AMPONSAH PREKO APPIAH

EVALUATION OF THE ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY IN MACROALGAE ASPARAGOPSIS ARMATA AND ASPARAGOPSIS TAXIFORMIS

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DECLARATION OF AUTHORSHIP

EVALUATION OF THE ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY IN MACROALGAE ASPARAGOPSIS ARMATA AND ASPARAGOPSIS TAXIFORMIS

Declaration of authorship of work**:**

I declare I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

(Amponsah Preko Appiah)

DEDICATION AND ACKNOWLEDGEMENT

I would like to dedicate this work to God Almighty who gave me strength and life to see this work through. Also, to my family, especially my parents; Victoria Adjei and Joseph Appiah as well as all my siblings for being supportive of me in the pursuit of my academic dreams. I say may God bless you all.

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ABSTRACT

In recent times, natural compounds have become widely employed in the treatment of human illnesses and health conditions such as cancer, neurological disorders, multiple sclerosis, diabetes, atherosclerosis, arthritis, and cardiovascular diseases all around the world. Plants have been the principal source of numerous pharmaceutical agents throughout history. However, in this modern era, much emphasis has been placed on the amazing biodiversity of life in the marine environment, which has proven to be an extraordinary repository of novel bioactive compounds with diverse structural and chemical properties, as well as a source of inspiration for new drug discovery. In the never-ending quest for novel safe and effective anti-inflammatory and antioxidant compounds beneficial to human health, this research focused on evaluating the ability of two distinct species of the macroalgae *Asparagopsis* spp (*Asparagopsis taxiformis*, as a gametophyte and as a sporophyte, and *Asparagopsis armata* as a gametophyte) to contribute for the treatment of anti-inflammatory diseases by assessing their antioxidant and anti-inflammatory activities.

Ethanol, ethyl acetate and hexane were employed as solvents for the extraction of bioactive compounds from *Asparagopsis* spp. These solvents cover a wide range of polarity indices with polarity ranging from 0.1 for hexane, 4.4 for ethyl acetate, and 5.2 for ethanol to maximize the spectrum of extracted compounds. In this study, the best extraction yields were obtained with ethanol across all species of *Asparagopsis* with yields of 12.4% in *A. armata*, 9.0% in *A. taxiformis* as a gametophyte and 6.8% in the same as a sporophyte. Hexane yielded the least amount of extracts across all the species with yields of 0.4% in *A. armata*, 1.1% and 1.0% in *A. taxiformis* as a gametophyte and sporophyte respectively. Ethyl acetate showed yields of 2.4% in *A. armata* and 2.5% and 1.8% in *A. taxiformis* gametophyte and sporophyte respectively.

Antioxidant properties were evaluated using tests such as ABTS, DPPH, iron & copper chelating activity, total phenolic content, and iron reducing activity while anti-inflammatory analysis was conducted specifically analysing for COX-2 inhibition. Results obtained suggests that *Asparagopsis* spp contain several bioactive compounds that exhibit good antioxidant abilities especially with metal chelating activities. Activity results in the DPPH assays were rather low (below 50% activity) across all species. For the ABTS assay, only the ethanolic extract of *A. armata* showed activity above 50% with an IC_{50} value of 0.24 mg/mL. The best

results for copper chelating activity were observed in the hexane extracts of *A. taxiformis* sporophyte with an IC_{50} of 0.12 mg/mL. This was followed by the hexane extract of *A. armata* with an IC_{50} of 0.28 mg/mL. The best iron chelating activity was seen in the hexane extract of *A. taxiformis* sporophyte once again with an IC_{50} of 0.09 mg/mL. The ethyl acetate extracts of *A. armata* and *A. taxiformis* sporophyte also showed IC_{50} values of 0.25 and 0.24 mg/mL respectively. The iron reducing antioxidant assay showed good results for ethyl acetate extracts across all species with IC_{50} values of 0.07 mg/mL recorded of *A. taxiformis* sporophyte, 0.16 mg/mL for *A. armata* and 0.34 mg/mL for the gametophyte life stage of *A. taxiformis*. These extracts were observed to show activities higher than the gallic acid positive control.

The macroalgae also demonstrated tremendous potential for COX-2 inhibition activity at an extract concentration of 1 mg/mL for all species (especially the hexane extracts showing over 50% activity across species) with the highest activity of 98.9 ± 0.4% observed in *A. taxiformis* gametophyte hexane extracts. Ethanol extract from *A. taxiformis* gametophyte also showed an activity of 79.1 ± 1.8% to take the second place whereas the hexane extract from *A. taxiformis* as a sporophyte showed inhibitory activity of 72.3 ± 5.1% taking the third spot. These activities compared to the ibuprofen positive control that was used at a concentration of 5 µg/mL and showed an activity of 55.0 ± 13.2% demonstrates that even as crude extracts *Asparagopsis* spp have great anti-inflammatory potentials and therefore in their highly refined states, extracts from *Asparagopsis* spp may be very competitive against other commercially known anti-inflammatory compounds on the market.

Total phenolic content analysis (TPC) conducted on the extracts confirmed the presence of phenolic compounds in all the species of *Asparagopsis* evaluated. The highest levels of TPC were observed in the ethyl acetate extracts of all the species. Levels in the ethyl acetate extracts ranged from 1.50 ± 0.12 mg-GAE/g for *A. armata*, 1.19 ± 0.03 mg-GAE/g for *A. taxiformis* gametophyte to 0.61 ± 0.07 mg-GAE/g for *A. taxiformis* sporophyte. The relatively higher phenolic content observed in the ethyl acetate extracts suggests that most of the phenolic compounds present in *Asparagopsis* spp may be phenolic compounds of lower polarity such as tocopherols and tocotrienol which have been demonstrated to be well extracted by ethyl acetate. In general, the levels of total phenolic compounds present in *Asparagopsis* spp were determined to be relatively low compared to other macroalgae in literature. Despite the relatively low phenolic contents demonstrated in *Asparagopsis* spp it still showed good antioxidant and anti-inflammatory activities thus the antioxidant activity observed in the crude

extracts were not solely dependent on the phenolic compounds but rather to a greater extent other bioactive compound(s) that may be present in the crude extracts of *Asparagopsis* spp.

Statistical analysis in the form of ANOVA at a 95% Confidence Interval (alpha = 0.05) and Tukey multiple comparison test ($P<0.05$) were performed on the results obtained for all the antioxidant and anti-inflammatory tests conducted. The analysis revealed significant differences between the *Asparagopsis* species (*A. taxiformis* and *A. armata*) considered in this study and most importantly between the life stages of *A. taxiformis* as a gametophyte and a sporophyte.

Multivariate data analyses in the form of principal component analysis (PCA) and cluster analysis (CA) were carried out between the antioxidant assay, metal chelating assays, COX-2 anti-inflammatory assay, and total phenolic content assay. Results of the analysis revealed that the solvent type used for extraction had a great influence on the antioxidant performance of the extracts. The analysis also explored the similarities between the tests and identified a good positive correlation between copper chelating activity test and COX-2 anti-inflammatory test. This observation is of great importance because COX-2 inhibition test is noted to be time consuming, complex, and very expensive to carry out. Hence in the event of a study where a large number of samples are to be tested it becomes challenging and puts a great strain on not only human resources, but financial resources and time as well. However, copper chelating activity (CCA) is comparatively simple and economical with time and funds hence could be a great way of pre-screening samples to determine which samples are likely to show good results with COX-2 analysis and hence reduce sample size which will eventually save time and other resources.

Overall, findings presented from this research work suggests that the macroalgae *Asparagopsis* spp demonstrates high anti-inflammatory and antioxidant potentials for use in the pharmaceutical and nutraceutical industries.

Keywords: *Asparagopsis* spp, Antioxidants, Macroalgae, Anti-inflammatory**,** Pharmaceutical

SUMÁRIO

Nos últimos tempos, os compostos naturais tornaram-se amplamente utilizados no tratamento de doenças humanas e condições de saúde como o cancro, perturbações neurológicas, esclerose múltipla, diabetes, aterosclerose, artrite, e doenças cardiovasculares em todo o mundo. As plantas têm sido a principal fonte de numerosos agentes farmacêuticos ao longo da história. Contudo, nesta era moderna, tem sido dada muita ênfase à espantosa biodiversidade da vida no ambiente marinho, que provou ser um extraordinário repositório de novos compostos bioativos com diversas propriedades estruturais e químicas, bem como uma fonte de inspiração para a descoberta de novos medicamentos. Na busca incessante de novos compostos anti-inflamatórios e antioxidantes seguros e eficazes e benéficos para a saúde humana, esta investigação centrou-se na avaliação da presença de compostos bioativos em duas espécies distintas da macroalga *Asparagopsis* (*Asparagopsis taxiformis*, como gametófito e esporófito, e *Asparagopsis armata*, como gametófito), avaliando as suas capacidades antioxidantes e anti-inflamatórias.

Etanol, acetato de etilo e hexano foram utilizados como solventes para extração de compostos bioativos de *Asparagopsis* spp. Estes solventes cobrem uma vasta gama de índices de polaridade com polaridade variando entre 0,1 para o hexano, 4,4 para o acetato de etilo, e 5,2 para o etanol para maximizar o espectro de compostos extraídos. Neste estudo, os melhores rendimentos de extração foram realizados com etanol em todas as espécies de *Asparagopsis* com rendimentos de 12,4% em *A. armata*, 9,0% em *A. taxiformis* como gametófito e 6,8% esporófito. O hexano originou a menor quantidade de extratos em todas as espécies com rendimentos de 0,4% em *A. armata*, 1,1% e 1,0% em *A. taxiformis* como gametófito e esporófito, respetivamente. O acetato de etilo apresentou rendimentos de 2,4% em *A. armata* e 2,5% e 1,8% em *A. taxiformis* como gametófito e esporófito respetivamente.

As propriedades antioxidantes foram avaliadas usando testes tais como ABTS, DPPH, atividade quelante do ferro e cobre, conteúdo fenólico total, e atividade redutora do ferro, enquanto que a análise anti-inflamatória foi conduzida especificamente para a inibição da enzima Ciclooxigenase-2 (COX-2). Os resultados obtidos sugerem que as espécies de *Asparagopsis* testadas contêm vários compostos bioativos que exibem boas capacidades antioxidantes, especialmente com atividades quelantes de metais. Os resultados da atividade nos ensaios DPPH foram bastante baixos (abaixo de 50%) em todas as espécies. Para o ensaio ABTS, apenas o extrato etílico de acetato de etilo de *A. armata* mostrou atividade acima de 50% com um valor de IC_{50} de 0,24 mg/mL. Os melhores resultados para a atividade quelante

do cobre foram observados nos extratos hexanos de *A. taxiformis* (esporófito) com um valor IC_{50} de 0,12 mg/mL. Seguiu-se o extrato de hexano de *A. armata* com um IC_{50} de 0,28 mg/mL. A melhor atividade quelante do ferro foi observada no extrato hexano de *A. taxiformis* (esporófito) mais uma vez com um IC_{50} de 0,09 mg/mL. Os extratos de acetato de etilo de *A. armata* e *A. taxiformis* (esporófito) também mostraram valores de IC_{50} de 0,25 e 0,24 mg/mL, respetivamente, no teste de quelação do ferro. O ensaio antioxidante redutor de ferro mostrou bons resultados para extratos de acetato de etilo em todas as espécies com valores de IC_{50} de 0,07 mg/mL registados de *A. taxiformis* (esporófito), 0,16 mg/mL para *A. armata* e 0,34 mg/mL para a fase de vida do gametófito de *A. taxiformis*. Estes resultados mostram que os extratos brutos demonstram um potencial bastante bom para a redução do ferro.

As macroalgas também demonstraram um enorme potencial de inibição da atividade da COX-2 com uma concentração de extrato de 1 mg/mL para todas as espécies (especialmente os extratos de hexano que mostram mais de 50% de atividade). O valor mais alto (98,9 ± 0,4% de inibição) foi observada para os extratos de *A. taxiformis* (esporófito) hexano. O extrato de etanol de *A. taxiformis* gametófito mostrou também uma atividade de 79,1 ± 1,8%, enquanto o extrato de hexano de *A. taxiformis* como esporófito mostrou uma atividade inibitória de 72,3 ± 5,1%. Estas atividades em comparação com o controlo positivo do ibuprofeno que foi utilizado numa concentração de 5 µg/mL e mostrou uma atividade de 55,0 ± 13,2% demonstra que mesmo como extratos brutos, *Asparagopsis* spp, têm grandes potenciais anti-inflamatórios e, portanto, nos seus estados altamente refinados, os extratos de *Asparagopsis* spp. podem ser muito competitivos contra outros compostos anti-inflamatórios comercialmente conhecidos no mercado.

A análise do conteúdo fenólico total (TPC) realizada nos extratos confirmou a presença de compostos fenólicos em todas as espécies de *Asparagopsis* avaliadas. Os níveis mais elevados de TPC foram observados nos extratos de acetato de etilo de todas as espécies. Os níveis de TPC nos extratos de acetato de etilo variaram de 1,50 ± 0,12 mg-GAE/g para *A. armata*, 1,19 ± 0,03 mg-GAE/g para *A. taxiformis* (gametófito) a 0,61 ± 0,07 mg-GAE/g para *A. taxiformis* (esporófito). O conteúdo fenólico relativamente mais elevado observado nos extratos de acetato de etilo sugere que a maioria dos compostos fenólicos presentes na *Asparagopsis* spp. podem ser compostos fenólicos não polares, tais como tocoferóis e tocotrienol, que se demonstrou serem bem extraídos pelo acetato de etilo. Em geral, os níveis de compostos fenólicos totais presentes nas espécies de *Asparagopsis* foram determinados como sendo relativamente baixos em comparação com outras macroalgas da literatura. Apesar dos teores fenólicos relativamente

baixos demonstrados em *Asparagopsis* spp, ainda apresentava boas atividades antioxidantes e anti-inflamatórias que poderão não depender apenas dos compostos fenólicos mas sim, em maior medida, de outros compostos bioativos que possam estar presentes nos extratos brutos de *Asparagopsis* spp.

A análise estatística sob a forma de ANOVA com um Intervalo de Confiança de 95% (alfa = 0,05) e o teste de comparação múltipla Tukey ($P<0,05$) foram realizados com base nos resultados obtidos para todos os testes antioxidantes e anti-inflamatórios realizados. A análise revelou diferenças significativas entre as espécies de *Asparagopsis* (*A. taxiformis* e *A. armata*) consideradas neste estudo e, mais importante, entre as fases de vida de *A. taxiformis* como um gametófito e um esporófito.

Foram realizadas análises de dados multivariados na forma de análise de componentes principais (PCA) e análise de agregados (CA) entre o ensaio de antioxidantes, ensaios de quelantes metálicos, ensaio de anti-inflamatórios COX-2, e ensaio de teor fenólico total. Os resultados da análise revelaram que o tipo de solvente utilizado para extração teve uma grande influência no desempenho antioxidante dos extratos. A análise também explorou as semelhanças entre os testes e identificou uma boa correlação positiva entre o teste de atividade quelante do cobre e o teste anti-inflamatório COX-2. Esta observação é de grande importância porque o teste de inibição do COX-2 é demorado, complexo e muito caro. No entanto, a atividade quelante de cobre (CCA) é comparativamente simples e económica, pelo que poderia ser uma ótima forma de pré-seleção de amostras para determinar quais as amostras suscetíveis de mostrar bons resultados na inibição da COX-2 e, consequentemente, reduzir o tamanho da amostra, o que acabará por poupar tempo a outros recursos.

Globalmente, os resultados apresentados neste trabalho de investigação sugerem que as macroalgas *Asparagopsis* spp demonstram elevados potenciais anti-inflamatórios e antioxidantes para utilização nas indústrias farmacêutica e nutracêutica.

Palavras-chave: *Asparagopsis* spp, Antioxidantes, Macroalgas, Anti-inflamatório, Farmacêutico

Part of work on digital media

- Excel sheets for the computation of antioxidant activity
- Excel sheets for the ANOVA computation
- Files for GraphPad Prism
- Files for Unscrambler X

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List of abbreviations and acronyms

ABTS	2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid),
COX	Cyclooxygenase Enzyme
CA	Cluster Analysis
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDGE	Energized Dispersive Guided Extraction
EDTA	Ethylenediaminetetraacetic Acid
EPR	Electron Paramagnetic Resonance
ET	Electron Transfer
FRAP	Ferric Reducing Antioxidant Power
HAT	Hydrogen Atom Transfer
NSAIDS	Nonsteroidal Anti-inflammatory Drugs
PCA	Principal Component Analysis
PG	Prostaglandins
PUFA	Polyunsaturated Fatty Acids
PV	Pyrocatechol Violet
ROS	Reactive Oxygen Species
TPC	Total Phenolic Content
TPTZ	Tripyridyltriazine
Atg - P -	*Asparagopsis taxiformis* (gametophyte) – collected from Portugal
Aag - P -	*Asparagopsis armata* (gametophyte) - collected from Portugal
Ats - S -	*Asparagopsis taxiformis* (sporophyte) – collected from Spain

1. INTRODUCTION & LITERATURE REVIEW

Recent studies have shown that most marine macroalgae (seaweeds), possess secondary metabolites (bioactive compounds) such as polyphenols (e.g., *Alaria esculenta*; Molina-Alcaide et al., 2017), isoprenoids (e.g., *Dictyota*; Machado et al., 2016), phlorotannins (brown seaweeds), and brominated halogenated compounds (e.g., *Asparagopsis*; Machado et al., 2016). Published studies suggest that these secondary metabolites are produced as a result of the complex nature of the habitat in which these marine organisms dwell. Due to the extreme environmental conditions they have to endure and the need for them to defend themselves from predators, competitors, and other threats, they are forced to adapt and develop chemical defences to survive (Ponte et al., 2022).

Macroalgae are mostly classified by their pigmentation (brown, green, and red) and by habitat (freshwater or marine) (Samanthi , 2020). Macroalgae may also be classified based on features such as cell wall composition, reproductive characteristics, and the chemical nature of their photosynthetic products. Additionally, structure, form and shape can equally be used to classify macroalgae (Putri, 2012).

Macroalgae and their secondary metabolites have a growing number of industrial and traditional uses in human culture, thanks to their diversity. Some forms of macroalgae are edible and have been traditionally consumed over the years as a delicacy in most parts of the world, especially Asia. Modern macroalgal uses include bovine feed, bioremediation or pollution management, fuel production, extraction of polymer compounds for used in industrial processes particularly in the food industry, and in medicinal and scientific applications for drug development. According to an assessment published in 2020, macroalgae might play a significant role in carbon sequestration to help reduce climate change while also producing attractive value-added goods for global economies (Paul et al., 2020).

Asparagopsis spp. are macroalgae that belong to the family Bonnemaisoniaceae, order Bonnemaisoniales, subclass Rhodymeniophycidae, class Florideophyceae, subphylum Eurhodophytina, phylum Rhodophyta. *Asparagopsis* is reported to have six species which include: *Asparagopsis delilei* (Montagne, nom. illeg. 1841.), *Asparagopsis taxiformis* ((Delile) Trevisan, 1845), *Asparagopsis armata* (Harvey, 1855), *Asparagopsis hamifera* ((Hariot) Okamura, 1921) which includes two infraspecific subspecies, *Asparagopsis hamifera f. sterilis Chemin* and *Asparagopsis sanfordiana f. amplissima* (Setchell & N.L. Gardner), and

Asparagopsis svedelii (W.R. Taylor, 1945). However only three of the above-mentioned species are officially taxonomically accepted. They include: *Asparagopsis taxiformis* ((Delile) Trevisan, 1845), *Asparagopsis armata* (Harvey, 1855), and *Asparagopsis svedelii* (W.R. Taylor, 1945) (Ponte et al., 2022).

Asparagopsis spp. are regarded as invasive species and considered to be one of the worse biological invaders. Abundance of these species in the marine world not only makes it a threat to local biodiversity but also a nuisance to fishing and aquaculture activities as they have the tendency to attach themselves to sailing vessels and also to fishing nets and other aquaculture structures within the ocean, hampering their efficient function (Streftaris & Zenetos, 2006). This implies the need for scientists to find an appropriate way to deal with this abundant marine macroalgae or, better still, make effective use of it as a natural resource for the benefit of humanity.

Emerging research on *Asparagopsis* spp. reports the presence of a number of bioactive compounds including brominated halogenated compounds which are able to demonstrate tremendous potential for CH_4 mitigation. As such, this macroalgae has been employed in livestock agriculture as dietary supplement to help reduce the production and release of methane gas by livestock and help prevent environmental degradation (Roque et al., 2019; Gao & McKinley, 1994).

Despite the awareness of the presence of bioactives in *Asparagopsis* spp., very little research has been reported on assessing properties such as antioxidant, anti-inflammatory, iron reduction, and metal chelating activities of these bioactive compounds in order to widen the scope of application of this macroalgae to other fields such as the pharmaceutical and cosmetic industries, among others. Therefore, further research is needed to assess these properties and possibly suggest the most effective species, the optimum conditions for their use, and the doses needed, as well as the potentially associated risks with their application.

1.1. Aims and Objectives

This research therefore focuses on evaluating if the macroalgae, *Asparagopsis taxiformis* in two life stages, as a gametophyte and as a sporophyte, and *Asparagopsis armata* as a gametophyte can be a source of antioxidant and anti-inflammatory compounds to be used in the pharmaceutical industry.

To achieve this objective, the following tasks have been outlined:

- Optimize and perform extractions from two species of *Asparagopsis* biomass (*A. taxiformis* (gametophyte/sporophyte), and *A. armata* (gametophyte)) employing three solvents of different polarities (Ethanol, Ethyl Acetate and Hexane) for each species.
- Check quality parameters such as repeatability and inter-day precision for the extraction process
- Assess the antioxidant and anti-inflammatory activities *(DPPH, ABTS, Ferric reducing antioxidant capacity, Iron (II) chelating activity, Copper chelating activity, COX-2 Inhibiting Activity)* of different extracts prepared from the selected macroalgae.
- Evaluate the Total Phenolic Content (TPC) of the extracts
- Analyse data using statistical tools.

1.2. Algae

Algae in general are large polyphyletic, photosynthetic organisms that include numerous groups of species. They are considered as a polyphyletic group owing to the fact that they do not share a common ancestor although their plastids appear to have a single origin, from cyanobacteria. They can be unicellular or multicellular in nature and therefore regarded as microalgae or macroalgae respectively (Samanthi, 2020). Algae are found mostly in aquatic habitats and are autotrophic, thus they have the ability to synthesize nutritional organic substances from inorganics nitrogen, phosphorous nutrients and carbon dioxide using light (Morris et al., 2019). Despite being photosynthetic, algae lack features such as stomata, xylem, and phloem morphologically as commonly seen in most plants.

1.2.1. Morphology and Reproduction in Algae

There are a variety of algal morphologies however, certain characteristics are common across unrelated groups. The majority of the simpler algae are unicellular flagellates or amoeboids, although some of the groupings have developed colonial and nonmotile forms independently. Algae have a wide range of lifecycles, each with its own set of characteristics. In general, the seaweed's cells are diploid during the asexual phase, haploid during the sexual phase, and the male and female gametes fuse during the sexual phase. Asexual reproduction allows for efficient population growth, but there is less variety. In sexual reproduction of algae, two specialized, sexually compatible haploid gametes establish physical contact and combine to generate a zygote. The growth and release of gametes is carefully coordinated and controlled to guarantee a successful mating; pheromones may play a significant role in these processes. (Johannes et al., 2014). Sexual reproduction allows for greater variety in features of the species

and also gives the advantage of effective recombinational repair of DNA damage during meiosis which is a critical stage of the sexual cycle.

1.3. Algal Division

1.3.1. Microalgae

Microalgae can be prokaryotic or eukaryotic, producing carbohydrates, proteins, and lipids as products of their photosynthesis. They grow rapidly and are able to survive in harsh environmental conditions. Prokaryotic microalgae include cyanobacteria (*Cyanophyceae*), and eukaryotic microalgae include the green algae *Chlorophyta*. There are also diatoms also known as *Bacillariophyta*. Due to the ability of microalgae to withstand harsh environmental conditions they are found not only in aquatic habitats but also terrestrial ecosystems implying a huge variety of species living in a wide range of environmental conditions (Dhal et al., 2012).

1.3.2. Macroalgae

Macroalgae on the other hand are larger in size and resemble plants in their morphology. They are not rapid growing as compared to microalgae (Dhal et al., 2012; Shackira et al., 2021). Macroalgae are commonly referred to as "seaweed" and may have features such as thallus, stipes, blades, fronds, and airbladders (Samanthi, 2020).

Reproduction in macroalgae happens in two main ways. The first occurs when a part of the algae breaks off from the parent and gets attached to a surface and regrows its entire structure with the same DNA as the parent. The second mode of reproduction is by meiosis in which specialized patches on the leaves produce haploid spores which in turn produces gametes. These gametes may mix with other gametes to produce new diploid seaweed (Putri, 2012). As photosynthetic sessile organisms, macroalgae respond directly to abiotic and biotic environmental factors exhibiting high sensitivity (Panayotidis et al., 2004).

1.3.2.1. Classification of Macroalgae

Macroalgae are mostly classified by their pigmentation (brown, green, and red) and habitat (freshwater or marine) (Samanthi, 2020). Macroalgae may also be classified based on features such as cell wall composition, reproductive characteristics, and the chemical nature of their photosynthetic products. Additionally, structure, form and shape can equally be used to classify macroalgae (Putri, 2012).

1.3.2.1.1. Brown Macroalgae

Brown macroalgae (figure 1.3), are also known as *Ochrophyta* (Phylum) and are mostly found in marine environments. They are specifically dominant in the temperate and polar regions of the earth as they thrive in colder waters. Their distinct greenish-brown colour is often attributed to the presence of the pigment fucoxanthin. Brown macroalgae range in size from just a few centimetres to several inches. Reproduction in brown macroalgae is by means of flagellated spores and gametes (Cock et al., 2011). Examples include: *Ascophyllum nodosum* (John Stackhouse, 1809), and *Macrocystis pyrifera* (Emil Friedrich August Walter Migula, 1909), which is the largest among macroalgae as well as one of the fastest growing organisms on the planet earth (Cribb, 1954).

A *B*

Figure 1.1 Examples of brown macroalgae A) Ascophyllum nodosum (John Stackhouse, 1809) Retrieved from: (florafinder.org, 2013) B) Macrocystis pyrifera (Emil Friedrich August Walter Migula, 1909) Retrieved from: (Ricciuti, n.d.)

1.3.2.1.2. Green Macroalgae

Green macroalgae (figure 1.2), belongs to the genus *Chlorophyta* indicating the presence of chlorophyllic-compounds. They vary in colour between species ranging from bright green to yellow as well as dark jade. Green macroalgae are considered as one of the most diverse and abundant group of macroalgae with extremely varied shapes and characteristics. Reproduction is either by fragmentation or by the release of reproductive spores (Kronenwetter, 2021). Examples of green macroalgae include: *Chaetomorpha antennina* (Jean-Baptiste Bory de Saint-Vincent, & Friedrich Traugott Kutzing, 1847). *Cladophora* (Friedrich Traugott Kutzing, 1843), and *Codium tomentosum* (John Stackhouse, 1797).

A

B

C

Figure 1.2 Examples of Green macroalgae A) Chaetomorpha antennina (Kutzing et al., 1847) Retrieved from: (Kripanand et al., 2015) B) Cladophora (Friedrich Traugott Kutzing, 1843) Retrieved from: (www.aquasabi.com, 2022) C) Codium tomentosum (John Stackhouse, 1797) Retrieved from: (www.ecured.cu, 2013)

1.3.2.1.3. Red Macroalgae

Red macroalgae (figures 1.4), are the most diverse group of all and are referred to as *Rhodophyta*. Red macroalgae are predominantly found in marine costal environment with just approximately 5% of the species found in freshwater sources in warmer climates (Dodds & Whiles, 2019). In terms of cell structure, red macroalgae never develop flagella and centrioles as well as a chloroplast endoplasmic reticulum. However, they do possess normal spindle fibres, microtubules, un-stacked photosynthetic membranes, phycobilin pigment granules, and pit connections between cells filamentous genera (www.vedantu.com, 2022). The pigments present in red macroalgae are phycobilins consisting of phycocyanobilin, phycoerythrobilin, phycourobilin, and phycobiliviolin. Other pigments that may be present include chlorophyll-*a*, carotene, lutein, and zeaxanthin. Reproduction can be via asexual or sexual means. Red macroalgae life cycle is characterized by two sporophyte generations namely, carposporophyte and tetrasporophyte. Carpospores are produced in the carposporophyte life stage and they in turn germinate into tetrasporophyte. The tetrasporophyte also generate spores known as tetrads which dissociate into gametophytes. The entire life cycle of red macroalgae is illustrated in the figure 1.3 below.

