A Comparative Study: Screening of Some Bangladeshi Medicinal Plants for Antibacterial Properties

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

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Dedicated to

All my beloved ones

DECLARATION

I hereby declare that the research work representing the results reported in this thesis entitled **"A Comparative Study: Screening of Some Bangladeshi Medicinal Plants for Antibacterial Properties"** submitted by the undersigned has been carried out under the supervision of Kashmery Khan, Lecturer, Biotechnology 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 Biotechnology, BRAC University, Dhaka and has not been submitted to any other institution for any degree or diploma.

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Afifa Noor
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ABSTRACT

Medicinal plants are the plentiful bioresource of drugs for traditional medicines, food supplements, modern medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs (Ishtiaq *et al.,* 2013). Nowadays, researchers are increasingly turning their attention in investigating herbal products due to the increased resistance of microorganisms against the currently used antibiotics and pharmaceutical companies are searching for alternatives for the high cost of manufacture of synthetic drugs. Medicinal plants can be one approach to alleviate this situation as most of them are safe with slight side effects, if any, are of low cost and affect a wide range of antibiotic resistant microorganisms (Zahra *et al.*, 2011). In the present study, ethanol, methanol and aqueous extracts of *Curcuma longa*, *Zingiber officinale*, *Cinnamomum verum*, *Nigella sativa*, *Azadirachta indica* and *Mentha longifolia* were subjected to microbial susceptibility assays using agar well diffusion method. The microorganisms employed were *Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri, Staphylococcus aureus, Streptococcus pyogenes, Proteus vulgaris and Pseudomonas aeruginosa*. The most susceptible microorganisms were *Klebsiella pneumoniae* and *Staphylococcus aureus*, while the least susceptible was *Proteus vulgaris*. Highest and remarkable antibacterial activity (zone of inhibition) was observed with methanolic extract of Black Cumin seeds against *Klebsiella pneumoniae* and *Staphylococcus aureus* (52mm and 35mm respectively). No antimicrobial activity was observed with aqueous extracts of all the six medicinal plants against the ten selected bacteria. Surprisingly, all the ten selected bacteria were susceptible to the ethanolic and methanolic extracts of only Cinnamon whereas the other five medicinal plant extracts did not show consistent antimicrobial activity against all the ten selected bacteria. However, the six standard antibiotic used as a positive control against each of the ten selected bacteria respectively had shown antimicrobial activity except Clindamycin against *Streptococcus pyogenes* and Cefoxitin against *Shigella flexneri*. Although *Streptococcus pyogenes* and *Shigella flexneri* were antibiotic resistant, but they were remarkably susceptible to the ethanolic and methanolic extracts of Cinnamon. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic extract of Black Cumin seeds against *Klebsiella pneumoniae* was found **34 mg/ml (MIC) and 44mg/ml (MBC)** respectively. Thus, the findings of this study may provide the possibility of developing antibacterial supplements from these medicinal plants.

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ABBREVIATIONS	ELABORATIONS
BHI	Brain Heart Infusion
cm	Centimeter
et al.	And others
ft	Feet
g	Gram
in.	Inches
MHA	Mueller-Hinton Agar
ml	Milliliter
mm	Millimeter
NA	Nutrient agar
ºC	Degree Celsius
rpm	Rotation per minute

Chapter One: Introduction

1.1 Background

Medicinal plants are a source of great economic value all over the world (Joshi *et al.,* 2011). Medicinal plants have played important roles in the treatment of diseases all over the world from a long time ago. A strategic plan for the development and promotion of traditional medicine has been recently published by the World health organization (WHO) in 4 areas which include: a) Identification of traditional medicine, presentation of a proper policy and plan, b) development of research and education, especially in the university level, c) Establishment of unity and cooperation between the employees of traditional and modern medicine d) Development of cultivation of the needed herbs to prevent destruction of natural resources (Rafieian-kopaei, 2012). Today, an estimation of about 80 % of people in developing countries still relies on traditional medicine based largely on species of plants for their primary health care. The use of herbal medicines is increasing day by day due to toxicity and side effects of allopathic medicines (Verma, 2008). Hence the plant products have been increasing worldwide, to lower side effects (Ds, 2014)

1.2 Description of the plants

1.2.1 *Curcuma longa* (Turmeric)

Curcuma longa is a perennial plant with a short stem and large oblong leaves and pyriform rhizomes, which are often branched and brownish-yellow in colour as shown in Fig 1(a). It is commonly known as turmeric which is a dietary spice belonging to the family Zingiberaceae (Tattari *et al.,* 2013).

Turmeric rhizome is used as a food additive (spice) preservative and colouring in Asian countries with a tropical climate, including India, China and Bangladesh (Arutselvi *et al.,* 2012). Its major constituent is curcumin which gives turmeric its unique aroma, flavor and medicinal properties (Arutselvi *et al.,* 2012).

Medicinal and healing properties of herbs are closely related to their phytochemical constituents which are classified into some major groups like alkaloids, acids, essential oils, steroids,

saponins, tannins etc. and getting these chemicals out into the herbal remedy depends upon the solubility of these compounds in various solvents (Al-daihan *et al.*, 2013).

In an investigation carried out by Sawant & Godghate (2013), methanolic extracts contained 16 phytochemicals and ethanolic extract contained 13 phytochemicals from the rhizomes of *Curcuma longa*.

Scientific Classification of *Curcuma longa*

Taxonomic hierarchy of *Curcuma longa* (Integrated Taxonomic Information System):

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants
Subdivision	Spermatophytina – spermatophytes
Class	Magnoliopsida
Superorder	Lilianae – monocots, monocotyledons
Order	Zingiberales
Family	Zingiberaceae – Ginger Family
Genus	*Curcuma L.* – hidden-lily
Species	*Curcuma longa L.* – turmeric

Fig 1 (a) The external morphology of Turmeric

(Retrieved from: http://www.shirleyprice.co.uk/ekmps/shops/nicearoma/images/turmeric-curcuma-longa-5ml-11679-p.jpg)

Fig 1 (b) Dried powder of Turmeric

Fig 1 (c) Extract of Turmeric

1.2.2 *Zingiber officinale* (Ginger)

Zingiber offcinale, commonly known as Ginger, belongs to Zingiberaceae family (Riaz *et al.*, 2015). Ginger is a perennial creeping plant, with thick tuberous rhizome, producing an erect stem 30-100cm (1-3 ft) tall. The lance-shaped leaves are bright green, 15 - 20 cm (6-8 in) long, with a prominent longitudinal rib, enclosing conical clusters of small yellow-green flowers marked with purple speckles (Adebowale *et al.,* 2014).

Ginger is native to Southern Asia, but it is now extensively cultivated in Jamaica, Nigeria, China, India, Fiji, Sierra Leone and Australia (Bhargava *et al.,* 2012). In many countries like Bangladesh, ginger is used in different boiled food preparations (Islam *et al.,* 2014).

Ginger is one of the most commonly consumed dietary condiments for various foods and beverages and has a long history of being an important traditional medicine herb for the treatment of stomach disorders (Bhargava *et al.,* 2012). Ginger is a herb whose rhizome (underground stem) is used as a spice as well as medicine either in fresh, dried and powdered, or as a juice or oil form (Taura *et al.,* 2014).

The main active phytochemicals present in ginger are gingerols, shogaols and paradols, and they have strong antioxidant and chemopreventive properties. Powdered ginger rhizome contains 3.6% fatty oil, 9% protein, 60-70% carbohydrates, 3.8% crude fiber, 8% ash, 9-12% water and other terpenes and terpenoids. Fresh ginger contains 80.9% moisture, 23% protein, 0.9% fat, 1.2% minerals, 2.4% fibre, and 12.3% carbohydrates (Tattari *et al.,* 2013). Phytochemical screening revealed the presence of alkaloids, saponins, tannins, flavonoids, terpenoid and phlobotannins in methanolic and ethanolic extracts of ginger. (Bhargava *et al.,* 2012)

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Scientific Classification of *Zingiber officinale*

Taxonomic hierarchy of *Zingiber officinale* (Integrated Taxonomic Information System):

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants
Subdivision	Spermatophytina – spermatophytes
Class	Magnoliopsida
Superorder	Lilianae – monocots, monocotyledons
Order	Zingiberales
Family	Zingiberaceae – Ginger Family
Genus	*Zingiber Mill.* – ginger
Species	*Zingiber officinale Roscoe* – garden ginger

Fig 2 (a) The external morphology of Ginger

(Retrieved from https://www.punmiris.com/himg/o.10171.jpg)

Fig 2 (b) Dried powder of Ginger

Fig 2 (c) Extract of Ginger

1.2.3 *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 e*t al*., 2016).

Scientific Classification of *Cinnamomum verum*

Taxonomic Hierarchy of *Cinnamomum verum* (Integrated Taxonomic Information System):

Kingdom	Plantae –plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants,
Subdivision	Spermatophytina – spermatophytes
Class	Magnoliopsida
Superorder	Magnolianae
Order	Laurales
Family	Lauraceae – laurels
Genus	*Cinnamomum Schaeff.* – cinnamon
Species	*Cinnamomum verum* J. Presl – cinnamon

Fig 3 (a) The external morphology of Cinnamon

(Retrieved from (http://www.heal-thy.com/wp-content/uploads/2016/09/Cinnamon_Verum_Bark13774324985219f3b27a35c-123.jpg)

Fig 3 (b) Dried powder of Cinnamon

Fig 3 (c) Extract of Cinnamon

1.2.4 *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).

Scientific Classification of *Nigella sativa*

Taxonomic hierarchy of *Nigella sativa* (Integrated Taxonomic Information System):

Kingdom	Plantae –plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants
Subdivision	Spermatophytina – spermatophytes
Class	Magnoliopsida
Superorder	Ranunculanae
Order	Ranunculales
Family	Ranunculaceae
Genus	*Nigella L.*
Species	*Nigella sativa L.* – black cumin

Fig 4 (a) The external morphology of Black Cumin seeds

(Retrieved from http://2.wlimg.com/product_images/bc-full/dir_38/1118339/black-cumin-seeds-1313686.jpg)

Fig 4 (b) Dried powder of Black Cumin seeds

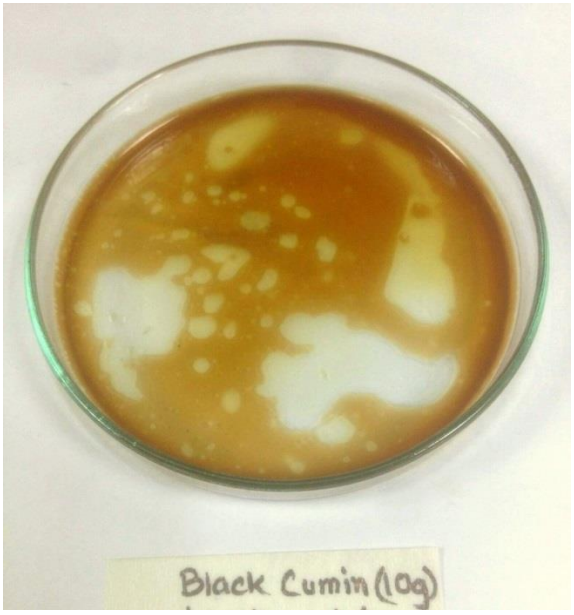


Fig 4 (c) Black Cumin seeds extract

1.2.5 *Azadirachta indica* (Neem)

Azadirachta indica, commonly known as Neem is a tall evergreen tree whose bark is hard, rough and scaly. Its leaves are alternate, flowers are small and white in color. It reaches up to 15–20 m (about 50–65 feet) tall, and sometimes even to 35–40 m (115–131 feet) (Chauhan, D., & Singh, 2014)

Azadirachta indica tree belongs to the family Meliaceae. Originally from Southeast Asia, Neem is also found in tropical and semitropical regions like India, Bangladesh, Pakistan and Nepal (Galeane *et al.,* 2017).

The most important active constituent of Neem is azadirachtin and the most characteristic metabolites of this family are called limonoids, which are tetranortriterpenoides having broad biological activity (Galeane *et al.,* 2017). The Chemical constituents contain many biologically active compounds that can be extracted from Neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones. Azadirachtin is actually a mixture of seven isomeric compounds labeled as azadirachtin A-G and azadirachtin E is more effective

Other compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin. Flavonoids, flavono-glycosides, dihydrochalocones, tannins and others are also important constituents of bark, leaves, fruits and flowers of Neem (Vinoth *et al.,* 2012).

Azadirachtin, a major compound of the neem has potent anti- fedent, growth and reproductive regulating properties. Likewise, nimbin, a limonoid from neem, is also involved in improving pesticide properties (Chauhan, D., & Singh, 2014).

Scientific Classification of *Azadirachta indica*

Taxonomic Hierarchy of *Azadirachta indica* (Integrated Taxonomic Information System):

Kingdom	Plantae –plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants
Subdivision	Spermatophytina – spermatophytes
Class	Magnoliopsida
Superorder	Rosanae
Order	Sapindales
Family	Meliaceae – mahogany
Genus	*Azadirachta A. Juss.*
Species	*Azadirachta indica A. Juss.* – neem

Fig 5 (a) The external morphology of neem tree (left) and neem leaves (right)

(Retrieved from http://www.medplants.net/wp-content/uploads/neem-tree.jpg)

Fig 5 (b) Dried powder of Neem leaves

Fig 5 (c) Extract of Neem leaves

1.2.6 *Mentha longifolia*

Mentha longifolia is commonly known as Mint which is aromatic and almost exclusively perennial, rarely annual herb. They have wide spreading, rhizomes and erect, branched stems. The arrangement of leaves is in opposite pairs, from simple oblong to lancetate, often soft and with a serrated margin (Ds, 2014).

There are about 25 species of fragrant herbs of the mint family Lamiaceae. It is native to Eurasia, North America, southern Africa, and Australia, mints are widely distributed throughout the temperate areas of the world and have naturalized in many places (Raja, 2012).

