Study of Antibacterial Activities of *Azadirachta indica* (Neem), *Ocimum sanctum* (Tulsi), and *Punica granatum* (Pomegranate peel) against *Vibrio cholerae*

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

Submitted By

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February 2022

Declaration

It is hereby declared that

1. The thesis submitted is my original work while completing my degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis titled "Study of antibacterial activities of *Azadirachta indica* (Neem), *Ocimum sanctum* (Tulsi), and *Punica granatum* (Pomegranate peel) against *Vibrio cholerae"* submitted by Hosne Ara Khandaker (14226005) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on February 2022.

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Ethics Statement

No animals were used in this study

Abstract

As multidrug-resistant (MDR) strains of *Vibrio cholerae* are emerging at an alarming rate, it has become a matter of importance to find and evaluate the already existing alternates to synthetic antibiotics. For several thousands of years, folks of the Indian Subcontinent, especially in Bangladesh have relied on plant-based medications to treat enteric diseases. As *Vibrio cholerae*, the causative agent of one of the biggest enteric diseases in Bangladesh, cholera; has been the subject of this present study where traditional plants like Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), and Pomegranate (*Punica granatum*) were evaluated against it in comparison to some conventional antibiotics. Among the three of the plant extracts, only the ethanolic extract of Tulsi showed significant antimicrobial activities, as it showed moderate efficacy with the Activity Index values of "1.16" and "0.7" against the pathogen in comparison to the antibiotics erythromycin and chloramphenicol respectively, which are often used to treat cholera. On the other hand, none of the aqueous extracts showed any sign of activity against the pathogen in concern.

Keywords: MDR; *Vibrio cholerae*; Plant extract.

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Sincerely,

Hosne Ara Khandaker

Febraury, 2022.

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List of Acronyms

Abbreviation	In full form
µl	Microlitres
AI	Activity Index
APW	Alkaline Peptone Water
MDR	Multi Drug Resistant
MIU	Motility Indole Urease
ml	Millilitres
MNA	Modified Nutrient Agar
MNB	Modified Nutrient Broth
MRVP	Methyl red- Voges Proskauer
TCBS	Thiosulfate-citrate-bile salts-sucrose
TSI	Triple sugar iron

Chapter 1

Introduction

1.1 Background Study

Cholera, an ancient and historically- feared disease with devastating acute illness, is caused by the notorious toxigenic *Vibrio cholerae* which has been posing serious global health problems for decades (Weil & Ryan, 2018). Approximately, in endemic countries, 2.8 million cases occur annually and in non-endemic countries, around 87000 cases occur with a fatal consequence for 2500, however, the frequency is estimated higher for children under five years old (Mark et al., 2018). Due to the severity of the disease, cholera remains a major public health concern in many parts of Asia, Africa, and Latin America, though mostly uncommon in developed countries (Weill et al., 2017). As it causes life-threatening secretory diarrhea resulting in rapid fluid loss and ultimately, death, if not treated promptly; marks it as an important infection worldwide that has been categorized as the *"emerging and re-emerging infection"* looming threat upon many parts of the world (Mandal et al., 2011). This waterborne disease is deeply connected with poverty-stricken socio-economic conditions, insignificant and rudimentary sanitary systems accompanied by the lack of basic public hygiene awareness in mostly developing and under-developing countries (Faruque et al., 1997).

1.2 Epidemiology

Vibrio cholerae was first identified by Filippo Pacini in 1854, an Italian scientist, nevertheless, the discovery was not well-known until Koch (1894), who eventually stated *Vibrio cholerae* being the causative agent of cholera infection (Mandal et al., 2011). However, records of a disease with symptoms similar to cholera have been found in some statements in Sanskrit dated

back thousands of years ago in the Indian Subcontinent (Mandal et al., 2011). Besides, many accounts of its description were retrieved also in India dating back to the time of Alexander the Great (Islam et al., 2020).

Cholera has been endemic in the deltas of Ganges and Brahmaputra in India, including West Bengal which has been known as the *"Homeland"* for cholera causing *Vibrio cholerae* (Sack et al., 2003). Since the early 1800s, seven cholera pandemics have been documented (Charles & Ryan, 2011). And the current seventh pandemic, which has set off in 1961 in Indonesia caused by the O1 El Tor variant (Weill et al., 2017), has been stuck around for more than 50 years with interposed episodes of emergence and re-emergence of the disease in areas scarce of modern sanitation. The largest outbreak of the current pandemic in South Africa in 1991, had prompted massive investment in sanitation systems (Weil & Ryan, 2018), which eventually ceased the outbreak. Though another larger outbreak took place in Zimbabwe in 2008, which lasted for a year instigating over 100 000 cases with at least 4000 deaths (Charles & Ryan, 2011). Unfortunately, in 2010, the outbreak in Haiti, have overshadowed the previous one. Only in 7 months after the explosive appearance of the first cases, it took away approximately 5000 lives with at least 300000 cases (Hill et al., 2011).

One of the largest epidemics of the 21st century with more than a million cases commenced during the ongoing civil war of Yemen due to the lacking of a functional health service. In Africa, the case fatality rate continues to remain highest with recurrent outbreaks (Weil & Ryan, 2018).

1.2.1 Cholera in Bangladesh

Bangladesh, being one of the most vulnerable countries of South East Asia for the cholera outbreak, have an estimated 352,000 cholera cases annually, including 3500-7000 deaths

occurring every year (Uddin et al., 2018). Cholera, being a constant endemic in the country, has maintained a regular seasonal pattern which has been explained by Swaroop et al. by identifying four common features of the cholera prevalent areas, such as the location around rivers with low lying lands, high population density, and an above-average humidity. Excluding the third possible reason, all of the attributes are associated with the environment indicating the role of the environment in sustaining the endemicity of the disease. In Bangladesh, the cholera outbreak reaches its peak during the pre-monsoon season with a second peak at some point in the post-monsoon stage (Islam et al., 2020).

1.3 Etiology

The causative agent for cholera; *Vibrio cholerae* is a highly motile curved or comma-shaped rod that is characterized by a single polar flagellum. It causes severe watery diarrhea by producing an enterotoxin named Cholera Toxin (CTX) (Faruque et al., 1998). This gram-negative, bacillus has over 200 serogroups, among which only O1 and O139 serogroups have been found culpable of causing endemic, epidemic, or pandemic in nature (Weil & Ryan, 2018). The O1 serogroup is further classified by serotype namely, Ogawa or Inaba, which is based on variations in the O-antigen on the surface of the bacterium, and biotypes such as El Tor or classical, based on phenotypic and genotypic differences (Charles & Ryan, 2011). According to typing method of Sakazaki and Shimada, there are in total 139 different O groups present, where *Vibrio cholerae* O1 and O139 are the most pathogenic and dominating strains (Maheshwari et al., 2011) and have been responsible for all of the major cholera pandemics documented so far (Kitaoka et al., 2011). However, the other non-O1, non-O139 serogroups are mostly associated with erratic gastroenteritis (Uddin et al., 2018).

1.3.1 Habitat and Transmission

Vibrio cholerae can be found naturally in both fresh water and marine environments such as estuaries and brackish waters as either free-living cells or associated with other aquatic organisms such as copepods (Hill et al., 2011). Fishes are also reservoirs of *Vibrio cholerae* as they can get infected with the bacterium through the consumption of copepods. Many studies found correlations between the occurrence of cholera and fish consumption or handling (Laviad-Shitrit et al., 2018). As this food-borne pathogen can contaminate food products at any level from production or processing to consumption, it poses a remarkable threat to the aquaculture industry and humans (Loo et al., 2020). It is also presumed that water contaminated with free-living *Vibrio cholerae* cells is possibly the main source of epidemics. Although to a lesser degree, food with contaminated water frequently plays a vital role in *Vibrio cholerae* transmission (Sánchez et al., 2013).