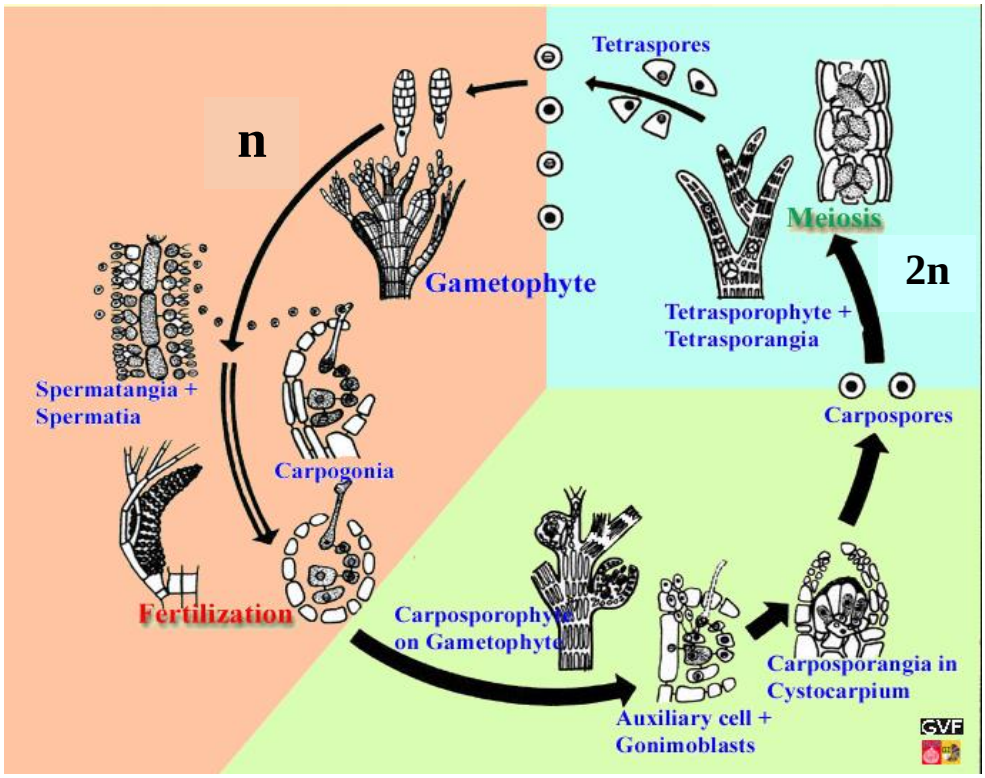


Figure 1.3 . General illustration of the life stages of a red macroalgae, Adapted from (www.vcbio.science.ru.nl, 2013)

Examples of the red macroalgae include: *Asparagopsis armata* (Harvey, 1855), *Chondrus crispus* (John Stackhouse), and *Palmaria palmata* (L. F. Weber & D. Mohr., 1805).

A *B* *C*

Figure 1.4 Examples of red macroalgae A) Asparagopsis armata (Harvey, 1855) Retrieved from: (Kern et al., 2019) B) Chondrus crispus (John Stackhouse) Retrieved from: (Wikipedia, 2022) C) Palmaria palmata (L. F. Weber & D. Mohr, 1805) Retrieved from: (Cwmhiraeth, 2022)

1.4. Uses of macroalgae

Macroalgae and its associated metabolites have huge biotechnological potentials such as application in the pharmaceutical industries where they are used for making drugs for the treatment of cancers and age-related functional decreases (Hassimotto et al., 2005). Numerous literature references make mention of bioactive extracts such as the tripeptides IPP and VPP used in commercial products, for example, Ameal-S 120® from Japan and Evolus® from Finland for the management of cardiovascular diseases and disorders including hypertension (Hayes & Tiwari, 2015). Other known extracts from macroalgae sources such as iodine and brominated compounds have reportedly been combined with other ingredients in the making of drugs for acne treatment (Kraan & Barrington, 2005). Pharmacological properties exhibited by these extracts which make them desirable for use in drug formulation include antioxidant, anti-inflammatory, neuroprotective, antiviral, antiprotozoal, anticarcinogenic, and antimicrobial properties. Nevertheless, their application in the pharmaceutical industry is not only limited to the properties listed above but also to characteristics such as binding activity, preservative tendencies, and moisturizing potentials (Ponte et al., 2022; Kalimuthu et al., 2021).

Bioactive extracts from macroalgae are also applied in the cosmetic industries to make dermatological preparations to help address a number of undesired skin conditions making use of bioactive compounds such as beta-carotene and phycocyanin. Apart from the treatment of skin ailments, cosmetic industry focuses on making dermatological preparations to achieve a broad number of effects such as anti-aging, photoprotective from ultraviolet radiations, skin hydration and skin toning and bleaching effects. Once again, some bioactive extracts from macroalgae have been found to exhibit antioxidant properties which help to address skin aging problems as well as photoprotective properties which protect the skin from ultraviolet radiations and hence are used in making such preparations. Moisturizing properties and toning effects have also been associated with some bioactive compounds such as flavonoids and hence they are specifically employed in the cosmetic industry for this purpose (Choi et al., 2013; Yong et al., 2009; Pandey et al., 2017; Yoon et al., 2009).

Applications of bioactive compounds extends to the food processing industry in the making of what we term “functional foods” or “super foods” which when consumed help to diminish the risk of numerous diseases, such as cancer, heart disease, stroke, Alzheimer’s, diabetes, and cataracts (Hassimotto et al., 2005). Functional foods are often supplemented with antioxidants

and with isoprenoids such as retinol (vitamin A), and tocopherol (vitamin E) with regards to their associated health benefits. The most often consumed fortified foods include fortified juices, dairy products (such as milk and yogurt) and eggs, as well as fortified milk alternatives including almond, rice, coconut, and cashew milk. Fortification of milk substitutes with vitamin D and calcium is a cost-effective strategy for reducing bone loss associated with aging, particularly in the elderly population who are at a higher risk of bone fractures. Another popular example is margarine spreads and eggs fortified with omega three fatty acids to support cardiovascular health (Gul et al., 2016). In addition to this, bioactive compounds are employed as additives to enhance food properties like texture, flavour, aroma, and colour and also as preservatives. (Vrancheva et al., 2018).

Furthermore, both the biomass of macroalgae and their bioactive extracts have been applied in animal nutrition to help prevent animal diseases as well as enhance animal nutrition. The potential health benefits of substances like laminarin and fucoidan found in various macroalgae and macroalgal products used as feed for monogastric animal species are attracting increasing interest. This is because macroalgae have been reported to be rich in distinctive bioactive components (Øverland et al., 2019). In terms of fish farming, it has been shown that adding algae and algal extracts to the diet of fishes might boost their immune systems and promote better development. Fish meals including various macroalgae have been found to have positive effects on productivity indices and overall health. Additionally, macroalgae and their extracts have lately been introduced as safer preventative and therapeutic medicines to manage the infectious illnesses afflicting farmed fish. Numerous studies have demonstrated that marine algae are a potential source of antibacterial compounds that are effective against both Gram-negative and Gram-positive pathogenic bacteria (Mohammed et al., 2021).

In recent times, macroalgae and macroalgae extracts have also been employed in environmental remediation practices especially for CH_4 mitigation and have proven to be remarkably effective for that matter (Roque et al., 2019). Recent batch-fermentation investigations indicate that certain macroalgae have the ability to reduce methane (CH_4) generation from beef cattle by up to 99% when added to animal feed. Fermentation in a semi-continuous in-vitro rumen system suggests that macroalgae, specifically *Asparagopsis* spp., can reduce methane production from enteric fermentation in dairy cattle by 95% when added at a 5% organic matter inclusion rate with no obvious negative effects on volatile fatty acid production (Roque et al., 2019). Emerging research over the last couple of decades speak of great promises for the use of

macroalgae and their extracts in the making of biofuels which inadvertently serve as better alternatives to fossil fuels in the bid to reduce CO_2 emissions (Gao & McKinley, 1994).

The benefits derived from macroalgae are not only limited to the bioactive compounds they contain but also to the entire organism itself. For instance, macroalgae have been grown and used directly as food for people for a long time, notably in several Asian and some European countries. Seaweeds grow in substantial numbers in the seas, many of which are edible and safe for human consumption. This is because they have been shown to contain many of the critical elements such as omega-3 fatty acids, amino acids, vitamins, and minerals. Seaweeds are becoming more widely acknowledged as a sustainable food source with the potential to play a significant part in ensuring food security around the world (Mahadevan, 2015). Macroalgae have also been investigated and reported to be very instrumental in the treatment of wastewater for the removal of excess nutrients (Gao & McKinley, 1994).

Reports by Otero et al. (2021) and Qiu et al. (2022) indicate that seaweeds have become a very vital source of bioactive polysaccharides which are used as immunomodulators, anti-obesity agents, and prebiotic ingredients in pharmaceutical and neutraceutical industries. Bioactive polysaccharides especially from red seaweeds are also employed as emulsifiers and thickners in the food industry. These polysaccharides are also noted to be essential tools for colloidal stabilization, fat reduction, and shelf-life extension for the industry (Katerina & Vassilis, 2019).

1.5. The *Asparagopsis* genus

Asparagopsis spp. are red seaweed that belongs to the family Bonnemaisoniaceae, order Bonnemaisoniales, subclass Rhodymeniophycidae, class Florideophyceae, subphylum Eurhodophytina, phylum Rhodophyta. Officially only three species are taxonomically confirmed and accepted, and they include: *Asparagopsis taxiformis* ((Delile) Trevisan, 1845), *Asparagopsis armata* (Harvey, 1855), and *Asparagopsis svedelii* (W.R. Taylor, 1945). (Ponte et al., 2022).

Geographical distribution of these macroalgae is dependent on the species in question thus, varied species of *Asparagopsis* can be found in different parts of the world. For instance, while *A. armata*, is a species that may be found in mild temperate areas, *A. taxiformis*, is spread out over the tropical and subtropical zones of the ocean (Bonin & Hawkes, 1987; Chualáin et al., 2004). However, due to the invasive nature of both species, they have been found to dominate the Mediterranean Sea.

The genus *Asparagopsis* has a triphasic and heteromorphic life cycle, which means that it alternates between two diploid and one haploid stage during its whole life cycle, like other seaweeds in the order Bonnemaisoniales. When a male gametophyte's spermatium (male gamete) fertilizes a female gametophyte's carpogonium (female gamete), reproduction has begun and an evolving zygote that finally develops into a diploid carposporophyte is the outcome of this. The carposporophyte invades the female plant as a parasite, spreading along the axes of the female branch and ingesting nutrition. Mature carpospores are released from the cystocarp depending on seasonal environmental factors including temperature. Tetrasporophyte are produced when carpospores settle and germinate. Tetrasporophyte eventually generate tetraspores, often in sets of four, with two of the spores developing into male and female gametophytes. The distribution of the spore among the sexes often occurs in a 50:50 ratio (Mickelson, 2013).

1.4.1. *Asparagopsis armata*

The discovery of *Asparagopsis armata* is attributed to Harvey, an Irish botanist who reported a detailed description of the species in the year 1855 (Andreakis et al., 2004). *Asparagopsis armata* is noted to be native to the south of Australia and New Zealand (Southern Hemisphere) and was initially discovered in the western Australian coast growing on stony infralittoral bottoms from the seawater's surface reaching to a depth of around 40 meters. However, due to the highly invasive nature of this species, it was reported to have gradually spread to the northern hemisphere through the Mediterranean and Atlantic Sea in the 1920s (Dixon & Irvine, 1977; Feldmann & Feldmann, 1942). *A. armata* is also regarded as the “red harpoon weed” in the English language and is often referred to as an "autogenic ecosystem engineer" owing to the fact that it is at the very bottom of the food chain in its natural habitat and hence has the tendency to manage resource availability to other creatures in the environment. A picture of *A. armata* is displayed in the figure1.5.

Figure 1.5 Asparagopsis armata (Harvey 1855) Retrieved from: (Kern et al., 2019)

1.4.1.1. Life cycle of Asparagopsis armata

A heteromorphic life cycle among the red algae group was originally noted in *A. armata*. The life stages have been determined to be three and they are the haploid carposporophyte, gametophyte, and diploid zygote. The filamentous "genus" Falkenbergia was discovered to be actually a part of the life cycle of *A. armata*. Based on *Polysiphonia rufolanosa*, it was determined that *A. armata* carpospores form a filamentous, free-floating, diploid, and an asexual tetrasporophyte that has been named *Falkenbergia rufolanosa* (Feldmann & Feldmann, 1942). Distinct phases with various morphologies and ploidies each contribute in a unique way to *A. armata's* capacity for proliferation. The gametophytes of this species are tiny carposoporophytes that split into tetrasporophytes and undergo meiosis to mature into the gametophyte.

When *A. armata* is completely mature, it has sparse branches on which long stolons with hook-like harpoons and upright shoots grow. The ramification of the branches, stolons, and shoots gives *A. armata* a thallus-like look (Andreakis et al., 2004). The largest branches are made up of a central medullary filament, a gelatinous matrix, and a cortex that is three to six cells thick. The final branchlets are filamentous and made up of three cell rows (Børgesen, 1913). Gametophytes are terete and have a height of about 200 mm. They cluster into tight, pink, interweaving bunches. Barbs that secure *A. armata* to ocean benthic substrates are a distinguishing feature of this species. The general life cycle of *A. armata* is illustrated in figure 1.6.

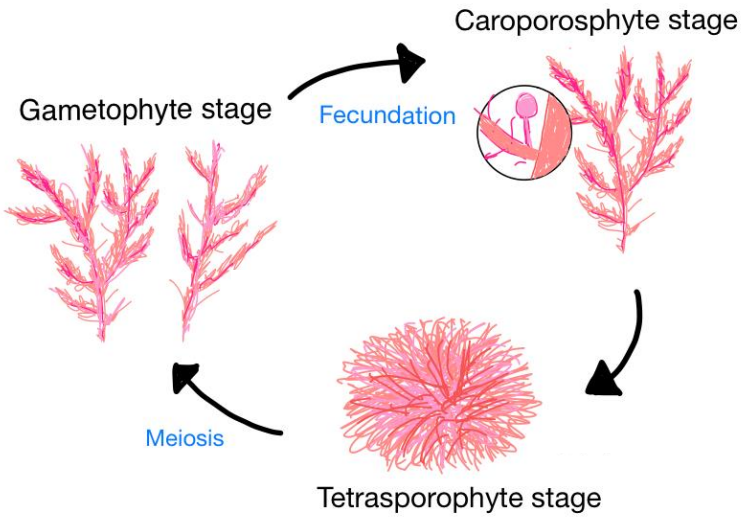


Figure 1.6 The Life Cycle of Asparagopsis armata (Harvey 1855) Adapted from: (Encyclopaedia of Life, 2021)

1.4.1.2. Studies about Asparagopsis armata

Despite the limited amount of research conducted with regards to *Asparagopsis* spp. in general, studies suggest the presence of bioactive compounds in the nature of halogenated metabolites mostly bromoform and minor amounts of other bromine-, iodine-, and chlorine-containing methane, ethanol, acetones, acetaldehydes, 2-acetoxypropanes, acroleins, propenes, epoxypropanes, and butenones especially in *A. armata*. They are generated and stored in vacuoles inside gland cells in the macroalgae. These compounds according to reports are produced in *A. armata* as a form of defensive mechanism against herbivores and other organisms in the marine environment that may threaten the survival of the seaweed (McConnell & Halogen, 1977). Due to the presence of these compounds in *A. armata*, various applications have been investigated including use in the mitigation of methane gas (CH_4) production in ruminants (Roque et al., 2019; Gao & McKinley, 1994). Application in the making of biopesticides (Duarte et al., 2021) have also shown promising results. This has vamped up interest in the red seaweed and hence it is expected that more research will be conducted in times to come.

1.5.2. Asparagopsis taxiformis

Delile Alire Raffeneau, a French botanist and Vittore Benedetto Antonio Trevisan de Saint-Léon, an Italian botanist are credited with the discovery of *Asparagopsis taxiformis* in the year 1845. However, it is reported that Delile completed the first taxonomic study on the holotype

species, *Asparagopsis taxiformis (Delile) Trevisan*, in 1813. He named it *Fucus taxiformis Delile* based on material he had collected in Alexandria, Egypt (Zanolla et al., 2022). Following Delile's description of the specimens discovered in Egypt, Jean Pierre François Camille Montagne, a French botanist, bryologist, and mycologist, named the holotype species *Asparagopsis delilei Montagne* and defined the genus *Asparagopsis* in 1840 from material discovered in the Canary Islands (Spain) because of the specimen's likeness to asparagus. Later on, Jakob Georg Agardh, a Swedish botanist and phycologist changed the species' name to *Lictoria taxiformis (Delile)* in 1841. In a brief time later, Trevisan described the same species under the name *Asparagopsis taxiformis (Delile)*. He did so in accordance with Montange and Delile's earlier descriptions, but he did not take Agardh's suggestion into account (Zanolla et al., 2022). Unlike *Asparagopsis armata*, this species of *Asparagopsis* is extensively present in tropical and subtropical areas and thought to be native to these regions. (Bonin & Hawkes, 1987; Chualáin et al., 2004). A picture of *A. taxiformis* is shown in figure 1.7.

Figure 1.7 Asparagopsis taxiformis ((Delile) Trevisan, 1845) Retrieved from: (Jean-Pascal , 2022)

1.5.2.1. Life cycle of Asparagopsis taxiformis

Both *Asparagopsis taxiformis* and *Asparagopsis armata* share a lot of similarities in their life cycles. *A. taxiformis* also goes through the haploid carposporophyte, gametophyte, and diploid zygote life stages and also develops a filamentous "genus". Based on *Polysiphonia hillebrandii*, the tetrasporophyte of *A. taxiformis* was identified as *Falkenbergia hillebrandii* (Dixon, 1964). Illustrated in figure 1.8 is the life cycle of *Asparagopsis taxiformis*.

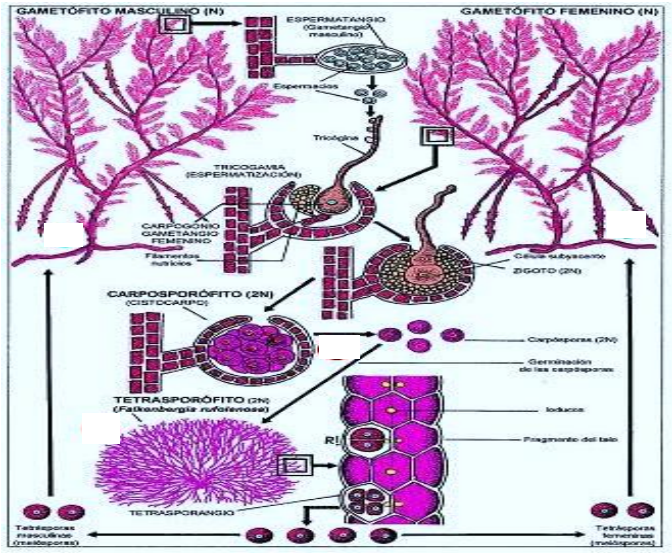


Figure 1.8. The life Cycle of Asparagopsis taxiformis ((Delile) Trevisan, 1845) Adapted from: (Zezinho, 2010)

With tall, indeterminate branches growing from entwined stoloniferous branches, *Asparagopsis taxiformis* gametophytes are up to 40 cm long, terete, and are conifer-like. The main shafts of the thalli can reach a diameter of 2 mm and are thickly covered by fluffy branches that are 1-2 cm long. Indeterminate branches are bare or have just rudimentary branches in the lowest third or half. The upper half to two thirds of the upright branches are covered with main heavily branched determinate branches that are radially organized, and are noted to be short, spinose subordinate branches. *A. taxiformis* has pairs of carpogonial branches, which are also known as cystocarps, that are positioned one next to the other across the bottom section of main determinate branches and short spinose branches, respectively. Transversely, major axes display a medullary zone covered in a multi-layered cortex as well as lengthy axial cells with inflated ends. The thallus is covered with extremely many, refractive glandular cells (Zanolla et al., 2022).

1.5.2.2. Studies about Asparagopsis taxiformis

Researchers at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and James Cook University demonstrated in 2014 that feeding ruminants a diet containing one to two percent red seaweed reduced methane emissions by more than 90% (Machado et al., 2014). Of the 20 species of seaweed studied, *A. taxiformis* showed the most promise, with over 99% efficacy (Battaglia, 2016). The findings piqued the interest of key academic and trade groups, who are now looking into the implications on ruminant animal production. According to these studies, the dichloromethane extract (discovered in *A. taxiformis*) was the most

powerful bioactive, lowering methane generation by 79%. Other bioactives discovered were bromoform, dibromochloromethane, bromochloroacetic acid, and dibromochloroacetic acid (Machado et al., 2016).

1.5.3. *Asparagopsis* spp sporophyte versus gametophyte

Not much studies have been conducted on comparing the life stages as noted in *Asparagopsis* spp. Studies conducted are mostly concerned with the physical and biological features of this seaweed in its alternating life stages and not its physiochemical characteristics (Wright et al., 2022). However, studies conducted on *A. armata* by Nicholas et al. (2006) seem to suggest that there happens to be a great statistical difference in the amounts of brominated compounds observed between the two life stages. The research seemed to account for only some major brominated compounds namely, bromoform ($CHBr_3$), dibromoacetic acid (DBA), bromo-chloroacetic acid (BCA), and dibromochloromethan ($CHBr_2Cl$). Among these compounds, bromoform and DBA were noted to be the major compounds responsible for the antibacterial activity associated with the extracts. However, the researchers stated the possibility of the presence of other compounds which might be collectively working together with the brominated compounds for this antibacterial effects (Nicholas et al., 2006). There is therefore the possiblity that these other compounds may differe entirely from one another between the gametophyte and sporophyte life stages of *Asparagopsis* spp. Hence, it is worth investigating properties such as antioxidant and anti-inflammation across the life stages of this seaweed.

1.6. Bioactive compounds

A bioactive substance is described in medical dictionaries as a chemical that has an impact on, induces a reaction, or stimulates a response in living tissue. In other words, it refers to a chemical (or substance) with biological activity if it has a direct influence on a live creature. A more comprehensive definition may be presented as follows, a bioactive compound is: “a compound which has the capability and the ability to interact with one or more component(s) of the living tissue by presenting a wide range of probable effects.” The origin of these substances can be natural-terrestrial or aquatic; a plant, animal, or other source (e.g., microorganisms) or synthetic: partially or totally (Guaadaoui et al., 2014). These effects might be good or negative depending on the drug, dosage, or bioavailability. Bioactive substances derived from plants, animals, microbes, and fungi are crucial in promoting health and curing/preventing disease. Seaweeds were previously primarily utilized as thickening and gelling agents in the food and pharmaceutical sectors, but recent studies have shown they have

the potential to be employed as alternative medicines due to the presence of bioactive compounds. The therapeutic benefits of red, brown, and green seaweeds for health and disease management have been demonstrated. These benefits include anticancer, anti-obesity, antihypertensive, antihyperlipidemic, antioxidant, anticoagulant, anti-inflammatory, immunomodulatory, antiestrogenic, thyroid stimulating, neuroprotective, antiviral, antifungal, and antibacterial properties (Khalid et al., 2018).

A number of bioactive substances have been found thus far. These substances are categorized based on how differently they operate and are chemically structured. Carotenoids, flavonoids, carnitine, choline, coenzyme Q, dithiolthiones, phytosterols, phytoestrogens, glucosinolates, polyphenols, and taurine are some examples of bioactive substances. Since vitamins and minerals have pharmacological effects, they can also be referred to as bioactive substances. The majority of bioactive substances demonstrate antibacterial, anti-inflammatory, anticarcinogenic, and antioxidant activities. Numerous epidemiological studies so claim that some of them also have preventive effects on cardiovascular illnesses (Hamzalıoğlu, & Gökmen, 2016).

1.6.1. Reported Bioactive in Macroalgae

Recent research has reported the presence of bioactive compounds (secondary metabolites) such as polyphenols (e.g., *Alaria esculenta*; Molina-Alcaide et al., 2017), isoprenoids (e.g., *Dictyota*; Machado et al., 2016), phlorotannins (brown seaweeds), and brominated halogenated compounds (e.g., *Asparagopsis*; Machado et al., 2016) in most marine macroalgae. Also worth mentioning is that natural pigments (NPs), polyunsaturated fatty acids (PUFAs), lipids, proteins, and polysaccharides are examples of commercial bioactive substances derived from algae. Evolutionary connections, ecological diversity, and chemical diversification may all be responsible for the natural heterogeneity in the composition of bioactive compounds. Natural algal populations' variations in bioactive marine chemical concentrations are regulated by environmental variables such as light, nutrients, pollutants, salinity, availability of CO_2, pH, temperature, and biotic interactions (Stengel et al., 2011). Some of these compounds are briefly discussed.

1.6.1.1. Polyphenols

Polyphenols are a broad class of organic chemical substances that include several phenol units. They are abundant in plants and structurally diverse. Polyphenols include flavonoids, tannic

acid, and ellagitannin (Quideau et al., 2011). Nearly all plants contain phenolic compounds, including their subgroup flavonoids, which have been widely discovered in grains, legumes, nuts, olive oil, tea, red wine, vegetables, and fruits. They primarily possess antioxidant qualities, and some studies have shown that they have positive impacts on risk factors for cardiovascular disease (Hamzalıoğlu, & Gökmen, 2016).

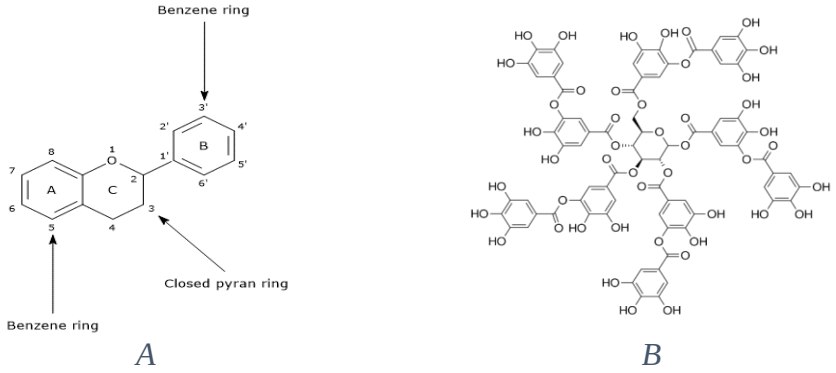


Figure 1.9 Examples of polyphenols A) Basic Skeletal Structure of Flavonoids Adapted from: (DR. Tazzini, 2014) B) Chemical structure of tannic acid Adapted from: (Abdel-Shafy, & Mansour, 2017)

1.6.1.2. Isoprenoids

The term "isoprenoids" refers to any group of organic compounds made up of two or more units of hydrocarbons, each of which has five carbon atoms with double bonds. In physiological processes of both plants and animals, isoprenoids have a wide range of functions which include functioning as pigments and fragrances to vitamins and precursors of sex hormones. They include carotene, phytol, retinol (vitamin A), tocopherol (vitamin E), dolichols, and squalene (Sharkey, 1996). Most isoprenoids such as vitamins are employed in the making of fortified foods or what we term “Functional foods” due to the health benefits associated with their antioxidant and anti-inflammatory activities.

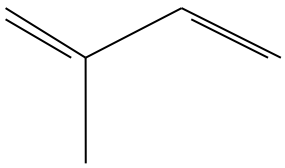


Figure 1.10. Basic Skeletal structure of Isoprenoids Adapted from: (Eastman & Kluger, 2018)

1.6.1.3. Phlorotannins

Despite being noted as polyphenols, phlorotannins have in recent years received a high amount of attention and research hence the need to highlight this group. Phlorotannins are a special

type of tannins which are oligomers of phloroglucinol. Phlorotannins have the ability to precipitate proteins and are an integral part of the structural component of cell walls in brown algae. Examples include fucodiphlorethol-G and phlorofucofuroeckol-B, among others. (Shibata et al., 2004; Ham et al., 2007; Sugiura et al., 2006). Phlorotannins are produced biosynthetically in nature via the acetate-malonate route. They are made of numerous phloroglucinol units connected to one another in diverse ways. Phlorotannin's potential cosmeceutical uses include reactive oxygen species scavenging, skin wrinkle prevention, and melanin production suppression (Noel Vinay et al., 2020).

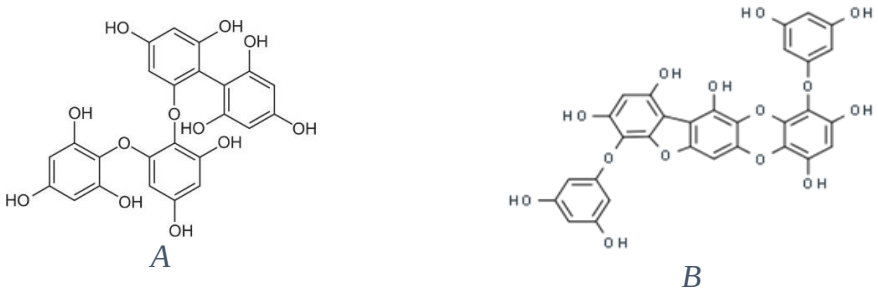


Figure 1.11 Examples of Phlorotannins A) Chemical structure of Fucodiphlorethol Adapted from: (Kim et al., 2014) B) Chemical structure of Phlorofucofuroeckol-B Adapted from: (www.chemspider.com, 2022)

1.6.2. Activities of bioactive compounds

1.6.2.1. Antioxidant activity

Antioxidants are a defence of organisms against oxygen and nitrogen reactive species (ROS & NOX). Reactive species are highly unstable free radicals in the form of atoms, molecules, or ions with unpaired electrons that occur due to endogenous factors such as biochemical reactions occurring in organisms or due to exogenous factors such as radiation, pollution, and other harsh environmental conditions. Due to these free radicals, cells have an optimal oxidant-antioxidant equilibrium. If this equilibrium shifts in favour of free radicals, cells will begin to experience the impacts of oxidative stress. Oxidative stress is a complicated process in which an imbalance exists between the creation of reactive oxygen and nitrogen species and the organism's ability to remove these reactive species. Antioxidant defences exist in cells to protect them from ROS, but when ROS production is elevated, antioxidant self-production might be hampered, which makes it difficult to ensure that cells are fully protected. Endogenous antioxidants are those made by the body itself; they can be either enzymes (such as glutathione peroxidase, catalase, and superoxide dismutase) or nonenzymatic substances (such as bilirubin, ascorbate,

glutathione, and albumin). Exogenous antioxidants are an alternate and external source of antioxidants that the body can employ to make up the deficiency when the internal antioxidant system is weakened (Lobo et al., 2010).

