EVALUATION OF ANTIOXIDANT PROPERTY OF

PURIFIED PHYCOBILIPROTEINS AND PHENOLIC

COMPOUNDS CONTAINING EXTRACTS FROM

BANGIA ATROPURPUREA

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By

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ABSTRACT

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RAJALAKSHMI PUNAMPALAM

Bangia atropurpurea is a freshwater red filamentous alga. It is one of the fast-growing algae with survival capacity. *B. atropurpurea* has high adaptation to a broad range of salinities over time and is able to tolerate desiccation and osmotic stress where other filamentous algae do not typically grow. The antioxidant property of this red alga was compared with *Chlorella vulgaris*, a freshwater green microalga. *C. vulgaris* is similar to most phototrophs as it absorbs light via the chloroplast to synthesise organic compounds for nutrition. In this study, the recovery yield of purified phycobiliproteins extracted from *B. atropurpurea* was evaluated and compared the antioxidant capacity with ascorbic acid, butylated hydroxytoluene (BHT) and phenolics extracted from *B. atropurpurea* and *C. vulgaris*. The crude extract of phycobiliproteins from *B. atropurpurea*

was purified by $(NH_4)_2SO_4$ saturation before further fractionation of the phycobiliproteins extract to R-phycoerythrin (R-PE), R-phycocyanin (R-PC) and allophycocyanin (APC) by gel filtration with Sephadex G-200. The separated R-PE (bright pinkish) and R-PC (purplish blue) proteins were identified by RP-HPLC and SDS-PAGE while APC was untraceable after gel filtration. The percentage of recovery yield of R-PE and R-PC from total protein extracted from *B. atropurpurea* increased proportionally with the purity index after each subsequent purification process. The recovery yields (%) of R-PE and R-PC after RP-HPLC were 94.4% at purity index (A_{562}/A_{280}) of 5.42 and 86.1% at purity index (A_{615}/A_{280}) of 3.95, respectively. A total of 85.9 mg of R-PE and 44.2 mg of R-PC were separated by RP-HPLC from 50 g of *B. atropurpurea* while from the total phycobiliproteins recovered, 66% was R-PE and 34% was R-PC. Therefore, R-PE is the predominant phycobiliprotein in *B. atropurpurea*. Phenolic compounds were extracted with solvents of different polarity. The Folin-Ciocalteu method was used to determine the TPC while 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric-reducing antioxidant power (FRAP) assays were used to determine the antioxidant capacity of the extracted phycobiliproteins and phenolic compounds. The phenolic compounds have high solubility in methanol solvent compared to other solvents used for extraction. The TPC in the methanol extract from *B. atropurpurea* (80.97 ± 0.53 mg GAE/g dry weight) was higher than *C. vulgaris* (62.13 ± 1.28 mg GAE/g dry weight). Similarly, the phenolics extracted from *B. atropurpurea* and *C. vulgaris* using methanol exhibited effective DPPH radical scavenging with the lowest IC_{50} ($30.82 \pm 0.92\,\mu g/mL$ and $34.28 \pm 0.79\,\mu g/mL$) and the highest FRAP (37.81 ± 0.04 mg GAE/g dry weight and 23.97 ± 0.61 mg GAE/g dry weight),

respectively. Analysis of the correlations between TPC and the antioxidant property measured by DPPH radical scavenging and FRAP assays showed good correlations with higher regression coefficient, $R^2 = 0.898$ and $R^2 = 0.925$, respectively. These data suggest that phenolic compounds are powerful free radical scavengers and effective metal ion reducing agents, however, this study has justified that the phenolic compounds were not the only contributor to the antioxidant capacity of this red alga. The purified R-PE and R-PC have contributed significantly in DPPH radical scavenging and metal ion reduction activity. The purified R-PE (IC_{50}, 7.66 ± 0.81 µg/mL) exhibited better radical scavenging activity compared to R-PC (IC_{50}, 9.42 ± 1.73 µg/mL), phenolic compounds in methanol extract (IC_{50}, 30.82 ± 0.92 µg/mL) and the synthetic antioxidant BHT (IC_{50}, 35.06 ± 1.15 µg/mL) while lower radical scavenging activity compared to ascorbic acid (IC_{50}, 6.78 ± 0.28 µg/mL). R-PE also exhibited higher FRAP (54.81 ± 0.31 mg GAE/g dry weight) compared to R-PC (42.18 ± 0.70 mg GAE/g dry weight), phenolic compounds in methanol extract (37.81 ± 0.04 mg GAE/g dry weight) and BHT (30.37 ± 0.12 mg GAE/g) while lower FRAP compared to ascorbic acid (65.77 ± 0.12 mg GAE/g). SDS-PAGE of purified R-PE and R-PC proteins showed single narrow bands at molecular weight of 20.5 kDa and 17.6 kDa, respectively. The findings of this study supported that *B. atropurpurea* could be a promising new source of potential antioxidants to replace the synthetic antioxidants used in food and pharmaceutical products due to its natural, non-toxic antioxidant capacity contributed by R-PE, R-PC and phenolics extract.

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This dissertation entitled "**EVALUATION OF ANTIOXIDANT PROPERTY OF PURIFIED PHYCOBILIPROTEINS AND PHENOLIC COMPOUNDS CONTAINING EXTRACTS FROM *BANGIA ATROPURPUREA***" was prepared by RAJALAKSHMI A/P PUNAMPALAM and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified that **Rajalakshmi A/P Punampalam** (ID No: **12ADM01364)** has completed this dissertation entitled "EVALUATION OF ANTIOXIDANT PROPERTY OF PURIFIED PHYCOBILIPROTEINS AND PHENOLIC COMPOUNDS CONTAINING EXTRACTS FROM *BANGIA ATROPURPUREA*" under the supervision of Dr. Khoo Kong Soo (Supervisor) from the Department of Chemical Science, Faculty of Science, and Dr. Sit Nam Weng (Co-Supervisor) from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(Rajalakshmi A/P Punampalam)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(RAJALAKSHMI A/P PUNAMPALAM)

Date _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

v/v	Volume per volume
w/v	Weight per volume
kDa	Kilodalton
g	Gravitational force
mL	Millilitre
μg	Microgram
μm	Micrometre
mM	Millimolar
nm	Nanometre
AEAC	Ascorbic acid equivalent antioxidant capacity
ANOVA	Analysis of variance
APC	Allophycocyanin
BSA	Bovine serum albumin
BHT	Butylated hydroxytoluene
DPPH	1,1-Diphenyl-2-picrylhydrazyl
Df	Degrees of freedom
DW	Dry weight
F-crit	Critical value of F
FRAP	Ferric-reducing antioxidant power
GAE	Gallic acid equivalent
IC_{50}	50% Inhibition concentration
MS	Mean square

PDA	Photodiode array
R^2	Regression coefficient
Rm	Relative mobility
R-PE	R-phycoerythrin
R-PC	R-phycocyanin
Sp.	Species
SS	Sum of squares
TFA	Trifluoroacetic acid
TPC	Total phenolic content
UV	Ultraviolet
A_{max}	Maximum absorbance
α	Alpha
β	Beta
γ	Gamma
ε	Epsilon
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

Bangia atropurpurea is a red filamentous alga from the family of Bangiaceae found in freshwater environments of many regions around the world, including Antarctica, Asia, Europe, and North America (Guiry, 2015). Earlier studies by Stewart and Lowe (2008) on *B. atropurpurea* showed that this species can adapt to a broad range of salinities over time and is able to tolerate desiccation and osmotic stress; these traits allow *B. atropurpurea* to the high survival *in vivo* where other filamentous algae do not typically grow. Though there is a study done on the production and survival capacity of *B. atropurpurea*, there was lack of information on the phytochemicals and biological activities of this alga species. Therefore, analysis of the antioxidant activities of phenolic compounds and phycobiliproteins extracts from *B. atropurpurea* is among the objectives of this present study. *Chlorella vulgaris* is a freshwater green microalga that has been widely analysed on biodiesel production due to its high lipid content (Mata et al., 2010; Lenka et al., 2015). Since *C. vulgaris* has easily breakable cell wall, hence it became the most preferred species among researchers to study on its properties. The phenolic compounds were extracted from *B. atropurpurea* and *C. vulgaris* to estimate the total phenolic content (TPC) and compare the antioxidant capacity of the extracts.

Phenolic compounds serve as antioxidants because of their ability to donate the hydrogen atoms or the electrons from the benzene rings and hydroxyls attached in their molecular structure to form a stable radical intermediate to retard the reactive oxygen species (ROS) (Mathew et al., 2015). The natural phenolic compounds from different families for example hydroxyphenyl, polyphenol, hydroxybenzoic, and phenylpropenoic have different solubility level (Singh et al., 2014) hence the TPC in *B. atropurpurea* and *C. vulgaris* were measured using five different extraction solvents with different polarities to determine the most suitable solvent for maximum phenolics recovery yield. The solubility of the phenolic compounds was not only influenced by the size and extent of the hydrogen bonding but also the energy associated with their crystal structures (Antonio et al., 2009). The TPC of algae extracts was determined by the Folin-Ciocalteu method (Andressa et al., 2013).

Other than phenolic compounds, phycobiliproteins were also extracted and evaluated for their antioxidant capacity in this research study. The aggregates of pigmented phycobiliproteins compose a water-soluble phycobilisome structure which is attached to the thylakoid membrane of the algae (Su et al., 2010). Each phycobiliprotein is connected by non-pigmented linker polypeptides which hold the phycobilisome structure firmly (Liu et al., 2005). Phycobiliproteins such as phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) are vital to absorb light energy while, the non-pigmented linker polypeptides are essential for the stability and assembly of the complex (Anderson and Grossman, 1990). The light-harvesting chromophores confer the pigments on their characteristic colours; red for PE while blue for PC

and APC (Glazer et al., 1975). According to Glazer et al. (1976), the intensely coloured PE, PC and APC proteins were identified at maximum absorption wavelength, λ_{max} ~ 562 nm, ~ 620 nm and ~ 650 nm, respectively.

PE, PC and APC comprise two non-identical polypeptide subunits, α (MW 12-19 kDa) and β (MW 14-21 kDa). Each phycobiliprotein contains one or more covalently linked open-chain tetrapyrrole chromophores (Glazer et al., 1976). These chains are generally organised in trimeric $(\alpha\beta)_3$ discs but larger phycobiliproteins aggregate in hexameric $(\alpha\beta)_6$ disc arrangement (MacColl, 1998). In PE of red algae, there are special polypeptides, designed for γ subunits with a molecular weight of MW 30-33 kDa, which are structurally different from the α and β subunits (Takemoto and Bogorad, 1975).

Phycobiliproteins combat ROS by different mechanisms associated with side chains of various constituting amino acids. The amino acids with hydrophobic side chain are good metal ion chelators and proton donors (Aftabuddin and Kundu, 2007). In ferric-reducing antioxidant power (FRAP) assay, the aromatic amino acids reduce the ferric ion to ferrous ion very efficiently by donating electron from the aromatic ring (Sarmadi and Ismail, 2010). On the other hand, the acidic property of amino acids contributes to the donation of hydrogen ions to scavenge 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals in DPPH assay analysis (Hwang and Thi, 2014). This indicates the combined involvement of both metal ions in reducing ability and the hydrogen ion donating ability of amino acids in phycobiliproteins to amplify the antioxidant activity.

Phycobiliproteins and phenolic compounds are significant antioxidants to retard oxidation process by scavenging free radicals or by acting as electron donors to suppress the progress of free radical-associated diseases (Valavanidis et al., 2009). To date, there have been considerable increases in the occurrence of oxidative stress-related diseases such as neurodegenerative diseases, atherosclerosis, arthritis, diabetes and cancer (Valavanidis et al., 2009; Samoylenko et al., 2013; Di Meo et al., 2016). Therefore, this study aimed to achieve the following objectives:

(a)   Extract the TPC and evaluate its antioxidant activity using five different extraction solvents (water, 50% aqueous methanol, methanol, ethyl acetate and hexane) from two different algae species (*B. atropurpurea* and *C. vulgaris*) using DPPH radical scavenging assay and FRAP assay.

(b)   Purify, identify and quantify the R-PE, R-PC and APC from *B. atropurpurea* by gel filtration, HPLC and SDS-PAGE.

(c)   Evaluate the antioxidant activity of the isolated R-PE, R-PC and APC from *B. atropurpurea* using DPPH radical scavenging assay and FRAP assay.

(d)   Compare and correlate the antioxidant activity of the extracted R-PE, R-PC, APC, phenolic compounds and synthetic antioxidants (ascorbic acid and BHT) using DPPH radical scavenging assay and FRAP assay.

The data gathered from this study will contribute towards a significant replacement for the needs of natural antioxidants in a large scale for health benefits. Many food industries use synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG)

and tert-butyl hydroquinone (TBHQ) as food additives. Studies have shown that the synthetic antioxidants are toxic and leave side effects in long-term usage, which increase the consumers' anxiety on the safety of food additives (Witschi et al., 1977; Witschi, 1986; Bauer et al., 2001). Replacing synthetic antioxidants with natural antioxidants may benefit the consumers via health implications and functionality.

CHAPTER 2

LITERATURE REVIEW

2.1   Algae

Algae are organisms ranging from unicellular microalgae to multicellular forms. The largest and most complex marine algae are called seaweeds. Algae are aquatic plants but lack of stomata and vascular tissues like xylem and phloem for water, gas and minerals transportation such as those found in plants on the land. Algae have phycobiliproteins and chlorophyll to absorb light and can make their own food through the process of photosynthesis. In this study, the bioactive compounds of *B. atropurpurea* (red alga) and *C. vulgaris* (green alga) were extracted and the antioxidant property of the extracts was analysed.

B. atropurpurea is a red filamentous alga (Figure 2.1). The *B. atropurpurea* cell contains chloroplast and thylakoid membrane, which are the common characteristics of the Rhodophyta division (Wright et al., 2003). The average diameter of *B. atropurpurea* filament is approximately 75 µm and the spore is 15.5 µm in diameter (Gargiulo et al., 2001). *B. atropurpurea* has small thalli with rapid growth and high reproductive output. *B. atropurpurea* exhibits behaviour characteristic of R-selected species. R-selected species is characterised by the production of numerous small offsprings in an exponential population growth. R-selected species require a very short gestation period and

mature quickly with minimum parental care. Light is a basic necessity for the

R-selected species spore germination (Spitale et al., 2012).

Figure 2.1: The alga *B. atropurpurea* in microscopic view. Image was derived from Cantonati and Lowe (2014).

Besides *B. atropurpurea,* another algal species *C. vulgaris* was extracted and

the TPC and the antioxidant capacity were evaluated and compared with those

of *B. atropurpurea*. *C. vulgaris* is a well-known green algal species (Figure 2.2)

for its high lipid content, which has been discovered to be most ideal microalga

for biofuel production due to its high growth rate (Griffiths and Harrison, 2009).

C. vulgaris is a green eukaryotic microalga from the family of Chlorellaceae

(Table 2.1). This organism is a unicellular microalga and has spherical cells with

the diameter of 2 to 10 µm (Liu et al., 2015).
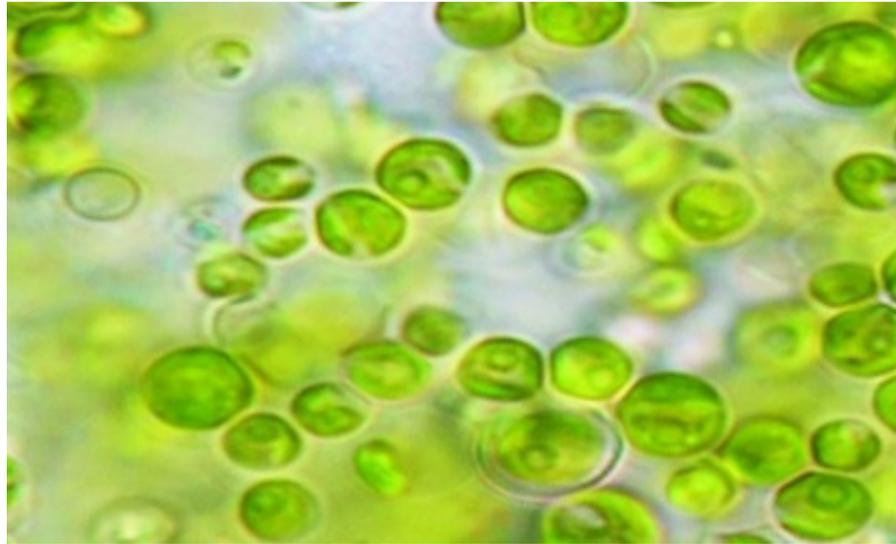
Figure 2.2: *C. vulgaris* in microscopic view. Image was derived from Shen et al. (2014).

Table 2.1: The scientific classification of the two algae studied.

	Chlorella vulgaris (Green alga)	*Bangia atropurpurea* (Red alga)
Domain	Eukaryota	Eukaryota
Kingdom	Plantae	Plantae
Division	Chloropyta	Rhodophyta
Class	Trebouxiophyceae	Bangiophyceae
Order	Chlorellales	Bangiales
Phylum	Chlorophyta	Rhodophyta

2.1.1 Red Algae

Red algae in general, show a wide range of colours from red, bright pink, dark purplish-brown to almost black. The range of colours is a result of the presence of two extra pigments namely phycoerythrin (red) and phycocyanin (blue) that mask the green chlorophyll and other photosynthetic pigments.

Red algae are usually smaller, generally ranging from microscopic size to about a meter in length (Johnston et al., 2014). Red algae are characterised by their high concentration of fibre and minerals and in certain algae, relatively high protein levels. Red algae contain various bioactive compounds such as polyphenols, carotenoids and tocopherols that can benefit the human health. Consumption of red algae including *B. atropurpurea* increases the intake of natural minerals that lower the occurrence of cancer cell growth and some chronic diseases such as diabetes, obesity and heart disease (Patarra et al., 2011).

2.1.2 Green Algae

Green algae are photosynthetic eukaryotes. The red algae possess complex composite cell walls made of cellulose, extensive matrix fibril and polysaccharides; thus, it is difficult to extract the bioactive compounds from red algae compared to the green algae, which have easily breakable cell walls (Johnston et al., 2014; Liu et al., 2017). Green algae are mostly in a spherical shape with 2 to 30 μm diameter. There are also green algae with few centimetres to about a metre in length (Liu et al., 2017). There are 80 species of green algae found in the Malaysian coastline. Among the algal divisions, green seaweeds

9

are found to reflect the least colour variation. They reflect green colour due to the presence of chlorophyll, which is not masked by any other colour pigments as in the red and brown algae.

2.2   Phycobiliproteins

Phycobiliproteins can be divided into three major groups based on their spectral properties; PE (A_{max} = 562 nm), PC (A_{max} = 615 nm) and APC (A_{max} = 652 nm). Each phycobiliprotein is composed of two non-identical polypeptide subunits α and β, which contain one or more covalently linked open-chain tetrapyrrole chromophores. The chromophores are known as phycobilin, which covalently bonded to amino acids by cysteinyl thioether linkages. There are mainly four types of phycobilins known as phycoerythrobilin (PEB), phycocyanobilin (PCB), phycourobilin (PUB) and phycobiliviolin (PXB) (Cole et al., 1967; Glazer et al., 1983).

Phycobiliproteins are arranged as a trimer $(\alpha\beta)_3$ and hexamer $(\alpha\beta)_6$ discs with the aid of linker polypeptides into phycobilisome structure, which are organised into two distinct structural domains known as the core and the rods. The core structure comprises discs of APC that form a physical connection with the outer surface of the thylakoid membrane. A series of rods is present above the core. The rods situated closer to the core proteins are phycocyanins, while those rods located further from the core are phycoerythrins (Figure 2.3). Linker polypeptides are believed to be bonded in the central cavity of the trimers and hexamers to stabilise the phycobilisome structure and optimise its absorbance as well as energy transfer characteristics (Koller et al., 1978; Glazer et al., 1983).
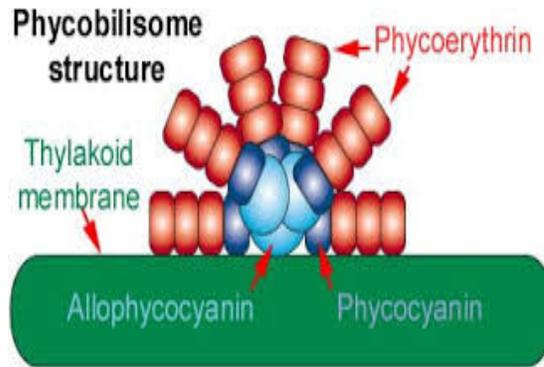
Figure 2.3: The phycobilisome structure with core and the rod phycobiliproteins attached to the outer surface of thylakoid membrane (Koller et al., 1978).

Phycoerythrin and phycocyanin confer red and purplish blue colours, respectively. The colours of phycobiliproteins are mainly due to covalently bound prosthetic groups that are open-chain tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins. They are either blue coloured (phycocyanobilin, PCB), red coloured (phycoerythrobilin, PEB), yellow coloured (phycourobilin, PUB) or purple coloured (phycobiliviolin, PXB) (Bongards and Gartner, 2010).

The phycobiliproteins are high molecular weight globular proteins. They are large water-soluble supramolecular protein aggregates in the stroma of the photosynthetic tissue involved in light harvesting (Overkamp et al., 2014). The phycobiliprotein in the photosynthesis process functions as the light absorber. The phycoerythrin traps the light energy efficiently and transfers it to phycocyanin and allophycocyanin, and eventually to chlorophyll (Yokono et al., 2011; Figueroa et al., 2012). Each phycobiliprotein has a specific absorption maximum in the visible range of light from 500 to 660 nm, which is inaccessible to chlorophyll (Wu, 2016).

2.2.1 Phycoerythrin

Three unique phycobilins are bonded to the subunits of phycoerythrin extracted from red algae. The α subunit has doubly bounded PEB, whereas the β subunit peptide bears a doubly linked PUB. The sequence of β subunit is identical to the peptide derived from a B-phycoerythrin, which has doubly linked PEBs (Schoenleber et al., 1984). This indicates that the doubly linked PUB and PEB tetrapyrroles are in isomeric structures. The different gamma subunits contain either three PEB and two PUB, or two PEB and one PUB (Nagy et al., 1985).

Based on this study on the antioxidant capacity of R-PE and R-PC, a higher antioxidant ability was observed in a hydrophilic medium compared to that in hydrophobic medium (Aftabuddin and Kundu, 2007). The R-PE displayed higher antioxidant activity in FRAP and DPPH radical scavenging assays compared to R-PC. Antioxidant activity of any protein may not be due to a single antioxidant mechanism because of the properties derived from different constituting amino acids favouring different mechanisms. For example, hydrophobic amino acids are good proton donors as well as chelators of metal ion (Sarmadi and Ismail, 2010). Ferrous ion chelating and DPPH radical scavenging activities of phycobiliproteins suggest that the antioxidant properties of phycobiliproteins (PBPs) are the combined consequence of both electron donating ability as well as metal ion chelating ability of the protein. Based on a study on 20 different types of protein, it has been concluded that the overall phycoerythrin has slightly lower chelating ability and higher reducing ability, while for phycocyanin and allophycocyanin, their antioxidant properties

were equally contributed by both reducing ability and chelating ability (Aftabuddin and Kundu, 2007).

