Potential Application of *Limnophila aromatica* Extracts as Sustainable Natural Emulsifier

July 2022

SOEUNG RASMEY

Potential Application of *Limnophila aromatica* Extracts as Sustainable Natural Emulsifier

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Bioresource Engineering

(Doctoral Program in Appropriate Technology and Sciences for Sustainable Development)

SOEUNG RASMEY

ABSTRACT

Emulsions are characterized based on the spatial distribution of the oil and water phases in the mixture. When oil droplets are dispersed in a water phase, an oil-in-water (O/W) emulsion (e.g., milk and mayonnaise) is formed. A water-in-oil (W/O) emulsion (e.g., butter and margarine) is created when the opposite happens. Emulsions made without a stabilizer are unstable, and depending on the density differences, the emulsion phase begins to separate into layers. In order to make a long-lasting stable emulsion, stabilizers must be added. Emulsion stabilizers are categorized based on how they keep the system stable. Emulsion stabilizers include emulsifiers, texture modifiers, weighing agents, and ripening retarders. Among these stabilizers, emulsifiers are essential components in the food industry because of their capacity to generate and stabilize emulsions. The majority of emulsifiers are derived from chemical and/or enzymatic processes. Natural food-grade emulsifiers are being extensively researched in order to address customer demand for green label food and beverage goods.

Rice paddy herb (*Limnophila aromatica)* belongs to the Scrophulariaceae family. *Limnophila aromatica* is a potential source of high-value-added additives with remarkable bioactivities such as antioxidant, anti-inflammatory, and antimicrobial properties. In the last decade, investigations of this plant's essential oil extraction and bioactivities have been conducted. Moreover, the polyphenolic compounds and starch isolation from this plant were also investigated by some researchers. However, no prior research has revealed the contents of saponin, protein, and other compounds linked to the emulsifying performance of the *Limnophila aromatica* extracts. This dissertation mainly focused on the suitability of *Limnophila aromatica* extract toward the sustainable production of natural emulsifiers.

First, the essential surface-active substances such as saponins, phenolic, and protein, as well as inorganic compounds of *Limnophila aromatica* extracts, were characterized. *Limnophila aromatica* extracts (LAEs) were extracted by solid-liquid extraction, using different concentrations of aqueous ethanol, including 0, 25, 50, 75, and 99.5% (*v/v*). The results showed that absolute ethanol extraction yielded the greatest total saponin (23.8%, *w/w*) and phenol content (12.7%, *w/w*), followed by 75%, 50%, 25%, and 0% aqueous ethanolic extraction. It indicated that raising the polarity of the extraction solvents by decreasing the water concentration related to a high yield of percentage of saponin and phenol content. Due to the precipitation of absolute ethanol, the optimum solvents for protein extraction were water and 20%, followed by 50%, 75%, and 99.5 % aqueous ethanolic extraction. The protein contents of *Limnophila aromatica* extracts ranged from 3.8 to 5.3% (*w/w*). Among all extracts, 75% (*v/v*) ethanolic *Limnophila aromatica* contained the lowest ash content, indicating less impurity. It was impossible to estimate the emulsifying performance of the extracts based on their chemical composition since the *Limnophila aromatica* consisted of complex compounds.

Second, the interfacial and emulsifying performances of aqueous ethanolic *Limnophila aromatica* extracts were evaluated. At the soybean oil/extracts (1 %, *w/w*) interface, all LAEs decreased interfacial tension between 12.5 and 16.1 mN/m independently of their chemical composition. Except for absolute ethanolic extract (LAE-99.5), which formulated emulsions with droplet sizes bigger than 3 µm, all ethanolic *Limnophila aromatica* extracts (LAEs) were feasible to produce submicron emulsions (273–747 nm) with a considerably negative charge. Surprisingly, just 75 % (v/v) aqueous ethanolic *Limnophila aromatica* extract (LAE-75) was able to keep emulsions stable for up to 7 days at 5 °C. As discussed above, LAE-75 contained the lowest ash content and did not contain the highest or lowest surface-active compound. These findings suggested that

emulsifying activity of LAEs not only relied on interfacial tension and/or surface-active elements. The instability of the emulsions was further affected by residual demulsifiers such as inorganic compounds. Lastly, the emulsion formed of 0.5 % (*w/w*) LAE-75 and 5% (*w/w*) soybean oil was maintained well in storage for up to 30 days at varying temperatures (5 or 25 °C).

In conclusion, *Limnophila aromatica* extract has the potential to be used as a novel source of natural emulsifier. To increase the emulsifying properties of the extracts to stabilize nanoemulsions (droplet size lower than 200 nm) with high oil concentrations, we propose employing purified *Limnophila aromatica* extracts as the emulsifiers by eliminating inorganic residuals in the future study.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

$d_{4,3}$	Volume mean diameter
LAEs	*Limnophila aromatica* extracts
MIC	Minimum inhibitory concentration
mg GAE/g	Milligram gallic acid equivalent per gram
mg QE/g	Milligram quercetin equivalent per gram
O/W	Oil-in-water
W/O	Water-in-oil

CHAPTER 1

INTRODUCTION

1.1 Background

Even though agricultural production could be increased, it may not be sufficient to fulfill global food demand. Therefore, Agri-produce processing is required to maximize food availability and shelf life, lowering losses [1]. Natural toxic materials are converted into more functional and pleasant foods or beverages throughout the food processing process. Food innovation should be emphasized for sustainable intensification and new food resources [2].

In the recent decade, research interest in environmentally friendly procedures and non-toxic additives has increased, particularly in the food and pharmaceutical industries. Moreover, the consumers are concerned about the accessibility to use and consume minimal process products containing natural ingredients. Thus, integrating natural preservatives and eliminating synthetic chemical additives for product formulation has recently been the trend toward satisfying consumer demand, as indicated by "clean label" ingredients [3].

The utilization of natural emulsifiers, which are significant ingredients for the effective formulation, provides many obstacles to product developers, owing to their inferior efficacy compared to synthetic counterparts. Chemical and synthetic emulsifiers continuously dominate the global market, contributing to 67% of total volume. The reason is that the industry could not identify natural emulsifiers as powerful and flexible as synthetic ones. So, they must use the combination of natural emulsifiers with other components to emulsify successfully. Nevertheless, research and development have substantially advanced in this field, and more innovative ingredients have been explored [4].

1.2 *Limnophila Aromatica*

Limnophila aromatica (Lam.) Merr. is also recognized as a rice paddy herb, one of the Scrophulariaceae family [5]. It is a tropical and aromatic herb approximately 30–50 cm high and can be harvested 40–45 days after sowing [6].

Table 1.1. The taxonomical classification of *Limnophila aromatica* [7].

Kingdom	Plantae
Sub-Kingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-Class	Asteridae
Order	Scrophulariales
Family	Scrophulariaceae
Genus	*Limnophila*
Species	*L. aromatica*
Binomial name	*Limnophila aromatica* (Lamk.) Merr.

This plant flourishes in hot weather and most commonly grows in wet situations, mainly in flooded rice fields. For a few months after the rainy season has passed, it thrives optimally on drained but still moist, sandy soil of harvested rice fields. After the rain stops

at the end of the monsoon season, the plant spreads like wildfire. It quickly dies after flowering. *Limnophila aromatica* is a decumbent fragrant herb with a turpentine odor, with sessile, opposite leaves that are oblong or lanceolate and finely serrated, purplish flowers in axillary and terminal racemes, long thin and glandular pedicels, and tiny obovoid-oblong capsules covered by the striate calyx [7].

Limnophila aromatica is widely grown in Southeast Asia, northern Australia, and other regions, including Bhutan, China, India, Japan, South Korea, New Guinea, and Micronesia [8–10]. The plant is native to Southeast Asia and is widely utilized as a species and medicinal herb among Southeast Asian communities. This plant adds flavor to soups, sauces, and other cuisines, particularly fresh fish dishes in Cambodian, Laotian, Thai, and Vietnamese cuisine, due to the flavor and aroma of both lemon and cumin. [9, 11, 12]. Because of the attractive aroma of its essential oil, *Limnophila aromatica* is widely utilized as a culinary plant in the form of teas and tight herbal balls for massage. The plant is also used in traditional folk medicine as an anti-inflammatory, orexigenic, antispasmodic, antimicrobial, diuretic, and galactagogue for various diseases. The decoction of plant leaves can cure fever and kidney stones, remove mucus from the bronchial tubes, and clean wounds. The plant's juice is used to treat fever and pharyngitis. It is also provided to nursing mothers when the milk gets sour [8, 11].

Farmers in Choeung Teuk commune, near Prey Veng city, Cambodia, have embraced crop diversification enthusiastically, switching from rice cultivation to rice paddy herb (*Limnophila aromatica*) production. In contrast to rice, the farmers would have a steady income because rice paddy herb (*Limnophila aromatica*) could be harvested shortly (almost every month) and suitable for planting in dry and rainy seasons. Farmers may only harvest 300 Kg of rice in the space of around 2,000 m^2 a season and sell the total

rice crop for approximately 200,000 to 300,000 riels (USD 50 to 75). However, they can harvest rice paddy herb (*Limnophila aromatica*) more than 10 times a year (almost every month) in the same single plot of land and get an income of approximately 1 million riels (USD 250) in each harvest. Based on the documentary of Prey Veng's provincial department of agriculture, 40 to 50 families in Choeung Teuk commune have shifted from growing rice to rice paddy herb (*Limnophila aromatica*). Farmers usually assist each other in harvesting their rice paddy herb to supply the market's demand in Phnom Penh and other regions. They could collect at least one to two tons of rice paddy herb daily [13].

Recently, a local small-scale enterprise in Cambodia named Hathkal Lab started to do business in essential oil production to support the local agricultural products. Rice paddy herb (*Limnophila aromatica*) essential oil is one of their popular products. The farmers could grow more rice paddy herbs and get more income through their business.

Searching across the Medline (National Library of Medicine) and Science Direct databases, a review study by Gorai *et al.* in 2014 reported that *Limnophila aromatica* contained fifty-four phytochemicals. Those chemicals mainly contain terpenoids, phenolics, and flavonoids [7]. Several research groups have researched the biological activity of *Limnophila aromatica* crude extracts and their phytoconstituents.

Using the disc diffusion technique, Nanasombat and Teckchuen evaluated the antibacterial effectiveness of methanol extract from *Limnophila aromatica* leaves against some bacteria described in Table 1.2 and found significant antibacterial activity with MIC (Minimum Inhibitory Concentration) values ranging from 2.6 to 41.7 mg/mL [14]. This extract was more effective against Bacillus cereus and Staphylococcus aureus (2.6 mg/mL MIC values).

Table 1.2. Influence of methanol extract of *Limnophila aromatica* on antibacterial activity [14].

Microorganism	Diameter of inhibition zone (mm)	MIC value (mg/mL)
Bacillus cereus	21.0 ± 5.2	2.6
Staphylococcus aureus	12.5 ± 2.5	2.6
Salmonella typhimurium	8.7 ± 0.6	10.4
Pseudomonas fluorescens	9.7 ± 4.2	20.8
Listeria monocytogenes	12.2 ± 3.4	20.8
Yersinia enterocolitica	11.2 ± 3.9	41.7

Several researchers have been interested in investigating the *Limnophila aromatic*a oil extraction in the last decade due to its unique scent and biological activity. The extraction yield and the main compounds of *Limnophila aromatica* essential oil differed depending on the growing region, season, plant portion, and extraction methods [8, 15–17].

Besides this, the *Limnophila aromatica* has been reported to contain high polyphenolic compounds, including the phenol content (40.5 mg GAE/g) and flavonoid content (31.11 mg QCE/g) [11] and antioxidant activity, which lead to the protection of vascular dysfunction [14, 18, 19]. As high-value-added additives with remarkable bioactivity, *Limnophila aromatica* could be a potential source of natural emulsifiers.

Table 1.3. Summary of *Limnophila aromatica* essential oil extraction by the previous study.

Plant Part	Extraction Method	Yield of Essential Oil	Main Compound	Reference
Aerial parts	Hydro-distillation 4 h	1% of dried	z-ocimene, terpinolene, Camphor	[15]
-	Hydro-distillation 8 h	2.17 % of fresh	Sabinene, Terpinene, α-humulene	[16]
Leave	Hydro-distillation 4 h	0.15 % of dried	methyl benzoate, pulegone, limonene	[8]

1.3 Emulsions

An emulsion is a thermodynamically unstable system of two insoluble liquid phases (commonly water and oil) in which one liquid is dispersed in the other and assisted by a stabilizer [20]. The spatial distribution of the oil and water phases in the combination determines how emulsions are classified. An oil-in-water (O/W) emulsion (e.g., milk and mayonnaise) is generated when oil droplets are dispersed in a water phase. In contrast, a

water-in-oil (W/O) emulsion (e.g., butter and margarine) is formed when the reverse occurs (Figure 1.1) [21].

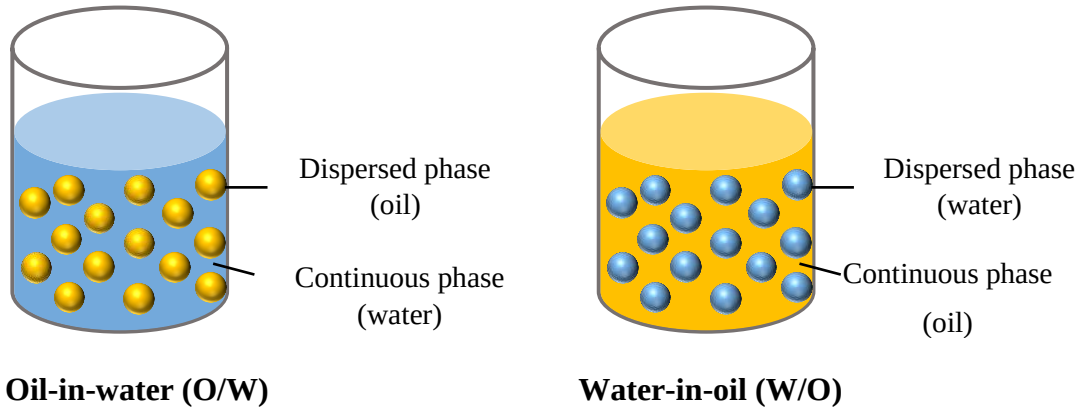


Figure 1.1. Concept of two-phase emulsions.

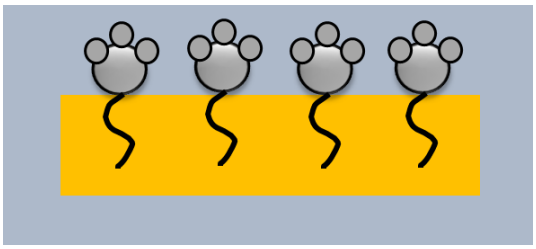
Emulsion stability is affected by physical and chemical factors since it is thermodynamically unstable. Ostwald ripening, creaming, flocculation, and coalescence are examples of recognized instabilities. Ostwald ripening refers to the formation of large droplets by continuous phase diffusion of emulsified monomers from tiny droplets to larger droplets. Creaming is the separation of emulsion droplets caused by gravity as a cream layer separates from the continuous phase. However, the creaming can also be inhibited by raising the aqueous phase's viscosity or the volume fraction of the dispersed phase to limit the droplet's mobility. Flocculation is the aggregation of droplets generated by the Brownian motion of droplet particles in the liquid. Droplets in close packing structures adjust their positions after meeting each other, resulting in flocculates with a more compact form and less continuous phase entrapped [20, 22].

1.4 Emulsifiers

A mechanical force is necessary to disperse one phase into another to produce an emulsion. However, emulsions formed without adding a stabilizer are unstable, and the

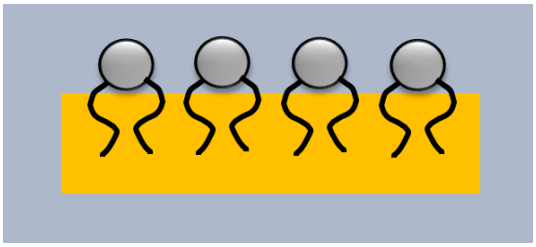
emulsion phase begins to break into various layers depending on the density differences. Thus, the addition of stabilizers is required to create a long-lasting stable emulsion. Emulsion stabilizers are classified according to how the system maintains stability. Emulsifiers, texture modifiers, weighting agents, and ripening retarders are known as emulsion stabilizers. 1) An emulsifier is a surface-active compound that adsorbs to the surface of an oil droplet during homogenization. Emulsifiers inhibit aggregation by adsorbing at the interface and facilitating oil disruption during emulsification. 2) A texture modifier is a molecule that has the ability to thicken or gel the aqueous phase. The system's stability is preserved by inhibiting droplet mobility. 3) A weighing agent is a material that permits a density match with the continuous phase, resulting in the elimination of creaming or sedimentation caused by gravitational separation. 4) A ripening retarder is a highly hydrophobic substance that inhibits Ostwald ripening when introduced to the dispersed phase [23].

