FOOD PRODUCT DEVELOPMENT OF ROSELLE SOY YOGURT

BY

MISS VARITHA ARIYABUKALAKORN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

IN APPLIED THAI TRADITIONAL MEDICINE

FACULTY OF MEDICINE

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ENTITLED

FOOD PRODUCT DEVELOPMENT OF ROSELLE SOY YOGURT

was approved as partial fulfillment of the requirements for
the degree of Master of Science in Applied Thai Traditional Medicine

on November 25, 2019

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ABSTRACT

Soy yogurt is a healthy functional food for consumers and becoming popular with it nutritious. Soy yogurt produced from fermentation between soymilk and lactic acid bacteria. Moreover, berry fruits are popular added into yogurt. Therefore, soy yogurt is developed to herbal soy yogurt products for increasing nutrients value. Roselle is an interesting herb for used to developed with soy yogurt. The objective of this study was to develop roselle soy yogurt. The specific aims were to compare biological activities and chemical content of extracts, to investigate roselle extract under stress conditions, to isolate lactic acid bacteria for soy yogurt production, to study optimal condition for soy yogurt fermentation and to develop product from soy yogurt and roselle extract.

Roselle aqueous extract displayed antioxidant activity and total phenolic content, but no potential to inhibit nitric oxide production in RAW264.7 cell and superoxide ions production in HL-60 cell. After being hydrolyzed, the hydrolyzed extract showed anti-inflammatory, antioxidant and total phenolic content more than the initial extract. The positive marker compound of roselle aqueous extract was chlorogenic acid, coumaric acid, ferulic acid, quercetin and cyanidin-3-o-

sambubiosides. In hydrolyzed extract, chlorogenic acid and cyanidin-3-osambubiosides were disappeared and become derivative compound. The results showed the increment of coumaric acid, ferulic acid and quercetin. Moreover, roselle aqueous extract under stress condition (thermal, moisture, acid, base and oxidation) demonstrated that the conditions of roselle extract for product development were thermal and moisture conditions. Therefore, roselle aqueous extract was developed in reverse spherification form and tested for antioxidant, total phenolic contents and bioactive compound during storage. Antioxidant and total phenolic contents of roselle alginate bead in storage period were significantly decreased in day 7. Besides, chemical fingerprint in roselle spherification was shown to be unstable and significantly decreased in day 14. Likewise, appearance of roselle spherification exhibited the highest scores in sensory evaluation and secondary grade was overall acceptance.

For soy yogurt production, *Lactobacillus plantarum* subsp. *plantarum* (ATCC 14917) and *Pediococcus acidilactici* (DSM 20284) were lactic acid bacteria that used for soy yogurt starters. The soymilk and two starter cultures on the various fermentation duration at 37°C was investigated for stability of physiochemical (viscosity, %acidity, pH, %syneresis, color and bacterial growth) and sensory qualities (appearance, smell, flavor, texture, overall acceptance and overall acceptance when eating with roselle beads). The bacterial growth of soy yogurt at fermentation time 10, 12 and 14 hr were reached to 10^8 CFU/g and slightly changed during storage. Viscosity and pH were reduced in storage time. Moreover, %acidity, %syneresis and color were increased during period. Organoleptic evaluation of all soy yogurt was not significantly altered at 95% confidence interval. The highest scores of overall acceptances were soy yogurt at fermentation time 10 and 12 hr. The combination of soy yogurt and roselle spherification was enhanced overall acceptance scores from participants. Consequently, fermentation duration was influenced to physiochemical and organoleptic qualities of soy yogurt. In addition, roselle spherification can improve flavor of plain soy yogurt.

Keywords: Roselle soy yogurt, Soy yogurt, Roselle spherification

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Miss Varitha Ariyabukalakorn

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
µg	Microgram
µl	Microliter
µM	Micromolar
ATCC	American type culture collection
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CFU	Colony forming unit
$CHCl_3$	Chloroform
$C_6H_8O_7$	Citric acid
$C_6H_9NaO_7$	Sodium alginate
$C_6H_{10}CaO_6$	Calcium lactate
cP	Centipoise
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EC_{50}	50% efficient concentration
EtOH	Ethanol
FBS	Fetal bovine serum
HCl	Hydrochloric acid
HL-60	Human promyelocytic leukemia cell
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HS	*Hibiscus sabdariffa* (Roselle)
IC_{50}	50% inhibitory concentration
LAB	Lactic acid bacteria

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
M	Molar
MeOH	Methanol
MRS	De Man, Rogosa and Sharpe agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltrazolium bromide
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
nm	Nanometer
NO	Nitric oxide
P	P-value
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Potential hydrogen
Rpm	Round per minute
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SEM	Standard error of mean
TAE	Tris-acetate
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Introduction

Nowadays, soymilk is a popular base milk to make yogurt. Soymilk is an aqueous extract from soybean. In 1999, U.S. Food and Drug Administration (FDA) demonstrated that consumed 25 grams soy protein or 100 mg isoflavones per day is suitable for the body. There are many benefits from this plant: it promotes metabolism, promotes bone health, anticancer and reduces risk of cardiovascular disease (Law, 1994; Messina, 2003; Boniglia *et al*., 2009). Moreover, soymilk product is a healthier choice for vegans. Vegans are people who consume only vegetables. There are about 375 million vegans in the world. Percentage of vegan consumers in India, Taiwan, New Zealand, United Kingdom and United States are 40%, 14%, 10.3%, 3.3%, 3%, respectively (The Vegetarian Resource Group, 2015; Roy morgan, 2016; The Vagan Society, 2016; Fleury *et al*., 2017).

Furthermore, soy products are alternative foods for people that are lactose intolerance. Briefly, lactose intolerance is a common condition in which the body is unable to digest lactose, a sugar in dairy products, such as butter, cheese, ice cream, mammalian milk. A previous study showed that North Americans, South Americans, Chinese, Japanese, Eskimos and Aboriginal people are very commonly lactose intolerance (Schaafsma, 2008). After infancy, 65% of people have reduced ability to digest lactose (National institute of Health (NIH), 2017). The undigested lactose is fermented by intestinal bacteria that produces carbon dioxide, hydrogen and methane. Fermentation products are the cause of symptoms including stomachache, flatulence, nausea and diarrhea (National Health Service, 2016). Because of these problems, some consumers who are lactose intolerant do not want to eat animal products, although they can be eaten. Therefore, a soymilk product is an interesting choice for consumer. Presently, soymilk is developed to many healthy products such as tofu, soymilk powder and yogurt.

Yogurt is a popular healthy food. The yogurts process requires that both soymilk and mammalian milk have the same fermentation. Lactic acid bacteria are microorganisms for yogurt production. Sugar in the milk is metabolized by the bacteria. This action coagulates milk into yogurt. In United States, FDA requires *Lactobacillus bulgaricus* and *Streptococcus thermophillus* as a standard culture for yogurt. However, other cultures have been used as starter culture in previous studies such as *Lactobacillus acidophilus* (Bedani *et al*., 2014; Pandey & Mishra, 2015; Farnworth *et al*., 2017), *Lactobacillus helveticus* (Yang & Li, 2010), *Lactobacillus johnsonii* (Farnworth *et al.*, 2007), *Lactobacillus reuteri* (Gu, 2015) and *Lactobacillus rhamnosus* (Farnworth *et al.*, 2007). Presently, yogurt product is developed to herbal-yogurt to be functional healthy product for consumer (Orano, Atanu, Prajapati & Suvera. 2017).

Hibiscus sabdariffa also called Roselle and Kra-Jeab (Thai). It has been used in Thai traditional medicine for a long time. Nowadays, the consumption of herbal food is increasing in a wellness trend. Roselle is used as a food ingredient, colorant in foods and beverages (Bako, 2009; Rocha, 2014). Previous research demonstrated that roselle shows antihypertensive, antihyperlipidemia, antiatherosclerotic, diuretics, digestive and anti-inflammatory activity (Chen *et al*., 2003; Abouzid & Mohamed, 2011; Alzweiri *et al*., 2011; Aziz, Wong, & Chong, 2013; Rocha, 2014). Roselle calyces contain a natural edible brilliant red color which is rich in anthocyanin, a strong antioxidant (Duangmal, Saicheua, & Sueeprasa, 2008; Ochani & Mello, 2009). Besides, flavonoid and phenolic group are chemical constituents in roselle (Mckay, 2009). Many researchers report that anthocyanin inhibits nitric oxide production in the inflammation process and displays antioxidant activity (Chen *et al*., 2004; Giriwono *et al*., 2011). The calyx of roselle has been shown to exhibit cytotoxicity to gram positive microbes, especially Lactobacillus spp. and Streptococcus spp. which one important for yogurt production (Sulistyani, Fujita, Miyakawa, & Nakazawa, 2016). The result has been unable to add roselle extract directly into yogurt. However, encapsulation is an interesting technique for containing roselle extract. Many previous research documents have suggested roselle is suitable for developing into healthy products. Therefore, the objectives of

this study are to investigate biological and chemical contents of roselle extract and to develop a roselle soy yogurt to be a functional food.

1.2 Objective

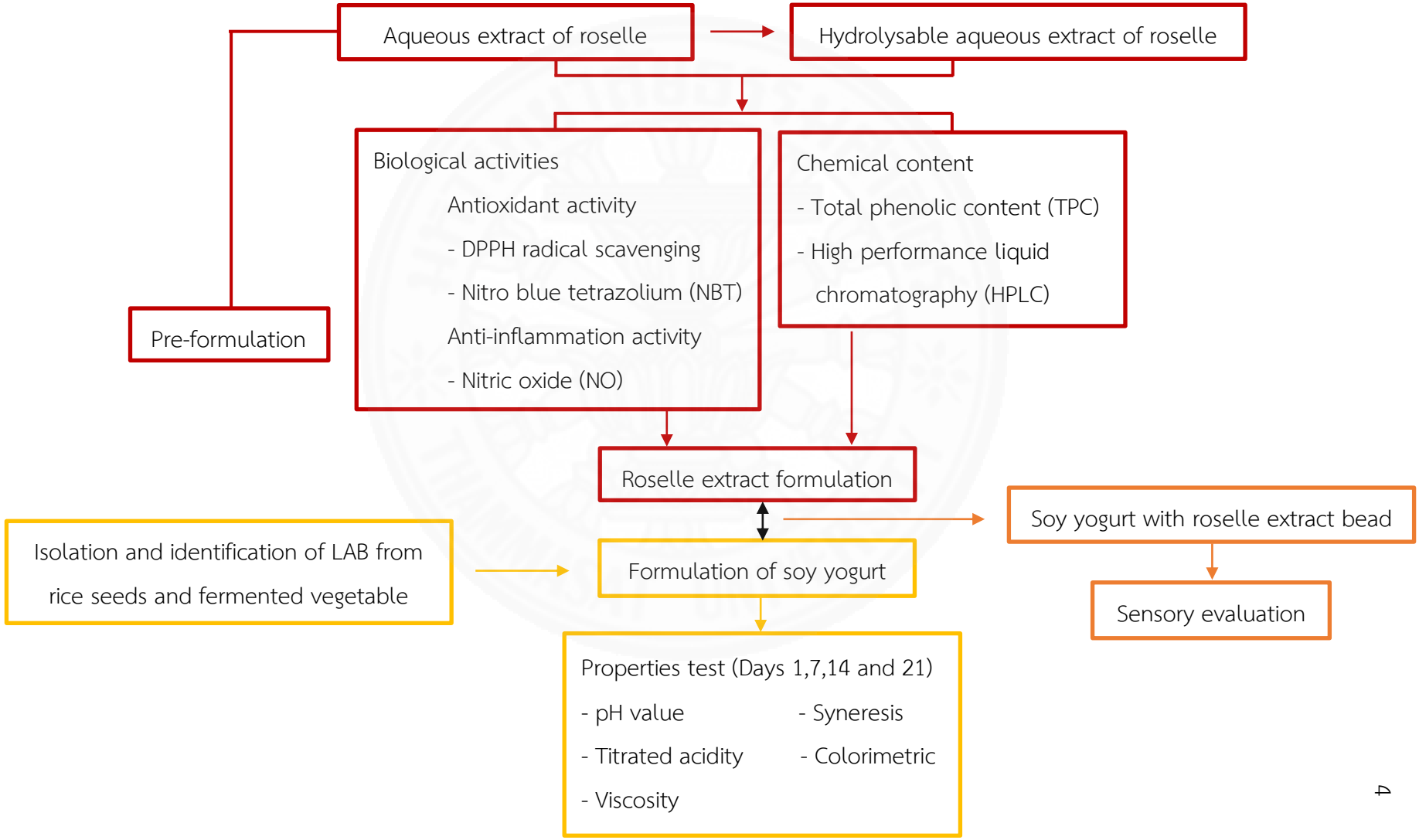
1.2.1 Overall aim

This research aims to develop roselle soy yogurt.

1.2.2 Specific aims

- To compare biological activities including antioxidant and anti-inflammatory activity of aqueous and hydrolyzed roselle extracts.
- To compare chemical content of aqueous and hydrolyzed roselle extracts.
- To investigate roselle extract pre-formulation.
- To isolate lactic acid bacteria for soy yogurt fermentation process.
- To investigate optimal conditions for soy yogurt fermentation.
- To develop a new product form of soy yogurt and roselle extract.

1.3 Conceptual Framework of Thesis



CHAPTER 2
REVIEW OF LITERATURE

2.1 *Hibiscus sabdariffa* L.

Figure 2.1 *Hibiscus sabdariffa* (Roselle)

Family name:	MALVACEAE
Common names	Roselle, Rosella, Red sorrel, Jamaica sorrel, Kharkade, Karkade
Part use:	Calyces, Leaves, Seeds, Roots
Flavor:	Sour
Species	Sudan

2.1.1 Botanical morphology

Hibiscus sabdariffa is a tropical shrub that can grow up to three meters. The stems are red, smooth cylindrical. The leaves are green ovate to lanceolate with a lobed margin, 7.5–12.5 centimeters long with reddish veins. The single flowers are white-pink trumpet shape, with five or more petals. Flowers can grow up to 18 centimeters in size. When the fruit is immature, the capsule is green and dry. The capsule turns to brown and splits open when mature and dry (Mahadevan *et al*., 2009; Mohamed *et al*., 2012).

2.1.2 Cultivation

The suitable altitude for roselle is 900 meters above sea level. Roselle requires rather warm temperatures between 18-35°C and suitable humid weather. Plant prefers short day period under 12 hr for growth (Ansari *et al*., 2013).

2.1.3 Bioactive constituents

2.1.3.1 Anthocyanins

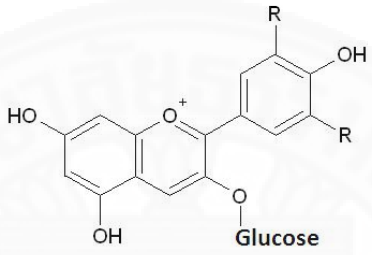


Figure 2.2 Chemical structure of anthocyanins

Anthocyanins are edible natural pigments. These compounds consist of anthocyanidin and an acyl group that derives from the flavonoid group. Replacement of hydroxyl and methyl groups forms glycosides that is called anthocyanidin. There are six groups in anthocyanidin compound: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. According to several previous studies, delphinidin-3-sambubiosides and cyanidin-3-sambubioside were presented as major anthocyanins in the calyx of roselle (Alarcon *et al*., 2007; Beltran *et al*., 2010; Peng *et al*., 2011; Herranz *et al*., 2012).

2.1.3.2 Flavonoids

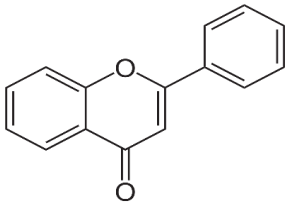


Figure 2.3 Chemical structure of flavonoids

McKay (2009) and Williamson *et al*. (2009) demonstrated that gossypitrin, gossytrin, hibiscitrin, sabdaritrin, quercetin and luteolin are flavonoid

compounds in *Hibiscus sabdariffa* L. extract. Comparison of quercetin and rutin in the aqueous extract showed that the amount of quercetin (3.2 mg/g) was more than rutin (2.1 mg/g) (Alonso *et al*., 2012). Several studies showed that quercetin is frequently found as well as rutin (Debon *et al*., 2010; Peng *et al*., 2011; Lopez *et al*., 2012).

2.1.3.3 Phenols group

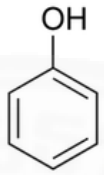


Figure 2.4 Chemical structure of phenols

McKay (2009) and Williamson *et al*. (2009) demonstrated that chlorogenic acid, protocatechuic acid, pelargonidic acid, b-sitosterol and ergosterol were present in roselle calyx. A study on the aqueous extract of roselle showed that chemical composition included: gallocatechin (2.44%), catechin (2.67%), caffeic (19.85%), protocatechuic acid (24.24%) and gallocatechin gallate (2.44%) were presented (Yang *et al*., 2010). Protocatechuic acid is an important compound in roselle (Lee et al., 2002; Williamson *et al*. 2009). In one study, 2.7 mg/g chlorogenic acid was present in calyx of roselle (Alarcon-Alonso *et al*., 2012).

2.1.4 Studies on biological activities of *Hibiscus sabdariffa*

There are many medicinal properties of roselle: antihypertensive, antioxidant, anti-inflammatory, antidiabetic, anticancer, antimicrobial and diuretic activities. Roselle displayed antihypertensive activity in mild to moderate hypertension patients by consuming roselle calyx tea twice a day for 1 month. The result exhibited roselle calyx tea reduced systolic (11.2%) and diastolic (10.7%) blood pressure (Faraji & Haji, 1999; Herrera-Arellano *et al*., 2004). In a clinical study, comparing efficacy of roselle aqueous extract and lisinopril in 193 patients with mild to moderate hypertension found that roselle extract had efficacy less than lisinopril, but was safer than lisinopril (Herrera et al., 2007). Furthermore, studies of roselle tea to reduce blood

pressure and blood sugar in patients by consuming 2 g of roselle tea 2 times/day in 1 month found that roselle can reduce blood pressure and blood sugar (Mozaffri *et al*., 2007). In addition, roselle juice can be used as a laxative drug. Roselle has high vitamin C which can remedy scurvy. In folk medicine, roselle can treat enlarged prostate gland. Roselle calyces have been used to increase the production of urine (diuretic). Previous research on isolation of protocatechuic acid from ethanolic roselle extract and antioxidant testing by DPPH assay found that 0.1 mg/ml can scavenge radicals to 82% efficacy (Tseng *et al*., 1996). This is consistent with other research that studied antioxidant activity of the ethanolic extract of *Hibiscus sabdariffa* by DPPH assay. *Hibiscus sabdariffa* had half maximal inhibition of extract concentration (IC_{50}) 1.10 µg/ml (Barhe & Tchouya, 2014). Studies on antioxidant activity by DPPH assay of ethyl acetate extract and chloroform extract found that ethyl acetate extract had half maximal efficacy of extract concentration (EC_{50}) value of 0.017 mg/ml and chloroform extract had EC_{50} 0.15 mg/ml (Tseng *et al*., 1997). Ethanol extract of dried calyces showed inhibition effects on superoxide anion radicals with value range 70-80% at dose 1 g for in vitro experiment (Mahadevan *et al*., 2009). Studied on HL-60 cells apoptosis in dose and time dependent of roselle methanolic extract by using MTT assay displayed the extract inhibited 50% of HL-60 cell viability at concentration value 2.49 mg/ml. Moreover, the extract at concentration 3 mg/ml cytotoxic to HL-60 cells by decreased cell number 75% at 1 day (Chang, Huang, Hsu, Yang & Wang, 2005). Using roselle extract from freeze dried technique, roselle seeds showed the highest total phenolic content more than roselle calyces (Esa *et al*., 2010). Roselle able to alleviate type 2 diabetic rat with roselle aqueous extract at concentration values 100 mg/kg and 200 mg/kg (Yang, Huang, Wang, Lee, Chen & Peng, 2013). The roselle ethanolic extract with concentration 72 mg/day/200 g body weight and 288 mg/day/200 g body weight against TNF-α in diabetic rat (Mardiah, Zakaria, Prangdimurti & Damanik, 2015). Moreover, adult male rats were fed roselle methanolic extract at concentration 500 mg/kg for 11 weeks. The results displayed Hibiscus extract maintained the ratio of IL-1β/IL-1ra plasma and hippocampus levels in rat and against impairments in spatial memory consolidation (Bayani *et al*., 2018). However, efficacy of roselle ethanolic

extract at concentration 400 mg/kg and 600 mg/kg less than glibenclamide drug at concentration 0.65 mg/kg (Rosemary, Rosidah & Haro, 2014). For anticancer study, roselle ethyl acetate and chloroform extracts against unscheduled DNA synthesis (UDS) induced by t-BHP in primary hepatocytes rat. Moreover, roselle chloroform extract (0.10 mg/ml) and roselle ethyl acetate extract (0.20 mg/ml) were decrease the leakage of lactate dehydrogenase (LDH) and the formation of malondialdehyde (MDA) induced by 1.5 mM t-BHP (Tseng et al., 1997). Male mouse was injected tumor cell (B16-F1) into body and fed roselle methanolic extract at concentrations 0.5, 1.0, and 2.0% of mouse body weight for 3 weeks. The results exhibited the most efficiency to against tumor metastatic was roselle extract at concentrations 2% (Su, Wang, Huang, Lee, Chan & Chang, 2018). In diuretic activity, male rat was orally roselle aqueous extract at concentration 500, 1000, 1500, 2000 and 2500 mg/kg for 5 hr. The results were significantly at concentrations 1500, 2000 and 2500 mg/kg with urine excretion values 3.0, 4.3 and 4.4 ml/h, respectively (Alarcón-Alonso *et al*., 2012). Roselle tea able to increase diuretic activity in renal stone patients by consuming roselle tea 1.5 g twice daily for 15 days (Prasongwatana, Woottisin, Sriboonlue & Kukongviriyapan, 2008). For anti-microbial study, the study showed that roselle methanolic extract was toxic against bacteria: *Streptococcus mutans*, *Streptococcus sanguinis*, *Lactobacillus casei*, *Actinomyces naeslundii*, *Actinomyces actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Streptococcus mutan*, *Streptococcus sanguinis*, *Lactobacillus casei*, *Actinomyces naeslundii*, *Actinomyces actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* (Sulistyani, Fujita, Miyakawa, & Nakazawa, 2016). In addition, roselle was also toxic against *Candida albicans* (Alshami & Alharbi, 2014).

Table 2.1 Bioactivity of *Hibiscus sabdariffa*

Bioactivity	Extraction	Result	Reference
Antihypertensive	Aqueous	- Consumed roselle tea twice a day for 1 month reduced systolic (11.2%) and diastolic (10.7%) blood pressure in mild to moderate hypertension patients.	Faraji & Haji, 1999
		- Comparing efficacy of roselle aqueous extract and lisinopril in 193 patients with mild to moderate hypertension found that roselle extract displayed efficacy less than lisinopril, but it was safer than lisinopril.	Herrera *et al*., 2007
		- Studied of roselle tea for reduce blood pressure and blood sugar in patients by consuming 2 grams of roselle tea 2 times/day in 1 month found that roselle can reduce blood pressure and blood sugar	Mozaffri *et al*., 2007.
		- Consumption *Hibiscus sabdariffa* at dose 100 mg for 1 month in metabolic syndrome patients significantly decreased total cholesterol and glucose level.	Gurrola-Diaz *et al*., 2010

Table 2.1 Bioactivity of *Hibiscus sabdariffa* (Cont.)

Bioactivity	Extraction	Result	Reference
Antihypertensive (Cont.)	Aqueous	- For roselle freeze dried technique, roselle seeds had the highest total phenolic content of 2.97±0.17 mg of GAE/g and roselle calyces had total phenolic content 1.85±0.11 mg of GAE/g.	Esa *et al*., 2010
Antioxidant	Ethanolic	- Isolation of protocatechuic acid from extract and antioxidant testing by DPPH assay found that 0.1 mg/ml can scavenge radicals to 82% efficacy.	Tseng *et al*., 1996
		- The extract against superoxide anion radicals with value range 70-80% at dose of 1 g.	Mahadevan et al., 2009
	Ethyl acetate and chloroform	- Studied on antioxidant activity by DPPH assay found that ethyl acetate extract showed activity with EC_{50} value 0.017 mg/ml and chloroform extract had EC_{50} 0.15 mg/ml.	Tseng et al., 1997

Table 2.1 Bioactivity of *Hibiscus sabdariffa* (Cont.)

