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**STUDY ON SEAWEED CHLOROPHYLLS AND LIPIDS:
DISTRIBUTION, BIOAVAILABILITY, AND FUNCTIONALITY**

(海藻のクロロフィルと脂質に関する研究：分布,生体利用
および機能性)

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List of Abbreviations

AA	= Arachidonic acids
ABCA1	= ATP-binding cassette transporter family A member 1
ABCB1	= ATP-binding cassette transporter family B member 1
ABCG2	= ATP-binding cassette transporter family G member 2
ABCG5	= ATP-binding cassette transporter family G member 5
ABCG8	= ATP-binding cassette transporter family G member 8
ADMA	= asymmetric dimethyl L-arginine;
AGEs	= advanced glycation end products;
AI	= Anthrogenic index
ALA	= Alpha linoleic acids
ALD	= Alcoholic liver disease
ARA	= Arachidonic acids
ATP	= Adenosine triphosphate
BCMO1	= Betacarotene 15,15'-monooxygenase1
BLT-1	= Block lipid transport-1
CAT	= Catalase
CCK	= Cholecystokinin
CD36	= Cluster of differentiations 36
cDNA	= Complementary DNA
CDs	= Chlorophylls and derivatives
Chl	= Chlorophyll
Chlds	= Chlorophyllades
CKD	= Chronic kidney diseases
CVD	= Chronic vascular diseases
CYP3A4	= Cytochrome P450 3A4

dCaco-2 cells	= Differentiated Caco-2 cells
DCFH-DA	= Dichloro-dihydro-fluorescein diacetate
DHA	= Docosahexaenoic acid
DMEM	= Dubelco's Modified Eagle's-Medium
DMSO	= Dimethyl sulfoxide
DW	= Dry weight
EPA	= Eicosapentanoic acids
Ez	= Ezetimibe
FAMES	= Fatty acids methyl esters
FAs	= Fatty acids
FBS	= Fetal bovine serum
Fx	= Fucoxanthin
GAPDH	= Glyceraldehyde-3-phosphate dehydrogenase
GC	= Gas chromatography
GLC	= Gas liquid chromatography
GPX	= Glutathione peroxidase 1
h/H	= Hypocholesterolemic/Hypercholesterolemic ratio
HDL	= High density lipoprotein
HDL-CE	= High density lipoprotein – cholesterol Ester
HEPG2 Cells	= Hepatocarcinoma cells
HMOX1	= Heme oxygenase 1
HPLC	= High performance liquid chromatography
IAA	= Intracellular antioxidant activity
LIMP-2	= Lysosomal integral membrane protein-2
Lut	= Lutein
MCT	= Monocarboxylate transporter
MDA	= Malondialdehyde

MEM	= Modified Eagle's-Medium
MPO	= Myeloperoxidase
mRNA	= Messenger RNA
MUFA	= Mono unsaturated fatty acids
NASH	= Non-alcoholic steato hepatitis
NDs	= Neurodegenerative diseases
NPC1L1	= Niemann-pick C1-like 1
NT	= Nitrotyrosine
NX	= Neoxanthin
OATP	= Organo anion transporter
ODS	= Octadecylsilyl
oxLDL	= Oxidized low-density lipoprotein;
PC	= Protein carbonyl;
PCA	= Principal component analysis
Pheo	= Pheophorbide
Phy	= Pheophytin
PL	= Phospholipids
Prx	= Peroxiredoxins;
PTFE	= Polytetrafluoroethylene
PUFA	= Poly unsaturated fatty acids
P-VASP	= Phosphorylated vasodilator-stimulated phosphoprotein;
RNS	= Radical nitrogen species
ROS	= Radical Oxygen species
RP HPLC	= Reverse phase High Liquid Chromatography
SDA	= Stearidonic acids
SFA	= Saturated fatty acids
SLC	= Solute carrier family

SOD1	= Superoxide dismutase (Cu-Zn) 1
SR-B1	= Scavenger receptor class B type 1
SREBP2	= Sterol regulatory element-binding protein 2
SSO	= Sulfosuccinimidyl Oleate
TBARS	= Thiobarbituric acid reactive substances
TI	= Thrombotic index
TL	= Total lipids
TRX 1	= Thioredoxin-1
TRX 2	= Thioredoxin-2
Trx	= Thioredoxin
V _x	= Violaxanthin
Z _x	= Zeaxanthin
α Toc	= Alpha tocopherol
α-car	= Alpha carotene
β-car	= Beta carotene

CHAPTER I

INTRODUCTION

1.1. Introduction

Global population growth has increased the enormous demand for energy, food, medicine, as well as other resources (Manirafasha *et al.*, 2016), and human diets play a role in sustaining health due to the increase in life expectancy (Lordan *et al.*, 2011). Furthermore, marine species are increasingly examined as a major source of healthy ingredients (Suleria *et al.*, 2016) for producing functional foods and nutraceuticals (Lordan *et al.*, 2011). The recent data showed the increase of global marine species production, particularly seaweeds (FAO, 2016) due to the diverse function as food ingredients and also bioactive compounds (Kim and Kim, 2012).

Algae including seaweeds have been estimated to include plants from 30,000 to 1 million species (Guiry, 2012). They are ubiquitous in Pacific and Asian and are also present in waters of France, Iceland, Ireland, Norway, Wales, as well as the Canadian Maritime provinces and Maine in the United States (Yuan, 2009). About 291 species of seaweeds are used worldwide as food, feed, paper, fertilizer, medicinal, and industrial product uses (White and Wilson, 2015).

Seaweeds have been exploited all over the world for centuries and are considered as food in the coastal community (Ferdouse *et al.*, 2018). Seaweeds have long been a staple diet in Japan, Korea, and China and the coastal areas of South East Asia countries (McHugh, 2003; Sho, 2001). More recently, the popularity and consumption in western countries have been raised (Bouga and Combet, 2015; Chen and Roca, 2019; Nova *et al.*, 2020) due to rich bioactive compounds and excellent sources of polysaccharide, amino acids, essential fatty

acids (FAs), which are nutritionally ideal in the ratio of n-6/n-3 polyunsaturated fatty acids (PUFAs) and the presence of minor components such as mineral, vitamins, trace elements, and pigments (Chen *et al.*, 2017; Nova *et al.*, 2020). These components are involved in the prevention and improvement of diseases by several mechanisms (Délérís *et al.*, 2016). In addition, the Japanese diet is documented in terms of eating habits with proven health benefits on increasing life expectancy and reducing the mortality rate from cancer (Shimazu *et al.*, 2007; Yamamoto *et al.*, 2016). The diet consists of high n-3 PUFAs from fish, seaweeds, soy products, and green tea with a low proportion of red meat and saturated fatty acids (SFAs) (Shimazu *et al.*, 2007; Tsugane and Sawada, 2014; Iwagaki *et al.*, 2017; Murakami *et al.*, 2018). Recently, epidemiological studies show a high consumption of seaweeds, which is protected against chronic disease. Historically, Japan and Korea consume a lot of seaweed in their daily diets (Yang *et al.*, 2010; Teas *et al.*, 2013). As a result of this consumption, there are reduced cases of breast cancer in their various communities, especially in Japan (Délérís *et al.*, 2016).

Generally, botanists have divided marine seaweeds (macroalgae) into three main phyla (singular, phylum) of green algae (Chlorophyta), brown algae (Ochrophyta), and red algae (Rhodophyta) based on pigment distribution (García-Poza *et al.*, 2020). Several kinds of pigments such as chlorophylls (Chls) and carotenoids in seaweeds have been described to show potential benefits in human health (Pangestuti and Kim, 2011). To produce and utilize the pigment for functional foods and nutraceuticals, the information on their composition and variation is essential.

The seaweed pigment composition is varied among different phyla (Pangestuti and Kim, 2011; Susanto *et al.*, 2019). In Chlorophyte, Chl a and Chl b are the major Chls. In additions, Chl a and Chl c are the main Chls in Ochrophyta, while Chl a is the dominant Chls in Rhodophyta. β -Carotene and fucoxanthin (Fx) were detected as major seaweed carotenoids

(Chan and Matanjun, 2017; Chen *et al.*, 2017; Susanto *et al.*, 2019). The composition of the pigments also differs according to the seasons. During the time of the year when there was prolonged exposure to sunlight, total Chls and carotenoids were lower than during brief exposure to sunlight (Sampath-Wiley *et al.*, 2008; Gerasimenko *et al.*, 2010; Ismail and Osman, 2016; Guihéneuf *et al.*, 2018).

It has been suggested that seaweeds pigments, Chls and carotenoids (Car), are all believed to contribute to the health benefits (Hosokawa *et al.*, 2004; Maeda *et al.*, 2007; Pangestuti and Kim, 2011). Historically, Chls have been consumed for millions of years ago by animals (Viera *et al.*, 2018) through the ingestion of the photosynthetic organisms. The daily ingestion has been stimulated about 26-28 mg Chls (Balder *et al.*, 2006). In mammals, they are converted into pheophytins (Phys), pheophorbides (Pheos), and chlorophyllades (Chlides) as a result of the digestion process (Solymosi and Mysliwa-Kurdziel, 2016; Chao *et al.*, 2018). The chlorophylls and their derivatives (CDs) have been reported accumulated in differentiated Caco-2 cells (dCaco-2 cells) (Ferruzzi *et al.*, 2001; Gallardo-Guerrero *et al.*, 2008; Chen and Roca, 2018a), animal tissues (Fernandes *et al.*, 2007; Hsu *et al.*, 2014), and human peripheral blood (Chao *et al.*, 2018).

The function of Chls in plants and seaweeds are related to light harvesting and protecting from oxidative stress (Thornber, 1975; Ilag *et al.*, 1994; Stenbaek and Jensen, 2010). Also, numerous bioactivities of CDs have been reported i.e. antioxidant properties (Ferruzzi *et al.*, 2002; Lanfer-Marquez *et al.*, 2005; Hsu *et al.*, 2013), anti-mutagenesis (Dashwood, 1997; Chernomorsky *et al.*, 1999; Ferruzzi *et al.*, 2002), induction of apoptosis (Cho *et al.*, 2014), anti-inflammatory (Islam *et al.*, 2013; Kang *et al.*, 2018). However, the role of CDs in the prevention of chronic diseases in humans is still unclear and limited in the anti-cancer, photodynamic therapy, and anti-inflammatory. Due to the widespread consumption, it is a

great interest to conduct a functional study on the health benefits of the seaweed pigment, Chl, since it is the most essential omnipresent pigment in seaweeds.

Therefore, this dissertation aims to address the composition, bioavailability, and functionality of CDs isolated and purified from seaweeds. The first chapter addresses the background of the study and the current situation of pigment utilization, bioavailability mechanism, and the beneficial effects on human health. Furthermore, it briefly reviews current literature regarding the distribution of Chls in seaweeds, bioavailability, and the biological function including antioxidant for protecting oxidative stress. The second chapter probe the composition of pigments, including Chls and carotenoids, and other lipid-soluble compounds in seaweeds collected from Indonesia and Japan. Meanwhile, the third chapter discusses the mechanism of intracellular uptake of Chls in human enterocyte cells dCaco-2 cells, and the fourth investigates the beneficial effects to protect hepatocellular, HepG2 cells from oxidative stress induced by H₂O₂. The fifth and sixth chapters provide general discussions and conclusions, respectively.

1.2. Chls structures, distributions and stability

Chls are conjugated, closed tetrapyrroles to which play essential roles in photosynthesis, in the absorption of sunlight and its conversion into chemical energy. Generally Chls contain magnesium in the central site of metal ion and a phytol (C₂₀) esterified with the propionic ion and moiety at C₁₇. Based on the saturation degree, it is divided into three main classes of Chls, i.e. chlorin, bacteriochlorin, and phytylphorphyrin (**Figure 1.1.**). The most common Chl structure is chlorin. This structure mainly composed in Chl a, Chl b, Chl d, and Chl f. In addition, bacteriochlorin is majorly present in bacteriachlorophyll a, b, and g, while phytylphorphyrin ring are mainly present in Chl c, an accessories Chl pigment in photosynthetic marine organism (Pérez-gálvez *et al.*, 2017). Chl c family is recognized of

several form, such as Chl c1, Chl c2, and Chl c3. They are characterized by lack of phytol ester (**Figure 1.2.**). Among Chl c family, Chl c1 and c2 are commonly detected in seaweeds (Fawley, 2008; Büchel, 2020). They have slightly different structure which makes them difficult to separate in reverse phase (RP) high performance liquid chromatography (HPLC). Chl c1 has an ethyl group at C4 in ring II, while vinyl group replaced ethyl group in Chl c2 at same position (**Figure 1.2.**) (Fawley, 1989; 2008).

About 100 different Chls structures are detected in photosynthetic organism such as terrestrial plants, algae, and bacteria (Solymosi and Mysliwa-Kurdziel, 2016). To be specific in seaweeds, Chen *et al.*, (2017) reported 31 different structures of CDs in three different seaweeds phyla. It is not only contained Chl a and Chl b which are commonly found in terrestrial plants but also Chl c and Chl d family. Chls distribution in seaweeds is related to the phyla dependent but not sampling location dependents (Susanto *et al.*, 2016; Chen *et al.*, 2017). Brown seaweeds (Ochrophyta) are consisted of Chl a and Chl c family, while major Chl of red seaweeds (Rhodophyta) is Chl a with a small portion of Chl d. On the other hand, green seaweeds contain Chl a and Chl b. This makes algae good source of diverse Chls for food and nutraceutical ingredients, cosmetics, and research (Humprey, 2004; Solymosi and Mysliwa-Kurdziel, 2016; Chen *et al.*, 2017).

Chls are extremely sensitive to pH and temperature which enable to convert into several derivatives through processing and human digestion. The exposure of heat and acidic conditions resulting in loss of central Mg^{2+} metal generating into Phy and phyropheophytin (Phyr). Furthermore, Pheo is resulted from the degradations of Chlides after thermal and acidification process (Ferruzzi and Blakeslee, 2007).

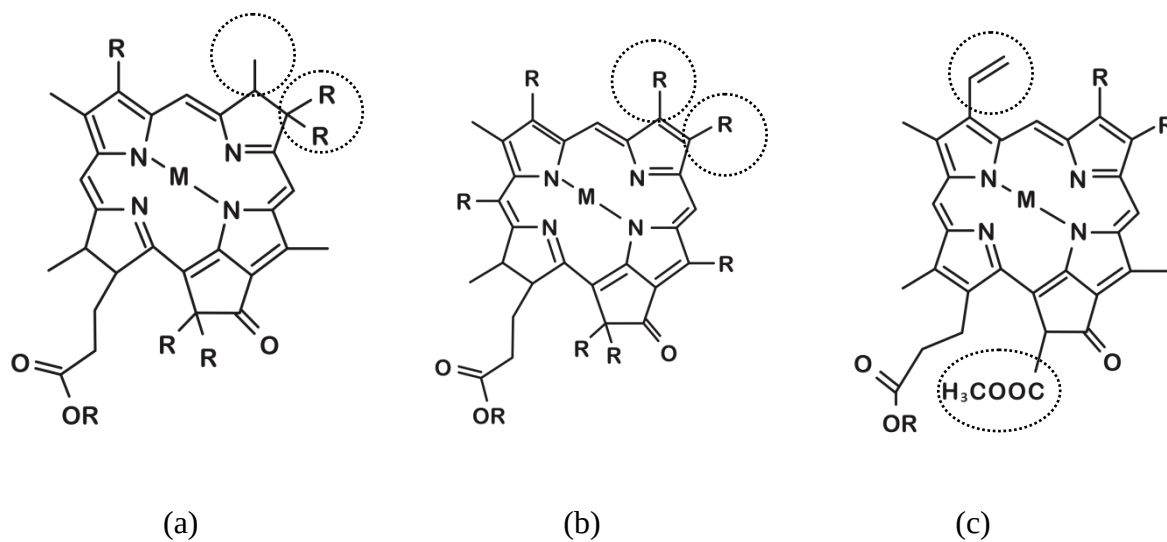


Figure 1.1. Structure of Chls and CDs, (a) chlorin type; (b) bacteriochlorin type, (c) porphyrin type (Pérez-gálvez *et al.*, 2017).

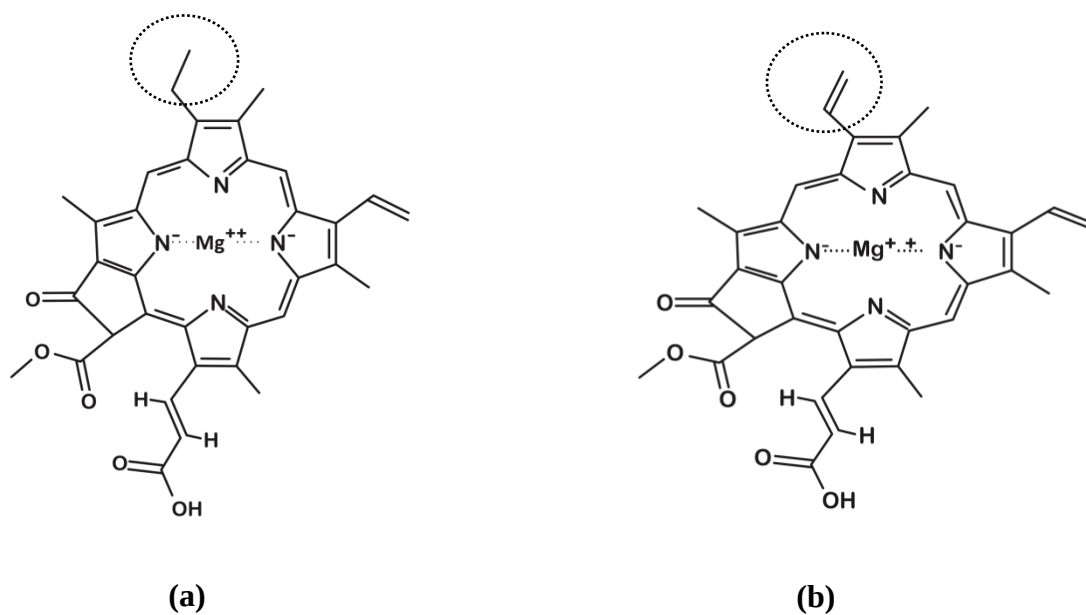


Figure 1.2. Structure of Chl c family in seaweeds (a) Chl c1 and (b) Chl c2 (Fawley, 1989; 2008)

1.3. Chls bioavailability and bio-distribution in tissue

Daily human diets contain carbohydrates, protein, lipids, minerals, and other phytochemicals, including pigments. Chls, the most abundant pigment in nature, are consumed by a human when ingested along with green vegetables, fruit, and algae (Hayes and Ferruzzi, 2020). Chls are consumed at 5 times higher than carotenoids (Ferruzzi and Blakeslee, 2007; Hayes and Ferruzzi, 2020). Furthermore, it has been known as a good source of antioxidants (Ferruzzi *et al.*, 2002; Lanfer-Marquez *et al.*, 2005; Hayes and Ferruzzi, 2020; Pérez-gálvez *et al.*, 2020). The consumption of Chls is related to the low incidence of immunological disease, cancer, and others (Mishra *et al.*, 2011; Park, 2013; Hayes and Ferruzzi, 2020). In order to utilize Chls for human health benefits, it needs to be satisfied with the bioavailability in the human body. Bioavailability of Chls in humans is important for their health beneficial applications such as functional foods and nutraceuticals (Fernandes *et al.*, 2007; Li *et al.*, 2015; Hayes and Ferruzzi, 2020).

Bioavailability is an important concept for the efficiency of absorption in humans (Fernandes *et al.*, 2007; Li *et al.*, 2015; Hayes and Ferruzzi, 2020). The other term is bioactivity, which includes the relationship between the transport of bioactive compounds and target tissue, the interaction with biomolecules, the resulting metabolism or biotransformation, and their physiological effects. Bioactivity determination is principally based on the activity during the interaction between compounds and biomolecules. The interaction results in signal transduction and biological response to express health benefits (Fernández-García *et al.*, 2009).

Studies on digestive behaviour, mechanism of bioavailability, and tissue distribution have been limited due to the common assumption that Chls cannot be absorbed by humans (Pérez-gálvez *et al.*, 2017). Recent study in the human enterocyte, dCaco-2 cells showed the

accumulation of parent Chls and derivatives after incubation with seaweeds digesta (Chen and Roca, 2018).

In vivo studies in mice, rabbits and humans, CDs have been detected in their organs and plasma (Hsu *et al.*, 2014; Viera *et al.*, 2018; Chao *et al.*, 2018). After ingesting 100 g of spinach, the rabbits were fasted for 24 h and killed after 2, 4, 8, 12, and 24 h. Chl a and Chl b, Chld a and Chld b, Phy a and Phy b, and Pheo a and Pheo b were reported in the liver as maximum amount after 8 h post-feeding. Furthermore, the gallbladder contained majorly Chlide a and a' (epimer of Chlide a), Pheo a, Pheo b and Pheo b' (epimer of Pheo b) in greatest amount after 2 h fed. Meanwhile, Pheo a and Pheo b were reported in plasma after 12 h feeding (Hsu *et al.*, 2014). It has been shown that Chl derivatives in the form of Phy a and Pheo a were incorporated into human plasma after the spinach diet (**Figure 1.4**).

Quantitative data on absorption and bioavailability of Chl in humans are known from the derivatives of Phy a and Pheo a in various age groups and sexes (Chao *et al.*, 2018). It was generally accepted that during digestion of Chl-containing foods, the Mg^{2+} and/or the phytol are lost, resulting in Phys, Pheos, and/or Chlide formation in human plasma (Solymosi and Mysliwa-Kurdziel, 2016). In addition, the conversion of parent Chls into derivatives acted by enzyme or microflora in the gut are absorbed in ileum and transported through enterohepatic circulation (Hsu *et al.*, 2014).

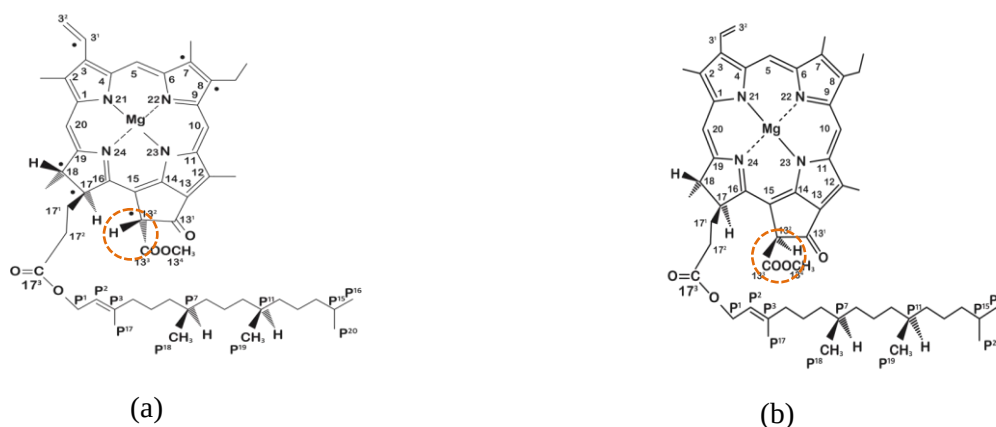


Figure 1.3. Structure of chlorophyll a and its epimer (a) chlorophyll a and (b) chlorophyll a' (Scheer, 1991).

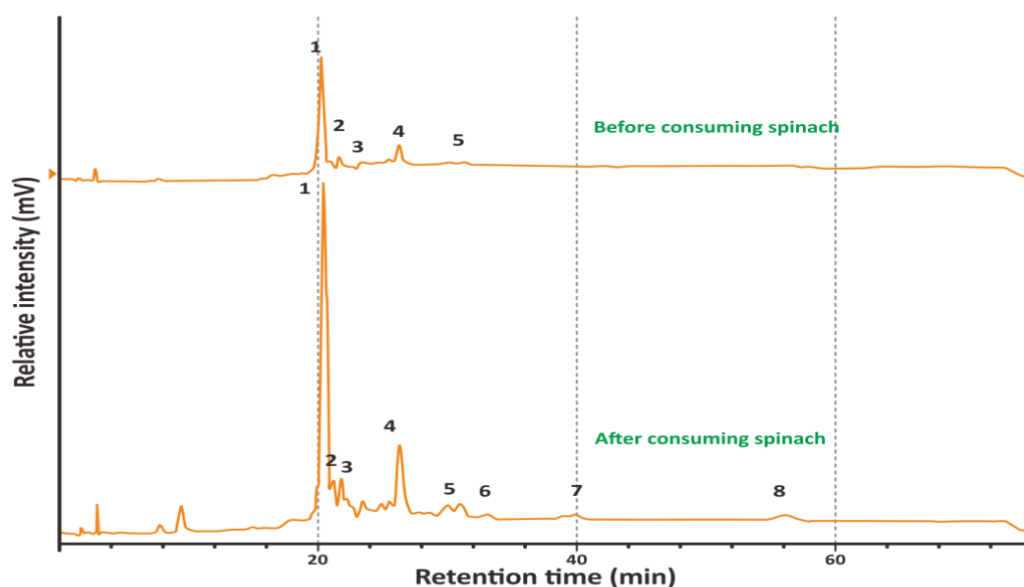


Figure 1.4. Changes in blood chlorophyll CDs and other pigments after 3 h spinach consumption. Identified peak (1) Lutein, (2) Zeaxanthin, (3-6) Phys, (7-8) Pheos (Chao *et al.*, 2018) (This figure was redrawn by the author without changing the meaning of the original picture).

1.4. Uptake of Chls into dCaco-2 cells

The basic aspects of Chl absorption in the intestine are unclear, such as quantitative information on the bioavailability rate of the different structures in time, dose, and temperature-dependent manner, the mechanism of intracellular uptake, and interaction with intestinal transporter. There are a few data on the mechanism of intracellular absorption of Chls into human intestinal cells (Caco-2 cells). However, a recent study has focused on the intracellular absorption and digesta metabolism of Chl-rich seaweeds in dCaco-2 cells (Chen and Roca, 2018a).

In vitro model, cultures of Caco-2 cells are widely used in various scientific fields such as nutrition, pathophysiology, pharmacology, and toxicology (Natoli *et al.*, 2012). It has been proved to express organic-anion-transporting polypeptides (OATP), monocarboxylate

transporters (MCT), and Solute carriers (SLCs) belonging to influx transporters (Estudante *et al.*, 2013). Lipophilic transporter (During *et al.*, 2005; Reboul, 2019) and efflux transporters such as ATP-binding Cassette Sub-family B Member 1 (ABCB1) (Silva *et al.*, 2015), ATP-binding cassette transporter G2/breast cancer resistance protein (BCRP/ABCG2) (Gutmann *et al.*, 2005), and multidrug resistance-associated proteins (MRPs) (Mandava *et al.*, 2010) are increasingly popular on the investigation of bioavailability mechanism of lipid-soluble compounds in the intestine (Meunier *et al.*, 1995).

It is interesting to investigate the mechanisms of CDs absorption, because it is essential to understand the biological aspects of CDs. Jonker *et al.*, (2002) have shown that the BCRP protein is involved in the protection of excessive amounts of Pheo a (dephytyllated Chls) in humans or animals. This study supported by Gandul-Rojas *et al.* (2009), who found that phytillated Chl absorption in Caco-2 cells was possibly based on passive pathway by simple diffusion. However, the dephytyllated one showed passive absorption by facilitated diffusion in lower concentrations. The more recent research by Viera *et al.*, (2018), shows the involvement of Scavenger Receptor Class B member 1 (SR-B1), which is encoded by SCARB1, in the Pheo a and uptake in Caco-2 cells.

There is no data on the intracellular uptake and transport mechanism of CDs from the apical membrane of the enterocyte to the basal membrane. Lipid-soluble compounds (hydrophobic) molecule crosses the aqueous intracellular without binding to transport of protein (Desmarchelier and Borel, 2017). However, intracellular ingestion of CDs is through a combination of two processes, passive diffusions and facilitated uptake by transport protein. Furthermore, intestinal lipid-soluble compounds uptake is regulated by several of transporter proteins and individual lipids absorption may be influenced by variation in intestinal protein levels (Tarling *et al.*, 2013). Among hundreds, facilitated protein in intestine, lipophilic and

ATP-binding Cassette (ABC) efflux transporters for cholesterol are the possible candidate for CDs absorption since these pigments belong to lipid-soluble pigments groups.

Intestinal transporters of the cholesterol group, ATP-binding Cassette Sub-family A Member 1 (ABCA1), ATP-binding cassette sub-family G member 5 (ABCG5), ATP-binding cassette sub-family G member 8 (ABCG8), and Niemann-Pick C1-Like 1 (NPCIL1), as well as SR-B1 (Masson *et al.*, 2010), which can bind or facilitated lipid soluble pigments, carotenoids (Reboul *et al.*, 2005; Sato *et al.*, 2012; During *et al.*, 2005) could be a good candidate (**Figure 1.5.**). In addition, the intestinal transporter ABC, which was involved in the efflux mechanism of fat-soluble compounds (Tarling *et al.*, 2013) also enabled the removal of protein for CDs. In the next paragraphs, the intestinal influx and efflux transporters of lipid-soluble compounds were explained.

SR-B1, NPC1L1, and cluster of differentiation 36 (CD36) may be involved in the uptake of lipid soluble compounds (Reboul *et al.*, 2005; During *et al.*, 2005; Sato *et al.*, 2012; Manabe *et al.*, 2019; Reoul *et al.*, 2011, Reboul and Borel, 2011; During *et al.*, 2007). Furthermore, SR-B1, NPC1L1, and CD36 have been known to mediate Chls counterpart, carotenoids, intracellular uptake in dCaco-2 cells (During *et al.*, 2008; Sato *et al.*, 2012; Manabe *et al.*, 2019; Reboul, 2019).

CD36, a heavily trans-membrane glycosylated protein, expressed in several tissues such as muscle, intestine, adipose tissue, and capillary endothelium (Bonen *et al.*, 2004; Cifareli and Abumrad, 2018). This protein is currently known to be a member of a superfamily of scavenger receptor proteins (class B), which also includes scavenger receptor SR-B1 and lysosomal integral membrane protein-2 (LIMP-2) (Glatz and Luiken, 2017; 2018). In small intestine, this protein abundantly express in brush border membrane of the proximal intestine which might be involved of lipids absorption (Yamanashi *et al.*, 2018). CD36 has function not only related to the lipid uptake and accumulation which is impact on lipids metabolism

but also several functions in digestal tract such as sensory fat perception and the cephalic phase digestion, fat-induced satiety, oleoylethanolamide (OEA), cholecystokinin (CCK), and secretin release, galistone formation, and intestinal homeostasis and inflammation (Cifareli and Abumrad, 2018). CD36 appears to be the predominant membrane protein facilitating not only FAs transport, at least in adipocytes, enterocytes, cardiac myocytes, and skeletal myocytes (Glatz and Luiken, 2017) but also several carotenoids such as lutein, β -carotene, lycopene, β -cryptoxanthin, and α -carotene in intestine (Reboul, 2019).

While NPC1L1, a regulator of sensing membrane cholesterol content, is abundantly expressed in the brush border membrane of small intestine of species. In the human NPC1L1 is expressed not only in the small intestine but also in the liver. NPC1L1 is mediated cholesterol absorption in the human intestines and counterbalances hepatobiliary cholesterol excretion (Jia *et al.*, 2011). This intestinal transporter may be regulated cellular cholesterol through Sterol regulatory element-binding protein 2 (SREBP2). Hepatocyte nuclear factor 4 α (HNF4 α) enhanced stimulated human NPC1L1 promoter activity through SREBP2 (Pramfalk *et al.*, 2010).

Another intestinal lipid transporter is high density lipoprotein (HDL) receptor, SR-BI, which is facilitated HDL and cholesterol ester uptakes or other lipoproteins. SR-B1 plays crucial role in cholesterol homeostasis, lipoprotein metabolism, and atherosclerosis (Gutiérrez-Rodríguez *et al.*, 2018). SR-B1 is abundantly identified in mammalian tissues and cell types, including intestine, macrophages, endothelial cells, smooth muscle cells, keratinocytes, adipocytes, and placenta. The expressions in these tissues were regulated by enzymes (Shen *et al.*, 2018). This protein has been proposed to be involved in vitamin, carotenoids, and Chls absorption (During *et al.*, 2005; Viera *et al.*, 2018; Yamanashi *et al.*, 2018). Different from other lipid intestinal transporters, SR-B1 has bidirectional activity (influx and efflux) for lipid soluble compounds including cholesterol, vitamin E, and vitamin

K1 (Yamanashi *et al.*, 2018). SR-B1 function is maintained by numerous posttranslational interaction and modifications (Shen *et al.*, 2018).

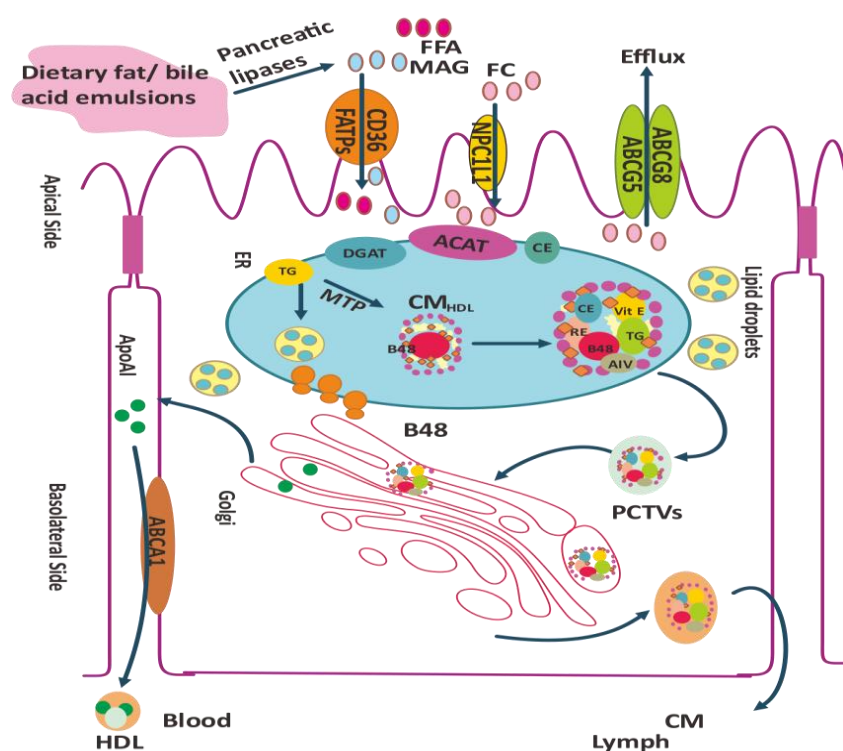


Figure 1.5. Intestinal lipid absorption mechanism (Source: Hussain, 2014; Iqbal and Hussain, 2009). (This figure was redrawn by the author without changing the meaning of the original picture).

Other transporter groups for lipophilic compounds are ABC intestinal transporter. The human ABC transporters, actively as efflux transporters, contain large member (49 members) with 7 sub families. ABC transporters act as outflow of many substrates such as amino acids, peptides, proteins, lipids, xenobiotics, etc. This superfamily transporters a wide range of substrates, including lipids, bile salts, sugars, amino acids, steroids, nucleotides, endogenous metabolites, ions and toxins including antibiotics and chemotherapeutics drugs (Cloos *et al.*, 2018; Li *et al.*, 2017).

P-glycoprotein (P-gp) or multiple drug resistance 1 (MDR1) gene which is expressed normal tissues has been shown to play important role in drug disposition and response. P-gp is a member of the adenosine triphosphate (ATP)-binding cassette family. The P-gp

transporter acts as a protective barrier to keep toxins out of the body by excreting these compounds into bile, urine, and the intestinal lumen and thereby prevents accumulation of such compounds in critical organs such as brain, gonads, and bone marrow, as well as, foetus (Marzolini *et al.*, 2004). The MDR1 gene (ABCB1) product P-gp is probably one of the most important ABC transporters for drug disposition in humans. It is assumed to be a protective mechanism against potentially toxic xenobiotics that are ingested with the diets (Schwab *et al.*, 2003).

BCRP/ABCG2 is a member of the ABC family that is capable of moving substrates against concentration gradients. It is expressed in a variety of normal tissues, including epithelial cells of the gastrointestinal tract and liver, placental syncytiotrophoblast cells, prostate epithelium, kidney cortical tubules, endothelial cells of the blood-brain barrier (BBB) and hematopoietic stem cells. Its function is similar to MDR1, which is protecting the body from toxic compounds (Cloos *et al.*, 2018). BCRP function as efflux transporters, moving heme and porphyrins from the intracellular to the extracellular environment (Krishnamurthy *et al.*, 2007). In addition, ABC intestinal transporters may also mediating in the Pheo a efflux (Jonker *et al.*, 2002).

ABCA1 is lipid efflux transporters which is abundantly found in basolateral side of intestine. This protein mediates transfer of cellular phospholipid (PL) and free (unesterified) cholesterol (FC) to apolipoprotein A1 (ApoA1) and related proteins present in the extracellular medium. ABCA1 plays substantive role in HDL biosynthesis and cellular cholesterol homeostasis (Phillips, 2014; 2018). ABCA1 is key transporter which is facilitating cellular cholesterol efflux (Neufeld *et al.*, 2001).

Furthermore, other ABC transporters, which were effluxed lipids soluble pigments are ABCG5 and ABCG8. The ABCG5 and ABCG8 genes encode proteins of 651 and 673 amino acids, respectively, which share 28% identity (Iida *et al.*, 2002). These genes are expressed

abundantly in the intestines and the liver (Li *et al.*, 2013). They are critically involved of non-cholesterol plant sterols, and as key protein to eliminate of cholesterol and plant sterol into the biliary lumen (intestinal lumen) (Hazard and Patel, 2007). These findings suggest that ABCG5 and ABCG8 work as sterol efflux pumps in the small intestine and liver (Wang, 2007). The cholesterol absorption in lumen was responsible by NPC1L1 whereas ABCG5 and ABCG8 may selectively 'pump' sterol out of the intestinal cells as a first-line defense to dietary input (Hazard and Patel, 2007).

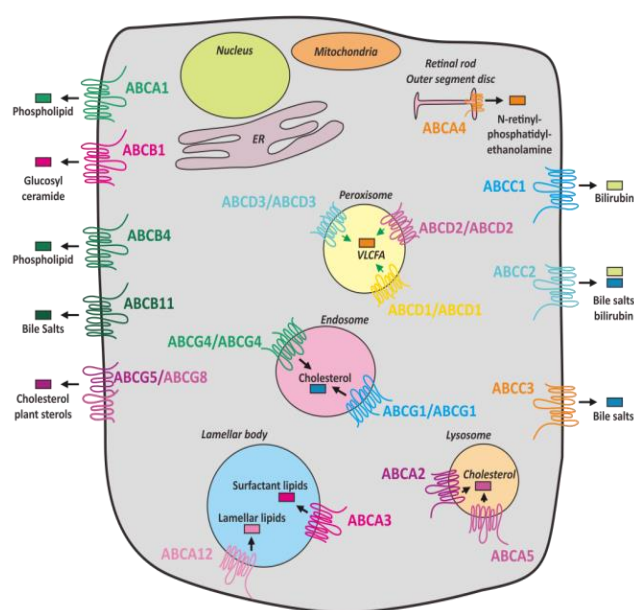


Figure 1.6. Localization of ABC lipid transporters (membrane localization of 20 of the 48 human ABC transporters). ABCA1, ABCB1 (MDR1), ABCB4 (MDR2/3), ABCB11 (BSEP), ABCG5/ABCG8, ABCC1 (MRP1), ABCC2 (MRP2), and ABCC3 (MRP3) localize to the plasma membrane. By contrast, ABCA2, ABCA3, ABCA4, ABCA5, ABCA12, ABCD1–3, ABCG1, and ABCG4 localize to specific intracellular organelles. (Source: Tarling *et al.*, 2013). (This figure was redrawn by the author without changing the meaning of the original pictures).

1.5. Biological functions of CDs

An understanding of intracellular absorption of CDs is required and the biological function is examined. Epidemiological studies related to metabolic syndrome and disease have promoted interest in seaweeds phytochemical as a part of dietary components (Shannon and Abu-Ghannam, 2019). The different chemical structure and physicochemical properties possess several biological functions that may produce a beneficial impact on human health (Pérez-gálvez *et al.*, 2017). These phytochemicals involve CDs originated from seaweeds;

that is why researchers are interested in the intracellular absorption of CDs and their biological functions.

Some researchers have reported the antioxidant activities of CDs (Lanfer-Marquez *et al.*, 2005; Hsu *et al.*, 2013; Pérez-gálvez *et al.*, 2020). The activities are affected by their structures and Chl b and the derivatives (Pheo b and Phy b) have been reported to be more active than Chl a derivatives. In the case of synthetic Chl (Cu Chlorophyllin), its antioxidant activity is likely to be higher than those of natural ones. The phenomenon may be due to the complex structure of Chlorophyllin and the difference between the central metal of the structure of Chl (Lanfer-Marquez *et al.*, 2005), since the central metal increased the electron density at the centre of the structure to elevate electron donation (Pérez-gálvez *et al.*, 2020). In addition, phytillated and dephytillated Chls showed different actions in radical scavenging activity. Their individual derivatives of IC₅₀ strength are as follows Chlds > Chlp > Chl > Phy > Pheo (Hsu *et al.*, 2013). The antioxidant capacity of CDs reduces the prooxidant and protects the body system through several mechanisms and systems. Furthermore, CDs have a polar and nonpolar regions, amphipathic, and are more hydrophilic in lipophilic (Hsu *et al.*, 2013). On the contrary, nonpolar structures are more effective in oil and water emulsion (Pérez-gálvez *et al.*, 2020).

Chl has potency as anti-carcinogenic agent by preventing bioavailability of some chemical carcinogens by a tight molecular complex of a dangerous chemicals when co-ingested (Pérez-gálvez *et al.*, 2017). This has been shown for Aflatoxin B1 (Dashwood, 1997; Dashwood *et al.*, 1998), benzo- α -pyrene (Terwel and van der Hoveen, 1985; Harttig and Bailey, 1998). Chl together with chlorine and other porphyrin has been used for photodynamic therapy of tumors which inhibits biomarker of cancer *in vivo* (Dashwood, 1997).

Furthermore, Chl inhibits the generation of cytotoxic factors in the gut due to their structural similarity between the porphyrin ring of Chls and heme. The human cohort study showed a connection between the consumption of heme iron (red meat) and Chls on the risk of colon cancer. The intake of Chls showed reducing colon cancer risk. Also, the intake of heme - iron is certainly linked with the risk of colon cancer (Balder *et al.*, 2006). A more recent studies also support the positive correlation between iron-heme and a higher risk of cancer (Fonseca-Nunes *et al.*, 2014).

The application of CDs also proposed a broad range of other biological functions such as reducing oxidative stress (Wang and Wink, 2016), anti-inflammation (Okai and Higashi-Okai, 1997; Islam *et al.*, 2013; Kang *et al.*, 2018), wound healing (Bowers, 1947), photodynamic therapy on some cancer or tumors (Moon *et al.*, 2017; Choi and Kim, 2020). The various health benefits of CDs as bioactive pigments elevate food containing Chls diets. The antioxidant potent in CDs possibly protect the human organs from the imbalance of pro-oxidant and antioxidant which can cause disorder of metabolism (Pérez-gálvez *et al.*, 2017; Pérez-gálvez *et al.*, 2020).

