STUDY OF THE EFFECTS OF PHARMACEUTICAL COMPOUNDS ON MARINE BACTERIA FROM THE COASTAL ENVIRONMENT

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I hereby declare that the data presented in this dissertation report entitled **"STUDY OF THE EFFECTS OF PHARMACEUTICAL COMPOUNDS ON MARINE BACTERIA FROM COASTAL ENVIRONMENT"**, is based on the results of investigations carried out by me **Anushka Shinde** at the **School of Earth, Ocean and Atmospheric Sciences, Goa**

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This is to certify that the dissertation report entitled as **"STUDY OF THE EFFECTS OF PHARMACEUTICAL COMPOUNDS ON MARINE BACTERIA FROM THE COASTAL ENVIRONMENT"** is a bonafide work carried out by **Ms. Anushka Ashok Shinde** under my supervision in partial fulfilment of the requirements for the award of the degree of Master's in the discipline **M.Sc. Marine Microbiology** at the **School of Earth, Ocean and Atmospheric Sciences,** Goa University.

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CHAPTER I

INTRODUCTION

1.1 Introduction

Pharmaceuticals are utilized worldwide for the prevention and treatment of disease. These medications fall into different categories such as antibiotics, non-steroid antiinflammatory drugs (NSAIDs), anti-histamines, lipid regulators, analgesics, antidepressants, β – blockers, selective serotonin reuptake inhibitors, anti-epileptics, anti-diabetics, hypertensive drugs, and anti-parasitic drugs. According to the chemical structure cardiac glycosides, fibrates, steroids, triptans, thiazide diuretics, benzodiazepines, and β-lactam antibiotics are the most common pharmaceutical classes. The mechanism of action of a drug is determined by its chemical structure, which means drugs belonging to the same chemical class will bind to the same biological target **(Besse and Garric, 2008)**. According to **(Jiang et al., 2014)** these pharmaceuticals and their breakdown products (degradates) are released into the surrounding environment through various sources such as from pharmaceutical industries, hospitals, agricultural sources, wastewater treatment plant effluents, and the mammalian excretion, un-metabolized, fully metabolized and partially metabolized. Altogether, these substances are collectively referred to as active pharmaceutical ingredients (APIs). Pharmaceuticals have physical and chemical properties such as low solubility, high hydrophobicity and strong attraction to organic

carbon which make them highly persistent in water and prone to binding sediments **(Pereira et al., 2020a, 2020b)**.

Throughout history Natural sources have been the primary origin of new pharmaceuticals compounds. At present more than half of the drugs available in the market are either derived from natural sources or synthesized using natural products as a foundation or a model. In ancient times, early societies have utilised natural medicines, in their crude form often derived from plants, to alleviate various ailments such as infection, inflammation and pain. Today in certain parts of the world, natural medicines remain provide the sole available treatment option. The study of ''natural'' ethnobotanical preparations resulted in the discovery and extraction of beneficial compounds that form the basis of today's pharmaceutical industry.

Pharmaceuticals substances have been found in various bodies of water such as freshwater, estuarine, and coastal sea waters with varying levels depending on the location and time in aquatic environment and ranging from below the detection limit of most analytical instruments **(Fram and Belitz, 2011)** to levels as high as 187.35 µg L− 1 **(Gomaa et al. 2020).** The Committee for Medicinal Products for Humans discovered about 113 pharmaceuticals and coastal waters have been found to contain their byproducts that result from the degradation of pharmaceuticals at concentrations ranging from 0.01 to 6800 ng L− 1. More than 61% of these pharmaceutical substances exceed the European Medicines Agency's threshold limit of having high concentrations above than the 0.01 mg L− 1 for predicted environmental concentrations in surface waters **(Gaw et al., 2014).** Coastal seawater and freshwater ecosystems have been found to contain various pharmaceutical drugs such as

acetaminophen, azithromycin, atenolol, carbamazepine, clarithromycin, diclofenac, erythromycin, gemfibrozil, ibuprofen, ketoprofen, naproxen, propranolol, roxithromycin, sulfamethoxazole, tetracycline, and trimethoprim. **(Bendz et al., 2005)** In Sweden's Hoje river carbamazepine, metoprolol, atenolol, gemfibrozil, sulfamethoxazole, and propranolol were detected. A study found that carbamazepine, benzotriazole, and caffeine were frequently detected and present in high concentrations **(Loos et al., 2009).** There are various other detections of propranolol, sulfamethoxazole, carbamazepine, indomethacin, clarithromycin, and diclofenac reported in freshwater ecosystems globally **(LopezSerna ´ et al., 2012; Osorio et al.,2016; Zhou et al., 2009).**

Sources of pharmaceuticals in Marine environment.

- Sewage

Effluents are a significant contributor to pharmaceuticals, including their metabolites, entering aquatic ecosystems. The effectiveness of wastewater treatment plants in removing drugs ranges from less than 10% to almost 100%, depending on the drug's physical and chemical characteristics and the treatment technology used. Pharmaceutical sources in wastewater come from community usage, hospital discharges, and, in some cases, pharmaceutical manufacturing wastewater. Effluent discharge into marine environments can occur through coastal and marine runoff, sewage treatment plant overflows, and rivers that receive treated sewage effluents. The Yangtze River in China carries sewage from 400 million people into the sea and discharges an estimated 152 tonnes of pharmaceuticals annually. Sewage effluent discharge into the marine environment can also come from boats.

- Aquaculture

The production of seafood through aquaculture is increasing globally, especially in Asia. To control diseases in marine aquaculture, antibiotics and other veterinary medicines are used, some of which are also approved for human use. However, up to 75% of the administered dose can be lost to the environment due to various reasons such as un-ingested pellets, excretion of drugs and their metabolites by marine organisms, and consumption of leftover food and feces by wild fish. This can lead to the spread of drugs and their byproducts to other marine organisms.

- Waste disposal

Disposing of waste in coastal regions is a way for pharmaceuticals to enter the marine environment. Waste from households and medical facilities can enter coastal waters through leachate from landfills and seafills. A study conducted in Mallorca revealed that landfill leachate contained a high concentration of drugs, reaching up to 27,000 nanograms per liter. In the past, pharmaceutical manufacturing waste, sewage sludge, and animal manure have been disposed of in the sea in some areas.

Categories of pharmaceutical compounds.

According to **(Chia et al., 2021)** and references therein, there are 7 chemical classes of pharamaceuticals. They are:

1. **ß-Lactam antibiotics**

   Structural feature: ß-Lactam ring

   Key examples: Antibiotics, penams, cephams, carbapenems, carbacephems

   Digoxin, Lanoxin, Digitoxin, etc.

   Solubility in water: Water soluble

   Mechanism of action: Inhibit cell wall biosynthesis of the bacterial peptidoglycan layer of the cell wall.

2. **Cardiac glycosides**

Structural feature: A steroid molecule linked to a sugar and an R group

Key examples: Digoxin, Lanoxin, Digitoxin, etc.

Solubility in water: Poor solubility in water

Mechanism of action: Acts as cellular sodium-potassium ATPase pump. They modulate electrophysiological properties of the heart and its contractile functions.

3. **Fibrates**

Structural feature: Amphipathic carboxylic acids

Key examples: Aluminium clofibrate, bezafibrate, ciprofibrate, etc.

Solubility in water: Lipophilic and virtually insoluble in water

Mechanism of action: Hypolipidemic agents - lower cholesterol levels.

