DURBAN UNIVERSITY OF TECHNOLOGY

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CREATIVE. DISTINCTIVE. IMPACTFUL.

Evaluation of toxicity and biochemical characterisation of a microalgal diatom

Submitted in fulfilment of the requirements for the degree of

Doctor of Food Science and Technology

Department of Biotechnology and Food Science, Faculty of Applied Sciences,
Durban University of Technology, Durban, South Africa

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Declaration

I declare that the thesis herewith submitted to the Department of Biotechnology and Food Science, Durban University of Technology for the award of the degree of Doctorate in Food Science and Technology is my work and has not been previously submitted for a degree at any other University or Higher Institution of Education.

As the candidate's supervisors, we agree to the submission of this thesis.

	06/04/2023
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	06/04/2023
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	06/04/2023
Signature of co-supervisor	**Date**

Dedication

In honour of my mum

In memory of my dad

I dedicate this thesis to my beloved dad (Mr D. Beekrum) and my mum (Mrs R. Beekrum) for their endless support and inspiration, and who were pivotal in influencing my educational journey.

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"Nutritional profile, bioactive compounds and antioxidant activity of microalgal strain, *Amphora* sp. isolated from the Cape coastal waters, South Africa"

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Safety evaluation of *Amphora* sp., a marine microalgal diatom isolated from the Cape coastal waters, South Africa

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ABSTRACT

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Abstract

One of the critical challenges that we face in the 21st century is the need to feed an ever-increasing human population with increasingly limited natural resources. Microalgae have emerged as a potential solution for global food security as a sustainable biological food source for humans due to their nutrient-rich composition, particularly rich protein and bioactive compounds that provide potential benefits for human health. By establishing microalgae as a new food platform, we can increase the supply of these essential products to address global demands in a more efficient and environmentally sustainable way. These under-exploited organisms have been consumed in the human diet for thousands of years. Microalgae cultivation does not compete with land and resources required for traditional crops and has a superior yield compared to terrestrial crops. Diatoms are a major group of microalgae in the phytoplankton community and have the potential to be engineered into cell factories for the sustainable production of bioactive compounds in food and nutraceutical industries.

This study aimed to characterise a rapidly growing marine diatom in terms of its toxicity and biochemical profile. This was done by evaluating the safety profile and biochemical composition, characterising the soluble protein, and investigating the carbohydrate profile with specific emphasis of β-glucan and its effect of cardioprotective properties on ferric-induced oxidative cardiac injury in a rat model.

Based on Basic Local Alignment Search Tool (BLAST) analysis, the strain showed the closest similarity to *Amphora* sp. (JF834543.1) with 99.5% and is therefore represented as *Amphora* sp., accession number MW721231. The bacterial reverse mutation assay found no evidence of mutagenicity on the methanolic, aqueous, and hexane extracts of *Amphora* sp. and was found to exert low levels of cytotoxicity against Peripheral Blood Mononuclear Cells (PBMC). A 28-day acute oral toxicity assessment on male Wistar rats showed an absence of adverse effects and mortality in the rats.

The biomass exhibited a low lipid profile, modest protein content, notable amino acid content, and excellent carbohydrate and mineral content. Results of this study for antioxidant assays displayed low to moderate activities. Protein extracted using three-phase partitioning (TPP) treatment showed that the protein concentration and total amino acid content were substantially higher in the protein-enriched biomass extract when compared to the dried biomass. The solubility of the protein-enriched biomass extract increased with the increase in pH within the

range of pH 2 to pH 12. The biomass consisted of a simple monosaccharide profile comprising glucose, rhamnose, and mannose, and a β-glucan content of approximately 9%.

The cardioprotective properties of the β-glucan extract on ferric-induced oxidative cardiac injury did not improve the glutathione (GSH) level significantly, it led to increased superoxide dismutase (SOD) and catalase activities, while depleting malondialdehyde (MDA), NO (nitric oxide), low-density lipoprotein cholesterol levels, and simultaneously elevating triglycerides and high-density lipoprotein (LDL) cholesterol levels. GC-MS analysis revealed a complete depletion of the lipid metabolites. Our results advocate the protective capabilities of the β-glucan extract against ferric-induced oxidative cardiac injury as portrayed by its ability to stall oxidative stress and modulate cardiac lipid metabolism while inhibiting the acetylcholinesterase and lipase activities. These results display that the β-glucan extract could be utilized as an alternative for the development of nutraceuticals for maintaining cardiac health.

The diversity of food bioactive molecules obtained from microalgae makes these micro-organisms a bioresource with full potential of exploitation in the food industry. The richness of compounds in microalgae can contribute to develop an algal-based food industry, focusing on producing and utilizing microalgae for innovative functional food products. Overall, this study demonstrated the potential utilization of the diatom, *Amphora* sp. as a potential ingredient and nutraceutical in foods.

Preface

This thesis is organized into nine chapters with Chapter one providing the general introduction to the thesis. Chapter two presents an overview of the composition of microalgae, a critical review of microalgal cultivation, and the application of microalgae in the food industry. Chapter three focuses on the identification and toxicity profile of the microalgal strain. Chapter four is based on screening the biochemical composition of biomass and extracts to determine the properties in terms of nutritional composition and bioactive components. Chapter five investigates the characterisation of soluble protein of the microalgal strain using a three-phase partitioning treatment. Chapter six discusses the preliminary characterisation of carbohydrates and its effect on cardioprotective properties of ferric-induced oxidative cardiac injury in a rat model. Chapter seven is a general discussion of the entire findings. Conclusions were drawn from the study and recommendations were proposed for future studies.

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List of Abbreviations

AAE	Ascorbic Acid Equivalent
ABTS	2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid)
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AOAC	Association of Official Analytical Chemists
AST	Aspartate Aminotransferase
ASW	Artificial Seawater
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
BRU	Biomedical Resource Unit
BUN	Blood Urea Nitrogen
BW	Body Weight
Ca	Calcium
CHOL	Cholesterol
CO_2	Carbon Dioxide
Cr	Creatinine
CSIR	Council for Scientific and Industrial Research
CV	Central Vein
DHA	Docosahexaenoic Acid
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DPPH	2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate
EAAs	Essential Amino Acids
EPA	Eicosapentaenoic Acid
FAs	Fatty Acids
FAMEs	Fatty Acid Methyl Esters
FRAP	Ferric-Reducing Ability of Plasma
FTIR	Fourier Transformed Infrared
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrophotometry

GLU	Blood Glucose
GSH	Glutathione
HCl	Hydrochloric Acid
HDL	High-Density Lipoprotein
IVPD	*In Vitro* Protein Digestibility
K	Potassium
LA	Linolenic Acid
LC	Lethal Concentration
LDL	Low-Density Lipoprotein
MDA	Malondialdehyde
MCF-7	Michigan Cancer Foundation-7
MUFAs	Monounsaturated Fatty Acids
N	Nitrogen
Na	Sodium
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TAC	Total Antioxidant Capacity
TPC	Total Phenolic Content
TPP	Three-Phase Partitioning
US FDA	United States Food and Drug Administration
UKZN	University of Kwa-Zulu Natal
UV	Ultraviolet
WHC	Water Holding Capacity
WHO	World Health Organisation
UPLC	Ultra-Performance Liquid Chromatography

List of Mathematical Symbols

Symbol	Full Term	Measurement
%	Percentage	Mathematics/Statistics
±	Approximately	Mathematics/Statistics
A	Absorbance	Spectrometry
ANOVA	Analysis of variance	Statistics
˚C	Degrees Celsius	Temperature
G	Gram	Weight
H	Hour	Time
Kg	Kilogram	Weight
L	Litre	Volume
Lhr^{-1}	Litre per hour	Flowrate
M	Molar	Concentration
mM	Millimolar	Concentration
M	Meter	Length
Mg	Milligram	Weight
mg/g	Milligram per gram	Concentration (*wt./wt.*)
mg/L	Milligram per litre	Concentration (*wt./v*)
Min	Minutes	Time
mL	Millilitre	Volume
N	Number of samples	Statistics
Rpm	Rotations per minute	Speed
s^{-1}	Per second	Time
SD	Standard deviation	Statistics
U/L Units per litre µg/mL	Microgram per millilitre	Concentration (*wt./v*)
µL	Microliter	Volume
µM	Micromolar	Concentration

Chapter 1: Introduction

The world population is currently 7.8 billion, with some demographers estimating that it will reach 8.5 billion by 2030 (Roser, Ritchie and Ortiz-Ospina, 2013). Global food security has deteriorated as a result of exponential population increase. Furthermore, the current COVID-19 epidemic has heightened the likelihood of famine and worldwide food instability, with an estimated 265 million people suffering from severe hunger (Laborde *et al.,* 2020). To meet rising global food demand, the agricultural industry must undergo massive transformation; yet, this comes at a significant cost (Timmer, 2017). With an increase in the world population, achieving adequate nutrition is a growing global concern. Microalgae are a sustainable and relatively novel source of nutritional compounds. They are an important source of a variety of valuable nutrients for human health (García, de Vicente and Galán, 2017).

Microalgae are a diverse group of photosynthetic marine and freshwater organisms with simple growing requirements such as light, sugars, carbon dioxide (CO_2), nitrogen (N), phosphorus (P), and potassium (K), and the ability to produce large amounts of lipids, proteins, and carbohydrates in a short period of time (Brennan and Owende, 2010). When compared to other energy crops, microalgae have better photosynthetic efficiency, higher biomass output, and quicker development (Miao and Wu 2006; Chiu *et al.,* 2009).

Microalgae have been widely employed as novel bioactive material sources. Along with this trend, there is a lot of interest in the prospect of replacing synthetic food components with natural ones (Ibañez and Cifuentes, 2013). In addition to their nutritional benefits, algae are increasingly being promoted as functional foods or nutraceuticals; in many countries, these phrases have no legal basis, but they represent foods that contain bioactive molecules, or phytochemicals, that may improve health in ways other than basic nutrition, such as anti-inflammatories and disease prevention (Hafting *et al.,* 2012; Bagchi, 2014). Overall, the global trend toward increased nutritional demand for algal products stems from a growing emphasis on health and a larger usage of food additives from natural sources.

The most predominant microalgal groups consists of Cyanophyceae (blue-green algae), Chlorophyceae (green algae), Bacillariophyceae (including diatoms), and Chrysophyceae (including golden algae) (Carlsson *et al.,* 2007). Diatoms are the most diverse group of eukaryotic organisms in aquatic environments. They are one of the most important producers of biomass in the ocean, accounting for around 40% of marine primary

production, Additionally, they are responsible for up to 20% of global CO_2 fixation in the biosphere. Diatoms develop faster than other microalgae (Furnas, 1990), which may be attributed to their silicic cell wall requiring less energy to build than organic barriers.

Diatoms are increasingly being used as a source of bioactive substances in sectors such as food and cosmetics (Gügi *et al.,* 2015). Diatoms have become significant sources of dietary supplements for humans due to their capacity to synthesize carotenoids, phytosterols, vitamins, and antioxidants (Becker, 2007). Furthermore, they may produce high amounts of polyunsaturated fatty acids (PUFAs), which are bioactive compounds that have been shown to benefit human health (e.g., reduce the incidence of cardiovascular illnesses and malignancies) (Lebeau and Robert, 2003; Kroth, 2007; Li *et al.,* 2014a).

Amphora sp. is a large genus of diatoms found in both saltwater and freshwater. According to El-Sayed *et al.* (2018), *Amphora* sp. contains bioactive components such as carotenoids, sulphated polysaccharides, PUFAs, -tocopherol, -glucans, and vitamins C and E. Several investigations have shown that *Amphora* sp. extracts can serve as protective and antioxidant agents (Lee *et al.* 2009b; Boukhris *et al.,* 2017; Mekkawy *et al.,* 2020). Because of the presence of β-carotene and fucoxanthin, which are frequently employed as food additives as well as in numerous nutraceutical applications, *Amphora coffeaeformis* has been regarded an effective radical scavenger (Jaswir *et al.,* 2011).

In recent years, microalgae have been receiving a lot of attention because of their rich metabolic composition. They create a wide range of bioactive molecules with diverse chemical structures and biological activity (Romano *et al*., 2017). Some diatom genera have been widely utilized as dietary supplements for both humans and animals because of their high nutritional value (Allen *et al.,* 2008; Sutak *et al.,* 2012). Although the potential for algal products/co-products for nutritional uses has long been recognized, commercial success has been limited, with just a few species (such as *Spirulina* and *Chlorella*) occupying niche markets (Brennan and Owende, 2010).

Increasing our understanding of the biochemical makeup of algae is essential for realizing the promise of algal products/co-products. Many forms of algae are nutritionally complete diets, their yields exceed most plant commodities, and a growing set of technologies is available to generate superior algal strains (Torres-Tiji, Fields and Mayfield, 2020). The existing food system has failed to safeguard the environment and has not ensured that everyone has access

to sufficient and nutritionally acceptable food. In 2019, it was reported that almost 690 million (8.9% of the world's population) people are anticipated to be undernourished internationally, with Africa accounting for 51.5% (433.2 million), the biggest number of undernourished people globally by 2030 (FAO and UNICEF, 2021). There is the possibility to enhance the supply of these vital items in a more effective and ecologically friendly manner by creating microalgae as a new food and feed platform (Torres-Tiji, Fields and Mayfield, 2020). Bearing this in mind, the purpose of this study was to investigate the bioactive properties of an underexploited, rapidly growing microalgal diatom isolated in South Africa for use in the food industry.

Chapter 2: Literature Review

A detailed nutritional characterisation of *Amphora* sp. grown in South Africa has not been established. In addition, the safety profile is yet to be investigated. This chapter presents the literature relevant to this study. The areas covered include an overview of microalgal classification, microalgal production systems and nutritional composition of microalgae. Knowledge of an insight into this unexplored diatom is therefore important to introduce this microalgal diatom to various food applications.

2.1 Overview of microalgal classification

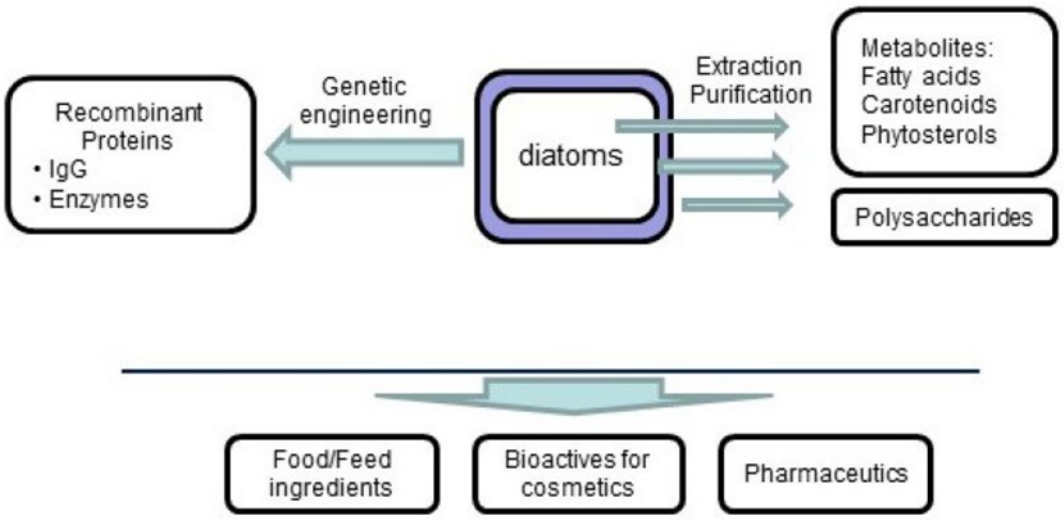
Microalgae is a general term that encompasses a large collection of a specific group of microorganisms. They can be defined to as photosynthetic microbes, which are broadly categorized as eukaryotes and prokaryotes. The prokaryotic microalgae are relatively simpler in form, they are also referred to as blue green algae and includes cyanobacteria. On the other hand, eukaryotic microalgae are more complex in structure and in their biochemistry, and includes the green algae and diatoms. In addition, biologists have also classified microalgae based on the type of photosynthetic pigments, the basic cellular structures as well as their life cycles. More recently, the microalgal classification system has been expanded to include factors such as the cell wall constituents, the cytological and morphological characteristics, as well as the biochemical nature of their storage products. Furthermore, microalgal organisms have also been grouped into ten major phyta based on deoxyribonucleic acid (DNA) sequence, *viz*., Cryptophyta, Chlorophyta, Dinophyta, Glaucophyta, Euglenophyta and Haptophyta. The remaining phyta include Heterocontophyta, Rhodophyta, Prochlorophyta as well as Cyanophyta which are the cyanobacteria (Groendahl, Kahlert and Fink, 2017).

Cyanobacteria have been playing a major function in the evolution of our planet, and these roles are still being played remarkably in our day-to- day existence. As a result of their inherent oxygenic photosynthesis, cyanobacteria have been described to be responsible for the earth's transition from a carbon dioxide-dominated atmosphere to the current relatively oxygen-rich one (Becker, 2004). In addition to being a major and critical contributor of the earth's oxygen gas, marine cyanobacteria, especially, have been identified as remarkable producers and sources of many important secondary metabolite. The marine cyanobacteria, which are also referred to as the blue green algae, are typically primary photosynthetic

microbes that can be found existing and colonizing various ecosystems which includes freshwater, oceans, seas as well as moist soil and bare rock (Whitton and Potts, 2012). It has been discovered that the variety of the secondary metabolites synthesized by these group of microalgae are very diverse and complex in nature; and they include pigments, fluorescent dyes and bioactive compounds with many potential pharmaceutical applications (Mazard *et al.,* 2016).

Diatoms are unicellular eukaryotic microalgae that constitute a large majority of the microalgal population and are well known for their siliceous coverings and their symmetrical structures. They are biologically classified into the phylum and class; Chrysophyta and Bacillariophyceae respectively. Diatoms are also typically noted to accumulate lipids especially in oily form as well as chrysolaminarin. In this regard, lipids have been identified as their main carbon storage compound with fatty acids (FAs) and triacylglycerides which comprised between 15 and 25% of their total dry biomass (Mangas-Sánchez and Adlercreutz, 2015). However, diatoms are continuing to attract scientific attention as a result of their potentials to secrete a wide variety of biologically active compounds as well as different fine chemicals with important applications in the industry (Vinayak *et al.,* 2015). They are predominantly endowed with pigments including carotenoids, which have been found immensely useful in human foods, animal feeds, cosmetics as well as pharmaceutical ingredients, especially in human health and food supplements (Vílchez *et al.,* 2011; Forján *et al.,* 2015; Fu *et al.*, 2017) (Figure 2.1).

Figure 2.1: Uses of active compounds from diatom in the health and food industries (Gügi *et al*., 2015)



2.2 Morphology of diatoms

Diatoms are microscopic unicellular microalgae that are well known for their silica-dominated cell wall. Their impervious, hydrated amorphous silica cellular wall, which is also referred to as the frustule, is their main physical distinguishing factor from all other microalgae. The frustule is made up of two bisected parts i.e., the hypotheca (smaller part) which sits perfectly on top of the epitheca (larger part) (Cristóbal *et al.,* 2020). There are many perceptile markings on the valves' surfaces which are commonly referred to as the striae, while the longitudinal slits that run along the thecae's middle are referred to as raphe. The differences in these fore-mentioned morphological features are the distinguishing factors between the different types of diatoms, hence they basically serve as robust bases for species identification within diatoms (Tomas, 1997; Leterme, 2015; Cristóbal *et al.,* 2020).

The two group of frustules, *viz*., centric and pennate, are morphologically grouped into three classes i.e., Bacillariophyceae (raphid diatoms), Coscinodiscophyceae (centric diatoms), and Fragillariophyceae (araphid diatoms) (Mishra *et al.,* 2017). The centric diatoms possess radial symmetries in valve view which can either be multipolar or unipolar. These class of diatoms contain plastids of small sizes, in addition they are oogamous, having eggs and mobile flagellated spermatozoids as gametes. Pennate diatoms, on the other hand, are elongated cells that have bilateral symmetry. The pennate diatoms usually show a slit along their long axis which are referred to as raphe, they secrete mucilage through this raphe to aid in their attachment to external structures or to glide over the structures. They may also have few plastids which are relatively larger and are isogamous in nature, having same shaped and non-flagellated gametes. Diatoms are known to exist independently as single cellular units; however, the planktonic diatoms have the ability to exist together as chains of cells. Their size are usually a few micrometres (e.g., *Cyclotella* sp. ~3 μm) to a few millimetres (e.g., *Ethimodiscus* ~2 mm) (Snoeijs, Busse and Potapova, 2002). There is a size disparity between the cells of freshwater and marine diatoms. While marine diatoms have been recorded to have a cell volume of above 109 μm^3, their freshwater counterparts are estimated to be thrice as small as their marine ones and possess cell size below 106 μm^3 (Litchman, Klausmeier and Yoshiyama, 2009).

2.3 Microalgal production systems

The two commonly used approaches for microalgae cultivation are the open raceway pond and the closed photobioreactor system (Brennan and Owende, 2010). The open pond production system is recorded as one of the earliest and easiest methods of cultivating microalgae biomass on a commercial level (Chisti, 2016). There are few variants of the open pond system, and these include natural water bodies (ponds and lakes), and the artificial water bodies (raceway and circular ponds). In some cases, it is not uncommon for containers such as tanks to be used in microalgae cultivation (Luque and Du, 2010).

The open raceway system entails microalgae production in circular oblong channels and recirculating ponds with semi-circular ends and their deflector baffles making sure that there is uniform flow and minimal dead zone formation. In the open raceway system, flow, recirculation as well as agitation are usually facilitated by a rotating paddle wheel. This system is relatively affordable and simple to construct as a cultivation technique; however, the drawbacks include low productivity and their vulnerability to the vagaries of weather as well as the introduction of constant contamination (Chisti, 2016). The currently available means of circumventing these concerns is by utilizing microalgae strains with the ability to survive and thrive under extreme alkaline or salt conditions to counteract the contaminants which are usually unable to thrive under these conditions (Stark and O'Gara, 2012; Ugwu and Aoyagi, 2012). Furthermore, as a result of the openness of this production system, it is more difficult to regulate specific growth factors especially the light intensity and temperature which will ultimately influence microalgal growth (Stark and O'Gara, 2012). However, the raceway pond system is still regarded as the microalgal system of choice in spite of the minimal productivity as the system requires a lower capital investment (Zhou *et al.,* 2014; Chisti, 2016), reduced energy demand, and the simplicity of scaling up (Costa and de Morais, 2014).

The closed photobioreactor system on the other hand is a production system where all the cultivation conditions including axenicity, CO_2, light, pH, etc. are tightly regulated. In addition, the cultivated cells are less likely to be prone to contamination as they are within a closed system. They can thus be positioned indoors or outside the building as long as illumination is sufficiently provided. However, this closed cultivation system is limited by its capital and power-intensive nature, which consequently constrains its economic competitiveness. When compared to an open raceway pond system, the main benefit of closed photobioreactor systems is their high biomass productivity. They are also more efficient in terms of quality since they

can operate under highly controlled settings and so avoid the drawbacks of the open culture method; they can be built, simulated, and optimized based on the individual algae strain chosen. The closed photobioreactor system is also not limited by space as it utilizes relatively reduced space, thus maximising light availability and remarkably reducing contamination. However, some challenges have been identified to include benthic algae growth, bio-fouling, cleaning concerns, overheating, high dissolved oxygen build-up leading to growth limits, and the extremely high costs of engineering, building, operation, and maintenance (Molina-Grima *et al.,* 1999; Chisti, 2008).

2.4 History of microalgae in food

The utilization of microalgae as food has been well known for many years (Wells *et al.,* 2017), in this regard they have been cultivated for human consumption in many countries in Asia, Europe, the Americas as well as in Oceania (Vigani *et al.,* 2015). Around the sixteenth century, during the Spanish invasion and conquest of Mexico, the colonizers discovered that the Aztecs were collecting a "new food" from a lake. The chroniclers recorded that the Aztecans made nets for collecting the "blue coloured techuitlatl" from the lagoons and making edible preparations from the biomass. Techuitlatl was severally referred to by many biologists until the close of the 16th century, but subsequently it was not being referred to, which probably signifies the loss of the lakes due to urban and agricultural development. However, Lake Texcoco, which is the only remnant lake from those days, has a copious algae *Spirulina* population within it. In West Africa, the Kanembu tribe that inhabit the Lake Chad bank, harvest wet algae, drain out the moisture through textile materials and lay them on the banks to dry. Subsequently, the dehydrated algae are segmentalized and moved to their living quarters, where the sun drying is finalized on mats (Abdulqader, Barsanti and Tredici 2000).

Nostoc and other cyanobacteria such as *Spirulina* and *Aphanizomenon* have been recorded as human food for centuries (Jensen, 2001; Spolaore *et al.,* 2006). Records show that about two millennia ago, the people in the Chinese subcontinent turned to the consumption of the microalgae, *Nostoc,* during a prolonged famine. In the 1950's, the geometrical rise in the global human population and predictions of limited protein supply propelled a search for alternative and unconventional sources of protein. However, microalgae large-scale production commenced during the 1960's with an establishment in Japan cultivating the green microalga, *Chlorella*. Subsequently, in the 1970's another cultivation plant was constructed in Mexico for *Spirulina* mass cultivation, and by the 1980s more facilities had sprung up in Australia, India

and United States among many other places (Enzing *et al.,* 2014). In spite of this prolonged history, only a limited sources of microalgae species have been domesticated and cultivated for human consumption which includes *Arthrospira platensis*, *Chlorella vulgaris* and *Aphanizomenon* (Spolaore *et al.,* 2006; Bleakley and Hayes, 2017; Caporgno and Mathys, 2018a). *Odontella aurita*, a marine diatom with French origin has also been approved as a new food ingredient since 2002 (Van der Spiegel, Noordam and Van der Fels-Klerx, 2013).

2.5 Nutritional composition of microalgae

Microalgae are highly rich in various nutritional components with immense potential as food products. Similar to plants, major classes of nutrients in microalgae have been identified such as lipids, protein, and carbohydrates (Table 2.1) (Levasseur, Perré and Pozzobon, 2020). In order to optimise the nutritional composition of these microalgae for enhanced human consumption, increased attention has been devoted to the environmental factors affecting algal cultivation; these include CO_2 supply, temperature value, pH value, light intensity, nutrient, mixing, etc (Kusmayadi *et al.,* 2020). Microalgal biomass have been noted to be made up of significant levels of carbohydrates, proteins and lipids. Furthermore, these microbes are inherently capable of synthesizing long-chain fatty acids, for example, arachidonic acid, docosahexaenoic and linolenic acids (Matos, 2016).

Similarly, a large repertoire of biological compounds are also produced by microalgal organisms, and these have been shown to be useful as potential antioxidants with examples ranging from carotenoids, phenolic compounds and vitamins (Ahmed *et al.,* 2014). Due to the chemical composition, microalgal species have also been described to be highly functional in the enhancement of the nutritional value of many foods. In addition, these organisms also possess applications as functional foods with the ability to deliver supplementary pharmacological and physiological benefits to humans (Matos, 2016).

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Table 2.1: Nutritional composition of microalgae species (% DW).

Microalgae	Carbohydrate (%)	Lipid (%)	Protein (%)	Ash (%)	Reference
Acutodesmus dimorphus	38.6	18.8	28.1	14.5	(Tibbetts, Milley and Lall 2015)
Botryococcus braunii	18.5	34.4	39.9	7.2	(Tibbetts, Milley and Lall 2015)
Chlorella spp.	25.2	15.7	53.3	5.8	(Tibbetts, Milley and Lall 2015)
Coelasteum sp.	30.0 ± 3.4	30.4 ± 0.9	28.1 ± 1.4	ND	(Mehrabadi, Farid and Craggs 2017)
Desmodesmus sp.	23.0 ± 1.0	40.2 ± 0.9	27.1 ± 3.0	ND	(Mehrabadi, Farid and Craggs 2017)
Isochrisis galbana	16.98 ± 0.05	17.16 ± 0.04	26.99 ± 0.08	16.08 ± 0.03	(Tokuşoglu and Üunal 2003)
Micractinium pusillum	29.2 ± 5.7	46.3 ± 3.6	16.8 ± 0.4	ND	(Mehrabadi, Farid and Craggs 2017)
Mucidosphaerium pulchellum	27.8 ± 6.9	48.2 ± 1.8	13.5 ± 1.5	ND	(Mehrabadi, Farid and Craggs 2017)
Nannochloropsis granulata	36.2	23.6	33.5	6.7	(Tibbetts, Milley and Lall 2015)
Neochloris oleoabundans	37.8	15.4	30.1	16.7	(Tibbetts, Milley and Lall 2015)
Pediastrum boryanum	28.5 ± 2.1	22.1 ± 0.4	47.4 ± 4.6	ND	(Mehrabadi, Farid and Craggs 2017)
Phaeodactylum tricornutum	25.2	18.2	39.6	17.0	(Tibbetts, Milley and Lall 2015)
Porphyridium aerugineum	45.8	13.7	31.6	8.9	(Tibbetts, Milley and Lall 2015)
Scenedesmus acutus	39.42 ± 0.08	38.55 ± 0.38	7.8	2.2	(Dong *et al.* 2016)
Spirulina platensis	15.81 ± 0.07	8.03 ± 0.06	61.32 ± 0.02	10.38 ± 0.05	(Tokuşoglu and Üunal 2003)
Spirulina spp.	22.2	14.2	55.8	7.8	(Tibbetts, Milley and Lall 2015)
Tetraselmis chuii	25.0	12.3	46.5	16.2	(Tibbetts, Milley and Lall 2015)

2.6 Proteins in microalgae

Microalgal organisms have been described as potential alternatives to meet the world's quest for a more sustainable food supply, especially with regards to the global protein demand. From a sustainability perspective, these promising protein sources are believed to be more beneficial when compared to the currently used raw materials (Caporgno and Mathys, 2018a). For instance, plant proteins have been the major proteins for both humans as well as animals. Although there has been an expansion in the plant cultivation area, changes in the frequency of cropping, as well as improvements in crop harvest would aid in meeting the rising food need; however, crop production is limited with regards to optimization. In addition, these approaches have the potential to deepen the current environmental problems that result from current cultivation systems, *viz*., deforestation, desert encroachment, land degradation, loss of biodiversity, and climate change (FAO, 2002). Additionally, the other popular alternative, animal-derived proteins are likewise dependent on the continuous supply of relatively inexpensive plant-derived proteins for their nutrition (FAO, 2002).

In comparison to the well documented scientific understanding of plant, animal and single cell proteins, little is known with regards to proteins from microalgae. Just as it is in other life forms, biosynthesis of particular proteins is dependent on the physiological state of the cell. Microalgae are known not to accumulate definite storage proteins; however, they contain many different types of proteins (Schwenzfeier, Wierenga and Gruppen, 2011). But due to high phylogenetic diversity, it is hard to oversimplify these microalgal proteins. However, the biology of various microalgae has shown that their proteins are responsible for different cellular functions which include catalysis, structure, signal transduction, motility, electron transfer as well as molecular chaperones and transporters. More specifically, a large proportion of these microalgal proteins serve as enzymes involved in photosynthetic reactions as well as many other activities within the cell (Wang *et al.,* 2004).

2.6.1 Proteins production in microalgae

Microalgal proteins have been demonstrated to be present in various compartments of the cell such as the cytoplasm, the cell wall, in organelles, in the nucleus as well as plastids (Safi et al., 2015). There is however, a considerable variation in the number of potential protein-coding genes in the microalgal genomes. For instance, *Arthrospira platensis* possess 18,545 protein- coding genes respectively,

while the *Chlorella* species have an average of 10,000 of these genes of interest (Wu *et al.,* 2019). It has also been shown that members of the diatom *Halamphora* genus such as *H. americana*, *H. coffeaeformis* and *H. calidilacuna* genomes share a set of 130 protein-coding genes in their genomes, as well as one tmRNA, three rDNAs, 27 tRNAs, and a closely related gene content to other pennate diatoms. Interestingly, it should be noted that protein-coding genes does not directly translate to the protein content. In this regard, some microalgae with relatively lower number of these genes displayed a high total protein content which range from 60% to 70% on dry weight (DW) basis. Typical examples include *Chlorella* (Liu and Hu 2013) and *Arthrospira* (El-Kassas, Heneash and Hussein, 2015).

One of the most studied and important proteins in photosynthetic organisms is the ribulose bisphosphate carboxylase-oxygen (Rubisco). Rubisco has been identified as the major photosynthetic enzyme that is involved in inorganic carbon fixation; in microalgae, it is usually located in a protein complex within the microalgal chloroplasts. This enzyme complex is mainly a 560 kDa hexadecameric quaternary protein structure which is formed from the association of 8 large (55 kDa) and 8 small (15 kDa) polypeptide chains (Taylor *et al.,* 2001). Although the main catalytic activity of Rubisco has been noted to be that of a carboxylase, however, its affinity for CO_2 is low. The Rubisco content of diatoms is considerably lower, with levels of 2–6% (w/w) of total cellular protein than the 20–50% (w/w) Rubisco content of the soluble protein in plant leaves (Losh, Young and Morel, 2013; Carmo-Silva *et al.* 2015). Interestingly, Rubisco has also been described as a protein with potential for use in human nutrition and medicine (Di Stefano *et al.,* 2018).

Although microalgae are known to synthesize the enzyme Rubisco, many microalgae show a relatively low content of this important enzyme, hence, wild strains are less likely to be viable sources of Rubisco (Losh, Young and Morel, 2013). Wang *et al.* (2018) have previously reported that *C. closterium* Rubisco operon transcription regulator was expressed in the 18-hour illumination sample, as observed in its 25 kDa protein bands. Earlier on, Schwenzfeier, Wierenga and Gruppen (2011) also described two distinct bands with molecular masses estimated at 50 kDa and 15 kDa as the respective large and small subunits of Rubisco in *Tetraselmis* sp., a commercial microalga. Most proteins in the microalgal extracts have also been noted to be enzymes involved in photosynthesis and many essential activities responsible for cellular continued existence and development (Wang *et al.,* 2003; Contreras *et al.,* 2008; Schwenzfeier, Wierenga and Gruppen, 2011).

The productivity of microalgal protein can be significantly facilitated by the adoption of specific growth strategies as well as the optimization of production conditions which may be specific to the microalgae strain (Zeng *et al.,* 2012; Rocha *et al.,* 2019). This is necessary as the protein content have been noted to differ vastly among microalgal species as well as strains and this has been shown to be greatly affected by the environmental conditions of growth. During cultivation, many microalgal species may accumulate considerable protein, which can range from 40–60% of dry matter (Wang *et al.,* 2021). In general, microalgal organisms exhibit high protein contents when cultured in growth media with elevated levels of a nitrogen source in addition to other well-conditioned growth conditions (Kratzer and Murkovic, 2021). These additional growth conditions include a carbon source, light intensity, availability of nutrients, temperature, halostress, and other climate conditions (Wan *et al.,* 2011). It is noteworthy that, Perez-Garcia *et al.* (2011) demonstrated that the source of nitrogen is the most determinant factor necessary for microalgal high-protein production. Ammonium when used in high concentrations can inhibit the cell growth (Soares *et al.,* 2018), on the other hand urea has been highlighted to be optimal for the nutrition of some microalgae species (Batista *et al.,* 2019).

2.6.2 Microalgae protein extraction

The extraction of proteins from microalgal biomass is a multi-step process. Firstly, the disruption of the microalgal cells is carried out, either by mechanical milling, chemical or enzymatic breakdown, high pressure homogenization, ultrasonication, or a combination of any of the methods (Soto-Sierra, Stoykova and Nikolov, 2018). Generally, the application of high-pressure homogenization leads to significant cell disruption efficiency relative to many other methods (Safi *et al.,* 2014). Ultrasonication has been demonstrated to be applicable with high ultrasonic energy inputs, it could significantly influence the desirable characteristics of microalgal proteins. Similarly, chemical lysis is also noted to result into considerable disintegration of the extracted proteins (Halim, Danquah and Webley, 2012). It has been observed that enzymatic lysis is not so simple to implement mainly because microalgae are known to possess very complex cell walls, thus the need arises for the utilization of complex mixtures of enzymes for effective degradation. However, the use of a combination of enzymes presents appreciable disadvantages mainly as a result of the side-activities of the different enzymes which may ultimately have detrimental effects on the structural integrity as well as the functionality of the targeted microalgal proteins (Grossmann, Hinrichs and Weiss, 2020).

In recent times, a lot of efforts have been focused on gentler methods of extraction of microalgal constituents, an important example of these methods is the pulsed electric field treatment (Luengo *et al.,* 2014). Efforts are also being made into the application of alternative techniques which are greener yet more efficient. In this regard, three phase partitioning (TPP) is an innovative approach with applications in the extraction and purification of important biomolecules (Gagaoua *et al.,* 2014). The TPP approach has been noted to be easily operable, relatively efficient as well as being scalable. It involves the addition of salts to an aqueous solution that contains product of interest, subsequently, t-butanol is added to the mixture which results into the formation of three phases. While the topmost phase of the three layers is t-butanol phase, the middle one is comprised of the protein precipitate, and the lowest layer signifies the aqueous layer where the solubility of water and t-butanol changes on salt addition. In this system, the product of interest may be partitioned in any of the phases, subject to characteristics of the targeted product as well as the different operational conditions (Avhad, Niphadkar and Rathod, 2014).

