Phytochemical Screening of ethanol and methanol extract of *Pimpinella anisum* and Comparison of Antibacterial Assays of prevalent organisms in Bangladesh

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

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Dedicated to
My beloved Parents and Brother

DECLARATION BY THE RESEARCHER

I do hereby declare that the thesis work entitled "**Phytochemical Screening of ethanol and methanol extract of *Pimpinella anisum* and Comparison of Antibacterial Assays of prevalent organisms in Bangladesh**" has been written and submitted by me, Faiza Noor-E-Rashid(ID-12136009) Department of Mathematics and Natural Sciences under the supervision of Ms. ZubaidaMarufee Islam, Lecturer,Department of Mathematics and Natural Sciences without the use of other sources than those mentioned. It is further asserted that this Bachelor's Thesis has never been submitted in the same or substantially similar version to any other examinations office.

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ABSTRACT

"There simply aren't enough new drugs in the pharmaceutical pipeline to keep pace with the evolution of drug-resistant bacteria, the so-called superbugs".

Medicinal plants synthesize a vast array of secondary metabolites that are important for human life. For medicinal purpose, antimicrobial activity of substances derived from plant extracts has been recognized for many years. The antimicrobial activity of the ethanol, methanol and aqueous extracts of the seeds of *Pimpinella anisum L.* (Apiaceae) was tested for their potential antimicrobial activities against *Salmonella typhii, Staphylococcus aureus, Shigella flexineri, Streptococcus pneumonae, Klebsiella sp., Bacillus cereus, Pseudomonas aeruginosa, Bacillus subtilis, Proteus vulgaris,* Enterotoxicogenic *E.coli* (ETEC)*, Klebsiella pneumonae* and *Streptococcus pyogenes.*

Extracts of different days had variable effects on the common pathogenic organisms chosen in context of Bangladesh. Positive antibacterial effect of aniseed was shownagainstfour types of bacteria, *Shigella flexineri, Bacillus Subtilis, Streptococcus Pneumoniae* and ETEC. Day 5 methanol extract showed the greatest activity against *Bacillus subtilis* and *Streptococcus pneumoniae*. Also the Day 5 methanol extract showed maximum activity against *Shigella flexineri, Bacillus subtilis* and Enterotoxicogenic *E.coli* (ETEC) indicating high sensitivity to the extract. However the ethanol extract had no effect on ETEC.

Phytochemicals such as tannin, saponin, phlobatanin, terpenoid, flavonoid and phenolic compounds were detected in both ethanol and methanol extracts as the extracts showed corresponding test result positive. Both the extracts yielded a negative result for the phytochemicals cardiac glycoside, phlobatannin and steroids. 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.

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CHAPTER ONE

INTRODUCTION

1.1 Descriptionof the Plant

Pimpinellaanisum is a flowering plant in the family Apiaceae, native to the India and southwest Asia. It is a herbaceous annual plant growing to 1m tall. The leaves at the base of the plant are simple, 2- 5 cm long and shallowly lobed, while leaves higher on the stems are feathery pinnate, divided into numerous leaflets. The flowers are white, 3 mm diameter, produced in dense umbels. The fruit is an oblong dry schizocarp, 3-5 mm long.

Anise (*PimpinellaanisumL.Apiaceae*) is an annual herb indigenous to Near East andwidely cultivated in the Mediterranean rim (Turkey, Egypt, Syria, Spain, *etc*.) and inMexico and Chile. It has been used as an aromatic herb and spice since Egyptian timesand antiquity and has been cultivated throughout Europe.

As a medicinal plant, *Pimpinellaanisum* has been used as a stimulating effect of digestion and antiparasitic, antifungal (Soliman and Badea, 2002) and antipyretic (Afifi*et.al*,1994). Additionally, the plant and especially its fruit essential oil have been used for treatment of some disease including seizures and epilepsy (Avicenna,1988;Abdul-Ghani*et.al.,*1987). Furthermore, it has been shown to have anticonvulsant effects and has been used for the treatment of constipation (Curtis*et.al,* 1996; Pourgholam*et.al.,* 1999; Chicouri and Chicouri, 2000) and possesses muscle relaxant effect (Albuquerque*et. al*,1995).Recently its oil has been reported to be used as antibiotic substitute in broiler ration (Mehmet *et.al*,2005). Aniseeds contain 1.5–5% essential oil. It is used in perfumery for its aromatic smell. It is used as a flavoring 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. It is used in the preparation of Ayurvedic medicine. Therefore, the present investigation was undertaken to evaluate antibacterial activity of *Pimpinellaanisum* dried fruits against some pathogenic bacteria mainly in context of the most

prevalent ones in Bangladesh and also to see the presence of certain phytochemicals in the ethanol and methanol extracts of the plant.

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 *Pimpinellaanisum L.*

Fig.1.1: Aniseed herb

Fig 1.2: The external morphology of aniseed

Fig 1.3: Dried Aniseed

1.3 Therapeutic Use of Aniseed

Aniseed enjoys considerable reputation as a medicine in coughs and pectoral affections. In hard, dry coughs where expectoration is difficult, it is of much value. It is greatly used in the form of lozenges and the seeds have also been used for smoking, to promote expectoration.

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 and flavonoids, which have been found to have antimicrobial properties in vitro (Khan *et al,* 2009). Aniseed-flavored 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).

The volatile oil, mixed with spirits of wine forms the liqueur Anisette, which has a beneficial action on the bronchial tubes, and for bronchitis and spasmodic asthma, Anisette, if administered in hot water, is an immediate palliative. For infantile catarrh, Aniseed tea is very helpful.

Because of the mentioned facts, searching for sources of new antimycotics is justified, with plants providing a promising source of new substances with antifungal activity.

