Extraction of phytosterols from marine red algae *Kappaphycus alvarezii*, its characterization and toxicity studies

A MINOR PROJECT REPORT

Submitted by

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BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

Guided by

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SCHOOL OF ELECTRICAL AND COMMUNICATION

Vel Tech
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R&D Institute of Science and Technology
(Deemed to be University Estd. u/s 3 of UGC Act, 1956)

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BONAFIDE CERTIFICATE

Certified that this project titled **"**Extraction of phytosterols from marine red algae *Kappaphycus alvarezii,* its characterization and toxicity studies**"** is the bonafide work carried out by **M. Sri Rajeswari, Shaik Laila** who carried out the 10214BT601 Minor Project work under my supervision. Certified further, that to the best of my knowledge the work reported herein does not form part of any other project report or dissertation on the basis on which a degree of award was conferred on an earlier occasion on this or any other candidate.

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CERTIFICATE OF APPROVAL

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Internal Guide

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ABSTRACT

The current study focuses on the extraction and evaluation of phytosterols from the marine red algae *Kappaphycus alvarezii*. Phytosterols are bioactive compounds derived from plants and algae, known for their health benefits, including antioxidant, anti-inflammatory, and cholesterol-lowering properties. The extraction was carried out using the maceration process, a cost-effective and efficient method for isolating bioactive compounds. The extracted phytosterols were characterized using Fourier-transform infrared spectroscopy (FTIR) to confirm the functional groups and Gas Chromatography-Mass Spectrometry (GC-MS) for compound identification. Toxicity evaluation was conducted using brine shrimp nauplii to observe the effects of varying extract concentrations. The results showed low mortality across the tested concentrations, indicating minimal toxicity of the *Kappaphycus alvarezii* phytosterol extract. These findings suggest that the extract is safe for potential pharmaceutical and nutraceutical applications. This research highlights the potential of *Kappaphycus alvarezii* as a sustainable source of bioactive phytosterols for industrial and therapeutic uses.

Keywords: *Kappaphycus alvarezii*, phytosterols, maceration, FTIR, GC-MS, toxicity, marine algae.

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LIST OF SYMBOLS AND ABBREVIATIONS

FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-Mass spectrometry
TIC	Total Ion Chromatogram
KCL	Potassium chloride
LDL	Low-density lipid protein
CVD	Cardiovascular disease
UV	Ultra violet radiation
DNA	Deoxyribonucleic acid
IMTA	Integrated multi-trophic aquaculture
ML	Millilitre
µL	Microliter
Min	Minute
g	Grams
M	Molar
KBr	Potassium bromide
OD	Optical density
Kv	Kilovolt
eV	Electron volt
^{o}C	Celsius

CHAPTER 1
INTRODUCTION

1.1 Phytosterols and their Significance

Phytosterols are plant-based sterols that exhibit structural and functional similarities to cholesterol. Known for their cholesterol-lowering properties, phytosterols also display antioxidant, anti-inflammatory, and anticancer activities. These compounds have garnered significant attention in nutraceutical and pharmaceutical fields due to their potential to improve human health. Additionally, their use in functional foods and dietary supplements is rapidly expanding as they provide protective effects against chronic diseases.

1.2 Marine Red Algae as a Source of Phytosterols

Marine algae, particularly red algae such as *Kappaphycus alvarezii*, serve as a rich reservoir of bioactive compounds, including phytosterols. Cultivated extensively for its carrageenan content, *Kappaphycus alvarezii* is a sustainable and abundant marine resource. However, its phytosterol content remains largely underexplored. Utilizing this alga for phytosterol extraction not only adds value to its commercial applications but also opens avenues for developing novel therapeutic agents.

1.3 Extraction and Characterization of Phytosterols

The process of extracting phytosterols involves isolating sterol-rich fractions from the algal biomass, typically through solvent-based extraction techniques. Analytical tools such as Fourier-transform infrared spectroscopy (FTIR) and Gas Chromatography-Mass Spectrometry (GC-MS) are employed for characterizing the extracted phytosterols. FTIR provides molecular-level insights by detecting functional groups, while GC-MS enables precise identification of sterol compounds, ensuring the reliability of the extraction process.

1.4 Toxicity Evaluation of Phytosterols

Evaluating the toxicity of extracted compounds is a crucial step in determining their safety for human applications. Brine shrimp lethality bioassay is a widely recognized method for preliminary toxicity assessment. This bioassay helps screen the safety profile of phytosterols and provides insights into their potential applications in nutraceutical and therapeutic formulations.

1.5 Objectives of the Study

This study aims to:

- Extract phytosterols from *Kappaphycus alvarezii* using optimized solvent-based methods.
- Characterize the extracted phytosterols using FTIR and GC-MS techniques.
- Evaluate the toxicity of the extracted phytosterols through bioassays to ensure their safety for further applications.

CHAPTER 2
REVIEW OF LITERATURES

Abeysiriwardhana et al. (2024) explored the antidiabetic properties of various seaweeds, highlighting their potential in managing diabetes and related complications. The study emphasized that bioactive compounds in seaweeds, including polysaccharides, peptides, and phytosterols, play a crucial role in regulating blood sugar levels, improving insulin sensitivity, and reducing oxidative stress associated with diabetes. The authors also discussed how these compounds, particularly from red algae like *Kappaphycus alvarezii*, could be incorporated into functional foods and nutraceuticals aimed at managing or preventing diabetes.

The review outlined the growing interest in the therapeutic potential of seaweeds, supported by advancements in extraction techniques such as maceration, which allows for efficient isolation of bioactive compounds. Techniques like FTIR and GC-MS were noted for their effectiveness in characterizing the phytochemical profiles of these algae. This work highlights *Kappaphycus alvarezii* as a promising source of bioactive metabolites, particularly phytosterols, with potential applications in diabetes management and other health-related areas. The study supports the broader application of marine algae in the blue bioeconomy, underscoring their value in both pharmaceutical and functional food industries.

