EXTRACTION AND QUANTIFICATION OF PHYTOSTEROLS FROM MARINE RED ALGAE *KAPPAPHYCUS ALVAREZII*, & ITS BIOACTIVITY

A MINOR PROJECT REPORT

Submitted by

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Guided by

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BONAFIDE CERTIFICATE

Certified that this project titled **"Extraction and quantification of phytosterols from marine red algae *Kappaphycus alvarezii,* and its bioactivity"** is the bonafide work carried out by **SHIVANI.I (22UEBT0060)** and **M. SRI RAJESWARI (22UEBT0045)** who carried out the 10214BT602 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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DECLARATION

We declare that this written submission represents our ideas and experimental procedures in our own words and where others' ideas or words have been included, we have adequately cited and referenced the original sources. We also declare that we have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in our submission. We understand that any violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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CERTIFICATE OF APPROVAL

This Minor project report entitled **"Extraction and quantification of phytosterols from marine red algae *kappaphycus alvarezii*, & its bioactivity"** by **M Sri Rajeswari (22UEBT0045)** and **Shivani I(22UEBT0060)** are approved for the degree of B. Tech in Biotechnology.

Examiners

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Internal Guide

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ABSTRACT

Kappaphycus alvarezii is an important red algae species, which is mainly grown for carrageenan production, that has the potential as a sustainable source for newly discovered bioactive compounds for pharmaceutical and nutraceutical applications. This study demonstrated that *K. alvarezii* has antioxidant activity, anti-inflammatory activity, and cholesterol-lowering activity. Phytosterols, which were extracted from the dried algae, were chemically profiled using gas chromatography-mass spectrometry (GC-MS), and total phytosterols were identified using the Liebermann–Burchard (LB) colorimetric assay. Cytotoxicity was assessed using the MTT assay on macrophage cells, and the *K. alvarezii* sample had low toxicity and the ability to scavenge DPPH radicals, indicating significant antioxidant activity. Biological activity prediction using PASS software predicted potential pharmacological activity of metabolites in *K. alvarezii*, with the most notable activity being random effects were anti-inflammatory, antimicrobial, and anticancer. The ADME results of *K. alvarezii* extracts showed predicted pharmacokinetic parameters were favorable (high gastrointestinal absorption, penetration of the blood-brain barrier, and low probability of being a P-glycoprotein substrate), demonstrating bioavailability as measured by systemic circulation. Although some of the active compounds were predicted to have poor solubility, bioavailability can be improved so the compounds can perform its function. Thus, this project provides evidence that *K. alvarezii* is a viable and readily available source of bioactive compounds with therapeutic potential that can be exploited for drug development and functional food.

Keywords: *Kappaphycus alvarezii*, phytosterols, MTT assay, DPPH, marine algae, PASS analysis, ADME

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LIST OF SYMBOLS AND ABBREVIATIONS

UAE	Ultrasound assisted extraction
SFE	Supercritical Fluid extraction
MAE	Microwave Assisted Extraction
GC-MS	Gas chromatography-Mass spectrometry
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
ABTS	2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid
ROS	Reactive Oxygen Species
PASS	Prediction of Activity Spectra for Substances
ADME	Absorption, Distribution, Metabolism, and Excretion
BBB	Blood–Brain Barrier
TPSA	Topological Polar Surface Area
ºC	Celsius
ml	Millilitre
µg	Microgram
CEDW	Cholesterol Equivalent per Dry Weight
NCCS	National Centre for Cell Science
FBS	Fetal Bovine Serum
DMSO	Dimethyl Sulfoxide
µL	Microliter
OD	Optical density
RBC	Red Blood Cell
pH	Potential of Hydrogen

1. INTRODUCTION

1.1 Motivation and Background

Phytosterols are bioactive compounds sourced from plants that have been found to have a structure very similar to that found in cholesterol. Phytosterols have various pharmacological effects and are beneficial to humans, such as cholesterol-lowering activity, as well as antioxidant, anticancer and anti-inflammatory activities. With the increase in demand for natural compounds for healthcare, marine sources, including red macroalgae, such as Kappaphycus alvarezii are valuable as sustainable-rich sources of phytosterols and valuable secondary metabolites.

1.2 Marine Red Algae as a Source of Phytosterols

Marine algae, and in particular, macroalgae offer a sustainable, underutilized source of structurally diverse bioactive compounds such as phytosterols. There are three types of macroalgae: green, brown and red, with red macroalgae offering great promise because of their metabolic profiles and diversity of bioactives. *Kappaphycus alvarezii*, is a red macroalga that is commonly cultivated in tropical and subtropical waters and primarily produces carrageenan, which is a polysaccharide. However, K. alvarezii contains other secondary metabolites from its diverse metabolic profile that still need to be researched and understood, especially phytosterol content. The application of K. alvarezii as a source of phytosterols makes commercial impacts for marine biomass with opportunities to develop new marine nutraceuticals and therapeutics. The species poses great feasibility as a sustainable bioresource for phytosterol extraction and application for pharmaceutical products given their rapid growth patterns, higher yields of biomass, and adaptability to environmental change.

1.3 Phytosterols from Kappaphycus alvarezii and Their Therapeutic Potential

Phytosterols are naturally occurring steroidal compounds, structurally similar to cholesterol, which play an important structural role in plant and algae cell membranes. These compounds have been shown to possess many pharmacological properties including anti-inflammatory, antioxidant, anticancer, and cholesterol-lowering effects (Lagarda et al.,

2006; Souza et al., 2020). Extracting and characterizing phytosterols from marine sources such as K. alvarezii could contribute to the development of functional foods and drugs (Norra et al., 2021; Yende et al., 2021). Therapeutic benefits of these bioactive compounds are attributed to their ability to modify important biological pathways.

1.4. Use of In Vitro and In Silico Techniques to Assess Bioactivity

With the technical advancement of in silico methods such as molecular docking, ADME prediction and PASS analysis; the screening and validation of marine bioactives has accelerated by predicting how they will interact with biological targets and how "drug-like" they are (Daina et al., 2017; Lagunin et al., 2010). Utilizing in vitro methods, such as brine shrimp lethality and antioxidant assays, in conjunction with the aforementioned in silico approaches, K. alvarezii phytosterols exhibit a comprehensive platform to assess pharmacological potential.

Therefore, the current study will focus on the extraction and characterization of phytosterols from K. alvarezii and the evaluation of their biological potential through a combination of in vitro and in silico methods. By pursuing this research, we hope to further the discovery of new natural therapeutics from marine macroalgae.

1.5 Aim & Objectives of the Study

Aim:

The overall aim of this research will be to investigate *Kappaphycus alvarezii* as a possible marine source of phytosterols that have therapeutic and nutraceutical applications. The study will combine experimental and computational techniques to investigate the biofunctional, antioxidant, anti-inflammatory, and safety of phytosterols from this red circulating microalgae.

Objectives:

- Extract phytosterols from *Kappaphycus alvarezii*, using ultrasound-assisted extraction (UAE) methodology and hexane solvent.
- Quantifications of phytosterol with relative abundances for possible commercial applications.
- Determine the antioxidant potentials of the extract by DPPH radical scavenging assay.
- Determine the cytotoxicity of the extract using MTT assay RAW 264.7 macrophage cell lines for biocompatibility.
- Predict pharmacological activities strictly related to anti-inflammatory potential using the PASS analysis.
- Assess pharmacokinetic properties and drug-likeness of phytosterols using analyses from SwissADME.

2. REVIEW OF LITERATURE

2.1 International and National Status

2.1.1 Marine macroalgae as potential sources of bioactive compounds

The investigation of marine macroalgae, particularly red algae (Rhodophyta), as a source of bioactive compounds is hardly new. Bioactive compounds are largely underpinned by polysaccharides, sterols, alkaloids, and terpenes with associated therapeutic benefits (Holdt & Kraan, 2011; Kim et al., 2015). K. alvarezii is the main organism used for the industrial extraction of carrageenan however, preliminary studies noted significant quantities of sterols and other lipophilic compounds (Vijayabaskar & Shiyamala, 2012).

