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Antimicrobial Activity of Microalgae and Medicinal Plants against Common Bacteria Causing Diseases in Fish and Shellfish

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects and that all revisions required by the thesis examination committee have been made

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List of Acronyms and Symbols Used

Abbreviation and symbols	Elaboration
mm	Millimeter
µm	Micrometer
ml	Milliliter
µg	Microgram
vol	Volume
AMR	Antimicrobial resistance
ARGs	Antibiotic resistance genes
WHO	World Health Organization
CVASU	Chattogram Veterinary and Animal Sciences University
PUFA	Poly-unsaturated fatty acid
C.	*Clinacanthus*
A.	*Aloe*
SDG	Sustainable development goal
AIDS	Acquired Immune Deficiency Syndrome
HIV	Human Immunodeficiency Virus
GLA	Gamma-Linolenic Acid
SQDG	Sulfoquinovosyl diacylglycerol
EDTA	Ethylene Diamine Tetraacetic Acid
L	Liter
^{0}C	Degree Celsius
mg	Milligram
gm	Gram
%	Percentage
CFU	Colony Forming Unit
µl	Microliter
h	Hour
BPW	Buffer Peptone Water
NB	Nutrient Broth
PBS	Phosphate buffer saline
TSA	Trypticase Soy agar

MSA	Mannitol Salt agar
MAC	MacConkey agar
TCBS	Thiosulfate-citrate-bile-salts-sucrose agar
EMB	Eosin Methylene Blue agar
MHA	Muller Hinton agar
TSI	Triple Sugar Iron agar
SIM	Sulfide Indole Motility agar
GP	Gram-positive
MIC	Minimum inhibitory concentration
MRS	Methicillin-Resistant Staphylococcus
OD	Optical Density
ELISA	Enzyme-linked immunoassay
nm	Nanometer
ANOVA	Analysis of variance
+	Plus
-	Minus
A	Antibiotic
B	Blank
Al	*Aloe vera*
Sp	*Spirulina*
Sn	Snake grass
Chl	*Chlorella*
P	Positive
N	Negative
C	Crab
S	Shrimp

Abstract:

Antimicrobial activity of the ethanolic extract of dried marine microalgae *Chlorella* sp., and *Spirulina* sp. and dried medicinal plant *Aloe vera (A. vera)* and *Clinacanthus nutans (C. nutans)* were studied against three Gram-negative bacteria *Aeromonas hydrophila*, *Vibrio* sp., *Escherichia coli* and one Gram-positive bacteria *Staphylococcus saprophyticus* isolated from local fish, shrimp and crab by using disk diffusion technique. In this study, *Spirulina* sp. showed the highest inhibition zone of 19.3±0.577 mm against *Vibrio* sp. isolated from crab, whereas *Chlorella* sp. exhibited a maximum inhibition zone of 11.41±0.65 mm against *S. saprophyticus*. *C. nutans* displayed antibacterial activity against *S. saprophyticus* and *E. coli* with the maximum zone of inhibition (14.21±1.075; 15.103±0.214 mm), whereas *A. vera* against *E. coli*, *Vibrio* sp. with significant inhibition zone (14.037±0.903; 15.363±1.11 mm) respectively. The Minimum Inhibitory Concentration (MIC) of the extracts was determined against the test bacteria and the lowest concentration was revealed by *Chlorella* sp. at 20 mg/ml against all the test bacteria. MIC value of the *Spirulina* sp., *A. vera, and C. nutans* was recorded from 20 mg/ml to 40 mg/ml against the test bacteria. These results indicate the presence of promising antibacterial compounds in the plants and microalgae studied. Further phytochemical studies are needed to elucidate the active component responsible for the antibacterial activity of these extracts.

Keywords:

Antibacterial properties, microalgae, medicinal plant, *Aeromonas hydrophila*, *Vibrio* sp., *Escherichia coli*, *Staphylococcus saprophyticus*

CHAPTER 1
INTRODUCTION

1.1 Background of the study

Aquaculture industries, and in particular the farming of fish and crustaceans, are major contributors to the economy of many countries and an increasingly important component of the global food supply. For instance, the annual global growth the fish consumption has been twice as high as the population growth over the past decades, and the global fish production peaked at about 171 million tons in 2016, according to the Food and Agriculture Organization (FAO, 2018). The primary constraint to the growth of the fish species in the field of aquaculture is a disease (Alday et al., 2006). Bacterial pathogens are disease organisms that cause most of the issues for aquaculture to manage the health of the fish culture (Schnick, 2001). Several antimicrobial drugs including antibiotics have been approved and used in many countries to treat bacterial diseases in aquaculture and the number of antibiotics being used in aquaculture systems has exerted a very strong selection pressure on resistant bacteria (Henriksson et al., 2018; Brunton et al., 2019). As a consequence, nearly 80% of antimicrobials (antibiotics and metals) used in aquaculture end up in uneaten medicated feeds; unabsorbed antibiotics and secretions of culture organisms enter aquatic environments closely related to aquaculture facilities (e.g., water, sediments, and other water-borne species), therefore provides a favorable environment for the development and enrichment of persistent aquatic antibiotic resistance genes (ARGs) (Tang et al., 2017).

Antimicrobials are used in animals to treat or prevent disease and also to promote growth. Therapeutic, metaphylactic, prophylactic, and sub-therapeutic are used as antimicrobial therapy apply in the animal body. The use of antibiotics in any setting contributes to the growing global threat of antibiotic resistance. Antimicrobial resistance (AMR) is the ability of a microorganism like bacteria, viruses, and some parasites to stop an antimicrobial such as antibiotics, antiviral, and antimalarials from working against it. As a result, standard treatments become ineffective, and infections persist and may spread to others. So, it is important to minimize the use of these drugs. This means eliminating unnecessary uses and finding other ways to prevent infections. In animal agriculture, alternative products play a crucial role in allowing farmers and veterinarians to reduce or largely phase out the use of antibiotics. Vaccines are among the most promising and widely

used of these alternatives, but prebiotics and probiotics and other innovative products like immune modulators, phages, phytochemicals, organic acid, anti-microbial peptides, etc. are also in use or currently being investigated. Many alternative products enhance animal productivity and prevent infection at the same time, which could make them particularly attractive for commercial operations (Hoelzer et al., 2017). In this regard, microalgal species and medicinal plants have the potential to benefit health as they contain some bioactive components. Different microalgal species such *as Chondrus crispus, Mastocarpus stellatus, Ascophyllum nodosum, Alaria esculenta, Spirulina platensis, Nannochloropsis oculata, Chlorella vulgaris,* and *Dunaliella salin* which are used to make products such as anti-irritant, antibacterial, antifungal, and anti-virus agent. Moreover, *Chlorella* sp., *Dunaliella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Tetraselmis* sp., *Spirulina* sp. and *Aphanizomenonflos aquae* have been used as an antimicrobial agent to cure aquatic animal disease (Patai et al., 2018). It has also been reported that lupeol, β-sitosterol, terpenoids flavonoids present in medicinal plants, *Clinacanthus nutans* possess antibacterial activity and *Aloe vera* contains six antiseptic agents: Lupeol, salicylic acid, urea nitrogen, cinnamonic acid, phenols and sulfur, which causes *Aloe vera* to have inhibitory effects against fungi, bacteria, and viruses (Surjushe et al., 2008; Yang et al., 2013).

1.2 Significance of the study

In the field of aquaculture, farmers face a severe outbreak of disease caused by bacteria pathogens which cause significant economic losses. Larvae fish or shrimp is more easily infected with various bacteria. To solve the problem, many scientists developed various chemicals that consist of the antimicrobial agents to prevent the propagation of bacteria under culture conditions. However, these chemicals which are used in fish or shrimp farming are highly costly and the most serious problem is they may cause the chemical residues to remain in the organism's body and the consequent effect on humans after ingestion of the contaminated fish. Therefore, it is very important to find out the other effective or alternative ways to prevent the propagation of bacteria it may not cause any effect on fish or shrimp.

The current study will make a paradigm in the field of antimicrobial drug design, if successful. This project might discover the bioactive compounds of microalgae and medicinal plants that inhibit bacteria to infect the animal body. Marine microalgae and

medicinal plants (*Clinacanthus nutans*, *Aloe vera)* might have potential components that may be used as substitutes for the expansive antimicrobial agent.

1.3 Objective

To determine the antimicrobial effect of microalgae and medicinal plant extracts against bacteria *Aeromonas hydrophila*, *Vibrio* sp., *Staphylococcus* sp., and *Escherichia coli* causing diseases in fish, shrimp, and crab.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Marine microalgae

Microalgae are defined as unicellular which convert the sun's energy into chemical energy by photosynthesis and can process like the mainland plant to produce complex organic compounds (Harun et al., 2010). There are over thousands or even millions of microalgae species existing in nature (Hannon et al., 2010). Microalgae are generally eukaryotic organisms, although cyanobacteria, such as *Spirulina*, which are prokaryotes, are included under microalgae due to their photosynthetic and productive properties (Ravishankar et al., 2012).

2.2. Marine microalgae available in Bangladesh

Marine microalgae are mainly found around the Cox's Bazar, Sundarban, and St. Martin's Island in Bangladesh. 45 taxa under 17 genera of green algae have been reported from Bangladesh coasts (Islam et al., 2000). They grow abundantly in winter, and spring growth is affected by rainwater runoff. The super-tidal zones or splash zone of the benthic marine environment is dominated by blue-green such as Calothrix. In the inter-tidal sub-tidal zones, brown algae dominated. Among the several green species of *Chlorella miminutissima, Tetrselmis chuii, Nannochloropsis* sp., *Arthrospira platensis, Isochrysis* sp., *Chondrus crispus, Mastocarpus stellallatus, Ascophyllum nodosum, Alaria esculentus, Spirulina platensis, Chlorella esculentus, Nannochloropsis oculata* and *Dunaliella salina* found in St. Martin's Island of Bangladesh.

2.3 *Chlorella*

Chlorella is a type of algae that grows in fresh and marine water. The whole plant is used to make nutritional supplements and medicine.

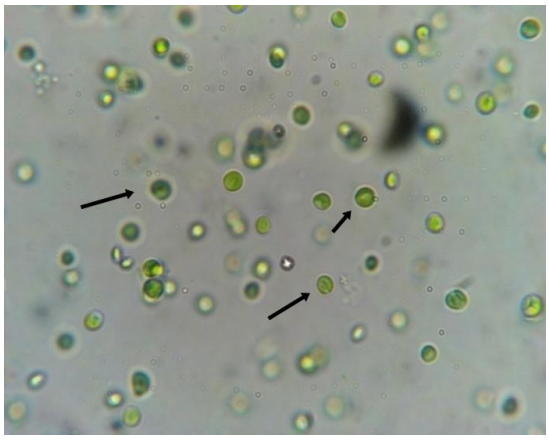

Figure 2.1 *Chlorella* sp. are spherical, single-celled green algae (indicated with arrows) with no flagella (Source K.M. EI-Khatib, Chemical Engineering, and Pilot Plant Department, National Research Centre)

2.3.1 Properties

The pharmaceutical importance of *Chlorella* is attributed to its medicinal properties. There is ample experimental evidence of its antitumor, anticoagulant, antibacterial, antioxidant, and anti hyperlipidemia effects in addition to a hepatoprotective property and the immune-stimulatory activity of enzymatic protein hydrolyzate (Korb et al., 1997; Lee et al., 2002; Ordog et al., 2004).

2.3.2 Bioactive compound

Many antioxidant compounds are thought to be responsible for *Chlorella's* functional activities. Antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid, and α-tocopherol, which are active against free radicals, have been identified. Some of these compounds not only are important as natural colorants or additives but also may be useful in reducing the incidence of cancer and in the prevention of macular degeneration (Korb et al.,1997; Kokou et al., 2012).

2.3.3 Uses

Chlorella sp. is an important species with a good bimolecular composition. Commercially, it is one of the most commonly used microalgae in aquaculture. The biomass of *Chlorella* is used in aquaculture as feed, growth enhancers, and immunostimulants. Despite claims of its benefits, *Chlorella vulgaris* is reported to have unfavorable effects when incorporated into diets at higher inclusion levels. In addition, its rigid cell wall might restrict the access of digestive enzymes to the intracellular components for proper digestion and assimilation. Several studies confirmed its ability to improve nutrition, immunity, aquatic bioremediation, amelioration of stress, disease resistance of fish and inhibits bacterial quorum sensing when used appropriately. The role of *Chlorella* sp. and its importance in aquaculture with emphasis on its environmental requirements, morphology, pigments, digestibility, dynamics on growth performance, antibacterial activity, bacterial quorum sensing, immunomodulatory effect, anti-stress effect, gut microbiome, aquatic bioremediation and its safety as food or feed (Ahmad, 2016).

2.4 *Spirulina*

Spirulina is a blue-green microalga that has a spiral cellular structure and has an extraordinary capacity to survive under conditions with many applications in food and

drug industries, such as food in humans, aquaculture, vet, and poultry industries (Ghaeni, 2010).

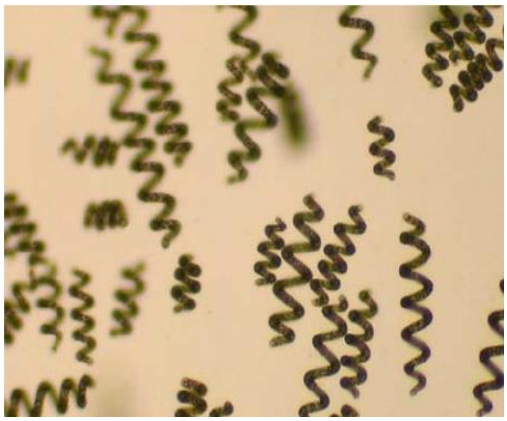

Figure 2.2 Microscopic view of microalgae *Spirulina* (Cyanobacteria). (Source, E. koru, Ege University)

2.4.1 Properties

The pharmaceutical importance of *Spirulina* is attributed to its medicinal properties. There is ample experimental evidence of its antibacterial, antioxidant, anticancer, anti-inflammatory, and effective against diabetes, obesity, hypertension, and antihyperlipidemia (Cintra et al., 1998; Hirahashi et al., 2002; Haque and Gilani, 2005; McCarty, 2007; Kumar et al., 2011).

2.4.2 Bioactive compound

The cyanobacterium, *Spirulina* has emerged as one of the most promising agents to synthesize potentially new therapeutic compounds, it contains large amounts of, carotenoid, *omega*-3, and *omega*-6 polyunsaturated fatty acids, Gamma Linolenic Acid (GLA), sulfolipids, glycolipids, polysaccharides (Varadhara et al., 2005). It is known to produce intracellular and extracellular metabolites with diverse biological activities such as antifungal, antiviral, and antibacterial activities (MacMillan et al., 2002; Kumar et al., 2011).

