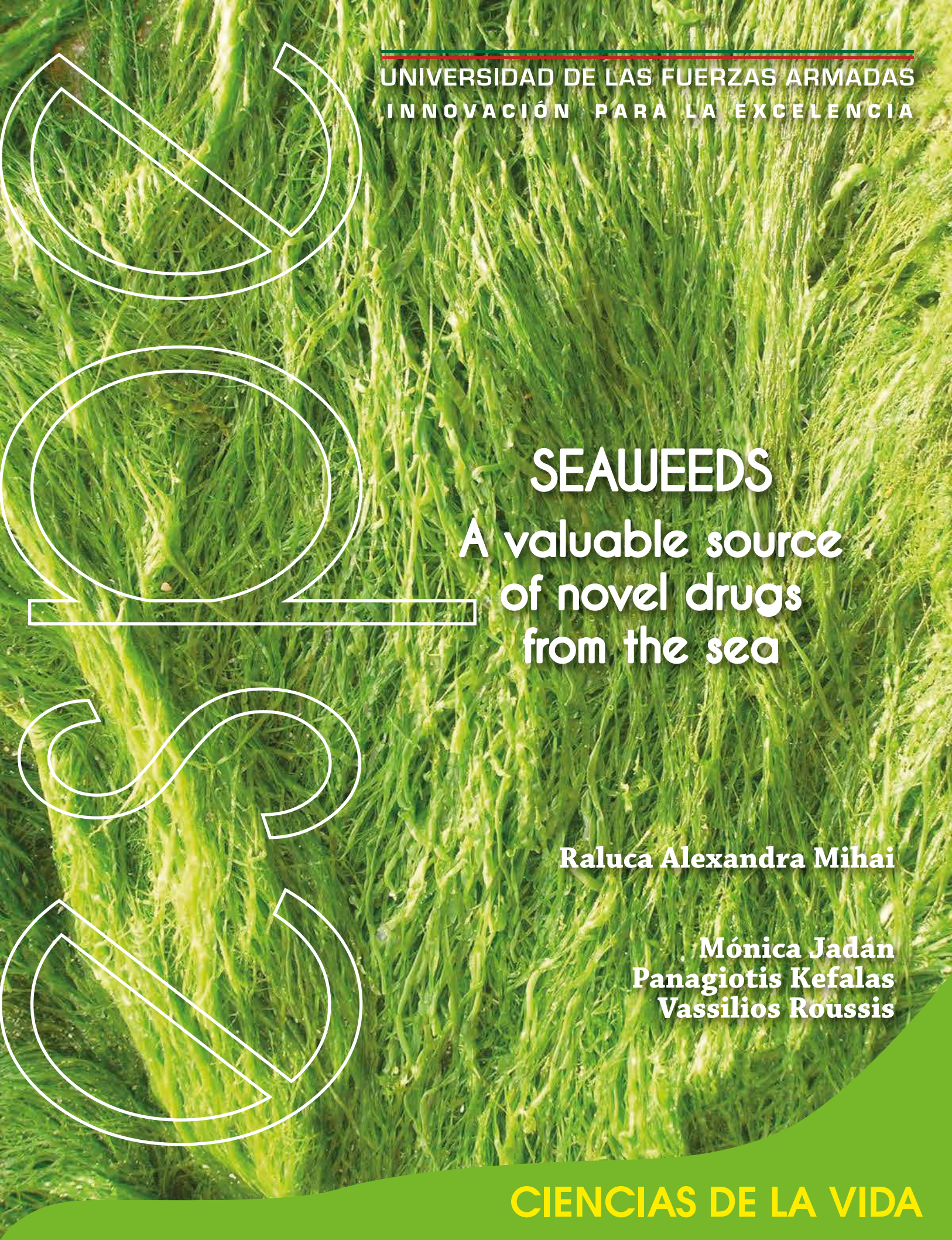

UNIVERSIDAD DE LAS FUERZAS ARMADAS
INNOVACIÓN PARA LA EXCELENCIA

SEAWEEDS
A valuable source of novel drugs from the sea

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CIENCIAS DE LA VIDA

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Seaweeds. A valuable source of novel drugs from the sea
Author: Raluca Alexandra Mihai, PhD.
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Primera edición electrónica **revisada**. Diciembre de 2016
ISBN: 978-9978-301-49-4

Pares revisión científica:
Javier Francisco Camacho; Ana Rosu.

Universidad de las Fuerzas Armadas ESPE
Grab. Roque Moreira Cedeño
Rector
Crnl. Ramiro Pazmiño
Vicerrector Académico General

Publicación autorizada por:
Comisión Editorial de la Universidad de las Fuerzas Armadas ESPE

Edición y producción
David Andrade Aguirre
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Diseño
Pablo Zavala A.

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Acknowledgements

The successfully completion of this thesis is owed to the thoughtful support and help of many persons from near or far to whom I want, without any particular order, to hereby kindly acknowledge bellow:

I would like to express my sincere gratitude to my supervisor, Dr. Vassilios Roussis, Associate Professor of Pharmacognosy at Athens University, for insightful scientific comments and suggestion, for useful guidance and help in achievement of this thesis, for being a valuable source of ideas and motivation as well.

I am very grateful to Dr. Antonios Makris, Coordinator of the Department of Natural Products and Biotechnology, for the scientific knowledge that I gained during two years, as student of this department.

I express my gratitude to Dr. Panagiotis Kefalas, Scientific Co-ordinator of the Departament of Food Quality Management, for his invaluable, pleasant and excellent assistance, as well as for sharing his scientific ideas and experience in the laboratories.

I wish to thanks to Mr. Abdelhak, officer in Food Quality laboratory for his generous assistance, for taking the trouble to share experience in the lab. with endless patience, and for his friendship.

The staff collaboration to complete the master thesis successfully is really acknowledged.

Above all, I would like to express my deepest gratitude, love and respect for my family for offering me in each moment of my life the

properly help and support, and for making me proud and happy for taking part from this family.

This book is dedicated to Dr. Panagiotis Kefalas, as symbol of my gratitude.

Preface

The intrinsic processes of conception, growth, and maintenance of living organisms are inexplicably associated with deleterious oxidative processes. In most living organisms, oxygen is not only necessary for life, but is also a major cause of damaging processes and death. The harmful effects of oxidative processes in living organisms, in addition to chemical and biochemical media, can be reduced by antioxidants (Simic, 1998).

Recently, many researchers have thought to find safe and effective natural antioxidants that can substitute current commercial synthetic antioxidants such as BHA and BHT, which can cause potential health hazards (Ahn, et al., 2004).

Most of the efforts made towards the discovery of new bioactive metabolites possessing antioxidant activity have focused for many years on the easily accessible terrestrial higher plants, animal and microorganisms (Roussis, et al., 2004).

Marine natural products have attracted the attention of biologists and chemists the world over for the last five decades. Deep water marine habitats constitute a relatively untapped resource for the discovery of novel antioxidants from natural products. To date approximately 16,000 marine natural products have been isolated several of the compounds from marine source exhibiting biological activity. The ocean is considered to be a source of potential drugs.

However, most natural antioxidants extracted from the terrestrial sources, i.e. carotenoids, phenolic compounds and vitamin E, have the important disadvantage of being water insoluble (Ahn, et al., 2004).

Seaweeds, like all photosynthesizing plants, are exposed to strong oxidizing agents that can cause photodynamic damage to the photosynthetic apparatus (Sukenik, 1993). The absence of such damage in seaweeds, in spite of the proximity of the photosynthetically produced oxygen and suitable targets within the photosynthetic apparatus, suggest that these cells have protective antioxidative mechanisms and compounds (Niki, 1997). In addition, seaweeds are rich in vitamins, minerals, natural bioactive compounds, and various functional polysaccharides. Particularly, the polysaccharides, which are plentifully present in brown seaweeds, such as alginates, fucans, and laminarans, are water-soluble dietary fibers and possess various bioactive properties. These polysaccharides are considered to be digested by some types of carbohydrases derived from microorganisms. These kinds of enzymes can convert water-insoluble seaweeds into water-soluble materials. It is expected that different bioactive properties, including antioxidant activities arise from the resulting enzymatic extracts of seaweeds (Ahn, et al., 2004)

This discovery, as well as the scientific advancement in the last two decades in the areas of chromatography, spectroscopy and marine technology, has allowed obscure and rare organisms to became accessible (Roussis, et al., 2004), and seaweeds, along with their extracts, to become good candidates as sources of natural water-soluble antioxidants (Ruperez, et al., 2002).

The attempts that have been made to explore the structurally unique chemistry of seaweeds for new antioxidant prototypes, have revealed the powerful antioxidant activity of seaweeds (Legarreta, 2004).

There has been a long tradition of consuming a wide range of seaweeds as sea vegetables, especially in the Far East and the Pacific (Mabeau, 1993), due to the recognized content of vitamins (A, B1, B2, and C), dietary fiber, and minerals (Nagai, 2003). Recent findings that seaweeds contain important antioxidant compounds have served to enhance its beneficial value as a human food (Yan, 1998), to the extent that it has the potential to be marketed worldwide (Nagai, 2003).

For this reason the investigation presented in this book is of real importance for the pharmaceutic and cosmetic industry finding a new source of important antioxidants.

CHAPTER 1

OXIDATIVE STRESS AND ANTIOXIDANTS

The related terms oxidative stress, oxidative damage, free, and antioxidant have become an integrated part of the scientific vocabulary and are often used in a variety of scientific discussions and issues by chemists, physicists, biologists, and researchers. (1)

In the biological systems during respiration, a significant fraction of oxygen is incompletely reduced. Such partially reduced oxygen, and their derivatives, are known as Reactive Oxygen Species (ROS), which are highly reactive pro-oxidants and toxic. (2) ROS represent a collective term that includes oxygen-centered radicals, such as superoxide and hydroxyl and some non-radical derivatives of oxygen, such as H_2O_2, singlet oxygen and hypochlorous acid. (3)

The cell is exposed to a large variety of ROS, from both exogenous and endogenous sources (Figure 1). (4) Some examples are represented by the exposure to di-oxygen, which, although a nonreactive biradical, is independently causing oxidation and damage to proteins and enzymes. Another source of ROS is represented by ozone which is not a radical like oxygen, characterized by its sharp odor and that can damage lungs, and can serve as a powerful oxidizing agent, that is oxidizing the biological components directly (5).

Exposure of living organisms to ionizing and nonionizing irradiation constitutes another major exogenous source of ROS (6). Exposure of the cell

to *c* -irradiation results in the production of a whole range of radical and nonradical species from ionization of intracellular water (eg, aqueous electron, OH. , H_2O_2). Even exposure to nonionizing irradiation such as UV-C (< 290 nm), UV-B (290–320 nm), and UV-A (320–400 nm) can indirectly produce a variety of ROS including, H_2O_2, and O., 2 radicals; hemolytic cleavage of H_2O_2 by UV radiation yields OH. radicals.

Air pollutants such as car exhaust, cigarette smoke, and industrial contaminants encompassing many types of NO derivatives constitute major sources of ROS that attack and damage the organism either by direct interaction with skin or following inhalation into the lung (7, 8).

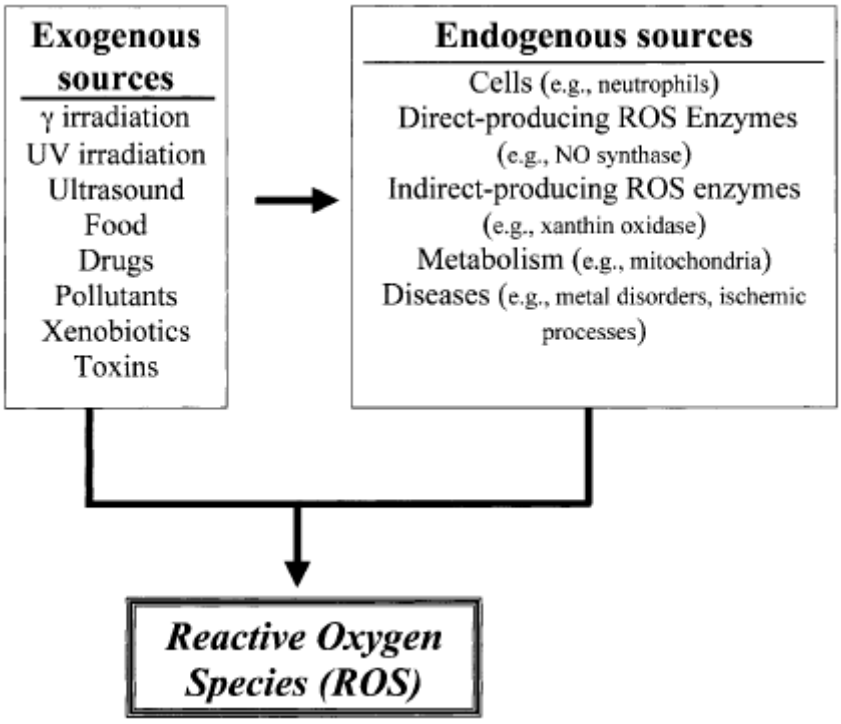

Figure 1. Exogenous and endogenous sources of reactive oxygen species (ROS). (1)

Natural resources such as radon, cosmic radiation, as well as cellular metabolisms (respiratory burst, enzyme reactions) also add free radicals to the environment. The most common reported cellular free radicals are hydroxyl ($OH\cdot$), superoxide ($O_2^{-}\cdot$) and nitric monoxide ($NO\cdot$). Even some other molecules like hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are not free radicals; they are reported to generate free radicals through various chemical reactions in many cases. (9) Radicals of oxygen (superoxide anion, hydroxyl, alkoxyl, and peroxyl), reactive non-radical species (H_2O_2 and singlet oxygen) and radicals of carbon, nitrogen, and sulphur constitute a variety of reactive molecules that cause oxidative stress to cells.

Free radicals (especially superoxide, O_2^{-}) and other reactive oxygen species, such as hydrogen peroxide (H_2O_2), are continuously produced *in vivo*. Free radicals are molecules capable of independent existence with unpaired electron in their outer orbit. Free radicals have very important role in origin of life and biological evolution, leaving beneficial effects on the organisms. (10) Oxygen radicals are involved in many biochemical activities of cells such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity. Nitric oxide (NO) is an important signaling molecule that essentially regulates the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics. (11)

Human body produce oxygen free radicals and other reactive oxygen species as by products through numerous physiological and biochemical processes. Oxygen related free radicals (superoxide and hydroxyl radicals)

and reactive species (hydrogen peroxide, nitric oxide, peroxynitrile and hypochlorous acid), are produced in the body, primarily as a result of aerobic metabolism. (12, 13) At the same time, antioxidants, such as glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A and tea polyphenols help to regulate the ROS thus generated.

An antioxidant (reductant or reducing agent), therefore, can be classifed as a compound capable of preventing the pro-oxidation process, or biological oxidative damage (14, 15). Halliwell (16, 17) suggested a definition for antioxidant, which states that this agent, when present in low concentration, significantly prevents or delays oxidation of an oxidizable substrate.

The organism must confront and control the presence of both pro-oxidants and antioxidants continuously. In a normal cell, there is an appropriate oxidant: antioxidant balance. There is know that the balance between these is tightly regulated and extremely important for maintaining vital cellular and biochemical

functions (18,19). This balance, often referred to as the redox potential, is specific for each organelle and biological site, and any interference of the balance in any direction might be deleterious for the cell and organism. Changing the balance towards an increase in the pro-oxidant over the capacity of the antioxidant is defined as oxidative stress and might lead to oxidative damage. Changing the balance towards an increase in the reducing power, or the antioxidant, might also cause damage and can be defined as reductive stress. (1)

Excessive amounts of ROS may be harmful because they can initiated bimolecular oxidations which lead to cell injury and death, and create oxidative stress which results to numerous diseases and disorders. (20)

There are various evidences that acute and chronic oxidative stresses have been implicated in a number of degenerative diseases, such as atherosclerosis, diabetes mellitus, ischemia/reperfusion (I/R) injury, Alzheimer's disease, inflammatory diseases (rheumatoid arthritis, inflammatory bowel diseases, and pancreatitis), carcinogenesis, neurodegenerative diseases, hypertension, ocular, pulmonary, and hematological diseases.(21, 22)

Damage caused by ROS may be due to their attack on membrane lipids, intracellular proteins/enzymes, carbohydrates, and nuclear DNA in cells and tissues. These include undesirable oxidation causing damage to membrane, protein modification, DNA damage, and cell death induced by DNA fragmentation and lipid peroxidation. (23)

Fortunately, free radical formation is controlled naturally by various beneficial compounds that are known as antioxidants. They protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by products of cell metabolism (24).

However, in food systems, antioxidants have been classified as substances, which are able to prevent or retard the oxidation of easily oxidisable materials, such as lipids in small quantities.

Antioxidants may intervene at any of the three major steps, of the free radical mediated oxidative process—initiation, propagation, and termination.

CLASSIFICATION OF THE ANTIOXIDANTS

Based on **chemical nature**, antioxidants can be classified as enzymatic and non-enzymatic antioxidants, preventive antioxidants, or scavenging, or chain breaking antioxidants.