Since synthetic emulsifiers are widely established, naturally derived emulsifiers must fulfill requirements to be considered a potential emulsifier. The commonly used natural emulsifiers are proteins, polysaccharides, phospholipids, and Saponin. Because of the appropriate quantities of polar and non-polar groups in their chemical structure (Figure 1.2), these emulsifiers have a high potential for absorption at the oil-water interface [3, 24]. Proteins (casein and whey protein, gelatins, pea, lupin, soy, and corn protein) contain hydrophilic and hydrophobic amino acids and polypeptide chains. It may be able to cause electrostatic repulsion due to the presence of the ($–COO^-$) or ($–NH_3^+$) group and steric repulsion because of the formation of a thick interfacial layer.



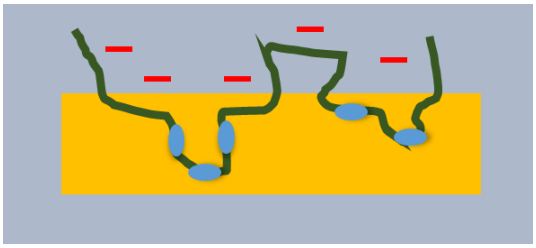
Biosurfactants

Saponin



Phospholipids

Lecithin

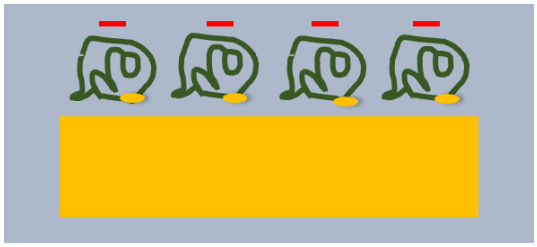


Random Coil Biopolymers

Starch granules, chitin crystals

Colloidal particles

Polysaccharides & Flexible protein



Compact Biopolymers

Globular proteins

Figure 1.2. Some natural emulsifiers use for stabilized emulsions [26].

Polysaccharides (gum Arabic, pectin, and galactomannans) generally have a low surface activity that could increase continuous phase viscosity leading to the inhibition of droplet movement. Phospholipids (lecithin) consist of an amphiphilic structure with a hydrophilic head (phospholipid acid esterified with glycerol) and a lipophilic tail (fatty acid). Lecithin has a thin interfacial layer prone to coalescence. The structures of saponin (secondary

metabolites of plants) contain the hydrophilic sugar group attached to the non-polar aglycone group. Saponins could produce electrostatic repulsion due to the presence of glucuronic acids [25].

Even though the proteins, phospholipids, and polysaccharides are commonly used as natural emulsifiers for preparing the emulsions, the emulsions formed by these compounds can be disrupted [26, 27]. So, several researchers have been interested in investigating the natural emulsifiers from other surface-active compounds such as saponin due to its potential to stabilize the emulsions [28, 29]. Saponins are typically found in various plants and crops. We believe that *Limnophila aromatica* might contain saponin, which is the potential for formulating and stabilizing emulsions.

1.5 Objective

The general objective of this dissertation is to understand the suitability of *Limnophila aromatica* extract toward the sustainable production of natural emulsifiers. To achieve this aim, the specific objectives of this study are listed as the following:

- To characterize the surface-active and inorganic compounds of *Limnophila aromatica* extracts.
- To understand the interfacial and emulsifying performance of *Limnophila aromatica* extracts.

1.6 Scope of the Study

- **Chapter 2: Physicochemical Characterization of *Limnophila Aromatica* Extracts.** In this chapter, the influence of aqueous ethanol (0, 25, 50, 75, 99.5%, *v/v*) on the yield and composition of *Limnophila aromatica* is

conducted. The surface-active substances, including protein, saponin, and phenolic compounds of the extracts, are determined due to their effectiveness in the stability of emulsions. The total ash content of each extract is also necessary to evaluate because of the significant impact of the residual substances on the destabilization of emulsion.

- **Chapter 3: Interfacial and Emulsifying Properties of *Limnophila Aromatica* Extracts.** First, the interfacial tension at the soybean oil/water interface is determined. To assess the influence of the various extracts on the formation and physical stability of oil-in-water (O/W) emulsions, the volume mean droplet diameter ($d_{4,3}$), electrical charge (ζ-potential), and droplet size distribution were determined. The chosen extract was then utilized to make emulsions with varying extract and oil concentrations. Finally, the stability of optimum emulsions was evaluated by storing for 30 days at either 5 or 25 °C.

CHAPTER 2

PHYSICOCHEMICAL CHARACTERIZATION OF *LIMNOPHILA AROMATICA* EXTRACTS

2.1 Introduction

Rice paddy herb (*Limnophila aromatica*) is native to Southeast Asia and widely utilized as a species and medicinal herb among the Southeast Asian community. The fresh *Limnophila aromatica* plants and essential oil have been brought to most parts of the world and are accessible in Asia, Europe, and North America [10, 30]. Rice paddy herb has been used for centuries to treat convulsions, anxiety, and stress, prevent vascular malfunction, and treat fever. Because of the flavor and aroma, similar to both lemon and cumin, it is a popular addition to fresh fish recipes [7, 8]. The antibacterial activities of 80% ethanol extract of this plant species have been shown to prevent the proliferation of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and Propionibacterium acnes [18]. Methanol extract from *Limnophila aromatica* leaves was also reported to contain significant antibacterial activity against bacteria such as *Bacillus cereus*, *Listeria monocytogens*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Yersinia enterocolitica* [14]. Certain researchers have described the anti-inflammatory, antimicrobial, antioxidant, and vascular protective properties of *Limnophila aromatica* extracts. [14, 18, 19]. Essential oil extractions from *Limnophila aromatica* were widespread because of its distinctive scent, and the main elements discovered were methyl benzoate, pulegone, limonene, z-ocimene, terpinolene, and camphor [8, 15, 16]. The investigations on phenol and flavonoid extraction and starch isolation from *Limnophila aromatica* have recently been published. The highest total phenol (4%) and flavonoid (3.1%) of the extract were obtained using absolute ethanolic extraction [11]. Wijaya and colleagues discovered that defatted and dephenolated *Limnophila aromatica* constituted 70.4% pure starch (55.1 % resistant starch) [6].

The food and pharmaceutical sectors have been particularly interested in bioactive constituents and secondary metabolites obtained from plant sources. Solid-liquid

extraction was used to isolate those substances of interest based on their relative solubility in the extraction solvent. The US Food and Drug Administration states that class 3 solvents, such as ethanol, should be used for solid-liquid extraction since they are non-toxic and provide a low hazard to public health. Due to its intrinsic toxicity, the use of Class 2 solvents like methanol should be minimized. Although the recovery yields in ethanol extraction may be lower than in methanol extraction due to the greater polarity of methanol, the safety of extract is guaranteed [31].

Recently, incorporating natural emulsifiers and avoiding synthetic chemicals for product formulation has become popular in fulfilling customer demand [4]. Therefore, investigating the new source of natural emulsifiers is currently trending. It is necessary to evaluate the contents of surface-active compounds and inorganic residues of plant extracts before using them as the natural emulsifier to understand their mechanism for emulsion stabilization. The contents of surface-active substances such as saponin and protein of *Limnophila aromatica* linked to the emulsifying ability of extracts have not been documented. Even though the determination of phenolic compounds of *Limnophila aromatica* extracts was conducted by other researchers [11], it is also important to evaluate this compound in this study because the different growing regions and seasons of the plants could be affected the composition of the extracts. Besides this, the measurement of residue substances (total ash content) is needed to investigate because it is strongly related to the emulsion instability [32]. Therefore, the objective of this chapter is to characterize the surface-active and inorganic compounds of *Limnophila aromatica* extracts obtained from different aqueous ethanolic extraction.

2.2 Materials and Methods

2.2.1 Materials and Chemicals

Limnophila aromatica were purchased from a local farm in Takeo province, Cambodia. Folin–Ciocalteu (FC) reagent was obtained from Sigma-Aldrich (Tokyo, Japan). Gallic acid, oleanolic acid, vanillin, acetic acid, perchloric acid (60%), ethyl acetate, sodium carbonate, and ethanol (99.5%) were acquired from FUJI-FILM Wako Pure Chemical Corporation (Osaka, Japan). The ultrapure water was generated by the Arium ® comfort II system (Sartorius AG, Göttingen, Germany).

2.2.2 Preparation of the Limnophila Aromatica Extracts

After harvested, aerial parts of *Limnophila aromatica* were cleaned with deionized water and dried for approximately three days in an open space at room temperature. The dried *Limnophila aromatica*) were packed in zip plastic bags and preserved at –20 °C until further usage. The dried plants were milled using the Ultra Centrifugal Mill ZM 200 at 6,000 rpm and sieved to a homogenous powder with a particle size of approximately 0.5 mm. The extraction was carried out by stirring the sample powder in various aqueous ethanol (0, 25, 50, 75, or 99.5% (*v/v*)) at a powder:solvent weight ratio of 1:10 using a magnetic stirrer for 3 h at room temperature. The mixtures were centrifuged for 30 min at 3,300 rpm using a Kubota Corp, Tokyo, Japan, and filtered through Whatman filter paper assisted by vacuum to eliminate the solid particles. After that, the solvents were eliminated using a rotary evaporator (EYELA Co., Ltd, Shanghai, China) at 49 hPa and 40 °C. The extracts were then further purified by re-dispersed in ultrapure water and centrifuged at 10,000 rpm for 30 min by high-speed refrigerated micro centrifuge (MX-307, TOMY, Japan) and filtered using syringe hydrophilic membrane filters (PTFE 0.45 μm) to separate the insoluble substances. Finally, the extracts were freeze-dried at –80 °C, 5 Pa, using an EYELA freeze drier to remove the water, and then kept at –20 °C until the

further experiment. The freeze-dried water-soluble *Limnophila aromatica* extracts were identified as LAE-0, LAE-25, LAE-50, LAE-75, and LAE-99.5 based on the ethanol concentration utilized throughout extraction. The extraction yield (EY) was calculated as the following equation (1):

$$\text{Extraction Yield (\%, dry basis)} = W_1/W_0 \quad (1)$$

W_1 is the weight of the freeze-dried *Limnophila aromatica* extract, whereas W_0 is the weight of *Limnophila aromatica* powder.

2.2.3 Chemical Composition Characterization of Limnophila Aromatica Extracts

a) Total Phenol Content

The total phenol content of each extract was measured spectrophotometrically using the method of Folin–Ciocalteu described by Sahu and Saxena, with slight modification [33]. The standard curve was created using gallic acid as the standard (0–80 µg/mL). In brief, 0.5 mL of each diluted extract (250 or 500 µg/mL) or standard was introduced into test tubes, followed by 2.5 mL of Folin–Ciocalteu reagent (10-fold diluted with ultrapure water) and mixed thoroughly by Vortex Mixer. Then, 2 mL of 7.5% (w/v) sodium carbonate was added to the mixture and mixed gently. The tubes were covered and kept for 30 min at room temperature. UV–VIS spectrophotometer (JASCO Co., Hachioji, Japan) was used to measure the absorbance of the mixture at 760 nm against the blank solution (prepared with the same process without the extract).

b) Total Saponin Content

The total saponin content of each extract was quantified spectrophotometrically using the colorimetric method [34]. The calibration curve was created using oleanolic acid as the standard (0–250 µg/mL). Briefly, 0.1 mL of each diluted extract (500 or 1,500 µg/mL) or standard was introduced into test tubes, followed by 0.1 mL of 5 % (w/v) vanillin–acetic

acid solution and mixed thoroughly by Vortex Mixer. 1.2 mL of 60% perchloric acid was added and mixed gently. The mixture was then incubated at 70 °C for 20 min and cooled down to room temperature. After that, 5 mL of ethyl acetate was added to the mixture and mixed thoroughly. The absorbance was immediately read by UV–VIS spectrophotometer (JASCO Co., Hachioji, Japan) at 550 nm against the blank solution (prepared with the same process without the extract).

c) Total Protein Content

The total protein content was estimated from total nitrogen content by multiplying the total nitrogen content with the nitrogen to protein conversion factor (6.25) [35]. The determination of the total nitrogen content of each extract was conducted by the Research Facility Center for Science and Technology of the University of Tsukuba using an organic elemental analyzer (C, H, N, S) (elementar (UNICUBE)).

d) Total Ash Content

The total ash content was estimated by combusting the organic matter. Before the burning process, the porcelain crucibles were clean, dried in the oven, and pre-weight. Approximately 2 g of *Limnophila aromatica* extract (LAE) was placed in those porcelain crucibles and combusted for 24 h using the furnace muffle at 600 °C [36] and then cooled down to room temperature by setting it in the desiccator for about 30 min. The crucible containing the ash was weighted, and the total ash content was computed as the following equation (2):

$$\text{Total ash content (\%, dry basis)} = W_1/W_0 \quad (2)$$

W_1 is the weight of the ash, whereas W_0 is the weight of freeze-dried *Limnophila aromatica* extract (LAE).

2.2.4 Statistical Analyses

All the experiments were conducted in three replications. One-way analysis of variance (ANOVA) with the Duncan test was conducted to assess significant differences among variables at a 95% confidence level using SPSS statistic software version 28.0 (IBM Corp., Armonk, New York).

2.3 Results and Discussion

2.3.1 Extraction Yield

Milling, grinding, homogenization, and extraction are the processes for phytochemicals extraction from plants and medicinal herbs. Extraction is the most important step in extracting and separating phytochemicals from plant materials. The chemical composition of phytochemicals, the extraction method utilized, sample particle size, the solvent used, and the presence of interfering compounds all impact extraction efficiency. The extraction yield is mainly affected by the solvent's polarity, pH, temperature, extraction time, and sample composition. However, the solvent and sample composition are the most relevant factors under the same extraction time and temperature [37]. This work focuses on the effect of different ethanol concentrations with the same sample, temperature, extraction time, and sample particle size. As shown in Figure 2.1, the extraction yield (EY) of *Limnophila aromatica* extracts using different aqueous ethanolic extraction was significantly different ($p \leq 0.05$). The result showed the maximized extraction yield ($9.53 \pm 0.15\%$, w/w) while increasing ethanol concentration to 50% (v/v) and stated to decrease when increasing the concentration of ethanol to 75% (v/v) and 99.5 %(v/v). It indicated that the extraction yield was various depending on the polarity of the solvent. The lowest polarity solvent (absolute ethanol) or highest polarity solvent (water) used for *Limnophila aromatica* extraction indicated a low yield. This result might be distributed to the higher solubility of proteins and carbohydrates in water than in

ethanol [38]. The combination of water and ethanol may contribute the extraction of soluble substances in water and/or organic solvent. The above findings agree with the previous study on the extraction yields of *Limnophila aromatica* [11] and some other medicinal plants [39].

2.3.2 Total Phenol Content of Limnophila Aromatica Extracts (LAEs)

The total phenol content was determined using the calibration curve $y = 0.01x + 0.0071$ with $R^2 = 0.9993$ (Figure 2.2), where x refers to the absorbance and y indicates the concentration of the gallic acid solution (µg/mL).

As shown in Figure 2.3, the total phenol content of *Limnophila aromatica* extracts (LAEs) using various aqueous ethanolic extraction was significantly different ($p \leq 0.05$) and ranged from 3.70 ± 0.04% (*w/w*) for water extraction to 12.79 ± 0.07% (*w/w*) for absolute ethanol. The total phenol content of *Limnophila aromatica* extracts (LAEs) increased in the following order: 0% < 25% < 50% < 75% < 99.5%. The results showed that when the polarity of the solvent was reduced by increasing the ethanol concentration, the phenol content increased significantly. It might imply that water extract contains more non-phenol compounds, such as carbohydrate and terpene, than other extracts. It might be attributed to the complex formation of some phenolic constituents in ethanol-soluble extracts. It is also widely understood that utilizing a much higher percentage of ethanol improves polyphenol extraction yield [11, 40].

2.3.3 Total Saponin Content of Limnophila Aromatica Extracts (LAEs)

The total saponin content was determined using the calibration curve $y = 0.0025x + 0.0018$ with $R^2 = 0.9957$ (Figure 2.4), where x refers to the absorbance and y indicates the concentration of the oleanolic acid solution (µg/mL).