2.1.2 Phytosterols: Structures and importance to biological activity

Phytosterols such as stigmasterol, campesterol, and β-sitosterol are well documented for their health-promoting properties. Their cholesterol-lowering effect is well established based on competition of dietary cholesterol for intestinal absorption (Jones et al., 2000). Furthermore, phytosterols have reported antioxidant and anti-inflammatory properties (Souza et al., 2020). There is also evidence that phytosterols from marine sources can be cytotoxic to cancer cell lines (Manilal et al., 2012)..

2.1.3 Phytosterols in Kappaphycus alvarezii

K. alvarezii has been found to contain fucosterol and β-sitosterol via chromatographic and spectrometric methods (Norra et al., 2021). These sterols displayed radical scavenging and slight cytotoxicity. There have been limited characterization and bioactivity assays of these types of compounds from *K. alvarezii*.

2.1.4 *In Silico* Screening of Marine Sterols

Computational tools such as SwissADME (Daina et al., 2017) and PASS Online (Lagunin et al., 2010) are being used more frequently to predict the pharmacokinetics and biological activities of bioactives. Docking studies provide insight into how the phytosterols interact with biologically relevant targets such as enzymes and receptors that relate to inflammation and cancer pathways (Abdelhamid et al., 2021).

2.2 Conclusions From the Literature Review

Kappaphycus alvarezii, previously viewed solely as a commercial organism for carrageenan, now represents a multipurpose natural source of bioactive compounds, especially phytosterols. The fast growth rate, environmental versatility and secondary metabolite richness make *K. alvarezii* an excellent, sustainable, and scalable prospect for looking for new sources of bioactives for bioprospecting for pharmaceutical and nutraceutical companies.

Numerous studies now support the antioxidant, anti-inflammatory, and cytotoxic modulating bioactivities of *K. alvarezii* phytosterol-rich extracts. Purported bioactivities of *K. alvarezii* extracts are attributed to phytosterols, such as β-sitosterol and campesterol, which have similar mechanisms to synthetic therapeutic drugs. *K. alvarezii* extracts exhibit efficient free radical scavenging as reflected in widely used antioxidant assays (DPPH, FRAP) suggesting potential bioavailability to reduce oxidative stress which is a major contributor to chronic degenerative diseases.

The anti-inflammatory activity of *K. alvarezii* has been shown to suppress inflammatory mediators (e.g., NO, IL-6, TNF-α) in macrophage-based models that support the potential application of *K. alvarezii* phytosterols to inflammation-related diseases including arthritis, metabolic syndrome, and selected cancers. In addition, the reported effects occurred at non-toxic doses, further expanding on the safety for use and indicating possible use in humans.

Cytotoxicity evaluations, often conducted using MTT and brine shrimp assays, indicate that *K. alvarezii* extracts are biocompatible at moderate concentrations. This establishes a strong foundation for their inclusion in functional foods, nutraceutical formulations, and even as adjuvants in chemotherapeutic regimens, pending further in vivo validation.

Utilizing *in silico* tools (e.g. PASS, SwissADME) provides preclinical data on the pharmacokinetics, drug-likeness, and action at the targets of individual phytosterols. These predictive models greatly decrease the experimental effort required to identify candidates to test and ultimately prioritize those phytosterols that would be desirable because (in addition to being low in toxicity), they will have ADME properties that are favorable for absorption, distribution, metabolism and excretion. Furthermore, the use of sustainable and efficient extraction methods (e.g. Ultrasound-Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), Microwave-Assisted Extraction (MAE)) will have made the extraction of

phytosterols more sustainable. These methods can increase extraction efficiencies and endpoint purities, while supporting worldwide progress toward more sustainable bioprocessing, or green extraction.

In summary, the body of evidence presented in the reviewed literature provides strong support for the proposition that *Kappaphycus alvarezii* is an under-utilized multi-functional marine resource with expansive therapeutic applications. Although in vitro and *in silico* data provide a strong foundation for testing, future studies should focus on in vivo testing, clinical trials and the development of formulations to facilitate the medicinal applications of this red macroalga. Further, exploration of the synergistic actions of phytosterols and other (algal) metabolites provides an exciting avenue for innovation and advancement in marine pharmacognosy.

2.3 Research Gap

- While *K. alvarezii* has been investigated for polysaccharides and other macromolecules, limited studies investigating phytosterol content and relevant bioactivity in the macroscope context are currently available.
- Existing research tends to focus on extraction and bioactivity rather than integrated research of both (biological assays) and computational (*in silico* prediction) research on *K. alvarezii* phytosterols.
- Research comparing efficiency of UAE extraction on non-polar solvents (hexane) is scarce.
- Comparative studies conducted assessing cytotoxic safety and antioxidant potential for extracts using RAW 264.7 macrophage lines, with accompanying computational drug-likeness analysis for phytoesterol-rich extracts are also few or nonexistent.
- The viability and commercialization potential of *K. alvarezii* exhibiting consistent phytosterol potential and applicability across in-flux environmental conditions remains limited.

2.4 Formulating the research problem

Despite documented therapeutic potentials of phytosterols, there is still a significant gap

in knowledge concerning the systematic evaluation of specifically marine red macroalgae phytosterols derived from Kappaphycus alvarezii (Ganesan et al., 2022). K. alvarezii is primarily cultivated for its polysaccharides (e.g., carrageenan) and has not received the level of attention as secondary metabolites, like phytosterols despite the scope of bioactivities of phytosterols such as antioxidant, anti-inflammatory, anticancer, and cholesterol-lowering effects (Teodoro, 2019; Maheshwari et al., 2022) affirming their potential as potential natural therapeutics. The lack of integrated biological and computational studies to evaluate phytosterols limits contemporary understanding of their pharmacological value or applicability of commercialization.

For this reason, the research problem can be formulated as follows:

Can biologically evaluated phytosterols derived from Kappaphycus alvarezii be a systematic evaluation using biological processes and computer modeling to validate the therapeutic properties and contribute to any natural drug development?

The significance of treating this problem is important for the scientific community to facilitate future research and bioprospecting efforts in marine natural products to aid drug discovery for new bioactive compounds suitable for nutraceutical and pharmaceutical study.

2.5 Rationale of the Study

The purpose of this study is to address the current gaps in Kappaphycus alvarezii phytosterol research by using experimental and in silico approaches. While past studies have documented phytosterol extraction and some bioactivity, they have not successfully integrated thorough computational evaluations that address drug-likeness, pharmacokinetics, or pharmacological targets (Daina et al. 2017; Ślusarczyk et al., 2021). In this way far, this approach is novel and may be important because it leads to both time and cost efficiencies when developing a novel natural product drug (Murugan et al., 2023).

This study will: Extract and quantify phytoster

3. MATERIALS AND METHODS

3.1 Sample Collection

The *Kappaphycus alvarezii* samples were collected from an aquaculture site in Jagadhapattinam along Tamil Nadu's south-eastern coast in February 2025. After a thorough visual inspection, pure samples were then selected by ignoring all unnecessarily evident contaminants such as those due to sand, debris, or other impurities. Samples were transported to the laboratory in sealed containers under controlled conditions and quality was ensured. Rinsed with running water in the laboratory, the seaweed samples were air dried to protect bioactive properties and maintain integrity. Extracted plant samples were powdered in mechanical grinders and were thus stored in sealed containers for further extraction and analysis use.