4. **Steroids**

Structural feature: Four carbon rings arranged in specific molecular configurations

Key examples: Prednisolone, Betamethasone, dexamethasone, hydrocortisone

Solubility in water: Hydrophobic and insoluble in water

Mechanism of action: Change membrane fluidity and act as signalling molecules.

5. **Triptans**

Structural feature: Hydroxy derivatives of primary amino acids

Key examples: Almotriptan, eletriptan, frovatriptan, naratritan, etc.

Solubility in water: soluble in water

Mechanism of action: Acts as agonists for serotonin 5-HT_{1B} and 5-HT_{1D} Receptors

6. **Thiazide diuretics**

Structural feature: Bicyclic heterocyclic benzene derivative containing nitrogens and one sulfur

Key examples: Chlorothiazide (diuril), chlorthalidone, hydrochlorothiazide (microzide), etc.

Solubility in water: Slightly soluble in water

Mechanism of action: Inhibit reabsorption of sodium and chloride ions from distal convoluted tubules in the kidney by blocking the thiazide-sensitve NA^+ Cl^- symporter.

7. **Benzodiazepines**

Structural feature: Fusion of benzene and diazepine rings

Key examples: Pyschoactive drugs: diazepam (Valium), midazolam, etc.

Solubility in water: General poor water solubility. Some members like prodrugs of diazepam and midazolem

Mechanism of action: Boost the efect of the neurotransmitter gamma

-aminobutyric acid; cause anterograde amnesia and dissociation.

Emerging trends in pharmaceutical research

Antibiotics are substances produced by microorganisms that can inhibit the growth or life of other microbes. Many marine organisms are known to have antibiotic properties, but only a small fraction of them have been tested for their potential medical uses. Bacteria in the marine environment produce bioactive substances to protect themselves from predators, and researchers have isolated bacteria with antibacterial properties from different water samples. Marine microbes have become increasingly important for the discovery of new microbial products with properties such as antibacterial, antiviral, antitumor, anticoagulant, and cardioactive effects. However, the overuse and misuse of antibiotics have led to the emergence of bacteria that are resistant to multiple drugs, which is now a global health problem. Additionally, no new chemical class of antibiotics has been discovered in the past 20 years, and modifying existing antibiotics can lead to bacteria developing resistance mechanisms against them.

For the past thirty years, research on the impact of chemical pollution on the environment has primarily focused on conventional "priority" pollutants, while a group of chemicals with the potential to cause harm - Active Pharmaceutical Substances (APS) - have received relatively little attention, particularly in developing countries. APS encompass a wide range of chemical compounds, including prescription drugs, over-the-counter drugs, veterinary drugs, and excipients used in pharmaceutical manufacturing, totaling over three thousand substances. **Oliver et al. (2003), Halling-Sørensen et al. (1997), Najat et al. (2010)** have all highlighted the issue of APS as

potential environmental pollutants **(EPA, 2010; Daughton and Ternes, 1999)**.Pharmaceuticals are continuously released into the environment through various sources, such as human activities, hospitals, and agribusinesses. Illicit drug use, veterinary drug use (specifically antibiotics and steroids), and the pharmaceutical industry also contribute to this. Since these drugs are intended to have a biological effect at certain doses, there is a possibility of unintended consequences at lower concentrations, especially with drugs like antineoplastics **(USEPA, 2002b; Daughton and Ternes, 1999; Erickson, 2002).** Certain

pharmaceuticals can disrupt the endocrine system and lead to negative reproductive and developmental effects, including reduced fertility, cancers, and other illnesses, in humans, animals, and wildlife. Exposure to a mixture of compounds can result in unknown health consequences. A single water sample has been found to contain as many as 38 organic wastewater compounds. Several countries, including Malaysia, Canada, Switzerland, England, USA, and Italy, have data on pharmaceutical residues in various water samples, but as of the time of this study, such information is not available in Nigeria **(Najat et al., 2010; Ternes et al., 2002; Golet et al., 2002; Buser et al., 1998; Ashton et al., 2004; Kolpin et al., 2002).** In Nigeria, commonly used drugs include analgesics, antibiotics, antimalarials, and antihypertensives. Synthetic steroids are also prescribed frequently, but the total amount sold annually is relatively low. According to **Akande and Ologe (2007), Oshikoya and Ojo (2007), Nwolisa et al. (2006),** and Odusanya (2005), the amount of pharmaceutical compounds found in water depends on how much is consumed and released into the environment. **Tauxe-Wuersch et al. (2005)** also support this claim.

Biological impacts in marine organisms

Marine ecotoxicology studies

Research on the effects of both human and veterinary pharmaceuticals on aquatic environments is increasing, but there is limited information available on the toxicity of these drugs to marine organisms. Only one study **Gaw et al., (2014)** has reported field ecotoxicity data for marine organisms, which found that exposure to the antimicrobial tylosin in sediment reduced microalgal biomass and primary productivity, as well as delayed diatom growth in benthic microalgal communities in the North Inlet Estuary in the USA. Laboratory ecotoxicity data is available for 22 compounds, but for most of these compounds, only one or two studies have been conducted on marine organisms. Out of the 20 drugs most commonly found in seawater, only seven have laboratory data available on their ecotoxicity, with fluoxetine being the exception and having data reported in seven studies. This highlights a divide between researchers studying environmental presence and those studying ecotoxicity. Additionally, only one study has examined the toxicity of these drugs to organisms living in sediment. The range of marine organisms tested so far has been limited to primary producers such as microalgae and diatoms, primary consumers such as bivalve mollusks and copepods, and consumers such as crustaceans and fish. The use of nominal rather than measured pharmaceutical exposure concentrations in most studies is a cause for concern. Ecotoxicology is concerned with understanding the impact of human activities that release pollutants into the environment, and how they can harm individual organisms, populations, and the biosphere as a whole. Pharmaceuticals are designed to trigger biological responses in target organisms but their chemical stability makes them difficult to degrade in the environment, leading to unintended consequences for non-target organisms and increasing concerns about water safety. Exposure to trace concentrations of persistent and accumulative pharmaceuticals over a long period can

result in unpredictable and undesirable outcomes. Pharmaceutical contaminants can affect both target and non-target organisms, including macro and microbiomes and fauna, with potentially harmful consequences. Despite being one of the most studied classes of contaminants in ecotoxicology, the impact of pharmaceutical contaminants on non-target aquatic organisms is still not well understood. Moreover, pharmaceuticals can undergo transformations that result in the formation of metabolites or by-products that may be more toxic than the parent molecule, further affecting non-target organisms. Studies have shown that exposure to various environmental contaminants can have significant effects on the microbiome of aquatic environments, as well as organisms at higher levels in the biological hierarchy, leading to changes in behavior that can impact evolutionary and ecological processes. It is important to recognize that pharmaceuticals, even at low concentrations, can be environmental stressors with significant consequences for aquatic organisms.

Methods to detect pharmaceutical compounds

Advancements in detection technology and analytical methods have led to an increase in reported detections of pharmaceuticals in low concentrations across various environmental matrices, including surface and groundwater, treated wastewater, and drinking water. Techniques such as gas chromatography with mass spectrometry (GC-MS) or tandem mass spectrometry (GC-MS/MS) and liquid chromatography with mass spectrometry (LC MS) or tandem mass spectrometry (LCMS/MS) are capable of detecting target compounds at levels as low as nanograms per liter, and are commonly used for detecting pharmaceuticals in water and wastewater **(Fatta et.al., 2007)**

The choice of method used depends on the physical and chemical properties of the target compound. LC-MS/MS is the preferred method for measuring target compounds that are more polar and highly soluble in water. Technological advancements have

enabled the detection of very low concentrations of pharmaceuticals in various environmental matrices, including water, using sensitive and accurate detection equipment and analytical methods such as GC-MS/MS and LC-MS/MS. However, it is essential to understand that detecting these compounds does not directly imply potential human health risks, which can only be evaluated using available human risk assessment methods. Furthermore, there is currently no standardized practice or protocol for sampling and analyzing pharmaceuticals in environmental media, which may affect the comparability and quality of data generated.

