

ISOLATION OF ANTIMICROBIAL ACTIVITY FROM MARINE SEAWEED FOR AQUACULTURE APPLICATIONS

PROJECT WORK

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
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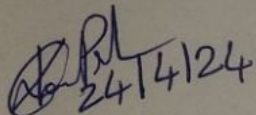
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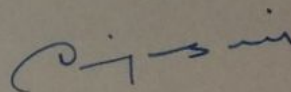
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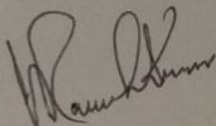
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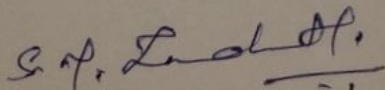
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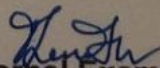
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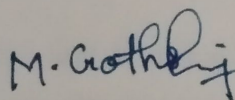
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DECLARATION

I, GOTHATHARSHINI M(41300010) hereby declare that the Project report entitled **ISOLATION OF ANTIMICROBIAL ACTIVITY FROM MARINE SEAWEED FOR AQUACULTURE APPLICATIONS** done by me under the guidance of **Ms. KAVI PRABA** (Internal) and **Dr. M. V. RAJESHWARI** (External) at **Centre For Aquaculture, Sathyabama Research Park**. Submitted in partial fulfilment of the requirements for the award of a Bachelor of Science degree in Microbiology.

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ABSTRACT

Marine seaweeds have emerged as promising sources of bioactive compounds with potential applications in various fields, including aquaculture. In this study, we aimed to isolate and characterize antimicrobial compounds from marine seaweed extracts for their potential use in controlling bacterial pathogens in aquaculture systems. The different solvent extraction methods were employed to obtain crude extracts from *Sargassum cristaefolium* seaweeds collected from coastal regions and characterised by FTIR and GC-MS. These extracts were then subjected to antimicrobial screening against a panel of aquaculture-relevant bacterial strains, including common pathogens such as *Aeromonas caviae*, *Aeromonas hydrophila* and *Edwardsiella tarda*. Experimental fish diets were prepared and supplemented by three different concentrations of *Sargassum cristaefolium* extract at rate of 0.1, 0.3 and 0.5 %. The goldfish *Carassius auratus* weighed about 8.4 ± 0.5 g were fed the different concentration of diets 3 times daily at the rate 3% of the body weight for 30 days. The results revealed that the growth of the *Carassius auratus* was significantly increased when compared to control. FTIR spectrum showed the presence of alcohol, alkene and carbonyl group. GC-MS confirmed as Dodecanoic acid, 1,2,3-Propanetriyl ester, (R)-(-)-14-Methyl-8-hexadecyn-1-ol, 9-Octadecenoic acid, and 2-[(1-Oxododecyl)Oxy]-1,3-Propanediyl. The compounds may act as highly responsible agent for their antimicrobial and antioxidant activity. The purified compounds showed potent antimicrobial activity against the tested bacterial strains, indicating their potential as natural alternatives to conventional antibiotics in aquaculture. Furthermore, the safety and compatibility of these compounds with aquaculture organisms were evaluated, revealing promising results for their application in controlling bacterial infections in aquaculture systems. Overall, this study highlights the potential of marine seaweed-derived antimicrobial compounds as sustainable and environmentally friendly alternatives for improving aquaculture health and productivity.

Keywords: *Carassius auratus*, *Sargassum cristaefolium*, Antimicrobial activity, *Aeromonas hydrophila*, Fish diets

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ADG	Average Daily Growth
GC-MS	Gas chromatography Mass Spectroscopy
FCR	Feed Conversion Ratio
FT-IR	Fourier Transform Infrared Spectroscopy
MAS	Motile Aeromonas Septicaemia
SGR	Specific Growth Rate
THC	Total Hemocyte Count

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CHAPTER 1

INTRODUCTION

1.1 AQUACULTURE

Aquaculture is more diverse today, with 40% more fish, shellfish, aquatic plant, and algal species cultivated in a wide variety of marine, brackish, and freshwater systems globally. Global production remains concentrated, however, with only 22 of all 425 species groups farmed in 2017 (5%) accounting for over 75% of global live-weight production. A small fraction of the 'aquatic plant and algae' category (~32 Mt) consisted of aquatic plants (1,639 tonnes) in 2017. Aquatic plants are listed by the Food and Agriculture Organization (FAO) under 'aquatic plants NEI' and are underreported given the informal nature of the harvests for household and local consumption (Naylor *et al.*, 2021).

1.2 INTERNATIONAL STATUS

Aquaculture is a rapidly growing fisheries sector in India with an annual growth rate of over 7%. Freshwater aquaculture contributes over 95% of the total annual aquaculture production of 5.77 million tone. Technologies of induced carp breeding and polyculture of the three Indian major carps (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*) as well as 'composite carp culture' with the addition of three exotic carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idella* and *Cyprinus carpio*) in ponds and tanks brought about perceptible upward shift in freshwater aquaculture production. Of late, the sector has been witnessing diversification with the inclusion of medium and minor carps, catfishes and murrels. Andhra Pradesh and West Bengal are the top producers of freshwater fish through aquaculture. System diversification has resulted in optimum productivity of 3-6 t ha⁻¹ yr⁻¹. Public and private carp hatcheries produce around 40 billion fry. Fish requirement by 2020 is expected to touch 15 million t, of which around 9 million t would need to come from freshwater aquaculture. Input-related, social and environmental constraints of aquaculture in India need to be tackled through horizontal and vertical expansion, technology as well as climate change mitigation and adaptation, for sustainable development (Jayasankar 2018).