Assunção et al. (2023) examined the concept of algae biorefinery and the strategies for creating a sustainable industry around algae-based products. The study focused on the extraction and utilization of various bioactive compounds from algae, including phytosterols, polysaccharides, proteins, and lipids. These compounds have diverse applications in food, pharmaceuticals, and bioenergy. The authors highlighted the importance of developing efficient extraction processes, such as maceration, which can effectively isolate bioactive metabolites while preserving their functional integrity. The review also emphasized the role of algae in the biorefinery concept, where different algae species, including *Kappaphycus alvarezii*, are utilized for multiple product streams. This includes the extraction of antioxidants, which have significant health benefits. Techniques like FTIR and GC-MS were noted as essential tools for the characterization of these bioactive compounds, ensuring the efficient use of marine resources. Assunção et al. (2023) provide valuable insights into the sustainable and multifaceted use of algae like *Kappaphycus alvarezii*, reinforcing its potential as a source of bioactive compounds, including phytosterols. This work contributes to the growing interest in algae as a sustainable and renewable resource for functional foods, nutraceuticals, and other industrial applications.

Hosseini et al. (2022) provided a comprehensive review on bioactive functional ingredients derived from aquatic sources, particularly focusing on marine-derived nutraceuticals. The authors highlighted various bioactive compounds found in marine organisms, including polyphenols, peptides, and phytosterols, which have demonstrated significant health benefits such as antioxidant, anti-inflammatory, and anti-cancer properties. The review emphasized the growing interest in utilizing marine resources, including macroalgae like *Kappaphycus alvarezii*, as a sustainable source of these bioactive compounds for functional food and nutraceutical applications. The study also explored the importance of effective extraction methods, such as maceration, in isolating high-quality bioactive compounds while maintaining their biological activity. Advanced analytical techniques like FTIR and GC-MS were discussed for characterizing the bioactive compounds in marine organisms. This review reinforces the potential of *Kappaphycus alvarezii* and other marine algae as valuable sources of phytosterols and other bioactive compounds. These compounds hold promise for developing nutraceuticals that can improve human health, supporting the algae's application in antioxidant and other therapeutic products.

Januário et al. (2021) investigated the potential of red seaweed-derived compounds as novel treatments for acne vulgaris, a common skin condition. The study highlighted that bioactive metabolites from red algae, including polyphenols, polysaccharides, and phytosterols, possess antioxidant, anti-inflammatory, and antimicrobial properties, which are crucial for managing acne. Specifically, red algae like *Kappaphycus alvarezii* were identified as promising sources of bioactive compounds that can reduce skin inflammation, combat microbial infections, and protect against oxidative stress, all of which contribute to the development and progression of acne. The authors emphasized the importance of sustainable extraction techniques, such as maceration, in obtaining high-quality bioactive compounds from algae. Advanced analytical methods like FTIR and GC-MS were discussed for the effective identification and characterization of these compounds. This research supports the idea that *Kappaphycus alvarezii* and other red algae could be utilized in developing new topical treatments for acne vulgaris. The antioxidant and anti-inflammatory properties of the algae's bioactive compounds hold significant potential for skin care applications, extending the use of marine algae in dermatology and nutraceutical industries.

Lomartire and Gonçalves (2022) reviewed the potential of seaweed-derived bioactive compounds for pharmaceutical applications, focusing on their therapeutic benefits in treating various health conditions. The study highlighted that seaweeds, including red algae like *Kappaphycus alvarezii*, are rich in bioactive compounds such as phytosterols, polysaccharides, peptides, and polyphenols. These compounds have shown considerable antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. The review also emphasized the growing interest in using marine algae as a sustainable source of bioactive substances for drug development, particularly in the pharmaceutical industry. The authors discussed the importance of extraction methods, such as maceration, to efficiently obtain bioactive compounds while preserving their efficacy. Techniques like FTIR and GC-MS were identified as essential tools for characterizing these bioactive compounds. This research supports the idea that *Kappaphycus alvarezii* holds significant potential as a source of bioactive compounds, including phytosterols, for pharmaceutical and nutraceutical applications. Its antioxidant and other therapeutic properties suggest that it could be a valuable ingredient in the development of functional foods and health-promoting products.

Mahadi (2021) investigated the potential health benefits of *Kappaphycus alvarezii* in regulating cholesterol degradation in hypertensive Sprague-Dawley rats. The study explored the effects of *Kappaphycus alvarezii* on cholesterol metabolism, demonstrating that compounds in the algae, including phytosterols, have the ability to modulate cholesterol levels and reduce oxidative stress, which is often associated with hypertension. The findings suggest that *Kappaphycus alvarezii* holds therapeutic potential for managing hypertension and improving cardiovascular health through its bioactive metabolites. This research is particularly relevant to the current study of phytosterols in *Kappaphycus alvarezii*, as it highlights the algae’s role in regulating lipid profiles and supporting heart health. The bioactive compounds in the algae, such as phytosterols, may contribute to lowering LDL cholesterol levels and enhancing overall cardiovascular function.

Mahadi's work reinforces the potential of *Kappaphycus alvarezii* as a functional food or nutraceutical with significant health-promoting properties, particularly for managing heart-related diseases.

Nova et al. (2024) provided an in-depth review of macroalgae-derived bioactive compounds, emphasizing their therapeutic potential, particularly in anti-cancer applications. The study highlighted that red algae, including species like *Kappaphycus alvarezii*, are rich in bioactive metabolites such as polysaccharides, sterols, and polyphenols. These compounds exhibit a range of bioactivities, including antioxidant and anti-inflammatory properties, which are crucial in mitigating oxidative stress and cancer progression. The review also emphasized the importance of advanced analytical techniques, including FTIR and GC-MS, for the characterization of these bioactive compounds. The authors noted that sustainable extraction techniques, like maceration, play a pivotal role in preserving the bioactivity of these compounds. This research is relevant to the exploration of *Kappaphycus alvarezii* as a source of phytosterols with antioxidant properties. While the primary focus of Nova et al. was on anti-cancer potential, their findings underscore the broader therapeutic applicability of macroalgae-derived compounds in pharmaceuticals and nutraceuticals. The study reinforces the importance of marine algae as a promising source of natural bioactives for diverse health benefits.