Bioactivity	Extraction	Result	Reference
Antioxidant (Cont.)	Methanolic	- Studied on HL-60 cells apoptosis in dose and time dependent of *Hibiscus sabdariffa* extract by using MTT assay displayed the extract inhibited 50% of HL-60 cell viability at concentration value 2.49 mg/ml. Moreover, the extract at concentration 3 mg/ml cytotoxic to HL-60 cells by decreased cell number 75% at 1 day.	Chang, Huang, Hsu, Yang & Wang, 2005
Anti-inflammatory	Ethanolic	- The extract with concentration 72 mg/day/200 g body weight and 288 mg/day/200 g body weight against TNF-α in diabetic rat.	Mardiah, Zakaria, Prangdimurti & Damanik, 2015
	Methanolic	- Adult male rats were fed *Hibiscus sabdariffa* extract at concentration 500 mg/kg for 11 weeks. The results displayed *Hibiscus* extract maintained the ratio of IL-1β/IL-1ra plasma and hippocampus levels in rat and against impairments in spatial memory consolidation.	Bayani *et al*., 2018
Antidiabetic	Aqueous	- Diabetic rat was treated HS extract 100 mg/kg and 200 mg/kg. The results exhibited insulin level decreased at HS concentration 100 mg/kg and HS 200 mg/kg able to reduce insulin level 20%.	Yang, Huang, Wang, Lee, Chen & Peng, 2013

Table 2.1 Bioactivity of *Hibiscus sabdariffa* (Cont.)

Bioactivity	Extraction	Result	Reference
Antidiabetic (Cont.)	Ethanolic	- The results displayed reduce blood glucose level in diabetes mice with concentrations 400 mg/kg and 600 mg/kg, but less than 0.65 mg/kg of glibenclamide drug.	Rosemary, Rosidah & Haro, 2014
Anticancer	Ethyl acetate and chloroform	- Both extracts against unscheduled DNA synthesis (UDS) induced by t-BHP at concentration value 0.20 mg/ml in rat primary hepatocytes. Moreover, roselle chloroform extract (0.10 mg/ml) and roselle ethyl acetate extract (0.20 mg/ml) able to decrease the leakage of lactate dehydrogenase (LDH) and the formation of malondialdehyde (MDA) induced by t-BHP (1.5 mM)	Tseng et al., 1997
	Methanolic	- Male mouse was injected tumor cell (B16-F1) into body and fed HS extract at concentrations 0.5, 1.0, and 2.0% of mouse body weight for 3 weeks. The results exhibited the most efficiency to against tumor metastatic was *Hibiscus sabdariffa* extract at concentrations 2% with the most body weight at value 22.7±1.29 g.	Su, Wang, Huang, Lee, Chan & Chang, 2018

Table 2.1 Bioactivity of *Hibiscus sabdariffa* (Cont.)

Bioactivity	Extraction	Result	Reference
Diuretic	Aqueous	- Male rat was orally HS extract at concentration 500, 1000, 1500, 2000 and 2500 mg/kg for 5 hr. The results were significantly at concentrations 1500, 2000 and 2500 mg/kg with urine excretion values 3.0, 4.3 and 4.4 ml/h, respectively.	Alarcón-Alonso *et al*., 2012
		- Comparison between healthy participants and renal stone patients were consumed roselle tea 1.5 g twice daily for 15 days. After intake, oxalate and citrate of healthy human were increased and significantly increased ($p<0.01$) in renal stone patients.	Prasongwatana, Woottisin, Sriboonlue & Kukongviriyapan, 2008
Antimicrobe	Methanolic	- The MIC and MBC of *Streptococcus mutan*, *Streptococcus sanguinis*, *Lactobacillus casei*, *Actinomyces naeslundii* and *Actinomyces actinomycetemcomitans* were 7.2 - 28.8 mg/ml and 57.6 - 57.6 mg/ml, respectively. Moreover, the MIC and MBC of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* were 7.2 – 14.4 mg/ml and 14.4 – 28.8 mg/ml.	Sulistyani, Fujita, Miyakawa, & Nakazawa, 2016
		- Roselle extract was toxic against *Candida albicans* with MIC ranging from 0.5 – 2 mg/ml.	Alshami & Alharbi, 2014

2.1.5 Thai Tradition medicinal use

There are many medicinal properties of roselle. Most traditional healers used decoction of roselle to increase the production of urine (diuretic effect). Moreover, roselle also has to anti-hypertensive properties. In research study showed that consumption of roselle calyx tea can reduce systolic (11.2%) and diastolic (10.7%) blood pressure in mild to moderate hypertension patients (Faraji & Haji, 1999; Herrera-Arellano *et al*., 2004). In addition, roselle tea can be used as a laxative drug and reduce fever. Consumption of roselle calyx tea 3 grams/day for 15 days can reduce uric acid in blood as a uricosuric agent (Prasongwatanaa, Woottisina, Sriboonluea, & Kukongviriyapan, 2008). Decoction of roselle roots is also used as a laxative drug. Roselle has high vitamin C which can be a remedy for scurvy. Moreover, infusion of leaves or calyces of roselle is used to remedy the common cold.

2.2 Spherification

William Peschardt is a scientist who discovered spherification techniques in 1942. Spherification is a cross-link formation of sodium alginate and calcium anion compound in order to produce a transparent gel-sphere form to coating solution inside (Chefsteps, 2017). The sphere capsules are able to release liquid after being chewed. Encapsulation has mainly 2 parts consisting of base solution and bath. There are 3 methods for molecular gastronomy: direct spherification, reverse spherification and frozen reverse spherification (Sen, 2017). Direct spherification uses sodium alginate mix in base juice, but reverse spherification uses calcium lactate or calcium lactate gluconate. In the bath solution part, the basic method uses calcium compound for cross-link with base solution. But reverse method uses sodium alginate to produce bath solution. Spherification is currently widely used, especially in collections of cultures, cuisine production and pharmaceutical (Laurienzo, 2010; Ching, Bansal, & Bhandari, 2017).

2.2.1 Ingredients of spherification

2.2.1.1 Sodium alginate

Sodium alginate ($NaC_6H_7O_6$) is an anionic sodium salt with alginic acid which is obtained from brown algae (Pubchem, 2005). L-guluronate and D-mannuronate are mainly components in a polysaccharide of the compound (Lee KY & Mooney DJ, 2012). This compound is often used as a food ingredient for stabilizer and texture jellification (Ching, Bansal, & Bhandari, 2017).

2.2.1.2 Calcium chloride

Calcium chloride is a bitter inorganic salt compound with empirical formula $CaCl_2$. It is a white crystal solid at room temperature and easily soluble in water. In solution, chloride anions can replace other ions in chemical interactions (Neyraud & Dransfield, 2004). There are many advantages of calcium chloride such as food stabilizer, food additive and food preservative.

2.2.1.3 Calcium lactate

Calcium lactate is a white powder with chemical formula $C_6H_{10}CaO_6$ that is composed of calcium and two lactate radicals. U.S. Food & Drug Administration of United States (2018) approved calcium lactate can be used as a flavor agent, food stabilizer, supplement and solidifier. Moreover, calcium lactate is used for reverse spherification in food molecular gastronomy by mixing with flavored liquid and dropping into sodium alginate solution (everydayhealth, 2018).

2.2.1.4 Citric acid

Citric acid is an organic acid with chemical formula $C_6H_8O_7$. It exists in fruits and vegetables. The most concentrated source of citric acid is citrus fruits, especially lemon and orange (Berovic & Legisa, 2007). Citric acid is an antioxidant natural substance for preservative and additive in foods and beverages with a sour acidifier (Ryan *et al*., 2019).

2.2.1.5 Sucrose

Sucrose is a sugar that is derived from photosynthesis in plants such as sugarcane or sugar beet. This compound is a disaccharide (glucose+fructose) sweetener additive in food and beverage production. Sucrose is a carbohydrate with

empirical formula $C_{12}H_{22}O_{11}$. Sucrose is a white crystalline solid, odorless and highly soluble in both cold and hot water (Onaolapo & Onaolapo, 2018).

2.2.2 Direct spherification

Direct spherification is a basic technique for creating a liquid in a squishy membrane. Preparation of basic spherification is by mixing flavored juice with sodium alginate and dropping in a calcium bath. Jellification of a flavorless thin membrane occurs in a short time and may be rinsed by using plain water. If the capsule is left in calcium bath for a long time, the liquid capsule will have a more bitter taste and a thicker layer. This basic technique produces small flexible sphere molecular food such as caviar. Direct spherification is an easy method and it doesn't take a long time to create spheres because alginate solution can be dropped into a calcium bath immediately (Molecularrecipes.com, 2014). However, encapsulation is not created in high acidity (pH < 3.6) and high calcium (e.g.milk and cheese) in palatable base. Moreover, it has a short storage period and deformation of beads will occur (Sen, 2017).

2.2.3 Reverse spherification

Reverse spherification is one popular technique in molecular gastronomy. The sphere liquid is obtained from chemical reaction between calcium in flavored based solution and sodium alginate in the bath. The calcium solution must freeze to solidify before dispersal in sodium alginate bath. Calcium lactate or calcium lactate gluconate are mostly used, but calcium chloride id rarely used in reverse technique because it leads to bitter food (Lee & Rogers, 2012). This method can determine size and quantity of spheres because flavored solution must freeze in a mold before placing into bath. Frozen reverse spherification is suitable for liquids that containing alcohol, calcium and have high acidity (Molecularrecipes, 2014). Frozen reverse spherification products can store longer than direct spherification products. In previous study, xanthan gum was a polymer used to preserve the form of beads (Tsai F, Chiang P, Kitamura Y, Kokawa M, & Islam MZ, 2017).

2.3 Yogurt production

2.3.1 Fermentation process of yogurt

Yogurt production must comply with the rules of the Food and Drug Administration (FDA, 2013). Yogurt is a fermentation between milk and main standard cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or other subspecies of *Lactobacillus*). For the yogurt process, the milk is pasteurized at temperature more than 72°C for 15 min to eliminate pathogen and spoilage organisms (yeast and molds) in milk. After that, the pasteurized milk is cooled down to the ideal temperature (42-45°C) for the growth of starter cultures. During the fermentation process, lactose sugar in milk is used as energy for the growth of the culture. The starter culture is digested the lactose sugar in milk become to lactic acid. The increment of lactic acid effect on pH reduction. Due to the more acidic, the protein in milk coagulates and precipitates out. The proteolytic activities denatured casein matrix by converting milk protein to peptides and essential amino acid (Burgain *et al*., 2014; Widyastuti, Rohmatussolihat & Febrisiantosa, 2014). This activity leading the coagulum network more sensitive to syneresis. After that, the curd formation is occurred and thickening the milk into a yogurt characteristic. Moreover, lactic acid also produces the unique flavor from the fermentation process. The process is taken several hours up to spices of bacteria and finish until the specific pH of yogurt is reached. The yogurt product is stored at 4°C. Furthermore, the yogurt is determined for quality control. Governmental regulations require a quality test to ensure that the product is safety (Table 2.2).

Table 2.2 Quality control of yogurt (FDA, 2013)

Parameters	Content
Protein (% by weight)	more than 2.7%
Butterfat (% by weight)	less than 15%
Acidity (% by weight)	more than 0.6%
Colony count (colonies/gram)	more than 10^7

Coliform bacteria (%/gram)	less than 3
Mold (colonies/gram)	less than 100
Yeast (colonies/gram)	less than 100

2.3.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are gram-positive facultative anaerobic microorganisms that found naturally and in dairy products. LAB was used as a preservative and to enhance the flavor of products. In milk fermentation, active bacteria metabolize lactose sugar in milk into lactic acid. LAB digests protein in milk to semi-solid state. There are many benefits of lactic acid bacteria: food fermentation, medicinal drug, cosmetic, solvent and plastic industries.

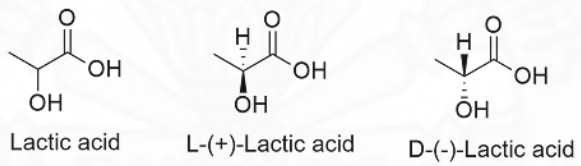


Figure 2.5 Structure of lactic acid bacteria

2.3.2.1 *Lactobacillus*

Lactobacillus represents a highly diverse genus of gram-positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacilli. Species within this genus are generally catalase-negative, although a few strains decompose peroxide by a non-heme-containing pseudo-catalase. *Lactobacillus spp.* are either homofermentative or heterofermentative with regard to hexose metabolism. Physiological characteristics are used to identify some species of *Lactobacillus* (Hayward *et al*., 1995).

2.3.2.2 *Lactococcus*

Lactococcus is a homofermentative gram-positive, catalase-negative and non-sporulating bacterium. They are characterized by spherical shape that appears in pairs or chains. Previous study reported that *Lactococcus* can

produce L-lactic acid. In addition, some studies have reported D-lactic acid can be produced when cultured at low pH (Aguirre & Collins, 1993).

2.3.2.3 *Pediococcus*

Pediococcus are characterized as being gram-positive, nonmotile, catalase-negative and aerobic to microaerophilic bacteria. Growing cultures commonly possess the ability to form l-lactate from l-malic acid. *Pediococcus* are chemo-organotrophs and require complex growth factors and amino acids. In addition, these are the only lactic acid bacteria that divide in two planes, which results in the formation of pairs, tetrads or large clumps of spherical cells (Gunther & White, 1961).

2.3.2.4 Benefits of lactic acid bacteria

Lactic acid bacteria have many benefits. Baricault *et al* (1995) studied the effect of fermented milks on colon cancer cell growth in cancer cell line (HT-29). HT-29 cells were cultured in milk fermented by one of the following bacterial genera: *Lactobacillus helveticus*, *Bifidobacterium*, *Lactobacillus acidophilus* or mix of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The most efficient strains in lowering 50% HT-29 growth rate was *Lactobacillus helveticus*. Moreover, researchers studied the anti-metastatic activity of orally administered *Lactobacillus casei* for 11 days by using an experimental model of hepatic metastasis. The result showed that *Lactobacillus casei* lowered the incidence of hepatic metastasis at dose 100 mg/kg per day (Tazawa *et al*., 1997). In a clinical study, Hata *et al* (1996) studied the effect of consuming sour milk with lactic acid microorganisms, *Lactobacillus* and *Zoocarcinamide* on blood pressure. It was a placebo-controlled study in hypertensive patients for 8 weeks. After consuming sour milk 95 ml/day, systolic blood pressure dropped in week 4 (9.4 ± 3.1 mmHg) whereas diastolic blood pressure decreased in week 8 (6.9 ± 2.2 mmHg). In 2005, researchers studied the effect of fermented milk with *Lactobacillus helveticus* in placebo-controlled double-blind study in moderate and mild hypertensive patients for 4 weeks. In the high hypertensive group, blood pressure changed from 137/85 to 133/80 mmHg in week 1, and 133/80 mmHg in week 4. In the mild hypertensive group, blood pressure changed from 149/93 to 142/89

mmHg in week 1 and 139/86 mmHg in week 4. Results of either moderate or mild hypertensive group were lower than the placebo group (Aihara *et al*., 2005).

2.3.3 *Glycine max* (L.) Merr (Soybean)

Figure 2.6 *Glycine max* (L.) *Merr* (Soybean)

Family name: FABACEAE (LEGUMINOSAE)

Common names Soya bean, Soya, Haba soya, Soja bean, Miracle bean, Utaw

Part use: Mature seeds

Flavor: Sweet

Soybean is widely consumed in many countries. The plant is a naturally excellent source of essential proteins, vitamins, minerals and fatty acids (Table 2.3). It contains no cholesterol and little or no saturated fat.

Table 2.3 Nutritive value of soybeans 100 grams (USDA, 2018)

Calories	446	Kcal
Carbohydrate	30.16	g
Protein	36.49	g
Total lipid	19.94	g
Fiber	9.30	g
Sugars	7.33	g

Calcium	277	mg
Iron	15.70	mg
Magnesium	280	mg
Phosphorus	704	mg
Potassium	1797	mg
Sodium	2	mg
Zinc	4.89	mg
Copper	1.66	mg
Manganese	2.52	mg
Selenium	17.80	µg
Thiamin	0.87	mg
Riboflavin	0.87	mg
Niacin	1.62	mg
Folate	375	µg
Choline	115.90	mg

2.3.3.1 Botanical morphology

Soybeans (legume plant) can grow up to 1.5 meters tall with an erect shape, especially successive in hot condition. The stems are green and covered with brown hair. The leaves are green alternate, trifoliate with ovate leaflets and entire edge. The single or cluster flowers are white-purple color with 5 petals. The fruit (pod) can grow up to around 10 centimeters with green straight shape and become to brownish color when it mature.

2.3.3.2 Bioactive constituents of soybeans

Table 2.4 Bioactive constituents of soybeans (Silva *et al*., 2013)

Bioactive constituents	Compound	Value (mg/kg)
Phenolic acid	Caffeic acid	25.9±0.9
	5-O-caffeoylquinic acid	5.4±0.0
	p-coumaric acid	9.5±0.0

	Ferulic acid	1.6±0.0
Flavonoid	Quercetin-3-O-rutinoside	29.5±0.3
	Kaempferol-3-O-glucoside	1.9±0.0
	Kaempferol-3-O-rutinoside	12.9±0.5
Isoflavones	Daidzein	9.8±0.4
	Genistein	5.7±0.1
	Daidzin	556.4±12.8
	Genistin	52.9±2.4
Sterols	Stigmasterol	3.5±0.3
	Campesterol	63.1±0.1
	β-Sitosterol	12.8±0.3
Organics acid	Oxalic acid	2730.4±13.2
	Aconitic acid	50.5±0.8
	Citric acid	5113.5±201.1
	Malic acid+quinic acid	18974.4±187.3
	Succinic acid	54928.2±111.1
	Fumaric acid	14869.2±174.0
Fatty acid	Lauric acid	8.5±0.8
	Tridecanoic acid	3.2±0.3
	Myristic acid	64.4±1.9
	Pentadecanoic acid	61.4±1.8
	Palmitic acid	729.8±44.1
	Palmitoleic acid	14.6±1.2
	Heptadecanoic acid	121.5±7.7
	Stearic acid	633.6±20.6
	Oleic acid	340.2±5.5
	Elaidic acid	16.3±1.3
	Linoleic acid	153.4±6.9
	Arachidic acid	184.1±3.4
	Heneicosanoic acid	33.8±0.1

	Behenic acid	206.9±3.7
	Tricosanoic acid	86.6±4.0
	Lignoceric acid	116.7±4.4

2.3.4 Soy yogurt

Soy yogurt has been popular through the Mediterranean and Middle East Asia for a long time. According to U.S. patent number US4664919, Yan & Peng studied the fermentation of soy yogurt by lactic acid bacteria (*Streptococcus sojalactis*) in 1987. Active bacteria were added to soymilk 4-6% and fermented at 35-40°c for 5-8 hr. In 1998, inventor studied mixtures of bacteria for soy yogurt production. There are four active strains in soy yogurt. The three major strains are *Lactobacillus bulgaricus*, *Lactobacillus casei* and *Lactobacillus germentum*. One minor strain from *Lactobacillus helveticus*, *Lactobacillus bifudus*, *Lactobacillus lactis*, *Lactobacillus thermophiles*, *Lactobacillus fermetti*, *Lactobacillus amylovorus*, *Lactobacillus pentosaceus*, *Lactobacillus salivaroes*, *Lactobacillus brevis*, *Lactobacillus leichmannii*, *Lactobacillus plantarum*, *Lactobacillus cellobiosus*, *Lactobacillus coryniformis*, *Lactobacillus curvatus*, *Lactobacillus buchneri*, *Lactobacillus fermentum* and *Lactobacillus viridescens* is included. In 2010, inventors studied the fermentation of soy-based products. The bacteria were separated into two groups. First group contained mesophilic cultures. The other group had *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Propionibacterium*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus casei*. The fermentation was incubated at 15-37°C for 5-10 hr with amounts of bacteria between 10^5 -10^9 CFU/ml (patent no. WO 2010136321). In 2012, a Chinese researcher (patent no. CN 102511562) developed soy yogurt from a mixture of strains (*Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus plantarum* and *Streptococcus thermophiles*). *Lactobacillus casei* was used to produce soy yogurt in 2013 (W02013113966 A1). Moreover, Tsuchimoto *et al*. (2015) improved soy yogurt flavor satisfactorily from *Lactobacillus brevis* (patent no. US20150164098 A1). The combination of microorganisms was digested monosaccharide into lactic acid. The protein in soymilk was precipitated and curd

forming. After that, soy yogurt displayed unique flavor and aroma. However, most of consumers is unpleasant beany flavors from soy-based product. Beany flavors are occurred during fermentation process. The unsaturated fatty acids were broken down into hexanal, and methanethiol by lipoxygenase enzyme (Wszelaki *et al.*, 2005; Lv, Song, Li, & Guo, 2011). Lipoxygenase enzyme containing the form of three isozymes (Lox-I, Lox-II and Lox-III) and conjugated with the break down unsaturated fatty acids that effected to the off-flavor in soy product (Wszelaki *et al.*, 2005).

Table 2.5 Nutritive value of soy yogurt 100 grams (USDA, 2016)

Calories	94 Kcal	% Daily Value
Total Fat	1.8 g	2%
Saturated fat	0.3 g	1%
- Polyunsaturated fat	1 g	
- Monounsaturated fat	0.4 g	
Cholesterol	0 mg	0%
Sodium	35 mg	1%
Potassium	47 mg	1%
Total Carbohydrate		5%
- Dietary fiber	0.2 g	0%
- Sugar	1.2 g	
Protein	3.5 g	7%
Calcium		11%
Magnesium		10%
Iron		6%

2.3.5 Determination of yogurt production

2.3.5.1 Analysis of microbial content

In order to determine the number of colonies in yogurt, the pour plate technique is used for colonies count. Take a 0.5 grams sample and warm MRS broth and add into petri dish. The mixture is combined by swirling. The plate is incubated at 37°C for 24 hr. After that, amounts of bacteria are determined by using ten-fold serial dilution technique.

2.3.5.2 Sensory evaluation

The most important parameters of the product is organoleptic characteristics. The objective is to determine the quality of the product. Sensory assessment is a survey of physical stimuli and sensory responses of consumers (Lim, 2011). Soy yogurt was evaluated by using a 5-point hedonic scale questionnaire. There are 5 points to evaluate: external appearance, smell, flavor, texture and overall acceptance. The evaluation rates using 5-point scale are: 1 = strongly dislike, 2 = slightly dislike, 3 = neither like nor dislike, 4 = slightly like and 5 = strongly like.

2.3.5.3 Properties evaluation

(1) pH value

pH value measures the acid amount in the product by using a pH meter. During fermentation, lactic acid bacteria produce lactic acid to metabolize protein. Bacterial activity is stopped by cooling. Decrease of pH results in growth of culture. The suitable pH for yogurt is between 4.0-4.5 (FDA, 2013).

(2) Titrated acidity

Titrated acidity is determined the concentration of total lactic acid by using neutralization reaction method (GPSIL, 2017). A burette is the basic titrator for this technique. The acidity of the sample is identified by titrating sodium hydroxide into a sample containing phenolphthalein indicator. Quantity of titrand is calculated and expressed as %lactic acid after chemical reaction is finished. %lactic acid has direct relation with pH of product.

(3) Viscosity

Viscosity is a parameter for measuring flow resistance of a substance (e.g. liquid, semi-solid and solid). The viscometer is an instrument to monitor efficacy and quality of a product. This parameter describes the resistance of yogurt texture. Low viscosity result demonstrated product is a fluid, whereas high viscosity exhibits more resistance of product (thickness), such as a gel or thick emulsion (brookfieldengineering, 2018).

(4) Syneresis

Syneresis is when a liquid is expelled from a product such as when liquid puddles on top of yogurt after fermentation process. In the thermal process, an imbalance of molecular reaction occurs while curd is created. It is a problem if product has an unpleasant texture by syneresis. Nowadays, there are many natural ingredients to maintain product structure: guar gum, xanthan gum, locust bean gum, and carrageenan. These are well-known stabilizers is in the food industry. But stabilizers can initiate change of texture and viscosity (WFS, 2013).

