสิทธิภาพในการห่อหุ้มสูงมากกว่าสูตร GM และสูตร GW ตามลำดับ การส่องกล้องอิเล็กตรอนแบบส่องกราดพบอนุภาคของไมโครแคปซูลสูตร GI (mGI) มีลักษณะเรียบและเป็นทรงกลมมากกว่าสูตรอื่น สารพฤกษเคมีหลักที่พบในสารห่อหุ้มหลังกระบวนการแอนแคปซูเลชั่น ได้แก่ ไทโมควีโนน เติร์ดบิวทิวไฮโดรควีโนนและลองจิโฟลีน ตามลำดับ ซึ่งพบใน mGI และ mGW ใกล้เคียงกับใน mGA สำหรับองค์ประกอบของกรดไขมันในอนุภาคของไมโครแคปซูลแต่ละสูตรไม่มีความแตกต่างกัน ผงไมโครแคปซูล mGI และ mGW มีความสามารถในการเปียกน้ำ 11 และ 14 นาที ตามลำดับ ซึ่งเร็วกว่าผงไมโครแคปซูลสูตรอื่นอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ในขณะที่ความสามารถในการละลายน้ำของผงไมโครแคปซูลสูตรผสมไม่แตกต่างกัน (ร้อยละ 87 - 93) แต่ละลายน้ำได้ดีกว่าผงไมโครแคปซูล mGA (79%) อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) การทดสอบฤทธิ์ต้านอนุมูลอิสระของผงไมโครแคปซูล mGI ด้วยวิธี DPPH พบว่ามีฤทธิ์เปอร์เซ็นต์การยับยั้งมากกว่าผงไมโครแคปซูลสูตรอื่นที่ทุกความเข้มข้น ค่าความเป็นพิษของ mGI ต่อ Caco-2 cells อยู่ที่ระดับความเข้มข้น 182.5 ไมโครกรัมต่อมิลลิลิตร เมื่อเสริมผงไมโครแคปซูล mGI หรือน้ำมันเทียนดำในผลิตภัณฑ์นมพร่องมันเนยและผลิตภัณฑ์นมถั่วเหลืองให้มีปริมาณไทโมควีโนน ~10 มิลลิกรัมต่อหน่วยบริโภค ผู้ทดสอบที่ผ่านการฝึกฝนสามารถแยกความแตกต่างระหว่างตัวอย่างประเภทเดียวกันที่เสริมผงไมโครแคปซูลหรือน้ำมันเทียนดำได้อย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) การยอมรับทางประสาทสัมผัสโดยรวมของตัวอย่างเสริมผงไมโครแคปซูลหรือน้ำมันเทียนดำไม่แตกต่างกัน โดยมีคะแนนเฉลี่ยความชอบอยู่ในระดับชอบเล็กน้อย - ปานกลาง (5.9 – 6.6 คะแนน) ดังนั้น จึงควรมีการพัฒนาผลิตภัณฑ์เสริมผงไมโครแคปซูลให้รสชาติเป็นที่ยอมรับมากขึ้นและมีคุณค่าโภชนาการเหมาะสมก่อนนำไปประยุกต์ใช้ในตลาดอาหารสุขภาพต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Nigella sativa Microencapsulation Spray drying Inulin Gum Arabic
Najwa Yanya Santiworakun : Microencapsulation of black cumin seed oil with hydrocolloids via spray drying. Advisor: Asst. Prof. Dr. TIPAYANATE ARIYAPITIPUN, Ph.D. Co-advisor: Assoc. Prof. Dr. Winai Dahlan, Ph.D.

Nigella sativa is one of historical and miracle herbs been used for many centuries in different civilizations. Although its oil provides high nutritious values of fatty acids and phytochemicals, the strong odor and peppery taste limits its application in food products. In additions, the black cumin seed oil also poor solubility and rapid deterioration. A wise strategy to enhance organoleptic property and promote oil solubility is encapsulating the cold press black cumin seed oil with suitable wall materials via spray drying. In this study, eight water soluble hydrocolloids were used for formulating the mixed microcapsules. Only four formulations of microcapsules were successfully spray dried including gum arabic alone (GA) or in combinations with inulin (GI), maltodextrin (GM), or whey protein (GW) at a ratio of 50:50. The microencapsulation efficiencies (ME) of all microcapsules were ranged from 75 - 88%. The ME of GI was superior to those of GM and GW, respectively. Based on the SEM image, the smooth and spherical shape microstructure was obtained from the GI microcapsule (mGI). The TQ, tertbutyl-hydroquinone (TBHQ), and longifolene were the main phytochemicals available in the mNSO powders, respectively. Amounts of TQ and TBHQ encapsulated by the GA, GI, and GW were no significant difference. Similar fatty acid profiles were observed in all microcapsules. The solubilities of the mixed microcapsules (87-93%) were significantly higher than that of mGA ($P < 0.05$). Also, the wettabilities of mGI and mGW (11 & 14 mins) were significantly faster than those of mGA and mGM. Based on the DPPH assay for antioxidant activity of microcapsules, the % inhibition of mGI was the highest at all concentrations. The cytotoxicity of mGI on Caco-2 cells was 182.5 µg/mL. The trained panelists could significantly distinguish sensory attributes of soybean milk and low fat cow milk containing 10 mg of TQ from mGI or NSO per serving size ($P < 0.05$). The overall mean acceptability scores of each fortified milk products were mild to moderate (5.9 – 6.6) but no significant difference among the samples. Therefore, the fortification of mNSO into health food products should be further study to improve acceptability and nutrition.

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Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

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Najwa Yanya Santiworakun

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CHAPTER I

INTRODUCTION

Many abundant medicinal plants have been exploited by human since the specie existed. The medicinal plants have been utilized for both minor and major ailments to meet basic survival needs. The knowledge of how to identify and utilize these plants has been passed from generation to generation. Many drugs derived from plants nowadays have been developed based on the medication provided by the ancient civilizations. The pharmacological attributes of medicinal plants lead to the incorporation of plant in daily diet as a functional food. Black cumin is one of those ideal plants which possess many pharmaceutical attributes. The crude oil obtained from the seed of black cumin is well known to be functional oil as a result of its high potential as a therapeutic agent (Kooti et al., 2016; Majdalawieh & Fayyad, 2016).

For therapeutic effects, it is recommended to consume black cumin seed or oil regularly in certain amounts. However, due to a bitter taste and very strong odor of black cumin seed and its oil, it is unacceptable in some groups of consumers, especially the consumers who are unfamiliar to the black cumin taste and odor. In additions, adding black cumin seed and its oil directly into food or nutraceutical products may effect on appearance, stability, and unpleasant taste and odor. Moreover, black cumin seed and its oil are potentially sensitive to acid, base, moisture, oxygen, light, and heat in food matrix and environment (Edris et al., 2016; Fernandes et al., 2014). An alternative approach for alteration of black cumin seed oil into a new form plays a critical role to maintain its functional properties while covering its strong odor and flavor, improve the shelf life as well as the stability of the oil. The microencapsulation is a method of choice to achieve that desired black cumin product. This process allows the enclosing of the core material, which is black cumin seed oil in this case, with the wall material shielding against surrounding environment in a micron size to provide the desired

characteristics of the products. Several methods have been developed to microencapsulate the bioactive oils including coacervation, spray drying, freeze drying, and extrusion (Kaushik et al., 2015; Rodríguez et al., 2016; Wen et al., 2014). Spray drying is the most feasible and competent method used in food industries. This method offers the transformation of the liquid oils into the powder form (Rodríguez et al., 2016). It had been extensively and successfully applied in many vitamins, bioactive oils, and oleoresins including fish oil, rice bran oil (Gupta et al., 2015), kaffir lime oil (Adamiec et al., 2012), rosemary oil (Fernandes et al., 2014), turmeric oleoresin(Delfiya et al., 2014), and black cumin oleoresin (Edris et al., 2016). It exposes high encapsulation efficiency (84.2-96.2%) and high wettability with acceptable moisture content used for food application. Moreover, it had been verified to be simple and cost effective over other methods. Ease of availability of equipment and wide range of the wall materials available in the market also are reasons for considering the spray drying technique for microencapsulation. High quality products with low water activities produced by the spray drying are convenient to use and easy for storage and transportation (Rodríguez et al., 2016).

In general, types of wall materials commonly used for microencapsulation are proteins and polysaccharides. Hydrocolloid is one type of polysaccharides commonly used to encapsulate bioactive compounds from plant and extract oil (Silva et al., 2014). The hydrocolloids derived from plants have been extensively applied in food products due to its beneficial effect as soluble fibers which promote the growth of good bacteria within the digestive system. In addition, it is also safe for Muslim consumers since it is from a plant origin.

Although recently the demand of usage of black cumin is high, the availability of the study on microencapsulation of black cumin is very few. Therefore, the exploitation of various types of hydrocolloids as wall materials for microencapsulation of black seed oil is necessary. Development of microencapsulated black cumin seed oil product via spray drying will facilitate the food formulation containing valuable black cumin seed oil. This study aims to microencapsulate black cumin seed oil via spray drying using different types of

hydrocolloids. The influence of different types of hydrocolloids as wall materials on the characteristics and properties of microcapsules are evaluated and compared. The desired microencapsulation of black cumin seed oil products is selected for further investigation on the cytotoxic effect on Caco-2 cells and compared with the non-encapsulated black cumin seed oil. The desired microencapsulation of black cumin seed oil products is applied in the commonly consumed food products. The physicochemical characteristics and nutritional profile of the microencapsulated products are investigated along with and sensory attributes.

Research questions

1. Do different hydrocolloids give different effects on microparticle characteristics of spray dried black cumin seed oil?
2. Do microencapsulated black cumin seed oil products toxic to Caco-2 cell, pathogenic bacteria, microflora, and probiotics?
3. Do the developed food products fortified with the microencapsulated black cumin seed oil products give an acceptable sensory attributes in terms of flavor and taste?

Objectives

1. To evaluate the effects of different types of hydrocolloids on the microparticle properties
2. To study the cytotoxicity of microencapsulated black cumin seed oil products against Caco-2 cells, pathogenic bacteria (*Salmonella* spp. and *Staphylococcus aureus*), *Escherichia* coli (microflora) and *lactobacillus rhamnosus* (a probiotic)?
3. To develop the functional food products derived from microencapsulated black cumin seed oil with acceptable flavor and taste

Hypotheses

1. Different types of hydrocolloids used for microencapsulation of black cumin seed oil might provide different effects on the microcapsule properties
2. Microencapsulated black cumin seed oil products do not have cytotoxicity property against Caco-2 cells
3. Microencapsulated black cumin seed oil products can inhibit growth of *Salmonella* spp. and *Staphylococcus aureus* (pathogenic bacteria) but no effect on growth of *Escherichia* coli (microflora) and lactobacillus rhamnosus (a probiotic)
4. The sensory attributes of developed food products from the microencapsulated black cumin seed oil are acceptable in terms of flavor and taste

CHAPTER ll

LITERATURE REVIEWS

2.1 Black cumin (Nigella sativa)

Black cumin (*Nigella sativa*) is one of the historical herbs cultivated in Mediterranean region especially Pakistan and India. Black cumin seeds and its oil are traditionally used for food consumption and medicinal purposes. The black cumin seeds and its oil have been applied in diet since the time of Al-Biruni and Ibn Sina (Avicenna) (Aljabre et al., 2015; Utami et al., 2016). In Islamic medicine, the black cumin is advised to apply daily. It is considered as one of the prophetic medicines stated for healing ability every illness except death(Ishtiaq et al., 2013). The therapeutic properties of black cumin seed and its oil have been extensively studied. It is shown to treat various illness, such as asthma, headache, toothache, diarrhea, common colds, influenza, gastrointestinal dysfunction nasal congestion, rheumatism, bronchitis, and skin disorders(Ahmad Fadzillah et al., 2011; Aljabre et al., 2015; Gharby et al., 2015; Harzallah et al., 2011). The crude oil obtained from the seed of black cumin is well known to be functional oil as it possesses phytochemical compounds. These phytochemical compounds claimed to display the properties of anti-tumor, anticancer, antioxidant, anti-diabetics, antimicrobial, anti-inflammatory, anti-diarrhea, and wound healing activities(Badary & El-Din, 2001; Halawani, 2009; Kooti et al., 2016; Majdalawieh & Fayyad, 2016; Padhye et al., 2008; Randhawa & Alghamdi, 2011). However, they are regularly sensitive to environmental factors, such as moisture, oxygen, light, and heat (Edris *et al*., 2016; Fernandes *et al*.; 2014). Moreover, the black cumin seed and its oil taste quite bitter and offer very strong odor so introducing it to some certain foods might give an unpleasant desire.

2.1.1 Botanical aspects

Nigella sativa Linn. is an annual herbaceous plant native to the Mediterranean region and grown widely in Pakistan and India for consumption and medicinal purposes. The plant is also called in other names as habbatus sawda, kalonji, black cumin, as well as ketzah (Aljabre et al., 2015; Ishtiaq et al., 2013; Utami et al., 2016). Scientists classify this plant under the Kingdom: Plantae, Division: Magnoliophyt, Order: Ranunculales, Family: Ranunculaceae, Genus: *Nigella*, Species: *Sativa*. The suitable time for the cultivation should be during November to April. It spends 10 - 15 days for germination after sowing seeds. This plant can be grown to as tall as a height of 60 cm. and generates foliage and white, pink, light blue and lavender flowers (Figure 1). The January to April is the flowering and fruiting periods of the plant. The seeds produced by this plant are black with a size of 1 - 5 mm. and give a bitter taste(Avula et al., 2010; Ishtiaq et al., 2013; Kiralan et al., 2014; Kooti et al., 2016; Piras et al., 2013).

Figure 1. Black cumin flowers and seeds (Kooti et al., 2016; Ramadan, 2016)

2.1.2 Prophetic Medicine

The black cumin has been used as one of important Islamic medicine. The Prophet has stated that “It is a cure for every type of disease”. In prophetic medicine it was fasten the absorption of an effective ingredient when apply together for cold medication at small dosages. The black cumin has been recommended to apply with honey to dissolve the kidney stone. It is cooked with vinegar for toothache relief. Many other treatments were mentioned in his book “At-Tibb an-Nabawi” and also in “Canon of medicine” by Avicenna(Aljabre et al., 2015).

2.1.3 Chemical compositions of *N. Sativa*

Most of bioactive compounds in *N. sativa* had been studied and isolated from different varieties of *N. sativa* seeds and its oil. The bioactive compounds possibly found in *N. sativa* seeds and oil are thymoquinone (TQ), p-cymene, longifolene, carvacrol, limonene, 4-terpineol, thymol, alpha-pinene, t-anethole benzene, alpha-thujene, alpha-hederin, nigellidine, nigellimine, nigellicine, and some of their chemical structures (Figure 2). *Nigella sativa* seeds are also rich in unsaturated fatty acids, such as linoleic acid, oleic acid, palmitic acid, and eicosadienoic acid (Edris et al., 2016; Gholamnezhad et al., 2016; Kooti et al., 2016). The fatty acid and sterol compositions of one varieties of *N. sativa* seed oil derived from Morocco were reported in Table 1 and Table 2. The major fatty acids present in Morrocan *N. sativa* seed oil are linoleic acid, oleic acid, and palmitic acid (Table 1). Beta-sitosterol, stigmasterol, and campesterol were the dominant sterols found in Morrocan *N. sativa* seed oil (Table 2). Other components of the seeds included saponins, flavonoids, indazole-type alkaloids, cardiac glycosides, vitamins, and some important minerals like calcium, phosphorus, and iron(Gholamnezhad et al., 2016). The different chemotypes in *N. sativa* seed oil depended on the geographical origin of the plant and environmental and climate conditions (Gharby et al., 2015; Piras et al., 2013). *Nigella sativa* from Iran gave high phenylpropanoid components and revealed to be a trans-anethole chemotype while *N. sativa* from Algeria and India tended to be a p-cymene/thymoquinone chemotype. On the other hands, *N. sativa* from Turkey and Egypt was a thymoquinone chemotype since it exhibited high thymoquinone levels. Many other chemotypes of N. sativa were also found in different parts of the world (Piras et al., 2013).

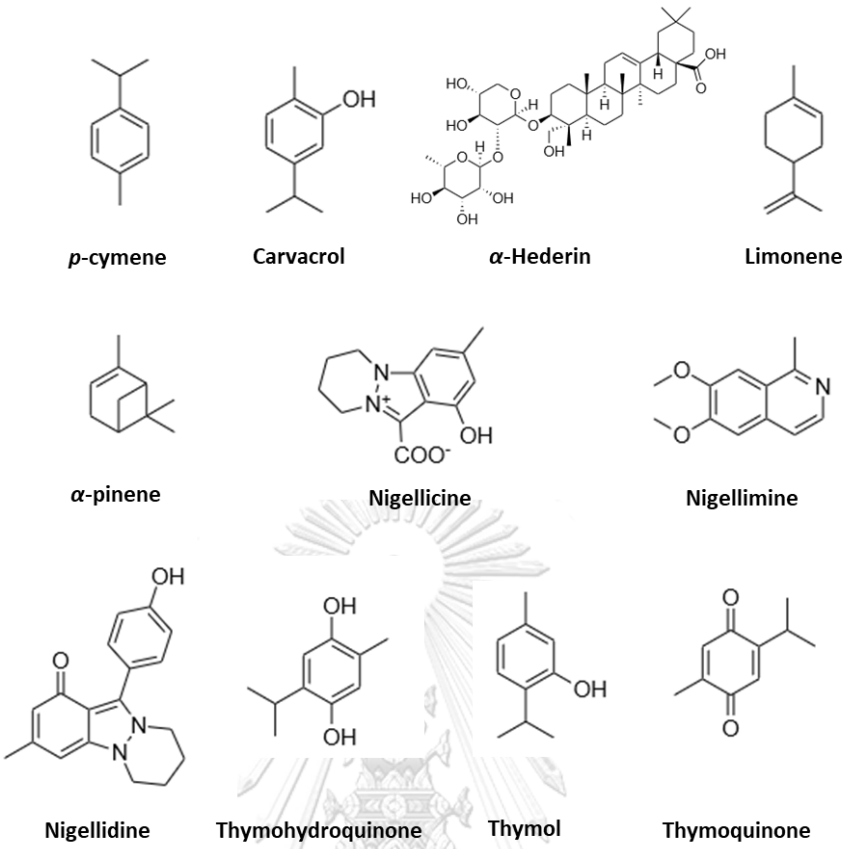


Figure 2. Chemical structures of some main constituents of N. sativa seeds (Modified from Kooti et al., 2016)

Table 1. Fatty acid compositions (%) in Morrocan *Nigella* seed oil

Fatty acids	Cold press extract	Solvent-extract
Myristic acid (C14:0)	1.0 ± 0.1	0.2 ± 0.1
Palmitic acid (C16:0)	13.1 ± 0.2	11.9 ± 0.2
Palmitoletic acid (C16:1 n-7)	0.2 ± 0.1	0.2 ± 0.1
Steric acid (C18:0)	2.3 ± 0.1	3.2 ± 0.1
Oleic acid (C18:1 n-9)	23.8 ± 0.1	24.9 ± 0.5
Linoleic acid (C18:2 n-6)	58.5 ± 0.1	56.5 ± 0.7
Linolenic acid (C18:3 n-3)	0.4 ± 0.1	0.2 ± 0.1
Arachidic acid (C20:3)	0.5 ± 0.1	0.2 ± 0.1
Total Saturated fatty acids	16.8 ± 0.5	15.5 ± 0.5
Total Unsaturated fatty acids	82.9 ± 0.5	82.1 ± 0.5

Source: Gharby *et al*., 2015.

Table 2. Sterol compositions (%) of the Moraccan *Nigella* seeds

Sterols	Cold press-extracted	Solvent-extracted
Cholesterol	0.9 ± 0.1	0.8 ± 0.4
Campesterol	13.1 ± 0.5	12.8 ± 0.5
Stigmasterol	17.8 ± 0.5	18 ± 1.0
ß-Sitosterol	49.4 ± 1.5	51.3 ± 2.5
Δ5-Avenasterol	12.4 ± 0.5	8 ± 1.0
Δ7-Stigmasterol	0.6 ± 0.1	0.7 ± 0.1
Δ7-Avenasterol	2.1 ± 0.2	1.3 ± 0.5

Source: Gharby *et al*., 2015.

2.1.4 Pharmaceutical attributes of *N. Sativa*

2.1.4.1 Antimicrobial activity

The antimicrobial activities of *N. sativa* essential oil are mainly attributed to the phenolic compounds present in the oil. The antimicrobial properties of phenolic compounds in *N. sativa* oil were first reported since 1965 (Aljabre et al., 2015). The antimicrobial properties of *N. sativa* extracts and oil toward a wide variety of pathogens had been investigated (Ishtiaq et al., 2013; Nair et al., 2005). *Nigella sativa* seed oil showed to inhibit 20 strains of *Listeria monocytogenes* (gram positive rod) by disc diffusion method impregnated with 10 µL of *Nigella sativa* seed oil and mean zones of inhibition was 31.50 mm (Figure 3 and Table 3). Furthermore, the *N. sativa* extracts revealed to inhibit human pathogenic bacterial strains and coliforms. Based on the disc diffusion method, 40 µL *of N. sativa* extracts could inhibit growth of gram positive cocci including *Enterococcus faecalis* and *Staphylococcus aureus,* and gram negative bacilli, such as *Escherichia coli, Proteus mirabilis* (Table 4). The antimicrobial activities of *N. sativa* by determination of minimal inhibition concentration (MIC) were also studied (Manju *et al.*, 2016; Piras *et al.*, 2013). The supercritical fraction extracted *N. sativa* volatile oil and fixed oil showed to inhibit growth of gram-negative bacterial strains including *E. coli*, *Pseudomonas aeroginosa*, and *Acinetobacer baumannii,* and gram positive bacterial strains which were *S. aureus*, *E. faecalis*, and *Bacillus subtilis* (Table 5). In additions, *N. sativa* essential oil was reported to

be effective against *Vibrio parahaemolyticus* Dahv2 via the agar disc diffusion assay with an inhibition zone of 23.9 mm. The correlation between antimicrobial activity and total phenolic content was reported (Manju et al., 2016). Compared to other medicinal plants, *N. sativa* essential oil presents the lowest MIC correlated to the high amount of total phenolic content in the oil as shown in Table 6 and Table 7.

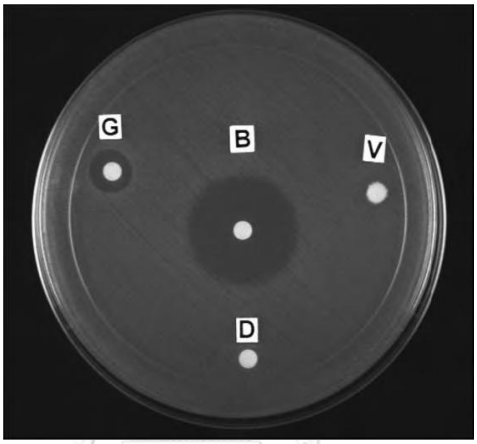


Figure 3. Effects of black seed oil (B), gentamicin (G), vegetable oil (V), and blank disc (D) on *Listeria monocytogenes* 19115

2.1.4.2 Antioxidant activity

The DPPH scavenging assay is one of the simple and convenience of the assay widely used for measuring antioxidant activity. The properties of antioxidant activity of *N. sativa* by the DPPH assay had been reported in many studies (Kooti et al., 2016; Manju et al., 2016). The antioxidant activities of essential oils from medicinal plants are mainly attributed to the active compounds present in them. Thymoquinone, carvacrol, t-anethole, and 4-terpineol are the main phenolic compounds found in the black cumin plant. The literature also exhibited that the high percentages of total phenolic contents in the black cumin promoted high free radical scavenging. The black cumin essential oil at a concentration of 300 μg/mL showed strongest radical scavenging effect (46.30%) by the DPPH radical scavenging assay compared to essential oils from

other plant species (Table 7) (Manju et al., 2016). This study was in agreement with Kooti *et al*. (2016) which reported that TQ could inhibit the lipid peroxidation by indirectly decreasing the production of reactive oxygen species in the test subjects. Thus, the antioxidant capacity is strongly related to the total phenolic contents in the black cumin plant.