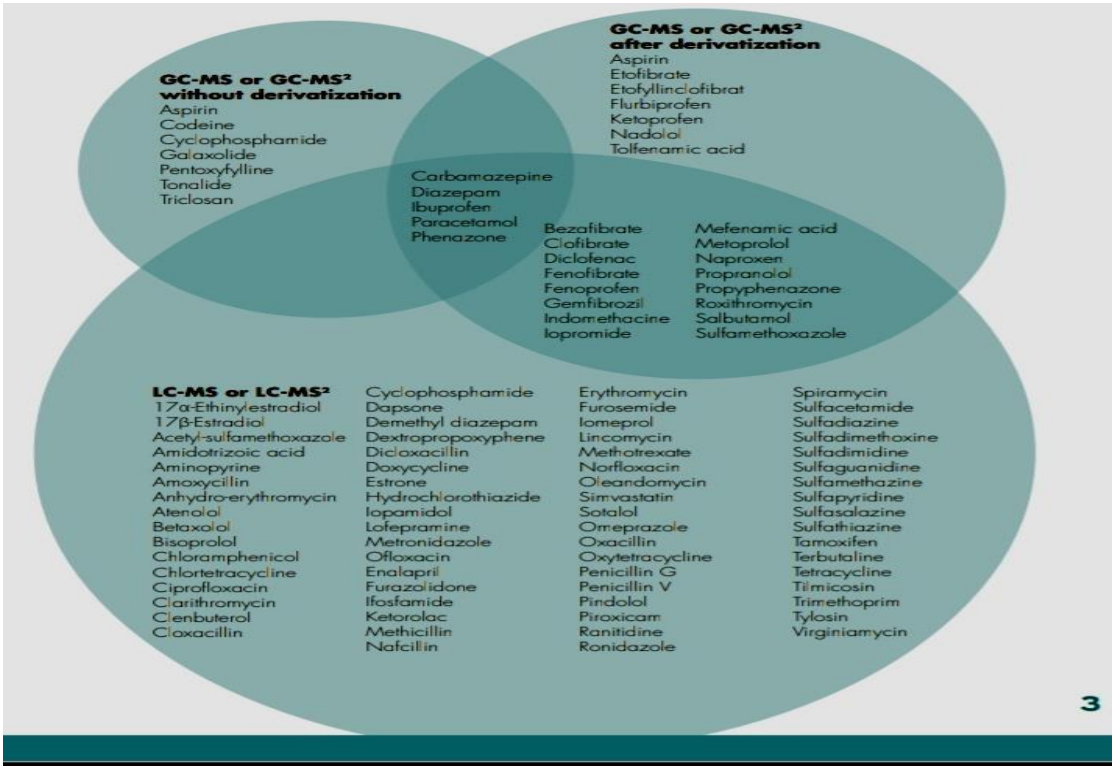

Fig 1: An illustration of analytical methods applied to detect pharmaceuticals in water and wastewater (adapted from **Fatta et.al., 2007**)

1.2 AIM AND OBJECTIVES

The presence of marine bacteria has been acknowledged for a long time, and it was confirmed to exist even in the Gulf of Naples at the depths exceeding 1000 meters **(Russell 1892).** Marine bacteria are a primary food source for many small aquatic animals, such as copepods and krill. These small organisms are in turn preyed upon by larger marine animals, which form the basis of the marine food web. The increasing and unregulated usage of pharmaceutical substances has emerged as a relevant environmental issue **(Dietrich et.al., 2002).** Considering the relevance of bacteria in the marine environment and the pharmaceutical compounds that are released into it, it would be interesting to study the effect of the pharmaceutical compounds on marine bacteria from the coastal environment.

AIM: To study the consequences of pharmaceutical compounds on marine bacteria.

OBJECTIVES:

- To isolate bacterial cultures from different coastal waters of Goa.
- To study the effect of pharmaceutical compounds on marine bacteria.

1.3 LITERATURE REVIEW

(Ferrari et.al., 2003) The study gathered data on the environmental impact and toxicity of six pharmaceuticals from various sources, including experiments and literature. They tested the European guideline on the environmental risk assessment of pharmaceuticals using measured concentrations in effluents and surface waters in France and Germany. All concentrations exceeded the cutoff value, requiring a second-tier assessment. However, the acute toxicity of the pharmaceuticals was not severe, and two of them were incorrectly identified as having no risks. The study highlights the

importance of appropriate ecotoxicity tests and raises concerns about the current European draft procedure on pharmaceuticals.

(Ferrari et.al., 2002) A research was carried out in four countries namely France, Greece, Italy and Sweden to study the occurrence and harmful impact of three pharmaceuticals namely carbamazepine, clofibric acid and diclofenac in sewage treatment plant (STP) effluents. The scientists conducted bioassays on different organisms to determine their predicted no-effect concentrations (PNEC) and evaluate the danger of exposure to these pharmaceuticals. The findings revealed that carbamazepine was the most dangerous compound based on PNEC values. While all three pharmaceuticals were present in the effluents, only carbamazepine was discovered in all the sewage treatment plants and in the highest concentrations. Moreover, the risk quotient calculations revealed that only carbamazepine was a threat to the water compartment.

(Devi et.al., 2010) Scientists conducted a study in Tamil Nadu, collecting water samples from Thiruchendur, Thoothukudi, and Kanyakumari coasts. They used marine agar to isolate 21 bacterial strains and screened them for antimicrobial properties against organisms like Salmonella typhi and Staphylococcus aureus. Out of these strains, six were found to be effective against the target organisms. The researchers identified two strains, Alteromonas sp. and Rhodopseudomonas sp., which showed antagonistic activity against all the test organisms in their cell-free extracts. The active compounds in these extracts were analyzed using Nuclear Magnetic Spectroscopy, revealing one compound similar to Drechslerine A and the other similar to cis-Sativenediol. The marine environment holds many mysteries yet to be uncovered, and this study highlights its potential as a source of valuable knowledge.

(**Mezzelani et.al., 2018)** Pharmaceuticals are increasingly being released into natural environments, particularly through wastewater treatment plants, which poses a significant threat to aquatic ecosystems. Even low concentrations of these compounds have the potential to harm aquatic species throughout their entire life cycle. While research has been conducted on the effects of pharmaceuticals on nontarget organisms in freshwater ecosystems, our knowledge on marine ecosystems and the majority of the 4000 classified pharmaceuticals is limited, and urgent prioritization of sustainability efforts is required. This review aims to summarize the harmful effects of various classes of pharmaceuticals on marine species exposed to them in both field and laboratory conditions. However, our understanding of the behavior of pharmaceuticals in chemical mixtures and their interactions with other environmental stressors is limited, and complex ecotoxicological effects are increasingly being documented. Therefore, multidisciplinary, integrated approaches are necessary to clarify the environmental hazards of these emerging pollutants in the marine environment.

