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Department of Food Technology

DOCTORAL THESIS

For the requirement of the degree of Doctor in Agronomic Sciences

Submitted by:

GHALIAOUI Nora

Theme

Seaweed natural pigments: Extraction, chemical characterization and study of biological properties.

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Département de Technologie Alimentaire

THESE DE DOCTORAT

En vue de l'obtention du diplôme de Doctorat en Sciences Agronomiques

Présentée par :

GHALIAOUI Nora

Thème

Pigments naturels des algues marines : Extraction, caractérisation chimique et étude des propriétés biologiques.

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LIST OF ABBREVIATIONS

Abs	Absorbance
ATCC	American type culture collection
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
Chl *c*	Chlorophyll *c*
DMSO	Dimethyl sulphoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl.
FA	Free Acidity
FeCl3	Ferric chloride
FTIR	Fourrier transform infra-red spectrophotometer
HCl	Hydrochloric acid
IT	Induction Time
FeSO4 * 7H2 O	Iron (II) sulfate heptahydrate
FeCl3 *6H2 O	Iron (III) chloride hexahydrate
mEq. O_2/kg	Milliequivalent oxygen per kilogramme
MH	Muller Hinton
PV	Peroxide value
PVs	Peroxide values
Rf	Retention factor
SDA	Sabouraud dextrose agar
SD	Standard deviation
IC50	The concentration providing 50% inhibition
TLC	Thin Layer Chromatography
TPTZ	2,4,6-tripyridyl-s-triazine
UV	Ultra violet

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General introduction

Natural pigments are an indispensable part of human life and play an important role in food, cosmetics, medical and textile industries, mostly they are used as additives or supplements. The first time use of natural pigments can be dated back to ancient Egypt for mainly decorative applications. However, due to the deficiency of natural pigments sources and the complexity of extraction process, they did not attract much attention until 1856, when Sir Willey Henry invented the first artificial colour "mauvine", after that began the age of synthetic pigments **(Ye et *al*., 2019)**. Unfortunately, synthetic pigments causes several side effects and health risks including carcinogenic, toxic, allergenic and irritant effects for human body **(Tanveer et *al*., 2018)**. For that reason, their use decreased, and therefore, natural pigments returned to the forefront as they are perceived safer, easily biodegradable and less toxic for use in food products, pharmaceuticals, cosmetics and many other applications **(Pardilhó et *al*., 2020; Shah, 2015)**.

Natural pigments are mainly extracted from plants, but their sources and production remain limited. Since the 1960's, prostaglandin precursors produced in algae were found highly bioactive in the soft coral species *Gorgonacea* and many reports demonstrated their potential health benefits, which lead natural product chemists to move their research from the land to the sea **(Ye et *al*., 2019)**.

Two third of the world is covered by oceans, they constitute the humanity's largest repository of natural resources **(Delgado-Vargas et *al*., 2000b; Ye et *al*., 2019)**. Seaweeds are the most abundant attached marine plants in the ocean. Most of them are green (Chlorophyta), brown (Phaeophyta) and red algae (Rhodophyta). Each group is characterized by specific combinations of photosynthetic pigments **(Yee, 2010)**.

In recent years, the importance of seaweed as important sources of bioactive natural substances and of functional ingredients has been well recognized due to their potential health effects. Therefore, a new trend on isolation and investigation of novel bioactive compounds from seaweed has emerged. In fact one particular interesting feature in seaweeds is their richness in natural pigments **(Pangestuti & Kim, 2011)**. There are three types of naturals pigments in seaweeds, namely chlorophyll, carotenoids and phycobiliproteins, which exhibit colours raging from green, yellow, brown to red **(Rozi et *al*., 2014)**.

In addition to their role in photosynthesis and pigmentation, seaweed natural pigments have also been reported to provide health benefits such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities, therefore, various pigments isolated from marine algae have found new safe applications in food, cosmetic and pharmacology fields **(Pangestuti & Kim, 2011).**

Against this background, the main objective of this doctoral dissertation was the extraction, the characterization and the evaluation of biological activities of natural pigments extracted from three brown seaweeds harvested in Algerian coast: *Padina sp, Sargassum vulgare* and *Phyllaria reniformis.* Then, the second objective was to investigate the effect of preprocessing on quality and quantity of extracted pigments with emphasis to their antioxidant activity. The third objective was to assess the potential application of the extracted pigments as healthy preservative in food applications. Thus, seaweed pigment extract with the highest antioxidant activity was added to vegetable oil and its antioxidant effect was investigated. This dissertation enters in the general topics investigated by team 3 "Valorisation des biopolymères d'agroressource locales" in the laboratory "Produits bioactifs et valorisation de la biomasse" in Ecole Normale Supérieure de Kouba, Algiers, Algeria. Some parts of experimental work was also achieved in Ecole Nationale Supérieure des Sciences Agronomiques et in Centre de Recherche sur les Analyses Physico-chimiques.

For this purpose, this dissertation was organized in two main parts literature review and experimental work, each part was divided in three chapters. Figure a summarizes the structure of this dissertation.

In the **literature review**, the first chapter reviews the three main families of seaweeds (red (Rhodophyceae), green (Chlorophyta), and brown algae (Phaeophyceae)) and the classes of metabolites produced by seaweeds as well as their bioactivities. The second chapter focuses and summarizes the main natural pigments derived from seaweeds with emphasis to their biological activities as well as their potential applications in foods and other many areas. The third chapter reports an overview of seaweeds studied in Algeria and a monography of the three seaweeds selected for this study: *Padina sp, Sargassum vulgare* and *Phyllaria reniformis*.

In the experimental part, three brown seaweeds were harvested from Algerian coast namely *Padina sp, Sargassum vulgare* and *Phyllaria reniformis*. and their natural pigments were extracted, chemically characterized by HPLC, UV-Visible spectrophotometry and ATR-

FTIR spectroscopy and their biological activities evaluated **(Chapter IV)**. A comparative study of pigments extraction quality and quantity within the three species was carried out. *Phyllaria reniformis* pigment extract showed the highest content of chlorophylls and carotenoids and was found to be the most potent antioxidant. Therefore, this brown seaweed was selected for the following studies **(Chapter V and VI)**.

The **fifth chapter** deals with the topic « Impact of freezing and drying preprocessing on pigments extraction from the brown seaweed *Phyllaria reniformis* collected in Algerian coast». This chapter was performed to find out the most efficient preprocessing method for pigment extraction.

In the **last chapter (Chapter VI),** the oxidative stability of soybean and sunflower oils enriched with pigment extract of *Phyllaria reniformis* was investigated.

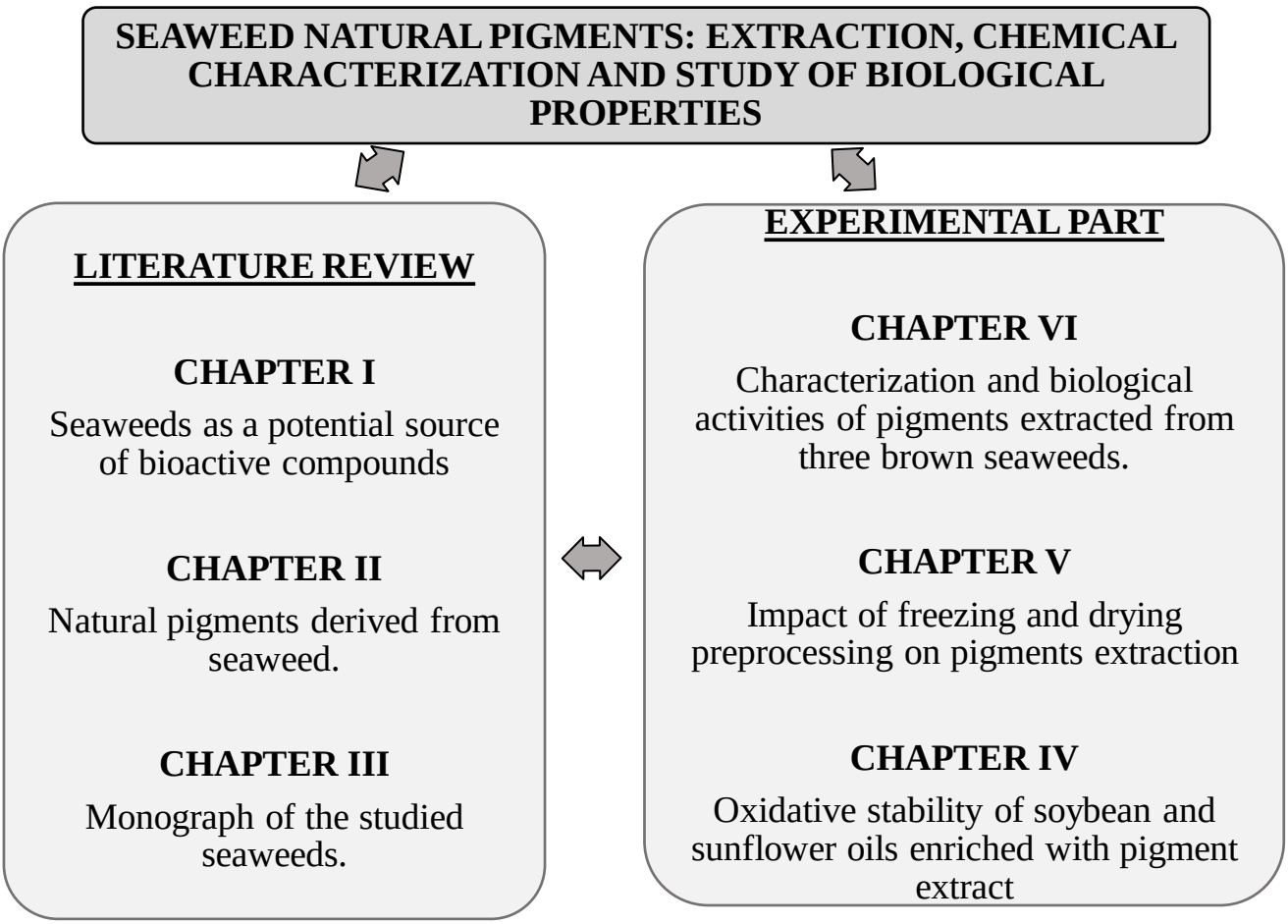


Figure a: Schematic overview of the chapters in this doctoral thesis.

PART 1
LITERATURE REVIEW

Chapter I

Seaweeds as a potential source of bioactive compounds

Chapter II

Natural pigments derived from seaweed.

Chapter III

Monograph of the studied seaweeds.

CHAPTER I

Seaweeds as a potential source of bioactive compounds

I.1. Introduction

The oceans cover more than 70% of the Earth's surface and contain a variety of marine species constituting approximatively half of the know worldwide biodiversity **(Se-kwon Kim & Wijesekara, 2010; Swing, 2003)**. This vast marine diversity is potential source of various functional ingredients such as polysaccharides, bioactive peptides, polyunsaturated fatty acids, minerals, natural pigments, vitamins, and enzymes **(Shahidi, 2008; Shahidi & Janak Kamil, 2001)**. Among marine organisms, marine algae are still identified as under-exploited plant resources although they have been used for thousands of years in China, Korea, and Japan and in all over the world for various food and non-food applications **(Heo et *al*., 2009; Pangestuti & Kim, 2011; Tiwari & Troy, 2015)**.

The term marine algae generally refers to marine macroalgae or seaweeds **(Pangestuti & Kim, 2011)**, they are mostly photosynthetic organisms **(Schmid, 2016)** with big morphological, taxonomical, and phylogenetic differentiation **(Baldauf, 2008; Norton et *al*., 1996)**. Macroalgae are taxonomically divided into red (Rhodophyceae), green (Chlorophyta), and brown algae (Phaeophyceae).

In recent years, several studies showed that marine algae are important sources of bioactive natural substances directly related to modulating chronic disease as shown in Figure I.1 **(Pangestuti & Kim, 2011)**. Several bioactivities of algal compounds were described to date ranging from antioxidant, anticancer, anti-inflammatory, antimicrobial, antifungal, antiviral to anti-obesity, and antidiabetic activities and against specific parasites **(Stengel & Connan, 2015)**. Therefore, a new trend to isolate and identify bioactive compounds and constituents from marine algae has emerged **(Pangestuti & Kim, 2011)**.

This chapter will review the classes of metabolites produced by this biochemically rich organism as well as their bioactivity.

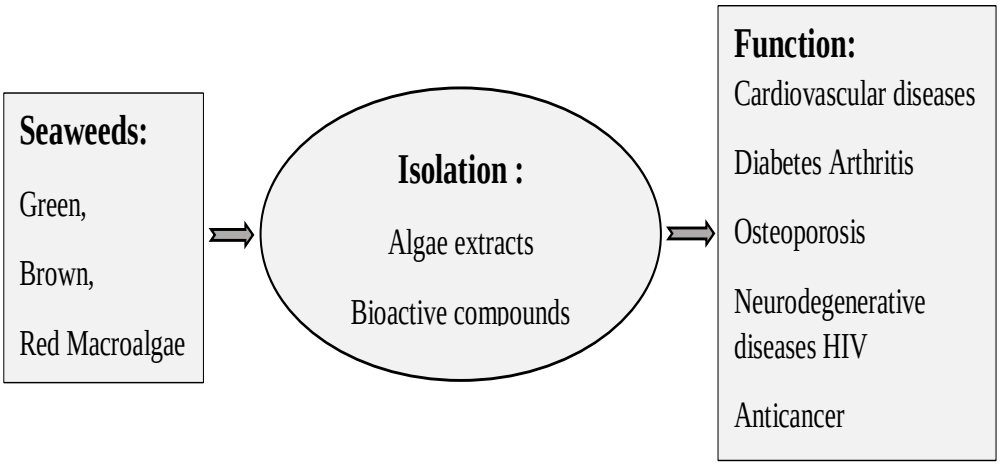


Figure I.1: Overview of seaweeds and their effect on some chronic diseases.

I.2. Seaweeds (Marine macroalgae)

Seaweeds or marine macroalgae are the oldest members of the plant kingdom, extending back to many hundreds of millions of years. They live either in marine or saltwater environment. They have little tissue differentiation comparing to plants, they did not contain roots, stems, leaves, flowers and vascular tissue **(Bocanegra et *al*., 2009; SeKwon Kim & Chojnacka, 2015)**.

Based on photosynthetic pigments, the literature agrees that marine macroalgae can be classified into three groups: green algae commonly known as Chlorophyta, brown algae or Phaeophyta and red algae also called Rhodophyta **(Garson, 1989; R. K. Gupta & Pandey, 2007; L. Pereira, 2016)**. This differentiation is traditionally based on differences in pigmentation but further research has shown that they also differ in biochemical composition, structural features, and life cycle **(Stengel et *al*., 2011)**.

In response to different kinds of environmental stress, marine algae developed defense strategies that resulted in a significant level of structural chemical diversity, from different metabolic pathways **(Barros et *al*., 2005)**.

Green seaweed (Chlorophyta): The green color characteristic of this algae is mainly due to the presence of chlorophyll a and b in the same amount like plants **(Husin, 2014; Se-kwon Kim, 2012)**.

Brown seaweed (Phaeophyta): Their brownish color results from the dominance of high percentages of fucoxanthin **(Husin, 2014; Se-kwon Kim, 2012; L. Pereira, 2016).**

Red seaweed (Rhodophyta): Red seaweed has reddish or purplish color that results from the dominance of phycoerythrin **(Husin, 2014; Se-kwon Kim, 2012).**

The presence of different pigments in seaweeds is related to their marine habitat. Thus, green macroalgae abound in coastal waters can absorb large amounts of light energy, while brown and red seaweeds dominate at greater depths where sunlight penetration is limited **(SeKwon Kim & Chojnacka, 2015)**.

I.3. Major bioactive compounds from seaweeds and their potential activities

Naturally, about 30 000 algae species are available for potential use in food, nutrition and bioactive resources **(SeKwon Kim & Chojnacka, 2015)**. Seaweeds contain minerals, amino acids, proteins, fatty acids, lipids, polysaccharides, dietary fibers, vitamins and various number of secondary metabolites. Many of these constituents possess high economical values and can be extracted to obtain antioxidative, anti-inflammatory, antimicrobial, anticancer, antihypertensive products **(Balboa et *al*., 2013; S. Gupta & Abu-Ghannam, 2011; J. C. Lee et *al*., 2013)**. A brief description of the most important seaweed compounds and their bioactivities will be presented in the following.

I.3.1. Proteins and amino acids

Various bioactive properties of algal proteins and peptides were reported to date by several studies such as antioxidant, anticancer, antihypertensive, anticoagulant, immunomodulatory, and antiproliferative activities **(Harnedy & Fitzgerald, 2011; Samarakoon & Jeon, 2012)**. A particular class of bioactive proteins "lectin" could be extracted from seaweed. Lectins are specific proteins able to irreversibly bind carbohydrate and are characterized by antibacterial, antiviral, anticancer, mitogenic, cytotoxic, anti- inflammatory, and antiadhesive activities. They can be found in some seaweeds species such as *Eucheuma*

serra, Ulva sp , Griffithsia sp. , Gracilaria sp. and *Boodlea coacta* **(Holdt & Kraan, 2011; Toshiyuki Mori et *al*., 2005; Sato et *al*., 2011)**.

Carnosine (β-alanyl-L-histidine) is a peptide found in *Ancanthophora dellei* (red seaweed) exhibited antioxidant activity and transition metals chelating ability **(Fleurence, 2004)** .

Seaweeds are good sources of essential amino acids. Thus, high concentrationS of glutamic acid, serine, and alanine were found in *Palmaria palmata* **(Galland-Irmouli et *al*., 1999)**. Tow amino acids : histidine and taurine with antioxidant and antihypertensive proprieties were also found in *Ulva pertusa* **(Houston, 2005; M. Zhang et *al*., 2004).**

I.3.2. Lipids

Lipids constitute 5% of dry seaweed weight, this amount can be much higher, it varies according to season, temperature, salinity and algae species. Phospholipids and glycolipids are the main classes of lipids found in algae **(Holdt & Kraan, 2011; Miyashita et *al*., 2013)**.

Seaweeds are a rich source of essential unsaturated fatty acids that have diverse activities, especially polyunsaturated fatty acids from group n-3(ω-3) and n-6 (ω-6) **(Dembitsky et al., 1990; Jamieson & Reid, 1972; Maeda, Tsukui, et al., 2008)**. The fatty acids composition in seaweeds has been explored to decrease risk of heart disease, thrombosis, atherosclerosis, they also act as anti-aging, anti-inflammatory, and regenerating agent **(Sánchez-Machado et *al*., 2004)**. While other fatty acids derived from various macroalgae, are applied in the treatment of psoriasis, eczema, hyperlipidemia, and some cancers. They are also effective against skin inflammation **(Bhaskar et *al*., 2004; Harada & Kamei, 1997; Stengel et *al*., 2011; Van Ginneken et *al*., 2011)**.

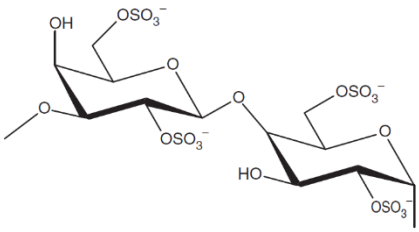
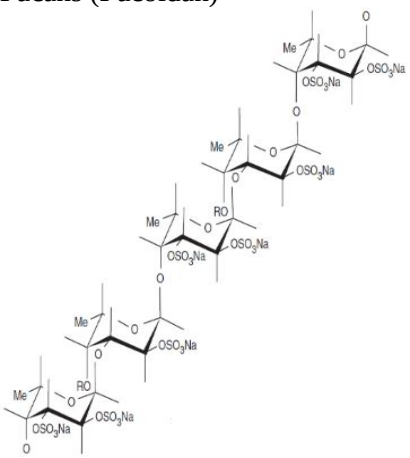
I.3.3. Sulfated polysaccharides

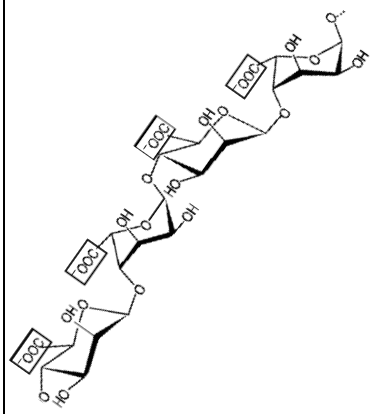
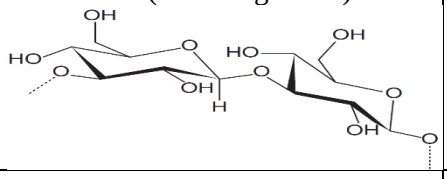
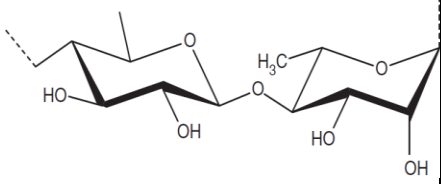
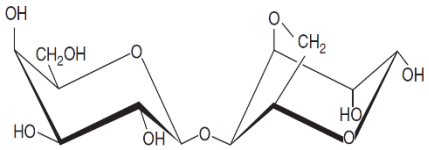
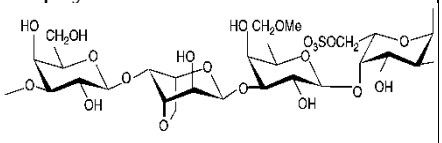
In the fields of food, biochemistry and pharmacology, sulfated polysaccharides isolated from marine algae, have attracted much more attention because of their efficiency as anti-HIV-1, antimalaria, antiparasitic, antioxidant, antithrombotic, antilipidemic, antiadhesive, anticoagulant, anti-cancer, and anti-inflammatory **agents (Hwang et *al*., 2011; Jiao et *al*., 2011; Sekwon Kim & Li, 2011; J. B. Lee et *al*., 2004; Mestechkina & Shcherbukhin, 2010;**

Wijesekara et *al*., 2011). Moreover, the inhibitory activities of algal sulfated polysaccharides against mumps and influenza virus were reported long time ago **(Deig et *al*., 1974)**.

Furthermore, a comparative study has reported the inhibition of herpes simplex virus and other viruses by polysaccharide fractions from various seaweed extracts. It is proposed that polysaccharides of 10 red algae are quite efficient in disrupting the viral peptide attachments that are supposed to be highly preserved in the drug-resistance mutation process. Therefore, polysaccharides are directed to affect these peptides as potential anti-HSV targets **(Ehresmann et *al*., 1977)**. More recently, **Wittine et *al*. (2019)** reported the human immunodeficiency virus (HIV) inhibiting potential activity of several polysaccharides extracted from seaweed. Thus, fucoidans isolated from three different brown seaweeds, exhibited ability to inhibit early steps of HIV infection. The negatively charged sulfated polysaccharides present in the alga cell wall exerted an antagonist effect with the HIV entry into cells. Table I.1 summarizes the major types of sulfated polysaccharides isolated from green, brown and red seaweeds and their biological activities.

Table I.1: Major sulfated polysaccharides isolated from seaweeds and their biological activities according to diverse studies reported in the literature

Seaweed	Sulfated polysacharides	Bioactivities	Reference
Green seaweed	Ulvans 	Antioxidant, Antiviral	**(Alves et *al*., 2013; Kaeffer et *al*., 1999; Lahaye & Robic, 2007; Qi, Zhang, et *al*., 2005; Qi, Zhao, et *al*., 2005)**
Brown seaweed	Fucans (Fucoidan) 	Antitumor, Anticoagulant Antithrombin Antiviral,	**(Bernardi & Springer, 1967; Berteau & Mulloy, 2003; Juan et *al*., 2008; E. J. Kim et *al*., 2010; B. Li et *al*., 2008; Pomin & Mourão, 2008; Queiroz et *al*., 2008; Springer et *al*., 1956; Wijesinghe & Jeon, 2012)**

	Alginates (both acid and salt forms) 	Immunization against virus. Treatment of esophagitis and urolithiasis, Cholesterol lowering, Antihypertensive Preventing absorption of toxic substances; Blood glucose regulating	**(Alves de Sousa et *al.*, 2007; Draget & Taylor, 2011; Goh et *al.*, 2012; K. Y. Lee & Mooney, 2012; Torsdottir et *al.*, 1991)**
	Laminarins (units of glucose) 	Antitumor,	**(Miao et *al.*, 1999; Vera et *al.*, 2011)**
Red seaweed	Carrageenan 	Anticoagulant, Antithrombotic Antiviral, Antitumor Immunomodulatory, hypocholesterolaemic Antiherpetic Anticoagulant	**(Buck et *al.*, 2006; Burges Watson, 2008; Campo et *al.*, 2009; Carlucci et *al.*, 1997; Necas & Bartosikova, 2013; Panlasigui et *al.*, 2003; Thomson & Fowler, 1981; Wijesekara et *al.*, 2011)**
	Agar (Agarobiose units) 	Antitumor Antioxidant Hypoglycemic Antiaggregating effect on red blood cells. Hepatoprotective,	**(H. M. Chen et al., 2005; T. Enoki et al., 2010; Fernández et al., 1989)**
	Porphyran 	Anticancer, Antioxidant, Antiaging Antiviral Antibacterial Anti-inflamator	**(Bhatia et al., 2008; Isaka et al., 2015; Kwon & Nam, 2006; Morrice et al., 1984; Venkatpurwar et al., 2011; Z. Zhang et al., 2009)**

Generally, biological activities of sulfated polysaccharides are dependent to their sugar composition, sulfate content, species and environmental factors **(SeKwon Kim & Chojnacka, 2015)**.

I..3.4. Vitamins

Similar to many vegetables, seaweeds contain both water and fat-soluble vitamins. The particularity of seaweed is the presence of vitamin B12, which is rare in vegetables. The vitamin composition of seaweed is variable, it depends on species, location, season, sea temperature, light, and salinity **(Den Berg et *al*., 1988; Guven et *al*., 1976; Ito & Hori, 2009; MacArtain et *al*., 2007; Škrovánková, 2011; Watanabe et *al*., 1999, 2000; Shoji Yamada et *al*., 1996)**. Besides to their biochemical functions and antioxidant activities, seaweed derived vitamins possess other health benefits such as reducing hypertension, preventing cardiovascular disease and reducing the risk of cancer **(Pereira, 2016; Škrovánková, 2011)**.

I.3.5. Phenols and phlorotannin

Phenolic compounds especially polyphenols and tannins extracted from seaweeds possess antimicrobial activities **(Glombitza, 1977)**. Bromophenols were isolated for the first time by **Hodgkin et *al*., (1966)** from the red seaweed *Polysiphonia lanora* and their antibacterial properties were demonstrated**.**

Eckol and eckol derevatives are polyphenolic compounds isolated from the brown alga *Ecklonia cava*, they demostrated antimicrobial and cytoprotective effect against oxidative stress **(Kang et *al*., 2005; Kang et *al*., 2013; Kim et *al*., 2014; MengYa Zhang et *al*., 2019)**.The structure of eckol is shown in Figure I.2.

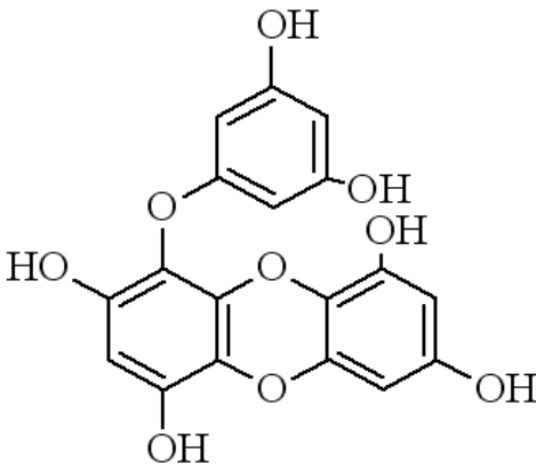


Figure I.2: Structure of Eckol which was isolated from *Ecklonia cava* (Yee, 2010).

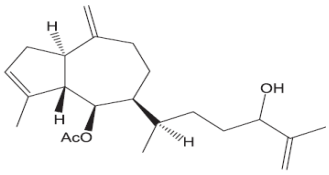
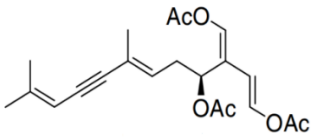
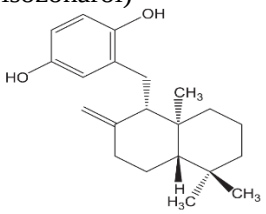
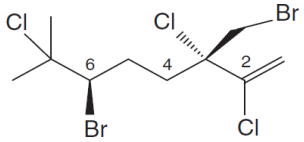
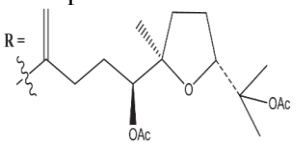
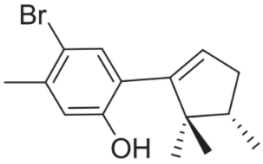
Because of their antioxidative properties, seaweeds' polyphenol may be successfully used as curative and preventive agents for treatment of numerous diseases. They act as anticancer agents, exhibit anti-inflammatory, antioxidant and antiproliferative activities. Polyphenols isolated from seaweed are known to protect the nervous and cardiovascular systems, they decrease blood glucose and limit diabetes occurrence and they are effective in the fight against obesity **(Cha et *al*., 2016; Kang et *al*., 2003; Sang Hoon Lee et *al*., 2009; Seung Hong Lee et *al*., 2010; Tadashi Mori et *al*., 2014; Murray et *al*., 2018; Namvar et *al*., 2012, 2013; Nwosu et *al*., 2011; O'Sullivan et *al*., 2011; Vijayabaskar & Shiyamala, 2012)**. **Yuan and Walsh, (2006)** proved that Laminaria and Porphyra sp. algae could reduce the risk of occurrence of mammary gland and intestine cancer.

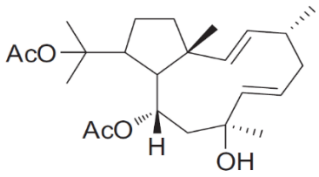
Besides, the methanol extract of brown seaweed is known to contain a large amount of phlorotannins (tannin derivatives) with bioactive proprieties such as antioxidant, antibacterial, anti-inflammatory, anti-HIV, antidiabetic, antiallergic and anti-matrix metalloproteinase activities **(Ahn et *al*., 2004; Eom et *al*., 2012; Erpel et *al*., 2020; Kim et *al*., 2006; Seung Hong Lee & Jeon, 2013; Nagayama et *al*., 2002; Nakamura et *al*., 1996).** Phlorotannin act as hypoglycemic agent, improve sensibility and secretion of insulin **(Seung Hong Lee & Jeon, 2013; Lopes et *al*., 2017; Zhao et *al*., 2017).**

I.3.6. Terpenes and terpenoids

Terpenes (diterpenes, triterpenes, tetraterpenes, hemiterpenes, and sesquiterpenes) are formed based on an isoprene structure and when they contain additional oxygen, they are termed terpenoids. Seaweeds contain many types of terpenes and terpenoids with several biological activities **(Fenical & Paul, 1984; Paul & Fenical, 1983)**. Table I.2 shows terpenes and terpenoids isolated from seaweeds and their biological activities. Brown algae of the genus *Dictyota* and *Dictyopteris* are a rich source of diterpenes and sesquiterpenes such as dictyols, dolabellane **(Manzo et *al*., 2009)** and zonarols **(Shimizu et *al*., 2015)** with several interesting biological activities including cytotoxic, antiviral, antifungal and antibacterial properties. On the other hand, red algae *Laurencia* genus are well known as secondary metabolites producers, mainly terpenoids. Also, the green algae *Caulerpa prolifera* contain sesquiterpenes (Caulerpenyne) that exhibited antibacterial, cytotoxic activities **(Yee, 2010)**.

Table I.2: Terpenes and terpenoids found in seaweeds and their bioactivities

Seaweed	Terpenes/terpenoids	Biological activities	References
Dictyota dichotoma var.implexa, *Dictyota menstrualis* (Brown algae)	Diterpenes (Dictyols) 	Antibacterial activity Inhibit virus replication	**(De-Paula et *al.*, 2012; N. Enoki et *al.*, 1983; Manzo et *al.*, 2009; H. S. Pereira et *al.*, 2004)**
Caulerpa prolifera (Green algae)	Sesquiterpene (Caulerpenyne) 	Antibacterial activity Cytotoxic on tumor cells Potent towards human nasopharyngeal carcinoma cells	**(Amico et *al.*, 1978; Bourdron et *al.*, 2006; Yee, 2010)**
Dictyopteris zonarioides, *Dictyopteris undulata* (Bown algae)	Sesquiterpene (Zonarol and isozonarol) 	Antifungal activity Prevents inflammation and Apoptosis Provides neuroprotection	**(Fenical et *al.*, 1973; K. Mori & Komatsu, 1986; Shimizu et *al.*, 2015; Sohsuke Yamada et *al.*, 2014)**
Portieria hornemannii ,*Plocamium cartilagineum*, (Red algae)	Polyhalogenated monoterpenes 	Antitumor activity Insecticide and acaricide activities	**(Fuller et *al.*, 1992, 1994; Mynderse & Faulkner, 1975; Shilling et *al.*, 2019)**
Laurencia obtuse (Red algae)	Triterpenoids 	Potential cytotoxic activity Anticancer agent	**(Gamal, 2010; Y. X. Li et *al.*, 2013; Suzuki et *al.*, 1987)**
Laurencia okamurai (Red algae)	Laurane-type sesquiterpenoids 	Antimicrobial activity	**(X. L. Li et *al.*, 2015)**

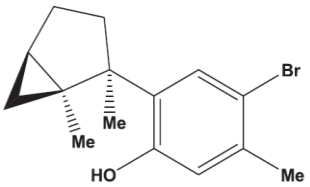
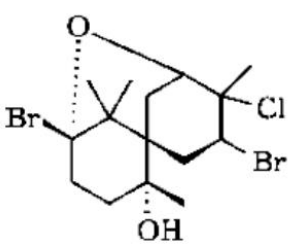
Dictyota pfaff, Dilophus Fasciola (Brown algae)	*Dolabellane diterpene* 	Antiviral activity	**(Barbosa et *al.*, 2003, 2004; J. Chen et *al.*, 2018; Cirne-Santos et *al.*, 2006; De Rosa et *al.*, 1984; H. S. Pereira et *al.*, 2004)**

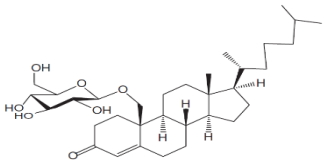
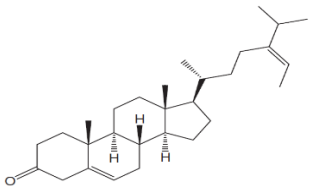
I.3.7. Steroids

Steroids are compounds possessing a characteristic tetracyclic carbon skeleton, named as perhydrocyclopenteno phenanthrene nucleus ore sterane **(Khan, 2010)**. Marine macroalgae are a good source of steroids. Therefore, a number of steroids have been isolated from various red, green and brown seaweeds. The red seaweed genus *Laurencia* is prolific source of steroids, including laurinterol and pecifenol. Laurinterol was reported as antiprotozoal and antiparasitic agent, while, pecifenol could be exploited for its antimicrobial, anti-allergic and inflammatory effects **(Arberas-Jiménez et *al.*, 2020; D'Orazio et *al.*, 2012)**.

Table I.3 summarizes the main steroid components extracted from seaweeds and their bioactivities.

Table I.3: Main steroids found in seaweeds and their bioactivities

Seaweed	Steroids	Biological activities	References
Laurencia intermedia, Laurencia okamurai, Laurencia johnstonii (Red algae)	Laurinterol 	Antibacterial activity Anticancer activity Antitumoral activity	**(Garcia-Davis et *al.*, 2019; García-Davis et *al.*, 2020; Irie et *al.*, 1970; M. M. Kim et *al.*, 2008)**
Laurencia sp (Red algae)	Pacifenol 	Antimicrobial activity Anti-inflammatory action	**(Compagnini & Toscano, 1986; D'Orazio et *al.*, 2012; San-Martín et *al.*, 2008; Sims et *al.*, 1975)**

Peyssonnelia sp (Red algae)	Sterol glycosides 	Inhibit the growth of human cancer cells	**(Lin et *al*., 2010)**
Tydemania expeditionis (Green algae)	Steroids 	Anticancer (prostate)	**(J. Zhang et *al*., 2012)**

I.3.8. Alkaloids

Alkaloids are chemical compounds that contain basic nitrogen atoms and are usually derived from amino acids. Most of the alkaloids are colourless and crystalline compounds **(Yee, 2010)**.