2.2.2 Phycocyanin

Phycocyanin is a water soluble purplish blue pigmented protein widely found in blue-green algae. The amino acid analyses of phycocyanin by separating its alpha and beta subunits based on absorption spectrum demonstrated that the alpha subunit carries a single PCB chromophore, while one PEB and one PCB chromophores were bonded to the beta subunit (Overkamp et al., 2014).

Phycocyanin is a powerful water soluble antioxidant. Recent study presented that phycocyanin was an effective free radical scavenger (Devi et al., 2011; Jiang et al., 2017). Phycocyanin in blue-green and red algae has not only been established as an energy-transfer pigment, but also as an electron-directing agent in trans-membrane migration of electrons (Kao et al., 1973; Tiwari et al., 2013).

2.3 Phenolic Compounds

Phenolic compounds extracted from plants are the combination of benzene rings and hydroxyls in their molecular structure. Each structure is substituted by at least one hydroxyl group. These compounds are classified as phenol rings binding to another long carboxylic acid chain. Examples of phenolic compounds include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans and quinones (Astello-García et al., 2015). These are essential for the growth and reproduction of algae, which act as antioxidants and antimicrobials

(Inderjit, 1996). Phenolic compounds are abundantly found in algae and some other medicinal plants.

In addition, phenolic compounds function as protective agents against ultraviolet (UV) light while building the cell walls at the same time, which are impermeable to gas and water to run photosynthesis process. These cell walls constructed with phenolic compounds are meant to give cell structural stability to algae (Kubanek et al., 2004; Chakraborty et al., 2015). High phenolic compound-containing food source is very important as a natural source of antioxidants. However, dietary intake of phenolic compounds is greatly affected by the eating habits and preferences of individuals. The average daily intake of dietary phenolics is about 1 g/day, which can be fulfilled by consuming beverages, fruits, vegetables and legumes (Fernandes et al., 2012). This overall intake of variable plants and beverages as a set of meal can be replaced by daily intake of one small portion of algae to fulfil the daily needs of a human body for antioxidants.

Many different algae species have been recognised to have medicinal properties and beneficial impact on health; for example, antioxidant activity and digestive stimulation action as well as anti-inflammatory, antimicrobial and anticarcinogenic potentials (Borowitzka, 1995; Sarmadi and Ismail, 2010). Algae rich in phenolic compounds are of increasing interest in the food industry since they retard ROS and thereby improve the quality and nutritional value of food. The composition of phenolic compounds might vary qualitatively and

quantitatively depending on the species, as well as on the environmental conditions and locations where the algae are collected (Haigh et al., 2015).

2.4.   Antioxidant Activity against Free Radicals and Reactive Oxygen Species to Overcome Cellular Oxidative Damage

Free radicals are atoms or groups of atoms with an odd number of electrons. A free radical can be defined as a molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron or accept an electron from another molecule, therefore causing the radicals to behave as oxidants or reductants (Huang et al., 2005; Ganesan et al., 2011).

The common oxygen containing free radicals involved in many diseases are hydroxyl radical ($\bullet$OH), superoxide anion radical (O_2^-), singlet oxygen (O_2), hypochlorite (ClO^-), nitric oxide radical ($NO^{\cdot}$) and peroxynitrite radical (ONO_2^-). These highly reactive radicals can start a chain reaction that could lead to a massive damage on cellular components including DNA and cell membrane. These damages cause the cells to function inefficiently or cause tissue death (Valavanidis et al., 2009; Kumar et al., 2014).

To prevent free radicals from causing uncontrollable damage to the body, the body needs antioxidants to protect against them. Antioxidant substances that scavenge free radicals play an important role in the prevention of free radical-induced diseases (Lobo et al., 2010). Antioxidants reduce primary radicals to

non-radical chemical compounds by donating protons. They are singlet oxygen quenchers and metal ion chelators (Poljsak et al., 2013). This action helps to protect the body from degenerative diseases. Studies have shown the beneficial effects of diets rich in phenolic compounds in reducing the risk of cardiovascular disease and certain cancers (Butterfield, 2014; Jiang et al., 2017).

Free radicals and other ROS are derived from either normal essential metabolic processes in the human body or external sources such as the exposure to X-rays, ozone, cigarette smoking, air pollutants, radiation, certain drugs, pesticides and other industrial chemicals (Suh et al., 2009). In the human body, free radicals are continuously formed in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions, which serve as a source of free radicals, include those involved in the respiratory chain, reperfusion injury and in phagocytosis process. Free radicals can also be formed in non-enzymatic reactions of oxygen with other organic compounds (Valavanidis et al., 2009; Nita and Grzybowski, 2016).

Free radicals attack important macromolecules leading to cell damage and homeostatic disruption, which result in various chronic diseases such as cancer, coronary heart disease, cataract, ageing, muscular dystrophy and some neurological disorders including Alzheimer's and Parkinson's diseases (Middleton et al., 2000).

The term "free radicals" and "ROS" are not similar, but have distinguishing properties. ROS are chemically reactive radicals containing oxygen. Examples

of ROS include peroxides (O_2^{2-}), superoxide (O_2^-), hydroxyl radical (•OH) and singlet oxygen (O_2). They are continuously generated by the mitochondrial electron-transport chain where molecular oxygen is reduced to O_2^- due to the escape of an active electron. The generated O_2^- is spontaneously or enzymatically converted to hydrogen peroxide, H_2O_2. Thereafter, H_2O_2 can be converted to •OH and OH^- by accepting an electron in a reaction catalysed by transition metal ions like Fe^{2+} or Cu^{2+}. The generated hydroxyl radicals are highly reactive and are believed to cause significant oxidative damage. In order to prevent the formation of these harmful hydroxyl radicals, H_2O_2 is converted to water via reactions catalysed by catalase (Liou et al., 2015).

The mechanisms of diseases and damages caused by ROS generally involve oxidative alteration of physiologically critical molecules including proteins, lipids, carbohydrates and nucleic acids along with the modulation of gene expression and inflammatory response. Oxidative stress caused by the imbalance between antioxidant systems and the production of oxidants including ROS seems to be associated with many multifactorial diseases (Halliwell and Cross, 1994). An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structures and functions. Oxidative stress induced by H_2O_2 was first thought to cause only lipid peroxidation and DNA and protein damage; however, it is now known that H_2O_2 activates various intracellular signalling pathways closely associated with neuronal cell death (Teare et al., 1994; Butterfield, 2014). Antioxidants are effective in protecting the body against ROS. According to Halliwell and Cross (1994), the term antioxidant refers to a substance that

neutralises ROS. ROS leads to ageing, arthritis, diabetes, cancer, cardiovascular diseases, inflammation, radioactive damage, atherosclerosis and neurodegenerative diseases in the presence of antioxidant compounds at low concentration. The antioxidant significantly delays or prevents oxidation of cell components.

Free radicals contain unusual and unpaired electrons since electrons typically come in pairs. The unpaired electrons make free radicals highly reactive, and in this excited state, the radicals can cause damage by attacking cellular components. Once free radicals are formed, they can create more free radicals by scavenging electrons from other molecules. Free radicals are neutralised either by providing the extra electrons needed or by breaking them to render them harmless (Samoylenko et al., 2013; Di Meo et al., 2016). Free radical neutralisation process cannot be achieved once all the antioxidants in the body system are used up, which leads to further cellular damage. Thus, a diet rich in antioxidants is crucial to ensure a constant supply.

Two principle mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism by which the primary antioxidant donates an electron to the free radicals continuously present in the system until the radicals are stabilised by a chain-breaking antioxidant or decayed into a harmless product. The second mechanism involves the removal of ROS initiators by quenching chain-initiating catalyst (Teare et al., 1994; Liochev, 2014).

Antioxidants including phycobiliproteins and phenolic compounds are integral part of the photosynthetic apparatus in algae and function as accessory pigments in the harvesting complex and act as protective agents against the ROS formed from photo-oxidation. The mechanism of biological effect due to illumination of ultraviolet involves endogenous photosensitisation and formation of ROS such as from singlet oxygen (O_2), superoxide radical (O_2^-) and hydroxyl radical (•OH) (Nita and Grzybowski, 2016). Algae develop a defence system against photo-oxidative damage by antioxidative mechanisms to detoxify and eliminate these highly ROS and other radicals. The elimination of ROS minimises the oxidative damage to living cells and oxidative deterioration of food (Teare et al., 1994). Algae derived compounds have been observed to exhibit effective antioxidant activity, which was attributed to the scavenging activity against superoxide and hydroxyl radicals, metal ion chelating ability and, quenching of singlet and triplet oxygen (Nita and Grzybowski, 2016).

2.5 *Bangia atropurpurea* Commercial Potential

Natural pigment proteins from plants are safe to be used in foods and drinks. The pigments are stable in mild heat, acidic or basic solutions (Gibert et al., 2007; Wu et al., 2016). Therefore, they can be utilised in foods and cosmetics as natural colouring agents. In addition, the natural colour pigment can be used under fluorescent light to detect an antibody, which works as a marker cell in immunological, cell biology and biochemical studies. Phycobiliproteins namely phycoerythrin and phycocyanin are the two currently used natural proteins that have been applied in the food and pharmaceutical industries (Kuddus et al., 2013; Mysliwa and Solymosi, 2017). In the intention to supply these natural

products at cheaper cost, a large number of more feasible algae cultivation and phycobiliprotein extraction techniques have been suggested. Additionally, since *B. atropurpurea* is a fast growing alga with high survival potential, it is possible for its cultivation to occur with minimum nutrient supply (Sekimoto et al., 2008).

At the current situation, it is important to identify, develop and utilise a safe and effective source of natural antioxidants. *B. atropurpurea* is one of the potential algae, which should be commercialised to replace the synthetic antioxidants widely used in the food industry since *B. atropurpurea* is able to cope with the high demand for antioxidants. Fulfilling the demand is possible with this species as it can quickly multiply with the slightest amount of nutrient supply and is less susceptible to contamination. *B. atropurpurea* can be cultured *in vitro* in a large scale to produce sterilise feedstock for medical purposes. Since culturing and extraction expenses are reduced thus, natural antioxidant products will be available at affordable price in the market. The antioxidants can be used in the food industry as food preservatives by preventing lipid peroxidation, which causes food spoilage. Hence, the food industry does not need to depend on side effect-inducing chemical preservatives.

CHAPTER 3

METHODOLOGY

3.1 *Bangia atropurpurea* and *Chlorella vulgaris*

The powdered *B. atropurpurea* and *C. vulgaris* samples were bought from Algae Bioresource Centre Sdn. Bhd. The samples were stored at 4 °C until further analysis. According to the supplier, the matured thallus of *B. atropurpurea* was cultivated in SWM-III medium, whereas the *C. vulgaris* was cultured in Bold's Basal Medium (BBM).

3.2 Chemicals and Equipment

Thermo Scientific™ Evolution 60S UV-Visible spectrophotometer was used for spectrum absorption reading. Shimadzu Class HPLC system SPD-M20A Prominence with photodiode array detector with C_{18} reversed-phase column (250 mm x 4.6 mm) (RP-HPLC-PDA) was used for chromatography separation in this study. The chemicals used in this study are listed in Table 3.1.

Table 3.1: The list of items purchased from respective manufacturers

Methods	Items	Manufacturers
Phycobiliproteins extraction and purification	Phosphate buffer, ammonium sulphate	Merck KGaA
	Sephadex G-200	Sigma Aldrich
	Trichloroacetic acid, acetonitrile, potassium dihydrogen phosphate, phosphoric acid (H_3PO_4)	Merck KGaA
SDS-PAGE	SDS-PAGE equipment	Bio-Rad
	Coomassie brilliant blue G-250, prestained protein ladder 250-10 kDa, commercial standard proteins (R-PE and R-PC) and SDS-PAGE electrophoresis chemicals	Sigma Aldrich
Bradford protein assay	Bradford reagent	Merck KGaA
	Bovine serum abumin (BSA)	Merck KGaA
TPC assay	Folin-Ciocalteu's reagent, methanol, ethyl acetate, hexane, sodium carbonate	Merck KGaA
DPPH assay	DPPH reagent, ascorbic acid, BHT	Sigma Aldrich
FRAP assay	Potassium ferric cyanide, iron (III) chloride-6-hydrate,	Merck KGaA
	Iron (II) sulfate-7-hydrate	Merck KGaA

3.3 Extraction of Phycobiliproteins from *Bangia atropurpurea*

Fifty grams of powdered *B. atropurpurea* (red alga) was added to 200 mL of 50 mM phosphate buffer (pH 7.2). This mixture was shaken on an orbital shaker for an hour at 100 rpm after sonication at room temperature for 10 minutes. The algae mixture was filtered through cheese cloths and the extracts were separated by centrifugation at $4500 \times g$ for 10 minutes at 4 °C (Senthilkumar et al., 2013). The procedure was repeated thrice with the pellet and supernatant pooled. The recovery yields of total protein, R-PE, R-PC and APC were determined by taking the absorbance readings at 280, 562, 615 and 652 nm, respectively.

The supernatant was precipitated with 35% saturated ammonium sulphate and the saturated mixture was centrifuged at $4500 \times g$ for 10 minutes at 4 °C. The supernatant was precipitated again with 65% saturated ammonium sulphate and centrifuged. The pellet was dialysed against 50 mM phosphate buffer (pH 7.2). The precipitated phycobiliprotein was suspended in 50 mM phosphate buffer (pH 7.2) and stored at 4 °C in the dark (Senthilkumar et al., 2013). The recovery yields of total protein, R-PE, R-PC and APC were quantitated again by taking the absorbance readings at 280, 562, 615 and 652 nm, respectively.

3.4 Separation of R-PE, R-PC and APC by Gel Filtration

Gel filtration with Sephadex G-200 column (3.7×65 cm) was employed for R-PE, R-PC and APC isolation from the phycobiliprotein extracts. The gel column was equilibrated with 50 mM phosphate buffer (pH 7.2) at 80 mL/h. Then, 5 mL of phycobiliprotein sample was loaded into the column. The loaded column was eluted at 60 mL/h with the same 50 mM phosphate buffer (pH 7.2) (Sun et

al., 2009). The eluted fractions were collected in 3 mL tubes. The bright pinkish

and purplish blue coloured protein fractions were collected in separate tubes and

the absorbance reading from wavelength 190 to 800 nm was determined by UV-

visible spectrophotometer. The phycobiliprotein was identified based on the

peak absorbance reading. The collected fractions were further purified through

a C_{18} column and the proteins identified by high performance liquid

chromatography (HPLC).

The separated R-PE, R-PC and APC fractions were examined with the

absorption spectrum from 190 nm to 800 nm. The maximum absorption

spectrum of the eluted coloured extract was identified. The absorption spectrum

at 280 nm determined the total protein in the extract. The high purity index ratio

of A_{562} to A_{280}, A_{615} to A_{280} and A_{652} to A_{280} would indicate that the sample has

lower impurities. The fractions with maximum absorption reading at

wavelength 562 nm were pooled and dialysed in the same buffer solution before

being stored at 4 °C for further use. The fractions of R-PC and APC identified

at the wavelength of 615 nm and 652 nm, respectively, were also pooled and

dialysed in 50 mM phosphate buffer before storage. At each purification stage,

the purity index of phycobiliprotein and the percentage of impurities were

calculated by the following formulae:

$$\text{Purity Index (R-PE)} = \frac{A_{562}}{A_{280}}$$

$$\text{Purity Index (R-PC)} = \frac{A_{615}}{A_{280}}$$

$$\text{Purity Index (APC)} = \frac{A_{652}}{A_{280}}$$

$$\text{Recovery R-PE from total protein (\%)} = \frac{\text{R-PE protein (mg)}}{\text{Total protein (mg)}} \times 100\%$$

$$\text{Impurities (\%)} = 100\% - \text{Recovery yield of R-PE (\%)}$$

(Senthilkumar et al., 2013)

3.5 Purification of R-PE, R-PC and APC by RP-HPLC

The R-PE, R-PC and APC fractions from the gel filtration were further purified by RP-HPLC. The RP-HPLC separation was analysed based on absorption detection and the phycobiliproteins separation was carried out through an analytical C_{18} column (250 mm × 4.6 mm). The instrument was equipped with a photodiode array (PDA) detector. For protein separation, 10 μL of phycobiliprotein extract was injected through the column, which had been previously equilibrated with 75% mobile phase A (0.1% trifluoroacetic acid (TFA) in water) and 25% mobile phase B (0.1% TFA in acetonitrile). The sample and mobile phase were filtered through 0.45 µm Millipore filter before being injected through the C_{18} column. The flow rate was set to 1 mL/min in a gradient from 30% to 100% of mobile phase B in 10 minutes (Cruz et al., 1997). The PDA detector then displayed the absorption of the eluates at 280 nm, 562 nm and 615 nm.

3.6   Bradford Protein Assay to Determine Total Protein

The total protein content in the sample was determined by the Bradford protein assay. BSA was used as the standard reference. Ten dilutions of the BSA standard from 0.2 mg/mL to 2 mg/mL concentration were prepared. The test tube with 200 µL distilled water served as blank. A volume of 50 µL of Bradford reagent was added to each tube containing 200 µL of BSA dilution and incubated for 15 minutes at room temperature (Bradford, 1976; Kruger, 2009). The absorbance was measured at 595 nm while the total protein content was estimated from the standard curve (Figure 3.1). The sample was assayed in triplicates.
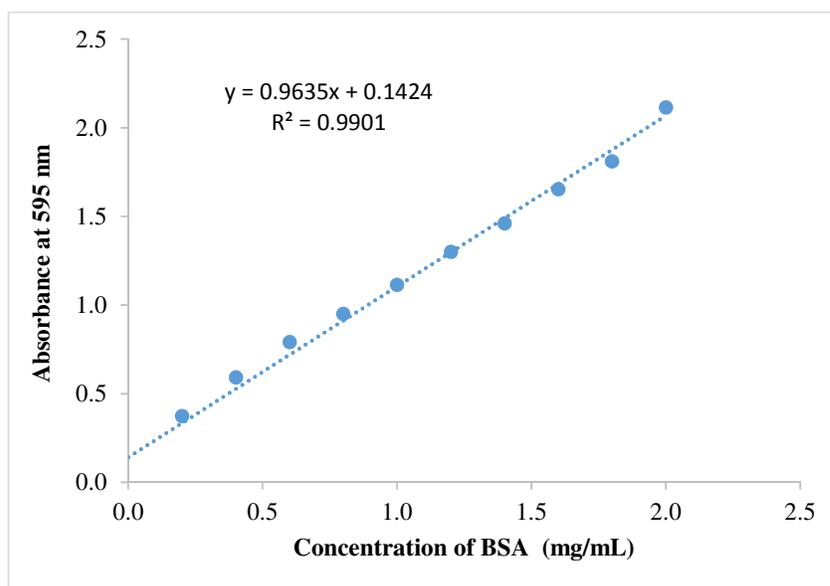
Figure 3.1: Calibration curve of Bradford protein assay.

The concentrations of R-PE and R-PC were estimated by spectrophotometry at the wavelength of 562 nm and 615 nm, and extinction coefficients, $E = 1.51 \times 10^5$ $M^{-1}cm^{-1}$ and $E = 1.17 \times 10^5$ $M^{-1}cm^{-1}$, respectively. The calibration equation for standard protein was y = 0.9635x + 0.1424, R^2 = 0.9901.

$$\text{Concentration (mg/mL)} = \frac{A_{280} \text{ x dilution concentration x molecular weight (Dalton)}}{\varepsilon_{280}} \times 100\%$$

(Bradford, 1976)

3.7 SDS-PAGE for Phycobiliproteins Analysis

The polypeptide components of the purified R-PE and R-PC samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresis was conducted in a vertical slab gel apparatus (Miniprotean III, Bio-rad) by the tricine buffer system where the gel was composed of 16.5% (w/v) separating gel in 374.5 mM Tris-HCl buffer (pH 8.8) containing 0.1% (w/v) SDS with a 4% (w/v) stacking gel in 61.8 mM Tris-HCl buffer (pH 6.8) and 0.2% (w/v) SDS. The electrode buffer was 192 mM Tris-glycine (pH 8.3) containing 0.1% (w/v) SDS. The R-PE and R-PC samples were first denatured with 20% (w/v) trichloroacetic acid, and the insoluble polypeptides were collected by centrifugation at $4500 \times g$ for 10 minutes under 4 °C. After the residual trichloroacetic acid was washed away, the precipitated polypeptide was suspended in 10 mM phosphate buffer (pH 7) containing 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.025% (w/v)

27

bromophenol blue, and 50 mM Tris-HCl buffer (pH 6.8), for 5 minutes at 95 °C. The incubated mixture was centrifuged at 4500 × *g* for 15 minutes to remove insoluble substances. After electrophoresis, the slab gel was washed two times with distilled water and then soaked in 0.2 M imidazole containing 0.1% (w/v) SDS for 10 minutes. Aliquots of 30 µL were loaded to the wells of a mini-slab gel. Gels were electrophoresed at room temperature. The electrophoresed gel was stained in Coomassie Blue G-250 solution for 30 minutes (Schagger et al., 1988). For calibration, protein marker was used to plot a standard curve.

3.8   Phenolic Compounds Extraction from *Bangia atropurpurea* and *Chlorella vulgaris* to Evaluate TPC

Phenolic compounds were extracted from *B. atropurpurea* and *C. vulgaris* using five different extraction solvents with different polarities such as water, 50% (v/v) aqueous methanol, methanol, ethyl acetate and hexane. These extracts were sequentially used to determine the TPC, FRAP and DPPH radical scavenging activity. Fifty grams of powdered *B. atropurpurea* (red alga) and *C. vulgaris* (green alga) were extracted in 200 mL of solvent. Five concentrations of the mixture from 0.2 mg/mL to 10 mg/mL were prepared. A conical flask containing algae powder and solvent was sonicated at room temperature for 10 minutes before being agitated on an orbital shaker at 100 rpm for an hour. The extract was then filtered with filter paper and the filtrate was evaporated to dryness by rotary evaporator before being stored at −20 °C until further analysis. The TPC extracted from *B. atropurpurea* and *C. vulgaris* using water, 50% (v/v) aqueous methanol, methanol, ethyl acetate and hexane solvents were measured by the Folin Ciocalteu's method. In order to make a valid comparison, the TPC of the extracts from *B. atropurpurea* and *C. vulgaris* was compared with that of

28

synthetic antioxidants, ascorbic acid and BHT. A volume of 1.5 mL of Folin

Ciocalteu's phenol reagent and 1.2 mL of 7.5% (w/v) Na_2CO_3 were added to

each 0.3 mL of extract and the reaction mixture was incubated in the dark for

30 minutes (Andressa et al., 2013). The absorbance of the mixture was then

measured at 765 nm. TPC was expressed in mg gallic acid equivalents (GAE)/g

dry weight. The calibration equation for gallic acid is y = 0.0024x and the R^2 is

0.9943 (Figure 3.2).
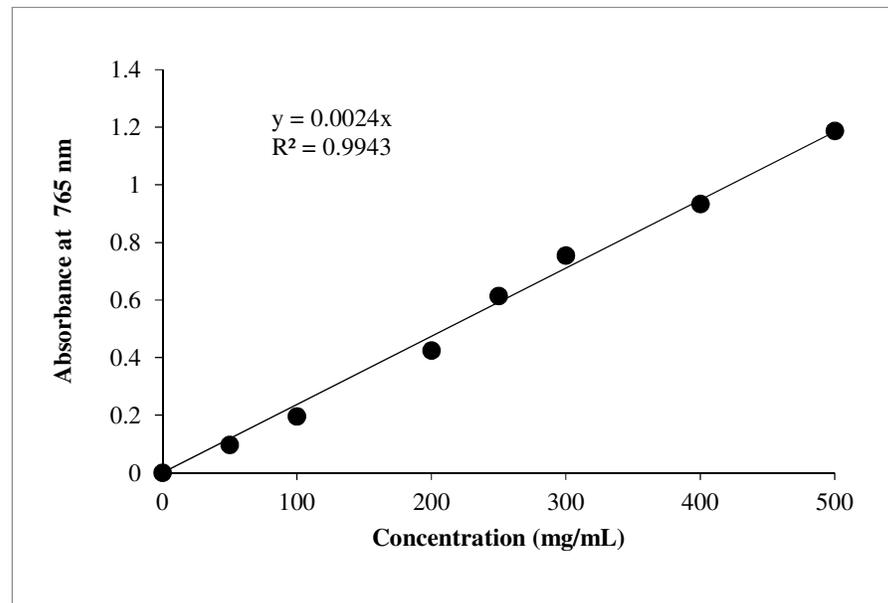
Figure 3.2: The standard calibration curve to estimate TPC.

