Determination of antibacterial activity of Cinnamon (*Cinnamomum verum*) and Black cumin (*Nigella sativa*) extracts, along with MIC\MBC against bacterial isolates and analysis of their phytochemical properties

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

Submitted by:

Tanha Mollic Moon

Student ID: 13326002

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University

Bangladesh

September 2018

DECLARATION

I hereby declare that the research work representing the results submitted in this thesis entitled "Determination of antibacterial activity of cinnamon and black cumin extracts, along with MIC\MBC against bacterial isolates and analysis of their phytochemical properties" submitted by me has been carried out under the supervision of Nazneen Jahan, Lecturer, Microbiology program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. I also declare that the research work presented here is original and any information or reference to research works performed by other researchers has been cited accordingly. This research paper has been submitted in the partial fulfilment for the degree of Bachelor of Science in Microbiology, BRAC University, Dhaka and has not been submitted to any other institution for any degree or diploma.

Tanha Mollic Moon

ID: 13326002

Certified by

_____

Nazneen Jahan

Lecturer

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University, Dhaka, Bangladesh

Acknowledgement

In the outset, I am thankful to the Almighty Allah to have enabled me to perform this thesis work by giving me patience, protection and good health.

The work I accomplished in pursuance of my B.Sc. study happens to be the first undertaking of this nature I have ever been exposed to. It may be a small step as such but for me it was a great opportunity. I needed help and inspiration not to be frustrated in the event of repeated failures in my experiments. Fortunately there were people around me who provided the needed supports.

I express my gratitude towards Prof. Dr. A. F. M Yusuf Haider, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for his kind cooperation, active support and constant supervision. Prof. Dr. Shah. M. Faruque, coordinator of Life Sciences, Department of Mathematics and Natural Sciences, for his exemplary guidance, monitoring and constant encouragement throughout the Project. He has been my moral guide throughout my student life.

My regards, gratitude, indebtedness and appreciation goes to my respected supervisor Nazneen Jahan, Lecturer, Department of Microbiology, BRAC University for her constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas and a good sense of humor and never ending motivation throughout the entire period of my research work.

It is a great pleasure to express my sincere gratitude to all the faculty members of Department of Mathematics and Natural Sciences, BRAC University especially Dr. Mahboob Hossain, whose knowledge and experiences shared during the coursework of my undergraduate program have made me capable of performing this research work by myself.

My sincere appreciation is extended to staffs of the department, who helped me even beyond their duty period to continue my research work.

ABSTRACT

Medicinal plants are nothing but a blessing to us as they are the ample bio-resource of drugs for traditional medicines, food supplements, modern medicines, pharmaceutical intermediates, and chemical units for synthetic drugs. In the present study, ethanol, methanol and acetonic extracts of *Cinnamomum verum* and *Nigella sativa*, were subjected to microbial susceptibility assays using agar well diffusion method. The microorganisms employed were *Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pyogenes,* and *Proteus vulgaris.* Five commercial antibiotics named gentamicin; clindamycin, vancomycin, meropenem and cefepime were also used for determining the Activity Index. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cinnamon and Black Cumin extracts were also detected against highly susceptible organisms by using tube dilution method. Here, the highest AI value was 1.68 for methamolic extract of black cumin to vancomycin against *S.aureus*. After that, we also examined the phytochemical properties (tannin, saponin, flavonoids, alkaloids, phenol, steroid and starch) of cinnamon and black cumin extracts. In result, the most susceptible microorganisms were *Bacillus subtilis* and *Staphylococcus aureus*, and *Bacillus cereus* while the least susceptible were *Escherichia coli* and *Klebsiella pneumoniae* for both extracts. The lowest MIC value (25mg\ml) and lowest MBC value (30mg\ml) were found for methanolic extract of both cinnamon and black cumin extracts against *B.subtilis* and *S.aureus* respectively. Three extracts of cinnamon showed positive result for saponin, alkaloid and steroid. On the other hand, all extracts of black cumin showed positive result for alkaloid, steroid and negative result for tannin and saponin. In short, the findings of this study may provide the possibility of developing antibacterial supplements from these medicinal plants.

Table of Contents

Lists of tables

List of graphs

List of figures

Chapter one: Introduction

1.1 Background:

In our environment there is a deep connection between plants and animal. This connection amongst plants and people is envisioned in ethnobotany, a field concentrating on the investigation of the indigenous information on how plants are seen, utilized and overseen (Chekole et al, 2015). Usually, medicinal plants are used to maintain people's health, as well as to prevent, diagnose, improve or treat physical and mental illnesses all over the world. Medicinal plants are accepted to be with healing powers, and individuals have utilized them for a long time. Expected to current medication revelation, customary therapeutic plants have been contemplated and created which is taken after the ethnobotanical lead of indigenous cures utilized by conventional medical system. Traditional medicinal knowledge, particularly utilizing therapeutic plants in the developing countries, has been in existence and use, and has been a part of therapeutic practices. Hence, the exploration of plants and their uses in medical purposes is a standout amongst the most essential human concerns and has been practiced in the world (Hong et al 2015).

1.2 Description of plants:

1.2.1 *Cinnamomum verum* (Cinnamon)

Cinnamomum verum, commonly known as cinnamon is used in the food industry because of its special aroma. It is an ever green tropical tree, belonging to the Lauraceae family (Vakilwala et al., 2017). The main commercial product of cinnamon trees is the dried bark of the stem in the form of quills, quislings and chips. The three major parts of the plant: leaf, stem-bark and root-bark yield three different types of essential oils (Abeysinghe et al., 2009).

Cinnamomum verum is one of the most important spice species in Sri Lanka and it contributes to 70% of the world bark production. There are 9 *Cinnamomum* species found in Sri Lanka (Abeysinghe et al., 2009). Many scientific pharmacological investigations have reported on anti-inflammatory potential of the bark of cinnamon. The anti-inflammatory action has been attributed to a series of tannins. The Phytochemical analysis of the various extracts from *Cinnamomum verum* showed presence of phenols, glycosides, and tannins in the methanolic and

chloroform extracts and alkoloids, flavonoid and saponins were absent in the extracts (Vakilwala et al., 2017). Cinnamon is high in antioxidant activity. The essential oil of Cinnamon also has antimicrobial properties, which is used in the preservation of certain foods (Sharma et al., 2016).

Fig 1.1: *Cinnamomum verum* (Cinnamon)

1.2.2 *Nigella sativa* (Black Cumin seeds)

Nigella sativa, commonly known as Black Cumin, is an indigenous herbaceous plant belongs to the Ranunculaceae family. This plant has finely divided foliage and blue flowers, which produce black seeds and it grows to a maximum height of about 60 cm (Ishtiaq et al., 2013). The delicate flowers of this plant have 5-10 petals (Yessuf, 2015).This plant is recognized by some other names in different countries such as kalonjiin in Urdu, habba-tusawda in Arabic, black cumin in English, shonaiz in Persian and kalajira in Bengali (Ishtiaq et al., 2013). *Nigella sativa* is an annual herbaceous plant grown in Western Asia and the Mediterranean region for its seeds (Zahra et al., 2011). Nigella sativa is also cultivated in many countries in the world like South Europe, Saudi Arabia, Turkey, Syria, Pakistan and India (Yessuf, 2015).The seeds contain fixed and essential oils, proteins, alkaloids. Much of the biological activity of the seeds has been shown due to thymoquinone, the major component of the essential oil, but which is also present in the fixed oil (Zahra et al., 2011). The black seeds contain 36–38% fixed oil, with proteins, alkaloids, saponins and essential oils making up the rest of the composition (Hasan et al., 2013).

Fig 1.2: ***Nigella sativa*** **(Black Cumin seeds)**

1.3 Therapeutic use of cinnamon and black cumin plants

Cinnamon is indicated as an analgesic and antipyretic agent against cold, fever, headache, myalgia (mascular pain), arthralgia (arthritic pain) and amenorrhea (failure of menstruation). Additionally, it has strong antibacterial properties, anticandidial, antiulcer, analgesic, antioxidant and hypocholesterolaemic activities (Vakilwala et al., 2017). Cinnamon has been reported to have remarkable pharmacological effects in the treatment of type II diabetes and insulin resistance (Hassan et al., 2012). The antinociceptive (analgesic) and antipyretic (fever reducing) activity were also reported (Pandey& Singh, 2014). It has also been used to treat toothache and bad breath (Sharma et al., 2016).

The Black Cumin seeds have been widely used for the treatment of different diseases and ailments. Seeds exhibit a wide spectrum of biological and pharmacological activities which include antihypertensive, antidiabetic, diuretics, anticancer, immunomodulator, analgesic, antioxidant, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepatoprotective, pulmonary protective, nephro-protective, gastro-protective, antioxytosis and anticonvulsant properties etc. It has got the place among the top ranked evidence based herbal medicines as it had showed miraculous power of healing (Yessuf, 2015). Different pharmacological effects such as cardiovascular disorders, antioxidant activity, anti-anxiety effect and anti-viral activity against cytomegalovirus have been reported for this medicinal plant (Ishtiaq et al., 2013).

1.4 Antimicrobial properties of cinnamon and black cumin:

As spices have the antimicrobial potential, the antimicrobial activity of *Cinnamomum verum* has been investigated as alternative to antibiotics. The MIC value of chloroform extract of *Cinnamomum verum* was found to be 3125 µg/ml against *Staphylococcus aureus*; 6250 µg/ml against *Bacillus cereus* and *Bacillus subtilis*; and 25,000 µg/ml against *E.coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Protease vulgaris*. In the antifungal study of methanolic extract of spice, maximum antifungal activity was shown by *Cinnamomum verum* extract against *Aspergillus niger* (Vakilwala et al., 2017). Disc diffusion method has been used to evaluate antibacterial activity of methanol extract of *Cinnamomum zeylanicum* against bacteria *B. subtilis* which gave its maximum size of zone of 25mm in case of *Bacillus subtilis*(0.5gm/ml) (Sharma et al., 2016).

Ethanolic extract of *Nigella sativa* showed inhibition zone against all the four bacterial strains *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pasturella multocida* between the range of 15 and 28mm (Zahra et al., 2011). Methanolic extract of *N.sativa* seeds shows antibacterial activity against all bacterial strains under investigation (Ishtiaq et al., 2013).

1.5 Some gram-positive and gram-negative bacteria selected for the study

Seven different bacteria are selected to observe antibacterial effect of ethanolic, methanolic and aqueous extracts of the six medicinal plants which are as follows:

1.5.1 ***Bacillus cereus***: *Bacillus cereus* is a gram-positive, rod shaped, motile, endospore-forming, aerobic or facultatively anaerobic bacterium that is commonly found in the environment and on many foods including meat, cereal dishes, vegetables, milk, products etc., but it does not usually pose a health risk. These cells grow in the body and secrete toxins to cause illness such as food poisoning when food is improperly cooked or stored in the temperature range of 41ºF to 135ºF for an extended period of time (Schneider et al., 2017).

1.5.2 ***Bacillus subtilis***: *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. They can contaminate food; however, they seldom result in food poisoning (Tam et al., 2006).

1.5.3 *Streptococcus pyogenes*: It is a gram-positive coccoid-shaped bacterium that grows in chains. Pathogenesis involves successful colonization of the upper respiratory mucosa or skin of human host. Streptococcus pyogenes infections include acute pharyngitis (strep throat) and localized skin infection (impetigo) in children and adolescents. It also produces a variety of other infections of the respiratory tract, including sinusitis of the skin and soft tissues including cellulitis, vaginitis, meningitis, pneumonia, neonatal sepsis, and surgical wound infections. It also is the proven cause of potentially serious acute rheumatic fever (ARF) (Nizet & Arnold, n.d.).

1.5.4 *Staphylococcus aureus*: *Staphylococcus aureus* is a gram-positive, non-motile, non-spore forming facultative anaerobes bacteria, characterized by cocci that divide in more than one plane to form grape-like clusters. It is a major cause of nosocomial infections worldwide, especially methicillin-resistant *Staphylococcus aureus* (MRSA). It often asymptomatically colonizes the skin and mucous membranes of healthy individuals (Costa et al., 2013).

1.5.5 *Proteus vulgaris*: *Proteus Vulgaris* is a rod-shaped gram-negative, chemoheterotrophic bacterium. Proteus vulgaris possesses peritrichous flagella, making it actively motile. It inhabits the soil, polluted water, raw meat, dust and gastrointestinal tracts of animals. In humans, Proteus spp., most frequently cause urinary tract infections, but can also produce severe abscesses and is widely associated with nosocomial infections (Park, n.d.).

1.5.6 *Klebsiella pneumoniae*: *Klebsiella pneumoniae* is a gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. *Klebsiella pneumoniae* is able to grow either with or without free oxygen, deeming it a facultative anaerobe. It is found in the normal flora of the mouth, skin, and intestinal tract of humans where it initially does not cause disease. *K.pneumoniae* can progress into severe bacterial infections leading to pneumonia, bloodstream infections, wound infections, urinary tract infections, and meningitis. Patients who require equipment such as catheters or ventilators are at high risk for infections (Legend, 2015).

1.5.7 *Escherichia coli*: *Escherichia coli (E. coli*) are gram-negative, normal gut micro flora that is found in the intestines of warm-blooded animals and humans. While many of the strains of *E. coli* are harmless, some are harmful (pathogenic) causing gastroenteritis, urinary tract infections, meningitis, and other more severe secondary illnesses in humans. *E. coli* that cause diarrhea are classified primarily by their pathogenicity and virulence properties (Tortora et al., 2010).

1.6 Antibiotics selected for the study

List of antibiotic disc used for this study are given below. [The brief overview has been taken from (PubMed Health)] :

1. Meropenem - Meropenem (Merrem, Meronem) is a broad-spectrum antibacterial agent of the carbapenem family, indicated as empirical therapy prior to the identification of causative organisms, or for disease caused by single or multiple susceptible bacteria in both adults and children with a broad range of serious infections.

