Evaluation of phytotoxicity of seaweed extracts from the Portuguese coast on tomato plants

Roberto Fernando Durán Clerque

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Resumo

Prevê-se que a população mundial mantenha um crescimento constante nos próximos anos, pelo que a gestão correcta da agricultura é imperativa para responder adequadamente à crescente procura de alimentos. Esta procura elevada levou à intensificação das actividades agrícolas, incluindo a implementação de produtos fitofarmacêuticos destinados a proteger os cultivos de organismos nocivos, pragas e doenças. Mas a utilização destas estratégias químicas apresenta efeitos nocivos para o ambiente e para a saúde humana. Neste contexto, o ambiente marinho representa uma fonte ampla e valiosa de novos compostos interessantes para substituir os atualmente utilizados. Embora os compostos de origem natural sejam considerados seguros para o consumo humano, o estudo do impacto real da sua aplicação continua a ser indispensável.

Assim, o objetivo desta dissertação foi avaliar o potencial antioxidante de vinte e quatro extractos diferentes produzidos a partir das algas marinhas *Asparagopsis armata, Codium* sp.*, Fucus vesiculosus e Sargassum muticum*, bem como a fitotoxicidade dos extractos aquoso e hidroetanólico.

A preparação dos extractos foi realizada através de uma extração sólido-líquido com água, etanol e água (75:25), apenas etanol, acetato de etilo e n-hexano como solventes. No que diz respeito às actividades biológicas, a atividade antioxidante dos compostos foi avaliada através dos métodos 2,2-difenil-1-picrilhidrazil (DPPH), capacidade de absorção do radical oxigénio (ORAC) e poder antioxidante redutor férrico (FRAP), bem como o conteúdo fenólico total (TPC). O efeito fitotóxico foi avaliado, em primeiro lugar, por um ensaio *in vitro* de punção das folhas, no qual folhas destacadas de tomateiro (*Solanum lycopersicum*; Solanaceae) foram aplicadas extractos previamente preparados em três concentrações diferentes (0,1; 0,5; e 1,0 mg/mL). Posteriormente, uma segunda abordagem foi avaliada em um ensaio *in vivo* em estufa, onde extratos aquosos liofilizados e hidroetanólicos foram aplicados em plantas de tomate por pulverização, semanalmente, durante 42 dias.

A análise dos extractos revelou valores baixos de conteúdo fenólico total. Os extractos hidroetanólico e etanólico de F. vesiculosus apresentaram os valores mais

elevados. No contexto da atividade de redução do radical DPPH, F. vesiculosus (hidroetanólico, etanólico e acetato de etilo), A. armata (acetato de etilo) e S. muticum (etanólico) apresentaram os resultados mais promissores, reduzindo mais de 50% do radical DPPH. Relativamente ao método FRAP, os resultados foram significativamente inferiores aos do BHT. Por outro lado, o resultado obtido no método ORAC foi significativamente superior ao do BHT, mas ainda muito baixo em comparação com outros compostos antioxidantes, como o ácido ascórbico. Em relação ao ensaio de punção foliar, os extractos não apresentaram diferenças significativas quando comparados com os controlos avaliados. Por outro lado, as plantas em estudo *in vivo* após 42 dias de tratamento não mostraram nem fitotoxicidade nem efeito bioestimulante.

Em conclusão, foram obtidos diferentes extractos de quatro algas marinhas diferentes e, globalmente, mostraram uma fraca capacidade antioxidante. Não se verificou qualquer efeito de fitotoxicidade nas plantas, no entanto, a temperatura elevada durante o período experimental na estufa foi um fator que afectou negativamente o crescimento das plantas de tomate. Embora nas condições apresentadas não tenha havido efeito positivo nas plantas, o facto de não ter apresentado efeito negativo e fitotoxicidade é favorável ao desenvolvimento de produtos com outras acções, como os biopesticidas.

Palavras-chave: Agricultura, macroalgas, atividade antioxidante, compostos bioativos, ensaio de punção de folha, fitotoxicidade, recursos marinhos.

Abstract

The global population is projected to maintain a steady growth the coming years, thus the correct management of agriculture is imperative to adequately address the escalating demand of food. This elevated demand has lead to the intensification of agriculture activities, including the implementation of plant protection products aiming to protect orchards from harmful organisms, pest, and diseases. But the use of these chemic strategies present harmful effects over the environment and human health. Within this context, marine environment represents a wild and valuable source of interesting new compounds to replace the current used. Even though natural origin compounds are considered safe for human consumption, the study of the real impact of its application remains indispensable.

Therefore, the objective of this dissertation was assessing the antioxidant potential of twenty-four different extracts produced from the seaweeds *Asparagopsis armata, Codium* sp., *Fucus vesiculosus* and *Sargassum muticum,* as well as the phytotoxicity of aqueous and hydroethanolic extracts.

The preparation of extracts was achieved through a solid-liquid extraction with water, ethanol and water (75:25), only ethanol, ethyl acetate and *n*-hexane as solvents. Regarding biological activities, antioxidant activity of compounds was evaluated through the 2,2-diphenyl-1- picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) methods, as well as total phenolic content (TPC). The phytotoxicity effect was assessed firstly by an *in vitro* leaf puncture assay, which detached tomato (*Solanum lycopersicum*; Solanaceae) leaves were submitted to extracts previously prepared at three different concentrations (0.1; 0.5; and 1.0 mg/mL). Subsequently, a second approach was evaluated in an *in vivo* assay in a greenhouse, where aqueous lyophilized and hydroethanolic extracts were applied in tomato plants by spraying, weekly for 42 days.

The analysis of the extracts revealed low values of total phenolic content. *F. vesiculosus* hydroethanolic and ethanolic extracts exhibiting the highest values. In the context of DPPH radical scavenging activity, *F. vesiculosus* (hydroethanolic, ethanolic and ethyl acetate), *A. armata* (ethyl acetate) and *S. muticum* (ethanolic) displayed the

most promising results, reducing more than 50% DPPH radical. Regarding FRAP method, results were significantly lower than BHT. In the other hand, the result obtained in the ORAC method was significantly higher than BHT, but still very low comparing to other antioxidant compounds, as ascorbic acid. Regarding the leaf puncture assay, extracts did not show significant differences when compared with controls assessed. On the other hand, the *in vivo* study plants after 42 days of treatment did not show nether a phytotoxicity or a bio stimulant effect.

In conclusion, different extracts were obtained from four different seaweeds and, globally, showed poor antioxidant capacity. No phytotoxicity effect was found on the plants, however, the high temperature during the experimental period in the greenhouse was a factor that negatively affected tomato plants growth. Although under the conditions presented there was no positive effect on the plants, the fact that it did not present a negative effect and phytotoxicity is favorable to the development of products with other actions, such as biopesticides.

Keywords: Agriculture, macroalgae**,** antioxidant activity, bioactive compounds, leaf puncture assay, marine resources, phytotoxicity.

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Abbreviation and acronyms

AA: *Asparagopsis armata*

AAPH: α,α′azodiisobutyramidine dihydrochloride

AUC: Area under the curve

CD: *Codium* sp.

CE: Circular economy

CFC: Commercial fertilizer control

DAT: Days after transplant

DMSO: Dimethyl sulfoxide

DPPH: Diphenyl-1(2,4,6-trinitrophenyl) hydrazyl

EA: Ethyl acetate

EQ: Equivalent

EU: European Union

FAO: Food and agriculture organization

FRAP: Ferric reducing antioxidant power

FV: *Fucus vesiculosus*

Ha: Hectares

NTC: Non-treatment control

ORAC: Oxygen radical absorbance capacity

PBS: Phosphate buffered saline

PGI: Protected geographical indication

ROS: Reactive oxygen species

SM: *Sargassum muticum*

TPC: Total phenolic content

TPTZ: Tripyridyl triazine

VC: Vacciplant control

WC: Water control

Introduction

1.1 Agriculture, issues, and future perspectives

In 2019, the global agricultural land area covered 4.8 billion hectares (ha) (FAO, 2021). Healthy, sustainable, and inclusive food systems are critical to achieve sustainable development goals. The global demand for agricultural products is predicted to increase during the coming decades due to human population growth and changes in consumption patterns (Hass et al., 2018).

Agriculture in Europe is tremendously diverse, ranging from vast, intense, and specialized commercial holdings to subsistence farming based mostly on traditional methods. It is also the main land user in the European Union with more than 47% of the total territory used and, consequently, it is expected that impacts of this activity on the environment vary in scale and intensity (Erisman et al., 2008; Giannakis & Bruggeman, 2015).

The existing food production and distribution strategy has significant negative consequences for people's lives and the environment. Pesticide and chemical fertilizer misuse, monocultures, deforestation, land grabbing, and rural depopulation are clear issues with a significant impact in our lives. Regarding environment, agriculture has a significant impact as primary source of nitrates, phosphates, and pesticide contamination in water. (FAO, 2002).

Aiming protect the plants from harmful organisms, pests, and diseases, it is common the use of plant protection products, which are substances or mixtures of substances that are primarily used in agriculture or in public health protection programs.

To protect crops, one of the most used components for agricultural management are insecticides, fungicides, herbicides, rodenticides, and plant growth regulators, to name a few examples of chemical substances. These products have been found in nearby subsurface aquifers in agricultural regions where pesticides are used. (Cerejeira et al., 2003; Laxmishree & Nandita, 2017; Nicolopoulou-Stamati et al., 2016).

Nevertheless, their inadequate use can have serious environmental consequences which can be potentiate by distinct factors. The dragging of these chemicals by the wind, leaching, and runoff are all forms of uncontrolled plant protection product distribution in the environment, resulting in soil and water contamination and loss of biodiversity.

Whereas soil is increasingly recognized as a non-renewable resource on a human life scale because, once degraded its regeneration is an extremely slow process (Bai et al., 2018). Soil quality is not limited to the degree of soil pollution, being commonly defined more broadly as "the capacity of a soil to function within ecosystem and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plan and animal health" (Bünemann et al., 2018). Indeed, soil quality is more complex than the quality of air and water, not only because soil constitutes solid, liquid and gaseous phases, but also because soils can be used for larger variety of purposes (Nortcliff, 2002). Charting environmentally sustainable pathways for agricultural development is of utmost importance, contributing to mitigate climate change.

Climate change is predicted to promote the most negative consequences in low- and middle-income nations, where millions of people rely on agriculture and are at risk of food insecurity. The Intergovernmental Panel for Climate Change estimated that this phenom, due to contamination, will have a "substantial negative impact" on per capita calorie availability, childhood undernutrition, and child deaths related to undernutrition (FAO, 2014).

The Fruit Sector stands as a crucial component of the European Agroindustry, contributing significantly to the EU agricultural production with a 14% value share, generating a substantial annual turnover exceeding 120 billion euros, and supporting approximately 550,000 employees across 1.4 million farm holdings. Notably, within the Portuguese West Region, the production of pears and apples plays a pivotal role, fostering a substantial number of direct and indirect employment opportunities (*Estatísticas Agrícolas 2020*, 2020).

1.1.1 Pear and apple fruticulture in Portugal

In Portugal, in 2019, 43% of the total area of the country were destined for agriculture increasing 3.2% since 2009, 3.9 million ha were destined for agriculture, of which 44 484 ha dedicated for fresh fruit production, yielding 15 419 Kg per ha. (*Estatísticas Agrícolas 2020*).

Portuguese fruit production has improved its efficiency, evolved into a dynamic, and advanced agricultural sector, which is organized under producer organizations and associations, reinforcing, and increasing sector´s competitiveness. Together with the National Strategy for the Fruit and Vegetable Sector it aims to face the principal challenges for the sector, such as climate change, water availability, extreme phenomena, and phytosanitary problems (Carmen Valverde, 2021; Martins et al., 2019). As stated, Duarte (2019), the fruticulture has been one of the Portugal´s most active economy industries in recent years. This development is the consequence of improved cultural methods and the modernization of orchards with more appropriate lands, more productive plants, irrigation, fertilization and control of pests and diseases. Portugal is today extremely well positioned in the global community and international market, being able to supply distinctive goods. In 2018, fruits accounted for 19% of the agricultural sector, with a commercial value of 1 405 million euros, having a steady growth over the previous ten years (Gabinete de Planeamento, 2020). Apples and pears represent 22.17% and peach 6% of fresh fruit production.

Two of the most iconic fruits not only from Portugal, but most specific from the West zone are the “Rocha” pear and “Alcobaça” apple. Pear and apple trees fill 11 325 ha and 14 311 ha, respectively. During 2020, the global production of apple and pear in Portugal was 287 000 tons, and 131 000 tons of pear, respectively, representing a decrease of production of 22.7% and 34%, respectively comparing with 2019 (*Estatísticas Agrícolas 2020*).

Pear tree dates from the middle of the 19th century, at Mr. Pedro Antonio Rocha´s property, “Fazenda Rocha”, situated in “Ribeira de Sintra”, in the West region close to Lisbon, Figure 1. In the West region exist near 11 000 ha of “Rocha” pear, 5 000 producers and around 15 000 workers involved in the harvest session. Portugal annually produces an average of 173 000 tons from which 60% of the production is for exportation (*Pêra Rocha – Flavor of Portugal*), produced and packaged in twenty-nine communes of the West region. The breeding, care, and harvesting of “Rocha” pear are all timed to the nature, as the climatic conditions of the location are needed for the fruit to properly grow.

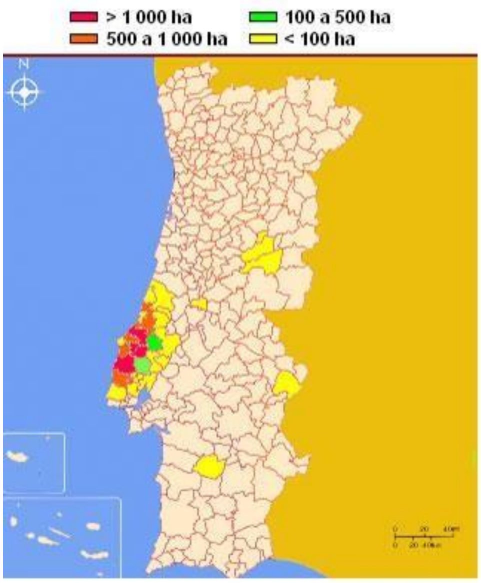


Figure 1 Earmarked territory to produce "Rocha" pear in Portugal (Pera Rocha Do Oeste DOP | Comisión Europea).

In addition, the West zone of Portugal possess another important orchard, the "Alcobaça" apple. The "Alcobaça Apple" belongs to the *Malus domestica Boekh* species, with "royal gala", "delicious", "jonagold", "fuji", "casanova de Alcobaça", "golden delicious", "granny smith" and "reineta parda" varieties (Silva, 2016).