- The human body uses an antioxidant defense system to neutralize the excessive levels of reactive oxygen. This system consists of *enzymatic* and *non-enzymatic antioxidants*. Some of the antioxidant enzymes that are found to provide a protection against ROS are superoxide dismutases, catalases, and glutathione peroxidases, in addition to numerous non-enzymatic small molecules distributed widely in the biological system and capable of scavenging free radicals (Figure 2). These non-enzymatic molecules include glutathione, tocopherol (vitamin E), vitamin C, β-carotene, and selenium. The enzymatic antioxidants act on specific ROS and degrade them to less harmful product, e.g., superoxide dismutases (SOD), catalase (CAT), and glutathione Peroxidase (GPx). These constitute the major intracellular antioxidants, while the extracellular antioxidants are mainly of the preventive and scavenging type. (25)

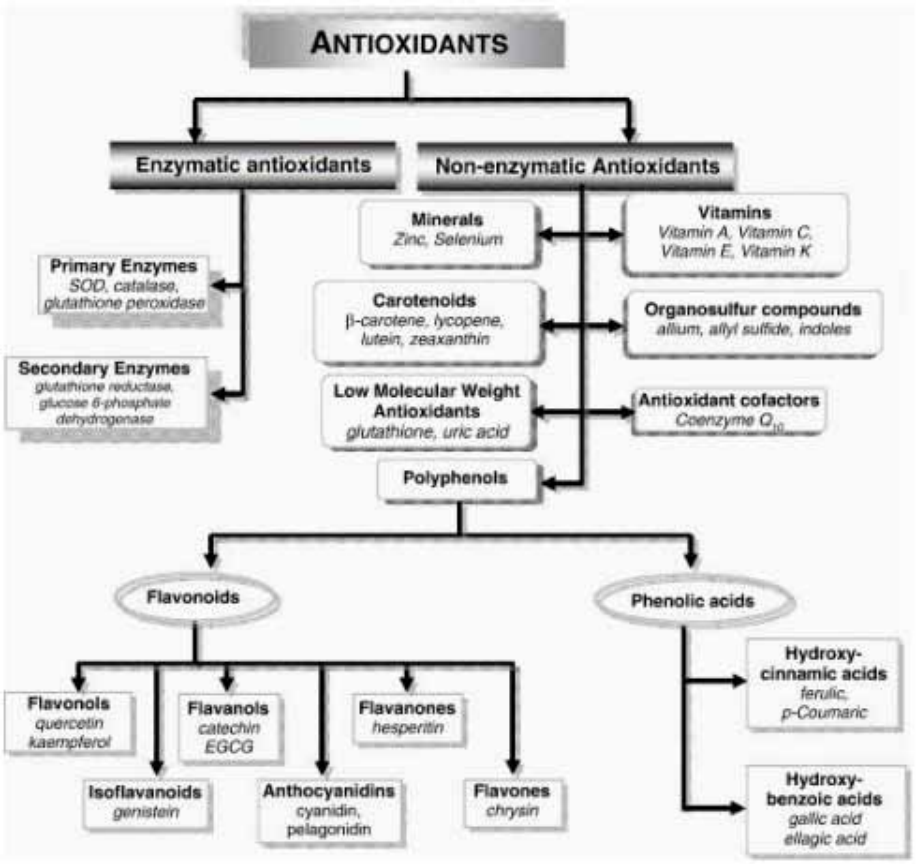

Figure 2. Classification of antioxidants. (25)

- *Preventive antioxidants* act by binding to and sequestering oxidation promoters and transition metal ions, which contain unpaired electrons and accelerate formation of free radicals, e.g., transferrin and lactoferrin (bind ferric ions), ceruloplasmin (binds copper ions), heptoglobins (binds hemoglobin), hemopexin (binds heme), and albumin (binds copper and heme). (26)
- *Scavenging or chain breaking antioxidants* act by presenting themselves for oxidation at an early stage in the free radical chain reaction and give rise to low energy products that are unable to propagate further. The major lipid soluble scavengers are Vitamin E (α-tocopherol), β-

carotene, and coenzyme Q (CoQ), while ascorbic acid, various thiols, uric acid, and bilirubin function in aqueous media. (27,28)

The antioxidants can also be classified **based on their source** as *endogenous* or *exogenous* antioxidants.

- GPx, CAT, and SOD are the major primary endogenous antioxidant enzymes, which metabolize toxic oxidative intermediates. These enzymes require micronutrients, such as selenium, iron, copper, zinc, and manganese, as cofactors for optimum catalytic activity and effective antioxidant defense mechanisms.

- Exogenous antioxidants are mainly from the dietary source, which include Vitamin C, E, and β-carotene. Vitamin C neutralizes ROS in the aqueous phase before lipid peroxidation is initiated while vitamin E, a major lipid soluble antioxidant, is the most effective chain breaking antioxidant within the cell membrane. β-carotene and other carotenoids also provide antioxidant protection to lipid rich tissues. (29,30)

Based on their **mode of action**, the antoxidants can be classified as *primary*, *secondary* or *co-antioxidants*.

- *The primary antioxidants* are the compounds that are able to donate a

hydrogen atom rapidly to a lipid radical, forming a new radical, which is more stable. (31, 32) For example, ascorbic acid, flavonoids, and tocopherol can stop chain reactions by donating an electron to the peroxyl radical of the fatty acid to stop propagation.

- *The secondary antioxidants* are the compounds that can react with the initiating radicals (or inhibit the initiating enzyme), or reduce the oxygen level (without generating reactive radical species). These can retard the rate of radical initiation reaction by elimination of initiators. This can be accomplished by deactivation of high energy species (e.g., singlet oxygen), absorption of UV light, scavenging of oxygen (thus reducing its concentration), chelations of metal catalyzing free radical reaction, or by inhibition of enzymes, such as peroxidases, NADPH oxidase, xanthine oxidase, etc.
- The combination of ascorbic acid and tocopherol can give rise to strong antioxidative effect. (33) The role of ascorbic acid is to restore the depleted concentrations of tocopherol, thus acting as *co-antioxidant*.

There has been a growing considerable interest to identify new sources of safe and inexpensive antioxidant and antimicrobial potential of natural origin (34).

The marine environment is known as a rich source of chemical structures with numerous beneficial health effects. Among marine organisms, marine algae have been identified as an under-exploited plant resource, although they have long been recognized as valuable sources of structurally diverse bioactive compounds.

Recently, much research attention has been focused on the free-radical-scavenging activity of metabolites from marine macro algae. Several studies have investigated the antioxidant activity of natural products in marine and freshwater algae. Considerable work has been done on algae natural products for the presence of nontoxic antioxidants that could be used in chemotherapy.

IN VITRO METHODS FOR THE DETERMINATIONS OF ANTIOXIDANT ACTIVITY OF DIFFERENT BIOLOGICAL SYSTEMS

- *Electron spin resonance (ESR) method*: These analytical methods measure the radical scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O2), the hydroxyl radical (OH), or the peroxyl radical (ROO). (25)
- *Enhanced chemiluminescence (ECL):* ECL has been used to measure antioxidant capacity in biological fluids. The assay involves the chemiluminescent substrate luminal. Light emission occurs when the luminal is oxidized by hydrogen peroxide that is generated in a reaction catalyzed by horseradish peroxidase (HRP). This method can quantify the antioxidant capacity of a substance which is sensitive to radical scavenging antioxidants that reduce the light output. (25)
- *4-2,2-Diphenyl-1-picrylhydrazyl (DPPH):* A rapid, simple and inexpensive method to measure antioxidant capacity that involves the use of the free radical 2,2-Diphenyl-1-picrylhydrazyl

(DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Figure 3), and to evaluate antioxidant activity. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. (25)

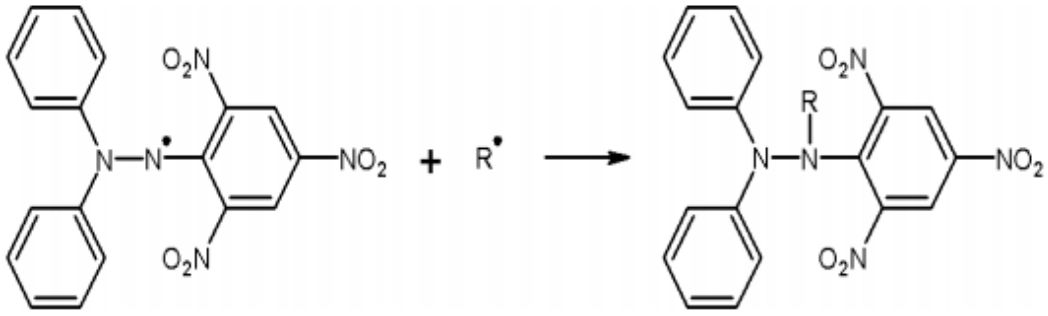

Figure 3. Mode of action of DPPH radical with antiradical compound (RH).

- *The ABTS [2,2'- azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation:* This has been used to screen the relative radical-scavenging abilities of flavonoids and phenolics. ABTS is a better choice than DPPH and more sensitive than DPPH. The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds. It is also useful for measuring antioxidant activity of samples extracted in acidic solvents. Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing

antioxidant activity of samples in different media and is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer solution containing 150 mM NaCl) (PBS). Another advantage of ABTS+ method was that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS), reaching a steady state within 30 min. The DPPH reacted very slowly with the samples, approaching but not reaching steady state after 8 h. This slow reaction was also observed when ABTS was reacted with samples in alcohol (Figure 4).(25)

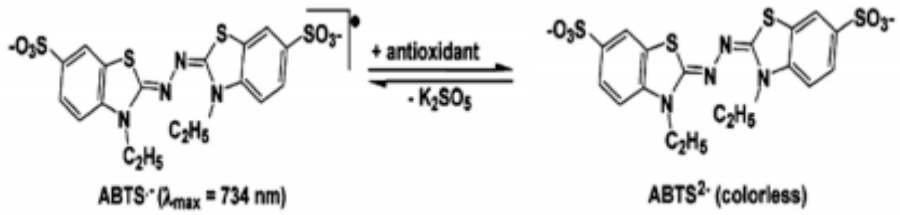

Figure 4. Reaction between ABTS radical and antioxidant compound.

- *The Oxygen Radical Absorbance Capacity (ORAC)*: This procedure is used to determine antioxidant capacities of fruits and vegetables. In the ORAC method, a sample is added to the peroxyl radical generator, 2,2′-azobis(2-amidinopropane) dihyd rochloride (AAPH) and inhibition of the free radical action is measured (35) using the fluorescent compound, B-phycoerythrin or R-phycoerythrin. Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, foods, and beverages and are usually quantified employing Folin's reagent. Vinson et al. (1998) (36)

have measured phenolics in fruits and vegetables calorimetrically using the Folin-Ciocalteu reagent and determined the fruit and vegetables antioxidant capacity by inhibition of low density lipoprotein oxidation mediated by cupric ions.

- *Cupric assay*: Cu (II) may act as a catalyst in the presence of excessive antioxidants, and the antioxidants Antioxidant reaction with ferric salt, $Fe(III)(TPTZ)_2Cl_3$ (TPTZ) 2,4,6-tripyridyls-triazine may act as pro-oxidants. Thus, Cu (II) is a questionable initiator for assaying the radical chain-breaking capacity of antioxidants (Figure 5). (25)

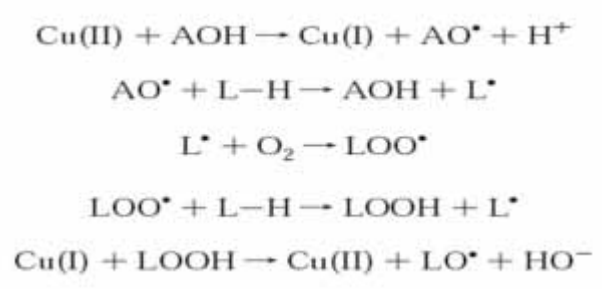

Figure 5. The reaction of cupric ion with antioxidant compound (AOH).

- *Ferric reducing antioxidant power (FRAP)*: FRAP assay also takes advantage of electron-transfer reactions. Here, a ferric salt, Fe (III) (TPTZ) 2Cl3 (TPTZ) 2,4,6-tripyridyls-triazine), is used as an oxidant. The redox potential of Fe (III) salt (-0.70 V) is comparable to that of ABTS (0.68 V). Therefore, essentially, there is no much difference between TEAC assay and the FRAP assay except that TEAC is carried out at neutral pH and FRAP assay under acidic (pH 3.6) conditions (Figure 6). (25)

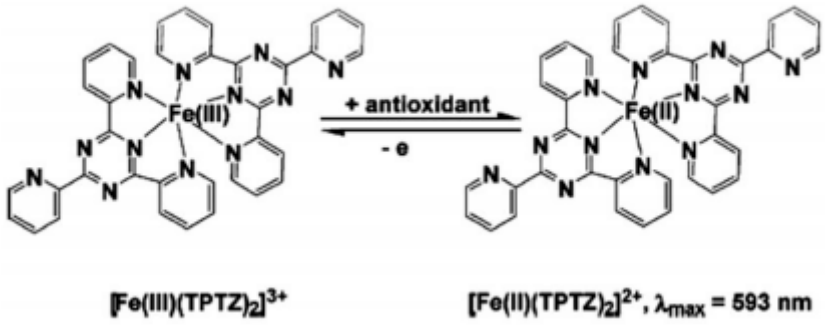

Figure 6. Antioxidant reaction with ferric salt, $Fe(III)(TPTZ)_2Cl_3$ (TPTZ) 2,4,6-tripyridyls-triazine.

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CHAPTER 2

ALGAE DEFINITION, CLASSIFICATION AND DESCRIPTION

Algae are a group which contain some of the most primitive members of the plant kingdom, simple structures that appeared on earth very early in geological history and haven't changed very much to the present day. (1)

Algae are very simple chlorophyll-containing organisms being considered by some researchers as plants or called by others *protists* or *protoctists*. According to the most recent phylogenetic studies, both are not quite correct. Some algae (most greens and the reds) are indeed related to the land plants, and some flagellated algae are related to the protists, but there is no justification for the including all algae in any generic term other than "algae". (2)

In the broadest sense, algae are oxygen-generating, photosynthetic organisms other than embryophyte land plants, fungi and lichens. They are an artificial and highly heterogeneous aggregation of organisms belonging to many different evolutionary lineages, and therefore highly diverse from a genetic point of view. This genetic diversity is reflected in the enormous diversity exhibited by algae in terms of morphological, ultrastructural, ecological, biochemical, and physiological traits.(2)

These organisms constitute a total of 25-30,000 species, with a great diversity of forms and sizes, and that can exist from unicellular microscopic organisms (microalgae) to multi cellular of great size (macroalgae).

The beginnings of algae research were dated in 1768 when Samuel Gottlieb Gmelin developed the work Historia Fucorum. By far the majority of the large, conspicuous form of attached marine plants is the seaweeds. In a more restrictive sense, the term seaweed is used referring only to macroscopic members of marine plant kingdom, only to macro-algae, representing plant-like organisms that generally live attached to rock or other hard substrata in coastal areas (3).

The colour of the thallus was introduced as a taxonomic character for the algae as long ago as 1838, and the modern classification of the algae still uses pigment composition as a primary character. Simply on the basis of their thallus colour due to specific combination of photosynthetic pigments, have been empirically distinguished since the mid-nineteenth century by the Irish botanist William Henry Harvey (1811-1866) so far three of the groups of marine macroalgae,: Red Algae (phylum Rhodophyta, represented by 4,500 species), Brown Algae (phylum Ochrophyta, class Phaeophycea, with 1,000 species) and Green Algae (phylum Chlorophyta, classes Bryopsidophyceae, Chlorophyceae, Dasycladophyceae, Prasinophyceae, and Ulvophyceae with a number of 900 species).(4)

Distinguishing these three groups, however, involves more substantial differences than indicated by this simple designation. In addition to the pigmentation, they differ considerably in many ultrastructural and biochemical features including photosynthetic pigments, storage compounds, composition of cell walls, presence/absence of flagella,

ultrastructure of mitosis, connections between adjacent cells, and the fine structure of the chloroplasts. In general, we can say that they are simple organisms composed of one cell, or grouped together in colonies, or as organisms with many cells, sometimes collaborating together as simple tissues. (2)

The green algae (including Chlorophyta, Charophyta, Euglenophyta), have a similar pigmentation and the bright grass-green colour characteristic of this division is due to the predominance of chlorophylls *a* and *b* over other accessory pigments such as β-carotene, phycocyanin and xanthophylls. (1)

The Chlorophyta or the green algae exhibit fantastic diversity in structure varying from simple filaments (Chaetomorpha), to flat sheets and diverse complex branching forms (Codium). (5) Most species live in fresh water, but several marine species attain larger size than most fresh water forms. The Chlorophyta have fewer marine species and some of the larger marine forms attach themselves to rocky substrate and form large mats. Some green algae live as comensals inside the shells of mollusks, and other live inside the shells of some protistans and animals.

The brown colored algae (Phaeophyta, Chrysophyta, Pyrrophyta and Cryptophyta) are the largest and longest of the seaweeds. The chlorophyll pigments of the Phaeophyta sometimes can be seen as a greenish hue. But more often the green is partially masked by the golden xanthophyll pigments, especially fucoxanthin, which is characteristic of this division.

The brown algae occur frequently as conspicuous algae that are almost exclusively marine. Some forms are simply branched filaments, other are

giant seaweeds that may be more than 60 m long. They grow primarily in the lower intertidal and subtidal zones and are fastened to the substrate. Kelps are brown algae with a plant body consisting of a basal holdfast region, a stripe or stem, and a broad blade. Brown algae, especially kelp are among the fastest-growing organism in the plan kingdom.

Red algae (Rhodophyta), with red and blue phycobilin pigments as well as chlorophyll, exhibit a wide range of colors. Most red algae living below low tide range from soft pinks to various shades of red.

The Rhodophyta, are mostly marine organisms and are common along rocky sea coasts. They are relatively small plants, usually less than a meter long, and almost all species are multicellular. They include sheets and simple branched filaments as well as lacy-branched forms. Many species occur in warm, tropical waters as small, inconspicuous organisms. In cooler waters, they are less abundant but often much larger.