Table 3. Inhibitory effects of black seed oil and gentamicin on *L. monocytogenes*

Strains	Sources	Black seed oil (zones in mm.)	Gentamicin (zones in mm.)
L. mono 19115	Human (ATCC)	39.5 ± 1.1	14.7 ± 0.3
L. mono EGDe	Rabbit	32.8 ± 1.7	15.7 ± 0.3
L. mono 315	Pork	32.2 ± 1.8	15.0 ± 0.0
L. mono 316	Pork	29.7 ± 1.6	14.7 ± 0.3
L. mono 116	Cheese	33.7 ± 2.0	14.7 ± 0.7
L. mono 598	Presque Isle culture	29.5 ± 1.0	15.0 ± 0.0
L. mono 1	Apple	32.8 ± 1.8	15.0 ± 0.0
L. mono 2	Apple	30.3 ± 2.4	15.3 ± 0.7
L. mono 7	Fish processing plant	32.7 ± 2.4	14.7 ± 0.3
L. mono 8	Fish processing plant	31.0 ± 1.7	14.7 ± 0.3
L. mono 9	Fish processing plant	25.5 ± 1.8	14.0 ± 0.0
L. mono 16	Fish processing plant	32.0 ± 0.9	15.3 ± 0.7
L. mono 17	Fish processing plant	28.8 ± 2.3	15.0 ± 0.0
L. mono 18	Fish processing plant	28.2 ± 2.0	14.0 ± 0.0
L. mono 19	Fish processing plant	32.8 ± 1.1	14.3 ± 0.3
L. mono 20	Goat	30.0 ± 3.0	14.0 ± 0.0
L. mono 21	Human	28.8 ± 1.1	16.7 ± 0.9
L. mono 23	Human	31.5 ± 3.2	14.7 ± 0.3
L. mono 24	Human	29.7 ± 2.3	13.7 ± 0.3
L. mono 25	Human	38.7 ± 1.2	16.7 ± 0.2

Mean ± SE. Source: Nair *et al*., 2005.

Table 4. Zones of inhibition of eight extracts against seven pathogenic bacterial strains identified by ribotyping

Extracts	Zones of inhibition (mm.)			
	E. coli	*S. aureus*	*P. mirabilis*	*E. faecalis*
Ethanolic				
100 mg/mL	13.50 ±1.30	NZ	NZ	NZ
50 mg/mL	12.00 ± 1.00	NZ	NZ	NZ
25 mg/mL	11.33 ± 1.20	NZ	NZ	NZ
10 mg/mL	10.00 ± 1.30	NZ	NZ	NZ
5 mg/mL	9.50 ± 1.00	NZ	NZ	NZ
Methanolic				
100 mg/mL	15.10 ± 1.00	14.16 ± 1.50	13.33 ± 2.30	15.33 ± 1.50
50 mg/mL	12.60 ± 0.50	12.00 ±1.50	11.66 ± 0.50	13. 33± 0.50
25 mg/mL	12.33 ± 0.51	9.30 ± 1.50	10.16 ± 0.70	12.66 ± 0.70
10 mg/mL	11.33 ± 1.20	NZ	9.30 ± 0.70	11.33 ± 0.50
5 mg/mL	10.80 ± 1.40	NZ	8.83 ± 1.00	8.67 ± 0.50
Chloroform				
100 mg/mL	12.00 ± 1.50	15.33 ± 1.10	14.00 ± 1.00	12.83 ± 1.00
50 mg/mL	11.66 ± 1.50	14.60 ± 1.00	13.33 ± 0.50	13.83 ± 0.20
25 mg/mL	11.00 ± 1.30	12.00 ± 2.00	10.50 ± 0.00	14.83 ± 0.20
10 mg/mL	NZ	9.10 ± 0.70	10.00 ± 0.50	15.50 ± 0.50
5 mg/mL	NZ	NZ	8.60 ± 0.50	16.33 ± 0.50
Hexane				
100 mg/mL	7.75 ± 0.30	15.80 ± 1.00	15.00 ± 1.00	14.86 ± 0.80
50 mg/mL	NZ	13.00 ± 1.00	12.60 ± 0.50	12.83 ± 2.00
25 mg/mL	NZ	NZ	9.70 ± 0.50	11.33 ± 1.50
10 mg/mL	NZ	NZ	NZ	11.00 ± 1.70
5 mg/mL	NZ	NZ	NZ	10.33 ± 2.50
Gentamycin 100µg/mL	24.00 ± 0.20	20.00 ± 0.20	28.00 ± 0.00	22.00 ± 0.50
Amoxycillin 100µg/mL	NZ	18.00 ± 0.00	18.00 ± 0.20	20.00 ± 0.00
Cefiniazone 100µg/mL	15.00 ± 0.30	32.00 ± 0.10	NZ	25.00 ± 0.00
Negative control	NZ	NZ	NZ	NZ

Mean ± SE, NZ = no zone, *E. coli* = *Escherichia coli*, *S. aureus* = *Staphylococcus aureus, P. mirabilis* = *Pseudomonas mirabilis*, *E. faecalis* = *Enterobacter faecalis* Source: Ishtiaq et al., 2013.

Table 5. The antimicrobial activity of *N. sativa* volatile oil against gram negative bacteria and *N. sativa* fixed oil against gram positive bacteria

Bacterial strains	Minimum inhibitory concentrations (µg/mL)		
	***N. sativa* volatile oil**	***N. sativa* fixed oil**	**Ampicillin**
Gram-negative bacteria			
A. baumannii	32	NT	0.12
E. coli	16	NT	< 0.12
P. aeruginosa	16	NT	0.5
Gram-positive bacteria			
B. subtilis	NT	32	0.12
E. faecalis	NT	32	0.50
S. aureus	NT	32	< 0.12

Source: Piras *et al.*, 2013. NT = not tested

Table 6. Minimum inhibitory concentrations (MIC) of the essential oils against pathogenic *V. parahaemolyticus* Dahv2

Source of essential oils	MIC of essential oils (µg/mL)
Acalypha indica	>500
Alternanthera sessilis	250
Cocos nucifera.	-
Euphorbia prostrata	>2,000
Hydnocarpus pentandra	500
Mentha longifolia	>1,000
Nigella sativa	100
Ricinus communis,	750
Schistosoma indicum	1,250

Source: Manju *et al.*, 2016.

Table 7. Total phenolic contents in the essential oils of medicinal plants

Medicinal plants	Phenolic content (µg/mL)	Reduction in DPPH (%)		
		100 (µg/mL)	**200** (µg/mL)	**300** (µg/mL)
Acalypha indica	523.7 ± 3.12	18.25 ± 3.45	29.56 ± 2.31	41.25 ± 2,51
Alternanthera sessilis	121.2 ± 0.20	8.32 ± 2.00	14.25 ± 2.31	25.63 ± 1.23
Euphorbia prostrata	142.3 ± 0.30	7.54 ± 1.20	10.52 ± 2.50	19.36 ± 2.50
Mentha longifolia	167.3 ± 0.20	10.23 ± 2.10	20.36 ± 1.23	31.54 ± 2.36
Nigella sativa	533.7 ± 3.07	19.37 ± 0.30	27.30 ± 2.50	46.30 ± 2.42
Ricinus communis	115.1 ± 0.15	5.52 ± 0.20	9.23 ± 1.32	20.56 ± 1,54
Schistosoma indicum	185.2 ± 0.30	13.56 ± 2.15	22.45 ± 2.41	35.62 ± 2.00
GAE* (100 µg/mL)	955.2 ± 3.87	-	-	-
BHT* (100 µg/mL)	-	-	27.00 ± 3.00	70.3 ± 2.0

Source: Manju *et al.*, 2016.

2.1.4.3 Anticancer properties

The anti-cancer properties of *N. sativa* in both *in vitro* and *in vivo* models have been extensively studied. The decoction (hot-water extract) containing *Nigella sativa* (seed) and other type of medicinal plants including *Hemidesmus indicus* (roots), and *Smilax glabra* (rhizome) have been traditionally applied to treat different types of cancer in Sri Lanka. The decoction of these plant mixtures by oral consumption for 10 weeks improved diethylnitrosamine-induced hepatocarcinogenesis in male Wistar rats at a dose of 4 - 6 g/kg BW/day. Oral administration (6 g/kg BW/day) of the herbal plant mixture of *N. sativa*, *H. indicus*, and *S. glabra* exhibits diethylnitrosamine-induced hepatocellular adenoma protection in Wistar rats. The pure *N. sativa* extracts also showed to inhibit skin carcinogenesis and postponed the onset of skin papilloma in mice treated with 7,12-dimethylbenzanthracene/croton oil (Majdalawieh & Fayyad, 2016). The anti-cancer mechanisms of *N. sativa* were reported to involve with the uses of free radical, interrupt enzyme activity, stop of cell proliferation, changes in intracellular glutathione, antioxidant activity, trapping free radicals and induction of apoptosis in cancer cells through a pathway dependent and independent of p53. In addition, the thymoquinone in *N. sativa* alone also exhibited the same mechanisms. The apoptosis of the cancer cell was occurred by increasing the expression of mRNAs of P53 and p21WAF1 and also inhibition of anti-apoptotic proteins (BCL-2). Moreover, thymoquinone has been reported to induce apoptosis in the human osteosarcoma cell line SaOS-2 by down regulating the NF-κB DNA-binding activity, XIAP, survivin, and VEGF in SaOS-2 cells(Kooti et al., 2016).

2.1.4.4 Antihyperlipidimic and antidiabetic properties

The antihyperlipidimic activity of *N. sativa* had been evaluated in both animal models and human subjects. The consumption of *N. sativa* showed to improve the lipid profiles. The black cumin seeds revealed to lower serum cholesterol in animal models. It provided beneficial effects in

treating dyslipidemia by inducing hepatic and intestinal apo A-I secretion, as well as apo A-I mRNA and gene promoter activity (Haas et al., 2014). Two months interventions of *N. sativa* in menopause women indicated better lipid profiles compared to placebo group. It reduced total cholesterol, low density lipoprotein cholesterol (LDL-c), triglyceride (TG) while the high density lipoprotein cholesterol (HDL-c) increased after *N. sativa* interventions (Ibrahim et al., 2014). The antihyperlipidimic effect of *N. sativa* had been evaluated in diabetic patients (Bamosa et al., 2010). The type 2 diabetes patients treated orally with *N. sativa* for 12 weeks (26.7 mg/kg BW/day) indicated to lower fasting blood glucose, 2-h postprandial blood glucose, glycosylated hemoglobin, and insulin resistance.

2.1.5 Cytotoxicity of *Nigella sativa*

In vitro model for toxicity study in replacing of animal studies had been a great of interest. It is no ethical consideration required and also cost effective over the animal studies. The antitumor and anticancer activities of *N. sativa* were also investigated via animal cell culture models. *Nigella sativa* extract (NSE) and *N. sativa* oil (NSO) were reported to exhibit in vitro cytotoxic effect. The alcoholic NSE and soxhlet extracted NSO at the concentrations of 0.25 - 1 mg/mL caused lost in the typical morphology of human lung cancer cell lines (A-549 cells) in a concentration dependent manner(Al-Sheddi et al., 2014). Similarly, the ethanolic NSE also exhibited in vitro cytotoxic effect on human renal cell carcinoma (ACHN) in a concentration and time dependent manner. The IC_{50} of ethanolic NSE against ACHN cell lines at 72 h by MTT assay was 636 µg/mL(Tabasi et al., 2015). Another studied also reported the cytotoxicity of *N. sativa* essential oil and TQ using MTT assay against human epithelial cell lines (Hep-2). The IC_{50} of *N. sativa* seed oil and TQ were 55.2 ± 2.1 and 19.25 ± 1.6 µg/mL, respectively(Harzallah et al., 2011).

2.1.6 Effective dose of *N. Sativa* and thymoquinone for therapeutic usage

The administrations of TQ and *N. Sativa* in different types of animal models were investigated. The thymoquinone and *N. Sativa* were given either by intraperitoneal, intravenous, or intragastric routes. The oral administration of TQ

was given to the rabbit with a concentration of 20 mg/kg body weight by which the TQ was reported to have slower absorption by oral administration (Alkharfy et al., 2015). Oral ingestion of TQ at concentration range from 10–100 mg/kg body weight indicated no toxic or lethal effects in rats and mice(Darakhshan et al., 2015). For rheumatoid arthritis study, arthritis scoring and bone resorption were significantly decreased after TQ administration at 5 mg/kg BW/day in arthritic rats. In additions, the result indicated the reduction of HNE, IL-1β, TNFα level and bone turnover markers in serums(Vaillancourt et al., 2011). The metabolic syndrome patients treated orally with NSO (60 mg/kg/day) as add-on therapy for 6 weeks showed to improve FBG and LDL-c values. This revealed the potential of NSO as therapeutic agent for hyperlipidemia and hyperglycemia patients (Najmi et al., 2008).

2.1.7 Safety assessment of *N. Sativa*

The *Nigella sativa* oil had been generally recognized as safe by US Food and Drug Administration (FDA)(USFDA, 2019). The degree of toxicity of *N. sativa* oil and seed were reported to be very low (Ali & Blunden, 2003). The safety assessment of *N. sativa* fixed oil and *N. sativa* essential oil was evaluated by which the animal model experimented was Sprague dawley rats. The data revealed that the liver and kidney parameters, serum protein profiles were found to be in normal range after treated with 4% of *N. sativa* fixed oil and 0.3% of *N. sativa* essential oil for 56 days of study(Sultan et al., 2009).

2.2 Caco-2 cell culture

Recently, the in vitro cell culture model had been widely used instead of animal studies for toxicity studies. The animal studies are costly, time consuming and required ethical concern. The human cell lines provide reliable and reproducible toxicity information which is an important requirement for toxicity study. The Caco-2 cells are colorectal adenocarcinoma cells commonly used in many laboratories as an in vitro model for studying chemical toxicity to represent the oral route of exposure. It is one of alternative ways to study the intestinal physiology and in vitro toxicology test in human intestine(Natoli et al., 2012). The

cytotoxicity of new drugs and active food compounds against Caco-2 cells were widely studied in order to understand its toxicity and absorption. The Caco-2 cells had been used to study the absorption of diterpenoid from Gancaofuzi decoction from China herbs (Ding et al., 2016). The cytotoxicity of in vitro digested casein peptide fraction was also evaluated by Caco-2 cells (Xie et al., 2015). Moreover, the toxicogenomic of nanoparticles and food related nanomaterials toxicity were also investigated via Caco-2 cells (Sahu, 2016). The cytotoxicity of TQ against Caco-2 cells was also investigated. The IC_{50} of TQ against Caco-2 cells was 15 μM for 24 h of TQ incubation. Surprisingly, no inhibition of growth and toxicity were observed in FHs74Int human normal intestinal cells even at 60 μM of thymoquinone (El-Najjar et al., 2010).

2.3 Microencapsulation

Microencapsulation can be defined as a process of coating the core material containing bioactive compounds within the wall materials to form microcapsules. It is an innovative technology which has been utilized in pharmaceuticals, cosmetics, and also food industries for a decade. It has been applied in the food industry in order to protect the sensitive ingredients, for example vitamins, flavors, and active oils, from environmental factors and food matrix (Rodríguez et al., 2016). The intervention of microencapsulation process into bioactive oils had shown to be very efficient to encounter the issues. It does not only protect the valuable of phytochemical residues but also mask the undesirable characteristics, improve the shelf-life of the oil, and enhance ease of handling.

Several methods have been developed to microencapsulate the bioactive oils including coacervation, spray drying, freeze drying, and extrusion (Kaushik et al., 2015; Rodríguez et al., 2016; Wen et al., 2014).

2.3.1 Coacervation

Coacervation is a chemical type of microencapsulation process whose name derived from Latin word “coacervare” means “to assemble together or cluster”. This method allows the phase separation of two liquids in colloidal solutions and alteration of the medium conditions to achieve deposition of polymer around the core material (Figure 4). The particle sizes of this process

usually range from 1 - 100 μm (Kaushik et al., 2015; Wen et al., 2014). There are two types of coacervation which are a simple coacervations and a complex coacervation. In case of applying only single polymer in the solution, it is considered as a simple coacervation. A complex coacervation is named if using two polymers of opposite charges in the solutions(Rodríguez et al., 2016). The advantages of this method are easy to operate, and cheap. However, the stability of the process only limited to some ranges of pH, ionic strength, and polymers. A positively charge polymer like gelatin which mainly derived from pig is not acceptable by vegetarians and some ethnic groups, especially Muslim consumers. However, the replacement of the pig gelatin by the fish gelatin increases in cost by 4 to 5 times(Kaushik et al., 2015).

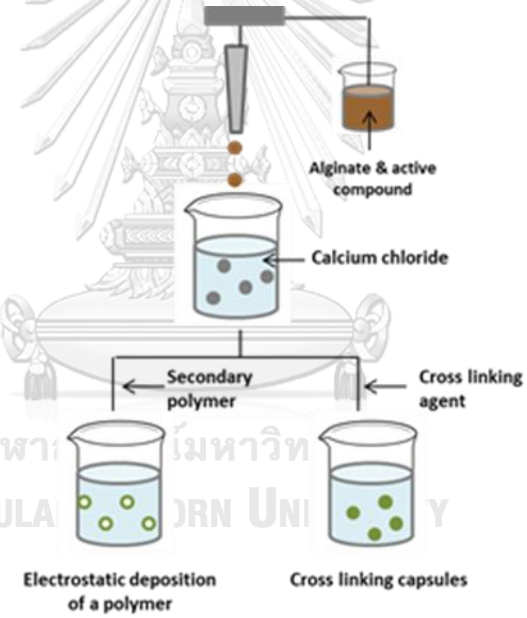


Figure 4. A schematic representation of the simple and complex coacervation processes (modified from Wen *et al.*, 2014).

2.3.2 Extrusion

Extrusion is a mechanical type of microencapsulation process which incorporates the core material within a sodium alginate solution and extrude into a hardening solution such as calcium chloride to form a capsule (Figure 5). This method relies on the ability of the wall materials or polysaccharide gels to

immobilize the core material when multivalent ion is associated. It tends to be the mildest method over other microencapsulation methods. It requires the lower pressure (100 psi) and temperature (< 118°C) for operation (Silva et al., 2014). This method is useful for heat labile product. It has been applied for flavours, vitamins, and colourants (Rodríguez et al., 2016). The main advantages of this method are the microcapsules produced are less porous, high density, and high stability compared to a spray drying method. On the contrary, it can reach twice the cost of the spray drying and the usage of screw extruders at high pressure tends to affect the stability of the core material. One of the limitations of the extrusion method is that the sizes of produced capsules are larger than 100 μm (500-1,000 mm). When introducing them into food products, they can impact the mouth feel (Kaushik et al., 2015).

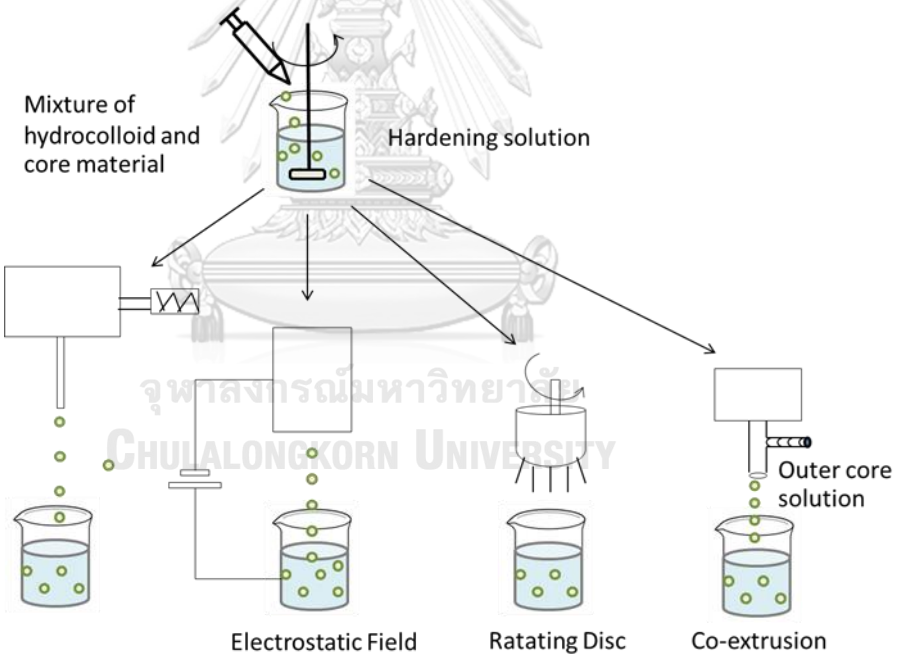


Figure 5. Extrusion technologies applied for the encapsulations of oils (modified from Wen *et al*., 2014)

2.3.3 Freeze drying

Freeze-drying is considered as one of simple and mild mechanical microencapsulation methods. This process involves with freezing of active compounds and subclimation by drying using vacuum. It is appropriated to be applied with heat labile core materials, especially volatile compounds. For the dehydration process, samples are operated with vacuum at low temperature. Oxygen is removed during the process in order to protect the oxidation and the alteration of chemical compositions of the final products. One of the disadvantages of this method is that the capsule is porous and need to be grinded after the drying process leading to the variability of the particle size. Rather than that the microcapsules produced by freeze drying gives lower encapsulation efficiency over spray drying (Silva et al., 2014). Similarly, it is time-consuming and requires high cost for operation compare to spray drying. Therefore, the spray drying process is more preferable for phytochemical encapsulation (Kaushik et al., 2015; Rodríguez et al., 2016; Wen et al., 2014).

2.3.4 Spray drying

Spray drying is a mechanical microencapsulation method. It is one of the most feasible and competent method used in many food industries. The process is initiated with the mixing of the wall materials and the core material such as oil by agitation. This emulsion will be then pumped and passed through the nozzle to form droplets. These droplets will be dried by hot air over the chamber. After that the finish particle product can be collected in the collection vessel (Figure 6). This method offers the transformation of the liquid oils into the powder form. Sizes of the final products are between the ranges of 10 – 100 μm (Wen et al., 2014). The main advantages of this method are simple operation, cheap, availability of the equipment, and provide a high quality product with low water activities. The final products are easy for storage and transportation (Rodríguez et al., 2016). The limitation of this method had also been reported. The microcapsules produced by the method can be porous when high temperature is applied. Moreover, the high temperature also causes the decrease oxidative

stability of the finish products. Selection of a good wall material is one of the key factors to successfully get a high quality of microencapsulated products by spray drying. The application of cross-linked wall materials like carbohydrate and protein is suggested to be an excellent solution(Kaushik et al., 2015).

Spray drying has been extensively used to encapsulate many types of food products including oils, flavours, and oleoresin(Kaushik et al., 2015). The black cumin oleoresin is successfully microencapsulated by this method. It exposed high encapsulation efficiency of black cumin oleoresin (84.2 - 96.2%). High wettability with acceptable moisture content of the spray drying end products is suitable for food application (Edris et al., 2016). The combination of modified starch and maltodextrin as wall materials for microencapsulated of black cumin seed oil was also reported. The resulted indicated the high thymoquinone stability was derived from the microcapsule formulated with modified starch and maltodextrin (50:50; w/w) with microencapsulation efficiency at 89.48% (Abdol-Samad et al., 2016). In additions, the previous work (Mohammed et al., 2017) also revealed that the microencapsulation by spray drying with the combination of maltodextrin DE10 and caseinate could prolong the shelf life of black cumin seed oil against oxidation.

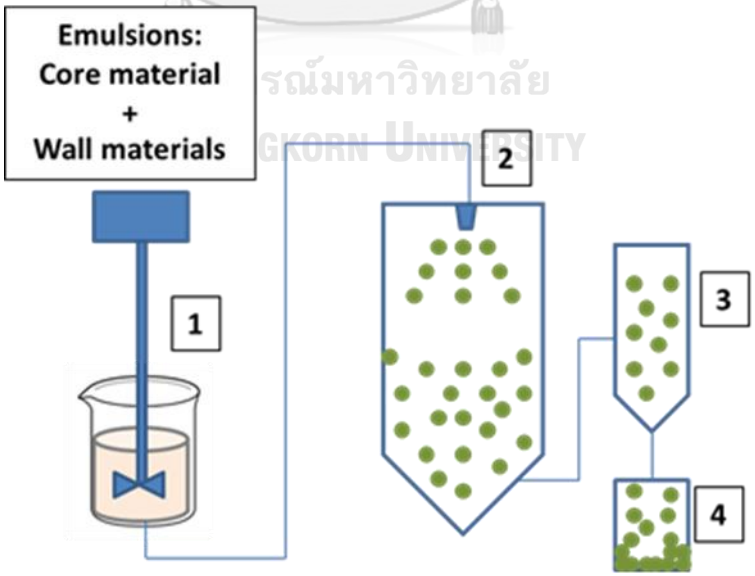


Figure 6. Spray drying process (modified from Rodríguez *et al.*, 2016).

2.4 Selection and type of wall materials used for microencapsulation by spray drying

Wall materials play a crucial role in microencapsulation. The wall materials impact stability of the emulsion, flow behavior of spray-dried particles and shelf-life of the spray-dried microcapsules(Gharsallaoui et al., 2007). The common wall materials used for microencapsulation in food industries generally derived from hydrocolloids, such as polysaccharides, proteins, and dietary fibers. Some examples of polysaccharides commonly used for microencapsulation are gum arabic, pectin, maltodextrin, glucomanan, and inulin. Whey and soy protein are proteins commonly used for microencapsulation. The criteria of choosing the wall materials depend on many reasons including chemical structures of the core materials, and the end use of the encapsulated microcapsules. Selections of the suitable wall materials result in good stability and high efficiency of the microcapsules. The excellent food grade wall materials should carry good emulsifying and film forming properties with low viscosity at high concentration. Moreover, the ability of wall materials to protect the core materials from environmental factors by maintaining a good shell and no reactivity with the core materials are also qualified. As a result that most of pre-spray drying samples are prepared in liquid solution, the water-soluble properties of the wall materials at acceptable level are required (Rodríguez et al., 2016; Silva et al., 2014). However, all of desired properties could not possibly obtained from just applying only one type of wall materials thus the combinations of two or more wall materials are generally applied in the encapsulation process.