(Almeida et.al., 2021) This chapter focuses on the impacts of pharmaceutical drugs on marine organisms, specifically in the presence of climate change-related factors such as temperature, pH, and salinity. There is currently limited research on this topic, but it is important to understand how these stressors can affect the toxicity of drugs and the sensitivity of marine organisms. Studies have shown that the combined effects of drugs and climate change-related stressors can lead to more severe responses than either stressor alone. However, more research is needed under realistic environmental conditions, including longer exposure tests and examination of individual and subindividual parameters, to better understand the effects of pharmaceutical drugs on marine organisms under actual and predicted climate change scenarios.

(Williams 2009) Marine bacteria are being recognized as an essential resource for discovering new therapeutic agents, particularly in the field of oncology and infectious diseases. Salinosporamide A and bryostatin are two examples of effective anti-cancer agents derived from marine bacteria, but there are numerous others yet to be explored. If utilized correctly, marine bacteria can be an essential source of drugs to fight against drug-resistant infectious diseases in the coming decades. This assessment focuses on the most recent compounds obtained from Actinobacteria and Cyanobacteria, which exhibit potential as therapeutic agents, mainly in oncology drug discovery programs. Even though the FDA has not yet approved any drugs from marine bacteria, the significant number of promising compounds currently in development indicates that this situation may change in the future. For a complete inventory of all marine bacterial compounds, refer to the comprehensive list.

(Nunes et.al.,2014) A study was conducted to evaluate the impact of paracetamol on various freshwater organisms using standardized assays. The results indicate that the toxicity of paracetamol differs significantly among species, even those that are closely related. Additionally, unpredictable physiological factors can affect the extent of paracetamol toxicity, making comparisons between species difficult. While paracetamol was found to be toxic to most of the test organisms at varying concentrations*, L. gibba* was found to be resistant to its effects at concentrations up to 1000 mg l−1. The toxicity ranking of the species tested, based on EC50 values, was as follows: *D. magna<D. longispina<V. fisheri<C. raciborskii<P. subcapitata<L. minor<L. gibba*, with daphnids being the most sensitive organisms to the pharmaceutical.

CHAPTER II

MATERIALS AND METHODS

2.1 STUDY SITE AND SAMPLING STRATEGIES

The first study site was Caranzalem beach, located to the west of Miramar beach along the mouth of the Mandovi river and is 3.5 km long which has stretches of white sand and clear water. It lies between the coordinates 15.4665Ñ and 73.8048Ê. The second study site was Vagator beach, the northernmost beach of Bardez Taluka, Goa. It is across the Chapora river from Morjim in Pernem and has black cliff rocks looking down on the shore. It lies between the coordinates 15º36’09”N and 73º44’01”E. The third study site was Anjuna beach located 18 km from Panaji and 8 km

to the west of Mapusa North Goa, the beach is a part of 30 km stretch of extended beach coastline along the west coast Goa by the Arabian Sea and lies between the coordinates 15.5833Ñ and 73.7333Ê.

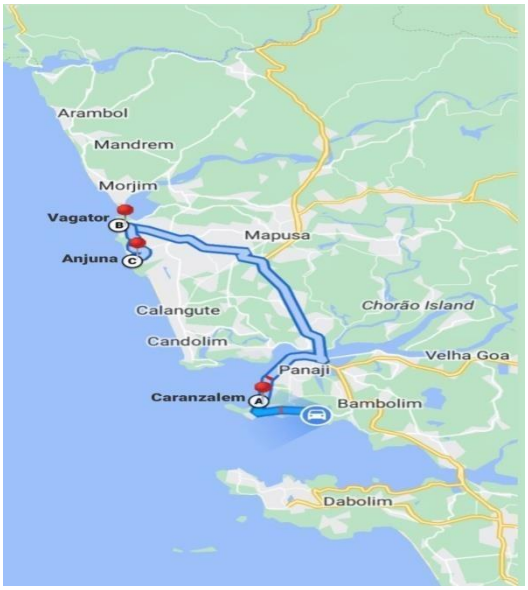

Fig 2: Geographical locations of (A) Caranzalem beach (B) Vagator beach and (C) Anjuna beach

Seawater sample from polluted areas of Caranzalem beach, Vagator beach and Anjuna beach was collected in sterile 50 ml centrifuge tubes. The centrifuge tubes was first rinsed with seawater from the site before collection of the sample. The samples were brought to the experimental site (laboratory) in an icebox to preserve the samples well. It was kept in the refrigerator till further experiments were performed within 24 hours of sampling. Proper care was followed while collecting the seawater sample in order to avoid contamination.

Analysis of Viable Count

A viable count is used to measure the number of cells of the sample that is able to form colonies on a suitable agar medium, each viable cell can yield one colony. For this particular reason viable count is also called as the colony count or the plate count. There are three ways of performing a colony count: the spread plate method, pour

plate method and membrane filtration. This experiment was performed using the spread plate technique. Spread plates are prepared by pipetting a volume of sample onto the Nutrient agar (NA) plates. Once growth is observed, the colonies are counted and viable count is calculated using the formula: CFU/ml = [(No of colonies) × (dilution factor)] ÷ (Volume of culture plated in ml)

Viable Count analysis involves serial dilution followed by spread plating or pour plating.

Serial Dilution: Serial dilution **(Koch, 1883)** techniques are routinely used in microbiology laboratories **(American Public Health, 2005; Hollinger, 1993; Taswell, 1984; Lin and Stephenson. 1998)** for microbes that grow on bacteriological media and develop into colonies. The objective off the serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria or viruses) of an unknown sample by counting the number of colonies cultured from serial dilutions of the sample.

Spread Plate Method: A method where bacteria is evenly distributed over the surface of an agar media is referred to as spread plate method. A small volume of bacterial suspension is spread all over the agar surface using sterile bent glass rod. The aim is to permit the growth of colonies and each plate is spread with a single inoculum of the bacterial suspension.

Methodology:

The viable count of all 3 samples (from Caranzalem, Vagator and Anjuna) was carried out. All the apparatus required in this experiment were autoclaved first and kept in the oven for drying (60℃). Labelling was done prior. Sterile saline was prepared (0.85% NaCl) according to the calculations and was autoclaved. Dilutions (10^{-1}, 10^{-2}, 10^{-3}) were done. 10^{0} is the water sample (from the centrifuge tube). In 10^{-1} , 10^{-2} and 10

$^{-3}$ tubes, 4.5 ml sterile saline was added. 0.5 ml of the water sample (10^{0}) was transferred into 10^{-1} and mixed well. After this, 0.5 ml of the mixture from 10^{-1} was transferred to 10^{-2} and similarly, from 10^{-2} to 10^{-3} and mixed well.

Nutrient agar (NA) was prepared according to the calculations done and was autoclaved. NA was then poured in the sterile petri plates (approx 15 ml) and was left to solidify at Room temperature for 24 hours. After 24 hours, 0.1 ml of inoculum from 10^{-1} ,10^{-2} ,10^{-3} was spreadplated onto each of 3 separate sterile petri plates. A glass rod dipped into the alcohol was flamed and was used to spread plate the bacterial suspension all over the agar surface. The plates were then incubated at room temperature for 24 hours. Colonies observed after 24 hours on the three plates, were counted, and viable count calculated based on the formula mentioned above.

Colony characteristics; Colonial characteristics is basically checking the qualities of the colonies that are formed. Colonies should be well isolated in order to determine the characteristics shape, size, colour, surface appearance and texture. The colonies observed on the media were characterized in terms of size (large, medium, small or pinpoint), surface appearance (smooth, rough, wrinkled or mucoid), colour (white, pink, yellow, orange etc). It should be noted that 24 hour old cultures were used for analysis of colony characteristics.

