Phytochemical Screening and Comparison of Antibacterial Assays of *Pimpinella Anisum* Through Extraction

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

Submitted by:
Shagoofa Rakhshanda
Student ID: 11236002
Biotechnology Program
Department of Mathematics and Natural Sciences
BRAC University
Bangladesh

December 2015

Dedicated to

My beloved parents and sister

DECLARATION BY THE RESEARCHER

This is to declare that the research work embodying the results reported in this thesis entitled **"Phytochemical Screening and Comparison of Antibacterial Assays of *Pimpinella Anisum* Through Extraction"** has been carried out by the undersigned under supervision of Zubaida Marufee Islam, Lecturer, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Bachelors of Science in Biotechnology, BRAC University, Dhaka and has not been submitted anywhere else for a degree or diploma.

Shagoofa Rakhshanda

Certified

Zubaida Marufee Islam

Supervisor
Lecturer
Department of Mathematics and Natural Sciences
BRAC University

ACKNOWLEDGEMENT

First and foremost, I express my deepest gratitude to Almighty for endowing me with health, patience, benediction, protection and mental power in all aspect of my life. I also express my sincere gratitude to Professor Naiyyum Choudhury, former Coordinator of the Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University, Chairman, Bangladesh Atomic Energy Regulatory Authority (BAERA); and Professor A. A. Ziauddin Ahmad, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for their cooperation and inspiration, prudent advice and encouragement in conducting this study.

This work was completed under the meticulous guidance of my Research Supervisor Zubaida Marufee Islam, Lecturer of BRAC University. She added new directions to my thinking, and acted as a continual stimulus for refinement and depth in the presentation of this work. I am much indebted and would like to express my sincere thanks to her for the expert guidance and constant support throughout the entire period of my research work.

I would also like to extend my gratitude to Kashmery Khan, Teaching Assistant, BRAC University for extending support throughout the research work. I extend my sincere gratitude to all the teachers of BRAC University, who shared their knowledge and experiences during the coursework of the program. I would specially like to mention the names of Dr. Aparna Islam, Associate Professor and Coordinator of Postgraduate Biotechnology program; Ms. Jebunnesa Chowdhury, Assistant Professor and Ms. Romanna Siddiqui, Senior Lecturer and current Coordinators of Undergraduate Biotechnology program; Dr. Mahboob Hossain, Associate Professor of the Department of Mathematics and Natural Sciences, BRAC University. I am indebted to all the other Teaching Assistants including Shaan Mahmeed, the Lab Officers and the Lab Assistants who had always been there to guide my shaky steps through the intricacies of laboratory work.

I am thankful to all of my friends, especially Fatima Amin and Ilma Rahman for their help and support throughout my work. Finally I would like to thank my family members for their spontaneous and enthusiastic support.

Shagoofa Rakhshanda

ABSTRACT

Increasing number of microbes is developing resistance to synthetic antibiotics which hinders combating infective health conditions. Assessing antimicrobial properties of naturally occurring *Pimpinella anisum* may help in the search for newer and less expensive antibiotics. Antibacterial effects of ethanol, methanol extracts taken of second, fifth and seventh days, along with aqueous extracts of aniseed were observed on selected bacteria. Extracts of different days had variable effects on the three bacteria (*Bacillus cereus, Bacillus subtilis* and *Streptococcus pneumonae*) showed positive antibacterial effect of aniseed. The methanol extract from the fifth day showed the greatest positive result against *Bacillus cereus*. The activity index of methanol extract of day five was the highest against *Bacillus subtilis* indicating high sensitivity to the extract. Phytochemicals such as tannin, saponin, terpenoid, flavonoid, cardiac glycoside, alkaloid, phenolic compounds and steroids were present in the aqueous extract of aniseed. Variation in the solubility of these phytochemicals in different solvents may have an effect leading to the difference in antibacterial action. It is expected that the findings of this study will stimulate researchers to design clinical trials that may lead to the development of less expensive antimicrobial agents.

TABLE OF CONTENTS

List of Figures

List of Tables

LIST OF ABBREVIATIONS

AI	Activity Index
$BaCl_2.H_2O$	Barium chloride dehydrate
BRAC	Bangladesh Rural Advancement Committee
et al	And others
g	gram
H_2SO_4	Sulphuric acid
ml	Milliliter
mm	Millimeter
MRSA	Methicillin resistant *Staphylococcus aureus*
NA	Nutrient Agar
NaCl	Sodium chloride
PAL	Phenylalanine Amonia Lyase

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 Description of the Plant

Pimpinella anisum is an annual grassy herb of height that ranges from 30 to 50 cm. It bears white flowers and has seeds of green to yellow colour (Shojaii and Fard, 2012; Ernst, 1989). The plant has a distinct aromatic smell with a sweetish taste. The seed is greyish-green in colour which has a pear-like shape and is 2-mm-long. The blossom is an umbel with filamentous involucral bracts with white and short petals (Ernst, 1989). *Pimpinella anisum* is mainly grown for its fruits which is commercially called 'aniseeds' (Gulcin *et al*, 2003).

The plant falls under the family of Umbelliferae. It is one of the oldest medicinal plants. The herb originated in the eastern Mediterranean region and is native to Asia Minor, Greece and Egypt (Shojaii and Fard, 2012; Ernst, 1989). It is commercially cultivated in Chile, China and the USA (Ernst, 1989).

Aniseeds contain 1.5–5% essential oil. It is used in perfumery for its aromatic smell. It is used as a flavouring agent because of its sweet taste. It is also used to relieve gastrointestinal spasms since it helps in digestion and it also has carminative properties. The production of milk in lactating women is also observed to increase after the consumption of aniseed. It also reduces the gastrointestinal problems of their children (Shojaii and Fard, 2012). Due to all these benefits, it is one of the oldest spices used in traditional medicine.

Aniseed is grown to a limited extent in Bangladesh. It is mostly imported, and widely used as flavoring spice in the preparation of food. The various medicinal properties are not widely known. It is used in the preparation of Ayurvedic medicine.

1.2 Scientific Classification of Aniseed

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta– Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae
Order	Apiales
Family	Apiaceae – Carrot family
Genus	*Pimpinella* L.
Species	*Pimpinella anisum* L.

Figure 1.1: The external morphology of aniseed

Source: Google "aniseed"

Figure 1.2: Aniseed herb

Source: Google "aniseed"

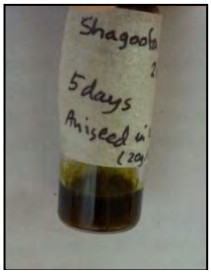


Figure 1.3: Extract of Aniseed

1.3 Therapeutic Use of Aniseed

For many years, various diseases were treated with natural products instead of antibiotics and other modern drugs. Different herbal extracts show different antimicrobial activities. Plants have a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found to have antimicrobial properties *in vitro* (Khan *et al*, 2009).

Extracts of the aniseeds are used as medicine for their diuretic and laxative effect, expectorant and anti-spasmodic action, and their ability to ease gastric pain and flatulence (Kreydiyyeh *et al*, 2003). Anise is used as an appetizer and tranquillizer drug. Anise-flavoured drinks like Pernod, Anisette, Raki are traditionally used after a heavy meal due to its antispasmodic effect on the digestive tract (Kosalec *et al*, 2005). It has also been reported that aniseed is a potent anti-peroxidative and anti-diabetic agent and thereby, possesses a vast spectrum of applications and exploitations in the food and drug industry (Shobha *et al*, 2013).

1.4 Antibacterial Properties of Aniseed

The spoilage of foods by microorganisms is a great problem that cannot be controlled even after the use of a large range of preservative techniques. Foods with such chemical preservatives are usually avoided by the consumers. Thus natural alternatives are needed so that the foods can be stored for a sufficiently long period of time without compromising the safety of the food (Gulcin *et al*, 2003). Hence, in a study, mixed extracts of aniseed were examined for an effective antimicrobial activity.

Dermatophyte fungi are the one of the main causative agents of skin diseases for any human. Many of the plant diseases occur due to some species of the fungi *Aspergillus*. They contaminate various plants and their products during the different stages of the plant's growth. Species of *Aspergillus* have the ability to produce mycotoxin in foods (Yazdani *et al*, 2009). Thus, in another study, antimicrobial activity of anise extract was examined against dermatophytes and saprophyte fungi.

The antibacterial activities of the aqueous, methanol, acetone and petroleum ether extracts of *Pimpinella anisum* fruits were tested against 4 pathogenic bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Escherchia coli, and Klebsiella pneumoniae*) by disc diffusion method (Akhtar *et al*, 2008), and the findings suggest that only aqueous and methanol extracts exhibited fair antibacterial activity against all of the test bacteria. The aqueous extract was found to be more effective than the methanolic extract. Acetone and petroleum ether extracts were incapable of inhibiting the growth of the organisms that were tested (Shojaii and Fard, 2012).

Antimicrobial effects of water and ethanolic extracts of aniseed were studied by Gulcin *et al* against 10 bacterial species, and *Candida albicans. The* disc diffusion method was used. The ethanolic extract showed positive results against all the bacteria that were tested. However, it did not show any positive result on *Candida albicans*. The aqueoous extract did not show any positive result against the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. However, it showed some positive results on *Candida albicans* (Gulcin *et al*, 2003). The alcoholic extracts of aniseeds showed antibacterial activity against *Micrococcus luteus* and *Mycobacterium smegmatis* (Ates and Erdogrul, 2003).

In another study by Al-Bayati, synergic antibacterial activity of essential oil and methanol extracts of *Thymus vulgaris* and *Pimpinella anisum* was tested against 9 pathogenic bacteria. Positive results were shown by most of the pathogens that were tested. This was done by measuring the zone of inhibition. The largest zone of inhibition was observed against *Staphylococcus aureus*, *Bacillus cereus*, and Proteus *vulgaris*. A combination of essential oil and methanol extracts of these plants showed a better effect against most of the tested bacteria namely *Pseudomonas aeruginosa* (Al-Bayati, 2008).

The antibacterial potential of aqueous extracts of a few spices, including aniseed was tested against 176 bacterial isolates. This was done through disc diffusion technique by Chaudhry and Tariq. The maximum antibacterial activities of the aqueous extract of aniseed were exhibited against *Micrococcus roseus* (Chaudhry and Tariq, 2006).

In a study by Kosalec *et al*, antifungal activities of aqueous extract and essential oil of aniseed was tested on seven species of yeasts and four species of dermatophytes. The process included agar diffusion method. It was seen that the aqueous extract of aniseed showed positive results against *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis*, *C. krusei*, *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis*, and *M. gypseum*. The essential oil of anise also showed strong antifungal activity against yeasts and dermatophytes. In comparison, the essential oil of aniseed exhibited a stronger antifungal activity against yeasts and dermatophytes as opposed to the aqueous extract (Kosalec *et al*, 2005).

Positive results for antifungal activity of essential oil from aniseed were also seen against *Aternaria alternata*, *Aspergillus niger* and *Aspergillus parasiticus* (Özcan and Chalchat, 2006). Methanolic extracts of aniseed was tested for its antifungal activity against four dermatophyte species and one saprophyte fungus. The extract gave positive results against the dermatophyte species only (Yazdani *et al*, 2009)

Many of the food-borne pathogens are becoming increasing resistant against antibiotics. Patients who regularly take antibiotics are at an increased risk for acquiring multidrug resistant pathogens that may be food borne or air bourn (Angulo *et al*, 2004). For this reason, alternative forms of antimicrobial agents are searched for within a spectrum of natural produces.