As follows, one can get cholera infection by ingesting contaminated water or food (Akond et al., 2009). However, for the onset of severe cholera, a high infectious dose of 108 bacteria is essential as this species is highly sensitive to the low pH in the human stomach (Kitaoka et al., 2011). After ingesting contaminated food or water the bacteria pass through the human gastric acid barrier into the small intestine where they colonize, multiply and begin to secrete cholera toxin (Chakraborty S, 2015).

1.3.2 Pathogenicity

The pathogenicity of *Vibrio cholerae* is primarily associated with two virulence factors, the toxin coregulated pilus (TCP) and the cholera toxin (Faruque et al., 1998). The cholera toxin binds to cellular receptors in the intestine of the host and releases an enzymatically active subunit which

eventually leads to an increased intracellular cyclic adenosine monophosphate (cAMP) production. The up-surged cAMP level causes secretion of electrolytes and water into the intestinal lumen in a large amount which ultimately causes vomiting, acidosis, and hypovolemic shock (Aminzare et al., 2018).

1.4 Treatment

Oral rehydration therapy is the focal point for cholera treatment. However, a combination of oral rehydration therapy with antibiotic treatment is recommended in severe cases, seeing as, antibiotics can minimize the volume of diarrhea (Laviad-Shitrit et al., 2018). Notably, for treating cholera in developing countries, where safe drinking water is scarce, as well as oral rehydration solutions, are in short supply (Kitaoka et al., 2011). A single dose of doxycycline co-administered with rehydration solution is recommended in severe cases, as this combination is adequate for the restoration. Otherwise, a multidose of tetracycline can also be given (Kitaoka et al., 2011). However, according to World Health Organization (WHO), liquid erythromycin treatment is preferable for children under five years old (WHO, 2004).

1.5 Antibiotic resistance

Even though individuals, who are treated with antibiotics get apparent benefits, however, the frequent use of these is not suggested by the World Health Organization since it increases the resistance to antibiotics among the bacterial species hence making them difficult to treat (Das et al., 2020). As strains of the *Vibrio* spp. have an enhanced ability to tolerate the effects of antibiotics to which they were previously susceptible (CDC 2018), many antibiotic-resistant, especially multidrug-resistant strains (MDR) have emerged over the past few decades (Loo et al., 2020). Such as, a variant of *Vibrio cholerae* O1 El Tor biotype was isolated from hospitalized

patients with more acute diarrhea than the usual El Tor strains, which have shown hypervirulence as well as multi-drug resistance (Chatterjee et al., 2016).

As a consequence, exposure to these resistant bacteria is resulting in prolonged and severe infections. And as per WHO, this may set in motion for increased mortality due to treatment failure as an outcome of antibacterial resistance (WHO 2017). Lately, *Vibrio* spp. isolated from both food and environmental sources have shown antimicrobial resistance to streptomycin, kanamycin, ampicillin, tetracycline, trimethoprim, and carbapenem (Loo et al., 2020). The mechanism behind *Vibrio cholerae* gaining drug resistance is by the genetic exchange between *Vibrio* and other pathogens, use of efflux pump, chromosomal mutations, or developing genetical resistance (Das et al., 2020).

As shown by recent studies, the emergence of MDR O1 El Tor variant strain is increasing at an alarming rate, and, as most of the common antibacterial agents are either bacteriostatic or bactericidal, which may further promote the development of more MDR strains, finding novel approaches to combat this phenomenon has become a matter of immense necessity (Abhijit Balasaheb Shinde & Yogini Ramkrishna Mulay, 2015). As the use of natural products as therapeutic has increased considerably in the last two decades, investigation of natural compounds as an alternative source needs to be considered as a potential therapeutic agent to treat cholera (Aminzare et al., 2018).

1.6 Plant sources

Since ancient times, natural products like herbs, fruits, spices, etc. have been regarded as effective components against diarrheal diseases (Chatterjee et al., 2016). Around 80% of the population from developing countries still rely upon plant-based medicinal remedies for primary healthcare (Abhijit Balasaheb Shinde & Yogini Ramkrishna Mulay, 2015). In Bangladesh and

the Indian subcontinent, diarrhea has been treated by medicinal plants for ages. Common plants like neem, tulsi, etc., and their extracts have shown medicinal properties against pathogenic organisms including toxigenic *Vibrio cholerae* (Chatterjee et al., 2016). Antimicrobials based on plant origin have massive therapeutic potential. These compounds are not only effective for treating disease, what is more, they can also minimize much of the side effects that are frequently associated with synthetic drugs (Aminzare et al., 2018). Plants are rich sources of various types of medicines as they produce a diverse range of bioactive molecules. These compounds are principally secondary metabolites, such as steroids, alkaloids, flavonoids, fatty acids, resins, tannins, phenol compounds, etc. (Abhijit Balasaheb Shinde & Yogini Ramkrishna Mulay, 2015).

Three of the common and traditionally used medicinal plants of importance have been evaluated in this study. The plants are described below:

1.6.1 Neem (Azadirachta indica)

For ages, *Azadirachta indica* commonly known as Neem in Indian Subcontinent has been used in traditional medicine as a source of several therapeutic agents (Raut et al., 2014). The tree belongs to the mahogany family native to Southeast Asia and grows well in tropical countries. Neem is a fast-growing tree that can reach up to a height of around 15-20 meters and sometimes even 35-40 meters. This is an evergreen tree that has wide and spreading branches that may shed nearly all of its leaves during severe drought (Harikrishnan et al., 2010).

From its leaves to roots, almost all parts of the tree are being used for medicinal purposes. Such as for treatment of skin disease, inflammation, infections, fever, and many more. Although the medicinal efficacy of the tree leaves is mostly described. The leaf and its components along have shown remarkable anti-inflammatory, immunomodulatory, antiulcer, antifungal, antimalarial, antibacterial, antiviral, antioxidant, antimutagenic, and anticarcinogenic properties (Raut et al.,

2014). One of the promising natural compounds amongst its biological products is Azadirachtin, an inactive compound, whose antifungal, antibacterial, antiviral, and insecticidal properties have been well known for years. More than 135 biologically active compounds of chemically diverse and structurally complex have been extracted from different parts of the neem tree. The extracts from the neem tree leaves have been successfully suppressed quite a few gram-positive pathogenic bacterial species and have arrested the growth of some gram-negative bacteria such as *Vibrio cholerae* (Harikrishnan et al., 2010).

1.6.2 Tulsi (Ocimum sanctum)

Ocimum sanctum, widely known as Tulsi or Holy Basil, is a member of the family Lamiaceae which is native to South Asia, Africa, Central America, and some parts of Europe. Mostly, in tropical regions, it grows well in warmer climates (N et al., 2017). Though the plant is almost legendary in South Asia for its religious and medicinal purposes. This 30-60, centimeter tall sub-shrub is fragrant with green and purple leaves is popular as a medicinal plant and herbal tea, and has been cultivated for ages. Tulsi comes across frequently in many domains of medicines, such as Ayurveda, Greek, Roman, Siddha, Unani medicine systems (Khurana, 2020). Tulsi has a complex chemical composition though some commonly known compounds are eugenol, ursolic acid, alkaloids, flavonoids; tannins and carbohydrates, and many more (Kumar et al., 2013). Tulsi has been used in folklore medicine to treat vomiting, diarrhea, heart disease, convulsions, malaria, fever, epilepsy, poisoning cases, several inflammatory problems, menstrual pains, to improve kidney function and to get relieved from typical cold and flu symptoms such as coughing, hoarseness, and bronchitis (Barbalho et al., 2012). Tulsi is also known as *"the elixir of life"* as it promotes longevity. Tulsi has been used therapeutically for the prevention of cardiac diseases, anti-diabetic, antioxidant, anticancer, antibacterial, and anti-inflammatory effects. The

alcoholic extract of the leaves has shown *Vibrio cholerae* growth inhibition. Along with that, this plant is also a water purifier with antibacterial and insecticidal properties (Khurana, 2020).