1.3 ORNAMENTAL FISH

Ornamental fish or living jewels became largest hobby worldwide due to their attractive colourful shape, generally tiny size, peaceful behaviour and ability to be accommodated in confined spaces. In recent past, 20th century onwards, aquarium keeping and rearing of ornamental fish got worldwide recognition. However, in present days this technique had developed in to an extensive and global component of international trade worth millions of dollars. Considering the diversity of ornamental fishes, India is not much behind from the other countries. A variety of fresh water and marine ornamental fishes are available of which about 600 species had been identified as potential fishes with ornamental value. In India, the culture of ornamental fish for the aquaria is a good economic activity which has not so far been popularized yet. Since, ornamental fish trade plays an important role for socioeconomic upliftment of the farmers and also a very profitable economic activity, the trade deserves scientific study and development (Saha and Bandyopadhyay, 2017).

Ornamental fish are considered among the most popular pets worldwide. Statistics indicate that in the United States one in every eight persons kept fish as pets, in Canada 1.2 million people have an aquarium, and in the UK 3.5 million people kept fish amounting to over 140 million fish. France's records indicates 34 million fish being kept by 2.1 million households while in Germany approximately 3.7 million households own fish (Rana 2002). Global fish markets records were established in 1976 with just 28 countries were known to be exporting ornamental fishes, later reaching 105 in 2004; presently more than 125 countries are involved this trade (Pierluigi Monticini 2010)

Singapore has been the ornamental fish capital of the world with an export value is US\$42.97 million, contributing to 12.7% of the total exports. Till today it remains the main trading hub in Asia, with more than 30% of the fish exported having been sourced from other countries. The second position was occupied by the Spain with exports worth US\$39.56 million, followed by Japan US\$33.10 million, Burma US\$32.05 million, Indonesia US\$24.64 million, Czech Republic US\$19.89 million, Malaysia US\$14.09 million, Netherlands US\$13.16 million, Sri Lanka US\$12.61 million and Colombia US\$10.68 million (Raja *et al.*, 2019).

1.3.1 Gold fish

Goldfish is a freshwater fish that belongs to the family Cyprinidae of order Cypriniformes, native to East Asia. As a member of the Cyprinidae family, goldfish is related to important ecological and genetic models, including zebrafish, and to carp, an economically important cultured species. Selective breeding over centuries has led to numerous breeds of goldfish, which vary in size, body shape, colouration, and fin and eye configuration (Smartt, 2001; Walker and Johansen, 1977).

Goldfish (*Carassius auratus*), historically considered as an ornamental species, is widely used as an important model organism for a wide variety of research applications, including biomedical research. For instance, it has proven a suitable model for studies on endocrinology, reproduction, and neurology. Studies on cardiac function and gut motility are other examples of biomedical research conducted using goldfish.

In the case of the goldfish other advantages can be considered. One is its size that makes it easy for maintenance and handling, allows tissue isolation and collection, as well as *in vivo*, *ex vivo* and *in vitro* cardiac functional studies (Blanco *et al.*, 2018). The electrical properties of the goldfish heart resemble that of larger mammals, and this makes it attractive also for mammalian-oriented translational studies.

CLASSIFICATION

Kingdom	:	Animalia
Phylum	:	Chordata
Subphylum	:	Vertebrata
Class	:	Actinopterygii
Order	:	Cypriniformes
Family	:	Cyprinidae
Genus	:	Carassius
Species	:	auratus

1.4 CURRENT BACTERIAL DISEASE OF SIGNIFICANT IMPORTANCE IN AQUACULTURE

In aquaculture, up to 50% of production loss is caused by diseases (Assefa and Abunna, 2018). High stocking densities and poor water quality present optimal conditions for the infestation and reproduction of parasites, leading quickly to pathogenic levels. Furthermore, spreading of infectious pathogens is supported by the inevitable transport of fish and equipment (Subasinghe *et al.*, 2001). Freshwater fish may be infected with various bacteria, including species of the genera *Vibrio*, *Aeromonas*, *Pseudomonas* and *Streptococcus*. In general, most fish diseases are induced by a stress factor, like a suboptimal environment, for instance, bad water quality, and this allows opportunistic bacteria including *Aeromonas hydrophila* to infect tilapia and cause disease. Moreover, many bacterial diseases are multifactorial. We should keep this in mind, when trying to understand the cause of and finding a way to cure a bacterial fish disease.

Regardless of the aquatic species an aquaculture venture produces, there are significant risks to success due to the ever-present threat of a disease outbreak. As reported by the World Bank (2014), disease results in an estimated economic impact of \$6 billion USD annually for the aquaculture industry on a global scale. Bringing the cost associated with disease outbreaks down is a major challenge, and in most cases, there is no easy solution. Therefore, aquaculture operations must explore solutions and implement various tools (products) that can work under the specific conditions associated with each facility or species they rear.

A disease can be characterized as infectious or noninfectious, which simply refers to an infection by a pathogenic organism that results in disease or other noninfectious factors such as genetics, environment, or nutrition that cause a disease (or abnormal) condition. The risk of pathogen exposure and disease is especially pronounced in aquatic systems (Oidtmann *et al.*, 2013) where animals are continually exposed to potential pathogens that are endemic or introduced to the water they are reared in.

Snieszko (1973) indicated that in most cases an infectious disease outbreak occurs when a virulent pathogen, a susceptible host, and an unfavorable environment are all concurrently present. Often, an infectious disease can be avoided if one of these components is missing, which emphasizes the importance of maintaining high biosecurity and optimum husbandry practices to avoid a virulent pathogen or unfavorable environment, respectively. However, even under the best conditions, a highly virulent pathogen may cause disease in a susceptible host.

1.5 CURRENT TREATMENT METHODS

Current treatment options such as triphenyl methane dyes, copper, acriflavine and formalin, as well as their effects on different life stages, are considered. Risks associated with many of these 'traditional' chemical therapeutants to fish, to the environment and to consumers have led to bans for their use in the aquaculture industry of many countries. The search for alternative environmentally friendly approaches for diseases has increased during the last decade and shall be considered in detail with emphasis on velvet disease where available (Lieke *et al.* (2019).