Typically, the obtained microalgal crude extract comprises of the microalgal proteins together with carbohydrates, lipids, cell wall components, salts, vitamins and all other important constituents. Subsequently, this crude extract may be fractionated into water-soluble and water-insoluble fractions by the use of centrifugation. Usually, the water-soluble portion does not contain solids and contaminants, however, it may have within it some other soluble compounds with the ability to negatively affect the functionality of the protein of interest (Grossmann *et al.,* 2018). As a consequence of this, the aqueous layer is usually purified specifically for proteins in order to retrieve the required protein isolate. Protein precipitation based on their isoelectric points, followed by further fractionation and the re-solubilization of the purified precipitate has been noted as an efficient approach for purifying such proteins. However, the demerit of this approach is mainly that only protein fractions with the same isoelectric points are retrieved while the ones with dissimilar isoelectric points are lost in the process (Grossmann, Hinrichs and Weiss, 2019).

Furthermore, the re-solubility of proteins may be impeded subsequent to precipitation. The other options entail protein precipitation with alternative compounds such as ammonium salts, deep eutectic solvents, ionic liquids or organic solvents, however, these solvents are more resource intensive (Grossmann *et al.,* 2018). For higher purity, protein isolates obtained from the precipitation method may be purified further via any of these methods or a combination thereof, *viz*., chromatography, membrane filtration, dialysis or ultrafiltration (Garcia *et al.,* 2018). It should be noted that microalgal protein extraction and purification may not necessarily give individual

protein fractions as multiple protein fractions and considerable quantities of polysaccharides may still be present (Teuling *et al.,* 2019).

It has been observed that protein-rich microalgae may be utilized in food applications in two basic ways. The first way is by being used as a whole-cell ingredient whereby the biomass serves as a passive filler with no additional important functionality. The second way is being applied as a protein extract, for their bulking, emulsifying or gelation properties and not necessarily nutritional functions. It has been reported that a large proportion of proteins are stationed within the cytoplasm of the cell. Thus, effective protein extraction techniques are required to obtain highly functional protein extracts. Hence, obtaining protein extracts with significant functionality involves a series of processes which include cell disruption, concentration, and/or dehydration (Figure 2.2).

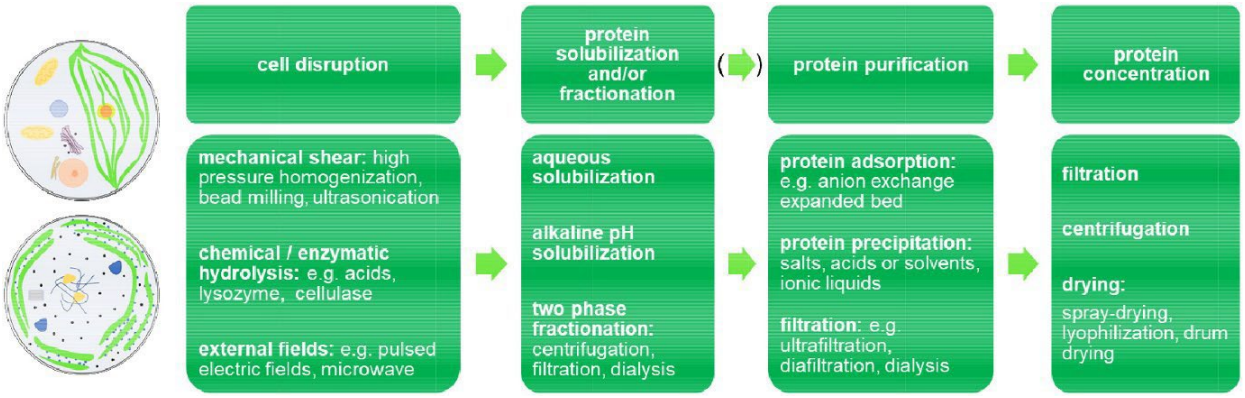


Figure 2.2: Typical workflow during microalgal protein extraction (Grossmann *et al.,* 2018).

2.6.3 Protein solubility

Protein solubility is identified as one of the major physicochemical characteristics in food applications and it has been noted to affect food technofunctional characteristics including emulsification and gelation. This protein property is necessary for gel formation, being that significant mobility is required for the protein of interest to interrelate considerably with the other proteins (Marangoni *et al.,* 2000). It has also been shown that protein solubility is very important for food products with low viscosity especially beverages, in preventing cloudiness and gravitational separation. Protein solubility can vary from zero to hundreds of mg/mL and it is specific for each protein (Kramer *et al.,* 2012). Since proteins are zwitterions, their solubility in water is affected by many innate factors such as amino acid composition, protein molecular weight, α-helix propensity

and external factors which includes the ionic strength of the solution, the temperature and the pH (Pelegrine and Gasparetto, 2005; Diaz *et al.,* 2010).

The relationship between the solubility of proteins isolated from microalgae, cyanobacteria and pH has been noted as a very important area of study. Many food products are frequently subjected to fluctuations in pH mainly because of fermentation and the external addition of acidic substances with the aim of increasing their stability and shelf life as well as for flavouring purposes. This has been noted to most likely limit the range of applications of food proteins, as proteins such as casein and soy are only sparingly soluble at mild acidity. This is the consequence of the property of many proteins to lose most of their overall net charge when in environments with pH close to their isoelectric point, which is between pH 4.0 and 6.0 for many of the common food proteins (Chove, Grandison and Lewis, 2001; Farrell Jr *et al.,* 2004). Gerde *et al.* (2013) studied the efficiency of the protein extraction process in *Nannochloropsis* spp. and recorded the highest yield of proteins at high pH values, thus confirming that proteins dissolve readily at higher pH values.

A study by Schwenzfeier, Wierenga and Gruppen (2011) also recorded an overall increase in the solubility of *Tetraselmis* sp. proteins within the pH range of 5.5 to 8.5. These studies all point to the fact that protein solubility patterns of microalgal proteins is relatively unusual when related to the patterns of proteins from other sources, such as the proteins in whey (animal) and soy (plant). Novel food proteins are believed to possess unique solubility patterns which are most likely to be divergent from those of the typical food proteins (Lam and Nickerson, 2013). Thus, microalgae with their complex protein composition are not unexpected to have protein solubility that differ remarkably from conventional proteins (Guarnieri *et al.,* 2013).

However, some studies have highlighted some microalgal protein extracts with remarkable solubility when compared to other proteins. In this regard, a typical protein solubility pattern of ≥80% within pH 2.0 and 12.0 for protein extracts was obtained from *Chlorella protothecoides* (Grossmann, Hinrichs and Weiss, 2019). Similarly, the solubility of *Tetraselmis* sp. protein extract was found to be unconstrained by ionic strength, in addition to the proteins totally dissolved at pH ≥5.5 (Schwenzfeier, Wierenga and Gruppen, 2011). *Nannochloropsis oculata* proteins was also observed to display significant solubilities at pH >5.5 (Cavonius, Albers and Undeland, 2015). These unusual protein behaviors in microalgal proteins have been ascribed to the increased presence of hydrophilic amino acid residues as well as an increased degree of glycosylation with charged polysaccharides which all facilitate the protein-aqueous solution interactions and also reduce the proteins' isoelectric point.

2.6.4 Emulsification properties

Another important property of proteins that is usually considered with regards to their real-time application is their inclination to stabilize emulsions. This is considered to be worthy of investigation because during the course of their food applications, proteins from microalgae may be dispersed in a wide variety of systems consisting of the aqueous and the oil phases. The characterization of the emulsifying potential of proteins is usually determined as the emulsifying capacity (EC) of the protein. EC is basically the highest quantity of oil that is able to dissolve in an emulsifier solution without resulting in the destabilization of the emulsion (McClements, 2005). Some proteins of microalgal origin have been shown to possess relatively high emulsifying capacities, a notable example is *Chlorella vulgaris* protein extract with an EC estimated at 3740 ± 20 mL oil/g protein (Ursu *et al.,* 2014). The recorded value was noted to be more than that of soy protein isolate and sodium caseinate under the same experimental conditions.

The protein extract of *Arthrospira platensis* demonstrated maximum EC at a pH range between 7.0 and 10.0 (Benelhadj *et al.,* 2016), whilst the lowest EC was recorded at pH 3.0. This might be associated to the isoelectric point precipitation, disrupting the emulsion droplets formed. For *Tetraselmis* sp., the concentration of protein adsorbed to the interface was estimated at 0.82 mg protein/m^2 at a protein extract concentration of 6 mg/mL (Schwenzfeier *et al.,* 2013). The protein extract was observed to be maintained at a concentration of >0.1 M NaCl within pH 5.0 to 7.0. In subsequent studies, it was confirmed that this remarkable stability was mainly due to the adsorption of charged sugars in the system. However, the stability of the emulsions was unstable in the presence of Ca^{2+} ions as the droplets became significantly larger at Ca^{2+} concentrations ≥ 5 mM, a phenomenon which results from a reduction in electrostatic repulsion as well as ion bridging. Furthermore, the presence of uronic acids, which are charged polysaccharides in the protein extract further confirmed the increased stability (Schwenzfeier *et al.,* 2014). Generally, the interactions of proteins and carbohydrates have been well demonstrated to facilitate the stability of emulsions under varying pH conditions (Grossman, 2016).

2.6.5 Foaming properties

Food products such as meringue, mousse and whipped cream have foams within them. Various scientific investigations have demonstrated that microalgal proteins possess the ability to form stable foams (Bertsch *et al.,* 2021). The stability of foam formed from *Tetraselmis* sp. protein extract was higher than that formed from albumin in egg white and proteins in whey within pH 5.0 and 7.0 (Schwenzfeier *et al.,* 2013). Additionally, Schwenzfeier *et al.* (2013) observed that

foams from the microalgal proteins were typically stabilized through the action of the proteins and other smaller sized polypeptides within the microalgal extract, with the electrostatically stabilized protein-polysaccharide interactions playing a less significant minor role.

The foaming capacity (FC) of *Arthrospira platensis* protein extract, at 1% and 3% concentration was recorded to be at the lowest level at pH 3.0, while an increase in the foaming capacities was observed to increase with increasing pH to approximately >150% at pH ≥ 7.0 (Benelhadj *et al.,* 2016). In a different study, the capacity of *Chlorella pyrenoidosa* protein at 3% to form foams was estimated at 95%. The *Chlorella pyrenoidosa* protein foams were observed to be stable for 3 hours with only a 3% reduction observed within this time (Waghmare *et al.,* 2016).

2.6.6 Protein digestibility

Digestibility is the term that denotes what quantity of a food's nitrogen content is absorbed in comparison to the total nitrogen. It has been shown that animal protein tends to possess a generally high digestibility which is usually above 90%. Although digestibility in plant proteins are also high, it has been noted to have a wide range. For example, protein from wheat flour and corn flour have digestibility estimated at 96% and 70% respectively. Some factors that have been identified to affect protein digestibility include food processing, the presence of various extraneous compounds which may inhibit enzyme activity and affect nutrient absorption such as anti-nutrients including tannins, trypsin inhibitors, and lectins. For enhanced industrial applications, it is expected that the amino acid profiles of microalgal proteins should be better than the current reference values. The few studies that have focused on the digestibility of microalgal proteins show that these proteins are much less digestible than casein, a typical reference protein (Kose *et al.,* 2017).

2.7 Amino acids in microalgae

Amino acids are classed as either non-essential or essential based on the body's capacity to synthesize adequate amounts of them (Hope and Salmond, 2021). In humans, the essential amino acids (EAAs) have to be sourced from external food sources as the human body lacks the ability to produce them, de-novo. The EAAs include cysteine, lysine, isoleucine, leucine, valine, phenylalanine, tryptophan, tyrosine, threonine, methionine and histidine. The most popular food sources that complement humans need for EAA are basically animal food sources which include meat, fish, other seafoods, eggs, mushrooms, milk and milk products. Different microalgae have also demonstrated to be remarkable EAAs sources (Matos, 2016; Terriente-Palacios and Castellari, 2022). According to the WHO, *Chlorella* sp. and *Spirulina* sp. are both microalgal

species that possess an adequately balanced EAA content necessary for human nutrition (Chronakis and Madsen, 2011).

Quantitative analysis of the proteins from the microalgal species have shown different amino acid profiles which are subject to the conditions of cultivation, in this regard it is believed that optimising the process of microalgae cultivation would facilitate the production of proteins with essential amino acids (James, Al-Hinty and Salman, 1989). Although the presence and proportion of essential amino acids in food is noted to be an important parameter in accessing protein quality, other factors such as protein bioavailability and digestibility are equally as important (Damodaran, Parkin and Fennema, 2008).

2.8 Lipids and fatty acids in diatoms

The recent decades have seen an upsurge in the application of microalgal lipids in the food, energy, pharmaceutical, chemical and cosmetic industries. Microalgal lipids are broadly categorised into storage lipids and structural lipids, and these specifically include neutral lipids, polar lipids, sterols, wax esters, as well as derivatives of prenyl such as carotenoids, tocopherols, terpenes, quinines and chlorophylls, which are derivatives of pyrrole. The lipid content is an important component of microalgae, for example, the lipid content in diatoms has been estimated to be around 25% of the total DW (Levitan *et al.,* 2014). Similar to all other biomolecules in microalgae, the content of microalgal lipids is usually a function of the growth conditions. Diatoms may produce a wide range of lipids, as well as other forms of bioactive molecules such as pigments (Michalak and Chojnacka, 2015) and halogen-containing compounds (Wichard *et al.,* 2005). The average lipid content in diatoms can reach 25% of DW (Levitan *et al.,* 2014) while lipid synthesis in diatoms might vary depending on culture circumstances.

2.8.1 Fatty acids

The fatty acid profile of diatoms comprises of various FAs from C14:0 to C22:6 (Table 2.3). Amongst the most common of these FAs are myristic acid, palmitic acid, palmitoleic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Jiang *et al.,* 2016). The fatty acid content of microalgal species is dependent on the species and growth conditions (Stonik and Stonik 2015; Jiang *et al.,* 2016).

Table 2.3: Lipid composition and bioactivity of diatoms

Lipids Class	Valuable compositions	Representation	Bioactivities	Diatom sources
Fatty acids[a]	HTA	16:3 (n-3)	Anti-bacterial	All the diatoms produce fatty acids but the content in each species varies.
	Palmitoleic acid	16:1 (n-7)	Anti-bacterial	
	Stearic acid	18:0	Antimicrobial	
	Remenic acid	18:2 (n-7)	Antimicrobial	
	EPA	20:5 (n-3)	Anticancer, antimalarial, anti-bacterial, anti-inflammatory	*Skeletonema menzelii* and *P. tricornutum* have the potential for commercial production of EPA and DHA.
	DHA	22:6 (n-3)		
TAG[b]	TAG		Biofuels	TAGs in *Thalassiosira weissflogii* and *C. cryptica* may account for 82% and 88% of total glycerolipids, respectively
Polar lipids[c]	Glycolipids		Anti-inflammatory, antitumor, antibacterial, antiviral	Glycolipids were characterized by mass spectrometry in diatom species, such as *T. pseudonana* and *Stephanodiscus* sp.
	Phospholipids		Valuable ingredient in functional foods, cosmetic, pharmaceutical industries	Phospholipids were identified in diatom species such as *P. tricornutum* and *Cylindrotheca fusiformis*
Steroids[d]	24-methylenecholesterol		Anti-inflammatory, antitrypanosomal, anti-mycobacterial	The sterol 24-methylenecholesterol accounts for 90% of total sterols in *Synedra acus*.
Oxylipins[e]	PUAs		Antimitotic, anti-inflammatory, antimicrobial	Some diatoms are a rich source of PUAs, such as *Thallasiosira rotula*, but some do not produce oxylipins, such as *Skeletonema pseudocostatum*.

[a](Kabara et al., 1972; Hallahan and Garland, 2005; Desbois et al., 2008; Lafourcade et al., 2011; Sapieha et al., 2011; Valenzuela et al., 2012; Hamilton et al., 2015; Jiang et al., 2016).
[b](d'ippolito et al., 2015).
[c](Yongmanitchai and Ward, 1992; Plouguerné et al., 2014; d'ippolito et al., 2015; da Costa et al., 2016).
[d](Volkman et al., 1998; Viegelmann et al., 2014).
[e](Paul and Fenical, 1986; Nappez et al., 1996; Girona et al., 1997; Caldwell, 2009; Lauritano et al., 2016).

Omega-3 FAs such as EPA and DHA has been identified as one of the well-studied FAs in microalgal diatoms as a result of their high nutritional value (Stonik and Stonik, 2015), C24–C28 polyunsaturated FAs are present in microalgae, though at a much lesser degree (Mansour *et al.,* 2005). Thus, various FAs sourced from microalgae have displayed remarkable bioactivities, especially the unsaturated FAs. For example, 16:3n-4 and 16:1n-7 are very effective against Gram-positive bacteria, though they are typically minor constituents of fatty acid fractions (Desbois *et al.,* 2008). EPA and DHA have also been demonstrated to be ameliorative against various human ailments including arteriosclerosis, hypertension, infections, inflammation, viral diseases, and various forms of tumours (Hallahan and Garland, 2005; Lafourcade *et al.,* 2011; Sapieha *et al.,* 2011; Peltomaa, Johnson and Taipale, 2017).

Although humans and many other animals have the inherent ability to synthesize many SFAs together with some monounsaturated fatty acids (MUFAs), they do not possess the essential enzymatic machinery to insert cis double bonds to the n-6 or the n-3 fatty acid positions (Trumbo, Schlicker and Yates, 2002). The human body has demonstrated the ability to synthesize EPA and DHA via a series of reactions that involves desaturation and elongation (Nakamura and Nara,

2004). However, EPA and DHA should be received externally via food sources due to the low conversion efficiency of the starting substrates to EPA and DHA (Yi *et al.,* 2017).

2.8.2 Triacylglycerols

Diatoms and other microalgae are highly sought after as their biomass have been identified as sustainable alternatives for biofuels production due to their remarkable accumulation of TAGs. Microalgal TAGs differ considerably among the different strains, species and genera (Hildebrand *et al.,* 2012). C16 FAs account for about 100% of the total FAs at the sn-2 position of TAGs in diatoms, which includes monounsaturated fatty acid palmitoleic 16:1 at a major level and unsaturated FAs, C16:2 and C16:3 at a minor level (Li *et al.,* 2014a).

2.8.3 Polar Lipids

Polar lipids are basically amphiphilic lipids possessing both a hydrophilic head and a hydrophobic tail. Diatom polar lipids are primarily composed of monogalactosyldiacylglycerol, digalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol, phosphatidylcholine, phosphatidyl-inositol, phosphatidylglycerol, and other minor elements such as 1-deoxyceramide-1-sulfate (Anderson, Kates and Volcani, 1978) (Figure 2.3). In microalgae, the glycolipids are important compounds mainly sequestered within chloroplasts; they have been shown to possess weak biological activities such as anti-inflammatory, antiviral, and antibacterial activities (Plouguerné *et al.,* 2014). On the other hand, phospholipids which are more commonly found across various organisms, especially in the cell membranes have been found to be very useful as functional food ingredients, cosmetic products and in medicine or as carriers of PUFAs (Yi *et al.,* 2017).

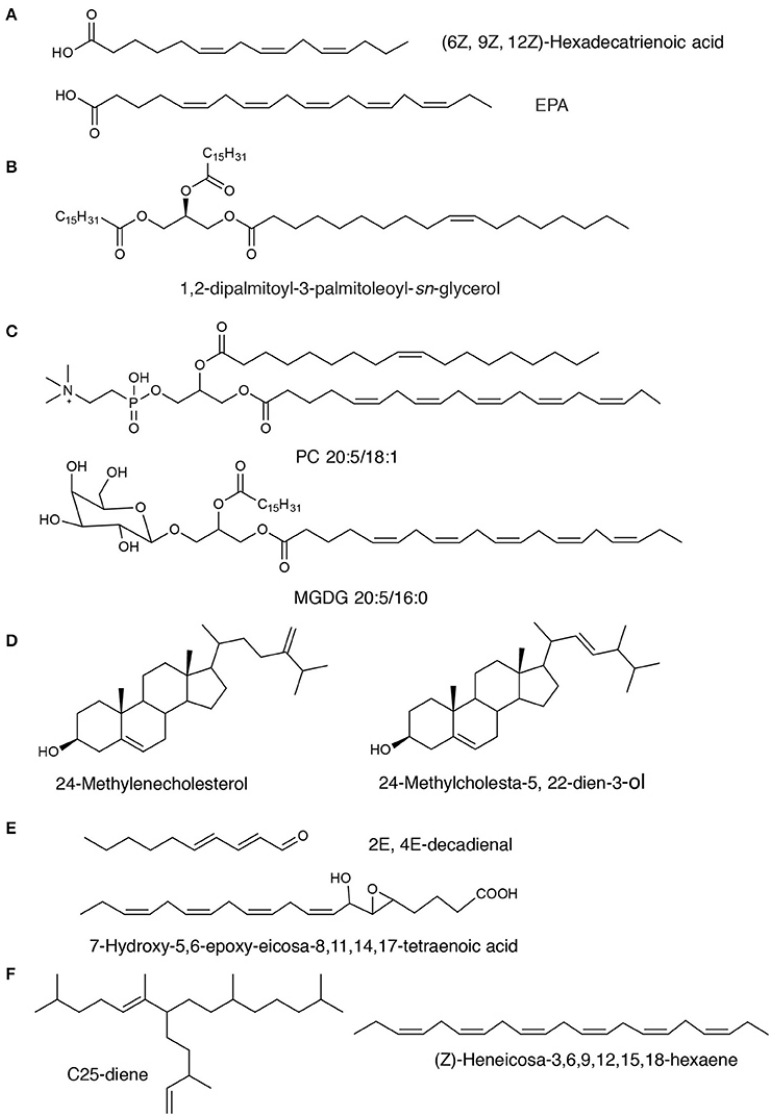


Figure 2.3: Chemical structure of microalgal lipids (Anderson, Kates and Volcani, 1978)

2.8.4 Steroids

Steroids are essential compounds for life in all complex organisms such as animals and plants. The Δ5-series with 24-methylencholestrol (Figure 2.3) as well as diatomsterol (24-methylcholesta-5,22E-dien-3β-ol) have been shown to be widely distributed in microalgae, especially in pennate diatoms (Rampen *et al.,* 2010). Most diatoms have also been shown to contain C28 sterols, even though C27 or C29 sterols are also predominant in some phylogenetic

groups. Although there exists a lack of data on the full chemical profiling of sterols in microalgae, the sterols and their various derivatives have been shown to be bioactive against cytotoxicity, inflammation, trypanosoma, bacteria and fungi (Viegelmann *et al.,* 2014). It is believed that microalgae have the ability to biotransform steroids via modification reactions such as isomerization, hydroxylation, reduction and side-chain degradation (Faramarzi, Adrangi and Yazdi, 2008).

2.8.5 Oxylipins

Oxylipins are formed from the oxidation of PUFAs and they typically serve the role of biochemical mediators in various physiological and ecological processes in microalgae. More than a quarter of marine diatoms are known to synthesize various oxylipin metabolites (Wichard *et al.* 2005; Sieg, Poulson-Ellestad and Kubanek, 2011). Like many other biomolecules, oxylipins biosynthesis is mainly condition-dependent (Figure 2.3) (d'Ippolito *et al.,* 2005; Cutignano *et al.,* 2011; Nanjappa *et al.,* 2014). Generally, the biosynthesis of oxylipin begins with oxidation action of lipoxygenases on the precursor PUFAs and membrane phospholipids (Orefice *et al.,* 2015). Subsequently, the lipoxygenase enzymes catalyse the incorporation of a hydroperoxide group into the carbon skeleton. Furthermore, the enzymes participate in the transformations which eventually result into the generation a wide array of oxylipins (Wichard *et al.,* 2005).

Studies on bioactive oxylipins have highlighted some polyunsaturated aldehydes from blooming diatoms to perform important biological functions like the inhibition of the growth of diatom predators and the induction of various responses in aquatic habitats (Caldwell, 2009; Lauritano *et al.,* 2016). Different studies have also demonstrated other biological activities which include anti-cancer activity (Sansone and Brunet, 2019), antimicrobial properties (Paul and Fenical, 1986) antimitotic (Nappez, Battu and Beneytout, 1996) and anti-inflammatory activity (Girona *et al.,* 1997). In summary, the chemical diversity of microalgal oxylipins is an important phenomenon especially with regards to diatom physiology and taxonomy (Yi *et al.,* 2017).

2.8.6 Extraction, fractionation and fatty acid profiling of lipids

The extraction of microalgae is basically done on a wet or dry mass basis, and it is one of the downstream processes involved in the valorisation of microalgal lipids. Usually extraction of these lipids employs the use of organic solvent extraction and could be done mechanically or by employing chemical methods (Lee *et al.,* 2021). Specifically, it is noted thatchloroform/methanol extraction are the optimum solvents for higher lipid yields from microalgae when compared to other solvents or solvent pairs (Gunnlaugsdottir and Ackman,

1993). Ryckebosch *et al.* (2012) highlighted the remarkable performance of chloroform-methanol (1:1) relative to the several solvent and solvents pairs. However, the drawbacks of these solvent systems include their toxicity, hence, the application of other solvents e.g. hexane (Danielewicz *et al.,* 2011) and methyl-tert-butyl ether (Matyash *et al.,* 2008). In addition to circumventing the toxicity concern, hexane also reduces the concentration of chlorophyll, a main contaminant during the extraction process (Danielewicz *et al.,* 2011).

Usually, the composition of microalgal lipids are routinely analysed by thin layer chromatography (Dunstan *et al.,* 1993). Lipid classes in many types of microalgae separated and analysed using the silica-based solid-phase cartridges method (Yongmanitchai and Ward, 1992), has been adopted subsequently (Danielewicz *et al.,* 2011; Ryckebosch *et al.,* 2012). Briefly, cartridges are required to be conditioned using hexane, subsequently the glycolipids, phospholipids and neutral lipids are eluted using acetone, chloroform or methanol based on the fraction's polarity. However, it has been observed that the column might retain compounds such as the choline-containing phospholipids and phosphatidylcholines (Danielewicz *et al.,* 2011).

Similarly, gas chromatography (GC) is also a routine and well-established technique applied in the profiling of microalgal FAs (Seppänen-Laakso, Laakso and Hiltunen, 2002). In this approach, the FAs that are released from the saponification of lipids are transformed to their corresponding methyl esters, after which they undergo GC separation. These FAs could be fractionated by flame ionization coupled with their respective retention times, while they would be specifically identified via mass spectrometry using their mass to charge ratios. Finally, 1H nuclear magnetic resonance has also been found instrumental in microalgal lipid profiling especially in determining the PUFAs to triacylglycerol ratio via the chemical shift of the diagnostic proton (Danielewicz *et al.,* 2011).

2.9 Carbohydrates

The exploration of carbohydrates from microalgae is an area of extensive study as a result of their potential applications in bioenergy, human nutrition, animal feed, pharmaceuticals and cosmetics (Schulze *et al.,* 2017). Photosynthetic organisms including microalgae trap the energy from the sun and transform it into other forms of energy, especially into chemical energy via photosynthesis, which is compartmentalized into the light and the dark reactions. Basically, the light reaction generates nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP), which are energy rich nucleotides using light from the sun, subsequently, CO_2 is reduced to carbohydrates by the energy from the nucleotides in a series of reactions involving the Calvin's

cycle (Taiz and Zeiger, 2010).

Microalgal carbohydrates are noted to have immense potential in the production of functional food. The proportion of carbohydrates in microalgae has been noted to differ widely between 10 and 50% of DW (Batista *et al.,* 2013). Microalgal carbohydrates comprise of different forms and could exist as cellulose, starch, sugars, and other polysaccharides functioning majorly as storage compounds or cell wall constituents (Batista *et al.,* 2013; Matos, 2016). Unlike plants, not all microalgal species contain cellulose, hemicellulose, or cell walls (John *et al.,* 2011). Many microalgae possess complex polysaccharides and recalcitrant components such as lignin and silica in red algae and diatoms, respectively (Popper and Tuohy, 2010). The cell walls may also contain exopolysaccharide-sulphated polysaccharides including fucoidans. A notable example is that of *Nannochloropsis* whose recalcitrant cell walls is made up of algaenans in addition to the typical cellulose, as well as diatoms which possess siliceous cell walls together with exude polymers (Wustman *et al.,* 1997; Richmond, 2004). The predominant simple sugars in microalgae include glucose, mannose, rhamnose, and xylose (Becker, 2007). However, there are some special carbohydrates which are specific to particular groups of microalgae, one of such is laminarin, a glucan, which is found in brown algae and in the diatom *Phaeodactylum tricornutum* (Menshova *et al.,* 2014; Caballero *et al.,* 2016).

Generally, beta glucans are widely found in diatoms, and these polysaccharides have been noted to differ in their grade of polymerization. They are basically complex sugars with monomeric glucose, with linear β-1,3- or β-1,4- glycosidic linkages which are interspersed and interconnected by β-1,6 linkages (Chen *et al.,* 2007). The β-1,3- or β-1,4- beta glucans are both effective sources of dietary fibre. However, while the former can elicit cellular immune responses, the latter does not exhibit such effects (Wismar *et al.,* 2011). In this regard, there is a significant inter-relationship between the conformation, molecular weight, and branching frequency of the glucans with immune response (Chen and Seviour, 2007; Stier *et al.,* 2014). In addition, various studies have also revealed the remarkable bioactivities of other polysaccharides sourced from microalgal sources. For instance, cellulose has demonstrated to be dietetic fibres, while sulphated polysaccharides are antioxidants and antitumoral agents (Gouda and Tadda, 2022).

2.10 Pigments

Pigments are basically compounds with the ability to trap light energy from the visible region of the electromagnetic spectrum. Various pigments have been used as food colorants, cosmetics,

nutraceuticals, and pharmaceuticals. Similarly, to other photosynthetic organisms such as plants, diatoms and other microalgae possess pigments in their cells for both primary and secondary metabolism. The pigments from microalgae have been broadly classified as carotenoids and chlorophylls (Begum *et al.,* 2016).

2.10.1 Chlorophylls

Chlorophylls are the most prevalent biological pigments responsible for photosynthesis, and they may be found in both higher plants, microalgae and macroalgae. Chlorophylls are critically significant in the "light harvesting" of photosynthesis, a process which is indispensable for the sustenance of all life forms on earth. This class of pigments absorb light selectively in the blue and red regions and consequently emits a green colour. The chemical structure of chlorophyll is basically a porphyrin macrocycle that is made up of four pyrrole rings as illustrated in Figure 2.4. An inclusion of a single isocyclic ring to one of pyrroles results into a phorbin structure (Humphrey, 2004). Out of the three types of chlorophylls, two are found in diatoms and other microalgae, *viz.*, chlorophyll a and chlorophyll c with chlorophyll a having the principal role of photochemical energy conversion during photosynthesis (Kuczynska *et al.,* 2015).

The absorption of chlorophyll under UV-Vis spectroscopy is largely influenced by the type of solvent that it is suspended in. For instance, the blue and red bands of chlorophyll a in acetone are typically observed at 661-663 nm and 430-431 nm, respectively. On the other hand, chlorophyll b ideally has its absorbance maximum at 644-647 nm and 453 - 457 nm, respectively (Nurachman *et al.,* 2015). It was also observed that the "red" absorption maximum of chlorophyll changes from 660 to 650 nm with increasing solvent polarity (Lichtenthaler and Buschmann, 2001). In microalgae, an additional pigment fucoxanthin forms the fucoxanthin-chlorophyll protein complex with chlorophyll a and chlorophyll c; this complex is synonymous to the light harvesting complexes in green plants playing the important light-harvesting role (Laurens *et al.,* 2012).

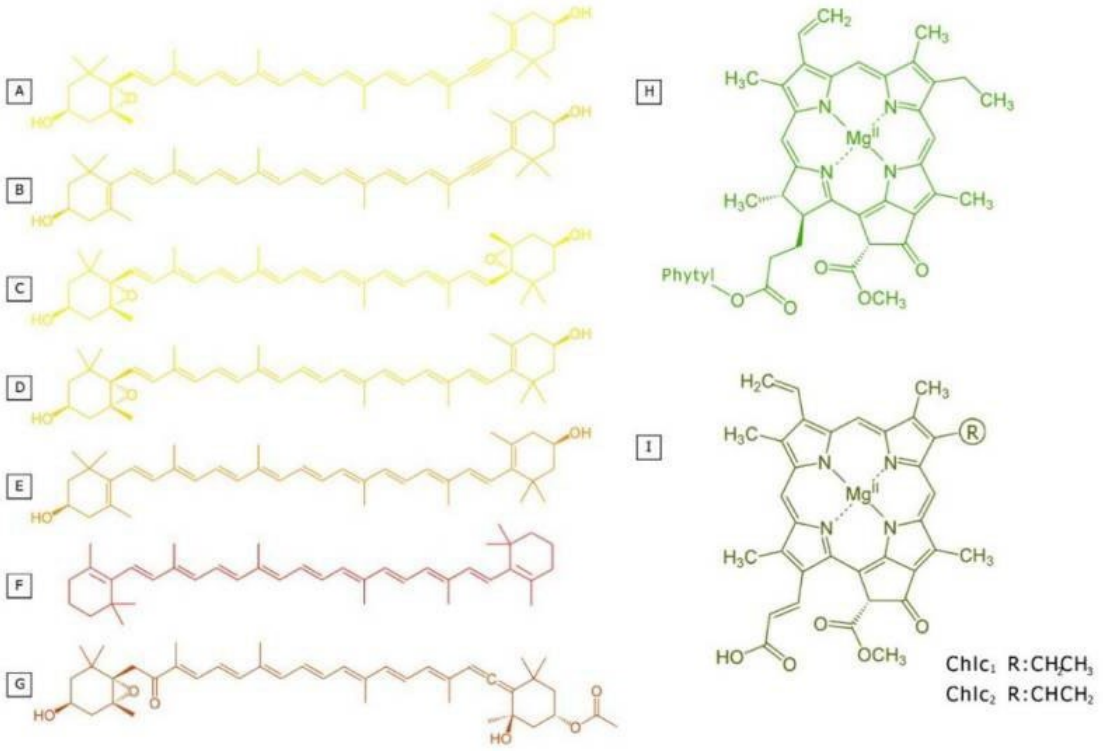


Figure 2.4: Structural formula of photosynthetic pigments in diatoms including all-trans carotenoids: (A) diadinoxanthin; (B) diatoxanthin; (C) violaxanthin; (D) antheraxanthin; (E) zeaxanthin; (F) β-carotene; (G) fucoxanthin; and chlorophylls: (H) chlorophyll a; (I) chlorophyll c (Kuczynska *et al.,* 2015).

2.10.2 Carotenoids

Carotenoids are basically terpenoid pigments found in plants, macroalgae, microalgae, and many other microbes. The most common examples include β-carotene, lutein, canthaxanthin and astaxanthin. This class of pigments is based on a 40-carbon polyene chain which confers on them their special molecular properties, especially, their ability to absorb light for photosynthesis. Furthermore, different carotenoids are complemented by oxygen-containing functional groups as well as cyclic groups. In this regard, carotenes are the collective term for hydrocarbon carotenoids while the xanthophylls are oxygenated derivatives of carotenoids (Del Campo *et al.,* 2007).

In diatoms, there are mainly seven types of carotenoids, namely, antheraxanthin, β-carotene, diadinoxanthin, diatoxanthin, fucoxanthin, violaxanthin, as well as zeaxanthin. Fucoxanthin has been identified as the major light-harvesting carotenoid in many microalgae including brown algae and diatoms. β-carotene, a major carotenoid in microalgae and in plants is well known for its multiple biological activities which have resulted in its constant demand in different industries. For example, β-carotene has been found very useful as a colouring agent, as an antioxidant,

anti-tumour agent, in aquaculture, cosmetics, human medicine and nutraceuticals (Del Campo *et al.,* 2004; Prieto *et al.,* 2011).

Similarly, to other biological compounds, the biosynthesis of the different pigments is greatly influenced by the prevailing environmental factors. Hence, factors including temperature, photoperiods, light intensity, pH, nutrient availability, salinity, and heavy metals stress do have significant effects on microalgal pigments biosynthesis (Fatma, 2009). For example, Grossman *et al.* (1993) specifically demonstrated the phycobiliprotein composition of a microalga was influenced by environmental conditions. It was also reported that cyanobacteria biosynthesis of tetrapyrroles was responsive to factors such as irradiances, light wavelength, nutrient availability, and temperature (Prasanna *et al.,* 2004).

2.11 Antioxidant compounds

Microalgae absorb carbon dioxide and light from the sun during photosynthesis. This process results into the generation of biomass and oxygen. However, the molecular oxygen that is produced is prone to the undesirable activation into reactive oxygen species (ROS), which includes superoxide anion, hydroxyl radicals and hydrogen peroxide, with the sunlight's ultraviolet radiation and/or heat acting as activators. Interestingly, nature has created an efficient means for algae and plants to suppress the amount of these ROS (Zandi and Schnug, 2022). In microalgae, this defense mechanism is made up of the numerous antioxidant compounds that are naturally synthesized by them. Subsequently, microalgae and other organisms have since emerged as effective sources of these natural antioxidants, that are very useful in medicine and in nutrition (Hajimahmoodi *et al.,* 2010). In humans specifically, antioxidants are very potent in their protective effects against diseases induced by ROS imbalance, including cancer, diabetes, cardiovascular disease and inflammation (Ngo *et al.,* 2011).

Lipid oxidation, which is caused by ROS is a major phenomenon that reduces the nutritional value of foods that are rich in lipids, resulting into rancidity, toxicity as well as undesirable taste, texture and appearance. In this regard, the food industry in particular have employed the use of various antioxidants, both natural and synthetic in their various production and packaging processes. Many, however, are of the opinion that natural antioxidants are the safer alternatives for human and animal consumption (Pena-Ramos and Xiong, 2001) as the artificial ones such as butylated hydroxyanisole and butylated hydroxytoluene are potentially considered to pose hazards to human and animal health (Safer and Al-Nughamish, 1999; Park *et al.,* 2001).

