DESIGN AND IMPLEMENTATION OF PHLOROTANNIN EXTRACTION PROCESSING TECHNIQUE FROM SEAWEED

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ABSTRACT

Upon the high demand of functional food and health supplement in the recent years, intensive investigation has been conducted by researchers either in academic area or professional field. In this work, the phlorotannin was extracted from brown seaweed using alcohol/salt liquid biphasic system (LBS). Liquid biphasic system is a novel sustainable technique that combined several processes into a step, where the separation and purification of bioactive compounds were completed in a unit operation. Non-toxic solvent was utilized and there was potentiality to recycle the solvent used in the system. The recycled solvent is able to recover similar amount of phlorotannin compared to the new solvent used. Phlorotannin is one of the beneficial bio-products found in brown algae and attracted huge interest of community owing to its health beneficial effect. Hence, the extraction of phlorotannin is lucrative as it has biological activities that can be applied into food, pharmaceutical and cosmeceutical products. The brown algae, *Padina australis* and *Sargassum binderi* were capable to recover 76.1% and 91.67% of phlorotannin by using 2-propanol/ ammonium sulphate system, with purification factor of 2.49 and 1.59, respectively. The recycling of salt phase was carried out and total amount of salt (g), 41.04% and 72.39% were recovered for *Padina australis* and *Sargassum binderi*, respectively. The system showed good recyclability, as comparable phlorotannin recovery (44.33% and 44.60% for *Padina australis*, 65.84 and 84.28% for *Sargassum binderi*) which was observed even twice extractions was performed. The LBS was concluded as an eco-friendly technique that has potential to be upscaled into industrial scale.

Keywords: Liquid Biphasic System, Algae, Phlorotannin, Recycle Study, Brown Seaweed

ABSTRAK

Kebelakangan ini, permintaan dan penawaran yang tinggi untuk makanan berkhasiat dan suplemen kesihatan menyebabkan penyelidikan yang intensif perlu dijalankan oleh penyelidik-penyelidik dari bidang akademik atau profesional. Dalam kajian ini, phlorotannin telah diekstrak daripada rumpai laut perang dengan teknik yang bernama alkohol/garam liquid biphasic sistem. Liquid biphasic sistem adalah salah satu teknologi hijau baharu yang mengintegrasikan beberapa proses kepada satu langkah, iaitu memekatkan, memisahkan dan membersihkan bioproduk yang telah diekstrak dalam hanya satu unit operasi. Kegunaan pelarut yang bukan toksik dalam kajian ini mempunyai potensi untuk pemulihan pelarut selepas pengekstrakan phlorotannin, dan juga memberi manfaat kepada alam sekitar dengan mengurangkan jumlah pelarut yang dituang ke dalam alam sekitar. Phlorotannin telah mendapat perhatian daripada komuniti kerana manfaat kesihatannya, contohnya mengurangkan kedutan, mengubati penyakit kencing manis dan anti radang. Oleh itu, pengasingan phlorotannin daripada rumpai laut sangat lumayan untuk aplikasi farmaseutikal, cosmeseutikal dan makanan berfungsi. Jumlah maksimum phlorotannin yang telah diekstrak daripada *Padina australis dan Sargassum binderi* adalah 76.1% dan 91.67%, dengan faktor penulenan, 2.49 dan 1.59, masing-masing. Kajian untuk mengitar semula garam dalam sistem telah dilaksanakan. Maksimum jumlah garam dipulihkan adalah 41.04% untuk *Padina australis* dan 72.39% untuk *Sargassum binderi*. Phlorotannin yang didapat selepas dua kitaran serupa dengan jumlah phlorotannin yang didapat selepas kitaran pertama (44.33% dan 44.60% untuk *Padina australis*, 65.84 dan 84.28% untuk *Sargassum binderi*). Ini menunjukkan sistem ini mesra alam sekitar dan mempunyai keupayaan untuk mengitar semula pelarut yang digunakan.

Keywords: Liquid Biphasic System, Algae, Phlorotannin, Recycle Study, Brown Seaweed

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LIST OF SYMBOLS AND ABBREVIATIONS

UV	:	Ultraviolet
LBS	:	Liquid Biphasic System
pH	:	photon of hydrogen
DNA	:	Deoxyribo nucleic acid
BHA	:	Butylated hydroxyanisole
PG	:	Propyl gallate
TBHQ	:	Tertbutylhydroquinone
BHT	:	Butylated hydroxytoluene
GLUT4	:	Glucose transporter type 4
AMPK	:	Adenosine monophosphate-activated protein kinase
MAE	:	Microwave-assisted extraction
TPC	:	Total phenolic content
SC	:	Supercritical
DW	:	Dry weight
DB	:	Dry basis
MCF-7	:	Michigan cancer foundation-7
$(NH_4)_2SO_4$	:	Ammonium sulphate
Na_2CO_3	:	Sodium carbonate
TLL	:	Tie-line length
V_R	:	Volume ratio
P_F	:	Purification factor
K	:	Partition coefficient
R%	:	Recovery
Y_A	:	Yield of alcohol recovered
Y_S	:	Yield of salt recovered

OH	:	Hydroxyl group
C-O	:	Carbon-oxygen bond
SLE	:	Solid-liquid extraction
m	:	Meter
mg	:	Milligram
g	:	Gram
µ	:	Microns
kDa	:	KiloDalton
GAE	:	Gallic acid
M	:	Molar
PGE	:	Phloroglucinol equivalent
Pa	:	Pascal
×g	:	times gravity
L	:	Litre

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Phlorotannin is a polyphenolic compound and a type of tannin which exists in brown algae (Phaeophyta), also known as seaweed. The phlorotannin in algae functions as a secondary defence metabolites in cell wall's development (Anaëlle et al., 2013). It is also able to protect algae from stress conditions, such as bacterial infection, UV radiation and grazing (Casas et al., 2016; Hyun et al., 2013). Phlorotannin has gain much attention because their anti-inflammatory, antioxidant, antidiabetic, and anti-bacterial properties (Namvar et al., 2013). It was proven that the interaction of protein and phlorotannin can result in protein precipitation (Stern et al., 1996). This is a significant discovery in downstream processing as the protein can be isolated from possible contaminants in blood stream. The formation of phlorotannin may vary due to different polymerization degrees of oligomers and polymers of phloroglucinols (1, 3, 5-trihydroxybenzene) (Montero et al., 2016). Bioactive compounds or bio-products in algae are extracted because of their non-poisonous properties and health effect. The extraction methods like supercritical fluid extraction, solid-liquid extraction and pressurised liquid extraction are the conventional methods in bio-product extraction (Anaëlle et al., 2013). However, these techniques are associated with long processing time and large volume of toxic organic solvent waste. Hence, a sustainable technique, namely liquid biphasic system (LBS) was suggested to extract and purify the bio-products from seaweed at the same time.

LBS is believed to be an alternative way in bio-products extraction, as better yield of purified product could be obtained through this technique. The phase forming components of LBS are usually made up of the combinations of polymer/polymer, alcohol/salt or polymer/salt (M. S. Tang et al., 2014; Walter, 2012). LBS has been studied intensively in the field of bio-separation over decades in order to separate or

purify the biological products from natural sources without denaturing the product. Researchers have reported that the extraction of biomolecules through LBS could be achieved in short mass transfer period, as well as obtaining high selectivity of biomolecules at less operational cost (Leong et al., 2017; Lin et al., 2013). The extraction and purification of particular bio-product can be affected by parameters such as pH, addition of inorganic salt, type of phase forming components and temperature. Besides, several studies have proven that LBS has successfully extracted and purified high value products (S. Y. Lee et al., 2017; Ng et al., 2014), like fucoxanthin (Gómez-Loredo et al., 2014), γ-cyclodextrin (Lin et al., 2016) and laccase (Rajagopalu et al., 2016).

Up to date, most studies are related to solvent and enzyme-assisted extraction in phlorotannin recovery. This is the first report of phlorotannin recovery using LBS, which has not been done before. The present study focused on the feasibility of alcohol/salt LBS technique in extraction/ purification of phlorotannin from *Padina australis* and *Sargassum binderi*. Ammonium sulphate was chosen as the bottom phase due to the versatility of ammonium sulphate forming two phases with other alcohols. Besides, it is a cheaper choice compared to the usage of polymer and copolymer as the bottom phase in the system. Ammonium sulphate is also known for its industrial usage especially as soil fertilizer where it can easily be obtained. An investigation on a better combination of alcohol with ammonium sulphate was conducted before optimising the system. Several studies were carried out to determine the capability of LBS in partitioning phlorotannin from brown macroalgae such as effect of tie-line length, volume ratio, sample concentration and pH study. Recycling study of phlorotannin recovery was also performed to determine the sustainability of LBS and feasibility in industrial application.

1.2 Problem Statement

The natural sources of functional ingredients that are isolated from could be a significant issue as the isolation of functional ingredients can result in competition among food and functional food market. On top of that, the countries with limited space of land or suitable type of land for agriculture are having issue in providing these natural sources for functional food production. These issues could be solved if an alternative source that does not compete with agriculture or required specific type of land could provide the functional ingredients is discovered. Hence, the algae are proposed as the alternative source due to their rapid growth rate. They are able to survive in any harsh conditions and contains various bio-products. They can be grown in sea or even land, in which sea, brackish even wastewater is utilised as water sources.

As a high value product, the isolation of biomolecules from algae requires multiple processing steps, including dewatering, cell disruption, extraction, additional processing or purification (repeated cycles of centrifugation and recovery using techniques like precipitation, ultrafiltration or chromatography) and final formulation. The costly and tedious processing requirements caused by numerous operation units have limited the industrial-scale production, where high amount of energy is consumed for the functional ingredients production. The conventional process has subsequently affected the operating cost and pricing of functional food in the market. Therefore, an alternative process that can obtain higher yield of purified product with low energy consumption is required.

1.3 Research Objectives

The scope of this research is to discover an energy-efficient extraction technique for isolating bioactive compounds from algae. Liquid biphasic system (LBS) has been found to be a potential technique to extract biomolecules with high efficiency and selectivity. There are three specific objectives to be achieved in the research:

1. To develop an eco-friendly and time-saving technique for phlorotannin extraction from algae.
2. To obtain maximum yield and high purity of phlorotannin by optimizing the operating parameters of the proposed extraction technique.
3. To evaluate cost efficiency and feasibility of the proposed extraction technique.

CHAPTER 2: LITERATURE REVIEW

This chapter is the literature review of current study. Most of the related information to my dissertation's topics and discussion of previous works are included in this chapter.

2.1 Introduction

The types of biomolecules exist in algae are given in the following section. Three major classes of biomolecules in algae are discussed in Section 2.2 as main compound to be extracted is Phlorotannins introduced in Section 2.3 and the structural properties and the functions of algae are also described. In addition, the potential applications of phlorotannins in various fields were tabulated due to their beneficial biological effects. High valuable compound like phlorotannins is worth to be extracted through an energy-efficient method that can produce high yield and purity of the product. Therefore, the inclusion of extraction technologies is essential to provide a good prospect in isolating phlorotannins from algae.

2.2 Types and biomolecules from Algae

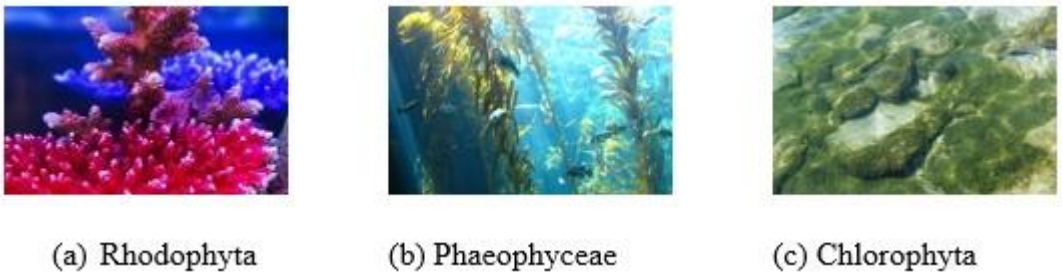
As part of the aquatic plant ecosystem, algae are one of the largest diverse group of photosynthetic organisms. Algae exist in various forms, from unicellular genera (diameter: 100-800 microns) to multicellular forms (length up to 50 m). They are classified into two categories, namely macroalgae and microalgae. Macroalgae are commonly known as seaweed in their most complex structure whereas microalgae's most complex form is *Charophyta*. In recent years, algae have attracted huge interests from the industrial community, especially from specialists in pharmaceutical, cosmeceutical and nutraceutical fields. The main reason of conducting algae research is due to the unique features of algae. Algae contain a variety of beneficial biomolecules that can be applied for various purposes. These beneficial biomolecules can be found in plants as well, but the competition with food and land usage poses an issue with the sustainability of utilizing plant-based biomolecules (Martins et al., 2017). Therefore, the

renewable and sustainable growth of algae sources has generated interest for investigation.

Several biomolecules that are commonly found in algae include vitamins, pigments, polyphenols and polyunsaturated fatty acids (Yaakob et al., 2014). These biomolecules exhibit various biological activities, such as anti-cancer, anti-inflammatory, anti-bacterial and anti-diabetic properties. Besides, the lipids and polysaccharides content within algae can be extracted and utilised as biofuel or bio-oil for the substitution of fuel and coal which will eventually deplete along with the fossil fuel and non-renewable sources. This shows the high potentiality of algae as functional food and energy source. The following sub-sections will elaborate some biomolecules of algae, particularly: pigments, polyphenols and vitamins.

2.2.1 Pigments

Algae are categorised into three types, namely *Rhodophyta* (red algae), *Chlorophyta* (green algae) and *Phaeophyceae* (brown algae), as shown in **Figure 2.1**. The pigments in these algae vary depending on the types of algae. For example, *Rhodophyta* has a higher amount of astaxanthin than the other two types of algae. *Chlorophyta* has more chlorophyll content in it. There are three main classes of natural pigments which can be found in algae: carotenoids, chlorophylls and phycobiliproteins (K. W. Chew et al., 2017). These pigments could serve as food colorant, functional ingredients for foods and poultry feed (N. Wang et al., 2018), additives in cosmetics and pharmaceutical products (Anis et al., 2017).



(a) Rhodophyta (b) Phaeophyceae (c) Chlorophyta

Figure 2.1: Types of algae

Carotenoids are one of the isoprenoid pigments which are synthesized by photosynthetic organisms (algae and plant) and some groups of non-photosynthetic organisms (bacteria and fungi) (Takaichi, 2011). The carotenoids are visualized as yellow, red or orange in colour and presented in 700 different chemical structures with diverse biological properties (da Costa Cardoso et al., 2017). Carotenoids could be generated from fats and stored in the fatty tissues of animals. The significant functions of carotenoids in algae include absorption of light energy for photosynthesis and prevention of photo-damage in chlorophyll. The properties of carotenoids such as photo-protection against UV radiation, has resulted in the intensive demands of carotenoids in cosmeceuticals field. In addition, carotenoids also act as an inhibitor of cancer due to their antioxidant properties and they are able to regulate the cell differentiation and cell cycle (Young, 1991).

Apart from carotenoids, chlorophylls are another green pigment found in the chloroplasts of algae and plants. Chlorophylls are lipid-soluble pigments which are responsible for the photosynthesis process. Similarly, chlorophyllic derivatives from algae vary depending on their growth environment. Chlorophyll oxidizes upon the exposure of weak acids, oxygen or light and forms degradation products. There are chlorophyll *a* and chlorophyll *b*, which can be identified through the colours due to the slight difference in structural formation, where the methyl group in ring II of chlorophyll *a* is replaced by a formyl group in chlorophyll *b*. Hence, chlorophyll *a* shows blue/green pigment while chlorophyll *b* shows green/yellow pigment (Hosikian et al., 2010). Chlorophyll is widely applied in pharmaceutical products, with its outstanding healing effect. It exhibits the ability to recover wounds faster by 25% in some studies (Bowers, 1947). The stimulation of tissue growth by chlorophyll can prevent the development of bacteria and be used for the interchange of carbon dioxide and oxygen in the body.

Phycobiliproteins are the aqueous soluble fluorescent pigments that are able to capture the radiation of visible spectrum (Chakdar & Pabbi, 2017). Phycobiliproteins help in the transaction of light energy during photosynthesis by connecting to the Photosystem II containing chlorophylls. Phycobiliproteins can be extracted from *Rhodophyceae* and specifically identified as R-phycoerythrin, R-phycocyanin and R-allophycocyanin. These components normally exist in low composition in algae but have high value for various applications. The fluorescent properties of phycobiliproteins are gaining importance in therapies or analytical purposes to serve as an immune-fluorescent probe (Mittal et al., 2017). Phycobiliproteins are widely used in beverages, snacks such as chewing gum and candies, and clinical laboratories (Verma et al., 2017).