Antimicrobials and other veterinary drugs are administered regularly as additives in fish food or sometimes in baths and injections and are used as prophylactics (prevent diseases before they occur), therapeutics (treat sick animals) or growth promoters (Rico *et al.*, 2013). Nevertheless, the use of veterinary drugs is becoming more restricted since they present numerous side-effects for the environment and health safety. For example, massive use of antibiotics have resulted in the development of resistant bacteria strains (Mirand and Zemelman, 2002; Seyfried *et al.*, 2010) or the presence of residual antibiotics in the muscle of commercialized fish and thus has potential consequences on human health (e.g. Cabello, 2006; Romero Ormazábal *et al.*, 2012). The use of drugs like trichlorfon or praziquantel in bath treatments for ectoparasites has also numerous disadvantages like development of resistance (Umeda *et al.*, 2006), being hazardous for animal health (Forwood *et al.*, 2013; Kiemer and Black, 1997) and environmental disadvantages. Vaccination has also been regarded as a potential treatment against disease outbreaks in aquaculture. However, commercial vaccines are too

expensive for widespread use by fish producers and they have the downside that a single vaccine is effective against only one type of pathogens (Harikrishnan *et al.*, 2011a; Pasnik *et al.*, 2005; Sakai, 1999).

Plant extracts have been reported to favor various activities like antistress, growth promotion, appetite stimulation, enhancement of tonicity and immune stimulation, maturation of culture species, aphrodisiac and anti-pathogen properties in fish and shrimp aquaculture due to active principles such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids or essential oils (Chakraborty and Hancz, 2011; Citarasu, 2010).

1.6 PLANT SOURCE

1.6.1 Seaweed

Seaweeds are important primary producers in the marine ecosystem, some with high economic value or ecological significance. Seaweeds are one of the most interesting organisms found in oceans around the earth, which are carrying great ecological importance and contribute to increase the biodiversity of ecosystems where they were originated and habitat. Within last few decades, discovery of secondary metabolites with biological activities from seaweeds has been significantly increased. Further, the unique secondary metabolites isolated from seaweeds including polysaccharides, carotenoids and polyphenols possess range of bioactive properties that make them potential ingredient for many industrial applications. Among those groups of compounds phlorotannins isolated from brown seaweeds have shown interesting bioactive properties including anti-cancer, anti-inflammation, anti-oxidant, anti-allergic, anti-wrinkling and hair growth promotion properties. Moreover, these properties associated with phlorotannins make them an ideal compounds to use as a functional ingredient in cosmeceutical products. Up to now no report has been reviewed about discuss properties of phlorotannins related to the cosmeceutical application. In the present review primary attention is given to the collect scientific data published about bioactive properties of brown algal phlorotannins related to the cosmeceutical industry (Sanjeewa *et al.*, 2016).

Marine macroalgae belong to three major classes or phyla: Chlorophyceae (green algae), Rhodophyceae (red algae), and Phaeophyceae (brown algae). The most extensively studied macroalgae are those of the Phaeophyceae family. Brown

macroalgae are well known to be rich in polyphenol compounds which potentially contribute to its antioxidant capacity. The brown seaweed *Sargassum* species have shown strong antioxidant activity (Prasedya *et al.*, 2021)

1.6.2 *Sargassum cristae folium*

Marine algae, particularly brown algae (*Sargassum* sp.), is a natural compound factory that has the capacity as a prophylactic and immunostimulant material. The active ingredients in *Sargassum* sp. were described in our previous study including tannin, saponin, β -carotene, flavonoids, alkaloids, phenolic, steroid and glucose. Alginic acid and fucoidan from brown algae and K-carrageenan from red algae showed a role as an immune trigger which helps aquatic animals in preventing various diseases. Provision of natural immunity triggers proven to be able to optimize the health profile of aquatic animals including various immunity parameters such as total hemocyte count (THC) and phagocytic activity (Mulyadi *et al.*, 2020).

Sargassum cristae folium is one of dominant seaweeds in the southern coast of Iran. Nowadays, it is a candidate for being used as an additive or supplement in shrimp feed. *Sargassum* as well as chemical drugs improved the defense system and survival rate of shrimp without negative effects. *Sargassum* is a genus of brown seaweed rich in bioactive compounds such as fucoidan which has various health promoting properties including anti-cancer activity, anti-viral activity, and anti-inflammatory activity.

SCIENTIFIC CLASSIFICATION

Kingdom	:	Chromista
Subkingdom	:	Harosa
Phylum	:	Ochrophyta
Class	:	Phaeophyceae
Subclass	:	Fucophycidae
Order	:	Fucales
Family	:	Sargassaceae
Genus	:	<i>Sargassum</i>
Species	:	<i>cristae folium</i>

CHAPTER 2

LITERATURE SURVEY

Many studies have reported that aquaculture could play an important role in providing the world's population with much-needed animal protein (*Tidwell and Allan, 2001*). Cultivable freshwater fish species including Catla (*Catla Catla*), rohu (*Labeorohita*) and mrigal (*Cirrhinus mrigala*), silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), and common carp (*Cyprinus carpio*) are the most economically important commercial freshwater food fish that widely distributed in the world (Jayasankar, 2018).

The market for ornamental fish is worth \$6 billion worldwide. Annually, millions of fish travel thousands of kilometers across the globe in a matter of days, typically without being subject to many limitations from governmental agencies for fish health (Ramos-Espinoza *et al.*, 2020). This is an important risk factor for spreading pathogens, not only for ornamental fish; these pathogens might affect cultured fish and even other animal species (Hoai *et al.*, 2019).