Thus, the production of antioxidants from natural sources has attracted increasing attention in recent times, with microalgae being identified as one of the most viable sources (Cornish and Garbary, 2010). In this regard, antioxidant activity has been investigated in numerous microalgae and cyanobacteria including species of the *Spirulina* (Miranda *et al*., 1998), *Chlorella* (Wu *et al*., 2005), and *Nostoc* (Li *et al*., 2007) genera. Furthermore, the scavenging ability of microalgal antioxidants has propelled them as candidates for alternative compounds against ROS-associated conditions (Goiris *et al.,* 2015; Raposo *et al.,* 2015).

In addition to carotenoids, which are the major antioxidants from microalgae, ascorbic acid has also been identified in both the cytosol and chloroplast, and has been demonstrated to effectively eradicate hydrogen peroxide, hydroxyl radicals, superoxides, as well as lipid hydroperoxides (Hajimahmoodi *et al.,* 2010). Similarly, tocopherols, such as α-tocopherols, which can be found within cell membranes of microalgae are also efficient in quenching lipid peroxides and singlet oxygen (Goiris *et al.,* 2012). Polyphenols, which work as antioxidants by hydrogen atom transfer and single electron transfer are naturally found in microalgae. They are bioactive compounds which includes flavonoids and phenolic acids that have been richly sourced from microalgae such as *Arthorspira platensis, Chlorella vulgaris,* and *Porphyridium purpureum* (Vieira *et al.,* 2021).

2.12 Toxicity of microalgae

Considering the great diversity of microalgae species, the need to select strains for industrial applications entails carrying out evidence-based risk assessment prior to their utilization as human food and animal feed. In this respect, many species including those belonging to the *Arthrospira*, *Chlorella*, *Haematococcus, Dunaliella* and *Schizochytrium* genera have been certified as GRAS (Generally recognized as safe) (Chacón-Lee and González-Mariño, 2010). *Odontella aurita*, a marine diatom from Innovalg (France), has also been authorized as a novel food since 2002 (Van der Spiegel *et al.,* 2013).

However, some concerns have been raised about the safety of microalgae in human nutrition, for example, dietary supplements which were produced from the microalgae, *Arthrospira* and *Aphanizomenon flosaquae* were recorded to contain microcystins at harmful levels (Roy-Lachapelle *et al.,* 2017). Further investigations revealed that these toxins might have arisen from the production by toxin-producing strains (e.g. *A. flosaquae* DC-1), the infection of pure cultures by toxin-producing microbes and the utilization toxin-infected water sources (Zhang *et al.,* 2016; Roy-Lachapelle *et al.,* 2017). Hence, it is key to adopt very stringent measures and continuous

monitoring of supplements derived from microalgae (Roy-Lachapelle *et al.,* 2017). It has since been shown that a majority of the toxin-producing microalgae are dinoflagellates and cyanobacteria (Qian, Kang and Ryu, 2015; Carmichael and Boyer, 2016). In fact, the rapid growth of these toxin-producing microalgae in different water bodies has resulted in the death of aquatic life, a phenomenon commonly referred to as harmful algal bloom (Richmond and Hu, 2013).

Besides the toxins produced by some microalgae, there are also concerns about the likelihood of commercial microalgae to serve as sources of heavy metals and purines (Becker, 2013; Henderson and Paterson, 2014; Moudříková *et al.,* 2017). High purine intake by humans and other animals may result into high uric acid concentrations in plasma leading to gout, an inflammatory disease as well as kidney defects (Choi, Liu and Curhan, 2005). In this regard, the quality of the water used in microalgae production should be devoid of high amount of biological toxins, chemical contaminants, heavy metals, purines, etc. (Becker, 2013; Chu and Phang, 2019). The safety of microalgae can also be ensured by streamlining the biorefinery process to obtain safe and high quality microalgal extracts and derivatives (Becker, 2013).

2.12.1 MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay

Cytotoxicity assays with cultured cells and *in vitro* cell viability are routinely utilized in toxicity evaluation of chemicals as well as in drug screening. Principally, the colorimetric assay is based on the quantification of a biochemical marker to assess the cells' metabolic activity. For example, the approaches are mainly based on various cellular functions including cell adherence, enzyme activity, co-enzyme production, cellular membrane permeability, ATP production, as well as nucleotide uptake activity (Ishiyama *et al.,* 1996).

The MTT assay is among the most efficient colorimetric assays to evaluate cellular cytotoxicity and viability (Mossman, 1983). Basically, the MTT assay utilizes mitochondrial enzymes (for example succinate dehydrogenase) to evaluate the mitochondrial function of cells and consequently determine cell viability (Stone *et al.,* 2009). The colourless colorimetric substance, MTT, is reduced to a formazan, which is purple in colour, by the action of nicotinamide adenine dinucleotide in the active mitochondrion and the formazan is subsequently quantified by light absorbance. In comparison, the MTT assay performs better than dye exclusion methods mainly as a result of its ease of use, non-toxicity and significantly high reproducibility (Stone *et al.,* 2009; Aslantürk *et al.,* 2017).

2.13 Aim and objectives

2.13.1 Aim

The aim of this research was to explore the toxicity, biochemical and health promoting properties of a locally selected microalgal diatom for potential use as a functional ingredient in foods.

2.13.2 Objectives

1. To identify a locally isolated microalgal diatom and to determine its toxicity profile using an *in vivo* rat model and *in vitro* studies.
2. To evaluate the biochemical and nutritional composition of *Amphora* sp.
3. To determine the effect of three phase partitioning treatment on the soluble protein of *Amphora* sp. biomass.
4. To characterise the carbohydrate profile in *Amphora* sp., and to investigate its protective effects against iron-induced oxidative cardiac injury in a rat model using biochemical approaches.

Chapter 3: Identification and Toxicity Evaluation of a Locally Isolated Marine Microalgal Diatom

3.1 Abstract

The geometrical increase in the human population has necessitated the need for alternative food sources without a compromise in the quality of nutrients provided. Thus, the utilization of different microalgae in human nutrition and animal feed has been gaining attention in the last two decades. Recent studies have highlighted the huge potential of a marine diatom, *Amphora* sp. as an affordable food supplement with significant therapeutic activities, however, the safety of the microalga for human consumption has not been evaluated. Therefore, in this study, the *in vitro* and *in vivo* toxicity profiles of *Amphora* sp. biomass and its extracts were investigated to facilitate its use in human nutrition. The *in vitro* toxicity profile was assessed through the brine shrimp lethality, cytotoxicity, and mutagenicity assays while the *in vivo* study was carried out using a rat model. The microalgal extracts displayed no cytotoxicity on the PBMC and Michigan Cancer Foundation-7 (MCF-7) cell lines. In the *Salmonella typhimurium* strains, there was no indication of mutagenicity (TA 98 and TA 100). Similarly, results from the acute toxicity assessment demonstrated no remarkable changes in body and organ weights, the biochemical parameters as well as in the histopathology of the murine liver, heart, and kidneys. Specifically, it was recorded that the oral administration of *Amphora* sp. to a dosage level of 4000 mg/kg/ body weight (BW) was relatively non-toxic, thus providing a scientific basis for selecting a safe dose in future studies aimed at highlighting the potential of the *Amphora* sp. in human nutrition.

3.2 Introduction

According to a recent report by the FAO (Food and Agriculture Organization), the global prevalence of malnutrition increased from 8.4% in 2019 to about 9.9% in 2020, indicating that between 720 and 811 million people battled hunger globally in 2020, with up to 160 million more individuals than the previous year (FAO and UNICEF, 2021). As a result, the noble aim of eliminating world hunger and malnutrition by 2030 is currently far-fetched as we continue to move in the opposite direction. Therefore, the need for alternative food sources with high nutritional value and environmentally sustainability is needed for the crowded world population. It is believed that these sources would contribute to global food security while developing the local economy. Many alternatives to plant-based foods and their sources have been identified and characterised in multiple studies and they include but are not limited to edible insects, single- cell proteins, products from bees (e.g. pot-honey and pot-pollen) as well as both macroalgae and microalgae (Mariutti *et al.,* 2021).

Microalgae, especially, have been noted to account for a large proportion of the Earth's primary production. They are major contributors to the carbon biogeochemical cycle as well as the nitrogen, phosphorus, and silica biogeochemical cycles (Litchman *et al*., 2015). Microalgae are resilient as they grow in poor quality waters including brackish water, freshwater, marine environments, and non-arable lands. Their prominent roles in food chain nutrient cycling and their very rapid growth rate have made them the focus of many research areas. Therefore, the almost unlimited resource of microalgal biodiversity has opened various avenues for their use in different areas of application, especially in renewable energy production, environmental bioindicators, food production, and many other value-added products. The exploration of microalgal biomass as an alternative protein source in food and animal feed has been reported to be well-established as many microalgal species have been shown to have protein contents that are comparable to those of many conventional protein sources (Amorim *et al*., 2021). It is also important to promote microalgae as a viable source of essential and physiologically active lipids, since they have been shown to contain significant amounts of omega-3 fatty acids, which compare well to those of many fishes (Conde *et al*., 2021).

Archaeological records from Chile and other written historical records dating back thousands of years are the basis of the long use of algae as food by humans (Torres-Tiji *et al.,* 2020). Along with their earlier highlighted significant protein and lipid content, microalgal biomass is also made up of substantial quantities of mineral nutrients, both macro and micro, as well as vitamins

(Hosseinkhani *et al.,* 2022). Many products derived from microalgae have also been highlighted for their wide range of biological activities, which include antioxidant (Capitani *et al.,* 2009; Lee *et al.,* 2010; Chen *et al.,* 2020; Dias *et al.,* 2020; Wan *et al.,* 2021), antimicrobial (Peng *et al.,* 2011), anti-inflammatory (Chen *et al.,* 2020; Dias *et al.,* 2020), cardioprotective (de Jesus Raposo, De Morais and de Morais, 2013) and hepatoprotective (Gammoudi *et al.,* 2019); which has raised their use as therapeutics, and prebiotics (Stirk and van Staden, 2022). Currently, many microalgal products are being marketed commercially as pills and capsules, while species such as *Arthrospira, Odontella aurita*, and *Chlorella* are now used as dietary supplements (Caporgno and Mathys, 2018b). Nevertheless, there is a gap regarding the safety, nutritional value, and other beneficial effects of microalgal species with most of the current information based solely on ethnobotanical records.

Toxicity studies of new products, both natural and synthetic, are regarded as essential in determining their approval for use by the final consumers. Only a handful of microalgae have GRAS classification, as recognized by the US Food and Drug Administration. The GRAS designation is typically applicable for the U.S. regulatory authorities and other countries/regions of the world may have their specific regulatory framework for toxicity. *Arthrospira platensis, Auxenochlorella protothecoides*, *Chlamydomonas reinhardtii, Chlorella vulgaris,* and *Euglena gracilis* are some microalgae with GRAS status. The key approach to measuring toxicants in food includes the real-time analysis of the effects of toxicants in various models that could range from *in vitro* biochemical systems to clinical settings as well as the systemic or organ-specific toxicity analysis (Torres-Tiji *et al.,* 2020).

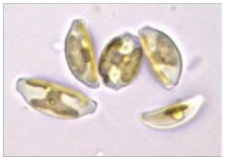
Species and strain selection are among the most important aspects in the bioprospecting of microalgae for any commercial application (Borowitzka, 2013). The *Amphora* genus is a prominent genus of diatoms of marine and freshwater origin whose potential utilisation in different industries has not been fully explored. It belongs to the Bacillariophyceae family, which has been identified as a versatile and abundant microalgal family, probably playing a prominent role in primary production in marine ecosystems. A few studies have highlighted some potential applications of *Amphora* strains in biofuel production (Hogan *et al.,* 2021), bioremediation (Dahmen-Ben Moussa *et al.,* 2018), and in the pharmaceutical sectors (Khumaidi *et al.,* 2020). However, according to available literature, there is no scientific evidence on the safety and toxicity profile of *Amphora* sp. for direct human consumption. This chapter specifically seeks to close this knowledge gap by determining the safety profile of *Amphora* sp., thus providing evidence for its future applications in different industries.

3.3 Material and Methods

3.3.1 Microalgal strain and cultivation

The marine microalgal culture, *Amphora* sp. WCA23.2 was isolated from Slangrivier, Western Cape, South Africa, and grown by the Council for Scientific and Industrial Research (CSIR), South Africa. The sampling location and description of the microalgal culture are presented in Table 3.1.

Table 3.1: Sampling site and description of microalgal culture

Sample site	Designation	Micrograph	Description
Western Cape Region A Isolate 23.2	"WCA 23.2"		Unicellular brown diatom. Marine species

Stock cultures of the species were retrieved from the culture collection unit of CSIR, South Africa, and maintained in transparent conical flasks (1 L) in a shaking incubator (Thermo Fisher Scientific, USA) under constant illumination of 100 µmol.$m^{-2}s^{-1}$. Thereafter, biomass production was carried out in a 50 L raceway pond fed with artificial seawater (ASW) medium (Appendix 1) over 23 days while agitating the culture at 80 rpm using a paddle wheel and illumination was via sunlight (Bayu *et al.,* 2022). The algal biomass was harvested via settling and it was then lyophilised and stored at -18˚C.

3.3.2 Microalgal extract preparation

Extracts of the dried biomass were prepared according to Frassini *et al.* (2019). An ultrasonic probe (VC 750, Sonics and Materials Inc., Newtown, USA) was used to immerse 10 g of dry biomass in 50 mL of hexane, distilled water, and methanol for 30 min at a constant frequency of 20 kHz and 20% amplitude (min). The temperature was monitored and did not surpass 18˚C. The extraction was carried out three times. The extracts were vacuum filtered through a 0.45 µm membrane and the solvents were concentrated using a Büchi RE Rotoevaporator (Büchi R-300 Labortechnik AG, Flawil, Switzerland).

3.3.3 DNA isolation and 18S RNA identification of *Amphora* sp.

DNA extraction was performed by using a method adapted from Melo *et al.* (2006). The microalgal culture was grown on potato dextrose broth for five days at 37˚C and under continuous shaking at 150 rpm. Equal volumes of phenol-chloroform-isoamyl alcohol were added to the reaction tube, this was followed by vortexing the suspension at high speed for 20 min and centrifugation (Eppendorf 5810R, Germany) for 10 min at 10 000 x g. The supernatant was transferred to a microcentrifuge tube and DNA was precipitated by the addition of absolute ethanol followed by incubation for 20 min at 70˚C. The samples were then centrifuged at 4˚C for 10 min. This was followed by resuspending the pellet in 1 mL of Tris-EDTA (pH 8) with 10 µL RNAse and incubation of the pellet for 1 h at 37˚C.

Polymerase Chain Reaction (PCR) was carried out in accordance with the method described by Zhao *et al.* (2001). Each of the reaction mixtures consisted of 2.5 µL of PCR buffer (100 mM Tris-HCL at 25°C, 15 mM $MgCl_2$, 500 mM KCl, 1.0% Triton X-100), 0.5 L of Taq DNA polymerase, 1 µL of deoxynucleoside triphosphates, 20 pmol of each primer, and 1 µL of isolate DNA. The forward and reverse primers were as follows:
Primer (18S-AB1): 5'- GGAGGATTAGGGTCCGATTCC-3' Reverse
primer (18S-TW4): 3′- CTTCCGTCAATTCCTTTAAG-5′.
The PCR was performed using a T100 thermocycler (Bio-Rad, USA). The PCR thermal cycling conditions used were as follows: 5 min at 95˚C, 30 s at 95°C, 30 s at 58˚C, 1 min at 72˚C (30 cycles), and a final extension of 5 min at 72˚C. The amplified PCR products were visualized with agarose gel (1%) electrophoresis which was stained with ethidium bromide. Sequencing of amplicons was performed at Inqaba Biotech, South Africa, using CLC Main Workbench 7 followed by a BLAST analysis on the National Centre for Biotechnology Information database.

3.3.4 Overview of toxicity evaluation

A chart displaying the toxicity assays (brine shrimp, cytotoxicity, bacterial reverse mutation, and acute oral toxicity) carried out in this study is shown in Figure 3.1.

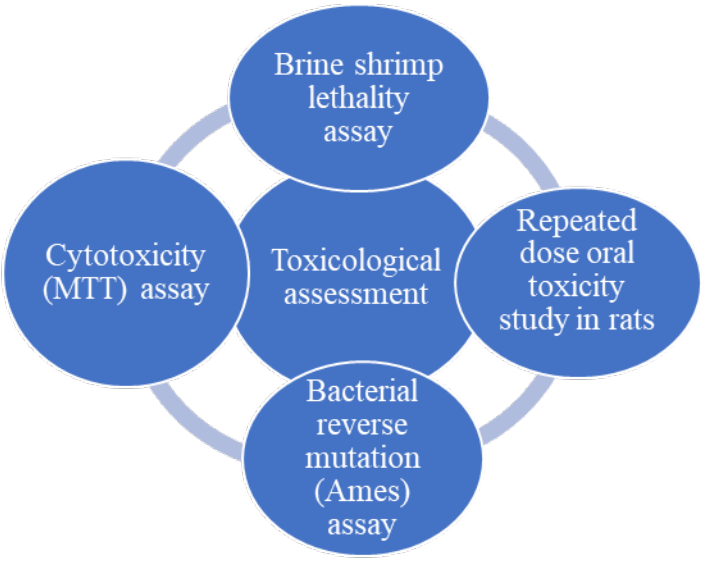


Figure 3.1: Schematic diagram of toxicological assays engaged in this study.

3.3.4.1 Brine shrimp lethality assay

The cytotoxic potential of the *Amphora* sp. biomass and its various extracts was investigated using the *in vivo* brine shrimp (*Artemia salina*) lethality assay in seawater (Meyer *et al.,* 1982). Brine shrimp eggs (Ocean Nutrition TM PET 152) purchased from Northlands Pets, Durban, South Africa were hatched under light conditions in a 500 mL beaker with seawater (23 g NaCl, 11 g $MgCl_2.6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2.2H_2O$, 0.7 g KCl in 1000 mL distilled water). To prevent nauplii mortality during incubation due to a decrease in pH at room temperature, a 0.1 M Na_2CO_3 solution was employed to adjust the seawater to pH 9.0. After 24 h, the nauplii were fed with 15 mL/L of yeast solution (3 mg in 15 mL sea water). Brine shrimp nauplii (n=10) were counted and transferred to vials using a Pasteur pipette and the volume was made up to 10 mL with artificial seawater.

Methanolic, aqueous, and hexane extracts of *Amphora* sp. biomass were dissolved in 1% DMSO (dimethyl sulfoxide) yielding final concentrations of 10, 100, and 1000 µg/mL in seawater. A constant volume of 100 µL of each extract was added to the vials containing the shrimp. After 24 h, the dead larvae were counted to determine the percentage lethality. Potassium dichromate ($K_2Cr_2O_7$) at concentrations of 10, 100, and 1000 µg/mL and seawater served as the positive and the negative control, respectively. For the extracts to be lethal, shrimp death >50% had to be recorded after 24 h.

3.3.4.2 Cytotoxicity evaluation of *Amphora* sp. extracts

3.3.4.2.1 Cell lines

MCF-7 and PBMC cell lines were investigated. Natasha Kolesnikova of the CSIR provided the MCF-7 cell line (Bioscience, CSIR). These cells were kept in 25 cm^2 tissue culture flasks and incubated at 37˚C in a humidified incubator with a 5% CO_2 atmosphere (SnjidersHepa, United Scientific, Cape Town, South Africa). The cells were subsequently transferred to two distinct 75 cm^2 flasks upon arrival (Greiner, Germany).

3.3.4.2.2 Cell maintenance

To ensure an aseptic environment, all cell culture maintenance studies were carried out in a laminar flow cabinet (Scientific Engineering, INC). The laminar flow cabinet was sterilized by exposure to UV light as well as swabbing frequently with 70% ethanol (Merck, South Africa) before performing cell culture experiments. MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) as two independent monolayers (consisting of glucose (4.5 g/L), 1 mM L-glutamine and 1 mM sodium pyruvate) (Sigma-Aldrich, Inc). The DMEM had been supplemented with 10% heat-inactivated foetal calf serum and 1% antibiotic (penicillin/streptomycin) solution (Sigma-Aldrich, Inc). The MCF-7 cell line was sub-cultured every 2-3 days, once the flasks had become 80% confluent to ensure they were in the exponential growth phase.

During sub-culturing, supplemented DMEM was removed from flasks and the monolayer of cells was washed with 5 mL phosphate saline buffer (PBS). Thereafter, an aliquot of 1 mL trypsin was added to the flasks. The monolayer of cells was incubated at 37˚C in a humidified incubator with a 5% CO_2 environment for three min. In order to detach the monolayer, the flasks were then tapped on the side for 30 s. Supplemented DMEM (10 mL) was added, and 1 mL of cell culture was thereafter transferred to each flask. A final aliquot of 20 mL DMEM was added to each flask, and this was followed by incubation at 37˚C in a humidified incubator that contained a 5% CO_2 atmosphere. The cells were monitored for contamination daily by noting medium colour and turbidity changes. Cell growth was examined with an inverted microscope (Nikon, Japan).

3.3.4.2.3 Storage of cells

Cell culture flasks that were 80% confluent were washed with 5 mL PBS and thereafter were treated with trypsin. A 10 mL aliquot of DMEM was added to each flask, and the cells were transferred to 50 mL tubes. The 50 mL tubes were then centrifuged at 1500 rpm for 10 min to collect pellets of cells. An aliquot of 2 mL of cryo-protective medium (10% DMSO, 20% Foetal

Bovine Serum, and 70% DMEM) was added to re-constitute the pellet in cryovials (Corning, South Africa). The cryovials were placed on ice before adding the cryo-protective solution to allow for slow cooling. The cryovials were transferred to thermos flasks and stored overnight at -20 C. The cells were then stored in a -80˚C bio freezer and thawed when they were required for experiments.

3.3.4.2.4 Cell regeneration

When no newly incubated cells were available for immediate use, cells were withdrawn from -80˚C storage and promptly thawed. Following that, the cells were moved to 75 cm^2 tissue culture flasks filled with 20 mL of pre-warmed supplemented DMEM. These flasks with cells were then incubated at 37˚C in a humidified incubator with a 5% CO_2 environment.

3.3.4.2.5 Cell enumeration

Cells were enumerated using trypan blue dye assay, this is an exclusion dye used for counting viable cells. This staining method is based on the principle that healthy (or intact) cells would not use the trypan blue dye, but non-viable cells (cells with compromised membrane integrity) would. Trypan blue staining allows scientists to visualize the morphology of cells since viable cells remain translucent, while non-viable cells appear blue. In this study, 100 µL trypan blue (Bio Whittaker, Wakersville, USA) was mixed with 100 µL cell suspension cultures in centrifuge tubes. These tubes were then incubated at room temperature for a minute. Thereafter, an aliquot of 10 µL trypan-suspended cell cultures was loaded into both chambers of the Neubauer haemocytometer. The cells within the middle square and in the four 1 mm corner squares of the two chambers were then counted. The equation below was used to evaluate the number of cells in suspension:

Total cell count:

16 squares x 4v = Cell counts in 4 sets of 16 squares

16 squares: 2×10^4/mL

Therefore, cells per mL = $\frac{\text{total cell count}}{4} \times 2 \times 10^4$ per mL

3.3.4.2.6 Isolation of PBMC cell lines

In the current investigation, PBMC were collected, separated, and isolated as described by Böyum (1967) with some modifications. Aseptic techniques were strictly employed during the isolation of monocytes from PBMC. The materials used were stored within a Class II Biological Safety Cabinet (Thermo Fisher Scientific, Inc). Furthermore, the reagents were sprayed with 70% ethanol before

being used within the laminar flow hood. This also ensured that they were at room temperature at the time of use.

To isolate the monocytes from PBMC, 50 mL of Buffy coat sample was obtained from the South African National Blood Services. The Buffy coat sample was diluted with an equal volume (50 mL) of pre-warmed Hanks' balanced salt solution (HBSS) (Sigma). Then, this mixture was carefully layered, in a 5:4 ratio, onto pre-warmed Histopaque 1077 (Sigma), with sterile centrifuge tubes (Greiner). The contents of the tubes were then centrifuged (room temperature) at 2000 rpm x g for 30 min. Centrifugation revealed the four different layers. The mononuclear cell (or monocytes) layer was then carefully aspirated using a sterile Pasteur pipette, from the interface between HBSS and histopaque. Once aspirated, monocytes were transferred to a sterile centrifuge tube. The mononuclear cells were then washed twice in HBSS, followed by centrifugation after each wash at 1200 rpm x g for 15 min at room temperature to remove any residual histopaque. Cell pellets were re-suspended in 1 mL RPMI 1640 (supplemented with 2 mM glutamine, 10% unheated foetal calf serum, 100 IU/mL penicillin and 100 Ig/mL streptomycin (Sigma-Aldrich) (Böyum 1967).

In this experiment, DMSO served as the negative control. It was introduced to the wells to see if it had any cytotoxic effects on the MCF-7 and PBMC cell lines. The cell-containing microtiter plates were cultured in a humidified incubator with 5% CO_2 for two days at 37˚C. Following incubation, a predetermined quantity of MTT reagent (5 mg/mL) was added to each well. The plate was then kept in an incubator with 5% CO_2 for a further 4 h at 37˚C. Following incubation, the media was removed, and each well received a 100 µL DMSO aliquot to dissolve the formazan crystals produced by metabolically active cells. The plates were then incubated for a further hour. The absorbance of the formazan was measured using an ELISA plate reader at 590-630 nm. The inhibitory concentration (IC_{50}) of the microalgal extracts was determined using Graph Pad Prism 6 software.

3.3.4.2.7 MTT assay

The MTT assay was performed to estimate the cytotoxicity of the methanolic, hexane, and aqueous extracts of *Amphora* sp. against the PBMC and MCF-7 cell lines (breast cancer) (Mosmann, 1983). Cells were enumerated using trypan blue, using a Neubauer counting chamber according to Lerma-Herrera *et al.* (2021). The test compounds/extracts, solvents, and negative control (DMSO) were all tested in triplicates.

This method (MTT) is simple, accurate, and reproducible, and by principle, it is based on the ability of a specific type of mitochondrial dehydrogenase known as succinate dehydrogenase to

cleave the tetrazolium ring structure of diphenyltetrazolium bromide in viable cells. The cleavage process converts the yellow-colored MTT to purple-colored formazan crystals, which are used to calculate the number of metabolically active (or surviving) cells (Mossman 1983). This implies that an increase or decrease in the number of viable cells is (or would be) a determinant of the number of formazan crystals produced. Mossman (1983) has indicated that the amount of formazan formed is, in turn, an indication of the degree of cytotoxicity induced/caused by a compound. The percentage cell viability was determined using the equation below:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.3.5 Ames mutagenicity assay (Bacterial Reverse Mutation)

The *Salmonella* mutagenicity experiment was performed with minor changes to the technique by Maron and Ames (1983) to determine the capacity of the extracts to produce spontaneous mutation. Strains TA 98 and TA 100 of *Salmonella typhimurium* were obtained on discs from the Medical Research Council in Durban. Both strains were grown from frozen disc cultures of broth that had been supplied. Using flamed forceps, the culture disks were aseptically removed, and they were then put into a sterile 250 mL flask with 25 mL of Oxoid nutrition broth and 78 µL of ampicillin (8 mg/mL). For 16 h, the flask was incubated at 37˚C in a shaking incubator (150 rpm). This was done to produce an optical density at 660 nm that varied from 1.2 to 1.4. *Amphora* sp. extracts were dissolved in DMSO at concentrations of 10, 100, and 1000 µg/mL to get them ready for analysis.

The active control and the negative control were, respectively, sodium azide (NaN_3) and DMSO. Triplicate analyses for each concentration of microalgal extract were performed. A sterile test tube was filled with 100 µL of bacterial culture, 100 µL of test extract, and 2.9 mL of soft agar, and the assay was run at 45°C. Before being placed into glucose minimal agar plates, the tube was quickly vortexed. The agar overlay was hardened, and the plates were then turned over and kept at 37˚C for 48 h. The mutant frequency was then determined as per the equation below, and reported, and revertant colonies (histidine dependent) were counted. Marghoob *et al*. (2022) state that the ratio between the number of revertant colonies and the number of colonies in the negative control was used to calculate the mutation frequency.

Mutant frequency = number of revertant colonies
number of colonies negative control

3.3.6 Oral toxicity study in rats

The acute oral toxicity study of *Amphora* sp. was evaluated using a rat model based on Organisation for Economic Co-operation and Development (OECD) Guideline 407 (OECD guideline 407 2008) with slight modifications. Figure 3.2 represents the experimental design of the study.

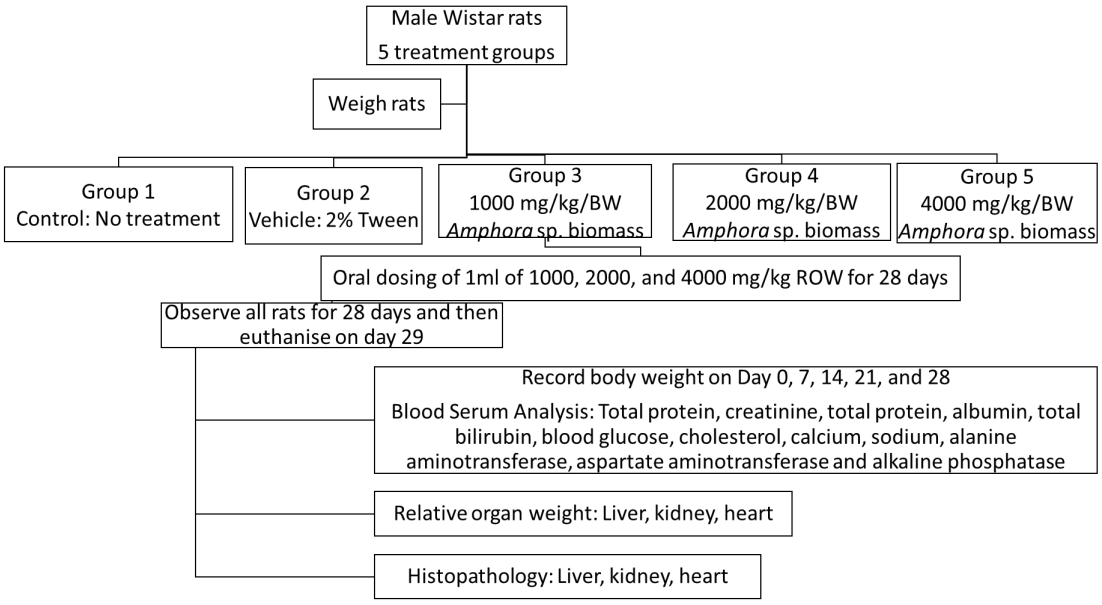


Figure 3.2: Experimental treatment design of oral acute toxicity study (OECD guideline 407, 2008).

3.3.6.1 Experimental design

Male Wistar rats (170–240 g in weight) were randomly selected and orally administered once daily with 1 ml of 1000, 2000, and 4000 mg/kg BW of *Amphora* sp. biomass for 28 days. The animals were divided into five treatment groups (n=5) as follows: control (no treatment), 2% Tween (vehicle), 1000 mg/kg/BW, 2000 mg/kg/BW, and 4000 mg/kg/BW Amphora sp. biomass. Traditional rodent laboratory diets with an unlimited supply of drinking water were employed for feeding. The rats were sacrificed by euthanasia, using isoflurane in an anaesthetic chamber. They were removed and then a cardiac puncture was performed for blood collection. Blood serum chemistry analysis was performed at Clinical & Viral Laboratory Health Labs Global (Durban, South Africa) while the liver, heart, and kidneys were carefully removed and processed for histopathological analysis at the Optics and Imaging Centre, University of KwaZulu-Natal (UKZN) (Durban, South Africa). All samples were fixed in 10% formaldehyde and stained

with.haematoxylin-eosin. All experimental rats were maintained according to the University of KwaZulu-Natal's Ethics Committee guidelines (Ethical approval reference number: AREC/021/018D) (Appendix 2). Between days 0 and 28, body weights were recorded at seven-day intervals, and all rats were inspected daily for mortality and overall condition.

3.3.6.2 Sampling and sample preparation

Healthy young adult male Wistar rats (Figure 3.3) belonging to the species *Rattus norvegicus* were supplied by the Biomedical Resource Unit (BRU), University of KwaZulu Natal, Westville Campus, South Africa. Each rat was randomly selected and weighed between 240 g each. The rats were labelled to enable for individual identification and maintained in their cages for five days prior to dosing to allow them to adjust to laboratory settings. The rats were maintained under laboratory conditions temperature (22-24˚C), humidity (40–60%), and 12 h light/ 12 h dark regime) at the BRU, (University of KwaZulu Natal, Westville Campus). Each treatment group was housed in separate cages.

Figure 3.3: Wistar rats belonging to the species *Rattus norvegicus*

A 2% solution of Tween 20 was prepared to dissolve the microalgal extracts and served as the carrier. The rats were randomly assigned by weight stratification and divided into five treatment groups consisting of five male Wistar rats in each group. The different groups were treated as follows: control (no treatment), 2% Tween 20, 1000, 2000, and 4000 mg/kg/BW *Amphora* sp. biomass, via oral administration with 1 mL once daily for 28 days. The trial also included a Humane Endpoint, which was carried out on a regular basis by veterinary support professionals. Through visual assessments, a table (Appendix 3) was utilized to measure the severity of the animals' well-being throughout the investigation.

3.3.6.3 Body weight (BW) and clinical observations

The BW of each rat was recorded on Day 0, 7, 14, 21, and 28. Food and water were provided *ad libitum*. All animals were observed daily for mortality and their general condition.

3.3.6.4 Blood serum chemistry

All blood samples were collected from the tail vein on Day 29 by collecting 500 µL of blood and centrifuged at 3200 × g for 10 min at 4˚C to extract serum. The serum was separated and blood urea nitrogen (BUN), creatinine, total protein, albumin (ALB), total bilirubin (TBIL), blood glucose (GLU), cholesterol (CHOL), calcium (Ca), sodium (Na), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated. The historical control range of Clinical & Viral Laboratory Health Labs Global (Durban, South Africa) was also placed into consideration for the evaluated biochemical parameters in this study.

3.3.6.5 Relative organ weight

On Day 29, all control and treatment rats were euthanised with isoflurane. The internal organs, specifically the liver, kidney, and heart were subjected to a thorough physical examination. They were examined for abnormalities and the presence of lesions. The organs were then meticulously dissected, washed, and weighed (absolute weight). Each organ's ROW was estimated using the equation below.

$$\text{ROW: Absolute organ weight (g) X } 100 \;=\; \text{BW of rat on sacrifice day (g)}$$

For histological analysis, each organ was kept in 10% buffered formalin. Tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin before being preserved as slices and examined under a microscope.

3.3.5.6 Histopathology

After blood collection on day 29, all the animals were euthanised and a comprehensive gross observation of the excised liver, kidney, and heart was carried out for any manifestation of abnormality and lesions, subsequently, the ROW were computed accordingly (Latif *et al.,* 2021). Representative liver, kidney, and heart tissue of each group were excised, trimmed of fat and other connective tissues, and further processed for histopathological studies according to Olaleye *et al.* (2014). Thin sections of the respective tissues were cut (3 µm) and stained using a standard haematoxylin and eosin protocol. The Axioscope A1 microscope (Carl Zeiss, Germany) was employed in viewing the prepared sections of tissue and a random selection of 4 fields of view per slide were analysed at an objective magnification of X20.

3.3.5.7 Statistical analysis

Results are reported as the average standard deviation of all trials, which were all carried out in triplicate (SD). The experimental data were analyzed by two-way ANOVA using the Windows (USA) version 6.0 program Graph Pad Prism. When $p < 0.05$, differences between groups were deemed significant.

3.4. Results and Discussion

3.4.1 18S rNA identification

The 18s rRNA gene sequence data displayed the closest similarity to *Amphora* sp. (JF834543.1) sharing 99.5% identity, whilst the next closest identification was *Amphora salina* (GQ330308.1), but only sharing 97% identity (Figure 3.4). Based on the sequencing data collected and submitted to GenBank, the microalgal strain in this study is represented as *Amphora* sp., accession number MW721231.

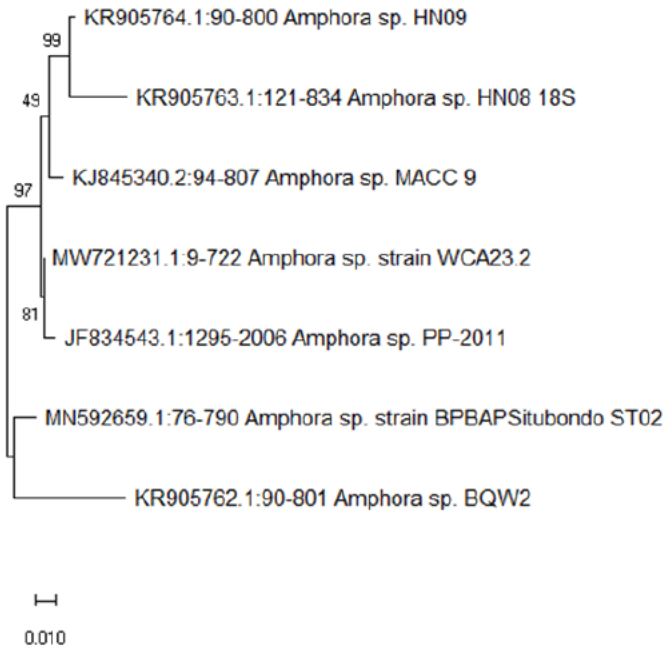


Figure 3.4: Phylogenetic assignment of *Amphora* sp. strain WCA23.2 using the neighbour joining method based on its 18S rRNA gene sequence.