The wall materials for spray drying purpose can be selected from a wide variety of biopolymers as follows:

2.4.1 Gum arabic

Gum Arabic is a natural gum derived from plant exudates of Acacia family plants, especially the *Acacia senegal* and *Acacia seyal* trees. It is a polymer which comprises of D-glucuronic acid, L-rhamnose, D-galactose and L-arabinose, with approximately 2% protein (Figure 7). This part of protein acts as

interface between oil and water, responsible for emulsifying properties of the gum while arabinogalactan is responsible for film forming properties of the gum (Adamiec et al., 2012; Edris et al., 2016; Li & Nie, 2016). It is one of the most common hydrocolloids used as the wall materials in microencapsulation of spray-dried products(Fernandes et al., 2014). It has been proved as "Generally Recognized As Safe (GRAS)" by US Food and Drug Administration (FDA) and considered as a soluble dietary fiber (Li & Nie, 2016). It offers advantages, such as less expensive, low viscosity at high solid concentrations, very high water solubility, formed stable emulsions in high concentrations, excellent emulsifying properties, and excellent retention of volatile during drying (Adamiec et al., 2012; Li & Nie, 2016). It has been applied for spray drying extensively including encapsulation of kaffir lime oil(Adamiec et al., 2012), rosemary oil(Fernandes et al., 2014), and black cumin oleoresin(Edris et al., 2016).

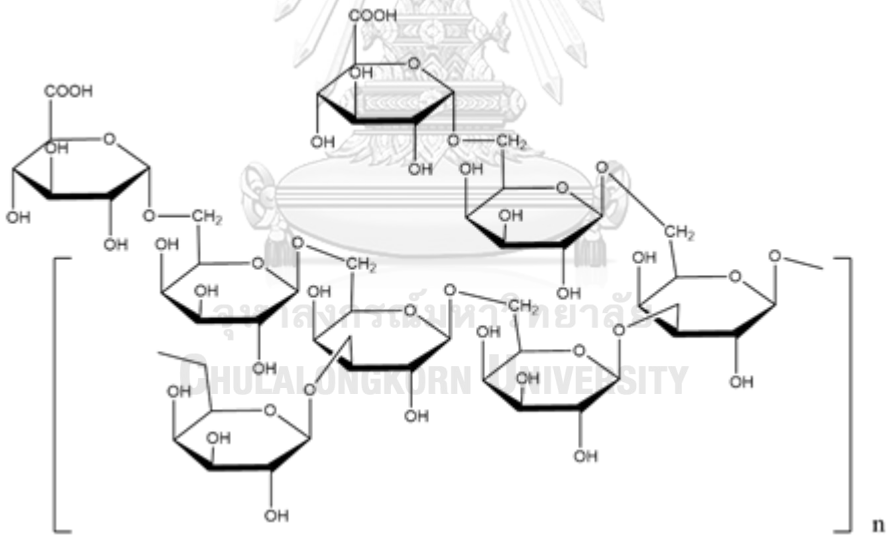


Figure 7. Chemical structure of gum Arabic (modified from Adamiec *et al*., 2012)

2.4.2 Guar gum

Guar gum is derived from the seeds of the plant *Cyamopsis tetragonoloba*, a member of Leguminosae family. It is a high molecular weight polysaccharide, mainly comprises of galactomannans in linear chain of mannose and galactose as well as 5-6% of proteins (Figure 8). The branching of guar gum is accounted for the formation of hydrogen bonding with water molecules in the solution. Guar gum is a soluble dietary fiber, very high water solubility and the solution gives very high viscosity even at very low concentration (Mudgil *et al*., 2014). In food application, it is used as a food additive considered as GRAS by US- FDA, less expensive, and only the small amount of guar gum at concentration less than 1% is added in the food products. It has been applied as a stabilizer, emulsifier, and thickener in various food products including ice cream, sauce, beverages, bakery, and meat industry. In additions, it is also used as a food supplement. Consumption of guar gum tends to reduce the cholesterol level leading to reduce the risk of heart disease, control diabetes, and maintains the bowel movement (Mudgil et al., 2014).

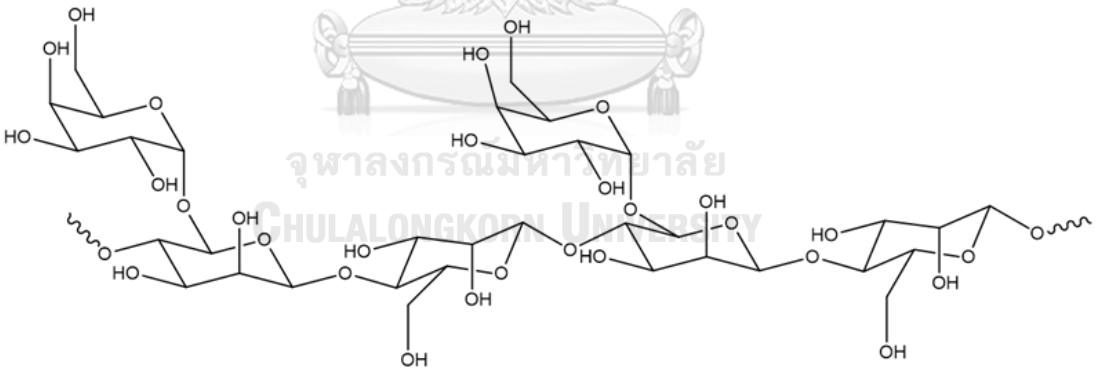


Figure 8. Chemical structure of guar gum (modified from Mudgil *et al*., 2014)

2.4.3 Xanthan gum

Xanthan gum is an anionic polysaccharide secreted from the fermentation of corn sugar by *Xanthomonas campestris*. It is considered as a soluble dietary fiber composed of glucose, mannose, and glucuronic acid with a trisaccharide side chain. This side chain interacts with the backbone by hydrogen bonding in order to protect the linkage from adverse conditions (Palaniraj &

Jayaraman, 2011). It is widely applied in food, cosmetic, pharmaceutical, and industries as a thickener, stabilizer, rheology modifier, emulsifier, and gelling and water binding agents. The xanthan gum offers highly stable against enzymolysis and acidolysis due to its very high molecular weight and its chemical structure (Figure 9). It has a significant property of soluble dietary fiber providing a highly viscous solution. Adding a very small amount of xanthan gum can prevent the separation of oils in emulsions. It has been proved as GRAS by US-FDA with no quantity limitations (Erten et al., 2014; Nikbakht et al., 2014).

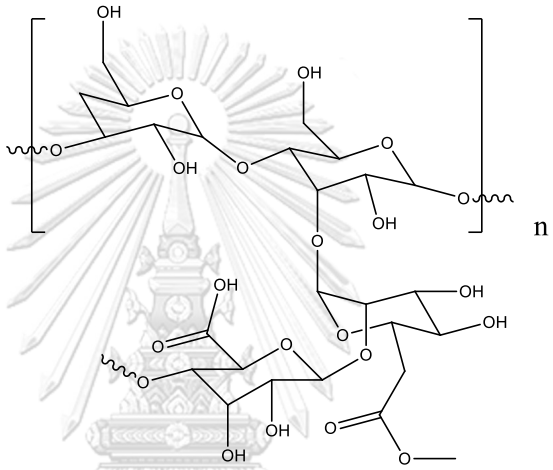


Figure 9. Chemical structure of xanthan gum (modified from Erten et al. 2014)

2.4.4 Pectin

Pectin is an anionic polysaccharide derived from the citrus fruits. It composes of linear chain of D-glucuronic acid forming a long homogalacturonic chains combined with rhamnose residues as shown in Figure 10(Abid et al., 2017). The presences of protein and acetyl groups in the chain are accounted for the emulsifying properties of the pectin. The stable emulsions could be obtained even at low concentration of pectin (Gharsallaoui et al., 2007). The pectin has traditionally been used as a gelling agent in jams and jellies and lately has been applied in many types of food, such as beverages, bakeries, and dairy products. The recent studies also showed that pectin was one of possible encapsulating agents for some core materials including fish oil, chia oil, probiotics (Chotiko & Sathivel, 2016; Noello et al., 2016; Tamm & Drusch, 2017). In additions, the pectin is also known as a soluble dietary fiber which can tolerate to

gastrointestinal conditions thus it is an ideal system for probiotic delivery system (Chotiko & Sathivel, 2016).

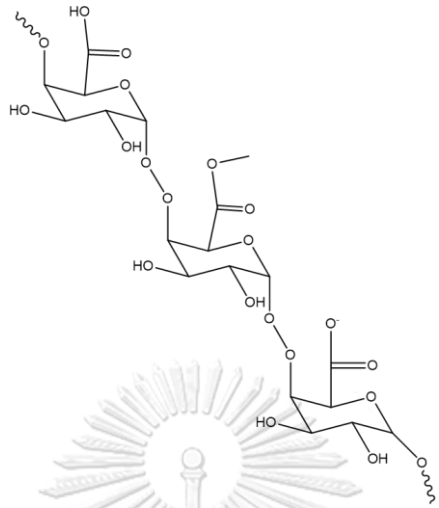


Figure 10. Chemical structure of pectin (modified from Abid *et al.*, 2017)

2.4.5 Carrageenan

Carrageenan is an anionic polysaccharide derived from marine algae. It is a high molecular weight polysaccharide which composes of linear repeating disaccharide that consists of β-(1, 3)-sulphated-D-galactose and α-(1, 4)-3, 6- anhydro -D-galactose (3-crosslinked-) as shown in Figure 11. Carrageenan is a soluble dietary fiber and had been applied as a stabilizer in dairy food products has been proved as GRAS. It can be divided into 3 types which are kappa-carrageenan, iota-carrageenan, and alpha-carrageenan. The first and second types of carrageenan have ability to form reversible gel by changes of temperature while the third type of carrageenan is not gelling type.

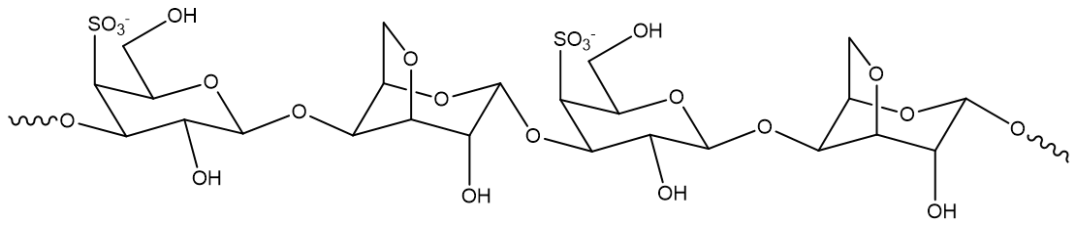


Figure 11. Chemical structure of carrageenan (modified from Hilliou, 2014)

2.4.6 Inulin

Inulin or fructan is a natural polyssaccharide derived from plants and considered as a soluble dietary fiber and prebiotic. It is found in high amounts in Jerusalem artichoke and chicory. Inulin is composed of a linear fructan linked by (2-1) glycosidic bonds with a terminal glucose residue unit (Figure 12) with the degree of polymerization (DP) of 2 – 60. It has been applied as a low calorie sweetener and texture modifier (Bayarri et al., 2010). Inulin is well recognized as one of the most common fat replacers(Laguna et al., 2014). The presence of inulin shows no interfering with casein network when introduced into whole milk yogurt. Moreover, the additions of inulin in low quantity improve the mouthfeel and creaminess in low-fat dairy and meat products. Approximately 4 g of inulin per day showed the bifidogenic effect against pathogenic bacteria. The fermentation of inulin in colon provided short chain fatty acids such as acetate, butyrate and propionate (Kaur & Gupta, 2003). The doses of inulin (10 g/day) may cause flatulence followed by mild bloating depends on the individual tolerance while high doses of inulin (≥20 g/day) may cause GI symptoms and diarrhea in some individuals. Surprisingly regular intake of inulin may increase GI tolerance and by dividing the inulin intake into two portions daily suggested to improve the inulin tolerance throughout the day (Bonnema et al., 2010).

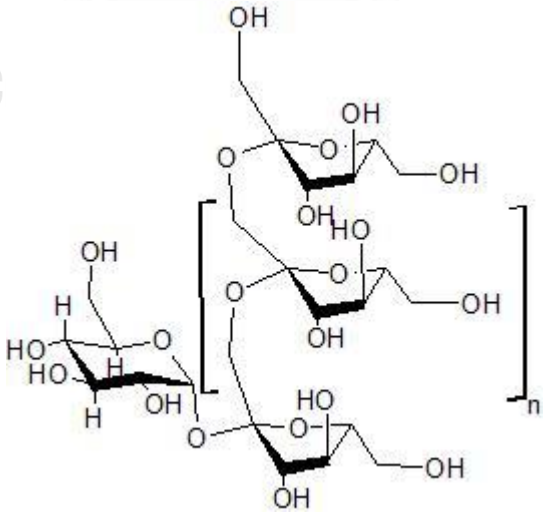


Figure 12. Chemical structure of Inulin (modified from Lambertz *et al*., 2017)

2.4.7 Hydroxypropyl methyl cellulose

Hydroxypropyl methylcellulose (HPMC) is nonionic water-soluble cellulose ethers obtained by synthetic modification of the naturally occurring polymer cellulose (Figure 13). It has been proved as GRAS by US-FDA. The HPMC has been applied as a fat replacer in many food products. It has also successfully incorporated with maltodextrin to protect the fish oil from oxidation (Al-Tabakha, 2010).

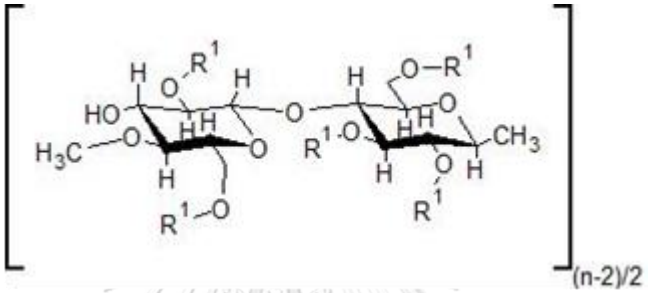


Figure 13. Chemical structure of Hydroxypropyl methyl cellulose (modified from Majumder et. al, 2016)

2.4.8 Maltodextrin

Maltodextrin is a hydrolyzed starch commonly used as wall material in microencapsulation of food ingredients (Fernandes et al., 2014). It is composed of long chain maltose (Figure 14). It provides many advantages including inexpensive cost, high water solubility, neutral aroma and taste, low viscosity at high solid concentrations, and good coverage to prevent oxidation. It has been used for encapsulation of vitamins, minerals, colorants, oils, and flavoring compounds (Gupta et al., 2015). However, it has a limitation due to its low emulsifying capacity as a result that it has been in cooperated with other types of wall materials, such as gum arabic, modified starches, and proteins for better microencapsulation by spray drying process (Marques et al., 2014).

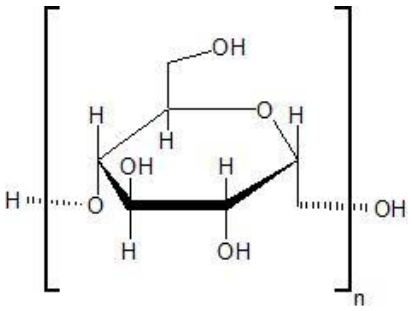


Figure 14. Chemical structure of maltodextrin (modified from Kirrane et. al, 2009)

2.4.9 Konjac glucomanan

Konjac glucomannan is a high molecular weight plant polysaccharide extracted from tubers of the *Amorphophallus* konjac plants which tropically found in China, Japan, and Thailand. Glucommanan consists of (1⟶4) linked β-D-mannose and β-D-glucose (Figure 15). The acetyl group of the backbone is responsible for solubility and gelling property. It is a healthy soluble dietary fiber which possesses anti-tumor, immunomodulation, and wound healing properties. It is commonly used as a gelling and thickening agent in many food and pharmaceutical applications due to its properties of thermal-irreversible gelling (Adamiec et al., 2012; Jian et al., 2016; Nualkaekul et al., 2013). The konjac glucomannan has been reported to exhibit oil retention capacity of sweet orange oil comparable to gum arabic and starch sodium octenyl succinate (SSOS). Compared to pure konjac glucomannan, likewise, the combinations of konjac glucomannan with other wall materials, such as gum arabic or SSOS tend to improve the yields of encapsulation products (Jian et al., 2016).

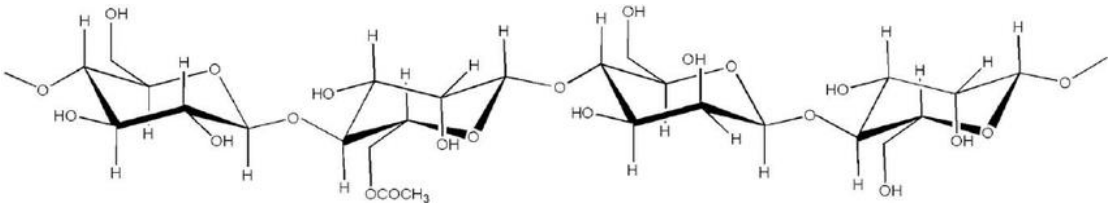


Figure 15. Chemical structure of konjac glucomannan (modified from Shah et. al, 2015)

2.4.10 Whey protein

Whey protein is a globular protein comprises of β-lactoglobulins, α-lactalbulins, immunoglobulins, serum albumins and lactoferrin. Whey, a by-product derived from dairy industry waste, exhibits a best source of natural branched chain amino acids (BCAAS) including leucine, isoleucine, and valine. Whey protein concentrate can be soluble in wide range of pH values. In additions, it also displays a good emulsifying property and so it has been applied in food processing as an emulsifier, fat replacer, antimicrobial film, delivery vehicle as well as encapsulating agent (Hu et al., 2003). The *Lactobacillus rhamnosus* CRL 1505 was encapsulated successfully by the combination of pectin and whey protein (Gerez *et al*. 2012). Encapsulating cardamom essential oil in microcapsules containing 30% of whey protein demonstrated a good microencapsulation efficiency (98.5%)(Mehyar et al., 2014).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 *Nigella sativa* seeds and hydrocolloids wall materials

Nigella sativa seeds were purchased from a local supplier (Bangkok, Thailand). Gum arabic and whey protein were purchased from Chemipan Co.Ltd., Inulin was purchased from Healthy Inc.Ltd. (Thailand), Maltodextrin was purchased from CT Chemical Co.Ltd. (Bangkok, Thailand).

3.1.2 Bacterial and animal cell line samples

All bacteria (*Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 25922, *Lactobacillus rhamnosus* ATCC 53103) and Caco-2 cell line were purchased from ATCC

3.1.3 Chemical reagents

Thymoquinone (99.9% purity) was purchased from Sigma-Aldrich (St. Louis, USA). Hexane and Methanol (HPLC grade) was purchased from Merck (Darnstadt, Germany). Other chemical reagents used were of analytical grade.

3.1.4 Instruments

High speed homogenizer (Ultra-Turrax IKA T25 basic, Wilmington, USA; IKA Labortechnik Staufen Germany), mini spray dryer B-290 (Büshi Labortechnik AG, Switzerland), scanning electron microscope (Quanta 250, FEI, USA), mastersizer 3000 (Malvern Panalytical Ltd, United Kingdom), aqualab 3 (Decagon Devices, Pullman, WA, USA), HPLC system with UV detector (Shimazu-20AD, Japan), MINOLTA Croma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan), ATR-FTIR with a crystal detector (BRUKER Tensor II, Germany), High capacity centrifuge (Himac CF7D2, Hitachi, Tokyo, Japan), gas chromatography system (GC-FID 2010, Shimazu), Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, USA) were applied in this study.

3.1.5 Consumable items and non-consumable items

The consumable items used in this study were pipette tips, aluminum foils, parafilms, filter papers, Petri dishes, microcentrifuge tubes, pyrex test tube, inoculating loop, latex and nitrile gloves. The non-consumable items used were mortars, pestles, cylinders, glass rods, beakers, conical flasks, forceps, duran bottles, 96- well plates.

3.2 Methods

3.2.1 Cold press oil from black cumin seed

Twenty kilogram of dried black cumin seed originally grown in India was purchased from the market. The sample was sent to the Transgenic Laboratory of Plant Transgenic Technology and Biosensor, Department of Botany, Faculty of Science at Chulalongkorn University for identification of species and variation. One kilogram of dried *N. sativa* seeds was slowly put in a cold press machine and the oil used as a core material was collected. The cold pressed *N. sativa* seed oil (NSO) was stored overnight for sedimentation and then filtered with Whatman paper No.1 using the suction flask couple with vacuum pump to get a clear sample. The extract oil was blown with nitrogen gas and stored in a brown Duran bottle at 4 °C. Then it was analyzed directly within 1-2 days after extraction.

3.2.2 Determination of thymoquinone in the NSO by high performance liquid chromatography (HPLC)

Methanol as a mobile phase was filtered through a 0.45 μm membrane filter (Millipore, Milford, MA) and degassed under vacuum before use. The chromatographic condition of high performance liquid chromatography was set up with methanol: water (70:30%, v/v) with a flow rate of 1.0 mL/min for thymoquinone analysis. The C18 reversed-phase column (150 x 4.6 mm, 5 μm particle size, GL Science) was applied in this study. The stock solution of thymoquinone standard was prepared and diluted to seven concentrations of thymoquinone (1 mg/mL, 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL, and 60 mg/mL). The TQ standard solutions were injected in the HPLC for making a calibration curve. For sample preparation, 2 g of *N. sativa* seed oil was dissolved in DMSO (1:9) and then 10 μL was further dissolved in 990 μL of

methanol (10:990). The injection volume of 20 uL of the sample was then injected in the HPLC system with UV detector set at 254 nm for thymoquinone analysis (Shimazu-20AD, Japan). The chromatogram obtained from the NSO sample was compared to the calibration curve for quantification of thymoquinone in the NSO oil.

3.2.3 Analysis of fatty acid compositions in the NSO by gas chromatography flam ionization detector (GC-FID)

The lipid of pure NSO was extracted according to Mahama *et al.*, (2020) with some modification. The 2 g of the black cumin seed oil sample was added into screw cap tube. The 10 mL of the methanol: hexane mixture (4:1, v/v) were added into the oil samples. The mixture was shaken every 15 min while incubation for 1 hour and the solution was filtered into a new test tube using Whatman paper no.1. The 0.1 M KCL solution with 20% of total volume was added into the filtrate by vortexing and the sample was further centrifuged at 2000 x g for 10 min (Himac CF7D2, Hitachi, Tokyo, Japan). Then, the lipid phase which is the lower phase was further converted to methyl esters (FAME) by the additions of 2 mL of 0.5 M NaOH-methanol into 200 µL of mixtures. The mixtures were heated at 100°C for 15 min and cool down at room temperature. The cap was closed tightly, further heated at 100°C within 1 min and again cool down to room temperature. The 500 µL of hexane and 5 mL of saturated NaCl were added into the mixture and later centrifuged at 1000 x g for 5 min. The upper phase was collected for the GC injection. Then the sample was determined using the GC–FID (Shimazu, Japan) equipped with a DB23 column, (30 m x 0.25 mm i.d. x 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA). The carrier gas was helium and the total gas flow rate was 1 mL/min 62.9 mL/min, a split ratio of 1: 50. The operating conditions were the following: the initial and final column temperature was 80°C and 220 °C, respectively. The injector and detector temperature were 250°C and 300°C, respectively. A comparison between the retention times of the samples with those of authentic standards, run on the same column under the same conditions, was made to facilitate identification. Results were expressed as the relative percentage of each individual fatty acid present in the sample.

3.2.4 Determination of phytochemical compositions in the NSO by gas chromatography–mass spectrometry (GC-MS)

The pure NSO was extracted using solvent-solvent extraction following the method as described in section 3.2.3. The mixture of Dichloromethane: methanol (2:1, v/v) was used for solvent extraction. After addition of 0.1 M KCL solution and removing of the upper phase, the remaining solution was blown with nitrogen gas to evaporate the solvent. Then, the sample was further trimethylsilylation using silylating agent namely BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamine] by which 100 µL of anhydrous pyridine was added to 20 µL of extract followed by the addition 100 µL of BSTFA. Then, the mixtures were heated at 60°C for 30 mins and so the 2 µL of mixtures was dissolve in 200 µL of hexane. The silylated NSO sample was submitted to Scientific and Technologiecal Research Equipment Centre for phytochemical determination. The 1 µL was injected into GC-MS (Triple Quadrupole GC-MS/MS equipped with an HP-5ms capillary column (30 m length, 0.25 mm i.d.; 0.25 µm film thickness). The operating conditions were: the injector temperature was 250◦C; the oven temperature was programmed to hold at 70◦C for 3 min, and from 70◦C to 280◦C at 4 min where it was held for 10 min, then further increased to 300◦C for 10 min; the helium carrier gas flowed at a linear velocity of 36 cm s-1; the split ratio was 20:1; the type of ionization used i was EI 70 eV; and the spectra were acquired by scanning at 33–600 m/z. The compounds were identified by comparison with spectra available in the GC-MS program and compared to linear retention indices relative to the series of n-hydrocarbons as well as NIST database. The component profile was expressed as percentage according to the relative peak area.