High stocking density has generated potential environmental stress to the fishes, which leads to high susceptibility to pathogenic microbes leading to severe damages and huge economic losses. These pathogenic microbes are responsible for causing a number of different diseases in freshwater fishes such as motile *Aeromonas* septicaemia (MAS) including swelling of tissues, dropsy, red sores, necrosis, ulceration, and haemorrhagic septicaemia (Ibrahim *et al.*, 2008). Globally, 6 billion dollars are estimated to be lost per annum due to the diseases caused by *Aeromonas hydrophila* freshwater fish aquaculture industry (Hasimuna *et al.*, 2020). During 2009-11, a disease outbreak of infectious salmon anemia in Chile reduced production value by more than 2 billion dollars and resulted in the layoff of 20,000 workers (Asche *et al.*, 2009).

Ornamental fish trade represents an important economic sector with an export turnover that reached approximately 5 billion US dollars in 2018. Despite its high economic importance, this sector does not receive much attention. Ornamental fish

husbandry still faces many challenges and losses caused by transport stress and handling and outbreak of diseases are still to be improved. Moreover, the role of different natural and sustainable microbial feed additives, particularly probiotics, prebiotics, and synbiotics on the health, reduction in transport stress, growth, and reproduction of farmed ornamental fish (Hoseinifar *et al.*, 2023).

Goldfish (*Carassius auratus*) is one of the most important domesticated ornamental fish in aquaria and are recently reported to be infected with various bacterial species like *Aeromonas*, *Acinetobacter*, *Edwardsiella*, *Lactococcus* etc. (Preena *et al.*, 2019) thereby interrupting the sustainable fish culture. Mass mortality of goldfish population infected with *Aeromonas hydrophila* (Dharmaratnam *et al.*, 2018), *A. Salmonicida* (Jin *et al.*, 2020), *Proteus hauseri* (Kumar *et al.* 2015), *Mycobacterium marinum*, (Hodgkinson *et al.*, 2012), *Flavobacterium columnare* (Verma *et al.*, 2015) etc. Was noticed in various fish farms.

In aquaculture operations, the current disease treatment protocols are often difficult to administer, not especially effective, costly, and sometimes even environmentally hazardous. Even though antibiotics and synthetic drugs have positive effects, they cannot be recommended in commercial aquaculture operations due to their negative aspects including relatively high cost, prohibited or uncertain regulatory status, unfeasible administration routes, poor absorption, toxicity, biomagnification, the possibility of consignment rejection and the fact that resistant bacteria may be transferred to humans through food handling and consumption (Thangaviji *et al.*, 2012).

The alternative herbal bio-medicinal products in the aquacultural operations, that have the characteristics of growth promoting ability and tonic to improve the immune system, act as appetite stimulators. They increase consumption, induce maturation, and have antimicrobial capability and also antistress characteristics that will be of immense use in the culture of shrimps and other fin fishes without any environmental and hazardous problems. Herbal compounds such as phenolics, polyphenols, alkaloids, quinones, terpenoids, lectines and polypeptides have been shown to be very effective alternatives to antibiotics and other synthetic compounds. The present paper is presented after a careful review of more than 50 herbal plants

for their biological effects such as growth promotion, immunostimulation, antistress, antibacterial, antifungal, antivirals, appetite stimulators and aphrodisiac(Citarasu2010).

One abundant source for obtaining new anticoagulants is the polysaccharide from marine algae. Marine algae are the main source of obtaining sulfate polysaccharides (SPs) from non-mammal groups. SPs (for example fucoidan, laminaran, carrageenan, ulvan) and fluorotanine from marine algae show potent anticoagulant activity based on several studies (Subhash *et al.*, 2014). In the last few decades, there is a tremendous growth in the area of herbal medicine. It is popular in developing as well as in the developed countries due to its natural origin treatment of internal diseases which are considered to be stubborn and incurable by other system of medicines. The previous study was to prove effect of *Sargassum cristaefolium* extract as anticoagulant by measuring blood clotting and bleeding time and also APTT and PT (Sudaryono *et al.*, 2018).

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To Isolate the antimicrobial products from marine Seaweed for Aquaculture Application

3.2 OBJECTIVES

- Collection of seaweeds from marine environment
- Extraction of antimicrobial products using various organic solvents
- Screening of antimicrobial activity against fish pathogens
- Characterization of active products by FT-IR and GC-MS
- Evaluation of immunomodulatory potential and disease resistance activity using fish model

CHAPTER 4

MATERIALS AND METHODS

4.1 SEAWEED COLLECTION

The marine seaweed was collected from Vaippar region, Tamil Nadu, India (Lat 9.02416; Long 78.264166) (Fig 4.1). The marine seaweed were validated at the R.K. Algae Project Centre, Gulf of Mannar herbarium and recognized as *Sargassum cristae folium* by identifying their morphological traits and examining their form using a light microscope and a field guide. Sample was washed with sea water to remove impurities, as well as to avoid evaporation from the sample. The sample was then rinsed twice using running water to remove the remaining salt and impurity and the next step the rinsing was done using distilled water to ensure no impurity of the sample. The seaweed was dried and then crushed into fine powder by using homogenizer. Then samples were sieved using mesh number sieves 18 to obtain coarse powder