3.4.2 Brine shrimp lethality assay

The brine shrimp lethality test was conducted on crude extracts of *Amphora* sp., *viz*., the methanolic, aqueous, and hexane extracts at different concentrations of 10, 100, and 1000 µg/mL.

None of the crude extracts evaluated displayed any level of toxicity, as they all demonstrated survival rates of 100% at 1000 µg/mL, which was the highest concentration tested (Table 3.2). As expected, $K_2Cr_2O_7$ which served as the positive control, and seawater, the negative control showed 100% and 0% mortality, respectively. These observations were in agreement with the previous reports where a 100% survival rate was also recorded for *Amphora* sp. (Jiménez-Valera and del Pilar Sánchez-Saavedra, 2016). The relative non-toxicity of algal extracts to brine shrimp larvae has also been demonstrated in many recent studies (Haniffa, 2021; Sankarapandian *et al.,* 2022).

Although the production of toxins by microalgae is a function of growth conditions including the pH, light intensity, temperature, salinity, and nutrients (Carballo-Cárdenas *et al.,* 2003; Scholz and Liebezeit, 2012), the conditions used in this study were the standard conditions used for the production of microalgal cultures in controlled environments according to Andersen (2005). Since these were adequate conditions, there was no stress on the *Amphora* sp. strain, thus impeding the production of toxic compounds, and highlighting their potential in food production. It has since been established that a highly toxic extract must display shrimp death (≥ 50%), while a slightly toxic extract should cause cell death of between 50-70% (Espíndola *et al.,* 2022).

Table 3.2: Toxicity of *Amphora* sp. methanolic, aqueous, and hexane extracts against *Artemia nauplii.*

Concentration (µg/mL)	% Survival rate against extracts		
	Methanolic	Aqueous	Hexane
10	100	100	100
100	100	100	100
1000	100	100	100
Positive control	100	100	100

3.4.3 Cytotoxicity of *Amphora* sp. extracts

In vitro cytotoxicity testing was performed to screen the *Amphora* sp. strain for potentially toxic compounds that may negatively influence basic cellular functions and morphology. Evaluating the cytotoxicity of the extracts against a normal (PBMC) and cancer (MCF-7) cell line was necessary to examine their effect on cell viability and proliferation. The percentage cytotoxicity of the methanolic, aqueous, and hexane extracts of Amphora sp. was investigated on PBMC and

MCF-7 cells at concentrations of 50, 100, and 1000 µg/mL, is presented in Table 3.3. An overall low cytotoxic effect of the methanolic, aqueous, and hexane extracts was observed against PBMC cells, with the most prominent toxic activity of 15.3% recorded with the methanolic extract at 1000 µg/mL. Although the cytotoxicity of the *Amphora* extract was observed to be generally low, a dose-dependent increase in cytotoxicity was recorded for each of the extracts, as the screening concentration increased from the lowest concentration to the highest. The low levels of cytotoxicity of *Amphora* sp. extracts against PBMC cells, therefore, indicate that the extracts do not have any significant adverse effect on the viability of the cells. Recently, moderate to low toxicities of extracts from microalgal biomass was demonstrated by Senousy, Abd Ellatif and Ali (2020), thus confirming the safety of microalgae for human use and also excluding the possibility that their inherent biological activities are due to their cytotoxic effects.

The concentration of the extracts improved the suppression of cell proliferation against the cancer cell lines. A previous study by Balaji *et al.* (2017) displayed that the methanol extracts of *Chlorella vulgaris*, *Desmococcus olivaceous* and *Chlorococcum humicola* induced concentration-dependent cytotoxic effects on MCF-7 cells at 100 µg/mL concentration. Similarly, the aqueous extract of *C. vulgaris* and *Artrospira plantesis* at 1000 µg/mL against HeLa cell lines induced a cell viability decrease of approximately 90% and 77%, respectively (Matos *et al.,* 2020).

Table 3.3: Cytotoxicity (%) and viability (%) of PBMC and MCF-7 cell lines at various concentrations.

Sample	Concentration	% Viability	
		PBMC	MCF-7
Methanolic	1000 µg/mL	84.7 ± 0.01	61.5 ± 0.02
	100 µg/mL	88.6 ± 0.01	65.7 ± 0.1
	50 µg/mL	91.3 ± 0.02	68.2 ± 0.02
Aqueous	1000 µg/mL	82.4 ± 0.1	53.7 ± 0.2
	100 µg/mL	87.2 ± 0.02	54.6 ± 0.04
	50 µg/mL	90.2 ± 0.0	58.8 ± 0.02
Hexane	1000 µg/mL	85.6 ± 0.2	70.4 ± 0.07
	100 µg/mL	87.8 ± 0.01	75.3 ± 0.1
	50 µg/mL	91.3 ± 0.05	77.8 ± 0.04

3.4.3 Ames mutagenicity assay

The mutant frequency caused by the microalgae extracts in *S. typhimurium* TA98 and TA100 is shown in Table 3.4. According to Maron and Ames (1983), mutagenesis potential might be assumed if the frequency of mutants is more than 2. Other options include that a probable mutagenic potential can be assumed if the mutant frequency is between 1.7 and 1.9, and no mutagenic potential can be regarded if the mutant frequency is less than 1.6. This study has demonstrated that all the microalgal extracts had no mutagenic activity against *S. typhimurium* TA98 and TA100 strains at all the tested concentrations including the highest concentration (Table 3.4). The tested extracts failed to induce any significant increase in the number of reverse mutant colonies in comparison to sodium azide, which served as the positive control (Figure 3.5). It was observed that the number of revertant colonies increased in direct proportion to the concentration of the extracts. Previous attempts at exploring the nutritional use of algal species have also demonstrated the non-mutagenicity of the prokaryotic species (Guedes *et al.,* 2013; Modeste *et al.,* 2019) as well as their different bioproducts (Athané *et al.* 2020; Matulka *et al.* 2021).

Table 3.4: Mutagenic response to *S. typhimurium* strain TA98 and TA100.

Microalgal extract	Mutant frequency of revertants at different concentrations (µg/mL)				
	5	10	20	100	1000
Methanolic	Na	0.210±0.540	Na	0.690±0.870	0.830±0.965
		0.247±0.180		0.570±0.600	0.722±0.085
Aqueous	Na	0.278±0.029	Na	0.691±0.088	0.725±0.067
		0.368±0.236		0.622±0.029	0.710±0.054
Hexane	Na	0.281±0.060	Na	0.428±0.043	0.495±0.072
		0		0	0.320±0.360
Sodium azide	1.059±0.620	2.086±0.750	3.210±0.450	Na	Na
	1.023±0.360	2.451±0.854	4.350±0.789	Na	Na

Values are expressed as mean ±SD, n=3, in black represents TA100 and in red indicates TA 98, Na = not applicable, 0 = no activity.

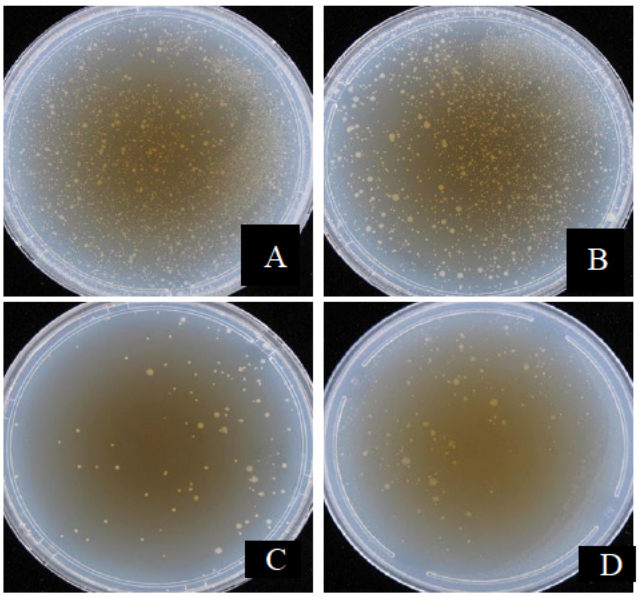


Figure 3.5: (A) Plate with T100 showing revertant colonies, (B) Plate with TA98 showing revertant colonies, (C) Plate showing revertant colonies of the methanolic extract against T100 at 10 µg/mL, (D) Plate showing revertant colonies of the methanolic extract against T98 at 10 µg/mL.

3.4.4 Oral toxicity study in rats

In this study, the repeated dose toxicity test on rats for 28 days using the dried biomass of *Amphora* sp. at dose levels of 0, 1000, 2000, and 4000 mg/kg BW/day displayed no mortality in the animals throughout the study. Furthermore, none of the animals demonstrated any physical sign of toxicity such as changes in eyes, mucous membranes, skin, and fur.

3.4.4.1 Body weight and relative organ weight

The BW of the control and rats treated with *Amphora* sp. biomass is represented in Figure 3.6. The changes in BW may be influenced by several factors, such as the environment of the rats and the type of treatment that may cause stress to the rats. However, in this study, an increase in BW was observed in all rats, with an average BW increase of approximately 70%, therefore, indicating the non-toxicity of the microalgal biomass to the rats. The toxicity assessment of *Nannochloropsis oculate* showed that the species displayed a similar trend in BW changes as observed in this study (Kagan and Matulka, 2015). Furthermore, according to numerous regulatory criteria, BW gain is an essential component of the traditional safety evaluation of the test substance (Schilter *et al*., 2003). As a result, the higher BW recorded confirmed the normal development of each rat, with no sign of toxicity.

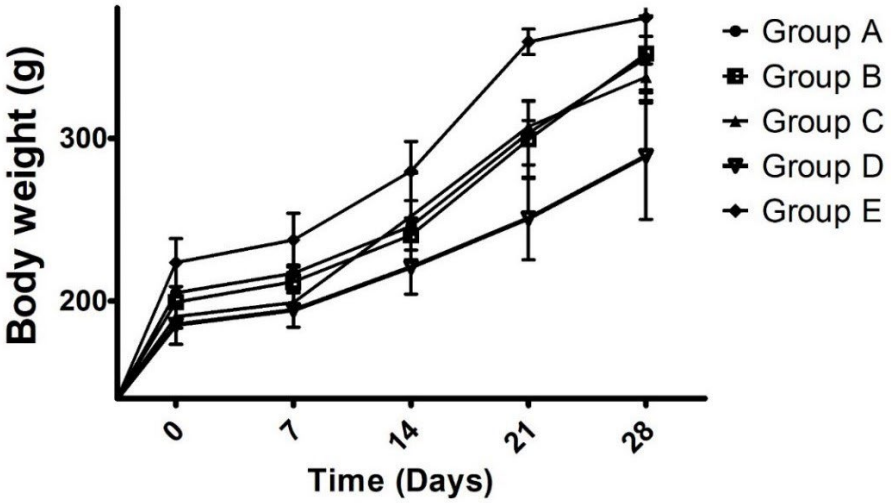


Figure 3.6: BW gains in the different animal groups during the 28-day experimental period. Data is expressed as a mean of n=5.

No treatment-induced changes of biological significance in the organ weights were observed in rats following administration of the microalgal biomass as the data for the liver, kidney, and heart ROWs showed no statistically significant difference ($P<0.05$) between the differently grouped rats as well as the control (Figure 3.7). Thus, the results demonstrate that the *Amphora* sp. biomass caused no damage or injury to the organs as impaired organs often have abnormal atrophy (Wang *et al.,* 2007). Internal organ weight loss is a simple and sensitive indicator of toxicity following exposure to any hazardous chemical (Bailey, Zidell and Perry, 2004).

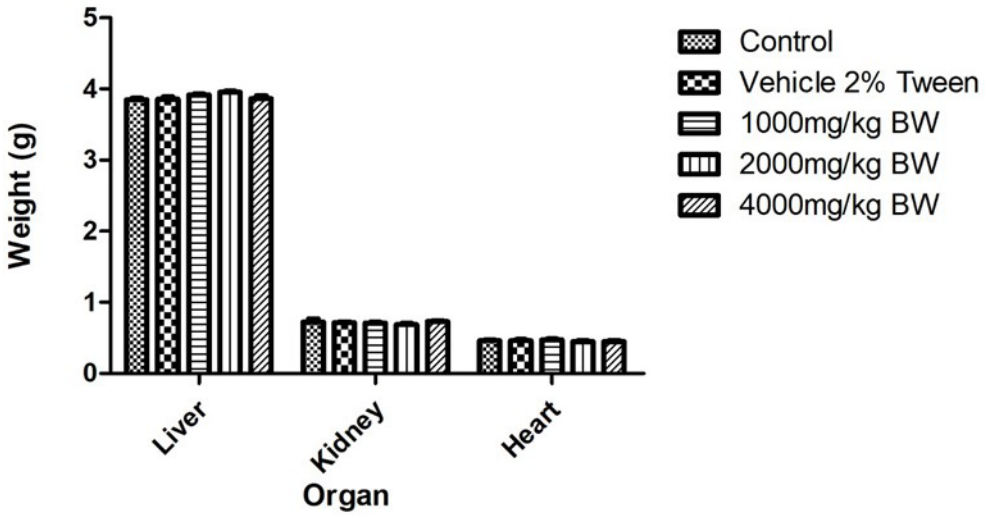


Figure 3.7: ROW of rats treated with different doses of *Amphora* sp. biomass.

3.4.4.2 Blood serum chemistry

The effect of the *Amphora* sp. biomass on blood serum parameters are summarised in Figure 3.8. Results showed no significant alteration in the level of BUN of the rats treated with *Amphora* sp. biomass (6.2-7.0 mmol/L) relative to the control group (6.26 mmol/L), therefore indicating the normal functional state of the treated rats. Furthermore, all treatment groups were within the standard laboratory historical control range, 5.12-10.22 mmol/L BUN. Elevated blood creatinine is highly regarded as a reliable indicator of nephrotoxicity, especially kidney function and impaired glomerular filtration (Waring and Moonie, 2011). The creatinine level in the experimental rats was observed to decrease in a dose-dependent manner at concentrations of 1000, 2000, and 4000 mg/kg BW/day when compared to the control, and thus, demonstrating no damaging effect on the kidneys.

An increase was recorded in the total protein concentration in all treated groups; however, this change was not statistically significant ($p>0.05$) between the groups fed with the microalgal biomass as well as when compared to the control; thus, they were within normal physiological ranges (Figure 3.8). The differences in the ALB concentration between all the groups were recorded to be minimal and not considered to be treatment-related. ALB levels of 34.7, 33.6, 34.2 and 35.5 g/L were recorded for the 1000, 2000 and 4000 mg/kg BW/day treatment groups as well as the control groups, respectively. These results revealed that the ALB levels of all the groups were within the historical control data range (31.2-38.5 g/L of ALB).

CHOL increased slightly in treatment groups 2000 and 4000 mg/kg BW/day with levels of 1.6 to 1.48 mmol/L, compared to the control (1.34 mmol/L) and vehicle control (1.38 mm/L). All treatment groups fell within the historical control range of 1.31-2.43 mmol/L, thus indicating the normal physiological functions of both the livers and the kidneys. An increase in GLU are an indication of diabetes, while low levels correspond with liver disease, infection, or certain types of tumours. Results from this study also revealed that the GLU levels for the control groups were all within the historical control range (5.27-11.12 mmol/L). Alterations in the levels of TBIL have also been associated with various liver functions. There was a significant decrease in TBIL levels in 1000, 2000, and 4000 mg/kg BW/day treatment groups (0.71, 0.74, and 0.70 µmol/L, respectively) when compared post-administration to the corresponding control level (0.79 µmol/L). However, all the treatment groups were within the historical control range (Figure 3.8).

In terms of serum minerals, slight increases in sodium have been observed, ranging from 139.4 mmol/L in the control and 139.6, 142.0 and 140.2 mmol/L in treatment groups 1000, 2000 and

4000 mg/kg. However, these alterations were very slight, and the values remained within the ranges of normal controls (132-145 mmol/L). On the other hand, decreased levels indicate possible damage to the pancreas, while increased levels serve as a possible indicator of kidney disease. All the groups demonstrated calcium levels ranging from 2.59-2.69 mmol/L and were within the historical control range (2.21-2.8 mmol/L).

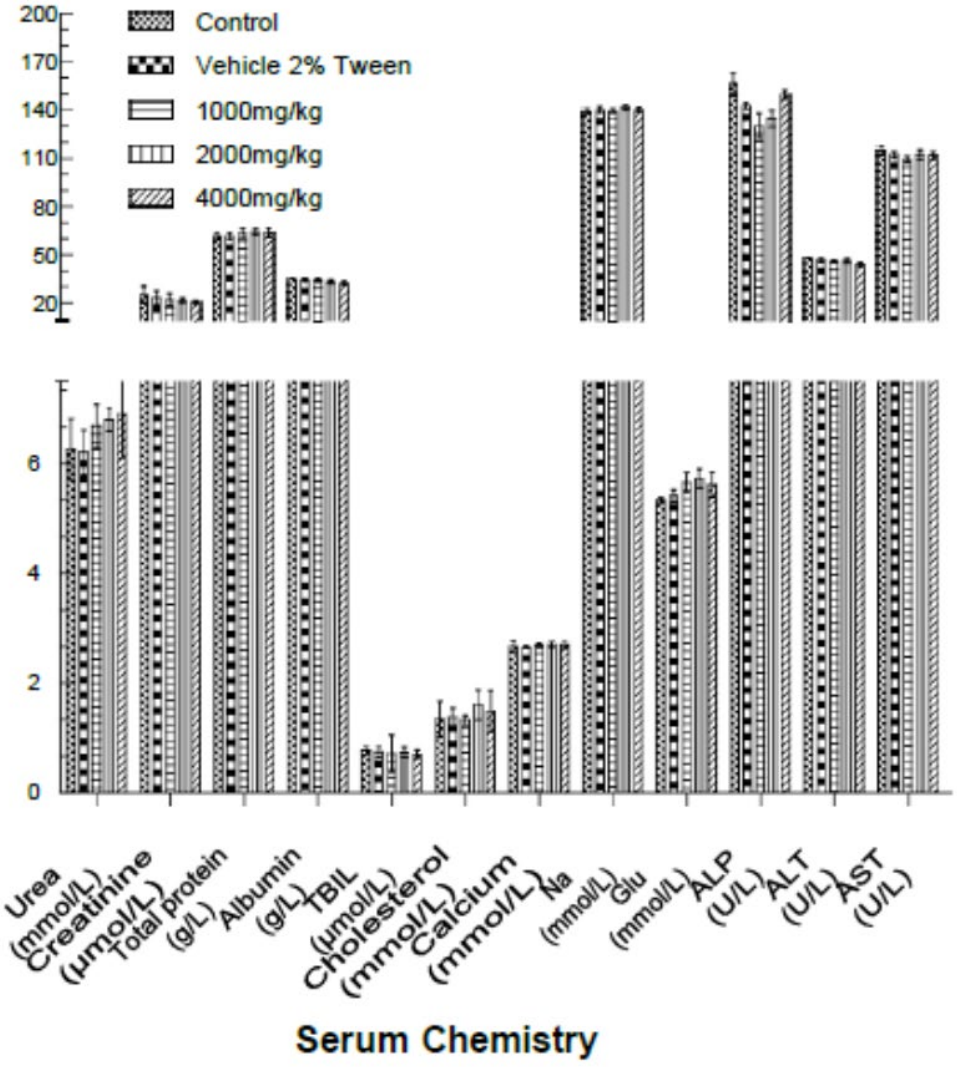


Figure 3.8: Effect of oral administration of *Amphora* sp. extracts on serum chemistry of male Wistar rats.

The results of plasma of ALP, ALT, and AST activities in the experimental animals are also highlighted in Figure 3.8. It was also observed that there were no significant changes in all the enzyme activities among all rat groups. Specifically, AST levels decreased in all treatment groups, 1000, 2000, and 4000 mg/kg with values of 110.28, 113.16, and 112.74 U/L respectively compared to the control (115.98 U/L) and vehicle (113.34 U/L). Similarly, a decrease in the ALT levels of the treatment groups was observed with values of 46.42, 46.93, and 44.62 U/L, compared to the

control (48.75 U/L) and vehicle (47 U/L). From the results, all groups were within the acceptable ALP limits of 112–322 U/L. The lack of significant changes in the activities of the enzymes, *viz*., ALT, AST, ALP as well as in creatinine level, suggests that the oral administration of the *Amphora* sp. biomass did not alter the normal physiological function and structural integrity of the liver and kidney of rats.

3.4.4.4 Histopathological analysis of liver, kidney and heart of rats

Histopathological examination of the liver sections revealed minor differences in cardiac, renal, and hepatic architectures between the control and the treatment groups (Figure 3.9 A-E). Liver histomorphology in the control group and vehicle group showed normal central vein (CV) and hepatic sinusoids (HS). Normal hepatocytes (blue arrow) radiating from the CV with centrally located nuclei. *Amphora* sp. biomass treatment group at 1000 mg/kg/BW showed infiltration of hematopoietic cells into CV with mild dilatation of the HS. However, the hepatocytes appear normal. *Amphora* sp. biomass treatment group at 2000 mg/kg/BW showed infiltration of hematopoietic cells into the CV, dilated HS and the hepatocytes appear to be clear. *Amphora* sp. biomass treatment group at 4000 mg/kg/BW displayed an infiltration of hematopoietic cells into CV, and disruption of the HS and normal hepatocytes. It can be suggested that any histopathological impairments noted had minor effects on renal function since the recorded levels of the biochemical profile were all within the normal physiological values.

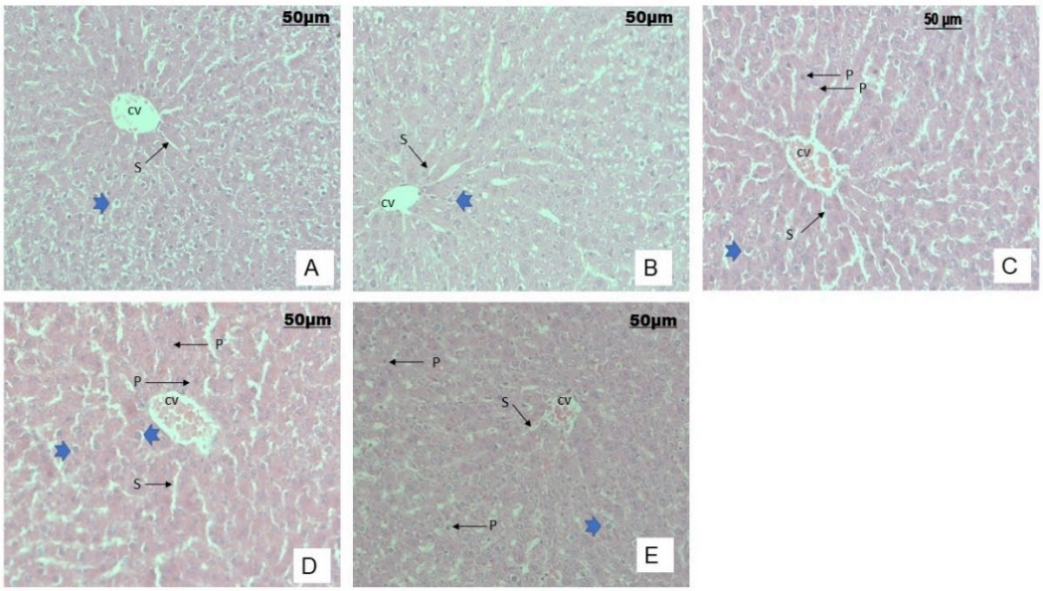


Figure 3.9: Histoarchitecture of liver sections (H&E 20X; 50µm). (A) Control, (B) 2% Tween, (C) *Amphora* sp. biomass 1000 mg/kg/BW, (D) *Amphora* sp. biomass 2000 mg/kg/BW, (E) *Amphora* sp. biomass 4000 mg/kg/BW.

Kidney histomorphology in the control and vehicle groups showed a normal glomerular (G) structure, bowman capsule (BC), and renal tubules (T). *Amphora* sp. biomass treatment group at 1000 mg/kg/BW showed a slight increase in G with a mild proliferation of the glomerular tuft constricting the BC space while the T appear normal (Figure 3.10 A-E). *Amphora* sp. biomass treatment groups in 2000 and 4000 mg/kg/BW showed normal G size and structure. The BC and T appear normal.

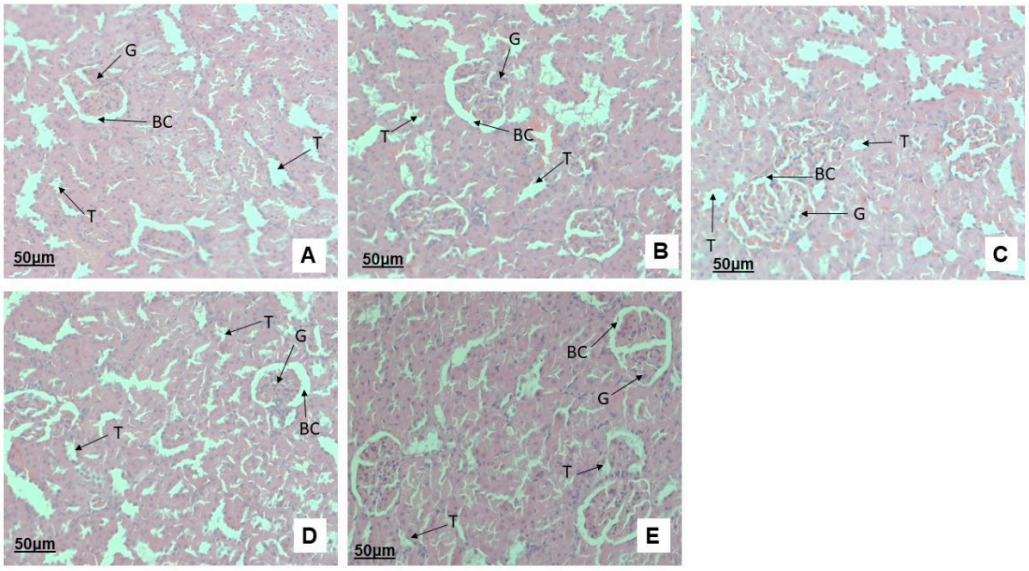


Figure 3.10: Histoarchitecture of kidney sections (H&E 20X; 50 µm). (A) control, (B) 2% Tween, (C) *Amphora* sp. biomass 1000 mg/kg/BW, (D) *Amphora* sp. biomass 2000 mg/kg/BW, (E) *Amphora* sp. biomass 4000 mg/kg/BW.

In this study, the cardiac histoarchitecture in the control and vehicle groups showed normal morphology with well-arranged multicellular cardiac muscle fibres (MF) connected by intercalated discs, clear transverse striations, and well-characterised cardiomyocytes with centrally located vesicular nuclei (Figure 3.11A-E). *Amphora* sp. biomass treatment group at 1000 mg/kg/BW showed normal size and shape of the cardiomyocytes. However, there is mild disorientation of the cardiac muscle fibres and intercalated discs, which might be due to the damage of structural protein in the membrane of the muscle fibres. The histoarchitecture in *Amphora* sp. biomass treatment groups in 2000 and 4000 mg/kg/BW showed normal cardiomyocytes and multicellular cardiac muscle fibres.

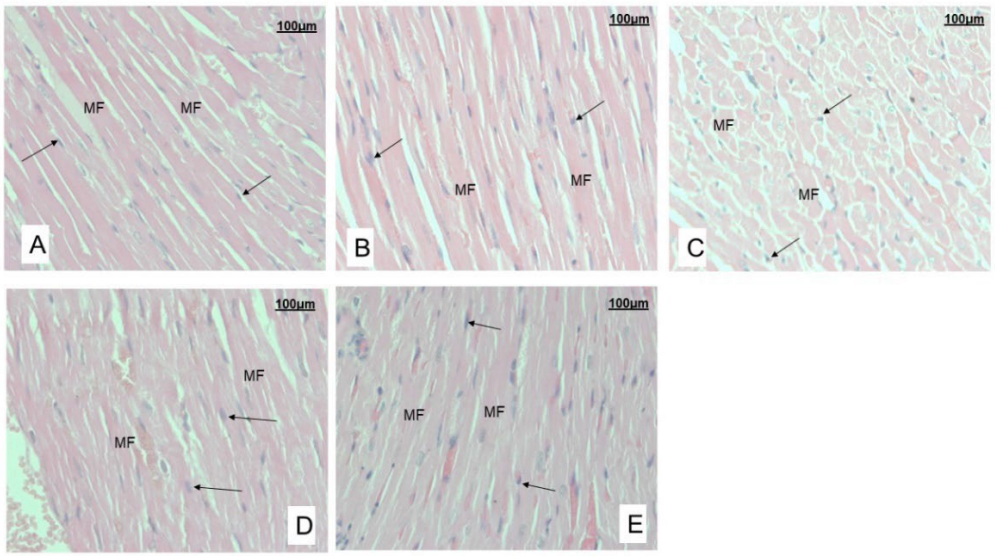


Figure 3.11: Histoarchitecture of heart sections (H&E 40X; 100 µm). (A) control, (B) 2% Tween, (C) *Amphora* sp. biomass 1000 mg/kg/BW, (D) *Amphora* sp. biomass 2000 mg/kg/BW, (E) *Amphora* sp. biomass 4000 mg/kg/BW.

3.5 Conclusion

The varying doses of *Amphora* sp. biomass and its various extracts did not exhibit any significant toxicity in all the assays performed. The toxicity study on the rats further established the safety of *Amphora* sp. and its metabolites after daily oral supplementation for 28 continuous days. The various biochemical parameters evaluated showed that there were no significant variations. Furthermore, there were no remarkable changes in the histopathology of the heart, liver, and kidney tissues of the treatment and control groups. In this investigation, the absence of mortality, changes in growth, vital organs, and serum chemistry markers in rats suggest that *Amphora* sp. biomass is non-toxic. The lack of physical and behavioural abnormalities, mortality, or any substantial variations in BW and ROW confirm these findings. All these results have established a scientific basis for the use of microalgae for human consumption, especially as food and medicine. However, future studies on *Amphora* sp. may be focused on the isolation, identification, and characterization of its various components and metabolites.

Chapter 4: Nutritional Profile and Antioxidant Activity of the Microalgal Strain, *Amphora* sp.

4.1 Abstract

Microalgae have the possibility to be a sustainable food supply, and interest in dietary supplements based on algae is growing internationally. However, a crucial step in creating value- added bioactive chemicals for the food business is choosing acceptable and indigenous microalgae species. The proximate composition, fatty acids, amino acids and mineral profile, as well as bioactive compounds and antioxidant activity in *Amphora* sp. were evaluated as a potential nutraceutical. The *Amphora* sp. biomass was found to be made up of 44.2% ash, 28% carbohydrates, 15% protein and 4% lipids. The fatty acid profile revealed that the diatom accumulates a significant amount of omega-7-MUFA palmitoleic acid (24.50 mg/g) while the amino acid profile demonstrated that it contained all the nine essential amino acids. The antioxidant activities of the diatom extracts showed that the methanolic extract displayed the highest DPPH radical scavenging activity (1.90 ±0.11 mg gallic acid equivalent (GAE)/g DW), and the lowest IC_{50} in all the antioxidant indices evaluated. These results suggest that *Amphora* sp. biomass and its extracts can be utilized as a potential source of ingredients and nutraceuticals in food systems for humans.

4.2 Introduction

There is an urgent need to provide a well-balanced diet with sufficient calories and nutrients essential for good health while circumventing the current challenges encountered with the current food production systems. In this regard, significant increase in food production as well as innovative approaches to provide alternate sustainable food sources to fulfil the global food crisis are imperative. One of these innovative approaches as an alternative solution has been noted to be the commercial production and utilization of marine microalgae as a basic food commodity, thus, in recent years, microalgae have attracted major interest as a source of natural nutrients (Mekkawy *et al.,* 2020).

Microalgae occupy both littoral and benthic habitats throughout the sea waters as phytoplankton, with the most abundant phytoplankton species being the diatoms. The diatoms are amongst the most productive microalgae and are the predominant primary producers in the marine ecosystems (Obata *et al.,* 2013). Microalgae have very high growth rates and photosynthetic efficiency. Additionally, their ability to grow indoors provides an opportunity for studying innovative nutritional products and their applications in the health and food industry (Dragone *et al.,* 2011). Bioactive compounds found in diatoms have been investigated in the food industry for different applications, especially in human health and as food supplements (Gügi *et al.,* 2015). Diatoms and other microalgae are well-known for their unique chemical composition which includes their components with proven health benefits, making them valuable as nutrient enhancing ingredients for foods (Borowitzka, 2018; Wang *et al.,* 2020).

Many marine chemicals have been discovered to have a variety of biological impacts depending on species, seasonality, and geographical location (Barkia *et al.,* 2019). The high nutritional value prompted increased interest in the production of microalgae-enriched foods, and the number of microalgae-containing foods introduced to the market grows on an annual basis (Lafarga, 2019). Microalgae have a variety of nutritious components that can be used to produce food products (Koyande *et al.,* 2019). Previous studies have shown the use of biomass microalgae supplementation in puddings and gelled desserts (Batista *et al.,* 2012), biscuits (da Silva *et al.,* 2021), and pasta (De Marco *et al.,* 2014) to add colouring and functional attributes, with possible health benefits (Batista *et al.,* 2013).

The literature from the past reflects an increase interest in algae-based dietary supplements in the form of complete biomass, making them viable sources of sustainable bioactivities throughout time (Zhou *et al.,* 2022). Notably, research has shown that the bioactive compounds found in diatoms can be discovered and isolated to have therapeutic qualities that can be advantageous to human health (Wong *et al.,* 2022).

Lipids, protein and carbohydrates are the primary nutritional components of algae species (Levasseur, Perré and Pozzobon, 2020). The average composition of proteins, fats, and carbohydrates in microalgae is 12-35%; 7.2-23%; 4.6-23% (DW), respectively (Becker, 2004). The protein content of algae can range from 40 to 60% (of dry matter) (Bleakley and Hayes, 2017). Microalgal proteins have long been considered as an alternative protein source in foods due to their abundance and amino acid composition (Spolaore *et al.,* 2006). According to research, microalgal proteins are prospective raw materials for the manufacture of protein-based food ingredients with both technological and bio-functional uses.

Microalgae lipid content typically ranges between 1 and 40% of DW, but can reach up to 85%. Microalgae are recognized as excellent sources of PUFAs, especially arachidonic acid, DHA, and EPA. These FAs have gained attention because of their association with the prevention and treatment of a range of diseases, including lowering cholesterol and the risk of heart disease (Chacón-Lee and González-Mariño, 2010).

Carbohydrates in the form of starch, cellulose, sugars and other polysaccharides are abundant in microalgae. Because accessible carbohydrates have a high overall digestibility, they have several applications in the food industry (Chacón-Lee and González-Mariño, 2010). Polysaccharides isolated from the microalgae, *Spirulina platensis* (Herrero *et al.,* 2005), *Rhodella reticulata* (Chen *et al.,* 2010) and *Schizochytrium* sp. (Wang *et al.,* 2011) have significant antioxidant properties. As an adaptive response to oxidative stress, microalgae can increase antioxidant defense mechanisms. Therefore, these organisms may be a source of natural antioxidants.

Antioxidants are often utilized as food additives to prevent lipid oxidation (Pokorný ,1991) and they are rapidly being commercialized as nutraceuticals and dietary supplements (Jain and Ramawat, 2013). Due to safety concerns, there is a rising trend in the food industry to replace synthetic antioxidants like butylated hydroxytoluene and butylated hydroxyanisole with natural alternatives. Microalgae are a promising new natural antioxidant source (Natrah *et al.,* 2007; Chacón-Lee and González-Mariño, 2010; Hajimahmoodi *et al.,* 2010; Lee *et al.,* 2010; Goiris *et*

al., 2012) with a high potential to accumulate numerous intracellular important bioactive molecules (Venkatesan *et al.,* 2015; Sansone and Brunet 2019). Their rapid growth rate and excellent efficiency under normal or stressed conditions, in particular, make them an appealing choice as a sustainable alternative of antioxidant sources with high nutritional content (Bulut *et al.,* 2019).

Currently, two species of microalgae are used commercially to produce carotenoid antioxidants: *Dunaliella* for beta-carotene production and *Haematococcus* for astaxanthin production. In recent years, the potential of diatoms as antioxidants (Hemalatha *et al.,* 2013; Xia *et al.,* 2014; Smerilli *et al.,* 2019); immunostimulants and natural antiviral agents (Bergé *et al.,* 1999; Manzo *et al.,* 2019) have also been investigated. Benthic diatoms are reported to be high in natural antioxidants (El-Sayed *et al.,* 2018).

Recognizing the chemical composition is critical when using screening procedures to uncover useful compounds (pigments, antioxidants, and PUFAs) in microalgae (Batista *et al.,* 2013). Microalgae are an enormously diverse group of organisms with widely varying chemical compositions, although their diversity is yet to be thoroughly investigated (Borowitzka, 2013). Diatoms have been studied as a source of bioactive metabolites, which have a variety of applications in the food industry, most notably in human health and dietary supplements; yet, *Amphora* sp. has minimal published evidence about its nutritional value and food applications. Therefore, this chapter investigates the nutritional composition, and relevant bioactivities such as antioxidant assays, fatty acid, mineral and amino acid profiles of the unexplored microalgal *Amphora* sp. strain as a potential novel food source.

4.3 Materials and Methods

All chemical analyses were carried out in triplicate on the *Amphora* sp. dried homogeneous biomass or the extracts. The biomass was sonicated in a sonicator (Scientz-1500F, 18 mm tip) with a power of 20 W for 15 min at 45˚C before nutritional and antioxidant analysis.