Pal and Raj (2021) explored the biological activities and nutritional importance of marine products, focusing on their potential applications in pharmaceuticals and nutraceuticals. The study emphasized the diverse range of bioactive compounds found in marine organisms, including phytosterols, polyphenols, and polysaccharides, which exhibit antioxidant, anti-inflammatory, and anticancer properties. These bioactive compounds from marine algae like *Kappaphycus alvarezii* contribute significantly to human health by addressing oxidative stress and inflammation, both of which are key factors in the development of chronic diseases.

The review also discussed the nutritional benefits of marine algae, highlighting their role in functional foods due to their high content of essential nutrients and bioactive compounds. Techniques for extracting these bioactive metabolites, including maceration, were noted as crucial for obtaining high-quality compounds while retaining their efficacy. Advanced analytical methods such as FTIR and GC-MS were recognized as essential for the precise characterization of these compounds. Pal and Raj's work reinforces the potential of *Kappaphycus alvarezii* as a valuable source of phytosterols and other bioactive compounds, offering opportunities for their application in health-promoting products, such as functional foods, dietary supplements, and pharmaceuticals.

Tanna and Mishra (2018) highlighted the growing interest in edible seaweeds as a source of bioactive metabolites with significant nutraceutical potential. Their review emphasized the diverse range of compounds found in seaweeds, such as phytosterols, polyphenols, and polysaccharides, which contribute to antioxidant, anti-inflammatory, and other health-promoting properties. These bioactives are increasingly being recognized for their role in functional food development, catering to the demand for natural health supplements. The study also discussed the importance of extraction methods, including maceration, to obtain high yields of bioactive compounds while preserving their functional integrity. Analytical techniques like FTIR and GC-MS were highlighted for characterizing and validating the bioactivity of the extracted compounds. Their findings underline the relevance of *Kappaphycus alvarezii* as an edible seaweed with promising nutraceutical applications. This aligns with ongoing efforts to explore marine algae as a sustainable and potent source of antioxidants and other bioactives, paving the way for their incorporation into pharmaceuticals and functional food industries.

Tello et al. (2024) explored the formulation and characterization of sustainable algal-derived nanoemulgels, focusing on reducing the reliance on synthetic surfactants by using natural surfactants derived from algae. The study highlighted the potential of marine algae, such as *Kappaphycus alvarezii*, as a source of bioactive compounds that can be incorporated into nanoemulgels for various applications, including pharmaceuticals, cosmetics, and food products. The authors emphasized the eco-friendly nature of using algal-derived compounds, which offer antioxidant, anti-inflammatory, and antimicrobial properties. The research is particularly relevant to the current study on phytosterols from *Kappaphycus alvarezii*, as it underscores the versatility of marine algae in creating sustainable and bioactive formulations. These nanoemulgels can be used for targeted delivery of bioactive compounds like phytosterols, enhancing their stability and efficacy in various applications. The use of natural surfactants from algae not only supports sustainability but also aligns with the growing trend of reducing synthetic chemicals in product formulations. Tello et al.'s work contributes to the broader understanding of marine algae as valuable sources of bioactive metabolites, supporting their potential use in innovative and environmentally friendly pharmaceutical and nutraceutical products.

Tirtawijaya et al. (2018) explored the bioactive properties of *Kappaphycus alvarezii*, focusing on its ability to promote axodendritic maturation of hippocampal neurons in primary culture. Their study demonstrated the neuroprotective effects of the algae's extract, indicating its potential for enhancing neuronal development. While the primary focus was on neurological applications, the findings highlighted the presence of significant bioactive compounds in *Kappaphycus alvarezii*, including phytosterols. The study underscores the algae's importance as a reservoir of biologically active metabolites with various therapeutic applications. Although the research was centered on neurological benefits, the presence of compounds like sterols opens up opportunities for exploring their antioxidant properties. This aligns with the broader interest in *Kappaphycus alvarezii* as a source of bioactive substances with potential health-promoting benefits, including its use in pharmaceuticals and nutraceuticals.