Mint extracts and menthol-related chemicals are used in food, drinks, cough medicines, creams and cigarettes. Chemical substances that can be extracted from wild mint include menthol, menthone, isomenthone, neomenthol, limonene, methyl acetate, piperitone, beta-caryophyllene, alpha-pinene, beta-pinene, tannins and flavonoids. In an investigation, the phytochemical analysis showed presence of flavonoid, alkaloid, tannin, phenol, glycosides and there it was reported that the identified phytochemical compounds may be the bioactive compounds and the various solvent extracts (Ethanol, Methanol, Diethyl Ether and Acetone) of Mint leaves used can be used as potential source of drugs in the treatment or control of intestinal disorders (Ds, 2014).

Scientific Classification of *Mentha longifolia*

Taxonomic Hierarchy of *Mentha longifolia* (Integrated Taxonomic Information System):

Kingdom	Plantae –plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants
Subdivision	Spermatophytina – spermatophytes
Class	Magnoliopsida
Superorder	Asteranae
Order	Lamiales
Family	Lamiaceae
Genus	*Mentha L.* – mint
Species	*Mentha spicata L.* – bush mint, spearmint

Fig 6 (a) The external morphology of mint leaves

(Retrieved from http://dailybouncer.com/wp content/uploads/2017/04/health_benefit_of_pudina.jpg)

Fig 6 (b) Dried powder of Mint leaves

Fig 6 (c) Extract of Mint leaves

1.3 Therapeutic use of these plants

Turmeric possesses many therapeutic properties and is used as a remedy for various problems especially in lowering cholesterol and triglyceride levels, inhibiting platelet aggregation, as antiseptic and also possesses anti-inflammatory and antioxidant properties (Al-daihan *et al.*, 2013). It is also recommended for treating diabetes, abdominal pains, menstrual disorder, wounds, eczema, psoriasis, jaundice, inflammations, cancerous symptoms and as a blood purifying activity (Sawant & Godghate, 2013). Curcumin is known for its inhibitory action on micro-organisms. Curcumin is a known bacteriostatic agent whereas the essential oil of turmeric is bactericidal and fungistatic (Tattari *et al.*, 2013). In Indian medicine, it is broadly used for the treatment of sprains and swelling caused by injury (Arutselvi *et al.*, 2012).

Ginger has a widespread range of action on the human body and has been found effective in the treatment of cataract, heart disease, migraines, struck amenorrhea, athlete's foot, bursitis, chronic fatigue, cold, flu, coughs, depression, dizziness, fever, erectile difficulties, kidney stones, renal disease and viral infection (Nayaka *et al.*, 2014). It is considered a safe herbal medicine with only few and insignificant adverse/side effects (Bhargava *et al.*, 2012). It serves as a soporific in fever and its natural diuretic stimulates the kidney to flush out toxins faster (Taura *et al.*, 2014). Rhizome or root part of ginger (genus Zingiber) is extensively employed in medicine for the

management of different diseased conditions like nausea, vomiting, motion sickness, gastrointestinal ulcers, diabetes, arterial tension, rheumatoid arthritis, dry mouth/ xerostomia, cancer, migraine headache, sore throat, minor respiratory ailments (Riaz *et al.*, 2015). Ginger extracts have been extensively studied for a broad range of biological activities including antibacterial, anticonvulsant, analgesic, antiulcer, gastric antisecretory, antitumor, antifungal, antispasmodic, antithrombotic, hypocholesterolemic, antiallergic, antiserotonergic, anticholinergic and other beneficial activities. Many studies have proved that ginger is endowed with strong antioxidant, antigenotoxic, antimutagenic and anticarcinogenic properties both in in vitro and in vivo studies (Tattari *et al.*, 2013)

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, pulmonaryprotective, nephro-protective, gastro-protective, antioxytocic 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).

Neem leaf is effective in treating eczema, ringworm, acne, anti-inflammatory, anti-hyperglycemic properties and it is used to heal chronic wounds, diabetic food and gangrene developing conditions. It is believed to remove toxins from the body, neutralize free radicals and purify the blood. It is used as anticancer agent and it has hepato-renal protective activity and

hypolipidemic effects. Neem extract has been reported to have anti-fungal, antibacterial, anti-protozoal, and antiviral activity. It is also considered as a natural insecticide/pesticide plant and the quality of pesticide and pharmacological products depend upon the contents of azadirachtin and nimbin in the plant. Accordingly, all parts of this plant are useful and have been used in treatment of diseases ranging from teeth decay, ulcers, swollen liver, malaria, dysentery, diarrhea etc. They possess astringent, purgative anti-inflammatory, moderate anti-tumor and bactericidal effects (Chauhan, D., & Singh, 2014). Almost every part of the tree has been in use since ancient times to treat a number of human ailments and also as a household pesticide. The extract from bark, leaves, fruits and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children (Vinoth *et al.*, 2012).

Nowadays Mint is used in many countries for various ailments. Mint is considered as an appetizer in Ayurveda and also useful in gastric troubles. The Native Americans used it in several traditional ways. In Europe, wild mint was traditionally used to treat flatulence, digestion problems, gall bladder problems and coughs. The oil was extracted and rubbed into the skin for aches and pains (Ds, 2014)**.**

1.4 Antimicrobial properties of these plants

Spices like turmeric and ginger, are vital for the preparation of our daily food and are reported to possess compounds, which have varied beneficial biological effects and also prevent the microbial spoilage of food. The medicinal, chemical and pharmacological properties of ginger have been extensively reviewed. Ginger has been shown to be effective against the growth of both gram-positive and gram-negative bacteria including *Escherichia coli, Proteus vulgaris, Salmonella typhi, Staphylococcus aureus* and *Streptococcus viridans* (Tattari *et al.*, 2013). In the study carried out by Tattari *et al.* (2013), the antimicrobial activity was found to be highest in turmeric, followed by ginger against *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* Ginger possesses a noticeable antimicrobial activity which was confirmed by checking the susceptibility of different strains of bacteria and fungus by measuring the zone of inhibition (Riaz *et al.*, 2015).

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).

The antimicrobial activity of Neem showed that the ethyl acetate extract and butanol fraction presented greater activity against *Streptococcus mutans* and *Streptococcus mitis* presenting a MIC = 50 µg/ml for these strains, and the strain *Enteroccocus faecalis*, the hydroethanolic extract and aqueous fraction were most promising samples with a MIC = 50 µg/ml and MIC = 25 µg/ml, respectively (Galeane *et al.,* 2017). Minimum Bactericidal Concentration (MBC) value of 5 mg/l was obtained with Azadirachta indica against *S. typhi* and *K. pneumoniae* (Joshi *et al.,* 2011). *Azadirachta indica* leaf extract has antibacterial activity against dental pathogens (Vinoth *et al.,* 2012).

The ethanol extract of Mint showed highest antimicrobial activity against *Shigella sonnei*, while lowest antimicrobial activity was observed by ethanol extract of Mint against *Citrobactor spp* and by Acetone extract of Mint against *Shigella dysentarie* (Ds, 2014). The minimal inhibitory concentration (MIC) of Mint extract was determined by broth microdilution assay. The highest (MIC) value (8, 16, 32, 32 and 32 µg/ml) was observed against *Bacillus fastidiosus, Staphylococcus aureus , Proteus mirabilis , Proteus vulgaris* and *Salmonella choleraesuis* respectively, while *Escherichia coli , Pseudomonas aeruginosa , Klebsiella pneumoniae* and

Serratia odorifera ranked next (MIC 64, 128, 256 and 512 µg /ml) respectively (Al-sum & Al-arfaj, 2013).

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

Ten 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, nonspore 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 *Pseudomonas aeruginosa:* *Pseudomonas aeruginosa* is a gram-negative, ubiquitous environmental bacterium with least requirements for survival and a significant capability to adapt to a variety of environmental challenges. It produces a soluble, blue-green pigment. It can cause urinary tract infections. It can also infect burns and wounds, and can cause blood infections (sepsis). It has a low permeability of the outer membrane which makes this pathogen resistant to a large range of harmful agents, including antibiotics. *Pseudomonas aeruginosa* is one of the main micro-organisms of nosocomial infections, and it is known for its resistance to a range of antimicrobial agents (Dantas, 2014).

1.5.6 *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.).

1.5.7 *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. *Klebsiella pneumoniae* 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).

1.5.8 *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).

1.5.9 *Salmonella typhi*: *Salmonella typhi* are gram-negative rod shaped bacteria. *Salmonella typhi* is a strain of bacteria that lives only in humans. It causes a bacterial infection of the intestinal tract and occasionally the bloodstream which is called typhoid fever. Antibiotics used on typhoid patients include ampicillin, trimethoprim- sulfamethoxazole, or chloramphenicol. Due to the overuse of such antibiotics, the species have started to develop drug resistance over the past few years (Pollack, 2003).

1.5.10 *Shigella flexneri*: *Shigella flexneri* is a gram-negative bacterium which causes the most communicable of bacterial dysenteries, shigellosis, causing abdominal cramps and fever. It is found only in humans. Studies show that the microbe is developing resistance against the common antimicrobial as ampicillin, chloramphenicol, streptomycin, trimethoprim-sulphamethoxazol and tetracycline (Jennison & Verma, 2004).

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. Ciprofloxacin - Ciprofloxacin belongs to the class of drugs known as quinolone antibiotics. It works by killing bacteria or preventing their growth. However, this medicine will not work for colds, flu, or other virus infections.
2. Imipenem - Imipenem, the first of a new class of carbapenem antibiotics, has potent activity against most clinically important species of bacteria, including isolates resistant to other antibiotics.
3. 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.
4. 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.

5. Cefoxitin – Cefoxitin is a semisynthetic, broad-spectrum, second-generation cephalosporin with antibacterial activity.
6. 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.

1.7 Effect of plant extracts on human body

Herbal treatment is still 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 influences the normal flora's niches 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 Effects of antibiotics on human body

Allopathic antibiotics are synthetic substances that destroy microorganisms or inhibit their growth and are used extensively to treat diseases in animals and humans. Some people may show allergic reactions to some antibiotics. These reactions may be mild like rashes appearing on the skin or may be very serious and can even be fatal. Allopathic system of medicine mostly makes use of chemicals as medicines. It takes more than a few years of testing and trials on animals and humans, before an allopathic medicine is made available in the market. Its effects, side-effects, efficiency, fixing recommended dose, etc. are extensively studied on scientific lines before it is made available in a market. Prolonged antibiotic treatment can lead to damaging side effects in patients, including ototoxicity, nephrotoxicity, and tendinopathy, yet the mechanisms underlying the effects of antibiotics in mammalian systems remain blurred (Kalghati *et al*., 2013). Some of

the antibiotics can create reactive oxygen species (ROS) throughout the body. The ROS is toxic to healthy cells and can possibly harm bacterial cells. The ROS start to kill energy sources as well as the mitochondria in human cells. The ROS are thought to bind to bacterial DNA and dissociate it, therefore killing the bacteria, as the bacteria no longer have genetic information. However, ROS are not terribly specific and can begin to damage all the cells and metabolic machinery in the body, not just in bacterial cells. This is known as oxidative tissue damage and can be especially harmful if a person's immunity is already compromised by a bacterial infection (Singh, 2013)

1.9 MIC and MBC measurement of plant extract

The potency of plant extracts can be determined by measuring the minimum inhibitory concentrations (MIC) value which is the lowest concentration of antimicrobials required to inhibit the growth of a test organism within a definite time period, usually within 18-24 hours (Turnidge *et al.,* 2003). MIC measurement by serial broth dilution method is the most preferred one which requires various dilutions of the antimicrobial compound in a suitable solvent. Selection of appropriate solvent is very important as it plays the most important role and has major influences in MIC measurements. The most common solvents used in MIC measurements are ethanol, methanol and DMSO. The minimum bactericidal concentration (MBC) value was determined by spread plating a small amount of broth from MIC containing tubes on to freshly prepared nutrient agar media. The plates were incubated further for 18 hours at 37°C. The highest dilution that produced no single bacterial colony on the nutrient agar plates was taken as MBC (Lawrence *et al.,* 2011).

1.10 Objectives of the study

There are huge benefits in the study of plants with medicinal value. The present study aims to compare the antimicrobial activity by agar well diffusion method of ethanolic, methanolic and aqueous extracts of six types of medicinal plant extracts on ten different bacterial strains and among them the most efficient extracts are identified, provided that the concentration of all the extracts were same (0.1g/ml) except Mint, whose concentration was 0.15g/ml.. In this investigation, the antimicrobial activity test of some common commercial antibiotics against the

selected bacterial strains was also done to serve as a positive control and to compare the antimicrobial potency of these plant extracts with that of the antibiotics. Considering these, the specific objectives of this study included the following:

1. Preparation of three different types of extracts for the six medicinal plants using three different solvents: ethanol, methanol and distilled water.

2. Observation of antimicrobial activity of the three types of extracts of the six medicinal plants against some gram-positive and gram-negative organisms.

3. Comparison of antimicrobial activity of the plant extracts with commercial antibiotics.

4. Determination of the most efficient plant extract among the six medicinal plants by the highest activity index value and then finding out the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of that extract

Chapter Two: Materials and Methods

2.1 Research Laboratory

This research work is laboratory based so it was carried out in the Microbiology and Biotechnology Laboratories of the Department of Mathematics and Natural Sciences at BRAC University, Dhaka, Bangladesh. In this laboratory, BSL-2 (Biosafety level 2) facility is followed and the entire microbiological works were done within laminar flow cabinet. In this research, the antimicrobial activity of ethanolic, methanolic and aqueous extracts of Turmeric, Ginger, Cinnamon, Black Cumin seeds, Neem and Mint was compared with the antibacterial activity of commercial antibiotics on some gram-positive and gram-negative bacteria.

2.2 Collection and Processing

2.2.1 Turmeric- It was purchased from the local market in Dhaka city. Turmeric was coarsely minced, air dried for few days and then crushed to a fine powder, weighed and stored in clean air tight container.

2.2.2 Ginger- It was bought from the local market in Dhaka city. It was peeled, washed, coarsely minced, air dried for few days and crushed with a blender to a fine powder, weighed and stored in clean air tight container.

2.2.3 Cinnamon- Barks were collected from the local market in Dhaka city. These barks were washed with distilled water to remove the adhering dust particles and air dried for few days place. The dried barks were powdered in a grinder and sieved into fine powder to be used for extraction. It was then weighed and stored in clean air tight container and kept at 4^{o}C for further use.