4.3.1 Standards and Reagents

Demineralized water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, USA). All reagents and standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2 Proximate composition

Moisture, fat, protein, fibre and ash contents were determined by the methods of the Association of Official Analytical Chemists (AOAC 2005) on the *Amphora* sp. dried biomass. Ash level was analysed by drying in a muffle furnace at 600˚C for 6 h. Moisture was determined by oven drying whilst fat was analysed using the Soxhlet method by petroleum ether extraction. Protein content was obtained through a distillation and titration method using the Kjeldahl technique. The nitrogen-to-protein conversion factor of 5.95 was used to calculate protein content (López *et al.,* 2010). Total carbohydrate content was calculated by subtracting the weight in grams of fat, protein, fiber, moisture and ash from 100%.

4.3.3 Fatty acid methyl esters profile by gas chromatography mass spectrophotometry

Fatty acid extraction was carried out according to Bligh and Dyer (2000). Briefly, 20 mg of biomass was mixed with 80 µL milli-Q water before 300 µL of chloroform–methanol (1:2) was added. Samples were vortexed for 2 min. Thereafter, 100 µL of chloroform was added; tubes were vortexed for 30 s; 100 µL milli-Q water was added to create a two-phase system and vortexed for 30 s more. Tubes were centrifuged (2,500 × g for 6 min), the clear aqueous phase discarded, the chloroform phase recovered, and the residue re-extracted with 100 µL chloroform, centrifuging as above and pooling the recovered chloroform with the first portion. The extract was filtered and then dried at 104˚C for 1 h. Lipids were trans-esterified to FAMEs via the direct transesterification method, modified from Lewis et al. (2000). Specifically, 20 mg of dried biomass and 3 mL of transesterification reaction mix [methanol/hydrochloric acid/chloroform (10:1:1 v/v)] were added. Cells were vortexed for 10 s to ensure even dispersal of biomass and were placed at 90˚C for 120 min. Once transesterification was complete, the samples were removed and allowed to cool to room temperature. Water (1 mL) was then added and vortexed for 10 s. FAMEs were then extracted via the addition of 3×2 mL aliquots of hexane and chloroform (4:1), vortexed for 10 s, and allowed to sit until clear liquid separations were achieved.

The resultant FAMEs were identified by GC-MS (Agilent Technologies, Santa Clara, CA, USA). Methanolic-HCl transesterification of lipids was performed, and the pooled organic layer was prepared for GC-MS analysis. The GC-MS system was used in conjunction with a CTC Analytics PAL autosampler. The FAs was separated on a ZB-5MS GUARDIAN with dimensions of 30 m, 0.25 mm ID, and 0.25 µm film thickness. The carrier gas, helium was used at a flow rate of 1 mL/min. The temperature of the injector was 280˚C and the split ratio was set at 10:1.

The oven temperature was programmed as follows: 100˚C for 1 min, 180˚C at a rate of 25˚C/min for 3 min, 200˚C at 4˚C/min for 5 min, 280˚C at 8˚C/min for 7 min and 310˚C at a rate of 10˚C/min and held for 5 min. The mass selective detector was operated in full scan mode and the source and quad temperatures were maintained at 230˚C and 150˚C, respectively. The transfer line temperature was maintained at 280˚C. The mass spectrometer was operated under electron impact mode at ionization energy of 70 eV, scanning from 35 to 500 m/z.

4.3.4 Amino acid composition

The amino acid content of the biomass was determined using the Pico-tag method (Bidlingmeyer, Cohen & Tarvin, 1984). About 0.12 ± 0.10 g of dried *Amphora* sp. biomass was added into a medium wall Pyrex test tube, with 15 mL of 6 M hydrochloric acid. The tube was quickly sealed and hydrolyzed under nitrogen environment in an oven at 110˚C for 24 h. After hydrolysis, the mixture was allowed to cool at room temperature, transferred to 50 mL volumetric flask and 10 mL α-aminobutyric acid was added as an internal standard. The volume was topped to 50 mL with deionised water. The mixture was filtered through a Whatman filter paper No. 1, and then, through a Whatman filter paper No. 42 before the derivatization process. About 10 µL of filtered sample were transferred into derivatization tube, and evaporated to dryness under vacuum for 30 min. Subsequently, a redrying reagent (a mixture of methanol–triethylamine and deionised water (2:1:2, w/w/w)) was added and re-dried for another 30 min.

After the final drying, a derivatization reagent (methanol–phenyltiocyanate–triethylamine and deionised water [7:1:1:1, v/v/v/v]) was added and the derivatization process was set for 20 min. It was then evaporated to dryness for another 20 min. About 100 µL of sample diluent (a mixture of disodium hydrogen phosphate, deionised water, 10% orthophosphoric acid and acetonitrile) were added to the sample. Of the prepared sample, 20 µL of aliquot and 8 µL of blank solution were injected into Pico-tag column (C18, 3.9 × 150 mm, Waters, Medford, MA) and the chromatographic separation on the hydrolysates was performed at 38°C using a UV detector at 254 nm. The solvent system consisted of two eluants: (A) an aqueous buffer and (B) 60% acetonitrile in water. Gradient elution was employed using two pumps programmed to deliver the mobile phases eluants A and B. A gradient which was run for the separation consisted of 10% B traversing to 51% B in 10 min. A set of amino acid standards (Sigma Chemicals) was analysed with the experimental samples. Identification of the amino acids in the samples was carried out by comparison with the retention times of the standards.

4.3.5 Mineral content

Approximately 0.5 g biomass was placed in a beaker with 1 mL nitric acid (HNO_3). The mixture was heated at 50˚C on a hot plate to allow the sample to be digested by HNO_3 in the fume hood. After acid digestion, the beaker was carefully removed from the hot plate and the contents were left to cool for 30 min, also allowing the acid to evaporate. After evaporation of the acid, the digested samples were transferred to a 50 mL volumetric flask with deionized water (1-5% acid concentration). Mineral elements (calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus and zinc) were analysed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500s, Agilent Technologies, Waldbronn, Germany). The element concentration was calculated using calibration curves for the standard elements (Hwang *et al.,* 2013).

4.3.6 Extraction of bioactive compounds

Extracts of the dried biomass were prepared according to (Frassini et al., 2019). An ultrasonic probe (VC 750, Sonics and Materials Inc., Newtown, USA) was used to immerse 10 g of dry biomass in 50 mL of hexane, distilled water, and methanol for 30 min at a constant frequency of 20 kHz and 20% amplitude (min). The temperature was monitored and did not surpass 18˚C. The extraction was carried out three times. The extracts were vacuum filtered through a 0.45 µm membrane and the solvents were concentrated using a Büchi RE Rotoevaporator (Büchi R-300 Labortechnik AG, Flawil, Switzerland). The biomass was suspended in deionized water prior to usage (Kagan & Matulka, 2015). All extracts were stored at –20˚C until analysis.

4.3.7 Bioactive compounds and antioxidant activity

The total carotenoid content was determined spectrophotometrically using the method described by Lichtenthaler, Packer and Douce (1987). In brief, 1 mL of extracts were diluted in water with 5 mL 90% (v/v) methanol and absorbances at 470, 652, and 665 nm were determined. The total phenolic content (TPC) was calculated using the Folin-Ciocalteu method. The absorbance at 750 nm was measured and expressed as GAE mg/g DW (Goiris *et al.,* 2012). Different assays were used to assess the antioxidant capability of the extracts, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferric reducing ability of plasma (FRAP) and total antioxidant capacity (TAC).

The DPPH test was carried out using the procedure reported by Brand-Williams, Cuvelier and Berset (1995) and measured at 515 nm. The ABTS radical cation decolorization test at 734 nm was used to assess the free radical scavenging activity of extracts (Re *et al.,* 1999). Furthermore, the antioxidant components were assessed using the FRAP assay at 593 nm (Benzie and Strain, 1996) and the total antioxidant capacity (TAC) of the extracts was calculated using the technique of Prieto, Pineda and Aguilar (1999) at 695 nm. The antioxidant activities and standards were expressed as inhibitory concentration 50 (IC_{50}) values (the concentration of extract that causes a 50% decrease in absorbance).

The results are presented as the average standard deviation (SD). Graph Pad Prism version 6.0 for Windows was used to analyze the experimental data (USA). Differences between groups were regarded statistically significant when $p < 0.05$.

4.4 Results and Discussion

4.4.1 Proximate composition

The proximate composition of *Amphora* sp. biomass is presented in Figure 4.1. Moisture is a key aspect in determining the quality of microalgae. The proximate analysis of *Amphora* sp. biomass revealed that the strain displayed a moisture content of 8.8% v/w which is in line with the ideal moisture content recommendation for microalgae i.e. less than 10% (Becker, 1994).

The protein content of the *Amphora* sp. biomass in this study was found to be 15% (w/w) DW, which was observed to be higher than that of the commercial strains, *Chlorella vulgaris* and *Hameatococcus pluvialis*, that have protein contents of 12.3% w/w and 10.2% w/w respectively (Batista *et al.,* 2013). However, the protein content in this study is much less than those of *Chlorella*, *Spirulina* and *Dunadiella* whose levels range above 50% w/w (Batista *et al.,* 2012). The diatoms studied by Renaud, Thinh and Parry (1999) consisted of a protein content of 19.4 - 36.7%. It is worth mentioning that the composition of microalgae is largely influenced by growth conditions and growth phase (Mata *et al.,* 2016).

The *Amphora* sp. biomass was also found to be made up of 4% w/w of fat, which is synonymous with previously reported values for *Spirulina maxima* (4% w/w) and *Chlorella vulgaris* green (5% w/w) (Batista *et al.,* 2013). However, the fat content of *Amphora* sp. was relatively lower

than the value of 11.14% DW which was published for another strain of *Amphora* i.e. (Boukhris *et al.,* 2017). It is believed that this disparity in fat content within the same genus may be due to differences in geographical location and/or processing methods. Microalgae cultivated under ideal circumstances produce a substantial amount of biomass but have a low neutral lipid content, whereas microalgae grown in nutrient deprivation accumulate a large amount of neutral lipids but grow slowly (Tan and Lee, 2016). Light intensity, pH, salinity, temperature, and nitrogen are all recognized physiochemical factors that impact microalgae lipid concentration (Liang *et al.,* 2011; Yeesang and Cheirsilp, 2011; Parjikolaei *et al.,* 2013).

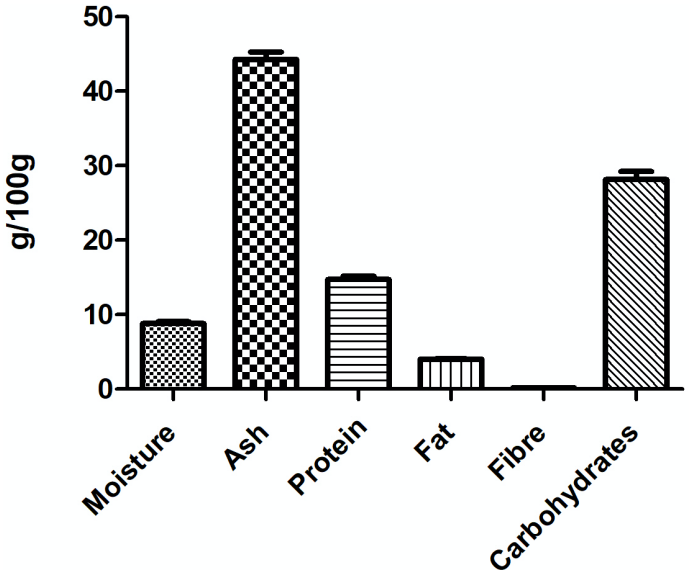


Figure 4.1: Proximate composition of *Amphora* sp. (g/100 g DW biomass) (n = 3).

Carbohydrates was found to be the second most prominent component of *Amphora* sp. biomass amounting to 28% w/w DW. This is significantly higher than in commercially produced nutrient rich microalgae such as *Nannochloropsis* sp. (9.6% w/w) and *Dunaliella* sp. (14.6% w/w) (Kent *et al.,* 2015) and the diatom *Phaedodactylum tricornutum* (11% w/w) (Niccolai *et al.,* 2017). Studies have shown that under ideal growth conditions, most microalgae tend to accumulate carbohydrates rather than lipids, which are synthesized more during stressed conditions as the biosynthetic pathways for both classes of compounds compete for precursor metabolites (Cheng *et al.,* 2017; Debnath *et al.,* 2021).

Ash was found to be the most abundant chemical component in this study, with a value of 44.2% w/w DW. It is generally known that ash content varies for microalgal species, however, diatoms are usually known to have ash content of above 40%, as a result of their high silica levels which are accumulated in their membranes, resulting in a distinct external feature called frustule (Fox

and Zimba, 2018). This recorded ash in combination with mineral content has also been ascribed to the environmental factors as well as the processing methods of the microalgal biomass (Qazi *et al.,* 2022). Mineral content is strongly dependent on physiological, environmental and processing variables (Rupérez, 2002). This might also be due to its growth in saline water, which contains more minerals and trace elements than freshwater (Sandgruber *et al.,* 2021). Previous works have reported that other marine microalgae used in human nutrition contained high ash values such as 30.9% in *Spirulina maxima*, 34.8% in *Chlorella vulgaris* (Batista *et al.,* 2013) and 55.6% in *Amphora coffeaformis* (Lee *et al.,* 2009a).

4.4.2 Mineral content

The biomass contains considerable levels of all the essential minerals for the body including iron, magnesium, manganese, potassium, calcium, phosphorus and zinc. The high ash content of *Amphora* sp. also signifies that the diatom adequately contains some of the essential minerals required for human nutrition and thus it meets the recommended daily allowances (RDA) for an adult male for these minerals according to the World Health Organization (WHO, 2004). For instance, the RDA for iron, zinc, and calcium are 8 mg/day, 11 mg/day and 1000-1200 mg/day respectively (West Suitor and Murphy, 2013), which is below the recorded values for *Amphora* sp. in this study; iron (298 mg/100g), zinc (12 mg/100g), and calcium (2205 mg/100g). Mineral elemental composition recorded in this study compare favourably with previously published work on microalgae (Table 4.1).

Table 4.1: Mineral composition of *Amphora* sp. (mg/100 g DW)

Mineral	mg/100g	Other microalgae (Tibbetts, Milley and Lall 2015)
Calcium	2025.00 ± 3.61^{a}	300-2100
Magnesium	1095.00 ± 9.54^{b}	100-1100
Phosphorus	929.00 ± 6.56^{c}	1700-3000
Potassium	1147.00 ± 4.58^{d}	600-1200
Sodium	1293.00 ± 12.77^{e}	700-1100
Copper	5.60 ± 0.17^{f}	1.2-65
Iron	298.00 ± 7.21^{g}	100-700
Magnesium	18.00 ± 1.00^{fhi}	3.7-59.2
Zinc	12.00 ± 0.71^{fi}	23.9-370

Results are presented as mean ± SD (n=3). Mean values in the same column with the same letter superscripts are not significantly different at $p < 0.05$.

In this regard, these results suggest that *Amphora* sp. might be highly beneficial therapeutically due to its mineral content, especially with addressing defects such as osteoporosis and anaemia.

4.4.3 Fatty acid profile

The fatty acid profile of *Amphora sp.* biomass is represented in Figure 4.2.

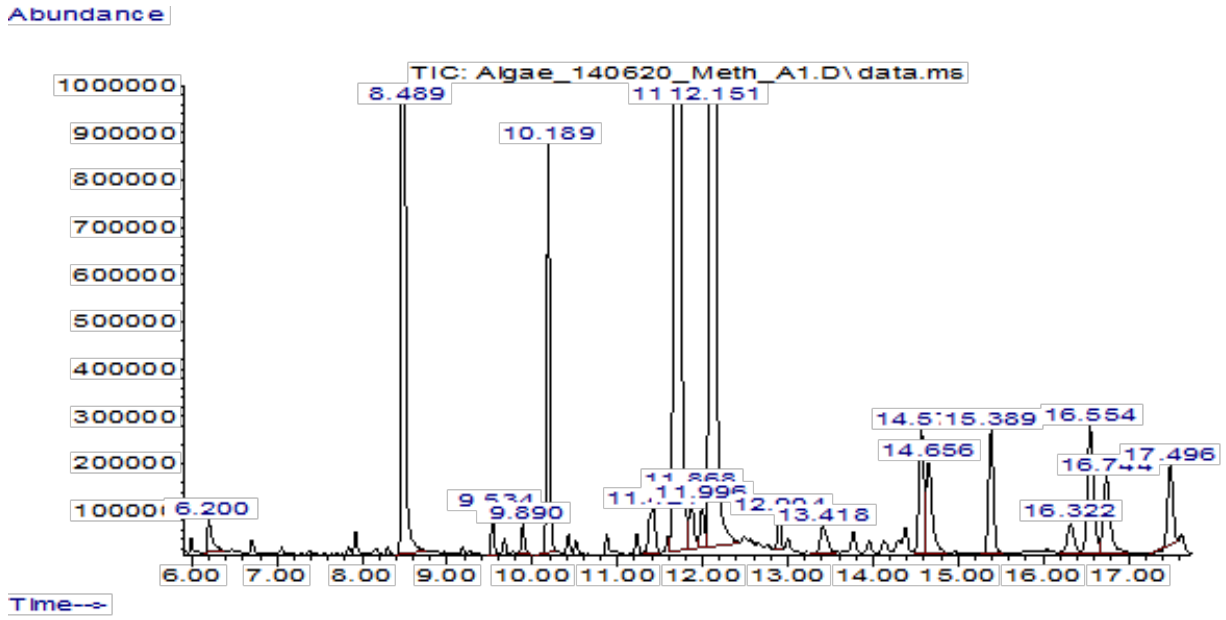


Figure 4.2: Chromatogram displaying the fatty acid profile of *Amphora* sp. biomass.

The fatty acid profile of the diatom showed that it included two MUFAs, seven saturated SFAs and four PUFAs (Table 4.2). MUFAs were the dominant class of FAs in *Amphora* sp., with a total of 25 mg/g, followed by SFAs with a total of 20.8 mg/g, whilst PUFAs was the lowest with an amount of 0.9 mg/g. According to Maltsev and Maltseva (2021), the C16:1 and C18:1 MUFAs are usually found in microalgae and cyanobacteria in significant quantities. Scientific evidence have since suggested that dietary MUFAs are beneficial to human health, especially as they enhance a healthy blood lipid profile, mediate blood pressure and positively modulate insulin sensitivity as well as glycaemic control (Gillingham *et al.,* 2011).

The fatty acid profile of *Amphora* sp. in this study is also remarkable as its major constituent fatty acid, palmitoleic acid (POA, C16:1Δ9) has been identified as an omega-7 MUFA with various beneficial effects in humans including the improvement of metabolic syndrome indications, the reduction of inflammation, protection against cardiovascular diseases and the inhibition of oncogenesis (Wu, Li and Hildebrand, 2012). It is worthy to note that the palmitoleic acid content in this study (24.50 mg/g) which amounts to ~2.5 % w/w compares favourably with

the 3.6% w/w recorded by Zhou *et al.* (2021) in their study which was focused primarily on the production of the fatty acids from the oleaginous *Scheffersomyces segobiensis*.

Table 4.2: Identification of fatty acids (mg/g DW) in *Amphora* sp.

Fatty acids	mg/g
Caproic acid	0.43 ± 0.01^{a}
Capric acid	0.24 ± 0.03^{ab}
Myristic acid	4.07 ± 0.07^{c}
Pentadecanoic acid	1.12 ± 0.01^{d}
Palmitic acid	13.73 ± 0.34^{e}
Stearic acid	0.71 ± 0.10^{abdf}
Lignoceric acid	0.50 ± 0.01^{abdfg}
Oleic acid	0.56 ± 0.02^{abdfgh}
Palmitoleic acid	24.50 ± 0.66^{i}
Linoleic acid	0.37 ± 0.02^{abfghj}
Arachidonic acid	0.17 ± 0.01^{abfghjk}
Eicosapentaenoic acid	0.18 ± 0.01^{abfghjkl}
Eicosatrienoic acid	$0.21 \pm 0.01^{abfghjklm}$

Results are presented as mean ± SD (n=3). Mean values in the same column with the same letter superscripts are not significantly different at $p < 0.05$

4.4.4 Amino acids content

Table 4.3 represents the amino acid composition of *Amphora* sp. biomass g/100 g protein DW. The biomass consisted of nine essential and nine non-essential amino acids with the most abundant amino acid being glutamine (7.87 mg/100 g protein), in contrast to the lowest, cysteine, with an amino acid content of 0.08 mg/100 g protein. Similar patterns of amino acids profile have been recorded in many microalgae (Brown, 1991). In terms of the contributions of the essential amino acids to the amino acid requirements (WHO, 2007), valine contributed 118%, followed by isoleucine (107%), threonine (100%), leucine (82%), phenylalanine (68%), histidine (55%), methionine (52%), and lysine (41%) (WHO, 2007). Tryptophan (1.49 g/100 g) exceeds the WHO/FAO/UNU amino acid requirements. *Amphora* sp. exhibited levels of essential amino acids that meet the nutritional requirement recommended the WHO for adults and children (2 - 5 years) (WHO, 2007).

Table 4.3: Amino acid composition of *Amphora* sp. biomass g/100 g protein DW.

Amino acid	*Amphora* sp.	FAO (1985) Adults/Children
Histidine	1.04	1.9
Threonine	3.39	3.4
Lysine	2.39	5.8
Methionine	1.31	2.5**
Valine	4.13	3.5
Isoleucine	3.01	2.8
Leucine	5.40	6.6
Phenylalanine	4.28	6.3*
Tryptophan	1.49	1.1
Total Essential Amino Acids	26.44	
Serine	3.12	
Arginine	3.82	
Glycine	4.47	
Aspartic acid	6.32	
Glutamic acid	7.87	
Alanine	4.20	
Proline	3.39	
Cysteine	0.08	
Tyrosine	3.32	
Total Non-Essential Amino Acids	36.59	

Amino acid requirement by FAO g/100g (WHO, 2007) for adults and children 2 - 5 years.
*Phenylalanine + Tyrosine; **Methionine + Cysteine

4.4.5 Bioactive compounds

Carotenoids are a well-known and significant family of lipophilic antioxidants, and microalgae are a rich source of these chemicals. These pigments are vital in quenching ROS produced during photosynthesis, particularly singlet oxygen, which is recognized to be a vital antioxidant for human health (Goiris *et al.,* 2012; Ahmed *et al.,* 2014). As shown in Table 4.4, the methanol extract of *Amphora* sp. displayed the highest total carotenoid content (1.62 ±1.12 mg/g DW),

followed by the hexane extract (0.85 ±0.95 mg/g DW) whereas the aqueous extract contained the lowest quantity (0.52 ±0.54 mg/g DW). These results are considerably lower than the findings of Ahmed *et al.* (2014) that reported the carotenoid content of some microalgal species extracts such as *Tetraselmis* sp. (5.8 mg/g DW), *Dunaliella tertiolecta* (1.1 mg/g DW) and *Isochrysis* sp. (5.0 mg/g DW). Goiris *et al.* (2012) also reported the carotenoid content in diatom, *Phaeodactylum tricornutum* and *Nannochloropsis* sp. (6.1 and 2.2 mg/g DW, respectively).

The results of this investigation also revealed that carotenoids in the *Amphora* sp. extracts may dissolve more readily in semi-polar solvents such as methanol than in more polar or less non-polar solvents such as water and hexane. The changes observed might be attributed to differences in growth phase or culture conditions, which have a direct impact on the quantity of carotenoids generated by microalgae cells (Jalal *et al.,* 2013).

Table 4.4: Carotenoid content and total phenolic content of *Amphora* sp. extracts

Extraction solvent	Carotenoid mg/g DW	Total phenolic content mg GAE/g DW
Methanol	1.62 ± 0.10^{a}	1.90 ± 0.11^{a}
Aqueous	0.52 ± 0.03^{b}	0.33 ± 0.06^{b}
Hexane	0.85 ± 0.03^{c}	0.23 ± 0.05^{b}

Results are presented as mean ± SD (n=3). Mean values in the same column with different letter superscripts are significantly different at $p < 0.05$

Similarly, the highest phenolic content was found in the methanol extract at 1.9 mg GAE/g DW, followed by the aqueous extract at 0.3 mg GAE/g DW and hexane extract at 0.2 mg GAE/g DW. These results are similar to those reported by Lee *et al.* (2009a), for *Amphora coffeaformis* as well as in line with those reported by Goiris *et al.* (2012) for *Chaetoceros calcitrans* (1.8 mg GAE/g DW) and *Nannochloropsis* sp. (1.4 mg GAE/g DW). The TPC results of this study were marginally higher than the TPC data reported by Hossain *et al.* (2016) in *Spirulina* (1.78 mg GAE/g). Phenolic compounds and terpenoids play the most significant role as strong antioxidants in diatoms, translating into biological activities such as anti-atherosclerotic, anti-inflammatory, and anticarcinogenic activities (Saxena *et al.,* 2021).

The DPPH test assesses a product's capacity to quench DPPH radicals by electron donation (Prior *et al.,* 2005). The inhibition percentages of the DPPH radicals by the *Amphora* sp. extracts and

the ascorbic acid (vitamin C) standard were found to be concentration-dependent (200-1000 µg/mL) as shown in Figure 4.3. According to the obtained results, all tested microalgal extracts possessed the ability of scavenging DPPH at various degrees. However, the methanol extract at 1000 ug/mL displayed the highest scavenging effect at 44%, followed by the aqueous extract at 32.7% and hexane extract (25.6%). These results are slightly higher than the methanol extract of *Amphora coffeaeformis* (22.7%) and *Navicula* sp. (31.6%) as reported by Lee *et al.* (2009a). Furthermore, these results are considerably higher than those reported by Hemalatha *et al.* (2013), that showed the methanol extract of *C. marina* to have an inhibition of 23.08% and *D. salina* (17.7%). Of noteworthy importance, is these results are significantly higher than those reported by Chu *et al.* (2010). The DPPH results of this study showed notable activities especially in the methanolic extracts, indicating a higher efficacy for scavenging free radicals.

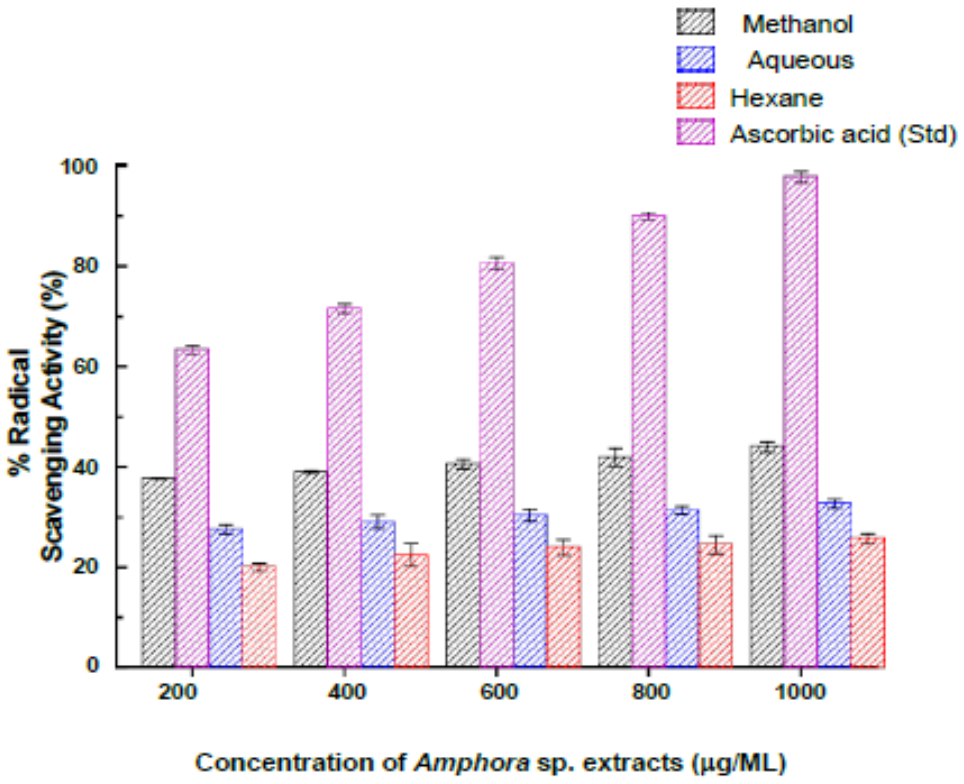


Figure 4.3: DPPH radical scavenging activity of *Amphora* sp. extracts and ascorbic acid standard. Each value represents mean ± SD (n = 3).

The half maximal inhibitory concentration 50 (IC_{50}) values are presented in Table 4.5. A lower value of IC_{50} indicates a high antioxidant activity. The methanol extract exhibited an IC_{50} value of 26.78 µg/mL for the DPPH scavenging activity, whilst the ascorbic acid standard displayed a high antiradical activity with an IC_{50} value of 5.51 µg/mL. Boukhris *et al.* (2017) reported an IC_{50} value of 0.23 µg/mL for the DPPH scavenging activity for the ethanol extract of *Amphora* sp. Boukhris *et al.* (2017) reported an IC_{50} value of 0.23 µg/mL for the DPPH scavenging activity of *Amphora* sp. ethanol extract. Similarly, with regards to ABTS scavenging ability, the IC_{50} value of 22.30 µg/mL recorded for the most active extract from the diatom was lower than 1.83 µg/mL observed for the standard antioxidant. Furthermore, the IC_{50} values for FRAP and TAC were also expectedly found to be lower than the respective standard compounds. It is suggested that the lower IC_{50} values relative to the different standard compounds is due to the fact that the standards are highly purified compounds with established antioxidant activities while the extracts are partially purified fractions that contain antioxidants and many other extraneous substances.

Table 4.5: IC_{50} values for antioxidant capacities of *Amphora* sp. extract compared to standards.

Antioxidant activity	IC_{50} (µg/mL) extract	IC_{50} (µg/mL) standard
DPPH	26.78 ±0.17	5.51 ±0.58
ABTS	22.30 ±0.74	1.83 ± 0.33
FRAP	49.64 ±0.63	6,59 ±1.64
TAC	49.72 ±1.24	8.47±1.29

The antioxidant properties of *Amphora* sp. extracts were assessed using the radical decolourisation test, one of the most commonly used techniques for assessing antioxidant capacity, which assesses a compound's ability to scavenge the ABTS radical. The ABTS assay uses a single electron transfer pathway and has been shown to be reliable for testing food items containing both hydrophilic and highly pigmented antioxidants (Prior *et al.,* 2005; Floegel *et al.,* 2011). When ABTS is combined with potassium persulphate and incubated under dark environments, stable radicals are produced that may be measured spectrophotometrically at 734 nm, which is the typical wavelength for ABTS radicals (Deepa *et al.,* 2013).

Fig. 4.4 displays the ABTS radical scavenging activity of the *Amphora* sp. extracts and the

ascorbic acid standard. The methanolic extracts (200-1000 µg/mL) showed the highest antioxidant activity ranging from 48.42 to 63.62%, followed by the aqueous extracts (32.03- 47.93%), whilst the hexane extracts displayed the lowest activity (19.92-31.59%).

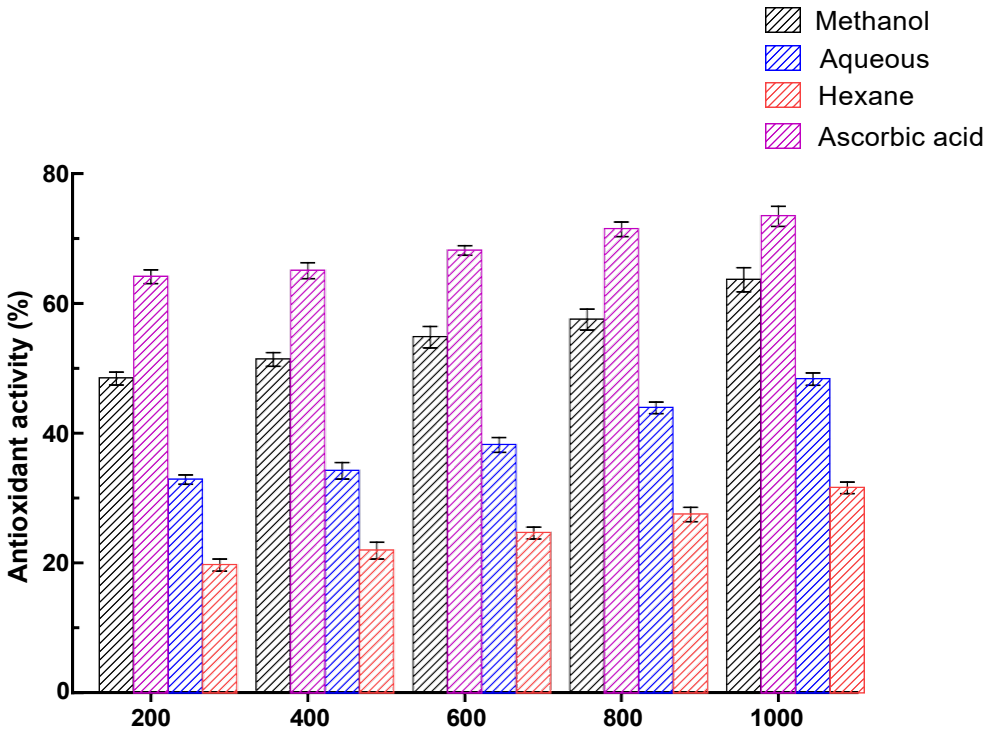


Figure 4.4: ABTS radical scavenging activity of *Amphora* sp. extracts and ascorbic acid standard. Each value represents mean ± SD (n = 3).

High molecular weight phenolics have a greater potential to reduce free radicals (ABTS), and their efficiency is governed by the molecular weight, the number of aromatic rings, and the kind of hydroxyl group substitutions rather than the particular functional groups (Hagerman *et al.,* 1998; Khan *et al.,* 2012). The current findings suggest that the extracts' free radical ABTS scavenging ability may be attributable to the presence of high molecular weight phenolic compounds and derivatives.

The FRAP test is based on phenols' capacity to convert yellow ferric tripyridyl triazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) via electron-donating antioxidants (Benzie and Strain, 1996). Figure. 4.5 presents the FRAP radical scavenging activity of *Amphora* sp. extracts and the standard Trolox. From the results, the FRAP activity is considerably lower than the standard Trolox (71 – 83 µmol TE/g).

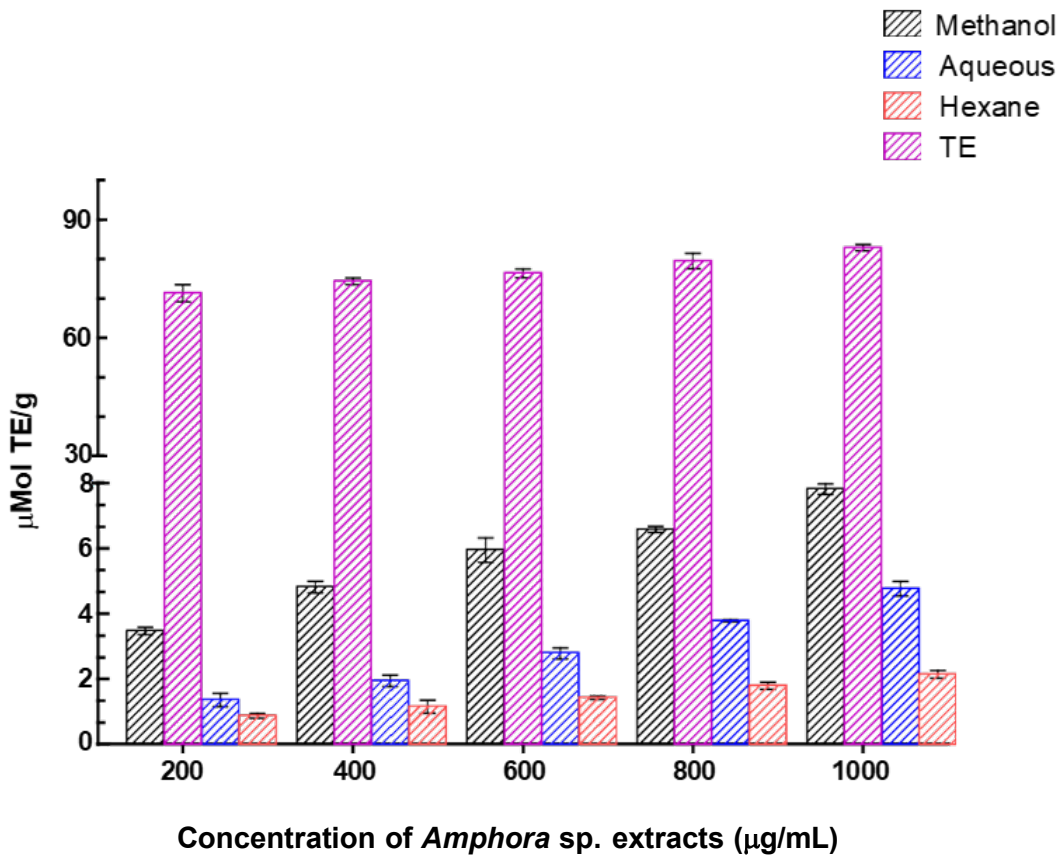


Figure 4.5: FRAP radical scavenging activity of *Amphora* sp. extracts and standard Trolox. Each value represents mean ± S.D (n = 3).