2.4.3 Uses

The nutritional value of *Spirulina* in addition to improving human nutrition can also be extended to the feeding of domestic animals and fish in aquaculture systems *Spirulina*-containing feed was found to reduce the cultivation time and mortality, and increase shell thickness of scallops. The study of Habib (2008), has shown that *Spirulina* can replace the feeding diets of fishmeal, groundnut meal, and soybean meal for domestic animals (poultry, cattle, and pigs) and fish. Several authors have documented the use of *Spirulina* sp. as a fish feed for various fish species mainly due to the rich nutrition and digestibility

(Habib, 2008; Zhang, 2015). *Spirulina* is a source of sulfolipids. Under laboratory conditions, in vitro experiments, it was shown a water-soluble extract of *Spirulina platensis* containing sulfoquinovosyl diacylglycerol (SQDG) inhibited herpes simplex virus type 1 (HSV-1) which are known reverse transcriptase inhibitor of human immunodeficiency virus (HIV) virus (Kwei, 2010). *Spirulina* contains improve its microbial balance. The probiotic efficiency of *S. platensis* is for lactic acid bacteria and also a potent antibacterial activity against human pathogenic bacteria (Bhowmik et al., 2009).

2.5 Marine microalgae and their potential in the pharmaceutical field

Pharmaceutically valuable products from microalgae and their industrial commercialization today is still in their infancy and can be seen as a gateway to a multibillion-dollar industry. Microalgae generally grow autotrophically and are ubiquitous. They represent a major untapped resource of genetic potential for valuable bioactive agents and fine biochemical. This proven ability of microalgae to produce these compounds places these microorganisms in the biotechnological spotlight for applications and commercialization as in the pharmaceutical industry. The immense chemical diversity of microalgae provides numerous applications in the food, feed, and pharmaceutical industries. Microalgae are cultivated for the production of whole biomass and valuable substances such as nutraceuticals, carotenoids, phycocyanin and poly-unsaturated fatty acids (PUFAs), which are utilized in the food and feed (notable aquaculture) industry. The production of biofuel from lipid- or carbohydrate-rich microalgae is underway. Microalgae comprise a vast group of photosynthetic, heterotrophic organisms which have an extraordinary potential for cultivation as energy crops. They can be cultivated under difficult agro-climatic conditions and can produce a wide range of commercially interesting by-products such as fats, oils, sugars, and functional bioactive compounds. Many valuable compounds can be extracted from microalgae, including pigments, lipids, proteins, polysaccharides, vitamins, or minerals (Encarnacao et al., 2015). The variety of compounds generated by microalgae can serve a broad spectrum of applications such as pharmaceuticals, cosmetics, human and animal nutrition, environmental restoration and protection, or bioenergy (Priyadarshani et al., 2012). Some strains of microalgae can produce metabolites with antibiotic activity aimed at killing or inhibiting bacterial growth. In some cases, this activity has only been identified in general extracts from the algal culture, without properly determining the chemical identity of the active compounds (Chu et al., 2004; Ordog et al., 2004). There are indications that antibiotics are more likely to

occur in strains isolated from environments polluted by bacteria than in strains isolated from cleaner environments. Several compounds have shown potent biological activities, such as antioxidants, anticoagulants, anti-inflammatory, antimicrobial or antitumoral (Raposo et al., 2013). The possible use of these compounds as a source of prebiotics, nutraceuticals, chemopreventive agents, or antimicrobial drugs was investigated and has demonstrated promising results (Amaro et al., 2011; Talero et al., 2015).

2.6 Antimicrobial activity of marine microalgae

Microalgae are present in almost all ecosystems around the world. They evolved in extreme competitive environments are largely grazed by highly diverse consumers and exposed to microbial pathogens such as bacteria, viruses, and fungi. Marine microalgae constitute attractive sources of novel and active metabolites, comprising proteins, enzymes, pigments, and polyunsaturated fatty acids (PUFA) that could be exploited in pharmaceutical, food, feed, and cosmetic industries (Cardozo et al., 2007; Surendhiran et al., 2014). Compounds with pharmaceutical characteristics, such as an antioxidative anti-inflammatory, antimicrobial or antitumoral properties, have been identified; some of them have been in the clinical trial state (Maadane et al., 2020). Microalgal species have the potential to benefit health as they contain some bioactive components such as lutein, β-carotene, fatty acids, phenol, phycocyanin, γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, etc. are also reduce cholesterol and improve skin health (Raposo et al., 2013). Different microalgae species such as *Chondrus crispus, Mastocarpus stellatus, Ascophyllum nodosum, Alaria esculenta, Spirulina plantesis, Nannochloropsis oculata, Chlorella vulgaris, Dunaliella salinwhich* are used to make products such as anti-irritant, antibacterial, antifungal and antivirus agent. Moreover, *Chlorella sp., Dunaliella sp., Scenedesmus sp., Nannochloropsis sp., Tetraselmis sp., Spirulina sp., and Aphanizomenon flosaquae* have been used as an antimicrobial agents to cure animal disease (Maadane et al., 2015). Eight marine microalgae *Chaetoceros sp., Chlorella sp., Dicrateria sp., Dunaliella sp., Isochrysis sp., Nannochloropsis sp., Synechococcus sp., Tetraselmis sp.* showed species-specific activity in inhibiting the growth of bacteria such as *Salmonella paratyphi, Pseudomonas fluorescens, Shigella boydi, Klebsiella pneumoniae and Escherichia coli* (Krishnika et al., 2017).

2.7 Medicinal plant

Natural products, such as medicinal plants, serve as a rich potential source of new therapeutic compounds. Herbal medicinal plants such as *Aloe Vera, Citrus hystrix, Zingibar officinale* and Sabah snake grass (*Clinacanthus nutans*) have been proved in the

previous study that the plant has antifungal properties. *Aloe vera* from the Aloe family has a long association with herbal medicine Anthraquinones are major substances found in aloe, and are known for their laxative, anti-microbial and anti-cancer activities (Young, 2006). *C. hystrix,* commonly known as kaffir lime, is a common tropical herb in the family Rutaceae found everywhere in Southeast Asia, the leaves and fruit peel contain natural antioxidants and active compound that is important in fighting the diseases and cancer (Srisukh et al., 2012). *Z. officinale* belongs to the family of Zingiberaceae, a perennial herb with thick tuberous rhizomes. *Z. officinale* extracts have medicinal properties, and antimicrobial activity as reported (Valverde et al., 2013) other than that as an antioxidant, anti-lipid, anti-diabetic, analgesic, and anti-tumor (Hasan, 2012). Sabah Snake Grass is suitable for plant growth in tropical weather such as in Malaysia and Thailand. This plant also has the antimicrobial activity similar to A. vera (Yahaya et al., 2015).

2.8 Medicinal plants available in Bangladesh

A survey was done in six communities in Bagerhat, Brahmanbaria, and Rajshahi districts of Bangladesh. 232 species were identified from the survey. Among them, are *Aloe vera, Bambusa arundinacea, Catharanthus roseus, Cocos nucifera, Clerodendrum indicum, Derris indica, Heritiera fomes, Hibiscus rosa-sinensis, Lannea grandis, Solanum indicum,* these medicinal plants are very popular in Bangladesh (Mollik et al., 2010). Besides, Sabah snake grass (*C. nutans*) is widely grown in tropical Asian regions. As having medicinal effects, this plant has been widely used in Thailand, Malaysia, and China (Sookmai et al., 2011). This plant is now being cultured at Mymensingh, at a very small scale for future research purposes.

2.9 *Clinacanthus nutans*

Clinacanthus nutans of the family Acanthaceae is an important medicinal herb in Malaysia, China and Thailand. This plant is locally known as Sabah snake grass" and "belalai gajah" in Malaysia because this plant is found in Sabah of East Malaysia and the the slightly curved stem looks like the curve of an elephant's trunk (Yahaya et al., 2015). Fresh leaves of *C. nutans* have long been used for traditional treatments in Asia.

Figure 2.3 Sabah snake grass (*C. nutans*) plant (Source flora and fauna webpage)

2.9.1 Properties

Clinacanthus nutans is a well-known medicinal plant in Asia. There is ample experimental evidence of its antiviral, anti-inflammatory, antivenom, antibacterial, antioxidant, and antiproliferative activity and the central mediated antinociceptive effect via the opioid and nitric oxide-mediated pathways (Uawonggul et al., 2006; Arullappan et al., 2014; Sulaiman et al., 2015; Chithra et al., 2016; Pongmuangmul et al., 2016; Rahim et al., 2016;).

2.9.2 Bioactive compound

C. nutans contain stigmasterol, botulin, lupeol and β-sitosterol. These chemical compounds were isolated from light petroleum extracts of the stems, roots, and leaves of *C. nutans* (Dampawan et al., 1977; Lin et al., 1983). Lupeol, β-sitosterol, and terpenoid flavonoids present in *C. nutans* possess antibacterial activity (Yang et al., 2013).

2.9.3 Uses

C. nutans has gained high popularity and have widely been used as remedies for various health ailments due to their high medicinal value in suppressing the advancement of cancer. In addition, various scientific reports had also proven the extract of *C. nutans* contains antioxidant and antiproliferative properties when tested with cancer cell lines (Yong et al., 2013; Arullappan et al., 2014; Sulaiman et al., 2015). *C. nutans* exhibited antimicrobial activity against *E. coli*, *Salmonella* sp., *Bacillus* sp., and *Streptococcus* sp. with a zone of inhibition from 7.00 - 15.66 mm (Chithra et al., 2016). These results indicated that the *C. nutans* extracts can inhibit the growth of certain microbes and is a potential anti-microbial agent.

2.10 *Aloe vera*

Aloe vera is a member of the Liliaceae family which contains about four hundred species of flowering succulent plants (Newall et al., 2002; Mohammad, 2003). *Aloe vera* is a typical xerophyte. It is a cactus-like plant with thick, fleshy, cuticularized spiny leaves that grows readily in hot, dry climates (Choi et al., 2002; Tan and Vanitha, 2004).

Figure 4.4: *Aloe vera* plant (Source flora and fauna webpage)

2.10.1 Properties

Aloe vera is a well-known medicated plant. There are ample experimental benefits of *Aloe vera* such as antioxidant, anticancer, antibacterial, wound healing, anti-aging, anti-inflammatory, antiviral, antitumor, and antiseptic effect. (Surjushe et al., 2008)

2.10.2 Bioactive compound

It has been reported that *Aloe vera* contains six antiseptic agents: Lupeol, salicylic acid, urea nitrogen, cinnamomic acid, phenols, and sulfur, which causes *Aloe vera* to have inhibitory effects against fungi, bacteria, and viruses (Surjushe et al., 2008). The anthraquinone aloin present in *Aloe vera* inactivates several enveloped viruses such as herpes simplex, varicella-zoster, and influenza (Sydiskis et al., 1991).

2.10.3 Uses

For thousands of years, *Aloe vera* has been used for medicinal purposes in several cultures: Greece, Egypt, India, Mexico, Japan, and China (Marshall, 1990). It is a well-known dietary supplement and chemopreventive agent, and its gel is also used for topical treatment of skin irritations (Bergamante et al., 2007). It has been reported that *Aloe vera* gel has a protective effect against radiation damage to the skin (Roberts and Travis, 1995). The gel also plays a role of a cohesive agent on the superficial flaking epidermal cells by

sticking them together, thereby softening the skin. The amino acids also soften hardened skin cells and zinc acts as an astringent to tighten pores (West and Zhu, 2003). Besides all these, *Aloe vera* is also known to have healing effects against ulcers, diabetes, inflammations, and tumors (Surjushe et al., 2008).

2.11 Medicinal plants (*C. nutans, Aloe vera)* and their potential in the pharmaceutical field

A significant number of new therapeutics are being derived from natural sources such as plants, as systemic and topical novel drugs and antiseptics to replace or to be used in collaboration with existing products (Woodford, 2005). Many plant materials used in traditional medicine have been proven to be more effective, and relatively cheaper than their modern counterparts (Mann et al., 2008). Antimicrobials of plant origin also alleviate many of the side effects that are often associated with synthetic ones (Iwu et al., 1999; Mukherjee and Wahile, 2006) *C. nutans* is listed in the National Key Economic Areas (NKEAs) proprietary list due to its medicinal properties (Narayanaswamy and Ismail, 2015). *C. nutans* leaves have long been used in herpes simplex treatment in Thailand and cream made from *C. nutans* extracts was shown to be as efficacious as Acyclovir in healing herpes simplex and herpex zoster (Vachirayonstien et al., 2010; Kunsorn et al., 2013). Reports from Farooqui et al. (2015) and Gan et al. (2015) showed that *C. nutans* is the most common herb being used in complementary and alternative medicine for cancer patients in Malaysia. In Malaysia and Singapore, the leaves of *C. nutans* are usually juiced with a green apple to mask the unpleasant grassy smell and consumed by cancer patients as a side treatment (Siew et al., 2014). Leaves are usually juiced or consumed as raw vegetables in Thailand and the pounded leaves have been applied to the affected site to treat skin rashes and insect bites (Makhija and Khamar, 2010). The virucidal activities of *C. nutans* extracts can inactivate intracellular and extracellular activity of HSV-2 and inhibit pre-infection (Sakdarat et al., 2009; Vachirayonstien et al., 2010). Wong et al. (2014) investigated the antioxidant properties of six tropical medicinal plants, namely *Clinacanthus nutans*, *Callicarpa formosana*, *Hedyotis diffusa*, *Vernonia amygdalina*, *Leonurus cardiac* and *Pereskia bleo*, and indicated *C. nutans* as having the strongest antioxidation potential with high metal chelating and radical scavenging activities compared to other medicinal plants at the concentration of 10mg/ml. *C. nutans* fresh leaves have been used traditionally in Thailand, Vietnam, and Malaysia to treat scorpion and snake bites. On the other hand, Uawonggul et al. (2006) studied the anti-venom activities of 64 plant extracts against scorpion venom activity on fibroblast cell lysis. The results

obtained indicated that *C. nutans* extracts can neutralize scorpion venom with an efficiency of 46.51%, indicating that the extracts can be used as an antiscorpion venom to prevent the cells from lysis. Rahim et al. (2016) experimented to determine the antinociceptive effect mechanism of *C. nutans* against animal nociceptive models and reported that the methanolic extract of *C. nutans* leaves was found to exhibit peripheral and central mediated antinociceptive effect via the opioid and nitric oxide-mediated pathways.

Aloe vera is now grown in large amounts in Bangladesh. People use *Aloe vera* as skin care products and also in the production of cosmetics and medicines. It has been reported that *Aloe vera* gel has a protective effect against radiation damage to the skin (Roberts and Travis, 1995). The exact mechanism of action is yet to be discovered, but after the administration of *Aloe vera* gel, metallothionein, which is an antioxidant protein, is generated in the skin. This scavenges hydroxyl radicals and prevents suppression of superoxide dismutase and glutathione peroxidase in the skin. This, in turn, reduces the production and release of skin keratinocyte-derived immunosuppressive cytokines such as interleukin-10, thereby preventing UV-induced suppression of delayed-type hypersensitivity (Byeon et al., 1998). The effects of *Aloe vera* on the immune system include Alprogen (Aloe single component) inhibiting calcium influx into mast cells, hence inhibiting the antigen-antibody-mediated release of histamine and leukotriene from mast cells (Ro et al., 2000). Anthraquinones present in the latex of *Aloe vera* is a potent laxative. It increases intestinal water uptake, stimulates mucus secretion, and increases intestinal peristalsis (Ishii et al., 1994). Mucopolysaccharides in *Aloe vera* gel help in binding moisture into the skin. Stimulating fibroblasts which produce collagen and elastin fibers, makmakee skin more elastic and less wrinkled (Surjushe et al., 2008).