2.2.4 Black Cumin seeds- Black cumin seeds were purchased from the local market in Dhaka city. First the dirty part from black cumin seed was removed by using hand and dried at room temperature, then grinded into fine powder by a grinder, weighed and stored in clean air tight container.

2.2.5 Neem- Leaves were collected from an *Azadirachta indica* tree in Dhaka city. It was ensured that the plant was healthy and uninfected. The leaves were washed under running tap

water, to eliminate dust and other foreign particles, then air dried for few days, then powdered in a grinder, weighed and stored in clean air tight container.

2.2.6 Mint- Mint leaves were obtained from a local market in Dhaka city. The leaves were first washed under running tap water, to eliminate dust and other foreign particles, then air dried for few days, then powdered in a grinder, weighed and stored in clean air tight container.

2.3 Preparation of extracts using different solvents

Three different types of extracts were prepared from all the six medicinal plant samples using two organic solvents- **ethanol** and **methanol**; and one inorganic solvent-**water**.

2.4 Ethanolic Extraction

Ethanol is widely used as a solvent. It is relatively safe, and can be used to dissolve many organic compounds which are insoluble in water.

2.4.1 Turmeric- Ten grams of powdered sample was dissolved in 100 ml of absolute ethanol in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 20 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.4.2 Ginger- Ten grams of powdered sample was dissolved in 100 ml of absolute ethanol in a conical flask, covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120

rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 10 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.4.3 Cinnamon- Ten grams of powdered cinnamon were weighed and mixed with 100 ml of absolute ethanol in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then concentrated using a rotatory evaporator for 10 minutes. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 15 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.4.4 Black Cumin seeds- Finely grinded ten grams of black cumin seeds powder was added to 100ml of absolute ethanol solvent in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator on continuous shaking at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then concentrated using a rotatory evaporator for 15 minutes. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 15 minutes. Finally, a sticky, oily extract appeared on the surface of the plate when all the solvent was evaporated. The crude oil extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of

the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.4.5 Neem- Ten grams of powdered Neem was dissolved in 100 ml of absolute ethanol in a conical flask, covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 10 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.4.6 Mint- Fifteen grams (15g) of Mint powder was dissolved in 100 ml of absolute ethanol in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 10 minutes. Finally, a dried, sticky, semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.5 Methanolic Extraction

Methanol is widely used as a solvent, because many of the compounds dissolve in it easily, which is important for the plant material, moreover methanol easily evaporates. So, it can be separated from the extract and it is also easily available at low cost.

2.5.1 Turmeric- Ten grams of powdered sample was dissolved in 100 ml of absolute methanol in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 20 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.5.2 Ginger- Ten grams of powdered sample was dissolved in 100 ml of absolute methanol in a conical flask, covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 10 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.5.3 Cinnamon- Ten grams of powdered cinnamon were weighed and mixed with 100 ml of absolute methanol in a conical flask, the mouth of the flask was covered with aluminium foil and

then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then concentrated using a rotatory evaporator for 10 minutes. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 15 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.5.4 Black cumin seeds- Finely grinded ten grams of black cumin seeds powder was added to 100ml of absolute methanol solvent in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator on continuous shaking at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then concentrated using a rotatory evaporator for 15 minutes. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 15 minutes. Finally, a sticky, oily extract appeared on the surface of the plate when all the solvent was evaporated. The crude oil extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.5.5 Neem- Ten grams of powdered Neem was dissolved in 100 ml of absolute methanol in a conical flask, covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 10 minutes. Finally, a sticky semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract

was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.5.6 Mint- Fifteen grams (15g) of Mint powder was dissolved in 100 ml of absolute methanol in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and then evaporated using an evaporator till the final volume was reduced to one-fourth of the original volume of the solvent used. Then the concentrated extract solution was poured on a sterile petri dish lid and kept in the incubator at 55ºC for 10 minutes. Finally, a dried, sticky, semi-solid extract appeared on the surface of the plate when all the solvent was evaporated. The extract was collected by scraping it off the plate and then stored in an autoclaved, weighed, McCartney bottle. The extract inside the bottle was weighed, recorded, and the exact amount of the extract was calculated by subtracting the mass of the empty bottle. Then the bottle was labeled with the name and amount of the extract and stored at 4°C in the refrigerator.

2.6 Aqueous Extraction

Water is capable of dissolving a variety of different substances because of its polarity and ability to form hydrogen bonds, for which it is a good inorganic solvent. However, water is good at dissolving ions and polar molecules, but poor at dissolving nonpolar molecules.

2.6.1 Turmeric- Ten grams of powdered turmeric sample was dissolved in 100ml of distilled water in a conical flask and boiled for 30 minutes on slow heat of a Bunsen burner. The residue was removed by filtering through autoclaved cheesecloth and then stored in an autoclaved McCartney bottle. Then the bottle was labeled with the name of the extract and stored at 4°C in the refrigerator

2.6.2 Ginger- Ten grams of powdered ginger was dissolved in 100 ml of distilled water in a conical flask, and boiled for 20 minutes on slow heat of a Bunsen burner. Using an autoclaved filter paper, the filtrate was collected slowly in a conical flask and stored in an autoclaved McCartney bottle. Then the bottle was labeled with the name of the extract and stored at 4°C in the refrigerator.

2.6.3 Cinnamon- Ten grams of powdered cinnamon were weighed and mixed with 100 ml of distilled water in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then stored inside autoclaved falcon tubes at 4°C in the refrigerator.

2.6.4 Black Cumin Seeds- Ten grams of powdered black cumin seeds were weighed and mixed with 100 ml of distilled water in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then stored inside autoclaved falcon tubes at 4°C in the refrigerator.

2.6.5 Neem- Ten grams of powdered Neem were weighed and mixed with 100 ml of distilled water in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then stored inside autoclaved falcon tubes at 4°C in the refrigerator.

2.6.6 Mint- Ten grams of powdered mint were weighed and mixed with 100 ml of distilled water in a conical flask, the mouth of the flask was covered with aluminium foil and then kept at 37ºC in a shaker incubator at 120 rpm for 24 hours. Using an autoclaved Whatman No.1 filter paper, the filtrate was collected slowly in a conical flask and then stored inside autoclaved falcon tubes at 4°C in the refrigerator.

2.7 Preparation of Extract Solution for Antibacterial Activity Test

All of the six types of collected crude extracts were dissolved in the same solvent which was used to obtain the extracts. For example, when 0.61g crude extract from 10g Neem powder was collected using 100ml absolute ethanol solvent, the extract solution was made by adding the amount of absolute ethanol obtained by the calculation as shown below. The amount of the solvent added to prepare each extract solution from each of the six crude medicinal plants extracts are determined by-

$$\text{Amount of solvent added} = \frac{100}{\text{amount of plant powder used}} \times \text{amount of extract obtained}$$

2.8 Storage and preservation of extracts

All the prepared extracts were stored in autoclaved 25 ml McCartney bottles which were then kept in 4°C refrigerator for preservation and prevention from external contaminants until use. The caps of all the bottles were closed tightly and properly labeled, showing the name of the extract, solvent used and the amount of extract obtained for antimicrobial assay.

2.9 Preparation of fresh nutrient agar (NA) plates

Nutrient agar is a general purpose medium which can support growth of a wide range of non-fastidious organisms. Usually, preparation of 1000 ml of NA requires 28g of the powder. By maintaining this concentration constant, the required volume of NA was prepared each time before the organisms were subcultured. The required mass of the NA powder was measured using an electronic balance and the required volume of distilled water was measured using a measuring cylinder. After pouring the distilled water into a conical flask containing the NA powder, continuous stirring using a sterile glass rod must be done to breakdown any lumps as the flask was heated on a Bunsen burner. After few minutes when the solution turned clear and bubbles appeared indicating the boiling point was reached, the heating was stopped. Then the flask was allowed to cool for a while and the top of the conical flask was sealed with aluminium foil and autoclaved for about 1.5 hours to remove any contamination present. Then, the nutrient agar solution was carefully poured in to sterile petri dishes inside a laminar flow cabinet as soon as it brought out of the autoclave machine. For each medium sized plate, 20 ml of nutrient agar solution is needed and for large sized plates, 35 ml is needed. The petri dishes were properly labeled holding the name of the media, the initials of the person who made the agar and the date of its preparation. Then it was kept in the refrigerator for getting solidified until use.

2.10 Subculture of bacteria

The stock cultures of the ten microorganisms were taken from the Laboratory sample of Department of Mathematics and Natural Sciences, BRAC University. Usually purity of the cultures was maintained by regular subculturing. For each organism, freshly prepared Nutrient

Agar (NA) plates were taken inside a laminar air flow chamber and then a loop was burned till red hot over a spirit lamp flame. A loopful of microbes was taken from the stock culture after the loop was cooled. The bacteria samples were streaked onto properly labeled NA plates inside a laminar air flow chamber and incubated at 37ºC for 24 hours before use. On the next day, when the growth of bacteria samples was clearly visible, the plates were wrapped with parafilm and stored at 4°C until further use. For each experiment of this study, the organisms were freshly subcultured and the 24-hour cultures were used.

2.11 Preservation and storage of selected bacteria samples

The selected bacterial samples were preserved in order to reuse them for this research purpose a number of times and so the selected bacterial strains were subcultured from the laboratory bacterial stock and then sealed with Parafilm to avoid getting contaminated with other samples. Finally, the bacterial samples were stored in the refrigerator at 4ºC until use.

2.12 Maintenance of aseptic condition

All antibacterial assays were conducted inside the biosafety hood under complete aseptic conditions. The laminar air flow chamber with HEPA filters was cleaned with 70% ethanol.

2.13 Preparation of 0.9% saline solution

0.9% saline solution was made by measuring 0.9g of sodium chloride (NaCl) using electronic balance and then it was added into a conical flask containing 100 ml of distilled water. Using a glass pipette, 10 ml of the saline solution were transferred in each test tube. In this way, several test tubes were prepared and autoclaved, with the screw cap opened through 1.5 turns. After autoclave was done, the screw caps were immediately turned to fully close the mouth of the test tubes to prevent contamination of the saline. These were used later, when required. To make 0.9% saline solution, 0.9 grams of sodium chloride (NaCl) was taken into 100 ml of distilled water.

2.14 Use of 0.5 McFarland standard solutions

Commercial samples of McFarland standards of different concentrations (i.e. 0.5, 1, 2, 3, 4, and 5) were available in the laboratory of MNS department, BRAC University. To visually compare the turbidity of each bacterial suspension with its adjusted standard turbidity, 0.5% McFarland standard solution was used in this study. This was done in order to keep the number of bacteria in the saline suspension within a given range for standardizing the lawn culture of antimicrobial tests.

2.15 Preparation of MHA media plates

Muller-Hinton agar (MHA) is a microbiological growth medium, usually used for antibiotic susceptibility testing. In this study, it was used for antimicrobial susceptibility testing of medicinal plant extracts by well diffusion method. To prepare 1000 ml of MHA, 38g of the powder is required. By maintaining this concentration constant, the required volume of NA was prepared each time before the organisms were subcultured. The required mass of the MHA powder was measured using an electronic balance and the required volume of distilled water was measured using a measuring cylinder. After pouring the distilled water into a conical flask containing the MHA powder, continuous stirring using a sterile glass rod must be done to breakdown any lumps as the flask was heated on a Bunsen burner. After few minutes when the solution turned clear and bubbles appeared indicating the boiling point was reached, the heating was stopped. Then the flask was allowed to cool for a while and the top of the conical flask was sealed with aluminium foil and autoclaved for about 1.5 hours to remove any contamination present. Then, the MHA solution was carefully poured in to sterile petri dishes inside a laminar flow cabinet as soon as it brought out of the autoclave machine. For each medium sized plate, 20 ml of MHA solution is needed and for large sized plates, 35 ml is needed. The petri dishes were properly labeled holding the name of the media, the initials of the person who made the agar and the date of its preparation. Then it was kept in the refrigerator for getting solidified until use.

2.16 Inoculation of test organisms

Standardized inoculum of 0.5 McFarland (approximate cell count density: 1.5x10^8) turbidity standard was prepared by taking with sterile loops about 1-2 colonies of organisms from 24 hour culture plates and then added in sterile saline solution in a test tube. The test tubes containing saline and organism were vortexed for homogeneous mixing and the turbidity compared to that of 0.5 McFarland standard solutions.

2.17 Antimicrobial assay using Agar well diffusion method

Agar-Well Diffusion Assay is an *in vitro* assessment of antimicrobial susceptibility testing and here it was used to study the antimicrobial activity of six medicinal plant extracts against the targeted ten bacteria. Standard antibiotic discs were used as positive control and further compared with the activity of the extracts on the targeted organisms. For each organism, the activity of six plant extracts was studied and each of the plant extracts had three different solvent solutions which were used to obtain its particular extracts. Ciprofloxacin was used as a positive control for *E. coli*; Vancomycin for *Bacillus subtilis*; Imipenem for *Bacillus cereus*, *Proteus vulgaris* and *Pseudomonas aeruginosa*; Clindamycin for *Streptococcus pyogenes* and *Staphylococcus aureus*; Cefoxitin for *Shigella flexneri*; and Meropenem for *Klebsiella pneumoniae*; Gentamicin for *Salmonella typhi*. An autoclaved cotton bud was dipped into the bacterial suspension in saline. This was done to perform lawn culture on properly labeled MHA petri dish plates which gives a uniform growth of bacteria. The lid of the petri dish was left half closed to allow inoculum to be absorbed before carrying out agar-well diffusion method. After lawn culture, holes were made on the MHA media with a sterile cork borer. 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 and the name of the selected antibiotic disc used as a positive control for a particular bacterial strain. Each of the plant extracts of about 60 µl per well were loaded in the wells using a micropipette. This was then incubated at 37°C temperature for 24 hours. The presence of a clear zone around the hole indicated a positive result for antimicrobial tests. This process was followed separately for each of the ten bacteria. Antimicrobial activities of the

extracts were determined by measuring the diameters of the zones of inhibition in millimeters produced against the bacteria.

2.17.1 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.