The methanolic extracts contained the highest FRAP activity (3.47 – 7.91 µmol TE/g) whilst the lowest FRAP activity was found in the hexane extracts (1.35 – 4.76 µmol TE/g) (Figure 4.6). The results in this study are similar to the reducing power of *Chlorella* (6.37 – 9.32 µmol TE/g), reported by Goiris *et al.* (2012). A substantially higher reducing power activity for *C. vulgaris* (42 – 64.65 µmol TE/g), *H. pluvialis* - green phase (41.34 µmol TE/g), *Isochrysis* ISO-T (46.69 µmol TE/g) and *Isochrysis* sp., (53.73 µmol TE/g), *N. oculata* (40.68 µmol TE/g), *Nannochloropsis* sp., (40.80 µmol TE/g), *P. tricornutum* (48.90 µmol TE/g) and *Tetraselmis* sp. (46.58 µmol TE/g) was published by Goiris *et al.* (2012). The results in this study are comparable to the reducing power of *Chlorella* (6.37 – 9.32 µmol TE/g), reported by Goiris *et al.* (2012). Hajimahmoodi *et al.* (2010) reported FRAP activity of 0.56 to 31.06 µmol TE/g for the hexane extract of *Microchaete tenera* and the aqueous extract of *Chlorella vulgaris*, respectively. FRAP activity was higher in the aqueous extract than the hexane extract and this trend is in agreement with Hajimahmoodi *et al.*(2010) and Goiris *et al.* (2012).

Molybdenum VI (Mo^{6+}) is reduced to generate a green phosphate/Mo^{5+} combination in the phosphomolybdenum technique. The TAC is compared with that of ascorbic acid. The methanol fraction at 1000 µg/mL demonstrated the highest ability for reducing Fe^{3+} (1.9 mg/g) at almost two times that of the aqueous extract 1.12 mg/g ascorbic acid equivalent (AAE) and approximately five times higher than the hexane extract (0.39 mg/g AAE). Results of this study exhibited similar trends with Hemalatha *et al.* (2013), who reported between 0.6 and 1.03 mg/g AAE for methanolic extracts of *Chlorella marina, Dunaliella salina and Navicula clavata*.

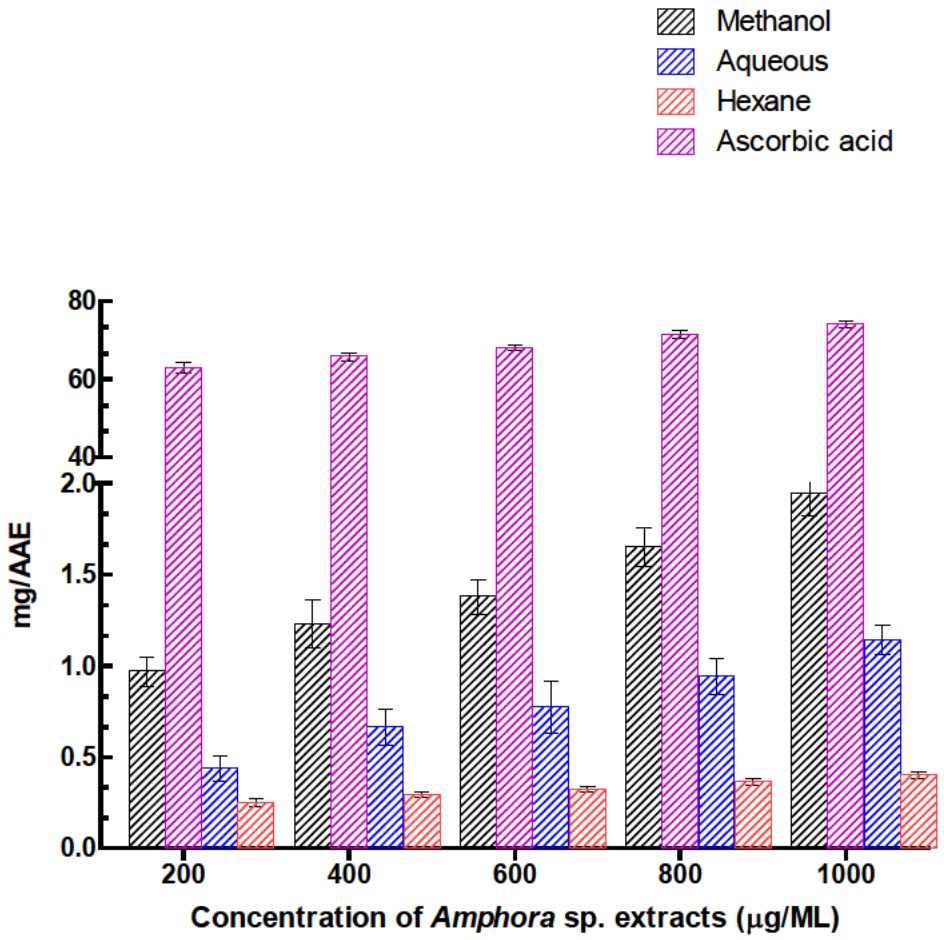


Figure 4.6: TAC of *Amphora* sp. extracts and ascorbic acid standard. Each value represents mean ± SD (n = 3).

The yield of chemical extraction is widely known to be dependent on the temperature and time of extraction, the kind of solvents used, and the chemical composition of the sample. The antioxidant components of microalgae may have various polarities; therefore, the extraction solvent has a substantial impact on the antioxidant capacity of microalgae (Li *et al.,* 2007; Wang *et al.,* 2007; Goh, Yusoff and Loh, 2010; López *et al.,* 2011). As a result, the findings of this study can be used

to improve future extraction methods, notably for antioxidant compounds from derived microalgae, which might be important components for applications in the functional food, fortified nutraceuticals and supplement sectors.

4.5 Conclusion

The *Amphora* sp. biomass was reported to contain a significant amount of protein and carbohydrate, with a remarkable amino acid, fatty acid and mineral profile. This is especially important since there is an urgent need to obtain protein and other important nutrients from alternative sources. It is quite notable that the concentration of five out of the nine essential amino acids in *Amphora* sp. meets the recommended nutritional guidelines (WHO, 2007), suggesting that microalgal proteins can be used in the production of foods, especially as a supplement. In addition, the antioxidant assays demonstrated that the methanol extracts consisted of the highest DPPH scavenging activity and a similar trend was observed for the ABTS assay. The appreciable antioxidative activities is a potential candidate as a natural antioxidant source in the food industry. This study has shown that *Amphora* sp. has the potential to be used as a food and nutraceutical source. However, there is still lots of scientific investigation to be carried out in order to unravel the full potential of this microalgae with the aim of its applications in the food and pharmaceutical industry. For instance, it is necessary to fully identify and enumerate the antioxidant compounds in the diatoms with the aim of meeting the huge demand for alternative source of natural antioxidants and healthy foods.

Chapter 5: Isolation and characterisation of soluble protein from *Amphora* sp.

5.1 Abstract

Microalgae are a high-protein source that may be used in functional, nutritional and medicinal applications. Microalgae are projected to play an important part in future food production systems as a sustainable protein source. As a result, it is critical to search for innovative protein sources that are also ecologically acceptable. This chapter aimed to characterise the soluble protein of *Amphora* sp. biomass using TPP precipitation. The biomass and protein-enriched biomass extract were analysed for their proximate content, amino acid profile, structural properties and functional properties such as water holding capacity (WHC), oil holding capacity (OHC), foaming capacity (FC) and foaming stability (FS) as well as *in vitro* protein digestibility (IVPD) and solubility profile. A comparison was made between the biomass and the protein-enriched biomass extract obtained via three-phase partitioning (TPP) treatment. The protein concentration and total amino acid content were substantially higher in the protein-enriched biomass extract. The solubility of the proteins was dependent on pH, and they were largely insoluble at pH values between 2 and 10. Differential scanning calorimetry (DSC) analysis demonstrated that the protein-enriched biomass extract was thermostable until 112˚C. It has been demonstrated that the WHC reported in the present study may make *Amphora* sp. a good candidate for applications in viscous foods or bakery products.

5.2 Introduction

The world faces a serious problem in maintaining enough food supply to meet the growing demand. Protein, in particular, is one of the nutrients that will be in limited supply in the near future. Plant-based proteins are the primary source of protein for food and feed at present. Traditional food production methods and techniques may soon be obsolete owing to greenhouse gas emissions, fertilizer run-off producing environmental pollution, soil deterioration and ecological disruption caused as a result of excessive harvesting of aquatic commodities (Tester and Langridge, 2010). Microalgae are a promising sustainable, alternative protein source. To fulfill global demand, substitution or alternative protein sources, as well as more efficient production processes, must be identified and developed.

Microalgae have long been recognised as a prospective source of biological components such as carbohydrates, lipids, pigments, vitamins, and polyphenols, especially proteins (Hsieh and Wu, 2009; Chia *et al.,* 2018). Because microalgae contain all necessary amino acids (Becker, 2004; Becker 2007; Safi *et al.,* 2014), they offer enormous promise as an alternative protein source (Salati *et al.,* 2017). Microalgae have attracted the attention of scientists due to their unusual growth requirements, such as the ability to thrive in saline water in the presence of carbon dioxide and sunlight on infertile ground. As a result, they have the potential to be a sustainable source of feedstock for the fuel, food, chemical, textile, polymer and even pharmaceutical industries (Viegas *et al.,* 2015).

The total protein content of microalgae biomass varies with species and can reach 70% of DW (Schade and Meier 2020; Amorim *et al.,* 2021) Proteins are utilized in food to form and stabilise food systems such as emulsions, foams, and gels as well as to bind water. Emulsions and foams are widely used in the food, cosmetic and pharmaceutical sectors, and they are frequently stabilised by synthetic surfactants or animal proteins. Microalgae cell walls are frequently disrupted in order to gain access to proteins, amino acids, and other components. Soluble proteins are found in the cytoplasm of certain microalgae, according to Chia *et al.* (2018) and El-Naggar *et al.* (2020). The number of studies on ways of processing microalgae and utilizing them as a source of protein has expanded (Ghribi *et al.,* 2015). It is difficult to scale up several techniques for concentrating and separating proteins from microalgae. Many researchers are interested in the TPP approach because it is rapid, easy and scalable for concentrating, isolating, and deactivating proteins from crude materials (Waghmare *et al.,* 2016).

Waghmare *et al.* (2016) investigated the influence of several factors on the TPP approach in order to optimise the protein separation process from *Chlorella pyrenoidosa*. Currently, the industry is looking for greener and more efficient alternatives to solve the aforementioned shortcomings. As a result, TPP was established as an effective method for isolating and purifying enzymes and biomolecules (Akardere *et al.,* 2010; Gagaoua *et al.,* 2017). Therefore, the present study focused on selecting the optimal protein extraction technique and to determine the biochemical composition, functional attributes, structure and IVPD of the extracted proteins and biomass as well as the solubility and thermostability profile of the extracted proteins.

5.3 Materials and methods

5.3.1 Materials

All chemicals were purchased from Sigma-Aldrich, South Africa. Dried biomass of *Amphora* sp. was stored at -18˚C.

5.3.2 Extraction of protein from microalgal biomass by three phase partitioning

The biomass (20 mL) was combined with ammonium sulphate (8 g) and ethanol (20 mL). The mixture was stirred at 28 ±2˚C for 20 min. Three phases were formed and the upper organic phase was removed first followed by piercing through the middle phase to pipette the lower aqueous phase. The intermediate phase containing concentrated proteins was dried at 50˚C for 12 h before being used for further analysis (Waghmare *et al.,* 2016).

5.3.3 Protein content of extract

The extracted protein was quantified through a modified Bradford method using BSA as a standard (Bradford 1976). The absorbance was read on a UV-Vis spectrophotometer (Shimadzu, Tokyo, Japan) at 595 nm.

5.3.4 Characterisation of the biomass and protein-enriched biomass extract

5.3.4.1 Proximate composition

Moisture, fat, protein, fibre and ash contents were determined by the methods of the Association of Official Analytical Chemists (AOAC, 2005). Ash content was analysed by ashing in a muffle furnace at 600˚C for 6 h. Moisture was determined by oven drying whilst fat was analysed using the Soxhlet method by petroleum ether extraction. Protein content was obtained through a distillation, and titration method using the Kjeldahl method. The nitrogen-to-protein conversion factor of 5.95 was used to

calculate protein content (López *et al.,* 2010). Total carbohydrate content was calculated by subtracting the weight in grams of fat, protein, fiber, moisture and ash from 100%.

5.3.4.2 Amino acid analysis

The amino acid content of the biomass was determined using the Pico-tag method (Bidlingmeyer, Cohen & Tarvin, 1984). About 0.12 ± 0.10 g of dried *Amphora* sp. biomass was added into a medium wall Pyrex test tube, with 15 mL of 6 M hydrochloric acid. The tube was quickly sealed and hydrolyzed under nitrogen environment in an oven at 110˚C for 24 h. After hydrolysis, the mixture was allowed to cool at room temperature, transferred to 50 mL volumetric flask and 10 mL α-aminobutyric acid was added as an internal standard. The volume was topped to 50 mL with deionised water. The mixture was filtered through a Whatman filter paper No. 1, and then, through a Whatman filter paper No. 42 before the derivatization process. About 10 µL of filtered sample were transferred into derivatization tube, and evaporated to dryness under vacuum for 30 min. Subsequently, a redrying reagent (a mixture of methanol–triethylamine and deionised water (2:1:2, w/w/w)) was added and re-dried for another 30 min.

After the final drying, a derivatization reagent (methanol–phenyltiocyanate–triethylamine and deionised water [7:1:1:1, v/v/v/v]) was added and the derivatization process was set for 20 min. It was then evaporated to dryness for another 20 min. About 100 µL of sample diluent (a mixture of disodium hydrogen phosphate, deionised water, 10% orthophosphoric acid and acetonitrile) were added to the sample. Of the prepared sample, 20 µL of aliquot and 8 µL of blank solution were injected into Pico-tag column (C18, 3.9 × 150 mm, Waters, Medford, MA) and the chromatographic separation on the hydrolysates was performed at 38°C using a UV detector at 254 nm. The solvent system consisted of two eluants: (A) an aqueous buffer and (B) 60% acetonitrile in water. Gradient elution was employed using two pumps programmed to deliver the mobile phases eluants A and B. A gradient which was run for the separation consisted of 10% B traversing to 51% B in 10 min. A set of amino acid standards (Sigma Chemicals) was analysed with the experimental samples. Identification of the amino acids in the samples was carried out by comparison with the retention times of the standards.

5.3.4.3 Emulsifying capacity (EC) and stability (ES)

The procedure previously described by Cano-Medina *et al.* (2011) was used for EC and ES. Biomass and protein-enriched biomass extract 2% (w/v) were vortexed for 3 min. After leaving

the tubes for 1 h, the EC and ES were determined using the following equations:

$$\text{Emulsion capacity \%} = \frac{\text{emulsion of portion (H)}}{\text{portion volume total (Ho)}} \times 100$$

$$\text{Emulsion stability \%} = \frac{\text{\% emulsification capacity at 24 hours}}{\text{\% emulsification capacity at 0 hours}} \times 100$$

where H is the height of the emulsion layer and Ho is the height of the total solution.

5.3.4.4 Foaming capacity (FC) and stability (FS)

Determination of FC and FS followed the method by Cano-Medina *et al.* (2011). Biomass and protein-enriched biomass extract, 3% (w/w) were dispersed in 50 mL water, the volume was recorded, and the mixtures were homogenized (APV2000 SPX, Germany) for 3 min. The volume of the homogenized material was measured immediately in a graduated cylinder. FC was expressed as the volume increase in percentage as a result of whipping.

$$\text{Foaming capacity (\%)} = \frac{\text{volume after} - \text{volume prior}}{\text{volume prior}} \times 100$$

For FS determination, the changes in foam volume in a graduated cylinder were recorded and represented as a percentage after 180 min.

5.3.4.5 Water holding capacity (WHC) and oil holding capacity (OHC)

WHC was determined as reported by Ghribi *et al.* (2015). Biomass and protein-enriched biomass extract (1 g) were added to 10 mL of distilled water, vortexed for 5 min and allowed to stand for 30 min. Thereafter, the mixtures were centrifuged (Eppendorf 5810R, Germany) at 3000 x g for 25 min. The supernatants were discarded and the surplus water was drained for 25 min at 50°C before reweighing. OHC was determined by weighing 0.5 g of either the biomass or protein- enriched biomass extract, soaking in oil or 30 min and then centrifuging at 3000 x g for 25 min. The oil was separated and the tubes were inverted for 25 min. The WHC and OHC were represented as grams of water or oil bound per 100 g of biomass or protein-enriched biomass on a DW basis.

5.3.4.6 Protein solubility

Solubility of protein-enriched biomass extract was measured using a technique modified from Beuchat, Cherry and Quinn (1975). The extract was prepared in ultrapure water at 1% (w/v) concentration. The pH was adjusted between 2 and 12 using either 1 M NaOH or 1 M HCl. Following that, each sample was mixed at room temperature for 45 min using a vibrating shaker (Thermo Fischer Scientific, South Africa) before being centrifuged at 4000 g x g for 30 min at 4˚C. The Lowry Assay was used to determine the quantity of soluble protein. The solubility (%) of the protein-enriched biomass extract at each pH point was estimated based on the total protein content at complete dispersion using the following formula:

$$\text{Solubility (\%)} = (C_{sup} / C_{tot}) \times 100\%$$

where S is the solubility, C_{sup} is the concentration of protein in the supernatant and C_{tot} is the concentration of protein in the total fraction.

5.3.4.7 Thermal properties

The thermal characteristics of the protein-enriched biomass extract was determined using DSC (DSC-25, TA Instruments, New Castle, USA) equipped with a TA 60 WS detector and computer-aided data processing system. Protein-enriched biomass extract (5.00 ±0.25 mg) was weighed into a DSC pan and sealed. An identical empty pan was placed on the reference side. Scans were carried out at 10-200˚C at a heating rate of 5˚C/min and a nitrogen gas purging rate of 70 m L/min.

5.3.4.8 Fourier Transformed Infrared assay

The FTIR spectra of the dried biomass and protein-enriched biomass extract was analysed on a Perkin Elmer model Spectrum 400 FTIR spectrometer equipped with a diamond crystal ATR (Waltham, MA, USA). A Scanning range of 4000-650cm^{-1} with a resolution of 1 cm^{-1} was used.

5.3.5 *In vitro* protein digestibility (IVPD)

The IVPD was determined using a multi-enzyme solution with minor modifications (Hsu *et al.,* 1977). Dried biomass and protein-enriched biomass extract (5 mg) was mixed well in 5 mL of distilled water, and the pH was adjusted to 8. A combination of trypsin (1.6 mg/mL) and α-chymotrypsin (1.6 mg/mL) enzymes was dissolved in 1 mL distilled water (pH 8). At 37˚C, pH fluctuations were observed for 10 min. The reference sample was sodium caseinate, which had a

100% IVPD value. The IVPD was calculated using the equation:

$$Y = 210.464 - 18.103\,x$$

x = pH of sample suspension after 10 min and 15 min

5.4 Results and Discussion

5.4.1 Effect of TPP on the protein concentration of the biomass

A protein concentration of 91.99% was recorded in the TPP-treated sample. Because of its salting-out capabilities and water solubility, ammonium sulphate was the salt of choice for TPP (Fleurence, 1999). Recently, the TPP approach was proposed for the isolation of microalgal proteins. Microalgal components are separated into nonpolar (upper phase) and polar (lower phase) phases upon TPP usage, whereas proteins remain in the intermediate phase. This is accomplished by combining ammonium sulphate, which precipitates the protein, with t-butanol, which enhances the buoyancy of the protein (Waghmare *et al.,* 2016).

5.4.2 Characterisation of the biomass and protein-enriched biomass extract

5.4.2.1 Proximate composition of dried biomass and protein-enriched biomass extract

A comparison of the proximate composition of dried biomass and protein-enriched biomass extract showed that TPP use concentrated the proteins in the biomass (Table 5.1). However, all the other parameters (fat, ash, moisture, and carbohydrate content) were reduced significantly after TPP treatment.

Table 5.1: Proximate composition of *Amphora* sp. biomass and protein enriched biomass extract.

Constituents	Dried biomass	Protein enriched biomass extract
Moisture (%)	8.82 ± 0.18^{b}	6.61 ± 0.15^{a}
Ash (%)	44.28 ± 0.05^{b}	40.36 ± 0.07^{a}
Protein (%)	14.72 ± 0.11^{a}	22.80 ± 0.18^{b}
Fat (%)	4.02 ± 0.04^{a}	2.51 ± 0.10^{b}
Fibre (%)	0.13 ± 0.05^{a}	0.07 ± 0.06^{a}
Carbohydrate (%)	28.04	27.65

Values are means ± SD (n=3). Different superscript within rows indicates significant differences ($p < 0.05$) in the dried biomass and protein-enriched biomass. Carbohydrates were determined by difference.

5.4.2.2 Amino acid composition of the dried biomass and protein-enriched biomass extract

Amino acid composition of the biomass and protein-enriched biomass extract is shown in Table 5.2.

Table 5.2: Amino acid composition of *Amphora* sp. biomass and protein-enriched biomass extract g/100 g DW.

Amino acid	**Biomass *Amphora* sp.**	**Protein enriched biomass**	**FAO (1985) Adults/Children**
Histidine	1.04 ± 0.092	1.22 ± 0.071	1.9
Threonine	3.39 ± 0.005	11.42 ± 0.007	3.4**
Lysine	2.39 ± 0.152	4.96 ± 0.007	5.8
Methionine	1.31 ± 0.134	2.06 ± 0.071	2.5**
Valine	4.13 ± 0.095	3.65 ± 0.071	3.5
Isoleucine	3.01 ± 0.089	5.34 ± 0.007	2.8
Leucine	5.40 ± 0.118	7.96 ± 0.001	6.6
Phenylalanine	4.28 ± 0.005	5.52 ± 0.000	6.3*
Tryptophan	1.49 ± 0.005	1.49 ± 0.071	1.1
Total Essential Amino Acids	**26.44**	**43.62**	
Serine	3.12 ± 0.003	1.22 ± 0.000	
Arginine	3.82 ± 0.005	5.06 ± 0.000	
Glycine	4.47 ± 0.014	4.68 ± 0.000	
Aspartic acid	6.32 ± 0.021	6.18 ± 0.000	
Glutamic acid	7.87 ± 0.026	10.30 ± 0.000	
Alanine	4.20 ± 0.013	5.34 ± 0.001	
Proline	3.39 ± 0.019	6.84 ± 0.004	
Cysteine	0.08 ± 0.027	1.59 ± 0.014	
Tyrosine	3.32 ± 0.038	3.65 ± 0.017	
Total Non-Essential Amino Acids	**36.59**	**44.86**	

Values are means ± SD (n=3).

EAAs were higher in the protein-enriched biomass extract (43.62%) as compared to the biomass (26.44%). The same trend was also observed for the non-essential amino acids. This suggested that the extraction process increased the quantity of amino acids. The EAAs of the protein-enriched biomass extract were comparable to reports for *Isochrysis* sp. (42.03%) and *Spirulina* sp. (45.18%) (Bleakley and Hayes, 2021). EAAs such as lysine, leucine, threonine, and methionine were substantially higher in protein-enriched biomass extract compared to the dried biomass. In comparison to previous reports, these levels were significantly higher, Liestianty *et al.* (2019) reported lysine and methionine contents of 30 mg/g and 14 mg/g, respectively. Threonine and methionine content in the protein-enriched biomass extract were 3.4 and 1.2 times higher than the levels recommended by the FAO guidelines for adults and children (WHO, 2007).

5.4.2.3 Emulsifying capacity and stability of dried biomass and protein-enriched biomass extract

The EC of the biomass was higher than that of the protein-enriched biomass extract (Table 5.3). The values were almost similar to EC results obtained by Bleakley and Hayes (2021) for protein from *Spirulina* sp. (approximately 21%).

Table 5.3: Functional properties of the biomass and protein-enriched biomass extract.

Parameters	Biomass	Protein-enriched biomass
Foaming capacity (%)	46.00 ± 0.92^{b}	29.00 ± 0.75^{a}
Foaming stability (%)	69.00 ± 0.24^{b}	52.00 ± 0.44^{a}
Emulsifying capacity (%)	35.00 ± 0.07^{b}	27.00 ± 0.92^{a}
Emulsifying stability (%)	70.00 ± 0.12^{b}	55.00 ± 0.73^{a}
Water holding capacity (g/mL)	2.78 ± 0.14^{b}	2.21 ± 0.12^{a}
Oil holding capacity (g/mL)	1.54 ± 0.02^{b}	0.98 ± 0.05^{a}
In vitro protein digestibility (%)	55.34 ± 0.18^{a}	68.64 ± 0.12^{b}
Thermal characteristics		
Denaturation temperature (°C)	ND	112°C

All the data are expressed as mean ± SD (n=3). Different superscript within rows indicate significant differences ($p < 0.05$) in the dried biomass and protein enriched biomass.
ND not determined

Although the EC was poor, the biomass had excellent ES (70%). Factors such as the pH, droplet size, net charge, interfacial tension, viscosity and protein conformation have all been shown to affect ES (Hayes and Tiwari, 2015). The biomass showed higher ES (70%), compared to the protein-enriched biomass extract (55%). Hayes and Tiwari (2015) emphasised that increased ES might be the cause for protein dissociation which leads to the formation of subunits with more hydrophobic groups and greater interactions with the lipid phase.

5.4.2.4 Foaming capacity and stability of dried biomass and protein-enriched biomass extract

FC and FS rely on the interfacial coating produced by proteins, which has the ability to keep air bubbles suspended and limit the pace of coalescence (Ma *et al.,* 2011). In addition, foaming properties also depend on other components such as carbohydrates. The biomass had the highest FC (46%) (Table 5.3). Waghmare *et al.* (2016) observed a 95% FC in a protein concentrate derived from the microalgae *Chlorella pyrenoidosa*. This significant foaming ability might be due to the high protein concentration (78.30%) reported in the study. Globular proteins are particularly resistant to surface denaturation, resulting in minimal foam.

The protein-enriched biomass extract from the biomass had the lowest FS (52%). FS is significant since the use of whipping agents is dependent on their capacity to keep the whip in place for an extended length of time. A study by Waghmare *et al.* (2016) reported a FS of 97% after 180 min of storage of *Chlorella pyrenoidosa* protein concentrate. Differences in the FS could be attributed to variations in the surface activeness of the soluble native proteins in the continuous phase (water). Kaur and Singh (2007), states that an excellent FS demonstrates that the natural proteins soluble in the continuous phase (water) are highly surface-active. Protein FC and FS have an effect on both sensory qualities in food formulations, providing the opportunity to increase the smoothness, lightness and palatability of a food product (Ngoc *et al.,* 2012).

5.4.2.5 Water holding capacity and oil holding capacity of dried biomass and protein-enriched biomass extract

The WHC of the biomass was higher than that of the protein-enriched biomass extract (Table 5.3). According to Chavan, McKenzie and Shahidi (2001), the water-binding ability of protein concentrates is due to protein structure, amino acid hydrophilic-hydrophobic balance and other intrinsic properties. WHC is affected by the presence of polar amino acids, reduced levels of water-soluble protein, and non-proteinaceous material in protein concentrates (Kaur and Singh, 2007). The WHC value of the protein-enriched biomass extract in this study was slightly lower than the

value of 3.1 g/mL reported for *Chlorella pyrenoidosa* protein concentrate (Waghmare *et al.,* 2016). WHC within the range of 2.02–2.87 g water/g protein was reported for microalgal proteins isolated from *Chlorella pyrenoidosa, Arthospira platensis* and *Nannochloropsis oceanica* (Chen *et al.,* 2019). Because the WHC reported in this study ranged from 2.21 to 2.78 g water/g protein, the protein may be a viable choice for viscous foods or bakery products (Aletor *et al.,* 2002).

The OHC demonstrates the EC capability of the protein, which is desired in products such as mayonnaise. OHC aids in flavour binding and slows the pace of oxidative rancidity. The presence of multiple non-polar side chains may bond the hydrocarbon chains of fats, resulting in increased oil absorption (Kaur and Singh, 2007). OHC of the biomass and protein-enriched biomass extract is given in Table 5.3. The reported OHC (0.98 g/mL) of the protein-enriched biomass extract was lower than the previously reported values of 1.3-4.1 g/mL for various protein concentrates (Waghmare *et al.,* 2016). The presence of non-polar side groups in the protein concentrate might explain the low OHC value. According to Kinsella and Melachouris (1976), the oil absorption process is due to physical trapping of oil and the binding of fat chains to the many non-polar side groups of proteins. Protein concentrates can be used in a variety of food applications, including meat replacement, meat extenders and sausages.

5.4.2.6 *In vitro* protein digestibility of dried biomass and protein-enriched biomass extract

The IVPD studies of the biomass and protein-enriched biomass were investigated using a pepsin-pancreatin enzyme system. The digestibility of the protein-enriched biomass extract was 68.6% (Table 5.3), slightly higher than that of the biomass (55.3%). Reported IVPD for *Chlorella* is 55-66%, *Spirulina* 70-85% and studies with other species has shown IVPD values between 29-93% (Tibbetts *et al.,* 2015). The IVPD of microalgal proteins varies according to species and season. There are other substances in microalgae, such as phenolic molecules or polysaccharides, that might impair protein digestion, mainly because test materials may be highly heterogeneous. Polysaccharides have been discovered to act similarly to soluble and insoluble fibres. Previous research has revealed that soluble fibres have a severe inhibitory influence on protein digestion (Fleurence, 1999). The lower IVPD results in this study may be related to the rigid cell wall of *Amphora* sp.

5.4.2.7 FTIR spectra of dried biomass and protein-enriched biomass

Based on the wavelength and intensity of the infrared radiation, FTIR spectra were utilized to analyze the chemical structure of dried biomass and protein-enriched biomass extract. The absorption strength of several distinctive peaks differed in the FTIR spectra of both samples. More of the three typical protein bands were observed in the protein-enriched biomass extract. For instance, the spectra of the protein-enriched biomass extract had two broader peaks, corresponding to amide I (1700-1600 cm^{-1}) at 1632 and 1699 cm^{-1} whilst the biomass had only one peak 1636 cm^{-1} (Figure 5.1). Both samples had the same broader peak within the amide II region (1600-1500 cm^{-1}) at 1543 cm^{-1}. Two broader peaks were observed within the amide III region (1200-1400 cm^{-1}) of the protein-enriched biomass extract (1244 and 1367 cm^{-1}) whilst the dried biomass had only one peak (1412 cm^{-1}). The protein enriched biomass had a broader peak within the 3207-3280 cm^{-1} range associated with the strong bending vibration of the -OH group linked to the presence of a polysaccharide and $-NH_2$ of the peptide chains, respectively (Zaleska, Ring and Tomasik, 2001). The band at 656 cm^{-1} observed in the dried biomass could be due to the phosphate group (Bahy, 2005).

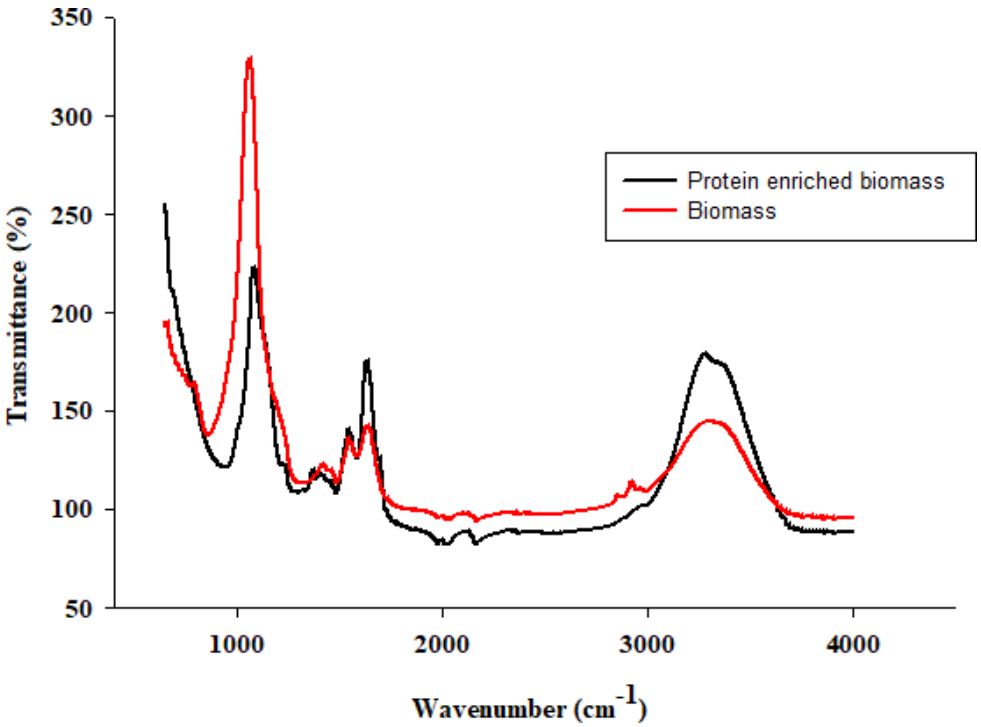


Figure 5.1: FTIR data of the dried biomass and protein-enriched biomass extract

5.4.3 Protein solubility of protein-enriched biomass extract

The solubility of protein-enriched biomass extract was assessed at a pH range of 2-12 and a concentration of 1% (w/v) in water (Figure 5.2). This range was chosen for two reasons: (i) low pH-values generally decrease the solubility of most proteins because acidic amino acids become protonated, resulting in an overall zero net charge (isoelectric point), and (ii) the highest solubilities are frequently observed at high pH to improve the solubility of insoluble proteins (Grossmann *et al.,* 2019).

The solubility of the protein-enriched biomass extract increased with an increase in pH (from pH 2 to 12), thereby suggesting that the pH affected the solubility. These results concur with that of *Phaedodactylum tricornum,* a diatom belonging to the same family as *Amphora* sp., as reported by Grossmann, Hinrichs and Weiss (2019). Furthermore, the trend of the results is in agreement with those recorded in *Spirulina* sp. which increased from 4.99% at pH 4 to 32.44% at pH 10 and 62.99% at pH 12 (Bleakley and Hayes, 2021). In the same study, the solubility ranged between 14.12% (pH 2) and 19.25% (pH 12) for the protein concentrate from an *Isochrysis* sp. strain. The difference in the solubilities is an indication of the variations in solubility between the various microalgae and could as a result of the differences in raw materials, extraction methods, protein isolate concentration and ionic strength (Chen *et al.,* 2019).

Spirulina sp. had a protein content of 85.5%, *Isochrysis* sp. 71.9% and the present study showed a protein content of 22.80% thereby confirming that the protein-enriched biomass extract had an effect on the protein solubility of the microalga. Aside from being a good measure of protein isolate performance, protein solubility is also important in food systems because it influences other functional aspects. Protein solubility is affected by protein fraction composition and protein denaturation in isolates. The presence of native proteins and low denaturation is indicated by high protein solubility (Chen *et al.,* 2019).

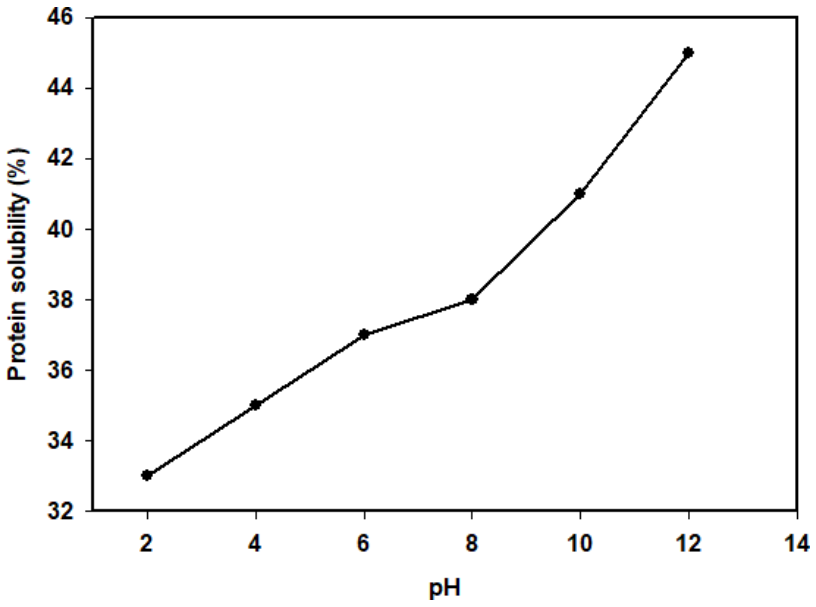


Figure 5.2: Soluble protein content in *Amphora* sp. protein-enriched biomass extract at pH 2 - 12.

5.4.4 Thermal properties of protein-enriched biomass extract

The thermal denaturation of the protein-enriched biomass extract was analysed using the DSC (Figure 5.3). In general, protein thermal stability indicated aggregation resistance and reaction to heating. The denaturation temperature (Td) in this study was 112˚C. Previous research has shown that thermal denaturation temperatures for numerous protein concentrates range between 75 and 95˚C (Ibanoglu, 2005; Ghribi *et al.,* 2015). Differences in denaturation temperature might be attributed to a variety of processes, including initial drying of the biomass, denaturation by the alcohol used for TPP and drying parameters following protein concentrate partitioning. The extraction process, pre-treatments such as heating and the use of solvents have all been documented to have an influence on protein thermal denaturation (Waghmare *et al.,* 2016).

