Antimicrobial activities of *Aloe vera* leaf and clove extracts, their comparison with commercial antibiotics and molecular analysis of genes involved with multidrug resistance in bacteria

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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For my beloved parents, sister and brother

Declaration

I hereby declare that this thesis entitled **"Antimicrobial activities of *Aloe vera* leaf and clove extracts, their comparison with commercial antibiotics and molecular analysis of genes involved with multidrug resistance in bacteria"** is submitted by me, Nusrat Afrin, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Dr. M. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

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Abstract

The need for new, potent antimicrobial agents from natural sources is increasing because of the growing cases of multidrug resistance in clinically relevant organisms. The purpose of this study was to investigate *in vitro* antimicrobial activity of *Aloe vera* leaf and clove extracts against microorganisms isolated from clinical samples. The antibacterial activities were then compared to commercial antibiotics, and the samples resistant to most antibiotics were screened for two multidrug resistant genes ($bla_{\text{NDM-1}}$ and $bla_{\text{OXA-2}}$). Antimicrobial susceptibility testing of a total of 21 clinical isolates (10 *Pseudomonas,* 10 *Klebsiella* and 1 *E.coli*) were carried out using crude ethanol, methanol, acetone and aqueous extracts of *Aloe vera* leaf and clove. Kirby-Bauer disk diffusion test of the same organisms was done using the following antibiotics: imipenem, meropenem, gentamicin, oxacillin, cloxacillin, ceftazidime, ampicillin, amikacin, kanamycin and ciprofloxacin. Strains highly resistant to most of the beta-lactam antibiotics were then screened for New Delhi metallo-beta-lactamase-1 (NDM-1) and OXA-2 genes using PCR. Extracts of both *Aloe vera* leaf and clove were effective against all the isolates, with the highest activity exhibited by acetone extracts. Aqueous plant extracts showed little to no activity against the isolates, except for three *Pseudomonas* samples whose growths were inhibited by aqueous clove extracts. All the bacterial isolates were highly resistant to most of the antibiotics except imipenem, meropenem and ciprofloxacin. It was also observed that 100% of the organisms were resistant to oxacillin, cloxacillin and ampicillin. While some samples were highly susceptible to some antibiotics, others showed little and moderate susceptibility. However, PCR with specific primers and gel electrophoresis revealed that none of the bacterial strains carried $bla_{\text{NDM-1}}$ or $bla_{\text{OXA-2}}$ genes.

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Abbreviations	Elaborations
WHO	World Health Organisation
DMSO	Dimethyl sulfoxide
PBP	Penicillin binding protein
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum β-lactamases
NDM-1	New Delhi metallo-β-lactamase-1
rpm	rotations per minute
g/L	grams per litre
NA	Nutrient Agar
MHA	Mueller-Hinton Agar
NIDCH	National Institute of Diseases of the Chest and Hospital
AI	Activity Index
PCR	Polymerase Chain Reaction
IPM	Imipenem
MEM	Meropenem
GEN	Gentamicin
OX	Oxacillin
OB	Cloxacillin
CAZ	Ceftazidime
AMP	Ampicillin
AK	Amikacin
K	Kanamycin
CIP	Ciprofloxacin
TE	Tris-EDTA
TBE	Tris-Borate-EDTA
EDTA	Ethylenediaminetetraacetic acid

Chapter 1: Introduction

Introduction

1.1 Background

The number of people using traditional or alternative medicine is increasing rapidly all over the world. Traditional medicine often includes herbal medicines, which consist of biologically active compounds from plant materials, or whole plants (WHO, 2002). According to the World Health Organization, around 65% of the world's population have incorporated plant medicinal agents into their primary aspects of healthcare. The key reasons behind using plants as sources of therapeutic agents include a) isolation of bioactive compounds for direct use as drugs, b) producing bioactive compounds of new or known structures as references for the synthesis of entities with higher activity and/or lower toxicity, c) using agents as pharmacological tools and d) using the whole plant or a certain portion of it as herbal remedy (Fabricant & Farnsworth, 2001). Consumer interest in herbal and alternative medicines arise from the fact that they consider these products to be both safe and effective, and this has prompted scientists to investigate the numerous bioactive compounds available in plants (Wendakoon et al., 2012).