THE ALGAL DESCRIPTION OF THE GREEK MAINLAND, INVESTIGATED FOR THE ANTIOXIDANT CAPACITY

The coastline (18,000 km) of the Greek mainland is located in the Eastern Mediterranean Sea, it is surrounded by the Aegean, Ionian and Cretan Seas and its morphological regime shows a variety of gulfs and semi-enclosed gulfs.

All these basins are eutrophic since they receive the waters and fertile material from large rivers and/or smaller water outfalls derived from agricultural and industrial activities.

This areas are comprising a huge biodiversity of algal species that where investigated from the point of view of the antioxidant capacity to be able to identify new and efficient sources of natural antioxidants.

A briefly and aleatoric description of these algae was done in the fallowing lines:

Taonia atomaria (Woodward) J. Agardh – **Figure 7**

Description: Thallus flat and leaf-like, to 300 mm long (usually 100-150 mm) and 10-40 mm broad; fronds thin and translucent, olive to yellow-brown, irregularly forked-lacinate, lacking a midrib; reproductive structures characteristically formed in wavy bands. **Habitat**: sunny, sandy pools in the lower intertidal in summer.

Distribution: Widespread in the Mediterranean.
Key characteristics: Absence of midrib, lacinate forking, reproductive structures in bands. (2)

Gelidium pulchellum (Turner) J.V. Lamouroux – **Figure 8**

Description:
Cartilaginous, regularly or irregularly bipinnate, dark red-brown fronds, 50-100 mm high, arising from a creeping base. Main axes narrow,

cylindrical, somewhat flattened above. Ultimate branches short, pointed at first, later spathulate, particularly when reproductive. Appearance variable with habitat and time of year.

Habitat: Abundant in and around tidal pools in mid- and lower intertidal in high-light situations. Widely distributed, common.

Key characteristics: Pinnate branching, mid- and lower-intertidal low-light habitats, and slighly flattened main axis. (2)

Laurencia obtusa (Hudson) J.V. Lamouroux – **Figure 9**

Description: Globose tufts of brittle, cartilaginous, narrow, cylindrical, reddish brown to yellowish red fronds, 150 mm long, from small discoid base. Axis simple, branches patent, often opposite, spirally arranged, shorter towards apex giving irregularly pyramidal outline. Ultimate ramuli very short, truncate. With special refringent inclusions in cortical cells (*corps en cerise*).

Habitat: Usually epiphytic, annual, lower intertidal, widely distributed, occasional to frequent. (2)

Ulva rigida C. Agardh – **Figure 10**

Description: Thallus sheet-like, emerald green, stiff at the base and more delicate near the growing edges, to 300 long, sometimes with irregular elliptical holes (above). Persists throughout the year.

Habitat: on rock and growing as an epipthyte, often developing "green tides"

Distribution: Common and widely distributed.

Key characteristics: emerald green colour and stiffness of thallus. (2)

Enteromorpha linza (Linnaeus) J.Agardh – **Figure 11**

Description: it is able to reach up to 45 centimeters in length. The plant is composed of clusters of long, unbranched, bright green ribbons about 5 centimeters wide and two cells thick with ruffled edges. Each blade of *Enteromorpha linza*

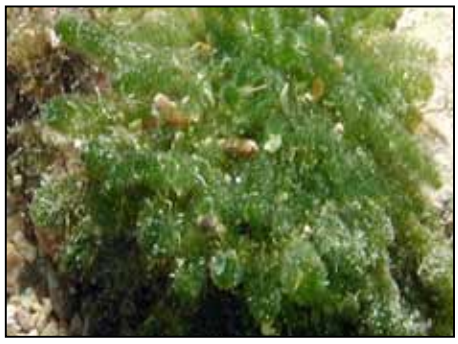
tapers into a small distinct tubular stipe which then cluster into a common holdfast area, and blade surfaces are uniquely smooth, often described as "silky."

Distribution: it is a common species in intertidal to shallow subtidal areas attached to any available substrate from pebbles to bedrock, often found in tide pools, but preferring more protected habitats than other species of *Enteromorpha*. Tolerant of a wide range of salinities, *Enteromorpha linza is* often found in estuary areas or places with freshwater run-off. (2)

Halimeda tuna (J.Ellis & Solander) J.V.Lamouroux – **Figure 13**

Description: The thallus is erect upto 9 cm tall, greenish to cream when dried; rhizoidal holdfast is not well developed and somewhat inconspicuous; basal portion consisits of one or two segments which may be fused, forming a barrel shaped stipe; branching dichotomous to tetra dichotomous. Segments are overlapping and are either spreading or compact; branched segments are moderately calcified, generally flabellate (fanshaped) to broadly reniform (kidney shaped), 13 - 33 mm across and 11-19.5 mm tall with dull glossy surface; segments are 0.5 - 1.7 mm thick, usually increasing from the base to the upper end. The distal margin of segments is significantly thicker appearing wavy and folded when pressed.

Dasycladus claviformis (Roth) C.Agardh – **Figure 14**



Cladostephus spongiosus (Hudson) C. Agardh
Figure 15

Caulerpa prolifera (Forsskal) LAMOUROUX – **Figure 16**

Sargassum vulgare C. Agardh Bonaire – **Figure 17**

Sargassum is a genus of brown (class Phaeophyceae) macroalga (seaweed) in the order Fucales. Numerous species are distributed throughout the temperate and tropical oceans of the world, where they generally inhabit shallow water and coral reefs. However, the genus may be best known for its planktonic (free-floating) species. While most species within the class Phaeophyceae are predominantly cold water organisms that benefit from nutrients upwelling, genus *Sargassum* appears to be an exception to this general rule. Any number of the normally benthic species may take on a planktonic, often pelagic existence after being removed from reefs during rough weather. ted

Dictyopteris membranacea (Stackhouse) Batters – **Figure 18**

Codium vermilara (Olivi)Delle Chiaje – **Figure 19**

Posidonia oceanica (L.) Delile – **Figure 20**

Posidonia oceanica, commonly known as Neptune Grass or Mediterranean tapeweed, is a marine flowering plant endemic to the Mediterranean Sea. Like other seagrasses, it forms large underwater meadows in the submerged photic zone of sheltered coastal waters (6). Due to its high rate of primary production and its ability to structure and stabilize the seabed, Neptune grass creates habitat for many other marine organisms and thus plays a significant role in littoral Mediterranean ecosystems (7, 8, 9).

Posidonia oceanica is one of the largest, slowest growing, and longest-lived plants. In a recent genetic study of 40 *P. oceanica* populations across the Mediterranean, Arnaud-Haond et al. (2012) (10) found individual clones spanning up to 15 km (9.3 miles). Based on the plant's known growth rate, such individuals are likely to be thousands, possibly tens of thousands of years old. (10)

With their origin possibly dating back to the Pleistocene, some *P. oceanica* meadows have shown great resilience, persisting through great environmental changes over millennia.

Corallina Mediterranea Areschoug – **Figure 21**

Laurencia papillosa (C. Agardh) Greville – **Figure 22**

Udotea petiolata (Turra) Børgesen –

Figure 23

Description: is a fan-shaped alga with rounded edges; they reach a height of 10-100mm and can be nearly as wide. The holdfast is typically composed of aggregated

colourless siphonous branches; the fan is often indented or tattered at the margins, with a pattern of concentric stripes. The texture of *U. petiolata* is weak and the alga has an olive green colouration (11)

Halopteris filicina (Grateloup) Kützing - **Figure 25**

Description: A small alga up to 10 cms long. Central axis surrounded by a cortex with alternately branched pinnate thalli. Very bushy and rigid.

Habitat: Epilithic and epiphytic in shaded pools of the low littoral and in the sublittoral to 20 m. (11)

1. ALGAL CONTENT

From earliest times man has used algae as food, both for direct consumption (fresh or dried), or as an ingredient in other foods. Today, seaweed is still used as food in many parts of the world, especially in the Far East and Japan.

The nutritional properties of seaweeds are incompletely known, and are estimated from their biochemical and chemical composition. (12)

Seaweeds are known for their richness in proteins, fatty acids, carbohydrates, vitamins and minerals. In the same time contain large amount of polysaccharides.

Most of these polysaccharides are not digested by humans endogenous enzymes, therefore, they can be regarded as dietary fibers. Edible seaweeds contain 33 – 50 % total fibers, higher than levels in the higher plants. The chemical nature and physicochemical properties of the seaweed dietary fibers are partly known for the edible seaweeds already used as sources of food additives. (12)

Seaweeds are known to possess a high mineral composition that varies according to phylum as well as various other factors (e. g. seasonal, environmental, geographical and physiological variation). Most of the macroalgae have high calcium, magnesium, phosphorus, potassium, sodium and iron contents. (12)

It is well known that red algae have very high content of minerals (15 – 30 % of dried algae) as compared to land plants.

Some other species notably *Ulva* and *Enteromorpha s*pp., are also characterized by high levels of particular elements with catalytic function such as magnesium (2 – 5,2 % of dry matter). (12)

The vitamin profile of seaweeds can vary according to algal species, season, algal growth stage and environmental parameters. Most red seaweeds contain large amount of provitamin A and significant quantities of vitamins B_1 and B_2, that are of major nutritional significance for human consumption.

The vitamin content of brown seaweeds appears less remarkable, but should be noted that recent data indicate high vitamin C levels (150 – 400

mg/Kg of dry weight: Centre d'Etudeet de Valorisation des Algues, unpublished) in fresh *Himanthalia elongate*.

The protein found in seaweed is a complete protein that contains all the eight essential aa. as in meat, but has virtually no calories, unlike this one. Seaweed protein contents also vary greatly from phylum to phylum. The proteic fraction of brown seaweeds is generally small (average: 5 – 15 % of the dry weight) whereas higher protein contents are recorded from green and red seaweeds (the total protein content of commercially grown *Prophyra* sp. ranges 30 – 50 %), the protein levels in this two phylum being comparable to those found in high protein vegetables such as soybeans.

Lipids represent 1 - 3% of algal dry matter. Red seaweeds contains significant quantities of polyunsaturated fatty acids, particular sterols are an interesting characteristic of some brown seaweeds, but their contribution as food energy source thus appears to be low.

More research is needed to evaluate the nutritional value of marine algae, especially in the fields of biochemical analysis by accurate modern methods and evaluation of their bioavailability. (12)

2. ALGAL USES

✓ *Industrial uses*

There have been two distinct phases in the industrial utilization of seaweeds: the first of these involved the burning of seaweeds and the extraction of sodium and potassium salts, and later iodine from the ash.

Before about 1800, seaweed ash was a major source of soda for the manufacture of soaps and glass but it was rapidly made obsolete for this purpose by the development of chemical process for sodium carbonate at the beginning of the 19th century. This industry became uncompetitive as a source of these relatively low- value products. (13)

The term" industrial gums" is a generic name for a range of products that are either manufactured artificially or extracted from animals or plants are used to achieve various levels of viscosity. These include polyethylenglycolate, xanthan gum, carboxymethylcellulose and gelatine. Industrial gums extracted from seaweeds fall into three categories: alginates (derivatives of alginic acid), cell wall structural polysaccharides from brown seaweeds, agars and carrageenans from red seaweeds.

Algin is a polysachharide specific to brown seaweeds and is not found in land plants, its localization varies among the varieties of brown algae (e.g. in *Laminaria* the algin is mainly in the stipe, in *Macrocystic*the most common source of algin the content being in the blade). (14)

Alginates have a wide range of industrial application, but the two major uses are in paper and textiles. For paper manufacture, alginate are used to improve ink holdout, to control the flow properties of coating and to improve smoothness of the sheet. In textiles alginates, especially sodium alginates form an excellent dressing and polishing material. In pharmaceuticals, alginates serve as protective coating suspending agents that increase the stability and bioavailability of the active ingredients. Their unique properties natural source and proven safety will increase the role of alginates in consumer products worldwide.

Agar is a gelling hydrocolloid obtained by extraction with water or alkali of certain species of red algae. It is a linear galactan, consisting primarily of alternating D – galactose and L – 3, 6 – anhydrogalactose monomers, with a small amount of charged substituting groups. The best quality agar is extracted from species of red algal genera Pterocladia and Gelium, lesser quality being extracted from Gracilaria and Hypnea species.

In 1881 it was discovered that agar could be used as gelling agent for microbial culture media. Modern agar is purified from consisting largely of the neutral fraction known as agarose; the non ionic nature of the latter makes it more suitable for a range of laboratory application. Agarose can be used for separation and purification of proteins such as enzymes. Agarose in a crude or purified form finds wide usage in the food industry where it is used as a stabilizer in various kinds of ices, canned foods and bakery products.

Carrageenan is a hydrocolloid consisting of the potassium, sodium, magnesium and calciumsulphate esters of galactose and 3,6 – anhydro-galactose copolymers. It is obtained by the extraction of certain species of red algae with water and alkali (15).

Carrageenan is widely used in gelled milk products, both in ready – to – consume desserts and in powder preparations for making flans or puddings. In meat-based products it is used to improve texture by adding firmness, increasing sliceability, decreasing liquid separation and producing juicer product.

There are many non- food application such as the thickening of tooth paste and shampoo. Carrageenan may be used as a tablet coating agent, and for bacteriological media.

A possible future use of carrageenan is as an agent that can prevent or inhibit development of viral infections transferred sexually. That is, carrageenan may help in the struggle against the HIV virus.

✓ *Agricultural uses*

In coastal agricultural regions of the world, seaweed was used for fertilizer in historic times.

Detached seaweeds or "total drift" have been used for many years in several European countries for making of "lazy beds" from layers of seaweed and send or sandy soil on bare rock. Both large algae and a number of crustose, calcareous red algae (*Corallinaceae*) have been collected for fertilizer/soil improvement. (16)

The effect of seaweeds on the soil and on the plants grown is due to the algal carbohydrates content that in the plant rod down and encourage soil bacteria to multiply.

Their soil conditioning action improves the structure, making a good "crumbs" aggregate which remains stable. This improves the soil`s ability to hold water. At the same time seaweed nutrients are released into the soil in form which plants can easily use. (1)

Seaweed products are much favored by organic farmers and horticulturists as it is belived to provide many trace elements that might otherwise need to be added in "chemical" form (17) and in the same time it is free of weed seeds and fungal spores which could harm future crops. (1)

A wide range of beneficial effects have been reported from the use of liquid seaweed extracts (18), including increased crop yields, resistance of plants to frost, increased uptake of inorganic constituents from the soil,

more resistance to stress conditions, and reductions in storage losses of fruits.

The presence of plant hormones has been suggested as being responsible for, at least some of the observed effects; it has been demonstrated that commercially – available seaweed extracts have high levels of cytokinin – like activity. (16)

Betain have been recorded for most of the species of marine algae used in the manufacture of seaweed extract. Blundel & Gordon 1986 (19) were of the opinion that these substances have an activity similar to that of cytokinins in certain respects.

✓ *For animal uses*

On small islands on the European side of the North Atlantic it has been common practice to feed sheep and cattle on harvested seaweeds of the intertidal brown algae *Ascophyllumnodosum* (20), which is the favorite of the commercial producer of animal feeding stuff.

Vitamins, minerals and other constituents present in seaweeds provide a healthy feed for animals. Feeding weed to animals and humans returns the valuable nutrients to the land, ending the cycle mineral loss due to natural erosion and poor farming practice to the benefit of all living things. (1)

✓ *Ecological uses*

Algae are very efficient in actively taking up ions from the surrounding water. This feature may be useful for cleanup of areas polluted with heavy

metals, or the algae may be used as monitoring organisms of environmental conditions. (21)

✓ *Medicinal uses*

Europe is rapidly opening its eyes when it comes to the potential role of seaweed in health and nutrition and a scientific basis in support of the many claims is rapidly amassing. (Table 1)

Table 1. Scientific support for the nutritional and health claims of seaweed derived components, symbols indicate where direct evidence is available, and numbers in brackets refer to listed references.

CARBOHYDRATES	FIBRE	▼ (7, 8)	✔ (3,8,17)	✚ (8)		
	LAMINARAN	▼(3)	✔ (17,18)	✚ (29,30)		
	FUCOIDAN	▼ (3, 12, 13)	✔ (21)	✚ (29)	⚷ (3, 13, 35,36)	
	ALGINIC ACID	▼ (3)	✔ (18,19,22)	✚ (26)	⚷	
	CARRAGEENAN	▼ (3)	✔	✚ (25)	⚷ (33)	
	PORPHYRAN	▼ (3)		✚		
	ULVAN	▼ (9)				
PROTEINS	ESSENTIAL AMINO ACIDS	▼ (12)				●●
	CAROTENOIDS	▼	✔ (21)	✚ (27,28)	⚷	
LIPIDS	OMEGA 3 & 6	▼ (11, 14)	✔	✚ (3)	⚷ (3)	
	STEROLS	▼ (10, 12)		✚		
ANTIOXIDANTS	POLYPHENOLS			✚		
	VITAMINS	▼		✚	⚷	●
MINERALS	CALCIUM	▼ (10)				●
	IRON	▼ (10)				
	MAGNESIUM	▼ (10)				●
	POTASSIUM					●
	ZINC				⚷	●
	SELENIUM				⚷	
	IODINE					

Reducing blood pressure

High blood pressure can result from an imbalance in sodium and potassium in the body and deficiency in other minerals, such as magnesium and calcium, can also lead to vasoconstriction. Seaweeds typically contain high concentrations of potassium, magnesium and calcium and have been shown to have a positive effect on regulating mineral balances and blood pressure (22). The soluble fiber component also promotes increased nutrient absorption and bioavailability in the small intestine (23).