Several alkaloids and other nitrogenous heterocyclic compounds have been obtained from seaweeds **(Güven et *al*., 2010; N'Diaye et *al*., 1994; C. R. M. Souza et *al*., 2020)**. *Caulerpa* are known as good seaweed sources of alkaloids such as caulerpin **(Figure I.3)**. Many biological activities were found in caulerpin such as anti-inflammatory, antinociceptive, anti-tumour, anti-microbial, neuroprotective and protein tyrosine phosphatase-1B inhibitory activities **(É. T. De Souza et *al*., 2009; Liu et *al*., 2013; Lucena et *al*., 2018; Mehra et *al*., 2019; Yang et *al*., 2014)**.

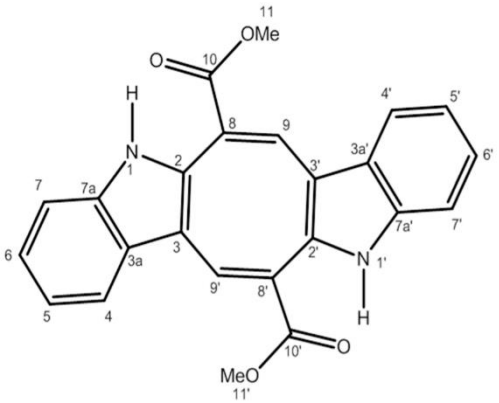


Figure I.3: The chemical structure of caulerpin (Lucena et *al*., 2018).

I.3.9. Pigments

Seaweeds are potentially a rich source of various natural pigments. The type of pigments varies among species, giving them different colours. Besides their important role in photosynthetic and pigmentation effects, these bio compounds has been explored to provide health benefits **(Pangestuti & Kim, 2011)**. Pigments, such as polyphenols, exhibit various strong bioactivities such as antioxidative, anti-inflammatory, anti-obesity, antiangiogenic, anticancer, neuroprotective and antiosteoporosis activities, that why they can be used in the treatment and prevention of numerous diseases **(Dumay & Morançais, 2016; Pangestuti & Kim, 2011)**. In the next chapter, seaweed pigments will be described in more detail.

I.3.10. Other Biologically Active Compounds

Other bioactive compounds were found in seaweeds with positive biological activities. Halogenated compounds such as indoles are the major secondary metabolites isolated from red and brown seaweeds, they are characterized by antifungal, antibacterial, and antiviral properties **(Ganesan et *al*., 2011; Marimuthu Antonisamy & Sankara Raj, 2016)**. Several enzymes with protective activities such as peroxidase, glutathione reductase, superoxide dismutase, and catalase were found in seaweed **(Jiménez et *al*., 2010)**. Saponins extracted from red seaweed exhibited many biological properties including antimicrobial and anti-inflammatory activities as reported by **Marimuthu Antonisamy & Sankara Raj (2016)**.

Diversity of bioactive compounds in seaweeds is a result of the aggressive environments in which they live **(Onofrejová et *al*., 2010)**.

I.4. Conclusion

In recent years, seaweed bio-compounds are attracting increasing attention because of their potential therapeutic effects to prevent or treat various diseases. Antioxidant, anticancer, antiangiogenic, anti-obesity, anti-inflammatory activities of different seaweed species extracts and their secondary metabolites have been extensively studied.

CHAPTER II

Natural pigments derived from seaweed

II.1. Introduction

Pigments are chemical compounds that absorb light in the wavelength range of the visible region. Pigments produce the colors that we see at each step of our lives **(Delgado-Vargas et *al*., 2000a; Hari et *al*., 1994; D. M. Pereira et *al*., 2014)**. Natural and synthetic pigments have been extensively used in various fields of daily life such as paper production, textile industries, food, pharmaceutic and cosmetic production, agricultural practices and researches, science and technology **(Kamla et *al*., 2012)**. In many countries, synthetic pigments are controversial and banned for use in food products because of safety concerns **(Tanveer et *al*., 2018)**. Because of their carcinogenic and toxic effects for human body, their use in non food applications has also decreased **(Boo et *al*., 2012).** As a result, there is an increasing demand for natural pigments as substitute of synthetic ones as they are perceived less toxic for use in food, pharmaceuticals and cosmetics products, in addition to their healthy biological activities such as antioxidant and anticancer **(Har Bhajan & Kumar Avinash, 2014)**.

Therefore, it is essential and important to explore various sources of natural pigments such as seaweeds which are known to contain a wide range of pigments with positive health benefits. Natural seaweed pigments offer many advantages such as cheaper and easy production, easier extraction and higher yields **(Pardilhó et *al*., 2020)**.

This chapter summarizes the main natural pigments derived from seaweeds with emphasis to their biological activities as well as their potential applications in foods and non-food products.

II.2. Natural pigments derived from seaweeds

Seaweeds contain a variety of natural pigment; their nature and concentration vary within species and give them their specific colours. To date, three basic classes of natural pigments were extracted from seaweeds: **chlorophylls**, **carotenoids** and **phycobiliproteins** which are all directly involved in photosynthesis**.** The major pigments present in seaweed are listed in table II.1. Chlorophylls and carotenoids are found in all classes of seaweeds as well as

in land plants, while phycobiliproteins are found only in Rhodophyta **(Ito & Hori, 2009; Schmid, 2016)**.

Table II.1: Distribution of pigments groups within seaweed classes (Bonanno & Orlando-Bonaca, 2018; Leliaert et *al*., 2012; Stengel et *al*., 2011).

Pigment group	Green seaweed	Brown seaweed	Red seaweed
Chlorophylls	Chlorophyll *a* Chlorophyll *b*, and derivatives	Chlorophylls *a* Chlorophylls *b* Chlorophyll *c*, and derivatives	Chlorophylls *a* Chlorophyll *d*, and derivatives
Carotenoids	α-, β- and γ-carotenes, Xanthophylls	Fucoxanthin β-carotene, zeaxanthin, violaxanthin	Xanthophylls α and β-carotene
Phycobiliproteins			phycocyanin, phycoerythrin, allophycocyanin,
Example of algae*	 *Caulerpa prolifera*	 *Fucus vesiculosus*	 *Palmaria palmata*

*Photos: (https://www.algaebase.org)

Green seaweeds are known to contain chlorophylls *a* (*Chl a*), chlorophylls b (*Chl b*), α-, β-, γ-carotenes and xanthophylls. Their green color is caused by the presence of *chl a*. Brown seaweeds contain *chl a*, *chl b*, chlorophylls c *(Chl c)*, fucoxanthin, violaxanthin, xanthophylls, zeaxanthin and β-carotene. They have a characteristic olive-green to dark brown color because of an abundance of fucoxanthin. Red seaweeds contain *chl a*, chlorophylls d *(Chl d)*, xanthophylls, α-carotene, β-carotene, phycocyanin, phycoerythrin and allophycocyanin. The red or pink color of this macroalga is caused by the presence of phycobilin pigments **(Dumay & Morançais, 2016)**.

II.2.1. Chlorophylls

Chlorophylls are a group of cyclic tetrapyrrolic pigments with common structures and functions, which exhibit maximum absorbance in the blue and red regions of the visible spectrum. they are the major photosynthetic greenish pigments found in algae, plants and cyanobacteria. Chlorophylls are greenish lipid-soluble pigments which contain a porphyrin ring with a central magnesium ion and usually a long hydrophobic chain. The molecular structure of the different chlorophylls varies by one or several side-chain substitutions, which affect the absorption characteristics **(Dumay & Morançais, 2016; Manivasagan et *al*., 2017; Pangestuti & Kim, 2011).**

The four major types of chlorophyll in seaweeds are *Chl a, Chl b, Chl c* and *Chl d.* These structures are constituted by a tetrapyrrole and a phytol chain, which can be short (*Chl c)* or long (*Chl d)* **(Benoît & Stéphane, 2018)**. An overview of the different chlorophylls present in seaweed taxonomic classes is shown in Table II.2. *Chl a* is present in all classes of seaweeds, while *Chl b* was detected only in green seaweeds and *Chl c* only in brown seaweeds, however *Chl d* was only found in red algae **(Ito & Hori, 2009)**.

Table II.2: Distribution of chlorophylls in Seaweeds (Ito & Hori, 2009).

Chlorophylls	Green seaweed	Brown seaweed	Red seaweed
a	+	+	+
b	+	-	-
c	-	+	-
d	-	-	*
e	-	-	-

*+: present, -: absent, *: Sometimes not detected*

- ***Chlorophyll a:***

This pigment is present abundantly in marine environment and always found in all seaweeds, it absorbs most energy from wavelength of violet blue and orange red light. This blue green color pigment has maximum absorbance from 660 to 665 nm. *Chl a* plays an important role in photosynthesis **(Dumay & Morançais, 2016; Holdt & Kraan, 2011; Hosikian et *al*., 2010)**.

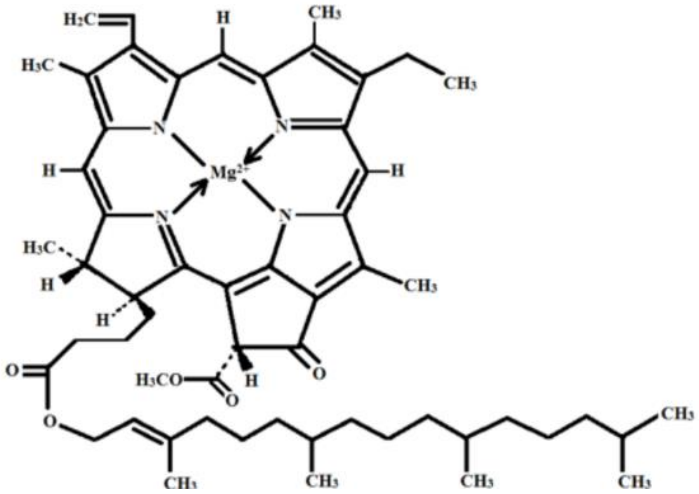


Figure II.1: Chemical structure of Chlorophyll a (Tanveer et *al*., 2018).

- ***Chlorophyll b:***

Chl b is a green yellow color pigment with maximum absorbance from 642 to 652 nm **(Ye et *al*., 2019)**. *Chl b* is an accessory pigment and acts indirectly during photosynthesis by absorbing and transferring it to *Chl a* **(Hosikian et *al*., 2010)**. *Chl a* and *Chl b* have a common basic structure. They differ, only slightly, in the composition of the side chain R2 where it is CH3 in *Chl a*, and CHO in *Chl b* as shown in Figure II.2 **(Dumay & Morançais, 2016)**.

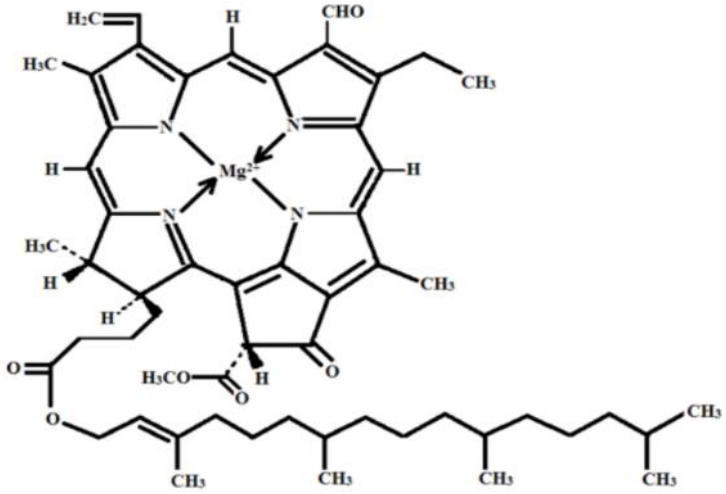


Figure II.2: Chemical structure of Chlorophyll *b* (Tanveer et *al*., 2018).

- ***Chlorophyll c:***

Chlorophyll *c* (Figure II.2) is an accessory blue greenish color pigment found in brown macroalgae with maximum absorbance from 447 to 452 nm and without phytyl chain **(Manivasagan et *al*., 2017)**. *Chl c* absorbs in the spectral region where *Chl a* and *Chl b* absorb

only weakly. Two types of *Chl c* are present in brown seaweeds: *Chl c1* and *Chl c2*. Their structures differ only in the composition of a side chain R3 (in *Chl c1*, it is CH_2CH_3; in *Chl c2*, it is $CHCH_2$) **(Dumay & Morançais, 2016)**.

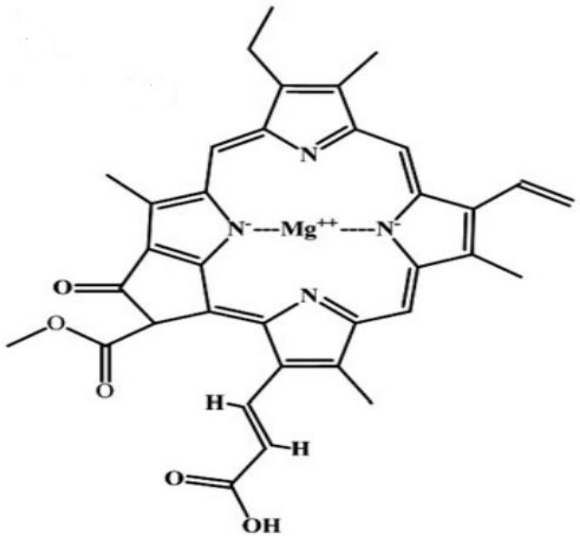


Figure II.3: Chemical structure of Chlorophyll *c* (Ye et *al.*, 2019).

- ***Chlorophyll d:***

Chl d is found in red algae and absorbs far- red light at 710nm. The existence of *Chl d* was in doubt until it was established in red seaweeds. The structure differs from *Chl a* only in the composition of the side chain R1 where in *Chl a*, it is $CHCH_2$; and in *Chl d*, it is CHO (Figure II.4) **(Dumay & Morançais, 2016; Larkum & Kühl, 2005)**.

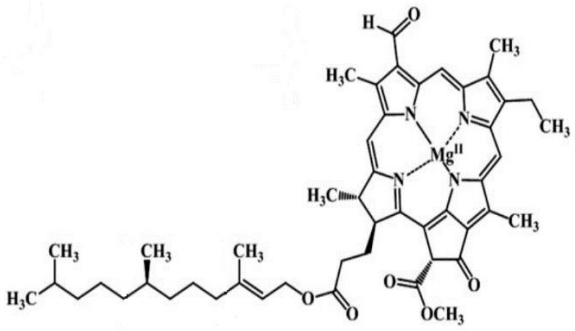


Figure II.4: Chemical structure of Chlorophyll *d* (Ye et *al.*, 2019).

- ***Chlorophylls derivatives:***

Natural chlorophylls are sensitive to extremes pH and temperature thereby they could be converted into various derivatives forms such as chlorophyllides, pheophins and pheophorbides. These derivatives show the main structural skeleton of chlorin and strongly

absorb light in the red band spectrum. Degradation and derivatization reactions of chlorophylls were thoroughly studied in several field particularly in food technologies **(Canjura et *al*., 1991; Ferruzzi & Blakeslee, 2007; Manivasagan et *al*., 2017)**. The main degradative reactions are summarized in Figure II.5.

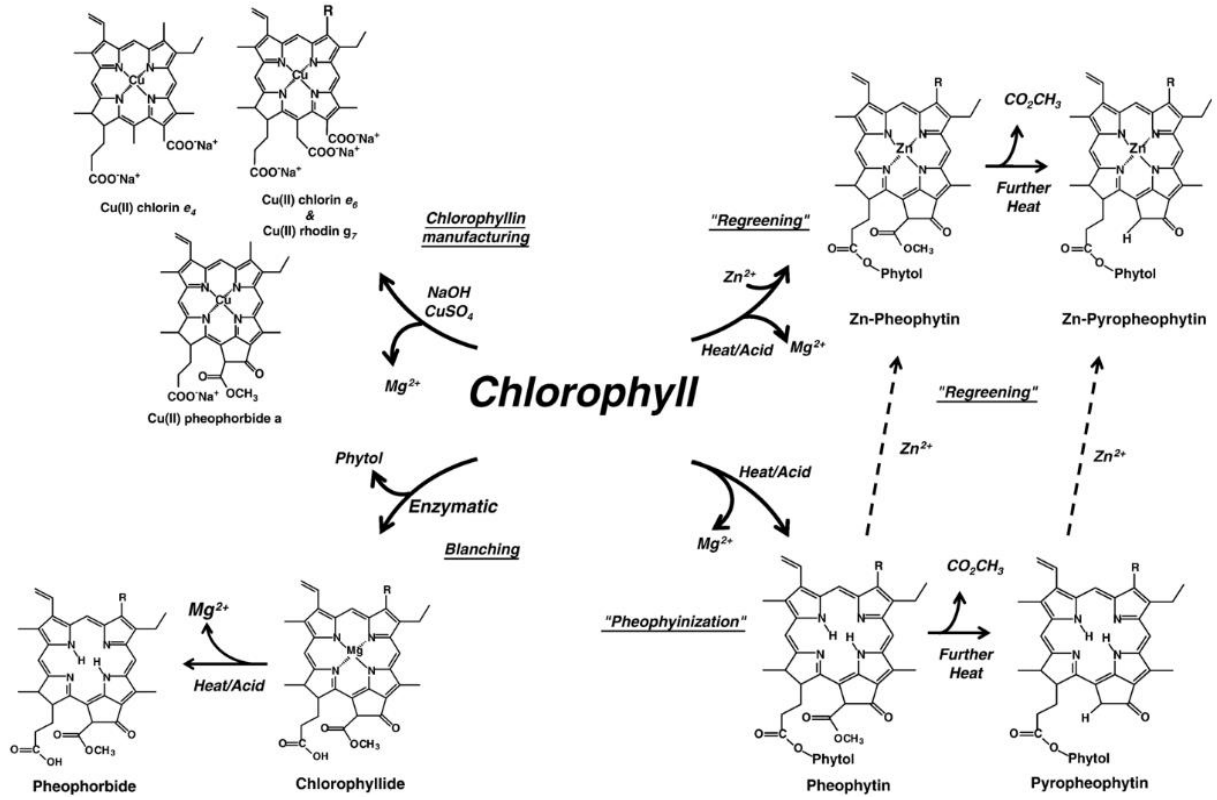


Figure II.5: Major chlorophyll derivatization and degradation reactions (Ferruzzi & Blakeslee, 2007).

Exposure to severe heat and/or acidic conditions results in a discoloration of vegetable tissue from green to brown, this phenomenon called pheophytinization results from the conversion of natural chlorophylls to Mg^{2+}-free derivatives such as pheophytins and pyropheophytins (Figure II.5). Chlorophyllase activity, induced by blanching operations, results in the formation of water-soluble chlorophyllides that further degrade during thermal processing and/or acidification to pheophorbide pigments **(Ferruzzi & Blakeslee, 2007).**

In the recent years, chlorophyll derivatives have opened numerous new avenues for biomedical applications because of their antibacterial, antioxidant, anti-inflammatory, and antimutagenic activities **(Ferruzzi & Blakeslee, 2007; Jelić et *al*., 2012; Lanfer-Marquez et *al*., 2005; W.-T. Li et *al*., 2007)**.

II.2.2. Carotenoids

Carotenoids are the most widespread class of pigments found in nature **(Bandaranayake, 2006)**. Their colors range is yellow, orange to red. They assist photoprotection and light energy harvesting therefore they play potential roles in the photosynthetic system **(Edge et *al*., 1999)**. Carotenoids are liposoluble linear polyenes made from isoprene units. Carotenoids are divided into two classes:

- Carotenes: α-carotene, β-carotene and γ-carotene.
- Xanthophylls: Lutein, violaxanthin, neoxanthin, astaxanthin, fucoxanthin, diatoxanthin, zeaxanthin and taraxanthin.

Carotenes are unsaturated hydrocarbons while xanthophylls present one or more functional groups containing oxygen **(Batista et *al*., 2006)**. Common carotenoids present in seaweeds include *β*-Carotene, Lutein, astaxanthin, zeaxanthin, violaxanthin, canthaxanthin and fucoxanthin **(Manivasagan et *al*., 2017)**.

- ***β-Carotene:***

Currently, *β*-Carotene extracted from algae is the most used for commercial purposes **(Shah, 2015)**. The content of *β*-carotene in algal dry mass ranges from 36 to 4500 mg kg^{-1} **(Holdt & Kraan, 2011)**. *β*-Carotene an orange pigment, is an isoprenoid compound possessing a long chain of conjugated double bonds with chemical formula $C_{40}H_{56}$ (Figure II.6).

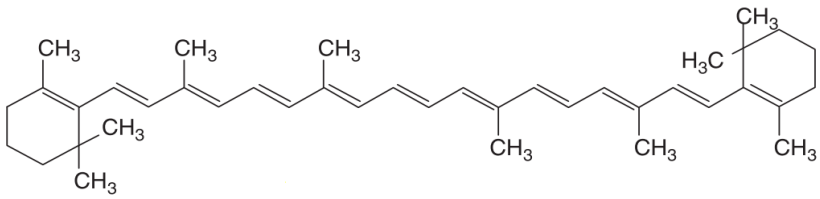


Figure II.6: Chemical structure of *β*-Carotene (SeKwon Kim & Chojnacka, 2015).

- ***Lutein:***

Lutein a yellow pigment, is member of the oxygenated carotenoids that consist of 40 carbons compounds with nine conjugated double bonds in the polyene chain. As shown in figure II.7, Lutein structure is characterized by the presence of two hydroxyl groups at the

terminal rings of the molecule on the basic carotene structure, thus it is referred to xanthophylls **(Ma & Lin, 2010; Teo et *al*., 2017)**.

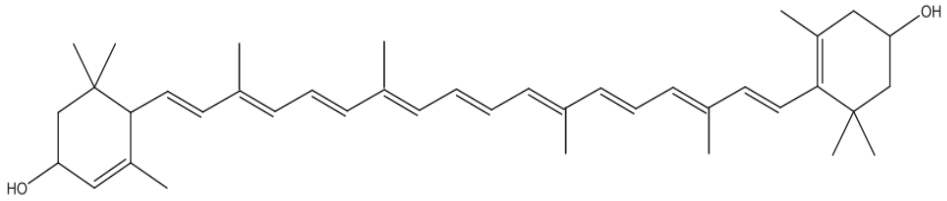


Figure II.7: Chemical structure of lutein (D. M. Pereira et *al*., 2014).

- ***Zeaxanthin:***

Zeaxanthin is one of the most common carotenoids pigments with yellow colors. It is a stereoisomer of lutein, differing only in the location of one double bond in one of the hydroxyl groups (Figure II.8). The hydroxyl groups are believed to provide unique biological function of xanthophylls as well they play a potential role in maintaining eye health **(Ma & Lin, 2010; Ye et *al*., 2019)**.

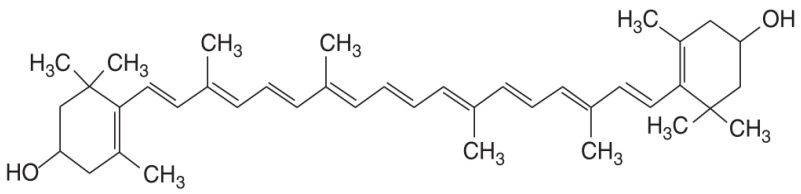


Figure II.8: Chemical structure of zeaxanthin (SeKwon Kim & Chojnacka, 2015).

- ***Astaxanthin:***

Astaxanthin is a red liposoluble pigment belonging to the xanthophyll class. Their structure possesses two identical asymmetric carbon atoms. Its properties are extremely unstable due to the long conjugated unsaturated double bond in the molecular structure **(Ye et *al*., 2019)**.

In recent year astaxanthin have been developed for use in foods, feeds, and pharmaceuticals. It is a ketone type protocarotenoid (no- vitamin A) with various biological proprieties including coloring, antioxidation, anticancer, enhancement of immunity, and antiinflammation. Therefore, it has great approved for application in nutrition and human health **(Boon & Jean Soon, 2004; Hussein et *al*., 2006; Palozza et *al*., 2009; J. P. Yuan et *al*., 2011).**

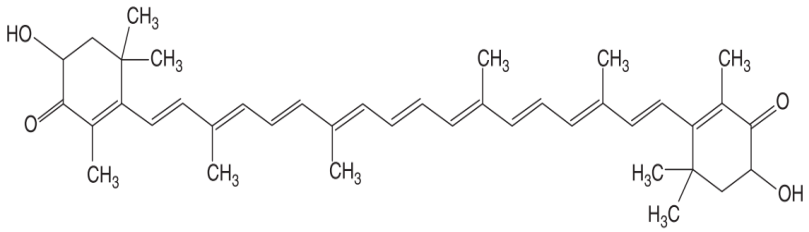


Figure II.9 : Chemical structure of astaxanthin (SeKwon Kim & Chojnacka, 2015).

- ***Canthaxanthin:***

Canthaxanthin is a xanthophyll pigment with a keto group in its ring. The chemical structure is shown in figure II.10. It is naturally found in a variety of green algae. This compound has an excellent antioxidant activity compared to other types of carotenoids. It is used as lipid-soluble natural pigment and has been developed as a food additive **(Manivasagan et *al*., 2017; Teo et *al*., 2017)**.

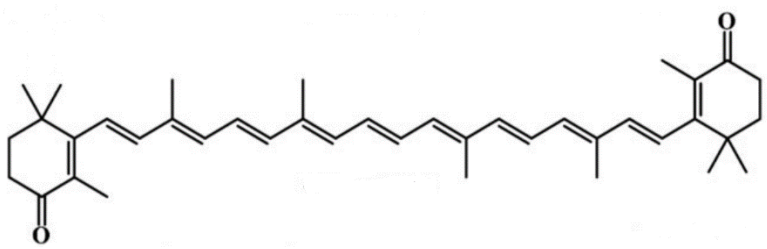


Figure II.10: Chemical structure of canthaxanthin (Manivasagan et *al*., 2017).

- ***Fucoxanthin:***

Fucoxanthin is one of the most abundant carotenoids in nature. It was first isolated from the marine brown seaweeds. It has a unique structure, including an unusual allenic bond and 5,6-monoepoxide. This orange pigment reaches 70% of total carotenoids in some algae and is responsible for the colour of brown seaweeds **(Maeda, Hosokawa, et *al*., 2008)**. Several bioactivities of fucoxanthin such as antioxidant, anticancer, anti-inflammatory, antidiabetic and antimalarial were reported **(Peng et *al*., 2011)**.

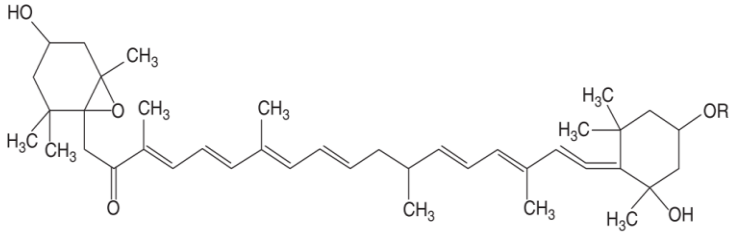


Figure II.11: Chemical structure of fucoxanthin (SeKwon Kim & Chojnacka, 2015).

Carotenoids contents for the three phyla of seaweeds are presented in Table II.3. In general, carotenoids in green seaweeds include α, β and γ-carotene, lutein, violaxanthin, astaxanthin, zeaxanthin, canthaxanthin and neoxanthin whilst red seaweeds contain α, β-carotene, lutein, zeaxanthin and taraxanthin are found in red seaweeds. The major pigments present in brown seaweeds are β-carotene, lutein, violaxanthin, diatoxanthin, zeaxanthin and fucoxanthin, whereas fucoxanthin is predominant.

Table II.3: Distribution of carotenoids in Seaweeds (Ito & Hori, 2009; Stengel et *al*., 2011).

Carotenoids	Green seaweed	Brown seaweed	Red seaweed
α-Carotene	a	-	+
β-Carotene	+	+	+
γ-Carotene	+	-	-
Lutein	+	+	+
Violaxanthin	+	+	+
Neoxanthin	+	-	-
Astaxanthin	+	-	-
Fucoxanthin	-	+	-
Diatoxanthin	-	+	-
Taraxanthin	-	-	+
Zeaxanthin	+	+	+
Canthaxanthin	+	-	-

[a] *Sometimes not detected*

II.2.3. Phycobiliproteins

Phycobiliproteins are water soluble pigments composed of a protein and a chromophore called phycobilin linked by covalent bonds. Phycobiliproteins are mostly present in cyanobacteria and some algal phyla like Rhodophyta, Cryptophyta, and Glaucophyta **(Benoît & Stéphane, 2018)**.

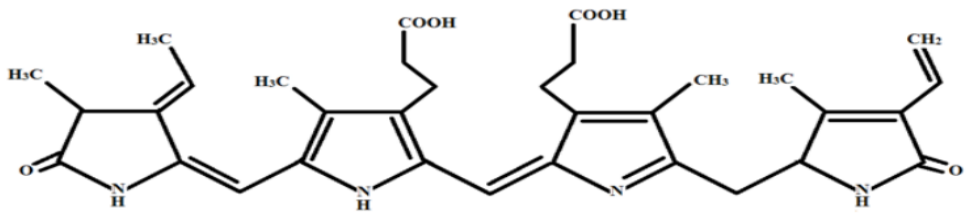


Figure II.12: Chemical structure of phycoerythrin (Tanveer et *al*., 2018).

Phycobiliproteins are divided into three main classes of phycobiliproteins: Phycoerythrins which are bright pink coloured pigments (γ_{max} 540–570 nm) (Figure II.12), phycocyanins with a dark blue-colour (γ_{max} 610–620 nm), and the brighter aqua blue coloured pigments, allophycocyanins (γ_{max} 650–655 nm) **(Ye et *al*., 2019)**.

II.3. Biological activities and health benefit proprieties of seaweed pigments

Seaweeds pigments are a valuable source of bioactive substances, which can be used for human health beneficial applications. According to the published literature, marine natural pigments possess potent therapeutic functions including: antioxidant, anticancer, antiangiogenic, anti-inflammatory, anti-obesity, and many other activities. Table II.4 gives an overview of the main biological activities of seaweed derivatives pigments.

II.3.1. Antioxidant activity

Beside their function as colorants, natural pigments derived from seaweeds are known as potent antioxidants. However, the antioxidant activity of natural pigments depends on their structural features such as porphyrin ring, phythyl chain and extended system of conjugated double bonds **(Pangestuti & Kim, 2011)**.

Several studies reported that Chl *a* and its derivatives including pheophorbide *a* of brown and green seaweed exhibited excellent antioxidant properties **(Cho et *al*., 2011; B. Le Tutour et *al*., 1998)**. Furthermore, according to their structure, chlorophyll derivatives which lack a central Mg^{2+} and phytyl chain had more potent antioxidant activity than chlorophyll **(Cahyana et *al*., 1993)**, as well as Chl *b* derivatives which showed stronger antioxidant activity than Chl *a* derivatives suggesting that the presence of aldehyde group in place of methyl group improves antioxidant activity **(Lanfer-Marquez et *al*., 2005)**. **Cahyana et *al*., (1992)**, reported that pyropheophytin *a* from the brown seaweed *Eisenia bicyclis* was more potent antioxidant than synthetic antioxidants such as BHT and α tocopherol.

Moreover, fucoxanthin isolated from brown seaweeds showed a great potential radical scavenging activities and reducing abilities compared to the commercial one **(Fung et *al*., 2013; Yan et *al*., 1999)**. Also, Phycoerythrobilin derived from *porphyra sp* exhibited strong antioxidant activity **(Yabuta et *al*., 2010)**.

Table II.4: Potential health benefit effects of some seaweeds-derived natural pigments (Pangestuti & Kim, 2011).

Natural pigments	Sources	Health benefit effects
Chl *a*	*Enteromorpha prolifera, Fucus vesiculosus*	Antioxidant
Pheophytin *a*	*Porphyra tenera*	Antimutagenic
	Sargassum fulvellum	Neuroprotective
	Enteromorpha prolifera	Antimutagenic
	Enteromorpha prolifera	Anti-inflammator
Pheophorbide *a*	*Enteromorpha prolifera*	Antioxidant
Lutein	*Porphyra tenera*	Antimutagenic
β-Carotene	*Porphyra tenera*	Antimutagenic
Fucoxanthin	*Porphyra tenera Undaria pinnatifida, Fucus serratus, Padina tetrastromatic*	Antioxidant
	Undaria pinnatifid	Anticancer
	Myagropsis myagroides	Anti-inflammator
	Undaria pinnatifid	Anti-obesity
	Undaria pinnatifid	Anti-angiogenic
	Hijikia fusiformis	Neuroprotective
	Laminaria japonica	Prevent osteoporosis
	Laminaria japonica	Photoprotective
Phycoerythrobilin	*Porphyra sp.*	Antioxidant

II.3.2. Anticancer activity

In the last few years, cancer became one of the most serious human health problems. Therefore, natural anticancer drugs for instance chemopreventive agents have gained a positive popularity in cancer treatment. In human body, the formation of cancer cells can be induced by free radicals, hence, radical scavenging substances such as natural pigments could be used indirectly to reduce cancer formation in human body **(Manivasagan et *al.*, 2017; Pangestuti & Kim, 2011)**.

Anticancer effect of chlorophyll and its derivatives were extensively studied, with specific emphasis on their antimutagenic effect against numerous dietary and environmental mutagens, additionally the potency of natural and commercial chlorophyll derivatives to act as photosensitizers have allowed their utilization in photodynamic therapy of cancer **(Ferruzzi & Blakeslee, 2007)**.

Various studies were published regarding the correlation between the protective effect of carotenoids against various forms of cancer such as breast cancer and lung cancer **(Socaciu, 2008)**. **(Goodman et *al.*, 2003; Parker, 1989; Toniolo et *al.*, 2001)**.

Moreover, a recent study from Japan reports that fucoxanthin acts on human leukemia by its apoptosis inducing effect **(M. Hosokawa et *al*., 1999)**. Meanwhile, antiproliferative effect and apoptosis induction by fucoxanthin in human colon and prostate cancer cells were observed **(Hosokawaa et *al*., 2004; Kotake-Nara et *al*., 2001, 2005)**.

II.3.3. Anti-inflammatory activity

Treatment of inflammatory diseases is mainly based on modulation of macrophages function which produces pro-inflammatory cytokines and mediators. Thus, inhibition of the production of these inflammatory mediators is a potential target in the treatment of inflammatory diseases **(Kanidta et *al*., 2017; Pan et *al*., 2011)**.

Metabolites derived from seaweeds are known to have anti-inflammatory activities **(Abad et *al*., 2008)**. However, few studies were focused on the potential anti-inflammatory activities of seaweed pigments. Thus, fucoxanthin and astaxanthin extracted from brown seaweeds exhibited anti-inflammatory activities **(Choi et *al*., 2008; Heo et *al*., 2010; Kim et *al*., 2010)**. In another study, **Shiratori et *al*., (2005)** reported that anti-inflammatory effect of fucoxanthin was comparable to the effect of the commercial steroidal anti-inflammatory drug predinisolone used in similar doses.

II.3.4. Antiangiogenic activity

Angiogenesis refers to the process of new blood vessel formation from a preexisting vasculature that occurs under, either physiological (ovary cycling, embryogenesis and wound healing) or pathological conditions (inflammatory disease, tumour growth and metastasis). Therefore, controlling angiogenesis is promising approach for treatment of cancer and other angiogenic related diseases **(Carmeliet, 2003; L. Zhang et *al*., 2017)**.

Sugawara et *al*., (2006), reported the antiangiogenic activity of brown seaweed fucoxanthin against human umbilical vein endothelial cells (HUVEC), the study suggested that fucoxanthin suppressed the differentiation of endothelial progenitor cells into endothelial cells involving new blood vessel formation. In the meantime, fucoxanthin was shown to inhibit micro-vessel outgrowth in ex vivo angiogenesis activity using a rat aortic ring.