2.12 Antimicrobial activity of Medicinal plant (*C. nutans, Aloe vera)*

Despite the numerous numbers of literature present on antimicrobial properties of plant extracts, not many plants derived chemicals have been successfully exploited for clinical use as antibiotics. A considerable part of the chemical diversity produced by plants is thought to protect plants against microbial pathogens. Hence, they have been proven to have antimicrobial importance both *in vivo* and *in vitro* (Gibbons, 2004). Several reports are available on the antimicrobial activity of hexane, ethanol, acetone, petroleum ether, and ethyl acetate extracts of *Aloe vera* gel and leaves (Agarry et al., 2005). Antibacterial activity of *Aloe barbadensis* was tested on certain clinically isolated bacterial pathogens such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonie,* etc. Antibacterial effects of ethanolic and aqueous extracts were tested by

examining the appearance of any zone of inhibition on bacterial culture plates. Ethanol extracts were found to be quite effective on both gram-positive and negative bacteria, but the aqueous extracts did not show any inhibitory effect (Pandey and Mishra, 2010). Another study was conducted to determine the antimicrobial activity of *Aloe vera* juice against gram-positive and negative bacteria, and the fungus *Candida albicans.* Making use of the disc diffusion method, the study revealed that the tested plant juice was most effective against gram-positive bacteria and *C. albicans* (Alemdar and Agaoglu, 2009). A study aims to investigate the antimicrobial activity of Dimethyl sulfoxide (DMSO) crude extracts of *Aloe barbadensis.* Miller gel against selected bacterial and fungal pathogens showed that *Aloe vera* extracts are effective against most of the microbial strains used. The maximum zones of inhibitions appeared against *E. coli, Proteus mirabilis, Pseudomonas aeruginosa, S. aureus, C. albicans,* and *Penicillium spp* (Devi et al., 2012). These results indicated that the *Aloe vera* extracts are a potential anti-microbial agent. The extract of *C. nutans* was also found to possess anti-microbial activities. *C. nutans* has also been reported to have antimicrobial activity against *Bacillus cereus* (ATCC11778), *Candida albicans* (ATCC10231), *Escherichia coli* (ATCC25922), and *Salmonella enterica* Typhimurium (ATCC14028) (Arullappan et al., 2014). Moreover, Ho et al. (2013) experimented to investigate the antibacterial activities of methanolic extract of *C. nutans* leaves against five selected bacteria, namely *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes*. They discovered that *C. nutans* extracts exerted a significant antibacterial effect against *E. coli* and *S. aureus* with minimum inhibitory concentrations (MIC) of 12.5 mg/ml (Ho et al., 2013). These results indicated that the *C. nutans* extracts can inhibit the growth of certain microbes and is a potential anti-microbial agent.

2.13 Bacterial diseases of aquaculture in Bangladesh

Farmers face a severe outbreak of disease caused by bacteria pathogens which cause significant economic losses. Over 13 bacterial genera have been reported to cause bacterial diseases in the aquaculture field worldwide (Pridgeon et al., 2012). Major bacterial pathogens include *Aeromonas, Salmonella, Edwardsiella, Vibrio, Streptococcus, Staphylococcus, and E. coli* species which affect fish (Baeck et al., 2006, Won and Park, 2008). Especially, the larval stages of several farmed aquatic animals are highly susceptible to bacterial diseases (Defoirdt et al., 2011). In pond-raised fish, bacterial infections are caused by motile members of the *Aeromonas* genus. *Aeromonas hydrophila, Aeromonas sorbia, Aeromonas caviae, Aeromonas schuberti* and *Aeromonas veronii* are

among others (Azad et al., 2001). In farmed fish, Edwardsiellosis is a serious bacterial septicemic disease and is caused by the Gram-negative bacterium, *Edwardsiella* sp. (Nadirahet et al., 2012). *Flavobacterium* sp. is another bacterial disease in Tilapia farms and is highly contagious, especially to fingerlings resulting in high mortality (IAAH, 2007). The bacterial illness caused by *Streptococcus* sp. is Streptococcosis. Freshwater-cropping trout is the most common pathogenic *Streptococcus* species that affects fish. *Streptococcus* sp. Gram-positive bacteria are organized in strings and cocci. Another parasite affecting tilapia is *Streptococcus agalactiae* which is associated with intense breeding of brood stock (Hernandez et al., 2009). Streptococcosis in tilapia farms can cause mortality of up to 70% (Laith et al., 2014) leading to dramatic economic losses from outbreaks (Fawzy et al., 2014). A wide number of freshwater fish species including tilapia are infected by *Pseudomonas* the etiological agent for red skin disease. It is suspected that *Pseudomonas anguilliaseptica* is one of the most effective pathogens in cultured fish (Mastan, 2013). Vibriosis is one of the major disease problems in shellfish and finfish aquaculture. Vibriosis is a bacterial disease responsible for the mortality of cultured shrimp worldwide (Chen et al., 2000). Bacterial diseases of crabs are not common but the genus *Vibrio* is frequently cited, especially *Vibrio parahemolyticus*, *Vibrio cholerae*, and *Vibrio vulnificus*, which commonly occur in crabs (Wang, 2011).

2.14 Common antimicrobials used in the aquaculture industry

Antimicrobials are used in animals to treat or prevent disease and also to promote growth. Therapeutic, metaphylactic, prophylactic, sub-therapeutic, etc. are used as antimicrobial therapy applies in the animal body. The use of antibiotics in any setting contributes to the growing global threat of antibiotic resistance (Milic et al., 2013). Different antimicrobial drugs are used for various purposes such as chloramphenicol, colistin, ceftriaxone, cefalexina, moxiolin, amitriptyline, cefpodoxime, cefliofur, chloramphenicol, ciprofloxacin, clavamox, clavaseptin, clavulanic acid, clindamycin, dichlorophene, doxycycline, enrofloxacin, neomycin, oxytetracycline, pirlimycin, etc are used as antibiotic for bacterial infection (Algama, 2012; Schwarz, 2016).

2.15 Antimicrobial resistance as a major obstacle in the aquaculture sector

Antimicrobial resistance (AMR) is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial such as antibiotics, or antiviral from working against it. As a result, standard treatments become ineffective; infections persist and may spread to others (WHO, 2020). There is concern about how antimicrobial resistance emergence in aquaculture will impact sustainable development goals (SDG), i.e., ensuring

healthy lives and promoting wellbeing for all, at all ages. With fish production set to increase from 200 million tons to 470 million tons by 2050, farmers will likely rely even more on antibiotics to prophylactically prevent disease to meet this expected demand. With the high proportion of poor-quality aqua drugs for therapeutic use in aquaculture, the problem of antibiotic overuse, particularly in low- and middle-income countries has increased. Numerous cases of antimicrobial resistance in humans have been traced to resistant microbes suspected to originating in fish, which is particularly concerning as infected stock, can be asymptomatic. Transmission of resistant bacteria from aquaculture to humans can occur through the consumption of meat, direct contact with colonized animals, or manure spread in the environment. The strongest correlation between interspecies and pathogens transmissions is observed in countries with policies to reduce agricultural antibiotic use. So, it is important to minimize the use of these drugs. This means eliminating unnecessary uses and finding other ways to prevent infections (WHO).

2.16 Spread of AMR and risks to public health

Every year, antimicrobial resistance causes the death of around 700 000 people, and this number is expected to rise to an estimated 10 million deaths annually by 2050 (Hoelzer et al., 2017). Antimicrobial resistance has the potential to affect almost all sustainable development goals (SDGs), particularly those targeting poverty, hunger, health, and economic growth. Although the reduction and eradication of antimicrobial resistance are not included as an individual SDG, paragraph 26 of Transforming our world: the 2030 agenda for sustainable development states: "We will equally accelerate the pace of progress made in fighting malaria, human immunodeficiency virus/acquired immunodeficiency syndrome AIDS, tuberculosis, hepatitis, Ebola and other communicable diseases and epidemics, including by addressing growing antimicrobial resistance and the problem of unattended diseases affecting developing countries. Antimicrobial resistance is a public health threat. Because antimicrobial consumption in food-producing animals contributes to the problem, policies restricting the inappropriate or unnecessary agricultural use of antimicrobial drugs are important. However, this link between agricultural antibiotic use and antibiotic resistance has remained contested by some, with potentially disruptive effects on efforts to move towards the judicious or prudent use of these drugs.

2.17 Alternatives to antibiotics

In aquaculture, alternative products play a crucial role in allowing farmers to reduce or largely phase out the use of antibiotics. Vaccines are among the most promising and widely

used of these alternatives, but prebiotics and probiotics, and other innovative products like immune modulators, phages, phytochemicals, organic acid, and anti-microbial peptides are also in use or currently being investigated. Many alternative products enhance fish productivity and prevent infection at the same time, which could make them particularly attractive for commercial operations (Hoelzer et al., 2017).

Pathogenic bacteria were chosen in this study which causes bacterial diseases in finfish and shellfish most often. Extracts of microalgae (*Chlorella* sp., *Spirulina* sp.) and medicinal plants (*C. nutans, Aloe vera*) were taken to observe the antimicrobial properties against those pathogenic bacteria to reduce the pressure on synthetic antibiotic which causes antimicrobial resistance and risk to public health. However, there is a very limited report of available microalgae in marine sources and medicinal plants, their activities against the microbial agents causing aquaculture, and human diseases in Bangladesh. So, the present work provides the eligibility of algae and medicinal plants commonly found in Bangladesh as prominent natural antibiotics against various pathogens.

CHAPTER 3

MATERIALS AND METHODS

Experimental design: My experiments were divided into four phases:

3.1 Culture of marine microalgae

3.2 Collection of medicinal plant

3.3 Isolation, identification, and culture of bacteria

3.4 Antimicrobial activity test

3.1 Culture of marine microalgae

Chlorella sp. was kindly supplied by Bangladesh Fisheries Research Institute (BFRI), Cox's Bazar. *Spirulina* sp. was obtained from University Malaysia Terengganu.

3.1.1 Conway medium preparation

Conway medium was used as a culture medium for the marine microalgae. Preparation of Conway medium needs to prepare the three stock solutions which were macronutrients (A), trace metal solutions (B), and vitamins (C). Each of the vitamins was dissolved separately in 100 ml of distilled water and stored in a refrigerator. Different reagent bottles were prepared for solutions A, B, and C. For each 1 ml of (A), 0.5 ml of (B), and 0.1ml of (C) were added to 1000 ml of filtered and sterilized seawater (James, 1996). The composition of the Conway medium is shown in Table 3.1

Table 3.1 Preparation of Conway medium:

Constituents	Quantities
Solution-A chemicals	
Potassium nitrate	100 g
Sodium orthophosphate	20 g
Sodium EDTA	45 g
Boric acid	33.4 g
Ferric chloride	1.3 g
Manganese chloride	0.36 g
Distilled water	1000 ml
Solution-B trace metals	
Zinc chloride	4.2 g
Cobalt chloride	4.0 g

Copper sulfate	4.0 g
Ammonium molybdate	1.8 g
Distilled water	1000 ml
Acidify with HCl to obtain a clear solution	
Solution-C vitamins	
Vitamin B (Thiamin)	200 mg
Vitamin B_{12} (Cyanocobalamin)	10 mg

3.1.2 Mass culture of microalgae

Mass culture of selected potential isolates was done on large scale in a tank using Conway medium. The culture was gradually scaled up from an initial starter culture volume of 20 ml to 20 L. Initially, 20 ml of microalgae stock cultures were mixed with 30 ml medium in each flask (total culture volume 50 ml), with batch cultures of increasing volume (250, 500) ml, 1 L, 10 L) as inoculum for the next step after which they were transferred to bigger container 20 L culture medium. After that, *Spirulina* sp. and *Chlorella* sp. were harvested at their stationary phase on day-12 and the microalgae were harvested by centrifugation at 5000 rpm for 5 minutes to get rid of the water content.

3.1.3 Preparation of microalgae extracts

The collected algae paste was dried at 40°C for 24 h using a hot air oven. 10 ml of the ethanol solvent was used for extraction of one gram of dried algal samples and it was soaked in the solvent in the sterile screw-capped bottles of 100 ml volume for two days (48 h) at room temperature. (Arun et al., 2012). After that, the extract was filtered through sterile Whatman no. 1 filter paper to remove all unextractable matter such as cellular material (Gonzalez et al., 2001) and the filtrate was concentrated under reduced pressure in a rotary evaporator. (Simic et al., 2012). The dry extract was stored at -20°C for the antimicrobial further studies.

3.2 Collection of medicinal plant

Medicinal plants (*C. nutans*) leaves were collected from a medicinal plant garden and, Mymensingh and *Aloe vera* leaves were collected from the local herbal plant selling market, Chattogram.

3.2.1. Preparation of medicinal plant extracts

Firstly, the *A. vera* and *C. nutans* leaves were washed with water to remove sand and dust particles. Then the cleaned leaves were dried in the oven at 80°C for 48 h. After that, the

samples that have been dried are crushed into small pieces using the blender until they became powdered. The solvent used is ethanol. For the preparation of ethanol, 20 g of each sample powder is soaked in 100 ml of the solvent and put on an incubator shaker for 24 h. The mixed solutions then filtered and were kept in the refrigerator until further use (Uda et al., 2018).

3.3 Isolation, identification, and culture of bacteria

3.3.1 Bacterial sample collection from fish

Fish samples (Tilapia, Bata) were collected from Fishery Ghat, Chattogram. Skin, intestinal, and gill swabs were taken from the fish using sterile swabs and inoculated into Buffered Peptone Water (BPW). The samples were incubated at 37°C overnight.

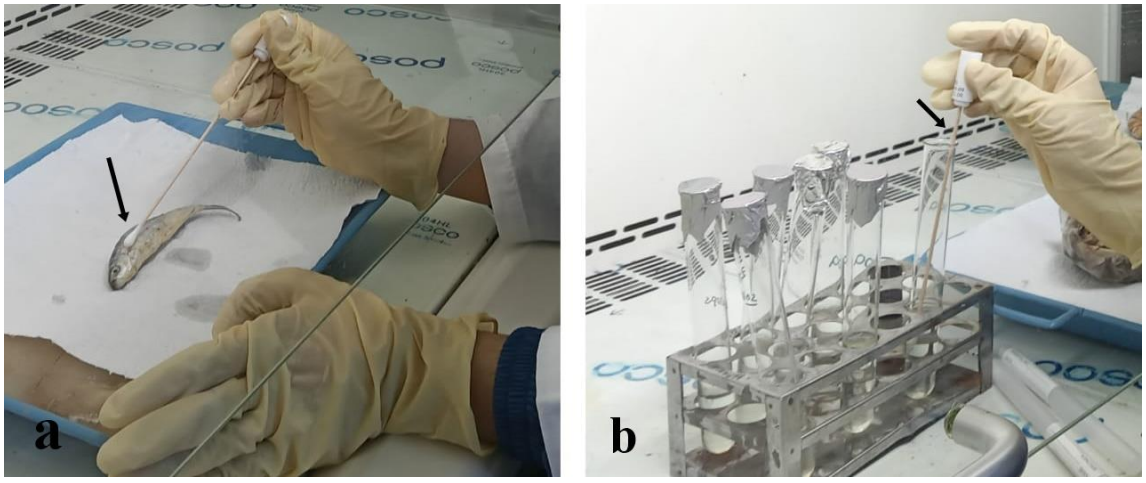

Figure 3.1 Swab were taken from the different organs of the fish sample (a) and (b) inoculated into buffer peptone water (arrow indicated swabs are placed in clear media)

3.3.2 Bacterial sample collection from Shrimp and crab

Shrimp sample was collected from the BFDC landing center and crab from the crab farm, Cox's Bazar. The swab was taken from the abdominal flap, mouth part, claw, and carapace region from the crab and joint of the appendages, uropod, endoskeleton, and cephalothorax region from shrimp using sterile swabs and inoculated into Nutrient Broth (NB). The samples were incubated at 37°C overnight.