Currently, due to their characteristics, it has earned the Protected Geographical Indication (PGI), being granted registration by the European Union in 1996 (*GIview.*). This classification implies that the apple is manufactured in compliance with the specifications, which include the conditions of production, harvesting, and packing of the product as well as the labeling, which must also meet the standards of the applicable legislation (Fernandes, 2007). This designation is granted with the traditional producers who match the standards of quality, appearance, flavor, aroma, density, and consistency of the fruits.

In 2015, the proposed amendment was adopted, allowing the municipalities of Batalha, Bombarral, Cadaval, Leiria, Lourinhã, Marinha Grande, Peniche, Rio Maior and Torres Vedras to participate in the production, Figure 2, processing and packaging (Silva, 2016). Its production is found between Aire-Candeeiros and Montejunto mountains and Atlantic Ocean, with a total area of 3 169 Km^2.

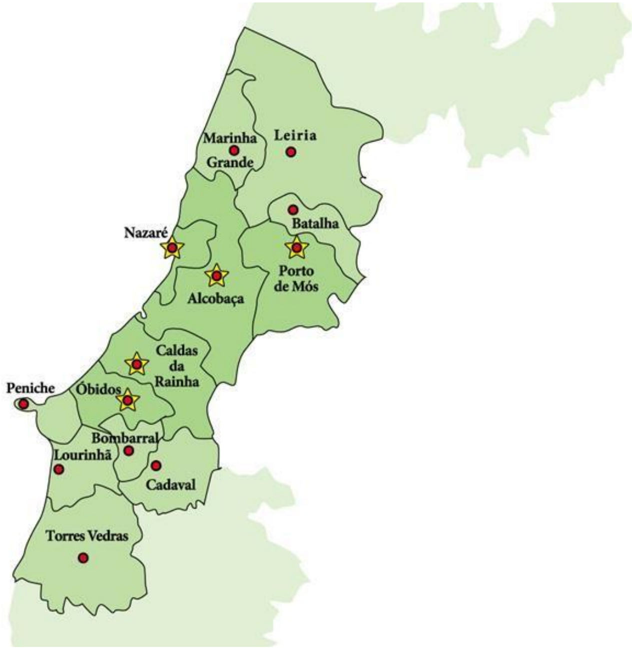


Figure 2 Geographic area of the "Alcobaça" apple production (Silva, 2016)

Apple and pear orchards, like many other crops, are plagued by a numerous of insect pests and microbiological diseases that threaten orchard viability and productivity. Fire blight and brown spot disease caused by *Erwinia amylovora* and *Stemphylium vesicarium* respectively, are pathogens that affects pear and apple orchards, and can cause up to 20% production losses (Erfani-Moghadam & Zarei, 2022; Loebler et al., 2020) with high impact in the production chain in the country, likewise these two species, exist more pathogens and diseases affecting orchards production.

To meet the requirements of the increasing world population, as well as yield production objectives, it is common the use chemical pesticides and synthetic fertilizers in order to protect plants. Yet, many of this chemical pesticides have been associated with health and environmental issues (Nicolopoulou-Stamati et al., 2016). For this reason, the study of new natural compounds to replace synthetic chemical products is important for health and development matters.

1.2 Macroalgae distribution in the Portuguese coast

Macroalgae or seaweeds are multicellular, macroscopic, eukaryotic, and autotrophic organisms (Leandro et al., 2020). Macroalgae are classified as Chlorophyta (green), Rhodophyta (red), and Phaeophyta (brown), according to the thallus color derived from natural pigments and chlorophylls. The pigment, growth, and chemical composition of macroalgae are affected by environmental parameters such as temperature, salinity, nutrients disposition, pollution, and even water movement, depending on their taxonomic groups and species (Jung et al., 2013).

Distribution responses may be caused by effects of temperature and other climatic and non-climatic physical factors (night summer temperature, maximum daily temperature, slope of the shore air temperature) on species (Martínez et al., 2012). The Portuguese coast is exposed to distinct biogeographic conditions, with climate effects from the Atlantic Ocean and the Mediterranean Sea determining unique species combinations in macroalgal ecosystems (Araújo et al., 2009).

The ecology of seaweeds is dominated by two environmental requirements, such as enough light for photosynthesis to occur and a fixation point, reason why marine algae live along the coast, and within that zone, on rocky seafronts rather than sand or gravel (L. Pereira, 2021). The coastline of Portugal measures approximately 2 587 Km, including the continental territory and archipelagos (Azores and Madeira), representing 2.7% of the total EU exclusive economic zone (DGMARE, 2013).

Red algae known as Rhodophyceae are phylogenetically the oldest division of lower plants and contain about 5 000 species, mainly marine multicellular organisms (Usov, 2011). This phylum shares some attributes as eukaryotic cells, lack of flagella, floridian starch, phycobiliprotein pigments (red and blue), unstacked thylakoids, and chloroplasts lacking and external endoplasmic reticulum (Sheath & Vis, 2015).

Green algae, Chlorophyceae, are photosynthetic eukaryotes bearing a double membrane-bound plastids containing chlorophyll *a* and *b*, accessory pigments found in embryophytes, and a unique stellate structure linking nine pairs of microtubules in the flagellar base (Lewis & McCourt, 2004).

Brown algae are a large and diverse class, *Phaeophyceae*, that range from small filamentous forms to large and complex, have plastids with girdle lamella, thylakoids in stacks of three, and chloroplast endoplasmic reticulum (Wehr, 2015). This group is responsible for the bulk of primary production, often forming extensive undersea forests hosting a high level of biodiversity (Cock et al., 2011).

According to endemism, species could be classified as endemic and native from the territory concerned, or invasive. Invasive species are often known as exotic or non- native species that have been purposefully or unintentionally brought into areas where they are not normally found (Pereira et al., 2021). Normally the introduction of invasive species on a habitat leads to a negative output to the host ecosystem, since this species will compete with native species for space and food, and also can produce secondary metabolites to confer advantages against native species. Non-indigenous marine species' expansion and establishment have a significant influence on community structure and function, ecological processes, and ecosystem services (Blanco et al., 2021).

Asparagopsis armata (Figure 3) is an invasive species introduced from the southern hemisphere, occurring from June or July to August or September; the plant grows up to 20 cm tall, has barbed branches, and is rosy, pink, yellowish pink in hue before being withdrawn from the water (M.D. Guiry in Guiry, M.D. & Guiry, 2022; Pinteus et al., 2018). The capacity of this species to adapt to the environment is such that could mean that their metabolites production might be different even within the same specie (Sacristán-Soriano et al., 2012). The vast production of different metabolites has drawn researchers' attention to study its ability for biotechnological applications, such as cosmetics, food and feed industry, therapeutics, antifouling, antibacterial, antifungal, and agriculture. A variety of primary and secondary compounds have been described for this algae, like vitamins, lipids, carbohydrates, polysaccharides and sulphated polysaccharides, phenolic compounds or halogenated secondary metabolites, (e.g. bromoform, dibromo acetic acid) (Félix et al., 2021;Pereira et al., 2021).

Figure 3 *Asparagopsis armata*, Funchal, Madeira, Portugal – 2019, (source: M.D. Guiry in Guiry, 2022)

Codium sp., a genus of green seaweed with 125 species found in marine habitats, is extensively dispersed around the world, mainly found in temperate and subtropical zones

(Sabry et al., 2019). This genus has been studied for the production of sulfated polysaccharides and its applications as immunostimulants, anticoagulants (de Oliveira-Carvalho et al., 2012).

Fucus vesiculosus (Figure 4) is one of the most common seaweeds in the north Atlantic Sea, usually found in the mid-intertidal on rocky shores and can tolerate a wide range of salinities. Its chemical composition depends on the harvest season, geographic location, and environmental factors, such as substrate firmness, exposure to ice and waves, salinity, wave force, light, or competition between macroalgae. However, it is essentially constituted by polyphenolic compounds, proteins, minerals, iodine, vitamins, fatty acids, and non-digestible polysaccharides (André et al., 2020; Bouga & Combet, 2015; Viana et al., 2015).

Figure 4 *Fucus vesiculosus*, Ria de Ferrol, Galicia, Spain – 2013, (source: M.D. Guiry in Guiry, 2022)

Sargassum muticum (Figure 5) is a seaweed native from the northwest Pacific region, appeared in Europe in the early 1970s and thanks to its considerable higher growth rate has been described as invasive or even the most "successful" invasive species (Milledge et al., 2016). It contains a variety of potentially bioactive compounds, including sulfated polysaccharides, fucoxanthin, steroids, terpenoids, and flavonoids. Compounds extracted from these algae have shown antifouling capabilities, alginate production, biosorption of toxic and heavy metals, pharmaceutical applications and antibacterial activity (El Atouani et al., 2016; Plouguerné et al., 2010). Anaerobic digestion of this algae could be a source to biogas production, and the digested algae used as fertilizer, soil conditioner or other further applications (Soto et al., 2015).

Figure 5 *Sargassum muticum,* Spiddal, Co. Galway, Ireland – 2020, (source Guiry in Guiry, 2022)

The use of marine macroalgae arouses great attention to the scientific community. In the industry could be the most viable in terms of technical variables, such avoiding the competition with the food market, the need of farmland, the use of freshwater, moreover, improving the management of this resource could be applicated for food and feed industry, cosmetic and pharmaceuticals products, fertilizers or soil improvers (Pardilhó, S. L., Machado, S., F. Bessada, S. M., F. Almeida, M., Oliveira, M. B., M. Dias, 2021).

1.3 Macroalgae as a source of novel natural products and their applications

Seaweeds have a long history of use in human diet, from a nutritional point of view. Edible seaweeds are low-calorie food and low lipid content, with a high concentration of minerals, proteins and vitamins such as A, C, D, and E along with B (Fitzgerald et al., 2011). Seaweeds are primary producers, the base of the marine food chain; they also compete for light, nutrients, and space, in addition to the need of carbon dioxide and water, they have also developed effective mechanisms to survive to many biotic threats, like bacteria, virus, or fungal infections (Leandro et al., 2020).

Edible seaweeds are a fascinating natural source of biologically active chemicals that might be exploited as functional components. Some algae thrive in complicated environments and are often exposed to harsh circumstances. Their metabolism is influenced by factors like as water temperature, salinity, light, and nutrients, and they are constantly forced to adapt to changing environmental conditions, therefore they create a wide range of physiologically active secondary metabolites in order to survive (Rodrigues et al., 2015).

Nowadays the use of macroalgae has been extended not just for feeding purposes but for cosmetic, industrial and pharmaceuticals purposes (Lourenço-Lopes et al., 2020). Macroalgae represent an inestimable source of biomolecules and micro-nutrients with biological and biochemical functions.

In the last years consumers are more concerned about the use of synthetic molecules and raised the demand to replace them for chemical compounds from natural origin. Considering the composition of macroalgae, they have been considered a promising source of compounds that could be employed to solve some of the current industry issues (Lourenço-Lopes et al., 2020).

Amino acids, terpenoids, phlorotannin's, steroids, phenolic compounds, halogenated ketones and alkanes, cyclic polysulphides, fatty acids, and acrylic acid are examples of algal chemicals with bacteriostatic and bactericidal action (Taskin et al., 2007).

Some seaweeds are prolific and often found regardless of geographical location, whereas others are exclusive to specific places. Although huge amounts of seaweed deposited on the shores regularly cause environmental impacts, there is also an opportunity to take innovative activities for the valorization of this biomass rather than just tossing it in landfills or allowing it to decompose (Ali et al., 2021).

Recent research has focused on seaweed-based extracts, which include a variety of biostimulator components such as distinct types of carbohydrates, amino acids, minor amounts of phytohormones, osmose protectants, and proteins. Seaweeds are also known for its antioxidant capacity, an antioxidant is any compound that considerably slows or prevents oxidation of an oxidizable substrate (Halliwell, 1990). Compounds with antioxidant capacity produced by seaweeds have shown an effective defense against lipid and protein oxidation, cell damage, and mutagenesis. This capacity makes antioxidant compounds useful in a range of industries, such as pharmaceutical, cosmetic, and nutraceuticals (Pinteus et al., 2018).

In Europe most of the seaweed production is bound to food industry (36%), food related uses (15%), feed (10%), accounting for 61% of the total uses, while other applications like fertilizers and bio stimulants contribute individually less than 11% (Araújo et al., 2021). It shows that the correct management algae industry in Europe has potential to grow and contribute to challenges as the EU carbon neutrality, innovate food system ensuring its access to nutritious and sustainable food in accordance with a circular bioeconomy.

The circular economy is gaining popularity as a strategy for attaining local, national, and global sustainability. It has gotten more attention from international corporations and policymakers in developed countries. Eco-design, reuse, refurbishing, remanufacturing, repair, product sharing, and industrial symbiosis are hence specific acts and behaviors that characterize it (Schroeder et al., 2019).

According to the circular economy principles, improper management of this seaweed resource does not allow for the creation of extra value in the economic sector such as new goods. As a result, it is critical and timely to investigate their recovery in nations with long coastlines and a heavy reliance on marine resources, such as Portugal (Pardilhó, S. L., Machado, S., F. Bessada, S. M., F. Almeida, M., Oliveira, M. B., M. Dias, 2021). Harvesting macroalgae in a sustainable manner is the key to a long-term viable business model using macroalgae.

1.3.1 Macroalgae components as agents to improve plants resilience to pest infections and abiotic factors

One of the oldest traditional applications of macroalgae are in agriculture, where have been applied as fertilizers. Fertilization improves the efficiency and quality of product recovery in agricultural operations. Non-organic fertilizers mainly contain phosphate, nitrate, ammonium, and potassium salts (Savci, 2012) however, their high consumption worldwide have been related with serious environmental problems.

Macroalgae offer elements such as magnesium, strontium, boron, and iron, which help increase crop output, also could help to balance the pH of acidic soils. Auxins, cytokinin’s, gibberellin, small levels of phytohormones present in the extracts as well as various stimulatory processes engaged in the plant system upon treatment with these extracts; seaweed extracts also showed the increase of plants’ endogenous cytokinin, isopentyl adenine, dihydrozeatin, and cis-zeatin which have all been linked to positive plant growth, proline and antioxidants are also increased endogenously in the plant in the presence of seaweeds extract (Ali et al., 2021; Mukherjee & Patel, 2020; L. Pereira, 2021).

Endogenous auxins have been found in brown, red, and green macroalgal species, shown to be a significant component in in the stimulating macroalgal rooting.

Foliar application of algae extracts has demonstrated quantifiable results in grapevine, watermelon, strawberry, apple, and tomato yield production. Auxins and cytokinin from brown, red and green algae have found to promote rooting, and act as plant growth regulator. Betaines extracted from macroalgae function protecting cells against osmotic stress, drought, salinity, and high temperature. In addition, some mono and polyunsaturated fatty acids play roles regulating permeability of cellular membranes and antimicrobial properties, as well as macro and micronutrients from brown algae has found to be rich in calcium, potassium, magnesium, etc. (Sharma et al., 2014).