2. Clindamycin - Clindamycin is used to treat bacterial infections. This medicine may be given to patients who have had an allergic reaction to penicillin. Clindamycin will not work for colds, flu, or other virus infections.

3. Gentamicin - Gentamicin belongs to the class of medicines known as aminoglycoside antibiotics. It works by killing bacteria or preventing their growth. However, this medicine will not work for colds, flu, or other virus infections.

4. Vancomycin- It is used to treat a number of bacterial infections. It is recommended intravenously as a treatment for skin infection, bloodstream infection, endocarditis, bone and joint infection and meningitis.

5. Cefepime- cefepime is a fourth generation cephalosporin antibiotic. Cefepime has an extended spectrum of activity against gram positive and gram negative bacteria.

1.7 Effect of plant extracts on human body

From the ancient time herbal treatment is being used for many health problems. Herbs are safe, less toxic, economical, and a reliable key natural resource of drugs all over the world (Al-daihan et al., 2013). Antibiotics, which are considered as a remedy against pathogens with ignoring their influences on the microflora, could lead to creating a new disease by disturbing the microbial ecosystem of the human body and develop new generations of antibiotics resistant pathogens. The biological interactions of the microflora in the human body are important in keeping the somatic eco-physiological balance. Many studies reported that antibiotics therapy directly impacts the normal flora's functions in the human body. In contrast, the phytotherapy using plant products could get rid the pathogens and maintains the normal flora of the human body (Abdallah, 2016).

1.8 Objectives of the study:

In this study, cinnamon and black cumin seed are used which are collected from local market of Bangladesh. The aim of this study is given below:

• Extraction of cinnamon and black cumin extracts by using suitable solvent system and determination of their antimicrobial activity against selected bacterial isolates

• Determination of their minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of these extracts

• Assessment of the phytochemical properties of cinnamon and black cumin extracts.

Chapter two:

Materials and method

2.1 Working laboratory:

The research project was wet laboratory based and the entire project work was performed in the microbiology laboratories of Department of mathematics and natural sciences, BRAC University, Dhaka, Bangladesh. In this laboratory, biosafety level 2 is followed and all the work was done under laminar flow inside laminar cabinet.

2.2 Collection of samples and processing:

The black cumin seeds and cinnamon were bought from the local market and washed 3 times with distilled water. They were sun dried for a day and then processed through a grinder to make powder form.

2.3 Extraction process:

Black cumin seeds and cinnamon were extracted in Ethanol, methanol and acetone.

2.3.1 Ethanolic extract: with the help of weight machine, 10 grams of black cumin seed powder was taken in a conical flask. The powder was mixed with 100 ml of ethanol and stirred for about 15 minutes. Then the flask was covered with foil paper and left it in shaker incubator for 24 hours at 37° c. After 24 hours the contents of the flask were filtered with Whattman no 1 filter paper and poured into a petri dish. It was then kept into incubator at 42°c. this was done till oily substance is visible on the plate. Then it was scooped out into a vial that was previously autoclaved. This was then labeled and kept in 4°c in refrigerator.

By the same way cinnamon were extracted in ethanol. In case of cinnamon, the final result is dried powered instead of oily substance.

2.3.2 Methanolic extract: with the help of weight machine, 10 grams of black cumin seed powder was taken in a conical flask. The powder was mixed with 100 ml of methanol and stirred for about 15 minutes. Then the flask was covered with foil paper and left it in shaker incubator for 24 hours at 37°c. After 24 hours the contents of the flask were filtered with Whatman no 1

filter paper and poured into a petri dish. It was then kept into incubator at 4°c and this was done till oily substance is visible on the plate. Then it was scooped out into a vial that was previously autoclaved. This was then labeled and kept in 4°c in refrigerator.

By the same way cinnamon were extracted in methanol. In case of cinnamon the final result is dried powder instead of oily substance.

2.3.3 Acetonic extract: with the help of weight machine, 10 grams of black cumin seed powder was taken in a conical flask. The powder was mixed with 100 ml of acetone and stirred for about 15 minutes. Then the flask was covered with foil paper and left it in shaker incubator for 24 hours at 37°c. After 24 hours the contents of the flask were filtered with Whatman no 1 filter paper and poured into a petri dish. It was then kept into incubator at 42°c. This was done till oily substance is visible on the plate. Then it was scooped out into a vial that was previously autoclaved. This was then labeled and kept in 4°c in refrigerator.

In the same way cinnamon were extracted in acetone. In case of cinnamon the final result is dried powder instead of oily substance.

2.4 Preparation of stock solution of the extracts:

To prepare the stock solution of the extracts, 1% Dimethylsulfoxide (DMSO) was used as solvent. Ten gram of each extract was dissolved in 100 ml of 1% DMSO. The solution was kept in aluminium foil wrapped McCartney bottles to avoid the molecular modification of DMSO in the presence of light. The stock solution was stored at 4° in a refrigerator.

2.5 Preparation of Nutrient agar plates:

Selected bacterial cultures were needed for testing antimicrobial activity of black cumin seed and cinnamon. For the purpose several media plates were prepared.

Preparation of nutrient agar media was done by adding 28 grams of media powder in 1000ml of distilled water. Keeping this proportion constant the amount of nutrient agar prepared when

required. For each small petri dish 15 ml of agar 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 reach into the boiling point. At this point visible small bubbles were formed at the bottom which rose up and gradually the solution became clear. The flask was then removed from the heat and let it cool down for a while. Then the mouth of the flask was covered with foil paper and autoclaved. In the laminar air flow the autoclaved media was poured quickly but cautiously onto small petri plate. This was then cooled down and leveled with the name with the agar.

2.6 Preparation of saline solution:

To prepare saline solution 0.9 grams of sodium chloride (Nacl) was mixed with 100 ml of distilled water. 10 ml of saline was then transferred into each test-tube as required and autoclaved with the screw cap opened 1.5 turn. When taken out of the autoclaved machine the caps were closed tightly so that the saline does not get contaminated. These were used later when required.

2.7 Sub-culturing of organism:

The stock cultures of eight organisms were taken. To subculture the stock organism were streaked onto nutrient agar plates inside the laminar flow chamber. For each organism the plates w7re taken inside the laminar and then a loop was burned red hot through a Bunsen burner. After cooling the loop, a loop full organism were taken from the stock and streaked onto a properly leveled NA plate. This was then incubated for 24 hours at 37°c before use.

2.8 Use of 0.5 McFarland standard:

For the visual determination of the turbidity of bacterial culture, different McFarland standards are used. In microbiology laboratory of Department of mathematics and natural sciences, BRAC University, Dhaka, Bangladesh, McFarland standards 0.5, 1, 2, 3, 4 and 5 were available. For this

experiment, McFarland standard 0.5 was used to determine the approximate number of bacteria by visual comparison.

2.9 Preparation of nutrient broth:

According to the media preparation guide labeled on the bottle, 13 grams of Nutrient Broth powder is needed for the preparation of 1 liter NB. Required amount of NB is measured using an electronic balance machine and dissolved in distilled water. The suspension is heated if needed to dissolve completely. The NB is then transferred to test tubes using a glass pipette. The caps of the test tubes were closed to 1.5 turns and then autoclaved for sterilization. After autoclave is done, the caps were closed tightly and stored in refrigerator until further use.

2.10 Preparation of Mueller Hinton Agar (MHA):

Mueller-Hinton agar (MHA) is the most effective medium to use for routine susceptibility testing using Kirby-Bauer disc diffusion technique for non-fastidious bacteria (both aerobe and facultative anaerobe). Use of media apart from Mueller-Hinton agar could end in inaccurate results. Mueller-Hinton agar is additionally the quality medium used for many broth dilution testing because the conditions of this medium (i.e. pH, cation concentration and thymidine contents) are well maintained. Mueller Hinton agar (MHA) may be purchased from industrial suppliers or may also be prepared from dehydrated medium.

According to the instruction stated on the bottle, 38 gram of MHA powder is needed to prepare 1 litre of media. Keeping this a constant, required amount of MHA is prepared each time. Required amount of MHA powder is measured on an electric balance machine, poured and suspended in distilled water in a conical flask. The flask is then placed in a bunsen burner with medium flame. Continuous stirring with a glass rod is required to break down any clump of powder in the flask. The appearance of bubbles indicates that the solution has reached boiling point. The solution must be heated until it is clear. The top of the flask is then covered with aluminium foil and autoclaved at 121° for 90-120 minutes. After that the agar medium is poured in sterile petri plates inside a laminar flow chamber. The MHA medium is let set for solidifying and then

labeled properly with name and preparation date. The media plates are then kept in refrigerator until further use.

2.11 Agar well diffusion method:

The agar diffusion assay is one technique for quantifying the ability of antibiotics to inhibit bacterial growth. Here it was used to determine the antimicrobial activity of different samples of cinnamon and black cumin against seven test microorganisms. Solvent 1% DMSO was used as a negative control to determine that solvent has no antimicrobial activity to enhance the potency of the plant extracts. For each organism, the activity of those sample extracts was studied. To prepare bacterial suspension, one or two colonies of each test microorganism was inoculated in 10 ml saline solution and compared with MacFarland standard 0.5. With autoclaved cotton swabs, lawn culture of each microbe was done on MHA plates inside a laminar flow cabinet. The plates were kept without lids for a little while to soak in the moisture in the media. With the help of a sterile cork borer, agar was cut to make well for extract and negative control DMSO. Four quadrants were drawn with marker on the outside of the petri dish plates where each quadrant was labeled accordingly with the name of the plant extract in its three respective solvent solutions, the name of the lawn culture bacteria. Each of the plant extracts of about 60 µl per well were loaded in the wells using a micropipette and fourth well was filled with DMSO. The plates were incubated for 24 hours at 37°C. This process was followed for all the seven test organisms against two extracts of six samples.

2.12 Measurement of zone of inhibition:

Presence of a clear area on the MHA plate around any antibiotic disc or around the agar well containing a medicinal plant extract represents the zone of inhibition which signifies the antibacterial activity of the antibiotic as well as of the extract. The diameter of the clear zone was measured three times in millimeter (mm) with a scale/ruler and the average value of zone of inhibition for each extracts was calculated and recorded.

2.13 Determination of MIC and MBC of the extracts:

The MIC is the lowest concentration of a drug that inhibits bacterial growth thus there'll be no turbidness within the culture media. However MBC is the lowest concentration that kills bacteria. Typically the concentration that is taken into account as MBC is above the concentration for MIC. On the other hand, MBC is sometimes conferred as MBC50 or MBC90 which implies the drug concentration that kills 50% and 90%, respectively, of initial bacterial population. So, to see the MBC one got to cultivate all of the clear tubes/wells on a solid medium like NA or MHA. Then the lowest concentration of a drug that inhibits bacterial growth is going to be considered as MBC.

To determine the MIC and MBC of the plant extracts, undiluted NB was used. In six test tubes each, 3 ml of NB was taken. To this, stock solution of plant extract was given in this following amount: 1600µl, 1400µl, 1200µl, 1000µl, 800µl, 600µl, 400µl, 200µl and 0µl. After that, 1000µl of bacterial suspension of McFarland standard 0.5 was added to each test tube.. The test tubes were incubated for 24 hours at 37°C. One NA plate is divided into eight section using scale and marker and marked 1-8 accordingly. After incubation one drop from each test tubes were placed on each section on NA plates for the determination of MBC.

The general procedure of finding MIC and MBC through a table chart are given below:

No of test tube	Nutrient Broth (ml)	Bacterial suspension (ml)	Extract volume (ml)	Concentration of extract (mg\ml)	Approximate Concentration Of Inoculum Transferred CFU/ ml
1	3	1	0.2	5	1.5×10^8
2	3	1	0.4	10	1.5×10^8
3	3	1	0.6	15	1.5×10^8
4	3	1	0.8	20	1.5×10^8
5	3	1	1	25	1.5×10^8

6	3	1	1.2	30	1.5×10^8
7	3	1	1.4	35	1.5×10^8
8	3	1	1.6	40	1.5×10^8

2.1 Table: Amount of NB, bacterial suspension, extract volume, final concentration of extract present in each test tube.

2.14 Phytochemical Tests

Seven different types of biochemical assays were done. These were for tannins, saponins, flavonoids, alkaloids, phenolic compounds, steroids and starch.

2.14.1 Test for tannins: One ml of water and Five to six drops of 5% of ferric chloride was added to 1 ml of the stock solutions. When there is a formation of blue to intense green color, it indicates positive results for the presence of tannins.

2.14.2 Tests for saponins: To 1ml of stock solution, 1ml of distilled water was added to dilute it, and shaken vigorously for 2 minutes. When frothing is observed, it indicates presence of saponins in the sample.

2.14.3 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.

2.14.4 Tests for alkaloids:

Dragendorff's test: To 1 ml of the extract solution, 1ml of dragendorff's reagents was added. Orange color formation indicates a positive result.

2.14.5 Tests for phenol: 1 ml of water and 1-2 drops of 10% of Ferric chloride was added with extract solution. If the solution turns blue to dark green color, it indicates a positive result.

2.14.6 Tests for steroids:

Salkwasky test: To 2 ml of extract, 2 ml of chloroform and few drops 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.

2.14.7 Tests for starch:

Tannic acid test: 2ml of stock solution was added with 20% of Tannic acid. Whitish precipitate indicates positive result.

Chapter three: Result

3.1 Observation of antibacterial activity of ethanolic, methanolic and acetonic extracts of Cinnamon and black cumin:

In this study, seven different bacteria were used for showing the antimicrobial properties of ethanolic, methanolic and acetonic extracts of cinnamon and black cumin using agar well diffusion method. As a negative control DMSO (1%) was used.