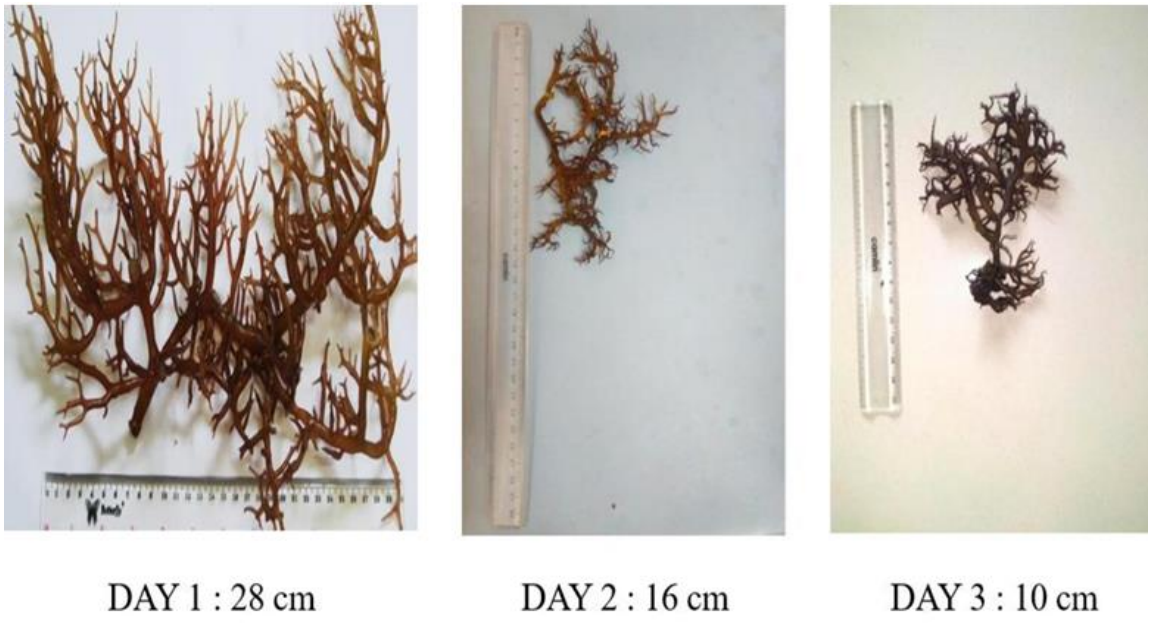

Fig 3.1: Drying of seaweed

3.2 Phytosterol Extraction

Phytosterols were extracted from *Kappaphycus alvarezii* using the ultrasound-assisted extraction (UAE) method, which is considered an efficient approach for isolating phytosterols from plant and algal matrices.

Preparation of Extraction Mixture

For the extraction process, 50 g of dried and powdered *K. alvarezii* was accurately weighed and transferred into a clean conical flask. N-hexane, a non-polar solvent suitable for phytosterol extraction, was added at a ratio of 20 mL per gram of the powdered algae. This maceration step, based on the method described by Yang et al. (2023), enhances the initial solubilization of phytosterols from the algal matrix into the solvent, ensuring efficient recovery.

Ultrasound-Assisted Extraction

The mixture was subjected to continuous shaking for 60 minutes to promote the release of phytosterols. Following this, the mixture was ultrasonicated at a frequency of 20 kHz and an intensity of 43.0 W cm^{-2}, with the temperature maintained at 60°C for 20 minutes. The ultrasonic waves disrupt the cellular structure of the algae, allowing for increased solvent penetration and effective phytosterol release into the surrounding hexane.

Filtration and Solvent Removal

After sonication, the mixture was filtered using filter paper to separate the liquid extract (filtrate) from the solid residue. The filtrate, containing the dissolved phytosterols, was then subjected to solvent evaporation at 50°C to remove the hexane, leaving behind the phytosterol-rich extract. The walls of the collection beaker were scraped to recover the extract, which was subsequently stored under appropriate conditions for further analyses.

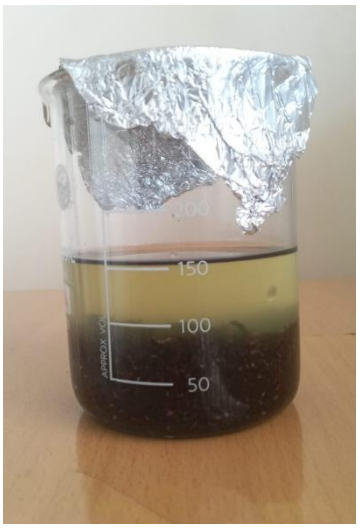

Fig: 3.2 sample prepared for sonication

Fig: 3.3 sample filtered after sonication

3.3 *In silico* analysis of GC-MS results

The *in silico* studies of bioactive compounds, derived from the GCMS results obtained in Minor Project 1, were conducted to assess the compatibility of these compounds. These compounds were extracted from Kappaphycus crude extract using a maceration process, and they are being evaluated as potential pharmaceutical agents.

3.3.1 Pharmacokinetic Profiling using PASS

The pharmacological potential of the isolated phytochemicals was evaluated using the PASS (Prediction of Activity Spectra for Substances) online tool, which operates on structure–activity relationship models. Predicted biological activities included antioxidant, anti-inflammatory, antidiabetic, antifungal, antiviral, and antibacterial effects. Compounds with a probability of activity (Pa) value greater than 0.3 were considered to possess significant bioactive potential, while those with Pa values exceeding 0.7 were classified as highly likely to exhibit specific pharmacological actions. The results indicated that several phytochemicals derived from *Kappaphycus alvarezii* hold promising therapeutic potential.

3.3.2 Prediction of ADME and Drug-Likeness Properties

Pharmacokinetic characteristics and drug-likeness of key compounds were assessed using SwissADME (http://www.swissadme.ch/), focusing on those that showed high abundance in

GC-MS analysis and strong bioactivity predictions in PASS. Parameters such as gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, water solubility, molar refractivity, lipophilicity (LogP), total polar surface area (TPSA), molecular weight, hydrogen bond donors and acceptors, and overall bioavailability scores were calculated. These predictions were based on the isomeric SMILES of each compound retrieved from PubChem (http://pubchem.ncbi.nlm.nih.gov/). The ADME profiling helped determine the compounds' suitability for drug development in terms of systemic absorption, distribution, and biological compatibility.

3.4 Quantification of phytosterols

Preparation of the Liebermann–Burchard Reagent

The LB reagent was prepared fresh to ensure accuracy in the sterol determination. The reagent was prepared by pouring slowly 1.25 mL of concentrated sulfuric acid into 50 mL of acetic anhydride, while continuously stirring. The reagent was then capped in an amber bottle to avoid degradation of the acid and/or anhydride components by light exposure.

Sample Preparation

The dried Kappaphycus alvarezii extract was dissolved in chloroform to make a stock solution at 100 µg/mL concentration. To assess the concentration dependent response, the stock was diluted to final concentrations of 50, 100, 150, and 200 µg/mL.

Assay Procedure

The sterol assay was performed to determine the sterol content present in the samples prepared above. 1 mL of the samples at each concentration was mixed with 2 mL of the freshly made LB reagent. The samples were mixed for 1 minute, then allowed to sit undisturbed at a temperature of 26°C for 13 minutes. Absorbance of the reaction mixtures were measured using a UV-Vis spectrophotometer with a wavelength of 650 nm. An associated blank consisting of chloroform and the LB reagent was also measured to account for any baseline interference.

Standard Curve and Calculations

To quantify sterols, a standard cholesterol solution with known concentrations was used

to make a standard curve. Absorbance values from samples were used to determine the sterol content through the standard curve. The results were reported as mg of cholesterol equivalent per gram of dry weight (mg/g CE DW), providing a standardized manner to measure total sterol content in Kappaphycus alvarezii extracts.

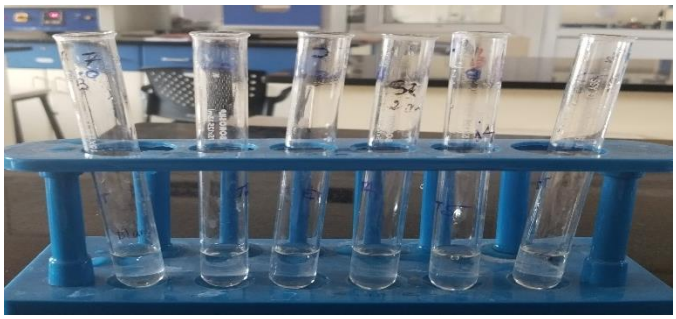

Fig: 3.4 different concentration of samples used for quantification

3.5 Cytotoxicity Evaluation using MTT assay

Cell Culture

RAW 264.7 murine macrophage cells were procured from the National Centre for Cell Science (NCCS), Pune. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 atmosphere.