(5) Colorimetric

Colorimeter theory based on human color perception. There are 3 colors receptors of eye retina: red, green, and blue which contribute to white light for eye vision (photonic, 2018). This method used for measured intensity color of specimen by using colorimeter. The instrument detect color passing directly specimen. Colorimetric is widely used in food industrial for food quality inspection.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Chemical reagents, material and equipment

Table 3.1 List of chemical reagents

Name	Source
Assay for antioxidant activity	
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Fluka, Germany
2,6-Di-tert-butyl-4-methylphenol (BHT)	Fluka, Germany
Absolute ethanol	RCL Labscan, Thailand
Distilled water	Milford, USA
Assay for Nitro blue tetrazolium (NBT)	
Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO)	RCL Labscan, Thailand
Hanks' Balanced Salt Solution (HBSS)	Gibco, USA
Nitrotetrazolium blue chloride (NBT)	Sigma, USA
PMA ($C_{36}H_{56}O_8$)	Sigma, USA
RPMI medium 1640	Gibco, USA
Trypan blue stain 0.4%	Gibco, USA
Assay for Anti-inflammatory activity	
Dimethyl sulfoxide [$(CH_3)_2SO$] (DMSO)	RCL Labscan, Thailand
Distilled water	Milford, USA
Fetal Bovine Serum (FBS)	Biochem, Germany
Hydrochloric acid (HCl)	Univar, Australia
Isopropanol	RCL Labscan, Thailand
Lipopolysaccharide from *E.coli* O55:B5	Sigma, USA
N-(1-Naphthy)ethylenediamine dihydrochloride	Sigma, USA

Table 3.1 List of chemical reagents (Cont.)

Penicillin-Streptomycin (P/S)	Gibco, USA
Phosphate Buffered Saline (PBS)	Amresco, USA
Phosphoric acid solution	Sigma, USA
Prednisolone ≥ 90%	Sigma, USA
DMEM medium	Gibco, USA
Sodium bicarbonate ($NaHCO_3$)	BHD, England
Sodium hydroxide (NaOH)	Univar, Australia
Sulfanilamide ($H_2NC_6H_4SO_2NH_2$)	Sigma, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
Trypan blue stain 0.4%	Gibco, USA
Trypsin - EDTA	Gibco, USA
Assay for Total Phenolic Content	
Folin-Ciocalteu's reagent	Fluka, Germany
Gallic acid [$(HO)_3C_6H_2CO_2H$]	Sigma, USA
Sodium carbonate anhydrous (Na_2CO_3)	Merck, Germany
HPLC technique	
Acetronitrile	RCL Labscan, Thailand
Chlorogenic acid	Aldrich, USA
Coumaric acid	Sigma, USA
Cyanidin-3-o-sambubiosides	Sigma, USA
Ferulic acid	Aldrich, USA
Methanol	RCL Labscan, Thailand
Phosphoric acid	RCL Labscan, Thailand
Quercetin	Aldrich, USA
Ultra-Pure water	RCL Labscan, Thailand

Table 3.1 List of chemical reagents (Cont.)

Isolation of Lactic Acid Bacteria (LAB)	
Agar powder	Pearl Mermaid, Thailand
Agarose powder	Thermo scientific, USA
Calcium carbonate	Unilab, USA
Deoxynucleotides (dNTPs)	Thermo scientific, USA
Gel/PCR Kit	Geneaid, Taiwan
Glycerine	Sigma, USA
Lactobacilli MRS broth	Difco, USA
Loading dye	Thermo scientific, USA
Magnesium chloride	Thermo scientific, USA
Skim milk	Difco, USA
Sodium chloride	Sigma, USA
TAE buffer	Thermo scientific, USA
Taq buffer A	Thermo scientific, USA
Taq DNA polymerase	Thermo scientific, USA
Yeast extract	Difco, USA
Spherification	
Calcium chloride	Merck, USA
Calcium lactate	Lab valley, Thailand
Citric acid	Mathawach, Thailand
Salt	Prung thip, Thailand
Sodium alginate	Lab valley, Thailand
Sucrose	Mitrphol, Thailand

Table 3.2 List of materials and equipment

Name	Source
96 well-plate sterile	Costar Corning, USA
96 well-plate non-sterile	Costar Corning, USA
Autoclave	Hirayama, Japan
Centrifuge machine	Boeco, Germany
Centrifuge tube 15, 50 ml	Costar Corning, USA
CO_2 incubator	Forma, USA
Colorimeter CR-400	Konica Minolta, USA
Cryogenic tube 2 ml	Corning, USA
Disposable pipette 5, 10 and 25 ml	Corning, USA
Eppendorf	Costar Corning, USA
Erlenmeyer flasks	Schott Duran, Germany
Filter paper no.1 (125 mmø)	Whatman, USA
Freezer	Sanyo, Japan
Glass bottle 250, 500 and 1000 ml	Schott Duran, Germany
Hematocytometer	Boeco, Germany
High Performance Liquid Chromatography (HPLC)	Agilent technology, USA
Hot air oven	Memmert, Germany
Hot plate	Thermolyne, USA
HPLC column	Phenomenax, USA
Inverted microscope	Nikon, Japan
Laminar air flow	Faster, Italy
Microcentrifuge tube	Costar Corning, USA
Micropipettes 1-20 µL, 20-200 µL, 100-1000 µL	Eppendrof, Germany
Microplate reader	Biotek, USA
Multi-channel pipette	Costar Corning, USA
pH buffer	Thermo Scientific, USA
pH meter	WTW inolab, Germany
Pipette tips	Costar Corning, USA

Table 3.2 List of materials and equipment (Cont.)

Pipette boy	Integra biosciences, Switzerland
Reagent reservoir	Costar Corning, USA
Rotary evaporator	Buchi, Switzerland
Sonicator	Elma, Germany
Syringe 5, 10 mL	Nipro, Thailand
Tissue culture flask 75 cm^3 with filter cap	Costar Corning, USA
Viscometer	Ametek Brookfield, USA
Vortex	Scientific industries, USA
Water bath	Memmert, Germany

3.2 Plant material and extraction

Calyces of *Hibiscus sabdariffa* L. or Sudarese roselle were collected from Songkla province. The specimen voucher number is SKP 109081901 that was identified at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Thailand. Aqueous extract of *Hibiscus sabdariffa* L. was prepared by decoction in water and evaporated by using spray dried technique. In the present study, roselle aqueous extract was obtained from Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University.

3.3 Determination of antioxidant activity

3.3.1 Determination by DPPH radical scavenging assay

3.3.1.1 Principle of DPPH radical scavenging assay

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a stable free radical that has an unpaired electron. DPPH has a purple color. Decolorization of DPPH will occur when it accepts an electron from an antioxidant compound. The activity will be

measured at absorbance 520 nm (Yamasaki, 1994; Molyneux, 2004; Kedare & Singh, 2011).

3.3.1.2 Preparation of sample solution

Roselle aqueous extract and roselle spherification was dissolved and adjusted to 1 mg/ml in distilled water. Then, sample solution was diluted into 1, 10, 50 and 100 µg/ml in distilled water. Moreover, hydrolysis extracts (HCl-$CHCl_3$, DI-$CHCl_3$ and HCl) and butylated hydroxytoluene (positive control) were diluted with absolute ethanol to the same concentrations.

3.3.1.3 Evaluation by DPPH radical scavenging assay

DPPH solution was prepared at concentration of $6x10^{-5}$ M by using absolute ethanol. Then, 100 µl of sample solution and BHT were added into each well of 96-well microplate followed by 100 µl of DPPH solution. After that, 96-well microplate was kept in dark room at room temperature for 30 min. Sample absorbance was measured at 520 nm by microplate reader. The percentage of inhibition was calculated by the formula below.

$$\%\text{Inhibition} = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}} \times 100$$

3.3.2 Determination by Nitro Blue Tetrazolium (NBT) assay

(Surarit *et al*., 2014)

3.3.2.1 Preparation of cell line

HL-60 (Human promyelocytic leukemia cell line) was purchased from ATCC, USA and cultured in RPMI 1640 medium contained with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained at 37°C incubation with 5% CO_2 atmosphere and 95% humidity. After that, HL-60 cells were induced by 1.3% DMSO in RPMI medium for 6 days.

3.3.2.2 Preparation of sample solution

Roselle aqueous extract was dissolved in sterile distilled water and filtered by 0.22 microns sterile filter, whereas hydrolyzed extract was dissolved by sterile dimethyl sulfoxide (DMSO) to 50 mg/ml concentration. Then, sample solution

was diluted into 50 and 100 µg/ml in RPMI medium. Propyl gallate was used as positive control and diluted to 1, 10, 50 and 100 µg/ml in RPMI 1640 medium.

3.3.2.3 Evaluation by Nitro blue tetrazolium (NBT)

The HL-60 cells line (7.5×10^5 cells/ml) were resuspended in HBSS and incubated with sample concentrations at 37°C for 15 min. Then, HL-60 cells were incubated with PMA 50 µl (250 ng/ml) and NBT 250 µl (1.25 mg/ml) for 1 hr. After that, 2 ml of HCl were added and centrifuged at 4,000 rpm for 10 min. Formazan residues were dissolved by DMSO 300 µl. The sample absorbance was measured at wavelength of 572 nm by microplate reader. The percentage of inhibition was calculated by the formula below.

$$\%\text{Inhibition} = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}} \times 100$$

Cytotoxicity of cell was also determined by using MTT colorimetric method. The inflamed HL-60 cells (7.5×10^5 cells/ml) were dissolved in HBSS and incubated with sample concentrations at 37°C for 15 min. After that, HL-60 cells were incubated in HBSS 250 µl and PMA 50 µl for 1 hr, centrifuged at 4,000 rpm for 10 min and the supernatant was removed. HBSS and MTT were added into cells and the tube was incubated for 2 hr. Then, the supernatant was removed and replaced with 300 µl DMSO. The sample absorbance was measured at wavelength of 570 nm by microplate reader. The percentage of cell survival was calculated by the formula below.

$$\%\text{Survival} = \frac{\text{ODsample}}{\text{ODcontrol}} \times 100$$

3.4 Determination of Anti-inflammatory activity

3.4.1 Principle of nitric oxide production inhibition

Nitric oxide is a free radical that is synthesized from amino acid L-arginine by nitric oxide synthases (NOS). In the NOS family, inducible nitric oxide

synthase (iNOS) is a main source of produce by macrophage during inflammation. When the cell is stimulated by LPS, nitric oxide is released from the cell and become to nitrite. After that, griess reagent will react with nitrite. If the cell is inflamed, the solution in the microplate will be pink. On the other hand, if the extract can resist inflammation, the color of the solution will soften.

3.4.2 Preparation of cell line

RAW 264.7 (Murine macrophage cell line was purchased from ATCC, USA) cells were cultured in DMEM medium containing with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained at 37°c with 5% CO_2 atmosphere and 95% humidity.

3.4.3 Preparation of sample solution

Roselle aqueous extract was dissolved in distilled water, whereas hydrolyzed extract was dissolved in sterile dimethyl sulfoxide (DMSO) to 50 mg/ml concentration. Then, sample solution was diluted into 1, 10, 30, 50 and 100 µg/ml in completed DMEM medium. Prednisolone was used as a positive control at same concentration and diluted to 0.1, 1, 10 and 50 µg/ml in DMEM medium.

3.4.4 Evaluated for nitric oxide production inhibition effect

Cultured RAW264.7 cells were seeded in 96-well microplate with $1x10^5$ cells/well for 24 hr at 37°C. After incubation, 100 µl of DMEM in each well was removed and replaced with 100 µl fresh DMEM medium containing 2 ng/ml of lipopolysaccharide (LPS) in A-D rows of 96 well plate and DMEM medium without LPS in E-H rows. Various sample concentration (1, 10, 50 and 100 µg/ml) were added into each well and incubated at 37°C for 24 hr. After that, 100 µl of supernatant was removed to another 96-well microplate and 100 µl of Griess reagent added to determine nitric oxide production. The sample absorbance was measured by microplate reader at wavelength of 570 nm. The percentage of inhibition was

calculated by the formula below and IC_{50} was calculated by statistic program (Tewtrakul & Itharat, 2007).

$$\%\text{Inhibition} = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}} \times 100$$

Cytotoxicity was also determined by MTT colorimetric method. MTT (10 µl/well) was added into 96-well microplate and incubated at 37°C for 2 hr. Then, supernatant liquid was removed and replaced with isopropanol containing 0.04 M HCl to dissolve formazan in the cells. Formazan production was measured at wavelength of 570 nm by microplate reader. The percentage of cell survival rate was calculated by the formula below

$$\%\text{Survival} = \frac{\text{ODsample}}{\text{ODcontrol}} \times 100$$

3.5 Determination for Total Phenolic Compound

3.5.1 Principle of Determination for Total Phenolic Compound

Total phenolic compound responds to Folin-Ciocalteu's reagent which contains phosphomolybdate. Phosphotungstic acid reagent is reduced by phenolic hydroxyl group of total phenolic compounds and becomes tungsten and molybdenum blue that gives blue color. It is inspected at wavelength 765 nm by using microplate reader.

3.5.2 Preparation of sample solution

10 mg of roselle aqueous extract, hydrolyzed extract and roselle spherification were weighed and adjusted to concentration 1 mg/ml. Aqueous extract and roselle spherification were dissolved in distilled water and diluted into 500, 1000 µg/ml. Hydrolyzed extract was dissolved in absolute ethanol and diluted to the same concentrations. Moreover, 1 mg of gallic acid was measured and concentration

adjusted to 1 mg/ml by using absolute ethanol. Standard stock was diluted by serial dilution technique to 5, 10, 25, 50, 100, 250 and 500 µg/ml.

Preparation of 75% w/v sodium carbonate (Na_2CO_3), was by weighing 3.75 g into 50 ml volumetric flask and adding distilled water to 50 ml. 5 ml (5000 µl) Folin-Ciocalteu's reagent was pipetted into 50 ml volumetric flask and distilled water added to 50 ml and mixed well. Solution was incubated at room temperature in a dark room.

3.5.3 Evaluated for Total Phenolic Content

20 µl sample solution and 20 µl gallic acid solution were added into 96-well microplate. After that, 80 µl of sodium carbonate and 100 µl of Folin-Ciocalteu's reagent were added in each well. The, 96-well microplate was kept in room temperature for 30 min. The sample absorbance was measured at 765 nm by using microplate reader.

3.6 Effect of acid hydrolysis on biological activity and chemical content of roselle aqueous extract (Ren *et al*., 2016; Jiang *et al*., 2018)

This study simulated gastric juice in the gastrointestinal tract (GI) system. There are 3 methods of study such as hydrochloric acid-chloroform, distilled water-chloroform and decoction in hydrochloric acid. All extracts that were obtained from three methods were investigated the inhibition of nitric oxide production, antioxidant activity and total phenolic content.

3.6.1 Hydrochloric acid-chloroform (HCl-$CHCl_3$) procedure

(Ren *et al*., 2016; Jiang *et al*., 2018)

Roselle aqueous extract (20 g) was boiled with 0.01 N hydrochloric acid at 80°C for 15 min and cooled down. Then, chloroform was added into solution at ratio 1:1. The hydrolysed roselle extract was obtained from the chloroform fraction which was evaporated and stored at -20°C.

3.6.2 Distilled water-chloroform (DI-$CHCl_3$) procedure

(Ren *et al*., 2016; Jiang *et al*., 2018)

Roselle aqueous extract (20 g) was decocted in 400 ml distilled water at 80°C for 15 min and cooled down. Then, chloroform equal to solution volume was added. After that, chloroform fraction was collected, evaporated and maintained at temperature -20°C. This extract was used as negative control.

3.6.3 Hydrochloric acid procedure (Jiang *et al*., 2018)

Roselle aqueous extract (20 g) was boiled with 0.01 N hydrochloric acid at 80°C for 15 min and cooled down. The extract was filtered through Whatman No. 1 filter paper. Then, solvent was neutralized with 0.01 N sodium hydroxide. The mixture was dried by a vacuum freeze dryer and kept at -20°C.

3.7 Determination of chemical substances in roselle extract

3.7.1 Principle of HPLC technique

High Performance Liquid Chromatography (HPLC) is a widely used technique to identify and separate components of mixtures. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). After injection analysis, the components move through mobile phase and stationary phase. High polar compounds were separated faster than non-polar compounds. The amount of a compound is determined from peak area.

3.7.2 Instruments and chromatographic conditions

The chemical fingerprint in roselle extract was determined by using High Performance Liquid Chromatography (HPLC) technique with modified method (Itharat & Sakoakdeejaroen, 2010). The HPLC system (Agikent® 1200) includes solvent degasser (G1322A), solvent pump (G1311A), autosampler (G1329A), column oven (G1316A) and photodiode array detector (G1315D). Chromatographic data was processed by using Chemstation software (B.04.01 SP1).

Chromatography was carried out along with C18 guard column (Phenomenax® Luna, 4.6 x 150 mm, 10 micron). Sample 10 µl were injected into HPLC system and separated by combination of 0.1% phosphoric acid (A) and 100% acetonitrile (B). There were 3 solvents in the elution program (De Ancos *et al*., 2000): isocratic elution with 6% solvent B from 0 to 10 min, linear gradient to 20% solvent B from 10 to 55 min and isocratic elution at 20% solvent B from 50 to 60 min at flow rate 1 ml/min. 5 bioactive markers were used for evaluated amount of compound in extract included chlorogenic acid (Aldrich, USA), coumaric acid (Sigma, USA), ferulic acid (Aldrich, USA), quercetin (Aldrich, USA) and cyanidin-3-o-sambubiosides (Sigma, USA). The detection wavelength of UV spectrum was screened for identified maximum absorbance of each compound. Therefore, the wavelength detection was selected at 325 nm as the detection of chlorogenic acid, coumaric acid and ferulic acid, 365 nm as the detection of quercetin and 520 nm as the detection of cyanidin-3-o-sambubiosides.

3.7.3 Preparation of standard chemical fingerprint

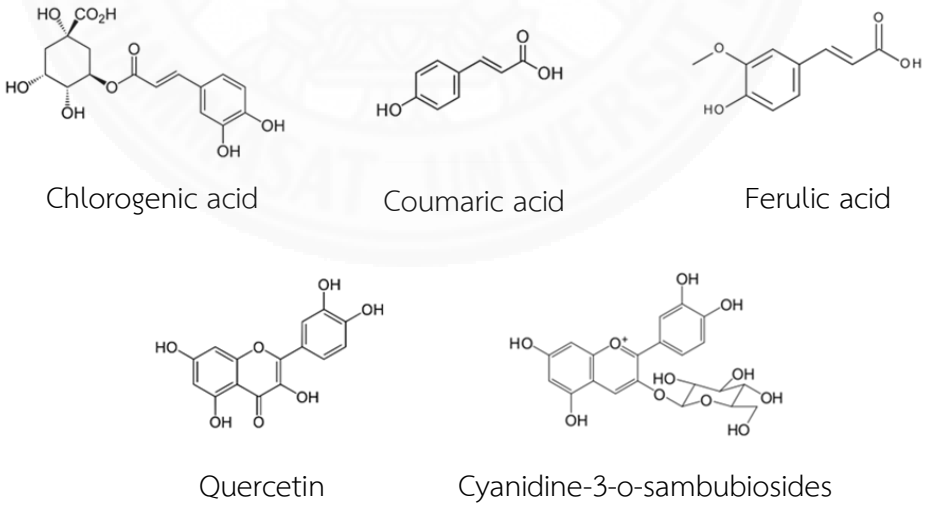


Figure 3.1 Structure of standard marker compounds

3.7.3.1 Chlorogenic acid

Chlorogenic acid 1 milligram was diluted into 10, 20, 40, 60, 80 and 100 µg/ml in 50% methanol (Labscan, Thailand).

3.7.3.2 Coumaric acid

Coumaric acid 1 milligram was diluted into 5, 10, 50, 100, 200 and 300 µg/ml in 50% methanol (Labscan, Thailand).

3.7.3.3 Ferulic acid

Ferulic acid 1 milligram was diluted into 10, 20, 40, 60, 80 and 100 µg/ml in 50% methanol (Labscan, Thailand).

3.7.3.4 Quercetin

Quercetin 1 milligram was diluted into 5, 10, 50, 100, 200 and 300 µg/ml in 50% methanol (Labscan, Thailand).

3.7.3.5 Cyanidin-3-o-sambubiosides

Cyanidin-3-o-sambubiosides 1 milligram was dissolved in 50% methanol (Labscan, Thailand) and diluted into 5, 10, 20, 30, 40 and 50 µg/ml in methanol.

3.7.4 Preparation of roselle solution

Roselle aqueous extract and hydrolyzed extract 5 mg were dissolved in 50% methanol (Labscan, Thailand) to concentration 5 mg/ml and diluted to 500 and 1000 µg/ml in 50% methanol.

3.8 Forced degradation study of aqueous extract of roselle

(Blessy M, Ruchi DP, Prajesh NP & Agrawal YK, 2014)

3.8.1 Temperature forced degradation

Roselle aqueous extract 50 mg was added in a test tube. After that, the extract was heated at 80°C for 3 hr and cooled down. Then, sample antioxidant activity by DPPH radical scavenging assay and total phenolic content were determined.

3.8.2 Moisture hydrolysis

Roselle aqueous extract 50 mg was added in a test tube. After that, 3 drops of deionized water were added and heated at 80°c for 3 hr. Then, sample antioxidant activity by DPPH radical scavenging assay and total phenolic content were determined.

3.8.3 Acid hydrolysis

Roselle aqueous extract 50 mg was placed in a test tube. After that, 3 drops of 3N hydrochloric acid was added and heated at 80°C for 3 hr. Then, 3N sodium hydroxide was added into each tube. After that, sample antioxidant activity by DPPH radical scavenging assay and total phenolic content were determined.

3.8.4 Alkaline hydrolysis

Roselle aqueous extract 50 mg was placed in a test tube. After that, 3 drops of 3N sodium hydroxide was added and heated at 80°C for 3 hr. Then, 3N hydrochloric acid was added into each tube. Sample antioxidant activity by DPPH radical scavenging assay and total phenolic content were determined.

3.8.5 Oxidation

Roselle aqueous extract 50 mg was placed in a test tube. After that, 3 drops of 30% hydrogen peroxide was added and heated at 80°C for 3 hr. Then, sample antioxidant activity by DPPH radical scavenging assay and total phenolic content were determined.

3.9 Soy yogurt production

3.9.1 Isolation and identification of lactic acid bacteria

Lactic acid bacteria were isolated form rice seed that were collected from 19 sources in Japan, Chiang Mai and Nakorn Pathom provinces in November 2016. The samples were kept in plastic bags at room temperature. Moreover, fermented

vegetable specimens were collected from Bangkok and Pathumthani provinces in May 2017. The samples were stored in a refrigerator at 4°C.

Table 3.3 Strain codes and sources of collected specimens

Sources	Sample code
Nakorn Pathom (Bang Len)	RN1
	RN2
	RN3
	RN4
Nakorn Pathom (Puttamonton)	RN5
	RN6
	RN7
	RN8
Nakorn Pathom (Puttamonton)	RN9
	RN10
Chiang Mai (San Kam Paeng)	RN11
Chiang Mai (Mae on)	RN12
Chiang Mai (Doi Saket)	RN13
	RN14
	RN15
	RN16
Japan	RN17
	RN18
	RN19
Bangkok (Saphan3 market)	FV1-1
	FV1-2
	FV2
	FV3
Pathumthani (Hospital market at Thammasat University)	FV4
	FV5

	FV6
Bangkok (Rung Charoen market)	FV7
	FV8
Bangkok (Siam)	FV9
	FV10
	FV11-1
	FV11-2

*RN = Rice seeds, FV = Fermented vegetable

3.9.1.1 Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from all specimens by enrichment the specimens in de Man, Rogosa and Sharpe (MRS) medium at 37°C for overnight. After that, lactic acid bacteria were isolated by using streak plate technique on MRS mixed with 0.3% $CaCO_3$ agar plate at the same condition (De man *et al*., 1960; Luangsakul *et al*., 2009). The colony in clear zone was recovered and homogenized in 0.85% NaCl. Homogenized mixture was re-streaked again on MRS+$CaCO_3$ agar plate. The pure cultures were stocked in cryotube vial with 40% glycerol at -20°C freezer.

