Study of the Antimicrobial Activities of the Bark Extract of *Cinnamomum verum* on Multi Drug Resistant *Klebsiella pneumoniae* and *Enterobacter agglomerans*

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

Submitted by
Mohammad Al Mahmud-Un-Nabi
Student Id.: 11326004
August, 2015

Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh.

DECLARATION

I hereby declare that the thesis project titled **"Study of the Antimicrobial Activities of the Bark Extract of *Cinnamomum verum* on Multi Drug Resistant *Klebsiella pneumoniae* and *Enterobacter agglomerans*"** submitted by me has been carried out under the supervision of Dr. Md. Kaiissar Mannoor, Scientist, Institute for Developing Science & Health Initiatives (ideSHi), Mohakhali, Dhaka as External Supervisor and Dr. Mahboob Hossain, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali, Dhaka as Internal Supervisor. It is further declared that the research work presented here is based on actual and original work carried out by me. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

(Mohammad Al Mahmud-Un-Nabi)
Candidate

Certified

(Dr. Md. Kaiissar Mannoor)
External Supervisor
Scientist
Institute for Developing Science & Health Initiatives (ideSHi)
IPH Building (2nd Floor)
Mohakhali, Dhaka-1212,
Bangladesh.
E-mail: kaiissar@ideshi.org

(Dr. Mahboob Hossain)
Internal Supervisor
Associate Professor
Department of Mathematics and Natural Sciences
BRAC University
66 Mohakhali, Dhaka-1212,
Bangladesh.
E-mail: mmhossain@bracu.ac.bd

ACKNOWLEDGEMENT

All praises be to the Almighty Allah.

The accomplishment of my thesis would not be possible without the help of some accommodative people. First of all, I would like to express my sincere gratitude to **Prof. A. A. Ziauddin Ahmad**, Chairperson, Department of Mathematics and Natural Sciences; **Prof. Naiyyum Choudhury**, Chairman at Bangladesh Atomic Energy Regulatory Authority (Ex. Coordinator of the Microbiology and Biotechnology Programme, Department of Mathematics and Natural Sciences) and **Dr. M. Mahboob Hossain**, Associate Professor, Department of Mathematics and Natural Sciences.

I would like to give my heartiest thanks to **Dr. Firdausi Qadri,** Director of Centre for Vaccine Sciences, Senior Scientist & Head of Immunology Unit, icddr,b and Executive Director, Institute for Developing Science & Health Initiatives (ideSHi), for giving me the excellent opportunity to complete my thesis in her laboratory.

I am highly indebted to my Supervisor **Dr. Md. Kaiissar Mannoor**, Scientist, ideSHi for guiding me and solved my problems which I have faced while doing the thesis. His new ideas helped me so much with the thesis work.

I am also thankful to all the laboratory assistant of ideSHi laboratory including **Suprovath Kumar Sarkar, Mohammad Amir Hossain, Tariqul Islam, Golam Sarower Bhuyain, Asifuzzaman Rahat** and **Grace Eckhoff** for their continuous support and advice for my thesis work. Without their help it would be really difficult for me to complete my thesis.

ABSTRACT

Infections caused by Multi Drug Resistant (MDR) organisms cause to stand a worldwide pandemic. MDR bacteria are an emerging threat with limited therapeutic options. Without having information about these strains, morbidity and mortality due to infections caused by these notorious bugs cannot be reduced. Alternative antimicrobial agent is needed beside the antibiotics to combat against these MDR organisms. Cinnamon is a spice obtained from the inner bark of trees of *Cinnamomum* sp. Traditionally it has also been used for medicinal purposes. In the current work we evaluated the antimicrobial activity of the cinnamon extract against MDR *Klebsiella pneumoniae* and *Enterobacter aggalomerans*, and also compared the results with drug sensitive organisms of the same species. Cinnamon extract was prepared with ethanol extraction procedure. The extract was dried and re-dissolved in Dimethyl sulfoxide (DMSO). The prepared extract was tested on the organisms by using agar diffusion technique followed by determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the cinnamon extract. From the agar diffusion test the minimum concentration of cinnamon extracts needed to visualise the zone of inhibition on the Mueller Hinton Agar was determined to be 40 mg/ml. The MIC of the cinnamon extract was found to be 1 mg/ml and the MBC was determined as 2 mg/ml. The results obtained from this study can act as the stepping stone in investigating the antimicrobial properties of cinnamon extract. This study could be the beginning of discovering a new antimicrobial agent against the MDR organisms.

CONTENTS

List of Tables

List of Figures

List of Abbreviations

API	Analytical Profile Index
AST	Antibiotic Sensitivity Test
BSL	Bio-Safety Level
CLSI	Clinical & Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
HGT	Horizontal Gene Transfer
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
ideSHi	Institute for Developing Science and Health Initiatives
Mac.	MacConkey Agar
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistant
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
Sen.	Drug Sensitive
STGG	Skimmed Milk, Tryptone, Glucose, and Glycerine
ULT	Ultra Low Temperature

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Overview

After the discovery of penicillin by Alexander Fleming in 1928, these antibiotics are being widely used for treatment against infection. However, bacteria are developing resistance against these antibiotics while they are being applied on them. These multi drug resistant (MDR) organisms are now becoming one of the major concerns throughout the world. The regularly prescribed antibiotics are not strong enough to kill or inhibit those organisms (Brown, 2007). On the other hand, MDR organisms can transfer various antibiotic resistant genes to the wild type organisms by Horizontal Gene Transfer (HGT) and making it resistant to the antibiotics (Gyles & Boerlin, 2014). MDR *Escherichia coli, Klebshiella pneumonia, Haemophillus influenzae* and other organisms spread easily from hospitals and can be isolated from community acquired infections (Akram, Shahid, & Khan, 2007). As microbes are getting stronger by developing resistance against many antibiotics, it is getting difficult to control them. New antibiotics are required to combat against these organisms.

Use of different plant parts as medicine has been a traditional practise all over the world. Herbalism is now the alternative solution beside antibiotics to fight against MDR organisms. Plant naturally develops antimicrobial properties and so plant extracts are used for medicinal purpose (Khan, et al., 2013).

Cinnamon is a spice used for cooking and also as herbal medicine. The use of cinnamon started from 2000BC in Egypt. In medieval time doctors started to use it as medicine in order to treat conditions such as coughing, arthritis and sore throats.

1.2 Background information on Cinnamon

Cinnamon is a dried inner bark of the plant *Cinnamomum* sp. The species of cinnamon found in the common market are *Cinnamomum cassia, C. burmannii, C. loureiroi* and *C. verum.* Among these species *C. verum* (also known as Ceylon cinnamon) is sometimes considered to be the true cinnamon. Leaves of cinnamon are used in tea for flavour. Cinnamon is a very popular culinary spice and is also used in candy, gum, incense, toothpaste and perfumes.

Domain	Eukarya
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Magnoliidae
Order	Laurales
Family	Lauraceae
Genus	*Cinnamomum*
Species	*Cinnamomum cassia, C. burmannii, C. loureiroi, C. verum, C. tamala, C. camphora, C. elongatum, C. mexicanum, C. montanum* and *C. sessilifolium*.

Table 1.1- Scientific classification of cinnamon (Plant Database, 2015).

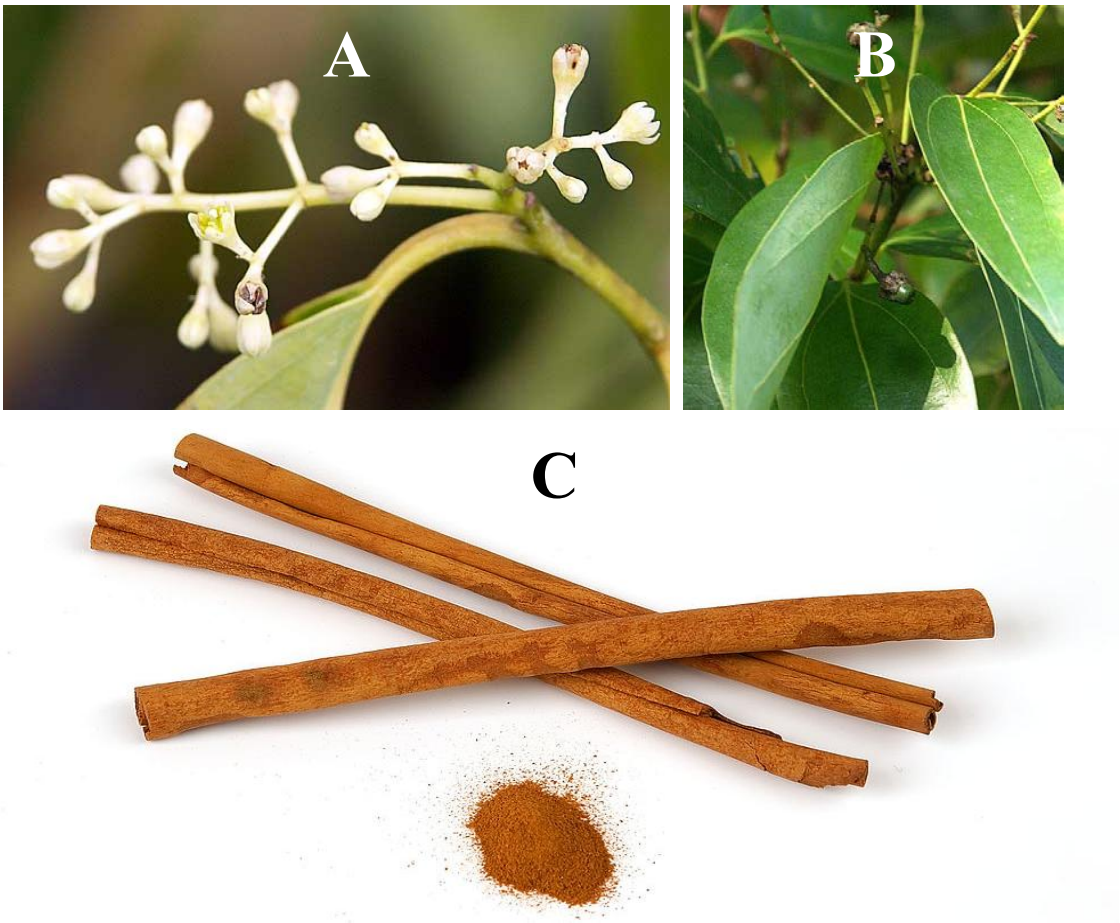

Figure 1.1- (A) Cinnamon bud; (B) Cinnamon leaves; (C) Cinnamon bark and its powder. (Photo Source: National Tropical Botanical Garden)

1.3 Medical importance of Cinnamon

Today's advance research has found many beneficial properties of this spice. Some of the beneficial properties of cinnamon are highlighted below (Leech, 2015) -

a. Traditionally, it is used to treat coughing, arthritis and sore throats
b. It contains antioxidants
c. It has anti-inflammatory properties
d. It may reduce risk of heart disease
e. It can improve sensitivity to insulin
f. It lowers blood sugar level and has a powerful anti-diabetic effect
g. It may have beneficial effects on neurodegenerative diseases
h. It may be protective against cancer
i. It helps fight bacterial and fungal infections
j. It may help fight the HIV virus

Other than the benefits, the cinnamon might have some side effects also (Cinnamon | NCCIH, 2011) like

a. Might be allergic to some people.
b. *Cinnamomum cassia* contains coumarin, the parent compound of warfarin, which prevents blood from clotting.
c. *C. cassia* contains some other chemicals which might harm liver. So, it is not suggested for liver disease patients.

1.4 History of antibiotics and bacterial resistance to it

The early history of antibiotics suggests that people unknowingly have been using antibiotics in order to heal wounds and treat infections. Mould and other plant parts were used to the site of wounds to heal. In 1928, Sir Alexander Fleming discovered penicillin from the fungus *Penicillium notatum* which was able to kill other microorganisms (Diggins, 1999). This was a great discovery in the history of science. Later, the use of antibiotics had started significantly and during the World War II, it was a life saver which helped to reduce the overall number of amputations and deaths (Bradley).

The use of antibiotics has increased day by day and more are being developed which are

much more efficient. Some of the antibiotics are no more effective on the organisms as they are developing resistance against the antibiotics due to indiscriminate use of them. As a consequence, pathogens resistant to two or more antibiotics are very common and numbers of Multi Drug Resistant (MDR) pathogens are increasing day by day.