Isolation and purification of bacterial cultures.

The colonies obtained were isolated by streaking on NA followed by subculturing on NA slants. The slants were stored at 4^{o}C till further use.

Streak plate method: The main aim of streak plate method is to obtain isolated colonies. When culture media is inoculated using a single isolated colony, the resulting culture grows from that single clone. One bacterial cell will create a colony as it

multiplies. The streak process is supposed to create a region where the bacteria are so dilute that when each bacterium touches the surface of the agar it is far away from the other cells so that an isolated colony can develop. Streaking techniques are of many types (T streaking, continuous streaking and quadrant streaking). Quadrant streaking method is more commonly used. Streaking is done on sterile agar medium using a nichrome loop.

Nutrient agar plates were prepared as per the calculations and were kept for 24 hours at RT for it to settle down completely. Nichrome loop was flamed and was allowed to cool. A well developed colony from the master plate was taken and was streaked using quadrant streaking method on the new sterile NA plate. This same method was repeated for the other well developed colonies. The streaked plates were than incubated at room temperature for 24 hours and growth was observed. After the first streaking technique, the next step was to subculture the plates to obtain pure cultured.

Subculturing: Subculturing is also known as picking off technique. The objectives of this technique is to obtain pure cultures from a mixed culture, to prevent contamination of cultures and to increase the life span of the cells. After incubation of streaked plates, now the further step is to subculture some colonies to a fresh medium using a nichrome loop. Each of these new cultures represents the growth of a single species and is called pure stock or cultures.

Nutrient agar plates were prepared according to the calculations and was kept to solidify for 24 hours at room temperature. Nichrome loop was flamed and was allowed to cool. The agar streak plate colony was picked using the nichrome loop and was streaked again on the newly prepared NA plates using quadrant streaking method. Subculturing was done 3 times using the same protocol until pure cultures were obtained.

Streaking on slants: Slant streaking was done using the subcultured plates. All the required apparatus were previously washed and autoclaved. Nutrient agar (NA) was prepared as per the calculations and was autoclaved. Approximately 10 ml of NA was poured in to the test tubes and the test tubes were later kept in a slanting position and were allowed to solidify for 24 hours. Using a nichrome loop, the colonies were streaked on the slants from the subcultured plates, and incubated for 24 hours at room temperature. Growth was observed after 24 hours of incubation at RT.

Characterisation of the cultures:

Gram staining

Gram staining is a laboratory technique used to differentiate bacterial species into two groups: gram-positive and gram-negative. The process involves applying a crystal violet stain, followed by a iodine solution to fix the stain in place, then rinsing with alcohol or acetone to decolorize the cells, and finally counterstaining with safranin. Gram-positive bacteria retain the crystal violet stain and appear purple under a microscope, while gram-negative bacteria lose the stain and appear pink or red. Gram staining is a useful diagnostic tool in microbiology and is commonly used to identify and classify bacterial species.

A 24 hour old inoculated culture was used for gram staining. On a clean grease free slide, loopful of saline and loopful of culture was added using a nichrome loop and a smear was made. Slide was air dried and heat fixed. Slide was stained with crystal violet for 1 minute. Stain was discarded. Slide was flooded with grams iodine for 1 minute. Decolourizer was added till the blue colour stopped flowing. The slide was gently washed with water. Counter stained with safranin for 2 minutes. The stain was discarded, gently washed and air dried completely. The slide was observed under oil immersion lens under 100x.

Biochemical tests

Catalase test

Biochemical tests are laboratory techniques used to identify and quantify various biochemical molecules in biological samples. These tests are widely used in microbiology laboratories. There are numerous biochemical tests available, each with its own set of protocols and procedures.

Catalase test: The catalase test is a technique to identify bacteria which produce the enzyme catalase. Catalase is an enzyme that breaks down hydrogen peroxide into water and oxygen. To perform catalase test hydrogen peroxide is added to a bacterial colony. If the bacteria possess catalase, then the addition of hydrogen peroxide will result in the release of oxygen gas bubbles which is an indication of a positive test. Conversely, if no bubbles are observed, it means that the bacteria do not produce catalase, which results in a negative test.

24 hour old bacterial cultures inoculated in Nutrient broth were used for this test. A loopful of culture was taken from the 24 hour old culture tubes and was streaked onto the slants. Slants were then incubated at room temperature for 24 hours for growth. After the growth, 2-3 drops of Hydrogen peroxide was added to the slants and checked for effervescence which indicated a positive test. Results were noted in

(+) or (-).

EXPERIMENTS: TO DETERMINE THE EFFECT OF PHARMACEUTICAL COMPOUNDS ON COASTAL MARINE BACTERIA

For the main experiment to determine the effect of pharmaceutical compounds on coastal marine bacteria, 3 categories of pharmaceutical drugs were selected based on their solubility in water: Cataspa (period pain tablet), Ondem (to control nausea and vomiting) and Paracetamol (headache tablet).

Table 1: Pharmaceutical drugs used and it's composition.

Pharmaceutical drug	Composition	Concentration used (mg/ml)
Paracetamol	500 mg	100
Cataspa	Diclofenac potassium 50 mg Dicyclomine hydrochloride 20 mg Total 70 mg	35
Ondem	4 mg	4

Protocol

(This same protocol was used for all the three pharmaceutical drugs but had different calculations and the experiments were performed separately)

In Experimental tubes : Culture + compound + Nutrient broth (9 tubes)×2 replicates

In Negative control : Compound + Nutrient broth (1 tube)×2 replicates

In Positive control : Culture + Nutrient broth (9 tubes)×2 replicates

Day 0 (Inoculation): A loopful of culture which were prepared and kept in the refrigerator was added to sterile Nutrient Broth tubes using a nichrome loop. The tubes were incubated at room temperature for 24 hours. After 24 hours growth was checked at 620 nm, using a colorimeter, to ensure their growth. These 24 hr old cultures were used for the experiments.

Day 1: For experimental tubes, addition of 100 ul of 24 hour old culture to sterile Nutrient Broth tubes containing the respective pharmaceutical compounds was done using pipette. The tubes were incubated at room temperature for 24 hours.

Day 2: The OD at 620 nm was checked using a calorimeter. Similarly, for the negative control and positive control tubes, OD was checked in a similar manner.

Fig 3: Experimental set up and working.

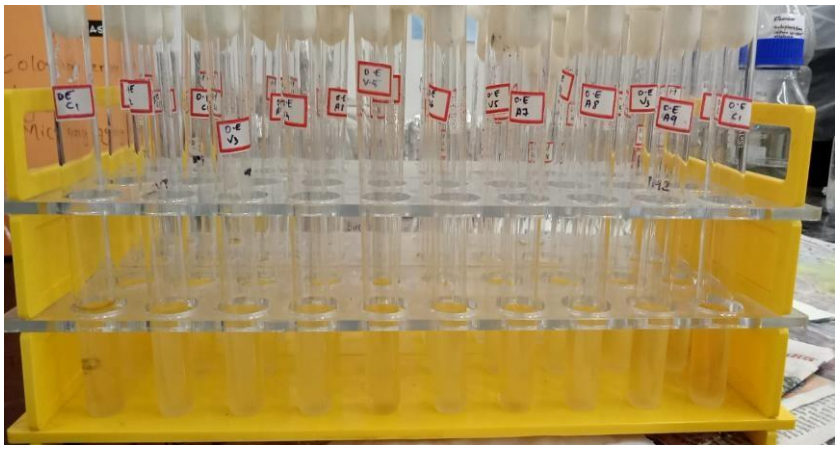

Fig 4 : Test tubes containing pharmaceutical compound, cultures and nutrient broth.