When oxidative stress lasts for an extended period of time in a biological system, ROS-activated chain reactions cause damages to a variety of cell components, including nucleic acids, DNA, and proteins. In certain cases, these damages even cause cell death and the long-term onset of illnesses (Sehwag & Das, 2013). In the human system, about 5% of the oxygen breathed by the human body is transformed into reactive oxygen species, which include the hydroxyl radical, superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and different lipid peroxides. All of them are capable of causing cellular harm by interacting with membrane lipids, nucleic acids, proteins and enzymes, and other tiny molecules. The primary function of antioxidants is to serve as free radical scavengers, preventing and repairing ROS-induced damage, as well as stabilizing ROS molecules and preventing them from reacting with other molecules. This helps to prevent chronic inflammation. Therefore, Antioxidants are essential for protecting the body (Sehwag & Das, 2013).

In food systems antioxidants refer to any chemical that, when present in lesser amounts compared to an oxidizable substrate, considerably slows down or stops the oxidation of that substrate. Oxidation of fats and oils causes rancid odour and flavour, resulting in a loss in nutritional quality, sensory appeal, and safety. This is due to the creation of primary hydroperoxides and subsequent potentially hazardous chemicals as a result of the auto-oxidation of unsaturated fatty acids via a free radical chain mechanism. Antioxidants are therefore essential for preserving the quality of food (Prenzler et al., 2021).

1.6.2.2. Production of Reactive Oxygen and Nitrogen Species

The primary metabolic activities that result in endogenous ROS generation are those that take place during respiration and photosynthesis in organelles such mitochondria, peroxisomes, and chloroplasts (Aglika, 2005; Lambert & Brand , 2009). Endogenous ROS production is predominantly sourced from the mitochondrial electron transport chain (ETC), which includes a family of membrane-bound NADPH oxidases (NOXs) and 5-Lipoxygenase (5-LOX). As a precursor of H_2O_2 and OH•, O_2 may be transformed into H_2O_2 by three isoforms of superoxide dismutases (SOD) (i.e., SOD1, 2, and 3) found in the cytoplasm, mitochondria matrix, and extracellular matrix. When oxygen (O_2) combines with nitric oxide (NO), peroxynitrite

(ONOO) is formed, which reduces antioxidant activity. OH• is produced from H_2O_2 through Fenton chemistry (Xiangbing et al., 2019).

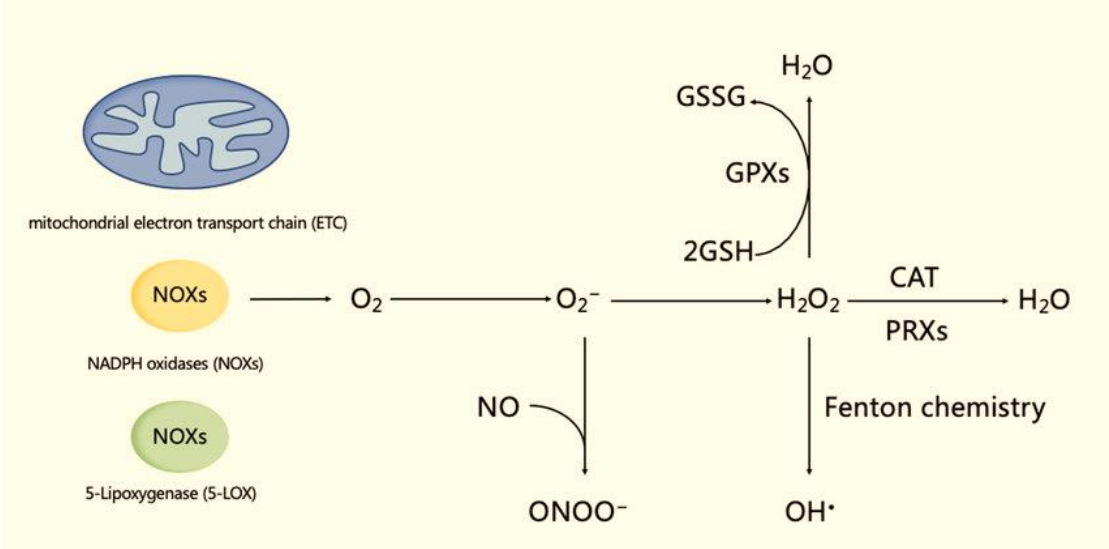


Figure 1.12. Formation of free radicals from biochemical reactions Adapted from: (Xiangbing, et al., 2019)

The exogenous generation of ROS is influenced by numerous substances, including toxins, heavy metals, cigarettes smoking, pharmaceuticals, xenobiotics, microplastics, and radiation. Environmental conditions such as high temperature and some interactions with other living things can also influence the production of ROS (Muthukumar & Nachiappan, 2010).

Ionizing radiation can produce harmful intermediates when it interacts with water, a process known as radiolysis. Water loses one electron in the process and becomes very reactive. Water is then successively transformed to hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), superoxide radical ($•O_2$), and finally oxygen (O_2) in a three-step chain reaction. The hydroxyl radical is very reactive and has the ability to remove electrons from any molecule in its path converting that molecule into a free radical and thus causing a chain reaction (Azzam et al., 2012).

1.6.2.3. Antioxidants Reaction Mechanisms

Depending on the reactivity and chemical structure of ROS, a variety of mechanisms can be used to prevent or slow down ROS generation. They include:

- Auto-oxidative chain-breaking

- Scavenging activities that can initiate peroxidation reactions
- Metal ions chelating activity to prevent the generation of reactive species or decompose lipid peroxides.
- Quenching O_2^- to avoid peroxide production
- Reducing localized O_2 concentrations

The mechanisms mentioned above can be conveniently put under three main reaction method which forms the bases for their evaluation, and they are as follows:

A) Chain Breakers or Free Radical Interceptors

Most mono- or polyhydroxy phenols with different ring substitutions are the principal antioxidants that function as free radical interceptors or chain breakers. They function on a hydrogen atom transfer mechanism as main antioxidants (AH). In doing so, the antioxidant reacts with the highly reactive radical lipid and peroxy radicals (ROO•) and transfers one hydrogen atom to the radical to create stable organic lipid derivatives and antioxidant radicals (A•) that are less likely to be readily available to take part in propagation reactions. Primary antioxidants react primarily with peroxy radicals and have greater affinity for peroxy radicals than lipids (Sehwag & Das, 2013). This mechanism is illustrated in figure 1.13.

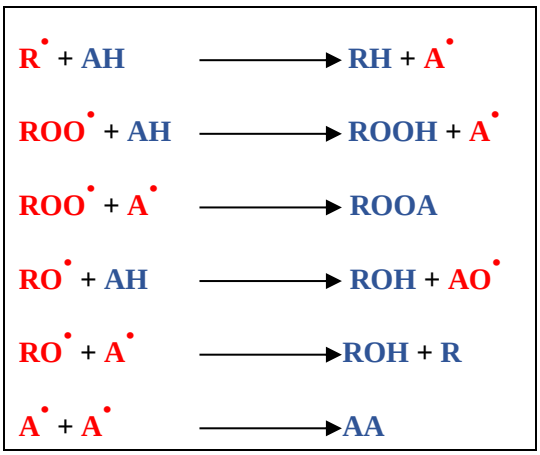


Figure 1.13 Illustration of the Chain Breaking mechanism of antioxidants

B) Single Electron Transfer Mechanism

In a single electron transfer process, the antioxidant creates a cation radical, which is also a less reactive species, while the free radical accepts one electron to create an energetically stable anion (Sehwag & Das, 2013). The Single Electron Transfer Mechanism is demonstrated in figure 1.14.

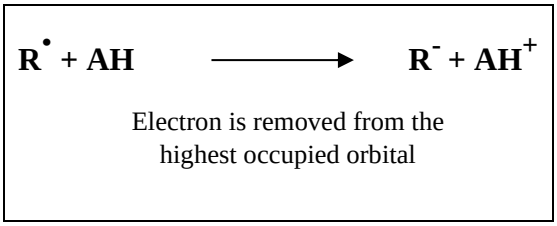


Figure 1.14.Illustration of a single electron transfer mechanism

C) Metal Chelation

Metal chelation refers to the phenomenon where transition metals function as both a catalyst and a proxidant in an oxidation process. By chelating metal ions, the antioxidant creates a stable compound and prevents the metal from catalysing and promoting the formation of oxyradicals or other reactive oxygen species (Sehwag & Das, 2013). Metal chelation mechanism is demonstrated in figure 1.15.

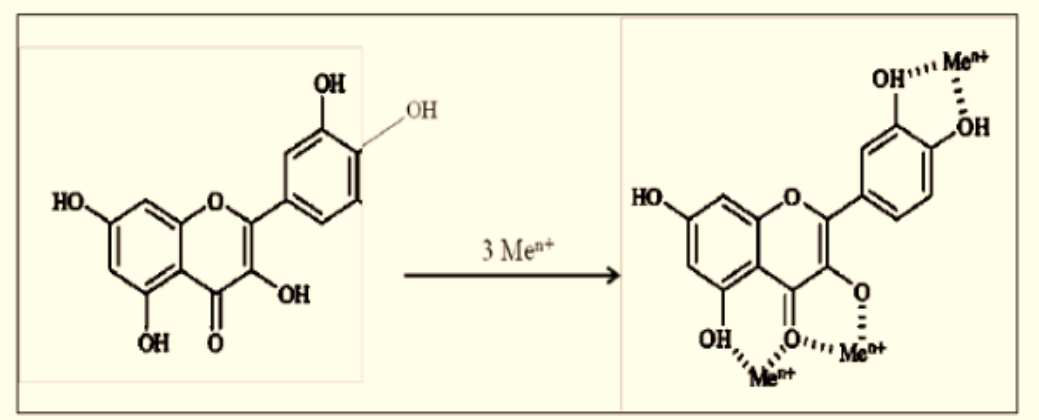


Figure 1.15. Metal chelating mechanism of primary antioxidants Adapted from: (Sehwag & Das, 2013)

Other Antioxidants

Some antioxidants are not capable of stabilizing free radicals to form stable compounds. Their mode of action is to donate protons to primary antioxidants, break down hydroperoxide into non-radical species, deactivate singlet oxygen, absorb UV radiation. They also operate as chelators for pro-oxidant or catalytic metal ions. They are noted to mostly increase the antioxidant activities of primary antioxidants (Sehwag & Das, 2013). Modes of action of these antioxidants and their examples are shown in the Table 1.1.

*Table 1.1 Antioxidants and modes of action (chain breakers excluded) **

Mode of Action	Antioxidant
Metal Chelating	Acids such as citric, malic, succinic, and tartaric Phosphates, ethylenediaminetetraacetic acid.
Singlet oxygen quenching	Carotenoids (including lutein, lycopene, and -carotene)
Oxygen scavenging and reducing agents	Sulphites, Erythorbic acid, Sodium erythorbate, Ascorbic acid, and Ascorbyl palmitate.

1.6.2.4. Classification of Antioxidants

Classification of antioxidants can be done based on certain factors such as their origin, mechanism of action and their chemical structures. On the bases of mechanism of reaction, antioxidants can be classified as follows:

- Primary Antioxidants

Primary antioxidants, also known as chain-breaking antioxidants, are chemicals, mostly phenolics, which act as hydrogen and electron donors while also breaking down free radical chains in lipid peroxidation.

- Secondary Antioxidants

Secondary antioxidants, also known as hydroperoxide decomposers, work to transform hydroperoxides into nonradical, nonreactive, and thermally stable compounds. They are frequently employed in conjunction with main antioxidants to produce synergistic stabilizing effects. Hydroperoxide decomposers inhibit the breakdown of hydroperoxides into highly reactive alkoxy and hydroxy radicals. As hydroperoxide decomposers, organophosphorus compounds and thiosynergist antioxidants are commonly utilized. Examples include phosphites, thioesters, and organophosphorus compounds (Shanina et al., 2002). A general reaction mechanism of this is displayed in figure 1.16. Thiosynergist such as thioesters are

among sulphur based hydroperoxide decomposers that proceed by decomposing hydroperoxide to alcohol, and the thiosynergist itself is converted into a range of oxidized sulphur compounds, including sulfenic and sulfonic acids. This reaction is demonstrated in figure 1.17.

Figure 1.16 Decomposition of hydroperoxides by organophosphorus. Adapted from: (Shanina et al., 2002)

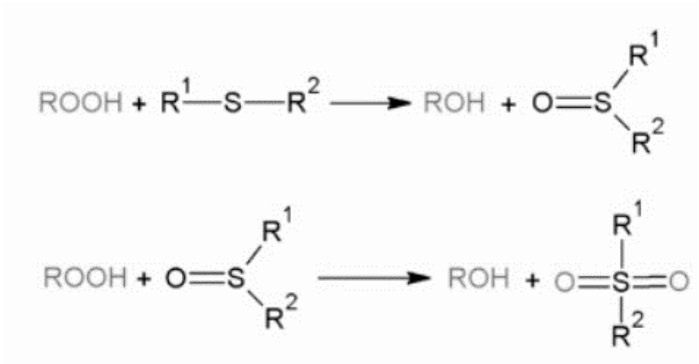


Figure 1.17. Decomposition of hydroperoxides by thiosynergist. Adapted from (Ambrogi et al., 2017)

- Enzymatic antioxidants

Enzymatic antioxidants in physiological systems include glutathione peroxidase, catalase, and superoxide dismutase. These enzymes are produced by the body and function to protect cells from radical attacks. Superoxide dismutase protects cells from radical attack by converting superoxide radical (produced in tissues as a by-product of oxygen metabolism) into hydrogen peroxide and molecular oxygen. Hydrogen peroxide in the body can cause cell damage at high concentrations, so catalase breaks peroxide down into water and molecular oxygen, limiting free radical-induced damage. Glutathione Peroxidase also converts hydrogen peroxides to water and lipid peroxides to alcohols. These enzymatic antioxidants have essential cofactors that help them in their functions, and they include, selenium, copper, iron, zinc, and manganese. Cofactors for the antioxidant enzymes catalase and superoxide dismutase include iron, copper, zinc, and manganese. Selenium is a cofactor for the enzyme glutathione transferase (Lobo et al., 2010; Eddaikra & Eddaikra, 2021).

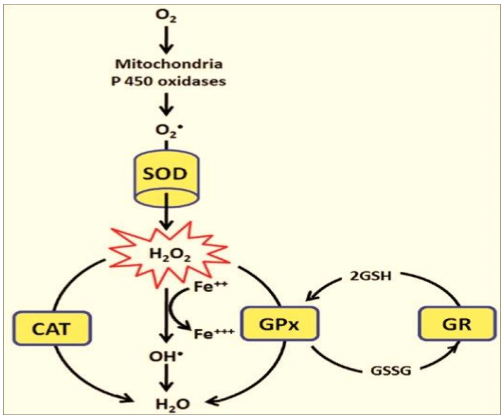


Figure 1.18. Role of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase in preventing oxidative stress. Adapted from: (Pandey & Rizvi, 2010)

In the food industries, Enzymatic antioxidants such as glucose oxidase, remove dissolved or head space oxygen from the environment. They also remove highly oxidative species, such as super oxide dismutase from a system to ensure that the food does not deteriorate with time.

- Metal chelators

These antioxidants chelate transition metals that function as a catalyst for lipid oxidation. Some antioxidants which are metal chelating agents are synergistic compounds that significantly increase the effectiveness of phenolic antioxidants. The majority of these synergists, such citric acid, amino acids, and phospholipids like cephalin, have negligible to no antioxidant action (Sehwag & Das, 2013).

Other classes of Antioxidants which are known to the food industry include:

- Oxygen scavengers

Oxygen scavengers are chemicals that react with oxygen and can subsequently eliminate it in a closed system. A typical example is ascorbic acid (vitamin C) (Sehwag & Das, 2013).

A more generalized classification of antioxidants is demonstrated in figure 1.19.

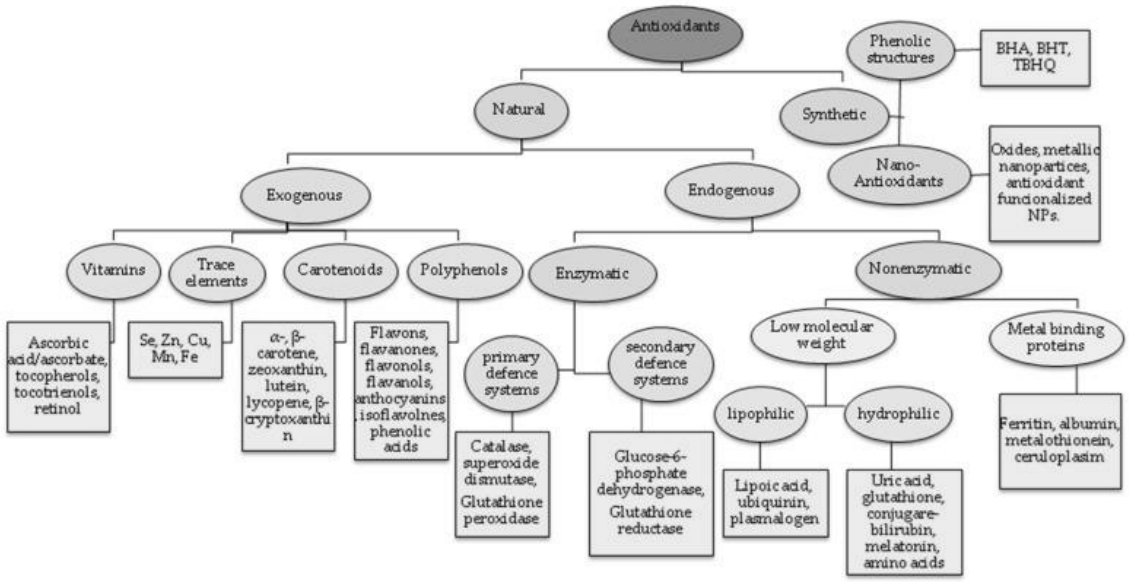


Figure 1.19. Generalized classification of antioxidants. Adapted from: (Flieger et al., 2021)

1.6.2.5. Anti-inflammatory Activity

The body's initial response to an infection or damage is inflammation, which is essential for both innate and adaptive immunity. Infectious microorganisms like bacteria, viruses, or fungi that invade the body, settle in certain tissues, or circulate in the blood and produce inflammation. Additionally, tissue injury, cell death, cancer, ischemia, and degeneration can all result in inflammation. In most situations, the development of inflammation is attributed to both innate and adaptive immune responses. The most crucial defence against invasive bacteria and cancer cells is the innate immune system, which is composed of macrophages, mast cells, and dendritic cells. In the adaptive immune system, more specialized cells, such as B and T cells, produce specific receptors and antibodies that are used to fight intruding pathogens and cancer cells. Numerous inflammatory mediators are produced and released during diverse types of inflammatory responses. The two main categories of inflammatory molecules are pro- and anti-inflammatory mediators (Pushpa, 2021).

Cyclooxygenase (COX) is an enzyme that is known to produce prostanoids (which include, prostaglandins, prostacyclins, and thromboxanes) which are responsible for the inflammatory response. The cyclooxygenase enzyme comes in two different varieties: COX-1 and COX-2. Both contribute to inflammation, however COX-1 can have a positive impact on the body (Ricciotti & FitzGerald, 2011). COX-1 is known for producing prostaglandins, which are involved in a variety of important bodily functions such as ovulation, blood coagulation, renal

function, wound healing, vasomotor tone, platelet aggregation, immune cell differentiation, neuron development, bone metabolism, and labour initiation. One important function of this prostaglandin system is to maintain the stomach lining intact. When this prostaglandin system is interrupted by using COX-1 medicines, stomach discomfort, digestive tract issues, intestinal or stomach haemorrhage, and even death may result (Ricciotti & FitzGerald, 2011).

The acute phase of inflammation, which characterizes the initial stage of a typical inflammatory event, is where the cascade starts with the immune and vascular systems' initial reaction to an infection or tissue injury. The acute phase often occurs before the immune response is developed. It is quick and lasts only for a short period of time (Ayertey et al., 2021). This phase is characterized by a sudden influx of blood granulocytes, usually neutrophils, followed quickly by monocytes that develop into inflammatory macrophages and then multiply, impairing the abilities of local tissue macrophages. The four essential symptoms of acute inflammation are rubor (redness), calor (heat), tumour (swelling), and pain. The inflammatory response might lessen and end if the initial noxious stimulus is eliminated by phagocytosis. Acute inflammation functions as a homeostatic mechanism that aids the host's healing process (Ricciotti & FitzGerald, 2011). Despite the fact that inflammation is a natural and useful reaction of the body, chronic inflammation can emerge when inflammation is not continuously managed for an extended period of time (longer than 6 weeks). Chronic inflammation in tissue happens when inflammatory reactions occur in the absence of an actual stimulus and is frequently related to an overproduction of pro-inflammatory mediators. Cytokines, chemokines, cyclooxygenase-2, prostaglandins (PGs), inducible nitric oxide synthase (iNOS), and nitric oxide (NO) are examples of pro-inflammatory mediators that may be increased.

Numerous chronic illnesses, including cancer, neurological disorders, multiple sclerosis, diabetes, atherosclerosis, arthritis, and cardiovascular diseases, are brought on by excessive levels of pro-inflammatory mediators and components. It has been determined that dysregulated inflammation plays a role in the origin of practically all human illnesses or disorders. Additionally, chronic inflammation brought on by endogenous causes is linked to greater levels of ROS generation, which primarily serve to destroy pathogens but become imbalanced when created in excess (Ricciotti & FitzGerald, 2011). Oxidative stress can cause DNA changes and cell damage, which can signal the activation of the inflammatory process. Inflammatory processes also create ROS, mostly as a result of leukocytes consuming oxygen while employing the NADPH oxidase system in phagocytic cells, which produces superoxide

ions, starting a chain reaction, and producing additional ROS molecules. In a very wide range of inflammatory diseases, both acute and chronic, an iron overload may intensify the harmful consequences of excessive superoxide generation (Cook, 2016). Therefore, a potent anti-inflammatory agent should be able to prevent the onset of inflammation without disrupting the body's natural homeostasis.

Chronic inflammation associated with cancer promotes unrestricted cell division, independent of growth hormones, resistance to growth inhibition, evasion of programmed cell death, accelerated angiogenesis, tumour extravasation, and metastasis. All phases of carcinogenesis are aided by inflammation, which is typically connected to the location of a place with a history of persistent infection or inflammation. Obesity, hyperglycaemia, or excessive lipid build-up can all cause inflammation, which is referred to as "low-grade chronic inflammation" and often has a systemic character. This increases the chance of developing cancer in general, including breast, colon, and liver cancers (Multhoff et al., 2012).

Neurodegenerative disorders including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and multiple sclerosis are all accompanied with inflammation of the central nervous system. Toll-like receptor signalling, which activates transcription factors and generates ROS, causes inflammation and changes in the morphology of microglia. These increase the production of inflammatory mediators like cytokines, boosting proinflammatory signals even more to cause neurotoxic consequences (Chitnis & Weiner, 2017).

Inflammation is also noted to contribute to the onset and manifestation of most cardiovascular diseases and atherosclerosis. In the case of the latter, the inflammatory cascade, which involves the over expression of cytokines and interleukins and the generation of reactive oxygen species, is crucial for the creation, modulation, and advancement of atherosclerotic plaque. The creation and instability of collagen in the fibrous cap are both influenced by inflammation, which also has a significant impact on the functional stability of complex atherosclerotic plaques. Thickening of the fibrous cap causes plaque instability with greater risk for rupture and potential acute events like stroke, which occurs in conjunction with lipid core development (Amin et al., 2020).

The primary purpose of anti-inflammatory drugs is to limit prostaglandin formation, which is known to be a mediator of inflammation, by inhibiting the enzyme cyclooxygenase (COX). Prostaglandins promote vasodilation and microvascular permeability during inflammation,

resulting in the traditional indications of redness and swelling and facilitating angiogenesis, which is a key component in cancer cell survival and reproduction. Cell damage (decreased ROS generation) and malignant cell survival are reduced by blocking this inflammatory pathway using anti-inflammatory drugs (Zarghi & Arfaei, 2011).

1.6.2.5.1. Biosynthesis of Prostaglandins (PGs) in inflammatory response

The production of the inflammatory response is significantly influenced by prostaglandins (PGs). Their production is greatly boosted in inflamed tissue, and they help to produce the classic symptoms of acute inflammation. Prostanoids (PGs and thromboxane A2 (TXA2)) are generated when phospholipases release arachidonic acid (AA), a 20-carbon unsaturated fatty acid, from the plasma membrane and it is processed by the successive activities of PGG/H synthase or cyclooxygenase (COX) and their respective synthases. In vivo, four major bioactive PGs are produced: prostaglandin E2 (PGE2), prostacyclin (PGI2), prostaglandin D2 (PGD2), and prostaglandin F2α (PGF2α). They are widely produced—typically, each cell type produces one or two major products—and operate as autocrine and paracrine lipid mediators in the body to maintain local homeostasis. PG production levels and profiles fluctuate drastically during an inflammatory reaction. In uninflamed tissues, PG synthesis is normally extremely low, but it increases rapidly in acute inflammation prior to the recruitment of leukocytes and the infiltration of immune cells (Ricciotti & FitzGerald, 2011). Figure 1.20 shows the production of PGs from the actions of PGG/H synthase or cyclooxygenase (COX) and their respective synthases.

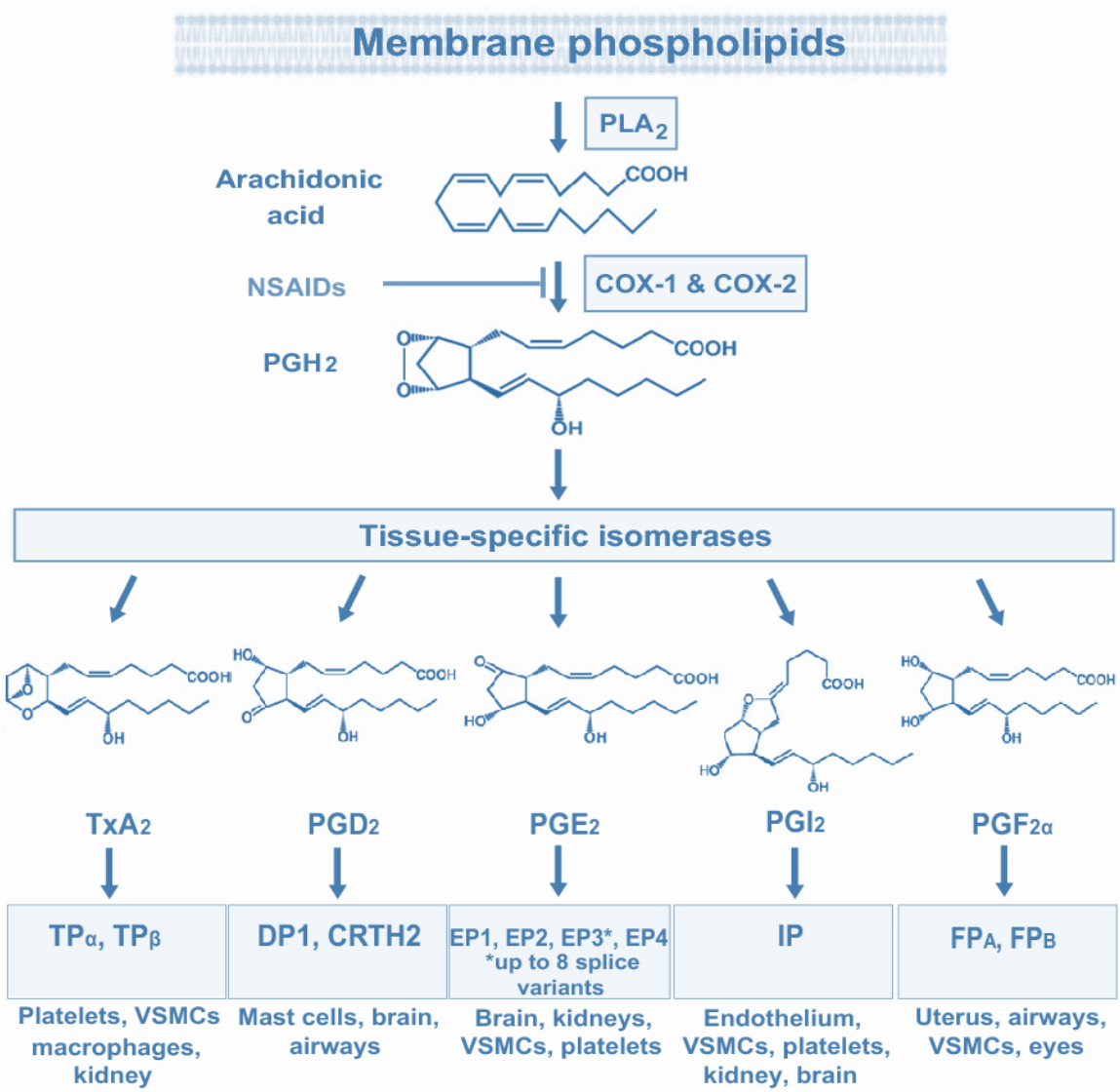


Figure 1.20. Illustration of the Biosynthetic pathway of prostanoids. Adapted from: (Ricciotti & FitzGerald, 2011)

1.6.2.5.2. Nonsteroidal anti-inflammatory medication and COX

Nonsteroidal anti-inflammatory medications (NSAIDs) are a widespread family of anti-inflammatory and analgesic compounds used to treat inflammation-related symptoms by inhibiting COX (1&2). COX-1 is noted to produce prostaglandins which are involved in essential processes such as ovulation, blood clotting, renal function, wound healing, vasomotor tone, platelet aggregation, immunological cell differentiation, nerve development, bone metabolism, and labour initiation. One of the most important tasks of PGs is to maintain the stomach lining intact. This is due to the acidity of the stomach which necessitates the fast replacement of stomach cells within days hence when your PG system is disrupted, stomach

discomfort, digestive tract issues, intestinal or stomach haemorrhage, and even death may occur. However, NSAIDs in their line of action are not selective to COX-1 enzyme as they inhibit COX in general. This tend to have adverse effects on the gastrointestinal lining, coagulation of blood and renal systems and therefore, alternate medication which are selective to COX-1 are desired. (Ayertey et al., 2021).