As shown in Figure 2.5, the total saponin content of *Limnophila aromatica* extracts (LAEs) using various aqueous ethanolic extraction was significantly different ($p \leq 0.05$) and ranged from 7.75 ± 0.20% (*w/w*) for 0% (*w/w*) ethanolic extraction (water) to 23.87 ± 0.54% (*w/w*) for 99.5% (*w*/w) ethanolic extraction (absolute ethanol). The total saponin content of *Limnophila aromatica* extracts (LAEs) decrease in the following order: 99.5% > 75% > 50% > 25% > 0%. This trend demonstrated that the total phenol content of *Limnophila aromatica* extracts (LAEs) dropped as the water concentration increased during extraction. Saponin extracted from medicinal plants is generally composed of several types of aglycone molecules (water-insoluble substances) and one or two sugar moieties (water-soluble substances). Hydrophobic saponins, in general, dissolve easily in low polarity solvents, whereas hydrophilic saponins dissolve readily in high polarity solvents [41]. As a result, *Limnophila aromatica* plants may contain significantly more semi-polar and polar saponins. Furthermore, our result agrees with Do et al., who discovered that low polarity solvents were better than high polarity solvents for saponin extraction from Codonopsis javanica root [42].

2.3.4 Total Protein Content of Limnophilla Aromatica Extracts (LAEs)

According to Figure 2.6 the lowest protein content was obtained from 99.5 % (*v/v*) ethanolic extraction (3.81 ± 0.29 %, *w/w*) while the highest protein contents were acquired by water extraction and 25% (*v/v*) aqueous ethanolic extraction (5.38 ± 0.27 and 4.97 ± 0.33 %, w/w, respectively). The result indicated that the protein content reduced as the polarity of the solvent enhanced, confirming protein precipitation by absolute ethanol and removal by centrifugation and filtration steps. Likewise, in high polarity solvents such as absolute ethanol, the protein was reported to be unstable and less soluble [43].

2.3.5 Total Ash Content of Limnophila Aromatica Extracts (LAEs)

The total ash content of *Limnophila aromatica* extracts (LAEs) was ranging between 14.74 ± 0.55% from 75% (*v*/v) ethanolic extraction to 30.86 ± 2.20% from absolute ethanol extraction (Figure 2.7). The ash content in *Limnophila aromatica* extracts might be related to the amount of inorganic material present, both internally and during the extraction processes. The inorganic residue that remains after removing the water and organic compounds by burning is known as ash and is often used to assess the total mineral content [44]. Nonetheless, the high ash concentrations reflect impurity elements that considerably impact emulsion formation and stabilization [32].

2.4 Conclusions

In summary, the highest total saponin and phenol contents were obtained from absolute ethanol extraction, followed by 75%, 50%, 25%, and 0% aqueous ethanolic extraction. It is indicated that the enhancement of polarity of the solvents by increasing ethanol concentration resulted in high saponin and phenol content. It might be attributed to the complex formation of some phenolic and saponin substances in ethanol-soluble extracts. In contrast, the best solvents for protein extraction were water and 20%, followed by 50%, 75%, and 99.5% aqueous ethanolic extraction due to the precipitation by absolute ethanol. The results showed that *Limnophila aromatica* extracts consisted of a high percentage of surface-active compounds, which could be the potential for forming and stabilizing emulsions. All extracts contained different amounts of surface-active compounds and inorganic substances. Therefore, we cannot select the extract for preparing emulsions based on their composition. All *Limnophila aromatica* extracts (LAEs) will be used for interfacial and emulsifying investigation.

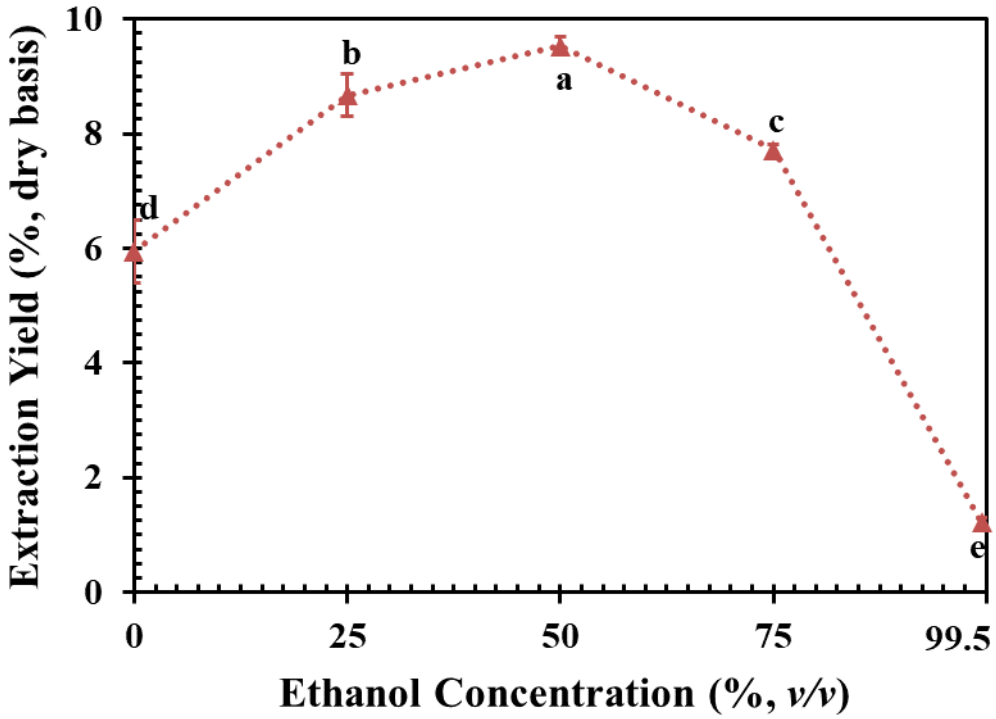


Figure 2.1. Extraction yields (%, *w/w*) of different aqueous ethanolic *Limnophila aromatica* extracts (LAEs). The average with the various letters means significantly different at a 95% confidence interval ($p \leq 0.05$).

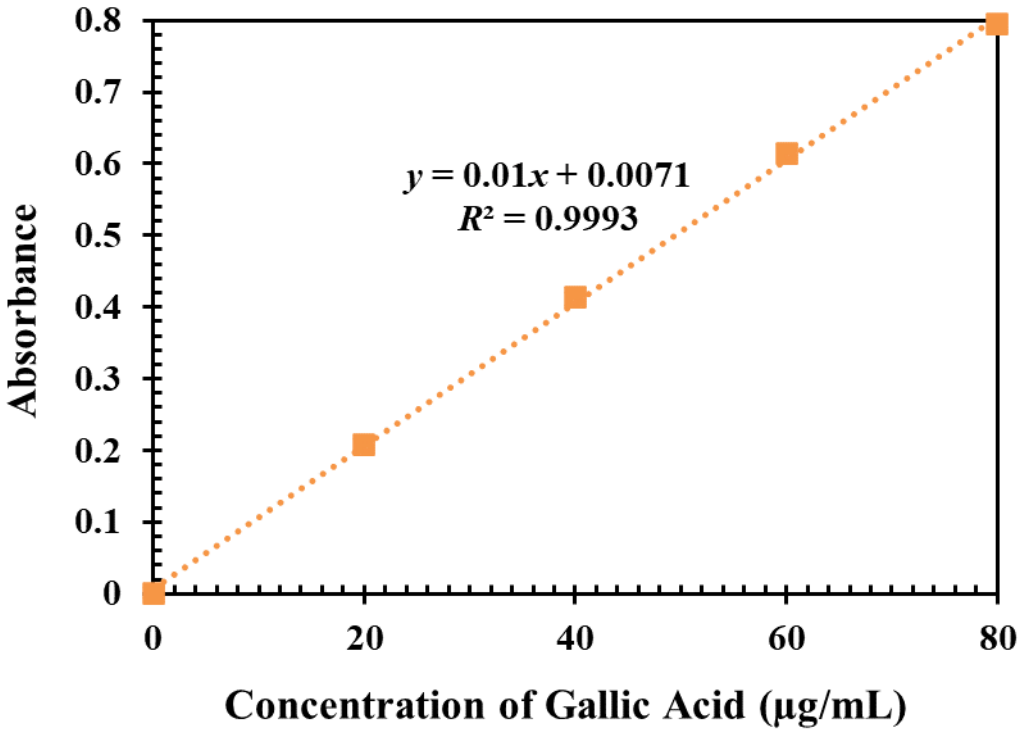


Figure 2.2. Calibration curve of gallic acid for total phenol content determination.

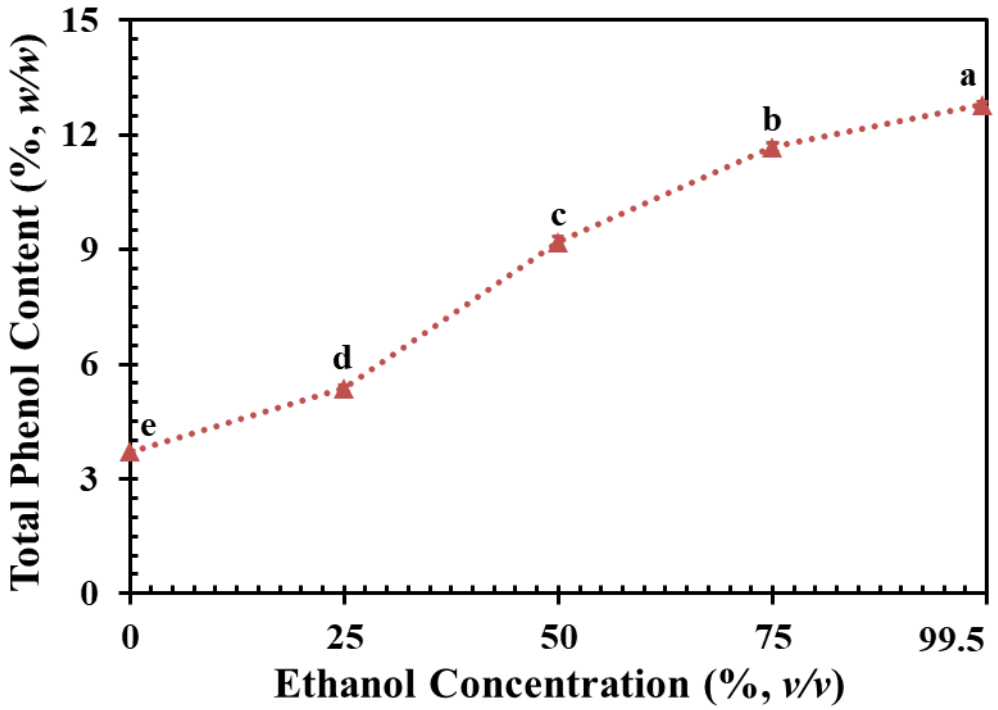


Figure 2.3. Total phenol content (%, *w/w*) of different aqueous ethanolic *Limnophila aromatica* extracts (LAEs). The average with the various letters means significantly different at a 95% confidence interval ($p \leq 0.05$).

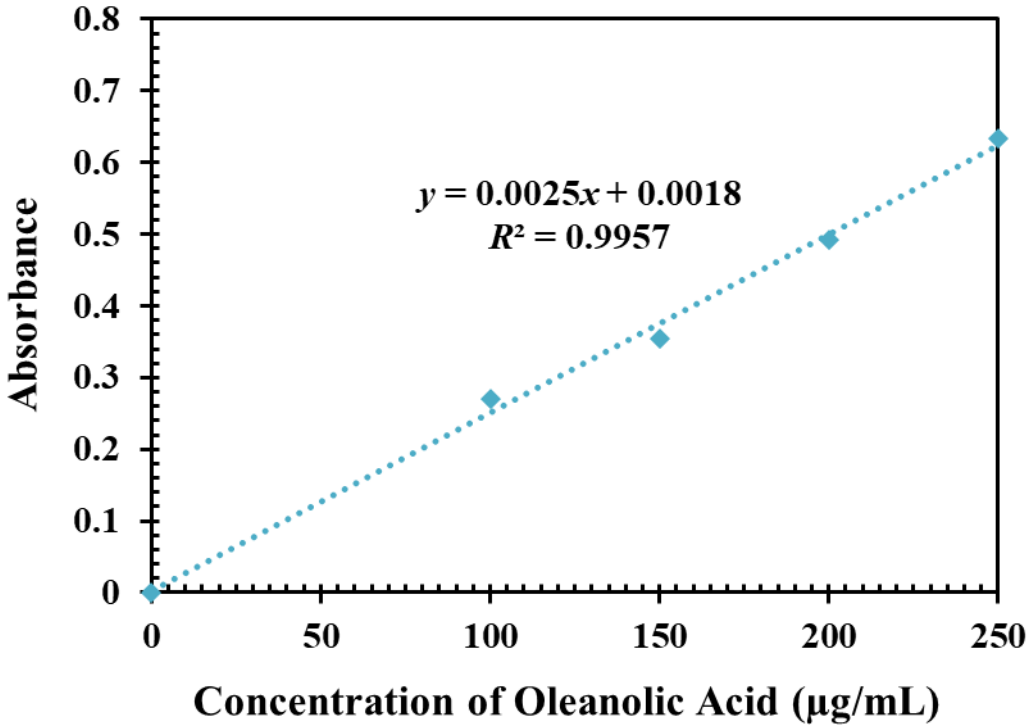


Figure 2.4. Calibration curve of oleanolic acid for total saponin content determination.

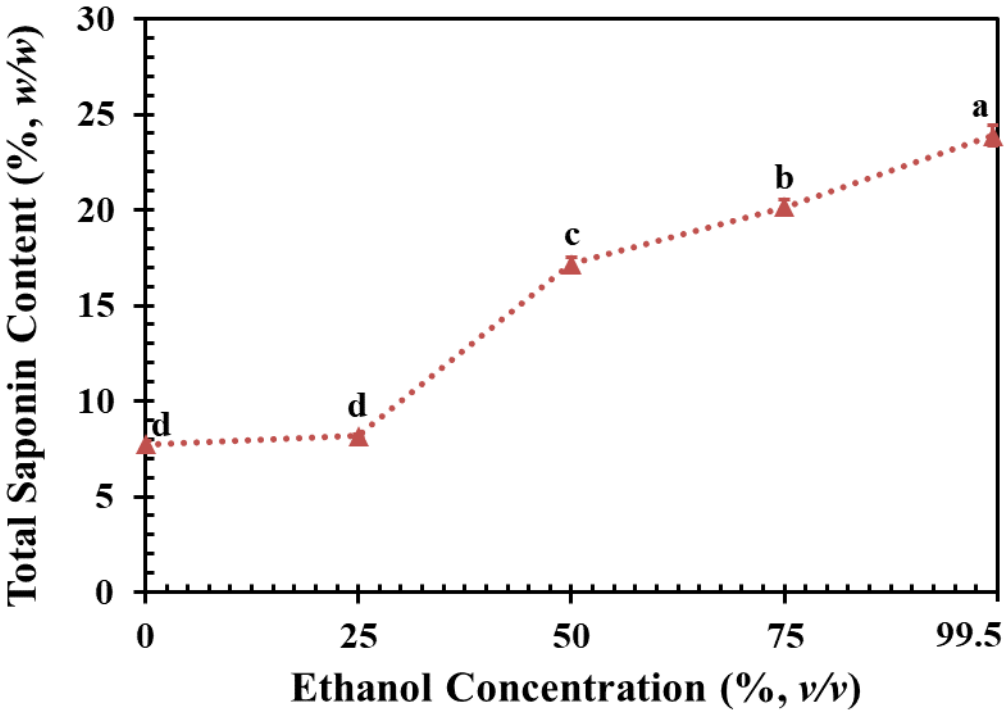


Figure 2.5. Total saponin content (%, *w/w*) of different aqueous ethanolic *Limnophila aromatica* extracts (LAEs). The average with the various letters means significantly different at a 95% confidence interval ($p \leq 0.05$).

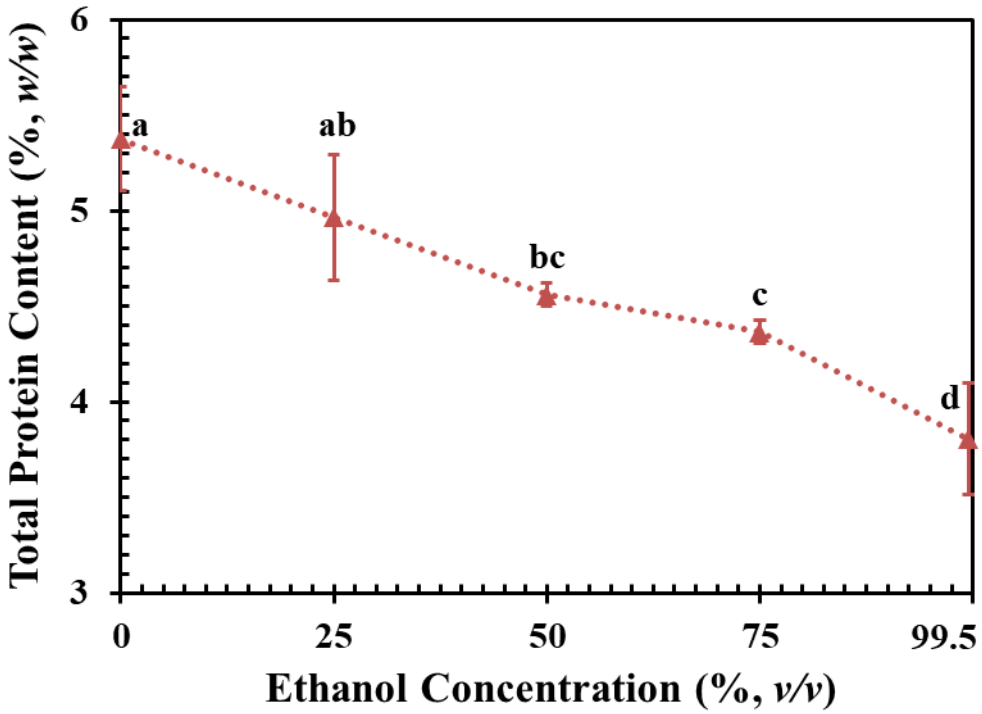


Figure 2.6. Total protein content (%, *w/w*) of different aqueous ethanolic *Limnophila aromatica* extracts (LAEs). The average with the various letters means significantly different at a 95% confidence interval ($p \leq 0.05$).