1.6.3 Pomegranate (Punica granatum)

Punica granatum Linn. Commonly known as Pomegranate is a scrumptious fruit of the Punicaceae family that has a rich history of traditional use in medicine. This shrub is native to Iran and has been long cultivated in Asia, Mediterranean Europe, and Africa (Akter et al., 2014). Medicinal usage of pomegranate has been found in Siddha, Ayurveda, and Unani medicine system notably for Gastrointestinal diseases (Haque et al., 2015). Pomegranate is known as *"a pharmacy unto itself"* in Ayurvedic medicine for its role as an antiparasitic agent, a *"blood tonic"*, and to treat aphthae, ulcers, and various other diseases such as hemorrhages, (Pradeep et al., 2008).

Besides, Hindu physicians of ancient times had used peel of the fruit and flower, mixed with cloves, cinnamon, coriander, and pepper to treat bowl astringent in diarrhea (Pradeep et al., 2008).

Various parts of the plant, like the fruit, fruit juice, peel, bark, leaves have been researched and found chemical components of medical significance such as, for their antibacterial, antifungal, anticancer, antioxidant, anti-inflammation, antidiarrheal, anti-glycemic, and anti-spermatogenic activities (Akter et al., 2014). Research on the chemical constituents of pomegranate has found the presence of ellagitannins, anthocyanin, punicic acid, flavonoids, flavonol, etc. (A, 2017). Peel of fruit has been extensively used in diarrhea and dysentery caused by enteric pathogens (Haque et al., 2015) and researchers have found that the peel extract of pomegranate has antibacterial activity against numerous pathogens responsible for enteric diseases including *Vibrio cholerae* (A, 2017). Through various investigations, it has been found that phytochemical compounds like

gallotinnins, ellagic acid, gallagic acid, punicalins, punicalagins etc. might be the source for its antibacterial activities (Al-Dhaher, 2013).

1.7 Research aim and Objectives

The current study has been conducted for the purpose of isolating potential *Vibrio cholerae* species from environmental sources and further assessing the antimicrobial activity of the selected plants of interest against the organisms isolated. For isolating the organism, two bazars in the Mohammadpur area where raw fish are sold on a daily basis have been selected. Water samples where freshly brought fishes are kept were collected to isolate *Vibrio cholerae* from them. The reason behind assessing water samples from where fishes are kept was to isolate and identify *Vibrio cholerae* from them as it is well known that, fishes are potential reservoirs of *Vibrio cholerae*. The samples were then subjected to various identification methods to find presumptive *Vibrio cholerae* species. To assess the antimicrobial activity of the selected plants, zone of inhibition was determined as well as were compared with commercial antibiotics. Taking into consideration these, certain objectives set for this study include:

1. Isolating and identifying presumptive *Vibrio cholerae* species from the collected water samples
2. Determining the antibacterial activity of the medicinal plants of interest against the isolated species
3. Comparing the antimicrobial activity of the plant extracts with conventional antibiotics.

Chapter 2

Method and Materials

2.1 Place of Work

The entirety of the research work was carried out in the Biotechnology and Microbiology Laboratory, Department of Mathematics and Natural Sciences, Brac University.

2.2 Site selection and sample collection

For conducting the study, Samples were collected in the early mornings during July 2019 to September 2019. Mohammadpur Townhall Kacha Bazar and Mohammadpur Krishi Market Kacha Bazar in the Mohammadpur area were chosen as sample collection sites. In total, 16 samples of water where raw fish are kept were collected. For the collection process, autoclaved plastic bottles were used. After collection, samples were transported to the laboratory within 2 hours of collection for further processing. A list of the sample types and their selection sites are given below:

Table 2.2.1: Sample collection; source and location of the samples

Sample no.	Source	Location
01	Rui fish water	Mohammadpur Townhall kacha Bazar
02	Mola fish water	Mohammadpur Townhall kacha Bazar
03	Chapila fish water	Mohammadpur Townhall kacha Bazar
04	Shrimp water	Mohammadpur Townhall kacha Bazar
05	Koi fish water	Mohammadpur Townhall kacha Bazar
06	Shing fish water	Mohammadpur Townhall kacha Bazar
07	Prawn water	Mohammadpur Townhall kacha Bazar
08	Kachki fish water	Mohammadpur Townhall kacha Bazar
09	Mola fish water	Mohammadpur Krishi Market Kacha Bazar
10	Prawn water	Mohammadpur Krishi Market Kacha Bazar
11	Shing fish water	Mohammadpur Krishi Market Kacha Bazar
12	Kachki fish water	Mohammadpur Krishi Market Kacha Bazar
13	Shol fish water	Mohammadpur Krishi Market Kacha Bazar
14	Rui fish water	Mohammadpur Krishi Market Kacha Bazar
15	Chapila fish water	Mohammadpur Krishi Market Kacha Bazar
16	Shrimp water	Mohammadpur Krishi Market Kacha Bazar

2.3 Isolation

Vibrio spp. are usually presumed to be in a viable but non-culturable (VBNC) state or the stressed condition in the environment (Shammi, 2016). Therefore, it is strongly advised to perform a pre-enrichment step for improved detection of the mentioned bacteria (Huq et al.,

2012). Thus, all the samples were enriched in sterile alkaline peptone water (APW) before culture. For that, 50ml of sample water was mixed with an equal volume of autoclaved alkaline peptone water in a sterile conical flask, thus making it a total of 100 ml of volume. Then the flasks were kept in a shaking incubator for 6 to 8 hours at 37°C before plating. After enrichment in APW, the surface growth, which appeared as a whitish layer on top was collected with a sterile metal loop and streaked on Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar; a highly selective medium for *Vibrio* spp. isolation. Also, a series of ten-fold serial dilutions were done up to 10^{-6} and 0.1ml of broth from each dilution were spread on the TCBS agar.

Moreover, direct plating of the sample water on TCBS agar was also done in parallel with APW enrichment, as it is found to be useful in occasions when there is an overgrowth of non-target bacteria on solid media that occurs after their overgrowth in the enrichment broth (Huq et al., 2012).

All plates were incubated overnight at 37°C. On the following morning, for obtaining pure cultures, loopful of yellow and green colonies from the plates were carefully taken with a sterile loop and re-streaked separately on TCBS agar and kept at 37°C overnight in an incubator. After incubation, loopful of small, flat, and sucrose-fermenting yellow colonies were sub-cultured on modified nutrient agar with a salt concentration of 3% (MNA).

For stock preparation, 1ml of modified nutrient broth with a salt concentration of 3% (MNB) was transferred in Eppendorf tubes and one loopful of the colony from MNA was added to the broth. After inoculation, the Eppendorf tubes were incubated at a temperature of 37°C overnight. The next day after incubation, the samples were stored at -20°C after adding 300 µl of autoclaved glycerol in each tube.

2.4 Identification

Gram-staining, along with a series of biochemical tests were performed for the confirmation of isolated organisms as *Vibrio cholerae* species. For each experiment, freshly sub-cultured colonies were used to ensure the validity of the research by experimenting with pure and viable organisms.

2.4.1 Gram-staining

Gram-staining was done to confirm whether the organism is gram-positive or gram-negative. *Vibrio cholerae* is a gram-negative, comma-shaped non-motile bacterium that can be observed under the microscope through gram staining.

2.4.2 Biochemical characterization

The following biochemical tests were performed according to the protocols from Microbiology: A laboratory manual (Cappuccino & Sherman, 2005).

2.4.2.1 Triple Sugar-Iron (TSI) Agar test

For the test, autoclaved TSI slants of 6ml volume were used. Using a sterile straight needle, a small amount of bacterial colony from a 24-hours old pure culture was inoculated by stabbing through the slant and finally streaking on the slanted surface of the medium. Then the slants were incubated overnight at 37°C and checked for sugar fermentation, gas, and H_2S production.

2.4.2.2 Catalase Test

For experimenting, sterile glass slides were labeled and placed on a petri dish. Then using a sterile loop, a small amount of the organism from each freshly sub-cultured modified nutrient agar were transferred on the glass slides carefully. Then one drop of the reagent (3% hydrogen

peroxide) was placed on each of them. The slides were checked for immediate bubble formation for a positive result.