1.6. Oxidative stress

Oxidative stress is a common disorder of human metabolism, and it is an imbalance between oxidants and antioxidants, leading to disruption of redox signaling and control or molecule damage including lipids, proteins, and nucleic acids (Stahl and Sies, 2003; 2017; Webb and Twedt, 2008; Auten and Davis, 2009; Lobo *et al.*, 2010; S. Li *et al.*, 2015). It occurs in human organs in the human.

Reactive oxygen species (ROS) are more reactive towards oxygen-containing species compared to molecular O₂ (Zhou *et al.*, 2014). In addition, it acts as cell signaling molecules for ordinary biological processes (Auten and Davis, 2009). ROS/Reactive Nitrogen Species

(RNS) trigger lipid peroxidation, provoke DNA strand breaks, and indiscriminately oxidize all molecules in biological membranes and tissues, disrupting normal physiology and injury leading to cell death (Auten and Davis, 2009; Li *et al.*, 2015). The injured tissue increases the production of free radical generating enzymes such as xanthine oxidase, lipoxygenase, cyclooxygenase, which are phagocytic to produce more ROS (Lobo *et al.*, 2010).

Under normal condition, the levels of ROS are low to be effectively removed by the natural defense mechanisms of the human cell (Brieger *et al.*, 2012). In the living organism system, the cell has two defense mechanisms against free radical damage i.e. enzymatic and non-enzymatic antioxidative system. Antioxidative enzymes primarily include super peroxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), while Glutathione Reductase (GR) is secondary. Conversely, non-enzymatic antioxidants primarily consist of vitamin C and E, polyphenols, and reduced glutathione (Rahman *et al.*, 2006; Rahman *et al.*, 2012).

Human cells models are widely used to investigate the protective effects of phytochemicals towards to oxidative stress. Study on chemoprevention and molecular mechanism of phytochemical and pigment action by simulation oxidative stress in different cell models and pro-oxidant are commonly used. The common human cells associated with oxidative stress, such as epithelial (Mukherjee *et al.*, 2004), Caco-2 cells (Wijeratne *et al.*, 2005; Carrasco-Pozo *et al.*, 2010), HepG2 (Marra *et al.*, 2011), HEK123 (Pujalté *et al.*, 2011; Guan *et al.*, 2012), SH-SY5Y (Fordel *et al.*, 2006; Zhang *et al.*, 2010), 3T3L1 (Rudich *et al.*, 1998) etc.

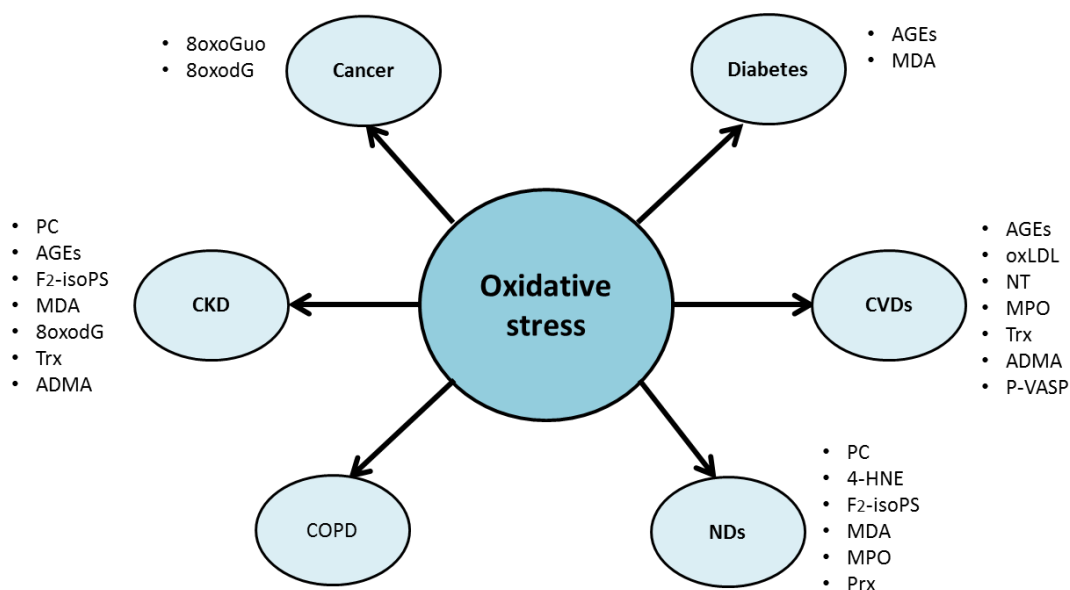


Figure 1.7. Oxidative stress, age-related diseases, and relative biomarker (Source: Liguori *et al.*, 2018). (This figure was redrawn by the author without changing the meaning of the original pictures).

Abbreviations: 4-HNE, *trans*-4-hydroxy-2-nonenal; 8oxodG, 7,8-dihydro-8-oxo-2'-deoxyguanosine; 8oxoGuo, 7,8-dihydro-8-oxoguanosine; ADMA, asymmetric dimethyl L-arginine; AGEs, advanced glycation end products; CKD, chronic kidney disease; CVDs, cardiovascular diseases; F₂-IsoPs, F₂-isoprostanes; MDA, malondialdehyde; MPO, myeloperoxidase; NDs, neurodegenerative diseases; NT, nitrotyrosine; oxLDL, oxidized low-density lipoprotein; PC, protein carbonyl; Prx, peroxiredoxins; P-VASP, phosphorylated vasodilator-stimulated phosphoprotein; Trx, thioredoxin (Sources: Liguori *et al.*, 2018).

Liver is an important organ which has a central role in metabolic homeostasis, because it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. Due to the high metabolic activity, free radicals are easily produced in the liver. In addition, liver enzymes, including diamine oxidase, aldehyde dehydrogenase, tryptophan dual oxidase, liver dehydrogenase and the cytochrome P450 enzyme system, induce oxidative stress (Zhu *et al.*, 2012). Therefore, liver produces low level of ROS even in normal condition. These ROS may damage the liver function through the oxidation of biomolecules such as PUFAs in cellular membrane (Jaeschke and Ramachandran, 2011).

Most phytochemicals are known to possess an ability to scavenge free radicals and ROS. For the measurement of the antioxidant activity of these phytochemicals, liver cells are used as a model system. Among the liver cells, HepG2 (human hepatoma cell-line) cell is widely used for *in vitro* study of xenobiotics metabolism in liver (Knasmüller *et al.*, 2004). Several studies have shown the protective effects of phytochemicals from fruits, vegetables, and seaweeds, against oxidative stress of HepG2 cells induced by H₂O₂ (Musonda and Chipman, 1998), tertbutylhydroperoxide (t-BOOH) (Alía *et al.*, 2006), ethanol (Xu *et al.*, 2003), palmitic acid (Alnahdi, John, and Raza 2019). These phytochemicals involve carotenoids (Martin *et al.*, 1996; Vardi *et al.*, 2010), phenolics and flavonoids (Lima *et al.*, 2006).

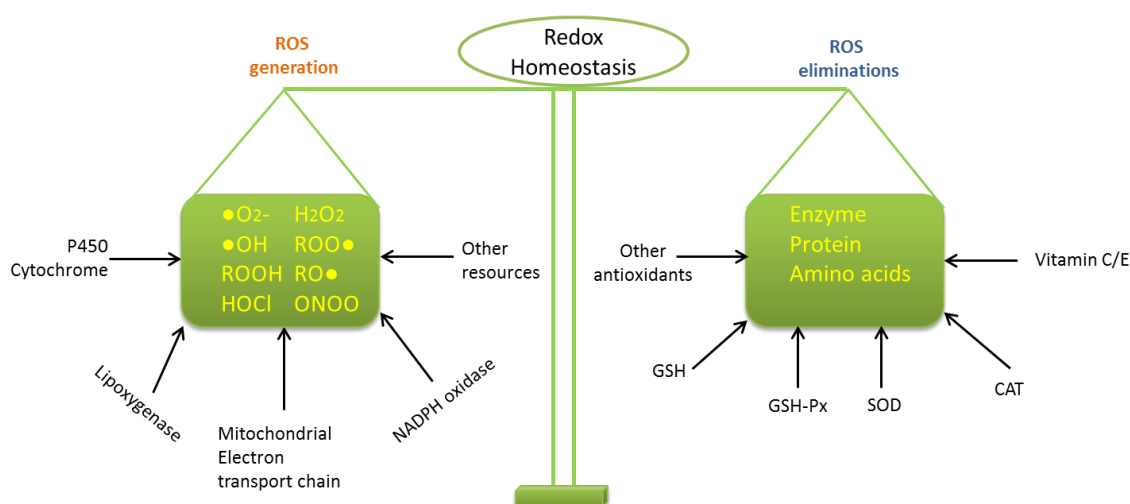


Figure 1.8. Redox homeostasis in the liver (Li *et al.*, 2015). (This figure was redrawn by the author without changing the meaning of the original picture).

Oxidative stress plays important role in pathophysiology of various liver diseases such as alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH), and hepatitis type C, where free radical attack to cell membranes, lipids, and protein leading to liver injury (Arauz *et al.*, 2016).

Antioxidants contained in green vegetables, fruits, and algae are able to protect and reduce the liver from oxidative stress (Li *et al.*, 2015). Some phytochemical from plants and

seaweeds has been proved to protect and recover liver cells and animal models from oxidative stress. Liver cells are able to develop advanced defenses system by regulating SOD1, SOD2, GPx, CAT, thioredoxin 1 and 2 (Trx1 & 2), and Peroxidoxins (Prx-I-V) (Jaeschke and Ramachandran, 2011).

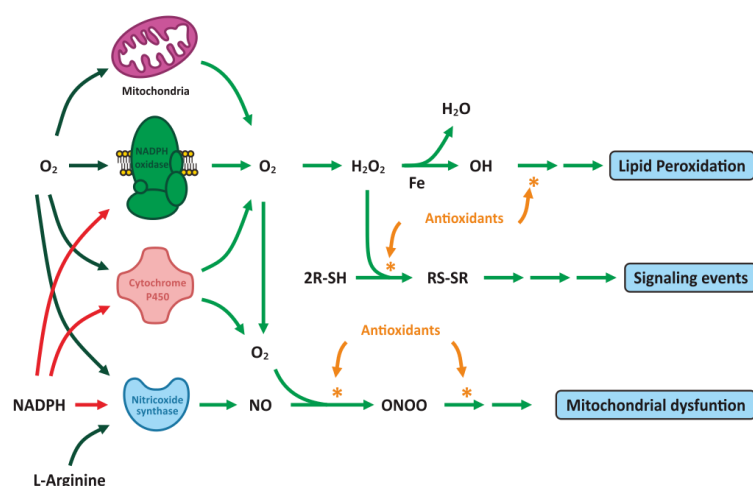


Figure 1.9. Biological significance of ROS and RNS (Singal *et al.*, 2011). (This figure was redrawn by the author without changing the meaning of the original pictures).

1.7. Significance of the proposed study

Seaweeds are one of the most promising marine organisms due to the availability and high nutritional value for food, industry, and other uses (Nova *et al.*, 2020). It has been recognized as unique sources of marine phytochemicals and pigments with a high amount of Chls. Seaweeds are a preeminent source of bioactive compounds that provide biological activities and health benefits. Therefore, much attention has been paid to the identification, isolation, and utilization of bioactive compounds from seaweeds (Pangestuti and Kim 2011).

Recently, marine natural pigments have been interested for the use of foods, cosmetics, and the pharmaceutical industry due to their biocompatibility, bioavailability, safety, and stability. Seaweeds are recognized as sources of natural pigments such as Chls,

carotenoids, and phycobiliprotein (Manivasagan *et al.*, 2018). Chls are ubiquitous in nature and seaweeds as well (Susanto *et al.*, 2019). The Chls production in biosphere is around 10^9 - 10^{12} tons per year (Solymosi and Mysliwa-Kurdziel, 2016), and has been taken by humans as part of diets through vegetable, fruits, and seaweeds consumption (Viera *et al.*, 2018). The daily intake of Chl is estimated to be about 26-86 mg (Balder *et al.*, 2006). Furthermore, about four CDs including Chl a, Chl b, Chl c, and Chl d family have been obtained in different seaweeds phyla (Chen *et al.*, 2017).

The epidemiological study has demonstrated the effects of ancient tradition and daily consumption of seaweeds in human health (Pangestuti and Kim, 2011). Following this study, *in vitro* and *in vivo* tests have shown the beneficial effects of CDs for health functions (Okai and Higashi-Okai, 1997; Ferruzzi *et al.*, 2002; Lanfer-Marquez *et al.*, 2005; Hsu *et al.*, 2008; Wang and Wink, 2016; Kang *et al.*, 2018).

Also, to determine a potential target in seaweeds as a source of Chls and other lipid-soluble compounds, information on the wide range of their composition is required. Despite the study indicating the composition of Chls and other lipid-soluble compounds our understanding of the intracellular uptake of Chls and their absorption mechanism is limited. This lack of understanding of the biological functions and bioavailability of Chls has deterred the study of the mechanism and bioactivity *in vitro* and *in vivo* due to the poor bioavailability perception.

Information on the bioavailability mechanism of CDs helps to decide their fate and functions. However, in the research on the bioavailability and efficacy of pigments, major concern has been focused on carotenoids (Reboul *et al.*, 2005; Maeda *et al.*, 2007; Airanthi *et al.*, 2011; Airanthi *et al.*, 2014; Widjaja-Adhi *et al.*, 2017; During *et al.*, 2005), while a little attention has been paid to Chl despite being the most abundant pigments on earth (Lanfer-Marquez *et al.*, 2005).

The CDs are grouped into 5 families with different structures and derivatives forms (Solymosi and Mysliwa-Kurdziel, 2016; Zepka *et al.*, 2019). The different structures are closely related to different molecular properties, biological, activity, and reactivity with targeted protein.

Most study on the bioavailability and functionality of CDs has been done using commercial CDs as well as the extract rich in Chls or only use some of the specific CD family (Ferruzzi *et al.*, 2001; Ferruzzi and Blakeslee, 2007; Gallardo-Guerrero *et al.*, 2008; Roca, 2012; Chen and Roca, 2018a; 2018b; Viera *et al.*, 2018; Hayes and Ferruzzi, 2020), and their purity was low due to the heterogeneous compounds in the samples. Therefore, it is difficult to elucidate the molecular mechanism of bioavailability and functionality of CDs. In the present study, each CD with high purity was successfully prepared, and then, analysed the absorption characteristics.

After measuring their bioavailability using enterocyte cells, further study was turned to their biological activities of CDs. The distinct CDs structure in seaweeds possessed different activities. Chls are known to be a good source of antioxidants. Antioxidant activity is important to protect living tissues against oxidative stress. Especially, liver is the center of the human metabolic organ. Therefore, in the present study, the efforts has been done to make clear the antioxidant activity of CDs in the liver cells. The expected results can contribute to the application of Chl to functional foods and nutraceuticals in the future.

1.8. Aim

This study aimed to investigate composition, *in-vitro* bioavailability, and functionality of CDs isolated and prepared from seaweeds. I focused on three research subjects:

1. Evaluation of the lipid-soluble pigment of 15 Japanese and Indonesian seaweeds, mainly paying the attention to their CDs composition.

2. Investigation on the intracellular uptake of CDs and their molecular mechanism, especially focusing on the interaction with intestinal transporters.
3. Investigation on the antioxidant activity of CDs in biological system, using HepG2 cells.

1.9. Philosophy approach

This study started with the investigation of lipid component composition in different phyla and sampling locations to obtain the potential target of seaweeds as sources of CDs for further experiments. Chls, the most abundant pigments in seaweeds, were the main target of this study. The highest potential as a source of CDs was then used for further investigation. Before evaluating the CDs efficacy, their bioavailabilities need to be evaluated. CDs were isolated from prospective seaweeds and their bioavailability characteristic and molecular mechanism were further investigated. This approach gave benefits, not only to specific compounds' characteristics but the molecular mechanisms. Meanwhile, after investigating their bioavailability, the biological function was evaluated in targeting to reduce ROS. Biological activities in cellular compartments using the most 4 absorbable compounds were also targeted. Therefore, the benefits for further utilization of CDs particularly from seaweeds as bio-functional materials for food and drinks, nutrients, and cosmetics are required.

CHAPTER II

VARIATION OF LIPID FUNCTIONAL COMPONENTS IN SEAWEEDS

2.1. Introduction

The consumption of seaweeds has existed for millennia, not only in Japan and Korea, but also in some Southeastern Asian countries, such as Malaysia, Philippines, and Indonesia (Dhargalkar and Pereira, 2005). Recently, global seaweed production has risen considerably with more than 291 exploited species for food, feed, paper, fertilizer, medicinal, and industrial product uses (White and Wilson, 2015). This might be because of the increasing interest in seaweeds as nutraceuticals, functional foods, cosmetics, and pharmaceuticals due to the presence of characteristic nutrients and bioactives in seaweeds (Buschmann *et al.*, 2017).

Seaweeds, promising marine products owing to their sustainability, contain valuable bioactive compounds that possess potential benefits for human health, such as anti-obesity, anti-diabetes, anti-cancer, and cardioprotective activities (Cardoso *et al.*, 2015; Chater *et al.*, 2015; Sharifuddin *et al.*, 2015; Xu *et al.*, 2017; Admassu *et al.*, 2018; Alves *et al.*, 2018; Circuncisão *et al.*, 2018; Gómez-Guzmán *et al.*, 2018; Seca and Pinto, 2018; Viera *et al.*, 2018). The most abundant nutrients in seaweeds are non-starch polysaccharides and minerals. The lipid content of seaweeds is low (1%–5% of the weight of a dry weight (DW) sample); however, the lipids comprise many kinds of bioactives, such as n-3 and n-6 PUFAs, Chls, carotenoids, terpenoids, and sterols (Terasaki *et al.*, 2009; Jiménez-Escrig *et al.*, 2012; Miyashita *et al.*, 2013; Aryee *et al.*, 2018; Ito *et al.*, 2018).

Seaweeds are marine algae that are rich sources of lipid soluble pigments, such as Chls and carotenoids. These natural pigments from seaweeds show several biological activities. Specifically, there have been many studies on the nutritional properties of seaweeds'

carotenoids, such as Fx (Miyashita and Hosokawa, 2018). Fx is a specific carotenoid found in brown seaweeds and known to prevent obesity and type 2 diabetes mellitus in animal model (Maeda *et al.*, 2005). CDs have a long-established history of use in traditional medicine and for therapeutic purposes (Ferruzzi and Blakeslee, 2007). Antioxidant and antitumor activities have been reported as general physiological activities of CDs (Ferruzzi and Blakeslee, 2007; Chen and Roca, 2018a). In addition, Chls from seaweeds exhibit therapeutic effects, such as anti-degranulation properties in RBL-2H3 cells (Yoshioka *et al.*, 2013; 2014), anti-inflammatory activity in RAW 264.7 cells (Okai and Higashi-Okai, 1997; Islam *et al.*, 2013), and neuroprotective activity in PC12 cells (Ina *et al.*, 2007). Because of their valuable health benefits, these natural pigments have attracted interest in their potential application in functional foods, cosmetics, and pharmaceuticals (Pangestuti and Kim, 2011), while more efforts are needed to clarify the detailed biological activities of Chl from seaweeds.

In addition to the pigments, seaweed lipids generally contain high levels of PUFAs, such as α -linolenic acid (18:3n-3, ALA), stearidonic acid (18:4n-3, SDA), and eicosapentaenoic acid (20:5n-3, EPA) as n-3 PUFAs, and arachidonic acid (20:4n-6, ARA) as an n-6 PUFAs (Terasaki *et al.*, 2009; Miyashita *et al.*, 2013; Ito *et al.*, 2018; Kumar *et al.*, 2011). Many studies, including substantial epidemiological, case-control, clinical, genetic, and nutrigenetic approaches, demonstrate a reduction in cardiovascular disease risk from intake of n-3 PUFAs, such as EPA and docosahexaenoic acid (22:6n-3, DHA), which are the active forms of n-3 PUFAs (Miyashita *et al.*, 2013; Gebauer *et al.*, 2006). ARA also plays an important role in biological systems, such as in the immune response, thrombosis, and brain function (Miyashita *et al.*, 2013). ARA and DHA are used as supplements in commercial infant formulas because both PUFAs are essential for infant neurodevelopment. Furthermore, the combination of ARA and DHA has also been found to improve age-related disorders of the brain and cognitive function (Kiso, 2011).

Tropical seaweed species have significantly lower lipid content than cold-water species (Miyashita *et al.*, 2013). Comparative studies have revealed that the total lipids (TL) content of a major brown seaweed family, Sargassaceae, was higher in subarctic zones (approximately 5% of the DW) than in tropical zones (0.9%–1.8% of the DW (Bhaskar *et al.*, 2004; Narayan and Miyashita, 2008; Susanto *et al.*, 2016; Terasaki *et al.*, 2009). In addition, different seaweed species have different TL contents, and some species have shown exceptionally high TL contents. For example, Gosch *et al.*, (2012), found higher TL contents in three tropical brown seaweed species (10.80%–11.91% of the DW) and two tropical green seaweed species (12.14%–13.04% of the DW) collected in tropical North Queensland, Australia. Higher TL contents have also been reported in brown seaweeds collected in the Indian Ocean (7%–8% of the DW) (Thinakaran *et al.*, 2012), as well as the Hawaiian coast (16%–20% of the DW) (McDermid and Stuercke, 2003). Therefore, seaweed lipids content varies greatly by species, geographical location, season, temperature, salinity, and light intensity, as well as interactions among these factors (Miyashita *et al.*, 2013; Susanto *et al.*, 2016; Nomura *et al.*, 2013; Khotimchenko and Yakovleva, 2005; Ganesan *et al.*, 1999; Zhao *et al.*, 2008). The content of lipid-soluble bioactive compounds in seaweeds also depends on several factors, such as species, sampling sites, environment, and seasonal variation (Miyashita *et al.*, 2013; Susanto *et al.*, 2016; McDermid and Stuercke, 2003; Nomura *et al.*, 2013; Khotimchenko and Yakovleva, 2005; Ganesan *et al.*, 1999; Zhao *et al.*, 2008; Kumari *et al.*, 2010; Chen *et al.*, 2017; Wong and Cheung, 2000; Burtin, 2003; Sánchez-Machado *et al.*, 2004; Marinho-Soriano *et al.*, 2006).

Although many papers have reported variations in the lipid components from different species of seaweeds collected in various areas, most of them have focused on the FAs composition (Van Ginneken *et al.*, 2011; Kumari *et al.*, 2013; Boulom *et al.*, 2014; Kumari *et al.*, 2010) and on specific bioactives, such as Fx (Terasaki *et al.*, 2009; Susanto *et al.*, 2016;

Nomura *et al.*, 2013). In finding a potential target in seaweeds as an ingredient for foods, animal feed, cosmetics, and nutraceuticals, information on the wide range of lipid and related compound profiles, especially on lipid soluble pigments, will be required. Among seaweed pigments, Chls play a key role in photosynthesis, while carotenoids serve as antenna pigments by absorbing and passing light energy to Chl. Both pigments are not only essential for photosynthesis, but also show various health benefits, as described above. However, little information is available on both lipid soluble pigment levels in Indonesia seaweeds. The investigation of lipid-soluble compounds in seaweed grown near the coast of Indonesia and northern coast of Japan is also limited (Susanto *et al.*, 2016).

Therefore, the primary aim of study chapter II was to determine Chls and carotenoids in 15 different species of seaweeds belonging to Ochrophyta (brown seaweeds), Chlorophyta (green seaweeds), and Rhodophyta (red seaweeds). In addition, the present study also revealed the FAs and α -tocopherol (α -Toc) profiles of these different seaweeds. Each sample was collected from tropical (Indonesia) or temperate (Japan) waters.

2.2. Materials and Method

2.2.1. Chemical

Standards for Chl a, Phy a, Phy b, and β -Carotene (β -Car) were obtained from Wako Pure Chemicals (Tokyo, Japan). Chl b and Chl c1 & c2 were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan) and DHI Laboratory Products (Hørsholm, Denmark), respectively. α -Carotene (α -Car), lutein (Lut), Zx (Zeaxanthin), Vx (violaxanthin), and Nx (neoxanthin) were purchased from Carote Nature GmbH (Münsingen, Switzerland). dl- α -Toc was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Fx standard for used in this study was purified from the TL content of *Sargassum horneri*, as described previously (Maeda *et al.*, 2005). Methyl tricosanoic (C23:0), used as an internal standard in the gas chromatography (GC) analysis, was obtained from Sigma-Aldrich (Tokyo, Japan). HPLC

grade solvents were used for the HPLC analysis and purchased from Wako Pure Chemicals, Ltd. (Osaka, Japan). All other solvents and chemicals used in the study were of analytical grade.

2.2.2. Samples

Seaweed samples which were used in this experiment were listed in **Table 2.1**.

Table 2.1. Seaweeds samples

Collecting sites	Phylum	Family	Seaweeds	Local name	Collection date
Tual, Indonesia ^{a,d}	Chlorophyta	Caulerpaceae	<i>Caulerpa lentillifera</i>	<i>Lat</i>	Feb-17
Hakodate, Japan ^{b,f}	Chlorophyta	Ulvaceae	<i>Ulva australis</i>	<i>Anaosa</i>	Jun-17
Hakodate, Japan ^{b,f}	Chlorophyta	Ulvaceae	<i>Ulva intestinalis</i>	<i>Bouaonori</i>	Jun-17
Tual, Indonesia ^{a,d}	Chlorophyta	Ulvaceae	<i>Ulva reticulata</i>	<i>Lumut daun</i>	May-17
Hakodate, Japan ^{b,f}	Ochrophyta	Agaraceae	<i>Costaria costata</i>	<i>Sujime</i>	May-17
Hakodate, Japan ^{b,f}	Ochrophyta	Alariaceae	<i>Undaria pinnatifida</i>	<i>Wakame</i>	May-17
Hakodate, Japan ^{b,f}	Ochrophyta	Laminariaceae	<i>Saccharina japonica</i>	<i>Konbu</i>	May-17
Tual, Indonesia ^{a,c,d}	Ochrophyta	Sargassaceae	<i>Sargassum aquifolium</i> ^a	<i>Pama</i>	Feb-17
Hakodate, Japan ^{b,f}	Ochrophyta	Sargassaceae	<i>Sargassum fusiforme</i>	<i>Hijiki</i>	May-17
Iwate, Japan ^{b,g}	Ochrophyta	Sargassaceae	<i>Sargassum horneri</i>	<i>Akamoku</i>	May-15
Hakodate, Japan ^{b,f}	Rhodophyta	Endocladaceae	<i>Gloiopeltis furcata</i>	<i>Fukurofunori</i>	May-17
Hakodate, Japan ^{b,f}	Rhodophyta	Gigartiniaceae	<i>Chondrus yendoii</i>	<i>Kurohaginnansou</i>	Jun-17
Hakodate, Japan ^{b,f}	Rhodophyta	Gigartiniaceae	<i>Mazzaella japonica</i>	<i>Akabaginnansou</i>	Jun-17
Jepara, Indonesia ^{a,e}	Rhodophyta	Gracilariaceae	<i>Gracilariopsis longissima</i>	<i>agar-agar</i>	May-17
Hakodate, Japan ^{b,f}	Rhodophyta	Rhodomelaceae	<i>Chondria crassicaulis</i>	<i>Yuna</i>	Jun-17

* The data value is expressed as the mean $\pm$ SD of three replicate measurements; ^a fresh samples collected, immediately washed, air dried, and kept in refrigerator (tropical seaweeds); ^b fresh samples collected, immediately kept in refrigerator (temperate seaweeds); ^c non-edible seaweeds; ^d latitudes/longitudes: 5° 37' 27.3396" S 132° 43' 22.3392" E; ^e latitudes/longitudes: 6.5868° S, 110.6444° E; ^f latitudes/longitudes: 41°45' N, 140°49' E; ^g latitudes/longitudes: 39°28'46.5"N 142°00'11.9"E. DW, dry weight.

2.2.3. Sample collection and handling

Seaweeds were collected from two different regions, Indonesia (tropic) and Japan (temperate). The 15 seaweeds including harvesting locations, phylum, family, scientific and local name, and date of collection are listed in **Table 2.1**. After collection, tropical seaweeds were washed, air-dried at room temperature (3–5 days; 27–30°C) to reduce their water content, and transported to the laboratory. Temperate seaweeds were kept in plastic bags,

stored on ice, and transported to the laboratory. These samples were then stored in a freezer (-30°C).

2.2.4. Moisture determination and total lipids extraction

Before moisture determination, all temperate seaweed samples were thawed and washed several times with running tap water until there were no debris, sand, or other contaminants. Before extraction, the moisture content of 15 seaweeds species was estimated with an oven-drying method, as described by Gómez-Ordóñez *et al.*, (2010), and used for expressing TL, Chls, carotenoids, n-3 PUFAs, and α -Toc content in seaweed samples on a DW basis. The TL content from the seaweeds was obtained by overnight extraction with a ten-fold portion of ethanol (w/v). For the extraction, the samples were crudely cut into small pieces. In the case of tropical seaweeds, a nine-fold portion of water was added to the washed sample before the ethanol extraction. After immersion for 1 h, the sample was subjected to the ethanol extraction, while the temperate raw seaweed sample was directly subjected to the ethanol extraction. The filtrate obtained by the extraction was filtered with Advantec No. 2 filter paper (Advantec Toyo Kaisha Ltd., Tokyo, Japan), and the residue was subjected to a further overnight extraction with the same solvent proportion. Both filtrates were combined and were vacuum-concentrated at 30°C using a rotary evaporator to obtain the ethanol extract (dark green mass). The ethanol extract was dissolved in a chloroform/methanol/water solution with a ratio of 10:5:3 (v/v/v), and the solution was placed into a separatory funnel. After allowing the funnel to stand overnight, the solution was separated into two layers. The lower layer evaporated under reduced pressure in a rotary evaporator. The last traces of the solvent and water were removed using nitrogen and using a high-powered vacuum to obtain the seaweed TL content. The TL content was dissolved in ethanol and stored at -30°C for further analysis.

2.2.5. Pigment analysis

The contents of CDs and carotenoids in the seaweed TL were analyzed using HPLC. HPLC was performed with a Hitachi HPLC La Chrome system (Hitachi Seisakusho Co., Tokyo, Japan) equipped with an auto sampler (L-2200), pump (L-2130), column oven (L-2300), and photodiode array detector (L-2455). An aliquot of the TL was weighed and dissolved in acetone. The sample solution was filtered with a 0.45 μm membrane filter of polytetrafluoroethylene (PTFE) (Ekicrodisc 13CR; Nippon Genetics Co. Ltd., Tokyo, Japan) and subjected to HPLC analysis. The analysis was performed on an octadecylsilyl (ODS) column (TSK-gel ODS 80-Ts, 250×4.6 mm i.d., 5 μm particle size; Tosoh, Japan) protected with a guard column (15 $\times$ 3.2 mm) with the same stationary phase. The mobile phase consisted of methanol/acetonitrile/1 M ammonium acetate (5:3:2, v/v/v) (A) and acetonitrile/ethyl acetate (1:1, v/v) (B) (Garrido and Zapata, 1993) with slight modification (**Table 2.2.**). The flow rate was kept at $1.0 \text{ mL} \cdot \text{min}^{-1}$. The UV/visible spectra absorption was recorded from 350 to 800 nm with a photodiode array detector, and sequential detection was carried out at 410, 430, 450, and 666 nm (Chen *et al.*, 2017). Quantification of CDs and carotenoids was performed with the corresponding calibration curves (**Tabel 2.2. and Figure 2.1.**). A calibration curve for each pigment was prepared using an authentic standard.

Table 2.2. Pigments standard

Pigments	Equation	R ²
Chl a	$y = 0.0000002164x - 0.4948217078$	0.9924
Chl b	$y = 0.0000002453x - 0.0075878186$	0.9999
Chl c1	$y = 0.0000011151x - 0.0069176122$	0.9966
Chl c2	$y = 0.0000003245x - 0.0990844827$	0.9957
Phy a	$y = 0.0000022496x - 0.0412254916$	0.9945
Phy b	$y = 0.0000040850x + 0.0368760047$	0.9970
Fx	$y = 0.0000001098x - 0.0028368723$	0.9997
Nx	$y = 0.0000000739x + 0.0090406558$	0.9937
Vx	$y = 0.0000000510x - 0.0000088918$	0.9999
Zx	$y = 0.0000001021x - 0.0222121627$	0.9997
Lut	$y = 0.0000000507x - 0.0140835850$	0.9998
α -Car	$y = 0.0000005622x + 0.0032425523$	0.9996
β -Ca	$y = 0.0000000739x + 0.0090406558$	1.0000

Table 2.3. HPLC gradients system of CDs and carotenoids analysis according to (Garrido and Zapata, 1993) with modification

Time (min)	Solvent		Gradient system
	A (Methanol:Acetonitrile:1 M Ammonium acetate, 5:3:2, v/v)	B (Acetonitrile:Ethyl acetate, 1:1, v/v)	
0	100		Injection
2	100		Isocratic
28		100	Linear
33		100	Isocratic
55	100		Stop

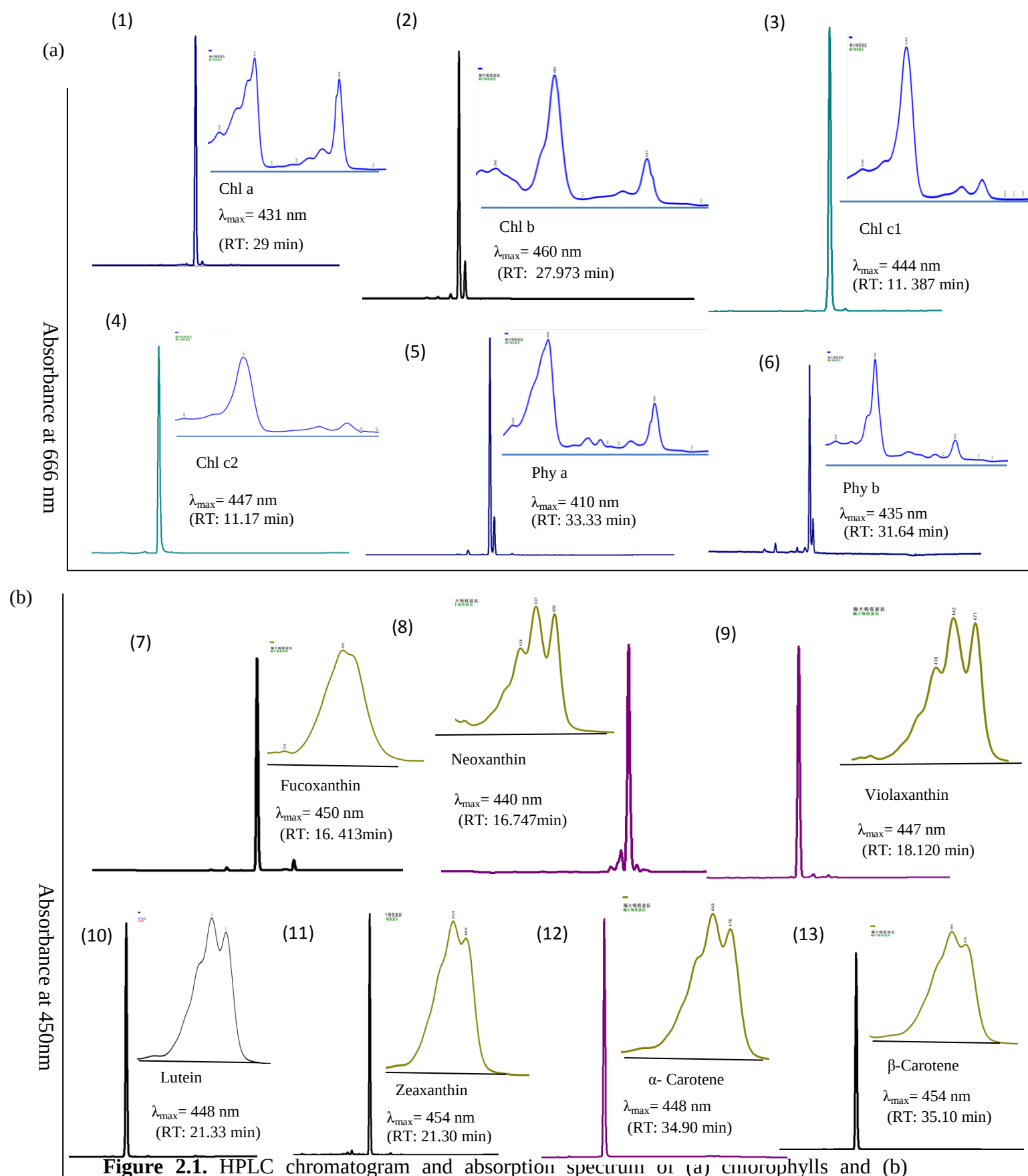


Figure 2.1. HPLC chromatogram and absorption spectrum of (a) chlorophylls and (b) carotenoids standard with their retention time (RT) and characteristics spectra with absorption maxima ($\lambda_{\max}$). HPLC condition is based on Susanto *et al.*, 2019.

2.2.6. FAs analysis

The FAs composition of the TL was determined by GC after conversion of fatty acyl groups in the lipid to their methyl esters. The fatty acid methyl esters (FAMES) were prepared according to the method by Prevot and Mordret (1979), with a slight modification. Briefly, 1 mL *n*-hexane containing an internal standard (methyl tricosanoic, C23:0) and 0.2 mL 2 M NaOH in methanol were added to an aliquot of the TL content (*ca.* 10 mg), vortexed for 10 s, and incubated at 50°C for 30 s. After the incubation, 0.2 mL 2 M HCl in methanol solution was added to the solution and vortexed for 60 s. The mixture was separated by centrifugation at $1000 \times g$ for 5 mins. The upper *n*-hexane layer containing FAMES was recovered and subjected to GC. The FAMES analysis was performed on a Shimadzu GC-2014 (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (Omegawax-320; 30 m $\times$ 0.32 mm i.d.; Supelco, Bellefonte, PA, USA). The detector, injector, and column temperatures were 260, 250, and 200 °C, respectively. The carrier gas was helium at a flow rate of $1.0 \text{ mL}\cdot\text{s}^{-1}$. FA content was expressed as the relative weight percentage of the total FAs weight. Each n-3 PUFAs level ($\text{mg}\cdot\text{g}^{-1}$ TL content) was quantified by comparing the peak ratio to that of the internal standard (23:0). Total n-3 PUFAs ($\text{mg}\cdot\text{g}^{-1}$ DW) were calculated from the content of each n-3 PUFAs in the TL content and the TL content g^{-1} DW. FAME peaks were identified by comparison of their retention time and log RRT with GLC-Reference standards by GC FID (Shimadzu GC-2014) after analysis. The FAs standard was prepared with GLC-Reference Standard, FAMES: GLC-462, and Echium oil, according to Susanto *et al.*, (2016).

Several factors predicting the nutritional quality of the seaweed TL were calculated based on the formula as below:

$$n-6/n-3 \text{ ratio} = \frac{\sum n-6 \text{PUFAs}}{\sum n-3 \text{PUFAs}} \quad (1)$$

$$\text{Atherogenicity index (AI)} = \frac{C12:0+4 \times C14:0+C16:0}{\sum \text{MUFAs} + \sum n-6 \text{PUFAs} + \sum n-3 \text{PUFAs}} \quad (\text{Gerasimenko and Logvinov, 2016}) \quad (2)$$

$$\text{Thrombogenicity index (TI)} = \frac{C14:0+C16:0+C18:0}{(0.5 \times \sum \text{MUFAs}) + (0.5 \times \sum n-6 \text{PUFAs}) + (3 \times \sum n-3 \text{PUFAs}) + \frac{\sum n-3 \text{PUFAs}}{\sum n-6 \text{PUFAs}}} \quad (\text{Matos et al., 2016}) \quad (3)$$

$$\text{FAs hypocholesterolemic/hypercholesterolemic ratio (h/H)} = \frac{C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3}{C14:0+C16:0} \quad (\text{Matos et al., 2016}) \quad (4)$$

$$\text{Unsaturation Index (UI)} = \sum (\% \text{PUFAs} \times \text{DB}); \text{DB is the number of double bonds} \quad (\text{Poerschmann et al., 2004}). \quad (5)$$

2.2.7. α -Toc analysis

The content of α -Toc in the TL content was analyzed by HPLC with a silica column (Mightysil Si 60 silica, 250 $\times$ 4.6 mm i.d.; Kanto Chemical Co., Ltd., Tokyo, Japan). HPLC was carried out with the same system as described in the pigment analysis, except that the fluorescence detector (Hitachi L-7485) was used for the peak detection. The mobile phase was *n*-hexane/2-propanol (99.2:0.8, v/v) at a flow rate of 1.0 mL $\cdot$ min⁻¹. The fluorescence detector was set at Ex. 298 nm and Em. 325 nm.

2.2.8. Data analysis

All analytical data were conducted in triplicate and the mean values are presented. The principal component analysis (PCA) was performed using JMP 14.0 statistical analysis. Two principal components based on the FAs composition and group (SFAs, MUFAs, PUFAs, n-3 PUFAs, and n-6 PUFAs) were used to make clear the relationship between different sampling areas and seaweed phyla. FAs composition (**Tables 2.6-2.8**) and FAs group (**Table 2.9.**) were

used for the multivariate analysis. However, an insignificant amount of FAs was not included in this analysis, these FAs were dodecanoic acid (C12:0), tridecanoic acid (C13:0), 7-tetradecenoic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), eicosatrienoic acid (C20:3), docosanoic acid (C22:0), docosaenoic acid (C22:1), docosadienoic acid (C22:2), tetracosanoic acid (C24:0), and tetracosanoic acid (C24:1).

2.3. Results

2.3.1. Total lipids

We investigated the TL and its soluble compounds in 15 species of seaweeds belonging to Ochrophyta (brown seaweeds), Chlorophyta (green seaweeds), and Rhodophyta (red seaweeds) Japan and Indonesia. The TL of seaweeds from Indonesia (tropical area) and Japan (temperate area) are described in **Figure 2.2**. The TL level varied among species, ranging from 6.91 to 62.48 mg·g⁻¹ DW. On average, the highest TL content was recorded in Ochrophyta (42.44 mg·g⁻¹ DW), followed by Chlorophyta (34.60 mg·g⁻¹ DW), and Rhodophyta (17.75 mg·g⁻¹ DW). In the present study, the lowest TL content was in *Chondrus yendoii* (6.91 mg·g⁻¹ DW), while *Ulva australis* had the highest total lipids content (62.48 mg·g⁻¹ DW) among all seaweeds tested. The unique results found in this study, *C. crassicaulis* and *U. australis* both classified in Rhodophyta and Chlorophyta, revealed a higher value of TL.