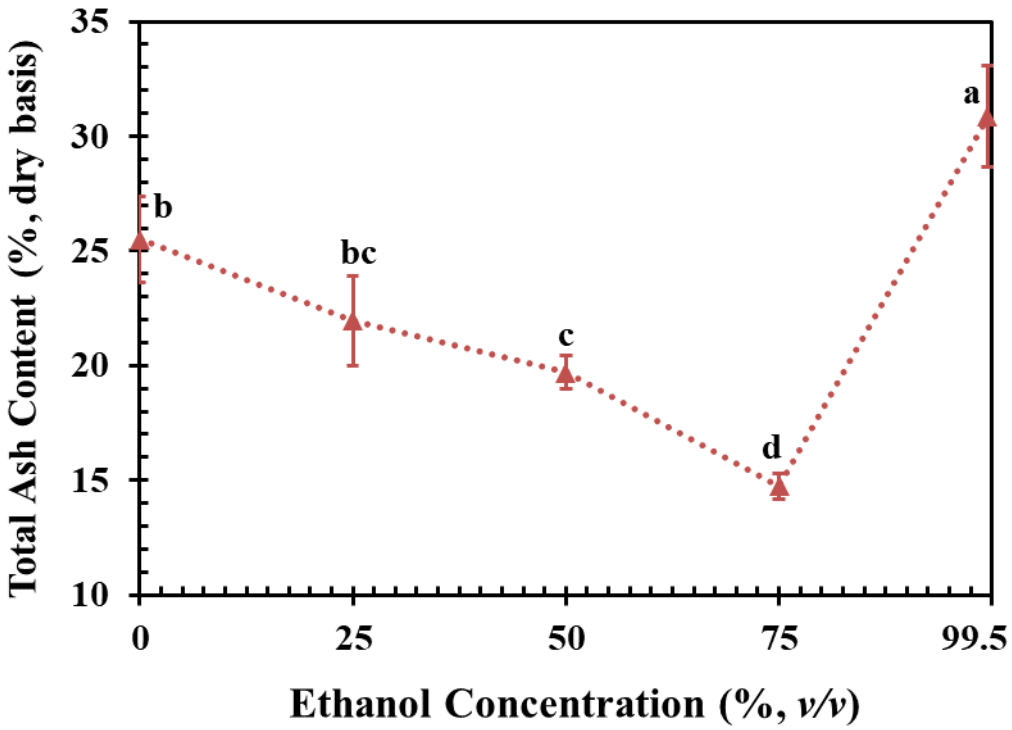


Figure 2.7. Total ash content (%, dry basis) of different aqueous ethanolic *Limnophila aromatica* extracts (LAEs). The average with the various letters means significantly different at a 95% confidence interval ($p \leq 0.05$).

CHAPTER 3

INTERFACIAL AND EMULSIFYING PROPERTIES OF *LIMNOPHILA AROMATICA* EXTRACTS

3.1. Introduction

Emulsions are commonly used in various industries, including agriculture, cosmetics, food, pharmaceutical, and petroleum [45]. A mechanical force is necessary to disperse one phase into another to produce an emulsion. However, emulsions formed without adding emulsifiers are unstable, and the emulsion phase begins to break into various layers depending on the density differences. Thus, the addition of emulsifiers is required to create a long-lasting stable emulsion [46].

Emulsifiers are essential elements in the food industry due to their abilities to generate and stabilize emulsions. Most emulsifiers are obtained from chemical and/or enzymatic reactions initially developed for large-scale and economical manufacturing. Natural food-grade emulsifiers are undergoing intensive research in order to meet the growing demand for green label food and beverage products among consumers [3].

Proteins (e.g., caseins, whey protein), polysaccharides (e.g., starch, pectin), and phospholipids (e.g., lecithin) are the most common natural emulsifiers used to produce emulsion-based products. However, emulsions produced by these substances break down quickly [26, 27]. As a result, researchers are interested in producing natural emulsifiers with better emulsifying characteristics using alternative surface-active components such as saponins [28, 29].

Saponins are tiny substances present in over 100 plant families and a few marine sources [47]. Plant species primarily influence the composition and concentration of saponin extracts. However, seasonal changes, plant portions, and extraction techniques affect saponin extract quality and quantity [48, 49]. According to some previous research, saponins derived from plant materials comprised a variety of various saponin components (e.g., over 100 saponins isolated from Quillaja Saponaria Molina) and botanical residue

substances, resulting in impurity saponins [50, 51]. Saponins are amphiphilic in nature, with hydrophilic carbohydrate chains connected to hydrophobic steroid or triterpenoid aglycon. Saponins' dispersion capacities are described by mixing polar and non-polar structural components in their molecules [52, 53]. Hence, researchers have been interested in using saponins (particularly quillaja saponins) as natural emulsifiers. The emulsions generated by quillaja saponins have a long shelf life and are stable against environmental challenges such as high temperature, pH change, ionic strength, and storage duration [28, 54–56].

Investigating various plant and agro-by-product sources (yucca, ginseng, red beet, oat bran, argan press cake, sugar beet, argan shell, olive oil cake, and liquorice) of saponins has become an important area of research because of the increasing demand for using saponin extracts as natural emulsifiers [32, 57–64]. Even though the extracts had lower saponin concentrations, their emulsion formulating performance was equivalent to quillaja saponins. It suggests that additional components, such as phenolic compounds and proteins, contribute significantly to the crude extracts' emulsifying properties. Generally, the researchers chose the extract for emulsion preparation based on its capacity to reduce interfacial tension and/or high surface-active substances [59, 60, 62]. The presence of residual particles in crude extracts, on the other hand, may significantly impact their physicochemical characteristics, leading to emulsion instability [32]. So, it's crucial to evaluate emulsions made from various extracts generated from the same material under different extraction conditions. The objective of this chapter was to understand the overall interfacial and emulsifying performance of *Limnophila aromatica* extracts.

3.2. Materials and Methods

3.2.1. Materials and Chemicals

Limnophila aromatica extracts were produced by ethanolic extraction using various concentrations of aqueous ethanol as described in Chapter 2.2.2. Sodium azide and soybean oil were acquired from FUJI-FILM Wako Pure Chemical Corporation (Osaka, Japan). All the ultrapure water utilized in this investigation was generated by the Arium ® comfort II system (Sartorius AG, Göttingen, Germany).

3.2.2. Measurement of Interfacial Tension of Limnophila Aromatica Extracts (LAEs)

The interfacial tensions between the *Limnophila aromatica* extracts (LAEs) and soybean oil were determined at 25 °C by a DMo-501 interfacial tension meter (Kyowa Interface Science Co., Ltd., Saitama, Japan) following the pendant drop method. First, 0.005–3% (*w/w*) freeze-dried *Limnophila aromatica* extracts (LAEs) were dissolved in ultrapure water at 25 °C using a magnetic stirrer for 12 h. A 22-gauge syringe needle (22 G) was used to inject the extract solution into the soybean oil. When the drop reached its full volume, a high-resolution camera snapped a photo of it right once to assess its size and shape. After that, the FAMAS analysis program calculated the interfacial tension automatically using the Young Laplace equation.

3.2.3. Preparation of Oil-in-Water (O/W) Emulsions

95% (*w*/w) continuous phase and 5% (*w*/w) dispersed phase (soybean oil) were pre-mix by a high-speed mixer (Polytron®, System PT 3100, Kinematica AG, Lucerne, Switzerland) at 10,000 rpm for 5 min to form the coarse emulsions. Each continuous phase consisted of 1% (*w/w*) *Limnophila aromatica* extract (LAE) in ultrapure water (pH ≈ 7) as an emulsifier and 0.02% (*w/w*) sodium azide as microbial proliferation inhibition agent. Coarse emulsions were homogenized four passes at 100 MPa throughout a single-

stage high-pressure homogenizer (NanoVater, NV200, Yoshida Kikai, Nagoya, Japan), illustrated in Figure 3.2. The obtained homogenous emulsions were kept at 5 °C until measurements. The optimum *Limnophila aromatica* extract (LAE) was then used as an emulsifier to investigate the influence of extract concentrations (0.1–2%, *w/w*) and oil mass fractions (0.5–20%, *w/w*) on the forming and stabilizing of oil-in-water (O/W) emulsions. The procedures to generate the homogenous emulsions were similar to the previous description.

3.2.4. Measurement of Droplet Size

The droplet size of each oil-in-water (O/W) emulsion was evaluated using a static laser diffraction particle size analyzer (LS 13,320, Beckman Coulter, Brea, CA). The dispersed and continuous phases' refractive indexes were set to 1.432 and 1.330, respectively. The emulsions were directly introduced into the measuring module without dilution for this analyzer. The droplet size measurement was expressed as the droplet size distribution, and the volume mean diameter, $d_{4,3} = \Sigma (n_i d_i^4) / \Sigma (n_i d_i^3)$, where n_i refers to the number of droplets with diameter d_i.

3.2.5. Measurement of Zeta-Potential

Electrophoretic mobility of oil droplets reported as ζ-potential of each emulsion was measured immediately after homogenization using a dynamic light scattering particle analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). In brief, the emulsions were diluted (1:100) with ultrapure water to prevent multiple scattering effects. 1 mL of diluted samples were then injected into disposal folded capillary cells (DTS 1070) and equilibrated at 25 °C for 60 s. The refractive index of the continuous and dispersed phases was set to 1.330 and 1.432, respectively.

3.2.6. Assessment of Emulsion Stability

The stability of the oil-in-water (O/w) emulsion was assessed by measuring the volume mean diameter ($d_{4,3}$), and the visual aspect of the emulsions changed over time. The optimum *Limnophila aromatica* and the selected extract concentration and oil mass fraction were prepared with the same procedure described in 3.2.3 and stored at two different temperatures (5 or 25 °C) for 30 days (Day 0, 7, 15, 21 and 30).

3.2.7. Statistical Analyses

All experiments in this chapter were carried out at least duplicated, with a minimum of three replicated. The presented values were the average with standard deviations computed using Microsoft Excel (One Microsoft Way, Redmond, Washington, U.S.).

3.3. Results and Discussion

3.3.1 Interfacial Properties of Limnophila Aromatica Extracts (LAEs)

The presence of surface-active molecules capable of quickly adsorbing at the oil/water interface was therefore attributed to the interfacial activities of *Limnophila aromatica* extracts. Saponins are small surfactants with a hydrophilic-to-hydrophobic configuration. As a result, they may adsorb more effectively at the oil/water interface, lowering the interfacial tension between the oil and water phases [28]. Phenolic substances are tiny molecules that displace quickly at the oil/water interface. Because they have lower surface activity than saponins, they are rarely utilized as primary emulsifiers [65]. On the other hand, proteins have larger particle sizes and take longer to adsorb at the oil/water interface. Proteins may bind to the droplet surface more efficiently due to their higher adsorption energy, enhancing emulsion stability [21]. Due to the complex composition of *Limnophila aromatica* extracts, it was hard to evaluate the exact component responsible for interfacial tension reductions in this study.

All *Limnophila aromatica* extracts (LAEs), as shown in Figure 3.3, can decrease interfacial tension at the soybean/water interface. When utilizing lower concentrations (0.01 percent, w/w) of the extracts, the interfacial tensions of LAEs were closed to ultrapure water, indicating poor adsorption of surface-active molecules at the oil/water interface. Absolute ethanol extracts achieved the lowest interfacial tension (12.58 mN/m) at the medium concentration (1%, *w/w*), whereas LAE-0 and LAE-25 produced the largest value of interfacial tensions (16.18 and 15.86 mN/m, respectively). As predicted, the greatest saponin and phenol concentration obtained from absolute ethanol substantially influenced the extract's interfacial characteristics. In contrast, the greatest protein concentrations of the extracts prepared by water and 25% (*v/v*) aqueous ethanol did not affect the extracts' interfacial activity. The interfacial tension was reduced to 10.1-13.2 mN/m when the extracts' concentration increased to 3% (*w/w*). This study showed that the interfacial tension values of all LAEs are in the same range as the minimal interfacial tension (7-16.3 mN/m) of other efficient natural emulsifiers isolated from the botanic.

3.3.2 Influence of Limnophila Aromatica Extracts (LAEs) on Emulsion Formation and Stabilization

The electrical charge of emulsions significantly impacts their stability under various storage environments. The high negative charge primarily suggests that the emulsifier layer generates stronger repulsive interactions between emulsion droplets, preventing coalescence and stabilizing emulsion [66]. Losso et al. found that emulsions exhibited good stability when their ζ-potential levels were between 41 and 50 mV [67]. The ζ-potential of LAE emulsions, on the other hand, did not respond to their stable features. Except for LAE-99.5, which had the largest negative charge (–67 mV) of all emulsions, all emulsions containing LAEs had a comparable ζ-potential value (–41 to –43 mV) (Figure 3.4). Surface-active components in the extracts, such as protein, saponin, and

phenolic compound, may generate a negative charge of emulsions. Proteins are composed of acidic and basic groups, which results in a significant negative charge (e.g., the ζ-potential of soy and chickpea proteins is about –40 mV) [68, 69]. Typically, the ζ -potential of saponin-prepared emulsions, such as quillaja, was strongly negative because their structures contained a carboxylic group [70]. However, additional compounds with anionic residues in the crude extracts influence the electrical charge of saponin emulsions. Böttcher et al. reported that purifying the extracts by eliminating anionic non-saponin components reduced the negative charge of the emulsion from 70 to 50 mV at pH 7 [71].

Except for emulsions utilizing LAE-99.5 (absolute ethanol), which exhibited the largest droplet diameter ($d_{4,3}$ = 3269 ± 29 nm) among the samples, all LAEs were successfully implemented as emulsifiers to generate emulsions ($d_{4,3}$ < 1 μm) with the volume mean diameter ($d_{4,3}$) ranging from 273 to 747 nm. The most effective emulsifier was LAE-50, which reduced the volume mean diameter ($d_{4,3}$) of fresh emulsion to approximately 273 nm, followed by LAE-75, LAE-25, LAE-0, and LAE-99.5, respectively (Figure 3.4). The stability of emulsions produced by LAE-99.5 was not observed since it did not effectively formulate submicron emulsions. Unexpectedly, after 7 days of storage at 5 °C, the droplet size ($d_{4,3}$) of emulsions generated by LAE-0, LAE-25, or LAE-50 increased considerably, but LAE-75 was able to maintain the emulsions without significant change in droplet size at the same storage conditions and periods (Figure 3.5). Bimodal droplet size distributions were seen regardless of the type of extract used to stabilize emulsions, demonstrating that emulsions may include varying droplet sizes (Figure 3.6). Nonetheless, there was nearly no change in the droplet size distribution of emulsions utilizing LAE-75 between Day 0 and Day 7, resulting in emulsion interface stability and coalescence inhibition. The visual appearance of the emulsions, which displayed significant flocculation and coalescence after 7 days of storage at 5 °C, validated these findings (Figure 3.7). A creaming layer

appeared on top of emulsions containing LAE-0, LAE-25, or LAE-50; the aqueous phase serum could also be visualized. However, emulsions containing LAE-75 as an emulsifier remained almost unchanged in visual appearance after storage. As a result, LAE-75 was chosen as the emulsifier for further tests.

The interfacial layer properties of oil droplets and the continuous phase composition have a significant impact on the physical stability of emulsions. [72]. Rapidly screening charges at the oil/water interfaces caused by increased salt concentration in electrostatically stabilized emulsions leads to flocculation and emulsion coalescence [58, 59]. Furthermore, flocculation is commonly produced by the depletion of non-adsorbing emulsifiers, which results in emulsion instability [73]. In this investigation, emulsions generated from extracts with a high concentration of inorganic components had worse emulsion stability, as determined by the total ash content in the previous chapter (Figure 2.7). Therefore, we hypothesize that the residual ionic composition of *Limnophila aromatica* extracts (LAEs) promotes electrostatic screening of droplet interfaces which significantly impacts emulsion stability.

