Antibacterial Effect of Black Cumin Seed (*Nygella sativa*) on the Specific Pathogen of *Vibrio cholerae* Isolated from Dried Fish

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By

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Antibacterial Effect of Black Cumin Seed (*Nygella sativa*) on the Specific Pathogen of *Vibrio cholerae* Isolated from Dried Fish

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DECLARATION

Antibacterial Effect of Black Cumin Seed (*Nygella sativa*) on the Specific Pathogen of *Vibrio cholerae* Isolated from Dried Fish

The results presented in this thesis with the above-mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor is it being concurrently submitted for any degree.

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DEDICATED TO

My Beloved Parents

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Abstract

This study aimed to analyze the antibacterial activity of black cumin seed (*Nigella sativa*) extracts in inhibiting the growth of *Vibrio cholerae* bacteria. This specific human pathogenic bacteria was isolated from three types of commercially valuable dried fish Lottya (*Harpodon nehereus),*Chingri *(Penaeus monodon) and* Churi *(Trichiurus haumela*). The extracts were made in ethanol and acetone solvents separately. The antibacterial activities of the extracts were determined by three different method of enumerating the bacterial load before and after extract treatment, disc diffusion method and tube dilution method. The average loads of the bacteria in Lottya and Chingri were 3.1×10^4 CFU/g and 2.67×10^3 CFU/g respectively while no *V. cholerae* was detected in dried Churi fish. Though both extracts showed strong sensitivity to the isolated bacteria but acetone extract of black cumin was found to be more effective than ethanol extract. The Minimum Inhibitory Concentrations (MIC) value of acetone and ethanol extracts in inhibiting the growth of *V. cholerae* was 25% and 30% respectively. So, black cumin seed extract can be used as an alternative medicine to prevent the enteric disease of consumers associated with dried fish consumption.

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Chapter one

INTRODUCTION

1. Introduction:

Fish and seafood constitute an important food component for a large section of world population. It has become an increasingly important source of protein and other element necessary for the maintenance of and healthy body. Fish and seafood constitute an important food component for a large section of world population. They come after meat and poultry as staple animal protein foods where fish forms a cheap source of protein (Adedeji *et al.*, 2012).

In Bangladesh, Fisheries is the second amongst the top earning export sectors and provide direct or indirect employment to about 10% of total population of the country. It contributes 3.74% in national GDP, 2.7% in export earnings and 22.23% in agriculture sector (FRSS, 2014). The contribution of fish and fishery products in national income, employment, health, nutrition and foreign exchange earnings is increasing. Some of the common forms of exported items are frozen shrimp and prawn, fresh live fish, frozen fish, dried fish, salted and dehydrated fish etc. However, due to insufficient attention towards the standard of hygiene and quality of the product, reasonable numbers of seafood products have gone out of business (Quaiyum *et al.*, 2012).

Drying is an ancient and simplest method to preserve fish. Salting and sun drying of fish is a traditional method of seafood preservation employed in many countries. In Bangladesh, dried fish is very demanding food stuff due to its high protein content and nutritional value. Dried fish is a very rich source of proteins; containing 80-85% protein (Jonsson *et al.*, 2007).This dried fish have long been consumed as a traditional food. Every year Bangladesh earns foreign currency by exporting dried fishes. During the year 2012-13, Bangladesh exported 1278 metric tons of dried fish and earned 36.03 cores taka (FRSS, 2014).

Dried products are usually considered shelf stable and are, therefore, often stored and distributed unrefrigerated. The characteristic of dried foods that makes them shelf stable is their low water activity (aw). Water activity is the measure of the amount of water in a food that is available for the growth of microorganisms, including pathogens. A water activity of 0.85 or below will prevent the growth and toxin production of all pathogens and is necessary for a shelf-stable dried product (FDA, 2011).

Though drying method is considered as the least expensive method of fish preservation, traditional drying is often rudimentary and good hygiene is rarely practiced. In Bangladesh, the process of drying is mainly performed by the households of artisanal fisherman who are mostly illiterate. There are frequent complaints from the consumers about the quality of the products. Lack of proper amenities like proper handling during loading and unloading, time and exposure of the fish to the high environmental temperature and besides, sufficient knowledge about scientific and hygienic methods of handling from the time of catch until it is processed into finished products contributes significantly to the loss of quality. Post harvest loss in dried fish product is estimated as 25% in Bangladesh (Mansur *et al.*, 2013).

Good quality raw material supply is essential for both domestic consumption and value-added product development for export market. It needs proper research support to produce safe and quality product for export. A better knowledge on quality and safety of sun-dried fish is important because a reasonable quantity of sun-dried fish is exported to international market every year. To continue export of this fishery product the quality and safety of the product should be assured. At the same time, the product should have desired quality and it should be safe for health of the domestic consumers.

The quality sun dried fishes are adversely affected by the occurrence of microorganisms. Determination of microbiological quality of such processed fishes is very important for guarding consumer's health and hygiene. The presence of the pathogenic loads in dried fishes is acquiring importance in view of the safety and quality of the seafood. The poor quality of dried fishes were mainly due to unhygienic processing, inadequate salting, unhygienic drying, use of spoiled fish for processing and lack of air tight packing of the dried fishes (Immaculate *et al.*, 2013) . Quality of fish and fishery product is strictly maintained and monitored for export to foreign countries but unfortunately, such quality control procedure is not maintained for local market (Huque *et al.*, 2013). So it is very important to assess microbiological quality of dried fishes in retail trade for guarding health and hygiene of local consumer.

It was studied the load of pathogenic and harmful bacteria in different dried fishes collected from retail market and khamar of different areas of Chittagong and Mymensingh districts of Bangladesh (Sultana *et al.*, 2010). *Vibrio cholerae* is one of the pathogenic bacteria that may result food intoxication by consuming the affected food. Although cholera is primarily known as a water-borne intestinal disease in the endemic regions including Bangladesh, contamination of foods can also be an imperative mode for cholera transmission (Mrityunjoy *et al.*, 2013). Infection due to *V. cholerae* begins with the ingestion of contaminated water or food (Nair *et al.*, 1996). *V. cholerae* can be classified into two strains: *V. cholerae* O1, cause cholera, whereas strains in other group, non-O1 *V. cholerae*, are generally associated with milder illness. The virulence of *V. cholerae* O1 is determined primarily by the presence of a protein enterotoxin, cholera toxin (CT). Strains that do not produce cholera toxin (i.e., are not toxigenic) tend to be avirulent or have reduced virulence but cause food poisoning with symptoms of diarrhoea, stomach cramps and vomiting (Ahmed., 1991).

The enterotoxin produced by this rod shaped bacteria causes copious, painless, watery diarrhea leading to vomiting, severe dehydration, and even death if treatment is not prompt (Sharma *et al.*, 2009).The subsequent loss of water and electrolytes leads to the severe diarrhoea characteristic of cholera. Thorough cooking of seafood products would virtually eliminate all microbial and parasitic pathogens; it will not destroy some microbial toxic metabolites (Ahmed, 1991).

Antibiotics are substances that have the ability to kill or prevent the growth and activity of micro-organisms (Romero *et al.*, 2012). Antibiotics can be obtained from two major sources, natural and synthetic. The natural sources of antibiotics are those that are obtained from herbs and any other living organisms (probiotics), while the synthetic sources are those that are obtained from chemicals.

Nevertheless, antibiotics should be safe (non-toxic) that is it must not pose any danger to the host, but toxic to the target organisms (Romero *et al.*, 2012). But the resistance to antimicrobial agents has become an increasing problem throughout the world. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity,

immune-suppression and allergic reactions. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants. With the alarming incidence of antibiotic resistance in bacteria of medical importance, there is increasing interest in plants as a source of agents for the treatment of microbial diseases. According to the World Health Organization more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The most significant bioactive compounds obtained from plants include alkaloids, flavonoids, tannins and phenolic substances (Sharma *et al.*, 2009). They act as chemotherapeutic, bactericidal and bacteriostatic agents. As a result anti-microbial substances derived from plants have received considerable attention than others in recent years.

A justified belief persists that traditional medicine is a lot cheaper and more effective than modern medicine. In developing countries, people belonging to the poor socioeconomic background use folk medicine for the treatment of common infections (Rojas *et al.*, 2006). Research on bioactive substances could possibly lead to the discovery of new compounds, resulting in the formulation of new and more potent antibacterial drugs, to overcome the problem of resistance to currently available antibiotics. The use of natural compounds from plants can provide an alternative approach against food-borne pathogens.

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. For examples, *Nigella sativa*, which produces black cumin seeds, popularly known in Bengali as Kalogira, is an essential ingredient in the Asian cuisine. The seeds of *N. sativa* (family: Ranunculaceae) has been used as a medicine and health-promoter for thousands of years. Black cumin seeds were found to exhibit antibacterial activity against wide range of gram-positive and gram-negative bacteria and also antiviral activity (Alam *et al.*, 2010).

Black cumin seed is recommended for a wide range of ailments, including fever, cough, bronchitis, asthma, chronic headache, migraine, dizziness, chest congestion, dysmenorrhea, obesity, diabetes, paralysis, hemiplagia, back pain, infection, inflammation, rheumatism, hypertension, and gastrointestinal problems such as dyspepsia, flatulence, dysentery, and diarrhea. It has been used as a stimulant, diuretic, emmenagogue, lactagogue, anthelmintic, and

carminative. Black Seed has also been used externally where it is applied directly to abscesses, nasal ulcers, orchitis, eczema, and swollen joints (Tariq., 2008). Black cumin seeds and black cumin seed oil have been widely used for reducing blood pressure, cleansing and tonifying the liver, reducing fluid retention, supporting healthy digestion, stimulating the appetite and treating skin disorders. Studies have confirmed numerous pharmacological benefits. They can be used to regulate the immune system, kill microorganisms, reduce inflammation, inhibit spasmodic activity, and open the tiny air passages in the lungs. Black seed oil protects the liver, the kidneys, and the stomach digestive system. It is a powerful antioxidant (Ahmed *et al.*, 2013).

Cumin seeds are also known to act synergistically with antibiotics, therefore, more dose-response preclinical studies can be done to assess the effectiveness of use of antibiotic/kalogira combination for bacteria that are difficult to eradicate (Gowsala., 2001). In this study, the antibacterial effects of two polar solvent extracts (acetone and ethanol) of black cumin seeds were assayed against the isolated *V. cholerae* from dried fish under laboratory conditions.

1.1. Objectives of the study:

- Isolation and identification of the *V. cholerae* from dried fish.
- Determination of the antibacterial effects of the black cumin seed extracts against the isolated *V. cholerae.*

Chapter two

LITERATURE REVIEW

2. Review of Literature

Mansur *et al.*, (2013) studied the market samples of three sun-dried freshwater fish species namely Indian major carp (*Labeo rohita*), snake headed fish (*Channa striatus*), and a type of eurasian catfish (*Wallago attu*). The safety aspect of the sun-dried fish samples were studied by the detection of heavy metal, total viable bacterial count (TBC), aerobic plate count (APC) and Total volatile base-Nitrogen (TVB-N). The total viable bacterial count (TBC) of the experimental samples ranged from 1.84×10^4 to 5.3×10^6 per g of the dried samples.

Sulieman *et al.*, (2014) estimated the microbiological characteristics as well as the safety of traditionally dried fish product, Kejeik, in Sudan and included that the higher aerobic bacterial load of the various Kejeik samples could be due to improper handling and sanitary condition during the preparation and their moisture contents. The study found the counts of aerobic bacteria in Kejeik prepared from Ijl, Nawk and Garmut (fish types) were 7.2×10^5, 4.1×10^5 and 3.4×10^5 cfu/gm, respectively.

Huque *et al.*, (2013) determined microbiological qualities (TVBC, TCC and TFC) of three different dried fishes (Chepa, Loitta and Chingri) collected from retails markets. Results of microbiology showed that total viable bacterial count (TVBC) was estimated in Chepa (5.58 ± 0.14 log cfu/g), Loitta (3.72 ± 0.09 log cfu/g) and Chingri (5.34 ± 0.15 log cfu/g) respectively.