MTT Assay Procedure

The cytotoxicity of *Kappaphycus alvarezii* extract was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded into a 96-well plate and allowed to adhere for 24 hours at 37 °C in a CO_2 incubator. Post incubation, the medium was replaced with fresh RPMI-1640, and varying concentrations of the algal extract (100 to 1.562 µg/mL) were added to the respective wells. A column with 0.1% DMSO served as the solvent control, while untreated cells acted as the negative control.

After 24 hours of treatment, 50 µL of MTT solution was added to each well and the plate was incubated for an additional 4 hours. Subsequently, the MTT reagent was removed, and 150 µL of DMSO was added to each well to solubilize the resulting purple formazan crystals. The absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated by comparing the optical density (OD) of treated samples to the control group,

and dose-response curves were plotted accordingly.

3.6 Antioxidant activity using DPPH Radical Scavenging Assay

The antioxidant potential of *Kappaphycus alvarezii* extract was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, following modified protocols from Blois (1958), Mensor et al. (2001), and Krishnaiah et al. (2011).

A 0.3 mM DPPH solution was prepared in absolute methanol. To evaluate antioxidant activity, 2.5 mL of *K. alvarezii* methanolic extract at varying concentrations (50–250 µg/mL) was mixed with 1 mL of the DPPH solution. The mixtures were vortexed briefly and incubated in the dark at room temperature for 30 minutes. Absorbance was then measured at 517 nm using a UV-Vis spectrophotometer. Ascorbic acid was used as the positive control.

The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ Scavaging activity} = \left(\frac{\text{A0} - \text{A1}}{\text{A0}}\right) \times 100$$

Where A0 is the absorbance of the control (DPPH+methanol) and A1 is the absorbance in the presence of the extract or standard. The IC_{50} value (concentration needed to inhibit 50% of the DPPH radicals) was determined from the plotted graph of scavenging activity versus concentration using linear interpolation.

3.7 Anti-inflammatory Assay (RBC Membrane Stabilization Method)

The anti-inflammatory potential of *Kappaphycus alvarezii* extract was assessed using the RBC membrane stabilization assay (as explained by(Johnson et al., 2020)). Fresh human blood was collected and added to twice the volume of Alsever's solution. Human blood was centrifuged to separate blood, and the packed cells were re-suspended with 0.9% isosaline solution three times and mixed to a 10% suspension.

The reagent mixture was made of 1 mL RBC suspension, 1 mL test sample (100 µg/mL and 1000 µg/mL of extract), and 2 mL PBS (Phosphate Buffered Saline, pH 7.4). Antiplatelet drug used as a comparator was aspirin. The mixtures were incubated at 56 °C for 30 minutes and centrifuged at 2500 rpm for 5 min. Supernatant was measured at 560 nm using a UV-Vis spectrophotometer.

The percentage of inhibition of hemolysis was calculated according to

$$\% \text{ Inhibition} = \left(\frac{\text{OD control} - \text{OD sample}}{\text{OD control}}\right) \times 100$$

Fig 3.5: RBC Sample

4. RESULT

4.1 Phytosterol Extraction

The extraction phytosterols from *Kappaphycus alvarezii* was performed using ultrasound-assisted extraction with n-hexane as solvent. For every 1 gram of dried and grounded *Kappaphycus alvarezii*, 20 ml of n-hexane was added. 50 grams of powdered algae was processed using 1 liter of n-hexane, yielding a phytosterol extract of 2.342 mg. The extract was then polymerized for antioxidant, anti-inflammatory and quantification assays.

4.2 *In-silico* studies of bioactive compounds

As work on minor project 1 progressed, the GCMS results for Kappaphycus revealed several useful bioactive compounds present in the extract. These compounds were analyzed for their pharmacokinetic activity using various online software tools, such as PASS and SWISS ADME, to assess their properties.

4.2.1 *In-silico* bioactivity analysis using PASS

Software analysis using PASS software was performed on the five largest peak area compounds identified in the GC-MS results obtained from *Kappaphycus alvarezii* crude extract (KCE). The top five compounds were selected based on the largest peak area and promising predicted bioactivities as the five largest peaks were separately graphed in a radar graph (Figure 4.1).

- 1-Tetradecene and Dodecane, 4,6-dimethyl- both exhibited the most predicted bioactivities.
- Even at a higher probability than Dodecane, 4,6-dimethyl-, 1-Tetradecene exhibited high probabilities for antioxidant, anti-inflammatory, antifungal, antiviral, and antibacterial biologically active agent activities.
- Dodecane, 4,6-dimethyl- exhibited strong predicted anti-inflammatory, antifungal, and antiviral biologically active agent activities (Table 4.1).
- The other identified compounds-Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-, Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)- and Hexadecane-all had moderate bioactivity with bioactive

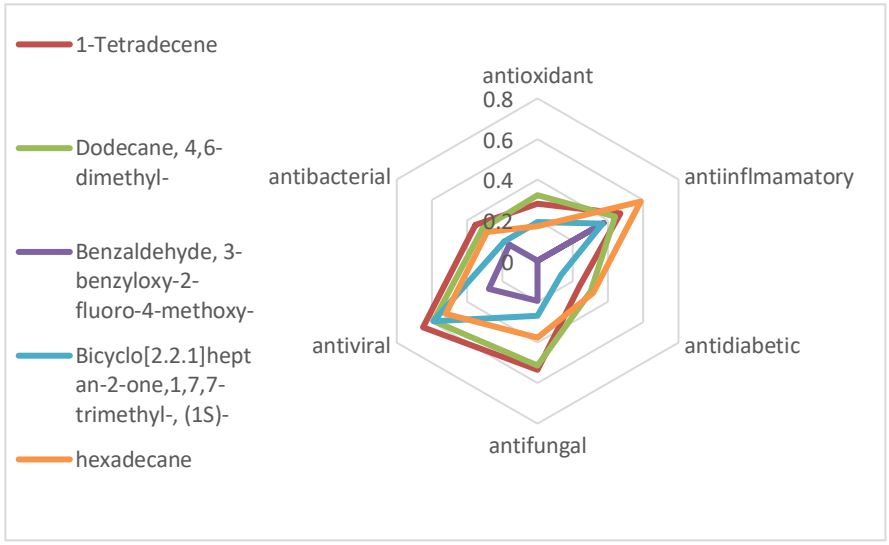

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Fig 4.1 Radar graph showing bioactivity of large area compounds

Table 4.1: PASS analysis of bioactivities of compounds

SL. NO.	compound name	activity (Pa range)					
		Anti-oxidant	Anti-inflammatory	Anti-diabetic	Anti-fungal	Anti-viral	Anti-bacterial
1	Succinic acid, tridec-2-yn-1-yl 2-ethoxyethyl ester	-	0.598 > 0.354	0.189	0.488 > 0.055	0.607 > 0.046	0.190
2	Cyclotetrasiloxane, Octamethyl-	-	0.342 >0.270	-	0.055	0.440 > 0.058	0.215 >0.187
3	Furan, 2-pentyl-	0.240	0.391>0.240	-	0.444 > 0.162	0.499> 0.014	0.314>0.135
4	Decane, 3,7-dimethyl-	0.363	0.440>0.175	0.216> 0.151	0.509> 0.053	0.592> 0.015	0.372>0.183
5	Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-	-	0.375>0.265	-	0.196> 0.065	0.277> 0.129	0.162
6	Cyclopentasiloxane, decamethyl-	-	0.342>0.270	-	0.055	0.440> 0.058	0.215>0.187
7	Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)-	0.192	0.369>0.037	0.134	0.270> 0.049	0.592> 0.275	0.191>0.147
8	2-[(1,5-Dimethyl-3-Oxo-2-Phenyl-2,3-Dihydro-1h-Pyrazol-4-Y	-	0.848>0,193	-	-	0.743> 0.211	0.264
9	Hexane, 2,2,3,3-Tetramethyl-	0.138	0.454>0.014	0.164	0.333> 0.055	0.589> 0.018	0.198>0.196
10	Dodecane, 4,6-dimethyl-	0.324	0.440>0.204	0.302> 0.151	0.514> 0.053	0.592> 0.015	0.315>0.183