TPC in solvent extracts expressed in GAE, was calculated by the following formula: $C = c. V/m$ where C is the total content of phenolic compounds (mg/g extract), c is the concentration of gallic acid established from the calibration curve (mg/mL), V is the volume of extract (mL) and m is the weight of extract (g).

3.9   Antioxidant Activities

3.9.1   DPPH Radical Scavenging Assay

The DPPH radical scavenging assay was carried out in triplicates by the method of Leong and Shui (2002) and Ashafa et al. (2010). A volume of 2 mL of 0.15 mM DPPH was added to 1 mL of extract and the reaction mixture was incubated for 30 minutes after which its absorbance was measured at 517 nm. The phenolic compounds containing extracts were prepared in different concentrations from 10 µg/mL to 200 µg/mL. The concentration of phenolic extracts required to inhibit 50% of DPPH radicals was recorded to determine the antioxidant capacity. The total DPPH radical scavenging activity in concentration-dependant manner was also evaluated. The synthetic antioxidants, ascorbic acid and BHT were used as controls in making comparison of antioxidant capacity with the extracted phenolic compounds from *B. atropurpurea* and *C. vulgaris*. The calibration equation for ascorbic acid was $y = 0.0057x$ (Figure 3.3).
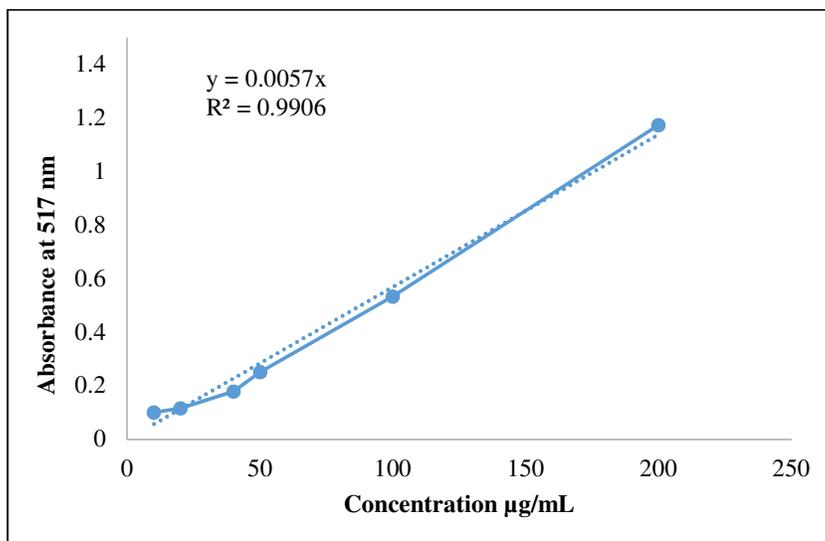
Figure 3.3: Calibration curve of ascorbic acid to determine equivalent antioxidant capacities.

The radical scavenging activity was expressed as a percentage and determined with the formula:

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

DPPH was expressed as ascorbic acid equivalent antioxidant capacity (AEAC) which was calculated based on mg ascorbic acid (AA)/100 g dry sample required to reduce DPPH radicals by 50% (IC_{50}).

$$\text{AEAC (mg AA/100 g)} = IC_{50 \text{ (ascorbic acid)}}/IC_{50 \text{ (sample)}} \times 10^5$$

(Sagar and Singh, 2011)

3.9.2 Ferric-Reducing Antioxidant Power Assay

The FRAP assay was determined using the method of Moniruzzaman et al. (2012) with modifications. A volume of 2.5 mL of 0.1 M potassium phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferric-cyanide were mixed with 1 mL of phenolic extracts from *B. atropurpurea* and *C. vulgaris* using different extraction solvents. The extracts were prepared in eight different concentrations from 50 mg/mL to 500 mg/mL. The reaction mixture was incubated at 50 °C for 20 minutes after which 2.5 mL of 10% (w/v) trichloroacetic acid was added. A volume of 2.5 mL of water and 0.5 mL of 0.1% (w/v) $FeCl_3$ were then added to 2.5 mL of reaction mixture. The solution was incubated for 30 minutes for colour development. The absorbance was then measured at 700 nm. The FRAP value was expressed as mg gallic acid equivalents (GAE)/g dry weight. Meanwhile, the synthetic antioxidants, ascorbic acid and BHT were used as controls to compare the antioxidant capacity with the phenolic extracts. The calibration equation for gallic acid was $y = 0.0017x$ (Figure 3.4).
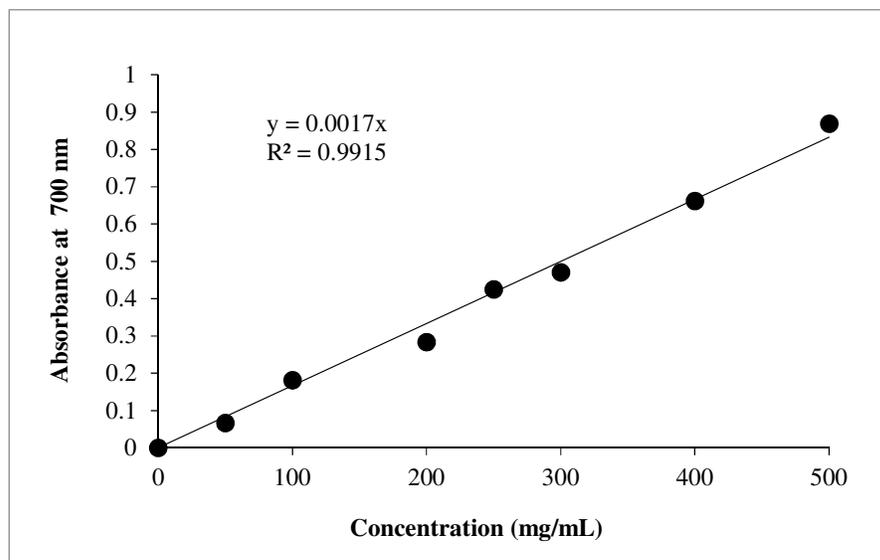
Figure 3.4: Calibration curve of gallic acid for FRAP assay.

3.10   Statistical Analysis

Data collected in this study were analysed by one-way analysis of variance (ANOVA) and Tukey Kramer's multiple comparison tests to determine the significant differences. For the Tukey's test, the critical value for the modified *t*-statistic was obtained by referring to the values in the distribution table of "Studentised Range". In order to determine whether or not the antioxidant activity has independent significance, two different sets of variables were fixed into a multivariate proportional regression analysis; (1) different extraction solvents used to extract the phenolic compounds (water, 50% (v/v) aqueous methanol, methanol, ethyl acetate and hexane); (2) phytochemical extracts of *B. atropurpurea* (R-PE, R-PC and phenolic compounds) and *C. vulgaris* (phenolic compounds) were compared with synthetic antioxidants (ascorbic acid and BHT). Data were expressed as mean value ± standard deviation of three replicates (n = 3) with statistical p value below 0.05 indicating a very strong evidence to reject the null hypothesis. The results in the table were marked with

different letters to show statistical significance. For all variables with the same letter, the difference between the means was seen statistically insignificant. If two variables have different letters, they are significantly different. The correlation coefficient, r, was determined for the calibration curves. Generally, correlation coefficient is a measure that determines the degree to which two variables' movements are associated, which is used to measure the linear relationship between two variables. In the correlation study between TPC with FRAP and DPPH radical scavenging activity, the coefficient of determination, R^2, was determined. The coefficient of determination is the proportion of the variance in the dependent variable predictable from the independent variable. The R^2 value of 1, indicates that the regression line has perfectly fitted the data. Results were analysed by Microsoft Excel 2013 and Statistical Package for the Social Sciences (SPSS 21).

CHAPTER 4

RESULTS

4.1   Evaluation of the Concentrations of R-PE and R-PC Extracted from *Bangia atropurpurea*

The crude extract of phycobiliproteins was obtained from powdered *B. atropurpurea* and further saturated with $(NH_4)_2SO_4$ prior to elution through a gel filtration column containing Sephadex G-200 beads. The R-PE and R-PC were separated by elution in a total of 93 different tubes based on colour differences. It was demonstrated that the bright pink fractions in the tubes (#63 to #71) were of those rich in R-PE, while the purplish blue fractions in the tubes (#69 to #79) were of those rich in R-PC, displaying peak absorption spectrum at 562 nm and 615 nm, respectively (Figure 4.1).
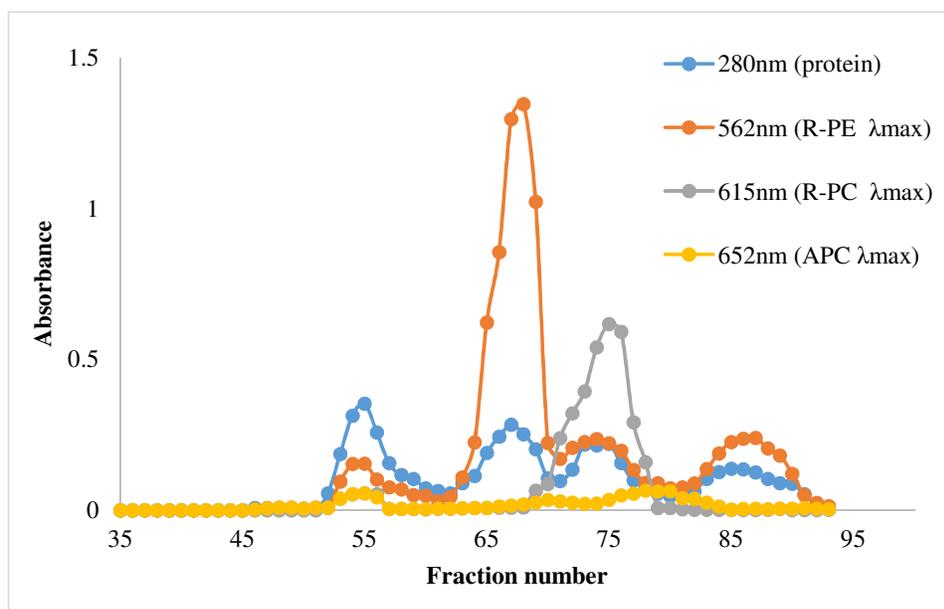
Figure 4.1: Absorption spectrum of isolated phycobiliproteins from *Bangia atropurpurea* by gel filtration with Sephadex G-200. (R-Phycoerythrin, R-PE; R-Phycocyanin, R-PC; Allophycocyanin, APC)

The phycobiliproteins found in *B. atropurpurea* were R-PE, R-PC and APC. The concentrations of R-PE, R-PC and APC were recorded after each purification stage as shown in Table 4.1. The concentrations of the extracted phycobiliproteins were determined by the Bradford protein assay. The Bradford protein assay is a procedure for determining the total protein concentration in the sample that relies on the change in absorbance based on the proportional binding of the Coomassie dye.

Referring to Table 4.1, 1.718 mg/mL R-PE was recovered by liquid chromatography. The concentration of R-PC was lower than R-PE; only 0.884 mg/mL was recovered, whereas the APC was found to be completely filtered off after gel filtration with Sephadex G-200. APC became almost untraceable in the pooled purplish blue fractions due to insignificant content of APC present in *B. atropurpurea*.

Table 4.1: Evaluation of the concentrations of phycobiliproteins in *Bangia atropurpurea* after each purification process.

	Crude extract	$(NH_4)_2SO_4$ saturated extract	Gel filtration	RP-HPLC
R-PE (mg/mL)	0.978	1.114	1.569	1.718
R-PC (mg/mL)	0.277	0.483	0.604	0.884
APC (mg/mL)	0.084	0.029	-	-

R-Phycoerythrin (R-PE); R-Phycocyanin (R-PC); Allophycocyanin (APC)

As can be seen in Table 4.2, the total content of R-PE recovered from *B. atropurpurea* after purification by gel filtration was the highest compared to R-PC and APC. The total content of R-PE recovered was 0.934 mg/g of dry weight compared to the concentration of R-PC recovered, which was only 0.315 mg/g, whereas the APC became untraceable after gel filtration. APC was recorded to present in a very minimum amount, 0.029 mg/g in the extract of the same algal species after saturating the crude extract with $(NH_4)_2SO_4$. Referring to Table 4.2, the phycobiliprotein content decreased as the subsequent purification process was conducted. The decrease in the total phycobiliprotein content in the extract was due to the enormous protein being leached off while eliminating contaminant proteins by purification process.

Table 4.2: The phycobiliprotein content in *Bangia atropurpurea* that was quantitated after each purification process.

	Crude extract	$(NH_4)_2SO_4$ saturated extract	Gel filtration	RP-HPLC
R-PE (mg/g)	1.569	1.114	0.934	0.778
R-PC (mg/g)	0.604	0.483	0.315	0.227
APC (mg/g)	0.084	0.029	-	-

R-phycoerythrin (R-PE); R-phycocyanin (R-PC); Allophycocyanin (APC)

4.2 Evaluation of the Purity Index and Total Recovery Yield of R-PE and R-PC Extracted from *Bangia atropurpurea*

The purity index of the separated compounds was demonstrated by recording the UV-visible spectrophotometric absorbance readings. The absorbance ratio of phycobiliproteins against total protein was calculated to determine the purity index. The purity index ratio for R-PE was expressed in fraction of A_{562}/A_{280} while, the purity index ratio of R-PC was measured at A_{615}/A_{280}. The purity index ratios of R-PE and R-PC were measured at A_{562} and A_{615} since the respective wavelength exhibited maximum absorbance reading. Peak absorbance at 280 nm indicates the concentration of total protein in the extract. Meanwhile, high purity index ratio indicates a high concentration of the purified R-PE and R-PC with minimum contaminant proteins in the extract.

The purity indices of R-PE and R-PC in the crude extract were 0.59 and 0.13, respectively, which were lower than 1 ($A_{562}/A_{280} < 1$, $A_{615}/A_{280} < 1$) (Tables 4.3 and 4.4). This indicates the presence of high impurities in the extract. As a part of the purification process, the crude extract was saturated by 35% $(NH_4)_2SO_4$,

saturated yield was subsequently saturated by 65% $(NH_4)_2SO_4$ to eliminate the remaining impurities. Gradual increase in degree of saturation with $(NH_4)_2SO_4$ will eliminate the impurities easily and effectively without eliminating much protein molecules. In this study, the recovery yield was evaluated from the very beginning stage of extraction. At crude extraction process, only 9.7% of the R-PE was recovered, whereas after the saturation process with $(NH_4)_2SO_4$, the recovery yield hiked to 31.8% while for R-PC extract, the recovery yield increased from 8.4% to 25.3%.

The saturated extracts were dialysed and further purified by gel filtration with Sephadex G-200 before analysed by RP-HPLC. These techniques efficiently eliminated a large amount of impurities, which have reflected a high absorption spectra of R-PE and R-PC at 562 nm and 615 nm, respectively. The purity index of R-PE and R-PC was much lower when saturated with $(NH_4)_2SO_4$; however, the gel filtration of saturated extract with Sephadex G-200 increased the purity index of R-PE and R-PC. The purity index of R-PE after saturation with $(NH_4)_2SO_4$ was 2.47, and elevated to 4.76 after further purification by gel filtration, while the purity index of R-PC had increased from the purity index of 0.77 to 2.80 resulted from the implementation of the same purification method.

As the purity index increased, the percentage of recovery yield of R-PE and R-PC extracted from *B. atropurpurea* increased proportionally. The gel filtration and RP-HPLC were the methods used that were not only aimed at the purification of phycobiliproteins, but also to increase the total recovery yield of phycobiliproteins. The overall recovery yields of R-PE and R-PC from the total

extracted protein in the final purification stage after RP-HPLC were 94.4% and 86.1%, respectively, which were very high. A total of 85.9 mg of R-PE and 44.2 mg of R-PC were separated by RP-HPLC from 142.3 mg of total protein extracted from 50 g of *B. atropurpurea*. Based on Table 4.3 and Table 4.4, 66% of R-PE extracted from the total phycobiliproteins recovered and the remaining 34% of phycobiliprotein was R-PC, hence determining that R-PE is the predominant phycobiliprotein in *B. atropurpurea*.

Table 4.3: Evaluation of purity and recovery yield of R-PE from *Bangia atropurpurea* after each purification process.

	Crude extract	$(NH_4)_2SO_4$ saturated extract	Gel filtration	RP-HPLC
A_{280}	1.497	0.424	0.283	0.268
A_{562}	0.887	1.048	1.348	1.453
Purity Index	0.59	2.47	4.76	5.42
Total R-PE (mg)	246.4	155.7	105.5	85.9
Total protein (mg)	2540.2	489.6	135.4	91.0
Recovery yield %	9.7	31.8	77.9	94.4
(R-PE from total protein)				
Impurities (%)	90.3	68.2	22.1	5.6

R-PE: R-phycoerythrin; A_{280}: max absorbance of total protein; A_{562}: max absorbance of R-PE

Table 4.4: Evaluation of purity and recovery yield of R-PC from *Bangia atropurpurea* after each purification process.

	Crude extract	$(NH_4)_2SO_4$ saturated extract	Gel filtration	RP-HPLC
A_{280}	1.497	0.424	0.220	0.198
A_{615}	0.2	0.325	0.618	0.784
Purity Index	0.13	0.77	2.80	3.95
Total R-PC (mg)	213.8	124.2	79.6	44.2
Total protein (mg)	2540.2	489.6	131.79	51.3
Recovery yield % (**R-PC from total protein)**	8.4	25.3	60.4	86.1
Impurities (%)	91.6	74.7	39.6	13.9

R-PC: R-phycocyanin; A_{280}: max absorbance of total protein; A_{615}: max absorbance of R-PC

4.3   SDS-PAGE for Phycobiliprotein Analysis

Phycobiliprotein sample extracted from *B. atropurpurea* was eluted by gel filtration and the pinkish eluates rich in R-PE were pooled before analysed by SDS-PAGE. The gel electrophoresis of phycobiliprotein band appeared to be narrower and clearer after purification by gel filtration. A single band with molecular weight of 20.5 kDa that was determined by SDS-PAGE appeared to be parallel with the standard commercial R-PE band (Figure 4.2). The phycobiliproteins bands generally vary in origination in molecular mass from 15 kDa to 22 kDa.
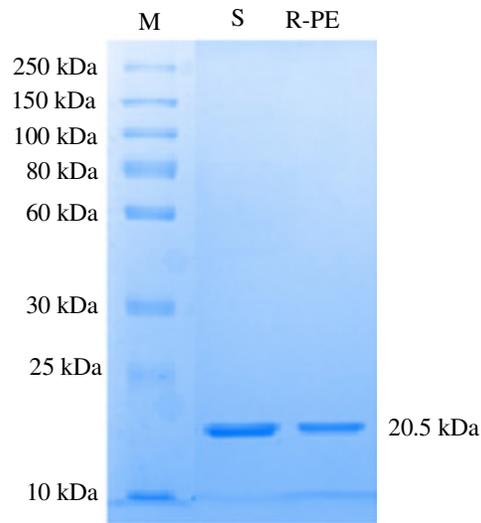
(A)
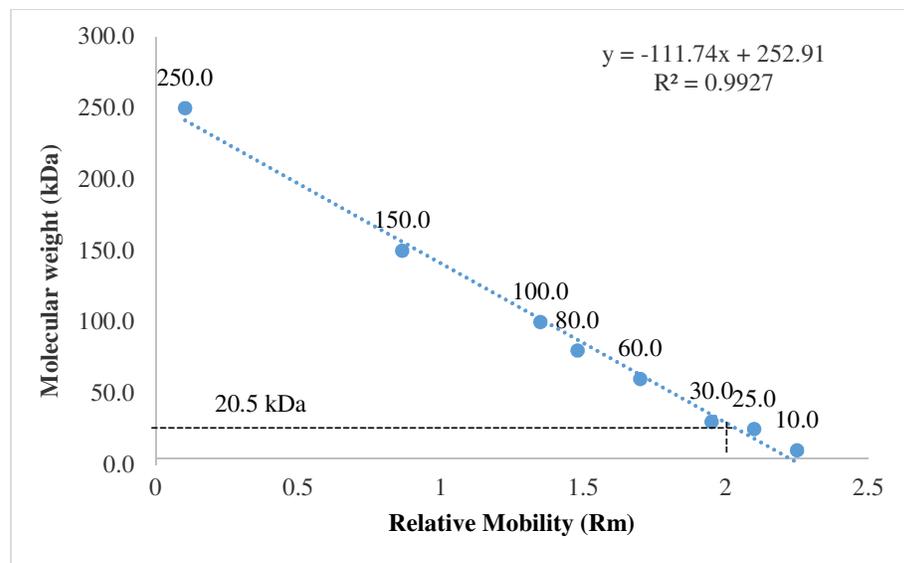
(B)

Figure 4.2: SDS-PAGE bands of purified R-PE from *Bangia atropurpurea* by gel filtration. **(A)** Lane M: Pre-stained precision marker protein, broad range (250-10 kDa); Lane S: Standard commercial R-PE protein; Lane R-PE: Purified sample of R-PE with single band at molecular weight 20.5 kDa. **(B)** Calibration curve to determine the molecular weight (kDa) against relative mobility (Rm) on electrophoresis gel.