2.17.2 Determination of activity index

The inhibitory effects of the methanolic, ethanolic and aqueous extracts were calculated and compared by measuring the activity index using the following formula:

$$\textbf{Activity Index (AI)} = \frac{\textit{Zone of inhibition of extract}}{\textit{Zone of inhibition of antibiotic}}$$

2.18 Preparation of Brain Heart Infusion (BHI) Broth

Brain Heart infusion broths were used in serial broth dilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts. The required amount of powder of the BHI broth was measured using an electronic balance and then mixed with distilled water. Then 10ml of broth solution was transferred into each sterile test tube and in this way 20 test tubes were prepared and autoclaved, with the screw cap opened through 1.5 turns. After autoclave was done, they were appropriately labeled, kept in a test tube holder and stored at room temperature.

2.19 Determination of MIC and MBC of the most effective plant extract

The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) was only determined for the plant extract that would show the highest diameter (in mm) of zone of inhibition or activity index. In determining the MIC and MBC, different concentration of plant extract solution and undiluted volumes of Brain Heart Infusion broth (BHI) were mixed in sterilized test tubes. The different concentrations of the plant extract solution that were added on different undiluted volumes of Brain Heart Infusion broth were: 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 6 mg/ml, 7 mg/ml, 8 mg/ml, 9 mg/ml, 10mg/ml, 14 mg/ml, 20mg/ml, 24 mg/ml, 30mg/ml 34 mg/ml, 40mg/ml and 44mg/ml. After that, 0.1 ml of inoculum of bacteria, which was highly susceptible to that plant extract, was taken from a sterile saline solution that was compared to 0.5 McFarland Standard solutions, was transferred into the broth. The final volume of each tube was made 10 ml. In a test tube containing BHI broth (growth medium) and plant extract was used as a positive control and a negative control tube was prepared with only BHI broth. A test tube containing BHI broth and 0.1ml of inoculum is kept as a growth control tube. The test tubes were then incubated at 37 °C for 24 hours. After incubation, the inoculated tube with the lowest concentration of the extract which showed no turbidity was considered as the minimum inhibitory concentration of the plant extract. To determine MBC, about 100µl of the inoculated broth of each tube were spread plated using a sterile glass spreader on the NA plates. The concentration at which there was no bacterial colonies formed on the surface of the agar plate was determined as the minimum bactericidal concentration of the plant extract.

Chapter Three: Results

3.1 Plant extracts obtained from different solvents

The table below summarizes the amount of crude extracts obtained from six different medicinal plant samples using three different solvents: ethanol, methanol and distilled water. It must be noted that for all the plant samples, the concentration of all the extracts was kept constant i.e. 100mg/ml except for Mint (Ethanolic and Methanolic extract) whose concentration was 150mg/ml.

Plant Sample	Type of Solvent	Volume of Solvent (ml)	Amount of powder (g)	Amount of Crude extract (g)
Turmeric	Ethanol	100	10	0.95
	Methanol	100	10	1.05
	Distilled water	100	10	3.90
Ginger	Ethanol	100	10	0.54
	Methanol	100	10	0.54
	Distilled water	100	10	4.50
Black Cumin	Ethanol	200	20	5.85
	Methanol	200	20	6.39
	Distilled water	200	20	5.80
Cinnamon	Ethanol	200	20	2.79
	Methanol	200	20	3.58
	Distilled water	200	20	6.20
Neem	Ethanol	100	10	0.48
	Methanol	100	10	0.92
	Distilled water	100	10	0.40
Mint	Ethanol	100	15	0.67
	Methanol	100	15	0.92
	Distilled water	100	10	2.10

Table 3.1: Amount of extracts obtained using ethanol, methanol and distilled water

All of these crude extracts were diluted in the method mentioned in **section 2.7** of this paper and the diluted extracts are shown as follows:

Fig 7: Three types of turmeric extracts

Fig 8: Three types of Ginger extracts

Fig 9: Three types of Black Cumin seed extract

Fig 10: Three types of Cinnamon extracts

Fig 11: Three types of Neem extract

Fig 12: Three types of Mint extracts

3.2 Observation of antibacterial activity of ethanolic, methanolic and aqueous extracts of selected plants with allopathic antibiotics:

In this study, ten different bacteria were used for comparing the antimicrobial properties of ethanolic, methanolic and aqueous extracts of the six medicinal plants: Turmeric, Ginger, Cinnamon, Black Cumin seeds, Neem and Mint.

During the antimicrobial assay, a positive control in the form of antibiotic disc was used on the petri plate along with different extracts of each of the six medicinal plants to evaluate and compare the activity of the extracts with that of antibiotic disc against the ten bacteria.

List of antibiotic disc used against the ten bacteria are as follows:

- Ciprofloxacin - *E. coli*;
- Vancomycin - *Bacillus subtilis*;
- Imipenem - *Bacillus cereus*, *Proteus vulgaris* and *Pseudomonas aeruginosa*;
- Meropenem - *Klebsiella pneumonia*;
- Clindamycin - *Streptococcus pyogenes* and *Staphylococcus aureus*;
- Cefoxitin – *Shigella flexneri*
- Gentamicin - *Salmonella typhi*

3.2.1 Turmeric

Ethanolic and methanolic extracts of Turmeric have shown higher zone of inhibition for the gram-positive bacteria than the gram-negative bacteria [as seen in Table 3.2.1 (a) & (b)]. The highest zone of inhibition was shown by the ethanolic extract of Turmeric against *Bacillus cereus* (**20mm**) and then against *Staphylococcus aureus* (**18.7mm**) as shown in (Fig 13). However, ethanolic extract of Turmeric had no antibacterial activity against *Streptococcus pyogenes* and methanolic extract had no antibacterial activity against *Salmonella typhi*. Although *Streptococcus pyogenes* and *Shigella flexneri* were resistant against the antibiotic Clindamycin and Cefoxitin respectively, but *Streptococcus pyogenes* was susceptible to methanolic extract and *Shigella flexneri* was susceptible to ethanolic and methanolic extracts of Turmeric to some extent. All the antibiotics have shown higher zone of inhibition against the ten bacteria. The aqueous extract of Turmeric has no zone of inhibition against the ten bacteria.

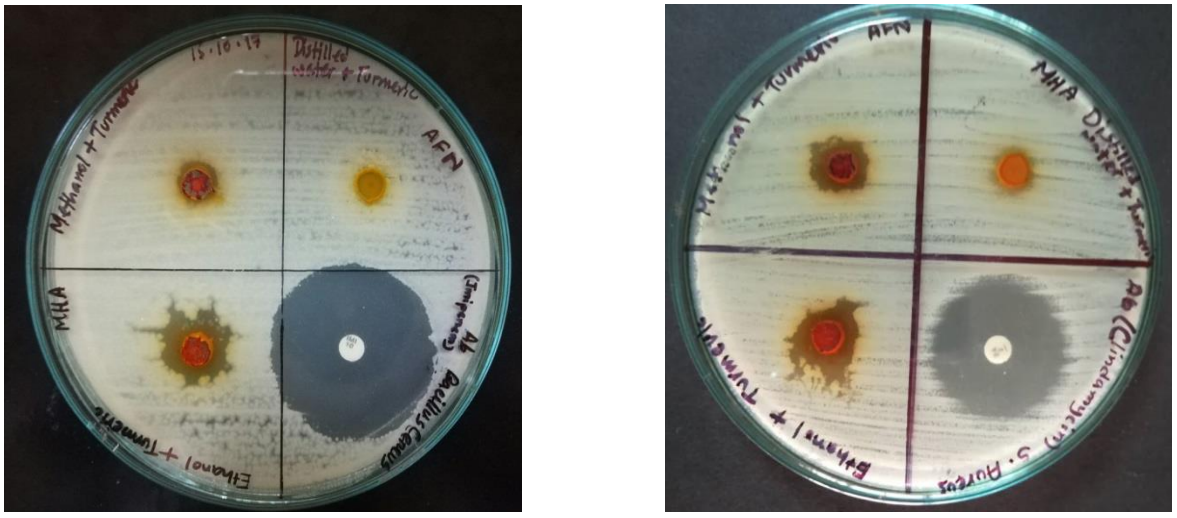
The following tables show the zone of inhibition of the antibiotic disc and various extracts of Turmeric against the four gram-positive and six gram-negative bacteria:

Gram (+)ve bacteria	Name of Antibiotic	Zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Staphylococcus aureus	Clindamycin	30	18.7	15	0
Bacillus cereus	Imipenem	42	20	11	0
Bacillus subtilis	Vancomycin	25	18	15	0
Streptococcus pyogenes	Clindamycin	0	0	8	0

Table 3.2.1(a): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Turmeric against the gram-positive bacteria

Gram (-)ve bacteria	Name of Antibiotic	Zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Proteus vulgaris	Imipenem	25	11.5	10	0
Pseudomonas aeruginosa	Imipenem	26	14	9	0
Klebsiella pneumoniae	Meropenem	25	15	12	0
Escherichia coli	Ciprofloxacin	35	12	12	0
Shigella flexneri	Cefoxitin	0	11.3	11	0
Salmonella typhi	Gentamicin	27	10	0	0

Table 3.2.1(b): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Turmeric against the gram-negative bacteria



(a) **(b)**

Fig 13: Zone of inhibition of Turmeric extracts against (a) *Bacillus cereus* and (b) *Staphylococcus aureus*

3.2.2 Ginger

Ethanolic and methanolic extracts of Ginger have shown higher zone of inhibition for the gram-positive bacteria than the gram-negative bacteria [as seen in Table 3.2.2 (a) & (b)]. The highest zone of inhibition was shown by the ethanolic extract (**20mm**) and then methanolic extract (**19mm**) of Ginger against *Klebsiella pneumoniae* as shown in Fig 14(a). The methanolic extract of Ginger has shown the third best zone of inhibition (**15mm**) against *Bacillus cereus* as shown in Fig 14(b). However, ethanolic and methanolic extract had no antibacterial activity against *Proteus vulgaris* and *Pseudomonas aeruginosa.* Although *Streptococcus pyogenes* and *Shigella flexneri* were resistant against the antibiotic Clindamycin and Cefoxitin respectively, but *Streptococcus pyogenes* and *Shigella flexneri* were susceptible to ethanolic and methanolic extracts of Ginger to some extent. All the antibiotics have shown higher zone of inhibition against the ten bacteria. The aqueous extract of Ginger has no zone of inhibition against the ten bacteria.

The following tables show the zone of inhibition of the antibiotic disc and various extracts of Ginger against the four gram-positive and six gram-negative bacteria:

Gram (+)ve bacteria	Name of Antibiotic	Diameter of zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Staphylococcus aureus	Clindamycin	30	14	11	0
Bacillus cereus	Imipenem	42	13	15	0
Bacillus subtilis	Vancomycin	25	13	12	0
Streptococcus pyogenes	Clindamycin	0	9	9	0

Table 3.2.2(a): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Ginger against the gram-positive bacteria

Gram (-)ve bacteria	Name of Antibiotic	Diameter of zone of inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Proteus vulgaris	Imipenem	25	0	0	0
Pseudomonas aeruginosa	Imipenem	26	0	0	0
Klebsiella pneumoniae	Meropenem	25	20	19	0
Escherichia coli	Ciprofloxacin	35	14	10	0
Shigella flexneri	Cefoxitin	0	11	11	0
Salmonella typhi	Gentamicin	27	5.5	11	0

Table 3.2.2(b): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Ginger against the gram-negative bacteria

(a) **(b)**

Fig 14: Best zone of inhibition of Ginger extracts against (a) *Klebsiella pneumoniae* and (b) *Bacillus cereus*

3.2.3 Cinnamon

Ethanolic and methanolic extracts of Cinnamon have shown higher zone of inhibition for the gram-positive bacteria than the gram-negative bacteria [as seen in Table 3.2.3 (a) & (b)]. The highest zone of inhibition was shown by the ethanolic extract (**28mm**) and then by the methanolic extract (**25mm**) of Cinnamon against *Shigella flexneri* as shown in Fig 15(a), whereas *Shigella flexneri* was resistant to Cefoxitin antibiotic. Although *Streptococcus pyogenes* and *Shigella flexneri* were resistant to Clindamycin and Cefoxitin antibiotics respectively but these bacteria were susceptible to the ethanolic and methanolic extracts of Cinnamon to a large extent. Moreover, the ethanolic and methanolic extracts of Cinnamon have shown antibacterial activity against all the ten bacteria. However, except the antibiotics Clindamycin and Cefoxitin, all the antibiotics have shown higher zone of inhibition against the ten bacteria. The aqueous extract of Cinnamon has no zone of inhibition against the ten bacteria.