3.2.5 Investigation of in vitro antimicrobial activity of the NSO

3.2.5.1 Agar disc diffusion method

The antimicrobial activity of the NS oil was investigated against four bacterial species including one gram-negative pathogenic bacterium (*Salmonella enterica* ATCC 14028*),* one gram-positive pathogenic bacterium (*Staphylococcus aureus* ATCC 25923), one coliforms (*Escherichia coli* ATCC 25922), and one gram-positive probiotic bacterium (*Lacobacillus rhamnosus* ATCC 53103). The agar disc

diffusion method was conducted following Manju *et al*., (2016) with some modification. Mueller-Hinton agar and broth (Difco Laboratories, Detroit, USA) was used for bacterial growth. The inoculum was an overnight culture of each bacterial species in Mueller-Hinton broth diluted in the same media to a final concentration of approximately 1 × 10^8CFU/mL. The 0.1 mL of each suspension was spread over control and test plates containing Mueller-Hinton agar. Paper discs of 6 mm diameter (Becton Dickinson Microbiology Systems, Maryland) were sterilized by autoclaving in a dry petri dish. The 400 mg of NSO was dissolved in 1 mL of dimethyl sulfoxide (DMSO). Under aseptic conditions, sterilized discs were impregnated with 20 µL of the respective NSO. The disk impregnated with standard antibiotics ceftriaxone 100 µg/mL was served as a positive control. The disk impregnated with DMSO was served as a negative control. The plates were left for 30 min at room temperature to allow diffusion of all samples. The plates inoculated with *S. enterica* ATCC 14028, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922 were incubated at 37 °C for 24 h. The plates inoculated with *L. rhamnosus* ATCC 53103 were incubated at 37°C for 48 h. The inhibition zones were observed and their diameters were measured in millimeters.

3.2.5.2 Broth microdilution method

The minimum inhibitory concentration (MIC) of the NSO was determined by broth microdilution method(Harzallah et al., 2011) with some modifications. This test was performed in sterile 96-well microplates. The NSO was sterile prepared and transferred to each microplate well in order to obtain a twofold serial dilution in DMSO. Briefly, the NSO was dissolved in DMSO (8 mg/mL) then diluted with DMSO with twofold microdilution into 9 different concentrations accordingly (4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, 0.3125 mg/mL and 0.15625 mg/mL). The inocula of each microorganism were adjusted to yield a cell concentration of 1 × 10^8CFU/mL. A final volume of 100 µL was dropped in each well. There were four control wells including 1) Culture control: one well with specific medium and inocula to check growth of bacteria 2) Media control: one well with the broth only to check the sterility of the media and the aseptic work conditions, 3) Negative

control: one well with DMSO, and 4) Positive control: one well with antibiotics. The microplates were prepared in triplicate and incubated at 37 °C for 24 h. After incubation, bacterial growth was evaluated by using 40 µL of 0.2 mg/mL INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) as a growth indicator according to the MIC method performed (Lall et al., 2013). The MIC was defined as the lowest concentration of the NSO that inhibited the growth of the bacteria. The results were expressed in milligrams per milliliters.

3.2.6 Preparation of emulsions

Nine formulas of emulsions (GA, GG, GC, GP, GK, GI, GH, GM, and GW) for microencapsulation of the NSO were prepared according to the previous study (Fernandes et al., 2014) with slightly modification. This study was formulate the wall materials with gum arabic alone and the combination of gum arabic with other eight hydrocolloids as shown in Table 8 and the combination was added in a ratio of 1:1 (Table 8). The mixture of wall materials was dissolved in distilled water in the concentration of 20% (200g : 1000g w/w) with constantly stirring overnight at room temperature. The homogeneity of each formula emulsion was observed. Only some emulsions were selected and then 40 g of the NSO as a core material was gradually added into the wall material solution with stirring at 14,000 rpm for 10 min. The emulsion was used as the feed liquid for the spray drying process.

Table 8. Proportions of the wall materials used prior to preparation of emulsions

Formulas	Types of hydrocolloids	Ratio (g:g)
GA	Gum arabic	200 : 0
GG	Gum arabic + Guar gum	100 : 100
GC	Gum arabic + k-carrageenan	100 : 100
GP	Gum arabic + Pectin	100 : 100
GK	Gum arabic + Konjac Glucomanan	100 : 100
GI	Gum arabic + Inulin	100 : 100
GH	Gum arabic + HPMC	100 : 100
GM	Gum arabic + Maltodextrin	100 : 100
GW	Gum arabic + Whey	100 : 100

3.2.7 Microencapsulation by spray drying

Four formulas of emulsions were dried using a spray - dryer with inlet temperature 170 °C and outlet temperature 60 °C. The dried powder was collected and kept in a brown Duran bottle with the lid was closed tightly and stored at 4 °C until further analysis.

3.2.8 Characterization of microcapsules

3.2.8.1 Moisture content

The moisture content of the different spray dried microencapsulated NSO (mNSO) powders was measured by oven-drying at 105◦C to constant weight (Fernandes et al., 2014). Then moisture content was calculated using the following formula:

$$\text{Moisture (\%)} = \frac{[W1-W2]}{W1} \times 100$$

where:

W1 = Weight of sample (g) before oven-dried

W2 = Weight of sample (g) after oven-dried

3.2.8.2 Water activity

The water activity was measure at 25°C using Aqualab 3 (Decagon Devices, Pullman, WA, USA).

3.2.8.3 Wettability

One gram of mNSO powder was sprinkled over the surface of 100 mL of distilled water at 20◦C without agitation. The time taken for the powder particles to sediment, sink, and disappear from the water's surface was recorded and used for a comparison of the extent of wettability of the samples (Fernandes *et al*., 2014).

3.2.8.4 Solubility

One gram of mNSO powder was resuspended into 100 mL of distilled water for 5 min using a magnetic stirrer. The solution was then centrifuged at 3000 × g for 15 min. An aliquot of 25 mL of the supernatant was

transferred to a 50 mL beaker and oven-dried at 105◦C for 24 h. The solubility (%) was calculated from the weight difference using the following equation:

$$\text{Solubility} = \frac{(P_a - P_b)}{0.25} \times 100$$

where,

P_a (g) = Mass of the beaker plus sample after dried

P_b (g) = Initial mass of the weighed beaker

3.2.8.5 Microencapsulation efficiency

The total oil content of the mNSO products were determined according to previous work (Bae & Lee, 2008). The 15 mL of hexane was added to 2 g of the microencapsulated powder in a 30 mL glass vial with a screw cap and shaken with vortex mixer for 2 min at ambient temperature to extract free oil. The mixtures were then filtered through Whatman No.1 filter paper. The remaining powder on the filter paper was rinsed three times with 20 mL Hexane. After evaporating the solvent at 60 °C to a constant weight, the surface free oil content was calculated as percentage by the weight difference in the powder before and after extraction and washing with hexane. Total oil was assumed to be equal to the initial oil. Microencapsulation efficiency (ME) was calculated using the following formula:

$$\text{Microencapsulation efficiency (ME)} = \frac{[(\text{Total oil} - \text{Surface oil})]}{\text{Total oil}} \times 100$$

3.2.8.6 Color measurement of mNSO

The microencapsulated NSO color was measured in triplicate were determined using MINOLTA Croma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan). The values of L, a and b were resolved.

3.2.9 Investigation of characteristics of NSO and mNSOs by FTIR

Approximate 1 mg of NSO or mNSO were placed into the attenuated total reflectance (ATR) accessory and investigated by Fourier transform infrared spectrophotometer (FTIR) with a crystal detector (BRUKER Tensor II, Germany).

The spectrum was recorded at the absorbance mode from 4,000 to 400 cm^{-1} (mid infrared region) at the resolution of 4 cm^{-1}. The fingerprint spectra of mNSOs were compared to the pure NSO and the spray dried powders without NSO (GA (100:0, w/w), GM, GI, and GW (50:50, w/w).

3.2.10 Determination of particle morphology and size distribution

The microstructural properties of the spray dried mNSO products were investigated using a scanning electron microscope (Quanta 250, FEI, USA). The microencapsulated NSO powders were placed on the SEM stubs coated with platinum in a vacuum evaporator using a 2-sided adhesive tape. The coated samples were then analyzed using the SEM operating at 10 kV. The pictures of all spray dried mNSO products were taken to characterize the microstructure of the products.

The particle size distribution was determined for all of the samples using mastersizer 3000 (Malvern Panalytical Ltd, United Kingdom). A small amount of the mNSO powder was suspended in distilled water with agitation. The particle size distribution was monitored during each measurement until successive readings were consistent.

3.2.11 Determination of thymoquinone in the mNSO products by HPLC

The mNSO powder samples were extracted following the method as described in section 3.2.4 using dichloromethane: methanol (2:1, v/v) as extract solvent. After addition of 0.1 M KCL solution and removing of the upper phase, the remaining solution was blown with nitrogen gas to evaporate the solvent. Then the sample was dissolved in DMSO (1:9) and then 10 µL was further dissolved in 990 µL of methanol (10:990). The injection volume of 20 uL of the sample was then injected in the HPLC system with UV detector set at 254 nm for thymoquinone analysis (Shimazu-20AD, Japan). The chromatogram obtained from the mNSO samples were compared to the calibration curve for quantification of thymoquinone in the mNSO samples.

3.2.12 Analysis of fatty acid compositions in the mNSO by GC-FID

Fatty acids in the mNSO powder samples were extracted and injected into gas chromatography system following the method as described in section 3.2.3. Triplicate samples were analyzed for each formulation. Fatty acid profiles in the mNSO samples were expressed as the relative percentage of each individual fatty acid present in the samples.

3.2.13 Analysis of phytochemical compositions in the mNSO by GC

The mNSO powder samples were analyzed for phytochemical compounds after microencapsulation using a gas chromatography mass spectrometer (Shimadzu GC-MS-QP2010 Plus) as described in 3.2.4. Triplicate samples were analyzed for each formulation and the compounds were identified by comparison with spectra available in the GC-MS program. The compounds were identified by comparison with spectra available in the GC-MS program and compared to linear retention indices relative to the series of n-hydrocarbons as well as NIST database. The component profile was expressed as percentage according to the relative peak area.

3.2.14 Determination of in vitro antimicrobial activity of the mNSO products

Both an agar dish diffusion method and a broth microdilution method for determining the antimicrobial activity of the mNSO products was investigated against four bacterial species as described in **3.2.5**. Inhibition zones and the minimum inhibitory concentration (MIC) of the mNSO products were determined and compare to those of the un-encapsulated NSO.

3.2.15 Antioxidant activity of NSO and mNSO by DPPH assay

The antioxidant activity was carried out according to previous study (Erkan et al., 2008). The NSO was dissolve in 1 mL of methanol in centrifuge tube. The NSO and mNSO sample were then centrifuged at 2000xg and the aliquot was further used for DPPH assay. The stock solution of 0.4 mM DPPH in methanol

was prepared. Then, the DPPH solution was diluted to 0.2 mM for working concentration. The 100 μL of sample was transferred to each microplate well in order to obtain a twofold serial dilution in methanol. Butylhydroxytoluene (BHT) was used as positive controls while the methanol was used as blank control. The 100 μL of 0.2 mM DPPH was transferred into the microplate. After 30 min of incubation, the absorbance of the samples was read at 517 nm using a spectrophotometer**.**

3.2.16 In vitro digestion of mNSO products and cytotoxicity of the mNSO products on Caco-2 cells by MTT assay

3.2.16.1 In vitro digestion of mNSO products

The in vitro digestion was performed (Liu et al., 2013) with some modification. For oral stage-saliva juice was made by dissolving the mixtures of sodium chloride (0.117 g), potassium chloride (0.149 g) and sodium bicarbonate (2.1 g) in 1 liter of deionized water. The saliva enzyme was prepared by dissolving 0.4 α-amylase in 100 mL of water with magnetic stirring until the mixture is fully dissolved. The one of selected mNSO (1.25 mg) was dissolved in 5 mL of saliva juice, transferred into a conical tube (50 mL) and heat at 37°C. The 2 mL of prepared α-amylase solution was then added into the tube and incubated in shaking water bath for 5 min at 37°C. The 2 mL of prepared α-amylase solution was then added into the tube and was incubated in shaking water bath for 5 min at 37°C. The pH of solution was adjusted to 1.9 using 6M HCL in order to in activate the enzyme activity. For in vitro digestion in the stomach, the sodium chloride (1.775 g) and 200 mg pepsin (Sigma-Aldrich, St. Louis, USA) were dissolved in 200 mL deionized water for gastric juice preparation and the pH was adjusted to 1.9 using 6 M HCl. Then, 10 mL of the gastric juice was further introduced into the food bolus in the first stage. The digestion bottle (25 mL) was then incubated for 90 min. The pH of the solution was adjusted to 8 using 6 M NaOH. For in vitro digestion in small intestine, 5 g of pancreatin (4xUSP) was dissolved in 500 mL of phosphate buffer (pH 8). Then, this intestine juice was transferred into digestion bottle and incubated for 12 h. The pH of solution was adjusted to 1.9 using 6M HCL in order to in activate the enzyme activity.

3.2.16.2 Caco-2 cell culture

The Caco-2 human colorectal adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC) company. The Caco-2 cells was cultured in plastic cell culture flasks in Minimum Essential Medium (MEM) supplemented with 3.7g/L $NaHCO_3$, 1% L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum at 37 °C and 5% CO_2. The cells were regularly maintained by regular passaging when the cells reached 80% confluency. The culture media was replaced with fresh media in every 72 h (Natoli *et al*., 2012).

3.2.16.3 MTT cytotoxicity assay

In the study of MTT, the digested mNSO was collected from section 3.2.16.1 and filtered with 0.2 μm sterile PES filter. The Caco-2 cells were seeded in 96-well culture plates at a density of $1x10^4$ cells per well and incubated in CO_2 incubator for 24 h. The digested microcapsule was added to 96-well culture plates and incubated for 72 h. The *in vitro* digestion solution without sample was used as a negative control. After 72h of incubation, the media was discarded and 10 μL of MTT (5 mg/mL) was added. Then, these cells were further incubated in the CO_2 incubator for 4 hours and the supernatants were discarded. Finally, 150 μL of DMSO was added. The absorbance of the 96-well culture plates was measured at 540 nm using a microplate reade(Ding et al., 2016). The IC_{50} values were calculated using equation (logarithmic line) in Microsoft Excel program to the curve formed from the data. The IC_{50} value is obtained from the equation y = 50 (50% of survival rate of cells).

3.2.17 Fortification of soymilk and cow milk with the mGI powder and sensory evaluation

3.2.17.1 Fortification of soymilk with the mGI powder

The soymilk was prepared according to previous work (Sengupta et al., 2013) with some modification. Soybean was carefully selected and soaked for overnight in distilled water. The soaked and peeled soybeans was mixed with distilled water (240 g: 1500 mL) in a ratio of 1: 6 (beans: water) and blended in a blender for 15 min. The slurry was boiled and cooled to destroy

trypsin inhibitor for improving flavor. The resultant slurry was filtered. The 10 g of mGI powder containing 11 mg of thymoquinone was added into 200 mL (1 serving size) of soymilk (mGI-SM) and stirred at 6,000 rpm for 15 minutes. Then, 5 g of pure NSO containing 11 mg of thymoquinone was added into 200 mL of soymilk (NSO-SM) and stirred at 15,000 rpm for 5 min. All fortified soymilks were pasteurized at 65°C for 30 min and immediately cool down at 4°C. Samples were kept in refrigerator at 4°C not longer than 48 hours for sensory evaluation.

3.2.17.2 Fortification of cow milk with the mGI powder

The low fat pasteurized cow milk (LFM) was purchased from the local market. The 10 g of mGI powder containing 11 mg of thymoquinone was added into 200 mL (1 serving size) of LFM (mGI-LFM) and stirred at 6,000 rpm for 15 minutes. Then, 5 g of pure NSO containing 11 mg of thymoquinone was added into 200 mL of LFCM (NS O-LFM) and stirred at 15,000 rpm for 5 min. All fortified LFM were pasteurized at 65°C for 30 min and immediately cool down at 4°C. Samples were kept in refrigerator at 4°C not longer than 48 hours for sensory evaluation.

3.2.17.3 Sensory evaluation

A descriptive analysis, triangle test, and preference test were conducted by ten trained panelists for the sensory analysis. The mGI powder was evaluated on a 15-cm unstructured line scale for the attributes as follow: overall acceptability, appearance (color, fine-coarse), flavor (black cumin oil, rancidity, floury), texture (smooth-coarse), taste (sweet, bitter, oily, spicy), and aftertaste. Lengths of marks from the left side on the line scales were measured. The first set of triangle test was used to discriminate the overall perception between the fortified mGI soymilk products (mGI-SM) and fortified NSO soymilk products (NSO-SM), while the second set of triangle test was the fortified mGI low fat cow milk products (mGI-LFM) and the fortified NSO low-fat cow milk products (NSO-LFM). Numbers of correct answer were count and used for statistical analysis. The preference test for all fortified products was performed by ten trained panelists to obtain more information on overall acceptability, appearance, color, flavor, taste, texture, and aftertaste of four fortified milk or soymilk products. The

attributes were scored on a 9- point hedonic scale ranging from 0 (attribute not detected or dislike extremely) to 8 (attribute very strong or like extremely).

3.2.18 Statistical analysis

One way analysis of variance (ANOVA) was performed using SPSS software (version 22, SPSS Inc, Chicago, IL, USA) to evaluate the significance differences of the four formulations on the characteristics of microcapsule samples and the attributes of the four fortified milk and soymilk products, followed by Tukey's means comparison test at a level of 5% significance ($P \leq 0.05$) when significant differences between the treatments were noted. The significance for triangle test was determined by reference total numbers of correct responses to a statistical table. Minimum numbers of correct responses required for significant difference ($P \leq 0.05$) between mGI-SM & NSO-SM or mGI-LFM & NSO-LFM were seven from ten panelists (ISO 4120:2004).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Identification of *Nigella Sativa* seed

Nigella Sativa seed was identified for confirmation of the species and the source of origin by Transgenic Laboratory of Plant Transgenic Technology and Biosensor, Department of Botany, Faculty of Science. The morphology of the *N. sativa* seed was inspected under microscope. Then, the *N. sativa* seed was compared with the morphology of the varieties from Egypt, Oman, India, Pakistan, Turkey, Tunisia, and Syria. The seed size measurement was done by measuring the maximum seed length and the maximum seed width. When compare the relation between the seed length divided by a half of seed width, the result of the test sample was 3.875 mm. The seed morphology studied by the previous studied (Sawarkar et al., 2016) revealed the seed size measurement of each varieties obtained from Pakistan, Egypt, Saudi Arabia, Oman, Turkey, India, Tunisia and Syria varieties were 2.6, 2.692, 2.904, 3.0, 3.473, 3.813, 4.143, and 4.21, respectively (Figure 16). Based on this measurement it indicated that the *N. sativa* seed morphology and size of the test sample was similar to the Indian variety.

Figure 16. The morphological of *N. sativa* seed from different varieties and the test sample

Comparing the result obtained based on riburose, 1, 5 biphosphate carboxylase/oxygenase gene using A and A1 primers with the nucleotide sequence from Genbank, it showed that this nucleotide sequence using A primer (Figure 17) had similarity to nucleotide sequence of *N. sativa* from South Asia countries including Iran, India, Pakistan with the score 1066 and 99.66% identity (Table 9). This score was higher than the previous report (Schori & Showalter, 2011). It is also similar to *Nigella arvensis* from England (KM360895.1 UK Flora DNA Bank Project). The nucleotide sequence using A1 primer (Figure 18) also gave the similarity that similar to the nucleotide sequence of *N. sativa* from South Asia countries including Iran, India, Pakistan with the score 1055 and 99.69% identity (Table 9).

Comparing the result obtained based on ITS gene with the nucleotide sequence from Genbank using Basic Local Alignment Search Tool for Nucleotide (Blastn), it showed that this nucleotide sequence using ITS primer (Figure 19) had similarity to nucleotide sequence of *N. sativa* from South Asia countries including Iran Pakistan India (eg. KY421056, KY421011) with the score 98.4% and 99.2% of identity (Table 11). Based on the result obtained from both seed size measurement and DNA sequencing it could be confirmed that the seed sample can be derived from India varieties.

TAGGGCGATTGGAGCTCCCGCGGTGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGCCCAAT
GTGGAATTCGCCCTTGTAAAATCAAGTCCACCGCGGAGACATTCATAAACCGCTCTACCATAGTTCTT
AGCAGATAATCCCAATTTTGGTTTAATAGTACATCCCAATAGGGGACGACCATACTTGTTCAATTTAT
CTCTCTCAACTTGGATGCCATGAGGCGGGCCTTGGAAGGTTTTAACATAAGCAACAGGGATTCTCAGA
TCCTCTAGACGTAGAGCGCGCAGCGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACATGTT
AGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTAGGCTACATAACAAATATATTGATTTTCTTCTC
CAGCAACGGGCTCGATGTGGTAGCATCGGCCTTTGTAACGATCAAGGCTGGTAAGTCCATCGGTCCAC
ACGGTTGTCCACGTACCTGTAGAAGATTCGGCAGCTACAGCAGCCCCTGCTTCTTCAGGCGGAACTCC
AGGTTGAGGAGTTACTCGGAATGCCGCCAAGGTATCCGTATCTTTGGTCACATACTCAGGAGTATAAT
AATTCAATTTGTAATCTTTAACACCTGCTTTGAAGCCAACACTTGCTTTAGTCTCTGTTTGTGGTGAC
ATAAGGGCGAATTCCACAGTGGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTAC
CCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCT
GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGGAGCATAAAGTGTAAAGCCTG
GGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA
ACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGC
TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA
CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAG
GCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTG
ACGAGCATCCCAAAAATCGACGCTCAGGTCAAAGGTGGCGAAACCCGANNGGACTT

Figure 17. Nucleotide sequence of *N. sativa* seed using Primer A rbcLa-F/ rbcLA-R for targeting riburose, 1,5 biphosphate carboxylase/oxygenase gene.

TAGGGCGATTGGAGCTCCCGCGGTGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGCCCAAT
GTGGAATTCGCCCTTATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGCTTCAAAGCAGGTGTTAA
AGATTACAAATTGAATTATTATACTCCTGAGTATGTGACCAAAGATACGGATACCTTGGCGGCATTCC
GAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCTGCTGTAGCTGCCGAATCTTCTACA
GGTACGTGGACAACCGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGCCGATGCTACCA
CATCGAGCCCGTTGCTGGAGAAGAAGATCAATATATTTGTTATGTAGCCTACCCTTTAGACCTTTTTG
AAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGCGCTGCGC
GCTCTACGTCTAGAGGATCTGAGAATCCCTGTTGCTTATGTTAAAACCTTCCAAGGCCCGCCTCATGG
CATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCTATTGGGATGTACTATTAAACCAA
AATTGGGATTATCTGCTAAGAACTATGGTAGAGCGGTTTATGAATGTCTCCGCGGTGGACTTGATTTT
ACCAAGGATGATGAGAACGTGAACTCCCAACCCTTTATGCGTTGGAGAGACCGTTTCAAGGGCGAATT
CCACAGTGGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTC
CCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTT
ATCCGCTCACAATTCCACACAACATACGAGCCGGGAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGA
GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA
GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCT
CGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA
ATACGGTTATCCCCANGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGG
CCAGGAACCGTAAAAAGGCCGCGTTGCTGGCCTT

Figure 18. Nucleotide sequence of *N. sativa* seed using Primer A1 rbcLa-F/ rbcLA-R for targeting riburose, 1,5 biphosphate carboxylase/oxygenase gene.