The Plants immunity is based on inducible defensive mechanisms that are activated in response to a threat. The plant defends itself against possible attackers or abiotic stressors while simultaneously building its defense mechanism ensuring a faster and/or stronger response. The state of enhanced capacity to activate stress-induced defense responses has been designed as ‘‘primed’’ (Conrath, 2009; Mauch-Mani et al., 2017).

Land plants evolved structural barriers, constitutive secondary metabolites, and inducible defensive systems to protect themselves from potentially harmful microorganisms and insects. When confronted with viruses, insects, or abiotic stress, primed plants exhibit more immediate and robust activation of defensive mechanisms. Several chemical signals, including salicylic acid, azelaic acid, and pipecolic acid, jasmonic acid, and/or ethylene have been linked to systemic signaling, priming, and direct activation of defense during plant immune responses (Conrath et al., 2015; Sharma et al.,2014).

Because of the current scenario in developing nations and public concern about the use of dangerous chemicals as fertilizers and pesticides for agricultural reasons, non-chemical eco-friendly bioagents are becoming increasingly significant in agricultural crop production. In recent years, rising public awareness over pesticide-related human health hazards has fueled interest in alternative eco-friendly ways of disease management (Abbasi et al., 2021).

Thousands of compounds have been extracted from marine sources so far, and hundreds of new compounds are found each year. Seaweeds components thereof have been demonstrated to improve productivity of crops, improving soil and disease management, water efficiency, drought tolerance, diversification of crops, and farming practices (Arioli et al., 2015).

Products derived from seaweed exhibit the capacity to enhance crop productivity significantly. Seaweeds represent a rich reservoir of essential nutrients, including fiber, minerals, proteins, vitamins, and fatty acids, which hold substantial importance in the realm of nutrition (Ali et al., 2021; Savci, 2012).

Reduction-oxidation (redox) alterations serve as critical triggers for the activation of immune functions, including the hypersensitive response, which results in the programmed cell death of challenged plant cells, this reactions controls cellular metabolism, prominently driven by NAD(H) and NADP(H) couples, while concurrently serving as a vital signaling mechanism that informs the cell of its environmental context (Frederickson Matika & Loake, 2014; Geigenberger & Fernie, 2014).

The study performed by Toledo et al.,(2023) has unveiled the promising antifungal potential of several extracts (*n*-hexane, ethyl acetate, and ethanolic) from *A. armata* in in vitro experiments targeting the growth and spore germination of *B. cinerea*, *F. oxysporum*, and *P. expansum*. Moreover, the aqueous extract from *S. muticum* displayed significant effectiveness in inhibiting *B. cinerea* infection in “Rocha” pears. Furthermore, both *in vitro* and *in vivo* assessments have revealed that extracts from the four seaweeds not only possess antifungal properties but also stimulate mycelial growth.

Natural compounds are usually regarded as safe for human health and are extensively utilized, although its definition is "unlikely to cause pain or injury," this is a phrase that is frequently connected incorrectly with natural (McGill, 2009; Tanna & Mishra, 2019). Given that over 1 000 new compounds have been reported from marine organisms each year, the sea's biotechnological potential is undeniably significant (Rangel & Falkenberg, 2015). However, there is a need for monitoring the negative impact of seaweeds, manage seaweed quality and negative seaweed aspects, which is also necessary in normal agriculture. Phytotoxic assays are adequate diagnostic tools to determine the effect of chemical agents on test organisms under specific and controlled experimental conditions.

In modern and basic plant science, *Arabidopsis thaliana* and *Lotus corniculatus* have been widely used as model plants (Motoichiro Kodama, 2007). In other matters, tomatoes are one of the most popular veggies in the world, however, its cultivation has been hampered by a plethora of illnesses caused by fungus, bacteria, viruses, and nematodes.

Tomato plants are easy to grow in the field or inside greenhouse, it is a fresh fruit plant, genome data its available (Su et al., 2021), plants grow flower, could be cultivated by different techniques, these has turned it a model plant for diverse studies.

The variety of infections demonstrates the importance of the tomato pathosystem as a suitable model for studying plant-pathogen and extract interactions (Motoichiro Kodama, 2007). The diverse range of infections observed within this system not only underscores its relevance but also highlights its versatility as a research tool. By diving into the mechanisms and responses of tomatoes to various pathogens and extracts, researchers can gather substantial insights and knowledge.

The information acquired through the study of model plants, like tomatoes, carries a broader implication. The findings and methodologies developed in this context hold the potential to be transposed and extrapolated to other commercially significant crops, like pear and apple orchards (Abbasi et al., 2021). This transfer of knowledge is immensely valuable as it can support in producing strategies for the management and protection of a wide array of crops.

As such, the tomato pathosystem not only serves as a first insight for understanding plant-pathogen interactions but also offers a gateway to enhancing the resilience and health of agriculture on a larger scale, thereby contributing to food security and sustainable farming practices (Motoichiro Kodama, 2007).

2. Research objectives

The development of the present study was integrated in the project “ORCHESTRA – add-value to ORCHards through thE full valoriSaTion of macRoalgAe“ activities, which the main goal is to find new, green, and sustainable solutions to control fungal diseases associated to the pear and apple cultivars, to work as biostimulants and biofertilizers, adding value to invasive and non-invasive abundant seaweeds from the Portuguese coast.

Thus, the main goal of this dissertation was the extraction of bioactive compounds from seaweeds and the characterization of their antioxidant and phytotoxicity (*in vitro* and *in vivo*) activities , to define the best active extracts may have potential for further studies.

3. Materials and Methods

3.1 Biological material

Four seaweeds were used for this study: Rhodophyta *Asparagopsis armata,* the Chlorophyta *Codium* sp., and the Phaeophyta *Sargassum muticum* and *Fucus vesiculosus.* Three marine seaweeds species were collected from the Portuguese coast, namely, *S. muticum* was collected in Praia do Norte (Peniche) in 2019, *A. armata* in Berlenga Natural Reserve (Peniche) in 2021, *F. vesiculosus* in Figuiera da Foz in 2021 and *Codium* sp. was obtained from an aquaculture, Algaplus (Ílhavo, Portugal).

Collected seaweeds were carefully rinsed with fresh water to remove any encrusting materials, detritus, sand, and other impurities, and then dried in a wind tunnel at 25 ºC to finally be milled to powder and stored in the dark at room temperature until use.

Phytotoxicity assays were conducted with tomato plants of the "Chucha Sir Elyan" variety. These plants were initially sown on February 8th, 2022, on a greenhouse to later be used on March 10th, 2022.

3.2 Extraction procedure

Four different solvents were used, namely, ethyl acetate (E.A.)(VWR Chemicals, Rosny-sous-Bois-cdex, France), *n*-hexane (n-hex) (VWR Chemicals, Rosny-sous-Bois-cdex, France), ethanol (AGA-Álcool e Géneros Alimentares, S.A., Prior Velho), and water Milli-Q, and a hydroethanolic solution (75:25).

Firstly, 100 g of each seaweed were suspended in each solvent at a 1:20 ratio, and the extraction was performed at room temperature, protected from the light, with mechanical agitation (OHS 20 Digital Overhead Stirrer, VELP SCIENTICA, Italy) at 625 rpm, for four hours.

3.2.1 Evaporation

After extraction, the suspension was filtered with a filter paper, 10-12 µm (PRAT DUMAS, France), to a flask with the help of a vacuum pump. The highest solvent volume was evaporated using a rotary evaporator (Heidolph, Merck, Germany) at 40 ºC and 150 rpm The residual volume of extracts was then transferred to a dark flask and dried using Vacufuge Plus Concentrator (Eppendorf, Hamburg, Germany).

The suspension of aqueous extract, due to its viscosity, was centrifugate at 3 220 *g* for 25 minutes at 10 ºC, and the supernatant was then evaporated using a rotary evaporator (Heidolph, Merck, Germany) at 60 ºC and 150 rpm A small aliquot of the extract was dried using a Vacufuge Plus Concentrator (Eppendorf, Hamburg, Germany), meanwhile the rest of the extract was freeze-dried (CoolSafe Freeze Dryer, ScanVac). The dried extracts were then stored in dark flasks at 4 ºC until further use.

3.3 Total phenolic content (TPC)

For the evaluation of the phenolic content of each extract, the Folin-Ciaucalteu method was used. This method is based on a color reaction between easily oxidized polyphenols or hydroxylated aromatic compounds and phosphotungsten-polymolybdic acid. (Zhang et al., 2006). A standard curve using gallic acid at different concentrations (1, 0.3, 0.1, 0.03, and 0.01 mg/mL) was performed.

The determination of TPC was performed using a 96-wells plate. Briefly 2 µL of sample was added with 158 µL of distilled water and 10 µL of Folin-Ciaucalteu reagent in each well. The samples were mixed and 30 µL of sodium carbonate (Na_2CO_3) 20% was added to each well, and the samples incubated for one hour at room temperature, protected from light. The TPC is expressed as mg equivalent of gallic acid per gram of extract (GAE/g extract).

3.4 Antioxidant activity of *Asparagopsis armata, Codium* sp. *Fucus vesiculosus and Sargassum muticum* extracts

Concerning the antioxidant assays, the following reagents were used: 2,2- Diphenyl-1(2,4,6-trinitrophenyl) hydrazyl reagent (Sigma, Steinheim, Germany) was used in DPPH radical scavenging assay; Fluorescein (Sigma-Aldrichh, St Louis, MO, USA), α,α′azodiisobutyramidine dihydrochloride (APPH) (Sigma-Aldrich, St Louis MO, USA), trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and phosphate buffer (Sigma, St Louis, MO, USA) were used in the Oxygen Radical Absorbance Capacity (ORAC) assay; Iron (II) sulphate ($FeSO_4$) (Sigma-Aldricht, St Louis, MO), 2,3,5-Triphenyltetrazolium chloride (TPTZ), Iron (III) chloride ($FeCl_3$) (Sigma-Aldrich, St Louis, MO, USA) and acetate buffer were employed as main reagents (Sigma, Karkanata, India) in Ferric Reducing Antioxidant Power (FRAP) assay.

3.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

DPPH radical scavenging assay was performed according to Brand-Williams and collaborators (1995) with slight modifications. The base of the method consists of this radical has an unpaired electron and is colored blue violet in this state, fading to pale yellow by the reaction at the presence of an antioxidant molecule. This color change can be measured spectrophotometrically at 517 nm to determine the capacity of antioxidant molecules (Llica, Q F Eva Ramos; Castañeda, Benjamín; Vásquez, 2008).

DPPH solution was prepared in ethanol at a concentration of 0.1 mM. Then 2µL of each extract (100 mg/mL) was added to 198 µL of DPPH solution in a 96-well plate. The samples were incubated at room temperature in the dark for 30 minutes. For blank samples was used ethanol instead DPPH solution. Control samples were prepared with 2 µL DMSO. For aqueous extracts, DPPH solution was prepared in a methanol/water solution (1:10), and this solution was also used for the blank samples.

Absorbance was measured at 517 nm (Synergy H1 Multi-Mode Microplate Reader Biotek® Instruments, Winooski, VT, USA). The antioxidant capability to scavenge DPPH were estimated in % of reduced DPPH from the control, following the equation:

$$DPPH\ radical\ scavenging\ capability\ (\%c\ control) = \left(\frac{Abs. sample - Abs.\ sample\ blank}{Abs. control - Abs\ control\ blanck}\right) * 100$$

(eq. 1)

3.4.2 Ferric reducing antioxidant power (FRAP)

For the evaluation of the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), which produce an intense blue color in the presence of antioxidant molecules. This method was performed as previously described by Benzie and Strain (1996) with minor modifications (Pinteus et al., 2017b).

The determination was performed using a 96-wells plate. A calibration curve was prepared with $FeSO_4$ µM at different concentrations (10, 8, 6, 4, 2, 1, 0.4 and 0.0 µM). FRAP reagent was prepared adding ferric tripyridyl triazine (Fe III TPTZ) prepared in 40 mM HCl, in 0.3 mM acetate buffer (pH=3.6) and 20 mM iron III chloride ($FeCl_3$). The FRAP reagent was heated at 37 ºC. Acetate buffer was used in blank samples. 96-wells plate was prepared using 2 µL sample and 198 µL FRAP reagent and incubate for 30 minutes protected from the light. After the incubation time, absorbance was measured in the microplate reader at 593 nm (Synergy H1 Multi-Mode Microplate Reader, Biotek® Instruments, Winooski, VT, USA).

Results were presented as µM equivalents of $FeSO_4$ per mg of extract (µM $FeSO_4$ EQ/ mg extract).

3.4.3 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according with Ou and collaborators (2002) adapted to microscale. Using fluorescein as the fluorescent probe and quantified by assessing the area under the fluorescent decay curve (AUC) of the sample as compared with the blank in which no antioxidant activity was present. A calibration curve was prepared, using Trolox (0-80 µM) as antioxidant, the assay was performed at 37 ºC in 75 mM phosphate buffer(pH=7.4) (Davalos et al., 2004).

Each well contained 20 µL of the different calibrate solutions and samples, following this, 120 µL fluorescein was added, meanwhile phosphate buffer to prepare the blanks. The first read was performed every minute for 15 minutes, and the temperature of the microplate reader was stablished at 37 ºC. Ending the first read, 60 µL of AAPH solution (20 µM) was added in the wells corresponding to calibrate curve concentrations and samples. Fluorescent was measured at $\lambda_{excitation}$: 458 nm and $\lambda_{emission}$: 520 nm wavelengths and recorded every minute for 240 min. The result was calculated using the difference of areas under the fluorescent decay curves, Area Under the Curve, and expressed as µmol Eq Trolox per mg of extract (µmol Eq Trolox/g extract) and extrapolated from AUC of a Trolox calibration curve by the means of the following equation.

$$AUC = \left(\frac{R1}{R1}\right) + \left(\frac{R2}{R1}\right) + \left(\frac{R3}{R1}\right) + \cdots + \left(\frac{R_n}{R1}\right)$$

(eq. 3)

Where R1 is the fluorescence measured at the beginning of the reaction and R_n is the final measurement. The Area Under the Curve were calculated using GraphPad Prism 8.0.1, by the following equations:

$$NET\ AUC = AUC_{sample-} - AUC_{blank}$$

(eq. 4)

$$ORAC = \left[\left(\frac{NET\ AUC.compounds}{m}\right)\right]$$

(eq. 5)

3.5 Phytotoxicity assays

3.5.1 Leaf puncture assay

For the leaf puncture assay, the produced extracts from seaweeds were considered. Extracts were dissolved in DMSO at 100 mg/mL (stock solution) and diluted in Phosphate Buffer Saline (PBS) at three different concentrations 1, 0.5 and 0.1 mg/mL. Young tomato plant (*Solanum lycopersicum,* Solanaceae*)* leaves with similar size were detached from the plants and used. The leaves were placed in Petri dishes and punctured with a sterile needle in three different zones of their adaxial side (Figure 6). Droplets of 20 µL of each extract at different concentrations were placed on the punctured wounds (three droplets per leaf). Each wound of every leave containing a different extract concentration.