1.5 Screening for the antimicrobial potential of the plant extracts

To develop an antibiotic is not an easy task and requires much effort, time and resources. Investigation of the antimicrobial properties of plants provides a promising ways and means to look for an alternative to antibiotics. For the pathogenic MDR organisms, where most of the antibiotics are not effective, some other antimicrobial substance has to be used as an alternative. Nature provides many antimicrobial substances which are effective against most of the organisms including the MDR pathogens. Plants products exhibiting natural antimicrobial activities including eugenol, thymol, carvacrol, cinnamaldehyde, allyl isothiocyanate, etc. are very effective against MDR organisms individually and are more effective when paired with an antibiotic. In a study, thymol and carvacrol were found to be highly effective in reducing the resistance of *Salmonella typhimurium* SGI 1 (tet A) to ampicillin, tetracycline, penicillin, bacitracin, erythromycin and novobiocin and resistance of Streptococcus pyogenes ermB to erythromycin. With *Escherichia coli* N00 666, thymol and cinnamaldehyde were found to have a similar effect in reducing the minimum inhibitory concentrations (MIC) of ampicillin, tetracycline, penicillin, erythromycin and novobiocin. Similarly, carvacrol, thymol and cinnamaldehyde were effective against *Staphylococcus aureus* blaZ and reduced the MIC's of ampicillin, penicillin and bacitracin. Allyl isothiocyanate was effective in reducing the MIC of erythromycin when tested against *S. pyogenes* (Palaniappan & Holley, 2010).

Currently, 25-50% of the pharmaceutical products are derived from plant, but they are not used as antimicrobials. The use of plant parts for the treatment was traditionally practiced with great success for a long period of time. Western medicine is trying to duplicate their successes. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids are known to have antimicrobial activity tested in vitro (Cowan, 1999). The crude ethanol extract of the powder of dried leaves of *Acacia nilotica,* dried bark of *Cinnamomum zeylanicum* (aka. *Cinnamomum verum*) and dried

buds of *Syzygium aromaticum* can be used against MDR organisms causing nosocomial and community acquired infections (Khan, et al., 2009).

1.6 Study subjects

In a study of Acute Respiratory Infection in Institute for Developing Science and Health Initiative (ideSHi) Laboratory, nasal swab specimens were collected from suspected children (age group between 0-5). It was found that the bacteria isolated from the nasal specimens were mostly resistant to more than one antibiotic. This finding gives us the idea that most of the bacterium isolated from the community acquired infections are resistant to any of the commonly prescribed antibiotics. In that study, out of 100 specimens two strains of bacteria were found to have MDR characteristics. These MDR bacteria were resistant to more than thirteen antibiotics, including first (Gentamycin, Tobramycin, Ceftriaxone, Cefixime, Ciprofloxacin, Imipenem, Meropenem and Azithromycin) and second line antibiotics (Amikacin, Netilmicin, Pipercillin/Tazobactam, Carbenicillin and Polymyxin B) according to Clinical and Laboratory Standard Institute (CLSI). Due to the MDR properties of the bacteria, the infections cannot be easily treated by the common antibiotics. Antibiogram test has to be done at first to find the antibiotic sensitivity pattern of the bacteria which could be beneficial for proper treatment of the patients.

1.7 Aims and objectives

Aim of this study was to find the antimicrobial activity of the ethanolic cinnamon extract on MDR bacteria isolated from the nasal swab specimens collected from acute respiratory infected children. At the same time, the sensitive bacterial strains of the same species were also studied to compare the result with MDR strains. The objectives of this study were set to:

a. Ethanolic extraction of the grinded cinnamon bark;
b. Use the extract to check the antimicrobial activity by agar diffusion method against MDR and sensitive strains;
c. Find the Minimum Inhibitory Concentration (MIC) of the cinnamon extract and
d. Find the Minimum Bactericidal Concentration (MBC) of the cinnamon extract.

Chapter 2

Methodology

Chapter 2: Methodology

2.1 Working laboratory

All the works were performed in the ideSHi Laboratory, IPH Building, Mohakhali, Dhaka, from February 2015 to June 2015. This Laboratory has got the Biosafety Level 2 (BSL-2) facility. All the microbiological works were done inside Biological Safety Cabinet.

2.2 Reference bacterial strains

In this study, I worked with four standard clinical strains of bacteria including Multi Drug Resistant (MDR) *Klebshiella pneumonia,* MDR *Enterobacter agglomerans and* their partially resistant strains which are referred as Drug Sensitive Strain (Sen.). The sensitive strain of *Klebshiella pneumoniae* was resistant to Azithromycin and Penicillin and the sensitive strain of *Enterobacter agglomerans* was resistant to Azithromycin and Amoxicillin antibiotics. The strains were obtained from the preserved bacterial stock of collected nasal swab samples from AREI study in ideSHi Laboratory.

2.2.1 Preparation for plating bacteria

The referred strains were taken from the STGG (a medium containing skim milk, tryptone, glucose and glycerin) stock culture from -80°C. They were streaked on MacConkey (Mac.) and Nutrient Agar (NA) Plate and incubated overnight at 37°C for growth in an aerobic incubator. The bacterial colony morphology was studied carefully. Isolated single colony obtained from the NA was enriched in Mueller Hinton Broth (MHB) for about 2 hours at 37°C in shaking incubator. Then they were prepared for Antibiotic Sensitivity Test (AST).

2.2.2 Confirmation of the reference strains

The strains were confirmed by several procedures. These procedures include-

1. Gram Staining
2. Biochemical Test
3. Analytical Profile Index (API® 20E)
4. Antibiotic Sensitivity Test

2.2.2.1 Gram Staining

Gram staining is a differential staining method which was used to differentiate between Gram positive and Gram negative bacteria by their cell wall composition. Gram positive bacteria retain the violet colour upon staining with crystal violet dye and Gram negative bacteria are counter strained with safranin which appears pink under microscope.

2.2.2.2 Biochemical Test

Different biochemical tests were used to differentiate between closely related bacteria. These tests were designed to identify various metabolic properties of different bacterial species. In this study the following biochemical tests were used-

1. Triple Sugar Iron (TSI)
2. Motility Indole Urease (MIU)
3. Citrate utilization

2.2.2.2.1 Triple Sugar Iron (TSI) Test

TSI Test was used to check the ability to ferment sugars and produce hydrogen sulphide. TSI Slant contains agar, pH sensitive dye (phenol red), 1% sucrose, 1% lactose and 0.1% glucose, sodium thiosulphate together with ferrous sulphate or ferrous ammonium sulphate. All of these ingredients were mixed together and allowed to solidify in a test tube in a slanted angle. This slanted shape of this medium provides an array of surfaces that are either exposed to oxygen-containing air in varying degrees or not exposed to air.

Bacteria fermenting any of these three sugars will produce acid as by-products. Therefore, the colour of the phenol red will turn red to yellow. Position of the colour change in the tube will determine the fermented sugar. For example, if the butt turns yellow, it represents that fermentation of glucose has occurred. If the whole agar turns yellow, it represents that all three sugars have been fermented. If the agar turned black, it confirms the production of hydrogen sulphide (H_2S) gas and production of bubbles confirms Carbon dioxide (CO_2) gas formation.

2.2.2.2.2 Motility Indole Urease (MIU) Test

This is a multi-purpose medium used for differentiation of the enterobacteriacae. It combines three individual tests into a single medium- motility of the organism, indole

production and presence of urease enzyme. A needle with a pure culture or a discrete single colony of the test organism was inoculated by making a single stab into the medium. If the organism was motile, it would be producing a hazy area around the stabbed region and if non-motile then the region will be sharp. Indole production will be detected by the indole reagent paper attached at the top of the test tube. Positive reaction will change the paper colour. If the bacteria has urease enzyme, it will degrade the urea present in the medium into ammonia. This ammonia will react with water to produce ammonium hydroxide and thus the colour of the medium will turn yellow to red.

2.2.2.2.3 Citrate Utilisation Test

This test used to check whether the organism was able to utilise the citrate present in the medium as sole carbon source for energy. For this study, the citrate agar medium was made with Simmons Citrate Agar powder. The positive result of the citrate utilization test changes the colour of the medium from green to blue and there would be no colour change for the negative result.

2.2.2.3 Analytical Profile Index

The Analytical Profile Index (API) is a mini-biochemical test which was used to distinguish between groups of closely related bacteria for their identification. There are 20 biochemical tests which are compiled together in a single test strip. Different test panels are prepared in dehydrated forms which are reconstituted upon addition of bacterial suspensions. The inoculated whole API® 20E test kit strips were incubated overnight at 37°C in an aerobic incubator. After the incubation period, test results are used to construct a 7-digits profile. Using this profile, the identity of the bacterium was derived from the database with the relevant cumulative profile code book or software.

2.2.2.4 Antibiotic Sensitivity Test

The antibiotic sensitivity test (AST) was used to find out the sensitivity of a bacterial strain against different types of antibiotic. For this purpose the bacterial strains were enriched for 2 hours at 37°C in incubator followed by swabbing the bacterial strains on Mueller Hinton Agar (MHA) with a sterile swab stick so that a mat of bacteria was produced on the agar plate. Then with a sterile forceps an antibiotic disc was picked up

from the cartage and applied on the agar plate. The plate was again incubated overnight at 37°C in an incubator. Next day the zone of inhibition (clear zone) was measured with a ruler and compared with the Antibiotic disc zone diameter interpretation.

2.2.3 Preservation of bacteria

To use the bacteria for a long period of time we needed to preserve them in a proper condition. We had to make sure that there were no characteristic changes in the strains. Accordingly, we stored the bacterial strains for short term as well as for long term preservation.

2.2.3.1 Short term preservation

For daily or weekly use of the bacteria short term preservation method was followed. There is a chance of the bacterial colony to get mutated when the culture is old because the competition for nutrition and space increases between the bacteria in the same colony. So the bacteria try to develop to get the most benefits. In this study, the bacteria were preserved for short time period in agar plates. NA and Mac. Agar plates were used for this purpose. A single colony of bacteria was taken in a loop and was streaked on the agar plates by quadrant streak method to get more single colonies. The plates were incubated overnight at 37°C to get the colonies. Following day, the plates were sealed with Parafilm® and stored at 4°C refrigerator for the next use (Thiel, 1999). The Parafilm® helps to avoid any contamination in the plate while storing and also it helps to maintain the moisture in the plate which prevents the bacterial colonies from drying. Using this method, the strains can be stored in the refrigerator for a month. For this study, the strains were sub-cultured on seventh day after culturing to maintain healthy bacterial colonies.

2.2.3.2 Long Term Preservation

Long term bacterial preservation is important because it helps to store the strain as stock sample. The bacteria could be revived again for later use if the short term preserved bacteria are very old with possibility of mutation. For this study, long term preservation of bacteria was done in a medium containing skim milk, tryptone, glucose, and glycerine (called STGG medium) and it was stored at -80°C in Ultra Low Temperature (ULT) freezer. It is an inexpensive medium and remains stable for at least 6 months after

sterilization (O'Brien, et al., 2001). Every 6 months the bacteria were thawed from the freezer, streaked on the agar plate and incubated overnight at 37°C to culture the bacteria. Next day, the bacterial colonies were ready for use or a few single colonies were picked up and inoculated in fresh STGG medium and restored in the ULT freezer for further preservation.

2.3 Preparation of extract

The cinnamon extracts were prepared by the following procedure-

1. The cinnamon barks were washed with deionised water and dried overnight in an oven.
2. The barks were then grinded using a grinder.
3. About 25g of grinded cinnamon was taken in a beaker and 250 ml of ethanol was added. The beaker was then sealed with a Parafilm® and stored overnight at room temperature.
4. Then the extract was filtered with a filter paper followed by filtering them with 0.2µm filter to remove any debris of cinnamon wood from the bark.
5. The extract was subjected to drying with vacuum dryer to remove all the ethanol from the extract.
6. The dry extract was then dissolved in dimethyl sulphoxide (DMSO).

2.4 Anti-microbial activity of cinnamon extract

The anti-microbial activity of cinnamon extract was tested by three methods.

1. Agar diffusion method,
2. Minimum inhibitory concentration and
3. Minimum bactericidal concentration

2.4.1 Agar diffusion method

The agar diffusion assay is a method for quantifying the capacity of anti-microbial agents to inhibit bacterial growth and it is the simplest one. It can be done in two ways, one is disk diffusion method and another is well diffusion method. In disk diffusion method, the disk is soaked in anti-microbial solution and dried. The dried disk is placed on microbe

matted agar plate. The anti-microbial agent from the disk gets diffused in the agar and creates a clear zone around disk. On the other hand, in well diffusion method, a hole is punched in the agar plate using a cork borer. A definite volume of the anti-microbial agent is added. To that hole the anti-microbial agent diffuses to the agar and creates a clear zone around the hole. If the organism is resistant to the anti-microbial agent no clear zone appears.

For this study the well diffusion method was followed. MHA was used for bacterial culture. Bacterial culture no more than 16-20 hours old was used. The organisms were enriched in MHB for 2-3 hours. Using a sterile cotton swab, the enriched inoculum was lawned on an agar plate. Holes were punched on the inoculated agar plate with sterile 0.2 ml micropipette tips. To those holes 50 µl of the cinnamon extract solution and control solution for the extract were added at different concentrations. The cinnamon extract and its control were diluted with 0.2 µm filtered and autoclaved deionised water to achieve definite concentration. After adding the cinnamon extract and control, the plate was incubated overnight at 37°C.

2.4.1.1 Quality control

It is important to set up a quality control while doing any experiment. Quality control helps to compare the results obtained with a positive response and a negative response to ensure there is no positive or negative impact on the result.