II.3.5. Anti-obesity activity

Obesity is one of the greatest public health problems affecting all age groups worldwide and leads to many serious diseases including: diabetes type 2, hypertension, hyperlipidemia, and cardiovascular disease **(Hasani-Ranjbar et *al*., 2013)**. Therefore, discovering alternative source of safe anti-obesity agents is necessary.

Diets containing fucoxanthin found in brown seaweeds might prevent obesity through the inhibition of adipocyte differentiation **(Maeda et *al*., 2006)**. Besides, other interesting studies revealed that oral treatment with fucoxanthin considerably reduced the abdominal white adipose tissue weight of obese mice and normal mice feed with high fat diet, however, no reductions were found on normal mice fed with normal diet. Results suggested that fucoxanthin might suppress adiposity in the obese mice **(Maeda et *al*., 2005; Maeda, Hosokawa, Sashima, & Miyashita, 2007; Maeda, Hosokawa, Sashima, Funayama, et *al*., 2007).**

In a clinical study, **Abidov et *al*., (2010)** reported that xanthigen , fucoxanthin and pomegranate seed oil promoted weight loss, reduced body and liver fat content and improved liver function tests in obese non diabetic women.

II.3.6. Neuroprotective effect

Neurodegenerative diseases are estimated to surpass cancer and be the second most common cause of death among elderly by 2040s. For this reason, a great deal of attention has been expressed by scientists regarding safe and effective neuroprotection. Several studies provided insight into neuroprotective properties of marine algae-derived pigments **(Pangestuti & Kim, 2011)**. For example, **Okuzumi et *al*., (1990)** demonstrated that fucoxanthin isolated from the brown seaweed *Hijikia fusiformis* inhibited N-myc expression and cell cycle progression of human neuroblastoma cell line.

To date, neuroprotective activities of natural pigments have been observed *in vitro*. Therefore, more research is needed to investigate pigments neuroprotective activities *in vivo* and in human subject **(Pangestuti & Kim, 2011)**.

II.3.7. Other biological activities

According to Das et *al*., 2010, dietary fucoxanthin may be useful for the prevention of osteoporosis and rheumatoid arthritis, which are known to be related to bone resorption. **Heo**

& Jeon (2009) revealed that fucoxanthin possessed photoprotective properties in human fibroblast cells via inhibition of DNA damage and enhancing antioxidant activity. Moreover, fucoxanthin has been demonstrated to suppress significantly skin mRNA expression related to melanogenesis, suggesting that fucoxanthin negatively regulated melanogenesis factor at transcriptional level **(Shimoda et *al*., 2010)**.

II.4. Current and potential application of seaweed pigments

Because of their non-toxic and antioxidant proprieties natural pigments have found application in many areas. seaweed pigments are generally considered as cheap, renewable and sustainable resources with little impact on environment **(Wu et *al*., 2020)**. Moreover, there is a great consumer interest toward natural bioactive pigments as alternatives to the synthetic ones. Natural pigments are widely used as natural colorants and powerful antioxidants associated with additional bioactivities in food, pharmaceutic, cosmetic, technologic and various other applications **(Pangestuti & Kim, 2011)**. They can be used in textile industries **(Rani et *al*., 2020)**.

Seaweed chlorophylls were used as natural, antimicrobial and deodorizing dye. Moreover, their strong antioxidant property and their ability to stimulate tissue growth qualifies these compound as valuable raw materials in cosmetics. β- carotene is considered as a sun care ingredient due to its skin protect effect against premature aging caused by UV radiation. It may also act as a prooxidant in the process of lipids peroxidation **(SeKwon Kim & Chojnacka, 2015)**. Zeaxanthin was used as nutritional supplement and colorant in wide range of foods **(Stankovic, 2004)**. The red-pink pigment R-phycoerythrin was widely used for colouring many cosmetic products **(Benoît & Stéphane, 2018)**.

II.5. Conclusion

Various naturel pigments derived from seaweeds proved to be one of the most useful biological compounds with great potential bioactivities (antioxidant, anticancer, anti-obesity, neuroprotective and many other powerful bioactivities). Seaweeds pigments are renewal, safe, healthy and non-toxicin addition to their relatively low production cost are their main advantages. For this reason, pigments may be used as functional ingredients in food, pharmaceutical and cosmetic products and in other applications.

CHAPTER III

Monograph of the studied seaweeds

III.1. Overview of seaweeds studied in Algeria

Algerian coast has a great biodiversity of algae, however research studies related to seaweeds harvested in Algeria were limited and their exploitation is still marginal. Generally, the first studies date back to the 19th century which performed inventories and checklist of seaweeds grouping the taxa and genus reported on the Algerian coast **(Ould-Ahmed et *al*., 1995; Ould-ahmed & Alexandre, 1990)** and continued in the recent years **(El Amine Bentaallah et *al*., 2017; Ould-Ahmed et *al*., 2019; Traiche et *al*., 2018)**. Other studies oriented on ecological side are carried by **Belhaouari and Bezzina (2019)**. According to these authors many algal florae are less studied.

Furthermore, nutritional value and chemical composition of some seaweeds of the coast of Algeria have been reported **(Laib & Leghouchi, 2012; Oucif et *al*., 2020; Zitouni et *al*., 2014)**. Otherwise, extraction and purification of some functional ingredients from the brown seaweed *Cystoseira sp*. , have been reported such as alginates **(Benchabane, 1988)**, and sterols **(Bouzidi et *al*., 2019)** that can be developed for number applications. In addition, biological activities of secondary metabolites extracted from seaweeds including polyphenols compounds have been demonstrated in some reports **(Fellah et *al*., 2017; Mellouk et *al*., 2017; Metidji et *al*., 2015; Othmani et *al*., 2014; Saidani et *al*., 2012)** . Moreover, any data found on pigments extraction or characterization of Algerian coast seaweeds.

III.2. Seaweeds used in the study

In the current study, three species of brown seaweeds are selected: *Padina* sp., *Sargassum vulgare* and *Phyllaria reniformis*. All these species are frequent macroalgae of the Mediterranean Sea, especially the Algerian littoral.

III.2.1. *Padina sp*.

III.2.1.1. Taxonomic classification and morphological characters

Table III.1: Taxonomic classification of *Padina sp.*

Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Order	Dictyotales
Family	Dictyotacea
Genus	*Padina*

Source : (https://www.algaebase.org)

The genus *Padina* belonging to the Dictyotaceae (Dictyotales, Phaeophyceae) is unique in being the only other genus of brown algae that is calcified. Thalli are generally fan-shaped with an inrolled margin enclosing a marginal meristem by which the growth is initiated. This genus is one of the economically important seaweeds and used as a source of algin, human food-prepared as salad and gelatine-like sweetmeat and fertilizer **(Ansari et *al*., 2019; Mya Kyawt & Soe-Htun, 2008)**.

III.2.1.2. Biochemical composition and bioactivities

Padina are a source of polysaccharides and minerals. It contains high amount of ashes (30-48%), carbohydrates (25- 39%) and total dietary fiber (27-39% on dry basis), 5-7% of protein, and 1.6-1.8% of lipids. Therefore various uses of the seaweed are in cosmetics for the protection from radiation and pharmacological use as antioxidants, antibiotics, anti-inflammatory, hypo-allergenic, antibacterial and antidiabetic **(Ansari et *al*., 2019)**.

III.2.1.3. Interest recent studies on *Padina sp*.

A resent research focused on silver nanoparticles synthesized using *Padina sp*. marine alga extract **(Bhuyar et *al*., 2020)**. Result revealed that the formation of Ag nanoparticles was

increased by the addition of marine alga. Also, the surface morphology and the size of synthetic nanoparticles was relatively uniform. In addition, Ag nanoparticles showed highly potent antibacterial activity, and therefore, in the future, its property can be well compatible for pharmaceutical and other biomedical applications.

III.2.2. *Sargassum vulgare*

III.2.2.1. Taxonomic classification and morphological characters

Sargassum vulgare belongs to phaeophycean class (brown seaweed) in the order of Fucale.

Table III.2: Taxonomic classification of *Sargassum vulgare*

Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophycea
Order	Fucales
Family	Sargassaceae
Genus	*Sargassum*
Species	*Vulgare*

Source: (https://www.algaebase.org)

The Thallus of *Sargassum vulgare* (10-200 cm or more in length) consist of a holdfast with one to several main stipes (1- 20 cm longer) with primary branches (10 cm to 200 cm or longer) forming specialized structures such as leaf-like laterals called blades, secondary branches, globose air bladders known as vesicles and fruiting branches known as receptacles. The alga is a monoecious or dioecious species (Receptacles are unisexual or bisexual **(Kumari & Sharma, 2012)**

III.2.2.2. Biochemical composition and bioactivities

Sargassum vulgare contains high carbohydrate, ash and crude fiber content of 34.18±0.32, 27.09±0.00, and 22.59±0.21% respectively. It represents 30 – 60% of the total dry

weight of brown seaweed which is mainly composed of fucoidan, alginates and laminarin. However, the protein content of the dried seaweed is 7.69% and the total lipids content is 0.56%.

Nutritional profile of *Sargassum vulgare* recommends that it can be used as alternative source of mineral and nutrition supplements with high carbohydrate and dietary fibre content **(Arguelles et *al*., 2019)**. Furthermore, many secondary metabolites derived from *Sargassum vulgare* including polyphenols and phlorotannin reported in many studies have been demonstrated various powerful biological properties such as : antioxidant, antibacterial, and anticancer activities **(Arguelles et *al*., 2019; Tannoury et *al*., 2016)**.

III.2.2.3. Interest recent studies on *Sargassum vulgare*

Beside the studies on antioxidant, antimicrobial and anticancer, many recent works reporting the importance of *Sargassum vulgare* are published recently.

Ibrahim et *al*., (2020) reported that the edible seaweed *Sargassum vulgare*, and its methanolic extract could be considered as a potentially functional food, with powerful antioxidant properties useful to alleviate oxidative stress and toxicity associated with consumption of the artificial sweetener aspartame.

Due to their physicochemical proprieties and biological activities, Zinc oxide nanoparticles have attracted considerable interest. According to **Karkhane et *al*., (2020)**, green biosynthesis of these nanoparticles using aqueous extract of *Sargassum vulgare* as a reducing and capping agent has been successfully achieved. In addition, antifungal, antioxidant and photocatalytic proprieties of Zinc oxide nanoparticles seemed effective and were highly desirable.

The Phyto-elicitor and Phyto-stimulatory properties of *Sargassum vulgare* alkaline extracts were investigated. Whereas foliar application of seaweed extracts at 0.5% concentration, reduced pathogen diseases either under greenhouse or field conditions. Moreover, treated plants showed enhanced plant growth and yield parameters. In line with this, *Sargassum vulgare* can be used for preparing of bio-stimulant products for crop production **(Ali et *al*., 2020)**.

The study of **Plouguerne et *al*., (2020)** highlighted the potential of glycoglycerolipids isolated from *Sargassum vulgare* as a new promising antifouling agents. The ability of *Sargassum vulgare* and its derived products, including fucosterol, to prevent the growth of

Leishmania parasites was investigated, and the results showed promising antileishmanial potency **(Tchokouaha Yamthe et *al*., 2020)**.

III.2.3. *Phyllaria reniformis*

III.2.3.1. Taxonomic classification and morphological characters

Table III.3: Taxonomic classification of *Phyllaria reniformis*

Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Order	Tilopteridales
Family	Phyllariacea
Genus	*Phyllaria*
Species	*reniformis*

Source: (https://www.algaebase.org)

III.2.3.2. Biochemical composition and bioactivities

Phyllaria reniformis or *Phyllariopsis brevipes* **(Henry & South, 1987)** is a species of brown algae that occurs in the Mediterranean Sea. However, until now, no published reports on the chemical characterization of this alga were found in the literature.

PART 2
EXPERIMENTAL PART

Chapter IV

Characterization and biological activities of pigments extracted from three brown seaweeds

Chapter V

Impact of freezing and drying preprocessing on pigments extraction

Chapter VI

Oxidative stability of soybean and sunflower oils enriched with Phyllaria reniformis pigments extracts.

CHAPTER IV

Characterization and biological activities of pigments extracted from three brown seaweeds

IV.1. Introduction

Marine algae, in particular the brown algae, were identified as an under-exploited plant resource and a source of functional food. They have also been recognized as rich sources of structurally diverse bioactive compounds such as pigments, fucoidans, phycocolloids, and phlorotannins with great pharmaceutical and biomedical potential. Several studies were focused on the isolation of these compounds and on their interesting biological activities **(Heo et *al*., 2009).**

Brown seaweeds like the other classes of algae are rich on photosynthetic pigments in particular fucoxanthin and chlorophyll c **(Haryatfrehni et *al*., 2015)**. Besides, their use as food colourants, they possess many health benefits such as antioxidant, anticancer, anti-inflammatory, and antidiabetic properties **(Aryee et *al*., 2018)**.

Algerian coast as part of Mediterranean Sea have a great biodiversity of algae, however research studies on seaweed harvested in Algeria were limited on few species **(Bentaallah et *al*., 2017; Fellah et *al*., 2017; Mellouk et *al*., 2017; Metidji et *al*., 2015; Saidani et *al*., 2012)**. Therefore, in the following chapter, three brown seaweeds were collected from Algerian coast, namely *Padina sp*., *Sargassum vulgare* and *Phyllaria reniformis* in order to identify and compare their pigments composition and their biological activities for their potential application in functional health foods.

IV.2. Materials and Methods

IV.2.1. Field Sampling and preparation

Fresh *Padina sp*., *Sargassum vulgare* and *Phyllaria reniformis* were collected by hand at more than 15 meters depth in June 2016 from Tipaza coast, north center of Algeria (36° 35' 50" N / 2° 27' 10" E) (Figure IV.1). Three independent groups were taken for each alga separately for further analysis. After a first rinse on-site with sea water, samples were taken to the laboratory in isothermal boxes. The Figure IV.2 shows the three collected Alga species.

The alga identification was made in the Laboratory of Biological Oceanography and Marine Environment, University of Science and Technology (USTHB), Bab Ezzouar, Algeria, by Mme Seridi Halima. After identification all samples were washed for a second time with fresh tap water to remove sand, epiphytes, shells and any sediments and then washed for a last time by distilled water. Afterwards, algae were stored at -18°C until pigment extraction.

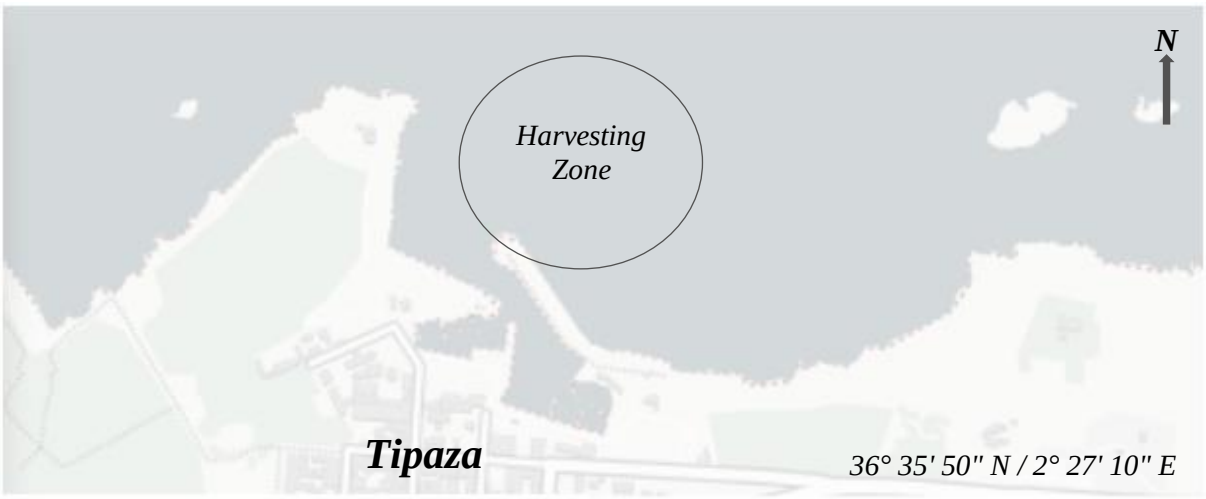


Figure IV.1: Harvesting location map of the three species.

IV.2.2. Pigments Extraction

Extraction was performed in the laboratory with low light intensity, at a temperature below 25 °C and as quickly as possible to prevent pigment decomposition. Seaweed samples were cut into small pieces and mixed with acetone at a ratio of 1/3 (w/v). Pigments were extracted in an ultrasonic bath (100W, 20 Hz, 24°C) for 90 min. Then, all the obtained extracts were filtered and the solvent was evaporated using rotary evaporator at 28°C. The obtained residues were lyophilized and stored at – 20°C in brown glass flasks for later analysis.

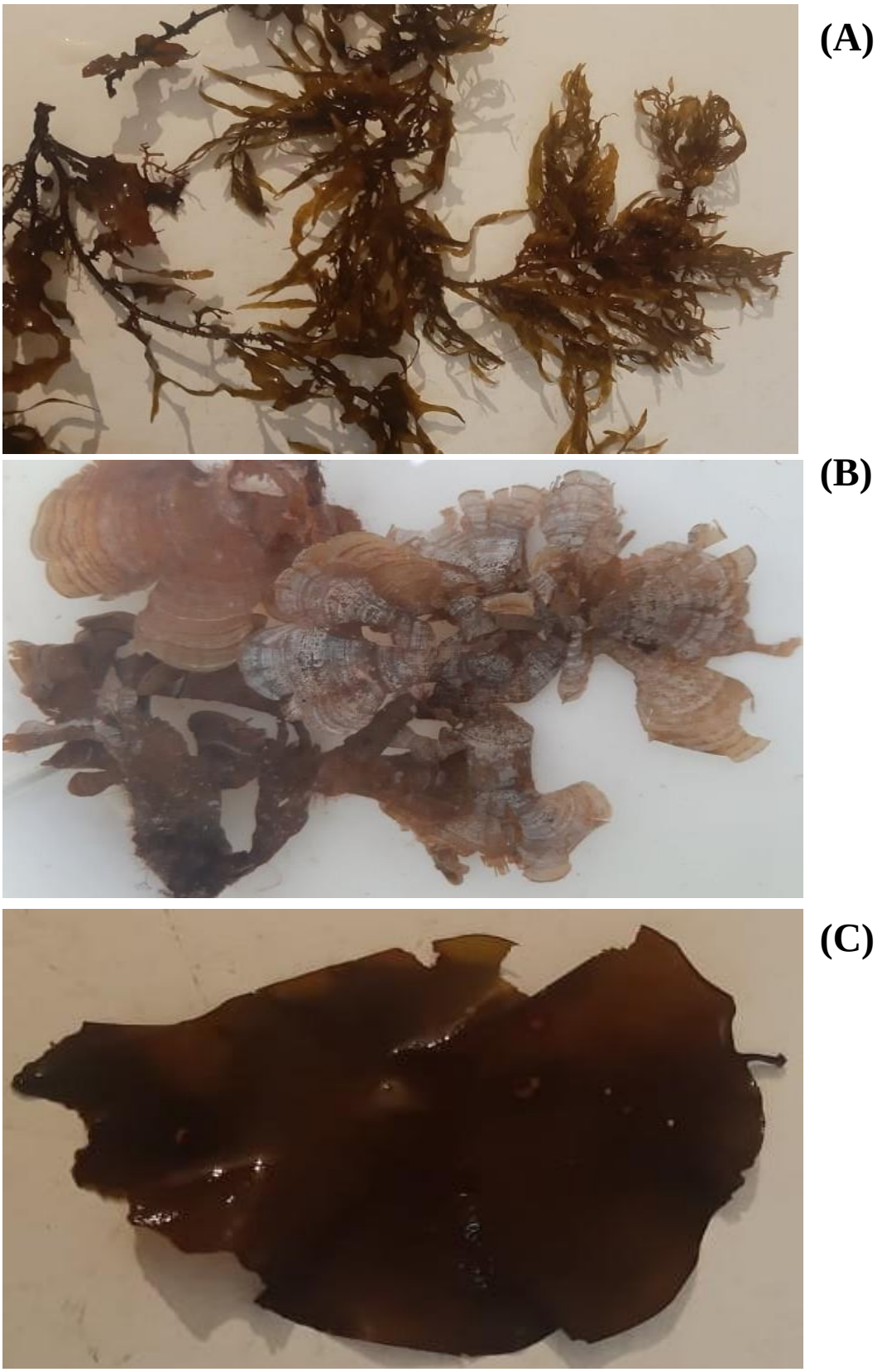


Figure IV.2: *Sargassum vulgare* (A), *Padina sp*. (B) and *Phyllaria reniformis* (C)

IV.2.3. Determination of photosynthetic pigments

Chlorophylls and carotenoids, the major photosynthetic pigments in seaweed were estimated by UV-Visible Spectrophotometry (SPECORD 210 PLUS 623F1138, Germany) as described in the following. 100 mg of dry extract were mixed with 2 mL of solvent (acetone) and filtered using Nylon microfilter (0.45 µm). The pigment absorbances (Abs) in the filtrate were recorded in wavelength range of 350-800 nm. The content of chlorophyll *a*, *b*, *c*, fucoxanthin and total carotenoids were calculated according to the equation of **Lichtenthaler and Wellburn (1983)** and **Seely *et al*., (1972)** .

$$[C_a] = 11.75 \times Abs_{662} - 2.35 \times Abs_{665}$$
$$[C_b] = 18.61 \times Abs_{645} - 3.96 \times Abs_{662}$$
$$[C_c] = (Abs_{631} + Abs_{581} - 0.3Abs_{664})/62.2$$
$$[Fx] = (Abs_{470} - 1.239 \times (Abs_{631} + Abs_{581} - 0.3 \times Abs_{664}) - 0.0275 \times Abs_{664})/141$$
$$[Tot\ Carot] = (1000 \times Abs_{470} - 2.27 \times C_a - 81.4 \times C_{ab})/227$$

Where:

Abs is the absorbance in the specified wavelength

C_a is the concentration of chlorophyll *a*

C_b is the concentration of chlorophyll *b*

C_c is the concentration of chlorophyll *c*

Tot Carot is the total carotenoids

Fx is the fucoxanthin

IV.2.4. Colour measurement

The colour parameters of the three seaweed pigment extracts were measured using a CM-700d colorimeter (Konica Minolta Sensing INC, New York, USA). Data were expressed as *L**, *a** and *b** values. *L** indicates lightness from 0 (absolute black) to 100 (absolute white). *a** is associated with redness or greenness changes, positives and negatives *a** values represent

red and green, respectively. *b** is associated with yellowness and blueness changes, positives and negatives b* values represent yellow and blue, respectively.

IV.2.5. High performance liquid chromatography pigment Analysis

The separation of seaweed pigments was conducted by analytical High-performance liquid chromatography (HPLC) (Agilent 1100, USA) equipped with UV-Visible detector. The column was C18, 5µm, 150× 4.6 mm. The injection loop size was 20 µl. The used method was inspired from the study of **Wright at *al*.,(1991)**. The column was equilibrated using a gradient of elution of solvent A (methanol: 0.5M ammonium acetate, 80:20 v/v) and solvent B (Acetonitrile: water, 90:10 v/v), solvent C (ethyl acetate). The flow rate was 1mL/min, and the gradient was as follows (minutes; % solvent A; % solvent B; % solvent C): (0; 100; 0; 0), (4; 0; 100; 0), (18; 0; 20; 80), (21; 0; 100; 0), (22;100;0;0), (25; 100; 0; 0). The column was equilibrated for 10 min. Pigments were detected by recording Abs at 440 nm. All these steps were carried out at room temperature. The obtained HPLC peaks were identified by comparing the retention times with those of standards pigments.

IV.2.6. Pigment standards

The authentic standard pigments Chlorophylls (*a, b,* c_1+c_2 and c_3), Chlorophyllides *a* and fucoxanthin were obtained from *Sargassum vulgare* by semi preparative HPLC and to confirm the purification, each pigment was chromatographed in two analytical column C18 and C8. The same gradient was used with a flow rate of 5mL/min. Each pigment was collected at the outlet of the detector, isolated immediately from solvent by evaporation. Pheophytin *a* was obtained by acidification of chlorophyll *a* with 1M of hydrochloric acid (HCl) **(Wright *et al.*, 1991)**. The identification of separated pigments was confirmed from their visible spectral absorption and compared with the literature (D. M. Pereira et al., 2014). Visible spectra were obtained with UV-Visible Spectrophotometer (SPECORD 210 PLUS 623F1138, Germany). *β*-Carotene was purchased from Sigma Aldrich.

IV.2.7. Thin-layer Chromatography (TLC)

Thin-layer chromatography (TLC) allowed pigments separation. This method was performed using Merck silica gel 60 F_{254} precoated aluminum plates (Darmstadt, Germany) as a stationary phase. The three collected seaweed freeze-dried pigment extracts were dissolved in acetone and were spotted separately over the same line around 1 cm above the bottom of the TLC plate using a micropipette. Then, TLC plate was put vertically inside a glass beaker containing the mobile phase and filled about 0.5 cm height of the solvent system. In order to select the most effective and appropriate solvents for TLC pigments separation, four different mobile phases were used in this study: acetone (100% and 90%), methanol (100%), and methanol (90%). Solvents flowed slowly up the plate by capillary action. The process stopped when the solvent reached the upper limit. Pigments eluted through the stationary phase and the separated pigments were clearly observed on the TLC plate with their colours. For better visualization, TLC plates were subjected to UV radiation at 365 nm Retention factors (R_f) were calculated for every spot applying the following formula:

$$R_f(spot) = (distance\ the\ spot\ has\ moved)/(distance\ solvent\ front\ moved)$$

IV.2.8. Fourier Transform Infrared Spectroscopy

Attenuated total reflectance Fourier Transform Infrared Spectroscopy (ATR- FTIR) was used to identify the functional groups present in the three seaweed pigment extracts. The FTIR spectras were recorded using FTIR spectrophotometer equipped with a Platinum ATR Module Diamond system (Bruker Alpha, Germany). A total of 32 scans were used on the samples with the spectral interval of 4000 to 400 cm^{-1} and a resolution of $1cm^{-1}$.

IV.2.9. DPPH Radical Scavenging Activity

The pigment extracts antioxidant activity was evaluated using a modified method previously described by **Menaceur *et al*. (2013)** and **Hazzit *et al*. (2009).** 25µL of each sample at different concentrations (from 0 to 100 µg/mL dissolved in methanol) were added to 975 µL of 2,2-diphenylpicrylhydrazyl (DPPH) solution (60µM) and incubated for 30 min in the dark at room temperature. The Abs was measured at 517 nm with UV-Visible spectrophotometer

(SPECORD 210 PLUS 623F1138, Germany). Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) were used as standards.

The DPPH radical scavenging activity was calculated using the following equation:

$$Scavenging\ activity\ (\%)\ =\ (Abs_b - Abs_s\ /\ Abs_b)\ \times 100$$

Where Abs_s is the sample Abs after 30 min and Abs_b is the sample Abs before reaction. Seaweed pigment extracts concentration providing 50% of inhibition (IC50) was calculated using the graph by plotting inhibition percentage against concentration.

IV.2.10. Ferric Reducing Antioxidant Power Assay (FRAP)

Measurement of ferric reducing antioxidant power of the pigment extracts was performed using **Benzie and Strain (1999)** procedure. The method is based on the reduction of a ferric-tripyridyl triazine complex to the bleu coloured ferrous form in the presence of antioxidants, associated with an increase in Abs at 593nm. In the following a brief description of the method is described, the FRAP reagent was freshly prepared at volume ratio of 10:1:1 from three different solutions: Sodium acetate buffer (300 mmol/L, pH 3.6), 10 mmol/L 2,4,6-tripyridin-2-yl-1,3,5-triazine (TPTZ) solution (40 mmol/L HCl as solvent) and 20 mmol/L iron (III) chloride solution. The obtained reagent was maintained at 37ºC. Then aliquots of 100 µL sample were mixed with 3 mL of FRAP reagent. After incubation at 37ºC for 4 min, reaction mixture Abs was recorded at 593 nm using UV-Visible spectrophotometer (SPECORD 210 PLUS 623F1138, Germany). The standard curve was made using $FeSO_4$ solution, and the results were expressed as mmol Fe (II)/g dry weight of extract.

IV.2.11. Evaluation of the antimicrobial activity

The agar disc diffusion method **(CLSI, 2009; Stephen et *al*., 2005)** was used to test the antimicrobial activity of pigment extracts against five pathogenic strains: *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6938), *Candida albicans* (ATCC 10231) and *Aspergillus basiliensis* (ATCC 16404). Samples were dissolved in two solvent: dimethyl sulfoxide (DMSO) and Tween 80 at three different concentrations (10, 20, 40mg/mL). Synthetic antibiotics (Primazol and Lamidaz) were used as positive controls for

bacteria and fungi, respectively. Muler Hinton (MH) was used as growth medium in bacteria test and Sabouraud agar in fungi. This part of experimental work was achieved in SAIDAL.

IV.2.12. Statistical Analysis

All measurements were conducted in triplicate. Results were presented as mean ± S.D. of three replicates. All statistical analysis was performed using R **(R Core Team, 2020)**. Data were subjected to one-way ANOVA and a *p* value ≤0.05 was considered statistically significant.

IV.3. Results and discussions

IV.3.1. Photosynthetic pigments

The major photosynthetic pigments, total chlorophyll and carotenoid contents were estimated in *Padina sp., Sargassum vulgare and Phyllaria reniformis* pigment extracts using spectrophotometric method.

Figure IV.3 illustrates chlorophyll *a*, *b*, *c* contents in the three selected seaweed extracts. The highest concentration of chlorophyll *a* (Chl *a*) was observed in *Phyllaria reniformis* (27.26±0.65 mg/mL of extract) and the lowest concentration was recorded in *Padina sp.* (16.66±0.29 mg/mL of extract). Chlorophyll *b* (Chl *b*) fluctuated from 1.51±0.36 to 8.76±1.33 mg/mL of extract, with a highest content in *Phyllaria reniformis*. Similarly, this seaweed species exhibited also the highest chlorophyll *c* (Ch *c*) content (1.36 ±0.13 mg/mL of extract), followed by *Sargassum vulgare* (3.95± 0.07 mg/mL of extract) and *Padina sp* (0.63± 0.08 mg/mL of extract). Amongst the brown seaweed species collected in the current study, *Phyllaria reniformis* exhibited significantly the highest content in Chl *a*, Chl *b* and Ch *c* ($p \leq 0.05$).

The total carotenoids and fucoxanthin contents were summarized in figure IV.4. Total carotenoids content varied from 30.19±2.07 to 22.19±0.26 mg/mL of extract. As found in the chlorophyll contents, *Phyllaria reniformis* was characterized by the highest content of carotenoids while *Padina sp*. revealed the lowest carotenoids level. Though one-way ANOVA total carotenoids results suggested no significant difference ($p > 0.05$). Maximum concentration of fucoxanthin was also noted in *Phyllaria reniformis* 19.21± 1.17 mg/mL of extract with a significant difference ($p \leq 0.05$) in comparison to the remaining species.

To the best of our knowledge, no published reports were found about using spectrophotometry for the evaluation of total chlorophyll and carotenoid contents in the three collected seaweed: *Padina sp. Sargassum vulgare and Phyllaria reniformis* pigment extracts. For that reason, a comparison with other brown seaweed species will be achieved. Similar trend in carotenoid contents was obtained in other brown seaweed species *Padina Gymnospora, Sargassum ilicifolium* and *Sargassum Polycustum* by **Kumar et *al.* (2009)**.

For better comparison, the ratios of Chl *b*/Chl *a*, Car/Chl *b* and Car/Chl *a* of the three brown seaweeds *Padina sp., Sargassum vulgare and Phyllaria reniformis* were calculated and are represented in Table IV.1.

Table IV.1: Ratios pigments of the selected brown seaweeds.

	Chl *b*/Chl *a*	**Car/Chl *a***	**Car/Chl *b***
Padina sp	0.09	1.33	14.68
Sargassum vulgare	0.23	1.72	7.45
Phyllaria reniformis	0.32	1.10	3.44

The obtained ratios Chl *b*/Chl *a*, Car/Chl *a* and Car/Chl *b* in the three selected seaweeds were higher than those obtained by **Kumar et *al.* (2009)** in brown seaweeds. According to our results Ch *b* was lower than Ch *a* and constituted almost 1/3, 1/4 and 1/10 in *Phyllaria reniformis*, *Sargassum vulgare* and *Padina sp.*, respectively. While, carotenoids were slightly higher than Chl *a* particularly in *Sargassum vulgare*. The high Car/Chl *b* ratios reflected the high carotenoids contents in the three algae especially *Padina sp*. (14.68).

Dere et *al.* (2003) reported that pigment contents are related to the algal taxa, geographical zones, and depth of collect in the sea. Furthermore, the successful quantitative analysis of pigments could be hampered by the fact that a part of seaweed pigments is not detected because it is bonded to various macromolecules (proteins, carbohydrates, cell wall, lipids, etc.) **(Terasaki et *al.*, 2012)**. For that reason, further analysis of pigments quality and quantity contents by HPLC will be presented in the following sections.

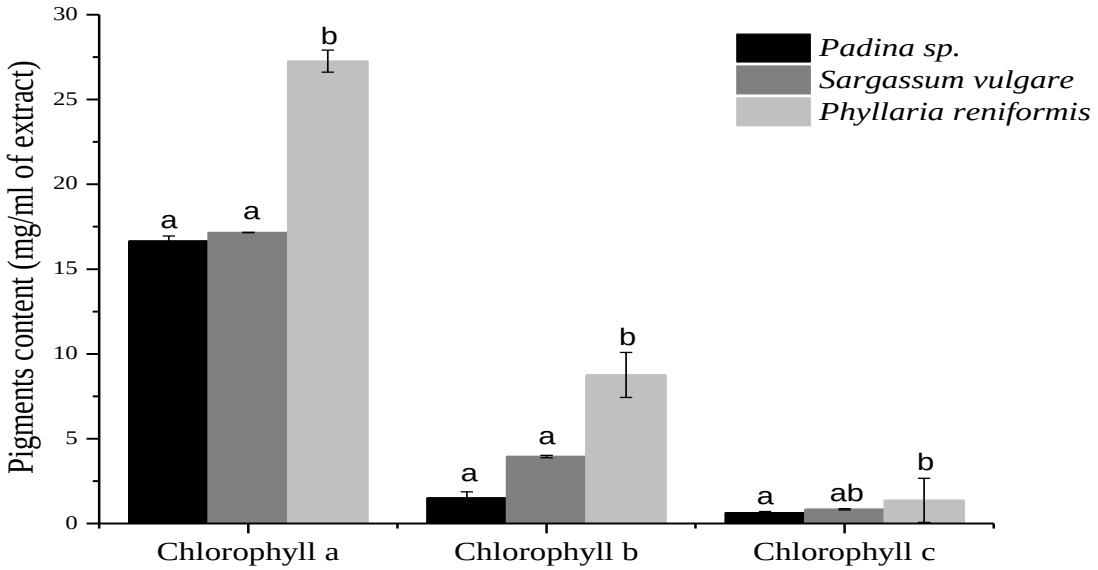


Figure IV.3: Chlorophylls content of pigment extracts of *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis* (Mean ± SD). Within any given pigment, bars with different letters indicate significant differences between alga species (*p*-value ≤ 0.05, Tukey's HSD test)

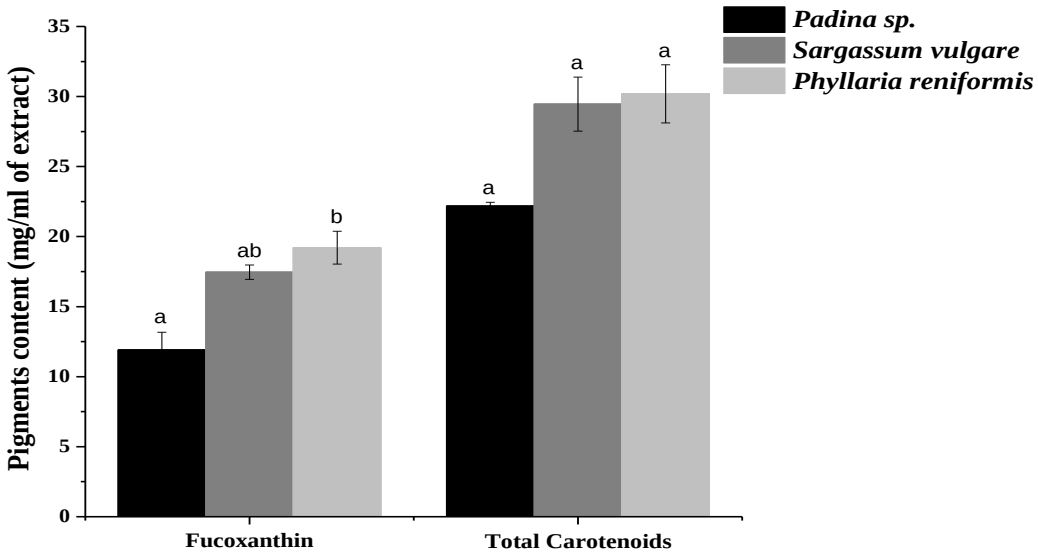


Figure IV.4: Total carotenoids and fucoxanthin content in pigment extracts of *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis* (Mean ± SD). Within any given pigment, bars with different letters indicate significant differences between alga species (*p*-value ≤ 0.05, Tukey's HSD test)

IV.3.2. Colour measurement

Colorimetric parameters (*L**, *a**, *b**) of pigment extracts of *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis* are given in figure IV.5. Results showed a significant difference between the pigment extracts of the three species of brown seaweeds, especially in *L** and *a** values ($p \leq 0.05$).