1.4 Antibacterial Properties of Aniseed

As a medicinal plant, *Pimpinellaanisum* has been used as a stimulating effect of digestion and antiparasitic, antifungal (Soliman and Badea, 2002) and antipyretic (Afif *et.al,*1994). Additionally, the plant and especially its fruit essential oil have been used for treatment of some disease including seizures and epilepsy (Avicenna1988;Abdul-Ghani *et.al,*1987). Furthermore, it has been shown to have anticonvulsant effects and has been used for the treatment of constipation (Curtis*et.al.,* 1996; Pourgholam *et.al,* 1999; Chicouri and Chicouri, 2000) and possesses muscle relaxant effect (Albuquerque*et. al,*1995).Recently its oil has been reported to be used as antibiotic substitute in broiler ration (Mehmet *et.al,*2005). There are few reports (Singh *et.al.*2002; Tabanca *et.al,* 2003) on systematic studies pertaining to antibacterial evaluation of *Pimpinellaanisum*. Hence, considering its therapeutic potential, it was essential to prove it for its exact rational use as medicine by scientific means. Therefore, the present investigation was undertaken to evaluate antibacterial activity of *Pimpinellaanisum* dried fruits against some pathogenic bacteria.

The antibacterial activities of the aqueous, methanol, acetone and petroleum ether extracts of *Pimpinellaanisum* fruits were tested against 4 pathogenic bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Escherchia coli,* and*Klebsiellapneumoniae*) 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).

In another study by Al-Bayati, synergic antibacterial activity of essential oil and methanol extracts of *Thymus vulgaris* and *Pimpinellaanisum* 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).

Positive results for antifungal activity of essential oil from aniseed were also seen against *Aternariaalternata, Aspergillusniger*and*Aspergillusparasiticus* (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)

Due to the increasing of human consumption demand for more natural nutrition, theabuse of toxic synthetic food substances and the increasing of resistance of pathogenicmicroorganisms against antibiotics, natural isolated substances from plants areconsidered as promising natural sources of food preservatives. 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 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 sesquiterpenes

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 day-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, Shigellaflexineri, Streptococcus pneumonae, Klebsiella spp, Bacillus cereus, Pseudomonas aeruginosa, Bacillus subtilis, Proteus vulgaris,* Enterotoxicogenic*E.coli*(ETEC),*Klebsiella pneumonae*and *Streptococcus pyogenes*.

1.7.1Enterotoxicogenic *Escherichia coli*(ETEC) 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 ***Shigellaflexineri*** 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*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).

1.7.8 *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.

1.7.9 *Proteus vulgaris* is a rod-shaped, nitrate-reducing, indole+ and catalase-positive, hydrogen sulfide-producing, Gram-negative bacteriumthat inhabits the intestinal tracts of humans and animals. It can be found in soil, water, and fecal matter. It is grouped with the Enterobacteriaceae and is an opportunistic pathogen of humans. It is known to cause wound infections and other species of its genera are known to cause urinary tract infections.

1.7.10 *Klebsiellapneumoniae* is a Gram-negative, non motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar.

Although found in the normal flora of the mouth, skin, and intestines, it can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum. In the clinical setting, it is the most significant member of the Klebsiella genus of the Enterobacteriaceae. In recent years, Klebsiella species have become important pathogens in nosocomial infections.

It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. As a free-living diazotroph, its nitrogen-fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions.

1.7.11 ***Streptococcus pyogenes*** is a species of bacteria. Like most other streptococci, it is clinically important in human illness and are round bacteria. It is an infrequent, but usually pathogenic, part of the skin flora. It is the predominant species harboring the Lancefield group A antigen, and is often called group A streptococcus (GAS). Group Astreptococcal infection can cause illness, which typically produces small zones of beta-hemolysis, a complete destruction of red blood cells.

1.8 Selected Phytochemicals

The phytochemical tests that were conducted were to determine the presence of saponins, phlobatannins, tannins, terpenoids, flavonoids, cardiac glycosides, 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 Phenolic compounds: Phenolics are chemical components that are found everywhere as natural color 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.

1.8.7 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).

1.8.8. Phlobotannins: Four members of a novel class of natural 'phlobaphene' condensed tannins, representing the products of stereospecific ring-isomerization (Journal of the Chemical Society, 1972 – 1995). Phlobaphenes (are reddish, alcohol-soluble and water-insoluble phenolic substances. They can be extracted from plants, or be the result from treatment of tannin extracts with mineral acids.

1.8.9 Carotenoids: Also called tetra terpenoids, are organic pigments that are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms, including some bacteria and some fungi. They can be produced from fats and other basic organic metabolic building blocks by all these organisms. Carotenoids serve two key roles in plants and algae: they absorb light energy for use in photosynthesis, and they protect chlorophyll from photodamage. Carotenoids that contain un-substituted beta-ionone rings have vitamin activity (meaning that they can be converted to retinol), and these and other carotenoids can also act as antioxidants. In the eye, certain other carotenoids (lutein, astaxanthin and zeaxanthin) apparently act directly to absorb damaging blue and near-ultraviolet light, in order to protect the macula of the retina, the part of the eye with the sharpest vision.

1.8.10 Reducing compounds:A reducing agent (also called a reductant or reducer) is an element or compound that loses (or "donates") an electron to another chemical species in a redox chemical reaction. Since the reducing agent is losing electrons, it is said to have been oxidized.