Loges *et al.*, (2012) investigated the quantum of microbial load in dried fishes processed by conventional method and found the presence of different group of faecal coliforms and *Vibrio* spp. was an alarming situations that warranted the need for incorporating hygienic and scientific ways of drying.

Acosta *et al.*, (2001) identified the risk factors and describe the pattern of spread of the 1997 cholera epidemic in a rural area (Ifakara) in southern Tanzania and found that eating dried fish were significantly associated with risk for cholera.

Prakash *et al.*, (2011) analyzed the microbial quality of six sun dried seafood species available at Tuticorin dry fish market in India during monsoon, post-monsoon and summer seasons and reported that the microbial quality parameters varied with different seafood's in different seasons and the quality were found to be poor during monsoon season. The study revealed the presence of *E. coli*, fecal *Streptococci*, *Salmonella* and *Vibrio* spp. in dried fishes.

Sultana *et al.*, (2010) conducted a study to isolate and identify different bacteria contaminating different dried fishes collected from retail market and khamar at Mymensingh in Bangladesh. Of the 25 dried fishes, *Bacillus* spp., *Staphylococcus* spp., *Salmonella* spp. and unidentified bacteria isolation rates were 56%, 80%, 48% and 20% respectively. Presence of bacteria in some dried fishes indicated the maintenance of low hygienic condition during dried fish processing.

Antimicrobials of plant origin have enormous therapeutic potential. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, phenol compounds and flavonoids etc. which are capable of producing definite physiological action on body (Joshi *et al.*, 2009).

The seeds of *N. sativa* have been thoroughly studied scientifically in the last 3-4 decades and have been reported to possess a number of medicinal properties (Randhawa and Al- Ghamdi, 2002; Ali and Blunden, 2003). Their crude extracts (Mouhajir and Pedersen, 1999; Ali *et al.* 2001) and essential oil (Halwani *et al.* 1999) have been shown to possess antibacterial activity against several bacteria. The oil and crude extracts of *N. sativa* were reported to have a promising effect on multi-drug resistant *S. aureus*, *P. aeruginosa* and *Candida albicans* (Mashhadian and Rakhshandeh, 2005), *Shigella* spp., *Vibrio cholerae* and *E. coli* (Ferdous *et al.*, 1992).

Adam., (2013) conducted a study to find the antimicrobial activity of bee honey, black cumin oil and green tea against multi-drug resistant pathogenic bacteria and found the bacterial strains that are investigated were sensitive or extremely sensitive to honey at concentrations 50% and 100 % and sensitive or extremely sensitive to black cumin oil at concentrations 30%, 50% and 100%.

Ramadan., (2007) stated that black cumin had been extensively studied for its nutritional value and biological activities. The black cumin oilseed had been shown to be anticancer, antidiabetic, antiradical and immune modulator, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepatoprotective, antihypertensive and renal protective. In his review paper, he summarized the nutritional value, functional properties and nutraceutical applications of black cumin (*Nigella sativa*) oilseeds.

Salman *et al.*, (2008) studied *Nigella sativa* (black cumin) seed oil and methanolic extract that were studied for antibacterial activity against various clinical isolates of bacteria resistant to a number of antibiotics, in varying concentrations by disc agar diffusion technique. Both the oil and extract showed remarkable dose dependent antibacterial activity against the gram positive and gram negative bacteria.

Masoud, *et al.*, (2012) evaluated the growth inhibitory effect of *Syzygium aromaticum (clove), Nigella sativa* (*black cumin*), *Commiphora molmol* (*myrrh*) and *Allium sativum* (*garlic*) and water, 80% ethanol and n-hexane plant extracts either alone or in combination were tested against 4 gram negative bacteria. The study concluded that Individual water, ethanolic and hexanic extracts of *N. sativa* did not show any growth inhibitory effect against all tested microorganisms.

The essential oil and the acetone extract of *N. sativa* were found to be potentially effective against *S. aureus* and *B.subtilis* (Singh *et al.* 2005). He used essential oil, alkaloids, phenolic compounds, butanol extract of the seeds and showed that the essential oil was effective against Gram-positive bacteria (*S. aureus, B. cereus*).

Tahir *et al.*, (2006) reviewed the effect of black cumin seed extract with different solvent (Aqueous,ethanolic,methanolic,ether,hexane, Ethereal) and revealed the bacterial species that were more susceptible included *Salmonella, Shigella shigae, Bacillus cereus, Vibrio cholerae, Pseudomonas aerogenosa.*

Nagi *et al.*, (2008) investigated the antimicrobial activity of *N. sativa* essential oil against Gram Positive and Gram negative strains, isolated from clinical specimens and noted that the crude extracts of *N. Sativa* had a promising effect on multi-drug resistant *S. aureus* and *Candida*

albicans, *Shigella* spp., *Vibrio cholerae* where *V. cholerae* 0139 Bengal was tested with different extract concentrations (100, 50, 25, 12.5 and 6.25%) producing small but clear zones of inhibition at 100 and 50% extract. For *V. cholerae* Ogawa strain, the extract used in methanol extracts, produced small but clear inhibition zones.

The anti-microbial agent has been isolated from the volatile oil, identified as thymohydroquinone and was found to be active against Gram-positive bacteria and yeasts (Toama *et al.* 1974). Farrag *et al.* (2000) found that the fixed oil of black cumin had an inhibitory effect against Gram positive such as *S. aureus* and *B. cereus* and Gram negative bacteria. Khan *et al.* (2003) reported that the extract from *N. sativa* seeds had antifungal activity against *Aspergillus parasiticus*, *Candida albicans* and *Saccharomyces cerevisiae* respectively.

Sandhu *et al.*, (2013) reviewed effect of black cumin seed extract for its antimicrobial effect against a wide range of bacterial, fungal and parasitic organisms. Alcoholic extract of the seeds showed antibacterial activity against *Micrococcus pyogenes*, *Shigella dysenteriae*, *S.boydii*, *S.Sonnei*, *Vibrio cholerae* and *Escherichia coli*. The ether extract showed in vitro anti microbial activity against Gram positive bacteria; e.g. *Staphylococcus aureus*, Gram negative bacteria; e.g. *Pseudomonas aeroginosa* and *Escherichia coli.*

Raza *et al.*, (1999) stated therapeutic uses of *N. sativa* in his review paper and noted that dried seed had the antibacterial effect against a number gram positive and gram negative bacteria. The study also revealed that seed essential oil on agar plate is active against *Vibrio cholerae* and *Shigella shiga* with MIC 50.0 µg/ml, against *Shigella dysenteriae* and *Shigella flexneri* with MIC 100.0 µg /ml and, against *Escherichia coli* and *Shigella flexneri* with MIC 200.0 µg /ml.

Gilani *et al.*, (2004) found that *Nigella sativa* seeds had been widely used in traditional medicine and Consequently, it had been extensively studied for its biological activities and had been shown to be antidiabetic, anticancer and immunomodulator, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepatoprotective, antihypertensive, renal protective and antioxidant properties. Alcoholic extracts of the seeds showed antibacterial activity against *Shigella dysenteriae, S. sonnei, S. boydii, Vibrio cholerae and Escherichia coli.*

Agrawal *et al.,* (1979) reported that antibacterial effects of the essential oil of black cumin showed pronounced activities even at 1:100 dilutions against several organisms that included *Staphylococcus albus, Escherichia coli, Salmonella typhi,* and *Vibrio cholerae*. It has been shown that the crude alkaloid extract and the water extract of seeds were effective against variety of organisms isolated from human patients suffering from septic arthritis, even those that were resistant to antibiotics (Ali and Blunden, 2003).

Alam *et al.*, (2010) analyzed chloroform and ethanol extracts of black cumin seeds (*Nigella sativa*) for antibacterial activity against five food and water borne pathogens, namely, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae*, *Klebsiella pneumoniae, Escherichia coli,* and one food spoilage bacteria *Bacillus subtilis*, all of which were previously found to be resistant to different antibiotics. All the bacterial strains except *E. coli and K. pneumoniae*, showed sensitivity to the chloroform extract as well as the bacterial strains except the *K. pneumoniae* showed sensitivities to ethanol extract. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of both extracts were also evaluated in the study.

Chapter three

MATERIALS and METHODS

3. Materials and Methods:

The present study was undertaken to isolate *V. cholerae* and determine the effect of black cumin seed extracts on *V. cholerae* of dried fish which were collected from local market (Shondha bajar, Moylapota) of Khulna, Bangladesh. The entire research work was conducted in the Quality Control Laboratory of the Department of Fisheries and Marine Resource Technology (FMRT) Discipline, Faculty of life Science, Khulna University. The study was conducted during the period from January to May, 2015.

3.1 Samples collection:

In this study, three types of commercially valuable dried fish were selected. Dried fish Lottya (*Harpodon nehereus*), Chingri (*Penaeus monodon*) and Churi (*Trichiurus haumela*) were collected in sterile plastic bag separately and the bags were teighed after collection to prevent extraneous contamination. Then the collected samples were carried out to the laboratory and preserved at 4°C.

3.2 Black cumin seeds collection

About 500 g black cumin seeds (*Nygella sativa*) were bought from the spice market and sorted for separation of dirt and unwanted materials. The seeds were washed thoroughly with clean water and air dried at room temperature. Then the seeds were brought to laboratory and preserved at room temperature in sterile plastic bag.

3.3 Preparation of extracts from black cumin seed *(N. sativa)*

The black cumin seed was heated in oven at 50° C for 15 minutes and grinded by using mixer grinder (Capacitor start motor, WUHU motor factory, China). Approximately 300 gm of powdered cumin seed was taken into two sterile conical flasks containing 150 gm each. Then 350 ml of 100% ethanol and acetone were added into flasks separately at a ratio of 1:3. The mixtures were kept at room temperature for 72 hours. The mixtures were stirred every 24 hour using sterile glass rod. Then the mixtures were filtered through Whatman® No.1 filter paper. Filtration procedures were done further twice for complete extraction of the bioactive compounds. The filtrates were then collected in separate beaker and concentrated by evaporating the solvents. After 96 hours the both solution took a liquid consistency and were considered at

stock solution of the extract. The liquid extracts were kept in a refrigerator (-20°C) for further study. The process was followed to (Yasni., *et al,* 2009) with slight modification. Total process of preparing the extracts is given below in short flow chart:

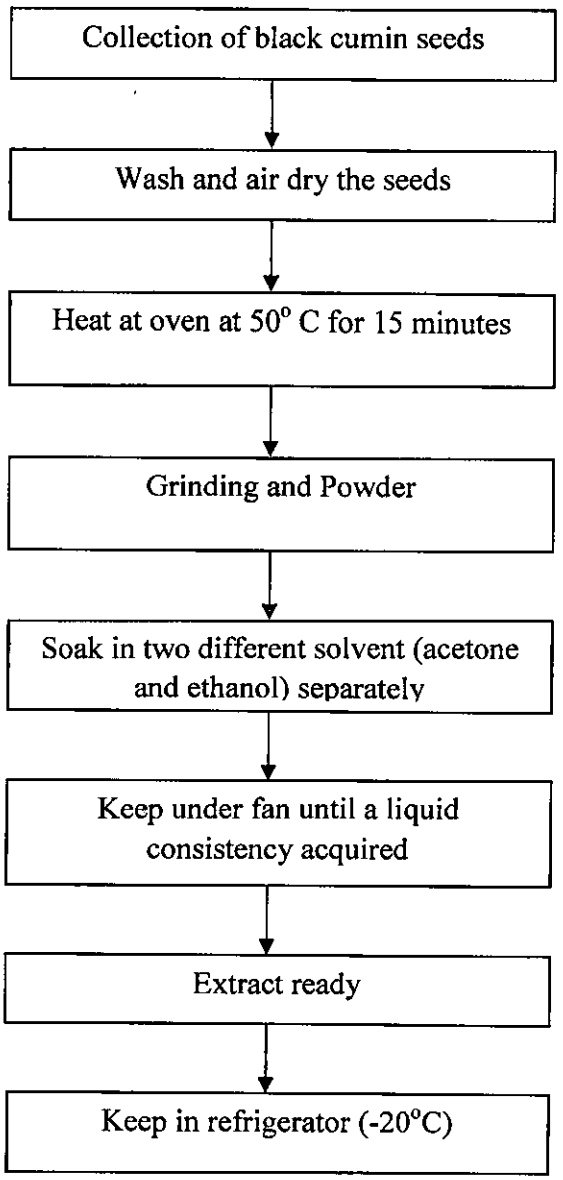

Figure 01: Flow chart of extract preparation of black cumin seed.