2.2.2 Polyphenol

Polyphenols are one of essential natural antioxidants formed by aromatic rings with hydroxyl groups. The structure of polyphenols is varied based on the number of aromatic rings and hydroxyl groups or characterized by the presence of phenol structural units. Polyphenol derivatives are common secondary metabolites found in various terrestrial plants and marine algal. The studies on polyphenol from terrestrial plants are thorough over few years. However, the studies on polyphenol from marine algal are scarce (Thomas & Kim, 2011).

Polyphenols are divided into few classes such as phenolic acids, flavonoids, isoflavonoids, lignans, stilbenes and phenolic polymers (Machu et al., 2015). These phytochemicals are often found not only in algae, but also in fruits and beverages such as tea, coffee and wine. Polyphenols in algae such as phlorotannins existed in abundance in *Phaeophyceae* and little amount in some of the *Rhodophyta*. In algae, the polyphenols' function is to protect the algae from ultraviolet radiation and pathogen. Therefore, the polyphenols are often synthesized during the infection from microbial, diseases, photo-oxidation stress, UV light and more (Klejdus et al., 2017). Polyphenols

are the integral structural components of the cell wall in algae and are important to the reproduction of algae besides contributing to the sensory characteristic and colour of the algae.

As a high value-added product in high demand, the isolation of polyphenols is studied mainly due to its variation of valuable biological effects. Several studies showed that polyphenols have antimicrobial (Eom et al., 2012), antidiabetic (S.-H. Lee & Jeon, 2013) and antiviral activities (Hardouin et al., 2014). The usage of polyphenols as natural antimicrobial agents can promote the prevention of antibiotic resistance and the anti-diabetic effects which can help to inhibit α-glucosidase production, hence lowering the uptake of glucose in skeletal muscle and subsequently lowering the postprandial hyperglycemia.

2.2.3 Vitamins

Vitamins are nutrients that are required for the proper functioning of our body system. Over the years, the market demand of vitamins has risen due to the population pursuing quality of life and enhancing the immunology system of human beings. The significance of improving health in the third world country has led to the discovery of cheaper sources of vitamin to reduce the mineral deficiencies among people. Vitamins can be in water-soluble and fat-soluble forms. Water-soluble vitamins need to be regularly replenished as they are easily removed through body fluids, while fat-soluble vitamins can be consumed in a smaller quantity as they will accumulate in the liver and fatty tissues. Vitamins' roles are to support the growth and development of cell, aid digestion, improve the health of heart and nervous system, and supply energy throughout the body (Fathima et al., 2017). As one of the micronutrient needed for growth, vitamin is essential for a balanced diet in order to avoid illnesses and malnutrition.

Algae contain many types of vitamins such as vitamin A, vitamin B (B_1, B_2, B_6 and B_{12}), vitamin E and provitamin A. Provitamin A, also known as β-carotene, is especially

found in unicellular algae such as *spirulina*, *chlorella* and *dunalliella* (G. Tang & Suter, 2011). Provitamin A is converted into active vitamin A after being processed in body and stored in liver. Besides, the composition of all vitamins contained in algae is higher than other terrestrial plant (Priyadarshani & Rath, 2012). It is believed that the brown algae contain higher composition of vitamin E than red and green algae. Lastly, the vitamin group B have been found in algae last few years, especially vitamin B_{12}. Vitamin B_{12} is involved in the synthesis of DNA and cellular energy production. A deficiency of vitamin B12 may result in the development of megaloblastic anaemia (O'Leary & Samman, 2010) which can cause intestinal malabsorption (Lachner et al., 2012).

2.3 Phlorotannin

The natural sources of phlorotannin are kelps, rockweeds or sargassaceae species that are categorized as brown algae and comparatively low amount of phlorotannin is present in red algae. In pharmaceutical and food industries, several artificial commercial antioxidants such as butylated hydroxyanisole (BHA), propyl gallate (PG), tertbutylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) have been widely used to hinder oxidation process. The synthetic ingredients for antidiabetic drugs and tannin acids may contain antioxidants but these compounds may exhibit possible health hazards. Nevertheless, strict regulations are set to control the over usage of synthetic compounds or ingredients due to potential health hazards. In this case, phlorotannin is a secured alternative in for pharmaceutical applications.

Phlorotannin is categorised under tannins and exists in soluble cell-wall-bound in algae. The interaction of phlorotannin with protein is similar with the interaction of other types of tannin with protein, which result in protein precipitation. The structure of phlorotannin is dictated by the polymerization of the monomer units – phloroglucinol (1,3,4-trihydroxybenzene). The linkage of several monomers to each other via different

degrees will result in various chemical structures shown in **Figure 2.2**. The example of phlorotannins are phloroglucinol, eckol, dieckol, 6,6'-bieckol, 8,8'-dieckol, phlorofucofuroeckol A and phlorofucofuroeckol B. They are named based on the chemical properties and structural arrangement of monomers. The formation of phlorotannin is recommended to be formed biosynthetically, by undergoing the acetate-malonate pathway (polyketide pathway) (S.-H. Lee & Jeon, 2013). It is reported that phlorotannin has a broad range of molecular sizes, ranging from 126 kDa to 650 kDa (Thomas & Kim, 2011).

Phlorotannin is often extracted from brown macroalgae and generally soluble in less polar solvents. This bioactive compound extracted from different type of brown algae demonstrates diverse biological activities such as eckol from Ecklonia stolinifera, which can be used in inhibiting matrix metalloproteinase-1 in human dermal fibroblasts and phlorofucofuroeckol A from Ecklonia kurome, which exhibits algicidal effect. Good quality of bioactive compounds has to be effectively attained in order to maximize the efficiency of phlorotannins production. Hence, the extraction and isolation methods are important and thorough investigation has to be performed to explore the techniques which are economically feasible and environment friendly.

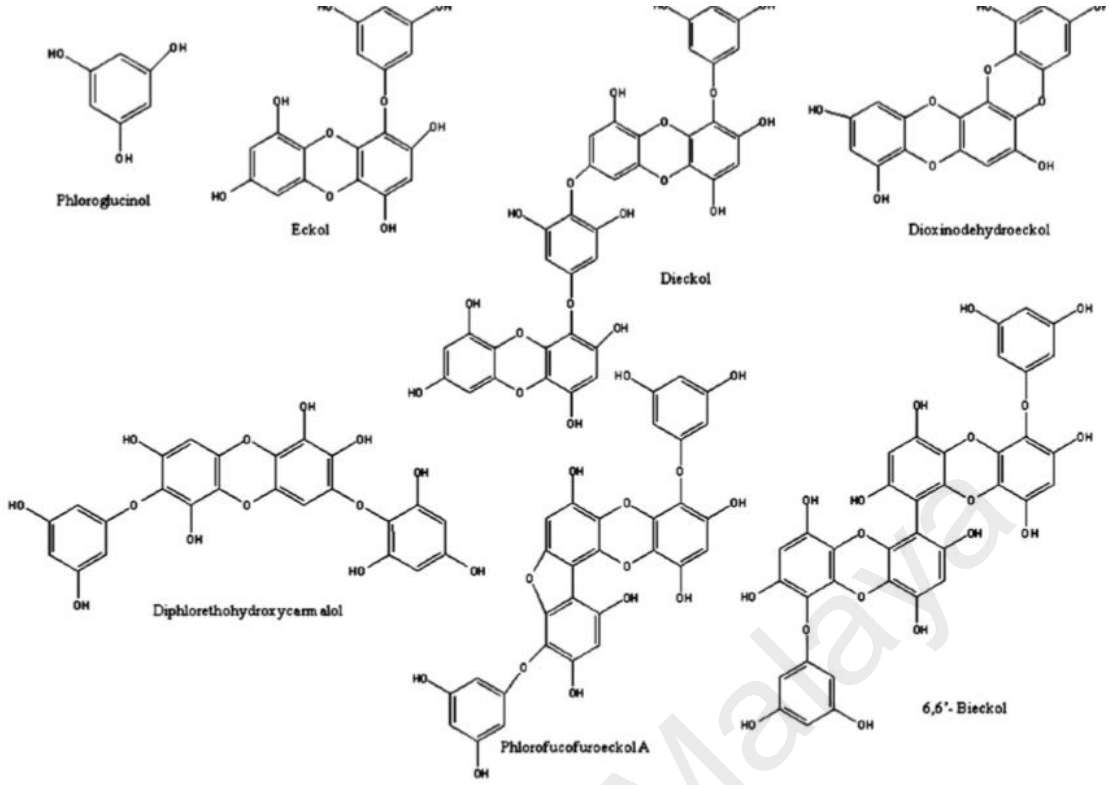


Figure 2.2: Chemical structures of phlorotannin (Shibata et al., 2015)

2.4 Biological Activity

A variety of bioactive compounds exist in algae have many commercial applications in the medical, pharmaceutical, food and agriculture industries. Various beneficial properties such as antioxidant, antifungal, antiviral and antimicrobial activities have been discovered in brown, green and red algae.

2.4.1 Antioxidant activity

The phenolic compounds found in algae can act as antioxidants through the chelation of metal ions which prevent the radical formation and improve the antioxidant endogenous system. The interest in natural antioxidants has increased in recent years as the demand has shifted away from synthetic antioxidants. Natural antioxidant substances have been successfully branded as a substitute for synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which have shown carcinogenic effects. Another advantage of natural antioxidants from plant origins is that they can react rapidly with free radicals and prevent the oxidative

deterioration. Natural antioxidants as the food additives are capable to increase the shelf life of food, thereby protecting the food and the consumers.

Phenolic compounds in brown algae are reported to be most effective antioxidants. The phenolic compounds are the secondary metabolites present in plants and algae and associated with the antioxidant activity. The phenolic content in brown seaweed extract was about 24.61 to 49.16 mg GAE/g seaweed extract while red seaweed can yield 1.5 to 4.1 mg GAE/g seaweed extract. This shows that brown algae contain much higher phenolic content than red seaweed (S.-H. Lee & Jeon, 2013).

2.4.2 Antibacterial activity

Antibacterial activities have also been found in tannins extracted from brown algae. This can broaden the application of tannins as natural preservatives in food products or as an antibacterial drug (Y. Chew et al., 2008). However, the level of defence against bacteria provided by the polyphenols in brown algae is rather low. The ability of high molecular weight phlorotannin from brown algae to provide antibacterial and antifungal effects appear to be mild, while the intermediate and lower molecular weight fraction are more responsible for the generation of these bioactivities. The antibacterial activities are vital to avoid bacterial slime from producing larval settlement and attracting grazers (Rocha et al., 2007). Several studies using brown algae for polyphenol extraction showed different findings in the antibacterial properties, as this activity was only reported in some work. There are authors that failed to find any antibacterial properties in the isolated polyphenols.

A study by Sandsdalen et al. (2003) deduced that the antibacterial activity in brown algae extract was caused by a polyhydroxylated fucophlorethol. This compound is effective against Gram-positive and Gram-negative bacteria. The antibacterial mechanism was tested against both the Gram-positive and Gram-negative bacteria and the presence of antibacterial compounds was confirmed (Sandsdalen et al., 2003). The

chemical structure formulated was also used in the characterization work on other extracts, which indicated that the phenolic compounds play a major role in the defence mechanism (Cox et al., 2010).

2.4.3 Anti-diabetic activity

Diabetes mellitus is the most severe chronic disease related to the aging and obesity in the population. The occurrence of diabetes mellitus is due to the abnormal metabolism of glucose. There are 2 types of diabetes mellitus, Type 1 diabetes which is insulin-dependent and Type 2 diabetes which is non-insulin dependent. An increasing number of patients was diagnosed with Type 2 diabetes in worldwide. The key to prevent diabetic complications is to control the blood glucose level effectively. Phlorotannin has the potential to serve as a glucose inhibitor. A study by Lee et al. (2016) stated that one of the phlorotannin compounds, octaphlorethol A, has successfully suppressed the GLUT4-mediated glucose uptake in the skeletal muscle of type 2 diabetic mice by the activation of AMPK in the muscle (S.-H. Lee et al., 2016). Besides, the phlorofucofuroeckol-A isolated from *Ecklonia cava* demonstrated inhibitory effects towards α-glucosidase and α-amylase activities, and it has higher inhibitory activities than acarbose (You et al., 2015). These findings corresponded to the study of Xu et al. (2012), where the phlorotannin was effective against Type 2 diabetes mellitus and could act as a potential source of therapeutic agents for clinical purposes (Xu et al., 2012).

2.4.4 Anti-inflammatory activity

Inflammation is a biological response of the body tissues towards stimulus such as physical injuries or pathogen and virus invasion. Phlorotannin demonstrates the ability in reducing inflammation and is suggested to be part of the ingredient for functional foods (Sugiura et al., 2013). Several studies showed that the investigated algae such as *Ecklonia sp.*, *Eisenia sp.* and *Myagropsis sp.* Wijesinghe et al. (2013) stated the nitric

oxide synthase and cyclooxygenase-2 expressions were inhibited by the phlorotannin-rich extracts from the fermented, lyophilized *Ecklonia cava* which was extracted using 80% ethanol in shaking incubator. The extract was capable to suppress the release of pro-inflammatory cytokines, interleukin-1β and interleukin-6 (Wijesinghe et al., 2013). The main components found to have anti-inflammatory activity are phlorofucofuroeckol A and B which depend on the inhibition of nitric oxide production (You et al., 2015). The effects of phlorotannin have been examined in-vivo using mice in a study by Sugiura et al. (2013). Examined phlorotannins such as eckol, 8,8'-bieckol and phlorofucofuroeckol had better performance in inhibiting inflammation after they were treated with arachidonic acid or oxazolone. This shows the potential of phlorotannin as anti-inflammatory ingredients and its anti-inflammatory effect can be improved after certain treatment.

2.5 Extraction Methods

Over decades, several techniques have been introduced in the extraction of phlorotannin. The conventional methods of phlorotannin extraction have been studied thoroughly. However, some improvements have to be made to increase the yield and the purity of phlorotannin. Several techniques for bioactive compounds extraction such as solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction and liquid biphasic system are discussed in the following sections.

2.5.1 Solvent Extraction

One of the conventional techniques used to extract phlorotannin from algal matrix is solvent extraction. The mechanism of solvent extraction is shown in **Figure 2.3**. Solvent extraction can be performed by mixing the sample extracts with solvents such as ethanol, hexane or methanol. A study conducted on the phlorotannin extraction using solvent extraction applies on the algal species, *Macrocystis pyrifera*. The algal samples were

pre-treated by washing the samples with hexane to remove pigments and lipid compounds. Incubation and centrifugation process were then carried out at 40 ^{o}C and 4 ^{o}C, respectively to obtain the supernatant for extraction (Leyton et al., 2016).

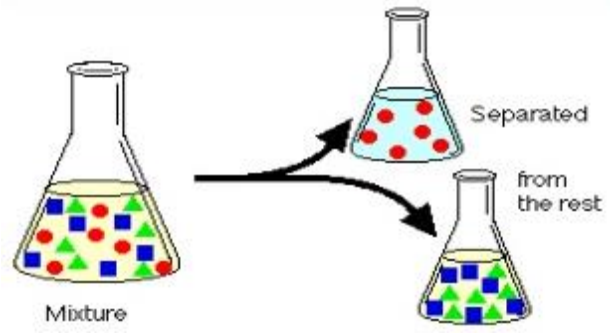


Figure 2.3: Mechanisms for solvent extraction

Eight different extraction solvents were studied by Leyton et al. (2016) to examine the extractability of phlorotannins from the samples. The solvents studied were methanol, ethanol, mixture of water and methanol, mixture of hexane and ethanol, mixture of ethanol and water, mixture of ethyl acetate and water, mixture of water and acetone and lastly, the mixture of methanol and chloroform. A total phlorotannin concentration of 147± 2.9 mg gallic acid equivalent (GAE)/100 g dry seaweed could be obtained by pre-treating the sample with hexane and extracting phlorotannin with water (Leyton et al., 2016), while the phlorotannin concentration of 200.5 mg GAE/ 100 g dry seaweed could be obtained through optimizing the process conditions. Another study conducted by Li et al. (2017) stated that a total phlorotannin content of 88.48 mg phloroglucinol equivalent/ 100 mg extract could be isolated from the source by using ethyl acetate (Yajing Li et al., 2017). However, this classical procedure is time consuming and requires large amount of solvent. Hence, further innovation in the extraction process is required to make it feasible.