The following tables show the zone of inhibition of the antibiotic disc and various extracts of Cinnamon against the four gram-positive and six gram-negative bacteria:

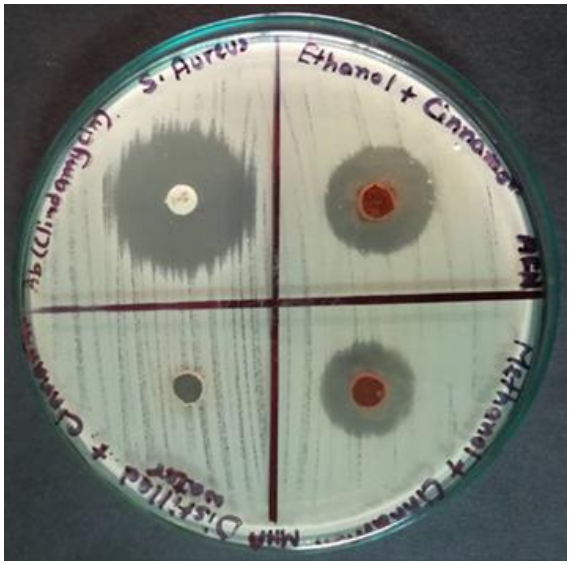
Gram (+)ve bacteria	Name of Antibiotic	Diameter of zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Staphylococcus aureus	Clindamycin	30	23	20	0
Bacillus cereus	Imipenem	42	13	17	0
Bacillus subtilis	Vancomycin	25	15	18	0
Streptococcus pyogenes	Clindamycin	0	20	22	0

Table 3.2.3(a): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Cinnamon against the gram-positive bacteria

Gram (-)ve bacteria	Name of Antibiotic	Diameter of zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Proteus vulgaris	Imipenem	25	20	20	0
Pseudomonas aeruginosa	Imipenem	26	10	12	0
Klebsiella pneumoniae	Meropenem	25	21	19	0
Escherichia coli	Ciprofloxacin	35	15	13	0
Shigella flexneri	Cefoxitin	0	28	25	0
Salmonella typhi	Gentamicin	27	20	16	0

Table 3.2.3(b): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Cinnamon against the gram-negative bacteria

(a)



(b)

(c)

(d)

Fig 15: Zone of inhibition of Cinnamon extracts against (a) *Shigella Flexneri*, (b) *Staphylococcus aureus*, (c) *Streptococcus pyogenes* and (d) *Klebsiella pneumoniae*

3.2.4 Black Cumin seeds

Ethanolic and methanolic extracts of Black Cumin seeds have shown remarkable zone of inhibition of **52mm** and **44mm** respectively against *Klebsiella pneumoniae* whereas, the antibiotic Meropenem had shown lower zone of inhibition (**25mm**) for this bacteria as shown in Fig 16(a). Also, the ethanolic and methanolic extract of Black Cumin seeds have shown higher zone of inhibition, **24mm** and **20mm** respectively, against *Bacillus cereus* as shown in Fig 16(b). Moreover, the methanolic extracts of Black Cumin seeds have shown higher zone of inhibition against *Staphylococcus aureus* (**35mm**) than that of the antibiotic Clindamycin (**30mm**) as shown in Fig 17(a). However, there was no antibacterial activity of ethanolic and methanolic extracts of Black Cumin seeds against *Streptococcus pyogenes* and *Proteus vulgaris*. Also, no antibacterial activity was observed by the methanolic extract of Black Cumin seeds against *Salmonella typhi.* The aqueous extract of Black Cumin seeds has no zone of inhibition against the ten bacteria.

The following tables show the zone of inhibition of the antibiotic disc and various extracts of Black Cumin seeds against the four gram-positive and six gram-negative bacteria:

Gram (+)ve bacteria	**Name of Antibiotic**	**Diameter of zone of Inhibition (mm)**			
		Antibiotic disc	**Ethanolic extract**	**Methanolic extract**	**Aqueous extract**
Staphylococcus aureus	Clindamycin	30	25	35	0
Bacillus cereus	Imipenem	42	24	20	0
Bacillus subtilis	Vancomycin	25	16	14	0
Streptococcus pyogenes	Clindamycin	0	0	0	0

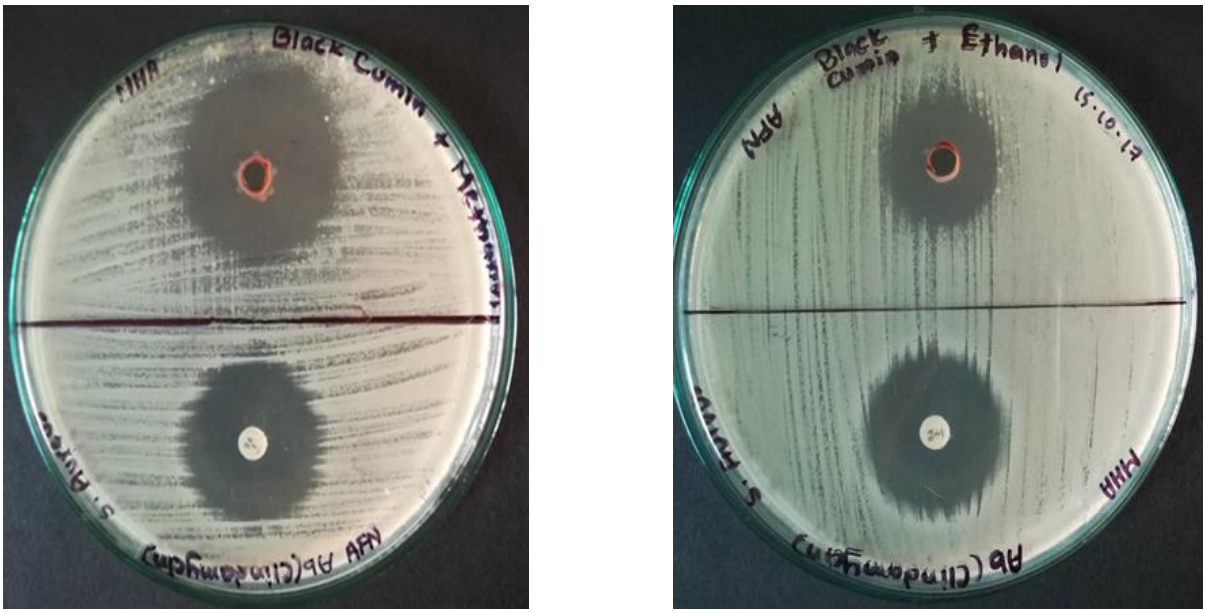
Table 3.2.4(a): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Black Cumin seeds against the gram-positive bacteria

Gram (-)ve bacteria	Name of Antibiotic	Diameter of zone of inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Proteus vulgaris	Imipenem	25	0	0	0
Pseudomonas aeruginosa	Imipenem	26	14	11	0
Klebsiella pneumoniae	Meropenem	25	44	52	0
Escherichia coli	Ciprofloxacin	35	10	15	0
Shigella flexneri	Cefoxitin	0	17	11	0
Salmonella typhi	Gentamicin	27	12	0	0

Table 3.2.4(b): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Black Cumin seeds against the gram-negative bacteria

(a) **(b)**

Fig 16: Zone of inhibition of Black Cumin seeds extracts against (a) *Klebsiella pneumoniae* and (b) *Bacillus cereus*



(a) **(b)**

Fig 17: Zone of inhibition of Black Cumin seeds (a) Methanolic extract and (b) Ethanolic extract against *Staphylococcus aureus*

3.2.5 Neem

Ethanolic and methanolic extracts of Neem have shown higher zone of inhibition for the gram-positive bacteria than the gram-negative bacteria [as seen in Table 3.2.5 (a) & (b)]. The highest zone of inhibition (**19mm**) was shown by the ethanolic extract of Neem against *Staphylococcus aureus* as shown in Fig 18(c). The methanolic extract of Neem has shown **16mm** zone of inhibition against *Bacillus subtilis* as shown in Fig 18(a) and the ethanolic extract of Neem has also shown **16mm** zone of inhibition against *Klebsiella pneumoniae* as shown in Fig 18(b). However, ethanolic extract had no antibacterial activity against *Proteus vulgaris* and *E.Coli;* also methanolic extract had no antibacterial activity against *Proteus vulgaris, Escherichia coli* and *Salmonella typhi.* Although *Streptococcus pyogenes* and *Shigella flexneri* were resistant against the antibiotic Clindamycin and Cefoxitin respectively, but *Streptococcus pyogenes* and *Shigella flexneri* were susceptible to ethanolic and methanolic extracts of Neem to some extent. All other antibiotics have shown higher zone of inhibition than the ethanolic and methanolic extracts of Neem against the ten bacteria. The aqueous extract of Neem has no zone of inhibition against the

ten bacteria. The following tables show the zone of inhibition of the antibiotic disc and various extracts of Neem against the four gram-positive and six gram-negative bacteria:

Gram (+)ve bacteria	Name of Antibiotic	Diameter of zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Staphylococcus aureus	Clindamycin	30	19	17	0
Bacillus cereus	Imipenem	42	15	14	0
Bacillus subtilis	Vancomycin	25	15	16	0
Streptococcus pyogenes	Clindamycin	0	9	9	0

Table 3.2.5(a): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Neem against the gram-positive bacteria

Gram (-)ve bacteria	Name of Antibiotic	Diameter of zone of inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Proteus vulgaris	Imipenem	25	0	0	0
Pseudomonas aeruginosa	Imipenem	26	10	10	0
Klebsiella pneumoniae	Meropenem	25	16	14	0
Escherichia coli	Ciprofloxacin	35	0	0	0
Shigella flexneri	Cefoxitin	0	10	9	0
Salmonella typhi	Gentamicin	27	9	0	0

Table 3.2.5(b): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Neem against the gram-negative bacteria

(a)

(b)

(c)

Fig 18: Zone of inhibition of Neem extracts against (a) *Bacillus subtilis*, (b) *Klebsiella pneumoniae* and (c) *Staphylococcus aureus*

3.2.6 Mint

Ethanolic and methanolic extracts of Mint have shown higher zone of inhibition for the gram-negative bacteria than the gram-positive bacteria [as seen in Table 3.2.6 (a) & (b)]. The highest zone of inhibition (16mm) was shown by the ethanolic extract of Mint against *Shigella flexneri.* However, methanolic extract of Mint had no antibacterial activity against *Proteus vulgaris* and *Salmonella typhi.* Although *Streptococcus pyogenes* and *Shigella flexneri* were resistant against the antibiotic Clindamycin and Cefoxitin respectively, but *Streptococcus pyogenes* was susceptible to ethanolic and methanolic extracts of Mint to some extent and *Shigella flexneri* was susceptible to ethanolic and methanolic extracts of Mint to large extent. All other antibiotics have shown higher zone of inhibition than the ethanolic and methanolic extracts of Mint against the ten bacteria. The aqueous extracts of Mint have no zone of inhibition against the ten bacteria.

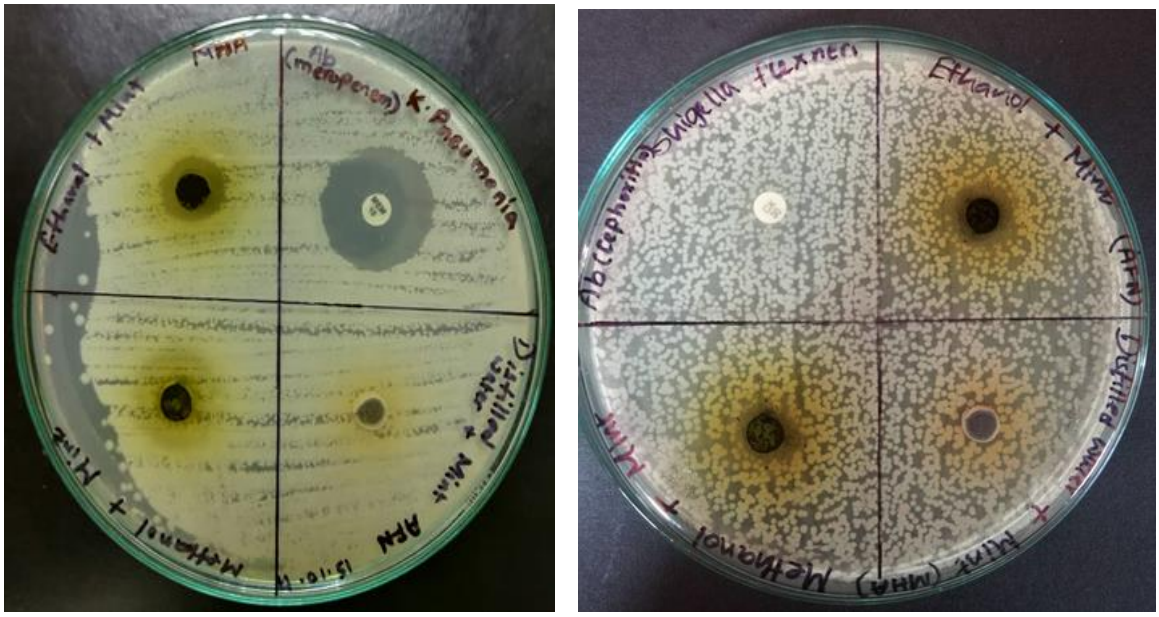
The following tables show the zone of inhibition of the antibiotic disc and various extracts of Mint against the four gram-positive and six gram-negative bacteria:

Gram (+)ve bacteria	**Name of Antibiotic**	**Diameter of zone of Inhibition (mm)**			
		Antibiotic disc	**Ethanolic extract**	**Methanolic extract**	**Aqueous extract**
Staphylococcus aureus	Clindamycin	30	12	11	0
Bacillus cereus	Imipenem	42	12	11	0
Bacillus subtilis	Vancomycin	25	9	9	0
Streptococcus pyogenes	Clindamycin	0	10	8	0

Table 3.2.6(a): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Mint against the gram-positive bacteria

Gram (-)ve bacteria	Name of Antibiotic	Diameter of zone of Inhibition (mm)			
		Antibiotic disc	Ethanolic extract	Methanolic extract	Aqueous extract
Proteus vulgaris	Imipenem	25	8	0	0
Pseudomonas aeruginosa	Imipenem	26	13	10	0
Klebsiella pneumoniae	Meropenem	25	15	10	0
Escherichia coli	Ciprofloxacin	35	11	10	0
Shigella flexneri	Cefoxitin	0	16	14	0
Salmonella typhi	Gentamicin	27	9	0	0

Table 3.2.6(b): Zone of inhibition produced by antibiotic, ethanol, methanol and aqueous extract of Mint against the gram-negative bacteria



(a) **(b)**

Fig 19: Zone of inhibition of Mint extracts against (a) *Klebsiella pneumoniae and*