1.11 Rationale of the Research

Currently there is an absence of clinical trials of aniseed, which require regulated experimental environment involving considerable resources. *In vitro* studies are required to establish the proof of evidence of the antimicrobial properties of various forms of aniseed extract. This current study will contribute to existing evidence of *in vitro* analysis of antimicrobial properties of aniseed against different pathogenic organisms specially ones that are prevalent in Bangladesh. Further in vivo clinical trials in limited resource settings may be warranted to confirm such tests.

1.12 Objectives of the Study

Antibiotics work by specifically interacting with a particular target molecule in the bacteria. For example, penicillin works by gumming up the assembly of a peptidoglycan that makes up the bacterial cell wall and the cells essentially burst due to osmotic shock when they can't form it properly.

As it turns out, over time bacteria, like most things, will experience minor mutations. Some of these mutations affect the structure of the molecular target of the antibiotic, rendering the bacterium resistant to the antibiotic effects. With time, the antibiotics become less effective. Resistance will eventually develop for all antibiotics whether that will be natural or synthetic; it is only a matter of time. "There simply aren't enough new drugs in the pharmaceutical pipeline to keep pace with the evolution of drug-resistant bacteria, the so-called 'superbugs,'" said Joseph R. Dalovisio, MD, president of the Infectious Diseases Society of America.

Mainly in a developing country like Bangladesh the infection is associated with malnutrition and poor health. This time the effect of infection can be delayed by prudent use of antibiotics, but it cannot be delayed forever. The selection pressure imposed by antibiotic use and the ubiquity of antibiotic resistance genes in the environment ensures that resistant strains of pathogens will eventually emerge.Looking at the antimicrobial properties of *Pimpinellaanisum* and its phytochemical contents can help in the search of newer antimicrobial 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 andor 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 and methanol extracts of aniseed (*Pimpinellaanisum*)
- Observation of the antimicrobial activity of the extracts on 12 selected pathogenic microbes mainly those prevalent in Bangladesh
- Determination of selected phytochemical/biochemical assay using the ethanol andmethanol extracts

CHAPTER 2

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 (*Pimpinella anisum*) 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. The powder was sieved through mesh to get the finest form of it.

2.2 Extraction

The aniseed powder was extracted in ethanol and methanol.

2.2.1 Ethanol extract: 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 using rotary evaporator. This was done till the contents of the beaker became approximately 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 extract: 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 approximately 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.

2.3 Preparation of Nutrient Agar (NA) Plates

Selected bacterial cultures were needed to observe the antimicrobial properties of aniseed extracts. 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 whenever required. For each medium sized petri dish plate 20 ml of nutrient agar media is needed. The required amount of nutrient agar media 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 left 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. These petri dishes with media were allowed to cool down to room temperature and were 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 McFarland Standard Solution (1.0 % v/v)

McFarland standard solutions were used to visually compare the turbidity of bacterial suspension with its adjusted standard turbidity. The supplied commercial standard solution was used to prepare different standard solutions. 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.

2.5 Preparation of Saline Solution

To make 0.9% (w/v) saline solution, 0.9 gm of sodium chloride (NaCl) was taken to make100 ml solution in 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 12 different species of bacteria were taken. To subculture, these were streaked on to the NA (nutrient agar) 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 loop full 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 loop full of bacteria was taken from the sub culture 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 cork borer. 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 both the ethanol and methanol 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 centimeter (cm) with the help of a scale. The width of the clear zone around the antibiotic disc and the well was measured and noted down.

2.8 Phytochemical Tests

Eight different types of biochemical assays were done. These were for tannins, saponins, terpenoids, flavonoids, cardiac glycosides, 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 filtrate.

2.8.3 Tests for terpenoids: To 5.0ml of extracts 2.0ml 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 1.0 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 5.0ml of extracts 2.0 ml of glacial acid (that contained 1 drop of ferric chloride solution) was added. Then 1.0 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 phenolic compounds: To 5.0 ml of extract, 5% of ferric chloride was added. If the solution turns to dark green color, it indicates a positive result.

2.8.7 Tests for steroids: To 2.0 ml of extract, 2.0 ml of chloroform and 2.0 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.

2.8.8 Test for phlobatannins: Seed extract was mixed with distill water in a test tube, then shaken well. Then to each extract, 1% aqueous hydrochloric acid was added and each extract sample was then boiled with the help of Hot plate stirrer. Formation of red colored precipitate confirmed a positive result for phlobatannins.

2.8.9 Test for reducing compounds: 1.0-2.0ml of water was added to 0.5ml of the extract the 0.5-1.0ml of Fehling solution were added. The mixture was then heated in a water bath. A brick red precipitate indicates the presence of reducing compound.

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 ethanolicandmethanolic extract of aniseed, nine different pathogenic microbes were used. Amongst these nine microbes, the ethanolicandmethanolic extract of aniseed showed remarkable positive results against only four. The rest either gave negative or nondescript results. Different combinations of extracts were put on petri dishes 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*)
- nitrofurantoin (for *S. flexneri*).

The four microbes against which the ethanolic and methanolic extract of aniseed showed positive results for were*Shigellaflexineri, Bacillus subtilis, Streptococcus pneumoniae*and Enterotoxicogenic *E.coli.*

3.1.1 Extract Results:

Antibacterial effect of 2nd, 5th and 7th day of both ethanol and methanol extract of aniseed is shown in the following table (table 1).