To evaluate the potential influence of DMSO and PBS (vehicles used), control leaves were treated with 3 concentrations of DMSO (1%, 0.5% and 0.1%) and PBS. The leaves were kept in a temperature-controlled room at 24 ºC, with a photoperiod of 16 h light/8 h dark for 12 days. All the leaves were regularly checked for hydration The diameter of the lesions was measured and expressed in mm.

Figure 6 Representation of the wound and application location in every tomato leave.

3.5.2 *In vivo* phytotoxicity assay

For the *in vivo* phytotoxicity evaluation, young tomato plants (2 months) were used. The experiment was carried out between August 11th and September 22nd, 2022. Each plant was transferred to pots containing 4 L of a mixture of peat and perlite (3:1 ratio peat to perlite) and they were randomly assigned in the greenhouse. A data logger (Datalogger EBI 2O-TH1, EBRO, Germany) was kept inside the greenhouse during the period of experiment to register the temperature and humidity along time. Seven plants (replicates) were used for each of the 20 conditions tested, described in the table below (Table 3.1).

Table 3.1. Test conditions and concentration assessed in tomato plants, applied by foliar pulverization weekly.

Seaweed	Test condition	Concentration (mg/mL)
Asparagopsis armata	Aqueous	0.1
		0.5
	Hydroethanolic	0.1
		0.5
Codium sp.	Aqueous	0.1
		0.5
	Hydroethanolic	0.1
		0.5
Fucus vesiculosus	Aqueous	0.1
		0.5
	Hydroethanolic	0.1
		0.5
Sargassum muticum	Aqueous	0.1
		0.5
	Hydroethanolic	0.1
		0.5
Controls	Non-treatment (NTC)	-
	Water (WC)	-
	Agri Xpert NPK 4-12-6 (CFC)	4 µL/mL
	Vacciplant®(VC)	1 µL/mL

All the treatments were applied simultaneously every week. A foliar application of the extracts (25 mL) by pulverization and controls was performed and the plants were daily checked for necessity of hydration. Multiple growth parameters, specifically the counts of both flowers and leaves, as well as the measurement of plant height, were recorded on a weekly basis over a 42-day period. This time span was designated as "Days After Treatment" (DAT), with the initial application conducted on young tomato plants. Plant height was ascertained by measuring the distance from the plant's base to the apex of its tallest leaf.

3.6 Data treatment and statistical analysis

At least three independent experiments were carried out in triplicate for antioxidants and leaf puncture assay. For greenhouse phytotoxicity assay the initial sample size was seven replicates.

One factor analysis of variance (ANOVA) was performed to evaluate the effect of extracts (ethanol, 75% ethanol/25% water, ethyl, acetate, *n*-hexane, water) in the TPC, FRAP, DPPH (%), ORAC and phytotoxicity assays. Preliminarily, all assumptions inherent to the execution of the analysis (namely, normality of data and homogeneity of variances) were validated. Whenever applicable, Tukey's multiple comparison test was performed. When the assumptions for carrying out the ANOVA failed, the non-parametric Kruskal-Wallis's test was used, followed by the Games-Howell test for multiple comparisons. For comparisons with the control group, Dunnett's test was used. All results were considered significant at the 5% significance level (*p-value* < 0.05). Whenever applicable, the results are presented as mean ± standard-error of the mean (SEM). All statistical analyses were performed using IBM SPSS Statistics 28. Graphic representations were performed using the software GraphPad 8

4. Results

4.1 Extraction of seaweed compounds

A solid-liquid extraction was performed using five different solvents or mixture of solvents (water, hydroethanolic solution (75% ethanol/25% water), ethanol, ethyl acetate and *n*-hexane) from 4 different seaweed species: *Asparagopsis armata, Codium sp, Fucus vesiculosus and Sargassum muticum*. The yield of each extraction (%) is presented in Table 4.1.

Table 4.1. Yield (%) of extraction of each seaweed using different solvents or mixture of solvents.

	Yield (%)				
Solvent / ***Seaweed***	Ethanol	75% Ethanol/25% Water	Ethyl acetate	*n*-Hexane	Water
Asparagopsis armata	1.92	4.57	0.42	0.20	7.35
***Codium* sp.**	2.69	32.59	7.47	1.01	21.52
Fucus vesiculosus	8.60	20.64	4.62	4.14	28.69
Sargassum muticum	1.43	6.16	1.25	0.48	22.73

.

It is possible to note that the aqueous extracts exhibited the highest yields among the different types of solvents for all the seaweeds used. On the other hand, *Codium sp.* was the one that displayed the higher yields for hydroethanolic and ethyl acetate extractions, presenting 32.59% and 7.47%, respectively. The organic solvents, ethyl acetate and *n*-hexane, showed the lower yield percentages in all cases, being the lowest yield attained with the *n*-hexane extraction, 0.2% for *A. armata* and the higher 4.14% for *F. vesiculosus* also *n*-hexane.

4.2 Total Phenolic Content (TPC)

The TPC of the extracts was estimated by the Folin-Ciocalteu method and the results are presented in Figure 7.

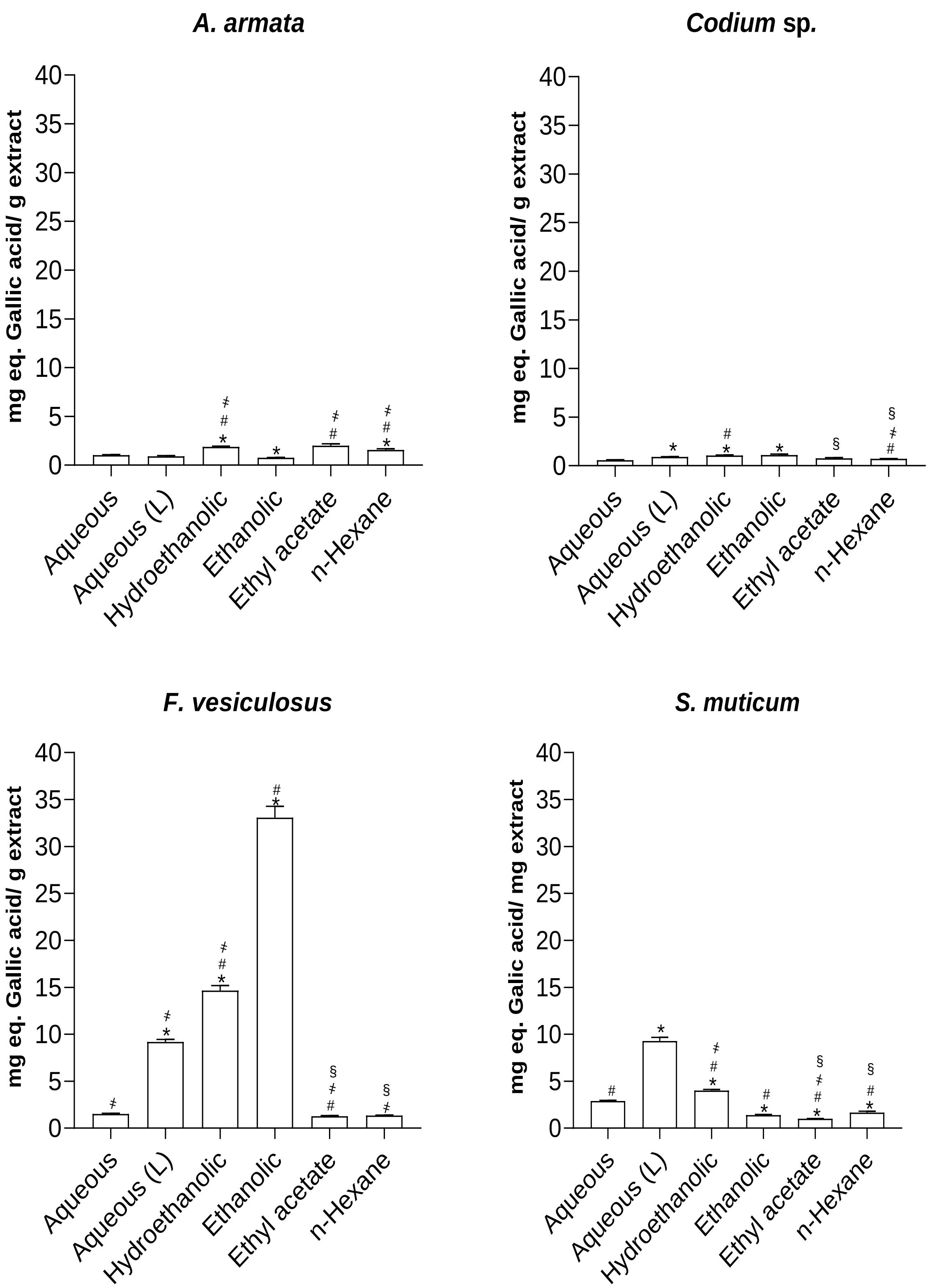


Figure 7 Total phenolic content of *Asparagopsis armata, Codium* sp*, Fucus vesiculosus and Sargassum muticum* extracts. Results are presented as mean ± SEM of at least three independent experiments carried out in triplicate. Symbol (*) represent significant differences (p-value<0.05) when compared to aqueous extract, symbol (#) represent significant differences when compared to aqueous lyophilized extract, symbol (ǂ) represents significant differences (p-value<0.05) when compared to ethanolic extract, symbol (§) represents significant differences (p-value<0.05) when compared to hydroethanolic extract (Kruskal Wallis, Games-Howell test, p-value<0.05)

As presented in Figure 7, the *F. vesiculosus* hydro ethanolic and ethanolic extracts exhibited the highest concentration of phenolic content, 14.64 ± 0.53 GAE/mg, and 33.07 ± 1.20 GAE/mg, respectively. On the other hand, all the other extracts did not show any remarkable result .

4.3 Antioxidant activity of *Asparagopsis armata, Codium* sp. *Fucus vesiculosus and Sargassum muticum* extracts

Antioxidant activity of all extracts obtained from the different seaweeds was evaluated through the following complementary methods: DPPH radical scavenging activity (DPPH), Oxygen Radical Absorbance Capacity (ORAC), and Ferric Reducing Antioxidant Power (FRAP). The results are presented in Topic 4.3.1 to 4.3.3.

4.3.1 DPPH radical scavenging activity

The ability of extracts to neutralize DPPH radicals was evaluated and the results presented as percentage (%) of available DPPH (Figure 8).

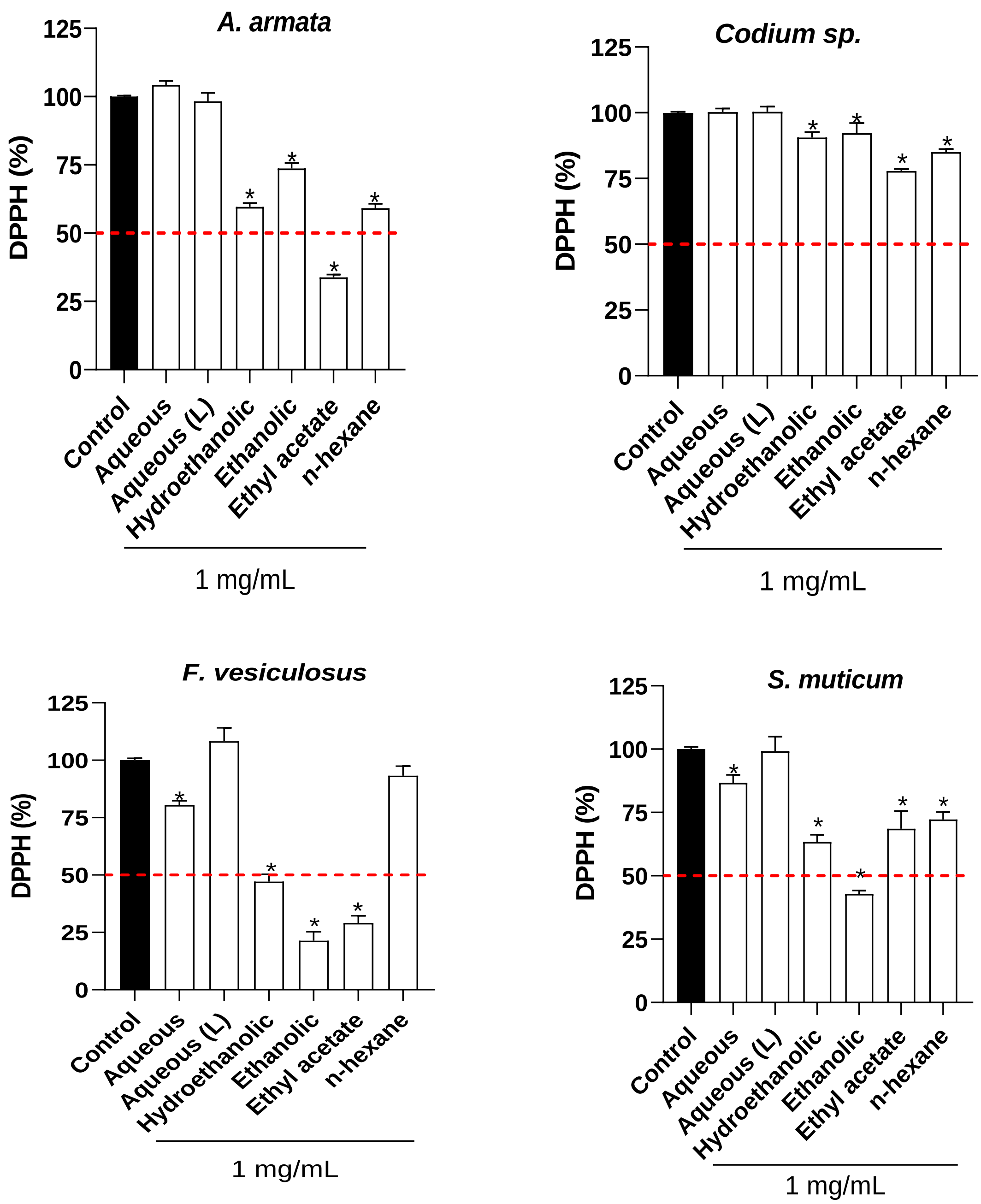


Figure 8 DPPH radical scavenging activity of extracts from *Asparagopsis armata*, *Codium* sp., *Fucus vesiculosus, Sargassum muticum*. Values are expressed as percentage of available DPPH. The measurements (λ = 536 nm) were made after 30 min incubation. Values are presented as mean ± SEM of at least three independent experiments carried out in triplicate. The vertical bars represent the standard error of the mean. Symbol (*) represent significant differences (ANOVA, Dunnett´s test, p-value<0.05) when compared to BHT.