Compared to *Sargassum vulgare* and *Padina sp,, Phyllaria reniformis* pigment extract was the highest in redness (*a** value =19.03±0.67) and in yellowness (*b**value=16.32±0.04), while it exhibited the lowest score in lightness (*L** value=20.44±1.30). The greener seaweed pigment extract was that obtained from *Sargassum vulgare* where *a** parameter was negative (-0.04±0.03).

Staumite et *al*. (2015) and **Itle and Kabelka (2009)** reported relationship between colour parameters and pigment concentration. According to **Indrawati et *al*. (2015)**, *b** value may represent the pheophytin a as well as chlorophyll c, while a* value may represent carotenoids. Similarly, in the present study, the high a* and b* values obtained, in *Phyllaria reniformis* pigment extract reflected its richness in chlorophyll c and carotenoids as shown in the pigment analysis using spectrophotometric method in section IV.3.1.

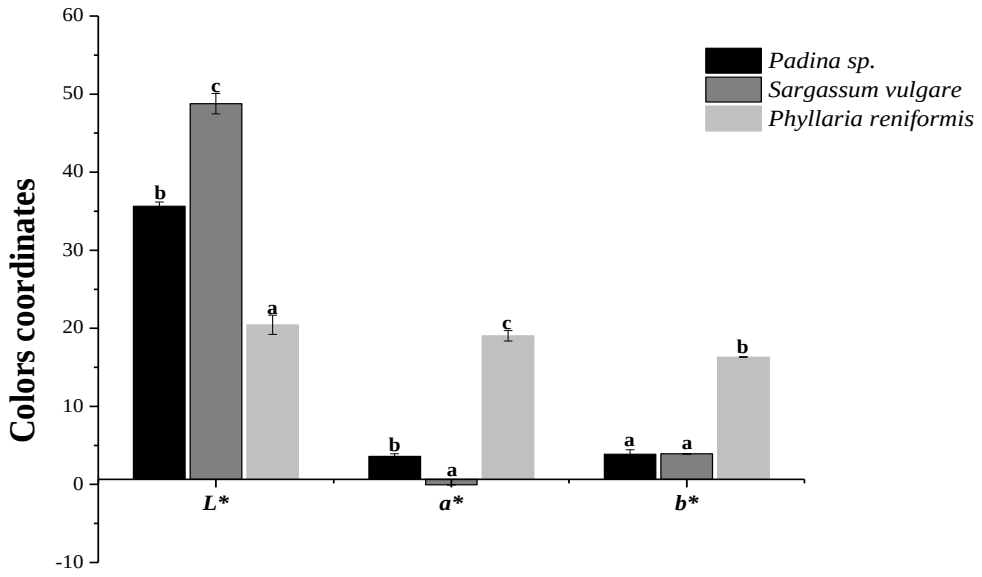


Figure IV.5: The *L, *a** and *b** colour parameters of *Padina sp. Sargassum vulgare*, and *Phyllaria reniformis* pigment extracts.**

IV.3.3. HPLC analysis

HPLC has been recognized as a reliable chromatography technique for the separation and identification of photosynthetic pigments. It was applied to separate pigments according to their polarity, from polar to nonpolar pigments such as chlorophyllide to pheophytin in chlorophyll group or xanthophyll to carotene in carotenoid group **(Brotosudarmo et *al*., 2018)**.

In the present study, pigments extracted from the three brown seaweeds *Padina sp. Sargassum vulgare and Phyllaria reniformis* were separated and identified according to their retention time using the RP-HPLC technique. Obtained chromatograms are illustrated in figure IV.6. Separated photosynthetic pigments of the three brown seaweed extracts and their retention times are summarized in table IV.2.

As shown in the chromatograms (Figure IV.6), sixteen individual photosynthetic pigment peaks were detected in *Sargassum vulgare* and *Padina sp* pigment extracts, and only thirteen peaks were found in *Phyllaria reniformis* pigment extract.

The same major pigment peaks were observed with a good resolution in the three pigment extracts which were from the polar to the non-polar end of the chromatogram as follow: chlorophyllides *a*, chlorophyll *c3*, and chlorophylls *c1*, *c2*, fucoxanthin, trans-neoxanthin, Chl *b*, Chl *a*, pheophytin *a* and *β*-carotene. Similar results were obtained previously in other brown seaweed species: *Padina australis* **(Brotosudarmo et *al*., 2018)**, *Sargassum horneri*, *Cystoseira hakodatensis*, and *Undaria pinnatifid* **(Terasaki et *al*., 2012)**. In agreement with seaweed pigment extracts analyzed in the present study, **Yalcin et *al*. (2020)** reported that the main pigments generally found in the brown seaweeds were fucoxanthin, Chl *a*, pheophytin *a*, β-cart and Chl *c*.

Several reports demonstrated that the most abundant and typical pigment produced by brown seaweed was fucoxanthin **(Brotosudarmo et *al*., 2018; Chandini et *al*., 2008; Masashi Hosokawa et *al*., 2009).** While, chlorophyll *c* was the characteristic chlorophylls of phaeophyta: The brown seaweeds family **(Hegazi et *al*., 1998)**. These conclusions agreed with the obtained results in the present study, where fucoxanthin constituted the major detected pigments in the three seaweeds *Padina sp., Sargassum vulgare and Phyllaria reniformis*, and chlorophyll *c* was present in the three extracts indicating the phylum of brown alga.

Table IV.2. Photosynthetic pigments of three brown seaweeds *Padina sp. Sargassum vulgare*, and *Phyllaria reniformis* pigment extracts

Peak	Retention time (min)	Pigment	*Padina sp.*	*Sargassum vulgare*	*Phyllaria reniformis*
0	1.8	Solvent	+	+	+
1	3.9	Chlorophyllide *a*	+	+	+
2	4.7	Chlorophyll *c3*	+	+	+
3	5.68	Chlorophyll *c1, c2*	+	+	+
4	6.68	UNK*	+	+	+
5	7.4	Fucoxanthin	+	+	+
6	8.5	Trans-neoxanthin	+	+	+
7	8.8	UNK	+	+	+
8	9.3	UNK	+	+	+
9	10.8	UNK	+	+	-
10	10.9	UNK	+	+	-
11	13.77	UNK	+	+	-
12	14.4	Chlorophyll *b*	+	+	+
13	14. 7	Chlorophyll *a*	+	+	+
14	15.00	Chlorophyll *a*	+	+	+
15	16.6	Phaeophytins	+	+	+
16	17.50	β Carotene	+	+	+

**UNK: unknow*

As shown in figure IV.6, the level of fucoxanthin and Chl *a* were in the following decreasing order *Phyllaria reniformis>Sargassum vulgare > Padina sp*. While chlorophyll *c1*, *c2* peak was highly present in *Phyllaria reniformis* compared to the remaining species. In contrast, the peak appearing at 8.5 min identified as trans-neoxanthin was highly present in both *Sargassum vulgare* and *Padina sp*.

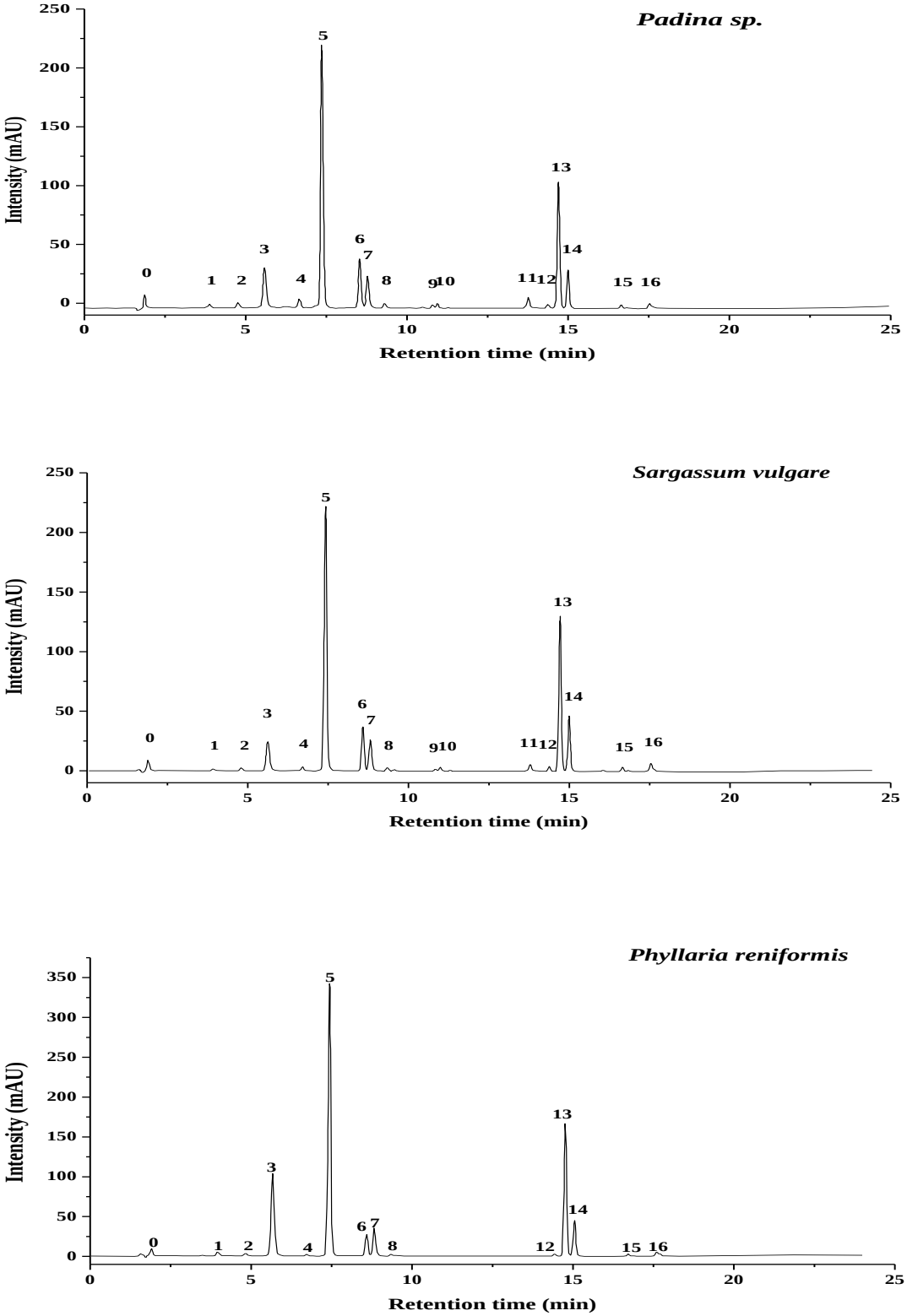


Figure IV.6. RP-HPLC Chromatogram separation of pigment extracts from *Padina sp.*, *Sargassum vulgare* and *Phyllaria reniformis*.

IV.3.4. Thin-layer Chromatography (TLC)

Pigments extracted from *Padina sp*., *Sargassum vulgare* and *Phyllaria reniformis* were separated through TLC using different solvents. Colour patterns formed on the TLC plate is the basis for the identification of pigments type contained in the three seaweed extracts. The chromatographic profiles were visualized under visible light and UV radiation at 365nm and the R_f values are presented in the figures IV.7 for 100% acetone and 100% methanol as eluting solvents and in figure IV.8 for 90% acetone and 90% methanol as eluting solvents. Obtained results showed that using acetone or methanol as solvent at the ratio of 100 or 90% allowed to separate pigment extracts.

Colour sequences that appeared in TLC plate were: green, yellow, yellowish green, bluish green, orange and gray. According to **Pesang et *al.* (2020)** Chl *a* expressed green blue colour, Chl *b* expressed yellow green colour and carotenoids expressed yellow, orange to red colours, whereas, gray spots was suspected to be a pheophytin *a*.

Three colour spots were obtained with pure acetone in *Phyllaria reniformis* extract yellow green (R_f=0.55), orange (R_f=0.78) and a strong green band (R_f=1.86), corresponding to Chl *b*, carotenoids and Chl *a*, respectively. While, only two colour spots (orange and green) were observed in *Padina sp*. and *Sargassum vulgare* extracts corresponding to Chl a and carotenoids, this difference is probably due to the higher level of Chl *b*, carotenoids and Chl *a* in *Phyllaria reniformis* pigment extract as shown previously in the HPLC and spectrophotometry analysis. These results could also be related to the type of solvent used, this is why other solvents were used at different concentrations.

Meanwhile, with pure methanol, four colour spots were formed in *Padina sp*., *Sargassum vulgare* and *Phyllaria reniformis* extracts gray (R_f=0.52), green blue (R_f=0.6), yellow green (R_f=0.65) and orange (R_f=0.78) probably indicating pheophytin *a*, Chl *a*, Chl *b*, and carotenoids, respectively. Likewise, when using 90% of aqueous acetone, four spots were obtained which were likely pheophytin a (R_f=0.54), Chl *a* (R_f=0.61), Chl *b* at yellow green spot (R_f=0.73) and carotenoids at orange spot (R_f=0.80). On the other hand, five main spots were observed in *Phyllaria reniformis* extract and four spots in *Padina sp*., and *Sargassum vulgare* extracts when using 90% aqueous methanol.

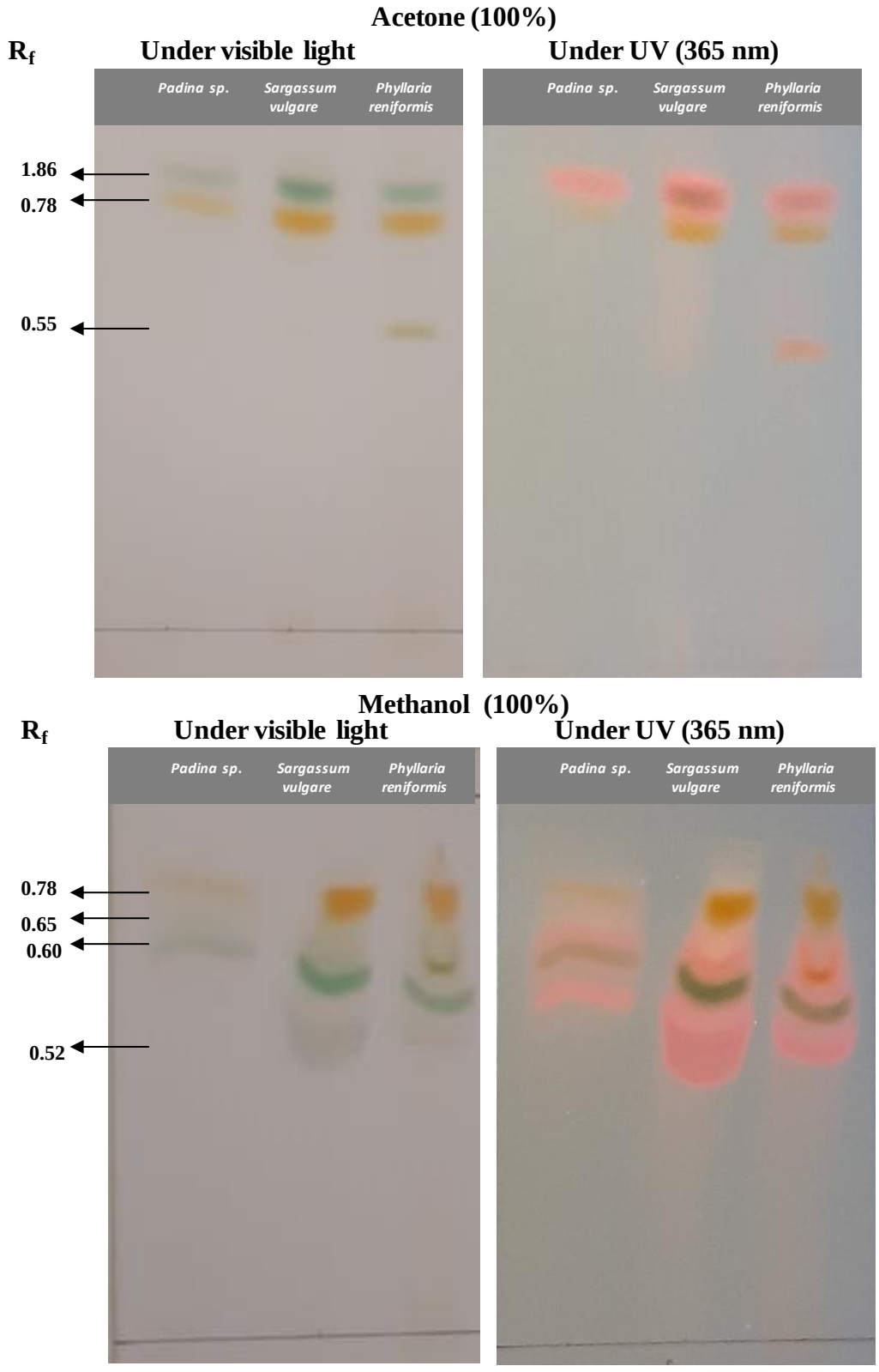


Figure IV.7: Thin-layer Chromatography plates of *Padina sp.*, *Sargassum vulgare* and *Phyllaria reniformis* extracted pigments eluted with 100% acetone and 100% methanol with indication to retention factor values under visible or UV light.

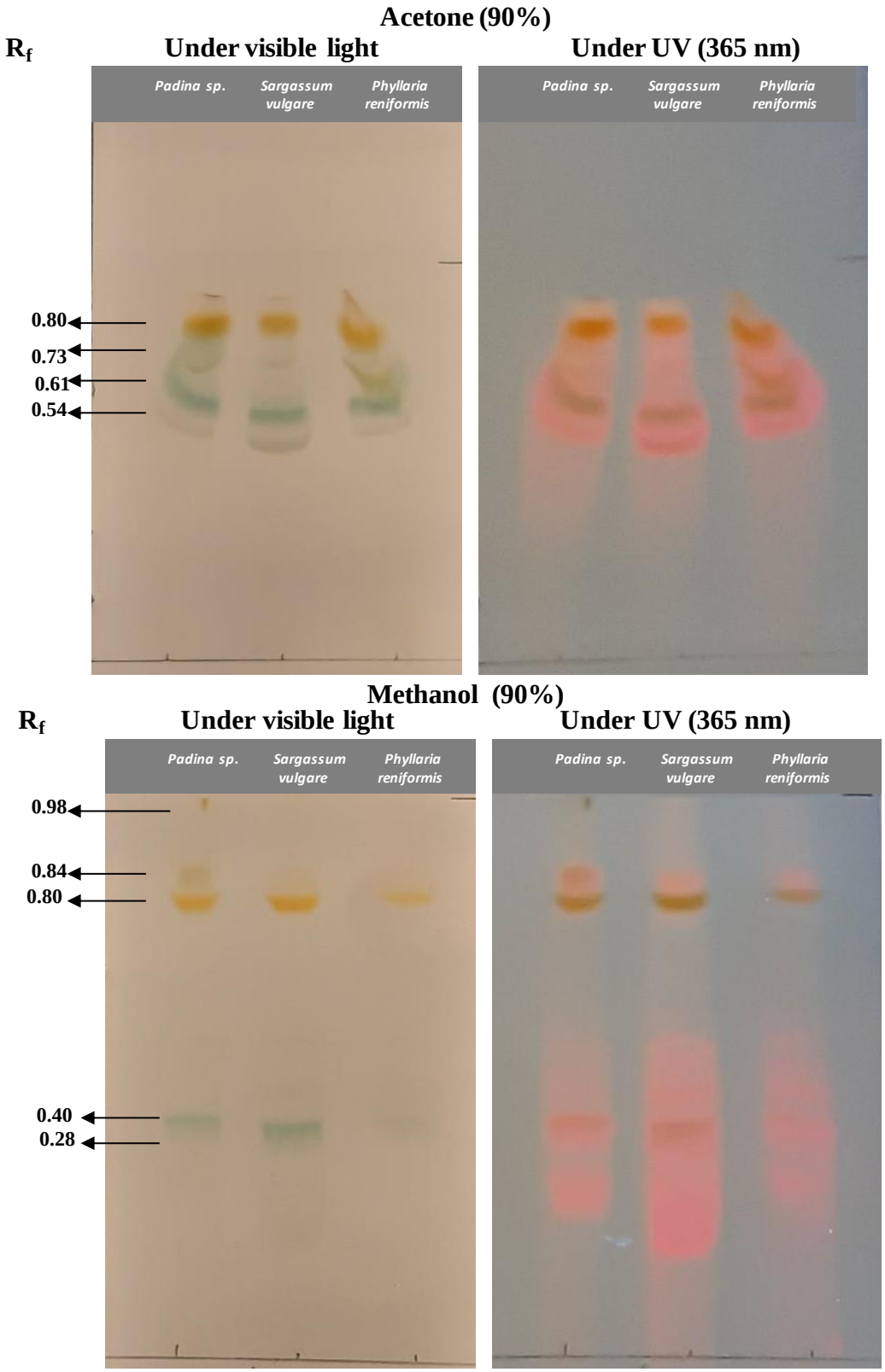


Figure IV.8: Thin-layer Chromatography plates of *Padina sp.*, *Sargassum vulgare* and *Phyllaria reniformis* extracted pigments eluted with 90% acetone and 90% methanol with indication to retention factor values under visible or UV light

IV.3.5. Fourier Transform Infrared Spectroscopy (FTIR)

ATR-FTIR spectroscopy of the pigment extracts of *Padina sp. Sargassum vulgare and Phyllaria reniformis.* was performed to detect the functional groups seaweed pigments. The main functional groups of the three brown seaweed pigment extracts are indicated with their wavenumber values in table IV.3 and figure IV.9. (A, B and C).

The three pigment extracts exhibited similar FTIR spectral profiles. The same common recorded peaks corresponded to the major functional groups of chlorophylls and carotenoids. Chlorophylls and their derivatives have characteristic infrared absorption bands of C=O, C=C and C-H groups **(A. S. Holt & Jacobs, 1955)**. The absorption bands situated in the region between 3391.29 and 3011.30cm^{-1} showed the presence of –OH bonds **(Yip et *al*., 2014)**. The peaks appearing around 2920 and 2850 cm^{-1} could be associated with asymmetric and symmetric C-H stretching vibrations of methyl (CH_2) groups, respectively **(A. S. Holt & Jacobs, 1955; Y. R. Kang et *al*., 2018; Merdekawati et *al*., 2019; Yip et *al*., 2014)**. The band near 1730 and 1280 cm^{-1} corresponded to the carbonyl group C=O **(Y. R. Kang et *al*., 2018; Xiaoli et *al*., 2018)**. The C=C stretching vibrations were observed between 1616 and 1510 cm^{-1}**(Y. R. Kang et *al*., 2018; Xiaoli et *al*., 2018)**. According to **Quijano-Ortega et *al*. (2020)**, the peak around 1550–1600 cm^{-1} could be attributed to C=C bond stretching vibrations of β-carotene.

The spectral responses near 1460 and 1370 cm^{-1} showed the presence of–C–H bonds **(Quijano-Ortega et *al*., 2020; Xiaoli et *al*., 2018; Yip et *al*., 2014).** The absorption bands situated in the region between 1100 and 1000 cm^{-1} could be attributed to the C–H bending or C–O or C–C stretching vibrations **(Anand & Suresh, 2015)**.

In *Padina sp. Sargassum vulgare and Phyllaria reniformis* pigment extractsFTIR spectra, it is also possible to identify characteristic bands located in the range of 980-500 cm^{-1}, these absorbance peaks may correspond to the C–H bending and stretching vibrations **(Anand & Suresh, 2015; Quijano-Ortega et *al*., 2020)**. **Quijano-Ortega et *al*. (2020)** reported that β-carotene produces a characteristic band around 968 cm^{-1}.

In the present study, ATR-FTIR analysis confirmed the presence of all functional groups corresponding to chlorophylls and carotenoids which were demonstrated in the previous analysis UV-VIS and HPLC results.

Table IV.3: FTIR peak assignment table of *Padina sp*., *Sargassum vulgare, Phyllaria reniformis,* and pigment extracts.

Functional groups	***Padina sp.***	***Sargassum vulgare***	***Phyllaria reniformis***	**References**
O-H	3279.83, 3010.11	3246.05, 3193.36, 3011.30	3391.29, 3335.18, 3282.77, 3010.78	**(Yip et *al*., 2014).**
C-H	2921.09	2922.28	2922.76	**(Y. R. Kang et *al*., 2018).**
C-H	2851.28	2851.80	2852.54	**(Merdekawati et *al*., 2019; Yip et *al*., 2014)**
C=O	1733.53	1736.59	1738.30	**(Y. R. Kang et *al*., 2018; Xiaoli et *al*., 2018; Yip et *al*., 2014).**
C=C	1608.34	1613.69	1616.48	**(Munawaroh et *al*., 2019; Xiaoli et *al*., 2018).**
C=C	1574.93, 1536.47	1575.82, 1538.42	1524.14	**(Merdekawati et *al*., 2019; Quijano-Ortega et *al*., 2020; Yip et *al*., 2014).**
C – H	1464.28	1459.37	1461.10	**(Yip et *al*., 2014)**
C – H	1378.43	1376.34 1302.89	1376.89	**(Yip et *al*., 2014).**
C=O	1282.62	1247.50	1276.46	**(Merdekawati et *al*., 2019).**
C-H, C=O, C=C	1157.77, 1056.46, 1032.96	1082.79, 1053.30, 1021.68	1147.38, 1053.15, 1033.96	**(Anand & Suresh, 2015)**
C – H	853.18, 713.98	967.19, 930.60, 853.59, 712.02, 623.12	880.74, 823.08, 701.78, 627.42	**(Anand & Suresh, 2015; Merdekawati et *al*., 2019; Quijano-Ortega et *al*., 2020)**

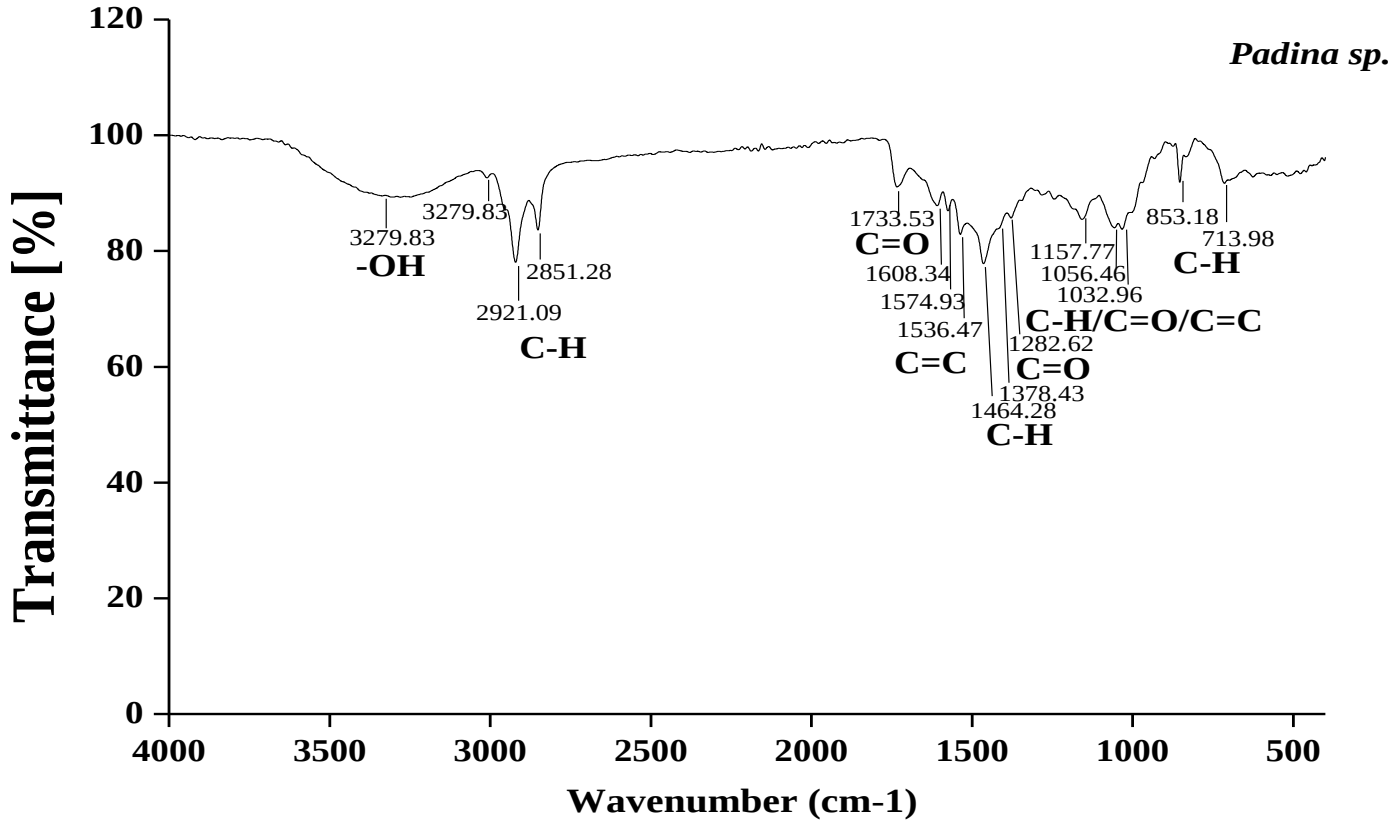


Figure IV.9.A: Transmission FTIR spectra of pigment extracts (*Padina sp.*)

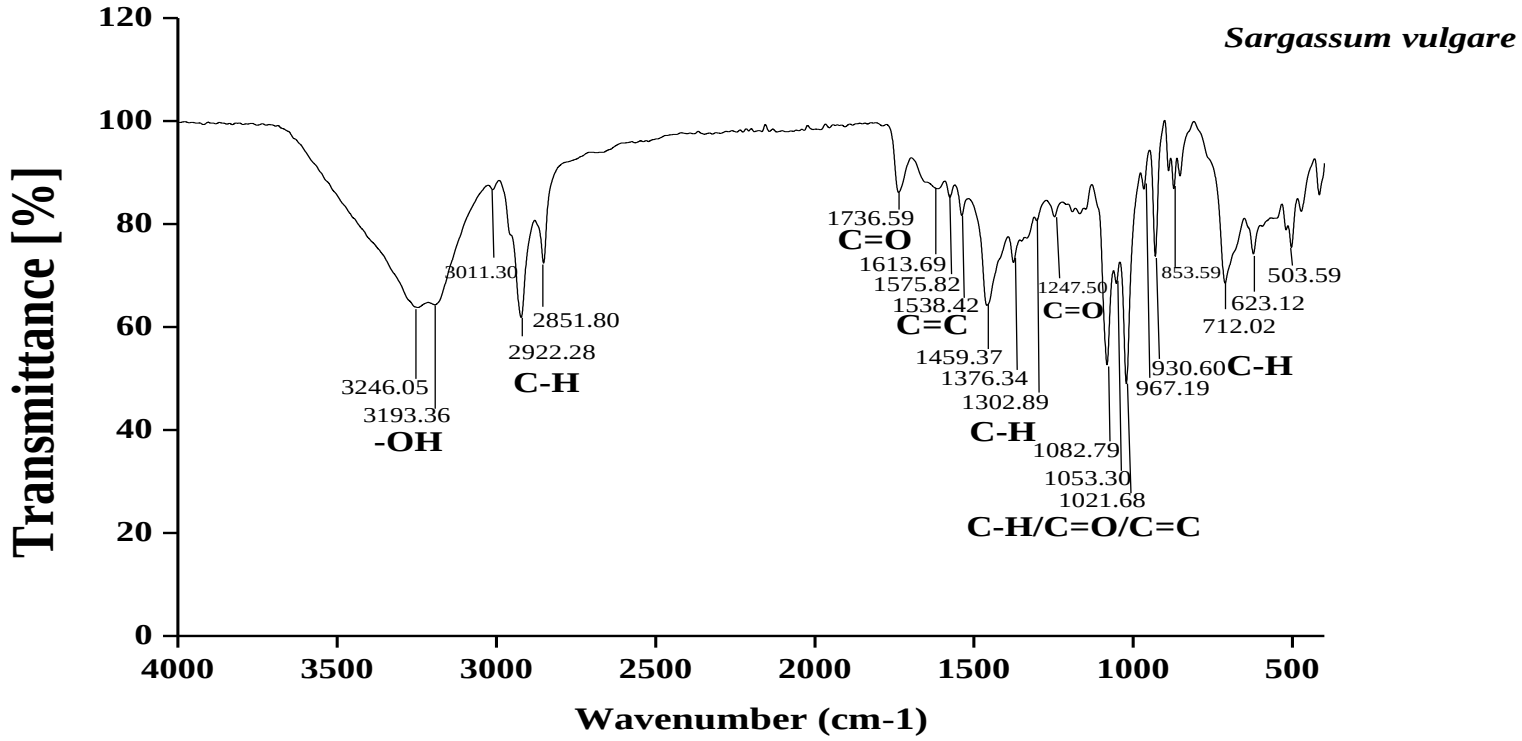


Figure IV.9.B: Transmission FTIR spectra of pigment extracts (*Sargassum vulgare*)

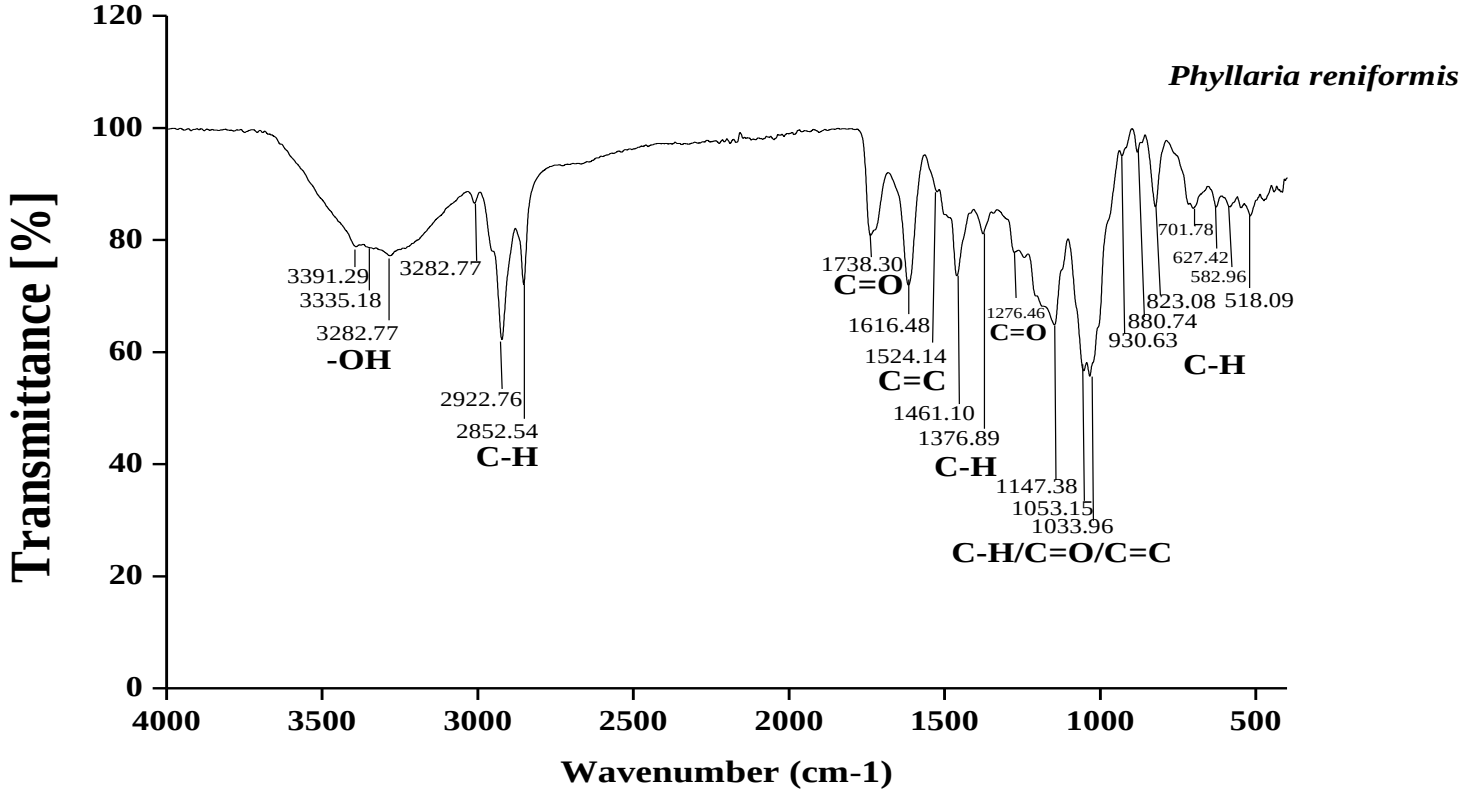


Figure IV.9.C: Transmission FTIR spectra of pigment extracts (*Phyllaria reniformis*)

IV.3.6. DPPH Radical Scavenging Activity

DPPH test has been used extensively for investigating the free radical scavenging activities of compounds **(Duan et *al.*, 2006)**. This method was also used to determine antioxidant activity in various species of alga **(Bianco et *al.*, 2015)**. In this study, antioxidant activity of synthetic antioxidant (BHA and BHT) likewise *Padina sp. Sargassum vulgare and Phyllaria reniformis* pigment extracts was evaluated by DPPH assay. Free radical-scavenging capacities of *Padina sp., Sargassum vulgare and Phyllaria reniformis* pigment extracts are shown in figure IV.10 and their IC50 values are illustrated in figure IV.11.