Table 1: Antimicrobial effects (average zone of inhibition in cm) produced by ethanol and methanol extract of aniseed, and that in positive controls

	Ethanol						**Methanol**					
	Day	Set1	Set2	Set3	Average	AI(e)	Day	Set1	Set2	Set3	Average	AI(m)
Shigellaflexineri	Control	2.4	2.5	2.4	2.43		Control	3	3	3	3	
	2	1.4	1.58	1.5	1.50	**0.62**	2	1.8	1.8	1.8	1.8	**0.3**
	5	0.8	1.0	1.0	0.93	**0.38**	5	1.5	1.4	1.5	1.47	**0.49**
	7	1.6	1.6	1.5	1.03	**0.42**	7	0.7	0.86	0.9	0.89	**0.27**
Bacillus subtilis	Control	2	2.1	1.9	2		Control	1.6	1.4	1.5	1.5	
	2	1.9	1.9	2	1.73	**0.87**	2	1.36	1.42	1.4	1.38	**0.92**
	5	1.8	1.9	1.85	1.85	**0.93**	5	1.6	1.6	1.6	1.6	**1.06**
	7	1.5	1.5	1.5	1.5	**0.75**	7	1.29	1.2	1.5	1.3	**0.88**
Streptococcus	Control	2.67	2.63	2.6	2.62		Control	2.5	2.0	2.7	2.4	

Pneumoniae	2	1.2	1.4	1.2	1.27	**0.48**	2	-	-	-	-	
	5	1.4	1.4	1.6	1.47	**0.6**	5	-	-	-	-	
	7	1.8	1.7	1.68	1.73	**0.66**	7	0.9	0.7	0.9	0.83	**0.34**
ETEC	Control	1.8	1.8	1.8	1.8		Control	1.7	1.8	1.8	1.76	
	2	-	-	-	-		2	-	-	-	-	
	5	-	-	-	-		5	0.46	0.38	0.45	0.43	**0.24**
	7	-	-	-	-		7	-	-	-	-	
Klebsiellaspp.	Control	1.4	1.5	1.5	1.46		Control	1.3	1.3	-	1.3	
	2	-	-	-			2	-	-	-		
	5	-	-	-			5	-	-	-		
	7	-	-	-			7	-	-	-		
Organism												
S. aureus	Control	3.4	3.4	3.1	3.3		Control	3.2	3.2	3.2	3.2	
	2	-	-	-			2	-	-	-		
	5	-	-	-			5	-	-	-		
	7	-	-	-			7	-	-	-		
K. pneumonea	Control	0.9	1.0	0.9	0.93		Control	1.0	0.8	0.8	0.86	
	2	-	-	-	-		2	-	-	-	-	
	5	-	-	-	-		5	-	-	-	-	
	7	-	-	-	-		7	-	-	-	-	
Salmonella typhi	Control	2.6	3	3	2.87		Control	2.9	2.9	2.9	2.9	
	2	-	-	-	-		2	-	-	-	-	
	5	-	-	-	-		5	-	-	-	-	
	7	-	-	-	-		7	-	-	-	-	

P. aeruginosa	Control	2.4	2.4	2.4	2.4		Control	2.4	2.4	2.4	2.4	
	2	-	-	-	-		2	-	-	-	-	
	5	-	-	-	-		5	-	-	-	-	
	7	-	-	-	-		7	-	-	-	-	
Streptococcus pyogenes	Control	0.9	0.9	1.1	0.96		control	0.85	0.9	1.0	0.92	
	2	-	-	-	-			-	-	-		
	5	-	-	-	-			-	-	-		
	7	-	-	-	-			-	-			

A positive result was shown only for *Shigellaflexineri, Bacillus subtilis, Streptococcus pneumonia* andETEC. None of the other organisms tested showed any clear zones, that is neither of the methanol or ethanol extract of aniseed had any effect on the organisms. Only the respective antibiotics showed a positive result in them.

Table 2: Positive antimicrobial effects (average zone of inhibition in cm) produced by ethanol extract of aniseed, and that in positive controls

Day of Extraction	Zone of Inhibition (cm)							
	ShigellaFlexineri		*Bacillus Subtilis*		*Streptococcus Pneumoniae*		*ETEC*	
	Per Trial	Average	Per Trial	Average	Per Trial	Average	Per Trial	Average
2nd Day	1.4	1.50	1.9	1.73	1.2	1.27	-	
	1.58		1.9		1.4		-	
	1.5		2.0		1.2		-	
5th Day	0.8	0.93	1.8	1.85	1.4	1.47	-	
	1.0		1.9		1.44		-	
	1.0		1.85		1.58		-	
7th Day	1.6	1.03	1.5	1.5	1.8	1.73	-	
	1.6		1.5		1.7		-	
	1.5		1.5		1.68		-	
Positive Control	2.4	2.43	2	2.0	2.67	2.62	1.8	1.8
	2.5		2.1		2.63		1.8	
	2.4		1.9		2.6		1.8	

3.1.2. Result of Ethanol extract: It is observed that the antibacterial effect of the ethanol extract of 2nd, 5th and 7th is observed as the zone of inhibition for the three microbes – *Bacillus subtilis,Shigellaflexineri* and*Streptococcuspneumoniae.* The maximum zone of inhibition was seen for the day 5 extract on*Bacillussubtilis* colonies, day 2 on *Shigellaflexineri* colonies and day 7 extract on *Streptococcus pneumonia* colonies. This result was constant in all 3 replicates of the experiment.The lowest average zone of inhibition was shown against *Shigellaflexineri* colonies using the day 5 extract.