TATAGGGCGAATTGGAGCTCCCGCGGTGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGCCC
AATGTGGAATTCGCCCTTTCCTCCGCTTATTGATATGCTTAAACTCAGCGGGTAGTCCCGCCTGACCT
GGGGTCGCAGGTTGAACGCCTTGATAACGGCTTTCAGGGGTCTGTCGTCCCAACTAACGAGGTTGCCG
CGTCTGCTGTCTTTGGGGGATGACAAATACAACCACCTCTGACCGCGACACTCGCCGACGGGACCATT
ATTTGAGCCGACCGTGCCTGGAAGGGCTCGGGGGGCCAATCTCCGATTCCCACCACATAAGTGGGTGG
GAAACGACGCTGTGCGTGACGCCCAGGCAGACGTGCCCTCAGCCTAGCGGCATCGGGCGCAACTTGCG
TTCAAAAACTCGATGGTTCACGGGATTCTGCAATTCACACCAAGTATCGCATAAGGGCGAATTCCACA
GTGGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTT
AGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG
CTCACAATTCCACACAACATACGAGCCGGGAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAG
CTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGC
ATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTC
ACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACG
GTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGA
ACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAAT
CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTTTCCCTTCGG
GAAACGTGGNNCTTTTCCCAAAGCTCNNGCTGAAGGTNTCTCAGTTCGGGGTAAGGTCGTTCGCTCCA
AGCTGGGCTGNGGGCCAAAACCCCCCGTTCAGCCCGACCNNTGGCCCTTNNCGGGAACTNNCGCCTTG
NNTCCACCCGGTTAANNNCCGANTTTATCCCCCTGGGCACNCCCCCTGGTTACCGGGTTTAACCAAAC
CGGGATTTAAGGCGGGGCCCAAAAATTCTTGAAGGGGGGCCCAAACCNGCCCNNCAAAAAAAAAATTT
TGGANTCCNNNCTCGGGAAACCATTTTCTTCGAAAAAGNTTGGNNNNTTTTNNGCAAAAAAAACCCGG
GGGGGGGTTTTTTT

Figure 19. Nucleotide sequence of *N. sativa* using Primer for targeting ITS inter transcribed spacer

Table 9. Comparison of the *N. sativa* with reference using Basic Local Alignment Search Tool with the NCBI database of riburose, 1,5 biphosphate carboxylase/oxygenase gene using A rbcLa-F/ rbcLA-R

Description	Max Score	Query Cover	Per. Ident	Accession
Nigella arvensis ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast	1056	70%	99.66%	MG946921.1
Nigella sativa ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast	1056	70%	99.66%	MG946894.1
Nigella arvensis ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; plastid	1046	69%	99.83%	KM360895.1
Nigella damascena ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	995	67%	98.77%	FJ626586.1
Nigella sativa voucher A1 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	985	65%	99.82%	KU499880.1

Table 10. Comparison of *N. sativa* with reference using Basic Local Alignment Search Tool with the NCBI database of riburose, 1,5 biphosphate carboxylase/oxygenase gene using A1 rbcLa-F/ rbcLA-R

Description	Max Score	Query Cover	Per. Ident	Accession
Nigella arvensis ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast	1150	93%	99.69%	MG946921.1
Nigella sativa ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast	1150	93%	99.69%	MG946894.1
Nigella damascena voucher DXS11-015 chloroplast, complete genome	1153	95%	99.08%	MK569488.1
Nigella damascena ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1089	90%	98.87%	FJ626586.1
Nigella sativa voucher A1 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1078	87%	99.83%	KU499880.1
Nigella damascena ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1060	92%	96.36%	HM850201.1
Nigella damascena ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1048	94%	96.50%	EU053918.1

Table 11. Comparison of *N. sativa* with reference using Basic Local Alignment Search Tool with the NCBI database of ITS gene using ITS primer

Description	Max Score	Query Cover	Per. Ident	Accession
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	665	45%	99.20%	KY421091.1
Nigella sativa small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	659	45%	98.94%	KY421011.1
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	656	45%	98.67%	KY421096.1
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	652	45%	98.40%	KY421092.1
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	652	45%	98.40%	KY421056.1
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	652	45%	98.40%	KY421042.1

Description	Max Score	Query Cover	Per. Ident	Accession
Nigella sativa small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	652	45%	98.40%	KY420909.1
Nigella sativa small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	647	45%	98.14%	KY421093.1
Nigella sativa small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	643	45%	97.87%	KY421094.1
Nigella sativa small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	643	45%	98.38%	KY421090.1
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	641	45%	97.88%	KY421095.1
Nigella sativa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	635	45%	98.12%	KY420924.1

4.2 Cold press extraction of *N. sativa* seed

Solvent extraction of conventional oil is generally done for commercial purpose as it offers high yield of final oil product. However, due to the safety concern, cold press extraction of the oil becomes an alternative over solvent extraction. In additions, cold pressed is an extraction technique which no heat or solvent involved therefore the oils derived from cold press extraction provide good source of lipophilic phytochemicals essential fatty acids. Cold press and solvent extraction of *N. sativa* seed oil have been well studied (Atta, 2003; Cheikh-Rouhou et al., 2007; Gharby et al., 2015; Kiralan et al., 2014; Lutterodt et al., 2010; Sultan et al., 2009). From the studied by Gharby et al. (2015), cold press extracted *Nigella* seed oil exhibits lower peroxide value (3.4 $MeqO_2$/kg) compared to solvent extracted oil (11.4 $MeqO_2$/kg). Similarly, the free fatty acid content of cold pressed oil in their study was shown to be lower than solvent extracted oil. Consequently, the cold press extracted black cumin seed oil is not only considered as safe but also proved to have a better quality over the solvent extracted black cumin seed oil. The yields obtained from cold press extraction of *N. sativa* seed oil in our study were 27.38 % (w/w). The result was in accordance with the previous studied (Gharby et al., 2015) which the oil content of *N. sativa* seed oil was 27% when using the cold press extraction. This cold pressed oil was further used in this study for developing microcapsule rich in lipophilic phytochemicals promoting health benefit.

4.3 Thymoquinone in the NSO

Thymoquinone is one of quinone compound found in many medicinal plant species including the plants in the families of Ranunculaceae, Lamiaceae, and Cupressaceae (Darakhshan et al., 2015). It is a major phytochemical compound found in NSO and thus the pharmaceutical properties of *N. sativa* are mainly attributed to this thymoquinone. In this study, the identification of the thymoquinone in NSO was relied on retention times and comparison of UV spectra with thymoquinone standard (Figure 20). The cold press NSO sample and thymoquinone standard were detected at 254 nm with similar retention time (Figure 21). The runtime required for thymoquinone analysis was less than 12

min with the retention time of 5.4. The HPLC chromatogram indicated well resolved peak with no interference.

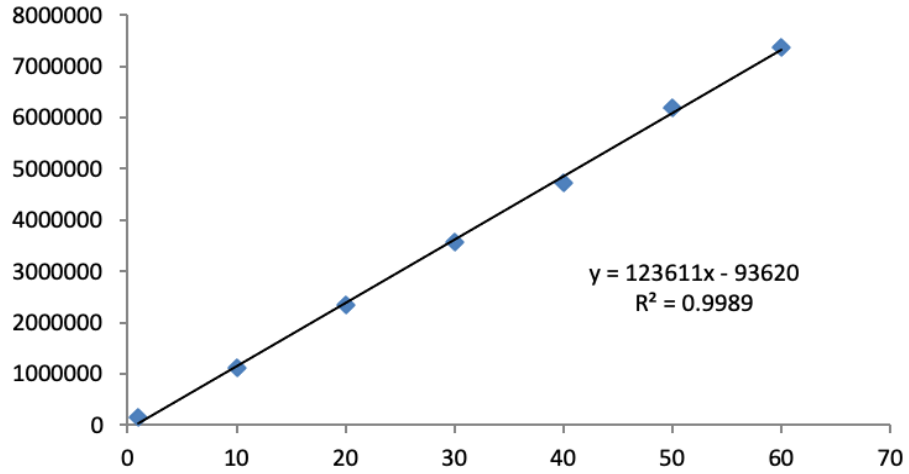


Figure 20. A standard curve of thymoquinone at concentrations 1, 10, 20, 30, 40, 50, and 60 mg/mL

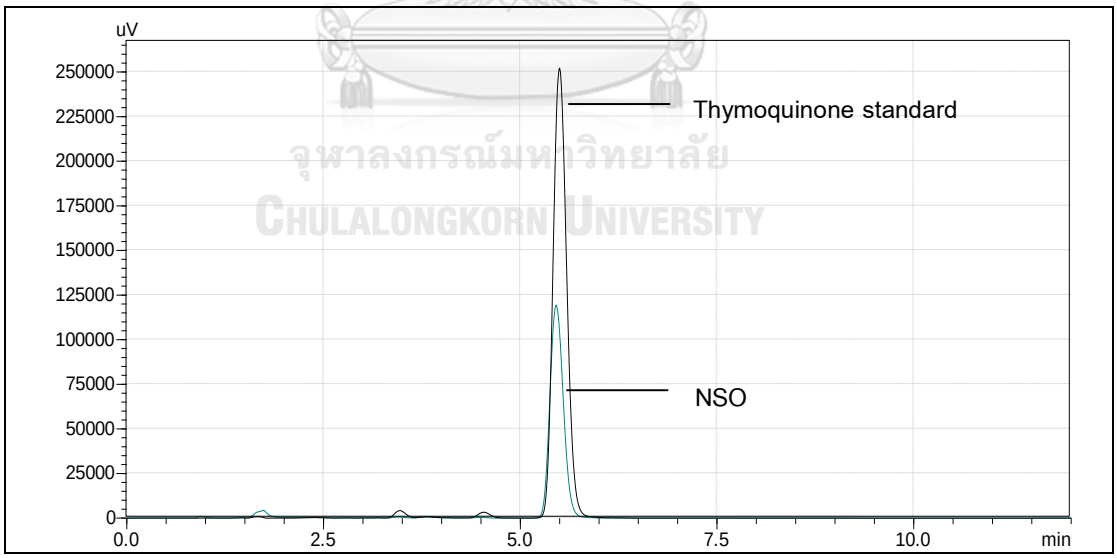


Figure 21. A HPLC chromatogram of thymoquinone in NSO compared with the thymoquinone standard with the retention time 5.4 detected at 254 nm

The thymoquinone content of NSO was calculated from the linear equation of thymoquinone standard curve. From the chromatogram obtained, the thymoquinone contents in cold pressed NSO were evaluated to be 4.4 mg/mL. When it was calculated by dilution factor the thymoquinone content was 2.2 mg g^{-1} in cold press black cumin seed oil. The previous study (Lutterodt et al., 2010) reported that the thymoquinone content of cold-pressed NSO was range from 3.48 - 8.73 mg g^{-1} while the study by Gosheh *et al.* (1999) revealed the thymoquinone content to be as lower as 0.526 mg g^{-1}. The source of the seed cultivation may influence the variation of active compounds (Gharby *et al*., 2015). *N. sativa* from India tended to be a p-cymene/thymoquinone chemotype. The seed derived from Turkey or Egypt was found to be thymoquinone chemotype and hence it exhibited high content of thymoquinone compound compared to other chemotypes (Piras et al., 2013).

4.4 Fatty acid compositions in the NSO

Individual percentages of the fatty acid composition determined in cold press NSO was given in Table 12 and Figure 22. In cold-pressed NSO fatty acid compositions (un-encapsulated NSO), linoleic acid (C18:2 n-6) was a major unsaturated fatty acid whereas palmitic acid (C16:0) was a major saturated fatty acid found in NSO. In this study, linoleic acid was accounted for 58.45%, oleic acid was 24.07% and palmitic was 13.01%. The NSO fatty acid compositions reported by other studies indicated that linoleic acid was range from 49.1 - 57.5%, oleic acid (C18:1 n-9) was 23.7 - 25.0% while palmitic acid (C16:0) was 9.9 - 18.4% (Cheikh-Rouhou et al., 2007; Gharby et al., 2015; Kiralan et al., 2014; Ramadan & Wahdan, 2012). The result obtained from this study was correlated to the cold-pressed Moroccan NSO which linoleic acid, oleic acid, and palmitic acid contents were 58.5%, 23.8%, and 13.1%, respectively (Gharby et al., 2015).

4.5 Phytochemical compositions in the NSO

A total of 32 phytochemical compounds were identified in cold press NSO (Table 13). Thymoquinone was the main components identified (22.89%). This

result was correlated with Burits and Bucar (2000) which indicated that the major bioactive components in NSO were thymoquinone (30 - 48%) followed by p-cymene (7 - 15%), carvacrol (6 – 12%), 4-terpineol (2 – 7%), t-anethole (1 –4%). The identification of NSO from a microwave assistant extraction (MAE) technique has also been reported (Liu *et al.*; 2013) with the thymoquinone was occupied for 38.23%, p-cymene for 28.61%, 4-isopropyl-9-methoxy-1-methyl-1-cyclohexene for 5.74%, longifolene for 5.33%, alpha-thujane for 3.88% and carvacol for 2.31%. However, p-cymene, carvacrol, t-anethole were not found in our study. On the other hand, the result obtained by other studies (Harzallah et al., 2011; Singh et al., 2005; Wajs et al., 2008) also reported the major bioactive components to be p-cymene which accounted for 36.20 - 60.2%. The different in phytochemical contents could be due to many factors, such as different methods of extraction, different origins of the seed, the cultivation and environmental factors. The GC-MS result obtained from our study clearly showed that the cold press NSO was a good source of many bioactive phytochemicals, especially thymoquinone playing an important role in pharmaceutical activities for wide varieties of diseases.

4.6 *In vitro* antimicrobial activity of the NSO

The antimicrobial activities of *N. sativa* essential oil are mainly attributed to the phenolic compounds present in the oil. Many studies showed that *N. sativa* extracts and oil had antimicrobial activities against various types of bacterial strains including *Acinetobacter baumanni*, *A. junii*, *Bacillus subtilis*, *Escherichia coli*, *Enterobacter faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *P. mirabilis*, and *Staphylococcus aureus* (Ishtiaq *et al*., 2013; Manju *et al.*, 2016; Nair *et al*., 2005; Piras *et al*., 2013). The results obtained by the disc diffusion method demonstrated that the methanolic cold press NSO provided an inhibition zone against *S. aureus* ATCC 25923, pathogenic bacterium (17.67±1.58 mm)(Table 14). For the minimal inhibition concentration (MIC) study, according to the working concentration (400 mg/mL) used in this study, the MIC of NSO against *S. aureus* was equal to 1 mg/mL (Table 15). The result was in agreement with the previous study by which the methanolic NSO was effective against *S. aureus* (MIC = 25 mg/mL, inhibition zone = 9.3 mm). Moreover, the MIC value of NSO extract against

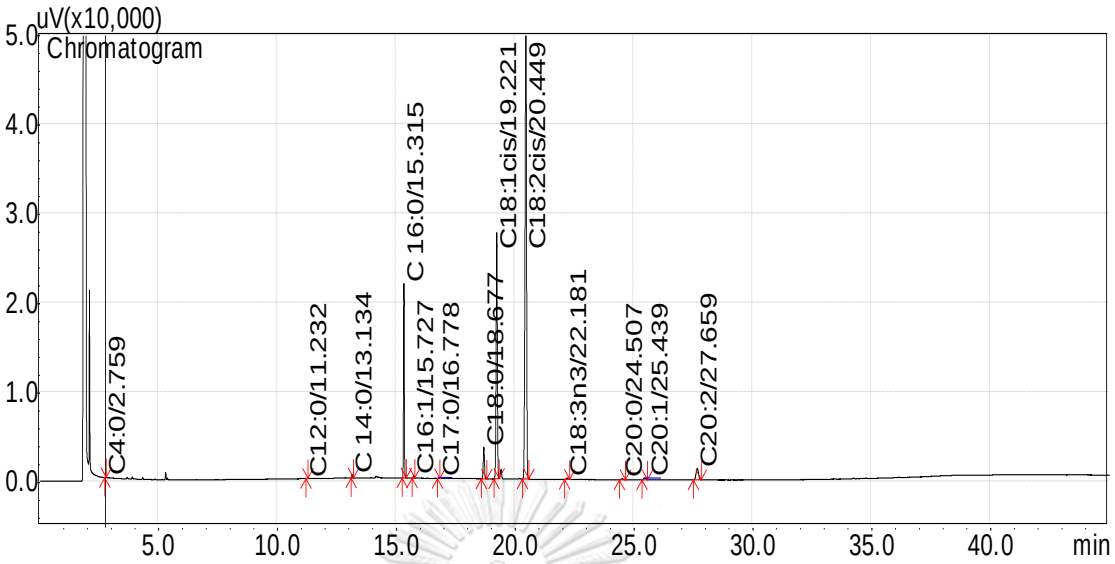


Figure 22. The GC-FID chromatogram of fatty acid compositions found in NSO

Table 12. The fatty acid compositions in cold press *Nigella Sativa* seed oil

Fatty Acids	**%** Peak Area
Lauric acid (C12:0)	0.06 ± 0.01
Myristic acid (C14:0)	0.21 ± 0.01
Palmitic acid (C16:0)	13.01 ± 0.11
Palmitoleic acid (C16:1 n-7)	0.22 ± 0.01
Stearic acid (C18:0)	3.26 ± 0.04
Oleic acid (C18:1 n-9)	24.07 ± 0.50
Linoleic acid (C18:2 n-6)	58.45 ± 0.58
Linolenic acid (C18:3 n-3)	0.23 ± 0.05
Arachidic acid (C20:0)	0.20 ± 0.01
Eicosenoic acid (C20:1 n-9)	0.36 ± 0.01
Polyunsaturated fatty acids	58.68
Monounsaturated fatty acids	24.65
Saturated fatty acids	16.74

Table 13.The phytochemical compositions in Nigella Sativa seed oil

	Compounds	% Peak Area	Identification
1.	alpha-phelladrene	9.53 ± 1.68	RI, MS
2.	(1R)-2,6,6-Trimehylbicyclo[3,1,1]hept-2-ene	2.08 ± 0.17	RI, MS
3.	beta-phellandrene	0.82 ± 0.11	RI, MS
4.	Bicyclo [3,1,1]heptane,6,6-dimethyl-2-methylene	1.63 ± 0.41	RI, MS
5.	Z-2-hydroxymethylcyclopentanol	0.46 ± 0.09	RI, MS
6.	D-Limonene	1.21 ± 0.14	RI, MS
7.	gamma-terpinene	4.52 ± 0.03	RI, MS
8.	D-(-)-Lactic acid	0.63 ± 0.19	RI, MS
9.	cis-4-methoxy thujane	0.63 ± 0.13	RI, MS
10.	Propanedionic acid	0.90 ± 0.28	RI, MS
11.	3,6,9- trioxa-2-silaundecane,2,2-dimethyl	0.40 ± 0.19	RI, MS
12.	Terpinen-4-ol	0.42 ± 0.04	RI, MS
13.	alpha-pinene	0.05 ± 0.08	RI, MS
14.	2-methyl-4-(2,6,6-trimethylcyclohex-2-enyl)but-3-en-2-ol	0.26 ± 0.04	RI, MS
15.	Thymoquinone	22.89 ± 3.58	RI, MS
16.	Silanamine	0.86 ± 0.19	RI, MS
17.	Linalool	0.56 ± 0.04	RI, MS
18.	Glycerol	1.16 ± 0.16	RI, MS
19.	Benzenepropanoic acid	0.25 ± 0.04	RI, MS
20.	Silane	0.43 ± 0.02	RI, MS
21.	5-Isopropyl-2methylphenoxy	7.94 ± 0.07	RI, MS
22.	Tricyclo[5.4.0.0(2,8)]undec-9-ene,2,6,9,9-tetramethyl	0.88 ± 0.05	RI, MS
23.	Longifolene	3.94 ± 0.03	RI, MS
24.	3,5-di-tert-butyl-4-hydroxyanisole	0.90 ± 0.18	RI, MS
25.	4-Methoxy-2,5-dimethyl-phenoxy	0.28 ± 0.03	RI, MS
26.	tert-butylhydroquinone	14.80 ± 1.15	RI, MS
27.	1-pentene	0.61 ± 0.23	RI, MS
28.	alpha-acorenol	0.20 ± 0.01	RI, MS
29.	1-naphthalenepropanol, alpha-ethenyldecahydro-alpha,5,5,8a-tetamethyl-2-methylylene	0.79 ± 0.12	RI, MS
30.	1H-cycloprop[e]azulane, decahydro-1,1,4,7-tetramethyl	0.98 ± 0.19	RI, MS
31.	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	9.84 ± 1.97	RI, MS
32.	beta-sitosterol	9.17 ± 3.09	RI, MS
	Total	**100.00**	

The compound has been identified by comparing mass spectra (MS), and retention indices, data from NIST (National institute of Standard and Technology), Wiley commercial library, chemistry web book (http://www.nist.org/chemistrywebbook)

S. aureus in this study showed more antimicrobial activity than that from previous study (Ishtiaq *et al*., 2013). However, there were no inhibition zone and MIC observed for *S. enterica* ATCC 14028 (pathogen), *E. coli* ATCC 25922 (coliform), and *L. rhamnosus* ATCC 53103 (prebiotic). In contrast to this study, it was demonstrated that *E. coli* and *E. faecalis, considered as* coliforms, were more sensitive to methanolic NSO extract (MIC = 5 mg/mL) than *S. aureus* (MIC = 25 mg/mL). This phenomenal might be due to the different among of active phenolic compounds presented in NSO.

Table 14. Antimicrobial activities of NSO against four bacteria species

Test samples	Inhibition zone (mm)*			
	S. aureus	*S. enterica*	*E. coli*	*L. rhamnosus*
NSO (400 mg/ml)	17.67±1.58	ND	ND	ND
Cefritaxone (100 µg/mL)	14.11±0.78	18.72±0.44	16.44±0.46	ND
Methanol	ND	ND	ND	ND

*Mean± SD, ND = not detected

Table 15. Minimum inhibitory concentrations of NSO against four bacteria species

Test samples	**MIC (mg/mL)**			
	S. aureus	*S. enterica*	*E. coli*	*L. rhamnosus*
NSO	1 mg/mL	ND	ND	ND

ND = not detected

4.7 Microencapsulation by spray drying

All formulations were prepared in small batch for preliminary study. The gum arabic was either formulated alone or combined with other wall materials (Table 16). According to the result, most of the formulations were semisolid when dissolved in water, they could not be further used for emulsion. Only four formulations, GA, GM, GI and GW could be further started as feed emulsions for the experiment.

Table 16. The appearance of emulsions prepared from different formulas of wall materials

Formulas	Ratio (g:g)	Physical Form
GA	20 g : 0 g	Liquid
GG	10 g : 10 g	Semisolid
GC	10 g : 10 g	Semisolid
GP	10 g : 10 g	Semisolid
GK	10 g : 10 g	Semisolid
GI	10 g : 10 g	Liquid
GH	10 g : 10 g	Semisolid
GM	10 g : 10 g	Liquid
GW	10 g : 10 g	Liquid

Four formulations of emulsions were successfully transformed to the powder form. According to the results, all the powder was white in color except of GW formulation which was cream in color. This phenomenon might be the color of whey protein which gives a color attribute to the final product. In this study, the percentage of total solids yield for mGA, mGM, mGI and mGW were 70.25%, 73%, 55.15% and 26.56% respectively. The product yield higher than 50% is considered a successful spray drying process. Several factors are involved in the product yield, such as the wall material selection, the core-wall materials ratio and the drying conditions (Bhandari et al., 1997).

4.8 Microencapsulation efficiency and characteristics of NSO and mNSOs

The microencapsulation efficiency values range from 75.31% to 88.22% and significantly different among the microcapsules (Table 17). The lowest value was obtained from the GW formulation while the highest value was obtained from the GA formulation. Replacing gum arabic with inulin or maltodextrin by 50% significantly reduced the ME by 2 – 5%, (mGI & mGA = 86.12% & 75.31%, mGM & mGA = 83.51% & 75.31%). The differences in percentages of microencapsulation efficiency were correlated to the different types of wall materials used in preparing emulsions for spray drying. Each wall material had different properties of retention and film-forming. Based on the result obtained from previous studied,

the microcapsule prepared by a combination of maltodextrin and whey protein for flaxseed oil encapsulation provided the poor microencapsulation efficiency (Carneiro et al., 2013; Charve & Reineccius, 2009). Similarly, the microcapsule prepared by whey protein combined with gum arabic has lower microencapsulation efficiency compare to the microcapsule powder prepared with modified starch for encapsulation of flavor and flaxseed oil(Charve & Reineccius, 2009).