As seen in Figure 8, it is possible to observe that the extracts prepared from *F. vesiculosus* presented the highest reduction of available DPPH (%). Hydroethanolic, ethanolic and ethyl acetate presented the higher capacity to reduce DPPH radical, as well as *S. muticum* ethanolic extract and *A. armata* ethyl acetate extract, which reduced more that 50% of DPPH radical. On the other hand, the remain extracts showed a weak capacity to reduce DPPH radicals. For extracts that reduced more than 50%, the EC_{50} was determined, and compared with butilhidroxitolueno (BHT) standard, results are presented in Table 4.2.

Table 4.2. EC_{50} of 2,2-diphenyl-1-picrylhydrazyl (DDPH) radical scavenging activity of the extracts presenting more than 50% of DPPH reduction, expressed as mg/mL. Symbol (*) represent significant differences (ANOVA, Dunnett´s test, p-value<0.05) when compared with BHT.

Algae	Extract	EC_{50} (mg/mL)
Asparagopsis armata	Ethyl acetate*	0.69 (0.61-0.78)
	Hydroethanolic*	0.48 (0.35-0.70)
Fucus vesiculosus	Ethanolic*	0.49 (0.43-0.57)
	Ethyl acetate*	0.56 (0.48-0.65)
Sargassum muticum	Ethanolic*	0.69 (0.61-0.78)
BHT	-	0.18 (0.16-0.21)

Five different extracts from three seaweeds were assessed for determination of their EC_{50} values (Table 4.2). *Fucus vesiculosus* (FV) hydroethanolic extract showed the lowest value of EC_{50} (0.48 (0.35-0.70) mg/mL) followed by FV ethanolic extract « (0.49 (0.43-0.57) mg/mL)». In addition, the extracts showed significant difference (ANOVA, Dunnett test *p-value<0.05)* when compared with BHT.

4.3.2 Ferric Reducing Antioxidant Power (FRAP)

The capacity of extracts to reduce ferric irons by donation was then carried out by this method. Results are expressed in µM of $FeSO_4$ equivalents per mg of extract (µM $FeSO_4$ EQ/ mg extract). The ability of seaweed extracts to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) was evaluated and the results shown in Figure 9.

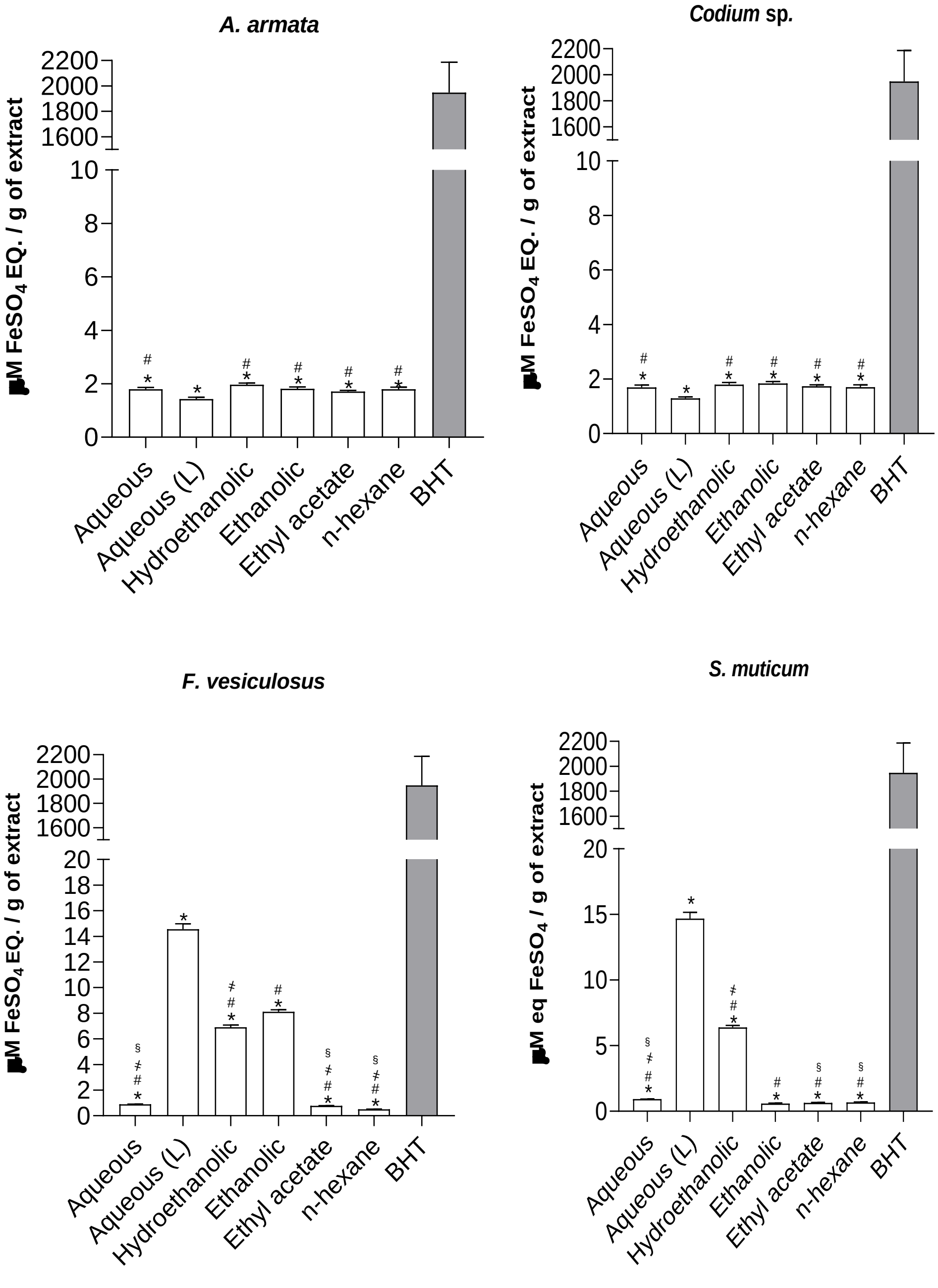


Figure 9 Ferric reducing antioxidant power (FRAP) of *Asparagopsis armata, Codium* sp., *Fucus vesiculosus and Sargassum muticum* extracts. Values are presented as mean ± SEM of at least three independent experiments carried out in triplicate. Symbol (*) represent significant differences (ANOVA, Dunnett's test, p-value<0.05) when compared with BHT standard, symbol (#) represent significant differences when compared to aqueous lyophilized extract, symbol (≠) represents significant differences when compared to ethanolic extract, symbol (§) represents significant differences when compared to hydroethanolic extract (Kruskal Wallis, Games-Howell test, p-value<0.05).

Results presented in Figure 9 showed that the higher capability to reduce ferric iron are mediated by *F. vesiculosus* hydroethanolic and ethanolic extracts and *S. muticum* hydroethanolic extract.

Nonetheless, all extracts displayed significant differences (ANOVA, Dunnett´s test *p-value*<0.05) when compared with BHT standard.

Regarding *F. vesiculosus* extracts, aqueous lyophilized extract displayed the highest value (14.56 ± 0.42) presenting significant differences with the rest of extracts (Kruskal-Wallis, Games-Howell test, p-*value*<0.05) , followed by the ethanolic extract (8.11 ± 0.16) and hydroethanolic extract (6.91 ± 0.16).

Asparagopsis armata aqueous lyophilized extract displayed the highest value for this algae (14.69 ± 0.48) followed by hydroethanolic extract (6.39 ± 0.15), presenting significant differences with the other extracts prepared from this algae(Kruskal-Wallis, Games-Howell test, p-*value*<0.05).

In the other hand, *A. armata* and *Codium* sp. extracts, aqueous lyophilized extract presented significant smaller values (Kruskal-Wallis, Games-Howell test, p-*value*<0.05) compared with other extracts from the respective algae.

4.3.3 Oxygen radical absorbance capacity (ORAC)

Extract's ability to reduce peroxyl radicals was evaluated by means of the ORAC method. Results are presented as µmol of Trolox equivalents per mg of extract (µmol Trolox EQ/ mg extract) and depicted in Figure 10.

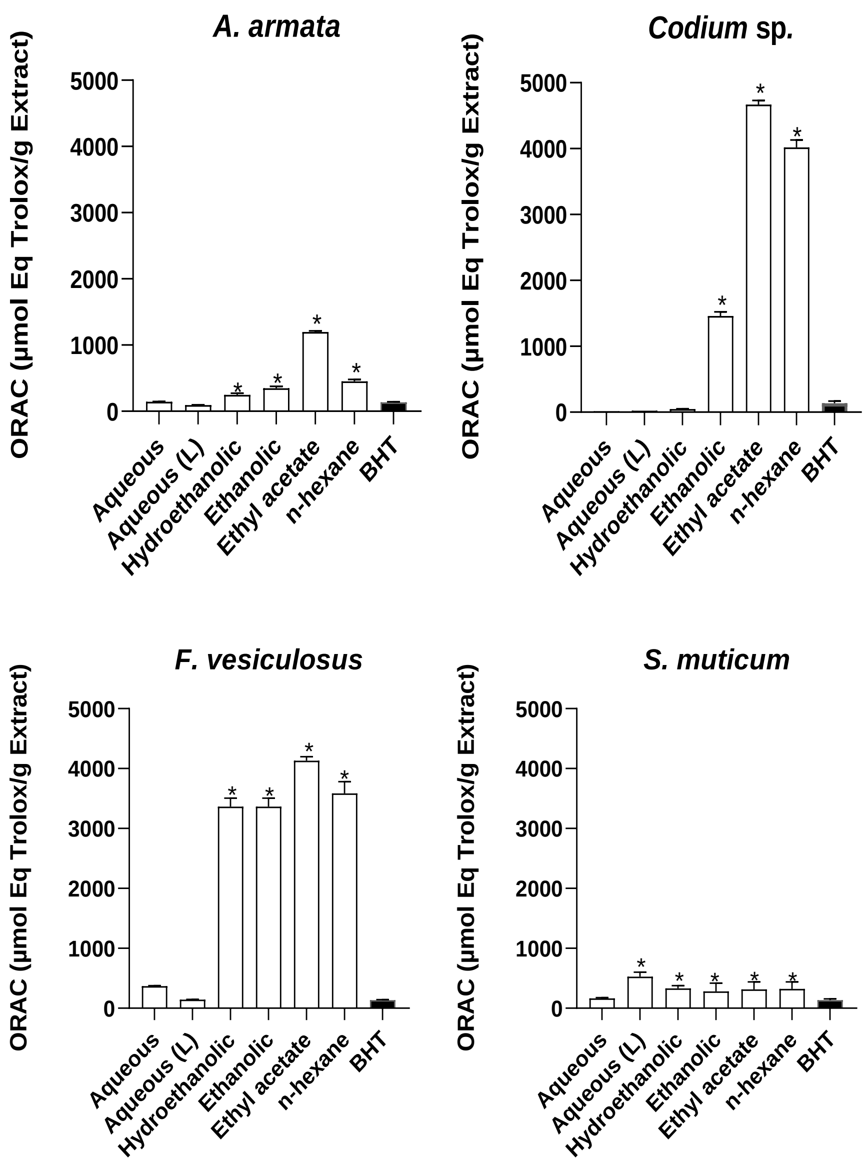


Figure 10 Oxygen Radical Absorbance Capacity of *Asparagopsis armata, Codium* sp*., Fucus vesiculosus, and Sargassum muticum* extracts. Values are presented as mean ± SEM of at least three independent experiments carried out in triplicate. Symbol (*) represent significant differences (ANOVA, Dunnett's test, p-value<0.05) when compared with BHT standard.

Regarding our extracts, *Codium* sp. ethyl acetate extract (4668 ± 61.81 µmol Trolox EQ/ mg extract) and *F. vesiculosus* ethyl acetate extract (4131 ± 65.68 µmol Trolox EQ/ mg extract) displayed the highest capacity to reduce peroxyl radicals following *Codium* sp. (4021 ± 108.6 µmol Trolox EQ/ mg extract) and *F. vesiculosus n*-hexane (3585 ± 195.5 µmol Trolox EQ/ mg extract) (ANOVA, Dunnett's test, p-value<0.05). It is possible to observe that for the ORAC assay method, the seaweeds with the most promising results were *F. vesiculosus* and *Codium sp.*, specifically the ethanolic, ethyl acetate and *n*-hexane extracts, when compared with BHT.

From *A. armata and S. muticum* extracts also presented capacity to reduce peroxyl radicals exhibiting significant differences compared with BHT standard (ANOVA, Dunnett's test, p-value<0.05), even so, values presented by these algae extracts are much lower than values reached with *F. vesiculosus and Codium* sp.

Regarding *A. armata* extracts, ethyl acetate extract (1 200 ± 13.91 µmol Trolox EQ/ mg extract) displayed the highest capacity to reduce peroxyl radicals, following *n*-hexane (450.10 ± 30.67 µmol Trolox EQ/ mg extract). Extracts from *S. muticum* displayed the lowest values, aqueous lyophilized extract (528.20 ± 34.13 µmol Trolox EQ/ mg extract) displayed the higher value for this algae, which as a matter of fact is the only aqueous extract that presented significant differences (ANOVA, Dunnett's test, p-value<0.05) when compared with BHT.

Regarding the solvent used, it is possible to note that aqueous un-lyophilized and lyophilized (except *S. muticum* extract) did not present significant differences (ANOVA, Dunnett's test, p-value<0.05) when compared to BHT.

4.4 Phytotoxicity assays

4.4.1 *In vitro* phytotoxic assay

Tomato plant is frequently used as a model plant because it is simple to cultivate, has a fast growth rate and it is a fruit plant. Two different approaches were assessed to evaluate the phytotoxicity of seaweed extracts, the first assay was on detached tomato leaves through the leaf puncture assay and the second one using tomato plants to deeper understand the effect of the aqueous and hydroethanolic extracts, since both type of extracts showed higher yields and thus, higher potential to scale-up.

The phytotoxicity data obtained in the leaf puncture assay of the extracts are presented in Table 4.3. When leaves treated with *A. armata n*-hexane extract, results showed the highest necrotic area at all concentrations (4.50, 5.00- and 6.25-mm necrotic area from the lower to the highest concentration respectively), while hydroethanolic extract from the same algae presented the smallest necrotic area (1.67, 1.33- and 2.00-mm necrotic area from the lower to the highest concentration respectively). Nonetheless, none of the extracts, at all concentration tested, resented significant differences (*p-value*>0.05) when compared with the respective DMSO control, as well as DMSO did not show significant difference when compared with PBS (*p-value*>0.05).