Results showed that increasing concentrations (0-100µg/mL) in the three seaweed pigment extracts, BHA and BHT induced raising DPPH scavenging activity (%). This improvement was different among samples, the order of DPPH scavenging activity (%) was as follows: BHA> *Phyllaria reniformis* pigment extract >BHT> *Sargassum vulgare* pigment extract >*Padina sp*. pigment extract. In comparison to BHT and pigment extracts, BHA showed the highest DPPH scavenging activity with low IC50 values 7.59±0.52 µg/mL.

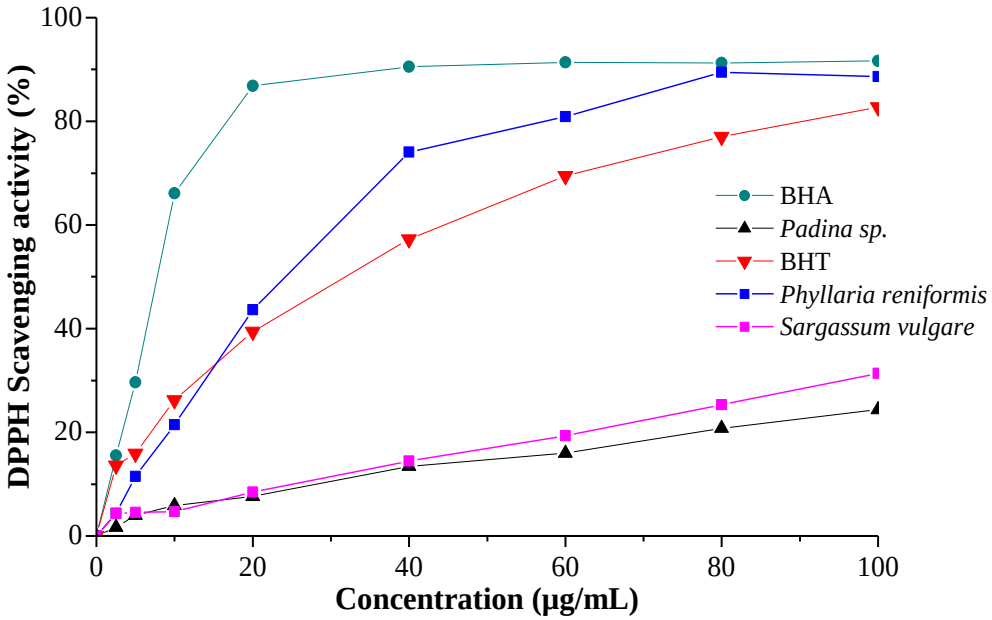


Figure IV.10: Free radical-scavenging capacities of reference antioxidant (BHA, BHT) and pigment extracts obtained from *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis*

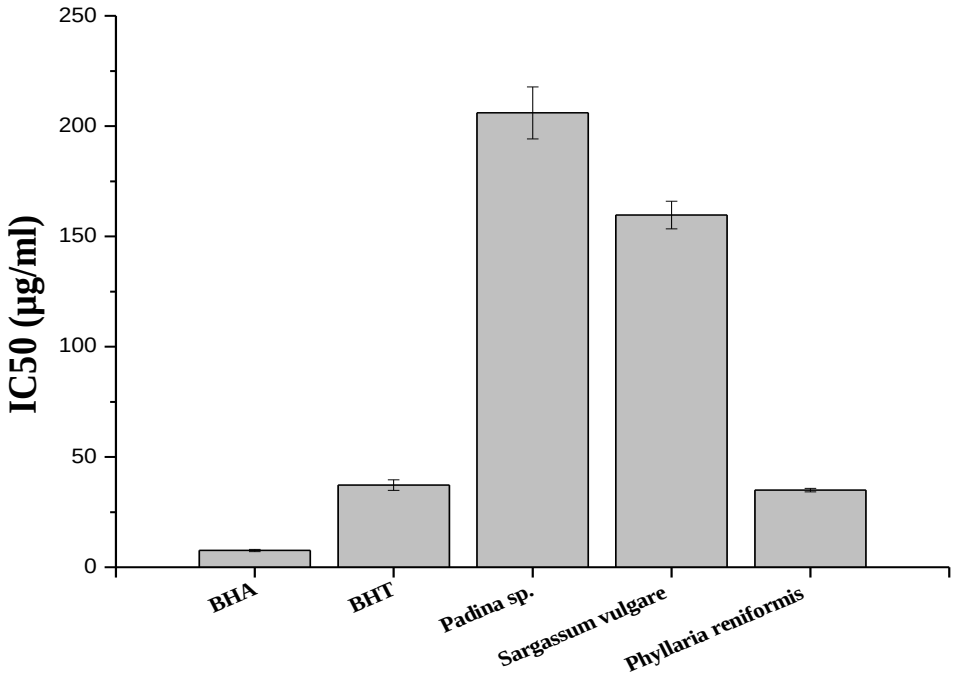


Figure IV.11: DPPH (IC50) values of reference antioxidants (BHA, BHT) and pigment extracts obtained from *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis* (Mean ± SD)

Phyllaria reniformis pigment extract exhibited higher antiradical activity (IC50 = 34.96 ± 0.84 µg/mL) in comparison to BHT and both remaining pigment extracts. This is probably due to the highest content of chlorophyll and carotenoids in *Phyllaria reniformis* pigment extract. According to **Hidayati et *al*. (2019)** chlorophyll and carotenoids especially fucoxanthin were the main pigments found in seaweed and have the potential effect to reduce the free radicals from DPPH. Whereas pigment extract from *Sargassum vulgare* and *Padina sp*. revealed a low antioxidant activity with IC50 values 159.67± 6.3 and 205.98± 11.74 µg/mL, respectively.

Nisa et *al*. (2020) reported that the IC50 value is divided into three categories: very strong (IC50<50 ppm), strong (IC50 50-100 ppm), moderate (IC50 100-250 ppm), weak (IC50 250-500 ppm) and inactive (IC50> 500 ppm). Based on the obtained results, the antioxidant activity of *Phyllaria reniformis* pigment extract is classified as very strong, while the two other piment extracts as moderate.

The present study suggests that the three brown seaweeds pigment extracts could constituted a rich source of antioxidant compounds, in particular *Phyllaria reniformis*.

IV.3.7. Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was used as second test to confirm the antioxidant activity of seaweed pigment extracts by DPPH method. The antioxidant potential was estimated from the ability of *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis* pigment extracts to reduce TPTZ-Fe $^{(III)}$ to TPTZ-Fe $^{(II)}$. Reducing activity of pigment extracts determined by FRAP is shown in figure IV.12. *Phyllaria reniformis* pigment extract was more reactive than *Sargassum vulgare* and *Padina sp*, the three pigment extracts antioxidant activities were lower but closer to BHT, while, BHA had a strong reducing power compared to other samples.

To date, no publications was found concerning the assessment of the antioxidant activity of brown seaweed pigment extracts using FRAP assay, which made it difficult to compare the results of the present study.

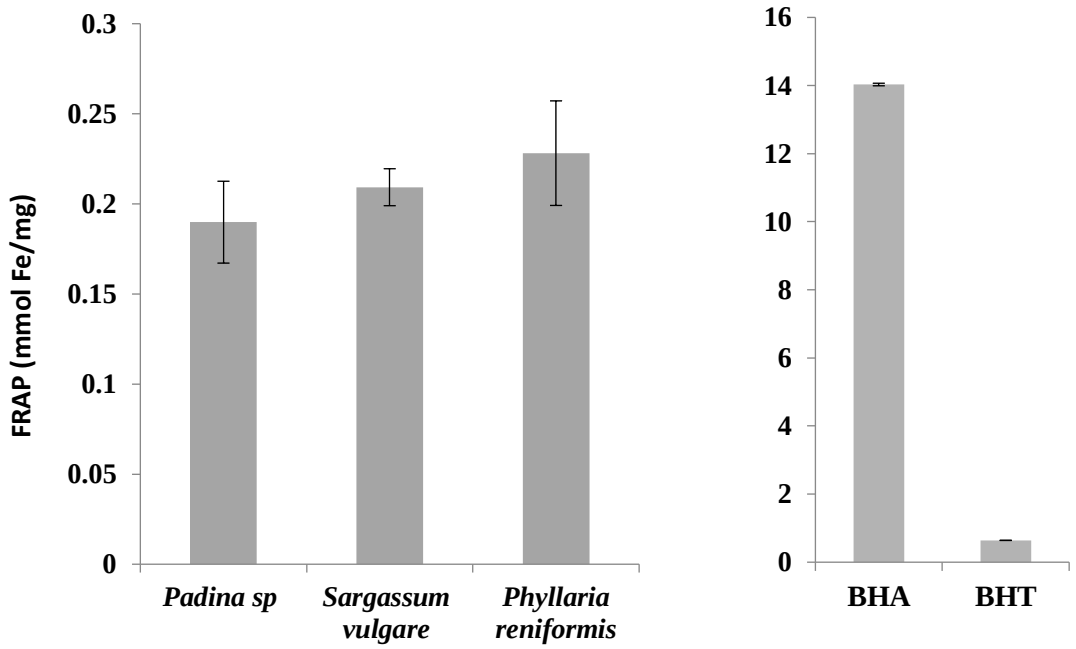


Figure IV.12: FRAP Assay of pigment extract of the selected seaweeds *Padina sp*. *Sargassum vulgare* and *Phyllaria reniformis* pigment extracts.

IV.3.8. Antimicrobial activity

Antimicrobial properties of pigment extracts were evaluated against five pathogenic strains: *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus*

(ATCC 6938), *Candida albicans* (ATCC 10231) and *Aspergillus basiliensis* (ATCC 16404). The agar diffusion technique was used to determine the bactericidal effect of the pigment extracts. Obtained results are summarized in Table IV.4 and figures IV.13- IV.16.

No antimicrobial activity was observed against the bacterial strains tested in this study, in the three pigment extracts at concentration range of 10 to 200 mg/mL. Whereas synthetic antibiotics (Primazol and Lamidaz) revealed great antimicrobial activities against bacteria and fungi, respectively. As shown in the three seaweed pigment extracts in this study, **Baraka et *al*. (2017)** reported that chlorophyll did not show any antimicrobial activity except for *Pseudomonas aeruginosa*. In another study, **Maekawa et *al*.(2007)** revealed that chlorophyll presented antifungal activity against *Candida albicans*.

Karpiński & Adamczak (2019) reported that fucoxanthin acted against 13 bacteria growing in aerobic condition including *Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella oxytoca*. While it was not active against strict anaerobic bacteria.

Table IV.4. Antimicrobial activities of pigment extracts of the three selected seaweeds and synthetic antibiotics against some pathogenic strains.

Pigment extracts [mg/mL] / Strains	*Padina sp.*				*Sargassum vulgare*				*Phyllaria reniformis*				Primazol	Lamidaz
	10	20	40	200	10	20	40	200	10	20	40	200	10	10
Escherichia coli (ATCC 8739)	-	-	-	-	-	-	-	-	-	-	-	-	+	/
Bacillus subtilis (ATCC 6633)	-	-	-	-	-	-	-	-	-	-	-	-	+	/
Staphylococcus aureus (ATCC 6938)	-	-	-	-	-	-	-	-	-	-	-	-	+	/
Candida albicans (ATCC 10231)	-	-	-	-	-	-	-	-	-	-	-	-	/	+
Aspergillus basiliensis (ATCC 16404)	-	-	-	-	-	-	-	-	-	-	-	-	/	+

-: no antimicrobial activity, +: antimicrobial activity, /: not tested

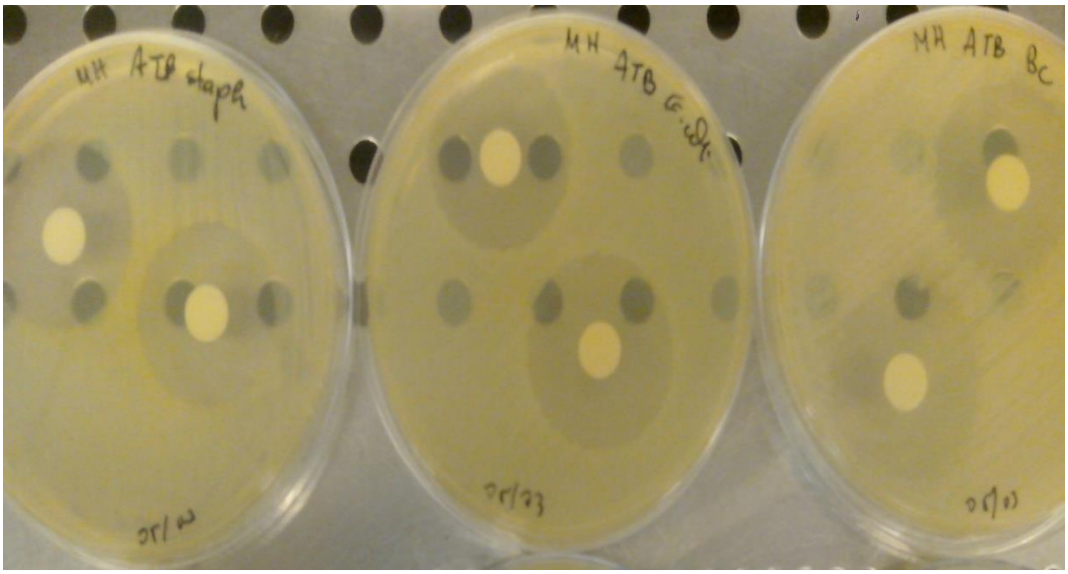


Figure IV.13: Antibacterial activity of antibiotics alone in the agar diffusion assay

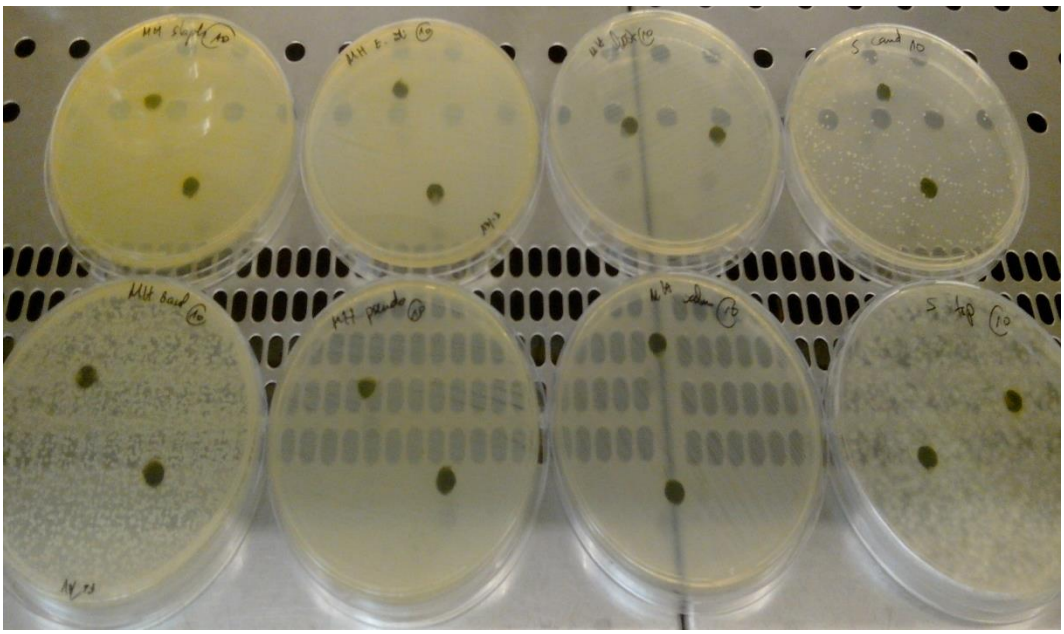


Figure IV.14: Antibacterial activity of *Padina sp*. in the agar diffusion assay

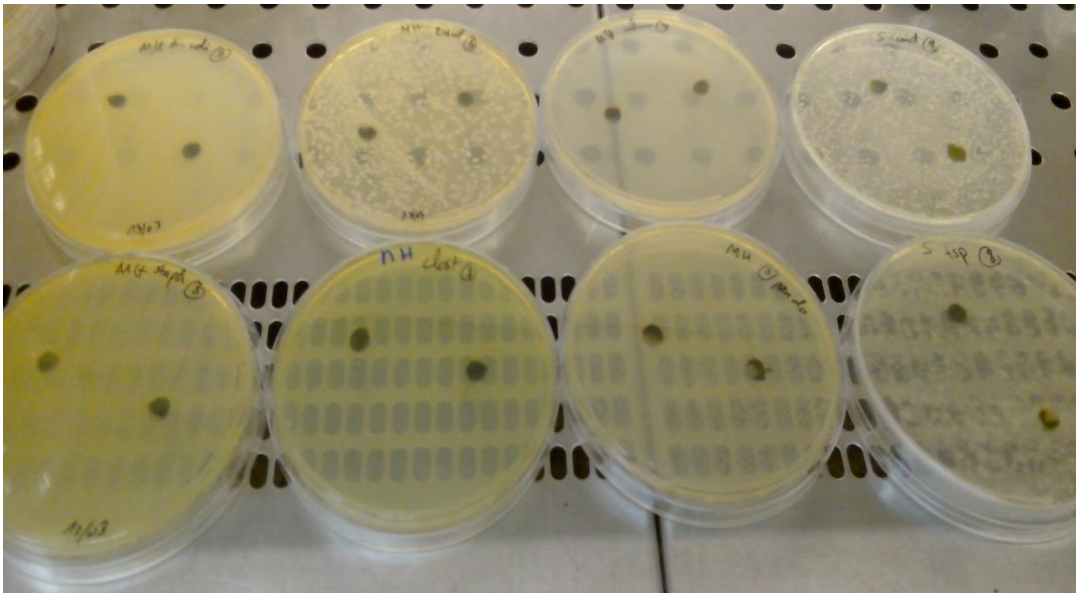


Figure IV.15: Antibacterial activity of *Sargassum vulgare* in the agar diffusion assay

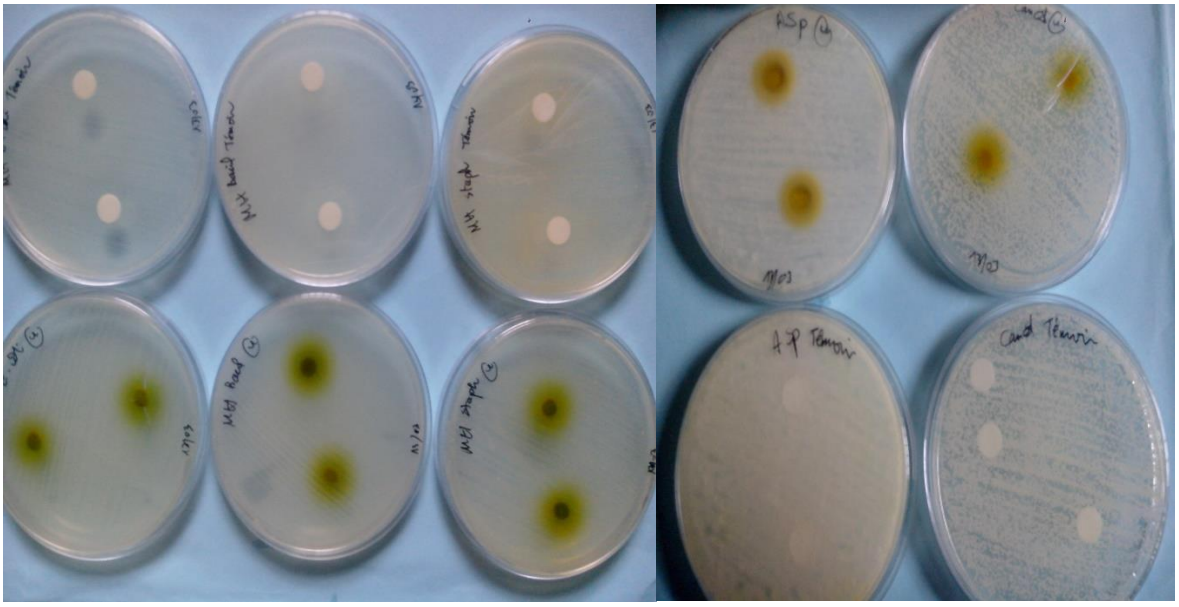


Figure IV.16: Antibacterial activity of *Phyllaria reniformis* in the agar diffusion assay

IV.4. Conclusion

Composition of pigments from *Padina sp. Sargassum vulgare and Phyllaria reniformis* brown seaweeds collected in Algerian coast was investigated with UV-Visible spectrophotometric method, RP-HPLC and ATR-FTIR spectroscopy. The experimental results showed that these species presented the same major pigments Fucoxanthin, Ch *a*, Ch *b*, Ch *c* and *β* carotene. Within the three selected seaweeds, *Phyllaria reniformis* contained significantly the highest amounts of pigments. In addition, antioxidant activity was assessed using two methods (DPPH and FRAP), results showed that *Phyllaria reniformis* pigment extract showed the highest antioxidant effect in both antioxidant assays DPPH and FRAP.

No antimicrobial activity was detected against bacteria and fungi strain in brown seaweeds pigments extracted from *Padina sp. Sargassum vulgare and Phyllaria reniformis.*

The present study suggests that the three collected brown seaweeds especially *Phyllaria reniformis* could be a potential source of pigments for industrial applications.

CHAPTER V

Impact of freezing and drying preprocessing on pigments extraction

V.1. Introduction

Nowadays marine macro-algae commonly known as seaweeds have been extensively used in food **(Bocanegra et *al*., 2009; Dulmaz et *al*., 2008; Maryam et *al*., 2017)**, agricultural **(Ramya et *al*., 2015)**, pharmaceutical **(Raman & Doble, 2015)** and cosmetic areas **(Fabrowska et *al*., 2015)**. They represent a natural source of bioactive compounds as they are able to produce a great variety of secondary metabolites such as pigments, flavonoids, polyphenols characterized by several biological proprieties **(Duan et *al*., 2006; Kuda et *al*., 2005; Lim et *al*., 2002; Lordan et *al*., 2011; Rajauria et *al*., 2013; Sivaramakrishnan et *al*., 2017; Vairappan et *al*., 2001).**

In the past decade, natural pigments were researched for their safety and health benefits compared to the synthetic ones. Macro-algae are renewable source of natural pigments such as chlorophylls and carotenoids **(Dulmaz et *al*., 2008; Hegazi et *al*., 1998)**. These Pigments have shown many biological activities as antioxidant **(Hsu et al., 2013; Sachindra et *al*., 2007; Yan et *al*., 1999)**, anti-obesity **(Maeda, Hosokawa, Sashima, & Miyashita, 2007)**, chemotherapeutic **(Hosokawaa et *al*., 2004)** and anti-inflammatory activities **(Shiratori et *al*., 2005)**. Brown seaweeds like the other classes of algae are rich on photosynthetic pigments in particular fucoxanthin and chlorophyll *c* **(Nirmal Kumar et *al*., 2017)**.

Several methods were used for pigments extraction: Conventional or advanced **(P. Kumar et *al*., 2010)** . For brown seaweed, the conventional method could be lengthy and difficult because of the thalli consistency mainly due to the polysaccharides. Therefore, innovative techniques allow obtaining algae pigments more quickly with higher yield and especially with reduced risk of their degradation. Multiple alternative extraction technologies have been suggested, such as ultrasounds, ultrasound-assisted enzymatic hydrolysis, microwaves, supercritical fluids, pulsed electric fields, high-pressure homogenization and liquid pressurization **(Le Guillard et *al*., 2016; Mittal et *al*., 2017; Poojary et *al*., 2016; Zhu et *al*., 2017)**.

Due to the high instability and easy degradation of pigments, new strategies for samples preprocessing before extraction must be suggested. Acid, enzymes, temperature, heat, light and oxygen, are the most important factors affecting the stability of naturals pigments. Although, many studies on algae pigments extraction and identification have been reported, little information is still available on the relation between pigments content and algae preprocessing. On the other hand, seaweed after their harvest are exposed to degradation, hence, drying and freezing are usually applied to minimize biological compounds degradation and conserve algae for long time.

Against this background, the main purpose of this work was the investigation of the effect of drying and freezing as preprocessing method on seaweed pigments quantity, quality and antioxidants activity. To the best of our knowledge, this is the first report on pigments characterization by spectrophotometer and by RP-HPLC analysis of *Phyllaria reniformis* collected from the Algerian coast and the effect of conservation method on seaweed pigments quality and quantity has also been understated.

V.2. Materials and methods

V.2.1. Seaweed collection and preprocessing

The brown seaweed *Phyllaria reniformis* was collected from Tipaza (Algeria) in June 2016 and washed three times as described in Section IV.2.1. The fresh alga samples were divided in three parts. One part was dried at 38 ± 1°C for one week, another part was frozen at -18°C for one week and the last part was immediately prepared for extraction. All these steps were performed in low light and as quickly as possible to prevent pigment degradation.

V.2.2. Extraction of seaweed pigments

The fresh, frozen and dried alga samples were cut into small pieces of 3 to 5 mm and mixed with acetone at a ratio of 1/3 (w/v). Pigments were extracted in an ultrasonic bath, concentrated, lyophilized and then stored at – 20°C for later analysis as described in Section IV.2.2.

V.2.3. Chlorophylls and carotenoids content

Chlorophylls and carotenoids contents in the fresh dry and frozen *Phyllaria reniformis* pigment extracts were determined by UV-Visible Spectrophotometry (SPECORD 210 PLUS 623F1138, Germany) as described in Section IV.2.3

V.2.4. High performance liquid chromatography pigments Analysis

Pigments separation and identification were performed by analytical HPLC (Agilent 1100, USA) equipped with UV-Visible detector as described in Section IV.2.5. Obtained peaks were identified by comparing the retention times with those of standards pigments, the authentic standard pigments Chlorophylls (*a*, *b*, *c1*, *c2* and *c3*), Chlorophyllides *a*, Fucoxanthin, were obtained from *Sargassum vulgare* and some terrestrial plants by semi preparative HPLC, Pheophytins was obtained by acidification of chlorophyll *a* with 1M HCl **(Wright, 1991)**, *β* carotene was purchased from Sigma Aldrich.

V.2.5. DPPH radical scavenging activity

Antioxidant activity of fresh, dried or frozen *Phyllaria reniformis* pigment extracts were assessed using DPPH radical scavenging method as described in Section IV.2.8

V.2.7. Statistical Analysis

All the analysis was run in triplicate. The data are presented as Mean ± Standard error. The Statistical Package for Social Science (SPSS Version: 20) was used for the analysis. One-way analysis of variance (ANOVA) was performed and comparison of data for significant differences (p-value ≤ 0.05) was made with Tukey's HSD test.

V.3. Results and discussions

V.3.1. UV-Visible absorption spectra of pigments extract

Chlorophylls and carotenoids represent the major group of photosynthetic pigments found in plants and in algae. Each group has multiple types of pigment that can be identified by

the specific wavelength. Pigments absorb on only specific wavelengths of visible light while reflecting the others; the reflected light is colour. The set of wavelengths absorbed by a pigment is its absorption spectrum.

Figure V.1 shows the absorption spectrum of the obtained pigment extracts recorded from 350 to 800 nm. All the pigment extracts absorb mostly in the blue (between 400 and 500nm) and red (between 600 and 700nm) visible spectral regions. A high Abs was observed in pigment extract obtained after freezing preprocessing followed by that obtained after drying preprocessing while the low Abs was recorded in the extract of fresh alga.

The broad absorption in the blue and red regions is probably due to the presence of carotenoids, chlorophyll *a* and chlorophyll *b* in the three pigments extracts. Each pigment has unique Abs spectra, whereas carotenoids absorb visual light broadly in the blue spectral range from 400 to 500 nm, whilst chlorophyll *a* and chlorophyll *b* absorb with narrow bands maximally in the blue (near 430 and 453nm) and red (near 662 and 642nm).

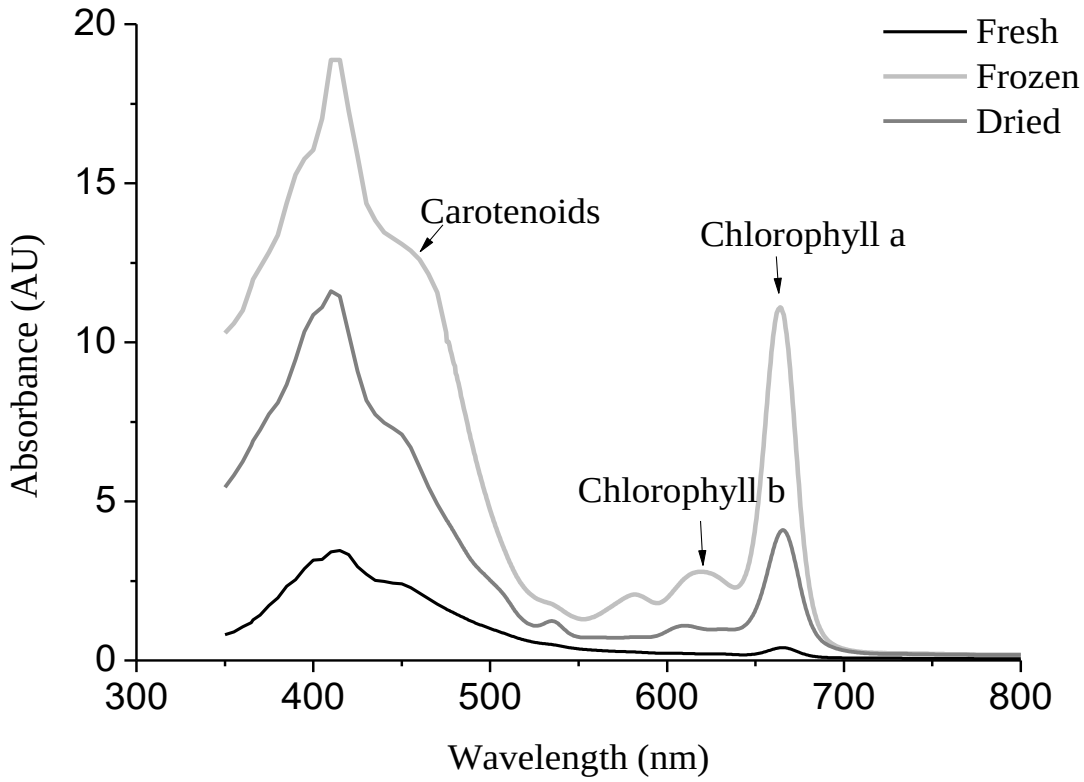


Figure V.1. Absorbance spectra of pigment extract from fresh, frozen and dried *Phyllaria reniformis.*

IV.3.2. Chlorophylls and carotenoids contents

Chlorophylls and carotenoids content in the three pigment extracts (dry, fresh and frozen) were determined by UV-Visible spectrophotometry and presented on figure V.2 for Chlorophyll *a*, *b* and *c* and figure V.3 for fucoxanthin and total carotenoids.

Results showed a variability of quantities for each pigment in relation to the applied preprocessing. For Chlorophyll *a*, *b*, fucoxanthin and total carotenoids, the highest amount were reported in frozen sample extract (7.00 ± 0.57, 2.09 ± 0.82, 2.79 ± 0.33, 3.88 ± 1.09 mg/mL respectively) followed by the dried one (4.78 ± 0.76, 1.57 ± 0.51, 2.58 ± 0.29, 3.49 ± 1.33 mg/mL respectively) and the lowest amount of these pigments were found in the fresh sample (1.19 ± 0.01, 0.73 ± 0.11, 0.66 ± 0.00, 1.31 ± 0.07 mg/mL respectively).

The highest value for chlorophyll *c* was found in the frozen extract (2.31 ± 0.16 mg/mL) followed by the fresh alga extract (1.31 ± 0.36 mg/mL), however the lowest have been demonstrated in extract obtained after drying preprocessing (0.91 ± 0.02mg/mL).