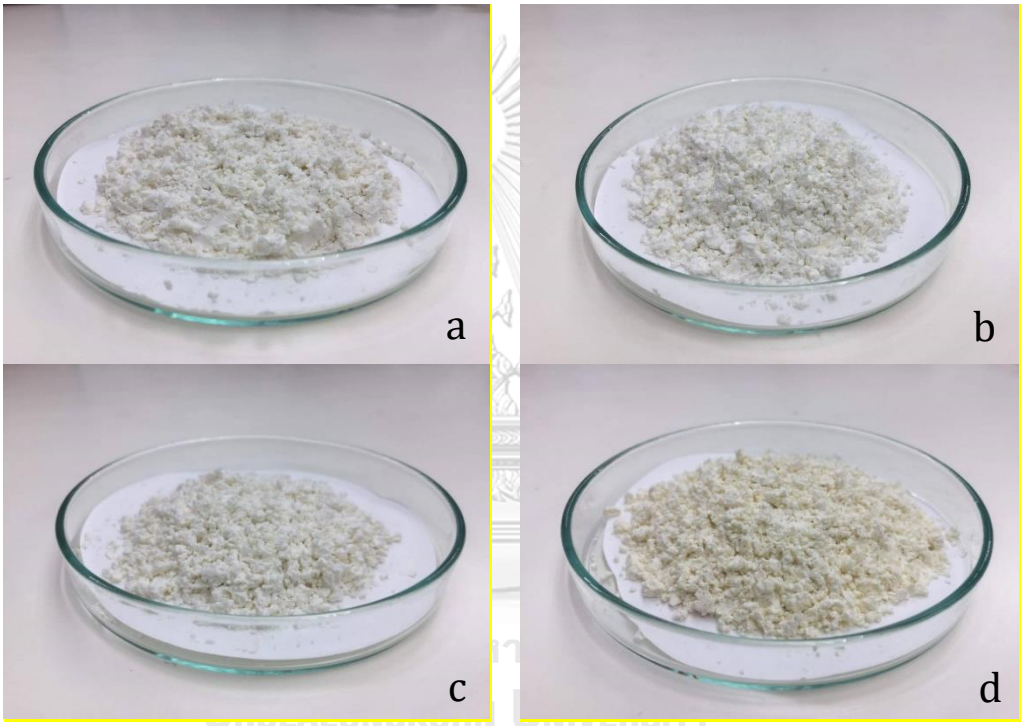


Figure 23. The powder form of four types of microcapsule a) gum arabic b) gum arabic + maltodextrin c) gum arabic + inulin d) GW = gum arabic + whey protein

Table 17. Physical properties of microcapsules containing *Nigella sativa* seed oil

Parameters	Formulas			
	GA	GI	GM	GW
Water activity (a_w)	0.21 ± 0.12^{c}	0.13 ± 0.01^{b}	0.14 ± 0.01^{b}	0.10 ± 0.00^{a}
Wettability (min)	52.88 ± 1.31^{d}	10.52 ± 0.64^{a}	25.65 ± 0.23^{c}	14.37 ± 0.15^{b}
Solubility (%)	79.14 ± 7.83^{a}	87.38 ± 3.42^{b}	88.71 ± 5.80^{b}	93.16 ± 2.23^{b}
ME (%)	88.22 ± 3.39^{c}	86.12 ± 4.96^{bc}	83.51 ± 3.16^{b}	75.31 ± 1.51^{a}
Thymoquinone (mg/mL)	2.18 ± 11.74^{a}	2.16 ± 14.25^{a}	2.49 ± 28.91^{b}	2.28 ± 6.66^{a}

GA = Gum arabic, GI = Gum Arabic + Inulin, GM = Gum arabic + Maltodextrin, GW = Gum Arabic + Whey Protein. Mean ± standard deviation. Values followed by the same letter did not differ statistically according to the Tukey test at 5% ($P \leq 0.05$).

The FTIR spectra of cold press NSO and mNSOs with or without oil were displayed in Figure 24 and 25. This method is shown to be one of the easiest screening methods to check the encapsulation status of microcapsule (). In order to ensure the encapsulation status of the oil, the surface oil should be extracted by hexane prior to FTIR analysis since the presence of oil inside or outside of microcapsule could not distinguish by FTIR analysis. The result obtained by FTIR provided a clear distinguish peak at 1750 cm^{-1} of pure NSO, GA and GM powder from powder without NSO. This peak represented the region of double bond stretching functional group. Other outstanding peaks are peak at 2924 and 2823 cm^{-1} of NSO which represented the region of hydrogen bond stretching of functional groups. This indicated that the core material was incorporated to microcapsules in all formulation.

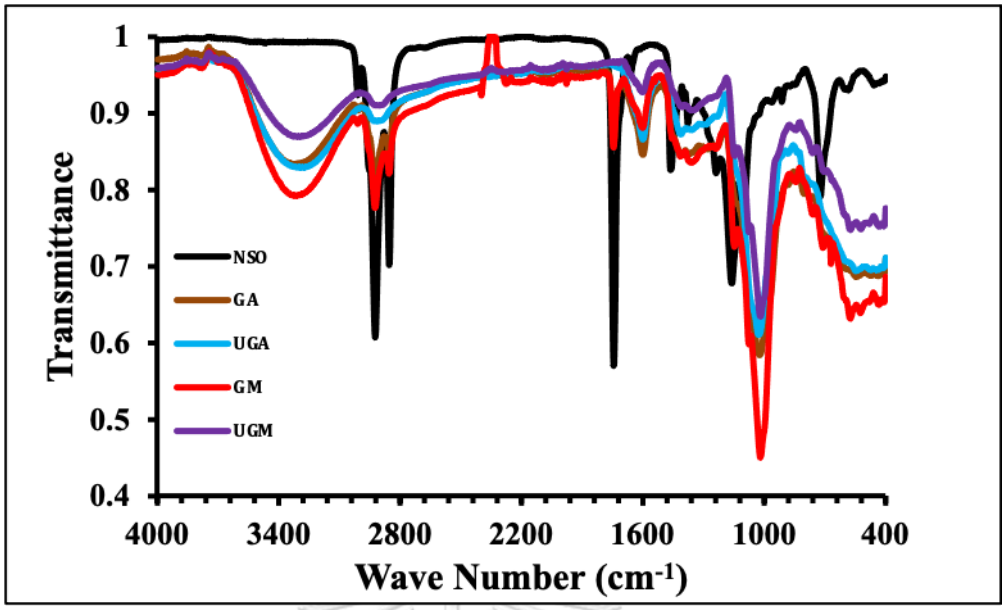


Figure 24. FTIR spectra of pure NSO and microcapsules prepared with 100/0 ratio of GA and 50/50 of GA: MD

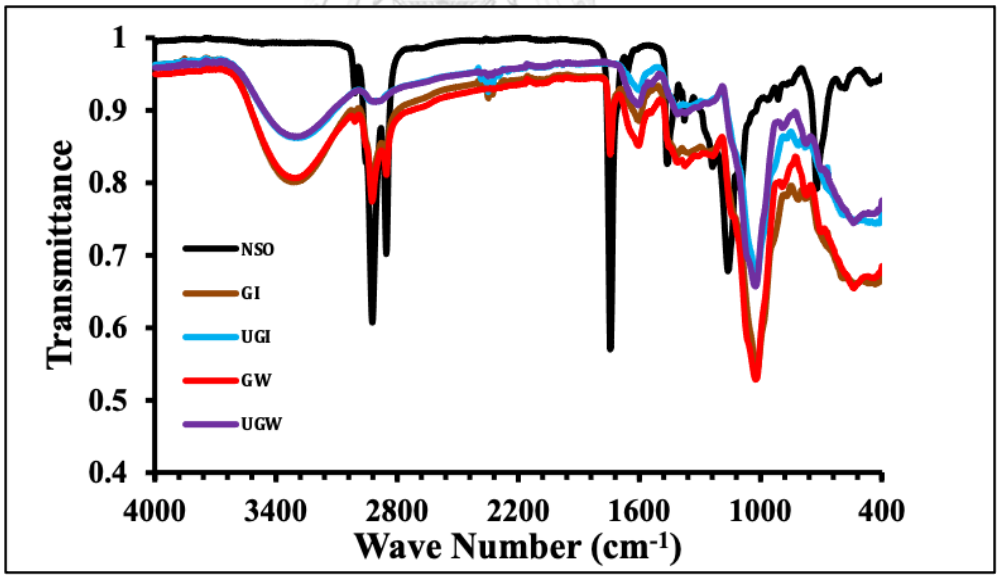


Figure 25. FTIR spectra of pure NSO and microcapsules prepared with 50/50 ratio of GA: IN and GA: WP

4.9 Characterization of microcapsules

4.9.1 Moisture content and water activity

The moisture content is one of the important parameters for evaluating a good quality of microcapsule powder. In general, a typical moisture content of spray-dried microcapsule powder is less than 5% (Tontul & Topuz, 2017). This is the major advantage of spray dried product for food industry since it is considered to be microbiologically safe and ensured the stability of the product. In this study, the moisture contents of all microcapsules were observed to be less than 5%. The moisture contents of microcapsules with GA, GM, GI, and GW formulations were 3.62 %, 1.60 %, 2.29 %, and 1.60 %, respectively. These low moisture content of the product also correlated to the inlet temperature used in this study (170°C). The high inlet temperature accelerated the moisture evaporation rate and lowered moisture content of the finish products(Aghbashlo et al., 2013).

Water activity is another parameter which also needed to concern for microcapsule products. It allows examining the free water availability which causes food deterioration and microbial contamination(Silva et al., 2020). The shelf life and storage time of food products also depend on the water activity. The use of different wall materials had significant influence on water activity ($P \leq 0.05$). The highest water activity was obtained from GA while the combinations of GA with other type of wall materials tend to significantly reduce the water activity of the products (Table 17). However, the water activities of all microcapsules were considered to be appropriate since the values were among acceptable level recommended in food industry and food regulation. This is very important since a high water activity value ($a_w > 0.5$) considered to be a risk of microbial spoilage and chemical reactions (Silva et al., 2020). The results obtained from both moisture content and water activity indicated that all microcapsules are suitable to be applied in food industry.

4.9.2 Wettability

In this study, the time taken for microcapsules to sediment, sink, and disappear from the water's surface was used as a measure of the degree of wettability (Table 17). There were many factors affect the wettability of

microcapsule including surface charge, surface area, porosity, density, particle size as well as the presence of amphipathic substance. The wetting times taken for the microcapsules' powder of GA, GM, GW, and GI were 52.88, 25.65, 14.37, and 10.52 respectively. There was a significance difference ($P \leq 0.05$) of wettability in all microcapsules. The wettability was reduced remarkably to 10.12 for the GI powder which indicated that the inulin could improve the hydrophilic character of microcapsule. The less time was taken to get wet, the greater the wettability of the powder (Edris et al., 2016).

4.9.3 Solubility

In general, the *Nigella Sativa* seed oil was poorly dissolved in water at room temperature. On the contrary, in this study the microencapsulated *Nigella Sativa* seed oil could be easily dissolved in the water. The solubility of each microcapsule was shown (Table 17) by which there was no difference in solubility observed from GW (93.16%), GM (88.71%), and GI (87.38%) ($P > 0.05$). The solubility value of GA was significantly lower than the other microcapsules ($P \leq 0.05$). This result was clearly shown that the solubility of microcapsule was improved when combining the GA with other types of wall materials.

4.9.4 Color

Color analysis of the microcapsule is necessary for its market purpose. The four microcapsules presented color parameter L*(Lightness), a* (red/green), and b* (yellow/blue) value using a Hunter lab colorimeter. The color of microcapsules presented by this study were significant different especially in term of L* and b*(Table 18). The difference between each microcapsule was clearly correlated to the type of wall materials used for spray drying since different combination of wall materials were applied in this study.

Table 18. Color of microcapsules containing *Nigella sativa* seed oil from spray drying process

Formulas	Color values		
	L*	a*	b*
mGA	91.95 ± 0.02[a]	-0.93 ± 0.00[a]	3.29 ± 0.02[c]
mGM	93.44 ± 0.02[b]	-1.41 ± 0.41[bc]	2.47 ± 0.01[a]
mGI	94.03 ± 0.07[d]	-1.56 ± 0.01[ab]	2.52 ± 0.00[b]
mGW	93.70 ± 0.03[c]	-2.02 ± 0.02[a]	5.43 ± 0.01[d]

Mean ± standard deviation. Values followed by the same letter did not differ statistically according to the Tukey test at 5% (P ≤0.05). GA = Gum arabic, GM = Gum arabic + Maltodextrin, GI = Gum Arabic + Inulin, GW = Gum Arabic + Whey Protein

4.10 Thymoquinone in the mNSO products after microencapsulation

Thymoquinone content in NSO had been evaluated by many studies including this study. However, only few studies reported on the thymoquinone content from a microencapsulation process. In this study, the thymoquinone content of microcapsules containing NSO were range from 2.16 - 2.49 mg/mL. The previous studied (Mohammed et al., 2017) revealed that the thymoquinone content of microcapsule was 5.45 mg/mL. Similarly, the other study showed that the microencapsulated NSO contained the thymoquinone at concentration of 6.59 mg/mL. After conversion of thymoquinone contents in microcapsules prepared in this study to mg per g of microcapsule powder, the thymoquinone content was range from 1.1- 1.2 mg/g powder. Comparing to Abedi et al. (2016), the thymoquinone content in their study was 3.87 mg/g. The difference of thymoquinone content in microcapsules might due to many factors. One of the factors is the origin of the seed (Gharby et al. 2015). The methods used to extract the NSO and the amounts of the NSO used for microencapsulation process were also factors influenced the amount of thymoquinone content in the microcapsules. The percentages of oil concentrations from previous studies (Abdol-Samad et al., 2016; Mohammed et al., 2017) were range from 6.25 - 20%, higher than our study (4%), resulting in high amount of thymoquinone accordingly.

According to the result obtained, there was a significant difference (P ≤0.05) in thymoquinone content of GM microcapsule from other microcapsules

(Table 17). Although GM microcapsule provided the highest thymoquinone content but the previous morphology observation in this study revealed that the very dents and irregular microstructure of GM microcapsule tend to be susceptible to oxidation and thus decrease protection of active ingredients in microcapsule.

4.11 Fatty acid compositions in the mNSO

For microencapsulated NSO in four microcapsule models, linoleic acid represented the major polyunsaturated fatty acids ranging from 57.36 - 57.69%, followed by oleic acid (24.06 - 24.29%), and palmitic acid (13.43-13.75%) respectively. There was no significant difference of linoleic acid presented in all microcapsules. The microencapsulated NSO using different combinations of wall materials (maltodextrin DE10: caseinate) was also investigated by Mohammed *et al.* (2017). Microencapsulated NSO in their studies exhibited high relative percentage of linoleic acid with the value of 60%. This might due to the different source of the seed cultivation may influence the variation of fatty acid composition.

4.12 Phytochemical compositions in the mNSO

The phytochemicals in microcapsules containing NSO were identified by GC-MS. The variation in the contents of four valuable components was found in microcapsules containing NSO (Figure 26). Three important compounds including tertbutylhydroquinone, longifolene, and thymoquinone were still remained in the microcapsule while alpha-phellandrene only found in GW formulation. Unfortunaltely, the result obtained could not be compare with other studies since there was still no report on phytochemicals investigation from mNSO by GC-MS.

Table 19. The fatty acid compositions in different NSO microcapsules

Fatty acids	Relative percentage composition of fatty acid (%)			
	mGA	**mGI**	**mGM**	**mGW**
Lauric acid (C12:0)	0.07 ± 0.14^{a}	0.39 ± 0.04^{a}	0.29 ± 0.03^{a}	0.84 ± 0.04^{a}
Myristic acid (C14:0)	0.25 ± 0.05^{b}	0.24 ± 0.01^{ab}	0.23 ± 0.01^{ab}	0.39 ± 0.01^{c}
Palmitic acid (C16:0)	13.39 ± 0.10^{b}	13.43 ± 0.05^{b}	13.43 ± 0.09^{b}	13.75 ± 0.03^{c}
Palmitoleic acid (C16:1 n 7)	0.23 ± 0.0^{ab}	0.23 ± 0.01^{b}	0.23 ± 0.02^{b}	0.24 ± 0.01^{c}
Stearic acid (C18:0)	3.30 ± 0.06^{a}	3.40 ± 0.19^{a}	3.32 ± 0.11^{a}	3.32 ± 0.11^{a}
Oleic acid (C18:1 n-9)	24.29 ± 0.14^{a}	24.29 ± 0.16^{a}	24.23 ± 0.07^{a}	24.06 ± 0.11^{a}
Linoleic acid (C18:2 n-6)	57.69 ± 0.22^{b}	57.61 ± 0.33^{b}	57.76 ± 0.20^{b}	57.36 ± 0.11^{b}
Linolenic acid (C18:3 n-3)	0.22 ± 0.04^{a}	0.21 ± 0.01^{a}	0.21 ± 0.01^{a}	0.22 ± 0.01^{a}
Arachidic acid (C20:0)	0.21 ± 0.01^{a}	0.21 ± 0.01^{a}	0.20 ± 0.02^{a}	0.21 ± 0.01^{a}
Eicosenoic acid (C20:1n-9)	0.34 ± 0.01^{b}	0.35 ± 0.01^{ab}	0.35 ± 0.01^{ab}	0.35 ± 0.01^{b}
SFA	17.22	17.67	17.47	18.51
MUFA	24.64	24.87	24.86	24.65
PUFA	57.91	57.82	57.97	57.58

Mean ± standard deviation. Values followed by the same letter did not differ statistically according to the Tukey test at 5% (P ≤0.05). GA = Gum arabic, GM = Gum arabic + Maltodextrin, GI = Gum Arabic + Inulin, GW = Gum Arabic + Whey Protein, PUFA = Polyunsaturated fatty acids, MUFA = Monounsaturated fatty acids, SFA = Saturated fatty acids

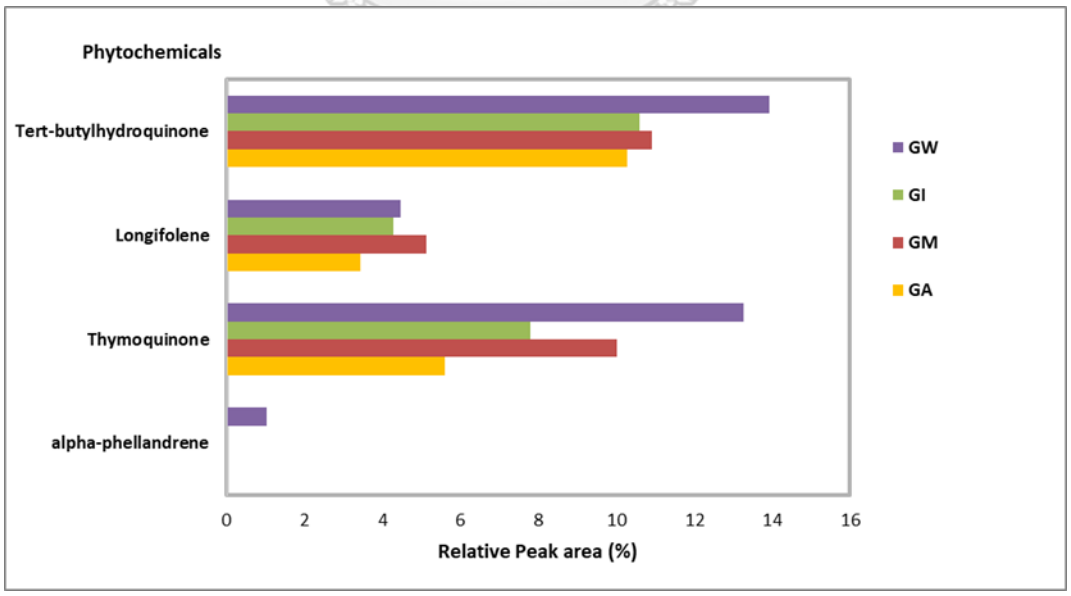


Figure 26. The percentage of main phytochemicals presented in microcapsules

4.13 *In vitro* antimicrobial activity of the mNSO products

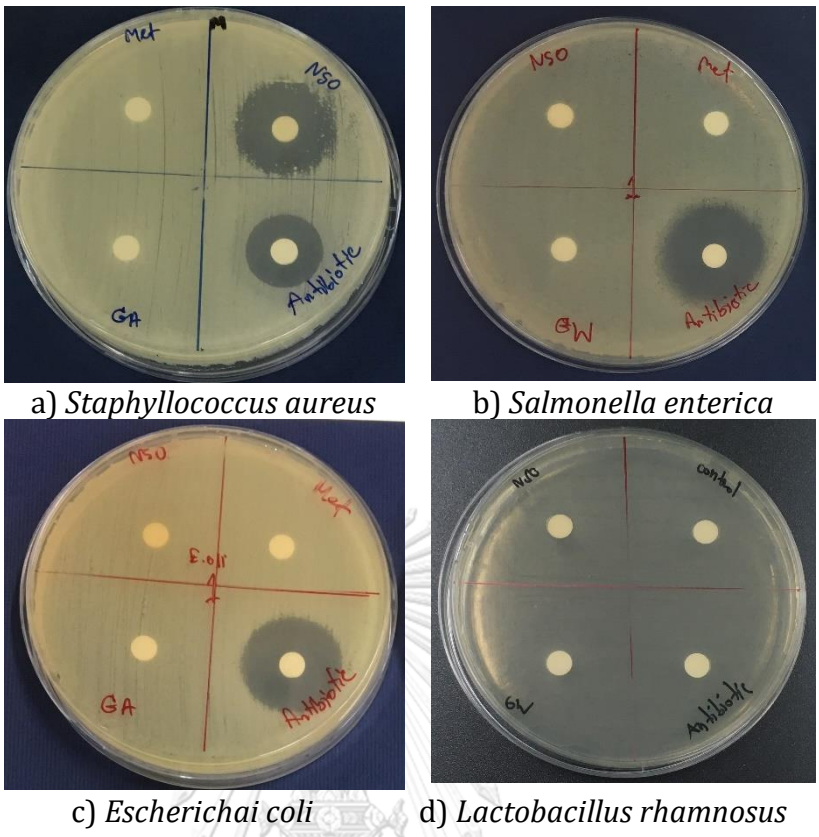
The antimicrobial of NSO after microencapsulation was studied. There was no zone of inhibition observed for all microcapsule powders (Table 20 and Figure 27). This might due to the bioactive compounds in microcapsules could not be released completely when dissolve in methanol. Thus, in order to confirm the antimicrobial activity of NSO in microcapsules, all microcapsules were extracted according to the method described in section 3.2.3. Both NSO and extracted oil from microcapsule were dissolved in DMSO. According to the result obtained (Table 21), this indicated that the antimicrobial activities of NSO in microcapsule still active comparing to the pure NSO.

For MIC study, the microcapsule powder was first dissolved in DMSO and serial dilutions of the sample with two-fold dilution. However, the result obtained (Table 22) indicated no MIC detection from the microcapsule samples. Therefore, the NSO was extracted from the microcapsule and dissolved in DMSO. The MICs of all extracted oil from microcapsules were observed. The MIC was only observed in mGI was 4 mg/mL (Table 23).

Table 20. Antimicrobial activities of mNSO powder against four bacteria species

Test samples	Inhibition zone (mm)			
	S. aureus	*S. enterica*	*E. coli*	*L. rhamnosus*
NSO (400 mg/mL)	17.67 ± 1.58	ND	ND	ND
mGA (400 mg/mL)	ND	ND	ND	ND
mGM (400 mg/mL)	ND	ND	ND	ND
mGI (400 mg/mL)	ND	ND	ND	ND
mGW (400 mg/mL)	ND	ND	ND	ND
Cefritaxone (100 µg/mL)	14.11±0.78	18.72±0.44	16.44±0.46	ND
Methanol	ND	ND	ND	ND

*Mean± SD, ND = not detected



a) *Staphyllococcus aureus* b) *Salmonella enterica*

c) *Escherichai coli* d) *Lactobacillus rhamnosus*

Figure 27. The disk diffusion of the mNSO against S. aureus, S. enterica, E. coli and L. rhamnosus.

Table 21. Antimicrobial activities of extracted mNSO against four bacteria species

Test samples	**Inhibition zone (mm)**			
	S. aureus	*S. enterica*	*E. coli*	*L. rhamnosus*
NSO (400 mg/mL)	12.33 ± 2.08	ND	ND	ND
mGA (400 mg/mL)	4.33 ± 0.57	ND	ND	ND
mGM (400 mg/mL)	3.67 ± 0.57	ND	ND	ND
mGI (400 mg/mL)	5.33 ± 1.15	ND	ND	ND
mGW (400 mg/mL)	6.33 ± 0.57	ND	ND	ND
Cefritaxone (100µg/mL)	14.11 ± 0.78	13 ± 0.81	12.33 ± 0.47	ND
DMSO	ND	ND	ND	ND

*Mean± SD, ND = not detected

Table 22. Minimum inhibitory concentrations of the NSO and mNSO against four bacteria species

Test samples	MIC (mg/mL)			
	S. aureus	*S. enterica*	*E. coli*	*L. rhamnosus*
NSO	1 mg/mL	ND	ND	ND
mGA	ND	ND	ND	ND
mGM	ND	ND	ND	ND
mGI	ND	ND	ND	ND
mGW	ND	ND	ND	ND

ND = not detected

Table 23. Minimum inhibitory concentrations of the NSO and mGI against four bacteria species

Test samples	MIC (mg/mL)			
	S. aureus	*S. enterica*	*E. coli*	*L. rhamnosus*
NSO	1 mg/mL	ND	ND	ND
mGI	4 mg/mL	ND	ND	ND

ND = not detected

4.14 Particle morphology and size distribution

The scanning electron microscope (SEM) has been one of useful analytical tools to directly evaluate a potential wall material from any particular microencapsulation process. The physical characteristics and functionality of microcapsule was correlated to its microstructure. The good quality of microcapsules could be selected successfully by combining the unique information provided by SEM with other chemical analysis (Chan et al., 2000; Rosenberg et al., 1985). The microstructures of powder produced with gum arabic or gum arabic combined with other wall materials were observed under scanning electron microscope (Figure 28). In this study, no cracks were observed for all microcapsule formulations which indicated a good covering protection of the core material by wall material and spray drying system. All of the microcapsule powders in this study were varieties in size which are one of the unique

characteristic of microcapsule derived from spray dry technology (Carneiro et al., 2013). The microstructure of microcapsules was influenced by the type of wall materials used to prepare the emulsions. This evidence was clearly seen when comparing the image of microcapsule prepared from gum arabic (Figure 28a, 28b) with the gum arabic combining with other type of wall materials (Figure 28c - 28h). The microstructure of gum arabic alone (mGA) was nearly spherical, smooth and many dented on the surface. Fernandes et al. (2014) observed the same morphology when encapsulating rosemary essential oil with GA. The microstructure for combination of gum arabic with inulin (mGI) in this study was different from their studies. The addition of inulin with gum arabic in this study was shown to improve the external surface of microcapsule with the smooth surface and spherical shape of microcapsules. The similar microstructure also obtained from GW formulation. The spherical shape and smooth surface have been suggested to give low gas permeability therefore increasing protection and retention of active ingredients within microcapsules (Abdol-Samad et al., 2016).