3.3.3 Influence of Extract Concentration on Emulsion Formation and Stabilization

The electrical charge of emulsions generated with different concentrations of LAE-75 varied from –40 to –52 mV for 0.1 and 2% (*w/w*), respectively (Figure 3.8). The high negative charge of emulsions significantly enhances emulsion stability by avoiding coalescence and flocculation [66]. In contrast, the emulsions containing 2% (*w/w*) LAE-75 showed the largest negative charge with minor stability. As described in earlier sections, the negative charge of emulsions can be caused by both the charge of surface-active substances and anionic residual compounds [73].

Figure 3.8 demonstrates that increasing the LAE-75 concentration from 0.1 to 1% (w/w) reduced the volume mean diameter ($d_{4,3}$) of emulsions from 836 nm to the minimal value of 424 nm. The droplet size ($d_{4,3}$) of emulsions was increased to 659 nm and 718 nm, respectively, by raising the concentration of LAE-75 to 1.5 and 2 % (w/w). Surprisingly, high extract concentrations, such as 1.5 and 2% (w/w), could not stabilize the emulsions, but 1% (*w/w*) could stabilize the emulsions for only 7 days at 5 °C. After a period of storage (15 days) at 5 °C, the emulsions containing low amounts of extract (0.5 %, *w/w*) maintained about the same droplet size ($d_{4,3}$) (Figure 3.9). As illustrated in Figure 3.10, the visual appearance of emulsions verified these findings. Oiling-off and creaming were seen in emulsions containing 1.5 and 2% (*w/w*) LAE-75 on Day 7 and 1% (*w/w*) on Day 15. However, emulsions containing lower concentrations did not change appearance after 15 days.

Consequently, while utilizing a low concentration of extract (< 0.5 % *w/w*) as the emulsifier, the droplet size of the emulsions was large because the surface-active substances to cover the oil interfaces were insufficient. Nevertheless, utilizing a high concentration of extract (> 0.5% *w/w*) resulted in flocculation and coalescence during storage due to an increase in un-adsorbed surface-active compounds and destabilizing agents (e.g., mineral) in the emulsions [64, 74].

3.3.4 Influence of Oil Concentration on Emulsion Formation and Stabilization

Oil-in-water (O/W) emulsions were formed by blending 0.5% (*w/w*) LAE-75 as an emulsifier with various concentrations of soybean oil (2.5 to 20%, *w/w*). The emulsion's droplet size ($d_{4,3}$) was raised when the oil concentration was increased (Figure 3.11). The value of $d_{4,3}$ varied between 250 and 2349 nm from 2.5 to 20% (*w/w*). The emulsions containing 20% (*w/w*) oil exhibited oiling-off a few hours after homogenization, whereas

the emulsions containing 10% (*w/w*) oil could be stabilized for 3 days and showed oiling-off on Day 7 (Figure 3.12). With a droplet size ($d_{4,3}$) of approximately 250 and 450 nm, respectively, LAE-75 (0.5 %, *w/w*) could stabilize 2.5 and 5 % (*w/w*) soybean oil for up to 7 days. However, with a droplet size ($d_{4,3}$) of approximately 250 and 450 nm, LAE-75 (0.5 %, *w/w*) could stabilize 2.5 and 5 % (*w/w*) soybean oil for up to 7 days.

The insufficient concentration of the extract and the growing emulsion viscosity produce instability in emulsions with high oil concentrations, resulting in disrupted efficiency during homogenization [60]. As a result, LAE-75 was able to stabilize oil-in-water (O/W) emulsions with up to 5% (*w/w*) oil concentration. We believe that raising the content of purified 75 % (*v/v*) aqueous ethanolic *Limnophila aromatica* extract might improve the stability of emulsions with high oil concentrations (LAE-75).

3.3.5 Oil-in-Water Emulsion Stability

The droplet size ($d_{4,3}$) of oil-in-water (O/W) emulsions produced by 0.5% (*w/w*) of 75% (*v/v*) aqueous ethanolic *Limnophila aromatica* extract (LAE-75) and 5% (*w/w*) soybean oil was monitored for 30 days at 5 or 25 °C. Figure 3.13 demonstrates that the droplet size was unaffected by storage at 5 or 25 degrees Celsius. Therefore, LAE-75 might be employed as a natural emulsifier in the formulation and stabilization of oil-in-water (O/W) emulsions.

3.4. Conclusions

In summary, all ethanolic *Limnophila aromatica* extract (LAEs) were able to produce submicron emulsions with a significantly negative charge, except the extract utilizing absolute ethanol (LAE-99.5), which formulated the emulsions with droplet size larger than 3 µm. Interestingly, only 75% (v/v) aqueous ethanolic *Limnophila aromatica* extract (LAE-75) was able to stabilize emulsions stored at 5 °C for up to 7 days. According to

chapter 2, the LAE-75 did not consist of the highest concentration of surface-active compounds and contained the lowest percentage of ash content. These findings showed that emulsifying characteristics of LAE were not dependent only on surface-active substances and interfacial activities. The residual compounds (e.g., mineral) of extracts might be the destabilized components promotes electrostatic screening of droplet interface (Figure 3.14) which induced emulsion instability. LAE-75 (0.5%, *w/w*) could maintain emulsions containing up to 5% (*w/w*) soybean oil for 30 days at either 5 or 25 ℃ without significantly affecting droplet size or visual appearance. Therefore, *Limnophila aromatica* extract has the potential to be used as a novel source of natural emulsifier.

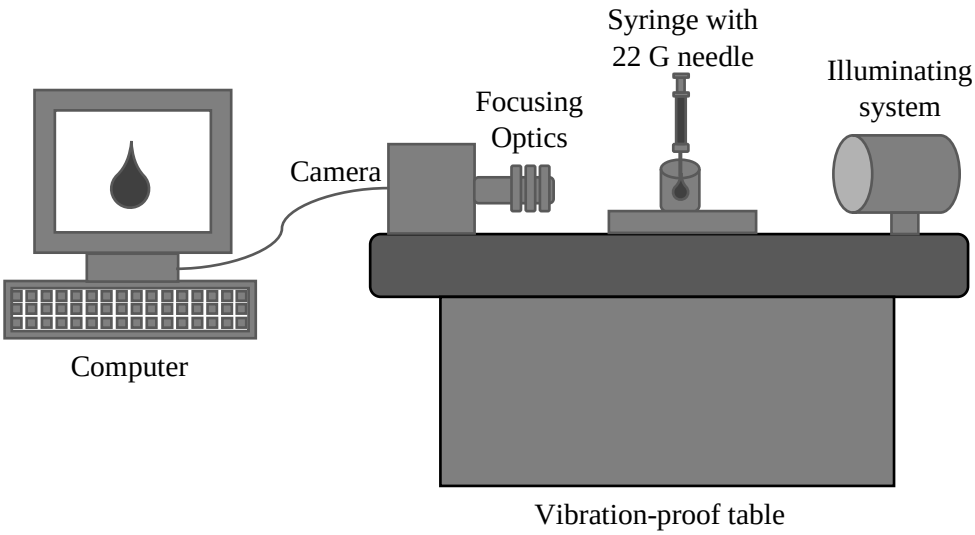


Figure 3.1. Pendant drop tensiometry apparatus.

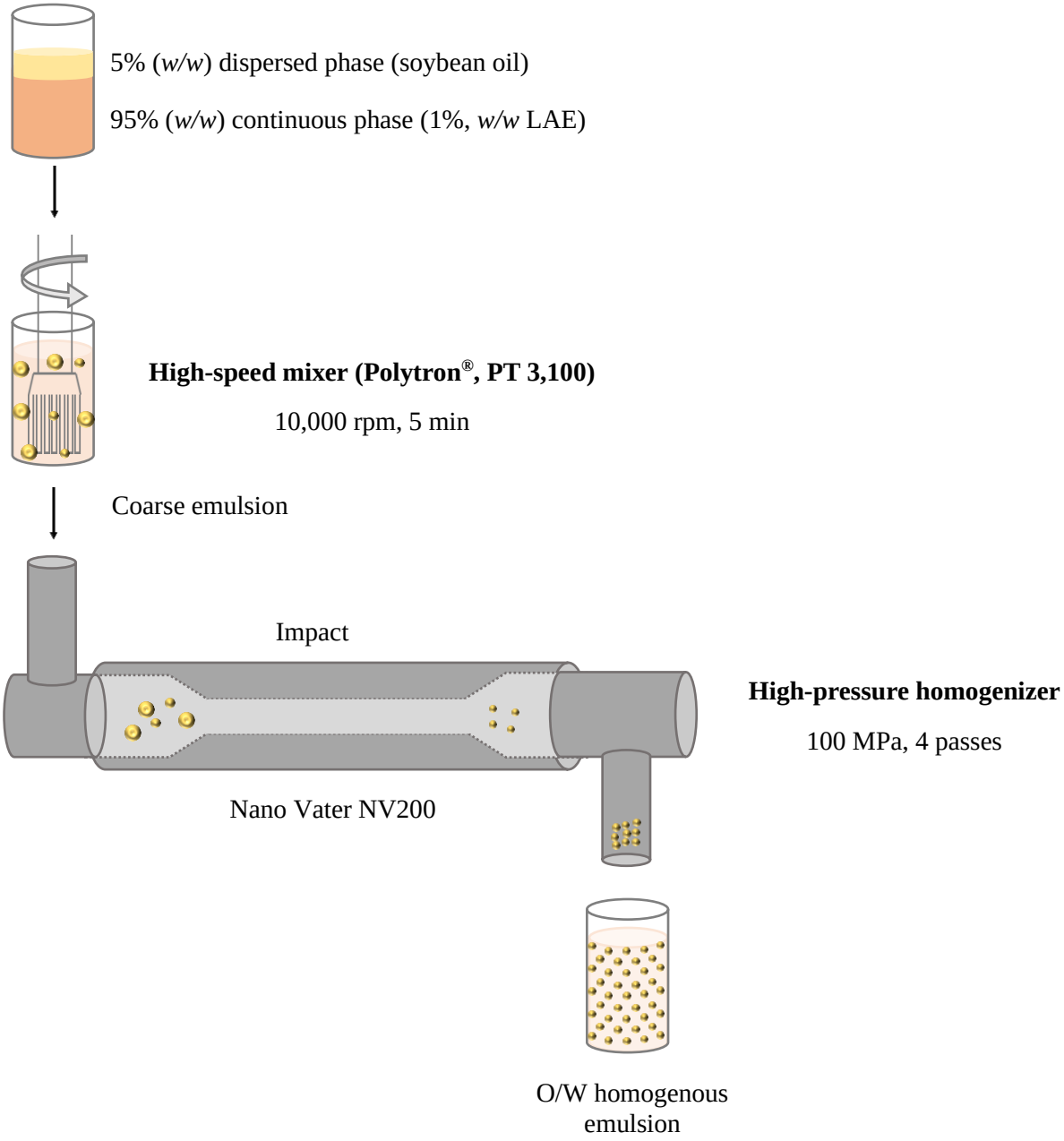


Figure 3.2. Preparation of oil-in-water emulsion containing 5% (*w/w*) soybean oil and 95% (*w/w*) aqueous phase (1%, *w/w Limnophila aromatica* extract) by high-speed mixture (Polytron®, PT 3,100) and high-pressure homogenizer.

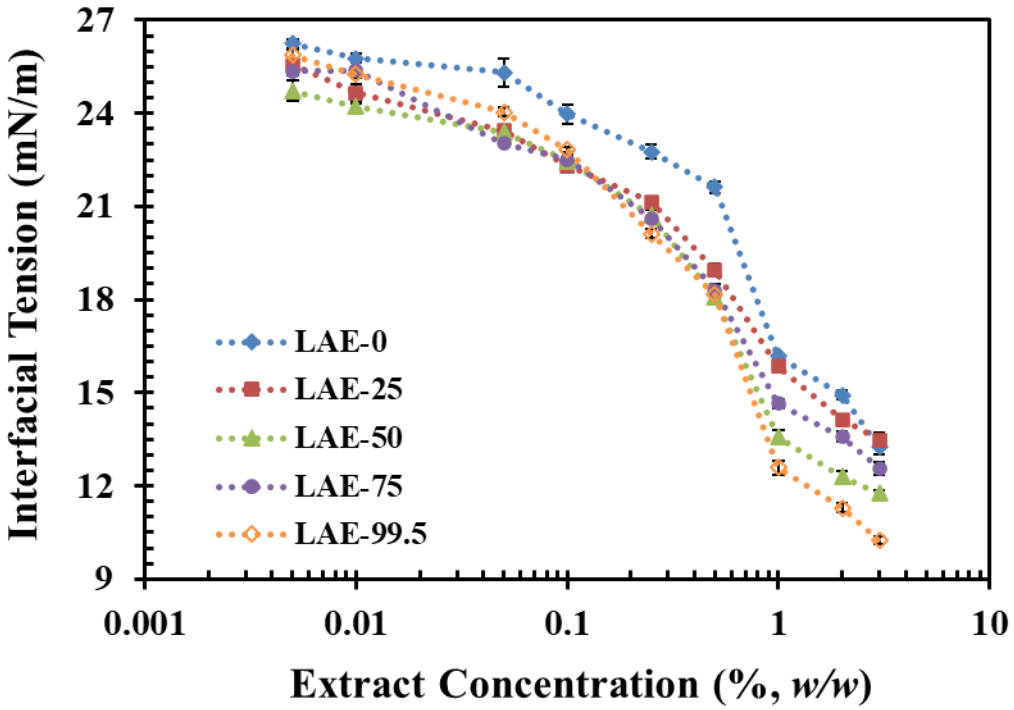


Figure 3.3. The interfacial tension at soybean oil/*Limnophila aromatica* extract interfaces as a function of concentrations (0.005–3%, w/w).

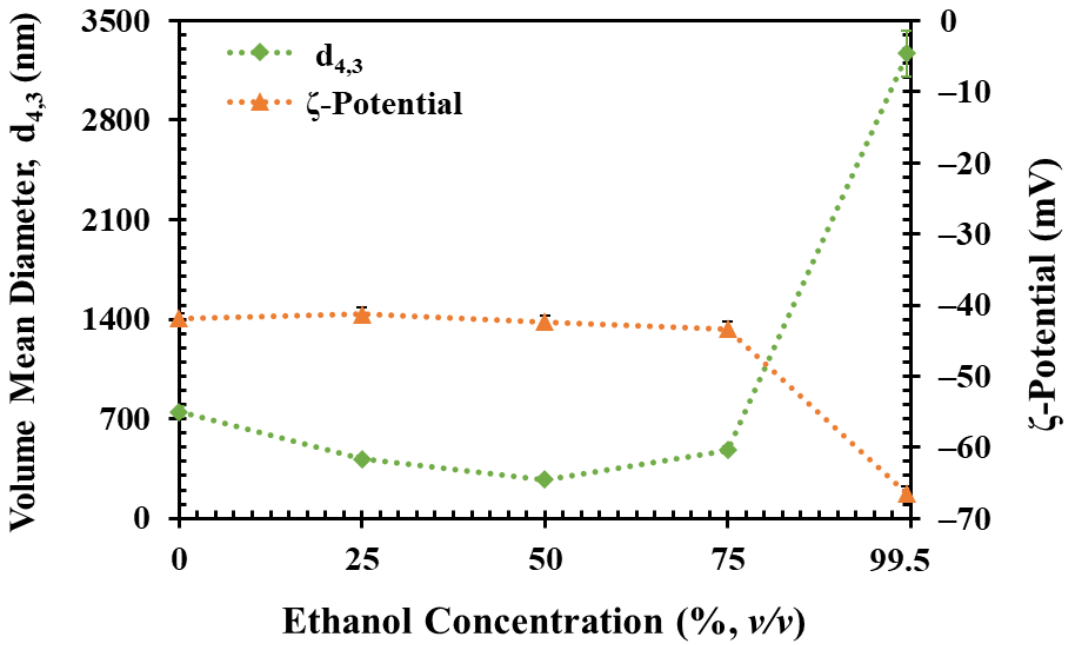


Figure 3.4. Influence of aqueous ethanolic *Limnophila aromatica* extracts (LAEs) on volume mean diameter ($d_{4,3}$) and ζ-potential of emulsions measuring immediately after homogenization.