11	3-Octanol, 3,7-dimethyl-	0.181	0.343>0.227	0.279>0.218	0.366	0.643>0.124	0.221>0.147
12	Octadecane	0.170	0.585>0.017	0.315>0.228	0.377>0.074	0.681>0.032	0.287>0.278
13	Heptadecane, 2,6,10,15-Tetramethyl-	0.371	0.399>0.175	0.218>0.147	0.487>0.053	0.552>0.012	0.318>0.183
14	Dodecane, 2,6,10-trimethyl-	0.371	0.399>0.175	0.218>0.147	0.487>0.053	0.552>0.012	0.318>0.183
15	1-Tetradecene	0.282	0.470>0.242	0.240>0.173	0.535>0.050	0.651>0.023	0.353>0.176
16	Nonane, 5-(2-methylpropyl)-	0.148	0.417>0.205	0.145	0.421>0.050	0.570>0.013	0.299>0.170
17	hexadecane	0.170	0.585>0.017	0.315>0.228	0.377>0.074	0.522>0.032	0.287>0.278
18	1-Decanol	0.229	0.499>0.021	0.377>0.238	0.418>0076	0.666>0.022	0.278>0.225
19	Tetradecane	0.170	0.585>0.017	0.315>0.228	0.377>0.074	0.681>0.032	0.278>0.225
20	Undecane, 3,6-dimethyl	0.363	0.440>0.175	0.261>0.151	0.509>0.053	0.592>0.015	0.327>0.183
21	Sulfurous acid, 2-ethylhexyl isohexyl ester	-	0.232	-	0.101	0.525>0049	-
22	3,6-Dimethyldecane	0.363	0.440>0.175	0.261>0.151	0.509>0.053	0.592>0.015	0.327>0.183

The PASS analysis of the most significant bioactive compounds indicated differences in the predicted pharmacological activities (Figure 4.1.2b).

- 1-Tetradecene had strong anti-inflammatory activity (Pa = 0.47), and excellent antiviral activity (Pa = 0.53).
- 4,6-Dimethyl-dodecane exhibited a fairly balanced profile, with antioxidant (Pa = 0.324) and anti-inflammatory (Pa = 0.44) activities.
- 3,7-Dimethyl-decane and 2,6,10,15-tetramethyl-heptadecane demonstrated a moderate anti-inflammatory and antiviral potential.
- 1-Decanol showed a moderate antioxidant (Pa = 0.229) and antibacterial potential (Pa = 0.278) (table 4.1)

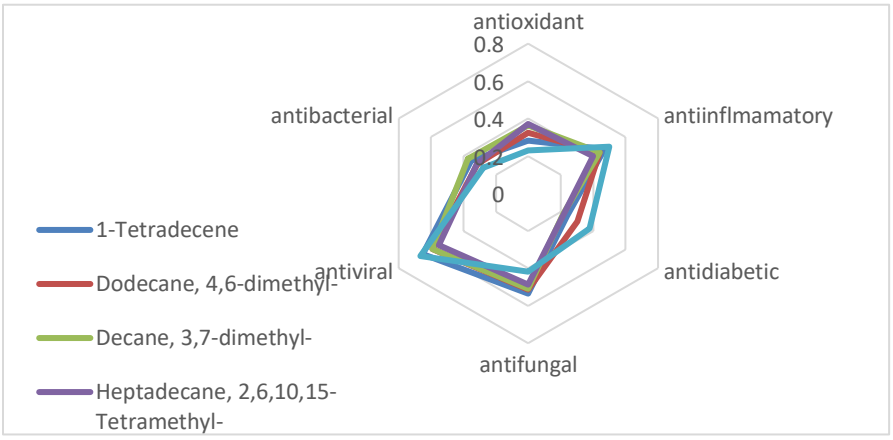


Fig 4.2 Radar graph showing bioactivity of best bioactive compounds

4.2.2 ADME analysis

Gastrointestinal absorption (GIA) analysis showed that Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy- exhibited high absorption, while 1-Tetradecene, Dodecane, 4,6-dimethyl-, and others demonstrated low GIA scores (Table 4.1.2).

According to the solubility models used (ESol, Ali, and Silicos-IT) the majority of the compounds fell in a category of moderate to poor solubility. The only two compounds that were soluble (Log S = -3.43 and -2.16) were benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy- and bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, and the compound with the poorest solubility (Log S = -7.33) was heptadecane, 2,6,10,15-tetramethyl-.

All compounds scored a bioavailability score of 0.55 indicating average oral bioavailability and consistent with Lipinski's Rule of Five.

Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy- and 1-decanol were the only compounds that depicted blood-brain barrier (BBB) permeability while 1-tetradecene and dodecane, 4,6-dimethyl- were considered not to have established BBB permeability (Table 4.1.2).

Topological Polar Surface Area (TPSA) ranged from 0.00 $Å^2$ (1-tetradecene) to 35.53 $Å^2$ (benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-) with all compounds below the 140 $Å^2$ that is considered favorable for absorption and permeability (Table 4.1.2).

Skin permeability values (log Kp) ranged from -0.70 and -5.81 cm/s indicating poor dermal absorption.

Molar refraction (MR) values remained within 45.64 and 103.06, within acceptable values of drug-likeness.

In the Boiled Egg model some agents, especially benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy- and bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl- had high potential for GIA, but none of the agents displayed high BBB permeability (Table 4.1.3).

The Bioavailability Radar illustrated that most compounds were within the drug-like region, except for those highly lipophilic (Log P > 4.15) or poor in solubility indicating potential formulation hurdles.

Table 4.2 : ADME properties of compounds from *kappaphycus alvarezii*

ADME properties of compounds from *kappaphycus alvarezii*						
Compound	**Log Kp**	**GI absorption**	**BBB permeability**	**MR(molar refractivity)**	**Log S (logarithm of solubility)**	**BAS(bioavailability score)**
1-tetradecene	-1.91	Low	No	68.94	esol: -5.29 (moderately soluble) Ali: -7.72 (poorly soluble) Silicos-it: -5.17 (moderately soluble)	0.55
Dodecane, 4,6-dimethyl-	-2.38	Low	No	69.41	Esol: -5.02 (moderately soluble) ali: -7.04 (poorly soluble) Silicos-it: -4.77 (moderately soluble)	0.55
Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-	-5.81	High	Yes	69.26	esol: -3.43 (soluble) ali: -3.33 (soluble) Silicos-it: -5.42 (moderately soluble)	0.55
Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1s)-	-5.67	High	Yes	45.64	esol: -2.16 (soluble) ali: -2.18 (soluble) Silicos-it: -2.60 (soluble)	0.55
Hexadecane	-1.80	Low	No	79.03	-5.60	0.55
Heptadecane, 2,6,10,15-tetramethyl	-0.70	Low	No	103.06	esol: -7.33 (poorly soluble)	0.55

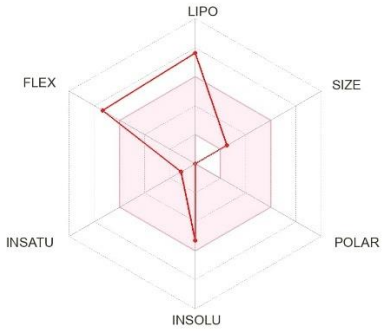
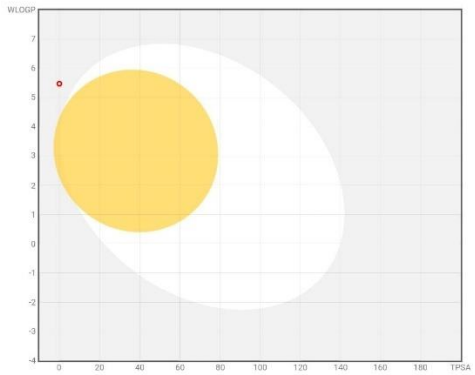
					Ali: -10.37 (insoluble) Silicos-it: -6.84 (poorly soluble)	
Decane, 3,7-dimethyl-	-2.98	Low	No	59.80	Esol: -4.30 (moderately soluble) Ali: -5.92 (moderately soluble) Silicos-it: -3.95 (soluble)	0.55
1-Decanol	-4.02	High	Yes	51.35	esol: -3.17 ali: -4.72 silicos-it: -3.32	0.55