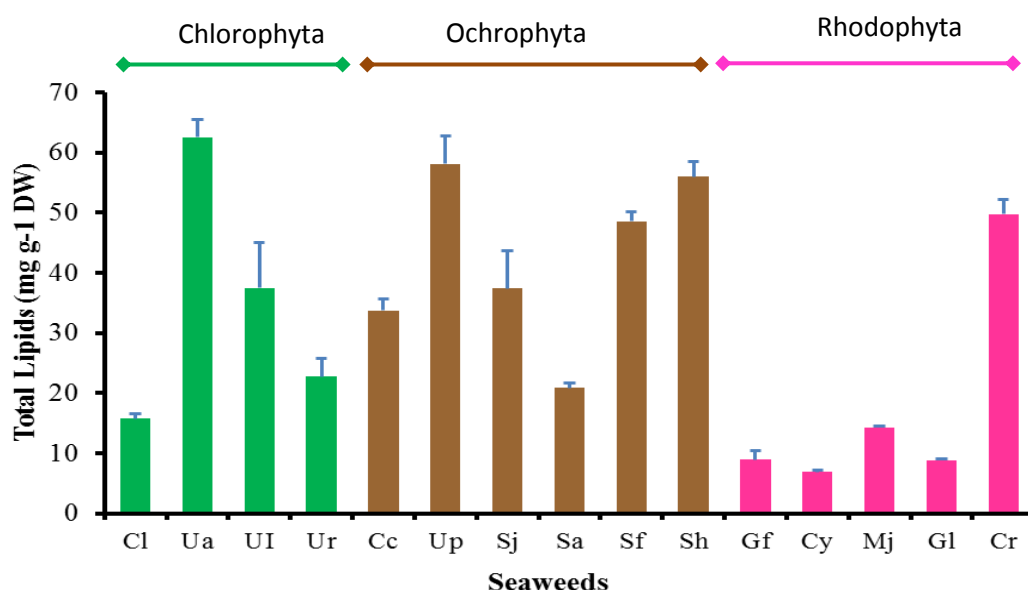


Figure 2.2. Total lipids of seaweeds collected from Japan and Indonesia (mg g⁻¹ DW). The data value express as treatments $\pm$ SD of three replicates. Abbreviation: Cl: *Caulerpa lentillifera*, Ua: *Ulva australis*; Ui: *Ulva intestinalis*; Ur: *Ulva reticulate*; Cc: *Costaria costata*; Up: *Undaria pinnatifida*; Sj: *Saccharina japonica*; Sa: *Sargassum aquifolium*; Sf: *Sargassum fusiforme*; Sh: *Sargassum horneri*; Gf: *Gloiopeltis furcate*; Cy: *Chondrus yendoii*; Mj: *Mazzaella japonica*; Gl: *Gracilariopsis longissimi*, Cr: *Chondria crassicaulis*.

2.3.2. Pigment profile

The primary photosynthetic pigments in seaweeds are comprised of Chls and carotenoids. In this study, we investigated the variation of these pigment contents in different phyla of seaweeds, which were collected from two regions (**Figure 2.3. and 2.4., Tables 2.4. and 2.5.**). Chls were the major pigment content in all seaweeds analysed. In average, the composition of Chls and carotenoids were in ratio 8:2 (w/w) (calculated from total Chls and carotenoids). The higher content of Chls was in Phaeophyta not in Chlorophyta and Rhodophyta. Chls easily decompose to produce their derivatives, Phys, by elimination of magnesium. This change from Chls to Phys is accelerated by heat treatment. In the dried samples from tropical seaweeds, Chl a and Chl b levels were very low or undetected, while

their corresponding derivatives, Phy a and Phy b, were major Chl components in these seaweeds (**Table 2.4. and Figure 2.4.**). The lower level of Chl a and Chl b in the tropical seaweeds was derived from the decomposition of these Chls to corresponding Phy during the drying process.

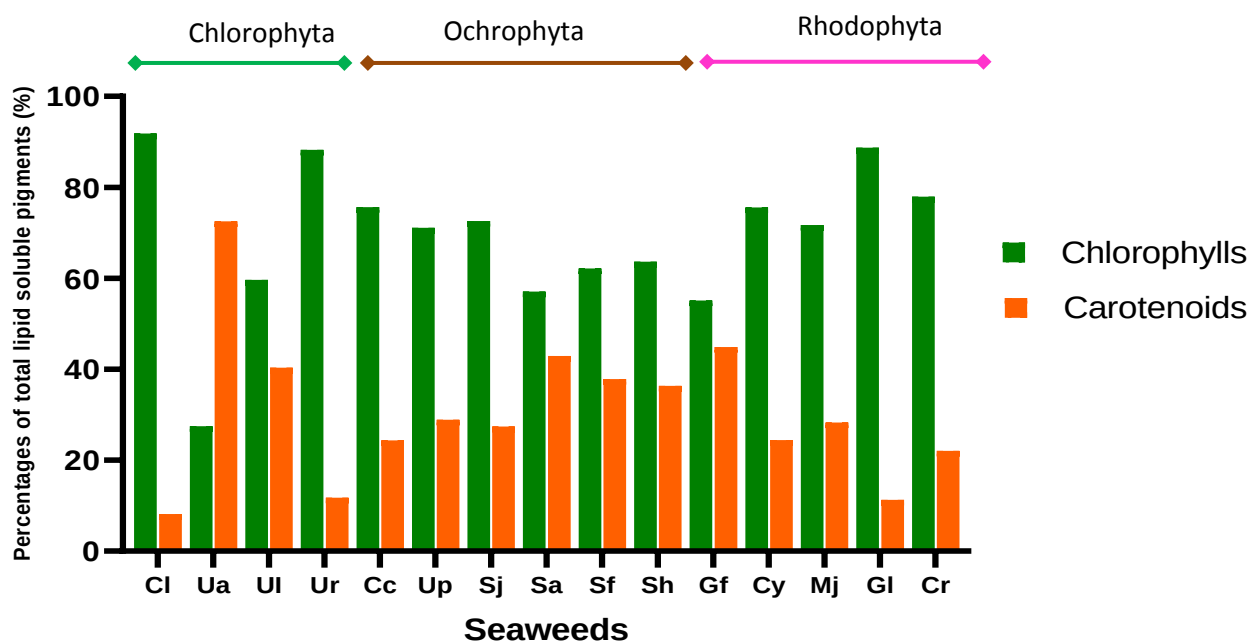


Figure 2.3. Pigments composition (%) of total pigments in seaweeds (calculated from total chlorophylls and carotenoids). Abbreviation: Cl: *Caulerpa lentillifera*, Ua: *Ulva australis*; Ui: *Ulva intestinalis*; Ur: *Ulva reticulata*; Cc: *Costaria costata*; Up: *Undaria pinnatifida*; Sj: *Saccharina japonica*; Sa: *Sargassum aquifolium*; Sf: *Sargassum fusiforme*; Sh: *Sargassum horneri*; Gf: *Gloiopeltis furcata*; Cy: *Chondrus yendoii*; Mj: *Mazzaella japonica*; Gl: *Gracilariopsis longissimi*, Cr: *Chondria crassicaulis*.

Although Chl a was detected in all seaweeds from the temperate area, its level widely ranged from 1.74 (*Chondria crassicaulis*) to 268.82 (*S. horneri*) mg·100 g⁻¹ DW (**Table 2.4.**). Chl b, the characteristic pigment in Chlorophyta, was present in small amounts and ranged from 16.83 (*Caulerpa lentillifera*) to 101.50 (*U. intestinalis*) mg·100 g⁻¹ DW (**Table 2.4.**). A unique finding was the occurrence of Chl c family, the characteristic accessory pigment in Ochrophyta. It was not only found in Ochrophyta, but also in Rhodophyta (*C. crassicaulis* and *Mazaella japonica*). Phy a was abundantly found in seaweeds of the

Laminariaceae family and Rhodomelaceae family, while Phy b was only found in Chlorophyta.

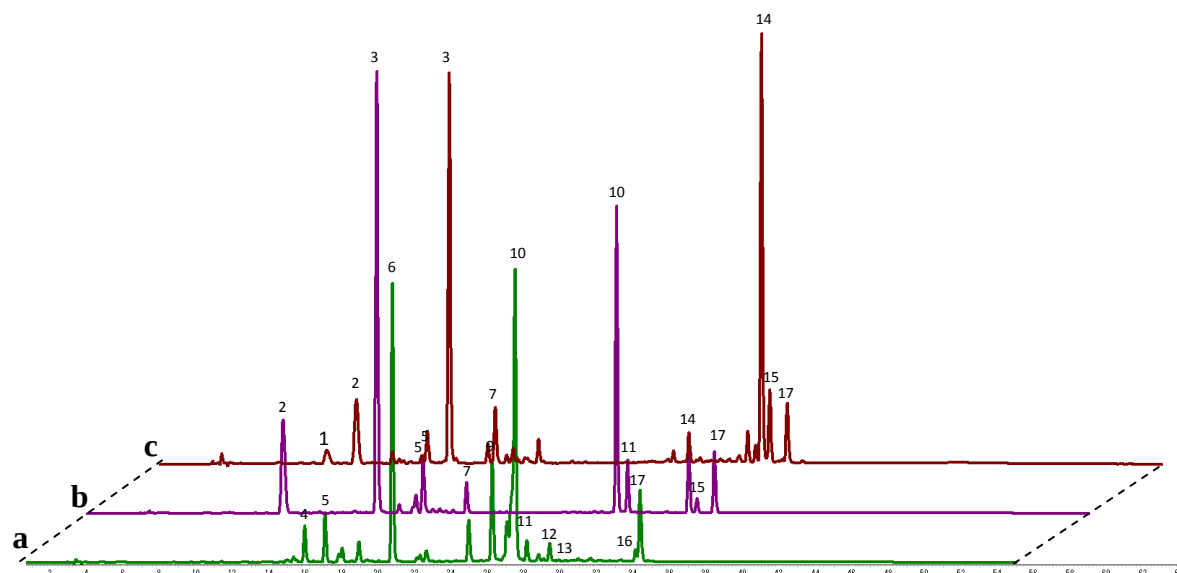


Figure 2.4. HPLC chromatogram (450 nm) of chlorophylls and carotenoids in different seaweeds total lipids (a) *Ulva australis* (Chlorophyta), (b) *Sargassum horneri* (Ochrophyta), (c) *Chondria crassicaulis* (Rhodophyta) at 450 nm. Pigments peak which were detected by HPLC La Chrome system: 1. Chlorophyll c1 (Chl c1); 2. Chlorophyll c2 (Chl c2); 3. Fucoxanthin (Fx); 4. Neoxanthin (Nx); 5. Violxanthin (Vx); 6. Lutein (Lut); 7. Zeaxanthin (Zx); 8. Chlorophyll b (Chl b); 9. Chlorophyll b' (Chl b'); 10. Chlorophyll a (Chl a); 11. Chlorophyll a' (Chl a'); 12. Pheophytin b (Phy b); 13. Pheophytin b' (Phy b'); 14. Pheophytin a (Phy a); 15. Pheophytin a' (Phy a'); 16. Alpha carotene (α -Car); 17. Beta-carotene (β -Car).

Carotenoids, accessory pigments in photosynthesis, also varied among seaweed species (**Table 2.5.**). Provitamin A carotenoids, α -carotene (α -Car) and β -carotene (β -Car), exhibited different patterns. β -Car was present in all species with a range from 1.12 to 58.24 mg·100 g⁻¹ DW, while α -Car was detected only in Chlorophyta and Rhodophyta. Fx was mainly found in Ochrophyta and found in a small amount in Rhodophyta, but not in Chlorophyta. In addition, zeaxanthin (Zx) content was relatively higher in two *Sargassum* species (*S. horneri* and *S. fusiforme*) and *Undaria pinnatifida* collected from the temperate area. Lutein (Lut) was found in Rhodophyta and Chlorophyta with a range from 0.62 mg·100 g⁻¹ DW (*M.*

japonica) to 124.82 mg·100 g⁻¹ DW (*U. australis*), but was not found in Ochrophyta. Violaxanthin (Vx) was only found in Ochrophyta and Chlorophyta collected from Japan with a range from 1.45 mg·100 g⁻¹ DW (*Costaria costata*) to 23.30 mg·100 g⁻¹ DW (*U. australis*). Neoxanthin (Nx), only present in Chlorophyta, also showed a wide range in its content from 30.63 mg·100 g⁻¹ DW (*U. reticulata*) to 185.56 mg·100 g⁻¹ DW (*U. australis*). Higher total carotenoid levels were found in Ochrophyta (*S. aquifolium*, *C. costata*, *S. japonica*, *U. pinnatifida*, *S. fusiforme*, *S. horneri*) from the two different areas. Chlorophyta species collected from the temperate area (*U. australis* and *U. intestinalis*) also showed higher total carotenoid levels, whereas Chlorophyta from the tropical area (*C. lentillifera* and *U. reticulata*) showed relatively low levels. The same trend was also found among Rhodophyta species collected from the temperate area (*C. crassicaulis*, *C. yendoi*, *Gloiopeltis furcata*, *M. japonica*) and those collected from the tropical area (*Gracilariopsis longissima*).

Carotenoids which are accessory pigment in photosynthesis, varied among seaweeds' classes. **Table 2.5.** shows the tremendous ranged carotenoids content in seaweed lipids. Provitamin A carotenoids; α -Car and β -Car, exhibited different pattern in seaweeds analyzed. α -Car was detected in Chlorophyta and Rhodophyta. Meanwhile, β -Car was discovered in all seaweeds tested with ranged from 0.021 to 0.582 mg·g⁻¹. The most abundance carotenoids, Fx, was found in significant amount in Phaeophyta and a trace amount in Rhodophyta. Zx content was considerably higher in Sargassum collected from Japan (*S. horneri* and *S. fusiforme*). All Rhodophyta and Chlorophyta species contain the variation of Lut with a range from 0.086 mg·g⁻¹ DW (*M. japonica*) to 1.248 mg·g⁻¹ DW (*U. australis*). Moreover, Vx was ranged from 0.015 to 0.233 mg·g⁻¹ DW. The higher level of content was found in *S. horneri*.

Table 2.4. Amount of Chls and Phys in 15 species of seaweeds (mg·100 g⁻¹ DW)*

Seaweeds	Collecting sites	Chl a	Phy a	Chl b	Phy b	Chl c (c1 + c2) ^b	Total chlorophylls
<i>Caulerpa lentillifera</i>	Indonesia	n.d.	118.68 ± 44.41	16.83 ± 4.60	90.28 ± 8.97	n.d.	614.153
<i>Ulva australis</i>	Japan	81.90 ± 4.94	26.17 ± 3.27	64.45 ± 9.36	n.d.	n.d.	2627.936
<i>Ulva intestinalis</i>	Japan	115.57 ± 51.28	165.98 ± 14.70	101.50 ± 29.21	64.38 ± 11.30	n.d.	352.318
<i>Ulva reticulata</i>	Indonesia	n.d.	115.85 ± 21.17	18.04 ± 0.61	267.69 ± 158.86	n.d.	707.76
<i>Costaria costata</i>	Japan	16.28 ± 3.80	291.92 ± 67.84	n.d.	n.d.	21.78 ± 0.84	1396.261
<i>Undaria pinnatifida</i>	Japan	54.67 ± 14.60	423.42 ± 57.72	n.d.	n.d.	38.58 ± 5.03	1872.709
<i>Saccharina japonica</i>	Japan	26.13 ± 16.79	425.42 ± 19.17	n.d.	n.d.	17.11 ± 2.91	2215.184
<i>Sargassum aquifolium</i> ^a	Indonesia	12.17 ± 4.02	149.64 ± 56.00	n.d.	n.d.	2.12 ± 0.21	1807.968
<i>Sargassum fusiforme</i>	Japan	210.72 ± 43.63	107.59 ± 30.30	n.d.	n.d.	18.20 ± 0.34	2621.332
<i>Sargassum horneri</i>	Japan	268.82 ± 59.93	220.86 ± 22.84	n.d.	n.d.	23.29 ± 4.9	326.26
<i>Gloiopeltis furcata</i>	Japan	2.98 ± 1.96	28.21 ± 4.92	n.d.	n.d.	n.d.	1336.487
<i>Chondrus yendoii</i>	Japan	4.77 ± 2.10	29.29 ± 2.21	n.d.	n.d.	n.d.	1356.211
<i>Mazzaella japonica</i>	Japan	6.70 ± 1.28	16.25 ± 1.85	n.d.	n.d.	7.63 ± 1.25	135.117
<i>Gracilariopsis longissima</i>	Indonesia	n.d.	14.66 ± 2.24	n.d.	n.d.	n.d.	124.536
<i>Chondria crassicaulis</i>	Japan	1.74 ± 1.19	322.72 ± 56.48	n.d.	n.d.	10.18 ± 1.29	109.265

*The data value is expressed as the mean ± SD of three replicate measurements; ^a non-edible seaweed; ^b Chl c composes of Chl c1 and Chl c2. However, both chlorophylls could not be separated on HPLC to be detected as one peak; therefore, Chl c1 and Chl c2 were expressed as Chl c altogether in the present study. Quantification of Chl c was done using the calibration curve of c1 standard, because Chl c1 has been reported to be the major chl c in brown seaweeds (Chen et al. 2017). n.d. = not detected. Abbreviations: Chl a: chlorophyll a; Chl b: chlorophyll b; Chl c: chlorophyll c; Phy a: pheophytin a; Phy b: pheophytin b.

Table 2.5. Carotenoids content of 15 species of seaweeds (mg·100g⁻¹ DW) *

Seaweeds	Collecting sites	β-Car	α-Car	Zx	Lut	Vx	Nx	Fx	Total carotenoids
<i>Caulerpa lentillifera</i>	Indonesia	1.84 ± 0.70	17.15 ± 2.96	n.d.	1.02 ± 0.53	n.d.	n.d.	n.d.	228.123
<i>Ulva australis</i>	Japan	58.24 ± 6.52	63.01 ± 0.04	n.d.	124.82 ± 13.14	23.30 ± 0.74	185.56 ± 59.65	n.d.	243.129
<i>Ulva intestinalis</i>	Japan	39.91 ± 9.54	17.83 ± 6.66	n.d.	77.78 ± 18.14	13.15 ± 4.19	153.70 ± 84.20	n.d.	16.0486
<i>Ulva reticulata</i>	Indonesia	3.89 ± 0.39	4.83 ± 0.35	n.d.	14.12 ± 2.85	n.d.	30.63 ± 7.84	n.d.	26.628
<i>Costaria costata</i>	Japan	7.31 ± 0.86	n.d.	n.d.	n.d.	1.45 ± 0.19	n.d.	97.60 ± 12.87	189.631
<i>Undaria pinnatifida</i>	Japan	25.58 ± 3.71	n.d.	12.03 ± 3.99	n.d.	3.51 ± 0.61	n.d.	169.48 ± 12.98	321.772
<i>Saccharina japonica</i>	Japan	18.10 ± 0.61	n.d.	3.04 ± 0.13	n.d.	1.47 ± 0.25	n.d.	154.71 ± 11.29	355.116
<i>Sargassum aquifolium</i> ^a	Indonesia	12.51 ± 1.24	n.d.	2.45 ± 0.32	n.d.	n.d.	n.d.	108.44 ± 9.17	325.665
<i>Sargassum fusiforme</i>	Japan	44.70 ± 7.52	n.d.	7.06 ± 1.65	n.d.	12.15 ± 2.45	n.d.	140.93 ± 12.98	486.009
<i>Sargassum horneri</i>	Japan	42.70 ± 6.70	n.d.	29.21 ± 2.72	n.d.	4.74 ± 0.98	n.d.	216.50 ± 31.97	304.185
<i>Gloiopeltis furcata</i>	Japan	8.99 ± 2.53	3.73 ± 0.70	0.47 ± 0.30	+(8.76 ± 2.31) ^c	n.d.	n.d.	3.43 ± 0.48	176.285
<i>Chondrus yendoii</i>	Japan	2.07 ± 0.19	4.60 ± 0.47	n.d.	1.78 ± 0.81	n.d.	n.d.	2.57 ± 0.07	136.987
<i>Mazzaella japonica</i>	Japan	2.23 ± 0.18	1.95 ± 0.27	n.d.	0.62 ± 0.12	n.d.	n.d.	7.26 ± 0.42	8.931
<i>Gracilariopsis longissima</i>	Indonesia	1.12 ± 0.25	n.d.	+(0.75 ± 0.16) ^b	n.d.	n.d.	n.d.	n.d.	23.1
<i>Chondria crassicaulis</i>	Japan	13.78 ± 2.40	8.19 ± 0.82	+(4.94 ± 1.66) ^b	n.d.	n.d.	n.d.	67.76 ± 9.63	9.885

*The data value is expressed as the mean ± SD of three replicate measurements; ^a non-edible seaweeds; ^b Zx and Lut were calculated from the calibration curve of Zx standard; ^c Lut and Zx were calculated from the calibration curve of Lut standard. n.d. = not detected. Abbreviations: β-Car: β-carotene; α-Car: α-carotene; Zx: zeaxanthin; Lut: lutein; Vx: violaxanthin; Nx: neoxanthin; Fx: fucoxanthin.

2.3.3. Fatty acids

The details of FAs composition in seaweed lipids are listed in **Tables 2.6 - 2.9**. All values are expressed as percentages of total FAs content. The absolute content of total n-3 PUFAs ($\text{mg}\cdot\text{g}^{-1}$ DW) is also shown in **Table 2.9**. The FAs composition varied widely among seaweed species and sampling locations.

In the three phyla, Chlorophyta, Ochrophyta, and Rhodophyta, palmitic acid (C16:0) was the predominant FAs (**Tables 2.6. – 2.8.**). The temperate seaweeds contained much more total PUFAs (28.15% (*C. crassicaulis*) to 51.28% (*S. japonica*)) than the tropical seaweeds (13.06% (*C. lentillifera*) to 25.75% (*U. reticulata*)) (**Table 2.9.**). Specifically, the absolute amounts of total n-3 PUFAs in the temperate seaweeds were much higher than those in the tropical (Indonesia) seaweeds. Total saturated fatty acids (SFAs) contents in the tropical seaweeds were relatively higher than those in the temperate seaweeds, except for *M. japonica*. Furthermore, monounsaturated fatty acids (MUFAs), dominantly comprising C16:1 and C18:1n-9, were higher in temperate Rhodophyta, namely, *C. crassicaulis*. Overall, in the tropical (Indonesia) seaweeds, SFAs accounted for 24.37% to 47.78%, followed by PUFAs (13.06% to 25.75%) and MUFAs (6.88% to 18.88%). In temperate seaweeds the most dominant FAs class was PUFAs (36.23% to 51.28%), except for *C. crassicaulis* (28.15%) and *M. japonica* (31.21%) (**Table 2.9.**).

Total PUFAs levels varied among species, ranging from 13.06% (*C. lentillifera*) to 51.28% (*S. japonica*) of total FAs. These seaweeds contained EPA (C20:5n-3), ARA (C20:4n-6), ALA (C18:3n-3), and SDA (C18:4n-3) as the major PUFAs; however, there was significant variation in the level of these PUFAs in the TL content from each species (**Tables 2.6 – 2.8.**). The main PUFAs in Ochrophyta collected from the temperate area were ARA (10.55% (*U. pinnatifida*) to 14.87% (*S. horneri*)) and EPA (8.36% (*C. costata*) to 13.04% (*S. japonica*)), whereas the main PUFAs in Ochrophyta collected from the tropical area

(*S. aquifolium*) was ALA (10.40%), and the EPA level of this seaweed was very low (0.96%) (**Table 2.7.**). The same trend was found in Rhodophyta (**Table 2.8.**). Rhodophyta collected from the temperate area contained relatively high levels of EPA (13.08% (*C. crassicaulis*) to 35.81% (*G. furcata*)) and of ARA (3.33% (*C. crassicaulis*) to 17.26% (*C. yendoii*)); however, the main PUFAs of Rhodophyta collected in the tropical area (*G. longissima*) was ARA (11.41%), and the EPA level was 0.21%. Chlorophyta collected from the two geographical locations contained ALA as its major PUFAs (**Table 2.6.**). Among the 15 seaweeds analyzed, *G. furcata* exhibited the highest value of total n-3 PUFAs (38.11%) with 35.81% EPA, whereas *U. intestinalis* exhibited the highest value of n-3 PUFAs expressed in $\text{mg}\cdot\text{g}^{-1}$ DW (9.84 $\text{mg}\cdot\text{g}^{-1}$ DW) (**Table 2.9.**).

Table 2.6. FAs composition (% of total FAs) of Chlorophyta

Fatty acids	<i>Caulerpa lentillifera</i>	<i>Ulva reticulata</i>	<i>Ulva australis</i>	<i>Ulva intestinalis</i>
12:0	0.06±0.02	0.01±0.01	0.08±0.06	n.d.
13:0	0.81±0.05	n.d.	0.02±0.01	0.003±0.01
14:0	1.68±0.36	0.49±0.02	0.54±0.01	0.93±0.15
14:1	n.d.	n.d.	n.d.	n.d.
15:0	0.21±0.04	0.31±0.00	0.08±0.01	0.12±0.02
15:1	n.d.	n.d.	n.d.	n.d.
16:0	25.70±6.02	40.86±0.53	23.19±0.19	20.90±1.47
16:1	10.43±3.71	3.80±0.22	1.18±0.16	2.36±1.20
17:0	0.19±0.17	0.22±0.02	0.17±0.01	0.21±0.08
17:1	n.d.	0.38±0.01	0.23±0.02	0.10±0.02
18:0	1.14±0.22	0.39±0.01	0.83±0.44	0.51±0.55
18:1n-9	0.94±0.19	2.23±0.15	1.54±0.06	1.41±0.11
18:2n-6	2.60±0.58	1.72±0.05	5.98±0.18	7.59±1.19
18:3n-6	1.04±0.46	0.31±0.02	0.70±0.03	1.86±1.75
18:3n-3	4.67±0.93	8.52±0.21	16.15±0.33	18.44±2.46
18:4n-3	1.43±0.80	10.78±0.15	10.21±0.28	6.80±1.15
20:0	n.d.	0.23±0.66	0.02±0.01	0.56±0.43
20:1n-9	0.03±0.01	0.03±0.03	0.15±0.01	0.20±0.06
20:2n-6	0.14±0.03	n.d.	0.05±0.01	0.03±0.02
21:0	0.09±0.05	0.09±0.01	0.18±0.01	0.61±0.04
20:4n-6	0.99±0.26	0.46±0.38	0.49±0.02	0.66±0.10
20:3	0.06±0.01	0.09±0.03	0.05±0.00	0.09±0.03
20:5n-3	0.37±0.10	0.82±0.07	0.60±0.03	2.20±0.47
22:0	0.07±0.01	0.75±0.07	0.35±0.02	0.33±0.06
22:1n-9	n.d.	n.d.	0.12±0.01	0.03±0.03
22:2n-6	n.d.	n.d.	n.d.	n.d.
22:5n-3	n.d.	3.03±0.08	1.98±0.05	1.44±0.17
24:0	0.39±0.21	0.05±0.05	0.01±0.01	0.20±0.08
24:1	0.03±0.03	0.44±0.09	0.23±0.00	0.14±0.12
Identified FAs	53.07	75.59	65.14	67.78
Unidentified FAs	46.93	24.41	34.86	32.22

Table 2.7. FAs composition (% of total FAs) of Ochrophyta

Fatty acids	<i>Sargassum aquifolium</i> ^a	<i>Costaria costata</i>	<i>Saccharina japonica</i>	<i>Undaria pinnatifida</i>	<i>Sargassum fusiforme</i>	<i>Sargassum honeri</i>
12:0	0.03±0.01	0.08±0.06	0.01±0.01	0.02±0.01	0.01±0.00	0.01±0.00
13:0	0.01±0.01	0.07±0.01	n.d.	0.01±0.00	n.d.	n.d.
14:0	4.82±0.76	12.47±1.35	7.53±0.67	5.23±0.21	3.63±0.10	2.65±0.17
14:1	0.004±0.01	0.12±0.02	0.06±0.01	0.01±0.00	0.06±0.00	0.03±0.01
15:0	0.37±0.05	0.34±0.03	0.33±0.01	0.44±0.01	0.40±0.01	0.20±0.02
15:1	n.d.	0.004±0.01	n.d.	n.d.	n.d.	n.d.
16:0	39.78±4.47	21.88±1.03	14.79±0.75	22.00±1.36	22.18±0.48	22.77±1.19
16:1	8.67±1.77	2.00±0.19	2.00±0.12	3.73±0.24	2.62±0.04	3.59±0.11
17:0	0.09±0.01	0.08±0.02	0.10±0.01	0.12±0.01	0.06±0.01	0.03±0.00
17:1	0.18±0.08	0.11±0.01	0.81±0.04	0.50±0.05	0.56±0.02	0.20±0.03
18:0	0.66±0.08	0.70±0.13	0.50±0.01	1.62±0.14	0.32±0.03	0.34±0.04
18:1n-9	9.91±0.45	13.24±1.32	12.70±0.60	9.43±1.10	6.32±0.10	7.97±0.14
18:2n-6	5.05±0.24	8.74±0.39	6.26±0.30	7.61±0.10	3.07±0.05	6.21±0.37
18:3n-6	0.98±0.30	1.30±0.10	1.28±0.10	0.90±0.01	0.22±0.01	0.76±0.05
18:3n-3	3.13±0.19	2.63±0.32	5.27±0.25	7.43±4.23	9.38±0.19	5.07±0.08
18:4n-3	2.11±0.20	4.23±0.51	12.84±0.44	11.33±1.23	7.71±0.26	9.91±0.72
20:0	0.20±0.10	0.53±0.04	0.40±0.02	0.81±0.07	0.18±0.00	0.17±0.06
20:1n-9	n.d.	n.d.	0.01±0.01	0.01±0.00	2.38±0.09	2.38±0.14
20:2n-6	0.22±0.01	0.12±0.01	0.06±0.04	0.06±0.00	0.12±0.01	0.19±0.02
21:0	1.02±0.04	1.31±0.07	0.69±0.07	0.78±0.06	0.49±0.00	0.51±0.04
20:4n-6	10.40±0.23	10.96±0.51	12.44±1.23	10.55±0.37	13.68±0.21	14.87±0.73
20:3	0.07±0.01	0.07±0.01	0.08±0.01	0.09±0.01	0.15±0.01	0.10±0.01
20:5n-3	0.96±0.06	8.36±1.37	13.04±1.34	10.08±0.58	12.96±0.35	11.89±0.92
22:0	0.30±0.05	0.01±0.01	n.d.	0.03±0.00	0.27±0.02	0.21±0.06
22:1n-9	n.d.	n.d.	0.004±0.01	0.02±0.01	0.02±0.01	0.01±0.01
22:2n-6	n.d.	0.10±0.02	0.01±0.01	0.12±0.18	0.01±0.01	0.01±0.00
22:5n-3	n.d.	0.10±0.03	n.d.	0.02±0.00	0.01±0.01	n.d.
24:0	0.18±0.03	0.01±0.01	0.01±0.01	0.07±0.00	0.10±0.01	0.09±0.03
24:1	0.12±0.02	0.45±0.01	0.18±0.08	0.37±0.04	n.d.	0.06±0.04
Identified FAs	89.26	90.01	91.40	93.05	86.91	90.23
Unidentified FAs	10.74	9.99	8.60	6.95	13.09	9.77

Tables 2.8. FAs composition (% of total FAs) of Rhodophyta

Fatty acids	<i>Gracillariopsis longissimi</i>	<i>Chondria crassicaulis</i>	<i>Chondrus yendoi</i>	<i>Goliopeltis fusrcata</i>	<i>Mazaella japonica</i>
12:0	0.10±0.06	0.10±0.03	0.11±0.01	0.05±0.02	0.12±0.01
13:0	0.08±0.02	0.04±0.01	0.01±0.000	0	0.01±0.00
14:0	1.87±0.19	7.68±0.57	2.53±0.17	2.50±0.24	3.41±0.18
14:1	0.48±0.05	0.45±0.02	0.21±0.01	0.13±0.01	0.22±0.02
15:0	43.03±0.83	26.75±1.04	32.40±0.49	21.40±1.25	38.31±0.27
15:1	3.41±0.87	11.66±0.71	0.72±0.02	1.95±0.07	2.19±0.19
16:0	0.19±0.07	0.09±0.01	0.15±0.02	0.07±0.03	0.19±0.02
16:1	0.15±0.06	0.27±0.04	n.d	n.d	n.d
17:0	1.83±0.06	1.27±0.43	1.06±0.02	1.08±0.03	1.81±0.02
17:1	7.10±0.48	8.04±0.16	8.96±0.15	15.42±0.11	11.45±0.08
18:0	0.54±0.03	3.05±0.09	1.55±0.03	1.70±0.08	1.71±0.01
18:1n-9	0.12±0.02	1.72±0.85	0.80±0.02	0.40±0.01	0.62±0.01
18:2n-6	0.53±0.04	1.93±0.08	0.10±0.01	0.62±0.05	0.35±0.02
18:3n-6	0.05±0.08	3.92±0.13	0.12±0.01	0.15±0.02	0.48±0.02
18:3n-3	0.10±0.01	0.14±0.01	0.004±0.01	0.01±0.00	0.09±0.01
18:4n-3	0.05±0.01	0.22±0.07	0.09±0.00	0.14±0.04	0.08±0.01
20:0	0.20±0.02	0.22±0.05	0.07±0.00	0.18±0.01	0.16±0.00
20:1n-9	0.66±0.08	0.27±0.02	0.79±0.03	1.54±0.07	0.89±0.00
20:2n-6	11.41±0.70	3.33±0.18	17.26±0.17	4.93±0.17	8.67±0.24
21:0	0.52±0.22	0.04±0.03	0.05±0.00	0.02±0.00	0.01±0.01
20:4n-6	0.21±0.17	13.08±0.49	23.67±0.83	35.81±2.02	18.28±0.49
20:3	0.09±0.01	0.02±0.02	0.003±0.01	0.01±0.00	0.05±0.02
20:5n-3	0.03±0.03	0.04±0.03	0.06±0.06	0.32±0.01	0.15±0.01
22:0	0.09±0.01	0.004±0.01	n.d.	n.d.	n.d.
22:1n-9	0.01±0.02	0.81±0.17	0.08±0.01	0.160±0.04	0.19±0.01
22:2n-6	0.32±0.08	0.05±0.04	0.20±0.01	1.36±0.07	0.74±0.04
22:5n-3	1.18±1.46	0.06±0.05	0.01±0.018	0.19±0.02	0.18±0.03
24:0	0.43±0.40	0.23±0.03	0.05±0.00	0.22±0.05	0.63±0.45
Identified FAs	74.78	85.51	91.05	90.36	90.99
Unidentified FAs	25.72	14.49	8.95	9.64	9.01

Table 2.9. FAs composition (weight % of total fatty acids (FAs))*

Seaweeds	Collecting sites	Σ SFAs	Σ MUFAs	Σ PUFAs	Σ n-3 PUFAs	Σ n-6 PUFAs	Σ n-3 PUFAs ^b
<i>Caulerpa lentillifera</i>	Indonesia	29.80 $\pm$ 1.65	9.08 $\pm$ 2.75	13.06 $\pm$ 0.32	7.31 $\pm$ 0.41	5.75 $\pm$ 0.10	0.01
<i>Ulva australis</i>	Japan	25.67 $\pm$ 0.47	3.45 $\pm$ 0.24	36.23 $\pm$ 0.82	29.00 $\pm$ 0.66	7.22 $\pm$ 0.22	6.83
<i>Ulva intestinalis</i>	Japan	24.37 $\pm$ 1.21	4.24 $\pm$ 0.87	39.16 $\pm$ 1.54	29.03 $\pm$ 2.02	10.14 $\pm$ 0.65	9.84
<i>Ulva reticulata</i>	Indonesia	43.01 $\pm$ 0.46	6.88 $\pm$ 0.42	25.75 $\pm$ 0.52	23.25 $\pm$ 0.36	2.50 $\pm$ 0.43	0.02
<i>Costaria costata</i>	Japan	37.46 $\pm$ 2.02	15.92 $\pm$ 1.46	36.59 $\pm$ 2.62	15.38 $\pm$ 2.15	21.21 $\pm$ 0.79	0.53
<i>Undaria pinnatifida</i>	Japan	31.12 $\pm$ 1.75	14.07 $\pm$ 0.90	48.19 $\pm$ 5.72	28.94 $\pm$ 5.71	19.25 $\pm$ 0.30	4.66
<i>Saccharina japonica</i>	Japan	24.35 $\pm$ 1.18	15.76 $\pm$ 0.64	51.28 $\pm$ 0.61	31.24 $\pm$ 1.91	20.04 $\pm$ 1.57	2.26
<i>Sargassum aquifolium</i> ^a	Indonesia	46.80 $\pm$ 5.31	18.88 $\pm$ 1.40	22.90 $\pm$ 0.89	6.26 $\pm$ 0.37	16.64 $\pm$ 0.52	0.10
<i>Sargassum fusiforme</i>	Japan	27.62 $\pm$ 0.58	11.95 $\pm$ 0.22	47.32 $\pm$ 1.08	30.22 $\pm$ 0.78	17.11 $\pm$ 0.27	2.66
<i>Sargassum horneri</i>	Japan	26.98 $\pm$ 1.49	14.24 $\pm$ 0.25	49.00 $\pm$ 2.36	26.97 $\pm$ 1.64	22.03 $\pm$ 1.16	4.03
<i>Gloiopeltis furcata</i>	Japan	26.97 $\pm$ 1.45	18.05 $\pm$ 0.23	45.33 $\pm$ 2.36	38.11 $\pm$ 2.12	7.22 $\pm$ 0.24	0.46
<i>Chondrus yendoii</i>	Japan	37.28 $\pm$ 0.62	9.89 $\pm$ 0.19	43.91 $\pm$ 0.85	24.23 $\pm$ 0.81	19.68 $\pm$ 0.21	0.68
<i>Mazzaella japonica</i>	Japan	45.29 $\pm$ 0.51	14.49 $\pm$ 0.61	31.21 $\pm$ 0.72	20.05 $\pm$ 0.50	11.15 $\pm$ 0.24	0.10
<i>Gracilariopsis longissima</i>	Indonesia	47.78 $\pm$ 2.36	11.18 $\pm$ 1.73	13.90 $\pm$ 0.64	1.63 $\pm$ 0.26	12.28 $\pm$ 0.71	0.01
<i>Chondria crassicaulis</i>	Japan	36.88 $\pm$ 1.23	20.49 $\pm$ 0.88	28.15 $\pm$ 1.37	19.82 $\pm$ 0.71	8.33 $\pm$ 0.82	2.73

*The data value is expressed as the mean $\pm$ SD of three replicate measurements; ^a non-edible seaweeds; ^b absolute content of total n-3 PUFAs (mg·g⁻¹ DW). Abbreviations: SFAs: saturated fatty acids; MUFAs: mono unsaturated fatty acids; PUFAs: polyunsaturated fatty acids.

2.3.4. Nutritional quality index

The nutritional quality index (Gerasimenko *et al.*, 2010; Matos *et al.*, 2016) based on the FAs composition was calculated in this study (**Table 2.10.**). The n-6/n-3 ratio was less than 3, except for *G. longissima* (7.69), and most of the ratios were less than 1.0. In general, the atherogenicity index (AI) and thrombogenicity index (TI) values increase with the decreasing degree of unsaturation of FAs, while the FAs hypocholesterolemic/hypercholesterolemic ratio (h/H) increases with the increasing unsaturated FAs composition. Therefore, AI and TI tended to be higher in the tropical seaweeds compared with those in temperate seaweeds. The h/H ratios of all temperate seaweeds were higher than those of the tropical seaweeds. In addition, the unsaturation index (UI) in all seaweeds was more than 3, and in Rhodophyta collected in the temperate area, it was more than 4.

Table 2.10. Nutritional quality index of different seaweeds judged from FAs composition.

Seaweeds	Collecting sites	n-6/n-3 PUFAs	AI	TI	h/H	UI
<i>Caulerpa lentillifera</i>	Indonesia	0.79 ± 0.05	1.53 ± 0.28	0.96 ± 0.13	0.44 ± 0.04	3.00 ± 0.07
<i>Ulva australis</i>	Japan	0.25 ± 0.01	0.64 ± 0.01	0.26 ± 0.01	1.04 ± 0.02	3.27 ± 0.01
<i>Ulva intestinalis</i>	Japan	0.35 ± 0.05	0.57 ± 0.04	0.23 ± 0.01	1.39 ± 0.13	3.19 ± 0.03
<i>Ulva reticulata</i>	Indonesia	0.11 ± 0.02	1.31 ± 0.05	0.35 ± 0.27	0.33 ± 0.02	3.67 ± 0.01
<i>Costaria costata</i>	Japan	1.40 ± 0.18	1.37 ± 0.16	0.54 ± 0.08	1.28 ± 0.12	3.63 ± 0.06
<i>Undaria pinnatifida</i>	Japan	0.68 ± 0.13	0.69 ± 0.08	0.28 ± 0.06	1.66 ± 0.23	3.71 ± 0.04
<i>Saccharina japonica</i>	Japan	0.65 ± 0.09	0.67 ± 0.04	0.20 ± 0.02	2.23 ± 0.08	3.88 ± 0.04
<i>Sargassum aquifolium</i> ^a	Indonesia	2.66 ± 0.07	1.42 ± 0.25	1.23 ± 0.20	0.67 ± 0.07	3.40 ± 0.01
<i>Sargassum fusiforme</i>	Japan	0.57 ± 0.01	0.62 ± 0.02	0.24 ± 0.01	1.76 ± 0.06	3.93 ± 0.00
<i>Sargassum horneri</i>	Japan	0.82 ± 0.05	0.53 ± 0.05	0.26 ± 0.03	1.82 ± 0.16	3.86 ± 0.02
<i>Gloiopeltis furcata</i>	Japan	0.19 ± 0.00	0.50 ± 0.05	0.19 ± 0.02	2.46 ± 0.26	4.75 ± 0.01
<i>Chondrus yendoii</i>	Japan	0.81 ± 0.03	0.79 ± 0.03	0.41 ± 0.02	1.48 ± 0.05	4.46 ± 0.01
<i>Mazzaella japonica</i>	Japan	0.56 ± 0.01	1.14 ± 0.03	0.58 ± 0.03	0.97 ± 0.03	4.49 ± 0.00
<i>Gracilariopsis longissima</i>	Indonesia	7.69 ± 1.58	2.03 ± 0.24	2.80 ± 0.25	0.44 ± 0.03	3.87 ± 0.02
<i>Chondria crassicaulis</i>	Japan	0.42 ± 0.03	1.19 ± 0.09	0.47 ± 0.03	0.86 ± 0.05	4.13 ± 0.03

^a Non-edible seaweeds. Abbreviations: AI: atherogenicity index; TI: thrombogenicity index; h/H: fatty acids hypocholesterolemic/hypercholesterolemic ratio; UI: unsaturation index.

2.3.5. α -Tocopherol

α -Toc content varied with seaweed species (**Table 2.11.**). Relatively lower α -Toc levels were found in Chlorophyta (*C. lentillifera*, *U. reticulata*, *U. australis*, *U. intestinalis*). Seaweeds belonging to Ochrophyta and Rhodophyta showed relatively higher levels of α -Toc, except for *C. crassicaulis*. Specifically, *C. yendoii* showed the highest α -Toc content ($9.34 \text{ mg} \cdot 100 \text{ g}^{-1}$), followed by two species of *Sargassum* (*S. fusiforme* and *S. horneri*).

Table 2.11. α -Toc content ($\text{mg} \cdot 100 \text{ g}^{-1}$ DW)*

Seaweeds	Collecting sites	α -Toc
<i>Caulerpa lentillifera</i>	Indonesia	0.87 ± 0.21
<i>Ulva australis</i>	Japan	0.44 ± 0.01
<i>Ulva intestinalis</i>	Japan	0.83 ± 0.06
<i>Ulva reticulata</i>	Indonesia	1.13 ± 0.08
<i>Costaria costata</i>	Japan	1.37 ± 0.10
<i>Undaria pinnatifida</i>	Japan	1.09 ± 0.05
<i>Saccharina japonica</i>	Japan	1.82 ± 0.02
<i>Sargassum aquifolium</i> ^a	Indonesia	2.40 ± 0.02
<i>Sargassum fusiforme</i>	Japan	3.56 ± 0.08
<i>Sargassum horneri</i>	Japan	3.65 ± 0.03
<i>Gloiopeltis furcata</i>	Japan	2.71 ± 0.34
<i>Chondrus yendoii</i>	Japan	9.34 ± 0.19
<i>Mazzaella japonica</i>	Japan	1.72 ± 0.01
<i>Gracilariopsis longissima</i>	Indonesia	2.58 ± 0.02
<i>Chondria crassicaulis</i>	Japan	0.54 ± 0.01

*The data value is expressed as the mean $\pm$ SD of three replicate measurements, ^a non-edible seaweeds.