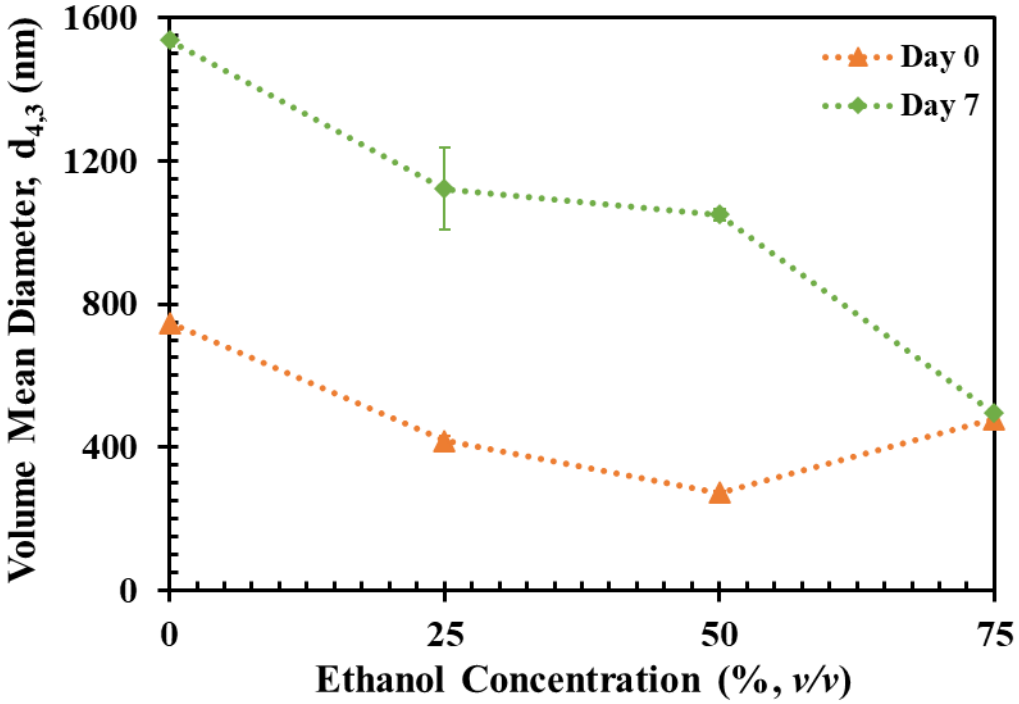


Figure 3.5. Influence of aqueous ethanolic *Limnophila aromatica* extracts (LAEs) on volume mean diameter ($d_{4,3}$) of fresh emulsions (Day 0) and after 7 days of storage (Day 7) at 5 °C.

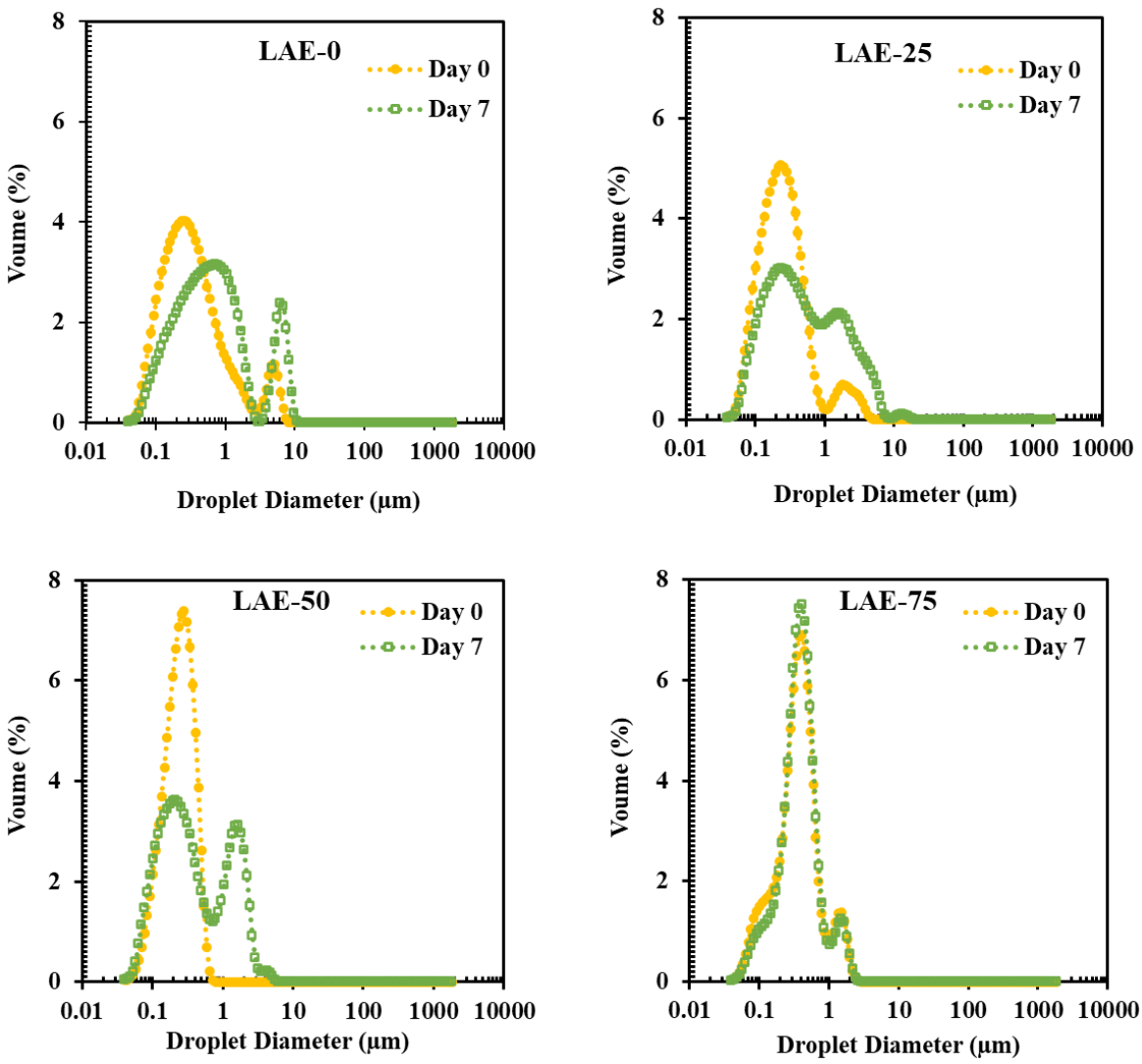
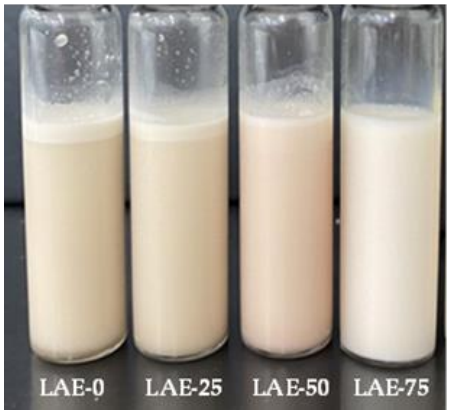


Figure 3.6. Influence of aqueous ethanolic *Limnophila aromatica* extracts (LAEs) on the droplet size distribution of fresh emulsions (Day 0) and after 7 days of storage (Day 7) at 5 °C.

Day 0



Day 7

Figure 3.7. Influence of aqueous ethanolic *Limnophila aromatica* extracts (LAEs) on the visual appearance of fresh emulsions (Day 0) and after 7 days of storage (Day 7) at 5 °C.

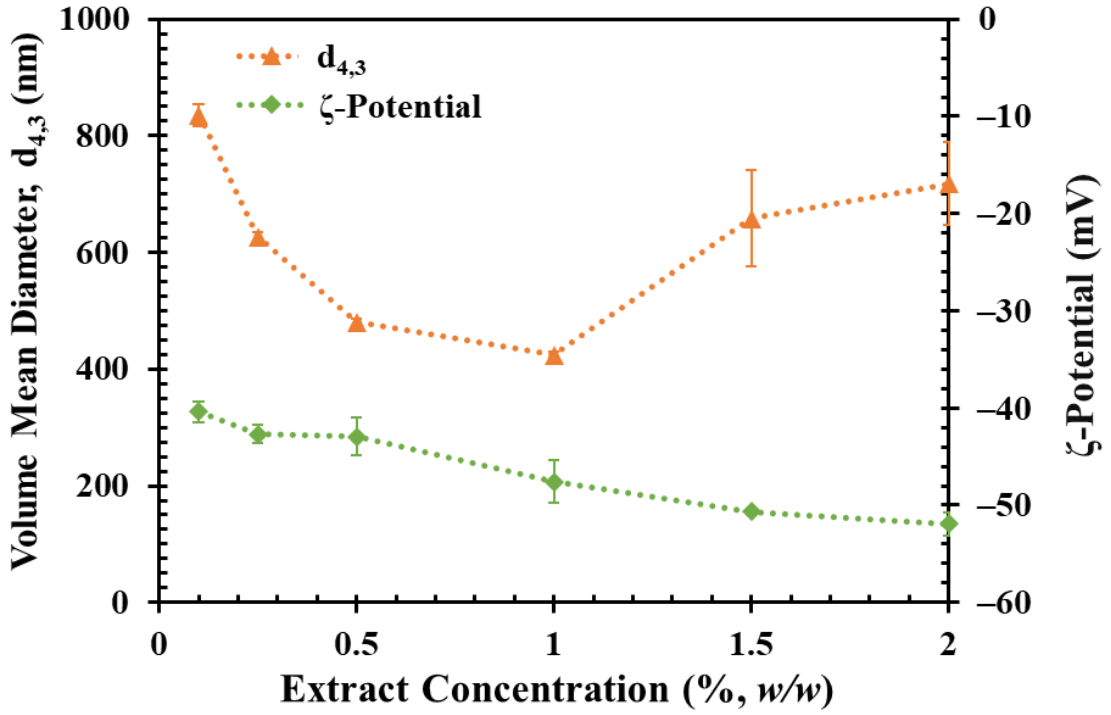


Figure 3.8. Influence of extract concentration on volume mean diameter ($d_{4,3}$) and ζ-potential of emulsions measuring immediately after homogenization.

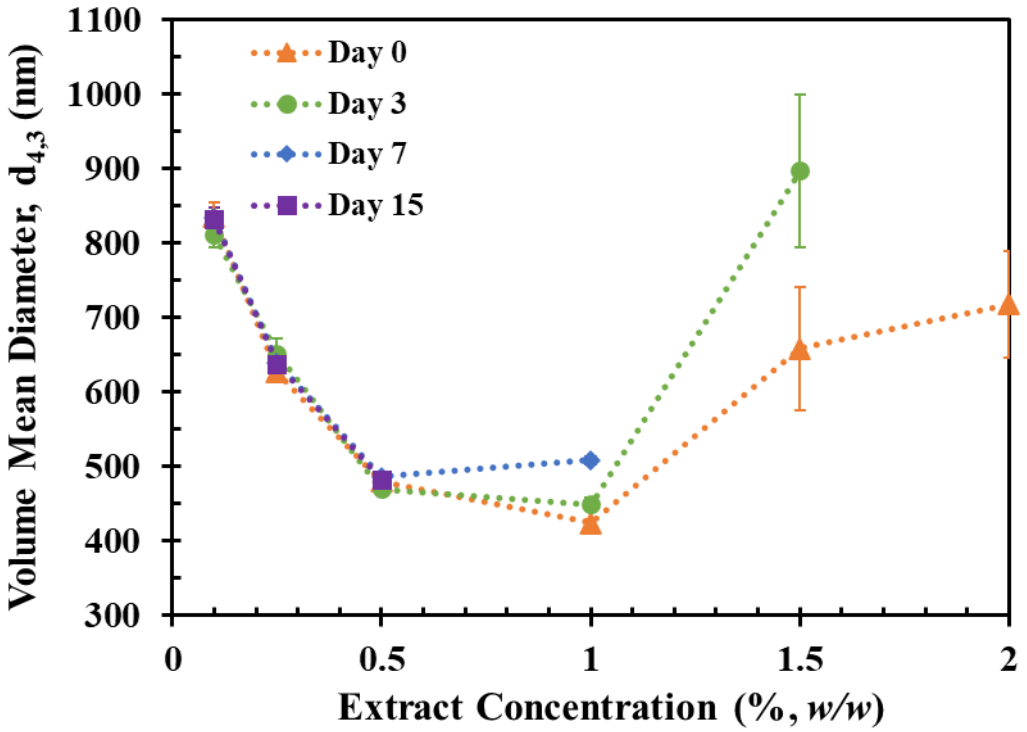
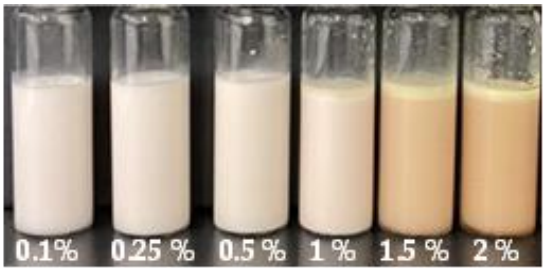


Figure 3.9. Influence of extract concentration on volume mean diameter ($d_{4,3}$) of fresh emulsions (Day 0) and Day 3, Day 7, and Day 15 at 5 °C.

Day 0

Day 7



Day 15

Figure 3.10. Influence of extract concentration on the visual appearance of fresh emulsions (Day 0) and after 7 days (Day 7) and 15 days (Day 15) of storage at 5 °C.

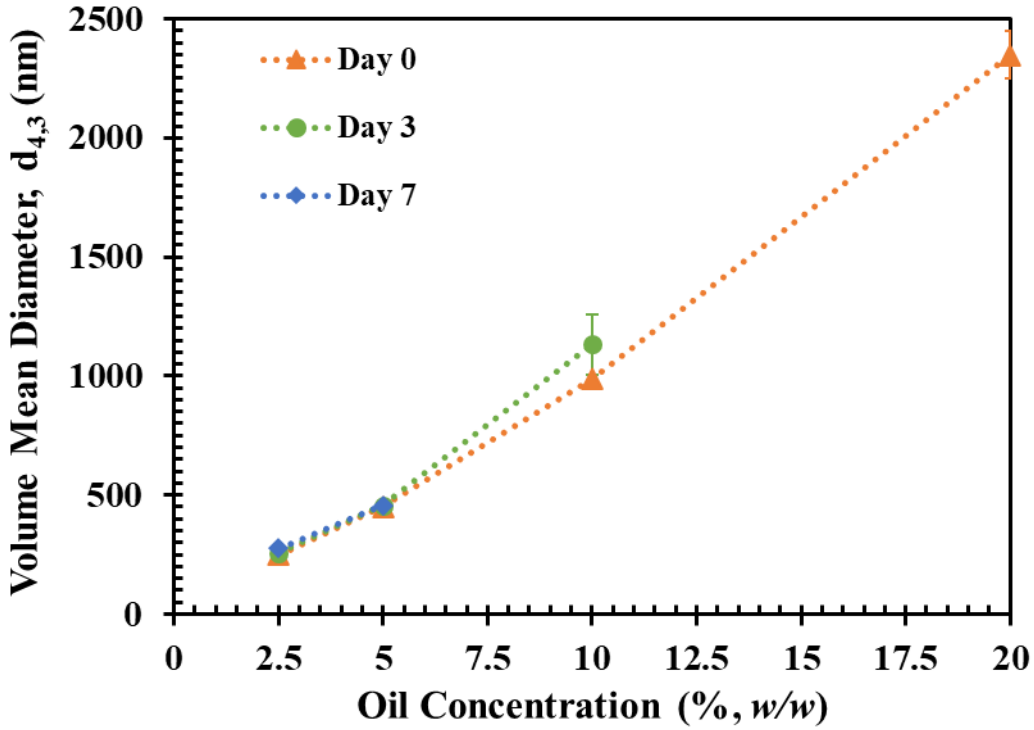
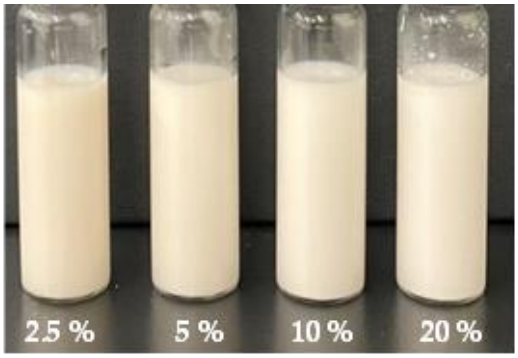
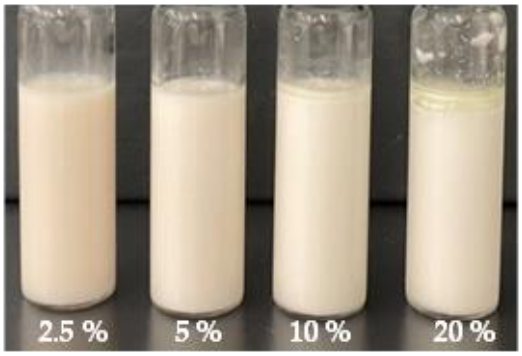


Figure 3.11. Influence of oil concentration on volume mean diameter ($d_{4,3}$) of fresh emulsions (Day 0) and after 3 days (Day 3) and 7 days (Day 7) of storage at 5 °C.