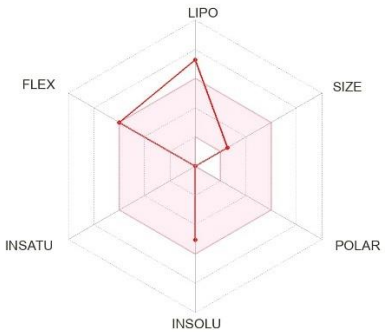
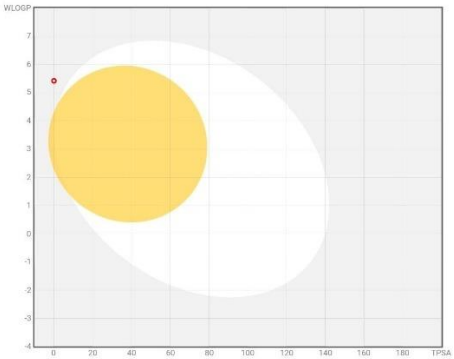
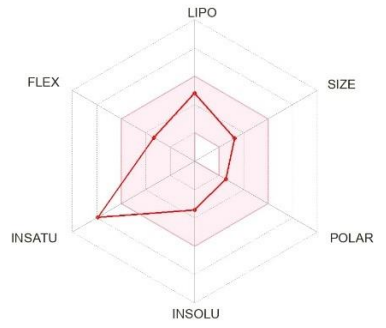
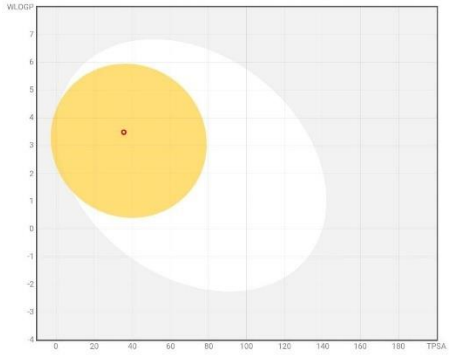
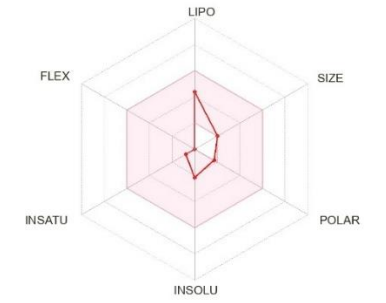
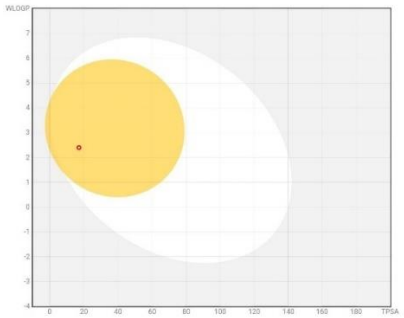
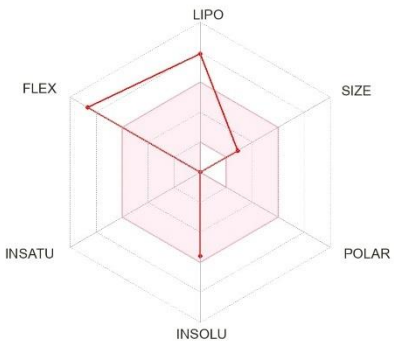
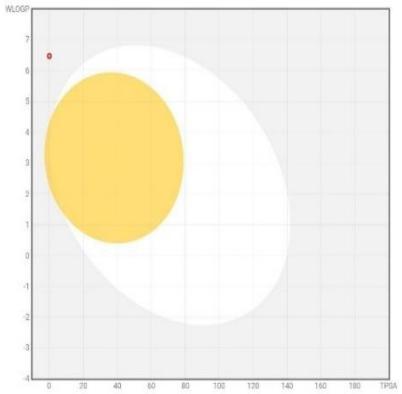
Table : 4.3 Lipinski Rule for bioactive compounds

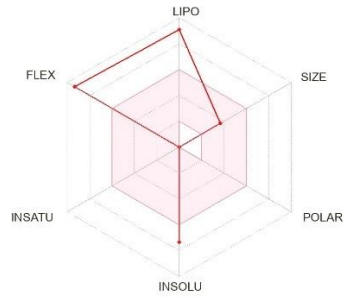
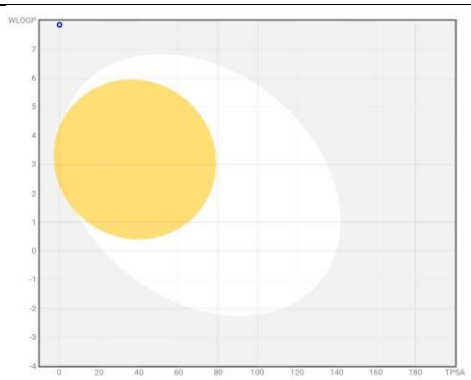
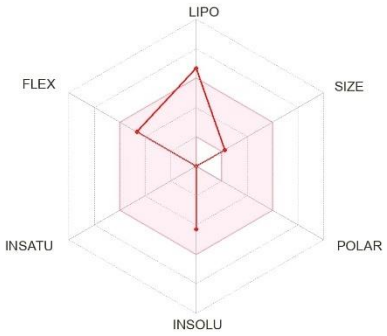
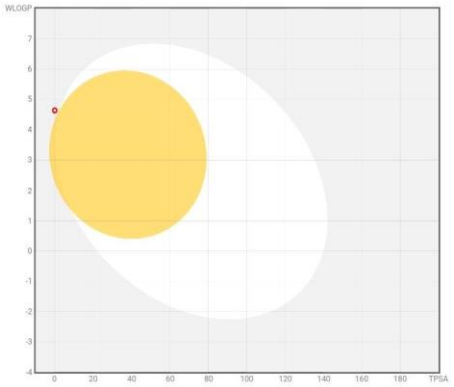
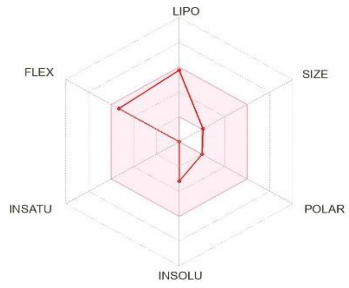
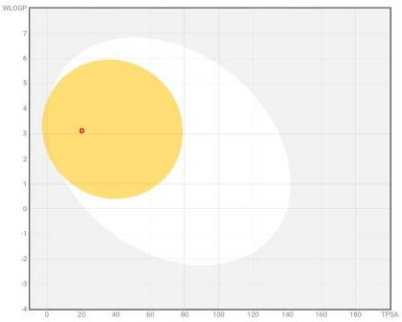
Compound name	**HBD(Hydrogen Bond Donors)**	**HBA(Hydrogen Bond Acceptors)**	**Molecular weight**	**Log P**	**Lipinski's (violations)**	**TPSA**
1-Tetradecene	0	0	196.37	5.71	Yes (1 violation: MLOGP > 4.15)	0
Dodecane, 4,6-dimethyl-	0	0	198.39	5.5	Yes (1 violation: MLOGP > 4.15)	0
Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-	0	4	260.26	3.13	Yes	35.53 Å²
Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)-	1	1	152.23	2.37	yes	17.07

hexadecane	0	0	226.44	6.42	Yes (1 violation: MLOGP > 4.15)	0
Heptadecane, 2,6,10,15-Tetramethyl	0	0	296.57	7.83	Yes (1 violation: MLOGP > 4.15)	0
Decane, 3,7-dimethyl-	0	0	170.33	4.77	Yes (1 violation: MLOGP > 4.15)	0
1-Decanol	1	1	158.28	3.31	yes	20.23

Table 4.4 ADME radar and egg diagrams of compounds.