3.1.1 Cinnamon

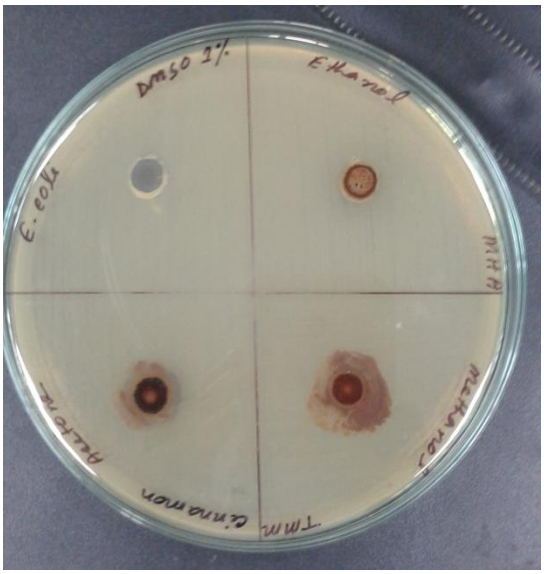
The highest zone of inhibition was observed for the ethanolic extract (27.5mm) and the acetonic extracts (24.5mm) against *Staphylococcus aureus*. On the other hand, methanolic extract showed highest zone of inhibition (25mm) against *Bacillus subtilis*.

The following table shows the zone of inhibition produced by Cinnamon against the selected bacteria:

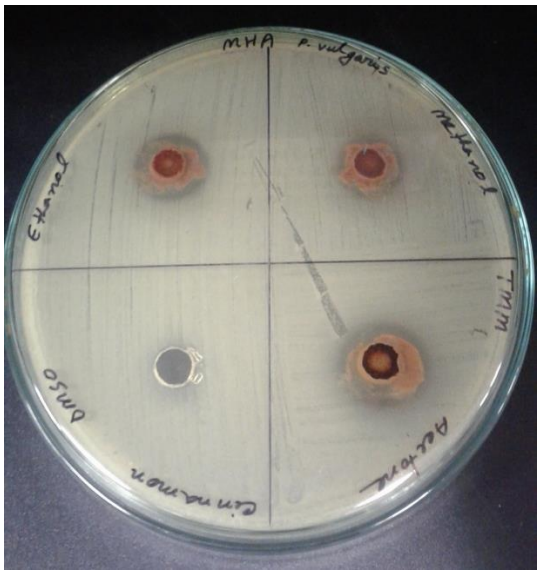
Name of organisms	Name of antibiotics	Zone of inhibition (mm)			
		Antibiotic	Ethanolic extract	Methanolic extract	Acetonic extract
Escherichia Coli	Gentamicin (10)	19	10	18	13.5
Proteus Vulgaris	Gentamicin (10)	20	17	15	19
Klebsiella Pneumoniae	Meropenem (30)	No zone	20	15.5	16
Bacillus Cereus	Gentamicin (10)	22	26	19.5	21
Staphylococcus Aureus	Clindamycin (2)	28	27.5	23	24.5

Bacillus Subtilis	Vancomycin (30)	20	20	25	23
Streptococcus Pyogenes	Clindamycin (2)	No zone	17	14.5	20

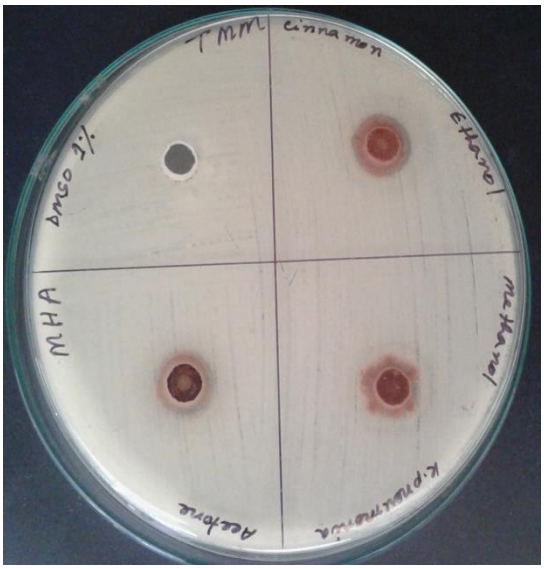
Table 3.1: zone of inhibition of various extracts of Cinnamon and antibiotics against the selected bacteria



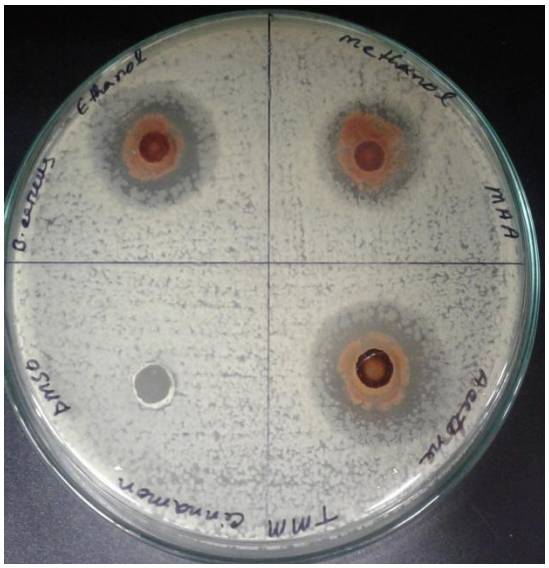
a



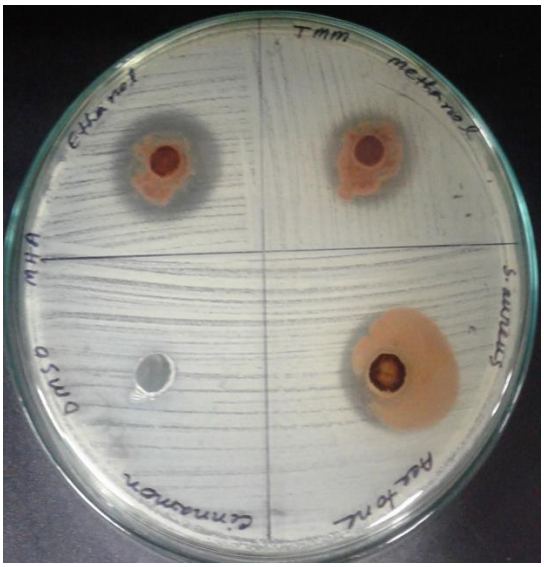
b



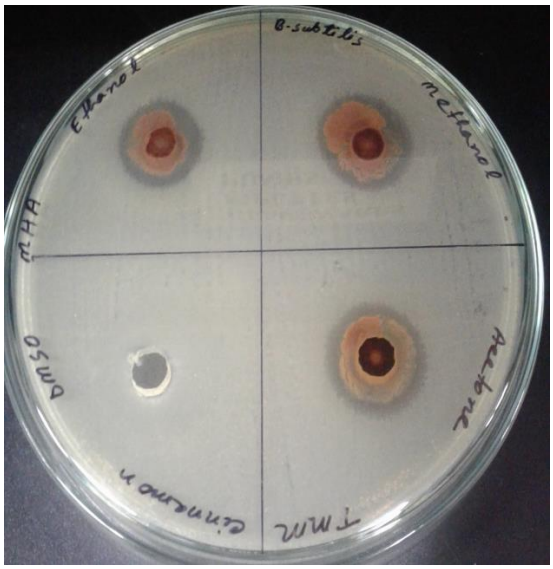
c



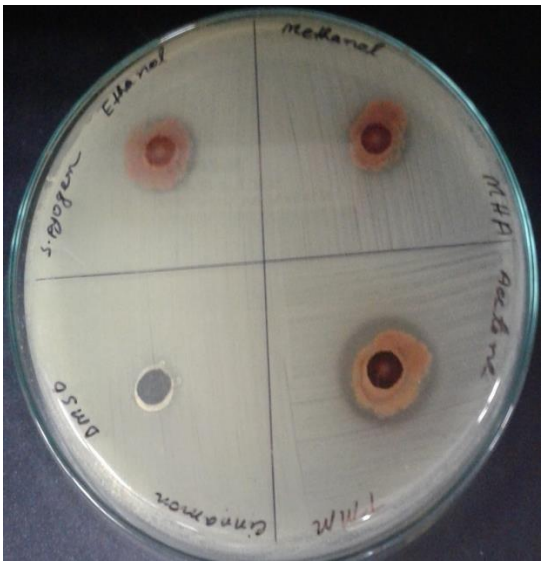
d



e



f



g

Fig 3.1: zone of inhibition of ethanolic, methanolic and acetonic extracts of Cinnamon against a) *Escherichia coli,* b) *Proteus vulgaris,* c) *K.pneumonia,* d)*B.cereus,* e) *S.aureus,* f) *B.subtilis,* g) *S.pyogen*

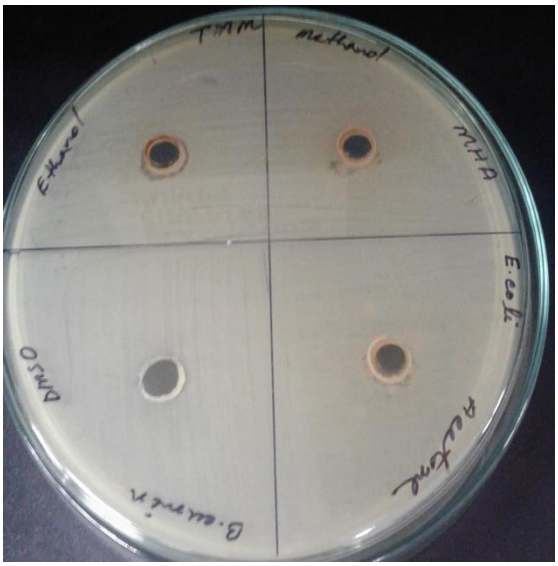
3.1.2 Black Cumin seeds

The highest zone of inhibition was observed for the ethanolic extract (31mm) and methanolic extracts (32mm) against *Staphylococcus aureus.* On the other hand, acetonic extracts showed highest zone of inhibition (25mm) against *Bacillus cereus*.

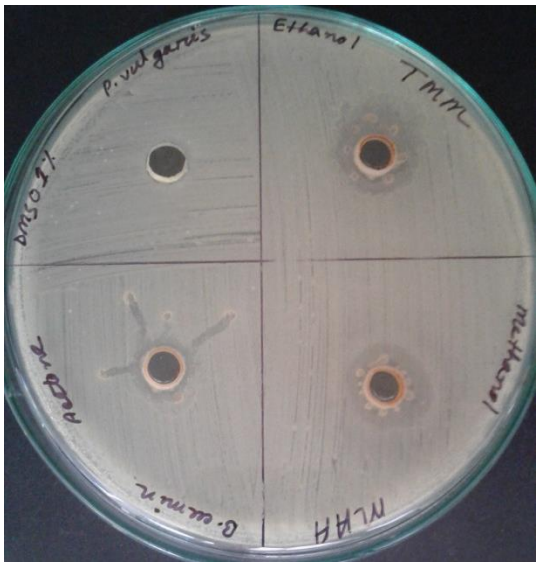
The following table shows the zone of inhibition produced by Black Cumin seeds against the selected bacteria:

Name of organisms	Name of antibiotics	Zone of inhibition (mm)			
		Antibiotic	Ethanolic extract	Methanolic extract	Acetonic extract
Escherichia Coli	Gentamicin (10)	19	11	No zone	No zone
Proteus Vulgaris	Gentamicin (10)	20	18	17	15
Klebsiella Pneumoniae	Meropenem (30)	No zone	No zone	No zone	No zone
Bacillus Cereus	Gentamicin (10)	22	27	26	25
Staphylococcus Aureus	Clindamycin (2)	28	31	32	20
Bacillus Subtilis	Vancomycin (30)	20	27	25	23
Streptococcus Pyogenes	Clindamycin (2)	No zone	11	10	12

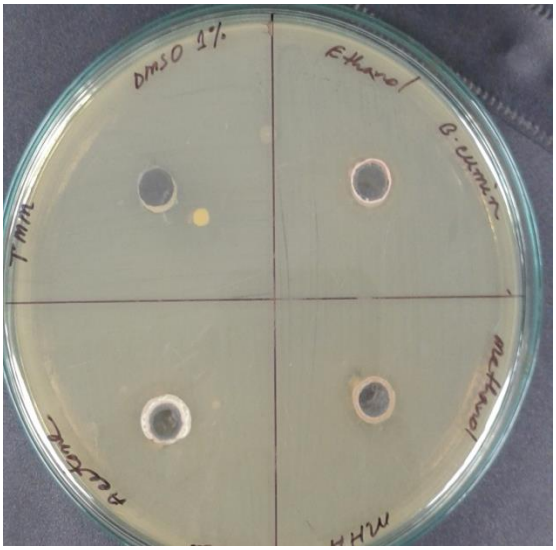
Table 3.2: zone of inhibition of various extracts of black cumin against the selected bacteria