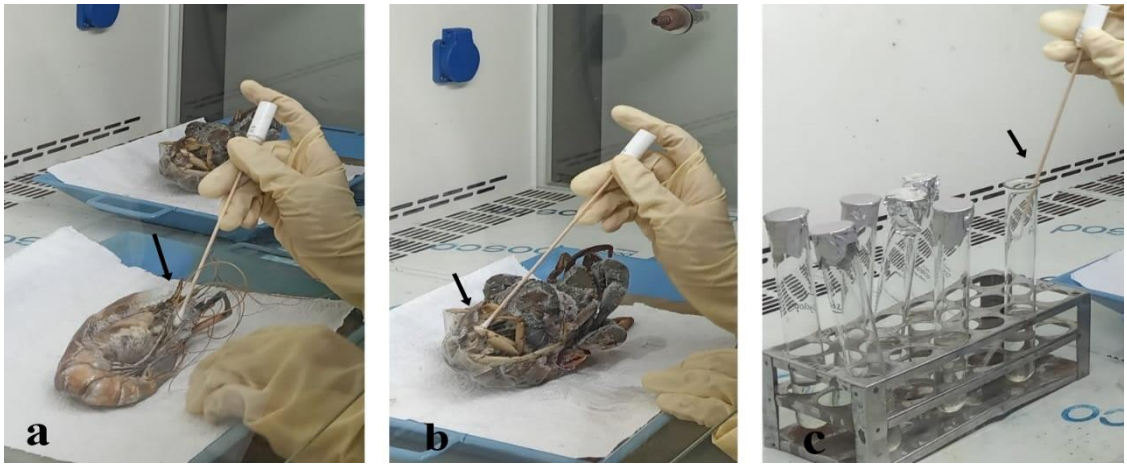

Figure 3.2: Swab were taken from (a) shrimp; (b) crab sample and (c) inoculated into the nutrient broth (arrow indicated swabs are placed in clear media).

3.3.3 Preparation of bacterial culture media:

Buffered peptone water

One gram of peptone bacteriological powder and 1 g of peptone powder were weighed and mixed in 1000 ml distilled water in a conical flask. The mixture was heated for proper mixing, autoclaved at 121℃ for 15 minutes for sterilization, and then cooled to room temperature. 5 ml of buffered peptone water was poured into the test tube for the collection of bacterial samples.

Nutrient broth

Suspended 13.0 g of NB powder in 1000 ml of distilled water. The mixture was boiled to dissolve the medium completely. Autoclaved at 121°C for 15-20 minutes.

Phosphate buffer saline (PBS)

Suspended 9.55 g of PBS powder into 1L of distilled water. Properly mixed well with the help of a glass rod. Autoclaved at 121°C for 20 minutes. Stored at 4°C in the refrigerator.

Trypticase soy agar (TSA)

Suspended 40g of TSA agar powder into 1000 ml of distilled water. The mixture was boiled to dissolve the medium completely. Autoclaved at 121°C for 15-20 minutes. Cooled to 45-50°C. Mixed well before pouring into sterile Petri plates.

Mannitol salt agar

Suspended 111.025 g of MSA agar powder into 1000 ml of distilled water. The mixture was boiled to dissolve the medium completely. Autoclaved at 121°C for 15-20 minutes. Cooled to 45-50°C. Mixed well before pouring into sterile Petri plates.

MacConkey agar

Suspended 49.53 g of MAC agar powder into 1000 ml distilled water. Heated to boil to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes. Cooled to 45-50°C. Mixed well before pouring into sterile Petri plates.

Eosin methylene blue (EMB) agar

Suspended 35.96 g of EMB powder in 1000ml distilled water. Heated to boil to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes. Cooled to 45-50°C and shook the medium to oxidize the methylene blue and suspend the flocculent precipitate. Mixed well before pouring into sterile Petri plates.

Thiosulfate-citrate-bile-salts-sucrose (TCBS) agar

Suspended 89.08 g of TCBS agar powder into 1000 ml distilled water. Heated to boil to dissolve the medium completely. Cooled to 45-50°C. Mixed well before pouring into sterile Petri plates.

Blood agar

Suspended 40 g of blood agar powder in 1000 ml of distilled water. Heated the mixture while stirring to fully dissolve all components. Sterilized by autoclaving at 121°C for 15 minutes. Cooled to 45-50°C in a water bath. Then aseptically added 5% (vol/vol) sterile defibrinated blood and mixed gently. Dispensed into sterile plates while liquid.

Muller Hinton agar (MHA)

Suspended 38.0 g of MHA agar powder into 1000 ml distilled water. Heated to boil to dissolve the medium completely. Cooled to 45-50°C. Mixed well before pouring into sterile Petri plates for a sensitivity test.

Triple Sugar Iron agar

Suspended 64.42 g of TSI agar powder in 1000 ml distilled water. Heated to boil to dissolve the medium completely. Mixed well and distribute into test tubes.

Sulfide indole motility (SIM) agar

Suspended 30 g of the medium in 1000 ml of distilled water. Heated to boil with agitation to dissolve completely. Dispense into tubes and sterilize by autoclaving at 121°C for 15 minutes.

3.3.4 Isolation and identification of bacteria by culture and staining

Bacterial culture from buffered peptone water and the nutrient broth was streaked onto MAC, MSA, TCBS, and blood agar and incubated at 37°C for 24 h. Bright-pink large colonies in MAC agar, green and yellow color colonies in TCBS agar, medium-sized yellow colonies in MSA, and round, smooth, and glistening, looking like dewdrops

with/without hemolysis in Blood agar indicated growth of *E. coli*, *Vibrio* sp., *S. saprophyticus*, and *A. hydrophila* respectively. Identification of bacteria was further confirmed by Gram's staining.

Gram staining of bacterial samples:

Preparation of Gram's stain:

Crystal violet

Firstly, 95% ethyl alcohol was dissolved into 20 ml of 2 g marked crystal violet. Then 8 g ammonium oxalate was dissolved into 80 ml of purified water. The two solutions were combined and allowed to stand at room temperature (25°C) overnight then filtered in a coarse filter paper and stored at room temperature.

Gram's iodine

One gram of crystalline iodine and 2 g potassium iodide were ground in a mortar and mixed in 300 ml distilled water. After that, stored at room temperature (25°C) in a foil-covered bottle (to protect the solution from light).

Decolorizer

To prepare the decolorizer, 500 ml acetone, 475 ml ethanol or methanol, and 25 ml distilled water were mixed thoroughly and stored at room temperature.

Safranin

Firstly, 2.5 g certified safranin was added to 95% ethyl alcohol. Then added a 10 ml mixture of safranin and ethanol alcohol solution to 90 ml of distilled water. Then stored at room temperature.

Gram staining procedure

Preparation of slide smear

One loop full of bacterial broth culture was taken onto a glass slide using a sterile inoculating loop. A drop of water was taken before bacterial samples were taken from agar plates and mixed well. The smear was left to air dry at room temperature. The smear was fixed by holding over the flame for a few seconds.

Staining protocol

In the slide, primary stain crystal violet was applied for 1 minute. To wash out the unbound crystal violet, rinsed the slide with a soft stream of water for up to 10 seconds. Added Gram's iodine for 1 minute, this is a mordant or argent that fixes the crystal violet to the bacterial cell wall. Rinsed the slide with acetone for 3 seconds and rinsed with a gentle stream of water. After that, secondary stain safranin was added to the slide for 1 minute and washed with a gentle stream of water. After air dried, slides were examined

under a light microscope. Gram-positive bacteria resembled blue/purple color while Gram-negative bacteria as pink color.

3.3.5 Biochemical tests

The bacterial samples were subjected to biochemical tests, performed in the Marine Biotechnology Laboratory at the University Malaysia Terengganu, Malaysia using the VITEK 2 system. Bacterial species of *A. hydrophila, S. saprophyticus, and E. coli* were confirmed by using VITEK 2 system. Several biochemical tests (Catalase, Production of H_2S, Triple Sugar Iron, motility tests) were done to confirm the *Vibrio* sp., at Disease and Microbiology lab, CVASU.

Principles of VITEK 2 biochemical identification method

VITEK 2 compact is one of the most widely used integrated and automated systems in bacterial identification based on biochemical profiles of tested strains. The VITEK 2 was used according to the manufacturing instructions; ID-Gram positive cards (ID-GP cards) were used for identification. The ID-GPC card is a 64 wells plastic card containing 18 empty wells and 46 wells for fluorescent and inhibitory tests that include pH change tests and derivatives to detect aminopeptidase and oxidizes. Results of detection are obtained in about 8h or less.

Preparation of test suspension

Three millilitres of sterile aqueous 0.45 to 0.50 % NaCl, pH (4.5- 7) were aseptically moved into polystyrene test tubes (12×75). A sufficient number of colonies were moved from 24h culture on the prescribed culture medium to the saline tube using a sterile stick or swab to obtain a density equal to McFarland 0.50 to 0.63 with the VITEK 2 DENSICHEK. The culture was tested by the VITEK 2 GP card system within 30 min of the suspended culture preparation. The culture tube and the VITEK 2 GP card were inserted into the VITEK 2 cassette and referred for guidance on the use of the instrument to the user manual (provided with the instrument). Results of recorded detection from the VITEK 2 system. Slash line or low discrimination identification were appropriate outcomes for the VITEK 2 GP process, as shown in the VITEK 2 GP product details given to end-users, requiring supplementary testing to better overcome the identification of the organism. Probabilities of bacteria species identification using this system is nearly 100%.

3.3.6 Biochemical tests for identification of *Vibrio* sp.

Selective media: TCBS agar, is a type of selective agar culture plate that is used in microbiology laboratories to isolate *Vibrio* species. TCBS agar is highly selective for the isolation of *V. cholerae* and *V. parahaemolyticus* as well as other *Vibrio* species.

Triple Sugar Iron (TSI) test

With a straight inoculating needle, touched the top of a well-isolated colony. Inoculated TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant. Left the cap on loosely and incubate the tube at 37°C in ambient air for 18 to 24 h. Following incubation, examined for color change in slant and butt, blackening, and cracks in the medium (Tille, 2014).

Catalase test

A small amount of organism is collected from a well-isolated 18 to the 24 h colony with a sterile inoculating loop or wooden applicator stick and placed onto the microscope slide. A drop of 3% H_2O_2 onto the organism on the microscope slide by using a dropper or Pasteur pipette. The formation of bubbles is observed against a dark background to enhance readability. If bubbles appear (due to the production of oxygen gas) the bacteria are catalase positive. If no bubbles appear, the bacteria are catalase-negative (Karen, 2016).

Motility test

Prepared Sulphide indole motility (SIM) agar medium which is semisolid in a test tube. Inoculated with a straight needle to a colony of a young (18- to 24-h) culture growing on an agar medium. Stabbed once to a depth of only 1/3 to ½ inch in the middle of the tube. Precautions are taken to keep the needle in the same line it entered as it is removed from the medium. Incubated at 37°C for 24 h. Observe for a diffuse zone of growth flaring out from the line of inoculation (Cappuccino and Sherman, 2008). The positive result indicates diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. A negative result indicates growth that is confined to the stab-line, with sharply defined margins and leaving the surrounding medium transparent.

3.4 Antimicrobial activity test

Disk diffusion testing was done according to the 2011 guidelines of the European Communities of Antimicrobial Activity Test using standard antibiotic disks and Mueller-Hinton agar (MHA) plates. 1 g of dried extracts were dissolved into 10 ml of extraction solvents. The bacterial suspension was adjusted by adding 0.85% physiological saline to match the turbidity of a 0.5 McFarland standard of approximately 1.5×10^8 CFU/ml (Yilmaz, 2012). Each bacteria species is inoculated in three replicates into the MHA plate for the use of antimicrobial activity by using sterile cotton swabs. Sterile filter paper discs (6mm) were immersed in the extraction solvents. After that, they were dried by normal evaporation in a contamination-free environment and placed on the Muller-Hinton agar

plate using sterilized forceps. The standard antimicrobial disks used for the *A. hydrophila, E. coli, and Vibrio sp., was* Colistin and for *S. saprophyticus* was Ceftriaxone as a positive control. A blank sterile Whatman filter paper was used as a negative control. The plates were incubated in an inverted position at 37°C overnight. Clear and circular zones surrounding the discs were the inhibition zones that were produced by the extracts and the zone of inhibitions were measured by using digital slide calipers (Robotics BD shipment) from one edge of the zone to the other edge. The comparison of the antimicrobial activity of the extract was a relative percentage inhibition and the following formula was used to monitor (Kumar et al., 2016).

$(X-Y) \div (Z-Y) \times 100$

Where,

X: total area of inhibition of the microalgae extracts

Y: total area of inhibition of the blank disc

Z: total area of inhibition of the standard antimicrobial agents

The total area of inhibition was calculated by using area = $\pi r2$; where, r = radius of inhibition zone.

3.4.1 Minimum inhibitory concentration (MIC)

The Minimal inhibitory concentration (MIC) of ethanol extract of microalgae and medicinal plants against bacteria was determined using the broth microdilution technique with slight modification (Dufour et al., 2003; Gary et al., 2003). For the preparation of *Chlorella, Spirulina, C. nutans, and Aloe vera* stock solutions, 2.5 g powder of each treatment was taken into a 15 ml PBS solution in a centrifuge tube and mixed vigorously using a vortex machine. After that, the tubes were placed in a Sonicator machine for 10minutes using pulsed, high-frequency sound waves to agitate and lyse the cells. The bacterial suspension was prepared and diluted to match the 0.5 McFarland standards. 150 µl bacterial suspensions were put in each well of a 96 well microtiter plate down the column (1-12). In each of the two replicates, microalgae and medicinal plant stock solution were put to make the concentration as 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml, and 50mg/ml (concentrations were determined based on our previous work in the lab). Bacterial suspensions with PBS act as the positive control and bacterial suspensions with antibiotics as the negative control. Incubated the 96 well plates overnight at 37^0C temperature. The presence of bacteria in each well was determined by color turbidity and OD value (630nm) by the ELISA test. The MIC value was determined as the minimum concentration of microalgae extract capable of inhibiting bacterial growth.

