Isolation of *E. coli* and *Shigella* spp. from raw Tilapia, elucidation of their antibiotic susceptibility pattern and evaluation of the antimicrobial efficacy of Oregano *(Origanum vulgare)*

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by:

Salma

Student ID: 13236006

Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University

66, Mohakhali, Dhaka-1212

Bangladesh

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Declaration

This is to declare that the research work embodying the results reported in this thesis entitled "**Isolation of *E. coli* and *Shigella* spp. from raw Tilapia, elucidation of their antibiotic susceptibility pattern and evaluation of the antimicrobial efficacy of Oregano *(Origanum vulgare)***" submitted by Salma to the Department of Mathematics and Natural Sciences (MNS), BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology is a record of work carried out by me under the joint supervision of my supervisors. I further declare that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma.

Candidate

_____

Salma

Certified:

Dr. M. Mahboob Hossain

Supervisor

Associate Professor

Microbiology Program

Department of Mathematics and natural Sciences

Dhaka, Bangladesh

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations	***Descriptions***
E. coli	*Escherichia coli*
Spp.	*Species*
HGT	*Horizontal Gene Transfer*
EMB	*Eosin Methylene Blue*
XLD	*Xylose Lysine Deoxycholate*
SS	*Salmonella-Shigella*
TSI	*Triple Sugar Iron*
mm	*Millimeter*
cm	*Centimeter*
L	*Liter*
g/L	*Grams per liter*
MAR index	*Multiple Antibiotic Resistance Index*
AI	*Activity Index*
DNA	*Deoxy ribonucleic acid*
H_2O_2	*Hydrogen peroxide*
H_2S	*Hydrogen sulfide*
MHA	*Mueller-Hinton Agar*
MIC	*Minimum Inhibitory Concentration*
MBC	*Minimum Bactericidal Concentration*

Abstract

Tilapia (*Oreochromis* spp.) has become the third most important fish in aquaculture because of their protein content, larger size and rapid growth to harvest. Due to improper handling and poor hygiene, fresh marketed raw fishes are becoming a potential reservoir of antibiotic-resistant bacteria. For this study, 28 tilapia fish samples from different markets of Dhaka city were investigated for the presence of *E.coli* and *Shigella* spp. Different organs such as skin, gill, intestine, and muscle were investigated from which it was revealed that maximum *E. coli* were isolated from gills (44%), whereas, *Shigella* spp. were isolated maximum from skin (36%) and intestine (36%). Out of 28 tilapia fish samples, a total of 25 *E. coli* isolates and 11 *Shigella* spp. isolates were identified. Nine groups of antibiotics were chosen: Beta-Lactam, Aminoglycoside, Lincosamide, Macrolide, Nitroimidazole, Quinolone, Sulfonamide, Chloramphenicol, and Tetracycline to study antibiotic-resistant pattern of the isolated bacteria. Antibiotic susceptibility test results of both the types of isolates showed that all the isolates were sensitive to Streptomycin, Levofloxacin, Ciprofloxacin, Imipenem, Kanamycin, Amikacin, and Norfloxacin. However, all of the 25 *E. coli* isolates were resistant to Oxacillin, Penicillin, Erythromycin, Clindamycin, Cephalexin, Amoxycillin and Metronidazole, and all of the 11 *Shigella* spp. isolates were resistant to Oxacillin, Ampicillin, Ceftazidime, Ceftriaxone, Co-Trimoxazole, Penicillin, Erythromycin, Clindamycin, Cephalexin, Amoxycillin, Azithromycin, Cefixime and Metronidazole. These are some common antibiotics used in day-to-day life. As plants are considered a new source of antimicrobial agents, the antibacterial property of Oregano (*Origanum vulgare*) was evaluated against the isolates. Among methanolic, ethanolic and aqueous extract, methanolic extract of oregano in 100% DMSO showed the best results for both *E. coli* and *Shigella* spp. isolates. An isolate of *Shigella* spp. isolated from the skin of a fish sample gave a high average zone of inhibition (19 mm) and the highest Activity Index (AI) for methanolic extract of oregano in 100% DMSO. Therefore, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of this isolate were measured. MIC was found to be 160 µg/ml and MBC was 165 µg/ml.

CHAPTER 01: INTRODUCTION

Introduction

1.1. Background:

Tilapia (*Oreochromis* spp.) farming is of great importance in Bangladesh as well as worldwide. In 2002, the worldwide production of tilapia exceeded 1,500,000 metrics tons that increased annually[1]. After carp and salmon, tilapia is the third most important fish in aquaculture because of their protein content, larger size and rapid growth (6 to 7 months)[2]. The natural production of fish has been decreased alarmingly for various factors, therefore, tilapia has become one of the most important fish species widely cultivated in Bangladesh to meet the increased protein demand[3]. Tilapia Mozambique (*Oreochromis mossambicus*) was the first imported tilapia species in Bangladesh from Thailand in 1970, later, Bangladesh Fisheries Research Institute imported *Oreochromis niloticus* in 1987[3]. The increase in production, overstocking, unbalanced physiology, poor water quality, and many stressors has increased the vulnerability of the fishes toward pathogens[4]. Bacterial infection is a serious threat to fish farmers as it can hamper fish production as well as infect users/handlers of raw fish. Also, with the increase in various kinds of cuisines that may include semi-cooked food, an increase in food-borne diseases has been reported.

Raw fish can be a potential reservoir of a number of pathogens such as *E. coli* and *Shigella.* Prolonged indiscriminate use of antibiotics to treat infections and promote growth has caused the development of resistant strains in bacterial populations which are hazardous to public health[6]. Antibiotic-resistant bacterial contamination of food is an alarming major threat to public health. This can also limit the available treatments against severe bacterial infections as the resistant gene can transfer from one pathogenic bacteria to another via Horizontal gene transfer[7]. Rocha (2017) has reported that food-borne strains resistant to antibiotics can pose a risk to consumers' health and favor in the transference of the phenotype to humans through the food chain. In recent years, an increase in multi-drug resistant bacteria has been reported.

Escherichia coli is a gram-negative, rod-shaped, coliform bacterium of genus *Escherichia*. *Shigella* is a genus of gram-negative rod-shaped bacteria genetically closely related to *E. coli*. Both are known to produce deadly Shiga toxin, also, the cause of diarrhea worldwide. The presence of *E. coli* and *Shigella* have been reported to be present in fish and farming waters as well as in contaminated ice used for storage[6]. Similar to *E. coli, Shigella* is one of the leading bacterial causes of diarrhea worldwide, causing an estimated number of deaths between 74,000 and 600,000 each year[9]. It has been reported that bacteria have an ability to transmit and acquire resistance to antibiotics. Since, plants are viewed as a new resource for producing antimicrobial agents, the efficacy of plant extracts against *E. coli* and *Shigella* needs to be checked to find an alternative to antibiotics.

The regeneration of newer and more deadly diseases due to the exposure of antibiotic-resistant microbial strains have called for the need to discover novel antimicrobials[8]. Plants are rich in a wide range of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found to have antimicrobial properties. This study focused on the antimicrobial property of *Origanum vulgare* (Oregano). Oregano essential oil has been used as folk medicine in some parts of the world, however, it is mostly used as dried culinary herb worldwide. Oregano has been used as a relaxant, antibacterial, antiseptic and fungicide, and also to boost the immune system since ancient times[10]. In Bangladesh, oregano is mostly imported as dried product and used in many food preparations.

1.2. Escherichia coli:

Escherichia coli is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium. *E. coli* were discovered in 1855 in human colon by German bacteriologist Theodor Escherich. The harmless strains of *E. coli* are part of normal flora in the human gut where they help the host to produce vitamin K_2. Though most strains of *E. coli* are harmless, certain strains can cause serious food poisoning and contamination.

The *E. coli* bacteria can be easily grown and cultured in a laboratory. Using *E. coli* many milestones in the medical field were successful such as Recombinant DNA technology, the invention of scientific fuel, many medicines, fighting cancer and even

Bio computer[11]. However, *E. coli* are evolving and are becoming responsible for creating major health hazards in the human body. They are responsible for various reports of contaminated foods and beverages, those that produce Shiga toxin. It is named Shiga toxin as the toxin is virtually identical to that produced by *Shigella dysenteria* type1[12]. The best known deadly strain of *E. coli* that produce Shiga toxin is *E. coli* 0157:H7[13].

1.3. Shigella spp.

Shigella is a gram-negative, facultative anaerobe, non-spore forming, non-motile, rod-shaped bacteria and is genetically closely related to *E. coli. Shigella* was first discovered in 1987 by Kiyoshi Shiga. It is the causative agent of shigellosis, which is a disease caused in only primates but not in other mammals. During Shigella infection, initially dysentery is noticed, but diarrhea can also be an after effect. Shigellosis infection can be caused by ingestion. Generally, it invades the epithelial lining of the colon, causing severe inflammation and death of lining cells of the colon. *Shigella flexneri* produces toxins such as ShET1 and ShET2, which may contribute to diarrhea. They can also result in stool containing blood, mucus or pus and in rare cases, it can cause seizures in children.

1.4. Phylogenetic tree of E. coli and Shigella:

E. coli and *Shigella* were reported to have exchanged genetic material with close relatives[14]. The phylogenetic tree is given below:

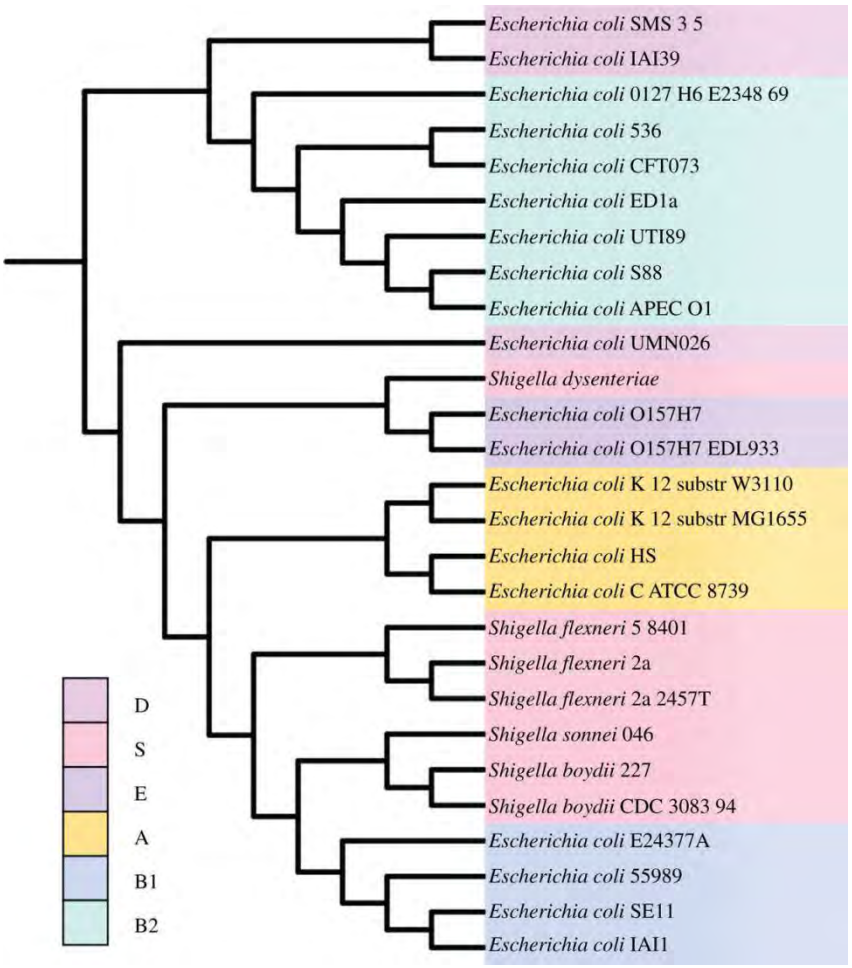


Figure 1.1: The Phylogenetic Tree containing *E. coli* and S*higella*.

1.5. Antibiotic Resistance:

The efficacy of antibiotics, which had transformed medicine and saved millions of lives, has become vulnerable due to the rapid emergence of resistant bacteria worldwide. The overuse of antibiotics has driven the evolution of resistance among bacteria. Among bacteria, genes can be inherited from relatives or can be acquired from non-relatives on mobile genetic elements such as plasmids, this can allow antibiotic resistance to be transferred among different species[15]. This is known as Horizontal Gene Transfer (HGT). Mutation can also allow resistance when bacteria are exposed to antibiotic drugs frequently, leading them to understand the drug mechanism. Resistant bacteria are left behind to reproduce as a result of natural selection when antibiotics remove drug-sensitive comptitors[15].