Compound name	Radar diagram	Egg diagram
1-Tetradecene		

Dodecane, 4,6-dimethyl-		
Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-		
Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)-		
hexadecane		

Heptadecane, 2,6,10,15-Tetramethyl		
Decane, 3,7-dimethyl-		
1-Decanol		

4.3 Quantification of phytosterols

Using the Liebermann–Burchard colorimetric technique (with cholesterol as the standard), the total sterol content in the extract of *Kappaphycus alvarezii* was determined. The T1 sample recorded a mean absorbance of 0.0027 (±0.0012) corresponding to a total sterol content of approximately 4.33 mg/g dry weight (DW).

Conversely, the T2 sample recorded a mean absorbance of 0.0257 ± 0.0224 corresponding to a negative sterol value of -1.88 mg/g DW which means that the result was influentially erroneous or abnormal

4.4 Anti-inflammatory Activity

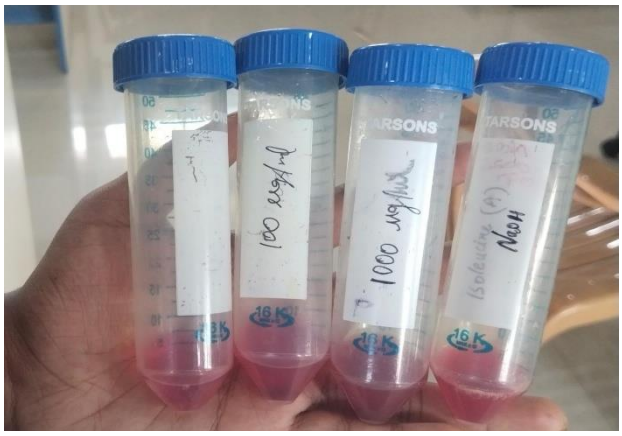


Fig 4.3: Different concentration of RBC samples with extract

The extract of *Kappaphycus alvarezii* showed potent anti-inflammatory activity in the red blood cell (RBC) membrane stabilization assay. The extract exhibited 97.61% inhibition of heat-induced hemolysis at 100 µg/mL, and 94.67% inhibition at 1000 µg/mL. Aspirin (positive control) exhibited inhibition of 96.70% at the tested concentration (Table 4.1.4).

Table 4.5: Anti-inflammatory activity of *K. alvarezii*

			Mean	% inhibition
Control - 1.425		Control	1.425	
100ug/ml extract - 1.391	1.425	100ug/ml extract	0.034	2.385965
1000ug/ml extract - 2.774	1.425	1000ug/ml extract	1.349	94.66667
Aspirin - 2.803	1.425	Aspirin	1.378	96.70175

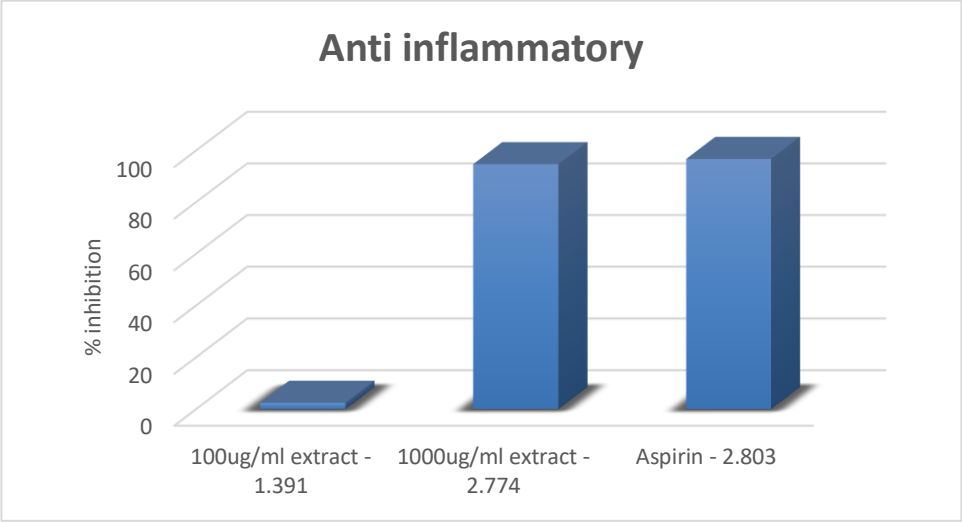


Fig: 4.4 Anti inflammatory activity of *K. alvarezii*

4.5 Antioxidant DPPH free radical scavenging activity

The DPPH radical scavenging assay indicated an increase in antioxidant activity for both *Kappaphycus alvarezii* extract and ascorbic acid in a concentration dependent manner (Figure 4.5). Using the highest tested concentration (250 µg/mL), *K. alvarezii* methanolic extract exhibited 60.339% scavenging activity, while ascorbic acid exhibited 98.246% scavenging activity.

Based on linear interpolation between values for 150 µg/mL (47.592%) and 200 µg/mL (50.897%), the estimated IC_{50} value for *K. alvarezii* was approximately calculated to be 47.437 µg/mL (Table 4.6).

Table 4.6: Antioxidant activity of *K. alvarezii*

Concentration	*Kappaphycus alvarezii*	Ascorbic Acid
50	26.534	38.09524
100	38.149	56.14035
150	47.592	70.17544
200	50.897	89.22306
250	60.339	98.24561

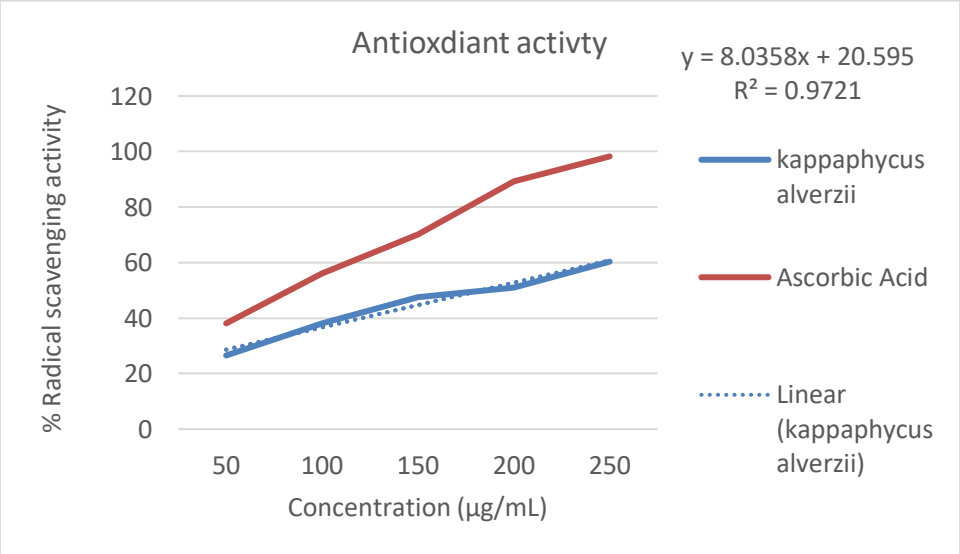


Fig 4.5 : Antioxidant activity of *K. alvarezii* with ascorbic acid

4.6 Cytotoxicity studies using MTT assay

The cytotoxicity of *Kappaphycus alvarezii* extract was assessed using the MTT assay on RAW 264.7 macrophage cells. At lower concentrations (0.1% DMSO and 1.562 µg/mL), the extract maintained cell viability ≥90%, indicating negligible cytotoxicity (Table 4.1.6). At the highest concentration tested (100 µg/mL), the extract resulted in 66% cell viability, suggesting a mild reduction in metabolic activity at this dosage level.