3.5 Bacteria preservation

3.5.1 Stock culture preservation

Selected a pure well-isolated colony showing the same colony characteristics and not overlapping. Stabbed each strain into 2 tubes of 1.2% TSA, labeled, and incubated. These will serve as stock cultures. Kept the stock cultures in the lowest compartment of the refrigerator (8-12°C) or at room temperature until use. Stock cultures in these conditions can be used for less than 6 months.

3.5.2 Long Term Preservation

Purified bacterial cultures may also be stocked in nutrient broth with 20% glycerol and stored at –80°C until use. Bacterial cultures may be stocked in this condition for 2 years.

3.6 Statistical analysis

All experiments were done in triplicates and the data presented are the averages of the mean of three independent experiments with standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) using SPSS and the *post-hoc* mean separations were performed by Duncan's multiple range test at $p < 0.05$ (Harter, 1960).

CHAPTER 4

RESULTS

4.1 Culture of marine microalgae

Chlorella and *Spirulina* were cultured in 20 L culture jars. After harvesting by centrifugation and drying process, finally harvested 7.2 g of *Chlorella* and 7.4 g of *Spirulina powder* from each 20 L bulk culture.

4.2 Collection of medicinal plant

Raw *Aloe vera* and *C. nutans* leaves were collected. After the drying process, finally harvested 40.72 g *Aloe vera* powder from four big leaves and 46.5 g of *C. nutans* powder from 100 g raw leaves.

4.4 Isolation and identification of bacteria

4.3.1 Isolation of fish sample

Samples collected from Bata and Tilapia fish were placed in buffer peptone water (BPW), and growth of bacteria was observed on the next day when the clear BPW turned cloudy (Figure 4.1) before inoculation into selective media. Turbid broth indicates bacterial growth. Bacteria were streaked onto different agar media for isolation and identification.

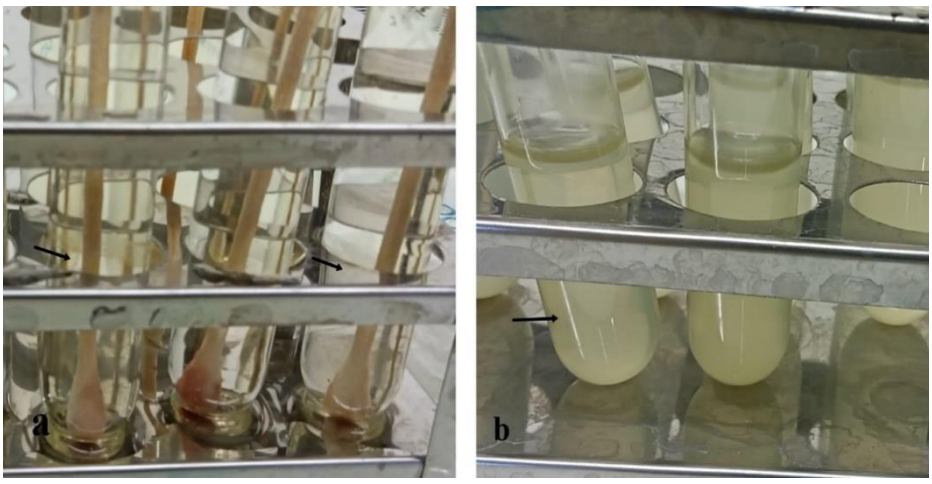

Figure 4.1 Sample collection and primary culture in buffer peptone water. a) Samples were collected using a sterile cotton swab inserted directly into the buffer peptone water (BPW, arrows indicated clear media). b) Cloudy appearance of BPW indicates the growth of bacteria (arrow indicated cloudy medium).

4.3.2 Isolation of Shellfish sample

Samples collected from Shrimp and Crab were placed in Nutrient Broth (NB), and the growth of bacteria was observed on the next day when the clear NB turned cloudy (Figure 4.2) before inoculation into selective media. Turbid broth indicates bacterial growth. Bacteria were streaked onto different agar media for isolation and identification.

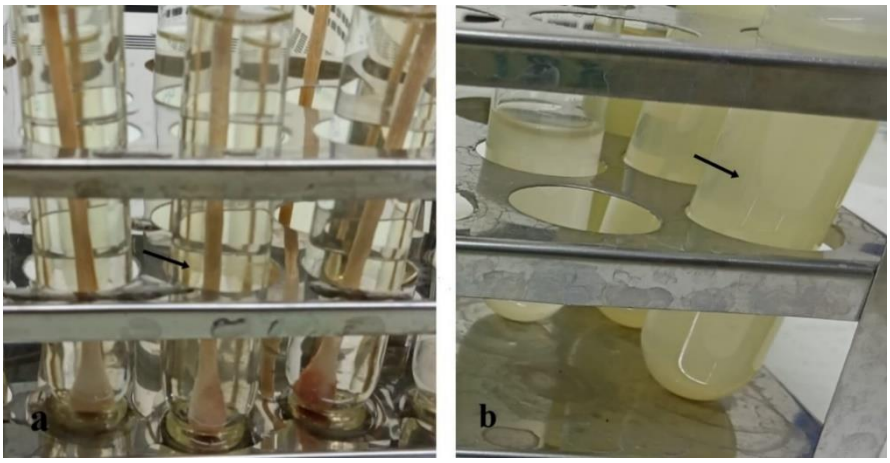

Figure 4.2 Sample collection and primary culture in Nutrient Broth. a) Samples were collected using a sterile cotton swab inserted directly into the NB (arrow indicated clear media). b) Cloudy appearance of NB indicates the growth of bacteria (arrow indicated cloudy medium).

4.3.3 *Aeromonas hydrophila*

All the samples collected from Bata and Tilapia fish were positive for *Aeromonas hydrophila*, and on the blood agar plate observed characteristics circular, grayish-white color colonies (Figure 4.3). Gram-negative rod-shaped, pleomorphic bacilli with a monotrichous flagellum were observed in Gram staining. *Aeromonas hydrophila* species was confirmed by a series of biochemical tests using a VITEK 2 system.

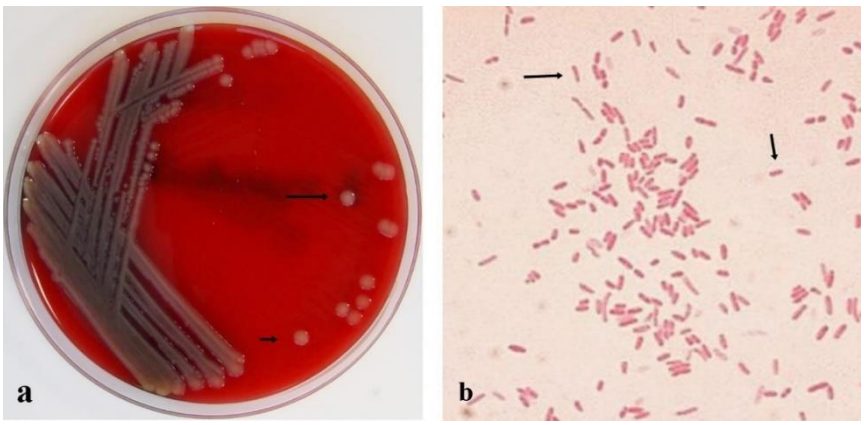

Figure 4.3 Isolation and identification of *Aeromonas hydrophila* by culture and Gram staining. a) On Blood agar, *Aeromonas* was observed as circular, grayish-white colonies indicated by arrows. b) Gram-negative bacilli were observed under a light microscope (100×) after Gram staining.

4.3.4 *Escherichia coli*

All the samples collected from Bata and Tilapia fish were positive for *Escherichia coli* as observed characteristics pink-colored colonies on MacConkey agar (Figure 4.4). Gram-negative rod-shaped bacilli were observed in Gram staining. *Escherichia coli* species were confirmed by a series of biochemical tests using a VITEK2 system.

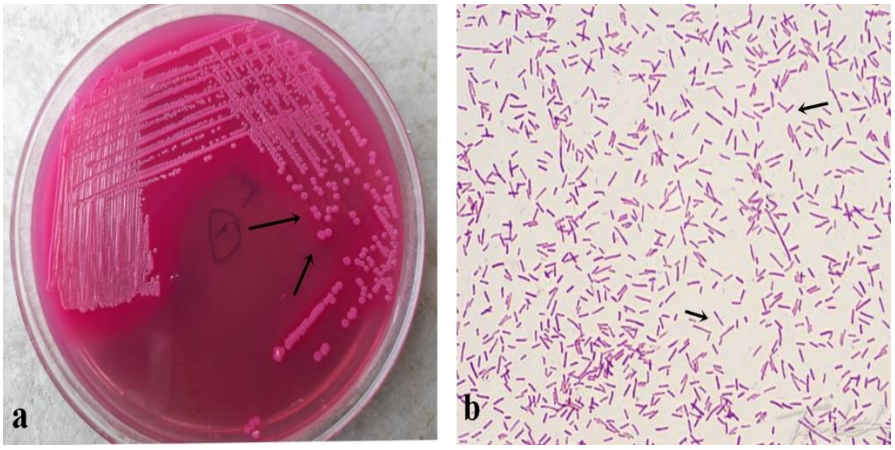

Figure 4.4 Isolation and identification of *Escherichia coli* by culture and Gram staining. a) On Mac Conkey agar, Escherichia coli was observed as pink-colored colonies indicated by arrows. b) Gram-negative bacilli indicated by arrows were observed under a light microscope (100×) after Gram staining.

4.3.5 *Staphylococcus saprophyticus*

Staphylococcus sp. was identified by colorless or yellow, glossy, large colonies onto mannitol salt agar (Figure 4.5). On Gram staining, uniform cocci with grapes-like arrangements were observed. *Staphylococcus saprophyticus* species was confirmed by several biochemical tests using a VITEK 2 system.

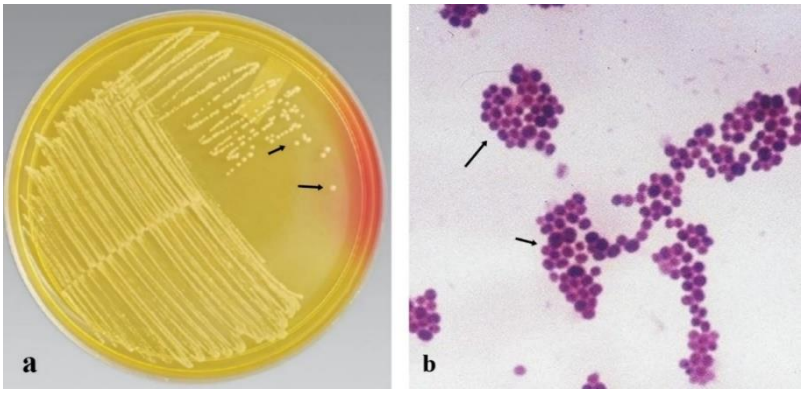

Figure 4.5 Isolation and identification of *Staphylococcus saprophyticus* by culture and Gram staining. a) On mannitol salt agar, *Staphylococcus* was observed as colorless or yellow, glossy, large colonies indicated by arrows. b) Gram-positive circular cocci with grapes-arrangement indicated by arrow were observed under a light microscope (100×) after Gram staining.

Results and interpretation of bacteria isolated from fish by VITEK 2

The VITEK 2 interprets the performance of a particular identifying pattern is identified; reported findings suggest a high likelihood fit for a single animal. If a potential pattern is not known the device will recommend additional tests to discriminate between 2 or 3 closely similar species or indicate the outcome as an unspecified organism (>3 species may demonstrate the pattern detected or the bio pattern is quite atypical and not shown in the database.

Table 4.1 Bacteria identification probability (%)

Bacteria	**Probability** (%)
Aeromonas hydrophila	99
Escherichia coli	99
Staphylococcus saprophyticus	95

4.3.6 *Vibrio* sp.

All the samples collected from shrimp and crab were positive for *Vibrio* sp., and on the TCBS plate observed characteristics were circular, green colonies (Figure 4.6, 4.7). Gram-negative curved bacillus, by means of a single flagellum, was observed in Gram staining. *Vibrio* species was confirmed by a series of biochemical tests.

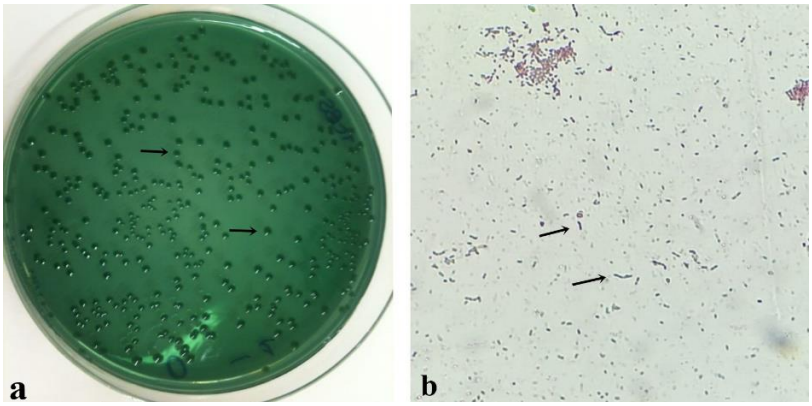

Figure 4.6 Isolation and identification of *Vibrio sp.* from shrimp by culture and Gram staining. a) On TCBS agar, *Vibrio* sp. was observed as green color colonies indicated by arrows. b) Gram-negative curved bacilli indicated by arrows were observed under a light microscope (100×) after Gram staining.

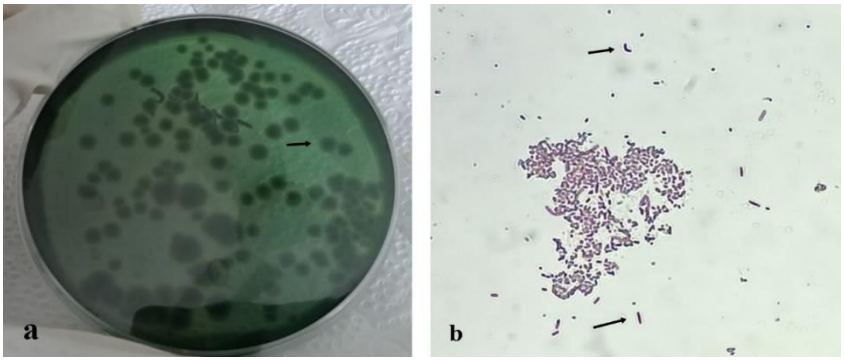

Figure 4.7 Isolation and identification of *Vibrio sp.* from crab by culture and Gram staining. a) On TCBS agar, *Vibrio* sp. was observed as green color colonies indicated by arrows. b) Gram-negative curved bacilli indicated by arrows were observed under a light microscope (100×) after Gram staining.

4.3.7 Biochemical test of *Vibrio* sp. isolated from shrimp and crab

Vibriosis is a very common disease in shellfish, that's why selective media TCBS were chosen to identify the bacteria. Besides, TSI, catalase test, and motility tests were performed to identify the genus of the bacterial inoculum (Figure 4.2).