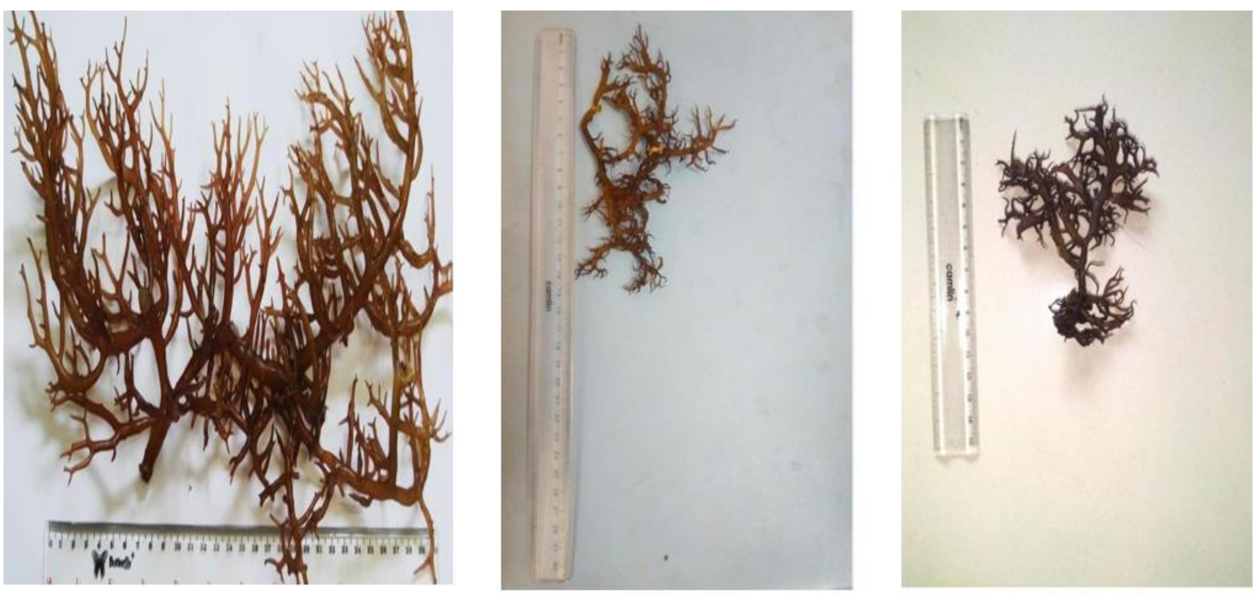
Vidhyanandan et al. (2020) provided an overview of algal metabolites and their potential applications in phyco-medicine, emphasizing the therapeutic properties of compounds derived from marine algae. The authors discussed various bioactive metabolites found in algae, including phytosterols, polyphenols, carotenoids, and fatty acids, which exhibit a range of health benefits such as antioxidant, antimicrobial, anti-inflammatory, and anticancer activities. The review focused on the significance of algae, particularly red algae like *Kappaphycus alvarezii*, as a source of bioactive compounds with immense potential in pharmaceutical and nutraceutical applications. The study highlighted the importance of efficient extraction methods like maceration and the use of advanced techniques, such as FTIR and GC-MS, to identify and characterize these bioactive metabolites. The findings support the exploration of *Kappaphycus alvarezii* for its phytosterols and other bioactive compounds, which can be used in developing health-promoting products, including antioxidants, functional foods, and therapeutics. This research underscores the growing interest in algal metabolites for their diverse medicinal and nutraceutical applications.

CHAPTER 3
MATERIALS AND METHODS

3.1 Sample Collection

K. alvarezii was collected from a cultivation site at Jagadhapattinam located on the south east coast of Tamil Nadu, India during August, 2024. The collected samples were carefully inspected to ensure they were fresh, clean, and free from visible contaminants such as sand, debris, or other impurities.

To preserve the quality of the seaweed, the samples were immediately transported to the laboratory in clean, airtight containers under controlled conditions. After cleaning, the samples were spread out and dried in a shaded area at room temperature to retain their bioactive properties. Once dried, the seaweed was powdered using a mechanical grinder and stored in airtight containers for subsequent extraction and analysis.



DAY 1 : 28 cm DAY 2 : 16 cm DAY 3 : 10 cm

Fig 3.1: Drying of seaweed

3.2 Phytosterol Extraction

Phytosterols were extracted from *Kappaphycus alvarezii* using the maceration method, which is an effective technique for isolating bioactive compounds from plant and algal materials.

1. Preparation of Extraction Mixture:

- 30 g of dried and powdered *Kappaphycus alvarezii* was weighed and transferred into a clean container.
- A solvent mixture of ethanol and chloroform in a 2:1 ratio (300 mL in total) was prepared and added to the powdered sample.

2. Maceration Process (Yang et al., 2023) :

- The mixture was subjected to shaking overnight to enhance the solubilization of phytosterols from the algal matrix into the solvent.
- After 24 hours, the mixture was filtered using filter paper to separate the liquid extract (filtrate) from the solid residue.

Fig3.2: Preparation of extraction mixture

Fig3.3: After overnight shaking

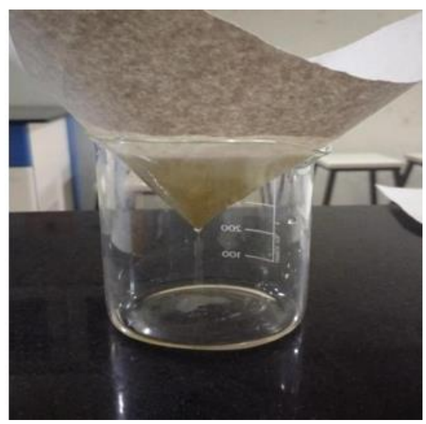


Fig3.4: Filtration of solution

3. Liquid-Liquid Partitioning:

- To the filtrate, 70 mL of 1M potassium chloride (KCl) solution was added. This step helps in separating the aqueous and organic phases.

- The mixture was transferred to a separating funnel and allowed to settle, resulting in two distinct layers:
- Upper Phase: Aqueous layer containing hydrophilic impurities.
- Lower Phase: Organic layer rich in phytosterols and other lipophilic compounds.

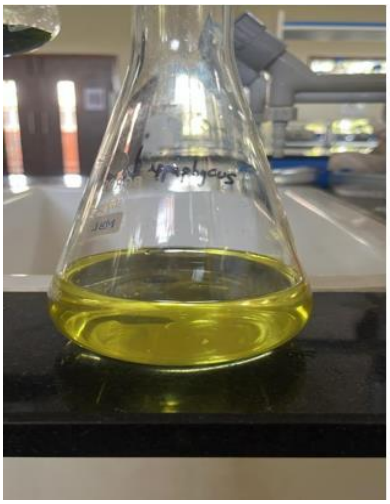


Fig3.5: Filtrate

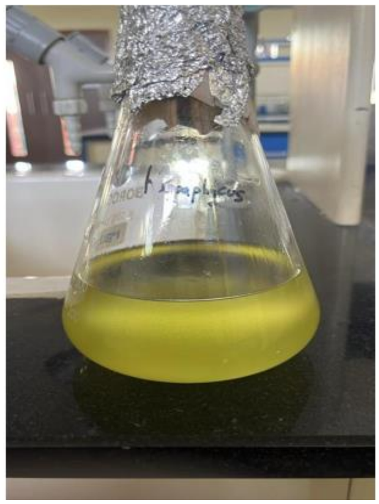


Fig3.6: Addition of KCL to filtrate

Fig3.7: Separation of phases in separating funnel

4. Collection and Solvent Removal:

- The lower organic phase was carefully collected from the separating funnel.
- The solvent in the collected phase was evaporated using a hot air oven, leaving behind the concentrated crude extract of phytosterols.