The liquid extracts were then re-suspended in ethanol and acetone before testing to get different concentration.

3.4 Preparation of extract at different concentrations

10%, 15% and 25% concentration of extract in both solvent (ethanol and acetone) was made separately by adding 100 µl, 150 µl and 250 µl both extracts into 900 µl, 850 µl and 750 µl of their relevant solvents (acetone and ethanol) respectively. After adding to the solvent, mixing was done in unidirectional manner by a vortex mixer.

A serial two fold dilution of extract was made to get concentrations of 50%, 25%, 12.5%, 6.25% and 3.12%. The ethanolic extract was diluted in ethanol solvent. 500 µl of ethanol was added into 500 µl of ethanolic extract was and mixed unidirectional manner using a vortex mixer. Then the dilution was made (2^{-1}, 2^{-2}, 2^{-3}, 2^{-4}, 2^{-5}) in ethanol.

Preparation of acetonic extract of black cumin seed at different concentration was done by the same procedure above. A serial two fold dilution of extract was made into acetone solvent to get concentrations of 50%, 25%, 12.5%, 6.25% and 3.12%.

Two more additional concentrations of 40% and 30% were also prepared by mixing 300 µl and 400 µl of extracts into 700 µl and 600 µl of relevant solvent respectively.

3.5 Processing of sample

The dried fishes were cut into small pieces with a sterile scissor and weighted in electric balance (HR-200). About 2 grams of samples were collected from each dried fish. The sample was then minced and grinded properly with alkaline peptone water using mortar and pestle. The mixture was taken into eppendorf tubes with alkaline peptone water used for isolating *V. Cholerae* (ISO/TS 21872-1). It was then homogenized using tissue homogenizer. Then a homogenized solution was centrifuged at 3000 rpm for 3 minutes (James & Hirsch, 1960) in Centrifuge (5415 D.). After centrifugation the supernatant liquid portion was collected with micropipette and taken into eppendorf tube and preserved in deep freeze.

3.6.4 Preparation of 0.5 McFarland Standards

0.5 Mcfarland standard was prepared by adding 0.5ml of 0.048M $BaCl_2$ (1.17 % w/v $BaCl_2.2H_2O$) to 99.5 ml of 0.18M sulphuric acid (H_2SO_4) (1 % w/v) with constant stirring. A barium sulphate precipitate was checked for optical density using matched curvettes with 1 cm path and distilled water as a blank standard. A UV-Vis (T-60) spectrophotometer was used to measure the absorbance at 625 nm. An absorbance of 0.1 was obtained which was in the accepted range of 0.08- 0.13. The suspension was distributed into tubes of the same size as those used for test inoculum's adjustment. Sealed tubes were stored in the dark at room temperature. The approximate cell density corresponding to 0.5 McFarland is 1×10^8 CFU/ml (Joshua and Takudzwa, 2013)

3.6.5 Sterilization of different equipments

Test tube, eppendorf tube, plastic tips and other glassware were sterilized by autoclaving in autoclave (VS-1221, Vision Scientific., LTD) at temperature 121°C and pressure of 15lbs/sq inch. For 15 minutes. Petri dishes, isolating loop, etc. were subjected to dry heat sterilization at 170°C for 1 hour in electric oven (Memmert).

3.7 Isolation and enumeration of *V. cholerae*

0.1ml stock solution of each dried fish was taken and mixed with 0.9ml Alkaline Peptone Water (APW) in sterilized test tubes. Then the mixture was incubated at 37°C for 6 ± 1. This was first selective enrichment. After that, the whole culture of test tube obtained in first selective enrichment was taken and transferred into other test tubes each containing 10ml APW. Then the solution was incubated at 41.5°C for 18h ± 1. This was the second selective enrichment. Then the serial dilution (tenfold) was done and 0.1ml suitable dilution of each culture was inoculated in TCBS agar plates. Then the inoculated TCBS Cholerae agar plates were incubated at 37°C. After 24 ± 3h of incubation in incubator (LSANYO), the plates were examined for the presence of typical colonies of presumptive *Vibrio* sp. (ISO/TS 21872-1, 2007). Then the standard plate was selected and counted the colonies. The total process was done according to the following flowchart.

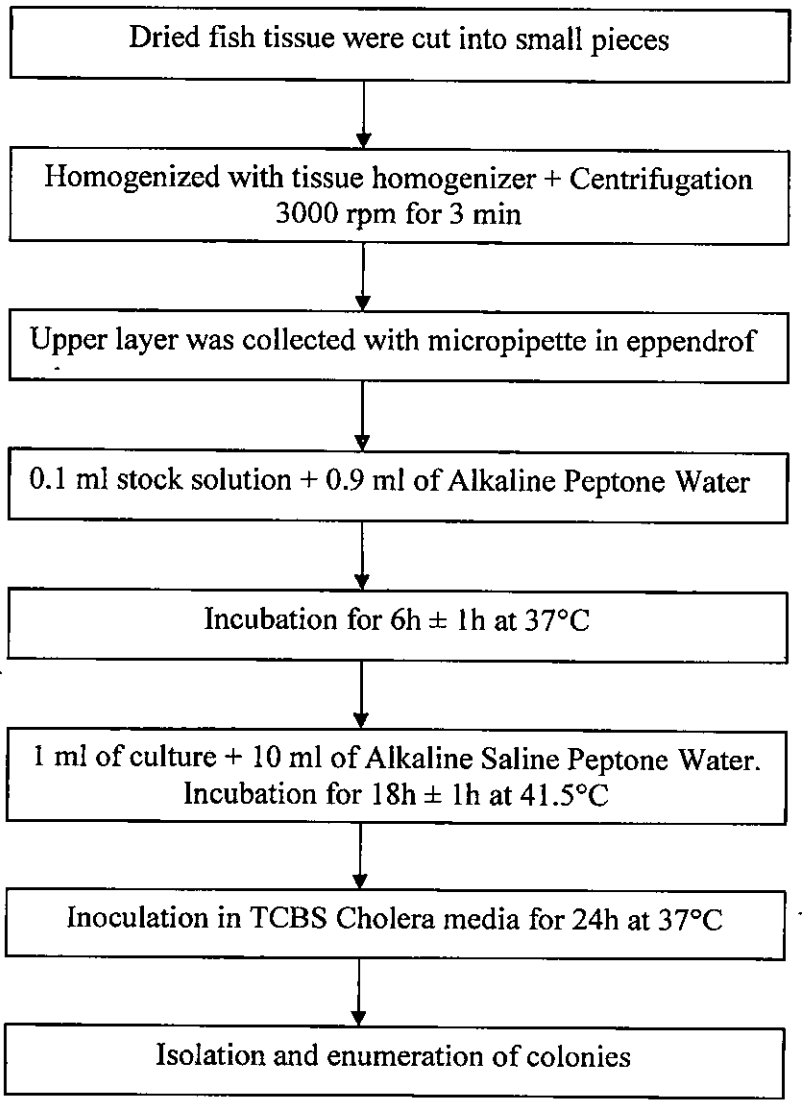

Figure 02: Flow chart of isolation and enumeration of *V. cholerae*

3.8 Identification of *V. cholerae*

Selective TCBS Cholerae agar media [OXOID LTD., Basingstoke, Hampshire, England] was used for the isolation of *V. cholerae*. Therefore, further identification was done by performing some biochemical tests viz. TSI test, Indole test, Oxidase test, MR test, Salt tolerance test.

3.8.1 Triple Sugar Iron (TSI) agar

TSI slants were inoculated with experimental isolates by stabbing the butt and streaking the surface of the medium. Slants were incubated at 37°C and examined after 24 hours. Caps on all tubes of biochemical were loosened before incubation. A change in color of the medium from reddish-orange (the original color) to yellow indicates the drop of pH, producing an acid slant and an acid butt (acid over acid; A/A). And produce neither gas nor blackening in the butt (i.e. H_2S Negetive). (CDC, 2014).

3.8.2 Indole test

The indole test was performed in an 8 h culture in peptone water adding about 1 ml ether, then shake, run 0.5 ml Ehrlich's reagent down the side of the tube. A red color in the solvent indicates positive reaction. (CDC, 2014)

3.8.3 Methyl red (MR) test

The methyl red reaction was tested by using MR-VP medium (Difco) incubated at 37°C for 48 h after inoculation. After incubation adding a few drops of methyl red solution to the culture, read immediately. A red color represents a positive test. (Kaysner and Depaola, 2004)

3.8.4 Oxidase test

Oxidase test was conducted with fresh growth from the nutrient agar medium. 2 to 3 drops of oxidase reagent (Gordon-McLeod Reagent, HiMedia) was placed on a piece of filter paper in a petri dish. The overnight growth was transferred into the wet paper by using a wooden toothpick. A dark purple color developing within 10 sec indicates a positive test growth. (Kaysner and Depaola, 2004)

3.8.5 Salt tolerance test

This test was done on nutrient agar media supplemented with varying amounts of NaCl (0%, 1%, 3%, 6% and 8%). The plates were incubated at 35° to 37°C and read at 18 to 24 hours. *V. cholerae* differs from other *Vibrios* by its ability to grow without any added salt as it is non holophilic. But the growth can be stimulated by adding salt upto 3%. (CDC, 2014).

3.9 Experimental design for *In-vitro* challenge test of black cumin against *V. cholerae*

At first the bacteria enrichment stock solution of each sample was done (ISO/TS 21872- 1, 2007). Then 0.5 ml of 50%, 40%, 35%, 30%, 25% and 15% extracts (acetone and ethanol) of black cumin was separately mixed with 0.5 ml of test solution. 0.1ml suitable dilution of the mixture was inoculated in petri dishes that containing TCBS Cholera agar media after at subsequent interval of 2 hour up to 6 hour. This procedure was done 2 times. Test solution of sample without extract also inoculated TCBS cholera agar media at 0 hour, 2 h, 4 h and 6 h subsequently. All the inoculated TCBS agar plates were incubated at 37° C for 24 ± 3 hours. Standard plates count was done after incubation and compare the plates with and without extract.

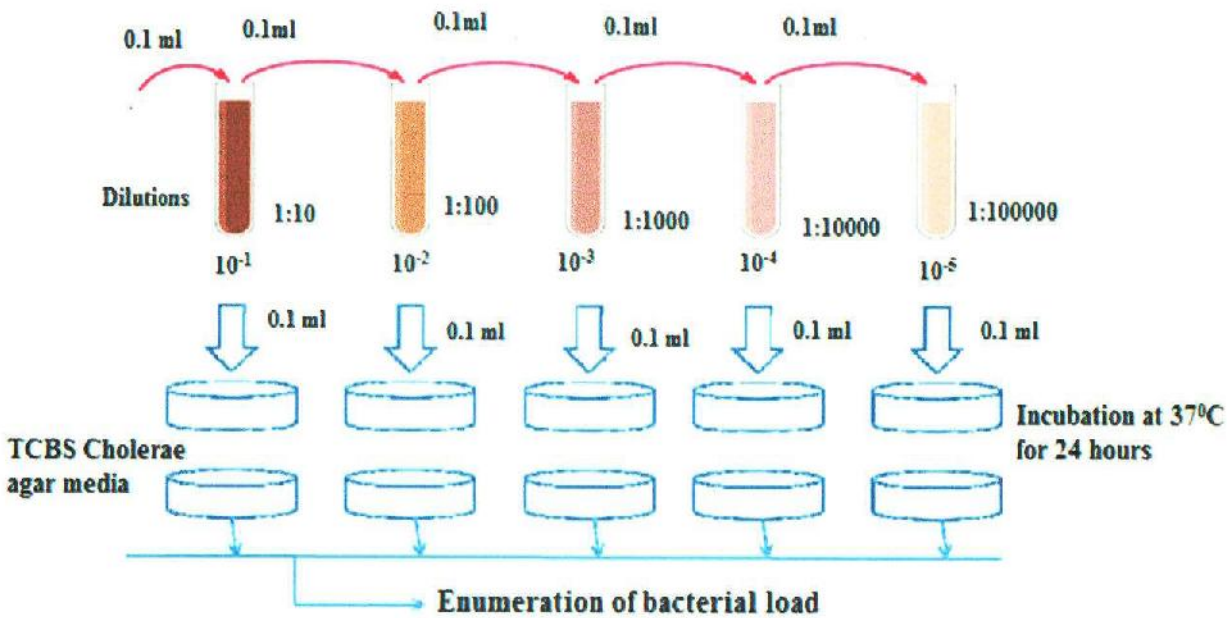

Figure 03: *In-vitro* challenge test and enumeration of *V. cholerae* load without extract.