3.9.1.2 Determination of fermentation characteristic

A durham tube was placed in MRS broth test tube and sterilized by autoclave at 121°C for 15 min. Each colony of lactic acid bacteria was inoculated into the test tube and fermented under aerobic conditions at 37°C for 24 hr (Tanasupawat *et al*., 2013).

3.9.1.3 Identification of LAB based on 16S ribosomal RNA gene sequence analysis (Ladda *et al*., 2015)

The pure lactic acid bacteria were analyzed by using 16S rRNA gene sequencing. The 16S rRNA gene was amplified by using primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-CGG TTA CCT TGT TAC GAC TT-3'. For colony template, colony was re-suspended with 30 µl of sterile ultrapure water. In a part of RCR mixer, 10X Taq Buffer A, $MgCl_2$ 25mM, Primer 10 pmol, dNTP 2 mM and Taq DNA polymerase 5 U were mixed in sterile ultrapure water. PCR product was obtained by

3 cycles: denaturation, annealing and extension. PCR product was purified by Geneaid Gel/PCR Kit. The sequence analysis of PCR products was performed by Macrogen company in Seoul, Korea. The nucleotide sequence was analyzed with 16s online database. The percent identity of the bacterial isolate was determined on the basis of the highest scores.

3.9.2 Soy yogurt fermentation

3.9.2.1 Preparation of starter culture

Three strains of lactic acid bacteria: *Lactococcus lactis* subsp. *lactis* RN19, *Lactobacillus plantarum* subsp. *plantarum* FV1-1 and *Pediococcus acidilactici* FV4 identified based on 16S rRNA gene analysis were used in this study (Table 3.4). Both strains in each experiment (Table 3.5) were cultivated in MRS broth at 37°C for 12 hr. After that, lactic acid bacteria volume 1% (1:1 of each bacteria) was seeded into fresh soymilk and incubated at the same conditions. This procedure was replicated 5 times in fresh soymilk. According to the procedure, starter culture was obtained and stored at 4°C.

Table 3.4 Isolate no. and species of lactic acid bacteria (LAB)

Isolate no.	Species of lactic acid bacteria (LAB)
RN19	*Lactococcus lactis* subsp. *lactis*
FV1-1	*Lactobacillus plantarum* subsp. *plantarum*
FV4	*Pediococcus acidilactici*

Table 3.5 Pairing of strains for experiments

Experiments	Species of lactic acid bacteria (LAB)
1	*Lactococcus lactis* subsp. *lactis* RN19 + *Pediococcus acidilactici* FV4
2	*Lactobacillus plantarum* subsp. *plantarum* FV1-1 + *Pediococcus acidilactici* FV4

3.9.2.2 Preparation of soymilk

The yellow soybeans were purchased from a local grocery in Bangkok, Thailand. Soybeans were washed and soaked in water with ratio 1:4 (w/v) at room temperature for 12 hr. The water was drain and the beans blended for 5 min. After that, soybeans were packed in a mesh bag and decocted in water with ratio 1:6 (w/v) at 80°C until it boiled for 15 mins. Then, the mesh bag was removed and heated soymilk for 20 min at temperature 40°C to sterilize. Soymilk was stored in a refrigerator at 4°C.

3.9.2.3 Preparation of yogurt starter

The stock of cultures (*Lactococcus lactis* subsp. *lactis* RN19, *Lactobacillus plantarum* subsp. *plantarum* FV1-1 and *Pediococcus acidilactici* FV4) were cultivated in MRS broth at 37°C for 24 hr. Each culture was transferred 3 times into new MRS broth. Then, culture volume 1:1 (1% v/v) was inoculated into fresh soymilk and incubated at 37°C for 12 hr. The starter was transferred into fresh soymilk 5 times. After that, the cell numbers and pH were evaluated (Champagne *et al*., 2009). Soy yogurt starters were stored at 4°C.

3.9.2.4 Preparation of soy yogurt

Starter cultures volume 1% (v/v) was added into 100 ml fresh soymilk and incubated at 37°C for 8, 10, 12 and 14 hr (Sokolinska & Pikul, 2004). The products were stored at 4°C for 21 days. Plain soy yogurt properties were evaluated.

3.9.3 Quality control of soy yogurt

3.9.3.1 Determination of chemical, physical and sensory properties of product on day 0, 7, 14 and 21.

(1) Determination of cell numbers

Enumerations of lactic acid bacteria were carried out at 0, 7, 14 and 21 days by using MRS agar plate. Spread plate technique was used for counting the colonies of bacteria. Serial dilution technique was used to assess the number of colony forming unit, which are presented as log CFU/g (Sokolinska & Pikul, 2004; Demirci *et al*., 2017).

(2) pH value

pH value was used to determine acid amount in a product by using pH meter. Sample 20 g was placed into a corning tube and centrifuged at 5,000 rpm for 10 min. After that, the pH meter was calibrated before measuring pH and the sample was measured by putting pH probe into the supernatant (Barkallah *et al*., 2017).

(3) Titrated acidity

Titrated lactic acidity determined by using the neutralization reaction method. Sample 20 g was mixed in distilled water 40 ml and centrifuged at 5,000 rpm for 10 min. The end point was identified by phenolphthalein indicator 3 drops into 10 ml supernatant. Then, the sample was titrated with 0.01 N sodium hydroxide into the supernatant until endpoint was pink. The results are presented as %lactic acid.

(4) Viscosity

The viscometer was used the disc-type spindle rotator No.5 with spindle speed set at 20 rpm. The rotator spindle was put into the center of soy yogurt sample container. The results were read and record 6 times in 1 min (10 seconds/time). The viscometer is a tool to determine stickiness of sample and result is reported as centipoise.

(5) Syneresis

Sample 20 g were placed in corning tube and centrifuged at 5,000 rpm for 10 min. After that, supernatant was poured out and sample weighed. Percentage of yogurt syneresis was calculated from formula below

$$\%\text{Syneresis} = \frac{\textbf{Weight of sample} - \textbf{Weight of supernatant}}{\textbf{Weight of sample}} \times 100$$

(6) Colorimetric

The colorimeter was examined sensor head ability before use by calibrated with white calibration plate. For color, value defines, tolerance was set

at 1 for measurement. After that, specimen 10 g was put in round cuvette container. Each specimen was measured 3 times to find average value by using CR-400 utility program (Konica Minolta, 2006).

(7) Sensory evaluation

Sensory characteristics of roselle soy yogurt and roselle spherification were evaluated by survey questionnaire. There were 50 participants from staff and students at Faculty of Medicine, Thammasat University. The questionnaire used a 5-point hedonic scale and there were 6 attributes to evaluate: appearance, smell, flavor, texture, overall attribute and overall acceptance of soy yogurt plus roselle bead. The evaluation rates using 5-point scale: 1 = strongly dislike, 2 = slightly dislike, 3 = neither like nor dislike, 4 = slightly like and 5 = strongly like (Lim, 2011; Demirci *et al*., 2017).

3.10 Spherification of roselle aqueous extract

3.10.1 Direct spherification

Roselle aqueous extract at various concentration (0.5, 1, 1.5, 2 and 2.5% w/v) were added into water and stirred moderately until dissolved. Other ingredients (sucrose 15% w/v and citric acid 0.3 % w/v) were mixed into roselle based juice. Roselle juice was stored in a dark container at 4°C until required. Calcium chloride solution 0.14 M was prepared by dissolving in distilled water and it was stored at 4°C until required (Arriola *et al*., 2016). After that, sodium alginate 4% (w/v) was dissolved in distilled water at 60°C and left in refrigerator until the bubbles disappeared. The solution was dropped by using a 5 ml syringe with 22 gauges metal needle under manual control into a calcium chloride bath. The beads were left in the calcium chloride bath for 15 min. After that, the beads were washed in clean water and stored in a container at 4°C (Arriola *et al*., 2016) for tested antioxidant, total phenolic content and bioactive compounds.

3.10.2 Reverse spherification

Calcium lactate (4% w/v) was dissolved in 100 ml water. After that, roselle extract (1, 1.5, 2 and 2.5% w/v), sucrose (15% w/v) and citric acid (0.5% w/v) were added into calcium lactate solution. The solution was dropped in a mold (500 µl/mold) and stored in a refrigerator at 4°C. For sodium alginate bath, sodium alginate 1% w/v was mixed in water by using a blender. Sodium alginate solution was kept at 4°C until the bubbles disappeared. After that, roselle beads were dropped in the sodium alginate solution and left for 10 min. Then, washed roselle beads in clean water. Roselle sphere beads were stored in a dark container at 4°C for tested antioxidant, total phenolic content and bioactive constituents.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 The percent yield of roselle extracts

In this research, roselle aqueous extract was obtained from Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University. Roselle were extracted by boiling with water and dried by spray dried technique. The percent yield of aqueous extract of roselle was 10%. After that, aqueous extract of roselle was performed acid hydrolysis by three methods such as hydrochloric acid-chloroform, distilled water and chloroform and hydrochloric acid. The percentage of yield of roselle extract was showed in Table 4.1. The results demonstrated that decoction aqueous extract by hydrochloric acid had the most value percent yield (28.65%) and the least percent yield value was decocted with distilled water-chloroform (0.60%). Besides, the aqueous extract that hydrolyzed by hydrochloric acid-chloroform obtained more %yield than distilled water-chloroform. The experiment demonstrated the polarity of the solvent relative to the percent yield of the extract. The polarity of water higher than hydrochloric acid solvent. To conclude, hydrochloric acid extraction able to get a better percentage of yield than water extraction

Table 4.1 Percentage of yield of roselle extract

Sample	Actual weight	%Yield
Aqueous extract	1 kg	10
Hydrochloric acid-chloroform extract	617 mg	3.09
Distilled water and chloroform extract	120 mg	0.60
Hydrochloric acid extract	5.73 g	28.65

4.2 DPPH scavenging activity of roselle extract

The results of DPPH radical scavenging activity of roselle extract are shown in Table 4.2. BHT was used as a positive control displayed antioxidant with EC_{50} value 13.58±0.25 μg/ml (61.62±1.13 μM). DI-$CHCl_3$ was against free radical more than and HCl-$CHCl_3$ with EC_{50} value 13.13±0.66 and 14.81±2.39 μg/ml. On the other hand, HCl decoction no potential against oxidant. Roselle aqueous extract showed the least ability for against oxidant. Chloroform extract of *Ziziphus jujuba* exhibited the maximum percentage inhibition at concentration 200 μg/ml in DPPH test with value 98.84% (Al-Saeedi, Al- Ghafri & Hossain, 2016). DPPH Study of Salalah ripe banana chloroform extract expressed the most %inhibition at concentration 200 μg/ml with value 98.1±0.22% (Amri & Hossain, 2018). Besides, chloroform extraction displayed the maximum of total flavonoid that calculated from quercetin calibration curve. Quercetin displayed antioxidant activity by DPPH assay with IC_{50} value 9.70±0.80 μM (Cos *et al*., 2002). In the case of DI-$CHCl_3$ and HCl-$CHCl_3$ extract, compounds of low to medium polarity that showed antioxidant activity may be soluble in chloroform. After concentration, these extracts have higher antioxidant activity than aqueous extract.

Table 4.2 Antioxidant activities of aqueous extract and hydrolysis extract

Type of extract	%Inhibitory of concentration (μg/ml): Mean±SEM				EC_{50} (μg/ml) (Mean±SEM)
	1	10	50	100	
Aqueous extract	2.01±0.32	10.32±1.34	49.79±1.28	62.31±0.89	50.29±1.41
Hydrolyzed extract					
- DI-$CHCl_3$	7.85±1.89	40.78±1.66	88.70±1.46	94.42±0.57	13.13±0.66
- HCl-$CHCl_3$	6.33±0.51	38.11±4.06	87.51±4.04	96.18±1.88	14.81±2.39
- HCl decoction	1.45±0.17	5.29±0.14	15.16±1.80	20.10±2.61	>100
BHT	7.06±3.61	39.91±0.77	76.08±0.89	80.55±1.17	13.58±0.25 (61.62±1.13 μM)

4.3 Determination of nitro blue tetrazolium (NBT) of roselle extract

Results of antioxidant activity by using NBT assay are shown in Table 4.3. Propyl gallate is a positive control with IC_{50} value 29.69±2.03 μg/ml (139.91±9.57 μM). Conversely, other extracts (HCl-chloroform, distilled water-chloroform and aqueous extract) had no activity in screen concentration (50, 100 μg/ml). Moreover, all of roselle extract and positive control were not toxic to HL-60 cell with %survival more than 70%. From documentary, chloroform solution able to extraction flavonoid from *Hibiscus* plant (Al-Saeedi, Al- Ghafri & Hossain, 2016). Quercetin is a flavonoid and cytotoxic activity against the HL-60 leukemic cells with IC_{50} value 14.00 mg/ml but no toxic to cell (Araújo *et al*., 2013).

Table 4.3 Determination for NBT assay of aqueous extract and hydrolysis extract of roselle in HL-60 cell lines

Type of extract	%Inhibitory of concentration (μg/ml): Mean±SEM (%Survival of concentration (μg/ml): Mean±SEM)				IC_{50} (μg/ml) (Mean±SEM)
	1	10	50	100	
Aqueous extract	-	-	20.20±2.02 (101.74±1.83)	42.82±5.61 (90.60±0.41)	>100
Hydrolyzed extract					
- DI-$CHCl_3$	-	-	22.15±1.36 (86.08±1.24)	48. 83±1.67 (74.59±1.18)	>100
- HCl-$CHCl_3$	-	-	25.44±1.86 (97.08±2.81)	45.62±2.66 (89.91±3.24)	>100
- HCl decoction	-	-	12.24±1.08 (93.06±1.32)	30.02±1.34 (89.27±1.13)	>100
Propyl gallate	15.52±6.12 (76.82±2.84)	30.72±4.71 (89.87±5.38)	62.96±4.07 (112.43±9.31)	85.02±1.30 (119.49±7.03)	29.69±2.03 (139.91±9.57 μM)

4.4 Nitric oxide production inhibition in LPS-induced RAW264.7 macrophages of roselle extract

Nitric oxide production inhibition assay was used to detected anti-inflammatory of roselle extract and cytotoxicity of RAW264.7 cell lines were studied by using MTT assay. Aqueous extract and HCl decoction had no activity, while HCl-$CHCl_3$ and Dl-CHCl3 extract displayed activity with IC_{50} value of 27.30±0.93 and 15.06±2.42 μg/ml at dose 50 mg/ml. Moreover, prednisolone was used as positive control and showed activity with IC_{50} value of 0.14±0.03 μg/ml (0.31±0.08 μM). All of extract had no toxic to cell lines (%survival more than 70%). Results for anti-inflammatory are displayed in Table 4.4. In previous study, *Hibiscus deflersii* that were fractionated using chloroform and found β-sitosterol and lupeol with content of 0.59±0.01 and 0.74±0.01 μg/mg extract, respectively (Alam *et al*., 2018). *H. sabdariffa* also contain β-sitosterol and lupeol that inhibited nitric oxide synthase and nitric oxide production (Da-Costa-Rocha *et al*., 2014; Giacoman-Martinez *et al*., 2019). Therefore, chloroform fraction (HCl-$CHCl_3$ and Dl-$CHCl_3$ extract) of roselle showed a higher activity of nitric oxide production inhibition than aqueous extract. Nevertheless, chloroform may dissolve and concentrate any low to medium polarity compounds that inhibit nitric oxide production and improve ability of roselle extract. Thus, roselle extract could be increased the biological activity by using chloroform extraction.

Table 4.4 Nitric oxide production inhibitory effect of aqueous extract, hydrolysis extract and stress test of roselle in RAW264.7 cell lines

Type of extract	%Inhibitory of concentration (µg/ml): Mean±SEM (%Survival of concentration (µg/ml): Mean±SEM)						IC_{50} (µg/ml) (Mean±SEM)
	0.01	0.1	1	10	50	100	
Aqueous extract	-	-	-	-	9.38±2.35 (110.51±9.74)	13.05±1.40 (115.33±8.61)	>100
Hydrolyzed extract							
- HCl-$CHCl_3$	-	-	21.42±1.76 (107.40±8.59)	29.61±1.74 (105.72±10.01)	75.99±1.32 (86.42±5.41)	93.53±1.26 (81.64±6.95)	27.30±0.93
- DI-$CHCl_3$	-	-	21.42±2.50 (106.99±4.27)	41.15±3.51 (98.17±4.14)	84.14±2.09 (83.59±8.65)	96.75±0.20 (74.22±7.74)	15.06±2.42
- HCl decoction	-	-	-	-	8.38±14.16 (89.18±0.81)	31.66±3.82 (96.98±6.22)	>100
Prednisolone	4.00±5.08 (89.46±0.68)	38.36±6.52 (84.81±1.21)	57.53±9.57 (75.80±0.58)	64.19±6.62 (84.44±3.61)	82.68±3.74 (72.77±1.09)	-	0.14±0.03 (0.31±0.08 µM)

4.5 Total phenolic contents of roselle extract

The results of total phenolic contents of extracts are shown in Table 4.5. The result of roselle aqueous extract displayed phenolic quantity with contents 46.51±2.58 mg GAE/g. After hydrolysis, DI-$CHCl_3$, HCl-$CHCl_3$ and HCl decoction extracts exhibited total phenolic compounds with value of 76.39±0.21, 69.10±0.60 and 49.71±1.72 mg GAE/g, respectively. Comparison between aqueous extract and aqueous-chloroform extract of *Ziziphus jujube* leaves found that aqueous-chloroform extract contains total phenols more than aqueous extract with value of 51.53 ± 0.92 and 22.33 ± 0.34 mg/g (Al-Saeedi, Al- Ghafri & Hossain, 2016).

Table 4.5 Total phenolic content of aqueous extract and hydrolysis extract

Type of extract	Total phenolic content (mg GAE/g): mean±sem		
	50	100	mg GAE/g
Aqueous extract	39.55±4.27	53.47±4.37	46.51±2.58
Hydrolyzed extract			
- DI-$CHCl_3$	74.88±0.29	77.90±0.19	76.39±0.21
- HCl-$CHCl_3$	66.62±1.06	71.58±0.30	69.10±0.60
- HCl decoction	43.98±3.52	55.44±0.36	49.71±1.72

4.6 Determination quantity of compound markers by HPLC technique

4.6.1 Determination quantity of compound markers of roselle aqueous extract

The comparison of HPLC chromatogram of 5 chemical fingerprints at different wavelength are shown in Figure 4.1, 4.2 and 4.3. The chemical marker content in roselle aqueous extract are shown in Figure 4.4. There are 5 chemical fingerprints as a marker of roselle aqueous extract to studied. The results found that chlorogenic acid showed the highest chemical content in roselle aqueous extract with value 5.76±0.05 mg/g, and the least compound in extract was ferulic acid with value 0.09±0.02 mg/g. For other marker compounds, coumaric acid, quercetin and cyanidin-3-o-sambubiosides were showed quantity with values 2.23±0.01, 0.57±0.01 and 0.56±0.01 mg/g, respectively.

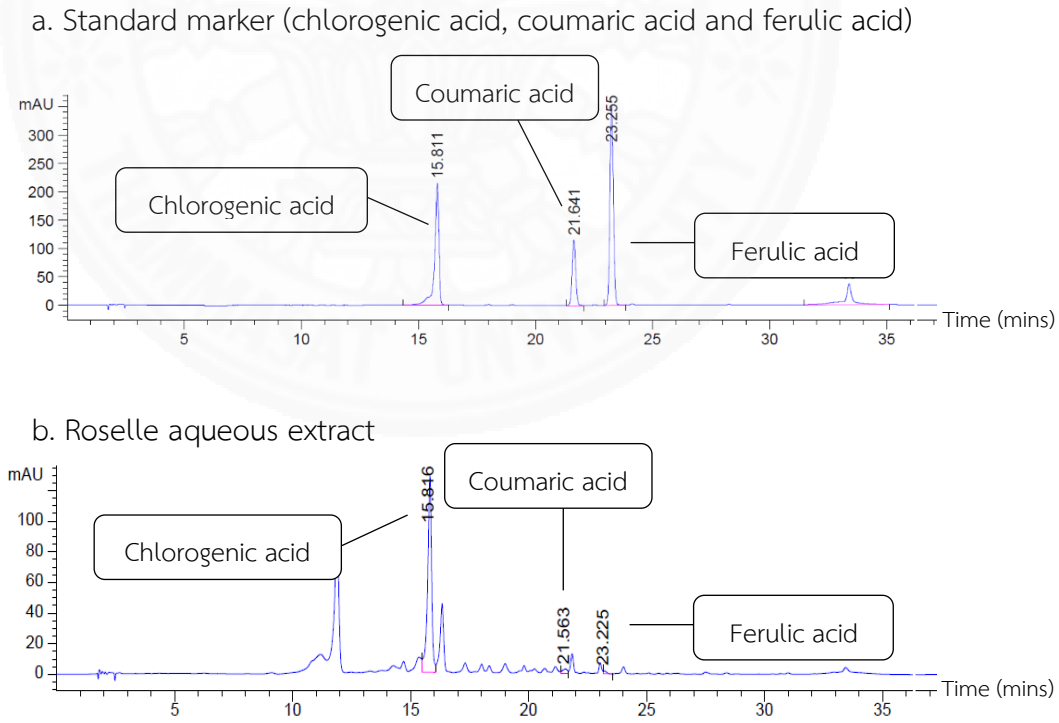
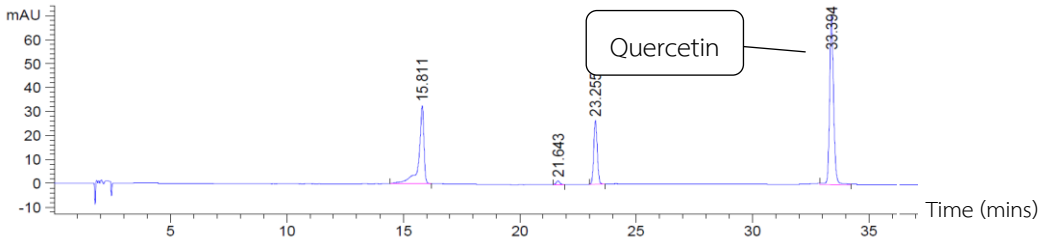


Figure 4.1 Comparison of HPLC chromatograms of standard marker and roselle aqueous extract at wavelength 325 nm

c. Standard marker (quercetin)



d. Roselle aqueous extract

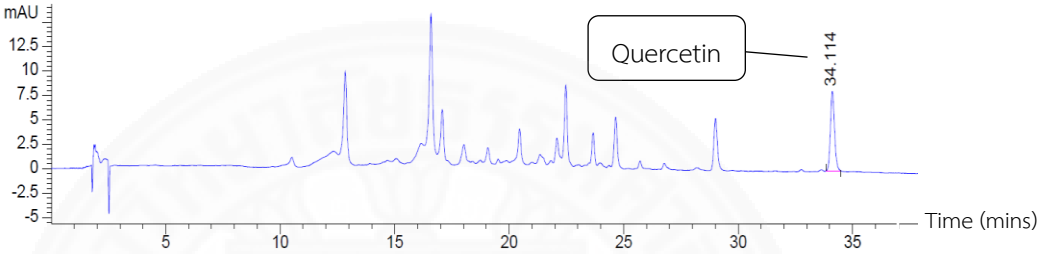
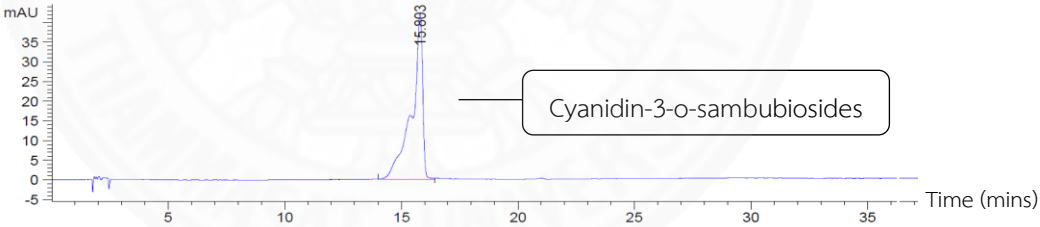


Figure 4.2 Comparison of HPLC chromatograms of standard marker and roselle aqueous extract at UV wavelength 365 nm.

e. Standard marker (cyanidin-3-o-sambubiosides)



f. Roselle aqueous extract

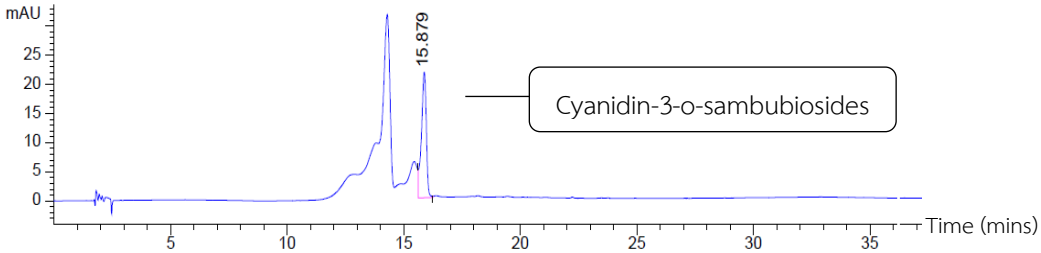


Figure 4.3 HPLC chromatograms of standard marker and roselle aqueous extract at wavelength 520 nm.