2.4.2.3 Oxidase Test

The experiment was done by first placing a filter paper on a sterile petri dish. Then using a sterile toothpick, bacterial colonies were picked from a 24-hours old pure subculture and smeared gently on the filter paper. Following the addition of oxidase reagent (tetramethyl-p-phenylenediamine) on it using a dropper. The filter paper was checked for any change in color. The formation of dark purple or violet color within a few seconds indicates the presence of cytochrome c oxidase enzyme within the bacterium that reacts with the reagent and produces this particular hue which ultimately gives a positive result. Whereas no change in color after a few seconds indicates the absence of the mentioned enzyme in the organism thus giving a negative result. The same procedure was repeated for each sample.

2.4.2.4 MIU (Motility, Indole, Urease) Test

MIU test was done to check the organism's ability to produce indole, degrade urea by using urease enzyme, and also, to determine whether the organism was motile or not. For the experiment, MIU agar was prepared and sterilized in test tubes. The solution was cooled down to approximately 50°-55°C. Afterward, 5% (v/v) urea solution, sterilized by syringe filtration, was added to each tube allowed to solidify until it forms a semi-solid medium. Each tube was stabbed by a sterile straight needle containing freshly cultured inoculum. Then, after incubation at 37°C overnight, the tubes were checked for any change in color and bacterial growth. Turning the medium's color to pink implied a positive utilization of urea and a positive motility test is indicated by producing a diffused, hazy growth away from the site of inoculation.

2.4.2.5 Indole Test

This particular test was done to determine the organism's ability to degrade tryptophan; an amino acid, by the enzyme tryptophanase. To experiment, a small amount of the selected isolates was inoculated in 6 ml of peptone water broth. The tubes were then incubated overnight at 37°C. After incubation, five drops of Kovac's reagent were added to each tube. Afterward, the cultures were observed for any color change. The presence of a cherry red reagent layer on top indicated a positive result. On the other hand, a presence of a brown or yellow layer indicated a negative result.

2.4.2.6 Methyl Red Test

Methyl Red (MR) test was done to determine the organism's ability to perform mixed acid fermentation when glucose is supplied in the culture medium. For performing the test, a loopful of bacterial culture was inoculated into separate test tubes containing 5 ml of MR-VP broth and incubated overnight at 37°C. On the following morning, five drops of methyl red indicator solution were added to each tube while being careful not shaking them. The appearance of red color within a few minutes, indicated a positive result, while the negative result was determined by the appearance of yellow color. However, the orange color of the culture indicated inconclusive results.

2.4.2.7 Vogues-Proskauer Test

For the experiment, test tubes containing 5 ml of MR-VP broth were inoculated with the bacterial samples and were incubated overnight at 37°C. After incubation, 6 drops of Barritt's reagent A were added and shaken. Subsequently, 6 drops of Barritt's reagent B were added and finally, the tubes were observed for 10 minutes. The appearance of a red ring indicated a positive result and a negative result was indicated by the absence of any such ring formation.

2.4.2.8 Citrate Utilization Test

A citrate utilization test was done to determine the microbe's ability to utilize citrate as the sole carbon source. For the test, Simmons's citrate agar slants of 2 ml were prepared in sterile glass vials. Using sterile technique, a small amount of the samples were inoculated and incubated at 37°C for 24 hours. After incubation, the slants were checked for both bacterial growth and change in color. A blue-colored medium indicated a positive citrate utilization test while no change in color after incubation indicated a negative result.

2.4.2.9 Antibiotic Disc Diffusion

An antimicrobial susceptibility test was performed to determine the antimicrobial susceptibility of the bacterial isolates to drugs of choice for infections caused by the bacteria of interest. To perform the test, the isolates were subjected to antimicrobial susceptibility testing by disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI, 2017) using commercial antimicrobial disks. The method described by Bauer and Kirby (1969) was followed. For the experiment, Muller-Hinton Agar, (MHA) plates were prepared for each isolate to test. A sterile cotton swab was dipped into the bacterial suspension. To get rid of excess broth, the cotton swab was pressed and rotated firmly against the inside wall of the test tubes above the fluid. Then the swab was streaked heavily on the MHA plate. The streaking was performed three times evenly in three directions over the entire surface of the agar plate by rotating the plate 90° each time to obtain a uniform inoculum. A final sweep was made on the agar rim with the cotton swab. The plate was allowed to absorb the inoculum for 5 minutes and then, the antibiotic discs were placed on it using sterile forceps. All disks were gently pressed down onto the agar with sterile forceps to ensure complete contact with the agar surface. The plates were then allowed to dry out. After that, the plates were incubated at 37°C for 24 hours. Following their incubation period, the plates were examined for zones of inhibition of bacterial growth. The clear zone

around each disc was observed through the back of the Petri plates and the diameter was measured to the nearest whole millimeter by a rule. The zone diameters for individual antimicrobial agents were then translated into susceptible, intermediate, or resistant categories according to the CLSI guidelines (2017).

For the purpose of this study, the following antibiotics impregnated discs were used: Ampicillin (25 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Tetracycline (30 µg).

Chapter 3

Results

3.1 Confirmation of *Vibrio cholerae*

In total 16 samples were collected. All the samples were enriched in APW and plated on TCBS agar. The samples were also plated directly on TCBS agar as well. Among 16 of them, 10 isolates produced characteristic yellow colonies on TCBS agar, and the rest of the 6 samples produced green and black colonies on the respective medium. Only the isolates that produced yellow colonies were further selected and sub-cultured on MNA before them being subjected to 8 biochemical tests along with gram staining.

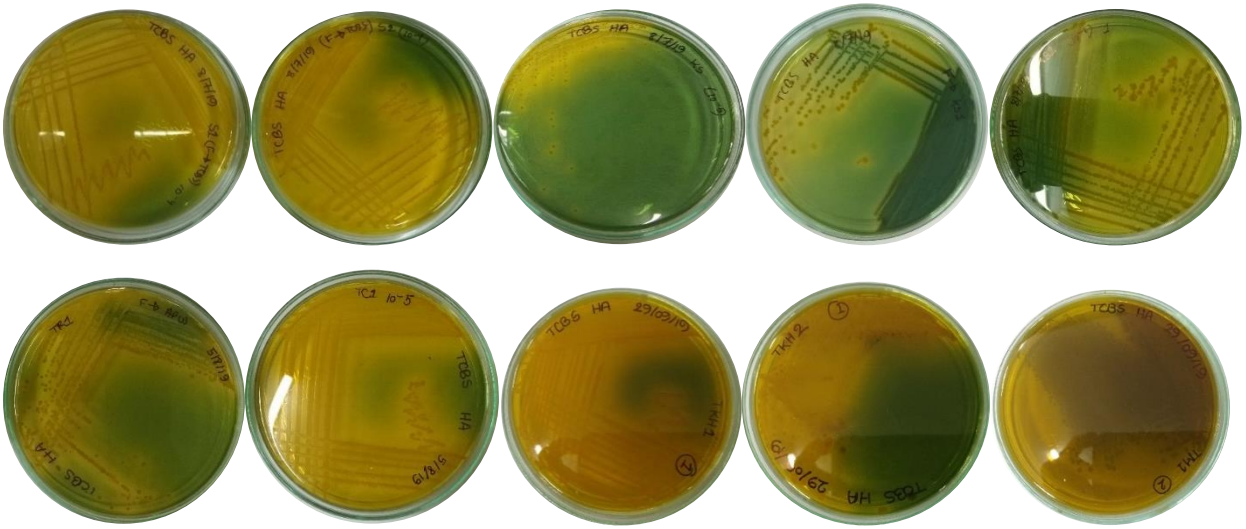

Figure 3.1.1 Growth of *Vibrio cholerae* on Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar media

Table 3.1.1 Colony characteristics of the 10 isolates on TCBS Agar selective media