2.3.6. Multivariate Analysis

A statistical analysis was performed using the 15 FAs composition measured in this study in order to establish the relationship between geographical location and phylum. The fifteen FAs variables explained the variability presented in the data, with principle component 1 (PC-1) accounting for 33.1% and principle component 2 (PC-2) for 22.5% of the variation (**Figure 2.5a.**). Although the total percentage was only 55.6%, the analysis

revealed a correlation of each seaweed within the phyla. Axis I separated the Rhodophyta species from the Chlorophyta, while Ochrophyta was positioned intermediately between these two, based on the principle component analysis (PCA) shown in the bi-plot data. However, these results could not determine whether the geographical location affected the FAs composition. Therefore, the PCA on the FAs groups was analyzed. The bi-plot of the FA groups' data matrix showed 81.5% of variance with PC-1 contributing 51.9% and PC-2 contributing 22.5% (**Figure 2.5b.**). These data exhibited a broad diversity of FAs in the seaweed samples. The analysis revealed that the tropical seaweeds were rich in SFAs, while temperate seaweeds were dominated by PUFAs, including n-3 and n-6 PUFAs, excluding *C. costata*, *M. japonica*, and *C. crassicaulis*. When PCA was used for clustering of the FAs group, the seaweeds could be grouped within the sampling location.

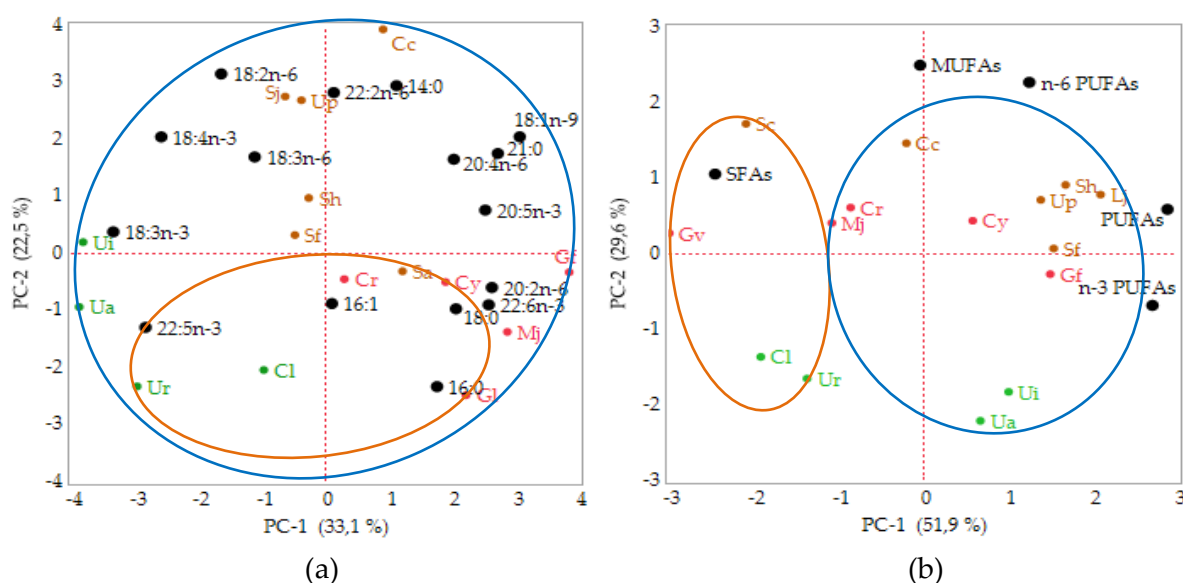


Figure 2.5. Bi-plot of the seaweeds' sample received from PCA analysis of (a) fatty acids (FAs) data composition (15 fatty acids), (b) FAs group (SFAs, MUFAs, PUFAs, n-3 PUFAs, and n-6 PUFAs). Abbreviations: Cl: *Caulerpa lentillifera*; Ua: *Ulva australis*; Ui: *Ulva intestinalis*; Ur: *Ulva reticulata*; Cc: *Costaria costata*; Up: *Undaria pinnatifida*; Sj: *Saccharina japonica*; Sa: *Sargassum aquifolium*; Sf: *Sargassum fusiforme*; Sh: *Sargassum horneri*, Cr: *Chondria crassicaulis*; Cy: *Chondrus yendoii*; Gf: *Gloiopeltis furcata*; Gl: *Gracilariopsis longissima*; Mj: *Mazzaella japonica*. The seaweeds species codes are colored based on the seaweeds' phyla (green: Chlorophyta; brown: Ochrophyta; red: Rhodophyta). The FAs composition and FAs group variable are colored in black. The circles were drawn based on geographical sampling location (orange circle: tropical seaweeds; blue circle: temperate seaweeds).

2.4. Discussion

Seaweeds have been an integral part of Japanese cuisine since hundred years ago, similar to the diet of coastal community in several Indonesian regions (Sho, 2001; McHugh, 2003). On the other hand, industries use seaweeds as marine hydrocolloids and ingredients for foods and animal feed. The utilization of seaweeds as staple diets is due to their characteristic nutrients which are proven to have therapeutic effect on humans. Seaweeds are a source of novel bioactive compounds, such as certain polysaccharides and antioxidants that are not found in terrestrial plants. The consumption of seaweeds has been linked to a lower incidence of chronic diseases such as cancer, hyperlipidemia, and coronary heart disease (Brown *et al.*, 2014). Recently, seaweed lipids have drawn increased interest because of the presence of many kinds of bioactives such as n-3 and n-6 PUFAs, carotenoids, sterols, and others (Miyashita *et al.*, 2013). Although seaweeds have the lower lipid contents than marine fish, they are still a potential source of functional lipids due to their large stock in coastal waters.

Among bioactive seaweed lipids, much attention has been paid to the lipid soluble pigments such as Chls and carotenoids (Pangestuti and Kim, 2011). However, a little information has been available on the relationship between Chls and carotenoids from different kinds of seaweeds. The present study analyzed the lipids from 15 different kinds of seaweed collected in two different regions, tropical (Indonesia) and temperate (Japan) areas, with a special reference to the content of bioactive components, Chls, carotenoids, n-3 and n-6 PUFAs, and α -Toc. The results confirmed that TL content and the composition greatly varied with species. Moreover, they were sometimes affected by the location. As shown in **Figure 2.2.**, TL level was higher in the temperate seaweeds than in the tropical seaweeds, although there were exceptions. It is generally accepted that TL of seaweeds varies with temperature, salinity, and light intensity. Especially the temperature strongly affects the TL

level of seaweeds. Several studies have revealed that the TL of temperate seaweeds is much higher than that of tropical seaweeds (Bhaskar *et al.*, 2008; Sánchez-Machado *et al.*, 2004; Susanto *et al.*, 2016; Terasaki *et al.*, 2009). The result on the TL variation obtained in the present study is consistent with these previous studies.

Although the major components of the seaweed TL are glycerolipids such as glyceroglycolipid (Miyashita *et al.*, 2013), other kinds of lipid related compounds such as photosynthetic pigments are also contained at considerably amount (Pangestuti and Kim, 2011). Chls and carotenoids are representative and major photosynthetic pigments found in seaweeds (Takaichi, 2013). Chls are the most abundant pigments on earth and allow seaweeds to convert light into biological energy (Chen and Roca, 2018). Carotenoids have also an important function as light energy harvesters in the photosynthesis by passing on light excitation to Chls. In addition, carotenoids act as antioxidants that inactivate reactive oxygen species formed by exposure to light and air in the photosynthesis. Both lipid soluble pigments have been known to show a variety of nutritional effects on humans (Fraser and Bramley, 2004; Fernández-García *et al.*, 2009; Vílchez *et al.*, 2011; Christaki *et al.*, 2013; Solymosi and Mysliwa-Kurdziel, 2016; Miyashita and Hosokawa, 2018; Hayes and Ferruzzi, 2020).

In seaweeds, the most important Chls is Chl a which absorbs the energy from wavelength of violet blue and orange-red light (Holdt and Kraan, 2011). Chl b and Chl c are also the important Chls found in seaweeds. They are participating as an accessory pigment in the antenna system of Chl a (Allakhverdiev *et al.*, 2016; Dougherty *et al.*, 1970; Fujiwara *et al.*, 2016; Katz *et al.*, 1978). The present study reveals that the variation of these three kinds of Chls was mainly affected by phyla, namely, Phaeophyta, Chlorophyta and Rhodophyta, but not by sampling location (**Table 2.4.**). In addition, the study also showed the lower levels of Chl a and Chl b in the tropical seaweeds as comparing with those in the temperate seaweeds, while the considerable amounts of corresponding Phy derivatives, Phy a and b,

were detected in the tropical seaweeds. Seaweeds from the tropical area were dried before the analysis; therefore, most of Chl a and Chl b in the tropical seaweed samples would be decomposed to produce corresponding derivatives, Phy a and Phy b, respectively. Therefore, considering the degradation of Chl a and Chl b during the treatment of the tropical seaweeds, major Chls could be considered as Chl a and Chl c for Phaeophyta, Chl a and Chl b for Chlorophyta, and Chl a for Rhodophyta, respectively.

In the present study, Chl c was found in two kinds of Rhodophyta, Cc and Mj (**Table 2.4.**). This finding will be noteworthy because Chl c has been regarded as specific Chls in Phaeophyta (Chen *et al.*, 2017; Jeffrey, 1972). Wilhelm (1987), detected Chl c in Chlorophyta, *Mantoniella comigrates*; however, there has been no report on the presence of Chl c in Rhodophyta. Chl c is also found in diatoms as major Chls. Diatoms use blue-green spectral region as photosynthetic energy. They have Chl a and Chl c as major Chls and Fx as major carotenoids. In the diatoms, the Fx-Chl protein complex is mainly responsible for the light-harvesting (Gelzinis *et al.*, 2015; Kuczynska *et al.*, 2015). The present study suggests that the Fx-Chl protein complex would be the key molecular complex for the light-harvesting not only in diatom but also in several kinds of seaweeds (Apt *et al.*, 1995; Gelzinis *et al.*, 2015).

As described above, the content of CDs mainly varied with the seaweed phyla but not with the different location. The same tendency was also found in the carotenoid composition (**Table 2.5.**). The carotenoid content in each phyla are related to the difference in the carotenoid biosynthesis of each species (Takaichi, 2013). Phaeophyta species were rich in Fx, with a considerable amount of β -Car and Zx, and with a small amount of Vx. On the other hand, α -Car and Lut were detected as major carotenoids in Chlorophyta and Rhodophyta. The presence of both carotenoids will be closely related to the presence of α -Car pathway in Rhodophyta and Chlorophyta (Mikami and Hosokawa, 2013). Two species (Cy and Mj) of

Rhodophyta analyzed in this study showed the absence of Zx. The lack of Zx may be related to the photo-acclimation in these species (Schubert *et al.*, 2006).

Chls and carotenoids have been considered as the most important biomolecules for the photosynthesis. On the other hand, both pigments have attracted major interest from biochemists and nutritionists alike because they are known to have significant biochemical and physiological effects and primarily exhibit a positive influence on human nutrition and health (Pangestuti and Kim, 2011). Especially, much attention has been paid to the antioxidant activity of Chls and carotenoids. Carotenoids are one of the most famous natural antioxidants together with tocopherols and polyphenols. They are regarded as the most efficient natural quenchers of singlet oxygen ($^1\text{O}_2$) and this effect has been attributed to physical mechanism, where the excess energy of singlet oxygen is transferred to conjugated double bonds of carotenoid (Miyashita and Hosokawa, 2018). In addition to the singlet oxygen quenching ability, carotenoids can scavenge free radicals. The reactions with free radicals are much more complex than those with singlet oxygen. Chls are also known to scavenge free radicals and the effect would be strongly related to their chemical structure, especially the structure of porphyrin ring, phytyl chain and extended system of conjugated double bonds (Pangestuti and Kim, 2011).

The strong ability of these seaweed pigments to quench singlet oxygen and/or to scavenge free radicals has been suggested as the main mechanism by which they afford their health benefits (Miyashita and Hosokawa, 2018; Chen and Roca, 2018; Pangestuti and Kim, 2011; Cho *et al.*, 2011). The seaweed lipids examined in the present study contained Chls and carotenoids as major lipid soluble antioxidants. In addition, α -Toc, the most popular natural antioxidant, was detected in these lipids. Therefore, the seaweed lipids are considered as a potential source of natural antioxidants that can be used as cosmetic and food ingredients (Kuczynska *et al.*, 2015; Sathasivam and Ki, 2018). α -Toc is a major tocopherol analogue

found in seaweeds (Burtin, 2003). In this study, the α -Toc content in two species of seaweeds from the Sargassaceae family (*S. fusiforme* and *S. horneri*) was higher than that of other seaweeds, except for *C. yendoi* (**Table 2.11.**). This result is in accordance with previous studies (Miyashita and Takagi, 1987; Santos *et al.*, 2015). The present study also suggests the relatively lower α -Toc level in Chlorophyta seaweeds (*C. lentillifera*, *U. reticulata*, *U. australis*, *U. intestinalis*) as compared with the other phyla, Ochrophyta and Rhodophyta.

Tables 2.6. - 2.9. show the FAs composition of seaweed lipids. The composition was strongly affected by the sampling location, as seen in the TL analysis. As shown in **Table 2.9.**, total PUFAs were the most dominant FAs class in the temperate seaweeds, and the level was higher than that in the tropical seaweeds. In the seaweeds collected from the tropical area, SFAs were a major FAs class. This result is consistent with other studies (Miyashita *et al.*, 2013; Van Ginneken *et al.*, 2011; Kumari *et al.*, 2013; Praiboon *et al.*, 2018). Gerasimenko and Logvinov (2016), reported that FAs content and composition in seaweeds differed not only by species, but also by seawater temperature. The lower temperature of seaweed habitats results in the high accumulation of n-3 PUFAs for accelerating cell metabolism, especially during the winter (Nomura *et al.*, 2013). The present study revealed that three phyla grown in the temperate area greatly accumulated EPA and SDA in Ochrophyta, ALA and SDA in Chlorophyta, and EPA in Rhodophyta, while these n-3 PUFAs levels were relatively lower in the corresponding phyla grown in the tropical area (**Tables 2.6. – 2.8.**). On the other hand, there was not much difference in the n-6 PUFAs levels between the seaweeds collected from the temperate and the tropical areas. The higher level of total PUFAs in temperate seaweeds may be derived from the higher accumulation rate of n-3 PUFAs.

It is known that TL content and FAs composition varies seasonally. For example, the seasonal changes of FAs composition have been determined in *Egregia menziesii* (Turner) (Nelson *et al.*, 2002), *S. japonica* (Honya *et al.*, 1994; Sanina *et al.*, 2008), *C. costata*

(Turner) (Gerasimenko *et al.*, 2010), *Stephanocystis hakodatensis* (Nomura *et al.*, 2013), *S. horneri* (Nomura *et al.*, 2013), *S. oligocystum* (Praiboon *et al.*, 2018), and *U. pinnatifida* (Harvey) (Gerasimenko *et al.*, 2011). In all cases, the higher level of total n-3 PUFAs has been found during winter or spring; this is the growing period for these seaweeds. Seasonal variation was also found in photosynthetic pigments, such as Chls and carotenoids (Nomura *et al.*, 2013; Altamirano *et al.*, 2000; Christaki *et al.*, 2013; Ismail and Osman, 2016; Sampath-Wiley *et al.*, 2008; Pereira *et al.*, 2012; Zavodnik, 1973). In the present study, no concern has been given to the seasonal changes in the seaweed pigments. These pigments are strongly related to the growing rate of each seaweed species. Therefore, much attention has been paid to the relationship between the growth rate of seaweeds and the level of lipid compounds, especially focusing on the photosynthetic pigments such as Chls and carotenoids.

The lower n-6/n-3 PUFAs ratio in dietary lipids has been considered to reduce the risk for many kinds of chronic diseases (Simopoulos, 2002). Although, a balanced ratio between n-6 and n-3 PUFAs, usually 1:2 to 1:4 (w/w) (Gebauer *et al.*, 2006), has been recommended. However, in modern society, intake of the n-6 PUFAs is much lower than that of the n-3 PUFAs, resulting in the increase in the n-6/n-3 PUFAs ratio. Most of the seaweeds, except for *G. longissima*, showed a low n-6/n-3 ratio, mostly less than 1.0 (**Table 2.10.**). In addition, TI and AI values in all seaweeds were less than 3, especially in the temperate seaweeds. The lower TI and AI values of temperate seaweeds are because of the considerable accumulation of n-3 PUFAs in the temperate seaweeds. TI and AI values found in the present study were lower than those in Indian *macrolagae*, namely, *Gracillaria salicornia*, *Sarconema cinaoides*, *Hypnea spinella*, and *Laurencia dendroidea* (Kumari *et al.*, 2013). Overall, all indices shown in **Table 2.9.** indicate that seaweed lipids analyzed in the present study, especially those from the temperate seaweeds, have a cardio-protective FAs composition.

Prabhasankar *et al.*, (2009), reported the change in the n-6/n-3 ratio of pasta lipids after incorporation of the powder of brown seaweed collected from the temperate area. When the brown seaweed powder was incorporated at 10% in the pasta, the n-6/n-3 ratio changed from 15.2 to 3.4. This result indicates that a drastic change could be found in the low-fat food by mixing seaweed powder rich in n-3 PUFAs.

2.5. Conclusion

In conclusion, this chapter showed that Chls and carotenoid contents varied among phyla, but not with the sampling location. Chl a and Chl b were the major Chls in Chlorophyta. Chl a and Chl c were the main Chls in Ochrophyta, while Chl a was the dominant Chls in Rhodophyta. β -Car and Fx were detected as major seaweed carotenoids. The TL content and FAs composition were strongly affected by sampling location. The TL and n-3 PUFAs levels tended to be higher in temperate seaweeds compared with those in tropical seaweeds.

Lipid soluble pigments such as Chl and carotenoids are the major concern on this study. Among seaweeds pigments, relatively little attention has been paid to Chls, despite the fact that Chls are abundant in our diet and their biological significance has been recognized for a long time (Ferruzzi and Blakeslee, 2007). This may be due to the lower bioavailability of Chls. In addition, the experiments on the bioavailability and functionality of Chls is required understanding their functionality to human. The following chapters will investigate and discuss in vitro study of the mechanisms of bioavailability and functionality of Chls.

CHAPTER III

STUDY ON CHLS BIOAVAILABILITY AND MECHANISMS IN DCACO-2 CELLS MONOLAYER

3.1. Introduction

Chls is lipid-soluble pigment that occurs naturally in parent and derivatives forms. For millions of years it has been consumed by animals and humans (Viera *et al.*, 2018) through the ingestion of fruits, vegetables, and seaweeds (Chen and Roca, 2018a). Balder *et al.*, (2006) stated that approximately 26-86 mg of Chls pigment are assimilated by human every day. The increase in its consumption for humans has been reported to be associated with a lower risk of cancer and metabolic syndrome (Viera *et al.*, 2018; Ferruzzi and Blakeslee, 2007; Hayes and Ferruzzi, 2020). CDs are among the trusted phytochemical compounds responsible for these associations (Hayes and Ferruzzi, 2020). They show several functions such as anti-oxidant (Kang *et al.*, 2018; Hsu *et al.*, 2013; Lanfer-Marquez *et al.*, 2005; Ferruzzi *et al.*, 2002), anti-inflammatory (Islam *et al.*, 2013; Okai and Higashi-Okai, 1997; Pangestuti and Kim, 2011; Subramoniam *et al.*, 2012), anti-mutagenic (Ferruzzi *et al.*, 2002), anti-carcinogenic (Dashwood, 1997; Dashwood *et al.*, 1998), and anti-gentoxication (Dashwood, 1997) properties.

Over the past decades, *in vitro* and *in vivo* studies on CDs bioavailability have increased (Ferruzzi and Blakeslee, 2007; Ferruzzi *et al.*, 2002; Gandul-Rojas *et al.*, 2009; Gallardo-Guerrero *et al.*, 2008; Roca, 2012; Chen and Roca, 2018a; Viera *et al.*, 2018; Hsu *et al.*, 2014; Chao *et al.*, 2018), proving the absorption, potential metabolism, and organ distribution in vertebrates (Viera *et al.*, 2018; Hsu *et al.*, 2014; Chao *et al.*, 2018; Fernandes *et al.*, 2007). Therefore, CDs has been reported to be absorbed in both animals and humans.

According to Chao *et al.* (2018), CDs in the portal vein is higher than those available in human peripheral blood through the lymphatic system and lipophilic compounds (Hayes

and Ferruzzi, 2020). The absorption is related to the chemical structures, with the mechanism of parent and phytillated Chls needed for miscelarisation as well as passive diffusion and facilitated transporter (Hayes and Ferruzzi, 2020; Gandul-Rojas *et al.*, 2009). SR-B1 is involved in the Pheo a absorption of dCaco-2 cells (Viera *et al.*, 2018). However absorption mechanism of each CD in dCaco-2 cells is not fully elucidated, although numerous bioavailability studies have been carried out *in vitro* and *in vivo*.

Algae, macro and microalgae, are the major sources of Chls in nature with those in algae found to be more diverse and complex than a terrestrial plant (Scheer, 1991) with approximately 31 found in different seaweeds phyla (Chen *et al.*, 2017; Zepka *et al.*, 2019). CDs in algae contain Chl a, Chl b, Chl c, Chl d, and Chl f family with the distinct variation responsible for making them a better candidate for structural absorption study (Chen and Roca, 2018b).

Research on Chl absorption is undeveloped compared to carotenoids, despite being the most abundant pigments in nature. Carotenoids absorption in Caco-2 cells involves SR-B1 (During *et al.*, 2005; Reboul, 2019; Widjaja-Adhi *et al.*, 2017), NPC1L1 (During *et al.*, 2005; Sato *et al.*, 2012; Manabe *et al.*, 2019), and CD36 (Moussa *et al.*, 2011; Borel *et al.*, 2013; Reboul, 2019). Therefore, the same approach used to facilitate transporter proteins has been applied in this study.

The specific pathway of bioavailability is dependent on the polarity and reactivity of the individual CDs molecules, which follows the lipophilic routes (Hayes and Ferruzzi, 2020). The investigation associated with its bioavailability focuses on the absorption and efflux mechanism that acts as facilitated proteins to protect the molecules from xenobiotics, which occurs in the absorption process. CDs are known in lower absorption, possibly efflux transporters in the intestine, which are involved in the accumulation process.

DCaco-2 cell, commonly used in studies related to transporter and absorption (Quan *et al.*, 2012), is spontaneously differentiated into the monolayer of polarized absorptive cells and displays morphological and biochemical characteristics in human small intestine cells (Moussa *et al.*, 2011). DCaco-2 cells possess hundreds of transporters that are suitable for absorption and identification of responsible dietary compounds due to their ability to reflect in the normal intestine epithelium (Sambuy *et al.*, 2005; Sun *et al.*, 2008; Zhang *et al.*, 2013; Manabe *et al.*, 2019; Glahn *et al.*, 1998; During *et al.*, 2008; Lea, 2015; Fang *et al.*, 2018).

This study focuses on the lipophilic intestinal transporter and ABC efflux inhibitors to reduce transporter activities to investigate CDs absorption. Furthermore, we examined the effects of CDs on the modulation of lipophilic intestinal transporter and related gene. The molecular docking was also conducted to determine the interaction between CDs and selected intestinal transporter. Therefore, this chapter focuses on of intracellular uptake of CDs and their molecular mechanism in order to discover facilitated proteins involved in the Phy b and Pheo a bioavailability. The results provide better understanding of the mechanism of cellular uptake of CDs in dCaco-2 cells, which is important to overcome the health strategy for better utilization by humans.

3.2. Materials and Methods

3.2.1. Material

Caco-2 cell was obtained from DS Pharma Biomedical (Suita, Osaka, Japan). Fetal Bovine Serum (FBS) was purchased from Biosera Co (France). Minimum Essential Medium (MEM) was purchased from Gibco, Thermofisher (Paisley, United Kingdom). Penicillin and streptomycin, Dimethyl sulfoxide (DMSO), and Dulbecco's phosphate buffered saline (PBS) were obtained from Fuji Film Wako Pure Chem Ltd. (Osaka, Japan). WST-1 was purchased from Dojindo Molecular Technologies, Inc (Kumamoto, Japan). RT high-capacity cDNA

Archive Kit were obtained from Applied Biosystem (Chiba, Japan), Gene Ace Probe a PCR Mix II was obtained from Nippon Gene Co. Ltd (Toyama, Japan). Micro Amp Optical Reaction Plate 0.1 mL was purchased from Applied Biosystem. Ezetimibe was obtained from Focus Biomolecules (Phi, USA). Sulfosuccinimidyl oleate (SSO), was purchased from Cayman Chemical (MI, USA), BLT-1 (Block Lipid transport-1), was purchased from Merck Milipore (MA, USA). Qiazol Lysis Reagent was purchased from Qiagen (Hilden, Germany). Sodium taurocholate was purchased from Sigma Aldrich (St. Louis, USA). Protein assay reagents were purchased from Bio Rad Laboratories (CA, USA). All protein targets were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). High-performance liquid chromatography (HPLC) grade solvents were used for the HPLC analysis and purchased from Fuji Film Wako Pure Chemicals, Ltd. (Osaka, Japan). Standards for Chl a, Phy a, and Phy b were obtained from Wako Pure Chemicals (Osaka, Japan). Chl b and Chl c2 were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan) and DHI Laboratory Products (Hørsholm, Denmark), respectively. All other solvents and chemicals used in the study were of analytical grade.

3.2.2. Methods

3.2.2.1. TL extractions, Chls isolation and its derivatization

TL extract from *S. horneri* collected from Usujiri, Hakodate, Japan and *Chlorella* sp were obtained according to Susanto *et al.*, (2019). Chls of *S. horneri* total lipids were isolated from TL with SiO₂ (Chromatores BW-80 S, Japan) column chromatography and eluted with *n*-hexane and acetone 7:3 (v/v) to obtain Chl a, then followed by 5:5 (v/v), and chloroform:methanol (8:2, v/v) to obtain Chl c2. Meanwhile, hexane and acetone with the proportion 7:3 (v/v) was used to isolate Chl b from *Chlorella* sp.

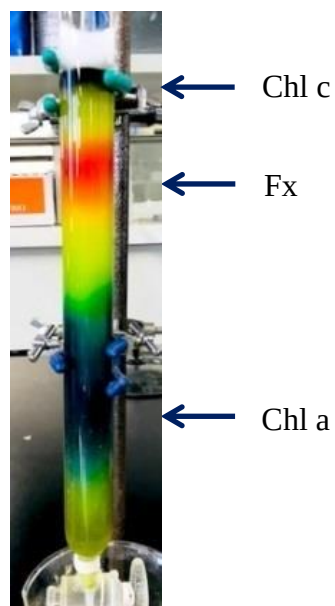
The Chl a and Chl b were generated to produce Phys according to Chen *et al.*, (2017) with modifications. Briefly, 50 mg of parent Chls (a and b) were diluted in diethyl ether and

reacted with HCl. Specifically, after addition of 20 drops of 5 M of HCl, the aliquot was vortexed for 5 min before washing with distilled water to remove remaining acid in the compounds. After completely washing, diethyl ether was evaporated in N₂ gas. The phytillated Chls were store in acetone at -30 °C for further purifications. Phytillated reaction of parent Chls into their derivatives was shown in **Figure 3.2**.

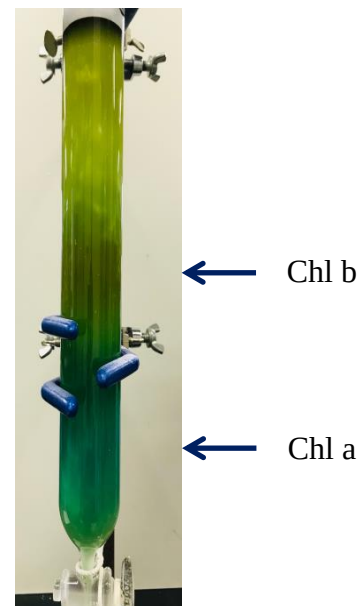
3.2.2.2. Purification of CDs

CDs were diluted in 100% acetone in specific concentrations followed by filtering through a 0.45 µm Torast Disc syringe filter (Shimadzu GLC Ltd., Kyoto, Japan). Preparative purification was performed with develosil column ODS-5 preparative column (5 µm, 20x250 mm, Nomura, Japan). The CDs were injected for purification into the Hitachi HPLC La Chrome system (Hitachi Seisakusho Co., Tokyo, Japan) equipped with pump (L-2130), UV detector (L-2400) and D-2500 chromato-integrator. The flow rate of mobile phase (acetone:methanol, 6:4 (v/v)) was 4 mL·min⁻¹. The specific amount of Chl a, Phy a, Chl b, and Phy b were collected in absorbance 430, 410, 460, and 435 nm, respectively. Meanwhile, Chl c2 were diluted in acetone by using column C18 (Waters Co. Ltd, USA) to remove Chl c contaminant before HPLC purification with ODS-UG 5 (250 × 4.6 mm, Nomura, Japan) using acetone (100%) in 450 nm.

The purity of each purified CDs were confirmed according to Susanto *et al.*, (2019). About > 97% of collected CDs were used in this study (**Figure 3.3**).



(a)



(b)

Figure 3.1. Chls separation. (a) *Sargassum horneri* Chls were isolated with SiO_2 (Chromatoprep BW-80 S) column chromatography and eluted with hexane and acetone 7:3 (v/v) followed by 5:5 (v/v), acetone, and chloroform:methanol (8:2, v/v) to obtain Chl c2. (b) separation of Chl b from *Chlorella* sp, total lipid of *Chlorella* was eluted by hexane and acetone with the proportion 7:3 (v/v).

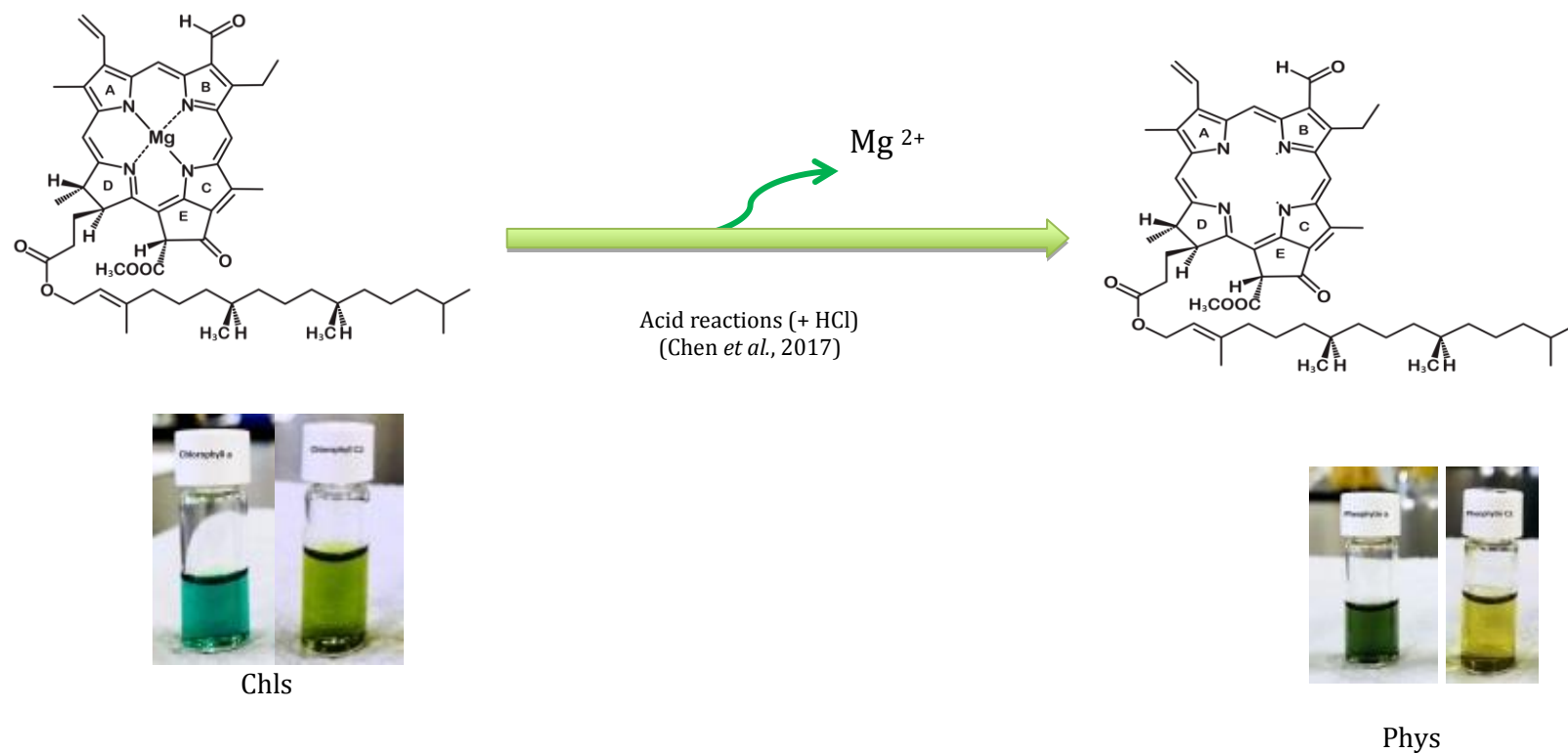


Figure 3.2. Pheophytinization reaction from parent Chls into phytyllated Chls (Chen *et al.*, 2017). Parent chlorophylls were reacted with 5 M of HCl for 5 min to get completely reactions.

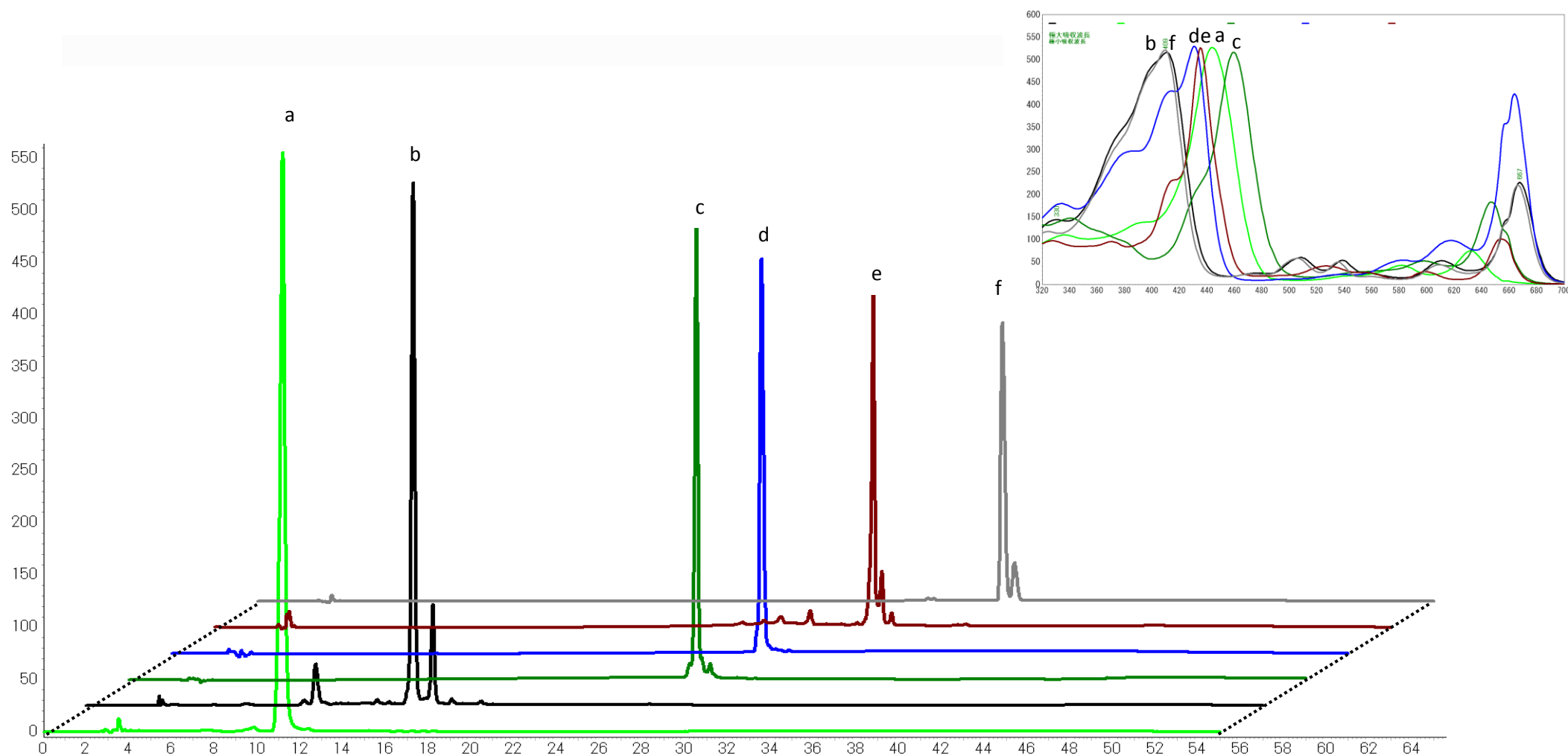


Figure 3.3. Chromatogram and UV spectra of purified CDs from brown seaweeds (*Sargassum horneri*) and green microalgae (*Chlorella* sp) and their derivatives after purification with HPLC La Chrome Systems. The isocratic purification was applied by using MeOH:Ac, 40:60, flow

rate : 4 mL min⁻¹ through ODS5 column in different wavelength (Chl a: 430 nm; Chl b: 465 nm; Chl c2: 450 nm; Phy a: 410 nm; Phy b: 465 nm). The purified CDs were confirmed with HPLC La Chrome system according to Susanto *et al.*, 2019. a. Chl c2; b. Pheo a and a`; c. Chl b and b`; d. Chl a; e. Phy b and b`; f. Phy a and a`.

3.2.2.3. Caco-2 cells culture

The adherent human colon cancer cell line Caco-2 cell originally obtained from DS Pharma Biomedical (Suita, Osaka, Japan). It was maintained in MEM containing 1% of NEAA, 10% of inactivated FBS, 100 U/mL penicillin, and 100 μ g streptomycin. Cells were grown in 200 cm² culture dish at 37 °C, 5% CO₂ and 95% humidity. Cell were subculture by adding 2 mL of trypsin contain ethylenediaminetetraacetic acid (EDTA) to detach cells from dish culture. Hereafter, passaged cells were seeded on 12 well-plates culture for intracellular uptake experiments and 24 well-plates for PCR analysis. The cells were maintained for 12 to 14 days post confluent. Growth medium were replaced every 2-4 days.

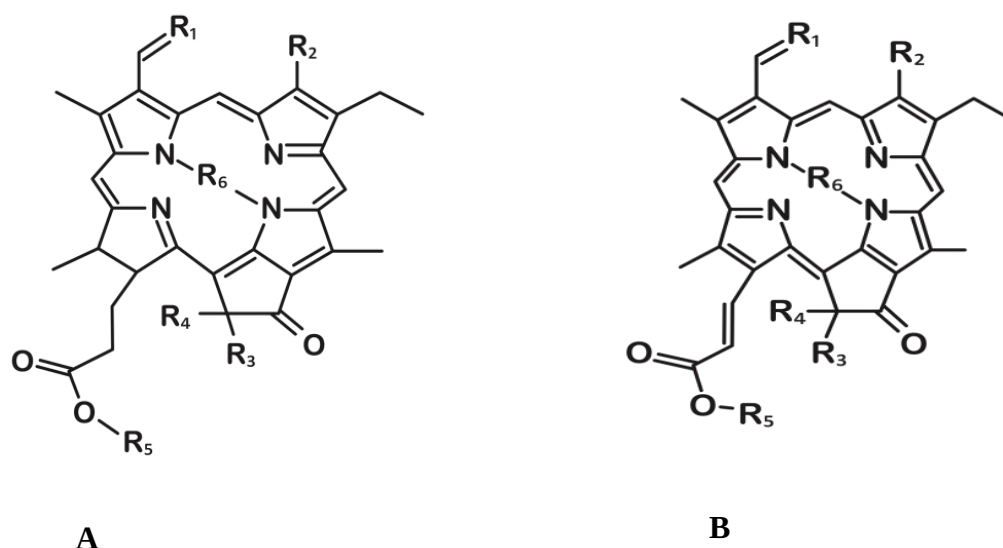


Figure 3.4. Chemical structures of CDs (A) chlorin and (B) porphyrin used in this study.

Table 3.1. Chemical structures of CDs and their sources used in this study

CDs	Source	Chemical formula	PubChem ID	Structure	R1	R2	R3	R4	R5	R6
Chlorophyll a	<i>Sargassum horneri</i>	C ₅₅ H ₇₂ MgN ₄ O ₅	12085802	A	C ₂ H ₃	CH ₃	H	COOCH ₃	C ₂₀ H ₃₇	Mg
Chlorophyll b	<i>Chlorella</i> sp	C ₅₅ H ₇₀ MgN ₄ O ₆	11593175	A	C ₂ H ₃	CHO	H	COOCH ₃	C ₂₀ H ₃₇	Mg
Chlorophyll c2	<i>Sargassum horneri</i>	C ₃₅ H ₂₈ MgN ₄ O ₅	16070018	B	C ₂ H ₃	CH ₃	H	COOCH ₃	-	Mg
Pheophytin a	<i>Sargassum horneri</i>	C ₅₅ H ₇₄ N ₄ O ₅	135398712	A	C ₂ H ₃	CHO	H	COOCH ₃	C ₂₀ H ₃₇	2H
Pheophytin b	<i>Chlorella</i> sp	C ₅₅ H ₇₂ N ₄ O ₆	135484751	A	C ₂ H ₃	CH ₃	H	COOCH ₃	C ₂₀ H ₃₇	2H
Pheohorbide a	Standard	C ₃₅ H ₃₆ N ₄ O ₅	253193	A	C ₂ H ₃	CH ₃	H	COOCH ₃	-	2H

3.2.2.4. Cell viability

Cell viability was examined by WST-1 (Dojindo, Tokyo, Japan) assays using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt. Caco-2 cells were seeded onto 96-well plates containing 2×10^4 cells per well in FBS supplemented MEM and cultured in 100 μ L per well for 24 h at 37 °C and CO₂ 5% before two times PBS washing. Concurrently, 100 μ L of the medium containing CDs was supplemented for another 24 h incubation. After 24 h exposure of sample, the medium containing CDs were changed with fresh MEM containing CDs. The 10 μ L of WST-1 reagent were added in each well and incubate for an additional 3 h. Each plate contained control (DMSO only), and substances in different concentrations with 8 replicates. The CDs (0-100 μ M) were prepared in MEM with $\leq 0.5\%$ dimethyl sulfoxide (DMSO). This concentration has been proven not to be toxic (data not shown). Cell viability was then measured spectrophotometrically at 450 nm and 650 nm (Spectramax, MS^e, Molecular device, USA) and was expressed as a percentage of cell viability (measured as WST-1 reduction) compared to control cultures, which were incubated 10 μ L of 0.5% dimethyl sulfoxide (DMSO) solution without the addition of CDs with the following equation:

$$\text{WST-1} = \frac{(C-B)}{(A-B)} \times 100$$

A = Absorbance of control with dCaco-2 cells

B = Absorbance of control/sample without dCaco-2 cells

C = Absorbance of sample with dCaco-2 cells

3.2.2.5. Stability of CDs in cell culture medium

Previous study exhibited the instability of CDs in the cells (Gallardo-Guerrero *et al.*, 2008; Gandul-Rojas *et al.*, 2009), therefore before intracellular uptake study we examined the stability of selected CDs in medium without the cells before conducting dose and temperature dependent characteristics. For stability experiments, 1 mL of 0.2% BSA in MEM-free serum containing CDs were added to 12 wells without dCaco-2 cells and incubated at 37 °C for 24 h. The medium were collected at 0, 2, 4, 8, and 24 h and then streamered with N₂ gas to store at -80 °C before extractions.