1.5 Phytochemical Properties of Aniseed

Medicinal and healing properties of herbs are related to their chemical compositions. These can be categorized into alkaloids, acids, essential oils, steroids, saponins and tannins. These compounds have different solubility in different solvents. Many plant extract contain highly volatile substances that can be isolated by physical methods (Al-Daihan *et al*, 2013). Aniseed contains 1.5–6.0 mass % of a volatile oil consisting primarily of *trans*-anethole and also as much as 8–11 mass % of lipids rich in fatty acids, such as palmitic and oleic acids, as well as approximately 4 mass % of carbohydrates, and 18 mass % of protein (Shojaii and Fard, 2012). Aniseed extracts contains a mixture of various compounds including sesqquterpens, phenolic compounds and alkenes. These compounds are responsible for the different antimicrobial and other such properties of aniseed (Albulushi *et al*, 2014).

1.6 Extraction from Aniseed

Extraction of aniseed is done in different ways for the purpose of research. For water extraction, 25g of the powdered sample was mixed with 500ml boiling water. A magnetic stirrer was used to agitate the mixture for 15 minutes. Later, the extract was filtered through the Whatman No. 1 filter paper (Gulcin *et al*, 2003). The filtrate was used for further research. In another study, the aqueous extract was prepared by adding 100 gm of dried powder sample to 1000 ml of boiled distilled water. This was left at room temperature for an hour. The suspension was agitated by alternatively stirring. This was then filtered through a filter paper. The extract was left to dry at 45ºC temperature. The dried extract was refrigerated at 4ºC until further use (Mahmood *et al*, 2010). Fifty grams of the powdered sample was soaked in 200 ml of distilled water for 2 days. This was agitated at a regular interval to get the aqueous extract. The same was done in methanol, acetone and petroleum ether to get the alcoholic, acetone and petroleum ether extract respectively. At the end of the 2 days time period, it was filtered. At first it was passed through a muslin cloth and then through a filter paper. The filtrate was collected and dried under room temperature. The extract was then stored at 40ºC until needed (Akhtar *et al*, 2008)

1.7 Effects of Aniseed on Selected Bacteria

The bacteria selected to observe antibacterial effect of aniseed extracts are: *Escherichia coli*, *Salmonella typhii*, *Staphylococcus aureus*, *Shigella flexinera*, *Streptococcus pneumonae*, *Klebsiella sp.*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. The brief overview follows (Tortora *et al*, 2010; Crump, *et al*, 2011; Replogle *et al*, 2000; Klugman, 1990; Bouza and Cercenado, 2002; Barbe *et al*, 2009; Entrez Genome Project; Tam, *et al*, 2006).

1.7.1 ***Escherichia coli*** are gram negative normal floral bacteria that are commonly found in the human intestinal tract. It can cause food poisoning and urinary tract infections. Some strains of *E.coli* produce enterotoxins that cause traveler's diarrhea and other foodborne disease.

1.7.2 ***Salmonella typhi*** are gram-negative rod shaped bacteria. They are normally found in the intestinal tracts of humans and animals. *Salmonella typhi* causes a serious illness which is called typhoid fever. Antibiotics used on typhoid patients include ampicillin, trimethoprim-sulfamethoxazole, or chloramphenicol. Due to the overuse of such antibiotics, the species have started to develop drug resistance over the past few years.

1.7.3 ***Staphylococcus aureus*** is a gram positive bacterium that causes food poisoning. It is usually found in grapelike clusters and it forms yellow pigmented colonies *(aureus* means golden). *S. aureus* can quickly develop resistance against antibiotics such as penicillin, methicillin, amoxicillin and oxacillin. Such strains are called methicillin-resistant *Staphylococcus aureus* (MRSA).

1.7.4 ***Shigella flexinera*** causes a disease that is called shigellosis that gives way to abdominal cramps and fever. It is found only in humans. Studies show that the microbe is developing resistance against the common antimicrobial as ampicillin, chloramphenicol, streptomycin, trimethoprim-sulphamethoxazol and tetracycline.

1.7.5 ***Streptococcus pneumonae*** is a gram-positive bacterium that causes pneumococcal pneumonia. It is also the leading cause of bacterial meningitis. It is found in the nasopharyngeal region. Almost everyone is a healthy carrier of this microbe. Pneumococcal meningitis occurs mostly among children between the ages of 1 month and 4 years. *S. pneumococcus is* getting resistant to antibiotics like penicillin, erythromycin, trimethoprim-sulfamethoxazole, and tetracycline.

1.7.6 ***Klebsiella*** **spp.** Members of the genus *Klebsiella* are commonly found in soil or water. In the human body, they are usually found in the respiratory tract. They are resistant to penicillins and can acquire resistance to third- and fourth-generation cephalosporin.

1.7.7 ***Bacillus cereus*** is a gram-positive, endospore-forming bacterium that is commonly found in soil and plants. It is generally considered harmless, but it may cause some forms of foodborne illness such as food poisoning due to the release of some toxins. Heating does not always kill the endospores that can later germinate on cool foods.

1.7.8 ***Pseudomonas aeruginosa*** is a gram-negative bacterium with flagellum that helps in its motility. It produces a soluble, blue-green pigment. It can cause urinary tract infections. It can also infect burns and wounds, and can cause blood infections (sepsis).

1.7.9 ***Bacillus subtilis*** is a gram-positive bacterium. It is naturally found in soil and plants and within the gastrointestinal tract of humans. It has the ability to survive under stressful conditions by forming stress-resistant endospores.

1.8 Selected Phytochemical Properties of Aniseed

The phytochemical tests that were conducted were to determine the presence of saponins, tannins, terpenoids, flavonoids, cardiac glycoside, alkaloids, phenolics and steroids.

1.8.1 Tannins: Tannins are found in the root, bark, stem and outer layers of plant tissue. They have a high molecular weight and are soluble in water and alcohol. Tannins have a characteristic feature to tan, that is, they can convert things into leather.

1.8.2 Saponins: Saponin is a word that was derived from *Saponaria vaccaria*, which is a plant that has an abundant amount of saponins and was once used as soap. Saponins therefore possess 'soaplike' behaviour in water, which is they produce foam.

1.8.3 Terpenoids: Terpenenoids are chemically diverse groups of natural products. They are flammable, unsaturated hydrocarbons. They exist in liquid form and are commonly found in essential oils, resins or oleoresins.

1.8.4 Flavonoids: Flavonoids are a type of polyphenols that are found in plants. They are used as antioxidants or free radical scavengers. They are derived another compound called flavans. Over four thousand flavonoids are known to exist.

1.8.5 Cardiac glycoside: The cardiac glycosides are steroids. When administered through injection, they have a very specific and powerful impact on the cardiac muscle.

1.8.6 Alkaloids: These are secondary metabolites which comprises mostly of ammonia compounds. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue.

1.8.7 Phenolic compounds: Phenolics are chemical components that are found everywhere as natural colour pigments, which are responsible for the colour of many fruits. Phenolics in plants are usually made from phenylalanine through the action of an enzyme called phenylalanine ammonia lyase (PAL). One of the most important roles of phenolics in the plant is in its defense against pathogens and herbivore predators. Thus, they also help in controlling human pathogenic infections via plants.

1.8.8 Steroids: Plant steroids are one of the most naturally occurring plant phytoconstituents. They have therapeutic applications as arrow poisons or cardiac drugs. Small doses of these may be enough to exhibit the required amount of stimulation on a diseased heart. Overdose may cause even death (Doughari, 2012).

1.9 Rationale of the Research

Currently there is an absence of clinical trials of aniseed, which require regulated experimental environment involving considerable resource. *In vitro* studies are required to establish the proof of evidence of the antimicrobial properties of aniseed. This current study will contribute to existing evidence of *in vitro* analysis of antimicrobial properties of aniseed. Further *in vivo* clinical trials in limited resource settings may be warranted to confirm such tests.

1.10 Objectives of the Study

Increasing number of bacteria is getting resistant to different synthetic antibiotics. Which is a grave problem in combating infective health conditions, more so when the infection is associated with malnutrition and poor health as found in developing countries like Bangladesh. Looking at the antimicrobial properties of *Pimpinella anisum*, and its phytochemical contents can help in the search of newer antibiotics extracted from locally available natural produce.

It is expected that the antimicrobial properties and the phytochemical assay of extracts of aniseed as found in this study will stimulate researchers to search further in-depth and/or design clinical trials to come up with less expensive antimicrobial agents to benefit mankind.

Therefore, the specific objectives considered for this study were:

- Isolation of ethanol, methanol and aqueous extracts of aniseed (*Pimpinella anisum)*
- Observation of the antimicrobial activity of the extracts on nine selected microbes
- Determination of selected phytochemical/biochemical assay

The aim of the present study was to investigate the antimicrobial activity of ethanolic, methanolic, and aqueous extracts of aniseed against selected nine bacterial strains. Also some phytochemical assays were conducted on the extracts of the seed to check the presence of secondary metabolites.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

This was a laboratory based exploratory descriptive study where selected antimicrobial activities of ethanolic, methanolic and aqueous extracts of aniseed were done. Selected phytochemical assay of the aqueous extracts was also done. The steps followed are as follows-

2.1 Collection and Processing

The aniseeds were bought from the local store and sundried for about 3 to 4 days. These were then processed through a grinder to turn the seeds into powder form.

2.2 Extraction

The seeds were extracted in ethanol, methanol and water.

2.2.1 Ethanol: With the help of an electric weighing machine, 20 gm of aniseed powder was taken. The powder was mixed in a beaker with 100 ml of ethanol and stirred for about 30 minutes. Then the beaker was covered with foil paper and left standing in a dark chamber for 2 days. The same procedure was followed for another two set of beakers as well and kept for 5 and 7 days respectively.

After 2, 5 or 7 days, the contents of the beakers were filtered, through Whatman No.1 filter paper, and then concentrated in a water-bath at about 80°C temperature. This was done till the contents of the beaker became about 20 ml. This was then poured into a petri-dish and put back in the water-bath. Once oily substances were visible, the dish was taken out of the water-bath and the extracts were scooped out into a vial that was previously washed with ethanol. This was then labeled for identification.

2.2.2 Methanol: With the help of an electric weighing machine, 100 gm of aniseed powder was taken. The powder was mixed in a beaker with 500 ml of methanol and stirred for about 30 minutes. Then the beaker was covered with foil paper and left standing in a dark chamber for 2 days. The same procedure was followed for another two set of beakers as well and kept for 5 and 7 days respectively.

After 2, 5 or 7 days, the contents of the beakers were filtered, through Whatman No.1 filter paper, and then concentrated in a water-bath at about 65°C temperature. This was done till the contents of the beaker became about 20 ml. This was then poured into a petri-dish and put back in the water-bath. Once oily substances were visible, the dish was taken out of the water-bath and the extracts were scooped out into a vial that was previously washed with ethanol. This was then labeled for identification.

2.2.3 Water: Into 500 ml of boiled distilled water, 50 gm of aniseed powder was added. This was incubated for an hour with occasional stirring and then filtered through a muslin cloth. After that, it was put in a water-bath at about 97°C temperature till the content of the beaker was about 20 ml. Then this was poured into a petri-dish and put back in the water-bath. Once the oily substances were visible, the dish was taken out of the water-bath and the extracts scooped out into a vial that was previously washed with ethanol. This was then labeled for identification.

All the three types of extracts (ethanol, methanol, and aqueous) collected on 2, 5 and 7 days were subjected to the same laboratory procedures.