Table 4.3. Phytotoxicity evaluation of seaweed extracts at three different concentrations (0.1, 0.5 and 1.0 mg/mL) by leaf puncture assay on tomato leaves. The necrotic area was measured after twelve days of treatment. Results are presented as area lesion (mm) ± SEM, n=3. No significant differences were found when compared between treatments (ANOVA, p-value>0.05).

		Lesion size (mm ± SEM)			
Extract	**Concentration (mg/mL)**	***F. vesiculosus***	***S. muticum***	***Codium* sp.**	***A. armata***
Aqueous	**0.1**	3.67 ± 0.47	3.00 ± 2.16	2.83 ± 0.24	2.67 ± 0.47
	0.5	4.00 ± 0.00	4.00 ± 2.94	3.67 ± 0.47	4.33 ± 0.47
	1.0	5.83 ± 0.24	5.33 ± 0.47	3.67 ± 0.47	4.33 ± 0.47
Hydro ethanolic	**0.1**	4.67 ± 0.94	2.67 ± 2.05	4.17 ± 1.03	1.67 ± 2.36
	0.5	4.67 ± 0.94	2.67 ± 0.94	3.67 ± 0.47	1.33 ± 1.89
	1.0	5.67 ± 1.31	3.67 ± 1.25	4.33 ± 0.47	2.00 ± 2.83
Ethanolic	**0.1**	3.50 ± 1.50	3.00 ± 2.16	3.67 ± 0.94	2.67 ± 0.47
	0.5	4.00 ± 1.00	4.00 ± 2.94	4.33 ± 0.47	3.67 ± 0.47
	1.0	4.50 ± 1.50	4.67 ± 0.94	4.00 ± 0.82	4.33 ± 0.47
Ethyl acetate	**0.1**	3.00 ± 1.41	2.33 ± 1.25	2.67 ± 0.47	4.00 ± 1.63
	0.5	1.67 ± 1.18	2.50 ± 1.22	4.00 ± 0.82	4.00 ± 0.82
	1.0	4.00 ± 0.82	4.00 ± 0.82	5.00 ± 0.82	4.33 ± 0.47
***n*-Hexane**	**0.1**	2.83 ± 2.46	4.67 ± 1.25	3.33 ± 0.94	4.50 ± 0.50
	0.5	4.67 ± 0.47	4.00 ± 0.82	3.67 ± 1.25	5.00 ± 1.00
	1.0	4.33 ± 0.62	4.33 ± 0.47	5.33 ± 0.47	6.25 ± 0.75
Control PBS	**-**	1.33 ± 1.89			
Control DMSO	**0.1**	1.67 ± 1.25			
	0.5	3.00 ± 2.45			
	1.0	2.33 ± 1.70			

Each leaf was treated with three different concentrations at the same time, as well as the control, as shown in Figure 11. Photographic evidence was taken and is presented (Table 4.4) as 1 day after the application and at the final day, 12 days after application.

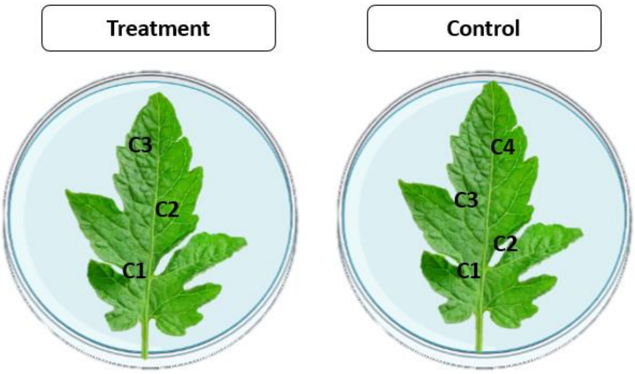


Figure 11 Illustration of the application zone on the leaves, for treatment leaves C1 stands for 0.1 mg/mL, C2 stands for 0.5 mg/mL and C3 stands for 1 mg/mL final extract concentration, while for control leaves C1 represent PBS, C2 represents 0.1% DMSO, C3 stands for 5% DMSO and C4 stands for 1% DMSO. All treatment was carried out in triplicate.

Table 4.4. Illustrative results obtained by leaf puncture assay of the seaweed extracts assessed at three different concentrations on detached tomato leaves. Photos were taken at day 1and 12 after treatment.

Extract	Time	*A. armata*	*Codium* sp.	*F. vesiculosus*	*S. muticum*
Aqueous	T1				
	T12				
Hydroethanolic	T1				

	T12				
Ethanolic	**T1**				
	T12				

Ethyl acetate	**T1**				
	T12				
***n*-Hexane**	**T1**				

T12				
Control	**R1**	**R2**	**R3**	
T1				
T12				

4.4.2 *In vivo* phytotoxic assay

Regarding the *in vivo* experiments using tomato seedlings, the hydroethanolic and aqueous extracts from the seaweeds were tested along 42 days to evaluate their phytotoxicity and ability to promote the growth and improve the health of plants. The aqueous and hydroethanolic extracts were chosen for the *in vivo* testing, primarily due to their higher yields results compared with the other solvents used, being this essential due to the higher volume of extract needed to conduct the assay, as well as being favorable to industrial scaled up. Furthermore, these extracts were chosen due to being green and environmentally friendly.

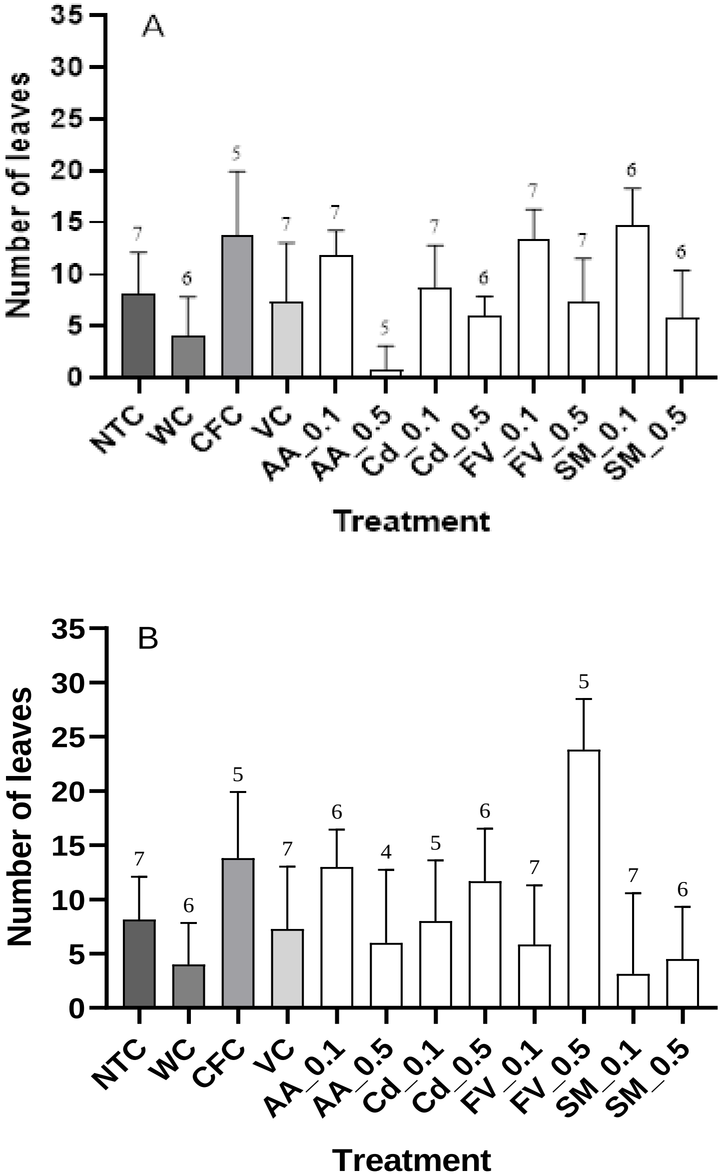


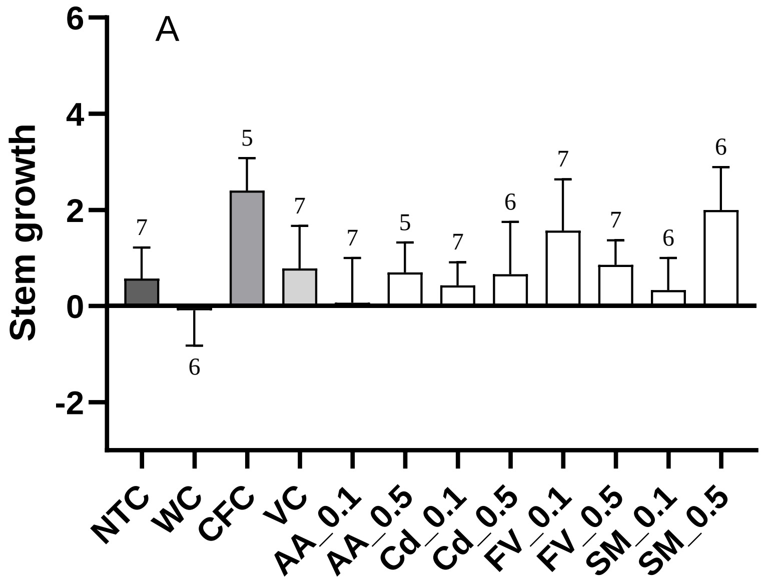
Figure 12 Effect of seaweed extracts on the development of new leaves on plant tomatoes at 42 days after treatment (DAT). Difference between number of leaves of the initial day and the last day of experiment was calculated. White bars represent seaweed treatment, *Asparagopsis armata (*AA), *Codium* sp. *(*Cd), *Fucus vesiculosus (*FV) and

Sargassum muticum (SM) at 0.1 mg/mL and 0.5 mg/mL. Grays bars represent different controls performed, namely non-treatment control (NTC), water control (WC), commercial fertilizer control (CFC), and Vacciplant® control (VC). (A) Aqueous extract; (B) Hydroethanolic extract. Numbers above bars represent the sample size, meanwhile missing samples are plants replicates which died during the experiment. Values are presented as mean ± SEM. No significant differences were found between treatments (Kruskal-Wallis, *p-value>0.05).*

All the test conditions and controls were applied weekly by pulverization. The difference on the number of leaves per plant, the growth and the number of flowers was evaluated along the experiment, and it is described on Figures 12 and 13 and Tables 4.5 and 4.6.

The number of new leaves varied over the six weeks of treatment, suffering some losses and the development of new ones. The control using commercial fertilizer presented the highest mean out of the different controls assessed, 13.80 ± 6.11 of new leaves compared with the day 0, followed by non-treatment control and Vacciplant® control with 8.14 ± 3.95 and 7.29 ± 5.74 of new leaves, respectively, and finally WC with 4.00 ± 3.84 of new leaves.

Regarding the aqueous extracts, it is possible to note that at higher concentration, the number of new leaves decreased, being the highest decrease observed in the presence of *A. armata* aqueous extract that at 0.1 mg/mL presented 11.86 ± 2.34 of new leaves. This value diminished to 0.80 ± 2.25 when the plants were treated with the concentration of 0.5 mg/mL. However, looking to the hydroethanolic extracts effect, the increase of concentration showed an inverted tendency, except for *A. armata* in which lower concentrations showed an elevated number of new leaves when compared with the highest concentration. Nevertheless, no significant differences were found when compared the different treatments assessed (Kruskal-Wallis, *p-value*>0.05).



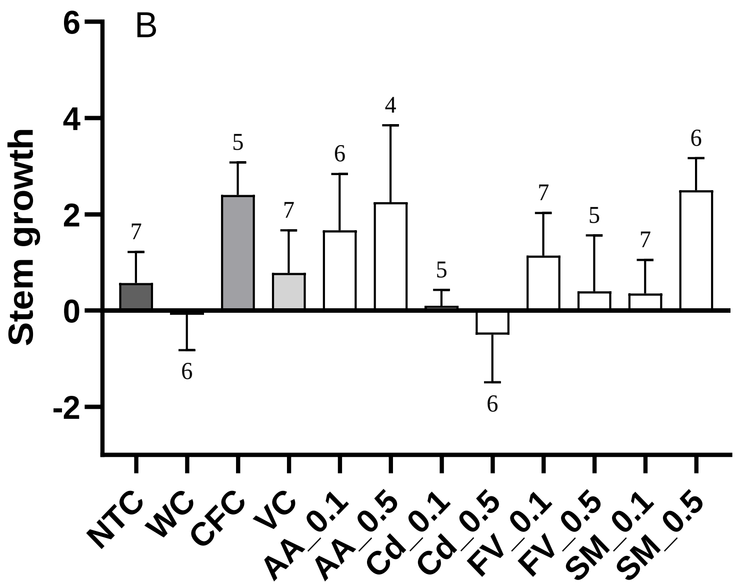


Figure 13 Effect of seaweed extracts on stem growth of plant tomatoes at 42 days after treatment (DAT). White bars represent seaweed treatment, *Asparagopsis armata (*AA), *Codium* sp. *(*Cd), *Fucus vesiculosus (*FV) and *Sargassum muticum* (SM) at 0.1 mg/mL and 0.5 mg/mL. Grays bars represent different controls assessed, non-treatment control (NTC), water control (WC), commercial fertilizer control (CFC), and Vacciplant® control (VC). (A) Aqueous extract (B); Hydroethanolic extract. Numbers above bars represent the sample size, meanwhile missing samples are plants replicate which died during the experiment. Values are presented as mean ± SEM. No statistically significant differences were found between treatments (Kruskal-Wallis, *p-value>0.05).*

Regarding stem growth, CFC showed one of the highest values with a total growth of 2.40 ± 0.68 cm, considering five plants, since two of them died during the experiment. *Codium* sp. hydroethanolic extract (0.5 mg/mL) presented the lowest value in all treatments followed by CFC. Regarding *Codium* sp. and *F. vesiculosus* there was a slight difference between the different extracts of the same seaweed, hydroethanolic induced a lower stem growth when compared with aqueous extract , tendency that was observed for both concentrations. Nevertheless, no significant differences were found when compared the different treatments assessed (Kruskal-Wallis, *p-value*>0.05).

Regarding the growth of flower cluster (FC), was measured at 21, 28, 35 and 42 DAT. Results are presented for aqueous (Table 4.5) and hydroethanolic (Table 4.6).

In the case of control plants, at least one FC grown at 21 DAT, with exception of the plant treated with water pulverization. The treatment with Vacciplant® control exhibited the highest number of FC until the end of the experiment (5 FC), followed by the fertilizer which presented the same number as the water control with a total of 3 FC. Finally, plants that were not treated displayed 2 FC.