Bacteria are reported to develop multiple antibiotic resistance and are referred to as "superbugs". In many parts of the world, antibiotics are overused and misused in people and animals, and frequently applied without professional advice[17]. Even though there are warnings against overuse, antibiotics are overly-prescribed worldwide[16]. For example, people are prescribed antibiotics for viral infections like common cold, flu, sore throat etc., and in animals' antibiotics are used as growth promoters[17]. These resistant microbes can spread between people and animals, and from person to person due to poor infection control, insufficient sanitary conditions and improper food-handling[18]. Hence, the prevalence of antibiotic-resistant bacteria in humans, animals, food, and environment is increasing creating a worldwide issue.

1.6. Antimicrobial property of Origanum vulgare (Oregano):

Origanum vulgare, commonly known as Oregano or wild marjoram, belongs to the Lamiaceae family. Oregano is a perennial plant that is known for its flavorful dried leaves and flowering tops. Oregano grows in warm temperate areas and grows from 20 – 80 cm tall, with opposite heart-shaped leaves 3 to 9 cm long, produced in erect spikes[19]. Oregano is used as a culinary herb and is also used as a medicinal herb in various parts of the world. Oregano is used as a relaxant, antibacterial, antiseptic and fungicide, and also to boost the immune system. It has also been used as herbal remedy for skin burns, cuts, and bruises, herbal remedy for a sore throat, asthma, colds, coughs, and flu. Oregano has also been found to be high in antioxidant that can help prevent cancer[10].

Scientific Classification Taxonomic hierarchy of Origanum vulgare (Oregano) (Integrated Taxonomic Information System):

Kingdom Plantae– plantes, Planta, Vegetal, plants

Subkingdom– Viridiplantae

Infrakingdom– Streptophyta – land plants

Superdivision– Embryophyta

Division– Tracheophyta – vascular plants, tracheophytes

Subdivision– Spermatophytina – spermatophytes, seed plants

Class– Magnoliopsida

Superorder– Asteranae

Order– Lamiales

Family– Lamiaceae

Genus– Origanum L.

Species– Origanum vulgare L. – oregano

Oregano is known to be important for maintaining human health with the increase of studies for natural therapies. Oregano is reported to contain carvacrol (it is a monoterpenoid phenol), which has antimicrobial properties. Along with carvacrol, some of the most important components are – limonene, gamma–cariofilene, rho–cymenene, canfor, linalol, alpha–pinene and thymol. Most of these components are responsible for its antioxidative, antimicrobial and antifungal effects. Thus, antibacterial effects of oregano have been reported against some gram-negative and gram-positive bacteria[20-21].

1.7. Objectives of the study:

The objectives and aims of the study are given below:

1. To isolate and identify *E. coli* and *Shigella* from tilapia fish samples from different parts of the Dhaka city.
2. To study the antibiotic resistance pattern of the bacterial isolates.
3. To determine the efficacy of the antimicrobial property of Oregano against the isolated bacteria.
4. To determine the MIC and MBC of the plant extract.
5. To determine the phytochemical components of Oregano extracts.

Chapter 02: Methods and Materials

Methods and Materials

2.1. Place of work:

The entire study was carried out in the laboratory of the Department of Mathematics and Natural Sciences (MNS) at BRAC University.

2.2. Collection of samples:

Twenty-eight specimens of tilapia fish were collected from different markets in Dhaka city. Skin surface, gills, intestine, and muscle of each raw fish were collected in sterile zipper bags. Each step was handled carefully and hand gloves were worn to prevent any contamination.

2.3. Sampling:

The skin sample was taken by rubbing a sterilized cotton swab over the skin and the homogenized in 0.85% peptone salt solution. Gills, intestine, and muscle samples were homogenized in 0.85% peptone salt solution using mortar and pestle. One ml of the samples were added to test-tubes containing6ml of EC broth, which is a selective medium for the growth of coliforms and *E. coli* from food and environmental samples, and incubated for 24 hours at 37°C.

2.4. Growth on MacConkey Agar:

MacConkey Agar is recommended for use as a selective and differential medium for the isolation of gram-negative bacilli (including coliform organisms and enteric pathogens), on the basis of lactose fermentation. For primary screening of *E. coli*, a loop full of broth culture was streaked on MacConkey agar and incubated at 37°C for 24 hours. *E. coli*, a lactose-fermenting bacterium, will form pink colonies surrounded by a zone of bile salt precipitation.

2.5. Growth on Eosin Methylene Blue (EMB) Agar:

The isolated pink colonies from MacConkey agar were streaked onto EMB agar and incubated at 37°C for 24 hours. EMB agar is a selective and differential medium

used to isolate fecal coliforms like *E. coli*, which produces a characteristic metallic green sheen.

2.6. *Growth on Xylose Lysine Deoxycholate (XLD) Agar:*

A loop full of the broth culture was streaked on XLD agar and incubated at 37°C for 24 hours for the primary screening of *Shigella*. The carbohydrate source is xylose which is fermented by most enteric except for *Shigella* species, and these colonies appear red on this medium as a result.

2.7. *Growth on Salmonella-Shigella (SS) Agar:*

The isolated red colonies from XLD agar were further streaked onto SS agar and incubated at 37°C for 24 hours. SS Agar is moderately selective and differential medium for the isolation and differentiation of *Salmonella* spp. and some strains of *Shigella* spp. *Shigella* colonies will appear colorless as they do not ferment lactose or produce hydrogen sulfide gas.

2.8. Culture Preservation:

Twenty-five *E. coli* and eleven *Shigella* isolates were obtained from the 28 samples and these were stored on EMB and XLD agar respectively and were sub-cultured every two weeks until the end of the study.

For long-time preservation, a loop-full of 24 hour bacterial culture inoculum was transferred into 5 ml nutrient broth and then incubated at 37°C for 24 hours. Later, 600µl of cultured broth was mixed with 400µl autoclaved glycerol and stored in -20°C refrigerator in 1.5ml centrifuge tubes.

2.9. Biochemical Tests:

Biochemical tests were performed to confirm whether the isolates were *E. coli* and *Shigella*. 24 hours of bacterial culture was used for all the biochemical tests. The tests are:

a. Gram Staining
b. Methyl Red (MR) test

c. Voges – Proskauer (VP) test
d. Citrate test
e. Oxidase test
f. Catalase test
g. Triple Sugar Iron (TSI) test
h. Urease test, Motility test and Indole test by MIU agar

2.9.1. Gram Staining:

Gram staining helps to distinguish between the two broad categories of bacteria: Gram-positive and Gram-negative. Gram-positive bacteria are distinguished by observing a purple stain while gram-negative bacteria are distinguished by observing a pink stain. The morphology of the bacteria can also be determined by this test.

There are four basic steps involved in the gram staining procedure. Firstly, a smear of bacteria is prepared onto a glass slide and it is heated for fixation. Then, Crystal violet is added to the smear. If the bacteria are gram positive their thick peptidoglycan layers retain the purple color. Secondly, Gram's iodine, a mordant, is applied which stabilizes the purple stain. Thirdly, 95% ethanol is used to decolorize the stain. In case of a gram-negative bacterium, its thin peptidoglycan layer is washed off. Finally, a counter dye safranin is added which is retained by decolorized gram-negative bacteria. Then, the slide is viewed under a compound microscope to analyze the color and shape of the bacteria[22].

2.9.2. Methyl Red (MR) test:

The bacterial cultures were inoculated in MRVP broth in clean test tubes and incubated overnight at a temperature of 37°C for 24 hours. After incubation, 5 drops of methyl red were added to broth cultures and were observed for the immediate color development. Appearance of a red color indicates a positive result[22].

2.9.3. Voges-Proskauer (VP) test:

The bacterial cultures were inoculated in MRVP broth in clean test tubes and incubated overnight at a temperature of 37°C for 24 hours. After incubation, 0.6ml

of Barrit's reagent A and 0.2ml of Barrit's reagent B were added along with a gentle shake to expose the medium to atmospheric oxygen. The tube was then kept undisturbed for 10-15minutes and the solution was then observed for color changes to determine whether the result is positive (pink-red) or negative(yellow)[22].

2.9.4. Citrate test:

Two milliliter of Simmon's citrate agar were added to clean vials and the vials were autoclaved. They were left to cool at a slanted position in order to create a butt and slant. Then, a loop of a single colony of 24 hours bacterial culture was then streaked on the slant of the media from bottom to top using a zigzag motion with the loop and the vials were incubated at 37°C for 24 hours. After incubation, color change to blue is considered to be positive and no color change was considered to be negative[22].

2.9.5. Oxidase test:

This test is performed to detect whether the bacteria produces cytochrome c oxidase, which is an enzyme of the bacterial transport system. All aerobic bacteria are oxidase positive. In positive cases, a deep blue or purple stain appears within 5-10 seconds. Kovac's Oxidase Reagent was used to perform this test[22].

2.9.6. Catalase test:

A sterile glass slide was placed on a petri dish and a small amount of organism was picked using a sterile inoculating loop. Then, 1 drop of 3% H_2O_2 was added on the organism using a dropper. Finally, an immediate evolution of oxygen bubbles was observed[22].

2.9.7. Triple Sugar Iron (TSI) test:

The sugar utilization test or carbohydrate utilization test is used to check whether the organism can utilize the carbon source. For this procedure, a straight inoculating needle was used to pick an isolated bacterial colony and inoculate the TSI agar slant by first stabbing the butt down to the bottom, and then streaking the surface of the slant. After incubating the test tubes for 24 hours at 37°C, the results were interpreted accordingly:

- Acid production: Changes the medium red to yellow. The organism can ferment the given carbohydrate and produce organic acids thereby reducing the pH of the medium into acidic.
- Acid and Gas production: Changes the medium red to yellow along with gas bubbles. The organism can ferment the given carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the cracks of the medium.
- The Absence of fermentation: The medium retains the red color. The organism cannot utilize the carbohydrate but continues to grow in the medium using other energy sources in the medium[22].

2.9.8. Urease test and Motility test using MIU agar:

For the preparation of MIU agar, MIU was prepared in test tubes and autoclaved. After autoclave, the test tubes containing agar was left to cool down to about 50°C and to it filtered 40% urea solution was added. The media was mixed properly and left to solidify.

After solidification, a colony from a 24 hours old bacterial culture was picked up by a sterile inoculating needle and inoculated in the medium by stabbing the needle down into the media. The test tubes were incubated at 37°C for 24 hours, after which appearance and color changes were observed[22].

2.9.9. Indole test:

For Indole test, tryptophan broth was inoculated with bacterial culture and incubated at 37°C for 24 hours. After incubation, 5 drops of Kovac's reagent was added to the broth culture. The color change was observed to determine whether the result is positive (cheery red ring) or negative (yellow)[22].

2.10. Antibiotic Susceptibility Test:

For antibiotic susceptibility test, Mueller-Hinton agar is primarily used in which lawn culture of tested bacteria are done. Commercially available antibiotic disks were used for this test. The procedure is called the Kirby-Bauer method.

In case the bacterium is sensitive to the antibiotic, a clear ring, or zone of inhibition will be observed and if the bacterium is resistant to any particular antibiotic it will grow over the antibiotic disks. Sometimes bacteria may gain resistance after a certain period of time and thus creating secondary zones of growth[23]. The list of commercial antibiotics used are given below:

Table 2.1: Antibiotic disks, their amount, and Zone of Inhibition size for Enterobacteriaceae.

Serial No.	Antibiotics	Disk content	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
1	Penicillin (P)	10 µg	10	11-21	22
2	Amoxycillin (AMX)	10µg	13	14-17	18
3	Streptomycin (S)	10µg	10	11-14	15
4	Levofloxacin (LE)	3µg	15	16-18	19
5	Oxacillin (OX)	1µg	10	11-12	13
6	Kanamycin (K)	30µg	13	14-17	18
7	Cephalexin (CL)	30µg	22	23-24	25
8	Ampicillin (AMP)	10µg	13	14-16	17
9	Ciprofloxacin (CIP)	5µg	19	20-21	22
10	Erythromycin (E)	15µg	13	14-17	18
11	Tetracycline (TE)	10µg	14	15-18	19
12	Chloramphenicol (C)	30µg	12	13-17	18
13	Ceftazidime (CAZ)	30µg	16	17-18	19
14	Ceftriaxone (CRO)	10µg	18	19-21	22
15	Nalidixic Acid (NA)	30µg	13	14-18	19
16	Imipenem (IMI)	10µg	13	14-15	16
17	Amikacin (AK)	30µg	14	15-16	17
18	Clindamycin (CD)	2µg	14	15-20	21
19	Norfloxacin (NOR)	10µg	12	13-16	17
20	Azithromycin (AZM)	15µg	13	14-17	18
21	Cefixime (CFM)	5µ	15	16-18	19
22	Co–Trimoxazole (COT)	1.25/23.75µg	10	11-15	16
23	Metronidazole (MT)	4µg	15	16-18	20

In accordance with performance standards for Antimicrobial Disk Susceptibility Tests,CLSI (formerly NCCLS).

