Isolation of *Salmonella spp.* from raw meat, elucidation of their antibiotic susceptibility pattern and evaluation of the antimicrobial efficacy of Oregano *(Origanum vulgare)* and Black Sesame *(Sesamum indicum)*

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

Submitted by:

Sunayna Hossain Moumi

Student ID: 14136004

Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University

66, Mohakhali, Dhaka-1212

Bangladesh

April, 2018

Declaration

I, Sunayna Hossain Moumi declare that this thesis and the work entitled **"Isolation of *Salmonella spp.* from raw meat, elucidation of their antibiotic susceptibility pattern and evaluation of the antimicrobial efficacy of Oregano *(Origanum vulgare)* and Black Sesame *(Sesamum indicum)*"** submitted 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. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Candidate

_____

Sunayna Hossain Moumi

Certified:

Shamira Tabrejee

Supervisor,
Lecturer, Biotechnology Program
Department of Mathematics and Natural Sciences
BRAC University

_____

Shamira Tabrejee

Dedicated to my Parents and Siblings

Acknowledgement

The completion of my undergraduate dissertation would not have been possible without the constant guidance of Almighty in every phase of my life.

I would like to thank my **Parents, Samiha Hossain, Nafees Hossain** and **Rahatul Amin Ananto** for everything and for being there for me all the time.

My deepest regards, gratitude and appreciation goes to my supervisor **Shamira Tabrejee**, Lecturer, Department of Mathematics and Natural Sciences, BRAC University for her constant supervision, constructive criticism, expert guidance, devoted involvement and enthusiastic encouragement throughout my entire research work. Without her supervision, cooperation and valuable suggestions, it would not have been possible to complete the study. I would also like to thank her for encouraging me throughout this period of time.

I would like to thank and express my heartiest gratitude to **Professor A F M Yusuf Haider**, Ph.D., Professor and Chairperson of Department of Mathematics and Natural Sciences, BRAC University for his active cooperation and encouragement. I express my deepest gratitude to late **Professor A. A. Ziauddin Ahmad**, Professor, Department of Mathematics and Natural Sciences, for his exemplary guidance, monitoring and constant encouragement.

I would also like to thank **Dr. Aparna Islam,** Professor, Department of Mathematics and Natural Sciences, BRAC University and **Dr. Mahboob Hossain,** Professor, Department of Mathematics and Natural Sciences, BRAC University for their enormous support and guidance.

I would like to extend deepest regards and my utmost appreciation to **Zubaida Marufee Islam**, Lecturer (On Leave), Department of Mathematics and Natural Sciences, BRAC University for her constant guidance, care and enthusiastic encouragement starting from my first semester at BRAC University. I would like to thank her for always motivating me to do better.

Thanks to **Romana Siddique**, Senior Lecturer, Department of Mathematics and Natural Sciences, BRAC University for her wise words and support. Also thanks to **Kashmery Khan**, Lecturer, Department of Mathematics and Natural Sciences, BRAC University for her innovative ideas, motivation, constant care and guidance.

I would like to extend my special thanks to **Asma Binte Afzal**, **Shamim Akhter Chowdhury**, **Nahreen Mirza** and **Salman Khan Promon** for their never-ending support, motivation and diligent guidance which helped pave my way through this research much easier.

Special regards to **Md. Furkan Mia** and **Md. Morshed-Al-Mamum** for helping me out each time I asked for help.

My cordial thanks and best wishes go to my friends **A.B.M. Rezwanul Kabir, Ehsan Sakib, Salma Noor, Shabnam Syeed and Sabiha Jahan Liya** for constantly helping me out throughout my thesis with their valuable support and company.

Sunayna Hossain

April, 2018

Table of Contents

1 **Chapter 01: Introduction**……..……**2**

1.1. Background of the study……..3

1.2. Objectives of the study…….....4

2. Chapter 02: Literature Review……**5**

2.1. Salmonella spp…….6

2.1.1.History and Nomenclature……6

2.1.2.Morphology and characteristics……6

2.2. Antibiotics Resistance…….……7

2.3. Horizontal Gene Transfer……8

2.4. Effect of Antibiotic on Human Being……..8

2.5. Origanum vulgare (Oregano) ……..9

2.6. Sesamum indicum (Sesame) ……10

2.7. Antimicrobial properties of these plants…..……..11

3. Chapter 03: Materials and Methods……..**12**

3.1. Working place……13

3.2. Collection of samples……13

3.2.1.Homogenization……...13

3.3. Incubation……..13

3.4. Growth on XLD agar……13

3.5. Growth on SS agar……14

3.6. Culture Preservation…...……...14

3.7. Biochemical Tests……14

3.7.1.Gram staining ……15

3.7.2.Methyl Red (MR) Test……15

3.7.3.Voges–Proskauer (VP) Test……15

3.7.4.Citrate Utilization Test……15

3.7.5.Oxidase Test……16

3.7.6.Catalase Test……...…16

3.7.7.Indole Test……...16

3.7.8.Nitrate reduction Test……..16

3.7.9.Triple Sugar Iron (TSI) Test……16

3.7.10. Urease test, Motility test and Indole test by MIU agar……..17

3.8. Antibiotic Susceptibility Test……17

3.8.1.Preparation of inoculums of the bacterial isolates……..19

3.8.2.Comparison with the McFarland Solution……..19
3.8.3.Inoculation on the MHA plates……...19
3.8.4.Placement of the antibiotic disks……19
3.8.5.Measurement of the zone of inhibition……...19
3.9. Casein hydrolysis test……20
3.10.Antimicrobial activity of Oregano and Sesame……20
3.10.1.Collection of the plant samples……20
3.10.2. Preparation of the plant extracts using different solvents……20
3.10.3. Extraction process……21
3.10.4. Storage and preservation of the extracts……...22
3.10.5. Preparation of inoculums of the bacterial isolates……22
3.10.6. Comparison with the McFarland Solution……...22
3.10.7. Agar well diffusion method……..22
3.10.8. Measurement of zone of inhibition……...23
3.10.9. Determination of activity index……23
3.11. Phytochemical test……..…23
3.11.1. Test for tannins……...23
3.11.2. Test for saponins……24
3.11.3. Tests for alkaloids……...24
3.11.4. Tests for phenolic compounds……24
3.11.5. Tests for steroids……24
4. Chapter 04: Results……**25**
4.1.Fifteen isolates showed characteristics of Salmonella spp. on XLD Agar and SS Agar……26
4.2.Eleven biochemical tests of the fifteen isolates further proved fourteen of them as Salmonella spp. ……..26
4.2.1. Gram Staining……...27
4.2.2. Methyl Red (MR) and Voges–Proskauer (VP) Tests……...27
4.2.2.1. Methyl Red (MR) ……27
4.2.2.2. Voges – Proskauer (VP) ……..27
4.2.3. Citrate Utilization Test……28
4.2.4. Oxidase Test……28
4.2.5. Catalase test……..29
4.2.6. Indole Test……29
4.2.7. Nitrate reduction Test……...29
4.2.8. Triple Sugar Iron Test (TSI) ……30
4.2.9. Urease test, Motility test and indole test by MIU agar……31

4.3.All the fourteen isolates showed the production of casease that hydrolyzes casein……...33
4.4.Antibiotic Susceptibility Test of the isolates……..33
4.4.1. Antibiotic susceptibility tests revealed all the isolates were resistant to nine common antibiotics……33
4.4.2. The isolated exhibited high Multiple Antibiotic Resistance (MAR) Index……..36
4.5. Antimicrobial properties of Oregano and Sesame……..37
4.5.1. Methanolic and Ethanolic extracts of Oregano showed antimicrobial activity against the Salmonella Spp.
Isolates……37
4.5.2. Aqueous extract of Sesame showed antimicrobial activity against the Salmonella spp. Isolates……38
4.5.3. Comparison of Activity Index showed methanolic extract of Oregano had a high activity index……..39
4.5.4. Results of Phytochemical Screening showing variability for Methanolic, Ethanolicc extracts of Oregano and Aqueous extract of Sesame……41
4.5.4.1. Test for tannins……41
4.5.4.2. Test for saponins……...41
4.5.4.3. Tests for alkaloids……42
4.5.4.4. Tests for phenolic compounds……..43
4.5.4.5. Tests for steroids……...44
5. Chapter 05: Discussion……..**45**
5.1.Discussion……46
5.2.Conclusion……...51
6. Reference……...……**52**

List of Figures

Fig 4.1: *Growth and Appearance on XLD Agar and SS Agar a) Growth on XLD Agar, Salmonella spp. has characteristic red colonies with black center b) Subsequent growth of the red colonies with black center on SS Agar, Salmonella spp. characteristic colorless colonies with black center.* _____ *26*

Fig 4.2: *Gram staining of one of the isolate showing the pink stain characteristic of Gram-negative bacteria and rod shaped morphology.* _____ *27*

Fig 4.3: *Appearance of red colour upon addition of Methyl Red indicating a positive result*_____ *27*

Fig 4.4: *VP test showing a) VP broth inoculated with S15 showing no colour change indicating negative result b) Control: VP broth with no bacteria.* _____ *28*

Fig 4.5: *Citrate Utilization test showing colour change of the citrate agar to blue colour indicating positive result on respective samples.* _____ *28*

Fig 4.6: *Oxidase test showing a) Filter paper inoculated with S12 showing no colour change of the filter paper indicating negative result b) Control: Filter paper with no bacteria.* _____ *28*

Fig 4.7: *Catalase test showing a) Hydrogen peroxide inoculated with S16 showing bubble of oxygen gas indicating positive result b) Control: Hydrogen peroxide with no bacteria.*_____ *29*

Fig 4.8: *No red/pink ring indicates a negative result for the indole test.*_____ *29*

Fig 4.9: *Nitrate reduction test a) Control: Nitrate broth with no bacteria b) Control: Nitrate broth with no bacteria but with addition of five drops of Sulphalinic acid reagent, Alpha napthylamine reagent and a pinch of Zinc powder indicating the Nitrate to Nitrite as the broth turns pink in color c) Nitrate broth inoculated with S16 showing reduction of Nitrate to Nitrite indicating positive result.*_____ *30*

Fig 4.10: *TSI test a) TSI agar inoculated with S14 showing red slant with black precipitate on the butt portion indicating fermentation of glucose and H2S production b) Control: TSI agar with no bacteria.*_*30*

Fig 4.11: *MIU Test a) Control: MIU agar with no bacteria b) MIU agar inoculated with sample S12 showing motility positive and urease negative result c) MIU agar inoculated with sample S6 showing motility positive and urease positive result.* _____ *31*

Fig 4.12: *Skim milk agar streaked with S17, S19, S20 and Bacillus*_____ *33*

Fig 4.13: *MHA plates showing the antibiotic susceptibility patterns of S16 and S17.* _____ *34*

Fig 4.14: *The number of Resistant, Intermediate Resistant and Susceptible isolates for each of the 24 antibiotics*_____ *36*

Fig 4.15: *Crude extracts from left corner – Methanolic extract of Oregano, Ethanolic extract of Oregano, Aqueous extract of Oregano, Methanolic extract of Sesame, Ethanolic extract of Sesame and Aqueous extract of Sesame.* _____ *37*

Fig 4.16: *MHA plates showing the antimicrobial assay of a) Methanolic, Ethanolic and Aqueous extract of Oregano along with Ciprofloxacin b) Methanolic extract of Oregano, Absolute methanol,*

Ciprofloxacin and Co – Trioxazole c) Ethanolic extract of Oregano, Absolute ethanol, Ciprofloxacin and Co – Trioxazole _____ *38*

Fig 4.17: *MHA plates showing the antimicrobial assay of a) Methanolic extract of Sesame, Absolute methanol, Ciprofloxacin and Co – Trioxazole b) Ethanolic extract of Sesame, Absolute ethanol, Ciprofloxacin and Co – Trioxazole c) Aqueous extract of Sesame, Distill water, Ciprofloxacin and Co – Trioxazole* _____ *39*

Fig 4.18: *Tannins test showing a) Presence of Tannins in Ethanolic extract of Oregano b) Presence of Tannins in Methanolic extract of Oregano c) Presence of Tannins in Aqueous extract of Oregano d) Absence of Tannins in Aqueous extract of Sesame* _____ *41*

Fig 4.19: *Saponins test showing a) Absence of Saponins in Ethanolic extract of Oregano b) Presence of Saponins in Methanolic extract of Oregano c) Absence of Saponins in Aqueous extract of Oregano d) Presence of Saponins in Aqueous extract of Sesame* _____ *42*

Fig 4.20: *Hager test showing a) Presence of Alkolids in Ethanolic extract of Oregano b) Absence of Alkolids in Methanolic extract of Oregano c) Presence of Alkolids in Aqueous extract of Oregano d) Presence of Alkolids in Aqueous extract of Sesame* _____ *42*

Fig 4.21: *Wagner's test showing a) Presence of Alkolids in Ethanolic extract of Oregano b) Presence of Alkolids in Methanolic extract of Oregano c) Presence of Alkolids in Aqueous extract of Oregano d) Presence of Alkolids in Aqueous extract of Sesame* _____ *43*

Fig 4.22: *Phenolic compounds test showing a) Presence of Phenolic compounds in Ethanolic extract of Oregano b) Presence of Phenolic compounds in Methanolic extract of Oregano c) Presence of Phenolic compounds in Aqueous extract of Oregano d) Absence of Phenolic compounds in Aqueous extract of Sesame* _____ *43*

Fig 4.23: *Steroid test showing a) Presence of Steroid in Ethanolic extract of Oregano b) Presence of Steroid in Methanolic extract of Oregano c) Absence of Steroid in Aqueous extract of Oregano d) Absence of Steroid in Aqueous extract of Sesame* _____ *44*