The bright pinkish fractions rich in R-PE protein were pooled after elution by gel filtration with Sephadex G-200, and UV-spectrophotometric reading was taken to find the purity index ratio A_{562}/A_{280} of 4.76. The R-PE exhibited a "single-peak" with an absorption maximum at 562 nm. It presented a typical absorption spectrum of R-PE (Figure 4.3). UV-visible absorption spectrum of the R-PE obtained from *B. atropurpurea* showed its peak at 530–570 nm indicating the presence of purified R-PE. The maximum absorption peak shoulder was recorded at 540 nm.
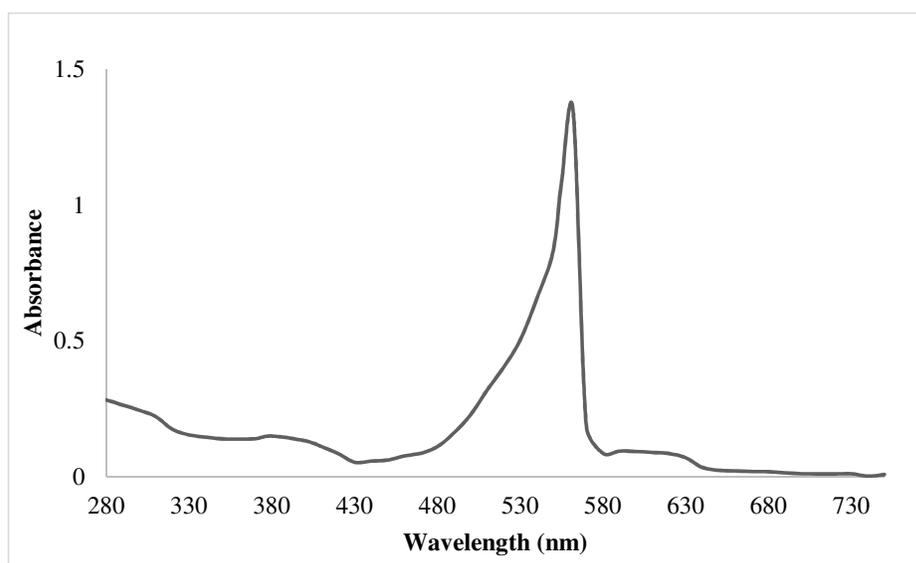
Figure 4.3: Absorption spectrum of the purified R-PE sample from *Bangia atropurpurea* by gel filtration.

The purplish blue fraction eluted by gel filtration was further analysed by SDS-PAGE. A single band was visualised at 17.6 kDa, which was expected to be the R-PC protein (Figure 4.4). The commercial standard R-PC protein band appeared parallel with the purified extract rich with R-PC protein, which were detected on the stained gel at 17.6 kDa. The clear narrow band appeared on

SDS-PAGE gel was determined by calibration curve of molecular weight (kDa) against relative mobility (Rm).
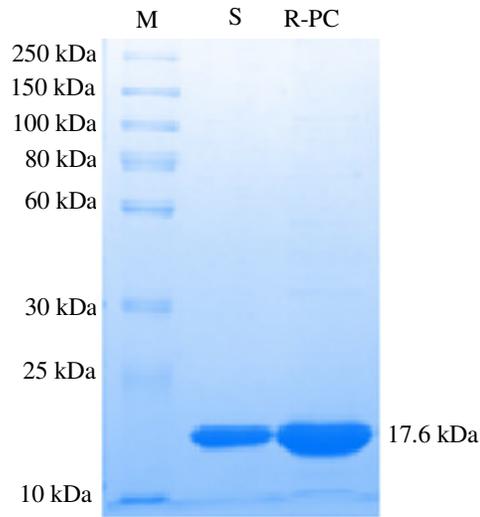
(A)
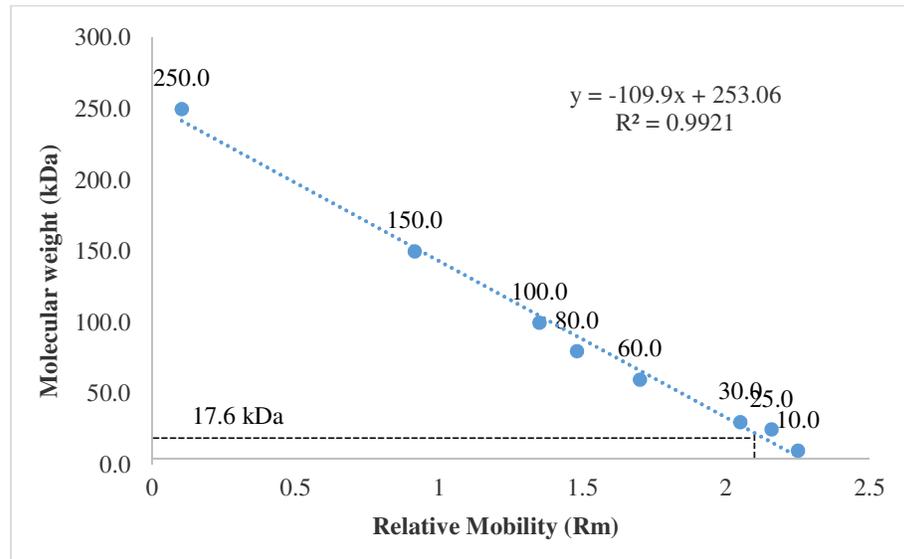
(B)

Figure 4.4: SDS-PAGE bands of purified R-PC from *Bangia atropurpurea* by gel filtration. **(A)** Lane M: Pre-stained precision marker protein, broad range (250–10 kDa); Lane S: Standard commercial R-PC protein; Lane R-PC: Extracted and purified sample of R-PC at molecular weight 17.6 kDa. **(B)** Calibration curve to determine molecular weight (kDa) against relative mobility (Rm) on electrophoresis gel.

The purplish blue fractions rich in R-PC protein were pooled after being eluted by gel filtration with Sephadex G-200, whereas UV-spectrophotometric reading indicated the purity index ratio A_{615}/A_{280} of 2.80. A single peak of R-PC at maximum peak absorbance at 615 nm was observed as in Figure 4.5. The maximum absorption peak shoulder was at 570 nm.
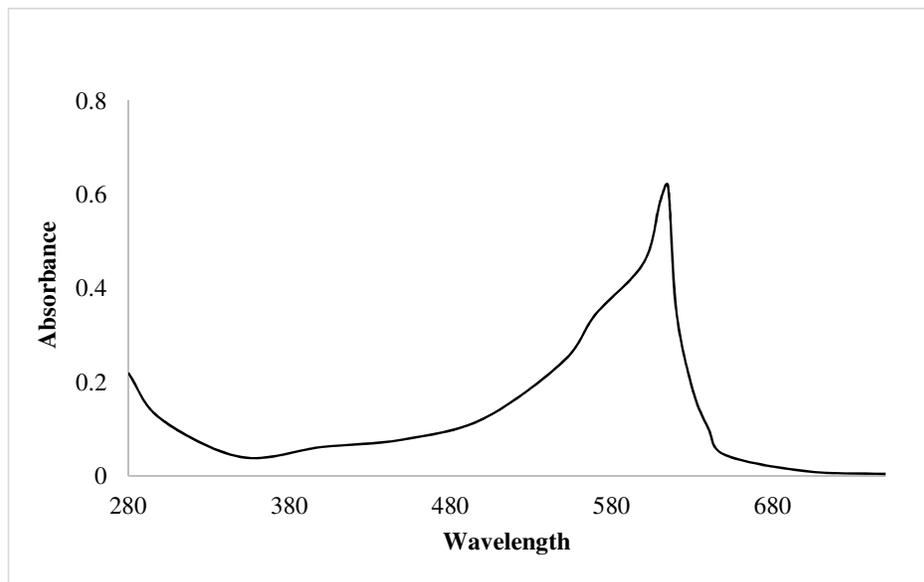
Figure 4.5: The absorption spectrum of purified R-PC extract from *Bangia atropurpurea*.

4.4   Identification of the R-PE and R-PC from *Bangia atropurpurea* by RP-HPLC

In the initial stage of purification process, the *B. atropurpurea* extract saturated with $(NH_4)_2SO_4$ showed few peaks of absorbance in the chromatogram (Figure 4.6A). These few peaks appeared after $(NH_4)_2SO_4$ saturation due to ineffective elimination of the contaminant proteins where the purity index (A_{562}/A_{280}) was only 2.47. As a result, multiple peaks at retention times of 4.3, 5.8 and 7.8 minutes appeared at 562 nm wavelength in the chromatogram. The *B. atropurpurea* extract saturated with $(NH_4)_2SO_4$ salt was further purified by gel filtration with Sephadex G-200. The eluted pinkish extract by gel filtration was further purified by RP-HPLC. As the extract was purified and the purity index increased from 2.47 to 5.42, the peaks at 4.4 and 7.8 minutes disappeared completely while the peak absorbance at minute 5.8 at 562 nm remained (Figure 4.6B). The peaks of contaminant proteins disappeared as the purity index increased.

The RP-HPLC retention time of the purified R-PE and R-PC from *B. atropurpurea* was compared with that of commercial R-PE and R-PC to confirm the identity of the extracted proteins. The commercial R-PE protein gave a single peak at retention time of 5.8 minutes at 562 nm (Figure 4.6C). The retention time of the peak obtained at minute 5.8 of purified R-PE was parallel with the commercial standard R-PE, which verified that the extracted R-PE from *B. atropurpurea* has a similar protein structure with commercial R-PE. The peaks of contaminants were eliminated and the remaining single peak at minute 5.8 was seen tally with the retention time of commercial R-PE. The purchased commercial R-PE protein was a protein extracted from an

unidentified species, which gave similar retention time of 5.8 minute at 562 nm and SDS-PAGE single narrow band at 20.5 kDa, which were similar to the results for R-PE protein extracted from *B. atropurpurea.*

This study determined that PE was the most abundant phycobiliprotein in *B. atropurpurea* characterised by strong absorption in the visible region of the spectrum from 480 nm to 570 nm. The maximum absorbance wavelength of purified PE is observed at 562 nm and *B. atropurpurea* is from the division of Rhodophyta and hence, the extracted PE from *B. atropurpurea* has the prefix R-PE.
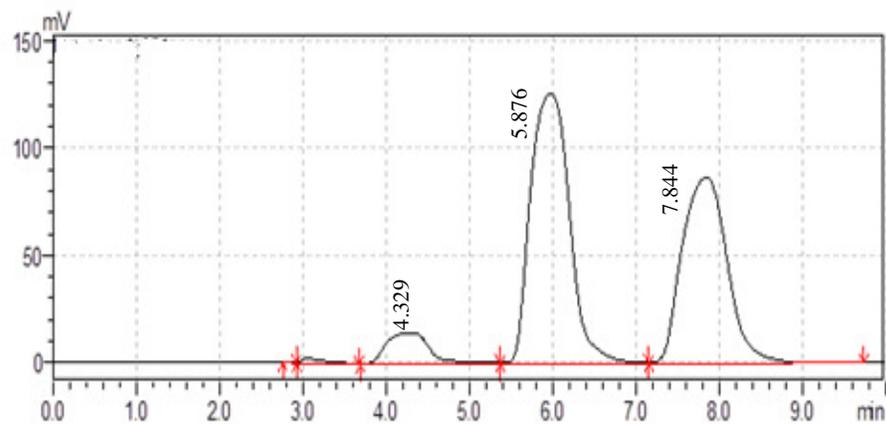
(A)
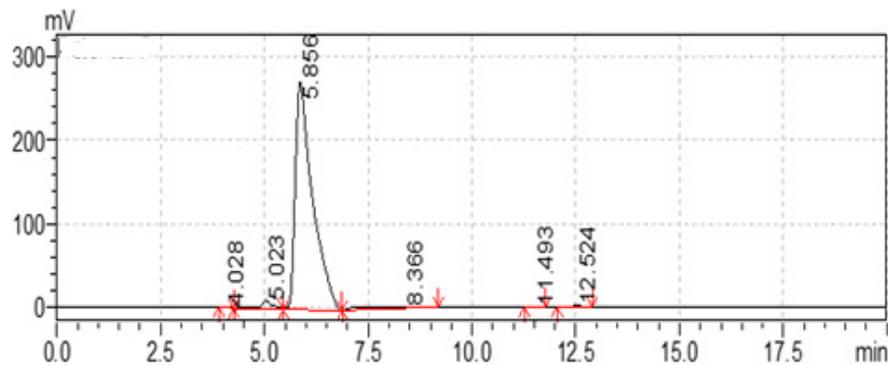
(B)
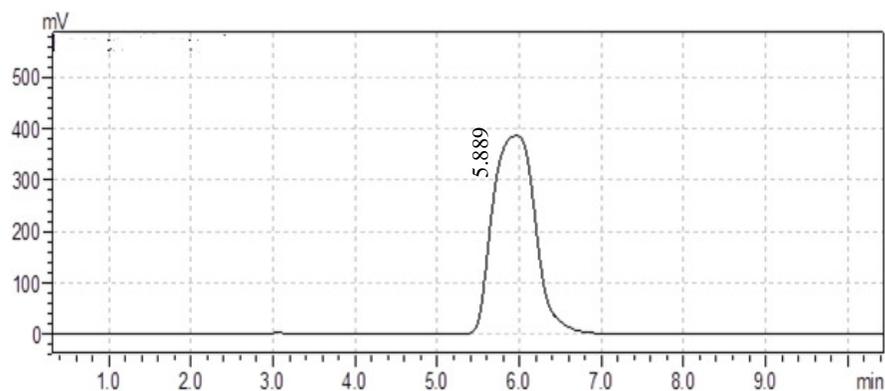
(C)

Figure 4.6: Chromatogram of R-PE. **(A)** Chromatogram of *Bangia atropurpurea* extracted protein, saturated with $(NH_4)_2SO_4$ analysed by RP-HPLC. **(B)** Chromatogram of extracted R-PE protein from *Bangia atropurpurea*, purified by gel filtration Sephadex G-200 and RP-HPLC. **(C)** Chromatogram of commercial standard R-PE protein analysed by RP-HPLC.

The total R-PC concentration at crude extraction was 0.277 mg/mL, then increased with subsequent purification process to 0.884 mg/mL after RP-HPLC. The increasing concentration of R-PC was proportional to the recovery yield of R-PC protein where the percentage of recovery yield increased from 8.4% to 86.1% after RP-HPLC purification. The *B. atropurpurea* extract saturated with $(NH_4)_2SO_4$ has a purity index of 0.77. The saturated extract was further purified by gel filtration with Sephadex G-200 where the purity index increased drastically to 2.80. The eluted purplish blue sample by gel filtration was further purified by RP-HPLC. As the sample became more purified, the purity index increased from 2.80 to 3.95. Meanwhile, the purity index increased, the percentage of recovery yield increased and the percentage of impurities decreased from 91.6% to 13.9%, thus indicating a more pure and contaminant-free extract produced with every subsequent level of purification. However, due to the elimination of unwanted contaminant proteins at every purification

50

process, for each 50 g of *B. atropurpurea* powder only 44.2 mg of R-PC protein remained at the final stage from a total 2540.2 mg of protein collected at the crude stage.

The number of peaks appeared on the chromatogram determined the purity index of the extract. The chromatogram of protein extract saturated with $(NH_4)_2SO_4$ was analysed by RP-HPLC, which shown three sharp peaks at minutes 1.8, 2.5 and 4.3, thus indicating the presence of contaminant proteins with lower purity index (Figure 4.7A). After saturation with $(NH_4)_2SO_4$, the extract was filtered by gel filtration followed by RP-HPLC where the purity index increased to 3.95; thus, only a single peak of R-PC at minute 2.5 remained (Figure 4.7B). The peaks at retention times of 1.8 minute and 4.3 minute disappeared after gel filtration followed by RP-HPLC purification. The retention time of the single peak of purified R-PC extracted from *B. atropurpurea* was parallel to the commercial R-PC, which indicates similar protein structure between extracted R-PC and the commercial R-PC (Figure 4.7C).
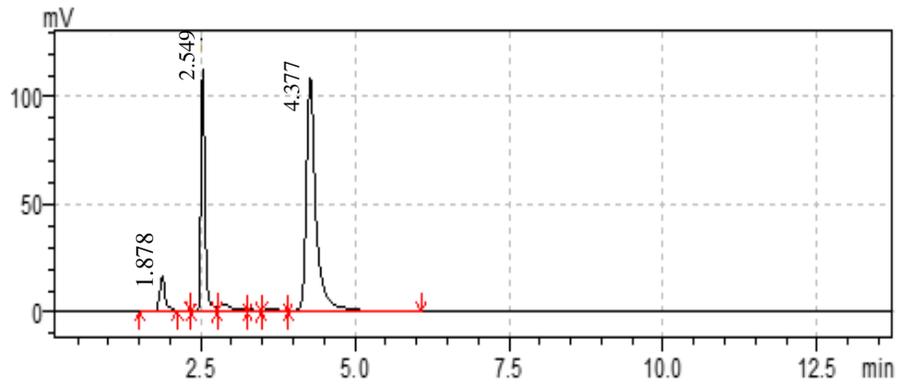
(A)
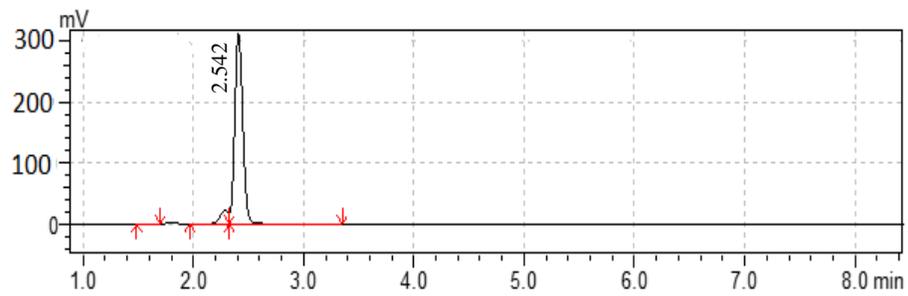
(B)
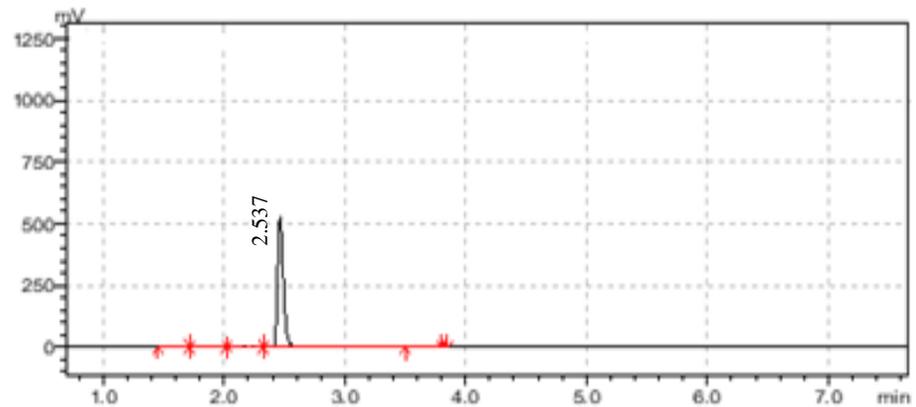
(C)

Figure 4.7: Chromatogram of R-PC. **(A)** Chromatogram of *Bangia atropurpurea* extracted protein, saturated with $(NH_4)_2SO_4$ analysed by RP-HPLC. **(B)** Chromatogram of extracted R-PC protein from *Bangia atropurpurea*, purified by gel filtration Sephadex G-200 and RP-HPLC. **(C)** Chromatogram of commercial standard R-PC protein analysed by RP-HPLC.

4.5   Extraction and Evaluation of TPC in *Bangia atropurpurea* and *Chlorella vulgaris* using Different Extraction Solvents

The comparison study of TPC in *B. atropurpurea* (red alga) and *C. vulgaris* (green alga) extracts using five different extraction solvents were summarised in Table 4.5. According to TPC analysis, the results indicated that the phenolic compounds extracted using methanol and ethyl acetate exhibited higher TPC compared to water, 50% aqueous methanol and hexane. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol showed the highest TPC at 80.97 ± 0.53 mg GAE/g dry weight and 62.13 ± 1.28 mg GAE/g dry weight, respectively. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using ethyl acetate was equally high with TPC at 61.84 ± 0.39 mg GAE/g dry weight and 44.15 ± 5.02 mg GAE/g dry weight, respectively. However, the non-polar hexane has lower solubility of phenolic compounds with the TPC for *B. atropurpurea* and *C. vulgaris* at 7.55 ± 0.14 mg GAE/g dry weight and 6.06 ± 0.24 mg GAE/g dry weight, respectively.

Table 4.5: Comparison of TPC in the extracts from *Bangia atropurpurea* and *Chlorella vulgaris* using extraction solvents of different polarity.

Extraction solvents	TPC (mg GAE/g dry weight)	
	Bangia atropurpurea	*Chlorella vulgaris*
Water	5.21 ± 0.03^{e}	4.37 ± 0.04^{i}
50% Aqueous methanol	37.58 ± 0.64^{c}	24.53 ± 0.93^{h}
Methanol	80.97 ± 0.53^{a}	62.13 ± 1.28^{b}
Ethyl acetate	61.84 ± 0.39^{b}	44.15 ± 5.02^{d}
Hexane	7.55 ± 0.14^{e}	6.06 ± 0.24^{i}

Mean value $\pm$ S.D. (n = 3), different letters in superscript indicate a significant difference at $p < 0.001$ between concentrations of extracts by one-way ANOVA *F*-test analysis.

Based on Figure 4.8, it can be concluded from the graph that the concentrations of the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol behaved in a dose-dependent manner. As the concentration of phenolic compounds increased, the concentration equivalent to gallic acid increased for both extracts from *B. atropurpurea* and *C. vulgaris*. At TPC concentration of 6 mg/mL, the extracts exhibited 422 ± 24.22 mg/mL GAE and 369 ± 15.23 mg/mL GAE for *B. atropurpurea* and *C. vulgaris*, respectively. Overall, *B. atropurpurea* has higher TPC compared to *C. vulgaris*; thus, it can be suggested that the red alga, *B. atropurpurea* has higher phenolic content compared to the green alga. However, the TPC readings between *B. atropurpurea* and *C. vulgaris* were almost parallel across the phenolic compound concentrations; for instance, at TPC concentration of 10 mg/mL, the extracts exhibited 577 ± 34.72 mg/mL GAE and 505 ± 37.53 mg/mL GAE for *B. atropurpurea* and *C. vulgaris*, respectively.
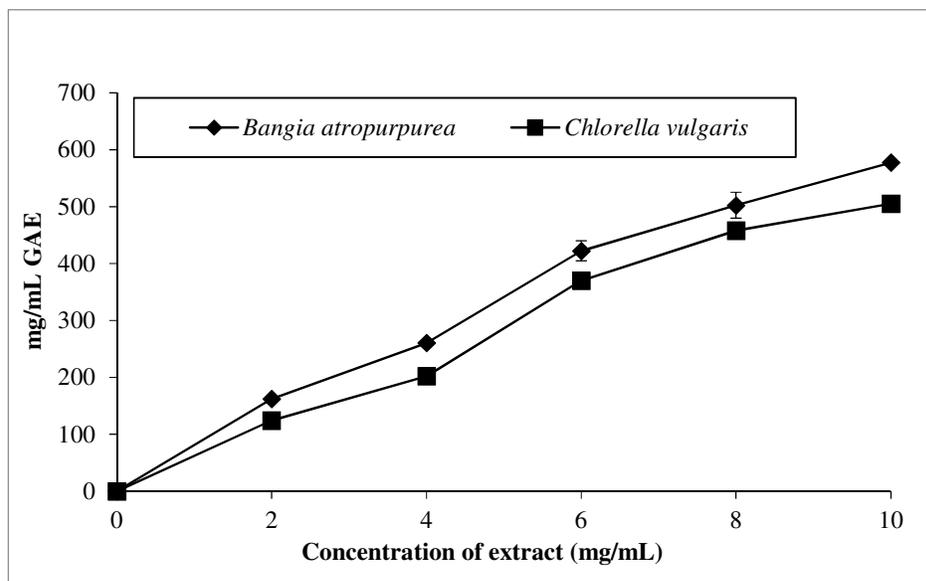
Figure 4.8: TPC extracted from *Bangia atropurpurea* and *Chlorella vulgaris* using methanol. Data were expressed as mean value ± S.D. (n = 3).