2.10.1. Inoculation on Mueller-Hinton Agar (MHA) plates:

Bacterial suspensions were prepared with 0.9% saline solution. Appropriate amount of bacteria were picked up from 24 hours old culture on nutrient agar plate using a sterile loop and suspended in the saline. Later, all the test tubes were vortexed properly to make the suspension homogeneous, the turbidity of the suspension was maintained and compared with McFarland 1.0 standard solution.

MHA was prepared, autoclaved and plated with care and proper labeling. An autoclaved cotton swab was dipped into the bacterial suspensions and rotated so that it absorbs a sufficient amount of the suspension. Then, the swab was streaked several times on the dried surface of the MHA plate to make a pure lawn ensuring all edges of the plate has been covered by the cotton swab. The agar plate was being rotated 90 degrees each time it was being streaked, to ensure the even distribution of the inoculate.

2.10.2. Placement of the antibiotic disks:

A sterile forceps was used to place the antibiotic disks on the surface of the inoculated MHA plate. Each of the disks was carefully placed and slightly pressed on the MHA plates using the forceps. The placement of the disks close to the edges of the plate was avoided to ensure zone measurement errors. The MHA plates were incubated at 37°C for 24 hours.

2.10.3. Measurement of the zone of inhibition:

The zone of inhibition for each of the antibiotics was observed on the incubated MHA plate. The size of zones for each antibiotic was measured carefully in millimeters (mm) using a ruler by observing the back of the petri dish. Each zone size was measured from three angles of the zone to ensure accuracy and was then recorded.

2.10.4. MAR Indexing:

The MAR index, when applied to a single isolate is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed[28]. MAR index value higher

than 0.2 is considered to have originated from high-risk sources of contamination like the human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used and MAR index value of less than or equal to 0.2 considered the origination of strain from animals in which antibiotics are seldom or never used[28].

2.11. Antimicrobial activity of Origanum vulgare:

2.11.1. Collection of plant samples:

Dried oregano leaves were purchased from a departmental store in Dhaka. Then, the dried leaves were crushed to a fine powder, weighed and stored in a clean air-tight container.

2.11.2. Preparation of plant extracts using different solvents:

Methanol and Ethanol organic solvents were used; hence, two different types of extracts were prepared using the two samples respectively.

Methanol as a solvent has the ability to dissolve many compounds present in the sample quite easily. Also, during the evaporation of the solvent to obtain a more concentrated form of the extract, methanol evaporates easily. Lastly, methanol is cost-friendly and is considered as a safe organic solvent.

Ethanol as a solvent also has the ability to dissolve many compounds present in the sample which may be insoluble in water. Ethanol is also considered to be relatively safer as an organic solvent.

2.11.3. Extraction process and preservation:

For preparing the methanolic and ethanolic extract of oregano sample, an appropriate amount of powdered oregano was measured and was carefully transferred in the extraction thimble, inside the Soxhlet apparatus. The round receiving flask of the apparatus was then filled with 250ml of extraction solvents such as methanol or ethanol. During the process, the Soxhlet was allowed to run at appropriate temperatures for different solvents. The boiling temperature of methanol and ethanol are 64.7°C and 78.37°C respectively. However, the process was carried at a temperature slightly lower than the exact boiling point of the solvents, to ensure the

chance of bumping to be reduced. The extraction cycles were repeated for three to four cycles consecutively and this took about 3 to 4 hours. Once the color of cotton inside the extraction thimble became colorless, the extraction process was stopped. Then, the contents of the round flask were transferred into the receiving flask of the rotary evaporator. Plants extract solvent evaporation and condensation is done in the rotary evaporator at low pressure and the temperature of 60°C. The plant extract solvent in rotary evaporator was evaporated to a point where the extract become concentrated. The extract was then scraped via sterile spatula and collected in sterile Petri plates and placed into the fume hood for 3 to 4 days until maximum evaporation of the solvent content in the extract was attained. A sticky semi-solid crude extract appeared on the surface of the plate when all the solvent was evaporated. Finally, the crude extract was transferred into an autoclaved McCartney bottle via sterile spatula.

McCartney bottles containing prepared extracts were then kept in 4°C refrigerator for preservation. The caps of all the bottles were tightly closed to ensure no external contaminants may enter. Also, the McCartney bottles were properly labeled according to the name of the extract, solvent used and the amount of extract obtained for antimicrobial assay. The extracts yield percentages were calculated using the following formula:

Extract yield% = R/S * 100 (where R; the weight of extracted plants residues and S; the weight of plant raw sample).

2.11.4. Dimethyl sulfoxide (DMSO) as a diluting agent:

The preserved extracts were diluted using 100% DMSO and 2% DMSO to prepare them for antimicrobial screening. DMSO is a colorless liquid that can dissolve both polar and non-polar compounds and is also miscible in various organic solvents as well as water. Stock solutions were prepared using both diluting agents at a concentration of 100mg/ml.

2.11.5. Agar well diffusion:

A loop of a single colony of the 24 hours old bacterial culture was then inoculated into test tubes containing 9ml of 0.9% saline and vortexed to obtain a homogenous suspension. The suspension turbidity was compared with McFarland 0.5 standard solution.

The surface of each of the Mueller-Hinton agar was streaked by a sterile cotton swab with the bacterial suspension from the inoculated saline. Then, the agar plate was punched with a sterile cork-borer to form a well of 4 mm size and 60 µl of stock solution was poured into the well using micropipette.

The Mueller-Hinton agar plate was divided into four quadrants, with two quadrants designated for each stock solutions. The other two quadrants were designated for 100% DMSO and 2% DMSO and these quadrants served as negative control for the test. The plates were then allowed to standby for 30 minutes and were later incubated at 37°C for 18-24 hours.

2.11.6. Measurement of the zone of inhibition:

A clear zone on the MHA plate around the agar well containing the stock solution represents the zone of inhibition which signifies the antimicrobial activity of the plant extract. No clear zone around the negative controls represents that the diluting agents do not affect the growth of tested bacteria. The diameter of the clear zone was measured three in millimeter (mm) with a scale and the test was triplicated. The average value of the zone of inhibition for each extract was then calculated and recorded.

2.11.7. Determination of activity index:

The antibacterial effects of the methanolic and ethanolic extracts were calculated and compared by measuring the activity index using the following formula:

Activity Index (AI) = zone of inhibition of extracts/ zone of inhibition of the antibiotics

2.11.8. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after 24 hours of incubation. For MIC, the most effective plant extract exhibiting a strong antimicrobial activity at 100mg/ml was manipulated using the broth dilution method. This biological assay was chosen because of its simplicity, reproducibility, sensitivity, and relatively low cost while being a rapid method at the same time[24].For the test, test tubes were taken containing 9ml of nutrient broth and 1ml of bacterial saline suspension (turbidity compared with McFarland 0.5 standard) was added which was highly susceptible to plant extract during agar well diffusion test. To each tube, plant extract of varying concentrations ranging from 100µg/ml to 270µg/ml was added. Then, the test tubes were incubated at 37°C for 24 hours. Later, the contents were sub-cultured as spot inoculation on MHA for MBC test.

MBC is defined as the concentration of plant extract that did not show any bacterial growth on MHA plates. The MBC was determined by sub-culturing samples from test tubes with concentrations above or equal to the MIC on fresh plates of MHA.

2.11.9. Phytochemical test:

Five different types of phytochemical assays were done to determine the presence of tannins, saponins, alkaloids, phenolic compounds, and steroids.

2.11.9.1. Test for tannins:

To 0.5 ml of the extract that was diluted in 2.5ml of distilled water, 2-3 drops of 10% of ferric chloride were added. The formation of bluish-black or brownish-green precipitate indicates the presence of tannins.

2.11.9.2. Test for saponins:

To 0.5ml of the extract, 2 ml distilled water was added to dilute it and also was shaken vigorously for 2 minutes. The observation of froth in the test tube indicates the presence of saponins.

2.11.9.3. Tests for alkaloids:

a. Hager test: To 0.5 ml of the extract, a few drops of picric acid were added down the side of the test tube. A creamy or white precipitate indicates the presence of alkaloids.

b. Wagner's test: To 0.5 ml of the extract, a few drops of Wagner's reagent (solution of iodine in potassium iodide) were added down the side of the test tube. A reddish-brown precipitate indicates the presence of alkaloids.

2.11.9.4. Tests for phenolic compounds:

To 1 ml of extract, 5% of ferric chloride was added. The color change of the extract to dark green color indicated the presence of phenolic compounds.

2.11.9.5. Tests for steroids:

To 1 ml of the extract, 1 ml of chloroform and 1 ml of sulphuric acid was added slowly down the side of the wall of the test tube. The red color produced in the lower chloroform layer indicates the presence of steroids.

All these methods and materials were used and performed to determine the isolates to be *E. coli* and *Shigella* and to determine the antibiotic resistance pattern of the isolates, and lastly to determine the antimicrobial properties of Oregano against these isolates.

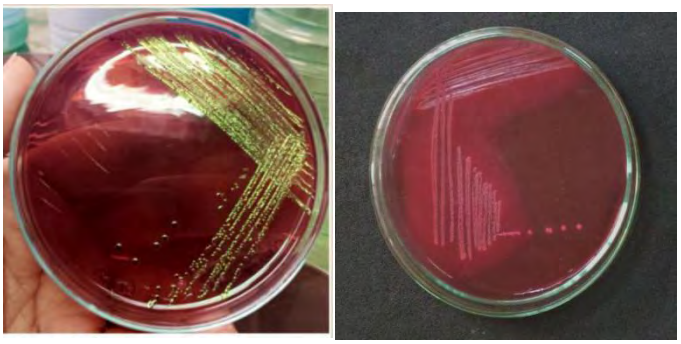
Chapter 03: Results

Results

3.1. Number of isolates:

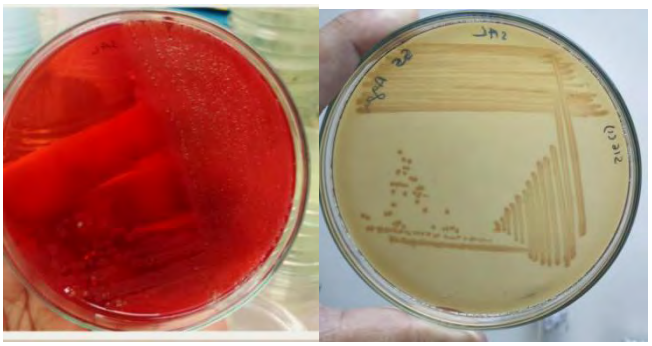
Twenty-six isolates of *E. coli* were found from different parts of the twenty-eight tilapia fish samples. After incubation at 37°C for 24 hours, these isolates produced pink colonies and green sheen on MacConkey agar and EMB agar plates respectively. These are the characteristics of *E. coli.*

Similarly, 14 isolates of *Shigella* spp. were found from different parts of the twenty-eight tilapia fish samples. These isolates produced red colonies and colorless colonies after 24 hours incubation on XLD agar and SS agar plates respectively. These are the characteristics of *Shigella* spp.



(a) **(b)**

Figure 3.1: Growth and Appearance on EMB Agar and MacConkey Agar. a) Development on EMB Agar, *E. coli* has characteristic metallic green sheen. b) Subsequent development on MacConkey Agar, *E. coli* has characteristic pink colonies.



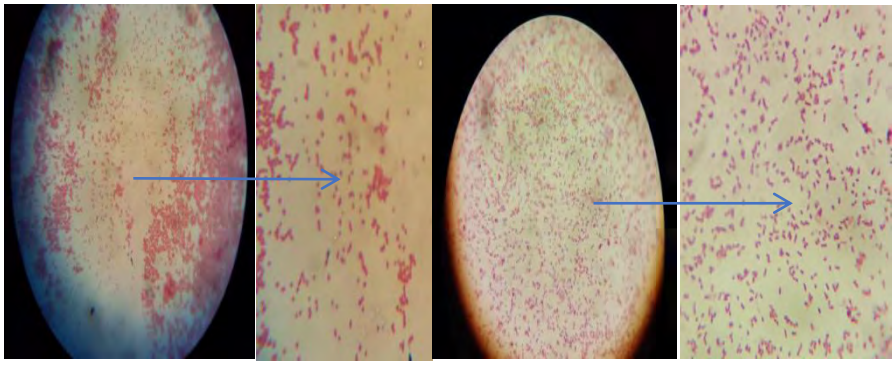
(a) **(b)**

Figure 3.2:Growth and Appearance on XLD Agar and SS Agar a) Development on XLD Agar, *Shigella* spp. has characteristic red colonies. b) Subsequent development on SS Agar, *Shigella* spp. *h*as characteristic colorless colonies.