2.4.1.1.1 Quality control for biochemical tests of the organisms.

For all the above mentioned biochemical tests performed, a negative control was set up. This was done without inoculating any organism to the test tubes. This helped to verify if any external source (contamination) was impacting the test results. The non-inoculated test tube (negative control) was kept in the same condition as test organisms test tubes. After overnight incubation, the results were compared. The negative control should not give any significant reaction.

2.4.1.1.2 Quality control for agar diffusion test

This control was set up to check whether any other components, for example, the solvent, DMSO, or the diluent water have had any effect on the bacterial strains or not. The

amount of DMSO used to dissolve the cinnamon extract and total volume of the dissolved extract were recorded. The same volume of DMSO was taken in a falcon tube and total volume of DMSO was adjusted with 0.2 µm filtered and autoclaved deionised water. Control for the extract was diluted in the same way as the extract itself. Several dilutions of the control were also prepared for the agar diffusion test.

2.4.1.1.3 Quality control for minimum inhibitory concentration of the cinnamon extract

The quality control for the minimum inhibitory concentration of the cinnamon extract was setup by keeping a negative control and a positive control for the test organisms. From the series of dilutions of the cinnamon extract, one concentration was set to 0 mg/ml, which means no cinnamon extract was added to those set vials. This concentration was used as control. From the control set, one of the five dram vials was not inoculated, which served as the negative control for the experiment. To the remaining four dram vials of the control set, four test bacteria were inoculated to serve as the positive control of the experiment. If visible growth was not observed in these vials, it could be indicative of the presence of a growth inhibiting factor or non-viability of the bacteria used.

2.4.1.1.4 Quality control for minimum bactericidal concentration of the cinnamon extract

The quality control for minimum bactericidal concentration was done by inoculating from the positive control culture medium, where no cinnamon extract was added. The purpose of the positive control was to ensure that there was no other external inhibitor other than the cinnamon extract affecting the secondary growth of the test organism on the MHA. After the inoculation of the of the organisms on MHA from different concentrations of the cinnamon extracts, all the MHA plates were incubated overnight in a 37°C aerophilic incubator to observe the growth of the organisms.

2.4.2 Minimum inhibitory concentration of cinnamon extract

Minimum inhibitory concentration or MIC is the minimum concentration of the cinnamon extract required to inhibit the growth of the bacterial strains.

2.4.2.1 Preparation of broth medium with different concentrations of extract

To find out the MIC, a set of different concentrations of cinnamon extract was prepared with the concentrations 8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml and 0 mg/ml in MHB. The stock extract concentration was 80 mg/ml. From the stock cinnamon extract concentration, 200 µl of the cinnamon extract was added to 1.8 ml of MHB to make 8 mg/ml concentration. For preparing next concentrations, the stock extract was serially diluted with 0.2 µm filtered and autoclaved deionised water to make 40 mg/ml, 20 mg/ml, 10 mg/ml, and 5 mg/ml of the concentrations of extract. From these later concentrations, 200 µl was taken and added to 1.8 ml MHB to produce 4 mg/ml, 2 mg/ml, 1 mg/ml, and 0.5 mg/ml concentration respectively. It was found that some precipitation had occurred after addition of the cinnamon extract and the mixture became cloudy. The mixture was mixed using vortex machine so that the anti-microbial agent got mixed with the MHB. Then the precipitate was centrifuged at 5000 rpm for 10 minutes. The supernatant was taken in a dram vial and the pellet was discarded.

2.4.2.2 Preparation of inoculums

The inoculums were prepared by taking a 16-24 hours culture of the organism on nutrient agar plate. One of the colonies was picked with a sterile loop and mixed with sterile normal saline. The bacterial suspension was prepared using a vortex machine. The optical density of the suspension was measured at 600 nm wavelength using a Spectrophotometer. The absorbance of light by the bacterial suspension was recorded and adjusted to 0.5 McFarland standards. The absorbance of 0.5 McFarland standards would be between 0.8-1.0 at 600 nm wavelength of light beam to travel a distance of 1 cm pathway. If the 0.5 McFarland Standard was set by the absorbance between the above mentioned ranges, then the bacterial suspension in the cuvette may contained around 1.5×10^8 CFU. The adjusted bacterial suspension was then transferred to a microcentrifuge tube. From the adjusted bacterial suspension, 20 µl was transferred to the specific dram vial containing 2 ml of MHB with cinnamon extract to dilute the bacterial CFU from 10^5 to 10^6. Then they were incubated overnight at 37°C at 200 rpm in shaking incubator. Following day the turbidity of the suspension was compared with the respective control extract dilutions in a 96-microwell plate by spectrophotometer at 600nm wavelength.

2.4.3 Minimum bactericidal concentration of cinnamon extract

Minimum bactericidal concentration (MBC) was required to find out the efficiency of any antimicrobial agent. It indicates the minimum concentration of the antimicrobial agent required to kill the bacteria. It was determined after determining the MIC of the antimicrobial by setting up a series of dilution of antimicrobial agent followed by inoculation of bacteria as in determining MIC. After overnight incubation at 37°C at 200 rpm in a shaking incubator, the cultures were sub-cultured by streaking from the solutions on MHA with a sterile cotton swab for bacterial growth. The streaked MHA were incubated overnight again in 37°C in an incubator. Next day, the bacterial growths of respective solutions were going to give the idea about the MBC of the extract.

Chapter 3

Results

3.1 Conformation of the strains

The strains used for this study were confirmed using certain laboratory test.

3.1.1 Colony Morphology on different agar plates

The following table and the images show the colony characteristics on MacConkey and Nutrient Agar Plates of different strains of bacteria used in this study.

Organism	Agar Plate	Bacterial colony characteristics					
		Size*	Margin	Elevation	Pigment	Form	Consistency
AREI-DN-005 (Sen. *K. pneumoniae*)	MacConkey	Small	Entire	Pulvinate	Pink	Circular	Smooth
	Nutrient	Large	Entire	Raised	White	Circular	Smooth
AREI-SN-014 (Sen. *E. agglomerans*)	MacConkey	Medium	Entire	Pulvinate	Pink	Circular	Mucoid
	Nutrient	Large	Entire	Umbonate	White	Circular	Mucoid
AREI-SN-044 (MDR *K. pneumoniae*)	MacConkey	Medium	Entire	Pulvinate	Pink	Circular	Smooth
	Nutrient	Medium	Entire	Convex	White	Circular	Smooth
AREI-SN-046 (MDR *E. agglomerans*)	MacConkey	Small	Entire	Pulvinate	Pink	Circular	Mucoid
	Nutrient	Medium	Entire	Convex	White	Circular	Mucoid

Table 3.1- Colony characteristics of the strains on different agar plates

*All the sizes of the colonies given bellow

Large: 4.5 mm (±0.5 mm)

Medium: 3 mm (±0.5 mm)

Small: 2 mm (±0.5 mm)

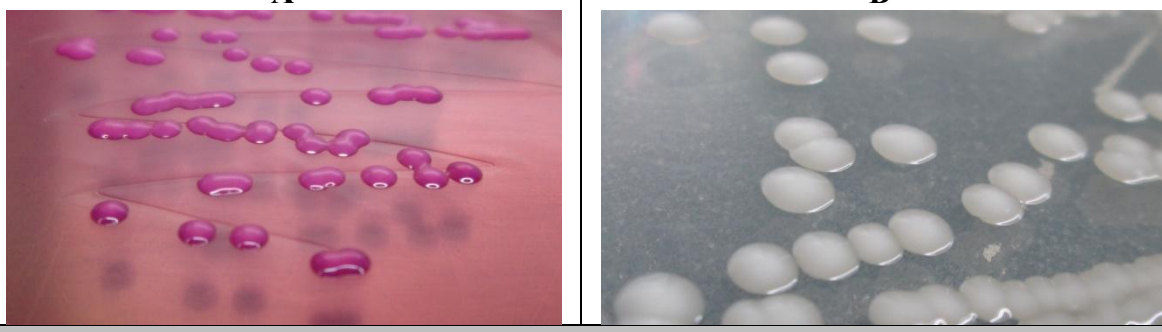

Figure 3.1- Colony appearance of AREI-DN-005 (Sen. *K. pneumoniae*) on (A) MacConkey Agar Plate and (B) Nutrient Agar Plate.

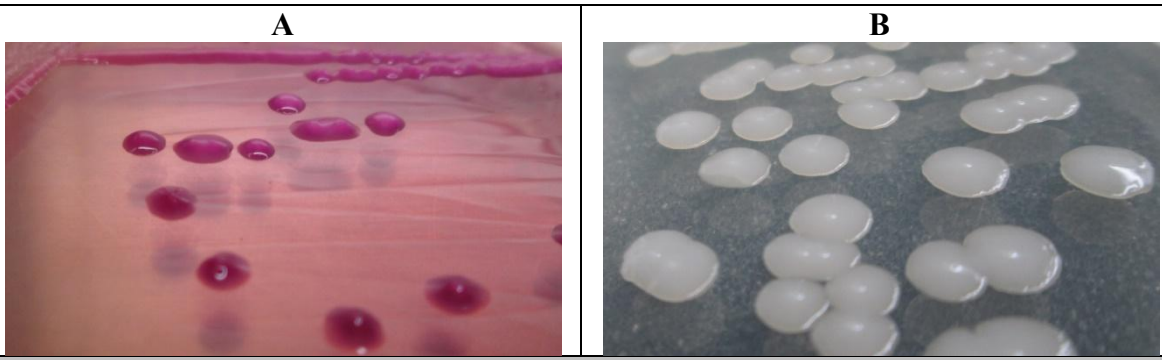

Figure 3.2- Colony appearance of AREI-SN-014 (Sen. *E. agglomerans*) on (A) MacConkey Agar Plate and (B) Nutrient Agar Plate.

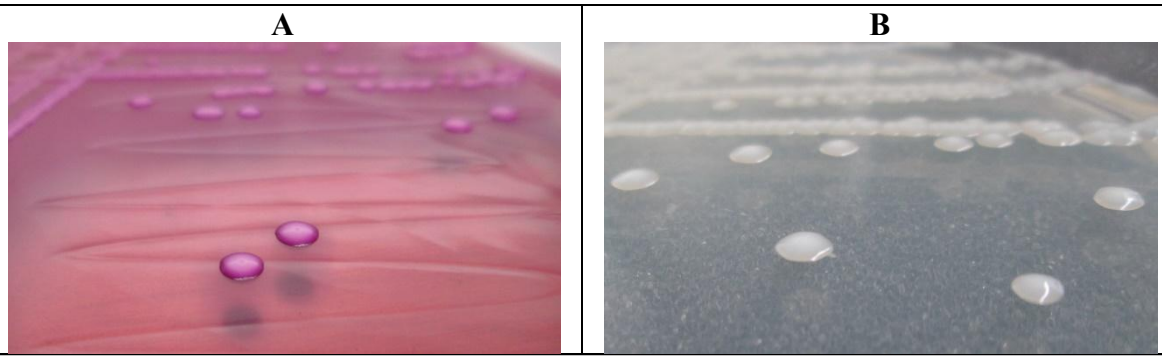

Figure 3.3- Colony appearance of AREI-SN-044 (MDR *K. pneumoniae*) on (A) MacConkey Agar Plate and (B) Nutrient Agar Plate.

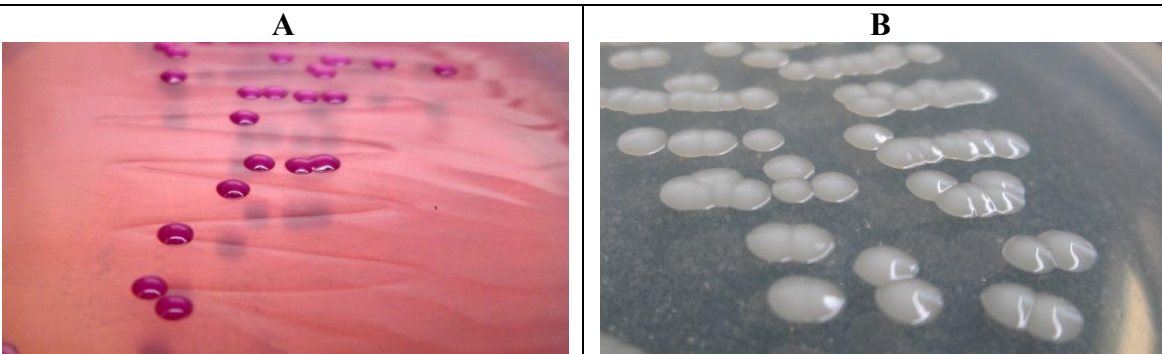

Figure 3.4- Colony appearance of AREI-SN-046 (MDR *E. agglomerans*) on (A) MacConkey Agar Plate and (B) Nutrient Agar Plate.

3.1.2 Gram's staining result of the strains

The following results are showing the Gram's staining result of the strains viewed under microscopic oil emersion lens.