4.6 Evaluation of Antioxidant Activity

Other than determining the total phycobiliproteins in the sample, the TPC of *B. atropurpurea* was also determined. The antioxidant activity of the extracted phycobiliproteins and phenolic compounds was analysed by the DPPH radical scavenging and FRAP assays.

4.6.1 DPPH Radical Scavenging Assay

The DPPH radical scavenging method was used to evaluate the antioxidant capacity of the extracted and purified phycobiliproteins from *B. atropurpurea*. Apart from that, the antioxidant capacity of the TPC extracted using different extraction solvents from *B. atropurpurea* and *C. vulgaris* algae were also evaluated. The DPPH free radical inhibition activity increased with the elevation in the concentration of the phycobiliproteins and TPC extracts.

DPPH scavenging activity was relatively correlated with the concentration of the phenolic compounds in the extract. The higher the TPC in the extract, the higher the DPPH free radical scavenging activity. The IC_{50} of the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using five different extraction solvents covered a wide range of scavenging activity. The two different species, *B. atropurpurea* and *C. vulgaris*, showed the highest DPPH radical inhibition with the minimum methanol extract concentration. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol required the lowest concentration of extracts for IC_{50}, 30.82 ± 0.92 µg/mL extract and 34.28 ± 0.79 µg/mL extract, respectively. The TPC extracted from *B. atropurpurea* and *C. vulgaris* using hexane and water were low hence high concentration of hexane and water extracts were required for IC_{50}. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* with hexane required 98.97 ± 1.66 µg/mL and 109.05 ± 0.57 µg/mL, respectively. Meanwhile, the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* with water required higher IC_{50}, 135.32 ± 0.87 µg/mL and 139.90 ± 0.99 µg/mL, respectively (Table 4.6). The phenolic compounds extract concentration was observed influencing the IC_{50} in DPPH assay since the TPC concentration was indirectly proportional to IC_{50}.

Table 4.6: Comparison of DPPH radical inhibition of phenolic compounds extracted from *Bangia atropurpurea* and *Chlorella vulgaris* using different extraction solvents.

Extraction solvents	DPPH 50% Inhibition Concentration, IC_{50} ($\mu g/mL$)	
	Bangia atropurpurea	*Chlorella vulgaris*
Water	135.32 ± 0.87^d	139.90 ± 0.99^f
50% Aqueous methanol	64.78 ± 1.01^c	74.37 ± 1.61^c
Methanol	30.82 ± 0.92^a	34.28 ± 0.79^b
Ethyl acetate	46.18 ± 0.15^b	57.77 ± 0.81^c
Hexane	98.97 ± 1.66^c	109.05 ± 0.57^e

Mean value $\pm$ S.D. (n = 3), different letters in superscript indicate a significant difference at $p < 0.001$ between concentrations of the extracts by Tukey Kramer's post hoc test.

Figure 4.9 displays an obvious comparison that the phenolic compounds extracted from the red alga, *B. atropurpurea* using methanol contributed to better scavenging activity compared to the phenolic compounds extracted from the green alga, *C. vulgaris*. However, the DPPH radical scavenging activity for extracts from both algal species was lower than the scavenging activity by the synthetic antioxidant ascorbic acid, but was higher than BHT. According to Figure 4.9, the DPPH radical inhibition of phenolic extract from *B. atropurpurea* at 100 µg/mL concentration was 89.63%, which was higher than *C. vulgaris* (83.68%) and BHT (76.85%) but lower than ascorbic acid (98.86%).
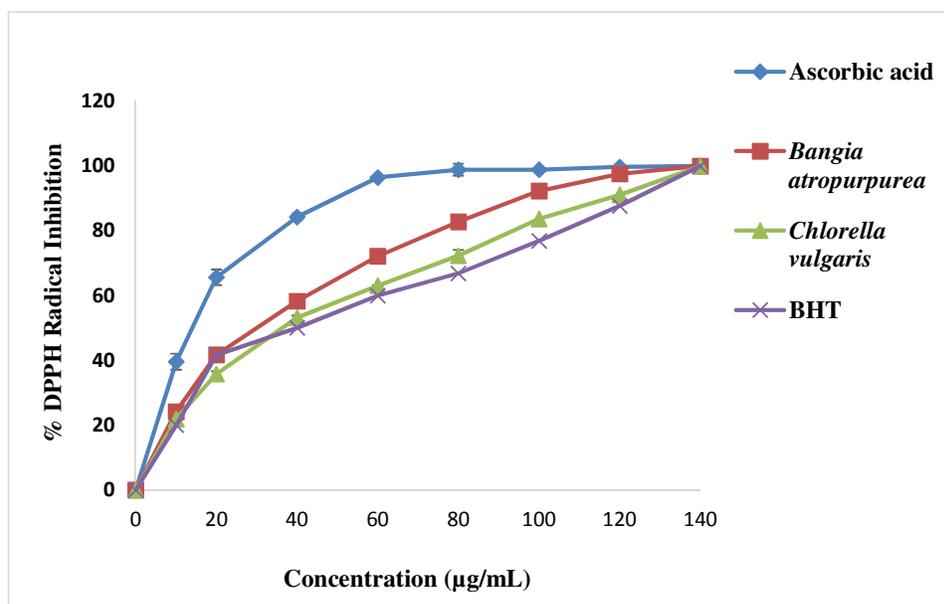
Figure 4.9: DPPH radical scavenging of phenolic compounds extracted from *Bangia atropurpurea* and *Chlorella vulgaris* using methanol. Data were expressed as mean value ± S.D. (n = 3).

The DPPH radical scavenging activity of phenolic compounds extracted from *B. atropurpurea* using methanol was compared with that of R-PE and R-PC extracts purified by gel filtration. The purified R-PE and R-PC extracts exhibited better scavenging activity compared to the phenolic extract and BHT (Figure 4.10). However, the ascorbic acid exhibited higher DPPH radical scavenging activity than R-PE and R-PC extracts. The purified R-PE and R-PC required 45.23 ± 0.13 µg/mL and 64.22 ± 0.78 µg/mL, respectively, to inhibit 80% of DPPH radicals, whereas a higher concentration TPC of 112.35 ± 0.21 µg/mL was required to inhibit the equal 80% of DPPH radical. The R-PE and R-PC did not display any drastic increment in the percentage of radical scavenging activity after 80 µg/mL extract concentration.

Referring to the same graph, it was determined that the ascorbic acid constantly has higher DPPH radical scavenging ability compared to the R-PE, R-PC and

phenolic compounds extracted from *B. atropurpurea*. The 100% DPPH radical inhibition was achieved by ascorbic acid at concentration of 80 µg/mL and the percentage of radical inhibition remained stable thereafter. The R-PE and R-PC extracts both achieved almost 100% DPPH radical inhibition at concentration of 120 µg/mL extract.
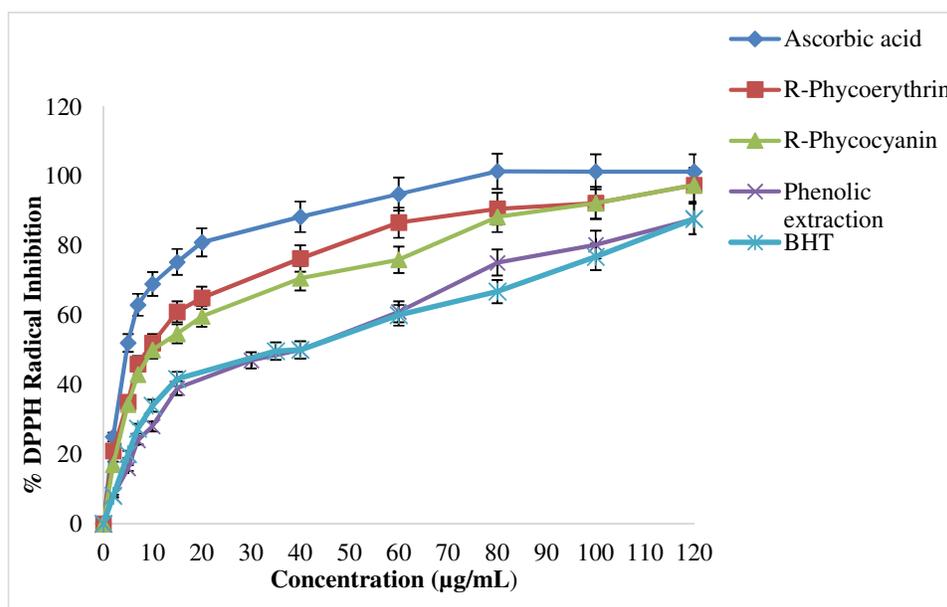
Figure 4.10: The percentage of DPPH radical inhibition by R-PE, R-PC and phenolic compounds extracted from *Bangia atropurpurea*. Data were expressed as mean ± S.D. (n = 3).

The antioxidant activity was expressed as IC_{50} and AEAC (ascorbic acid equivalent antioxidant capacity). When a minimum concentration of extract is required to inhibit DPPH radicals, the IC_{50} value will be lower and the AEAC value will be higher, which indicate that the extract has greater antioxidant capacity. The R-PE and R-PC extracts from *B. atropurpurea* showed an effective radical inhibition with IC_{50} of 7.66 ± 0.81 and 9.42 ± 1.73 µg/mL, respectively. Indirectly, the R-PE and R-PC extract exhibited very high AEAC

values, which were 2583.57 ± 423 mg AA/100g and 2336.78 ± 605 mg AA/100g, respectively, which suggests that R-PE and R-PC have a high antioxidant capacity (Table 4.7). The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* with methanol exhibited AEAC values of 1511.07 ± 487 mg AA/100g and 1234.28 ± 110 mg AA/100g, respectively, which were lower than AEAC values of R-PE, R-PC and ascorbic acid. Therefore, R-PE can be assumed an effective antioxidant, which presented greater antioxidant activity compared to the active compounds, R-PC and phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris*.

Table 4.7: The 50% of DPPH radical inhibition by R-PE, R-PC and phenolic compounds extracted from *Bangia atropurpurea* and *Chlorella vulgaris*.

Extracts	Species	DPPH scavenging activity	
		IC_{50} (µg/mL)	AEAC (mg AA/100g)
Phenolic extracts with Methanol	*B. atropurpurea*	30.82 ± 0.92^d	1511.07 ± 487
Phenolic extracts with Methanol	*C. vulgaris*	34.28 ± 0.79^d	1234.28 ± 110
R-PE	*B. atropurpurea*	7.66 ± 0.81^a	2583.57 ± 423
R-PC	*B. atropurpurea*	9.42 ± 1.73^b	2336.78 ± 605
BHT (control)	n/a	35.06 ± 1.15^e	n/a
AA (control)	n/a	6.78 ± 0.28^a	n/a

Mean value $\pm$ S.D. (n = 3), different letters in superscript indicate a significant difference at $p < 0.05$ between IC_{50} of the extracts by Tukey Kramer's post hoc test. AEAC: ascorbic acid equivalent antioxidant capacity; BHT: butylated hydroxytoluene; AA: ascorbic acid; n/a: not applicable

4.6.2 FRAP Assay

The FRAP of the extracts from *B. atropurpurea* and *C. vulgaris* was determined and expressed in mg GAE/g dry weight to make a comparison among the extracted phenolic compounds from *B. atropurpurea* and *C. vulgaris* using five different extraction solvents as listed in Table 4.8. The phenolic compounds extracted using methanol exhibited the highest reducing activity compared to the phenolic compound extracts by other extraction solvents. As shown in Table 4.8, the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol gave the highest reducing power of 37.81 ± 0.04 mg GAE/g dry weight and 23.97 ± 0.61 mg GAE/g dry weight, respectively. However, the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using 50% aqueous methanol and hexane solvent showed low reduction potential which is positively correlate with low TPC extracted from *B. atropurpurea* and *C. vulgaris* using 50% aqueous methanol and hexane solvent. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using 50% aqueous methanol exhibited ferric ion reduction potential at 18.95 ± 0.59 mg GAE/g dry weight and 11.98 ± 0.27 mg GAE/g dry weight, respectively. Whereas the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using hexane exhibited ferric ion reduction potential at 14.49 ± 0.28 mg GAE/g dry weight and 7.75 ± 0.22 mg GAE/g dry weight, respectively.

Table 4.8: Comparison of FRAP of phenolic compounds extracted from *Bangia atropurpurea* and *Chlorella vulgaris* using different extraction solvents.

	FRAP (mg GAE/g dry weight)	
Extraction Solvents	*Bangia atropurpurea*	*Chlorella vulgaris*
Water	5.22 ± 0.33^e	4.50 ± 0.15^e
50% Aqueous methanol	18.95 ± 0.59^c	11.98 ± 0.27^h
Methanol	37.81 ± 0.04^a	23.97 ± 0.61^f
Ethyl acetate	29.86 ± 0.91^b	15.93 ± 1.00^c
Hexane	14.49 ± 0.28^c	7.75 ± 0.22^i

Mean value±S.D., (n = 3), different letters in superscript indicate a significant difference at $p < 0.01$ between extracts by Tukey Kramer's post hoc test.

Figure 4.11 displays a ferric-reducing activity by phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol. The graph showed an obvious difference in the capacity of the two different species upon ferric ion reduction. Based on the graph, it was concluded that overall, the phenolic compounds extracted from *B. atropurpurea* have better antioxidant property than *C. vulgaris* especially when the concentration of the phenolic compounds extracted from *B. atropurpurea* was above 4 mg/mL where the reduction potential drastically increased and the effectiveness of ferric ion reduction activity doubled. The ferric ion reduction capacity by phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* at concentration of 8 mg/mL is far diverged, 317.61 ± 0.74 mg/mL GAE and 168.59 ± 0.69 mg/mL GAE, respectively (Figure 4.11).
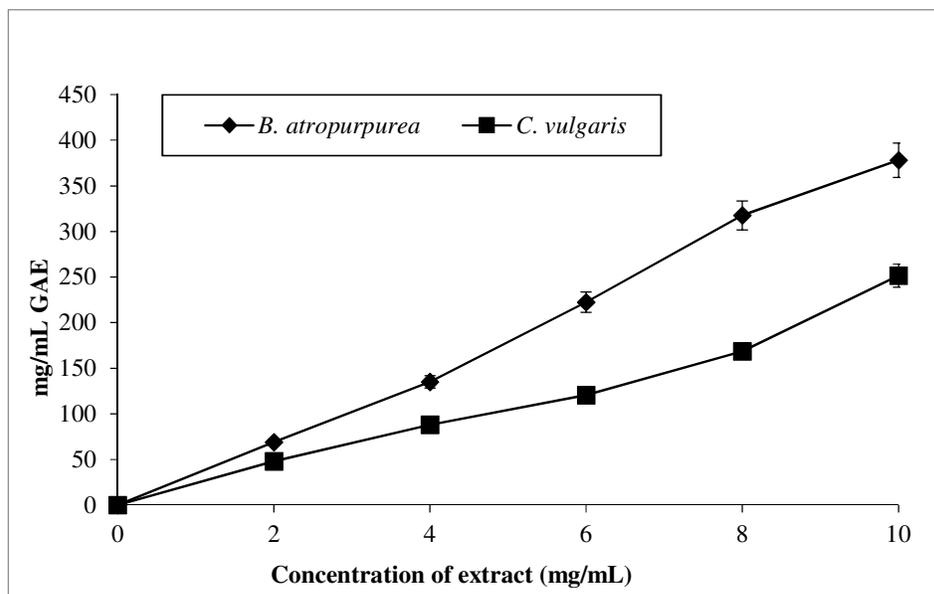
Figure 4.11: FRAP of phenolic compounds extracted from *Bangia atropurpurea* and *Chlorella vulgaris* using methanol. Data were expressed as mean value ± S.D. (n = 3)

The ferric-reduction potential of phenolic compounds extracted from *B. atropurpurea* using methanol was compared with those of R-PE and R-PC extracts purified by gel filtration. According to Figure 4.12, it was determined that the purified R-PE and R-PC extracts exhibited far greater reduction activity compared to BHT. However, ascorbic acid exhibited higher reduction activity than R-PE and R-PC extracts. The purified R-PE and R-PC exhibited equal reduction capacity at 348.96 ± 0.93 mg/mL GAE at the concentration 8 mg/mL, while BHT and ascorbic acid exhibited 54.46 ± 0.27 mg/mL GAE and 522.49 ± 0.67 mg/mL GAE at the concentration 8 mg/mL. Thus, it was concluded that R-PE and R-PC have a better reduction capacity compared to BHT however, lower reduction capacity compared to ascorbic acid. The FRAP of phenolic compounds in methanol was 255.45 ± 0.83 mg/mL GAE at the concentration of 8 mg/mL extract which is five times more effective ferric-reduction potential compared to BHT.
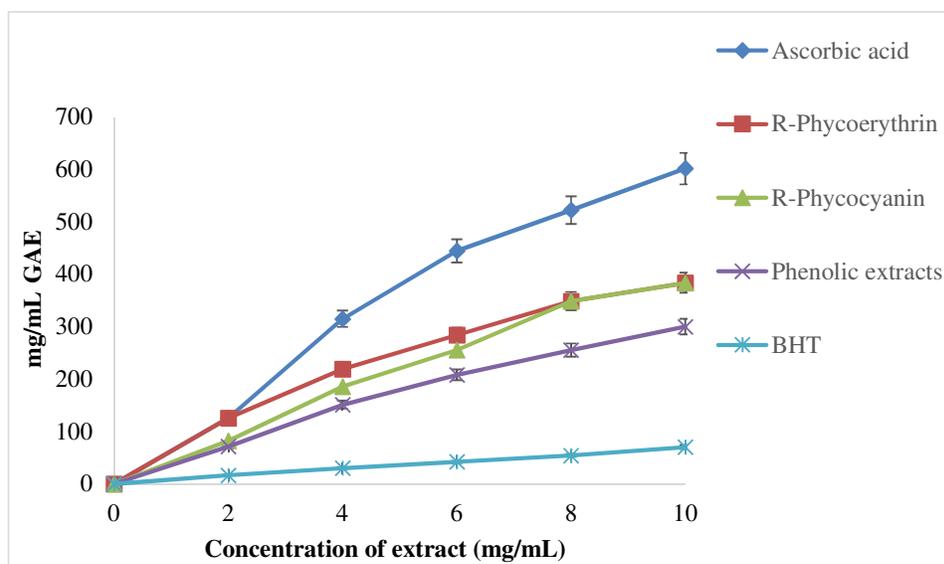
Figure 4.12: FRAP of R-PE, R-PC and phenolic compounds extracted from *Bangia atropurpurea*. BHT: Butylated hydroxytoluene

The antioxidant activity was expressed by comparing the concentration equivalent to gallic acid. Table 4.9 indicates that the R-PE and R-PC exhibited higher antioxidant capacity with the FRAP of 54.81 ± 0.31 mg GAE/g dry weight and 42.18 ± 0.70 mg GAE/g dry weight, respectively. Since the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol exhibited very high TPC, only the methanol extracts were used to compare the reduction potential with extracted R-PE and R-PC. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* showed two times lower reduction capacity compared to R-PE and R-PC. Thus, R-PE and R-PC were considered effective antioxidants presenting greater ferric ion reduction capacity compared to the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris*. The reliability of the results was evaluated by comparing the R-PE, R-PC and phenolics methanolic extract with two other synthetic antioxidants namely ascorbic acid and BHT as shown in Table 4.9.

Table 4.9: The FRAP of R-PE, R-PC and phenolic compounds extracted from *Bangia atropurpurea* and *Chlorella vulgaris*.

| Extracts	Species	(mg GAE/g dry weight)
Phenolic extracts with methanol	*Bangia atropurpurea*	37.81 ± 0.04^a
Phenolic extracts with methanol	*Chlorella vulgaris*	23.97 ± 0.61^b
R-PE	*Bangia atropurpurea*	54.81 ± 0.31^c
R-PC	*Bangia atropurpurea*	42.18 ± 0.70^c
BHT (control)	n/a	$30.37 \pm 0.12a^b$
AA (control)	n/a	65.77 ± 0.12^d

Mean value ± S.D. (n = 3), different letters in superscript indicate a significant difference at $p < 0.01$ between extracts by *t*-test analysis. BHT: butylated hydroxytoluene; AA: ascorbic acid; n/a: not applicable

4.7   The Correlation between TPC with DPPH and FRAP Results

A comparison on TPC among all five extraction solvents was determined for *B. atropurpurea* and *C. vulgaris* separately. Then, the TPC comparison was carried out between that of the two different algal species. Based on the comparison, it was found that the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol exhibited the highest TPC value compared to the TPC in the four other extraction solvents namely water, 50% aqueous methanol, ethyl acetate and hexane. Meanwhile, comparing the TPC between *B. atropurpurea* and *C. vulgaris* extracts determined that on average, *B. atropurpurea* contains higher TPC compared to *C. vulgaris*. For instance, phenolic compounds extracted from *B. atropurpurea* using methanol exhibited higher TPC, 80.97 ±

0.53 mg GAE/g dry weight compared to *C. vulgaris* TPC, 62.13 ± 1.28 mg GAE/g dry weight only (Table 4.5).

The TPC of *B. atropurpurea* and *C. vulgaris* was correlated with FRAP and DPPH radical scavenging activity in Table 4.10. The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using five different extraction solvents were analysed with the FRAP and DPPH assays. The DPPH assay indicated that the phenolic compounds extracted from *B. atropurpurea* using methanol showed the greatest DPPH radical inhibition compared to DPPH assay with phenolic compounds extracted from *B. atropurpurea* using the four other extraction solvents. With TPC of 80.97 ± 0.53 mg GAE/g dry weight extracted from *B. atropurpurea* using methanol effectively exhibited radical inhibition with minimum concentration, which was IC_{50}, 30.82 ± 0.92 µg/mL. Similarly with TPC, 62.13 ± 1.28 mg GAE/g dry weight extracted from *C. vulgaris* using methanol exhibited the radical inhibition with the lowest IC_{50} of 34.28 ± 0.79 µg/mL compared to DPPH assay test with phenolic compounds extracted from *C. vulgaris* using four other extraction solvents. The FRAP assay analysed with phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol, showed the greatest ferric ion reduction of 37.81 ± 0.04 mg GAE/g dry weight and 23.97 ± 0.61 mg GAE/g dry weight, respectively, compared to the FRAP assay analysis with phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using the four other extraction solvents.