Table 4.5. Effect of aqueous extracts tested at two different concentrations (0.1 and 0.5 mg/mL), weekly applied by pulverization, over the total number of flower clusters in tomato plants grown over the experimental time. Starting at 21 days after treatment (DAT) until 42 DAT. (NTC: non-treatment control, WC: water control, CFC: commercial fertilizer control, VC: Vacciplant® control).

Treatment	Concentration (mg/mL)	21 DAT	28 DAT	35 DAT	42 DAT
NTC	-	1	5	1	2
WC	-	0	1	1	3
CFC	4 µL/mL	1	2	1	3
VC	1 µL/mL	1	2	1	5
A. armata	0.1	3	5	5	5
	0.5	1	1	0	0
***Codium* sp.**	0.1	0	0	0	0
	0.5	2	4	1	1
S. muticum	0.1	3	6	4	3
	0.5	2	3	2	2
F. vesiculosus	0.1	3	3	4	3
	0.5	3	3	2	2

Table 4.5 displays the results obtained with the application of aqueous extract. All the treatment exhibited FC at 21 DAT, with exception of *Codium* sp. at the smaller concentration. The number of FC in plants treated with seaweed extract are higher than control plants.

The *A. armata* extract presented clear differences between the two concentrations tested, where the smaller concentration (0.1 mg/mL) began the flowering stage with a total of 3 FC, value that increased until 28 DAT to 5 FC and maintained this value until the end of the treatment. Meanwhile, the highest concentration (0.5 mg/mL) at 21 DAT grew 1 FC and kept it until 28 DAT from this point on, value decreased to 0 FC and maintained until the end of the treatment.

In the case of *Codium* sp. at 0.1 mg/mL, the plants did not exhibit any FC during all the experimental time. On the other hand, when tested at 0,5 mg/mL 2 FC were observed at 21 DAT and double fold this number until the 28 DAT. However, after this time at the end day, the plants loss the most of FC, finishing with one FC.

Both *S. muticum and F. vesiculosus* conditions were marked with a high number of FC at the beginning of the stage (3 FC), in the case of *S. muticum* both concentrations followed an increase until the 28 DAT. After this day the number of FC decreased until the end of the experiment.

The *F. vesiculosus* extract maintained 3 FC until 28 DAT for both concentrations, then the smaller concentration grew 1 more FC until 35 DAT and finished with 3 FC at the end of treatment; the higher concentration decreased to 2 FC until the 35 DAT and ended the treatment with this value.

The effect of hydroethanolic extracts is presented in Table 4.6, the results slightly differ from aqueous extracts. Not all the treatment exhibited FC at 21 DAT, *S. muticum* did not present any FC, *F. vesiculosus* and did not grow FC and the lowest concentration (0.1 mg/mL). Meanwhile *Codium* sp. did not grow FC at higher concentration (0.5 mg/mL).

Table 4.6. Effect of hydroethanolic extracts tested at two different concentrations (0.1 and 0.5 mg/mL), weekly applied by pulverization, over the total number of flower clusters in tomato plants grown over the experimental time. Starting at 21 days after treatment (DAT) until 42 DAT. (NTC: non-treatment control, WC: water control, CFC: commercial fertilizer control, VC: Vacciplant® control).

Treatment	Concentration (mg/mL)	21 DAT	28 DAT	35 DAT	42 DAT
NTC	-	1	5	1	2
WC	-	0	1	1	3
CFC	4 µL/mL	1	2	1	3
VC	1 µL/mL	1	2	1	5
A. armata	0.1	3	3	2	2
	0.5	2	5	3	2
Codium sp.	0.1	1	2	0	0
	0.5	0	1	1	2
S. muticum	0.1	0	0	0	0
	0.5	0	0	0	1
F. vesiculosus	0.1	0	0	1	2
	0.5	1	5	3	3

Asparagopsis armata firstly showed better results (3 FC) at lower concentration (0.1 mg/mL) compared with its higher concentration (2 FC). At a lower concentration the value kept the same until 28 DAT, to eventually decrease to 2 FC until the end of treatment. While at higher concentration (0.5 mg/mL) displayed a great increase of FC until 28 DAT (5 FC), then this value decrease to 3 FC at 35 DAT and finished the treatment with 2 FC.

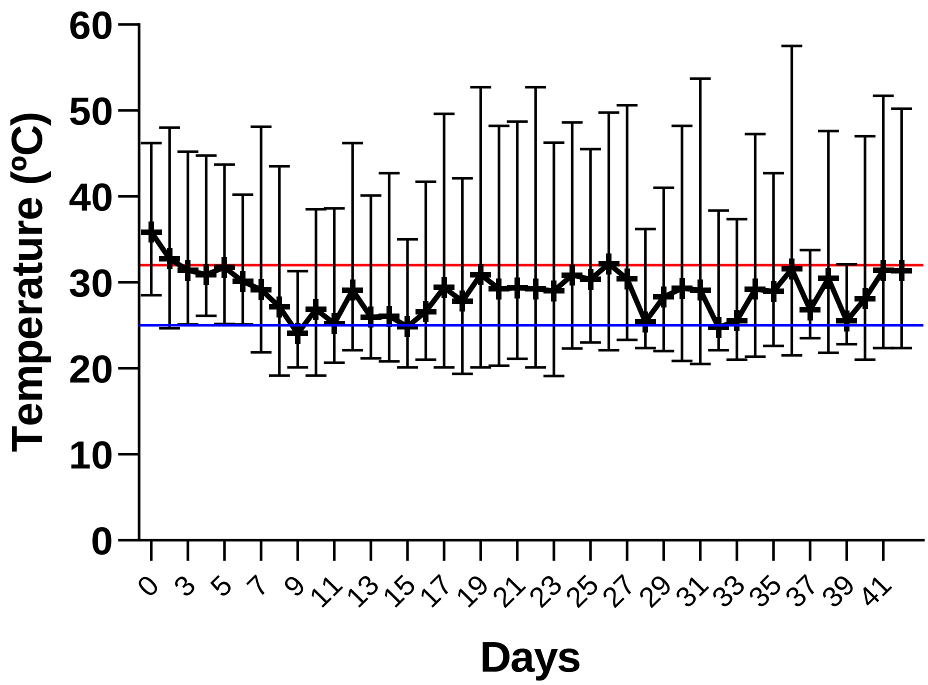
In the case of *Codium* sp. at lower concentration (0.1 mg/mL) displayed 1 FC at 21 DAT, increased to 2 FC for 28 DAT, then suffered a decreased losing all FC until the end of treatment. Meanwhile at higher concentration the first FC appeared at 28 DAT and double fold this number until 42 DAT.

Sargassum muticum extracts showed the lowest values regarding FC, at lower concentration (0.1 mg/mL) plants were not able to grow any FC during the entire time of treatment, meanwhile at higher concentration (0.5 mg/mL) the first and only FC grew at 42 DAT.

Fucus vesiculosus at lower concentration (0.1 mg/mL) displayed 1 FC at 35 DAT, and double fold this number for 42 DAT. When assessed at higher concentration (0.5

mg/mL) 1 FC grew at 21 DAT and rapidly increase until 28 DAT (5 FC), eventually this number decrease to 3 FC, and maintained until the end of treatment. Between 28 and 35 DAT it is possible to observe a marked decrease of the number of FC in all cases, and just a few plants managed to grow new FC until end of experiment. Notwithstanding VC lost 1 out of 2 FC between 28 DAT and 35 DAT, but at 42 DAT achieve to grow 4 new FC and ended up with a total of 5 FC.

Additional to the plant growth parameters assessed, two another conditions were considered and measured over the experiment time (42 days), relative humidity and greenhouse temperature. Relative humidity (RH) and temperature were measured every 30 minutes with a temperature and humidity registerer. Relative humidity presented a mean of 57.59% at the end of the experimental period and the temperature values, including minimum and maximum values registered are presented in Figure 14.



1. **Figure 14** Temperature (°C) inside the greenhouse along the experimental period, measured every 30 minutes. Points represent mean and, bars correspond to range (minimum and maximum). Line in blue (25 ºC) represent the ideal temperature for tomato development and r red line (32 ºC) represent the maximum temperature tomato plant can endure.

The temperature was measured every 30 minutes to have a most accurate perception of the conditions inside the greenhouse and the values are presented as the mean and **range** (minimum and maximum) of the day (Figure 14). The blue line (25 ºC) represents the ideal temperature necessary for tomato developing, while the red line (32 ºC) shows the maximum temperature that tomato plants can support without their morphology and correct development be affected. It is also possible to observe that the average temperature was rounding the maximum level, and regarding the highest temperature, this are between 45 ºC and 57 ºC, much above the proper values.

5. Discussion

Agriculture and fruticulture have been increasing in the last years, due to the increased demand stimulated by the growth of human population. In Portugal the fruticulture sector has a high relevance, being "Alcobaça" apple and "Rocha" pear iconic crops in the West region, taking a considerable part of the economic development of the country. In 2020, the global production of apple in Portugal was 287 000 tons, and 131 000 tons of pear, meaning a 22.7% and 34% decrease when compared with 2019 production, respectively (*Estatísticas Agrícolas 2020*).

Due the consumers demand and the challenges of the fruit sector, such as climate change, water availability, extreme phenomena and phytosanitary problems, the need and use of plant protection chemical products have increased as well, which can have a negative impact on human and animal health. Thus, it is critical to discover and develop new solutions. Furthermore, one of the sustainable development goals of 2030 agenda are Good Health and Well-Being, aiming substantially reduce the number of deaths and illnesses from hazardous chemicals and air, water and soil pollution and contamination (*Health - United Nations Sustainable Development*).

When compared to terrestrial natural products, the marine ecosystem offers a virtually untouched environment, receiving growing attention from research community as a source of novel compounds with distinct chemical characteristics and mechanisms of action. Along of the last decades, the number of new marine naturals products have been documented, indicating a minor hint of a greater possibility, especially given that the marine environment is a unique source for producing a vast quantity of secondary metabolites with high biological activity.

Seaweeds such as *A. armata, Codium* sp., *F. vesiculosus and S. muticum* have been analyzed for their total phenolic content and antioxidant capacity in previous studies (Balboa et al., 2014, 2015; Bouhlal Rhimou, 2013; Pinteus et al., 2017a). Seaweeds have been studied also for their content in a variety of carbohydrates, plant growth regulator such as auxins, cytokinin's, gibberellins betaines, abscisic acid (ABA) jasmonic acid (JA), polyamines and minerals (Sharma et al., 2014). Demonstrating the high capacity of these species to produce a wild variety of chemical with potential use in agriculture as bio stimulants and biofertilizers.

As previously mentioned, the vast use of plant protection products to prevent plant infection and losses in crop yield and production, has become one important threat to human health, as well as to the conservation of the environment, making evident the need of new products to replace the current used. In this way, macroalgae has proven to be a valuable source for natural products, nevertheless, its necessary to perform studies to understand the effect of these resources before their implementation.

In this dissertation, using 4 different species of seaweeds and 5 different solvents to extract different components, including bioactive compounds, 24 extracts were produced. Regarding the solvent used, aqueous extract yielded greater values with all seaweeds, but *Codium* sp. which hydroethanolic extract (32.59 %) is higher than aqueous (21.52 %). The order of the yield of different extracts from *A. armata, F. vesiculosus* and *S. muticum*. was aqueous > hydroethanolic > ethanolic > ethyl acetate > *n*-hexane, meanwhile *Codium* sp. was hydroethanolic > aqueous > ethanolic > ethyl acetate > *n*- hexane.

This study used two methods to guarantee dryness of the extracts: freeze-drying and vacuum drying.

Freeze-drying, despite its many advantages in preserving the quality and homogeneity, does come with some notable disadvantages. One of the primary is its significant cost, both in terms of equipment and energy consumption, making it less economically viable for large-scale production. Additionally, freeze-drying is a time-consuming process, which might not be suitable for process where an elevated quantity of extracts is needed (Shukla, 2011).

In contrast, vacuum drying, although a useful method for moisture removal, also has its own set of disadvantages. One notable disadvantage is the time-consuming nature of the process. Vacuum drying typically takes a longer time to remove moisture from materials, and this extended duration can be a limiting factor, another disadvantage of vacuum drying is the challenge it presents in achieving uniform results across different samples. Therefore, its time-consuming nature and potential variability in results should be taken into consideration when choosing a drying method for specific applications (LA Bazyma and VA Kutovoy, 2006).

Asparagopsis armata yielded smaller values compared with the other seaweeds. Aqueous extract from this seaweed (7.35 %) was the higher value, in the other hand *n*-hexane yield (0.2 %) is the lowest value for this seaweed, as well as solvent used.

The phenolic content of the extracts was estimated by the means of Folin-Ciocalteu method, where the highest phenolic content was mediated by *F. vesiculosus* hydroethanolic and ethanolic extracts with 14.64 and 28.35 mg eq. Gallic acid/g extract, respectively, which could be possibly associated to the presence of phlorotannin's in the composition of this alga. Previous studies carried out with brown algae from the *Fucus* genus also revealed a significant phenolic content when compared to other brown algae, such as *Ascophyllum nodosum* (Agregán et al., 2018; Bielski S.; Falkowski J., 2017; Hermund et al., 2018).

The DPPH method, developed by Blois (1958) is based on the scavenging capacity of antioxidants towards DPPH. The reduction of color is proportional to the reduction of the radical by the antioxidant molecules present in the samples. The DPPH technique was used to assess the antioxidant activity of different standards to compare with the results obtained with the extracts, exhibiting the ascorbic acid the highest reductive capability of 84.02% at 30 µM (Leaves, 2014; O. P. Sharma & Bhat, 2009).

In this study, when compounds were tested by the means of DPPH assay, from all algae, the aqueous extracts showed the lowest DPPH reductive capacity. Lyophilized extracts did not show significant differences (p-*value*<0.05) compared with BHT, meanwhile un-lyophilized extracts from *F. vesiculosus* and *S. muticum* showed significant differences (p-*value*<0.05) compared with BHT, on the contrary, *A. armata* and *Codium* sp. aqueous un-lyophilized extracts did not display significant differences compared with BHT (ANOVA, Dunnett´s test, p-*value*<0.05). Generally, all other extracts (hydroethanolic, ethanolic, ethyl acetate and *n*-hexane), except for *F. vesiculosus n*-hexane, showed significant differences compared with BHT (ANOVA, Dunnett´s test, p-v*alue*<0.05). Furthermore, some extracts were capable to reduce DPPH radical in more than 50%: *F. vesiculosus* hydroethanolic, ethanolic and ethyl acetate extracts are the most prominent extracts, displaying 47.14%, 21.37% and 29.08% respectively, *A. armata* ethyl acetate extract left 33.84% and *S. muticum* 42.89% of available DPPH.