(b) Shigella flexneri

3.3 Comparison of antibacterial activity by different solvent extracts

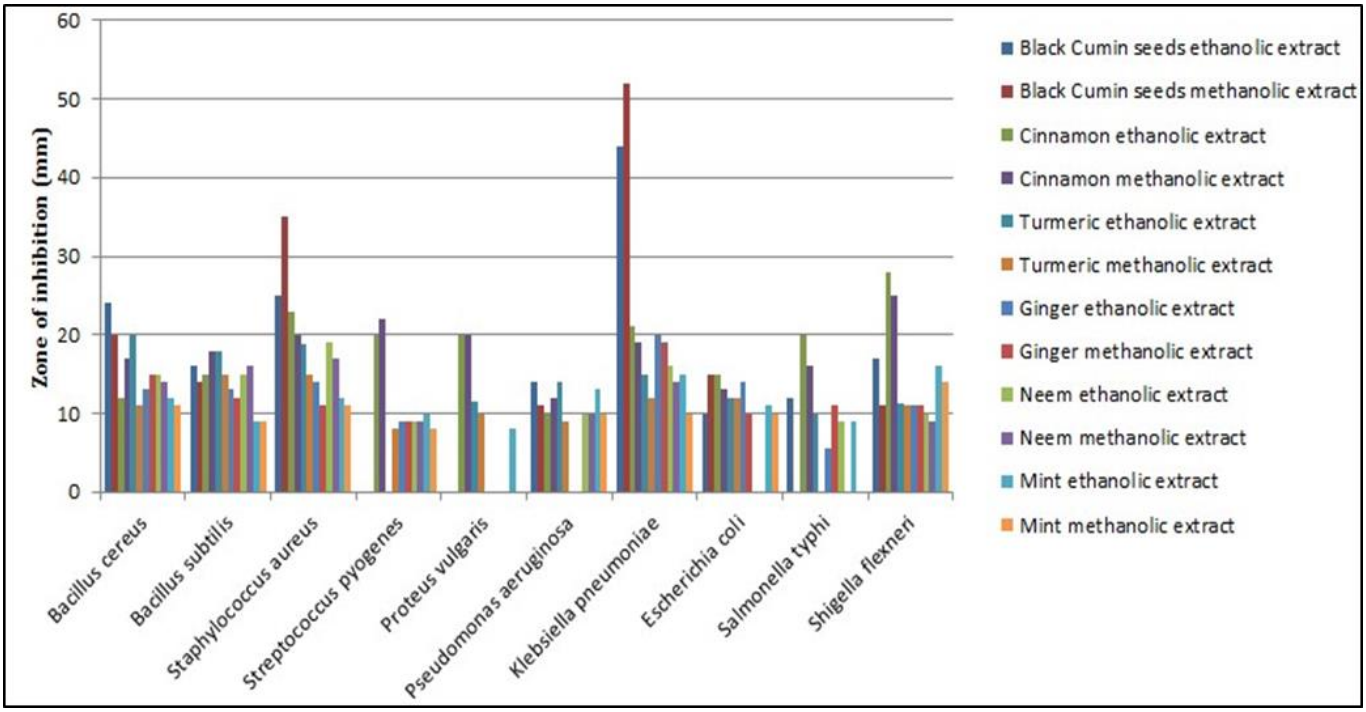


Fig 20: The antibacterial activities of ethanolic and methanolic extracts of six medicinal plants against the ten bacteria

In the above figure, it is observed that among the ethanolic and methanolic extracts of all the six medicinal plants, the highest zone of inhibition (**52mm**) was shown by the **methanolic extract** of Black Cumin seeds against the bacteria *Klebsiella pneumoniae*, then against *Staphylococcus aureus* (**35mm**) and *Bacillus cereus* (**20mm**). The **ethanolic extract** of Black Cumin seeds has also shown large zone of inhibition against *Klebsiella pneumoniae* (**44mm**), *Staphylococcus aureus* (**25mm**) and *Bacillus cereus* (**24mm**).

Two bacteria had shown resistance against their respective antibiotic and those were *Shigella flexneri* and *Streptococcus pyogenes* to Cefoxitin and Clindamycin respectively. These two bacteria were susceptible to the various extracts of the six medicinal plant samples and the largest zone of inhibition against these two bacteria were observed for the ethanolic and methanolic extracts of Cinnamon. Both the ethanolic and methanolic extracts of Cinnamon have shown clear zone of inhibition against all the ten bacteria. The best zone of inhibition was shown by the **ethanolic extract** of Cinnamon against *Shigella flexneri (***28mm)** and the **methanolic**

extract of Cinnamon against *Shigella flexneri* (**25mm**). However, the aqueous extracts of all the six medicinal plant samples did not show any antibacterial activity against the ten bacteria and thus taken to be a negative control for this study.

3.4 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 extracts of six different medicinal plants against seven different antibiotics, named: Clindamycin, Ciprofloxacin, Vancomycin, Gentamicin, Cefoxitin, Imipenem and Meropenem.

Using the formula**, Activity Index (AI) =** $\frac{Zone\ of\ inhibition\ of\ extract}{Zone\ of\ inhibition\ of\ antibiotic}$**,** the AI values as shown in the following graphs have been calculated for the ethanolic and methanolic extracts of all the six medicinal plants against all the selected bacteria except *Streptococcus pyogenes* and *Shigella flexneri* as these two bacteria have shown antibiotic resistance.

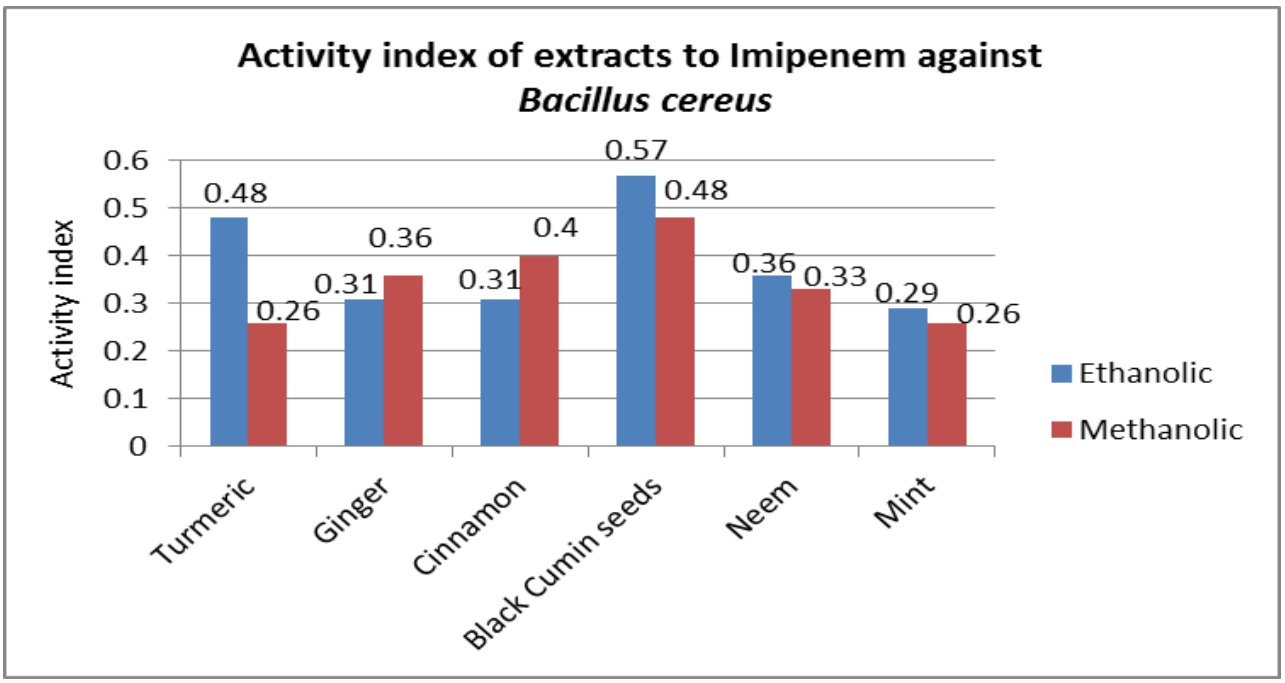


Fig 21: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Imipenem against *Bacillus cereus*

In **Fig 21**, the AI value of ethanolic extract of Black Cumin seeds to Imipenem was the highest (**0.57**) for *Bacillus cereus* compared to the other medicinal plant extracts.

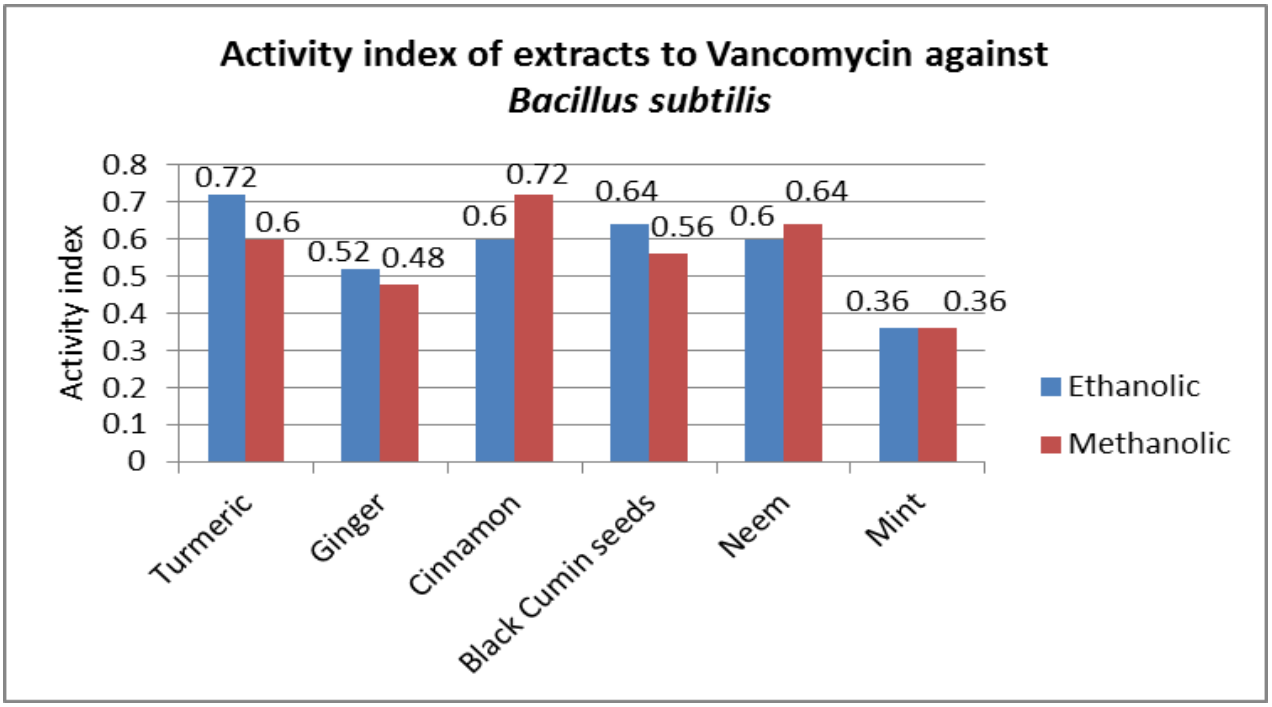


Fig 22: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Vancomycin against *Bacillus subtilis*

In **Fig 22**, the AI value of ethanolic extract of Turmeric to Vancomycin and the AI value of methanolic extract of Cinnamon to Vancomycin were the same and the highest (**0.72**) for *Bacillus subtilis* compared to the other medicinal plant extracts.

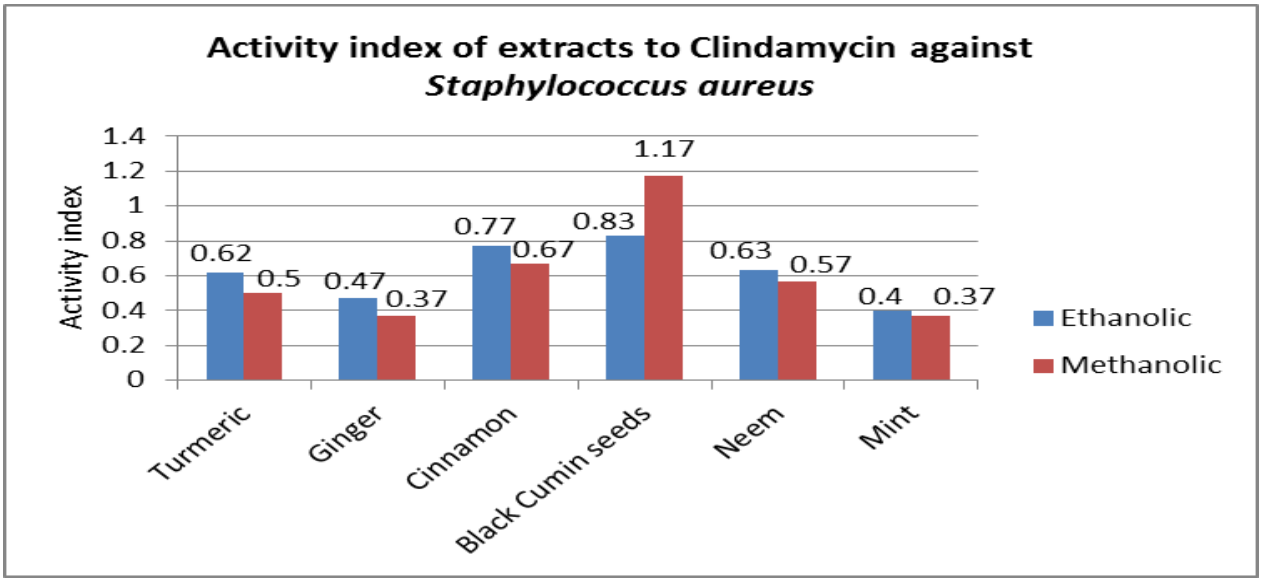


Fig 23: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Clindamycin against *Staphylococcus aureus*

In **Fig 23,** the AI value of methanolic extract of Black Cumin seeds to Clindamycin was the highest (**1.17**) for *Staphylococcus aureus* compared to the other medicinal plant extracts.