2.3 Preparation of Nutrient Agar (NA) Plates

Selected bacterial culture was needed to observe the antimicrobial properties of aniseed. For the purpose several plates of culture media were prepared.

Preparation of nutrient agar media was done by adding 28 gm of nutrient agar powder in 1000 ml of distilled water. Keeping this proportion constant, the amount of nutrient agar required was prepared when required. For each medium sized plate 20 ml of agar is needed.

The required amount of agar was prepared in a conical flask, and put onto a bunsen burner and stirred with a glass rod until the boiling point was reached. At this point, visible small bubbles formed at the bottom of the conical flask which rose up and the solution gradually turned clear. The flask was then removed from the heat and let to cool down for some time. Then the mouth of the flask was covered with aluminum foil and autoclaved. In the laminar air flow chamber, the autoclaved nutrient agar solution was quickly but cautiously poured into previously labeled petri-dishes – about 20 ml per medium sized plates or 30 ml per large sized plates. This was then kept

in the refrigerator to solidify. The petri-dishes were labeled with the name of the agar, the name or initials of the person who made the agar, and the date when it was made.

2.4 Preparation of 0.5% McFarland Standard Solution

McFarland standard solution was used to visually compare the turbidity of bacterial suspension with its adjusted standard turbidity. This was done so that the number of bacteria in the suspension can be within a given range for standardizing the microbial tests. By using McFarland solutions of different concentration, the number of bacteria in a given suspension could be altered as well.

To make the McFarland standard solution, 0.05 ml of 1.175% barium chloride dehydrate ($BaCl_2.H_2O$) was mixed with 9.95 ml of 1% sulphuric acid (H_2SO_4).

To make 1 ml of 1.175% barium chloride dehydrate, 0.01175 gm of barium chloride dehydrate is dissolved in 1 ml of distilled water. The laboratory has 97% of sulphuric acid. The formula used to make 1% sulphuric acid (H_2SO_4) from the 97% sulphuric acid is given below:

$$\text{Concentration 1} \times \text{Volume 1} = \text{Concentration 2} \times \text{Volume 2}$$

Where, concentration 1 is 97% of sulphuric acid and volume 1 is unknown volume of the same acid, concentration 2 is 1% sulphuric acid and volume 2 is 9.95 ml of the corresponding acid solution. According to the formula, 0.10258 ml of 97% sulphuric acid is taken and distilled water is added to this till the volume reaches 9.95 ml. Then 0.05 ml barium chloride dehydrate is added to the sulfuric acid to make the standard solution.

2.5 Preparation of Saline Solution

To make 0.9% saline solution, 0.9 gm of sodium chloride (NaCl) was taken into 100 ml of distilled water. About 10 ml of the saline solution were put in each test tube. Several such test tubes were prepared and autoclaved, with the screw cap opened through 1.5 turns. When taken out of the autoclave machine, the screw caps were turned fully to close the mouth of the tube so that the saline does not get contaminated. These were used later, when required.

2.6 Sub-culturing of Microbes

The stock cultures of the nine microorganisms were taken. To subculture, these were streaked on to the NA plates inside a laminar air flow chamber. For each organism, the plates were taken inside the chamber and then a loop was burned till red hot over a bunsen burner flame. After cooling the loop, a loopful of microbes were taken from the stock culture and streaked onto a properly labeled NA plate. This was then incubated at 37°C temperature for 24 hours before use.

2.7 Antimicrobial Tests

For conducting antimicrobial tests, agar (or well) diffusion method was used. The microbes from the 24 hours incubated subculture were taken and a bacterial suspension made. After burning the loop till red hot, a loopful of bacteria was taken and suspended into the saline solution in the test tubes. This was then vortexed for homogenous mixing. Then the tube was visually compared to the McFarland standard solution by holding both of the tubes against a dark background. The turbidity of the suspension was adjusted to match that of the McFarland solution. When the suspension was less turbid more bacteria were added and when it was more turbid more saline solution was added.

An autoclaved cotton bud was then taken and dipped into the bacterial suspension. This was done to do lawn culture on properly labeled NA agar plates. This gives a uniform growth of bacteria. After making the lawn culture, holes were made on the media with the back of an autoclaved micropipette tip. The holes were marked and accordingly the samples of aniseed extracts were poured into the holes with the help of separate autoclaved micropipettes, taking care that a positive control in the form of an appropriate antimicrobial disc is included in each plate. This was then incubated at 37°C temperature for 24 hours, at the end of which the presence of a clear zone around the hole indicated a positive result for antimicrobial tests. This process was followed separately for the ethanol, methanol, and aqueous extracts for each of the nine microorganisms.

The activity index (AI) values of the different extracts of aniseed were calculated for the microbes against which positive results were seen. The following formula was used to calculate the AI value:

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

The zone of inhibition was measured in millimeter (mm) with the help of a scale. The width of the clear zone around the antibiotic disc and the well was measured thrice and the average of the three value was noted down.

2.8 Phytochemical Tests

Eight different types of biochemical assays were done. These were for tannins, saponins, terpenoids, flavonoids, cardiac glycosides, alkaloids, phenolic compounds and steroids.

Around 10 gm of the powdered sample was taken in a beaker along with 100 ml of distilled water. This was boiled for about 10 minutes. The solution was filtered while still hot. Then the filtrate was let to cool down. This was then used to conduct further tests.

2.8.1 Test for tannins: Five to six drops of 10% of ferric chloride is added to 1 ml of the filtrate that was previously diluted with 5ml of distilled water. When there is a formation of bluish-black or brownish-green precipitate, it indicates positive results for the presence of tannins.

2.8.2 Tests for saponins: To 2.5ml of filtrate 10ml with distilled water was added to dilute it, and shaken vigorously for 2 minutes. When frothing is observed, it indicates presence of saponins in the filterate.

2.8.3 Tests for terpenoids: To 5ml of extracts 2ml of chloroform was mixed. Then 3 ml of concentrated sulfuric acid was added to form a layer. If reddish brown precipitates are observed at the interface between the two layers, it implies a positive result for the presence of terpenoids.

2.8.4 Tests for flavonoids: To 1 ml of extract, a few drops of 20% sodium hydroxide solution were added. A change of color to yellow indicates a positive result. To reconfirm the test, acid was added and the solution turned back to its original color.

2.8.5 Tests for cardiac glycoside: To 5ml of extracts 2ml of glacial acid (that contained 1 drop of ferric chloride solution) was added. Then 1 ml of sulphuric acid was added slowly down the side of the test tube. The presence of a brown ring at the interface indicates the deoxysugar characteristics of cardiac glycoside. There may also be a presence of violet ring below the ring while in the acetic acid layer; a greenish ring may be formed.

2.8.6 Tests for alkaloids: 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 a positive result.

2.8.7 Tests for phenolic compounds: To 5 ml of extract, 5% of ferric chloride was added. If the solution turns to dark green color, it indicates a positive result.

2.8.8 Tests for steroids: To 2 ml of extract, 2 ml of chloroform and 2 ml of sulphuric acid was added slowly down the side of the wall of the test tube. Red color produced in the lower chloroform layer indicates a positive result.

These methods were followed to check on the antimicrobial and phytochemical properties of aniseed. The results of both these tests are discussed in the following chapter.

CHAPTER THREE

RESULTS

RESULTS

3.1 Results of Antibacterial Assay

For the test of antimicrobial properties of ethanolic, methanolic and aqueous extract of aniseed, nine different microbes were used. Amongst these nine microbes, the ethanolic, methanolic and aqueous extract of aniseed showed remarkable positive results against only three. The rest either gave negative or nondescript results.

Different combinations of extracts were put on petridishes to compare the results. Three replicates were made for better accuracy. Antimicrobial discs were used as positive controls. The antibacterials used were ampicilin (for *S. typhii* and *S. aureus*), gentamycin (for *E.* coli and *P. auriginosa*), tetracycline (for *B. cereus*), kanamycin (for *B.subtilis* and *Klebsiella* spp.), chloramphenicol (for *S. pneumonae*) and nitrofurantoin (for *S. flexneri*). The three microbes against which the ethanolic, methanolic and aqueous extract of aniseed showed positive results were *B. cereus*, *B.subtilis* and *S. pneumonae*. The zone of inhibition of chloramphenicol against *S. pneumonae* was the greatest, while that of kanamycin was the least.

3.1.1 Methanol Extraction:

Antibacterial effect of 2^{nd}, 5^{th} and 7^{th} day of methanol extract of aniseed is shown in table 3.1. It is observed that the zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for the 5^{th} day extracts in all three replicates. The methanol extract from the 5^{th} day showed the highest positive result against *B. cereus*.

Table 3.1: Positive antimicrobial effects (average zone of inhibition) produced by methanol extract of aniseed, and that in positive controls

Day of Extraction	Zone of inhibition (mm)					
	Bacillus cereus		***Bacillus subtilis***		***Streptococcus pneumonae***	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
2nd day	19.60	**19.03**	19.30	**18.77**	11.33	**12.07**
	20.20		18.20		13.67	
	17.30		18.80		11.20	
5th day	20.50	**20.13**	18.00	**19.27**	14.67	**14.5**
	20.00		20.40		14.33	
	19.90		19.40		15.60	
7th day	19.60	**18.43**	15.40	**15.63**	14.50	**14.87**
	17.30		16.90		16.00	
	18.40		14.60		14.20	
Positive controls	28.17	**28.61**	24.00	**23.44**	31.00	**31.67**
	29.67		23.33		32.67	
	28.00		23.00		31.33	

The lowest positive result was shown against *S. pneumonae* from the 2nd day extract. The results found for different days of methanol extract of aniseed, compared to the positive controls are seen in figure 3.1.