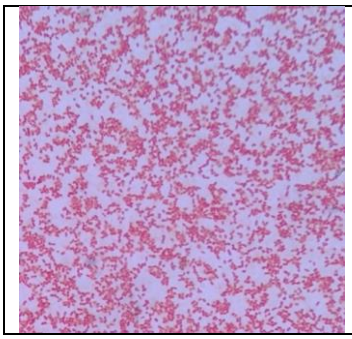	**AREI-DN-005 (Sensitive *K. pneumoniae*)** **Cell wall type:** Gram Negative **Shape:** Rod Shaped (Bacillus) **Figure 3.5-** Showing microscopic view of AREI-DN-005 (Sensitive *K. pneumoniae*)

Table 3.2- Microscopic characteristics of Sensitive *K. pneumoniae* after Gram's staining

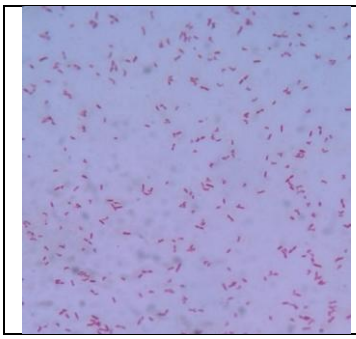	**AREI-SN-014 (Sensitive *E. agglomerans*)** **Cell wall type:** Gram Negative **Shape:** Rod Shaped (Bacillus) **Figure 3.6-** Showing microscopic view of AREI-SN-014 (Sensitive *E. agglomerans*)

Table 3.3- Microscopic characteristics of Sensitive *E. agglomerans* after Gram's staining

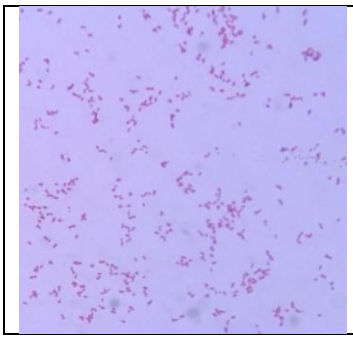	**AREI-SN-044 (MDR *K. pneumoniae*)** **Cell wall type:** Gram Negative **Shape:** Rod Shaped (Bacillus) **Figure 3.7-** Showing microscopic view of AREI-SN-044 (MDR *K. pneumoniae*)

Table 3.4- Microscopic characteristics of MDR *K. pneumoniae* after Gram's staining

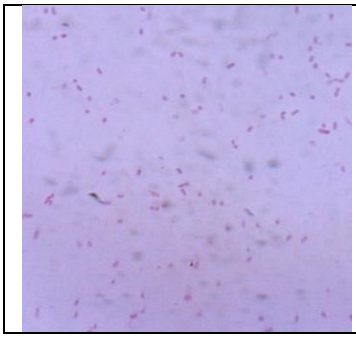	**AREI-SN-046 (MDR *E. agglomerans*)** **Cell wall type:** Gram Negative **Shape:** Rod Shaped (Bacillus) **Figure 3.8-** Showing microscopic view of AREI-SN-046 (MDR *E. agglomerans*)

Table 3.5- Microscopic characteristics of MDR *E. agglomerans* after Gram's staining

3.1.3 Result of biochemical tests of the strains

3.1.3.1 Triple Sugar Iron Test

The following table and the figure show the result of the Triple Sugar Iron (TSI) test of the stains.

Organism	Characteristics			
	Butt	Slant	Gas Production	H_2S Production
AREI-DN-005 (Sen. *K. pneumoniae*)	Acid	Acid	Positive	Negative
AREI-SN-014 (Sen. *E. aggalomerans*)	Acid	Acid	Positive	Negative
AREI-SN-044 (MDR *K. pneumoniae*)	Acid	Acid	Positive	Negative
AREI-SN-046 (MDR *E. aggalomerans*)	Acid	Acid	Positive	Negative

Table 3.6- Triple sugar iron test result of the organisms

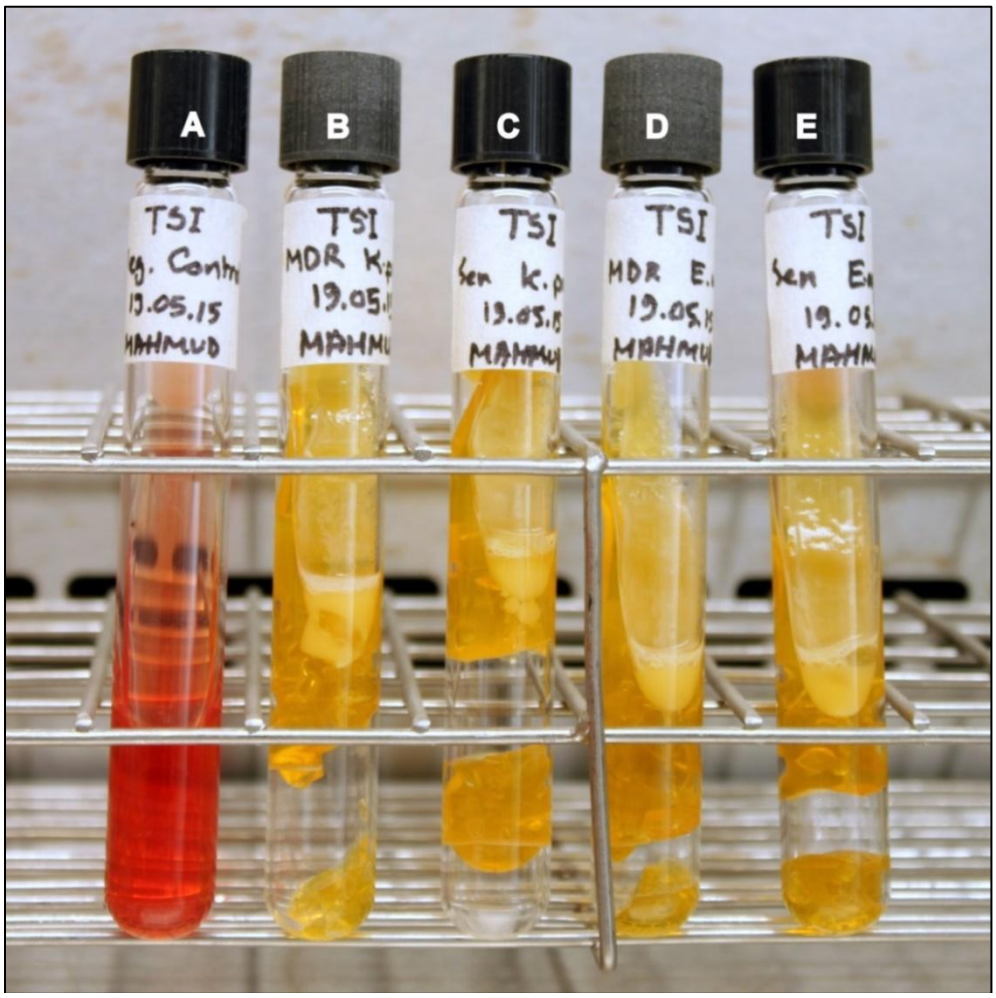

Figure 3.9- Showing the results of Triple sugar iron test of the organisms. (A) Negative Control, (B) MDR *K. pneumoniae, (C)* Sensitive *K. pneumoniae, (D)* MDR *E. agglomerans and (E)* Sensitive *E. agglomerans*

3.1.3.2 Motility Indole Urease Test

The following table and the figure show the result of the Motility Indole Urease (MIU) test of the stains.

Organism	Characteristics		
	Motility	**Indole**	**Urease**
AREI-DN-005 (Sen. *K. pneumoniae*)	Negative	Negative	Positive
AREI-SN-014 (Sen. *E. aggalomerans*)	Positive	Negative	Negative
AREI-SN-044 (MDR *K. pneumoniae*)	Negative	Negative	Positive
AREI-SN-046 (MDR *E. aggalomerans*)	Positive	Negative	Negative

Table 3.7- MIU test result of the organisms.

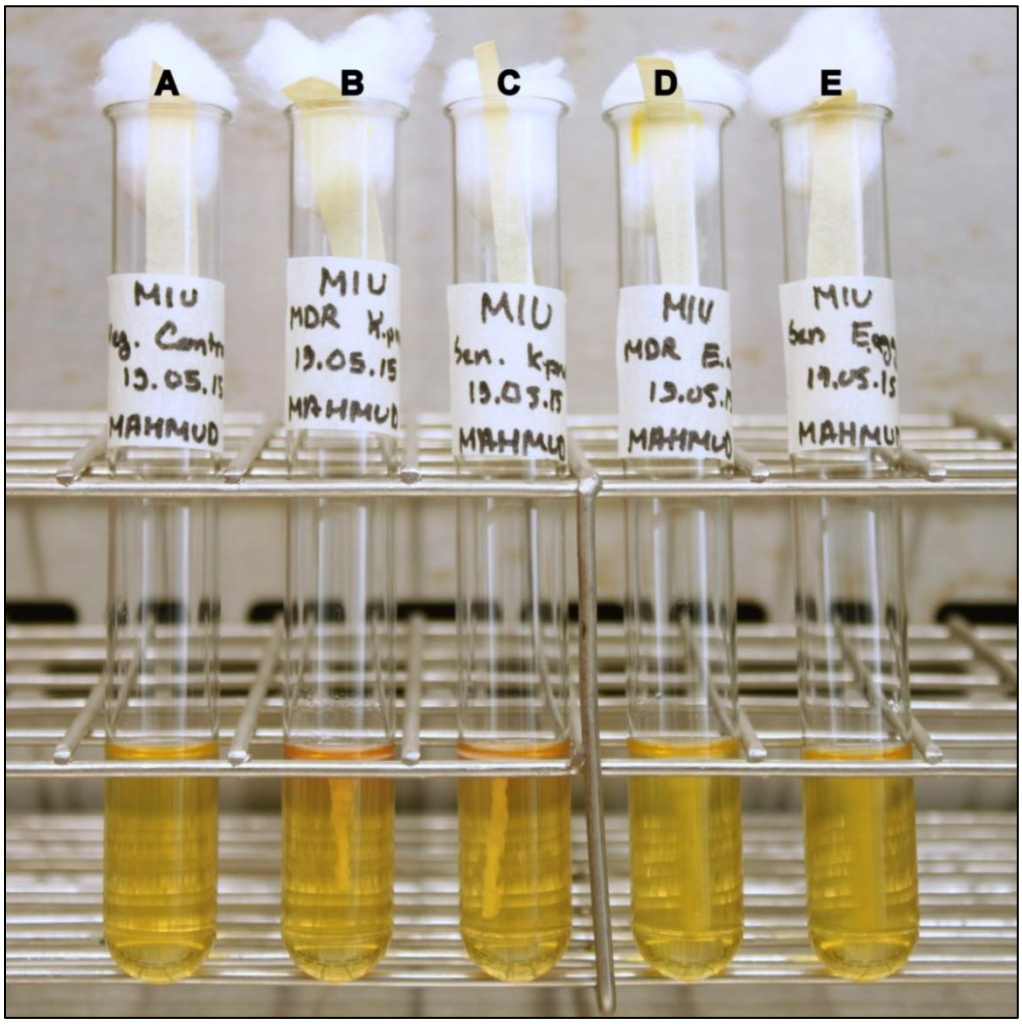

Figure 3.10- Showing the results of MIU test of the organisms. (A) Negative Control, (B) MDR *K. pneumoniae, (C)* Sensitive *K. pneumoniae, (D)* MDR *E. agglomerans and (E)* Sensitive *E. agglomerans*

3.1.3.3 Citrate Utilisation Test

The following table and the figure show the result of the Citrate Utilisation Test of the stains on Simmon's citrate agar.

Organism	Reaction
AREI-DN-005 (Sen. *K. pneumoniae*)	Positive
AREI-SN-014 (Sen. *E. aggalomerans*)	Positive
AREI-SN-044 (MDR *K. pneumoniae*)	Positive
AREI-SN-046 (MDR *E. aggalomerans*)	Positive

Table 3.8- Citrate utilization test result of the organisms.