However, there is another important reason behind people turning to natural sources in search of compounds with potent antimicrobial activities these days - the alarming rate at which microbial resistance to synthetic antibiotics is growing. Owing to the side effects and resistance that pathogenic microorganisms develop against common, commercially available antibiotics, more attention is being paid to extracts and bioactive components that can be isolated from plants used in herbal medicine (Essawi & Srour, 2000). Antibiotic and multi-drug resistance is now a world-wide problem in hospitals, long-stay residential centres and also in the community (Livermore, 2000). Unselective and extensive use of antibiotics and selective pressure on bacterial strains is highly considered to be the reason behind such evolution (Goudrazi et al., 2015; Habeeb et al., 2007). Bacteria are not only able to acquire resistance through mutation, but also by plasmid spread through different strains (Robicsek et al., 2006). A deficiency in new drugs, vaccines and diagnostic aids is also recognized as a major problem in the management of drug resistant infections (Finch & Hunter, 2006). To address all these shortcomings, a significant number of

new therapeutics is being derived from natural sources such as plants, as systemic and topical novel drugs and antiseptics to replace or to be used in collaboration with existing products (Woodford, 2005). Many plant materials used as traditional medicine have been proven to be more effective, and relatively cheaper than their modern counterparts (Mann et al., 2008). Antimicrobials of plant origin also alleviate many of the side effects that are often associated with synthetic ones (Iwu, et al., 1999; Mukherjee & Wahile, 2006).

1.2 *Aloe vera*

Aloe barbadensis Miller (*Aloe vera*) is a member of the Liliaceae family which contains about four hundred species of flowering succulent plants (Newall et al., 1996; Mohammad, 2003). *Aloe vera* is a typical xerophyte. It is a cactus-like plant with thick, fleshy, cuticularized spiny leaves that grows readily in hot, dry climates (Choi et al., 2002; Tan & Vanitha, 2004). *Aloe vera* plants are stem less or sometimes very short-stemmed plants that grow up to 60-100 cm tall. The thick leaves are green, with some variants that show white flecks on the upper and lower stem surfaces. The serrated margin of the leaves have small, white teeth, and the flowers are produced in summer. Each pendulous flower has a yellow, tubular corolla 2-3 cm long. *Aloe vera* forms arbuscular mycorrhiza, a symbiotic mechanism that allows the plant better access to mineral nutrients present in soil (Gong et al., 2002).

The fresh leaves of this perennial, drought resistant plant is used to obtain two distinct products: a bitter, yellow latex (exudate) and a mucilaginous gel from the parenchymatous tissues in the leaf pulp. The gel is revealed after removal of the thick outer cuticle (Surjushe et al., 2008). *Aloe vera* gel is 99.3% water and the remaining 0.7% consists of a range of active compounds including polysaccharides such as glucose and mannose, vitamins, amino acids, phenolic compounds and organic acids. These compounds give *Aloe vera* the special property as a skin-care product (Crew et al., 1939; Borrelli & Izzo, 2000 and Agarry et al, 2005).

The name of the plant was derived from the Arabic “alloeh” meaning “shining bitter substance” because of the bitter liquid found in the leaves. The word “vera” is Latin for “truth”. It is also known as “lily of the desert”, the “plant of immortality” and the medicinal plant that has the qualities to serve as alternate medicine (Arunkumar & Muthuselvam, 2009).

Products derived from *Aloe vera* are primarily used in cosmetics, pharmaceuticals, nutraceuticals and food industries (Klein & Penney, 1988). The gel has the ability to stimulate cell growth and enhance the restoration of damaged skin. Its moisturizing ability arises from its water holding capacity (Eshun & He, 2004). The first reference to *Aloe vera* in English was a translation by John Goodyew in A.D. 1655 of Dioscorides' Medical treatise De Materia Medica (Surjushe et al., 2008).

The plant grows mainly in the dry regions of Africa, Asia, Europe, America and India. Because of its increasing demand these days, *Aloe vera* is now grown in large amounts in Bangladesh. People use *Aloe vera* as skin care products and also in the production of cosmetics and medicines.