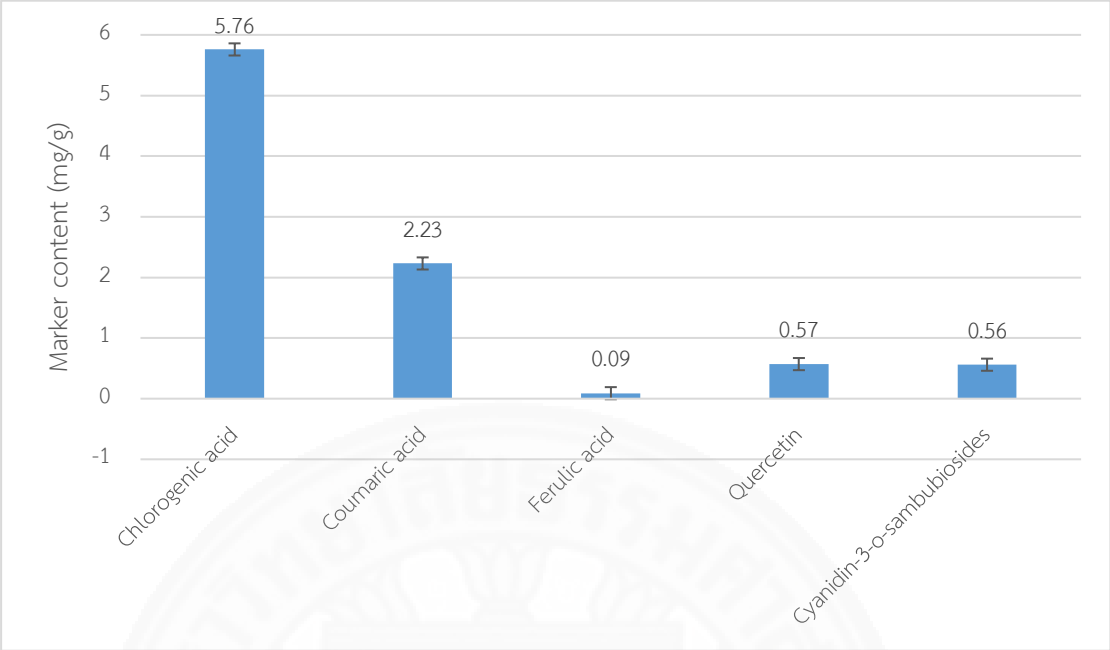


Figure 4.4 Marker content in roselle aqueous extract

4.6.2 Determination of chemical markers of HCl-$CHCl_3$, distilled water-$CHCl_3$ and HCl decoction of roselle aqueous extract

Bioactive compound markers of HCl-$CHCl_3$, DI-$CHCl_3$ and HCl decoction are shown in Table 4.6 and HPLC chromatogram comparison between standard marker, DI-$CHCl_3$ and HCl-$CHCl_3$ displayed in Fig. 4.7-4.9. Five bioactive markers that contain in roselle extract displayed biological activities such as antioxidant and anti-inflammation activities. The antioxidant was tested by DPPH radical scavenging assay. In previous study, chlorogenic acid, coumaric acid, ferulic acid and quercetin were showed anti-radical activity with IC_{50} values of 22.8±1.50, >100, 61.90±0.01 and 9.70±0.80 μM, respectively (Cos *et al*., 2002). Cyanidin-3-o-sambubiosides showed against oxidation with EC_{50} value at 7.29 μM (Lima, Sussuchi & Giovani, 2007). For anti-inflammatory effect, chlorogenic acid displayed no ability to inhibit TNF-α production and no cytotoxic on RAW264.7 cells (concentration range 16-500 μM). Conversely, quercetin inhibited TNF-α production at concentration values 125, 250 and 500 μM without cytotoxic effect (Wang & Mazza, 2002). Coumaric acid and ferulic acid were against inflammation with EC_{50} values 17 and 8.3 μM and cyanidin-3-o-sambubiosides were inhibited with value range 6.4-6.7 μM (Ogiwara, Satoh, Negoro, Okayasu, Sakagami & Fujisawa, 2003; Cheng, 2009).

The chemical contents of aqueous and hydrolysis extract are shown in Table 4.6. For chemical content analysis, HCl-$CHCl_3$ and distilled water-$CHCl_3$ had a similar content of all markers. Chlorogenic acid and cyanidin-3-o-sambubiosides could not be detected in HCl-$CHCl_3$ and distilled water-$CHCl_3$, while other markers have been detected a higher content than aqueous extract. HCl-$CHCl_3$ displayed chemical compound as following ferulic acid, coumaric acid and quercetin with value 13.84±0.20, 10.84±0.03 and 2.03±0.11 mg/g, respectively. Distilled water-$CHCl_3$ presented marker compound as following ferulic acid, coumaric acid and quercetin with value 13.56±0.16, 10.35±0.10 and 1.86±0.15 mg/g, respectively. HCl decoction still showed chlorogenic acid content with 0.30±0.05 mg/g that was lower than aqueous extract. While coumaric acid and quercetin content were detected in HCl decoction with values 3.00±0.05 and 1.29±0.05 mg/g dried extract, ferulic acid and cyanidin-3-o-

sambubiosides could not be detected. The results in Table 4.6 showed that chlorogenic acid was unstable under acid condition. Chlorogenic acid or caffeoylquinic acid was stable in pH 3-4 while it was degraded in the pH range 1-2 and 5-8.5 (Narita & Inouye, 2013; Kan, Cheung, Zhou & Ho, 2014). However, roselle extract was tested with 0.01 M HCl that showed pH 2.0. Therefore, the cause of a low amount of chlorogenic acid in HCl-decoction may be the degradation of chlorogenic acid under acid condition. Moreover, chlorogenic acid was insoluble in chloroform that was used for partition of HCl-$CHCl_3$ and distilled water-$CHCl_3$. Thus, chlorogenic acid was undetected in chloroform extract. For degradation of chlorogenic acid, documentary demonstrated that combination of time and thermal digested aglycone in chlorogenic acid to coumarin glucoside derivative (Zoric, Dragovic-Uzelac, Pedisic, Kurtanjek & Garofulic, 2014). Moreover, Fig. 4.10 displayed esterification between chlorogenic acid and acid solution changed isomer of chlorogenic acid to derivative compound such as caffeoylquinic acid, p-coumaroylquinic acid and feruloylquinic acid (Dawidowicz & Typek, 2017). In addition, chlorogenic acid was also changed isomer into its derivative compounds such as ferulic acid-4-o-sulfate and isoferulic acid-3-o-glucuronide in metabolites process (Stalmach *et al*., 2009). Therefore, quantities of coumaric acid and ferulic acid were increased. Ferulic acid was unstable in thermal conditions (Chitgar, Aalami, Kadkhodaee, Maghsoudlou & Milani, 2018). Consequently, ferulic acid was undetected in HCl decoction extract. In the next bioactive compound, quantity of quercetin has been also decreased at high temperature (Tiwari & Cummins, 2011; Harris, Brunton, Tiwari & Cummins, 2015; Prikryl, 2018). From hydrolysis process, the acid decoction was digested sugar in aqueous extract. After that, chloroform solvent was also concentrated the bioactive compound. Accordingly, quercetin content was increased in acid decoction and more increased after concentrated by using chloroform solvent. Previous documentary demonstrated anthocyanin (cyanidin-3-o-sambubiosides) and phenolic compounds (chlorogenic acid) were unstable under thermal condition and time storage (Keenan *et al*., 2010; Zoric, Dragovic-Uzelac, Pedisic, Kurtanjek & Garofulic, 2014; Riaz & Chopra, 2018). Likewise, Fig. 4.11 showed cyanidin-3-o-sambubiosides was transformed into cyanidin, protocatechuic acid,

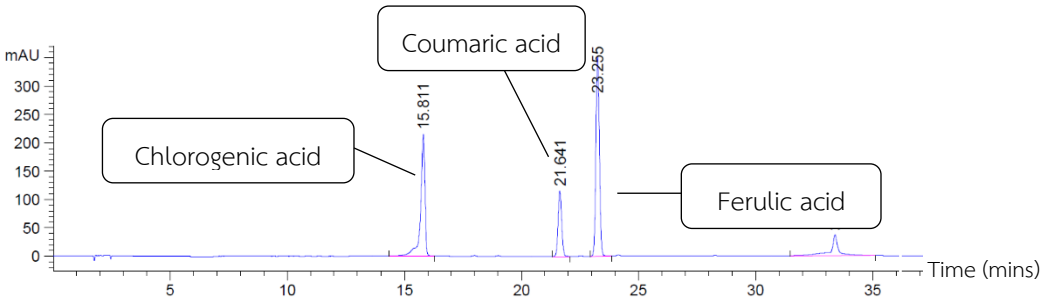
phloroglucinaldeyde and a few quercetin sambubioside under thermal process (Sinela, Rawat, Mertz, Achir, Fulcrand & Dornier, 2017). Therefore, cyanidin-3-o-sambubiosides was disappeared after HCl decoction and hydrolysis process.

Table 4.6 Chemical fingerprint of aqueous extract and hydrolyzed extract (HCl-$CHCl_3$, distilled water-$CHCl_3$ and HCl decoction) by HPLC technique (Mean ± SEM)

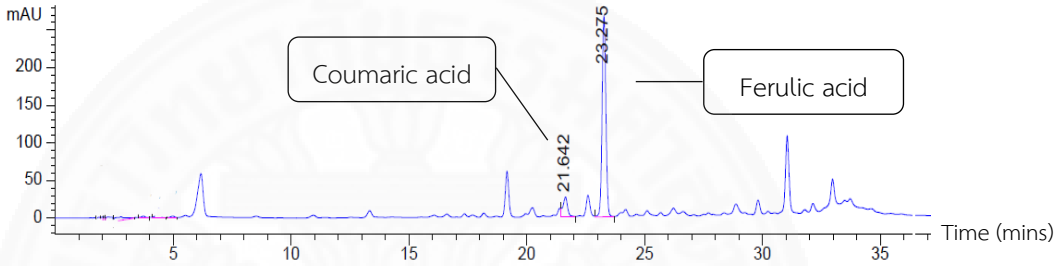
Chemical compound (mg/g)	**Aqueous extract**	**Hydrolyzed extract**		
		HCl-$CHCl_3$	**Distilled water-$CHCl_3$**	**HCl decoction**
Chlorogenic acid	5.76±0.05	ND	ND	0.30±0.05
Coumaric acid	2.23±0.01	10.84±0.03	10.35±0.10	3.00±0.05
Ferulic acid	0.09±0.02	13.84±0.20	13.56±0.16	ND
Quercetin	0.57±0.01	2.03±0.11	1.86±0.15	1.29±0.01
Cyanidin-3-o-sambubiosides	0.56±0.01	ND	ND	ND

* ND: Not detectable

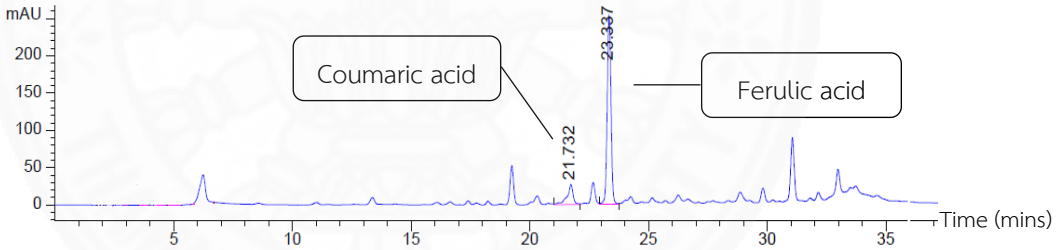
a. Standard marker (chlorogenic acid, coumaric acid and ferulic acid)



b. Distilled water-$CHCl_3$ of roselle extract



c. Acid hydrolysis of roselle extract



d. Acid decoction of roselle extract

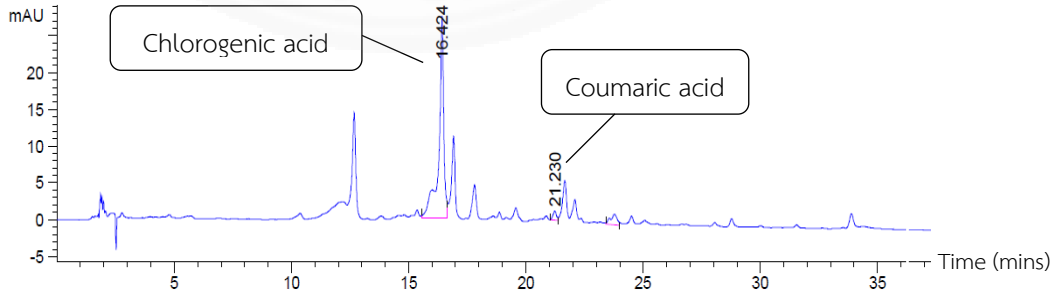
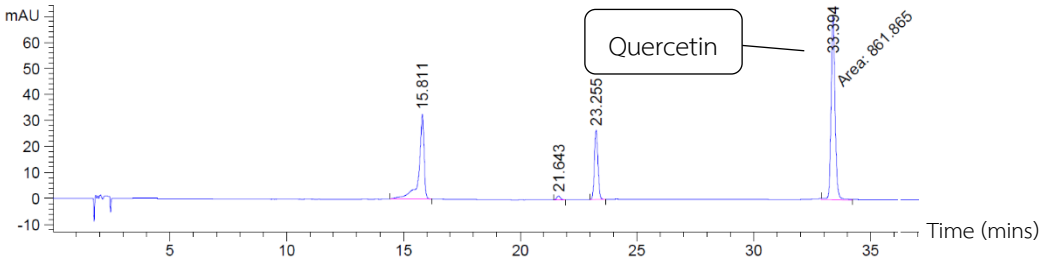
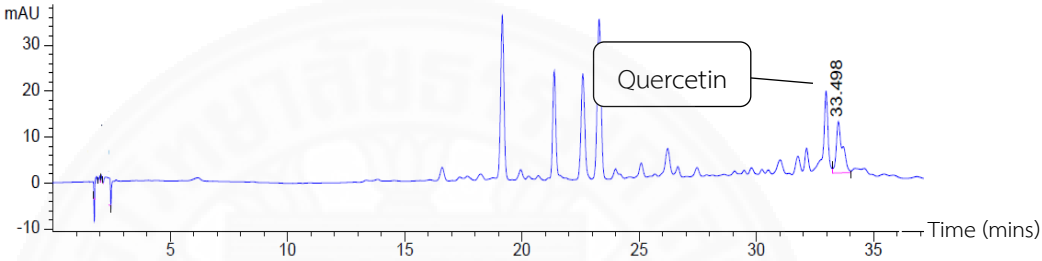


Figure 4.5 HPLC chromatograms of chlorogenic acid, coumaric acid, ferulic acid, DI-$CHCl_3$ and HCl-$CHCl_3$ at wavelength 325 nm. Extraction conditions: a-chlorogenic acid, coumaric acid and ferulic acid; b-DI-$CHCl_3$; c-HCl-$CHCl_3$; d-Acid decoction.

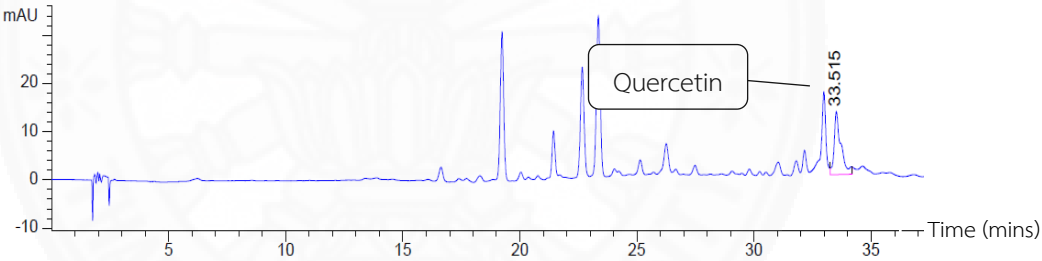
a. Standard marker (quercetin)



b. Distilled water-$CHCl_3$ of roselle extract (Continued)



c. Acid hydrolysis of roselle extract (Continued)



d. Acid decoction of roselle extract (Continued)

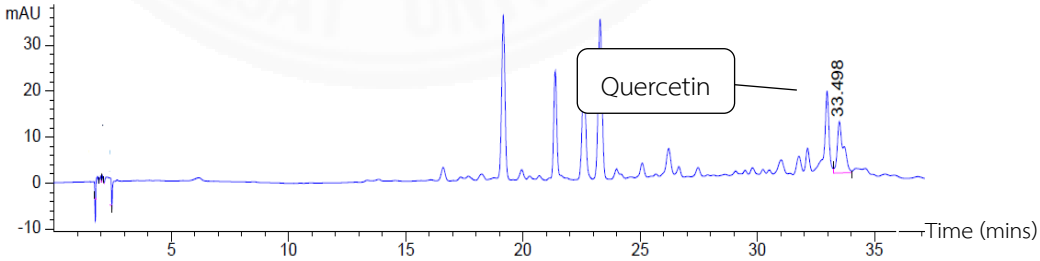
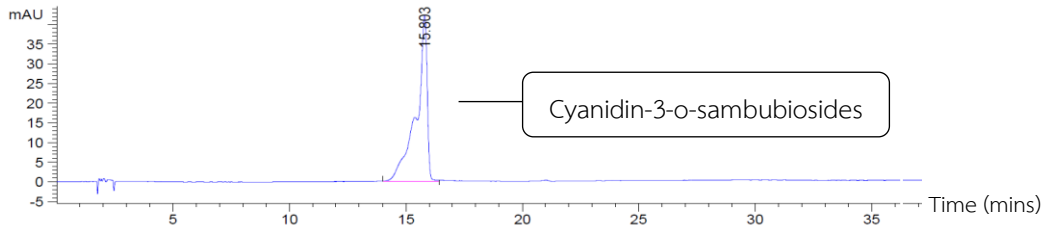
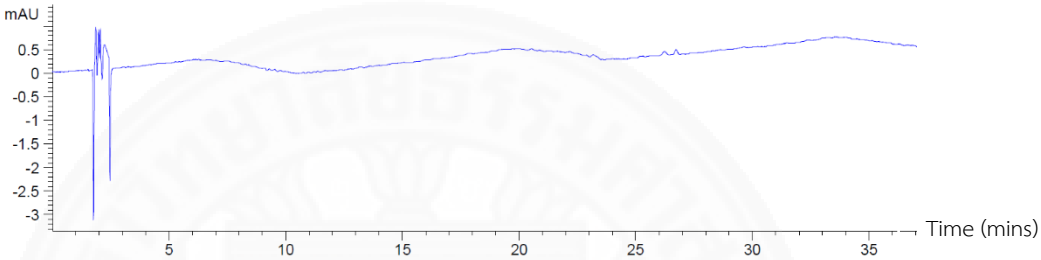


Figure 4.6 HPLC chromatograms of quercetin, DI-$CHCl_3$ and HCl-$CHCl_3$ at wavelength 365 nm. Extraction conditions: a-quercetin; b-DI-$CHCl_3$; c-HCl-$CHCl_3$; d-Acid decoction.

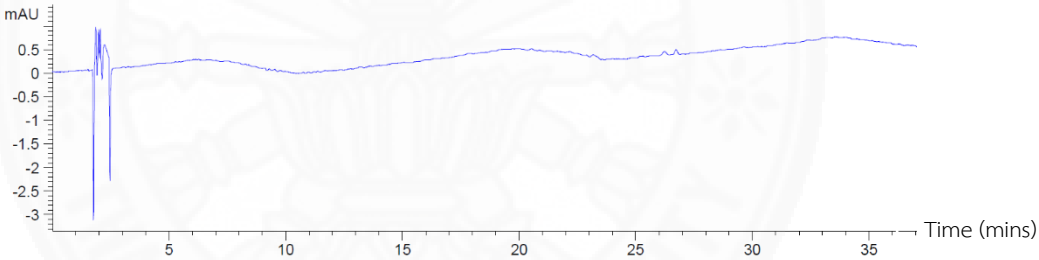
a. Standard marker (cyanidin-3-o-sambubiosides)



b. Distilled water-$CHCl_3$ of roselle extract (Continued)



c. Acid hydrolysis of roselle extract (Continued)



d. Acid decoction of roselle extract (Continued)

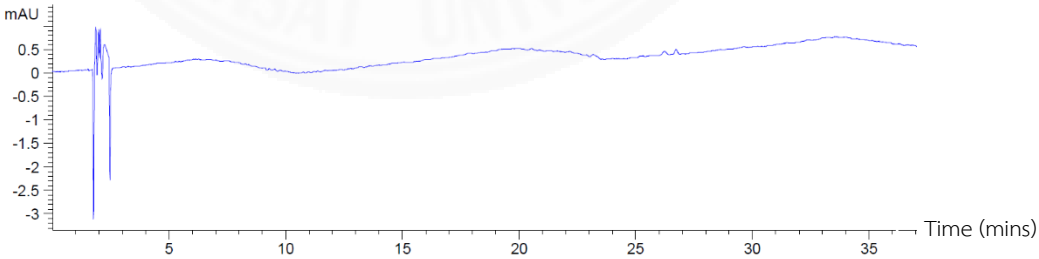


Figure 4.7 HPLC chromatograms of cyanidin-3-o-sambubiosides, DI-$CHCl_3$ and HCl-$CHCl_3$ at wavelength 520 nm. Extraction conditions: a-cyanidin-3-o-sambubiosides; b- DI-$CHCl_3$; c-HCl-$CHCl_3$; d-Acid decoction.