Nonsteroidal anti-inflammatory medications (NSAIDs) target the two COX isoforms, COX-1, and COX-2 (NSAIDs). These medications are both competitive active site inhibitors of COXs. Although both COXs exist as homodimers, only one partner is employed for substrate binding at a time. COX-1/COX-2 heterodimers may possibly occur, although their biological relevance is unknown. Only one of the monomers of the COX dimer is bound to and inactivated by NSAIDs, which is enough to prevent prostanoid production. The other monomer appears to have an allosteric role. NSAIDs have no effect on the peroxidase capability of either protein (Ricciotti & FitzGerald, 2011).

1.6.3. Methods for Assessment of Antioxidant Activity.

Several assays using various processes, such as hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, and metal chelation, among others, can be used to measure antioxidant activity. It is critical to choose the right method(s) for a reliable assessment of antioxidant potential in the targeted applications by being aware of the basic mechanisms, benefits, and limits of measuring assays (Shahidi & Zhong, 2015). Antioxidant assessment methods can be broadly categorized as chemical assays, food model system evaluations, and biological model systems assays. Each of these categories have a number of assays classified under them. A few of these assays are discussed as follows:

1.6.3.1. Radical/ROS scavenging methods

In straightforward "lipid free" systems, a wide variety of assays are available for the precise determination of hydrogen atom or electron transfer from putative antioxidants to free radicals. The antioxidant activities described by this category of methodologies are typically linked to their ability to scavenge particular radical species, some of which may be synthetic and unrelated to biology. They have so drawn criticism for failing to accurately represent the condition in an in-vivo setting. However, the information on their hydrogen atom or electron donating ability that may be collected using these techniques gives valuable insight into their inherent antioxidant potential with the least amount of external disturbance. These tests do not

need a lipid substrate and typically use a chemical solution that includes an oxidant (free radicals or other ROS), an oxidizable probe (though this is not always required), and the antioxidants that are being tested. These tests may be divided into two groups: those based on the single electron transfer (ET) reaction and those based on the hydrogen atom transfer (HAT) reaction. Regardless of the mechanism involved, antioxidants can neutralize radicals or other ROS (such as hydrogen peroxide and lipid peroxides) through HAT and ET, producing the same effects, albeit the kinetics and risk of side effects vary (Prior et al., 2005).

The prevailing mechanism in a particular system depends on the antioxidant's structure and characteristics, solubility, partition coefficient, and solvent system in the case of proton-coupled ET and HAT reactions. Antioxidant activities are either represented as a suppression of ROS-mediated oxidation of the probe or as equivalents of a chosen reference antioxidant, such as Trolox, ascorbic acid, or another antioxidant. Different detecting technologies that are improving with the accessibility of cutting-edge equipment that can measure the oxidation of the probe. These include, among others, spectrophotometric, fluorometric, chemiluminescent, EPR (electron paramagnetic resonance), FT-IR (Fourier transform infrared), NMR (nuclear magnetic resonance), and amperometric techniques (Shahidi & Zhong, 2015). Some of the major reactive oxygen species (ROS) scavenging assays and redox potential-based assays are summarized in Table 1.2.

Table 1.2. Major ROS Scavenging and Redox Potential Assay for Antioxidant Assessment

Assay	**Main Mechanism**	**Oxidant**	**Probe**	**Detection Technique**
ROS Scavenging assays				
DPPH Scavenging	ET	DPPH radical	DPPH radical	Spectrophotometry or EPR
TEAC (ABTS Assay)	ET	ABTS radical cation	ABTS radical cation	Spectrophotometry
ORAC	HAT	Peroxyl radical generated by AAPH	Fluorescein	Fluorometry

Chemiluminescence	HAT	Hydrogen peroxide	Luminol	Fluorometry
Redox Potential Assays				
FRAP	ET	Fe^{3+}	Ferricyanide	Spectrophotometry
CERAC	ET	Ce^{4+}	Indigo carmine dye	Spectrophotometry
CHROMAC	ET	Cr^{6+}	Cr^{3+} complex	Spectrophotometry
Au^{3+} reducing	ET	Au^{3+}	Au nanoparticle	Cyclic voltammetry
Ag^{+} reducing	ET	Ag^{+}	Ag nanoparticle	Surface plasmon resonance

NB: DPPH = 2,2-Diphenyl-1-picrylhydrazyl, ABTS = 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid), ET = single electron transfer, HAT = hydrogen atom transfer, EPR = electron paramagnetic resonance

1.6.3.2. Metal chelation capacity

Although heavy metals such as copper, iron, and zinc are necessary for many biological activities, such as cell development and reproduction, biomolecule synthesis, several enzymatic reactions, and the body's immune system, an excessive amount of them can be hazardous. They specifically induce oxidative stress (OS) and produce free radicals and reactive oxygen species (ROS) in metabolism. To prevent this, metals may be effectively eliminated from the body after being chelated with the right chelating agents, preventing any harm to metabolism. The interaction between the ligand and a central metal atom creates a complicated ring-like structure, which is the basis for metal ion chelating (Shahidi & Zhong, 2015).

Metal chelation is widely regarded as the most promising and widely used antioxidant technique. Chelation is the process of joining existing ions or molecules of a ligand to a central

metal atom or ion through an acyclic or ring-like coordination bond. A ligand is a molecule or ion having two or more atoms that may easily give two electrons to create a covalent bond. Ligands are categorized into three types based on the nature of the bond between the ligand and the covalent atom. The stability of the complexes varies according to metal ion and ligand interactions. Chemical species bond with varying affinities. In actuality, functional biomolecules can serve as numerous linker site systems. Binding selectivity can therefore be produced by a reagent on two or more substrates or by two or more sites on the same substrate. Electronic and steric variables between receptors and substrates contribute to this. One method for determining chelation capacity is to test free ions using a chelating agent that generates a spectroscopically observable complex. Metal chelators produce compounds that inhibit the reactivity of metals like iron and copper, thereby rendering them inert. As a result, metal chelation capacity is frequently utilized as a measure of antioxidant activity, typically in conjunction with other antioxidant tests (Wettasinghe & Shahidi, 2002).

1.6.3.2.1. Iron Chelating Activity

The capacity of ferrous iron (Fe^{2+}) to transfer a single electron allows for the formation of many radical reactions, even from relatively non-reactive radicals. By creating soluble, stable complexes that are later eliminated through faecal matter, iron chelators help mobilize iron from tissues. Since the ability of bioactives to chelate metal ions is crucial to the antioxidant process, the iron chelating activity test is frequently employed to assess the secondary antioxidant activity of bioactive compounds. Additionally, transition metals like Iron (Fe) cause the Fenton and Haber-Weiss reactions as well as the production of ROS like OH•. The well-known Fenton reaction is a process wherein H_2O_2 and metal ions combine to generate OH•. The Haber-Weiss process, on the other hand, uses ferrous ions as its catalyst to create OH• from O_2• and H_2O_2. It was Fritz Haber and his student who initially proposed the reaction. Later research established that these processes are the primary producers of radicals and the main causes of cellular deterioration (Haber & Weiss, 1934).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \ (\text{Fenton reaction})$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + H_2O + OH^{\bullet} \ (\text{Haber–Weiss reaction})$$

Figure 1.21. Fenton and Haber-Weiss reaction mechanisms Adapted from (Haber & Weiss, 1934).

A stable iron (II) chelate molecule is formed in the presence of iron chelating substances and antioxidants, such as ferrozine, which lowers the concentration of free ferrous ions, inhibits the production of OH•, and lowers the concentration of the transition metal (ferrous ion) that catalyzes lipid oxidation. Be a result, substances with a greater metal chelating activity might also be indirectly referred to as antioxidant substances (Canabady-Rochelle et al., 2015).

1.6.3.2.2. Copper Chelating Activity

It has long been understood that copper ions are necessary in biological processes. Copper ions are integral parts of certain important protein structures and play a key role in catalytic and regulatory processes (Uauy et al., 1998). Copper ions often occur in two oxidation states in biological systems: cupric ($Cu^{2+,}$ oxidized) and cuprous ($Cu^{1+,}$ reduced). Numerous enzymes have used this redox activity for catalysis. Copper has biologically beneficial chemical characteristics, yet they may also be poisonous. Copper participates in redox processes that produce the hydroxyl radical and have the ability to seriously harm lipids, proteins, and DNA (Halliwell & Gutteridge , 1984).

1.6.3.3. Total Phenolic content (TPC)

Total phenolic content (TPC) is a crucial component of total antioxidant capacity (TAC) and is frequently used to assess antioxidant extracts from, among other things, cereals, legumes, fruits, and spices. The widely used technique for determining TPC is the Folin-Ciocalteu test. The Folin-Ciocalteu test, which makes use of the phenolic amino acid tyrosine in proteins, was first developed for the study of proteins (Bonanni et al., 2007).

There have been reports of TPC measurements using techniques other than the conventional Folin-Ciocalteu test. In their 2007 study, Bonanni, et al. showed how to employ a biosensor to detect the presence of phenolic compounds, which tyrosinase converted to quinones while consuming oxygen. The amperometric measurement of oxygen consumption and the presence of phenolic compounds were associated. The same authors also suggested an OXY-adsorbent approach, which makes use of the reactivity of phenolic antioxidants with hyperchlorous acid (HClO) and measures the TPC by spectrophotometrically detecting the excess unreacted hyperchlorous acid through a chromogenous agent (Bonanni et al., 2007)

1. MATERIALS AND METHODS

2.1. Chemicals and kits

Solvents and reagents used for the extraction and analysis of bioactives from *Asparagopsis* spp are tabulated below (Table 2.1).

Table 1.1 Solvent and Reagent list and their respective sources

SOLVENT	SOURCE
Extraction Solvents	
Pure Ethanol 96% v/v (Analytical grade)	Valente e Ribeiro Lda (Alcanena)
Ethyl Acetate (Analytical grade)	Fisher Chemical
Hexane (Commercial grade)	Fisher Chemical
Reagents used for analysis	
Ultra-Pure water (Distilled Water)	Milli-Q® Advantage A10 Ultrapure Water Purification System (Merck, Germany).
DMSO 99.9% (Analytical grade)	Fisher Chemical
EDTA	Fluka Chemika (Buchs, Switzerland)
Iron (II) Chloride	VWR (Portugal)
Ferric Chloride	VWR (Portugal)
Potassium Persulfate	Biochem Chemopharm (Cosne Sur Loire, France)
Ferricyanide	Acros Organics (Geel, Belgium)
Pyrocatechol Violet	Acros Organics (Geel, Belgium)
Ferrozine	Acros Organics (Geel, Belgium)
Trichloroacetic Acid	Sigma-Aldrich (Munich, Germany)
Sodium Acetate	Sigma-Aldrich (Munich, Germany)
DPPH	Sigma-Aldrich (Munich, Germany)
ABTS	Sigma-Aldrich (Munich, Germany)
Copper (II) Sulphate pentahydrate	Merck (Germany)
Sodium Carbonate	Merck (Germany)
Pure Methanol 96% v/v (Analytical grade)	Valente e Ribeiro Lda (Alcanena)
Methanol HPLC grade	Fisher Chemical
Chloroform HPLC grade	Fisher Chemical
Acetonitrile HPLC grade	Fisher Chemical
Folin and Ciocalteu's phenol reagent	Sigma-Aldrich (Munich, Germany)
Assay Kits and Drugs	
Cyclooxygenase (COX) Inhibitor Screening Assay Kit	Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA)
Ibuprofen (≥98% (GC))	Sigma–Aldrich (Munich, Germany)

2.2. Pre-treatment of biomass

All three samples namely, *Asparagopsis armata* (gametophyte), *Asparagopsis taxiformis* (gametophyte), and *Asparagopsis taxiformis* (sporophyte) were dried to remove excess moisture that may have been present in the samples using freeze drying method. The dried

biomasses were then pulverized into fine particulate powder using a ball mill (Retsch PM-100 Ball mill). Parameters employed for the milling are as follows: 20 mm size stainless steel grinding balls and 500 mL stainless steel jar were used. For each 10 g of biomass, an RPM of 450 for 15 min in 3 cycles of 5 min each. Milling time was broken into three cycles of 5 min to prevent the increase of temperature within the system that may lead to the deterioration of any heat sensitive compounds that may be present in the biomass. This pre-treatment phase was critical for ensuring correct biomass mixing and promoting the breakdown of cell walls, allowing for improved solvent access to intracellular components and a more effective extraction. Figure 2.1 shows the ball mill that was used for the milling process.

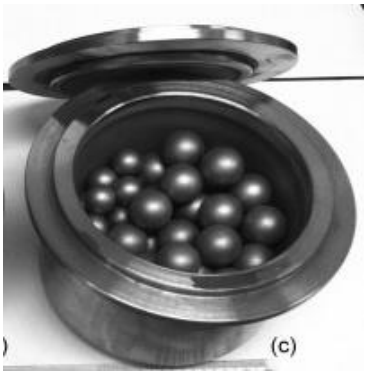
a) Grinding jar and balls	b) Milling unit
	

Figure 2.1. Retsch PM 100 Ball Mill Machine and accessories.

2.3. Solvent Extractions

Solvent-based extraction procedures were used to produce the algal extracts, as solvent-based extraction methods are required for the separation of analytes prior to analysis. When it comes to algal and plant extractions, solvent extraction has shown to be the most practical approach (Grima et al., 2013).

In this work, a brand-new, revolutionary extraction tool for sample extraction called the "EDGE" was employed for the solvent extractions of the biomasses. EDGE is an acronym which stands for Energized Dispersive Guided Extraction. This device is an automated solvent extractor that combines the pressured fluid extraction with the dispersive solid phase extraction techniques.

2.3.1. Configuration and operation of the Energized Dispersive Guided Extraction System (EDGE)

The configuration of the EDGE system includes a position rack for holding glass vials (for collecting extracts) and Q-cups (a cup which holds the biomass to be extracted), a reaction chamber (with a pressure cap and heating core), a syringe attached to a mobile robotic arm, extraction solvent tubes (connected to solvent containers), a waste container (for holding solvent waste), and a functional touchscreen platform for operationality. The apparatus can withstand temperatures of up to 200 ^{o}C, and its maximum pressure depends on the solvent used because heating the solvent increases the pressure inside the vessel chamber. The extraction is carried out in a Q-cup (extraction vessel) with two detachable parts, where a filter sandwich (S1 Q-disc) made of two cellulose filters (high retention capacity but low structural capacity) and a glass filter (high structural capacity) is sandwiched between the two parts to create a universal filter (to avoid any sample particles to block lines).

A *B*

Figure 1.1 EDGE Extraction setup A) EDGE Extractor B) Q-Cup

In the operation of the EDGE system, the user specifies the maximum temperature, solvent type, cycle time, number of cycles (up to five), and cooling temperature for the extraction process. Following the selection and initiation of an extraction process, the EDGE robotic arm picks up the selected Q-cup and transports it to the reaction chamber, where the pressure cap establishes a pressured seal on the top of the Q-Cup. When the reaction chamber is closed, the appropriate solvent is introduced, and the extraction begins at the specified temperature, time, and cycle number. As the reaction chamber walls heat up and the pressure inside the reaction chamber rises, the pressurized fluid extraction component is obtained, pushing the solvent to disperse into the sample. The addition of solvent from both the top and bottom results in the dispersive effect as well. The extracted material is automatically transferred to the appropriate

collecting container. The robotic arm then returns the Q-cup to its original position. Additionally, EDGE has the capacity to carry out supported liquid extraction and liquid-liquid extraction. With EDGE, it should be feasible to "reduce extract preparation time and produce rapid, simple, and efficient extractions" based on these concepts (CEM Corporation, 2019).

1.6.4. Extraction of *Asparagopsis* spp. extracts.

Extraction of the *Asparagopsis* biomasses was done using the EDGE extractor. The EDGE extraction process was optimized for number of extraction cycle for each solvent employed (Ethanol, Hexane, and Ethyl Acetate) to ensure that a good extract yield was obtained. Data from the optimization indicated that for each solvent that the most appropriate number of cycles was three. The parameters employed for the extraction are displayed in Table 2.2. The powdered biomass was put into the Q-Cups pre-fitted with a specialized filter paper and sand to enhance the surface area and give more room for solvent-biomass interaction. Biomass and sand were installed in the Q-Cups in the ratio of 1:1 (w/w) for each case. Every other parameter was set as shown in Table 2.2, and the extraction was carried out. The temperature, time of extraction and ratio of biomass: solvent as stated in Table 2.2 were specifically selected to ensure optimal yield of extracts were obtained from the biomass without any form of thermal degradation taking place. Between extractions, the EDGE solvent lines were washed with the next solvent to be used. After the extraction, the collecting vial was immediately removed from the rack and covered with aluminium foil as well as placed in an ice-filled box to prevent photo- and thermal degradation of sensitive compounds that may be present in the extract. The extracts were then transferred into a pre-weighed round bottom flask and the solvents evaporated by means of a rotary evapourator. The concentrated extract was further dried under a gentle nitrogen flow until constant weight. They were then transferred into amber vials and stored in the fridge at a temperature of -18 ^{o} C until analysis.

Table 1.2. Extraction Parameters

Biomass Name	*Asparagopsis* spp.			
Biomass General Code	ATg-P, ATs-S, AAg-P			
Pre-treatment Conditions	*Ball milling (RPM 450 /15mins in 3 cycles of 5 mins each)*	Ethanol	Ethyl Acetate	Hexane
Extraction conditions	*Temperature*	30°C	30°C	30°C
	Pressure	28 psi	28 psi	28 psi
	Hold time per cycle	10 mins	10 mins	10 mins
	Volume of solvent per cycle	20mL	20mL	20mL
	Total number of cycles	3	3	3
Evapouration conditions	*Temp = 40°C*	40°C	40°C	30°C *
	Pressure = 175 mbar (Ethanol)	175 mbar	240 mbar	260 mbar *
	RPM= 110	110	110	110
Drying Conditions	*Time under nitrogen flow*	until con. Mass	until con. Mass	until con. Mass

2.4. Antioxidant Assay

Prior to all antioxidant assays, dimethyl sulfoxide (DMSO) was used to resuspend the dried extracts at a concentration of 20 mg/mL. This stock solution was stored at a temperature of -18 °C and during the time of analysis serial dilutions were made in a concentration range from 5 mg/mL to 0.16 mg/mL.

2.4.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

One of the most used techniques and the initial tool for assessing antioxidant activity is the DPPH radical scavenging test. It is an ET-based approach, and the assay only uses the HAT mechanism as a minor reaction route (Prior et al., 2005).

The stable chromogen radical DPPH has a deep purple colour. The DPPH scavenging test relies on antioxidants to provide electrons to counteract the DPPH radical. The discoloration of the DPPH to a yellowish colour at a range from 515 nm to 517 nm, which occurs together with the

reaction, serves as a gauge of the effectiveness of the antioxidant. Mechanism of this reaction is illustrated in figure 2.2.

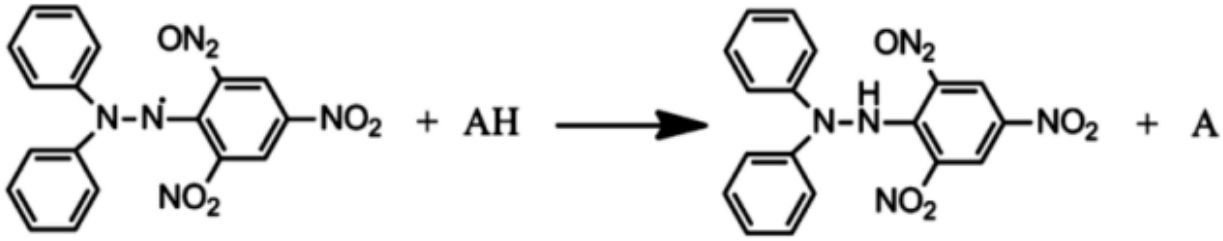


Figure 1.2. Reduction of DPPH by an Antioxidant. (Retrieved from Marbiotech DPPH Assay Protocol)

The IC_{50}, which is defined as the effective concentration of the antioxidant required to reduce the initial DPPH concentration by 50%, is frequently used to measure the antioxidant activity via DPPH scavenging. The DPPH test is a straightforward procedure that just requires a UV spectrophotometer or an EPR (electron paramagnetic resonance) spectrometer. However, it has been noted that the DPPH scavenging process does not mirror the radical scavenging mechanism of antioxidants in actual food or biological systems since the assay lacks oxygen radicals (Benzie & Strain, 1999). As a result, this technique is primarily dependent on the notion that antioxidant activity is proportional to electron donating capacity, also known as reducing power.

In this research work, DPPH assays were carried out in 96-well microplates in sextuplicate for 3 consecutive days (n=3) at room temperature. All spectrophotometric measurements were performed using a Synergy HT MultiDetection Microplate Reader (BioTec Instruments, Inc., USA). To each well, 200 µL of the DPPH solution (0.1 mM DPPH in methanol) and 22 µL of the sample, with concentrations ranging from 1.6 mg/mL to 5 mg/mL, were added.

The positive control was gallic acid (2 mg/mL), while the negative control was DMSO, each added to the DPPH solution instead of the sample. Two hundred microlitres of methanol was used as a colour control, and 22 µL of sample was also added. The colour control was used to ensure that the natural colour (if any) of the sample being analysed does not interfere with the spectrophotometric readings as the absorbance is dependent on colour intensity of the samples. To eliminate this interference, the colour control absorbance is subtracted from the overall absorbance of the samples under analysis. A 30-minute period of darkness and room temperature incubation were given to the reaction mixture. At 517 nm, discoloration was

measured in sextuplicate. Equation 1 was used to compute the percentage of DPPH radical scavenging activity (A_{DPPH}, %).

$$A_{DPPH}, \% = \frac{A_O - A_1}{A_O} \times 100\% \qquad (Equation\ 1)$$

where A_O = absorbance of the negative control,

A_1 = absorbance of the extract corrected for the absorbance of the colour control.

in the event where radical scavenging activity was higher than 50% at a concentration of 5 mg/mL (DMSO), six serial dilutions in the ranges of 5 mg/mL DMSO to 0.1 mg/mL were performed, and the IC_{50} was calculated.

2.4.2. 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) Assay

The ABTS radical scavenging test is a commonly used spectrophotometric technique for determining the antioxidant activity of various phenolic compounds present in complicated biological matrices. The test assesses antioxidants' capacity to scavenge the stable radical cation $ABTS^{\bullet+}$ (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)), a blue-green chromophore with maximum absorption at 734 nm that reduces in intensity when antioxidants are present. Antioxidants can neutralize the radical cation $ABTS^{\bullet+}$ either by direct reduction via electron donation or through radical quenching via hydrogen atom donation, and the balance of these two methods is mainly regulated by antioxidant structure and medium pH (Prior et al., 2005). This process is fast, and the end result is used to calculate antioxidative efficiency. The reaction mechanism is shown in figure 2.3.

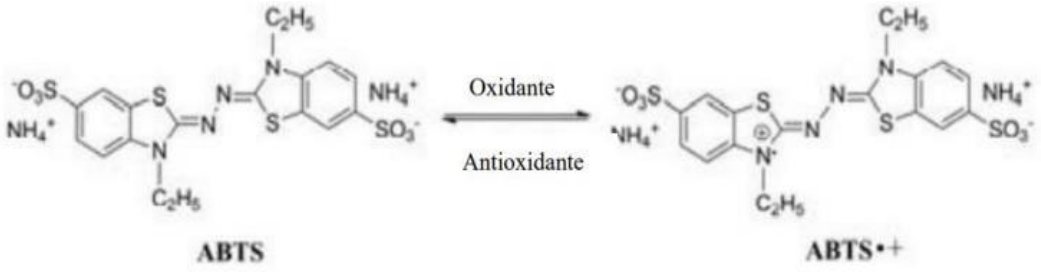


Figure 1.3. ABTS Assay reaction mechanism. (Retrieved from Marbiotech ABTS Assay Protocol)

The time of the reaction, the intrinsic antioxidant activity, and the quantity in the sample all affect how much the blue-green colour fades, as measured by a drop in absorbance at 734 nm.

In this work, ABTS assays were carried out in 96-well microplates in sextuplicate for 3 consecutive days (n=3) at room temperature. The ABTS test was carried out using the method developed by Re et al. (1999), in which a 7 mM aqueous ABTS solution was mixed with a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution to produce the radical ABTS•+. The radical cation solution was further diluted in ethanol until an absorbance value of 0.7 at 734 nm was obtained. The test was carried out in a microtiter plate containing 190 µL of ABTS solution and 10 µL of sample at concentrations ranging from 0.16 mg/mL to 5 mg/mL. The positive control in each case was gallic acid (2 mg/mL), whereas the negative control was DMSO. Equation 1 was once again used to compute the percentage of ABTS radical scavenging activity (A_{ABTS}, %).

$$A_{ABTS}, \% = \frac{A_O - A_1}{A_O} \times 100\% \quad (Equation\ 1)$$

whereA_O = absorbance of the negative control,

A_1 = absorbance of the extract corrected for the absorbance of the colour control.

in the event where radical scavenging activity was higher than 50% at a concentration of 5 mg/mL (DMSO), six serial dilutions in the ranges of 5 mg/mL DMSO to 0.1 mg/mL were performed, and the IC_{50} was calculated.

2.4.3. Metal Chelating Activity

2.4.3.1. Iron chelating Activity

The Ferrozine assay is a quick and reliable colorimetry assay for measuring iron levels. When linked to ferrous iron, the chelator 3-(2-pyridyl)-5,6-bis (4-pheylsulfonic acid)-1,2,4-triazine (Ferrozine) absorbs at a range of 550 nm to 562 nm, enabling measurement (Fish, 1988). By generating complexes with ferrous iron that are pinkish-red in colour and that significantly absorb at 550 nm, ferrozine is frequently used to determine the amount of iron in biological samples. The Fe (II)-ferrozine complex is disrupted in the presence of chelating agents, which results in a reduction in the red colour (Canabady-Rochelle et al., 2015). The measurement of colour reduction enables assessment of the concurrent chelator's chelating activity. The reaction mechanism is illustrated in figure 2.4.

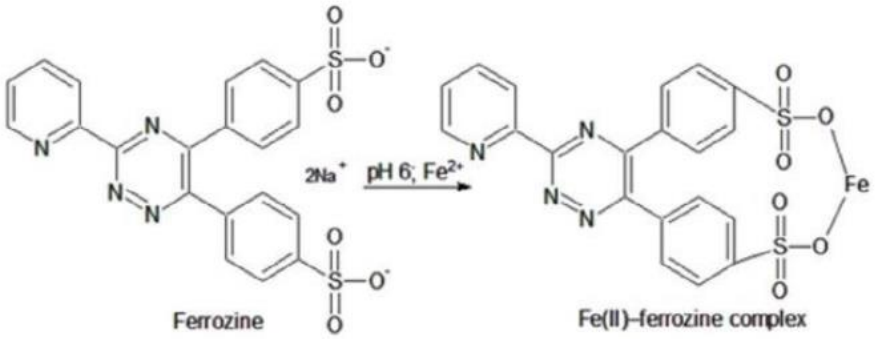


Figure 1.4. Reaction mechanism in an Iron chelating Activity Assay (Retrieved from Marbiotech Laboratory Protocol)

According to the procedure described by (Dinis et al., 1994), the iron (II) binding capacity of the chelators was assessed on microtiter plates, with the exception that ferrous sulphate was replaced with ferrous chloride. In a nutshell, 200 µL of distilled water, 30 µL of extract working concentrations (ranging from 0.16 to 5 mg/mL), and 30 µL of ferrous chloride (0.8 mM) were added to each well. The mixture was then left to sit at room temperature for 30 minutes. Then, 12.5 µL of ferrozine (40 mM) was added to each well to initiate the reaction. The finished combination was let to sit at room temperature for 10 minutes. The solution's absorbance was measured at 562 nm. Positive control was provided by the synthetic metal chelator EDTA (1 mg/mL). Equation 1 was here also used to compute the percentage of iron chelating activity ($I_{\text{Iron Chelating}}$, %).

$$\text{Iron Chelating}, \% = \frac{A_O - A_1}{A_O} \times 100\% \qquad (Equation\ 1)$$

where A_O = absorbance of the negative control,

A_1 = absorbance of the extract corrected for the absorbance of the colour control.

in the event where iron chelating activity was higher than 50% at a concentration of 5 mg/mL (DMSO), six serial dilutions in the ranges of 5 mg/mL DMSO to 0.1 mg/mL were performed, and the IC_{50} was calculated.

2.4.3.2. Copper Chelating Activity

The copper chelating activity was determined using the pyrocatechol violet (PV) method developed by (Saiga et al., 2003) in which the complex of PV with $CuSO_4$ is blue, and the colour changes to yellow when PV dissociates a Cu ion in the presence of chelating agents, so the copper chelating activity can be estimated by measuring the rate of colour reduction.