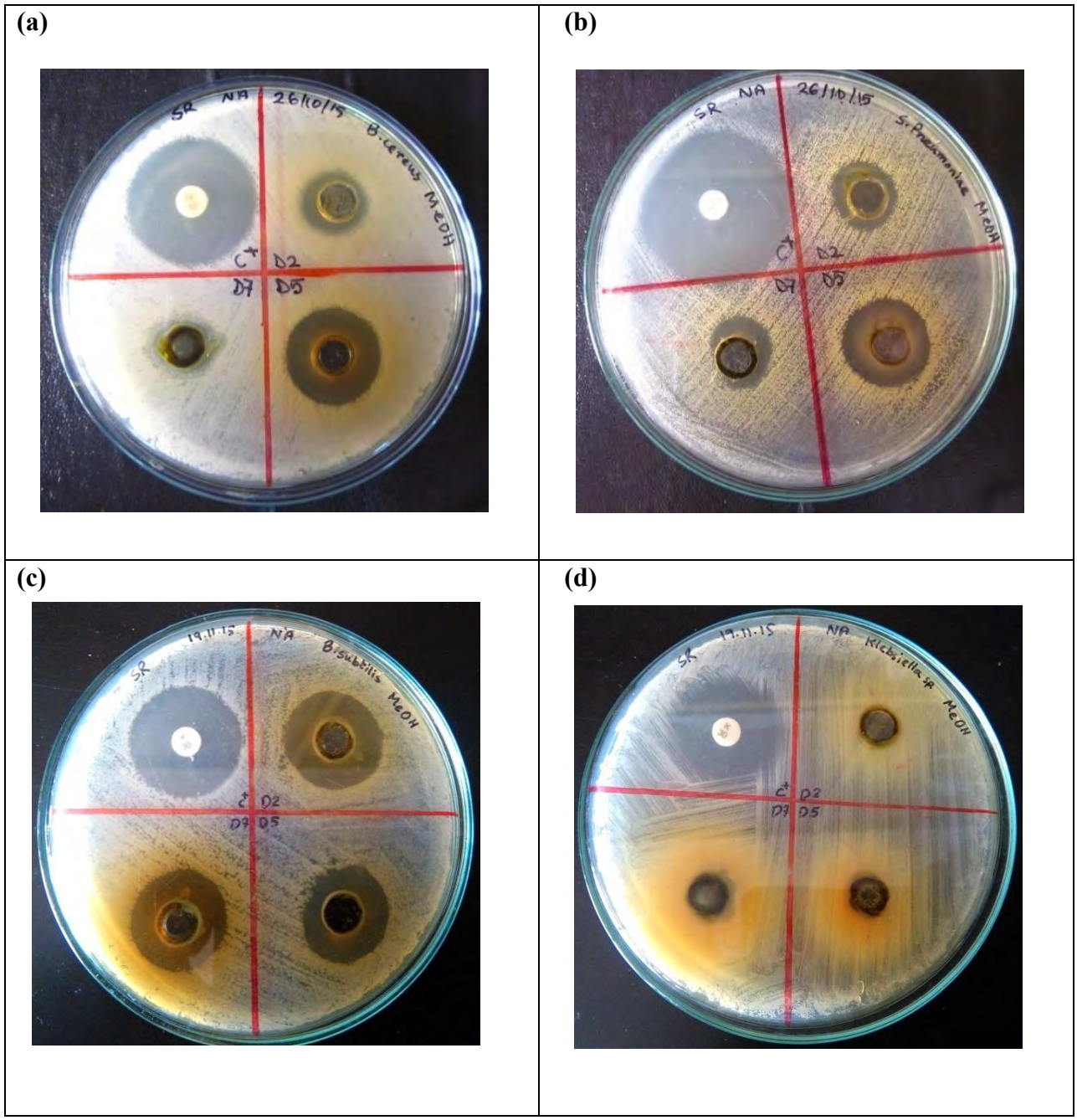


Figure 3.1: The antibacterial effect of methanol extract of aniseed of 2nd, 5th and 7th day of extraction along with the positive control against: **(a)** *B. cereus*, **(b)** *S. pneumonia*, **(c)** *B. subtilis* and **(d)** *Klebsiella*

3.1.2 Ethanol Extraction: In table 3.2, antibacterial effect of 2nd, 5th and 7th day of ethanol extract of aniseed is shown. It is seen that the zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for the 7th day extracts in all three replicates.

Table 3.2: Positive antimicrobial effects (average zone of inhibition) produced by ethanol extract of aniseed, and that in positive controls

Day of Extraction	Zone of inhibition (mm)					
	Bacillus cereus		***Bacillus subtilis***		***Streptococcus pneumonae***	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
2nd day	15.20	**14.90**	13.50	**14.01**		
	15.00		13.70		12.50	
	14.50		14.83		12.67	
5th day	13.80	**14.52**	13.00	**12.19**	12.17	**13.00**
	15.60		12.40		13.33	
	14.17		11.17		13.50	
7th day	14.50	**14.78**	14.50	**16.08**	13.33	**14.44**
	14.00		16.90		15.67	
	15.83		16.83		14.33	
Positive controls	29.60	**28.20**	22.90	**23.80**	32.00	**31.39**
	27.00		23.00		32.67	
	28.00		25.5		29.50	

The ethanol extract from the 7th day showed the highest positive result against *B. subtilis*. The lowest positive result was shown by the 2nd day extracts against *S. pneumonae*. The results found for different days of ethanol extract of aniseed, compared to the positive controls are observed in figure 3.2.

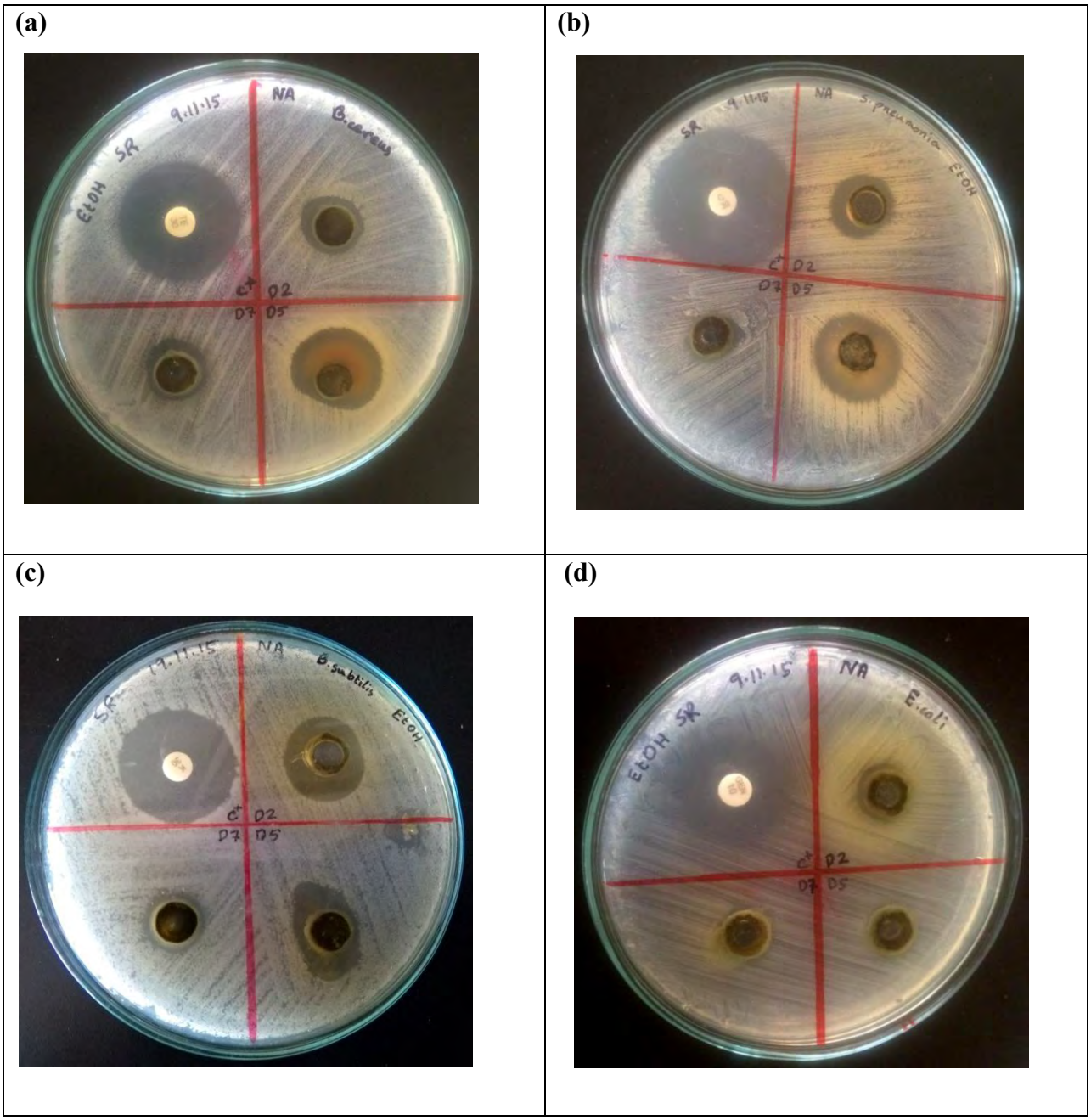


Figure 3.2: The antibacterial effect of ethanol extract of aniseed of 2nd, 5th and 7th day of extraction along with the positive control against **(a)** *B. cereus*, **(b)** *S. pneumonia*, **(c)** *B. subtilis* and **(d)** *E. coli* (negative result)

3.1.3 Aqueous Extraction: The results found for aqueous extract of aniseed, compared to the positive controls are shown in table 3.3. The aqueous extract of aniseed had no antibacterial effect on *S. pneumonae*. Against *B. cereus* and *B. subtilis*, it had very little effect.

Table 3.3: Positive antimicrobial effects (average zone of inhibition) produced by aqueous extract of aniseed, and that in positive controls

Extraction	Zone of inhibition (mm)					
	Bacillus cereus		***Bacillus subtilis***		***Streptococcus pneumonae***	
	Per trial	**Average**	**Per trial**	**Average**	**Per trial**	**Average**
Aqueous	10.83	**11.05**	10	**10.83**		
	11.83		11.67		**-**	
	10.50		-		**-**	
Positive controls	30.17	**29.56**	23.83	**24.83**	29	**29**
	30		25.83		29.67	
	28.5		24.83		28.33	

3.1.4 Comparison of Different Extraction:

Antibacterial effect of 2nd day of extracts of ethanol and methanol along with the aqueous extract of aniseed is shown in figure 3.3. It is observed in figure 3.6 that the average zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for methanol extract. The methanol extract from the 2nd day showed the highest positive result against *B. cereus*. The aqueous extract of aniseed showed unremarkable result against *S. pneumonae*.

Antibacterial effect of 5th day extracts of ethanol and methanol along with the aqueous extract of aniseed is shown in figure 3.4. It is observed in figure 3.7 that the average zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for methanol extract. The methanol extract from the 5th day showed the highest positive result against *B. cereus*. The aqueous extract of aniseed did not show mentionable result against *S. pneumonae*

Antibacterial effect of 7th day extracts of ethanol and methanol along with the aqueous extract of aniseed is shown in figure 3.5. It is observed in figure 3.8 that the average zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* –was maximum for methanol extract. The methanol extract from the 7th day showed the highest positive result against *B. cereus*. The aqueous extract of aniseed did not show mentionable result against *S. pneumonae*.

Considering figures, 3.6, 3.7 and 3.8, it is observed that the average zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for methanol extract from the 5th day which showed the highest positive result against *B. cereus*. The average zone of inhibition for *B. subtilis* was also maximum for methanol extract from the 5th day. The average zone of inhibition for *S. pneumonae* was maximum for methanol extract from the 7th day.