Isolate no.	Isolate code	Source of Isolate	Colony color	Colony form	Elevation of the colony	Size of the colony (mm)	Edge of the colony
01.	S1	Shrimp water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	3	Entire
02.	S2	Shing fish water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	3	Entire
03.	KS	Shol fish water from Mohammadpur Krishi Market Kacha Bazar	Yellow	Circular	Convex	2	Entire
04.	KS1	Shrimp fish water from Mohammadpur Krishi Market Kacha Bazar	Yellow	Circular	Convex	3	Entire
05.	KS2	Shing water from Mohammadpur Krishi Market Kacha Bazar	Yellow	Circular	Convex	2	Entire
06.	TR1	Rui fish water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	2	Entire
07.	TC1	Chapila fish water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	2	Entire
08.	TKH1	Kachki fish water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	4	Entire
09.	TKH2	Koi fish water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	3	Entire
10.	TM1	Mola Fish Water from Mohammadpur Townhall kacha Bazar	Yellow	Circular	Convex	2	Entire

Table 3.1.2 Total Aerobic count of the 10 isolates on Modified Nutrient Agar (MNA)

Isolate no.	CFU for different dilutions						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	CFU/ml
01.	TNTC	TNTC	TNTC	TNTC	TNTC	167	$1.67x10^{9}$
02.	TNTC	TNTC	TNTC	TNTC	98	58	$3.39x10^{8}$
03.	TNTC	TNTC	132	87	21	0	$1.48x10^{7}$
04.	TNTC	TNTC	TNTC	194	105	77	$4.37x10^{8}$
05.	TNTC	TNTC	TNTC	98	63	0	$3.64x10^{7}$
06.	TNTC	TNTC	TNTC	TNTC	TNTC	114	$1.14x10^{9}$
07.	TNTC	TNTC	TNTC	157	129	61	$3.69x10^{8}$
08.	TNTC	TNTC	TNTC	178	80	28	$1.8x10^{8}$
09.	TNTC	TNTC	113	51	0	0	$3.1x10^{6}$
10.	TNTC	TNTC	TNTC	TNTC	179	42	$2.99x10^{8}$

TNTC= Too numerous to count. CFU/ml= Colony-forming unit per ml

* 0.1ml sample from each dilution was plated. CFU/ml was determined by taking an average of the last two dilutions where colonies were found.

3.2 Biochemical Characterization

The biochemical test results were evaluated with comparison to standard references mentioned in Microbiology Laboratory Manual (Cappuccino & Sherman, 2005) and also analyzed with the help of an online laboratory tool for bacterial identification, Advanced Bacterial Identification Software (ABIS).

Table 3.2.1 Biochemical Test Results

Isolate no	Indole	Methyl Red	Voges Proskauer	Citrate Utilization	Oxidase	Catalase	Motility	Urease	TSI				Gram Staining
									Slant	Butt	H_2S	Gas	
01.	+	+	+	-	+	+	+	-	Y	Y	-	-	Gram-negative, curved rod
02.	+	+	+	-	+	+	+	-	Y	Y	-	-	Gram-negative, curved rod
03.	-	+	+	-	-	-	+	-	Y	Y	-	-	Gram-negative, rod
04.	+	+	+	-	+	+	+	-	Y	Y	-	-	Gram-negative, curved rod
05.	-	+	+	-	-	-	+	+	Y	Y	-	-	Gram-negative, rod
06.	+	+	+	-	-	+	+	-	Y	Y	-	-	Gram-negative, rod
07.	+	+	+	+	+	+	+	-	Y	Y	-	-	Gram-negative, curved rod
08.	+	+	+	-	+	+	+	-	Y	Y	-	-	Gram-negative, curved rod
09.	+	+	+	-	-	+	+	+	R	Y	-	-	Gram-negative, rod
10.	-	+	+	-	-	+	+	+	R	Y	-	-	Gram-negative, rod

KEY: + = Positive result; - = Negative result

Y=Yellow color (Acidic condition); R= Red color (Alkaline condition)

3.3 Biochemical test results image

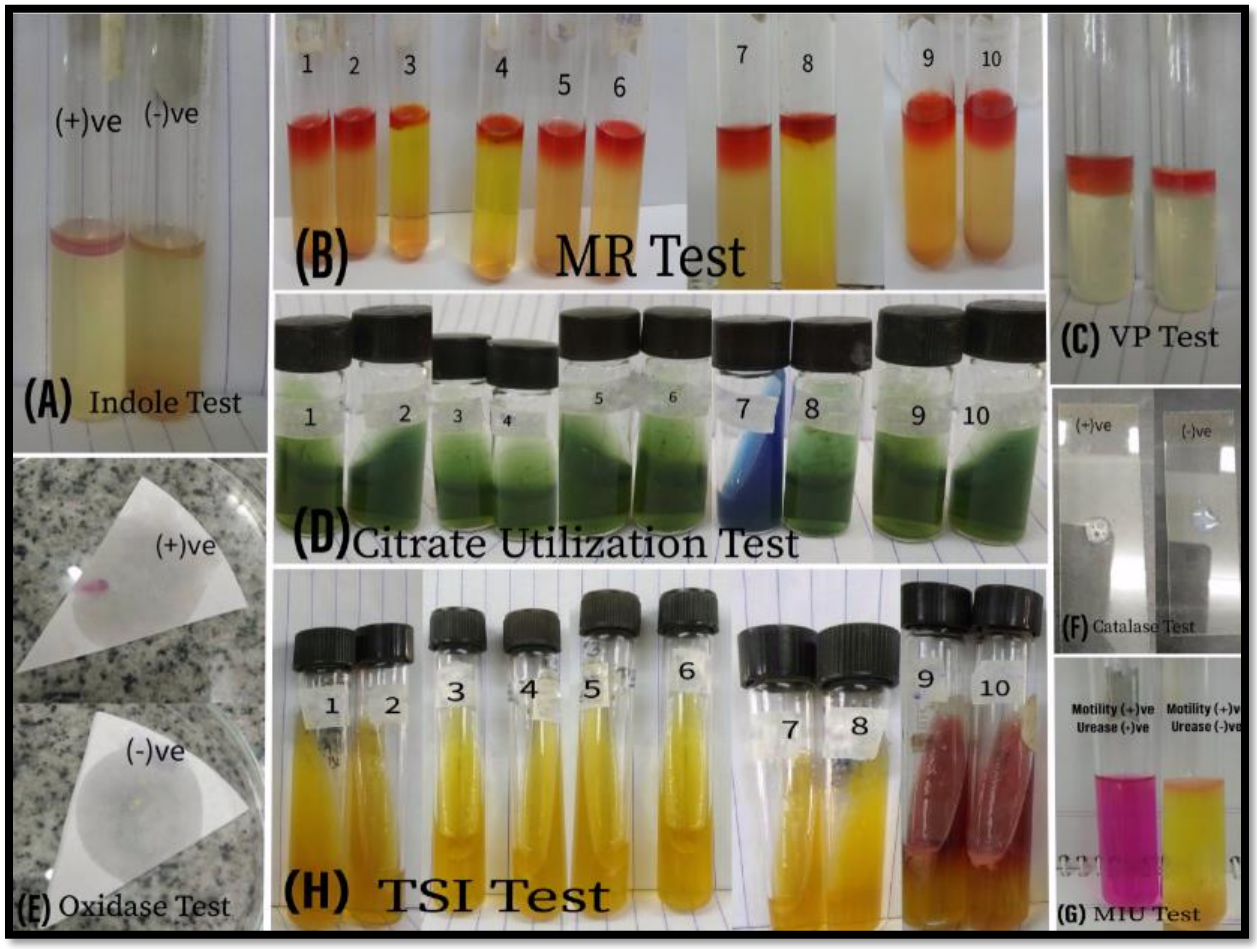

Figure 3.3.1 (A) Indole test result: sample no. 1,2,4,6,7,8,9 showed positive result; sample no. 3,5,10 showed negative results. (B) MR test result: All the samples produced positive results (C) VP test result: All the samples showed positive results (D) Citrate utilization test: All the samples except no.7 showed negative results; only sample no.7 produced a positive result. (E) Oxidase test: Sample no. 1,2,4,7,8 produced positive results; sample no. 3,5,6,9,10 produced negative results. (F) Catalase test: Sample no. 1,2,4,6,7,8,9,10 produced positive result; sample no. 3,5 produced negative results. (G) MIU test result: All the samples produced positive results for motility; sample no. 5,9,10 were positive for urease; sample no. 1,2,3,4,6,7,8 were negative for urease. (H) TSI test result: Sample no. 1,2,3,4,5,6,7,8 went through the acid formation with

yellow slant and butt; in sample no. 9,10 only dextrose fermentation occurred, yellow butt, red slant; none of the samples produced any gas nor H_2S.