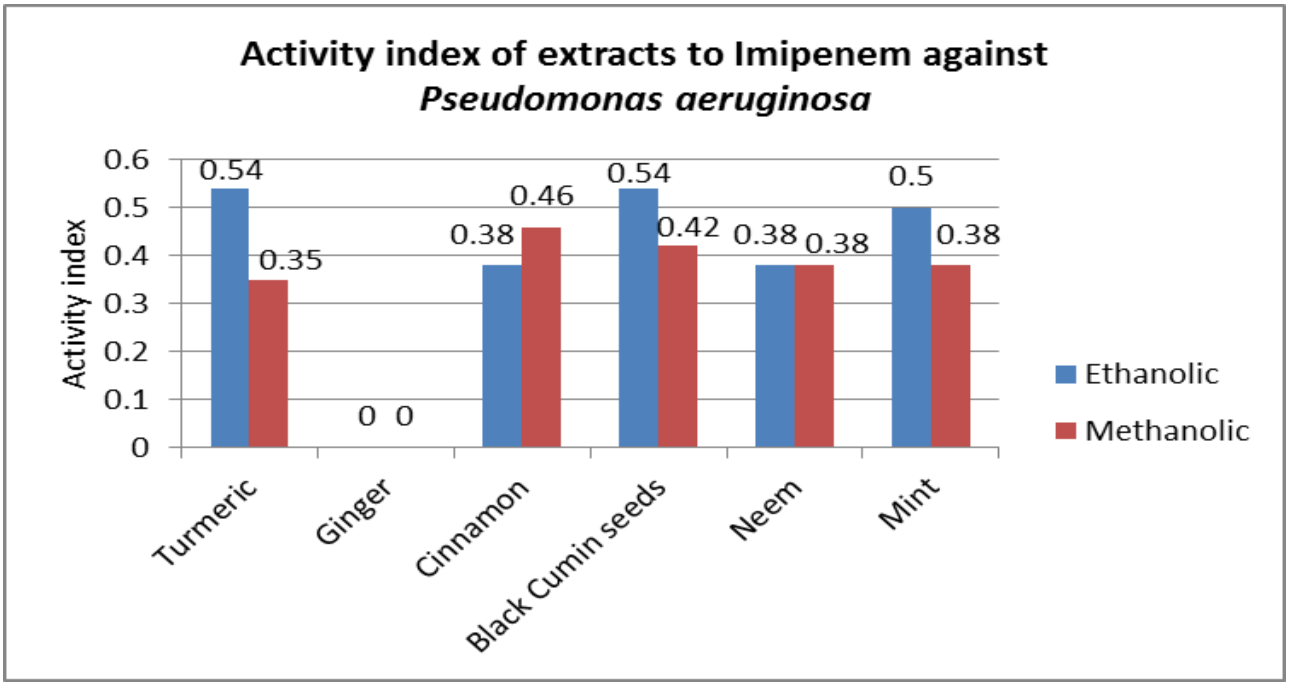


Fig 24: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Imipenem against *Pseudomonas aeruginosa*

In **Fig 24**, the AI value of ethanolic extract of Turmeric to Imipenem and the AI value of ethanolic extract of Black Cumin seeds to Imipenem were the same and the highest (**0.54**) for *Pseudomonas aeruginosa* compared to the other medicinal plant extracts.

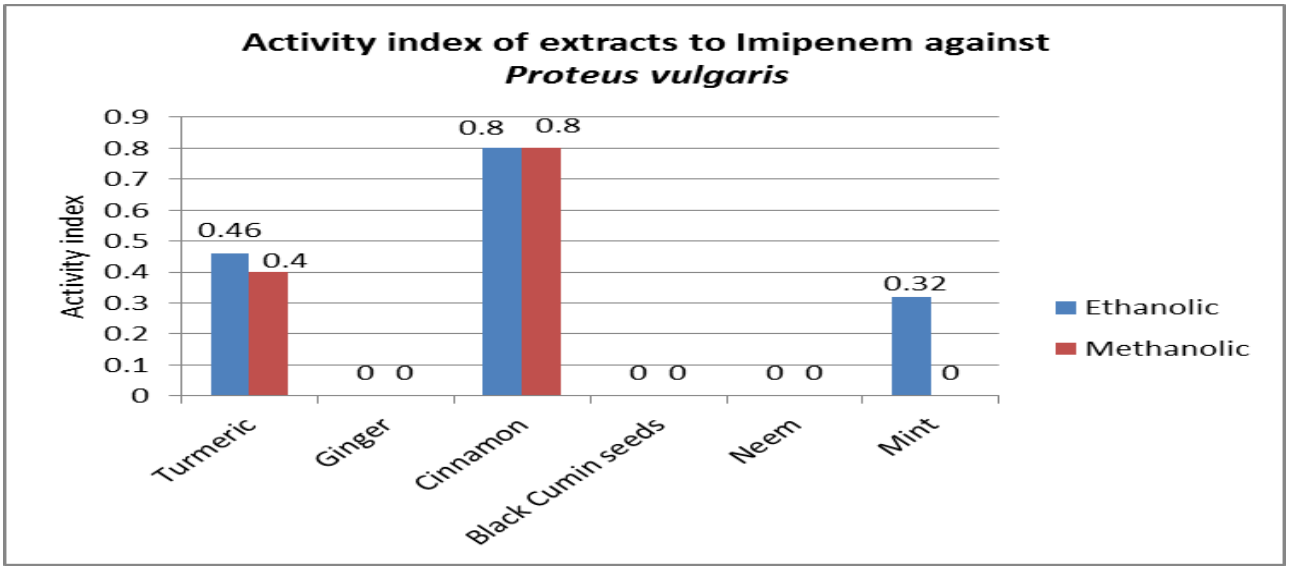


Fig 25: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Imipenem against *Proteus vulgaris*

In **Fig 25**, the AI value of ethanolic and methanolic extracts of Cinnamon to Imipenem was the same and the highest (**0.8**) for *Proteus vulgaris* compared to the other medicinal plant extracts.

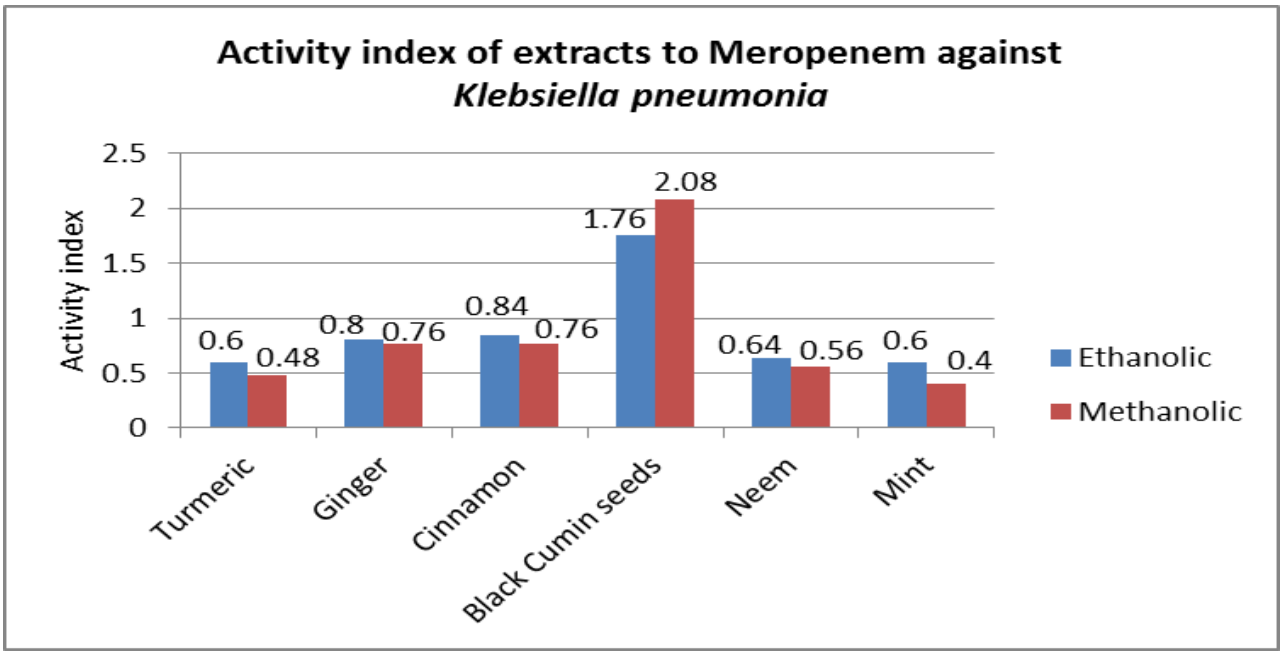


Fig 26: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Meropenem against *Klebsiella pneumoniae*

In **Fig 26**, the AI value of methanolic extract of Black Cumin seeds to Meropenem was the highest (**2.08**) for *Klebsiella pneumoniae* compared to the other medicinal plant extracts.

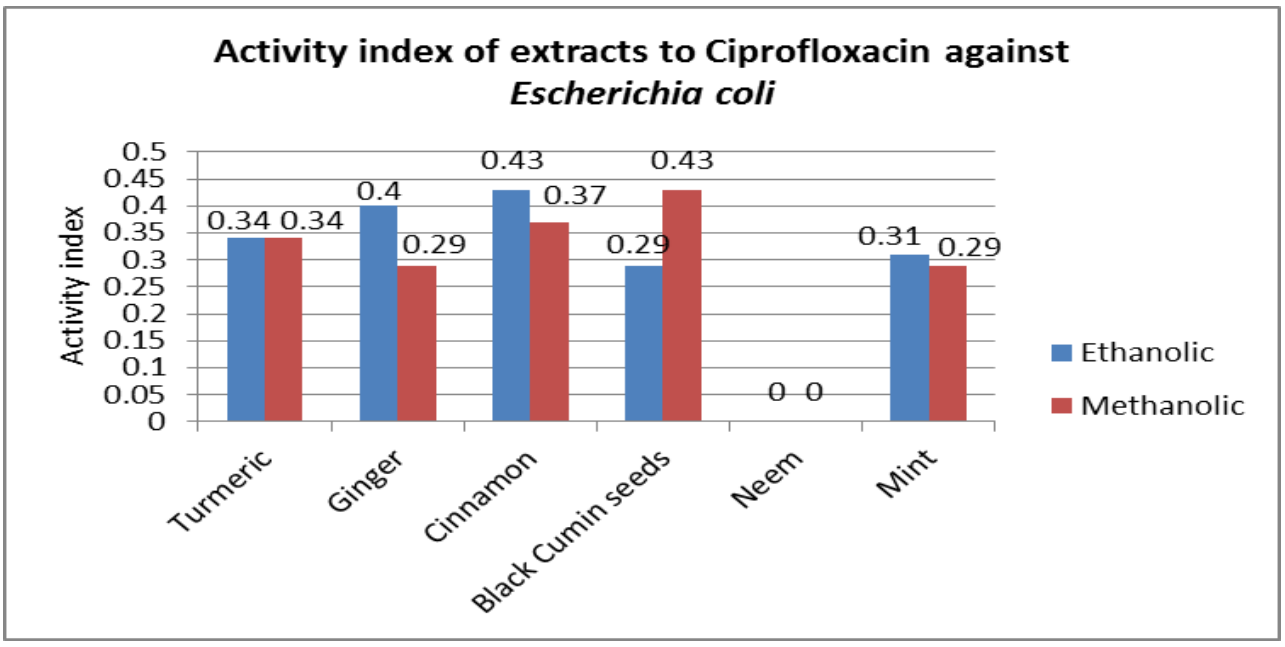


Fig 27: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Ciprofloxacin against *Escherichia coli*

In **Fig 27**, the AI value of ethanolic extract of Cinnamon to Ciprofloxacin and the AI value of methanolic extract of Black Cumin seeds to Ciprofloxacin were the same and the highest (**0.43**) for *Escherichia coli* compared to the other medicinal plant extracts.

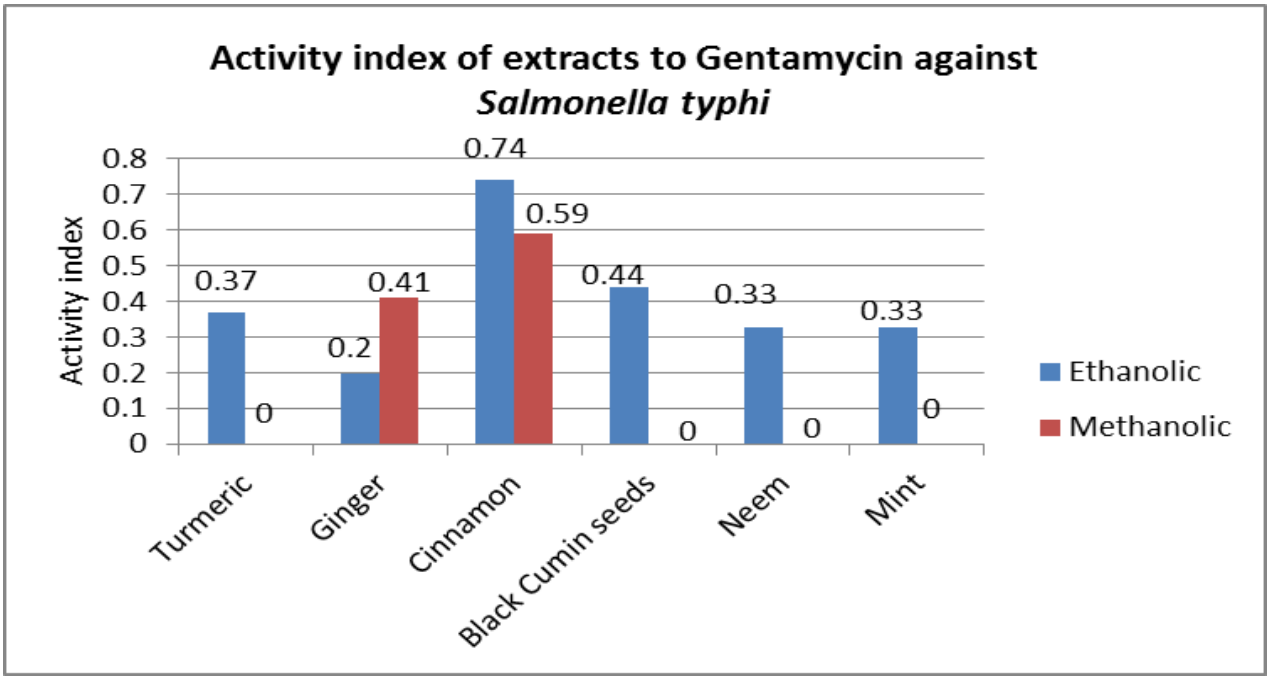


Fig 28: The activity index of the ethanolic and methanolic extracts of the six medicinal plants to Gentamycin against *Salmonella typhi*

In **Fig 28**, the AI value of ethanolic extract of Cinnamon to Gentamycin was the highest (**0.74**) for *Salmonella typhi* compared to the other medicinal plant extracts.