List of Tables

Table		Page No.
Table 3.1	Antibiotic disks, their amount, and Zone of Inhibition size for Enterobacteriaceae.	18
Table 4.1	Results of the Biochemical Test of the 15 isolates.	32
Table 4.2	Results of the Antibiotic Susceptibility Test of the 14 isolates. Red coloured box indicating Resistance, Green coloured box indicating Intermediate Resistance and Yellow coloured box indicating Susceptible results respectively.	35
Table 4.3	MAR Index and Level of Resistance of the 14 isolates.	36
Table 4.4	Results of the Oregano Extracts	38
Table 4.5	Results of the Sesame Extracts	39
Table 4.6	Comparison of Activity Index	40
Table 4.7	Phytochemical assay of Oregano and Sesame	44

List of Abbreviations

Abbreviations	Descriptions
S.enterica	*Salmonella enterica*
Spp.	Species
HGT	Horizontal Gene Transfer
XLD	Xylose Lisine Deoxycholate
SS Agar	*Salmonella Shigella* Agar
MR	Methyl Red
VP	Voges – Proskauer
i.e.	That is
pH	Negative logarithm of hydrogen ion
LPS	Lipopolysaccharide
ROS	Reactive oxygen species
DNA	Deoxy ribonucleic acid
LB broth	Luria – Bertani broth
TSI	Triple Sugar Iron test
MIU	Motility, Indole and Urease test
H_2O_2	Hydrogen peroxide
MHA	Muller – Hinton Agar
AI	Activity Index
MAR Index	Multiple Antibiotic Resistance Index
H_2S	Hydrogen sulfide
Mm	Millimeter
µg	Microgram
H	Hour
%	Percent
^{O}C	Degree Celsius
Cm	Centimeter
g/L	Grams per liter
mL	Milliliter
µL	Microliter

Abstract

With the increasing consumption of beef all over the world, the prospect of *Salmonella spp.* as a potential reservoir of antibiotic resistance was investigated. As plants are considered new sources of antimicrobial agents against antibiotic resistant bacteria, the antibacterial activity of Oregano (*Origanum vulgare) and* Black Sesame *(Sesamum indicum)* were evaluated against the *Salmonella spp.* isolates. A total of 14 *Salmonella spp.* isolates were obtained from 20 raw beef samples collected from different areas of Dhaka city. Antibiotic susceptibility test of these isolates was done using 24 antibiotics belonging to eight groups – Aminoglycoside, Beta–Lactam, Lincosamide, Macrolide, Nitroimidazole, Quinolone, Sulfonamide and Tetracycline. The antibiotic susceptibility test reported that all the isolates were resistant to Oxacillin, Erythromycin, Tetracycline, Clindamycin, Co-Trimoxazole, Metronidazole, Sulfamethoxazole, Norflaxacin, and Azithromycin. The results also revealed that the isolates exhibited resistance to Cephalexin and Ceftriaxone (86%), Levofloxacin and Penicillin (79%), Kanamycin (71%), Ceftazidime (64%), Amikacin (43%) and Ciprofloxacin (7%) respectively. In addition, the results revealed that the isolates were intermediately resistant to Streptomycin (71%), Amoxicillin and Ampicillin (64%), Imipenen (43%), Ceftazidime (36%), Penicillin (21%), Cephalexin and Ceftriazone (14%), and Kanamycin (7%). No isolate was resistant to two antibiotics which were Chloramphenicol and Cefixime. As an alternative, the Methanolic, Ethanolic and Aqueous extracts of Oregano and Sesame respectively were subjected against the isolates to evaluate the antimicrobial activity. Remarkable antimicrobial activity was observed by the Methanolic extract of Oregano with average zone of inhibition of 23.6 mm, followed by the Ethanolic extract of Oregano with average zone of inhibition of 17.6 mm and least by the Aqueous extract of Sesame with average zone of inhibition of 10.6 mm. The Aqueous extract of Oregano and the Methanolic and Ethanolic extracts of Sesame did not show any antibacterial activity. This study showed that the *Salmonella spp.* isolates are becoming resistant to 17 antibiotics that are commonly used for *Salmonella spp.* illness – Amoxicillin, Levofloxacin, Cephalexin, Ampicillin, Ciprofloxacin, Ceftazidime, Ceftriazone, Nalidixic Acid, Sulfamethoxazole, Imipenen, Amikacin, Clindamycin, Norflaxacin, Azithromycin, Co-Trimoxazole, and Metronidazole. However, commonly used Cefizime and Chloramphenicol were reported to be effective antibiotics in this study. Lastly, the presence 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.

Chapter 01: Introduction

CHAPTER 01: INTRODUCTION

1.1 Background of the study:

The worldwide production of meat has been observed to increase triple its range over the past four decades, more specifically there has been an increase of meat production to about 20 percent in the last 10 years. The world consumed about 129.5 billion pounds of beef in 2016[2]. Developed countries have been statistically observed to consume double the quantity of growing amount of meat compared to developing countries[1]. Since Bangladesh falls under the category of the UN developing countries, the concern of the increase in consumption and production of beef in Bangladesh can be logically addressed. With the increase of various types of cuisines containing beef served, which may also include semi-cooked dishes, an increase of food borne diseases causing foodborne illnesses has been reported. The prevalence of food-borne pathogens in raw meats have also been studied as raw meats are potential medium for transmitting these pathogens[3]. Since food borne illnesses are the most serious public health issues in Bangladesh, proper precaution must be taken[4]. As raw beef can be a potential reservoir of a number of microorganisms such as pathogenic strain of *Salmonella spp.*, which may be multidrug resistance, the concern over food safety arises. Food safety check will help ensure non-contaminated use of food products which can facilitate in decreasing the food borne illnesses in Bangladesh.

Salmonella spp. is a rod shaped Gram negative bacteria which is mostly studied for its food-borne illness causing agents. *Salmonella spp.* causes – Typhoid fever, food poisoning, gastroenteritis, enteric fever, and salmonellosis. *Salmonella* is a major cause of human bacterial infections in the United States (U.S.). According to the Centers for Disease Control and Prevention (CDC), it affects around 1 million Americans every year, leading to 19,000 hospitalizations and 380 deaths[5]. Although *Salmonella* gastroenteritis is a self-limiting illness, severe cases may require antimicrobial treatment and/or therapy[6].

Contamination of food with antibiotic-resistant bacteria is an alarming major threat to public health, the transfer of the antibiotic resistance gene from one pathogenic bacteria to another can be determined via Horizontal Gene Transfer which can limit the available treatment against the severe bacterial infections[6]. In the recent decades, an increase in multidrug resistance bacteria has been reported[7].

Various types of plants contributing to the medicinal aspect has been derived from ancient times. Plants contain various compounds within themselves which enhances its antimicrobial properties. The development and promotion of traditional medicine published by the World health organization (WHO) talks about how to identify traditional medicine, formation of proper policy and plan to use them, have a proper research and education on them, establish a bond between the employees of traditional and modern medicine, and lastly develop a method to cultivate the needed herbs without the destruction of natural resources[8]. With the increase of multidrug resistant bacteria, the efficacy of many antibiotics have been reported to not be able to efficiently treat severe bacterial infections. As an alternative source, the use of plant products containing antimicrobial activities have been increasing worldwide to treat bacterial infections. Plant products are natural products, thus have lower side effects compared to antibiotics[9].

Origanum vulgare (Oregano) along with being a useful culinary herb, it is also used as medicinal herb in various parts of the world. Oregano is used as relaxant, antibacterial, antiseptic and fungicide, and also to boost the immune system[26]. *Sesamum indicum* (Sesame) is used for nutritional, medicinal and industrial purposes, as they have energizing, carminative and diuretic. They are also used to cure dysentery and relax the bowels[32].

Oregano is grown and also used to a limited extent in Bangladesh. It is mostly imported, and widely used as culinary herb in the preparation of food. Hence, the various medicinal properties may not widely be known. On the other hand, Sesame is grown in various states of Bangladesh. It is commonly used for both culinary and medicinal purposes.

1.2 Objectives of the study:

The specific aims and objectives of the study are as follows:

- To isolate and identify *Salmonella spp.* from raw beef samples from different parts of the Dhaka city.
- To study the antibiotic resistance pattern of the *Salmonella spp.* isolates.
- To determine the efficacy of the antimicrobial property of Oregano and Sesame against the isolated *Salmonella spp.*
- To determine the presence of phytochemicals in the efficacious plant extracts.

Chapter 02: Literature Review

CHAPTER 02: LITERATURE REVIEW

2.1.*Salmonella spp.*

Salmonella spp. are Gram-stained negative rod-shaped bacteria having tail-like structure, i.e., flagella, which helps in locomotion[10]. *Salmonella spp.* belongs to the Enterobacteriaceae group and have two sub-species; *Salmonella enterica* and *Salmonella bongori*[11].

Based on the specific proteins on the bacterial and flagellar surface, the different types of S*almonella spp.* are characterized. Different combination of protein coats is termed as a "serovar", which are determined by immunologic tests by special laboratories[10].

2.1.1. History and Nomenclature:

In 1885, Theobald Smith, research – assistant of Dr. Salmon, had discovered the first stain of *Salmonella* (*Salmonella enterica* var. Choleraesius, that causes hog cholera); but since Dr. Daniel E Salmon, an American veterinary scientist, was in charge of the research, he was given the credit for the discovery. Hence, the genus of *Salmonella spp.* was named after Dr. Salmon[12].

The nomenclature of *Salmonella spp.* have been altered over time now. At present, there are over 2000 strains of *Salmonella spp.* grouped into *Salmonella enterica*[14]. This species is further divided into six subgroups (*S. enterica* subsp.*enterica, S. enterica* subsp.*salamae, S. enterica* subsp.*arizonae, S. enterica* subsp.*diarizonao, S. enterica* subsp.*houtenae, and S. enterica* subsp.*indica*) based on host range specificity[15]. All strains that are pathogenic to humans are in species *Salmonella enterica*, subgroup 1[13].

2.1.2. Morphology and characteristics:

Salmonella spp. belongs to the Enterobacteriaceae group[11]. They are Gram-stain negative rod-shaped (0.7 – 1.5 micrometers by 2.0 – 5.0 micrometers) bacteria which are facultative anaerobic in nature[10]. They have both a respiratory and fermentative type metabolism, hence are Chemoorganotrophic. Hence, glucose and other carbohydrates are catabolized with the production of acid and usually gas. Therefore, they are also Aerogenic, expect for *Salmonella choleraesuis* , subsp. *typhi.*[16]

They normally grow on an optimum temperature of 37ºC but can also grow at above 5ºC to 47ºC. Likewise, the optimal growth occurs at pH 7 but can also grow at minimum pH 5.4 with acetic acid to 4.5 with hydrochloric and citric acids[17].

Salmonella spp. have tail-like structure to facilitate motility, i.e., flagella. However, non-motile mutants can occur too. *Salmonella gallinarum / Salmonella pullorum*, are a type of *Salmonella spp.* that is always non-motile. They are structurally covered with short hair-like structures that helps them in cellular attachment, i.e., pilli[16].

Salmonella spp. possess three major antigens: H or flagellar antigen, O or somatic antigen, and Vi antigen. H antigen can occur in phase 1 and/or phase 2. O antigens are determined based on the specific sugar sequences on the surface of the cell and also occurs on the surface of the outer membrane. Vi antigen overlaps the O antigen as they are considered to be superficial antigen.

The cell envelope of *Salmonella spp.* contains a form of complex lipopolysaccharide (LPS) structure that is released on the lysis of the bacterial cell. LPS moiety may possess endotoxin properties which could help to determine the level of virulence of the bacteria. An outer O-polysaccharide coat, a middle portion (the R core), and an inner lipid A coat – are the three components of the macromolecular endotoxin complex. . The structure of Lipopolysaccharide is hence important for various reasons: the sugar variation in the outer O-polysaccharide helps determining the virulence level of the bacteria as it is responsible for O antigen specificity; the role of endotoxin component of the cell wall in pathogenesis. Endotoxins evoke fever, activate the serum complement, clotting systems, depress myocardial function, and alter lymphocyte function[17].

2.2.Antibiotics Resistance

With the increase intake of antibiotics in today's time that to without professional advice, the occurrence of antibiotic resistance is mostly likely to process via genetic variation. The number of locations of the world overdosing or misusing the intake of antibiotics for both humans and animals have shown great rise over the period of time. Antibiotic resistance occurs when microorganisms are exposed to antibiotics frequently, leading them to understand the mechanism of the antibiotic drug and hence can alter its defense mechanism against the antibiotic. On developing multiple antibiotic resistance, the microorganisms is then referred to as "superbugs". Therefore, certain

antibiotics become unproductive, weakening the medicinal support and thus resulting in the infections to stays back in the body. This further risk the spread of the infection to others. The spread of antibiotic resistance microbes in the environment can occur due to – Weakening medicinal control, inefficient infection control, low sanitary conditions and improper food-handling managements. With the increase in antibiotic resistance, we are most likely to head towards the "post – antibiotic" era where common infections and/or minor injuries could ones again lead to dead. Henceforth, it is a worldwide issue[18-20].

2.3.Horizontal Gene Transfer

Horizontal gene transfer (HGT) is a process that involves the movement of genetic information from one organism to another. This process also involves the spread of antibiotic resistance genes between the organisms (expect for the Vertical Gene Transfer, i.e., from parents to offspring). This further results in the increase of pathogens around us[21].

Any sort of genes may be horizontally transferred and proliferate by natural selection. It is not necessary that only virulent determinant gene may get horizontally transferred[22].

HGT process with three genetic mechanisms –

- Transformation: Bacteria take up DNA from their environment,
- Conjugation: Bacteria directly transfer genes to another cell, and
- Transduction: Bacteriophages move genes from one cell to another.

2.4.Effect of Antibiotic on Human Being

Antibiotics interfere with the bacterial functions in the Human body to ensure the degradation of the bacterial cell. Some antibiotics also can create reactive oxygen species (ROS) in the body which is lethal to healthy cells and helps in further degradation of the pathogenic bacterial cells. The ROS is toxic to healthy cells and can possibly harm bacterial cells. The ROS start to kill energy sources as well as the mitochondria in human cells. The ROS are thought to bind to bacterial DNA and dissociate it, therefore killing the bacteria, as the bacteria no longer have genetic information. However, ROS are not terribly specific and can begin to damage all the cells and metabolic machinery in the body, not just in bacterial cells. This is known as oxidative tissue damage and can be especially harmful if a person's immunity is already compromised by a bacterial infection[23].