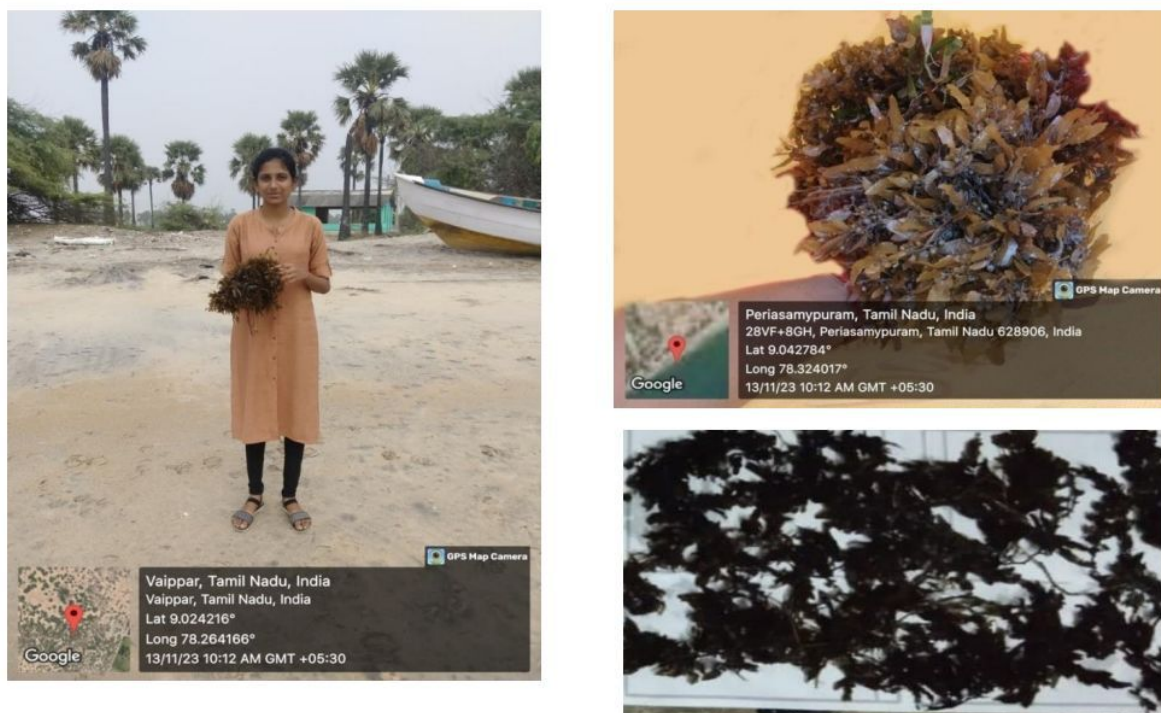


Fig 4.1 *Sargassum cristae folium*

4.2 EXTRACT PREPARATION

The extraction was carried out as follows: grounded *Sargassum cristaefolium* 10g were mixed with different solvent such as ethanol, methanol, water and acetone and kept it in shaking incubator for 24 hours (Fig 2). After incubation, different solvent extract were filtered through Whatman No.1 filter paper and transferred into petri dish for air dry, the extract were collected in glass vials. The high amount of extract were collected from ethanol extract compared to the rest of the extract.



Fig 4.2 Solvent extraction of *Sargassum cristaefolium*

4.3 CHARACTERIZATION OF BIOACTIVE COMPOUND

4.3.1 GCMS Analysis

The bioactive compounds were extracted from the *Sargassum cristae folium* by using methanol solvent and characterized by Gas Chromatography-Mass Spectrometry (GC-MS) analysis to identify the active compounds. The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260 °C during the chromatographic run. The 1 µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min⁻¹; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 230 °C; ion source temperature 230 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectra of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

4.3.2 FTIR Analysis

The methanol extracted bioactive compound from the *Sargassum cristae folium* was analyzed qualitatively for the active compounds by Fourier Transform Infra-Red (FT-IR) method. Two milligram of dried fish fecal sample was mixed with 100 mg KBr and compressed in the form of KBr pellet. The infrared (IR) spectrum was recorded in the wave length ranging between 4000 and 400 cm⁻¹ using FTIR spectrometer (Bruker spectrophotometer).

4.4 ANTIMICROBIAL SUSCEPTIBILITY TEST

4.4.1 Isolation of Microorganisms

Four aquatic fish pathogens (*Aeromonas hydrophila*, *Aeromonas caviae*, *Staphylococcus aureus*, *Edwardsiella tarda*) were collected from Centre for Aquaculture, Sathyabama Institute of Science and Technology, Chennai. The

subculture plates was prepared by inoculating the mother culture of four bacterial pathogen in the nutrient agar plates. Then the cultured plates were incubated at 37°C for 24 hours.

4.4.2 Agar Well Diffusion Method

Suspend 1.3 g of nutrient broth in 100 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. A loop of 4 different bacterial culture is inoculated in 10ml of nutrient broth in a test tube and incubated at 37°C for 24 hours. Suspend 3.9g of Mueller Hinton Agar in 100ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. Pour the liquid in petri dishes and leave for solidifying.

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Magaldi et al., 2004; Valgas et al., 2007). Similarly to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. The standardized suspension was used to inoculate the microorganisms on the surfaces of Mueller Hinton agar plates using sterile cotton swab. The plates were left for 20 minutes; using a 100 µL pipette tip make a hole with a diameter of 6mm and extract solution at desired concentration (10µL, 20µL and 50µL) is introduced into the well. Then the plates were incubated at 37°C for 24 hrs and then were examined for the zone of inhibition around the well. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the bacterial pathogens.

4.5 EXPERIMENTAL FISH

A total of 100 apparently healthy live goldfish (*Carassius auratus*) with an average body weight of 8.4 ± 0.5 g were obtained from a private fish farm in Kolatur, Chennai, India. The fish were kept in a recirculation system. High water quality was ensured, and the system was thermostatically controlled at $25 \pm 1^\circ\text{C}$. The fish were divided into 3 groups, each of which was divided into subgroups. Each subgroup had three replicates (10 fish/replicate). The fish were allowed to acclimate to the laboratory conditions for three weeks before the start of the experiment. The

experiment carried out at form Centre for Aquaculture, Sathyabama Institute of Science and Technology, Chennai.