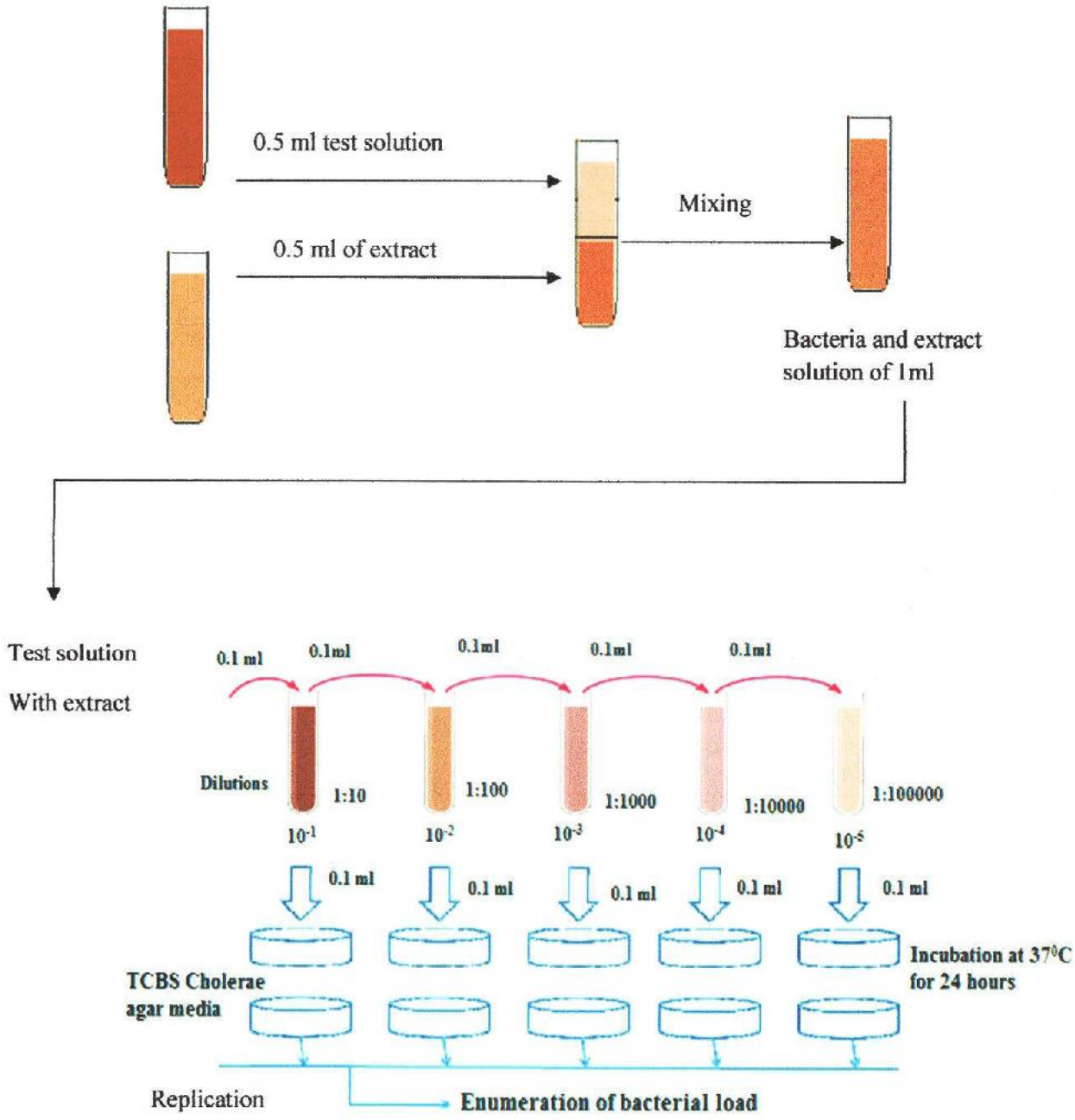

Figure 04: *In-vitro* challenge test and enumeration of *V. cholerae* load with extracts

3.10 *In-vitro* Challenge Test and Determination of Antagonistic Activity of black cumin extracts against *V. cholerae*

Process A: Enumerate the load before and after treatment.

Process B: Determining the zone of inhibition of black cumin extracts (Disc diffusion)

Process C: Determining the Minimum Inhibitory Concentration (MIC) of the both extracts in both solvent.

3.10.1 Process A: Enumerate the Load before and after Treatment

At first one loop of single colony of *V. cholerae* was dissolved in separately 1 ml alkaline peptone water (APW) in eppendorf tube. Then serial dilution (ten fold) was done. After that 0.15 ml of 50%, 40%, 35%, 30%, 25%, 20%, 15% of both ethanolic and acetonic extracts of black cumin seed were mixed with 0.85 ml of bacterial solution separately. Then 0.1ml of the mixture was inoculated in petri-dishes that containing TCBS Cholera agar media. Test bacterial solution without extract also inoculated in the TCBS Cholera agar media. All inoculated TCBS agar plates were incubated at 37° C for 24 ± 3 hours. Standard plates count was done after incubation and compare the plates with and without extracts.

3.10.2 Process B: Determining the Zone of Inhibition of black cumin extracts against *V. cholerae*

Antibacterial activity of the black cumin extract was evaluated using disc diffusion method (Bauer et al, 1966) with slight modification. The discs 4 mm of Whatman® paper were prepared for negative control. Standard antibiotic discs were used as a positive control to compare the antibacterial activity of black cumin. TCBS Cholera agar media was prepared and raised temperature up to 100° C in hot plate. Then the media transferred into the petri dishes and kept for cooling. 0.1 ml of bacterial stock solutions were placed on separate petri dishes and spread throughout the plate by spread plate technique. The antibiotic and the discs that's were loaded with 20 µl of different concentration (50%, 40%, 30%, 20%) of both ethanolic and acetonic extracts separately were placed on the bacterial solution inoculated plate with help of sterile forceps carefully with adequate spacing between each other. The plates were kept at room temperature for 30 min, which helps to diffuse the extract on the medium. Later the plates were then incubated at 37° C for 24 hrs in incubator to determine the antibacterial activity of ethanolic

and acetonic extracts of black cumin. Karamycin was used for Positive control and sterile Whatman® paper was used for negative control. After incubation, zone of inhibition in diameter was measured by a slide calipers (Tricle Brand) and recorded.

3.10.3 Process C: Determining the Minimum Inhibitory Concentration (MIC) of black cumin extracts

Minimum Inhibitory Concentration (MIC) is the lowest antibiotic concentration where growth of bacteria is inhibited. MIC was determined in broth dilution method. It is a technique in which test tubes holding identical volumes of broth with antimicrobial solution in incrementally increasing concentration are inoculated with known number of bacteria (EUCAST Discussion.5.1, 2003). Nutrient agar and McFarland standard was prepared by standard method (Hudzicki., 2009).

3.10.3.1 Preparation of inoculum

Colony suspension method was used for the preparation of inoculum. 30 hour old cultures from non selective media (nutrient agar) were touched with a loop and the growth was transferred to sterile nutrient broth. The suspension was adjusted to give a turbidity equivalent to the 0.5 McFarland standards. . If the suspension was too light more bacteria was added or if it was too heavy it was diluted by nutrient broth. This resulted in a suspension containing approximately 1.5×10^8 colony forming units (CFU/ml). The inoculum prepared above was diluted in nutrient broth to give a final bacterial density of 5×10^5 CFU/ml. This was done by transferring 0.1 ml of bacterial suspension to a tube containing 9.9 ml of nutrient broth which, when mixed with an equal volume of extract solution (acetonic and ethanolic extract) in tubes, gave a result in a final inoculum of 5×10^5 CFU/ml. (EUCAST. Dis. 5.1, 2003).

3.10.3.2 MIC determination by Broth/Tube Dilution Method

The procedure described by Rollins, *et al.*, (2003) was followed with slight modification.

Procedure:

1. A series of sterile capped test tubes 1 through 9 were taken and marked with number. All of the following steps were carried out using aseptic technique.
2. 2.0 ml of extract solution was added to the first tube and 1.0 ml of sterile nutrient broth was added to all other tubes (1-8).
3. 1.0 ml was transferred from the first tube to the second tube.
4. By using a separate pipette, the contents were mixed of this tube (2^{nd} tube) and 1.0 ml was transferred to the third tube.
5. Dilutions were continued in this manner to tube number 8, was being certain to change pipettes between tubes to prevent carryover of extract on the external surface of the pipette.
6. 1.0 ml from tube 8 was removed and discarded. The ninth tube, which serves as a control, received no extract.
7. 1.0 ml of the diluted culture suspension was added to each of the tubes. The final concentration of extract was then one-half of the original concentration in each tube.
8. Two additional concentrations (40% and 30%) were also prepared aside this procedure.
9. All tubes were incubated at 35°C overnight.
10. Then tubes were examined for the minimum inhibitory concentration (MIC) and minimum lethal concentration by a spectrophotometer (T 60 UV-Visible spectrophotometer).

Chapter four

RESULTS

4. Results:

4.1 *V. cholerae* load in fish tissue of the collected dried fish

A selective media (T.C.B.S Cholera medium) was used for isolation of *V. cholerae*. After sample preparation, fish tissues were taken into eppendorf tubes and APW (alkaline peptone water) was used for isolating *V. cholerae*. After the incubation, colonies for *V. cholerae* on the agar media appeared as yellow, shiny colonies, 2 to 4 mm in diameter .Then the colonies were counted. The average load of *V. cholerae* in the dried fish tissue of lottya was 3.1×10^4 and in dried Chingri was 2.67×10^3. No *V. Cholerae* was found in Churi shutki. The *V. cholerae* load in three dried fishes is shown in Table 1.

Table 01: *Vibrio cholerae* load in dried fish

Dried fish	SL. No.	Bacterial Load (CFU/g)	Avg. Bacterial load (CFU/g)
Lottya (*H. nehereus*)	01.	1.29×10^4	3.1×10^4
	02.	7.1×10^4	
	03.	9.2×10^3	
Chingri (*P. monodon*)	01.	3.17×10^3	2.67×10^3
	02.	1.83×10^3	
	03.	3.00×10^3	
Churi (*T. haumela*)	01.	NF	NF
	02.		
	03.		

N.B. NF= Not found

4.2 Biochemical tests for the identification of *V. cholerae*

Though a selective media (TCBS Cholerae) was used for the isolation of *V. cholerae*, five biochemical tests were performed for further confirmation. The isolated colonies of the bacteria were inoculated in different biochemical media and incubated overnight at 37^0c. The TSI agar slant, Indole test, Oxidase test, MR test, Salt tolerance test were performed by following the procedures of Centers for Disease Control and Prevention (CDC), 2014.

Table 02: Biochemical tests for the confirmation of *V. cholerae*.

Test		Lottya (*Harpodon nehereus*)							Chingri (*Penaeus monodon*)						
		C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_1	C_2	C_3	C_4	C_5	C_6	C_7
TSI test		A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH	A/A, NG, NH
Indole formation		+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR test		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in NaCl	0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	3%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6%	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	8%	-	-	-	-	-	-	-	-	-	-	-	-	-	-

N.B: A/A= Acid over Acid, NG=No gas, NH= No H_2S, + = Positive, - = Negative, $C_{1, 2, \ldots\ldots, 7}$ = Number of colonies.

All the isolated bacteria from the both dried fish showed positive result to Indole test, MR test, Oxidase test.