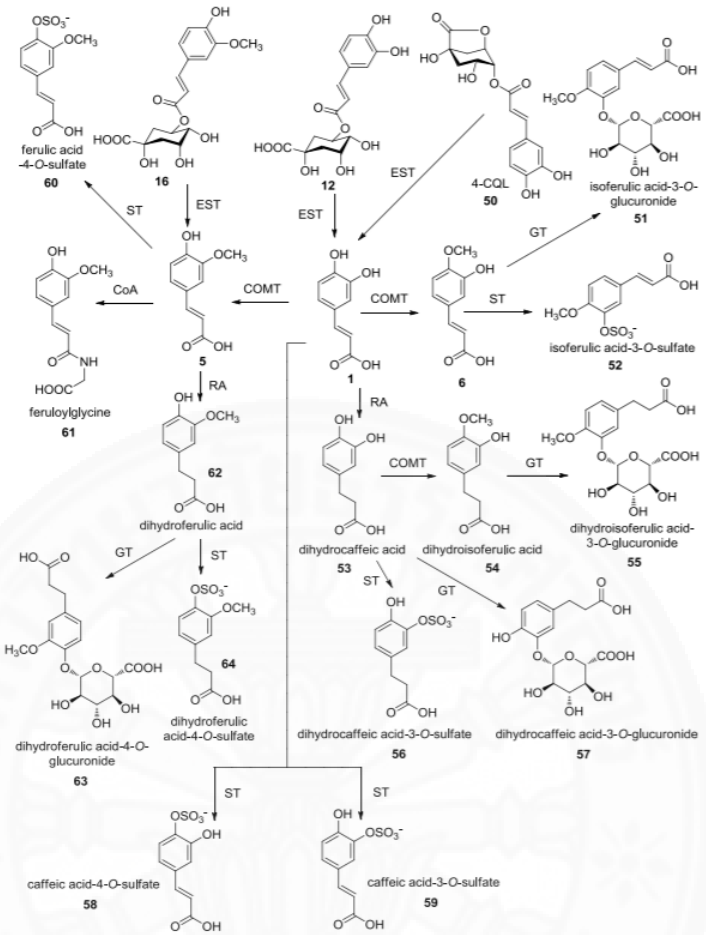


Figure 4.8 Chlorogenic acid and enzymes affecting in metabolism process (COMT = catechol-omethyl transferase; EST = esterase; RA = reductase; GT = UDP-glucoronyl transferase; ST = sulfate-o transferase) (Stalmach *et al*., 2009)

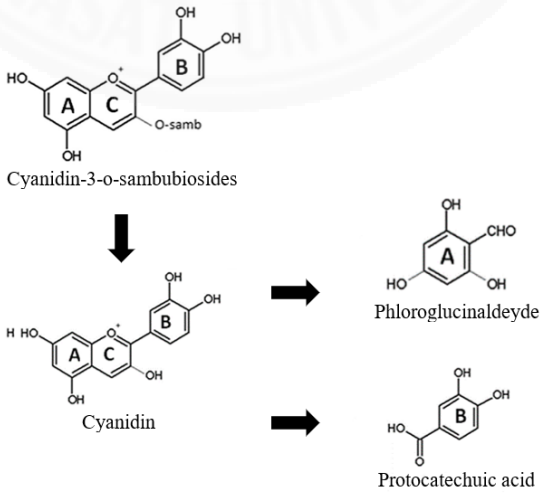


Figure 4.9 Transformation of cyanidin-3-o-sambubiosides structure after thermal process (Sinela, Rawat, Mertz, Achir, Fulcrand & Dornier, 2017)

4.7 Stress test condition of roselle aqueous extract

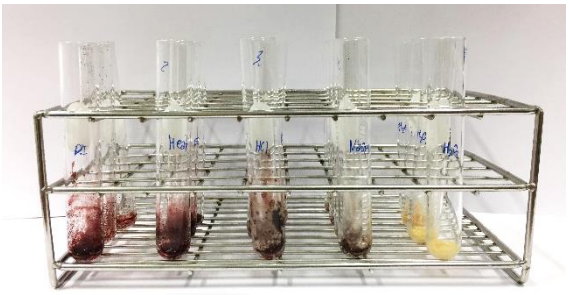


Figure 4.10 Stress test of roselle aqueous extract

Roselle extract were tested degradation under stress condition study that use for product development. There are five stress conditions of exposure to high temperature, moisture, acid, alkaline and oxidation (Fig. 4.12). Moreover, HPLC chromatograms of roselle extract under stress conditions are shown in Fig. 4.13-4.15. After stress test, each extract was investigated for DPPH scavenging assay, nitric oxide production inhibition, total phenolic contents and chemical constituent analysis as showed in Table 4.7 and 4.8. After the high temperature condition, roselle extract showed higher antioxidant activity than no stress control with EC_{50} of 16.27±1.18 µg/ml. Moreover, almost all chemical contents were lower than no stress control, except ferulic acid content was higher than no stress control. Similarly, results can also be observed in moisture, acid and base conditions. One possibility is ferulic acid may be changed from chlorogenic acid and cyanidin-3-o-sambubiosides. Thus, ferulic acid content in roselle extract under stress condition was higher than no stress control (Sinela, Rawat, Mertz, Achir, Fulcrand & Dornier, 2017). All 0stress condition showed a low coumarin content. Coumarin is phenolic compound that can be found in cereals and plant. It is transformed to p-hydroxybenzaldehyde under high temperature and pH change (Boz, 2015). All stress condition may affect to coumarin transformation. Similar results are observed in quercetin. Quercetin is unstable in many conditions such as temperature, pH value and oxygen condition. Although, quercetin was stored at 20°, it still maintained loss of 40% quercetin content (Wang et al., 2016). Quercetin

that was found in roselle extract was unstable under all condition. Moreover, roselle aqueous extract under stress conditions were tested for total phenolic content and calculated by calibration curve (R^2=0.999). The results displayed in Table 4.8. Roselle aqueous extract was used as control. The most phenolic content was thermal condition with content 26.85±0.06 mg GAE/g. Roselle aqueous extract under oxidation condition no potential in total phenolic compound. However, roselle extract that was tested antioxidant activity under stress condition still showed antioxidant activity against free radical except oxidation condition. It may able to develop product in various condition such as high temperature, moisture, acid and alkaline conditions. Furthermore, oxidation condition such as light, air atmosphere should be avoided for the development of roselle product. Moreover, roselle extract showed antioxidant activity under all stress condition. Thus, antioxidant agents are not necessary for roselle product.

Table 4.7 Anti-inflammatory, antioxidant and total phenolic content under stress condition (Mean±SEM)

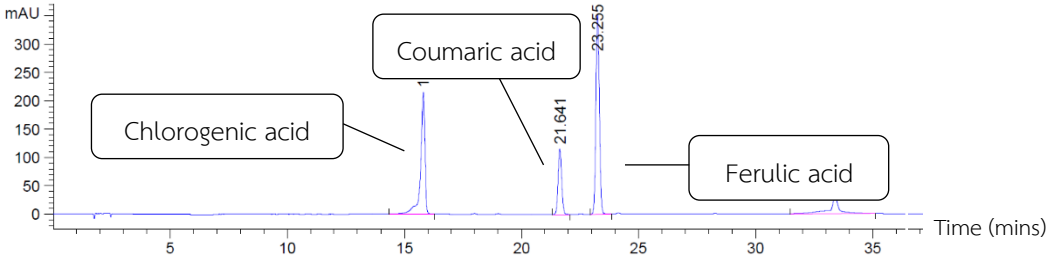
Stress condition	**Nitric oxide production inhibitory effect**	**DPPH scavenging radical**	**Total phenolic content**
	IC_{50} (μg/ml)	EC_{50} (μg/ml)	mg GAE/g
- Aqueous extract (No-stress control)	>100	50.29±1.41	46.51±2.58
- Heat	>100	16.27±1.18	26.85±0.06
- Moisture	>100	18.66±0.39	24.52±0.21
- Acid	>100	27.97±2.55	11.89±0.54
- Base	>100	38.83±1.36	4.54±0.21
- Oxidation	>100	51.46±0.88	-2.80±0.25
- Positive control	Prednisolone 0.14±0.03 (0.31±0.08 μM)	BHT 13.58±0.25 (61.62±1.13 μM)	-

Table 4.8 Chemical marker of roselle aqueous under stress condition (Mean±SEM)

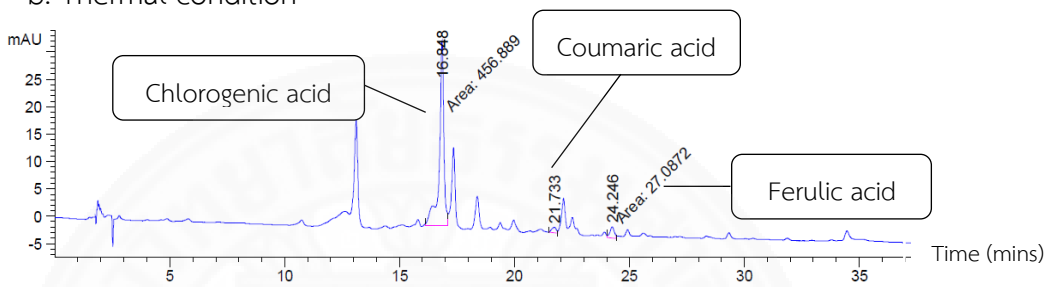
Stress condition	Chemicals content in roselle extract (mg/g dried extract)				
	Chlorogenic acid	Coumaric acid	Ferulic acid	Quercetin	Cyanidin-3-o-sambubiosides
- Aqueous extract (No-stress control)	5.76±0.05	2.23±0.01	0.09±0.02	0.57±0.01	0.56±0.01
- Heat	1.89±0.58	0.11±2.69	0.56±1.11	0.13±2.27	ND
- Moisture	1.75±0.70	0.10±0.22	0.53±0.15	0.15±1.32	ND
- Acid	0.32±13.62	0.01±0.46	0.49±0.74	0.01±0.40	ND
- Base	0.60±9.62	0.09±0.56	0.52±0.23	ND	ND
- Oxidation	ND	ND	ND	ND	ND

* ND = Not detectable

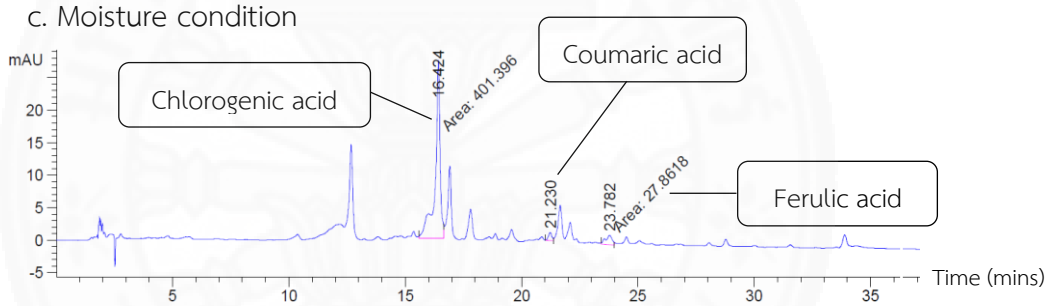
a. Standard marker (chlorogenic acid, coumaric acid and ferulic acid)



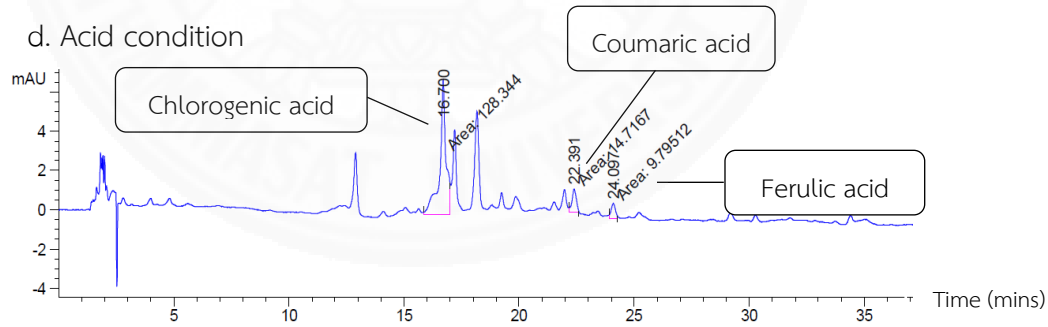
b. Thermal condition



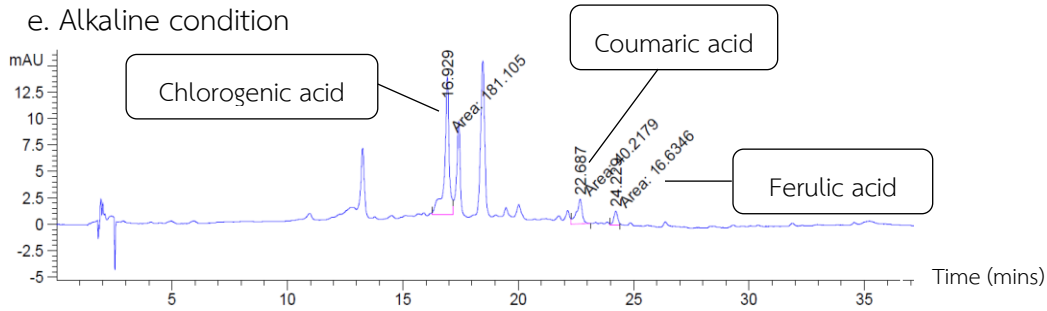
c. Moisture condition



d. Acid condition



e. Alkaline condition



f. Oxidation condition

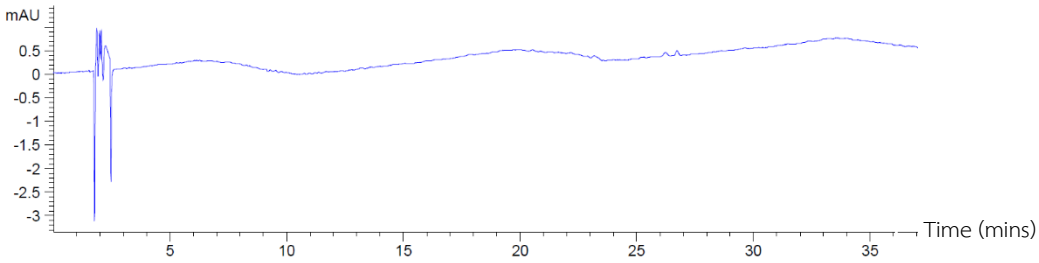
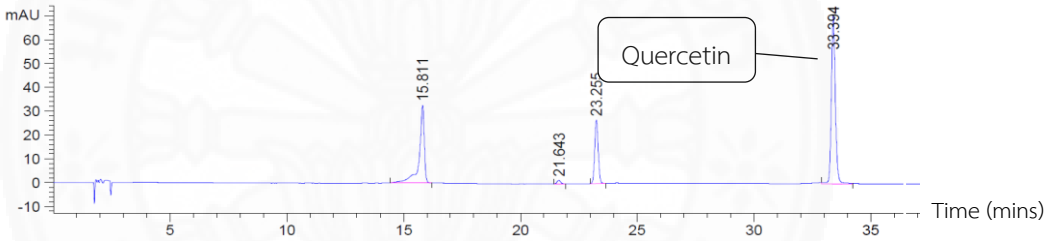
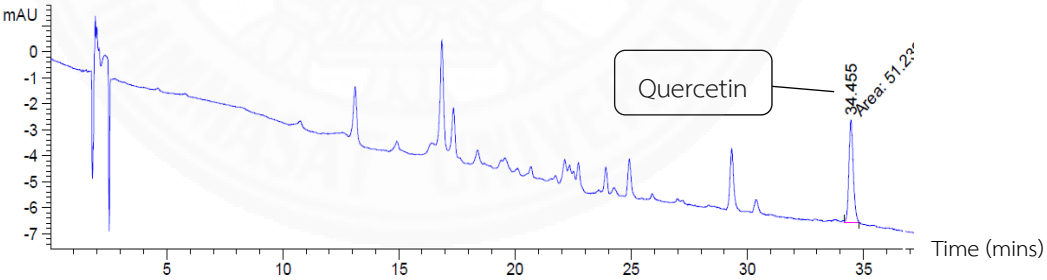


Figure 4.11 HPLC chromatograms of chlorogenic acid, coumaric acid, ferulic acid and stress test conditions at wavelength 325 nm. Extraction conditions: a-chlorogenic acid, coumaric acid and ferulic acid; b-thermal condition; c-moisture condition; d-acid condition; e-alkaline condition; f-oxidation condition.

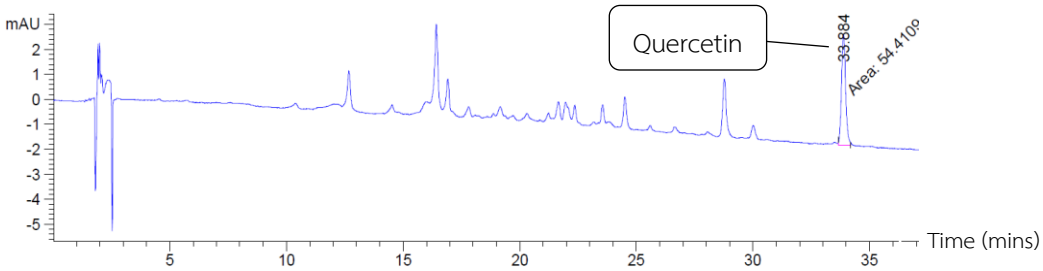
a. Standard marker (quercetin)



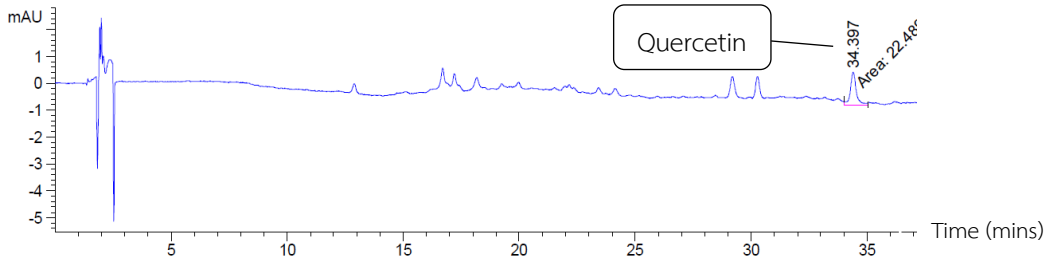
b. Thermal condition (Continued)



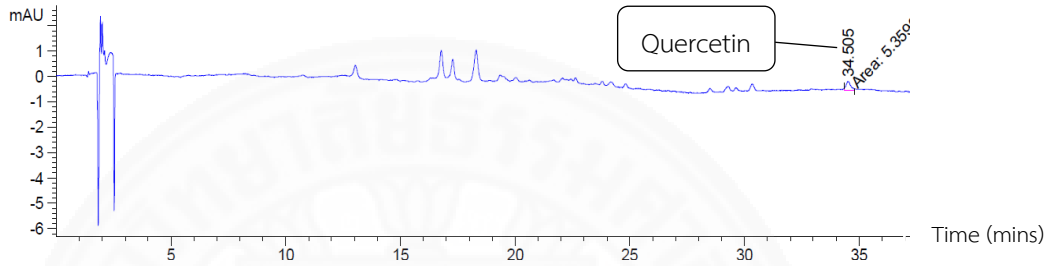
c. Moisture condition (Continued)



d. Acid condition (Continued)



e. Alkaline condition (Continued)



f. Oxidation condition (Continued)

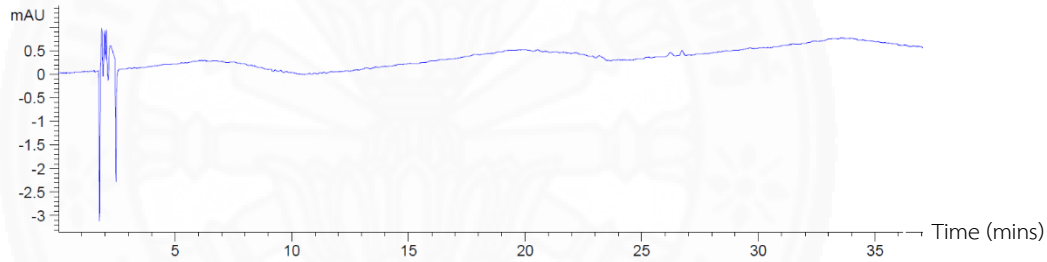
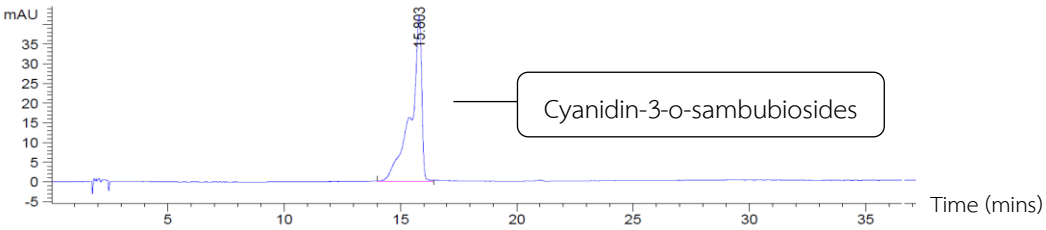
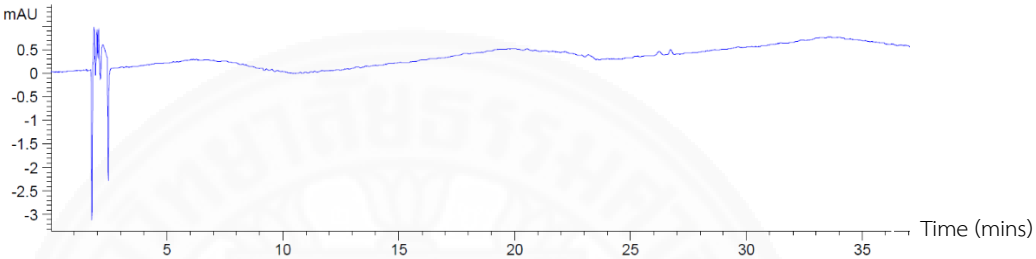


Figure 4.12 HPLC chromatograms of quercetin and stress test conditions at wavelength 365 nm. Extraction conditions: a-quercetin; b-thermal condition; c-moisture condition; d-acid condition; e-alkaline condition; f-oxidation condition.

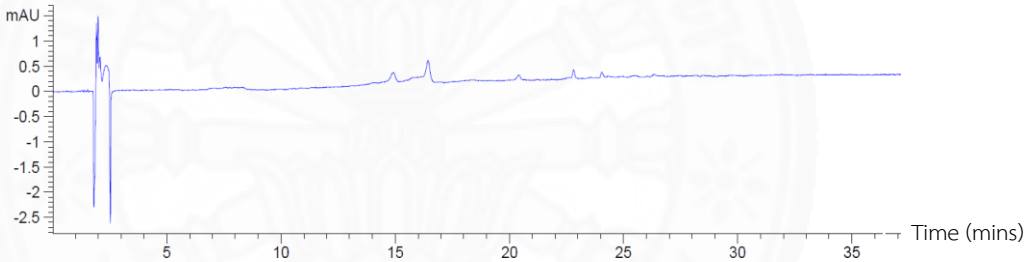
a. Standard marker (cyanidin-3-o-sambubiosides)



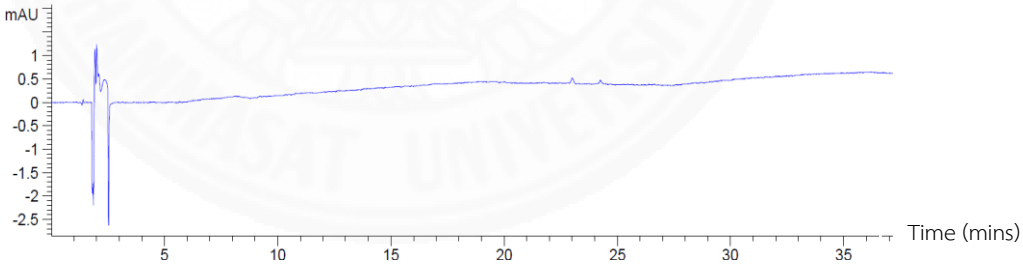
b. Thermal condition (Continued)



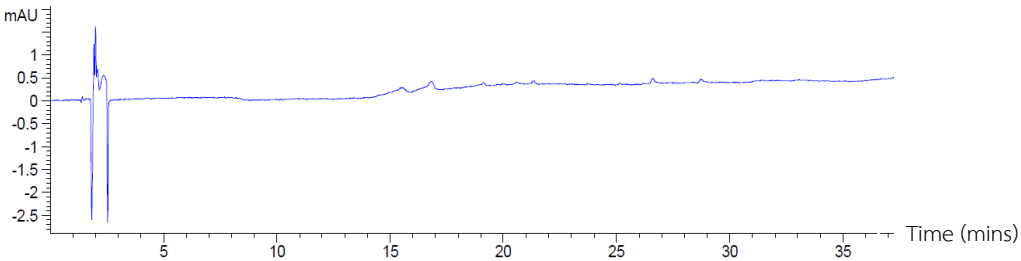
c. Moisture condition (Continued)



d. Acid condition (Continued)



e. Alkaline condition (Continued)



f. Oxidation condition (Continued)

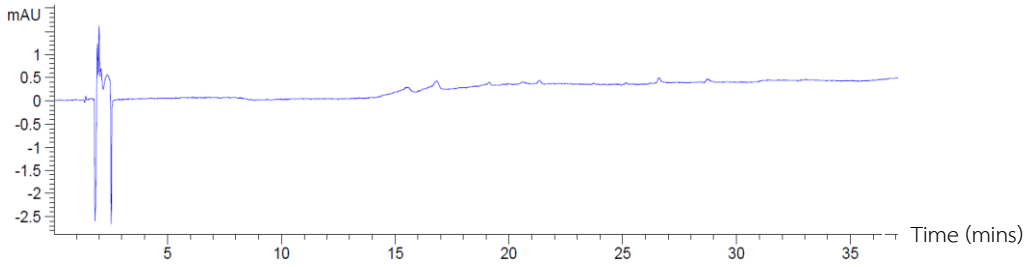


Figure 4.13 HPLC chromatograms of cyanidin-3-o-sambubiosides and stress test conditions at wavelength 520 nm. Extraction conditions: a- cyanidin-3-o-sambubiosides; b-thermal condition; c-moisture condition; d-acid condition; e-alkaline condition; f-oxidation condition.