Some antibiotics have the affinity towards synthetic substances that can completely destroy or inhibit the growth of a microorganism. These antibiotics are known as Allopathic antibiotics and are highly used to treat infected animals and human. It has also been observed that allergic reactions (mild or fetal) could occur due to certain type of antibiotics. This is due to the chemicals used in preparation of the Allopathic antibiotic. The use of antibiotics for a prolonged period of time can lead to ill effects in patients, such as – ototoxicity, nephrotoxicity, and tendinopathy[24].

2.5.*Origanum vulgare* (Oregnao)

Origanum vulgare, commonly known as Oregano or wild marjoram, belongs to Lamiaceae family. Oregano is a perennial plant known for its flavorful dried leaves and flowering tops. Oregano grows in warm temperate areas. They grow from 20 – 80 cm tall, with opposite heart-shaped leaves 3 to 9 cm long, produced in erect spikes[25].

Oregano is native in India, western and southern Eurasia and Mediterranean countries, only later it was cultivated in the Philippines and other Asian countries. It is an important culinary herb as it is used to for its aroma and slight bitter taste. Oregano is mostly used in Mediterranean and Mexican foods. Other than oregano being used as culinary herb, it is also used as medicinal herb in various parts of the world. Oregano is used as relaxant, antibacterial, antiseptic and fungicide, and also to boost the immune system. It also has been used as herbal remedy for skin burns, cuts and bruises. The minty flavor of oregano enhanced its herbal remedy for sore throat, asthma, colds, coughs and flu. According to studies, it has also been observed that high in antioxidant can help prevent cancer[26].

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

2.6.*Sesamum indicum* (Sesame)

Sesamum indicum, commonly known as Sesame, is an oilseed crop. They are annual herb with hollow stem and branches that grow in an erect manner. They can be branched or unbranched based on its variety. The growth of the plant is about 2 meters in height. It has an appearance similarity with foxglove. The flower of the plant can be either white, yellow, pink or violet in color. The flowers are usually said to follow self-pollination. In most sesame plants, only the middle fruit of the group fully ripens[28-31].

The long taproot of the plant has many lateral roots. The plant was first originated in Africa. These plants are cultivated throughout the tropical and subtropical parts of the world as it requires moist and warm weather conditions for its growth, and dry weather conditions for the seed to ripen. Ones the seed capsules turn brownish black, they burst and release the ripe seeds, which again can be white, brown or black in color depending on the variety[28-31].

Sesame can be used for nutritional, medicinal and industrial purposes. They are said to be energizing, carminative and diuretic. They also help in an increase of breast milk in female and sperms in male. They have also been useful to cure dyspepsia and dysentery. Sesame is also used to tonify the liver and the kidney, replenish vital essence and blood, and to relax the bowels[32].

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 – Pedaliaceae – pedaliums

Genus – *Sesamum L.* - sesame

Species – *Sesame indicum L.* – sesame

2.7.Antimicrobical properties of these plants

Oregano is known to be one important source of medicinal plant for maintaining human health for a while now, with the increase of studies for natural therapies. Oregano is said to contain carvacrol (it is a monoterpenoid phenol), which is said to have antimicrobial properties. Along with that, some of the most important components oregano possess 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 few gram negative and gram positive bacteria[33-34].

Seasame on the other hand, is rich in phytochemicals such as lignans (they are methylene dioxyphenyl compounds). Along with lignans, sesame has a wide range of phenolic compounds – phenolic acids, flavonoids, anthocyanins, tannins, lignans, catechin, and others – these are also known have antioxidant activities. These components are said to provide protection against harmful free-radicals. Since, phenolic antioxidants can react directly with free radicals and convert them into stable products, these components have been known to reduce the risk of certain types of cancer, cardiovascular disease (CVD), coronary heart disease (CHD), stroke, atherosclerosis, and other degenerative diseases associated with oxidative stress. The primary antioxidants functions by donating a hydrogen atom. The secondary antioxidants lower the rate of oxidation by several mechanisms. Natural phenolic compounds can function as both primary and secondary antioxidants[35]. Thus, antibacterial effects of sesame have been reported against gram negative and gram positive bacteria.

Chapter 03: Materials and Methods

CHAPTER 03: MATERIALS AND METHODS

3.1.Working place

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

3.2.Collection of samples

From different areas in Dhaka city, twenty raw meat samples were collected from different butchers shop. Each sample that was collected from different butchers shop was kept in sterile zipper bags and labelled accordingly. Each sample was homogenized without minimum delay. To prevent contamination, care was taken and hand gloves were worn at each steps while handling the samples.

3.2.1. Homogenization:

With the help of mortar and pestle, the samples were homogenized on 0.85% peptone salt water. The equipment used was sterilized with 70% ethanol beforehand and care was taken while homogenization to avoid any sort of contamination.

3.3.Incubation

500 µl of the sample was then put into test tubes containing LB broth (Luria-Bertani Broth), which is a nutritionally rich medium, is primarily used for the growth of bacteria, and incubated at 37 ºC for 24 h.

3.4.Growth on XLD agar

A loop full of the broth culture was then streaked on to Xylose Lysine Deoxycholate (XLD) agar and incubated at 37 ºC for 24 h for the primary screening of *Salmonella spp* colonies. XLD Agar is highly recommended selective medium for observing the growth of *Salmonella* and *Shigella*[36].

3.5.Growth on SS agar

A loop of single colony of 24 h bacterial culture (red-pink colonies with black center) from the XLD agar was then streaked on to Salmonella – Shigella (SS) agar and incubated at 37 ºC for 24 h for the secondary screening of *Salmonella spp* colonies. As for the name suggests by itself, SS Agar is yet another highly recommended selective medium for observing the growth of *Salmonella* and *Shigella*. The resulting colonies (colorless colonies with black center) were further cultured into new XLD agar plates and this step is triplicated in order to increase the chances of obtaining a pure culture of *Salmonella spp.*[36]

3.6.Culture Preservation

15 *Salmonella spp.* isolates were obtained from the 20 samples and these were stored on XLD agar and sub-cultured every two weeks until the end of the study.

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

3.7.Biochemical Tests

The 15 isolates that are considered to be *Salmonella spp.* were further tested via a set of biochemical tests in order to confirm that they were indeed *Salmonella spp.* For all the biochemical test, 24 h bacterial culture was used. The following biochemical tests were performed:

- Gram Staining,
- Methyl Red (MR) test,
- Voges – Proskauer (VP) test,
- Citrate test,
- Oxidase test,
- Catalase test,
- Triple Sugar Iron (TSI) test,
- Urease test, Motility test and Indole test by MIU agar, and
- Nitrate Reduction test.

3.7.1. Gram staining:

Gram staining is a test that helps to distinguish between the two broad categories of bacteria: Gram positive and Gram negative. Gram positive bacteria is observed by the display of a purple stain while Gram negative bacteria is observed by the display of a pink stain. The morphology of the bacteria can also be determined.

Gram staining involves four basic steps. Firstly, a smear of bacteria is prepared onto a glass slide and heat treated. Gram's iodine is then added to the smear. In case the bacteria are gram positive their thick peptidoglycan layers retain the purple color. Secondly, mordant is applied which stabilizes the purple stain. Thirdly, 95% ethanol is used to wash the stain; in case the bacterium is negative its thin peptidoglycan layer is washed off. Lastly, a counter dye safranin is added which is retained by gram negative bacteria. The slide is then viewed under a compound microscope to analyze the color and shape of the bacteria[36].

3.7.2. Methyl Red (MR) Test:

The bacterial culture are inoculated into MRVP broth in a clean test tube and incubated overnight at a temperature of 37°C for 24 h. After incubation, 5 drops of methyl red was added and the medium was observed for the immediate color development. Appearance of a red colour indicates a positive result[36].

3.7.3. Voges–Proskauer (VP) Test:

The bacterial culture are inoculated MRVP broth in a clean test tube and incubated overnight at a temperature of 37°C for 24 h. After incubation, 0.6ml (12 drops) of Barrit's reagent A and 0.2ml (4drops) of Barrit's reagent B was added along with a gentle shake of the medium to atmospheric oxygen. The tube was then kept in still position for 10-15mins. The solution was then observed for color changes to determine whether the result is positive (pink-red) or negative (yellow) [36].

3.7.4. Citrate Utilization Test:

Firstly, the citrate agar was prepared. 2 ml media was then added to clean vials. The vials were autoclaved and then left to cool at a slanted position in order to create a butt and slant. A loop of a single colony of the bacterial culture from a 24 h bacterial culture was then streaked on the slant of the media from bottom to top using a zigzag motion with the loop. The vials were incubated at

37°C for 24 h. The colour of the media was observed after incubation. Colour change to blue is considered to be positive and no colour change was considered to be negative[36].

3.7.5. Oxidase Test:

The oxidase test is performed to determine where 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. To perform this test, Kovacs Oxidase Reagent was used. Its composition is 1% tetra-methyl-p-phenylenediamine dihydrochloride, in water[36].

3.7.6. Catalase Test:

A sterile microscopic slide was placed on a petri dish and a loop of the organism was picked using a sterile inoculating loop. Then 1 drop of 3% H_2O_2 was added on the organism on the microscopic slide by using a dropper. Finally, it was observed for the presence of bubbles of oxygen gas[36].

3.7.7. Indole Test:

To perform the indole test, tryptophan broth was inoculated with bacterial culture and incubated at 37°C for 24 h. After incubation, 0.5 ml of Kovac‘s reagent was added to the broth culture. The color changes was observed to determine whether the result is positive (cheery red ring) or negative (yellow) [36].

3.7.8. Nitrate reduction Test:

The nitrate broth was prepared, boiled and 5 ml of the broth was added to each test tube using a glass pipette. The broth was autoclaved and left to cool. Using an inoculating loop, a loop of single colony of the bacterial culture was inoculated into the broth in each test tube respectively. The test tubes were incubated at 37°C for 24 h. After incubation, 5 drops of each reagent A and reagent B was added to the test tubes. Formation of a red colour was observed as positive result[36].

3.7.8. Triple Sugar Iron (TSI) Test:

The sugar utilization test or carbohydrate utilization test is basically used to check whether carbon source is utilized by the organisms present. To perform the test, a straight inoculating needle was used to pick an isolated colony and inoculated the TSI 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 h at 37°C the results were observed and interpreted accordingly:

- Acid production: Changes the medium into yellow color- organism ferments the given carbohydrate and produce organic acids there by reducing the pH of the medium into acidic.

- Acid and Gas production: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the inverted Durham tubes.

- The Absence of fermentation: The broth retains the red color. The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium[36].

3.7.9. Urease test, Motility test and Indole test by MIU agar:

To conduct this test, the MIU was prepared, boiled and autoclaved. Empty test tubes were autoclaved as well. After autoclave, the media was left to cool so that the temperature went down to 50°C. In a separate flask, 40% urea solution was made and filtered. To the cooled media, the urea solution was added and mixed properly.

A sterile glass pipette was used to transfer 6ml of the media to the autoclaved test tubes. The media was left to cool down completely until it had a semi solid consistency.

Using an inoculating needle, a colony from a 24 h bacterial culture was picked up and inoculated in the medium by stabbing the needle down into the media. The test tubes were incubated at 37°C for 24 h. The appearance and colour of the media was observed after incubation[35].

3.8. Antibiotic Susceptibility Test

The antibiotic disk diffusion test is conducted to detect antimicrobial susceptibility in the bacterial isolates. Kirby-Bauer antibiotic testing was used to test whether particular bacteria are susceptible to specific antibiotics.

In this study, the effect of 24 different commercially available antibiotics was determined. The list of antibiotics used is as follows:

Table 3.1: Antibiotic disks, their amount, and Zone of Inhibition size for Enterobacteriaceae.

Disk diffusion diameter (in millimeter, mm)

S. No:	Antibiotics	Disc Content	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
1.	Penicillin (PEN)	10 µg	10	11-21	22
2.	Amoxicillin (AMX)	10 µg	13	14-17	18
3.	Streptomycin (STR)	10 µg	10	11-14	15
4.	Levofloxacin (LVX)	3 µg	15	16-18	19
5.	Oxacillin (OXA)	1 µg	10	11-12	13
6.	Kanamycin (KAN)	30 µg	13	14-17	18
7.	Cephalexin (LEX)	30 µg	22	23-24	25
8.	Ampicillin (AMP)	10 µg	13	14-16	17
9.	Ciprofloxacin (CIP)	5 µg	19	20-21	22
10.	Erythromycin (ERY)	15 µg	13	14-17	18
11.	Tetracycline (TET)	10 µg	14	15-18	19
12.	Chloramphenicol (CHL)	30 µg	12	13-17	18
13.	Ceftazidime (CAZ)	30 µg	16	17-18	19
14.	Ceftriazone (CRO)	10 µg	18	19-21	22
15.	Nalidixic Acid (NAL)	30 µg	13	14-18	19
16.	Trimethoprim/Sulfamethoxazole (SXT)	25 µg	12	13-15	16
17.	Imipenem (IPM)	10 µg	13	14-15	16
18.	Amikacin (AMK)	30 µg	14	15-16	17
19.	Clindamycin (CLI)	2 µg	14	15-20	21
20.	Norfloxacin (NOR)	10 µg	12	13-16	17
21.	Azithromycin (AZM)	15 µg	13	14-17	18
22.	Cefixime (CFM)	5 µg	15	16-18	19
23.	Co – Trimoxazole (COT)	1.25/23.75 µg	10	11-15	16
24.	Metronidazole (MT)	4µg	15	16-18	20

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

3.8.1. Preparation of inoculums of the bacterial isolates:

The test tubes were labeled carefully. 85% saline solution was prepared and taken on the test tubes. Using an inoculating loop, one or two colonies of the bacterial isolates were picked up from sub cultured nutrient agar plates and suspended on the saline .Then all the test tubes were vortexed properly to make the suspension homogenous, the turbidity of the suspension was maintained.