4.3 *In-vitro* Challenge test of black cumin against *V. cholerae*

The present study showed that there was a significant antagonistic effect of black cumin seed (*Nygella sativa*) against *V. Cholerae*. *In-vitro* challenge test of dried Lottya fish, the potential antagonistic effect of black cumin extract (both acetone and ethanol) was gradually obtained at 0 h, 2 h, 4 h and 6 h after treatment.

4.3.1 *In-vitro* Challenge test of black cumin acetone extract against *V. cholerae*

Average load of *V. cholerae* without extract were 7.1×10^4, 6.2×10^4, 8×10^4 and 1.64×10^4 CFU/g at 0 h, 2h, 4h and 6h respectively. With 5%, 10% and 25% concentration of extract the average load were 3.5×10^4, 8×10^3 and 2.5×10^3 CFU/g at 0 h. Average *V. cholerae* load without extract gradually increased with time but the average load of the bacteria with different concentration of extract gradually decreased.

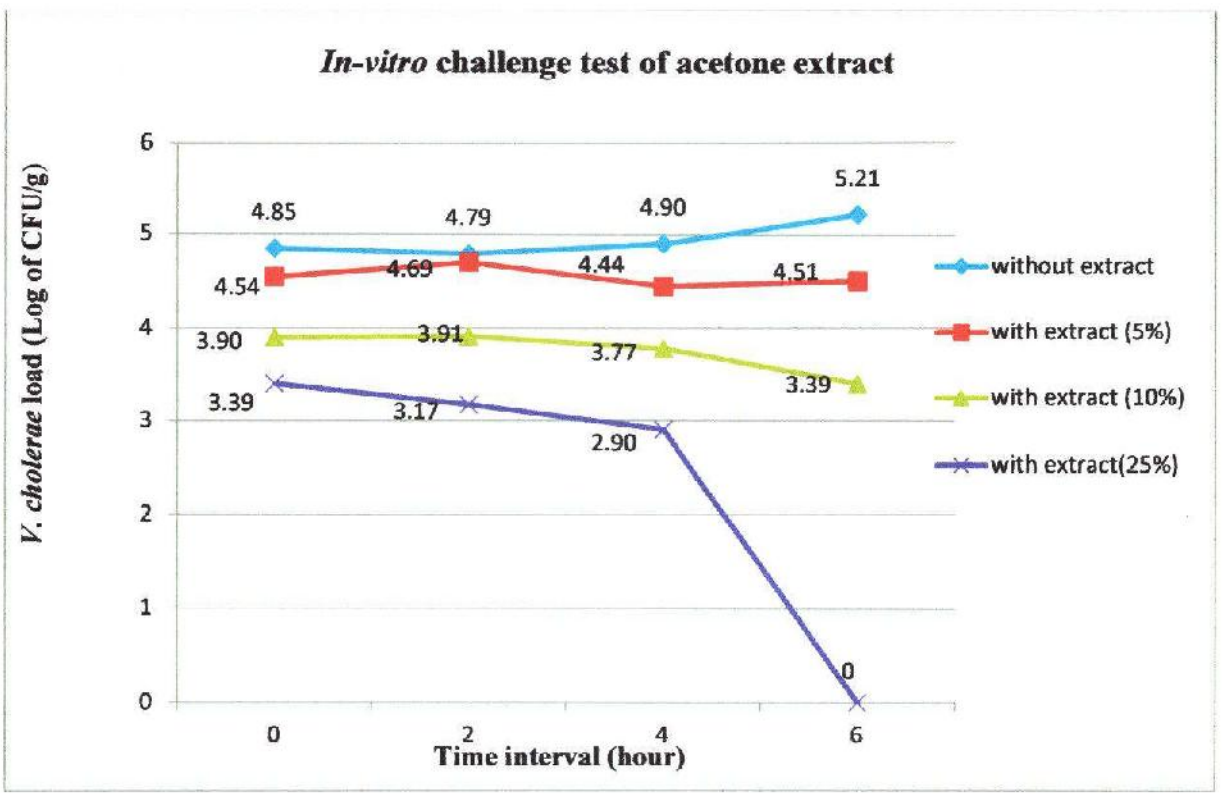

Figure 05: *In-vitro* challenge test of black cumin extract in acetone against *V. cholerae*

With 5%, 10% and 25% concentration of extract the average load were 5×10^4, 8.2×10^3 and 1.5×10^3 CFU/g at 2 hours and 2.8×10^4, $6\times10^3$and 8×10^2 CFU/g at 4 hours respectively. With the treatment of 25% or 250 µl/ml acetonic extract of black cumin, the growth totally stopped at 6 h. From the result it could be said that more concentration of extract was more effective against *V. cholerae*. 25% black cumin extract was more effective to use concentration basis.

4.3.2 *In-vitro* Challenge test of black cumin ethanolic extract against *V. cholerae*

Average load of *V. cholerae* without extract were 7.1×10^4, 6.2×10^4, 8×10^4 and 1.64×10^4 CFU/g at 0 h, 2h, 4h and 6h respectively. With 5%, 10% and 25% concentration of extract the average load were 3.3×10^4, 2.6×10^4 and 1.93×10^3 CFU/g at 0 h. Average *V. cholerae* load without extract gradually increased with time but the average load of the bacteria with different concentration of extract gradually decreased.

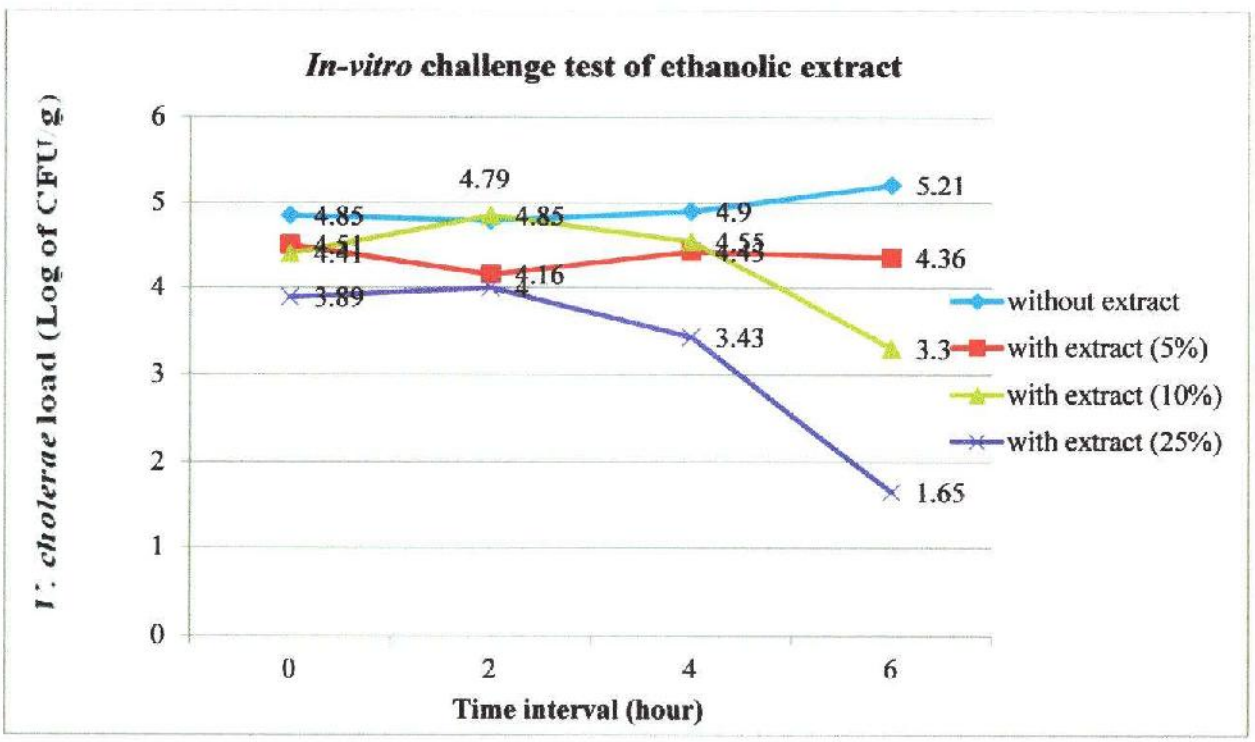

Figure 06: *In-vitro* challenge test of black cumin extract in ethanol against *V. cholerae*

With 5%, 10% and 25% concentration of extract the average load were 1.46×10^4, 7.2×10^4 and 1.2×10^4 CFU/g at 2 hours and 2.7×10^4, $3.6\times10^4$and 2.5×10^3 CFU/g at 4 hours respectively. With

the treatment of 25% or 250 µl/ml ethanolic extract of black cumin, the growth drastically reduced at 6 h to 4.5×10^1 CFU/g.

4.4 *In-vitro* Challenge test and Determination of Antagonistic Activity of Black cumin extracts against *V. cholerae.*

Process A. Enumerate the load before and after treatment

4.4.1 The antibacterial activity of Black cumin extracts in acetone against *V. cholerae*

The antibacterial activity of black cumin extracts in acetone is shown in Table 3 by comparing the average load of the bacteria without and with black cumin acetonic extract at 50%, 40%, 30% and 20% concentration. No growth of *V. cholerae* was found up to 30% concentration of extract. In 20% concentration the load found was 2.41×10^4 that decreased in 94.6% comparing the primary load.

Table 03: *V. cholerae* load before and after treatment with black cumin acetone extract

SL. No	Without extract (CFU/ml)		With black cumin acetone extract at different concentration (CFU/ml)							
	Load	Avg. Load	50%		40%		30%		20%	
			Load	Avg. Load	Load	Avg. Load	Load	Avg. Load	Load	Avg. Load
01.	3.96×10^5	4.5×10^5	N	N	N	N	N	N	2.7×10^4	2.41×10^4
02.	2.68×10^5		N		N		N		1.92×10^4	
03.	6.86×10^5		N		N		N		2.58×10^4	

N.B. Avg. = average; SL. No. = Serial number; N = No growth, CFU = Colony Forming Unit. Each time 100 µl solution was inoculated in Petri dish.

The procedure was repeated twice and additionally, three more concentrations of the extract (35%, 25% and15%) were prepared to determine the antibacterial activity. The average load found in 25% of acetonic extract was 5.6×10^2 which indicated the reduction of *V. cholerae* load in 99.8%. But at the 15% concentration average load found was 3.21×10^5 that indicated a merely reduction of load in 28.6%.

Table 04: *V. cholerae* load before and after treatment with black cumin acetone extract at 25% and 15% concentration.

SL. No	Without extract		With black cumin acetone extract at different concentration			
			25%		15%	
	Load (CFU/ml)	Avg. Load (CFU/ml)	Load (CFU/ml)	Avg. Load (CFU/ml)	Load (CFU/ml)	Avg. Load (CFU/ml)
01.	3.96×10^5	4.5×10^5	8×10^2	5.6×10^2	3.56×10^5	3.21×10^5
02.	2.68×10^5		4×10^2		2.07×10^5	
03.	6.86×10^5		5×10^2		4.08×10^5	

N.B. Avg. = average; SL. No. = Serial number; CFU = Colony Forming Unit. Each time 100 µl solution was inoculated in Petri dish

4.4.2 The antibacterial activity of Black cumin ethanolic extracts against *V. cholerae*

The antibacterial activity of black cumin extracts in ethanol is shown in Table 5 by comparing the average load of the bacteria without and with black cumin ethanolic extract at 50%, 40%, 30% and 20% concentration. No growth of *V. cholerae* was found up to 40% concentration of extract. The average load of the bacteria reduced at 99.7% and 12.8% in 30% and 20% concentration of the extract respectively.

Table 05: *V. cholerae* load before and after treatment with black cumin ethanol extract

SL. No	Without extract (CFU/ml)		With black cumin ethanolic extract at different concentration (CFU/ml)							
	Load	Avg. Load	50%		40%		30%		20%	
			Load	Avg. Load	Load	Avg. Load	Load	Avg. Load	Load	Avg. Load
01.	3.96×10^5	4.5×10^5	N	N	N	N	9×10^2	1.06×10^3	3.56×10^5	3.92×10^5
02.	2.68×10^5		N		N		12×10^2		4.20×10^5	
03.	6.86×10^5		N		N		11×10^2		4×10^5	

N.B. Avg. = average; SL. No. = Serial number; N = No growth, CFU = Colony Forming Unit. Each time 100 µl solution was inoculated in Petri dish.