3.4 Morphological confirmation through Gram-stain

The morphology of the isolates that were confirmed through biochemical tests further went through gram-staining to determine their morphological characteristics. *Vibrio cholerae* is usually a gram-negative curved rod, which appears pinkish-red color upon staining and observing under a microscope.

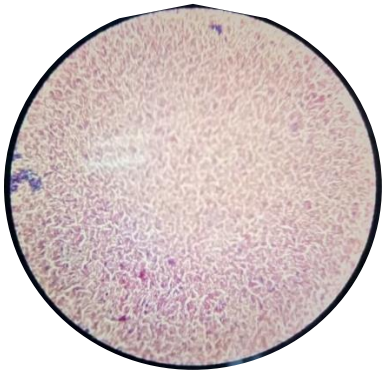

Figure 3.4.1 Gram-negative curved rods were observed under the microscope indicates the presence of *Vibrio cholerae*

Once, all the biochemical and morphological characterization was done, four presumptive isolates were selected which gave similar results to the standard ones. Isolate no. 2,4,7 and 8 were chosen for further research purposes to assess their antibacterial resistance pattern compared with conventional antibiotics and plant extracts.

3.5 Antimicrobial sensitivity test using antibiotic disc diffusion method

The confirmed isolates were subjected to the antibiotic disc diffusion method. This experiment was conducted using 5 commercially available antibiotic discs: Ampicillin (25 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Tetracycline (30 µg). The size of the zone of inhibition was considered to evaluate the resistance or sensitivity of the microorganism for the selected antibiotics.

The criteria for determining the sensitivity of the organisms to the different antibiotics and the results obtained from the disc diffusion test are presented in tables 3.5.1 and 3.5.2 respectively.

Table 3.5.1 Interpretative criteria used to determine the antibiotic susceptibility of *Vibrio cholerae* with disc diffusion method

Antimicrobial agent	Disc potency	Diameter of zone of inhibition (mm)		
		Susceptible	Intermediate	Resistant
Ampicillin	25 µg	≥17	14-16	≤13
Chloramphenicol	30 µg	≥18	13-17	≤12
Erythromycin	15 µg	≥17	13-16	≤12
Gentamycin	10 µg	≥15	13-14	≤12
Tetracycline	30 µg	≥15	12-14	≤11

Table 3.5.2 Antibiotic susceptibility profile of the 4 selected isolates

Sample no.	Isolate code.	AMP (25 µg)	C (30 µg)	E (15 µg)	GEN (10 µg)	TET (30 µg)
02.	S2	R	S	R	S	S
04.	KS1	R	S	I	S	S
07.	TC1	R	S	R	S	S
08.	TKH1	R	S	R	S	I

R=Resistant; S=Sensitive; I=Intermediate

3.6 Plant extract obtained from ethanolic and aqueous solvents

Table 3.6.1 presents the amount of crude extract obtained from the three selective plants using two types of solvents: ethanol and distilled water.

Table 3.6.1 Amount of plant extract obtained using ethanol as solvent

Plant sample	Type of solvent	The volume of solvent (ml)	Amount of powder (gm)	Amount of crude extract (gm)
Neem	Ethanol	100	10	0.49
Tulsi	Ethanol	100	10	0.69
Pomegranate peel	Ethanol	100	10	0.54

Table 3.6.2 Amount of plant extract obtained from using distilled water as solvent

Plant sample	Type of solvent	The volume of solvent (ml)	Amount of powder (gm)	Amount of crude extract (gm)
Neem	Distilled water	100	10	0.41
Tulsi	Distilled water	100	10	0.62
Pomegranate peel	Distilled water	100	10	2.10

3.7 Observation of the antimicrobial activity of the ethanolic and aqueous extracts of the plants compared to conventional antibiotics

For the study, *Vibrio cholerae* isolates were used for assessing the antimicrobial activity of the ethanolic and aqueous extract of the following plants: Neem leaves, Tulsi leaves, and Pomegranate peel. An antibiotic disc, as a positive control and the extracts, were each applied on the Petri plate to observe and compare their antimicrobial activity against *Vibrio cholerae.*

Table3.7.1 Zone of inhibition produced by antibiotic, ethanolic, and aqueous extract of Neem against *Vibrio cholerae*

Name of the organism	Name of Antibiotic	Zone of Inhibition (mm)		
		Antibiotic disc	Ethanol extract	Aqueous extract
Vibrio cholerae	Ampicillin	10	10	0
	Erythromycin	12		
	Chloramphenicol	20		

Table3.7.2 Zone of inhibition produced by antibiotic, ethanolic, and aqueous extract of Tulsi against *Vibrio cholerae*

Name of the organism	Name of Antibiotic	Zone of Inhibition (mm)		
		Antibiotic disc	Ethanol extract	Aqueous extract
Vibrio cholerae	Ampicillin	10	14*	0
	Erythromycin	12		
	Chloramphenicol	20		

*The Highest zone of inhibition by the plant extract

Table3.7.3 Zone of inhibition produced by antibiotic, ethanolic and aqueous extract of Pomegranate peel against *Vibrio cholerae*

Name of the organism	Name of Antibiotic	Zone of Inhibition (mm)		
		Antibiotic disc	Ethanol extract	Aqueous extract
Vibrio cholerae	Ampicillin	10	8	0
	Erythromycin	12		
	Chloramphenicol	20		

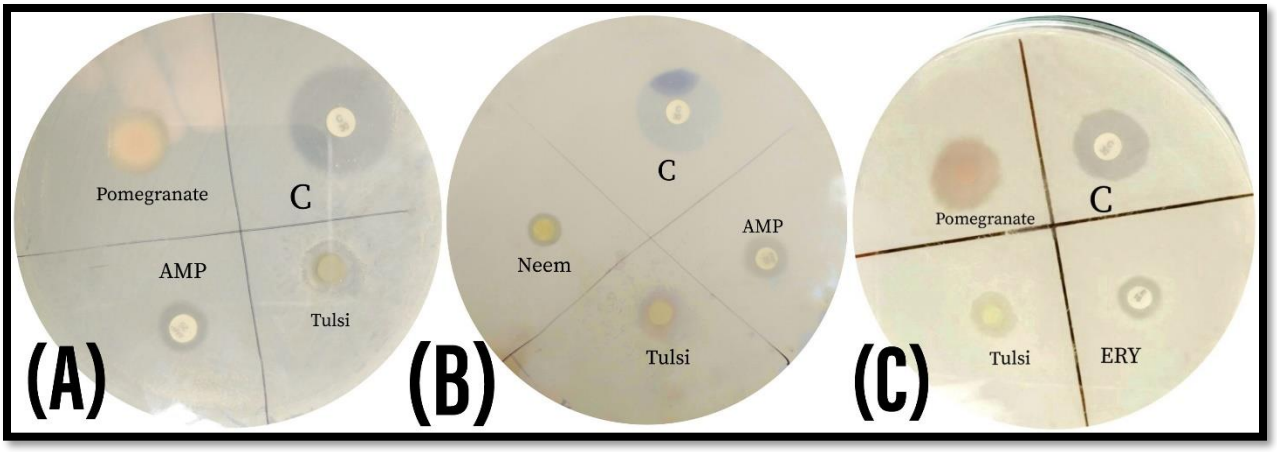

Figure 3.7.1 Antimicrobial activity of the plant extracts in comparison to conventional antibiotic discs. (A) Antibacterial activity of pomegranate peel and tulsi compared to conventional antibiotics ampicillin and chloramphenicol. (B) Antibacterial activity of neem and tulsi in comparison with chloramphenicol. (C) Antibacterial activity of pomegranate peel and tulsi compared to erythromycin.