3.2. Biochemical tests:

Even though the 26 isolates and the 14 isolates were suspected to be *E. coli* and *Shigella* spp. respectively after being observed in selective media, a set of biochemical tests were conducted in order to confirm that they were indeed the suspected organisms. From the tests, it was found that 25 isolates approved to be *E. coli* and 11 isolates approved to be *Shigella* spp.

Both *E.coli* and *Shigella* are gram-negative, rod-shaped bacteria. Therefore, all the isolates which were suspected to be observed as pink stained after gram staining was performed. The morphology of the isolates was observed to be rod-shaped under the microscope. Hence, all the isolates were considered to be rod-shaped gram-negative bacteria.



(a) **(b)**

Figure 3.3: Gram staining of one of the isolate of *Shigella* (a) and *E. coli* (b) showing the pink stain characteristic of Gram-negative bacteria and rod shaped morphology.

Figure 3.4: Biochemical tests for identification of the isolates

Table 3.1: Biochemical tests of the suspected 26 *E. coli* isolates

Isolates	MRVP		MIU			Catalase	Oxidase	Citrate	TSI						Gram staining	Presumptive bacterial genus
	MR	VP	Motility	Urease	Indole				Butt/ Slant	Glucose	Lactose	Sucrose	H_2S	Gas		
E1(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E1(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E1(4)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E5(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E5(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E6(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E6(4)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E8(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E10(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E10(4)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E11(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E11(4)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E12(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E12(4)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E16(4)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E17(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E24(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E24(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E23(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E23(4)	+	-	+	+	+	+	-	-	Y/Y	+	+	+	-	+	-	*Leclercia*
E25(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E26(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E26(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E27(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E28(2)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*
E28(3)	+	-	+	-	+	+	-	-	Y/Y	+	+	+	-	+	-	*Escherichia coli*

*Y= yellow; G+= Gas production; + = positive; - = negative; H_2S= H_2S production; Gas= Gas production

Table 3.2: Biochemical tests of the suspected 14 isolates of *Shigella* spp.

Isolates	MRVP		MIU			Catalase	Oxidase	Citrate	TSI						Gram staining	Presumptive bacterial genus
	MR	VP	Urease	Motility	Indole				Butt/ Slant	Glucose	Lactose	Sucrose	H_2S	Gas		
S10(3)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S11(3)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S14(1)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S16(1)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S17(1)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S17(4)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S18(2)	+	-	-	-	+	+	-	-	Y/Y	+	+	-	-	-	-	*Yersinia intermedia*
S19(4)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S20(1)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S20(3)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S21(3)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S21(4)	+	-	-	-	+	+	-	-	Y/R	+	-	-	-	-	-	*Shigella* spp.
S23(3)	+	-	-	-	+	+	-	-	Y/Y	+	+	-	-	-	-	*Yersinia intermedia*
S25(4)	+	-	-	-	+	+	-	-	Y/Y	+	+	-	-	-	-	*Yersinia intermedia*

*Y= yellow; G+= Gas production; + = positive; - = negative; H_2S= H_2S production; Gas= Gas production

Table 3.3: Number and percentage of *E. coli and Shigella* spp isolated from 28 samples of four parts of collected fishes.

Organism	Skin	Gills	Intestine	Muscle	Total No. Of Isolates
E. coli	0	11 (44%)	8 (32%)	6 (24%)	25 (69%)
***Shigella* spp.**	4 (36%)	0	4 (36%)	3 (28%)	11 (31%)
Total	4 (11%)	11 (31%)	12 (33%)	9 (25%)	36

3.3. Antibiotic Susceptibility Test:

3.3.1. For Shigella isolates:

The antibiotic susceptibility test was carried on the 11 isolates using 23 antibiotics and the results showed that all the isolates were resistant to Oxacillin, Ampicillin, Penicillin, Erythromycin, Amoxycillin, Co-Trimoxazole, Cefixime, Metronidazole, Ceftazidime, Ceftriaxone, Clindamycin, Cephalexin, and Azithromycin. All the isolates showed intermediate resistance to Chloramphenicol.

Three isolates were intermediately resistant to Nalidixic Acid (27%) and rest 8 isolates were resistant to Nalidixic Acid (73%).

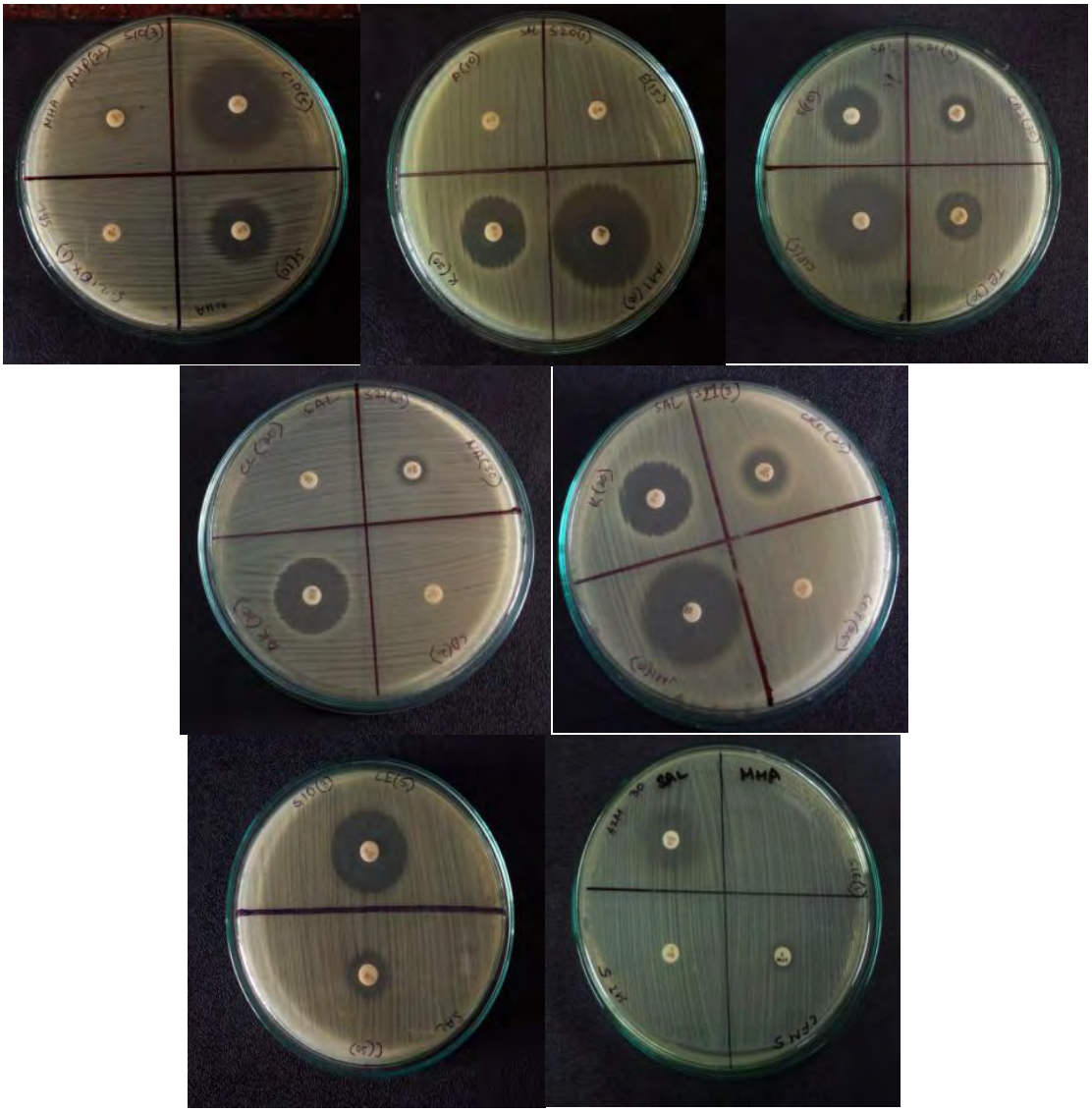


Figure 3.5: MHA plates showing antibiotic susceptibility pattern of some *Shigella* isolates.

Table 3.4: Antibiotic Susceptibility Test Pattern of the *Shigella* spp. isolates.

Antibiotics	S10(3)	S14(1)	S20(3)	S17(4)	S20(1)	S19(4)	S21(4)	S21(3)	S16(1)	S17(1)	S11(3)
Streptomycin	S	S	S	S	S	S	S	S	S	S	S
Levofloxacin	S	S	S	S	S	S	S	S	S	S	S
Oxacillin	R	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S
Ceftazidime	R	R	R	R	R	R	R	R	R	R	R
Ceftriaxone	R	R	R	R	R	R	R	R	R	R	R
Co-Trimoxazole	R	R	R	R	R	R	R	R	R	R	R
Penicillin	R	R	R	R	R	R	R	R	R	R	R
Erythromycin	R	R	R	R	R	R	R	R	R	R	R
Imipenem	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S	S
Clindamycin	R	R	R	R	R	R	R	R	R	R	R
Amikacin	S	S	S	S	S	S	S	S	S	S	S
Cephalexin	R	R	R	R	R	R	R	R	R	R	R
Nalidixic acid	R	I	I	I	R	R	R	R	R	R	R
Amoxycillin	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol	I	I	I	I	I	I	I	I	I	I	I
Norfloxacin	S	S	S	S	S	S	S	S	S	S	S
Azithromycin	R	R	R	R	R	R	R	R	R	R	R
Cefixime	R	R	R	R	R	R	R	R	R	R	R
Metronidazole	R	R	R	R	R	R	R	R	R	R	R

*S=Sensitive; R=Resistant; I=Intermediate

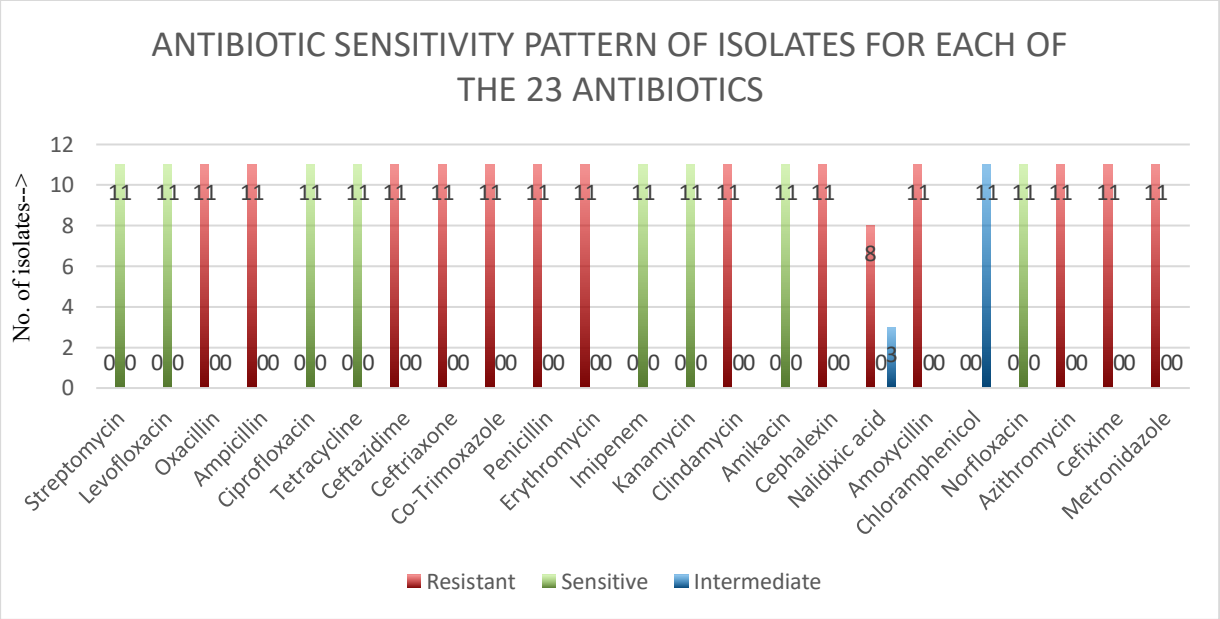


Figure 3.6: The number of resistant, intermediate and sensitive *Shigella* isolates.