Additionally, two more concentrations of the extract (35% and 25%) were prepared to determine the antibacterial activity. At 35% concentration of ethanolic black cumin extract, not any growth of the bacteria observed. But at the 25% concentration average load found was 2.4×10^5 that indicated a reduction of load in 46.6%

Table 06: *V. cholerae* load before and after treatment with black cumin ethanol extract at 35% and 25% concentration.

SL. No	Without extract		With black cumin ethanolic extract at different concentration			
			35%		25%	
	Load (CFU/ml)	Avg. Load (CFU/ml)	Load (CFU/ml)	Avg. Load (CFU/ml)	Load (CFU/ml)	Avg. Load (CFU/ml)
01.	3.96×10^5	4.5×10^5	N	N	1.2×10^5	2.4×10^5
02.	2.68×10^5		N		3.2×10^5	
03.	6.86×10^5		N		2.8×10^5	

N.B. Avg. = average; SL. No. = Serial number; N = No growth, CFU = Colony Forming Unit. Each time 100 µl solution was inoculated in Petri dish.

Process B: By determining the zone of inhibition of black cumin extract (both in acetone and ethanol) against *V. cholerae*. (Disc diffusion)

The efficacy of different concentrations of ethanolic and acetone extracts of black cumin seed against *V. cholerae* is shown in figure 09. Both extracts of black cumin seed exhibited antibacterial activity. Antibacterial activity was evaluated by measuring the zone of inhibition in mm.

It was observed in the study, with the increase of the concentration of extract from 20% to 50%, the diameters of inhibition zone (mm) of black cumin seed against the bacteria were increased. The zone of inhibition of acetone black cumin extract ranged from 6.15 ± 0.05 mm to 13.275 ± 0.025mm. At 50%, 40%, 30%, 25% and 20% concentrations the zones of inhibition (mm) were 13.3, 11.05, 13.05, 12 and 6.15 respectively.

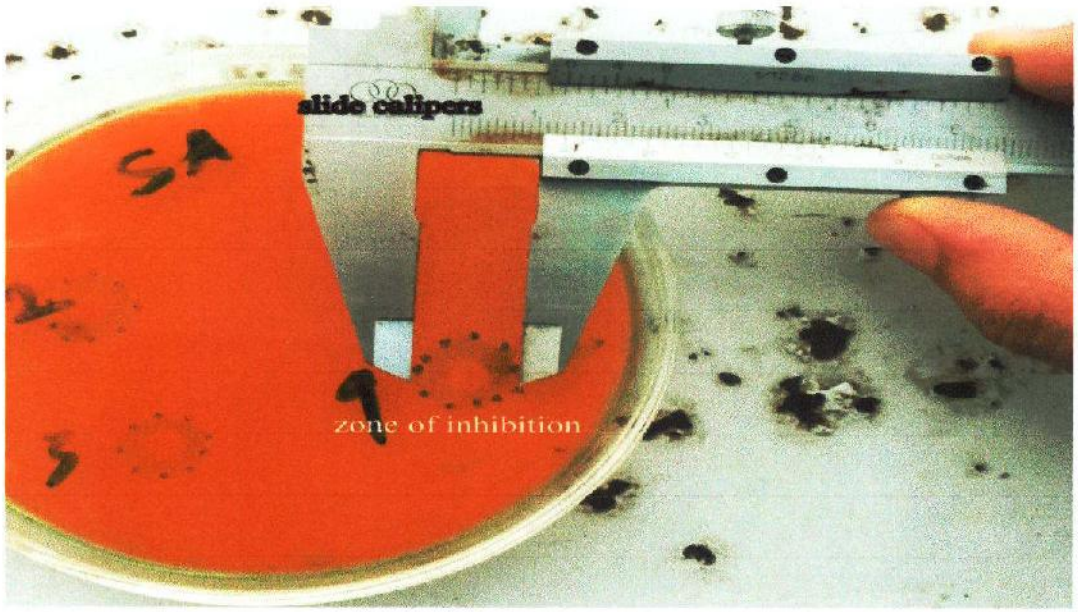

Figure 07: Measuring zone of inhibition at different concentrations of Acetone extracts.

On the other side, at 20% concentration black cumin ethanol extract, no inhibition zone was found. The zone of inhibition of ethanol black cumin extract ranged from 5.75 ± 0.00 mm to 11.15± 0.0 mm up to 50% concentration. At 50%, 40%, 30% and 25% concentrations the zones of inhibition (mm) were 11.15, 10.1, 9.5 and 5.75 respectively.

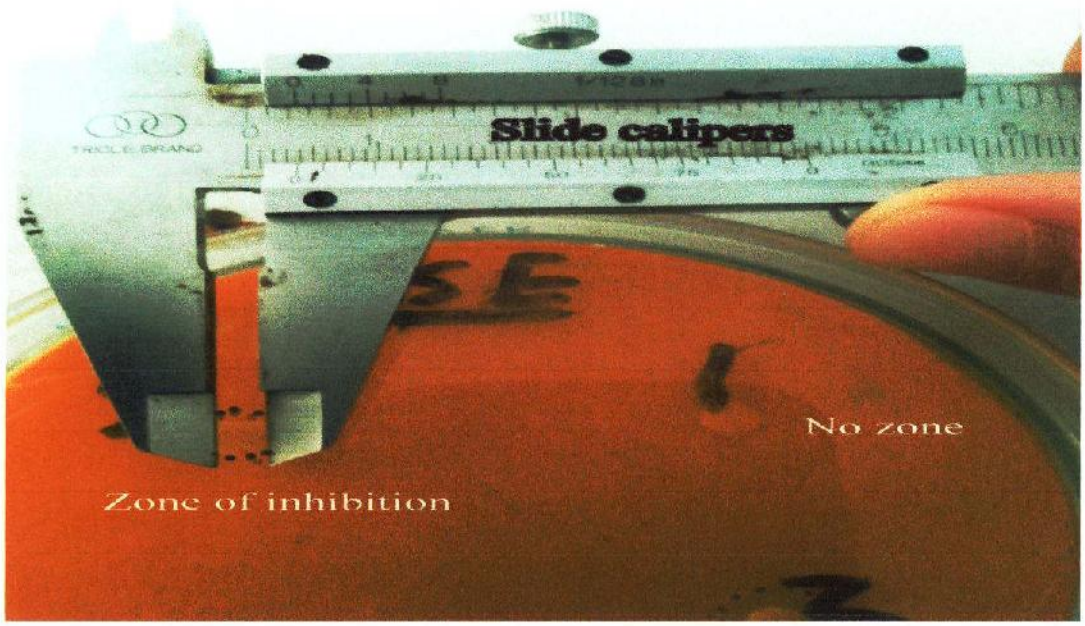

Figure 08: Measuring zone of inhibition at different concentrations of Ethanol extracts.

These results are shown in figure 09. The result reveal that zone of inhibition produced by the both extracts of black cumin seed had a relationship with the concentration of extracts.

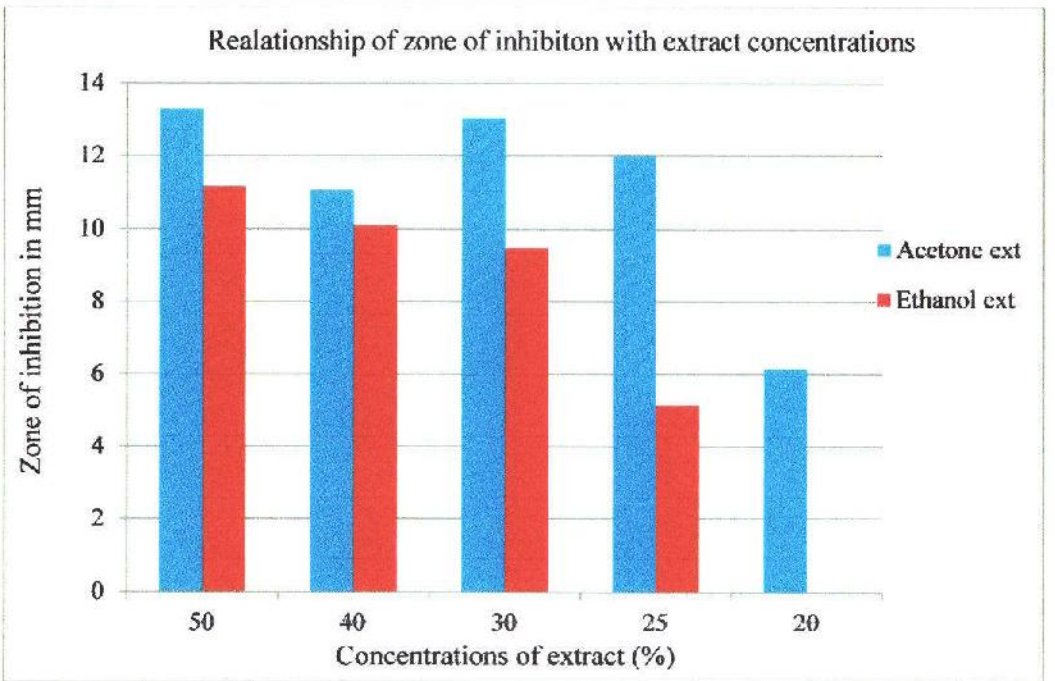

Fig 09: Effect of *N. sativa* in different concentrations of two extracts against *V. cholerae*

Process C: By determining the minimum inhibitory concentration (MIC) on the test organisms (*V. cholerae*)

The MIC of the cumin (*N. sativa*) seed extract was determined by tube dilution techniques in nutrient broth. Growth of the bacteria was assessed by recording optical density using a spectrophotometer (T 60 UV-Visible spectrophotometer). For this study the optical density was set as 600 nm because at this wavelength, absorbance of light by other molecules in the cell, such as flavins and carotenoids, is minimal (MMBB255 week 5).

4.4.3 Determination of minimum inhibitory concentration (MIC) of the acetone extract

The MIC value of black cumin acetonic extract is shown in Table 8.The result revealed that at the concentration of 50% 40%, 30% and 25%, bacterial growth was inhibited. So 25%

(250µl/ml) was the highest dilution at which growth was minimal. So the MIC value for black cumin extract in acetone was 250µl/ml (25%).

Table 07: Minimum inhibitory concentration (MIC) of black cumin acetone extract against *V. cholerae* on nutrient broth determined by a spectrophotometer (OD_{600})

Tube no.	1	2	3	4	5	6	7	8	9
Concentration (µl/ml)	500	400	300	250	125	62.5	31.25	15.6	control
Absorbance (Abs)	0.030	0.058	0.062	0.089	0.134	0.379	0.575	0.623	0.850

4.4.4 Determination of minimum inhibitory concentration (MIC) of the ethanol extract

The MIC value of black cumin ethanolic extract is shown in Table 9.The result revealed that at the concentration of 50% 40% and 30%, bacterial growth was inhibited. So 30% (300µl/ml) was the highest dilution at which growth was minimal. So the MIC value for black cumin extract in ethanol was 300µl/ml (30%).

Table 08: Minimum inhibitory concentration (MIC) of black cumin ethanol extract against *V. cholerae* on nutrient broth determined by a spectrophotometer (OD_{600})

Tube no.	1	2	3	4	5	6	7	8	9
Concentration (µl/ml)	500	400	300	250	125	62.5	31.25	15.6	control
Absorbance (Abs)	0.027	0.058	0.062	0.122	0.361	0.327	0.537	0.716	0.850

Chapter five

DISCUSSION

5. Discussion:

The spoilage and degradation of dried fish and its shelf life were depended largely on microbial population and other factors. All the preservation technologies involving the use of heat, radiation and/ or chemicals aim at reducing and eliminating this spoilage factors. Both short term and long term preservations of dried fish and fisheries product have been attempted. In the present study, three dried fish samples had been analyzed for microbiological parameter, focused on *V. cholerae* load which is a devastating pathogen of shrimp and human being and the antimicrobial effect of black cumin extracts (*N. sativa*) upon the isolated bacteria was observed.