2.5.2 Microwave-assisted Extraction

Microwave-assisted extraction (MAE) is the application of microwave along with other types of extraction methods. The microwave irradiation serves to generate

molecular movement to create a rotation of the liquid with permanent dipole during the extraction process (Castro-López et al., 2016). **Figure 2.4** shows a simple setup to recover phenolic compound from grape fruit using MAE, where the extraction flask was placed inside a microwave. A control unit was used to control the temperature and irradiation supply. The resulting product was extracted and measured to determine its yield.

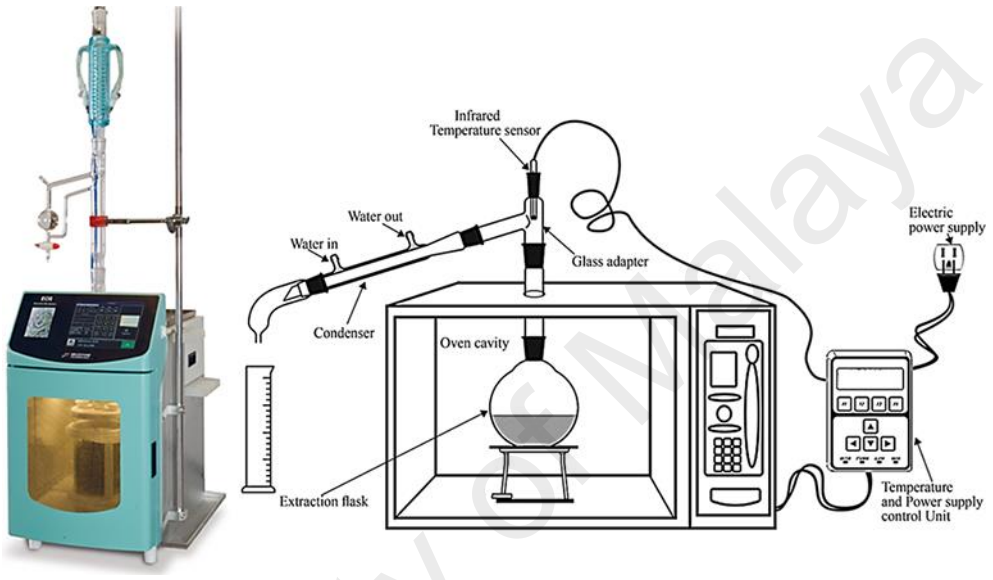


Figure 2.4: Setup to recover phenolic compound from grape fruit (Castro-López et al., 2016)

A comparison study between MAE and conventional method was carried out by He et al. (2013). It was proven that MAE has a better efficiency in extracting phlorotannin compared to solvent extraction. The total phlorotannin content (TPC) of 0.644 mg phloroglucinol was extracted out using MAE, while a yield of 0.585 mg phloroglucinol was attained using conventional method (He et al., 2013). Besides, typical enzymatic extraction and microwave-assisted enzymatic extraction of phlorotannin were performed by Charoensiddhi et al. (2015). The enzymatic extraction method with microwave irradiations resulted in higher yield of phlorotannin with antioxidant activities compared to the conventional enzymatic extraction (Charoensiddhi et al., 2015). The promising result indicates that MAE is a faster and better technique in phlorotannin extraction. Besides, it requires less amount of organic solvent and is more

cost effective. However, the phlorotannin production will decline at longer irradiation time, as it is likely to cause the degradation of phlorotannin after constant exposure to high irradiation rates during the process.

2.5.3 Ultrasound-assisted Extraction

Ultrasound as another energy source was also introduced for phlorotannin extraction. The schematic diagram of ultrasound-assisted extraction is shown in **Figure 2.5**. The ultrasonic probe is inserted into the sample extract and the depth of probe can be adjusted depending on the location desired. The frequency and time of ultrasonic process can be adjusted from the ultrasound generator. A jacketed glass beaker is suggested to be used to avoid the breakage of container caused by ultrasonic waves during the extraction process.

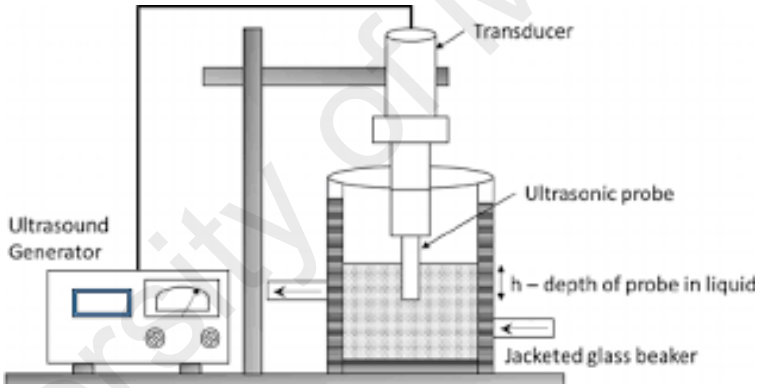


Figure 2.5: Setup example for ultrasound-assisted extraction (Kadam et al., 2013)

Kadam et al. (2015) conducted a study on the solvent extraction assisted with ultrasound. This study was carried out in adiabatic conditions using a T-type thermocouple, and the ultrasonic power dissipation of ultrasonic device was calculated and recorded at each amplitude level. From the study, the highest yield of total phenolics compound was achieved with the ultrasound amplitude of 114 µm and 0.03 M HCl was used as solvent. However, the yield of phlorotannin was found to decrease with concentration of solvents (Kadam, Tiwari, Smyth, et al., 2015). It was suspected to be occur due to the decomposition and acid hydrolysis effect. Although the usage of ultrasound altered or degraded the biological products, it was observed that ultrasonic

wave had no effect on degrading the phlorotannin. In the presence of ultrasound wave, the extraction by using acid as solvent exhibited higher yield and molecular weight of phlorotannin than by using water, representing the potential of ultrasound-assisted extraction to be applied in enhancing the extractability of targeted compound. However, some drawbacks of ultrasound-assisted extraction included difficulties in integration with other instruments and possible depolymerisation effect of polysaccharides required asolution to overcome the mentioned issues (Ebringerová & Hromádková, 2010).

2.5.4 Supercritical Fluid Extraction

Supercritical fluid extraction is utilised in the extraction of various bioactive compounds from many natural sources. The common solvent used in this technique is carbon dioxide, as carbon dioxide is cheaper than other solvents. **Figure 2.6** is a schematic diagram explaining the mechanisms of supercritical fluid extraction. In supercritical fluid extraction, supercritical solvents such as carbon dioxide are used and the condition is adjusted using cooler and pump to create supercritical fluid prior to the extraction process. Solvent such as methanol is also used with the supercritical carbon dioxide during the extraction.

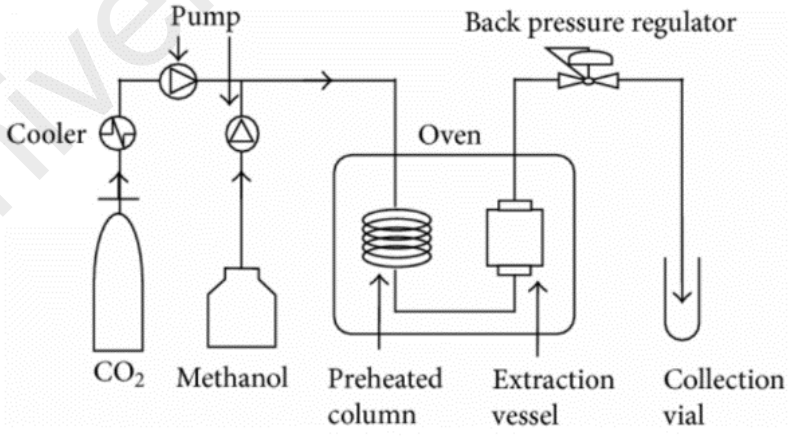


Figure 2.6: Schematic diagram for supercritical fluid extraction apparatus (Juliasih et al., 2015)

Some studies were conducted by Anaëlle et al. (2013) using supercritical fluid extraction on brown algae, *Sargassum muticum*. The preparation of raw material was

similar for all techniques (Anaëlle et al., 2013). For supercritical fluid extraction, ethanol was mixed with the supercritical carbon dioxide, CO_2 (SC-CO_2) at high pressure to form a compressed fluid mixture. The compressed fluid mixture was heated before being fed into the extraction cell which contained algae and dispersing agent (sea sand) to avoid clogging. The yield of phlorotannin by using CO_2-ethanol as the extraction solvent in this method was the lowest yield amongst all types of solvent, which was due to high polarity of the mixture that forbidding the extraction. However, this method only had optimal efficiency in extracting phlorotannin using ethanol as extraction solvent (Yajing Li et al., 2017). After discussing all the current techniques in extracting phlorotannin, the information are tabulated to show the yield of phlorotannin obtained from algae by using various extraction methods in **Table 2.1**.

Table 2.1: Yield of phlorotannin from algae via various techniques

Extraction Methods	Yield	Description/ Remarks	Reference
Solvent Extraction	200.5 ± 5.6 mg GAE[1]/ 100 g dry seaweed	Water with the pre-treatment of hexane	(Leyton et al., 2016)
	88.48 mg PGE[2]/100 mg extract	Ethanol Fractioned by: Ethyl acetate	(Yajing Li et al., 2017)
	63.61 mg PGE per g extract	30% Ethanol	
	0.585 mg	55% Ethanol Temperature: 60 °C	(He et al., 2013)
Microwave-assisted Extraction	0.644 mg	Extraction: Solvent extraction	(He et al., 2013)
	4.4 g PGE/ 100 g DW[3]	Extraction: Enzymatic extraction	(Charoensiddhi et al., 2015)
	0.923 mg/ g	40 % Ethanol Time: 25 min	(Luo et al., 2010)
Ultrasound-assisted Extraction	143.12 mg GAE/ g DW	Extraction: Acid concentration: 0.03 M HCl Ultrasonic amplitude: 114 μm	(Kadam, Tiwari, Smyth, et al., 2015)
	82.70 mg GAE/ g DB[4]	Extraction: Solvent extraction	(Kadam, Tiwari, O'Connell, et al., 2015)
	47.4%	Extraction: Solvent extraction	(Blanc et al., 2011)

[1]GAE is gallic acid equivalent, [2]PGE is phloroglucinol equivalent, [3]DW is dry weight and [4]DB is dry basis

Table 2.1, continued

Extraction Methods	Yield	Description/ Remarks	Reference
Supercritical Fluid Extraction	0.927 mg/g	Pressure: 300 bar Temperature: 48.98 °C	(Saravana et al., 2017)
	0.74 mg TPC[5]/ g DW	Pressure: 15.2 MPa Temperature: 60 °C	(Anaëlle et al., 2013)

[5]TPC is total phenolic content.

2.5.5 Liquid Biphasic System (LBS)

In the exploration of a better extraction technique, liquid biphasic system (LBS) emerges as a potential alternative to the conventional extraction techniques for its simplicity, efficiency and environmental friendliness. LBS is a liquid-liquid extraction technique that utilizes two water-soluble polymers or polymer and salt combination to separate and purify biological products like nucleic acids, virus, proteins, enzymes and antibodies. Numerous advantages have been revealed in applying this technique such as high rate of mass transfer, ease of scale-up, separation with high quality product (low probability of denaturation), cost effectiveness and enrichment of product which make LBS technique to be more outstanding than other extraction techniques. Besides, the non-toxicity environment of this technique enables the extraction of high quality product by minimizing the product denaturation.

Once the two liquid phases achieved a certain concentration, the formation of two immiscible phases can be observed. **Figure 2.7** shows the formation of phase separation after LBS. The partitioning of biomolecules often depends on surface properties (hydrophobicity and charge) and physicochemical properties of the two liquid phases that can be controlled by ionic strength, polymer molecular mass and concentration, pH and type of phase forming salt. Furthermore, the partition coefficient of biomolecules is

related to partitioning the targeted product, this represents that thorough studies have to be carried out to obtain the highest yield of good quality biological product.

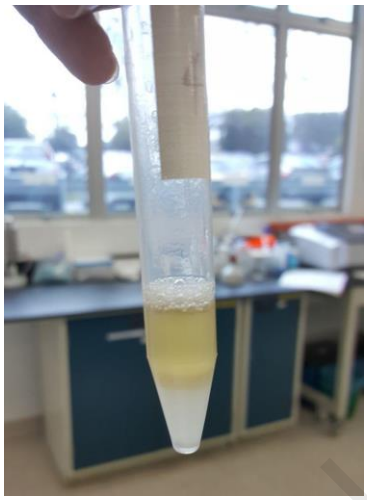


Figure 2.7: Phase separation after LBS

2.5.6 Selection of Alcohol/Salt

The phase forming component is important because the type of alcohol and salt used must be able to form LBS, as well as suitable to be applied in extracting phlorotannin. The alcohols such as methanol, ethanol, 1-propanol and 2-propanol, are chosen as phase forming components combined with ammonium sulphate in extracting phlorotannin from macroalgae. Several studies showed phlorotannin activity remains stable after the extraction with these common alcohols (Fairhead et al., 2006; Lordan et al., 2013). Ammonium sulphate is selected as the bottom phase due to the feasibility and stability in forming two phases with the chosen alcohols. Studies showed ammonium sulphate is the best salt in extraction of various bioactive compounds, such as anthocyanin from fruit residues of *Vaccinium uliginosum Linn* (Hua et al., 2013), phenolic compounds from eucalyptus (*Eucalyptus globulus*) wood industrial wastes (Xavier et al., 2017) and lithospermic acid B from *Salvia miltiorrhiza* Bunge (Guo et al., 2012).

2.6 Applications

As one of the bioactive compounds from algae, phlorotannin has gained much attention due to its biological effect exhibits. The beneficial health effect of phlorotannin has resulted in the application of phlorotannin in different areas as tabulated in **Table 2.2**. There are three main fields that phlorotannin can contribute to, namely pharmaceutical, food and cosmeceutical areas. It can be served as additives or functional ingredients mainly to control the undesired activities in body or by inhibitor. In comparison with phlorotannin, the studies have showed that synthetic phenolic compound such as butylated hydroxytoluene is carcinogenetic and harmful to murine and human health (Carocho et al., 2014). Butylated hydroxytoluene is commonly utilised as antioxidant and food additive to prevent ranciditication and lipid peroxidation. Hence, phlorotannin shows the potentiality in substituting the artificial phenolic compounds utilised in our daily life. It is because the phlorotannin has similar functionality to artificial phenolic compounds, safer to be consumed and associated with no toxicity compared to artificial phenolic compounds which lead to possible side effects such as carcinogenesis (Caleja et al., 2017).

Table 2.2: Applications and benefits of phlorotannin

Field	Purpose	Benefit	References
Pharmaceutical	Functional ingredients	Reduce intracellular reactive oxygen species generated by gamma-ray radiation	(Heo et al., 2009)
	Dietary for curing cancer	Reduce growth of MCF-7 (breast cancer cells by stimulation of apoptosis	(Kong et al., 2009)
	Curing allergies	Inhibit the release of histamine from human basophilic leaukimia	(Yong Li et al., 2008)
Food	Ingredients in beverages	High radical scavenging activity to overcome cardiovascular and diabetes	(Y.-X. Li & Kim, 2011)
	Natural preservatives	Avoid the growth of positive and negative Gram bacteria	(Jaiswal & Jaiswal, 2014)
	Soluble dietary fiber in bread	Lower down the starch bioavailability	(Gupta & Abu-Ghannam, 2011)
Cosmeceutical	Matrix metalloproteinases inhibitor	Controlling the metabolism of collagen in dermis	(Kim et al., 2006)
	Whitening ingredient	Reduce melanin synthesis and tyrosinase activity	(CHA et al., 2011)

CHAPTER 3: METHODOLOGY

In this work, I have collected two seaweeds, *Padina sp.* and *Sargassum sp.,* from Prof. Phang Siew-Moi and prepared them prior to extraction. The prepared algae was stored inside the refrigerator to avoid the death of algae and maintain the activity of biomolecules within the algae. The extraction technique, Liquid Biphasic System (LBS) was proposed and several parameters have assessed in this research. The parameters determined are type of alcohols, tie-line length of the system, volume ratio, pH study as well as crude load of sample. Besides, the recycling study has been conducted to determine the feasibility of technique in cost-saving as well as reducing the environmental impact during the extraction process. Finally, a rough cost estimation has been carried out to compare a conventional technique, solid-liquid extraction with the current study.