4.8 Isolation of lactic acid bacteria (LAB)

The coccal isolates, RN17, RN18, RN20 and FV4 and the rod-shaped isolates RN19, FV1-1, FV1-2, FV7, FV10 and FV11-1-2 were isolated from specimens (rice seeds and fermented vegetable). On the basis of 16S rRNA gene analysis, isolate RN17 was closely related to *Weissella confusa* JCM 1093^T with 100% similarity and was identified as *Weissella confusa* (Collins, Samelis, Metaxopoulos & Wallbanks, 1993). The nearest sequence analysis of isolates RN18 and RN20 were *Lactococcus lactis* subsp. *hordniae* NBRC 100931^T with 99.56% similarity. Therefore, they were identified as *Lactococcus lactis* subsp. *hordniae* (Schleifer, Kraus, Dvorak, Kilpper, Collins & Fischer, 1985). Isolate RN19 was closely related to *Lactococcus lactis* subsp. *lactis* JCM 5805^T with 100% of similarity and it was identified as *Lactococcus lactis* subsp. *lactis* (Schleifer, Kraus, Dvorak, Kilpper, Collins & Fischer, 1985). Isolates FV1-1, FV1-2 and FV7 were closely related to *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917T with 100% similarity and therefore they were identified as *Lactobacillus plantarum* subsp. *plantarum* (Bringel, Castioni, Olukoya, Felis, Torriani & Dellaglio, 2005). Isolate FV4 was closely related to *Pediococcus acidilactici* DSM 20284^T with 99.78% similarity and it was identified as *Pediococcus acidilactici* (Tanasupawat, Okada, Kozaki & Komagata, 1993). Isolates FV10 and FV11-1-2 were closely related to *Lactobacillus pentosus* DSM 20314^T with 100% similarity and they were identified as *Lactobacillus pentosus* (Zanoni, Farrow, Phillips & Collins, 1987). The results of identification of isolates are shown in Table 4.9.

In this study, *Lactococcus lactis* subsp. *lactis* RN19, *Lactobacillus plantarum* subsp. *plantarum* FV1-1 and *Pediococcus acidilactici* FV4 are selected to use as starters starter culture for plain soy yogurt because these bacteria are frequently used for milk fermentation (Heller, 2001; Gemechu, 2015). However, *Weissella confusa* strain was not widely used as a dairy starter because it was isolated from human feces (Lee, Park, Jeong, Heo, Han & Kim, 2012). In addition, *Weissella confusa* strain was not resistant under the acid condition in the human body. Besides,

several infection cases in human that related to *Weissella confuse* strain, it was resistance to the drug, especially vancomycin (Salimnia, Alangaden, Bharadwaj, Painter, Chandrasekar & Fairfax, 2011). *Lactobacillus pentosus* unpopularly used to yogurt production.

Table 4.9 Isolate no, fermentation type and nearest relatives based on 16S rRNA gene similarity

Isolate no.	Sources	Fermentation type	Nearest relatives	%Similarity
RN17	Chiang Mai (Doi Saket)	Heterofermentative	*Weissella confusa* JCM 1093^{T}	100%
RN18	Japan	Homofermentative	*Lactococcus lactis* subsp. *hordniae* NBRC 100931^{T}	99.56%
RN19	Japan	Homofermentative	*Lactococcus lactis* subsp. *lactis* JCM 5805^{T}	100%
RN20	Japan	Homofermentative	*Lactococcus lactis* subsp. *hordniae* NBRC100931^{T}	100%
FV1-1	Don Muang	Homofermentative	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^{T}	99.15%
FV1-2	Don Muang	Homofermentative	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^{T}	99.15%
FV4	Don Muang	Homofermentative	*Pediococcus acidilactici* DSM 20284^{T}	99.78%
FV7	Bangkok (Saphan3)	Homofermentative	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^{T}	100%
FV10	Bangkok (Sathupradit6)	Homofermentative	*Lactobacillus pentosus* DSM 20314^{T}	100%
FV11-1-2	Bangkok (Sathupradit6)	Homofermentative	*Lactobacillus pentosus* DSM 20314^{T}	100%

4.9 Development of roselle soy yogurt

4.9.1 Selection starter of soy yogurt

Two experiments of the culture starter of soy yogurt that are shown in Table 4.10. Both starters were inoculated with proportion 1:1 (v/v) and incubated at 37°C for 12 hr. After fermentation, a starter from experiment 2 exhibited results better than experiment 1. Selection of microorganisms were chosen following the U.S. FDA and previously reported. Food and Drug Administration (FDA) of the United State allowed *Lactobacillus* species and lactic acid-producing bacteria as microorganism for yogurt production (FDA, 2018). *Lactococcus lactis subsp. lactis* (JCM 5805), *Lactobacillus plantarum* subsp. *plantarum* (ATCC 14917) and *Pediococcus acidilactici* (DSM 20284) strains are widely used as a probiotic microorganism that promoting natural intestinal flora. Probiotic initiated regular health system, especially the digestive and immune system (Sotoudegan, Danialib, Hassani, Nikfar & Abdollahi, 2019). Besides, the lactic acid represented cytotoxicity activity of bacteria including *Bacillus cereus*, *Escherichia coli*, *Acinetobacter baumannii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter cloacae*, *Listeria monocytogenes*, *Klebsiella pneumonia* (Kumar *et al*., 2016; Porto, Kuniyoshi, Azevedo, Vitolo & Oliveira, 2017; Nehal *et al*., 2019). Moreover, three lactic acid bacteria in the yogurt displayed antioxidant activity by DPPH assay with value 75.00%, 53.05%, and 31.04% (Li *et al*., 2012; Ozdogan, Akcelik, Aslim, Suludere & Akcelik, 2012; Yeong & Dong, 2015).

Table 4.10 Cell numbers and pH of soy yogurt starter after 12 hr fermentation

Experiments	Starter	Cell numbers (CFU/g)	pH
1	*Lactococcus lactis* subsp. *lactis* RN19 + *Pediococcus acidilactici* FV4	4.8×10^{7}	4.72
2	*Lactobacillus plantarum* subsp. *plantarum* FV1-1 + *Pediococcus acidilactici* FV4	5.6×10^{8}	4.54

4.9.2 Stability test of soy yogurt

The plain soy yogurts were tested physiochemical stability that contains viscosity, % lactic acid, syneresis, color, pH and amounts lactic acid bacteria of plain soy yogurt. The data were analyzed by using ANOVA statistical are displayed in Table 4.11. Day 0 in each group was used as control to compare the other days of storage. Microorganism, fermentation time and temperature were influenced to physiochemical of soy yogurt (Somkuti & Steinberg, 2010; Filho *et al*., 2016). In MRS medium, *Lactobacillus plantarum* was reduced pH value from 6.48 to 4.43 under condition at 37°C for 16 hr (Sawitzki *et al*., 2009). Moreover, combination of microorganisms (*Pediococcus acidilactici* and *Lactobacillus bulgaricus*) fermented in skim milk for 10 hr at 37°C exhibited pH with value 5.32 (Somkuti & Steinberg, 2010). The different of maximal growth of bacteria effected to incompatibility strains during fermentation (Pereira *et al*., 2011). The results demonstrated that viscosity and pH of soy yogurt were decreased. On the other hand, acidity and syneresis were increased. Fermentation time was affected to texture of soy yogurt. Viscosity is a yogurt gel formation under thermal fermentation. The hydrophilic peptides were removed from soymilk and became to curd formation. The viscosity was increased depend on increment of fermentation time. However, texture of sample 2 (fermentation time 10 hr) was harder than other sample and the hardness was significantly decreased during storage in day 14 and 21 ($p<0.05$). Moreover, viscosity of every samples were significantly decreased under 21 days storage. The microorganisms in soy yogurt were growth and effect to protein precipitated (viscosity decreased). The growth of microorganisms in sample 1, 2, 3 and 4 (fermentation time 8 hr, 10 hr, 12 hr and 14 hr) were slightly changed during of storage. The lactic acid bacteria were produced acid during fermentation. The acid in sample were displayed in pH value which is inversely to %acidity. If sample was low pH value, the %acidity was increased. The least pH was sample 4 (fermentation time 14 hr). On the other hand, the most %acidity was sample 4. pH of all samples was decreased during 21 days of storage. The percent syneresis of samples were increased depend on fermentation time. After fermentation, %syneresis of samples were significantly increased during storage period. The results

demonstrated that soy yogurt at fermentation time 8 to 12 hr had not significantly changed at day 7, but significantly increased %syneresis in day 14. For fermentation time 14 hr, soy yogurt had not significantly changed within 14 day and %syneresis significantly increased in day 21. Syneresis was problem of yogurt after fermentation. The reduction of peptide bond lead to %syneresis increment (Mousavi, Heshmati, Garmakhany, Vahidinia & Mehdi Taheri, 2019). The high syneresis values showed the low capability of the protein/peptides to hold water in the gel. This can cause the lower viscosity during storage due to more water released into the yoghurt system. Acidity of soy yogurt in storage period also lead to protein denaturation in soy-based product. Moreover, time storage also affected color of soy yogurt. The color of soy yogurt was analyzed by using CR-400 utility program. The program was displayed color index result in form of L* (black (-) to white (+)), a* (green (-) to red (+)) and b* (blue (-) to yellow (+)). ΔE*ab was set at tolerance = 1 and the results displayed color stability of each sample. The color measurement of samples 1 was changed in day 14 (ΔE*ab > 1). Besides, color stability of sample 2, 3 and 4 were changed in day 21. To conclude, all of samples more darken during storage (ΔE*ab increased). In control (day 1), the rising time fermentation increased lactic acid production. Many commercial yogurts carried bacteria more than 100 million CFU/g in product (Science, 2018). However, FDA of Thailand determined microorganism after fermentation not less than 10 million CFU/g (FDA, 2013). There were 4 different time: 8 hr, 10 hr, 12 hr and 14 hr at 37°C were reached to 4.5×10^6 CFU/g, 1.1×10^8 CFU/g, 2.3×10^8 CFU/g and 2×10^8 CFU/g, respectively. The most bacterial growth was plain soy yogurt at fermentation time 14 hr. Moreover, plain soy yogurt at fermentation time 8 hr containing bacteria less than criteria of fermented milk. From the result, amounts of bacteria at fermentation time 12 hr in during storage period that containing the most bacteria. Besides, the amount of microorganism in soy yogurts (8 – 14 hr) were decreased during storage period like previous reported (Zhi *et al*., 2018). In storage period, lactic acid bacteria metabolite nutrients for survival. The acid was released from bacteria and suppressed other lactic acid bacteria (Izadi *et al*., 2015; Zhi *et al*., 2018). In addition, this reason effected to change of pH, %acidity, %syneresis and color index of plain soy yogurt.

Figure 4.14 Soy yogurt at various fermentation time (8, 10, 12 and 14 hr.)

Table 4.11 Soy yogurt at each time fermentation

Yogurt	Viscosity (cP)	% Acidity	pH	% Syneresis	Colorimeter				Bacterial cell numbers (CFU/g)
					L*	a*	b*	ΔE*ab	
Incubation time 8 hours (Sample 1)									
Day 0	9514.17±409.73	0.01±0.00	4.83±0.00	44.67±0.20	73.84±0.01	-3.84±0.01	9.55±0.00		$4.5x10^6$
Day 7	10625.83±357.83	0.02±0.00	4.82±0.00	46.22±0.49	74.16±0.01*	-3.32±0.00*	9.74±0.00*	0.64	$4.5x10^6$
Day 14	8854.67±436.64	0.02±0.00	4.80±0.00*	48.22±0.20*	75.08±0.01*	-3.36±0.00*	10.17±0.01*	1.47	$4.3x10^6$
Day 21	5826.00±322.04*	0.03±0.00*	4.74±0.00*	51.32±0.74*	75.45±0.00*	-3.49±0.00*	10.83±0.01*	2.09	$6.1x10^6$
Incubation time 10 hours (Sample 2)									
Day 0	18456.67±370.56	0.03±0.00	4.58±0.00	45.32±0.54	74.47±0.00	-3.95±0.00	9.65±0.01		$1.1x10^8$
Day 7	18000.00±257.94	0.04±0.00*	4.57±0.00	47.88±0.85	74.59±0.01*	-3.42±0.01*	9.66±0.00	0.54	$4.3x10^7$
Day 14	16443.33±337.70*	0.05±0.00*	4.53±0.00*	51.73±0.99*	74.63±0.00*	-3.33±0.01*	9.54±0.00*	0.65	$6.1x10^7$
Day 21	15980.00±369.07*	0.05±0.00*	4.51±0.00*	53.00±0.63*	75.28±0.00*	-2.96±0.00*	9.82±0.0*	1.29	$1.1x10^8$
Incubation time 12 hours (Sample 3)									
Day 0	18106.67±308.53	0.05±0.00	4.50±0.00	46.25±0.28	75.62±0.01	-2.77±0.01	9.49±0.00		$2.3x10^8$
Day 7	17116.67±218.57*	0.07±0.00*	4.46±0.00*	47.18±0.23	76.32±0.00*	-2.43±0.00*	9.77±0.01*	0.83	$2.9x10^8$

*Significantly at 95% confidence interval ($p<0.05$)

Table 4.11 Soy yogurt at each time fermentation (Cont.)

Yogurt	Viscosity (cP)	% Acidity	pH	% Syneresis	Colorimeter				Bacterial cell numbers (CFU/g)
					L*	a*	b*	ΔE*ab	
Day 14	16233.33±294.88*	0.09±0.00*	4.45±0.00*	49.27±0.67*	76.27±0.01*	-2.42±0.00*	9.82±0.00*	0.81	2.6×10^8
Day 21	10181.67±86.66*	0.10±0.00*	4.45±0.00*	53.57±0.50*	76.56±0.00*	-2.38±0.01*	10.20±0.01*	1.24	2.5×10^8
Incubation time 14 hours (Sample 4)									
Day 0	17653.33±354.20	0.08±0.00	4.34±0.00	46.82±0.36	75.06±0.00	-3.12±0.00	10.04±0.00		2.0×10^8
Day 7	17080.00±315.76	0.07±0.00	4.32±0.00*	48.42±0.54	75.91±0.01*	-2.84±0.00*	9.77±0.00*	0.93	1.3×10^8
Day 14	16121.67±426.31*	0.09±0.00	4.29±0.00*	48.75±0.23	76.26±0.00*	-2.77±0.01*	10.46±0.00*	0.98	4.0×10^7
Day 21	14953.33±390.17*	0.12±0.00*	4.25±0.00*	54.02±0.95*	76.27±0.00*	-2.14±0.01*	11.56±0.00*	1.54	2.4×10^7

* Significantly at 95% confidence interval ($p<0.05$)

4.10 Roselle aqueous extract spherification

4.10.1 Direct spherification

The roselle alginate bead was developed by using direct spherification technique. The formulae were included roselle aqueous extract (0.5%, 1%, 1.5%, 2% and 2.5%), sucrose 15% w/v, citric acid 0.3 % w/v, sodium alginate 4% and calcium chloride 0.14 M. The result of formulae at various concentration of roselle extract are shown in Figure 4.10. However, the result demonstrated roselle alginate bead exhibited unpleasant with bitter taste. Because of roselle is fully of bioactive constituents with a sour taste, proportion of calcium chloride (originated bitter) was increased to entrap roselle solution within carrier. The method unsuitable for acidity substances at pH below 3.6 (Molecularrecipes, 2014). Moreover, the carrier uncontained the active solution more than 4 days. The bead from direct spherification technique must be served immediately because the sphere was unstable (Molecularrecipes, 2014). According to the result, roselle alginate bead from direct spherification were not to determined stability of antioxidant and bioactive compound.

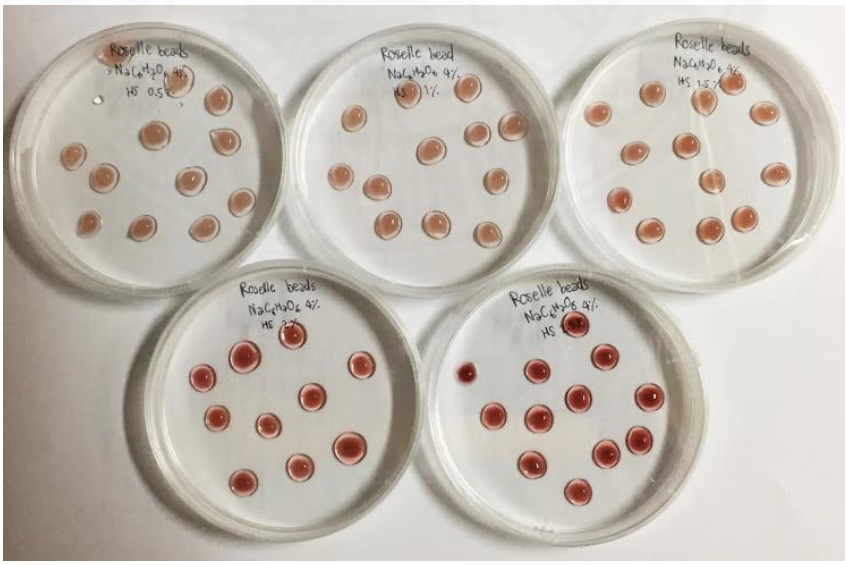


Figure 4.15 Direct spherification of roselle extract at various concentration (0.5%, 1%, 1.5%, 2% and 2.5%) of roselle extract

4.10.2 Reverse spherification

The ingredients for roselle reverse spherification included roselle aqueous extract (1%, 1.5%, 2% and 2.5%), sucrose 15% w/v, citric acid 0.3 % w/v, calcium lactate 0.14 M and sodium alginate bath 4%. After reverse spherification, the results are shown in Figure 4.11. The figure 4.11 demonstrated concentration of roselle extract effect to color appearance of roselle bead. Roselle bead with roselle concentration 1 and 1.5% were showed fade color and were not appealing to the tester. Moreover, roselle bead with more roselle concentration was given sore taste and not prefer to tester. Therefore, the roselle bead containing 2% of roselle aqueous extract was used to evaluated stability of antioxidant, total phenolic content and bioactive constituents for 21 days storage at 4°C. Sensory evaluation of roselle reverse spherification was also tested by using 5-points hedonic scale questionnaire.

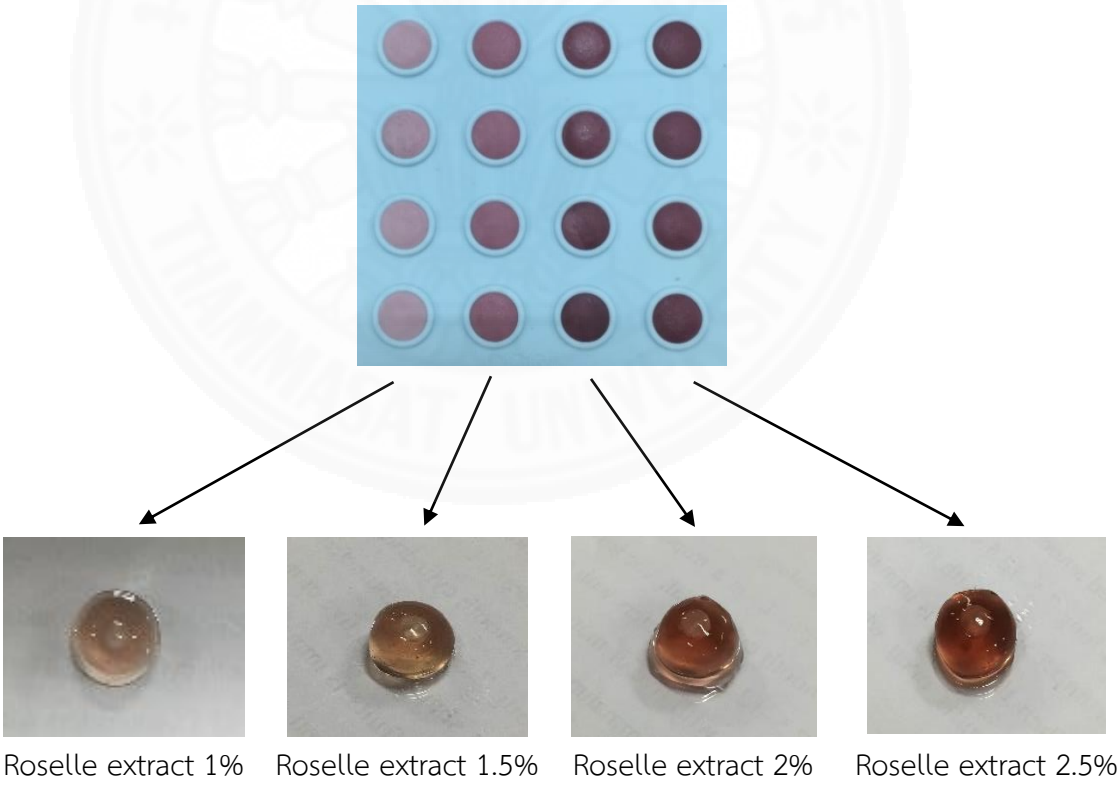


Figure 4.16 Reverse spherification of roselle extract at concentration 1, 1.5, 2 and 2.5%

4.10.3 Stability test of roselle reverse spherification

After reverse spherification process, the roselle concentration 2% of roselle alginate bead was chosen from the tester and tested for antioxidant by using DPPH radical scavenging assay and studied total phenolic content. The values are shown in mean±sem. According to Table 4.12, roselle alginate bead in first day was used to compare with another day during storage period. Roselle spherification of day 0, 7, 14 and 21 showed significantlys ($p<0.05$) decreased in antioxidant compared to day 0 (control group). In antioxidant activity, stability of roselle alginate bead at day 0, 7, 14 and 21 exhibited with EC_{50} values 25.89±1.38 (control group), 41.23±0.88, 42.74±0.81 and 51.22±2.36 μg/ml, respectively. In addition, phenolic contents in roselle spherification were reduced in during storage. The total phenolic results of day 0, 7, 14 and 21 were exhibited with values 75.97±3.31 (control group), 53.20±0.86, 53.20±2.16 and 50.22±0.48 mg GAE/g, respectively. All of the total phenolic results were also significantly ($p<0.05$) decreased in during storage.

Roselle aqueous extract containing bioactive constituents, especially anthocyanins, flavonoids and phenolic compound (Herranz *et al*., 2012). The stability of roselle spherification were analyzed chemical content by using HPLC technique, as displayed in Table 4.12. The highest chemical compound showed in day 0 of all sample. After storage, the chemical compound significantly decreased in storage period. The reduction of chemical compound related with decrement of antioxidant and total phenolic content in Table 4.13. The result illustrated the stability of roselle alginate bead in this study able to storage for 7 days. Previous documentary reported caffeic acid, delphinidin, cyanidin, myricetin and quercetin in roselle based product at 6°C decreased gradually in during period (Ifie *et al*., 2018). Quercetin in berry juice was also degraded 46.1% under darkness storage at 4°C for 56 days (Odriozola-Serrano, Soliva-Fortuny & Martin-Belloso, 2008). Bioactive constituents in roselle can be degraded by many factors such as oxidation, light, storage and temperature. Besides, polysaccharides were applied with sodium alginate to improve stability of encapsulation in food industry. Documentary described natural polysaccharide (carrageenan, cellulose, chitosan, konjak, gum arabic, pectin and zein)

and protein (whey protein and gelatin) have been used for prolonged self-life of bioactive molecule in solution (Devi, Sarmah, Khatun & Maji, 2017; Jiang & Zhu, 2019; Martynova, Maceichik & Lomovskiy, 2019).