Antihypertensive activity has also been reported in rats and rabbits through ingestion of seaweed fibre (24) and laminine, a brown seaweed protein (25)

General heart health

Seaweeds are generally low in fat (<2%) but much of this can comprise polyunsaturated fatty acids (26) including the essential omega (n)-3s LNA, EPA and DHA (*resp.* α-linoleic, Eicosapentaenoic and Docosahexaenoic acids) and omega-(n)-6 LA (linoleic acid). n-3 and n-6 fatty acids have opposing physiological functions that require a balance for normal growth and development in humans (26). A ratio of 5:1 (omega n-6: n-3) is recommended but most European diets are overly high in n-6 and low in n-3 (27).

Brown and red seaweeds provide a good balance with ratios that are comparable with cold fish sources (26). Omega-3 fatty acids are not thought to have a direct effect on blood cholesterol levels but they show an ability to reduce blood triacylglycerol (storage fats) levels and hence help to maintain

general heart health. Long chain *n*-3 fatty acids have been shown to reduce the risk of having a fatal heart attack (27). Although precise mechanisms are not clearly understood, protection against blood clot formation (thrombosis), protection against heart arrhythmias and a beneficial impact on blood pressure are likely (28).

Lowering digestive tract disease

Dietary fiber is key to healthy digestion, it is required to clear the digestive tract, protect surface membranes in the stomach and intestine against ulcers and carcinogens, and promotes a healthy gut flora. The recommended daily intake of fibre or Guideline Daily Amount is 24g (29) approximately 12.5% of which could be provided by an 8g (dry weight) portion of selected seaweeds (22).

Some seaweed fiber is fermented in the lower intestine but in general, soluble and insoluble seaweed fiber tends to pass through the gut without being digested (30,31), this results in decreased bowel transit time and increased fecal bulking and water retention.

A lowering in bowel transit time can reduce the risk of colo-rectal cancer, ulcers and inflammatory bowel diseases, and the incorporation of seaweed fiber into the diet has been shown to actively reduce the above.

Research has shown that the non-starchy polysaccharides of seaweeds in general can have a prebiotic effect *i.e.* they selectively stimulate and activate useful gut flora microorganisms. Prebiotic ingredients, or those that boost the growth of beneficial probiotic bacteria in the gut, are worth about € 90 million in the European market place (32).

As little as a 2.5% (wt/vol) addition of alginate oligosaccharides can significantly increase the levels of bifidobacteria and lactobacilli in rats and humans (32).

Fighting obesity

Seaweeds high in dietary fiber have also been shown to have positive effects on reducing hyperglycemia and hyperinsulinemia and thus are useful in relation to controlling diabetes and obesity (31).

A range of seaweed based preparations are commercially available to help with weight control. One might be sceptical when reading advertisements such as "*Lose Weight With a High-Fibre Seaweed Supplement That Supports Your Liver, Slows Digestion, and Makes You Feel Full"* but in fact, the bulking effect of seaweed fiber is known to prolong postprandial feelings of satiety and reduce the absorption of metabolites in the intestine (31, 33). For these reasons, researchers at Newcastle University have even experimented with adding alginate fiber to burgers in an attempt to make "healthier" junk food (34).

The brown algal pigment, Fucoxanthin and its metabolite fucoxanthinol have been shown to induce the expression of UCP1 a protein that suppresses fat accumulation, particularly around internal organs in rats and mice, resulting in an increase of <10% fat oxidation (35).

Diabetes

An estimated 19 million people in the EU and 20 million in USA suffer from diabetes (36). The presence of soluble alginate fiber in the diet is

known to regulate the absorption of metabolically available nutrients, thus reducing glycaemic load and the resultant insulin response (31, 37).

Fighting cancer

The anti-cancer properties of seaweeds have been well documented since ancient times, the Chinese and Egyptians used brown seaweed to treat breast cancer. Seaweeds contain large amounts of antioxidants and polyphenols which constitute their own protective antioxidative defence systems and many studies have demonstrated *in vitro* antioxidant activity in a range of them (24).

Seaweed derived oligo and polysaccharides have been demonstrated to induce apoptosis (programmed cell death) in cancer cell lines (38) and some, including carrageenan (39) and alginate (40) can inhibit the growth of tumours in rodents through immunomodulating activity.

The Takara Bio Inc., Japan produces a range of seaweed based products that are active against leukaemia, stomach and colon cancer. The active ingredient is U-Fucoidan™, a guluronic rich polysaccharide extracted from kelps. Modiflan™ (www.modiflan.com) boasts similar properties and is a polysaccharide based extract from the kelp *Laminaria japonica.* The active components are fucoidan, laminaran and alginate.

Fucoxanthin (photosynthetic pigment) extracted from the *kelp Undaria* can, significantly reduce the viability of human prostate cancer cells (41) and colon cancer cells (42).

Japanese women typically suffer from very low levels of estrogen related cancers (breast and cervical) and it is thought that this is due, in

part, to the high levels of brown seaweed that they ingest. Incorporating the brown seaweeds *Laminaria* and *Fucus vesiculosus* into the diet has been shown to prolong the length of the menstrual cycle in women and to exert anti-estrogenic effects that may be responsible for the reduced risk of estrogen related cancers (43, 44).

Seaweeds are also a good source of the trace element selenium which is currently attracting a lot of attention regarding its potential antitumoral.

Anti-inflammatory

Polysaccharides, in particular the sulphated ones like fucoidan are well known for their anti-inflammatory activity. Modiflan™ which contains fucoidan, is marketed as an antiinflammatory.

The fucoidan component promotes the growth of fibroblasts, in turn repairing inflamed cells (www.modiflan.com).

Normal metabolism of polyunsaturated fatty acids in seaweeds can lead to the production of eicosanoids which are important for range of physiological functions, including anti-inflammatory activity (24). An ecosanoid like compound isolated from the brown *seaweed Caulocystis cephalornithos* has shown anti-inflammatory activity akin to salicylic acid (24) and Caulerpeyne, isolated from the green seaweed *Caulerpa* has been shown to be effective against bees stings (24).

In Japanese women, high intake of seaweed has been shown to reduce the prevalence of allergic rhinitis – hay fever (45).

Antimicrobial protection

The range of seaweed derived compounds that show antimicrobial activity is diverse and well documented (24, 46). Seaweeds naturally employ antimicrobial systems for wound defence and prevention of disease. Most compounds employed are halogenated substances, terpenoids or phenolics (24) but also sulphated polysaccharides.

The red seaweeds in particular are rich in carbohydrates with antiviral activity. In a study of 40 different species of red seaweed, carbohydrate extracts from 37 showed
significant suppression of retroviral growth (46).

Commercial antiviral preparations are readily available however, Carraguard™ (containing 3% carrageenan) is probably the best known and this is still under clinical trial. It has demonstrated antiviral activity against sexually transmitted viruses including HIV – Human Immunodeficiency Virus.

Sulphated polysaccharides from brown seaweeds, namely fucoidan, are also active against a range of gram positive and gram negative bacteria and viruses (24)

Viracle™ (Marine BioMedical Research Pty. Ltd., Australia) is a product containing an extract from the kelp *Undaria* that shows confirmed inhibitory activity against HSV – Herpes Simplex Virus and potential activity
against HIV.

Seaweeds also have antifungal properties. Proteins isolated from the red alga *Hypnea musiformis* were shown to be active against human pathogenic yeasts (47) but in general, the antifungal properties of seaweeds

are utilised more in the horticultural industry for disease and pest control (48).

Seaweeds naturally produce chemical deterrents in response to grazing activities of invertebrates and fish (49). Some of these compounds have been responsible for human deaths (24) but a few amino acid derivatives are utilised as vermifuges. (24)

Healthy bones, joints & skin

Seaweeds are naturally high in minerals thatare essential for healthy bones and joints.

They are also high in vitamins and antioxidants - important components of cosmetic preparations (50).

Calcium can comprise up to 7% of dry weight (51) and is also found in association with with polysaccharides, such as alginate and carrageenans. The calcareous red seaweeds such as *Lithothamnion* spp. can contain as high 35% calcium. An Irish Product AquaminTM is a multi-mineral preparation from *Lithothamnion* spp. It has been shown to be high in bioavailable calcium, more so than the standard calcium carbonate that is used in supplements, and is effective against mobilisation of bone calcium through parathyroid hormonal activity. Similar activity has been observed with a mix of oystershell and the brown seaweed *Cystophyllum fusiforme* (52).

Three genera of seaweeds are routinely used in cosmetics -*Laminaria, Fucus* and

Chondrus - for their ability to nourish and rehydrate the skin (53). Topical application of fucoidan has been shown to have anti-aging activity by increasing the moisture and elasticity of the cells (54, 55). Fucoidan is

known to stimulate the production of HGF – Heparin Growth Factor which promotes growth in a range of cells and tissues and this is exploited commercially by the Takara-Bio Company in Japan.

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CHAPTER 3

1. NEED FOR ANTIOXIDANTS

It has been reported in epidemiological studies that many of antioxidant compounds posses anti inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities to greater or lesser extent (1, 2, 3). In many cases, increased oxidative stress is a widely associated in the development and progression of diabetes and its complications which are usually accompanied by increased production of free radicals or failure of antioxidant defense (4).

Though the intake of natural antioxidants has been reported to reduce risk of cancer, cardiovascular diseases, diabetes and other diseases associated with aging, there is considerable controversy in this area. (5, 6, 7, 8) Leukocytes and other phagocyte destroy bacteria, parasites and virus-infected cells with NO, O_2, H_2O_2, and OCl, those are powerful oxidants and protect humans from infection. However, they cause oxidative damage and mutation to DNA and participate in the carcinogenic process if unchecked. In many cases, it is concluded that antioxidants modulate the pathophysiology of chronic inflammation up to some extent. (9, 10, 11, 12, 13, 14)

Moreover, experiments and studies infer that antioxidants are needed to scavenge and prevent the formation of ROS and reactive nitrogen species (RNS); out of them, some are free radicals while some are not. (15) There is growing evidence that oxidative damage to sperm DNA is increased when there is ascorbate insufficiency in diet. (16) This strongly suggests the protective role of antioxidant in our daily diet.

2. SOURCES OF ANTIOXIDANTS

Four endogenous sources appear to account for most of the oxidants produced by cells. (17) Normal aerobic respiration in which mitochondria consume O_2, reduces it by sequential steps to produce O_2, H_2O_2, and -OH as byproduct. (18) Bacteria or virus infected cells get destroyed by phagocytosis with an oxidative burst of nitric oxide (NO), O_2^-, H_2O_2 and OCl. (19) Peroxisomes produce H_2O_2 as a by-product of fatty acid and other lipid molecular degradation, which is further degraded by catalase. Evidence suggests that, certain conditions favor escape of some of the peroxide from degradation, consequently releasing it into other compartments of the cell and increasing oxidative stress leading to DNA damage. (20) Animal Cytochrome P_{450} enzymes are one of the primary defense systems that provides protection against natural toxic chemicals from plants, the major source of dietary toxins. Even these enzymes are protective against acute toxic effects from foreign chemicals, yet they may generate some oxidative byproducts that damages DNA. (21)

Various antioxidants are supplied to human body through diet, both vegetarian as well as non vegetarian. Vitamins C and E, β-carotene and coenzyme Q are the most famous antioxidants of diet, out of which, Vitamin E is present in vegetable oils and found abundantly in wheat germ. Being lipid soluble, vitamin E can effectively prevent lipid peroxidation of plasma membrane. (22, 23)

Plants (fruits, vegetables, medicinal herbs) may contain a wide variety of free radical scavenging molecules such as phenolic compounds (Phenolic acids, flavonoids, quinons, coumarins, lignans, stilbenes, tannins etc.), nitrogen compounds (alkaloids, amines, betalains etc.), vitamins, terpenoids (including carotenoids) and some other endogenous metabolites which are rich in antioxidant activity. (24, 25, 26, 27)

Marine natural products have attracted the attention of biologists and chemists the world over for the last five decades. To date approximately 16,000 marine natural products have been isolated from marine organisms capable of synthesizing a variety of secondary metabolites with unusual structure and interesting biological activities. The chemistry and biological activities of these metabolites have been reviewed by many workers.(28) Several hypotheses have been put forward to explain the biogenesis of these metabolites and some experimental evidences have been procured for biogenetic theories. It is generally believed that the origin and mode of formation of secondary metabolites of marine organisms do not differ substantially from the well documented biosynthetic pathways of the secondary metabolites of terrestrial plants and animals. However,

biosynthetic experiments are yet to confirm this assumption. The marine environment provides different biosynthetic conditions to organisms living in it. (29)

The buffering action of sodium carbonate and bicarbonate maintains the pH of the sea water between 8.2 and 8.5. Sea water contains up to 40% salt and has an osmotic pressure of 15-20 atm. The cell structure, particularly the membrane composition of marine organisms, is expected to differ from their terrestrial counterparts. (28)

Besides, there are some striking differences between the metabolism of marine and terrestrial organisms. For example, halogens and isocyanide functions are frequently found in the metabolites of algae and sponges, whereas these are rarely observed in the metabolites of terrestrial plants and animals. Moreover, the absolute stereochemistry of the metabolites of marine organisms often differs from that of terrestrial organisms.

For these reasons marine secondary metabolites can constitute a novel and interesting source of medicines especially of antioxidants.

3. MARINE NATURAL PRODUCTS AS NOVEL DRUGS CANDIDATES

The term "natural product" is commonly reserved for those organic compounds of natural origin that are unique to one organism or common to a small number of closely related organisms and that are conferring a real evolutionary advantage.

Nature has continuously provided mankind with a broad and structurally diverse arsenal of pharmacologically active compounds (natural products) that continue to be utilized as highly effective drugs to combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature. (30)

Natural products chemistry is in danger being widespread belief that most species have already studied chemically and most of the interesting naturally occurring compounds have been characterized.

Traditionally, higher plants and terrestrial m.o. have proven to be the richest sources of natural drugs that are indispensable especially for the treatment of fatal diseases such as cancer. (30)

Deep water marine habitats constitute a relatively untapped resource for the discovery of drugs derived from natural products.

Marine organisms comprise over a half million species and inhabit very unusual places when compared with terrestrial organisms. Because of the immense biological diversity in the sea as a whole, it is increasingly recognized that a huge number of natural products and novel chemical entities exist in the ocean, with biological activities that may be useful in the quest for finding drugs with greater efficacy and specificity for the treatment of many human diseases.

From 1969-1999 approximately 300 patents on bioactive marine natural products were issued. From humble beginnings the number of compounds isolated from various marine organisms has virtually soared and now exceeds 10,000, with hundreds of new compounds still being discovered every year. (31)

An analysis of the phyletic distribution of these compounds shows that the majority (93 percent) are confined to four groups (macro-algae, coelenterates, echinoderms, and sponges), largely due to the abundance and ease of collection of these organisms.

Through the combined efforts of marine natural products chemists and pharmacologists a number of promising compounds have been identified that are either already at advanced stages clinical trials (most of them in the treatment of cancer) or have been selected as promising candidates for extend preclinical evaluation.(32)

Looking back over the past several decades, biomedical investigations of marine natural products have focused mainly on a few areas: CNS-membrane active toxins and ion channel effectors, anticancer and antiviral agents, and metabolites that control microfilament- mediated processes.

✓ *Marine natural products targeting ion channels*

The huge resource of marine natural products has played a key role in the explosive growth of biomedical science over the last two decades especially in the development and formulation of ion channel and tumor promotion models.

Perhaps the most important molecules (from cellular physiology and pharmacology standpoint) derived from marine sources to date are the very potent and specific sodium (Na+) channel blockers tetradotoxin and saxitoxin. These compounds act as Na+ occluders, with no effect on the gating mechanism of the blocked channels. They interact only on the

external surface of Na+ channels, interrupting passive inward flux of Na+ ions. (33) These toxins have proven extremely useful and popular chemical tools for neurophysiology and neuropharmacology studies. The toxins have also proven applicable in monoclonal antibody studies of lymphocyte activation. (34)

The venoms of the predatory cone snails (*Connus* spp), contain pharmacologically active peptides, which are target to various ion channels and receptors.(34)

Ω-conotoxins are calcium channels blockers, K-conotoxins are potassium –channel blockers and μ-, as well as μ o-conotoxins , are sodium channel blockers , and all this form conotoxin families that are potentially lethal to humans, but may , in the future be turned to our advantage by pharmacologists who have been investigating their potential uses as adjuncts in anaesthesia, analgesics or as drugs for the treatment of conditions such as epilepsy, cardiovascular disease and psychiatric disorders.

Ziconotide, the synthetic form of ω-conotoxin MVIIA, a neuron-specific N-type calcium channel blockers, was developed as an intrathecal treatment for chronic pain. Unlike morphine it does not induce the development of tolerance, constipation or respiratory suppression. It occurs along with other peptides in the venom of the predatory marine mollusks *Conus magus*. (35)

Maitotoxin, produced by the marine dinoflagellate *Gambierdiscustoxicus* increased cellular calcium uptake, neurotransmitters/ hormone release,

phosphoinositide breackdown, contraction of smooth and skeletal muscle and stimulation effects on the heart.(36) Most effects of maitotoxin appear to be due to either its interaction with extra- cellular calcium or the enhanced influx of calcium. The potential of maitotoxin as a unique pharmacological tool for studying calcium transport is yet to be fully realized.

GTS21, 3- (2,4- dimethoxybenzylidene) - anabaseine, isolated from the worm *Amphiporuslactifloreus*is a selective α7 nAChR (the nicotinic/ acetylcholine receptor) partial agonist in clinical development to treat Alzheimer disease and schizophrenia.