From all the AI values, the methanolic extract of Black Cumin seeds to Meropenem has got the highest AI value (2.08) for *Klebsiella pneumoniae*. The second highest AI value (1.17) was also seen for the methanolic extract of Black Cumin seeds to Clindamycin for *Staphylococcus aureus.* The third highest AI value (0.8) was seen for the ethanolic and methanolic extracts of Cinnamon to Imipenem for *Proteus vulgaris.*

3.5 MIC and MBC of the most effective plant extract

Since the methanolic extract of Black Cumin seeds to Meropenem has got the highest AI value (2.08) for *Klebsiella pneumoniae*, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic extract of Black Cumin seeds against *Klebsiella Pneumoniae* was found out and the value was 34 mg/ml (MIC) and 44mg/ml (MBC) respectively as shown in the table and figure below:

Table 3.5: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of the methanolic extract of Black Cumin seeds against *Klebsiella Pneumoniae.*

Extract Vol. (ml)	BHI undiluted Vol. (ml)	Inoculum Vol. (ml)	Final Vol. (ml)	Concentration Of extract Per ml After Dilution (mg)	Approximate Concentration of *Klebsiella Pneumoniae* Inoculum Transferred CFU/0.1 ml
0.1	9.8	0.1	10	1	1.5×10^7
0.2	9.7	0.1	10	2	1.5×10^7
0.3	9.6	0.1	10	3	1.5×10^7
0.4	9.5	0.1	10	4	1.5×10^7
0.5	9.4	0.1	10	5	1.5×10^7
0.6	9.3	0.1	10	6	1.5×10^7
0.7	9.3	0.1	10	7	1.5×10^7
0.8	9.1	0.1	10	8	1.5×10^7
0.9	9.0	0.1	10	9	1.5×10^7
1	8.9	0.1	10	10	1.5×10^7
1.4	8.5	0.1	10	14	1.5×10^7
2	7.9	0.1	10	20	1.5×10^7
2.4	7.5	0.1	10	24	1.5×10^7
3	6.9	0.1	10	30	1.5×10^7
3.4	6.5	0.1	10	34 (MIC)	1.5×10^7
4.4	5.5	0.1	10	44 (MBC)	1.5×10^7

Fig: 29 Determination of the minimum inhibitory concentration (MIC) of the methanolic extract of Black Cumin seeds through serial dilution.

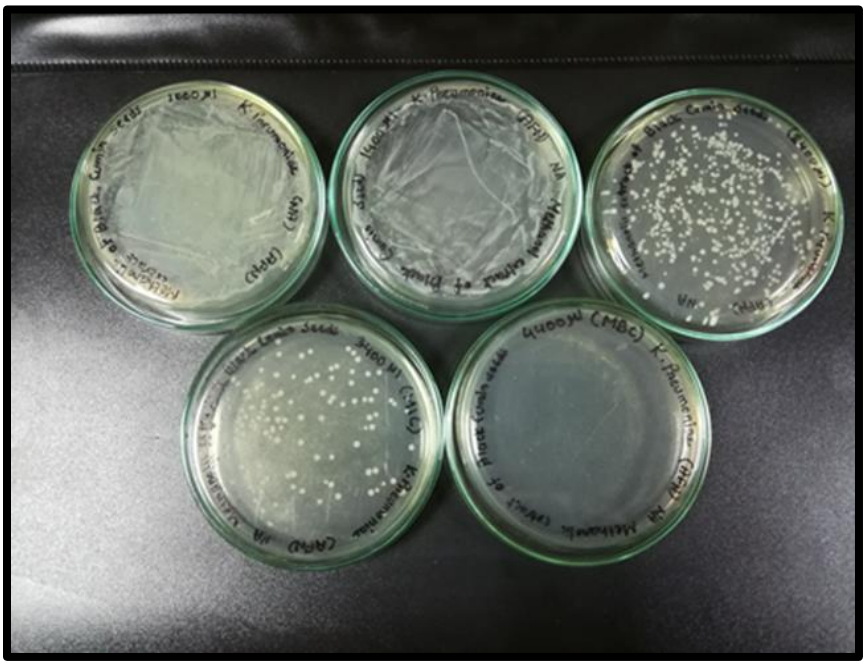


Fig: 30 Determination of the minimum bactericidal concentration of the methanolic extract of Black Cumin seeds against *Klebsiella pneumoniae* when spread plated on agar plates from diluted 10 ml BHI broth.

Chapter Four: Discussion

The purpose of this study was to prepare three different types of extracts from Turmeric, Ginger, Cinnamon, Black Cumin seeds, Neem and Mint using three different solvents: ethanol, methanol and distilled water to investigate the antimicrobial activity of the various extracts of the six medicinal plants against some gram-positive and gram-negative organisms. Nowadays, researchers are increasingly turning their attention in investigating herbal products in order to fight the increasing occurrences of microbial drug resistance (Rahman *et al.,* 2011).

In this study, the antibacterial activity of ethanolic, methanolic and aqueous extracts of the six different medicinal plants was tested against ten different bacteria, where four of them were gram-positive bacteria- *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus* and *Streptococcus pyogenes;* and six of them were gram-negative bacteria- *Proteus vulgaris, Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Shigella flexneri*. The antibacterial tests showed that some of the plant extracts may be used most effectively as an antibiotic agent against microorganisms such asgram-positive *Staphylococcus aureus* and gram-negative *Klebsiella pneumoniae*.

The comparison of the zone of inhibition by the **methanolic extracts** of all the six medicinal plants showed that the highest zone of inhibition (**52mm**) was done by **Black Cumin seeds** against *Klebsiella pneumoniae*. The comparison of the zone of inhibition by the **ethanolic extracts** of all the six medicinal plants showed that the highest zone of inhibition (**44mm**) was done by **Black Cumin seeds** against *Klebsiella pneumoniae*. 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.*

Among all the six medicinal plants, the **ethanolic** extract of **Black Cumin seeds** has shown the best zone of inhibition (when compared to the ethanolic extract of the other medicinal plants) against four bacteria in which two were gram-positive bacteria: *Staphylococcus aureus* (**25mm**) and *Bacillus cereus* (**24mm**); and the other two were gram-negative bacteria: *Klebsiella pneumoniae* (**44mm**) and *Pseudomonas aeruginosa* (**14mm**). 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*. Also, the **ethanolic** extract of **Cinnamon** has shown the best zone of inhibition against five bacteria, in which one

was gram-positive bacteria: *Streptococcus pyogenes* (**20mm**) and other four were gram-negative bacteria: *Shigella flexneri* (**28mm**), *Salmonella typhi* (**20mm**), *Proteus vulgaris* (**20mm**) and *Escherichia coli* (**15mm**). Previously, ethanolic extract of *Cinnamomum verum* was not tested on the bacteria used in this study. The ethanolic extract of Turmeric has shown the best zone of inhibition (when compared to the ethanolic extract of other medicinal plants) against the gram-positive bacteria: *Bacillus subtilis* (**18mm**) and the gram-negative bacteria: *Pseudomonas aeruginosa* (**14mm**). Arutselvi *et al.,* (2012) showed that the ethanolic extract of Turmeric had shown around **18mm** zone of inhibition against *Bacillus subtilis.*

Among all the six medicinal plants, the **methanolic** extract of **Black Cumin seeds** has shown the best zone of inhibition against four bacteria in which two were gram-positive bacteria: *Staphylococcus aureus* (**35mm**) and *Bacillus cereus* (**20mm**); and two were gram-negative bacteria: *Klebsiella pneumoniae* (**52mm**) and *Escherichia coli* (**15mm**). Another experiment by Hasan *et al.,* (2013) showed that the methanolic extract of Black Cumin seeds at concentration of 100 mg/mL, the antibacterial activity of **19 mm** was shown for *Streptococcus pyogenes*, and an inhibition zone measuring around **15 mm** was recorded for *Pseudomonas aeruginosa* and *Proteus vulgaris*. Also, the **methanolic** extract of **Cinnamon** has shown the best zone of inhibition against six bacteria, in which two were gram-positive bacteria: *Streptococcus pyogenes* (**22mm**) and *Bacillus subtilis* (**18mm**); and other four were gram-negative bacteria: *Shigella flexneri* (**25mm**), *Proteus vulgaris* (**20mm**), *Salmonella typhi* (**16mm**) and *Pseudomonas aeruginosa* (**12mm**). 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*.

Although the ethanolic and methanolic extracts of all the six medicinal plants have shown antibacterial activity, the aqueous extract of these medicinal plants did not show any antibacterial activity. This may be due to the presence of some of the chemical components in these plant extracts whose activity may depend on their solubility in different solvents, giving rise to the different results for ethanolic, methanolic and aqueous extracts. In a study by Al-daihan *et al.,* (2013) it was mentioned that, against some clinical bacterial isolates, methanolic extract of turmeric sample had shown much better antibacterial activities in contrast to aqueous extract,

where they considered that, it may be because of organic nature of methanol and also for its high capacity to dissolve more organic and active antimicrobial compounds.

However, in the common households of Bangladeshi people, Black Cumin seeds, Cinnamon, Mint, Turmeric and Gingerare usually eatenin raw form, are used as spice and cooked or usually eaten in mixture with water. Usually it is not eaten in mixture with ethanol or methanol. Neem leaves are usually not eaten but used externally on the body as a homemade herbal remedy for skin allergies by either boiling the leaves in water to make an aqueous extract or made into a semi-solid paste using waterby mortar and pestle. So the *in vitro* antimicrobial effect shown in this study may reflect that these plant extracts may not be as effective against any of these organisms *in vivo*.However, in this investigation, only crude extracts of the medicinal plants were used for antimicrobial activity test. The concentrations of the extracts can be changed, or different extraction methods can be performed to produce different significant results. So by considering the overall outcomes of the antimicrobial effects of the six medicinal plant extracts of this study, it can be stated that further thorough experimental *in-vitro* study has to be done which may lead to clinical trials, giving rise to production of herbal medicines using these medicinal plant extracts.

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 “2.08” obtained from the methanolic extract of Black Cumin seeds to Meropenem for *Klebsiella pneumoniae* and the activity index value “1.17” obtained from the methanolic extract of Black Cumin seeds to Clindamycin for *Staphylococcus aureus*, were the remarkable ones which indicated that the Black Cumin seeds extracts are much more effective against the tested bacteria than the allopathic antibiotics. Although the selected allopathic antibiotics have shown antibacterial activity, there were two bacteria: *Streptococcus pyogenes* and *Shigella flexneri*, which showed complete resistance to Clindamycin and Cefoxitin antibiotics respectively. So the AI values could not be calculated for these two antibiotics. While

these two allopathic antibiotics were not effective against *Streptococcus pyogenes* and *Shigella flexneri*, the ethanolic and methanolic extracts (except aqueous) of the six medicinal plants have shown positive antibacterial activity against these two strains.

Hence, in comparison with the allopathic antibiotics, the plant extracts which have shown higher AI values indicated that those plant extracts are more efficient against the bacteria than the antibiotic. However, the AI values were very low which were obtained for the antibiotics that were highly effective against the test bacteria and this indicated that the antibiotics can be more effective than the natural plant extracts unless the microorganisms develop resistance to them. This difference in the efficiency of bacterial growth inhibition by the natural plant extracts and the allopathic antibiotics can be there for the different mechanism of interactions of these antimicrobial agents on the bacteria. Due to the hydrophobicity of the plant extracts and its chemical components, they are able to interact with the lipids of the bacterial cell membrane and mitochondria, resulting in changes of the structure and function of the membranes, which in turn, may impair growth and activity of the bacterial cells (Sikkema *et al.,* 1994).

Conclusion:

Overall, the organic solvent extracts of Black Cumin seeds were the most effective antibacterial agent compared to allopathic antibiotics against *Klebsiella pneumonia* and *Staphylococcus aureus* and have also shown good antibacterial activity against the other test bacteria of this study. The organic solvent extracts of Cinnamon were effective against all the ten bacteria and have shown "**20mm"** zone of inhibition and sometimes even more against most of the test bacteria. While the organic solvent extracts of the other four medicinal plants- Turmeric, Ginger, Neem and Mint, have shown positive antibacterial activity, they were not more effective when compared to the antibacterial activity of the allopathic antibiotics. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic extract of Black Cumin seeds against *Klebsiella pneumoniae* was found 34 mg/ml (MIC) and 44mg/ml (MBC) respectively.

The organic solvents- ethanol and methanol were the most effective solvents for this procedure while the aqueous extracts of all the six medicinal plants possessed no antibacterial activity.

It can be concluded that, instead of using allopathic antibiotics, if organic solvent extracts of Black Cumin seeds or its combination with extracts from other spices with similar antibacterial properties are used , then there would be potential benefits to humans. Since the organic solvent extracts of various medicinal plants have shown positive antibacterial effect, the potential value of these medicinal plant extracts is on the rise when compared to that of allopathic antibiotics as certain bacteria have developed resistance against allopathic antibiotics but are susceptible to the natural plant extracts. It is hoped that the outcomes of this study may stimulate other researchers to design clinical trials and to come up with a less expensive antibacterial agent that may be beneficial for the people from developing countries like Bangladesh.

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APPENDIX-I

Media composition

Compositions of the media used in this study are provided below. The media were autoclaved at 121°C for 15 min at 121psi.

1. Nutrient Agar (HiMedia, India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.5
Sodium chloride	5.0
Yeast extract	1.5
Agar	15.0

2. Mueller-Hinton Agar (HiMedia, India)

Ingredients	Amounts (g/L)
Beef infusion	300
Casamino acids	17.5
Starch	1.5
Agar	17.0

3. Brain Heart Infusion Broth (HiMedia, India)

Ingredients	Amounts (g/L)
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride 5.000	5
Disodium phosphate	2
Final pH (at 25°C)	7.4±0.2

APPENDIX-II

Instruments

Autoclave	Wisd Laboratory Instruments Made in Korea
Water Bath WiseBath^{R}	Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea
Incubator	Model: DSI 3000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Vortex Mixer	Model: VM-2000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Electronic Balance	RADWAG WagiELEktroniczne Model: WTB 200
Refrigerator (40ºC)	Model: 0636 Samsung
Laminar flow chamber	SAARC Engineering
Rotary evaporator	Heidolph Made in Germany
Fume hood chamber	-