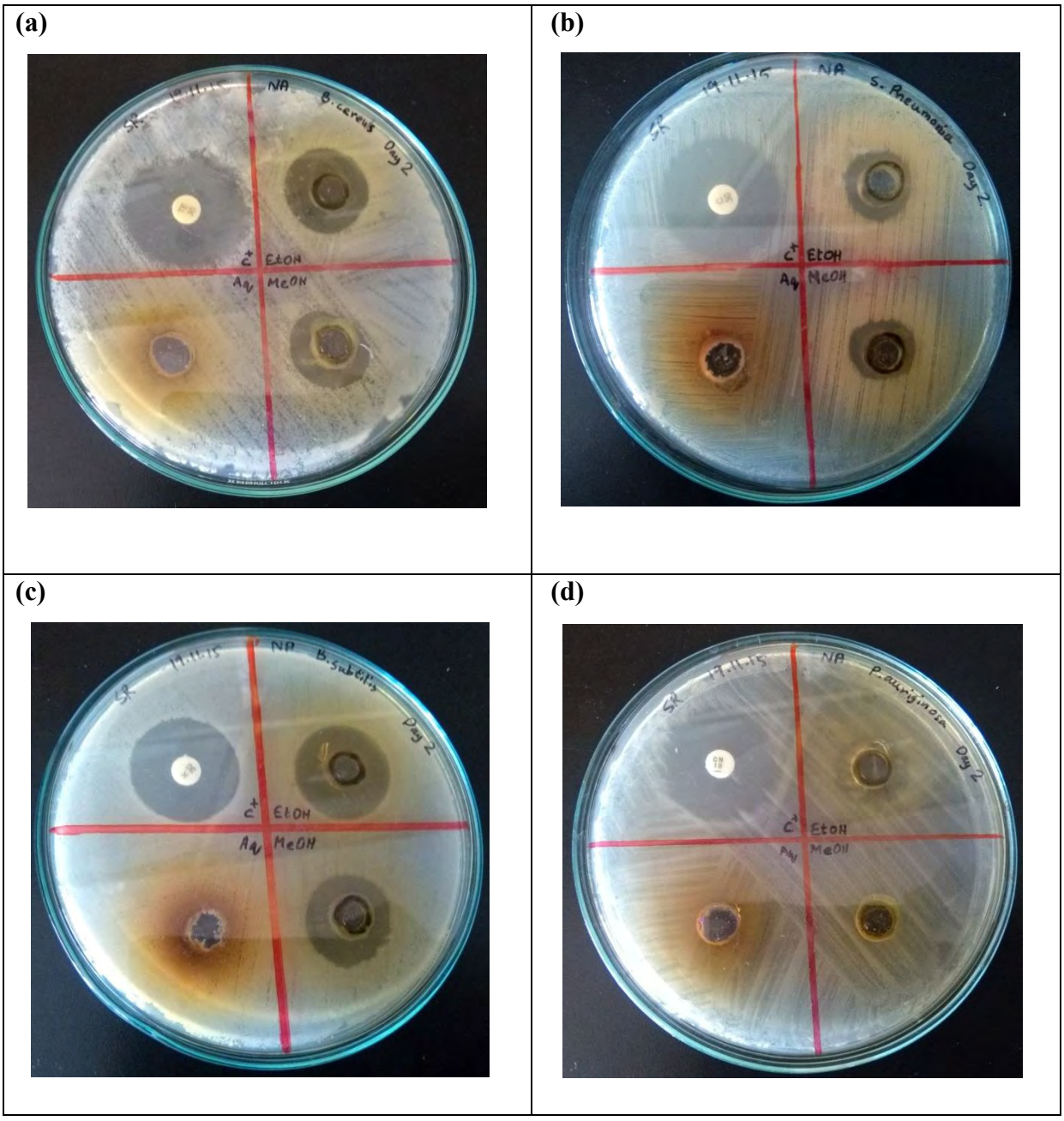


Figure 3.3: The antibacterial effect by 2nd day of ethanol and methanol extraction of aniseed compared to the aqueous extract of aniseed and the positive control against **(a)** *B. cereus*, **(b)** *S. pneumonae*, **(c)** *B. subtilis* and **(d)** *P. auriginosa* (negative result)

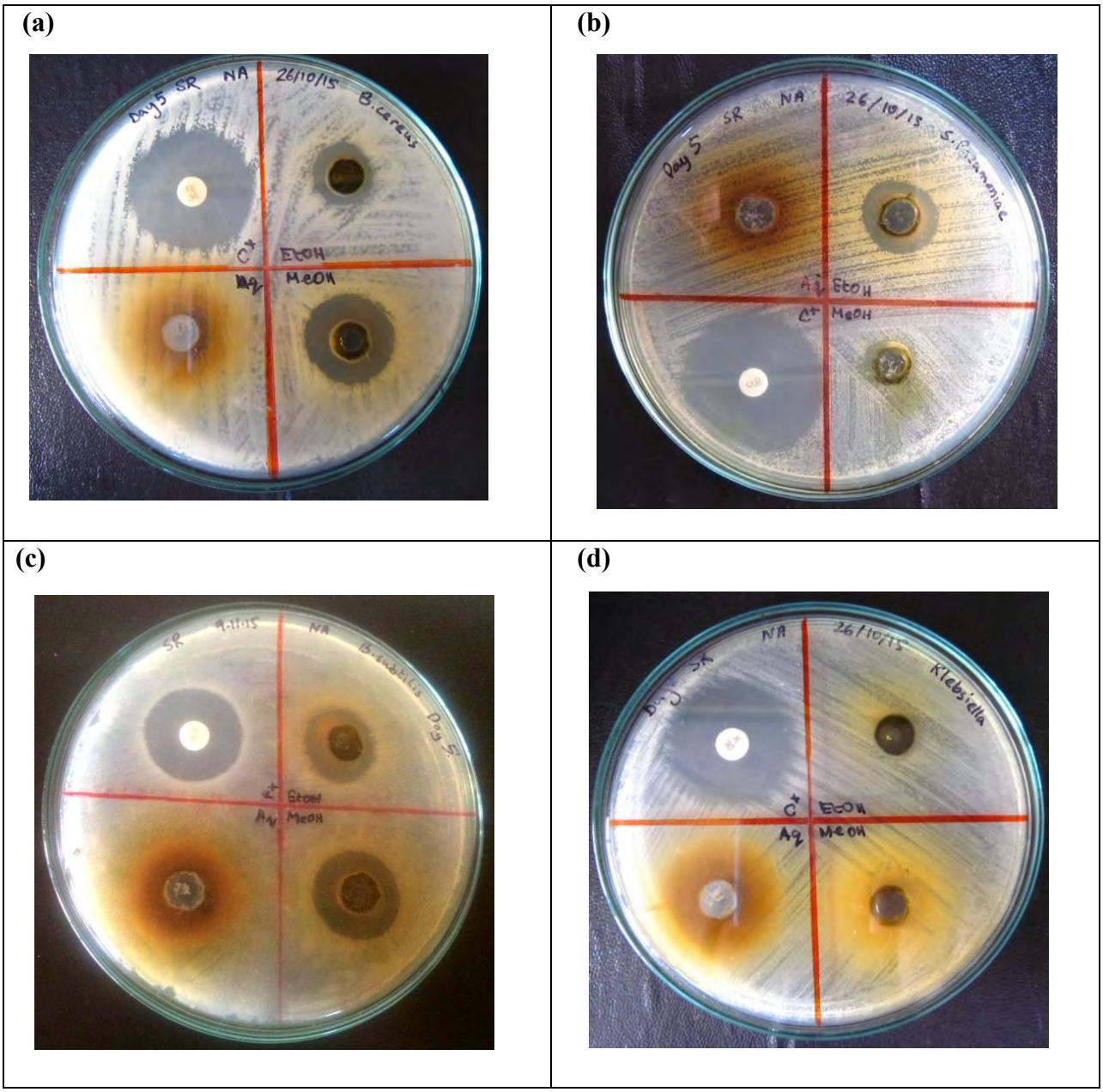


Figure 3.4: The antibacterial effect by 5th day of ethanol and methanol extraction of aniseed compared to the aqueous extract of aniseed and the positive control against **(a)** *B. cereus*, **(b)** *S. pneumonae*, **(c)** *B. subtilis* and **(d)** *Klebsiella* spp. (negative result)

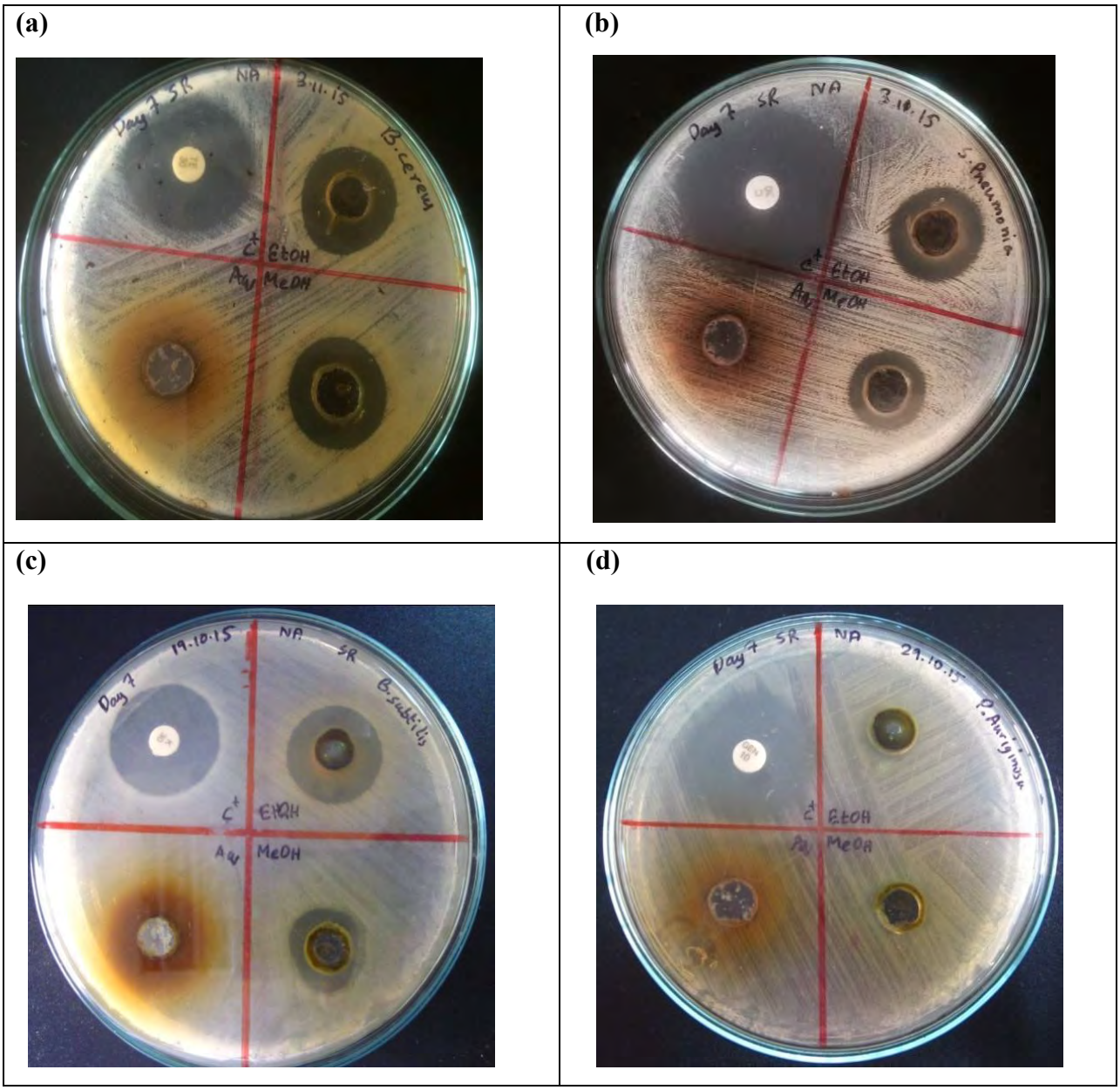


Figure 3.5: The antibacterial effect by 7th day of ethanol and methanol extraction of aniseed compared to the aqueous extract of aniseed and the positive control against **(a)** *B. cereus* **(b)** *S. pneumonae*, **(c)** *B. subtilis* and **(d)** *P. auriginosa* (negative result)