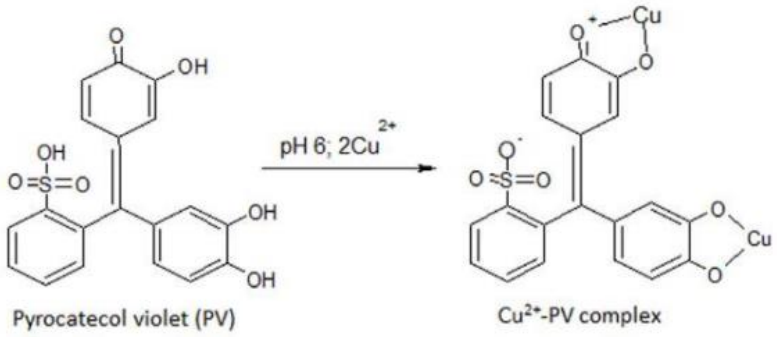


Figure 1.5.Formation of Cu^{2+} - PV Complex (Retrieved from Marbiotech Laboratory Protocol)

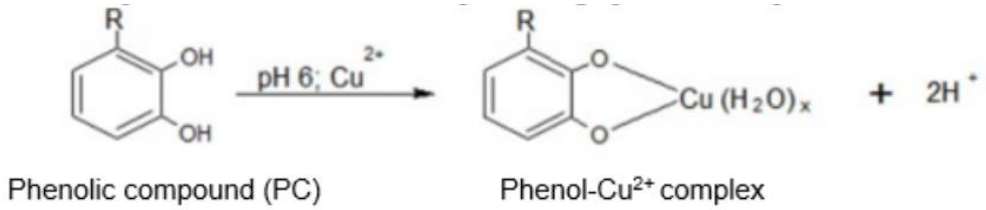


Figure 1.6. Formation of Cu^{2+} - Antioxidant Complex (Retrieved from (Santos , et al., 2017))

In a 96-well microplate, samples (30 µL at concentrations ranging from 0.16 to 5 mg/mL) were combined with 200 µL of Na acetate buffer (50 mM, pH 6), 6 µL PV (4 mM) in the same buffer, and 100 µL $CuSO_4$. The synthetic metal chelator EDTA (1 mg/mL) was employed as a positive control, whereas DMSO was utilized as a negative control. A microplate reader was used to measure the change in colour of the solution at 632 nm. Equation 1was used to compute the percentage of iron chelating activity ($A_{\text{copper Chelating}}$, %).

$$\text{Copper Chelating}, \% = \frac{A_O - A_1}{A_O} \times 100\% \qquad (Equation\ 1)$$

where A_O = absorbance of the negative control,

A_1 = absorbance of the extract corrected for the absorbance of the colour control.

in the event where copper chelating activity was higher than 50% at a concentration of 5 mg/mL (DMSO), six serial dilutions in the ranges of 5 mg/mL DMSO to 0.1 mg/mL were performed, and the IC_{50} was calculated.

2.4.4. Ferric reducing antioxidant power (FRAP) assay

The FRAP test is a typical ET-based technique that assesses the degree to which antioxidants in acidic environment reduce ferric ion (Fe^{3+})-ligand complex to the strikingly blue ferrous (Fe^{2+}) complex. Increases in absorbance at 593 nm are used to measure antioxidant activity, and the results are represented as micromolar equivalents of Fe^{2+} or in relation to an antioxidant standard (Antolovich et al., 2002). In contrast to other ET-based techniques, the FRAP test is conducted in an acidic pH environment (pH 3.6) to retain iron solubility and, more critically, to promote electron transfer (Hegerman et al., 1998). This will raise the redox potential and change the reaction mechanism that is dominant. Tripyridyltriazine (TPTZ) is the iron-binding ligand used in the original FRAP test, but other ligands, such as ferrozine for the assessment of ascorbic acid reducing power, have also been used (Molina-Diaz et al., 1998).

The ferric reagent most often employed in FRAP experiments in recent years has been potassium ferricyanide. The final product in the latter scenario, Prussian blue, is measured spectrophotometrically and shows the reducing power of the antioxidants examined. Prussian blue may be produced using two distinct methods and yet have the same effect. Either the ferricyanide is reduced to ferrocyanide by antioxidants, which binds the free Fe^{3+} in the solution, or the ferricyanide is reduced to ferricyanide by antioxidants, which binds the free Fe^{3+} in the solution, yielding Prussian blue (Berker et al., 2010). The simplified strategy for these two reactions is shown in figure 2.7.

$$\text{Antioxidant} + Fe^{3+} \rightleftarrows Fe^{2+} + \text{oxidized antioxidant}$$

$$Fe^{2+} + Fe(CN)_6{}^{3-} \rightleftarrows Fe[Fe(CN)_6]^{-} \text{ or,}$$

$$\text{Antioxidants} + Fe(CN)_6{}^{3-} \rightleftarrows Fe(CN)_6{}^{4-} + \text{oxidized antioxidant}$$

$$Fe(CN)_6{}^{4-} + Fe^{3+} Fe[Fe(CN)_6]^{-}$$

Figure 1.7. FRAP Mechanism (adapted from (Berker et al., 2010))

The FRAP assay does not need specific equipment and is easy, quick, and economical. Its usage has been expanded to include evaluating the antioxidant activity of different bodily fluids, foods, and plant extracts. It was first used to quantify the reducing power in plasma. A

modified version of this assay as described by (Oyaizu, 1986) was employed in this work. A 96-well microplate was filled with samples (50 µL, at concentrations ranging from 0.16 to 5 mg/mL), 50 µL of 0.2 M phosphate buffer (pH 6.6), and 50 µL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. After adding 50 µL of 10% trichloroacetic acid and 10 µL of 0.1% ferric chloride, the plates were incubated at 50 °C for an additional 10 minutes. As a positive control, gallic acid (2 mg/mL) was utilized. At 700 nm, absorbance was measured. Equation 2 below was used to compute the iron reducing capacity (R_{Fe}, %).

$$\text{Fe Reducing Capacity } = 100 - \left(\frac{A_O - A_1}{A_O}\right) \times 100\% \qquad (Equation\ 2)$$

where A_O = absorbance of the negative control,

A_1 = absorbance of the extract corrected for the absorbance of the colour control.

in the event where Fe reducing capacity was higher than 50% at a concentration of 5 mg/mL (DMSO), six serial dilutions in the ranges of 5 mg/mL DMSO to 0.1 mg/mL were performed, and the IC_{50} was calculated.

2.5. Anti-inflammatory Activity Assay

2.5.1. Cyclooxygenase (COX) Inhibiting Activity

Utilizing a COX Inhibitor Screening Assay Kit per manufacturer's instructions, the COX inhibition assay was performed. With the use of $SnCl_2$ reduction of COX-derived PGH2, which is a product of the COX reaction, this kit monitors variations in PGE2 levels. The direct COX products PGG2 and PGH2 are unstable, hence this is a typically used approach. Using a widely specific antiserum that binds to all the main PG components, the prostanoid product is measured using an enzyme immunoassay (ELISA). Recombinant human COX-2 was the enzyme employed in the test.

The AChE competitive ELISA technology is based on PGs and a PG-AChE conjugate (hence referred to as PG tracer) competing for a small quantity of PG antiserum. The quantity of PG tracer that can bind to the PG antiserum will be inversely proportional to the concentration of PG in the well since the amount of PG tracer is maintained constant while the amount of PG fluctuates. A mouse monoclonal anti-rabbit antibody that has previously adhered to the well wall is what the rabbit antiserum PG (free or tracer) complex binds to (pre-coated 96-well plate). After washing the plate to get rid of any unbound reagent, the well is filled with Ellman's Reagent, which includes an AChE substrate. By assessing the tracer's AChE activity with

Ellman's Reagent, which is made up of acetylthiocholine and 5,5'-dithio-bis(2-methoxyethyl) (2-Nitrobenzoic Acid). Thiocholine is created when AChE breaks down acetylcholine, and 5-thio-2-nitrobenzoic acid is created when thiocholine interacts non-enzymatically with 2,5'-dithio-bis (2-nitrobenzoic acid). The end result of this enzyme process is clearly yellow in colour and significantly absorbent at 412 nm. The quantity of PG tracer attached to the well is inversely proportional to the amount of free PG present in the well throughout the incubation, and this relationship is used to quantify the colour intensity using spectrophotometry. Therefore, less PG tracer will be able to bind to the antibody linked to the well and less yellow colour will be seen when the greater the PG concentration in a sample.

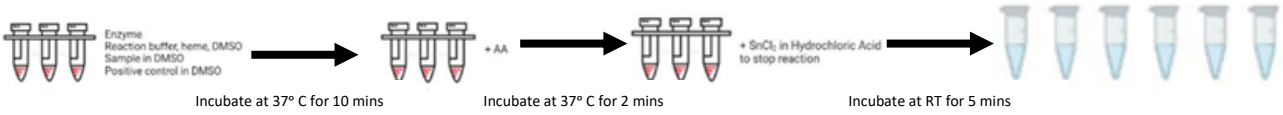


Figure 1.8 PGE_2 Production

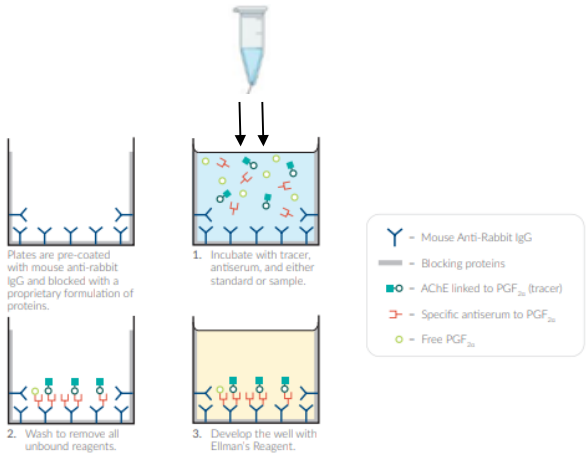


Figure 1.9. PGE_2 Quantification Adapted from: (Cayman Chemical Company, 2018)

In a nutshell, Heme (0.065 mM) and the reaction solution (0.1 M Tris-HCl buffer, pH 8.0, including 5 mM EDTA and 2 mM phenol) were put in reaction tubes. The reaction tubes were divided into three types: background tubes (BCK), 100% Initial Activity tubes (100% IA), and Sample Inhibition tubes (SI). 10 µL inactivated enzyme (enzyme in boiling water for 3 minutes), 160 µL reaction buffer, and 10 µL heme are combined in the BCK tubes. 160 µL reaction buffer, 10 µL heme, 10 µL of the enzyme in issue, and 10 µL DMSO are introduced to the 100% IA tubes. 160 µL reaction buffer, 10 µL heme, 10 µL inhibitor (1 mg/mL and 0.1 mg/mL DMSO), 10 µL DMSO, and 10 µL enzyme are introduced to the SI tubes. The tubes

were incubated at 37 °C for 10 minutes. The tubes were then incubated for two minutes with 10 µL of the arachidonic acid and potassium hydroxide combination at a final concentration of 200 M. 30 µL of an HCl and stannous chloride solution (final concentration of 0.13 M) was used to halt the reaction, trap the reaction product, and reduce it to a more stable state. At room temperature, the tubes underwent one last incubation for five minutes.

ELISA Buffer was used to dilute each BCK, 100% IA, and SI reaction tube, yielding a 1:100 dilution of the original material (BC tubes). While 100% IA and SI tubes were still diluted twice to produce dilutions of the original sample of 1:2.000 and 1:4000, BCK tubes were no longer diluted (IA2, IA3 and SI2 SI3, respectively). After diluting the reaction liquid to 1/4000, the enzyme immunoassay (ELISA) of the same kit was used to quantify the concentration of prostaglandin PGF2α (the end product), which was then derived from a standard curve.

The plate design contained wells for blanks, total activity, nonspecific binding, maximal binding, background COX (1:2000 and 1:4000 dilutions), standards, and inhibitor dilutions in the provided ninety-six well plate coated with mouse anti-rabbit IgG. (1:2000 and 1:4000). Except for the total activity well, different quantities of sample, ELISA buffer, tracer, and antiserum were applied to each well. The plate was wrapped in plastic wrap and incubated in the dark at room temperature at 400 rpm on an orbital shaker for eighteen hours (Eppendorf Thermomixer comfort). When the plate was ready for development, it was washed five times with wash buffer, developed with Ellman's Reagent, added 5 µL of tracer to the total activity well, covered with plastic film, and read on a microplate reader at 410 nm and 420 nm, when maximal binding wells exhibited absorbance 0.3-0.8 A.U. With kinetic readings every five minutes, the microplate reader (Biotek Synergy Neo2, BioTek Instruments, USA) was set on orbital shaking for 40 minutes.

In total, 9 extracts (*A. armata*- ethanol, *A. armata*- hexane, *A. armata*- ethyl acetate, *A. taxiformis* (gametophyte)- ethanol, *A. taxiformis* (gametophyte)- hexane, *A. taxiformis* (gametophyte)- ethyl acetate, *A. taxiformis* (sporophyte)- ethanol, *A. taxiformis* (sporophyte)- hexane, *A. taxiformis* (sporophyte)- ethyl acetate) were tested at a concentration of 1 mg/mL, with 4 analytical replicates. The positive control used was Ibuprofen (≥98% (GC)) from Sigma–Aldrich (Munich, Germany) with different concentrations (5-25 µg/mL).

By subtracting the average values of nonspecific binding and maximal binding from the samples' absorbance values, the percent inhibition of the samples was calculated. Determine the percentage sample or the maximum or standard bound. The COX sample levels were

multiplied by the dilution parameters (BC = 100; IA2 = 2.000; and IA3 = 4000). Taking the BC values away from the IA2, IA3, and SI2, SI3 values in order to get the percent inhibition, remove each inhibiter's sample from the sample with 100% starting activity, divide the result by 100% initial activity, and then multiply by 100.

$$\%Inhibition = \frac{100\%\ Initial\ Activity - Sample\ Activity}{100\%\ initial\ Activity}\ X\ 100\ \ (equation\ 3)$$

2.6. Total phenolic content (TPC)

The reduction of the Folin-Ciocalteu reagent by phenolic compounds in an alkaline environment is the foundation of the Folin-Ciocalteu test. The Folin-Ciocalteu reagent's precise chemical makeup is unknown, although it is thought to contain complexes of phosphomolybdic and phosphotungstic acids, which when reduced, produce a blue chromophore with a maximum absorption wavelength of 765 nm. It is generally acknowledged that the complex's molybdenum centre serves as the reduction site, where the Mo^{6+} ion is converted to Mo^{5+} by absorbing an electron from the phenolic antioxidant. Folin-Ciocalteu test is an ET-based assay that measures the phenolic antioxidants' ability to reduce a substance. TPC can also be used to quantify the amount of phenolic compounds present in a sample and in this case a calibration curve of a known standard at different concentrations is necessary to be developed for the computation. Gallic acid is a frequently used benchmark, and TPC values are typically given as gallic acid equivalents.

F-C reagent was diluted at a dilution factor of 10. Five microlitres of each extract at a concentration of 10 mg/mL was mixed with 100 µL of the diluted F-C reagent in 96-well microplates. 100 µL of Sodium Carbonate (75 g/L in distilled H_2O) was added to each well and the plate was incubated for 90 min at room temperature. The absorbances was read at 725 nm. Gallic acid at concentration range of 0.00098 mg/mL to 2 mg/mL was used to develop a standard curve. The total phenolic content of each extract was then determined using the standard curve.

2.7. Statistical Analysis

All calculations, charts, and graphs were done using Microsoft excel (Office 365). The data obtained was tested for homogeneity and then treated to eliminate outliers using the interquartile range method. The data was then analysed statistically using the statistical toolpak in Microsoft excel and GraphPad prism (version 9.3.0) for the analysis of variance (ANOVA).

For multiple statistical comparison, a Tukey test; $P<0.05$ (95% Confidence Interval), was conducted with GraphPad Prism to access the difference between samples. IC_{50} which is defined as the effective concentration of the antioxidant required to achieve half maximal inhibitory activity was determined using GraphPad Prism and the data analysis toolpak of Microsoft excel. Multivariate data analysis in the form of principal component analysis (PCA) and cluster analysis (CA) were conducted using the software Unscrambler (version X 10.4., 2016) from Camo Analytics. The results were then and discussed.

3. RESULTS AND DISCUSSIONS

3.1. Extraction Cycle optimization

Previous work done using the Energized Dispersive Guided Extraction (EDGE) for algal extraction as reported by Martins Silva M. (2021) showed that 20 mL of solvent and 10 minutes of extraction time per each gram of biomass used was effective for the extraction of bioactives from microalgae. Temperature was also reported to have a positive linear relation on the extraction yield. However, the drawback of high temperature use for the extractions is the possible degradation of thermally sensitive compounds that may be present in the extract thus affecting bioactivity (Martins Silva M., 2021).

Based on this and the fact that the nature of compounds that may be present in the *Asparagopsis* extract is unknown, the lowest and yet effective extraction temperature possible with this method was used, that is 30 ^{o}C. With all these parameters being accounted for, the method was then optimized for the number of extraction cycles necessary for each solvent. Experiments for the cycle optimization were carried out in triplicates and average results are reported with standard deviation (Error bars) in figures 3.1, 3.2, and 3.3.

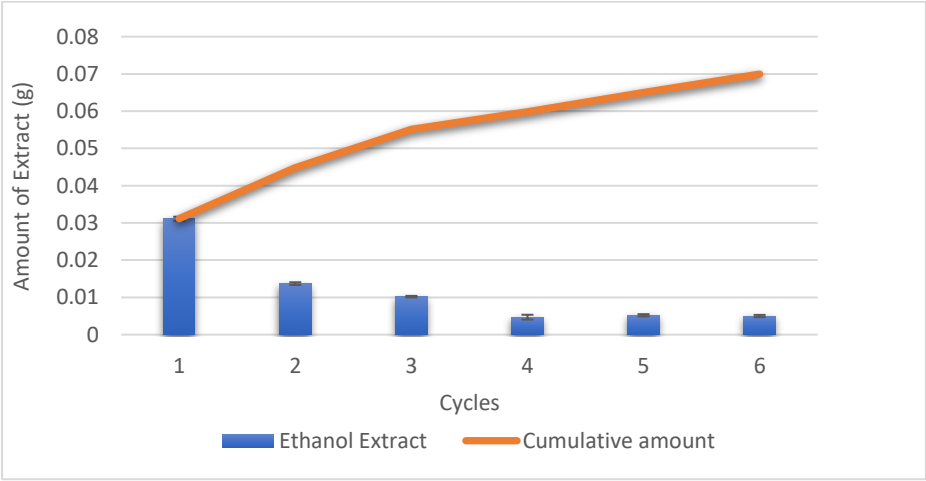


Figure 0.1.Ethanol extraction profile showing the average extract obtained in each extraction cycle with ethanol and cumulative amount of extracts obtained

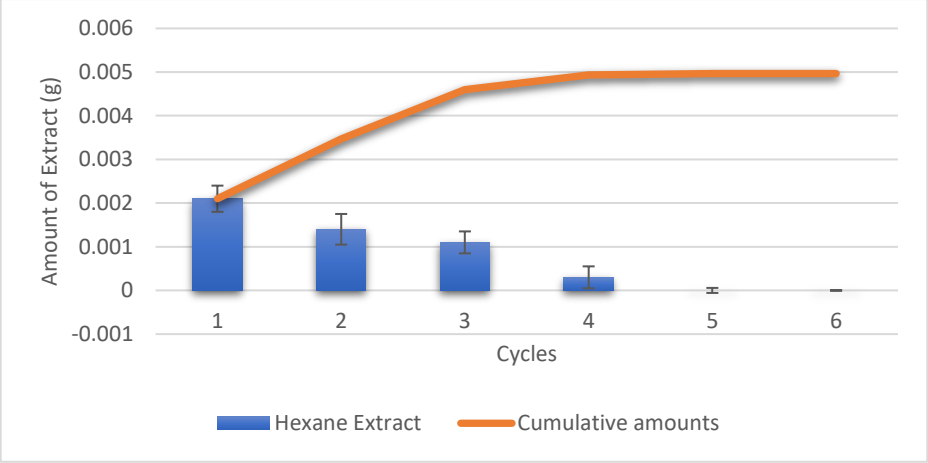


Figure 0.2. Hexane extraction profile showing the average extract obtained in each extraction cycle with hexane and cumulative amount of extracts obtained

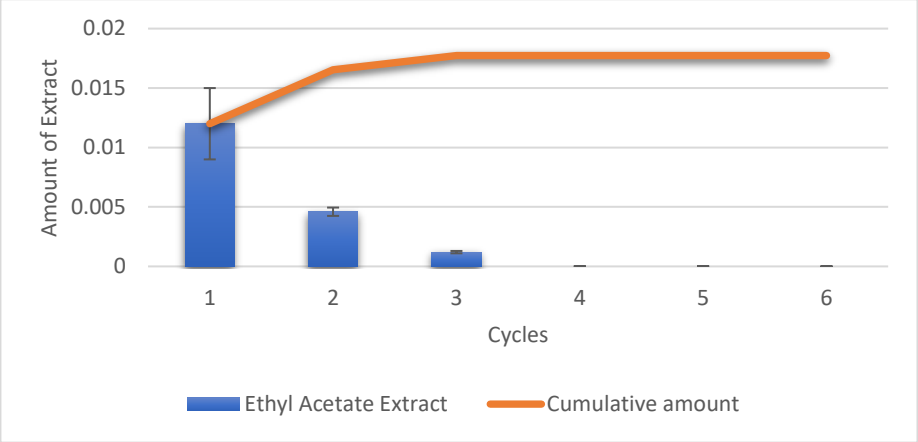


Figure 0.3. Ethyl acetate extraction profile showing the average extract obtained in each extraction cycle with ethyl acetate and cumulative amount of extracts obtained

It was observed that the yield of extracts obtained from the biomass decrease with increasing number of extraction cycle for each solvent employed (Ethanol, Hexane, and Ethyl Acetate). Visual inspection of the data suggests that after the third cycle, extractions yielded very small amounts of extract which are comparatively insignificant to the values of extracts obtained among the first three extraction cycles. From this data, three was determined to be the optimum number of cycles to be employed for the extraction process across all solvents.

3.2. Quality Parameters for Extraction (Repeatability, and Inter day Precision)

Quality parameters such as pooled repeatability, grand mean and inter day precision were computed for the extractions carried out. Extraction data for three consecutive days of extraction for each solvent in the case of each of the three specimen was used. However, in the

case of *Asparagopsis taxiformis* (sporophyte), inter day precision and pooled repeatability was not calculated as multiple day extractions could not be carried out due to the limited quantity of biomass. Instead, the standard deviation and relative standard deviations were calculated. Formulae used for the computations are stated as follows:

$$Mean(X) = \frac{sum\ of\ extraction\ yield}{number\ of\ extractions} \quad (Equation\ 6)$$

$$\text{Sample Standard deviation (S)} = \sqrt{\frac{\sum(x-X)^2}{n-1}} \quad (Equation\ 7)$$

$$\%\ Relative\ Standard\ Deviation(RSD) = \frac{S}{mean} \times 100 \quad (Equation\ 8)$$

$$Repeatability_{pooled} = \sqrt{\frac{(n_1 - 1)RSD_1^2 + (n_2 - 1)RSD_2^2 + \ldots + (n_k - 1)RSD_k^2}{n_1 + n_2 + \ldots + n_k - k}} \quad (Equation\ 9)$$

$$Inter\ day\ Precision = \frac{S_{inter\ days}}{Grand\ mean} \quad (Equation\ 10)$$

Where x = yield

X = yield mean

n = Total number of extractions

k = the nth term / number of terms

$S_{inter\ days}$ = Standard deviation across days

Grand mean = mean of individual day means

Results of the quality parameters are displayed in Tables 3.1, 3.2, and 3.3.

Table 0.1. Quality parameters for Asparagopsis taxiformis (gametophyte)

Asparagopsis taxiformis (gametophyte)									
	Ethanol Extract			Ethy Acetate Extract			Hexane Extract		
	Day 1	Day 2	Day3	Day 1	Day 2	Day3	Day 1	Day 2	Day3
Mean %Yield within Day	9.00	7.92	9.23	2.55	3.37	3.20	1.14	1.14	1.11
Standard deviation Within Day (±)	0.47	0.42	0.82	0.25	0.20	0.34	0.07	0.09	0.07
RSD Within Day (±)	0.05	0.05	0.09	0.10	0.06	0.11	0.06	0.08	0.06
% RSD (Repeatability Within Day) (±)	5.21	5.33	8.89	9.89	5.98	10.69	5.88	8.05	5.91
Pooled Reapeatability (Multiple days) (±%)	6.70			8.98			6.73		
Grand mean	8.72			3.04			1.13		
Standard deviation across days (±)	0.70			0.44			0.02		
Inter Day Precision (±%)	7.98			14.34			1.41		

Table 0.2. Quality parameters for Asparagopsis armata (gametophyte)

Asparagopsis armata (gametophyte)									
	Ethanol Extract			Ethy Acetate Extract			Hexane Extract		
	Day 1	Day 2	Day3	Day 1	Day 2	Day3	Day 1	Day 2	Day3
Mean %Yield within Day	13.54	12.13	15.58	2.44	2.93	2.83	0.36	0.37	0.38
Standard deviation Within Day (±)	0.79	0.88	1.81	0.15	0.16	0.11	0.02	0.01	0.01
RSD Within Day (±)	0.06	0.07	0.12	0.06	0.06	0.04	0.05	0.04	0.02
% RSD (Repeatability Within Day) (±)	5.86	7.27	11.64	6.12	5.50	3.88	5.10	3.95	1.64
Pooled Reapeatability (Multiple days) (±%)	8.61			5.07			3.74		
Grand mean	13.75			2.74			0.37		
Standard deviation across days (±)	1.74			0.26			0.01		
Inter Day Precision (±%)	12.62			9.36			2.40		

Table 0.3. Quality parameters for Asparagopsis taxiformis (Sporophyte)

Asparagopsis taxiformis (sporophyte)			
	Ethanol	Ethyl Acetate	Hexane
Mean % yield within Day	6.77	1.79	1.03
Standard deviation Within Day (±)	2.06	0.06	0.17
RSD Within Day (±)	0.30	0.03	0.17

Pooled repeatability values calculated for *A. taxiformis* (gametophyte) and *A. armata* (gametophyte) for all three solvents show values all well below ± 15% which implies that there is a good degree of consistency in the extraction method employed. The best pooled repeatability was observed with the hexane extraction of *A. armata* (gametophyte) specimen with a ± 3.74% pooled repeatability score and the worst was with the ethyl acetate extraction of *A. taxiformis* (gametophyte) at ± 8.98%. Once again, the individual daily repeatability scores were observed to be well below ± 15% for the two species in question with the best score

observed in *A. armata* hexane extraction at ± 1.64% and the worse observed with *A. armata* ethanol extraction at ± 11.64%. The reason for hexane extraction having the best repeatability score could be attributed to the relatively higher number of extractions conducted with hexane as a large data size has a positive impact on the accuracy of repeatability scores. The relatively higher number of extractions observed with hexane is due to the very low yields obtained per cycle hence more extractions were required to gain an appreciable amount of extract from each specimen. In general, repeatability refers to the level of consistency in outcome value between several processes conducted progressively under the same conditions. In other words, repeatability relates to how precise a sequence of measurements of a particular object or region are when made consecutively, by the same operator, and with the same tool or equipment (Higher precision, 2018). The higher the value of repeatability is, the more spread out the values of the sequence of processes are and the less precise the process is. High values of repeatability could also imply the occurrence of errors during the process and hence is a great indicator for determining the quality of a process. It is therefore desired that repeatability values be as low as possible and for this work the extraction method seem to satisfy this objective.

The inter day precision scores likewise were all of good standing well below ± 15% with the best score observed in the hexane extraction of *A. taxiformis* (gametophyte) at ± 1.41% and the worst recorded for *A. armata* ethanol extraction at ± 12.62%. Just like repeatability (also known as intra-day precision), inter day precision also follows a similar principle but however measures the consistency of several measurements made across a series of consecutive days. This to some extent gives an indication of how stable a method or process is or was conducted with respect to time and also seems to be a good indication of process quality.

The standard deviation and RSD calculated for the extraction process of *A. taxiformis* (sporophyte) shows satisfactory deviation values for the ethanol extractions thus ± 2.06 and ± 0.30, respectively.

The general satisfactory scores recorded for both individual repeatability and the pooled repeatability as well as inter day precision could be attributed to the fact that the extraction was carried out in a precise manner devoid of many errors and this is the case as the EDGE process was fully automated. Also, the sample pre-treatment (milling) before the extraction could be said to also contribute to the consistency of the extraction process. The use of the ball mill (Retsch PM-100 Ball mill) seems to have ensured homogenous mixing of compounds across the entire biomass resulting in a consistent extraction series.

3.3. Extraction results

The results of the extractions are tabulated below.