Day 0



Day 7

Figure 3.12. Influence of oil concentration on the visual appearance of fresh emulsions (Day 0) and after 7 days (Day 7) of storage at 5 °C.

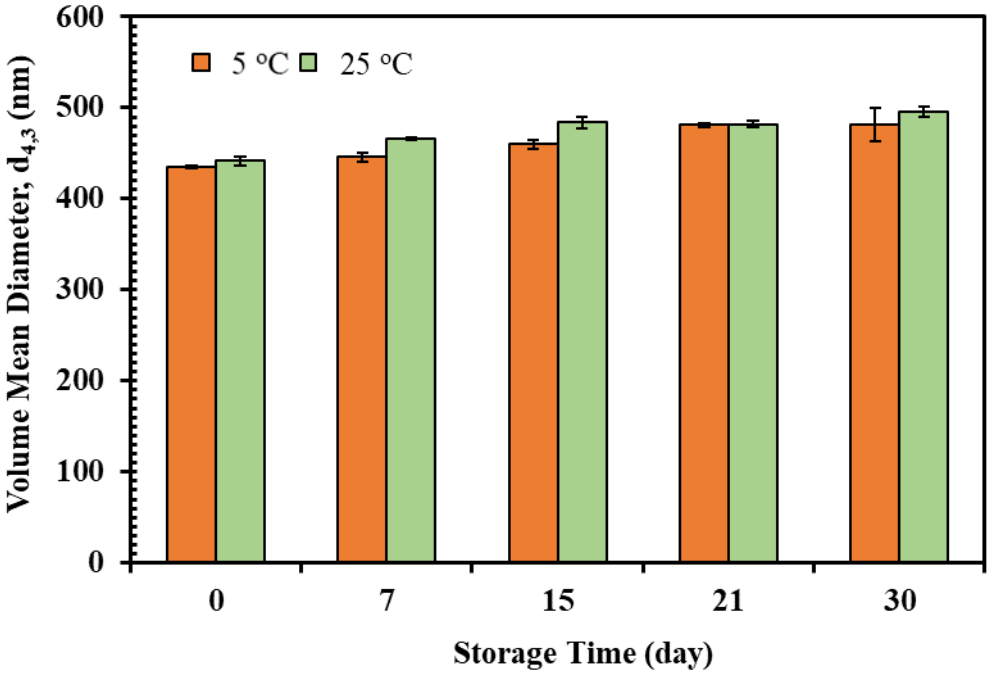


Figure 3.13. Volume mean droplet diameter, $d_{4,3}$ (nm), of oil-in-water (O/W) emulsions stabilized by 0.5% (*w*/*w*) LAE-75 and 5% (*w*/*w*) soybean in a period of storage (30 days) at 5 or 25 °C.

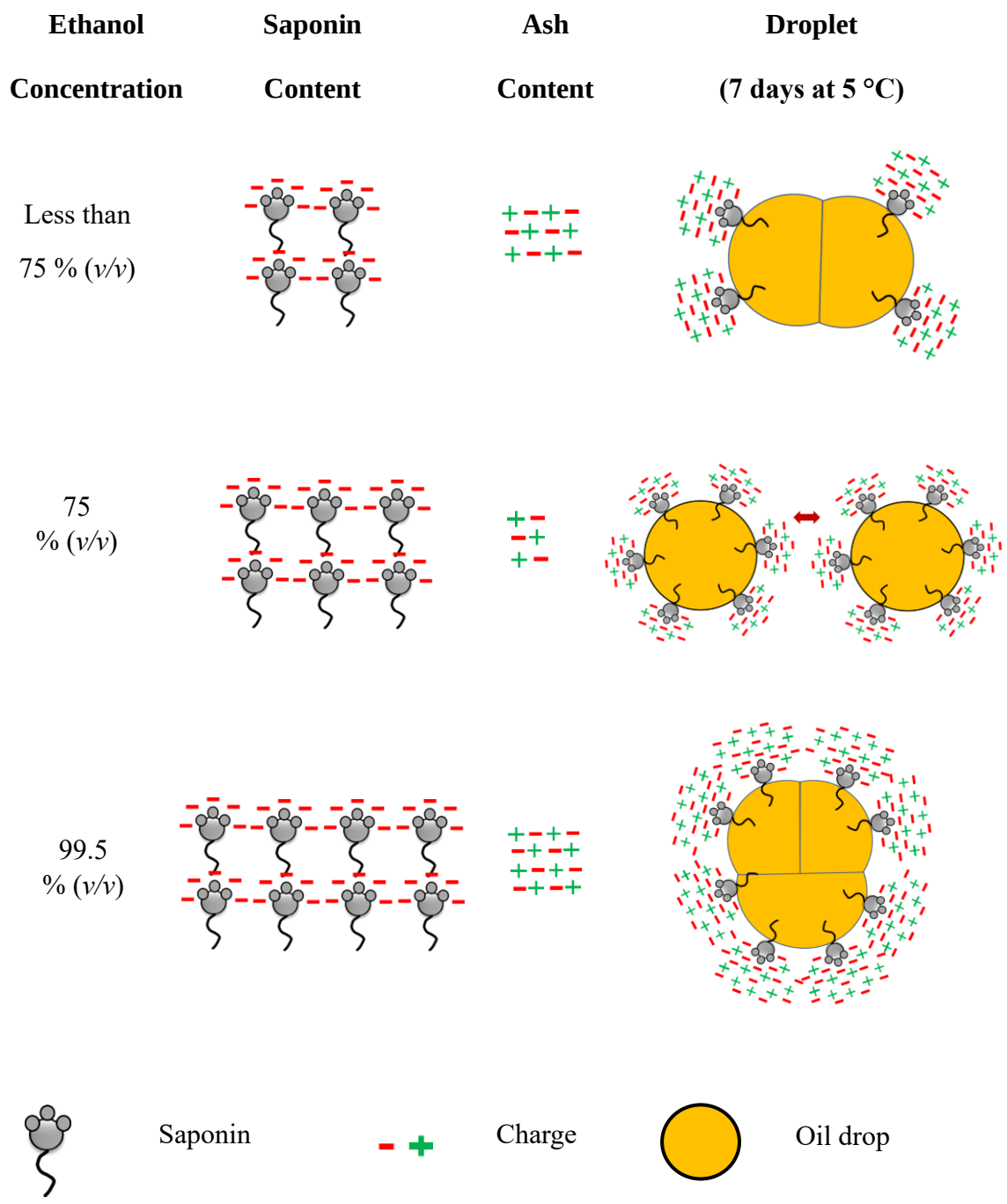


Figure 3.14. Proposed mechanism for the impact of saponin and inorganic substances of *Limnophila aromatica* extracts (LEAs) on droplet stabilization.

CHAPTER 4

GENERAL CONCLUSIONS AND FUTURE PROSPECTIVES

Because of their ability to form and stabilize emulsions, emulsifiers are crucial components in the food industry. Most emulsifiers are manufactured by chemical or enzymatic reactions. Natural food-grade emulsifiers are being widely explored to meet customer demand for environmentally friendly food products. Rice paddy herb (*Limnophila aromatica*) is a known medicinal herb traditionally used to cure particular diseases. The potential health benefits can be attributed to the presence of phenolic and flavonoids as well as other bioactivity performance.

This dissertation was focused on the overall emulsifying performance of *Limnophila aromatica* extract. However, each extract's surface-active compound, inorganic substances, and interfacial activity were evaluated to support this objective. The extraction method used in the study was solid-liquid extraction using different concentrations of ethanol since it is reported to be a safe solvent with low risk to human health and the environment.

In summary, the emulsifying performance of *Limnophila aromatica* extract was not only highly reliant on interfacial tension and/or surface-active components. The emulsions' destabilization occurred further by remaining demulsifiers such as inorganic chemicals in the extract. 0.5% (w/w) LAE-75 could stabilize emulsions up to 5% (w/w) soybean oil for 30 days. Therefore, we hypothesize that *Limnophila aromatica* extract has potential application as a sustainable natural emulsifier.

In future work, it would be ideal to find the method to remove the inorganic residual from the *Limnophila aromatica* extracts (e.g., membrane technology) since this impurity could disturb the emulsifying performance of the extract. We believe that this purification will enhance the extract's quality. The emulsion stabilized by this purified extract will successfully produce nanoemulsions with high oil concentration and resistance to

environmental stress. Since the *Limnophila aromatica* plants contain much bioactivity performances, the study on the encapsulation of nutritional compounds (e.g., carotenoid, lycopene) in oil-in-water emulsions assisted by *Limnophila aromatica* extract should be investigated.

REFERENCES

1. Acharya, V.; Shukla, S.; Jain, S. Agri-Produce Processing and Value Addition for Nutrition Security. *Int. J. Appl. Home Sci.*, **2017**, *4*, 611–616.
2. Ingram, J. S. I.; Wright, H. L.; Foster, L.; Aldred, T.; Barling, D.; Benton, T. G.; Berryman, P. M.; Bestwick, C. S.; Bows-Larkin, A.; Brocklehurst, T. F.; et al. Priority Research Questions for the UK Food System. *Food Secur.*, **2013**, *5*, 617–636. https://doi.org/10.1007/s12571-013-0294-4.
3. Ozturk, B.; McClements, D. J. Progress in Natural Emulsifiers for Utilization in Food Emulsions. *Curr. Opin. Food Sci.*, **2016**, *7*, 1–6. https://doi.org/10.1016/j.cofs.2015.07.008.
4. Andres-Brull, M. The Quest for Natural Emulsifiers; A Leatherhead Food Research white paper, 2016; pp 1–5.
5. Philcox, D. A Taxonomic Revision of the Genus Limnophila R . Br . (Scrophulariaceae). *Kew Bull.*, **1970**, *24* (1), 101–170. https://doi.org/10.2307/4103255.
6. Wijaya, C.; Do, Q. D.; Ju, Y. H.; Santoso, S. P.; Putro, J. N.; Laysandra, L.; Soetaredjo, F. E.; Ismadji, S. Isolation and Characterization of Starch from Limnophila Aromatica. *Heliyon*, **2019**, *5*, e01622. https://doi.org/10.1016/j.heliyon.2019.e01622.
7. Gorai, D.; Jash, S. K.; Singh, R. K.; Gangopadhyay, A. Chemical and Pharmacological Aspects of Limnophila Aromatica (Scrophulariaceae): An Overview. *AJPCT.*, **2014**, *2* (3), 348–356.
8. Dai, D. N.; Thang, T. D.; Thai, T. H.; Ogunwande, I. A. Chemical Constituents of Leaf Essential Oils of Four Scrophulariaceae Species Grown in Vietnam. *J. Essent. Oil Res.*, **2015**, *27* (6), 481–486. https://doi.org/10.1080/10412905.2015.1067650.

9. Minh, N. P.; Son, D. T.; Buu, L. T.; Nga, N. H. Efficacy of Drying and Stir-Frying to Purple Rice Paddy Herb (Limnophila Aromatica) Tea. *J. Glob. Pharm. Technol*, **2019**, *11* (8), 110–114.
10. Tucker, A. O.; Maciarello, M. J.; Hendi, M.; Wheeler, K. A. Volatile Leaf and Stem Oil of Commercial Limnophila Chinensis (Osb.) Merrill Ssp. Aromatica (Lam.) Yamazaki (Scrophulariaceae). *J. Essent. Oil Res.*, **2002**, *14* (3), 228–229. https://doi.org/10.1080/10412905.2002.9699831.
11. Do, Q. D.; Angkawijaya, A. E.; Tran-Nguyen, P. L.; Huynh, L. H.; Soetaredjo, F. E.; Ismadji, S.; Ju, Y. H. Effect of Extraction Solvent on Total Phenol Content, Total Flavonoid Content, and Antioxidant Activity of Limnophila Aromatica. *J. Food Drug Anal.* 2014, pp 296–302. https://doi.org/10.1016/j.jfda.2013.11.001.
12. Houdkova, M.; Urbanova, K.; Doskocil, I.; Rondevaldova, J.; Novy, P.; Nguon, S.; Chrun, R.; Kokoska, L. In Vitro Growth-Inhibitory Effect of Cambodian Essential Oils against Pneumonia Causing Bacteria in Liquid and Vapour Phase and Their Toxicity to Lung Fibroblasts. *S. Afr. J. Bot.*, **2018**, *118*, 85–97. https://doi.org/10.1016/j.sajb.2018.06.005.
13. Mom, S. From rice to a rice paddy herb. https://www.khmertimeskh.com/77638/rice-rice-paddy-herb/ (accessed May 9, 2022).
14. Nanasombat, S.; Teckchuen, N. Antimicrobial, Antioxidant and Anticancer Activities of Thai Local Vegetables. *J. Med. Plant Res.*, **2009**, *3* (5), 443–449.
15. Bhuiyan, M. N. I.; Akter, F.; Chowdhury, J. U.; Begum, J. Chemical Constituents of Essential Oils from Aerial Parts of Adenosma Capitatum and Limnophila Aromatica. *Bangladesh J. Pharmacol.*, **2010**, *5*, 13–16. https://doi.org/10.3329/bjp.v5i1.4019.

16. Vairappan, C. S.; Nagappan, T. Major Volatile Hydrocarbons of Rice Paddy Herb, Limnophila Aromatica Lam. Merr as Possible Chemotaxonomic Marker. *J. Trop. Biol. Conserv.*, **2014**, *11*, 41–48.
17. Yingngam, B.; Brantner, A.; Treichler, M.; Brugger, N.; Navabhatra, A.; Nakonrat, P. Optimization of the Eco-Friendly Solvent-Free Microwave Extraction of Limnophila Aromatica Essential Oil. *Ind. Crops. Prod.*, **2021**, *165*, 113443. https://doi.org/10.1016/j.indcrop.2021.113443.
18. Rattanasena, P. Antioxidant and Antibacterial Activities of Vegetables and Fruits Commonly Consumed in Thailand. *Pak. J. Biol. Sci.*, **2012**, *15* (18), 877–882. https://doi.org/10.3923/pjbs.2012.877.882.
19. Kukongviriyapan, U.; Luangaram, S.; Leekhaosoong, K.; Kukongviriyapan, V.; Preeprame, S. Antioxidant and Vascular Protective Activities of Cratoxylum Formosum, Syzygium Gratum and Limnophila Aromatica. *Biol. Pharm. Bull.*, **2007**, *30* (4), 661–666. https://doi.org/10.1248/bpb.30.661.
20. McClements, D. J. *Food Emulsions Principles, Practices, and Techniques*, Third Edit.; CRC Press, 2016.
21. Tadros, T. F. *Emulsion Formation and Stability*; Wiley-VCH Verlag GmbH & Co. KGaA, 2013. https://doi.org/10.1002/9783527647941.
22. McClements, D. J. Theoretical Analysis of Factors Affecting the Formation and Stability of Multilayered Colloidal Dispersions. *Langmuir*, **2005**, *21* (21), 9777–9785. https://doi.org/10.1021/la0512603.
23. McClements, D. J. Emulsion Design to Improve the Delivery of Functional Lipophilic Components. *Annu. Rev. Food Sci. Technol.*, **2010**, *1* (1), 241–269. https://doi.org/10.1146/annurev.food.080708.100722.
24. Dickinson, E. Towards More Natural Emulsifiers. *Trends Food Sci. Technol.*, **1993**,

4 (10), 330–334. https://doi.org/10.1016/0924-2244(93)90103-H.

25. McClements, D. J.; Bai, L.; Chung, C. Recent Advances in the Utilization of Natural Emulsifiers to Form and Stabilize Emulsions. *Annu. Rev. Food Sci. Technol.*, **2017**, *8*, 205–236. https://doi.org/10.1146/annurev-food-030216-030154.
26. McClements, D. J.; Gumus, C. E. Natural Emulsifiers — Biosurfactants, Phospholipids, Biopolymers, and Colloidal Particles: Molecular and Physicochemical Basis of Functional Performance. *Adv. Colloid Interface Sci.*, **2016**, *234*, 3–26. https://doi.org/10.1016/j.cis.2016.03.002.
27. McClements, D. J. Protein-Stabilized Emulsions. *Curr. Opin. Colloid Interface Sci.*, **2004**, *9* (5), 305–313. https://doi.org/10.1016/j.cocis.2004.09.003.
28. Yang, Y.; Leser, M. E.; Sher, A. A.; McClements, D. J. Formation and Stability of Emulsions Using a Natural Small Molecule Surfactant: Quillaja Saponin (Q-Naturale ®). *Food Hydrocoll.*, **2013**, *30* (2), 589–596. https://doi.org/10.1016/j.foodhyd.2012.08.008.
29. Chung, C.; Sher, A.; Rousset, P.; Decker, E. A.; McClements, D. J. Formulation of Food Emulsions Using Natural Emulsifiers: Utilization of Quillaja Saponin and Soy Lecithin to Fabricate Liquid Coffee Whiteners. *J. Food Eng.*, **2017**, *209*, 1–11. https://doi.org/10.1016/j.jfoodeng.2017.04.011.
30. Dogan, M. The Effectiveness of Light Emitting Diodes on Shoot Regeneration in Vitro from Shoot Tip Tissues of Limnophila Aromatica (Lamk.) Merr. and Rotala Rotundifolia (Buch-Ham. Ex Roxb) Koehne. *Biotech. Histochem.*, **2020**, *95* (3), 225–232. https://doi.org/10.1080/10520295.2019.1670359.
31. U.S. Department of Health and Human Services Food and Drug Administration; (CBER), C. for D. E. and R. (CDER) C. for B. E. and R. *Guidance for Industry Q3C*; 2017; Vol. 9765.