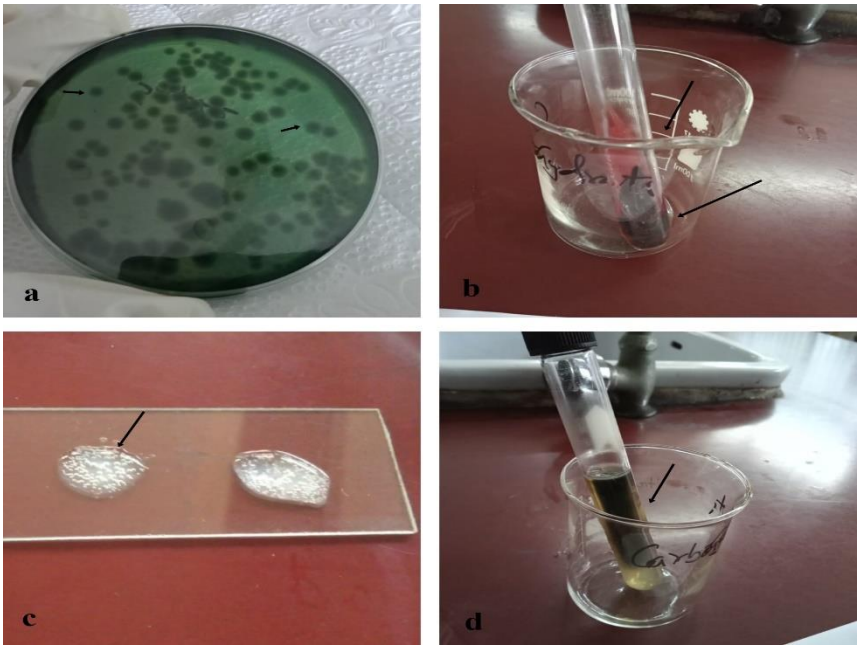

Figure 4.8 Biochemical test of *Vibrio* sp. a) Uniform green colony on TCBS agar; b) TSI test after incubation at 37^0C for 24 hours c) Catalase test d) Motility test after incubation at 37^0C for 24 h

Table 4.2 Description of the biochemical test results from Figure 4.8

Biochemical test	Properties	Result (Figure 4.8)	Presence of *Vibrio* sp.
TCBS agar	On TCBS agar, *Vibrio parahemolyticus* appears green colony, and *V. cholera* appears yellow colony	Uniform green color colonies are observed in both samples (shrimp, crab)	Present
TSI test	Yellow slant, yellow butt in *V. cholera* and Red slant, yellow butt in *Vibrio parahemolyticus*	Red slant, yellow butt, produced H_2S gas.	Present
Catalase test	If bubbles appear the bacteria are catalase positive. If no bubbles appear, the bacteria are catalase negative. Both	Bubbles appeared, and the bacteria are catalase positive.	Present

	Vibrio sp. are catalase positive.		
Motility test	*V. cholera* and *V. parahemolyticus* are motile bacteria. The positive result indicates diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.	Results indicated diffuse, hazy growth that spread throughout the medium rendering it slightly opaque	Present

From biochemical tests, a genus of *Vibrio* is confirmed and the species is also suspected to be *V. parahemolyticus* as it matches the criteria from the test results and literature of Silva et al. (2013).

4.4 Extracts that showed antimicrobial activities

In this experiment, ethanol was used to extract the medicinal plant and marine water species of microalgae. The result showed that *Aloe vera, C. nutans, and Chlorella* sp. had antimicrobial properties against the selected bacteria. *Spirulina* sp. had antimicrobial properties toward *Vibrio sp., S. saprophyticus, and A. hudrophila* but was *absent in E. coli* (Table 4.3).

Table 4.3 Ethanolic extracts of *Aloe vera, C. nutans, Chlorella* sp. *Spirulina* sp. and its antimicrobial properties on selected bacteria

Selected bacterial	**Antimicrobial properties**			
	Aloe vera	*C. nutans*	*Chlorella*	*Spirulina*
***Vibrio* sp. ©**	+	+	+	+
Vibrio* sp. *(s)	+	+	+	+
S. saprophyticus	+	+	+	+
A. hydrophila	+	+	+	+
E. coli	+	+	+	-

*+ Present, – Absent; Crab (c); shrimp (s)

4.4.1 Antimicrobial activity test

The antimicrobial activity of ethanol extracts of *Aloe vera, C. nutans, Chlorella* sp., *Spirulina* sp. were observed against *Vibrio* sp., *S. saprophyticus, A. hydrophila, and E. coli*. Except for *Spirulina* sp., all the extracts showed a higher zone of inhibition against the entire test bacterial. Meanwhile, the antibiotic disc which was colistin (10 µg/disc) had shown a clear zone toward *Vibrio* sp., *A. hydrophila*, *E. coli*, and ceftriaxone (30 µg/disc) had shown a clear zone toward *S. saprophyticus*. Antibiotic disc acted as positive control and blank disc as the negative control had showed no inhibition zone against the entire test bacterial. The details of the antimicrobial activity are stated in Table 4.4

Table 4.4 Inhibition zone of test bacterial at a concentration of 100 mg/ml of *Aloe vera, C. nutans, Chlorella* sp., *Spirulina* sp. with antibiotic agents

Diameter zone of inhibition (mm)					
Bacteria	***Aloe vera***	***C. nutans***	***Chlorella***	***Spirulina***	**Control**
***Vibrio* sp. ©**	15.363±1.11^{b}	11.38±0.778^{a}	11.59±1.888^{a}	19.193± 0.577^{c}	14.637±1.898^{b}
Vibrio* sp. *(s)	12.997±0.49^{b}	12.317±0.739^{a,b}	13.225±1.039^{b}	11.385±0.601^{a}	15.1±0.255^{c}
S. saprophyticus	9.043±0.474^{a}	14.21±1.075^{d}	11.41±0.658^{b}	13.05±0.035^{c}	10.44±0.121^{b}
A. hydrophila	11.53±1.212^{c}	8.783±0.869^{b}	7.55 ± 0.778^{a,b}	8.61±0.014^{a,b}	13.375±0.007^{d}
E. coli	14.037±0.903^{b,c}	15.103±0.214^{c}	11.633 ± 0.891^{a}	----------	14.027±1.155^{b}

Values are expressed as mean ± SD. Values are not sharing similar superscripts within the same column are significantly different (P<0.05) as determined by one-way ANOVA.

4.4.2 Antimicrobial index of microalgae extracts

The antimicrobial index of microalgae and medicinal plant extracts was calculated by using the formula stated above which was used to compare the antimicrobial effect of the microalgae and medicinal plant extracts with the effect of known standard antibiotic agents. The results of antimicrobial activity of the extracts were compared to the antibiotic agents (positive control) are shown in Table 4.5

Table 4.5 Relative percentage of inhibition of the microalgae and medicinal plant extracts compared to antibiotics

Test bacteria	**Plant Extract**		**Microalgae extract**	
	Aloe vera	*C. nutans*	*Chlorella* sp.	*Spirulina* sp.
Vibrio sp.©	112.2193	52.45	55.17	186.472
Vibrio sp. *(S)*	69.23	60.26	72.34	48.76
S. saprophyticus	63.04	228.51	129.71	184.96
A. hydrophila	67.84	28.79	14.69	26.69
E. coli	100.17	119.49	61.79	------

4.4.3 Minimum Inhibitory Concentration(MIC)

In the experiment, *A. vera, C. nutans, Chlorella* sp., and *Spirulina* sp. extracts had shown the strong antimicrobial properties of the test bacteria stated in Table 4.2. All the extracts had been chosen to carry on for minimum inhibitory concentration (MIC). The MIC value was determined as the minimum concentration of microalgae capable to prevent bacterial growth. Two separate methods were used to determine the MIC value; color and turbidity by the naked eye and ELISA reader (OD_{630} nm). *Chlorella* showed the lowest MIC value (20 mg/ml) against all the test bacteria whereas the other extracts had a different ranges of MIC value (20-40) mg/ml (Table 4.6).

Table 4.6: MIC value of *Aloe vera, C. natuns, Chlorella* sp., *Spirulina* sp. extracts

Treatments	**MIC Value (mg/ml)**				
	Vibrio sp. (crab)	*Vibrio* sp. (shrimp)	*S. saprophyticus*	*A. hydrophila*	*E. coli*
Aloe vera	30	40	20	20	20
C. nutans	30	40	30	20	20
Chlorella sp.	20	20	20	20	20
Spirulina sp.	20	30	20	20	----

CHAPTER 5

DISCUSSION

In this study microalgae (*Chlorella sp., Spirulina sp*.), and medicinal plants (*A. vera, C. nutans*) were chosen to find whether they have antimicrobial properties against pathogenic bacteria causing diseases in fish and shellfish. Mass culture of microalgae was done using Conway medium (Table 3.1.2) and from 20 L bulk culture, got 7.2 g,7.4 g of dry powder of *Chlorella sp., Spirulina sp.* Raw leaves of *Aloe vera* and *C. nutans* were taken and after drying, got 40.72 g, and 46.5 g of dry powder respectively. The four samples were extracted using ethanol to demonstrate the bacterial varieties collected from fish, shrimp, and crab and whether they have an antibacterial effect on them. It was observed that all the samples collected from fish's skin, gill, intestine; crab's abdominal flap, mouth part, claw, carapace region, and shrimp's joined appendages, uropod, endoskeleton, cephalothorax region were positive for *A. hydrophila, E. coli, S. saprophyticus, Vibrio* sp. (Figure 4.1 to 4.7). All these three identified bacteria are important in aquaculture and cause a wide variety of infectious diseases such as motile Aeromonas septicemia, Strptococcosis, Vibriosis, etc. (Haenen, 2017). Although most bacterial infections are treated successfully with antibiotics, antimicrobial resistance is a major growing threat, for example, methicillin-resistant *Staphylococcus* (MRS) are very hard to treat.

The ethanolic extract of the *Chlorella, Spirulina, A. vera, and C. nutans* was tested against *A. hydrophila, E. coli, S. saprophyticus, and Vibrio* sp. Because the ethanolic extract exhibited significant antibacterial activity against all the tested bacteria in previous studies (Uda et al., 2018; Ozdemi, et al., 2001). The result showed that antimicrobial properties were present in *Aloe vera, C. natuns, and Chlorella* sp. against the selected bacteria. *Spirulina* sp. showed positive antimicrobial properties toward *Vibrio sp., S. saprophyticus, and A. hudrophila* except for *E. coli.* (Table 4.3).

The antibacterial activity was evaluated by the disc diffusion method. The test was carried out using a 100 mg/ml concentration of each extracts. The zones of inhibition were measured by digital slide calipers. The zones of inhibition of *Vibrio(c) sp.* were measured and shown in Table 4.4. It was observed that there was an inhibition zone present in the pathogenic bacteria of *Vibrio(c) sp.* tested with four different types of algae and plant extractions. The inhibition zone of the *C. nutans, Chlorella* sp. are quite similar ranging from 11.38±0.778 mm to 11.59±1.888 mm whereas control showed 14.637±1.898 mm.

Spirulina and *Aloe vera* significantly inhibited Vibrio sp. as well as from control isolated from crab with 19.193± 0.577 and 15.363±1.11 mm respectively. Das and Pradhan (2010) found that ethanolic extracts of the *S. platensis* showed maximum zone size, ranging from 15.6 to 16.3 mm and Chlorella showed 12.0±1.0 mm against *Vibrio* sp. The results of the study by Coopoosamy and Magwa (2007) also revealed that the lowest concentrations of ethyl acetate and ethanol crude extracts of *Aloe vera* resulted in complete inhibition of visible growth of pathogenic bacteria compared with the control antibiotics.

The zones of inhibition of *Vibrio sp.* were measured isolated from shrimp. All the treatments showed antimicrobial activity ranging from 11.385±0.601 to 13.225±1.039 mm compared to the control (15.1±0.255 mm). The highest inhibition zone was observed in *Chlorella* sp. Though the data is not significant but all the treatments showed antimicrobial activity (Table 4.4). Das and Pradhan (2010) established that ethanolic extract of *C. vulgaris* showed a maximum zone of inhibition (12.0±1.0) against Vibrio sp. which corresponds to our result.

Ethanolic extract of *C. nutans, Chlorella* sp.*, and Spirulina sp. significantly inhibited S. saprophyticus* as well as from control isolated from fish with a mean diameter of an inhibition zone 14.21±1.075; 11.41±0.658; 13.05±0.035 mm respectively. *Aloe vera* showed an inhibition zone of 9.043±0.474mm whereas control showed 10.44±0.121mm. From the comparison, it could be said that all the extracts significantly inhibited *S. saprophytucus* (Table 4.4). Ho et al., (2013) discovered that *C. nutans* extracts exerted a significant antibacterial effect against *S. aureus.* Velichkova et al., (2018) found that *C. vulgaris* ethanol extract showed a zone of inhibition against *S. aureus* (10.0±1.0). Chakraborty et al., (2015) studied ethanol extract of *S. platensis* against bacteria and reported zones of inhibition of *S. aureus* (13 mm) which corresponds to our results although the species are not similar. A study conducted by Begum et al., (2016) showed that *Aloe vera* extracts inhibited the growth of *S. saprophyticus* 20 mm and the findings are less similar to our result.

The zones of inhibition of *A. hydrophila* were measured isolated from fish. All the treatments showed antimicrobial activity ranging from 7.55 ± 0.778 to 11.53±1.212 mm compared to the control (13.375±0.007mm). The highest inhibition zone was observed in *Aloe vera* against *A. hydrophila*. Though the data is not significant but all the treatments showed antimicrobial activity (Table 4.4). Das and Pradhan (2010) found that ethanolic extract of *Chlorella vulgaris* and *Spirulina platensis* against *Aeromonas hydrophila* was 11.03±0.03 and 10±0.05 mm respectively. Razak et al., (2019) found that *C. nutans*

methanol water extract shows a zone of inhibition against *Aeromonas hydrophila* was 6.00 mm which corresponds to our result though the extracts are different. Iqbal and Ahmed (2021) found that ethanolic extract of *Aloe vera* shows a zone of inhibition against *Aeromonas hydrophila* was 9.6±1.06 mm.

Among four treatments, *Aloe vera, C nutans., and Chlorella* sp. showed antimicrobial activity against *E. coli. Aloe vera* and *C. nutans significantly* inhibited *E. coli* as well as from control with a mean diameter of inhibition zone 14.037±0.903 and 15.103±0.214 mm individually. Sekar and Rashid (2016) found that The methanolic extract of *Clinacanthus nutans* leaves showed the maximum antibacterial activity against *E. coli* at 100 mg/ml (8.00±2.00 mm). The extracts of *Spirulina* could not inhibit *E coli.* Ahmed (2016) found that *S. platensis* crude extract had no effect on *Pseudomonas* and *E. coli,* which also corresponds to our results. A study conducted by Begum et al., (2016) showed that *Aloe vera* extracts inhibited the growth of *E. coli* 12.5 mm. *Chlorella* sp. extracts showed an 11.633 ± 0.891 mm inhibition zone which was not significant as opposed to control (14.027±1.155 mm) (Table 4.4). Velichkova et al., (2018) found that *C. vulgaris* ethanol extract shows a zone of inhibition against *E. coli* (9.1±0.02 mm).