Table 0.4. Extraction yields and total extracts obtained

Species	Ethanol Extraction		Ethyl Acetate Extraction		Hexane Extraction	
	%Yield	**Total Extract (g)**	**%Yield**	**Total Extract (g)**	**%Yield**	**Total Extract (g)**
A. taxiformis (gametophyte)	9.0%	1.21	2.5%	0.86	1.1%	0.59
A. armata (gametophyte)	12.4 %	1.07	2.4%	1.10	0.4%	0.27
A. taxiformis (Sporophyte)	6.8%	0.28	1.8%	0.09	1.0%	0.08

From Table 3.4, we see that generally, the ethanolic extractions of all the species showed relatively higher extraction yields among the selected solvents used for this study. This was followed by the ethyl acetate extractions and lastly hexane extractions respectively. *A. armata* (gametophyte) ethanol extraction had the highest extraction yield of 12.4% whereas the hexane extraction of the same species demonstrated the lowest extraction yield of 0.4%. Ethyl acetate yields ranged from a maximum of 2.5% for *A. taxiformis* (gametophyte) to a minimum of 1.8% for *A. taxiformis* (sporophyte) and *A. armata* (gametophyte) falling in between with 2.4% ethyl acetate extraction yield.

Different extraction yields have been reported in several studies involving the extraction of bioactives from macroalgae (Alghazeer et al., 2013; Bianco et al., 2015; Chernane et al., 2014; Elangovan & Anantharaman, 2019; Maadane et al., 2015). These studies suggest that extract yields are dependent on factors such as the extraction technique and associated parameters like temperature, pressure etc, type of solvents employed, ratio of biomass to solvent and type of macroalgae used among others. Reports from a study conducted by Nunes et al. (2018) involving the extraction of bioactives from *A. taxiformis* indicate an extraction yield of 10.92 ± 1.77% for ethanol and 5.32 ± 0.67% for ethyl acetate solvent using cold maceration and soxhlet extraction methods (Nunes et al., 2018). These results are consistent with findings of this current study.

The relatively high ethanolic yields realised compared to that of the other selected solvents in this study suggests that most of the compounds present in *Asparagopsis* are likely to be relatively polar in nature as ethanol is noted to have a polarity index of PI 5.2 (Big Chemical Encyclopedia, 2019). Most studies have also reported that ethanol as an extraction solvent demonstrates an affinity for certain groups of compounds which include phenolics such as iso-flavonoids, flavonoids, and tannins (Blicharski & Oniszczuk, 2017). However, due to its relatively high polarity (compared with non-polar solvents), ethanol can also extract other compounds such as small peptides and sugars, polar lipids and carotenoids. Total phenolic content analysis performed in this work (to be discussed in a subsequent sub section) shows that phenolic compounds constitute a fraction of the composition of all the extracts (including the ethanolic extracts) and hence suggests the presence of compounds other than phenolics. Contrary to reports by Blicharski & Oniszczuk (2017) concerning ethanol being more effective in extracting phenolic compounds, the TPC analysis showed that despite the fact that phenolics were realised in all the extracts the greatest amount of these were seen mostly in the ethyl acetate extracts across all the 3 specimen.

Hexane which has a polarity index of PI 0.1 showed the least yields of extracts across all the species implying the presence of non-polar compounds in *Asparagopsis* spp. Hexane is mostly noted for its ability to extract non-polar lipids from most substrates hence the low yields of hexane extracts suggests that *Asparagopsis* spp may be very low in non-polar lipid content (Saini et al., 2021).

Ethyl acetate as a solvent with a relatively medium polarity index (PI 4.4) among the three solvents was noted to have an extraction yield relatively lower and higher than ethanol and hexane, respectively. The biphasic action of this solvent just like the ethanol gives it the ability to extract both polar and non-polar compounds when used for extraction (Javad, 2013) and hence this could possibly be the reason why it performed better than the hexane solvent which is only limited to the extraction of non-polar compounds.

In general, the percentage yields realised in this study seem to be in conformity with findings reported by (Ghada & El-Sikaily, 2013) which stated that red seaweeds (including *Asparagopsis* spp), as part of their structure, contain about 80% to 90 % water, which could possibly imply that a large amount of polar compounds may be present and hence explains the relatively higher yields obtained for the ethanol extractions. Furthermore, the dry weight of this species was reported to comprise of 7–38% minerals, 1% fats, and 50% carbohydrates hence

this explains the very low percentage yields realised for the hexane extractions (Ghada & El-Sikaily, 2013).

3.4. Antioxidant and Chelating Activity

Antioxidant activity screening assays, specifically ABTS and DPPH assays were conducted alongside metal chelating activity assays (copper and iron (II)), for the ethanol, ethyl acetate and hexane extracts obtained from the three species thus, *A. taxiformis* (gametophyte), *A. armata* (gametophyte), and *A. taxiformis* (sporophyte). Iron reducing assay was also performed in this regard. Results of the assays were compared to positive controls thus Gallic acid in the case of ABTS, DPPH and Iron reducing activity, and EDTA in the case of iron and copper chelating activities. The results were expressed in percentages and statistical analysis was conducted. The key for the tabulated results for all the antioxidant and chelating activity assays is displayed in Table 3.5.

Table 0.5. Key for Tabulated Antioxidant and chelating activity results

KEY	
Atg - P -	*Asparagopsis taxiformis* (gametophyte) – collected from Portugal
Aag - P -	*Asparagopsis armata* (gametophyte) - collected from Portugal
Ats - S -	*Asparagopsis taxiformis* (sporophyte) – collected from Spain
ET	Ethanol Extract
EA	Ethyl Acetate Extract
H	Hexane Extract
N/A	Not Applicable

3.4.1. ABTS Assay

Results obtained for the ABTS assays for each species are presented with their standard deviation in Table 3.6.

Table 0.6. ABTS Assay Results

Sample	In-well Conc. (mg/mL)	% Activity	Std Deviation ±	IC_{50} (mg/mL)
Atg - P - ET	0.25	16.5	1.9	-
	0.0625	8.29	0.59	
Atg - P - EA	0.25	14.2	2.4	-
	0.0625	6.57	0.78	
Atg - P - H	0.25	-	-	-
	0.0625	-	-	
Aag - P - ET	0.25	54.7	5.3	0.24
	0.0625	13.0	1.7	

Aag - P - EA	0.25	14.5	1.9	-
	0.0625	5.77	0.91	
Aag - P - H	0.25	5.37	1.66	-
	0.0625	-	-	
Ats - S - ET	0.25	15.8	1.9	-
	0.0625	4.84	0.74	
Ats - S - EA	0.25	18.0	2.1	-
	0.0625	7.67	0.59	
Ats - S - H	0.25	17.1	1.6	-
	0.0625	7.12	1.47	
Gallic acid (+)	0.1	99.5	1.4	0.00019

Results of the ABTS assays displayed in Table 3.6 shows that activity is dependent on extract concentration. Thus, the higher the extract concentration the higher the percentage activity observed. Comparative analysis of the percentage activity for each extract at a concentration of 0.25 mg/mL was done and plotted in the bar graph displayed in figure 3.4.

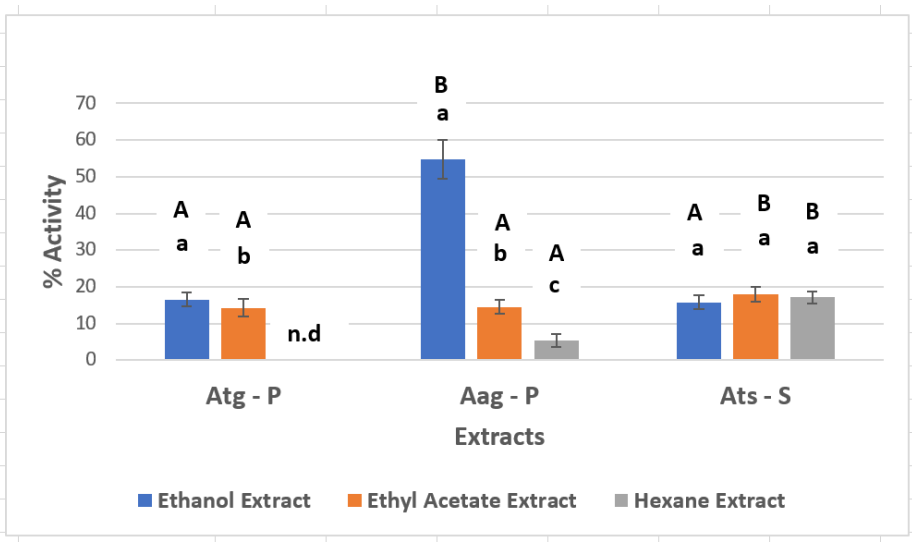


Figure 0.4. Comparative Analysis for Extracts at 0.25 mg/mL for ABTS assay *Note: n=3, alpha = 0.05, Tukey P = 0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.*

From figure 3.4, it can be observed that the extract that displayed the highest percentage activity for ABTS assay was the ethanol extract of *A. armata* (gametophyte) showing over 50% activity (54.7 ± 5.3%, IC_{50} = 0.24 mg/mL). Despite the fact that other extracts showed activity for the assay, they were significantly less as compared to the former and were all well below 50%. Comparing all the observed activities to the gallic acid control which showed a 99.5 ± 1.4% activity (IC_{50} = $1.9x10^{-4}$ mg/mL), we see a vast difference in performance with the gallic acid

performing better even at a concentration of 0.1 mg/mL with IC_{50} = $1.9x10^{-4}$ mg/mL as compared to the highest concentration of all the extracts thus 0.25 mg/mL with IC_{50} = 0.24 mg/mL. In practice, a lower IC_{50} value is desired as it implies a more effective activity. Lower activities in the extracts compared with the positive control are generally expected as gallic acid is a pure compound whereas the extracts are not highly refined. Therefore, the concentration of the extracts are the collective concentration of all other compounds present plus the actives observed. The IC_{50} values presented are expressed in terms of the in-well concentrations of the samples. In-well concentration refers to the final concentration of the sample or extract within the microplate wells taking into account the dilution resulting from the analytical process.

A two-way ANOVA conducted on the data (figure 3.5) suggests that statistically, the type of solvent used for the extraction has a significant influence on the performance of the extract in the ABTS assay. This observation is in accordance with reports by Pérez-Jiménez & Saura-Calixto (2006) in which they state that the antioxidant capacity of an extract is dependent on the solvent used for the extraction process. The analysis once again suggests that statistically, there is a significant difference between the nature of the species and their performance in the ABTS assay. This inference was drawn from the fact that the F_{stat} for ANOVA between the solvents which was 857.80 and the F_{stat} for the ANOVA between the species which was 390.61 were all far greater than the $F_{critical}$ value of 3.55 in both cases. Also, the P values recorded for Analysis between the solvents and also between the species which were $1.4025x10^{-18}$ and $1.49096x10^{-15}$ respectively, were significantly lesser than the alpha value of 0.05 (95% Confidence Interval). Statistically, this affirms the observation that performance of the extracts in the ABTS assay is highly influenced by the type of species and the type of solvent employed for the extraction. Similar findings are reported in a study by Kiana et al. (2017) in which different speices of the red macroalgae (*Gracilaria corticata, Acanthophora muscoides, Palisada perforata, Laurencia dendroidea,* & *Galaxaura rugosa*) and brown macroalge (*Sargassum boveanum, Sargassum vulgare, Sargassum angustifolium, Polycladia myrica,* & *Sirophysalis trinodis*) were extracted with various solvents and assessed for their antioxidant (ABTS assay) and antidiabetic properties. Observations from the study indicated that performance of extracts were influenced by the solvent type and species of macroalgae.

Further analysis with a one-way ANOVA (Tukey $P<0.05$) showed that there was a statistical difference in ABTS assay performance within all the species across all solvents except *A. taxiformis* (sporophyte) which showed no significant difference across the three extraction

solvents. Tukey test also revealed that *A. taxiformis* gametophyte and sporophyte were not so different in activity with regards to the ethanol extracts. However, with the ethyl acetate extract there is an observed statistical difference with a P value of 0.04 which is slightly lower than the alpha value of 0.05. Also, the hexane extract of the two life stages showed a great significant difference with *P<0.0001* implying a great magnitude of statistical difference. This observed difference could be attributed to the fact that these two specimens are of the same species but at different life stages hence there might be differences in the nature of compounds present as well as their abundance at different life stages. In a similar study by Nicholas et al. (2006) where *A. armata* in its life stages (gametophyte & sporohyte) was investigated, it was discovered that the amount of bioactive compounds (specifically bromoform) in the extracts varied depending on the life stage and other factors such as sampling time. This finding is in agreement with the inference of the observations made from this analysis.

Interactions between solvents and species as demonstrated by the two-way ANOVA also suggests that there is a relation between the amount and nature of extract obtained and the solvent and type of species used for the extractions. The result of the two-way ANOVA is tabulated and displayed in figure 3.5. All other ANOVA results can be found in the Annex of this report.

ABTS ASSAY AT 0.25 mg/mL			
	Atg-P	Aag-P	Ats-P
Ethanol	17.6	55.1	15.5
	16.3	52.8	15.3
	15.6	56.2	16.5
Ethyl Acetate	14.2	14.8	19.03
	14.8	14.4	15.4
	13.4	14.4	19.6
Hexane	n.d	4.38	17.1
	n.d	5.84	16.8
	n.d	5.90	17.3

Note: n.d. implies not detected

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (Solvent)	2123.401	2	1061.701	857.8022	1.4025E-18	3.554557
Columns	966.9119	2	483.456	390.6088	1.49096E-15	3.554557
Interaction	2494.713	4	623.6782	503.9015	3.30809E-18	2.927744
Within	22.27857	18	1.237699			
Total	5607.304	26				

Figure 0.5. ANOVA Analysis for ABTS Assay

The activities observed in the ABTS assay for the extracts implies the presence of bioactive compounds in each of the three species. This observation is in conformity with reports from Zubia et al. (2009) which made claims to the presence of bioactive compounds present in red seaweeds including *Asparagopsis* spp. With the exception of *A. taxiformis* (gametophyte), all

the other species showed activity across all three extraction solvents. *A. taxiformis* (gametophyte) hexane extract failed to show any activity in the ABTS assay. This does not necessarily imply that the hexane extract may not contain any non-polar bioactives that have antioxidant properties in the ABTS assay but however, it may be that the amount of bioactive compounds present in the hexane extract at the 0.25 mg/mL concentration might not be much to show significant antioxidant activity for the assay.

Ethanol as an organic solvent is noted for its ability to extract relatively polar compounds including phenolics such as flavonoids and tannins and other compounds such as peptides, sugars, polar lipids, and carotenoids whereas ethyl acetate is noted to extract to a greater extent relatively less polar compounds such as some alkaloids and sterols (Pintać et al., 2018). Hexane on the other hand has been reported to be very effective in the recovery of compounds such as alkanes, essential oils and neutral & non-polar lipids (Pintać et al., 2018).

A study by Alghazeer et al. (2013) showed that red macroalgae such as *Gelidium latifolium, Hypnea musci-formis, Jania rubens, Jania* spp. and *Laurencia obtuse,* all demonstrated the presence of bioactive compounds such as alkaloids, tannins, saponins, flavonoids, terpenes, anthraquinones, and coumarins in varying abundance and hence suggests that these compounds may also be present in *Asparagopsis* spp since they all belong to the same group Rhodophyta. Total phenolic content (TPC) analysis conducted on *Asparagopsis* spp shows that there are some phenolic compounds present in this species across all solvent extracts and hence confirms the claim of the presence of phenolics. However, the amount of phenolic content realised among the extracts suggest that the composition of the extracts might not only be phenolic compounds as the amounts represent but a fraction of the total weight of the extract in each case. Therefore, the antioxidant activity observed might partly be due to the phenolics present but also to a greater extent other bioactive compounds which may be present in the extracts. A study on the red macroalgae *Gracilaria domingensis* by Torres et al. (2022) reported that despite the high values of antioxidant activity observed among extracts, total phenolic content analysis (TPC) conducted by GC-MS and HPLC could not detect the presence of any phenolic compounds in both methanol and hexane solvent extracts of the named red macroalgae, hence further emphasising the observation that to a greater extent, antioxidant activity may be due to the presence of other bioactive compounds rather than just the phenolic compounds (Torres et al., 2022). Therefore, findings of this current study suggests that the antioxidant activities witnessed in the ABTS assay of the various extracts from the three species could be a result of

the presence of bioactive compounds including phenolics and other unknown antioxidants such as pigments in the extracts (Goiris et al., 2012).

3.4.2. DPPH Assay

Results obtained for the DPPH assays for each species are presented with their standard deviation in Table 3.7.

Table 0.7. DPPH Assay Results

Sample	In-well Conc. (mg/mL)	% Activity	Std Deviation ±
Atg - P - ET	0.495	23.0	2.0
	0.124	15.8	1.5
Atg - P - EA	0.495	35.0	3.0
	0.124	16.4	0.5
Atg - P - H	0.495	17.9	1.0
	0.124	12.5	0.6
Aag - P - ET	0.495	48.9	1.2
	0.124	20.7	1.3
Aag - P - EA	0.495	39.9	1.6
	0.124	24.5	2.9
Aag - P - H	0.495	26.9	1.4
	0.124	10.3	0.7
Ats - S - ET	0.495	9.80	2.06
	0.124	2.27	0.45
Ats - S - EA	0.495	18.0	1.4
	0.124	3.19	0.66
Ats - S - H	0.495	16.1	2.1
	0.124	4.16	0.78
Gallic acid (+)	0.20	95.1	0.5

Results of the DPPH assays as displayed in Table 3.7 once again shows that activity is dependent on extract concentration. Comparative analysis of the percentage activity for each extract at a concentration of 0.495 mg/mL was done and plotted in the bar graph displayed in figure 3.6

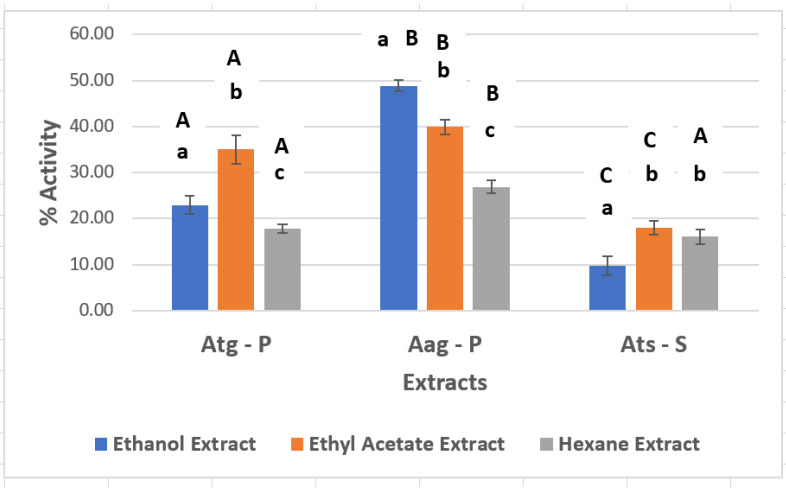


Figure 0.6 .Comparative Analysis for Extracts at 0.495 mg/mL for DPPH assay *Note: n=3, alpha = 0.05, Tukey P<0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.*

It can be observed from figure 3.6 that the extract with the highest percentage activity was the ethanol extract of the *A. armata* (gametophyte) demonstrating 48.9 ± 1.2% activity. The ethanol extract of *A. taxiformis* (sporophyte) was observed to show the lowest percentage activity of 9.8 ± 2.1%. Unlike the ABTS assay, all solvent extracts from all three specimen showed some form of activity though they were well below 50% activity hence IC_{50} values for the DPPH assay in all the extracts was not determined. The values of DPPH radical scavenging activity observed across all extracts is consistent with reports from a study by Custódio et al. (2016) which also showed low percentages of antioxidant activities below 50% of methanol extracts of *A. armata* applied at a concentration of 10 mg/mL in a DPPH assay (Custódio et al., 2016).

Among all the solvent extracts across the three species in this current study, *A. armata* (gametophyte) had the highest percentage activity observed in each case. Similarly, *A. taxiformis* (gametophyte) took the second spot in this regard as all activities observed across all solvent extracts were the second highest. Values recorded for *A. taxiformis* sporophyte places it last on this ranking. Gallic acid as a positive control once again out classed the activities observed among all the extracts by far as expected, showing a 95.1 ± 0.5% activity at a concentration of 0.20 mg/mL in the DPPH assay.

Two-way ANOVA statistics conducted on the results of DPPH assay (figure 3.6) shows the same trend as observed in the ABTS assay where the performances in the assay are significantly influenced by both the type of solvent used and the type or nature of the species used. The F_{stat} for ANOVA between the solvents for the DPPH assay is 162.54 and the F_{stat} for the ANOVA between the species which is 791.39. These are all far greater than the $F_{critical}$ value of 3.55 in

both cases. Also, the P values recorded for analysis between the solvents and also between the species which are $3.01301x10^{-12}$ and $2.87394x10^{-18}$ respectively, are significantly lesser than the alpha value of 0.05 (95% Confidence Interval). Another study with similar observations is the study by Elangovan & Anantharaman (2019) in which DPPH radical scavenging activity screening of selected red and green macroalgae show performances dependent on type of extraction solvent and species of macroalgae used (Elangovan & Anantharaman, 2019).

Further statistical analysis using one-way ANOVA (Tukey test $P<0.05$) shows that there is significant differences within each species and also among the species in the case of each solvent. However, similarities were observed between the hexane extract of *A. taxiformis* gametophyte and sporophyte as the Tukey multiple comparison test showed no significant differences between these particular extracts. With regards to ethanol and ethyl acetate extract among the two life stages of *A. taxiformis*, the Tukey multiple comparison test reported great statistical differences between the life stages in the magnitude of $P<0.0001$ for each solvent extract. This follows to show that there exist some physiochemical differences between the two life stages of *A. taxiformis* just as observed and discussed in the ABTS assay.

Within species, Tukey multiple comparison test also shows that the ethyl acetate and hexane extract of the *A. taxiformis* sporophyte have no statistically significant differences. Interactions as observed from the two-way ANOVA suggests the same inferences as was drawn from the ABTS assay which shows that there is a relation between the amount and nature of extract obtained and the solvent and type of species used for the extractions. The result of the two-way ANOVA is tabulated and displayed in figure 3.7. All other ANOVA results can be found in the Annex of this report.

DPPH ASSAY AT 0.495 mg/mL			
	Atg-P	Aag-P	Ats-P
Ethanol	23.2	48.6	8.52
	23.0	48.9	12.8
	22.8	49.1	8.66
Ethyl Acetate	36.1	41.7	17.9
	35.9	39.4	17.7
	33.1	38.7	18.2
Hexane	18.1	28.5	16.5
	17.8	26.7	15.1
	17.6	25.5	16.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	529.268	2	264.634	162.5353	3.01E-12	3.554557
Columns	2577.017	2	1288.508	791.3878	2.87E-18	3.554557
Interaction	771.1117	4	192.7779	118.4021	1.14E-12	2.927744
Within	29.30693	18	1.628163			
Total	3906.703	26				

Figure 0.7. ANOVA Analysis for DPPH Assay

Results obtained for the DPPH assay shows similar trends to that of the ABTS assay. Literature reports of similar observations made in a study by Shah & Modi (2015) where different species of mushrooms were tested in a comparative study of DPPH, ABTS and ferric reducing antioxidant power assay (FRAP) which confirmed similar trends between ABTS and DPPH among species (Shah & Modi, 2015). Similarities as observed between DPPH and ABTS assays could be attributed to the fact that the mechanism of action of these assays are the same (electron transfer (ET) / hydrogen atom transfer (HAT)) (Amorati & Valgimigli, 2014). The ethanolic extract of *A. armata* shows the highest percentage activity. This observation is similar to reports from DPPH assay of Maadane et al. (2015) where ethanolic extracts from microalgae showed the highest activity in DPPH assay.

Despite the fact that similar trends are observed between the DPPH and ABTS assay results, the percentage of radical scavening activity obtained for the species differs in magnitude from one another across the two tests. This observation is also reported in a study by Floegel et al. (2010) and also in another study by Martysiak-Żurowska & Wenta (2012). These studies attribute this difference to the variations in sensitivity between the two tests and also the stability of the ABTS and DPPH radicals. These studies pointed ABTS as being more sensitive than DPPH.

As previously stated in the case of the ABTS assay, the activities observed across all species could be attributed to the presence of bioactive compounds such as phenolics (flavanoids,

tannins etc) which was confirmed to be present in varying amounts across all extracts. It is also possible to say that pigments such as carotenoids and phycobilins as well as polyunsaturated fatty acids (omega-3 and omega-6 fatty acids) and other compounds may be present in the ethyl acetate extracts and hexane extracts and also the ethanol extracts and these may contribute to the activities observed.

3.4.3. Iron (II) chelating Activity

Results obtained for the iron chelating activity assays for each species are presented with their standard deviation in Table 3.8.

Table 0.8. Iron (II) Chelating Activity Assay Results

Sample	In-well Conc. (mg/mL)	% Activity	Std Deviation ±	IC_{50} (mg/mL)
Atg - P - ET	0.55	33.6	5.3	-
	0.138	8.94	1.48	
Atg - P - EA	0.55	27.4	2.6	-
	0.138	6.97	1.28	
Atg - P - H	0.55	23.2	3.4	-
	0.138	6.95	1.21	
Aag - P - ET	0.55	35.0	2.5	-
	0.138	12.9	1.7	
Aag - P - EA	0.55	71.8	4.3	0.25
	0.138	26.0	4.6	
Aag - P - H	0.55	36.6	4.0	-
	0.138	18.5	2.1	
Ats - S - ET	0.55	43.8	7.1	-
	0.138	21.9	4.2	
Ats - S - EA	0.55	89.9	5.7	0.24
	0.138	39.7	7.1	
Ats - S - H	0.55	87.8	2.1	0.09
	0.138	56.5	3.2	
EDTA (+)	0.11	98.0	0.6	0.0068

Results of the iron (II) chelating assays displayed in Table 3.8 shows that chelating activity is dependent on extract concentration. Comparative analysis of the percentage activity for each extract at a concentration of 0.55 mg/mL was done and plotted in figure 3.8.

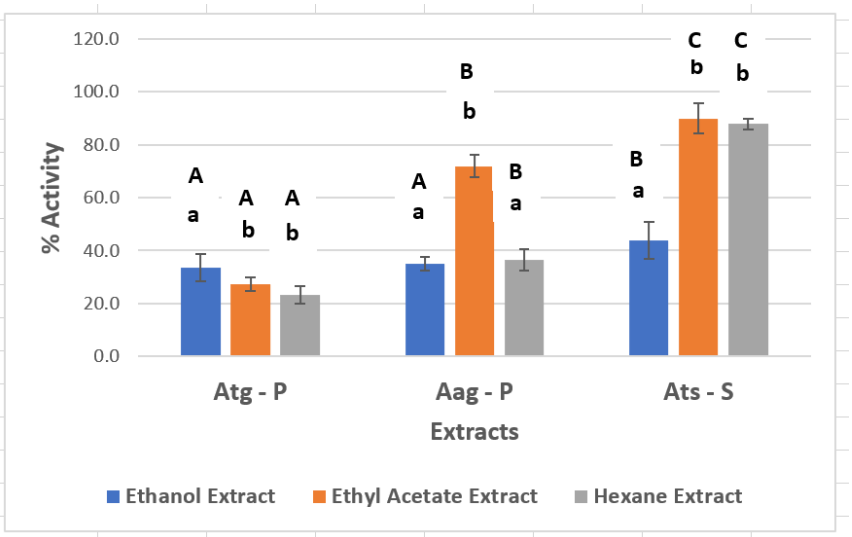


Figure 0.8. Comparative Analysis for Extracts at 0.55mg/mL for iron chelating activity. *Note: n=3, alpha = 0.05, Tukey P < 0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.*

From figure 3.8, we observe that all three species demonstrated iron (II) chelating activities at various levels. The best chelating activity was witnessed in *A. taxiformis* (sporophyte) in all solvent extracts. *A. armata* (gametophyte) follows in this order while the chelating activity of *A. taxiformis* (gametophyte) is not promising and falls behind the former species. With the exception of *A. taxiformis* (gametophyte), the ethyl acetate extracts of all the other species showed chelating activities well above 50% with IC_{50} values of 0.25 mg/mL and 0.24 mg/mL for *A. armata* (gametophyte) and *A. taxiformis* (sporophyte) respectively. However, the highest chelating activity was observed in the hexane extract of *A. taxiformis* (sporophyte) with an IC_{50} value of 0.09 mg/mL. Comparing the observed IC_{50} values with that of the EDTA positive control (IC_{50} = 0.0068 mg/mL), we observe that the extracts of *Asparagopsis* spp especially the hexane extracts of *A. taxiformis* (sporophyte) show tremendous chelating potentials even in their unrefined states.

Comparing the findings in this work to other studies, we see that the iron chelating activity results obtained for *Asparagopsis* spp were significantly higher than what has been reported among some red macroalgae namely, *Hypnea musciformis*, *H. valentiae*, and *Jania rubens* in a study by Chakraborty et al. (2015), where extracts were obtained using hexane, dichloromethane, and ethyl acetate as solvents. The best IC_{50} value reported in that study was that of the ethyl acetate extracts of *Hypnea musciformis* which showed a value of 0.70 mg/mL (Chakraborty et al., 2015). This value is however significantly outclassed by the best IC_{50} value recorded in the current study by the ethyl acetate extract of *A. armata* (gametophyte) (IC_{50} =

0.09 mg/mL), implying that *Asparagopsis* spp may be a better source of iron (II) chelating compounds compared to the mentioned red macroalgae and others alike.

Two-way ANOVA results (figure 3.9) reveals that there is a statistically significant difference in the performance of the various extracts in the iron (II) chelating activity assay. Once again, the analysis suggests that this difference in performance could be partly influenced by the type of solvent employed for the extraction and the type of species used as was the case with findings reported by Pérez-Jiménez and Saura-Calixto in their 2006 study. Statistical evidence for this observation lies in the fact that the F_{stat} for ANOVA between the solvents which was 353.72 and the F_{stat} for the ANOVA between the species which was 1135.39 were all far greater than the $F_{critical}$ value of 3.55 in both cases.