EC_{50} was determined as the concentration that induce the reduction of radical in 50%. *F. vesiculosus* extracts showed the lowest concentration to reduce 50% of DPPH radical exhibiting a EC_{50} value of 0.48 mg/mL and 0.49 mg/mL for hydroethanolic and ethanolic extracts, respectively, and ethyl acetate with 0.56 mg/mL. *A. armata* ethyl acetate and *S. muticum* ethanolic extracts presented the highest concentration needed to reduce half of the DPPH compound with a value of 0.69 mg/mL.

The above-mentioned results displayed that ethanolic, ethyl acetate and *n*-hexane extracts presented the higher scavenging capability. In the case of the brown macroalgae *F. vesiculosus*, the antioxidant capacity of the hydroethanolic and ethanolic extracts could be associated to the presence of phenolic compounds, phlorotannin and peptide derivatives, known to be present in this species and to show antioxidant activity (André et al., 2020).

Regarding the FRAP assay, it determines the capability of antioxidant compounds to reduce ferric iron (Fe) in a colorimetric oxidation-reduction reaction based on electron transfer. During this work, the extracts analyzed displayed a slight capacity to reduce ferric iron (III). The effect was significant smaller when compared with BHT (Figure 5). A weak reducing power has been reported previously for these algae, normally this antioxidant capability has been associated with phenolic compounds, nonetheless, has been reported that these compounds and their antioxidant activity can be influenced by seasonal variations (Julião et al., 2021; O'Sullivan et al., 2011; Pedro et al., 2022; Rupérez et al., 2002; Silva et al., 2021). However, in the case of *F. vesiculosus* and *S. muticum* aqueous lyophilized extracts showed significantly higher values (*p-value*<0.05) compared with the other extracts, followed by hydroethanolic extract. Moreover, the antioxidant activity of aqueous un-lyophilized extracts its clearly diminished compared with the lyophilized process. Meanwhile, *A. armata* and *Codium* sp. aqueous lyophilized extract are significantly lower than the rest of extracts and no remarkable results were found when studied these extracts.

The ORAC method was used to assess the capability of extracts to scavenge peroxyl radicals that induce the oxidation of fluorescein. Our results (Figure 10) demonstrated their capability to scavenge peroxyl radicals being the effects more marked than BHT standard. The practice of presenting ORAC values as micromolar equivalents of Trolox creates very large figures, giving the false impression that extracts and other natural materials include components that are several orders of magnitude more reactive. Juan José Córdoba Granados (2021) evaluated a different standard for antioxidant positive control, ascorbic acid, presenting a value of 53 887.10 ± 651.86 µmol Trolox EQ/mg compound. For this case the author evaluates pure isolated compounds with known antioxidant capability, but still the values presented are very high compared with result presented in this study for crude extracts. It would be also interesting to perform the fractionation of the crude extracts obtained in this study in order to understand if the antioxidant activity is being masked by the presence of other compounds.

Concerning the assessment of the phytotoxicity activity, the main goal was to understand if the produced extracts have potential to be further studied for the agricultural field, since, although natural extracts are being used, none of them is free from causing toxicity in plants. This way, it is of extreme importance to take as one of the first steps, the evaluation of phytotoxicity, and in this case, in tomato plants, considered model plants for scientific research due to their intrinsic characteristics.

For that, young tomato leaves (*Solanum lycopersicum,* Solanaceae*)* were analyzed in a leaf puncture assay. This method has been widely used to assay phytotoxicity of toxins and secondary metabolites produced by different organisms (Capasso et al., 1996; Cuq et al., 1993), where phytotoxins caused widespread chlorosis and necrosis when tested on tomato leaves at different concentrations, showing a relationship between the dose applied and size of the necrosis.

It was possible to observe that 3 days after inoculation slight necrosis occurred in some conditions, and after a week almost all conditions presented a brown area around the application zone. After twelve days, the diameter of the necrotic area was measured, being the results presented in Table 4.3. *Asparagopsis armata n*-hexane extract showed the highest necrotic area, ranging between 4.50 mm at 0.1 mg/mL and 6.25 mm at 1 mg/mL, as well as the aqueous extracts from *F. vesiculosus and S. muticum*, where the bigger values were achieved at the highest concentration (5.83 ± 0.24 and 5.33 ± 0.47 mm respectively). However, it is possible to note a slightly trend for *n*-hexane extracts, forming a bigger necrotic area when compared with the other seaweeds. In the other hand, *A. armata* hydroethanolic (0.5 mg/mL) and *F. vesiculosus* ethyl acetate (0.5 mg/mL) extracts presented the smaller necrotic area at all (1.33 ± 1.89 and 1.67 ± 1.18 mm respectively).

Anyway, any of the extracts or corresponding concentrations did not show significant difference when compared with DMSO or PBS (ANOVA, *p-value*>0.05). Thus, it is possible to conclude that any of extracts are responsible for phytotoxicity in tomato plant leaves, since the cause of the small areas of necrosis formed may be mainly caused for the wound performed.

Previous studies have used this method to understand the associated toxicity to marine fungi phytotoxins (Huang et al., 2018) and other natural origin metabolites (Dalinova et al., 2021; Masi et al., 2019) with the main propose to find new pesticides, showing no harmful effects to the host plant.

As a second approach to evaluate the phytotoxic effect of seaweed extracts on host plant, an *in vivo* assay was performed, cumulative data of growth parameters such as stem length, number of leaves, and number of flower clusters were studied (Figures 12 and 13 and Table 4.5 and 4.6). Measurements were recorded along the experiment and at the end of 42 days after transplantation of tomato plants under the application of different treatments with seaweed extracts and control as well.

The tomato plants cultivated in greenhouse were treated with a commercial fertilizer, the biological fungicide/priming agent Vacciplant® and aqueous and hydroethanolic extracts of four different seaweeds via pulverization.

Vacciplant® is a substance derived from Laminarin, a plant self-defense inducer. Laminarin is a substance derived from the algae *Laminaria digitata*, which contains a high concentration of the chemical, it is a natural biological fungicide that works as an inducer and activator of plant self-defense systems (*Vacciplant® | UPL*).

The use of Vacciplant® treatment (VC) presented smaller values in the number of new leaves and stem growth than commercial fertilizer treated plants (7.27 ± 5.74 and 0.79 ± 0.89 respectively) and greater values of FC (5 FC). When used a commercial fertilizer (CFC) treatment, the number of leaves achieved the second higher stem of all conditions (13.80 ± 6.11) , as well as the second highest stem growth (2.40 ± 0.68) as well as a total of 3 flower cluster at 42 DAT. By this means that the positive control applied are showing an effect of the physiological conditions and development of the plants. Regarding the case of the water control that shows a negative value, this could be due by the loss of shoot tip leaves until the last measure, affecting the results.

F. vesiculosus hydroethanolic extract at the concentration 0.5 mg/mL showed the maximum growth of new leaves at 42 DAT (23.80 ± 4.68 new leaves), yet the stem growth did not vary significantly (0.40 ± 1.17 cm), even in this treatment the sample size its just 5 out of 7, by means of more data could lead to greater values. However, no significant differences were found between treatments (Kruskal-Wallis, $p>0.05$).

Flowering stage started at 21 DAT, and for almost treatments at 28 DAT plants reached the greatest number of flower clusters. Until 35 DAT was observed a decrease in the number of flower cluster.

From 28 DAT it was recorded an increase of temperature, 25 ºC (mean) and ranging between 22 ºC at its minimum and 36 ºC maximum, until 31 DAT when the mean temperature was 29 ºC (20 ºC – 50 ºC), even reaching one of the highest values for all the experimental time, then until 35 DAT the temperature slightly decreased to 29 ºC mean but stay still a range between 22 ºC to 42 ºC. The mean value recorded during these days were above the ideal temperature of 25 ºC and almost reaching the maximum for tomato plants of 32 ºC. Then, a maximum temperature was recorded at 36 DAT with 57 ºC maximum and a minimum of 22 ºC, and mean values kept between 26 ºC and 31 ºC, as well as the minimum and maximum values ranged from 22 ºC to 57 ºC until the end of experiment at 42 DAT.

It has been proved that the application of algal extracts increases the germination, growth and biochemical composition of tomato plants, according to Baroud and collaborators (2021). The plants treated with aqueous extracts of three brown algae had considerably greater levels when compared to the respective control: the concentrations of 0.5% and 1% of *F. spiralis*, as well as 0.5% of *B. bifurcata*, favored the length of the tomato aerial parts.

Hussain and collaborators (2021) also concluded that the effect of brown seaweed *Durvillaea potatorum* and *Ascophyllum nodosum* extracts on plants was observed to greatly improve a variety of plant growth metrics, including the number of flower clusters and blooms, fruit number, dry weight of leaves and roots, and root length, increased tomato plant productivity and total soluble solid concentration in tomato fruit.

Seaweed extract treatment considerably increased chlorophyll levels in tomato leaves during the blooming period (Mzibra et al., 2021). Results showed that using polysaccharide-enriched extracts attained from seaweeds would be beneficial to improve vegetative and reproductive parameters such as early flowering, fruit set precocity, yield, and quality of tomato plants and fruits. Extracts also showed the ability to increase the water holding capacity of the soil and thus improve plant growth, resulting in significantly longer stems and a higher number of leaves and flowers per plant.

Considering that there are several factors that may be interfering the development of tomato plants, the real effect of the seaweed extracts tested was not truly achieved, raising the question how these factors can be involved in the effects observed.

Conditions in a greenhouse have an impact on a number of variables, including photosynthetic rate, morphology, yield, and disease incidence. It is recommended that the lowest acceptable and ideal air temperatures be maintained between 7 ºC and 22 °C, respectively, for the rate of leaf appearance (Baudoin et al., 2017). Indeed, tomato crops that experience heat stress are projected to have yields that decline by 12.6% for every 1.2 °C increase in temperature above 25 °C. Furthermore, it is recommended that the relative humidity range for tomato plants during all stages of growth be between 50 and 70%. If the air around plant leaves is too hot and humid in a greenhouse, transpiration at the leaf surface will be ineffective, and the root and stem system may not be able to adequately hydrate the leaves (Shamshiri et al., 2018).

During 2022, continental Portugal registered air temperature values higher than normal on summer months, average temperature was 23.30 ºC and 20.64 ºC during August and September, respectively. Between August 1st and August 19th, registered the hottest days, with a mean of maximum temperature in continental Portugal around 35 º(IPMA, 2022b, 2022a). According to Shamshiri et al., (2018) when air temperature can rise up to 38 ºC, the temperature inside a greenhouse may be 20-30 ºC higher than outside. This is reflected in Figure 10, the average temperature in most of the days of experiment were above the ideal temperature of 25 ºC for tomato plants. In addition, it was possible to verify that the highest temperature during the experiment reached 57.50 ºC recording the hottest day of experiment inside the greenhouse.

Conclusion

In conclusion, the work performed in this dissertation lead to the preparation of a total of 24 different crude extracts, by the used of four solvents (organic and non-organic solvents) and a hydroethanolic solution (75:25), from four seaweeds, namely *A. armata, Codium* sp., *F. vesiculosus, and S. muticum*. Aqueous and ethanolic solvents are considered as green extractions and displayed the highest yields, therefore, are more appealing to scale-up to industrial scale. On the other hand, organic solvents displayed very low yields, turning them more difficult to use on a large scale.

Regarding the antioxidant capability, extracts presented a weak activity determined by FRAP method, meanwhile in DPPH and ORAC assays, the extracts showed a better activity when obtained with organic solvents and ethanolic based extracts. Lyophilization process guarantees a more uniform extract, and facilitates the dry process, notwithstanding it represent an increase in the production values. In the case of *A. armata* and *Codium* sp., the lyophilization process did not affect the antioxidant results, meanwhile in the case of *F. vesiculosus* and *S. muticum* the lyophilized extracts presented greater values than un-lyophilized.

Before the use of a new product, even if it is from a biological origin, it is necessary to perform assays to ensure the safety of their use. In this study, a first screening assay where extracts were applied did not show a phytotoxic effect on detached tomato leaves. Moving forward, to a better understanding of the extracts effect, a greenhouse phytotoxicity assay with tomato model plants was performed, also showing no phytotoxicity associated to the extracts. However, this study did not provide enough data to conclude the real effect of the extracts since it was affected by the presence of high temperatures during the experimental time, reason that seemed to be negatively affecting the results obtained. Still, this was a study integrated in a wider project aiming to understand the potential of these seaweeds in a myriad of agriculture applications, and thus, even in the conditions here testes, despite not improving growth, the apparent non-negative effect and absence of phytotoxicity is favorable to the development of other bioactive such as biopesticides of priming agents, which are currently being developed

Future perspective

Marine resources have awakened the interest of the scientific community due to their great potential on the production of a wild variety of new chemical compounds and its applications in different fields. Accordingly, this study was based on the assessment of the antioxidant capability of 24 extracts and phytotoxicity from aqueous and hydroethanolic from *A. armata*, *Codium* sp., *F. vesiculosus*, and *S. muticum*. This study represents the first step to understand the actual effect of these extracts for further applications in agriculture as plant biofertilizer, biostimulants, biopesticides, and other applications.

Despite the differences in the results of antioxidant capabilities of extracts, another interesting approach, considering that this study only worked with crude extracts, more could be achieved if isolation or partitioning would be performed, looking forward to isolate fractions or compounds with higher antioxidant capabilities as previous works already demonstrated.

In addition of the antioxidant capability, more can be study from seaweed extracts, like the identification and quantification of phytohormones by ultra-performance liquid chromatography, to determine the presence of some plant growth regulators such as oxins, cytokinins, gibberelins, etc. Moreover, the study of the priming effect of seaweed extracts could provide an insight in the way the extracts could act as biostimulants of plant developing.

On the other hand, it should be also relevant to assess other perspectives related with the phytotoxicity of extracts that have not been evaluated in this study, for instance, the effect over the plant photosynthetic apparatus. In this aspect, biological assays more able to represent the intracellular interaction involver should be performed, methods like «Image Pulse Amplitude Modulation» could provide a better understanding of the extract presence in the photosynthetic process in a non-invasive manner.

Considering that the *in vivo* phytotoxic study suffered from abiotic stress, namely elevated temperatures derived from heatwaves, keeping in mind that plant tomatoes are mainly affected by this condition, the *in vivo* assay should be replicated in more suitable weather conditions for plant development. Accordingly, further experimental assays should be considered to ensure the effectiveness of the methodology applied and to the understanding of the actual effect of seaweed extracts for their uses on fruticulture sector.

The overall aim of this work was to highlight the use of these seaweed extracts in agriculture, evaluating their safety for plants, still, in a circular economy approach, the use of these seaweeds is intended for a biorefinery approach, where the same biomass may be fractionated for several uses and the activities here addressed being part of a wider scope on their use for agriculture and also other industries, and that is the path that is being followed and should be followed to create mechanisms for the most valuation of biomass, aiming greener industries and a zero-waste approach.

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