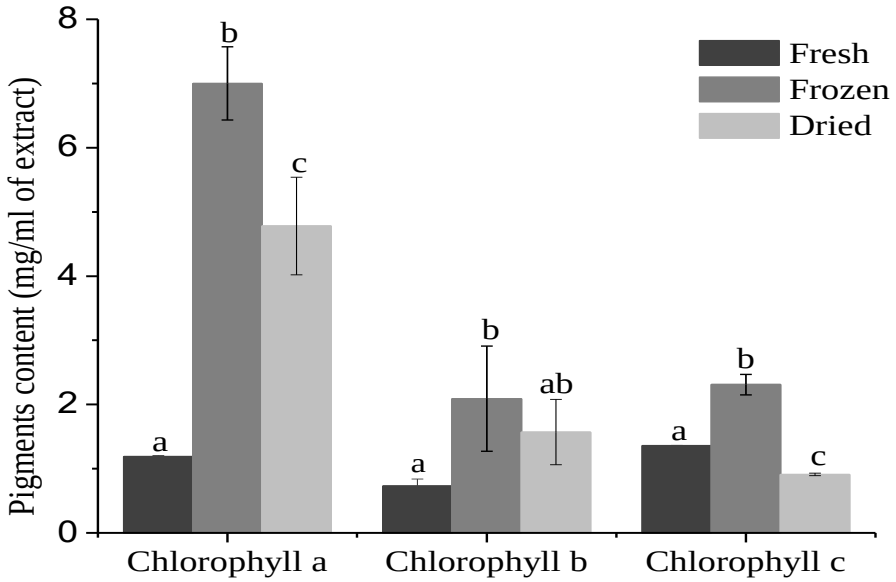


Figure V.2. Chlorophylls content in pigment extracts obtained from the fresh, frozen and dried brown alga *Phyllaria reniformis* (Mean ± SD). Within any given pigment, bars with different letters indicate significant differences between alga preprocessing types (*p*-value ≤ 0.05, Tukey's HSD test)

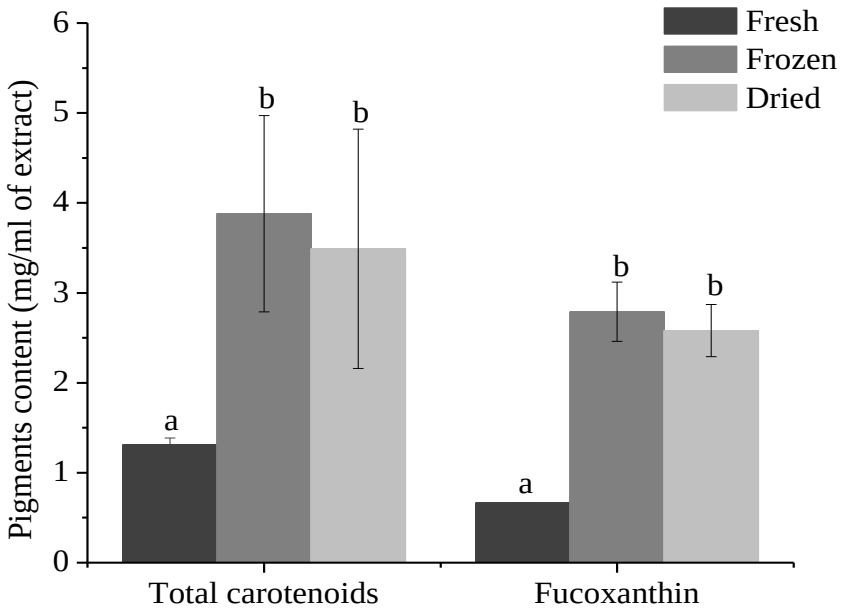


Figure V.3. Total carotenoids and fucoxanthin content in pigment extracts obtained from the fresh, frozen and dried brown alga *Phyllaria reniformis* (Mean ± SD). Within any given pigment, bars with different letters indicate significant differences between alga preprocessing types (*p*-value ≤ 0.05, Tukey's HSD test)

The analysis of variance showed a significant effect of preprocessing on pigments contents (*p*-value ≤ 0.05). However, the pairwise comparisons using Tukey's HSD test revealed that for both, total carotenoid and fucoxanthin, no significant difference was found between drying and freezing preprocessing, but for chlorophylls (*a* and *c*) it was statistically significant. This test revealed also a significant difference between fresh and frozen samples extracts in chlorophylls (*a*, *b*, *c*), fucoxanthin and total carotenoid.

The freezing preprocessing of alga before pigments extraction gave the highest yields; this is probably due to the degradation of the thalli by freezing effect. Whilst the drying preprocessing revealed also an important pigments yield compared to the fresh one, but chlorophyll *c* was underestimated due to the low water content in the dried alga.

The use of UV-Visible spectrophotometry for quantitative determination of chlorophylls and carotenoids is complicated. Due to a similarity in the Abs spectra of some pigments, there could be an underestimation or an overestimation, therefore concentration of total chlorophylls and total carotenoids could accurately be estimated, however individual pigment concentration was difficult to be resolved **(Thrane et *al*., 2015)**.

Furthermore, the determination of the pigment content may have unfair value due to the formation of new products such as pheophytins and chlorophyllides resulting from pigment degradation and having similar wavelength absorption to the original pigment. For that reason, the high content of chlorophyll *a* may be related to chlorophyll *c* that was abounded in brown seaweeds, and it may result to their degradation to pheophytin *a* and chlorophyllide *a*. Moreover, the accuracy of UV-Visible spectrophotometric method is also affected by other facts such as the solvent used for extraction, the type of sample, the sample preprocessing and also the spectrophotometer used **(Haryatfrehni et *al*., 2015; Ritchie, 2018)**.

V.3.3. HPLC analysis

HPLC is considered as an efficient method for measuring pigment concentrations in plant and algae. This technique can resolve most chlorophylls and carotenoids, including their degradation products such as pheophytins **(Mantoura & Llewellyn, 1983).**

Table V.1 lists the photosynthetic pigments separated of samples extracts and their retention times. Figure V.4 shows typical chromatograms (A, B, C) resulting from RP-HPLC analysis of pigment extracts from respectively fresh, frozen and dried alga samples.

Table V.1. Photosynthetic pigments of *Phyllaria reniformis* extract (Fresh, Frozen, Dried)

Peak	Retention time (min)	Pigment	Fresh	Fozen	Dried
0	1.55	Solvent	+	+	+
1	3.92	Chlorophyllide *a*	+	+	+
2	4.84	Chlorophyll *c3*	+	+	+
3	5.68	Chlorophyll *c1, c2*	+	+	+
4	7.38	Fucoxanthin	+	+	+
5	8.48	Trans-neoxanthin	+	+	+
6	8.78	UNK*	+	+	+
7	9.34	UNK	+	-	+
8	14.38	Chlorophyll *b*	+	+	-
9	14. 96	Chlorophyll *a*	+	+	+
10	15.08	Chlorophyll *a*	+	+	+
11	16.73	phaeophytins	+	+	+
12	17.50	*β* Carotene	+	+	+
a	6.46	UNK	-	-	+
b	16.25	UNK	-	-	+
c	17,11	UNK	-	-	+

**UNK: unknow*

For each sample a good resolution of the major pigments was achieved. Twelve peaks indicating pigments were resolved, as shown in table V.1. At the polar end of the chromatogram, chlorophyllides *a*, chlorophyll *c3*, and chlorophylls *c1*, *c2* were almost resolved, however in the central region of the chromatogram, fucoxanthin, trans-neoxanthin and two unidentified components were presented; while at the non-polar end of chromatogram, chlorophylls *a* and *b*, pheophytin *a* and *β*-carotene were resolved.

In all, chromatogram of fresh alga (Figure V.4.A) was dominated by fucoxanthin, followed by chlorophyll *a*, then chlorophyll *c1+c2*. These three pigments are the main pigments in brown algae, which impart a greenish brown color to the algae **(Kadam et *al*., 2013)**.Chlorophyll *c1* and *c2* are only found in phaeophyceae. Smaller amounts of chlorophyllides *a*, chlorophyll *c3*, chlorophyll *b*, pheophytin *a* and *β*-carotene were also resolved. The same resolution was found in the frozen alga sample (Figure V.4.B) but with higher peaks.

Chromatogram of the dried pre-proceeded sample (Figure V.4.C) shows also a high amount of fucoxanthin and chlorophyll *a*, and a lower amount of chlorophyll *b*, with lower peak intensity in comparison to that of freezing preprocessing but superior to that of the fresh sample. However, in the same sample, chlorophyll *c1+c2* was less abundant compared to the fresh and frozen ones, this might be caused by the high polarity of chlorophylls *c*. Another possible reason may be the percentage of water missing during solvent extraction by aqueous acetone in the dried sample which may lead to a lower diffusion of chlorophylls *c* than that in both fresh and frozen samples. Therefore, drying sample before extraction might be suitable for extraction of hydrophobic compounds (nonpolar) probably because of the lower water content.

β-carotene is completely hydrophobic hence, it was presented by the highest intensity peak compared to fresh and frozen samples. According to **Seely et *al*., (1972)** dimethyl sulphoxide (DMSO) a more polar solvent was shown to extract much of the chlorophyll *c* and fucoxanthin from the intact thalli of brown algae, while subsequent extraction with acetone rapidly removes most of the chlorophyll *a* and *β*-carotene.

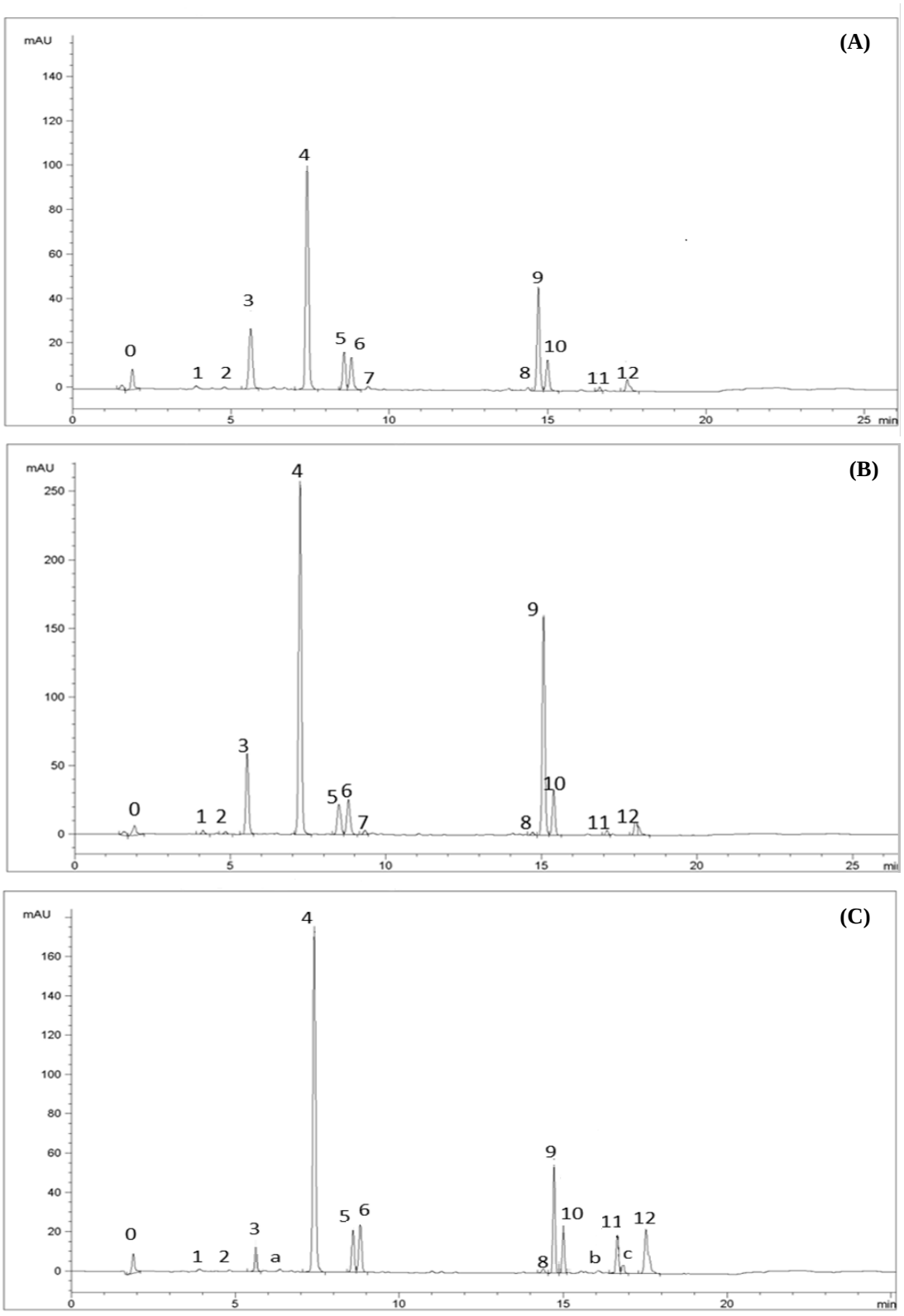


Figure V.4. Chromatogram separation of pigment extracts from the fresh (A), the Frozen (B) and the dried (C) brown algae *Phyllaria reniformis*.

Similar result was observed for pheophytin *a*, a hydrophobic pigment highly present in the dried sample probably due to the degradation of chlorophyll *a*. When the chlorophylls were exposed to heat or acidic conditions, the magnesium ion is lost from their structure and the resulting molecule was (pheophytin) which exhibits olive-green colour **(Mohamed et *al*., 2012)**. Other traces of pigments are present in the dried sample chromatogram such as the unknown (peaks a, b, c) which may indicate that a little degradation had occurred.

Based on these results it can be assumed that *Phyllaria reniformis* drying before pigments extraction can lead to a selection of pigments especially *β*-carotene. However, **Hynstova et *al.*, (2018)** concluded that the processing of *Chlorella vulgaris* and *Spirulina platensis* dried powder will lead to a decrease *β*-carotene content, probably due to heat or light exposure. The study of **Tang and Chen (2000)**on the stability and degradation of freeze-dried carotenoids powder showed that the amount of *β*-carotene and lutein decreased with increasing storage temperature. Several researchers demonstrated that carotenoids tend to decrease with increasing drying time due to oxidation and isomerization **(Anguelova & Warthesen, 2000; Karabulut et *al*., 2007)**. According to **Chan et *al*.,(1997)** the nutritional composition including pigments of seaweed *Sargassum hemiphyllum* is greatly affected by different drying methods.

V.3.4. Antioxidant activity

In comparison with red and green seaweeds, brown seaweeds are characterized by higher antioxidant potential. Several researches demonstrated that brown algae extracts and especially algae pigments are comparatively similar or superior to synthetic antioxidants due to the presence of carotenoid and fucoxanthin **(Kosanić et *al*., 2019; B. Le Tutour et *al*., 1998; Sudhakar et *al*., 2013)**. Moreover, chlorophylls, pheophytins and carotenoids are known to act as antioxidants to prevent oxidative DNA damage and lipid peroxidation **(Heo et *al*., 2008; Hsu et *al*., 2013; Lanfer-Marquez et *al*., 2005; Sindhu et *al*., 2010).** In the present study the antioxidant abilities of pigment extracts were evaluated by scavenging of DPPH radical. The scavenging effect increased with the increasing sample concentrations as shown in figure IV.5.

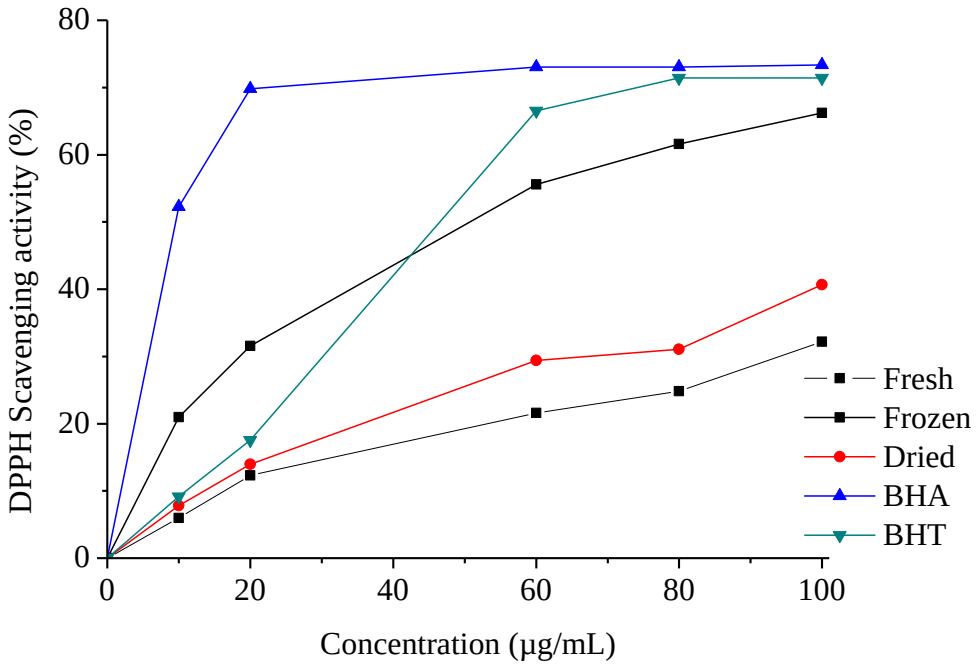


Figure V.5. Free radical-scavenging capacities of reference antioxidant (BHA, BHT) and pigment extracts obtained from the fresh, frozen and dried brown alga *Phyllaria reniformis*, (Mean ± SD)

All pigment extracts from fresh, dried and frozen alga exhibited antioxidant activity. The frozen sample extract (at a concentration exceeding 60µg/mL) showed significant activity almost similar to BHA and BHT. In the same sample, the maximum alga pigment extract concentration used (100µg/mL) exhibited more than 80% of radical inhibition while those extracted from the dried and fresh sample extracts showed lower activities 50.82% and 32.17%, respectively.

The effectiveness of antioxidant properties is inversely correlated with their IC50 values representing the concentration of extracts at which they scavenge the 50% of the DPPH solution. The lower the IC50 value of an antioxidant the higher would be its free radical scavenging power. Figure V.6 displays comparison of the IC50 values of BHA and BHT as standards with those of pigments extracts.

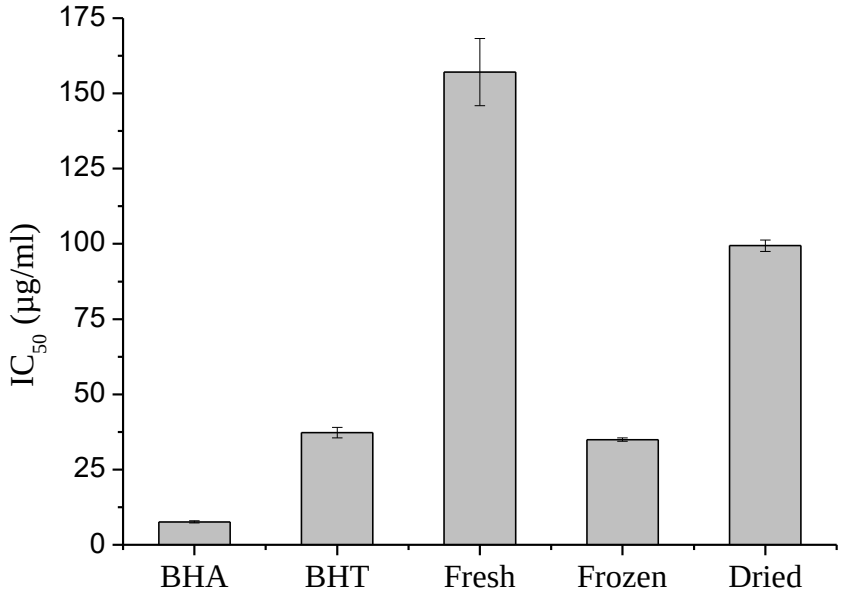


Figure V.6. DPPH (IC50) values of reference antioxidants (BHA, BHT) and pigment extracts obtained from the fresh, frozen and dried brown alga *Phyllaria reniformis*, (Mean ± SD)

Pigment extract obtained after frozen preprocessing was the most efficient by the lowest IC50 values of 34.96±0.6 µg/ml among all extracts and BHT reference antioxidant, and it was less efficient compared to BHA reference antioxidant. A low antioxidant activity was observed in the fresh sample extract with 157.09 ±11.14 µg/ml of IC50 value. While the IC50 of the dried sample extract was 99.39±1.90µg/ml. The analysis of variance showed a significant difference between the pigment extracts (*p*-value ≤ 0.05). This difference is probably due to the effect of the preprocessing of the alga before pigments extraction.

V.4. Conclusion

Marine algae are an excellent source of biologically active compounds for pharmaceutical, food, cosmetic sectors. Seaweed could be exploited as a good source of natural pigments. Consequently, for appropriate pigments extraction method, the preprocessing step of algae before extraction remains the most important because of the highest sensitivity of pigments. This study showed the effect of drying and freezing preprocessing on quantity, quality and antioxidant activity of pigments extracts. Based on the obtained results from the spectrophotometric determination of chlorophylls and carotenoids and their separation by

chromatography method (RP-HPLC), the freezing preprocessing of alga was the most efficient technique to isolate high level of chlorophylls and carotenoids. The drying preprocessing gave also a fairly large amount of pigments compared to fresh alga especially for hydrophobic pigments such as *β*-carotene. This may be due to the small content of water in sample, in spite of that, drying could contribute to a loss of pigments justified by the presence of pheophytin *a* probably produced after chlorophylls degradation and other pigments traces. According to DPPH scavenging activity results, *Phyllarira reniformis* could constitute a natural source of antioxidant substances of high importance. The highest activity was obtained in frozen sample extract. To sum up, this study offers to *Phyllarira reniformis* the opportunity to be used as a natural source of biocompounds in different fields, because of its richness in antioxidant pigments especially fucoxanthin. On the other hand, and from an economical point of view, freezing preprocessing is an appropriate method for pigments extraction with high efficiency. Freezing before pigments extraction could be employed to recover more pigments from algae in term of quality, because of alga thalli degradation, while the drying preprocessing led to the extraction of higher contains of the most stable pigments such as *β*-carotene. The choice of the suitable preprocessing technique before pigments extraction could direct the researcher to a specific pigment.

CHAPTER VI

Oxidative stability of soybean and sunflower oils enriched with pigments extracts

VI.1. Introduction

During the past decade, consumer's interest and preference for natural substitutes of synthetic additives increased mainly for health benefic reason. Moreover, these natural additives are nontoxic, biodegradable and do not leave damaging residues, however, they show lower efficiency compared to the synthetic ones **(SeKwon Kim & Chojnacka, 2015; Sydney et *al*., 2020)**. Therefore, there is an urgent need to develop new and safe products of natural origin, with similar properties to the synthetic ones, in particular antimicrobial, antifungal, and antioxidative. **(SeKwon Kim & Chojnacka, 2015).**The incorporation of natural bioactive additives into food products (i.e., beverages, bakery, oils and dairy products) is growing on worldwide market. Among commercial functional foods, enriched vegetable oils take the major part in all the food categories **(Blasi & Cossignani, 2020; Lourenço et *al*., 2019)**.

Edible vegetable oils, the ideal cooking media today, hold an important part of human diet for multiple viewpoints such as nutritional value, organoleptic characteristics and functional properties within the food matrix **(Czaplicki et *al*., 2016; Hannachi & Elfalleh, 2020; B. Holt, 2016; Makni et *al*., 2015)**. Soybean and sunflower oils belong to the popular vegetable oils utilized worldwide in food, cosmetic, and pharmaceutical industries because of their high fatty acids and liposoluble vitamins contents **(Kozłowska & Gruczyńska, 2018)**. However, lipid oxidation is a major factor affecting their nutritional and sensorial qualities **(Siraj et *al*., 2019)**. Thus, enrichment of vegetable oils with antioxidants seems to be a solution to prevent the oxidation process **(Şahin et *al*., 2017, Saoudi et *al*., 2016)**. Various synthetic antioxidants were used within regulated limits to reduce deterioration, rancidity and oxidative discoloration in vegetable oils. Butylated hydroxyl anisol (BHA) and butylated hydroxyl toluene (BHT) are two widely used synthetic antioxidants, however, they are volatile and decompose easily at high temperatures **(Ammari et *al*., 2012)**. Besides, recent reports revealed that these compounds may have harmful side effects **(Yao et *al*., 2020)**. So far, many natural pigments were used as additives in food systems, they induced nutritional advantages in

addition to an appealing colour associated with good functional properties in particular antioxidant effects **(Batista et *al*., 2006; Gouveia et *al*., 2007)**.

To the best of our knowledge, the addition of *Phyllaria reniformis* pigment extracts to vegetable oils as natural antioxidant was not reported to date.

Against this background, the aim of this chapter is to investigate and to improve oxidative stability of soybean and sunflower oils enriched with natural pigment extracts of the brown seaweed *Phyllaria reniformis*.

VI.2. Materials and methods

VI.2.1. Seaweed collection

The brown seaweed *Phyllaria reniformis* was collected from Tipaza (Algeria) in June 2016 as described in Section IV.2.1.

VI.2.2. Extraction of seaweed pigments

Phyllaria reniformis pigments were extracted using ultrasound assisted acetone extraction from frozen alga sample as described in Section IV.2.2.

VI.2.3. Vegetable oils

Refined sunflower (Lesieur, France) and soybean oil (Labelle, Algeria) were purchased from a local market in 1 and 2-L packs, respectively. Both are edible vegetable oils extensively used in Algeria.

VI.2.4. Preparation of enriched vegetable oils

Two concentrations of *Phyllaria reniformis* pigment extract (200 and 1000 ppm) were partially dissolved in sunflower or soybean oils and mixed vigorously using ultrasonic bath (Bioblock Scientific TS 540, Germany) at the following conditions: Power 100W and 20Hz for 30 min at 24°C. 200 ppm of a synthetic antioxidant (BHA) was added to vegetable oils for comparison. Native and enriched oils were kept in amber glass bottles at 6°C for further analysis.

VI.2.5. Free Acidity

Free acidity (FA) content was determined using the standard method **(ISO 660 2nd edition 15-05-1996)**. 10 g of vegetable oil were weighed into a 250 mL glass Erlenmeyer. 75mL of neutralized ethanol and 1% (w/v) of phenolphthalein as indicator were added. The mixture was titrated with 0.1N NaOH until pink colour appeared and persisted (10 seconds). The FA content was calculated as percentage of oleic acid according to the following formula:

$$FA\ \ (as\ oleic\ acid)\ (\%) = \frac{V \times N \times 28.2}{m}$$

Where:

V was the volume of NaOH consumed (mL),
N was the normality of NaOH
m was the mass of the test sample (g).

VI.2.6. Peroxide value

The primary oxidation compounds of oils were evaluated by the peroxide value (PV) using the standard method **(ISO 3960 4th edition 2007**) and briefly described in the following. 5 g of sunflower or soybean oils were weighed into a 250 mL glass Erlenmeyer. 12 mL of chloroform and 18mL of acetic acid were added, then 1mL of saturated potassium iodide (KI) was incorporated into this solution. After 1 min of incubation in dark, 75mL of distilled water were added with stirring. The mixture was titrated with 0.01N of $Na_2S_2O_3$ in the presence of starch solution (1% (w/v)) until the solution is completely discolored. PV is given by the following formula:

$$PV\ (meq.peroxide/Kg\ sample) = \frac{(V_1 - V_0) \times N \times 1,000}{m}$$

Where:

V_1 was the volume of $Na_2S_2O_3$ consumed (mL),
V_0 was the volume of $Na_2S_2O_3$ of the blank test,

N was the normality $Na_2S_2O_3$ solution used

m was the mass of the test sample (g).

VI.2.7. Chlorophylls and carotenoids content in oil

Chlorophylls and carotenoids content in native and enriched oils were determined according to the procedure described by **Mosquera *et al*. (1991)**. A sample of oil (7.5 g) was dissolved in 25 mL of cyclo-hexane. The amount of chlorophylls and carotenoids was measured using UV spectrophotometer (SPECORD 210 PLUS 623F1138, Germany) at 670 nm and 470 nm, respectively. The concentrations of total chlorophylls and total carotenoids in the enriched and native oils were expressed using the following equations:

$$Chlorophylls\ (mg/kg) = \frac{Abs_{670} \times 10^6}{613 \times 100 \times d}$$

$$Carotenoids\ (mg/kg) = \frac{Abs_{470} \times 10^6}{2000 \times 100 \times d}$$

Where:

Abs_{670} is the absorbance at 670 nm,

Abs_{470} is the absorbance at 470 nm,

d is the optical pathlength (1 cm),

613, 100, and 2000 are specific coefficients.

VI.2.8. Colour measurement

The colour coordinates (*a**, *b** and *L**) of the native and enriched oils by seaweed pigment extracts or BHA were measured using a CR-10 colorimeter (Konica Minolta Cr-10 Tristimulus, Japan).

VI.2.9. DPPH Radical Scavenging Activity of enriched oil

The scavenging effects of the native and enriched oils was measured using the method of **Hazzit *et al*., (2009)** as described in chapter IV (Section IV.2.8). Isooctane was used as solvent for preparing DPPH and oil samples dilution.

VI.2.10. Oxidative stability

The oxidative stability of the enriched and native oils was evaluated by measuring the induction time (IT), using a Rancimat apparatus (Metrohm, model 743, Switzerland). This method is based on the detection of the electrical conductivity in water caused by the volatile degradation compounds. The time taken to reach the conductivity inflection point, was recorded and expressed as IT (h). In this study, 3 g of vegetable oil were heated at 100°C in a thermostated electric heating block and subjected to dried air at a flow rate of 10 L/h previously filtered and cleaned. IT was determined from the conductivity curve at the inflection point between the horizontal (conductivity, µS. min^{-1}) and vertical (time, h) tangents.

VI.2.11. Statistical analysis

All the analyses were performed in triplicate and results were presented as average of at least three replicates ± Standard deviation. An analysis of variance (ANOVA) was performed using the Statistical Analysis System R 4.0.2. (R Core Team, 2020). ANOVA statistical tests were performed using Tukey's multiple comparison procedure on a 5% significance level.

VI.3. Results and discussions

VI.3.1. Free Acidity

Oil acidity expressed as FA is the most frequently used quality test for vegetable oils. However, FA increases with free fatty acids mainly formed during triacylglycerol hydrolysis and by oxidation **(Neves *et al*., 2020).** The vegetable oil oxidation process was induced by the reaction with moisture initialy present or moisture formed during other deterioration reactions **(Al-Harbi & Al-Kahtani, 1993)**.

FA was determined, in order to assess the effect of *Phyllaria reniformis* pigment extracts addition in soybean and sunflower oils quality. Table VI.1 shows the FA content of supplemented or non-supplemented soybean and sunflower oils. The control oil sample without any additive and the enriched oil samples after the addition of 200 or 1000ppm pigment extract of *Phyllaria reniformis* or BHA (200ppm) were compared.

The control and enriched soybean oil with BHA or pigment extract (200 ppm and 1000ppm) exhibited closer FA values which ranged from 0.42 to 0.63%. A slight increase of FA was observed in enriched soybean oils with BHA and with *Phyllaria reniformis* pigment extracts. Meanwhile, sunflower oil with or without additives showed approximatively the same FA, about 0.56±0.00, 0.42±0.14, 0.56±0.00 and 0.63±0.07%, for the control and enriched soybean oil with BHA or pigment extract (200 ppm and 1000ppm), respectively.

Table VI.1: Free acidity (%) of enriched soybean and sunflower oils (200, 1000ppm of pigment extract or 200 ppm of BHA) and the control sample.

Sample	Free acidity (%)	
	Soybean oil	**Sunflower oil**
Control	0.42±0.14 [a]	0.56±0.00 [a]
BHA (200ppm)	0.56±0.28 [a]	0.42±0.14 [a]
Pigment extract (200ppm)	0.62±0.08 [a]	0.56±0.00 [a]
Pigment extract (1000ppm)	0.63±0.06 [a]	0.63±0.07 [a]

Values indicate the mean of three triplicate ± Standard Deviation, Values in one column followed by different superscript letters are significantly different ($p<0.05$ Tukey's HSD test).

Compared to the control oil samples, no significant differences ($p>0.05$) in FA were observed in both vegetable oils used in this study (p-value =0.797 for soybean oil and p-value =0.401 for sunflower oil). Therefore, addition of pigment extract to vegetable oils seemed not affecting FA. Previously, similar results were obtained by **Sousa et *al.* (2015),** in their study about the effect of adding flavourings (hot chili peppers, laurel, oregano and pepper) to olive oils.

In opposition, other studies reported that the addition of natural additives led to increase significantly the vegetable oils FA. Thus, **Sousa et *al.* (2015)**, showed that the addition of garlic to olive oils induced an increase in FA values from 0.6 to 0.8%. In a similar study, **Gambacorta et *al.* (2007)** showed that FA results of extra virgin olive oils flavored with herbs and spices were not affected after 7 months of storage. While, a significant increase was observed by **Ayadi et *al.* (2009)** in FA values of enriched oils by aromatic plants (rosemary, lavender, sage, lemon and thyme). Similar results were reported by **Ammar et *al*. (2017)** during their study of

the effect of *Opuntia ficus-indica* flowers addition to two virgin olive oils **(Ammar et *al*., 2017)**.

VI.3.2. Peroxide value

PV is one of the most widely used quality parameters in food. It is measured to specify the concentrations of peroxides and hydroperoxides produced in the first stage of lipids oxidation **(Delfanian et *al*., 2016)**. PVs of soybean and sunflower oils before and after addition of *Phyllaria reniformis* pigment extract (200 ppm and 1000ppm) or BHA (200 ppm) were determined and presented in table VI.2.

PVs of soybean and sunflower oils obeyed the Codex Alimentarius limit for refined oils **(Codex Alimentarius, 1999)** which is equivalent to 10 mEq. O_2/kg**.** When soybean oil was enriched with 200 or 1000 ppm of pigment extract or with 200 ppm BHA, PVs results varied between 3.96±0.03 and 4.95±1.00 mEq. O_2/kg. On the other hand, PVs of sunflower oil samples enriched with 200 or 1000ppm seaweed pigment extract were 8.92±1.01 and 8.94 ±0.97 mEq. O_2/kg, respectively. These values were lower than the control sample (10.47 ±1.47 mEq. O_2/kg) and the sunflower oil sample enriched with 200ppm BHA (10.38 ±0.44 mEq. O_2/kg). In all cases, comparing to the two control sample oils, no significant ($p \leq 0.05$) changes were observed in PVs of enriched soybean and sunflower oils.

Hence, in the present study, addition of seaweed pigments or BHA to soybean and sunflower oils seems to not affect their PVs. Meanwhile, a recent study reported that the addition of *Opuntia ficus-indica* flowers induced a slight increase in the formation of peroxides of olive oil **(Ammar et *al*., 2017).** However, olive oils flavoured with garlic and oregano exhibited lower PV comparatively to the control sample **(Sousa et *al*., 2015).**

Table VI.2: Peroxide values (mEq. O_2/kg of oil) of enriched soybean and sunflower oils with 200, 1000ppm of pigment extract or 200 ppm of BHA and the control samples.

Sample	Peroxide values (mEq. O_2/kg of oil)	
	Soybean oil	**Sunflower oil**
Control	3.96±0.02 [a]	10.47 ±1.47 [a]
BHA (200ppm)	4.44 ±0.48 [a]	10.38 ±0.44 [a]
Pigment extract (200ppm)	3.96±0.03 [a]	8.92±1.01[a]
Pigment extract (1000ppm)	4.95±1.00 [a]	8.94 ±0.97 [a]

Values indicate the mean of three triplicate ± Standard Deviation, Values in one column followed by different superscript letters are significantly different ($p \leq 0.05$ Tukey's HSD test).

VI.3.3. Chlorophyll and carotenoids content

VI.3.3.1. Carotenoids

Enriched soybean and sunflower oils with *Phyllaria reniformis* pigment extract exhibited richer contents of chlorophylls and carotenoids than the control samples. Figures VI.1 and VI.2 illustrate the total carotenoids content in soybean and sunflower oils, respectively.