The result of the present experiment showed that, the average *V. cholerae* load in Lottya (*H. nehereus*) and Chingri (*P. monodon*) were 3.1×10^4 CFU/g and 2.67×10^3 CFU/g. No *V. Cholerae* was found in Churi shutki (*T. haumela*).

According to WHO, 1991, *V. cholerae* has been associated with consumption of numerous types of fishery products including: crustaceans (shrimp, crab, lobster), shellfish (oysters, clams, mussels, scallops, abalone) and finfish, including dried processed fish.

Blake *et al.,* (1983) stated that the principle environment of *V: cholerae* is man, estuaries and shelfish. The study showed that dried fishes sold in fish market were contaminated with the pathogenic bacteria. This might be due to the unhygienic handling of the fisher folks, improper processing and unhygienic vendors and, venting area. Prakash *et al.*, (2011) stated that incidence of pathogens in the dry fish samples of fish market may be attributed to external contamination and poor sanitation in fish handling at ambient temperature which supports the presence of *V. cholerae* in dried fish.

All the *V. cholerae* isolates from both dried fish sample, produced the same results in biochemical tests. Reactions in TSI agar slant revealed yellow slant and butt as well as no production of gas and hydrogen sulphide gas was observed as described by the Centers for Disease Control and Prevention (CDC), 2014. All the isolates showed positive results in indole formation, oxidase test ans MR test which support the findings of Kaysner and Depaol (2004). According to CDC (2014), *V. cholerae* differs from other *Vibrios* by its ability to grow without

any added salt as it is non halophilic. But the growth can be stimulated by adding salt up to 3% which support the result of salt tolerance test of this study.

The present study has found the potential antibacterial effect of the medicinal plant, black cumin (*N. sativa)* seed on the isolated human pathogenic bacteria *V. cholerae* from dried fish. Black cumin seed was chosen in this study as it is digestible to human and its tremendous health benefit as it was described earlier.

Extracts of the seed were prepared in two non-polar (ethanol and acetone) solvent by following appropriate procedure. Then the antimicrobial effects of the both extracts were examined in three different processes.

Process A. Enumerate the load before and after treatment

The average load of *V. cholerae* was enumerated with and without treatment of black cumin extract. In acetone extract of the seed, no growth of the bacteria was found up to 30% concentration and the average load found in 25% of acetone extract was 5.6×10^2 which indicated the reduction of *V. cholerae* load in 99.8%.

In ethanolic extract of black cumin seed, the study revealed that up to 40% concentration of the extract, no bacterial growth was found. The average load of the bacteria reduced at 99.7% in 30% concentration of the extract.

This result indicated the high susceptibility of *V. cholerae* against the black cumin seed extracts. Tahir *et al.*, (2006) reviewed the effect of black cumin seed extract with different solvent (Aqueous, ethanolic, methanolic, ether, hexane, ethereal) and revealed the bacterial species that were more susceptible included *Salmonella, Shigella shigae, Bacillus cereus, V. cholerae, Pseudomonas aerogenosa* which supports the findings of present study.

Process B: By determining the zone of inhibition of black cumin extracts (both in acetone and ethanol) against *V. cholerae*.

In the present study, it was observed that with the increase of the concentration of extract from 20% to 50%, the diameter of inhibition zone of black cumin seed against the bacteria were increased. The zone of inhibition of acetone black cumin extract ranged from 6.15 ± 0.05 mm

to13.275 ± 0.025mm. The zone of inhibition of ethanolic black cumin extract ranged from 5.15 ± 0.00 mm to 11.15± 0.0 mm up to 30% concentration.

On the other side, at 20% concentration ethanolic black cumin extract, no inhibition zone was found. That indicated that, *V. cholerae* was resistant at this concentration in ethanolic extract.

Mandal, *et al.,* 2014, evaluated the antibacterial activity of *Mimusops elengi (M. elengi)* seed (MSE) and *Bauhinia variegate (B. variegata)* seed (BSE) extracts against *Salmonella enterica* serovar Typhi *(S. typhi)* and *V. cholerae* by agar diffusion method in ethanolic extract. The *V. cholerae* BSE (500 µg) and MSE (500 µg) had the Zone Diameter Inhibition was 12-17 mm amongst the different isolates. These results have a similarity with the present study as the zone of inhibition ranges from 11- 14 mm in the both extracts of black cumin seed.

Process C: By determining the minimum inhibitory concentration (MIC) on the test organism (*V. cholerae*)

The MIC of the cumin (*N. sativa*) seed extract was determined by tube dilution techniques. The tube with lowest concentration yielding no bacterial growth was considered as MIC. Growth of the bacteria was assessed by recording optical density using a spectrophotometer (T 60 UV-Visible spectrophotometer).

In black cumin acetonic extract, 25% (250µl/ml) was the highest dilution at which the reading of the spectrophotometer (absorbance) was 0.089. That denotes the transmission of light was almost 100% at this concentration as no bacterial cell was on that tube to absorb the light.

In black cumin ethanolic extract, 30% (300µl/ml) was the highest dilution at which the reading of the spectrophotometer (absorbance) was 0.062. That denotes the transmission of light was almost 100% at this concentration as no bacterial cell was on that tube to absorb the light.

Alam *et al.*, (2010) analyzed the antibacterial effect of chloroform and ethanol extracts of black cumin seeds (*N. sativa*) on five water and food borne pathogens. The study found the MIC value of the ethanolic extract was 2250 µg/ml and concluded that the extract could be used against multiple drug resistant *V. cholerae* bacteria as it showed high susceptibility to this extract. Apart

from this, different experiments were carried out to determine MIC of black cumin seed extracts of different pathogenic bacteria by various methods (appendix-1).

From all the result found above, it can be summarized that black cumin acetone extract is more effective than ethanol. Sharma *et al.,* (2009) stated that different extracts differed significantly in their vibriocidal properties with respect to different solvents. About 16 medicinal plants of India were selected for his study and the plant extracts were made in water, acetone and ethanol to determine the antimicrobial effect (MIC) against *V. cholerae* and *V. parahaemolyticus*. The study found that, The MIC values in ethanolic extracts were a minimum against *V.cholerae, V. cholerae non-O1, and V.parahaemolyticus.*

Singh *et al.*, (2013) choose fifteen types of Indian spices and prepared their extract in three types of solvents (ethanol, methanol and acetone) with the concentration of 70% against three *Vibrio species (V. cholerae, V. alginolyticus, V. parahaemolyticus)* in which the extract in acetone showed maximum inhibitory effect in all fifteen different spices while minimum inhibitory effect was seen in case of ethanol. This research support the present study as acetone extract of black cumin was found more effective than ethanol.

The detection of *V. cholerae* had brought up from the concern that dried fish samples might have the possible risk to public well being upon consumption; and hence highlighting the need for improvement of hygiene and sanitation practice.

Apart from that, the present study also revealed the antimicrobial susceptibility patterns of the bacteria against black cumin seed extracts. In a study conducted in India showed that among 94 isolates, 43 strains of *V. cholerae* contained multiple-drug resistant plasmids and exhibited resistance to ampicillin, neomycin, tetracycline, gentamicin, streptomycin, sulfonamide, furazolidone and chloramphenicol. (Thungapathra *et al.*, 2002). A substantial health risk is the antibiotic resistance properties of *V. cholerae* and is a major clinical hindrance in treating infections caused by the pathogen and ultimately leading to an increase of morbidity (Tenover, *et al.*, 2006). So, black cumin extracts can be an alternative solution in this regard.

6. Conclusion:

The availability of microorganisms e.g. *V. cholerae*, in dried fish indicates the external contamination and poor sanitation in processing procedures as well as the market conditions. Since these dried fish products are commonly consumed, their consumption may pose a risk of food borne infection and intoxication to the consumers. Black cumin (*N. sativa*) is a medicinal plant, whose seeds have been used for centuries to treat various infectious diseases. From this study, it can be concluded that 25% acetone extract and 30% ethanol extract of black cumin are the minimum concentrations at which bacterial (*V. cholerae*) growth can be hindered. So, it can be an alternative solution to the farmers for ensuring the microbial safety by using the extracts during the processing of dried fish. Therefore, further work is needed to improve the extraction procedure of the black cumin seeds and to separate the components of cumin seeds that are responsible for its antibacterial activity.

Chapter seven

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APPENDIX

Appendix-1

Table: MIC of black cumin seed extracts of different types of bacteria

Extract	Bacteria	Method	Minimum inhibitory concentration (MIC) dose	(MIC) in Percentage (%)	Researcher
Ethanol extract	Methicillin resistant *Staphylococcus aureus*	Disc diffusion and Agar Dilution	0.2-0.5 mg/ml	20-50%	Forouzanfar, *et al.*, 2014
Black cumin seed oil	Gram-negative bacilli: *Escherichia coli, Salmonella enteric, Vibrio parahaemolyticus* ,	Broth microdilution	8 to 32 µg/ml	0.8-3.2%	
Aqueous and methanol extracts	*Gram-positive bacteria: Pseudo. aeroginosa, Klebseilla pneumoniae, Proteus vulgaris*	Disc diffusion	20 mg/ml and 100 mg/ml	200%	
Black cumin seed oil	*Vibrio Cholarae, Shigella shiga*	Disc diffusion	50 µg/ml	5%	Raza, *et al.*, 1999.
Seed oil	*Escherichia coli, Salmonella*	Disc diffusion	800 µg/ml	80%	Halawani, E. 2009.

	typhimurium				
Volatile oil	*S. aureus and E. coli*	Broth micro dilution	187µg/ml and 375µg/ml	18.7% and 37.5%	Ara, *et al.*, 2005.
Ethanol extract	*Salmonella typhimurium,*		0.084%	0.084%	Yasni, *et al.*, 2009.
Ethyl acetate	*Staphylococcus aureus*		1.88%	1.88%	
Ethanol extracts	*V. cholerae*	Broth microdilution	2250 µg/ml	225%	Alam, *et al.*, 2010.

Appendix-2 Photo gallery

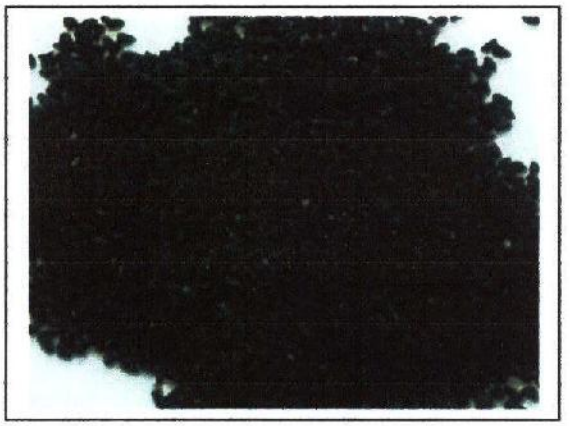

Photo 1: Black cumin seed/ kalojira (*N. sativa*)

Photo 2: Dried Lottya (*H. neherus*)

Photo 3: Dried Churi (*T. haumela*)

Photo 4: Dried shrimp (*P. monodon*)