(a) **(b)**

(c) **(d)**

Fig 3.1 : The antibacterial effect of ethanol extract of aniseed of 2nd, 5th and 7th day of extraction along with the positive control against (a) *Shigella flexineri (b) Bacillus subtilis (c) S. pneumonia* and *(d) E. coli (negative result)*

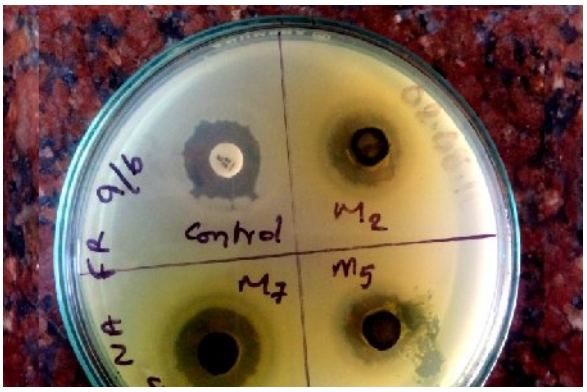
Table 3: Positive antimicrobial effects (average zone of inhibition in cm) produced by methanol extract of aniseed, and that in positive controls

Day of Extraction	Zone of Inhibition in cm							
	ShigellaFlexineri		*Bacillus Subtilis*		*Streptococcus Pneumoniae*		*ETEC*	
	Per Trial	Average	Per Trial	Average	Per Trial	Average	Per Trial	Average

2nd Day	1.8	1.8	1.36	1.38				
	1.8		1.42					
	1.8		1.4					
5th Day	1.5	1.47	1.6	1.6			0.46	0.43
	1.4		1.6				0.38	
	1.5		1.6				0.45	
7th Day	0.7	0.89	1.29	1.33	0.9	0.83		
	0.86		1.2		0.7			
	0.9		1.5		0.9			
Positive Control	3	3.0	1.6	1.5	2.5	2.4	1.7	1.76
	3		1.4		2.0		1.8	
	3		1.5		2.7		1.8	

3.1.3. Result of Methanol Extract: It is observed that the antibacterial effect of the methanol extract of 2nd, 5th and 7th is observed as the zone of inhibition for the two microbes – *Bacillus subtilis*and*Shigellaflexineri*. The largest clear zone shown constantly over the three replicates was shown by the 2nd day methanol extract on *Shigellaflexineri* and day by day 5 extract in bacillus subtilis. The day 7 and 5 methanol extracts showed a positive effect on *Streptococcus pneumonia* and ETEC respectively only and constantly over all the three replicates of it as well.Thesmallest average zone of inhibition was shown day 7 extract on*Shigellaflexineri*and by day 5 extract on ETEC.

(a) **(b)**



(c) **(d)**

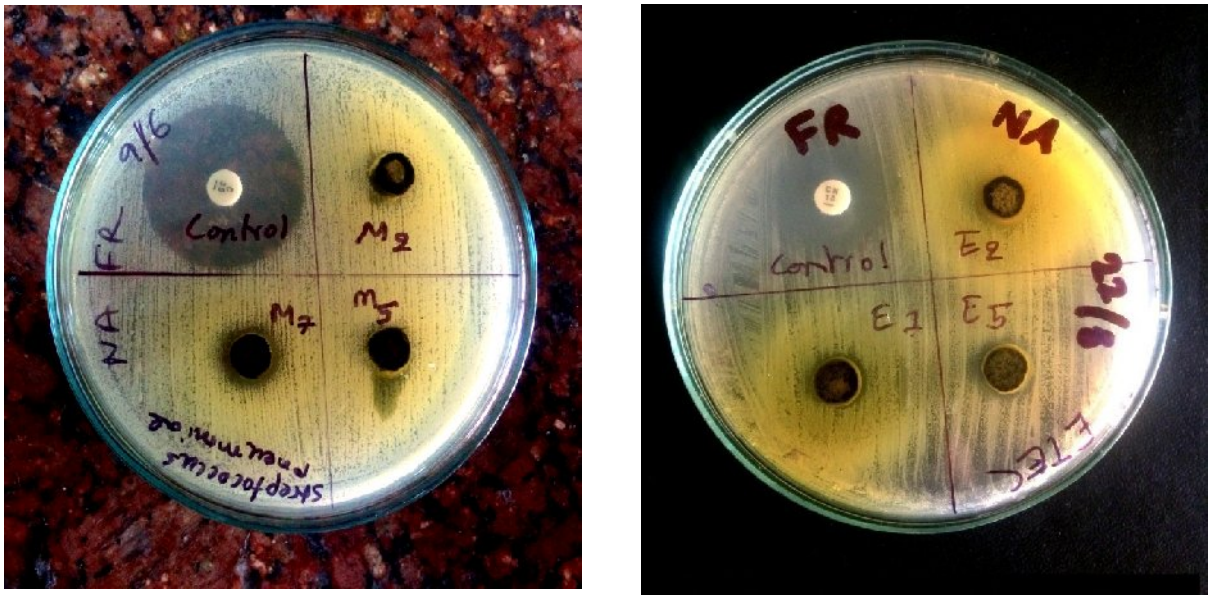


Fig 3.2 : The antibacterial effect of methanol extract of aniseed of 2nd, 5th and 7th day of extraction along with the positive control against (a) *Shigella flexineri* (b) *Bacillus subtilis*(c) *S. pneumonia* and(d) *E. coli* *(negative result)*