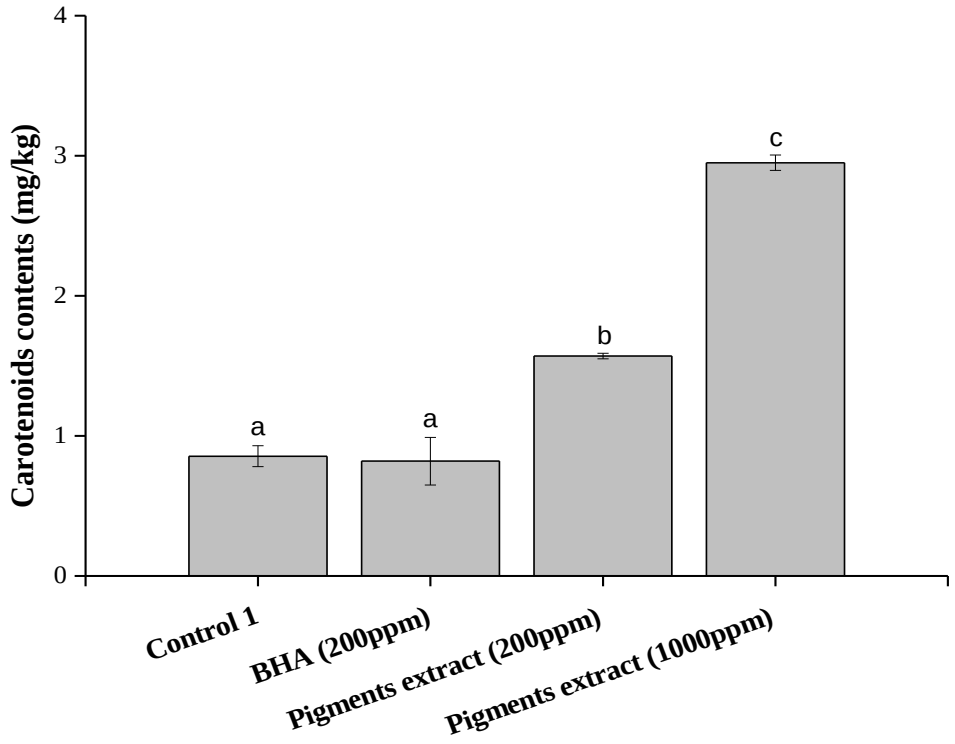


Figure VI.1: Carotenoids content of enriched and non-enriched soybean oils (Mean ± SD). Different letters indicate significant differences (*P*≤0.05, Tukey's HSD test)

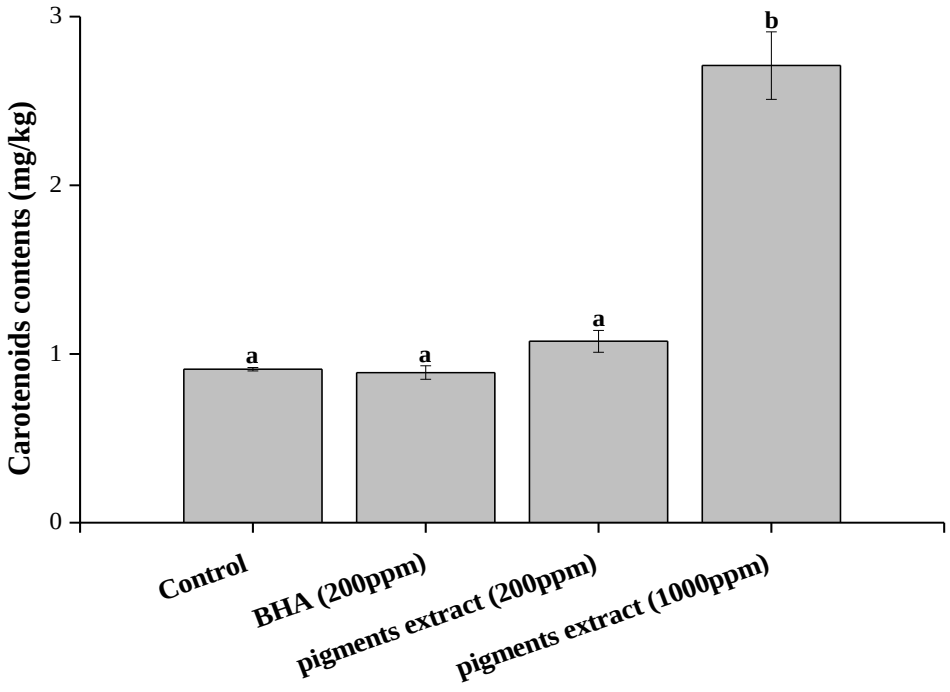


Figure VI.2: Carotenoids content of enriched and non-enriched sunflower oil (Mean ± SD). Different letters indicate significant differences (*P*≤0.05, Tukey's HSD test)

In comparison to both the control and the BHA soybean oil samples, total carotenoids content in the enriched soybean oil increased about 2 times (1.57± 0.02 mg/kg of oil) and 3 times (2.95± 0.05 mg/kg of oil) when adding 200 ppm or 1000ppm of seaweed pigment extract, respectively (Figure VI.1).

Statistical analysis showed a high significant difference (*p*-value = 0.000293 ***) between total carotenoids content of enriched soybean oils with seaweed pigment extract and total carotenoids content of soybean oils without pigment extract.

The same results were observed with sunflower oil (Figure VI.2), where total carotenoids content in control sample (0.91± 0.01 mg/kg of oil) and BHA enriched sample (0.51 ±0.01 mg/kg of oil) were two times lower than the enriched sample with 200ppm of seaweed pigment extract (1.07 ±0.06 mg/kg of oil), and much lower approximately 3 times than the enriched sample with 1000ppm of seaweed pigment extract (2.71± 0.2 mg/kg of oil).

Results showed a highly significant difference (*p*-value =0.000701 ***) in the total carotenoids content of sunflower sample supplemented with 1000ppm compared to the remaining samples.

In the present study, total carotenoids content in soybean and sunflower oils increased significantly by increasing incorporation of seaweed pigment extract. Moreover, this result revealed that the addition of seaweed pigment extract to soybean and sunflower oils may improve their oxidative stability.

VI.3.3.2. Chlorophyll content

Chlorophylls content followed a similar trend to that of carotenoids. Figure VI.3 and VI.4 show chlorophylls content of soybean and sunflower enriched oils, respectively.

The concentration of chlorophylls in soybean oils enriched with 200 or 1000ppm of *Phyllaria reniformis* pigment extract were 2.03±0.34 and 6.6±0.4 mg/kg of oil, respectively. These values were 10 and 33 times higher than the control (0.19 mg/kg) and the BHA enriched oil (0.22 ±0.09 mg/kg) (Figure VI.3).

The analysis of variance showed a significant difference on chlorophylls content (*p-value* = 0.000211 ***), between the supplemented soybean oil with 1000ppm seaweed pigment extract and the remaining studied samples.

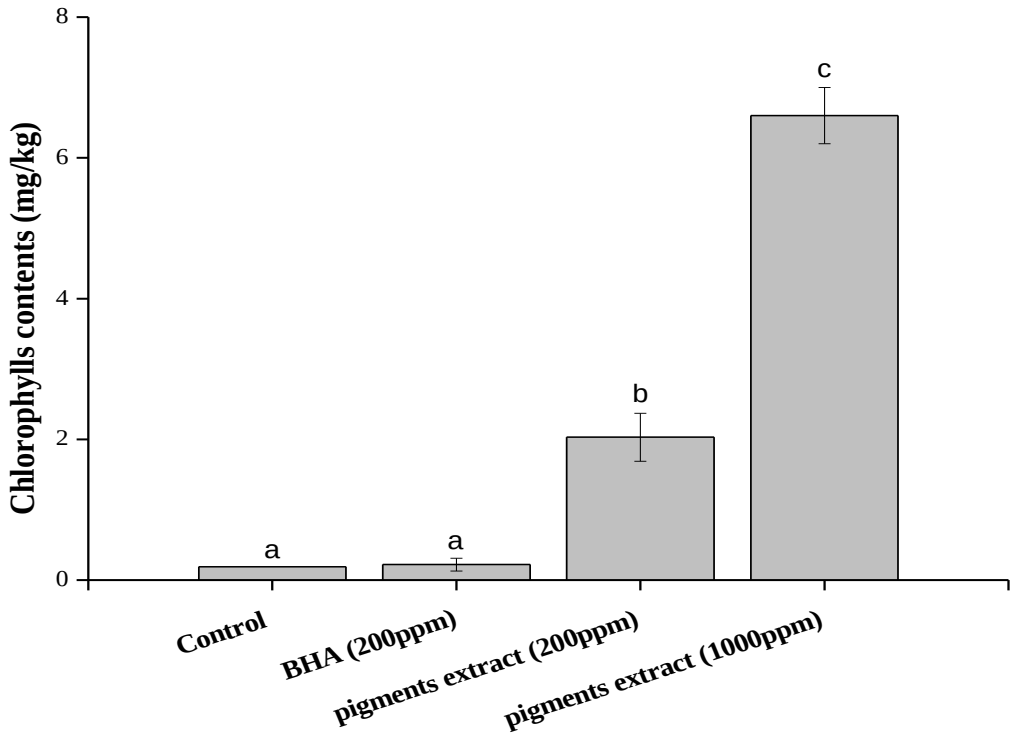


Figure VI.3: Chlorophylls content of enriched and non-enriched soybean oil (Mean ± SD). Different letters indicate significant differences (*P*≤0.05, Tukey's HSD test)

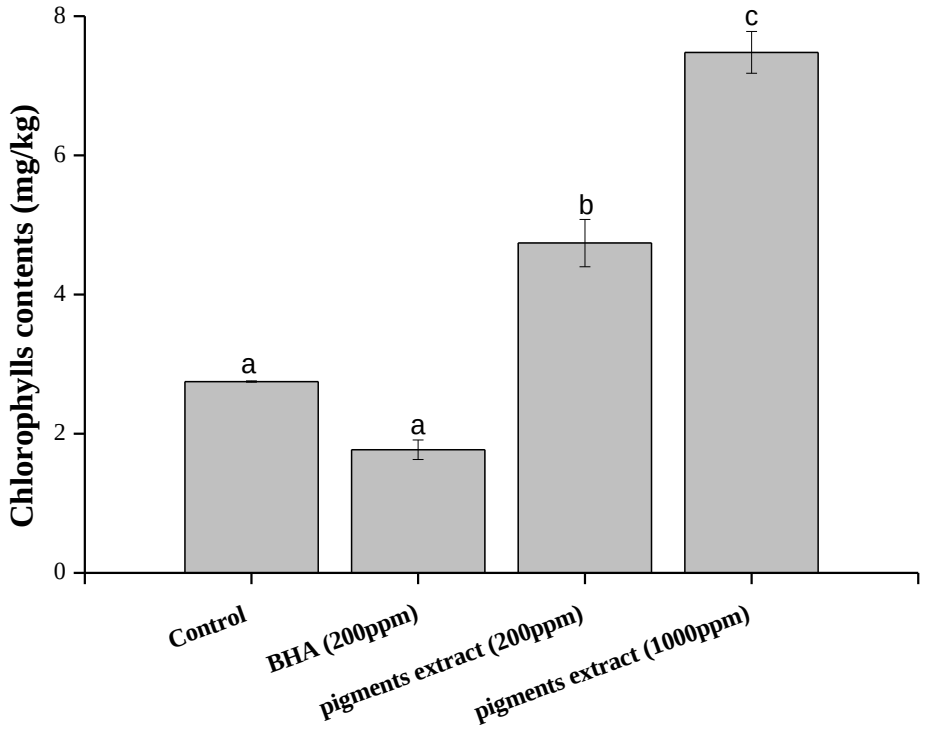


Figure VI.4: Chlorophylls content of enriched and non-enriched sunflower oil (Mean ± SD). Different letters indicate significant differences (*P*≤0.05, Tukey's HSD test)

As shown in figure IV.4, the concentration of chlorophylls in sunflower oil enriched with *Phyllaria reniformis* pigment extract increased in comparison to the control and BHA samples. The highest total chlorophylls content was observed in 1000ppm pigment enriched sunflower oil (7.48 ±0.3 mg/kg) followed by that of 200ppm (4.74 ±0.34 mg/kg). High significant difference was observed (0.000278 ***) in the chlorophyll's contents in all samples.

Results suggest that the carotenoids and chlorophylls contents in soybean and sunflower oils were deeply related to the concentration of seaweed pigment extracts incorporated and in the meantime the vegetable oils qualities may be improved with increasing antioxidant natural additives such as *Phyllaria reniformis* pigment extract.

VI.3.4. Colour measurement

Colour, is an important factor for consumer appeal and acceptability **(Gouveia et *al*., 2007)**. As shown in figure VI.5 and based on the visual analysis, the enrichment of soybean and sunflower oils with *Phyllaria reniformis* pigment extract influenced their colour and gave them more greenness. However, for more detailed insight into this colour change, colours parameters *L** (lightness), *a** (redness) and *b** (yellowness) of analysed samples was obtained by colorimetric measurement. Results of colours (*a**, *b** and *L**) measurement are illustrated in figures VI.6 and VI.7 for soybean and sunflower oils, respectively.

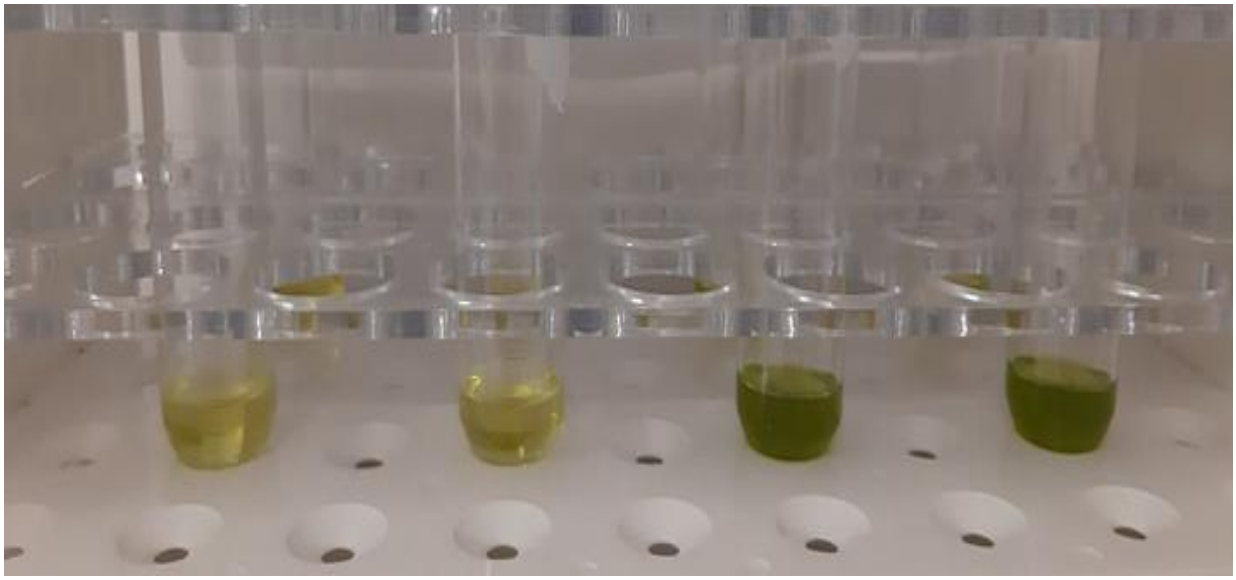


Figure VI.5: Photography of soybean oil before and after enrichment, from left to right: Control, BHA (200ppm), Pigment extract (200ppm), Pigment extract (1000ppm)

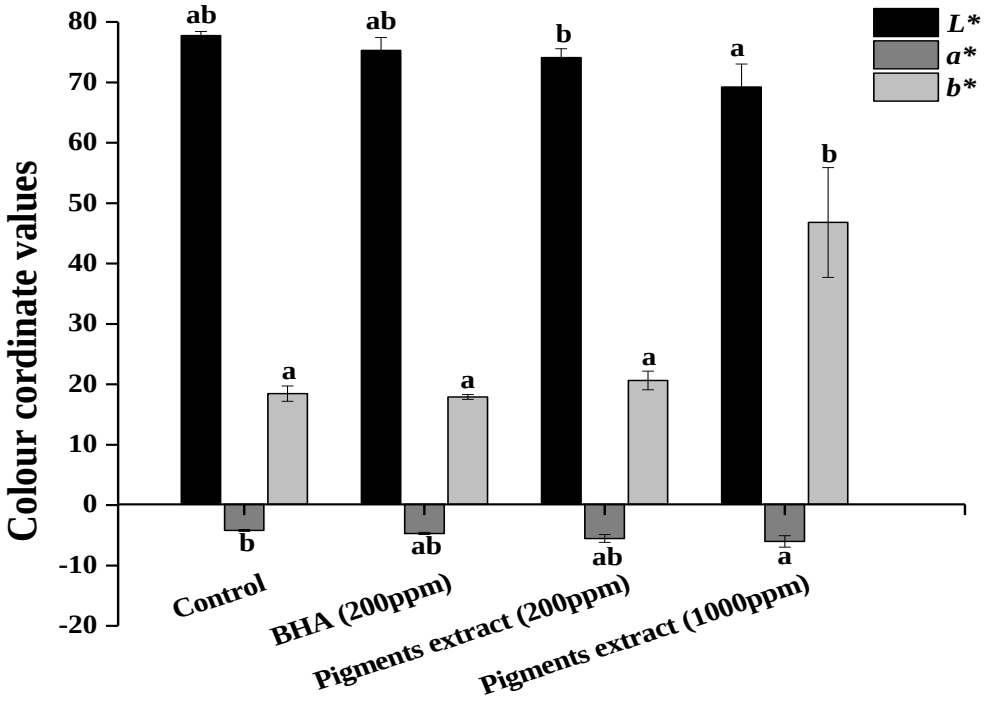


Figure VI.6: Colour (*L, *a**, and *b**) of soybean oil supplemented with *Phyllaria reniformis* pigment extract at two concentration (200 and 100ppm) and soybean oil with or without BHA. Different letters indicate significant differences (*P*≤0.05, Tukey's HSD test)**

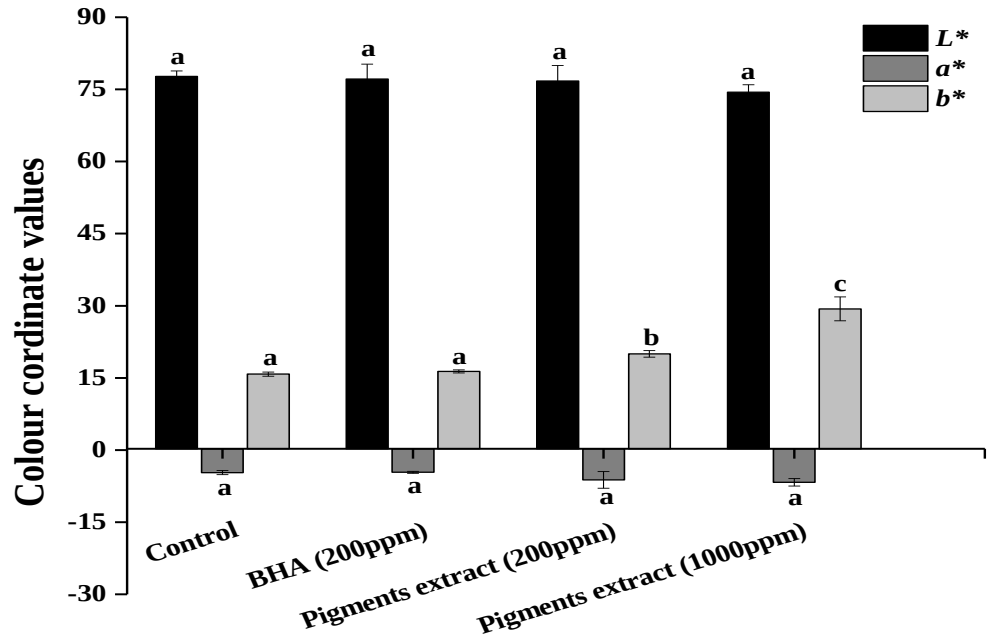


Figure VI.7: Colour (*L, *a**, and *b**) of sunflower oil supplemented with *Phyllaria reniformis* pigment extract at two concentration (200 and 100ppm) and sunflower oil with or without BHA. Different letters indicate significant differences (*P*≤0.05, Tukey's HSD test)**

As shown in figures VI.6, the value *L**, which indicates the lightness was higher in native soybean oil without additives than the remaining supplemented samples. Enrichment of soybean oil caused a slight decrease of *L** from 77.73± 0.70 to 69.23±3.80. The obtained value of *a** were all negative corresponding to the green zone, they decreased from -4.2±0.17 for the control to -6.03±0.95 for enriched soybean oil with 1000ppm of pigment extract. The *b** values were all positive indicating the yellowness. The highest value of *b** was obtained in oil sample supplemented with 1000ppm (+46.8 ±9.08), and the lowest *b** value was observed in the control (+18.46±1.27). For sunflower oil samples, the same observation was found (figures VI.7) where *L** decreased from 77.7±1.15 (control) to 74.43 ± 1.55 (sample supplemented with 1000ppm of pigment extract). a* value decreased from -4.7±0.43 (control) to -6.7± 0.78 (sample supplemented with 1000ppm), while *b** increased from +15.8±0.43 (control) to +29.36± 2.47 (sample supplemented with 1000ppm).

Results of statistical analysis showed significant difference ($P \leq 0.05$) between the enriched soybean oil by 1000 ppm of pigment extract and the remaining oil samples in all determined colour coordinates. While in sunflower oil, no significant difference ($P > 0.05$) was observed between samples for the two colour coordinates *L** and *a**, whereas, *b** values of enriched sunflower oil at the concentration of 200 and 1000ppm were significantly different compared to the enriched BHA oil and the control.

Hence, the addition of *Phyllaria reniformis* pigment extract to both oils caused a notable change in the colour coordinates (*a**, *b** and *L**). Consequently, the oil enriched with pigment extract became less luminous, greener and yellower. This variation could be attributed to the high content of chlorophylls and carotenoids in the pigment extract. According to **Corbu et *al.* (2020)**, the improvement of the colour parameters of oils may increase consumer attractiveness.

VI.3.5. DPPH Radical Scavenging Activity of enriched oil

Among several methods for vegetables oils antioxidant activities evaluation, the DPPH radical scavenging procedure was the most common used. In this study, the effect of adding *Phyllaria reniformis* pigment extract on antioxidant capacity of soybean and sunflower oils was assessed.

Figures VI.8 and VI.9 show the DPPH scavenging activity (%) and the IC50 values of native or enriched soybean oil, respectively. The obtained results showed that DPPH activity

of the enriched soybean oil was improved when the concentration of pigment extract increased from 200 to 1000ppm. The highest DPPH radical-scavenging capacity was observed in the soybean oil sample containing 1000 ppm of *Phyllaria reniformis* pigment extract with the lowest IC50 (5.23±0.10 mg/mL), followed by those enriched with 200 ppm of BHA or 200ppm pigment extract with IC50 of 5.58±0.11 and 5.75±0.03mg/mL, respectively. The control oil showed the lowest activity (IC50= 6.20±0.03 mg/mL). Significant difference (P=0.00312 **) was noted between the supplemented soybean oil and the control oil.

Concerning sunflower oil, control sample showed the lowest DPPH radical-scavenging capacity with IC50 of 10.06±0.31mg/mL, followed by 200ppm pigment extract enriched oil (IC50 = 9.58±0.09mg/mL) then 1000ppm (IC50 = 9.21±0.13mg/mL). However, BHA as a synthetic antioxidant, exhibited the best efficiency ($P \leq 0.05$) with IC50 value of 7.56±0.16mg/mL (Figures VI.10 and VI.11). DPPH antioxidant capacity before enrichment of oils with pigment extract or BHA was lower.

In all cases, the enrichment of soybean or sunflower oils increased their antioxidant capacities, especially for soybean oil when adding 1000ppm of seaweed pigments.

In previous study, **Gouveia et *al*. (2007)** evaluated the stability of soybean oil containing pigment extract of a microalga *Chlorella vulgaris*. They reported that pigments could contribute to oil stability due to their antioxidant effect. A similar result was found when using xanthophylls isolated from orange peel as antioxidant additive in soybean oil **(Yen & Chen, 1995)**.

Yao *et al.* (2020) evaluated the antioxidant capacity of zeaxanthin in soybean oil. They proved that the addition of zeaxanthin enhanced the ability of soybean oil to scavenge the free radical. In another study, supplementation of commercial oils (olive, sunflower and palm oils) by adding olive leaf extract may contribute to the increase of radical scavenging activity **(Salta et *al*., 2007)**. More recently, **Tinello et Lante (2020)** reported that the antioxidant activity of soybean oil increased after adding ginger and turmeric freeze dried powders.

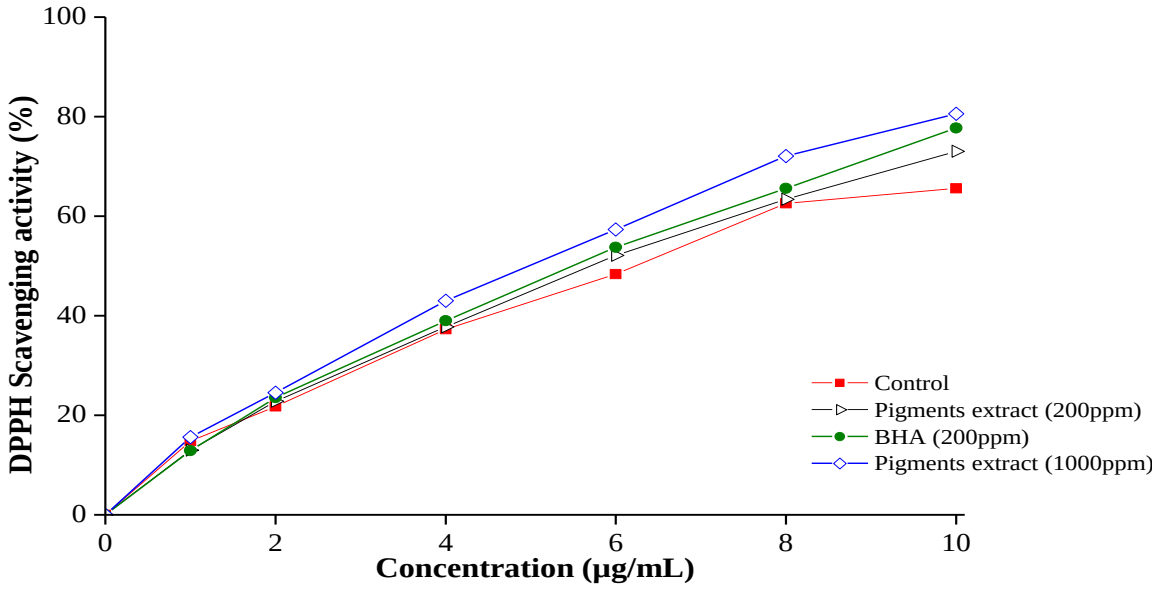


Figure IV.8: Free radical-scavenging capacities of soybean oil enriched by BHA and pigment extract obtained from the brown alga *Phyllaria reniformis* .

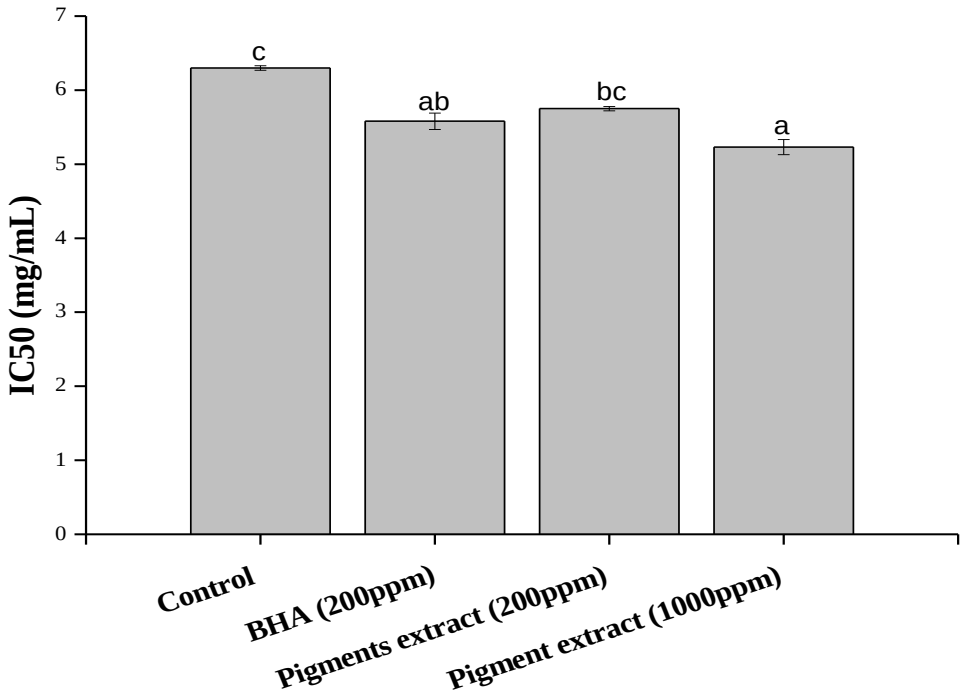


Figure VI.9: DPPH (IC50) values of soybean oils enriched by BHA and pigment extract obtained from the brown alga *Phyllaria reniformis* (Mean ± SD). Different letters indicate significant differences ($P \leq 0.05$, Tukey's HSD test).

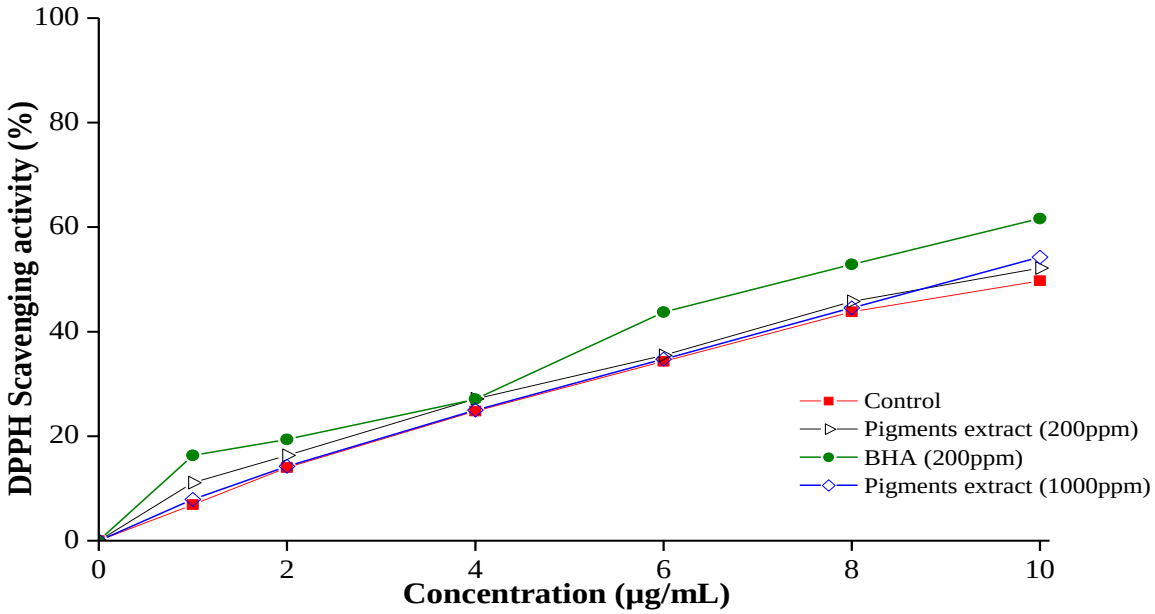


Figure IV.10: Free radical-scavenging capacities of sunflower oils enriched by BHA and pigment extract obtained from the brown alga *Phyllaria reniformis* .

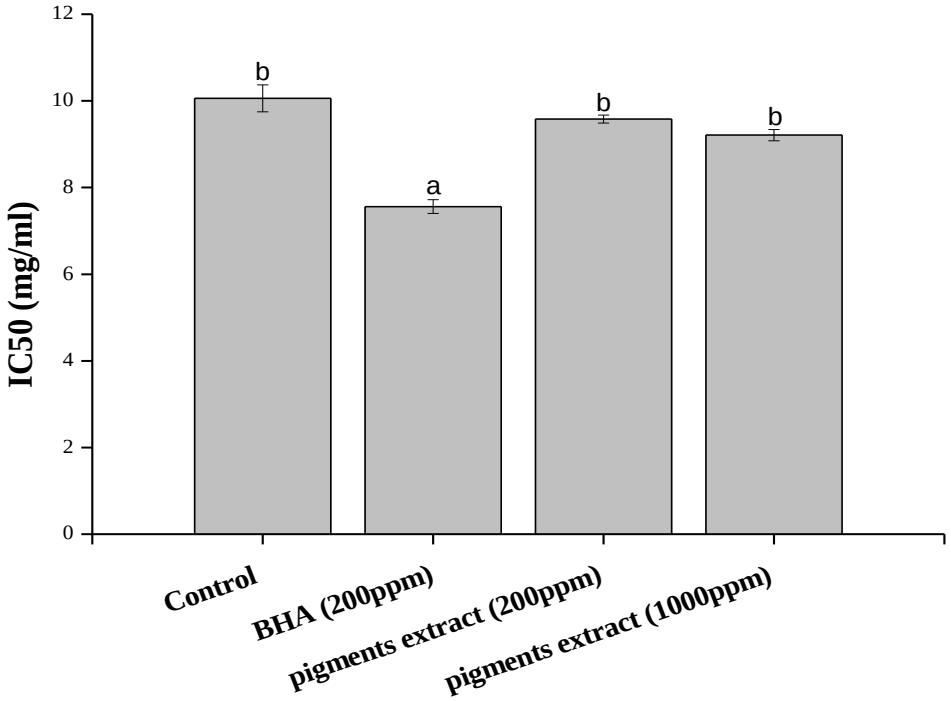


Figure VI.11: DPPH (IC50) values of sunflower oils enriched by BHA and pigment extract obtained from the brown alga *Phyllaria reniformis* (Mean ± SD). Different letters indicate significant differences ($P \leq 0.05$, Tukey's HSD test).

VI.3.6. Oxidative stability by Rancimat test

The oxidative process of vegetable oils could be accelerated by the Rancimat test. This method is usually used to assess the oxidative stability of edible oils. In this study, the Rancimat analysis was performed at 100°C and the IT(h) was evaluated for soybean and sunflower oils enriched with *Phyllaria reniformis* pigment extract or with BHA or without enrichment as control oil.

Figures VI.12 and VI.13 illustrate the oxidative stability of soybean oil expressed by the ID and the total oxidative stability curve (conductivity versus time), respectively. Results showed that both BHA and pigment extract had a strong antioxidant activity in soybean oil compared to the control one (8.95± 0.54 h). Thus, the presence of pigment extract retarded the oxidation of soybean oil. The highest stability was observed in soybean oil sample containing 1000ppm of extract (12.95± 0.43h) and those containing 200ppm of pigment extract or BHA gave IT value of 12.06± 0.7 and 12.47± 0.12 h, respectively. The addition of additives (pigment or BHA) to soybean oil increased significantly ($P \leq 0.05$) the IT compared to the control sample.

IT of enriched sunflower oil with 200 ppm of BHA was significantly higher (9.27± 0.13h) than all remaining samples. Enriched sunflower oil with 200 or 1000 ppm of *Phyllaria reniformis* pigment extract led to increase IT compared to the control sample as shown in Figures VI.14 and VI.15. Statistical analysis showed a high significant difference ($P \leq 0.05$) between sunflower oil sample enriched with BHA and the remaining samples.

Interestingly, the obtained results showed that oxidative stability of soybean and sunflower oils enriched with *Phyllaria reniformis* pigment extract was improved compared to control oil samples. The oxidative stability of both oils tended to increase with the addition of BHA or pigment extracts. Consequently, the ability of an additive to stabilize vegetable oils may depend on the nature of oil, the type and concentration of additives.

Due to the lack of published works on the effect of enrichment of vegetable oils with seaweed pigment extracts on their oxidative stability, it was very difficult to compare the obtained data with other studies, nevertheless, we tried to compare our results to other natural additives enrichment of vegetable oils.