3.2.2.6. Measurement of CDs intracellular uptake into dCaco-2 cells

Intracellular CDs uptake was evaluated in dCaco-2 cells. Caco-2 cells were cultured according to the report by Chen and Roca (2018) with modification. The Caco-2 cells were cultured 12-14 days after confluent in 12 wells plate (RPP, USA) in MEM supplemented with 1% NEAA (Gibco, UK) to form dCaco-2 cells. Medium was replaced every 2-4 days. Evaluation of CDs characteristics of intracellular uptake were carried out in structural-, concentration-, and temperature-dependent. Twenty four hours prior to sample subjection, the growth medium was change in the serum free MEM (Gibco, UK). All CDs were used in this study diluted in DMSO and then applied to the 0.2% BSA (Sigma-Aldrich, Missouri, United States). DMSO is also used for carotenoids transport study in Caco-2 cells (Sato *et al.*, 2012). Therefore the same additions were conducted as DMSO solution of CDs.

The intracellular uptake experiments were performed with 50 μM concentrations of various CDs (Chl a, Chl b, Chl c2, Phy a, Phy b, and Pheo a) at 37 °C for 24 h. The purpose of this study was to investigate the intracellular uptake rate among CDs in dCaco-2 cells. Only the four most absorbable of CDs were used for further experiments.

The stability of CDs (Chl c2, Phy a, Phy b, and Pheo a) in the culture medium were monitored (**described in 3.2.2.5.**), to decide the optimum time for the following experiments. The 4 h incubation was chosen due to insignificant degradation of CDs in medium (**Figure 3.4.**).

The uptake of CDs (Chl c2, Phy a, Phy b, and Pheo a) in dCaco-2 cells was measured in concentrations and temperature dependent. Then, CDs were subjected in different specific concentrations (0, 1, 5, 10, and 50 μM) for 4 h. To determine the effects of incubation temperature on the CDs intracellular uptake the dCaco-2 cells were incubated with CDs at a concentrations 25 μM at 4°C and 37°C for 4 h. All experiments were done in triplicates.

3.2.2.7. Bi-direction transport studies

This purpose of this experiment is to evaluate the transport direction for CDs to predict the proper intestinal transporters which are involvement in CDs uptake. To measure CDs bi-direction transport assay using dCaco-2 cells, the cell was seeded at density of $1 \times 10^5 \text{ cell} \cdot \text{cm}^{-2}$ on trans well (12 well, pore size 0.4 μm , surface area 1.32 cm^2) millicell culture inserts (Corning, NY, USA).

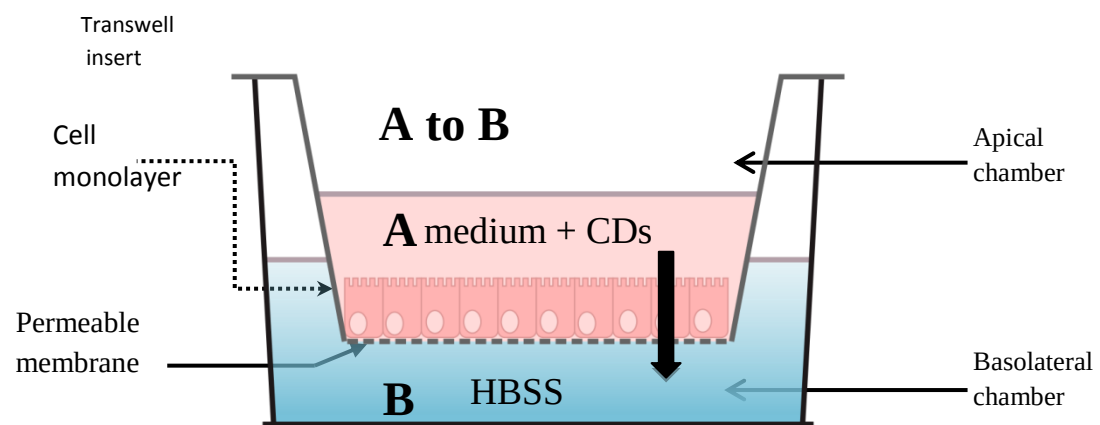
dCaco-2 cells monolayer reached the transepithelial electrical resistance (TEER) reached more than 350 $\Omega \cdot \text{cm}^{-2}$ (about 18 to 21) were used to ensure the bi-directional transports of CDs. TEER was determined by inserting the chopped stick of electrodes of millicells ERS-2 volt-ohm meter (Millipore, USA) resistance of an electrical current between

electrodes. The TEER measurement was conducted according to manufacture protocols. The value of TEER were documented in **Figure 3.5**.

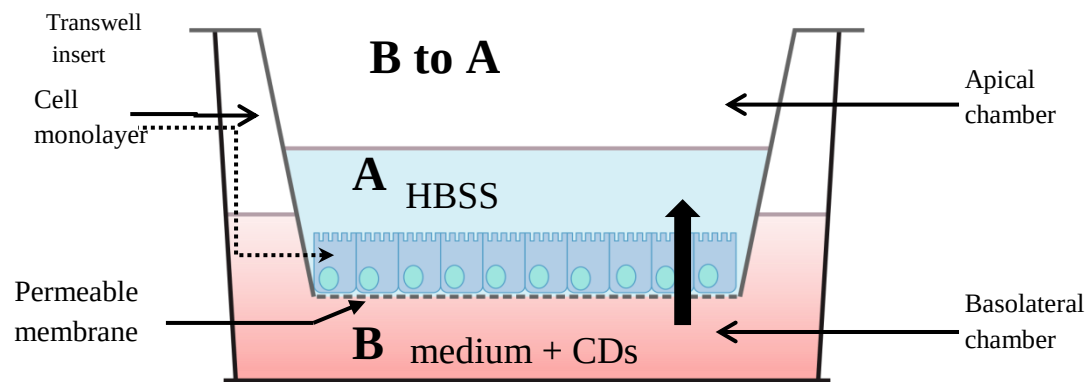
The cells were incubated with fresh medium and used for transport experiments of Phy b and Pheo a. Transport experiments were conducted to investigate the prepared CDs, according to Sato *et al.*, (2012) with modification. Phy b and Pheo a were added in the apical side (A) or basolateral side (B) to evaluate the transport characteristics of CDs into dCaco-2 cells **Figure 3.5**. Wash the apical layer with HBSS (+) (pH 7.4) phenol red free buffer. After washing, the apical compartments were loaded with 500 μ L of medium containing CDs, the basolateral compartment were loaded with 1000 μ L 1% FBS containing HBSS + free phenol.

In the direction of B to A, the basolateral compartments were loaded with 1000 μ L of medium containing CDs, whereas the apical side were loaded with 500 μ L of HBSS + phenol red free buffer (pH 7.4). After 24 h incubation, both apical and basolateral layer were collected for CDs analysis. The dCaco-2 cells in permeable membrane were extracted with 500 μ L of RIPA buffer, 450 μ L was used for the CDs extractions. The rest of dCaco-2 cells was used for protein determination (**method 3.2.2.12.**).

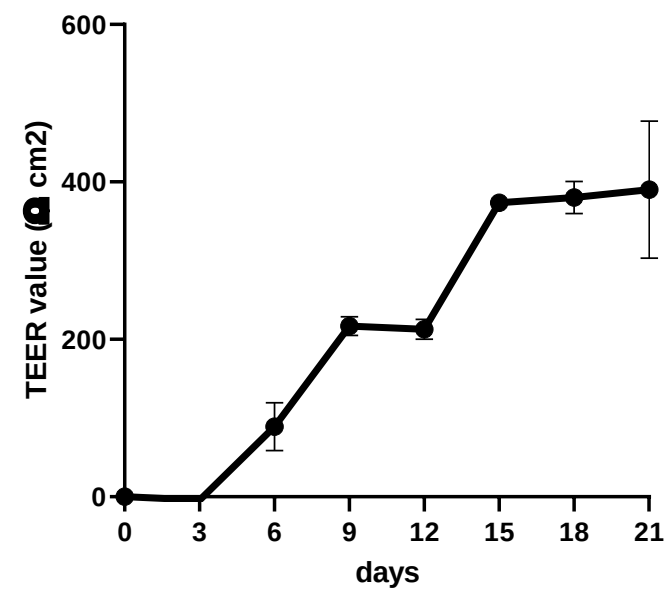
The medium and cell containing CDs were storage at -80°C after streaming with N₂ gas until HPLC analysis.



(a)



(b)



(c)

Figure 3.5. Transport of Phy b and Pheo a through dCaco-2 cells; (a) transport from apical to basolateral (A to B), (b) transport from basolateral to apical (B to A), (c) TEER value and bi-direction of Phy b and Pheo a dCaco-2 cells differentiated for 21 days were used for bi-direction assay.

3.2.2.8. Lipophilic transporter inhibitors effect on the cellular uptake of CDs

Lipophilic transporters have been known to facilitate carotenoids intracellular uptake in dCaco-2 cells (During *et al.*, 2005; Reboul and Borel, 2011; Desmarchelier and Borel, 2017). Therefore, we focused on lipophilic transporter for the CDs intracellular uptake in dCaco-2 cells. In order to assess the involvement of lipophilic transporters, the lipophilic transporter inhibitors were used to confirm their implication. The dCaco-2 cells were cultured according to the methods mention elsewhere on this chapter in 12 well plates (Iwaki, Tokyo, Japan). The inhibitors study was conducted according to Manabe *et al.*, (2019) method with modification. For uptake experiment, dCaco-2 cells were washed with 1 mL of free serum MEM, followed by incubation in 0.6 mL of 0.2% BSA in free-serum MEM-containing 50 μ M BLT-1 (SR-B1 inhibitor) (Sato *et al.*, 2012; Manabe *et al.*, 2019), 50 μ M ezetimibe (NPC1L1 inhibitor) (Sato *et al.*, 2012), and 400 μ M of SSO (CD36 inhibitors) (Borel *et al.*, 2013; Smith *et al.*, 2008) for 30 min. Three inhibitors were delivered using DMSO as a vehicle at a final concentration of 0.1% for BLT-1 and Ezetimibe, and 0.5% for SSO. After a 30-min pre-incubation, 0.4 mL of MEM containing the CDs (the final concentration was 25 μ M) was added to each well, and the cells were further incubated for 4 h. After incubation, the cells were washed PBS 2 times, harvested, and extracted as described in this chapter.

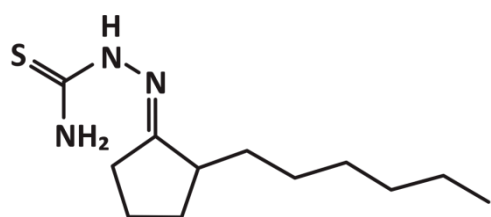
The *Bcrp1*^{-/-} mice has been reported increase the accumulation of CDs, Pheo a, in the animal study (Jonker *et al.*, 2002). From this study, the lipids ABC transporters may influence on the CDs accumulation in the dCaco-2 cells. Therefore, to confirm the involvement of ABC intestinal transporter on the intracellular uptake of CDs, the ATP depletion reagent and ABC transporter inhibitors were utilized. About 50 μ M of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Funakoshi, Tokyo, Japan) (Sato *et al.*, 2012) in DMSO were used. After confirmation the positive effects of ATP depleting reagents, the further steps was attempted using specific ABC transporter inhibitor to dCaco-2 cells. To

confirm the involvement of specific ABC transporters 100 μ M of Verapamil (Vera), a P-glycoprotein inhibitors, was used (Bansal *et al.*, 2009). To knowing the modulation by BCRP, BCRP inhibitor, Fumitremorgin C (FC, 20 μ M) was also used (Henrich *et al.*, 2007). In order to know the ABCG5/ABCG8 involvement in the CDs uptake, 5 mM of sodium vanadate (SV) were used (Johnson *et al.*, 2010). ABC transporter inhibitors were pre-incubated for 30 min and followed by 4 h incubations with Phy b and Pheo a, however the results were not significantly different (data not shown). Therefore, the pre-incubation was prolonged into 60 min to get maximum results and 4 h incubation of both CDs. The maximum concentration of DMSO were added at 0.5% without toxicity for dCaco-2 cells (data not shown).

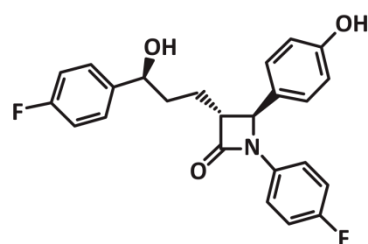
3.2.2.9. TL extraction from medium and cell lysate

Prior to TL extraction, the cells were washed with 1 mL of cold PBS containing 5 mM of sodium taurocholate (Sigma-Aldrich, Missouri, USA) to remove extracellular pigment. Then, the cells were washed with 1 mL of cold PBS (Fujifilm Wako, Osaka, Japan) to remove remaining culture media and extracellular pigments. The dCaco-2 cells were detached after suspending in 1 mL of PBS. 0.9 mL of PBS containing dCaco-2 cells was used for TL extraction and the rest for protein determination.

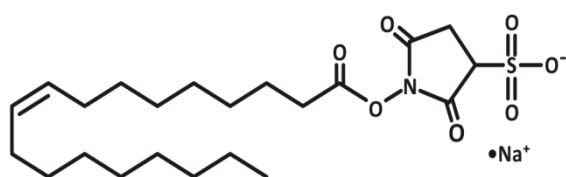
The dCaco-2 cells lipids were extracted according to Bligh and Dyer (1959) in the presence 0.1% BHT containing methanol, 0.1% BHT containing chloroform, and PBS (1:1:0.9, v/v/v). The extraction process was repeated for 2 times to obtain the CDs extract. The lower layer containing total lipids were collected and dried under a stream of N₂ gas at 30°C. The medium were extracted with the same condition with the same proportion of solvents.



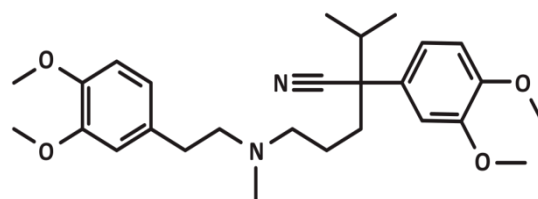
(a)



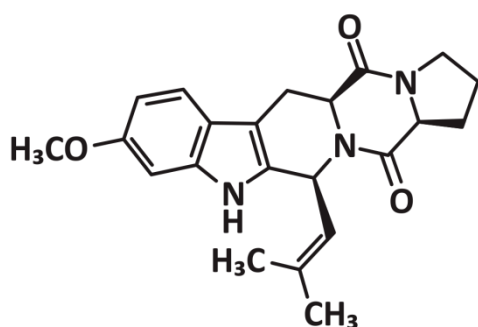
(b)



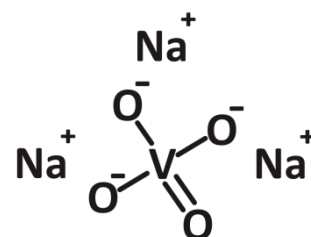
(c)



(d)



(e)



(f)

Figure 3.6. Chemical structures of intestinal transporters inhibitors were used in the study (a) BLT-1 (PubChem ID : 5719399); (b) Ezetimibe (PubChem ID: 150311); (c) SSO (Sulfosuccinimidyl oleate) (PubChem ID: 46782948); (d) Verapamil (PubChem ID : 2520); (e) Fumitremorgin C (PubChem ID : 403923); (f) Sodium Vanadate (PubChem ID : 159677).

3.2.2.11. HPLC analysis and quantification of CDs

The total lipids of dCaco-2 cells were diluted in 200 μ L of methanol containing 0.1% BHT followed by filtration with 0.45 μ m Torast Disc syringe filter (Shimadzu GLC, Ltd). Then, the solutions (90 μ L) were injected HPLC for CDs quantification in the dCaco-2 cells according to Susanto *et al.*, (2019). Identification of CDs was made by HPLC analysis using the authentic standard and their absorption spectral characteristics. Quantification of CDs was performed with the corresponding calibration curves by comparing the peak area using an authentic standard (**Figure 2.1.**). The CDs uptake quantification in dCaco-2 cells was expressed in $\text{mg}\cdot\text{g}^{-1}$ of cellular protein.

3.2.2.12. Protein determination

The protein content of dCaco-2 cell monolayers was determined by the method of Lowry using bovine serum albumin (BSA) (Sigma Aldrich, Missouri, USA) as standard. The remaining 0.1 mL of cell suspension was centrifuge at 1000 x g 4°C. Cells were lyzed with RIPA buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1 mM VNa_3O_4 , 2.5 mM sodium pyrophosphate, 0.01% phenylmethylsulfonyl fluoride, 1 mM β -glycerilphosphate, 1% tritonX-100, protease inhibitor cocktail (Sigma Chemical Co. St. Louis, USA). Cell lysate were incubate at 4°C and then centrifuged at 1200 x g, 4°C for 20 min. The cellular protein concentrations were quantified by using DC protein assays kit Bio-rad Laboratories (Hercules California, USA).

3.2.2.13. Effect of CDs on the mRNA expressions of cholesterol and ABC lipid transporters

Caco-2 cell monolayers (0.5×10^5 cells per well) were grown in 24 well plates and were treated with Phy b and Pheo a for 4 h. After treating with both CDs, dCaco-2 cells were washed two times with PBS. After washing, total RNA was extracted from dCaco-2 cells by using QiAzolTM, lysis reagent (Qiagen, Hilden, Germany).

3.2.2.13. RNA isolation

After 4 h incubation at 37°C, the cells were harvested and used for total RNA extraction using QiAzol™, lysis reagent. mRNA extraction from dCaco-2 cell was conducted based on the Company manual (Thermo Fisher Co. Ltd). After incubation of dcaco-2 cells FBS-free MEM containing sample (4 h incubations) was removed from the cells and washed with PBS twice. QiAzol™ lysis reagent (Qiagen, Hilden, Germany) was added to cells in the 24 well collagen type I plate surface. Chloroform (10%) was added to the Qiazol and gently mixed to make 2 layers. The tube containing cells was centrifuge at 12,000 rpm for 10 min, at 4°C. The upper layer containing mRNA were taken and added equal amount of iso propyl alcohol (IPA), then gently mix. Furthermore, the RNA containing fractions were centrifuge for another 10 min at 12,000 rpm at 4°C. 75% of ethanol was added to decanting the cells. After drying up, 40 µL of RNA free water was added as to prepare total RNA aliquot.

Then, cDNA was synthesized from total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems Japan Ltd, Tokyo, Japan) according to manufacture's instruction.

Master mix 10 µL of (Toyobo Inc., Japan) and 10 µL of total RNA were transferred to 0.5 mL Eppendorf tube. Then the mixed reagent was convert to cDNA by Astec system machine for 75 min. Step 1 was carried out in 30°C for 10 mins and 47°C for 60 mins in other steps, the last steps was 99°C for 5 mins. The cDNA was stored at -30°C until further use. The cDNA was diluted 1:20 (v/v) with H₂O before its used in RT-qPCR experiment

3.2.2.14. qRT-PCR analysis

qRT PCR was performed in 96-well plates using 4.3 µL of cDNA and 5.7 µL of master mix II (Nippon Genetics, Tokyo, Japan) in a final volume 10 µL. Five serial dilutions of the standard was set up to design to determine all primers and reaction of all primer pairs. Gene specific primer (Thermo Fisher Scientific, Tokyo, Japan) used in this experiment are listed in **Table 3.2**. RT-qPCR analysis was performed with StepOnePlus real time PCR

system (Applied Biosystems Japan Ltd, Tokyo, Japan). According to GeneAmp Probe qPCR Mix II (Nippon gene, Tokyo, Japan), PCR cycling conditions were performed at 50 °C for 2 min, 95 °C for 10 mins, and cycled at 95 °C for 30 s and 60 °C for 1 min for 40-50 cycles. The housekeeping GAPDH gene was used as a standard for all treated and untreated samples. All RT-qPCR were carried out in triplicate. The mRNA expression levels were measured using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Frederick, MD, USA) (Table 3.2.).

Table 3.2. Intestinal transporters protein were used in the experiments

Primer	Code	Gene of specific primers for drug transporters
Hs01053790_m1	ABCB1	Multidrug Resistance (MDR) 1
Hs01053790_m1	ABCG2	Breast cancer Receptor Protein (BCRP)
Hs00604506_m1	CYP3A4	P450 cytochrome 3A4
Hs02786624_g1	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Hs00354519_m1	CD36	Cluster of differentiation 36
Hs00969821_m1	SCARBI	Scavenger receptor class B, type I (SR-BI)
Hs00905233_m1	NPC1L1	Niemann-Pick C1-Like 1 (NPC1L1)
Hs06363176_m1	BCMO1	Beta-carotene monooxygenase 1
Hs01088679_m1	SREBP2	Sterol regulatory element-binding protein 2

3.2.2.15. Molecular docking analysis

The purpose of docking experiment is to investigate the interaction of CDs and proteins at the atomic levels and to describe the action of small molecules in the binding site of target protein (Meng *et al.*, 2011). We choose only Pheo a was analyzed for docking experiments, due to higher absorption compared to other CDs. We applied this method for knowing the interaction between Pheo a (Pubchem ID: 253193) with intestinal cholesterol transporter (SR-B1 and NPC1L1) protein. Crystal structure of an active form of mammalian SR-B1 and NPC1L1 were acquired from the Protein Data Bank (PDB) (www.rcsb.org), with

the accession ID: 5KTF (SR-B1) and 3QNT (NPC1L1). All simulated computing was performed on the Windows 10 operating system, the Intel Core i5-8th Gen as a processor with 4 GB of RAM. Molecular docking studies include activity predictions with online PASSES, docking simulations, RMSD calculations, and visualization interactions between proteins and CDs performed using PyRx and Biovia Discovery Studio Visualizer 2019. The reserve docking is to knowing the interaction between ligand and selective protein transporters through the binding of protein target were simulated with Autodock vina and Discovery studio 2016.

3.2.2.16. Data analysis

$P_{app} (AP - BL)$ and $P_{app} (BL - AP)$ (efflux assay) were calculated according to the following equation:

$$P_{app} = \frac{Vr}{C \times S} \times \frac{dC}{dt}$$

Where Vr = is the volume of medium in the receiver chamber, c is the concentration of drug in the donor chamber, S is the surface area of the monolayer and dC/dt is the linier slope of the CDs concentration-time plot in the receiver chamber after correcting for dilution. dC/dt must be constant for accurate determination of P_{app} .

The efflux ratio (ER) was defined by the following equation:

$$ER = \frac{P_{app} (BL - AP)}{P_{app} (AP - BL)}$$

All analyses were run in triplicate and the mean $\pm$ SD value is shown. For comparison between groups, analysis of variance (ANOVA) was performed and followed by multiple comparisons either using the *Scheffe* or *Tukey Post Hoc test* with $p < 0.05$ taken as significant. The graph and data in this study was draw and analyzed using GraphPad Prism 8 software (SD, USA).

3.3. Results

3.3.1. Influence of CDs on dCaco-2 cells viability

The viability of dCaco-2 cells was measured with WST-1 assay in the absence (DMSO only) and presence of CDs in various concentrations (0 to 100 μ M). The cell viability of the cells treated with CDs by WST-1 assay is shown in **Fig 3.2**. The cell viability was expressed as percentages of control (absence of CDs).

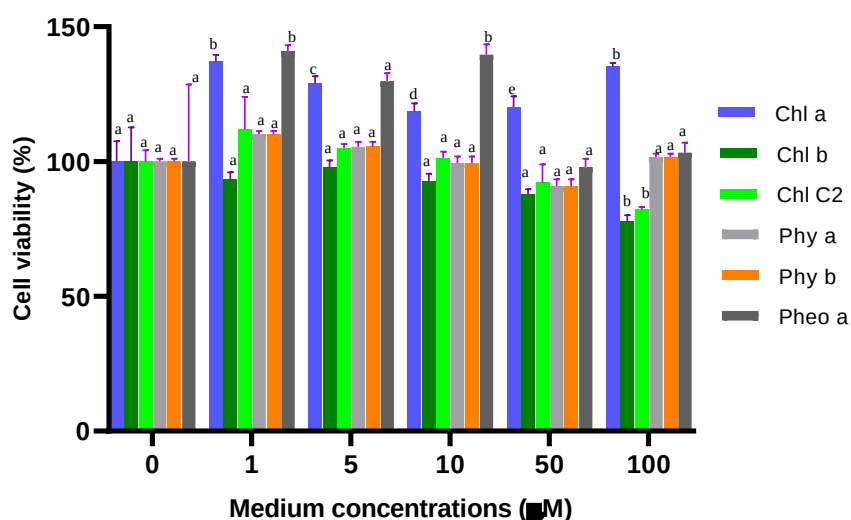


Figure 3.7. Cell viability of dCaco-2 cells treated with CDs (CDs) for 24 h incubation. The cell viability was evaluated by WST-1 test with the range concentration of each compounds 0-100 μ M. $n \geq 6$ replications. Values with different letters are significantly different ($P < 0.05$) by *Tukey post hoc test*.

As shown in **Figure 3.7**. CDs treatments ($\leq 50 \mu$ M) did not showed toxicity in dCaco-2 cells, while higher concentration of Chl b and Chl c2 significantly ($p < 0.05$) reduced cell viability after incubation 24 h. Hence, CDs concentrations below 50 μ M with $\geq 90\%$ of cell viability were selected for the further experiments.

3.3.2. Accumulation of Chls and effect of structural, concentrations and temperature on accumulation in dCaco-2 cells

The concentrations of 50 μM of CDs were applied to the structurally-dependent intracellular uptake experiments. Initially, the relationship between CDs structure and intracellular uptake was evaluated by dCaco-2 cells to determine their characteristics. The intracellular uptake results of various CDs using dCaco-2 cells in 0.2% BSA of free serum MEM for 24 h are shown in the **Figure 3.8**. Furthermore, large variations of intracellular uptake among CDs were determined, with Phy showing the highest absorption rate of 3594% than Chl b. The extracellular CDs content in the medium are oppositely correlated with the content in dCaco-2 cells which varies from 0.06 to 5.80 mg g^{-1} of protein.

The structural differences of CDs intracellular uptake is in descending order of Phy a > Pheo a > Chl c2 > Phy b > Chl a > Chl b. The dephytyllated and phytyllated uptake increased by 4 folds of parent Chls with Chl c2 showing higher absorption rate.

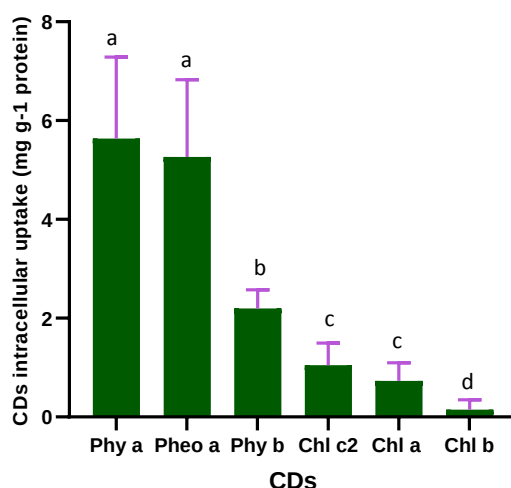


Figure 3.8. Intracellular uptake of various CDs. Differentiated Caco-2 cell monolayer after 14 days culture was used after culturing in 10% FBS containing-MEM. Then culture media was changed to serum free MEM containing 0.2% BSA containing DMSO at 0.5% and either Chl a, Chl b, Chl c2, Phy a, Phy b, and Pheo a at initial concentration 50 μM (3 well per treatment) and dCaco-2 cells were incubated for 24 h. CDs were extracted from dCaco-2 cells monolayer and MEM then analyzed by HPLC LaChrome system according to Susanto *et al.*, (2019). Data are means $\pm$ SD obtained from three replications of two independent experiments. Values with different letters are significantly different ($p < 0.05$) by *Tukey post hoc test*.

In order to determine the intracellular behaviour uptake, this research utilized four absorbable CDs. The stability of selected CDs was evaluated in the same medium conditions for intracellular uptake experiments. The background of the study was associated with the instability and degradations of previous experiments (Gallardo-Guerrero *et al.*, 2008; Ferruzzi *et al.*, 2001). Serum-free medium is enriched with 0.2% of BSA containing 50 μ M of individual CDs without cells for 24 h incubation. The Phy a and Pheo a are stable during the experiments for 24 h, however, Chl c2 and Phy b significantly decreased after 4 h compared to 0 h. The incubation period from 1-4 h was chosen because during this time there were significant no degradations of CDs in the medium (**Figure 3.9**). Therefore for the following experiments, 4 h incubation periods were chosen for studying the characterization of CDs in dCaco-2 cells.

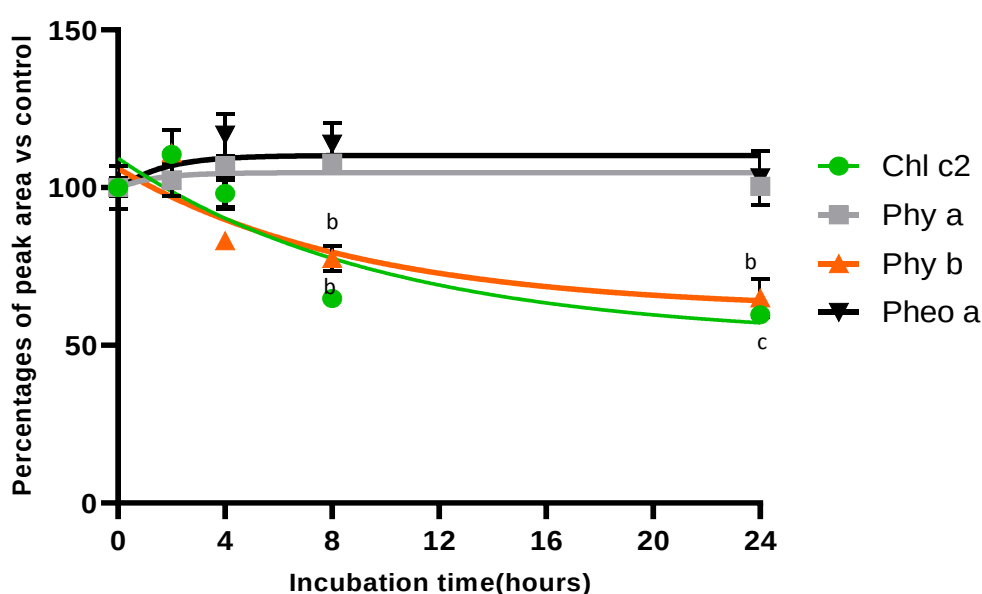


Figure 3.9. The stability of selected CDs in the serum free 0.2% BSA medium (MEM) incubated for 24 h at 37°C. The concentration of CDs were determined using HPLC Shimadzu System according to Susanto *et al.*, (2019). Each point represents the mean with SD of 3-4 measurements. The ANOVA were analyzed to knowing the significant interactions of between the treatments. Different letter above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

This study evaluated the comparison of kinetic uptake of Chl c2, Phy a, Phy b, and Pheo a to clarify the effects of the uptake of different CDs concentrations, which are incubated using dCaco-2 cells. A lower concentration of CDs (1-10 μM) indicated the linear uptake followed by saturation at higher concentrations (50 μM). This indicates during absorption combination of carrier mediated process and passive diffusions were occurred (**Figure 3.10a.**). In contrast, Phy a indicates an increase in intracellular uptake with a rise in CDs concentrations. This phenomenon is in agreement with the previous study by Gandul-Rojas *et al.*, (2009).

CDs were incubated at 37°C compared to 4°C. When CDs uptake were evaluated at different temperature, it showed significantly higher intracellular accumulation at 37°C incubation compared to 4°C. The results showed the significance of Phy a, Phy b, and Pheo a uptake in different temperatures. This indicates that the CDs uptake facilitated diffusion and actively in the energy-dependent process of the absorption.

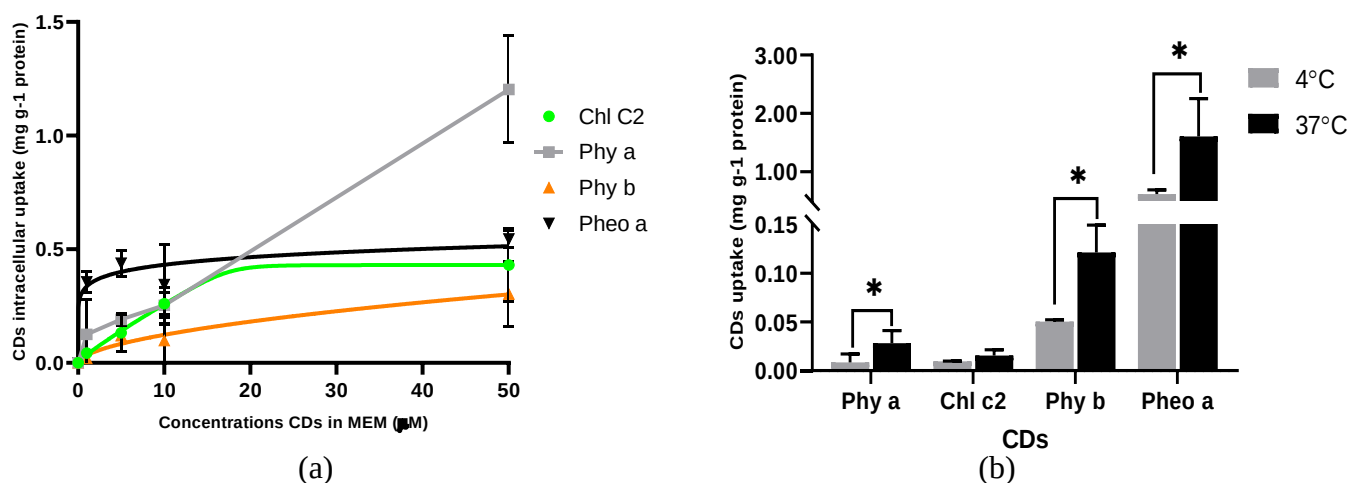


Figure 3.10. The uptake of CDs by dCaco-2 cells. The uptake of CDs (25 μM) was measured in (a) different concentrations of CDs at 37°C; (b) at different temperatures (4 °C and 37 °C) after 4 h incubation. The concentration of CDs were determined using HPLC Shimadzu System according to Susanto *et al.*, (2019). Each point represents the mean with SD of 3-4 measurements. * significantly from 4°C ($p < 0.05$).

The hypothesis associated with passive diffusions is a linear uptake by compounds/xenobiotics, while facilitated protein were associated with the saturation

absorptions process (Reboul and Borel, 2011; Holander and Ruble, 1978; Gandul-Rojas *et al.*, 2009). This research showed that the characteristics of CDs accumulation in dCaco-2 cells varied in each CDs. The Phy a was linearly increased from lower to higher concentrations, which demonstrated the simple diffusion mechanism in the passive uptake (**Figure 3.10b**), while other CDs displayed combination of passive uptake and facilitated uptake (**Figure 3.10a**).

3.3.3. Bi-direction of CDs transport across dCaco-2 cells monolayer

After determining the intracellular uptake of CDs in dCaco-2 cell, we investigated the uptake direction by monitoring the apical to basolateral side and vice versa. This data helps us to understand that the facilitated transporter family is responsible for the uptake and efflux of CDs in dCaco-2 cells. In order to better characterize the involvement of influx and efflux transporters, two CDs (Phy b and Pheo a) were assessed.

Table 3.3. Permeability of CDs (Phy b and Pheo a; 25 μM) during apical to basolateral (A to B) in figure 3.5a. and basolateral to apical (B to A) in figure 3.5b. (P_{app} ($\times 10^{-10} \text{ cm}\cdot\text{s}^{-1}$) efflux after 24 h incubation at 37°C. Each point represents the mean with SD of 3-4 measurements.

CDs	A to B ($\times 10^{-10} \text{ cm}\cdot\text{s}^{-1}$)	B to A ($\times 10^{-10} \text{ cm}\cdot\text{s}^{-1}$)	Efflux Ratio (ER)
Phy b	0.68 $\pm$ 0.04	2.10 $\pm$ 0.11	3.07
Pheo a	2.20 $\pm$ 0.06	3.02 $\pm$ 0.16	1.3

Bi-direction transport of CDS showed that P_{app} value was influenced by structures of CDs. The efflux ratio showed that Phy b exhibit higher efflux ratio compare to Pheo a with the tendency to obtain a higher rate from basolateral to apical than basolateral to apical. In Phy b transport, the results suggested that efflux transporters potentially higher contribution compared to influx (ER $\geq$ 2) (**Table 3.3.**).

3.3.4. Effects of selective transporter inhibitors on the CDs accumulation in dCaco-2 cells

The following experiment was carried out to investigate whether intracellular uptake of CDs was facilitated or not by the carrier. The experiment showed that specific mRNA expressions facilitated the intracellular uptake of CDs, therefore the inhibitor was used to determine their involvement on CDs uptake. The same approach with the carotenoids study was applied in this study. Before investigating the transporter involved in CDs accumulation by dCaco-2 cells, mRNA expressions of lipophilic and ABC intestinal transporters were also evaluated by PCR, which demonstrated their occurrence (**Fig. 3.11.**).

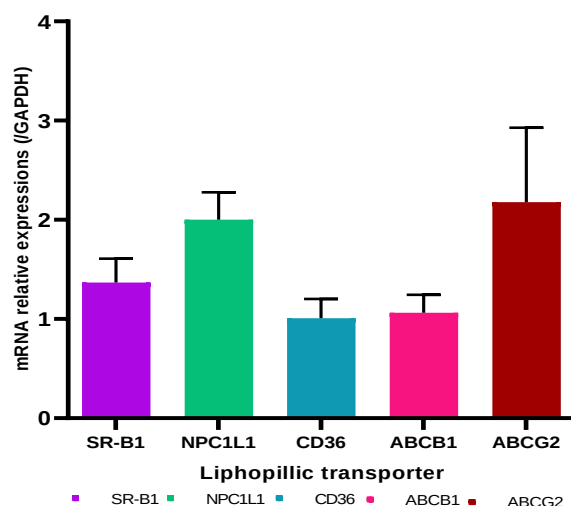


Figure 3.11. mRNA expression of SR-B1, NPC1L1, CD36, ABCB1, and ABCG2 in dCaco-2 cells. RT-PCR was performed with total RNA isolated from dCaco-2 cells. Each point represents the mean with three independent experiments.

Furthermore, the effects of lipophilic and ABC transporter inhibitors in dCaco-2 cells were evaluated. SR-B1 is known to play a role in the cellular uptake of Pheo a (Viera *et al.*, 2018). BLT-1, Ezetimibe and SSO are known reducing carotenoids absorption in dCaco-2 cells (Reboul *et al.*, 2005; Sato *et al.*, 2012; During *et al.*, 2005). These inhibitors were able to blockade the cholesterol transporters in dCaco-2 cells. In order to investigate specificity of

the facilitated carrier such as intestinal transporters, Ezetimibe or Zetia (EZ), an inhibitor of NPC1L1 (Davis *et al.*, 2007; Patel, 2004), Block Lipid Transport-1 (BLT-1), an inhibitor of SR-B1 inhibitor (Raldia *et al.*, 2007; Faloon *et al.*, 2014; Gutierrez-Pajares *et al.*, 2016), and SSO, a specific inhibitor of CD36 (Kuda *et al.*, 2013; Mansor *et al.*, 2017) were evaluated upon the CDs uptake in dCaco-2 cells.