32. Taarji, N.; Bouhoute, M.; Chafai, Y.; Hafidi, A.; Kobayashi, I.; Neves, M. A.; Tominaga, K.; Isoda, H.; Nakajima, M. Emulsifying Performance of Crude Surface-Active Extracts from Liquorice Root (Glycyrrhiza Glabra). *ACS Food Sci. Technol.*, **2021**, *1* (8), 1472–1480. https://doi.org/10.1021/acsfoodscitech.1c00208.
33. Sahu, R.; Saxena, J. Screening of Total Phenolic and Flavonoid Content in Conventional and Non Conventional Species of Curcuma. *J. pharmacogn. phytochem.*, **2013**, *2* (1), 176–179.
34. Zhaobao, X.; Chunhong, T.; Chen Gang, S. Z. Studied on Corlorimetric Determination of Oleanolic Acid in Chinese Quince. *Natural Product Research and Development*, **2001**, *13* (4), 23–26.
35. Mariotti, F.; Tomé, D.; Mirand, P. P. Converting Nitrogen into Protein - Beyond 6.25 and Jones' Factors. *Crit. Rev. Food Sci. Nutr* ., **2008**, *48* (2), 177–184. https://doi.org/10.1080/10408390701279749.
36. Liu, K. Effects of Sample Size, Dry Ashing Temperature and Duration on Determination of Ash Content in Algae and Other Biomass. *Algal Res.*, **2019**, *40*, 101486. https://doi.org/10.1016/j.algal.2019.101486.
37. Stalikas, C. D. Extraction, Separation, and Detection Methods for Phenolic Acids and Flavonoids. *J. Sep. Sci.*, **2007**, *30* (18), 3268–3295. https://doi.org/10.1002/jssc.200700261.
38. Zieliński, H.; Kozłowska, H. Antioxidant Activity and Total Phenolics in Selected Cereal Grains and Their Different Morphological Fractions. *J. Agric. Food Chem.*, **2000**, *48* (6), 2008–2016. https://doi.org/10.1021/jf990619o.
39. Sultana, B.; Anwar, F.; Ashraf, M. Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts. *Molecules*, **2009**, *14* (6), 2167–2180. https://doi.org/10.3390/molecules14062167.

40. Tan, S. P.; Stathopoulos, C.; Parks, S.; Roach, P. An Optimised Aqueous Extract of Phenolic Compounds from Bitter Melon with High Antioxidant Capacity. *Antioxidants*, **2014**, *3* (4), 814–829. https://doi.org/10.3390/antiox3040814.

41. El Aziz, M. M. A.; Ashour, A. S.; Melad, A. S. G. A Review on Saponins from Medicinal Plants: Chemistry, Isolation, and Determination. *J. Nanomed. Res.*, **2019**, *7* (4), 282–288. https://doi.org/10.15406/jnmr.2019.07.00199.

42. Do, T. H.; Huynh, T. D.; Vo, K. A.; Nguyen, K. A.; Cao, T. S.; Nguyen, K. N.; Nguyen, H. A. H.; Nguyen, T. T. T.; Le, N. P. N.; Truong, D. H. Saponin-Rich Fractions from Codonopsis Javanica Root Extract and Their in Vitro Antioxidant and Anti-Enzymatic Efficacy. *J. Food Process. Preserv.*, **2022**, *46* (1), 1–13. https://doi.org/10.1111/jfpp.16113.

43. Pace, C. N.; Treviño, S.; Prabhakaran, E.; Scholtz, J. M. Protein Structure, Stability and Solubility in Water and Other Solvents. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, **2004**, *359* (1448), 1225–1235. https://doi.org/10.1098/rstb.2004.1500.

44. Pomeranz, Y.; Meloan, C. E. Ash and Minerals. In *Food Analysis*; Springer, Boston, MA, 1994; pp 602–624. https://doi.org/10.1007/978-1-4615-6998-5_35.

45. Ichikawa, T. Electrical Demulsification of Oil-in-Water Emulsion. *Colloids Surf. A Physicochem. Eng. Asp.*, **2007**, *302* (1–3), 581–586. https://doi.org/10.1016/j.colsurfa.2007.03.036.

46. Akbari, S.; Nour, A. H. Emulsion Types, Stability Mechanisms and Rheology: A Review. *Int. J. Innov. Res. Sci. Stud.*, **2018**, *1* (1), 14–21.

47. Kregiel, D.; Berlowska, J.; Witonska, I.; Antolak, H.; Proestos, C.; Babic, M.; Babic, L.; Zhang, B. Saponin-Based, Biological-Active Surfactants from Plants. In *Application and Characterization of Surfactants*; 2017. https://doi.org/10.5772/68062.

48. Moses, T.; Papadopoulou, K. K.; Osbourn, A. Metabolic and Functional Diversity of Saponins, Biosynthetic Intermediates and Semi-Synthetic Derivatives. *Crit. Rev. Biochem. Mol. Biol.*, **2014**, *49* (6), 439–462. https://doi.org/10.3109/10409238.2014.953628.

49. Fenwick, G. R.; Price, K. R.; Tsukamoto, C.; Okubo, K. Saponins. In *Toxic Substances in Crop Plants*; 1991; pp 285–327. https://doi.org/10.1533/9781845698454.285.

50. Maier, C.; Conrad, J.; Carle, R.; Weiss, J.; Schweiggert, R. M. Phenolic Constituents in Commercial Aqueous Quillaja (Quillaja Saponaria Molina) Wood Extracts. *J. Agric. Food Chem.,* **2015**, *63* (6), 1756–1762. https://doi.org/10.1021/jf506277p.

51. Kite, G. C.; Howes, M. J. R.; Simmonds, M. S. J. Metabolomic Analysis of Saponins in Crude Extracts of Quillaja Saponaria by Liquid Chromatography/Mass Spectrometry for Product Authentication. *Rapid Commun Mass Spectrom.,* **2004**, *18* (23), 2859–2870. https://doi.org/10.1002/rcm.1698.

52. Sparg, S. G.; Light, M. E.; Van Staden, J. Biological Activities and Distribution of Plant Saponins. *J. Ethnopharmacol*, **2004**, *94* (2–3), 219–243. https://doi.org/10.1016/j.jep.2004.05.016.

53. Vincken, J. P.; Heng, L.; de Groot, A.; Gruppen, H. Saponins, Classification and Occurrence in the Plant Kingdom. *Phytochemistry*, **2007**, *68* (3), 275–297. https://doi.org/10.1016/j.phytochem.2006.10.008.

54. Uluata, S.; McClements, D. J.; Decker, E. A. Physical Stability, Autoxidation, and Photosensitized Oxidation of ω-3 Oils in Nanoemulsions Prepared with Natural and Synthetic Surfactants. *J. Agric. Food Chem.*, **2015**, *63* (42), 9333–9340. https://doi.org/10.1021/acs.jafc.5b03572.

55. Schreiner, T. B.; Santamaria-Echart, A.; Ribeiro, A.; Peres, A. M.; Dias, M. M.; Pinho, S. P.; Barreiro, M. F. Formulation and Optimization of Nanoemulsions Using the Natural Surfactant Saponin from Quillaja Bark. *Molecules*, **2020**, *25* (7), 1–14. https://doi.org/10.3390/molecules25071538.

56. Ozturk, B.; Argin, S.; Ozilgen, M.; McClements, D. J. Formation and Stabilization of Nanoemulsion-Based Vitamin e Delivery Systems Using Natural Surfactants: Quillaja Saponin and Lecithin. *J. Food Eng.*, **2014**, *142*, 57–63. https://doi.org/10.1016/j.jfoodeng.2014.06.015.

57. Bouhoute, M.; Taarji, N.; Vodo, S.; Kobayashi, I.; Zahar, M.; Isoda, H.; Nakajima, M.; Neves, M. A. Formation and Stability of Emulsions Using Crude Extracts as Natural Emulsifiers from Argan Shells. *Colloids Surf. A*, **2020**, *591*, 124536. https://doi.org/10.1016/j.colsurfa.2020.124536.

58. Ralla, T.; Salminen, H.; Edelmann, M.; Dawid, C.; Hofmann, T.; Weiss, J. Oat Bran Extract (Avena Sativa L.) from Food by-Product Streams as New Natural Emulsifier. *Food Hydrocoll.*, **2018**, *81*, 253–262. https://doi.org/10.1016/j.foodhyd.2018.02.035.

59. Taarji, N.; Rabelo da Silva, C. A.; Khalid, N.; Gadhi, C.; Hafidi, A.; Kobayashi, I.; Neves, M. A.; Isoda, H.; Nakajima, M. Formulation and Stabilization of Oil-in-Water Nanoemulsions Using a Saponins-Rich Extract from Argan Oil Press-Cake. *Food Chem.*, **2018**, *246*, 457–463. https://doi.org/10.1016/j.foodchem.2017.12.008.

60. Ralla, T.; Salminen, H.; Wolfangel, T.; Edelmann, M.; Dawid, C.; Hofmann, T.; Weiss, J. Value Addition of Red Beet (Beta Vulgaris L.) by-Products: Emulsion Formation and Stability. *Int. J. Food Sci. Technol.*, **2019**, *54* (3), 619–625. https://doi.org/10.1111/ijfs.13886.

61. Ralla, T.; Herz, E.; Salminen, H.; Edelmann, M.; Dawid, C.; Hofmann, T.; Weiss,

J. Emulsifying Properties of Natural Extracts from Panax Ginseng L. *Food Biophys.*, **2017**, *12* (4), 479–490. https://doi.org/10.1007/s11483-017-9504-5.

62. Ralla, T.; Salminen, H.; Edelmann, M.; Dawid, C.; Hofmann, T.; Weiss, J. Stability of Emulsions Using a New Natural Emulsifier: Sugar Beet Extract (Beta Vulgaris L.). *Food Biophys.*, **2017**, *12* (3), 269–278. https://doi.org/10.1007/s11483-017-9482-7.
63. Ralla, T.; Salminen, H.; Tuosto, J.; Weiss, J. Formation and Stability of Emulsions Stabilised by Yucca Saponin Extract. *Int. J. Food Sci. Technol.*, **2018**, *53* (6), 1381–1388. https://doi.org/10.1111/ijfs.13715.
64. Fainassi, F.; Taarji, N.; Benkhalti, F.; Hafidi, A.; Neves, M. A.; Isoda, H.; Nakajima, M. Emulsion Formation and Stabilizing Properties of Olive Oil Cake Crude Extracts. *Processes*, **2021**, *9*, 1–13. https://doi.org/10.3390/pr9040633.
65. Karefyllakis, D.; Octaviana, H.; van der Goot, A. J.; Nikiforidis, C. V. The Emulsifying Performance of Mildly Derived Mixtures from Sunflower Seeds. *Food Hydrocoll.*, **2019**, *88*, 75–85. https://doi.org/10.1016/j.foodhyd.2018.09.037.
66. Lu, G. W.; Gao, P. Emulsions and Microemulsions for Topical and Transdermal Drug Delivery. In *Handbook of Non-Invasive Drug Delivery Systems*; 2010; pp 59–94. https://doi.org/10.1016/b978-0-8155-2025-2.10003-4.
67. Losso, J. N.; Khachatryan, A.; Ogawa, M.; Godber, J. S.; Shih, F. Random Centroid Optimization of Phosphatidylglycerol Stabilized Lutein-Enriched Oil-in-Water Emulsions at Acidic PH. *Food Chem.*, **2005**, *92* (4), 737–744. https://doi.org/10.1016/j.foodchem.2004.12.029.
68. Hu, M.; McClements, D. J.; Decker, E. A. Lipid Oxidation in Corn Oil-in-Water Emulsions Stabilized by Casein, Whey Protein Isolate, and Soy Protein Isolate. *J. Agric. Food Chem.*, **2003**, *51* (6), 1696–1700. https://doi.org/10.1021/jf020952j.

69. Can Karaca, A.; Nickerson, M. T.; Low, N. H. Lentil and Chickpea Protein-Stabilized Emulsions: Optimization of Emulsion Formulation. *J. Agric. Food Chem.*, **2011**, *59* (24), 13203–13211. https://doi.org/10.1021/jf203028n.

70. Böttcher, S.; Drusch, S. Saponins — Self-Assembly and Behavior at Aqueous Interfaces. *Adv. Colloid Interface Sci.*, **2017**, *243*, 105–113. https://doi.org/10.1016/j.cis.2017.02.008.

71. Böttcher, S.; Keppler, J. K.; Drusch, S. Mixtures of Quillaja Saponin and Beta-Lactoglobulin at the Oil/Water-Interface: Adsorption, Interfacial Rheology and Emulsion Properties. *Colloids Surf. A Physicochem. Eng. Asp.*, **2017**, *518*, 46–56. https://doi.org/10.1016/j.colsurfa.2016.12.041.

72. Goodarzi, F.; Zendehboudi, S. A Comprehensive Review on Emulsions and Emulsion Stability in Chemical and Energy Industries. *Can. J. Chem. Eng.*, **2019**, *97* (1), 281–309. https://doi.org/10.1002/cjce.23336.

73. Dickinson, E. Structure, Stability and Rheology of Flocculated Emulsions. *Curr. Opin. Colloid Interface Sci.*, **1998**, *3* (6), 633–638. https://doi.org/10.1016/S1359-0294(98)80092-7.

74. Dickinson, E. Hydrocolloids at Interfaces and the Influence on the Properties of Dispersed Systems. *Food Hydrocoll.*, **2003**, *17*, 25–39. https://doi.org/https://doi.org/10.1016/S0268-005X(01)00120-5.

ACKNOWLEDGMENT

First and foremost, I would like to express my deep and sincere gratitude to my supervisor, Assoc. Prof. Dr. Marcos Antonio das Neves for his supervision, guidance, patience, motivation, enthusiasm, attention, and constant support throughout the laboratory work and thesis writing that have contributed to the success of this research.

I am also truly grateful to Prof. Mitsutoshi Nakajima and Dr. Isao Kobayashi for their invaluable guidance, immense knowledge, and insightful and constructive comments.

Thanks to Kawamoto San for all administrative support and assistance around the laboratory.

Deep thanks to all seniors, Meryem, Noamane, Vodo, Samar, Lenin, Grace, and all lab mates, Teetach, Lorena, Sohan, Aymen, Kubra, Chizie, and Barbara for their stimulating discussions, kindness, and moral support from the beginning till the end of this research.

Profound thanks to all the co-authors of my article, especially Meryem, Noamane, and Lorena, for their technical support, advice, and discussion. Without their never-end support, it would not have been possible for me to publish my paper successfully.

Special appreciation also goes to my family. My mom for her encouragement, love, and motivation. Punleu for being such a wonderful younger sister who handles and settles all issues during my absence. I cannot thank you enough, my beloved husband, Vuth, who is willing to leave his comfort zone to accompany me through this difficult time. You are a real blessing in my life, and you have been here and helped me to complete my journey. Thanks to my parents-in-law, sister-in-law, brother-in-law, and all my relative for their abiding love, motivation, and support.

Thanks to all my friends and juniors for their encouragement and motivation during my hard time.

I also would like to express my sincere thanks to my home university, the Royal University of Agriculture, for nominating me and providing the opportunity for me to participate in this doctoral program.

Last but not least, I would like to acknowledge the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan for providing the scholarship to conduct the current research under the MEXT Special Scholarship Program on Trans-world Professional Human Resources Development Program on Food Security & Natural Resources Management (TPHRD) for a Doctoral Course.

LIST OF PUBLICATIONS

This thesis is based on the following publication:

Soeung, R.; Felipe, L.d.O.; Bouhoute, M.; Taarji, N.; Nakajima, M.; Neves, M.A. *Limnophila aromatica* Crude Extracts as Natural Emulsifiers for Formation and Stabilizing of Oil-in-Water (O/W) Emulsions. Colloids Interfaces 2022, 6, 26. https://doi.org/10.3390/ colloids6020026