TMM
methanol
MHA
E.coli
Acetone
B.subtilis
DMSO

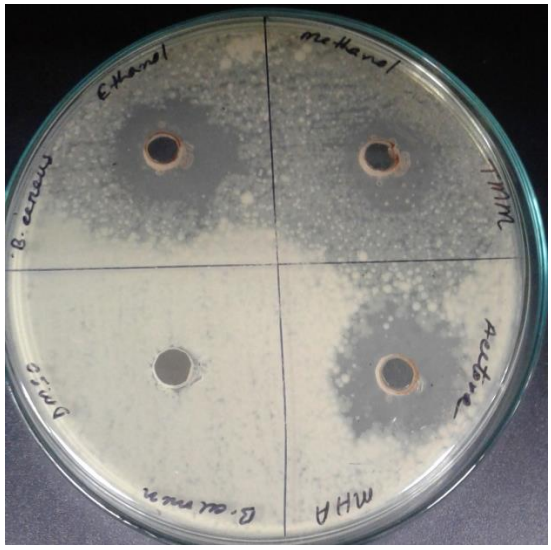
a


P.vulgaris
Ethanol
TMM
DMSO 1%
Acetone
methanol
B.subtilis
MLHA

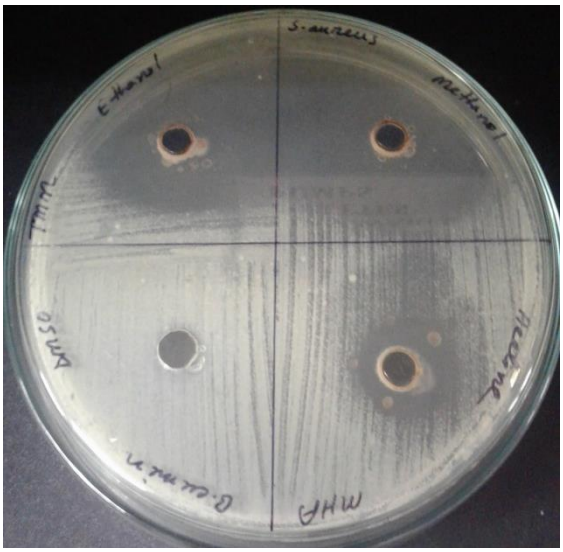
b


DMSO 1%
Ethanol
B.cereus
TMM
Acetone
methanol
MHA

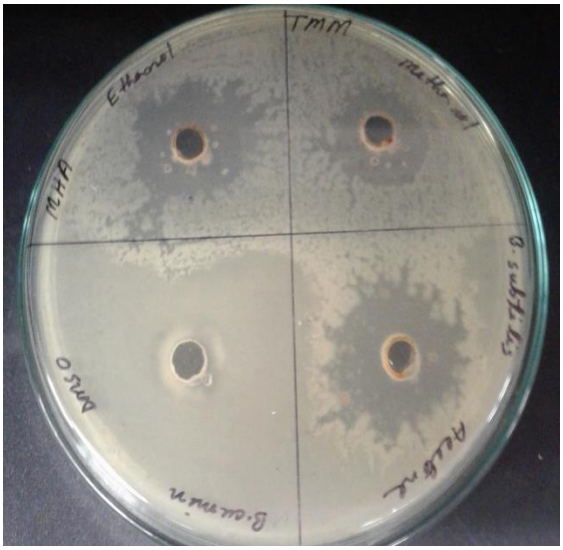
c


Ethanol
methanol
B.cereus
TMM
DMSO
Acetone
B.subtilis
MHA

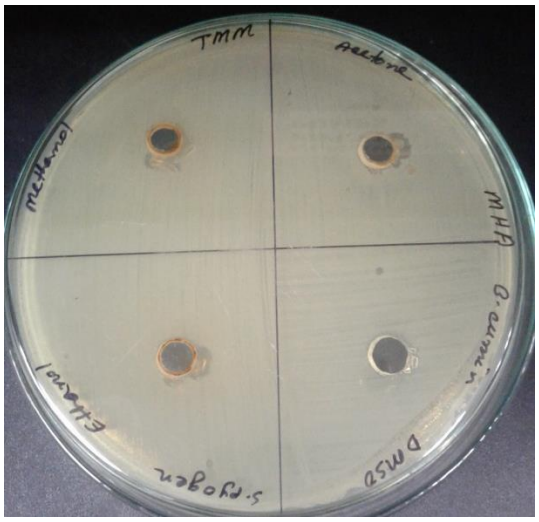
d



e



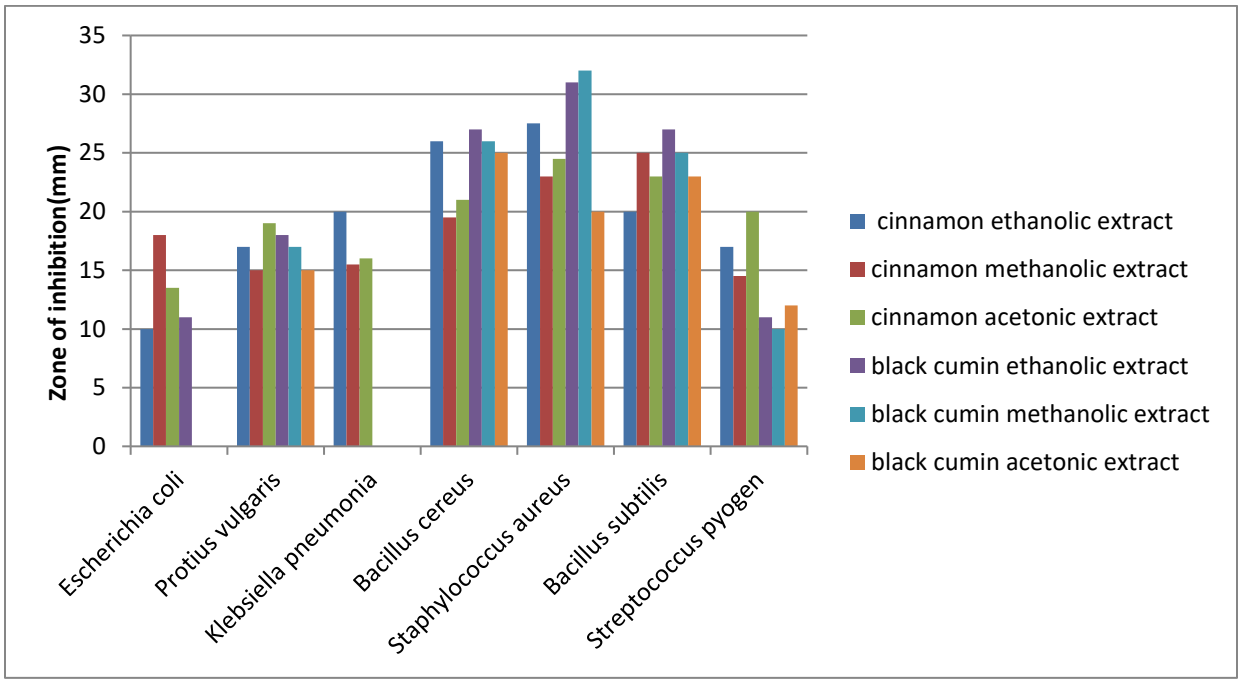
f



g

Fig 3.2: zone of inhibition of ethanolic, methanolic and acetonic extracts of black cumin against a) *Escherichia coli,* b) *Proteus vulgaris,* c) *K.pneumonia,* d)*B.cereus,* e) *S.aureus,* f) *B.subtilis,* g) *S.pyogen*

3.1.3 Comparison of antibacterial activity by different extracts

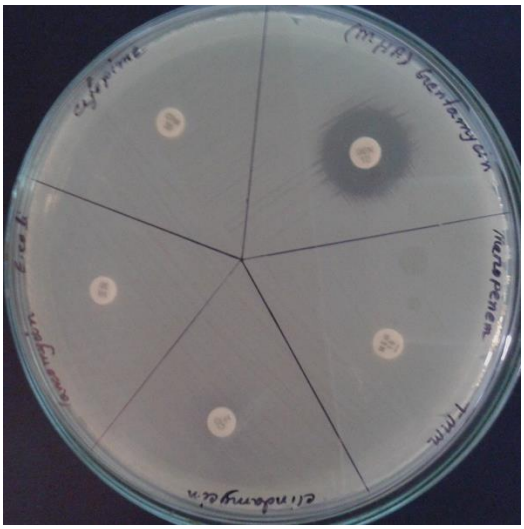


Graph 3.1: antibacterial activity of ethanolic, methanolic and acetonic extracts of Cinnamon and black cumin

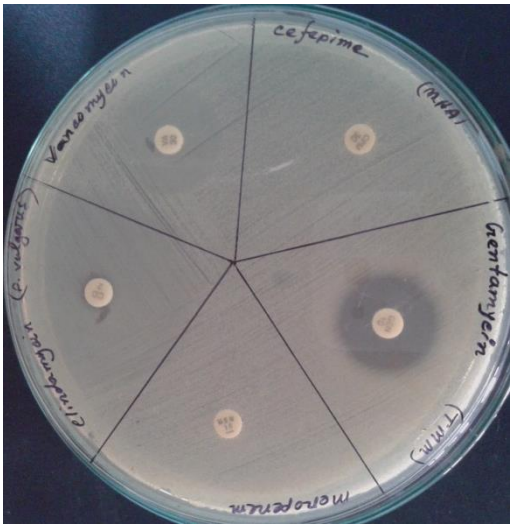
3.1.4 Comparable study of antibacterial activity by showing activity index

Activity index (AI) values are the estimated measure of the potency of antimicrobial activity of plant extracts by quantitatively comparing them to the respective standard antibiotics (Nimmakayala et al., 2014). In this study, the AI values are calculated for the ethanolic and methanolic and acetonic extract of cinnamon and Black cumin against highest inhibition zone producing organisms. Here five different antibiotics, named: Clindamycin, Vancomycin, Gentamicin, cefepime and Meropenem were used to find out the antibiogram.

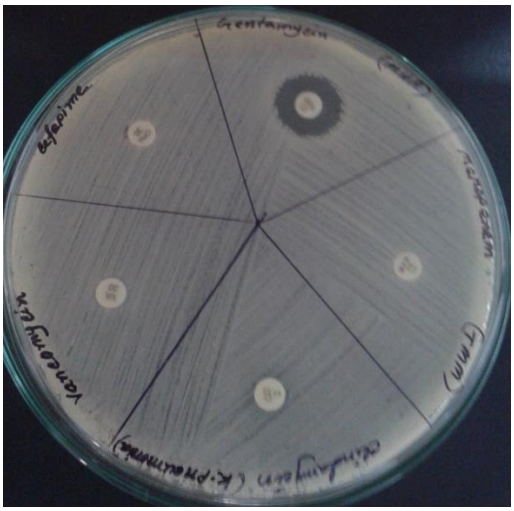
Using the formula, Activity Index (AI) == $\frac{\text{zone of inhibition of extract}}{\text{zone of inhibition of antibiotic}}$, the AI values as shown in the following graphs have been calculated for the highest zone of ethanolic, methanolic and acetonic extract of cinnamon and black cumin.