CHAPTER |||

RESULTS AND DISCUSSION

Viable count

Viable count of the seawater sample from each stations was studied.

The viable count of bacteria ranged from 25.15×10^3 to 44.3×10^2 (CFU/ml) in the 3 study areas (Caranzalem, Vagator and Anjuna). A total of 9 cultures were isolated (details in Fig. 5 and Table. 3,4 & 5).

Stations and number of cultures

Table 2: Number of cultures isolated from each stations.

Stations	**Number of cultures**
Caranzalem	2
Vagator	3
Anjuna	4

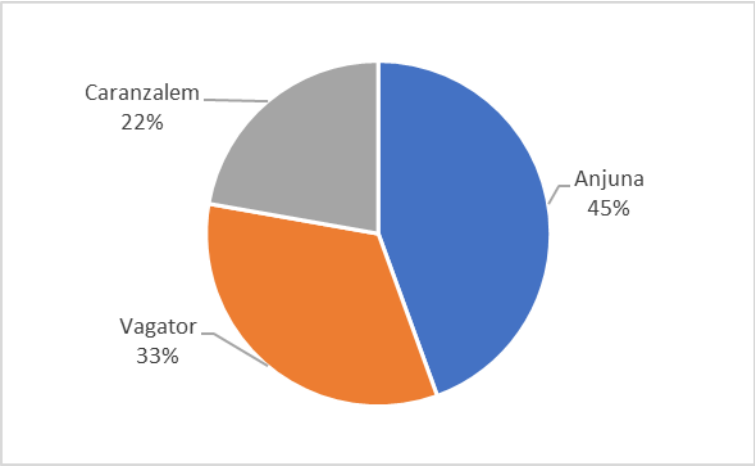

Fig no 5 : Distribution of bacterial isolates with respect to their place of isolation.

This pie chart depicts the number of cultures isolated from each study area in %.

More number of cultures were isolated from Anjuna 45% and Vagator 33% .

Caranzalem with 22% was the least (Fig.5).

Colony characteristics

Morphological characteristics of bacterial isolates from 3 different study areas.

Table 3 : Characteristics of bacterial isolates from **Caranzalem**.

Dilutions and Culture codes	10^{-1} (C1)	10^{-2}	10^{-3} (C2)
Media	Nutrient agar		Nutrient agar
Time	24 hours		24 hours
Temperature	RT		RT
Shape	Round	Matt growth	Round
Colour	White		White and Yellow
Elevation	Flat		Flat
Margin	Entire		Entire
Surface	Smooth		Smooth

Table 4: Characteristics of bacterial isolates from **Vagator**.

Dilutions and Culture codes	10^{-1} (V3)(V4)(V5)	10^{-2}	10^{-3}
Media	Nutrient agar	Nutrient agar	
Time	24 hours	24 hours	
Temperature	37°C	37°C	
Shape	Round	Round	No growth
Colour	White and yellow	Yellow	
Elevation	Flat	Flat	
Margin	Entire	Entire	
Surface	Smooth	Smooth	

Table 5: Characteristics of bacterial isolates from **Anjuna**.

Dilutions and cultures codes	10^{-1} (A7)(A8)	10^{-2} (A6)	10^{-3} (A9)
Media	Nutrient agar	Nutrient agar	Nutrient agar
Time	24 hours	24 hours	24 hours
Temperature	37°C	37°C	37°C
Shape	Round	Round	Round
Colour	White	White and Yellow	White
Elevation	Flat	Flat	Flat
Margin	Entire	Entire	Entire
Surface	Smooth	Smooth	Smooth

Gram Staining

Table 6: Gram staining and catalase test results of the bacterial isolates from different seawater samples.

Study area	Cultures	Gram character	Shape	Catalase test
Caranzalem	C1	Gram positive bacteria	Bacilli	Positive
	C2	Gram positive bacteria	Bacilli	Positive
Vagator	V3	Gram positive bacteria	Bacilli	Positive
	V4	Gram positive bacteria	Bacilli	Positive

	V5	Gram positive bacteria	Bacilli	Positive
Anjuna	A6	Gram negative bacteria	Cocci	Positive
	A7	Gram negative bacteria	Cocci	Positive
	A8	Gram positive bacteria	Bacilli	Positive
	A9	Gram positive bacteria	Bacilli	Positive

Gram staining revealed that cultures C1, C2, V3, V4, V5, A8 and A9 were Gram positive bacilli whereas, cultures A6 and A7 were Gram negative cocci. Catalase test was positive for all the cultures (Table 6).

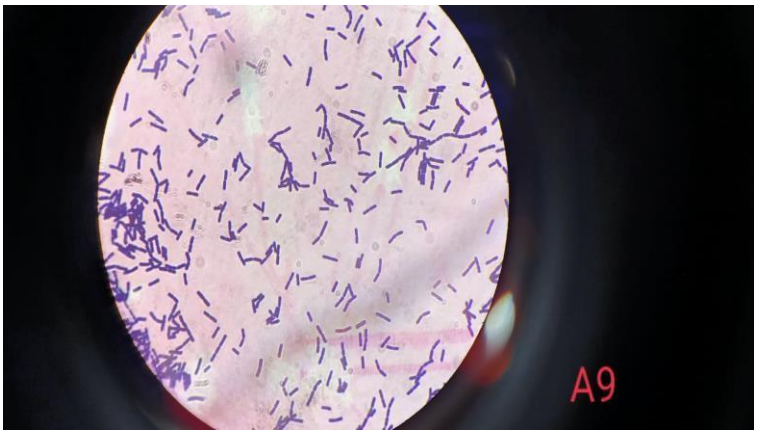

Fig 6 : Gram positive bacilli, culture A9.

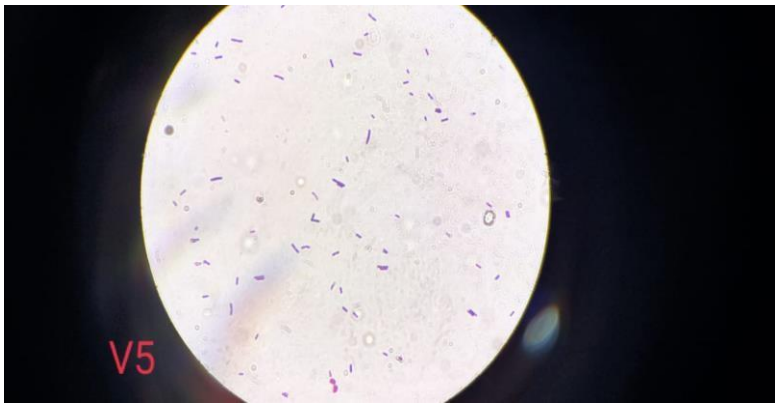

Fig 7 : Gram positive bacilli, culture V5.

OD at 620 nm of bacterial isolates.

Table 7: OD at 620 nm of bacterial isolates grown in Nutrient Broth at RT for 24 hours.