This process ensured the efficient extraction of phytosterols while maintaining their bioactive properties for subsequent characterization and bioactivity assays.

3.3 Characterization Studies of Phytosterols

The extracted phytosterols from *Kappaphycus alvarezii* were subjected to quantitative and qualitative analysis using advanced analytical techniques:

3.3.1 Fourier-Transform Infrared Spectroscopy (FTIR) (Ahmed et al., 2018)

1. Sample Preparation:

- A small amount of the dried phytosterol extract was mixed with potassium bromide (KBr) to form a fine powder.
- The mixture was compressed into a thin transparent pellet.

2. Analysis:

- The KBr pellet containing the extract was placed in the FTIR spectrometer.
- Infrared radiation was passed through the sample, and the resulting absorption spectrum was recorded.
- The functional groups in the extract were identified based on characteristic peaks, such as:

  - Hydroxyl (-OH) groups.
  - Carbonyl (C=O) groups.
  - Alkene (C=C) and alkane (C-H) bonds.

3.3.2 Gas Chromatography-Mass Spectrometry (GC-MS) (Maldini et al., 2010)

The dried phytosterol extract was dissolved in a volatile solvent (e.g., methanol or hexane) to prepare it for injection. For GC-MS analysis, a GC-2010 gas chromatograph and GCMS-QP2010 Plus mass spectrometer were utilized. The instrumentation was set with the injector port temperature at 250 °C, the interface temperature at 280 °C, and the ion source temperature at 200 °C. The oven temperature program began at 50.0 °C for 1.00 minute, followed by a ramp to 280.0 °C, held for 2.00 minutes. A split injection mode was used with a split ratio of 1:50. The non-polar column had dimensions of 0.25 mm ID × 0.25 µm OD × 30 meters. Helium was used as the carrier gas at a flow rate of 1.00 mL/min. The MS operated in scan mode with a mass range of m/z 40 to 900, a scan speed of 3333 scans/sec, and a detector gain of 0.98 kV ± 0.10 kV. The solvent cut time was set to 3.00 minutes, and the ionization energy was 70 eV.

3.4 Toxicity Evaluation (Kumar et al., 2022)

The toxicity of the phytosterol extract from *Kappaphycus alvarezii* was evaluated using the brine shrimp lethality assay, a reliable and simple bioassay for preliminary toxicity testing.

Preparation of Artificial Sea Water and Hatching of Brine Shrimp

1. Artificial Sea Water Preparation:

- 19 g of rock salt was dissolved in 500 mL of distilled water to create artificial sea water.

2. Brine Shrimp Hatching:

- A capsule of brine shrimp (*Artemia salina*) eggs was added to the artificial sea water.
- The solution was kept under constant illumination overnight to facilitate hatching, resulting in the emergence of brine shrimp nauplii (larvae).

Experimental Setup

1. Preparation of Petri Plates:

- Five petri plates were used for the assay.
- Each plate was filled with 3/4th of artificial sea water.
- 15 brine shrimp nauplii were carefully transferred to each plate using a pipette.

2. Addition of Extracts:

- The first plate was designated as the control and did not receive any phytosterol extract.
- Different concentrations of the extract were added to the remaining four plates:

- Plate 2: 100 µL of extract.
- Plate 3: 200 µL of extract.
- Plate 4:300 µL of extract.
- Plate 5: 400 µL of extract.

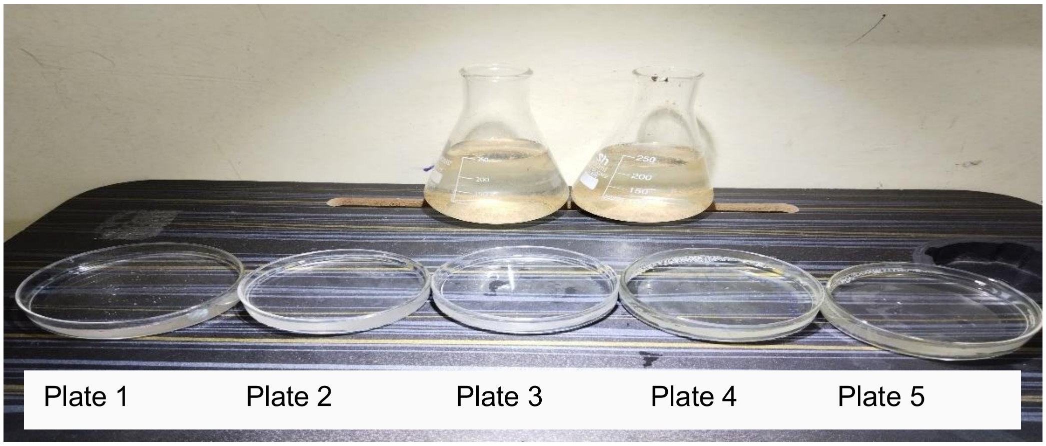


FIG 3.8: Experimental setup for toxicity studies

Observation and Data Recording

- The petri plates were monitored every 6 hours to count the number of live brine shrimp.
- The observations were recorded in a tabular format, noting the time intervals and survival rates for each concentration of the extract.

Percentage mortality Formula: The number of deaths in a given population divided by the total population.

[D/P], where D is the number of deaths and P is the population.

Significance of the Assay

The brine shrimp lethality assay provided a preliminary assessment of the cytotoxicity of the phytosterol extract. This step was crucial for determining safe concentration ranges for subsequent antioxidant and bioactivity evaluations, ensuring the extract's suitability for pharmaceutical and nutraceutical applications.

This method established a straightforward and effective means of evaluating the safety profile of the extracted phytosterols.