cefepime
(MRSA) Gentamycin
Meropenem
TMP
clindamycin
Teicoplanin

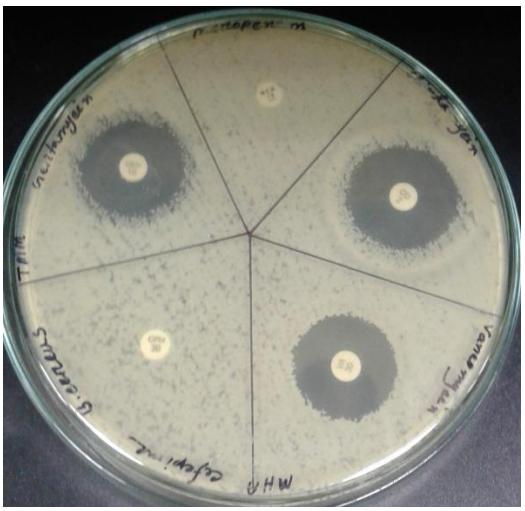
a


cefepime
(MRSA)
Gentamycin
(TMP)
Meropenem
clindamycin
(S. aureus)
Vancomycin

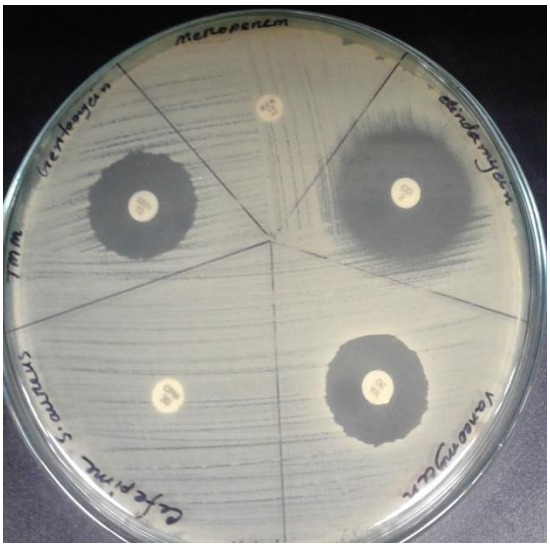
b


Gentamycin
cefepime
(TMP)
clindamycin (K. pneumoniae)
Vancomycin

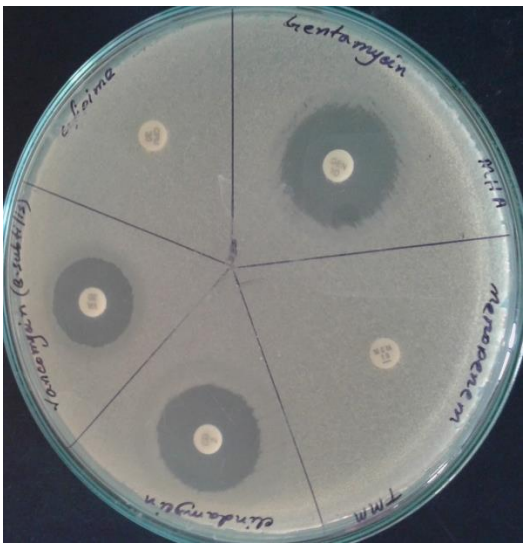
c


Gentamycin
TMP
(S. aureus)
Vancomycin
MHA

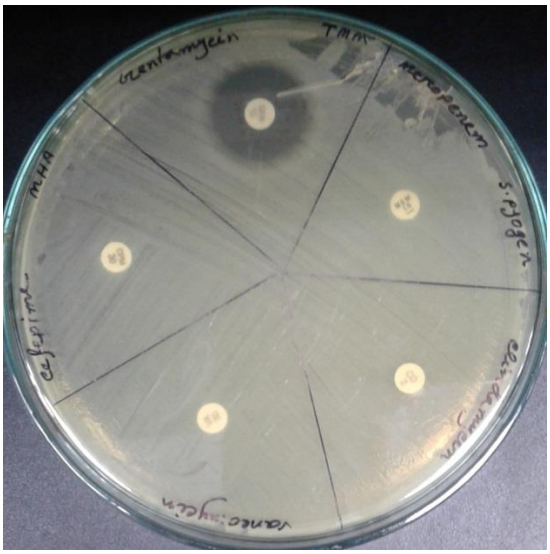
d



e



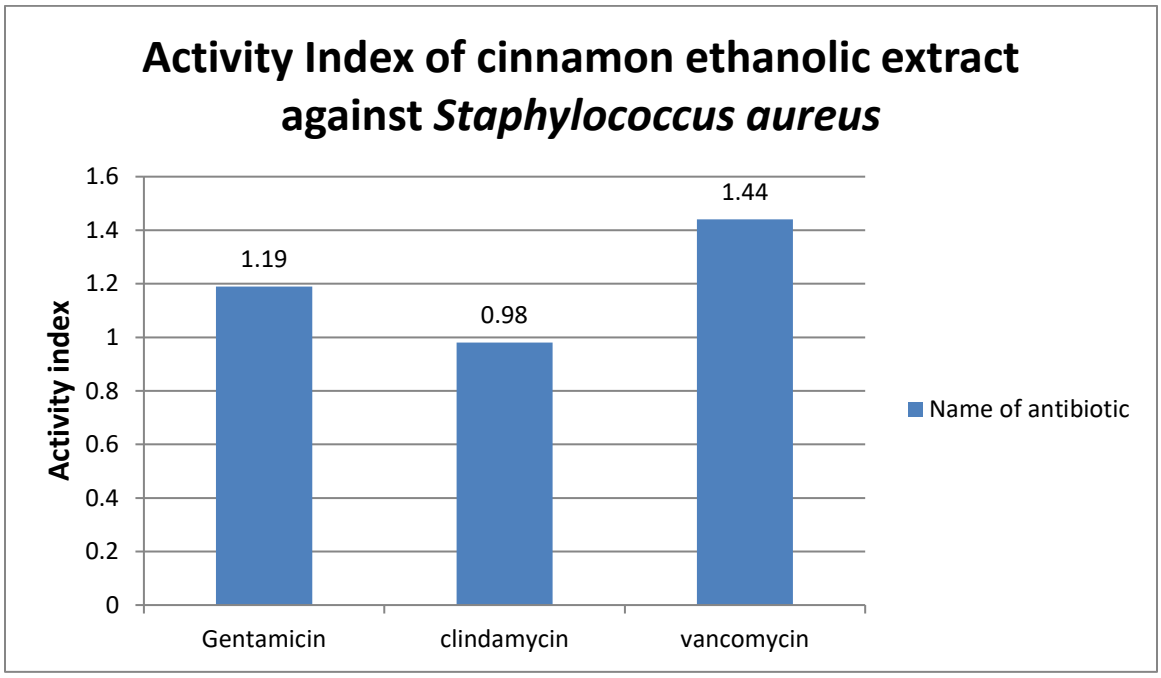
f



g

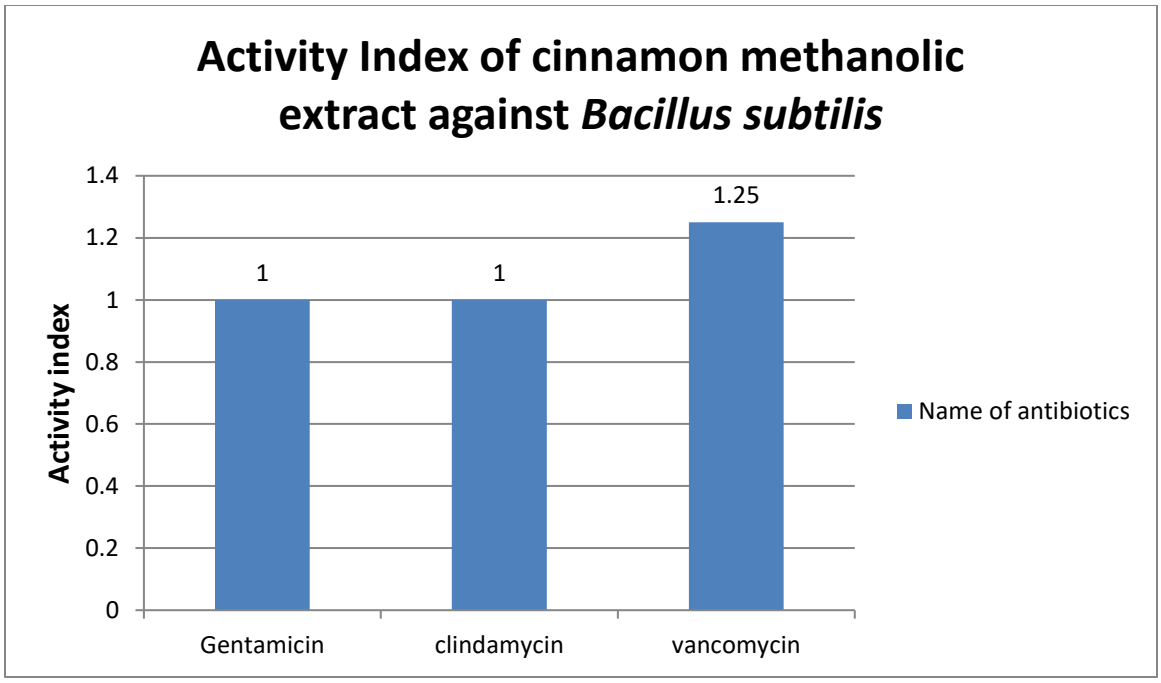
Fig 3.3: antibiogram using gentamicin, meropenem, clindamycin, vancomycin and cefepime against a) *Escherichia coli,* b) *Proteus vulgaris,* c) *K.pneumonia,* d)*B.cereus,* e) *S.aureus,* f) *B.subtilis,* g) *S.pyogen*

3.1.4.1 Activity Index of cinnamon extracts:



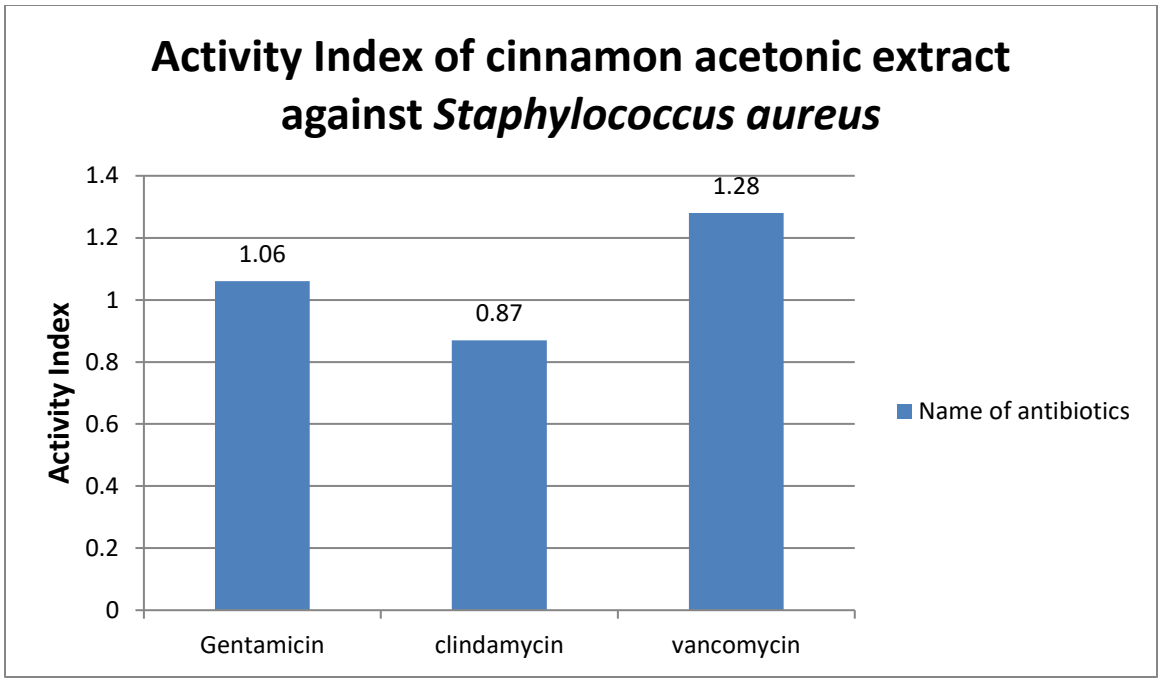
Graph 3.2: The activity index of ethanolic extracts of cinnamon to gentamicin, clindamycin and vancomicin against *S.aureus.*

In this graph, the AI value of cinnamon ethanolic extract to vancomycin is highest(1.44) for *S.aureus* compared to other antibiotics.



Graph 3.3: The activity index of methanolic extracts of cinnamon to gentamicin, clindamycin and vancomicin against *B.subtilis*.

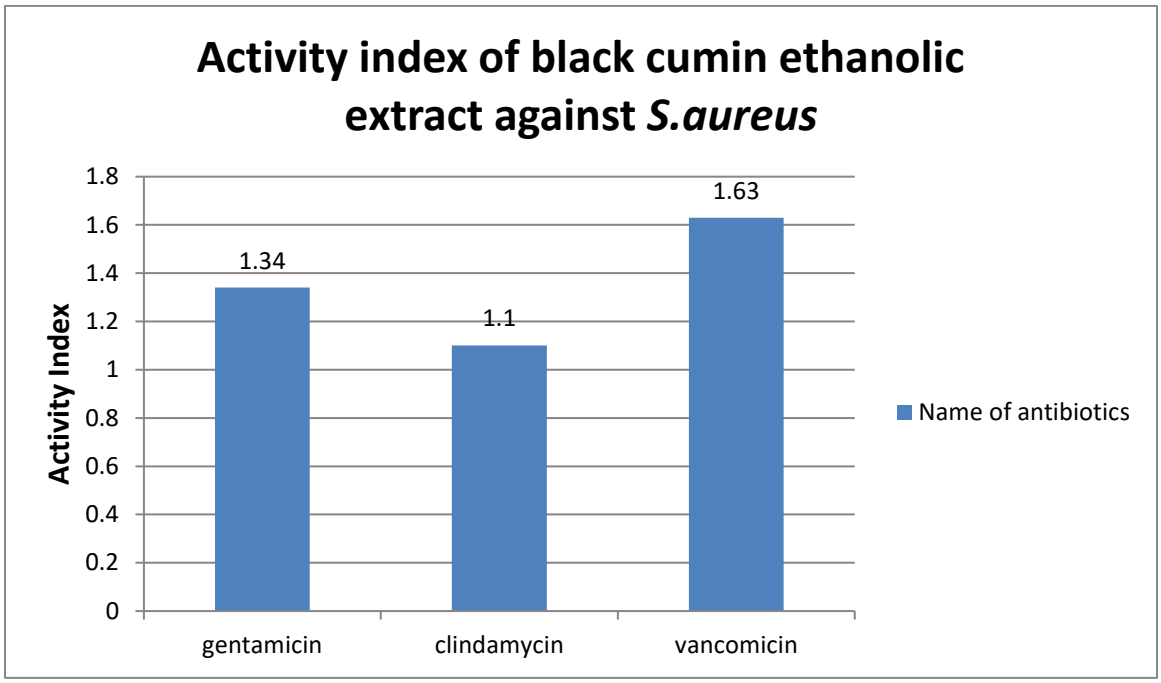
In this graph, the AI value of cinnamon methanolic extract to vancomycin is highest (1.25) for *B.subtilis* compared to other antibiotics.



Graph 3.4: The activity index of acetonic extracts of cinnamon to gentamicin, clindamycin and vancomicin against *S.aureus*.

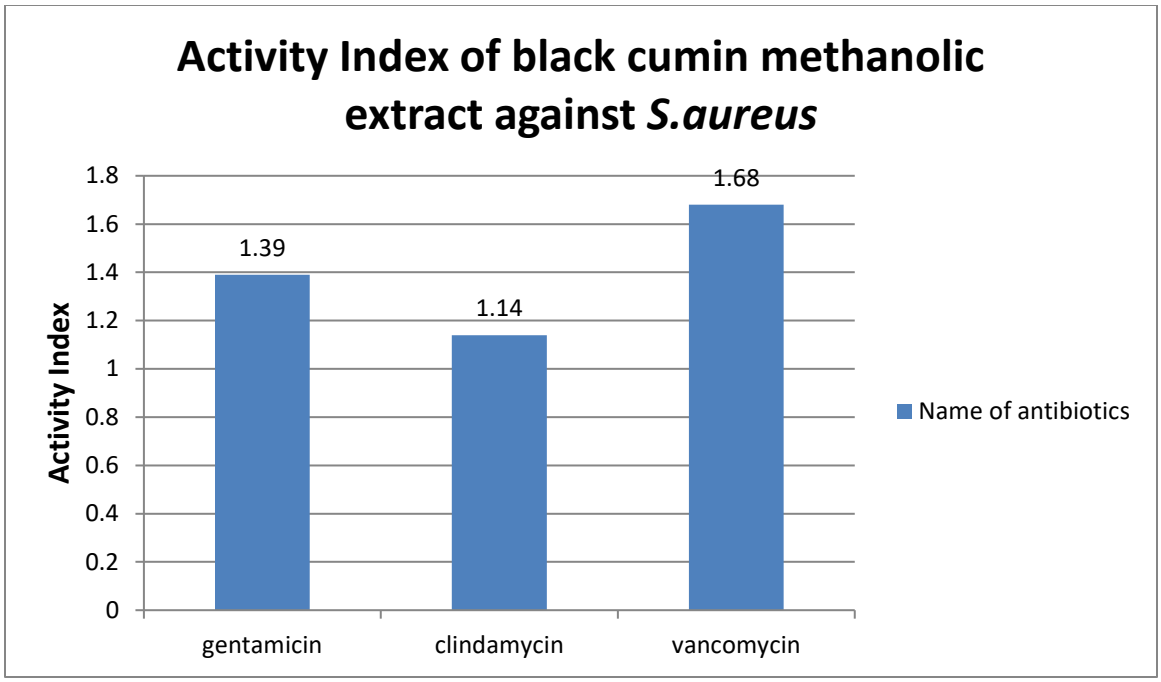
In this graph, the AI value of cinnamon acetonic extract to vancomycin is highest (1.28) for *S.aureus* compared to other antibiotics.