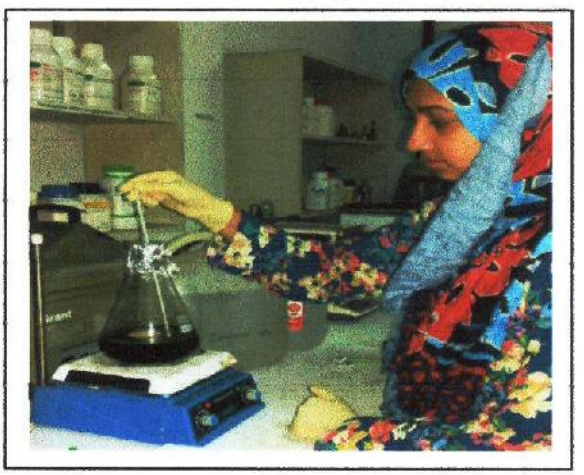

Photo 5: Media preparation

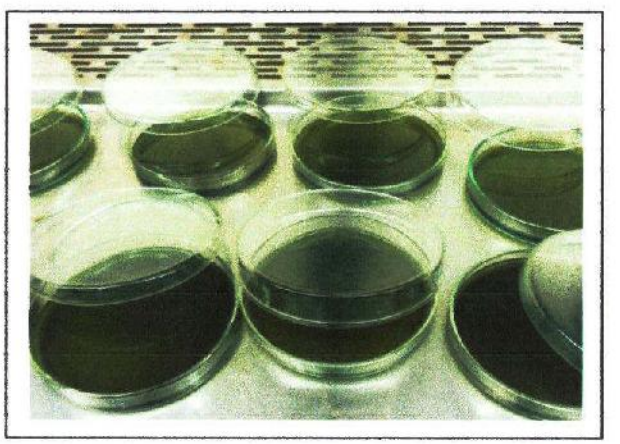

Photo 6: Petri dish with media

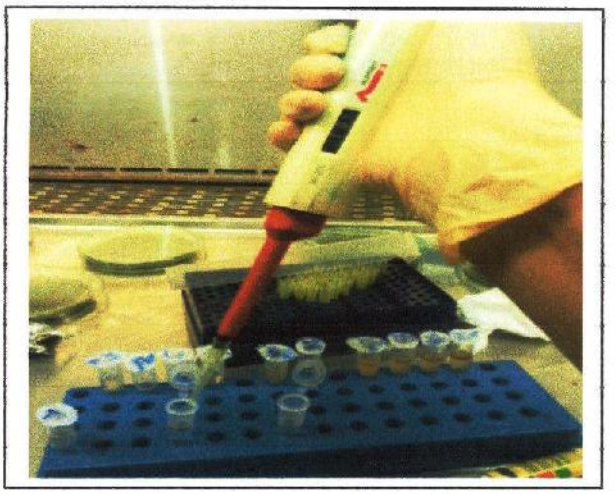

Photo 7: Serial dilution

Photo 8: Mixing in vortex

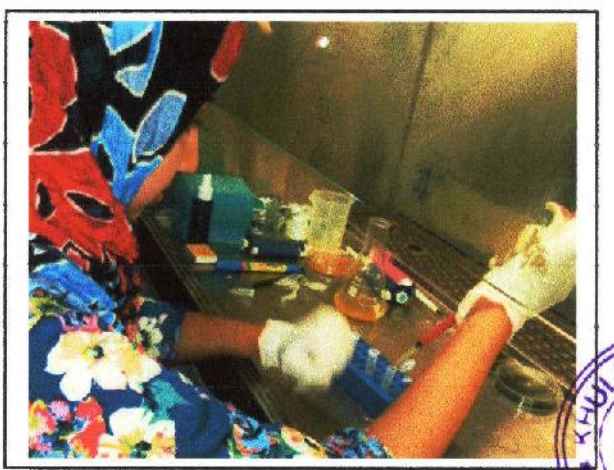

Photo 9: Inoculation

Photo 10: Spreading solution on media

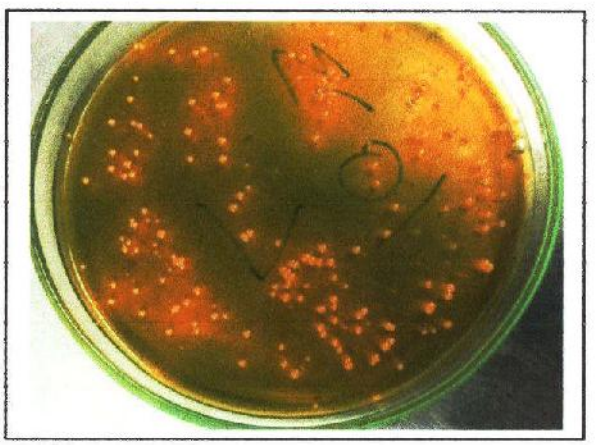

Photo 11: Colony of *V. cholerae*

Photo 12: Taking single colony

Photo 13: Result of salt tolerance test

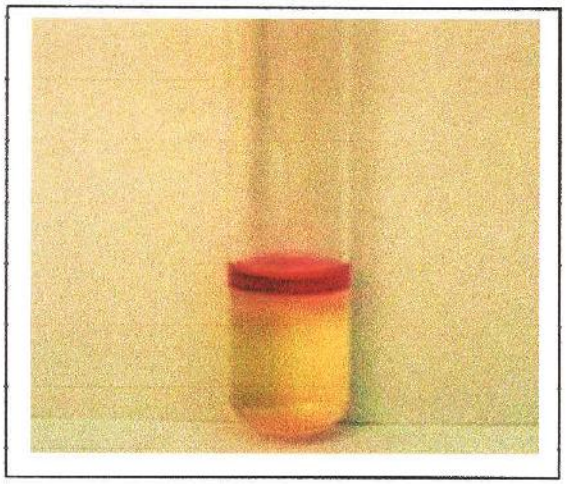

Photo 14: Result of Indole test

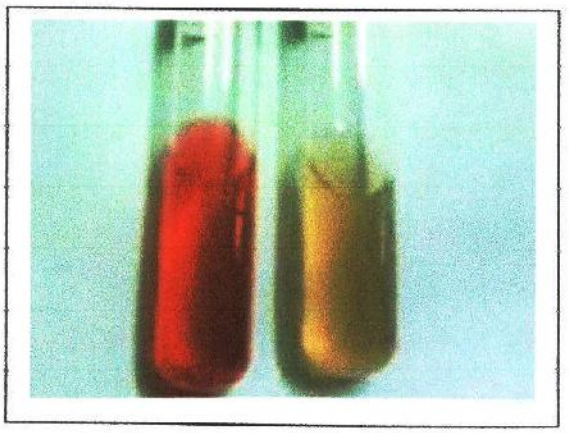

Photo 15: Result of MR test

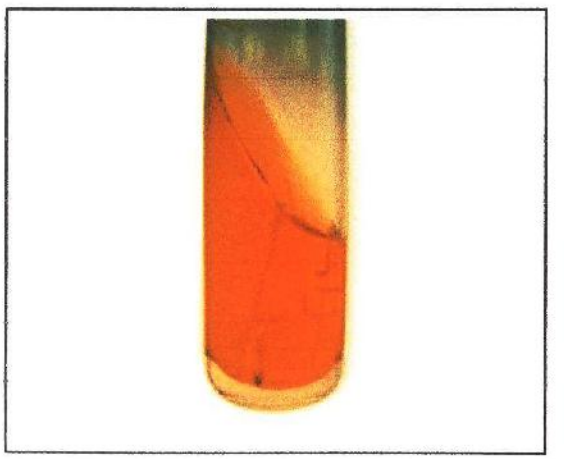

Photo 16: Result of TSI test

Photo 17: Extracts of *Nygella sativa*

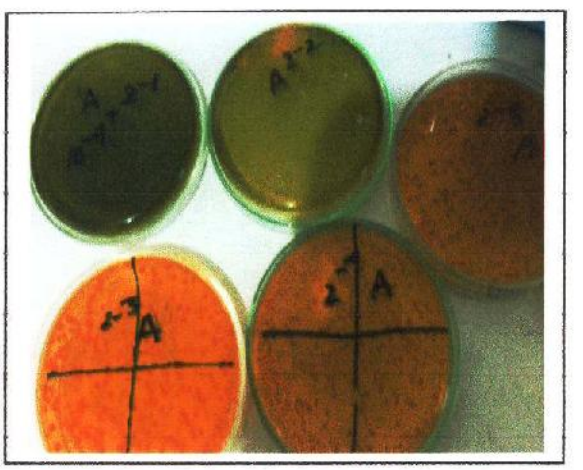

Photo 18: Treatment with different dose of Acetone extract

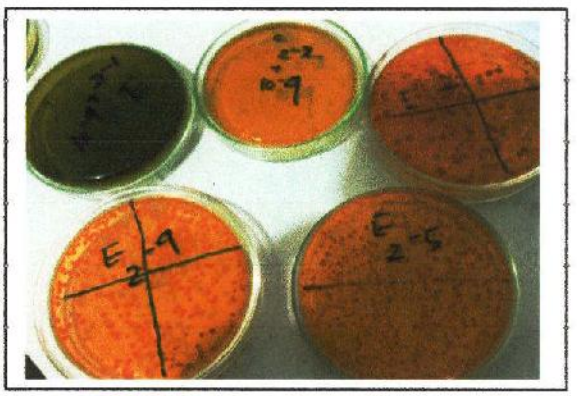

Photo 19: Treatment with different dose of Ethanol extract

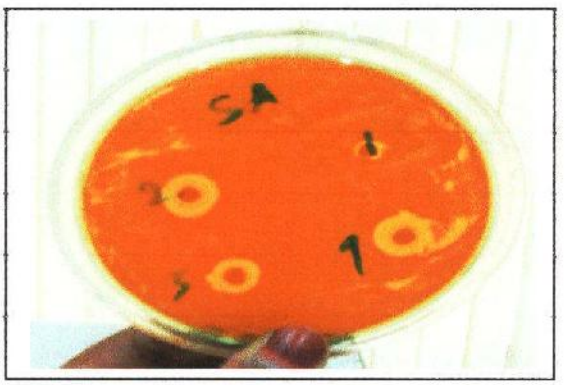

Photo 20: Zone of Inhibition

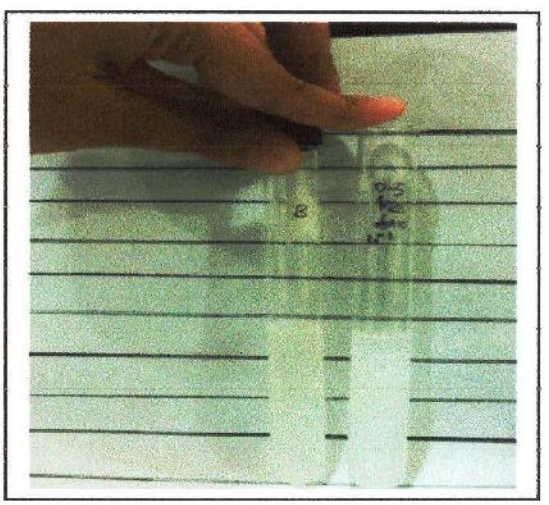

Photo 21: Adjusting Turbidity of bacterial solution with 0.5 Mcfarland

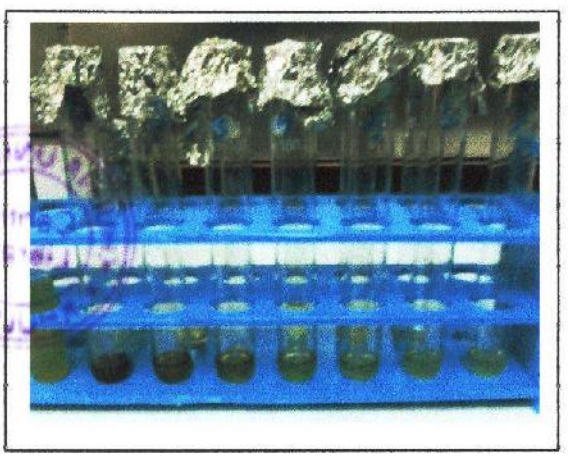

Photo 20: Tube dilution for MIC

Photo 21: Visual growth of the bacteria in nutrient broth

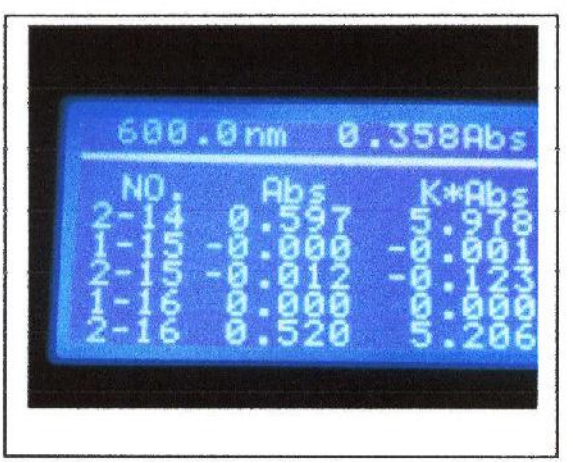

Photo 22: Spectrophotometer Reading