Cultures	**OD at 620 nm**
C1	0.13
C2	0.22
V3	0.18
V4	0.21
V5	0.32
A6	0.36
A7	0.31
A8	0.27
A9	0.24

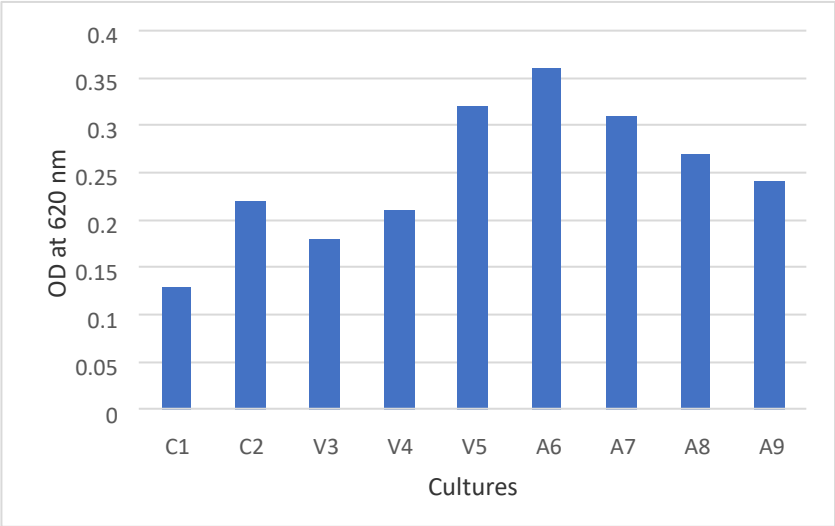

Fig 8 : Bacterial isolates and its OD at 620 nm

The growth of bacterial isolates (in terms of OD at 620 nm) ranged from 0.13 to 0.36 (Fig 8). These cultures were used as inoculum for the subsequent experiments.

Experiments:

To determine the effect of pharmaceutical compounds on coastal bacteria

1) **Paracetamol**

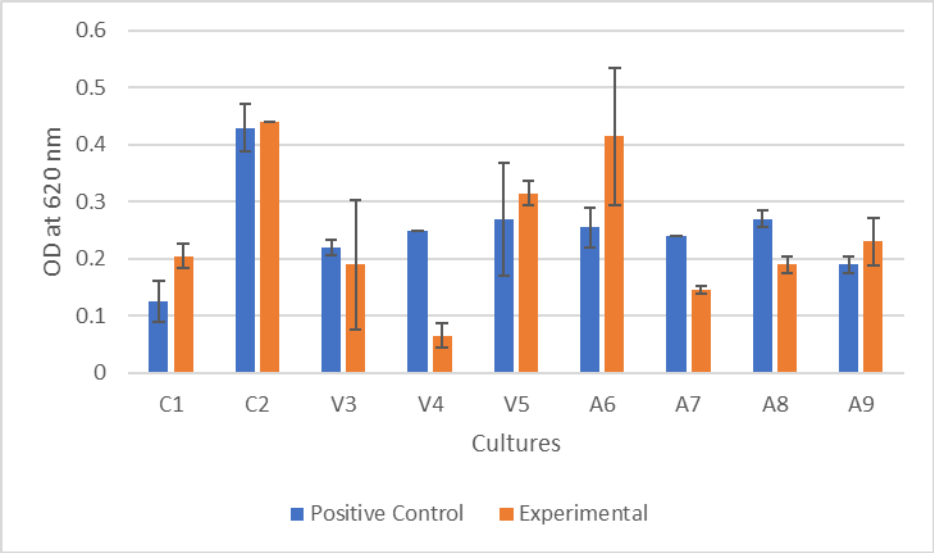

Fig 9: Effect of Paracetamol on growth of bacterial isolates, compared to control. This graph shows comparison between Positive control and experimental and also the effect on Paracetamol on bacteria. Maximum reduction of growth was seen in cultures V4, A7 and A8 whereas, minimum reduction was observed in C1, V3, V5 and A9. Cultures C2 and A6 showed enhancement in growth (Fig 9).

2) **Ondem**

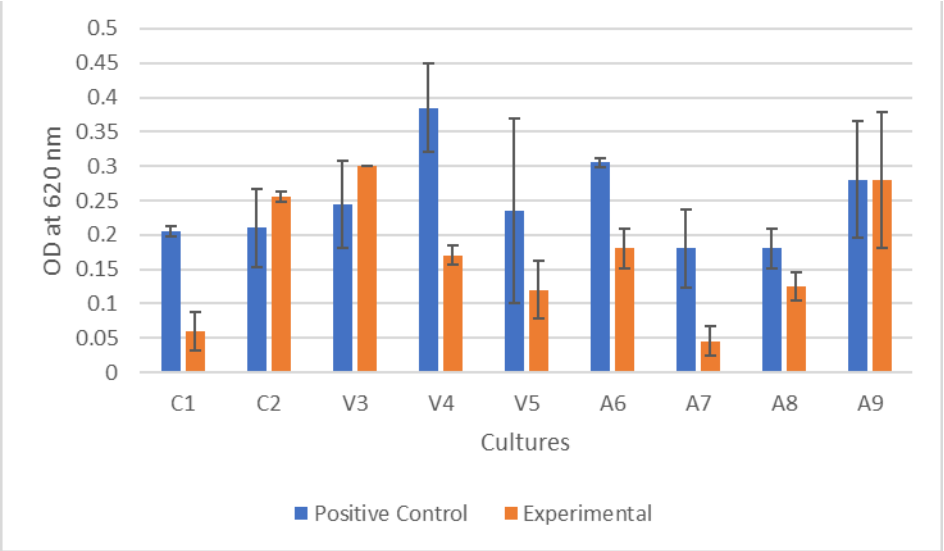

Fig 10: Effect of Ondem on growth of total culturable bacteria, compared to control.

This graph shows comparison between Positive control and experimental and also the effect on Ondem on bacteria. Maximum reduction of growth was seen in cultures C1, A7 and V5 whereas, minimum reduction was observed in V4, A6 and A8.

Cultures C2, V3 and A9 showed enhancement in growth (Fig 10).

3) **Cataspa**

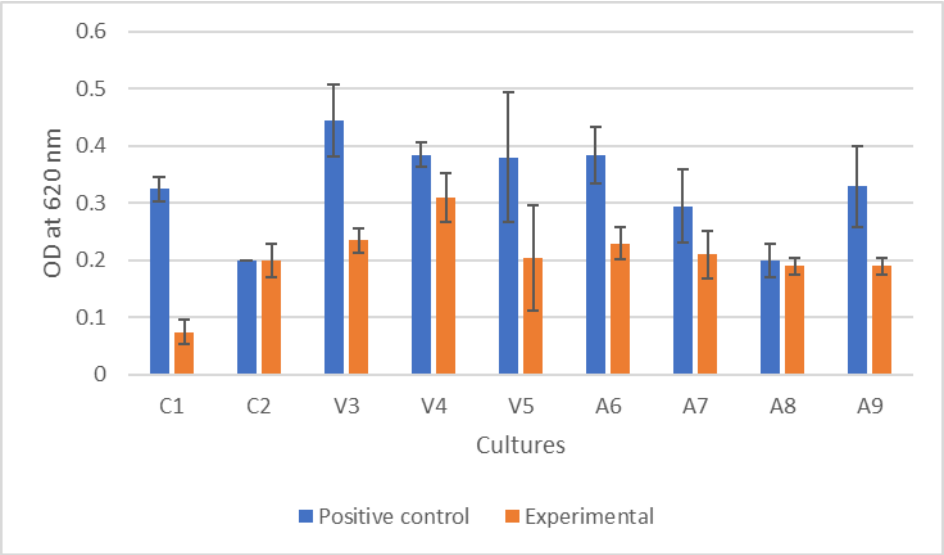

Fig 11: Effect of Cataspa on growth of total culturable bacteria, compared to control. This graph shows comparison between Positive control and experimental and also the effect on cataspa on bacteria. Maximum reduction of growth was seen in culture C1, A8 and A9 whereas, minimum reduction was observed in C2, V3, V5, A7. Cultures V4 and A6 showed enhancement in growth (Fig 11).