3.8.2. Comparison with the McFarland Solution:

The inoculums turbidity were then compared with standard McFarland 1.0.

3.8.3. Inoculation on the MHA plates:

MHA (Muller-Hinton Agar) agar was prepared, autoclaved and plated with care and proper labeling. Autoclaved swab was dipped into the bacterial suspensions and rotated so that it is contained a sufficient amount of the suspension. Before dipping the cotton swab, the test tubes containing the bacterial suspension were vortexed. The swab was then 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 of the swab. The agar plate was being rotated 90 degrees each time it was being streaked, to ensure the even distribution of the inoculums.

3.8.4. Placement of the antibiotic disks:

A burnt sterile forceps was used to insert the antibiotic discs on the MHA plate. Antibiotic discs were placed on the surface of the inoculated MHA plates. Each of the discs were carefully placed and slightly pressed on the MHA plates using the forceps. The placement of the discs close to the edges of the plate was avoided to ensure zone measurement errors. The MHA plates were inverted and incubated at 37ºC for 24 h.

3.8.5. Measurement of the zone of inhibition:

After the incubation period, the zone of inhibition for each of the antibiotics was observed on the MHA plate. The size of zones for each antibiotic were measured carefully in millimeters (mm) using a ruler. All the measurements were taken viewing the back of the Petri dish. Each zone size was measured from three angles of the zone to ensure accuracy. The zone size was then recorded on the recording sheet in a chart.

3.9. Casein hydrolysis test

To determine the ability of a microorganism that can degrade the protein casein by excreting hydrolytic extracellular enzyme that enables the degradation. Skim milk agar is prepared, autoclaved and plated carefully ensuring the aseptic conditions. A loop of the single colony of the bacterial culture is streaked on the skim milk agar. The plates are then incubated at 37°C for 24 h. On the following day, if the organisms secrete proteases, it will exhibit a zone of proteolysis which is determined by observing the clear (zone) area surrounding the bacterial growth. This is interpreted as a positive result. A negative result is interpreted when the protease activity is absent, i.e., there would be no clear (zone) area surrounding the bacterial growth, instead the medium surrounding growth of the organism remains opaque[36].

3.10. Antimicrobial activity of Oregano and Sesame

3.10.1. Collection of the plant samples:

- **Oregano:**

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

- **Sesame:**

Black sesame seeds were purchased from the local market in Dhaka city. They were air dried for a day and then crushed to a fine powder, weighed and stored in clean air tight container.

3.10.2. Preparation of the plant extracts using different solvents:

Methanol and Ethanol, these two organic solvents were used. Along with that one inorganic solvent was used, i.e., distill water. Hence, three different types of extracts were prepared using the two samples respectively.

In case of methonolic extract, methanol is used as the solvent. Methanol 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 from of the extract, methanol easily evaporates. Lastly, methanol is cost friendly and is considered as a safe organic solvent.

In case of ethanolic extract, ethanol is used as the solvent. Ethanol 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.

In case of aqueous extract, distill water is used. Water has the ability to dissolve various substances present in the sample because of its polarity and ability to form hydrogen bonds, for which it is considered to be one of the efficient inorganic solvent. Water is just poor at dissolving nonpolar molecules which is the only drawback.

3.10.3. Extraction process:

To prepare the extracts (methanolic extract, ethanolic extract) of the two samples, 70 grams of the powder was measured via electronic balance and then the powder was carefully transferred on the extraction thimble, inside the Soxhlet apparatus. The round receiving flask of the apparatus was then filled with 250 ml of extraction solvent such as methanol or ethanol respectively. During the process, the Soxhlet was allowed to run at different temperatures. As the boiling temperature of the solvents varied. The boiling temperature of methanol and ethanol are 64.7 ℃ and 78.37 ℃ repectively. However, while operating the Soxhlet apparatus, the process was carried at a temperature which was little 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 h – 4 h. Ones the color of cotton inside the extraction thimble became colorless, the extraction process was stopped. The contents of the round flask was then transferred into the receiving flask of the rotary evaporator. Plants extract contents evaporation and condensation is done into the rotary evaporator at the temperature of 80 ℃. The solvent of the plant extract contents that recovered from Soxhlet was evaporated to a point where the extract become concentrated. The extract was then scraped via spatula and collected in the sterile petri plates and stored into the fume hood overnight until maximum evaporation of the solvent content in the extract was attained. A sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The crude extract was then again transferred into an autoclaved McCartney bottle via spatula. The process is then repeated for each plant sample using the respective solvents.

To prepare the aqueous extract of the two samples, 70 grams of the powder was measured via electronic balance and then the powder was carefully transferred into a beaker containing 250 ml

of distill water. This mixture was stirred continuously and heated for about 1h – 2h. The mixture was then let to cool down until it reached the room temperature. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. The concentrated extract solution was then poured on a sterile petri plates and stored into the fume hood overnight until maximum evaporation of the solvent content in the extract was attained. A sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The crude extract was then again transferred into an autoclaved McCartney bottle via spatula. The process is then repeated for each plant sample using distill water.

Both the process to obtain the methanolic, ethanolic and aqueous extract of the samples were performed ensuring sterile conditions.

3.10.4. Storage and preservation of the extracts:

All the prepared extracts were stored in autoclaved 25 ml McCartney bottles which were then kept in 4°C refrigerator for preservation. The caps of all the bottles were tightly closed to ensure no external contaminants until use. 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.

3.10.5. Preparation of inoculums of the bacterial isolates:

To prepare bacterial suspension, 0. 9 g NaCl was dissolved in 100 ml distilled water in clean conical flask. The saline prepared was then heated and transferred into clean test tubes. After autoclaving the tubes, they were labelled accordingly. A loop of single colony of the 24h bacterial culture was then inoculated into the saline and vortexed to obtain a homogenous suspension.

3.10.6. Comparison with the McFarland Solution:

The inoculums turbidity were then compared with standard McFarland 1.0.

3.10.7. Agar well diffusion method:

The agar surface of each of the Muller-Hinton Agar was streaked by a sterile cotton swab with the bacterial suspension from the physiological inoculated saline receptively. After that, the agar plate was punched with a sterile cork borer of 4 mm size. 60 µL of each plant extract sample was poured into the well-formed via micropipette.

The Muller-Hinton Agar was divided into four quadrants, with two quadrants designated for the plant extract obtained and 100% of the respective solvent. The other two quadrants were designated for the placement antibiotic disc (a sensitive antibiotic disc interpreted for positive result, and a resistance antibiotic disc for negative result) based on the results obtained during Kirby-Bauer antibiotic testing.

The plates were then allowed to standby for 30 min. The plates were incubated at 37°C for 24 h.

3.10.8. Measurement of zone of inhibition:

A clear zone on the MHA plate around the agar well containing the plant extract represents the zone of inhibion which signifies the antimicrobial activity of the antibiotic activity of the extract. The positive control, i.e., the antibiotic disc used showing a clear zone also ensures the procedure being followed accurately. The diameter of the clear zone was measured three times in millimeter (mm) with a scale from three different angles of the zone of inhibition. The average value of zone of inhibition for each extracts was then calculated and recorded.

3.10.9. Determination of activity index:

The inhibitory effects of the methanolic, ethanolic and aqueous 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

3.11. Phytochemical test

Biochemical assays of five different types were done to determine the present of: tannins, saponins, alkaloids, phenolic compounds and steroids.

3.11.1. Test for tannins:

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

3.11.2. 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 frothing observation in the test tube indicates the presence of saponins.

3.11.3. Tests for alkaloids:

- **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.

- **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.

3.11.4. 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.

3.11.5. 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.

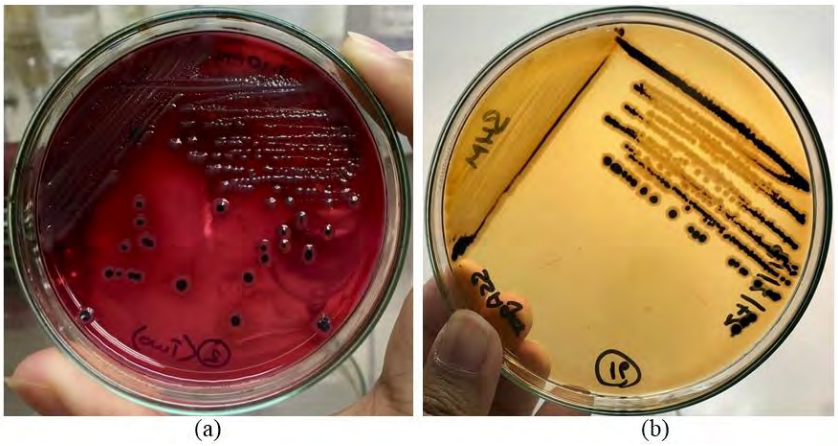
All these material and methods were used and performed to determine the isolates to be *Salmonella spp.*, to determine the antibiotic resistance pattern of the isolates, to determine its ability to produce hydrolytic extracellular enzyme that enables the degradation of protein casein, and lastly to determine the antimicrobial properties of Oregano and Sesame against these isolates.

Chapter 04: Results

CHAPTER 04: RESULTS

4.1.Fifteen isolates showed characteristics of *Salmonella spp.* on XLD Agar and SS Agar

Both XLD Agar and SS Agar are selective and differential media for *Salmonella spp*. After an incubation period of 24 hours, 15 isolates from the 20 samples produced red colonies with black center on the XLD Agar. These 15 isolates were further streaked on SS Agar, where it produced colorless colonies with black center. All of these are characteristic for *Salmonella spp*. However, sample 08, 09, 10, 13 and 18 did not produce any red colonies with black center and neither colorless colonies with black center when cultured on XLD Agar and SS Agar respectively. Hence the samples 08, 09, 10, 13 and 18 were discarded from the investigation.



(a) (b)

Fig 4.1: Growth and Appearance on XLD Agar and SS Agar a) Growth on XLD Agar, *Salmonella spp.* has characteristic red colonies with black center b) Subsequent growth of the red colonies with black center on SS Agar, *Salmonella spp.* characteristic colorless colonies with black center.

4.2.Eleven biochemical tests of the fifteen isolates further proved fourteen of them as *Salmonella spp.*

Even though these 15 isolates were suspected to be *Salmonella spp.,* a set of biochemical tests were performed in order to confirm that they were indeed *Salmonella spp.*

4.2.1. Gram Staining:

All the 15 isolates which were suspected to be *Salmonella spp.* was observed to be stained pink after Gram staining was performed. The morphology of the isolates were also checked under the microscope and all of them were observed to be rod shaped. Hence, all the 15 isolates were considered to be rod shaped Gram-negative bacteria.

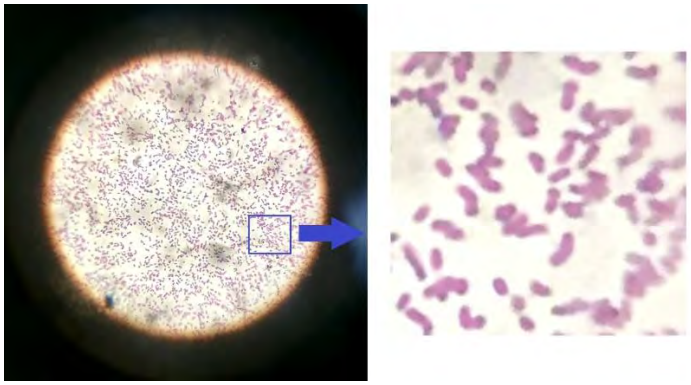


Fig 4.2: Gram staining of one of the isolate showing the pink stain characteristic of Gram-negative bacteria and rod shaped morphology.

4.2.2. Methyl Red (MR) and Voges–Proskauer (VP) Tests:

4.2.2.1. Methyl Red (MR):

All the 15 isolates gave red colour upon addition of a few drops of Methyl red. Hence all the isolates gave positive result for *Salmonella spp.*

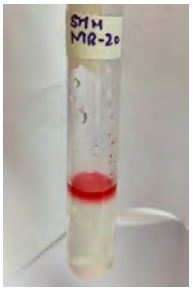


Fig 4.3: Appearance of red colour upon addition of Methyl Red indicating a positive result

4.2.2.2. Voges – Proskauer (VP):

All the 15 isolates gave a negative result for the *Salmonella spp.* Upon addition of the solution containing alpha-naphthol and potassium hydroxide, no apparent colour change was observed.

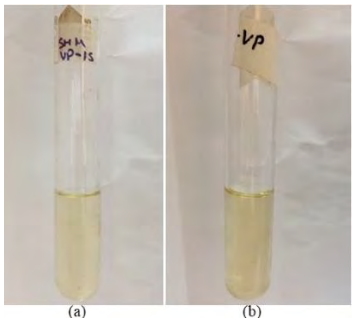


Fig 4.4: VP test showing a) VP broth inoculated with S15 showing no colour change indicating negative result b) Control: VP broth with no bacteria.

4.2.3. Citrate Utilization Test:

All the 15 isolates showed positive result for the citrate utilization test. The green colour of the citrate changed to blue upon incubation for 24 hours.

Fig 4.5: Citrate Utilization test showing colour change of the citrate agar to blue colour indicating positive result on respective samples.

4.2.4. Oxidase Test:

All the 15 isolates gave a negative result upon addition of Kovac's reagent.

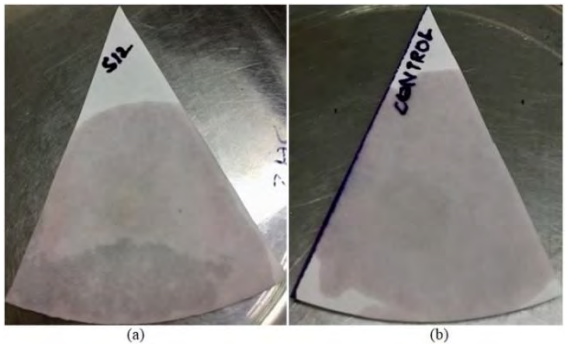


Fig 4.6: Oxidase test showing a) Filter paper inoculated with S12 showing no colour change of the filter paper indicating negative result b) Control: Filter paper with no bacteria.