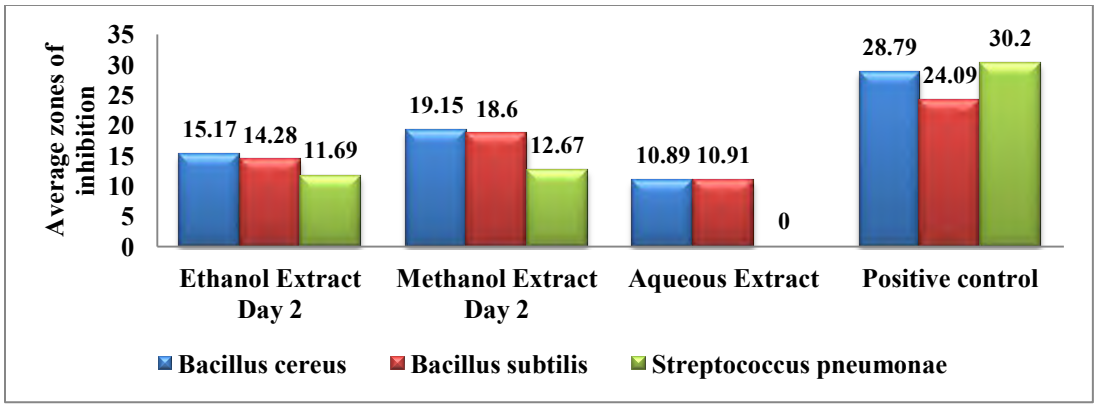


Figure 3.6: Average zones of inhibition produced by ethanol, methanol from 2nd day of extraction, along with the aqueous extracts of aniseed and the positive controls

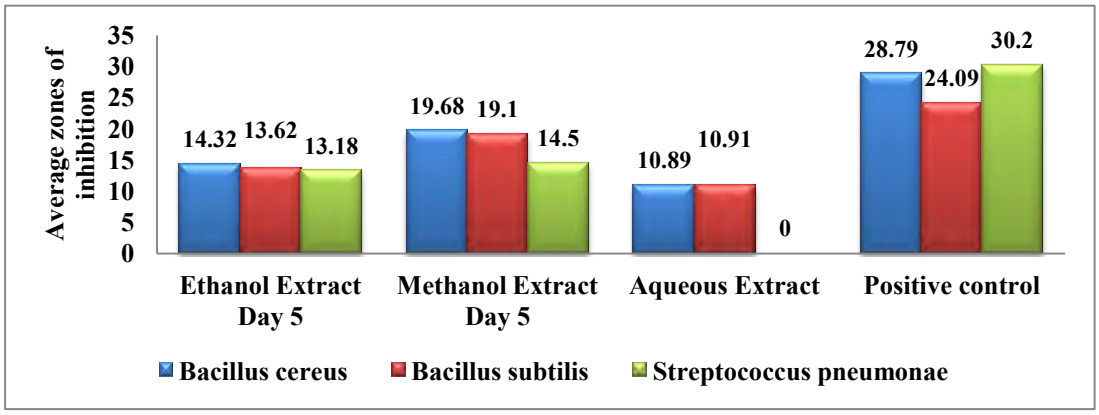


Figure 3.7: Average zones of inhibition produced by ethanol, methanol from 5th day of extraction, along with the aqueous extracts of aniseed and the positive controls

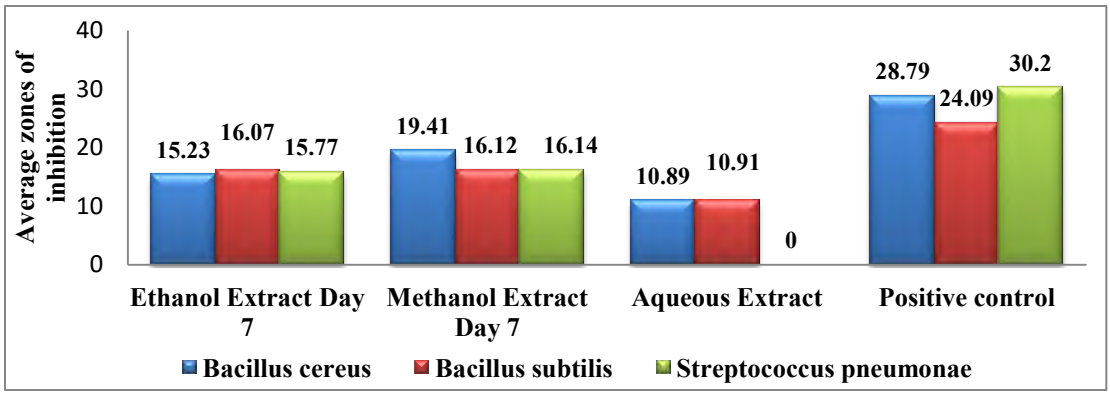


Figure 3.8: Average zones of inhibition produced by ethanol, methanol from 7th day of extraction, along with the aqueous extracts of aniseed and the positive controls

3.1.5 Comparison of 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

The activity index value is a measure of the antimicrobial activity which is quantitatively compared to the respective standard antibiotics. The activity index (AI) values for the three microbes were maximum in methanol extract from the 5th day for *B. cereus*. The AI value for *B. subtilis* was also maximum for methanol extract from the 5th day. The activity index value for *S. pneumonae* was maximum for methanol extract from the 7th day. The aqueous extract of aniseed showed no value for activity index against *S. pneumonae*, as a consequence of the previous inconsequential antibacterial result against *S. pneumonae.* This is shown in Figure 3.9.

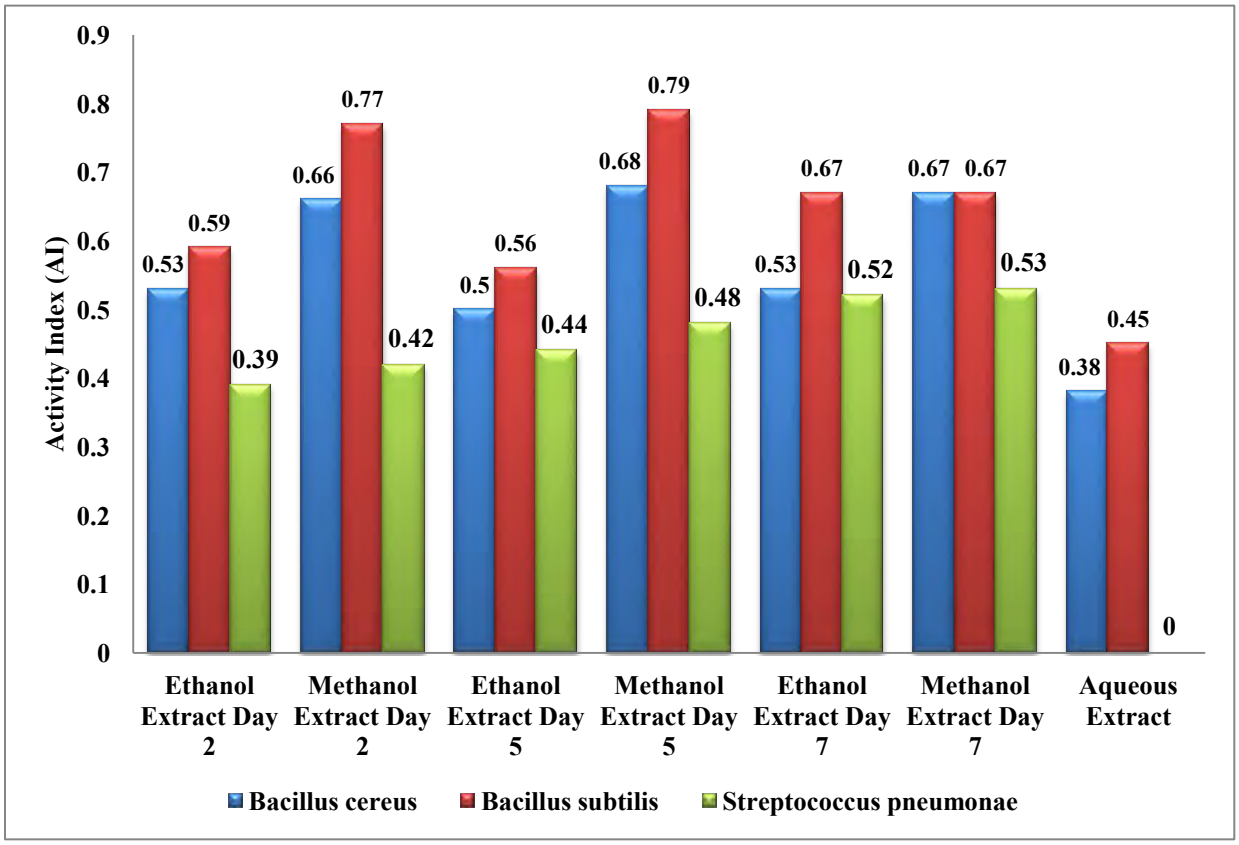
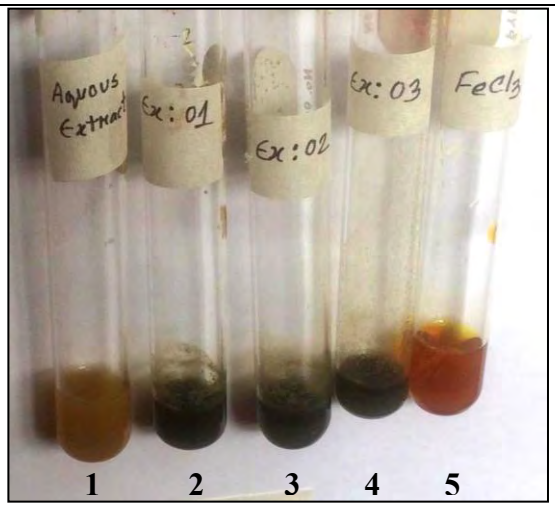
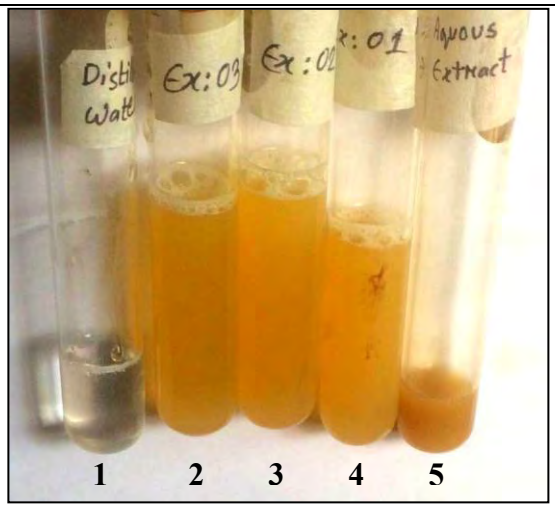