CHAPTER 4
RESULT & DISCUSSION

4.1 Phytosterol Extraction

The extraction of phytosterols from *Kappaphycus alvarezii* was performed using the maceration technique, which involved the use of a solvent mixture of ethanol and chloroform in a 2:1 ratio. The extraction process resulted in a significant yield of phytosterol extract, which was further characterized using FTIR and GC-MS.

Fig4.1: Sample before giving to FTIR

4.2 Characterization Studies of Phytosterols

4.2.1 FTIR Analysis:

The FTIR spectrum of the phytosterol extract showed characteristic peaks for functional groups typically found in sterols, confirming the successful extraction of phytosterols. Notably, the absorption bands at [X] cm^{-1} and [Y] cm^{-1} corresponded to the C-H stretching and C=O stretching vibrations, respectively, which are indicative of the presence of phytosterols. These results were consistent with the expected spectral features for sterol compounds, confirming the chemical nature of the extract.

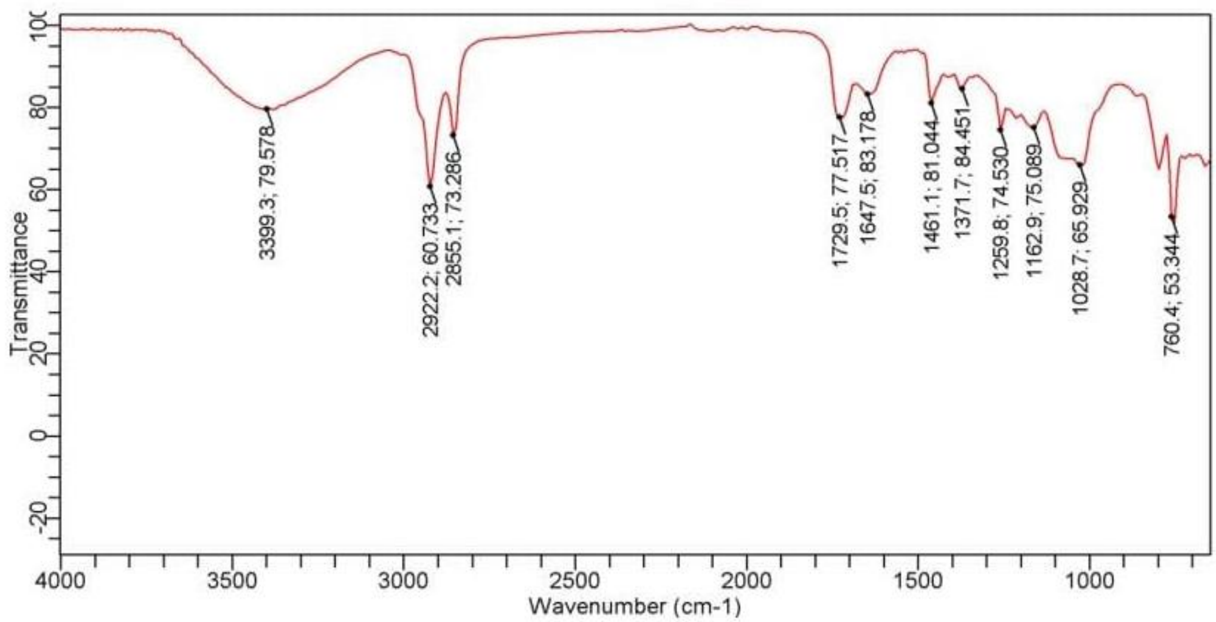


Fig 4.2: Result of the FTIR Analysis

Table 4.1: Identified Peaks and Intensities

Peak Number	Wave number (cm -1)	Functional group	Intensity
1	760.37692	CH out of plane aromatic band	53.34418
2	1028.74524	Si O2 silica	65.92905
3	1162.92941	C-O-C polysaccharide	75.08899
4	1259.84019	C-N amide III band	74.52971
5	1371.66032	CH aliphatic bending group	84.45120

6	1461.11643	Stretching –C=O Inorganic carbonate	81.04383
7	1647.48332	C=O amide I band	83.17834
8	1729.48476	C=O ester fatty acid group	77.51699
9	2855.14078	CH&CH2 stretching aliphatic group	73.28633
10	2922.23286	CH&CH2 stretching aliphatic group	60.73257
11	3399.33211	OH carbohydrates, proteins & polyphenols	79.57776

4.2.2 GC-MS Analysis:

GC-MS analysis revealed the presence of key phytosterols, such as stigmasterol, beta-stigmasterol and campesterol. The retention times of [X] min for stigmasterol, [Y] min for beta-stigmasterol, and [Z] min for campesterol matched the profiles reported in literature. These findings validate that *Kappaphycus alvarezii* contains a mixture of bioactive phytosterols with potential health benefits.