Table 4.10: The TPC correlation with DPPH radical scavenging and FRAP results

| Species | TPC (mg GAE /g dry weight) | Antioxidant activity (AOA) | |
		DPPH IC$_{50}$ (µg/ml)	FRAP (mg GAE /g dry weight)
Bangia atropurpurea	80.97 ± 0.53	30.82 ± 0.92	37.81 ± 0.04
Chlorella vulgaris	62.13 ± 1.28	34.28 ± 0.79	23.97 ± 0.61

A positive relationship was observed between phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* with the antioxidant activity observed based on the FRAP and DPPH radical scavenging assays (Figures 4.13 and 4.14). Analysis of the correlations between TPC and the antioxidant properties measured with FRAP and DPPH radical scavenging assays showed good correlations by exhibiting high regression coefficients of $R^2 = 0.925$ and $R^2 = 0.898$, respectively. The data provided suggest that the phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* were seen exhibiting as powerful scavengers of free radicals and effective reducing agents.
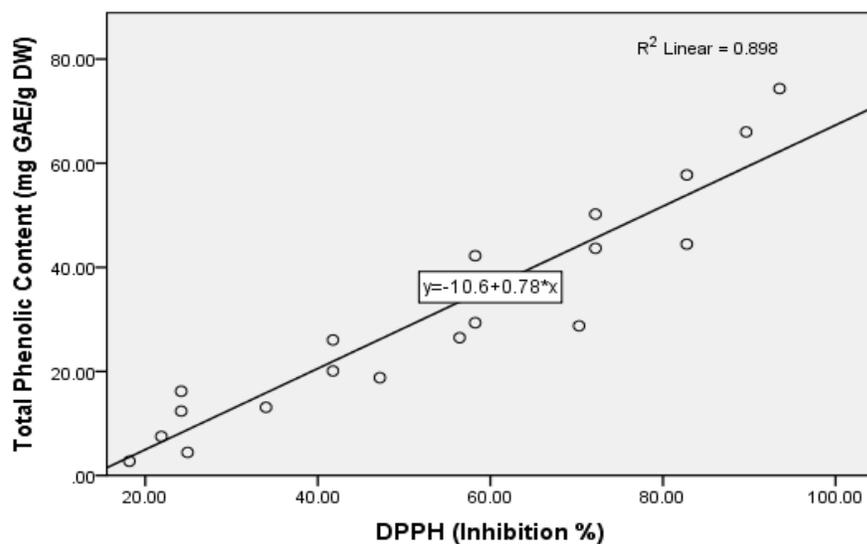
Figure 4.13: The correlation of TPC extracted from *B. atropurpurea* and *C. vulgaris* with DPPH radical inhibition. The statistical analysis (SPSS 21) indicates positive correlation between TPC and antioxidant activity with significant difference at $p < 0.001$.
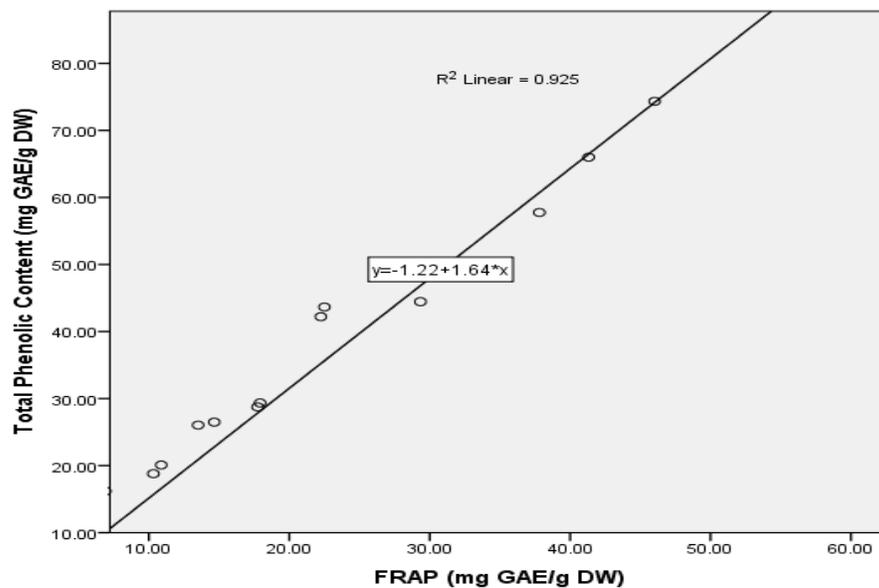
Figure 4.14: The correlation of TPC extracted from *B. atropurpurea* and *C. vulgaris* with FRAP. The statistical analysis (SPSS 21) indicates a positive correlation between TPC and antioxidant activity with significant difference at $p < 0.01$.

CHAPTER 5

DISCUSSION

5.1 Evaluation of the Concentrations of R-PE and R-PC Extracted from *Bangia atropurpurea*

The phycobiliproteins concentration in *B. atropurpurea* was quantified. The phycobiliproteins in this red alga comprised of R-PE, R-PC and APC. Phycobiliproteins such as R-PE and R-PC are soluble in water since they have hydrophilic amino acids on their surfaces that attract water molecules that interact with them. The solubility of the protein in water depends on the ionic strength and pH of the buffer (Aftabuddin and Kundu, 2007). The phosphate buffer used for extraction and elution of R-PE and R-PC through the gel filtration column was maintained at pH 7.2. The pH was maintained since the R-PE and R-PC can be easily destroyed under alkaline condition at pH above 7.5 (Niu et al., 2006).

According to Kim et al. (2011), the apoprotein of phycoerythrin is readily broken in water to release the chromophore phycoerythrobilin. The peroxy radical scavenging capacity of phycoerythrobilin is twofold greater than phycoerythrin. Meanwhile, the chromophore phycocyanobilin of phycocyanin is further reduced to phycocyanorubin under acidic condition (Jing et al., 2013), which can inhibit the formation of superoxide radicals (Matthew et al., 2007). In conclusion, phycoerythrin and phycocyanin can be broken down to phycoerythrobilin and phycocyanobilin chromophores, respectively, to function

as efficient antioxidants to impose various therapeutic activities in the human body (Wu et al., 2015).

The peptide chain of phycobiliproteins is able to inhibit the generation of ROS which is associated with aging, neurodegenerative diseases and other oxidative activity related diseases (McCarty et al., 2010). The essential amino acid compositions in the peptide chain contribute to such applications. Amino acids with hydrophobic branch chains are good proton and electron donor. Similarly, acidic, basic and aromatic amino acids facilitate DPPH radical scavenging by donating proton and reducing ferric metal ions by donating electron (Sarmadi and Ismail, 2010). In particular, it was reported by Sonani et al. (2015), that PE has low chelation capability but a high reduction capacity when compared with those of PC and APC, which indicates that PE plays an important role in oxidation-reduction reactions. According to the study by Harnedy and Fitz (2011), the amino acids synthesised from PE exhibit higher ROS removal activities compared with the amino acids synthesised from PC or APC. ROS include free radical species namely superoxide anions, hydroxyl radicals, singlet oxygen and non-radical species, including hydrogen peroxide.

The concentration of R-PE increased from 1.569 mg/mL to 1.718 mg/mL when the fraction obtained by gel filtration with Sephadex-G-200 was further purified by RP-HPLC. Meanwhile, the concentration of R-PC increased from 0.604 mg/mL to 0.884 mg/mL using the same purification technique. Sampath-Wiley and Neefus (2007) used the same filtration method, which is gel filtration followed by RP-HPLC to determine R-PE and R-PC of *Porphyra purpurea*, a

medium-sized red alga in the division of Rhodophyta. The results showed that the concentrations of R-PE and R-PC increased from 0.464 mg/mL to 0.872 mg/mL, respectively, which are lower compared to the concentrations of R-PE and R-PC achieved with *B. atropurpurea*.

It has been reported by other studies that the phycoerythrin contents obtained from purified red algae namely *Portieria hornemannii, Gracilaria corticata* and *Gelidiella acerosa,* ranged from 0.39 mg/g to 1.23 mg/g of dry weight, while the contents of phycocyanin ranged from 0.18 mg/g to 0.36 mg/g (Ganzon, 1997; Kumar et al., 2010; Senthilkumar et al., 2013). This shows that *B. atropurpurea* is a species that abundantly contains phycoerythrin and phycocyanin compared to other red algae species surveyed earlier by other researchers. This indicates that R-PE is the predominant phycobiliprotein in *B. atropurpurea* species. As such, the R-PE is the major component in the phycobilisome structure attached to the thylakoid membrane.

In this study, the APC was completely filtered out after gel filtration with Sephadex G-200 (Table 4.1). The APC became untraceable in the fraction collected by gel filtration, which indicates an insignificant amount of APC presented in *B. atropurpurea* species. Similar observation was reported by the study of Sampath-Wiley and Neefus (2007), which confirmed that APC was untraceable after gel filtration of *P. purpurea* extract.

Phycobiliproteins are brilliantly coloured, highly fluorescent components of the photosynthetic light-harvesting antenna complexes of red algae, cyanobacteria

(blue-green algae) and cryptomonads. The phycobiliprotein content analysis was not performed on *C. vulgaris* since this green alga does not have the fluorescent components like R-PE and R-PC. Instead, green algae generally contain an abundance of the green pigment chlorophyll located within the thylakoid membrane and stroma (Ortiz et al., 2014).

5.2 Evaluation of the Purity Indices and Total Recovery Yields of R-PE and R-PC Extracted from *Bangia atropurpurea*

The absorption spectrum of the extracted phycobiliproteins from *B. atropurpurea* have determined the maximum absorbance wavelengths of R-PE, R-PC and APC to be 562 nm, 615 nm and 652 nm, respectively, while the maximum absorbance wavelength of the total protein in the extract was determined at 280 nm. From Tables 4.3 and 4.4, the maximum absorbance readings of R-PE and R-PC elevated at every subsequent stage of the purification process, which were indirectly proportional with the absorbance reading of the total protein. This implies that the purity index increased with subsequent purification processes (Punampalam et al., 2018).

Referring to Tables 4.3 and 4.4, after each purification stage, the purity index and the recovery yield of the extracts obtained were quantified. Sedimentation of crude extract was gradually purified with 35% followed by 65% $(NH_4)_2SO_4$ saturation, which exhibited 31.8% of the recovery yield of R-PE from the total protein extracted with the purity index of 2.47. Meanwhile, 25.3% of R-PC was recovered from the total protein extracted from *B. atropurpurea* with the purity index of 0.77. Kozhummal and D. Kaushik (2005) reported 20% of recovery yield of R-PE from *Nostoc muscorum* species with 55% $(NH_4)_2SO_4$ saturation,

73

whereas the purity index was 2.89. Even though a higher purity index was achieved, a lower recovery yield was attained with 55% $(NH_4)_2SO_4$ saturation. In addition, Kozhummal and Kaushik (2005) and Sara et al. (2015) obtained a very high recovery yield for R-PC. Sara et al. (2015) reported, 80% of recovery yield for R-PC from the blue-green algae, *Phormidium* sp., *Lyngbya* sp. and *Halomicronema* sp. after saturating the crude extracts with 70% $(NH_4)_2SO_4$. However, the purity index was only 0.66, which indicates that the high recovery yield obtained might have been contributed by contaminant proteins.

The study involving RP-HPLC analysis after gel filtration was considerably a simple and efficient purification method to purify R-PE. The purity index of R-PE increased from 4.76 to 5.45 with the total recovery yield increased from 77.9% to 94.4%. Meanwhile the purity index of R-PC increased from 2.80 to 3.95 with the total recovery yield increased from 60.4% to 86.1%. However, Tchernov et al. (1999) achieved a high recovery yield of 60% for the R-PE from *Nostoc* sp. with purity index of 3.2 using frozen extract, which was dialysed in phosphate buffer with the rivanol compound and further purified by chromatographic method. Moreover, Galland-Irmouli et al. (2000) indicated that 18% R-PE was recovered from the red alga, *Palmaria palmate*, when the extract was frozen with liquid nitrogen and extracted with a phosphate solution before further purification by electrophoresis technique. Besides, Hilditch et al. (1991) proposed a method based on three-step chromatography from *Corallina officinalis* with a recovery yield lower than 1%. A total recovery yield of 49.5% for phycocyanin with the purity index of 4.18 was obtained from *Arthrospira platensis* after further purification by gel filtration with Sephadex G-100 and

ion-exchange chromatography (Ratana et al., 2018). Meanwhile, Devendra et al. (2014) reported the recovery yield of phycocyanin from *Spirulina platensis* by single step chromatography using DEAE-Cellulose-11 achieved 80% with the purity index of 1.5 which indicate a low purity level.

Since the purification of phycobiliproteins is a burdensome process involving a huge investment on an industrial scale, achieving maximum purity with minimum investment would be a feasible option (Hafting et al., 2015). The methods implemented in the final purification stage were very effective. The gel filtration with Sephadex G-200 and RP-HPLC contributed to a higher percentage of recovery yield from the total protein extracted concurrently with the increase of purity index of the extracted phycobiliproteins (Tables 4.3 and 4.4). Based on the comparison with the findings reported by the earlier researchers, it can be concluded that *B. atropurpurea* naturally has higher R-PE and R-PC contents than other species. This is because other researchers who employed the same purification technique, gel filtration and RP-HPLC, failed to establish a high recovery yield as achieved in this study with *B. atropurpurea.*

5.3 SDS-PAGE of Purified R-PE and R-PC Extracted from *Bangia atropurpurea*

Generally, the purity of R-PE and R-PC was evaluated based on the absorbance ratio $A_{max\ visible}/A_{280}$. However, the purity of the sample can be verified following gel electrophoresis analysis. Electrophoresis analysis showed more precise fractions of R-PE and R-PC by presenting a single narrow band on slab-gel. At the same time, the commercial R-PE and R-PC were also analysed to compare if the purified R-PE and R-PC bands are parallel with their respective commercial phycobiliproteins.

The molecular weight of R-PE and R-PC extracted from *B. atropurpurea* was determined by SDS-PAGE. A typical red and blue pigmented R-PE and R-PC complex of red algae were held by various originations in molecular mass from 15 kDa to 22 kDa (Bermejo et al., 2003; Sun et al., 2009; Wang et al., 2014). Based on an earlier report by Senthilkumar et al. (2013), the molecular mass of R-PE from *Portieria hornemannii* obtained through SDS-PAGE has a protein band with the size of 21 kDa. Similar results were observed for *Porphyridium cruentum* (Bermejo et al., 2003; Benavides and Rito, 2004) and *Porphyra yezoensis* (Sakai et al., 2011). The molecular mass of the R-PE of *P. cruentum* was 18 kDa, while the R-PE fraction eluted from *Porphyra yezoensis* extracts presented protein band on the slab-gel at 19 kDa. The molecular mass of purified R-PC from the red macroalga *P. urceolata* determined by SDS-PAGE has one protein band at 17.5 kDa (Wang et al., 2014). Devendra et al. (2014) reported, the SDS-PAGE band of purified PC from *S. platensis* has double PC protein band isotopes at 16 kDa and 17 kDa.

In this study, the molecular mass of the R-PE and R-PC proteins extracted from *B. atropurpurea* was 20.5 kDa and 17.6 kDa, respectively (Figures 4.2 and 4.4). The single protein bands of R-PE and R-PC appeared on the slab-gel were narrower as the total R-PE and R-PC contents in the sample became more concentrated and purified after a few filtration processes with $(NH_4)_2SO_4$ saturation and eluted through gel filtration. In order to obtain a higher resolution of phycobiliprotein bands on the gel, a higher percentage of polyacrylamide gel was used. The protein bands of R-PE and R-PC from *B. atropurpurea* were separated by SDS-PAGE on 16.5% polyacrylamide gel as referring to the method of Pushparajan et al. (2017) to obtain a clearer vision of bands under Coomassie stain instead of 12% as mentioned in the other studies by Bermejo et al. (2003) and Wang et al. (2014) where the protein bands appeared thicker and merged.

5.4   Analysis of the Novelty of the Purified R-PE and R-PC by Comparison with Commercial Phycobiliproteins

Generally, the types of phycobiliproteins can be determined based on their absorption spectra. The bright pinkish coloured phycobiliproteins fall into three distinct species; R-PE (λmax ~ 495-565 nm), B-PE (λmax ~540-560 nm) and C-PE (λmax ~543 and ~492 nm). The red pigmented phycoerythrin extracted from *B. atropurpurea* has the maximum wavelength of 562 nm. Thus, the phycoerythrin isolated from *B. atropurpurea* was R-PE. The prefixes R-, B- and C- are used for indicating the organisms from which the pigment proteins were originally extracted; for instance, R-PE from Rhodophyta (red algae), B-PE from Bangiales and C-PE from Cyanophyta (blue-green algae) (Glazer et al., 1976; Bryant, 1982; Tandeau de Marsac, 2003). The blue pigmented

phycocyanin was subdivided into three different species according to the maximum wavelength of R-PC (λmax~615 nm) mainly in red algae and C-PE (λmax~615-620 nm), which exclusively exists in blue-green algae (Glazer et al., 1976; Bryant, 1982; Tandeau de Marsac, 2003).

The high resolution of HPLC is able to separate the proteins based on very small differences in their structure (Cruz et al., 1997). Hence, slight differences in phycobiliprotein structure and presence of any other contaminant proteins were detected on the chromatograms and appeared as different peaks. Multiple peaks on the chromatograms appeared very obvious for extract with lower purity indices. The R-PE and R-PC extracted from *B. atropurpurea* were injected into RP-HPLC after $(NH_4)_2SO_4$ saturation achieved purity indices of 2.47 and 0.77, respectively. Thus, at a lower purity index with the presence of high percentage of impurities, multiple peaks with retention times of 4.3, 5.8 and 7.8 minutes were observed for R-PE and retention times of 1.8, 2.5 and 4.3 minutes were observed for R-PC. Nevertheless, the contaminant protein peaks were eliminated from RP-HPLC analysis by gel filtration with Sephadex G-200. The RP-HPLC analysis reflected only a single peak for R-PE at the purity index of 5.42 and retention time of 5.8 minute. The clear sharp single peak of R-PC was observed at the retention time of 2.5 minute with a high purity index of 3.95.

5.5   TPC Extracted from *Bangia atropurpurea* and *Chlorella vulgaris* using Different Extraction Solvents

The highest TPC has been found in those algae species with long exposure to solar radiation (Henrik and Elisabet, 2000). The algae naturally produce phenolic compounds within their cell membrane to overcome the oxidative stress due to long exposure to photo-radicals (Kubanek et al., 2004). Phenolic compounds serve as antioxidants because of their ability to donate the hydrogen atoms or the electrons from the benzene rings and hydroxyls attached in their molecular structure to form a stable radical intermediate to retard ROS. (Mathew et al., 2015). The natural phenolic compounds from different families, for example hydroxyphenyl, polyphenol, hydroxybenzoic and phenylpropenoic, have different solubility levels, hence the TPC in *B. atropurpurea* and *C. vulgaris* was extracted using five different extraction solvents with different polarity, namely water, 50% aqueous methanol, methanol, ethyl acetate and hexane to determine the best extraction solvent to yield the highest concentration of phenolic compounds from these species.

The extraction efficiency of five different solvents was enhanced by sonication of the mixture with high intensity sound waves to disrupt cells, allowing to extract phenolic compounds from ruptured cell membrane and further shaking the mixture using orbital shaker after sonication to increase the solubility of the phenolic compounds in the solvent (Bushra et al., 2009; Zou and Hou, 2017). Phenolic compounds are soluble in water to some extent due to their ability to form hydrogen bonding with water molecules. Phenolic compounds have a diversity of structures, from rather simple structures, for example phenolic acids and complex structures such as flavonoids. Since extraction of phenolic

compounds were limited by sonication and subsequently shaking at room temperature hence, the solubility of large phenolic molecules was limited in water because high temperature is required to break the strong hydrogen bonds between water molecules before readily dissolve the large phenyl groups of phenolic compounds (Hiba et al., 2014). Thus, in this study the solubility of phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* was lower in water (TPC, 5.21 ± 0.03 mg GAE/g dry weight and 4.37 ± 0.04 mg GAE/g dry weight) and 50% aqueous methanol (TPC, 37.58 ± 0.64 mg GAE/g dry weight and 24.53 ± 0.93 mg GAE/g dry weight), respectively, though these solvents have high polarity. According to Lou et al. (2014), the extraction of certain phenolic compounds such as tangeretin, ferulic acid, p-coumaric acid increased with heat water treatment (150°C), however, the extraction yields of phenolics like flavonoids, tannins and anthocyanins decreased drastically in heat water treatment as degradation of phenolics occurs. Thus, extraction of phenolic compounds using water at room temperature or with heating will only able to extract certain phenolic compounds, thus recovering the maximum phenolic yields with water was found to be complicated. Indeed, strong interactions of some phenolic compounds with plant cell walls could also limit the phenolics extraction (Véronique, 2012).

Thus, many extraction solvents have to be used for extraction in order to identify the most suitable solvent with appropriate polarity to dissolve maximum phenolic compounds. In this study, methanolic extraction yielded the highest TPC compared to the four other solvents used in this study. The phenolic compounds extraction from *B. atropurpurea* and *C. vulgaris* using methanol

showed the highest TPC of 80.97 ± 0.53 mg GAE/g dry weight and 62.13 ± 1.28 mg GAE/g dry weight, respectively (Table 4.5). Meanwhile, ethyl acetate extract exhibited TPC of 61.84 ± 0.39 mg GAE/g dry weight and 44.15 ± 5.02 mg GAE/g dry weight of the *B. atropurpurea* and *C. vulgaris*, respectively. Based on the current study, hexane extract contained moderately lower phenolic compounds with TPC of 7.55 ± 0.14 mg GAE/g dry weight and 6.06 ± 0.24 mg GAE/g dry weight for *B. atropurpurea* and *C. vulgaris*, respectively.

Cho et al. (2011) reported that, the TPC varies among algae species and extraction solvents. The TPC of the crude extract and solvent-partitioned fractions of *Enteromorpha prolifera*, a type of green algae, ranged from 46.2 mg GAE/g dry weight to 80.4 mg GAE/g dry weight. These values were considerably similar to the results obtained by this study. The phenolic content of the ethyl acetate fraction of *Polysiphonia urceolata*, a red alga, was 73.7 mg GAE/g dry weight (Duan et al., 2006) whereas, the phenolic content of the ethanol extract of *Papenfussiella kuromo*, a brown alga, was only 0.18 mg GAE/g dry weight (Kuda et al., 2005). Devi et al. (2011) reported that the total phenol concentrations of methanol and ethyl acetate extracts of *Turbinaria conoides* were 1.23 mg GAE/g dry weight and 1.19 mg GAE/g dry weight, respectively. Several studies have reported the antioxidant activity of extracts from various types of algae that might be correlated with the total phenolic content in the extract. Based on this study and as reported by Farasat et al. (2013) as well as Chakraborty et al. (2015), the red algae have higher TPC as compared to the green algae.

5.6   Antioxidant Activity by DPPH Radical Scavenging Assay

Marine algae are a rich source of bioactive metabolites, including phenolic compounds which contribute to their antioxidant activity (Fernando et al., 2016). Reactive oxygen and free radicals are produced during oxygenic photosynthesis by microalgae. Meanwhile, antioxidant compounds are produced by microalgae as their defence to avoid oxidative damage (Lu and Foo, 1995) and serve as potent chemical blockers of UV radiation (Bongards and Gartner, 2010). Antioxidant activity is an important property of algal bioactive compounds, which has been ascribed to their ROS scavenging ability, singlet oxygen quenching ability, reducing power and chelating ability (Andrade et al., 2013; Maharana et al., 2015). The presence of different types of phenolic compounds in extracts therefore requires different assays to measure the antioxidant property of algal extracts. In this study, DPPH and FRAP assays were used to evaluate the antioxidant property of phycobiliproteins and phenolic compounds extracts.