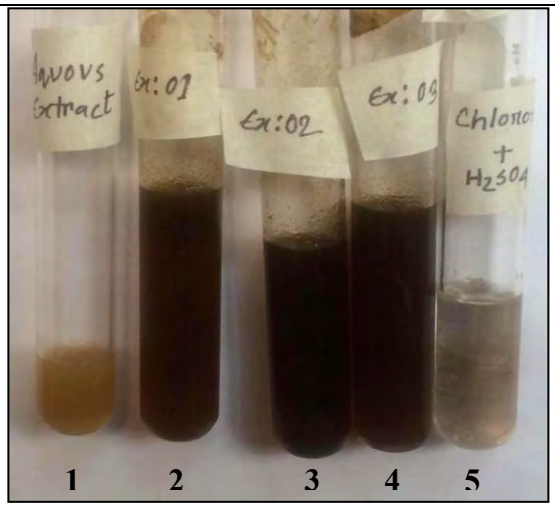
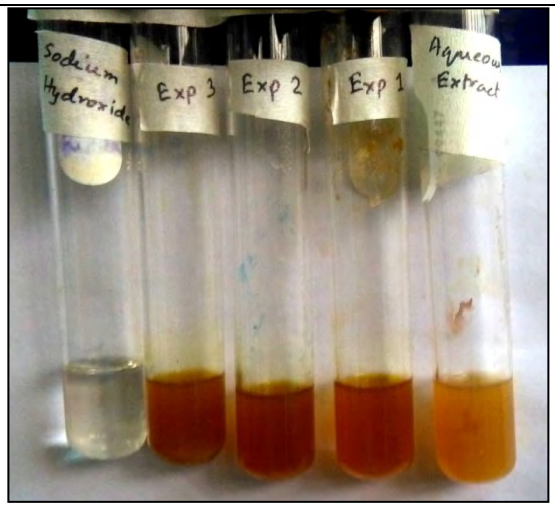
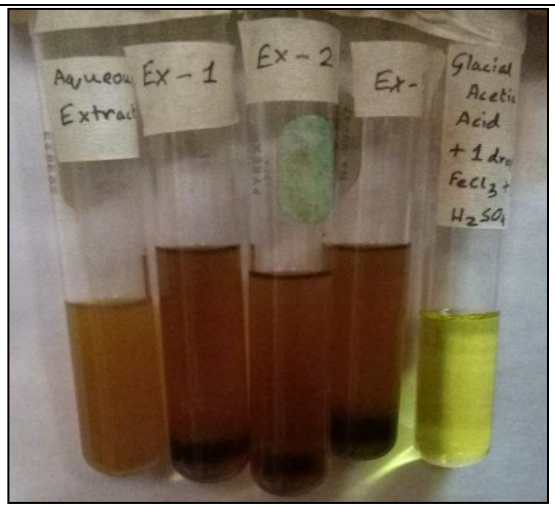
Figure 3.9: Activity Index of the ethanol, methanol and aqueous extracts of aniseed that showed positive antibacterial effects

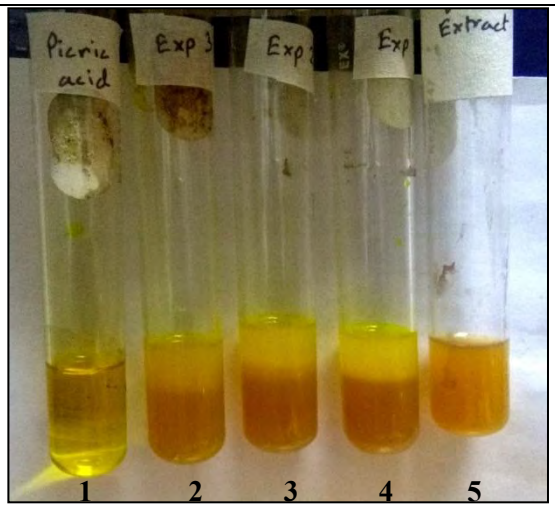
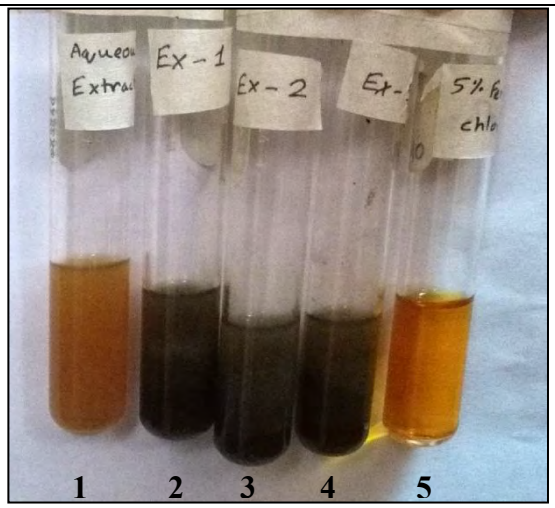
3.2 Results of Phytochemical Screening

The results obtained for the phytochemical assay is shown below.

Table 3.4: Phytochemical assay of aniseed

Name of tested chemical	Figure	Description of result
Tannins		Tube 1 contains the aqueous extract. The tubes 2, 3 and 4 contain the aqueous extract along with few drops of 10% ferric chloride, showing the bluish-black colour. Tube 5 contains 10% ferric chloride.
Saponins		Tube 1 contains distilled water. The tubes, 2, 3 and 4 contain the aqueous extract along with 10 ml of distilled water, showing the frothing. Tube 5 contains the aqueous extract

Terpenoids		Tube 1 contains aqueous extract. The tubes, 2, 3 and 4 contain the aqueous extract along with chloroform and sulphuric acid, showing the reddish-brown colour. Tube 5 contains a mixture of only chloroform and sulphuric acid
Flavonoids		Tube 1 contains 20% sodium hydroxide solution. The tubes, 2, 3 and 4 in the centre contain the aqueous extract along with the sodium hydroxide solution, showing the reddish yellow colour. Tube 5 contains aqueous extract of aniseed.
Cardiac Glycoside		Tube 1 contains the aqueous extract of aniseed. The tubes, 2, 3 and 4 contain the aqueous extract along with 2 ml glacial acid, a drop of ferric chloride and 1 ml of concentrated sulphuric acid, showing a brown ring on the interface. Tube 5 contains 2 ml glacial acid, a drop of ferric chloride and 1 ml of concentrated sulphuric acid.

Alkaloids		Tube 1 contains picric acid. The tubes, 2, 3 and 4 contain the aqueous extract along with picric acid, showing the turbid effect on the top layer of the mixture. Tube 5 contains aqueous extract of aniseed.
Phenolics		Tube 1 contains aqueous extract of aniseed. The tubes, 2, 3 and 4 contain the aqueous extract along with 5% ferric chloride, showing dark green colour. Tube 5 contains 5% ferric chloride.
Steroids		Tube 1 contains chloroform along with sulphuric acid. The tubes, 2, 3 and 4 contain the aqueous extract mixed with 2 ml chloroform along with 2 ml sulphuric acid, showing red colour. Tube 5 contains aqueous extract of aniseed.

CHAPTER FOUR

DISCUSSION

DISCUSSION

For years now, antibiotic resistance is a vital challenge for the world. It affects not only the pharmaceutical industry, but also the food and beverage industries. As such, it has become one of the main concerns of treating the ever increasing variety of infective diseases in human. Many bacteria are getting resistant to more than a single antibiotic. Such resistant pathogens are hard to treat (Gilchrist *et al*, 2007). These pathogens are also called multi-drug resistant bacteria. They may develop due to the unintentional misuse and/or overuse of antibiotics (McEachran *et al*, 2015). As a result, alternative sources of more natural antibiotics, in the form of medicinal plants, are being researched these days. Many medicinal plants possess natural antimicrobial compounds. The extracts of such plants have the potential to be used as new agents that are effective against many infections (Wendakoon *et al*, 2012). Such herbal medicines are used worldwide for the treatment of many infectious diseases. Medicinal plants are safer, more readily available, and cause much less side effects (Hoque *et al*, 2011).

Aniseed is one such plant that has many therapeutic properties. The antibacterial properties of aniseed are not yet fully understood, and so are still being researched. *Pimpinella anisum* is a spice that comes from a plant that falls under the Umbelliferae family. It is sometimes used in cooking due to its distinct anise-like flavor and smell. It helps in relieving gastrointestinal spasms and it has carminative properties (Shojaii and Fard, 2012). It is one of the many traditionally used medicines. Not much of the cultivation of aniseed is done in Bangladesh. The spice is usually imported from other countries. But, because of its many therapeutic properties, it can be a great asset for the country, if its cultivation increased.

A study at Maharashtra Animal and Fishery Sciences University revealed antimicrobial effects after 48 hours of mixing aniseed powder with 50% methanol, and with distilled water. The results showed that the aqueous extract was slightly more effective than the methanol extract against all bacteria that was tested using the disc diffusion method (Akhtar *et al*, 2008). In this study, the zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for the methanol extract from the 5th day of extraction. The aqueous extract of aniseed was not as effective as the ethanol or methanol extracts for any of the three microbes. Another study by Gulcin *et al* (2006) showed that the aqueous extract was made by mixing the

aniseed sample with boiling water for only 15 minutes. Both the ethanol and aqueous extracts showed strong antibacterial effect against *Staphylococcus aureus* (Gulcin *et al*, 2003). But, in this research, no antibacterial effect was found against *Staphylococcus aureus* for any of the three extracts that were tested. This differences in results may be due to any or all of the reasons, including the slight variation in the aniseed grown in the different soil type, the difference in environmental and/or experimental conditions, genotype, and the concentrations of the alcohols used, all of which may have an impact on the extract and its composition.

In another study, aniseed sample was mixed with boiled distilled water and left for an hour while alternatively stirring the mixture. The aqueous extract of aniseed had no effect on any of the tested microbes – *E. coli*, *S. typhii* and *Candida albicans* (Mahmood *et al*, 2010). The same study by Gulcin *et al* (2003), also found that the aqueous extract of aniseed showed no antibacterial effect against *P. auriginosa* and *E. coli.* In the test of synergic antibacterial activity of methanol extracts of *Pimpinella anisum* against nine pathogenic bacteria, one of the largest zones of inhibition was observed against *Bacillus cereus* (Al-Bayati, 2008). These results were in coherence with this study, since no results were observed against either of the two microbes, *E. coli* and *S. typhii* for any of the three types of extracts.

Although the ethanol and methanol extracts of aniseed from different days of extraction showed good results against *Bacillus cereus*, *B. subtilis* and *S. pneumonae*, the aqueous extracts of aniseed was not as effective on any of these organisms. However, in the common households of Bangladesh, the people usually eat the aniseed in raw form or is used as a spice and cooked. Either way, it is not eaten in mixture with ethanol or methanol. But rather, it is usually eaten in mixture with water. The *in vitro* antimicrobial effect may reflect that aniseed extract may not be as effective against any or all of these organisms *in vivo*.

There have been other studies that showed positive antifungal activities of aqueous extract of aniseed against species of yeasts, dermatophytes and saprophytes. The essential oil of anise also showed strong antifungal activity against yeasts and dermatophytes (Kosalec *et al*, 2005; Özcan and Chalchat, 2006; Yazdani *et al*, 2009). Antifungal effects of aniseed extracts were not in the purview of this study, but may be undertaken by other researchers.

Another finding of the study is the activity index (AI) values of aniseed from different extraction methods. The AI values are used to find the potential of antimicrobial activity of an extract that is quantitatively compared to the respective standard antibiotics. High AI values imply that the extracts have a good activity against the bacteria in comparison with the standard antibiotics (Sridhar *et al*, 2014). In a study by Awan *et al* (2013), the significant use of chloroformic and isoamyl alcohol extracts of a few selected medicinal plants with standard antibiotics was calculated through activity index. The results from this research suggested that the growth of bacterial pathogens was inhibited by the crude extract of the medicinal plants. The results obtained through activity index were consistent with another study by Shekhawat and Vijayvergia (2010). The activity index value of *Justicia neesii* was calculated by Sridhar *et al* (2014). It was observed that the plant extract showed higher AI values against Gram negative bacteria implying that the extracts have good activity against the Gram negative bacteria compared to the standard.

From this study, it was found that *B. subtilis* has the highest AI value and the overall AI value of *B. subtilis* is higher than that of *B. cereus*. The AI values of *S. pneumonae* were lower than the AI value of the other two microbes. However, it was also found that on an average, the zone of inhibition against *B. cereus* was much more than that against *B. subtilis*. This is due to the fact that the zone of inhibition of tetracyclin against *B. cereus* was greater than that of kanamycin against *B. subtilis*. Hence, in comparison with the antibiotics, the effect of the aniseed extracts against *B. cereus* was lower than that against *B. subtilis*.