3.1.4.2 Activity Index of black cumin extracts:



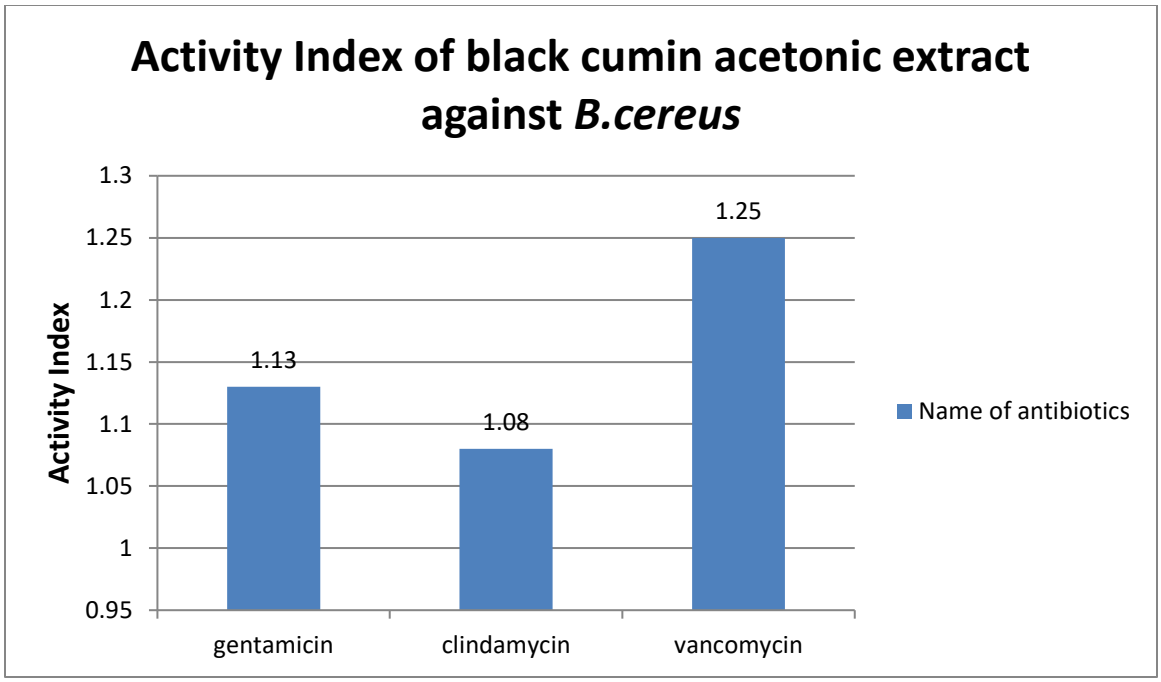
Graph 3.5: The activity index of ethanolic extracts of black cumin to gentamicin, clindamycin and vancomicin against *S.aureus.*

In this graph, the AI value of cinnamon ethanolic extract to vancomycin is highest (1.63) for *S.aureus* compared to other antibiotics.



Graph 3.6: The activity index of methanolic extracts of black cumin to gentamicin, clindamycin and vancomicin against *S.aureus.*

In this graph, the AI value of black cumin methanolic extract to vancomycin is highest (1.68) for *S.aureus* compared to other antibiotics.



Graph 3.7: The activity index of acetonic extracts of black cumin to gentamicin, clindamycin and vancomicin against *B.cereus.*

In this graph, the AI value of cinnamon acetonic extract to vancomycin is highest (1.25) for *B.cereus* compared to other antibiotics.

3.2 MIC and MBC of the most effective plant extract

3.2.1 MIC and MBC of cinnamon extract:

MIC and MBC value are measured using tube dilution method and the results are given below through a table:

Name of extracts	MIC value	MBC value
Ethanolic extract against *S.aureus* (mg\ml)	30	35
Methanolic extract against *B.subtilis* (mg\ml)	25	30
Acetonic extract against *S.aureus* (mg\ml)	30	35

Table 3.3: this table shows the result of MIC and MBC of highest zone producing extracts of cinnamon

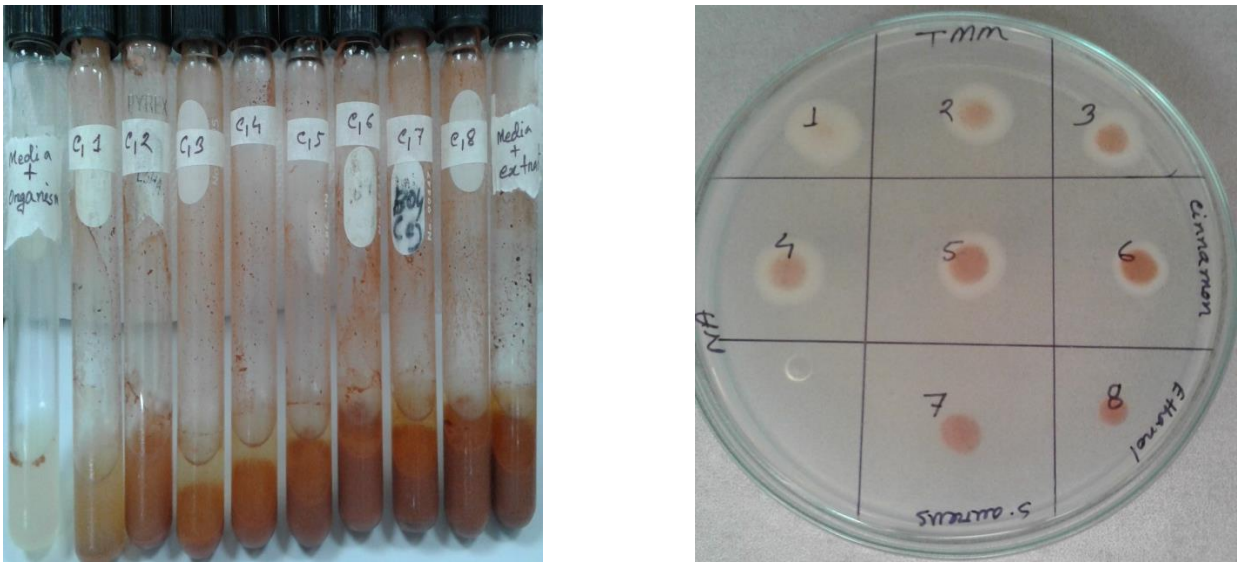


Fig 3.4: MIC and MBC of Ethanolic extract of cinnamon against *S.aureus*

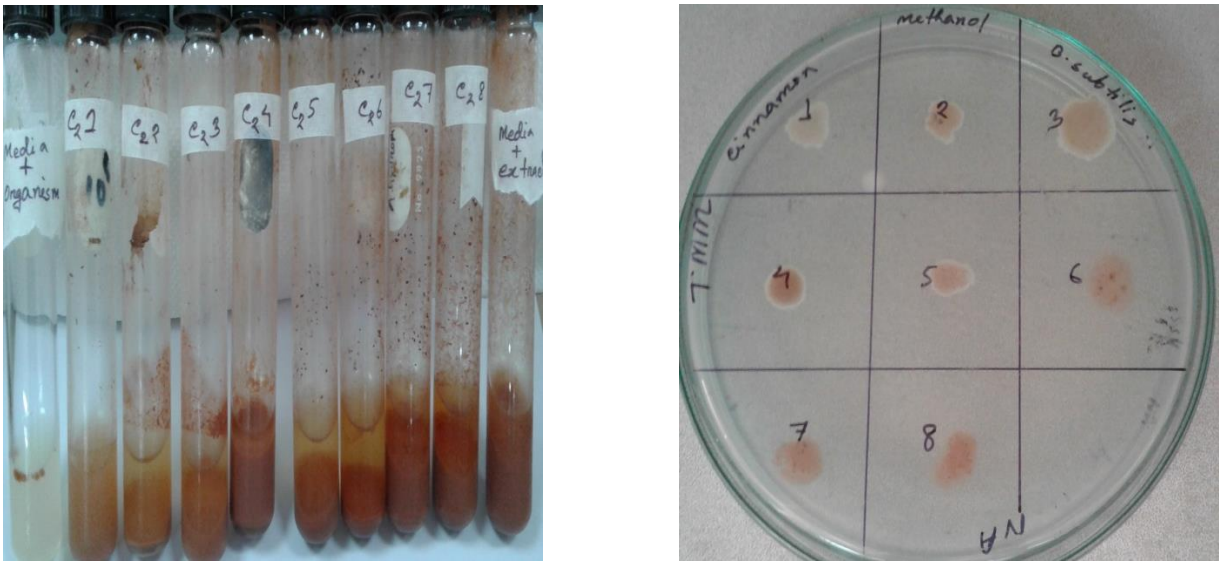


Fig 3.5: MIC and MBC of methanolic extract of cinnamon against *B.subtilis.*

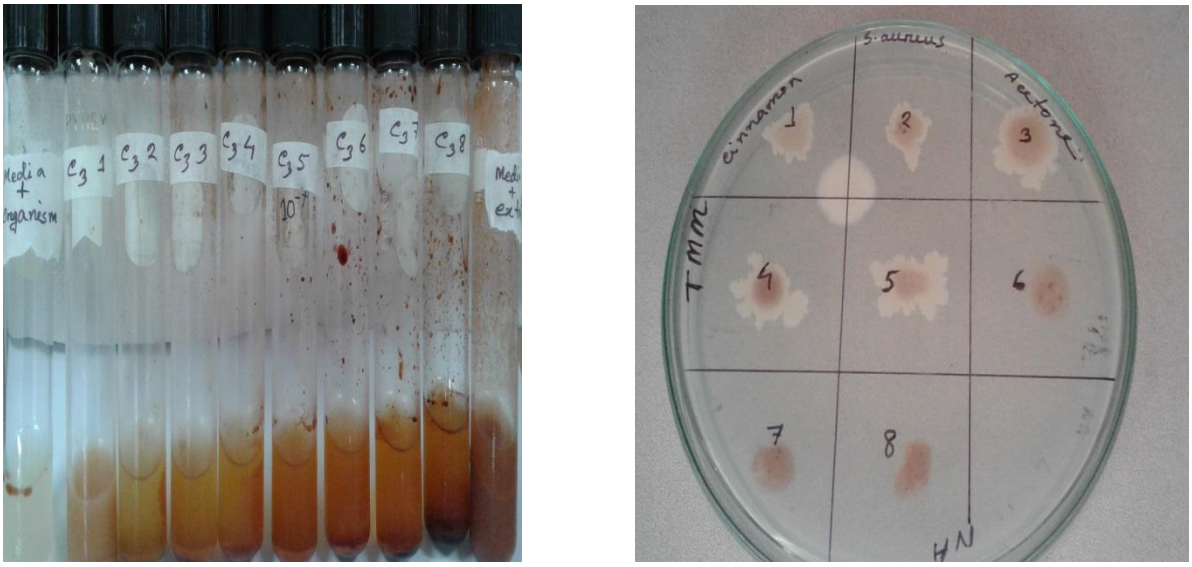


Fig 3.6: MIC and MBC of acetonic extract of cinnamon against *S.aureus.*

3.2.2 MIC and MBC of black cumin extract:

MIC and MBC value are measured using tube dilution method and the results are given below through a table:

Name of extracts	MIC value	MBC value
Ethanolic extract against *S.aureus* (mg\ml)	35	40
Methanolic extract against *S.aureus* (mg\ml)	25	30
Acetonic extract against *B.cereus* (mg\ml)	30	35

Table 3.4: this table shows the result of MIC and MBC of highest zone producing extracts of black cumin.