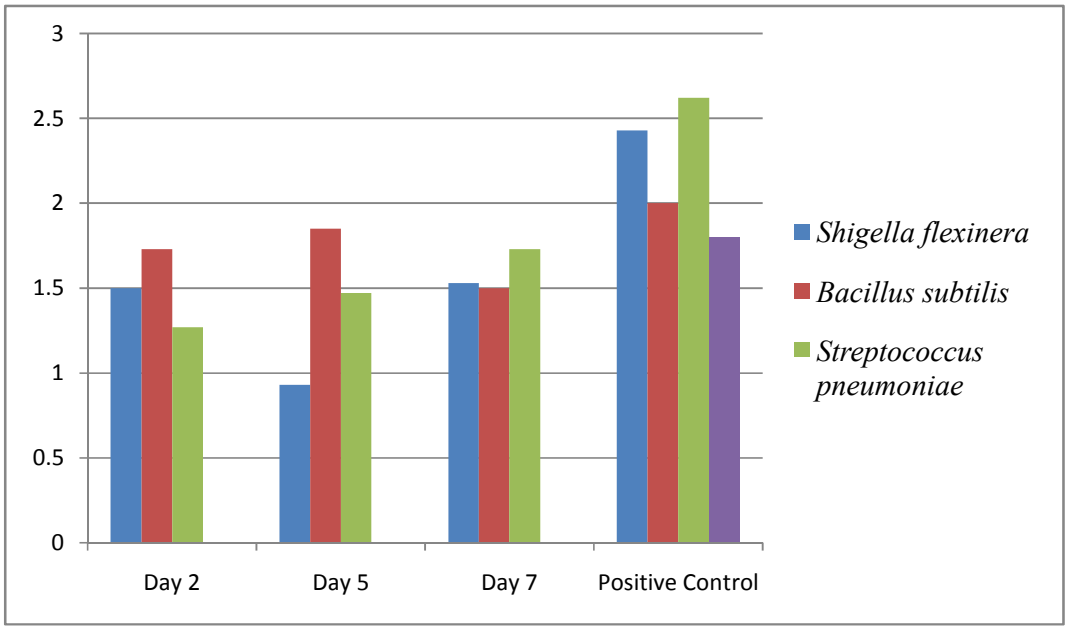


Figure 3.3 : Average Zone of Inhibition in cm by Ethanol Extract

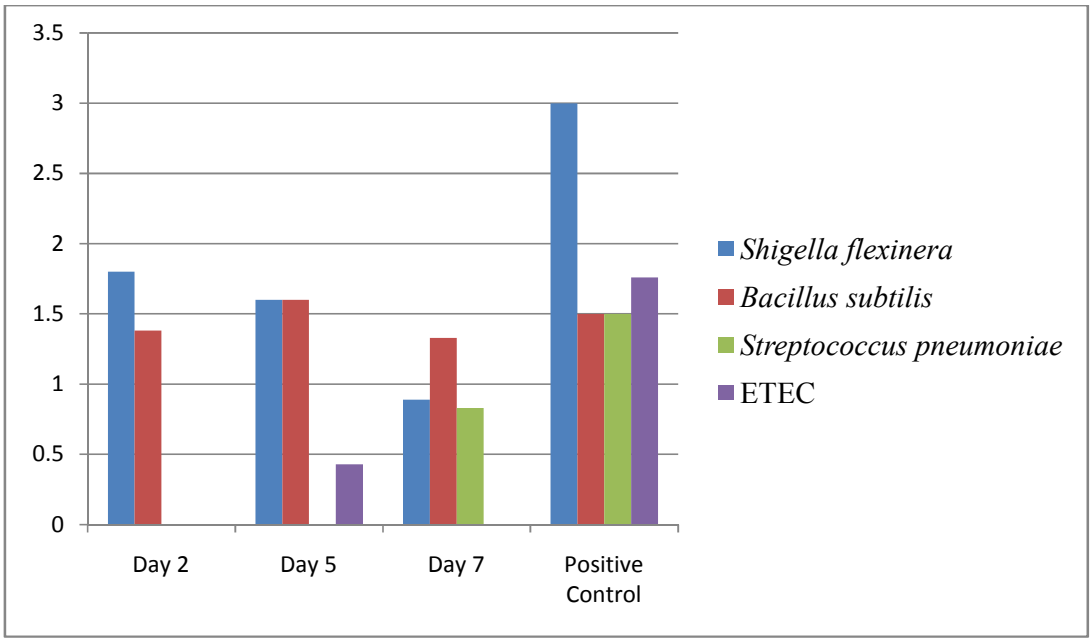


Figure 3.4 : Average Zone of Inhibition in cm by Methanol Extract

3.1.4 Comparison of the positive result (zone of inhibition in cm) of Different Extracts:

From table 1 it can be clearly seen that day 2 extract of both ethanol and methanol had the greatest positive result on *Bacillus subtilis*. The replicates showed a more or less constant zone of inhibition for both and the activity index too was the greatest. Both the day 2 ethanol and methanol extract had positive effects, that is, showed zones of inhibition on both *Shigellaflexineri* as well. However the day 2 ethanol extract showed a clear zone on *Streptococcus pneumoniae* but not the day 2 methanol extract. No effect of day 2 extract of either ethanol or methanol was visible on ETEC.

The best day 5 result of both the ethanol and methanol extract was also seen in petri dishes containing *Bacillus subtilis*; the methanol extract showed a better activity index too. Alongside the second best result was seen in *Shigellaflexineri* for boththe day 5 ethanol and methanol extracts. Positive result of day 5 ethanol extract was also seen in *Streptococcus pneumoniae* but the methanol day 5 extract had no effect on it. But then again, a positive and constant effect was seen by the 5^{th} day methanolic extract on ETEC.

Bacillus subtilis continued to be the most susceptible organism tested. Both the day 7ethanol and methanol extract too had the best result on *Bacillus subtilis*. Next best effect was seen on *Shigellaflexineri*. The day 7 ethanol extract had no effect on *Streptococcus pneumoniae* but the methanol extract did show clear zones of inhibition. Neither of the day 7 ethanol and methanol extract had any effect on ETEC although.

The rest of the organisms tested were not susceptible to the any of the 2^{nd}, 5^{th} or 7^{th} day extract.

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.