4.2.5. Catalase test:

All the 15 samples were positive for the catalase test. Upon addition of hydrogen peroxide (H_2O_2), bubbles of oxygen gas was produced.

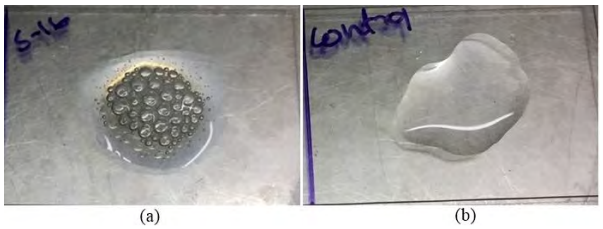


Fig 4.7: Catalase test showing a) Hydrogen peroxide inoculated with S16 showing bubble of oxygen gas indicating positive result b) Control: Hydrogen peroxide with no bacteria.

4.2.6. Indole Test:

All the 15 isolates showed negative result for the indole test as no red/pink ring was formed at the top of the solution.

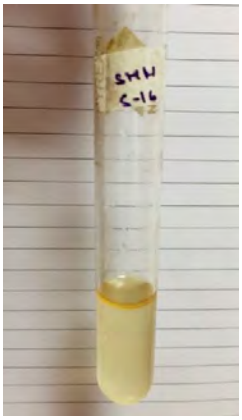


Fig 4.8: No red/pink ring indicates a negative result for the indole test.

4.2.7. Nitrate reduction Test:

All the 15 isolates showed positive result for the nitrate reduction test, i.e., reduction of nitrate (NO3) to nitrite (NO2) would be observed.

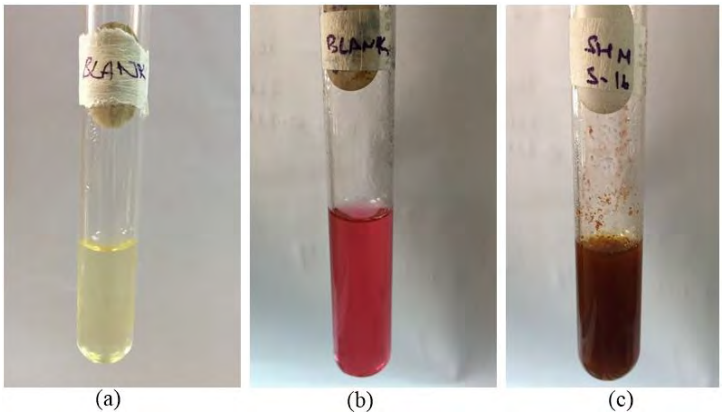


Fig 4.9: Nitrate reduction test a) Control: Nitrate broth with no bacteria b) Control: Nitrate broth with no bacteria but with addition of five drops of Sulphalinic acid reagent, Alpha napthylamine reagent and a pinch of Zinc powder indicating the Nitrate to Nitrite as the broth turns pink in color c) Nitrate broth inoculated with S16 showing reduction of Nitrate to Nitrite indicating positive result.

4.2.8. Triple Sugar Iron Test (TSI):

All the 15 isolates produced red slants and black precipitate on the butt portion. Indicating the production of hydrogen sulphide (H2S) gas and fermentation of glucose.

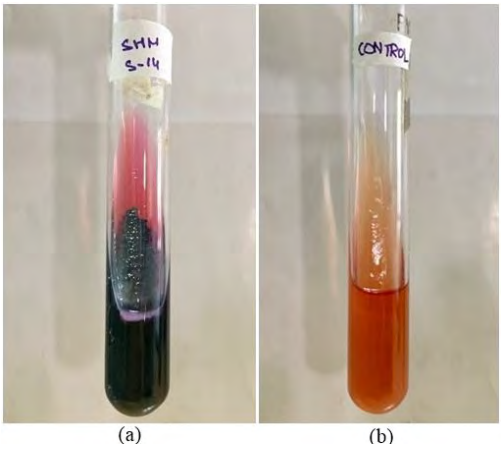
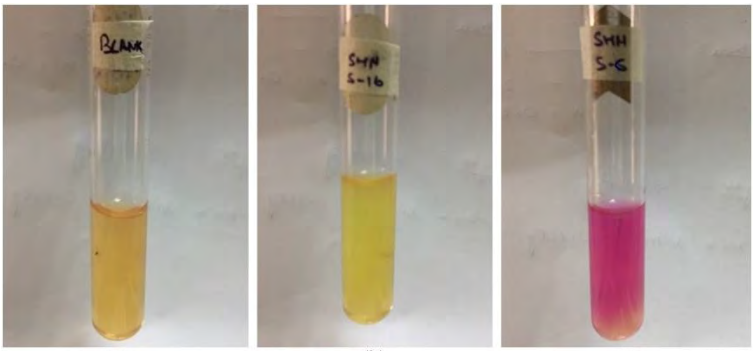


Fig 4.10: TSI test a) TSI agar inoculated with S14 showing red slant with black precipitate on the butt portion indicating fermentation of glucose and H_2S production b) Control: TSI agar with no bacteria.

4.2.9. Urease test, Motility test and indole test by MIU agar:

In the MIU test, all the 15 isolates showed signs of motility. But, out of the 15 isolates, 14 of the isolates were urease negative.



(a) (b) (c)

Fig 4.11: MIU Test a) Control: MIU agar with no bacteria b) MIU agar inoculated with sample S12 showing motility positive and urease negative result c) MIU agar inoculated with sample S6 showing motility positive and urease positive result.

Table 4.1: Results of the Biochemical Test of the 15 isolates.

Samples	Gram Staining	Methyl Red (MR)	Voges–Proskauer (VP)	Citrate	Oxidase	Catalase	Triple Sugar Iron (TSI)				MIU			Nitrate Reduction	Suspected organism
							Slant	Butt	Gas	H2S	Motility	Indole	Urease		
Desired	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 1	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 2	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 3	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 4	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 5	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 6	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	+	+	*Citrobacter werkmanii*
S 7	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 11	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 12	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 14	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 15	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 16	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 17	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 19	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*
S 20	+	+	-	-	-	+	R/Y	Black	✓	×	+	-	-	+	*Salmonella ssp.*

4.3.All the fourteen isolates showed the production of casease that hydrolyzes casein.

Hydrolysis of casein was carried out on the 14 isolates using skim milk agar. The production of exoenzyme, casease, was observed as it hydrolyzed the casein protein present in the agar. An ATCC strain of *Bacillus* was streaked on the agar as a control strain.

Fig 4.12: Skim milk agar streaked with S17, S19, S20 and *Bacillus*

4.4.Antibiotic Susceptibility Test of the isolates

4.4.1. Antibiotic susceptibility tests revealed all the isolates were resistant to nine common antibiotics:

The antibiotic susceptibility test was carried out on the 14 isolates using 24 antibiotics and the results showed that all the isolates were fully resistant to Oxacillin, Erythromycin, Tetracycline, Clindamycin, Co–Trimoxazole and Metronidazole, i.e., these antibiotics when tested by all the isolates produced no zone of inhibition. Sulfamethoxazole, Norflaxacin and Azithromycin also were reported that 100% of the isolates were resistant to them. The results showed that all the isolates were intermediate resistant to Nalidixic Acid.

12 isolates were resistant to Cephalexin and Ceftriazone (86%), 11 isolates to Levofloxacin and Penicillin (79%), 10 isolates to Kanamycin (71%), 9 isolates to Ceftazidime (64%), 6 isolates to Amikacin (43%) and 1 isolate was resistant to Ciprofloxacin (7%) respectively.

10 isolates were intermediate resistant to Streptomycin (71%), 9 isolates to Amoxicillin and Ampicillin (64%), 6 isolates to Imipenen (43%), 5 isolates to Ceftazidime (36%), 3 isolates to Penicillin (21%), 2 isolates to Cephalexin and Ceftriazone (14%), and 1 isolate was intermediate resistant to Kanamycin (7%) respectively.

No isolate was resistant to two antibiotics which were Chloramphenicol and Cefixime.

Sample S12 and S14 showed maximum resistance against 16 out of the 24 antibiotics that were tested.

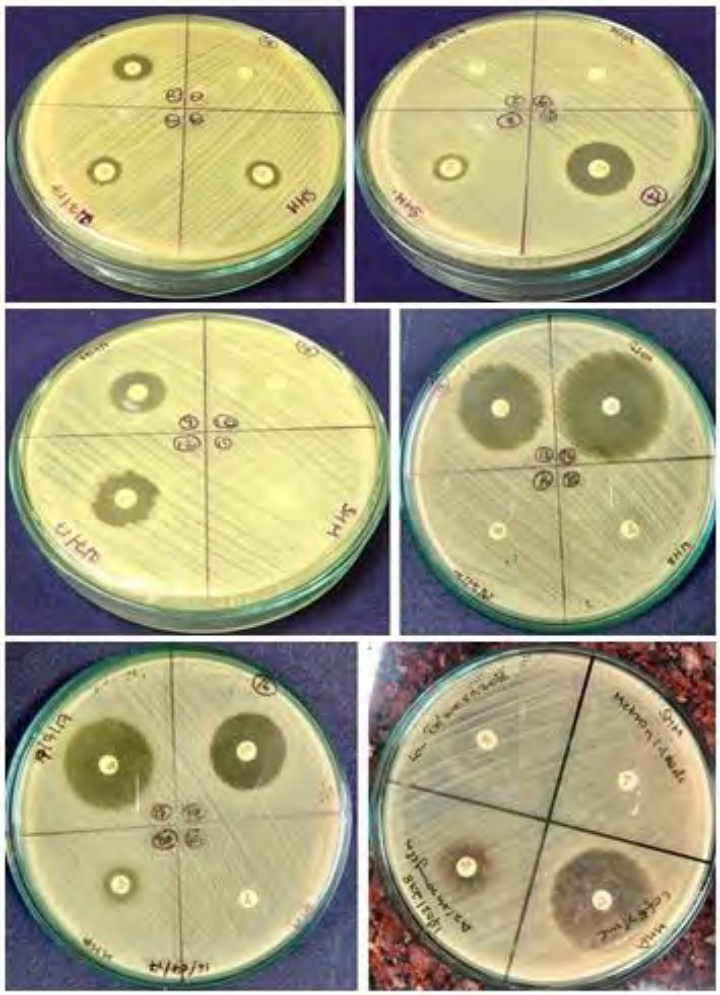


Fig 4.13: MHA plates showing the antibiotic susceptibility patterns of S16 and S17.

Table 4.2: Results of the Antibiotic Susceptibility Test of the 14 isolates. Red coloured box indicating Resistance, Green coloured box indicating Intermediate Resistance and Yellow coloured box indicating Susceptible results respectively.

Measurement of the zone of inhibition = millimeters

S.No:	Anitbiotics	S1	S2	S3	S4	S5	S7	S11	S12	S14	S15	S16	S17	S19	S20
1.	Penicillin (PEN)	00	00	19	20	00	00	00	00	00	00	00	00	00	00
2.	Amoxicillin (AMX)	00	00	27	26	00	00	26	00	00	22	00	00	27	00
3.	Streptomycin (STR)	11	11	17	18	11	13	19	12	11	12	11	12	19	12
4.	Levofloxacin (LVX)	12	13	35	36	12	11	27	12	12	12	12	15	12	13
5.	Oxacillin (OXA)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
6.	Kanamycin (KAN)	00	00	18	17	00	00	18	00	00	00	00	00	18	00
7.	Cephalexin (LEX)	21	22	20	20	23	23	22	21	22	22	22	22	22	20
8.	Ampicillin (AMP)	00	00	25	25	00	00	00	25	00	00	25	00	25	00
9.	Ciprofloxacin (CIP)	22	22	39	40	23	22	38	23	19	23	22	23	42	23
10.	Erythromycin (ERY)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
11.	Tetracycline (TET)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
12.	Chloramphenicol (CHL)	19	18	20	21	29	19	22	18	19	19	19	20	22	20
13.	Ceftazidime (CAZ)	16	16	16	16	17	17	16	16	16	16	16	17	17	17
14.	Ceftriazone (CRO)	18	18	18	17	17	18	18	18	17	20	18	20	17	17
15.	Nalidixic Acid (NAL)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
16.	Sulfamethoxazole (SXT)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
17.	Imipenen (IPM)	15	15	16	16	16	16	15	16	16	15	15	15	16	16
18.	Amikacin (AMK)	17	14	17	14	14	18	17	14	17	14	18	14	18	17
19.	Clindamycin (CLI)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
20.	Norflaxacin (NOR)	05	03	05	05	05	05	05	05	05	03	03	05	05	03
21.	Azithromycin (AZM)	11	12	11	11	11	09	11	11	11	10	11	11	12	11
22.	Cefixime (CFM)	35	36	35	35	32	32	35	35	36	35	35	40	32	36
23.	Co – Trimoxazole (COT)	00	00	00	00	00	00	00	00	00	00	00	00	00	00
24.	Metronidazole (MT)	00	00	00	00	00	00	00	00	00	00	00	00	00	00

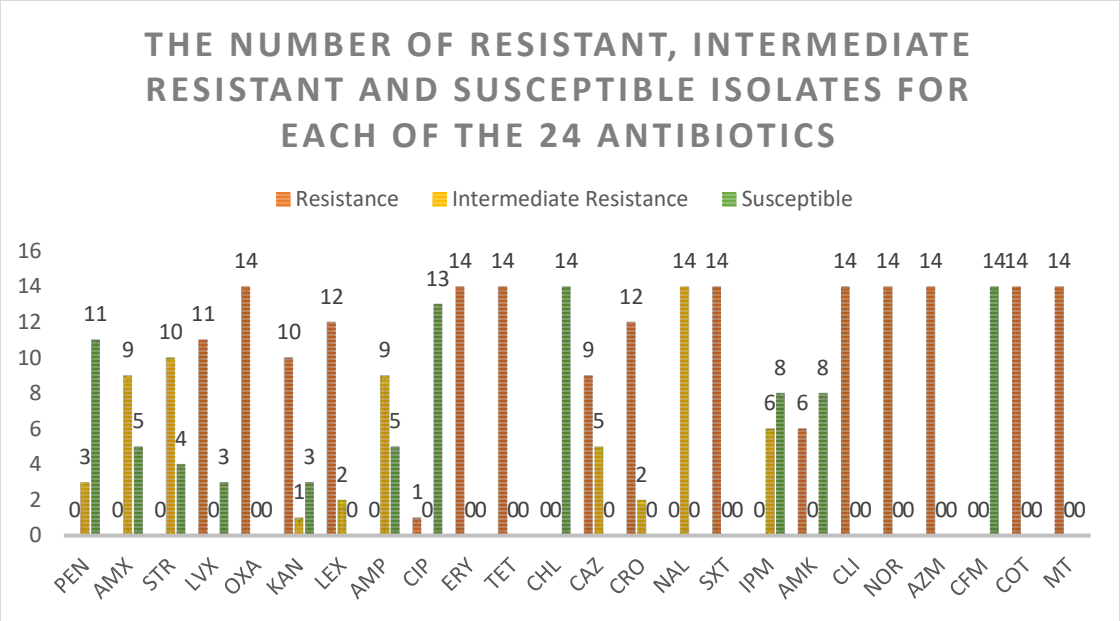


Fig 4.14: The number of Resistant, Intermediate Resistant and Susceptible isolates for each of the 24 antibiotics

4.4.2. The isolated exhibited high Multiple Antibiotic Resistance (MAR) Index:

The ratio of the number of antibiotic to which an organism is resistant to total number of antibiotics to which the organism in exposed, is determined as the multiple antibiotic resistance (MAR) index.