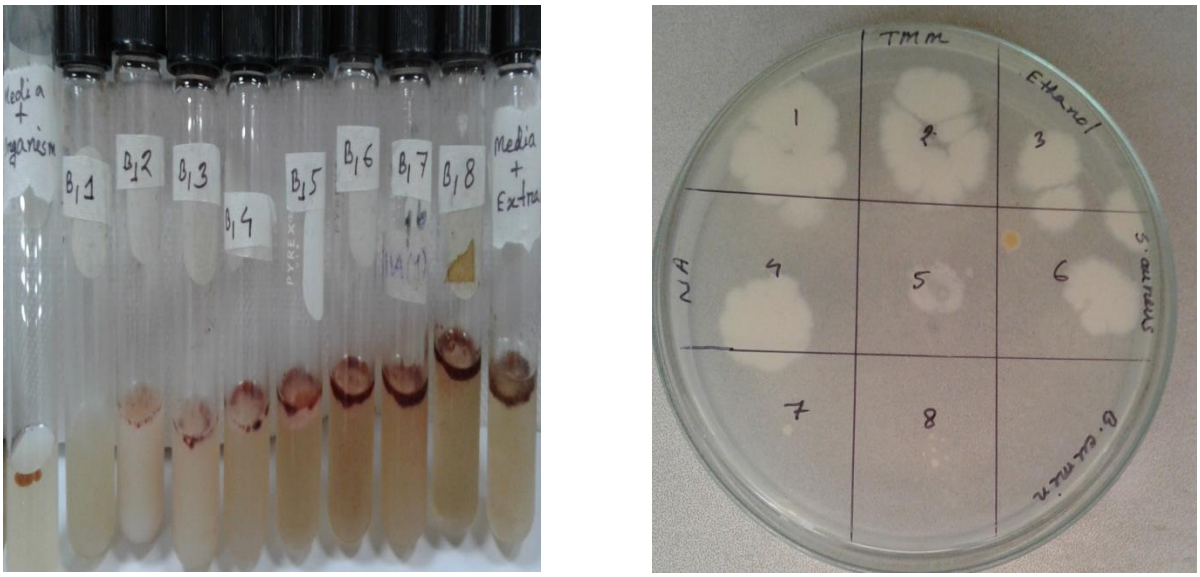


Fig 3.7: MIC and MBC of black cumin ethanolic extract against *S.aureus*

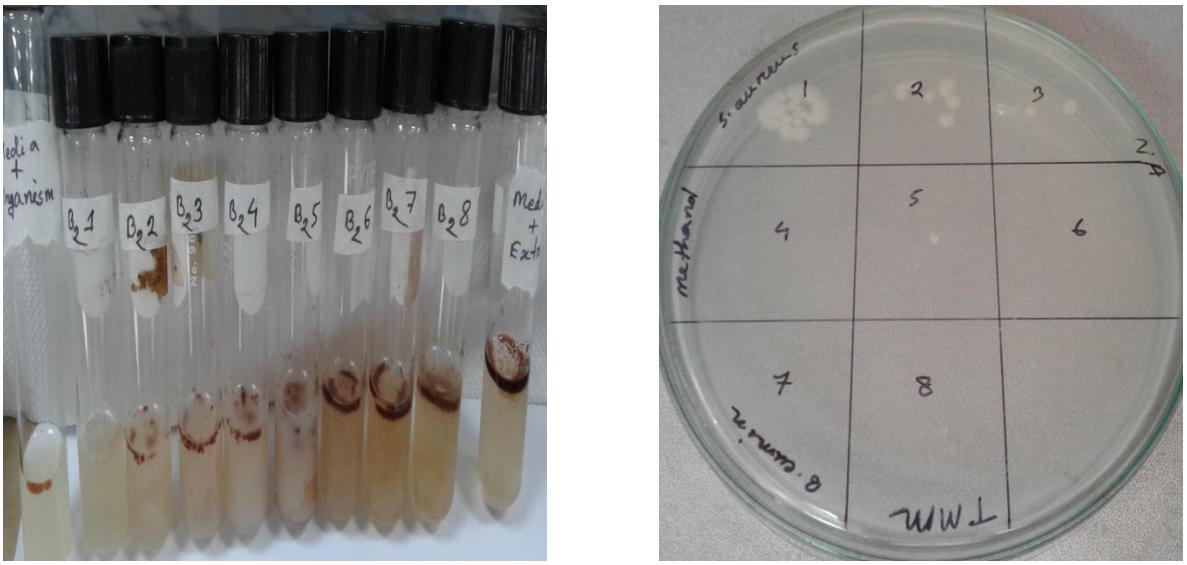


Fig 3.8: MIC and MBC of black cumin methanolic extract against *S.aureus*

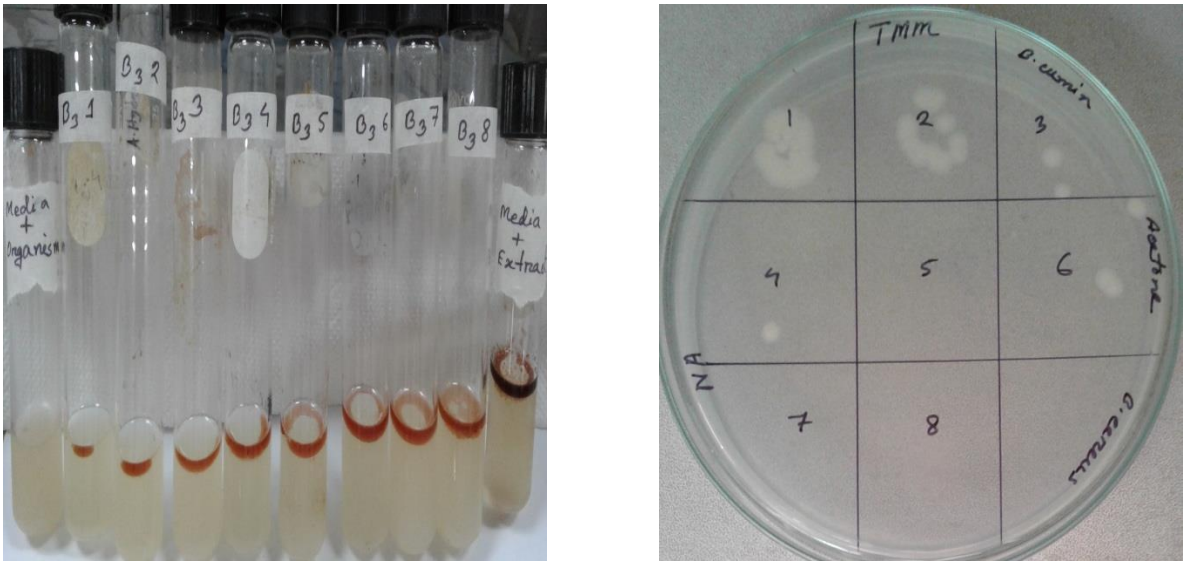


Fig 3.9: MIC and MBC of black cumin acetonic extract against *B.cereus*

3.3 Results of phytochemical screening

3.3.1 Results of phytochemical screening of different extracts of cinnamon:

Name of Chemical group test	Specific test method	Observation of result		
		Ethanolic extract	Methanolic extract	Acetonic extract
Tannin	5% Ferric chloride test	Negative	Negative	Positive
Saponin	Frothing test	Positive	Positive	Positive
Flavonoids	20% sodium hydroxide test	Positive	Positive	Negative
Alkaloid	Dragendorff's test	Positive	Positive	Positive
Phenol	10% Ferric chloride test	Negative	Negative	Positive
Steroid	Salkwasky test	Positive	Positive	Positive
Starch	20% tannic acid test	Positive	Positive	Negative

Table 3.5: phytochemical properties of different extracts of cinnamon

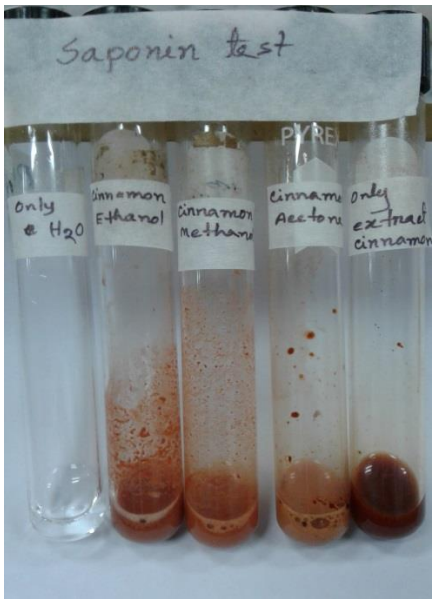


Fig 3.10: Tannin and Saponin test

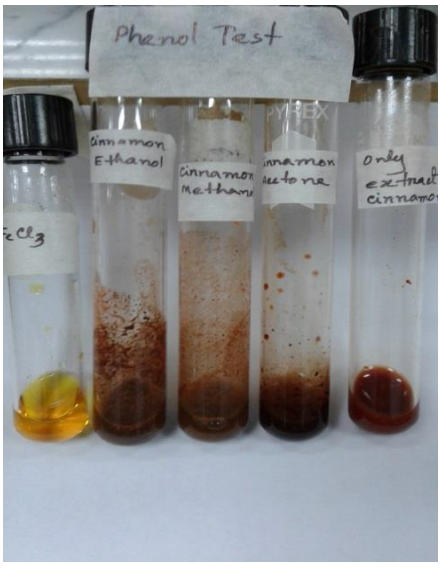


Fig 3.11: Flavonoids and Phenol test

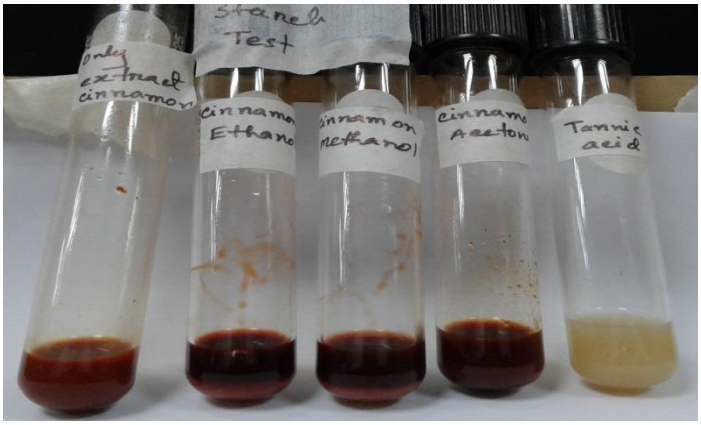


Fig 3.12: Starch test

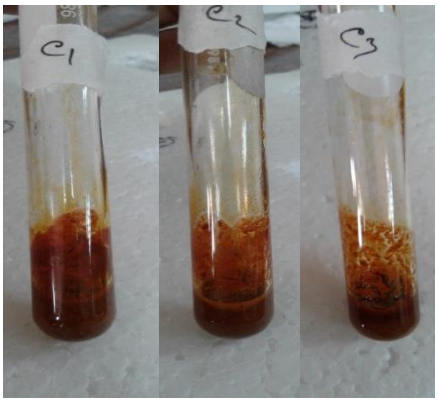


Fig 3.13: Alkaloid test. c1, c2, c3 indicate ethanolic, methanolic and acetonic extract.

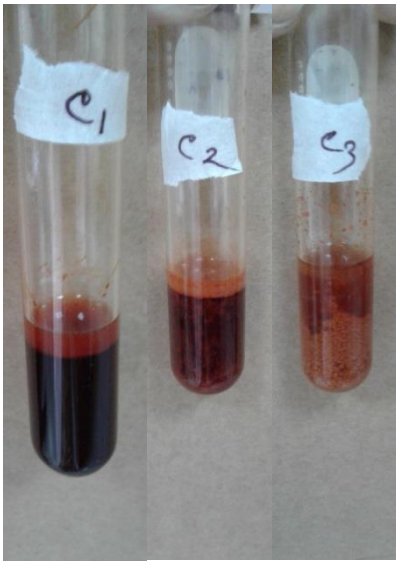
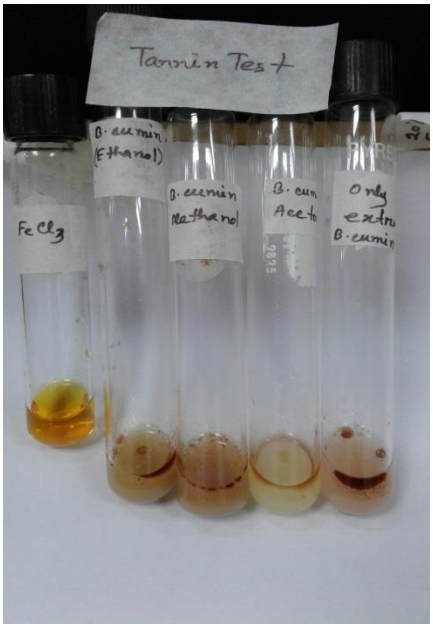


Fig 3.14: Steroid test. c1, c2, c3 indicate ethanolic, methanolic and acetonic extract.