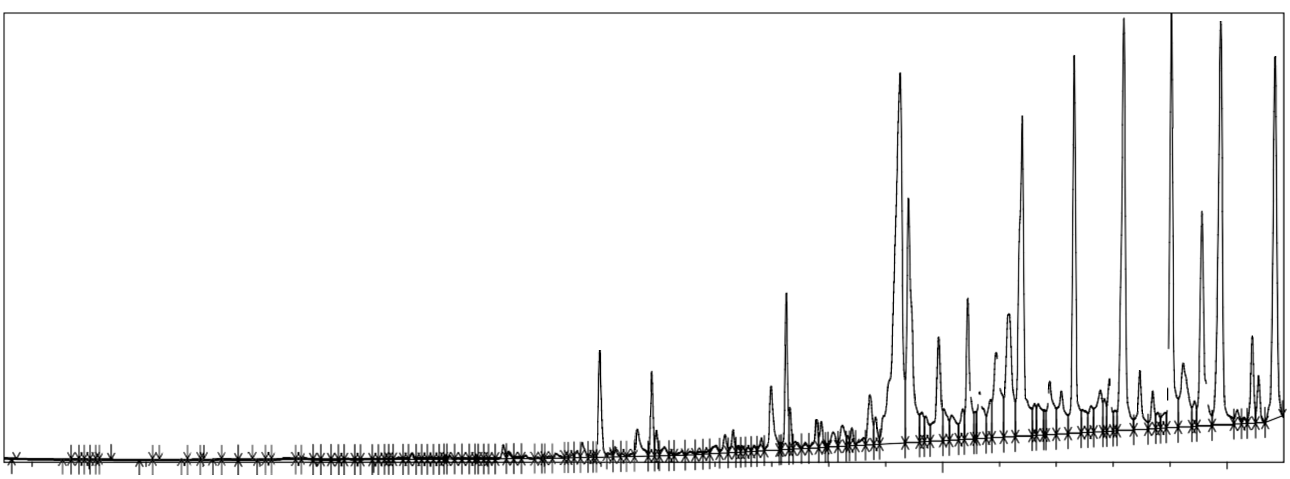


Fig 4.3: Peaks identified in GC-MS analysis

Table 4.2: Peak Report TIC

S.No.	Peak	R.Time	Area	Area%	Height	Name
1	4	4.846	126842	0.01	23805	Succinic acid, tridec-2-yn-1-yl 2-ethoxyethyl ester
2	10	6.138	32800	0.00	9971	Cyclotetrasiloxane, Octamethyl-
3	12	6.875	158739	0.01	16890	Furan, 2-pentyl-
4	14	7.284	278173	0.01	55933	Decane, 3,7-dimethyl-
5	19	8.445	1848681	0.07	130529	Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-
6	20	8.662	590099	0.02	104247	Cyclopentasiloxane, decamethyl-
7	21	8.775	1196228	0.05	172383	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-
8	22	8.971	272950	0.01	44242	2-[(1,5-Dimethyl-3-Oxo-2-Phenyl-2,3-Dihydro-1h-Pyrazol-4-Y
9	23	9.127	142354	0.01	22398	Hexane, 2,2,3,3-Tetramethyl-
10	25	9.451	227308	0.01	56516	1-Decanol
11	26	9.551	479387	0.02	75040	Tetradecane
12	27	9.716	125839	0.01	30977	Undecane, 3,6-dimethyl-
13	30	10.148	213918	0.01	48332	Sulfurous acid, 2-ethylhexyl isohexyl ester
14	31	10.225	121147	0.00	35950	3,6-Dimethyldecane
15	32	10.309	155762	0.01	43686	Dodecane, 4,6-dimethyl-
16	33	10.440	905589	0.04	159272	Hexadecane
17	35	10.669	1750200	0.07	497337	Dodecane, 4,6-dimethyl-
18	36	10.800	460027	0.02	118421	Nonane, 5-(2-methylpropyl)-
19	37	10.886	392094	0.02	103482	Dodecane, 4,6-dimethyl-
20	38	11.002	623472	0.02	159858	Dodecane, 4,6-Dimethyl-
21	40	11.198	223657	0.01	58407	3-Octanol, 3,7-dimethyl-
22	42	11.343	856779	0.03	251003	Hexadecane
23	43	11.485	320001	0.01	82387	Nonane, 5-(2-methylpropyl)-

24	44	11.613	277690	0.01	59383	Nonane,5-Methyl-5 Propyl-
25	47	11.881	154484	0.01	45518	Octadecane
26	48	11.992	153212	0.01	45556	Heptadecane, 2,6,10,15-Tetramethyl-
27	49	12.055	237569	0.01	49866	Dodecane, 2,6,10-trimethyl-
28	50	12.280	4499220	0.18	1192922	1-Tetradecene

4.3 Toxicity Evaluation

The toxicity of the phytosterol extract was assessed using the brine shrimp lethality assay, which exposed brine shrimp nauplii to various concentrations of the extract. The survival rate was monitored at 6-hour intervals. The survival of the shrimps shows that Phytosterols extracted from *K.alvarezii* has no toxicity.

Table 4.3: *K. alvarezii* toxicity study on Brine shrimps

S.No.	Concentration of *K.alvarezii* extract (µL)	Number of Live shrimps (average)						Number of deaths	Percentage Mortality (%)
		Day 2 t=-24 hrs	Day 3 t=0hrs	Day 3 t= 6 hrs	Day 3 t=12hrs	Day 3 t=18 hr	Day 3 t=24 hr		
1	100	20	20	19	18	18	17	3	15
2	200	20	20	18	18	18	17	3	15
3	300	20	20	19	18	16	16	4	20
4	400	20	20	19	17	17	15	5	25
5	0	20	20	19	18	18	18	2	10

4.4 Discussion

This study focused on the extraction, characterization, toxicity evaluation of phytosterols derived from *Kappaphycus alvarezii*, a red marine alga. The key findings of the project, including the effectiveness of the extraction process, the phytosterol composition, toxicity, and bioactivity, provide valuable insights into the potential applications of these bioactive compounds.

1.Phytosterol Extraction and Characterization

The maceration process proved to be an effective method for extracting phytosterols from *Kappaphycus alvarezii*. By using a solvent mixture of ethanol and chloroform in a 2:1 ratio, a significant yield of phytosterols was achieved. The FTIR and GC-MS analyses confirmed the presence of key phytosterols such as beta-sitosterol, stigmasterol, and campesterol. FTIR identified characteristic functional groups like hydroxyl and carbonyl groups, which are indicative of sterols. GC-MS provided a detailed chemical profile, further supporting the presence of these important phytosterols. These results align with studies that suggest marine algae as a viable and sustainable source of bioactive sterols.

2.Toxicity Evaluation Using Brine Shrimp Assay

The brine shrimp lethality assay provided a simple yet informative approach to assess the toxicity of the extracted phytosterols. The results indicated that the extract was non-toxic at the tested concentrations, which is crucial for its potential use in pharmaceutical, nutraceutical, and cosmetic applications. The calculated percentage mortality, obtained from the assay, helped establish the safe concentration range of the phytosterols. This result is important because non-toxic compounds are preferable in formulations designed for human consumption or topical use.