Table 4.12 Stability test of roselle spherification by using DPPH scavenging radical assay and Total phenolic content

Stability of roselle bead	DPPH scavenging radical assay EC_{50} (µg/ml)	Total phenolic content mg GAE/g
- Day0	25.89±1.38	75.97±3.31
- Day7	41.23±0.88*	53.20±0.86*
- Day14	42.74±0.81*	53.20±2.16*
- Day21	51.22±2.36*	50.22±0.48*

* Significantly at 95% confidence interval ($p<0.05$)

Table 4.13 Stability of chemical fingerprint in roselle spherification during storage by using HPLC technique

Chemical compound (mg/g±sem)	Day 0	Day 7	Day 14	Day 21
Chlorogenic acid	5.55±0.01	5.24±0.13	4.89±0.13*	4.81±0.03*
Coumaric acid	2.08±0.01	2.00±0.00*	1.99±0.00*	1.97±0.00*
Ferulic acid	0.15±0.00	0.14±0.00	0.13±0.00	0.08±0.00*
Quercetin	0.27±0.00	0.24±0.01	0.20±0.00*	0.11±0.00*
Cyanidin-3-o-sambubiosides	1.03±0.01	0.92±0.01*	0.45±0.00*	0.42±0.00*

* Significantly at 95% confidence interval ($p<0.05$)

4.11 Sensory evaluation of roselle soy yogurt and roselle spherification

4.11.1 Sensory evaluation of roselle soy yogurt

Sensory evaluation (appearance, smell, flavor, texture, overall acceptance of plain soy yogurt and overall acceptance of plain soy yogurt plus roselle beads) of soy yogurt at different incubation times (10, 12 and 14 hr) are shown in Table 4.14 by stata 14.2 program. The soy yogurt fermented for 8 hr was excluded from sensory test because the pH more than 4.5 and the texture of yogurt was not as good as it should be. There were 6 parameters: appearance, smell, flavor, texture, overall acceptance and overall acceptance with roselle bead for sensory analysis of soy yogurt. The results were analyzed by using one-way ANOVA statistical and presented in value mean±sd. The sensory evaluation demonstrated that all of soy yogurts were not significantly difference at 95 percent confidence level. Appearance of all samples were nearby value and showed the highest satisfied from participants. The smell of sample 1 (fermented 10 hr), 2 (fermented 12 hr) and 3 (fermented 14 hr) were 3.22±1.08, 3.42±0.99 and 3.08±1.19 scores, respectively. After fermentation, beany smell was increased that it was identity odor of soy fermentation. The smell of soybean is caused by the oxidation of unsaturated fatty acids such as linoleic and linolenic (Siedow, 1991). This reaction is stimulated oxygen molecules into unsaturated fatty acids and released lipoxygenases isozymes. Participants were given flavor score for sample 1, 2 and 3 with value of 3.20±1.21, 3.26±1.19 and 3.32±1.08 scores, respectively. The acidification was occurred during the growth of microorganisms, but the LAB was not affected to bean flavor of soy yogurt. Beany of flavor and odor are the same caused by lipoxygenase reaction of unsaturated fatty acids in soybean (Lv, Song, Li, & Guo, 2011). Soybean infusion with $NaHCO_3$ was eliminated beany flavor and odor in soy product (Endo, Ohno, Tanji, Shimada, & Kaneko, 2004). The $NaHCO_3$ was inhibited lipoxygenase activity during soaking soybeans that cause of unpleasant bean odor. Grinding soybeans in water under temperatures between 80-100°C able to get rid of unpleasant odor.

Moreover, soaking soybean under thermal condition for 15 min before blending or soaking soybeans for 8-12 hr and immersion in boiling water for 30 min able to eliminate beany smell (inspire world of main, 2011). Besides, the inventor (U.S. patent 4929451) suggested glucose and glucose oxidase able to eliminate bean odor by adding in soaked soybean water (free patents online, 1990).

Texture analysis of sample 1, 2 and 3 were 3.76±0.96, 3.66±0.85 and 3.8±0.98 scores, respective. The highest score was presented in sample 3 (fermentation time 14 hr). The texture of soy yogurt was more soften when the time of fermentation was increased (Zuo, Peng, Shi & Guo, 2016). For overall attributes, the results of samples 1 and 2 were equal at value 3.44±0.81 scores and nearby sample 3 with value 3.36±1.05 scores. Moreover, overall acceptance when eating soy yogurt with roselle bead were assessed. The highest satisfaction was sample 3 (fermentation time 12 hr) with value 3.84±0.93 scores. The result of soy yogurt mixed with roselle bead tend to more satisfaction than consumed only plain yogurt in every samples, but not significantly different ($p>0.05$).

Table 4.14 Sensory evaluation of soy yogurt

Variable	Sample	Participants (persons)	Minimum score	Maximum score	Mean±SD	p-value between groups
Appearance	1	50	3	5	4.34±0.59	0.98
	2	50	3	5	4.32±0.62	
	3	50	3	5	4.32±0.62	
Smell	1	50	1	5	3.22±1.08	0.30
	2	50	2	5	3.42±0.99	
	3	50	1	5	3.08±1.19	
Flavor	1	50	1	5	3.20±1.21	0.88
	2	50	1	5	3.26±1.19	
	3	50	2	5	3.32±1.08	
Texture	1	50	2	5	3.76±0.96	0.69
	2	50	2	5	3.66±0.85	
	3	50	2	5	3.80±0.98	

Table 4.14 Sensory evaluation of soy yogurt (Cont.)

Variable	Sample	Participants (persons)	Minimum score	Maximum score	Mean±SD	p-value between groups
Overall acceptance	1	50	2	5	3.44±0.81	0.88
	2	50	2	5	3.44±0.81	
	3	50	2	5	3.36±1.05	
Overall acceptance with roselle bead	1	50	2	5	3.64±1.08	0.60
	2	50	2	5	3.68±0.91	
	3	50	2	5	3.84±0.93	

* Sample 1 = fermented 10 hours, Sample 2 = fermented 12 hours and Sample 3 = fermented 14 hours

4.11.2 Sensory evaluation of roselle spherification

Sensory quality of roselle spherification are shown in Table 4.15. The one-way anova was statistic that used for calculated for this study. Appearance of roselle bead exhibited the highest scores with value 4.32±0.62 scores. The results of smell, texture and overall acceptance were displayed with values 4.06±0.82, 4.10±1.97 and 4.12±0.82 scores, respectively. The least scores of roselle bead assessment was flavor with value 3.96±0.92 scores. Many participants suggested increment of sweetness influence to increase roselle alginate bead admiration.

Table 4.15 Sensory evaluation of roselle spherification

Variable	Participants (persons)	Minimum score	Maximum score	Mean±sd
Appearance	50	3	5	4.32±0.62
Smell	50	3	5	4.06±0.82
Flavor	50	2	5	3.96±0.92
Texture	50	2	5	4.10±1.97
Overall acceptance	50	2	5	4.12±0.82

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Roselle calyx (*Hibiscus sabdariffa*) has widely used in food product with its antioxidant effect. It is mainly containing anthocyanin, flavonoid and phenolic acid. The bioactive constituents unstable under thermal, acid and time storage. Aqueous extract of roselle displayed antioxidant activity (EC_{50} 50.40 µg/ml) and total phenolic compound (46.51 mg GAE/g). The extract was neither potential for nitric oxide production inhibition in RAW264.7 nor superoxide production inhibition from HL-60 leukemia cell. Roselle aqueous extract 1 g. displayed chlorogenic acid, coumaric acid, ferulic acid, quercetin and cyanidin-3-o-sambubiosides with quantity 5.76, 2.23, 0.09, 0.57 and 0.56 mg., respectively. The document reported some phenolic compound degraded under acid condition. Therefore, modification of roselle hydrolysis in gastro-intestinal tract techniques was used to investigated antioxidant, anti-inflammation and bioactive constituents. This process was concentrated bioactive substances in roselle aqueous extract. As a result, the bioavailability (anti-inflammation and antioxidant) of roselle aqueous extract under HCl-$CHCl_3$ and DI water-$CHCl_3$ condition were expressed potential after this process.

Stress test of roselle aqueous extract were studied for suitable condition of extract before product development. The results displayed the roselle aqueous extract stable in thermal, moisture and acidity conditions, but unstable under alkaline and oxidation conditions. As a result, direct and reverse spherification techniques were used to develop roselle product by entrapping roselle solution in carrier. For result of direct spherification, this technique unable to contain roselle solution. On the other hand, reverse spherification technique able to entrap the solution longer than direct spherification technique. Therefore, roselle solution was entrapped in sphere form by using reverse spherification technique. Stability of antioxidant and chemical compound were tested for 21 days storage. As a result, loss in bioactive compound in roselle

spherification leading to loss of antioxidant activity potential. According to statistic calculation, the roselle beads unable to maintained bioactive constituents more than 14 days. The sensory evaluation questionnaire was tested for acceptability of roselle spherification. The most acceptance was appearance of roselle bead. The participants claimed that color of roselle bead look like the ruby. For the flavor, the participants suggested more sweetness able to improve flavor of roselle bead. Leguminous odor in soy yogurt was unpleasant from the tester. However, the overall acceptance of roselle bead was accepted from the participants.

Lactobacillus plantarum subsp. *plantarum* FV1-1 and *Pediococcus acidilactici* FV4 were isolated from rice seeds and fermented vegetable. The cultures were lactic acid bacteria and used as a starter cultures in dairy product. The interaction between soy yogurt starter and time fermentation effect on quality parameter of soy yogurt during storage period (21 days). The results indicated viscosity and pH of soy yogurt at all fermentation time were decreased which relation to increment of syneresis, %lactic acid and growth of bacteria. Syneresis of soy yogurt was an important problem after fermentation. The quality parameter leading to sensory evaluation. The sensory testing was determined soy yogurt at various fermentation time including 10, 12 and 14 hr. The most acceptability was soy yogurt at fermentation 14 hr. The addition of roselle bead into soy yogurt seem to improve acceptability of soy yogurt product. Therefore, the roselle soy yogurt should be developed for further study.

5.2 Recommendations

The results of this research should be considered for further study and to develop characteristics and quality of soy yogurt. Moreover, roselle spherification method should be developed for storage and maintain bioactive constituents. The further study should be concerned following below:

1. The strain of bacteria is mainly optimization for soy yogurt starter. The strain of lactic acid bacteria should be carefully selected for soy yogurt production. Difference strain get difference chemical and physical of product.
2. To reach the minimum requirement of the amount of the microorganisms in the yoghurt, increased quantity of the starter cultures at the time of inoculation is recommended.
3. To make rounder-shape roselle beads, increased viscosity of the solutions and rotation speed used in the spherification step are recommended.
4. The roselle beads should be coated with chitosan to protect the leakage of roselle solution and develop non-vegan product.
5. In term of characteristic of soy yogurt, temperature and fermentation time should be considered in the fermentation process.
6. Beany odor in soy yogurt is a consumer obstruction. Elimination of leguminous smell is an important in soymilk process.
7. For reducing bias, sample size of sensory evaluation should be 30 participants/group and separate to two groups that base on vegetarian and non-vegetarian.
8. Carrier of spherification process should be developed by using polysaccharides to maintain structure of roselle spherification.

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APPENDICES

APPENDIX A

Chemical Reagents

1. 0.14 M Calcium chloride ($CaCl_2$)

Prepared 1.4 M calcium chloride by weighing 15.54 g calcium chloride and dissolved in 100 ml DI water. Mixed well and kept at temperature 4°C. 0.14 M calcium chloride was prepared by using 10 ml 1.4 M calcium chloride diluted with 90 ml DI water.

2. 40% Glycerol

Glycerol 40 ml were mixed in distilled water 100 ml. After that, pipetted the solution 1 ml into cryotube. The cryotubes were purified at temperature 121°C for 15 min. Then, cooled down and stored at room temperature.

3. Griess's reagent

Phosphoric acid 2.5%, sulfanilamide 1% and N-(1-naphthy) ethylenediamine dihydrochloride 0.1% were dissolve in DI water. After that, the solution was adjusted volume to 500 ml and were stored at temperature 4°C.

4. Dulbeco's modified eagle medium (DMEM)

DMEM powder 13.4 g were dissolved in 1,000 ml sterile DI water under sterile condition. After that, $NaHCO_3$ 3.7 g and 10% HCl 1.2 ml were added into the DMEM medium. The medium was filtered by using 0.2 microns sterile membrane filter. The DMEM media 1,000 ml were mixed by using FBS 100 ml and P/S 10 ml. The, the DMEM media were stored at temperature 4°C.

5. Roswell Park Memorial Institute medium (RPMI)

Preparation of RPMI 1640 medium used a same technique for preparation of DMEM media. But $NaHCO_3$ was changes to 2 g.

6. 0.01 M Hydrochloric acid (HCl)

37% hydrochloric acid 0.49 ml was added into volumetric flask and adjusted volume to 500 ml.

7. 1% Phenolphthalein

Phenolphthalein 1 g was dissolved 50% ethanol solution (50 ml ethanol mixed with 50 ml DI water).

8. Phosphate buffer saline (PBS)

PBS 1 tablet was placed into the bottle containing DI water 100 ml. The PBS solution was purified by using autoclave (121°C, 20 min).

9. 0.1% Phosphoric acid

500 µl of phosphoric acid was added into volumetric flask and adjusted volume to 500 ml by using ultrapure water. The solution was kept in glass container.

10. 1% Sodium alginate ($C_6H_9NaO_7$)

Blended 1 g of sodium alginate with 100 ml of DI water. The sodium alginate bath was stored at temperature 4°C until the bubbles disappeared.

11. 0.1 M Sodium hydroxide (NaOH)

Sodium hydroxide 0.4 g was dissolved in DI water 100 ml.

APPENDIX B

16S rRNA gene sequence of representative isolates

1. RN17 (*Weissella confusa*)

GGTTCAACTGATTTGAAGAGCTTGCTCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAA
CCTACCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGA
TGGTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGATGATGCATAGC
CGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACA
ATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAAGAATGAC
ATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTAT
GTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACT
GAGGAATTGCTTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATA
TGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAG
ATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATTAAG
CACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGT
TGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTAC
GTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATA
CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAG

2. RN18 (*Lactococcus lactis* subsp. *hordniae*)

TACTTGTACCAACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAAC
GAATGCTAATACCGCATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTG
TATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTG
AGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGA
GTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACT
ACCCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAA
AGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTG
CAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTG
GCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATACTCGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAACGAGACTG
CCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGAT
GGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTAC
ATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC
CACGGGAGTTGGGAGTACCCGAAGTAGGTTGCC

3. RN19 (*Lactococcus lactis* subsp. *lactis*)

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGGTTGGTACTTGTACCGACTGGATGAGCAGCGAACGG
GTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCGCATAAAAACTTTAAACAC
AAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACC
AAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGT
TGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGT
AAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGC
GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCTCTGTAT
CGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATTCCTAGAGATAGGAA
GTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGA
CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTA
GCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATC
AGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTG
CCTAACCGCAAGGAGGGCGCTTCCTAAGGTAAGACCGATGACTGGGGTG

4. RN20 (*Lactococcus lactis* subsp. *hordniae*)

TACTTGTACCAACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAAC
GAATGCTAATACCGCATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTG
TATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTG
AGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGA
GTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACT
ACCCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAA
AGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTG
CAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTG
GCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAGATGTAGGGAGCTATAAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGGAGTACGACCGCAAGGTTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAAGCATGGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATACTCGTGCTATTCCTAGAAAAATAGGAAGTTCCTTCGGGACACGGGAAACAAGTGGGTGGCAAGGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAA
CGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT
ACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGTAGGCTGCAA
CTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCCTAACCGC

5. FV1-1 (*Lactobacillus plantarum* subsp. *plantarum*)

TGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACAC
CTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATG
GTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCA
CATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCA
ACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATT
GACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGG
AAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAG
GCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTA
AACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCC
GCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACC
TTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTC
TGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTG
CAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGAGA

6. FV1-2 (*Lactobacillus plantarum* subsp. *plantarum*)

TGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACA
ACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGG
GTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGC
TCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTT
TTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGA
ACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAAGCGGCTGTCTGGTCTGTAACTGACGCTGA
GGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTT
CCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGGCCGCAAGGCTGAAACTCAAAGGAATTTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAA
TCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCG
AACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGC
TAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACA
CCCAAAGTCGGTG

7. FV4 (*Pediococcus acidilactici*)

TGTGCTTCATGATATTATAACACCAAGGGAGTGGCGGACGGGTGAGTAACACGTTGGGTAACCTGCCCAGAAGCAGGGGGATA
ACAACACTGGAAACAGATGCCTAAGTACCGGTATAACAGAAAGAAAACCCGCCTGGTGTTTCTTTTTTAAAAGAATGGCTCTTG
CCCTATCACTTCCTGGGGATGGACCCCCGCGGCGGCCATTAGCTTTAGTTGGTGGAGGGTAAACCGGCTTCCACCAAGGGCGA
AATGATGCCGGTTAGGCCGAACCTGAAGAAAGGGGTAAAATCGGCCCCACATTTGGGGAACTTGAAGAAACACGGGGCCCCAG
AACTTTCCCTACGGGAAGGGGCAGCCAGGTTAGGGGAAATCTTTTCCCACAATTGGGAACGGGCAAGTTCTTGAAATGGAAGCC
AAACGGCCCCGCGGTGAAGGTGGGAAGAAGGGGTTTTTCGGCCTTTCGTAAAGCTTCCTTGTTGTTTTAAAAGAAAGAAACGTT
GGGGGTGGAGAAGGTTAACTTGGTTTCCACCCAGGTGGACGGGTTATTTTTAACCCCAGAAAAAGCCAACGGGCTTAAACTTAC
CGTTTGCCAGGCCAAGCCCGCGGGTTAAATACGTTAGGGGTGGGGCAAAGCGGTTATCCCCGGAATTTTATTGGGGGCCGTAA
AAGGCGGAGGCCGCAGGGCGGGTTCTTTTTTAAGGTCCTAAATGTTGAAAAGCCCTTTCGGGCTTCAAACCCGAAAAGAAAGTG
CCATTTGGGAAAACTTGGGGAGAACTTTGGAGTTGCAAGAAAGAGGGACAGGTGGGAACCTTCCATTGTTGTAGCGGGTGAAA
ATGCCCGTAGATATTATGGGAAAGAAACACCCAGGTGGGCGAAGGGCGGGCTTGTTCTGGGTCTTTGTAACTGGAACGCTGAG
GGCTCCGAAAAGCAATGGGGTAGCCGAAACAGGGATTAAGATACCCCTGGGTAGTCCCATGCCCGTAAACGGATGATTACTAA
GTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
AAAAGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGAC
ATCTTCTGCCAACCTAAGAGATTAGGCGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGT
ACAACGAGTCGCGAAACCGCGAGGTTTAGCTAATCTCTTAAAACCATTCTCAGTTCGGA

8. FV7 (*Lactobacillus plantarum* subsp. *plantarum*)

TTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGG
GATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACT
TTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTA
ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
ATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTT
CAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC
GGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGA
AACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGT
GGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCC
ATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGA
GTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGC
GAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTT
GGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT
ACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATT
GTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCG

9. FV10 (*Lactobacillus pentosus*)

CCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACAT
TGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACG
CCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGAC
GGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG
GCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAA
CTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCG
GCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAAC
GATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTA
CCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGG
TGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT
ACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAAT

10. FV11-1-2 (*Lactobacillus pentosus*)

CTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAG
AAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCG
GCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTG
AGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAG
TAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGT
GCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGA
ACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCT
GGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCG
CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
AGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGC
ATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAG
TTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGT

APPENDIX C

Sensory evaluation questionnaire

Sensory evaluation of roselle soy yogurt

1. Gender ❒ Male ❒ Female

2. Ages ❒ 16-20 yrs ❒ 21-30 yrs ❒ 31-40 yrs ❒ 41-50 yrs ❒ 51-60 yrs

Please rate the score in the blank following your opinion by fill ✓

(1=Strong dislike, 2=Dislike, 3=Neither like nor dislike, 4=Like, 5=Strong like)

Sample 1	1	2	3	4	5
Color	❒	❒	❒	❒	❒
Odor	❒	❒	❒	❒	❒
Flavor	❒	❒	❒	❒	❒
Texture	❒	❒	❒	❒	❒
Overall acceptance	❒	❒	❒	❒	❒
Overall acceptance with roselle beads	❒	❒	❒	❒	❒
Sample 2					
Color	❒	❒	❒	❒	❒
Odor	❒	❒	❒	❒	❒
Flavor	❒	❒	❒	❒	❒
Texture	❒	❒	❒	❒	❒
Overall acceptance	❒	❒	❒	❒	❒
Overall acceptance with roselle beads	❒	❒	❒	❒	❒
Sample 3					
Color	❒	❒	❒	❒	❒
Odor	❒	❒	❒	❒	❒
Flavor	❒	❒	❒	❒	❒
Texture	❒	❒	❒	❒	❒
Overall acceptance	❒	❒	❒	❒	❒
Overall acceptance with roselle beads	❒	❒	❒	❒	❒
Roselle spherification					
Color	❒	❒	❒	❒	❒
Odor	❒	❒	❒	❒	❒
Flavor	❒	❒	❒	❒	❒
Texture	❒	❒	❒	❒	❒
Overall acceptance	❒	❒	❒	❒	❒

Suggestion

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แบบสอบถามการทดสอบคุณภาพทางประสาทสัมผัสของโยเกิร์ตนมถั่วเหลืองกระเจี๊ยบแดง

1. เพศ ❒ ชาย ❒ หญิง

2. อายุ ❒ 16-20 ปี ❒ 21-30 ปี ❒ 31-40 ปี ❒ 41-50 ปี ❒ 51-60 ปี

กรุณาให้คะแนนลงในช่องที่ตรงกับความพึงพอใจของท่านมากที่สุด

(1=ไม่ชอบมากที่สุด, 2=ไม่ชอบ, 3=เฉยๆ, 4=ชอบ, 5=ชอบมากที่สุด)

ตัวอย่างที่ 1	1	2	3	4	5
สี	❒	❒	❒	❒	❒
กลิ่น	❒	❒	❒	❒	❒
รสชาติ	❒	❒	❒	❒	❒
เนื้อสัมผัส	❒	❒	❒	❒	❒
ความชอบโดยรวม	❒	❒	❒	❒	❒
ความชอบเมื่อทานกับอัลจิเนตกระเจี๊ยบแดง	❒	❒	❒	❒	❒
ตัวอย่างที่ 2					
สี	❒	❒	❒	❒	❒
กลิ่น	❒	❒	❒	❒	❒
รสชาติ	❒	❒	❒	❒	❒
เนื้อสัมผัส	❒	❒	❒	❒	❒
ความชอบโดยรวม	❒	❒	❒	❒	❒
ความชอบเมื่อทานกับอัลจิเนตกระเจี๊ยบแดง	❒	❒	❒	❒	❒
ตัวอย่างที่ 3					
สี	❒	❒	❒	❒	❒
กลิ่น	❒	❒	❒	❒	❒
รสชาติ	❒	❒	❒	❒	❒
เนื้อสัมผัส	❒	❒	❒	❒	❒
ความชอบโดยรวม	❒	❒	❒	❒	❒
ความชอบเมื่อทานกับอัลจิเนตกระเจี๊ยบแดง	❒	❒	❒	❒	❒
อัลจิเนตกระเจี๊ยบแดง					
สี	❒	❒	❒	❒	❒
กลิ่น	❒	❒	❒	❒	❒
รสชาติ	❒	❒	❒	❒	❒
เนื้อสัมผัส	❒	❒	❒	❒	❒
ความชอบโดยรวม	❒	❒	❒	❒	❒

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BIOGRAPHY

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Publication

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