3.3.2. For E. coli isolates:

The antibiotic susceptibility test was carried out on 25 isolates using 23 antibiotics and the result showed that all the isolates were fully resistant to Oxacillin, Penicillin, Clindamycin, Amoxycillin and Metronidazole, i.e., these antibiotics when tested against all the isolates produced no zone of inhibition. Also, all the isolates were 100% resistant to Erythromycin and Cephalexin. Cephalexin showed secondary growth to no clear zone of inhibition. Seven isolates were resistant to Tetracycline (28%), 7 isolates to Co-Trimoxazole (28%), 4 isolates to Ampicillin (16%), and 2 isolates were resistant to Nalidixic Acid (8%). All the isolates were intermediate to Azithromycin, 7 isolates were intermediate to Ceftazidime (28%), 4 isolates to Tetracycline (16%), 2 isolates to Ceftriaxone (8%), and 1 isolate was intermediate to Nalidixic Acid (4%). Isolates E16(4), E26(3), and E23(2) showed maximum resistance against 10 out of 23 antibiotics that were tested.

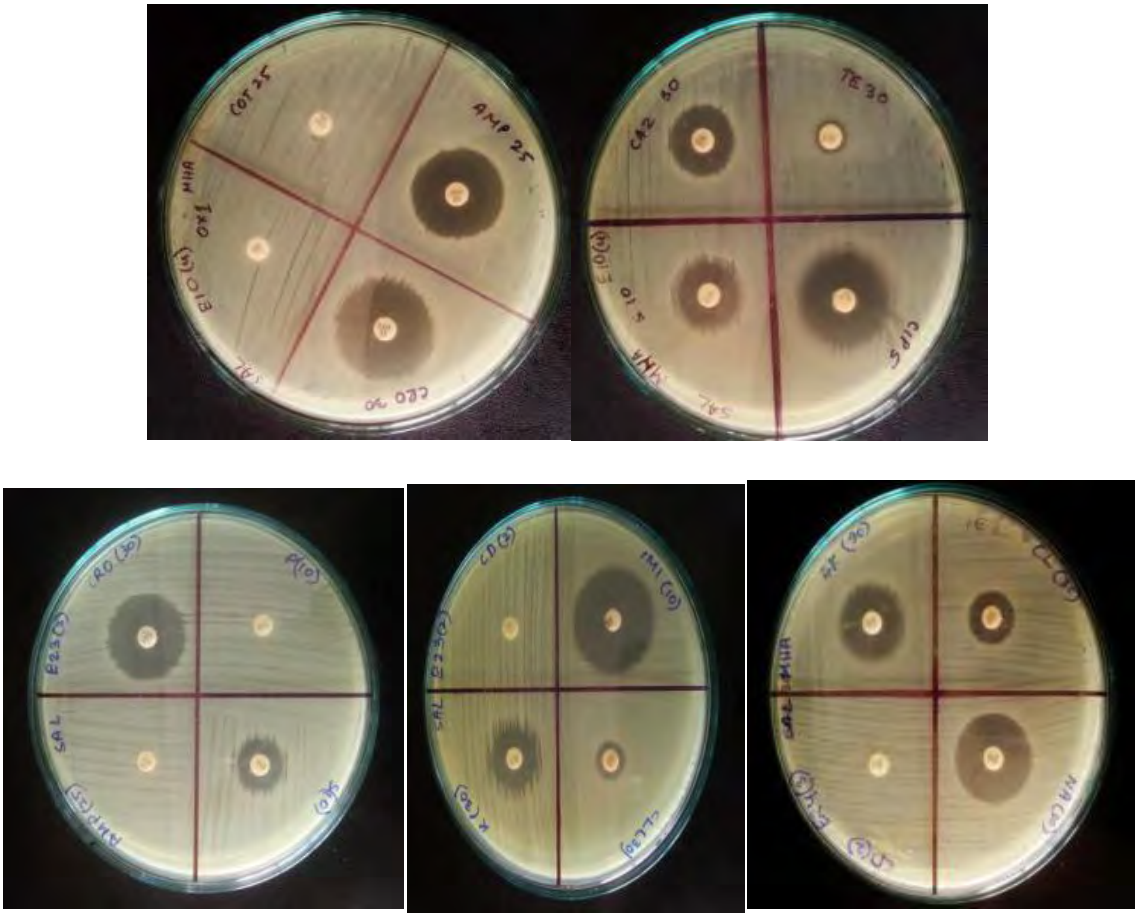


Figure 3.7: Antibiotic Susceptibility pattern of some *E. coli* isolates

Table 3.5: Antibiotic Susceptibility Test Pattern of the *E. coli* isolates.

Antibiotics	E1(3)	E1(4)	E5(2)	E5(3)	E12(4	E1(2	E27(3	E24(3	E10(2	E10(4	E8(2)	E11(4	E25(3	E6(4)	E12(2	E26(2	E24(2	E16(4	E17(3	E11(2	E28(3	E6(2)	E26(3	E28(2	E23(2
Streptomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Levofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Oxacillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	R
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	R	S	S	S	I	S	I	I	R	R	S	R	R	R	I	R
Ceftazidime	S	S	S	S	S	I	S	S	S	S	S	S	I	S	S	I	I	I	S	S	S	S	I	I	S
Ceftriaxone	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	I	S	S	S
Co-Trimoxazole	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	S	R	S	R	S	R
Penicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Erythromycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Clindamycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cephalexin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Nalidixic acid	S	S	S	S	S	R	S	S	S	S	S	S	S	S	R	S	S	I	S	S	S	S	S	S	S
Amoxycillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Norfloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Azithromycin	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Metronidazole	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefixime	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

*S=Sensitive; R=Resistant; I=Intermediate

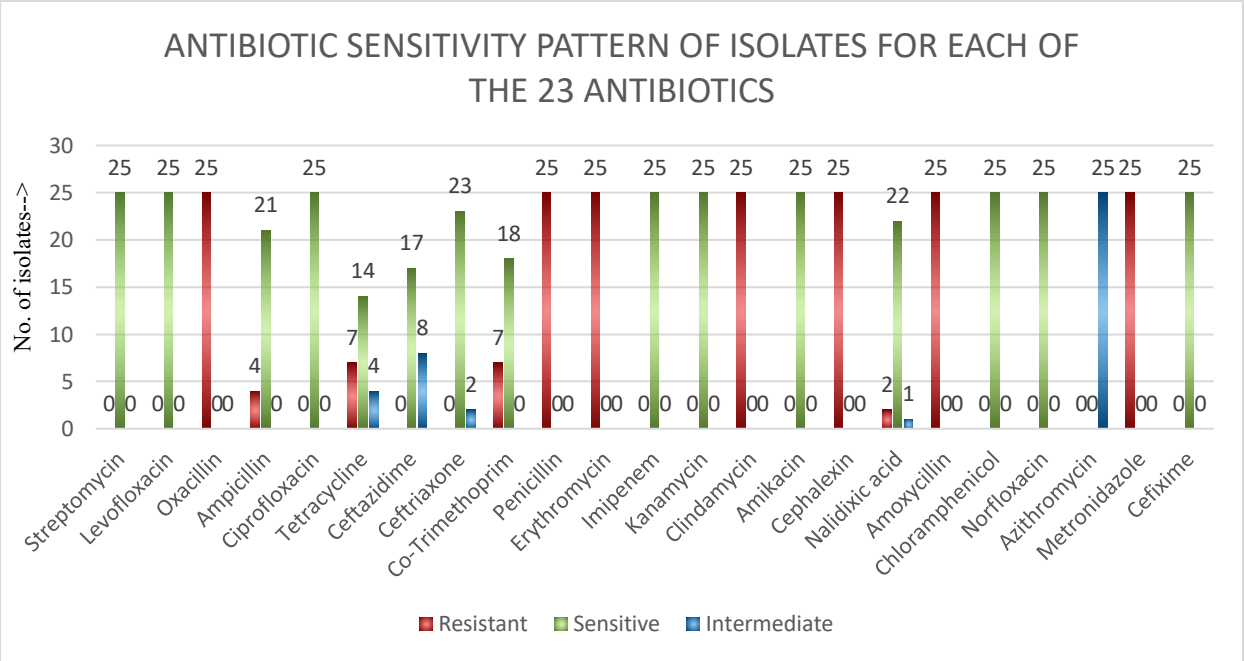


Figure 3.8: The number of resistant, susceptible and intermediate *E. coli* isolates.

3.3.3. The isolates exhibited high Multiple Antibiotic Resistance (MAR) Index:

The ratio of the number of antibiotics to which an organism is resistant to the total number of antibiotics to which the organism is exposed is determined as the Multiple Antibiotic Resistance (MAR) Index.

The Multiple Antibiotic Resistance (MAR) Indices of the *Shigella spp*. isolates showed that all the isolates had a MAR Index of 0.50 and greater value, thus, all of the isolates had a High level of Resistance.

The Multiple Antibiotic Resistance (MAR) Indices of the *E. coli* isolates showed that all the isolates had a MAR Index of between 0.30 to 0.50, thus, all of the isolates had a Moderate level of Resistance.

Table 3.6: MAR Index and Level of Resistance of the *Shigella spp*. isolates.

***Shigella spp*. isolates**	Resistant (a)	Tested (b)	MAR(a/b)	Level of resistance
S10(3)	14	23	0.60	High
S11(3)	14	23	0.60	High
S14(1)	13	23	0.56	High
S16(1)	14	23	0.60	High
S17(1)	14	23	0.60	High
S17(4)	13	23	0.56	High
S19(4)	14	23	0.60	High
S20(1)	14	23	0.60	High
S20(3)	13	23	0.56	High
S21(3)	14	23	0.60	High
S21(4)	14	23	0.60	High

Table 3.7: MAR Index and Level of Resistance of *E. coli* isolates

***E. coli* isolates**	Resistant (a)	Tested (b)	MAR(a/b)	Level of resistance
E1(2)	8	23	0.35	Moderate
E1(3)	7	23	0.30	Moderate
E1(4)	7	23	0.30	Moderate
E5(2)	7	23	0.30	Moderate
E5(3)	7	23	0.30	Moderate
E6(2)	8	23	0.35	Moderate
E6(4)	7	23	0.30	Moderate
E8(2)	7	23	0.30	Moderate
E10(2)	7	23	0.30	Moderate
E10(4)	8	23	0.35	Moderate
E11(2)	7	23	0.30	Moderate
E11(4)	7	23	0.30	Moderate
E12(2)	8	23	0.35	Moderate
E12(4)	7	23	0.30	Moderate
E16(4)	10	23	0.43	Moderate
E17(3)	9	23	0.39	Moderate
E24(2)	9	23	039	Moderate
E24(3)	7	23	0.30	Moderate
E23(2)	10	23	0.43	Moderate
E25(3)	7	23	0.30	Moderate
E26(2)	8	23	0.35	Moderate
E26(3)	10	23	0.43	Moderate
E27(3)	7	23	0.30	Moderate
E28(2)	7	23	0.30	Moderate

3.4. Extracts Yield % of Oregano:

Crude extracts were obtained from dried oregano plant samples using ethanol and methanol.

Methanolic Extract yield% = 21%

Ethanolic Extract yield% = 17%

Each crude extract was dissolved in 100% DMSO and 2% DMSO to prepare stock solutions maintaining a constant concentration of 100 mg/ml.

3.4.1. Methanolic and Ethanolic extracts of Oregano showed antimicrobial activity against the isolates:

The antimicrobial property of methanolic and ethanolic extracts of oregano using 100% and 2% DMSO as solvents were tested against the isolates. In both cases, *E. coli* and *Shigella spp*., methanolic extract in 100% DMSO showed the most remarkable positive results against the isolates. Three replicates were made for better accuracy and interpretation. Also, the solvents used in the preparation of stock solutions were used as negative controls to determine whether they could display any antimicrobial property. *Shigella spp.* isolates showed larger zone of inhibition than *E. coli* isolates, isolate S17(4) being the highest.

3.4.2.Comparison of Activity Index (AI) showed methanolic extract of Oregano in 100%DMSO had a high activity index:

The activity index values were calculated using the following formula:

Activity Index (AI) = zone of inhibition of extracts/ zone of inhibition of the antibiotics.