4.6 FEED PREPARATION

The experimental feeds were prepared with basic ingredients such as wheat flour, rice bran, tapioca flour, ragi flour, fish meal and groundnut oilcake (Table 1 and Fig. 1). They were split into three equal portions. These ingredients were mixed until very soft biscuit-textured dough was obtained. The material was then steam sterilized (autoclaved at 120 °C for 20 min) and pelletized by hand to get pellets of 3 mm size. The pellets were dehydrated in a forced-air oven at 55–60 °C for 48 h when the final moisture content <15%. After drying, the basal diet supplemented with *Sargassum cristaefolium* extract at rates of 0.1, 0.3 and 0.5% (G1, G2 & G3) respectively. After that, dough was heated in a drying oven at 35 °C for 30 min and asked by crumbling and then stored at room temperature.

Table 4.1 Shows Formulation of Experimental Diets (g/100g)

Control	Group1 (G1)	Group2 (G2)	Group3 (G3)
Soybean (15.22)	Soybean (15.22)	Soybean (15.22)	Soybean (15.22)
Fishmeal (15.22)	Fishmeal (15.22)	Fishmeal (15.22)	Fishmeal (15.22)
Groundnut oil cake(11.28)	Groundnut oil cake(11.28)	Groundnut oil cake(11.28)	Groundnut oi lcake(11.28)
Wheat flour (13.04)	Wheat flour (13.04)	Wheat flour (13.04)	Wheat flour (13.04)
Tapioca flour (13.04)	Tapioca flour (13.04)	Tapioca flour (13.04)	Tapioca flour (13.04)
Rice bran (12.2)	Rice bran (12.2)	Rice bran (12.2)	Rice bran (12.2)
Vitamins (5. 0)	Vitamins (5. 0)	Vitamins (5. 0)	Vitamins (5. 0)
Nil	Seaweed Extract (0.1%)	Seaweed Extract(0.3%)	Seaweed Extract(0.5%)



Fig 4.3 Experimental fish diet preparation

4.7 GROWTH PERFORMANCE AND CONDITION FACTOR OF GOLDFISH

For each experimental treatment, 10 fish were randomly selected bi-weekly to estimate the periodical gain in weight and length. At the end of 30 days experimental period, koi carp fish were anesthetized by clove oil (Himedia, Mumbai, India, 50 $\mu\text{L L}^{-1}$), collected, counted, and weighed. The growth performance and feed utilization parameters were determined as follows:

- Weight gain (g fish^{-1}), $\text{WG} = \text{Wt} - \text{W}_0$;

- Average daily gain ADG $\text{g fish}^{-1} \text{ day}^{-1} = \frac{W_t - W_0}{\text{days}}$;
- Specific growth rate ($\% \text{ day}^{-1}$);
- $\text{SGR} = 100 \times \frac{\ln W_t - \ln W_0}{\text{experimental period}}$, where: W_0 : initial fish weight (g), W_t : final fish weight (g), $\ln$: natural logarithm;
- Survival rate (%) = $100 \times (\text{final number of fish} / \text{initial number of fish})$.

4.8 STATISTICAL ANALYSIS

Statistical analyses were carried out on various parameters that were measured on the plants to evaluate the effect of each treatment. Data was presented as mean value $\pm$ standard deviation (error bars). Analysis of variance (ANOVA) was used and Tukey's honest significant difference ($P < .05$) multiple range test was conducted to determine differences between treatments.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 DIFFERENT SOLVENT EXTRACTIVE VALUES OF *Sargassum cristaefolium*

The extractive values of *Sargassum cristaefolium* extract using different solvents such as aqueous, ethanol, acetone, and methanol. The extractive value was higher in methanol extract of *S.cristaefolium*(16.2%) followed by ethanol extract of *S.cristaefolium*(13.7%), acetone extract of *S.cristaefolium* (6.8%),and water extract of *S.cristaefolium*(5.4%).

Table 5.1 Different solvent extractive values of *Sargassum cristaefolium*

Solvent	Extract value
Methanol	16.2%
Ethanol	13.7%
Acetone	6.8%
Water	5.4%

The previous study revealed that the edible red seaweed, *Gracilariachangii* was extracted using five solvent systems: 80 % ethanol, 80 % methanol, 80 % acetone, ethyl acetate and water. The result revealed that methanol extract had the highest yield followed by ethanol > water > acetone > ethyl acetate extract (Chan et al., 2015).

5.2 FT-IR ANALYSIS

The IR spectrum of the methanol extract of *Sargassum cristaefolium* sample showed in Table 5 and Fig. 1. The main absorption band at around 3335.28 cm^{-1} , which is strong, broadband and attributed to OH stretching showed the presence of

alcohol. The absorption band at 2945.73 cm^{-1} and 2834.85 cm^{-1} were identified as C-H stretching, the band at 1653.66 cm^{-1} was recognised as C=C bending exhibiting the presence of alkene and the band at 1017.27 cm^{-1} was recognised as C-O-C bending exhibiting the presence of carbonyl group.