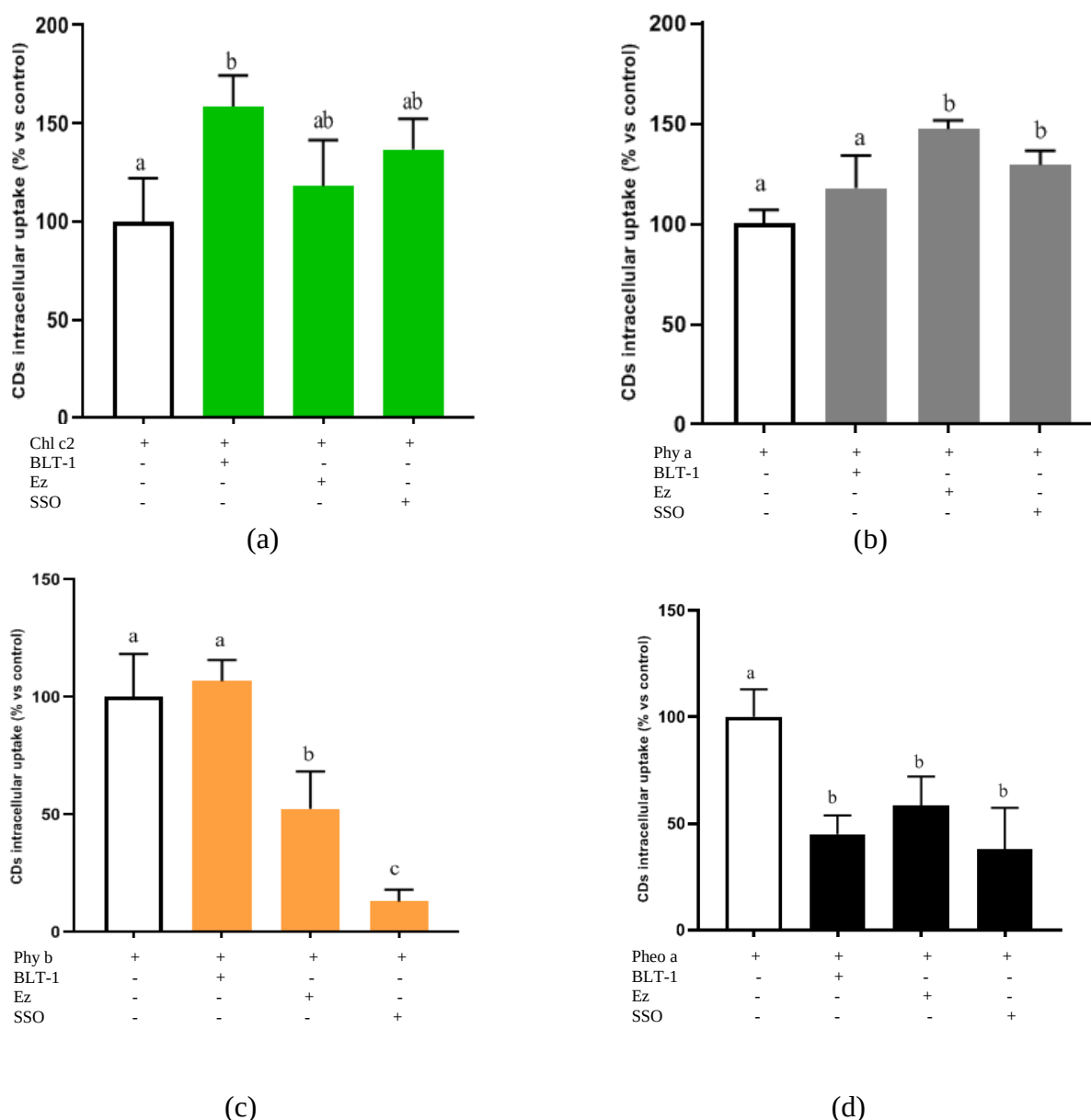


Figure 3.12. Inhibitory effects of 50 μM ezetimibe; 50 μM BLT-1; 100 μM sulfosuccinimidyl oleate on the accumulation of CDs in dCaco-2 cells. Effects of cholesterol inhibitors on the intracellular uptake of (a) Chl c2, (b) Phy a, (c) Phy b, and (d) Pheo a. The accumulation of CDs (50 μM) was measured at 37°C in 0.2% BSA containing FBS-free MEM. The concentration of CDs were determined using an HPLC La Chrome system according to method by Susanto *et al.*, (2019). Each column is represented the mean of three

measurements. The ANOVA were analyzed to knowing the significant interactions of between the treatments. Different letter above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

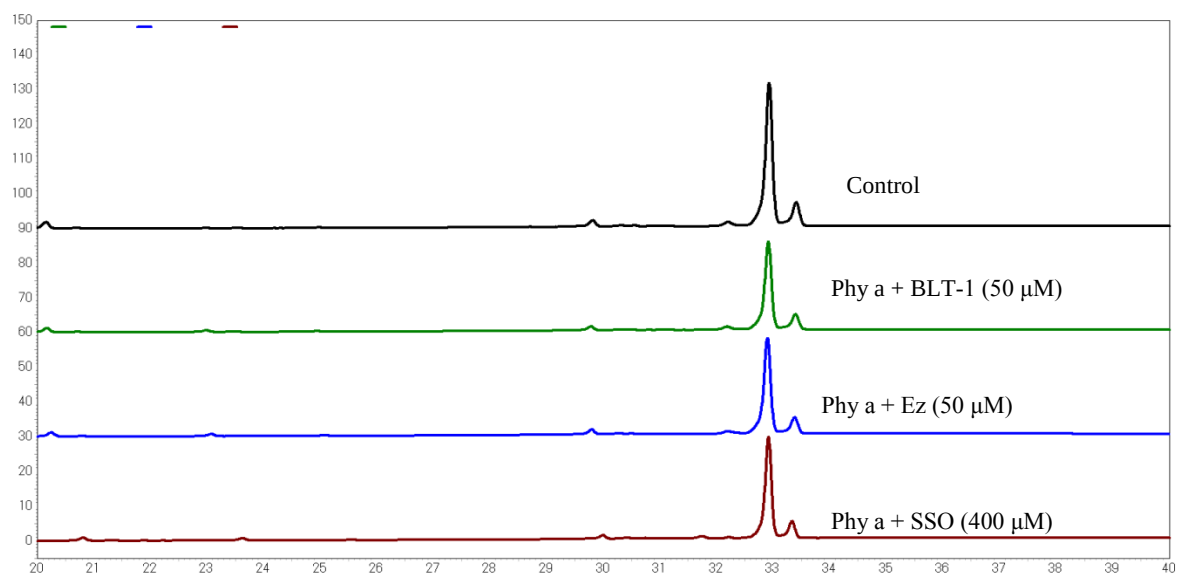


Figure 3.13. Representative chromatogram of Phy a analysis in dCaco-2 cells treated with BLT-1 (50 μM), Ezetimibe (50 μM); and SSO (400 μM) on the accumulation of Phy a.

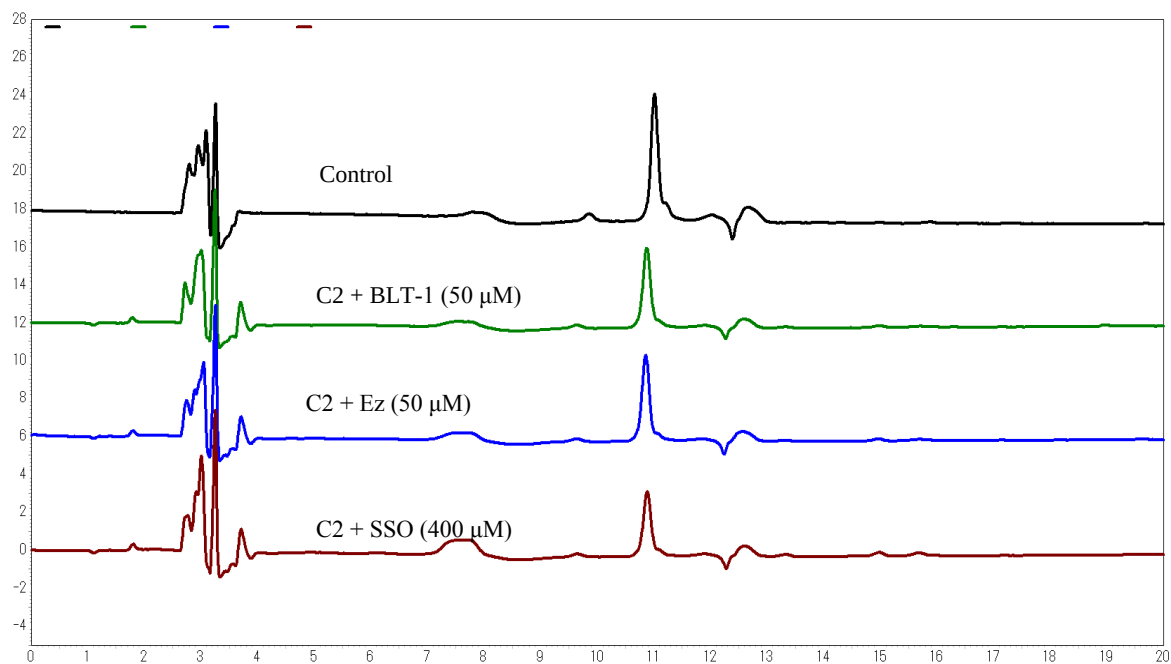


Figure 3.14. Representative chromatogram of Chl c2 analysis in dCaco-2 cells treated with BLT-1 (50 μM), Ezetimibe (50 μM); and SSO (400 μM) on the accumulation of Chl c2.

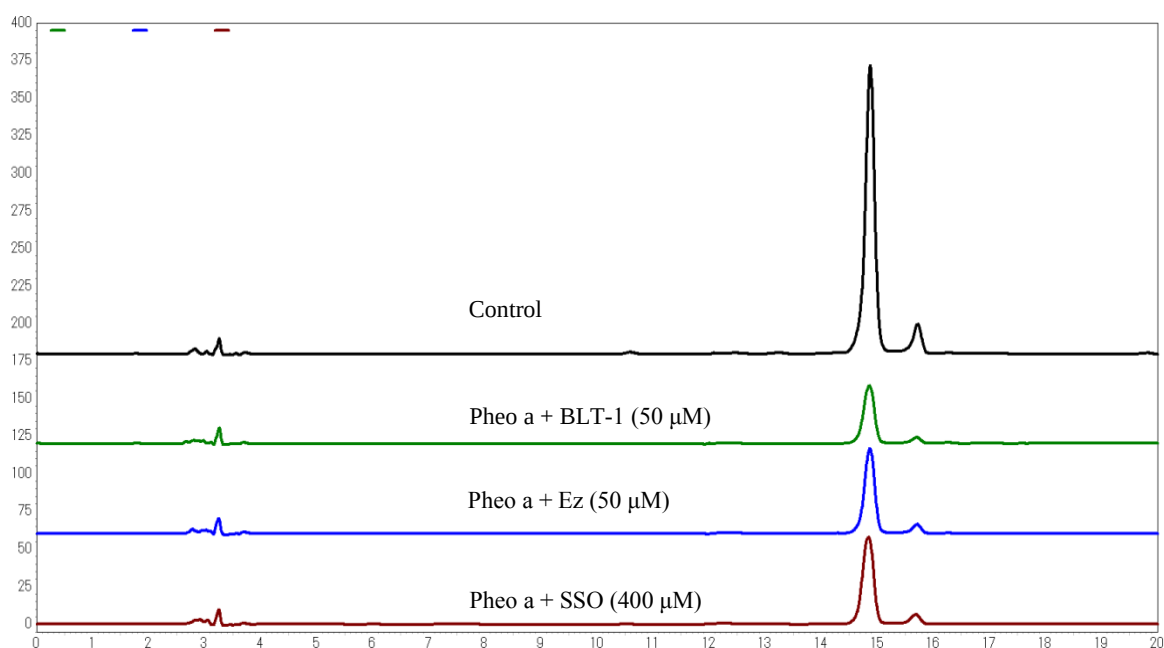


Figure 3.15. Representative chromatogram of Pheo a analysis in dCaco-2 cells treated with BLT-1 (50 μ M), Ezetimibe (50 μ M); and SSO (400 μ M) on the accumulation of Pheo a.

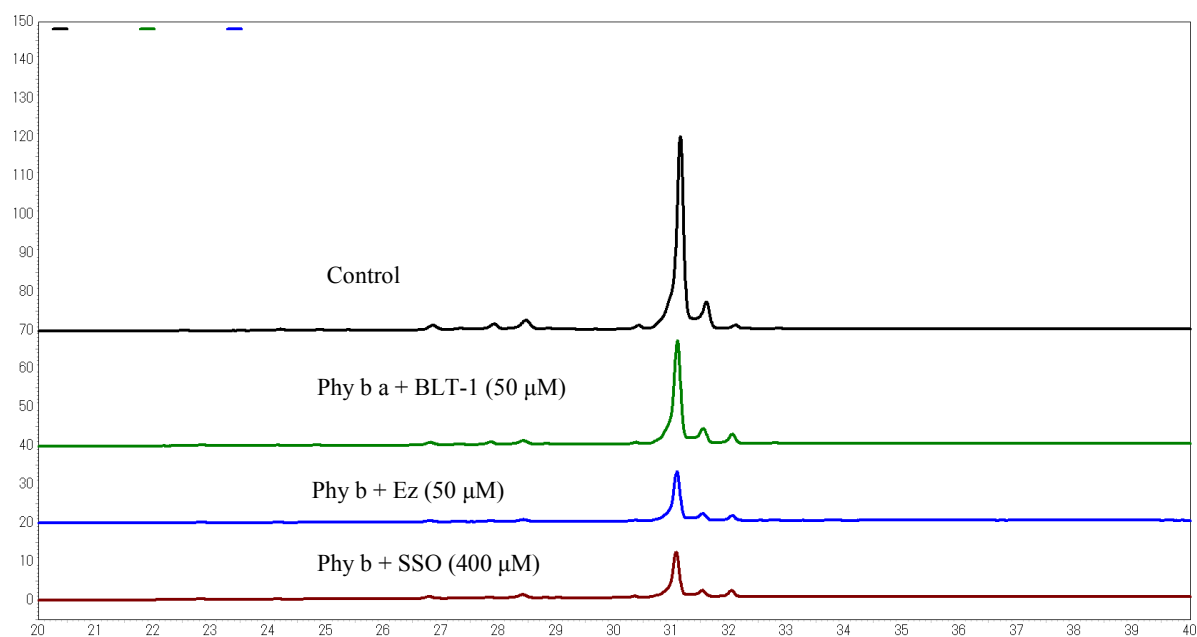


Figure 3.16. Representative chromatogram of Phy b analysis in dCaco-2 cells treated with BLT-1 (50 μ M), Ezetimibe (50 μ M); and SSO (400 μ M) on the accumulation of Phy b.

The pre-incubated with cholesterol inhibitors significantly ($p<0.05$) increased the intracellular uptake of Chl c2 and Phy a and showed the different effects on the absorption of selected CDs. However, these inhibitors provided a significant ($p<0.05$) reduction in the intracellular uptake of Phy b and Pheo a (**Figures 3.12. a-d**). As shown in **Figures 3.12. (c-d) and Figures 3.13 – 3.16**. Ezetimibe and SSO inhibited Phy b intracellular uptake by dCaco-cells. All cholesterol inhibitors were significantly reduced by Pheo a intracellular uptake in dCaco-2 cells. Therefore, it is possible that Chl c2 and Phy a absorption is not mediated by intestinal cholesterol transporter or dominated by passive diffusion (Phy a) as shown in **Figure 3.10a**.

3.3.5. Effects ATP-depleting conditions and ABC-transporters inhibitors

The results of bi-direction showed that the efflux transporter contributed to the CDs bioavailability in dCaco-2 cells. The research also examined the effects of the energy-dependent transporter (ABC transporters) with the same uptake study conditions. On the efflux study, Phy b and Pheo a were investigated, to clarify the involvement of lipid intestinal uptake transporter.

ABC intestinal transporters are intended to efflux the lipid-soluble compounds (Tarling *et al.*, 2013). Therefore, to determine the involvement of energy-dependent transporters in the intracellular uptake of the selected CDs, several ABC transporters inhibitors such as ABCB1, ABCG2, and ABCG5/ABCG8 were assessed. To confirm their involvements, in the beginning the study evaluated the effects of carbonyl cyanide m-chlorophthnyl dehydration (CCCP; an inhibitor of oxidative phosphorylation) (Sato *et al.*, 2012) as well as the accumulation of Phy b and Pheo a. After determining the significant results, further steps were used for specific ABC transporter inhibitors, namely verapamil (Ver; a specific inhibitor of ABCB1) (Bansal *et al.*, 2009), fumitremorgin C (FC; a inhibitor of ABCG2) (Gonzalez-Lobato *et al.*, 2010; Toyoda *et al.*, 2019), and Sodium Vanadate

(SV; an inhibitor of ABCG5) (Johnson *et al.*, 2010) were used. The ABC transporter inhibitors did not show significant results in the accumulation of CDs in dCaco-2 cells after 30 min pre-incubation period (data not shown). Therefore the timeframe was extended to 60 min.

The specific ABC transporter inhibitors were investigated to determine the particular transporters responsible for the intracellular uptake of Pheo a and Phy b. Vera (100 μ M), FC (20 μ M), and SV (5 mM) acted as p-glycoprotein inhibitors, BCRP/ABCG2 inhibitors and ABCG5/ABCG8 inhibitors, respectively were pre-incubated for 1 h before Pheo a and Phy b incubation.

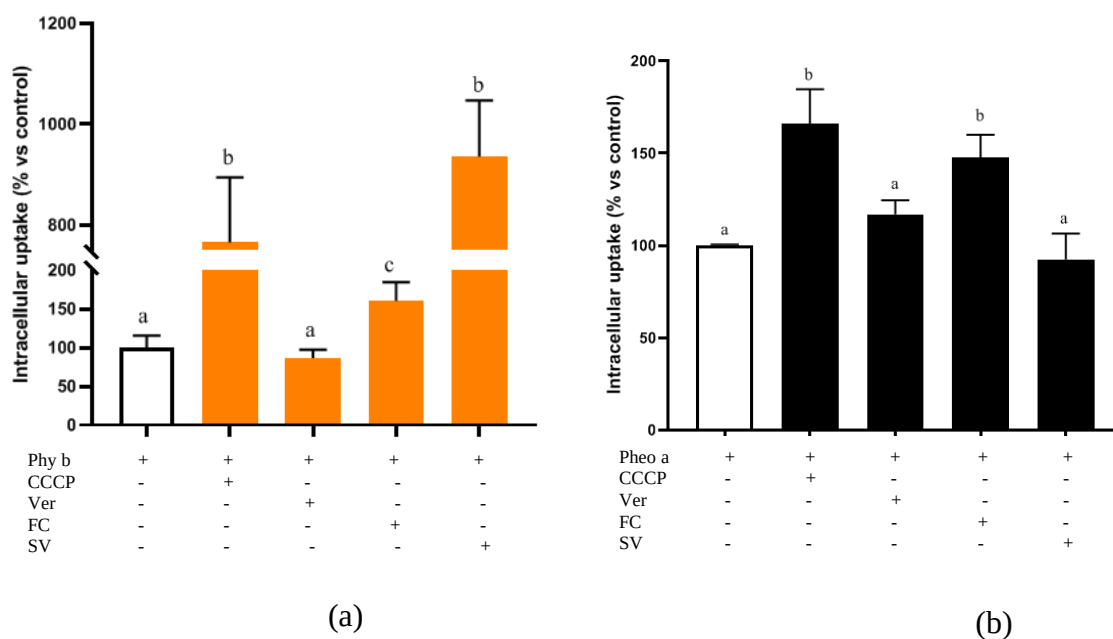


Figure 3.17. Accumulation of (a) Phy b and (b) Pheo a; after 1 h pre-incubation with CCP (50 μ M), verapamil (100 μ M), fumitremorgin C (20 μ M) and sodium vanadate (5 mM). The accumulation of Phy b and Pheo a (50 μ M) was measured at pH 7.4 at 37°C in 0.2% BSA containing FBS-free MEM. The concentration of both CDs was determined using an HPLC LaChrome system according to Susanto *et al.*, (2019). Each column represents the mean of three measurements. Different letter above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

Data showed the different accumulation of CDs after 1 h pre-incubation with specific ABC transporter inhibitors. In addition, 20 μ M of FC was significantly ($p < 0.01$) increase the

accumulation of Pheo a, this is possibly that ABCG2/BCRP plays important role on the efflux of Pheo a. This result supports the study conducted by Jonker *et al.*, (2002) study. In addition, Phy b, derivatives products of Chl b, were significantly ($p<0.05$) accumulated after pre-incubated with 5 mM of SV for 1 h. This is the first finding associated with ABCG5/ABCG8, which possibly involves in the efflux of Phy b in dCaco-2 cells.

3.3.6. The effect of CDs on the mRNA expression of selected transporters in dCaco-2 cells

After determining the responsible facilitated protein in the Pheo a and Phy b uptake, gene expressions of the responsible protein are investigated. Alteration of the gene related to absorption of CDs (NPC1L1, SR-BI, CD36, SRBEP2, and BCMO1) and efflux transporters including ABCB1, ABCG2 and CYP3A4 were evaluated after the supplementation of Phy b and Pheo a for 4 h. Surprisingly, the expressions of selected lipophilic uptake and ABC transporters seemed to increase after incubation with both CDs. SR-BI was significantly upregulated by 2 and 4 folds of Phy b and Pheo a, while NPC1L1 was upregulated by 4 and 8 folds of Phy b and Pheo a compared to the control. The ABC transporters such as ABCB1 after Phy b and Pheo a showed a significantly up-regulated mRNA expression up to 8 and 10 folds, as shown in **Figure 3.18**. In the case of cholesterol transporters, the gene was up-regulated from 3 to 8 folds compared to untreated groups. From these results, the different patterns of up-regulation of both cholesterol and ABC transporters were confirmed.

This study showed that both CDs also increased the BCMO1 and SREBP2 mRNA levels. The increase in SREBP2 transcription factors leads to the stimulation of NPC1L1 by both CDs. These data suggest that CDs up-regulated NPC1L1 mRNA expression by increasing the transcription factors of SREBP2 in intestinal dCaco-2 cells. In addition, BCMO1 which acts as a downstream of SR-BI (Lobo *et al.*, 2010; Borel, 2012), contributes to the SR-BI up-regulation after CDs supplements.

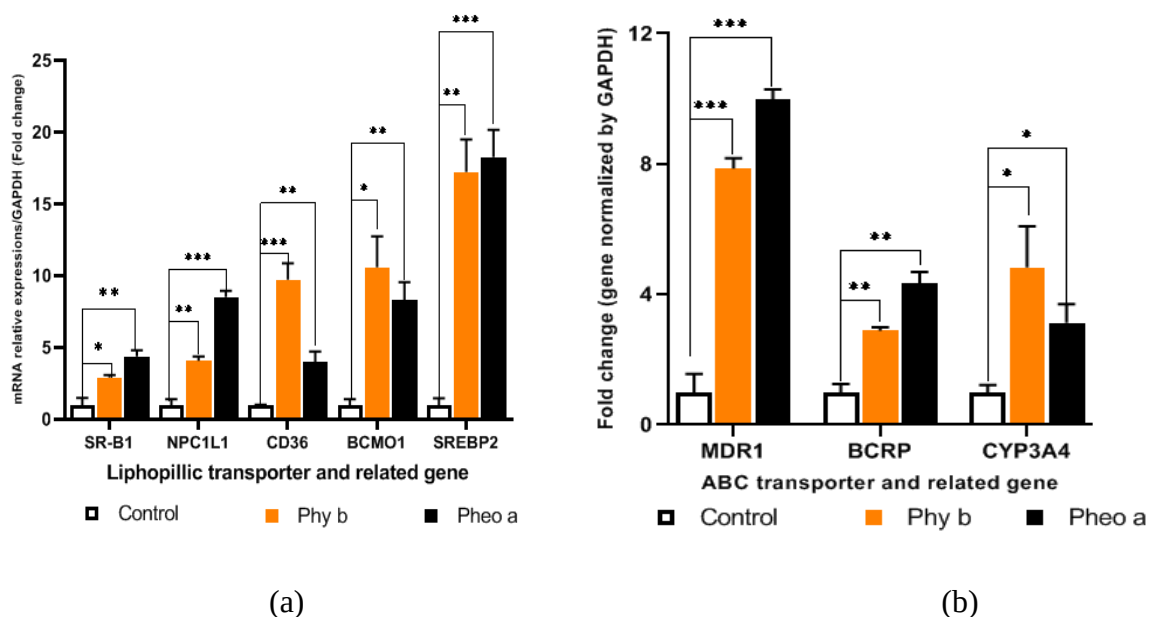


Figure 3.18. The effects of Phy b and Pheo a (25 μ M) in the expression of mRNA (a) intestinal cholesterol transporters and related gene (NPC1L1, SR-BI, CD36, BCMO1, SREBP2); (b) ABC intestinal transporters and related gene (ABCB1, ABCG2, and CYP3A4). Phy b and Pheo a were incubated for 4 h incubation at 37°C, 5% CO₂ in 12 well plates. The expression was normalized to GAPDH. The results were obtained from two independent experiments. The results are expressed as the means $\pm$ SD of triplicates samples. * denotes significantly different ($p < 0.05$), ** denotes significantly different ($p < 0.01$), and ***denotes significantly different ($p < 0.001$).

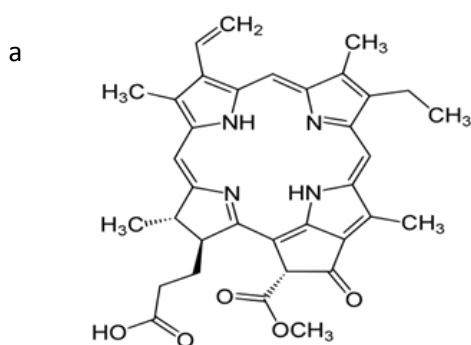
3.3.10. Docking experiments

The molecular simulation results regarding the interaction between ligands (Pheo a) and cholesterol transporter (SR-B1 and NPC1L1) were depicted with computer simulations used to examine the possible binding site of Pheo a. Molecular docking was performed on the uptake to study the lowest binding energy of residues related binding mechanism and regarding the interactions between ligands and cholesterol transporter as described in **Figure 3.11**. The interactions pocket of Pheo a was bound to SRB1 and NPC1L1 located in the regulatory domain of the active side, which is constituted to AA.

In the 2D conformations, the conventional hydrogen bond was used to charge the polar interactions, denoted by light green circles. In addition, the alkyl and Pi-alkyl interactions

were denoted by light pink circles, while Pi-anion was by orange. The interactions between the hydrogen bond and the AA in the intestinal transporters are indicated by light green dotted arrow, while the side chains are indicated by pointing out the electron donors. Pi-alkyl interactions is the interaction of between Pi-electron with aromatic group and electron group of any alkyl group (Ribas *et al.*, 2002; Matthews *et al.*, 2014). While Pi-anion is non-covalent contact between anionic systems and electron-deficient aromatics (Schottel *et al.*, 2008; Robertazzi *et al.*, 2011). Furthermore, the hydrogen bond is the electromagnetic attractive interaction between a hydrogen atom from a molecule or a molecular fragment (Arunan *et al.*, 2011).

The active site of NPC1L1 successfully forms two hydrogen bonds, conventional as well as Pi-anion with the active amino acids asparagine (ASP A:208), cysteine (CYS A:189), and asparagine (ASN A:211), while Pi-Anion shares the interactions in ASP amino acids. The interaction between Pheo a and SR-B1 is build up by interacting with alkyl and Pi-Alkyl using AA, such as tyrosine (TYR A:446), leucine (LEU A:449), and valine (VAL A:447), while tyrosine (TYR A:432) interacted with conventional hydrogen bonds such as Pi-Alkyl.



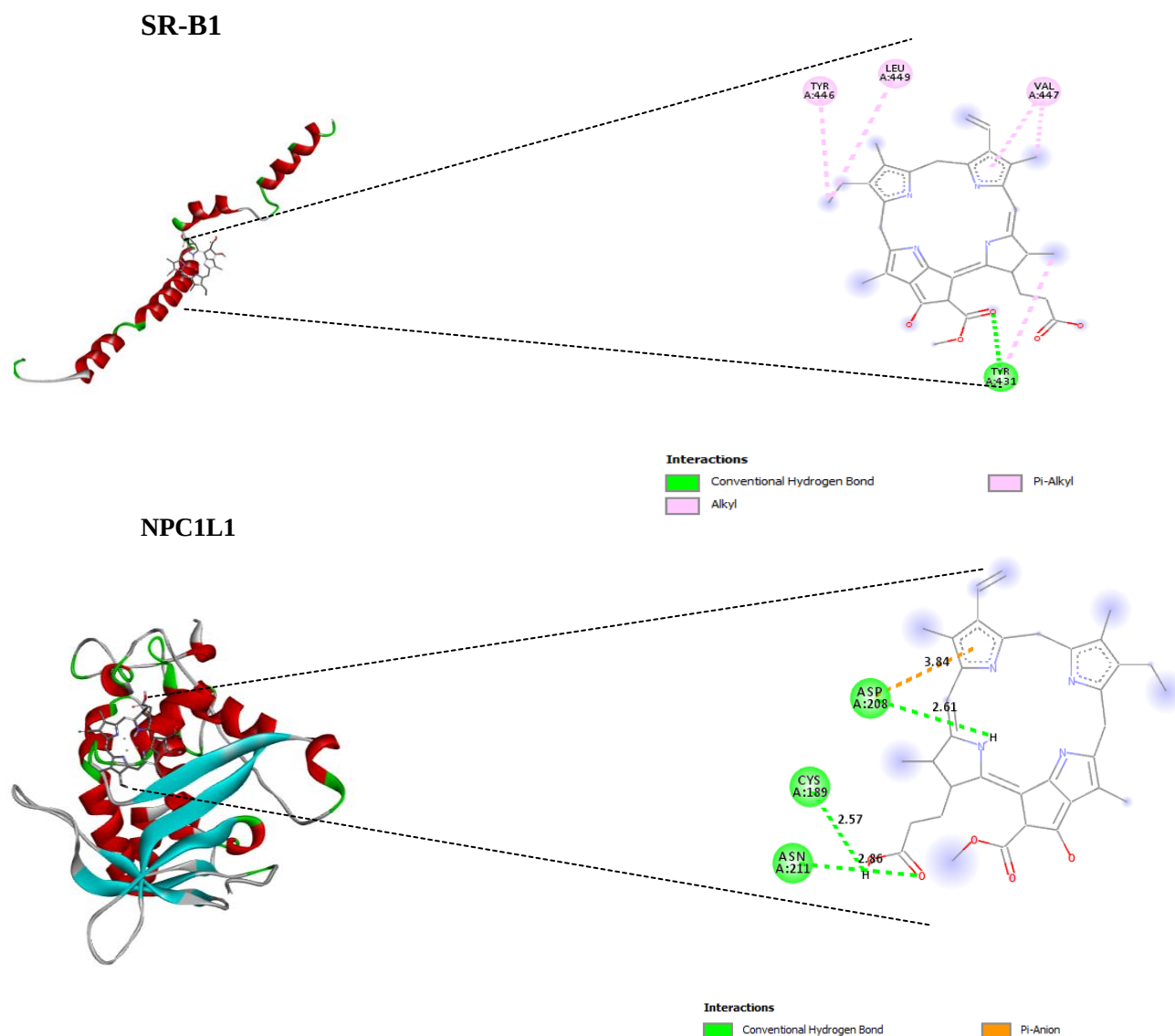


Figure 3.19. The molecular docking models: (A) chemical structure of Pheo a; (b) molecular docking study of Pheo a to SR-B1 (5KTF) and NPC1L1 (3QNT) docking simulated with 3D (left) and 2D (right).

3.4. Discussion

The intake of CDs can be associated with lower incidences of non-communicable diseases such as cancer, inflammation, and oxidative stress (Ferruzzi *et al.*, 2002; Park *et al.*, 2007; Pérez-gálvez *et al.*, 2020; Hayes and Ferruzzi, 2020). However, the CDs absorption and molecular their mechanism were not adequately defined because of lower bioavailability assumptions. Therefore, this research evaluated the CDs uptake in different structures of CDs,

concentrations, temperature-dependent paying the attention to transporter involvements. The principal of this study are as follows: (1) intracellular accumulation of CDs observed in structural, temperature, and dose dependent manners, (2) involvement of NPC1L1, SR-B1, and CD36 on the intracellular uptake of CDs, meanwhile, ABCB1, ABCG2, and ABCG5 were shared in the intracellular efflux of Phy b and Pheo a. (3) up-regulation of the lipophilic intestinal and ABC transporters related gene by Phy b and Pheo a. (4) the interaction between Pheo a and lipophilic transporter protein. As a result, the study suggests that when CDs is structurally distinct, it display a specific mechanism on their absorptions and efflux.

The efficacy of CDs in humans is related to their bioavailability in tissue. CDs bioavailability involves several factors such as CDs structures, the time release, concentrations, pH, miscelar composition, etc. (Solymosi and Mysliwa-Kurdziel, 2016). Therefore, this study evaluated the bioavailability of 6 CDs solubilized in DMSO in human intestinal epithelial cells as a model for intracellular uptake. This study also proved the effects of different CDs structure greatly affected their uptake by dCaco-2 cells, (**Figure 3.3.**) which attributed to the CDs structure, molecular weight, solubility, absorption mechanism, and facilitated protein (Gandul-Rojas *et al.*, 2009). Phytillated and dephytillated CDs showed higher absorption compared to parent CDs. This result is consistent with previous studies (Gallardo-Guerrero *et al.*, 2008; Gandul-Rojas *et al.*, 2009; Chen and Roca, 2018).

Pheo a and Chl c possessed almost similar structure without phytol chain, while Chl c has a magnesium ion. This study showed that the lower absorption of Chl c2 had better ability than the Phys molecules in the parent Chls. The central ion and phytol tail in the CDs structures affect absorption (Stawoska *et al.*, 2008), with the CDs were equipped with Mg^{2+} central ion and phytol chain, which showed lower absorption compared to those that lose Mg^{2+} central ions and phytol chain. The presence of phytol chain in the CDs structure increased the hydrophobicity of the molecules, modifying the π -electron system, peripheral

position in each structure of CDs are able to modify the π -electron system and absorption properties (Zepka *et al.*, 2019). In addition, the number of conjugated double bonds and different substituents on the pyrrole strongly affected the energy levels. Therefore, CDs absorption is suggested to be dependent on each structure (Groce and Amarungan, 2004).

In order to compare the uptake characteristics in each CDs, the four most absorbable CDs were chosen for the experiments. The saturation of intracellular uptake of Phy b and Pheo a was found after the supplementation of higher concentrations. Absorptions of Phy a, Phy b and Pheo a was significantly affected by the temperature. The saturation rate of the CDs uptake is the basis for the protein-mediated transport experiment (Reboul and Borel, 2011) with receptors/transporters capable on absorbing CDs in dCaco-2 cells.

A recent study has indicated that the intestinal absorption of Pheo a is facilitated by the use of specific transporters (Viera *et al.*, 2018) which plays a significant role in the distribution and elimination of many clinically important therapeutic agents in the body (Sukhai and Piquette-Miller, 2000). Intestinal uptake and efflux are two major factors for determining the bioavailability of the compound. Efflux and influx drug transporter is responsible for compounds absorption (Liu and Liu, 2013). Therefore, this study evaluated two different groups of facilitated protein, in accordance with the CDs intracellular uptake and efflux in dCaco-2 cells.

The result showed the involvement of lipophilic transporters in the cellular uptake of Pheo a, while NPC1L1 and CD36 possibly facilitated Phy b uptake. This study is consistent with the study by Viera *et al.*, (2018) which confirmed the reduction in the accumulation of Pheo a in the pre-incubation process using BL-T1. The SR-B1 transporter mediates the lipophilic substrate, such as cholesterol (Shen *et al.*, 2018), carotenoids (Airanthi *et al.*, 2014; Knauss *et al.*, 2017; Toomey *et al.*, 2017; Widjaja-Adhi *et al.*, 2017), and Pheo a (Viera *et al.*, 2018). This study also proved that other lipophilic transporters such as CD36 and NPC1L1

were involved in the Pheo a uptake. Meanwhile, Phy b, which is structurally composed of phytol chain and lack of Mg^{2+} central ion showed different facilitated involvement. NPC1L1 is the main substance used for the transportation of cholesterol and phytosterol. It also involves the absorption of carotenoids, vitamins, and pigments (Davis *et al.*, 2004; Davis and Altmann, 2009; Reboul and Borel, 2011; Reboul *et al.*, 2011; Sato *et al.*, 2012; Manabe *et al.*, 2019). Meanwhile, CD36, is a protein that is involved in the FAs uptake in the intestine (Lobo *et al.*, 2001). The results in the Pheo a and Phy b uptake in this study are plausible and used to support the detailed mechanism in dCaco-2 cells, thereby establishing the PCR study and molecular docking is needed.

In contrast, the increase of Phy a concentration was positively increase correlated the Phy a uptake which is acted as by passive uptake diffusion. This hypothesis is also supported by the inhibitor study (**Figure 3.12.**) which is in accordance with the data by a previous study conducted by Gandul-Rojas *et al.*, (2009).

After absorption by epithelium cells, some bioactive lipids are transported back to the apical side of the cells and into the GIT lumen due to the presence of specific efflux transporters (McClements *et al.*, 2015). As results of bi-direction study, the efflux transporter may majorly contribute to the CDs bioavailability.

Therefore, to elucidate the possible involvement of those transporters, the depleting ABC transporter of CCCP was used. The absorption of Pheo a and Phy b significantly increased ($p<0.05$) after pre-incubation with Ver and FC. This study consistent with Jonker *et al.*, (2002) which denote the accumulation of Pheo a in the mice^{-/- BCRP}. In addition, the results on the accumulation of Phy b after pre-incubation with ABCG5/ABCG8 inhibitors significantly increased. ABCG5/ABCG8 is for the lipophilic inhibitor ABC transporter, which are located in the apical side of Caco-2 cells (Hazard and Patel, 2007; Johnson *et al.*, 2010), therefore, these ABC transporters were involved in the Phy b and Phy a efflux

mechanism. This data also supported PCR results, which displayed up-regulation after CDs incubation for 4 h. However, this data needs to be confirmed with *in vivo* research using knockout, and supplemented transporters inhibitors in mice feed.

The research showed that the ABC transporters inhibitors significantly affected the intracellular accumulation of Pheo a and Phy b. ABC intestinal transporters play an essential role in cancer drug resistance, protection against xenobiotics, and the passage of drugs through cellular and tissue barrier. MDR-ABC transporter key is a physiological cellular defense system used to protect the accumulation of a foreign chemical in the human body. Foreign compounds are removed through the energized ATP hydrolysis (Szakács *et al.*, 2008; Quan *et al.*, 2012; Cloos *et al.*, 2018) with ABCG2 important for xenobiotics protection and regulation of oral bioavailability (Kanako *et al.*, 2006). This research is in line with previous studies that proved BCRP transporter plays an essential role in the efflux of Pheo a (Dietrich *et al.*, 2003; Viera *et al.*, 2018; Jonker *et al.*, 2002; Gandul-Rojas *et al.*, 2009). ABCG2 is essential as an efflux pump for a variety of hydrophobic xenobiotics, localized at the brush border membrane of dCaco-2 cells, together with ABCB1 (Uchida *et al.*, 2015). This study further showed the share of ABC transporter in the efflux of Pheo a with ABCB1 and ABCG2 as well as Phy b with ABCG5/ABCG8 and ABCG2.

Molecules or their metabolites regulate the cellular uptake of numerous molecules by the modulation of membrane transporter, which involves their uptake and efflux (Borel *et al.*, 2013). Therefore, this research examined whether Phy b and Pheo a modulate the levels of SR-B1, NPC1L1, CD36, ABCB1, ABCG2, and their related genes. The study showed that both Phy b and Pheo a significantly ($p < 0.05$) up-regulated all genes, therefore, suggesting that the absorption and efflux in dCaco-2 cells via effect modulation selected the transporter. Both CDs significantly upregulated BCMO1 which is a key enzyme in vitamin A metabolism (Lietz *et al.*, 2010; Airanthi *et al.*, 2014; Babino *et al.*, 2015; Knauss *et al.*, 2017; Widjaja-

Adhi *et al.*, 2017). Therefore, further study is needed to elucidate the mechanism Chls up-regulated BCMO1 in dCaco-2 cells. In addition, the up-regulation of NPC1L1 results from modulation of gene transcription factor SREBP2 with the cholesterol uptake mediated by NPC1L1 through the up-regulation of SREBP2 (Levy *et al.*, 2006; Sudhop *et al.*, 2005). The results clearly showed that increasing the SRBEP2 stimulates the up-regulation of NPC1L1 expressions in dCaco-2 cells.

This is the first study to examine the facilitated uptake transporters of CDs and molecular docking to explain and binding mechanism into cellular facilitated protein. Furthermore, more effort is needed to explore the efficacy of Chls, as the most abundant pigment in nature, and to get more clinical studies on the CDs used in healing humans from non-communicable diseases. The next chapter discusses the biological activity of CDs in HepG2 cells.

3.5. Conclusion

In conclusion, this study showed the facilitated protein involved in the absorption and efflux of CDs in dCaco-2 cells. CDs bioavailability in dCaco-2 cells involves more than one transporter, while the passive and facilitated diffusions are required to elucidate the clear mechanism. In addition, knockout mice are a better strategy used to determine the specific transporters regulated on the CDs absorptions instead of supplementing the transporters inhibitors during animal feeding. Therefore, the bioavailability study was carried out to determine the accumulation of CDs in dCaco-2 cells with the efficacy study needed for their human cell activity.

CHAPTER IV

CYTOPROTECTIVE EFFECTS OF CDs FROM *Sargassum horneri* AGAINST H₂O₂-INDUCED OXIDATIVE STRESS IN HepG2 CELLS

4.1. Introduction

In chapter 3, the *in vitro* characteristics of CDs uptake was evaluated using in intestinal epithelial cells (dCaco-2 cells).). The results demonstrated that CDs are incorporated into dCaco-2 cells. Then, this chapter investigates the biological activity of CDs to protect cells from oxidative stress.

In the body, normal cells tend to produce ROS and other free radicals because they play important physiological roles in the cells (Ma *et al.*, 2016). For example, liver is an important organ in humans used to maintain homeostasis and responsible for the various endogenous and exogenous compounds during metabolism (Casass-Crajkess, 2017). The liver is constantly exposed to different oxidizing agents and H₂O₂ among those that cause oxidative injury (Zhang *et al.*, 2010; Jiang *et al.*, 2014). H₂O₂ has a longer half-life than other oxidant compounds and can easily be transformed into the most destructive free radicals, such as hydroxyl radical (Clarkson and Thompson, 2000). Furthermore, it can diffuse freely inside and outside the cells and tissue (Jiang *et al.*, 2014). This compound has been used for oxidative stress study in several tissues cell such as keratinocytes (Svobodová *et al.*, 2006), human lymphosites (Hashim *et al.*, 2005), Caco-2 cells (Wijeratne *et al.*, 2005), human neuroblastoma cells (Zhou *et al.*, 2018), and lens ephitellial cells (Zheng *et al.*, 2010). H₂O₂ also has been used on the liver cells (HepG2) for oxidative stress studies (Musonda and Chipman, 1998; Jiang *et al.*, 2014; Salla *et al.*, 2016; Wang *et al.*, 2019; Yang *et al.*, 2020a; Yi *et al.*, 2020). Therefore, in this research, H₂O₂ was used to induce oxidative stress in HepG2, and then, protective effects of CDs were analysed.

ROS sometimes occurs in the human body as a result of imbalances between pro-oxidants and antioxidants, thereby leading to molecular damage which causes higher productions (Sies, 1991; Stahl and Sies, 2003; Halliwell, 2012). ROS results in intracellular oxidative stress and damages the intracellular structures, such as DNA, lipids, and protein leading to a variety of degradation processes (Zhao *et al.*, 2005). The radicals and non-radical are involved in various cell signaling pathways leading to gene transcriptions, mitogenic response, and protein synthesis. Furthermore, it leads to the development of several diseases such as cancer, diabetes, cardiovascular, hypertension, and kidney diseases (Khan, 2012; Matsuda and Shimomura, 2013; Incalza *et al.*, 2018). These "free radicals" are usually removed or inactivated *in vivo* by antioxidants which are spread to prevent the generation of ROS and eliminate pro-oxidants (Benzie and Strain, 1996). In recent years, epidemiological studies reported that natural and synthetics antioxidants play a pivotal role in protecting the human organs against oxidative stress (Augustyniak *et al.*, 2010).

Antioxidants substances can prevent or reduce oxidation of other molecules (Zhang *et al.*, 2017) and regulate the biological process to reduce the occurrence of several diseases (Reuter *et al.*, 2010). Although both natural and synthetic antioxidants can inhibit the excessive amount of free radicals in the human body (Horáková *et al.*, 2000; Augustyniak *et al.*, 2010; Montazerifar *et al.*, 2010; Lobo *et al.*, 2010), the synthetic one may provide negative effects to the body mainly in the livers (Franco *et al.*, 2019; Estévez *et al.*, 2006). On the other hand, the natural antioxidants contained in fruits, vegetables, and algae can reduce the excessive free radicals in the human body without adverse effect.

Most of phytochemical contained in plants and seaweeds possess antioxidant activities and they are sometimes used in protecting and the therapeutics of humans from a variety of diseases (Ganesan *et al.* 2019; Shannon and Abu-Ghannam 2019; Collins *et al.* 2016). In recent years, more attention has been paid to the antioxidants contained in

seaweeds because epidemiologically they reduced the occurrence of cancer (Yang et al. 2010; Moussavou et al. 2014). Chls are the most abundant phytochemicals in seaweeds. Recently, tremendous attention has been paid to CDs due to their antioxidant activity which are effective in healing and reducing oxidative stress (Gandul-Rojas et al. 2009; Hsu et al. 2013; Lanfer-Marquez, Barros, and Sinnecker 2005; Pérez-gálvez, Viera, and Roca 2020; Park et al. 2007; Hsu et al. 2008; Wang and Wink 2016).