The Multiple Antibiotic Resistance (MAR) Indices of the 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.

Table 4.3: MAR Index and Level of Resistance of the 14 isolates.

Salmonella spp. isolates	Resistant (a)	Tested (b)	MAR (a/b)	Level of resistance
S1	14	24	0.58	HIGH
S2	15	24	0.62	HIGH
S3	12	24	0.50	HIGH
S4	13	24	0.54	HIGH
S5	14	24	0.58	HIGH
S7	13	24	0.54	HIGH
S11	13	24	0.54	HIGH
S12	16	24	0.66	HIGH

S14	16	24	0.66	HIGH
S15	15	24	0.62	HIGH
S16	15	24	0.62	HIGH
S17	14	24	0.58	HIGH
S19	12	24	0.50	HIGH
S20	14	24	0.58	HIGH

4.5. Antimicrobial properties of Oregano and Sesame

The amount of crude extracts were obtained from two different plant samples using three different solvents: ethanol, methanol and distilled water. The concentration of all the extracts was kept constant, i.e., 280mg/ml.

Fig 4.15: Crude extracts from left corner – Methanolic extract of Oregano, Ethanolic extract of Oregano, Aqueous extract of Oregano, Methanolic extract of Sesame, Ethanolic extract of Sesame and Aqueous extract of Sesame.

4.5.1. Methanolic and Ethanolic extracts of Oregano showed antimicrobial activity against the Salmonella spp. isolates:

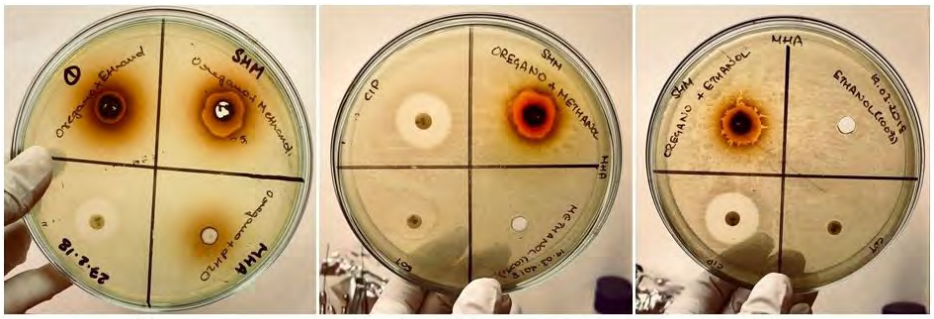
The antimicrobial properties of ethanolic, methanolic and aqueous extract of oregano were tested against all the 14 *Salmonella spp.* isolates. The methanolic extract showed the most remarkable positive results against the isolates. The ethanolic extract also showed positive results against the isolates. The aqueous extract however did not show any antimicrobial activity against any of the isolates. Three replicates were made for better accuracy and interpretation. Antibiotic Ciprofloxacin and Co – Trimoxazole were used as the positive and

negative control respectively. Also, the solvent used for the preparation of the extract respectively were examined to determine whether without the plant sample they could display any antimicrobial property.

Table 4.4: Results of the Oregano Extracts

Measurement of the zone of inhibition = millimeters

Oregano Extracts	**S1**	**S2**	**S3**	**S4**	**S5**	**S7**	**S11**	**S12**	**S14**	**S15**	**S16**	**S17**	**S19**	**S20**
Methanolic Extract	25	22	23	25	25	25	22	20	22	22	25	24	26	24
Ethanolic Extract	18	15	15	17	18	19	19	16	16	16	20	20	18	19
Aqueous Extract	00	00	00	00	00	00	00	00	00	00	00	00	00	00



(a) (b) (c)

Fig 4.16: MHA plates showing the antimicrobial assay of a) Methanolic, Ethanolic and Aqueous extract of Oregano along with Ciprofloxacin b) Methanolic extract of Oregano, Absolute methanol, Ciprofloxacin and Co – Trioxazole c) Ethanolic extract of Oregano, Absolute ethanol, Ciprofloxacin and Co – Trioxazole

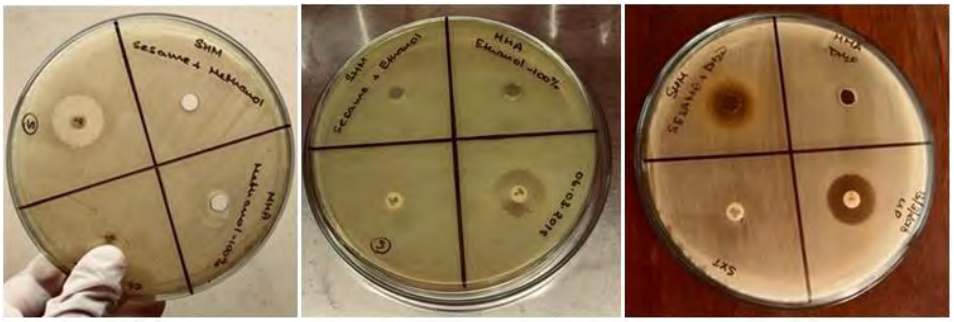
4.5.2. Aqueous extract of Sesame showed antimicrobial activity against the Salmonella spp. isolates:

The antimicrobial properties of ethanolic, methanolic and aqueous extract of sesame were tested against all the 14 *Salmonella spp.* isolates. The aqeuous extract showed the antimicrobial activity against the isolates. The methanolic and ethanolic extracts however did not show any antimicrobial activity against any of the isolates. Three replicates were made for better accuracy and interpretation. Antibiotic Ciprofloxacin and Co – Trimoxazole were used as the positive and negative control respectively. Also, the solvent used for the preparation of the extract respectively were examined to determine whether without the plant sample they could display any antimicrobial property.

Table 4.5: Results of the Sesame Extracts

Measurement of the zone of inhibition = millimeters

Sesame Extracts	S1	S2	S3	S4	S5	S7	S11	S12	S14	S15	S16	S17	S19	S20
Methanolic Extract	00	00	12	10	10	10	00	12	00	00	00	00	00	00
Ethanolic Extract	00	00	11	10	12	10	00	12	00	00	00	00	00	00
Aqueous Extract	10	10	10	12	10	11	11	11	11	11	11	10	11	10



(a) (b) (c)

Fig 4.17: MHA plates showing the antimicrobial assay of a) Methanolic extract of Sesame, Absolute methanol, Ciprofloxacin and Co – Trioxazole b) Ethanolic extract of Sesame, Absolute ethanol, Ciprofloxacin and Co – Trioxazole c) Aqueous extract of Sesame, Distill water, Ciprofloxacin and Co – Trioxazole

4.5.3. Comparison of Activity Index showed methanolic extract of Oregano had a high activity index:

The activity index values of all three types of extracts were calculated using the following formula:

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

Methanolic extract of Oregano had an AI of 1 or above 1 for eight isolates, and the methanolic extract of Oregano had an AI of below 0.5 for none of the isolates. Ethanolic extract of Oregano had an AI of 0.5 or above 0.5 for eleven isolates, and the ethanolic extract of Oregano had an AI of below 0.5 for three of the isolates. Aqueous extract of Sesame had an AI of 0.5 or above 0.5 for three isolates, and the aqueous extract of Sesame had an AI of below 0.5 for eleven of the isolates.

Table 4.6: Comparison of Activity Index

Measurement of the zone of inhibition = millimeters

***Salmonella spp.* isolates**	**Plant Extract (a)**		**Ciprofloxacin (b) (mm)**	**AI (a/b)**
	Name of Extract	**Zone of inhibition (mm)**		
S1	Methanolic Extract of Oregano	25	22	1.13
	Ethanolic Extract of Oregano	18		0.81
	Aqueous Extract of Sesame	10		0.45
S2	Methanolic Extract of Oregano	22	22	1.00
	Ethanolic Extract of Oregano	15		0.68
	Aqueous Extract of Sesame	10		0.45
S3	Methanolic Extract of Oregano	23	39	0.58
	Ethanolic Extract of Oregano	15		0.38
	Aqueous Extract of Sesame	10		0.25
S4	Methanolic Extract of Oregano	25	40	0.62
	Ethanolic Extract of Oregano	17		0.42
	Aqueous Extract of Sesame	12		0.30
S5	Methanolic Extract of Oregano	25	23	1.08
	Ethanolic Extract of Oregano	18		0.78
	Aqueous Extract of Sesame	10		0.43
S7	Methanolic Extract of Oregano	25	22	1.13
	Ethanolic Extract of Oregano	19		0.86
	Aqueous Extract of Sesame	11		0.50
S11	Methanolic Extract of Oregano	22	38	0.57
	Ethanolic Extract of Oregano	19		0.50
	Aqueous Extract of Sesame	11		0.28
S12	Methanolic Extract of Oregano	20	23	0.86
	Ethanolic Extract of Oregano	16		0.69
	Aqueous Extract of Sesame	11		0.47
S14	Methanolic Extract of Oregano	22	19	1.15
	Ethanolic Extract of Oregano	16		0.84
	Aqueous Extract of Sesame	11		0.57
S15	Methanolic Extract of Oregano	22	23	0.95
	Ethanolic Extract of Oregano	16		0.69
	Aqueous Extract of Sesame	11		0.47
S16	Methanolic Extract of Oregano	25	22	1.13
	Ethanolic Extract of Oregano	20		0.90
	Aqueous Extract of Sesame	11		0.50
S17	Methanolic Extract of Oregano	24	23	1.04
	Ethanolic Extract of Oregano	20		0.86
	Aqueous Extract of Sesame	10		0.43
S19	Methanolic Extract of Oregano	26	42	0.61
	Ethanolic Extract of Oregano	18		0.42
	Aqueous Extract of Sesame	11		0.26
S20	Methanolic Extract of Oregano	24	23	1.04
	Ethanolic Extract of Oregano	19		0.82
	Aqueous Extract of Sesame	10		0.43

4.5.4. Results of Phytochemical Screening showing variability for Methanolic, Ethanolicc extracts of Oregano and Aqueous extract of Sesame:

The results obtained for the phytochemical assay is shown below:

4.5.4.1. 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. Aqueous extract of Sesame showed negative results.

Fig 4.18: Tannins test showing a) Presence of Tannins in Ethanolic extract of Oregano b) Presence of Tannins in Methanolic extract of Oregano c) Presence of Tannins in Aqueous extract of Oregano d) Absence of Tannins in Aqueous extract of Sesame

4.5.4.2. Test for saponins

Aqueous extract of Sesame and 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.

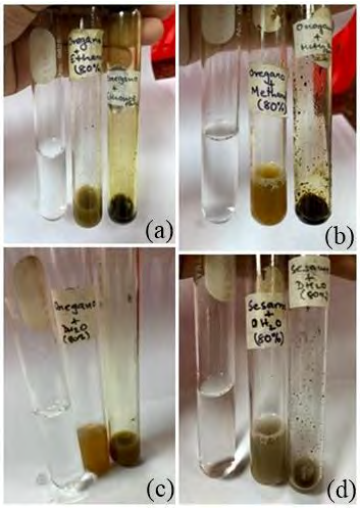


Fig 4.19: Saponins test showing a) Absence of Saponins in Ethanolic extract of Oregano b) Presence of Saponins in Methanolic extract of Oregano c) Absence of Saponins in Aqueous extract of Oregano d) Presence of Saponins in Aqueous extract of Sesame

4.5.4.3. Tests for alkaloids:

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

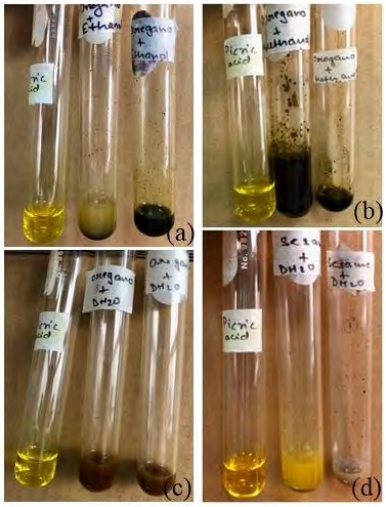


Fig 4.20: Hager test showing a) Presence of Alkolids in Ethanolic extract of Oregano b) Absence of Alkolids in Methanolic extract of Oregano c) Presence of Alkolids in Aqueous extract of Oregano d) Presence of Alkolids in Aqueous extract of Sesame

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

Fig 4.21: Wagner's test showing a) Presence of Alkolids in Ethanolic extract of Oregano b) Presence of Alkolids in Methanolic extract of Oregano c) Presence of Alkolids in Aqueous extract of Oregano d) Presence of Alkolids in Aqueous extract of Sesame

4.5.4.4. Tests 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. Aqueous extract of Sesame showed negative results.

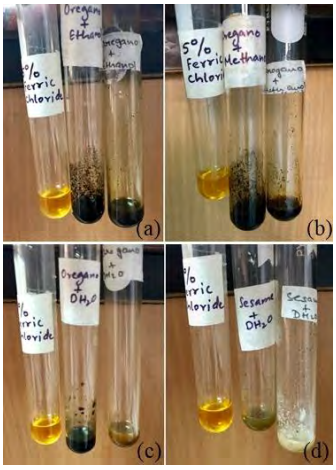


Fig 22: Phenolic compounds test showing a) Presence of Phenolic compounds in Ethanolic extract of Oregano b) Presence of Phenolic compounds in Methanolic extract of Oregano c) Presence of Phenolic compounds in Aqueous extract of Oregano d) Absence of Phenolic compounds in Aqueous extract of Sesame

4.5.4.5. Tests 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 Sesame and Aqueous extract of Oregano showed negative results.