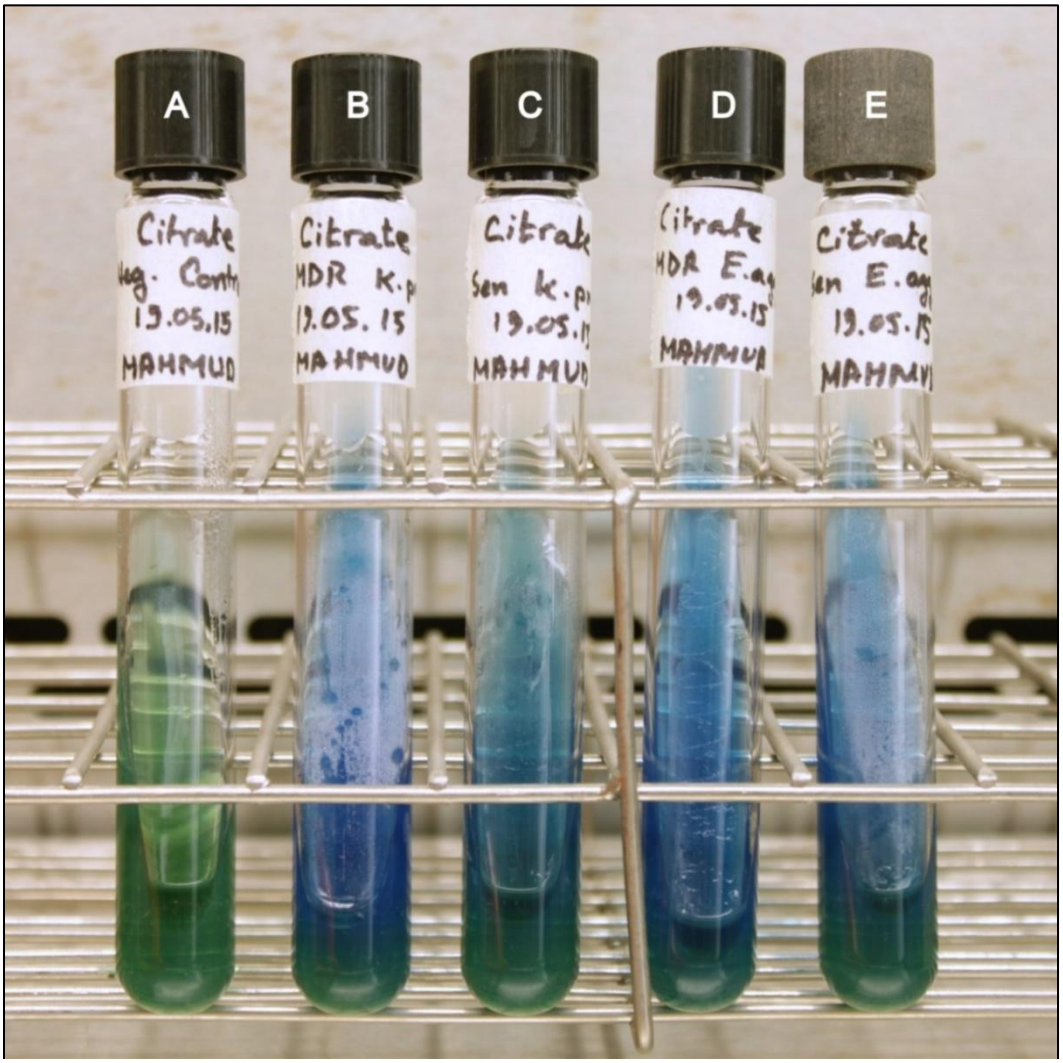

Figure 3.11- Showing the results of citrate utilization test of the organisms. (A) Negative Control, (B) MDR *K. pneumoniae, (C)* Sensitive *K. pneumoniae, (D)* MDR *E. agglomerans and (E)* Sensitive *E. agglomerans*

3.1.4 Analytical Profile Index

The following shows the result of the Analytical Profile Index of the strains used in this study. The result is based on several biochemical reactions of the strains in API® 20E strips.