Considering the overall findings of antimicrobial effects of aniseed extracts in this study, it can be stated that further intensive empirical in-vitro investigation leading to clinical trial may be undertaken.

Medicinal values of many spices lie in the presence of chemical substances that have a definite physiological action on the human body. Different phytochemicals have been found to possess different activities. For example, alkaloids protect against chronic diseases and saponins protect against hypercholesterolemia and also possess antibiotic properties. In a study conducted at Andhra University, various extracts from some spices, including aniseed were screened for the presence of alkaloids, flavonoids, steroids, saponins, tannins and triterpenoid (Harsha *et al*,

2013). In a different study by Prof. Hayder M Alkuraishy (2012), phytochemical analysis of aniseed extracts showed the presence of tannin, saponins, terpenoids, phenolic agents, flavonoids and alkaloids. The results of this study are in conformity with these studies as it showed positive results for the presence of all of the phytochemical properties, saponins, tannins, terpenoids, flavonoids, cardiac glycoside, alkaloids and phenolics.

The purpose of this study was to collect the ethanol and methanol extracts from different days of extraction along with the aqueous extracts of aniseed and to investigate the presence of phytochemicals in *Pimpinella anisum*. Antibacterial tests showed that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *B. cereus*, *B. subtilis* and *S. pneumonae.* The comparison of the different extracts showed that methanol extracts from the 5^{th} day of extraction has the greatest antimicrobial effect against the *B. cereus*, *B. subtilis* and *S. pneumonae*. The phytochemical analysis of the aqueous extract revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, phenolic compounds and cardiac glycosides. However, the aqueous extract of aniseed did not contain any steroid. This shows that these phytochemicals could be responsible for the observed antimicrobial properties.

The results from this article imply that *Pimpinella anisum* extracts have a few antibacterial properties. The different method and day of extraction had a considerable antibacterial effect on a few of the tested organisms. This may be due to the presence of some of its chemical components. The activity of these components may depend on their solubility in different solvents, giving rise to the different results for ethanol, methanol and aqueous extracts. Substituting the commercial antibiotics with extracts of *Pimpinella anisum* or its combination with extracts from other spices with similar antibacterial properties would have potential benefits on human.

From the study, it can be concluded that the traditional use of aniseed for infectious diseases is promising against many bacteria and disease causing pathogens. It is expected that the findings of this study may help or stimulate other researchers of Bangladesh to design clinical trials to come up with a less expensive antimicrobial agent that may further benefit people from developing countries like Bangladesh.

REFERENCES

Akhtar, A., Deshmukh, A. A., Bhonsle, A. V., Kshirsagar, P. M. and Kolekar, M. A. (2008). *In vitro* antibacterial activity of *Pimpinella anisum* fruit extracts against some pathogenic bacteria. *Veterinary World*, *1*(9), 272-274.

Al-Bayati, F. A. (2008). Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. *Journal of Ethnopharmacol*, *116*(3), 403-6.

Albulushi, S. M. A., Saidi, H. A., Amaresh, N. and Mullaicharam, A.R. (2014). Study of physicochemical properties, antibacterial and GC-MS analysis of essential oil of the aniseed (Pimpinella anisum Linn.) in Oman. *Research and Reviews: Journal of Pharmacognosy and Phytochemistry, 2*(4), 24-33.

Al-Daihan, S., Al-Faham, M., Al-Shawi, N., Almayman, R., Brnawi, A., Zargar, S. and Bhat, R. S. (2013). Antibacterial activity and phytochemical screening of some medicinal plants commonly used in Saudi Arabia against selected pathogenic microorganisms. *Journal of King Saud University - Science*, *25*(2), 115-120.

Alkuraishy, H.M. (2012). Evaluation the antibacterial activity of aniseed: *in vitro* study. *Webmed Central, 3*(6), 1-7.

Ates, D. A., Erdogrul, Ö. T. (2003). Antimicrobial activities of various medicinal and commercial plant extracts. *Turkish Journal of Biology*, *27*(2003), 157–162.

Awan, U. A., Andleeb S., Kiyani, A., Zafar, A., Shafique, I., Riaz, N., Azhar, M. T. and Uddin, H. (2013). Antibacterial screening of traditional herbal plants and standard antibiotics against some human bacterial pathogens. *Pakistan Journal of Pharmaceutical Sciences*, *26*(6), 1109-1116.

Barbe, V., Cruveiller, S., Kunst, F., Lenoble, P., Meurice, G., Sekowska, A., Vallenet, D., Wang, T., Moszer, I., Medigue, C. & Danchin, A. (2009). From a consortium sequence to a unified sequence: the Bacillus subtilis 168 reference genome a decade later. *Microbiology, 155*(Pt 6), 1758–1775.

Bouza, E., Cercenado, E. (2002). Klebsiella and enterobacter: antibiotic resistance and treatment implications. *Seminars in Respiratory Infections*, *17*(3), 215-30.

Chaudhry, N. M. and Tariq, P. (2006). Bactericidal activity of black pepper, bay leaf, aniseed and coriander against oral isolates. *Pakistan Journal of Pharmaceutical Sciences*, *19*(3), 214-8.

Crump, J. A., Medalla, F. M., Joyce, K. W., Krueger, A. L., Hoekstra, R. M., Whichard, J. M., Barzilay, E. J., Emerging Infections Program NARMS Working Group. (2011). Antimicrobial resistance among invasive nontyphoidal Salmonella enterica isolates in the United States: National Antimicrobial Resistance Monitoring System, 1996 to 2007. *Antimicrobial Agents and Chemotherapy*, *55*(3),1148-54.

Doughari, J. H. (2012). *Phytochemicals: Extraction methods, basic structures and mode of action as potential chemotherapeutic agents*.

Ernst, D. (1989). *Pimpinella anisum* L. (anise): cell culture, somatic embryogenesis, and the production of anise oil. In Y. P. S. Bajaj (Eds.). *Biotechnology in Agriculture and Forestry: Medicinal and Aromatic Plants II* (Volume 7, pp 381-397).

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.

Gulcin, I., Oktay, M., Kıreccı, E. & Kufrevıoglu, O. I. (2003). Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts. *Food Chemistry*, *83*(3), 371–382.

Harsha, N., Sridevi, V., Lakshmi, M.V.V. C., Rani, K. And Vani, N. D. S. (2013). Phytochemical analysis of some selected spices. *International Journal of Innovative Research in Science, Engineering and Technology, 2*(11), 6618-6621.

Hoque, M. M., Rattila, S., Shishir, M. A., Bari, M. L., Inatsu, Y. and Kawamoto, S. Antibacterial activity of ethanol extract of betel leaf (*Piper betle* L.) against some food borne pathogens. *Bangladesh Journal of Microbiology, 28*(2), 58-63.

Khan, R., Islam, B., Akram, M., Shakil, S., Ahmad, A., Ali, S.M., Siddiqui, M., & Khan, A. U. (2009). Antimicrobial activity of five herbal extracts against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. *Molecules*, *14*(2), 586-597.

Klugman, K. P. (1990). Pneumococcal resistance to antibiotics. *Clinical Microbiology Reviews*, *3*(2), 171-96.

Kosalec, I., Pepeljnjak, S. & Kustrak, D. (2005). Antifungal activity of fluid extract and essential oil from anise fruits (*Pimpinella anisum* L., *Apiaceae*). *Acta Pharm. 55*, 377–385.

Kreydiyyeh, S.I., Usta, J., Knio, K., Markossian, S. & Dagher, S. (2003). Aniseed oil increases glucose absorption and reduces urine output in the rat. *Life Sciences*, *74*(5), 663-673.

Mahmood, M. A., Al- Dhaher, Z. A., AL-Mizraqchi, A. S. (2010). Antimicrobial activity of aqueous extracts of pomegranate, sumac, sage, anise, hand bull tongue, thyme, cloves, lemon and mint against some food-borne pathogens. Iraqi Journal of Veterinary Medicine, *34*(2), 85-94.

McEachran, A. D., Blackwell, B. R., J. Delton Hanson, J.D., Wooten, K. J., Gregory D., Mayer, G. D., Cox, S. B., ,[2] and Smith, P. N.[1,] Antibiotics, bacteria, and antibiotic resistance genes: aerial transport from cattle feed yards via particulate matter. *Environmental Health Perspective, 123*(4).

Nimmakayala, S., Duggirala, S. L and Puchchakayala, G. (2014). Antimicrobial activity of ethanolic extracts of *Justicia neesii*. *Bangladesh Journal of Pharmacology*, *9*(4), 624-627.

Özcan, M. M. and Chalchat, J. C. (2006). Chemical composition and antifungal effect of anise (*Pimpinella anisum* L.) fruit oil at ripening stage. *Annals of Microbiology*, *56*(4), 353-358.

Replogle, M. L., Fleming, D. W. & Paul R. Cieslak. (2000). Emergence of antimicrobial-resistant shigellosis in Oregon. *Clinical Infectious Diseases 2000*, *30*(3), 515–9.

Shekhawat, N. and Vijayvergia, R. (2010). Evaluation of antimicrobial potential of some medicinal plants against plant and human pathogens. *Journal of Pharmacy Research, 3*(4), 700-702.

Shobha RI, Rajeshwari CU, Andallu B. (2013). Anti-peroxidative and anti-diabetic activities of aniseeds (*Pimpinella anisum* l.) and identification of bioactive compounds. *American Journal of Phytomedicine and Clinical Therapeutics, 1*(5), 516-527.

Shojaii, A. & Fard, M. A. (2012). Review of pharmacological properties and chemical constituents of *Pimpinella anisum*. *International Scholarly Research Notices Pharmaceutics*, *2012*, Article ID 510795, 8 pages.

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.

Tam, N. K. M., Uyen, N. Q., Hong, H. A., Duc, L. H., Hoa, T. T., Serra, C. R., Henriques, A. O., & Cutting, S. M. (2006). The intestinal life cycle of *Bacillus subtilis* and close relatives. *Journal of Bacteriology, 188(7),* 2692-2700.

Tortora, G. J., Funke, B. R. & Case, C. L. (2010). *Microbiology: An introduction*. Boston: Benjamin Cummings.

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.

Yazdani, D., Rezazadeh, S., Amin, G., Abidin M. A. Z., Shahnazi S. and Jamalifar H. (2009). Antifungal activity of dried extracts of anise (*Pimpinella anisum* L.) and star anise (Illicium verum Hook, f.) against dermatophyte and saprophyte fungi. *Journal of Medicinal Plants*, *8*(5), 24–29.

APPENDIX – I

Reagents

1. **10% Ferric chloride**

   1 g of Ferric chloride in 10 ml distilled water

2. **5% Ferric chloride**

   1 g of Ferric chloride in 20 ml distilled water

3. **20% sodium hydroxide solution**

   2 g of sodium hydroxide in 10 ml distilled water

4. **Glacial acid**

   Acetic acid

APPENDIX – II

Instruments

The important equipment used through the study are listed below:

Instrument	**Manufacturer**
Autoclave	SAARC
Freeze (-20ºC)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200 µl)	Eppendorf, Germany
Oven, Model :MH6548SR	LG, China
Refrigerator (4ºC) Model: 0636	Samsung
Safety Cabinet Class II Microbilogical	SAARC
Vortex Mixture	VWR International
Water Bath	Korea
Weighing Balance	ADAM EQUIPMENTTM, United Kingdom