✓ *Antitumor compounds*

Of the marine natural products that are currently under clinical investigation as potential new anti- cancer drugs the marine alkaloid ecteinascidin 743(ET-743) isolated from the tunicate *Ecteinascidia turbinate*, is by far the most advanced compound. The drug has a broad-spectrum anti-tumor activity and is especially effective against solid tumors such as sarcomas and breast cancer (37). The alkaloid was shown to be a minor-groove alkylator of DNA (Minuzzo et al. 2000) and to cause inhibition of MDR1 gene transcription (Jin et al.2000), the latter being responsible for the well- known phenomenon of multi-drug resistance, which causes tumors to became insensitive to anti- cancer drugs and is a severe obstacle for chemotherapy.

In the series of peptides found in marine organisms, the most interesting and important two substances are the potent antitumor agents didemin B, cyclic depsipeptide isolated from a Caribbean tunicate, an dolastatin 10, a linear peptide obtained from the Indean Ocean sea hare *Dolabellaauricularia*.(d)

Didemnin B, provoked interest back in the 1980s due to its pronounced anti-tumor activity which can be traced back to its interference with protein synthesis (Ahuja et al 2000)(a)

Dehydrodidemnin B (also called aplidine) isolated from the Mediterranean tunicate *Aplidiumalbicans* appears to be more effective than didemninB (five to six times more active)(Geldof et al.1999)(a)with a broad spectrum activity both *in vitro* and *in vivo* against various types of cancer diseases such as colorectal, lymphoma, tyroid and renal cancer.(Rinehart 2000)

KahalalideFis a marine natural product anticancer drug candidate, a cyclic depsipeptide isolated from the sea slug *Elysiarufescens* but most probably derived from *Bryopsis* sp., its green algae diet. The compound has shown antitumor activity, and it is currently in phase I trials for prostate cancer and other solid tumors.(1)

An increasing number of polyethers and macrolides, that exhibit potent cytotoxic and antitumor activities, have been found in various marine organisms. Some of them seem quite promising and may be further investigated as developing anticancer agents.

✓ *Anti- inflammatory/ Analgesic compounds*

Several marine natural products with anti-inflammatory and analgesic properties have entered in clinical trials and holds considerable economic potential.

Extracts of *P.elisabethae* show anti- inflammatory activity and are nowadays used as an ingredient for cosmetic skin care products.

Manoalide, a non- steroidal sesquiterpene isolated from sponge *Luffariellavariabilis* (Silva and Scheur, 1980) emerged as a potent tool for studying inflammation. The most important factor contributing to the anti-inflammatory activity of manolide was attributed to its inhibitory effect on calcium channels (Wheeler et al.1988)(c)

Hymenialdisine is a sponge alkaloid named after *Hymeniacidonaldis*, indicating anti-inflamatory activity. (c)

Pseudopterosins A and E, members of a family of diterpeneribosides isolated from gorgonians *Pseudopterogorgiabipinata* and *P.elizabethae*, exhibited potent anti-inflammatory and analgesic activities and act as reversible inhibitors of lipoxygenase and PLA_2. (Luedke 1900)(c)

✓ *Antiviral agents*

The didemins, depsipeptides isolated from Carribean ascidian *Tridemnumsolidum*, exhibited *in vitro* and *in vivo* antiviral properties. (c)

PatellazoleB, isolated from the ascidian *Lissoclinum patella* proved to have very potent antiviral activity against *Herpes simplex* viruses. (Ireland et al.1990)

The eudistomins, a class of β- carbolines isolated from the Caribbean ascidian *Eudistomaolivacea*, were active in shipboard antiviral assays. (Rinehart et al.1981)(c)

4. Algal natural products and their bioactive potential

Marine microorganisms are perhaps the last barely tapped source of novel bioactive compounds, including antitumor, antibacterial, antiviral, antifungal, anti-inflammatory, and other compounds. The structural novelty of these compounds is potentially infinite, but a significant number of known compounds are frequently isolated from marine algae.(J.Np 2001,64.)

Algae secondary products have attracted investigation, and information about their structure, biosynthetic origin and pharmaceutical and cosmetic use has accumulated steadily over this period.(Faulkner 2002)PP52

The panel of natural products produced by marine Chlorophyta (green –algae), Phaeophyta (brown algae) and Rhodophyta (red algae) includes: alkaloids (aplysinopsins, bromopyrroles, bromotyrosines, brominated indole alkaloids, makaluvamines), lipopeptides (cyclicand linear, malyngamides), furanocumarins, oxylipins (eicosanoids, prostanoids, carbocyclic and heterocyclic), peptides (simple and cyclic depsipeptides),

phenolics (simple, polyphenolics, polyhaloganated, quinines) and terpenoids(oxygenated C10- C30, polyhalogenated C_{10}- C_{20}, cyclic, linear and terpene quinines).(3)

Compounds with antiviral, antihelmitic, antifungal, anticoagulant, anti-inflammatory and antibacterial activities have been detected in green, brown and red algae.(PP53)

Brown macrophytic algae, notably those from temperate regions, are rich in phenolic metabolites while tropical species contain characteristic non polar metabolites, notably diterpenes.(f). Brown algae of the family *Dictyotaceae* are known to produce diverse diterpenoid secondary metabolites with a wide variety of carbon frameworks, e.g.selinane, cubebane, bourbonane, and perhydroazulene skeletons, with an additional prenyl unit on the side chain. Some of these diterpenes function as chemical deterrents against marine herbivores.(J.Np65)

Monoterpenes in the form of loliolides, which are carotenoid metabolites, have been isolated from land plants and marine algae and are well known to have immunosuppressive, germination inhibitory and antirepellent activities among others. From a component analysis of the brown alga Undaria which is very popular in the japonese diet, there were isolated three new loliolidederivates as well as four known carotenoid metabolites, loliolide, isololiolide, β-ionone, and fucoxanthinone.(J.Np, 65)

The brown alga Carpophyllumangustifolium is very reach in phloroglucinol derivatives, substances which have antibiotic, antifungal, antialgal and toxic activity.(J.Np.62)

Algal-sulfated polysaccharides, including the Laminariaspkelp(brown algae) metabolite fucoidinwere shown to inhibit a variety of DNA- and RNA-enveloped viruses, including Herpes simplex virus and HIV(Baba et al,1988). In the same time fucoidin exhibited antithrombin, anticoagulant, fibrinolytic, onco- inhibitory and antitumor activities, and was used as a probe of lymphocyte membranes.(c)

Brown algae have been also the source of several proteintyrosinekinaseinhibitors.The brown alga Stypopodiumzonale has yielded stypoquininic acid which is a novel inhibitor of the Src-family protein tyrosine kinaseLcK (23,241).

Brown algae appear to detoxify inorganic arsenic by converting it to methylated arsenates and arsenolipids.The arsenic content of edible brown seaweeds is of concern.(f)

The members of the family Sargassaceae are inedible due to the inherently great concentration of polyphenolic substances(J Np 62) and to certain biological activities attributed, including antitumor, antibacterial, antifungal, antiviral, insecticidal and insect- antifeedant activities .(PP55)

The natural products chemistry of red algae (Rhodophyta) has been studied extensively over the past two decades. Many species of red algae have the ability to synthesize organic halogen-containing compounds that incorporate bromine, chlorine and occasionally iodine from seawater. Many novel halogenated terpenes are known from red algae, including sp. of genera Laurencia, Plocamium, Portieria and Ochtodes. (J. Np2000)

Algae of the genus Laurencia are known to be prolific sources of different terpenoid and nonterpenoid secondary metabolites including a C_{15} family of halogenated cyclic ether enynes and related

allenes.(PP44).Eleven sesquiterpenes have been isolated from the CH_2Cl_2 extract of red algae Laurencia scoparia that showed anthelmintic activity against the parasitant stage of Nippostrongylusbrasiliensis.(J.Np2001, 64))

Among the red seaweeds, the genera Portieria and Ochtodes are known to contain large amounts of both acyclic and cyclic polyhalogenated monoterpenes. Continued studies of the natural products chemistry of these seaweeds have recently been stimulated by the discovery of the acyclic monoterpene, halomon [6(R)-bromo-3(S) – (bromomethyl)-7-methyl -2,3,7-trichloro-1-octenel] which exhibits selective antitumor activity in the National Cancer Institute`s human tumor and disease oriented in vitro screen.(J.Np1999)

Pocaniumcartilagineum is a red alga that is found throughout the warld and it is a ricc source of diverse polyhalogenated monoterpenes.(1,2 PP45)

The red alga Asparagopsisarmata and the tetrasporophyte phase Falkenbergiarufolanosa, are rich sources of polyhalogenatedcompounds,(1,5PP47). The pungent aroma of these algae is due to their halogenated volatile constituents, mainly brominated iodinated hydrocarbons. A. armata and F. rufolanosa have been reported in the past to possess antibacterial activity and antifungal activity on some dermatophyte strains and pathogenic yeasts.(PP47)

The Indonesian red alga was identified as a candidate for fraction because its crude lipid extract showed activities in a mechanism- based anticancer assays.(J. Np 2002.65)

Thirty years of research into natural products from marine algae have led to the conclusion that, while red and brown algae possess physiological and biochemical mechanisms with the ability to produce a great variety of secondary metabolites with vary different skeleton types and functionalities, in green algae these mechanisms do not seems to be so widely diversified. The genera Caulerpa, Halimeda and Bryopsis from tropical and subtropical habitats have been more thoroughly investigated because of the abundant metabolites they contain, which may point to the need for chemical defence against intense feeding pressure by herbivores in the aforementioned habitats.

In 1976, the "cymopoles" were described as the first halogenated natural products isolated from a green alga, isolation and characterization of new secondary metabolites from this same genus, despite evidence that it is a rich source of pharmacologically active compound exhibiting antifungal, antitumor, antimicrobial and antimutagenic properties, in addition to other biological properties of a defensive and antifeedant nature.(J.Np2002, 65)

The isolation of 2(R)-hydroxyhexadecanoic acid and 2-oxohexadecanoic acid from green alga *Ulva perfusa* is of interest as long-chain aldehydes exemplified by (8Z.11Z.14Z) – heptatrienal has been implicated as contributors to the flavor of this alga which is consumed commercially in Japan. (f)

Halogenated sesquiterpenes have been isolated from the calcareous green alga Neomerisannulata, and are of biosynthetic interest since 1(R) – bromo-ent-maaliol has been proposed as a biosynthetic precursor of heterocladol and oppositol. (f)

It is important to note that the number of compounds reported annually in increasing steadily, indicating that marine organisms will continue to be significant sources of natural products receiving the greatest attention from the perspective of drug development. (c)

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CHAPTER 4

MATERIALS AND METHODS followed to complete the procedure for the antioxidant evaluation of the Mediterranean algae and the identification of the metabolites responsible for this character.

- ✓ **Collection of algae material.**

Field work was performed at several coastal sites around Grrece, that are specifically showed in the following table, by free diving in rocky midlittoral and upper sublittoral levels (0–3 m depth) and by scuba diving down to 42 m depth.

All specimens were collected by destructive sampling (picking or scraping the macroalgae off the substratum). A part of algal material collected was preserved in 4% buffered formalin/seawater and/or mounted on Bristol paper, pressed, air dried, and prepared as herbarium specimens, also used for a further taxonomic identification by Professor V. Roussis from Department of Pharmacy, University of Athens, Greece, specimens being observed under dissecting or compound microscopes. When necessary, they were sectioned manually with a razor blade.

Herbarium specimens have been deposited in the East Mediterranean Seaweed Herbarium at the Department of Botany, Faculty of Biology, University of Athens (Greece).

For the antioxidant evaluation the different seaweeds specimens collected, were transported to the Laboratory of Analytical and Organic Chemistry of Maich (Mediterranean Agronomic Institute of Chania), in net nylon bags. Within the laboratory the algae were rinsed carefully in fresh water and then dried at room temperature in a shady place, used further for different chemical and analytical investigations.

Table (3) **The identification, the distribution and the date of collection of the harvested seaweeds:**

Nr.	**Organism**	***Type***	**Collection site**	**Date of collection**
1	Ulva rigida	Green	Salamis island (Kanakia)	7/03/2003
2	***Halimeda tuna***	Green	Salamis island (Kanakia)	8/02/2003
3	***Udotea petiolata***	Green	(Agios Cosmas) Saronicos gulf	8/02/2003
4	***Codium vermilara***	Green	Salamis island (Kaki vigla)	8/02/2003
5	***Dassycladus clavaeformis***	Green	Lemnos island	20/07/2003
6	***Caulerpa prolifera***	Green	Salamis island (Kanakia)	5/07/2003
7	***Enteromorpha rinza***	Green	(Ag. Marina) Saronicos gulf	13/06/2003
8	***Coralina mediterranea***	Red	Salamis island (Kaki vigla)	2/08/2003
9	***Gelidium sp***	Red	(Legrena) Saronicos gulf	7/05/2003

10	***Laurencia obtusa***	Red	Patmos island	28/07/2003
11	***Sargasum vulgare***	Brown	Salamis island (Kanakia)	15/08/2003
12	***Dictyopteris membranaceae***	Brown	Salamis island (Kanakia)	8/02/2003
13	***Taonia atomaria***	Brown	Patmos island	17/06/2003
14	***Posidonia oceanica***	seaweed	(Agios Cosmas) Saronicos gulf	28/10/2003
15	***Halopteris (filicina)***	Brown	Lemnos island	7/02/2003
16	***Cladostephus spongiosus***	Brown	Lemnos island	7/02/2003
17	***Laurencia papillosa***	Red	Paros island	8/02/2003

✓ Chemicals and standards used in the experiment

- Ascorbic acid, Sigma, Chemical Co. (Germany)
- Boric acid, Sigma, Chemical Co. (Germany)
- Cobalt (II) [$CoCl_2 \cdot 6H_2O$], E. Merck (Germany)
- Dichloromethane, Chemical Co. (Germany)
- DPPH (2,2-diphenyl-1-picryhydrazyl radical), Sigma Chemical Co. (Germany)
- EDTA (Ethylene diamine tetra acetic acid), Sigma Chemical Co. (Germany)
- Ethyl acetate, pa, lab-Scan

- Ethyl ether, pa, lab-Scan
- N-Hexane, pa, lab-Scan
- Cellulose plates, Merck (Germany)
- Hydrochloric acid, minimum 37%, Merk (Germany)
- Luminol (3-aminophtahydrazyne), Sigma, Chemical Co. (Germany)
- Methanol, pa, Readel-de Haen (Germany)
- Quercetin, Sigma, Chemical Co.
- Silica gel powder, Merck (Germany)
- TLC paper (SiO_2), Aluminium sheets 20 x 25 cm, Merk (Germany)
- Trolox, Sigma, Chemical Co. (Germany)

Distilled water was used throughout this entire study.

✓ **Equipments used in the experiment**

- Fluorimeter, 6200, Jenway (UK)
- Balance, AT 261 Deltarange, Mettler
- Balance, PM 2000, Mettler
- Centrifuge, T 23 D, M1W
- Hot plate with magnetic steering, Heidolph, MR 3001
- ^{1}H-NMR, Bruker DRX (Department of Pharmacy, Division of Pharmacognosy, University of Athens, Greece)
- Mass Spectra obtained from a Hewlett Packard 5973-6890 GS-MS system
- pH meter, Universal Pocket Meter Multiline Pu SET 3, Germany
- Rotatory evaporator, Buchi R
- Rotatory evaporator, WB 2001, Heidolph

✓ **Preparation of algal extracts**

For each sample, on this experimental step three solvents of different polarities (CH_3OH 40%, CH_2Cl_2, MeOH) were used for the exhaustive extraction of compounds from the solid algal tissue into the solution.

Three times 1 g of dried material from each sample were weighed on the precision balance and each gram placed in three different glass tubes.

5 ml of each solvent were added to the algae tissue that previously was grinded to a dry powder within the tubes. Homogenization was accomplished using an orbital shaker at 24º for about 1 hour.

To minimize the loss of the algal compounds the extraction was repeated three times. The obtained homogenate was clarified by centrifugation at 3.400 rpm for 10 min.

The solvent in the supernatant was then evaporated under vacuum at 30º C. The volume of remaining algal residues was weighted, in order to calculate the yield and finally re – dissolved in 5 ml of the initial solvent. The algal extract of each species was subject to further bioassays for AOA (antioxidant activity).

All chemicals were of analytical grade.

✓ **AOA evaluation of polar extracts using luminol chemiluminescence**

Co (II) / EDTA / induced luminol chemiluminescence assay was used for the polar extracts that were prepared using as solvent MeOH and aqueous solution of MeOH. The solutions for the preliminary evaluation of

algal antioxidant activity were prepared based on the method that has been described previously by Parejo at al. 2000.

The procedure of the method consist on the preparation of 3 dilutions for each sample using the same solvent of the extract, the concentrations of dilutions depending on the measured activity of the initial extract alone.

In a test tube reaction mixture containing 2 ml of buffer solution (boric acid 0.05 M, pH of 9, Co (II), 0.2 mg/ml, EDTA 1 mg/ml and 200 µl of the luminol solution $5.6x10^{-4}$ M luminol dissolved in borate buffer), were first mixed well (vortex during 15 sec.)

Then 50 µl of H_2O_2 aqueous solution ($5.4x10^{-3}$ M) were added into the previous mixture and vortexed for another 15 sec.

On the top of the chemical cocktail was quickly added 50µl of the sample and vortexed for 30 seconds in order to initiate the chemiluminescence reaction. Immediately afterward the mixture was loaded in a 10x10 quartz cuvette and the instantaneous decrease in luminol intensity was recorded.