On the other hand, the undesirable morphology of microcapsules which very collapsed at the surface and irregular in shape was obtained from the combination of gum arabic with maltodextrin (mGM). The presence of collapsed surface of the powder revealed to give adverse effect on the flow properties of microcapsules which result in susceptibility of products to oxidation (Li et al., 2017; Rosenberg et al., 1985). From the result obtained there were a significance difference ($P< 0.05$) in particle size ($D_{4,3}$) of mGA, mGM, mGI and mGW with values 2.67, 3.39, 4.10 and 2.93 μm, respectively. The difference of particle sizes was due to the different types of wall materials prepared for microcapsules.

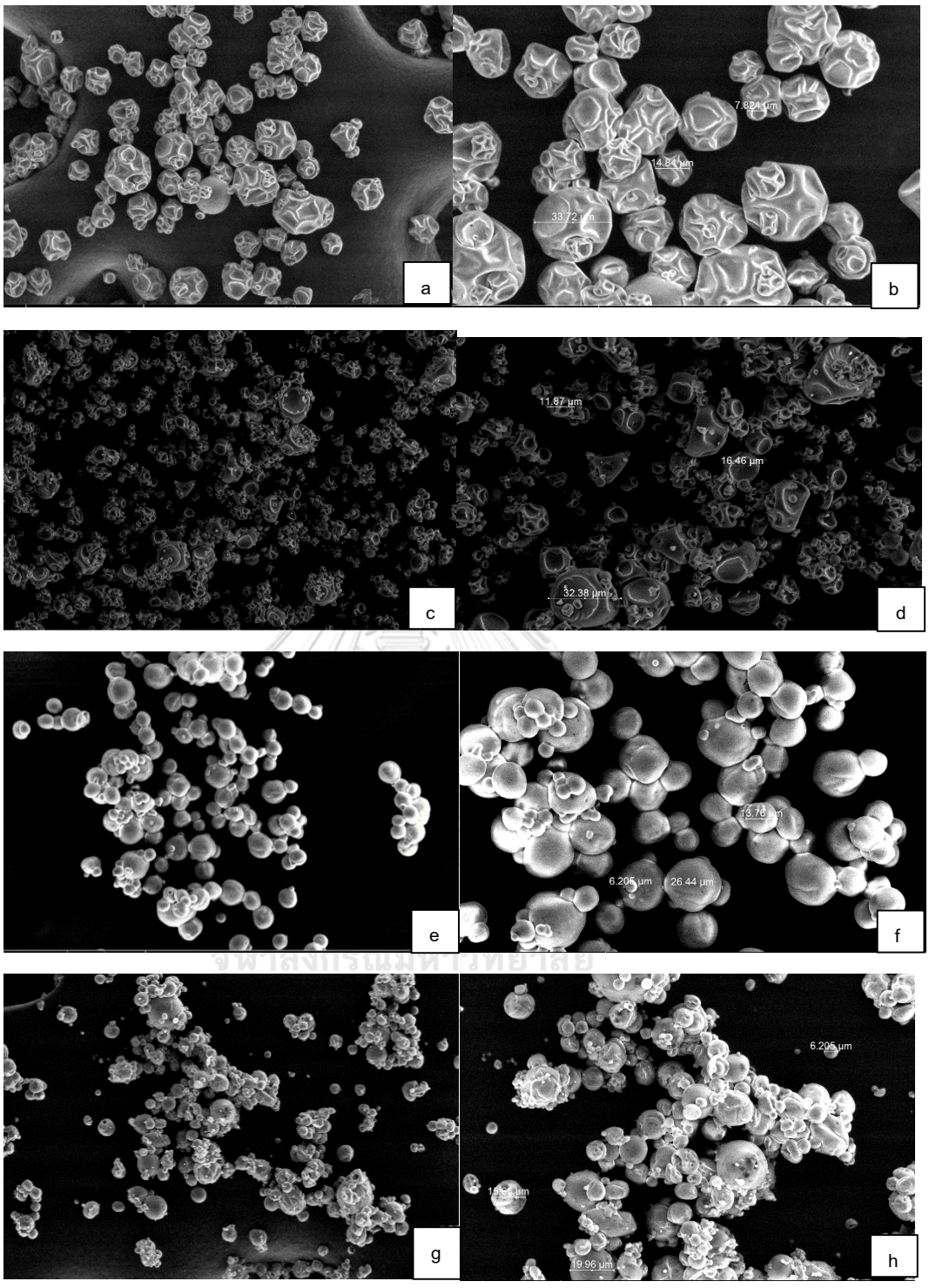


Figure 28. Microstructure of four different formulations of microcapsules with 1000x and 2000x. a) GA microcapsules at 1000x b) GA microcapsules at 2000x c) GM microcapsules at 1000x d) GM microcapsules at 2000x e) GI microcapsules at 1000x f) microcapsules at 2000x g) GW microcapsules at 1000x and h) GW microcapsules at 1000x

4.15 Antioxidant activities of NSO and mNSO by the DPPH assay

The antioxidant activities of both NSO and mNSO were investigated in this study. The powders of all microcapsules were directly diluted in methanol. According to the result, the NSO provided the percent inhibition value similar to BHT at the concentration of 62.5 mg/mL which was 15% higher than BHT (4 mg/mL) (Table 24). For microcapsule formulations, the % inhibition of mGA, mGI, mGM, and mGW at concentration of 125 mg/mL were 40.65, 71.94, 60.25, and 65.55, respectively (Table 25).

The mGI formulation provided highest % inhibition among all microcapsules. However, the microcapsule powder at the same weight as NSO gave lower value of inhibition. This might due to the bioactive compounds could not release completely when the microcapsules dissolved in methanol. There was the same evidence reported from the antimicrobial experiment by this study. It showed that the oil extracted from powder prior to dissolve to methanol provided better antimicrobial activity compared to the microcapsule directly dissolved in methanol. Thus, in order to compare the antioxidant activity of NSO with microcapsules at the equivalent concentration, the microcapsule should be extracted prior to dissolve in methanol. The antioxidant activity was correlated the amounts of phenolic compounds present in the sample. Thus, the mGI indicated the highest phenolic contents among the microcapsules.

4.16 In vitro digestion of mNSO products and cytotoxicity of the mNSO products on Caco-2 cells by MTT assay

The in vitro digestion is one of the tools to artificially digest the test sample as closely to the natural environment especially when combining with the Caco-2 cells. The Caco-2 cells are colorectal adenocarcinoma cells commonly used in many laboratories as an in vitro model for studying chemical toxicity in human intestine. It is one of alternative ways to study the intestinal physiology and in vitro toxicology test (Natoli *et al.*, 2012). The uses of Caco-2 cells were first proposed since 1989. It was isolated from human colon cancer cells known as adenocarcinoma of a 72-year-old caucasian man. Upon cell-cell interaction, Caco-2 cells can spontaneously differentiate in normal culture condition after reaching

confluency. Although it was isolated from colon tissue but after differentiation it loses its phenotype and forms dense cell monolayer and cell polarity which exhibited the morphology and function that similar to human intestinal epithelial cells (Sahu *et al*., 2016). The Caco-2 cell was first seeding with MEM containing 10 % FBS. Then, the media of Caco-2 was replaced until reach 80 % confluency (Figure 29). After counting of the viable cells, the Caco-2 cells were transferred to 96-well plate for cytotoxicity analysis.

Comparing to the other animal cells, the cytotoxicity of microcapsules containing NSO on Caco-2 cells were not well established. In this study, the cytotoxicity of NSO and microcapsule were investigated. The cytotoxicity of mGI was first investigated in order to know the IC_{50} of the microcapsule product prior to the preparation of starting concentration used in in vitro digestion. The result obtained in this study revealed that the IC_{50} of NSO on caco-2 was 13.63 μg/mL while the IC_{50} of mGI was 182.5 μg/mL (Figure 30 and Figure 31). It was reports that the cytotoxicity of NSO against human epithelial cell lines (Hep-2) using MTT was 55.2 μg/mL (Hazallah *et al.*. 2011). The Caco-2 cells were treated with digested mGI containing NSO approximately 29.76 μg/mL to check the cytotoxicity of mGI on Caco-2 cells at this concentration. The results revealed that the percent survival of the cells was as closed as the percent survival of the control media. (Figure 29 - Figure 31). This result indicated that after the microcapsule got through the *in vitro* digestion process, the microcapsule become less toxic to the cell lines. The study of NSO and microcapsule treated on Caco-2 cells were beneficial for those who interested in implement NSO and microcapsule containing NSO in food formulation.

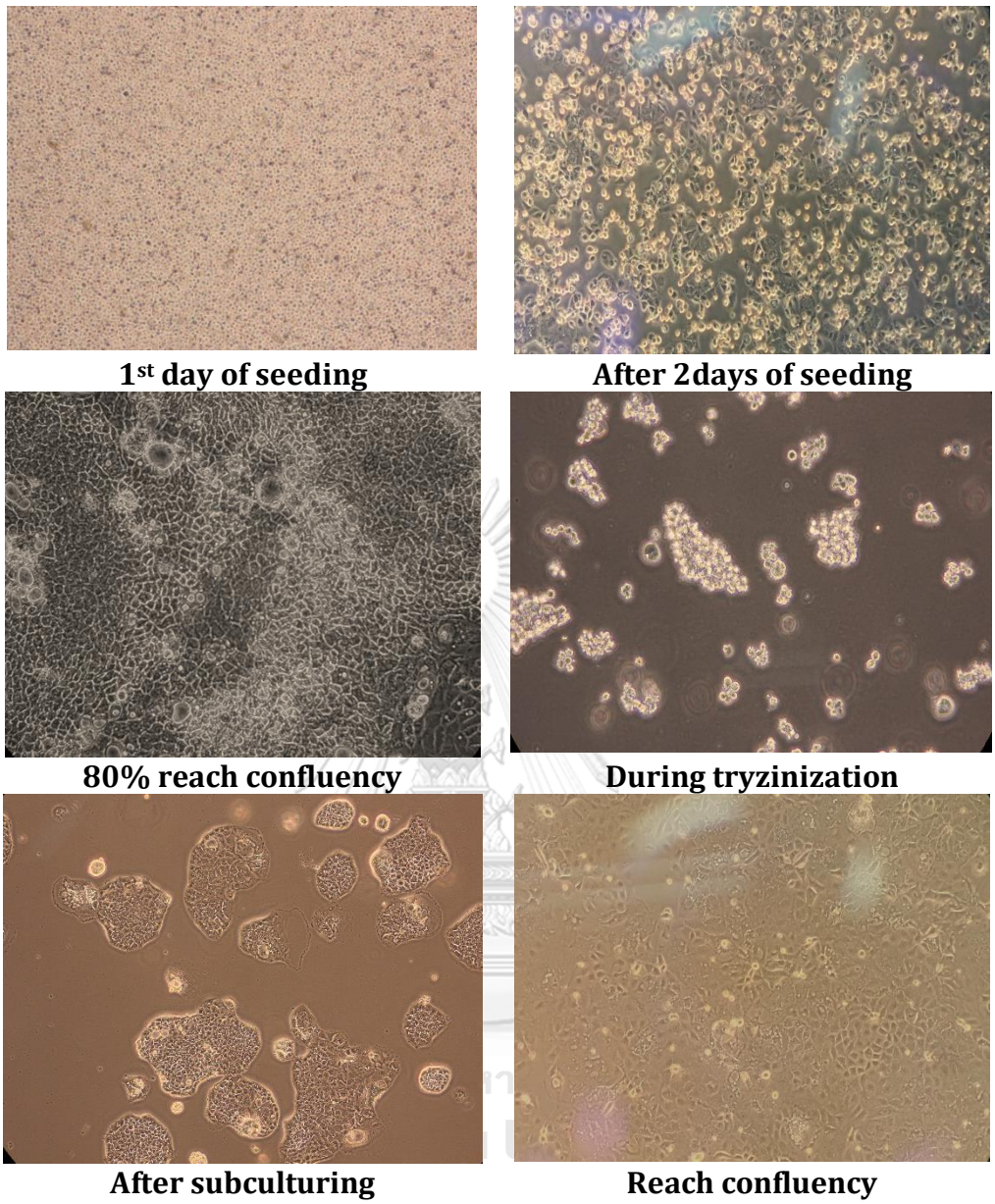


Figure 29. The sequence of culturing Caco-2 cells starting from the first day of seeding until the cells were ready to harvest

Table 24. The % inhibition of BHT, NSO and microcapsules

Test samples	% inhibition		
BHT (4.0 mg/mL)	87.75 ± 0.00		
NSO (62.5 mg/mL)	87.00 ± 0.00		
Microcapsules	Concentrations of microcapsules		
	31.25 mg/mL	62.5 mg/mL	125 mg/mL
mGA	3.83 ± 0.01	12.69 ± 0.00	40.65 ± 0.02
mGI	19.73 ± 0.01	42.05 ± 0.01	71.94 ± 0.02
mGM	12.56 ± 0.03	34.61 ± 0.09	60.25 ± 0.07
mGW	13.89 ± 0.02	31.96 ± 0.05	65.55 ± 0.08

*Mean± SD (n = 9)

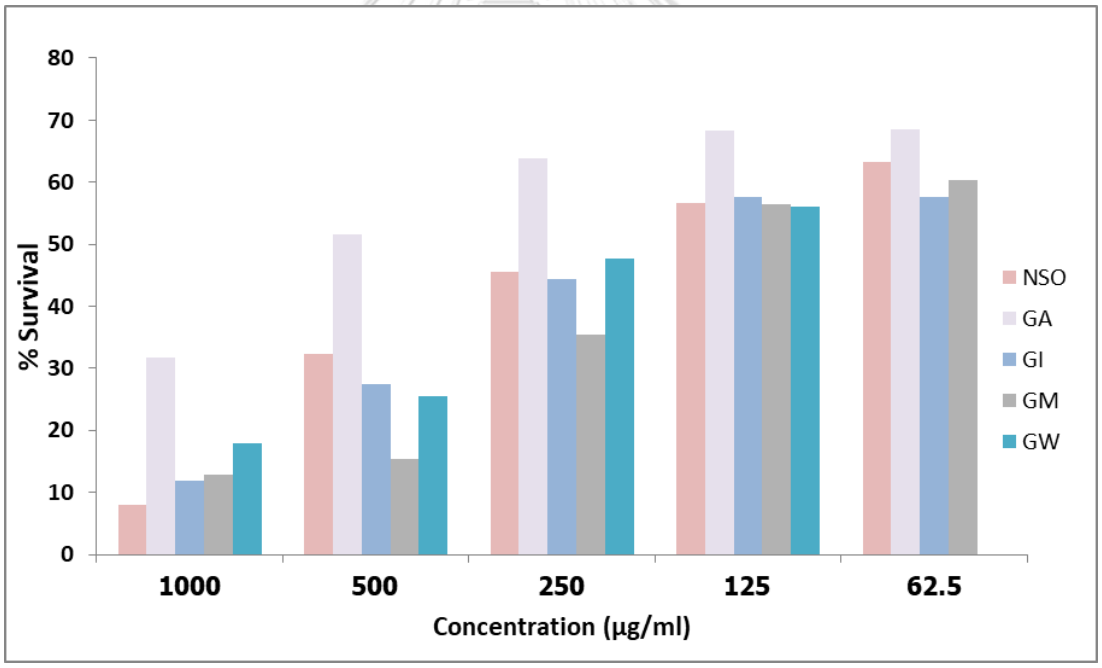


Figure 30. The cytotoxicities of NSO and microcapsules on Caco-2 cells (n=4)

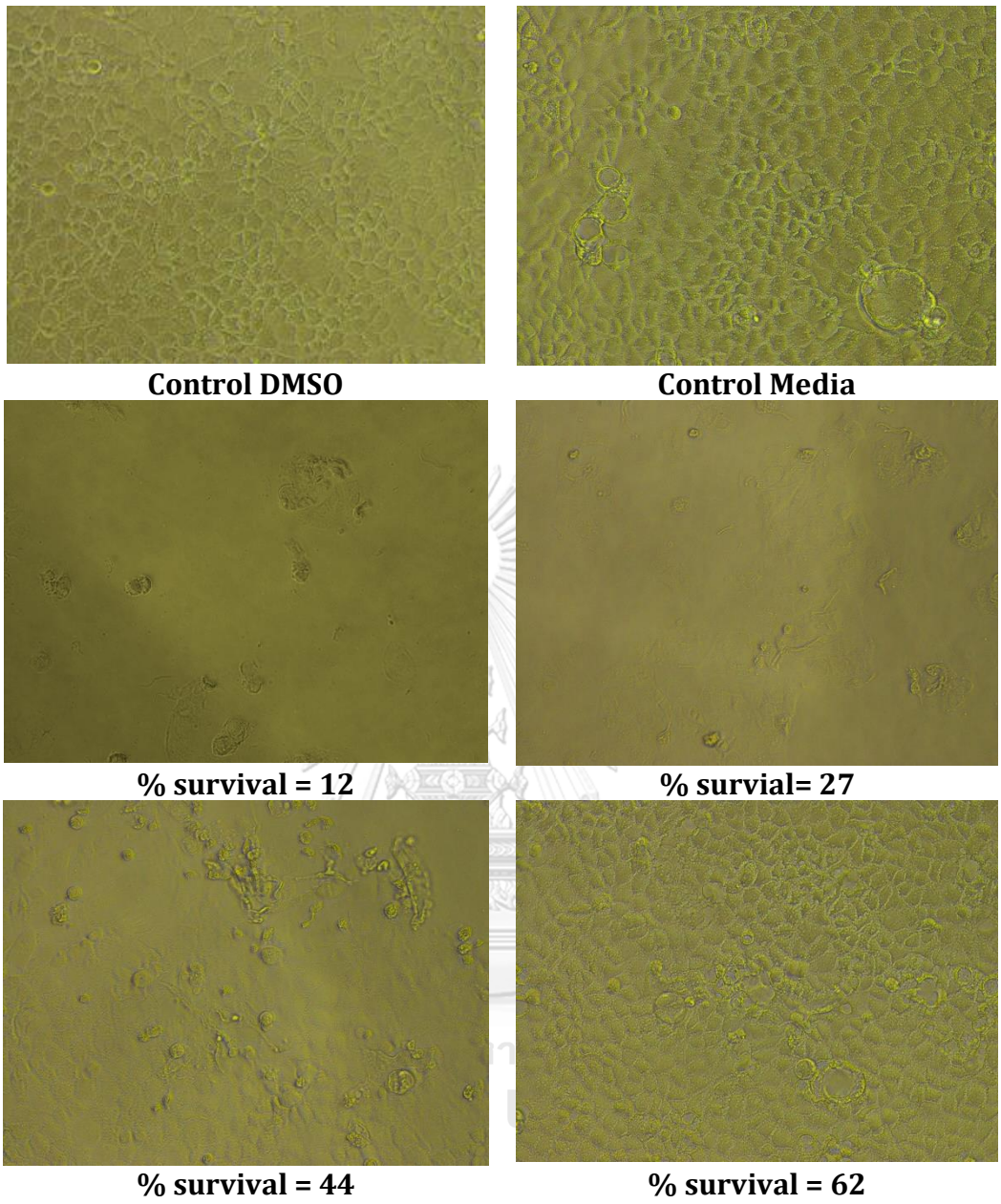


Figure 31. The morphology of Caco-2 cells after treated with the mGI microcapsule for 72 hours

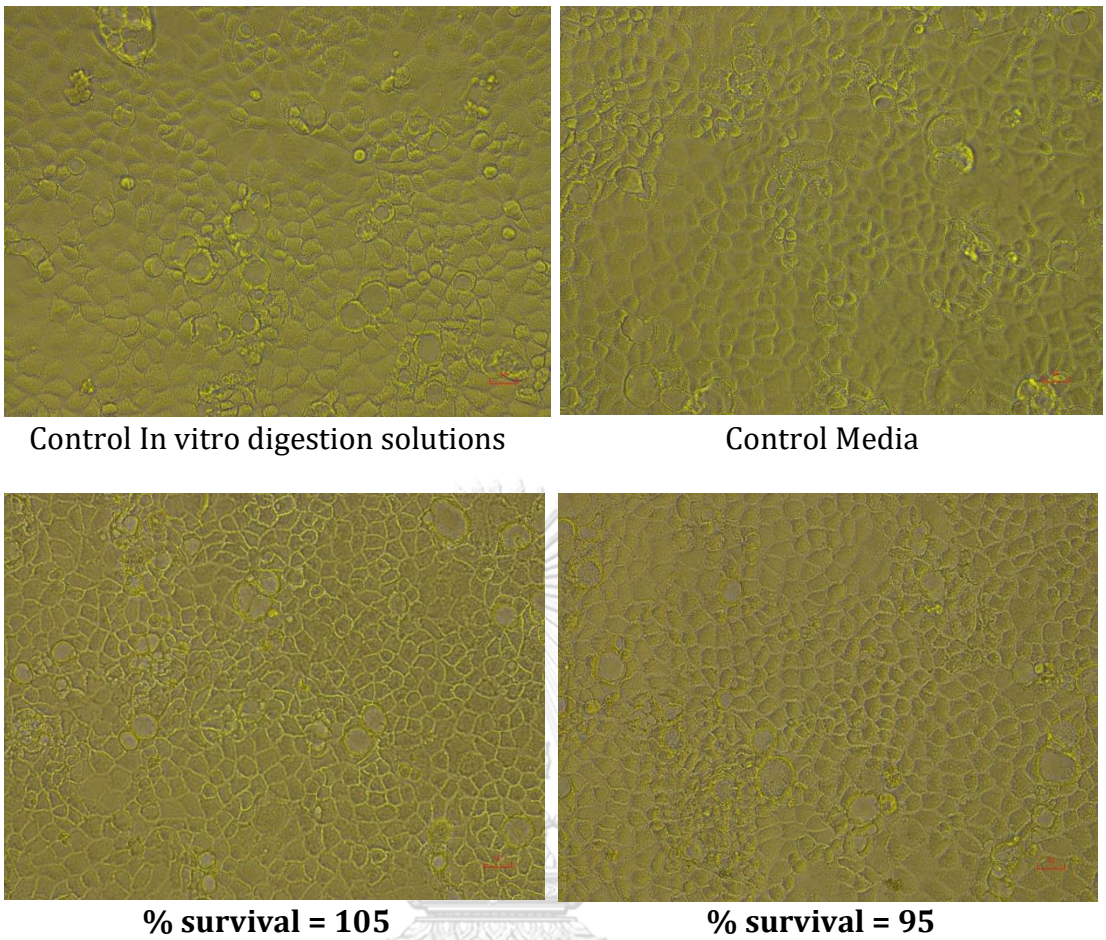


Figure 32. The morphology of Caco-2 cells after treated with the digested mGI microcapsule for 72 hours

4.17 Sensory evaluation of fortified soymilk and cow milk containing mNSO

Microencapsulated NSO powder using gum arabic plus inulin (mGI) as a wall material was selected as a model for conducting sensory analysis. A descriptive analysis data from ten trained panelists indicated that the mGI powder could mask the bitter taste but not strong odor of NSO (Figure 33). The unique flavor of black cumin oil in mGI powder could moderately release to the products. After adding mGI to the milk samples, the soymilk and cow milk samples were evaluated for sensory perception with ten trained panelists using the triangle test. It required the panelist to differentiate the one sample from three samples according to triangle criteria. According to the result obtained, all the panelists were able to differentiate the fortified microcapsule products from NSO products with p-value < 0.01 according to minimum numbers of correct judgements apply

for sensory study for both two set of triangle tests (Table 25). In additions, all fortified samples were scores in terms of appearance, color, odor, taste, texture, after taste and overall acceptability (Table 27 and Figure 34).

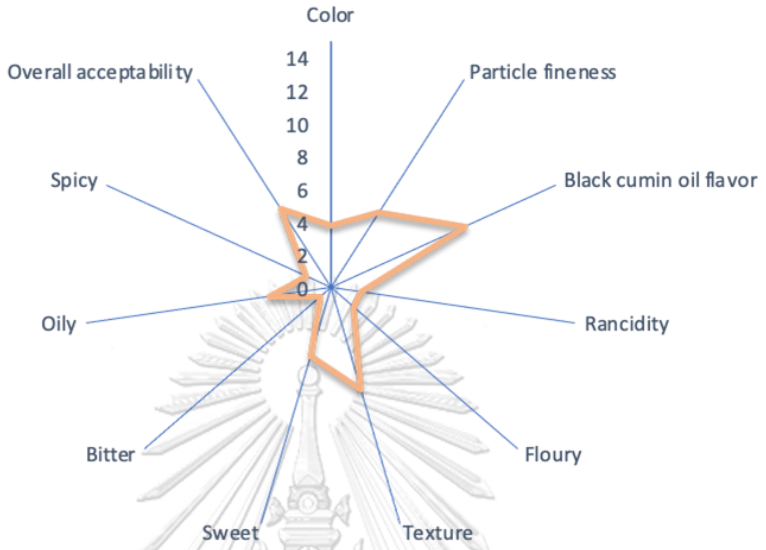


Figure 33. Sensory scores from the descriptive analysis data for the microencapsulated NSO powder using gum arabic and inulin (mGI) as a wall material

Table 25. The 15-cm unstructured line scale for the attributes of mGI powder.