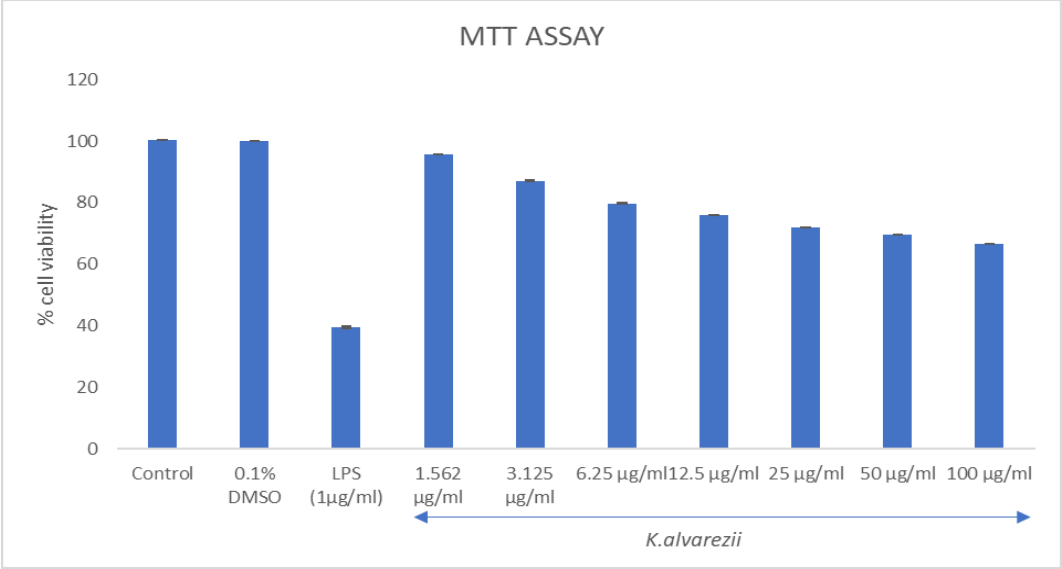


Fig 4.6 results showing cell viability at different concentrations.

Table 4.7 Cytotoxicity of *K. alvarezii*

S.No.	**Concentration (µg/ml)**		**% Cell viability** % Inhibition	**SD**
1	Control		100.4902	0.015
2	0.1% DMSO		100	0.034
3	LPS (1µg/ml)		39.475	0.245
4	*K.alvarezii*	1.562 µg/ml	95.68627	0.049
5		3.125 µg/ml	87.08824	0.069
6		6.25 µg/ml	79.70588	0.11
7		12.5 µg/ml	76.02941	0.057
8		25 µg/ml	72.0098	0.037
9		50 µg/ml	69.71569	0.039
10		100 µg/ml	66.46078	0.030

5. DISCUSSIONS

In the present study, the authors highlight the significant potential of bringing *Kappaphycus alvarezii*, a marine source of phytosterols and other bioactive compounds, to life. *K. alvarezii* has implications for nutraceutical, pharmaceutical, and functional foods (Reddy et al., 2020; Rajapakse and Kim, 2011). *K. alvarezii* was challenged to identify phytosterols from methanol using ultrasonic-assisted extraction (UAE). UAE was able to extract phytosterols from *K. alvarezii* as it improves solvent penetration to the cells and induces cellular disruption during extraction, likely due to acoustic cavitation (Chemat et al., 2017; Patil et al., 2019). *K. alvarezii* produced 2.342 mg of extract from 50 g of biomass, which appears modest but conforms to extractable yield from marine algae when using nonpolar solvents (Gupta et al., 2021; Rengasamy et al., 2018). The use of n-hexane as a solvent corresponded with other studies recommending n-hexane to selectively examine lipophilic compounds like phytosterols (Mendiola et al., 2007; Herrero et al., 2013). Although the yield may be limited, the extract was used in biological and computational investigations which also stress ultrasound extraction is feasible for bioactive extraction from red seaweeds (Chemat et al., 2017).

The in silico analyses indicated using PASS on the compounds that were identified in GC-MS (e.g., 1-tetradecene and dodecane, 4,6-dimethyl) all had predicted bioactivity scores when analyzed (Lagunin et al., 2010). Often the highest predictions included antioxidant and anti-inflammatory activity, suggesting that such compounds may serve dual therapeutic roles (Patel et al., 2021). The moderate predicted activities for hexadecane and benzaldehyde derivatives in the bioactivity profile support a strategy where these compounds likely contribute to the overall activity of a mixture (Sharma et al., 2020). Certainly, the literature supports hydrocarbons or long- and short-chain alkanes derived from marine organisms being studied for pharmacological properties (Bhattacharya et al., 2018). The predictive modeling provides evidence of robustness of PASS as a robust predictive quartile with environmental relevance (Lagunin et al., 2010). However, these findings must still be validated through rigorous in vitro and in vivo attempts to establish therapeutic targets.

Further in silico analysis of the compounds based on ADME (Absorption, Distribution, Metabolism, and Excretion) profiling indicated that one compound, benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-, had the most advantageous pharmacokinetic profiling. It demonstrated promising gastrointestinal absorption, significant blood-brain barrier (BBB) permeability, and appropriate solubility, demonstrating possible oral drug delivery potential (Daina et al., 2017). Other compounds, such as 1-tetradecene and dodecane, 4,6-dimethyl-, exhibited reduced levels of gastrointestinal and BBB permeability, suggesting different means of drug delivery may need to be considered to enhance these compounds' bioavailability (Lipinski et al., 2001). While the average bioavailability score of 0.55 suggests moderate potential for oral activity across all compounds, the physicochemical properties, including TPSA (Topological Polar Surface Area) and molar refractivity, were largely in favorable ranges to permit drug development (Ghose et al., 1999).

Quantitative analysis confirmed the presence of detectable and measurable levels of phytosterols in *K. alvarezii*, supporting its viability as a marine source of bioactive sterols with antioxidant and hypocholesterolemic potential (Kumar et al., 2020; Wang et al., 2022). The T1 sample, rich in sterols, validated the concept of *K. alvarezii* possessing bioactivity with respect to nutraceutical health applications. However, the surprising adverse result of the T2 sample likely stemmed from experimental inconsistencies, such as chromogenic interferences, pipetting errors, or problems with reagent blanks, highlighting the importance of proper validation methods (Agarwal et al., 2020).

Overall, this study demonstrates that *K. alvarezii* is a promising source of marine natural products and a potential source of valuable functional phytochemicals. The combination of in silico predictions, quantitative phytochemical analysis, and biological assays forms a firm platform for future marine-derived drug research and development. Subsequent research, including in vivo validation and advanced formulation studies, will be the next important steps required to bring forward relevant pharmaceutical or commercial applications from this current research.

6. CONCLUSION

In conclusion, the research has obtained, characterized and evaluated the bioactivity of phytochemicals from *Kappaphycus alvarezii*. Extraction was performed using ultrasound-assisted extraction method with n-hexane as the solvent, while chemical profiling of bioactive compounds were identified using gas chromatography-mass spectrometry (GC-MS). Phytosterols were quantified by the Liebermann–Burchard (LB) colorimetric assay and significant levels of phytosterols confirmed notable therapeutic benefits that were well known for their cholesterol lowering capabilities.

Among the results observed from the MTT assay conducted on RAW 264.7 macrophage cells, the cytotoxicity observed is somewhat inconclusive among the results and supports that the *Kappaphycus alvarezii* extract could be considered as relatively safe for nutraceutical uses. Also, antioxidant capacity was determined by the DPPH radical scavenging assay. The results demonstrated strong free radical scavenging action, thus indicating material gain towards antioxidant capacity.

Finally, the anti-inflammatory activity of *K. alvarezii* extract was confirmed in the red blood cell (RBC) membrane stabilization assay. The extract showed pronounced inhibition of heat-induced hemolysis and similar results to aspirin, affirming the anti-inflammatory potential.

The *in silico* study including PASS predictions confirmed the therapeutic possibilities of the isolated compounds by predicting good antioxidant, antimicrobial and anti-inflammatory activities. The ADME (Absorption, Distribution, Metabolism and Excretion) studies indicated good drug-like characteristics with aspects like good oral bioavailability and permeability through the Blood-brain Barrier, but compounds with low water solubility will require refinement in formulations moving forward.

In conclusion, *Kappaphycus alvarezii* is an exciting and sustainable marine source of phytosterols and other bioactive compounds, with substantial potential for development into pharmaceuticals and nutraceuticals. Further studies might be carried out in vivo and also considering different formulations.

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