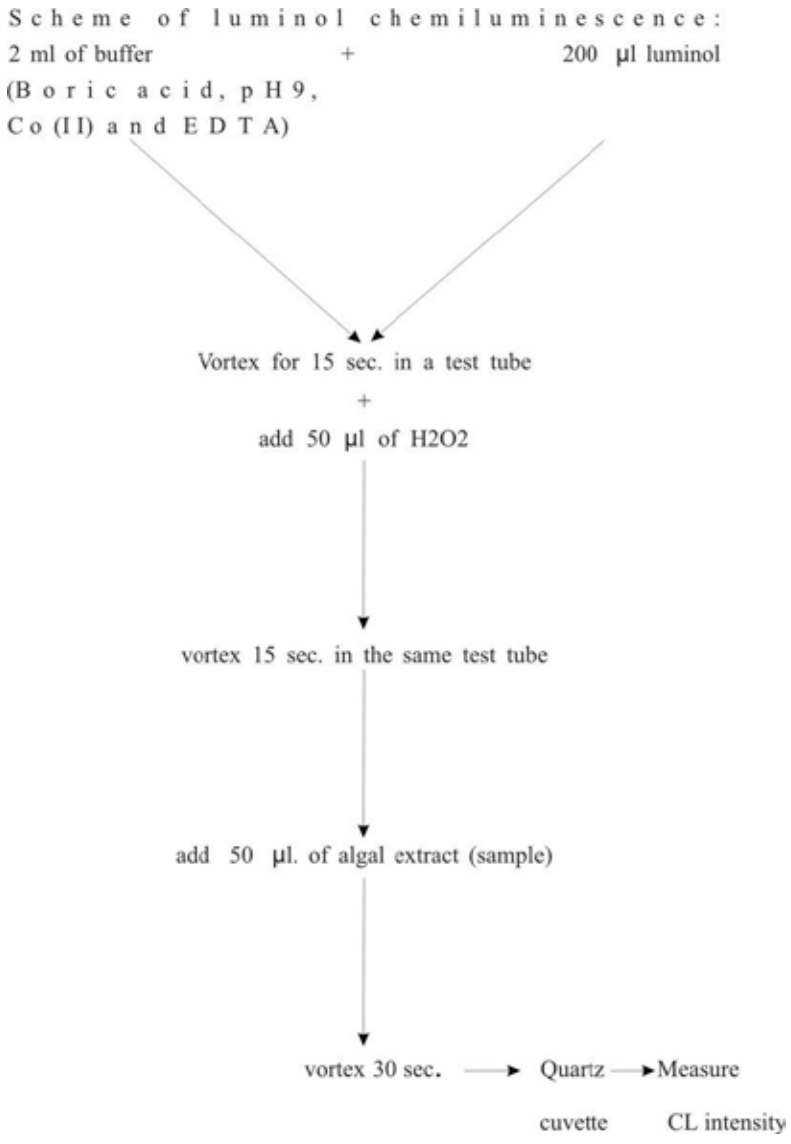
Chemiluminescence measurements were carried out on a Model 6211 Fluorimeter, Jenway (Jenway Gransmore Green Felsted Dunnow Essex CM6 31B) keeping the lamp off and using only the photomultiplier of the apparatus.

A fluorimeter calibration was preceded prior to chemiluminescence measurements using the procedure described above but in the absence of algal sample.

All solution measurements were carried out using precision pipettes.

"I" represent the symbol for instantaneous reduction in luminol intensity elicited by the addition of the sample meanwhile "$\mathbf{I_0}$" was considered the light intensity in the absence of any sample.

The amount of sample needed to decrease the initial chemiluminescence intensity by 50% (EC_{50}) was calculated by plotting the ratio I_0/I for each sample vs. the concentration (mg/ml) of alga extract sample, work elaborated using the equation of linear regression (y = bx + a) of the computer program Excel 2000.



✓ POCL method for the evaluation of nonpolar algal extract

Peroxyoxalate Chemiluminescence Assay has been suggested to evaluate antioxidant activity of the nonpolar extracts (using EtOAc as solvent) containing the algal metabolite.

Solution used for POCL assay:

- EtOAc saturated with H_2O

EtOAc and deionised water were shaken in a separator funnel and left overnight to separate. The upper layer was collected and used for all dilutions and dissolution.

- EtOAc saturated solution : acetonitrile – 9:1
- Fluorophore solution ($5x10^{-4}$ M)

5 mg of 9, 10 – dimethylanthracene were dissolved by ultrasonication in 50 ml of solution 2.

Imidazole solution (4.5×10^{-3}M)

15 mg Imidazol were dissolved by ultrasonication in 50 ml of EtOAc saturated solution.

- Oxalate solution (TCPO) (4.5×10^{-4}M)

10 mg of oxalic acid bis (2,4,6 – triclorophenyl) ester were dissolved by ultrasonication in 50 ml of EtOAc saturated solution.

All chemicals used for POCL assay were of analytical grade and were used without further purification.

Chemiluminescent measurements were carried out on a Jenway 6200 Fluorimeter.

Scheme of POCL procedure for evaluation of AOA:

1800 µl of Fluorophore solution in the 5 x 10^{-4} M concentration range, were mixed well with 200 µl Imidazol solution (4.5 x 10^{-3}M) in a glass vial. Then 25µ H_2O_2 (2.25 x 10^{-3}M) were added on the top of the previous mixture and vortexed for 15 sec. 200µl of TCPO solution (4.5 x 10^{-4}M) were added drop wise to 50 µl of sample containing the antioxidant compounds that were loaded in a 1 cm path length cuvette.

The previous solution was immediately added on the top and thoroughly mixed using a Pasteur pipette.

The resulting emission spectrum was recorded on JenWAY™ 6200 fluorimeter in chemiluminescence mode (with excitation source off).

The ratio I_0/I was calculated, were Io symbolized the light intensity elicited in the absence of sample and plotted vs the concentration of antioxidant for defining the antioxidant parameter EC_{50}.

Calibration of the apparatus was completed prior to POCL measurements on the same way described above but in the absence of the sample and using a different quantity of H_2O_2 (50µl).

Solutions were of the highest purity available.

- ✓ **Estimation of the antioxidant activity using DPPH method for polar algal extracts**

Procedure:

a) Preparation of the DPPH working solution.

1 DPPH mother solution containing 20 mg DPPH in 50 ml MeOH. was prepared. From this solution was obtained the DPPH working solution of 5 x 10^{-5}M concentration. 6 different concentration of the DPPH were used for the determination of the standard curve.

All spectrophotometrical data were acquired using an UV – Visible diode array spectrophotometer 8452, Hewlett – Packard in a 10 mm quartz cuvette.

b) DPPH measurements

After preliminary experiments that were carried out in order to determine the time necessary to reach the steady state in absorbance an aliquot of methanol (25µl) solution containing different concentrations of samples (algal extract) was added to 975 µl DPPH (5 x 10^{-5}M in methanol).

The decrease in absorbance was determined at 515 nm after 90 min when the reaction reached a plateau.

Methanol was used to zero the spectophotometer.

The exact initial DPPH concentration in reaction medium (CDPPH) was calculated from the calibration curve with the equation:

$$Abs515nm = -0.0065 \times 29.3112(CDPPH) \quad (3)$$

as determined by linear regression (r = 0.9999)

It was calculated the percentage of DPPH remaining at the steady state, for each antioxidant concentration tested, following the equation:

$$\% \text{ DPPH rem} = [\text{ DPPH} \cdot]_T / [\text{ DPPH} \cdot]_{T=0}$$

were „T "symbolizes the time necessary to reach the plateau. The values obtained were plotted vs mg algal extract / µl DPPH· leading to the amount of antioxidant necessary to decrease the initial DPPH· concentration by 50% (EC_{50}).

✓ **Evaluation of AOA of nonpolar compounds from algal extract using hydrogen donating method with EtOAc.**

From the mother solution (20 mg DPPH dissolved in 50 ml EtOAc) were taken 6 ml and mixed with100 ml EtOAc for the preparation of working DPPH solution.

For calibration curve were used 5 additional concentrations of DPPH solution.

All spectrophotometric data were acquired using a UV-spectrum and disposable cuvettes for visible absorbance measurements. EtOAc was used to zero the spectrophotometer. The same procedure was followed for the polar compounds.

✓ **Second extraction of algae number 5, *Posidonia oceanica***

Due to the high AOA showed during all the previous bioassays, a second large scale extraction was applied to the remaining *Posidonia oceanica* alga material.

For this extraction were measured 38 g of *Posidonia oceanica* dry material and putted in a flask were methanol solvent was added on the top. The extraction procedure was achieved 3 times, each time the duration

being 6 hours. The methanol extracts were evaporated to dryness under vacuum and the residuum weighted in order to calculate the yield.

After the quantitative measurement the drug extract was collected in a glass tube and kept in refrigerator for further analyses.

✓ **Preliminary TLC of the extract**

TLC method was applied for estimation of the extract content before column chromatography.

Few mg of *P.oceanica* extract were redissolved in a small quantity of methanol and spotted on TLC plates (using silica gel and aluminum support).

Were used 2 eluents of increasing polarities: hexane, CH_2Cl_2, EtOAc, MeOH.

For this procedure was used developing solution 5% phosphomolybdic acid in ethanol (95%), followed by heating at 80^0 C for 5 minutes.

✓ **Separation of *Posidonia oceanica* extracts using flash chromatography.**

In this preparative chromatography the stationary phase was represented by small particles (0.063 to 0.2 nm) of silica (SiO_2).

15 cm from the cylindrical volume of the glass column with 40 mm diameter were filled with silica gel particles washed earlier with 80 ml solution CH_2Cl_2: MeOH (3:1).

1.5 g of the extract and 3 g of silica gel were dissolved in MeOH and evaporated under vacuum. The powder obtained was added to the glass column on the top of the wet silica. Sand was poured for protection on the top of the extract powder. Different proportion between CH_2Cl_2: MeOH (Showed in the table nb.) were used as gradient eluents and carefully poured into the column.

The elution was carried out under pressure regulated by a nanometer in order to obtain a flow rate of about one drop /second.

The fractions containing algal compounds of different polarities were collected in glass tubes (100 ml / tube).

Tabel (4):**The proportion between the solvents used for flash chromatography.**

Eluent order	*Eluent proportion*
1	60ml Ch_2Cl_2 : 20ml MeOH
2	40ml Ch_2Cl_2 : 40ml MeOH
3	20ml Ch_2Cl_2 : 60ml MeOH
4	80ml MeOH

✓ **TLC, to monitor the eluted compounds from column chromatography**

The presence of the algal compounds in the fractions obtained from column chromatography was monitored using TLC method with the eluent system CH_2Cl_2: MeOH (3:2).

TLC was carried out using:

1. silica gel on aluminum support,
2. developing solution 5% phosphomolybdic acid in ethanol 95%,

It was followed the same procedure as the previous one described.

The fractions that indicated the presence of algal constituents were concentrated in 3 volumetric flasks according to their polarity. The eluent from each volumetric flask was evaporated in a rotator evaporator under vacuum, measured the yield of the dry extract and redissolved in:

- 5 ml of MeOH, for the fractions with predominant contain of polar compounds: fractions F2 and F3
- 5 ml of EtOAc, for fraction containing nonpolar compounds: fraction F1

✓ **Second separation of nonpolar fraction F1 by Flash Chromatography**

A further separation of the nonpolar fraction F1 was performed for separating the chlorophylls content of this fraction, using flash chromatography method.

The same column used for the previous flash chromatography was washed in this case with the eluent system Hexane: Acetone using the proportion 9 : 1.

Tabel (5): **The composition of the eluents used in fractionation**

Eluent order	Eluent composition
1	100 ml of n-hexane
2	90 ml of n- hexane: 10 ml of acetone
3	80 ml of n- hexane: 20 ml of acetone
4	70 ml of n- hexane: 30 ml of acetone
5	60 ml of n- hexane: 40 ml of acetone
6	50 ml of n- hexane: 50 ml of acetone

Was followed the same procedure as for the previous flash chromatography fractionation.

The eluents were collected in glass tubes (100 ml/tube) and TLC of the obtained fractions monitored the elution.

The fractions that indicated the presence of algal constituents were concentrated in two flasks, one containing F1 (polar part) and the other containing F2 (non polar part, that supposed to be the chlorophylls), and after were evaporated the solvents and measured the yields.

✓ **Antioxidant activity tested for the isolated groups**

The constituted 5 fractions (F1, polar and non polar parts, F2, F3) were submitted to luminol and POCL chemiluminescence assays in order to detect the group of compounds indicating the highest antioxidant activity.

Was applied the same experimental protocol described previously for POCL and luminol chemiluminescence.

✓ **Structural profile of fractions using H-NMR.**

The spectra of fraction number 1 (polar part), 2, 3 were recorded on a Bruker DRX 400 MHz in the Department of Pharmacy, University of Athens, Greece.

✓ **Separation of *Posidonia oceanica* fraction 2 using vacuum chromatography.**

Fraction F2 of *Posidonia oceanica* was further purified by applying the vacuum chromatography method. For this was used a büchner funnel that was dry – packed with silica gel under vacuum in order to achieve the maximum packing density.

The vacuum was released and low polarity solvent represented by CH_2Cl_2 was poured onto the surface of the adsorbent and the vacuum was reapplied. The column was sucked dry and let it ready for loading.

The Fraction F2 dissolved in MeOH was applied directly on the top of silica gel in the büchner funnel and was drawn gently into the packing by applying the vacuum.

The column was eluted with solvent mixtures, starting with solvent of low polarity (CH_2Cl_2) and gradually increasing the polarity, as showed in the table nb., pulling the column dry between each fraction collected.

Tabel (6): ***The composition of the eluents used for vacuum chromatography***

Eluent order	*Eluent composition*
1	100ml CH_2Cl_2
2	90mlCH2Cl2 : 10mlMeOH
3	80mlCH2Cl2 : 20mlMeOH
4	70mlCH2Cl2 : 30mlMeOH
5	60mlCH2Cl2 : 40mlMeOH
6	50mlCH2Cl2 : 50mlMeOH
7	40mlCH2Cl2 : 60mlMeOH
8	30mlCH2Cl2 : 70mlMeOH
9	20mlCH2Cl2 : 80mlMeOH
10	10mlCH2Cl2 : 90mlMeOH
11	100mlMeOH

The fractions obtained after the vacuum chromatography were collected into büchner vessels and recollected each one in different flask for a further evaporation of eluent solvent in rotovapor and measuring of the yields.

✓ **TLC, to monitor the eluted compounds from vacuum chromatography.**

The adsorbent silicagel was the component of TLC plates used for this method.

The fractions obtained by vacuum chromatography were spotted using Pasteur pipettes, on TLC plates, that after were dried and eluted with solvent mixtures of different proportion as follows:

CH_2Cl_2 : MeOH (3:1)

CH_2Cl_2 : MeOH (1:1)

CH_2Cl_2 : MeOH (1:3)

✓ **Structural profile of the fraction number 6 using ^{1}H- NMR.**

^{1}H NMR spectra were recorded using Bruker AC 200MHz and DRX 400MHz spectrometers. Chemical shifts are given in *ä* (ppm) scale using TMS as internal standard.

✓ **Identification of major components from F6 on the basis of ^{1}H- NMR, and MS spectra.**

Low-resolution EI mass spectral data were recorded on a HP 5890 mass selective detector.

The GC analyses were performed on a Varian 3300 Gas Chromatograph equipped with an on column injector (200^{o}C) and a flame

ionization detector (250°C). As carrier gas was used He (2mL/min) and the capillary columns used were a DB-5 (30m x 0.32mm) and a HP-Innowax (30m x 0.25mm). The initial temperature of the columns was 60°C and then was heated to 280°C with a 3°C/min rate. Mass spectra were obtained from a Hewlett Packard 5973-6890 GC-MS system operating on EI mode (equipped with a HP 5MS 30m x 0.25mm; 0.25ìm film thickness capillary column, same temperature program). The identification of the chemical constituents was based on comparison of the R_t values and mass spectra with those obtained from authentic samples and/or the NIST/NBS and Wiley library spectra. The quantification of the components was performed on the basis of their GC peak areas, without corrections for response factors.

Column chromatography was performed with Kieselgel 60 and 60H (Merck). TLCs were performed with Kieselgel 60 F_{254} aluminum support plates (Merck).

✓ **Purification of acetylated fraction F6 by column chromatography.**

The TLC system was used to guide the further column chromatography separation for the acetylated metabolites of fraction F6.

The column chromatography was performed under reduced pressure. The particle size of the stationary phase (SiO_2) in this case is 0.040 to 0.063nm. The procedure followed was the same with the previous described for flash chromatography.

The TLC system used for purification:

ELUENT COMPOSITION	**PROPORTION**
Isopropanol: acetic acid (15%)	7.5 : 1.5
Isopropanol:Acetic acid (15%)	9.5 : 0.5
Isopropanol:Hexane: Acetic acid (15%)	8.5 : 1: 0.5
Isopropanol:Hexane: Acetic acid (15%)	7.5 : 2: 0.5

- ✓ **Final bioassay on the fraction F6 containing the pure metabolites**.

The separated acetylated metabolites of fraction F6 were submitted to the chemiluminescence bioassay to determine their antioxidant capacity.

- ✓ **Check of silica influence on the antioxidant activity of *Posidonia oceanica* extract.**

365.4 mg of *Posidonia* dried extract were solved in 15 ml of MeOH, and the extract was subjected to chemiluminescence bioassay in order to screen the antioxidant activity properties of it.

After the antioxidant activity of the extract was checked, 4 mg of silica gel were added into the extract. For 10 minutes the extract remains in direct contact with the silica. The procedure was followed by a separation of the extract from the silica in a Pasteur pipette containing cotton on the bottom. The extract separated by the silica was evaporated, measured the yield and resolved in 5 ml of MeOH. This extract was submitted to chemiluminescence assay in order to detect the stability of metabolites in the presence of silica compared to the initial extract.