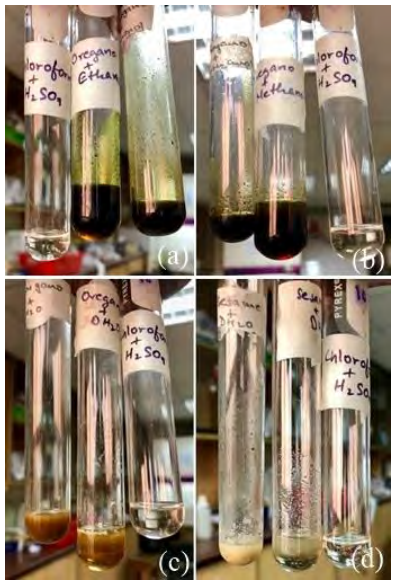


Fig 4.23: Steroid test showing a) Presence of Steroid in Ethanolic extract of Oregano b) Presence of Steroid in Methanolic extract of Oregano c) Absence of Steroid in Aqueous extract of Oregano d) Absence of Steroid in Aqueous extract of Sesame

Table 4.7: Phytochemical assay of Oregano and Sesame

Name of Tested chemical		**Methanolic Extract of Oregano**	**Ethanolic Extract of Oregano**	**Aqueous Extract of Oregano**	**Aqueous Extract of Sesame**
Test for Tannins		Presence of Tannins	Presence of Tannins	Presence of Tannins	Absence of Tannins
Test for Saponins		Presence of Saponins	Absence of Saponins	Absence of Saponins	Presence of Saponins
Test for Alkaloids	**Hager test**	Absence of Alkaloids	Presence of Alkaloids	Presence of Alkaloids	Presence of Alkaloids
	Wagner's test	Presence of Alkaloids	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	Absence of Phenolic compounds
Test for steroids		Presence of steroids	Presence of steroids	Absence of steroids	Absence of steroids

Chapter 05: Discussion

CHAPTER 05: DISCUSSION

5.1.Discussion

Raw beef has been a potential reservoir of food-borne pathogens. The consumption of beef has been reported to increase with the increase of different types of cuisines at present and raw beef is a prime source of *Salmonella spp.*, which also happens to be one of the leading foodborne pathogen responsible for causing salmonellosis and gastroenteritis in human. In Bangladesh, the prevalence of *Salmonella spp.* is predicted to be in abundance due to the poor management of handling and processing of beef. The tendency of multidrug-resistant *Salmonella spp.* has also been reported to emerge with the misuse of antibiotics in animals to raise the yield of food production[37].

In this investigation, 20 raw beef samples were collected from different butcher shops situated in different parts of Dhaka city. Out of the 20 samples, *Salmonella spp.* was isolated from 14 samples, with a high prevalence of 70.0% for this investigation. The high prevalence of *Salmonella spp.* in raw beef corresponds to an investigation held in Vietnam that stated the retail raw meat and poultry samples from markets and supermarkets were heavily contaminated with 60.8% (n = 180) prevalence on *Salmonella spp.*[38] On the other hand, the prevalence of *Salmonella spp.* was found to be fairly low in Tehran, Iran where 33.0% of both the beef and chicken samples were positive for *Salmonella spp.* which was obtained from retail outlets across the state[39]. In addition to that, the prevalence of *Salmonella spp.* was found to be fairly low in Washington, D.C., where 20.5% (n = 200) of the ground meat samples were positive for *Salmonella spp.* which was again obtained from supermarkets across the state[37].

On confirming that the isolates were *Salmonella spp.*, the next step of the investigation was to check their antibiotic susceptibility. Eight groups of antibiotics were used – Aminoglycoside, Beta–Lactam, Lincosamide, Macrolide, Nitroimidazole, Quinolone, Sulfonamide and Tetracycline. The antibiotic susceptibility test reported that 100% (n=14) of the isolates were fully resistant to Oxacillin, Erythromycin, Tetracycline, Clindamycin, Co-Trimoxazole and Metronidazole, i.e., these antibiotics when tested by all the isolates produced no zone of inhibition. The isolates were also reported to be resistant against Sulfamethoxazole, Norflaxacin, and Azithromycin. The results also revealed that the isolates exhibited resistance to Cephalexin and

Ceftriaxone (86%), Levofloxacin and Penicillin (79%), Kanamycin (71%), Ceftazidime (64%), Amikacin (43%) and Ciprofloxacin (7%) respectively. In addition, the results revealed that the isolates were intermediately resistant to Streptomycin (71%), Amoxicillin and Ampicillin (64%), Imipenen (43%), Ceftazidime (36%), Penicillin (21%), Cephalexin and Ceftriazone (14%), and Kanamycin (7%). No isolate was resistant to two antibiotics which were Chloramphenicol and Cefixime. With the increase in consumption of beef in various forms of cuisines, where some of the cuisines might require undercooked or semi cooked form of beef, which could further leave a possibility of infection to occur in a very high rate. Also, witnessing the vegetable's and butcher's shops being situated nearby, along with the poor maintained culture of the butcher's shops from which the raw beef were collected, 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 *Salmonella spp.* infections really dangerous and even worst in case these pathogens turn out to be highly resistant. This cross contaminated may occur via Horizontal Gene Transfer (HGT). HGT if occurred, 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. This trend of the increase of antibiotic resistance is potentially caused due to the uncontrolled usage of antibiotics in the treatment of animals and their integration in animal feeds[40].

In the investigation, it was found that the resistance of all the 14 *Salmonella spp.* isolates to beta-lactam group of antibiotics (Penicillin, Oxacillin, Cephalexin, Ceftazidime, Ceftriazone, Amoxicillin, Ampicillin and Imipenen) is high. Though, all the isolates were observed to be susceptible towards one of the third generation antibioitc of the same group, i.e., Cefixime. Amoxicillin, Ampicillin, Ceftazidime and Ceftriazone – are also third generation antibiotics of the beta-lactam group of antibiotics, against which the isolates were found to be resistant. In correspond, a study in China reported high Multiple Antibiotic Resistance index (MAR) (15.8%) against the 3rd generation of beta-lactam group of antibiotic[41]. MAR value basically determines the ratio of the number of antibiotic to which a bacterium is resistant to total number of antibiotics to which the organism in exposed. Thus higher the MAR value, the more chances it is for the

bacterium to carry antibiotic resistant gene. Also in this investigation, it was found that all the 14 *Salmonella spp.* isolates had a high MAR index (*MAR* > 0.50) which indicated that each of the respective bacterium was resistant towards many of the antibiotics that were used against it. This is a matter of concern, as the prevalence of high resistance of the isolates towards Quinolone group of antibiotic is in line with a study based in Vietnam where the study reported a moderate to high resistance against Quinolone group of antibiotic[42]. In addition, a study in Southern Brazil also reported high resistance rate towards both Beta-lactam and Quinolone antibiotics[43]. On the other hand, Chloramphenicol was found to be one of the most effective antibiotics against the isolates as it can prevent the protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome[44]. In this investigation also, Chloramphenicol has been found to be one of the antibiotics towards which all the isolates showed susceptibility. Thus, both Cefizime and Chloramphenicol were reported to be effective antibiotics in this study.

Imipenem (Carbapenum) which is beta-lactamase inhibitor binds irreversibly to the beta-lactamases produced by many bacteria thus inactivating the enzymes by leaving the microorganisms sensitive to beta-lactamase susceptible antibiotics[45]. In clinical practice, the beta-lactamase inhibitors are often administered in combination with β-lactam antibiotics to extend the spectrum of antibacterial activity of the antibiotics[46]. Hence, Imipenem and other β-lactamase inhibitors are the drug of choice to treat serious *Salmonella spp.* based illness or kill highly resistant *Salmonella spp.* However, unfortunately, in the investigation, 43% of the isolates have been found to be intermediate resistant towards Imipenem which thereby is a matter of concern.

Upon checking the Level of Resistance in view of the estimation of MAR list, a High level of Resistance was discovered in all isolates. Out of the 24 antibiotics used during the investigation, 17 antibiotics are commonly used for *Salmonella spp.* illness – Amoxicillin, Levofloxacin, Cephalexin, Ampicillin, Ciprofloxacin, Ceftazidime, Ceftriazone, Nalidixic Acid, Sulfamethoxazole, Imipenen, Amikacin, Clindamycin, Norflaxacin, Azithromycin, Cefixime, Co-Trimoxazole, and Metronidazole. As per the investigation, high level of resistance against these commonly used antibiotics except Cefixime is an alarming sign. Thus, it can be considered that raw beef is a potential source for the danger of procuring and being contaminated with different antibiotic-resistant Salmonella spp.

The next step of the investigation was to determine the efficacy of the antimicrobial property of Oregano and Sesame against the isolated *Salmonella spp.* 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 is the prime goal[47]. Plants can be considered as a more natural alternative form of antibiotic and can be titled as medicinal plants. These medicinal plants, however, contain antimicrobial compounds which can be extracted using respective solvent. The extracts of these plants can further be a potential source of treatment against many infections[487]. Oregano and Sesame are two such plants that have many antimicrobial properties. Though the antimicrobial properties of Oregano are not completely understood as compared to Sesame, it is being researched.

In this investigation, it has been found that the Methanolic and Ethanolic extracts of Oregano showed antimicrobial activity against the *Salmonella spp.* isolates. The Methanolic extract of Oregano was observed to show the best antimicrobial activity with an average zone of inhibition of 23.6 mm, where the biggest zone was 26 mm against one isolate and the smallest zone was 20 mm against one isolate. The average zone of inhibition by Ethanolic extract of Oregano was 17.6 mm with the biggest zone measured as 20 mm against two isolates and the smallest zone was 15 mm for two isolates. Aqueous extract of Oregano did not show any antibacterial activity. 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 the Methanolic extract was discovered in eight of the isolates, a Medium Activity Index of the Ethanolic extracts of Oregano was discovered in eleven of the isolates, and three of the isolates were observed to have a Low Activity Index for the Ethanolic extract of Oregano. A study in the USA reported that Oregano oil to be an effective alternative against *Salmonella Newport* on organic leafy greens[48]. In addition, a study in North-West of Sanandaj city in Iran reported that *Salmonella spp.* showed more sensitivity than *E.coli* and *S.aureus* against the essential oil extracted from both the flower and the leaves of Oregano respectively[49]. In this investigation, it has been found that the Methanolic extract of Oregano 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 saponin might be 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 steroids

and saponins in the Aqueous extract of Oregano could be a potential reason as to why it showed no antimicrobial activity. A study in Tamil Nadu, India 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 of the extract[50]. Another study in Pakistan reported that presences of alkaloid and flavonoids, as well as other compounds with antimicrobial potential, can be extracted and further purified from *Oregano* which can then be used against multidrug-resistant bacteria[51].

On the other hand, in this investigation, the Methanolic and Ethanolic extract of Sesame surprisingly showed no antimicrobial activity against any of the *Salmonella spp.* isolates. However, the Aqueous extract of Sesame showed low antimicrobial activity against the isolates with an average zone of inhibition of 10.6 mm, where the biggest zone was 12 mm against one isolate and the smallest zone was 10 mm against six isolates. On contrary, a study in Agra, India reported that the Methanolic extract of Sesame seeds showed antimicrobial activity and antioxidant activity which could be due to the presence of phenolic compounds[52]. In addition, a study in China reported that sesame seeds to have great antimicrobial activity, and black sesame seeds have a higher phenolic compounds presence than compared to white sesame seeds[53]. Black sesame were used in this investigation which leaves a contradictory observation compared to the study carried out in China. However, in this study, since only the Aqueous extract of Sesame showed low antimicrobial activity against the isolates, the phytochemical screening of this extract was only examined. Only the presence of saponins and alkaloids were found. In line with this, a study in India reported that the presences of alkaloids in both white and black variety of Sesame is one of the main factors to enhance the antimicrobial activity of the extract[54].

Upon checking the Activity Index (AI) of the Methanolic and Ethanolic extracts of Oregano and the Aqueous extract of Sesame, High AI value was observed in case of the Methanolic extract of Oregano followed by Medium AI value being observed for the Ethanolic extract of Oregano and Low AI value being observed for the Aqueous extract of Sesame. The AI value is basically to check and determine the potential of antimicrobial activity of an extract that is quantitatively compared to the respective standard antibiotics to which the bacterium is susceptible. High AI values imply that the extracts have a good activity against the bacteria in comparison with the standard antibiotics[55].

To sum up, the overall observation from the study determines that the use of Oregano extracts for treating foodborne illnesses against *Salmonella spp.* could be a potential and promising alternative to antibiotics. Thus, on 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.