3.1 Materials

The marine algae, *Padina sp.* and *Sargassum sp.* were contributed by Prof. Phang Siew-Moi, Institute of Ocean and Earth Sciences, University of Malaya. The marine algae were collected from Cape Rachado, Port Dickson, Malaysia in September 2016. The samples were washed thoroughly three times with tap water to remove the sediments and marine salts attached on the surface of samples. The samples were rinsed with distilled water before drying in oven. The cleaned samples were oven-dried at 50 ^{o}C for three to five days. The dried samples were cut into small fragments, which is around 0.5 to 1 cm long prior to further analysis. Besides, phloroglucinol (1, 3, 5-trihydroxybenzene), ammonium sulphate ($(NH_4)_2SO_4$), Folin-Ciocalteu reagent and sodium carbonate (Na_2CO_3) were purchased from Merck (Selangor, Malaysia). The alcohols such as ethanol, methanol, 1-propanol and 2-propanol were purchased from R&M chemicals (Malaysia). All chemicals and reagents used in this study were analytical grade.

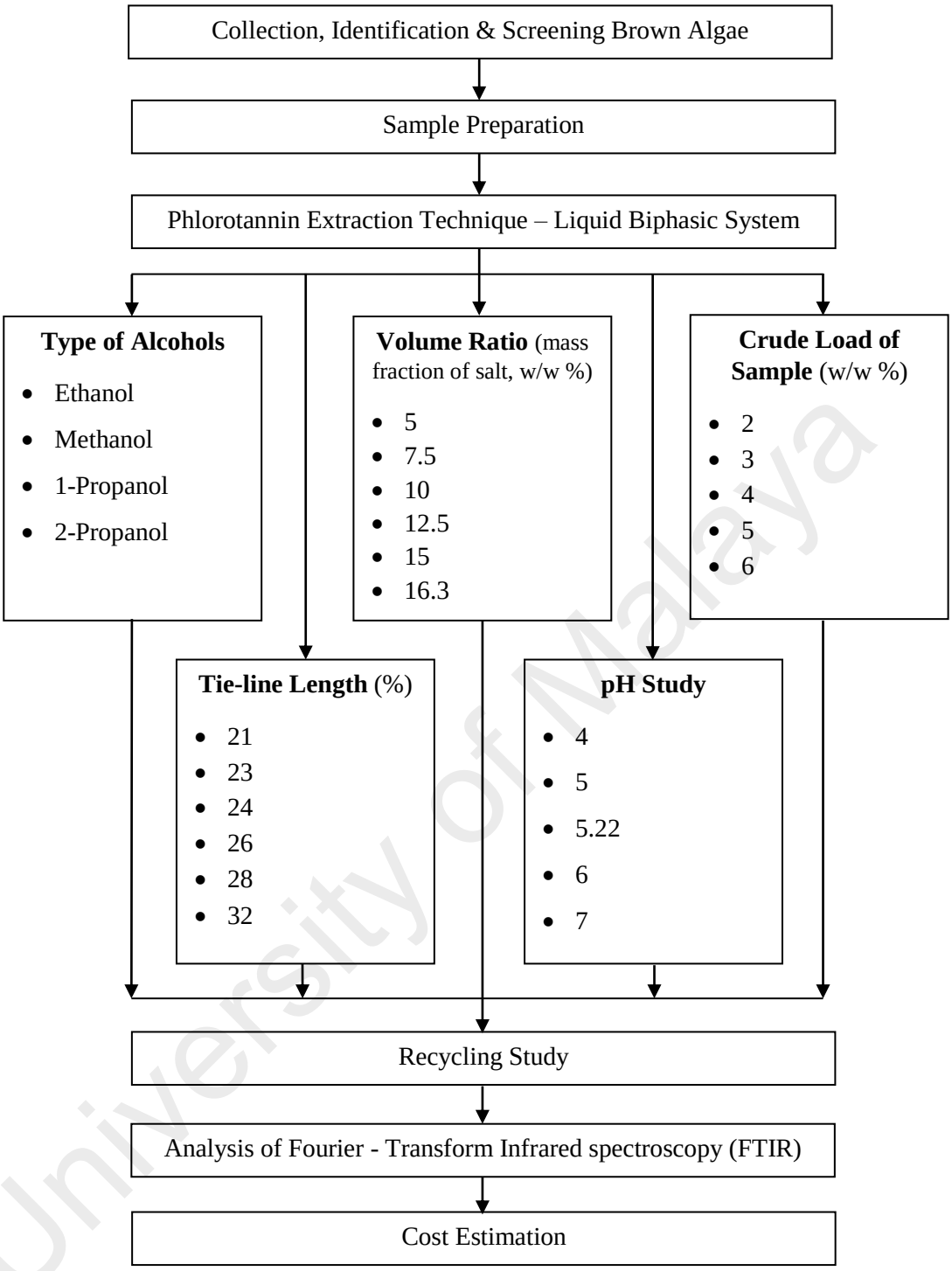


Figure 3.1 Research flow chart of current work

3.2 Partitioning of phlorotannin in LBS

The composition of LBS was prepared on weight percentage (%, w/w) basis according to the alcohol-ammonium sulphate phase diagram reported by Khayati and Shahriari (2016) (Khayati & Shahriari, 2016) and Wang et al. (2010) (Y. Wang et al., 2010). A concentrated stock solution of ammonium sulphate (40%, w/w) was prepared by dissolving appropriate quantity of chemicals in distilled water. The alcohol stock solutions solution like ethanol, methanol, 1-propanol and 2-propanol were prepared at a weight basis of 80% prior to usage. The LBS was prepared in a 1.5 mL centrifuge tube by adding the appropriate quantity of 80% (w/w) alcohol stock, 40% (w/w) $(NH_4)_2SO_4$ stock solution, distilled water and 5% (w/w) of samples to a final mass of 1.5 g. The components were mixed homogenously by gentle agitation and subjected to centrifugation. The mixture was centrifuged using a Sigma 1-14 microcentrifuge (Sartorius, Germany) at 4000 ×g for 10 min to ensure a complete separation. After phase separation, the top and bottom phases were collected and the concentration of phlorotannin within the mixture was analysed. All the experiments were performed triplicate at room temperature.

3.2.1 Tie-line Length (TLL)

The weight of sample biomass, alcohol solution (2-propanol), salt solution (ammonium sulphate) and total amount of the system were fixed for various tie-line length studied. This study was performed after examining the most suitable alcohol for phlorotannin extraction. The tie-line length of system was varied according to the phase diagram report by Khayati and Shahriari (2016). The tie-line length of system was calculated using the formula below:

$$TLL = \sqrt{\Delta X^2 + \Delta Y^2} \times 100\% \quad (4)$$

where ΔX and ΔY represent the difference of 2-propanol and ammonium sulphate in mass fraction.

The mass fraction of salt was fixed at 10% (w/w) whereas the mass fraction of alcohol was adjusted accordingly to obtain different TLL.

3.2.2 Volume Ratio

The volume ratio was performed by dividing the mass fraction of 2-propanol over mass fraction of ammonium sulphate solution in the system. The suitable TLL obtained in **Chapter 3.2.1** was used, 0.26 for *Padina sp.* and 0.24 for *Sargassum sp.* The range of volume ratio studied for *Padina sp.* and *Sargassum sp.* were dissimilar, such that the volume ratio of 1.04 to 9.50 was examined for *Padina sp.* and a range of 0.89 to 9.00 was determined for *Sargassum sp.*

3.2.3 pH Study

A range of pH 4 to 7 was examined to study the effect of acidic pH value to neutral pH value on phlorotannin extraction. The original pH value of system was determined using pH meter before any adjustment was made. The pH value without any adjustment is pH 5.22. The pH of the system was adjusted using sodium hydroxide and sulphuric acid to the desired pH values.

3.2.4 Crude Load of Sample

Various weight of biomass sample were weighed using an analytical balance for *Padina sp.* and *Sargassum sp.* A tweezers was used to pick up the small fragments of algae and put into the microtubes. The crude load of sample was studied in a range of 2% (w/w) to 6% (w/w) of the total system. The TLL, volume ratio and pH value that obtained highest recovery for *Padina sp.* and *Sargassum sp.* have been applied in this study, which were listed as followed:

Padina sp.: 0.26, 3.35 and pH 6

Sargassum sp.: 0.24, 2.00 and pH 6

3.3 Determination of phlorotannin content

The total phlorotannin content in brown macroalgae was examined by a modified Folin-Ciocalteu method described in the work by Ahmad et al. (2016) (Ahmad et al., 2016). A standard stock solution of phloroglucinol (20,000 mg L^{-1}) was prepared by dissolving 0.2 g of phloroglucinol into distilled water and made up to 10 mL of solution. Then, the stock solution was diluted into a range of 20 to 600 mg L^{-1} as working solutions, in order to produce a calibration curve to measure the phlorotannin concentration. The working solutions were prepared in a measurable range of Epoch microplate reader (BioTek, USA). Approximately 0.2 mL of sample was extracted from top and bottom phases and was mixed with 1.0 mL of 10% (v/v) Folin-Ciocalteu reagent (which the reagent was diluted with distilled water) in a test tube. The mixture was allowed to stand for 5 min and followed by the addition of 0.8 mL of 7.5% (w/v) Na_2CO_3 to the mixture. Each test tubes was cap-screwed and vortexed for 20 sec. The mixture was incubated at room temperature to react for 2 h in dark and measured at wavelength of 740 nm using microplate reader. The absorbance of the sample extract was measured against a blank sample which contained similar mixture without the sample extract. The concentration of the total phlorotannin content in sample extract was determined by comparing with the calibration curve of phloroglucinol.

3.4 Determination of phase volume ratio (V_R), purification factor (P_F), partition coefficient (K) and recovery (R%)

Phase volume ratio is the volume ratio of alcohol and salt phase after the centrifugation of system. The volume ratio (V_R) was calculated as the ratio of volume of the top phase (V_T) to the volume of the bottom phase (V_B) by using the Equation (5) as below:

$$V_R = \frac{V_T}{V_B} \quad (5)$$

The purification factor of the bottom phase was calculated by using the formula below:

$$P_F = \frac{SA\ of\ phase\ sample}{SA\ of\ crude\ feedstock} \quad (6)$$

where SA is the specific activity and P_F is the purification factor.

The partition coefficients of the phlorotannins between the phases were calculated as followed:

$$K = \frac{C_T}{C_B} \quad (7)$$

where C_T and C_B are equilibrium concentrations of the partitioned phlorotannin in the alcohol-rich top phase and the salt-rich bottom phase, respectively. The recovery of phlorotannin in the bottom phase was examined using the formula as followed:

$$R(\%) = \frac{100}{\left(1+\left[\frac{1}{V_R \times K}\right]\right)} \quad (8)$$

where K is the partition coefficient and V_R is the volume ratio.

3.5 Recycling Study

The recycling studies were performed in 50 mL test tubes, with known mass. A total mass of 20 g for both systems containing different macroalgae species were mixed, followed by centrifugation (Eppendorf Centrifuge 5430, Malaysia) at 4000 ×g for 10 min to separate the phases. The upper and bottom phases were separated and measured after extraction. Both phases were subjected to a recycle process. The alcohol was recovered from the remaining water by using Heidolph Rotary Evaporator, Laborota 4003 (Germany). The volume of recovered alcohol was recorded and its refractive index was measured. The yield of recovered 2-propanol was defined as followed:

$$Y_A = \frac{V_1}{V_0} \times 100\% \quad (9)$$

where V_1 and V_0 is the volume of 2-propanol recovered from the top phase and original volume of 2-propanol used in the system.

Recovery of salt was performed by adding methanol to the bottom phase (0.5 to 2 times the volume of bottom phase). The crystallized ammonium sulphate was recovered through filtration. The yield of ammonium sulphate recovered was defined as followed:

$$Y_S = \frac{M_1}{M_0} \times 100\% \quad (10)$$

where M_1 and M_0 is the mass of ammonium sulphate recovered from the bottom phase and original mass of ammonium sulphate used in the system.

3.6 Characterization of phlorotannin extract

The characterization of phlorotannin extract was performed using Fourier transform infrared spectroscopy (FTIR). The sample extracts were collected after the extraction using the optimized parameters from the studies. The sample extracts from *Padina sp.* and *Sargassum sp.* were pre-fractioned with methanol to induce the precipitation of the co-extracted carbohydrates, and subsequently concentrated in a Heidolph Rotary Evaporator, Laborota 4003 Control (Germany) at 40 ^{o}C. The operating pressure of rotary evaporator was below 0.1 mbar (Leyton et al., 2016). All the extracts were stored in 4 ^{o}C refrigerator immediately after evaporating the alcohols. The analysis was performed at room temperature and the IR spectra obtained for the sample extracts were recorded using Perkin Elmer IR spectrometer (Germany). The analysis was performed triplicate under similar extraction conditions. The region of 4,000 to 400 cm^{-1} was used for the analysis.

3.7 Cost Estimation

A comparison of cost estimation between this study and the study by Tierney et al. (2013) was discussed. The cost was estimated using the raw materials used, which were 2-propanol and ammonium sulphate for this study; ethanol for the study of Tierney et al. (2013) without including operating and capital cost. In this study, raw materials were purchased from local companies as mentioned in Section 3.1 and the price of raw materials was taken from the local company, R&M chemicals as standard price. The price was converted into USD for reference and below is the price list for raw materials calculated for this study:

Table 3.1: Price for raw materials in both studies

Raw Material	Unit	Price
Ammonium sulphate	1 kg	$ 12.5
2-propanol	2.5 L	$ 10
Ethanol	2.5 L	$ 17

The amount of raw material used in both studies was shown as below:

Table 3.2: Usage of raw materials in both studies

	Raw Material	Unit
Current Study	Ammonium sulphate	695.15 g
	2-propanol	1.39 L
Tierney et al.'s Study	Ethanol	5.13 L

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Selection of Alcohol/Salt LBS

Methanol, ethanol, 1-propanol and 2-propanol, were examined in extracting phlorotannin from macroalgae. In general, *Padina sp.* and *Sargassum sp.* have showed the highest affinity towards the combination of 2-propanol/ammonium sulphate system among all systems. Higher interaction of hydrogen bond between alcohol and water resulted in the lower solubility of targeted product in alcohol phase (Y. Wang et al., 2010). Therefore, phlorotannin was preferentially partitioned to the salt phase, which is the bottom phase of the system.