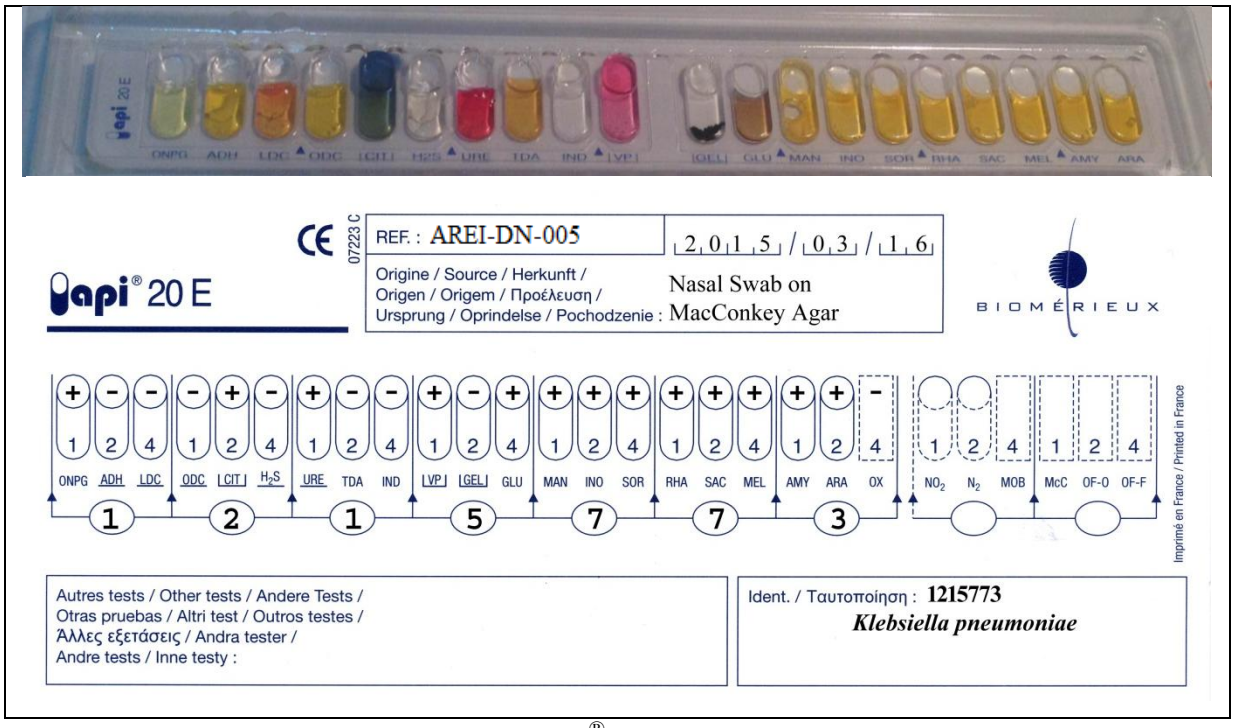

Figure 3.12- Showing API® 20E strip of AREI-DN-005

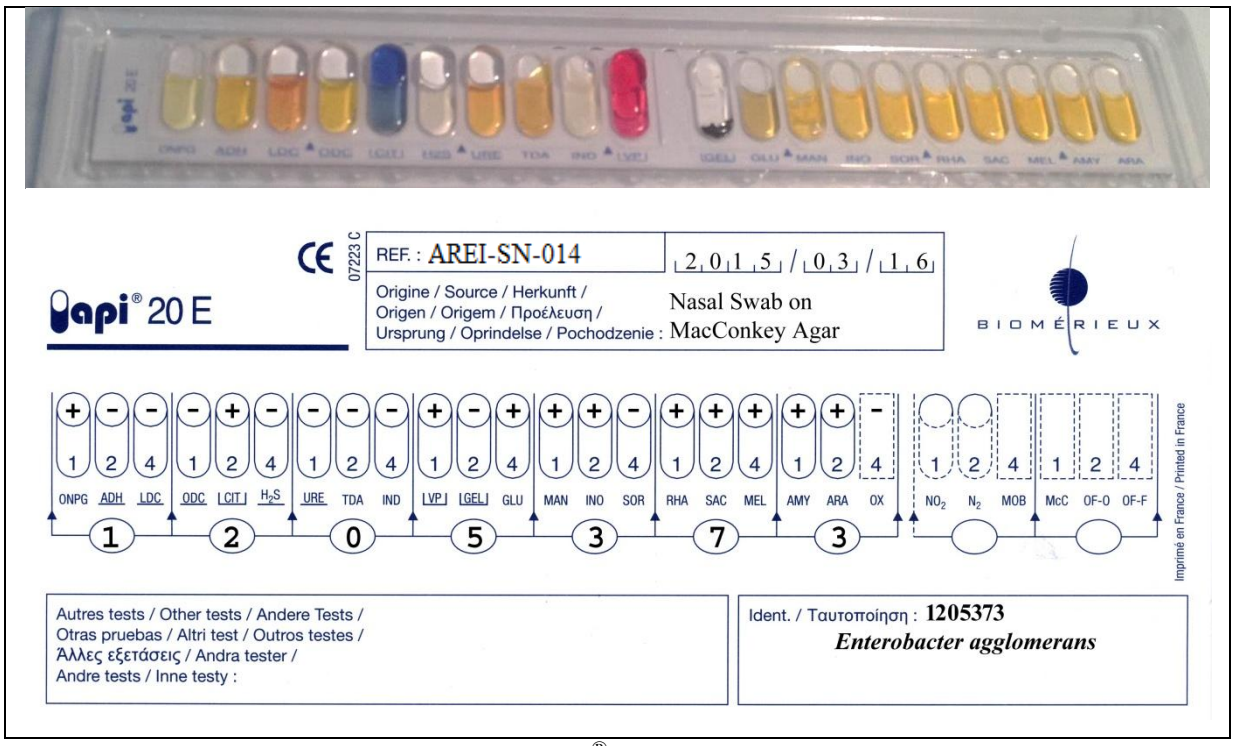

Figure 3.13- Showing API® 20E strip of AREI-SN-014

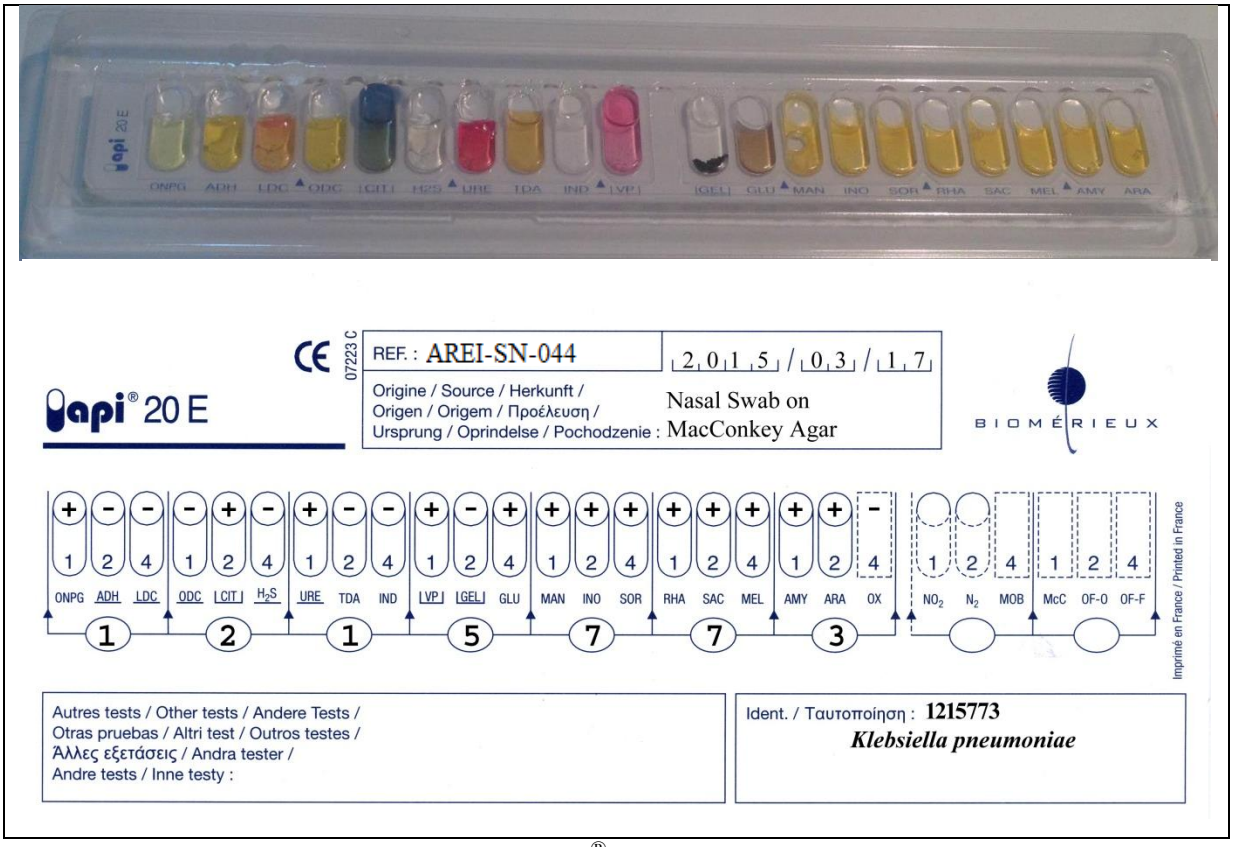

Figure 3.14- Showing API® 20E strip of AREI-SN-044

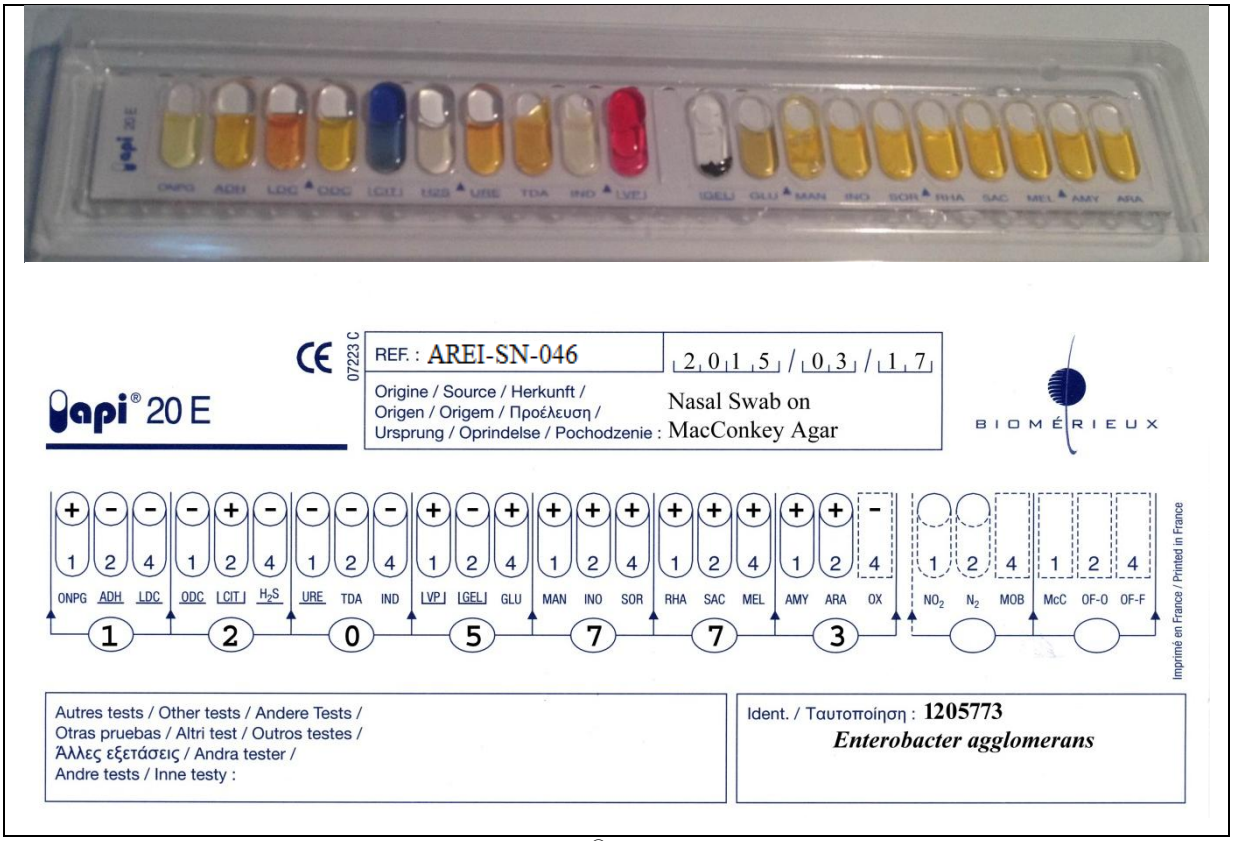

Figure 3.15- Showing API® 20E strip of AREI-SN-046

From the API Results we get a 7-digits profile for the tested organisms. The identity of the organisms used in this study were found from the database code book by using the 7-digits profile which are tabulated below-

Organism Id.	**API® 20E**	**Org identified.**
AREI-DN-005	1215773	*Klebsiella pneumoniae*
AREI-SN-014	1205373	*Enterobacter agglomerans*
AREI-SN-044	1215773	*Klebsiella pneumoniae*
AREI-SN-046	1205773	*Enterobacter agglomerans*

Table 3.9- Showing the identified organisms from the Analytical Profile Index results

3.1.5 Antibiotic Sensitivity Test Results

After overnight incubation of the organisms on Mueller Hinton agar plate with the antibiotic discs the results are as following

Organism Id.	**First Line Antibiogram**								**Second Line Antibiogram**				
	CN 10	TOB 10	CRO 30	CFM 5	CIP 5	IMP 10	MEM 10	AZM 15	AK 30	NET 10	TZP 110	CAR 100	PB 50
AREI-DN-005	S	S	S	S	S	S	S	R	-	-	-	-	-
AREI-SN-014	S	S	S	S	S	S	S	R	-	-	-	-	-
AREI-SN-044	R	R	R	R	R	S	S	R	R	R	R	R	R
AREI-SN-046	R	R	R	R	R	R	R	R	R	R	R	R	R

Table 3.10- Antibiotic sensitivity data of the organisms according to the Clinical and Laboratory Standard Institute (CLSI).

From the Antibiotic Sensitivity Test (AST), it was found that AREI-DN-005 and AREI-SN-014 were sensitive to most of the first line antibiotics except Azithromycin 15 µg (AZM-15). These organisms were referred as sensitive (Sen) organisms. As the organisms were sensitive to most of the first line antibiotics, their second line antibiogram was not done. On the other hand, AREI-SN-044 was resistant to most of the first line antibiotics except Imipenem and Meropenem 10 µg. The AREI-SN-046 was resistant to all of first line as well as second line antibiotics. Therefore, AREI-SN-044 and AREI-SN-046 are referred as Multi Drug Resistant (MDR) Organisms.

3.2 Anti-microbial activity of cinnamon extract

3.2.1 Result of agar diffusion method

The results of anti-microbial activity of the cinnamon extract against MDR and Sen. *Klebsiella pneumoniae* and *Enterobacter agglomerans* are given below.

Organisms	Concentration	Diameter of zone of inhibition/mm											
		Extract						Control					
		E_1 (160 mg/ml)	E_2 (80 mg/ml)	E_3 (40 mg/ml)	E_4 (20 mg/ml)	E_5 (10 mg/ml)	E_6 (5 mg/ml)	C_1 (Control for E_1)	C_2 (Control for E_2)	C_3 (Control for E_3)	C_4(Control for E_4)	C_5 (Control for E_5)	C_6 (Control for E_6)
MDR *Klebsiella pneumonia*		16	11	9	-	-	-	-	-	-	-	-	-
MDR *Enterobacter agglomerans*		14	9	8	-	-	-	-	-	-	-	-	-
Sen. *Klebsiella pneumoniae*		16	11	9	-	-	-	-	-	-	-	-	-
Sen. *Enterobacter agglomerans*		18	14	10	-	-	-	-	-	-	-	-	-

Table 3.11- Clear zone size of the organisms with different concentrations of cinnamon extracts.

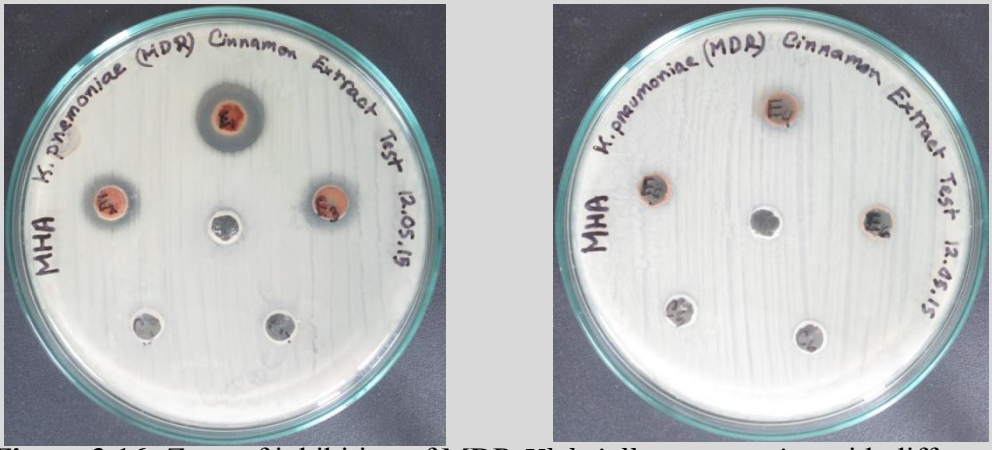

Figure 3.16- Zone of inhibition of MDR *Klebsiella pneumoniae* with different concentrations of cinnamon extract.

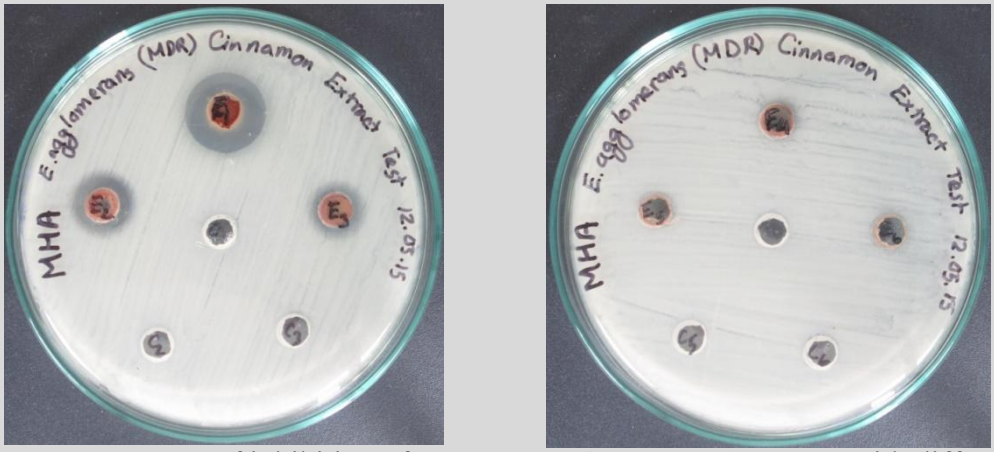

Figure 3.17- Zone of inhibition of MDR *Enterobacter agglomerans* with different concentrations of cinnamon extract.

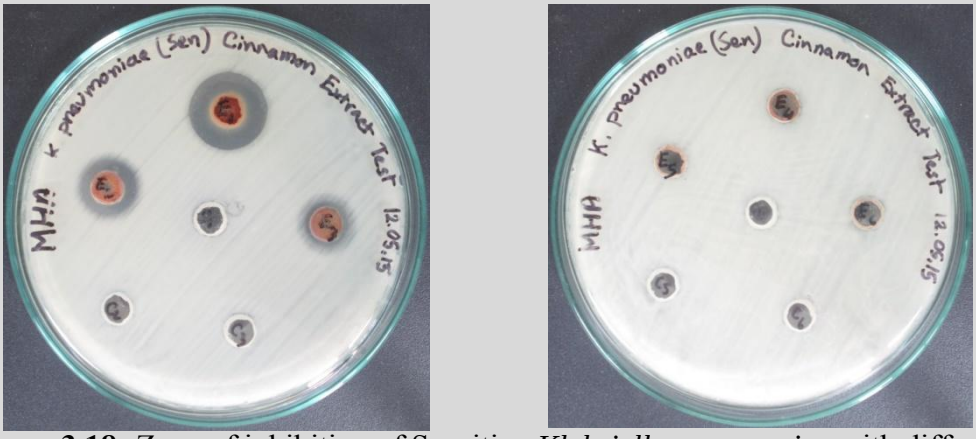

Figure 3.18- Zone of inhibition of Sensitive *Klebsiella pneumoniae* with different concentrations of cinnamon extract.

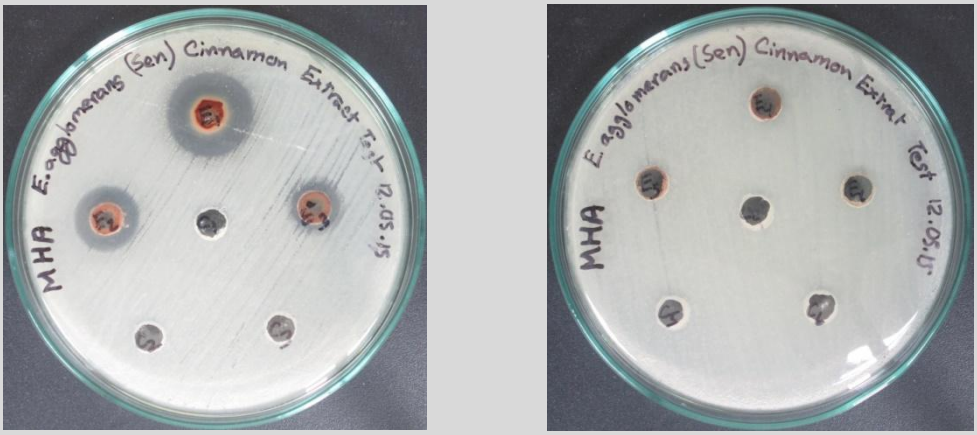

Figure 3.19- Zone of inhibition of Sensitive *Enterobacter agglomerans* with different concentrations of cinnamon extract.

The results suggest that there was a significant effect of the cinnamon extract on inhibition of the growth of the above-mentioned bacteria until the minimum concentration of 40 mg/ml was achieved to visualize a zone of inhibition on MHA.

3.2.2 Result of Minimum Inhibitory Concentration (MIC) of the cinnamon extract on the organisms.

After inoculation of the organisms in various concentrations of the cinnamon extract in Mueller Hinton Broth, next day the optical densities of the broth mediums were measured in 96-microwell plate using spectrophotometer at wavelength 600 nm. The mean optical densities of the broths are shown below.