Furthermore, Chls are known to be converted to pheophytin, pyropheophytin, and pheophorbide in processed vegetable foods, and after ingestion. Judging from their potential biological potency, these CDs may give beneficial health effects (Chernomorsky et al. 1999). The derivatives products of CDs possess a higher level of antioxidants, however, the fate of Chls in the human body and their potential biological effects remains essentially unexplored (Marková et al. 2018). In addition, the study showed that the CDs from seaweeds are diverse and more complex than terrestrial plants. Therefore, the attempt to make clear the biological activities of CDs originated from seaweeds will be challenging.

Therefore, in this chapter, antioxidant activity of CDs prepared from seaweeds was investigated to know their biological activities. For the analysis, oxidative stress was induced in HepG2 by adding H₂O₂ to the cell culture, and then, the protective effect of CDs was measured on the cell viability, ROS, thiobarbituric acid reactive substances (TBARs), and antioxidant enzymatic activities. In addition, chemical ability of CDs to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals was also analysed.

4.2. Materials and Methods

4.2.1. Material

HepG2 (human hepatocellular carcinoma) cell line was obtained from DS Pharma Biomedical (Suita, Osaka, Japan). FBS was purchased from Biosera Co (Nuaille, France).

Dubelcco's Modified Eagle Media (DMEM) was purchased from Nisui Pharmaceuticals Co. Ltd. (Tokyo, Japan). Penicillin and streptomycin, DMSO, and Dulbecco's PBS were obtained from Fuji Film Wako Pure Chem Ltd. (Osaka, Japan). MTT was purchased from Dojindo Molecular Technologies, Inc (Tokyo, Japan). RT high-capacity cDNA Archive Kit and Micro Amp Optcal Reaction Plate 0.1 mL were obtained from Applied Biosystem (CA, USA). Gene Ace Probe a PCR Mix III was obtained from Nippon Gene Co. Ltd (Toyama, Japan). Ezetimibe was obtained from Focus Biomolecules (PHI, USA). QiAzolTM, Lysis Reagent was purchased from Qiagen (Hilden, Germany). Sodium taurocholate was purchased from Sigma Aldrich (MO, USA). Protein assay reagents were purchased from Bio Rad Laboratories (CA, USA). All protein targets were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). High-performance liquid chromatography (HPLC) grade solvents were used for the HPLC analysis and purchased from Fuji Film Wako Pure Chemicals, Ltd. (Osaka, Japan). '2,7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and MDA assay kits were purchased from Cayman Chemical (MI, USA). All other solvents and chemicals used in the study were of analytical grade.

4.2.2. Methods

4.2.2.1. TL extractions and CDspreparations

The procedures of TL extractions and isolation of CDs was the same as shown in **Chapter 3, section 3.2.2.1.**

4.2.2.2. Derivatives Chls

The preparation of CDs was the same as the previous shown in **Chapter 3, section 3.2.2.2.**

4.2.2.3. Purification of CDs

The purifications of CDs was the same as the previous shown in **Chapter 3, section**

3.2.2.2.

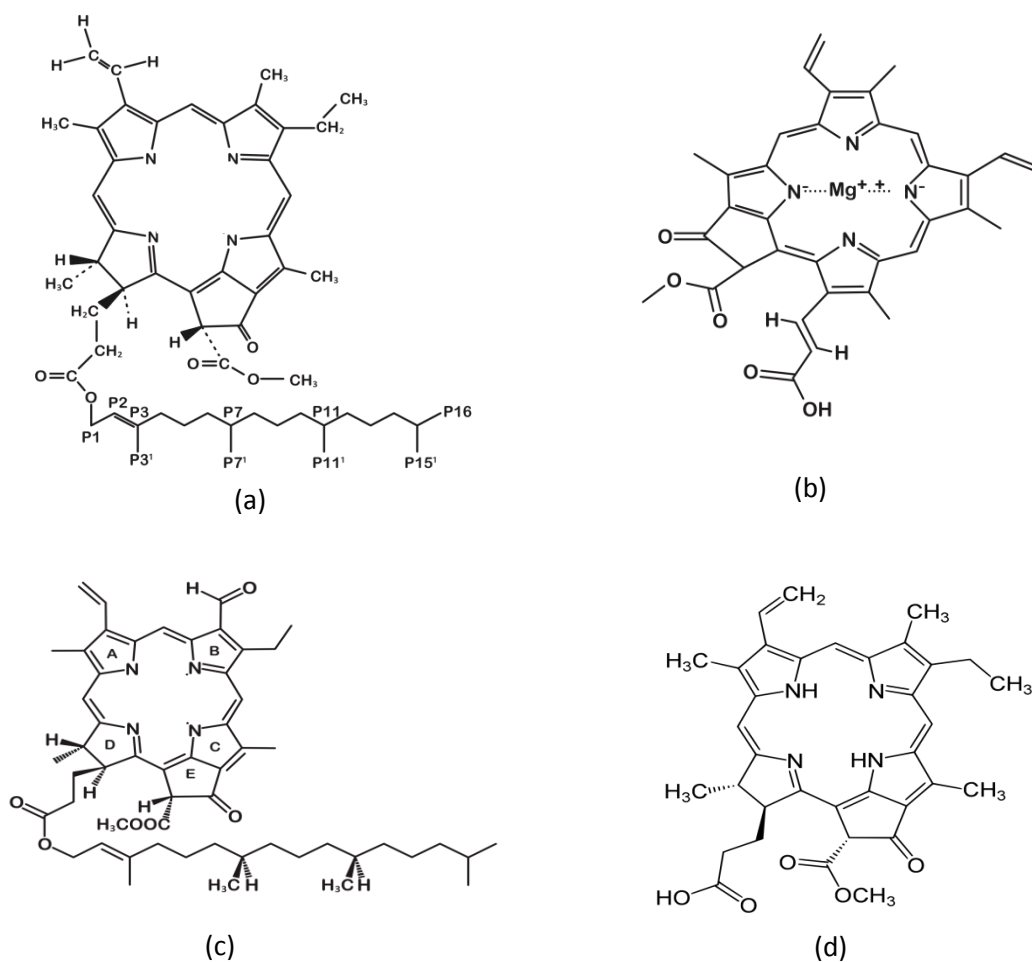


Figure 4.1. CDs used in oxidative stress study. (a) Chl c2; (b) Phy a; (c) Pheo b; (d) Pheo a.

4.2.2.4. HepG2 cells culture

HepG2 cells were maintained DMEM containing 10% of inactivated FBS, 100 U/mL penicillin, and 100 µg streptomycin. HepG2 cells were subculture by detaching from culture dish with adding 2 mL of trypsin contain EDTA for 5-7 min after 70-80% confluence. HepG2 cells were cultivated at 37°C, 5% CO₂, and 95% humidity.

Cells were treated with different concentration of Chl c2, Phy a, Phy b, and Pheo a. Oxidative stress was induced by the exposure of H₂O₂ to cells for 24 h at 37 °C. Cells were then lysed in ice cold solubilisation buffer and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was collected and antioxidant enzymes activities were analysed.

4.2.2.5. Cell viability and cytotoxicity assays

Cell viability was carried out by MTT methods. HepG2 cells were cultured with density 1x10⁴ cells in 100 µL of medium in clear 96 wells plate. Cells were incubated in 37°C and CO₂ 5% for 24 h. After 24 h incubation, 100 µL of the medium was changed with the medium and samples and then incubated for additional 24 h. After incubation, the medium were changed new medium adding H₂O₂ and cells were incubated for additional 24 h. The 10 µL of MTT solution reagent (Dojindo, Kumamoto, Japan) were added in each well and incubate for another 3 h. After 3 h, cells were wash with 150 µL PBS for 1 min. 100 µL of DMSO was added to cells in the well. Then absorbance was measured by plate reader (Spectramax, MS^e, Molecular device, USA) at 535 nm. The MTT (%) was calculated with the following equation:

$$\text{MTT (\%)} = \frac{(C-A)}{(B-A)} \times 100$$

A = Absorbance of blank without HepG2 cells

B = Absorbance of control with HepG2 cells

C = Absorbance of sample with HepG2 cells

4.2.2.6. Antioxidant capacity

4.2.2.6.1. DPPH

DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. In the presence of antioxidant, it will reduce the color and change into uncolored ethanol solution (Clarke *et al.*, 2013). 10 µL of extract diluted appropriately in DMSO was

mixed with 190 μL of DPPH in methanol (0.1 mM DPPH) in a 96-well plate. The plate was kept in the dark and mixed for 30 min at room temperature. Thereafter, the absorbance of each samples was measured at 517 nm (A_{samples}) using multidetection of microplate reader, where the blank was prepared with 0.5 mL ethanol and 2.0 mL of 0.1 mM DPPH reagent (A_{blank}) (Spectramax, MS^e, Molecular device, USA). Due to the higher variation on the color, before DPPH addition, the absorbance of samples was check.

Appropriate blanks (DMSO) and standards (alpha tocopherol solutions in DMSO) were run simultaneously. The inhibition of DPPH radical-scavenging activity in percent ($I\%$) was calculated according to the following equation:

$$I \% = \left[\frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \right] \times 100$$

Where:

A_{sample} is the absorbance of a sample solution

A_{blank} is the absorbance of the blank solution (containing all reagents except the test sample).

4.2.2.6.2. ABTS radical scavenging activity

The ABTS is spectrophotometric which is widely used for the antioxidant activity of various substances (Yang *et al.*, 2020). The methods is based on the capacity of CDs to scavenge the ABTS radical cations ($\text{ABTS}^{\bullet+}$) compared to standard trolox (Trolox). Addition of antioxidants to the preformed radical cation reduces it to ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction (Re *et al.*, 1999).

The ABTS measurements were conducted according to ABTS radical solution. It was adjusted with methanol to an absorbance of 0.700 at 734 nm before use. To 190 μL of $\text{ABTS}^{\bullet+}$ solution was added of 10 μL of sample or Trolox or solvent upon a 96-well plate.

The mixture was incubated for 5 min at 30°C, and the absorbance at 734 nm was measured with a microplate reader. The inhibition percentage of the radical scavenging activity was calculated using the equation:

$$\text{Inhibitions (\%)} = \left\{ 1 - \left(\frac{A_s}{A_o} \right) \right\} \times 100$$

where:

A_o = absorbance of the control

A_s = absorbance of the sample at 734 nm

The radical stock solution was freshly prepared, and all analyses were made in triplicate. All solution should be prepared in the dark light.

4.2.2.6.3. Intracellular antioxidant assays (IAA)

The IAA of CDs was assessed by the IAA assay according to the method described by Sun *et al.*, (2019).

HepG2 cells (6×10^5 cells per well in 96-well plate) were cultured in a 96-well black plate for 24 h. Removed DMEM and washed 2 times with PBS. DCFH-DA working solution (50 μ L of 25 μ M) was added to each well and mixed with 50 μ L of serum-free media without and with antioxidant. The plate was placed in the incubator at 37°C for 1 h. After 1 h, the solution was removed, HepG2 cells were washed three times with PBS, followed by addition of 100 μ L of 600 μ M AAPH in HBSS.

The plate was immediately incubated in plate reader (SpectraMax MS^e, Molecular Devices) at 37°C. Measurements were performed every 5 min for 1 h at 538 nm emission wavelength and 485 nm excitation wavelength.

After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the IAA value at each concentration of sample as follows:

$$\text{IAA unit} = \left\{ 1 - \left(\frac{\int SA}{\int CA} \right) \right\} \times 100$$

where:

$\int SA$ = the integrated area under the sample fluorescence versus time curve

$\int CA$ = the integrated area from the control curve.

4.2.2.7. CDs uptake in HepG2 cells

HepG2 cells were seeded 3×10^5 cells in 2 mL in 6 well plates (TPP, Trasadingen, Switzerland). After 80-90% confluence, the cells were added samples with the following concentration, 10, 25, and 50 μM . The CDs were diluted in DMSO before supplemented to the HepG2 cells. The maximum DMSO concentration ($\leq 1\%$) was added to the HepG2 cells (showing did not affect to the viability of cells, data not shown). After 24 h incubation, cells were washed with 0.5 mL of cold PBS containing 5 mM of sodium taurocholate (Sigma Aldrich, Germany) to remove pigment in extracellular cells. Then, it washed with PBS to remove remaining media. Cold PBS (1 mL) was added to detach the cells from the dish with a scraper, then take 100 μL for protein analysis and 900 μL for CDs extractions. The CDs from HepG2 cells were extracted according to Bligh and Dyer method (1959) with modification. The cells were suspended in 900 μL of PBS, and then chloroform (containing 0.2% BHT) and methanol (0.2% BHT) with ratio (1:2, v/v) was added, then vortexed for 30 s. After overnight extraction, 1 mL of chloroform containing 0.2% BHT was added, then centrifuged 3,000 rpm for 15 min. Pipette the chloroform layer before addition of 1 mL

chloroform containing 0.2% BHT, then centrifuge with the same procedure. Chloroform layer was removed and added the collected chloroform phase. The chloroform phase was dried under N₂ before dilution in acetone to further investigation. CDs quantification is expressed in per protein content of HepG2 cells. The HepG2 cells protein content was quantified by using DC protein assays kit Bio-rad Laboratories (Hercules CA, USA). The CDs uptake in HepG2 cells was expressed in $\mu\text{g}\cdot\text{mg}^{-1}$ of protein cells.

4.2.2.8. Induction of oxidative stress

HepG2 cells were treated with different concentration of Chl c₂, Phy a, Phy b, and Pheo a (10, 25, and 50 μM) for 24 h. Oxidative stress in HepG2 was induced by treating cells with different concentrations of H₂O₂ (0.4 mM) for another 24 h at 37°C. After 24 h incubation with H₂O₂, HepG2 cells were measured for further analysis.

4.2.2.9. Determination of intracellular ROS accumulation

The production of intracellular ROS in CDs-treated cells was determined with DCFH-DA, an oxidation sensitive and cell membrane penetrating probe. HepG2 cells (1×10^5 cells per well) were cultured in a 96-well black plate for 24 h and treated with or without samples for 24 h prior to induction of H₂O₂. After induction H₂O₂ for 24 h, HepG2 cells were supplemented with 10 μM DCFH-DA in PBS for 45 min at 37 °C in the dark. The generation of ROS was quantitatively detected using a microplate reader (SpectraMax MS^e, Molecular Devices) with excitation: 485 nm and emission: 535 nm (Wojtala *et al.*, 2014) .

4.2.2.10. 2-Thiobarbituric acid reactive substances (TBARS) assays

Lipid peroxidation of HepG2 cells was assessed by measuring production of a lipid peroxidation end product, MDA, by the TBARS assay kits (Cayman, MI, USA).

4.2.2.11. RT-PCR

Diluted cDNA was used as template reaction for each primer (4.3 µL) and master mix (Nippon Genetics, Tokyo, Japan) (5.7 µL) in a final volume 10 µL. Five serial dilutions of the standard were set up to design to determine all primers and reaction of all primer pairs. PCR amplification was performed in a StepOne plus RT PCR System machine (Applied Biosystem, California, USA). For cDNA denaturation, 40 to 50 cycles of 95°C for 30 s, 45 s for final amplification, and one cycle for 75 s. Each assay included a no template control for each primer pair. All RT-qPCR were carried out in triplicate. The gene were used in this study (Hs00156308_m1; CAT, Hs00533490_m1; SOD-1, Hs00829989_gH; GPX1, Hs02786624_g1; GAPDH).

4.2.2.12. Statistical analysis

All analyses were run in triplicate and the mean value is shown. Statistical significance of differences in means was determined using ANOVA, followed by multiple comparison *Tukey post hoc test* at $p < 0.05$. The graph and data in this study were draw and analyzed using GraphPad Prism 8 software (SD, USA).

4.3. Results

4.3.1. Antioxidant activity of CDs

Several studies have been carried out to measure and screen the antioxidant activity of natural and synthetic compounds or samples (Wang et al. 2009; Huang et al. 2005). To know the antioxidant activity of CDs (Lanfer-Marquez 2005; Ferruzzi et al. 2002; Hsu et al. 2013), in the present study, different procedures were adapted. The radical quenching activity was measured using DPPH and ABTS reagent. In addition, intracellular-based (IAA) antioxidant activity of different CDs was also evaluated. CDs used in the present study was isolated from seaweeds.

4.3.1.1. DPPH radical scavenging activity

In this method, the antioxidants were able to reduce the stable DPPH radicals, resulting in a decrease in its absorbance at 517 nm (Behgar et al. 2011).

As shown in **Figure 4.2.**, the antioxidant activity of Phe a was the highest followed by Phy b, Phy a, and Chl c2. Furthermore, the antioxidant property of CDs is based on the family and degraded compounds polarity of the dephytillated Mg^{2+} and phytol chain free Chls. The exhibited antioxidant activity was higher compared to phytillated and parent Chls (**Figure 4.1.**).

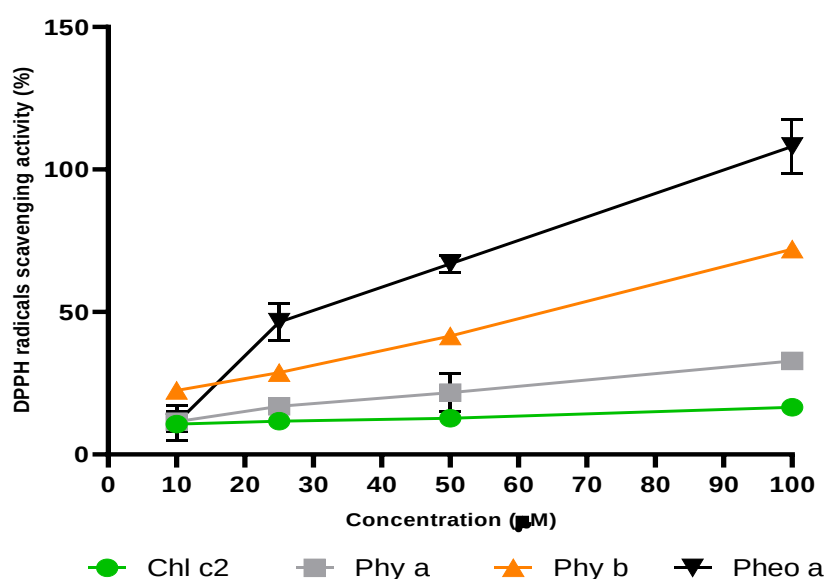


Figure 4.2. DPPH radical scavenging activities of CDs in different concentrations (10, 25, 50, and 100 µM).

4.3.1.2. ABTS scavenging activity

ABTS is considered to be appropriate in evaluating antioxidant properties of diversified pure compounds or a complex mixture (Cano *et al.*, 2002; Ben Sghaier *et al.*, 2011). The scavenging activity of the compounds on free radical ABTS, generated by

potassium persulfate was compared with a standard amount of Trolox (Ben Sghaier *et al.*, 2011).

In the present study, the antioxidant activity of CDs isolated from seaweeds and algae was also evaluated using the ABTS radical. All CDs showed scavenging ability on ABTS radical (**Figure 4.2.**). Among the CDs evaluated, Phy b showed a relatively lower activity, while there was a little difference between other three kinds of CDs. This result is inconsistent with the effect of these CDs on DPPH radical scavenging activity.

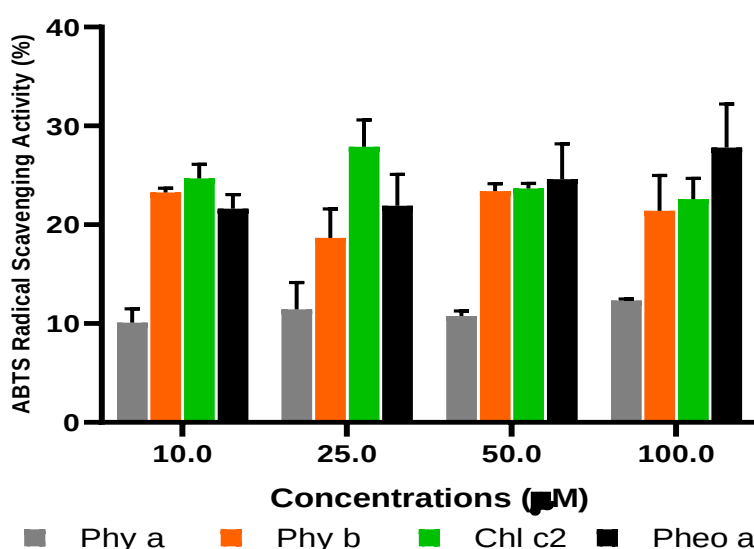


Figure 4.3. ABTS radical scavenging activities of CDs (10, 25, 50, and 100 μM).

The activity of CDs in ABTS radical scavenging obtained by the ABTS discoloration increased with polarity in the order Pheo a > Chl c2 > Phy b > Phy a. This result is inconsistent with the DPPH concentrations used to measure the lower activity of in ABTS systems.

4.3.1.3. IAA assay

IAA was carried out to examine the intracellular antioxidant activity of CDs in HepG2 cells. Chemical antioxidant assays are commonly used in the individual study of phytochemical or mixture of biological assays without using the cell culture (Phan et al., 2019). The IAA is a more biological pertinent method than the chemical analysis because it is estimated to determine some factors such as uptake metabolism, and the spot of antioxidant compounds inside the cells. The IAA assay utilizes a DCFH-DA probe, which is cleaved to DCFH by cellular esterase after diffusing into cells and being oxidized to DCF by peroxy radicals produced by ABAP. ROS scavengers, such as antioxidants, reduce intracellular peroxy radicals, thereby, inhibiting the formation of DCF used as a measure of the scavenging activity and intracellular ROS via the CAA assay (Wolfe and Rui, 2007; Hwang *et al.*, 2016).

This study utilized the HepG2 cells with DCFH-DA as a probe and ABAP as the radical generator. The probe deacetylates inside the cells and oxidized in the existence of hydroxyl radicals to fluorescent DCFH-DA, with the presence of antioxidants used to extinguish the reactions to prevent the generation. This can be undertaken on the surface or within the cells membrane for successful uptake of antioxidant compounds (Chen *et al.*, 2017). The kinetic oxidation of DCFH by peroxide radical assessed in this study shows an increase of fluorescence from DCF formation inhibited by CDs in dose dependents manner.

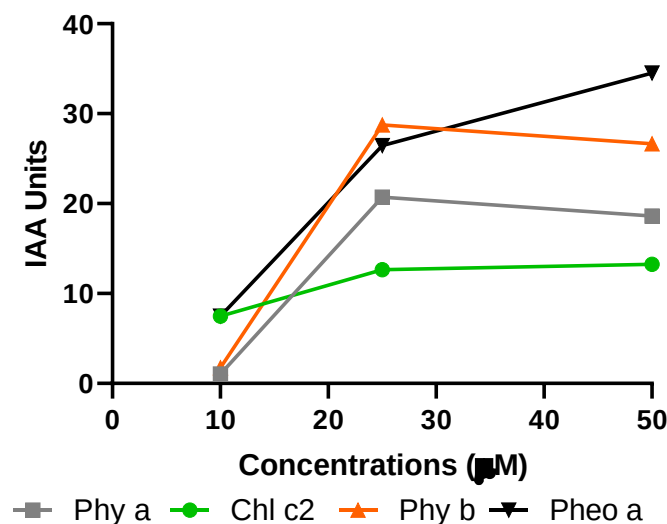


Figure 4.4. The IAA of the CDs in HepG2 cell treated with 10, 25, and 50 μM of CDs for 24 h. Values were exposed as IAA units and presented as means, $n=8$.

In this study, the different concentrations of CDs were evaluated with the oxidation probe at 37°C for 60 min. Cellular response was induced by supplementing the medium with 600 μM ABAP and the results showed a dose-dependent reaction, with a decrease of cellular fluorescence. The Pheo a has a high value of IAA compared to other CDs in accordance with their hydrophilic contents, which lead to the higher cells membrane-permeability of Pheo a over Chl c2 (**Figure 4.4.**).

4.3.2. Cytotoxic of H_2O_2 induced toxicity

H_2O_2 has been used in the oxidative stress injury models in hepatocyte cells, and before *in vitro* hepato-protective studies, the cytotoxic effect of pro-oxidants H_2O_2 was examined in human hepatoma HepG2 cells. Cells were incubated at $1 \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$ with various H_2O_2 concentrations (10, 100, 200, 400, and 800 μM) and incubation time (1, 3, 6, 12, and 24 h) of H_2O_2 , respectively. The purpose of the analysis was to choose the appropriate concentrations to induce cellular damage (**Figure 4.5.**) The present results showed that the cell viability decreased significantly when the concentration of H_2O_2 was more than 10 μM

H₂O₂ concentrations and with a decrease in its doses dependent-manner. The study also showed that 400 μ M decreased the cell viability by 50.8%, while 800 μ M was significantly decreased by 94.1% after 24 h treating with H₂O₂. In the presence of 400 μ M H₂O₂, the cell viability demonstrated moderate damage. Therefore, treating the HepG2 cells with H₂O₂ at a final concentration of 400 μ M for 24 h was selected for subsequent experiments to establish oxidative stress injury models.

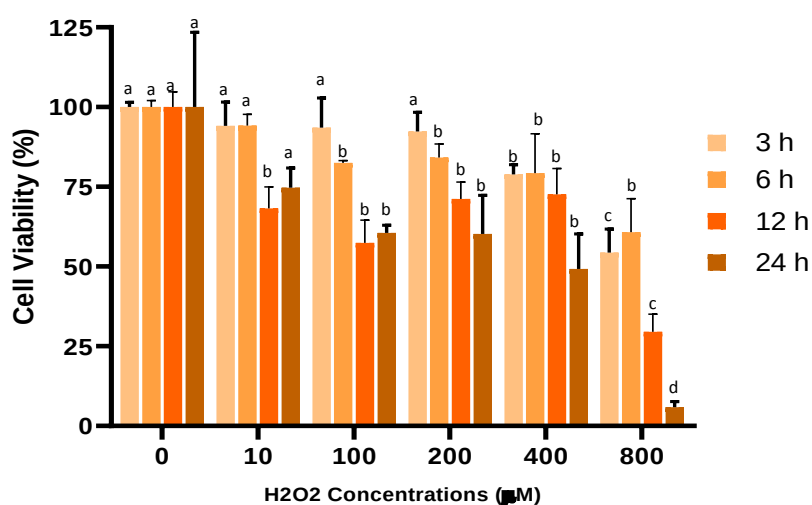


Figure 4.5. Cytotoxicity of H₂O₂ on HepG2 cells. Cell viability was measured by WST-1 assays as describe in methods. HepG2 cells treated with various concentrations (0, 10, 100, 200, 400, and 800 μ M) and incubation time (3, 6, 12, and 24 h). Results are expressed as percent control and represent means $\pm$ SD of three to four independent experiments. Different letters above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

4.3.3. Effect of H₂O₂ on ROS productions

HepG2 cells are easy to culture cells, with fast reproductions and ideal to estimate the oxidative stress. HepG2 cells contain a high level of ROS, which causes cell injury and apoptosis in the lipid and protein peroxidation (Yang *et al.*, 2020).

ROS are depicted as molecules, ions, or radicals, including superoxide anion, organic and hydroxyl radicals, singlet oxygen and hydrogen peroxide, which are by-products of mitochondrial metabolism and redox signaling (Afrin *et al.*, 2017). ROS, which is a product

of the respiration cycle of oxidative phosphorylation, rises to cellular DNA breaks and mutation that further cause cell mortality, aging, or tumor (Yi *et al.*, 2020). ROS can be produced in HepG2 cells by H₂O₂. Therefore, to determine their level as an effect of H₂O₂ inductions, the HepG2 cells related to ROS productions were carried out in different time exposure (12 h and 24 h).

This study further investigates the suppression effects of CDs on HepG2 cells after H₂O₂. The level of intracellular ROS was monitored using DCFH-DA (Liang *et al.* 2020), which is the most commonly used detections probe for intracellular ROS.

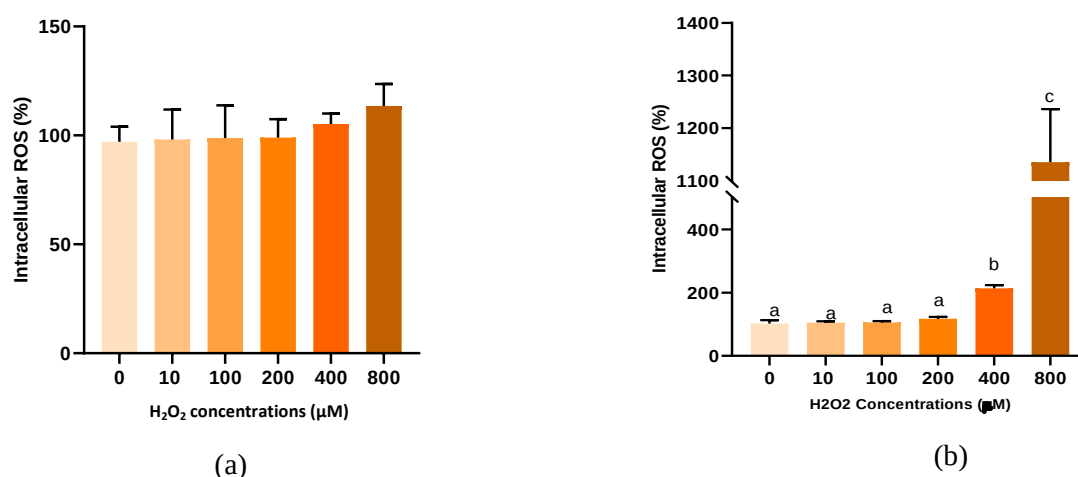


Figure 4.6. Intracellular ROS produced by H₂O₂ after (a) 12 h; (b) 24 h incubation of HepG2 cells. Intra-hepatocellular was monitored by using DCFH-DA fluorescence method as describe in methods. Each value is expressed as the mean $\pm$ SD (n=3-4). Different letters above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

A 12 h incubation period, showed no significant differences in ROS products after incubations from 0 to 800 μ M of H₂O₂. However, a 24 h incubation period of H₂O₂ showed a significant increase in ROS to 400 and 800 μ M. Among the groups, a 24 h incubation period of H₂O₂ was significant ($p < 0.05$) while at 12 h it was insignificant ($p > 0.05$). Therefore, 24 h incubation of H₂O₂ was chosen for further experiments (**Figure 4.6.**).

4.3.4. Concentration screening of CDs

In parallel MTT assay, HepG2 incubated with CDs resulted in a dose-dependent decrease of cell viability. Therefore, MTT was carried out with various concentrations (0 to 200 μM) to establish an optimal concentration of CDs on the viability of HepG2 cells. The cellular toxicity of samples by MTT assay, as exhibited in **Figure 4.7.** showed that 1×10^4 cells per well of HepG2 treated with 0, 10, 50, 100, and 200 μM . All CDs did not show any toxicity up to 200 μM except Chl c2, indicating moderate and acute damage after 100 and 200 μM Chl c2 treatments. In general, they survived within the treatment concentrations of < 200 μM , therefore further experiments were done at 10 to 100 μM of CDs.

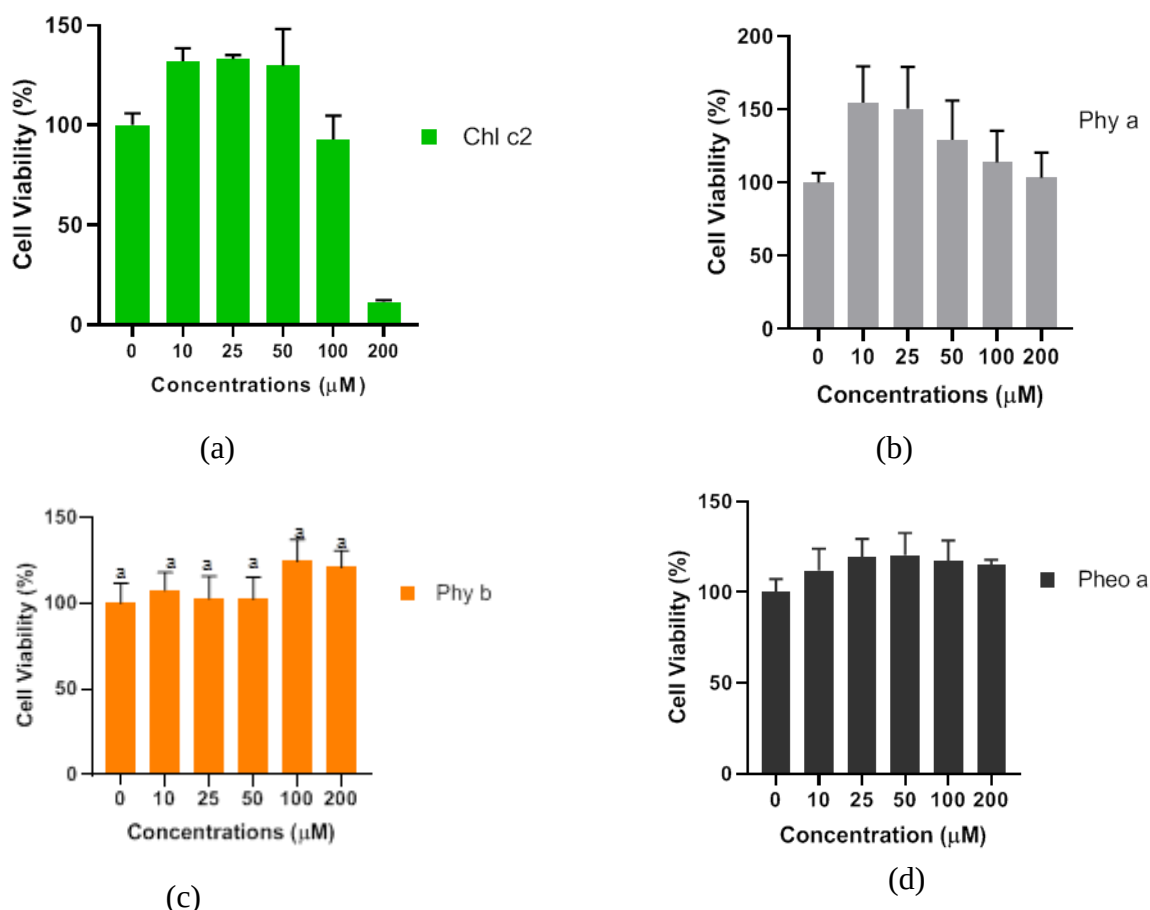


Figure 4.7. Cytotoxicity of (a) Chl c2; (b) Phy a; (c) Phy b; and (d) Pheo a on cultured HepG2 cells with various concentrations (0-200 μM). Cell viability was measured by MTT assays as describe in methods. Cells were treated with indicated concentrations CDs for 24 h. Different letters above bars indicate a significant difference at $p < 0.05$ among each value and the same letter were not significantly different ($p > 0.05$) determined by *Tukey post hoc test*.

4.3.5. Preventive effects of CDs on H₂O₂-treated HepG2 cells

Cell viability is a pivotal marker of its mortality after exposure to toxic substances and stimulation. Therefore, to examine whether the observed protective effects of CDs on HepG2-cells after induced 400 μ M of H₂O₂. The MTT assay was evaluated to potentially exempt the cytotoxic effects revealed by CDs and H₂O₂. The results showed that the cell viability had negative effect on HepG2 proliferation as measured in MTT assays after being treated with maximum concentrations (100 μ M) (**Figure 4.8.**). Further experiments were carried out using at different concentrations of CDs to determine the different exposure effects of CDs (10, 25, 50, and 100 μ M) to H₂O₂.

Figure 4.8. showed that the effects of CDs on the viability of H₂O₂-treated HepG2 cells exhibited different dose-dependent manners. Among the various CDs tested, Phy b exerted the better protecting effects on HepG2 cells treated with H₂O₂.

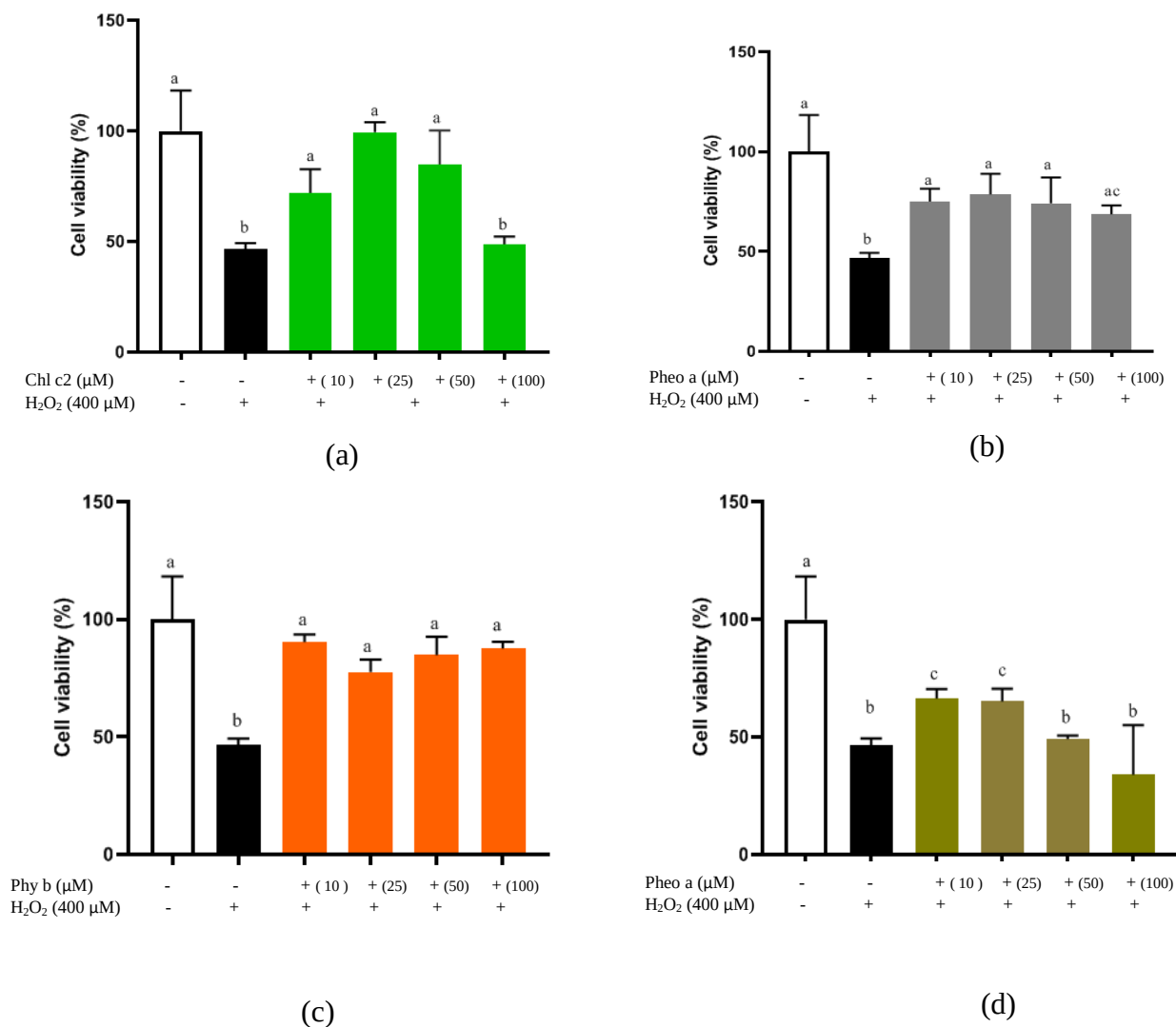


Figure 4.8. Protective effects of CDs on the viability of HepG2 cells induced oxidative stress by adding 400 μM H₂O₂. Each value is expressed as the mean ± SD (n=3-4). Con: control; H₂O₂: data were presented as means ± SD, n=3-4. HepG2 cells pre-treated with various concentrations (a) Chl c2; (b) Pheo a; (c) Phy b; and (d) Pheo a with the range concentration of each compounds 0-100 μM. Different letters above bars indicate a significant difference at $p < 0.05$ among each value and the same letter were not significantly different ($p > 0.05$) determined by *Tukey post hoc test*.

4.3.6. Bioavailability of CDs in HepG2 cells

The suppression effects of CDs against H₂O₂ induced oxidative stress are related to the antioxidant activity and availability of CDs in the HepG2 cells. Therefore, the bioavailability of CDs in HepG2 was investigated to determine the accumulation of CDs in

HepG2 cells. The results showed that CDs accumulated in dose dependent manner. The maximum level accumulation of Pheo a, Phy a, Phy b, and Chl c2 in the HepG2 cells were 28.08, 5.73, 1.08, 0.4 mg·g⁻¹ protein, respectively. The increase in CDs concentrations led to a higher bioavailability of CDs in HepG2 cells. Overall, Pheo a is highly accumulated in the HepG2 cells, followed by Phy a, Phy b, and Chl c2, with CDs structurally dephytillated than the parent Chls. This study is also in agreements with previous studies on bioavailability in dCaco-2 cells which showed that the dephytillated Chls had a better absorption rate compared to the phytillated and parent Chls (**Figure 4.9.**).

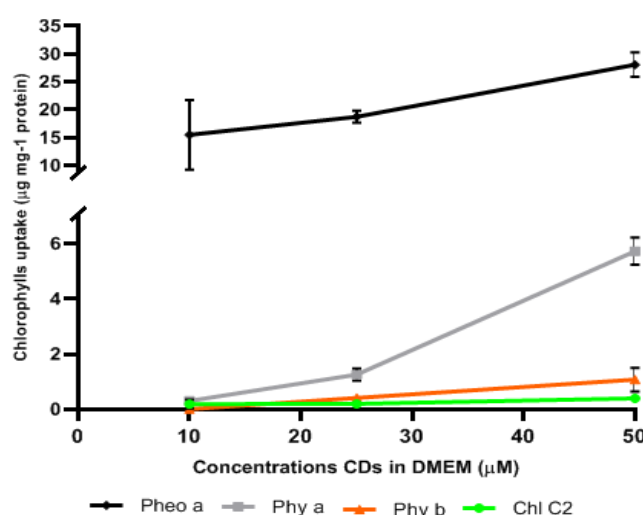


Figure 4.9. The bioavailability of CDs in HepG2 cells. HepG2 (2×10^5 cell/2 mL) in 12-wells were incubated with each CD at indicated concentrations (10, 25 and 50 µM) for 24 h incubation at 37°C, 5% CO₂. The concentration of CDs were determined using Shimadzu UHPLC System according to Susanto *et al.*, (2019). Closed four equal angles, Pheo a; closed square, Phy a; closed triangle, Phy b; closed circle, Chl c2. Each point represents the mean with SD of 3-4 measurements.

From this result, dephytillated Chls showed better absorption compare to phytillated and parent Chls.

4.3.7. The effects of CDs on the suppression of ROS

The CDs attenuated in the oxidative stress in HepG2 cells treated with 400 μM H_2O_2 for 24 h, ROS level was measured by a DFH-DA probe. The intracellular ROS was visualized by detecting dichlorofluorescein (DFC) derived from the oxidations of the DCF. After 24 h exposure to H_2O_2 (400 μM), the HepG2 cells significantly increases the intracellular ROS accumulations ($p < 0.05$). **Figure 4.10.** shows that the percentages of ROS induced by H_2O_2 were much higher than control groups ($p < 0.05$), while those in the pre-treated groups were significantly reduced ($p < 0.05$) (**Figure 4.10.**).