3.3.2 Results of phytochemical screening of different extracts of black cumin:

Name of Chemical group test	Specific test method	Observation of result		
		Ethanolic extract	Methanolic extract	Acetonic extract
Tannin	5% Ferric chloride test	Negative	Negative	Negative
Saponin	Frothing test	Negative	Negative	Negative
Flavonoids	20% sodium hydroxide test	Positive	Positive	Negative
Alkaloid	Dragendorff's test	Positive	Positive	Positive
Phenol	10% Ferric chloride test	Positive	Positive	Negative
Steroid	Salkwasky test	Positive	Positive	Positive
Starch	20% tannic acid test	Negative	Positive	Positive

Table 3.6: phytochemical properties of different extracts of black cumin



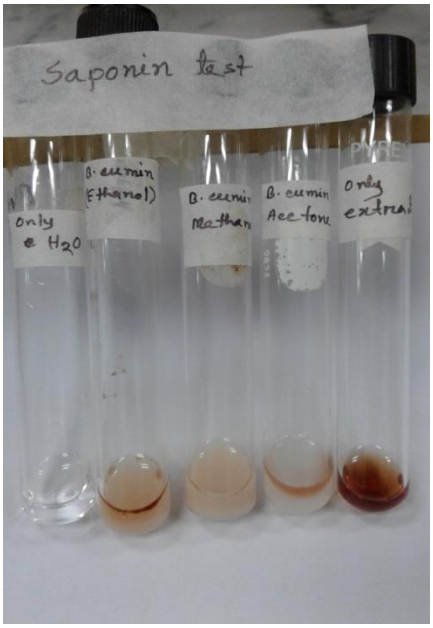


Fig 3.15: Tannin and Saponin test

Fig 3.16: Phenol and Flavonoid test

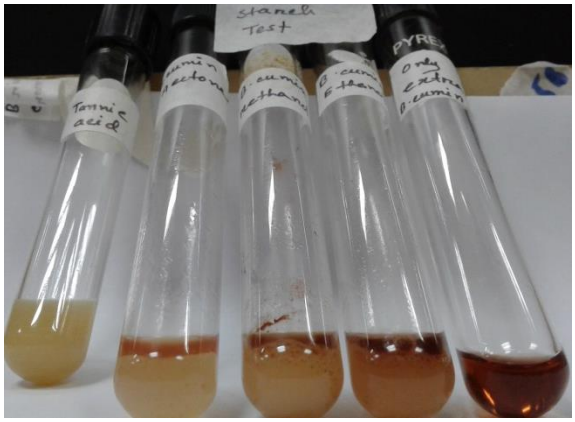


Fig 3.17: Starch test

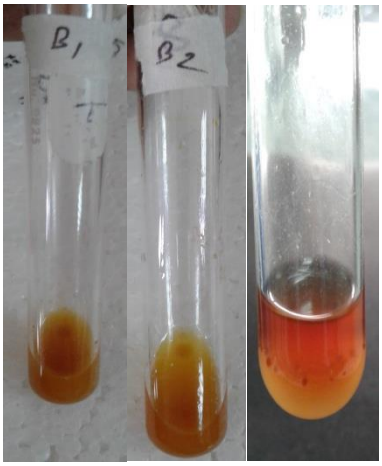


Fig 3.18: **Alkaloid test. b1, b2, b3 indicate ethanolic, methanolic and acetonic extract.**

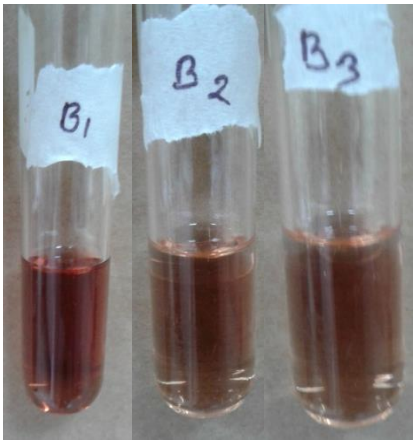


Fig 3.19: Steroid test. b1, b2, b3 indicate ethanolic, methanolic and acetonic extract.

Chapter four:

Discussion and conclusion

Medicinal plants are universally valuable sources of new drugs. Furthermore, up to 80% of people in developing countries are totally dependent on herbal drugs for their primary healthcare, and over 25% of prescribed medicines in developed countries are derived from wild plant species. With the increasing demand for herbal drugs, natural health products, and secondary metabolites of medicinal plants, the use of medicinal plants is growing rapidly throughout the world (Chen et al, 2016). People of Bangladesh traditionally use different types of spices in their daily life. The purpose of this study was to observe the antimicrobial activity of Bangladeshi cinnamon and black cumin extracts against some bacterial isolates along with MIC\MBC value and phytochemical properties.

In this study, the antibacterial activity of ethanolic, methanolic and acetonic extracts of cinnamon and black cumin were tested against seven bacteria named *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes*; *Proteus vulgaris, Escherichia coli*, and *Klebsiella pneumoniae*. The antibacterial tests showed that both cinnamon and black cumin extracts may be used most effectively as an antibiotic agent against microorganisms such as *Staphylococcus aureus, B.subtilis* and *B.cereus*.

ethanolic, methanolic and acetonic extracts of cinnamon showed antibacterial activity more or less against all the selected bacteria (table 3.1). The highest zone of inhibition was observed for the ethanolic extract (27.5mm) and the acetonic extracts (24.5mm) against *Staphylococcus aureus*. Whereas, methanolic extract showed highest zone of inhibition (25mm) against *Bacillus subtilis*. Vakilwala et al., (2017) observed that methanolic extract of *Cinnamomum verum* against *Klebsiella pneumonia, Proteus vulgaris, Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus* have shown zone of inhibition ranging from 21-33 mm but did not show an inhibition against *Escherichia coli*. On the other hand, in our study methanolic extracts of cinnamon showed 18mm zone against *Escherichia coli*.

Here, ethanolic, methanolic and acetonic extracts of black cumin showed higher zone of inhibition against gram positive bacteria than gram negative bacteria. The highest zone of inhibition was observed for the ethanolic extract (31mm) and methanolic extracts (32mm) against *Staphylococcus aureus.* On the other hand, acetonic extracts showed highest zone of inhibition (25mm) against *Bacillus cereus*. Similar work of Zahra et al.,(2011) provided the information that, ethanolic extract of Black Cumin seeds at concentration of 100 mg/ml gave

13mm zone of inhibition against *Staphylococcus aureus*. All extracts of black cumin showed no zone against *Klebsiella pneumonia*. On the other hand in an experiment carried out by Hasan et al., (2013), it was found that, methanolic extract of Black Cumin seeds at concentration of 100 mg/ml, around 15 mm zone of inhibition was shown against *Klebsiella pneumoniae*.

Comparison of the antibacterial activity of the medicinal plant extracts and allopathic antibiotics were done in this study by measuring the activity index values. The AI values are the estimated potency of antimicrobial activity of plant extracts by quantitatively comparing them to the respective standard antibiotics (Nimmakayala et al., 2014). High AI values denote that the extracts have a good activity against the bacteria in comparison with the standard antibiotics (Sridhar et al., 2014).

From this study, the activity index value "1.44" was obtained from the ethanolic extract and "1.28" was obtained from acetonic extract of cinnamon to Vancomycin for *Staphylococcus aureus*. Again, the activity index value "1.25" was obtained from the methanolic extract of cinnamon to Vancomycin for *Bacillus subtilis*. This result indicated that cinnamon extracts are much more effective against the tested bacteria than the allopathic antibiotics.

In case of black cumin the activity index value "1.63" was obtained from the ethanolic extract and "1.68" was obtained from methanolic extract of black cumin to Vancomycin for *Staphylococcus aureus*. Again, the activity index value "1.25" was obtained from the acetonic extract of black cumin to Vancomycin for *Bacillus cereus*. In this experiment meropenem and cefepime showed resistance against all tested bacterial isolates. Moreover, meropenem and clindamycin also showed resistance against *Klebsiella pneumoniae* and *Streptococcus pyogenes* respectively. This result indicated that the Black Cumin seeds extracts are much more effective against the tested bacteria than the allopathic antibiotics.

The MIC and MBC were measured for highest zone producing extracts of cinnamon and black cumin. From table 3.3 it can be acknowledged that the lowest value of MIC (25mg\ml) and MBC (30mg\ml) is of methanolic extract against *Bacillus subtilis*. On the other hand, for ethanolic and acetonic extracts of cinnamon MIC\MBC value are same against *Staphylococcus aureus* (MIC is 30mg\ml and MBC is 35mg\ml). In a study by Vakilwala et al., 2017 found that MIC value of cinnamon methanolic extract was 25mg\ml against *Bacillus subtilis* which is similar to our result.

From table 3.4 it can be observed that MIC\ MBC of ethanolic extract (35mg\ml)\ (40mg\ml) and methanolic extracts (25mg\ml)\ (30mg\ml) was found against *Staphylococcus aureus* of black cumin. Again MIC and MBC of acetonic extract was found 30mg\ml and 35mg\ml respectively against *Bacillus cereus*. According to present study, our observation coincides with Ishtiaq et al., 2013, the MIC of methanolic extract is 25 mg/ml against *S. aureus*.

In this study all extracts of cinnamon showed positive result for saponin, alkaloid and steroid (table3.5). Then, ethanolic and methanolic extract showed positive result for flavonoids and starch while acetonic extract showed negative result for those tests. On the other hand, tannin and phenol test showed negative result for ethanolic, methanolic extracts and positive result for acetonic extract. Our observation coincides with Snehlata et al., (2014) which showed the presence of sapinin, alkaloid and steroid in all extracts of cinnamon.

Here, all the extracts of black cumin showed positive result for alkaloid, steroid and negative result for tnnin, saponin (table 3.6). Then, ethanolic and methanolic extract showed positive result for flavonoids and phenol while acetonic extract showed negative result for those tests. On the other hand, starch was present in methanolic and acetonic extracts while it was absent in ethanolic extract. Our observation coincides with Ishtiaq et al., (2013) which showed the presence of alkaloid and steroid in all extracts of black cumin.

Conclusion

The aim of this study is to see the effectiveness of Bangladeshi cinnamon and black cumin extracts. Seeing the results of the antibacterial activity of both cinnamon and black cumin, we can say that those extracts can be used as antibacterial agent against those tested bacteria specially against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*. This antibacterial activity depends on concentration of extracts. From this study other researchers would be motivated to do further research on in vivo and in vitro experiment to use this antibacterial activity of cinnamon and black cumin in medical sciences.

Chapter five: Reference

Al-daihan,S., Al-faham, M., Al-shawi, N., Almayman, R., Brnawi, A., & Bhat, R. (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. https://doi.org/10.1016/j.jksus.2012.11.003

Hasan, N. A., Nawahwi, M. Z., & Malek, H. A. (2013). Antimicrobial activity of nigella sativa seed extract. Sains Malaysiana, 42(2), 143–147.

Hassan, S. A., Barthwal, R., Nair, M. S., & Haque, S. S. (2012). Aqueous Bark Extract of Cinnamomum Zeylanicum : A Potential Therapeutic Agent for Streptozotocin- Induced Type 1 Diabetes Mellitus (T1DM) Rats, 11(June), 429–435.

Ishtiaq, S., Ashraf, M., Hayat, M. Q., & Asrar, M. (2013). Phytochemical Analysis of Nigella sativa and its Antibacterial Activity against Clinical Isolates Identified b y Ribotyping, 7, 1151–1156.

Abbas, R.Z., D.D. Colwell and J. Gilleard, 2012a. Botanicals: An alternative approach for the control of avian coccidiosis. World Poultry Sci. J., 68: 203–215

Abbas, R.Z., Z. Iqbal, A. Khan, Z.U.D. Sindhu, J.A. Khan, M.N. Khan and A. Raza, 2012b. Options for integrated strategies for the control of avian coccidiosis. Int. J. Agric. Biol., 14: 1014–1020

Arunkumar, S. and M. Muthuselvam, 2009. Analysis of phytochemical constituents and antimicrobial activities of Aloe vera L. against clinical pathogens. World J. Agric. Sci., 5: 572–576

Boskabady, M.H., N. Mohsenpoor and L. Takaloo, 2010. Antiasthmaticeffectof Nigella sativa in airways of asthmatic patients. Phytomedicine, 17: 707–713

Criagg, G.M. and J.N. David, 2001. Natural product drug discovery in the next millennium. J. Pharm. Biol., 39: 8–17

El-Abhar, H.S., Abdallah, D.M. and S. Saleh, 2003. Gastroprotective effect of black seed. J. Ethnopharmacol., 84: 251–258

Eloff, J.N., 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharmacol., 60: 1–8

El-Sayed, M.M., H.A. E1-Bana and E.A. Fathy, 1996. The use of Nigella sativa oil as a natural preservative agent in processed cheese spread. Egypt. J. Food Sci., 22: 381–396

Enomoto, S., R. Asano, Y. Iwahori, T. Narui, Y. Okada, A.N. Singab and T.Okuyama, 2001. Hematological studies on black cumin oil from the seeds of Nigella sativa L. Biol. Pharmaceut. Bull., 24: 307–310

Eugene, A.R., Y.I. Oshchepkova, T.I. Odintsova, N.V. Khadeeva, O.N. Veshkurova, T.A. Egorov, E.V. Grishin and S.I. Salikhov, 2011. Novel antifungal defensins from Nigella sativa L. seeds. Plant Physiol. Biochnol., 49: 131-137

Harborne, J.B., 1973. Phytochemicals Methods. Chapman and Hall Ltd., London

Hashem , F.M. and M.A. El-Kiey, 1982. Nigella sativa seeds of Egypt. J. Pharm. Sci., 3: 121-133

Hosseinzadeh, H., M. Eskandari and T. Ziaee, 2008. Antitussive effect of thymoquinone a constituent of Nigella Sativa Seeds in guinea pigs. Pharmacologyonline, 2: 480-484

Javed, S. and A.A. Shahid, 2010. Nutritional, phytochemical potential and pharmacological evaluation of Nigella sativa (Kalonji) and Trachyspermum ammi (Ajwain). J. Med. Plants Res., 6: 768–775

Karapinar, M. and S.E. Aktug, 1987. Inhibition of food borne pathogens by thymol, eugenol, menthol and anethole. Int. J. Food Microbiol., 4: 161-166

Nostro A, Germano MP, Dangelo V, Marino A, Cannatelli MA. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett Appl.Microbiol 30: 379-384, 2000.

Chitravadivu C., Manian S.,and Kalaichelvi K.. Qualitative Analysis of Selected Medicinal Plants. Tamilnadu. India Midd-East j of Scienti Resea. 4 (3): 144-146, 2009.

Mabrouk SS, El- ShayebNMA. Inhibition of aflatoxin formation by some spices. LebensmUntersForsh 171: 344-347, 1980.

Ramadan FM, El-Zanfaly HT, El- Wakeil FA, Alian AM.On the antibacterial effects of some essential oils.I. Use of agar diffusion method.ChemMikrobiolTechnol.Lebensm 25: 51-55,1972.

Barbosa Canovas GV, Pothakamury VR, Paulo E, Swanson BG. Non-thermal preservation of foods. Marcel Dekker Inc New York , 1998.

Nyarko A. A., Addy M. E.Effects of aqueous extract of Adenia cissampeloideson blood pressure and serum analyze of hypersensitive patients. Phytotherapy Res. 4(1): 25-28, 1990.

Amin Mir M., Sawhney, S.S. and Jassal, M.M.S. Qualitative and quanditative analysis of phytochemicals of Taraxacumofficinale.Wudpecker J. Phar. and Pharmaco. 2 (1): 001-005, 2013.