The antimicrobial index of microalgae and medicinal plant extracts was calculated by using the formula stated above (3.4). It was done to compare the antimicrobial effect of the microalgae with the standard antibiotic agents. The results showed that *Spirulina* extract exhibited maximum relative percentage inhibition against *Vibrio sp.* isolated from crab, with minimum against *Vibrio sp.* isolated from shrimp while *C. nutans* extracts exhibited maximum relative percentage inhibition against *S. saprophyticus and E. coli* followed by *Vibrio* sp. isolated from the crab. The antimicrobial index of extracts *Chlorella* sp. showed *Vibrio sp.* (isolated from shrimp) 72.34% more sensitive compared to *A. hydrophila* (14.69%). Meanwhile, *A. hydrophila* (67.84%) was relatively more sensitive compared to *S. saprophyticus* (63.04%) on *Aloe vera* extract. (Table 4.5)

Furthermore, MIC was conducted to study the antimicrobial activity of the four extracts due to the largest inhibition zone against the test bacteria. MIC value of *Chlorella* sp. had a result at a concentration of 20mg/ml to inhibit all the test bacterial (1.5×10^{-8} CFU/ml or 0.5 McFarland standards) that this extract can control well the growth of the bacteria. *Spirulina* was able to inhibit the growth of *A. hydrophila, S. saprophyticus, Vibrio* sp. (c) *at* a concentration of 20mg/ml and *Vibrio* sp. (s) at a concentration of 30mg/ml. Besides, *Aloe vera, and C. nutans* showed better antimicrobial capacity against *A. hydrophila, and*

E. coli at a concentration of 20 mg/ml, while 30 mg/ml and 40mg/ml concentration against *Vibrio* sp. (crab and shrimp). The growth of *S. saprophyticus* was inhibited at a concentration of 20 mg/ml and 30 mg/ml by *Aloe vera, and C. nutans* individually (Table 4.6) Ali et al., (2020) found that MIC values of *Spirulina* sp. against E. coli are 250 mg/ml. MIC values of *Chlorella vulgaris* against pathogenic bacteria determined by broth macro dilution were between 125 µg/ml to 1000 µg/ml (Ibrahim et al., 2015). Ho et al., (2013) discovered that *C. nutans* extracts exerted a significant antibacterial effect against *E. coli* and *S. aureus* with minimum inhibitory concentrations (MIC) of 12.5 mg/ml. The lowest concentration of the extractions where the effect is smaller is considered the minimal inhibitory concentration. zone of inhibition of ethanol *A. vera* extract against pathogenic bacteria increases from 9.22 mm at 5 mg/ml to 9.56 mm at 30 mg/ml, zone of inhibition of ethanol Sabah Snake Grass extract against pathogenic bacteria increases from 10.00 mm at 5 mg/ml to 11.21 mm at 30 mg/ml which corresponds to our result (Uda et al, 2018). The difference in methodology, chemical nature, and quantity of bioactive metabolite compound present in the extracts and their mode of action to test organisms might be the reason behind the difference in MIC values.

At last, it can be concluded that bioactive compounds possessed by the microalgae and medicinal plants are potential for antimicrobial activity and should be looked into for natural antibiotics. However, it is required to identify these active compounds in further study.

CHAPTER 6

6.1 Conclusion

In the present study, it was concluded that *Aloe vera, C. nutans, Chlorella,* and *Spirulina* sp. extracts had shown antimicrobial properties among the selected bacteria. Microalgae and medicinal plants can be represented as a new source of antimicrobial properties that can use in the pharmaceutical industries. The stability of bioactive compounds isolated from the microalgae and medicinal plants are suitable to use for substituted antimicrobial agents. The bioactive compounds obtained from the microalgae and medicinal plant are natural resources which it is healthy and good compared to the antibiotic agents that may cause side effects, and resistance problem. The use of microalgae and plant extracts with known antimicrobial properties can be of great significance in disease treatments.

6.2 Recommendations and future prospective

The bioactive compounds of the microalgae and medicinal plant will need to study and identify the chemical structures and benefits effect for inhibition of pathogenic bacteria. The bioactive compounds of the microalgae and medicinal plant may be suitably used for the other pathogenic bacteria.

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APPENDIX I

A. Inhibition zones exhibited by the *Aloe vera, C. natuns, Chlorella and Spirulina* sp. extract on the test bacterial. [[A= antibiotic; B= Blank; Al= Aloe vera; Sp= Spirulina; Chl= Chlorella; Sn= Snake grass (C. natuns)]

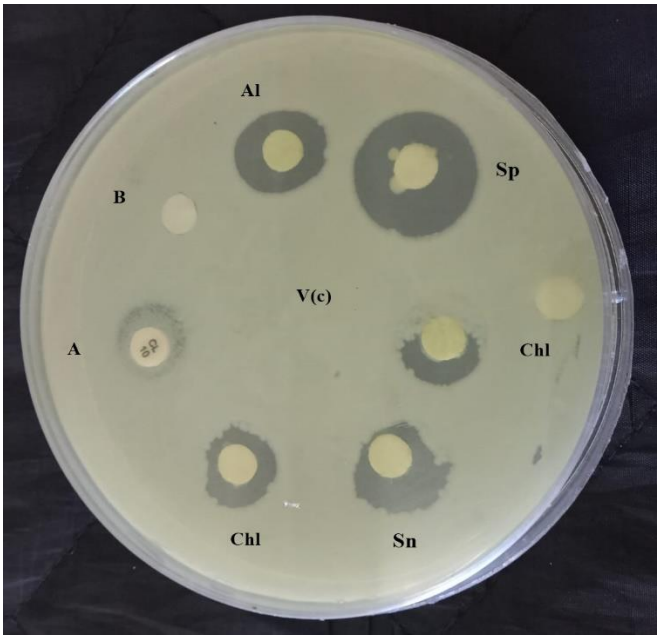

Figure 1 Inhibition zone of the extracts against *Vibrio* sp. isolated from crab

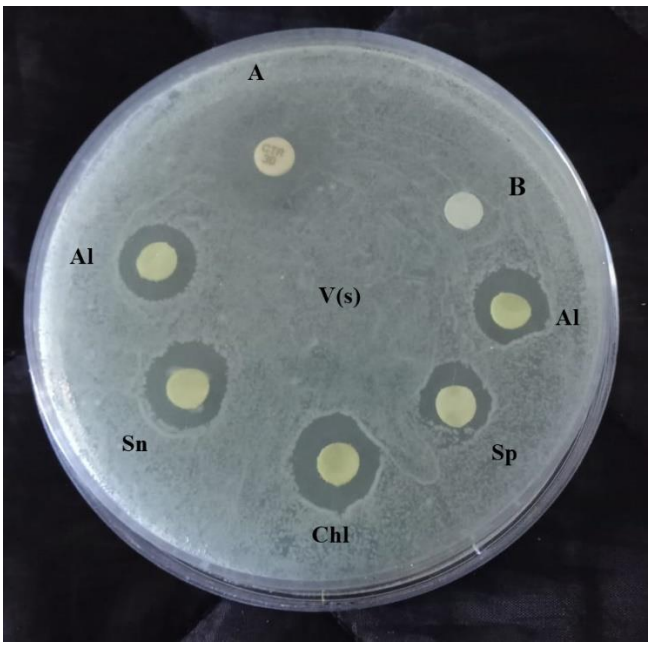

Figure 2 Inhibition zone of the extracts against *Vibrio* sp. isolated from shrimp