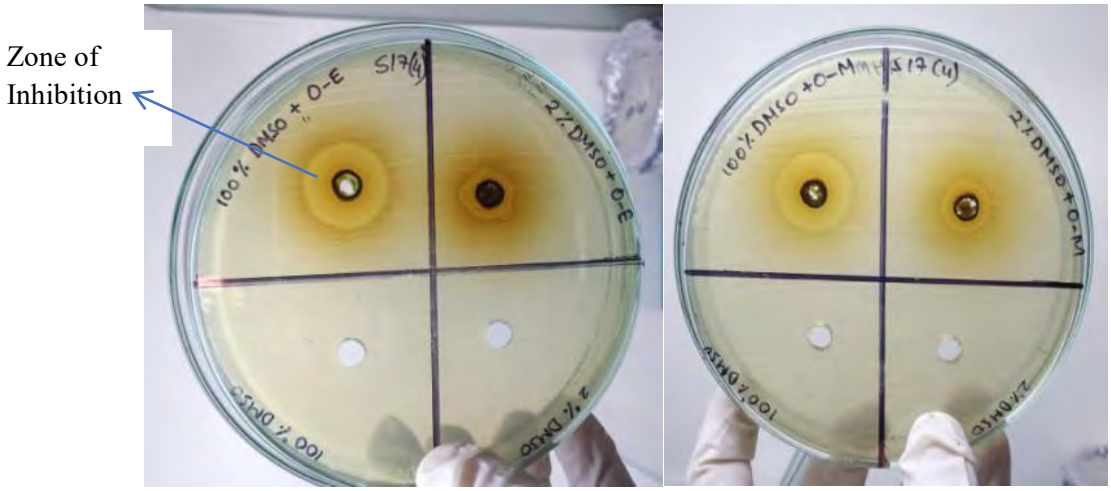
Results of antibiotic Ciprofloxacin were used to calculate the AI as all the isolates were most susceptible to this antibiotic. Methanolic extract of oregano in 100% DMSO gave higher AI for all the isolates. S20(1) among *Shigella spp.* isolates and E12(2) among *E. coli* isolates showed the highest activity index (AI).

Table 3.8: Antimicrobial activity of Methanolic Extract against *Shigella spp.* isolates.

***Shigella spp.* Isolates**	**100% DMSO as solvent**		**2% DMSO as solvent**	
	Zone of inhibition (mm)	Activity Index (AI)	Zone of inhibition (mm)	Activity Index (AI)
S10(3)	17	0.56	14	0.46
S11(3)	18	0.66	14	0.52
S14(1)	18	0.47	14	0.37
S16(1)	17	0.53	15	0.47
S17(1)	19	0.63	14	0.47
S17(4)	20	0.58	15	0.44
S19(4)	15	0.47	10	0.31
S20(1)	19	0.68	14	0.50
S20(3)	19	0.56	14	0.41
S21(3)	18	0.64	14	0.50
S21(4)	18	0.64	14	0.50

Table 3.9: Antimicrobial activity of Ethanolic Extract against *Shigella spp.* isolates.

***Shigella spp.* Isolates**	100%DMSO as solvent		2% DMSO as solvent	
	Zone of inhibition (mm)	Activity Index (AI)	Zone of inhibition (mm)	Activity Index (AI)
S10(3)	17	0.56	10	0.33
S11(3)	14	0.52	7	0.26
S14(1)	15	0.39	7	0.18
S16(1)	16	0.50	7	0.22
S17(1)	19	0.63	12	0.40
S17(4)	19	0.56	12	0.35
S19(4)	14	0.44	7	0.22
S20(1)	19	0.68	11	0.39
S20(3)	16	0.47	11	0.32
S21(3)	17	0.60	12	0.43
S21(4)	17	0.60	12	0.43



(a) **(b)**

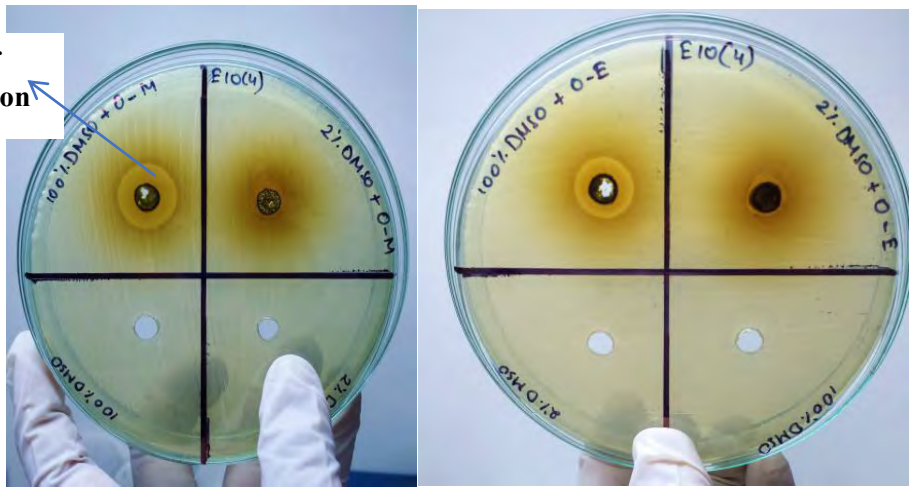
Figure 3.9: Antimicrobial activity of (a) Ethanolic Extract of Oregano and (b) Methanolic Extract of Oregano against *Shigella spp.* isolate S17(4). S17(4) produced largest zone of inhibition among *Shigella spp.* isolates.

Table 3.10: Antimicrobial activity of Methanolic Extract against *E.coli* isolates

***E.coli* Isolates**	**100% DMSO as solvent**		**2% DMSO as solvent**	
	Zone of inhibition (mm)	Activity Index (AI)	Zone of inhibition (mm)	Activity Index (AI)
E1(2)	16	0.53	11	0.37
E1(3)	16	0.57	11	0.39
E1(4)	15	0.52	11	0.38
E5(2)	14	0.48	11	0.38
E5(3)	14	0.50	11	0.39
E6(2)	13	0.46	8	0.28
E6(4)	13	0.50	8	0.31
E8(2)	16	0.53	11	0.37
E10(2)	16	0.47	11	0.32
E10(4)	16	0.57	11	0.39
E11(2)	14	0.54	11	0.42
E11(4)	14	0.54	11	0.42
E12(2)	16	0.64	11	0.44
E12(4)	16	0.59	11	0.41
E16(4)	15	0.55	10	0.37
E17(3)	15	0.53	10	0.36
E24(2)	15	0.53	10	0.36
E24(3)	15	0.43	10	0.28
E23(2)	15	0.62	11	0.46
E25(3)	15	0.53	11	0.39
E26(2)	14	0.58	11	0.46
E26(3)	13	0.52	10	0.40
E27(3)	15	0.40	11	0.30
E28(2)	13	0.45	8	0.27
E28(3)	13	0.46	8	0.28

Table 3.11: Antimicrobial activity of Ethanolic Extract against *E. coli* isolates.

***E.coli* Isolates**	**100% DMSO as solvent**		**2% DMSO as solvent**	
	Zone of inhibition (mm)	Activity Index (AI)	Zone of inhibition (mm)	Activity Index (AI)
E1(2)	13	0.43	8	0.27
E1(3)	14	0.50	9	0.32
E1(4)	13	0.45	8	0.27
E5(2)	12	0.41	8	0.27
E5(3)	12	0.43	8	0.28
E6(2)	11	0.39	7	0.25
E6(4)	11	0.42	8	0.31
E8(2)	12	0.40	8	0.27
E10(2)	14	0.41	9	0.26
E10(4)	14	0.50	9	0.32
E11(2)	11	0.42	9	0.35
E11(4)	13	0.50	8	0.31
E12(2)	14	0.56	9	0.36
E12(4)	14	0.52	9	0.33
E16(4)	12	0.44	7	0.26
E17(3)	13	0.46	8	0.28
E24(2)	13	0.46	8	0.28
E24(3)	12	0.37	9	0.23
E23(2)	13	0.54	8	0.33
E25(3)	13	0.46	8	0.28
E26(2)	12	0.50	8	0.33
E26(3)	13	0.52	8	0.32
E27(3)	13	0.35	8	0.22
E28(2)	11	0.38	7	0.24
E28(3)	11	0.39	7	0.25



(a) **(b)**

Figure 3.10: Antimicrobial activity of (a) Methanolic Extract of Oregano and (b) Ethanolic Extract of Oregano against *E. coli* isolate E10(4). E10(4) produced largest zone of inhibition among the *E. coli* isolates.

3.4.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of isolate S20(1):

MIC and MBC were performed to isolate S20(1) as it had the highest AI value. The stock solution was prepared using 100 mg methanolic extract of oregano dissolved in 1 ml of 100% DMSO to a concentration of 100 mg/ml. The MIC of the methanolic extract of oregano against S20(1) was found to be 0.160 mg/ml and MBC was found to be 0.165 mg/ml.

Table 3.12: MIC and MBC of isolate S20(1) using stock solution of concentration 100mg/ml. Test-tube No. 16 was used as negative control containing 1ml nutrient broth and 1ml of stock solution.

Test-tube No.	NB broth (ml)	Bacterial suspension McFarland standard 0.5 (ml)	Stock solution Added (µl)	Concentration of Methanolic Extract (µg/ml)
1	9	1	10.5	105
2	9	1	11	110
3	9	1	11.5	115
4	9	1	12	120
5	9	1	12.5	125
6	9	1	13	130
7	9	1	13.5	135
8	9	1	14	140
9	9	1	14.5	145
10	9	1	15	150
11	9	1	15.5	155
12	9	1	16	160
13	9	1	16.5	165
14	9	1	17	170
15	9	1	17.5	175

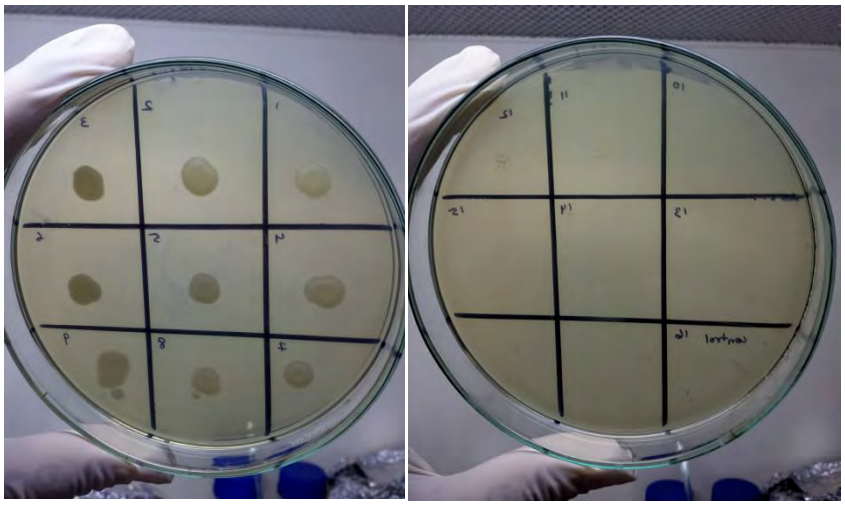


Figure 3.11: MIC and MBC of methanolic extract of oregano against *Shigella spp.* isolate S20(1). MIC can be seen on grid 12 and MBC on grid 13. MIC was 160 µg/ml and MBC was 165 µg/ml.

3.4.4. Results of Phytochemical Screening showing variability for Methanolic, Ethanolic and Aqueous extracts of Oregano :

3.4.4.1. Test for tannins:

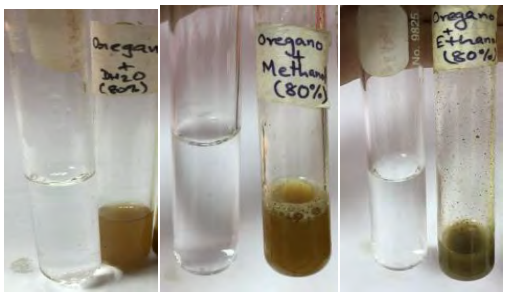
Ethanolic extract of Oregano, Aqueous extract of Oregano and Methanolic extract of Oregano showed the formation of bluish-black or brownish-green precipitate indicating the presence of tannins.

(a) (b) (c)

Figure 3.12: Results for Tannins. (a) Positive for Ethanolic Extract, (b) Positive for Methanolic Extract and (c) Positive for Aqueous Extract of Oregano.

3.4.4.2. Test for saponins:

Methanolic extract of Oregano showed frothing observation in the test tube indicating the presence of saponins. Formation of bubbles would be observed on the top of the solution in the test tube indicating froth formation. Ethanolic extract of Oregano and Aqueous extract of Oregano showed negative results.