REFERENCES:

3. Harborne, J.B.; Williams, C. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504.

CHAPTER 5

RESULTS AND DISCUSSION

1. Algal extract yields

The yields of the algal extracts obtained during the preliminary extractions using methanol, dichloromethane and aqueous methanol (40%) as solvents, are shown in the following table:

Table 7. Yields of preliminary extractions

Name of species	Dry algae weight (g)	Methanol extraction yield (mg)	Methanol 40% extraction yield (mg)	Dichloromethane extraction yield (mg)
Taonia atomaria	1	220	370	50
Laurencia obtusa	1	100	320	30
Enteromorpha linza	1	80	80	20
Halimeda tuna	1	130	140	10
Posidonia oceanica	1	80	200	30
Udotea petiolata	1	190	250	30
Dasycladus clavaeformis	1	70	160	30

Corallina mediteraneea	1	50	100	10
Caulerpa prolifera	1	250	210	60
Ulva rigida	1	60	180	10
Halopteris filicina	1	40	70	20
Gelidium sp.	1	440	870	30
Sargassum vulgare	1	110	250	10
Dictyopteris membranacee a	1	130	320	40
Cladostephu s spongiosus	1	40	100	20
Codium vermilara	1	160	400	30
Laurencia papillosa	1	60	380	10

2. AOA evaluation of polar extracts using luminol chemiluminescence

Co(II)/ EDTA luminol- enhanced chemiluminescence was used for this study as a sensitive assay for monitoring free radicals, and in particular to determine the ability of algal extracts to inhibit the presence of free radicals.

The addition of free radical scavengers to this chemiluminescent solution led to a decrease in the luminol intensity, allowing evaluation of the antioxidant activities of the extracts.

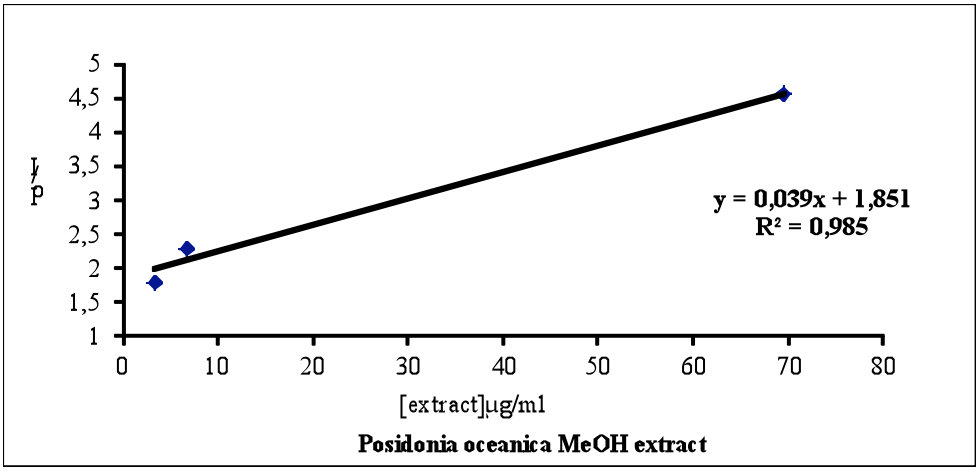
The light intensity in the absence of algal sample (I_0) was decreased by the addition of the sample antioxidant concentration to a value I.

Table 8. Antioxidant activity of the polar extracts, using luminol chemiluminescence (EC_{50} expressed in mg extract / ml)

Name of specie	*EC_{50} of methanol extract*	*EC_{50} of methanol 40% extract*
Ulva rigida	**185.6**	**503.5**
Enteromorpha linza	**107.9**	**461.5**
Halimeda tuna	**40.6**	**895**
Caulerpa prolifera	**15.0**	**1047.6**
Codium vermilara	**161.4**	**5094**
Corallina mediteraneea	**53.8**	**173.9**
Laurencia obtusa	**195**	**248.1**
Taonia atomaria	**2.3**	**27.7**
Dictyopteris membranacea	**154.3**	**266.4**
Sargassum vulgare	**89**	**318.2**
Cladostephus spongiosus	**34.4**	**73.7**
Halopteris filicina	**103.7**	**137.6**
Laurencia papillosa	**26.2**	**525**
Gelidium sp.	**202.7**	**754**
Posidonia oceanica	**3.8**	**13.8**
Dasycladus clavaeformis	**37.6**	**22.2**
Udotea petiolata	**27.5**	**365.5**

The slope of the ratio I_0/I vs. mg/ml plotted, as shown in the Fig. 23, is a measure of the reactivity of the algal extract (sample) toward the hydroxyl radicals.

Fig. 23 Example of a curve of the CL results obtained from the dry extract of *Posidonia oceanica* using methanol solvent



A comparison between the samples slopes allowed the evaluation of the antioxidant efficiency.

From all the data obtained in this work, the scavenging activity for all samples tested was in the following order:

For the methanol extracts:

Taonia atomaria* > *Posidonia oceanica* > *Caulerpa prolifera* > *Laurencia papillosa* > *Udotea petiolata* > *Cladostephus spongiosus* > *Dassycladus clavaeformis* > *Halimeda tuna* > *Corallina mediteraneea* > *Sargassum vulgare* > *Halopteris filicina* > *Enteromorpha linza* > *Dictyoperis membranaceea* > *Codium vermilara* > *Ulva rigida* > *Laurencia obtusa* > *Gelidium sp.

For the aqueous methanol extract:

Posidonia oceanica > Dassycladus clavaeformis > Taonia atomaria > Cladostephus spongiosus > Halopteris filicina > Corallina mediteraneea > Laurencia obtusa > Dictyoperis membranaceea > Sargassum vulgare > Udotea petiolata > Enteromorpha linza > Ulva rigida > Laurencia papillosa > Gelidium sp > Halimeda tuna > Caulerpa prolifera > Codium vermilara

3. POCL results for antioxidant evaluation of non polar algal extracts.

For the algal extracts, EC_{50} values representing the concentration needed to decrease the initial chemiluminescence intensity (Io) by 50% were determined to estimate their relative hydroxy radical scavenging activities compared to that of the reference compound (trolox).

Table 9. Antioxidant activities of non polar (dichloromethane) extracts using POCL method (EC_{50} expressed in mg extract / ml EtOAc)

Name of species	*EC_{50} of dichloromethane extract*
Ulva rigida	**61.9**
Enteromorpha linza	**58.1**
Halimeda tuna	**19.7**
Caulerpa prolifera	**15.0**
Codium vermilara	**267**
Corallina mediteraneea	**1500**
Laurencia obtusa	**328**
Taonia atomaria	**94.3**
Dictyopteris membranacea	**92.6**
Sargassum vulgare	**282.1**

Cladostephus spongiosus	**399**
Halopteris filicina	**38.1**
Laurencia papillosa	**681**
Gelidium sp.	**75.5**
Posidonia oceanica	**97.7**
Dasycladus clavaeformis	**69.5**
Udotea petiolata	**15.9**

The results of the assays showed that antioxidant activity for the samples tested decreased in the following order:

Caulerpa prolifera > Udotea petiolata > Halimeda tuna > Halopteris filicina > Enteromorpha linza > Ulva rigida > Dassycladus clavaeformis > Gelidium sp> Dictyoperis membranacea > Taonia atomaria > Posidonia oceanica > Codium vermilara > Sargassum vulgare > Laurencia obtusa > Cladostephus spongiosus > Laurencia papillosa > Corallina mediteraneea

4. Results of DPPH radical scavenging method for polar and nonpolar extracts.

The concentration of antioxidant needed to decrease the initial DPPH · concentration (EC_{50}) by 50% was a parameter used to measure the antioxidant activity.

The lower the EC_{50}, the higher the antioxidant power. Another parameter AE = 1/ EC_{50} was defined as antiradical efficiency: The higher the AE, the higher the antioxidant activity.

The data obtained are shown in the table below:

Table 10. Antioxidant activity of the methanol extracts (DPPH)

(EC_{50} expressed in mg extract/g DPPH)

Name of species	EC_{50} methanol extract
Taonia atomaria	3.295
Posidonia oceanica	3.280
Gelidium sp.	4.162
Dictyopteris membranacea	4.519
Dasycladus clavaeformis	3.948

Table 11. Antioxidant activity of the aqueous methanol extracts (DPPH)

(EC_{50} expressed in mg extract/g DPPH)

Name of species	EC_{50} aqueous methanol extract
Taonia atomaria	2.514
Posidonia oceanica	2.053
Dasycladus clavaeformis	2.122
Sargassum vulgare	1.65
Dictyoperis membranaceea	2.644
Cladostephus spongiosus	2.727

Table 12. Antioxidant activity of the dichloromethane extracts (DPPH)

(EC_{50} expressed in mg extract/g DPPH)

Name of species	EC_{50} dichloromethane extract
Taonia atomaria	4.056

The data obtained in this study indicate that the extracts of *Posidonia oceanica* and *Taonia atomaria* were more efficient than the remaining extracts in decreasing the DPPH · initial concentration. The algae that do not appear in this table, showed noteworthy radical scavenging activity during the DPPH test.

5. Results of *Posidonia oceanica* second extraction

The yield of the second extraction in the polar solvent MeOH was 385 mg .The extract residue was dissolved in 20 ml of MeOH and evaporated again. The dried extract was kept in the refrigerator for further studies.

6. Estimation of the extract content by TLC

4 Eluents of increasing polarities were used to estimate the extract content before column chromatography.

7. Fractionation of *Posidonia oceanica* extract by flash chromatography.

Two different eluents (CH_2Cl_2 and MeOH) were used in different proportions for the separation of the *Posidonia* extract. The presence of compounds in the fractions obtained during flash chromatography was monitored by TLC using the mobile phase CH_2Cl_2 and MeOH (3:2).

The fractions containing algal compounds of different polarities were collected and concentrated in 3 fractions according to their polarity.

Immediately after the fraction collection, each fraction was evaporated on the rotary evaporator; the residues were weighted on the balance, and then redissolved in the solvent according to the polarity of compounds contained. Table 13 shows the weight of each fraction extract derived from the flash column chromatography:

Table 13. Yield of fraction by silica gel flash chromatography

Fraction number	Weight (g)
1	0.08
2	0.52
3	0.49

8. Second separation of nonpolar fraction F1 by flash chromatography

For a better isolation of the active metabolites a second fractionation of F1 was performed to separate the chlorophyll content of this fraction.

The fractions showing the presence of algal constituents were concentrated in two flasks, one containing F1, the polar part and the other containing F2, the non polar part, mainly the chlorophylls. The yields of the fractions were than calculated.

Table 14. The yields of fractions separated from F1

Fractions	Yield (mg)
F1 nonpolar part	3
F1 polar part	11

9. Evaluation of antioxidant activities of the fractions obtained by flash chromatography.

The resulting fractions were subjected to luminol and POCL chemiluminescence assays to identify the group of compounds with the highest antioxidant activity.

Table 15. Antioxidant activities of chromatography fractions

Fraction number	EC_{50}
F1 nonpolar part	0.0024
F1 polar part	0.0084
F2	0.0174
F3	0.0163

10. Separation of *Posidonia oceanica* fraction 2 using vacuum chromatography.

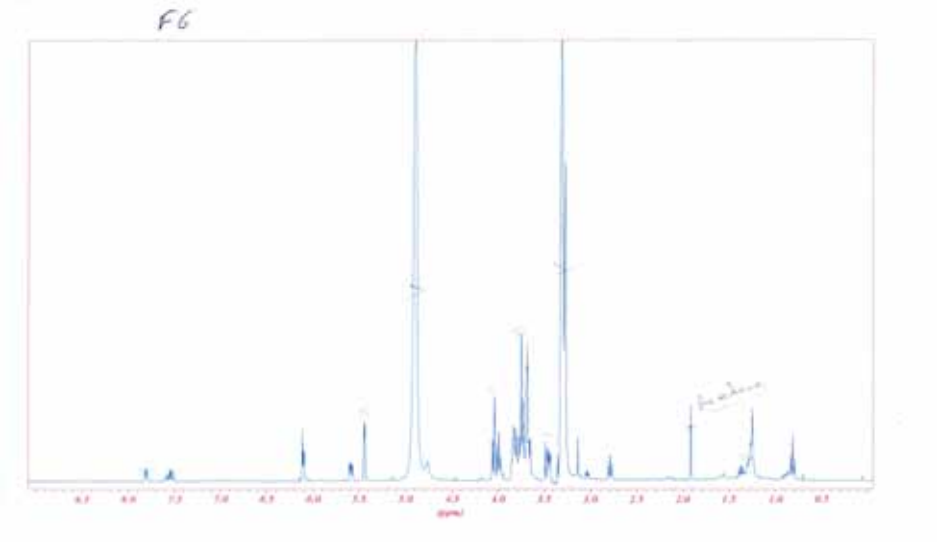
The LC-MS, as well as the ^{1}H-NMR methods used for the fourth fractions, indicated an interesting chemical profile for fraction number 2, which was also supported by the high antioxidant activity showed by this fraction. For this reason, fraction F2 was further purified by applying the vacuum chromatography method. Eleven fractions were obtained, which were monitored by TLC, using the eluent system CH_2Cl_2: MeOH (in different proportions), for the presence of active metabolites.

The presence of compounds was identified by TLC in fraction F6.

11. Spectral profile of Fraction 6 (^{1}H-NMR)

Fraction F6 contained polar constituents, as was analysed by TLC with different mobile phases.

Fig. 25 : ^{1}H- NMR spectrum of Fraction 6



The ^{1}H NMR spectrum of Fraction 6 showed several resonances in chemical shifts that were characteristic of oxygenated methines. The complex spin systems in the region of 3.0 ppm – 4.1 ppm is indicative of carbohydrates. The deshielded protons at 5.4 ppm, 5.6 ppm and 6.1 ppm are the anomeric protons of these carbohydrates.

The up field region did not show any peaks for aliphatic protons, apart from resonances at 1.25ppm and 0.85 ppm that are due to some fatty acid solvent contaminants.

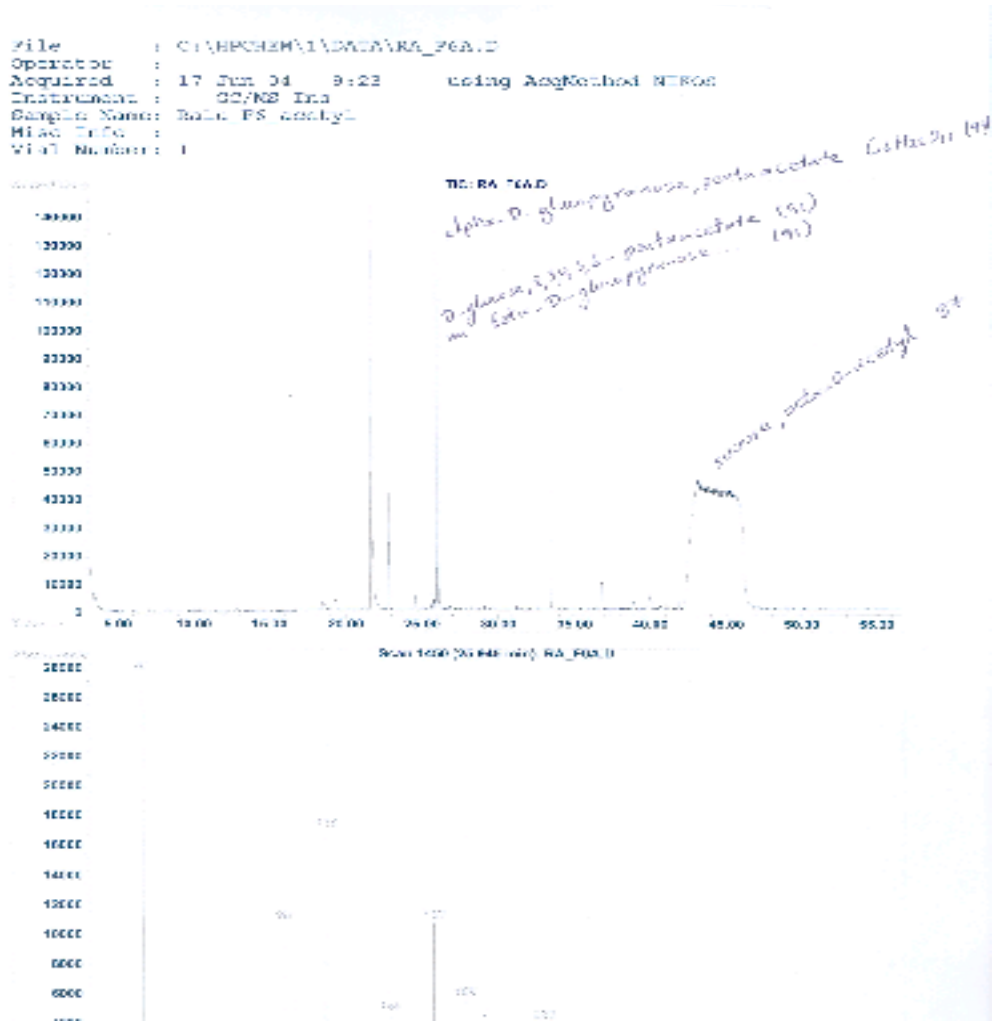
12. Identification of the major metabolites from fraction 6 by GC-MS.

Since the polarity of the oligo / polysaccharides is prohibitive for their analyses by GC-MS, it was decided to derivative them in order to reduce their polarity and allow better separation on the Gas chromatograph.