Organisms	Concentration of the Cinnamon Extract						Keys
	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	0 mg/ml (Control)	
Control (No Organism)	0.126	0.1	0.08	0.08	0.07	0.046	No Growth
MDR *Klebsiella pneumoniae*	0.102	0.086	0.091	0.079	0.922	1.087	Medium Growth
MDR *Enterobacter agglomerans*	0.113	0.085	0.081	0.084	0.885	0.914	
Sen. *Klebsiella pneumoniae*	0.097	0.086	0.077	0.082	0.948	1.084	Heavy Growth
Sen. *Enterobacter agglomerans*	0.111	0.08	0.081	0.083	0.456	0.431	

Table 3.12- The mean absorbance of the broth mediums at spectrum of 600nm wavelength

The absorbance results in the Table 3.12 suggest that no significant growth of bacteria were observed in the concentrations 8, 4, 2 and 1 mg/ml. Therefore, the MIC of the cinnamon extract for all above mentioned bacteria was 1 mg/ml.

3.2.3 Result of Minimum Bactericidal Concentration (MBC) of the cinnamon extract on the organisms.

The minimum bactericidal concentration (MBC) of the extract was done by culturing the organisms in different concentrations of cinnamon extracts in Mueller Hinton Broth medium in a shaking incubator after overnight culture of the organisms. The broth mediums was then streaked with a sterile cotton swab on Mueller Hinton Agar and again incubated overnight for the surviving bacteria to grow. The results are given bellow.

Organisms	Concentration of the Cinnamon Extract					
	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	0 mg/ml (Control)
Control (No Organism)	-	-	-	-	-	-
MDR *Klebsiella pneumoniae*	-	-	-	+	+	+
MDR *Enterobacter agglomerans*	-	-	-	+	+	+
Sen. *Klebsiella pneumoniae*	-	-	-	+	+	+
Sen. *Enterobacter agglomerans*	-	-	-	+	+	+

Table 3.13- Growth of the organisms after sub-culturing them from different concentrations of the cinnamon extracts in Mueller Hinton Broth.

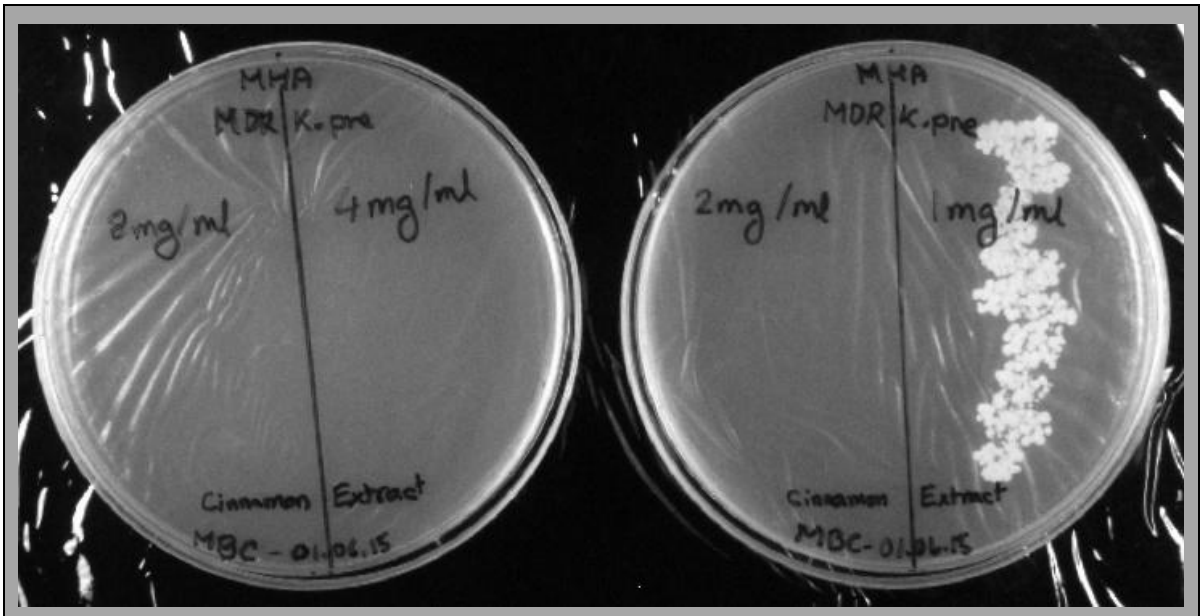

Figure 3.20- Checking the growth of MDR *Klebsiella pneumoniae* for Minimum Bactericidal Concentration with different concentrations of cinnamon extract.

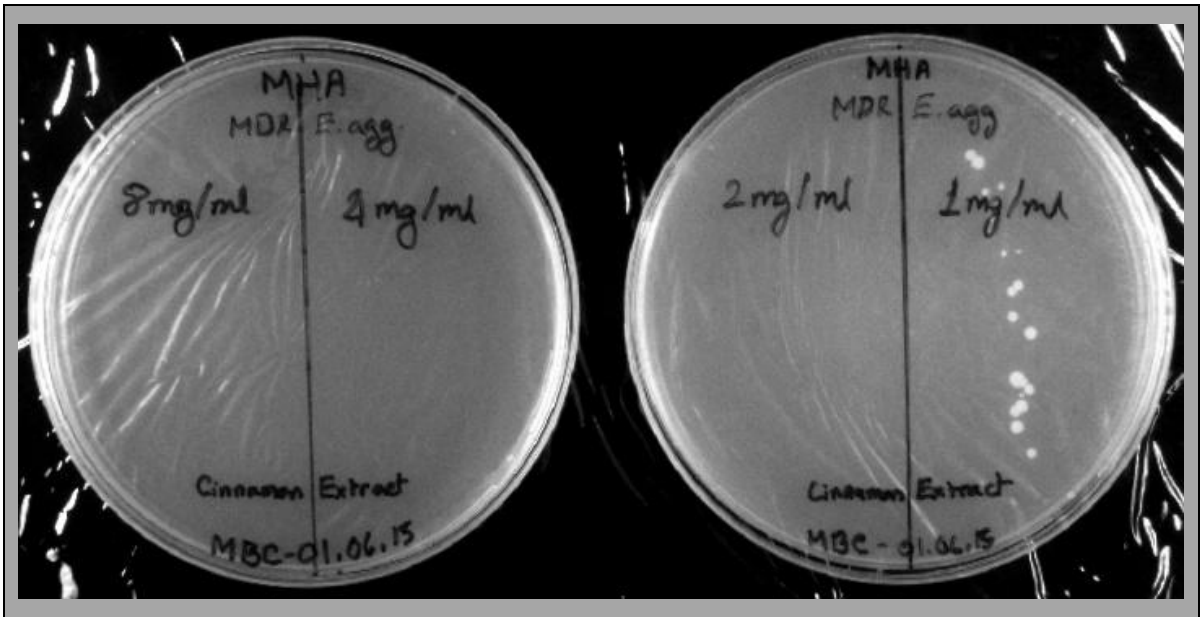

Figure 3.21- Checking the growth of MDR *Enterobacter agglomerans* for Minimum Bactericidal Concentration with different concentrations of cinnamon extract.

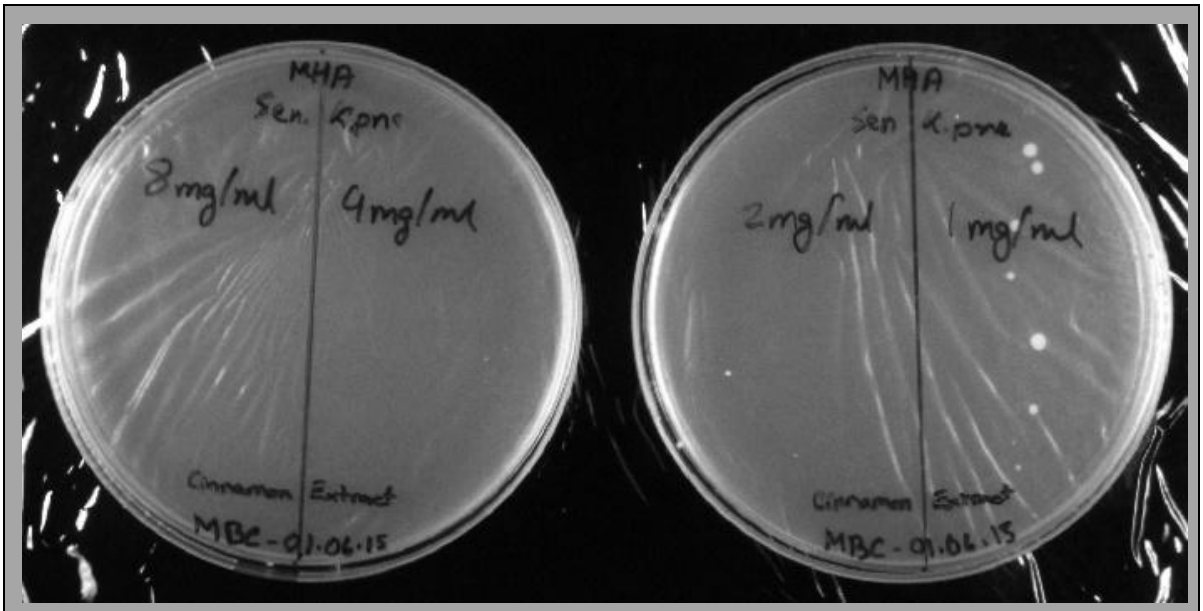

Figure 3.22- Checking the growth of Sensitive *Klebsiella pneumoniae* for Minimum Bactericidal Concentration with different concentrations of cinnamon extract.

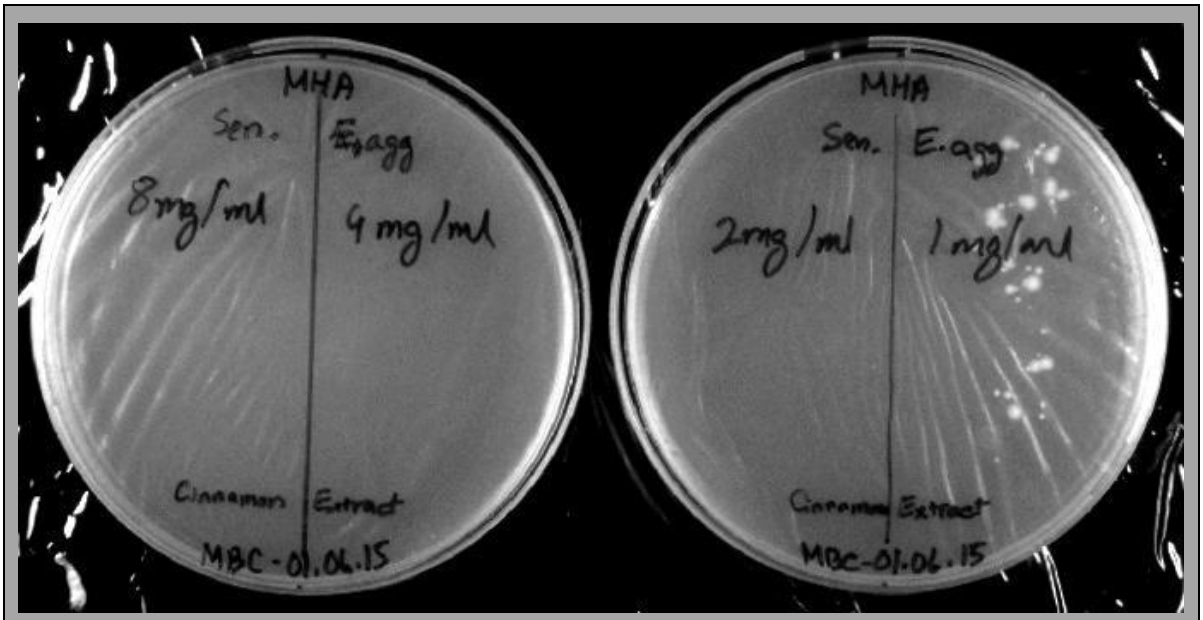

Figure 3.23- Checking the growth of Sensitive *Enterobacter agglomerans* for Minimum Bactericidal Concentration with different concentrations of cinnamon extract.