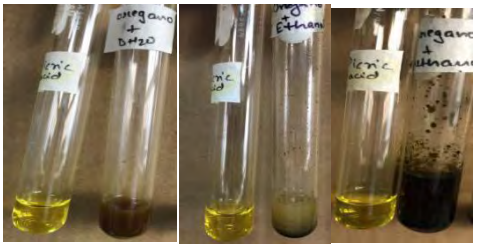


(a) (b) (c)

Figure 3.13: Results for Saponins (a) Negative for Aqueous Extract, (b) Positive for Methanolic Extract and (c) Negative for Ethanolic Extract of Oregano.

3.4.4.3. Tests for alkhaloids:

- ***Hager test:*** Ethanolic extract of Oregano and Aqueous extract of Oregano showed creamy or white precipitate indicating the presence of Alkaloid. Methanolic extract of Oregano showed negative results.



(a) (b) (c)

Figure 3.14: Results for Hager test. (a) Positive for Aqueous Extract, (b) Positive for Ethanolic Extract and (c) Negative for Methanolic Extract of Oregano.

- ***Wagner's test:***Ethanolic extract of Oregano, Methanolic extract of Oregano and Aqueous extract of Oregano showed a reddish brown precipitate indicating the presence of alkaloids.

(a) (b) (c)

Figure 3.15: Results for Wagner's test. (a)Positive for Methanolic Extract, (b) Positive for Ethanolic Extract and (c) Positive for Aqueous Extract of Oregano.

3.4.4.4. Test for phenolic compounds:

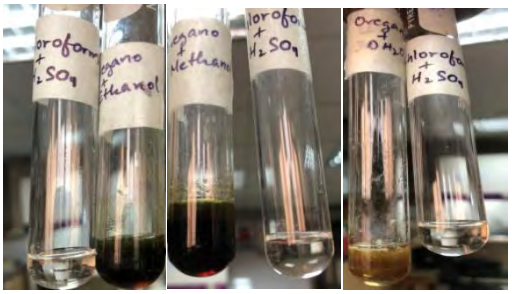
Ethanolic extract of Oregano, Methanolic extract of Oregano and Aqueous extract of Oregano showed the color change of the extract to dark green color indicating the presence of phenolic compounds.

(a) (b) (c)

Figure 3.16: Results for Phenolic Compounds. (a) Positive for Methanolic Extract, (b) Positive for Ethanolic Extract and (c) Positive for Aqueous Extract of Oregano.

3.4.4.5. Test for steroids:

Ethanolic extract of Oregano and Methanolic extract of Oregano showed the red color produced in the lower chloroform layer indicating the presence of steroids. Aqueous extract of Oregano showed negative results.



(a) (b) (c)

Figure 3.17: Results for Steroids. (a) Positive for Ethanolic Extract, (b) Positive for Methanolic Extract and (c) Negative for Aqueous Extract of Oregano.

Table 3.13: Phytochemical assay of Oregano

Name of the tested chemical		Methanolic Extract of Oregano	Ethanolic Extract of Oregano	Aqueous Extract of Oregano
Test for Tannins		Presence of Tannins	Presence of Tannins	Presence of Tannins
Test for Saponins		Presence of Saponins	Absence of Saponins	Absence of Saponins
Tests for Alkaloids	**Hager Test**	Absence of Alkaloids	Presence of Alkaloids	Presence of Alkaloids
	Wagner's Test	Presence of Alkaloids	Presence of Alkaloids	Presence of Alkaloids
Test for Phenolic Compounds		Presence of Phenolic Compounds	Presence of Phenolic Compounds	Presence of Phenolic Compounds
Test for Steroids		Presence of Steroids	Presence of Steroids	Absence of Steroids

Chapter 04: Discussion

Discussion

In this investigation, 28 tilapia fish samples were collected from different markets situated in different parts of Dhaka city. Four organs: skin, gills, intestine, and muscle of the fish were tested for bacterial contamination. It was revealed that maximum *E. coli* were isolated from gills (44%), whereas, *Shigella* spp. were isolated maximum from skin and intestine (36%). Out of the 28 samples, *E. coli* was isolated from four different organs of 17 tilapia fish samples, with a high prevalence of 60.7%, and *Shigella* was isolated from four different organs of 8 fish samples, with a low prevalence of 28.5%. The high prevalence of *E. coli* and low prevalence of *Shigella* corresponds to an investigation held in Sudan that stated that *E. coli* was highly dominant with 23.2% and *Shigella* spp. were lowest with 2.2% (n=150), investigated from marketed fish samples[25]. Also, the highest presence of *E. coli* isolates (70%) from seafood from retail markets was investigated in a study held in Thailand[26]. Another report in Kenya had found a prevalence of *Shigella* species to be 39.7% isolated from fish samples[27].

On confirming that the isolates were *E. coli* and *Shigella* species, the next step of the study was to check their antibiotic susceptibility. Nine groups of antibiotics were chosen: Beta-Lactam, Aminoglycoside, Lincosamide, Macrolide, Nitroimidazole, Quinolone, Sulfonamide, Chloramphenicol and Tetracycline. In the case of 25 *E. coli* isolates, the antibiotic susceptibility test reported the isolates to be resistant to Oxacillin, Penicillin, Erythromycin, Clindamycin, Cephalexin, Amoxycillin and Metronidazole. All the isolates were also intermediate to Azithromycin. Furthermore, the results also revealed that the *E. coli* isolates showed resistance to Ampicillin and Nalidixic Acid (8%), and to Tetracycline and Co-Trimoxazole (28%). The results also revealed to be intermediate to Nalidixic Acid (4%), Ceftriaxone (8%), Ceftazidime (28%) and to Tetracycline (16%). Moreover, all the 25 *E. coli* isolates were susceptible to Streptomycin, Levofloxacin, Ciprofloxacin, Imipenem, Kanamycin, Amikacin, Chloramphenicol, Norfloxacin and Cefixime. In case of 11 isolates of *Shigella* spp., the antibiotic susceptibility test reported them to be resistant to Oxacillin, Ampicillin, Ceftazidime, Ceftriaxone, Co-Trimoxazole, Penicillin, Erythromycin, Clindamycin, Cephalexin, Amoxycillin, Azithromycin, Cefixime and Metronidazole. Also, all the isolates showed intermediate zone for

Chloramphenicol. Additionally, these isolates revealed to be resistant to Nalidixic Acid (73%) and intermediate to Nalidixic Acid (27%). All the *Shigella* isolates were susceptible to Streptomycin, Levofloxacin, Ciprofloxacin, Tetracycline, Imipenem, Kanamycin, Amikacin and Norfloxacin. Antibiogram results of both the types of isolates showed that all the isolates were sensitive to Streptomycin, Levofloxacin, Ciprofloxacin, Imipenem, Kanamycin, Amikacin and Norfloxacin.

The investigation also revealed that all the isolates of *Shigella* spp. had high MAR values (MAR>0.50) than the *E. coli* isolates which had moderate MAR values (between 0.30 and 0.50). Multiple Antibiotic Resistance Index (MAR) determines the ratio of the number of antibiotics to which a bacterium is resistant to the total number of antibiotics the bacterium is subjected to. Since *Shigella* spp isolates had a MAR value more than 0.50, it can be said that each of the isolates was resistant towards many antibiotics that were used against the isolate. Furthermore, it was revealed that most isolates were resistant and/or intermediately resistant to many Beta-Lactams, Tetracycline, Co-trimoxazole and to some Quinolones. A study in Hong Kong has reported the presence of Beta-lactamase enzyme in *Shigella* isolates and the isolates have shown to be multiple resistance to many Beta-Lactams[29]. *Shigella* species are progressively acquiring resistance to antibiotics.Additionally, the vegetable and fish vendors are being situated nearby, a possibility of cross-contamination of the vegetables is most likely to be possible. Many of these vegetables are also considered to be consumed raw e.g. capsicum, cucumber, lettuce, tomato, and etc. Thus making bacterial infections dangerous and even worst in case these pathogens turn out to be highly resistant. This cross-contamination may occur via Horizontal Gene Transfer (HGT) and might lead to the increase of highly resistant bacteria in the surrounding. Since the misuse of antibiotics in a great number has led to the emergence of antibiotic resistant bacteria, it would be wise to investigate whether the antibiotic-resistance is plasmid-mediated or chromosomally mediated, to plan and design efficient drugs accordingly. These developments of antibiotic resistance can be a serious threat to both humans and other animals.

The antibiotic to which both *E. coli* and *Shigella* spp. isolates were most susceptible was Ciprofloxacin. Ciprofloxacin is a broad-spectrum antimicrobial fluoroquinolone. Its bactericidal action results from inhibition of the enzymes DNA gyrase and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair and recombination[30]. However, upon checking the Level of Resistance in view of the estimation of MAR list, a High level of Resistance was discovered in *Shigella* isolates than in *E. coli* isolates. Prevalence of *E. coli* was high, but most of the isolates showed moderate Level of Resistance except three isolates that were resistant to 10 out of 23 antibiotics. Although the prevalence of *Shigella* species was very low, however, all the isolates were found to be resistant to eight commonly used antibiotics to treat *Shigella* infections. These are Ampicillin, Amoxicillin, Cefixime, Ceftriaxone, Nalidixic acid, Azithromycin, Tetracycline, and Co-trimoxazole. Therefore, it can be considered that raw fish can be a potential source for the danger of procuring and being contaminated with different antibiotic-resistant *Shigella* spp. and *E. coli,* pathogenic to the human body.

The next step of the investigation was to determine the efficacy of the antimicrobial property of Oregano.Since antibiotic resistance has become a matter of concern for the world, treating the ever raising variety of infectious diseases caused by pathogens with an efficient alternative has become the prime goal[31]. Plants can be considered as a more natural alternative form of antibiotic and many are titled as medicinal plants. These medicinal plants, however, contain antimicrobial compounds which can be extracted using respective solvents. The extracts of the plants can further be a potential source of treatment against many bacterial infections.

In this investigation, it has been found that the methanolic and ethanolic extracts of oregano showed antimicrobial activity against the *E. coli* and *Shigella* isolates. Both the extracts were dissolved in 100% DMSO and 2% DMSO to a concentration of 100mg/ml. In the case of *Shigella* isolates, the methanolic extract of oregano in 100% DMSO was observed to show the best antimicrobial activity with an average zone of 19.6 mm, where the biggest zone was 20 mm and the smallest was 19 mm against one isolate. The average zone of inhibition by ethanolic extract of oregano in 100% DMSO was 18.6 mm, where the biggest zone was 19 mm and the smallest was 18 mm for three isolates. Upon

checking the Activity Indices of the methanolic and ethanolic extracts of oregano, in view of the estimation of AI list, a High Activity Index of methanolic extract in both solvents was observed in 10 out of 11 *Shigella* isolates. Activity Indices of ethanolic extract in 100% DMSO was found to be high, however, in 2% DMSO, the AI of the ethanolic extract against *Shigella* isolates were relatively very low. In the case of *E. coli* isolates, the methanolic extract of oregano in 100% DMSO was observed to show the best antimicrobial activity with an average zone of 15.3 mm, where the biggest zone was 16mm and smallest was 14 mm. The average zone of inhibition by ethanolic extract of oregano in 100% DMSO was 13.6 mm, where the biggest zone was 14 mm and the smallest was 13 mm. The AI of methanolic extract of oregano in 100% DMSO against *E. coli* isolateswas high compared to AI of methanolic extract of oregano in 2% DMSO which was found to be very low. Against *E. coli* isolates, AI of ethanolic extract in 100 % DMSO was moderate, however in 2% DMSO, the AI was relatively lower. This corresponds to an investigation held in Turkey which reported *E. coli* to be most resistant to oregano oil with a MIC of 250 µg/ml[32].

The isolate that showed highest Activity Index of 0.68 among both *E. coli* and *Shigella* isolates was S20(1), an isolate of *Shigella* spp. isolated from the skin of a fish sample. This *Shigella* isolate also gave a high average zone of inhibition of 19.6 mm for methanolic extract of oregano in 100% DMSO. Therefore, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of S20(1) was measured. MIC was 160 µg/ml and MBC was 165 µg/ml. Finally, in this investigation, it has also been found that the methanolic extract of oregano (dried leaves) contained compounds such as – Tannins, Saponins, Alkaloids, Phenolic compounds, and Steroid. Similarly, the ethanolic extract of oregano also contained the mentioned compounds except for saponins. So, it can be concluded that saponin might be the responsible agent of possessing better antimicrobial properties and this could be a possible reason as to why the efficacy of the methanolic extract of oregano showed better antimicrobial activity than that of the ethanolic extract.Also, the absence of both steroidsand saponins in the Aqueous extract of Oregano could be a potential reason as to why it showed no antimicrobial activity. A study in India has reported that the presence of tannins, saponins, alkaloids, phenolic compounds, steroid, glycosides triterpenoids, resins,

carbohydrate, sterol, flavonoids, gums, and mucilage in the methanolic extract of oregano were all responsible for the antimicrobial activity[33].