DPPH assay is an easy and rapid method. In the presence of an antioxidant, a yellow coloured solution will be observed when antioxidant reacts with the deep-violet coloured 1,1-diphenyl-2-picrylhydrazyl (DPPH). Methanol has a suitable polarity to dissolve maximum phenolic compounds, thus the TPC for *B. atropurpurea* methanol extracts was the highest. The methanol extract with the highest TPC, exhibited AEAC 1511.07 ± 487 mg AA/100g. Similar trend was reported in earlier studies by Lai and Lim (2011), which stated the high correlation of TPC readings with AEAC values.

The DPPH free radical inhibition activity increased with increasing concentration of TPC, which were the potential antioxidant compounds in the extracts. This can be determined with absorbance readings measured by UV-visible spectrophotometer. According to Figure 4.9, the DPPH scavenging activity of methanol extract at 100 µg/mL concentration for *B. atropurpurea* (89.63%) was found to be higher than *C. vulgaris* (83.68%). The concentration of 100 µg/mL of methanol extract of *Spatoglossum asperum* presented DPPH inhibition activity of 74.67%, which is similar to that in earlier findings by Vinayak et al. (2011). According to Paola et al. (2017), the DPPH radical scavenging activity at the concentration of 100 µg/mL of phenolics in the ethanol extract from the red alga, *Cystoseira osmundacea* was the most active (67.9%) compared to the other algae analysed. Other red algae, which gave considerably high antioxidant activities were *Padina concrecens* (62.8%), *Eisenia arborea* (58.8%) and *Acanthophora spicifera* (50.4%) (Paola et al., 2017). Based on the comparison done, the algal extracts showed antioxidant activity at various degrees in which green algae *Chaetomorpha linum* exhibited relatively high antioxidant activity (83.6%), which was significantly different compared to those of *Chaetomorpha aerea* (57.2%), *Chaetomorpha brachygona* (48.9%) and *Chaetomorpha crassa* (22.98%) with 100 µg/mL of methanol extract (Massoumeh et al., 2013). In conclusion, the data collected would certainly reflect the high potency of *B. atropurpurea* as natural antioxidants, which displayed the highest percentage of radical inhibition at the same concentration.

DPPH radical scavenging activity of the partially purified PE from the red algae, *Leptolyngbya* sp. and *Porphyra haitanensis* prepared by $(NH_4)_2SO_4$ precipitation and Q-Sepharose column chromatography resulted the IC_{50} of 10.67 ± 1.51 µg/mL and 18.12 ± 1.96 µg/mL, respectively (Chayakorn et al., 2011). The R-PE and R-PC extracts from *B. atropurpurea* showed effective radical inhibition with IC_{50} of 7.66 ± 0.81 µg/mL and 9.42 ± 1.73 µg/mL, respectively in the study.

5.7   Antioxidant Activity by FRAP Assay

The antioxidant capacity was also evaluated by the FRAP assay. In this study, the methanol extract displayed the highest reducing power, while the water and hexane extracts showed equally lower potency. In this assay, the antioxidant activity was determined based on the ability of antioxidant compounds present in algal extracts to reduce ferric iron (III) to ferrous iron (II) in a colourimetric redox reaction that simply involves the transfer of electrons (Sapan et al., 1999; Moniruzzaman et al., 2012). Reducing agents present in the extracts promoted the reduction of the ferrocyanide, Fe^{3+} complex to the ferrous form (Fe^{2+}) in which the concentration of Fe^{2+} ion was measured at the absorbance of 700 nm (Ganesan et al., 2011).

The phenolic compounds extracted from *B. atropurpurea* and *C. vulgaris* using methanol, displayed the highest FRAP at 37.81 ± 0.04 mg GAE/g dry weight and 23.97 ± 0.61 mg GAE/g, respectively. This high FRAP of *B. atropurpurea* and *C. vulgaris* extracts was in correspondence to the fact that phenolic compounds have high solubility in methanol by displaying high TPC value of

80.97 ± 0.53 mg GAE/g dry weight and 62.13 ± 1.28 mg GAE/g dry weight, respectively. Referring to the data provided by Daniel et al. (2016), the phenolic compounds from the red algae, *Osmundaria obtusiloba* and *Pterocladia capillacea* with 70% aqueous ethanol gave the FRAP of 18.76 ± 0.54 mg GAE/g dry weight and 13.06 ± 2.21 mg GAE/g dry weight, respectively. Further survey on FRAP of green algae found that methanol extracts from *Desmococcus olivaceous* and *Chlorococcum humicola*, exhibited lower antioxidant capacities with the FRAP of 9.82 mg GAE/g dry weight and 8.77 mg GAE/g dry weight, respectively (Uma et al., 2011).

In this study, other than phenolic compounds, the antioxidant capacity of *B. atropurpurea* was exerted by the presence of phycobiliproteins. The R-PE and R-PC extracted from *B. atropurpurea* showed an average FRAP of 54.81 ± 0.31 mg GAE/g dry weight and 42.18 ± 0.70 mg GAE/g dry weight, respectively, compared to a very high FRAP exhibited by the synthetic antioxidant, ascorbic acid at 65.77 ± 0.12 mg GAE/g dry weight. Comparing to the results by other researchers for purified PE extracts, the FRAP of *Leptolyngbya* sp. and *Porphyra haitanensis* was far lower, which was 7.44 ± 0.14 mg GAE/g dry weight and 3.89 ± 0.08 mg GAE/g dry weight, respectively (Chayakorn et al., 2011).

5.8   Limitations of Study

In this study the phycobiliproteins, R-PE, R-PC and APC have been exclusively investigated for their antioxidant or free radical scavenging potentials after a few separation and purification techniques employed. The separated phycobiliproteins were further analysed by two different antioxidant assays to determine their antioxidant properties. However, there were several limitations to the present study. The sampling size was limited in this study where the findings may evade from being generalised. Thus, increasing the number of red algae, green algae and cyanobacteria species in the study may amplify the differences detected and emphasise statistical correlation between different species.

Apart from that, the phycobiliprotein structure may be further explicated. The phycobilisome structure attached to the thylakoid membrane has been extracted and separated into specific phycobiliproteins. However, the phycobiliproteins, R-PE, R-PC and APC have rather complicated structures. Phycobiliproteins are composed of apoproteins providing chromophores, which give unique spectral features to them. Phycobiliprotein apoprotein comprises two dissimilar peptides namely α- and β- subunits. The α- and β- subunits were further associated to form trimers ($\alpha_3\beta_3$) and hexamers (($\alpha_3\beta_3$)$_2$) and sequentially stacked to construct the giant light harvesting complex, phycobilisome with 160 to 180 amino acid residues, respectively, which is responsible for the antioxidant activity (Anderson and Grossman, 1990). Therefore, it is necessary to further elucidate the structure of the phycobiliproteins extracted from *B. atropurpurea* and evaluate the potential of these peptides as antioxidant agents.

5.9   Future Studies

The study has laid a strong foundation for future investigations on the α- and β-subunits of phycobiliproteins. Now that the antioxidant potentials of the phycobiliproteins extracted from *B. atropurpurea* have been elucidated, effort should be directed towards unravelling the specific subunit mechanism responsible for conferring the selective antioxidant properties in addition to further the understanding the roles of the extracted peptides in the prevention of ageing caused by free radicals. Basically, antioxidants are useful for anti-ageing, thus future aims should be focused on analysing the signalling pathways involved in the mechanism of action of peptides synthesised from phycobiliproteins, demonstrating their antioxidant activities in cells and addressing their potential value as antioxidant agents.

Ageing is defined as systematic decreases in physiological functions, including biochemical functions, occurring in majority of organisms. According to the free radical theory of ageing, a major cause is activated oxygen species and, therefore, removal of such species is being investigated for the prevention of ageing. Further study on the anti-ageing potential of phycobiliproteins may reveal a better understanding on their mode of action in averting the ageing process. Establishment of the antioxidant nature of phycobiliproteins will be of great importance in the therapeutics of ROS-associated disorders.

CHAPTER 6

CONCLUSIONS

The aims of this study were to find a good natural source of R-PE and R-PC by a simple purification procedure hence, R-PE has been purified from a red alga, *B. atropurpurea* by gel filtration with Sephadex G-200 and RP-HPLC. Chromatography technique has shown many advantages including safe and rapid purification procedure to separate maximum R-PE and R-PC from the total protein yield in a short period with high purity indices of 5.42 and 3.95, respectively. This method can be effectively and economically used in large-scale production. The efficiency of this method has been reflected by high recovery yields of R-PE (94.4%) and R-PC (86.1%) from the total proteins extracted from *B. atropurpurea*. Precisely, from 50 g of powdered *B. atropurpurea* alga, 85.9 mg of R-PE and 44.2 mg of R-PC protein have been recovered. In conclusion, 66% of R-PE and 34% of R-PC were separated from total recovered phycobiliproteins as such R-PE is the predominant phycobiliprotein in *B. atropurpurea*.

Additionally, the phenolic compounds were extracted from *B. atropurpurea* and *C. vulgari*s using five different extraction solvents, including water, 50% aqueous methanol, methanol, ethyl acetate and hexane. Methanol was identified as the most suitable extraction solvent as the fraction exhibited the highest TPC, 80.97 ± 0.53 mg GAE/g dry weight and 62.13 ± 1.28 mg GAE/g dry weight

extracted from *B. atropurpurea* and *C. vulgaris*, respectively. Similarly, the phenolic extracts from *B. atropurpurea* and *C. vulgaris* using methanol also exhibited the highest DPPH radical scavenging (IC_{50}, 30.82 ± 0.92 µg/mL and 34.28 ± 0.79 µg/mL) and FRAP (37.81 ± 0.04 mg GAE/g dry weight and 23.97 ± 0.61 mg GAE/g dry weight) compared to the phenolic extracts by the other solvents. The results obtained show a significant positive correlation between TPC with DPPH radical scavenging activity and FRAP. Thus, it can be concluded that phenolic extracts in methanol have the highest TPC and contributed to effective antioxidant activity.

Based on the overall antioxidant activity analysis among R-PE, R-PC and phenolic compounds methanol extract from *B. atropurpurea*, R-PE is the most effective DPPH radical scavenger with IC_{50} of 7.66 ± 0.81 µg/mL and most effective metal ion reducing agent with FRAP value of 54.81 ± 0.31 mg GAE/g dry weight. Since *B. atropurpurea* naturally has abundant R-PE content (0.778 mg/g) compared to other red algae, hence *B. atropurpurea* extract can be widely used as a main source of antioxidants.

B. atropurpurea has a high antioxidant capacity in positive correlation with TPC. However, studies have justified that the phenolic compounds were not the only contributors to the antioxidant capacity of this red alga. *B. atropurpurea* contains phycobiliproteins, which are the other antioxidant compounds present in this alga that contribute to its vast antioxidant property. The findings of this study supported that *B. atropurpurea* could be a promising new source of potential antioxidants to replace the synthetic antioxidants used in food and

pharmaceutical products. The use of phycobiliproteins as non-toxic and non-carcinogenic natural food additives is gaining importance worldwide in view of the potential toxicity and carcinogenicity of synthetic food additives. Meanwhile, natural products are gaining importance as safe and effective alternatives for synthetic products.

REFERENCES

Aftabuddin, M. and Kundu, S., 2007. Hydrophobic, hydrophilic, and charged amino acid networks within protein. *Biophysical Journal,* 93, pp. 225-231.

Anderson, L. K. and Grossman, A. R., 1990. Structure and light-regulated expression of phycoerythrin genes in wild-type and phycobilisome assembly mutants of *Synechocystis* sp. strain PCC 6701. *Journal of Bacteriology,* 172**,** pp. 1297-1305.

Andrade, P.B., Barbosa, M., Matos, R. P., Lopes, G., Vinholes, J., Mouga, T. and Valentão, P., 2013. Valuable compounds in macroalgae extracts. *Food Chemistry*, 138(2), pp. 1819-1828.

Andressa, B., Gisely, C. L. and João, C. P., 2013. Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense* L. *Journal of Molecules,* 18, pp. 6852-6865.

António, J. Q., Fátima, L. M., Simão, P. P. and Eugénia, A. M., 2009. Solubilities of biologically active phenolic compounds: Measurements and modeling. *The Journal of Physical Chemistry*, 113 (11), pp. 3469-3476.

Ashafa, A. O. T., Grierson, D. S. and Afolayan, A. J., 2010. *In vitro* antioxidant activity of extracts from the leaves of *Felicia muricata* (Thunb.) an underutilized medicinal plant in the Eastern Cape province, South Africa. *African Journal of Traditional, Complementary and Alternative Medicines,* 7**,** pp. 296-302.

Astello-García, M. G., Cervantes, I., Nair, V., Santos-Díaz, M. D. S., Reyes-Agüero, A., Guéraud, F., Negre-Salvayre, A., Rossignol, M., Cisneros-Zevallos, L. and Barba De La Rosa, A. P., 2015. Chemical composition and phenolic compounds profile of cladodes from *Opuntia spp.* cultivars with different domestication gradient. *Journal of Food Composition and Analysis,* 43**,** pp. 119-130.

Bauer, A. K., Dwyer-Nield, L. D., Hankin, J. A., Murphy, R. C. and Malkinson, A. M., 2001. The lung tumor promoter, butylated hydroxytoluene (BHT), causes chronic inflammation in promotion-sensitive BALB/cByJ mice but not in promotion-resistant CXB4 mice. *Journal of Toxicology,* 169**,** pp. 1-15.

Benavides, J. and Rito-Palomares, M., 2004. Bioprocess intensification: a potential aqueous two-phase process for the primary recovery of B-phycoerythrin from *Porphyridium cruentum. Journal of Chromatography,* 807**,** pp. 33-38.

Bermejo, R., Acien, F. G., Ibanez, M. J., Fernandez, J. M., Molina, E. and Alvarez-Pez, J. M., 2003. Preparative purification of B-phycoerythrin from the microalga *Porphyridium cruentum* by expanded-bed adsorption chromatography. *Journal of Chromatography,* 790**,** pp. 317-325.

Bongards, C. and Gartner, W., 2010. The role of the chromophore in the biological photoreceptor phytochrome: an approach using chemically synthesized tetrapyrroles. *Accounts of Chemical Research,* 43**,** pp. 485-495.

Borowitzka, M. A., 1995. Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology,* 7**,** pp. 3-15.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72**,** pp. 248-254.

Bryant, D. A., 1982. Phycoerythrocyanin and phycoerythrin: Properties and occurrence in cyanobacteria. *Journal of Microbiology,* 128**,** pp. 835-844.

Bushra, S., Farooq, A. and Muhammad, A., 2009. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Journal of Molecules,* 14, pp. 2167-2180.

Butterfield, D. A., 2014. The 2013 discovery award from the society for free radical biology and medicine: selected discoveries from the butterfield laboratory of oxidative stress and its sequelae in brain in cognitive disorders exemplified by Alzheimer's disease and chemotherapy induced cognitive impairment. *Free Radical Biology and Medicine,* 20**,** pp. 157-174.

Cantonati, M. and Lowe, R. L., 2014. Lake benthic algae: Toward an understanding of their ecology. *Freshwater Science,* 33**,** pp. 475-486.

Chakraborty, K., Joseph, D. and Praveen, N. K., 2015. Antioxidant activities and phenolic contents of three red seaweeds (Division: Rhodophyta) harvested from the Gulf of Mannar of Peninsular India. *Journal of Food Science and Technology,* 52**,** pp. 1924-1935.

Chayakorn, P., Panmuk, V., Yuwadee, P., Pimporn, L., Walailuck, B., Masaharu, I. and Chartchai, K., 2011. Thermostablility of phycobiliproteins and antioxidant activity from four thermotolerant cyanobacteria. *Phycological Research*, 59, pp. 166-174.

Cho, M., Lee, H. S., Kang, I. J., Won, M. H. and You, S., 2011. Antioxidant properties of extract and fractions from *Enteromorpha prolifera*, a type of green seaweed. *Food Chemistry,* 127**,** pp. 999-1006.

Cole, W. J., Chapman, D. J. and Siegelman, H. W. 1967. Structure of phycocyanobilin. *Journal of the American Chemical Society,* 89**,** pp. 3643-3645.

Cruz, E., Euerby, M. R., Johnson, C. M. and Hackett, C. A., 1997. Chromatographic classification of commercially available reverse-phase HPLC columns. *Chromatographia,* 44**,** pp. 151-161.

Daniel, B. A., Fátima, C., Telesde, C., Rosa H. R. and Daniel R. S., 2016. Bioactive extracts of red seaweeds *Pterocladiella capillacea* and *Osmundaria obtusiloba* (Floridophyceae: Rhodophyta) with antioxidant and bacterial agglutination potential. *Asian Pacific Journal of Tropical Medicine*, 9, pp. 372-379.

Devendra, K., Dolly, W. D., Sunil, P., Neeraj, K. and Suresh W., 2014. Extraction and purification of C-phycocyanin from *Spirulina platensis* (CCC540). *Indian Journal Plant Physiology*, 19(2), pp. 184-188.

Devi, G. K., Manivannan, K., Thirumaran, G., Rajathi, F. A. A. and Anantharaman, P., 2011. *In vitro* antioxidant activities of selected seaweeds from Southeast coast of India. *Asian Pacific Journal of Tropical Medicine,* 4**,** pp. 205-211.

Di Meo, S., Reed, T. T., Venditti, P. and Victor, V. M., 2016. Role of ROS and RNS sources in physiological and pathological conditions. *Oxidative Medicine and Cellular Longevity,* 2016**,** pp. 1245-1249.

Duan, X. J., Zhang, W. W., Li, X. M. and Wang, B. G., 2006. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata. Food Chemistry,* 95**,** pp. 37-43.

Farasat, M., Khavari-Nejad, R. A., Nabavi, S. M. and Namjooyan, F., 2013. Antioxidant properties of two edible green seaweeds from Northern Coasts of

the Persian Gulf. *Jundishapur Journal of Natural Pharmaceutical Products,* 8**,** pp. 47-52.

Fernandes, S. A., Bassani, L., Nunes, F. F., Aydos, M. E., Alves, A. V. and Marroni, C. A., 2012. Nutritional assessment in patients with cirrhosis. *Arquivos de Gastroenterologia,* 49**,** pp. 19-27.

Fernando, S. I. P., Kim, M., Kwang-Tae S., Jeong Y. and You-Jin J., 2016. Antioxidant activity of marine algal polyphenolic compounds: A mechanistic approach. *Journal of Medicinal Food*, 20, pp. 234-239.

Figueroa, M., Martínez-Oyanedel, J., Matamala, A. R., Dagnino-Leone, J., Mella, C., Fritz, R., Sepúlveda-Ugarte, J. and Bunster, M., 2012. In silico model of an antenna of a phycobilisome and energy transfer rates determination by theoretical Förster approach. *Journal of Protein Science,* 21, pp. 1921-1928.

Galland-Irmouli, A. V., Pons, L., Lucon, M., Villaume, C., Mrabet, N. T., Gueant, J. L. and Fleurence, J., 2000. One-step purification of R-PE from the red macroalga *Palmaria palmata* using preparative polyacrylamide gel electrophoresis. *Journal of Chromatography,* 73**,** pp. 117-123.

Ganesan, K. C. S., Kumar, P.V.S. and Rao, S. T., 2011. Comparative assessment of antioxidant activity in three edible species of green seaweed, *Enteromorpha* from Okha, Northwest coast of India. *Innovative Food Science Emerging Technology*, 12 (1), pp. 73-79.

Ganzon, F. E. T., 1997. Influence of tidal location on morphology, photosynthesis and pigments of the agarophyte, *Gelidiella acerosa*, from Northern Philippines. *Journal of Applied Phycology*, 9, pp. 525-534.

Gargiulo, G. M., Genovese, G., Morabito, M., Culoso, F. and De Masi, F., 2001. Sexual and asexual reproduction in a freshwater population of *Bangia atropurpurea* (Bangiales, Rhodophyta) from eastern Sicily (Italy). *Journal of Phycologia*, 40(1), pp. 88-96.

Gibert, J.-M., Peronnet, F. and Schlötterer, C., 2007. Phenotypic plasticity in *Drosophila* pigmentation caused by temperature sensitivity of a chromatin regulator network. *Journal of Genetics,* 3**,** pp. 30-36.

Glazer, A. N., Apell, G. S., Hixson, C. S., Bryant, D. A., Rimon, S. and Brown, D. M., 1976. Biliproteins of cyanobacteria and Rhodophyta: Homologous family of photosynthetic accessory pigments. *Proceedings of the National Academy of Sciences,* 73**,** pp. 428-431.

Glazer, A. N. and Hixson, C. S., 1975. Characterization of R-PC. Chromophore content of R-PC and C-phycoerythrin. *Journal of Biology and Chemistry,* 250**,** pp. 5487-5495.

Glazer, A. N., Lundell, D. J., Yamanaka, G. and Williams, R. C., 1983. The structure of a "simple" phycobilisome. *Annals of Microbiology (Paris),* 134**,** pp. 159-180.

Griffiths, M. J. and Harrison, S. T. L., 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, 21(5), pp. 493-507.

Guiry, M. D., 2015. *Bangia atropurpurea* (Mertens ex Roth) C.Agardh, 1824: Accessed through World Register of Marine Species. *Journal of Algae Base*, 144, pp 423-444.

Haigh, R., Ianson, D., Holt, C. A., Neate, H. E. and Edwards, A. M., 2015. Effects of ocean acidification on temperate coastal marine ecosystems and fisheries in the Northeast Pacific. *Journal of Phycology,* 10**,** pp. 533-542.

Halliwell, B. and Cross, C. E., 1994. Oxygen-derived species: Their relation to human disease and environmental stress. *Environmental Health Perspectives,* 102**,** pp. 5-12.

Hafting, J. T., Craigie, J. S., Stengel, D. B., Loureiro, R. R., Buschmann, A. H., Yarish, C., Edwards, M. D. and Critchley, A. T., 2015. Prospects and challenges for industrial production of seaweed bioactives. *Journal of Phycology*, 51, pp. 821-837.

Harnedy, P. A. and Fitz, G. R. J., 2011. Bioactive proteins, peptides and amino acids from macroalgae. *Journal of Phycology*, 47, pp. 218-232.

Henrik, P. and Elisabet, B., 2000. Extrinsic factors influencing phlorotannin production in the brown alga *Ascophyllum nodosum. Marine Ecology Progress Series,* 193**,** pp. 285-294.

Hiba, N., Rajha, N., Zeina, H. and Nicolas, L., 2014. Extraction of total phenolic compounds, flavonoids, anthocyanins and tannins from grape byproducts by response surface methodology. Influence of solid-liquid ratio, particle size, time, temperature and solvent mixtures on the optimization process. *Food and Nutrition Sciences*, 5(04), pp. 397-409.