Attributes	Un-structured line scale (mm)
Color	3.729 ± 1.73
Particle fineness	5.429 ± 4.81
Black cumin oil flavor	8.956 ± 3.24
Rancidity	1.862 ± 2.27
Floury	1.847 ± 2.47
Texture (smooth-coarse)	6.444 ± 4.50
Sweet	4.392 ± 3.52
Bitter	0.885 ± 1.10
Oily	3.799 ± 3.21
Spicy	1.618 ± 2.11
Overall acceptability	5.647 ± 3.40

Mean ± standard deviation (n = 10)

Table 26.The triangle test of the fortified NSO or mGI soymilk and cow milk**.**

Triangle set	No. sample	Correct/Total response	False/Total response
1 (fortified NSO and mGI in soymilk)	30	30/30	0/30
2 (fortified NSO and mGI in cow milk)	30	30/30	0/30

The trained panelists could significantly distinguish sensory attributes of low fat cow milk and soybean milk containing 10 mg of TQ from mGI or NSO per serving size ($P < 0.05$). There was no significant difference observed for overall acceptability of all fortified milk products. However, the overall acceptance scores of the fortified milks were moderate (5.9 – 6.6 scores). It was required some improvement for better taste as well as higher amount of TQ for health promotion.

Table 27. Acceptability scores of the fortified NSO or mGI soymilk and cow milk

Sensory attributes	Soy milk		Low fat cow milk	
	mGI-SM (n= 40)	NSO-SM (n=50)	mGI-LFM (n=43)	NSO-LFM (n=47)
Appearance	5.90 ± 1.80^{a}	7.28 ± 1.36^{b}	7.47 ± 1.28^{b}	7.64 ± 1.34^{b}
Color	6.45 ± 1.68^{a}	7.48 ± 1.23^{b}	7.65 ± 1.02^{b}	7.77 ± 1.15^{b}
Odor	6.35 ± 1.37^{a}	5.68 ± 1.75^{ab}	6.81 ± 1.44^{ab}	6.43 ± 1.68^{b}
Taste	5.98 ± 1.23^{ab}	5.86 ± 1.34^{a}	6.67 ± 1.32^{b}	6.17 ± 1.77^{ab}
Texture	6.33 ± 1.80^{a}	6.94 ± 1.43^{ab}	7.20 ± 1.39^{b}	7.02 ± 167^{ab}
After taste	5.43 ± 1.50^{a}	5.56 ± 1.5^{a}	6.44 ± 1.50^{b}	6.19 ± 1.65^{ab}
Overall	5.90 ± 1.64^{a}	5.98 ± 1.50^{a}	6.65 ± 1.63^{a}	6.40 ± 2.10^{a}

Mean ± standard deviation. Values followed by the same letter did not differ statistically according to the Tukey test at 5% ($P < 0.05$).

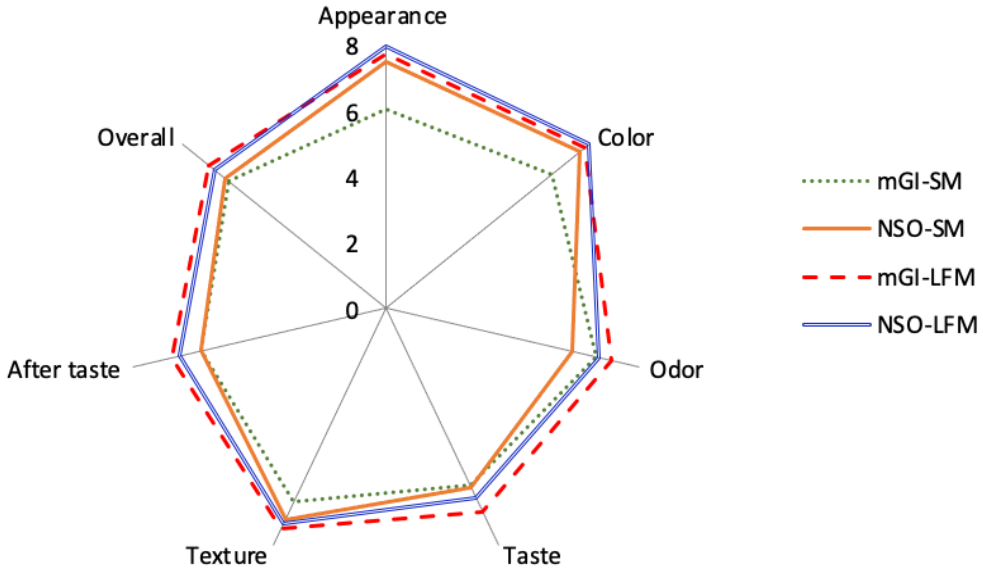


Figure 34. Sensory evaluations of products: 1) mGI-SM = soymilk fortified with mGI microcapsules 2) NSO-SM = soymilk fortified with NSO 3) mGI-LFM = low fat cow milk fortified with mGI microcapsules 4) NSO-LFM = low fat cow milk fortified with NSO.

CHAPTER V

CONCLUSION

Black cumin (*Nigella sativa*) is one of potential plants that possess many benefits to human as food and therapeutic agents. Creation of new formulation of black cumin is found to be attractive for many food industries since the encapsulated product could mask the undesired characteristics of the oils. In this study, the NSO seed was identified and reported to be India origin. The NSO was extracted by cold press extraction with the 27% yields of NSO. This cold pressed NSO was encapsulated successfully via spray drying technique by four formulations. The four formulations of microcapsules were gum arabic alone (GA) or in combination with inulin (GI), maltodextrin (GM), or whey protein (GW) at percent ratio of 50:50.

Prior to the microencapsulation process, the NSO was evaluated for phytochemicals, fatty acids profiles, antimicrobial activity, antioxidant activity as well as cytotoxicity against Caco-2 cells. According to the GC-MS analysis, 32 phytochemical compounds were identified in cold press NSO. The thymoquinone was found to be a main phytochemical compound in NSO. By HPLC analysis, the thymoquinone was 4.4 mg/mL. The fatty acid profiles were evaluated by GC-FID and the three main abundant fatty acids were linoleic acid (58.45%), oleic acids (24.07%) as well as palmitic acid (13.01%). The cold-pressed NSO had antimicrobial activity against *Staphylococcus aureus* with a inhibition zone of 17.67±1.58 mm by disc diffusion and the minimal inhibition concentration (MIC) of 1mg/mL by broth dilution. The % inhibition of NSO by DPPH at concentration of 62.5 mg/mL was 87%. The result obtained in this study revealed that the IC_{50} of NSO on Caco-2 cells using MTT was 13.63 μg/mL which shown to be higher than the cytotoxicity of NSO against human epithelial cell lines (Hep-2).

After microencapsulation process, the physicochemical properties of four formulations of microcapsules were evaluated. The moisture contents of microcapsule with GA, GM, GI, GW formulations were 3.62 %, 1.60 %, 2.29 %, and 1.60 % accordingly. The moisture content of all microcapsule was lower than 5%

which demonstrated the microbiological safe and ensured the stability of the powder. In additions, the water activity of all microcapsules was lower than 0.5. The highest water activity was obtained from GA formulation. Both moisture content and water activity were important parameters to evaluate a good quality of microcapsule product. Thus, all microcapsules were considered to be lower risk of food deterioration and microbial contamination and were favorable by food industry. By FTIR analysis, all microcapsules were shown to contain the distinguish peak at 1750 cm^{-1}. This peak represented the region of double bond stretching found in NSO. The result demonstrated that the NSO was incorporated in four formulations of microcapsules. The combinations of GA with other hydrocolloids (inulin, maltodextrin, and whey protein) demonstrated to improve wettability and solubility of microcapsules. The best wettability was obtained from GI formulation with value 10.52 min while the best solubility was obtained from GW formulation. These wettability and solubility are very beneficial since the hydrophobicity of NSO was improved and the NSO could be introduced into many varieties of food product due to these properties. The microencapsulation efficiency is another parameter needed to concern in microencapsulation process. The microencapsulation efficiency was significantly different among the microcapsules. The microencapsulation efficiency of all microcapsules was in acceptable range (75-88%). The difference in percentage of microencapsulation efficiency was correlated to the different types of wall materials used in preparing emulsions for spray drying. By observing of all microcapsule formulations, the GI revealed a good spherical shape microstructure with a smooth surface which demonstrated the best candidate among other microcapsules.

The phytochemical compounds, fatty acids composition, antimicrobial activity, antioxidant activity as well as cytotoxicity against Caco-2 cells of microcapsules were also evaluated. Based on GC-MS data, thymoquinone, longifolene, tertbutylhydroquinone were the main phytochemical remained in all microcapsules while alpha-phellandrene only found in GW formulation. The thymoquinone was further quantified by HPLC analysis, the thymoquinone content of all microcapsules were range from 2.16 - 2.49 mg/ml. The significant

different of thymoquinone content was seen in GM formulation ($p < 0.05$). However, the morphology of GM microcapsule was undesirable with irregular shape which will later result in susceptible to oxidation. The fatty acid compositions of all microcapsules were no different among four formulations of microcapsules ($p > 0.05$) and well remained with linoleic acid range from 57.36-57.69%, followed by oleic acid which was range from 24.06-24.29%, palmitic acid was range from 13.43-13.75%. All microcapsules were remained to active against *S. aureus* with the clear zone of inhibition ranging from 3.67-6.33 mm by disc diffusion technique and the MIC of GI was 4 mg/mL by broth dilution technique. The highest % inhibition by DPPH of microcapsules was obtained from GI formulation at concentration of 125 mg/mL with 71.90 of % inhibition. The cytotoxicities of all microcapsules against Caco-2 cells were lower than NSO with the IC_{50} of GI was 182.5 μg/ml.

Based on the physicochemical analysis, SEM inspection, as well as antioxidant activity, GI microcapsule was selected to be a suitable microcapsule applied in food formulation since it provides good wettability, solubility with a smooth and spherical shape of microstructure as well as strong antioxidant activity. In additions, after encapsulation process, the phytochemical like thymoquinone, longifolene, tertbutylquinone were still available. The nutritious value of linolenic acids was also not much different from the cold press NSO.

The GI microcapsule was further fortified in soymilk and milk products. There was no significant difference in sensory attribute in terms of overall. The different in sensory attributes were observed in fortified soymilk with NSO ($p < 0.05$), especially in terms of odor and taste. By encapsulation of NSO, the odor and taste of the product were improved although the appearance score of soy milk containing encapsulated NSO product seem to be lower than other formulations. The overall acceptance scores of the fortified milks were moderate (5.9 – 6.6). Therefore, the formulation of mNSO into food product should be further study in order to satisfy the consumer needs with remain the nutritious value.

APPENDIX A
Preparation of thymoquinone standard for HPLC analysis

1. Preparation of thymoquinone standard

- 1 mg of thymoquinone is dissolved in 1ml of methanol
- The thymoquinone is then diluted according to the concentration required (1 mg/mL, 10 mg/mL , 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL and 60 mg/mL) as followed:

$M_1V_1 = M_2V_2$

$1000 \times V_1 = 10\ (1000\ \mu L)$

$V_1 = 10\ \mu L \rightarrow 990\ \mu L\ (\text{methanol})$

$1000 \times V_1 = 10\ (1000\ \mu L)$

$V_1 = 10\ \mu L \rightarrow 990\ \mu L\ (\text{methanol})$

$1000 \times V_1 = 20\ (1000\ \mu L)$

$V_1 = 20\ \mu L \rightarrow 980\ \mu l\ (\text{methanol})$

$1000 \times V_1 = 30\ (1000\ \mu L)$

$V_1 = 30\ \mu L \rightarrow 970\ \mu L\ (\text{methanol})$

$1000 \times V_1 = 40\ (1000\ \mu L)$

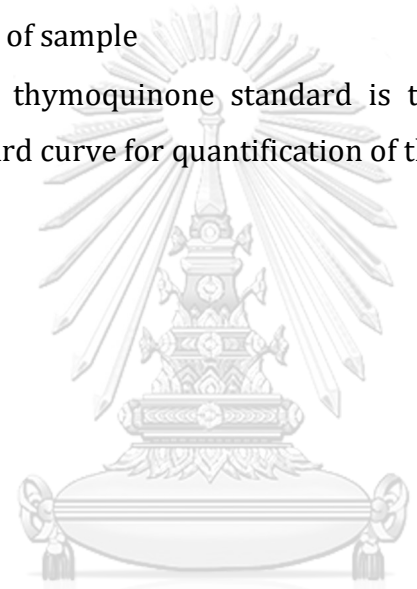
$V1 = 40\ \mu L \rightarrow 960\ \mu L\ (\text{methanol})$

$1000 \times V_1 = 50\ (1000\ \mu L)$

$V1 = 50\ \mu L \rightarrow 950\ \mu L\ (\text{methanol})$

2. <u>HPLC operatinon</u>

- All mobile phase should be filtered with 0.45 μm membrains filter (Millipore) deganed under vaccuum before using for operate HPLC
- C_{18} reversed – phase column (150 x 4.6 mn , 5 μm particle size , GL Science) is inserted into the column chamber
- The chramatographic condition is set with methanol : H_2O (70 : 30 %, v/v) of and run until the pressure is stable
- Single injection of one of thymoquinone concentration to check the retention time of sample
- The prepared thymoquinone standard is then injected into HPLC for making standard curve for quantification of thymoquinone



APPENDIX B

Bacterial culture preparation

1. **Trypic soy agar and slant agar media preparation**
    - Weight and dissolve 10 g of agar in 1000 mL of DW with magnetic stirring.
    - Sterilize by autoclaving the media at 121˚C for 15 min
    - Let it cool at 45-50˚C and mix well before transferring 25 mL into aseptic petri dishes
    - Close the lid with parafilm and write the date and invert the plate to prevent moisture
    - Pour the media solution into petri dish estimated 25 mL each per plate
    - Prepare the slant agar from tryptic soy agar by transferring 5 mL of the media into an aseptic test tube
    - Put one of it in 37 ^{0}C incubator and observe for contamination in the next day

2. **Mueller Hinton agar media preparation**
    - Weight and dissolve 38 g of Mueller Hinton agar in 1000 mL DW with magnetic stirring
    - Sterilize by autoclaving the media at 121˚C for 15 min
    - Let it cool at 45-50˚C and mix well before transferring 25 mL into petri dishes
    - Close the lid with parafilm and write the date and invert the plate to prevent moisture
    - Put one of it in 37 ^{0}C incubator and observe for contamination in the next day

3. **Mueller Hinton broth media preparation**

- Weight and dissolve 21 g of Mueller Hinton broth in 1000 mL DW with magnetic stirring.
- Sterilize by autoclaving the media at 121˚C for 15 min.
- Let it cool at 45-50˚C and mix well before transferring 25 mL into petri dishes.
- Close the lid with parafilm and write the date and invert the plate to prevent moisture.
- Put one of it in 37 ^{0}C incubator and observe for contamination in the next day.

4. **Activate the bacterial culture from the stock culture of *S. aureus*, *S. enterica* and *E. coli***

- Thaw the stock cultures and streak it on tryptic soy agar plate
- Grow overnight at 37˚C and observe the growth
- Observe the single colony and transfer the inoculum from streak plate culture to the slant agar plate
- Incubate at 37˚C incubator and observe the growth on the next day

5. **Lactobacilli MRS agar and broth media preparation**

- Weight and dissolve 70 g of Lactobacilli MRS Agar and 55 g of Lactobacilli MRS Broth in 1000 mL DW with magnetic stirring.
- Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
- Sterilize by autoclaving the media at 121˚C for 15 min.
- Let it cool at 45-50˚C and mix well before transferring 25 mL into petri dishes.

- Close the lid with parafilm and write the date and invert the plate to prevent moisture.
- Put one of it in 37 °C incubator and observe for contamination in the next day

6. <u>Activate the bacterial culture from the stock culture of *L. Rhamnosus*</u>

- Thaw the stock cultures and streak it on Lactobacilli MRS agar plate
- Grow at 35˚C for 48 h and observe the growth
- Observe the single colony and transfer the inoculum from streak plate culture to the MRS slant agar plate
- Incubate at 35 ^{0}C incubator and observe the growth on the next day

7. <u>Preparation of paper disc for disc diffusion</u>

- Sterilize the 6 mm paper disc and transfer 20 µL of 400 mg/mL of NSO, microcapsules containing NSO and 100 µg/mL of cefritaxone in 6mm paper disc
- Let it dry at least 30 minutes before using and spread 0.1 mL bacterial culture on the Mueller Hinton agar plate
- Place the discs on the Mueller Hinton agar plate where bacteria culture have been placed

8. <u>Plate count agar media preparation</u>

- Weight and dissolve 23.5 g of Plate Count Agar in 1000 mL DW with magnetic stirring
- Sterilize by autoclaving the media at 121˚C for 15 min
- Let it cool at 45-50˚C and mix well before transferring 25 mL into petri dishes

- Close the lid with parafilm and write the date and invert the plate to prevent moisture
- Put one of it in 37 ^{0}C incubator and observe for contamination in the next day

9. <u>Peptone water preparation</u>

- Weight and dissolve 15 g of peptone in 1000 mL DW with magnetic stirring
- Sterilize by autoclaving the media at 121˚C for 15 min
- Let it cool at 45-50˚C and mix well before transferring test tube for colony counting

10. <u>Colony count by serial dilution of the sample using spread plate technique</u>

- Dilute the bacterial culture with 10x dilution factor until 1:10^4 to 1:10^6 then pipette the 0.1 ml of sample for spread plate
- Incubate at 37˚C for 24-48h and observe the colony

11. <u>Preparation of INT (2 – p – iodophenyl – 3 – p – nitrophenyl – 5 – phenyl tetrazolium chloride) indicator used for bacterial growth</u>

- 2 mg of INT is dissolved in water
- The concontration used for the MIC is 0.2 mg/mL (dissolve 0.2 mg in dH_2O)
- For indicating bacterial growth (40 µL of 0.2 mg/mL INT/P – Iodonitrotetrazolium chloride(INT) was added to each well and incubate for 30 min). The INT is changed to red once the bacteria is present

clear colorless	=	inhibition of bacterial growth
pink - red color	=	presence of growth
MIC	=	the lowest conc showing no colour change

APPENDIX C

Antioxidant activity by DPPH assay

1. **Preparation of DPPH**

   - The 0.4 mM of DPPH will be prepared accordingly:

     MW of DPPH = 394.32 g

     1 M = 394.32 g

     0.4 mM = (0.4 x 394.32 g x 10-3) / 1M

     = 0.1577

     = 15.8 mg in 100 ml MeOH

   - 0.2 mm DPPH (working reagent) is prepared freshly when needed

2. **Preparation standard BHT**

   - 200 mg BHT is dissolved in 50 ml MeOH to get a concentration of 4 mg/mL

3. **Preparation of NSO and microcapsules for DPPH assay**

   - Weight 500 mg/mL of NSO and microcapsule containg NSO samples in methanol compared with spray dried microcapsule without NSO
   - Vortex and centrifuge of 1000 xg and transfer an aliqout to 96 well plate (100 μL)
   - Then add a control and all sample in the well
   - Measure the absorbance at 517 nm
   - Calculate the % inhibition form the equation:

     $$\% \text{ inhibition} = \frac{(A_{blank} - A_{sample}) \times 100}{A_{blank}}$$

APPENDIX D

Reagents used for *in vitro* digestability test

1. **Preparation in vitro digestibility test**

- Preparation of Saliva juice 1 L (use 5 mL for each sample)
  - sodium chloride 0.117 g
  - potassium 0.149 g
  - sodium bicarbonate 2.1 g
- Gastric juice (200 mL) (use 10 mL for each sample)
  - sodium chloride 1.775 g
  - 200 mg pepsin
- Preparation of phosphate buffer (pH8) 500 mL
  - NaCl 40 g
  - KCI 2 g
  - Na2NPO4 14.4 g
  - KH2PO4 2.4 g

APPENDIX E

Caco-2 culture

1. <u>Media preparation for Caco2</u>

Complete MEM (500 mL) consist of

- 15% FBS	75	mL
- Sodium pyrurate (1 mm from 100 mm)	0.5	mL
- 1% L – glutamine (glutamax)	5	mL
- 1% Non – essential amino acids	5	mL
- 1% pen – strep	5	mL
- 0.2% amphotericin	1	mL
	91.5	mL
- Add MEM	408.5	mL
Total Volume	500	mL

APPENDIX F

Master sheet used for sensory analysis

Master sheet การจัดตัวอย่างในการทดสอบแบบ triangle

ผลิตภัณฑ์ ....Fortified Soy Milk..... วันที่ทดสอบ ....20/11/2020..... เวลา .....

Permutation numbers (Perm #)	NEE = 1 ENE = 2 EEN = 3 NNE = 4 NEN = 5 ENN = 6

รหัสผู้ประเมิน	Perm. #	E	E	N	N
001	4	120[3]		168[1]	145[2]
001	5	131[2]		127[1]	155[3]
001	6	165[1]		190[2]	163[3]
002	3	184[1]	112[2]	143[3]	
002	1	156[2]	132[3]	170[1]	
002	4	110[3]		189[1]	123[2]
003	5	136[2]		185[1]	117[3]
003	2	130[1]	113[3]	183[2]	
003	5	145[2]		111[1]	109[3]
004	6	*175*[1]		188[2]	150[3]
004	2	*153*[1]	101[3]	164[2]	
004	1	*105*[2]	114[3]	149[1]	
005	4	*104*[3]		161[1]	137[2]
005	2	*148*[1]	*121*[3]	151[2]	
005	5	*134*[2]		108[1]	178[3]
006	4	173[3]		106[1]	*116*[2]
006	6	180[1]		146[2]	*167*[3]
006	2	104[1]	170[3]	124[2]	
007	4	201[3]		154[1]	182[2]
007	5	166[2]		139[1]	125[3]
007	1	129[2]	158[3]	171[1]	
008	2	174[1]	131[3]	128[2]	
008	3	122[1]	186[2]	159[3]	

รหัสผู้ประเมิน	Perm. #	E	E	N	N
008	4	142[3]		138[1]	164[2]
009	5	152[2]		182[1]	135[3]
009	4	144[3]		176[1]	126[2]
009	2	118[1]	100[3]	177[2]	
010	4	141[3]		169[1]	119[2]
010	5	187[2]		147[1]	160[3]
010	2	133[1]	179[3]	157[2]	

ตัวยกคือ ลำดับตัวอย่างที่จัดวางในถาด จากซ้ายไปขวา

master sheet การจัดตัวอย่างในการทดสอบแบบ triangle

ผลิตภัณฑ์ ....Fortified Cow Milk..... วันที่ทดสอบ ....20/11/2020..... เวลา .....

Permutation numbers (Perm #)	NEE = 1 ENE = 2 EEN = 3 NNE = 4 NEN = 5 ENN = 6

รหัสผู้ประเมิน	Perm. #	E	E	N	N
001	4	173^{3}		106^{1}	116^{2}
001	6	180^{1}		146^{2}	167^{3}
001	2	104^{1}	170^{3}	124^{2}	
002	4	201^{3}		154^{1}	182^{2}
002	5	166^{2}		139^{1}	125^{3}
002	1	129^{2}	158^{3}	171^{1}	
003	2	174^{1}	131^{3}	128^{2}	
003	3	122^{1}	186^{2}	159^{3}	
003	4	142^{3}		138^{1}	164^{2}
004	5	152^{2}		182^{1}	135^{3}
004	4	144^{3}		176^{1}	126^{2}
004	2	118^{1}	100^{3}	177^{2}	
005	4	141^{3}		169^{1}	119^{2}
005	5	187^{2}		147^{1}	160^{3}
005	2	133^{1}	179^{3}	157^{2}	
006	4	120^{3}		168^{1}	145^{2}
006	5	131^{2}		127^{1}	155^{3}
006	6	165^{1}		190^{2}	163^{3}
007	3	184^{1}	112^{2}	143^{3}	
007	1	156^{2}	132^{3}	170^{1}	
007	4	110^{3}		189^{1}	123^{2}
008	5	136^{2}		185^{1}	117^{3}
008	2	130^{1}	113^{3}	183^{2}	
008	5	145^{2}		111^{1}	109^{3}
009	6	175^{1}		188^{2}	150^{3}

รหัสผู้ประเมิน	Perm. #	E	E	N	N
009	2	153[1]	101[3]	164[2]	
009	1	105[2]	114[3]	149[1]	
010	4	104[3]		161[1]	137[2]
010	2	148[1]	121[3]	151[2]	
010	5	134[2]		108[1]	178[3]

ตัวยกคือ ลำดับตัวอย่างที่จัดวางในถาด จากซ้ายไปขวา

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