To sum up, the overall observation from the study determines that the use of Oregano extracts for treating foodborne illnesses against *E. coli* and *Shigella spp.* could be a potential and promising alternative to antibiotics. Therefore, a further analysis and research of the specific components responsible for the efficacy displayed by oregano may lead to the development of a less expensive yet efficient antimicrobial agent that may further benefit people from developing countries like Bangladesh.

4.1. Conclusion:

To conclude, this study has strengthened the fact that raw tilapia fish samples were a prominent reservoir of *E. coli* and *Shigella spp.* On an alarming point, all of theisolates were observed to possess high resistance to many of the antibiotics that were commonly used in day-to-day life. Moreover, after elucidating the antibiotic susceptibility pattern of these isolates, the study evaluated an alternative source to treat illnesses caused by these pathogenic isolates. Oregano was observed to show great potentials exhibiting antimicrobial activity against the isolates. In addition, the presence of specific components responsible for the efficacy displayed by Oregano could yield in the formulation and research of an effective drug to treat highly antibiotic-resistant bacteria. Thus, the objectives that were set at the beginning of the study were well achieved.

References:

1. Fessehaye, Yonas (2006). Natural mating in Nile tilapia (*Oreochromis niloticus* L.) Implications for reproductive success, inbreeding and cannibalism (PDF). Wageningen: Wageningen UR. pp. 150pp.

2. Richard Xiao, Mr Trade Group. "Tilapia farming, tilapia nutrient, healthy seafood, protein, omega-3". www.mytilapia.com. Retrieved 6 April 2018.

3. Rahman M.M., *et al*(2012) Economics of Tilapia Culture in Watershed Pond in Bangladesh. J Aquacult Res Dev 3:141. doi:10.4172/2155-9546.1000141

4. Sichewo, P. R.,*et al* (2013). Isolation and Identification of Pathogenic Bacteria in Edible Fish: A Case Study of Fletcher Dam in Gweru, Zimbabwe. International Journal of Science and Research (IJSR),2(9).

5. Masud, Z. (2017, March). Food borne illnesses and Bangladesh situation | Bangla ... Retrieved fromhttp://www.bing.com/cr?IG=79D1F2D532794E7BBAE18AF96BA9B444&CID=177C1750F55B6603214D1C8FF4F467B6&rd=1&h=qg3leIaEKy5bgJFsyBV0j3Px2ZzyeGyMt5f-3rmRfAw&v=1&r=http://zuranazrecipe.com/news_event/food-borne-illnesses-and-bangladesh-situation/&p=DevEx.LB.1,5069.1

6. Rocha, R. D., *et al* (2014). Antimicrobial Susceptibility of *Escherichia coli* Isolated from Fresh-Marketed Nile Tilapia (*Oreochromis niloticus*). Journal of Pathogens. http://dx.doi.org/10.1155/2014/756539

7. Van, T. T., *et al*(2007). Detection of *Salmonella* spp. in Retail Raw Food Samples from Vietnam and Characterization of Their Antibiotic Resistance. Applied and Environmental Microbiology,73(21), 6885-6890. doi:10.1128/aem.00972-07

8. Reghu R., Sahadevan P., Sugathan S. (2017) Antimicrobial Agents from Plants. In: Sugathan S., Pradeep N., Abdulhameed S. (eds) Bioresources and Bioprocess in Biotechnology. Springer, Singapor.

9. Bowen A (2016). "Chapter 3: Infectious Diseases Related to Travel". The Yellow Book: Health Information for International Travel. CDC. ISBN 978-0-19-937915-6. Retrieved 22 June 2016.

10. Oregano. (2018) Retrieved from https://www.britannica.com/plant/oregano

11. Feltman, R. (2013). Is There Nothing Science Can't Do With *E. coli*? Retrieved from http://www.popularmechanics.com/science/health/a8832/is-there-nothing-science-cant-do-with-e-coli-15408934/

12. Griffin P.M. and Tauxe, R.V., The Epidemiology of Infections Caused by *Escherichia coli* O157:H7, Other Enterohemorrhagic *E. col*i, and the Associated Hemolytic Uremic Syndrome. Epidemiological Review, Vol. 13, pp. 60-98 (1991).

13. Lim J.Y. Jang W. Y., and Carolyn J. H., (2010). A Brief Overview of *Escherichia coli* O157:H7 and Its Plasmid O157. J Microbiol Biotechnol. 20(1): 5–14.

14. Skippington, E., & Ragan, M. A. (2012, September 01). Phylogeny rather than ecology orlifestyle biases the construction of *Escherichia coli–Shigella* genetic exchangecommunities. Retrieved June 13, 2017, from http://rsob.royalsocietypublishing.org/content/2/9/120112.

15. Read AF, Woods RJ. Antibiotic resistance management. Evol Med Public Health. 2014;2014(1):147.

16. The antibiotic alarm.Nature. 2013 Mar 14; 495(7440):141.

17. World Health Organization (WHO), (2000). Survey of non-prescribed use of antibiotics for children in an urban community in Mongolia.

18. Antibiotic Resistance Questions and Answers. Centers for Disease Control and Prevention. Retrieved from https://www.cdc.gov/antibiotic-use/community/about/antibiotic-resistance-faqs.html

19. Oregano Herbal Medicine, Health Benefits, Side Effects. (2012). Retrieved from http://www.medicalhealthguide.com/articles/oregano.html

20. Chaudhry, N. M., Saeed, S., Tariq, P. (2007). Antibacterial effects of Oregano (*Origanum vulgare*) against gram negative Bacilli. Pak. J. Bot., 39((2): 609-613, 2007.

21. Coccimiglio, J., *et al*(2016). Antioxidant, Antibacterial, and Cytotoxic Activities of the Ethanolic *Origanum vulgare* Extract and Its Major Constituents. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4804097/

22. Cappuccino, J. G., and Sherman N. (2005). Microbiology: A Laboratory Manual.

23. Bauer A. W, Kirby W. M. M., Sherris J. C., Turck M., (1997). Antibiotic susceptibility

testing by a standardised single disk method. Am J Clin Pathol 1997; 45:493-496.

24. Elisha et al. BMC Complementary and Alternative Medicine (2017) 17:133. DOI10.1186/s12906-017-1645-z.

25. Yagoub, SO. (2009). Journal of Bacteriology Research Vol. 1(7), pp. 085-088, October, 2009

26. Ananchaipattana, C.,*et al*(2012): Bacterial Contamination in Retail Foods Purchased in Thailand, Food Science and Technology Research 18, 705-712.

27. David, O.M., *et al*(2009): Isolation of Salmonella and Shigella from fish harvested from the Winam Gulf of Lake Victoria, Kenya. Journal of Infection in Developing Countries, 3, 99-104

28. R. Subashkumar , Tha. Thayumanavan , G. Vivekanandhan and P. Lakshmanaperumalsamy , 2007. Occurrence of Multiple Antibiotic Resistance among Children Diarrhoeal Isolates of *Aeromonas hydrophila* . *Research Journal of Microbiology, 2: 58-65.*

29. Sui, L. K., *et al* (2000).Lactamases in Shigella flexneriIsolates from Hong Kong and Shanghai and a Novel OXA-1-Like Lactamase, OXA-30. Antimicrob. Agents Chemother.August 2000 vol. 44 no. 8 2034-2038.

30. Lebel, M. Ciprofloxacin: chemistry, mechanism of action, resistance, antimicrobial spectrum, pharmacokinetics, clinical trials, and adverse reactions.Pharmacotherapy. 1988;8(1):3-33.

31. Wendakoon, C., Calderon, P. and Gagnon, D. (2012). Evaluation of selected medicinal plants extracted in different ethanol concentrations for antibacterial activity against human pathogens. *Journal of Medicinally Active Plants, 1*(2), 60-68.

32. Birol, O. (2010). The antibacterial activity of essential oil of oregano (*Origanum vulgare L.*). Journal of Food, Agriculture & Environment Vol.8 (2): 2 7 2 - 2 7 4 . 2 0 1 0

33. Prathyusha, P. (2009). Pharmacognostical and phytochemical studies on *Origanum vulgare L.*(Laminaceae). Ancient Science of Life, Vol. 29, No.2 (2009) Pages 17 – 23.

Appendix - I

Media Composition

The following media was used during the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise.

Nutrient Agar:

Component	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Sodium Chloride	5.0
Agar	15.0
Final pH	7.0

Luria Bertani Broth:

Component	Amount (g/L)
Tryptone	10.0
Sodium Chloride	10.0
Yeast extract	5.0

EC Broth:

Components	Amount (g/L)
Tryptone	2.0
Lactose	5.0
Bile Salts No.3	1.5
Di-potassium Phosphate	4.0
Mono-potassium Phosphate	1.5
Sodium Chloride	5.0

Simmon's Citrate Agar:

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Methyl Red Voges- Proskauer (MRVP) Media:

Component	Amount (g/L)
Peptone	7.0
Dipotassium hydrogen phosphate	5.0
Dextrose	5.0
Final pH	7.0

Tryptophan Broth:

Component	Amount (g/L)
Peptone	10.0
Sodium Chloride	5.0

Nitrate Reduction Broth:

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Motility Indole Urease Agar (MIU):

Components	Amount (g/L)
Tryptone	1.0
Phenol red	0.1
Agar	2.0
Sodium Chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Triple Sugar Iron Agar (TSI):

Component	Amount (g/L)
Bio-polytone	20.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium Chloride	5.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.125
Agar	13.0
Final pH	7.3

Saline:

Components	Amount (g/L)
Sodium Chloride	9.0

Appendix - II

Reagents

The following reagents were used throughout the study:

Barritt's reagent:

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

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

Crystal violet Stain (2%):

Two gram of crystal violet was dissolved in 20 ml of 95% ethyl alcohol. 0.8 g of ammonium oxalate monohydrate was then dissolved in 80 ml distilled water. The two solutions were mixed and filtered into sterile reagent bottle.

Iodine solution (Gram's):

Potassium iodide of 6.7 g was dissolved in 100 ml distilled water. To this, 3.3 g of iodine was added, stirred, and the solution made up to 1 L with distilled water. The reagent bottle was then covered in aluminium foil and stored at room temperature.

Kovac's reagent:

Five gram para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. To this, hydrochloric acid (1M) was added to make up the final volume 25 ml. The reagent bottle was then covered with aluminium foil and stored at 4°C.

Malachite green (0.5%):

Malachite green of 0.5 g was dissolved in 100 ml of distilled water. The solution was then stored at room temperature by covering the reagent bottle with aluminium foil.

Methylene blue solution (1%):

One gram of methylene blue was dissolved in 75 ml distilled water, and diluted to make100 ml. The solution was then filtered out and stored in reagent bottle.

Methyl red reagent:

Methyl red of 0.1 g was dissolved in 300 ml of 95% ethyl alcohol. To this, distilled water was added to make up the final volume500 ml. The reagent was then covered with aluminum foil and stored at 4°C.

Oxidase reagent:

N, N, N^1, N^1-tetramethyl-p-phenyldiamine-dihydrochloride of 100 mg was dissolved in 10 ml of distilled water. The solution was then covered with aluminum foil and stored at 4°C.

Safranin:

Safranin of 0.1 g was dissolved in 75 ml of distilled water. The solution was diluted to100 ml, filtered and stored in clean reagent bottle.

Appendix - III

Instruments

Instrument	Company
Autoclave	SAARC
Cellulose filter paper (0.9)	Whatman
Colorimeter, ISO 9001	Labtronics, India
Freeze (-20°C)	Siemens
Incubator	SAARC
Hotplate stirrer	LabTech
Micropipette (10-100 µl)	Eppendorf, Germany
Micropipette (100-1000 µl)	Eppendorf, Germany
Microscope	Optima
pH meter, Model: E-201-C	Shanghai Rousuaa Technology Company, China
Pipette (5 ml, 10 ml)	Eppendorf, Germany
Safety Cabinet	SAARC
Vortex Mixture	VWR International
Weighing balance	ADAM EQUIPMENT™, United Kingdom
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Surgical Millipore syringe fillter	Millex-GS