Hilditch, C. M., Balding, P., Jenkins, R., Smith, A. J. and Rogers, L. J., 1991. R-PE from the macroalga *Corallina officinalis* (Rhodophyceae) and application of a derived phycofluor probe for detecting sugar-binding sites on cell membranes. *Journal of Applied Phycology,* 3**,** pp. 345-354.

Huang, D., Ou, B. and Prior, R. L., 2005. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry,* 53**,** pp. 1841-1856.

Hwang, E. S. and Thi, N. D., 2014. Effects of extraction and processing methods on antioxidant compound contents and radical scavenging activities of Laver (*Porphyra tenera*). *Preventive Nutrition and Food Science,* 19**,** pp. 40-48.

Inderjit, 1996. Plant phenolics in allelopathy. *The Botanical Review,* 62**,** pp. 186-202.

Jiang, L., Wang, Y., Yin, Q., Liu, G., Liu, H., Huang, Y. and Li, B., 2017. Phycocyanin: A potential drug for cancer treatment. *Journal of Cancer,* 8**,** pp. 3416-3429.

Jing, Z., Toyoshi, I., Shuji, S., Yasutaka, M., Mark, F. M., Masakazu, F., Noriko, I., Kunihisa, K., Noriyuki, S. and Ryoichi, T., 2013. Phycocyanin and phycocyanobilin from *Spirulina platensis* protect against diabetic nephropathy by inhibiting oxidative stress. *American Journal of Physiology, Regulatory, Integrative, Comparative and Physiology*, 304, pp. 110-120.

Johnston, E. T., Lim, P. E., Buhari, N., Keil, E. J., Djawad, M. I. and Vis, M. L., 2014. Diversity of freshwater red algae (Rhodophyta) in Malaysia and Indonesia from morphological and molecular data. *Journal of Phycologia,* 53**,** pp. 329-341.

Kao, O. H. W., Berns, D. S. and Town, W. R., 1973. The characterization of C-phycocyanin from an extremely halo-tolerant blue–green alga, *Coccochloris elabens. Biochemical Journal,* 131**,** pp. 39-50.

Kim, E. Y., Choi, Y. H. and Nam, T. J., 2011. Identification and antioxidant activity of synthetic peptides from phycobiliproteins of *Pyropia yezoensis*. *International Journal of Molecular Medicine*, 42(2), pp. 789-798.

Koller, K. P., Wehrmeyer, W. and Morschel, E., 1978. Biliprotein assemble in the disc-shaped phycobilisomes of *Rhodella violacea.* On the molecular composition of energy-transfering complexes (tripartite units) forming the periphery of the phycobilisome. *European Journal of Biochemistry,* 91**,** pp. 57-63.

Kozhummal, R. and Kaushik, B., 2005. Purification of phycobiliproteins from *Nostoc muscorum. Journal of Scientific and Industrial Research*, 64, pp. 372-375.

Kruger, N. J., 2009. The Bradford method for protein quantitation. *Journal of Protein***,** 2, 17-24.

Kubanek, J., Lester, S. E., Fenical, W. and Hay, M. E., 2004. Ambiguous role of phlorotannins as chemical defenses in the brown alga *Fucus vesiculosus. Marine Ecology Progress Series,* 277**,** pp. 79-93.

Kuda, T., Tsunekawa, M., Goto, H. and Araki, Y., 2005. Antioxidant properties of four edible algae harvested in the Noto Peninsula, Japan. *Journal of Food Composition and Analysis,* 18**,** pp. 625-633.

Kuddus, M., Singh, P., Thomas, G. and Al-Hazimi, A., 2013. Recent developments in production and biotechnological applications of C-Phycocyanin. *BioMedical Research International,* 13**,** pp. 74-85.

Kumar, A., Chen, S.H., Kadiiska, M. B., Hong, J. S., Zielonka, J., Kalyanaraman, B. and Mason, R. P., 2014. Inducible nitric oxide synthase is key to peroxynitrite-mediated, LPS-induced protein radical formation in murine microglial BV2 cells. *Free Radical Biology and Medicine,* 20**,** pp. 51-59.

Kumar, M., Kumari, P., Gupta, V., Reddy, C. R. K. and Jha, B., 2010. Biochemical responses of red alga *Gracilaria corticata* (Gracilariales, Rhodophyta) to salinity induced oxidative stress. *Journal of Experimental Marine Biology and Ecology,* 391**,** pp. 27-34.

Lenka, B., Alica, B. and Kristína G., 2015. Cultivation of microalgae (*Chlorella vulgaris*) for biodiesel production. *The Journal of Slovak University of Technology*, 36(23), pp. 87-95.

Leong, L. P. and Shui, G., 2002. An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chemistry*, 76, pp. 69-75.

Liochev, S. I., 2014. Free Radicals: How do we stand them? Anaerobic and aerobic free radical (chain) reactions involved in the use of fluorogenic probes and in biological systems. *Medical Principles and Practice,* 23**,** pp. 195-203.

Liou, J. W., Hung, Y. J., Yang, C. H. and Chen, Y. C. 2015. The antimicrobial activity of gramicidin a is associated with hydroxyl radical formation. *Journal of Biochemistry,* 10**,** pp. 70-75.

Liu, L. N., Chen, X. L., Zhang, Y. Z. and Zhou, B. C., 2005. Characterization, structure and function of linker polypeptides in phycobilisomes of cyanobacteria and red algae: An overview. *Biochimica et Biophysica Acta,* 17**,** pp. 133-142.

Liu, N., Li, F., Ge, F., Tao, N., Zhou, Q. and Wong, M., 2015. Mechanisms of ammonium assimilation by *Chlorella vulgaris* F1068: Isotope fractionation and proteomic approaches. *Bioresource Technology,* 19**,** pp. 307-314.

Lobo, V., Patil, A., Phatak, A. and Chandra, N., 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews,* 4**,** pp. 118-126.

Lou, S. N., Lin Y. S., Hsu, Y. S., Chiu, E. M. and Ho, C. T., 2014. Soluble and insoluble phenolic compounds and antioxidant activity of immature calamondin affected by solvents and heat treatment. *Food Chemistry Journal*, 15, pp. 246-253.

Lu, F. and Foo, L. Y., 1995. Phenolic antioxidant components of evening primrose: Packer, nutrition, lipids, health and disease. *American Oil Chemists' Society*, 2, pp. 86-95.

Maccoll, R., 1998. Cyanobacterial phycobilisomes. *Journal of Structural Biology,* 124**,** pp. 311-334.

Maharana D., Das P. B., Verlecar, X. N., Pise, N. M. and Gauns, M., 2015. Oxidative stress tolerance in intertidal red seaweed *Hypnea musciformis* (Wulfen) in relation to environmental components. *Environmental Science Pollution Research International Journal*, 22, pp. 18741-18749.

Massoumeh, F., Ramazan-Ali, Khavari-Nejad, Seyed, M. B. N. and Foroogh, N., 2013. Antioxidant properties of some filamentous green algae (*Chaetomorpha* Genus). *Brazilian Archives of Biology and Technology Journal*, 56, pp. 921-927.

Mata, M. T., Martins, A. A. and Caetano, N. S., 2010. Microalgae for biodiesel production and other applications: A review. *Renewable Sustainable Energy Review*, 14, pp. 217-232.

Mathew, S., Abraham, T. E. and Zakaria, Z. A., 2015. Reactivity of phenolic compounds towards free radicals under *in vitro* conditions. *Journal of Food Science and Technology,* 52**,** pp. 5790-5798.

Matthew, D. M., Ronald, B., Cindy, L. and Marilyn, L. F., 2007. Amino acid nitrogen isotopic fractionation patterns as indicators of heterotrophy in plankton, particulate and dissolved organic matter. *Geochimica et Cosmochimica Acta*, 71, pp. 4727-4744.

McCarty, M. F., Barroso-Aranda, J. and Contreras, F., 2010. Oral phycocyanobilin may diminish the pathogenicity of activated brain microglia in neurodegenerative disorders. *Medical Hypotheses,* 74**,** pp. 601-605.

Middleton, E. Jr., Kandaswami, C. and Theoharides, T. C., 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. *Pharmacology Review,* 52(4), pp. 673-751.

Moniruzzaman, Khalil, M. I., Sulaiman, S. A. and Gan, S. H., 2012. Advances in the Analytical Methods for Determining the Antioxidant Properties of Honey: A Review. *African Journal Traditional Complement Alternative Medicine*, 9(1), 36-42.

Mysliwa, K. B. and Solymosi, K., 2017. Phycobilins and phycobiliproteins used in food industry and medicine. *Mini Reviews in Medicinal Chemistry,* 17**,** pp. 1173-1193.

Nagy, J. O., Bishop, J. E., Klotz, A. V., Glazer, A. N. and Rapoport, H., 1985. Bilin attachment sites in the alpha, beta and gamma subunits of R-PE. Structural studies on singly and doubly linked phycourobilins. *Journal of Biological Chemistry,* 260**,** pp. 4864-4868.

Nita, M. and Grzybowski, A., 2016. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxidative Medicine and Cellular Longevity,* 20**,** pp. 734-740.

Niu, J. F., Wang, G. C. and Tseng, C. K., 2006. Method for large-scale isolation and purification of R-PE from red alga *Polysiphonia urceolata* Grev. *Protein Expression and Purification,* 49**,** pp. 23-31.

Ortiz M. E. Y., Casazza, A. A., Aliakbarian, B., Perego, P., Converti A. and de Carvalho, J. C., 2014. Production of *Chlorella vulgaris* as a source of essential fatty acids in a tubular photobioreactor continuously fed with air enriched with CO_2 at different concentrations. *Biotechnology Progress,* 30(4), pp. 916-922.

Overkamp, K. E., Langklotz, S., Aras, M., Helling, S., Marcus, K., Bandow, J. E., Hoef-Emden, K. and Frankenberg-Dinkel, N., 2014. Chromophore composition of the phycobiliprotein Cr-PC577 from the cryptophyte *Hemiselmis pacifica*. *Photosynthesis Research,* 122**,** pp. 293-304.

Paola, A. Tenorio-Rodriguez, Jesús, Murillo-Álvarez, Ángel, Campa-Cordova and Carlos Angulo, 2017. Antioxidant screening and phenolic content of ethanol extracts of selected Baja California Peninsula macroalgae. *Journal Food Science Technology*, 54(2), pp. 422-429.

Patarra, R. F., Paiva, L., Neto, A. I., Lima, E. and Baptista, J., 2011. Nutritional value of selected macroalgae. *Journal of Applied Phycology,* 23**,** pp. 205-208.

Poljsak, B., Suput, D. and Milisav, I., 2013. Achieving the balance between ROS and antioxidants: When to use the synthetic antioxidants. *Oxidative Medicine and Cellular Longevity,* 13**,** pp. 792-800.

Pushparajan, C., Goswami, S. K., Mcadam, C. J., Hanton, L. R., Dearden, P. K., Moratti, S. C. and Cridge, A. G., 2017. A mechanically strengthened polyacrylamide gel matrix fully compatible with electrophoresis of proteins and nucleic acids. *Electrophoresis*, 39, pp. 824-832.

Punampalam, R., Khoo K. S. and Sit N. W., 2018. Evaluation of antioxidant properties of phycobiliproteins and phenolic compounds extracted from *Bangia atropurpurea*. *Malaysian Journal of Fundamental and Applied Sciences*, 14(2), pp. 78-87.

Ratana, C., Nattayaporn, C., Veara, L., Suvit, T. and Boosya, B., 2018. Stepwise extraction of high-value chemicals from *Arthrospira (Spirulina)* and an economic feasibility study. *Biotechnology Reports*, 20, pp. 245-250.

Sakai, S., Komura, Y., Nishimura, Y., Sugawara, T. and Hirata, T., 2011. Inhibition of mast cell degranulation by phycoerythrin and its pigment moiety phycoerythrobilin, prepared from *Porphyra yezoensis. Food Science and Technology Research,* 17**,** pp. 171-177.

Sagar, B. K. and Singh. R. P., 2011. Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science Technology*, 48(4), pp. 412-422.

Samoylenko, A., Hossain, J. A., Mennerich, D., Kellokumpu, S., Hiltunen, J. K. and Kietzmann, T., 2013. Nutritional countermeasures targeting reactive oxygen species in cancer: From mechanisms to biomarkers and clinical evidence. *Antioxidants and Redox Signaling,* 19**,** pp. 2157-2196.

Sampath-Wiley, P. and Neefus, C. D., 2007. An improved method for estimating R-PE and R-PC contents from crude aqueous extracts of *Porphyra* (Bangiales, Rhodophyta). *Journal of Applied Phycology,* 19**,** pp. 123-129.

Sapan, C. V., Lundblad, R. L. and Price, N. C., 1999. Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry,* 29 (2)**,** pp. 99-108.

Sara, P., Cuellar-Bermudez, Aguilar-Hernandez, I., Diana L., Cardenas-Chavez, Ornelas-Soto, N., Miguel, A., Romero-Ogawa and Parra-Saldivar, R., 2015. Extraction and purification of high-value metabolites from microalgae: Essential lipids, astaxanthin and phycobiliproteins. *Journal of Applied Phycology*, 8(2), pp. 190-209.

Sarmadi, B. H. and Ismail, A., 2010. Antioxidative peptides from food proteins: A review. *Journal of Peptides,* 31**,** pp. 1949-1956.

Schagger, H., Aquila, H. and Von Jagow, G., 1988. Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis. *Analytical Biochemistry,* 173**,** pp. 201-205.

Schoenleber, R. W., Lundell, D. J., Glazer, A. N. and Rapoport, H., 1984. Bilin attachment sites in the alpha and beta subunits of B-phycoerythrin. Structural studies on a doubly peptide-linked phycoerythrobilin. *Journal of Biological Chemistry,* 259**,** pp. 5481-5484.

Sekimoto, S., Yokoo, K., Kawamura, Y. and Honda, D., 2008. Taxonomy, molecular phylogeny and ultrastructural morphology of *Olpidiopsis porphyrae* sp. novae (Oomycetes, straminipiles), a unicellular obligate endoparasite of *Bangia* and *Porphyra spp.* (Bangiales, Rhodophyta). *Mycological Research,* 112**,** pp. 361-374.

Senthilkumar, N., Suresh, V., Thangam, R., Kurinjimalar, C., Kavitha, G., Murugan, P., Kannan, S. and Rengasamy, R., 2013. Isolation and characterization of macromolecular protein R-PE from *Portieria hornemannii*. *International Journal of Biological Macromolecules,* 55**,** pp. 150-160.

Shen, Y. F., Liu, L., Gong, Y. X., Zhu, B., Liu, G. L. and Wang, G. X., 2014. Potential toxic effect of trifloxystrobin on cellular microstructure, mRNA expression and antioxidant enzymes in *Chlorella vulgaris*. *Environmental Toxicology and Pharmacology,* 37**,** pp. 1040-1047.

Singh, M., Jha, A., Kumar, A., Hettiarachchy, N., Rai, A. K. and Sharma, D., 2014. Influence of the solvents on the extraction of major phenolic compounds (punicalagin, ellagic acid and gallic acid) and their antioxidant activities in pomegranate aril. *Journal of Food Science and Technology,* 51**,** pp. 2070-2077.

Sonani, R. R., Rastogi, R. P. and Madamwar, D., 2015. Antioxidant potential of phycobiliproteins: Role in anti-aging research. *Journal of Biochemical Analysis*, 4, pp. 2172-2181.

Spitale, D., Scalfi, A., Angeli, N. and Cantonati, M., 2012. Fine-scale spatial patterns of mature and immature filaments and bioorganic compounds of the Rhodophyte *Bangia atropurpurea* in the supra and eulittoral zones of a large lake. *Journal of Phycology,* 48**,** pp. 1530-1534.

Stewart, T. W. and Lowe, R. L., 2008. Benthic algae of Lake Erie (1865-2006): A review of assemblage composition, ecology and causes and consequences of changing abundance. *Ohio Journal of Science,* 108(5), pp. 82-94.

Su, H. N., Xie, B. B., Zhang, X. Y., Zhou, B. C. and Zhang, Y. Z., 2010. The supramolecular architecture, function and regulation of thylakoid membranes in red algae: An overview. *Photosynthesis Research,* 106**,** pp. 73-87.

Suh, Y. W., Buettner, G. R., Venkataraman, S., Treimer, S. E., Robertson, L. W. and Ludewig, G., 2009. UVA/B-Induced formation of free radicals from decabromodiphenyl ether (Deca-BDE). *Environmental Science and Technology,* 43**,** pp. 2581-2588.

Sun, L., Wang, S., Gong, X., Zhao, M., Fu, X. and Wang, L., 2009. Isolation, purification and characteristics of R-PE from a marine macroalga *Heterosiphonia japonica*. *Protein Expression and Purification,* 64**,** pp. 146-154.

Takemoto, J. and Bogorad, L., 1975. Subunits of phycoerythrin from *Fremyella diplosiphon*: Chemical and immunochemical characterization. *Journal of Biochemistry,* 14**,** pp. 1211-1216.

Tandeau De Marsac, N., 2003. Phycobiliproteins and phycobilisomes: The early observations. *Photosynthesis Research,* 76**,** pp. 193-205.

Tchernov, A. A., Minkova, K. M., Houbavenska, N. B. and Kovacheva, N. G., 1999. Purification of phycobiliproteins from *Nostoc* sp. by aminohexyl–Sepharose chromatography. *Journal of Biotechnology,* 69**,** pp. 69-73.

Teare, J. P., Greenfield, S. M., Watson, D., Punchard, N. A., Miller, N., Rice-Evans, C. A. and Thompson, R. P., 1994. Lipid peroxidation in rats chronically fed ethanol. *Journal of British Society of Gastroenterology,* 35**,** pp. 1644-1647.

Tiwari, V., Peters, W. K. and Jonas, D. M., 2013. Electronic resonance with anticorrelated pigment vibrations drives photosynthetic energy transfer outside the adiabatic framework. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** pp. 1203-1208.

Uma, R., Sivasubramanian, V. and Niranjali D. S., 2011. Evaluation of *in vitro* antioxidant activities and antiproliferative activity of green microalgae, *Desmococcus olivaceous* and *Chlorococcum humicola*. *Journal of Algal Biomass Utilization*, 2 (3), pp. 82-93.

Valavanidis, A., Vlachogianni, T. and Fiotakis, K., 2009. Tobacco smoke: Involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. *International Journal of Environmental Research and Public Health,* 6**,** pp. 445-462.

Véronique, C., 2012. Phenolic compounds: from plants to foods. *Phytochemistry Reviews Journal,* 11(3), pp. 153-177.

Vinayak, R. C., Sabu, A. S. and Chatterji, A., 2011. Bio-prospecting of a few brown seaweeds for their cytotoxic and antioxidant activities. *Evidence-Based Complementary and Alternative Medicine,* 11**,** pp. 83-96.

Wang, Y., Gong, X., Wang, S., Chen, L. and Sun, L., 2014. Separation of native allophycocyanin and R-PC from marine red macroalga *Polysiphonia urceolata* by the polyacrylamide gel electrophoresis performed in novel buffer systems. *Journal of Phycology,* 9**,** pp. 369-378.

Witschi, H., Williamson, D. and Lock, S., 1977. Enhancement of urethan tumorigenesis in mouse lung by butylated hydroxytoluene. *Journal of the National Cancer Institute,* 58**,** pp. 301-305.

Witschi, H. P., 1986. Enhanced tumour development by butylated hydroxytoluene (BHT) in the liver, lung and gastro-intestinal tract. *Food Chemistry and Toxicology,* 24**,** pp. 1127-1130.

Wu, H., 2016. Effect of different light qualities on growth, pigment content, chlorophyll fluorescence and antioxidant enzyme activity in the red alga *Pyropia haitanensis* (Bangiales, Rhodophyta). *BioMed Research International,* 16**,** pp. 918-922.

Wu, H. L., Wang, G. H., Xiang, W. Z., Tao, L. and Hui, H., 2016. Stability and Antioxidant Activity of Food-Grade Phycocyanin Isolated from *Spirulina platensis*. *International Journal of Food Properties*, 19(10), pp. 2349-2362.

Wu, Q., Fu, X. P., Sun, L. C., Qian, Z. L., Cao, M. J. and Cai, Q. F., 2015. Effects of physicochemical factors and *in vitro* gastrointestinal digestion on antioxidant activity of R-phycoerythrin from red algae *Bangia fusco-purpurea*. *International Journal of Food Science and Technology*, 50, pp. 1445-1451.

Yokono, M., Uchida, H., Suzawa, Y., Akiomoto, S. and Murakami, A., 2011. Stabilization and modulation of the phycobilisome by calcium in the calciphilic freshwater red alga *Bangia atropurpurea*. *Biochimica et Biophysica Acta*, 181(2), pp. 306-311.

Zou, Y. and Hou, X., 2017. Sonication enhances quality and antioxidant activity of blueberry juice. *Food Science and Technology*, 37(4), pp. 599-603.

Appendix A

ANOVA for total phenolic content (TPC). The *F*-test analysis was done based on the comparison of the TPC extracted from *Bangia atropurpurea* among five different extraction solvents (Within Groups) and compared between *Bangia atropurpurea* and *Chlorella vulgaris* (Between Groups)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	15065.13	9	1673.90	3.65	0.001	2.04
Within Groups	27534.00	60	458.9			
Total	42599.14	69				

Critical value (9, 40) is 33.71.

Appendix B

Tukey Kramer's post hoc test by *t*-statistic analysis of TPC extracted from *Bangia atropurpurea* and *Chlorella vulgaris* with five different extraction solvents in correlation with FRAP and DPPH analysis

TPC with extraction solvents	*Bangia atropurpurea*				*Chlorella vulgaris*			
	FRAP (mg/mL GAE)		DPPH (IC$_{50}$, µg/mL)		FRAP (mg/mL GAE)		DPPH (IC$_{50}$, µg/mL)	
	t-value	p-value	t-value	p-value	t-value	p-value	t-value	p-value
Methanol	30.05	1.67E-06	6.24	4.06E-10	21.61	1.16E-05	5.94	7.31E-10
Ethyl acetate	35.36	2.98E-05	5.10	3.64E-09	9.90	0.0001	4.43	6.74E-09
50% Aqueous Methanol	49.81	4.29E-06	5.76	1.01E-09	8.31	4.94E-05	4.57	1.87E-09
Hexane	98.58	7.13E-05	5.02	2.31E-08	31.72	0.001	4.25	5.12E-08
Water	4.98	0.01	3.64	2.37E-07	5.07	0.01	4.25	2.05E-07

Appendix C

t-test analysis of antioxidant activity for *Bangia atropurpurea* extracts and synthetic antioxidants

	FRAP (mg/mL GAE)			DPPH (IC$_{50}$, µg/mL)		
	Mean	t-value	p-value	Mean	t-value	p-value
Phycoerythrin	251.17 ± 122	5.890	0.002	68.62± 26	9.099	9.42E-07
Phycocyanin	224.45 ±127	4.587	0.005	65.29± 26	8.487	1.85E-06
Phenolic compounds	197.18± 89	4.920	0.004	58.43 ±25	8.057	3.05E-06
Ascorbic	272.33± 103	3.954	0.008	79.39± 24	11.493	9.05E-08
BHT	42.79± 21	4.585	0.005	52.17 ±27	6.670	1.76E-05