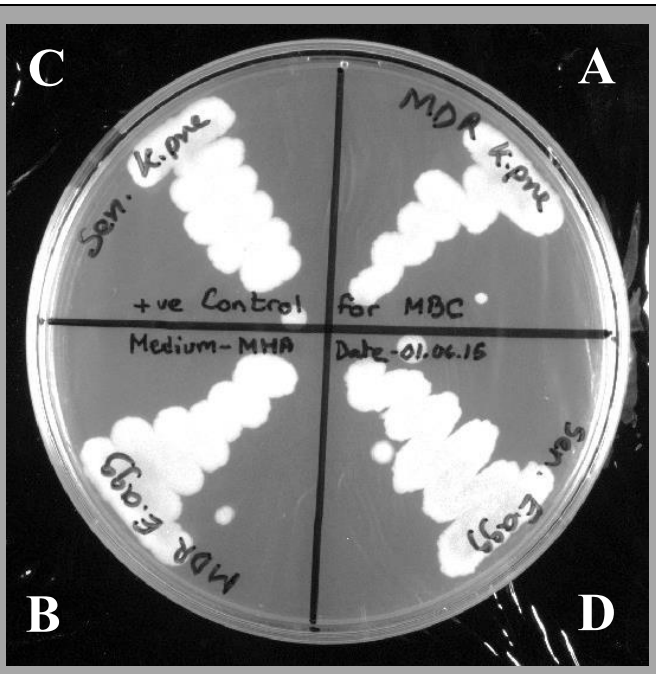

Figure 3.24- Checking the growth of (A) MDR *Klebsiella pneumoniae;* (B) MDR *Enterobacter agglomerans;* (C) Sensitive *Klebsiella pneumoniae;* and (D) Sensitive *Enterobacter agglomerans* as positive control for Minimum Bactericidal Concentration with different concentrations of cinnamon extract.

Although there was no growth from the concentrations 8, 4 and 2 mg/ml, growth was observed from 1 mg/ml broth. These results suggest that the MBC of the cinnamon extract for all the above mentioned bacteria was 2 mg/ml.

Chapter 4

Discussions

Chapter 4: Discussion

Multi Drug Resistant (MDR) organisms are emerging threat because existing antibiotics are not able to combat these organisms. New antibiotics need to be discovered or existing antibiotics have to be modified so that these can have broad spectrum activity. However, both the procedures are time consuming and also costly. Use of anti-microbial activity of natural products like different extract of plants, herbs can be an alternative option to fight against those microorganisms.

The use of herbal medicine is becoming popular due to toxicity and side effects of allopathic medicines. This has led to sudden increase in the number of herbal drug manufacturing (Agarwal, 2005). Medicinal plants play a vital role for the development of new drugs. Besides production of synthetic drugs, the biopharma industries in Bangladesh like Square Pharma, ACME and others, are producing herbal medicines also.

This study was intended to find out a substance which can be an alternative solution to antibiotics to combat against a broad spectrum of bacteria, especially the MDR varieties. Cinnamon extract was known to have some antimicrobial properties and therefore, the ethanolic extracts of the cinnamon were tested on the MDR *Klebshiella pneumoniae*, MDR *Enterobacter agglomerans*, Sensitive *Klebshiella pneumoniae* and Sensitive *Enterobacter agglomerans* strains.

Cinnamon contains 0.5-2% essential oil which is very much effective against microbes. The cinnamon oil consists of two major antimicrobial compounds Cinnamaldehyde (aldehyde) and Eugenol (phenol) (Shelef, 1984). Around 85% of this essential oil consists of cinnemaldehyde. This compound is very effective to inhibit both Gram positive and Gram negative bacteria. In addition, this compound can also inhibit the growth of mould, fungi too (Kartik, 2011). The mechanism of bactericidal activity of cinnamaldehyde and eugenol are still unknown. There is evidence supporting both membrane interaction and inhibition of specific cellular processes or enzymes (Gill & Holley, 2004).

The result of agar diffusion assay (Table-3.11) suggests that there is significant inhibition of all strains irrespective of antibiotic resistant (MDR) or sensitive bacteria for the concentration between 40-160 mg/ml (E_1-E_3) of Cinnamon extract. However, no significant inhibition was observed for the concentration between 5-20 mg/ml (E_4-E_6).

From Figure 3.16 to Figure 3.19, we can observe a clear zone on the agar plate representing the inhibitory effect of the extract. On the other hand, the negative controls (C_1-C_6) of the extracts did not produce any significant clear zone. Thus, we can say that dimethyl sulphoxide (DMSO) which used to re-dissolve the cinnamon extract did not have any significant effect on the bacteria.

As there was a good response in agar diffusion assay, the minimum inhibitory concentration (MIC) of the cinnamon extract for all the organisms were determined. The result (Table 3.12) indicates that the MIC for all the organisms was 1 mg/ml. No growth of organisms was observed in the MHB medium from the concentration 8 mg/ml to 1 mg/ml MHB.

Also, minimum bactericidal concentration (MBC) was determined. The result (Table 3.13) indicates that the MBC for all the organisms was 2 mg/ml. No bacterial growth was found when the cinnamon extract concentration was from 8 mg/ml to 2 mg/ml.

From the result of this study, it was found that the crude ethanolic extract of the Cinnamon was very much effective against both MDR and sensitive pathogens. From other studies on cinnamon extract, it was learned that it is effective against broad spectrum of bacteria, mould and fungi too. Therefore, cinnamon extract could be one of the antimicrobial alternate to the antibiotics to decrease the use of antibiotics. The result of this study is based on *in vitro* test with the organisms. *In vivo* test with animal model has to be done first before implication.

Probably, the antimicrobial effects of the cinnamon extracts were due to the combined effect of different antimicrobial compounds such as cinnamaldehyde, eugenol and other compounds present in the extract. However, it would be great if the exact antimicrobial compounds could be identified from the crude cinnamon extract. Accordingly, the compounds need to be identified and purified using high performance liquid chromatography (HPLC) or other high throughput technique. If the major antimicrobial compound is identified, the compound could be further tested *in vitro* using mammalian cell line and *in vivo* test using an animal model.

Chapter 5

Conclusion

Chapter 5: Conclusion

The crude ethanolic extract of cinnamon could be a source to obtain new and effective herbal medicine beside the antibiotics to treat infections caused by MDR organisms from community acquired and nosocomial infection. Beforehand, it is necessary to find out all the side effects of the extract and pharmaco-kinetic properties of the cinnamon extract before it is to be implemented. Further phytochemical analysis of the cinnamon extract will be helpful for isolation and characterization its active compounds. Moreover, these plants extract should be investigated *in vivo* to better understand their safety, efficacy and properties.

References

References

1. Akram, M., Shahid, M., & Khan, A. U. (2007). Etiology and antibiotic resistance patterns of community-acquired urinary tract infections in J N M C Hospital Aligarh, India. *Annals of Clinical Microbiology and Antimicrobials*.

2. Agarwal, A. (2005). *Pharma Times* , 9-11.

3. Bradley, J. (n.d.). *How Did the Invention of Penicillin Affect World War II?* Retrieved July 28, 2015, from Synonym.com: http://classroom.synonym.com/did-invention-penicillin-affect-world-war-ii-8709.html

4. Brown, T. A. (2007). *Genomes 3* (3rd ed.). New York: Garland Science Pub.

5. Cappuccino, J. G., & Sherman, N. (2005). *Microbiology: A Laboratory Manual.* New Delhi: Pearson Education, Inc. and Dorling Kindersley Publishing, Inc.

6. Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews*, 564-582.

7. Diggins, F. W. (1999). The true history of the discovery of penicillin, with refutation of the misinformation in the literature. *Br J Biomed Sci.*, 83-93.

8. Gill, A. O., & Holley, R. A. (2004). Mechanisms of Bactericidal Action of Cinnamaldehyde against *Listeria monocytogenes* and of Eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Applied and Environmental Microbiology*, 5750–5755.

9. Gyles, C., & Boerlin, P. (2014). Horizontally Transferred Genetic Elements and Their Role in Pathogenesis of Bacterial Disease. *Veterinary Pathology*, 328-340.

10. Kartik, K. (2011). *Amazing Antimicrobial Properties of Cinnamon*. Retrieved July 29, 2015, from BENEFITS FROM CINNAMON!: http://www.benefitsfromcinnamon.com/benefits/amazing-antimicrobial-properties-of-cinnamon

11. Khan, R., Islam, B., Akram, M., Shakil, S., Ahmad, A., Ali, S. M., et al. (2009). Antimicrobial Activity of Five Herbal Extracts Against Multi Drug Resistant (MDR) Strains of Bacteria and Fungus of Clinical Origin. *Molecules*, 586-597.

12. Khan, U. A., Rahman, H., Niaz, Z., Qasim, M., Khan, J., Tayyaba, et al. (2013). Antibacterial activity of some medicinal plants against selected human pathogenic bacteria. *European Journal of Microbiology and Immunology*, 272–274.

13. Leech, J. (2015). *10 Evidence-Based Health Benefits of Cinnamon*. Retrieved July 26, 2015, from Authority Nutrition: http://authoritynutrition.com/10-proven-benefits-of-cinnamon/

14. O'Brien, K. L., Bronsdon, M. A., Dagan, R., Yagupsky, P., Janco, J., Elliott, J., et al. (2001). Evaluation of a Medium (STGG) for Transport and Optimal Recovery of *Streptococcus pneumoniae* from Nasopharyngeal Secretions Collected during Field Studies. *Journal of Clinical Microbiology*, 1021–1024.

15. Palaniappan, K., & Holley, R. A. (2010). Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. *International Journal of Food Microbiology*, 164–168.

16. Shelef, L. A. (1984). Antimicrobial Effects of Spices. *Journal of Food Safety*, 29-44.

17. Thiel, T. (1999). *Maintenance of Bacterial Strain.* Retrieved 05 26, 2015, from University of Missouri–St. Louis: http://www.umsl.edu/~microbes/pdf/maintenanceofbacteria.pdf

18. *Cinnamon | NCCIH*. (2011). Retrieved from National Institute of Health: https://nccih.nih.gov/health/cinnamon

19. *Plant Database*. (2015). Retrieved from USDA:NRCS: http://plants.usda.gov/java/ClassificationServlet?source=display&classid=CINNA2

Appendices

APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121°C for 15 min.

1. Nutrient Agar

Ingredients	Amount (g/L)
Peptone (Himedia, India)	5.0
Yeast Extract (Himedia, India)	2.5
NaCl (Sigma, Germany)	5.0
Agar (Himedia, India)	15.0

2. Nutrient Broth / Luria-Bertani Broth

Ingredients	Amount (g/L)
Peptone (Himedia,India)	5.0
Yeast Extract (Himedia,India)	2.5
NaCl (Sigma, Germany)	5.0

3. MacConkey Agar (Difco™)

Ingredients	Amount (g/L)
Peptone	17.0
Proteose Peptone	3.0
Lactose	10.0
Bile Salts No. 3	1.5
Sodium Chloride	5.0
Agar	13.5
Neutral Red	0.03
Crystal Violet	0.001

4. Mueller Hinton Agar (Oxoid, England)

Ingredients	Amount (g/L)
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar (Himedia, India)	15.0

5. Mueller Hinton Broth (Oxoid, England)

Ingredients	Amount (g/L)
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5

6. Triple Sugar Iron Agar (Difco™)

Ingredients	Amount (g/L)
Beef Extract	3.0
Yeast Extract	3.0
Pancreatic Digest of Casein	15.0
Proteose Peptone No. 3	5.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous Sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Agar	12.0
Phenol Red	0.024

7. Motility Indole Urease Agar

Ingredients	Amount (g/L)	
NaCl (Sigma)	5	Prepare up to 900ml for **autoclave**
Agar (Himedia, India)	4	
KH_2PO_4 (Fisher Chemical, USA)	2	
Peptone (Himedia, India)	30	
Phenol Red (0.25%) (Sigma, India)	2 ml/L	
Urea (Amresco, USA)	20	Prepare up to 100ml for **filter sterilization**

8. Simmon's Citrate Agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

APPENDIX-II

List of Antibiotics

Name	Concentration	Short Form	Manufacturer
Gentamycin	10 µg/disc	CN10	Oxoid
Tobramycin	10 µg/disc	TOB10	Oxoid
Ceftriaxone	30 µg/disc	CRO30	Oxoid
Cefixime	5 µg/disc	CFM5	Oxoid
Ciprofloxacin	5 µg/disc	CIP5	Oxoid
Imipenem	10 µg/disc	IMP10	Oxoid
Meropenem	10 µg/disc	MEM10	Oxoid
Azithromycin	15 µg/disc	AZM15	Oxoid
Amikacin	30 µg/disc	AK30	Oxoid
Netilmicin	10 µg/disc	NET10	Oxoid
Pipercillin/Tazobactam	110 µg/disc	TZP110	Oxoid
Carbenicillin	100 µg/disc	CB100	Oxoid
Polymyxin B	50 units/disc	PB50	Oxoid

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Name	**Manufacturer**
Autoclave	WiseClave
Refrigerator	Electra, Samsung (+4°C)- to store bacteria; Vestfrost (+4°C)- to store bacterial medium;
Freeze	Vestfrost (-20°C) to store stock antibiotics; ESCO (-80°C) to store stock bacteria.
Incubator	Memmert
Shaking Incubator	WiseCube
Oven	WiseVen
Water bath	WiseBath
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-Safety Cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Weighing Machine	OHAUS®
Weighing Paper	**Fisher**brand®
Vacuum Drier	Savant SpeedVac® SC110
Vacuum Pump	MILLIPORE
Spectrophotometer	Eon™ BioTek®
96-Well Plate	Nunc™ 96F Microwell Plate
Centrifuge Machine	Thermo SCIENTIFIC
Light Microscope	OLYMPUS CX41
Grinder	Miyako