In general maximum activity index was seen in *Bacillus subtilis* by both the ethanol and methanol extract. The highest was shown by day 5 extract of both ethanol and methanol on *Bacillus subtilis*. The second most susceptible organism in the study turned out to be *Shigella flexineri.* Though both type of extract showed significant effect on *Bacillus subtilis*, I general the ethanol extract worked a lot better on this organism. Further comparing the overall effect of ethanol and methanol extract on *Streptococcus pneomoiae* it can be clearly seen that day 2 and day 5 methanolic extract had no effect on streptococcus pneumonia. However it all the ethanolic extract of the 3 days had effect on the same organism. Maximum activity index shown in this organism was by day 7 ethanolic extract. As for ETEC the activity index result was inconsistent too as the ethanolic extract of no single day had any effect on the organism; but the 5^{th} day methanolic extract had effect on ETEC.

	Ethanol				Methanol			
	Shigella flexineri	*Bacillus subtilis*	*Streptococus pneumoniae*	*ETEC*	*Shigella flexinera*	*Bacillus subtilis*	*Streptococcus pneumoniae*	*ETEC*
Day 2 AI_E	0.62	0.87	0.48	0.0	0.3	0.92	0.0	0.0
Day 5 AI_E	0.38	0.925	0.56	0.0	0.49	1.06	0.0	0.24
Day 7 AI^E	0.42	0.75	0.66	0.0	0.27	0.88	0.34	0.0

Table 4: Activity Index of 2nd, 5th and 7th day ethanol and methanol extract of *Pimpinella anisum*

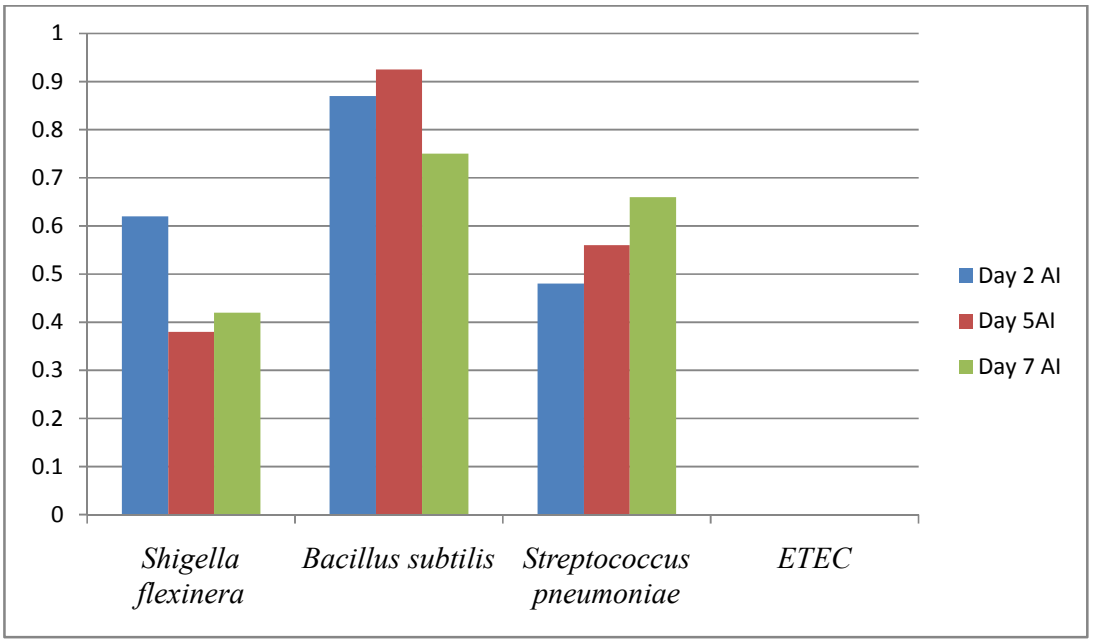


Fig 3.5: Activity index from ethanol extract of 2nd,5th and 7th day.

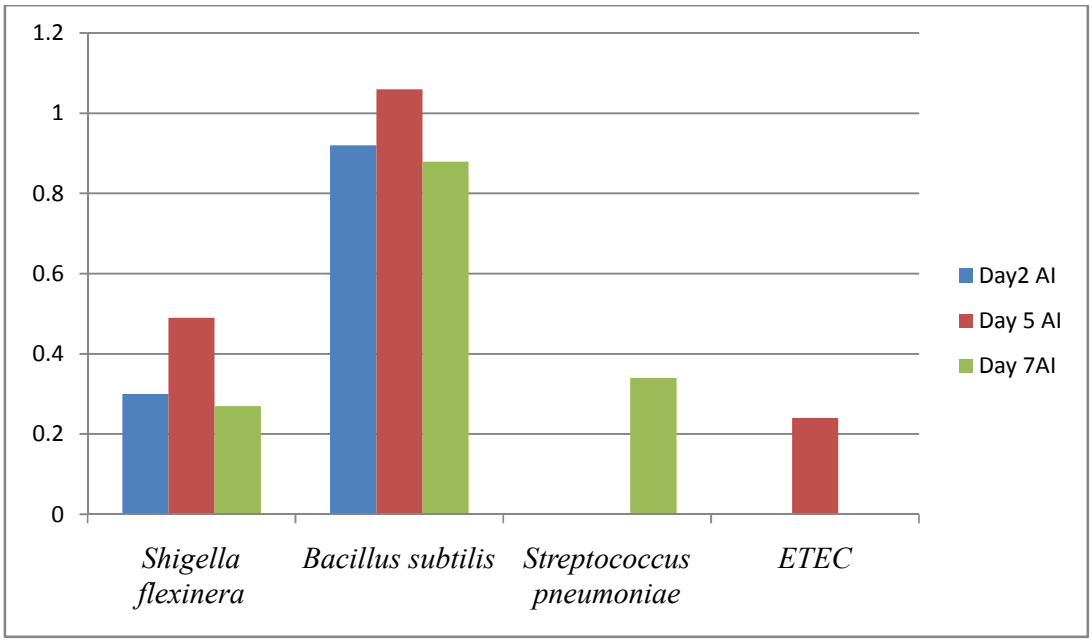


Fig 3.6: Activity index from methanol extractof2nd,5th and 7th day

3.2Results of Phytochemical Screening

Name of tested chemical	Figure		Result	
	Ethanol	Methanol	Ethanol	Methanol
Tanin			Positive	Positive
Saponin			Positive	Positive
Phlobatannin			Negative	Negative