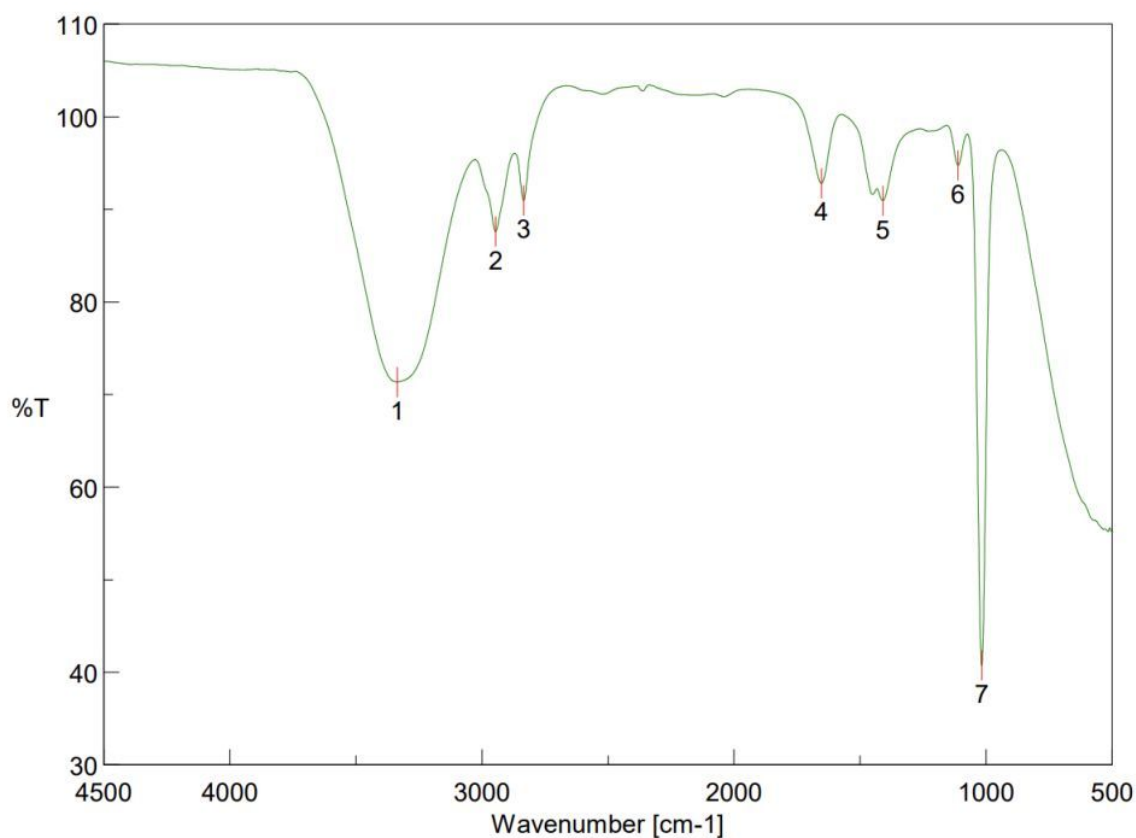


Fig 5.1 Structural characterization of *Sargassum cristaefolium* active fraction by FITR analysis.

Table 5.2 FTIR absorption frequencies of organic functional groups of *Sargassum cristae folium* extract using FTIR

No.	Position	Intensity	Functional group
1	3335.28	71.329	OH
2	2945.73	87.5923	CH
3	2834.85	90.9372	CH
4	1653.66	92.8018	C=C, conjugated
5	1409.71	90.9204	CH ₂
6	1111.76	94.7189	C=C, Trans
7	1017.27	40.7577	C-O-C

The result consistent with previous study that the broad band centered at 3435 cm⁻¹ assigned to hydrogen bonded O–H stretching vibration. The band at 2939 was attributed to the C–H stretching pyrenoid ring and C6 of fucose and/or galactose units (Wang et al. 2014). The band at 1614 cm⁻¹ indicated the stretching of asymmetric carboxylate O-C-O vibration. The band at 1424 cm⁻¹ suggested C–OH deformation vibration with contribution of O–C–O symmetric stretching vibration of carboxylate group (Immanuel et al. 2012). The presence of O=S=O stretching vibration of sulfate esters was indicated by a band at 1258 cm⁻¹ (Pielesz and Binias 2010; Immanuel et al. 2012). The band at 1040 cm⁻¹ corresponded to stretching vibration of C–O–C/C–OH (Wang et al. 2014).

5.3 GC-MS ANALYSIS

The GC-MS study revealed that the presence of chemical compounds in the *Sargassum cristae folium* extract was identified with the help of the NIST library (Table 2) (Fig. 3). The peak at the retention times of 43.87, 44.21, 44.31, 47.53 were confirmed as Dodecanoic acid, 1,2,3-Propanetriyl ester, (R)-(-)-14-Methyl-8-hexadecyn-1-ol, 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-and

Hexadecanoic acid and 2-[(1-Oxododecyl)Oxy]-1,3-Propanediyl. The compounds may act as highly responsible agent for their antimicrobial and antioxidant activity.

Table 5.3 Major components from the GC-MS analysis of the methanol extract of *Sargassum cristae folium*.

Peak	R.Time	Area	Area%	Name
1	43.874	10848175	31.45	Dodecanoic acid, 1,2,3-Propanetriyl ester
2	44.218	7271788	21.08	(R)-(-)-14-Methyl-8-hexadecyn-1-ol
3	44.313	11141952	32.31	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-
4	45.795	3568087	10.35	Dodecanoic acid, 1,2,3-Propanetriyl Ester
5	47.534	1658320	4.81	Hexadecanoic acid, 2-[(1-Oxododecyl)Oxy]-1,3-Propanediyl

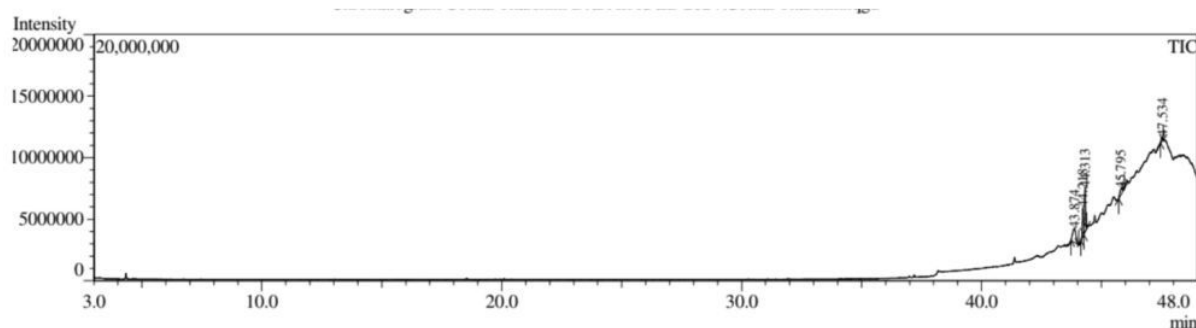


Fig 5.2 Structural characterization of *Sargassum cristae folium* active fraction by GC-MS analysis.