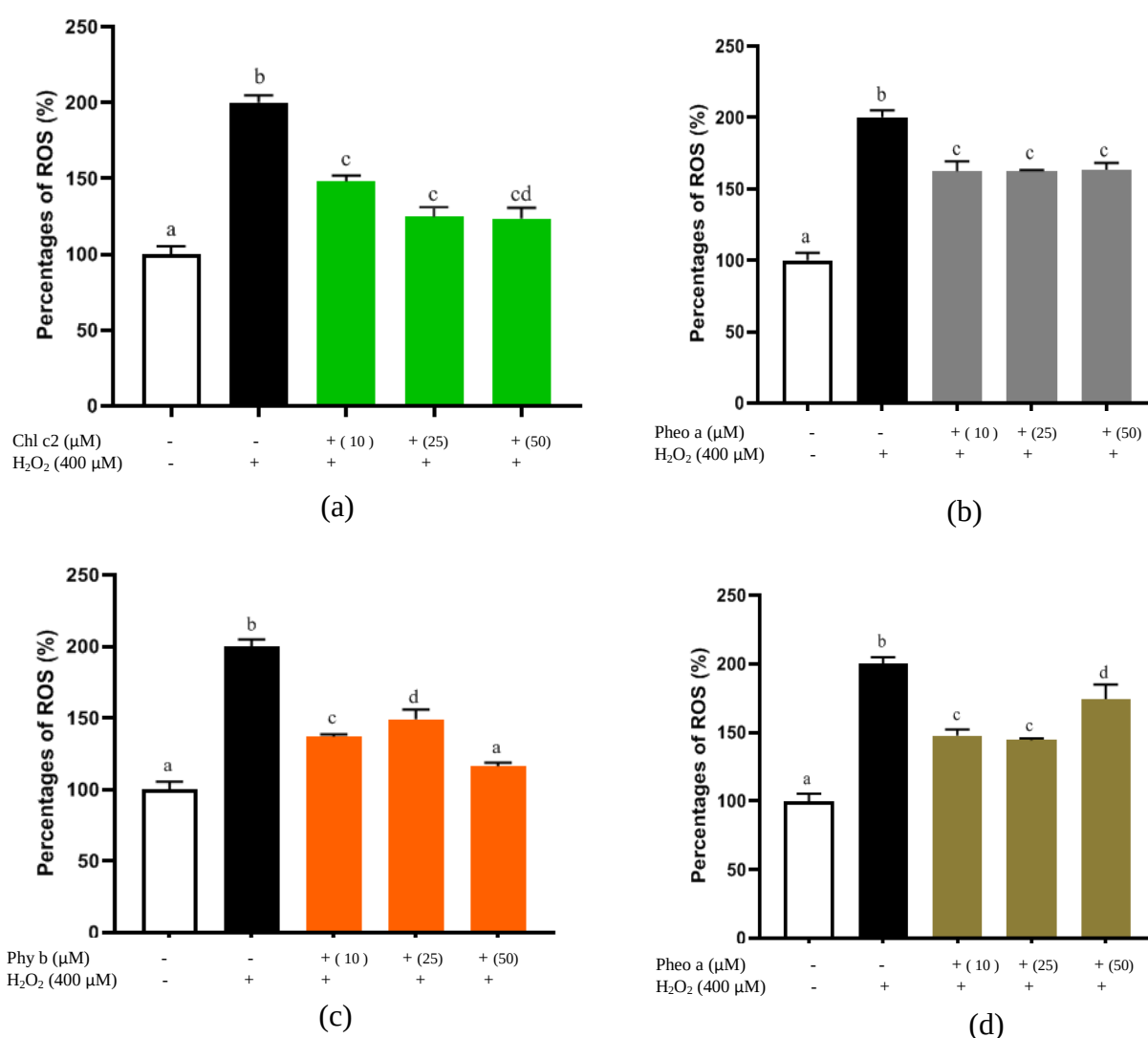


Figure 4.10. The effect of (a) Chl c2; (b) Phy a; (c) Phy b; and (d) Pheo a on the accumulation of intracellular ROS in HepG2 cells after 24 h incubation with 400 μM . Intra-hepatocellular was monitored by using DCFH2-DA fluorescence method as describe in methods. Each value is expressed as the mean $\pm$ SD ($n=3-4$). Different letters above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

The addition of CDs was able to reduce the ROS productions in structurally and dose-dependent manners as shown in **Figure 4.10**. Furthermore, the pre-incubations with CDs were effectively used to reduce ROS in HepG2 cells compared to H₂O₂ treatments.

4.3.8. MDA formation

The HepG2 cells were exposed to oxidative stress induced by H₂O₂ treatments. Therefore, to determine the ability of cytoprotective effect to incorporated CDs resulting from scavenging of intracellular free radicals or activations of the cellular antioxidant enzyme, MDA levels in the cells and medium were measured at first. **Figure 4.11**. showed that incubations of HepG2 with H₂O₂ increased the MDA productions compared to untreated groups, while CDs effectively reduced the MDA levels. The protective effects of different concentrations of CDs on H₂O₂-induced peroxidation were also comparable in dose dependent manners. H₂O₂ were significantly ($p < 0.0001$) increase concentration of MDA compared to control cells. In contrast, the supplementation of CDs 24 h prior to H₂O₂ exposure reduced the level of MDA and show the protective effects of CDs. This suggest that CDs treatments able to inhibits MDA productions in HepG2 cell culture.

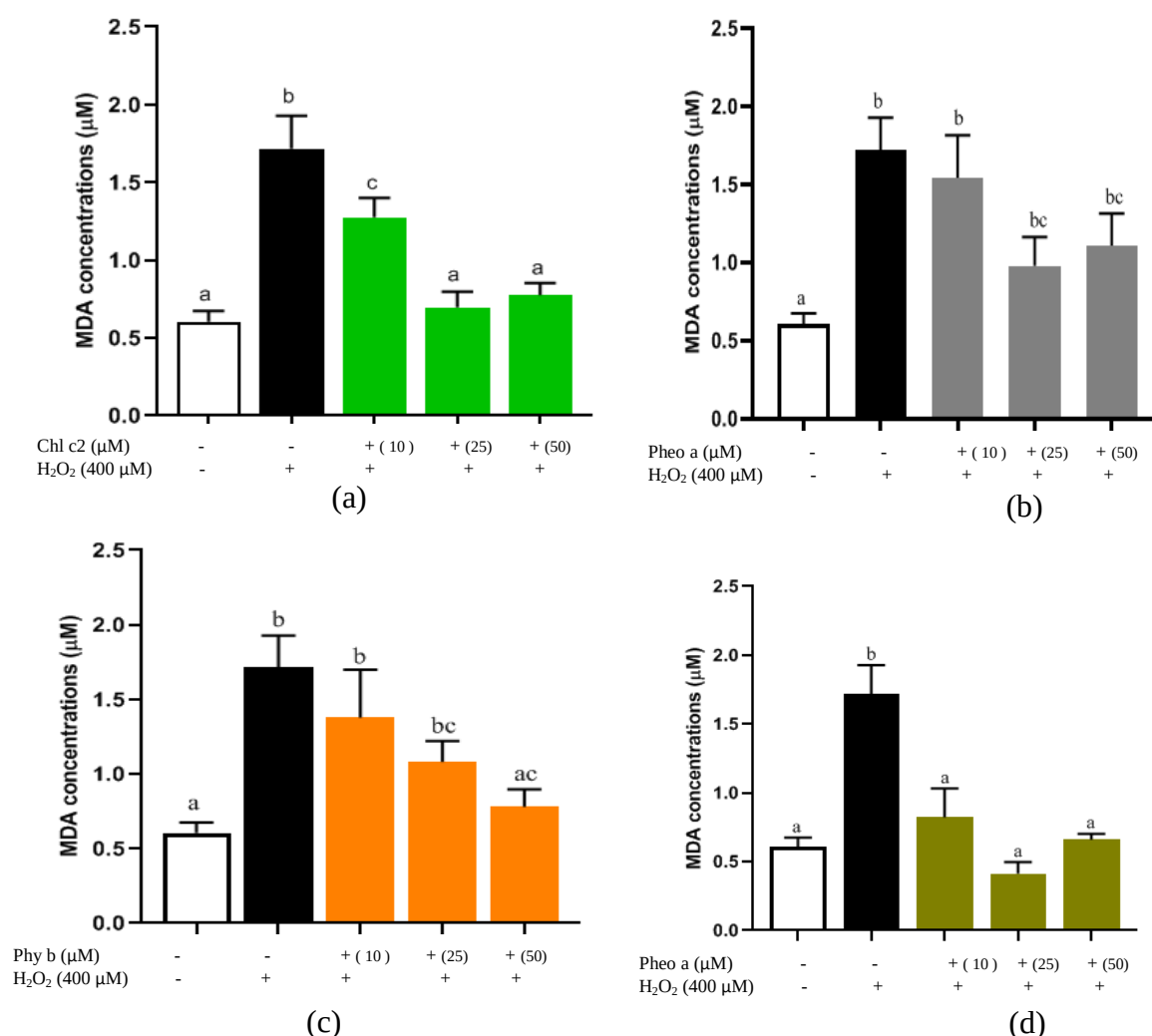


Figure 4.11. The effects of (a) Chl c2, (b) Phy a, (c) Phy b, (d) Pheo a on MDA production in H₂O₂-induced HepG2-cells. The bars shown in each graph are from a single experiment (values are means $\pm$ SD, $n = 6$ of two independent experiments). Different letters above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

4.3.9. mRNA expressions of antioxidant enzyme in HepG2 cells induced oxidative stress by H₂O₂

The function of oxidative agents depends on the balance between antioxidants and pro-oxidant (Yi et al., 2020). To prevent possible damage of biological molecules particularly DNA, lipids, and proteins from oxidative stress, the integrated antioxidant system including

enzymatic and non-enzymatic components were activated in biological system. Several enzymes such as CAT, GSH-Px and SOD can scavenge ROS to protect cell mortality. The activities of the antioxidant enzymes in the cells can be considered as an antioxidant response (Alía et al. 2005; (Surh, Kundu, and Na 2008).

Therefore, the effect of CDs on the antioxidant enzymes activity was measured in the present study by analysing the mRNA expression of each enzyme. As shown in **Figure 4.12.-4.14.**, mRNA expressions of all antioxidant enzyme activity were downregulated by H₂O₂ treatment. On the other hand, treatment of HepG2 cells with CDs increased the mRNA expression of SOD1, GPX1, and CAT in dose-dependence manners.

The different CDs exhibited varying modulation of antioxidant enzymes activity in HepG2 cells with a significant increase in their mRNA expressions. These results suggested that the antioxidant activities of CDs were mediated through the regulation of antioxidant enzymes in HepG2 cells as well as cellular activity of antioxidants compounds.

These results showed that H₂O₂ induced a strong oxidative stress response and ROS generation which inhibits the expression of antioxidant enzymes. Furthermore, there is an increase of antioxidant enzyme in the cells pre-treated with CDs against a reduction of antioxidant enzymes in HepG2 cells exposed H₂O₂.

After treatment for HepG2 cells with CDs in variations of 0, 10, 25, and 50 µM and 400 µM H₂O₂ for 24 h, the mRNA expression of SOD1, GPX1, and CAT increased in dose-dependence manners of CDs as shown in **Figures 4.12.-4.14.** After being pre-treated with CDs of 10, 25, and 50 µM relative mRNA levels of SOD-1, GPX-1, and CAT were suppressed compare to control.

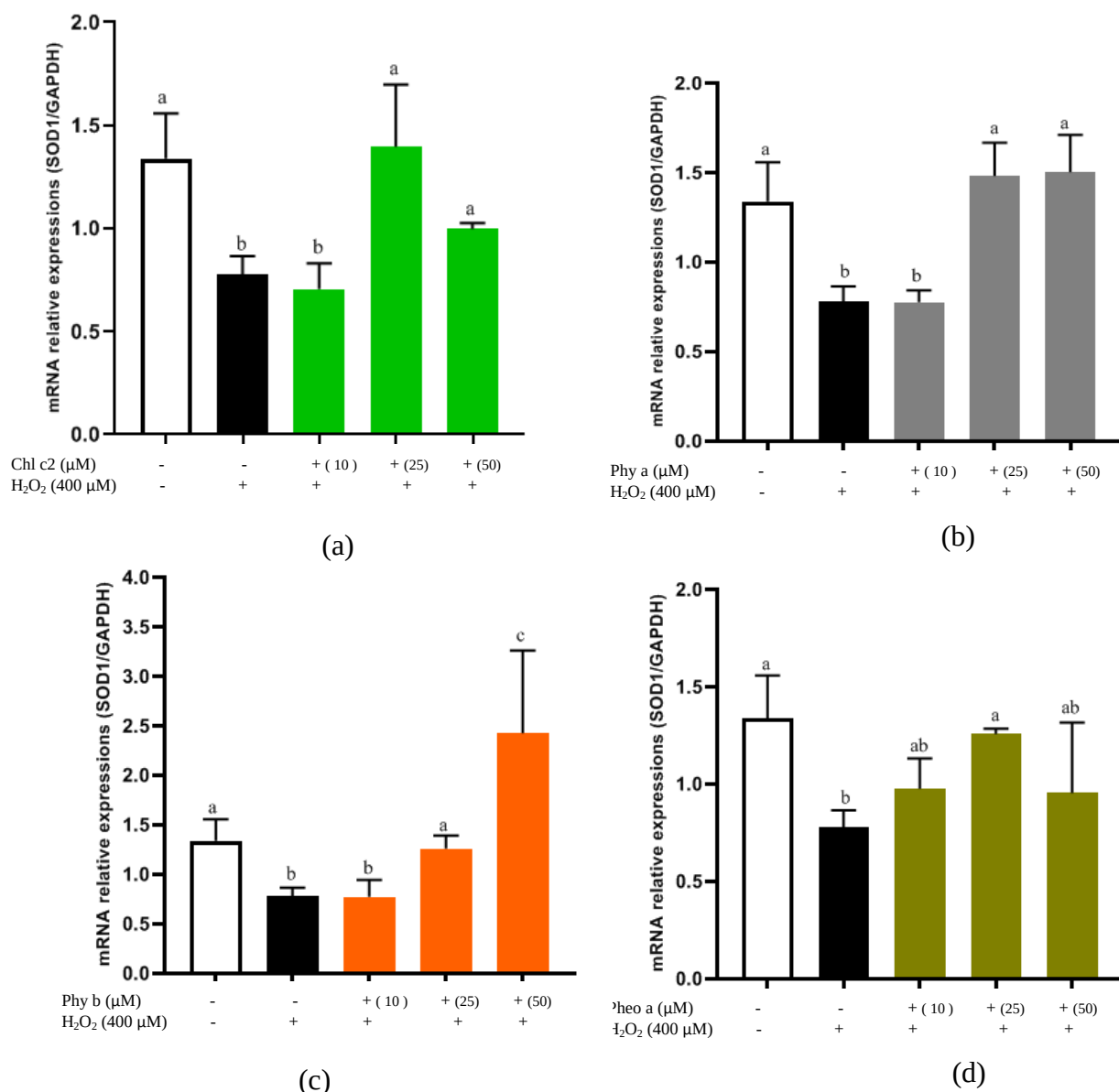


Figure 4.12. The mRNA expression of SOD1 in HepG2 cells after induction treatment with 400 μM of H_2O_2 for 24 h. HepG2 cells pre-treated with (a) Chl c2; (b) Phy a; (c) Phy b; and (d) Pheo a with the range concentration of each Chls 0-50 μM . Each mRNA level was normalized to that of GAPDH and expressed relative to the control level. Data were expressed as mean $\pm$ SD. Different letter above bars indicate a significant difference at $p < 0.05$ among each value determined by Tukey post hoc test.

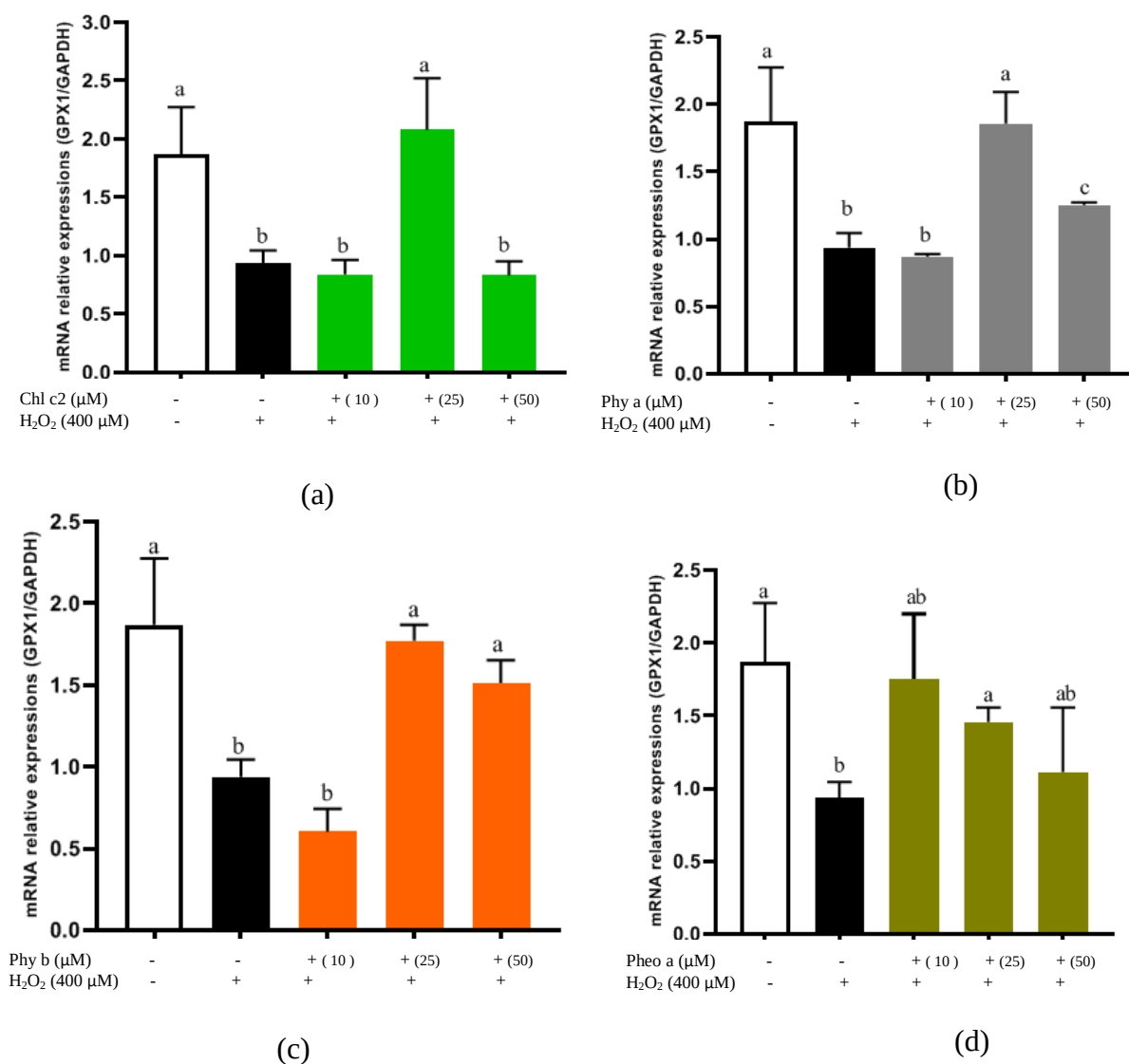


Figure 4.13. The mRNA expression of GPX1 in HepG2 cells after treated with 400 μM of H_2O_2 for 24 h. HepG2 cells pre-treated with (a) Chl c2; (b) Phy a; (c) Phy b; and (d) Pheo a with the range concentration of each Chls 0-50 μM . Each mRNA level was normalized to that of GAPDH and expressed relative to the control level. Data were expressed as mean $\pm$ SD. Different letter above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

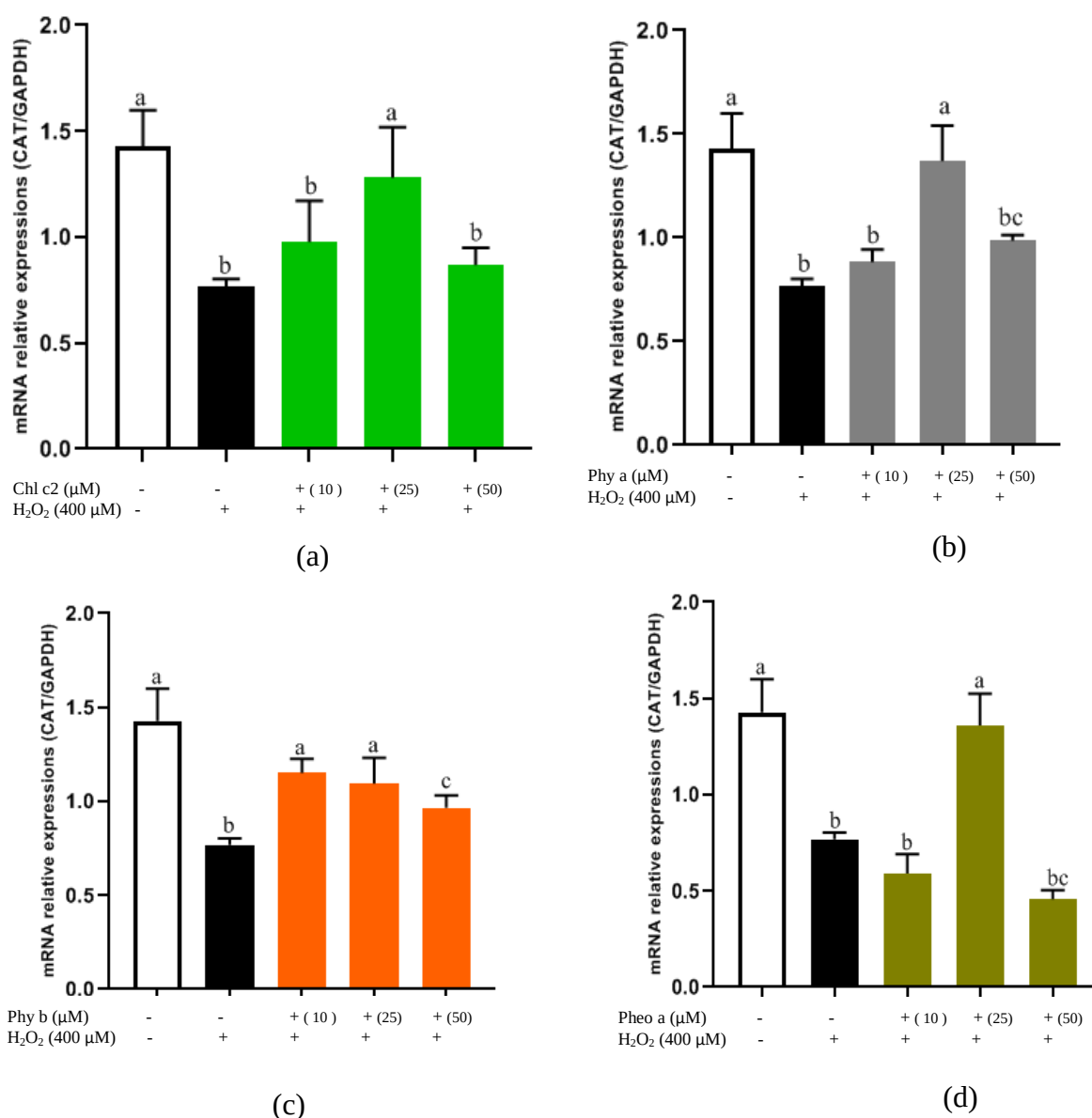


Figure 4.14. The mRNA expression of CAT in HepG2 cells after treatment with 400 μM of H₂O₂ for 24 h. HepG2 cells pre-treated with (a) Chl c2; (b) Phy a; (c) Phy b; and (d) Pheo a with the range concentration of each Chls 0-50 μM. Each mRNA level was normalized to that of GAPDH and expressed relative to the control level. Data were expressed as mean ± SD. Different letter above bars indicate a significant difference at $p < 0.05$ among each value determined by *Tukey post hoc test*.

The different CDs exhibited varying modulation of antioxidant enzymes in HepG2 cells with a significant increase in the expressions of antioxidant enzymes compared to H₂O₂

alone treatments. These results suggested that the antioxidant activities of CDs were mediated through the regulation of antioxidant enzymes in HepG2 cells as well as cellular activity of antioxidants compounds.

4.4. Discussion

Oxidative stress is a key mechanism in the etiology of liver disease and damage. Some treatments are able to repair oxidative stress through the consumption of natural antioxidants (Fiedor and Burda 2014; Guerin, Huntley, and Olaizola 2003; Hughes 2000; Pérez-gálvez et al. 2020). Natural pigments can be available from vegetables, fruits, and also seaweed, and most of them are known to show antioxidants activities (Pérez-gálvez et al. 2020). Several potential molecular mechanisms for the prevention of diseases by CDs have been elucidated in their anti-inflammatory and anti-cancer action (Kang et al. 2018; Lin et al. 2013; Subramoniam et al. 2012; Choi and Kim 2020; Dashwood 1997). However, there is limited information about their cellular antioxidant activity and its mechanism in reducing oxidative stress in HepG2 cells.

This study demonstrates that CDs from seaweeds and algae have the ability to protect human liver cells against oxidative stress by inhibiting ROS generation and MDA production, and modulating mRNA expressions of antioxidant enzymes. The CDs used in this study exert antioxidant activity, both chemical- and cellular-based, by directly quenching the ROS and activating the cellular antioxidant enzyme (**Figure 4.2.- 4.4.; 4.12-4.14.**). The DPPH radical scavenging ability of CDs were higher than those on ABTS radicals, and it is comparable to those found in CDs extracted from spinach (Hsu et al. 2013). This implies that seaweed CDs are potent as an alternative source of antioxidants.

Surprisingly, our CDs extracted from seaweeds not only showed potent antioxidant activity in a chemical-based system but also in a cellular one (HepG2 cells), and Pheo a

displayed higher antioxidant activity. The antioxidant activity, both chemical- and cellular-based, is related to structure. Structures with an absence of a phytol tail displayed higher antioxidant capacity than did those with a phytol tail. The basic porphyrin and central metal ion in the structure of CDs is crucial in the reduction of free radicals, which are important for antioxidant activity (Endo, Usuki, and Kaneda 1985; Endo, Usuki, and Kaneda 1985). The bioactivity of CDs is similar to that reported in other pigments, such as phycobiliprotein and carotenoids, in the prevention of scavenging active oxygen (Hsu et al. 2013; Lanfer-Marquez et al. 2005), anti-inflammatory (Kang et al. 2018; Lee et al. 2017; Lin et al. 2013; Okai and Higashi-Okai 1997b; Subramoniam et al. 2012), anti-oxidative stress (Park et al. 2007; Wang and Wink 2016), and anti-cancer activities (Dashwood 1997).

The cellular antioxidant activity is related not only to the structure but also to the bioavailability of CDs for HepG2 cells. The antioxidant compounds need to be absorbed in the cells before scavenging free radicals (Zhang *et al.*, 2017). Our study has revealed that CDs were absorbed in HepG2 cells, which is in concordance with a previous study by Viera *et al.*, (2018). Direct antioxidant activity of CDs needs higher levels of accumulation/bioavailability in HepG2 cells in order to react ROS. Chemical-based antioxidants could play a direct role in radical scavenging, while cells are protected by an endogenous defense antioxidant that maintains oxidative stress (Chen *et al.*, 2017).

Our study showed that all CDs do not show antioxidant activity in high doses, since elevated CDs may also act as pro-oxidants in HepG2 cell cultures. Thus, we selected the range of CDs after cell viability assays showed that 0 – 100 μ M did not alter HepG2 cell viability. However, after the preliminary evaluation with 100 μ M of all CDs for protection from oxidative stress, the 100 μ M concentrations showed reduced cell viability. In the present study, pre-treatment with CDs prior to oxidative stress induced by H₂O₂ indicated that cells

treated with CDs were fully protected against oxidative stress at concentrations of 0 – 50 μM . Therefore, the maximum concentration used was determined 50 μM for this study.

As a home of human metabolism, the liver is challenged by various pro-oxidants, such as H_2O_2 . H_2O_2 can initiate the excess of ROS formations, which causes DNA damage, reduces defense anti-oxidant activity, and produces MDA, a lipid peroxidation product. (Janero, 1990; Romero *et al.*, 1998). The evaluation of ROS as the result of oxidative stress is a good indicator of the oxidative damage to living cells (Martín *et al.*, 2008; Lushchak, 2014). A pro-oxidant, such as H_2O_2 , can generate lipid peroxides and ROS (Lismont *et al.*, 2019; Wang *et al.*, 2020). Here, the ROS generation in cultured HepG2 cells was significantly reduced after pre-treatment with various concentrations of CDs.

Intracellular ROS can be scavenged by exogenous and endogenous antioxidants. (Liang *et al.*, 2020; Zheng *et al.*, 2018). Our experiments showed that treated cells produced an excessive amount of ROS and MDA and down-regulated antioxidant defense mRNA expressions. In addition, we have recently shown that the CDs plays an important role in reducing oxidative stress by the inhibition of ROS and MDA excess. Together, these biological activities make CDs an attractive candidate for chemoprevention of oxidative stress. The liver is treated as a major site of xenobiotic metabolism instead of the main target for dietary antioxidant effects (Martin *et al.*, 2008). Natural antioxidant pigments protect the liver against oxidative damage (Kasdallah-Grissa *et al.*, 2007; Sebai *et al.*, 2010; Vardi *et al.*, 2010; Aydın *et al.*, 2016).

The reactions of ROS to PUFAs in HepG2 cells produced lipid hydroperoxide as a result of cell membrane degradation that formed aldehyde (Girrotti, 1998; Gęgotek and Skrzydlewska, 2019). MDA was produced from PUFAs by chemical and enzyme-catalyzed reactions as a product of lipid peroxidation (Marnett, 1999; Tsikas, 2017). Our results showed that the treated HepG2 cells with 400 μM H_2O_2 were able to increase MDA levels, while pre-

treatment with CDs was able to reduce MDA concentrations in HepG2 cell cultures (Menezes *et al.*, 2018; Jaweleker *et al.*, 2010). Increasing of MDA content in the cells was related to the elevation of various diseases, while reducing MDA concentration was suggest to protection from disease (Gorelik *et al.*, 2008; Han *et al.*, 2017). The CDs demonstrated in the inhibitions of lipid hydroperoxide formations were dependent on the structure (Hosina *et al.*, 1998) and central metal ions (Ferruzzi *et al.*, 2002; Lanfer-Marquez *et al.*, 2005).

The antioxidant benefits are obtained from the indirect activity of changing cell signaling and related gene transcriptions in order to increase the activity of endogenous antioxidant activity (Chen *et al.*, 2017).

Under H_2O_2 treatment, three enzymatic antioxidant mRNA expressions were significantly downregulated as compared to those that were untreated cells. Furthermore, CDs also suppressed the downregulation of antioxidant enzyme mRNA expressions by H_2O_2 . Natural compounds effectively ameliorate oxidative stress by cytoprotection of defense enzymes (Liang *et al.*, 2018). In additions, our data showed the variations of mRNA expression in their structurally and dose dependent manners. The significant upregulation of mRNA expressions of SOD1, GPX1, and CAT clearly indicates a positive response in reducing ROS and MDA levels in HepG2 cell cultures. In the other studies, using carotenoids and polyphenols protects against cell damage to prevent the modulations of mRNA expression induced H_2O_2 (Alía *et al.*, 2006; Martín *et al.*, 2008; Sun *et al.*, 2019). Lack of antioxidant enzymes makes tissue prone to damage. The liver possesses and maintains massive antioxidant machinery because it handles the oxidative metabolism of various substances. The significant upregulation of mRNA expressions of SOD1, GPX1, and CAT clearly indicates a positive response in reducing ROS and MDA levels in HepG2 cell cultures. CDs as well as carotenoids and polyphenols protect against cell damage by preventing the modulation of mRNA expression induced H_2O_2 .

Our study showed low levels of mRNA expression of endogenous antioxidant defense enzymes. In biological systems, the observed effect on mRNA expression of an enzyme would thus cause the synergistic and antagonistic effects of interaction between the enzyme and compounds present in the cellular environments (Wijeratne *et al.*, 2005). The modulations of enzymatic mRNA expressions can be considered as a biomarker of cellular antioxidant response in human cells (Virgili and Marino, 2008; Limón-Pacheco and Gonsebatt, 2009). The important function of antioxidant enzymes here is in the reduction of ROS and maintaining the cellular conditions in their appropriate redox status (Oktyabrsky and Smirnova, 2007; Martín *et al.*, 2008; Takahashi, 2012). The antioxidant enzyme systems play a crucial role in the defense against oxidative stress.

There are three forms of SOD in mammals that catalyze the dismutations of superoxide radical anion—MnSOD in mitochondria, Cu/Zn SOD in cytosol, and extracellular SOD (Alía *et al.* 2005; Zelko *et al.*, 2002; Moon Bin Yim *et al.* 1996; Röhrdanz *et al.* 2002), while GPx catalyzes GSH oxidations to oxidize glutathione (GSSG) at the expense of H₂O₂ and other peroxides (Helmy *et al.* 2000; Ighodaro and Akinloye 2018; Yang, Chan, and Yu 2006). In addition, catalase (CAT) is the antioxidative enzyme that regulates H₂O₂ concentrations in cells (Ighodaro and Akinloye 2018). SOD enzymes convert a highly reactive superoxide anion (O₂•-) into hydrogen peroxide (H₂O₂).

Antioxidant effects are among the most firmly established activities performed by CDs (Marková *et al.*, 2018). Antioxidant molecules and enzymes potentially reduce the oxidant burden of cells. Pro-oxidant generation and scavenging by antioxidant defense mechanisms is a very delicate process (Carocho and Ferreira, 2013). Antioxidant molecules and enzymes play a critical role in neutralizing the oxidant injury (Kurutas, 2016).

4.5. Conclusion

The present results showed the effectiveness of CDs prepared from seaweeds in the protection of HepG2 cells from oxidative stress induced by H_2O_2 . Furthermore, CDs upregulated the expressions of antioxidant enzymes, reducing MDA productions, and suppressing H_2O_2 excess in HepG2 cells. Further study in vitro and in vivo study will be needed to explore the protein expressions and enzyme activity in oxidative stress. This finding provides the information on the effectiveness of CDs extracted from seaweeds for health promoting effects. This is based on the reduction of H_2O_2 -induced oxidative stress by CDs.

CHAPTER V

GENERAL DISCUSSIONS

Seaweeds have been an integral part of diets in East Asia, South Asia, Europe, America, Australia, New Zealand, and other parts of the world. They are known as a source of numerous novel bioactive compounds such as lipids soluble compounds, protein, polysaccharides, and others. The consumption of seaweeds has been linked to a lower incidence of chronic diseases such as cancer, hypertension, heart diseases, diabetes mellitus, etc (Lordan et al. 2011; Brown et al. 2014; Figueiredo et al. 2016; Shannon and Abu-Ghannam 2019; Délérís et al. 2016).

Research associated with the utilization of seaweeds as health ingredients is continuously growing due to their high potency to exploits. The health beneficial effects are, in part, due to the presence of phytochemicals such as carotenoids and CDs. Among them, information on CDs is relatively less than those of carotenoids; therefore, the present study is mainly focused on CDs from seaweeds. They are the composition of CDs, their bioavailability, new biological functions and the molecular mechanism. The previous study exhibited the variations of CDs in seaweeds phyla and their absorption by using small intestine like cells, dCaco-2 cells (Chen *et al.*, 2017; Chen and Roca, 2018a). In addition, the specific protein (SR-B1) has been reported to facilitate Pheo a absorption in dCaco-2 cells (Viera, Chen, et al. 2018). There is a possibility that other lipophilic transporters are likely to be involved in the absorptions of lipophilic CDs (Susanto et al. 2019). The lipid absorption involves influx and efflux transporters with CDs comprising of low bioavailable in humans. Therefore, the effluxes transporter protects the body from the accumulations of xenobiotic in humans. The bioavailability mechanisms are important and used to set a strategy for boosting the biological activity.

Three studies were carried out to determine the availability, bioavailability, and bio-efficacy of CDs from seaweeds. The first study used 15 different seaweeds collected from temperate (Japan) and tropical (Indonesia) areas. The compositions of the lipid-soluble pigments, FAs, and α -Toc varied in seaweeds phyla and sampling locations. These results are in accordance with other previous studies (Narayan and Miyashita 2008; Nascimento et al. 2014; Nomura et al. 2013; Susanto et al. 2019).

CDs were the less exploited lipophilic compounds in seaweeds. The brown algae, displayed a higher level of CDs compared to other seaweeds phyla, thereby, making it a potential source of CDs. *S. horneri* showed a higher level of CDs which are grouped in two different families, namely Chl a and Chl c. It is one of the prospectus seaweeds widely growth in Japan (from Hokkaido to Kyushu areas), Korea, and China (Uwai *et al.*, 2009; Hu *et al.*, 2011). Aquacultures to produce these seaweeds, which are good sources of phytochemicals. Overall, these findings show that the CDs varied based on seaweeds phyla, with *S. horneri* possessing a higher level of CDs. Further study on the *in vitro* bioavailability study of seaweeds CDs in dCaco-2 cells was subsequently conducted to investigate their absorptions and molecular mechanism.

The second study focused on the bioavailability of CDs in dCaco-2 cells and their molecular mechanism because its bioavailabilities as key steps in ensuring their bio-efficacy. Therefore a better understanding of CDs bioavailability is needed.

Therefore, it is important to examine CDs bioavailability for adequate absorption of the mechanism. Furthermore, its broad multiplicity has assisted in studying its absorption rate which involves several different characteristics and mechanisms. The absorption rate of 6 different CDs was evaluated in dCaco-2 cells. Parent Chls exhibited lower absorption rates compared to those that are phytillated and dephytillated. These results might be related to the absence of Mg^{2+} and phytol tail (Chen and Roca 2018a; Ferruzzi and Blakeslee 2007;

Gallardo-Guerrero et al. 2008a). The Phy a linearly increased with increasing its concentration; therefore, this CD will be absorbed through passive diffusions. On the other hand, other examined CDs increased with increasing their concentrations, but the increase rate was saturated at higher concentrations, showing that the absorption mechanism of these CDs would be based on a facilitated uptake.

The efflux ratio was calculated in order to determine the proposed facilitated transporter involved in CDs absorptions. The efflux ratio showed 3.07 and 1.3 for Phy b and Pheo a, which defined the involvement of ABC efflux transporter. In this study, the pharmacological study showed the increasing accumulation of CDs after pre-treated with ABC transporter inhibitors. In this study, the pharmacological study using lipophilic intestinal transporter inhibitors showed a decrease in Phy b and Pheo a absorptions of dCaco-2 cells. These results indicate that Pheo a and Phy b would have similar active sites for the lipophilic transporter inhibitors. Therefore, their absorption and efflux mechanism were dominated by single facilitated transporter and involved in several facilitated processes. This data complements the previous study on the involvement of CDs absorptions (Jonker *et al.*, 2002; Viera *et al.*, 2018). Furthermore, the CDs absorption modulates membrane protein through a direct effect or their metabolites. The results of qPCR measurements of the selected transporter showed that incubation of Phy b and Pheo a affected their expressions. These results are in contrast with the lycopene absorptions study which does not modulate the expression of lipophilic intestinal transporter in dCaco-2 cells (Moussa *et al.*, 2008; 2011).

Furthermore, the result suggested that the components modulate the absorption to determine the effect on the expression levels of lipophilic transporter and ABC efflux transporter. After investigating their bioavailability, the next step is associated with understanding the bio-efficacy of CDs in HepG2 cells of the human body. This cell line is home to human metabolism and ROS production.

The third study was designed to compare the effects of the selected 4 absorbable CDs in the second project on the protection of HepG2 from oxidative stress. Different structures of CDs were used because their biological properties were closely related (Zepka *et al.*, 2019). They were also tested to determine their chemical and cellular-based activities following their effects on protecting HepG2 cells from oxidative stress.

The results showed that CDs show antioxidant activity in different assays, DPPH, and ABTS radical scavenging activities. Also, the cellular-based antioxidant represents an advanced approach for testifying physiological effects that exhibit the antioxidant activity of CDs. This study is similar to other previous research carried out by Vagliante *et al.*, (2019) which stated that the CDs tests exhibited the potential antioxidant status in HepG2 cells. The results were further compared to the findings of previous research where the antioxidant activity of CDs extracted from spinach showed a higher level in Chl b family compared to Chl a. In addition, the present study revealed that the dephytillated CDs exhibited higher level antioxidants activity which is supporting by the previous study's results (Lanfer-Marquez *et al.*, 2005; Hsu *et al.*, 2013).

Also, CDs significantly reduced the ROS and MDA levels as compared to the treated group (H_2O_2) where almost similar to the control groups. The molecular study showed that pre-treated with CDs significantly increased mRNA expressions of the enzymes antioxidative (SOD1, GPX1, and CAT) in HepG2 cells.

This study had some limitations and challenge, which tends to affect the research findings. For example in Chapter 2, only dried seaweeds were used as samples from tropical area, because it is difficult to have fresh seaweeds, while those samples from temperate area were fresh. The different sample treatment might affect the composition and quantity of each CD. Furthermore, the different times of collections influenced the pigments and FAs compositions because they were collected in varying sampling sites.

In Chapter 3, the current study only used different CDs from seaweeds to compare the absorptions rates. To obtain clear results of absorptions characteristics and mechanisms of each CDs, other kinds of are needed for discussions and conclusions. limitation of the present study is diluted CDs with DMSO in cellular absorptions. As other studies did by using oily matrix, micellar digestions, and other lipophilic carrier have been used to increase their bioavailability. CDs polarity requires the incorporation in micellar before absorptions which were incorporated in food matrixes using digesta. This is similar to the real absorptions in humans since the compounds are bonded in food matrixes.

In Chapter 4 the limitations is less information on the endogenous defences of antioxidants activity, content, and western blotting. The study powered the protective effects to determine the clear mechanism of the CDs protections.

The overall strengths of this study are as follows:

- a. It describes the usefulness of CDs in seaweeds from upstream to downstream.
- b. It used high purity of CDs from a different family which was difficult to prepare and limited commercially available.
- c. It can compare lipid-soluble compositions in two different areas and phyla which were difficult in the collections, preparations, and delivery of laboratory due to its cost and limited access.

CHAPTER VI

GENERAL CONCLUSIONS AND PROSPECTIVE

This research aims to investigate composition, *in vitro* bioavailability, and functionality of CDs isolated from seaweeds. Based on these three attributes, the finding can be concluded as follows:

1. The present study clearly shows the major factors affecting the variation of seaweed lipid bioactives such as CDs with its distribution not related to geographical locations, rather the seaweeds phyla. Among the seaweeds tested, is *S. horneri* which showed a quantitatively higher level of CDs among others.
2. The different structures of CDs showed varying absorption rates, characteristics, and pathways. The lipophilic intestinal transport was involved in the Phy b and Pheo a absorption, while lipophilic ABC intestine transporter comprises of the efflux mechanism of CDs in dCaco-2 cells. This result displayed the different mechanisms of each CD which is partly facilitated by proteins.
3. Although the previous studies showed that CDs were poorly absorbed, however, the present study provides evidence that those from seaweeds were observed into dCaco-2 and HepG2 cells. They had the ability to increase cellular antioxidant status in HepG2 cells and reduce excess ROS and MDA. This research shows that CDs modulate the mRNA expressions of defense enzymes antioxidative.

Overall, although it may be difficult to conclude the characteristics of the bioavailability and bio-efficacy of CDs completely, the present study shows important knowledge about absorption and functions of CDs to expect health benefits.

In conclusion, CDs extracted from seaweeds with a potential effect on human health can be applied in the formulations of functional foods and nutraceuticals. It can be added and

fortified in foods with numerous provide health benefits to humans and increase consumer preferences in food products.

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