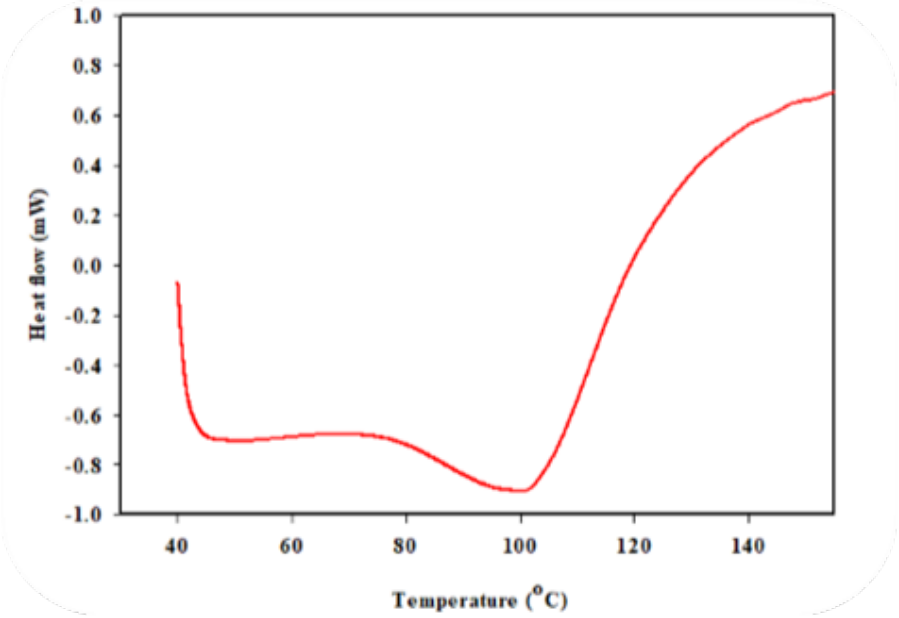


Figure 5.3: DSC heating curve of protein enriched biomass

5.5 Conclusion

The use of microalgae has recently gained attention in the food industry mainly due to the need for valuable, sustainable, unconventional, non-climate-dependent protein sources for food and feed. *Amphora* sp. has the potential to be used as an alternative valuable protein source for humans based on the quality of its protein. The biomass exhibited noteworthy WHC when compared to the protein-enriched biomass. This study demonstrated that microalgal biomass and proteins have potential in food formulations.

Chapter 6: Preliminary Characterisation of Carbohydrates Isolated from *Amphora* sp. and its Cardioprotective Properties on Ferric-Induced Oxidative Cardiac Injury

6.1 Abstract

Microalgae have attracted considerable interest worldwide because of their remarkable potential as a nutrient source. Microalgae have been shown to contain a significant amount of polysaccharides in their cells, including the widely reported β-glucans which are notable for their therapeutic properties. Hence, microalgae are now considered important sources of bioactive natural compounds, thus, a lot of effort has been directed towards the isolation and characterisation of these bioactive polysaccharides from various microalgae. In this study, the β- 1,3-glucan from *Amphora* sp. was characterized and its protective effects on $FeSO_4$-induced cardiotoxicity in rats were investigated. Results revealed that the β-glucan extracted from *Amphora* sp. was a β-type polysaccharide, with a yield of ~9%, and that it was principally made of glucose, rhamnose, and mannose units. Treatment of the challenged tissues with the β-glucan extract depleted previously elevated MDA, NO, low-density lipoprotein (LDL-c), and cholesterol levels. The $FeSO_4$-induced increase in the indicative lipid metabolites were also attenuated by *Amphora* sp. β-glucan. Hence, this work revealed the protective capabilities of *Amphora* sp. β-glucan against ferric-induced cardiac injury as portrayed by its capacity to stall oxidative stress, and modulate cardiac lipid metabolism while inhibiting the acetylcholinesterase and lipase activities, thus highlighting the potential of microalgae in the development of novel functional foods.

6.2 Introduction

Recently, microalgal carbohydrates have attracted considerable attention as renewable carbohydrate sources with potential applications in the food as well as the pharmaceutical industry (Barkallah *et al.,* 2019; Ravindran and Rajauria, 2021). Microalgae produce these carbohydrates through carbon fixation and the photosynthetic processes. However, despite being amongst the most abundantly produced molecules, there is a dearth of information about the structural types and concentrations of microalgal glycans (Becker *et al.,* 2020). Diatoms, for example, have been reported to have carbohydrate contents of between 10 and 70% of their total organic matter. These diatom carbohydrates are mainly polysaccharides that can be classified into three different groups: the storage β (1,3) glucans, the cell wall polysaccharides and extracellular polysaccharides (Granum *et al.,* 2002).

With the ever-growing human population, there is an urgent need to develop foods with the propensity to improve human health by lowering the risk of chronic diseases, promote lifespan, improve immunity to infections, or possibly reduce the aging process. β-glucans have been widely reported to improve human health and nutrition (Wang *et al.,* 2017), particularly, β-1,3- glucans from microalgae are useful in this regard (Schulze *et a*l., 2016). These microalgal β- glucans are of interest because of their various inherent bioactivities which include but are not limited to antioxidative, antibacterial, anti-diabetic, anti-tumour, anti-hypertensive, anti-hypercholesterolemic and immunomodulatory effects (Murphy *et al.,* 2020). Furthermore, glucans from diatoms such as *Synedra acus* was revealed to inhibit the proliferation of colon tumour cells in humans (Kusaikin *et al.,* 2010). Similarly, *Odontella aurita* derived glucans showed remarkable antioxidant activity therefore highlighting their potential in aquaculture, food, as well as in pharmaceutical industries (Xia *et al.,* 2014).

Generally, the economic relevance of β-glucans has increased due to their potential in the pharmaceutical industry as health foods and dietary products (Vo, Ngo and Kim, 2012). For example, their use as nutraceuticals resulted in reduced cholesterol levels (Caz *et al.,* 2015; Ho *et al.,* 2016), hence, they are being given attention as one of the ideal soluble fibres for nutritional supplementation with the aim of improved health. Their application as therapeutics against cardiovascular diseases has since raised a lot of scientific attention (Chen and Raymond, 2008). Cardiovascular dysfunction, which ultimately leads to cardiac or heart failure, has remained a global health issue that spans different ages, gender, race, social class and regions. Several factors have been shown to occur recurrently in the pathogenesis of cardiovascular diseases.

These include oxidative imbalance, lipotoxicity, aggravated angiotensin-converting enzyme (ACE) activity, as well as disrupted bioenergetics. Recent studies revealed that mixed-linked β-glucans such as the (1 → 3) (1 → 6)-β-D-glucans - fungal, as well as the (1 → 3) (1 → 4)-β-D-glucans-cereal, have demonstrated cardiovascular protective effects (Wouk *et al.,* 2021).

In light of the above, this study was designed to characterise the β-glucans from *Amphora* sp., and to investigate its protective effects against $FeSO_4$-induced oxidative cardiac damage. This was accomplished by using biochemical approaches, such as the measurement of antioxidant activities, cardiac metabolic profiling and pathway metabolic analysis.

6.3 Materials and Methods

6.3.1 Materials

The research was conducted in accordance with the Animal Ethics Committee of the UKZN in Durban, South Africa (Protocol approval number: AREC/021/01 cardiac tissue lysate 8D). Three male Wistar rats were obtained from the UKZN BRU, Westville Campus, Durban, South Africa, for the cardiotoxicity study. The rats were euthanised with isofor the same day and sacrificed. To eliminate blood stains, the hearts were extracted and washed in a NaCl solution (0.9%) and homogenized in a 50 mM sodium phosphate buffer at pH 7. The homogenates were centrifuged (Eppendorf 5810R, Germany) at 15,000 x g for 10 min at 4˚C. *Ex vivo* experiments were conducted using the lysates (supernatants).

6.3.2 Extraction and purification of *Amphora* sp. cell wall polysaccharide

The cell-wall polysaccharide was isolated using the method described in Ford and Percival (1965). Soxhlet extraction with ethanol was used to extract pigments and lipids for period of 4 days. The β-glucans were dissolved in water at 80˚C for 2 h, and thereafter centrifuged (Eppendorf 5810R, Germany) at 6000 x g for 15 min. The remaining insoluble material was bleached at 80˚C for 3 h using 200 mL of chlorite solution, 200 mL of acetate buffer and 1 L of deionised water. The material was then centrifuged (Eppendorf 5810R, Germany) with 1% NaOH and left in the alkaline solution for 1 h with agitation. Three bleaching and alkaline treatments were performed. The cell wall polysaccharide was dissolved in 4% NaOH containing 20 mM sodium borohydride for 45 min at 100˚C. After dialysis against distilled water (3.5 - 13 kDa cut off), the recovered cell-wall polysaccharide was freeze-dried (Le Costaouëc *et al.,* 2017). Finally, the recovered polysaccharide was lyophilized to produce a slightly white powdered polysaccharide extract for future investigation.

Following the manufacturer's instructions, the β-Glucan assay kit (K-YBGL 04/08, Megazyme, Ireland) was used to determine the β-glucan content in accordance with the manufacturer's instructions. To solubilize the glucans, the polysaccharide extract was placed in 12 M H_2SO_4 at -4˚C for 2 h. The extracts were hydrolysed in 2 M H_2SO_4 for 2 h at 100˚C. Following incubation, any leftover glucan fragments were quantitatively hydrolysed to glucose using a combination of exo-1,3-β glucanase and β-glucosidase, yielding a total glucan measurement. A glucose oxidase peroxidase reagent was used to detect glucose using amyloglucosidase and invertase. The difference in each value was used to calculate β-glucan.

6.3.3 Monosaccharide profile of β-glucan extract

The hydrolysed extract was diluted in 1 mL of distilled water, and monosaccharide analysis was carried out using a Shimadzu UHPLC LC-2010 C system (Shimadzu, Kyoto, Japan), which was equipped with a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Li *et al.* 2021). Isocratic separation using a mobile phase of water-acetonitrile (80:20 v/v) was carried out at 50˚C for 15 min with a flow rate of 0.5 mL/min and an injection volume of 10 µL. Shimadzu Evaporative Light Scattering Detector (ELSD) (Shimadzu, Kyoto, Japan) was used to monitor the elution. For monosaccharide quantification of the β-glucan extract, the peak areas were compared to the standard calibration curves. Sugar standards used were glucose, galactose, mannose, rhamnose, arabinose, xylose, galacturonic acid and glucuronic acid.

6.3.4 FTIR determination

Infrared spectra were acquired using a FTIR spectrometer (Perkin Elmer model Spectrum 400, Massachusetts, USA), fitted with a diamond crystal ATR (Waltham, MA, USA). The spectra of the β-glucan extract were acquired from the scan range 380–4000 cm^{-1} with a resolution of 1 cm^{-1}. Data were analysed using Perkin Elmer Spectrum software.

6.3.5 Induction of oxidative cardiac injury

The hearts of three male Wistar rats were harvested after being euthanised with isofor and rinsed in NaCl solution (0.9%). Subsequently, the organs were homogenised in a 0.05 M phosphate buffer at pH 7.5 and centrifuged at 10 000 x g at 4°C for 15 min. The lysates were collected and stored until further use at 4°C. The lysate (100 µL) was incubated with 30 µl of pro-oxidant (0.1 mM $FeSO_4$) and then with varying concentrations of the β-glucan extract (30, 60, 120, and 240 µg/mL) for 30 min at 37˚C (Erukainure *et al.* 2017). Lysates without the extract and $FeSO_4$ represented the normal control, and the negative control was the reaction mixture devoid of the β-glucan extract.

6.3.6 Assessment of cardiac antioxidant status

6.3.6.1 Determination of antioxidative activities

Ellman's method (Ellman, 1959) was used to determine the GSH level in the cardiac tissues. The tissues were deproteinised with 10% TCA and then centrifuged at 3500 x g for 5 min. The supernatants were transferred to a 96-well plate, followed by addition of 50 µL of Ellman's reagent. The absorbance of the tissues was measured at 415 nm. The lipid peroxidation content which was represented as MDA level was measured using thiobarbituric acid reactive substances (Chowdhury and Soulsby, 2002). Aliquots of 75 µL of 20% acetic acid, 100 µL of 8.1% SDS solution, and 1 mL of 0.25% thiobarbituric acid was combined with the supernatants and boiled for 1 h before being transferred to a 96-well plate. The absorbance was measured at 532 nm.

A previously established procedure was used to evaluate the catalase activity of the cardiac tissues (Aebi, 1984). Cardiac tissues were treated with 50 mM sodium phosphate buffer at pH 7.0 and incubated with 2M hydrogen peroxide for 5 min. The absorbance was measured at 240 nm at 1 min intervals for 3 min. The SOD activity based on the oxidation of 6-hydroxydopamine by hydrogen peroxide from $O_2^{\cdot-}$ dismutation (Gee and Davison, 1989). Cardiac tissues (15 µL) were combined with 170 µL of 0.1 mM diethylenetriaminepentaacetic acid and transferred to a 96-well plate. The reaction mixture was then treated with 15 µL of 1.6 mM 6-HD and the absorbance was measured at 492 nm every 1 min for 5 min.

6.3.6.2 Determination of NO level

A modified Griess technique was used to determine the NO levels in the lysates (Ghozali *et al.,* 2021). The Griess reagent was made by combining equal amounts of 2% sulfanilamide in 5% HCl and 0.1% N1-(1-naphthyl) and N2-diethylethylenediamine oxalate in water. A total of 20 µL of cardiac tissues were combined with 100 µL of Griess reagent and water. After 5 min of stirring, the absorbance at 540 nm was measured.

6.3.6.3 Determination of angiotensin-converting enzyme activity

This was carried out spectrophotometrically with N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as a substrate (Holmquist, Bünning and Riordan, 1979). Briefly, 50 µL of the cardiac tissues were incubated with 500 µL of 0.5 mM FAPGG at 37˚C for 10 min. Absorbance was read at 345 nm at 2 min intervals.

6.3.6.4 Determination of acetylcholinesterase activities

Acetylcholinesterase activity was measured using a colorimetric technique to detect the cholinergic state of cardiac tissue lysates (Ellman *et al.,* 1961). The reaction mixture was made using 2 L of 100 mM phosphate butter pH 8.0, 100 mL of cardiac lysate stock solution in methanol (a final concentration of 42.5 µg/L), 100 mL of enzyme AChE solution at a final concentration of 0.03 U/mL, and 100 mL of DTNB (0.3 mM) prepared in 100 mM phosphate buffer pH 7.0 that contained 120 mM sodium bicarbonate. The mixture was vortexed and then incubated for 30 min at 37˚C. ATCI (0.5 mM) served as the negative control. The change in absorbance at 412 nm was measured at room temperature for 5 min.

6.3.6.5 Determination of purinergic enzymes activities

The enzyme activities of adenylpyrophosphatase (ATPase) of the tissue lysates was determined according to Erukainure *et al.* (2017). A 100 µL of cardiac tissue supernatant was added to a mixture of 100 µL of 5 mM KCl, 650 µL of 0.1 M Tris-HCl buffer, and 20 µL of 50 mM ATP. The reaction mixture was incubated for 30 min in a shaker at 37˚C. Then, 500 µL of distilled water and 1.25% ammonium molybdate were added to terminate the reaction. A 500 µL of a freshly prepared 9% ascorbic acid was then added to the mixture and allowed to stand for 30 min. Absorbance was measured at 660 nm.

The ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase) was determined in the tissue lysates according to Akomolafe *et al.* (2017). A 20 µl of tissue lysate was incubated for 10 min with 200 µL of the reaction buffer containing a mixture of 1.5 mM $CaCl_2$, 5mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl at 37˚C. After which 20 µL of 50 mM ATP was added to the reaction mixture and further incubated for 20 min in a shaker at 37°C. The reaction was terminated with 200 µL of 10% TCA. A 200 µL of 1.25% ammonium molybdate and freshly prepared 9% ascorbic acid was then added and allowed to stand for 10 min on ice. Absorbance was measured at 600 nm.

6.3.6.6 Determination of lipase activity

Lipase activity was evaluated as described by Ullah *et al.* (2022). In brief, cardiac tissue lysates (100 µL) were treated with 190 µL of Tris buffer at 37˚C for 15 min. Subsequently, 10 µL of p-nitrophenyl butyrate (10 mM) was added and further incubated for 15 min at the same temperature. The absorbance was read at 405 nm at 1 min intervals.

6.4 Cardiac lipid profile

The cardiac lysates in addition to $FeSO_4$ (0.1 mM) and β-glucan extract or commercial β-glucan (240 µg/mL) were incubated for 12 h, and thereafter centrifuged at 12000 x g for 20 min at 4˚C. Subsequently, the lipid profile as well as the total cholesterol, HDL-cholesterol and triglycerides levels of the supernatants were determined using Randox kits (Midrand, South Africa) according to the manufacturer's guidelines.

6.5 Metabolite extraction and GC-MS analysis

Lipid metabolites were isolated according to Ralston-Hooper *et al.* (2011). The chloroform phase was analysed using GC-MS (Agilent Technologies 6890). The operating parameters were set as outlined by Salau *et al.* (2020). The metabolites were detected using an inbuilt US National Institute of Standards and Technology database.

6.6 Metabolic pathway analysis

The enumerated lipid metabolites were subjected to MetaboAnalyst 5.0 facilitated pathway enrichment to highlight the most relevant metabolic pathways (Zhao *et al.,* 2022).

6.7 Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA) and given as mean standard deviation (SD). Tukey's HSD-multiple range post-hoc test was employed to determine if there were significant differences between means ($p<0.05$). The IBM Statistical Package for Social Sciences (SPSS) for Windows, version 23.0, was used for statistical analyses (IBM Corp., Armonk, NY, USA).

6.8 Results and Discussion

6.8.1 *Amphora* sp. β-glucan yield

The β-glucan content of the *Amphora* sp. biomass was found to be 8.97%, which is in line with the previous findings of Schulze *et al.* (2016). A study by Schulze *et al.* (2016) had shown that 31 microalgae have β-glucan contents ranging between 5 and 10% DW. In addition, the β- glucan content of *Amphora* sp. was significantly higher than in most cereals such as oats (8%), rye (2.7%), maize (1.7%), wheat (1.0%) and rice (0.13%) (Bacic, Fincher and Stone, 2009). One of the most important factors in microalgal cultivation is light, as it influences the growth and the accumulation of primary metabolites in the biomass. Ho, Chen and Chang (2012) reported that

elevated irradiances resulted in an increase in lipids and carbohydrates. Similarly, *S. obliquus* SAG 276-7 (currently *Acutodesmus obliquus*), showed an increase in carbohydrate output when there was an increase in irradiance from 50 to 200 µmol photons m^2/s (Gris *et al*., 2014). According to Markou, Angelidaki and Georgakakis (2012) and Schulze *et al.* (2016), modifying culture conditions to optimize carbohydrate synthesis resulted in an increase in total glucan content as well as an increase in the number of β-glucans in microalgae biomass.

6.8.2 Monosaccharide profile of *Amphora* sp. β-glucan extract

The monosaccharide composition of the polysaccharide extract of *Amphora* sp. is displayed in Figure 6.1. The results showed glucose as the major component (50%) followed by rhamnose (28%) and mannose (22%). Glucans from diatoms *Halamphora* and *Odontella aurita* were described to be mainly composed of glucose (Xia *et al.,* 2014; Daglio *et al.,* 2018). However, the isolated glucan from the model diatom *Phaeodactylum tricornutum* was entirely composed of glucose (Caballero *et al.,* 2016). The significant presence of glucose and mannose is also in agreement with earlier phylogenetic reports for diatoms (Templeton *et al.,* 2012). Furthermore, the higher amount of glucose in the *Amphora* sp. polysaccharide is most probably a result of the presence of chrysolaminarin, which is a well-characterized reserve carbohydrate in diatoms that is made up of monomeric glucose units (Caballero *et al.,* 2016).

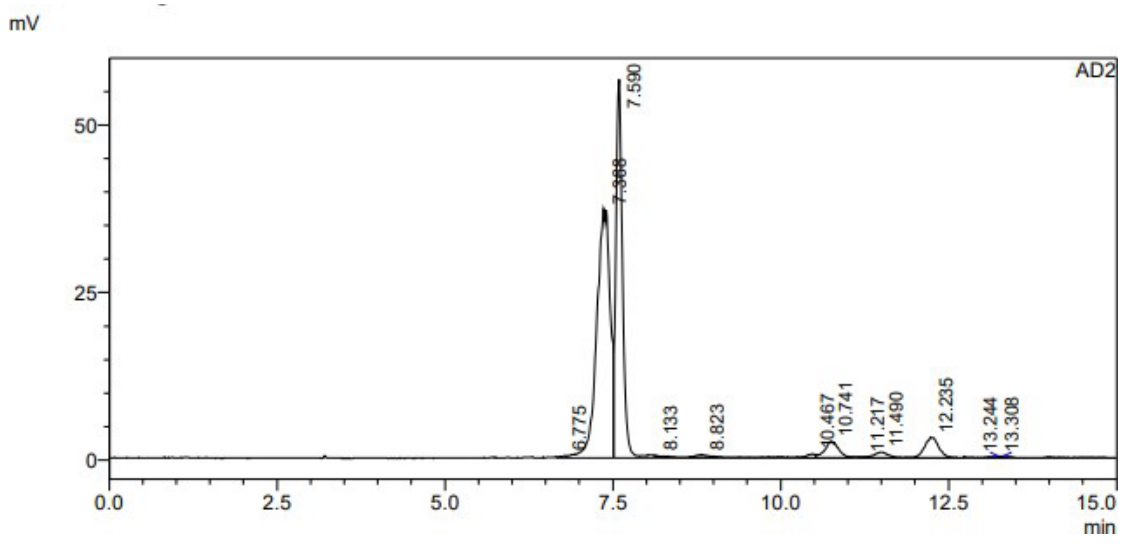


Figure 6.1: HPLC chromatograph of monosaccharide profile for purified *Amphora* sp.

6.8.3 FTIR assay

The structural composition of the *Amphora* sp. glucan extract was characterised using FTIR spectroscopy between 4000 and 380 cm^{-1} as shown in Figure 6.2. The first polysaccharide

absorption peak was recorded at 3411.27 cm^{-1}, which correlates to the O-H groups stretching vibration (Wang *et al.,* 2012) while the 2946.85 cm^{-1} peak was due to vibration of C-H (You *et al.,* 2014). Carboxyl groups were recorded to be present because of the stretching absorption peak at 1367.63 cm^{-1} (Zhang *et al.,* 2012). The absorption peak observed at 1710.63 cm^{-1} corresponds to ester C=O bond formations (Machana *et al.,* 2020). The prominent peak at 1078.62 cm^{-1} is a resultant effect of vibrations from the pyranose rings present (Barker *et al.,* 1954), that are lapped over with stretching vibrations of C-O glycosidic band vibration (Zou *et al.,* 2010; Machana *et al.,* 2020). The characteristic peak observed at 889.15 cm^{-1} was because of β-glycosidic linkage, suggesting that the *Amphora* sp. had a β-type polysaccharide (Zhang *et al.,* 2012).

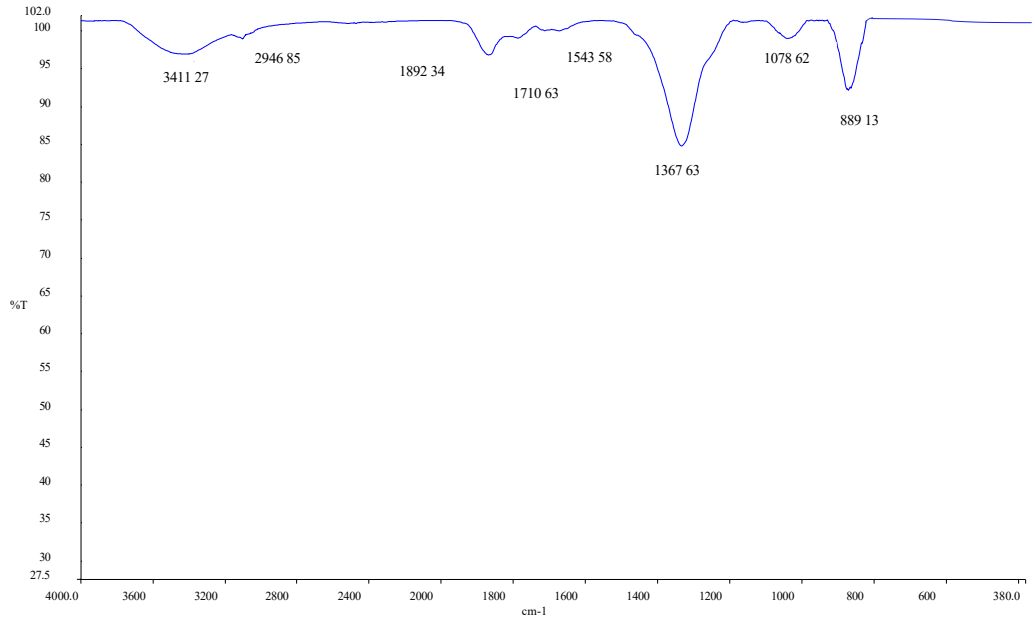
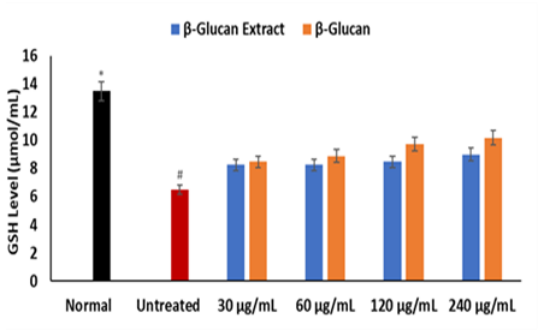


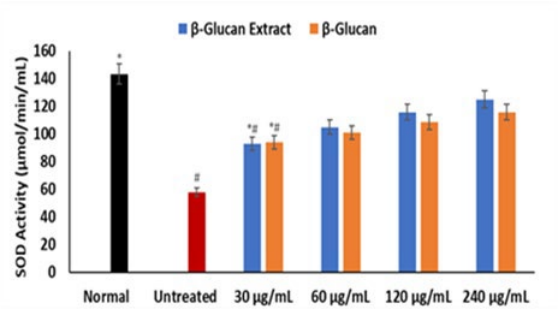
Figure 6.2: FTIR spectra of purified polysaccharides from *Amphora* sp.

6.8.4 Cardiac antioxidant status

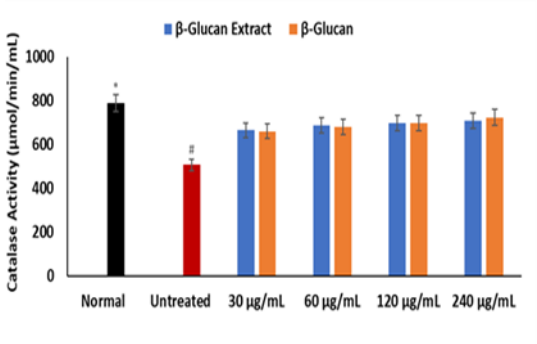
The $FeSO_4$ –induced cardiac injury was observed to cause a significant depletion of the GSH level, reductions in the SOD, and catalase activities, as well as elevation of the MDA level ($p<0.05$) (Figures 6.3A–D). Apart from MDA, the quantities and activities of the biochemical biomarkers are representative of the occurrence of oxidative stress and corroborate in oxidative- mediated cardiovascular damage (Mansouri *et al.,* 2020).



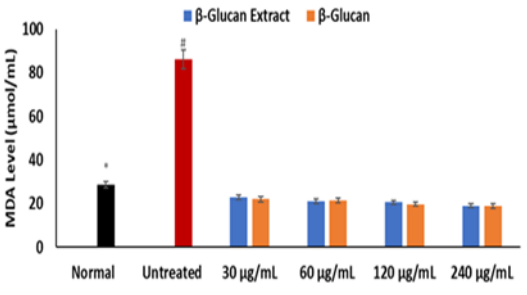
(A)



(B)



(C)



(D)

Figure 6.3 Effect of β-glucan extract on (A) GSH level, (B) SOD, (C) catalase and (D) MDA level in oxidative cardiopathy. Data represented as a mean ± SD (n = 3). *Statistically significant compared to untreated tissue lysates; #statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

The toxicity of $FeSO_4$ in the cardiac tissues can be ascribed to Fe-catalysed Haber Weiss and Fenton's reactions which eventually caused an increased production of superoxide (O_2^-) (Latunde-Dada, 2017). The subsequent treatment with *Amphora* sp. β-glucan extract resulted in a significantly increased catalase and SOD activities ($p<0.05$), with the concomitant depletion of the level of MDA. The reversion of the induced states depicts the notable antioxidative effect of the extract as was observed in many studies including recent ones by Patel, Atar and Ali (2019) and Moutabian *et al.* (2022). The depleted MDA level specifically indicates an anti-peroxidative activity. Thus, suggesting the tendency of the β-glucan to halt oxidative stress in oxidative cardiac injury. However, the β-glucan extract treatment did not significantly change the previous GSH level.

The induction of oxidative injury caused a significant rise in cardiac NO level ($p<0.05$) as highlighted in Figure 6.4. This is mainly due to the fact that exacerbated O_2^- levels, resulting

from reduced SOD activity, convert NO to peroxynitrite (ONOO−), a potent radical that has been well-reported for its pro-inflammatory activities (Pérez de la Lastra *et al.,* 2022) The treatment of the lysates with the extracted β-glucan was observed to deplete the NO level, thus, indicating the anti-proinflammatory potential of the extract as recently shown by Iqbal *et al.* (2022).

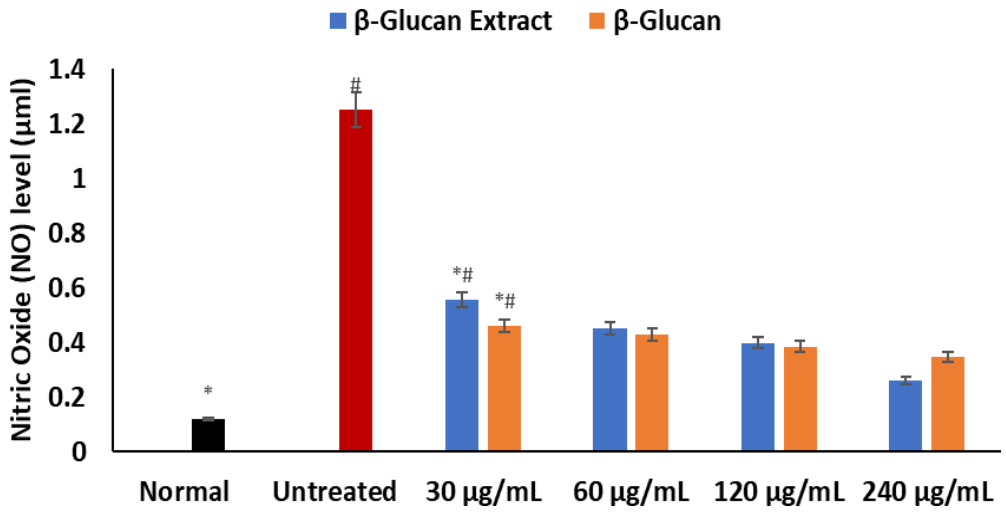


Figure 6.4: Effect of β-glucan extract on NO level in oxidative cardiopathy. Data represented as a mean ± SD (n = 3). *Statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

Similarly, there was a remarkable elevation in ACE activity following the induction of tissue damage in the cardiac tissues ($p<0.05$) (Figure 6.5). Exacerbated ACE activity is related to the onset and progression of cardiovascular dysfunction (Nikolaidis *et al.,* 2002; Deshmukh *et al.,* 2004). The increased ACE activity also demonstrates an occurrence of oxidative injury, as recently described by Aziz *et al.* (2022). The declined activity of ACE recorded on the treatment with the extracted β-glucan depicts a therapeutic effect of the extract in the management of the oxidative exacerbated cardiac ACE activity. This validates previous findings on the application of natural products as therapeutics for cardiovascular diseases (Nikolaidis *et al.,* 2002; Agunloye *et al.,* 2019).

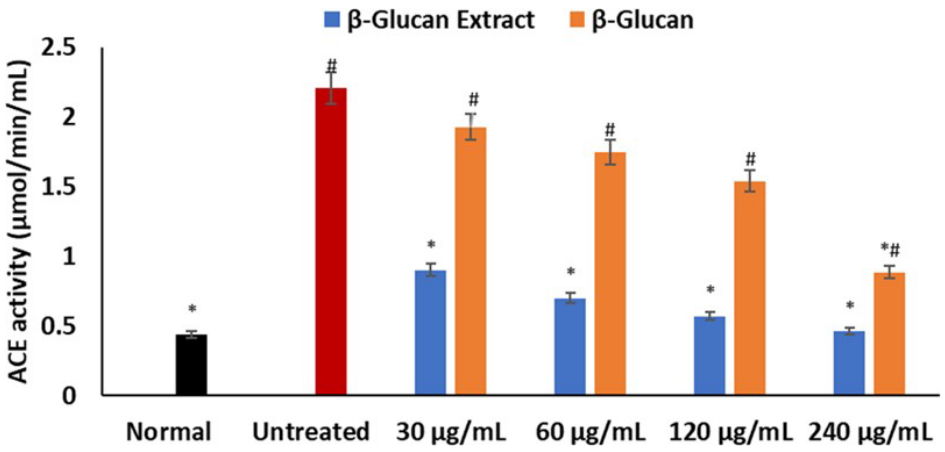


Figure 6.5: Effect of β-glucan extract on ACE activity in oxidative cardiopathy. Data represented as a mean ± SD (n = 3). *Statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

Cholinergic dysfunction was deduced in the tissues judging from significant elevation of cardiac acetylcholinesterase activity ($p<0.05$), as shown in Figure 6.6. Cholinergic dysfunction characterized by exacerbated activities of acetylcholinesterase is well linked with the pathophysiology of cardiovascular diseases. Acetylcholinesterase catalyses the conversion of acetylcholine to acetate and choline, thereby reducing acetylcholine's availability for cholinergic induced vasodilation (Agunloye *et al.,* 2019). Treatment with the β-glucan extract significantly depleted the activity ($p<0.05$), consequently depicting the ability of the extract to improve cholinergic function. Thus, corroborating previous findings on the use of acetylcholinesterase inhibitors derived from natural sources as therapeutics for cardiovascular diseases (Estrada-Nieto *et al.,* 2021).

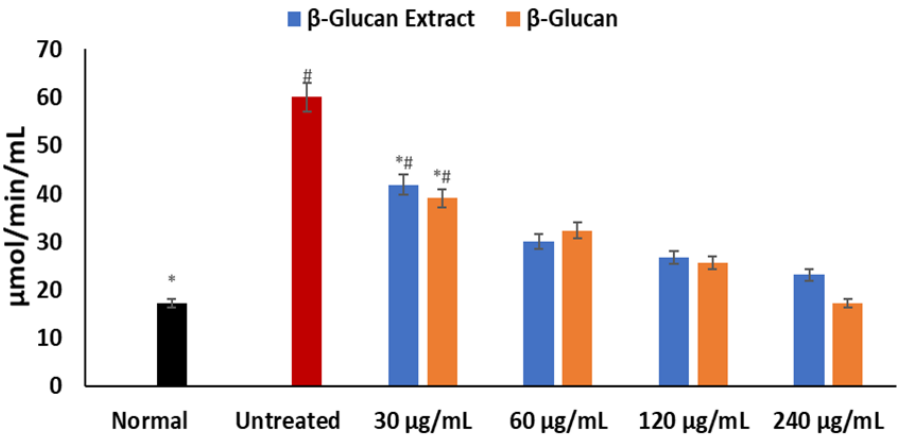


Figure 6.6: Effect of β-glucan extract on acetylcholinesterase activity in oxidative cardiopathy. Data represented as a mean ± SD (n = 3). *Statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

As shown in Figures 6.7A and 6.7B, the cardiac purinergic activities were significantly altered on induction of oxidative stress as given the observed elevated and depleted activities of ATPase and ENTPDase, respectively ($p<0.05$). These alterations are known to be related to depleted levels of adenosine and ATP. ATP has been shown to possess mitigative effects on hypertension by facilitating vasodilation via the action of the P2 receptors to release NO (Burnstock, 2017). While adenosine has been demonstrated as a potent coronary vasodilator (Burnstock and Ralevic, 2014; Burnstock, 2017), there was a dose-dependent decrease in ATPase as well as an increase in ENTPDase activities on treatment with the β-glucan extract. Thus, signifying the property of the extract to modulate altered purinergic activities in oxidative mediated cardiovascular dysfunction.