Table 4.1: Effect of alcohol types on the partition coefficient (K) of phlorotannin

Type of Alcohols	*Padina sp.*	*Sargassum sp.*
Methanol	0.324	0.101
Ethanol	0.559	0.529
1-Propanol	0.574	0.138
2-Propanol	0.534	0.100

The partition coefficient (K) indicates the solubility of phlorotannin in both alcohol and salt phase of the system. **Table 4.1** describes the K values of phlorotannin in different type of alcohols/ammonium sulphate systems. The lowest K value was observed in the combination of methanol with ammonium sulphate whereas similar partitioning behaviour was observed in other combinations for *Padina sp.* This was due to the precipitation of ammonium sulphate caused by methanol where similar phenomena were observed in *Sargassum sp*. Unlike *Padina sp.*, the phlorotannin in *Sargassum sp.* was partitioned easily in ethanol/ammonium sulphate system compared to other combinations. A big difference of K was observed between various systems. The recovery of phlorotannin was, however, not affected much by the K values as the 2-propanol/ammonium sulphate system was able to recover high amount of phlorotannin

in *Sargassum sp.* with the low K value obtained in **Figure 4.1**. Besides, the separation of each alcohol was obvious except in methanol where cloudy suspensions were formed. The separation between methanol and ammonium sulphate was faint, furthermore precipitation was occurred in methanol/ammonium sulphate system. It is not favourable as this might affect the product recovery. The highest K value was found in the 1-propanol/ammonium sulphate system for *Padina sp.*, while the highest K value for *Sargassum sp.* was the ethanol/ammonium sulphate system. The K values of phlorotannin from *Padina sp.* were generally higher than phlorotannin from *Sargassum sp.* in all the systems. This represents the concentration of phlorotannin in *Padina sp.* was higher in alcohols than in the salt phase and vice versa for *Sargassum sp.*

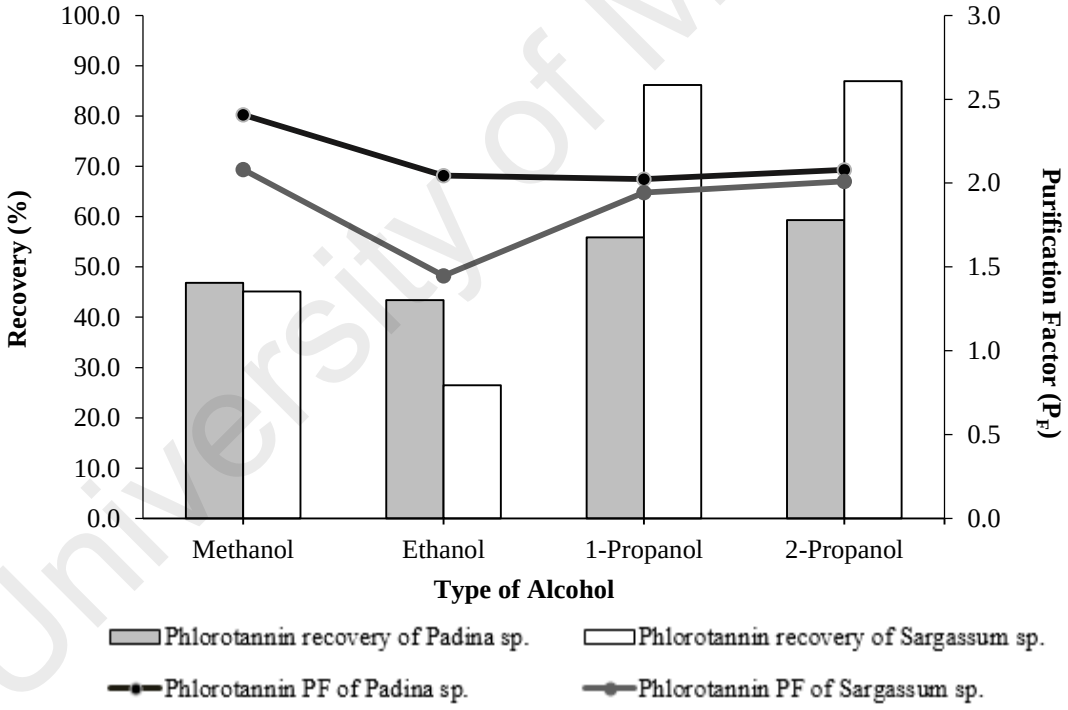


Figure 4.1: Effect of alcohol types on phlorotannin recovery and purification factor (P_F) at bottom phase

Apart from the partition behaviour, the recovery and purification factor (P_F) of phlorotannin were taken into consideration in this study. The correlation between the types of alcohol and the P_F and recovery of phlorotannin in bottom and top phase were illustrated in **Figure 4.1** and **Figure 4.2**, respectively. In **Figure 4.1**, 2-

propanol/ammonium sulphate system had the highest recovery of phlorotannin obtained from *Padina sp.* was 59.3%, which have comparable recovery with the combination of 1-propanol/ammonium sulphate system. However, the combinations of ethanol and methanol with ammonium sulphate recovered around 43 to 48% of phlorotannin respectively. The P_F calculated for all systems were in the range of 2.02 to 2.4, where there was only 0.38 difference between the different alcohol/salt combinations. There is only small difference between P_F in various systems due to the interaction of different alcohols with ammonium sulphate, causing different extraction rate and hence affecting the yield. The P_F of phlorotannin in methanol/ammonium sulphate was the highest amongst all systems for *Padina sp.*, although the second lowest recovery of phlorotannin was obtained using this system. This indicates that the phlorotannin obtained from this system had the highest purity among all other systems. The lowest P_F of phlorotannin was obtained with 1-propanol/ammonium sulphate system which had considerably high phlorotannin recovery. This may resulted by higher extraction efficiency of this system has extracted other biomolecules within the samples along with phlorotannin, resulting the low purity of phlorotannin obtained.

Higher recovery of phlorotannin was observed in *Sargassum sp.* than in *Padina sp.*, as around phlorotannin recovery of 86.9% was obtained as shown in **Figure 4.1**. The combination of 2-propanol and 1-propanol with ammonium sulphate had similar phlorotannin recovery compared to ethanol and methanol with ammonium sulphate systems. 2-propanol was chosen over 1-propanol due to its slightly higher recovery of product. Better recovery of phlorotannin was found in 1-propanol and 2-propanol because these alcohols are less polar than ethanol and methanol, resulting phlorotannin to be solubilised in the bottom phase. The P_F of 2-propanol in LBS was found to be the second highest, while that of methanol was the highest. The precipitation in the methanol/ammonium sulphate system did not affect the P_F of phlorotannin much. The

lowest P_F and recovery of phlorotannin was obtained in the ethanol/ammonium sulphate system while the highest P_F and recovery of phlorotannin was obtained in 2-propanol/ammonium sulphate system for *Sargassum sp*. A slightly wider range of P_F was observed in extracting phlorotannin from *Sargassum sp.*, which ranged from 1.4 to 2.1. The phlorotannin from *Sargassum sp.* at the bottom phase was able to be recovered more, although the highest P_F of phlorotannin was slightly lower than *Padina sp.* The systems using methanol, 1-propanol and 2-propanol had comparable P_F but the system with ethanol has much lower P_F of phlorotannin. However, the methanol/ammonium sulphate system was not chosen for both algae species as the amount of phase components reduced due to the precipitation, resulting in lower amount of recovered phlorotannin. The 2-propanol had higher recovery and P_F of phlorotannin due to the longer hydrocarbon chain than methanol, ethanol and 1-propanol. As the carbon chain increases, the polar OH group becomes smaller part in the molecule and the solubility of alcohol in water will decrease accordingly.

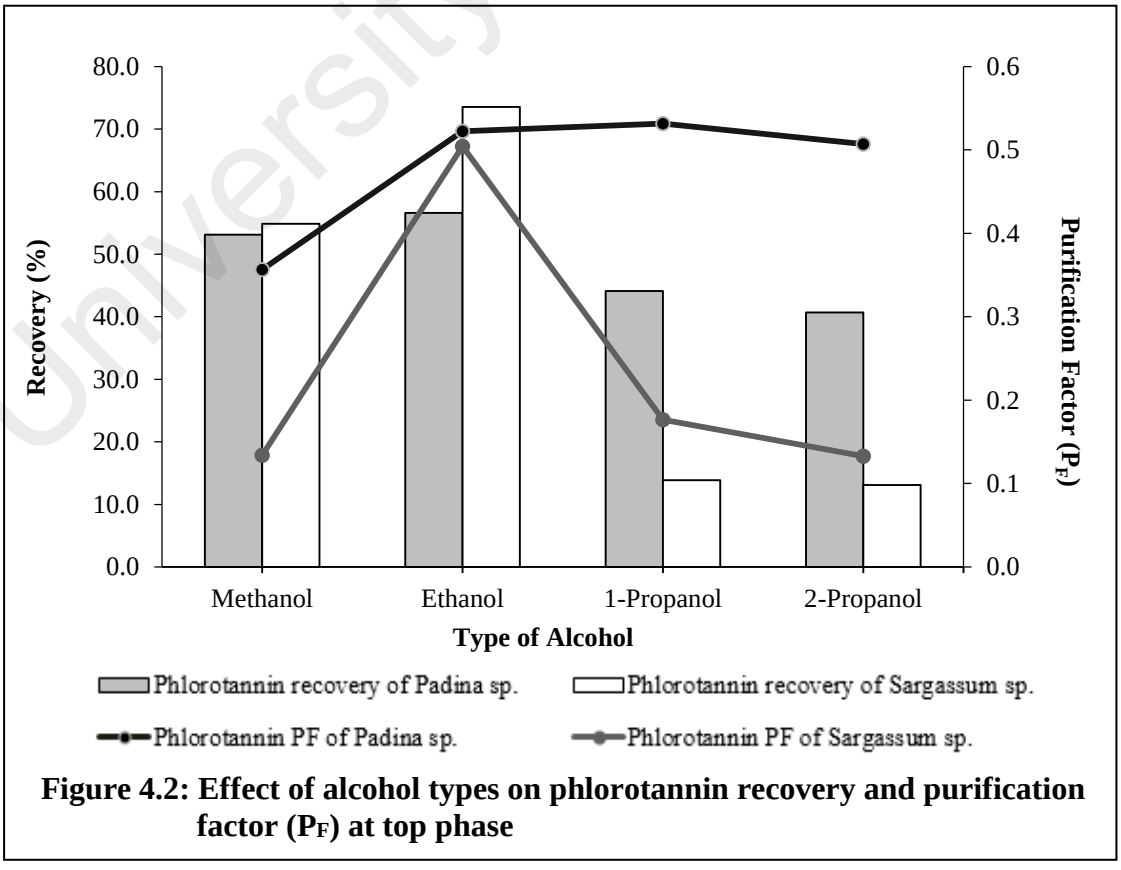


Figure 4.2: Effect of alcohol types on phlorotannin recovery and purification factor (P_F) at top phase

From **Figure 4.2**, comparable phlorotannin recovery at the top phase was observed for methanol and ethanol systems from *Padina sp.* The phlorotannin recovery fell between 40 and 56% and slight decrease yield of phlorotannin was observed in 1-propanol and 2-propanol/ammonium sulphate systems. This indicates the amounts of phlorotannin recovered using all systems were not different much. However, the P_F of phlorotannin from *Padina sp.* recovered from the methanol/ammonium sulphate system differed much from the other three systems, with the lowest value of 0.357. The range of phlorotannin P_F in *Padina sp.* was 0.357 to 0.532, with the highest P_F obtained in 1-propanol/ammonium sulphate system. By comparing the P_F of phlorotannin in top and bottom phase, the phlorotannin recovered in top phase for *Padina sp.* has lower P_F, and lower recovery of phlorotannin was obtained in top phase too. Therefore, the phlorotannin recovered from *Padina sp.* favoured the bottom phase rather than the top phase in the system.

For *Sargassum sp.*, the recovery of phlorotannin were over 50% for both methanol and ethanol/ammonium sulphate system. Nevertheless, the 1-propanol and 2-propanol/ammonium sulphate systems were only able to recover less than 20% of phlorotannin. The phlorotannin recovery through methanol/ammonium sulphate system was comparatively higher than 1-propanol and 2-propanol might be due to the increased volume of top phase after extraction. This phenomena was observed due to the partitioning of phlorotannin towards the bottom phase in 1-propanol and 2-propanol/ammonium sulphate systems, where more of phlorotannins were partitioned to the top phase of ethanol and methanol/ammonium sulphate systems. Besides, a significant difference of P_F at the top phase was observed by using various systems to recover the phlorotannin from *Sargassum sp.* The highest P_F of phlorotannin was obtained by using ethanol/ammonium sulphate system (0.504) whereas the P_F values around 0.13 to 0.17 were obtained by using the other three alcohols. By determining

both top and bottom phases of the system, the phlorotannin was found to be partitioned in the bottom phase rather than the top phase. The P_F of phlorotannin in bottom phase for both species were generally higher than P_F of top phase as well.

4.2 Tie-line Length (TLL)

Based on previous study, the LBS of 2-propanol and ammonium sulphate was chosen to determine the effect of tie-line length in recovering phlorotannin. The combination of 2-propanol and ammonium sulphate was chosen due to the high recovery of phlorotannin obtained compared to other systems. The mass fraction of 10% (w/w) salt was chosen as a fixed parameter, while the mass fraction of alcohol was altered accordingly in the system. The results related to the partition coefficient of phlorotannin from both species in the study of tie-line length was tabulated and shown in **Table 4.2**.

Table 4.2: Effect of TLL on partition coefficient (K) of phlorotannin for *Padina sp.* and *Sargassum sp.*

Mass fraction of alcohol (% w/w)	TLL (%)	*Padina sp.*	*Sargassum sp.*
28.5	21	0.461	0.124
30.0	23	0.410	0.149
32.0	24	0.567	0.053
33.5	26	0.370	0.112
35.5	28	0.384	0.125
40.0	32	0.506	0.104

The interaction of phlorotannin with 2-propanol could be observed through the K values calculated using the concentration of phlorotannin in alcohol and salt phase. The K values in *Padina sp.* were generally higher than those in *Sargassum sp.*, indicating that higher amount of phlorotannin was separated to the top phase in *Padina sp.* than to *Sargassum sp.* All the tie-lines were parallel to each other in the phase diagram reported

by Khayati & Shahriari (2016). A system with similar tie-line length may result in different partitioning coefficient due to the type of sample. The highest K value of 0.567 was found in *Padina sp.* using 32% (w/w) of alcohol and 0.149 was found in *Sargassum sp.* using 30% (w/w) of alcohol. The results indicate that lower mass fraction of alcohol is required to partition phlorotannin from *Sargassum sp.* compared to *Padina sp.* to obtain the highest K value within the selected range of TLL. A comparable K value of phlorotannin (0.506) from *Padina sp.* was observed with the highest K value (0.567) using 32% (w/w) of alcohol. The K value of phlorotannin from both species varied with different TLL. It is concluded that the mass fraction of alcohol did affect the partitioning effect of phlorotannin in the system.

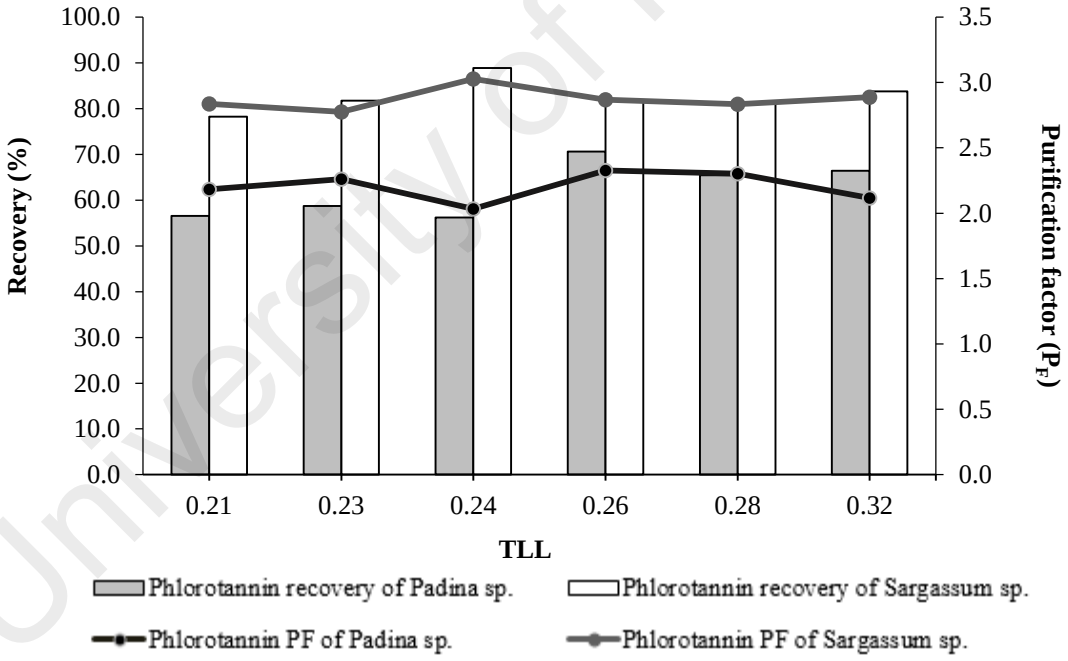


Figure 4.3: Effect of TLL on phlorotannin recovery and purification factor (P_F) at bottom phase

Padina sp. had the highest phlorotannin recovery of 71% and purification factor using the mass fraction of 33.5% (w/w) 2-propanol. With the increase of alcohol fraction in the system, the phlorotannin recovery in the bottom phase increased as well. The increment of the 2-propanol concentration represents that more water molecules are

needed around the molecules of 2-propanol in the top phase. Hence, higher amount of phlorotannin was partitioned in the bottom phase, resulting in the higher phlorotannin recovery (Tianwei et al., 2002). The recovery of phlorotannin started to decrease along the higher mass fraction of alcohol than 33.5% (w/w). Comparing to *Padina sp.*, the values of phlorotannin recovery from *Sargassum sp.* with different tie-lines were higher than those of *Padina sp.* The highest phlorotannin recovery of 89% was obtained in the system with 32% (w/w) of 2-propanol is around 89%. The phlorotannin recovery was fluctuating along the increment of alcohol mass fraction. However, similar phlorotannin recovery was observed at 30, 33.5 and 35.5% (w/w) 2-propanol. The P_F of *Sargassum sp.* was higher than *Padina sp.*, ranging from 2.77 to 3.02.