5.2.Conclusion

To conclude, this study has strengthen the fact that raw meat samples (beef in this case) is a prominent reservoir of *Salmonella spp.* On an alarming point, all of the *Salmonella spp.* isolates were observed to possess high resistant to many of the antibiotics that are commonly used in day-to-day life. However, after elucidating the antibiotic susceptibility pattern of these isolates, the study evaluated an alternative source to treat illnesses caused by these pathogenic isolates. Out of the two potential plants used to evaluate its efficacy, Oregano was observed to show great potentials exhibiting antimicrobial activity against the isolates. In addition, the presence 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 during the beginning of the study were well met.

References

1. Global Meat Production and Consumption Continue to Rise. (2018). Retrieved from http://www.worldwatch.org/global-meat-production-and-consumption-continue-rise
2. Cook, R. (2016). *World Beef Consumption Per Capita (Ranking of Countries)*. Retrieved from http://beef2live.com/story-world-beef-consumption-per-capita-ranking-countries-0-111634
3. Zhao, C. (2001). Prevalence of Campylobacter spp., Escherichia coli, and Salmonella Serovars in Retail Chicken, Turkey, Pork, and Beef from the Greater Washington, D.C., Area. *Applied and Environmental Microbiology,67*, 5431-5436.
4. Masud, Z. (2017, March). Food borne illnesses and Bangladesh situation | Bangla ... Retrieved from http://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
5. Nordqvist, C. (2017, September 06). Salmonella: Symptoms, causes, and treatment. Retrieved from https://www.medicalnewstoday.com/articles/160942.php
6. Van, T. T., Moutafis, G., Istivan, T., Tran, L. T., & Coloe, P. J. (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
7. Doublet, B., Weill, F., Fabre, L., Chaslus-Dancla, E., & Cloeckaert, A. (2004). Variant Salmonella Genomic Island 1 Antibiotic Resistance Gene Cluster Containing a Novel 3-N-Aminoglycoside Acetyltransferase Gene Cassette, aac(3)-Id, in Salmonella enterica Serovar Newport. *Antimicrobial Agents and Chemotherapy,48*(10), 3806-3812. doi:10.1128/aac.48.10.3806-3812.2004
8. Rafieian-kopaei, M. (2012). Medicinal plants and the human needs, 1(1), 1–2.
9. Verma, S. (2008). Current and future status of herbal medicines, 1(11), 347–350.
10. Davis, C. P. (2017). Salmonella Poisoning: Symptoms,Treatments & Causes. Retrieved from https://www.emedicinehealth.com/salmonella/article_em.htm#what_is_salmonella
11. World Health Organization (WHO), (2018). Salmonella (non-typhoidal). Retrieved from http://www.who.int/en/news-room/fact-sheets/detail/salmonella-(non-typhoidal)

12. Falkenstein, D. (2015). All You Need To Know About Salmonella. *Food Poison Journal*. Retrieved from http://www.foodpoisonjournal.com/food-poisoning-information/what-you-need-to-know-about-salmonella-2/
13. Meštrović, T. (2017, November 06). Salmonella History. Retrieved from https://www.news-medical.net/health/Salmonella-History.aspx
14. D'Aoust, J., & Maurer, J. (2007, January 01). Salmonella Species. Retrieved from http://www.asmscience.org/content/book/10.1128/9781555815912.ch10
15. Porwollik, S., Boyd, E. F., Choy, C., Cheng, P., Florea, L., Proctor, E., & Mcclelland, M. (2004). Characterization of Salmonella enterica Subspecies I Genovars by Use of Microarrays. *Journal of Bacteriology,186*(17), 5883-5898. doi:10.1128/jb.186.17.5883-5898.2004
16. SALMONELLA. Retrieved from https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/Salmonella.htm
17. Giannella, R. A. (1996, January 01). Salmonella. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK8435/
18. Antibiotic / Antimicrobial Resistance. (2018, April 09). Retrieved from https://www.cdc.gov/drugresistance/threat-report-2013/index.html
19. Antibiotic Prescribing and Use in Doctor's Offices. (2017, December 07). Retrieved from https://www.cdc.gov/antibiotic-use/community/about/antibiotic-resistance-faqs.html
20. World Health Organization (WHO), (2018). Antibiotic resistance. Retrieved from http://www.who.int/en/news-room/fact-sheets/detail/antibiotic-resistance
21. Burmeister, A. R. (2015). Horizontal Gene Transfer. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4536854/
22. Soucy S. M., Huang J. & Gogarten J. P. (2015). Horizontal gene transfer: building the web of life. *Nature Reviews Genetics* **16**, 472–482 doi:10.1038/nrg3962
23. Singh, A. (2013, July 03). Antibiotics Harm Healthy Cells And Vital Functions In The Body; Are Medicines Helping Patients At All? Retrieved from https://www.medicaldaily.com/antibiotics-harm-healthy-cells-and-vital-functions-body-are-medicines-helping-patients-all-247367
24. Kalghatgi, S., Spina, C. S., Costello, J. C., Liesa, M., Morones-Ramirez, J. R., Slomovic, S., Collins, J. J. (2013). Bactericidal Antibiotics Induce Mitochondrial Dysfunction and Oxidative

Damage in Mammalian Cells. *Science Translational Medicine*, 5(192), 192ra85. http://doi.org/10.1126/scitranslmed.3006055

25. Oregano Herbal Medicine, Health Benefits, Side Effects. (2012). Retrieved from http://www.medicalhealthguide.com/articles/oregano.htm
26. Oregano. (2018) Retrieved from https://www.britannica.com/plant/oregano
27. Origanum vulgare L. (n.d.). Retrieved from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=32632#null
28. Ali, M. (2016, August 21). Flora of Bangladesh. Retrieved from http://www.floraofbangladesh.com/2016/05/til-sesamum-indicum.html
29. Kapoor, S., Parmar, S. S., Yadav, M., Chaudhary, D., Sainger, M., Jaiwal, R., & Jaiwal, P. K. (2015). Sesame (Sesamum indicum L.). Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25416247
30. GER, H. (n.d.). Sesame, Sesamum Indicum L., Pedaliaceae (sesame family) - Dr. Hauschka Cosmetics - Nature's treasures transformed for you. Retrieved from https://www.dr.hauschka.com/en_DE/knowledge-base/medicinal-plant-facts/sesame/
31. Amoo, S. O., M. Okorogbona, A. O., Du Plooy, C. P., & Venter, S. L. (2017). Sesamum indicum. *Medicinal Spices and Vegetables from Africa,*549-579. doi:10.1016/b978-0-12-809286-6.00032-7
32. Basic Botanical Information of Black Sesame. (2015, November 30). Retrieved from https://www.mdidea.com/products/new/new06701.html
33. 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.
34. Coccimiglio, J., Alipour, M., Jiang, Z., Gottardo, C., & Suntres, Z. (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/
35. Kumar, C. M., & Singh, S. A. (2015, May). Bioactive lignans from sesame (Sesamum indicum L.): Evaluation of their antioxidant and antibacterial effects for food applications. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4397349/
36. Cappuccino, J. G., and Sherman N. (2005). Microbiology: A Laboratory Manual.

37. White, D. G., et al. (2001). The Isolation of Antibiotic-Resistant Salmonella from Retail Ground Meats. *N Engl J Med* 2001; 345:1147-1154. DOI: 10.1056/NEJMoa010315
38. Van, T. T., Moutafis, G., Istivan, T., Tran, L. T., & Coloe, P. J. (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
39. Dallal, M. M., Doyle, M. P., Rezadehbashi, M., Dabiri, H., Sanaei, M., Modarresi, S., Sharifi-Yazdi, M. (2010). Prevalence and antimicrobial resistance profiles of Salmonella serotypes, Campylobacter and Yersinia spp. isolated from retail chicken and beef, Tehran, Iran. *Food Control,21*(4), 388-392. doi:10.1016/j.foodcont.2009.06.001
40. Galland J. C., Hyatt D.R., Crupper S.S., Acheson D.W. (2001). Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. *Appl Environ Microbiol.*; 67(4):1619-27. ; World Health Organization (WHO), (2000). *Survey of non-prescribed use of antibiotics for children in an urban community in Mongolia.*
41. Yang, L., Li, W., Jiang, G., Zhang, W., Ding, H., Liu, Y., Jiang, H. (2017). Corrigendum: Characterization of a P1-like bacteriophage carrying CTX-M-27 in Salmonella spp. resistant to third generation cephalosporins isolated from pork in China. *Scientific Reports,7*, 46728. doi:10.1038/srep46728
42. Nhung, N. T., Van, N. T., Cuong, N. V., Duong, T. T., Nhat, T. T., Hang, T. T., . . . Carrique-Mas, J. (2018). Antimicrobial residues and resistance against critically important antimicrobials in non-typhoidal Salmonella from meat sold at wet markets and supermarkets in Vietnam. *International Journal of Food Microbiology,266*, 301-309. doi:10.1016/j.ijfoodmicro.2017.12.015
43. Giuriatti, J., Stefani, L. M., Brisola, M. C., Crecencio, R. B., Bitner, D. S., & Faria, G. A. (2017). Salmonella Heidelberg: Genetic profile of its antimicrobial resistance related to extended spectrum β-lactamases (ESBLs). *Microbial Pathogenesis,109*, 195-199. doi:10.1016/j.micpath.2017.05.040
44. Hahn, F. E., et al. (1954). Mode of Action of Chloramphenicol. *Department of Virw and Rickett8ial Diseases, Army Medical Service Graduate School, Washington, D. C.*
45. Moellering R.C., (1991). Current treatment options for community-acquired methicillin-resistant Staphylococcus aureus infection. *Clin Infect Dis*.;46(7):1032-7. doi: 10.1086/529445.

46. Gilchrist, M. J., Greko, C., Wallinga, D. B., George W. Beran, G. W., David G. Riley, D. G., and Thorne, P. S.. (2007). The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environ Health Perspect, 115*(2), 313–316.
47. 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.
48. Moore-Neibel, K., Gerber, C., Patel, J., Friedman, M., Jaroni, D., & Ravishankar, S. (2013). Antimicrobial activity of oregano oil against antibiotic-resistant Salmonella enterica on organic leafy greens at varying exposure times and storage temperatures. *Food Microbiology,34*(1), 123-129. doi:10.1016/j.fm.2012.12.001.
49. Moradi1, M., et al. (2014). Phytochemical and Antibacterial Properties of Origanum vulgare ssp. gracile Growing Wild in Kurdistan Province of Iran. *Journal of Food Quality and Hazards Control 1* (2014) 120-124.
50. Prathyusha, P. (2009). Pharmacognostical and phytochemical studies on Origanum vulgare L.(Laminaceae). *Ancient Science of Life*, Vol. 29, No.2 (2009) Pages 17 – 23.
51. Mehreen, A., Waheed, M., Liaqat, I., & Arshad, N. (2016). Phytochemical, Antimicrobial, and Toxicological Evaluation of Traditional Herbs Used to Treat Sore Throat. *BioMed Research International,2016*, 1-9. doi:10.1155/2016/8503426
52. Nigam, D., et al. (2014). Evaluation of in vitro study of antioxidant and antibacterial activities of methanolic seed extract of Sesamum indicum. *Journal of pharmacognosy and phytochemistry.*
53. Zhou, L., Lin, X., Abbasi, A. M., & Zheng, B. (2016). Phytochemical Contents and Antioxidant and Antiproliferative Activities of Selected Black and White Sesame Seeds. *BioMed Research International,2016*, 1-9. doi:10.1155/2016/8495630
54. Neeta, M. P., et al. (2015). Comparative Qualitative Phytochemical analysis of *Sesamum indicum L. International Journal of Current Microbiology and Applied Sciences.* Special Issue-2: 172-181
55. Sridhar, N., Duggirala, S. L. and Puchchakayala G. (2014). Antimicrobial activity of ethanolic extracts of *Justicia neesii*. *Bangladesh Journal of Pharmacology*, *9*(4), 624-627.

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
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline:

Component	**Amount (g/L)**
Sodium chloride	9.0

Luria – Bertani Broth:

Component	**Amount (g/L)**
Tryptone	10.0
Sodium chloride	10.0
Yeast extract	5.0
Agar	15.0
Final pH	7.0

Simmon's Citrate Agar:

Component	**Amount (g/L)**
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0

Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Nutrient Broth:

Component	**Amount (g/L)**
Nutrient Broth	13.02

Methyl Red Voges- Proskauer (MRVP) Media:

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

Triple Sugar Iron Agar:

Component	**Amount (g/L)**
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar:

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Nitrate Reduction Broth:

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

Appendix II

Reagents

The following reagents were used throughout the study:

1. Barritt's reagent

Solution A: 5 g alpha-naphthol was dissolved in 95% ethanol. 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 cooled, creatine was added. Final volume was adjusted with distilled water and the reagent covered with aluminum foil was stored at 4°C.

2. Crystal violet Stain (2%)

2 g of crystal violet was dissolved in 20 mL of 95% ethyl alcohol. 0.8 g ofammonium oxalate monohydrate was next dissolved in 80 mL distilled water. The two solutions were mixed and filtered into sterile reagent bottle.

3. Iodine solution (Gram's)

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

4. Kovac's reagent

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

5. Malachite green (0.5%)

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

6. Methylene blue solution (1%)

1 g of methylene blue was dissolved in 75 mL of distilled water, and then diluted to make 100 mL. The solution was filtered out and stored in reagent bottle.

7. Methyl red reagent

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

8. Oxidase reagent

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

9. Safranin

0.1 g of safranin was dissolved in 75 mL of distilled water. The solution was diluted to 100 mL, filtered and stored in clean reagent bottle.

Appendix III

Instruments

Instrument	Company
Autoclave	SAARC
Cellulose filter paper (9.0 cm)	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 Ruosuaa, Technology company, China
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Refrigerator (4°C), Model: 0636	Samsung
Safety cabinet, Class II Microbiological	SAARC
Surgical Millipore syringe filter (0.22µm)	Millex-GS
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Weighing balance	ADAM, EQUIPMENT™, United Kingdom
Rotary evaporator	Heidolph, Made in Germany
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea