Investigation of *in-vitro* antimicrobial activities of *Nigella sativa*'s oil sample

A project submitted

by

Mairin Afroz Ummi

ID: 13346051

Session: Summer 2013

to Spring 2017

The Department of Pharmacy

In partial fulfillment of the requirements for the degree of

Bachelor of Pharmacy (Hons.)

Department of Pharmacy

Dhaka, Bangladesh

October 2017

This work is dedicated to my parents and siblings for their love and constant support…

Certification statement

This is to certify that this project titled “Investigation of *in-vitro* antimicrobial activities of *Nigella sativa*'s oil” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Shahana Sharmin, Senior Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

_____

Countersigned by the supervisor,

_____

Acknowledgement

First of all, I would like to express my gratitude to Almighty for endowing me with health, patience, protection and faith in all aspect of my life and for the help in completion of this research and preparation of this dissertation.

I would like to thank, Shahana Sharmin, Senior Lecturer, Department of Pharmacy, BRAC University, for providing me with continuous support and guidance since the very first day of my project-work. As a person, she has continuously inspired and motivated me with her knowledge on pharmacology, photochemistry and medicinal chemistry which made me more passionate about this project-work when it began. I would also like to thank her for her unwavering patience in me during every stage of project-work. My deepest and utmost gratitude for her positive attitude and specially for her scholastic knowledge in helping me to complete my project paper. Without her, my study and research could not be complete.

I would also like to thank Dr. Eva Rahman Kabir, Chairperson of Department of Pharmacy, BRAC University, for providing me with an opportunity to carry out the project at an individual level. I would also like to thank Arifur Rahman, Lecturer, Department of Pharmacy, Jagannath University, Dhaka, who advised me to do my research on this topic and has suggested me in collecting the sample from Shariatpur, Dhaka, Bangladesh.

Further I would like to thank Dr. Mahboob Hossain, Associate Professor of Department of MNS, BRAC University, for his inspiration, prudent advice, and immense patience in guiding me and for giving me the opportunity to collect bacteria from the Microbiology Lab, BRAC University.

Additionally, I would like to thank all the lab officers and lab assistants for their continuous guidance pertaining to lab-work.

Finally, I would like to thank the faculties of Department of Pharmacy at BRAC University, my friends and my family for their moral support, motivation and patience that altogether enabled me to complete my project-work successfully.

Abstract

Medicinal plants and its miracle healing property surprised the world from the beginning of medical science and pharmaceutical science. Before the dramatic change and invention of modern medicine and synthetic drugs people were completely depending on medicinal plants, still, 80% population of the world somehow depend on the medicinal plant. Among the medicinal plants, *Nigella sativa* is one of the most popular and diversely used plants. *Nigella sativa* seed's oil and extract have a long history of folklore usages in the various systems of medicines like Unani, Ayurveda etc. In this project, based on *Nigella sativa* seed's popularity and acceptance among general people and recent rising threat of antimicrobial drug resistance, I select a market preparation of its seeds oil to screen its anti-microbial property by using popular disk diffusion and Agar dilution methods. Three gram-positive bacteria's named *Staphylococcus aureus, Staphylococcus pyogenes, Bacillus subtilis* and five gram-negative bacteria's named *Vibrio cholera, E. coli, Shigella flexneri, Proteus vulgaris* and *Salmonella typhi* were used for the screening. The antimicrobial activity was tested over four different concentration of oil sample on Disk diffusion method and three different concentration on Ager dilution method. I observed strong antimicrobial property against *Bacillus subtilis, E. coli, Proteus vulgaris, Vibrio cholera* and *Salmonella typhi* on Disk diffusion method and did not show any activity against *Shigella flexneri, Staphylococcus pyogenes* and *Staphylococcus aureus*. On Ager dilution method, a strong inhibition was observed against the growth *Salmonella typhi* by the higher concentration of oil and the Moderate inhibition was observed against the growth of *Vibrio cholera* by all the concentrations of oil samples. Moderate inhibition of the growth of *E. coli* also witnessed by the highest concentration of oil and minimum inhibition was showed by rest of the samples. Minimum inhibition of growth of *Bacillus subtilis* also showed over all the concentrations of oil samples. Hence *Proteus vulgaris, Shigella flexneri, Staphylococcus pyogenes* and *Staphylococcus aureus* did not show any inhibition activity. Therefore, this result represents a positive response against the biggest challenge and threat of antimicrobial drug resistance and furthermore intensive and molecular study needed to achieve the success.

Table of Contents

Pages

Certification Statement I

Acknowledgement II

Abstract III

List of Contents IV

List of Tables VIII

List of Abbreviation IX

List of Contents

CHAPTER 1: INTRODUCTION

1.1. Medicinal plant 1

1.2. History of medicinal plant 2

1.3. Medicinal plant in Bangladesh 5

1.4. Antibacterial agent 10

1.4.1. Classification of Antibacterial agents 10

1.4.2. Risk Factors of Antibacterial agents 11

1.5. Alternative to conventional antibiotics 12

1.6. Oils as treatment agent ……12

1.7. General Characteristics of *Nigella sativa* seed ……13

1.7.1. Classification of *Nigella sativa* (Khare ., 2004) ……14

1.7.2. Medicinal uses of *Nigella sativa* ……14

1.7.3. Chemical composition of *Nigella sativa* oil ……15

1.8. Rationale of the study ……15

1.9. Aim of the study ……16

1.10. Objectives of the study ……16

CHAPTER 2: LITERATURE REVIEW

2.1. Pharmacological properties of *Nigella sativa* oil ……17

2.1.1. Antimicrobial actions ……17

2.1.2. Antioxidant effect ……18

2.1.3. Antidiabetic action ……18

2.1.4. Anti-inflammatory and analgesic actions ……19

2.1.5. Respiratory and immunological actions ……20

2.1.6. Effect on cardiovascular system and blood ……20

2.1.7. Antiulcer action ……21

2.1.8. Antiparasitic actions ……21

2.2. Toxicological Properties ……22

CHAPTER 3: MATERIALS & METHODOLOGY

3.1. Working Place ……24

3.2. Collection of sample ……24

3.3. Chemicals Used ……24

3.4. Bacterial Strains ……24

3.5. Methods for Evaluating the Antibacterial activity ……24

3.5.1. Disk diffusion method ……24

3.5.1.1. Antibacterial activity study by disc diffusion method ……25

3.5.1.2. Preparation of oil sample ……25

3.5.1.3. Preparation of agar medium ……25

3.5.1.4. Pre-culturing the bacterial strains ……26

3.5.1.5. Preparation of bacterial suspension in nutrient broth medium ……26

3.5.1.6. Preparation of disc ……26

3.5.1.7. Procedure ……27

3.5.2. Agar dilution method ……27

3.5.2.1. Antibacterial activity study by agar dilution method ……28

3.5.2.2. Preparation of bacterial suspension in nutrient broth medium ……28

3.5.2.3. Preparation of oil and agar mixture ……29

3.5.2.4. Preparation of agar medium ……29

3.5.2.5. Procedure ……29

CHAPTER 4: RESULTS

4.1. Antibacterial activity study by disc diffusion method 30

4.2. Antibacterial activity study by agar dilution method 38

CHAPTER 5:

DISCUSSION 48

CHAPTER 6:

CONCLUSION 50

REFERENCES 51

List of Tables

Pages

Table: 1.1. List of Medicinal plant around the world ……3

Table: 1.2. List of Medicinal plant in Bangladesh…… 6

Table 4.1.1: Antimicrobial activity of *Nigella sativa* oil against *Bacillus subtilis* ……30

Table 4.1.2: Antimicrobial activity of *Nigella sativa* oil against *E. coli.* ……31

Table 4.1.3: Antimicrobial activity of *Nigella sativa* oil against *Vibrio cholera* ……32

Table 4.1.4: Antimicrobial activity of *Nigella sativa* oil against *Proteus vulgaris* ……33

Table 4.1.5: Antimicrobial activity of *Nigella sativa* oil against *Salmonella typhi* …… 34

Table 4.1.6: Antimicrobial activity of *Nigella sativa* oil against *Shigella flexneri* ……35

Table 4.1.7: Antimicrobial activity of *Nigella sativa* oil against *Streptococcus pyogenes* ..36

Table 4.1.8: Antimicrobial activity of *Nigella sativa* oil against *Staphylococcus aureus* ..37

Table 4.2.1: Antimicrobial activity of *Nigella sativa* oil against Bacteria by using agar dilution method 39

List of Abbreviation

B.C. - Before Christ

WHO- World Health Organization

E. coli -Escherichia coli

KCl- Potassium chloride

AA- Arachidonic acid

GSH- Glutathione

MIC- Minimum inhibitory concentration

MBC- Minimum bactericidal concentration

mm- Millimeter

g- Gram

mg- Milligram

µL- Microliter

µg/disk- Microgram per disk

IC50: Half maximum inhibitory concentration

CHAPTER ONE

INTRODUCTION

Chapter 1: Introduction

Maintaining healthy life or leading a life which is free from any kinds of disease are always being the main concern among the people. It is a very common practice from the very beginning of the human existence in the earth. From the ancient period of time humans mostly depend on nature for their daily survival. In that time people had to utilize all the material which they found easily near to their hand and took attempt to use those materials in a very productive way for their struggle of existence. From foodstuffs to shelter, fertilizer, clothing, transportation, medicine people are highly dependent with natural stuffs. Human body is designed in a way that is connected to nature. Plants are the fundamental part of nature. As plants are present throughout the time of human history, it continues for the important source of medicine.

1.1. Medicinal plant:

From the very beginning of the human civilization plants mostly the medicinal plants play a very important role in the human being welfare. It is a backbone of modern and traditional medicine. Medicinal plants are those plants which have medicinal property and those can be used for treatment purpose. The synthesize metabolites of the medicinal plant can be used to produce modern medicine. Medicinal plant use as a traditional treatment system and around the world it is gaining a great popularity. This is a common practice from prehistoric period. It is a very effective ancient method of healing. Different countries used the medicinal plant as like folk medicine and used for prevention of disease in a different way. Medicines from medicinal plant are commonly known as herbal medicine. Herbal medicines are used for prevention or cure of disease and this medicine refers to the pants of fraction of the plants (Hassan, 2012).

People are more dependent on medicinal plant as well as herbal medicine from past 30 years and the uses of this natural medicine are increased drastically day by day. According to the survey which was done by WHO only 20% of the people worldwide dependent on the modern medicine primarily for their healthcare needs. Rest of the people primarily chooses herbal medicine for their health care treatment. Also according to their survey almost 21000

plant species have medicinal property. This system of treatment considers as the safest method of treatment and it has a very least chance of side effects. For this reason, this system achieves a huge popularity around the world. On the other hand, the medicinal plant contains very impressive number of the ingredient which can use for modern medicine synthesis and their development (Abeloff et al., 2008).

1.2. **History of medicinal plant:**

The foundation or history of medicinal plant are still unclear. Time of invention, disease purpose, first time of use etc. still mystery for the scientists. But it is said that the use of the medicinal plant was started at first by our ancestor. When they were searching for food they found so many plants and they tried to investigate and justify each of the plant to understand their property so that they can use those plants as food. They discovered some of the plants have poisonous effect, some plants were good for some specific health related condition like get relief from pain, reduce inflammation etc. some plants had potential to producing sugar (Traditional Remedies with Plants, 2016).

According to a myth long ago, a Spanish soldier was suffering from fever. He drank water from a specific pool. A bark of Cinchona was fallen in that pool. It was observed that after drinking water from that pool, the soldier was cured of the fever. Quinine, which is the main component of Cinchona was discovered to be a cure for periodic fever after this incident.

The first written format of medicinal plants uses was found to be as old as 400 years. The writers of the text were a tribe of antique people from Sumerian culture. They lived beside the rivers named Euphrates and Tigris. They used of small clay plates for writing and Researchers of Iraq discovered those plates later (Traditional Remedies with Plants, 2016)

Huge number of principles of medicinal plants was written in documents called Papyrus of Ebers by the Egyptians. More than 700 formulas were found which were written around 1700 A.C (Traditional Remedies with Plants, 2016)

'Pen Tsao' is an admirable documented form of medicinal plant use which contains the use of more than 300 medicinal plants. Ayurveda, the Indian medicinal system has left references of medicinal plant use, from 800 A.C (Traditional Remedies with Plants, 2016).

The analytical history of medicinal plant unveils that our ancestor such as Assyrians, Babylonians, and Egyptians were accustomed with the medicinal properties of the herbs and

trees. A huge portion of medicinal plant was familiar to the Babylonians (about 300 B.C) and it stated that modern medicine still utilizes some plants just like Babylonians did (Ghani, 2003).

The apply medications misuse stimulating plants flourished most all through the Greek advancement once chronicled personalities like Hippocrates (born in 460 BC) and Theophrastus(born in 370 BC) working on enhancing solution.

The utilization of plants and animal substances, secretions and minerals are involved in multiple outline of medicine in Egypt and ancient China. These important evidences are the confirmation of their tendency in using medicinal plants and their secretion in the past epoch. However, the Chinese people are using 'Chinese Herbal'- a separate orthodox of remedies. It is seen that botanical origins are in the support of Chinese herbalism. Plants as many as more than 1200 are implicated in the traditional treatment system and about 500 plants are very frequently used for the treatment and cure of several diseases (Li, 2000).

People from China in recent time make use of the herbal medicine according to the same manner that they had used in the earlier eras. It is observed that 20% of the pharmaceutical industries of Chinese market consist of almost 5000 traditional remedies (Li, 2000)

The utilization of medicinal plants in treating diseases is slowly increasing day by day in developed countries. Use of plants on the purpose for curing illnesses have been recognized by certain different well-known organization such as ESCOP (European Organization Cooperative On Phototherapy, 1999), German Commission E (Bluemental et.al, 1989) and WHO.

Table: 1.1. List of Medicinal plant around the world (Ross, 2005):

Serial Number	**Botanical name**	**Plant parts used**	**Traditional use**	**Picture**
01	*Cannabis sativa*	Seed, Leaves	To clear the blood; to cool the temperature; to relieve fluxes; for rheumatism; for migraine and for cancer.	

02	*Coffea Arabica*	Seed, Fruit, Leaf, Root	For asthma; use as a cardiotonic and neurotonic; for anemia, influenza, edema, asthenia, and rage; for hepatitis and liver troubles; for nervous shock; to increase mother milk production; for stomach pain; for cuts and hemorrhage.	
03	*Daucus carota*	Seed, Root, Fruit, Leaf, Flowers	For diabetes; use as a nerve tonic, stimulant and as an anthelmintic and cicatri-zing agent; for leucorrhea and to improve sight; for cystitis; for cancer of the stomach, bowel, and uterus; for ulcers, jaundice and inflammation; for dermatitis and burns; for urinary tract infections,	
04	*Nicotiana tabacum*	Leaves, Root, Seed	Used for a hair treatment purpose to prevent baldness; to treat dysmenorrhea; use to destroy worms in sores; applied ophthalmically as drops for bloodshot eyes; for rheumatism and to treat hoarseness; for myasis, headache, and wounds; applied for ring-worms, fungal diseases of the skin, wounds, ulcers, bruises, sores, mouth lesions, stomatitis, and mucosa; for kidney diseases.	
05	*Plantago ovate*	Seeds, Leaf	For diarrhea; use as an emollient poultice; for gastric complaints; for constipations and used as a diuretic; for rheumatoid arthritis and gout; for urinary tract inflammations.	

06	*Sesamum indicum*	Seeds, Fruits, Leaves	For tuberculosis; for asthma; for controlling diabetes; to promote hair growth; for eye diseases, ulcers, piles, and biliousness; used externally to prevent premature graying of hair; for laxative effect; to expel placenta; for increasing milk flow in nursing mothers; for dislocations, chest pains, and contusions.	

1.3. Medicinal plant in Bangladesh:

Bangladesh is considered as a country which is rich in biodiversity. Being a semitropical country, Bangladesh is enriched with more than 5000 angiosperm of 200 families. It has a comfortable weather for raising and nourishing medicinal plants. As recognized in the 'Materia Medica' (Fakir, 2015) the divisional cities like Dhaka, Rajshahi and the hilly areas like Chittagong and Sylhet are blessed with about 500 different species of medicinal plant.

In Bangladesh, traditional therapies are very popular from the very beginning of its existence. Conventional medicines have cured around 500 disease among 2000 ones with the help of this remarkably growing environment of medicinal plants (Fakir, 2015).

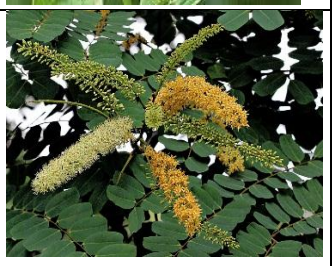
Added that in the recent years most of companies and industries have been using medicinal plants at a large scale. Even some of the leading pharmaceutical companies of Bangladesh are now using medicinal plants of various categories. Digitoxin which produced digitalis, vincristine which extracted from *Terminalia arjuna,Catharanthus roseus, T. chebula, T. bellerica, Withania somnifera, Cassia angustifolia, Aegle marmelos, Saraca asoca* are some of the most widely used medicinal plants, which are mainly used in Ayurvedic and Unani medicine since the existence of Bangladesh (Fakir, 2015).

The life of the remote rural part of Bangladesh has been greatly influenced by the role of wild medicinal plants. The reason behind this is the basic health treatment of the folk people is mainly dependent on wild medicines. In order to have primary treatment most of the tribal

and rural people of Bangladesh greatly depend on the medicinal plant. The primary reason may be their belief which is that nature won't cause any herm to their health. Various parts of plants like steam, barks, flower, fruit, rhizome etc. are used by them depending on their faith and belief (Fakir, 2015).

Table: 1.2. List of Medicinal plant in Bangladesh (Hasan et al., 2014, Roy et al., 2016, Azam et al., 2014):

Serial number	Botanical name	Local Name	Plant parts used	Picture
01	*Azadirachta indica*	Neem	Bark, leaf, seed	
02	*Abelmoschus moschatus*	Mushakdana	Root, leaves, seed, fruit	
03	*Abroma augusta*	Ulatkambal	Root, leaves, barks, fruits ,seeds	
04	*Acalypha indica*	Muktajhuri	Root, leaves, barks, fruits ,seeds	

05	*Achyranthes aspera*	Apang	Root, leaves, barks, fruits ,seeds	
06	*Adenanthera pavonina*	Raktakanchan, Ranjan	Leaves, seeds, woods, barks	
07	*Bunium persicum*	Kalo Jeera	Seed, whole plant	
08	*Bacopa monnieri*	Brahmishak	Root, leaves, fruits, seeds	
09	*Bauhinia acuminita*	Shet Kanchan,	Root, leaves, barks, fruits ,seeds	
10	*Benincasa hispida*	Chalkumra	Leaves, fruits, seeds	

11	*Bauhina purpurea*	Rakta Kanchan	Root, leaves, barks, fruits, seeds	
12	*Brassica nigra*	Kalo sarisha	Root, leaves, fruits, seeds.	
13	*Boerhaavia diffusa*	Punnarnava	Root, leaves, fruits, seeds.	
14	*Cajanus cajan*	Arhar	Root, leaves, fruits, seeds	
15	*Calotropis gigantea*	Boro akanda	leaves,fruits, seeds	
15	*Carica papaya*	Pepe	Fruit, seed	
17	*Capsicum frutescens*	Marich	Root, leaves, barks, fruits, seeds	

18	*Citrus aurantifolia*	Lebu, pati lebu	Leaves, fruits, seeds	
19	*Datura stramonium*	Dhotura	Seed	
20	*Dalbergia sissoo*	Sissoo gach	Root, leaves, barks, fruits, seeds	
21	*Foeniculum vulgare*	Mouri, Sop	Fruits and seeds	
22	*Gardenia jasminoides*	Gandharaj	Leaves, fruits, seeds	
23	*Gloriosa superba*	Ulatchandal, Bishlanguli	seeds, flowers	

24	*Helianthus annuus*	Surjamukhi	Leaves, seeds, flowers	
25	*Impatiens balsamina*	Dupati	Flowers and seeds	

1.4. Antibacterial agent:

Antibacterial' signifies a natural or semi-synthetic or synthetic compound which terminates or restrains the development of bacteria without harming the host. The term antibacterial was originates from Greek words where anti meaning against and bios means life which concerns all entities that functions microbial organisms.

Bacteria are a unicellular organism which have variant nature. All bacteria are not deleterious to human health, some resides within the human body and facilitate metabolism to sustain life.

Microorganisms like Bacteria and viruses are microorganisms that are quite vulnerable to new drugs. Moreover, the likelihood is less to develop new drugs to be consistent with the raising demand of antibacterial agent. Therefore, the significance of inventing new drugs is heightening in order to prevent the antibacterial diseases.

Therapeutic effects and antibacterial effect can be generated by compounds which are abundant in medicinal plants (Prasad et al. 2015).

1.4.1.Classification of Antibacterial agents:

Antibacterial agents are classified into 3 groups on the basis of their mechanism of action. These are-

1.Cell wall synthesis inhibitors,

2. Protein synthesis inhibitors and

3. Nucleic acid synthesis inhibitors (Whalen et al. 2014).

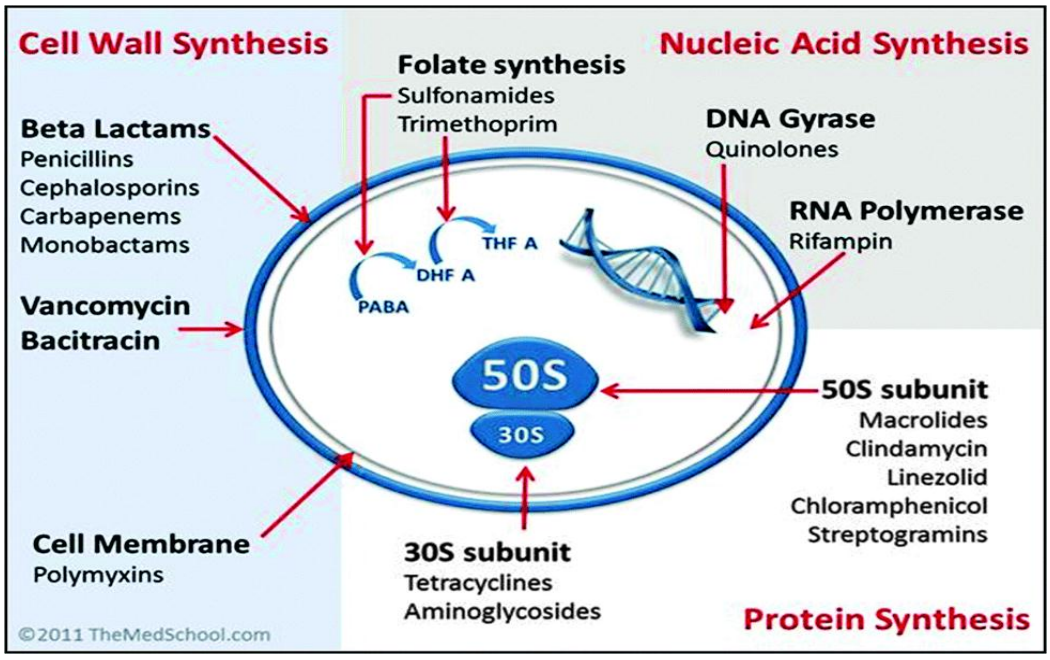


Figure 1.1: Mechanism of action of Antibacterial agents.

1.4.2. Risk Factors of Antibacterial agents:

Antibacterial agents are works against invading microorganism. Though they are selectively toxic to those microorganisms but they also affect host cell. As an example, the antibacterial agent sometimes causes allergic reaction or may be in a way which is unrelated to antimicrobial activity.

The most frequently occurring risk factors are hypersensitivity reaction or immune reactions to antimicrobial agents. As an example, penicillin causes serious hypersensitivity reactions despite their selective microbial toxicity and it ranging from urticaria to anaphylactic shock.

Sometimes antibiotics with high serum level can cause toxicity reaction by directly affect the cellular process of the hosts. Antimicrobial agents can also cause superinfections. The combination of antimicrobial agents or broad spectrum antimicrobials can lead to alteration of a normal microbial flora of the genitourinary tracts, oral, intestinal and upper respiratory

tracts, permitting the opportunistic organism to overgrowth mostly resistant bacteria or fungi (Whalen et al., 2014).

1.5. Alternative to conventional antibiotics:

Nowadays antibiotic resistant bacteria and nosocomial infection are considered as a major health issue around the world. Most of the time antibiotics come with some potential side effects. Oil can be a good source for treatment of many diseases and it can fight against bacteria without creating much severe potential side effects. In fact, oils are much safer compared to prescribed antibiotics. Moreover, it will also help to address the worldwide antibiotic resistance problem (Warnke et al., 2009).

At present situation, antibiotic resistance becomes one of the biggest threat to human life. Scientists demonstrated that antibiotics are nowadays more disable to treat illness and it is the biggest challenge to overcome the antibiotic resistance in the recent century (Deckard. 2015).

In Romm's opinion, antibiotic resistance in the U.S. has been responsible for causing more than 2 million illness in the year 2013. This has been reported by the Centers for Disease Control and Prevention. Moreover, they have predicted approximately 23,000 deaths and as extra healthcare costs of around $20. Beside these, it is getting worse day by day. A recent report presented by U.K. government states that drug-resistant microbes are capable to cause more than 10 million deaths and can also cost the global economy to around $100 trillion within 2050 (Rodreguez. 2015).

1.6.Oils as treatment agent:

The essential oil has the property which can be used for the hilling purpose. Some people realize that oil has the ability to fight against bacteria, fungi, yeast, it protects the host cell from infection, it can help to prevent various skin disease and much more. Essential oil possesses antibacterial, antioxidant, antifungal, antiviral, insecticidal properties (Burt, 2004). For cancer treatment, some essential oil has been used. In fragrance industry, for aromatherapy also for some food preservation, some other essential oils have been used.

The essential oil contains a great number of biologically active compound. Day by day the interest towards the antimicrobial property of various plant extract particularly towards

essential oil are significantly increased. Therefore, it is rational to expect a variety of compounds in these oils samples and these samples contain specific as well as general antimicrobial activity and antibiotic potential (Sylvestre et al., 2005).

1.7. General Characteristics of *Nigella sativa* seed:

Nigella sativa is one of the most widely used medicinal plants around the world. It is very famous and widely used as a traditional system of medicine, for example, Tibb and Unani, Ayurveda and Siddha. *Nigella sativa* seeds and oil have an ancient history of usage as a food and also various system of medicine. The seed of this plant used widely for the treatment of different disease and also for various ailments. In the Islamic literature, it is described as the greatest form of medicine which can be used for the various healing purpose. It is suggested to use regularly in Tibb-e-Nabwi (Prophetic Medicine). It has been used widely as liver tonics, antidiarrheal, antihypertensive, diuretics, appetite stimulant, digestive, antibacterial, analgesics and in skin disorders (Ahmad et al., 2013).

Nigella sativa is a flowering plant which is about 20-90 cm tall and these plants grow annually. The leaves of these plants are finely divided. And also the leaf narrowly segments linear to threadlike. The flowers normally have 5-10 petals and usually, the color of the flowers are yellow, white, pink, pale purple or pale blue. The size of the fruits are large and each capsule contains 3 to 7 united follicles and each of the follicles contain numerous seeds. The odor of the seeds are slightly aromatic, taste is bitter and the seeds are small dicotyledonous, angular, trigonous, tubercular, internally white and externally black in color (Goreja ., 2003).

By various scientists, extensive research on *Nigella sativa* has been carried out and they explored a wide spectrum of the pharmacological property of this plant. These properties include anticancer, antidiabetic, immunomodulator, antimicrobial, anti-inflammatory, bronchodilator, spasmolytic, hepato-protective, gastro-protective, renal-protective properties etc. As *Nigella sativa* have the miraculous power of healing, it has become the highest ranked evidence based herbal medicine (Ahmad et al., 2013).

Figure 1.2: *Nigella sativa*.

1.7.1Classification of *Nigella sativa* (Khare ., 2004) :

Kingdom - Plantae

Subkingdom - Tracheobionta
Superdivision - Spermatophyta
Phylum - Magnoliophyta
Class - Magnoliopsida
Subclass - Magnoliidae
Order - Ranunculales
Family –Ranunculaceae
Genus - *Nigella*
Species - *N. sativa*

1.7.2. Medicinal uses of *Nigella sativa*:

Numerous diseases and disorders concerned with the respiratory system, liver, cardio vascular system, immune system and kidney functions have been treated customarily by *Nigella sativa*. General health disorders have also been treated by *Nigella sativa*.

In Arabian and Indian civilization as remedies and food, black seeds and their oil have been used from several centuries. Traditionally they have been used for treating asthma, bronchitis, rheumatism and related diseases in Middle Eastern countries and Southeast Asian countries.

Nigella sativa has been esteemed with the name, 'Habbatulbarakah', meaning the seed of blessing due to its variant applications. For treating indigestion, diarrhoea, dropsy, amenorrhoe, loss of appetite and dysmenorrhoea, skin eruptions and worms,a solution produced from the seeds is used.

For external usage the oil is applied and antiseptic and local anesthetic. Provision of roasted black seeds prevent vomiting tendency (Ahmad et al., 2013).

1.7.3. Chemical composition of *Nigella sativa* oil:

Till date, numerous active compounds have been separated, distinguished and assessed in diverse categories of *Nigella sativa*. The most prominent out of all these active compounds are thymoquinone (30%- 48%), dithymoquinone, p-cymene (7%-15%), thymohydroquinone, 4-terpineol (2%-7%), carvacrol (6%-12%),sesquiterpenelongifolene (1%-8%) α-pinene ,t-anethol (1%-4%), and thymol etc. Other compounds are also present in *Nigella sativa* in scarcely detectable proportions. Two dissimilar categories of alkaloids are present in seeds; they are- isoquinoline alkaloids (for example: nigellicimine and nigelliciminne-N-oxide) and pyrazol alkaloids or indazol ring bearing alkaloids i.e. nigellidine and nigellicine. Furthermore, alpha-hederin is also reported to be present in *Nigella sativa*. The alpha-hederin is a water soluble pentacyclictriterpene and saponine-considered to be a possible anticancer agent. Carvone, limonene, citronellol are among the other compounds that were detected in scarce amounts (Ahmad et al., 2013).

Fixed oil takes up 28 to 36% *of Nigella sativa* seed. The fixed mainly constitute of unsaturated fatty acids (arachidonic, linoleic, eicosadienoic, and linolenic) and saturated fatty acids (stearic, palmitic, and myristic) . Cholesterol, stigmasterol, campesterol, α-spinasterol, β-sitosterol, , (+)-citronellol, (+)-limonene, citronellyl acetate, p-cymene carvone, arachidic, nigellone, linoleic, linolenic, oleic, myristic, palmitoleic, palmitic, and stearic acids are some of the compounds that are present in the seed oil. Fixed oils for example, linoleic acid (55.6%), oleic acid (23.4%), and palmitic acid (12.5%) and volatile oils like trans-anethole (38.3%), limonene (4.3%), p-cymene (14.8%), and carvone (4.0%) are found in seed oil (Dinagaran et al., 2016)

1.8. Rationale of the study:

From the previous literature review, it has been found that there is a significant number of work has been done on my selected plant oil *Nigella sativa*. But all those studies performed with the *Nigella sativa* sample which is not originated in Bangladesh. However previous

studies performed with a various sample which was originated in a different location around the world and reported a potential antimicrobial activities. My oil sample is collected from Shariatpur, Dhaka, Bangladesh. So now the main objective of this study is to find out the biological activity of this oil sample. The investigation is also performed to find out any unknown property of this sample and opportunities of the selected oil sample in the goodness to the planetary healthcare.

1.9. Aim of the study:

The aim of this study is to identify *in-vitro* antimicrobial activities of this *Nigella sativa* oil sample.

1.10. Objectives of the study:

The study protocol is consists of the following steps:

1. Antimicrobial evaluation of the oil sample for the identification of the major activities of the sample against specific Bacteria by disk diffusion method.
2. Antimicrobial evaluation of the oil sample for the identification of the major activities of the sample against specific Bacteria by agar dilution method.

CHAPTER TWO

LITERATURE REVIEW

Chapter 2: Literature Review

2.1. Pharmacological properties of *Nigella sativa* oil:

During last two decades, there are so many studies have been conducted in vivo or in vitro on the human body system on the effect of *Nigella sativa* seed extract and its active compounds like thymoquinone.

2.1.1Antimicrobial actions:

There are several reports that have been published on the effects of *Nigella sativa* seed extracts or its oil on various bacterial isolates. It was found that the extract and the oil have a broad spectrum of antimicrobial activity against a great number of microbes. As an example this oil showed marked in vitro antibacterial effects even in 1:100 dilutions against a number of microbes that includes Escherichia coli, Staphylococcus albus, Salmonella typhi, Vibrio cholera and Shigella niger (Agrawal et al., 1979). *Nigella sativa* oil was found to be more active against Gram-positive organisms rather than Gram-negative organisms. However this oil has also a very notable antifungal activity and it was especially against *Aspergillus* species (Agrawal et al., 1979).

Among the other researchers, El-Kamali et al. (1998), used plate diffusion method for their research to confirmed the previous report and found that the essential oil of *Nigella sativa* has a great effect against Gram-negative bacteria (Pseudomonas aeruginosa and Escherichia coli) and Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus). When Gram-positive Bacillus subtilis was used the antibacterial effect was found maximal.

There were several research conducted and found that the both the water extract of the seeds and the crude alkaloid extract were equally effective against a number of organisms which were isolated from human patients who were suffering from septic arthritis, even those individuals who were resistant to particular antibiotics (Morsi, 2000). It was founded that Gram-negative organisms were influenced more than Gram-positive organisms. However, like some other antimicrobial agents, the extracts strive more antibacterial actions at lower concentration rather than higher concentrations. Physico-chemical factors as an example diffusion and solubility might be responsible for this. *Nigella sativa* oil shown powerful effects towards murine cytomegalovirus infection in mice when it has been given

intraperitoneally. It was recommended that this action has a relation to potentiate the activity of the oil on innate immunity (Salem and Hossain, 2000).

2.1.2. Antioxidant effect:

It has been identified that non-enzymatic lipid peroxidation in liposomes was inhibited by the fixed oil of *Nigella sativa* and thymoquinone which is the main compound of the essential oil (Houghton et al., 1995). By using thin-layer chromatographic (TLC) technique, it has been found that compounds isolated from *Nigella sativa* have a very good amount of free radical scavenging properties. thymoquinone, carvacol, t-anethole and 4-terpineol- these are some compound isolated from *Nigella sativa* (Burtis and Bucar, 2000).

The various compound which was found in the oil acted in a synergistic manner. For this reason, the whole oil or its crude extract of the seeds are very much important in pharmacological studies. With a number of species this property has been noted before (Beckstrom-Sternberg and Duke, 1994). *Nigella sativa* does not involve in iron-complexing activity although its antioxidant property is multifunctional (Burtis and Bucar, 2000). Free radical generation is the partial basis of many human disease or conditions. Thus in folk medicine, the antioxidant actions of *Nigella sativa* explain its usefulness. This antioxidant property of this oil explains its action against liver fibrosis and cirrhosis (Türkdogan et al., 2000), CCl4 hepato-toxicity (Nagi et al., 1999), and hepatic damage which was induced by Schistosoma mansoni infection (Mahmoud et al., 2002).

2.1.3. Antidiabetic action

A number of workers have reported about the response of *Nigella sativa* on alloxan-induced diabetes mellitus in rabbits model has been investigated by (e.g. Al-Hader et al., 1993; Meral et al., 2001; El-Zawahrawy and Al-Zahraa, 1998;).According to the report of Al-Hader et al. (1993) after 4 -6 h of intraperitoneal administration of the volatile oil of Nigella sativa seeds (50 mg / kg) it possessed noticeable effect on reducing (by about 15%–23%) the fasting blood glucose level in normal- and hyperglycaemic rabbits. The unchanged condition of the insulin by the treatment possibly referring to the hypoglycemic effect that has been mediated by an unknown mechanism which does not involve insulin.

It was suggested that extract has the effect on diabetic rats in repairing the structural damage of pancreas suggested by El-Zawahrawy and Al-Zahraa (1998). The potential effects of the plant extract on the histology of the liver and pancreas in diabetic rats along with lipid peroxides, glutathione, ceruloplasmin and glucose was investigated by Meral et al. (2001). The results were referring to the fact that 2 months of treatment with the extract can significantly decrease the elevated level of glucose and lipid peroxides, and decrease that of glutathione and ceruloplasmin, and ameliorate the biochemical and histological signs of liver damage. It was considered that the positive effect of *Nigella sativa* in diabetes might be because of its antioxidant property. The above results are found to be in contrast to earlier reports from Kuwait, which was likely to refer that *Nigella sativa* seeds have no effect in streptozotocin-induced diabetes in rats (Al-Awadi and Gumaa, 1987). It was also described, that a five plant mixture (including Nigella sativa) is generally used by Kuwaiti diabetics to antagonize the control of hyper-glycemia. Experimentally the combination of plants was found to change glucose tolerance positively in both streptozotocin diabetic and normal rats (Al-Awadi and Gumaa, 1987; Al-Awadi et al., 1991).

2.1.4. Antiinflammatory and analgesic actions:

Khanna et al. (1993) used three antinociceptive tests in mice and rats which were a tail-pinched test, hot plate test, and acetic acid-induced writhing. According to their research, they concluded that the fixed oil of *Nigella sativa* seeds is enriched with strong antinociceptive actions. These actions were due to an opioid principle in the oil because they were antagonized by naloxone.

Again, according to these authors, the *Nigella sativa* oil has remarkable CNS depressing properties. These results were confirmed by Abdel-Fattah (2000). He used four different models of analgesia. These were tail-pinched test, hot plate test, formalin-induced pain and acetic acid-induced writhing where the oil and thymoquinone were given either by injection or orally (p.o.); either intracerebroventrically (i.c.v.) or intraperitoneally (i.p.).

By using general opioid antagonist naloxone, and a number of kappa, mu and delta receptor antagonists, the mechanism of the analgesia was examined.

Nigella sativa is very useful in inflammatory conditions in the human body. To test this claim, the aqueous extract of *Nigella sativa* seeds was tested in mice for its analgesic, anti-

inflammatory, and antipyretic action (Al-Ghamdi, 2001). Using the hot plate reaction time as a model of nociception and carrageenan-induced paw edema as a model of inflammation, it is found that the extract was possessed remarkable analgesic and anti-inflammatory action. But it was failed to induce yeast-induced pyrexia (Abdel-Fattah et al., 2000).

2.1.5. Respiratory and immunological actions:

The study showed that when the volatile oil of *Nigella sativa* administered intravenously dose-dependently increases the respiratory rate as well as the intratracheal pressure of guinea-pigs (El-Tahir et al., 1993). However, without much affecting respiratory rate thymoquinone increased the intratracheal pressure. The actions of the volatile oil referred to muscarinic and histaminergic mechanisms which are based on the effects of certain antagonists. These authors pointed that if thymoquinone were removed from *Nigella sativa* volatile oil then it could be a useful for respiratory stimulant. This experimental work is very useful for the asthmatic patients.

In Pakistan, Gilani et al. (2001) found that the crude extract of *Nigella sativa* seeds on guinea-pig tracheal preparations and isolated rabbit jejunum. In the rabbit jejunum, The dose-dependent relaxation of spontaneous contractions and inhibition of KCl-induced contractions was caused by the extract.

These actions which were produced here were similar to the actions which were produced by verapamil, a calcium-channel antagonist. In the preparation of tracheal, the extract antagonized the contractions which were induced by carbachol, histamine, and KCl. The bronchodilator, spasmolytic effects were observed and it is referred to be mediated via calcium channels.

2.1.6. Effect on cardiovascular system and blood:

The actions of the volatile oil of *Nigella sativa* and thymoquinone (active constituent of *Nigella sativa*) on the heart and arterial blood pressure of anesthetized rats were investigated by El-Tahir et al. (1993b). When these both agents were injected intravenously, heart rate and arterial blood pressure decreased dose-dependently. The mentioned effects were antagonized by atropine which is a cholinergic (M) receptor antagonist, Cyproheptadine which is a serotonin and histamine (H1) receptor antagonist, reserpine which is an

adrenergic depleting agent, hexamethonium which is a ganglionic nicotinic (N) receptor antagonist and by spinal pithing. It was found that cardiovascular actions of these agents are centrally mediated via indirect or direct mechanisms which involve cholinergic and tryptaminergic mechanisms as the action of these agents are multifunctional.

It was recommended that *Nigella sativa* oil could be used as a centrally acting antihypertensive drug. *Nigella sativa* oil showed inhibitory effects when it is solubilized with methanol.and this methanol soluble portion give its effects on blood coagulation and arachidonic acid (AA) induced-platelet aggregation (Enomoto et al., 2001). There are several compounds that contain anti-coagulation effect could be isolated from this oil. These isolated compounds were more potent than aspirin (known therapeutic agent for thrombosis).

2.1.7. Antiulcer action:

There is a single report in rat where it has been found that the aqueous extract of *Nigella sativa* seeds was very useful against reducing the ulcer index which is induced by aspirin by about 36% (Akhtar et al., 1996). The treatment though did not change any mucin activity but it reduced the acid production and peptic activity. These finding suggested that the folkloric use of this plant may not be founded to treat peptic ulcer.

On the other hand recently El-Dakhakhny et al. (2000a) conducted a research and obtained an opposite results where it was found that administration of 0.88kg of *Nigella sativa* oil per day for two weeks reduced histamine content, increased gastric mucin, and glutathione content, but it did not affect the peptic activity and free acidity of the gastric juice. This report is in line with the previous report where according to El-Kadi et al., 1987, it was suggested that cytoprotective action of *Nigella sativa* oil and lends have some credence towards the folkloric use of this oil for using as an antiulcer agent. Hence the further work is warranted on the action of the extract of the seeds and the oil on experimental duodenal and gastric ulcer.

2.1.8. Antiparasitic actions:

Nigella sativa oil has anticestodal and antinematodal properties and it is comparable to those of piperazine (Agrawal et al., 1979). In a study, Mahmoud et al. (2002) identified that

Nigella sativa oil has a great effect in reducing the number of Schistosoma mansoni worms in the liver and also the total number of ova deposited in both the liver and the intestine were decreased if 2.5 and 5.0 mL/kg was taken orally for 2 weeks. Moreover, it increased the amount of dead ova in the wall of intestinal and significantly reduced the granuloma diameters. Continuous administration of *Nigella sativa* oil with praziquantel lowered the number of dead ova rather than praziquantel was given alone. It indicates that the *Nigella sativa* oil potentiates the action of praziquantel.

2.2. TOXICOLOGICAL PROPERTIES:

Nigella sativa seed extract and its other constituents contain the low amount of toxicity. When 50mg/kg *Nigella sativa* seed extract intraperitoneally administered to rats for five days, it did not extremely affects the activities of a number of enzymes and also metabolites indicative of hepatic and renal function (El Daly, 1998).

When 10 mL/kg seed oil administered orally in rats and mice it did not cause any overt toxicity or any mortality during 48 hours observation period (Khanna et al., 1993). It has been confirmed recently, when it was found that 10 mL/kg of *Nigella sativa* administered orally for up to 12 weeks it did not cause any significant alteration or any mortality of the key hepatic enzymes in rats (Zaoui et al., 2002b). It was found that the LD50 value of thymoquinone was 2.4 g/kg (range 1.52–3.77) (Badary et al., 1998). If it was administered in a high dose (2 g/kg or more) then it might cause hypoactivity and also difficulty in respiration. Biochemically, these high doses Biochemically reduced GSH concentrations in liver, heart, and kidney and damaged kidney and liver, as it was demonstrated by significant increases in enzymes and plasma metabolites (Badary et al., 1998). When thymoquinone added in the drinking water of the mice at the concentrations of up to 0.03% for 90 days, this did not cause any sign of toxicity except for a remarkable lowering plasma glucose concentration (Badary et al., 1998).

Two case report has been found where the allergic contact dermatitis was reported after topically using pure oil of *Nigella sativa* for two persons who suffered maculopapular eczema. But the *Nigella sativa* oil was marketed to prevent skin disorders, skin dysfunction, inflammation, acne or eczema. In the past contact dermatitis cases have been revealed with

the use of essential oils which were present in perfumes and cosmetics. By using topical corticosteroids these cases could be treated (Steinmann et al., 1997; Zedlitz et al., 2002).

CHAPTER THREE

MATERIALS AND METHODOLOGY

Chapter 3: Materials and Methodology.

3.1. Working Place:

The overall research was performed in the Microbiology Laboratory, Department of Pharmacy, BRAC University. All the microbiological tests were done under laminar air flow, Biological Safety Cabinet.

3.2. Collection of sample:

Good quality *Nigella sativa* oil were collected from Shariatpur, Dhaka, Bangladesh. The sample I collected was fully preservative free or free from other additives.

3.3. Chemicals Used:

1. Mueller Hinton agar
2.0 .2% agar powder
3. Nutrient broth
4. Ethanol

3.4. Bacterial Strains:

In this study I used 8 bacterial species, they were *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Vibrio cholera, E. coli, Shigella flexneri, Proteus vulgaris and Salmonella typhi.* All those species were obtained from BRAC University stock culture.

3.5. Methods for Evaluating the Antibacterial activity:

I used two most popular methods for this test. They are:

1. Disk diffusion method
2. Agar dilution method

3.5.1. Disk diffusion method:

This is the most common and standard method for Antibacterial activity test. Mueller Hinton has been seeded with a bacterial suspension for 24 hours in this method. 6mm of filter paper disk which is sterile previously soaked with oil sample and unloaded on the agar with the help of the

sterile tweezers.If the oil sample which is used in this study are capable enough to inhibit the growth of microorganism than after 24 hours of incubations a clear halo can be observed around the disks. Around the disk as large the zone is observed it means the more germ is sensitive (Boukhraz et al., 2016)

3.5.1.1. Antibacterial activity study by disc diffusion method:

To determine the antibacterial activity of *Nigella sativa* oil sample, the oil was used against 8 bacterial strains. The list of these bacteria are given below-

Name of Bacterial Strains	**Type of Bacteria**
Staphylococcus aureus	Gram (+)ve Bacteria
Streptococcus pyogenes	Gram (+)ve Bacteria
Bacillus subtilis	Gram (+)ve Bacteria
Vibrio cholerae	Gram (-)ve Bacteria
E. coli	Gram (-)ve Bacteria
Shigella flexneri	Gram (-)ve Bacteria
Proteus vulgaris	Gram (-)ve Bacteria
Salmonella typhi	Gram (-)ve Bacteria

3.5.1.2. Preparation of oil sample:

For the antimicrobial activity tests, we used Ethanol as a solvent to dissolve the oil. We prepared four different concentrations of oil and ethanol mixture proportion (1:1, 1:10, 1:20 and 1:40 µl/ µl.) by serial dilution. Here 1 µl was oil and the rest were ethanol.

3.5.1.3. Preparation of agar medium:

At first 7.6gm of Mueller-Hinton agar was added into 200 mL of distill water. Agar powders were properly dissolved into water by vigorous shaking. Then at 121°C the mixture was autoclaved for 60 min and cooled up to 45°C -50°C. Afterward sterile Mueller-Hinton agar solution poured equally into eight sterile Petri dishes. Each 100mm petri dish got

approximately 25ml of Mueller-Hinton agar solution. Then the solution was set at room temperature to cool and solidify.

3.5.1.4. Pre-culturing the bacterial strains:

Bacterial Strains were collected from long term preserved medium which was preserved in a very low-temperature freezer. For the regeneration of those bacteria, nutrient agar medium was prepared by adding 5.6gm of nutrient agar to 200ml of distill water and autoclaved. After that, the sterile nutrient agar solution was poured equally into eight sterile Petri dishes. Then with the help of sterile loop, the bacterial strains were taken from the previously cultured medium and streaked into the freshly prepared nutrient agar medium. Then those bacteria containing nutrient agar medium were allowed to incubate for 24 hours to regeneration those bacteria.

3.5.1.5. Preparation of bacterial suspension in nutrient broth medium:

For the preparation of bacterial suspension, nutrient broth medium was prepared by adding 0.25gm of nutrient broth into 10ml of distill water. To prepare the eight different bacterial suspensions, eight different broth medium was prepared in the conical flasks by this same manner. Then these entire broth medium allowed to autoclaved for 60 minutes at 121°C. After that the broth medium was cool down at room temperature. Finally, every strain was collected from the nutrient agar media and dipped into the nutrient broth medium and mixed rigorously to prepare the bacterial suspension. The freshly prepared bacterial suspension set into shaking incubator for 24 hours. So that bacteria could grow on that suspension. After 24 hours the absorbance of those bacterial suspensions was taken out. As the desired value of absorbance was about 0.1-0.2 so the suspension had been diluted with freshly prepare nutrient broth and achieved the desired concentration.

3.5.1.6. Preparation of disc:

The disc was 6 mm diameter and made of Whitman paper. All the disks were autoclaved in a test-tube. Then a mixture of oil and ethanol of different concentration which was made previously were added to the discs respectively. After that, it was allowed to soak all the solution for 10-15 minute.

3.5.1.7. Procedure:

To observe the antibacterial activity of *Nigella sativa* oil in eight different microorganisms, a freshly prepared Mueller-Hinton agar medium was taken. Then the suspensions of the bacteria in nutrient broth medium were taken and a cotton swab was dipped into a bacterial suspension. To remove extra fluid, the swab was mildly squeezed against the tube. Firstly, the swab was used to streak the bacterial suspension to the nutrient agar plate in one direction. After that, it was streaking in right direction and then diagonally. To streak the strains at the end of the diameter of the nutrient plate the swab was used. This same procedure was followed for each of the eight Petri dishes. Afterward, the agar plates were allowed to get dry for 5 minutes. Then 5 minutes later, the discs containing oil and ethanol mixture were placed individually on the surface of the plate with the help of forceps. For each plate, one antibiotic disk was used. Finally, all the Petri dishes were incubated at 37°C for 24 hours to get the visible growth of bacteria. All whole procedure was done under laminar airflow which is a biosafety cabinet.

3.5.2. Agar dilution method:

Agar dilution method is not as much as popular than other methods for the antimicrobial test. In this method, the test sample is prepared by using 0.2% of agar solution and the desired amount of oil sample is diluted in the solution. 13.5 ml of Mueller-Hinton are taken each conical flask and sterilized these conical flask autoclave and cooled at 45 ° C. Then 5ml dilution of test samples is added aseptically to this Mueller-Hinton solution. Then the conical flask is properly agitated so that before pouring into Petri dishes, oils are dispersed in the culture medium properly. The control cultures are also prepared which contain the Muller Hinton culture medium plus .02% agar solution.

The minimum inhibitory concentration (MIC) is the inhibition of any growth which is visible to the naked eye by the lowest concentration of oils after 16 to 20 hours of incubation at 37 ° C.

The minimum bactericidal concentration (MBC) is determined by seeding a sample of the Petri dish where there is no growth of any bacteria on agar Mueller-Hinton. After 24 hours of incubation at 37 ° C, the minimum concentration of essential oils which are capable to kill

99.99% of the bacteria are corresponds to the MBC (Boukhraz et al., 2016).

3.5.2.1. Antibacterial activity study by agar dilution method:

To determine the antibacterial activity of *Nigella sativa* oil sample, the oil was used against 8 bacterial strains. The list of these bacteria are given below-

Name of Bacterial Strains	**Type of Bacteria**
Staphylococcus aureus	Gram (+)ve Bacteria
Streptococcus pyogenes	Gram (+)ve Bacteria
Bacillus subtilis,	Gram (+)ve Bacteria
Vibrio cholerae,	Gram (-)ve Bacteria
E. coli	Gram (-)ve Bacteria
Shigella flexneri	Gram (-)ve Bacteria
Proteus vulgaris	Gram (-)ve Bacteria
Salmonella typhi	Gram (-)ve Bacteria

3.5.2.2. Preparation of bacterial suspension in nutrient broth medium:

For the preparation of bacterial suspension, nutrient broth medium was prepared by adding 0.25gm of nutrient broth to 10ml of distill water. To prepare the eight different bacterial suspensions, eight different broth medium was prepared in the conical flasks by this same manner. This entire broth medium was autoclaved for 60 minutes at 121°C. After that the broth medium was allowed to cool down at room temperature. Finally, every strain was collected from the nutrient agar media and dipped into the nutrient broth medium and mixed rigorously to prepare the bacterial suspension. The freshly prepared bacterial suspension then allowed to set on shaking incubator for 24 hours. So that bacteria could grow on that suspension. After 24 hours the absorbance of those bacterial suspensions were taken. As the desired value of absorbance was about 0.1-0.2.So the suspension had been diluted with freshly prepare nutrient broth and achieved the desired concentration.

3.5.2.3. Preparation of oil and agar mixture:

For the antibacterial activity test by agar dilution method at first oil and agar mixture of different concentration had to prepare. Three different concentration of oil and agar mixture (1:10, 1:25, and 1:50) were used here. 0.4gm of nutrient agar was dissolved into 200ml of distill water and then autoclave at 121°C for 60 minutes. After that three another sterile conical flask was taken and each of those flasks oil and sterile agar was added respectively to make the desired concentration.

3.5.2.4. Preparation of agar medium:

Mueller-Hinton agar was used here for the preparation of agar medium. 30.4gm of Mueller-Hinton agar added into 800 mL of distill water. After that, the solution was shaken thoroughly so that the agar was dissolved completely into the water. Then at 121°C the mixture was autoclaved for 60 min and cooled up to 45°C -50°C. Afterward 5ml of oil and agar mixture of those three different concentrations was added into each 25ml sterile Mueller-Hinton agar solution and poured into 32 sterile Petri dishes. So that each 100mm petri dish got approximately 25ml of Mueller-Hinton agar solution plus 5ml of oil and agar mixture. Then the solution was set at room temperature to cool and solidify.

3.5.2.5. Procedure:

To observe the antibacterial activity of *Nigella sativa* oil in eight different microorganisms, a freshly prepared Mueller-Hinton agar medium containing oil and agar mixture was taken. Then the diluted suspensions of the bacteria in nutrient broth medium were taken and a glass spreader was dipped into a bacterial suspension. The spreader was then applied thoroughly on the agar medium so that the spreader could touch the each corner of that medium. This same procedure was followed for each of the 32petri dishes for that eight different bacteria. Afterward, the agar plates were allowed to get dry for 5 minutes. Then 5 minutes later, all the Petri dishes were allowed to incubate at 37°C for 24 hours to get the visible growth of bacteria. All of this work was done under laminar airflow which is a biosafety cabinet.

CHAPTER FOUR

RESULTS

Chapter 4: Results

4.1Antibacterial activity study by disc diffusion method:

In vitro antimicrobial screening of *Nigella sativa* were carried out using four different concentrations of oil and ethanol mixture proportion (1:1, 1:10, 1:20 and 1:40 µl/ µl.) to have the extent of antimicrobial activity. Kanamycin, Amoxicillin, cefixime, azithromycin, and chloramphenicol were used as positive control for this study.

Bacillus subtilis showed the zone of inhibition which increase with the concentration. The results are shown in Table 3.1.1. In case of *Bacillus subtilis*, the positive control disk showed the largest zone of inhibition. After that the zone of inhibition increased gradually with the increased concentration (Figure 4.1.1).

Table 4.1.1: Antimicrobial activity of *Nigella sativa* oil against *Bacillus subtilis*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Bacillus subtilis*
1	Ethanol	5 µl	0
2	Kanamycin (positive control)	30(µg/disk)	22
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	18
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	11
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	12
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	9

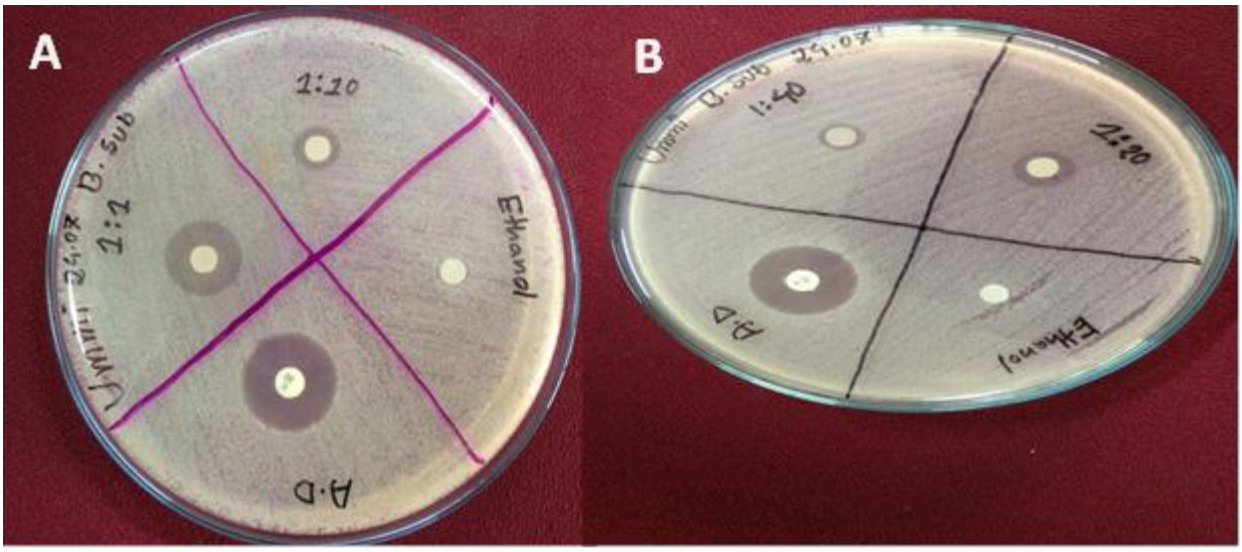


Figure 4.1.1: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Bacillus subtilis* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Bacillus subtilis* in Mueller-Hinton agar.

E. coli showed the zone of inhibition which increase with the concentration. The results are shown in Table 3.1.2. In case of *E. coli,* the highest concentrated disk showed the largest zone of inhibition. After that the zone of inhibition increased gradually with the increased concentration (Figure 4.1.2).

Table 4.1.2: Antimicrobial activity of *Nigella sativa* oil against *E. coli.*

Group	Treatment	Concentration	Zone of inhibition (mm) of *E. coli*
1	Ethanol	5 µl	0
2	Cefixime (positive control)	5(µg/disk)	18
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	30
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	15
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	13
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	8

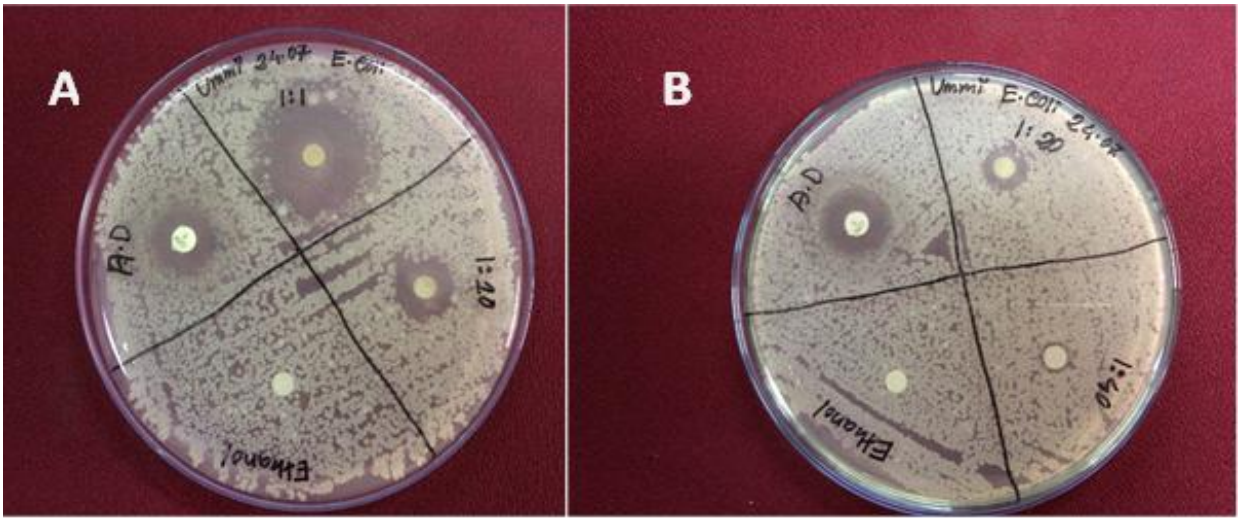


Figure 4.1.2: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *E. coli* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *E. coli* in Mueller-Hinton agar.

Vibrio cholerae. Showed the zone of inhibition which increase with the concentration. The results are shown in Table 3.1.3. In case of *Vibrio cholerae*, the positive control disk showed the largest zone of inhibition. After that the zone of inhibition increased gradually with the increased concentration (Figure 4.1.3).

Table 4.1.3: Antimicrobial activity of *Nigella sativa* oil against *Vibrio cholera*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Vibrio cholerae*
1	Ethanol	5 µl	0
2	Amoxicillin (positive control)	10(µg/disk)	36
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	13
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	10
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	8
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	7

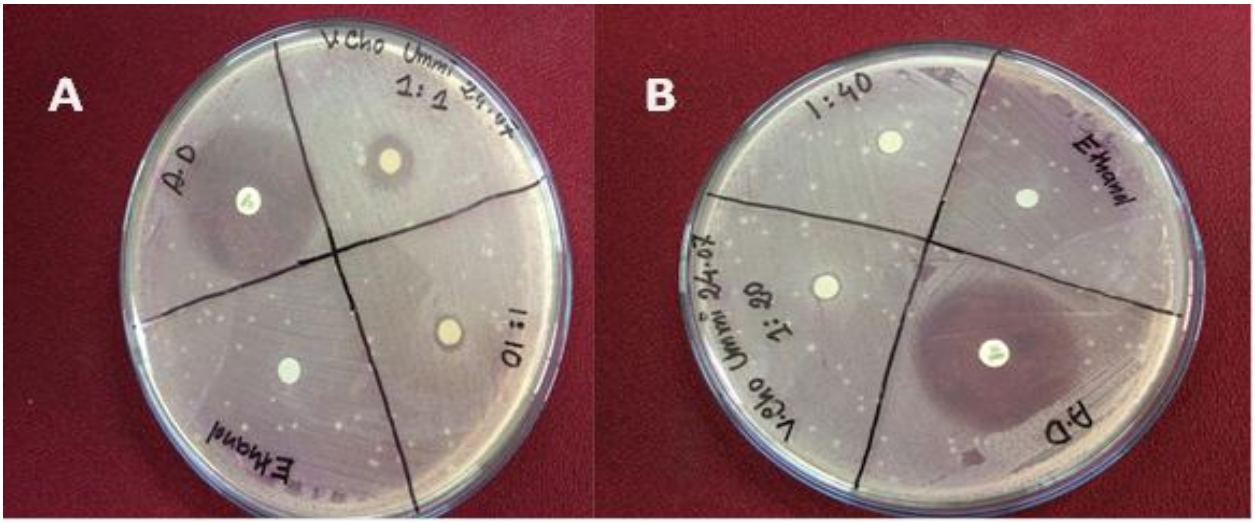


Figure 4.1.3: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Vibrio cholerae* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Vibrio cholerae* in Mueller-Hinton agar.

Proteus vulgaris Showed the zone of inhibition which increase with the concentration. The results are shown in Table 3.1.4. In case of *Proteus vulgaris*, the positive control disk showed the largest zone of inhibition. After that the zone of inhibition increased gradually with the increased concentration. But the 1:20 concentrated disk show large zone of inhibition than 1:40 concentrated disk (Figure 4.1.4).

Table 4.1.4: Antimicrobial activity of *Nigella sativa* oil against *Proteus vulgaris*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Proteus vulgaris*
1	Ethanol	5 µl	0
2	chloramphenicol (positive control)	30(µg/disk)	30
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	11
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	12
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	14
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	10

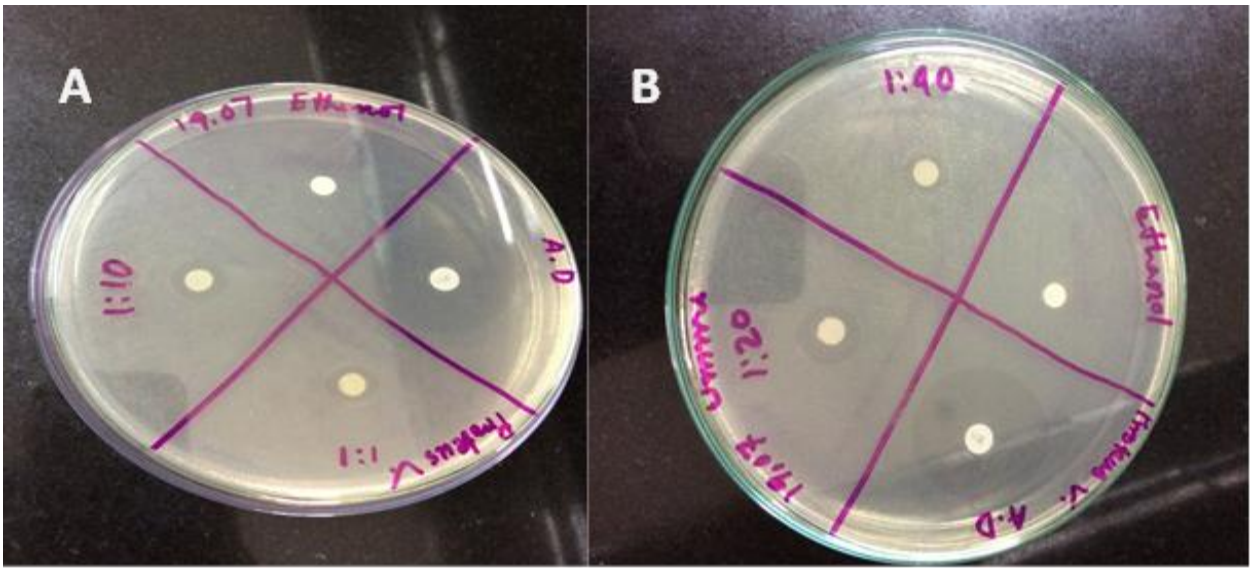


Figure 4.1.4: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Proteus vulgaris* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Proteus vulgaris* in Mueller-Hinton agar.

Salmonella typhi Showed the zone of inhibition which increase with the concentration. The results are shown in Table 3.1.5. In case of *Salmonella typhi*, the 1:1 concentrated disk showed the largest zone of inhibition. After that the zone of inhibition increases gradually with the increased concentration (Figure 4.1.5).

Table 4.1.5: Antimicrobial activity of *Nigella sativa* oil against *Salmonella typhi*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Salmonella typhi*
1	Ethanol	5 µl	0
2	Amoxicillin (positive control)	10(µg/disk)	20
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	25
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	14
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	12
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	8

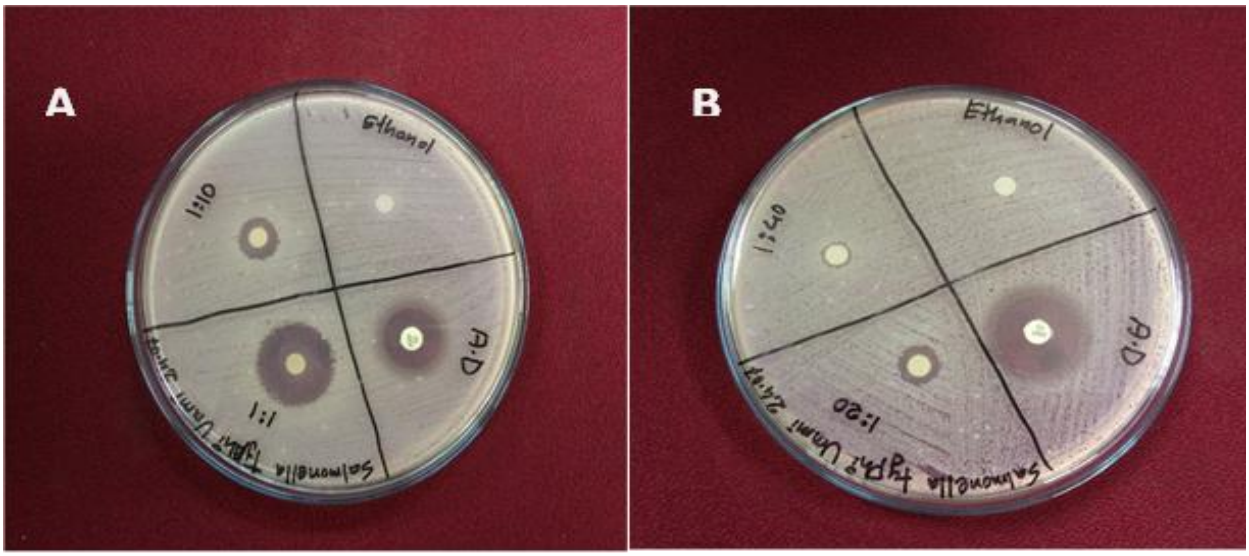


Figure 4.1.5: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Salmonella typhi* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Salmonella typhi* in Mueller-Hinton agar.

Shigella flexneri did not Show any zone of inhibition with the increased concentration. The results are shown in Table 4.1.6. In case of *Shigella flexneri*, only the positive control disk shows the largest zone of inhibition (Figure 4.1.6).

Table 4.1.6: Antimicrobial activity of *Nigella sativa* oil against *Shigella flexneri*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Shigella flexneri*
1	Ethanol	5 µl	0
2	Cefixime (positive control)	5(µg/disk)	23
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	0
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	0
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	0
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	0

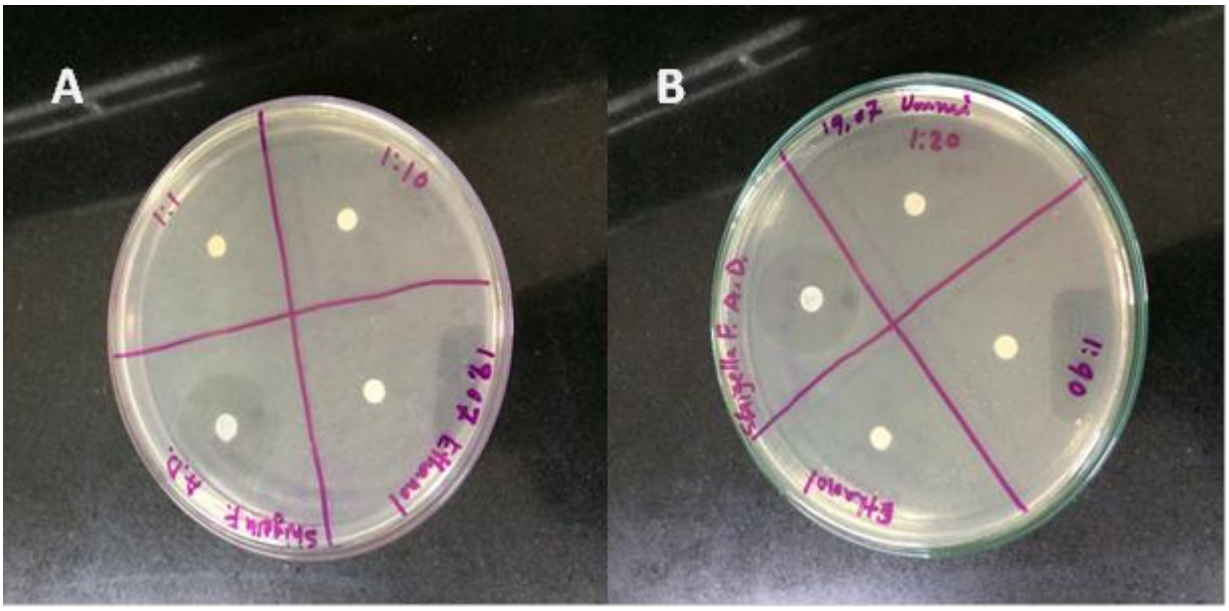


Figure 4.1.6: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Shigella flexneri* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Shigella flexneri* in Mueller-Hinton agar.

Streptococcus pyogenes did not Show any zone of inhibition with the increased concentration. The results are shown in Table 3.1.7. In case of *Streptococcus pyogenes* only the positive control disk shows the largest zone of inhibition (Figure 4.1.7).

Table 4.1.7: Antimicrobial activity of *Nigella sativa* oil against *Streptococcus pyogenes*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Streptococcus pyogenes*
1	Ethanol	5 µl	0
2	Azithromycin (positive control)	15(µg/disk)	22
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	0
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	0
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	0
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	0

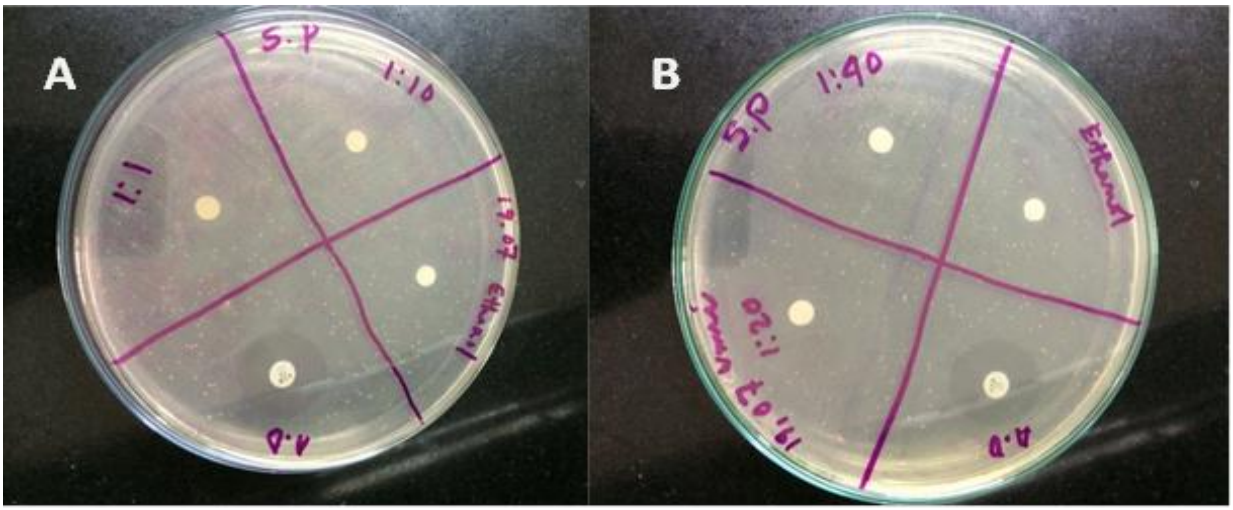


Figure 4.1.7: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Streptococcus pyogenes* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Streptococcus pyogenes* in Mueller-Hinton agar.

Staphylococcus aureus did not Show any zone of inhibition with the increased concentration. The results are shown in Table 3.1.8. In case of *Staphylococcus aureus* only the positive control disk shows the largest zone of inhibition (Figure 4.1.8).

Table 4.1.8: Antimicrobial activity of *Nigella sativa* oil against *Staphylococcus aureus*

Group	Treatment	Concentration	Zone of inhibition (mm) of *Staphylococcus aureus*
1	Ethanol	5 µl	0
2	Cefixime (positive control)	5 (µg/disk)	26
3	*Nigella sativa* oil+ Ethanol	1:1(µl/ µl)	0
4	*Nigella sativa* oil+ Ethanol	1:10(µl/ µl)	0
5	*Nigella sativa* oil+ Ethanol	1:20(µl/ µl)	0
6	*Nigella sativa* oil+ Ethanol	1:40(µl/ µl)	0

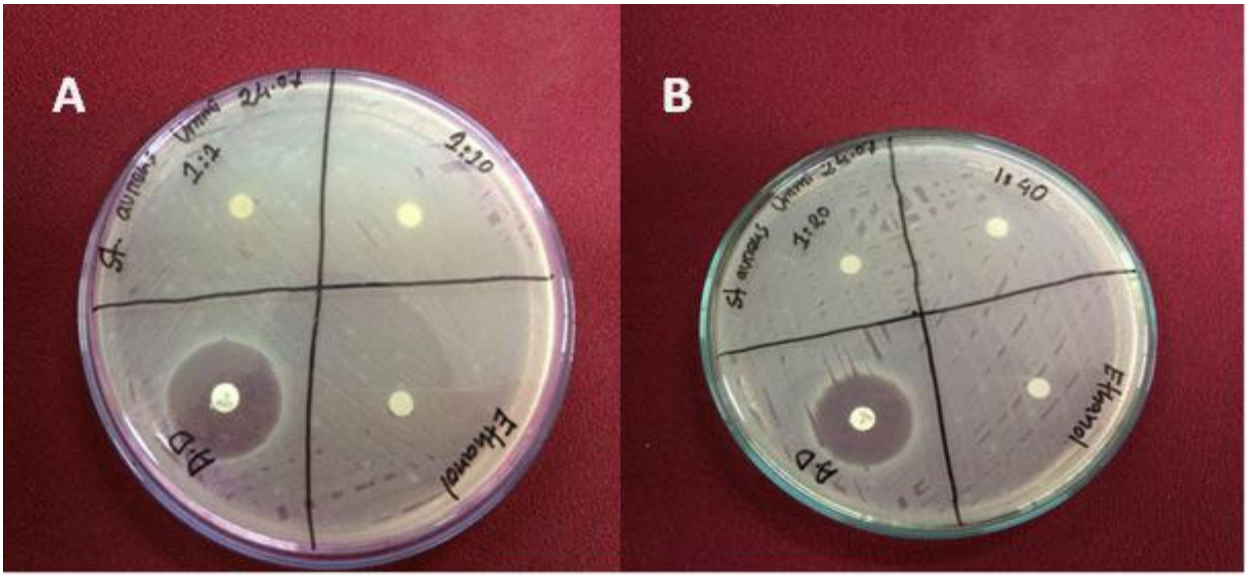


Figure 4.1.8: (A) Effect of *Nigella sativa* oil (1:1; 1:10) on the growth of *Staphylococcus aureus* in Mueller-Hinton agar. (B) Effect of *Nigella sativa* oil (1:20; 1:40) on the growth of *Staphylococcus aureus* in Mueller-Hinton agar.

4.2Antibacterial activity study by agar dilution method:

In vitro antimicrobial screening of *Nigella sativa* by agar dilution method were carried out using three different concentrations of oil and agar mixture proportion (1:1, 1:25 and 1:50 ml/ ml.) to have the extent of antimicrobial activity. Here also a blank solution was used which did not contain oil and agar mixture.

This method shows that *Nigella sativa* has significant antibacterial activity on the tested strains. However, the studied microorganisms did not show the same sensitivity against this oil. Every microorganisms were grow in the highest amount on blank preparation. Then the other concentrated disks were compare to their respective blank preparation. The results are shown in Table 4.2.1.

In the case of *Nigella sativa* oil, the strains *Bacillus subtilis* showed the same degree of inhibition for every concentration (Figure 4.2.1).While the growth of *E. Coli* were decreased at concentrations of 1:10 ml/ml and then it increased respectively with the decreased concentration (Figure 4.2.2). *Vibrio cholera* showed the same degree of inhibition for every concentration (Figure 4.2.3). *Salmonella typhi* showed the complete inhibition at 1:10 ml/ml concentration and few bacteria was grow on 1:25 ml/ml

concentarion and 1:50 ml/ml concentration did not show any inhibitory effect (Figure 4.2.5). However *Proteus vulgaris*, *Shigella flexneri*, *Streptococcus pyogenes* and *Staphylococcus aureus* did not show any inhibitory effects for this method (Figure 4.2.4, Figure 4.2.6, Figure 4.2.7 and Figure 4.2.8).

Table 4.2.1: Antimicrobial activity of *Nigella sativa* oil against Bacteria by using agar dilution method.

Concentration (ml/ml)	**Name of the bacteria**							
	Bacillus subtilis	***E. coli***	***Vibrio cholerae***	***Proteus vulgaris***	***Salmonella typhi***	***Shigella flexneri***	***Streptococcus pyogenes***	***Staphylococcus aureus***
Blank	+++	+++	+++	+++	+++	+++	+++	+++
1:10	++	+	+	+++	_	+++	+++	+++
1:25	++	++	+	+++	+	+++	+++	+++
1:50	++	++	+	+++	+++	+++	+++	+++

(+++): Strongly growth

(++) : Moderate growth

(+) : Modest growth

(-) : Inhibition

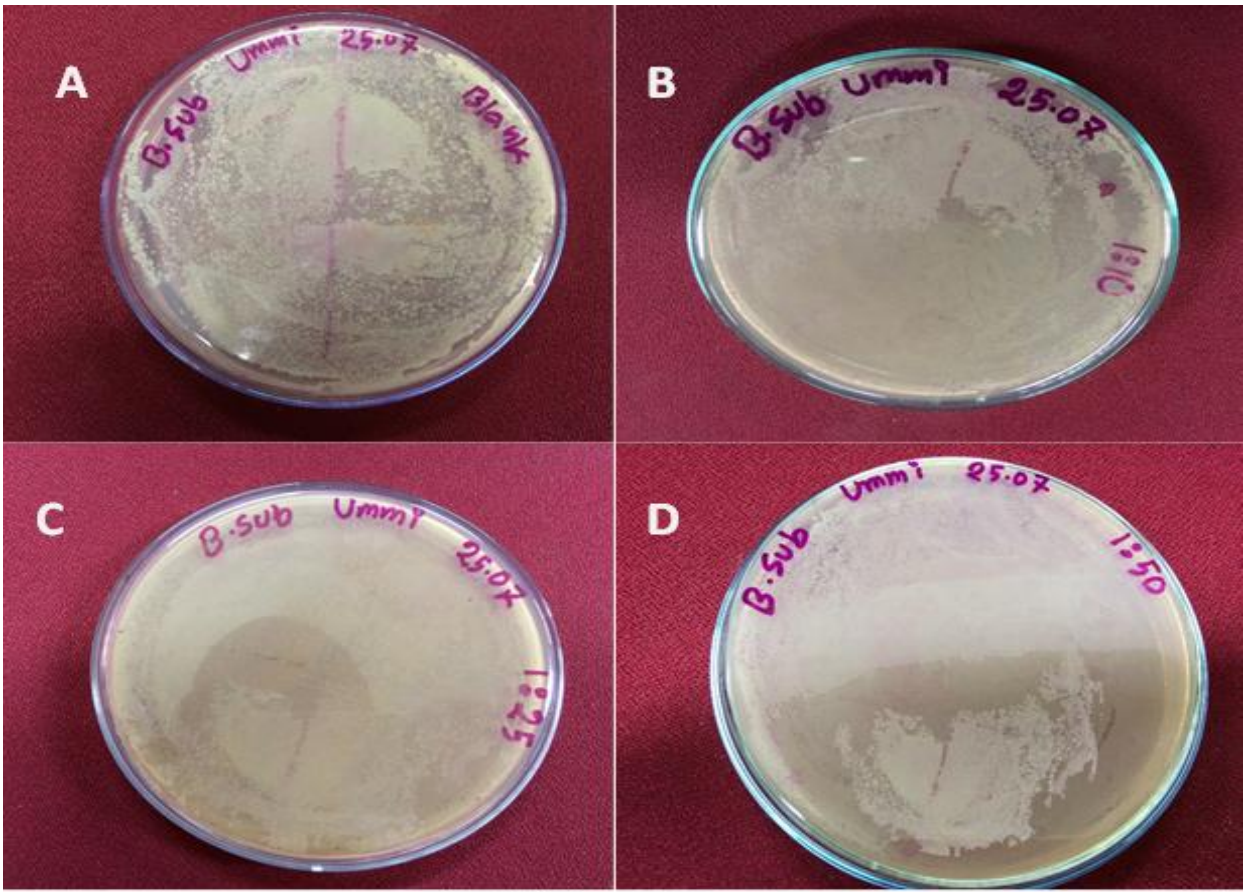


Figure 4.2.1: (A) Growth of *Bacillus subtilis* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Bacillus subtilis* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *Bacillus subtilis* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Bacillus subtilis* in Mueller-Hinton agar.

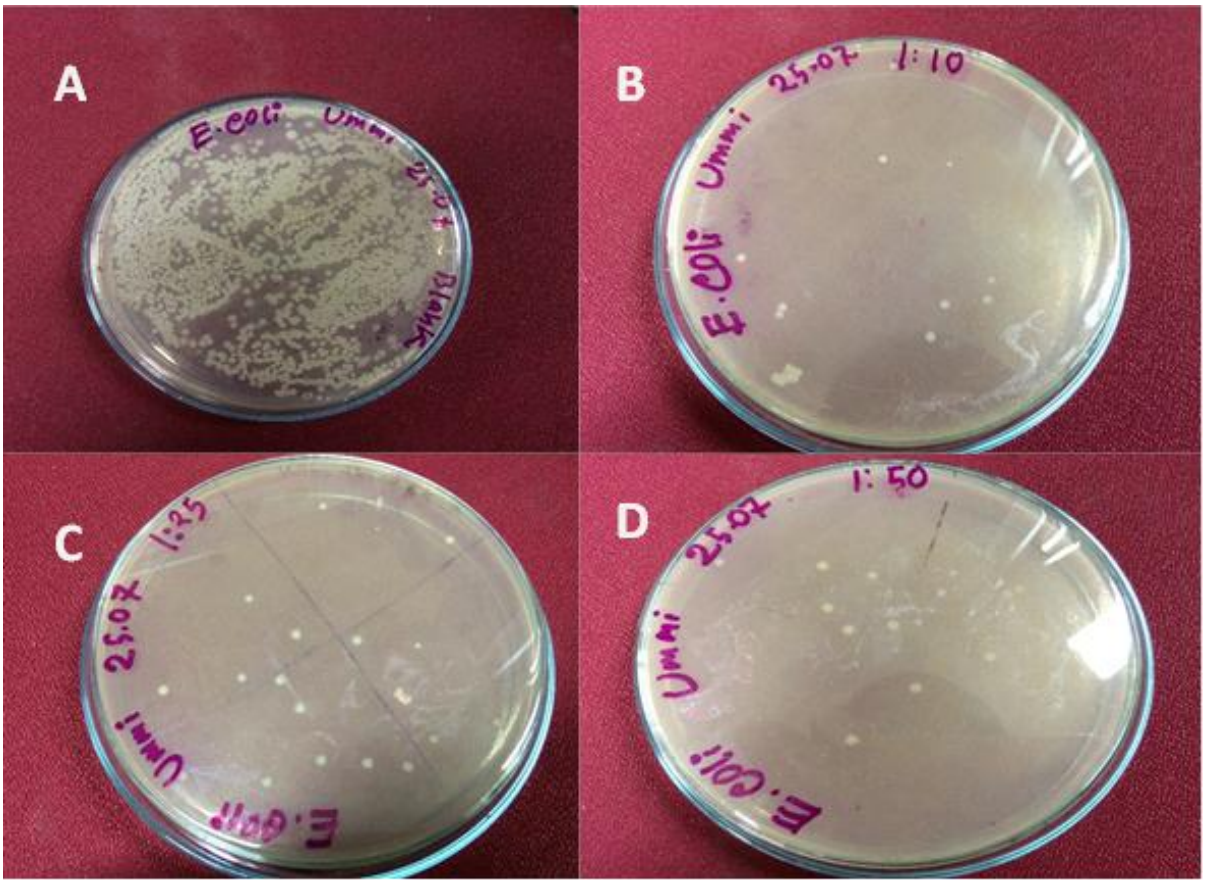


Figure 4.2.2: (A) Growth of *E. coli* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *E. coli* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *E. coli* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *E. coli* in Mueller-Hinton agar.

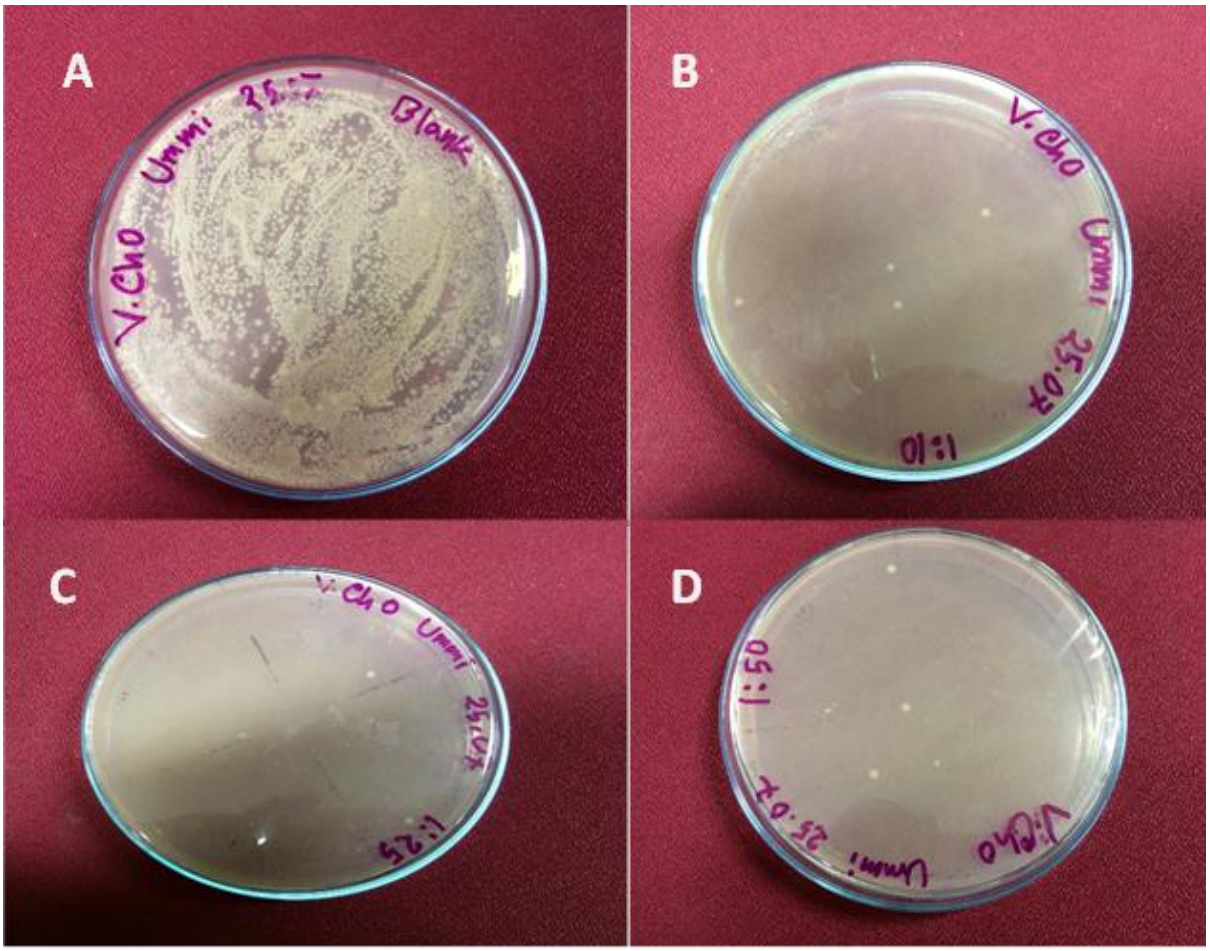


Figure 4.2.3: (A) Growth of *Vibrio cholerae*, in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Vibrio cholerae*, in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *Vibrio cholerae*, in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Vibrio cholerae*, in Mueller-Hinton agar.

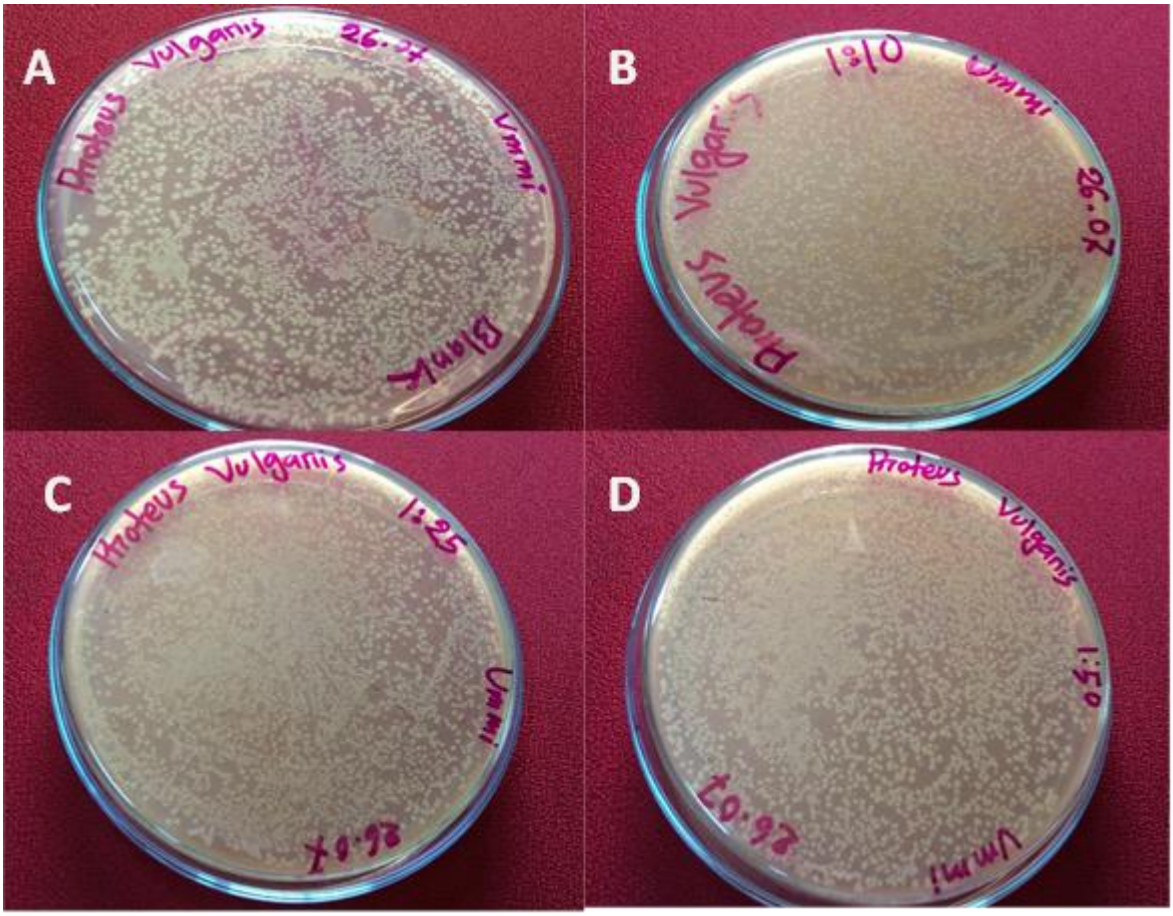


Figure 4.2.4: (A) Growth of *Proteus vulgaris* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Proteus vulgaris* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth *Proteus vulgaris* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Proteus vulgaris* in Mueller-Hinton agar.

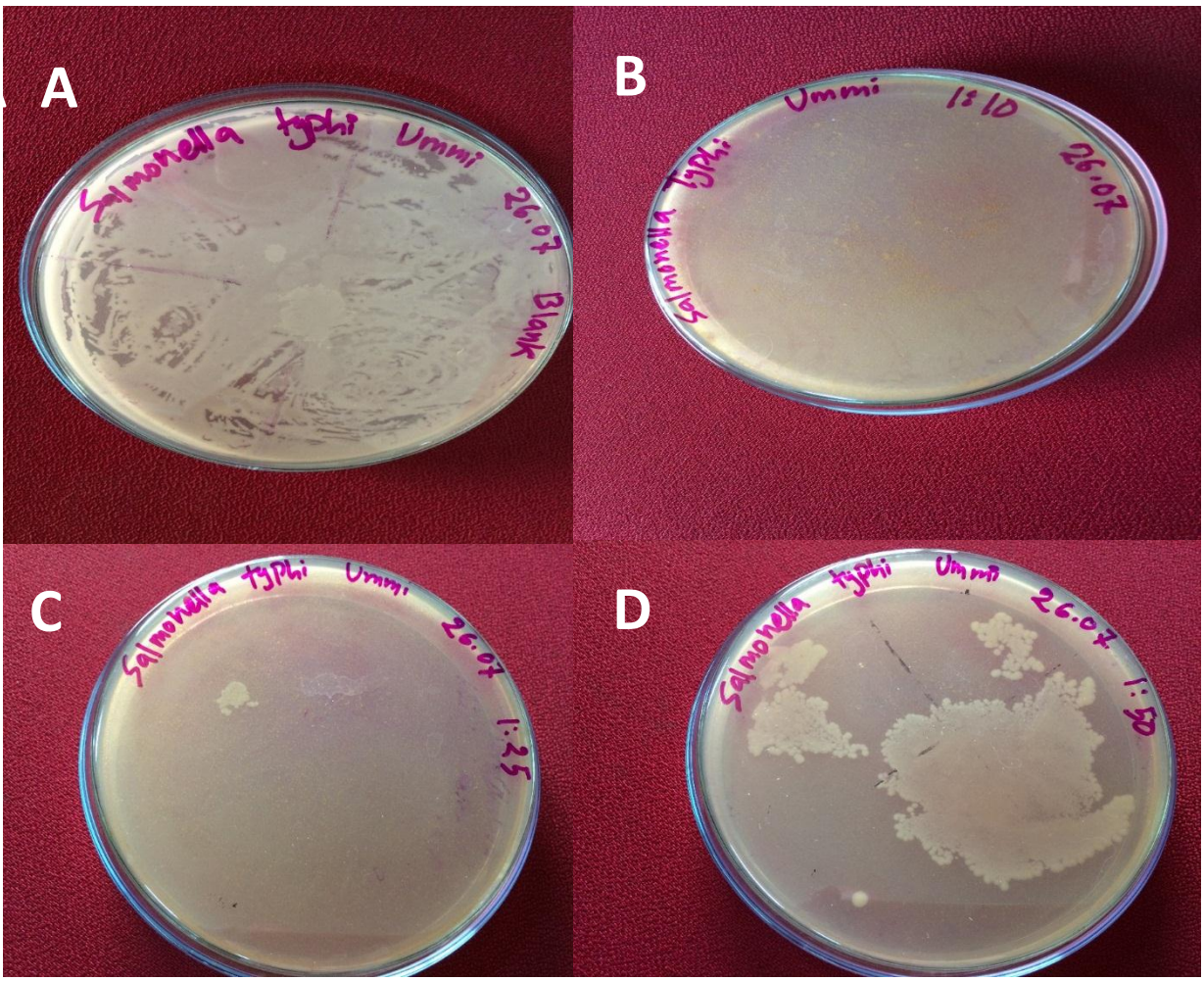


Figure 4.2.5: (A) Growth of *Salmonella typhi* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Salmonella typhi* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *Salmonella typhi* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Salmonella typhi in* Mueller-Hinton agar.

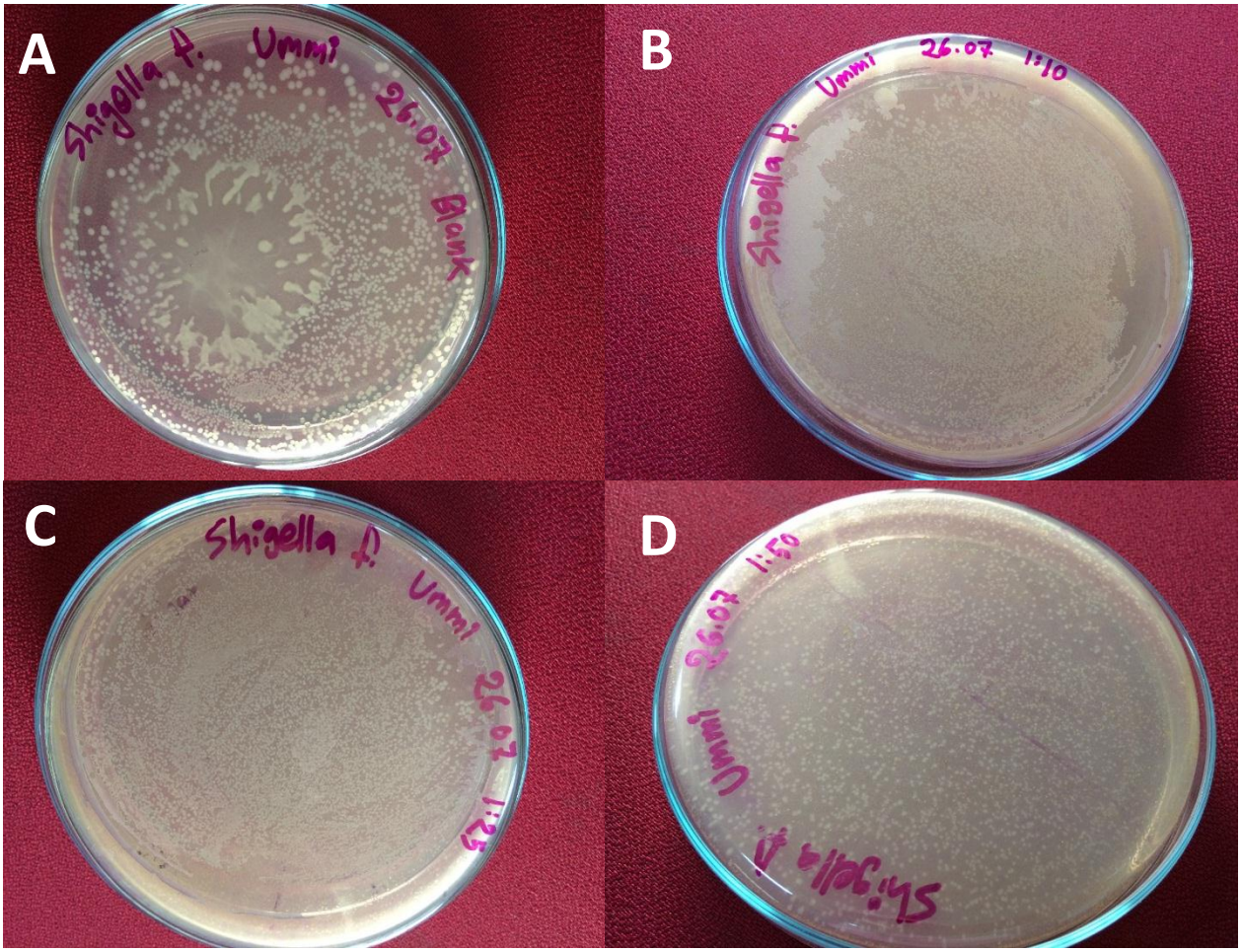


Figure 4.2.6: (A) Growth of *Shigella flexneri* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Shigella flexneri* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *Shigella flexneri* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Shigella flexneri* in Mueller-Hinton agar.

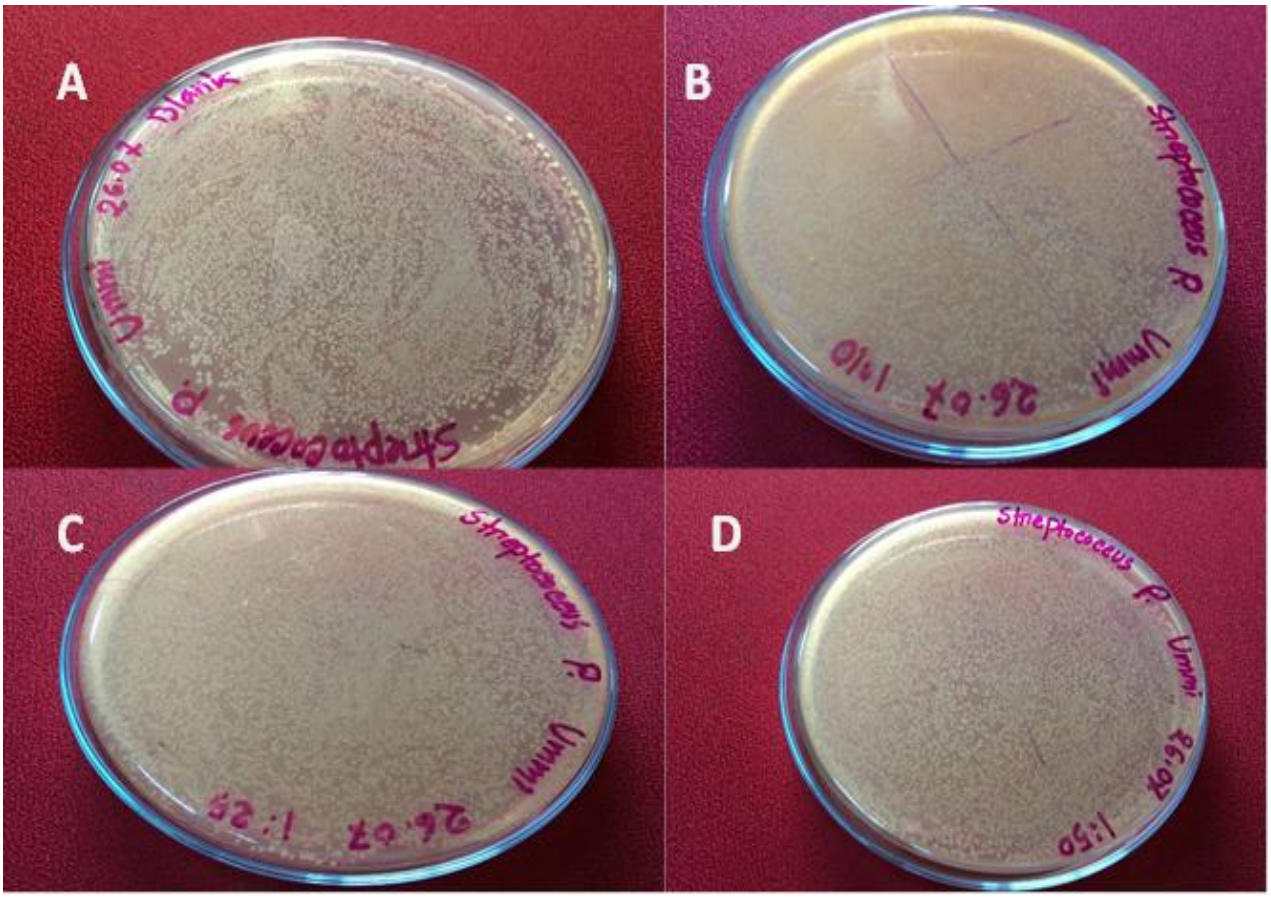


Figure 4.2.7: (A) Growth of *Streptococcus pyogenes* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Streptococcus pyogenes* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *Streptococcus pyogenes* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Streptococcus pyogenes* in Mueller-Hinton agar.

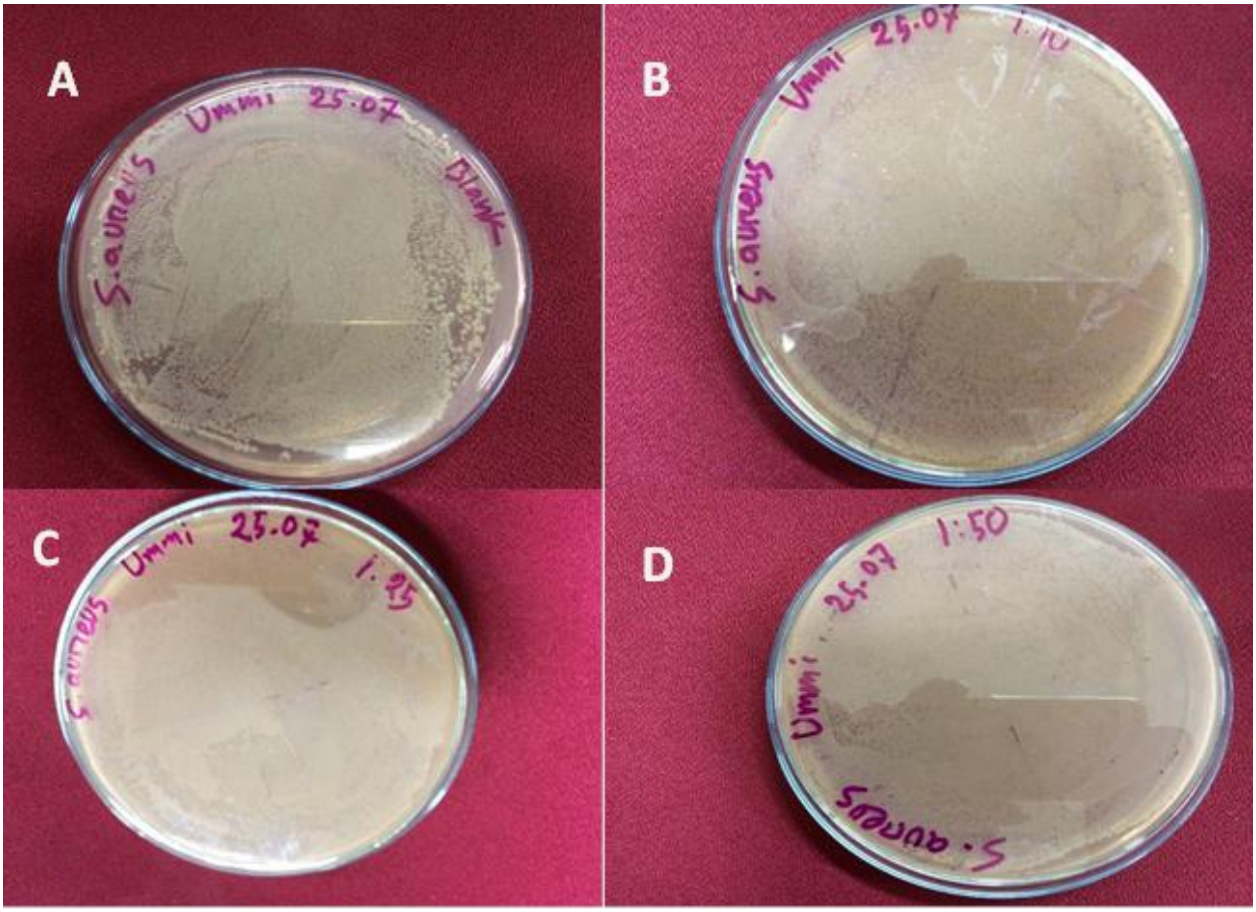


Figure 4.2.8: (A) Growth of *Staphylococcus aureus* in Mueller-Hinton agar {Blank sample}. (B) Effect of *Nigella sativa* oil (1:10) on the growth of *Staphylococcus aureus* in Mueller-Hinton agar. (C) Effect of *Nigella sativa* oil (1:25) on the growth of *Staphylococcus aureus* in Mueller-Hinton agar. (D) Effect of *Nigella sativa* oil (1:50) on the growth of *Staphylococcus aureus* in Mueller-Hinton agar.

CHAPTER FIVE

DISCUSSION

Chapter 5: Discussion:

As a complementary medicine, the use of herbal drugs is gaining worldwide popularity. There are numerous drugs which are straightly collected from plants origin, while the rest of them are chemically reformed natural products.

Nigella sativa specially its seed and oil are used in various traditional medicines system and also as a folk medicine around the world for the prevention and treatment of a variety of diseases. As it has the miraculous power of healing, *Nigella sativa* has been widely used as anti-bacterial, analgesics, antihypertensives, diuretics, liver tonic, digestive, appetite stimulant, antidiarrheal, and in skin disorders.

There are considerable research studies have been carried out on *Nigella sativa* by lots of scientists and a wide range of its pharmacological activity have been found which include antimicrobial, antidiabetic, antioxidant, anticancer, anti-inflammatory, immunomodulator, spasmolytic, bronchodilator, analgesic, hepatoprotective, renal protective, and gastroprotective properties (Ahmad et al., 2013).

In previous research, it has been found that, alcoholic extracts of *Nigella sativa* seeds exhibit antimicrobial activity against *Micrococcus pyogenes var. aureus.* This extracts also showed antimicrobial activity against *S. sonnei, Shigella dysenteriae, s. boydii, Vibrio cholerae* and *E. coli.* On the other hand the ether extracts of *Nigella sativa* exhibit in vitro antimicrobial activity against gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa* and gram positive bacteria such as *Staphylococcus aureus.* In another research, *Nigella sativa* was found to possess antimicrobial activity especially against *Bacillus subtilis, Bacillus pumilus, Streptococcus mutans, Staphylococcus aureus, Staphylococcus lutea* and *Pseudomonas aeruginosa* (Gilani et al., 2004).

This study is based on the antimicrobial property of *Nigella sativa* seed oils. Here, inhibition rate of *Nigella sativa* was measured. Preservative-free marketed preparation of this seed oil is collected from Shariatpur, Dhaka, Bangladesh for the antimicrobial screening.

Over disk diffusion method, *Nigella sativa* oil exhibited a strong antimicrobial property against *Bacillus subtilis, E. coli, Proteus vulgaris, Vibrio cholera and Salmonella typhi.*

The strongest response was observed 33 mm zone of inhibition against *E. coli* in 1:1 concentration of oil. Alike same concentration showed second strongest response spotted 25

mm zone of inhibition against *Salmonella typhi*. In *Vibrio cholera* and *Proteus vulgaris* culture, 13 mm and 11 mm zone of inhibition was spotted by the same concentration of oil. On the other hand, only *Salmonella typhi* growth was completely inhibited by 1:10 concentration of oil at Agar dilution method. But in all those three different concentration *Bacillus subtilis* was grown moderately and *Vibrio cholera* showed modest growth. However *E. coli* showed modest growth on 1:10 concentration and average growth on both 1:25 and 1:50 concentration. The other four bacteria *(Proteus vulgaris***,** *Shigella flexneri, Staphylococcus pyogenes* and *Staphylococcus aureus*) did not exhibit any inhibition property in this agar dilution method. Thus the overall screening demonstrates that *Nigella sativa* oil contains potential antimicrobial activity and offering us an opportunity to develop natural antimicrobial agent. The further study requires screening the unique chemical compounds that are responsible for the antimicrobial property.

CHAPTER SIX

CONCLUSION

Chapter 6: Conclusion:

Plant derivative's including oils are used for diet, nutrition, raw medicinal source and the natural healing agent from the beginning of civilization. Among all the plants *Nigella sativa* specially its seed carried most potent medicinal value. *This in vitro* study showed that *Nigella sativa* seed oil inhibited bacterial progression in dose depending way nevertheless oil antimicrobial response varied with bacterial strain. This work is devoted to determining the antimicrobial properties of *Nigella sativa* oil which was harvested in the region of Shariatpur, Dhaka, Bangladesh. The study of the antibacterial activity of *Nigella sativa* oil on gram-negative bacteria and gram-positive bacteria showed the presence of a strong antibacterial activity. Therefore the study showed that *Nigella sativa* oil might be a significant contributor to drug development with its potential antimicrobial activity. This study opens new vistas for the use of *Nigella sativa* seed extract and its oil and also it's bioactive compound thymoquinone which leads to the new drug designing and development opportunity and new hope against multi-drug resistant bacterial infection.

REFERENCE

Abeloff, M. (2008). *Abeloff's Clinical Oncology* (4th ed.). Churchill Livingstone

Ahmad, A., Husain, A., Mujeeb, M., Khan, S. A., Najmi, A. K., Siddique, N. A., Damanhouri, Z. A., & Anwar, F. (2013). A review on therapeutic potential of Nigella sativa: A miracle herb. *Asian Pacific Journal of Tropical Biomedicine, 3*(5), 337-352.30. Goreja, W. G. (2003). *Black Seed: Nature's Miracle Remedy*. New York: NY 7 Amazing Herbs Press.

Ali, B. H., & Blunden, G. (2003). Pharmacological and toxicological properties of Nigella sativa. *Phytotherapy Research, 17*(4), 299-305.

Aruoma OI. (1998). Free radicals, oxidative stress, and anti-oxidants in human health and disease. J Am Oil Chem Soc, 75, 199–212.

Auudy, B., Ferreira, F., Blasina, L., Lafon, F., Arredondo, F., Dajas, R. & Tripathi, P.C. (2003). Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. *J. Ethnopharmacol*, 84, 131-138.

Azam, M. N. K., Mannan, M. A., & Ahmed, M. N. (2014). Medicinal plants Used by The Traditional Medical Practitioners of Barendra and Shamatat (Rajshahi & Khulna Division) Region in Bangladesh for treatment of Cardiovascular Disorders. *Journal of Medicinal Plants Studies, 2*(2), 09-14.

Bandoniene, D., Pukalskas, A., Venskutonis, P.R., & Gruzdiene, D. (2000). Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil. *Food Res Int*,**33**,785–791.

BOTANICA. (n.d.). Retrieved September 08, 2017, from http://www.botanical-online.com/

Boukhraz, A., Elhartiti, H., Barrahi, M., Sedki, H., Elayne, N. S., Lakhrissi, B., Rhaiem, N., & Ouhssine, M. (2016). Evaluation of the Bacteriostatic and Bactericidal Activity of Essential Oil of Thymus Satureioides. *International Journal of Research Studies in Science, Engineering and Technology , 3*(3), 24-28.

Braca, A., Tommasi, N. D., Bari, L. D., Pizza, C., Politi, M., & Morelli, A. (2001). Antioxidant Principles from Bauhinia tarapotensis. *J. Nat. Prod., 64*(7), 892-895.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology, 94*(3), 223-253.

Couladis, M., Tzakou, O., Verykokidou, E., & Harvala, C. (2003). Screen-ing of some Greek aromatic plants for antioxidant activity. *Phytother Res*, **17**, 194–195.

Deckard, A (2015). 11 Powerful Antibacterial Essential Oils.

Dinagaran, S., Sridhar, S., & Eganathan, P. (2016). Chemical composition and antioxidant activities of black seed oil (Nigella sativa L.). *International Journal of Pharmaceuticals Sciences and Research, 7*(11), 4473-79.

Fakir, M. M. (2015). Biodiversity of Medicinal Plants in Bangladesh: Prospects and Problems. 2-8.

Ghani, A. (2003). *Medicinal plants of Bangladesh with chemical constituents and uses*. Dhaka: Asiatic Society of Bangladesh.

Gilani, A. H., Q. J., & Khan, M. A. (2004). A Review of Medicinal Uses and Pharmacological Activities of Nigella sativa. *Pakistan Journal of Biological Sciences, 7*(4), 441-451

Goreja, W. G. (2003). *Black Seed: Nature's Miracle Remedy*. New York: NY 7 Amazing Herbs Press.

Harbertson, J., & Spayd, S. (2006). Measuring Phenolics in the winery. Am. J. Enol. Vitic.,280-288.

Hasan, M. M., Hossain, S. A., Ali, M. A., & Alamgir, A. N. (2014). Medicinal plant diversity in Chittagong, Bangladesh: A database of 100 medicinal plants. *Journal of Scientific and Innovative Research , 3*(5), 500-514.

Hassan, B. A. (2012). Medicinal Plants (Importance and Uses). *Pharmaceutica Analytica Acta, 03*(10).

Inatani, R., Nakatani, N., & Fuwa, H. (1983). Antioxidative effects of the constituents of rosemary (*Rosmarinus officinalis*) and their derivatives. *Agricultural and Biological Chemistry*, 47, 521-528.

Jayaprakasha, G. K., & Jaganmohan Rao, L. (2000). Phenolic constitu-ents from lichen Parmotrema stuppeum (Nyl.) Hale and their antioxidant activity. Zeitschrift fu¨r Naturforschung, 56, 1018–1022.

Khare, C. P. (2004). *Indian herbal remedies rational Western therapy, ayurvedic, and other traditional usage, botany*. Berlin: Springer.

Li, L. (2000) Opportunity and Challenge of Traditional Chinese Medicine in Face of the Entrance to WTO (World Trade Organization). Chinese Journal of Information on Traditional Chinese Medicine, 7, 7-8.

Mraihi, F., Journi, M., Chérif, J. K., Sokmen, M., Sokmen, A., & Trabelsi-Ayadi, M. (2014). Phenolic Contents and Antioxidant Potential ofCrataegusFruits Grown in Tunisia as Determined by DPPH, FRAP, andβ-Carotene/Linoleic Acid Assay. *Journal of Chemistry*, 1-6.

Pal, D., Sannigrahi, S., & Mazumder, U. (2009). Analgesic and anticonvulsant effects of saponin isolated from the leaves of Clerodendrum infortunatum Linn. in mice. *Indian Journal of Experimental Biology*, **47**, 743-747.

Pieroni, A., Janiak, V., Dürr, C.M., Lüdeke, S., Trachsel, E., & Heincrich, M. (2002). *In vitro* antioxidant activity of non-cultivated veget-ables of ethnic Albanians in Southern Italy. *Phytother Res,* **16**, 467–473.

Prasad, M. V., Sudha, G. S., Khadri, C. H., & Raizunnisa, D. K. (2015). In vitro Antibacterial activity and phytochemical studies of leaf extracts of Adhatoda vasica and Crotolaria verrucosa. *WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES, 4*(6), 506-513.

Rodriguez, T. (2015). Essential Oils Might Be the New Antibiotics.

Ross, I. A. (2005). *Medicinal Plants of the World, Volume 3 Chemical Constituents, Traditional and Modern Medicinal Uses*. Totowa (NJ): Humana Press.

Roy, S. K., & Roy, D. K. (2016). Use of Medicinal Plant and Its Vulnerability Due to Climate Change in Northern Part of Bangladesh. *American Journal of Plant Sciences, 07*(13), 1782-1793.

Shahidi, F., Janitha, PK., & Wanasundara, P.D. (1992). Phenolic anti-oxidants. *Crit Rev Food Sci Nutr,* **32**, 67–103

Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. (2002). Lyon, France: International Agency for Research on Cancer.

Steinhoff, B. (1999). The Contribution of the European Scientific Cooperative on Phytotherapy and World Health Organization Monographs. *Drug Information Journal, 33*(1), 17-22.

Sylvestre, M., Pichette, A., Longtin, A., Nagau, F., & Legault, J. (2006) . Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. Journal of Ethnopharmacology, **103**(1), 99-102.

The world health report 1999 - making a difference. (n.d.). Retrieved September 08, 2017, from http://www.who.int/whr/1999/en/

Traditional Remidies with Plants. (n.d.). Retrieved September, 2017, from http://www.botanical-online.com/english/historyofmedicinalplants.htm#listado

Whalen, K., Finkel, R., & Panavelil, T. A. (2014). *Lippincott illustrated reviews: pharmacology* (6th ed.). LWW.

Wiegand, I., Hilpert, K., & Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163-175.