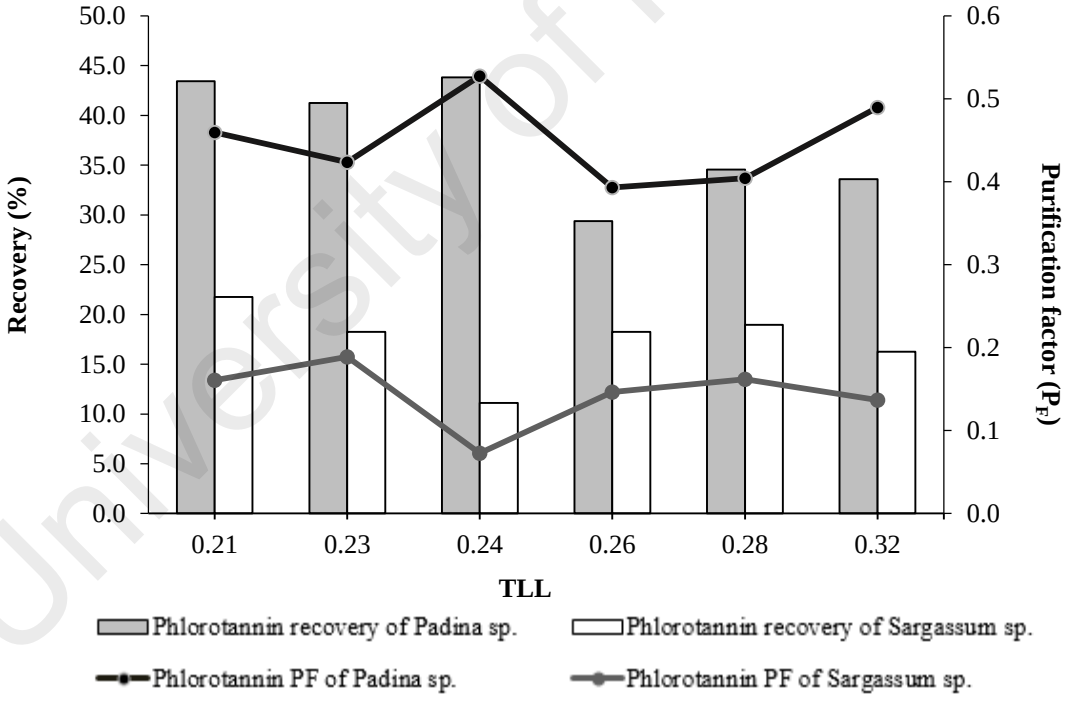


Figure 4.4: Effect of TLL on phlorotannin recovery and purification factor (P_F) at top phase

From **Figure 4.4**, the highest recovery and P_F of phlorotannin in *Padina sp.* were obtained through the system containing 32% (w/w) of 2-propanol, which was different from the results obtained at the bottom phase of the system. The lowest recovery and P_F of phlorotannin was obtained in the system with 33.5% (w/w) of alcohol. For

Sargassum sp., the highest recovery of phlorotannin was obtained with least mass fraction of alcohols while the highest P_F of phlorotannin was obtained with 30% (w/w) of alcohol. The least amount of recovered phlorotannin from *Sargassum sp.* was partitioned to the top phase with 32% (w/w) of alcohol. The P_F of phlorotannin in *Padina sp.* ranged from 0.4 to 0.52, whereas the P_F of phlorotannin in *Sargassum sp.* ranged from 0.07 to 0.19.

4.3 Volume Ratio

With the optimum TLL determined in the previous study (26% for *Padina sp.* and 24% for *Sargassum sp.*), different volume ratios were studied which aided to optimise the phlorotannin recovery. In this study, the mass fraction of ammonium sulphate was ranged from 5% to 16.3% (w/w) for both species. The mass fraction of alcohol chosen for *Padina sp.* was ranged from 17 to 47.5% (w/w) whereas a range from 14.5 to 45% (w/w) of alcohol was chosen for *Sargassum sp.* to form the system. The value of volume ratio is calculated as mass fraction of alcohol over mass fraction of salt.

Table 4.3: Effect of volume ratio on partition coefficient (K) of phlorotannin for *Padina sp.* and *Sargassum sp.*

Mass fraction of salt (% w/w)	Mass fraction of alcohol (% w/w)		Volume Ratio		Partition Coefficient (K)	
	Padina sp.	***Sargassum sp.***	***Padina sp.***	***Sargassum sp.***	***Padina sp.***	***Sargassum sp.***
5	47.5	45.0	9.50	9.00	0.391	0.050
7.5	41.0	38.0	5.47	5.07	0.356	0.081
10	33.5	32.0	3.35	3.20	0.367	0.199
12.5	27.0	25.0	2.16	2.00	0.567	0.084
15	20.0	18.0	1.33	1.20	1.009	1.029
16.3	17.0	14.5	1.04	0.89	0.905	0.864

In term of K value, a wide range was obtained for both species of algae and tabulated in **Table 4.3**. The K values ranging from 0.356 to 1.009 for *Padina sp.* and K values of

0.05 to 1.029 for *Sargassum sp.* were obtained. Higher amount of phlorotannin was attracted to the top phase using the system of 15% (w/w) of salt and 20% (w/w) of 2-propanol for *Padina sp.* However, the phlorotannin recovery in *Sargassum sp.* favoured to the system consisting of 15% (w/w) of salt and 18% (w/w) of alcohol. The K values of the chosen volume ratio for next study are 0.367 for *Padina sp.* and 0.084 for *Sargassum sp.* The optimum condition for both species was chose based on the phlorotannin recovery and P_F. The volume ratio of the systems has been chosen to be studied in the next parameter are 3.35 for *Padina sp.* and 2.00 for *Sargassum sp.*

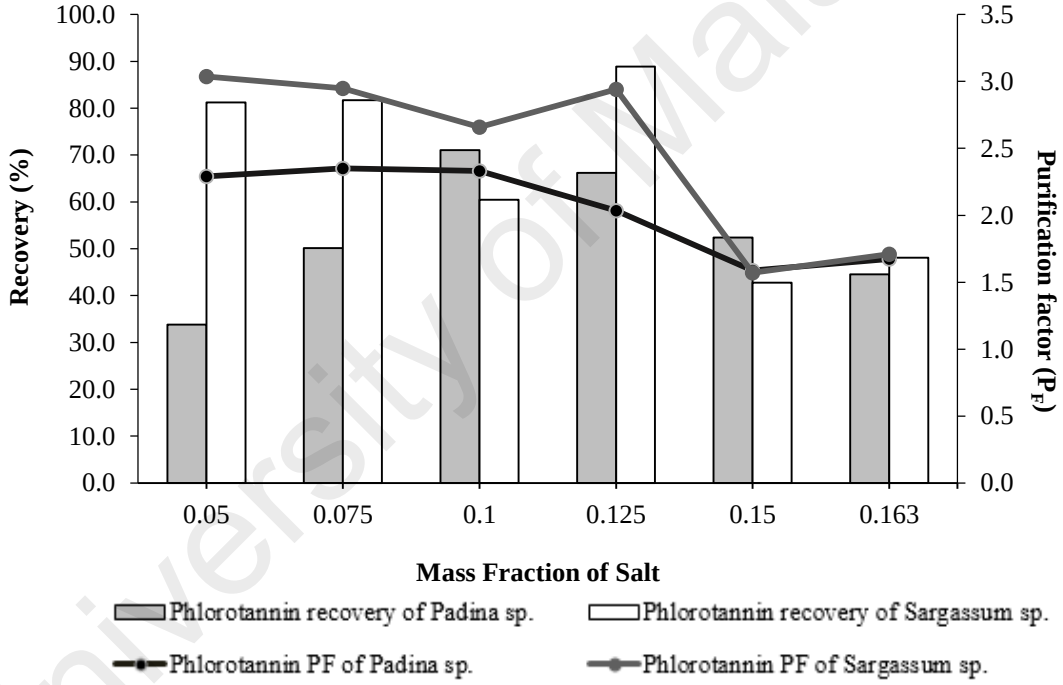


Figure 4.5: Effect of volume ratio on phlorotannin recovery and purification factor (P_F) at bottom phase

The effect of volume ratio of alcohol to salt on the P_F and phlorotannin recovery at the bottom phase was shown in **Figure 4.5**. As the mass fraction of alcohol decreased, the mass fraction of salt added into the system increased. However, the increment of bottom phase volume did not necessarily increase the phlorotannin recovery for both brown algae species. The phlorotannin recovery was decreased after reaching0.1 mass fraction of salt (*Padina sp.)* and 0.125 mass fraction of salt (*Sargassum sp.)* in the

system. The highest phlorotannin recovery of 71% in *Padina sp.* was obtained from 33.5% (w/w) of alcohol and 10% (w/w) of salt LBS along with a P_F of 2.33. For *Sargassum sp.*, the highest recovery of 88.85% was achieved in a system containing 25% (w/w) of alcohol and 12.5% (w/w) of salt. The P_F obtained at this volume ratio was the highest as well. The lowest recovery and P_F of phlorotannin were found in system which consisted of the highest salt mass fraction and lowest alcohol mass fraction. Gradual decrement of phlorotannin recovery was observed in higher mass fraction of salt, e.g. 12.5 and 15% (w/w) in the system. These results can be explained by the "ion-dipole" interactions between water molecules and salts that led to the hydration of ions, and influence the solubility of target product in bottom phase (Y. Wang et al., 2010).

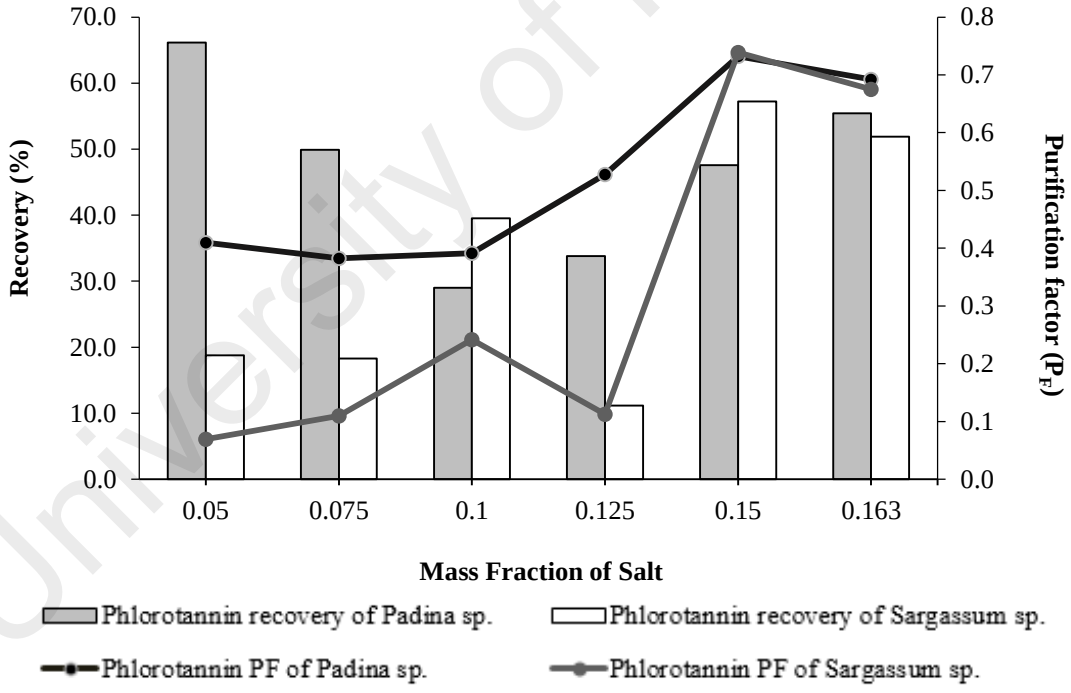


Figure 4.6: Effect of volume ratio on phlorotannin recovery and purification factor (P_F) at top phase

The P_F and recovery of phlorotannin at the top phase were illustrated in **Figure 4.6**. In **Figure 4.6**, the highest recovery of phlorotannin was obtained at 5% (w/w) of salt and the highest P_F was obtained at 15% (w/w) of salt for *Padina sp.* The lowest recovery of phlorotannin was obtained with considerably low P_F of phlorotannin with 10% (w/w)

of salt. The recovery of phlorotannin was increased from 33.8% to 47.6% using 12.5% (w/w) of salt and 15% (w/w) of salt, which corresponded to the P_F value of 0.53 to 0.73 respectively. However, the systems obtained higher recovery of phlorotannin from *Padina sp.* was not able to obtain high P_F of phlorotannin.

For *Sargassum sp.*, similar P_F of phlorotannin were obtained in the systems consisting of 15% and 16.3% (w/w) of salt. The recovery of phlorotannin from *Sargassum sp.* was generally lower than *Padina sp.*, despite the systems consisted of similar mass fraction of salt. The lowest recovery of phlorotannin was obtained at 12.5% (w/w) of salt and the highest recovery was obtained at 15% (w/w) of salt. The gradual increment of phlorotannin recovery was coupled with increasing purity of phlorotannin especially in the system with 15% (w/w) of salt. It was observed that the systems with higher mass fraction of salt (15% and 16.3% w/w) had recovered more than 50% of phlorotannin in *Sargassum sp.*, whereas the highest and lowest mass fraction of salt had recovered more phlorotannin in *Padina sp.*

4.4 pH Study

This study was performed by using the volume ratio of 3.35 and 2, for *Padina sp.* and *Sargassum sp.*, respectively, which was determined in previous section. The pH value of the system was believed to influence the partition coefficient (K), purification factor (P_F) and recovery of phlorotannin. The chemicals used to adjust pH to base or acid neutralization are sodium hydroxide and sulphuric acid. In **Table 4.4**, the K values of phlorotannin at different pH were shown. The pH values chosen were neither very acidic such as pH 2 nor very alkaline pH like pH 10, as the extreme pH might result in the denaturation of phlorotannin. Hence, the pH in the system ranged from 4 to 7 at 298 K. The pH without adjustment (pH 5.22) was considered in this study as well. The K values of phlorotannin recovered from *Padina sp.* were generally higher than the K

values of phlorotannin recovered from *Sargassum sp.* The K values in the pH study were obtained as low as 0.34 in *Padina sp.* and around 0.1 in *Sargassum sp.*

Table 4.4: Effect of pH value on the partition coefficient (K) of phlorotannin for *Padina sp.* and *Sargassum sp.*

pH Value	*Padina sp.*	*Sargassum sp.*
4	0.670	0.093
5	0.515	0.169
5.22	0.454	0.291
6	0.339	0.095
7	0.456	0.125

The highest K values were obtained at pH 4 for *Padina sp.* and pH 5.22 for *Sargassum sp.* The exact values were 0.67 and 0.29 for *Padina sp.* and *Sargassum sp.*, respectively. This indicates the partitioning behaviour of phlorotannin was favourable under slightly acidic condition for both species of algae. Phlorotannin recovery and P_F were favourable at pH 6 in the bottom phase of LBS for both brown algae species. pH 6 was the optimum pH value for the extraction. This was probably due to the positive and negative potential of the top and bottom phase, respectively (Cheng et al., 2016). Most of the phenolics present amphoterism, which can react as acid in presence of a base and as a base in presence of an acid. The phlorotannin compounds absorbed H^+ and carried positive charge. Positively charged compounds partition to the lower salt phase, therefore the phlorotannin was recovered and purified in the bottom phase. If the targeted compound is negatively charged, the compound would be partitioned to the top phase, which is alcohol-rich phase (Y. Wang et al., 2010).