3.8 Comparative study of the antibacterial activity through Activity Index

Activity index (AI) values are used to determine the effectiveness of plant extracts quantitively by comparing them with conventional antibiotics. In this study, Activity index values were calculated only for the ethanolic extracts of the plants against three conventional antibiotics: Ampicillin, erythromycin, and chloramphenicol using the following formula:

$$\text{Activity Index (AI)} = \frac{Zone\ of\ inhibition\ of\ extract}{Zone\ of\ inhibition\ of\ antibiotic}$$

The following graph represents the difference in the antibacterial potency of the plant extracts against the chosen antibiotics through activity index value.

It is important to mention that, the higher the value of activity index of an extract, the higher their potency against the pathogen compared to the allopathic antibiotic.

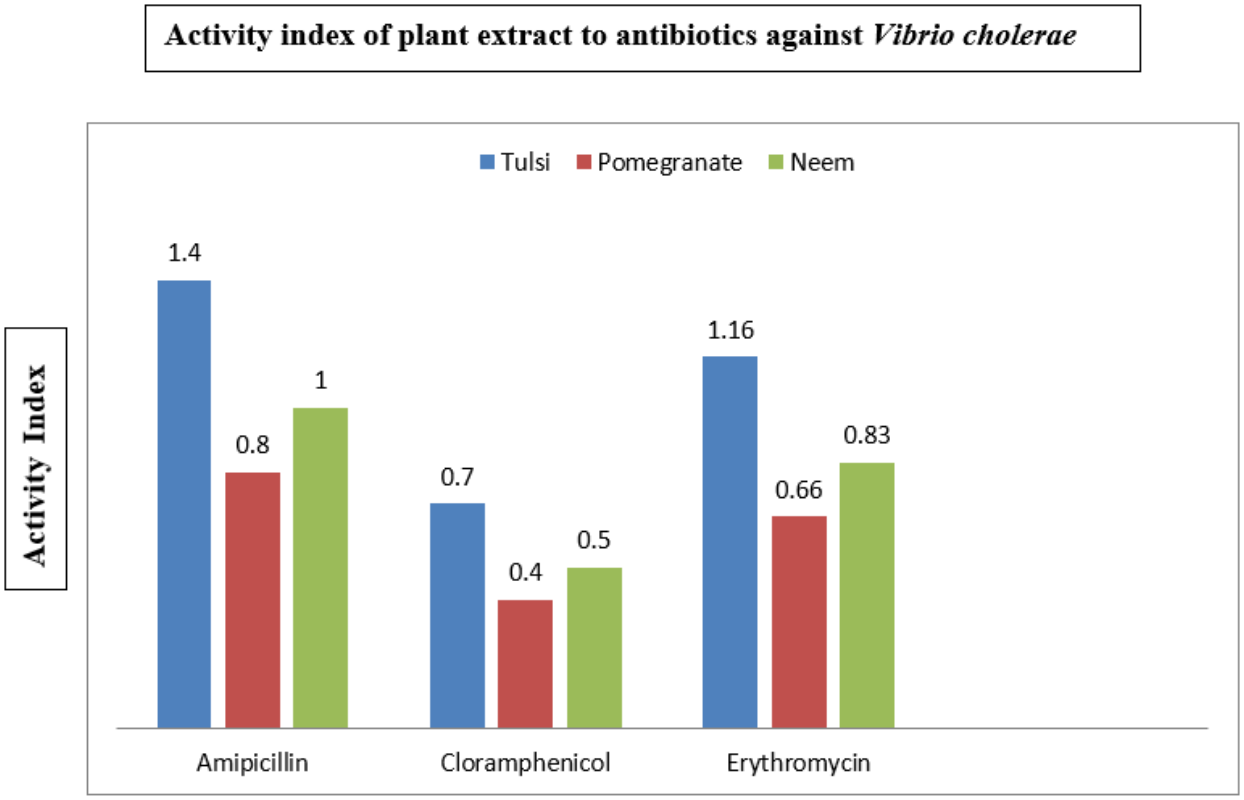

Figure 3.8.1 Activity index values for the ethanolic extracts of neem, tulsi, and pomegranate peel against *Vibrio cholerae*. The activity index of tulsi against the pathogen shows an effective result in comparison to the antibiotic ampicillin with an AI value of 1.4. Also, the activity index value of 1.16 for erythromycin and 0.7 for chloramphenicol respectively, show effective results for tulsi leaves extract. The activity index value of neem leaves extract to antibiotic ampicillin and erythromycin show effective results with the values of 1 and 0.83 respectively. However, activity index values for pomegranate peel extract, except for ampicillin with an AI value of 0.8 do not show any value of significance against the other antibiotics.

Chapter 4

Discussion

Since, the emergence of MDR *Vibrio cholerae* strains, concerns have risen as an impending threat for the future of cholera treatment, which has resulted in a strong interest among the scientific community to find alternates using herbal medicines that have strong antibacterial activities against the pathogen of concern. In many cultures, medicinal plants have been used as natural antimicrobial agents in food and for controlling the common health of folks for several thousands of years. Tannins, saponins, phenolic compounds, flavonoids, and essential oil are believed to be the reasons behind the antimicrobial potency of plant origins (Akter et al., 2014). The present study focuses on evaluating the antibacterial activities of some well-known medicinal plants that have been used for ages to fight enteric diseases like cholera. For the purpose of the study, samples have been collected from environmental sources to isolate *Vibrio cholerae* from and later on, to assess antibacterial activities of the selected plants against four isolates of *Vibrio cholerae* in comparison to conventional antibiotics. The current study primarily focuses on evaluating the antibacterial potential of the already used traditional medicinal plants such as neem, tulsi, and pomegranate peel against cholera pathogen. For the study, both aqueous and ethanolic extracts of the mentioned plants were prepared and the antimicrobial activity of them was determined in comparison to antibiotics Ampicillin (25µg), Erythromycin (15µg), and Chloramphenicol (30µg).

From the results obtained from the study, it can be seen that aqueous extracts of any of the plants have not shown any result of consequences (Table 3.7.1, Table 3.7.2, and table 3.7.3). It could be because the solubility of various phytochemical compounds in the extract fluctuates with

different solvents. Among the two extraction solvents for each plant, only ethanolic ones produced any results of significance, which may be because ethanol has a high aptitude for dissolving organic compounds that are found in the plant extracts. From the results above, it seems that Tulsi leaves have the strongest antibacterial activity against *Vibrio cholerae*. Scientists have proved before that, *Ocimum sanctum* Linn. have shown development hindrance for *Vibrio cholerae* (Khurana, 2020). Moreover, according to Barbalho et al., (2012), Tulsi had previously exhibited high antibacterial activity, primarily by damaging the membrane of the organism and resulting in the death of the bacterial cell (Barbalho et al., 2012). It was also observed by Pingale et. al., (2012) that Tulsi has shown significant antibacterial activity against fish pathogens like *Vibrio* spp. (Pingale et al., 2012). Pingale et al., also showed that when mixed with neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*) produces better results against the enteric pathogens (Pingale et al., 2012). Luthra (2010) reported that the alcoholic extract of Tulsi produces a wider zone of inhibition than other extraction methods (Luthra, 2010). According to the researchers, the antibacterial property of Tulsi might be due to the presence of phytochemical components like eugenol, carvacrol, methyl eugenol, caryophyllene (Deshmukh et al., 2015).

However, in this present study, even though Tulsi has produced the highest zone of inhibition compared to the other plant extracts (which is 14 mm), (Table 3.7.2) however, it can be seen from the results that, the zone of inhibition was only greater than the zone of inhibition produced by ampicillin but less than the zones of inhibition produced by the other two antibiotics (Ampicillin produced a zone of 10 mm, Erythromycin 12 mm and Chloramphenicol 20 mm). This indicates that the efficacy of conventional antibiotics is superior to the plant extract here

except for ampicillin as the pathogen of concern has been already been resistant to this particular antibiotic.

After Tulsi, neem leaves have produced the second-highest zone of inhibition compared to the other plant extract. Though the inhibition zone produced by neem (Table 3.7.1) was lesser than the zones produced by allopathic antibiotics in comparison which again established conventional antibiotics being more competent in treating cholera than neem.

However, various studies done on the efficacy of neem plant extracts, have shown better results with a moderately higher zone of inhibitions. A study conducted by Chakraborty S (2015), reported that among various plant extracts, the ethanolic extract of neem has shown promising antibacterial activity against *Vibrio cholerae* (Chakraborty S, 2015). Another study by Sharma et al., (2009) have found moderate efficacy of neem extract against the cholera pathogen. It was also reported by Sharma et al., (2009) that the extract of neem can halt the growth of *Vibrio cholerae* (Sharma et al., 2009). Furthermore, another study conducted by Harikrishnan et al., (2010) have reported that, when combined with the *Ocimum sanctum* (Tulsi), the ethanolic extract of neem yields better results in combating *Vibrio cholerae* than the extract of neem alone. Harikrishnan et al., (2010) also stated, though the mechanisms behind this specific organism's survival against antibacterial agents are not fairly understood, however, the combined effect of *Azadirachta indica* + *Ocimum sanctum* extract may disrupt the organism's cell membrane (Harikrishnan et al., 2010). According to researchers, the antibacterial activity of neem may be due to the presence of limonoids, protomeliacins, azadirone, and its derivatives such as gedunin and vilasinin, and components like several proteins, carbohydrates, sulfurous compounds, and flavonoids. Besides, Harikrishnan et al., (2010) also reported newly found chemical components like 6-azabicyclo, octane, cyclohexane,1-ethyl-1-methyl-2,4-bis(1-methylenyl)-,[1S-(1α,2β,4β)]-

βElemen, eudesma-4(14), 11-diene and globulol have shown higher antibacterial activity than the earlier components found (Harikrishnan et al., 2010).

Lastly, the peel extract of pomegranate has produced the lowest zone of inhibition among all the extracts (Table 3.7.3) which is only 4 mm in diameter. All the antibiotics tested against this extract produced a higher zone of inhibition. On the other hand, many researchers have confirmed the strong antibacterial activity of the peel extract against *Vibrio cholerae* (Al-Dhaher, 2013). It was reported by Doostkam et al., (2019) that pomegranate peel extract has exhibited a fair amount of antibacterial activity against water-borne pathogens like *Vibrio cholerae* (Doostkam et al., 2019). An investigation by Chakraborty S (2015) reported the antibacterial efficacy of tea infusion of pomegranate peel against *Vibrio cholerae* (Chakraborty S, 2015). Antibacterial activity of the plant extract may be due to the presence of phytochemical constituents, specifically secondary metabolites like flavonoids, tannins, alkaloids, glycosides (Akter et al., 2014), also, Ali et al., (2018) suggested that gallic acid in the peel extract might be a crucial antibiotic agent (Ali et al., 2018). On the contrary, Sharma et al., (2009) reported that the alcoholic extract of Pomegranate peel has shown poor biocidal activity (Sharma et al., 2009). Moreover, an investigation by Pradeep et al., (2008) showed that the methanolic extract of the pomegranate peel resulted in a greater antibacterial activity as compared with other extractions of the component (Pradeep et al., 2008).

Figure 3.8.1 demonstrates the comparison between the antibacterial activity of the plant extracts against synthetic antibiotics by calculating the activity index (AI) values. AI values help to comprehend the approximate effectiveness of the plant extracts by quantitively evaluating them to the corresponding antibiotics of standard use. The higher the value, the more efficient an extract is against the standard antibiotic. A higher activity index value of “1.4” has been obtained

from the ethanolic extract of Tulsi against the antibiotic ampicillin, though *Vibrio cholerae* has been long resistant to ampicillin, this value does not hold any impactful significance for the study. However, from the figure, it can be observed that Tulsi leaves has given a decent value of "1.16" against erythromycin, an antibiotic to treat cholera. Also, extract of Tulsi leaves produced higher activity index value against chloramphenicol as well, which is greater than the values obtained from the other two extracts. then again, the AI values were lower enough than the antibiotics in comparison. The low efficacy of the plant extracts might be for the difference in mechanism on how the antimicrobial agents interact with the bacteria.

It is a matter of consideration that, the results found in this study fluctuate with the hypothesis and investigations by other researchers regarding the mentioned medicinal plants. The reasons behind the not-so-efficient results obtained in this study can be of many. The optimum concentration of the crude extract, use of a different extraction method, the quality of the plant materials and reagents, the viability of the isolates used in this study are some of the facts that might be considered. Nonetheless, experiments should be carried out for seemly in-vitro investigations that may precedent clinical trials leading to the production of impactful herbal medications using these mentioned medicinal plants.

Conclusion

To sum up, it can be said that the results obtained from this study about evaluating the antibacterial effects of three common and traditionally used medicinal plants, such as neem, tulsi, and pomegranate peel against *Vibrio cholerae* in comparison to some conventional antibiotics produced moderate results opening the path for further research in this area. Approaches suggested by the researchers about taking an infusion of pomegranate peel, green tea, and bay leave prepared in water to treat *Vibrio cholerae* infections should be considered. Also, an edible alternate of ethanolic extract of neem and tulsi in combination should be investigated for treating cholera as it has also shown promising results. The strong recommendation about alternate, and effective herbal-based medication is of great significance in the context of Bangladesh, as the expense of getting medical care is high here and alternatives to antibiotics are cost-effective. Hence, purification and characterization of the active phytochemical components may lead a path to novel agents of significance to treat cholera.

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Appendixes

Appendix A: Media composition

All the media mentioned below except TCBS Agar was sterilized by autoclaving for 15 minutes at 121oC at 15 psi pressure.

Alkaline Peptone Water

Ingredients	Grams/ litre
Bacteriological peptone	20.0
Sodium chloride	20.0
Final pH (at 25°C) 7.4 ± 0.2	

Mueller-Hinton Agar

Ingredients	Grams /litre
Beef, hydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Motility Indole Urease (MIU) Agar

Ingredients	Grams /litre
Tryptone	10.0
Phenol red	0.1
Sodium chloride	2.0
Agar	5.0
Final pH (at 25°C) 7.0	

Modified Nutrient Agar

Ingredients	Grams /litre
Peptone	5.0
Beef extract	3.0
Sodium chloride	10.0
Agar	15.0
Final pH (at 25^{o}C) 7.0	

MR-VP broth

Ingredients	Amount(g /L)
Peptone	7
Dextrose	5
Potassium phosphate	5

Saline

Ingredients	Grams/ litre
Sodium chloride	9.0

Simmon's citrate agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bactobromothymol blue	0.08

TCBS Agar

Ingredients	Grams /litre
Beef extract	3.0
Peptone	20.0
Yeast extract	30
Lactose	10.0
Sucrose	10.0
Dextrose monohydrate	1.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

Triple sugar iron agar (Himedia, India)

Ingredients	Amount(g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0

Appendix B: Reagents and Buffers

Barritt's Reagent

Solution A: 5 g alpha-naphthol was dissolved in 95% ethanol. The reagent was covered in aluminium foil and stored at 4°C.

Solution B: 40 g KOH was dissolved in distilled water. Once the mixture cooled, creatine was added. Final volume was adjusted with distilled water and the reagent covered with aluminium foil was stored at 4°C.

Methyl Red Reagent

0.1 g methyl red was dissolved in 300 mL of 95% ethyl alcohol. To this, distilled water was added to make up the final volume of 500 ml. The reagent was covered with aluminium foil and stored at 4°C.

Oxidase reagent

100 mg of N, N, N1, N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in

10 mL distilled water. The solution was covered with aluminium foil and stored at 4°C.