Furthermore, a one-way ANOVA (Tukey $P<0.05$) revealed that there is a statistically significant difference within species and also across species with the same solvent. Within species, it can be observed that the hexane and ethyl acetate extracts of *A. taxiformis* are statistically identical in chelating activity. This observation is true for both gametophyte and sporophyte life stages of the aforementioned specie. However, Tukey multiple comparison analysis between specimen in the iron chelating activity assay highlights the already noted physiochemical differences between the two life stages (gametophyte and sporophyte) of *A. taxiformis* as seen in previous antioxidant assays (ABTS & DPPH). The magnitude of the observed difference was in the range of $P<0.0001$ across all solvents implying a high degree of statistical difference between the life stages. This same observation and inference was made by Nicholas et al. (2006) in their study of bioactive compounds present in *A. armata* across its two life stages (Nicholas et al., 2006).

In the current study, other similarities observed within species include similarties in chelating activity between the ethanol and hexane extracts of *A. armata* (gametophyte). All other ANOVA results can be found in the Annex of this report.

Iron Chelating Activity AT 0.55mg/mL			
	Atg-P	Aag-P	Ats-P
Ethanol	33.4	36.5	42.1
	33.8	34.5	44.9
	33.6	34.1	44.5
Ethyl Acetate	28.1	71.7	95.6
	26.7	72.9	86.1
	27.5	71.0	88.1
Hexane	24.7	38.8	88.6
	23.0	36.3	86.9
	21.9	34.6	87.9

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	2952.529	2	1476.265	353.7208	3.56E-15	3.554557
Columns	9477.168	2	4738.584	1135.39	1.15E-19	3.554557
Interaction	3876.39	4	969.0976	232.2009	3.19E-15	2.927744
Within	75.12356	18	4.173531			
Total	16381.21	26				

Figure 0.9 ANOVA results for Iron (II) chelating Activity Assay

High levels of chelating activity were observed in the hexane extract of *A. taxiformis* (sporophyte) and the ethyl acetate extract of *A. taxiformis* (sporophyte) and *A. armata* (gametophyte) at the concentration of 0.55 mg/mL. The high values of chelating activity with respect to ethyl acetate extract is suggestive of the presence of peptides and small proteins which have been reported to demonstrate metal chelating activities specifically in seaweeds by Toyosaki & Iwabuchi (2009). These findings are consistent with reports by Ebrahimzadeh et al. (2018) who also observed high metal chelating activity with ethyl acetate extracts from marine algae.

The high chelating activity observed with the hexane extract of *A. taxiformis* (sporophyte) is suggestive of the presence of polyunsaturated fatty acids (PUFAs) and sterols which are easily extracted with hexane and have been reported to demonstrate unprecedented metal chelating activity especially with iron (II) (Custódio et al., 2012). This activity observed in hexane extracts is consistent with findings reported in a study on the red macroalgae *Gracilaria domingensis* by Torres et al. (2022). Iron chelating activity as observed in ethanolic extracts of the three species could be attributed to the presence of polar compounds such as sulphated polysaccharides and some mycosporine-like amino acids (MAAs) which are known to possess metal chelating activities (Rupérez et al., 2002; Torreset al., 2022). Other polar compounds such as phenolics have been known to also possess metal binding abilities especially with

transition metals and hence it could be possible that these phenolics could also be present in the extracts and contribute to the chelating activities observed (Santos et al., 2017).

Chelating activities as observed with extracts from *Asparagopsis* spp are very promising and this could prove vital in the making of pharmaceuticals and nutraceuticals to address health issues associated with iron overload. An iron overload may intensify the harmful consequences of excessive reactive oxygen species generation. Additionally, long-term oxidative stress may alter iron intake and storage, triggering a self-perpetuating cycle of cytotoxic and mutagenic events. One of the main causes of aging is iron excess. It is related to numerous degenerative disorders, such as type II diabetes, and it also causes Alzheimer's disease, hence the need for iron chelating activity (Cook, 2016).

3.4.4. Copper chelating activity

Results obtained for the copper chelating activity assays for each species are presented with their standard deviation in Table 3.9.

Table 0.9. Copper Chelating Activity Assay Results

Sample	In-well Conc. (mg/mL)	% Activity	Std Deviation ±	IC_{50} (mg/mL)
Atg - P - ET	0.446	34.9	5.5	-
	0.112	11.4	2.1	
Atg - P - EA	0.446	62.5	5.8	0.32
	0.112	30.7	5.5	
Atg - P - H	0.446	57.4	8.8	0.37
	0.112	21.3	2.1	
Aag - P - ET	0.446	31.1	1.2	-
	0.112	10.9	1.2	
Aag - P - EA	0.446	62.7	1.6	0.34
	0.112	25.1	1.7	
Aag - P - H	0.446	73.0	2.0	0.28
	0.112	25.6	1.2	
Ats - S - ET	0.446	29.3	2.4	-
	0.112	10.8	2.1	
Ats - S - EA	0.446	53.5	1.3	0.41
	0.112	19.6	1.4	
Ats - S - H	0.446	75.9	0.9	0.12
	0.112	45.7	1.8	
EDTA (+)	0.089	96.6	0.1	0.017

Results of the copper chelating assays displayed in Table 3.9 show that chelating activity is dependent on extract concentration. Comparative analysis of the percentage activity for each extract at a concentration of 0.446 mg/mL was done and plotted in the bar graph in figure 3.10.

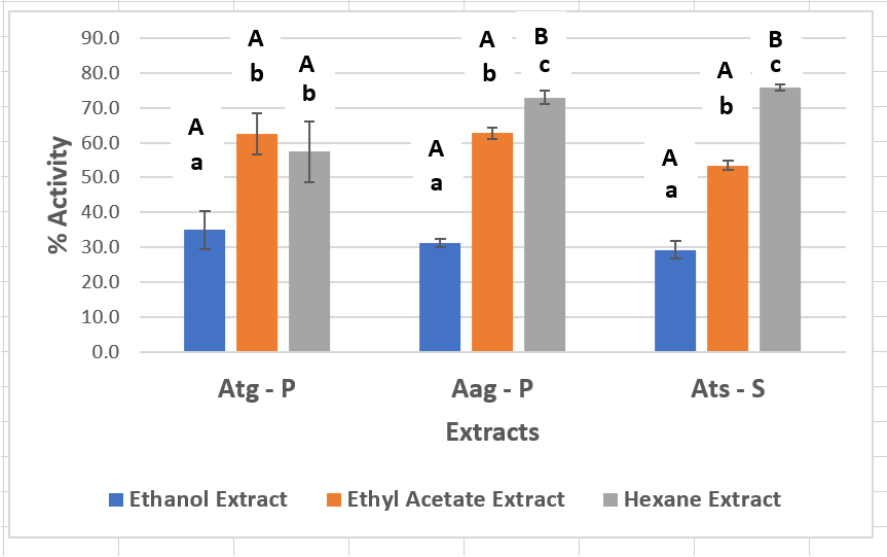


Figure 0.10 Comparative Analysis for Extracts at 0.446 mg/mL for copper chelating activity.
Note: n=3, alpha = 0.05, Tukey P < 0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.

Figure 3.10 shows copper chelating activities across all the species. The highest copper chelating activity was seen in the hexane extract of *A. taxiformis* (sporophyte), and the lowest activity was seen in the ethanol extract of the same. It was observed that at the concentration 0.446 mg/mL, chelating activity for both ethyl acetate and hexane extracts across all species were well above 50% whereas that of ethanol extracts were all below 50%. In a similar study by Silva et al. (2022) where the chelating activity of selected microalgae species was assessed, a similar observation was made with the ethyl acetate extracts showing activity above 50% across most of the species studied (Silva et al., 2022). In general, studies have reported that macroalgae including red macrolage show great potential for copper chelating activity (dos Santos Silvestre, 2017; Chernane et al., 2014) and this is also observed in the chelating activity studies of this work. IC_{50} values observed across the ethyl acetate and hexane extracts of *Asparagopsis* spp range from 0.12 to 0.37 mg/mL and are significantly higher than the IC_{50} value (0.017 mg/mL) of the EDTA positive control. In practice, a lower IC_{50} value is desired as it implies a more effective inhibition activity hence the EDTA control performs better comparatively as expected. Nevertheless, the observed IC_{50} values for the extracts is indicative of the tremendous chelating potentials of *Asparagopsis* spp. This is because the concentration observed with regards to the crude extracts are not necessarily the absolute concentration of

the actives that may be present in the extracts but however the collective concentration of all other compounds present plus the actives observed. The relatively low levels of chelating activity observed in the ethanol extracts of *Asparagopsis* spp suggests that bioactive compounds that show better chelating activity are most likely compounds of relatively lower polarity to no polarity at all. This observation is consistent to findings reported in the study by Torres et al. (2022) and Chernane et al. (2014) among others.

Statistical analysis (two-way ANOVA) (figure 3.11) suggests that there is a significant difference in activity across solvents in general. However, in terms of ethanol and ethyl acetate extracts among species, a one-way ANOVA (Tukey $P<0.05$) suggests that there is no significant difference between these extracts of the three species whereas for the hexane extracts there was notable statistical differences between species. It is worth mentioning that the hexane extract of *A. armata* (gametophyte) and *A. taxiformis* (sporophyte) were identical in activity however that of *A. taxiformis* (gametophyte) showed significant difference statistically in comparison to the former two extracts. The statistical difference in the hexane extract between *A. armata* (gametophyte) and *A. taxiformis* (gametophyte) had a P value of 0.0425 which is very close to the alpha value of 0.05 (95% Confidence Interval) implying a relatively small degree of difference. The same is observed between the hexane extract of *A. taxiformis* (sporophyte) and *A. taxiformis* (gametophyte), showing a P value of 0.0207 which is once again close to the alpha value of 0.05 (95% Confidence Interval). These differences suggest the existence of physiochemical differences between species (*A. armata* & *A. taxiformis*) and also between the life stages of *A. taxiformis* (gametophyte and sporophyte) as earlier discovered. On the other hand, similarities observed across species as identified in the two-way ANOVA analysis could imply that the same bioactive compounds are responsible for the observed chelating activity as in the case of the ethanol and ethyl acetate extracts across all the species.

All other ANOVA results can be found in the Annex of this report.

Copper Chelating Activity AT 0.446 mg/mL			
	Atg-P	Aag-P	Ats-P
Ethanol	42.1	31.8	28.0
	29.6	31.6	32.0
	33.1	29.9	27.9
Ethyl Acetate	69.7	63.4	54.2
	61.3	61.5	52.6
	56.5	63.3	53.6
Hexane	51.6	73.2	77.1
	69.3	73.0	75.2
	51.3	72.8	75.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	6673.171	2	3336.586	148.8521	6.37E-12	3.554557
Columns	75.07795	2	37.53897	1.674693	0.21527	3.554557
Interaction	739.275	4	184.8188	8.245154	0.000582	2.927744
Within	403.4779	18	22.41544			
Total	7891.002	26				

Figure 0.11. ANOVA results for copper chelating Activity Assay

The copper chelating activity of the species as observed in the hexane and ethyl acetate solvent extracts can be attributed to the presence of compounds such as peptides, small proteins, polyunsaturated fatty acids (PUFAs) and sterols which have all been shown to possess chelating abilities and are well extracted by these solvents (Ebrahimzadeh et al., 2018; Custódio et al., 2012). Also, the activities observed in the ethanol extracts suggest that polar compounds such as bioactive polysaccharides and phenolic compounds which are known to show metal chelating activities may be present.

The results obtained for copper chelating activity assay show some similarities with that of the iron chelating activity assay meaning a consistent metal chelating activity can possibly be attributed to *Asparagopsis* spp. This means that *Asparagopsis* spp may contain antioxidants that can quickly neutralize pro-oxidant metal ions, halting or delaying the oxidation of lipids brought on by metal ions. This makes them perfect resources for the mitigation of numerous degenerative disorders, such as type II diabetes, and Alzheimer's disease.

3.4.5. Iron reducing antioxidant capacity

Results obtained for the iron reducing antioxidant capacity assays for each species are presented with their standard deviation in Table 3.10.

Table 0.10. Iron reducing antioxidant capacity Assay Results

Sample	In-well Conc. (mg/mL)	% Activity	Std Deviation ±	IC_{50} (mg/mL)
Atg - P - ET	1.19	14.2	0.4	-
	0.298	13.2	1.3	
Atg - P - EA	1.19	133.5	5.6	0.34
	0.298	45.7	9.0	
Atg - P - H	1.19	29.9	2.3	-
	0.298	20.4	1.3	
Aag - P - ET	1.19	28.9	3.0	-
	0.298	15.9	1.9	
Aag - P - EA	1.19	186.1	6.7	0.16
	0.298	78.1	3.1	
Aag - P - H	1.19	25.5	2.5	-
	0.298	19.4	1.3	
Ats - S - ET	1.19	30.5	6.0	-
	0.298	21.8	4.3	
Ats - S - EA	1.19	166.7	16.7	0.07
	0.298	73.8	3.6	
Ats - S - H	1.19	63.2	2.5	0.88
	0.298	26.7	2.3	
Gallic acid (+)	0.476	99.8	3.6	0.0004

Results of the iron reducing assays as displayed in Table 3.10 shows a dependence between extract concentration and activity. Comparative analysis of the percentage activity for each extract at a concentration of 1.19 mg/mL was done and plotted in the bar graph in figure 3.12.

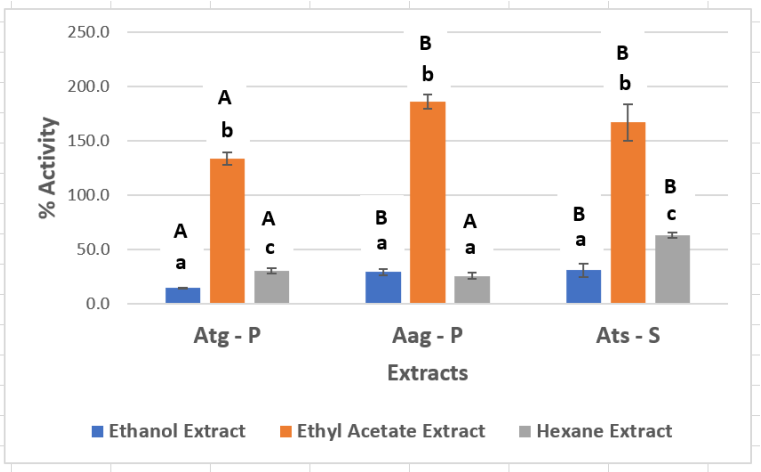


Figure 0.12. Comparative Analysis for Extracts at 1.19 mg/mL for Iron reducing activity *Note: n=3, alpha = 0.05, Tukey P < 0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.*

Figure 3.12 shows iron reducing activity across all the specimens, an observation which is consistent with several studies on macroalgae including studies by Ganesan et al. (2008),

Bianco et al. (2015) and Zubia et al. (2017). However, notable iron reducing activities (well above 50%) were observed in only the ethyl acetate extract of the three species. The highest activity was observed in the ethyl acetate extract of *A. armata* (gametophyte) with a value of 186.1 ± 6.7% and IC_{50} of 0.16 mg/mL whereas the lowest activity was observed in the ethanol extract of *A. taxiformis* (gametophyte) with a value of 14.2 ± 0.4%. The best IC_{50} value among the extracts was recorded at 0.07 mg/mL for the ethyl acetate extract of *A. taxiformis* (sporophyte). IC_{50} value for the gallic acid control was calculated as $0.04x10^{-2}$ mg/mL which shows gallic acid to be the better performer in reducing activity compared to the extracts. Nevertheless the activity observed in Asparagopsis spp shows that it has good iron reducing potential.

A two-way ANOVA (figure 3.13) conducted on the data reveals that in general there was a statistically significant difference in the performance of the various extracts in the iron reducing activity assay. In details, it was noted that there was a significant difference in performance among the solvents and among the species. This was highlighted with an F_{stat} for ANOVA between the solvents which was 946.96 and the F_{stat} for the ANOVA between the species which was 34.52 and were all far greater than the $F_{critical}$ value of 3.55 in both cases respectively.

Tukey multiple comparison test revealed some interesting observations within and between species. Within species, the test revealed that ethanol and hexane extracts of *A. armata* were identical in iron reducing activity. However, there was a significant difference observed in the activity between the ethyl acetate extract and the former two extracts of this specie. Significant differences we also observed within species for *A. taxiformis* (gametophyte & sporophyte) across all solvents. Between species, ethanol and ethyl acetate extracts of *A. armata* and *A. taxiformis* (sporophyte) showed no significant difference in activity. The same was observed for the hexane extracts of *A. taxiformis* (gametophyte) and *A. armata*. Finally, significant differences were observed across all solvents between the two life stages of *A. taxiformis* once again highlighting their physiochemical differences.

Results of the two-way ANOVA are displayed in figure 3.13. All other ANOVA results can be found in the Annex of this report.

Iron Reducing Power AT 1.19 mg/mL			
	Atg-P	Aag-P	Ats-P
Ethanol	14.0	25.8	39.0
	14.0	28.8	26.2
	14.5	32.0	27.0
Ethyl Acetate	133.0	179.0	187.2
	130.5	189.0	163.0
	137.0	191.0	150.0
Hexane	32.4	28.5	61.5
	29.9	25.0	63.6
	27.3	22.8	63.9

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	102509	2	51254.513	946.9591	5.81E-19	3.554557
Columns	3737.208	2	1868.6039	34.52362	6.91E-07	3.554557
Interaction	3520.323	4	880.08081	16.26004	8.5E-06	2.927744
Within	974.2567	18	54.125372			
Total	110740.8	26				

Figure 0.13. Results of two-way ANOVA for Iron reducing activity assays

Iron reducing activity as observed in ethanol extracts of the three species could be attributed to the presence of phenolics and pigments such as carotenoids and phycobilins. Reports by Yarnpakdee et al. (2018) also inidcated iron reducing activity observed with ethanol extracts from macroalgae only that the percentage activities obtained in their studies for ethanolic extracts were relatively higher than that of this study. *Asparagopsis* spp ethyl acetate extracts shows very high iron reducing activity similar to that reported by Pereira et al. (2020) where ethyl acetate and acetone extracts of the microalgae *T. striata* CTP4. In this report, ethyl acetate and acetone extracts showed higher iron reducing activity than extracts such as aqueous, ethanolic and hexane. The iron reducing activity observed with the ethyl acetate extracts of *Asparagopsis* spp could be an indication of the presence of phenolics and pigments such as carotenoids and phycobilins which are noted to demonstrate such antioxidant abilities (Tingjing et al., 2016; Wilhelm & Helmut, 2004; Mysliwa-Kurdziel & Solymosi, 2017). Once again the hexane extracts of *Asparagopsis* spp showed iron reducing activities implying the presence of non-polar compounds such as polyunsaturated fatty acids (PUFAs) and sterols which could be responsible for this antioxidant activity observed.

Ethyl acetate extracts of *Asparagopsis* spp demonstrated a tremendous potential for iron reducing antioxidant activity. The values observed for these extracts were well over 100% suggesting that they were more effective in reducing ferric (Fe^{3+}) to ferrous (Fe^{2+}) than the positive control (gallic acid) which was used for the assay. This suggests that *Asparagopsis* spp could be a great resource for the pharmaceutical and nutraceutical industries in the making

of products to regulate iron in the human system. This is particularly important because in a very wide range of inflammatory diseases, both acute and chronic, an iron overload may intensify the harmful consequences of excessive reactive oxygen species generation. Iron in itself is very vital to the function of the human body and is absorbed in the body in its ferrous (Fe^{2+}) form or bonded to a protein like heme. However, iron is found in its oxidized, ferric (Fe^{3+}) form at normal pH levels. It is therefore paramount that iron be reduced into its absorbable form to be used by the body else it may add up to the so-called labile pool, (an ill-defined and reactive pool of iron) that generates reactive oxygen species via the Fenton reaction and intensifies the negative effects of inflammation in the body (Ems et al., 2022).

3.5. Anti-inflammatory Activity (Cyclooxygenase (COX-2) inhibition)

The inhibition of COX-2 was assessed by a reduction in prostaglandin synthesis. ELISA was used to measure prostaglandins (PG). The standard concentrations of PG employed for the calibration curve ranged from 2000 to 15.625 pg/mL. Calibration curves were developed over the time range between absorbance units of 0.3 to 0.8 AU thus from 20 to 45 minutes in the case of this COX inhibition assay. The average linearity (R^2) of the calibration curves across the time range was calculated to be 0.988 which shows a very good correlation. The sample data were converted into concentrations (pg/mL) and then into the percentage of COX inhibition using the linear regression of the calibration curve at each time. Four analytical replicates for each of the extracts at a concentration of 1 mg/mL were done and the averages and standard deviations of the measurements were determined. Ibuprofen (≥98% (GC)) at a concentration range of 5 to 25 µg/mL was used as a positive control. Results of the COX-2 inhibition assay are presented in Table 3.11 and graphically represented in figure 3.14.

Table 0.11 COX-2 Inhibition results for Asparagopsis spp

Samples	Average % Inhibition	Std Deviation ±
Atg - P- ET	79.1	1.8
Atg - P- EA	61.1	15.9
Atg - P- H	98.9	0.4
Aag - P- ET	26.1	7.8
Aag - P- EA	48.3	2.9
Aag - P- H	57.1	5.1
Ats - S- ET	65.2	3.0
Ats - S- EA	21.5	5.7
Ats - S- H	72.3	5.1
Ibuprofen (5 µg/mL) (+)	55.0	13.2

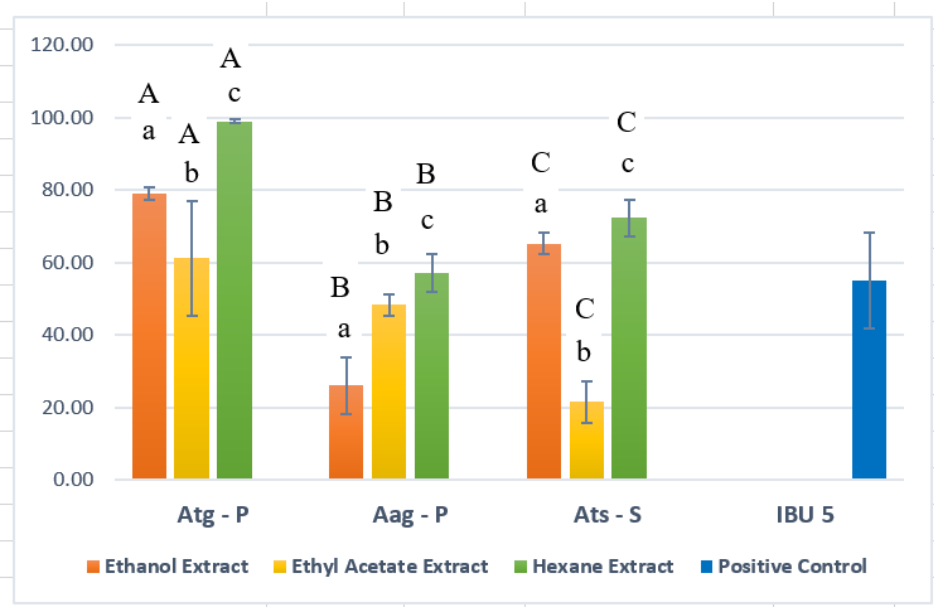


Figure 0.14. COX-2 Percentage inhibition for Asparagopsis spp *Note: n=4, alpha = 0.05, Tukey P < 0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.*

From the COX-2 inhibitory analysis, it was observed that all the extracts showed anti-inflammatory properties in the inhibition of COX-2. ANOVA (Tukey P<0.05) multiple comparison test reveals that generally there are significant differences within species and also among the species across all the solvents at a 95% Confidence Interval.

Within species, the magnitude of the observed differences varied with comparison of individual solvent extracts. Great statistical differences (P<0.0001) were observed for the comparison of the individual solvent extracts activity (that is; ethanol vs ethyl acetate, ethyl acetate vs hexane, and ethanol vs hexane) for *A. taxiformis* (gametophyte) extracts. Similar observations (P<0.0001) were made with respect to comparisons between the ethanol vs ethyl acetate and the ethyl acetate vs hexane extract activity for *A. taxiformis* (sporophyte). However, the magnitude of the differences for the comparison of activity for ethanol vs hexane extracts for *A. taxiformis* (sporophyte) was less (P= 0.0148) but still some distance below the set alpha value of 0.05. This implies that despite there are observed differences between the activities of ethanol and hexane extract of *A. taxiformis* (sporophyte), the difference is quite minimal. This can be visually observed in the graph (figure 3.14). Once again, the magnitude of the differences observed within species for *A. armata* extracts was also great (P<0.0001) with regards to activity comparison made between ethanol vs ethyl acetate and ethanol vs hexane. However, the magnitude of the observed difference between ethyl acetate vs hexane extract activity for this same specie was lesser (P=0.0072) than the ones previously mentioned for the

other solvent comparison. The implication of this observation is that the observed differences between the activities of ethyl acetate and hexane extract of *A. armata* is quite minimal. This is visually evident in figure 3.14.

Between species, Tukey multiple comparison (*A. armata* vs *A. taxiformis* (gametophyte), *A. armata* vs *A. taxiformis* (Sporophyte) and *A. taxiformis* (gametophyte) vs *A. taxiformis* (Sporophyte)) were significantly different across all solvents in the magnitude of $P<0.0001$. This suggests that all species were physiochemically different from one another.

From the COX-2 Inhibition assay (figure 3.14), the extract that showed the highest activity was the hexane extract of *A. armata* (gametophyte) with 98.9 ± 0.4% inhibition activity. Ethyl acetate extract of *A. taxiformis* (sporophyte) showed the lowest activity with 21.5 ± 5.7%. It was observed from the assay that the hexane extracts in the case of each of the three species, had the best COX-2 inhibitor activity and were all well above 50% activity. This observation is in conformity with reports by Lopes et al. (2020) where hexane extracts of some selected red macroalgae (*Palmaria palmata*, & *Porphyra dioica*), green macroalgae (*Ulva rigida* & *Codium tomentosum*) and brown macroalage (*Fucus vesiculosus*) all showed COX-2 inhibitor activity with the highest activity observed to be 89.5 ± 0.9% in the red macroalgae *P. palmata* at a concentration of 500 µg/mL (Lopes et al., 2020). A study by Wol Soon et al. (2010) reports that most often, lower polarity index organic solvents like hexane are found to provide the best anti-inflammatory effects (Wol Soon et al., 2010). This observation with hexane extracts have been associated to the presence of lipids and lipophilic substances as well as polyunsaturated fatty acids which have been reported to show anti-inflammatory properties (Simopoulos, 2002; Monmai et al., 2018). This also explains why higher inhibition activities were observed in the hexane extracts as compared to the other solvent extracts.

Anti-inflammatory activities as observed in the ethanolic and ethyl acetate extracts of *Asparagopsis* spp may be as a result of the presence of polyphenolic compounds in this macroalgae. There is also the possibility that the presence of pigments such as carotenoids and phycobillins could be contributing to the anti-inflammatory effects observed in *Asparagopsis* spp as these compounds have been noted to demonstrate anti-inflammatory activities (Hussain et al., 2016; Choe et al., 2020).

Comparing the COX-2 inhibition percentages realised from the *Asparagopsis* spp to the that of the ibuprofen positive control reveals promising prospects for the extracts especially the hexane extracts. Logically, the ibuprofen positive control at a concentration of 5 µg/mL (equivalent to

0.005 mg/mL) with a percentage inhibition of 55.0 ± 13.2% will be recognised as a far better performer than the hexane extract of *A. taxiformis* (sporophyte) at a concentration of 1 mg/mL with a percentage of 98.9 ± 0.4%. However the narrative changes when it is considered that the extracts in question are unrefined and hence the observed concentration is a resultant of all other compounds plus the bioactive(s) present which all sum up to the concentration of the extract. Therefore the concentration of the bioactive(s) present in the extract might be significantly lower on refining. This implies that highly refined extracts from *Asparagopsis* spp may be very competitive against other commercially known anti-inflammatory compounds on the market.

The observed anti-inflammatory properties of extracts from *Asparagopsis* spp makes them a great potential resource for the making of anti-inflammatory drugs with COX-2 inhibition properties. This is particularly important as numerous chronic illnesses, including cancer, neurological disorders, multiple sclerosis, diabetes, atherosclerosis, arthritis, and cardiovascular diseases, are brought on by excessive levels of pro-inflammatory mediators and components and therefore require anti-inflammatory drugs to limit prostaglandin formation, as a mediator of inflammation, by inhibiting the enzyme cyclooxygenase (COX). However COX occurs in two isoforms (COX-1 & COX-2). COX-1 is noted to produce prostaglandins which are involved in essential body processes such as ovulation, blood clotting, renal function, wound healing, vasomotor tone, platelet aggregation, immunological cell differentiation, nerve development, bone metabolism, and labour initiation. It is also noted to produce PGs which maintain the stomach lining intact. This is due to the acidity of the stomach which necessitates the fast replacement of stomach cells within days hence when the PG system is disrupted, stomach discomfort, digestive tract issues, intestinal or stomach haemorrhage, and even death may occur (Ayertey et al., 2021).

3.6. Total Phenolic content

The total phenolic contents of the extracts were determined using the Folin-Ciocalteu test. Gallic acid at concentration range of 0.00098 mg/mL to 2 mg/mL was used to develop a standard curve (figure 3.15) and total phenolic content of each extract was determined using the standard curve. Measurements were conducted in triplicates. Results obtained are displayed in Table 3.12. and figure 3.16.