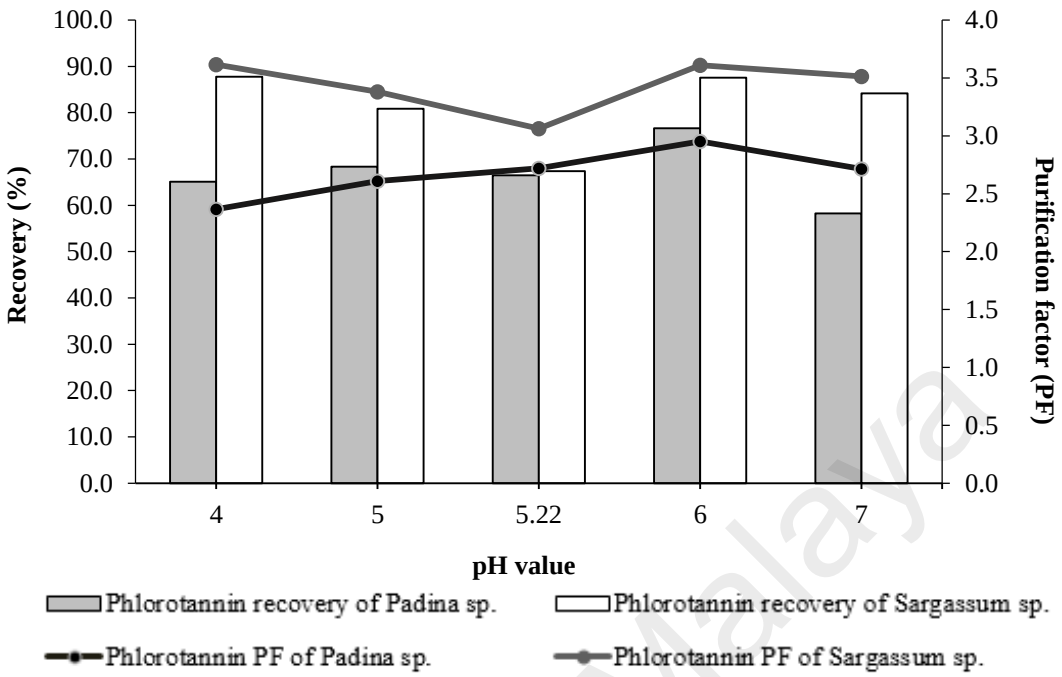


Figure 4.7: Effect of pH value on phlorotannin recovery and purification factor (P_F) at bottom phase

Optimum pH value obtained for *Padina sp.* was pH 6 where the recovery and purification factor were outstanding compared to other pH values as shown in **Figure 4.7**. The phlorotannin recovery and P_F were decreased at slightly more alkaline pH (pH 7). As in *Sargassum sp.*, the recovery was found to be lowest at pH 5.22, while comparable recovery was observed at other pH. However, lowest K values were observed in both species of macroalgae at pH 6. The P_F of phlorotannin obtained was similar for the pH range tested. The pH values plays a role in partition of targeted product by affecting the electrical charge form of targeted product, which is negative, positive or neutral charge. As the electrical charge form of targeted product changes, the solubility of targeted product in bottom phase will alter subsequently based on the hydrophobicity or hydrophilicity of targeted product (Y. Wang et al., 2010).

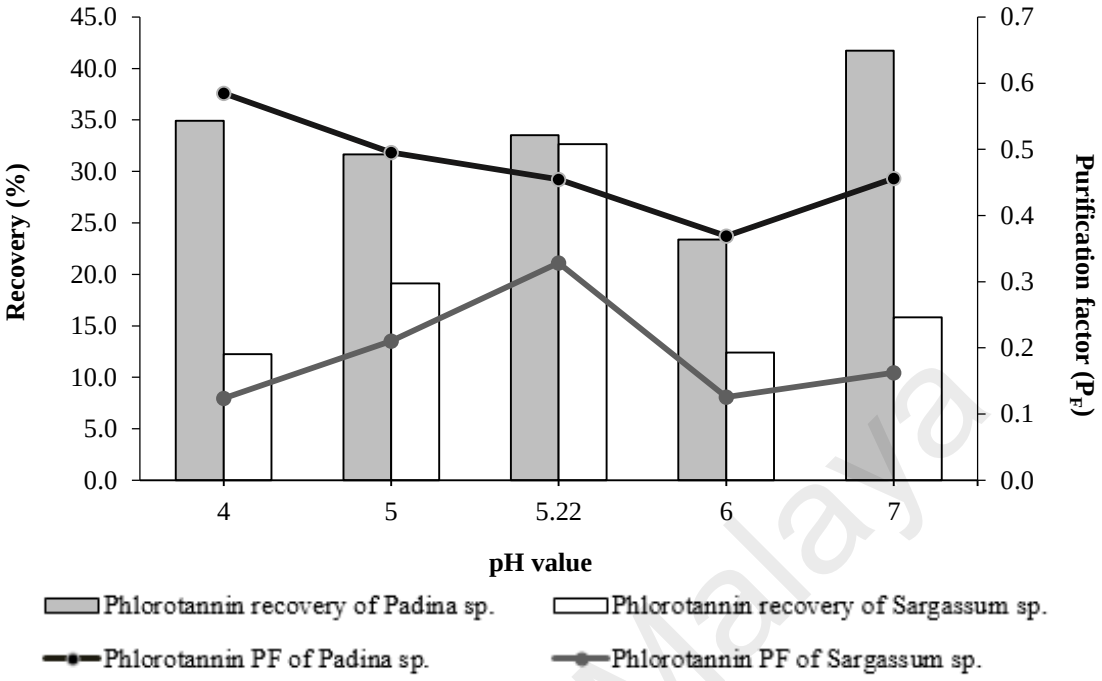


Figure 4.8: Effect of pH value on phlorotannin recovery and purification factor (P_F) at top phase

The phlorotannin recovery and P_F at the top phase from both species were shown in **Figure 4.8**. The highest recovery of phlorotannin for *Padina sp.* was obtained at a neutral pH while the system at slightly acidic pH was also able to recover comparable amount of phlorotannin from *Padina sp.* The lowest recovery of phlorotannin was observed at pH 6 with the lowest P_F of phlorotannin as well, which was dissimilar to the results obtained at bottom phase. Apart from *Padina sp.*, the recoveries of phlorotannin from *Sargassum sp.* were generally lower than 35%, where the solubility of phlorotannin to the top phase was highly unfavourable at the tested pH. The P_F of phlorotannin ranged from 0.124 to 0.328, where the highest P_F value and recovery were obtained at unadjusted pH. The charge of phlorotannin may have altered at different pH, resulting in the decreased solubility of phlorotannin in more alkaline solutions for both algae species. In summary, the partition behaviour of phlorotannin was significant influenced by pH of the LBS and the pH 6 was shown to be the optimal pH for both species as highest phlorotannin recovery and purification factor were obtained.

4.5 Crude Load of Sample

The weight percentage of the algae in system was varied in this study using the optimised tie-line length, volume ratio and pH value from the previous studies. The weight percentage pf biomass ranging from 2 to 6% (w/w) in the system was tested for *Padina sp.* and *Sargassum sp*. 5% (w/w) was the weight percentage of sample used in the previous studies. Therefore, lower weight percentage of sample and slightly higher weight percentage of sample were tested to optimise the system. The effect of algae weight percent on the partition coefficient (K) of phlorotannin was tabulated in **Table 4.5**.

Table 4.5: Effect of crude load of sample on the partition coefficient (K) of phlorotannin for *Padina sp.* and *Sargassum sp.*

Weight percentage of sample	*Padina sp.*	*Sargassum sp.*
2	0.777	1.000
3	0.337	0.540
4	0.689	0.067
5	0.283	0.137
6	0.475	0.089

It was found that the phlorotannin was partitioned easily at lower sample loading for both species of algae. The K values for *Padina sp.* could be as low as 0.28 to as high as 0.78. The highest K value of *Padina sp.* was obtained at 2% (w/w) of sample, where K value was 0.78, with a considerably low recovery percentage of phlorotannin, 41.53% as shown in **Figure 4.9**. The K values of phlorotannin might be varied due to the different composition which had been altered with the increasing or decreasing weight percentage of sample in the system. The extraction process may be influenced by the sample weightage as the area of biomass sample exposed to the solvent (alcohols and salt) was varied with different crude load of sample. On the other hand, the *Sargassum sp.* had obtained a higher K value than *Padina sp.* at weight percents of 2 and 4¸ but the

average K value was lower than *Padina sp.* The results explained that the higher amount of phlorotannin from *Sargassum sp.* solubilised in the bottom phase more than top phase compared to *Padina sp.* Unlike *Padina sp.*, the highest K value of samples in *Sargassum sp.* was having the highest phlorotannin recovery, where K value was 1.00 with 91.67% of phlorotannin recovery as shown in **Figure 4.9**. Furthermore, the K value of phlorotannin from *Sargassum sp.* varied much for the system consists of 4% (w/w) and 6% (w/w) sample compared to other weight percentage of samples.

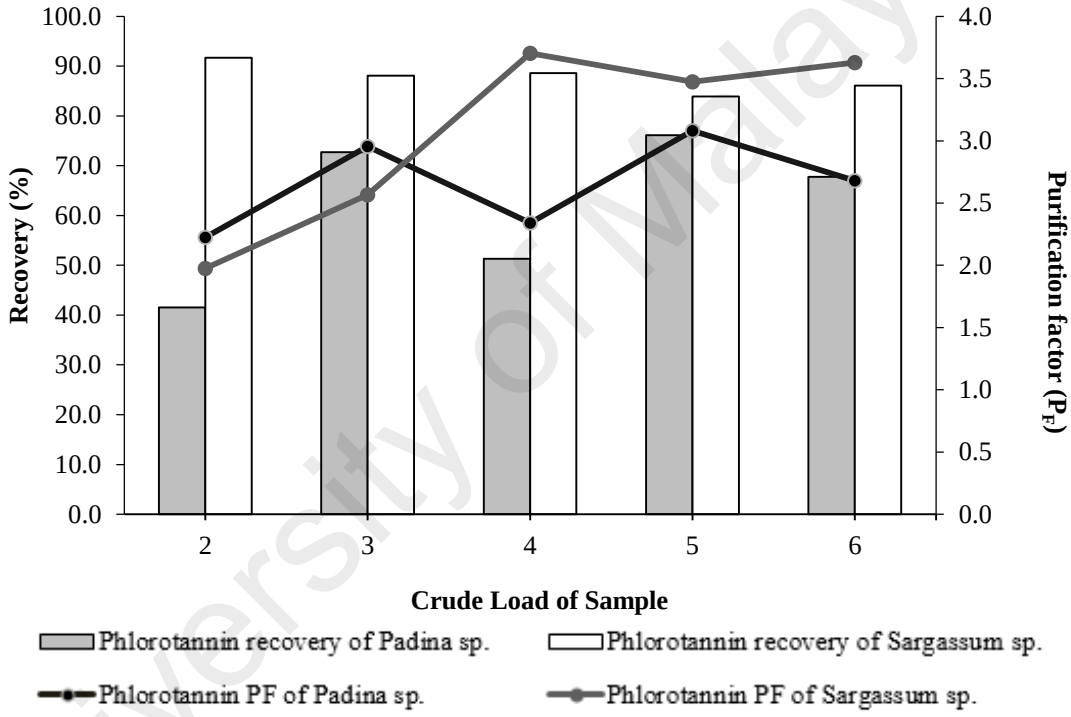


Figure 4.9: Effect of crude load of sample on phlorotannin recovery and purification factor (P_F) at bottom phase

From **Figure 4.9**, the crude load of 5% (w/w) was found to be producing the highest recovery with 76.1% of phlorotannin in *Padina sp.* at bottom phase. Moreover, the highest recovery at this weight percent was coupled with the highest P_F of phlorotannin. It was observed that the P_F was slightly decreased from 2.49 to 2.16 and 2.99 to 2.8 with increasing sample loading for *Padina sp.* and *Sargassum sp.*, respectively. This might be due to the higher the amount of biomass sample, the harder to purify phlorotannin by the system. Unlike *Padina sp.*, the lowest sample loading (2% w/w) of *Sargassum sp.*

had recovered the highest yield of phlorotannin (91.67%) and lowest P_F (1.59). However, the recovery percentage of phlorotannin was having insignificant difference along with the increment of sample concentration, ranging from 83.9% to 91.67%. The P_F in *Sargassum sp.* were increased gradually from 2 to 4% (w/w) and slightly decreased at 5 and 6% (w/w). In summary, the recovery yield of phlorotannin and P_F of phlorotannin in *Sargassum sp.* were higher than the recovery yield and P_F of phlorotannin in *Padina sp.*

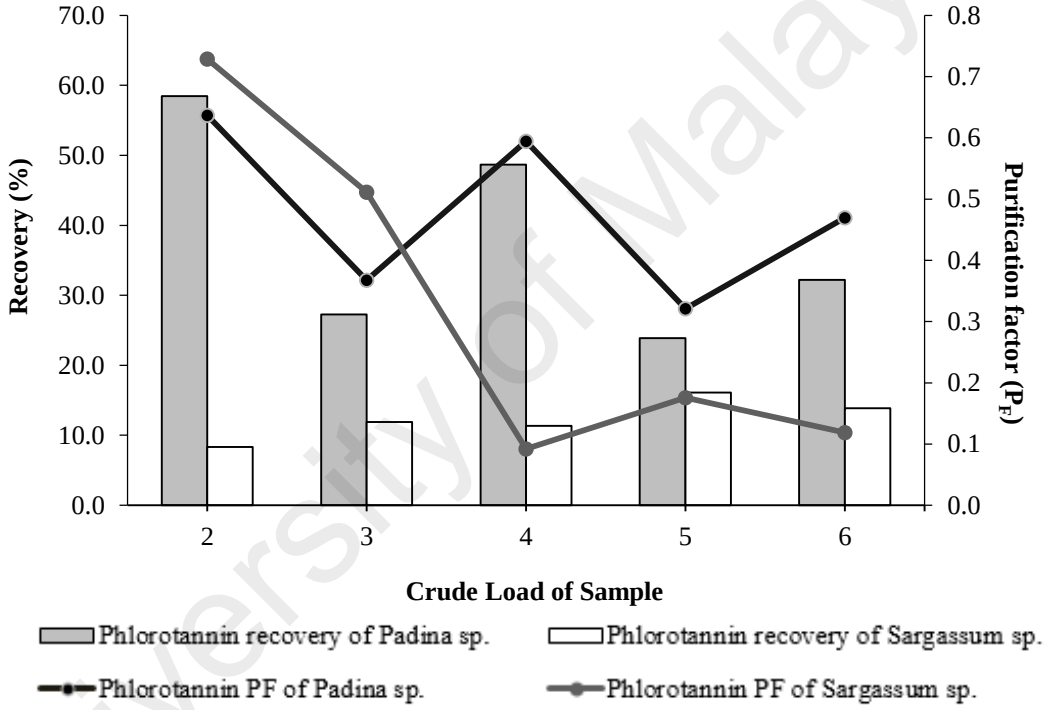


Figure 4.10: Effect of crude load of sample on phlorotannin recovery and purification factor (P_F) at top phase

From **Figure 4.10**, the best sample weightage to recover phlorotannin from *Padina sp.* at top phase was 2% (w/w) to obtain highest recovery and P_F, whereas the phlorotannin recovery from *Sargassum sp.* were similar of all sample weightage. The P_F of phlorotannin in *Sargassum sp.* were varied much compared to *Padina sp.* and the recovery of phlorotannin in *Padina sp.* were higher than *Sargassum sp.* The lowest P_F of phlorotannin was obtained at 5% (w/w) for *Padina sp.* and 4% (w/w) for *Sargassum sp.*

In conclusion, the optimum sample loading for *Padina sp.* was 5% (w/w) and 2% (w/w) for *Sargassum sp.*

4.6 Recycling Study

This study was performed by the system with the pH value of 5.22, 5 and 2% (w/w) of sample concentration for *Padina sp.* and *Sargassum sp.*, respectively; whereas 10% (w/w) of salt and 33.5% (w/w) of alcohol for *Padina sp.*; 12.5% (w/w) of salt and 25% (w/w) of alcohol were for *Sargassum sp.* From **Table 4.6**, the phlorotannin recovered from *Padina sp.* was similar for first extraction, first recycling and second recycling of salt phase. On the other hand, the phlorotannin recovery from *Sargassum sp.* slightly varied in times of recycling bottom phase. The yields of salt recovered in *Sargassum sp.* were generally higher than *Padina sp.* for all the recycled bottom phases. Overall, the amount of phlorotannin could be recovered from *Sargassum sp.* was higher than *Padina sp.*

Table 4.6: Recycling of salt phase

Macroalgae species	Times of Recycling	Recovery (%)	Amount of Recovered Salt (g)	Yield of Salt Recovered (%)
Padina sp.	First Extraction	44.31	0.82	41.04
	Recycle I	44.33	0.44	22.08
	Recycle II	44.60	0.69	34.51
Sargassum sp.	First Extraction	73.72	1.81	72.39
	Recycle I	65.84	0.91	36.32
	Recycle II	84.28	1.31	52.52

However, the amount of salt recovered from the bottom phase was not similar for both algae species. The salt recovered from first extraction was higher than the salt recovered from bottom phase of first recycling. The decrement of salt recovery after first recycling experiment may be due to less addition of fresh salt solution (ammonium sulphate) into the system. For the second recycling of salt phase, more salt was

recovered because higher amount of fresh salt solution was added into the system compared to first recycling of experiment. Overall, the percentage of salt recovered from first extraction was found to be highest compared to the recycled phases. The salt phase is encouraged to recycle for once only as recycling the salt phase for second time will require similar amount of salt solution to the first extraction. Apart from the salt phase of LBS, the importance of alcohol phase had to consider as well. Although the mass fraction of alcohol phase utilised in the system was higher than the salt phase, only small amount of alcohol can be recycled (around 2 ml out of 17.5 ml). The amount of recycled alcohol was not sufficient for another cycle of evaporation, therefore the alcohol was only recycled once. A total yield of 25% 2-propanol has been recovered using rotary evaporator from the top phase of LBS. The recycling of alcohol phase requires further investigation in order to observe the recyclability of top phase.