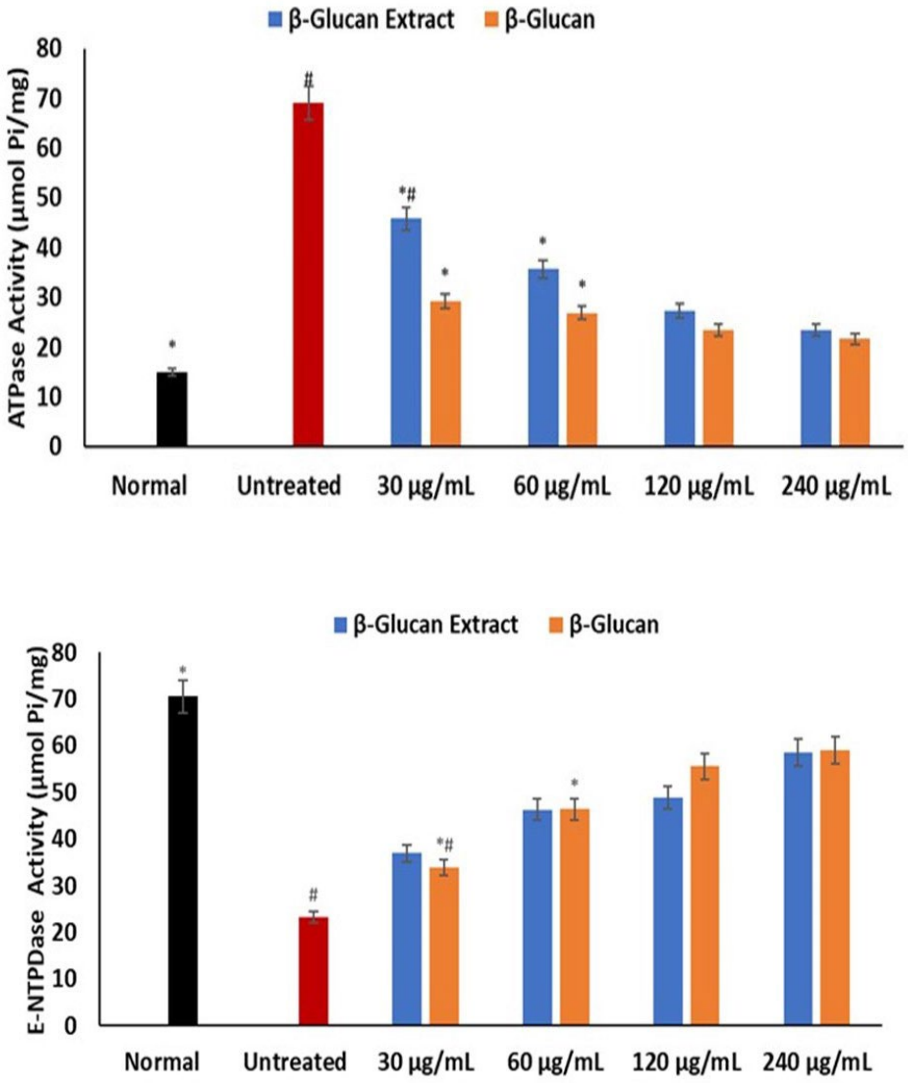


Figure 6.7: Effect of β-glucan extract on ATPase and ENTPDase activities in oxidative cardiopathy. Data represented as a mean ± SD (n = 3). *Statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

An increase in the lipase activity was recorded in the tissue's sequel to the induction of oxidative damage (Figure 6.8), which agrees with previous research on elevated lipolytic activities in cardiovascular dysfunctions leading to altered cardiac levels of fatty acid as well as its utilization (Swamy *et al.,* 2013).

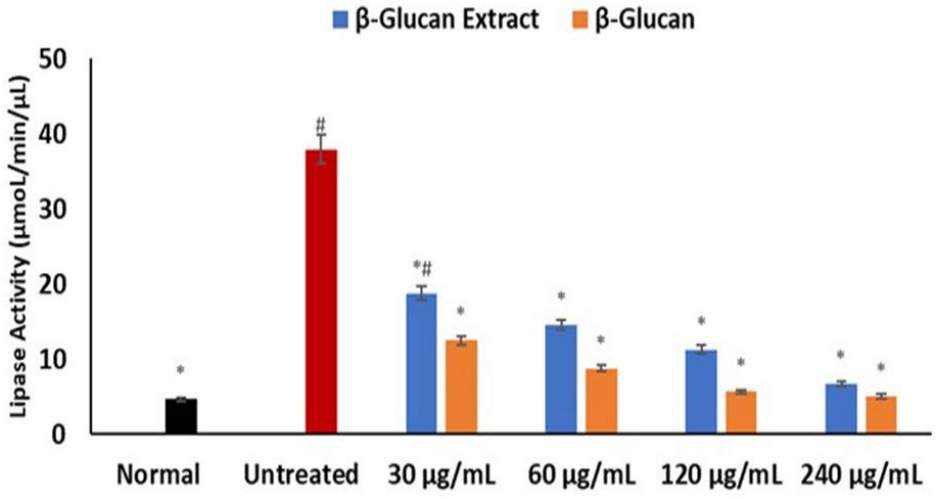


Figure 6.8: Effect of β-glucan extract on lipase activity in oxidative cardiomyopathy. Data = mean ± SD; n = 3. *Statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

β-glucan extract administration resulted in a remarkable decline in the activity of lipase ($p<0.05$), thereby indicating the mitigating capabilities of the extract on lipotoxic activities observed in cardiovascular dysfunction.

6.8.5 Cardiac lipid profile

Significant increase in cardiac cholesterol and LDL-c values were recorded following the oxidative injury in the lysates, with concomitant depletion in HDL-c and triglycerides as described in Figure 6.9. Thus, observations are pointers to the fact that disruption of the cardiac lipid spectrum is associated with the pathogenesis and the progression of cardiovascular dysfunctions (Poulter, 2003). The roles of cholesterol and LDL-c in coronary vasoconstriction when in high concentrations have been well-reported (Mayala *et al.,* 2019). The depletion in triglyceride level sequel to oxidative injury is linked with the elevated activity of the lipase enzyme. Treatment with the β-glucan (240 µg/mL) significantly reversed the lysates' lipid spectrum as demonstrated by the reduction in the levels of LDL-c and cholesterol, as well as the simultaneous elevation of triglycerides and HDL-c in the lysates.

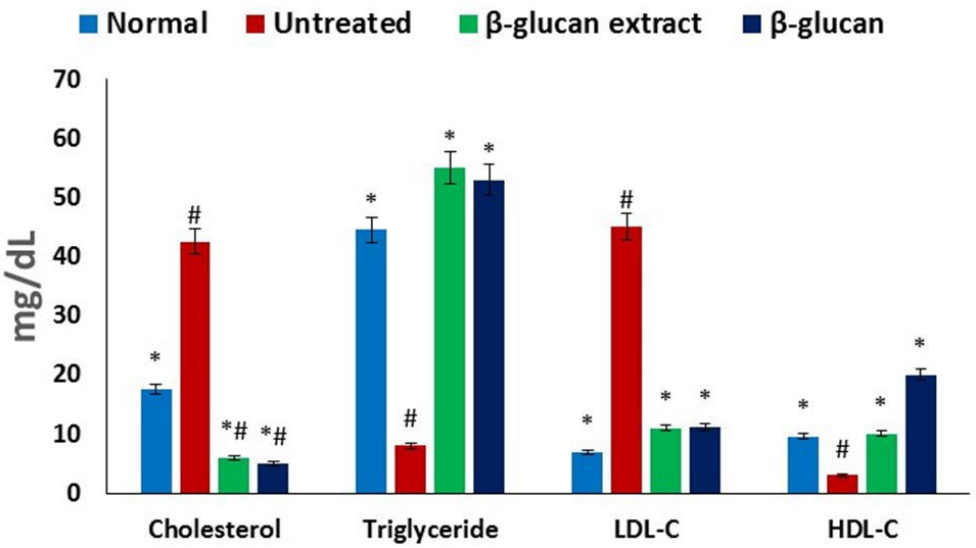


Figure 6.9: Effect of β-glucan extract on lipid profile in oxidative cardiomyopathy. Data = mean ± SD; n=3. *Statistically significant compared to untreated tissue lysates; #statistically significant compared to normal tissue lysates.

6.8.6 Lipid metabolite profiling

The analyses of the lipid metabolites present in the lysates showed complete depletion in the amount of 2,6,10,15-tetramethylheptadecane, cis-9-tricosene, pentadecanoic acid, cis,cis- linoleic acid, cis-10-heptadecenoic acid, ethyl palmitate, glycyl-L-tryptophylglycine, 1-O-(22-Hydroxydocosyl)-d-mannitol and retinol in response to $FeSO_4$-induced oxidative damage. The concomitant generation of spinacene, palmitic acid, (6Z)-6-octadecenoic acid, arachic acid, palmitic acid.beta.-monoglyceride, etc was recorded (Table 6.1). It also revealed an elevation of cholesterol levels. These alterations in the lipid metabolites insinuate disturbances in cardiac lipid metabolism following oxidative damage in the tissues. The disturbances observed in this study have been implicated in defects in cardiac energy metabolism, which is a major mechanism of cardiovascular dysfunctions (Olorundare *et al.,* 2020; Wang *et al.,* 2021). The generated lipid profiles, especially the SFAs and elevated cholesterol may insinuate lipotoxicity, as their high concentrations have been associated with coronary vasoconstrictions (Dow *et al.,* 2014).

Table 6.1: GC-MS identified fatty acid metabolites in experimental cardiac tissues.

Lipids	Metabolites	Normal	Untreated	β-glucan extract	β-glucan
Fatty alkanes	Eicosane	2.35	0.39	ND	5
Fatty acyls	Tetradecane	1.81	4.35	1.38	2.47
	2,6,10,15-Tetramethylheptadecane	3.99	ND	4.13	0.13
	cis-9-Tricosene	0.32	ND	ND	ND
	Spinacene	ND	0.24	ND	ND
	Pentadecane	ND	ND	1.87	ND
	2,4-Dimethylicosane	ND	ND	ND	0.11
Fatty alcohol	Pentadecanol	0.40	0.51	0.42	0.33
	n-Heptadecanol-1	0.92	0.83	1.06	0.62
	6-Tridecanol	ND	ND	ND	0.13
Fatty acid	Pentadecanoic acid	6.27	ND	5.43	3.04
	cis,cis-Linoleic acid	3.67	ND	3.82	0.86
	Palmitoleic acid	2.01	2.78	ND	ND
	cis-10-Heptadecenoic acid	0.97	ND	ND	ND
	Octadecanoic acid	5.29	0.51	4.68	0.23
	Arachidonic acid	0.88	1.22	0.65	ND
	Palmitic acid	ND	6.73	ND	ND
	(6Z)-6-Octadecenoic acid	ND	1.63	2.03	ND
	Arachic acid	ND	5.4	ND	ND
	Octadec-9-enoic acid	ND	ND	0.94	ND
	trans-2-Dodecenoic acid	ND	ND	ND	0.11
Fatty acid ester	Ethyl palmitate	1.51	ND	1.63	1.21
	Ethyl pentadecanoate	3.38	1.31	3.49	1.98
	cis-11,14-Eicosadienoic acid, methyl ester	ND	4.66	ND	ND
	Ethyl tridecanoate	ND	ND	ND	0.18
Glycerol	Palmitic acid .beta.-monoglyceride	ND	0.53	ND	ND

Glycol	Glycyl-L-tryptophylglycine	1.41	ND	ND	0.25
	1-O-(22-Hydroxydocosyl)-d-mannitol	6.06	ND	ND	ND
Steroid	Cholesterol	2.9	3.36	2.36	0.55
	14-Methylcholest-7-en-3-ol	ND	ND	0.51	ND
	2-(Cholest-5-en-3-yloxy)ethyl acetate	ND	ND	ND	0.15
	25-Hydroxycholesterol, dimethyl ether	ND	ND	ND	3.95
Non-fatty acids	Retinal	0.15	ND	ND	ND

The complete depletion of cis-11,14-eicosadienoic acid, spinacene, palmitic acid, arachic acid, methyl ester and palmitic acid.beta.-monoglyceride, with the concomitant restoration of 2,6,10,15-tetramethylheptadecane, pentadecanoic acid, cis,cis-linoleic acid and ethyl palmitate on treatment with the β-glucan extract, may depict a protective effect of the *Amphora* sp. β- glucan against oxidative mediated lipid disturbances in the cardiac tissues.

The oxidative species-mediated cardiac damage led to the disruption of the retinol metabolism pathway (Figure 6.10), with concomitantly activating the metabolism of glycerolipid, the extension of FA in the mitochondria, the biosynthesis of FA, as well as other relevant FA metabolic pathways. Activation of these pathways insinuates an increased lipid metabolism and may be an indication of energy dysmetabolism.

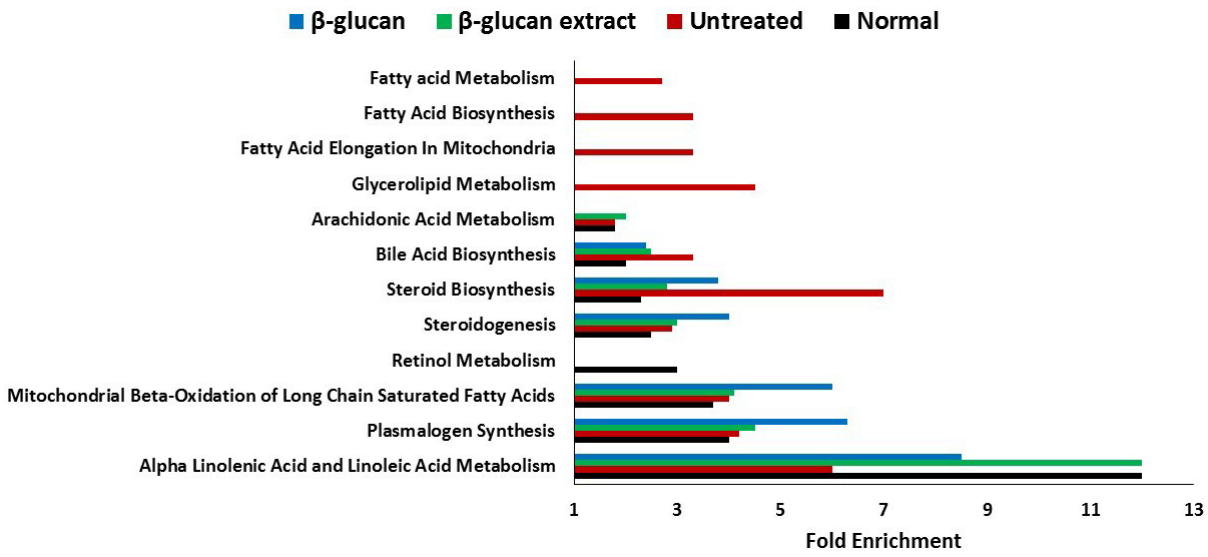


Figure 6.10: Fold enrichment of identified lipid metabolic pathways.

The heart has been reported to be dependent on oxidative phosphorylation and β-oxidation for energy metabolism (Antozzi and Zeviani, 1997). The activation of the specific pathways also insinuates an increased generation of NADH and $FADH_2$, which are the major electron donors into the respiratory chain to produce energy (ATP) (Marín-García and Goldenthal, 2002). However, the exacerbated production of NADH and $FADH_2$ from the activated lipid metabolic pathways can cause an undesirable increase in the membrane potential of the mitochondria (Dambrova *et al.,* 2021). This in turn would block electron transport at complex II, while oxygen (O_2) undergoes reduction to superoxide (O_2^-), resulting in oxidative stress (Brownlee, 2001).

6.9 Conclusion

The β-glucan content from *Amphora* sp. presented a low value of approximately 9%, with a monosaccharide composition consisting of glucose, rhamnose, and mannose. The purified β-glucan was determined as a β-type polysaccharide. These results, when considered together, indicate the protective potentials of the *Amphora* sp. β-glucan extract against $FeSO_4$-induced cardiac injury as portrayed by the capacity to delay oxidative damage, inhibit lipase and acetylcholinesterase enzyme activities while regulating cardiac lipid metabolism. However, additional pre-clinical and clinical studies are required to substantiate these highlighted potentials.

Chapter 7: General Discussion

Microalgae have attracted the interest of researchers, companies, and governments all over the world due to their capacity to create a diverse variety of essential compounds with several potential applications. Furthermore, diverse microalgae serve as unexplored resources for food applications. Only a few microalgae, such as *Dunaliella*, *Spirulina*, *Chlorella*, and *Haematococcu*s, have been commercially grown so far (Borowitzka, 2013). Bioprospecting and screening of prospective novel species for the production of certain compounds are thus a key part of any future microalgae enterprise.

This chapter provides a comprehensive discussion of this research, which includes the safety assessment of *Amphora* sp., its biochemical composition and protein characterisation as well as its characterisation and application of carbohydrates.

7.1 The biochemical composition and safety assessment of *Amphora* sp.

Despite the fact that microalgae have been used since ancient times, they are regarded as unique products that must undergo a battery of toxicity tests to ensure their safety. The evaluation of chemical composition, biogenic and non-biogenic toxic compounds, protein quality assessments, biochemical nutritional studies, supplementary value of algae to conventional food sources, sanitary analysis, safety evaluations (feeding trials with animals), clinical studies (tests for safety and suitability of the product for human consumption) are some of the fundamentals for the utilization of microalgal biomass for human and animal consumption (Becker, 2004).

Choosing appropriate local microalgal strains is a critical step toward attaining dependable and viable commercial production of bioproducts. It requires algae to produce the desired bio compounds under the local environmental conditions under which large-scale cultivation is envisaged (Borowitzka, 2013). DNA sequence analysis has become a more potent approach in diatom research, thus opening a new avenue into their systematic evolution. Genetic examination using 18S rDNA analysis confirmed the strain used in this study was *Amphora* sp. (Genbank accession: MW721231).

Toxicity testing or safety assessment was conducted to determine if *Amphora* sp. could potentially damage test organisms. Although it does not give appropriate information about the mechanism of toxic action, the brine shrimp lethality test provides preliminary screening data and is a highly effective approach for assessing the hazardous potential of different extracts

(Gadir, 2012; Naidu *et al.,* 2014). The extracts of *Amphora* sp. did not exhibit toxic activity against the brine shrimp in the brine shrimp lethality assay. Cytotoxicity data demonstrated a cell viability decrease of >80% suggesting low toxicity against a healthy PBMC cell line. Further analysis indicated that there was no evidence of mutagenic properties in the bacterial reverse mutagenic assay which indicated a mutant potential ranging from 0.20-0.83. The Ames test confirms a test item's mutagenic potential if the mutant frequency is 2.0 or above (OECD, 1997) Additionally, there was no thinning of the bacterial lawn. Toxic effects of the test item are often shown by the lack of a bacterial lawn, which can be partial or total (Hamel, Roy and Proudlock, 2016).

The results for the various parameters such as BW, ROW, blood serum chemistry, and histopathology evaluated in the repeated dose oral toxicity test conducted on Wistar rats for 28 days were generally negative. There were no significant changes in body/organ weight, biochemical parameters and histopathology. Histopathological examination revealed some variations in the lower dose groups; however, no changes were observed in the 4000 mg/kg/day groups. These findings were considered adaptive and were therefore considered test article related. Overall, it can be concluded that administration of the biomass of *Amphora* sp. up to a dose level of 4000 mg/kg BW did not result in toxicity. At present, studies on reproductive toxicity, neurotoxicity, and long-term exposure were not performed. Because of the wide range of natural metabolites generated by these microorganisms, biochemical characterisation of microalgal biomass is critical. This strategy provides an excellent prospect to separate and identify novel species from natural habitats. This opens up the prospect of discovering appropriate species for the manufacture of nutraceuticals, food components, metabolites, vaccines and fuels.

According to Yamaguchi (1996), microalgae can biosynthesize, metabolise, store and also release a wide spectrum of primary and secondary metabolites. Diatoms in particular offer valuable bioactive compounds however their use in the food industry is limited compared to blue-green and green microalgae such as *Spirulina* sp. and *Chlorella* sp., respectively (Gouveia *et al.,* 2008). Algae, like higher plants, has a variable chemical composition that is influenced by environmental conditions such as temperature, pH value, mineral content, CO_2 supply, population, density, growth phase, and algae physiology. Many specific properties, such as digestibility of cell wall (Epifanio *et al.,* 1981), cell size and biochemical profile (Fernández- Reiriz *et al.,* 1989) are considered to determine the nutritional value of microalgae.

Proximate composition, fatty acids, amino acids and mineral profile, as well as antioxidant activity, carotenoid and TPC of *Amphora* sp. biomass were evaluated. Due to the silicon-based

frustules of many diatom species, ash was the most abundant component of the biomass. Overly high ash levels in foods is typically undesirable because it compromises food quality such as flavour; but, algal ash can improve to reaching the necessary daily mineral intake in human nutrition (Sánchez-Machado *et al.,* 2004). Certain pre-treatments, such as a moderate acid wash of algal biomass before inclusion into foods might mitigate the high ash concentration. Further improvement of the culture and environmental conditions may minimise the ash content, due to rapidly dividing cells which have thinner frustules (Lewin and Guillard, 1963). A high ash level indicates a high mineral content, as demonstrated in this study, with Ca, P, and Na being the most prevalent minerals. The mineral content is largely dependent on physiological and environmental conditions, as well as processing and mineralization procedures that involve dry mineralization in a 550˚C oven (Rupérez, 2002).

Microalgal carbohydrates are complex, consisting of a mixture of neutral sugars, amino sugars, and uronic acids, and their composition varies depending on species and growing parameters (Takeda and Hirokawa, 1978; Kapaun and Reisser, 1995; Pieper *et al.,* 2012). Hildebrand *et al.* (2013) reported that insoluble starch, hydrosoluble glycogen and water-soluble β-1,3-glucans like chrysolaminarin are all examples of carbohydrate storage. *Amphora* sp. biomass consisted of a substantially high amount of carbohydrates (28%) compared to microalgae reported by Laurens *et al.* (2012) which consisted of about 20% DW. Carbohydrate and starch content varies according to microalgae species, culture parameters and duration of cultivation (Zhao, Brück and Lercher, 2013; Laurens *et al.,* 2014).

The biomass consisted of moderate to low levels of lipids, proteins, antioxidants and carotenoids compared to commercially produced microalgae as well as other diatoms. This is attributed to the species and cultivation parameters (Becker, 2004; Becker, 2007; Seyfabadi *et al.,* 2011; Khatoon *et al.,* 2014; Waghmare *et al.,* 2016; Menegol *et al.,* 2017; Teuling *et al.,* 2019). Microalgae require nutrients such as nitrogen, phosphorus, potassium and others in addition to solar energy and carbon dioxide to grow. If a nutrient is depleted or removed from the growth medium, the microalgae modify their metabolic pathways, causing a shift in biomass composition (Markou, Angelidaki and Georgakakis, 2012). This implies that artificially manipulated cultivation conditions can induce the production of certain nutrients. Nitrogen concentration has been shown to have a significant influence on lipid production, with nitrogen deficiency increasing lipid content in microalgae (Viegas *et al.,* 2015; Sulochana and Arumugam, 2020).

Hu and colleagues discovered that the lipid content of microalgae changes from 10 to 20% under nitrogen restricted circumstances (Hu *et al.,* 2008). Under typical conditions, microalgae produce a substantial quantity of biomass, but they do not produce high-value compounds such as lipids (Piligaev *et al.,* 2019). Microalgae cultivated under nutrient-stressed conditions produce a higher amount of TAGs. This contributes up to 80% of the total lipid content in the cell because of a modification in their FA metabolism (Ratomski and Hawrot-Paw, 2021). When nitrogen is lacking in the growth medium, microalgae accumulate a significant amount of lipids, but the cells do not synthesize enough amounts of proteins, resulting in lower biomass accumulation (Udayan *et al.,* 2022).

Under nutrient-stressed conditions, chlorophyll content fluctuates, which is a sign of photosynthesis and photochemical activities that release the energy stored in ATP (Rai, Gautom and Sharma, 2015). As a result, substantial nutrient supply is required to achieve high lipid production as well as high biomass production efficiency, in addition to ideal culture conditions such as temperature, pH, light and mixing (Ratomski and Hawrot-Paw, 2021).

The nature and concentrations of nitrogen sources, as well as the intensity of light have an impact on increased pigment and protein production (Benavente-Valdés *et al.,* 2016; Schüler *et al.,* 2020; Eze *et al.,* 2022). The increase in protein and carotenoid content may be related to species differences or the period of cultivation (Eze *et al.,* 2022). Nutrient stress also causes the formation of free radical species in the cell, which can lead to alterations in antioxidant levels (Goiris *et al.,* 2015). As a result, more research is required to optimize the growth and production of the components of interest, by manipulating various cultural conditions during the growth phase as discussed above.

7.2 Characterisation of soluble protein and carbohydrates in *Amphora* sp.

Proteins were extracted using TPP treatment and the biomass and TPP protein extracts were investigated for their proximate content, amino acid profile, structural properties, functional properties as well as IVPD and solubility profile. Traditional separation procedures such as membrane separation, column chromatography, precipitation and crystallization frequently demand many unit operations and a large amount of harmful organic solvents. Due to various processing stages, more time is used and product is lost during the process, resulting in lower proportions of the final products. Currently, the industry is looking for alternate methods to address the aforementioned shortcomings by employing more environmentally friendly and

effective methods. As a result, TPP was established as an effective method to extract and purify enzymes and biomolecules (Akardere *et al.,* 2010; Gagaoua *et al.,* 2017). TPP is a basic, economical and scalable process that involves adding salt and t-butanol to an aqueous solution to produce three phases. The desired product may separate to either phase depending on operational circumstances and physicochemical qualities (Avhad *et al.,* 2014).

Protein extraction by TPP increased the proteins and amino acids such as histidine, threonine, lysine, methionine, isoleucine, leucine, phenylalanine, tryptophan in the protein-enriched biomass extract compared to the biomass. Since most of the proteins are within microalgal cells, all the protein could not be extracted using the traditional method compared to the TPP method. TPP uses a group of principles involved in a variety of procedures such as salting out, isoionic precipitation, cosolvent precipitation, osmolytic and kosmotropic protein precipitation. Furthermore, the presence of salt forces the protein out of the solution, forming an interfacial precipitate layer between the aqueous and organic layers (Dennison and Lovrien, 1997). However, other parameters such as fat, ash, moisture and carbohydrate contents were reduced significantly after TPP treatment. The functional properties of the biomass such as FC, FS, EC, ES, WHC and OHC displayed higher values compared to the protein-enriched biomass extract, however, the IVPD was higher in the protein-enriched biomass extract.

The stabilisation of emulsions and foams by several microalgae species has been established, and it has been demonstrated that crude microalgae extracts, i.e. the complete ruptured cells, may be used to form emulsions (Devi and Venkataraman, 1984; Nirmala, Prakash and Venkataraman, 1992; Guil-Guerrero *et al.,* 2004; Shimada, Fonseca and Petri, 2017; Law *et al.,* 2018; Caporgno *et al.,* 2019) and foams (Devi and Venkataraman, 1984; Nirmala, Prakash and Venkataraman, 1992; Buchmann *et al.,* 2019) without further fractionation or purification. When compared to soy flour, crude extracts of microalgae could generate stable emulsions and foams with higher EC and foam overrun (Nirmala, Prakash and Venkataraman, 1992; Guil-Guerrero *et al.,* 2004). Functional properties such as FC, FS, EC, ES, WHC and OHC were reduced in the protein-enriched biomass extract. Although the EC was poor, the biomass had excellent ES (70%). OHC, FC, and FS displayed much lower values compared to other reported microalgae, however, the WHC reported in the present study is comparable to other microalgae indicating that the protein may be a good candidate for viscous foods or bakery products (Aletor *et al.,* 2002).

The utilization of crude extracts is a potential method for employing the entire microalgae biomass with minimal waste and energy input. However, following purification, such as extraction into a soluble component or protein isolation, the emulsification and foaming capabilities of microalgae

fractions are improved (Devi and Venkataraman, 1984; Nirmala, Prakash and Venkataraman, 1992; Guil-Guerrero *et al.,* 2004; Shimada, Fonseca and Petri, 2017; Law *et al.,* 2018; Pereira, Lisboa and Costa, 2018; Buchmann *et al.,* 2019; Caporgno *et al.,* 2019). The lower IVPD results in this study are comparable to other microalgae and may be attributed to its frustule-based cell wall.

The FTIR secondary structure of the protein-enriched biomass extract revealed a typical protein footprint. The most prominent polypeptide bonds band in the spectra was detected at 1600-1700 cm^{-1} (amide I), followed by amide II (1200-1400 cm^{-1}) and amide III (1200-1400 cm^{-1}). The tested samples exhibited a unique FTIR spectrum, with distinctive peaks appearing at defined wavelengths that vary according to environmental conditions (Stehfest, Toepel and Wilhelm, 2005; Driver *et al.,* 2015). In this study, DSC revealed a T_d of 112˚C which is notably higher than those reported by Ibanoglu (2005), Ghribi *et al.* (2015), and substantially lower than *Spirulina platensis* protein (173˚C) reported by Ramírez-Rodrigues *et al.* (2021).

The thermal stability of the proteins indicates their resistance to aggregation in response to heating. According to Waghmare *et al.* (2016), the method of protein extraction, pre-treatments, heating and solvents all have an impact on thermal denaturation. Heat-induced protein denaturation is determined by structural changes in the molecule. Differences in denaturation temperature may be related to structural properties of the protein as a result of extraction processing parameters and protein purity (Li *et al.,* 2014b).

Protein resistance to unfolding during heating is an important characteristic in the food industry; as the protein structure changes, solubility may decrease owing to the development of aggregates, thus compromising food product quality (Ramírez-Rodrigues *et al.,* 2021). The insoluble microalgae protein-rich components do not dissolve readily in water and is therefore a challenge to create complex food structures such as emulsions, gels, or foams. The lack of molecular mobility and cross-reactivity results in the inability of the material to cross-link and form networks, adsorb at surfaces, or self-assemble into larger macromolecular structures with anisotropic geometries, such as fibrils (Dai *et al.,* 2020).

The *Amphora* sp. biomass was characterised in terms of its β-glucan content, monosaccharide profile and structural components. A monosaccharide profile was noted with glucose being the most predominant sugar. The β-glucan isolated from *Amphora* sp. presented a value of approximately 9% which is in agreement with microalgae as reported by Schulze *et al.* (2016). FTIR analysis revealed a characteristic peak observed at 889.15 cm^{-1} confirming that *Amphora* sp. possessed a β-type polysaccharide (Zhang *et al.,* 2012). The protective effects of β-1,3-glucan

against iron-induced cardiotoxicity were investigated in Wistar rats. The findings showed that it is possible to delay oxidative stress, suppress acetylcholinesterase and lipase activities, and modulate myocardial lipid metabolism. As a result, the β-glucan extract may be used as an alternative in the production of nutraceuticals for the maintenance of heart health.

7.3 General Conclusions

This is the first study that provides a detailed investigation of *Amphora* sp. grown under normal conditions in terms of safety, toxicity and biochemical profile, and further characterisation of protein and carbohydrate components for possible applications in the food industry. Results of the 28-day oral toxicity study revealed an absence of mortality, and an alteration in growth, vital organs and serum chemistry parameters in a rat model. The dried biomass exhibited appreciable amounts of amino acids, minerals and carbohydrates. The protein, antioxidant and carotenoid content were moderate compared to commercially available microalgae whilst low antioxidant activities were observed. Proximate composition of dried biomass and protein-enriched biomass extract showed that the use of TPP concentrated the proteins and amino acids in the biomass but significantly reduced all the other parameters such as ash, fat, ash moisture. The β-glucan isolated from *Amphora* sp. was 9% and this was confirmed by FTIR analysis. Treatment with β glucan extract on the cardiac tissue led to increased SOD and catalase activities, while depleting MDA, NO, LDL-c and cholesterol levels, while simultaneously elevating triglycerides and HDL-c levels. Our results advocate the protective capabilities of the β-glucan extract against ferric-induced oxidative cardiac injury in a rat model. Overall, the results of this study suggest that *Amphora* sp. showed promising results as a suitable process organism for applications as a direct food ingredient under normal cultivation conditions, however, its maximum utilization and nutritional benefits can be enhanced by the manipulation of its culture conditions.

7.4 Recommendations

This study provides valuable preliminary data on the toxicity profile of *Amphora* sp. biomass. The utilization of *Amphora* sp. is still in its early phases of study, since better understanding of the processes and functions of the various components present is required. Further investigations involving sub-chronic and chronic, as well as pre-clinical and clinical studies of the biomass and extracts, will be necessary to determine the safe dose before it is prescribed as a food ingredient. Several cultivation approaches may be used to control the composition of microalgal biomass in order to increase the production of microalgal biomass and the production of specific molecules.

These cultivation techniques are mostly connected to culture and environmental aspects that may be managed (nutrients, light and temperature) and which have been shown to influence algal growth and biomass composition. This strain has the potential to be developed for food applications with an emphasis on increasing its nutritional value. This study established the foundation for identifying high-value natural compounds from potential local microalgae for food and feed applications.

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Appendices

1. ASW Preparation

Amphora sp. grown on ASW, composed of: 13.98 g/L NaCl; 2.60 g/L $MgCl_2.6H_2O$; 3.56 g/L; $MgSO_4.7H_2O$; 0.77 g/L $CaCl_2.2H_2O$; 0.39 g/L KCl; 0.01 and 0.03 g/L H_3BO_3. A volume of 1.0 mL/L of trace elements solution composed of 19.60 g/L $CuSO_4.5H_2O$; 44.0 g/L $ZnSO_4.7H_2O$; 20.0 g/L $CoCl_2.6H_20$; 360.0 g/L $MnCl_2.4H_2O$ and 12.60 g/L $NaMoO_4.2H_20$ was added to the media. A volume of 1.0 mL/L of Fe/EDTA solution composed of 5.71 g/L $FeC_6H_5O_7$ and 17.35 g/L $Na_2EDTA.2H_2O$ was added to the media. A volume of 1.0 mL/L of the vitamin solution composed of 200.00 mg/L Thiamine-HCl; 1.0 mg/L Biotin and 1.0 mg/L B-12 was added to the media (after autoclaving). Media components were weighed out and made up to the appropriate volume. Media was then sterilised for 20 min at 121°C. Filter sterilised Fe/EDTA (1 mL) and vitamin solution (1 mL) were added from the stock solutions.

2. Ethics approval

UNIVERSITY OF KWAZULU·NATAL
INYUVESI YAKWAZULU-NATALI

05 October 2018

Ms Sharlynn Beekrum (40003220)
Department of Biotechnology and Food Technology Durban
University of Technology
Steve Biko Campus

Dear Ms Beekrum,

Protocol reference number: AREC/021/0IBD
Project title: Characterisation and evaluation of a marine microalgal strain for food applications

Full Approval - Research Application With regards to your revised application received on 16 May 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS:

- A maximum of 250 µL blood can be allowed via tail prick.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before OS October 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached In the event of any unanticipated event involving the animals' health/ wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor Eric Amonsou
Cc BRU - Dr Linda Bester
Cc Registrar: Mr Simon Mokoena

Animal Research Ethics Committee (AREC)
Ms Marlette Snyrnan (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000

3.Humane endpoint form

DATE OF STUDY				**ANIMAL NO:**			
WEIGHT (g)							
DATE							
DAY							
TIME							
UNDISTURBED OBSERVATION							
Inactive							
Mobility							
Hunched posture							
Grooming							
Alertness							
Presence of a mass							
Ruffeld coat							
ON HANDLNG							
Not inquisitive and alert							
Not eating							
Not drinking							
Vocalization on gentle palpatoin							
Body weight (g)							
% of baseline weight							
Dehydration							
Type of breathing *							
Condition scoring 4 to 1 **							

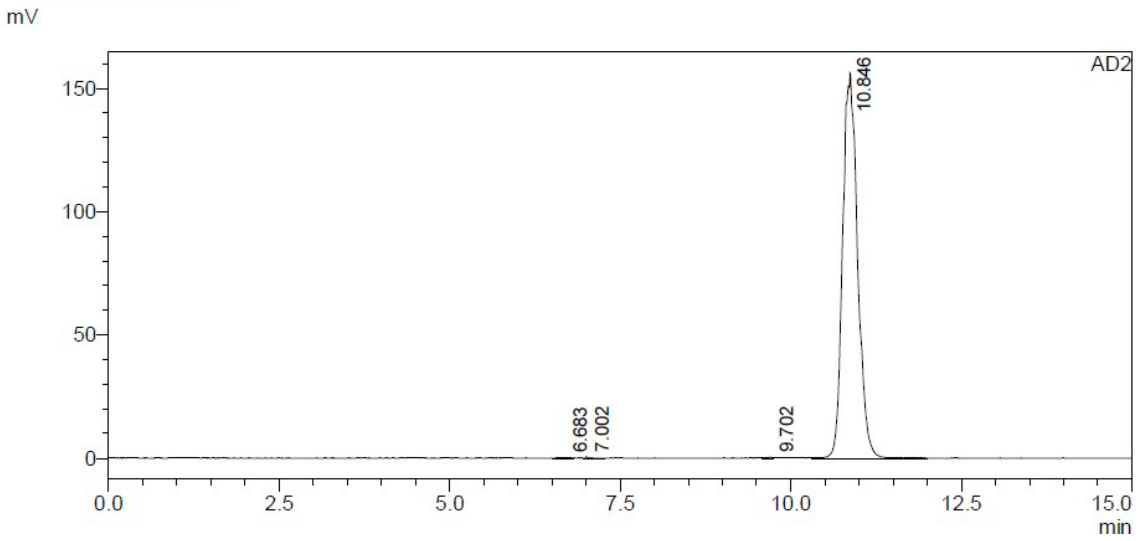
SPECIFIC CLINICAL SIGNS							
Size of tumour							
Necrosis of tumour							
Bleeding of tumour							
Ulceration							
Nothing Abnormal Detected (NAD)							
OTHER							
SIGNATURE							

4. Monosaccharide standards

<Sample Information>

Sample Name	: Glu-stock		
Sample ID	: UNK-0021		
Data Filename	: Glu-stock.lcd		
Method Filename	: Aminex HPX -ELSD Sugars.lcm		
Batch Filename	: Second run 30 August.lcb		
Vial #	: 1-21	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 2018/08/30 09:56:30 PM	Acquired by	: System Administrator
Date Processed	: 2018/08/30 10:11:31 PM	Processed by	: System Administrator

<Chromatogram>



<Sample Information>

Sample Name	: Man-stock		
Sample ID	: UNK-0015		
Data Filename	: Man-stock.lcd		
Method Filename	: Aminex HPX -ELSD Sugars.lcm		
Batch Filename	: Second run 30 August.lcb		
Vial #	: 1-15	Sample Type	: Unknown
Injection Volume	: 10 uL		
Date Acquired	: 2018/08/30 08:24:11 PM	Acquired by	: System Administrator
Date Processed	: 2018/08/30 08:39:12 PM	Processed by	: System Administrator

<Chromatogram>