5.4 ANTIMICROBIAL SUSCEPTIBILITY TEST

The *Sargassum cristae folium* extract exhibited most significant activity against aquatic fish bacterial pathogens. The extract showed maximum activity against the tested pathogens. The significant zone of inhibition was observed against the bacterial pathogens *Aeromonas caviae* (8 mm), *Edwardsiella tarda* (7 mm) and *Aeromonas hydrophila* (5 mm) (Fig 6).

Table 5.4 In vitro antagonistic screening of *Sargassum cristae folium* extract against bacterial pathogens

Bacterial Pathogens	Zone of inhibition (mm)		
	50 µg	100 µg	150 µg
<i>Aeromonas hydrophila</i>	-	-	5 ± 0.25
<i>Aeromonas caviae</i>	-	-	8 ± 1.35
<i>Edwardsiella tarda</i>	-	-	7 ± 1.35
<i>Staphylococcus aureus</i>	-	-	-



(a)



(b)



(c)



(d)

Fig 5.3 Antimicrobial activity of bioactive compound by Agar well diffusion method (a) *Aeromonas hydrophila* (b) *Aeromonas caviae* (c) *Edwardsiella tarda* (d) *Staphylococcus Aureus*

The results consistent with previous study that the maximum inhibition zone (24.66 ± 11.06 mm) was recorded in ethanolic extract of *Sargassum cristaefolium* against *A. hydrophila*, and minimum activity (1 ± 1.73 mm) was observed in aqueous extract against *P. aeruginosa* (Bolanos et al., 2017). Similarly, methanolic extract of *Sargassum cristaefolium* showed antibacterial activity against *E. coli* and *aureus* (Susilo et al., 2022).

5.5 GROWTH PERFORMANCE

Results of growth performance are illustrated in Table 5. There were significant ($P < 0.05$) differences among treatments in terms of final weight, weight gain, average daily gain (ADG), specific growth rate (SGR), final length, length gain and condition factor (K). Fish fed with experimental diet showed the highest values of growth performance, and lowest values of condition factor.

Adult fish can grow on vegetable diets, however juvenile fish require a greater protein content in their feeds for better growth (Mohanta, K. & Subramanian 2002). Furthermore, improved growth performance may be attributed to feeding fish live natural food rather than commercial diets which are dependent on the palatability, quality, and adequate quantity of feed, size, type, and physical appearance of the

feed, as well as other factors such as attractiveness, feed acceptability, and assimilation of nutrients.

Table 5.5 Growth performance, Survival rate and feed utilization of gold fish fed with experimental diets for 30 days

Parameters	Control	Group 1	Group 2	Group 3
Initial weight (g)	8.26±0.64	8.4±0.25 ^a	8.32±0.83 ^b	8.4±0.75
Final weight (g)	8.74±0.06	9.5±0.65 ^a	9.7±0.24 ^b	10.12±0.42 ^a
Weight gain	5.81±0.23	13.09±0.13 ^a	16.58±0.31 ^b	20.47±0.53 ^a
Specific growth rate	1.6±0.34	3.6±0.33 ^a	4.6±0.13 ^b	5.73±0.28 ^a
FCR	2.74±0.01	1.21±0.40 ^a	0.96±0.15 ^b	0.77±0.02 ^a
Survival	80	85	90	90

Values are represented as mean ± SD (n = 3), Significant difference ($p < 0.05$) vs control

In contrast, shrimp fed with the diets supplemented with various levels of 200-1000 mg *S. cristaefolium* extract kg⁻¹ diet have resulted significantly ($p < 0.05$) increased growth performance and a better feed utilization (FCR) than those fed not supplemented with *S. cristaefolium* extract. Survival and the number of moulting shrimp of all the treatment groups were high (80-97% and 36-48shrimp, respectively) and not affected by *S. cristaefolium* extract supplementation levels (Sudaryono et al., 2015).

CHAPTER 6

SUMMARY AND CONCLUSION

The bioactive compound extractive value was higher in methanol solvent extract of *Sargassum cristaefolium* (18.2%) followed by ethanol, acetone, and water. The GC-MS study revealed that the presence of chemical compounds in the *S. cristaefolium* extract was identified as 9-Octadecenoic acid, cis-9-Hexadecenal, and 9Z,11E,13Z-1,3,12-Nonadecatriene-5,14-diol. The compounds may act as highly responsible agent for their antimicrobial and antioxidant activity. The FTIR spectrum of the ethanol extract of *S. cristaefolium* sample showed the presence of aliphatic primary amine, conjugated alcohol, alkene and Alkyl Halide. The *S. cristaefolium* extract exhibited most significant activity against aquatic fish bacterial pathogens such as *Aeromonas caviae*, *Edwardsiella ictaluri*, and *Aeromonas hydrophila*. The extract showed maximum activity against the *Aeromonas hydrophila* pathogens. Experimental fish diet prepared supplemented with *Sargassum cristaefolium* extract at rates of 0.1, 0.3 and 0.5% (G1, G2 & G3) respectively. Fish fed with experimental diet showed the highest values of growth performance, and lowest values of condition factor.

In conclusion, this study declared that the addition of *Sargassum cristaefolium* extract in goldfish diets adverse effects on growth parameters or health status. In addition, it contains bioactive compounds responsible for antimicrobial and antioxidant activity that improved immune status. *Sargassum cristaefolium* has been suggested as immunestimulant due to their biological effects.

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