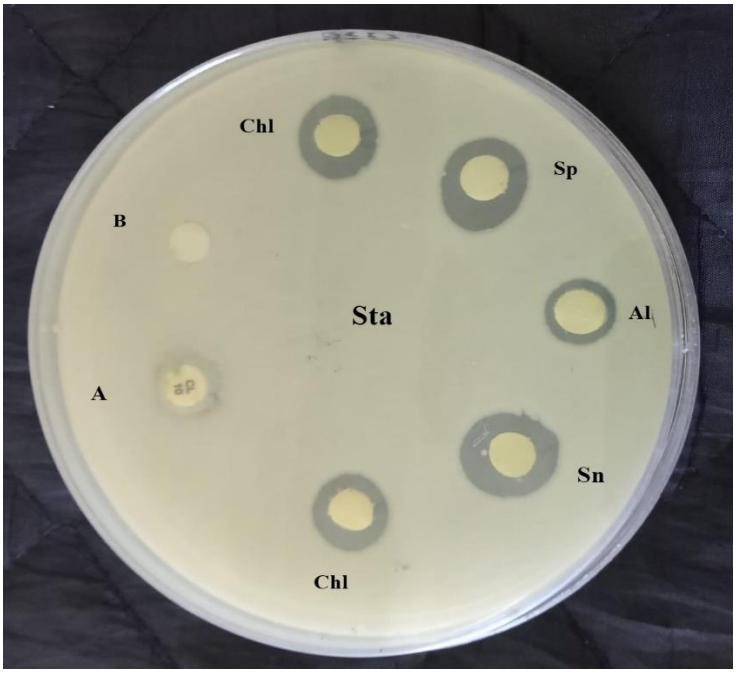

Figure 3 Inhibition zone of the extracts against *Staphylococcus saprophyticus*

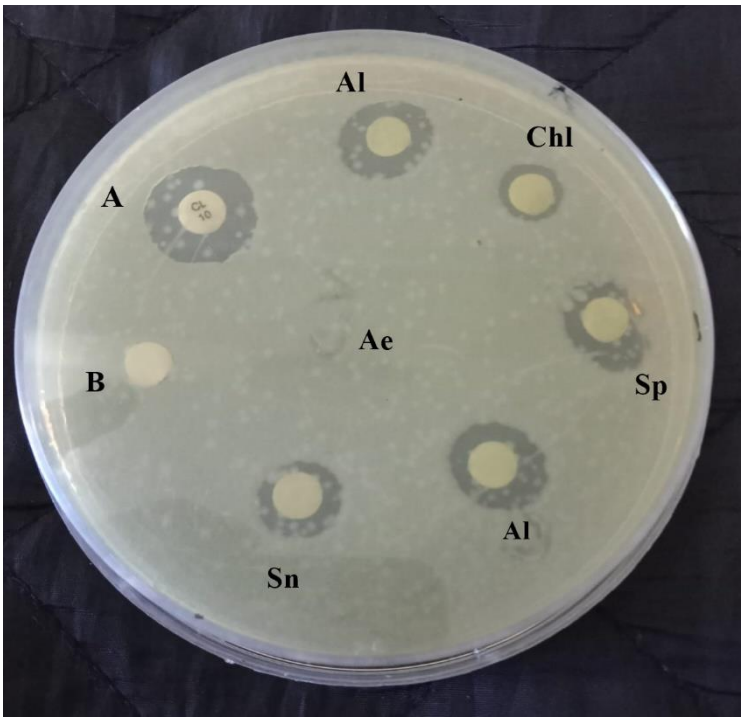

Figure 4 Inhibition zone of the extracts against *Aeromonas hydrophila*

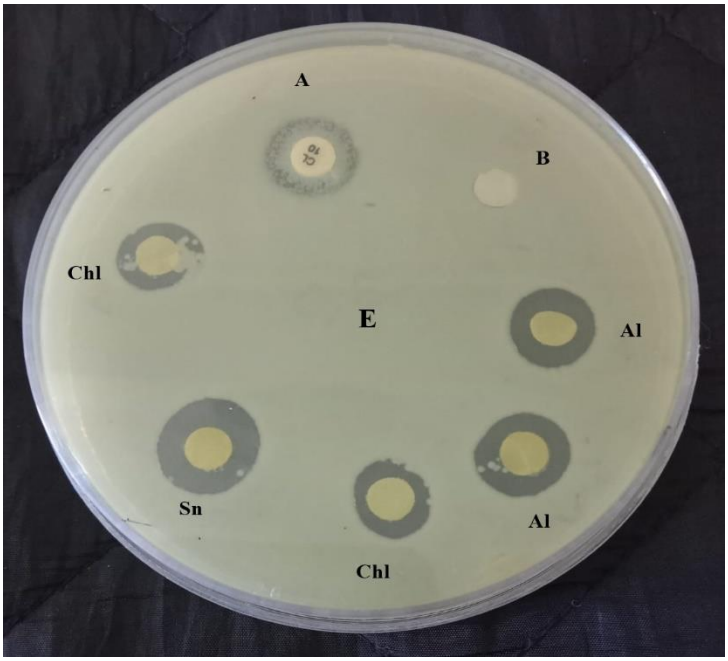

Figure 5 Inhibition zone of the extracts against *Escherichia coli*

B. MIC of the different extracts against test bacteria

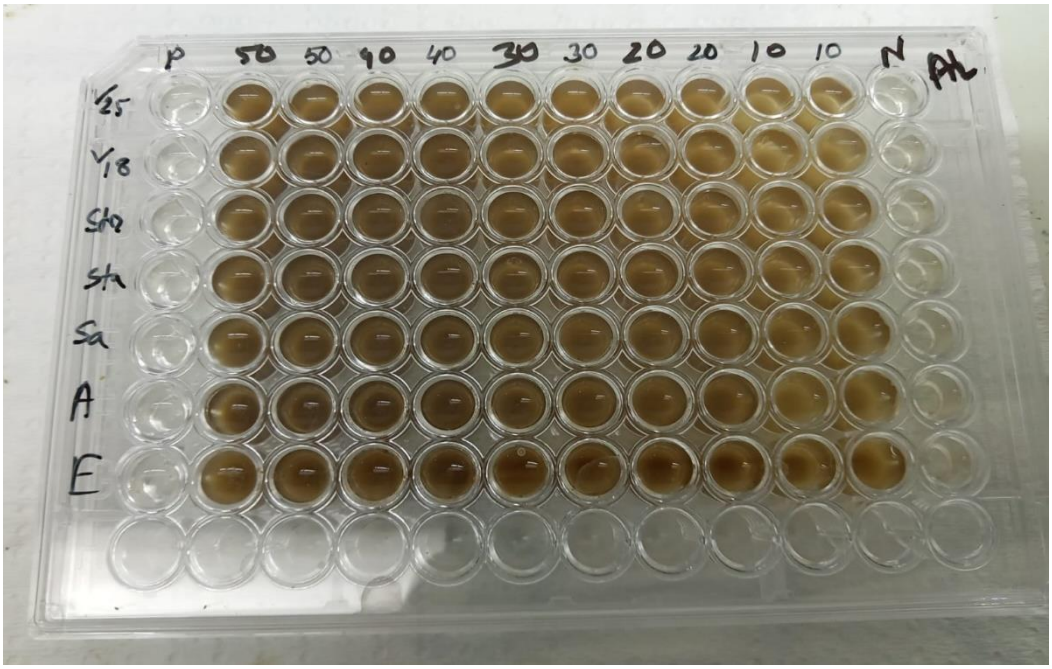

Figure 6 MIC of *Aloe vera* on test bacteria

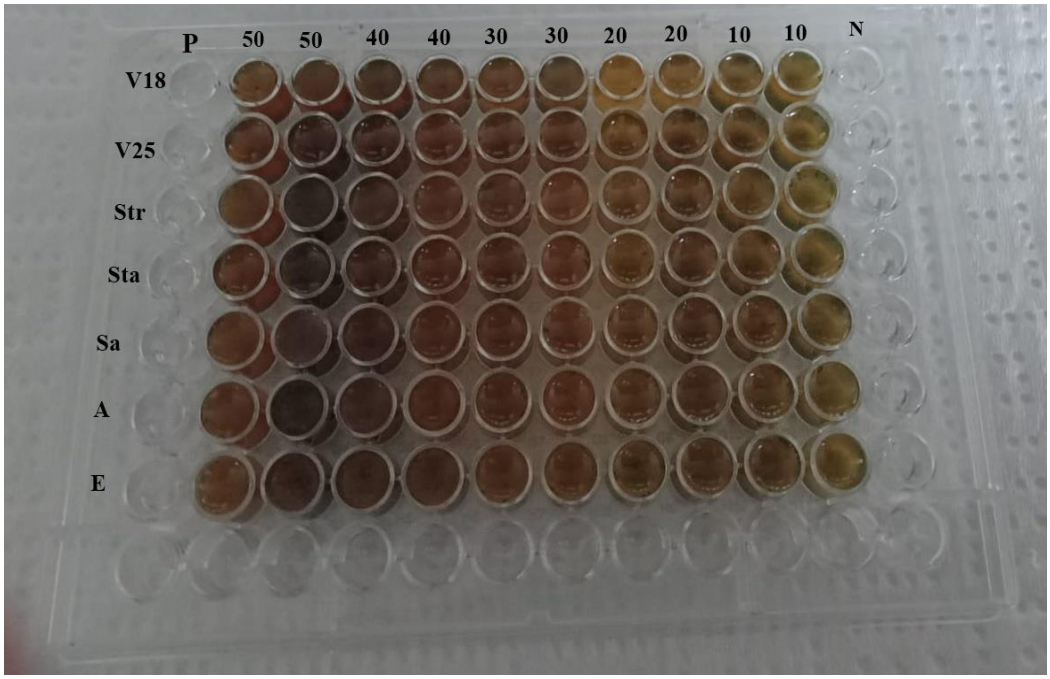

Figure 7 MIC of Snake grass *(C. nutans)* on test bacteria

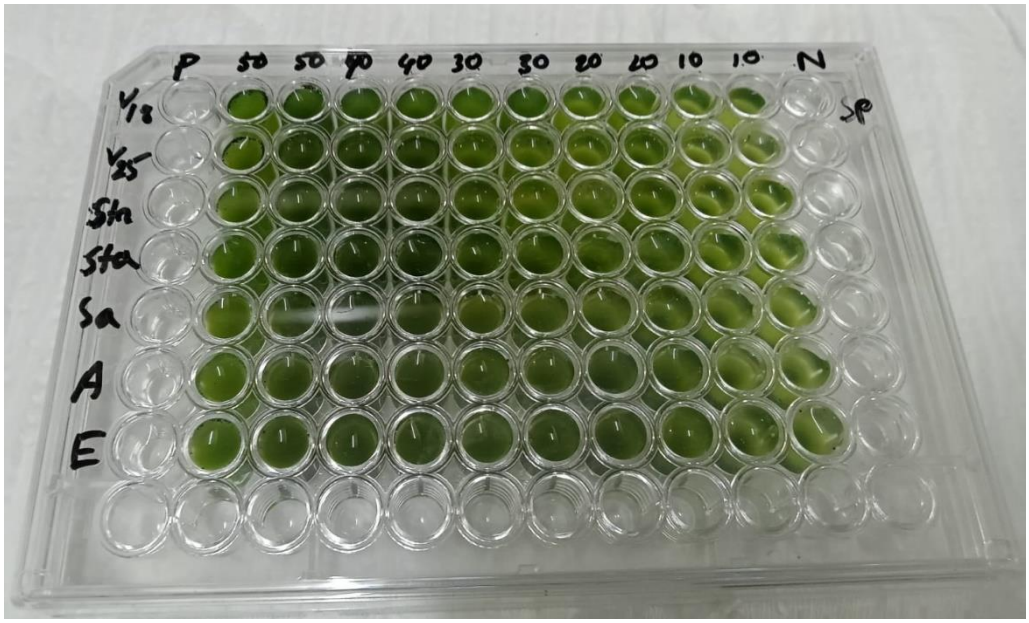

Figure 8 MIC of *Spirulina* sp. on test bacteria

Figure 9 MIC of *Chlorella* sp. on test bacteria

APPENDIX II

Figure 1 Culture of microalgae. Small volume culture of a) *Chlorella* sp. on left and *Spirulina* sp. on the right side; b, c) Gradual increase of culture; d) Mass culture of microalgae in a 20-liter jar

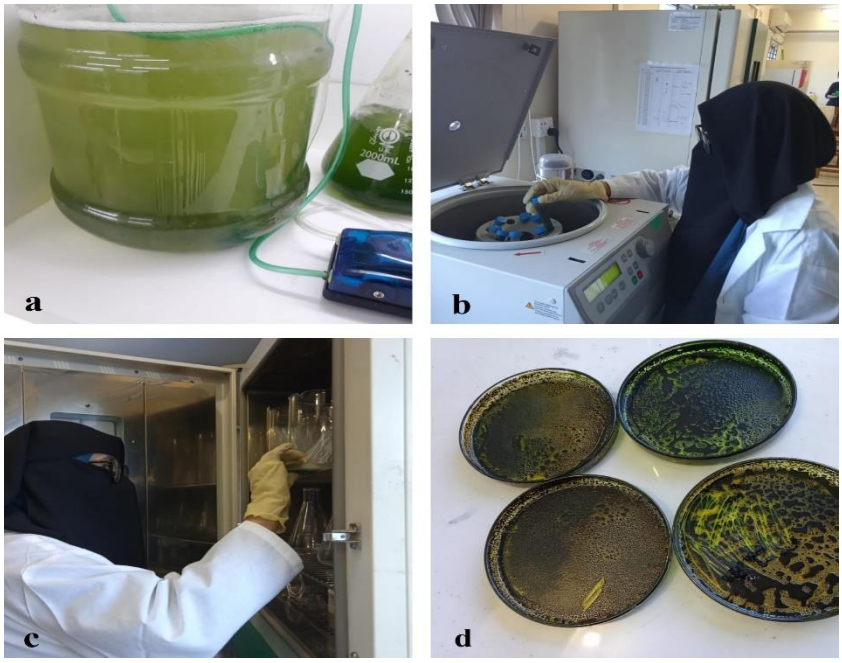

Figure 2 a) 20 L jar are brought to harvesting lab b) Harvesting of microalgae using centrifuge machine c) Drying of algal paste at 40^0c for 24 hours d) Dry algal powder in Petri dish

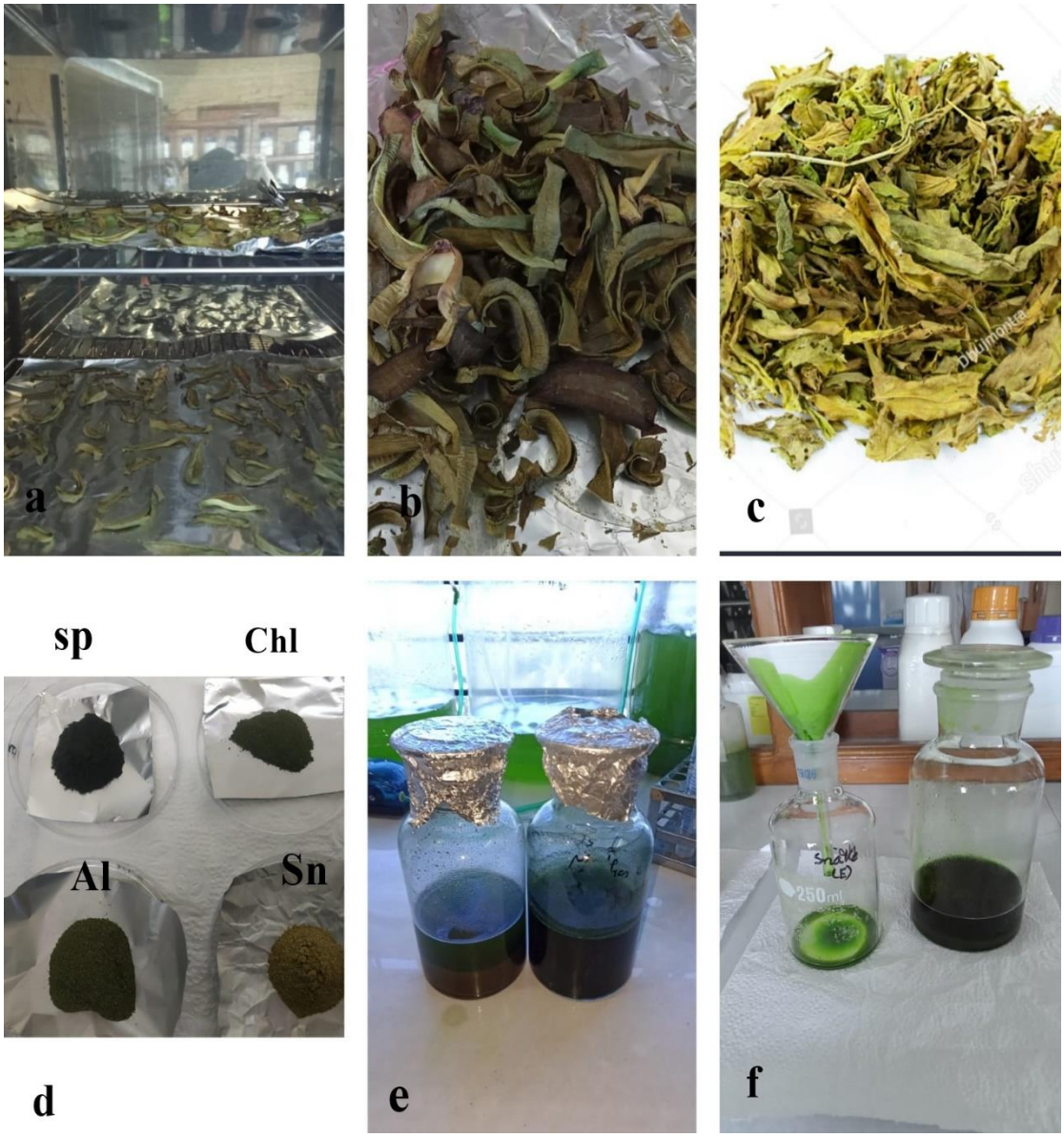

Figure 3 a) aloe vera leaves were cut into pieces and dried for 48 hours at 80^0c b) Dried chunk of aloe vera leaf c) Raw snake grass leaves were dried as the same process as aloe vera c) Used blender to make powder form of the leaves, here sp means Spirulina; Chl meant Chlorella; Al meant Aloe vera; Sn meant Snake grass e) All dried powder was soaked in ethanol for 48 hours f) After extraction, Whatman filter paper used to remove unextractable matter and kept in the refrigerator for further use

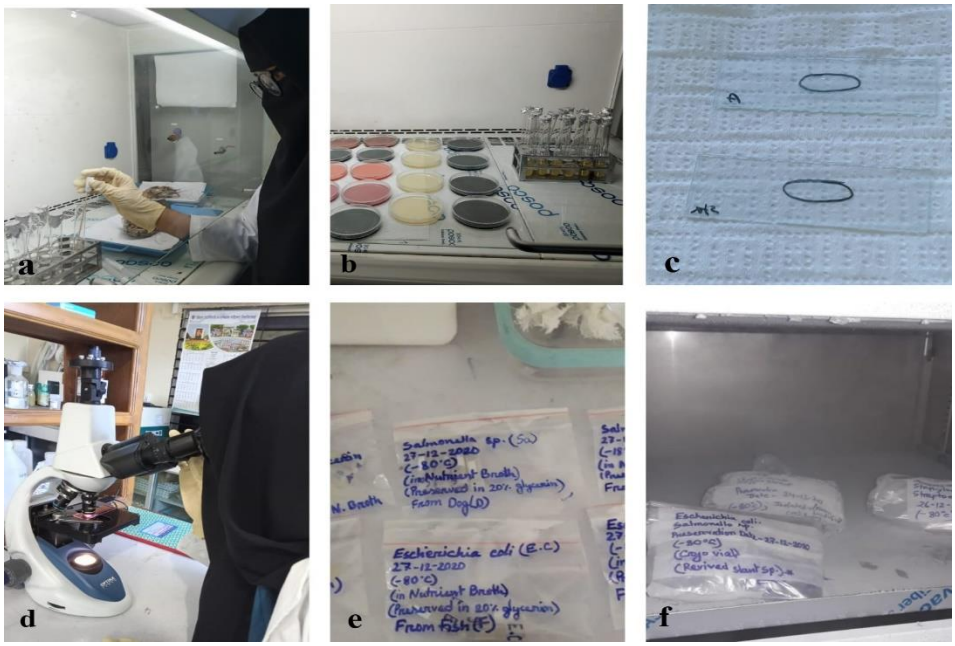

Figure 4: a) Taking swab from the fish sample and inoculated into broth b) Stread onto agar plate from turbid broth media c) Single colony from agar plate are taken slightly using inoculating loop to perform gram staining d) Identification of bacteria by microscopic identification e) Preservation of identified bacteria with 20% glycerol f) Preserved at (-(-80^0c)

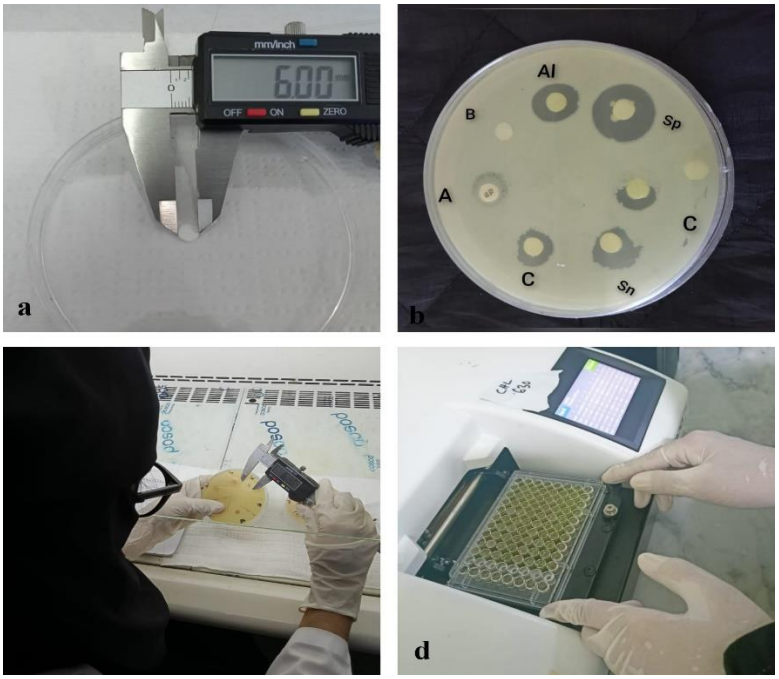

Figure 5 a) Using 6mm disk for AMR test b) Clear inhibition zone c) Measuring inhibition zone by slide calipers d) Taking OD value to determine MIC