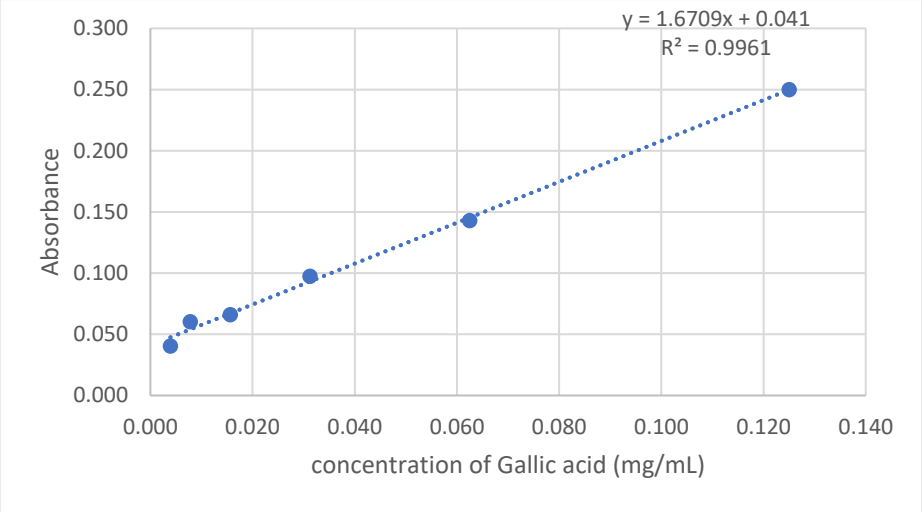


Figure 0.15. Calibration curve for Total Phenolic Content Assay

Table 0.12. Total Phenolic content of Asparagopsis spp extracts

Extract	Average TPC (mg-GAE/g)	Standard deviation (±)	Average TPC gGAE/100g (extract)	Average TPC gGAE/kg (biomass)
Atg-P-ET	0.30	0.04	0.03	0.03
Atg-P-EA	1.19	0.03	0.12	0.03
Atg-P-H	0.65	0.11	0.07	0.01
Aag-P-ET	0.54	0.04	0.05	0.07
Aag-P-EA	1.50	0.12	0.15	0.03
Aag-P-H	0.54	0.06	0.05	0.02×10^{-1}
Ats-S-ET	0.41	0.05	0.04	0.03
Ats-S-EA	0.61	0.07	0.06	0.01
Ats-S-H	0.09	0.08	0.01	0.01×10^{-1}

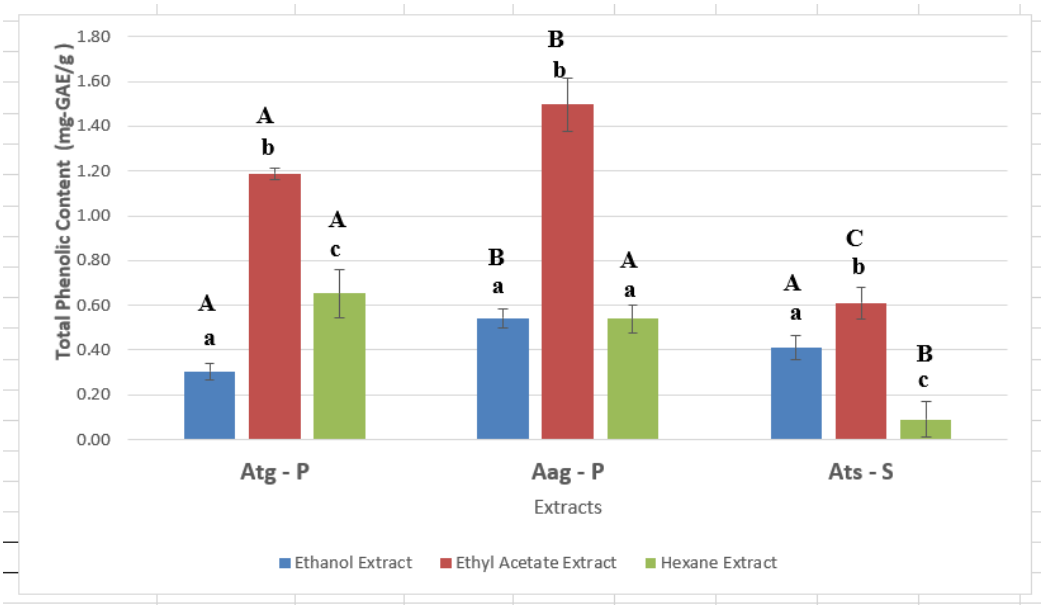


Figure 0.16. Total Phenolic content of Asparagopsis spp extracts *Note: n=3, alpha = 0.05, Tukey P < 0.05, lower case letters (a, b, c,) refers to statistical differences within species across solvents, Upper case letters (A, B, C) refer to statistical differences across species in the same solvent.*

Total phenolic content analysis of *Asparagopsis* spp confirms the presence of phenolic compounds in the species. In general, higher TPC values were observed in ethyl acetate extracts than the other solvent extracts of all the species. The highest TPC value (1.50 ± 0.12 mg-GAE/g) was observed in the ethyl acetate extract of *A. armata* (gametophyte) whereas the lowest value (0.09 ± 0.08 mg-GAE/g) was seen in the hexane extract of the sporophyte life stage of *A. taxiformis*.

A One-way ANOVA (figure 3.17) conducted on the data shows that there is a statistically significant difference in the TPC values across all the species. Tukey multiple comparison test ($P<0.05$) also revealed that generally there were significant differences in the observed TPC values within the species. However individual extract comparison for ethanol vs hexane extract for *A. armata* showed no significant difference between the two. Analysis between species reveals that the ethanol extracts of *A. taxiformis* are identical in phenolic content in its two life stages (gametophyte & sporophyte). A similar observation was seen between the hexane extracts of *A. taxiformis* (gametophyte) and *A. armata* (gametophyte) which showed no significant difference in their TPC.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Atg-P-ET	3	0.914676721	0.30489224	0.001362404
Atg-P-EA	3	3.567937439	1.18931248	0.000638088
Atg-P-H	3	1.957827119	0.65260904	0.012011318
Aag-P-ET	3	1.626668263	0.542222754	0.001793756
Aag-P-EA	3	4.491591358	1.497197119	0.013922185
Aag-P-H	3	1.621281944	0.540427315	0.003769467
Ats-S-ET	3	1.228080675	0.409360225	0.002843019
Ats-S-EA	3	1.835338241	0.611779414	0.004988957
Ats-S-H	3	0.27131087	0.090436957	0.006055532

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.571378795	8	0.571422349	108.5328864	1.224E-13	2.510157895
Within Groups	0.094769453	18	0.00526497			
Total	4.666148248	26				

Figure 0.17 One-Way ANOVA results for TPC

Generally, different amounts of TPC have been reported in various studies involving extracts from different types of macroalgae. However, the levels of TPC observed in this study are comparatively lower than what was reported in a study by Yarnpakdee et al. (2018) where TPC levels ranged between 6 to 12 gGAE/kg across selected red, green, and brown macroalgae species (Yarnpakdee et al., 2018). Another study involving TPC analysis of extracts from *A. taxiformis* by Nunes et al. (2018) reports similar low values of TPC across all solvent extracts (water = 0.62 ± 0.01 gGAE/(100g), ethanol = 1.71 ± 0.03 gGAE/(100g), methanol = 0.57 ± 0.02 gGAE/(100g), & ethyl acetate = 0.07 ± 0.01 gGAE/(100g)) (Nunes et al., 2018). This suggests that *Asparagopsis* spp might containt relatively small amounts of phenolics by nature when compared to some macroalgae. In relation to this, Stengel et al. (2011) reports that diversity in bioactives in macroalgae are due to factors such as evolutionary connections, ecological diversity, and chemical diversification (Stengel et al., 2011). This therefore explains the observed differences in phenolic content between *Asparagopsis* spp and other forms of macroalgae.

The quantity of phenolic content discovered in the extracts in this study suggests that phenolic compounds are but a fraction of the composition of the entire crude extracts for each solvent.

As a result, the antioxidant activity reported may be attributable in part to the phenolics present, but also, to a greater extent, to other bioactive chemicals contained in the extracts. This inference is emphasized by reports from studies by Torres et al. (2022), Yarnpakdee et al. (2018), Chernane et al. (2014), and Heo et al. (2005) who all indicated that no direct relationship was established between TPC values of crude extracts and antioxidant activities and hence observed activities may be also influenced by the presence of other compounds apart from phenolics in the crude extracts.

Findings about the distribution of phenolic content in the solvent extracts of this study were contrary to that of studies by Ganesan et al. (2008) and Chakraborty et al. (2015) who suggested that TPC values were directly correlated with solvent polarity, thus solvents of higher polarity index are able to extract more phenolic compounds as compared to those of lower polarity. However, observations in this current study show that despite the fact that ethanolic solvent extracts where relatively higher in TPC than the Hexane extract, they were lower when compared to ethyl acetate solvent extracts which also demonstrated the highest TPC values across all species in this study. In general, the higher TPC values observed with ethyl acetate could be partly attributed to the biphasic action of this solvent which gives it the ability to extract both polar and non-polar compounds as well as the fact that it has a relatively medium polarity index of PI 4.4 (Javad, 2013). Once again, the relatively higher amount of TPC found in ethyl acetate extract could be indicative of an appreciable amount of lower polarity phenolic compounds such as tocopherols and tocotrienol which have been demonstrated to be well extracted by ethyl acetate (Beldean-Galea et al., 2010). A look at the TPC values obtained for hexane extracts show a good amount of phenolic content. This observation could further suggest the presence of phenolic compounds of varying polarities in *Asparagopsis* spp.

Phenolic compounds are a type of secondary metabolite found in plants and other organisms like macroalgae. They are classified as phenolic acids and polyphenols. These molecules can be found in combination with monosaccharides and polysaccharides, connected to one or more phenolic groups, or as derivatives such as ester or methyl esters. Among the several types of phenolic compounds, phenolic acids, flavonoids, and tannins are considered the most important dietary phenolic compounds (Minatel et al., 2017).

The antioxidant properties of polyphenols are related to their ability to scavenge a wide variety of ROS. The processes behind polyphenol antioxidant ability include reduction of ROS

creation by inhibition of enzymes involved in their synthesis, scavenging of ROS, or upregulation or protection of antioxidant defences. Polyphenols may have anti-inflammatory effects by scavenging free radicals, regulating cellular activity in inflammatory cells, and modulating the activities of enzymes involved in arachidonic acid metabolism (phospholipase A2, COX) and arginine metabolism (NOS), as well as modulating the production of other proinflammatory molecules (Hussain et al., 2016). These properties of phenolic compounds make them very important and therefore their presence in extracts of *Asparagopsis* spp suggest the macroalgae to be a potentially great resource for the pharmaceutical and nutraceutical industries.

3.7. Multivariate Analysis

Correlation studies were carried out between the antioxidant assay, metal chelating assays, COX-2 anti-inflammatory assay, and total phenolic content assay. Data obtained from these tests for *Asparagopsis* spp were organised into a table as shown in Table 3.13 and analyzed using the chemometic software Uscrambler, version X 10.4., 2016. Principal component analysis (PCA) as well as cluster anlaysis were performed and the results are discussed as follows.

Table 0.13. %Activity of the various Asparagopsis spp specimen across the various tests

Assays	Atg-P-ET	Atg-P-EA	Atg-P-H	Aag-P-ET	Aag-P-EA	Aag-P-H	Ats-S-ET	Ats-S-EA	Ats-S-H
ABTS	16.5	14.2	n.d.	54.7	14.5	5.37	15.8	18.0	17.1
DPPH	22.98	35.0	17.9	48.9	39.9	26.9	9.8	18.02	16.1
ICA	33.6	27.4	23.2	35.02	71.8	36.6	43.8	89.9	87.8
CCA	34.9	62.5	57.4	31.1	62.7	72.98	29.3	53.5	75.9
IRP	14.2	133.5	29.9	28.9	186.1	25.5	30.5	166.7	63.2
COX-2	79.08	61.09	98.97	26.05	48.25	57.13	65.17	21.52	72.30
TPC	0.30	1.19	0.65	0.54	1.50	0.54	0.41	0.61	0.09

Note: n.d. implies not detected.

3.7.1. Principal Component Analysis (PCA)

PCA was carried out using Nepals algorithm to determine the latent variables that possibly explains the variations observed among all the tests. In all, five principal components were obatined with PC 1 explaining 68% of the variations in the data and PC 2 explaining 23% of the variations. PC 3, 4, and 5 were also seen to explain 5%, 2%, and 2% of the data respectively. Based on these percentages, only the first and second principal components (PC 1 and 2) were deamed necessary for the analysis as eliminating the other principal components will not result in any significant loss of information. The loadings and score plots for the data were then generated and are displayed in figures 3.18 and 3.19 respectively.

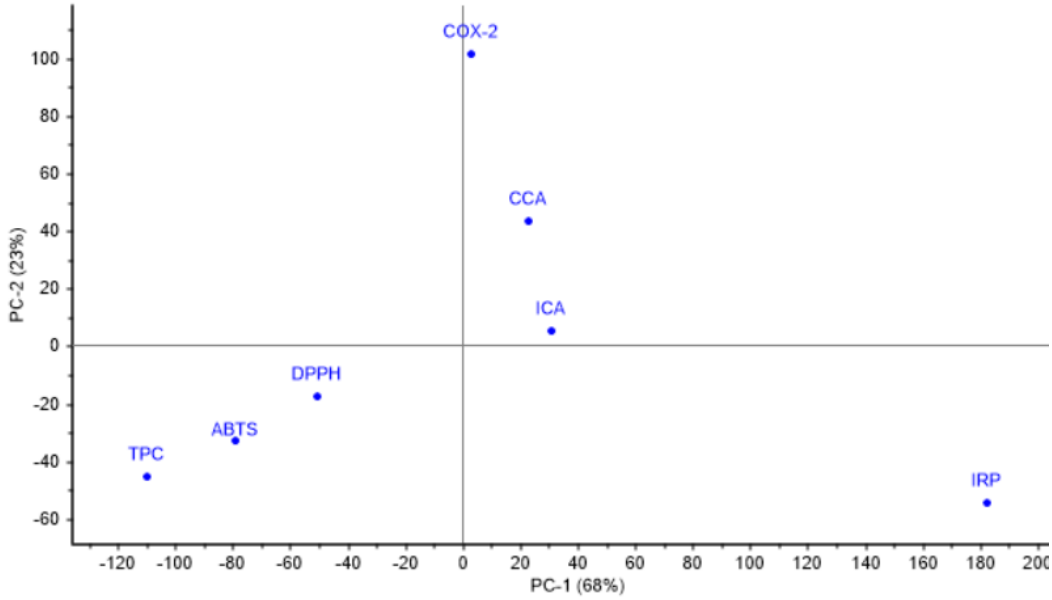


Figure 0.18. Loadings plot for principal components analysis of the data

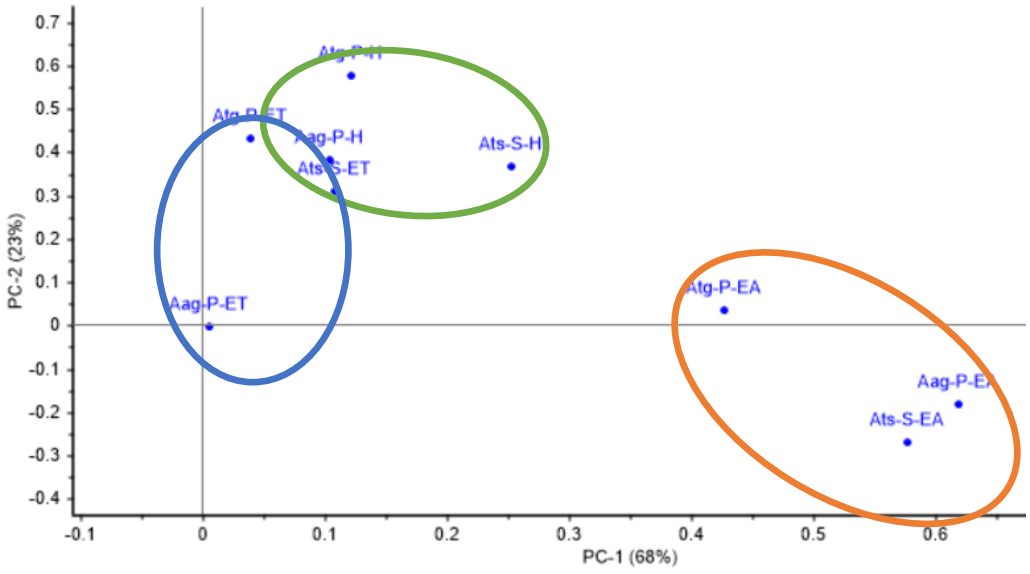


Figure 0.19. Scores plot for principal components analysis of the data

A critical look at the loadings plot (figure 3.18) of the principal component analysis reveals that PC1 is mostly influenced by the iron reducing antioxidant test (IRP) whereas PC2 is mostly influence by COX-2 test. Total phenolic content test (TPC) had the least influence on the principal components (PC 1& 2) among all the tests. This suggests that antioxidant activity as witnessed in our samples was the least influenced by the TPCs of the samples. This finding is consistent with reports from studies by Heo et al. (2005) and Yarnpakdee et al. (2018). Another study by Torres et al. (2022) on extracts from red macroalgae reported of the observation of

high antioxidant activity in extracts but however no phenolic compounds were detected on GC-MS and HPLC analysis of the extracts (Torres et al., 2022). This once again confirms the earlier observations made in this current study which suggests the presence of other bioactive compound(s) other than phenolics which contribute to the antioxidant activities seen in extracts of *Asparagopsis* spp.

Furthermore, observations from the loadings plot (figure 3.18) show what seems to be some groupings among the activity tests. For instance, it is observed in the right upper quadrant the grouping of copper chelating activity test (CCA), iron chelating test (ICA), and the COX-2 tests. In the same light, observations are made of the grouping of the tests ABTS, DPPH and total phenolic content test (TPC) in the right lower quadrant of the loadings plot. These groupings as observed is suggestive of the possibility that the tests in the various groups might be influenced by specific extract content(s) in the case of each group. From a broader pespective, the groupings could also be said to be indicative of some form of correlation among tests in the same groups. Thus base on this, it can be suspected that there is some similarities between tests like the cooper chelating test (CCA), iron chelating test, and to some extent the COX-2 test and also that there is some similarities between ABTS and DPPH tests with respect to *Asparagopsis* spp analysis. Similarities in these tests could be attributed to the mechanism of action employed by the tests. In the case of ABTS and DPPH, these tests are known to employ electron transfer mechanism (ET) and in some cases hydrogen atom transfer (HAT) in their operations and this explains the observed similarities. Also in the case of CCA and ICA, these tests are classified as metal chelating tests implying a similar mechanism of action for these two tests (Shahidi & Zhong, 2015; Prior et al., 2005).

In general, the strength of the correlation between tests is demonstrated by the magnitude of the distance between the tests as well as the quadrants in which they lie. Tests in opposite quadrant can be said to be negatively correlated whereas those in the same quadrant are positively correlated. For this reason, it can be said that the scores plot suggests that tests such as COX-2 is negatively correlated to total phenolic content (TPC). This observation further hightlights the possibility of other bioactive compound(s) other than phenolic compounds contributing to the observed antioxidant activity in the extracts of *Asparagopsis* spp. Also, CCA shows greater signs of a positive correlation with COX-2 than ICA and all the other antioxidants tests used in this study. This also suggests the possibility of employing CCA as a preliminary screening test for samples prior to COX-2 analysis. This observation is of great importance because COX-2 inhibition test is noted to be time consuming, complex, and very

expensive to carry out. Hence in the event of a study where a large number of samples are to be tested it becomes challenging and puts a great strain on not only human resources, but financial resources and time as well. However, cooper chelating activity (CCA) is comparatively simple and economical with time and funds hence could be a great way of pre-screening samples to determine which samples are likely to show good results with COX-2 analysis and hence reduce sample size which will eventually save time and other resources.

Observations from the scores plot (figure 3.19) shows the distribution of the extracts along the prinicipal components axis (PC 1 & 2). This observation shows how the extracts project on to the principal components. Extracts to the extreme right of the loadings plot demonstrate a high relation to PC1 where as extracts to the upper part of the scores plot demonstrate a high relation to PC2. This observation implies that principal components 1 & 2 could be some specific group(s) of bioactive compounds that are distributed across the extracts in varying quantities and are actually responsible for the antioxidant activities observed in the extracts.

Furthermore, the formation of clusters among the extracts and along the principal component axis is observed in the scores plot. A pattern is observed with this clustering which is that; extracts of the same extraction solvent cluster together. From this, a clear grouping of ethyl acetate extracts at the extreme right of the scoress plot along the axis of PC1 is identified. Similarly, groupings of other solvent extracts (ethanol and hexane) are observed in the upper quadrant of the scores plot along the PC2 axis. However the groupings in the case of the ethanol and hexane extracts are not clearly defined. This could be attributed to inteference of other components in the extracts since they are only crude extracts and not highly refined. The solvent pattern observed in the groupings could imply that the type of solvent used for the extraction of extracts from *Asparagopsis* spp has a significant influence on the nature of bioactive compound(s) found in the crude extracts. In the case of the clear grouping observed with extracts of ethyl acetate, a possible explanation of this is that ethyl acetate is distinct from the other solvents in the sense that it is able to extract compounds which are not present in the other solvents and display a specific activity, such as observed in iron reducing activity test. This observation is in conformity with reports from the studies by Pintać et al. (2018) and Pérez-Jiménez and Saura-Calixto (2006), who reported that the antioxidant capacity of an extract is affected by the solvent utilized for the extraction process.

3.7.2. Cluster Analysis

Cluster analysis is used to analyse the occurance of natural groupings or cluster formation among a set of items. Clustering is done using the distances between items based on information (factors) obtained from the set. The distances can be calculated using different methods however in this study the Bray-curtis distance (dissimilarity) was used as we sort to explore the similarities between the tests used.The occuring groupings observed with cluster analysis are usually represented by a dendrogram where each item in the set is linked to the item which is closest to it in terms of distance and is also in turn linked to the next closest item. Linkages can be assessed in different ways but for this study the average linkage clustering method was used.

Cluster analysis conducted on the data in Table 3.13 revealed the same nature of groupings observed in the principal component analysis between ABTS and DPPH as well as COX-2 and CCA tests. The dendrogram seen in figure 3.20 shows that the test most closely related to COX-2 is CCA. It also shows that ICA and CCA tests are closely related to each other at almost the same distance on the relative distance scale and this happens to be true as both tests are metal chelating activity tests. ABTS and DPPH tests were also seen to be clustered together at the same scale distance impling these two test are of the same caliber. This observation is affirmative as both ABTS and DPPH test are antioxidant tests that function on the same mechanisms. The associations observed between the tests could also be attributed to the possibility of these tests being sensitive to the same group(s) of bioactive compounds in the extracts as also previously discussed.

TPC test is observed to be the least connected test in the set as it connects at a further distance than all the other tests along the relative distance scale. This further emphasizes the earlier made inference which suggests that antioxidant activities as observed in the *Asparagopsis* spp extracts might be greatly influenced by the presence of other bioactive compound(s) other than phenolic compounds.

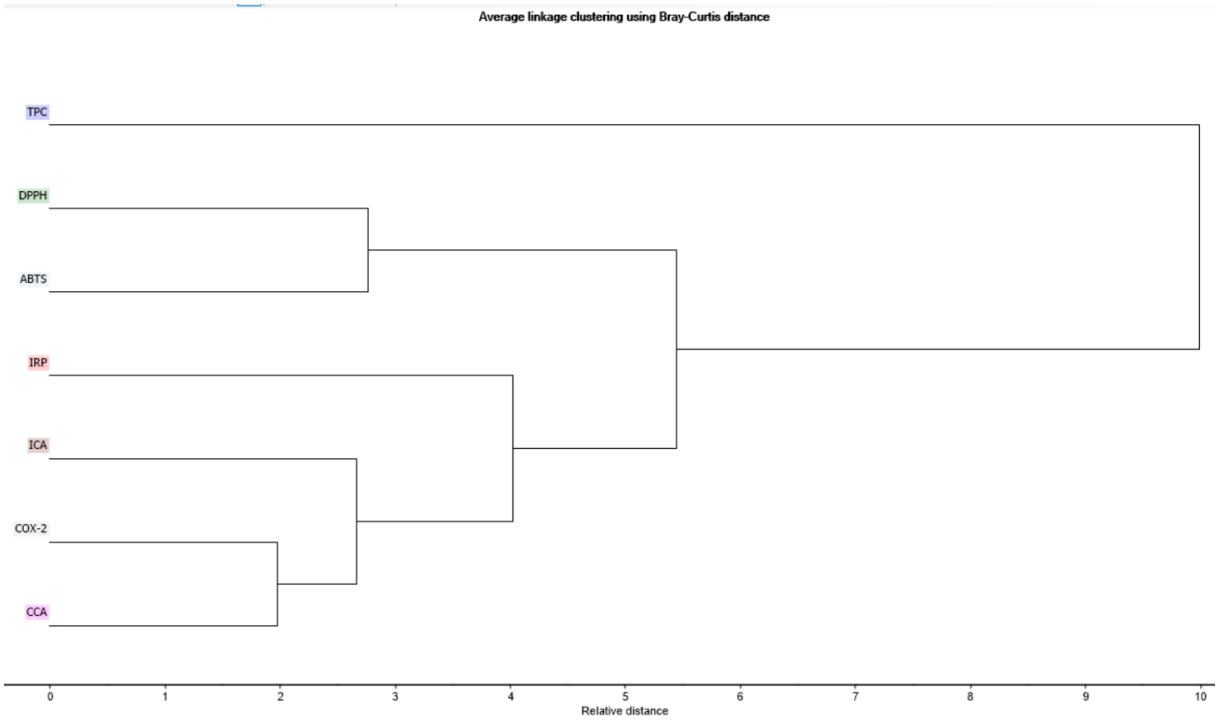


Figure 0.20. Dendrogram for Cluster analysis of ABTS, DPPH, CCA, ICA, COX-2, TPC, and IRP tests

Statistical analysis conducted on the tests performed suggest that some of the tests are closely related to each other. Thus, ABTS and DPPH assays are closely related with regards to principal component analysis and cluster analysis whereas copper chelating activity and iron chelating activity assays are also closely related on the cluster analysis. An interesting discovery is that of the positive correlation observed between COX-2 assay and copper chelating activity assay in both the principal component analysis and the cluster analysis. The observation theoretically suggests that to a greater extent, copper chelating activity assay can be used as a preliminary screening test among samples prior to COX-2 assay as it is mostly likely that samples that show good activity with copper chelating assay will also show good inhibitory activity with COX-2 assay.

4.0 CONCLUSION AND RECOMMENDATIONS

This research focused on evaluating the presence of bioactive compounds in two distinct species of the macroalgae, *Asparagopsis taxiformis*, as a gametophyte and as a sporophyte, and *Asparagopsis armata* as a gametophyte by assessing their antioxidant and anti-inflammatory capabilities. Antioxidant properties were evaluated using tests such as ABTS, DPPH, Iron and copper chelating activity, total phenolic content, and iron reducing activity while anti-inflammatory analysis was conducted specifically for COX-2 inhibition.

Results obtained suggests that *Asparagopsis* spp contain several bioactive compounds that exhibit good antioxidant abilities especially with metal chelating activities for copper and iron. The macroalgae also demonstrated tremendous potential for iron reducing activity and COX-2 inhibition activity for all species. These antioxidant and anti-inflammatory activities as observed have been reported to be influenced by the presence of bioactive compounds such polyphenols, pigments, sterols, and polyunsaturated fatty acids, among others. Factors such as type of species, the life stage of the species and the type of solvent used for the extractions as well as the amount of extract concentrations used in the various assays were observed to have significant impact on the results obtained for each assay.

The presence of phenolic compounds was confirmed for *Asparagopsis* spp through the evaluation of the total phenolic contents (TPC) in this work. However, the levels of total phenolic compounds present in *Asparagopsis* spp were determined to be relatively low compared to other macroalgae in literature. Further analysis revealed that the antioxidant activity observed in the crude extracts were not solely dependent on the phenolic compounds but rather to a greater extent other bioactive compound(s) that may be present in the crude extracts.

Multivariate analysis studies in this work revealed that copper chelating activity assay has a good positive correlation with COX-2 inhibitory assay and therefore could be used as a preliminary screening test in the case where selective COX-2 inhibitory testing is desired to be conducted on a large set of extracts. This observation is of great importance because COX-2 inhibition test is noted to be time consuming, complex, and very expensive to carry out. Hence in the event of a study where a large number of samples are to be tested it becomes challenging and puts a great strain on not only human resources, but financial resources and time as well. However, cooper chelating activity (CCA) is comparatively simple and economical with time and funds hence could be a great way of pre-screening samples to determine which samples

are likely to show good results with COX-2 analysis and hence reduce sample size which will eventually save time and other resources.

Overall, findings presented from this research work suggests that the macroalgae *Asparagopsis* spp demonstrates high anti-inflammatory and antioxidant potentials for use in the pharmaceutical and nutraceutical industries for making drugs for the treatment of inflammatory based disease such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, arthritis, and cardiovascular diseases.

Recommendations for the follow-up of this study include:
the performance of an activity guided fractionation of crude extracts to quantify, identify and characterize the specific bioactive compound(s) responsible for the observed activities in *Asparagopsis* spp associated with each antioxidant and anti-inflammatory test.

It is also recommended that further correlation studies be carried between the antioxidant and anti-inflammatory tests and other tests that were not used in this research to properly establish a group of tests which could be used as preliminary tests for more expensive and involving tests.

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