4.7 Characterization of Phlorotannin Extract

The phlorotannin extracts were characterized through the analysis of Fourier Transform Infrared spectroscopy (FTIR) as this is a common method to characterise phlorotannin (Girija et al., 2013). The result obtained by Leyton et al. (2016) was shown in **Figure 4.11** as a comparison to the result of current study.

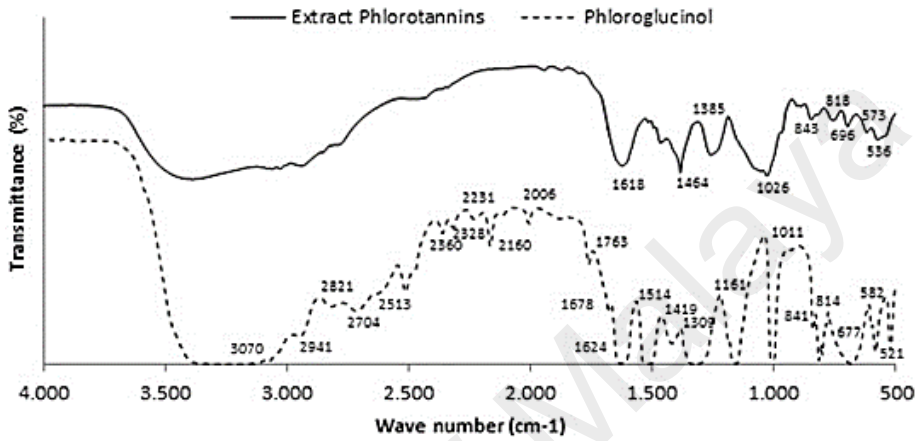


Figure 4.11: FTIR analysis of phloroglucinol and phlorotannin extracts from *Mycrocystis pyrifera* (Leyton et al. 2016)

The chemical composition of phlorotannin extracted from *Padina sp.* and *Sargassum sp.* under optimal condition were shown in **Figure 4.12** and **Figure 4.13**. The analysis of FTIR was performed using water phlorotannin extracts from *Padina sp.*, *Sargassum sp.* and *Mycrocystis sp.* Similar peaks range was observed for both phlorotannin extracts, where the major peaks existed in between 3845 and 424 cm^{-1}. There were 13 major peaks observed for phlorotannin extracts from *Padina sp.*, whereas the extracts from *Sargassum sp.* gave rise to nineteen major peaks within the range. The stretching bands around 1470 to 1450 cm^{-1} were corresponding to the aromatic nuclei in both phlorotannin extracts. This result is in agreement with the results obtained by Leyton et al. (2016). In phlorotannin extracts, the peaks in between 3420 and 3250 cm^{-1} were indicating the –OH bonds in phenols, which caused by the stretching vibration of OH bond. On the other hand, the bands at 1101.11 and 1097. 97 cm^{-1} of phlorotannin extracted from *Padina sp.* and *Sargassum sp.*, respectively, corresponded to the

glycosidic linkage vibrations of C-O-C, indicating the presence of some carbohydrates in the extracts, which is similar to past studies. The stretch vibration around 1015 cm^{-1} for both extracts indicates C-O bond stretching, representing the presence of some 2-propanols in the sample extracts. Phlorotannin extracts from both species showed the ring in benzene derivatives with medium strength of vibrations at 580 to 420 cm^{-1} where the in-plane and out-of-plane ring deformations with two bands were observed. The study by Leyton et al. (2016) was presenting the characteristic stretch band of carboxyl groups at a peak of 536 cm^{-1}, falls into the same range of this study (Leyton et al., 2016). The strong intensity of C=O stretch in benzophenones was observed as well at the wavenumber of 1646.56 cm^{-1} for *Sargassum sp.* and the C=O stretch was observed at 1636.36 cm^{-1} for *Padina sp.* The peaks in the range of 2000 to 1650 cm^{-1} indicate that several bands from combinations bands, where substituted benzene rings were present in the sample extracts. Phlorotannin extracts from both species have similar peaks; however, phlorotannin extract from *Sargassum sp.* has more peaks in the range of 2000 to 1400 cm^{-1} compare to *Padina sp.* Overall, the phlorotannin extracts in current study have more peaks compared to the results obtained in Leyton et al. (2016).

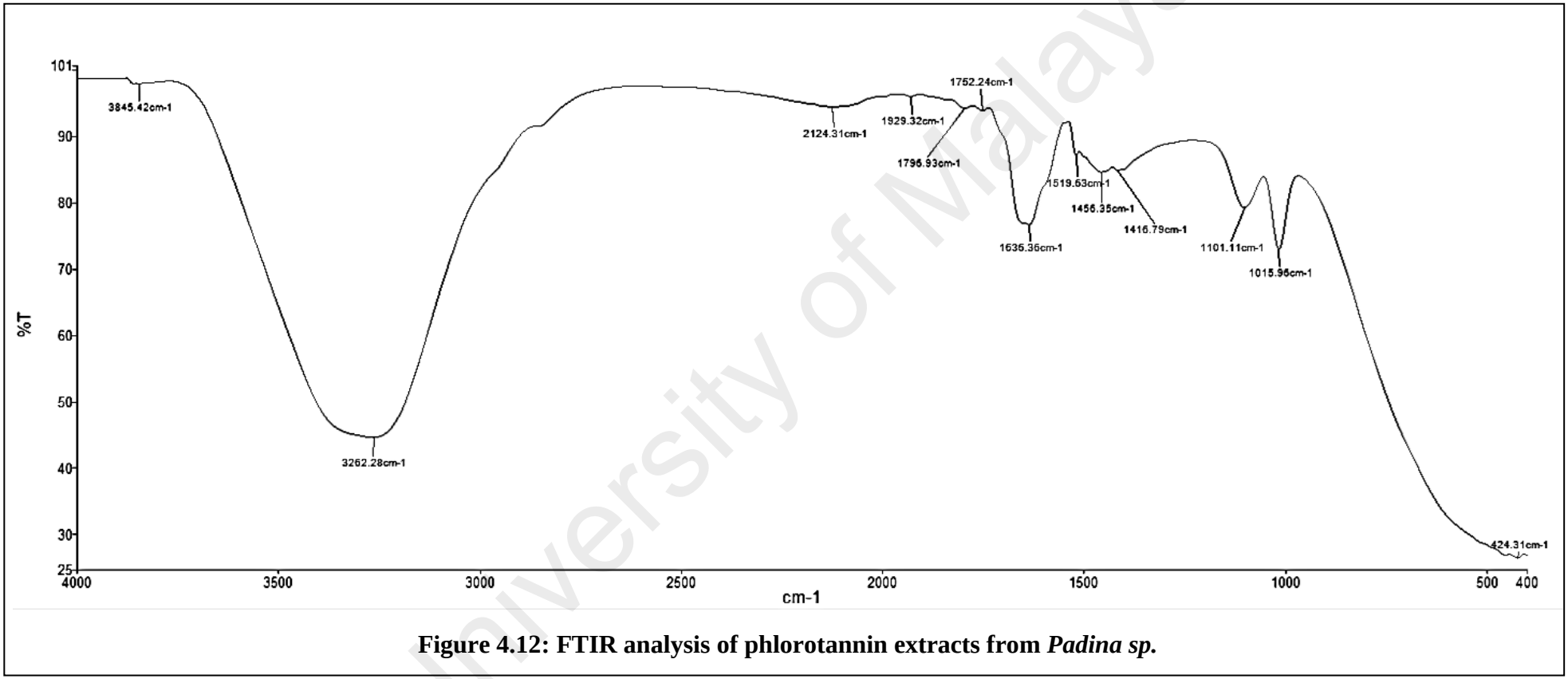


Figure 4.12: FTIR analysis of phlorotannin extracts from *Padina sp.*

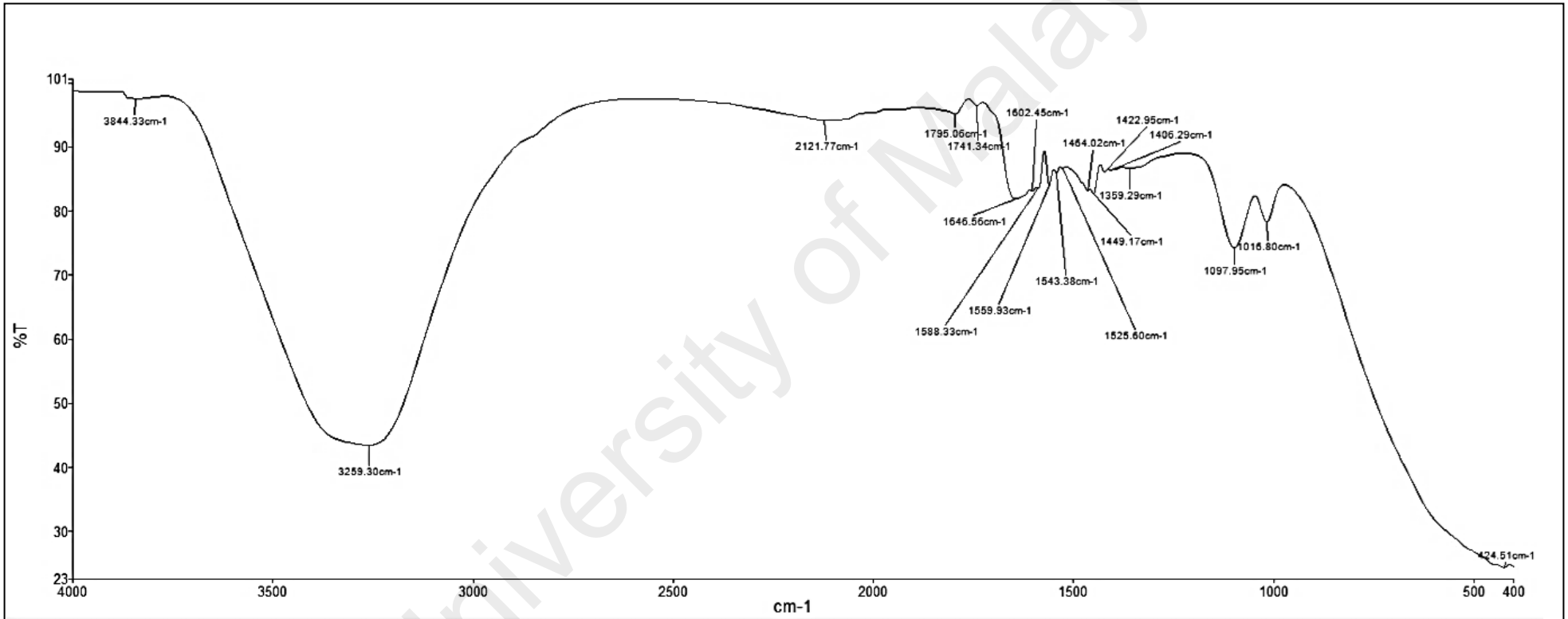


Figure 4.13: FTIR analysis of phlorotannin extracts from *Sargassum sp.*

4.8 Cost Analysis

The cost analysis of phlorotannin extraction was performed by estimating the cost needed in this study and compared with the study using solid-liquid extraction (SLE) by Tierney et al. (2013) (Tierney et al., 2013). The simple cost estimation was conducted based on the optimum extraction conditions of phlorotannin using both the LBS and solid liquid extraction (SLE) processes. The cost estimation for LBS mainly consisted of the raw materials prices since there was little involvement of expensive pre-treatment and operation processes in the extraction process. In current study, 2-propanol was chosen as the raw material due to the better yield obtained among alcohols studied. The cost of 2-propanol purchased from a local company was $4 per litre and the cost of ammonium sulphate was $12.5 per kg. To produce 1 g of phlorotannin from the crude stock using LBS, the total cost of $14.25 was required for purchasing 1.39 L of 2-propanol and 695.15 g of ammonium sulphate.

In solid liquid extraction (SLE), the samples were subjected to freeze drying pre-treatments before extraction. An estimation was performed according to the solvent used based on the yield of phlorotannin extracted. The yield of phlorotannin extracted was 35.86 μg per mg of sample extract. It was estimated that a total volume of ethanol ranging from 5.13 L was needed to extract 1 g of phlorotannin. The cost of ethanol purchased from local company was $6.8 per litre; hence the price of $35.03 will be required for purchasing ethanol. By comparing the methods, the total cost of LBS is about 2.45 times cheaper than SLE based on estimations of the amount of solvent used. Further cost additions from the energy consumed by pretreatment processes in SLE will also elevate the total cost needed for phlorotannin production. The higher yield of phlorotannin from LBS and the simplicity of its operation show that LBS is a feasible technique with low cost operation.

CHAPTER 5: CONCLUSION

The scope of this research has been achieved, which is to discover an energy-efficient extraction technique for isolating phlorotannin from algae. It is also concluded that the three objectives proposed in Section 1.3 have been successfully completed within this work. The objectives were achieved as followed:

1. An eco-friendly and time saving technique for phlorotannin extraction from algae was developed. The technique is liquid biphasic system (LBS) which utilized combination of alcohols and salt to isolate phlorotannin from brown algae. LBS is a green technique as the phase components used could be recycled (as shown in Section 4.6) and have short processing time.

2. The maximum yield and high purity of phlorotannin have been obtained through the optimization of LBS parameters. The extraction and purification of phlorotannin from *Padina australis* and *Sargassum binderi* were performed by using alcohol/ammonium sulphate LBS. The 2-propanol/ammonium sulphate LBS was found to be the most suitable phase system for both brown algae. The optimum result for *Padina sp.* through the studies was achieved by LBS was the condition with following parameters: 33.5% (w/w) of 2-propanol, 10% (w/w) of ammonium sulphate, pH of 6 and sample loading of 5% (w/w). For *Sargassum sp.*, the optimum condition for obtaining phlorotannin was as followed: 25% (w/w) of 2-propanol, 12.5% (w/w) of ammonium sulphate, pH 6 and 2% (w/w) of sample loading.

3. The cost efficiency and feasibility of LBS were analysed and discussed. LBS was highly suggested for phlorotannin extraction due to high efficiency and low cost required. This showed that LBS is feasible to be applied in phlorotannin extraction for pharmaceutical application. In addition, the system is easy to scale up for future studies and large scale production. The recycling of

salt phase could be recycled for once to cut cost and not for twice as the fresh salt solution required in second recycle was similar with first extraction.

In conclusion, the high valuable biomolecule, phlorotannin has been isolated from the brown algae, *Padina sp.* and *Sargassum sp.* The studied system in this work is small compared to industrial scale. A scaled-up system can be studied as a future work to verify the compatibility and feasibility of LBS for large production purpose. Besides, the algae biomass after extraction can be utilized as the source for bioenergy production such as biochar and bioethanol via pyrolysis and fermentation in future study.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of Publications

1) Chia, S. R., Ong, H. C., Chew, K. W., Show, P. L., Phang, S. M., Ling, T. C., Nagarajan, D., Lee, D. J., Chang, J. S. (2018). Sustainable approaches for algae utilisation in bioenergy production. Renewable Energy, 129, 838-852.
2) Chia, S. R., Chew, K. W., Show, P. L., Yap, Y. J., Ong, H. C., Ling, T. C., Chang, J. S. (2018). Analysis of economic and environmental aspects of microalgae biorefinery for biofuels production: a review. Biotechnology Journal, 1700618.
3) Chia, S. R., Ong, H. C., Show, P. L., Phang, S. M., Ling, T. C. (2018). Sustainable approach in phlorotannin recovery from macroalgae. Journal of Bioscience and Bioengineering, 126 (2), 220-225.

List of Conference Proceedings

1) "Bioactive compounds from marine macroalgae"- Postgraduate Colloquium of Environmental Research 2017 (POCER)
2) "Phlorotannin extraction from brown algae"- 4th International Conference of Energy Materials and Environment Engineering 2018 (ICEMEE)