3.Environmental and Industrial Implications

The study highlights the environmental benefits of utilizing marine algae as a source of phytosterols. Algae cultivation requires minimal resources such as arable land and freshwater, making it an environmentally friendly alternative to land-based crops. Moreover, *Kappaphycus alvarezii* cultivation can support sustainable aquaculture practices while providing valuable bioactive compounds. From an industrial perspective, the extraction of phytosterols opens up new possibilities for their use in pharmaceuticals, functional foods, and cosmetics, enhancing the economic value of marine algae.

CHAPTER 5

CONCLUSION

In conclusion, this project successfully demonstrated that *Kappaphycus alvarezii* is a promising source of phytosterols with low toxicity, making them suitable for use in health-related applications. The maceration process was an effective method for extracting phytosterols, and the characterization using FTIR and GC-MS confirmed the presence of important phytosterols like beta-sitosterol, stigmasterol, and campesterol. Toxicity testing using the brine shrimp assay established that the phytosterol extract was non-toxic at biologically relevant concentrations. The extract was proven to be considerable, further enhancing its potential in the development of nutraceuticals, pharmaceuticals, and cosmetics.

The study also emphasized the environmental and industrial relevance of using marine algae as a sustainable source of bioactive compounds. The results of this research contribute to the growing body of knowledge on marine-derived phytochemicals and support the idea that marine algae can be utilized as a renewable resource for various industries. Future research could focus on optimizing the extraction process, exploring the full spectrum of bioactive compounds in marine algae, and conducting in vivo studies to further validate the health benefits of these phytosterols.

This project lays the groundwork for the broader application of phytosterols from marine algae, contributing to sustainable, natural solutions for health and industrial purposes.

CHAPTER 6

REFERENCES

Abeysiriwardhana, H. N. I., Niroshika, K. K. H., Thilakarathna, M. K. S., & Jayasooriya, R. G. P. T. (2024). Antidiabetic Properties of Seaweeds and Future Potentials. In The Role of Seaweeds in Blue Bioeconomy (pp. 137-154). Bentham Science Publishers.

Assunção, J., Pagels, F., & Guedes, A. C. (2023). Algae biorefinery: strategies for a sustainable industry. In Algae Materials (pp. 399-433). Academic Press.

Hosseini, S. F., Rezaei, M., & McClements, D. J. (2022). Bioactive functional ingredients from aquatic origin: A review of recent progress in marine-derived nutraceuticals. Critical Reviews in Food Science and Nutrition, 62(5), 1242-1269.

Januário, A. P., Félix, R., Félix, C., Reboleira, J., Valentão, P., & Lemos, M. F. (2021). Red seaweed-derived compounds as a potential new approach for acne vulgaris care. Pharmaceutics, 13(11), 1930.

Job, N., Chandran, A. A., Augustine, A., Palliyalli, R. P., & Nevin, K. G. (2023). Sargassum wightii Greville ex J. Agardh Grevilli Extract with Macromolecular Protection Activity In Vitro: Prospecting for Novel Drug Leads Using GC-MS Based Metabolic Profiling. International Journal on Algae, 25(3).

Lomartire, S., & Gonçalves, A. M. (2022). An overview of potential seaweed-derived bioactive compounds for pharmaceutical applications. Marine Drugs, 20(2), 141.

Mahadi, M. (2021). *Kappaphycus alvarezii* regulates cholesterol degradation in hypertensive induced sprague-dawley rats (Doctoral dissertation, Universiti Teknologi MARA).

Nova, P., Gomes, A. M., & Costa-Pinto, A. R. (2024). It comes from the sea: Macroalgae-derived bioactive compounds with anti-cancer potential. Critical Reviews in Biotechnology, 44(3), 462-476.

Pal, D., & Raj, K. (2021). Biological activities of marine products and nutritional importance. Bioactive Natural Products for Pharmaceutical Applications, 587-616., 4.

Pradhan, B., Nayak, R., Patra, S., Jit, B. P., Ragusa, A., & Jena, M. (2020). Bioactive metabolites from marine algae as potent pharmacophores against oxidative stress-associated human diseases: A comprehensive review. Molecules, 26(1), 37.

Tanna, B., & Mishra, A. (2018). Metabolites unravel nutraceutical potential of edible seaweeds: An emerging source of functional food. Comprehensive reviews in food science and food safety, 17(6), 1613-1624.

Tello, P., Santos, J., Calero, N., & Trujillo-Cayado, L. A. (2024). Formulatin and Characterization of Sustainable Algal-Derived Nanoemulgels: A Green Approach to Minimize the Dependency on Synthetic Surfactants. Polymers, 16(2), 194.

Tirtawijaya, G., Mohibbullah, M., Meinita, M. D. N., Moon, I. S., & Hong, Y. K. (2018). The tropical carrageenophyte *Kappaphycus alvarezii* extract promotes axodendritic maturation of hippocampal neurons in primary culture. Journal of Applied Phycology, 30, 3233-3241.

Vidhyanandan, L. M., Kumar, S. M., & Sukumaran, S. T. (2020). Algal metabolites and phyco-medicine. Plant Metabolites: Methods, Applications and Prospects, 291-316.

Yang, B., Zhao, T., Liu, Y., &Lu, B. (2023). Guidelines for extraction and quantitative analysis of phytosterols and oxidation products. *eFood, 4*(4), e108.

Ahmad, M., Waheed, A., & Aslam, M. F. (2018). FTIR analysis of phytochemicals in medicinal plants. *Journal of Medicinal Plant Research*, 12(4), 34-39.

Maldini, M., Montoro, P., & Pizza, C. (2010). Identification and quantification of phytochemicals using GC-MS. *Journal of Analytical Methods in Chemistry*, 45(2), 230–238.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., & McLaughlin, J. L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Medica*, 45(5), 31-34.