Discussion

Pharmaceutical compounds are found in many water bodies, primarily as a result of their use by humans and their subsequent discharge into waterways. These

compounds enter the marine environment through various pathways. These compounds can have a negative impact on the marine microorganisms such as bacteria. In our study the pharmaceutical compound paracetamol was not toxic to all the bacterial isolates however in some cultures it had shown much reduction in growth ,compared to positive control. In a study conducted by **(Nunes et.al.,2014)** the results indicated that the toxicity of Paracetamol varied significantly among the species eventhough they were closely related. They found out that paracetamol was toxic to all the test organisms except for one *L. gibba*. No effects were found on this species even at higher concentrations.

CHAPTER |V
SUMMARY AND CONCLUSIONS

SUMMARY

The study of Pharmaceutical compounds and its effects on marine bacteria was the topic selected. It was important because marine bacteria are important for maintaining the health and functioning of marine ecosystems and have a wide range of potential applications in various industries. I wanted to study the effect of pharmaceutical substances on coastal bacteria to better understand their potential risks and benefits. To experimentally understand this, seawater samples from 3 different stations Caranzalem, Vagator and Anjuna, Goa-India were collected. Viable count analysis which involves serial dilutions and spread plating was done, after which the colony

characteristics of the same were studied in terms of size, shape, colour, surface, margin and elevation. The colonies obtained were isolated by streaking on Nutrient agar (NA) followed by subculturing on NA slants. The slants were stored at 4°C till further use. OD at 620 nm of the cultures was taken after few days of isolation in order to ensure the growth of the cultures. It was observed that culture A6 had maximum growth, culture C1 was minimum and the growth of bacterial isolates (in terms of OD at 620 nm) ranged from 0.13 to 0.36. Gram staining and biochemical characteristics (Catalase test) of the cultures were also observed. Gram staining revealed that cultures C1, C2, V3, V4, V5, A8 and A9 were Gram positive bacilli whereas, cultures A6 and A7 were Gram negative cocci. Catalase test was positive for all the cultures. To test the effect of pharmaceuticals on coastal bacteria, three pharmaceutical compounds, that were water-soluble, were selected (Paracetamol, Ondem and Cataspa). Experiments were conducted separately for each compound in replicates (positive control, negative control and experimental). OD at 620 nm was taken for all the cultures and noted down. It was observed that for Paracetamol the maximum reduction of growth was seen in cultures V4, A7 and

A8 whereas, minimum reduction was observed in C1, V3, V5 and A9. Cultures C2 and A6 showed enhancement in growth. In Ondem maximum reduction of growth was seen in cultures C1, A7 and V5 whereas, minimum reduction was observed in V4, A6 and A8. Cultures C2, V3 and A9 showed enhancement in growth and for Cataspa it was observed that Maximum reduction of growth was seen in culture C1, A8 and A9 whereas, minimum reduction was observed in C2, V3, V5, A7. Cultures V4 and A6 showed enhancement in growth. It was noted that in all the three pharmaceutical compounds cultures C1 and A7 showed much inhibition in growth as compared to other cultures. The effect of pharmaceutical compounds was different in each cultures.

CHAPTER V
APPENDIX

MEDIA AND COMPOSITIONS

1) Nutrient agar

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Distilled water	1000 ml

2) Nutrient broth

Beef extract	5.0 g
Peptic digest of animal tissue	5.0 g
Sodium chloride	1.50 g
Yeast extract	1.50 g
Distilled water	1000 ml

3) Gram stain

a) Crystal violet (grams/100 ml)

Crystal violet	2.0
Ethyl alcohol (95%)	20 ml
Ammonium oxalate	0.800
Distilled water	80 ml

b) Gram's iodine (grams/300 ml)

Iodine	1.000

Potassium iodide	2.00
Distilled water	300 ml

c) Gram's decolouriser (100 ml)

Ethyl alcohol (95%)	50.0 ml
Acetone	50.0 ml

d) Saffranine (0.5% grams/100 ml)

Saffranine O	0.500
Ethyl alcohol	100 ml

Table 8: Viable count of the seawater sample from different stations

Stations	**Media**	**Dilutions**	**Number of colonies**	**Average**	**Dilution factor (CFU/ml)**
Caranzalem	Nutrient agar	10^{-1}	3	2.515	25.15×10^{3}
		10^{-2}	Matt growth		
		10^{-3}	5		
Vagator	Nutrient agar	10^{-1}	10	1	10×10^{2}
		10^{-2}	1		
		10^{-3}	No growth		
Anjuna	Nutrient agar	10^{-1}	3	13.3	44.3×10^{2}
		10^{-2}	3		
		10^{-3}	1		

1) Paracetamol

Table 9a and 9b: OD at 620 nm of cultures in replicates of positive control and experimental.

a) Positive control

Cultures	Set | (OD at 620 nm)	Set || (OD at 620 nm)
C1	0.10	0.15
C2	0.46	0.40
V3	0.21	0.23
V4	0.25	0.25
V5	0.34	0.20
A6	0.28	0.23
A7	0.24	0.24
A8	0.26	0.28
A9	0.18	0.20

b) Experimental

Cultures	Set | (OD at 620 nm)	Set || (OD at 620 nm)
C1	0.22	0.19
C2	0.44	0.44
V3	0.11	0.27
V4	0.05	0.08
V5	0.33	0.30
A6	0.50	0.33
A7	0.15	0.14
A8	0.18	0.20
A9	0.26	0.20

2) Ondem

Table 10a and 10b: OD at 620 nm of cultures in replicates of positive control and experimental.

a) Positive control

Cultures	Set | (OD at 620 nm)	Set || (OD at 620 nm)
C1	0.20	0.21
C2	0.25	0.17
V3	0.20	0.29
V4	0.34	0.43
V5	0.33	0.14
A6	0.30	0.31
A7	0.22	0.14
A8	0.16	0.20

A9	0.22	0.34

b) Experimental

Cultures	**Set | (OD at 620 nm)**	**Set || (OD at 620 nm)**
C1	0.08	0.04
C2	0.26	0.25
V3	0.30	0.30
V4	0.16	0.18
V5	0.09	0.15
A6	0.16	0.20
A7	0.06	0.03
A8	0.14	0.11
A9	0.35	0.21

3) Cataspa

Table 11a and 11b: OD at 620 nm of cultures in replicates of positive control and experimental.

a) Positive control

Cultures	**Set | (OD at 620 nm)**	**Set || (OD at 620 nm)**
C1	0.34	0.31
C2	0.20	0.20
V3	0.49	0.40
V4	0.37	0.40
V5	0.30	0.46
A6	0.42	0.35
A7	0.25	0.34
A8	0.22	0.18
A9	0.28	0.38

b) Experimental

Cultures	**Set | (OD at 620 nm)**	**Set || (OD at 620 nm)**
C1	0.06	0.09
C2	0.18	0.22
V3	0.22	0.25
V4	0.28	0.34
V5	0.14	0.27
A6	0.21	0.25

A7	0.18	0.24
A8	0.18	0.20
A9	0.18	0.20

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