A quantity of Fraction 6 (30 mgs) was taken in a round bottom flask and, after evaporation of the residual solvents, was diluted with 5 mls of pyridine. Subsequently, 2 mls of acetic anhydride were added and the

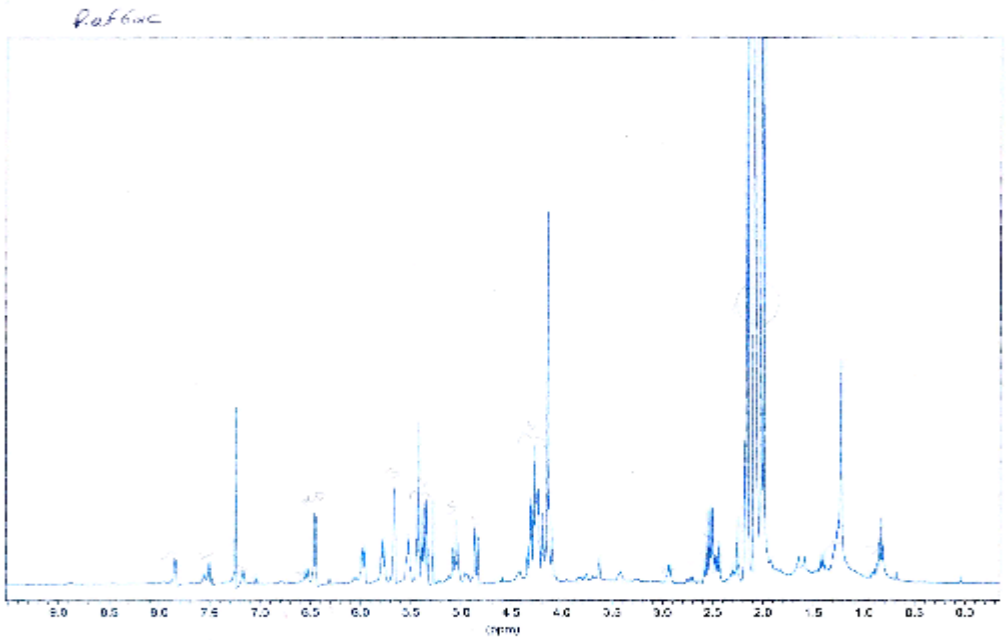
reaction vessel, equipped with a water condenser was refluxed for 3 hours. The reaction mixture was loaded in a separation funnel and was partitioned between water and dichloromethane. The organic layer was washed 3 times with water and then dehydrated by Na_2SO_4. The solvents were evaporated in vacuum and the reaction products were taken in $CDCl_3$ for NMR analyses. The same reaction mixture was also analysed by GC-MS.

Fig. 26: GS-MS spectrum of the acetylated Fraction 6



The GC-MS spectrum showed the presence of 2 major constituents which, when compared with the library spectra were identified with a good confidence as α-D-Glucose pentaacetate and Sucrose octaacetate.

Fig. 27 ^{1}H- NMR spectrum of acetylated Fraction 6



The ^{1}H NMR spectrum of the acetylated Fraction F6 showed the presence of a number of Acetyl groups (1.9 – 2.2 ppm), supporting the assumption of the oligosaccharide presence. The region of the methine protons was better resolved in the acetylated derivatives and the anomeric protons showed a notable down field shift.

Based on the information obtained by the GC-MS and that in the relevant literature, it was attempted to justify the observed peaks on the NMR spectrum by the proposed metabolites. The literature spectra of the detected metabolites (α-D-Glucose pentaacetate and Sucrose octaacetate) were found and compared with the experimental data.

Fig. 28 ^{1}H NMR spectrum of metabolite 1(α-D-Glucose pentaacetate)

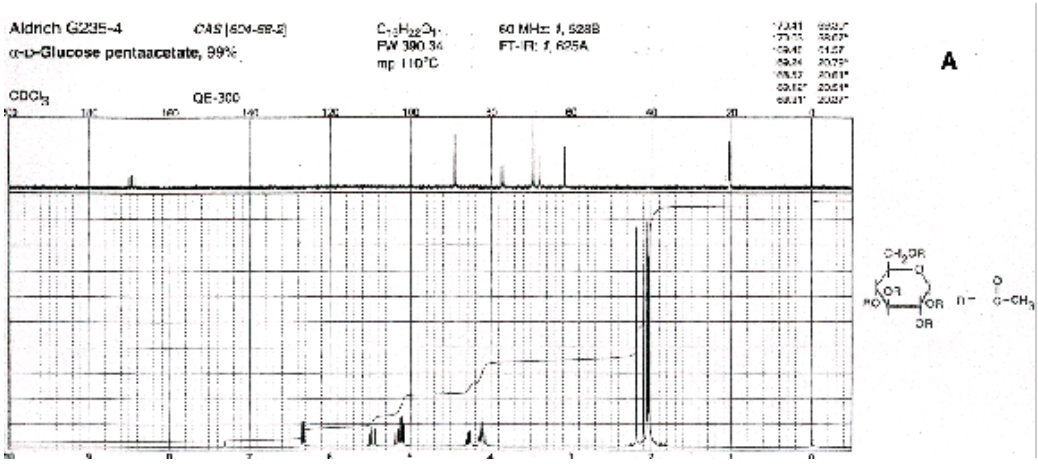


Fig. 29 GC-MS spectra of metabolite 1(α-D-Glucose pentaacetate)

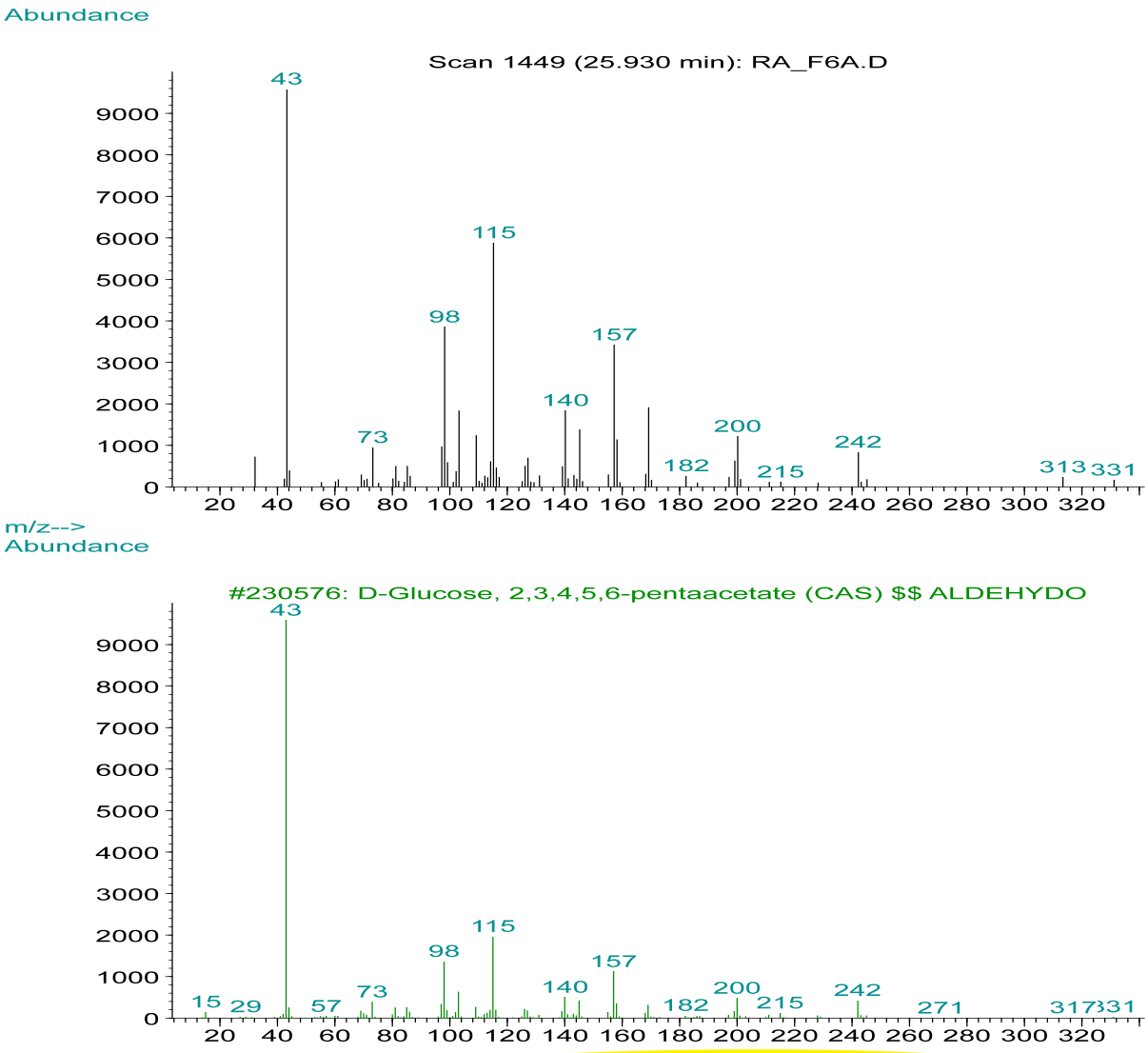


Fig. 30 ^{1}H NMR spectrum of metabolite 2(Sucrose octaacetate)

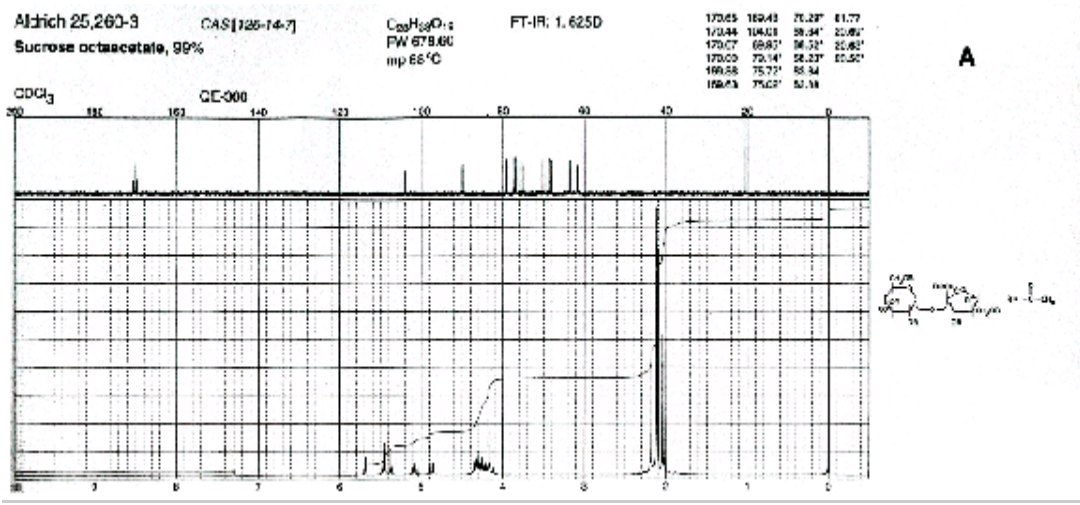
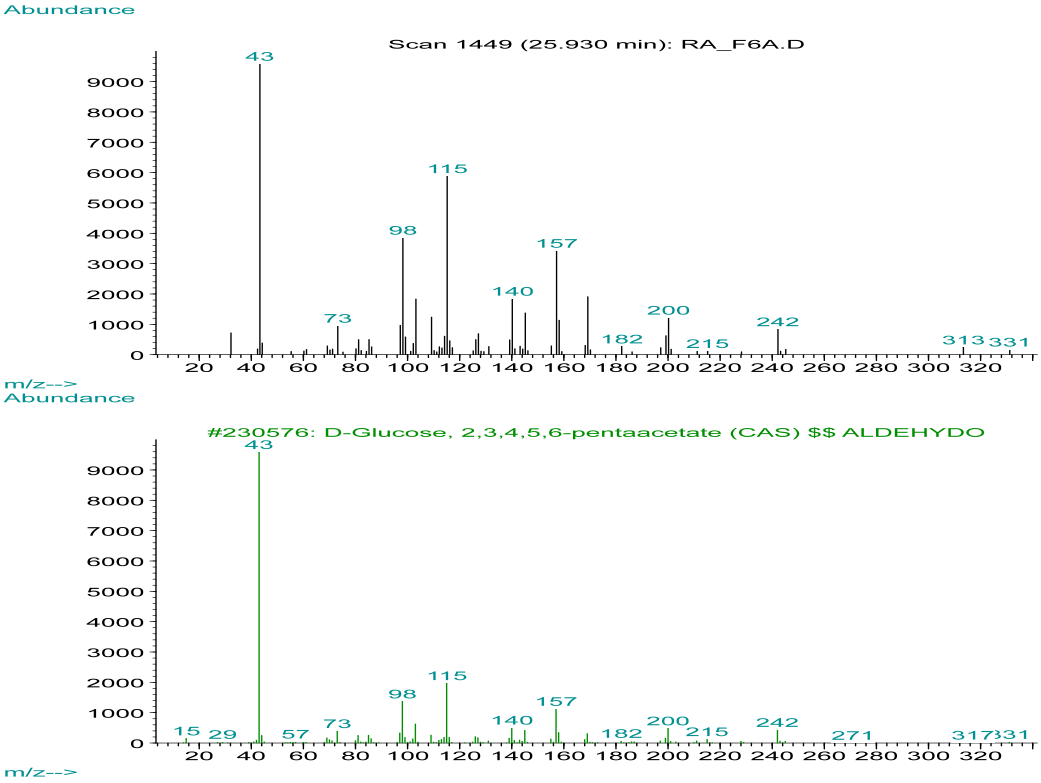


Fig. 31 GC-MS spectra of metabolite 2 (Sucrose octaacetate)



Even though the analysed residue was a mixture of oligosaccharides, the identity of the constituents was clear, confirmed by their characteristic proton resonances clearly shown in the spectra.

13. Purification of acetylated Fraction 6 by column chromatography

The TLC system for the acetylated Fraction 6, which was used during column chromatography for the purification of the fraction, was represented by:

7.5 isopropanol: 2 hexane: 0.5 acetic acid.

0.902 g extract were collected during the purification and were used for further analysis.

14. Evaluation of antioxidant activity of purified acetylated Fraction 6

The acetylated fraction F6, after purification, was subjected to the final chemiluminescence test to discover the source of antioxidant activity.

The result showed that its antioxidant activity was not comparable with Fraction 2 or with that of the crude extract, proving in this way that the observed antioxidant power activity came from the synergistic effect of metabolites, destroyed during subsequent fractionations.

15. The stability of active metabolites in the presence of silica.

The scavenging activity of the crude (initial) *Posidonia* extract was proven again using a methanol extract containing 365.4mg of *Posidonia*, for chemiluminescence studies.

Table 16. The results of antioxidant test for the crude *Posidonia* extract

Name of species	**IC_{50}**	**AE**
Posidonia oceanica	0.014265335	70.1

After the addition of 4 mg of silica gel to the crude extract of *Posidonia oceanica* and a further purification of the extract from the silica (obtaining 143.8 mg of extract that was dissolved in 5 ml MeOH), the results of the chemiluminescent test indicated a persistence in the antioxidant activity of the extract, proving the stability of active metabolites in the presence of an acidic medium such as silica.

Table 17. Evaluation of antioxidant activity of the *Posidonia* extract treated with silica

Name of species	**IC50**	**AE**
Posidonia oceanica	0.010525967	95.00315126

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

The present study on the antioxidant activity of 17 Mediterranean algae species using both the chemiluminescence and DPPH methods, proved that algae *Taonia atomaria* and *Posidonia oceanica* possessed high antiradical scavenging activity compared to the other species. In addition of the high antioxidant capacity, the widespread presence of *Posidonia oceanica*, made us proceed with investigation on the extract for the identification of the metabolites responsible of the significant antiradical scavenging activity.

TLC guided column chromatography was selected to perform the isolation of metabolites. The result of this preliminary separation was the collection of three fractions of different polarities that were submitted to the above mentioned screening method, chemiluminescence.

According to the results of the radical scavenging assay, LC-MS, and NMR , fraction number 2 proved to have an interesting chemical profile that also proved antioxidant properties. This result prompted us to continue with further chromatographic separation, obtaining other 11 fractions. The analyse of TLC and H-NMR showed an interesting and important chemical composition of fraction 6 (F6). The identification of metabolites contained by this fraction was proceeded by GC-MS and NMR, the two methods indicating the presence of 2 polysacchrides: α-D-Glucose pentaacetate and Sucrose octaacetate.

The chemiluminescence assay on fraction F6 proved a decrease in radical scavenging activity compared to the initial *Posidonia* extract. This result suggest that the antioxidant activity of *Posidonia oceanica* comes from all the metabolites working together synergically , this synergy being destroyed during fractionation.

We would like to emphasize that the remarkable antioxidant activity displayed by the extract , the widespread presence *of Posidonia* in

Mediterranean area and the presence of polysaccharides in the extract makes this alga an important object for further chemical studies.

In recent years , algal polysaccharides have been demonstrated not only to play an important role as free- radical scavengers and antioxidants for the prevention of oxidative damage in living organisms, but also to have diverse physiological activities including anticoagulant, antiviral and antitumor activities (Zhang, Q., 2004).

For this reason the polysaccharides content of *Posidonia* extract trigger out a future work focused on an evaluation of the toxicity of the extract and each of its compounds in order to determine the safety of their use as pharmacological products or as additives in edible products.

The presence of *Posidonia oceanica* in high amount in the Mediterranean area constitute an important advantage from financier point of view for further chemical studies as well as for a possible commercialisations of derivatives products of *Posidonia* extract.

LIST OF FIGURES

LIST OF TABLES

Índice

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ISBN: 978-9978-301-49-4

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