Name of Chemical	Figure		Result	
	Ethanol	Methanol	Ethanol	Methanol
Terpenoid			Positive	Positive
Flavonoid			Positive	Positive
Cardiac glycosides			Negative	Negative

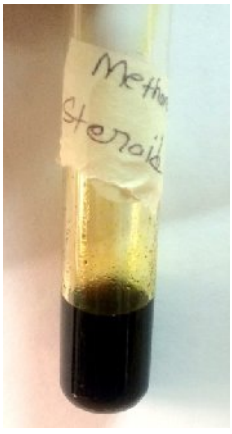
Name of Chemical	Figure		Result	
	Ethanol	Methanol	Ethanol	Methanol
Phenolic Compounds			Positive	Positive
Reducing Sugar			Positive	Positive
Steroids			Negative	Negative

Table 5: Results yielded for each phytochemical tested in *Pimpinella anisum*

CHAPTER 4

DISCUSSION

4.1 DISCUSSION

Plants are a large source of new bioactive molecules with therapeutic potentials. Only a small percentage of living plants on Earth have been phytochemically investigated. 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). Medicinal plants are increasingly gaining acceptance even among the literates in urban areas.

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* isaspice 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. As mentioned earlier 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.

The purpose of this experiment was to investigate the phytochemical properties of the selected plant species and also to find its antimicrobial properties against the most prevalent organisms in context of Bangladesh.

In general, the results showed that *Pimpinellaanisum* had desirable effects on quite a few organisms that are mostly prevalent in perspective of Bangladesh and also showed to contain quite a few phytochemicals in both the ethanol and methanol extracts of it.

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 namely the four bacteria *Shigella flexineri, Bacillus subtilis, Streptococcus pneumonia* was maximum. Day 5 methanol extract showed the greatest activity against *Bacillus subtilis* and *Streptococcus pneumoniae*. Also the day 5 ethanol extract showed maximum activity against *Shigellaflexineri*, *Bacillus subtilis* andEnterotoxicogenic*E.coli* (ETEC) indicating high sensitivity to the extract.

On comparing, the aqueous extract of aniseed was not as effective as the ethanol or methanol extracts for any of the microbes that were seen to be sensitive (Rakshanda, 2015). However, the the average zone of inhibition for both ethanolic and methanolic extract on *Bacilus subtilis* was more or less similar to that mentioned in the study by (Rakshanda, 2015). This one common organism in both the studies yielded a similar result. 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 two 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.

All in all on comparison of the ethanol and methanol extracts with the aqueous extract it can be seen that the ethanolic and methanolic forms of it had better effect but sadly even though we do consume this plant it is usually either eaten raw or cooked down with food or used as a flavoring agent coming down to being used in its aqueous form. As such we cannot expect to have any of the expected results that we are getting in vitro as in vivo.

Further there had 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*).* Unfortunately I could not perform the antifungal effects of aniseed extracts in this study due to limitations in the lab, 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).Both theethanol and methanol yielded maximum activity on*Bacillus subtilis* and *Shigella flexineri* showed maximum result and also it was specifically the day 5 extract in each case.

However, there was no activity of the ethanol extract on *ETEC* and the methanolic extract had no effect on *S. pneumoniae* apart from the day 7 extract of it. Also only the day 5 methanolic extract had some effect on ETEC but it was too insignificant.

As for the phytochemical testing part both the ethanol and methanol extracts showed to contain tanin, saponin, reducing compounds, phenolic compounds, terpenoids and flavonoids whereas cardiac glycosides and steroids were absent in the extracts. 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. So it is clearly seen that none of the three types of extracts of this plant contained steroid.

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 terpenoid (Harsha *et al,* 2013). In a different study by Prof. Hayder M Al Quraishy (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, alkaloids and phenolics. I also did a test for cardiac glycosides, phlobatannin and steroids results for which was negative for both ethanol and methanol extracts.

Only cardiac glycoside was out of the line as it showed absent in my extracts. This may have been because of the reagents used not being up to par and also because the strain of pimpinella anisum used was different based on the soil it was grown in.

The purpose of this study was to collect the ethanol and methanol extracts from different days of extraction and compare to the aqueous extracts of aniseed and to investigate the presence of phytochemicals in *Pimpinellaanisum*. Antibacterial tests showed that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *S. flexineri*, *B. subtilis*, ETEC and *S. pneumonae*. The comparison of the different extracts showed that the 5th day of extraction of both the ethanolic and methanolic extraction has the greatest antimicrobial effect against B. subtilis. The phytochemical analysis of the aqueous extract revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, phenolic compounds but no phlobatannins, cardiac glycosides and steroids. However, the aqueous extract of aniseed did not contain any steroid as well. This shows that these phytochemicals could be responsible for the observed antimicrobial properties.

To sum it up it can be said that *Pimpinellaanisum* seems to be a promising source of antimicrobial properties in a time when resistant bacteria are on the rise. Based on 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 to not only help Bangladesh but any developing countries as a matter of fact. This is an inexpensive and abundant source and should be resorted to.

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