According to **Shadyro et *al*. (2020)**, the addition of carotenoids (b-carotene, lutein, zeaxanthin) in flaxseed oil showed an increase of the IT value compared to the oil without

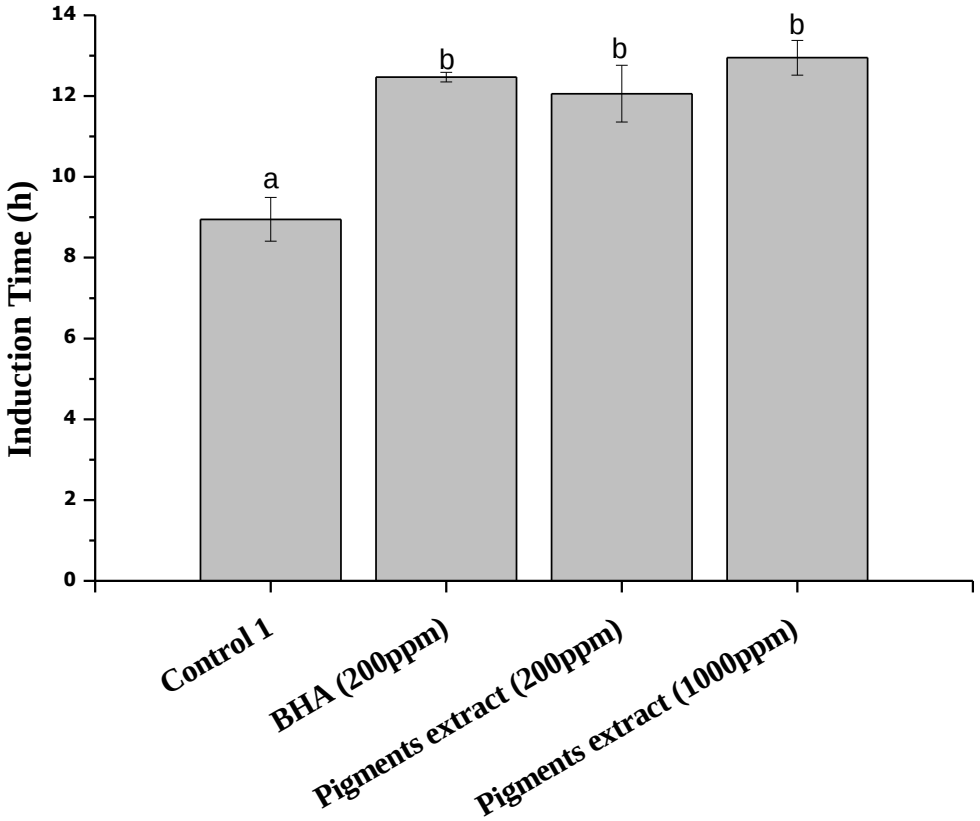


Figure VI.12: Induction time of the control and enriched soybean oils (Mean ± SD). Different letters indicate significant differences ($P \leq 0.05$, Tukey's HSD test).

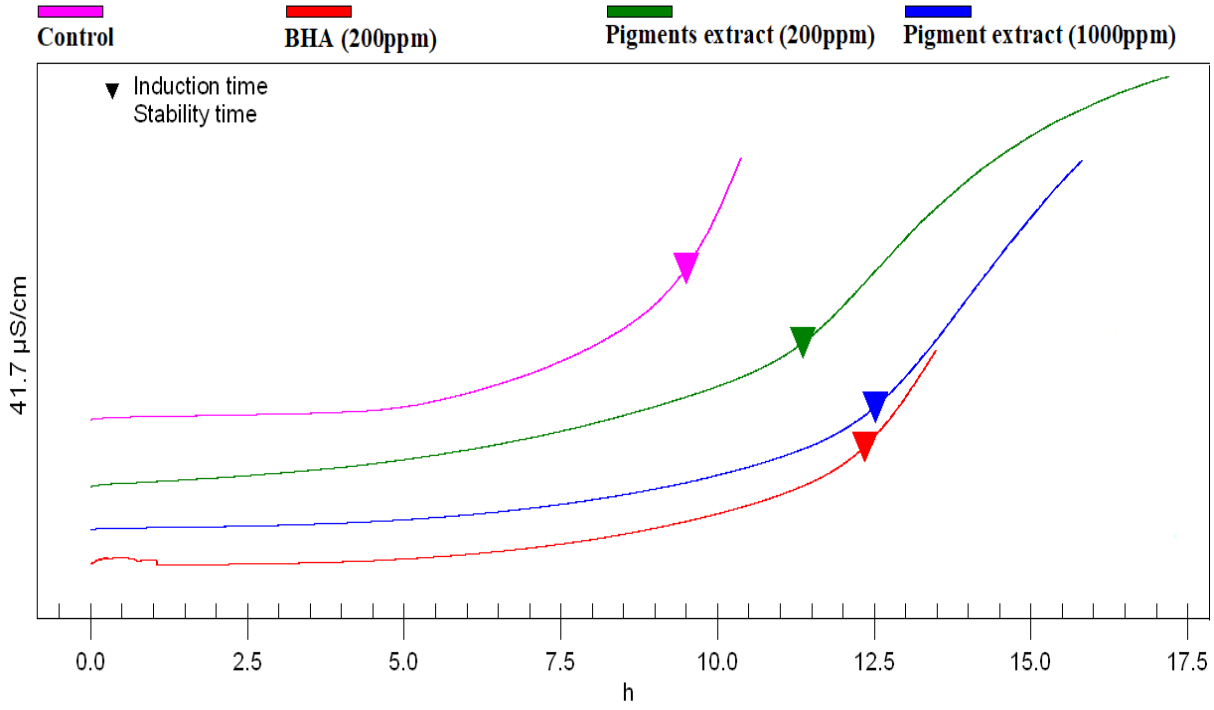


Figure VI.13: Oxidative stability curve (Induction Time) of the control and enriched soybean oils.

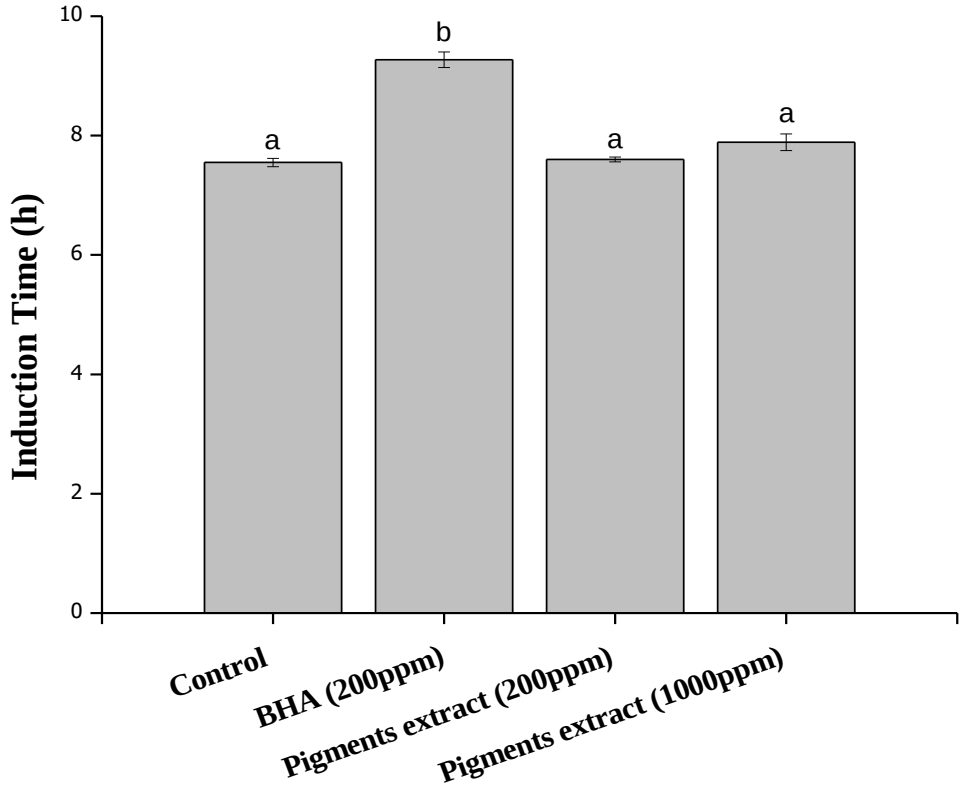


Figure VI.14: Induction time of the control and enriched sunflower oils (Mean ± SD). Different letters indicate significant differences ($P \leq 0.05$, Tukey's HSD test).

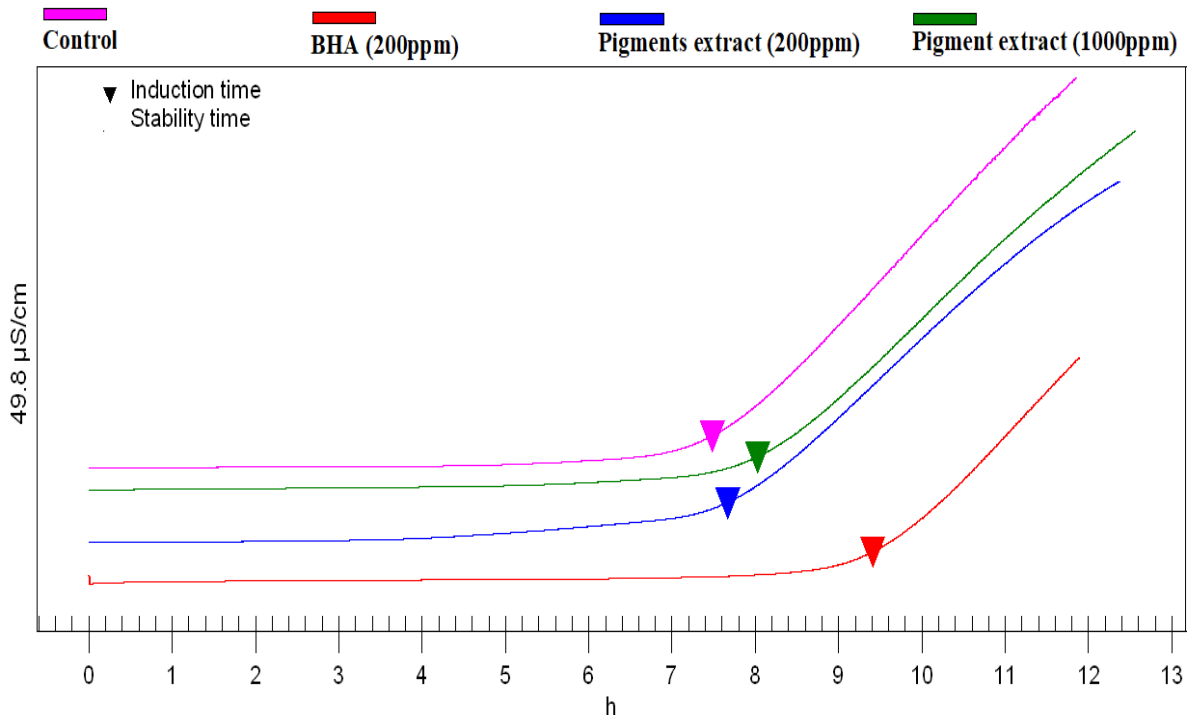


Figure VI.15: Oxidative stability curve (Induction Time) of the control and enriched sunflower oils.

additives (3.8 -5.6 h), whereas, at concentration higher than 10 mg of carotenoids per 100 g of flaxseed oil had a pro-oxidant effect, and then decreased the oxidation stability of flaxseed oil.

Le Tutour (1990) studied the antioxidant effect of the methanol-chloroformic extract of seven species of seaweed on the oxidation stability of sunflower oil. Results showed that within the seven-seaweed species, *Laminaria digitata* and *Himanthalia elongata* extracts were the most effective in extending the IT of the enriched oil. Similarly, in more recent studies, lipidic oxidation was inhibited by the addition of the brown seaweed *Fucus vesiculosus* ethanolic or acetonic extracts to fish oil enriched granola bars **(Karadağ et *al*., 2017)**, milk or mayonnaise **(Hermund et *al*., 2015; Honold et *al*., 2016)**.

Likewise, **Alavi and Golmakani (2017)** reported that the supplementation of olive oil by increasing percentage of microalga *Spirulina* powder (0.5-1.5%) could improve the oxidative stability 12.69 to 22.24 % in comparison to the control sample and then extended the shelf life of olive oil ..

Several studies demonstrated that the use of aromatic plants improved the oxidative stability of the formulated oils **(Jabri-Karoui & Marzouk, 2014; Karoui et *al*., 2011; Saoudi et *al*., 2016)**.

Furthermore, the effectiveness of adding various herbal plant extracts (olive leaf, marjoram, thyme, oregano, *Bifurcaria bifurcata*, beetroot, carrot, tomato, swede, ginger, turmeric, and *Opuntia ficus-indica*) on oxidative stability of edible oils (sunflower, soybean, canola, rapeseed, and olive oil) was evaluated in many studies **(Agregán et *al*., 2017; Ammar et *al*., 2017; Kozłowska & Gruczyńska, 2018; Salta et *al*., 2007; Tinello & Lante, 2020; Tundis et *al*., 2017; Zribi et *al*., 2013).**

Delfanian et *al*. (2016) showed that *Eriobotrya japonica* skin extracts could retard the oxidation of soybean oil, where the highest IT was observed in oils containing 400 or 1000ppm with IT values of 4.69 and 4.49 h, respectively. While the control oil exhibited only 3.32 h. The IT values obtained in the present study were two to three times higher, this may indicate that enrichment of soybean or sunflower oils with *Phyllaria reniformis* pigment extracts allowed more oxidative stability than *Eriobotrya japonica* skin extracts. Therefore, plant extracts **(Taghvaei & Jafari, 2015; Yanishlieva & Marinova, 2001)** and particularly seaweed pigment extracts could be recommended as a potent source of natural antioxidants replacing synthetic antioxidants for protection of edible oils against oxidation

VI.4. Conclusion

The present study was an opportunity to highlight the effectiveness of *Phyllaria reniformis* pigment extracts on reducing soybean and sunflower oils oxidation. Experimental results showed that the addition of pigment extract did not affect quality parameters of vegetable oils (FA and PV values), besides, it improved the carotenoid and chlorophyll contents. Both antioxidant effect and oxidative stability were improved after oils supplementation compared to the control oils.

Therefore, it is possible to obtain coloured functional extract from *Phyllaria reniformis* to be used in the food industry, particularly in oil as natural antioxidants preservative.

General Conclusions and Perspectives

As photosynthetic organisms, seaweed contains various pigments responsible for their brown, green and red colours. Seaweed pigments can be divided into three main groups: chlorophylls, phycobiliproteins and carotenoids and have a number of health benefits when consumed. Research concerning seaweed-derived bioactive compounds has increased significantly in recent years and there is currently considerable interest in the antioxidant, anti-obesity and anti-cancer activities of macroalgal pigments.

The main objective of this study was to extract, to characterize and to investigate biological activities (antioxidant and antimicrobial) of Algerian coast seaweed pigments. The second objective was to investigate the effect of seaweed pre-processing (drying, freezing and fresh) on the quality, quantity and antioxidant activity of extracted pigment. The third objective was to assess the potential use of these extracted pigments as food additives for preserving two vegetable oils from oxidation.

Therefore, in the first experimental chapter (Chapter IV), three brown seaweeds *Padina sp*. *Sargassum vulgar*e and *Phyllaria reniformis* collected in Algerian coast were selected for pigments extraction. RP-HPLC, UV-Visible, TLC and ATR-FTIR techniques were used to characterize pigment extracts, besides, antioxidant and antimicrobial activities were evaluated. The obtained results showed that the three selected seaweeds were riche in chlorophylls and carotenoids and they all exhibited high antioxidant activities. *Phyllaria reniformis* contained the highest amount of chlorophylls and carotenoids and showed a strong antioxidant activity compared to *Padina sp*. and *Sargassum vulgare*. Hence, *Phyllaria reniformis* was chosen for the second and the last studies in this thesis. However, all seaweed pigment extracts did not exhibit antimicrobial activity against five selected pathogenic strains: *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6938), *Candida albicans* (ATCC 10231) and *Aspergillus basiliensis* (ATCC 16404).

In the second experimental chapter (Chapter V), the impact of the brown seaweed *Phyllaria reniformis* freezing or drying pre-processing on pigments quantity, quality and antioxidant activity was assessed. Based on UV-visible spectrophotometry and HPLC results, alga pre-processing before extraction affected the quality and quantity of extracted pigment, a high variability on pigments content was shown. Freezing pre-processing exhibited the most efficient pigment extraction in term of quantity. While, drying pre-processing demonstrated higher amount of *β*-carotene and pheophytin *a*. The highest and most efficient antioxidant activities were obtained in the frozen samples. The quality, quantity and antioxidant activities of *Phyllaria reniformis* pigment extract was found to be deeply related to the pre-processing step.

In the third and last experimental chapter of this thesis (Chapter VI), *Phyllaria reniformis* pigment extract was added as natural antioxidant and colorant additive to soybean and sunflower oils for preserving them from oxidation. Results indicated that adding pigment extract increased induction time of both vegetable oils and in the meantime their stability against oxidation without affecting their physicochemical proprieties (Acidity and peroxide contents). Therefore, beside their health benefits, pigment extracts could be used as alternative to synthetic antioxidant additive for preserving food or non-food products from oxidation and in the same time as natural, renewal and healthy dyeing products.

This study could be a starting point to extend pigment seaweed extraction to other alga species and to optimize the conditions of pigment extraction process for higher extraction yields. As future work, seaweed pigments recovery and study of their biological effects as separate biocompounds would be much more relevant, particularly fucoxanthin which was highly present in all the three seaweed selected in this study. Besides, it may be helpful to try understanding the seaweed pigments antioxidant mechanism and to extend the assessment of other biological properties such as anti-cancer, anti-obesity and anti-inflammatory.

As concluding perspective, pigments extracted from Algerian seaweeds especially *Phyllaria reniformis* exhibited higher antioxidant proprieties and might find several potential applications as healthy ingredient in food products.

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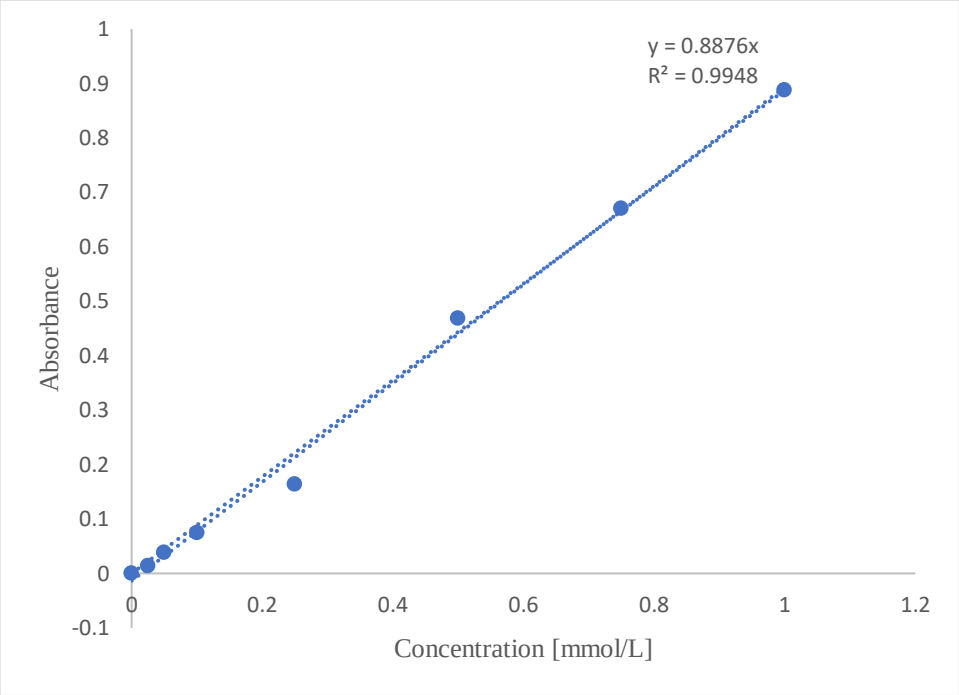
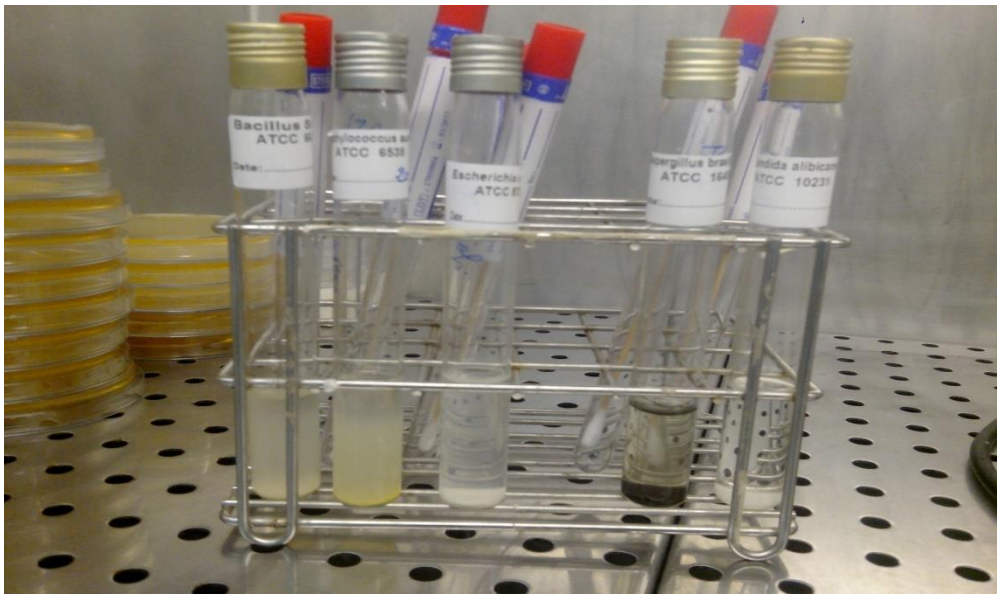
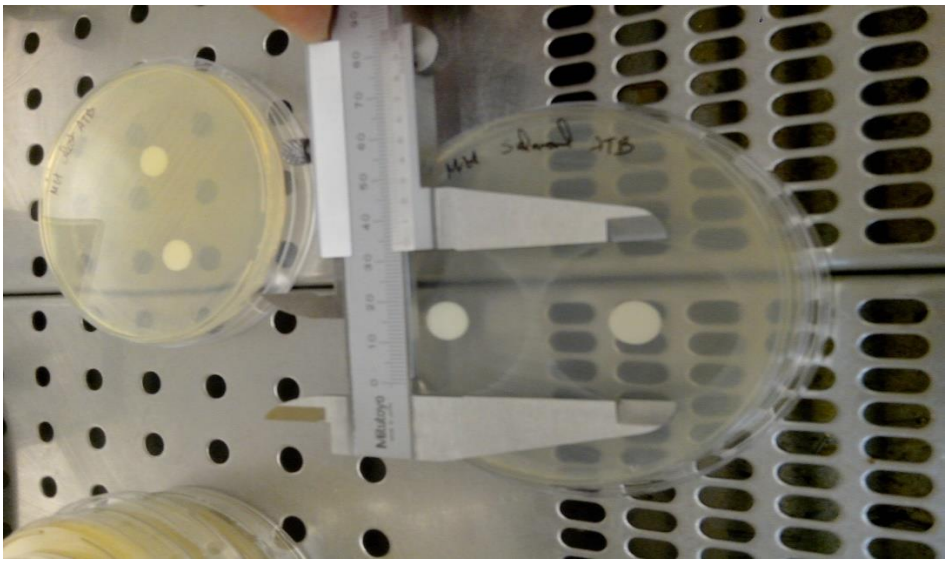


Appendix 01 : Standard curve (FRAP)



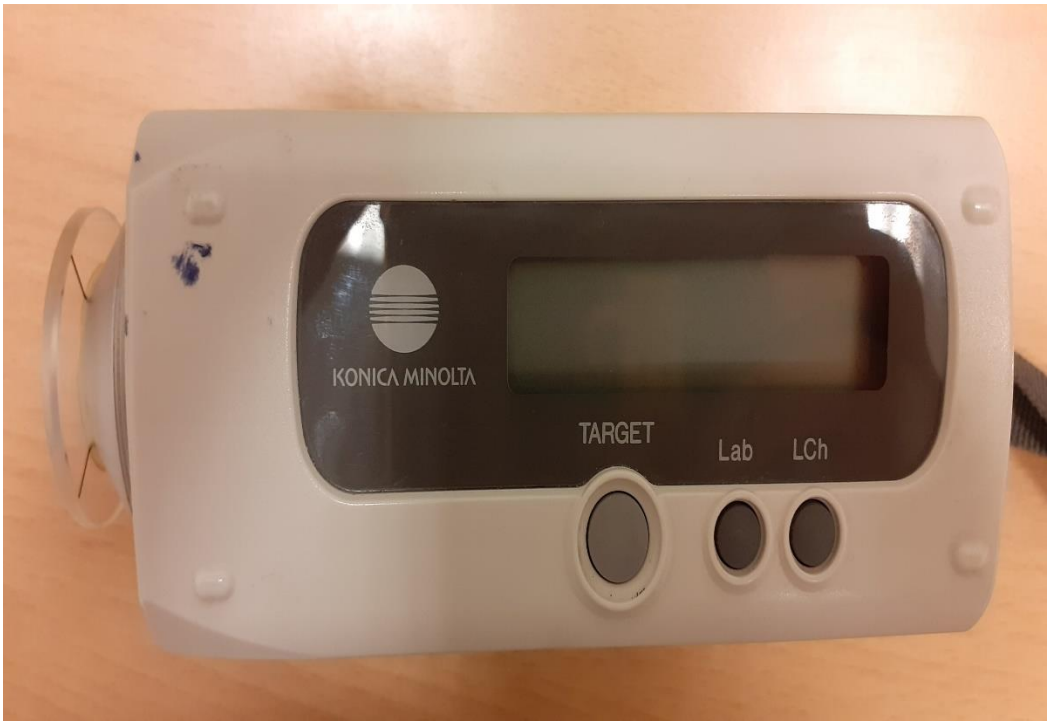
Appendix 02: ATTC strains



Appendix 03

Appendix 04: RP-HPLC

Appendix 05: ATR-FTIR Spectroscopy



Appendix 06: Colorimeter

Appendix 07: Rancimat

LIST OF PUBLICATIONS AND COMMUNICATIONS

I -Publications :

Nora GHALIAOUI, Hind MOKRANE, Mohammed HAZZIT, Mohammed HADJADJ, Fayçel SAID OTMANI, Souad TOUATI, Halima SERIDI. Impact of freezing and drying preprocessing on pigments extraction from the brown seaweed « Phyllaria reniformis» collected in algerian coast. Carpathian journal of food science and technology, 2020, 12(3), 81-94. https://doi.org/10.34302/crpjfst/2020.12.3.6

Nora GHALIAOUI, Lilya BOUDRICHE, Aromatisation des huiles végétales : Etude de l'effet de la méthode d'aromatisation sur les propriétés physicochimiques de l'huile végétale, Abstract book in The North African Journal of Food and Nutrition Research (2019) Vol. 03 (06), pp. A1- A127.

Nora GHALIAOUI, Amel HADJ ZIANE, 2015. " Caractérisation des algues vertes marines du littoral algérien à valoriser comme ingrédient d'intérêt nutritionnel ", BIOSEC2015, UMBB / LRTA, 617-621.

Nora GHALIAOUI, Amel HADJ ZIANE, 2015. " Caractérisation biochimique de certaines algues vertes méditerranéennes pour leur valorisation en nutrition humaine ", Nutr. Health, Vol. 04 No. 01 (suppl.), S1-S129.

II – Communications orales :

Nora GHALIAOUI, Lilya BOUDRICHE, Aromatisation des huiles végétales : Etude de l'effet de la méthode d'aromatisation sur les propriétés physicochimiques de l'huile végétale, 1ST International Conference Biodiversity In the Service of Biotechnologies, 09-10 Mars, Mila, Algeria

Nora GHALIAOUI Contribution à la protection de consommateur algérien : Enquête sur l'utilisation des additifs alimentaires dans les produits agroalimentaires, séminaire international des sciences alimentaires (SISA 2018) , Constantine le 15 et le 16 octobre.

Nora GHALIAOUI Étude du Potentiel d'application des huiles essentielles dans la conservation des aliments : Cas de l'huile essentielle de Lemon grass dans la sardine commune, séminaire international sur l'agroalimentaire (SIA 2018), Guelma le 16 et le 17 octobre.

III – Communications affichées:

Nora GHALIAOUI Caractérisation biochimique de certaines algues vertes de la mer Méditerranée pour leur amélioration en nutrition humaine. 1st International Congress of the

Algerian society of nutrition CI-SAN 2015, October 2015, Sheraton Club des Pins, Algiers, Algeria.

Nora GHALIAOUI Caractérisation des algues vertes algériennes à valoriser comme ingrédient d'intérêt nutritionnel ". 1st National Seminar on Biodiversity Environment and Food Security, Biosec2015, Octobre 2015, Boumerdes, Algeria.

Nora GHALIAOUI Macération (conventionnelle et assistée par micro-ondes) de feuilles de thym dans une huile végétale : mise en œuvre de la microscopie électronique à balayage . 1st International Congress of Biotechnology at the Service of Sustainable Development. CIBSDD 2017, Octobre 2017, Boumerdes, Algeria.

Nora GHALIAOUI Influence de la technique de macération (conventionnelle, assistée par micro-ondes et assistée par ultrasons) sur l'enrichissement des huiles végétales en polyphénols, chlorophylles et caroténoïdes ", International Seminar on Medicinal Plants (SIPM_2018), Janvier 2018, El Oued, Algeria.

Abstract

Most of seaweeds are green (Chlorophyta), brown (Phaeophyta) and red algae (Rhodophyta). Each group is characterized by specific combinations of photosynthetic pigments. In this study, three species of brown seaweed were harvested in Algerian coast: *Phyllaria reniformis*, *Sargassum vulgare* and *Padina sp*. and selected for pigments extraction. The aim was to investigate chemical composition, biological activities and potential use as antioxidant and natural dying additive in vegetable oil. The quantitative and qualitative analysis of the extracted pigments was determined by spectrophotometry, RP-HPLC, Thin Layer Chromatography and ATR-FTIR. The three seaweeds contained almost the same pigments composition but at different concentrations: Fucoxanthin was the most abundant directly followed by chlorophyll *a*, then chlorophyll *b*, *c* and *β* carotene. Extracted pigments exhibited high antioxidant activities, however, no antimicrobial effect was observed against all pathogenic strains used in this study. Among these brown seaweeds, *Phyllaria reniformis* revealed the highest content of chlorophylls and carotenoids and demonstrated the best antioxidant activity. The impact of seaweed preprocessing (fresh, freezing or drying) on pigments extraction indicated that freezing preprocessing exhibited the most efficient pigment extraction in term of quantity and showed highest antioxidant activities. Adding *Phyllaria reniformis* pigment extractto soybean and sunflower oils increased carotenoids, chlorophylls contents and antioxidant activities in both vegetable oils without affecting their physicochemical properties (acidity and peroxide contents) and led to improve their oxidative stabilities. .

This study could be a starting point to extend pigment seaweed extraction and use them as alternative to unhealthy synthetic antioxidant additives for preserving food or non food products from oxidation and in the same time as natural, renewal and healthy dyeing products.

Key words: Seaweed pigments, Chemical characterization, Antioxidant activity, Preprocessing, vegetable oil, Oxidative stability.

ملخص

أغلبية الأعشاب البحرية أو الطحالب، خضراء (Chlorophyta)، بنية (Phaeophyta) أو حمراء (Rhodophyta). كل مجموعة تتسم بنسب متفاوتة من الأصباغ الطبيعية. في هذه الدراسة، تم إختيار و جني ثلاثة أنواع من الأعشاب البحرية البنية من الساحل الجزائري *Phyllaria reniformis*, *Sargassum vulgare* و *Padina sp.* لإستخلاص أصباغها و تحليلها الكيميائي و تقدير فعالياتها البيولوجية و البحث في إمكانية إستعمالها كمضافات مضادة للأكسدة وملونات طبيعية في الزيوت النباتية. تم التحليل الكمي و الكيفي للأصباغ المستخلصة من الأعشاب البحرية بالمطيافية المرئية و الكروماتوغرافيا السائلة العالية الجودة للطور المعاكس (RP–HPLC) ، كروماتوغرافية المستوى طبقة رقيقة (Thin Layer Chromatography) و المطيافية التحت الحمراء بتحويل فوريي (ATR–FTIR). أظهرت النتائج أن الأعشاب البحرية الثلاثة تحتوي تقريبا على نفس الصبغات لكن بنسب متفاوتة: حيث الفوكوكسانثين موجود بوفرة يليه الكلوروفيل أ ثم الكلوروفيل ب ، ج و الـ *β* كاروتين. أظهرت الأصباغ المستخلصة من الأعشاب البحرية الثلاث نشاطات مضاد للأكسدة مرتفعة، لكنها لم تبرز أي تأثير مضاد للميكروبات إزاء جميع البيكتيرا و الفطريات الخطيرة المستخدمة في هذه الدراسة. من بين هذه الأعشاب البحرية، إتسمت *Phyllaria reniformis* بأعلى محتوى من الكلوروفيل والكاروتينات و أكبر فعالية مضادة للأكسدة. بينت نتائج تأثير طريقة التحضير (الإستعمال المباشر أو التجميد أو التجفيف) قبل استخلاص الأصباغ، أن التجميد هو الأكثر فعالية لاستخلاص كمية كبيرة من الاصباغ مع نشاط مضاد للأكسدة أكبر. أدت إضافة مستخلص أصباغ *Phyllaria reniformis* لزيت فول الصويا وزيت عباد الشمس، إلى زيادة في محتويات الزيتين من الكاروتينات و الكلوروفيل و رفع تأثيرهما المضاد للأكسدة بدون التأثير على خصائصهما الفيزيائية والكيميائية (الحموضة و نسبة البيروكسيدات) ، مما أدى إلى زيادة في إستقرارهما ضد الأكسدة.

يمكن لهذه الدراسة أن تشكل نقطة إنطلاق لتوسيع إستخلاص أصباغ الطحالب و سبل إستعمالها كبدائل للمضادات للأكسدة الصناعية و غير الصحية لحماية المنتجات الغذائية و الغير غذائية من الأكسدة و في نفس الوقت كمواد ملونة طبيعية، متجددة و صحية.

الكلمات الاساسية : أصباغ الأعشاب البحرية ، التحليل الكيميائي ، نشاط مضاد للأكسدة ، طرق التحضير، زيوت نباتية ، الاستقرار مضاد الاكسدة.

Résumé

La majorité des algues marines sont des algues verte (Chlorophyta), brune (Phaeophyta) ou rouge (Rhodophyta). Chaque groupe est caractérisé par une combinaison spécifique de pigments photosynthétiques. Dans cette étude, trois espèces d'algues brunes ont été récoltées de la côte algérienne : *Phyllaria reniformis*, *Sargassum vulgare* and *Padina sp*. Pour l'extraction de leurs pigments et la détermination de leur composition chimique et leurs activités biologiques ainsi que pour leur utilisation potentielle comme additif antioxydant et colorant dans l'huile végétale. L'analyse quantitative et qualitative des pigments extraits a été effectuée par spectrophotométrie, RP-HPLC, Chromatographie en Couche Mince et ATR-FTIR. Les trois algues contiennent approximativement la même composition en pigments mais à différentes concentrations : Fucoxanthine était le plus abondant directement suivi par la chlorophylle *a*, puis la chlorophylle *b*, *c* et le *β* carotène. Les pigments extraits ont exhibé des activités antioxydantes élevées, cependant, aucun effet antimicrobien n'a été observé contre toutes les souches pathogènes utilisées dans cette étude. Parmi ces algues brunes, *Phyllaria reniformis* a révélé la plus forte teneur en chlorophylles et caroténoïdes et a montré la meilleure activité antioxydante. L'étude de l'impact du prétraitement (fraiches, congelées ou séchées) sur l'extraction des pigments a indiqué que le prétraitement par congélation permettait l'extraction de pigment la plus efficace en termes de quantité avec des activités antioxydantes élevées. L'ajout des extraits de pigment de *Phyllaria reniformis* à l'huile de soja et de tournesol a permis d'augmenter les taux de caroténoïdes et de chlorophylles et des activités antioxydantes des deux huiles végétales sans affecter leurs propriétés physicochimiques (acidité et taux de peroxyde) et a mené à l'amélioration de la stabilité à l'oxydation des deux huiles végétales enrichies.

Cette étude pourrait constituer un point de départ à l'extension de l'extraction des pigments des algues marines et leurs utilisations comme substituant aux antioxydants synthétiques pour la conservation des produits alimentaires ou non alimentaires et en même temps comme colorants renouvelable et naturels.

Mots clés : Pigments des algues marines, Caractérisation chimique, Activité antioxydante, prétraitement, Huiles végétales Stabilité à l'oxydation.