Figure 1.2: *Aloe vera* plant with flower detail inset (Retrieved from: https://commons.wikimedia.org/w/index.php?curid=5084561)

1.2.1 Taxonomic hierarchy of *Aloe vera*

Taxonomic Hierarchy of *Aloe vera* (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	Asparagales
Family	Xanthorrhoeaceae, liliaceae, asphodelaceae
Genus	Aloe L-aloes
Species	*Aloe vera* (L.)

1.2.2 Therapeutic uses of *Aloe vera*

For thousands of years, *Aloe vera* has been used for medicinal purposes in several cultures: Greece, Egypt, India, Mexico, Japan and China (Marshall, 1990). It is a well-known dietary supplement and chemopreventive agent, and its gel is also used for topical treatment of skin irritations (Bergamante et al., 2007). It has been reported that *Aloe vera* gel has a protective effect against radiation damage to the skin (Roberts & Travis, 1995). The exact mechanism of action is yet to be discovered, but after administration of *Aloe vera* gel, metallothionein, which is an antioxidant protein, is generated in the skin. This scavenges hydroxyl radicals and prevents suppression of superoxide dismutase and glutathione peroxidase in the skin. This, in turn, reduces the production and release of skin keratinocyte-derived immunosuppressive cytokines

such as interlukin-10, thereby preventing UV-induced suppression of delayed type hypersensitivity (Byeon et al., 1998).

The effects of *Aloe vera* on the immune system includes Alprogen (Aloe single component) inhibiting calcium influx into mast cells, hence inhibiting the antigen-antibody-mediated release of histamine and leukotriene from mast cell (Ro et al., 2000). Anthraquinones present in the latex of *Aloe vera* are a potent laxative. It increases intestinal water uptake, stimulates mucus secretion and increases intestinal peristalsis (Ishii et al., 1994). Mucopolysaccharides in *Aloe vera* gel help in binding moisture into the skin. By stimulating fibroblasts which produce collagen and elastin fibres, it makes the skin more elastic and less wrinkled. The gel also plays a role of a cohesive agent on the superficial flaking epidermal cells by sticking them together, thereby softening the skin. The amino acids also soften hardened skin cells and zinc acts as an astringent to tighten pores (West & Zhu, 2003). Besides all these, *Aloe vera* is also known to have healing effects against ulcer, diabetes, inflammations and tumors (Surjushe et al., 2008).

1.2.3 Antimicrobial properties of *Aloe vera*

Despite the numerous number of literature present on antimicrobial properties of plant extracts, not many plant derived chemicals have been successfully exploited for clinical use as antibiotics. A considerable part of the chemical diversity produced by plants is thought to protect plants against microbial pathogens. Hence, they have been proven to have antimicrobial importance both *in vivo* and *in vitro* (Gibbons, 2004). A number of reports are available on antimicrobial activity of hexane, ethanol, acetone, petroleum ether and ethyl acetate extracts of *Aloe vera* gel and leaves (Agarry et al., 2005). Antibacterial activity of *Aloe barbadensis* was tested on certain clinically isolated bacterial pathogens such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* etc. Antibacterial effects of ethanolic and aqueous extracts were tested by examining the appearance of any zone of inhibition on bacterial culture plates. Ethanol extracts were found to be quite effective on both gram positive and negative bacteria, but the aqueous extracts did not show any inhibitory effect (Pandey & Mishra, 2010). Another study was conducted to determine the antimicrobial activity of *Aloe vera* juice against gram positive and negative bacteria, and the fungus *Candida albicans.* Making use of

disc diffusion method, the study revealed that the tested plant juice was mostly effective against gram positive bacteria and *C. albicans* (Alemdar & Agaoglu, 2009).

A study aiming to investigate the antimicrobial activity of Dimethyl sulfoxide (DMSO) crude extracts of *Aloe barbadensis* Miller gel against selected bacterial and fungal pathogens showed that *Aloe vera* extracts are effective against most of the microbial strains used. The maximum zones of inhibitions appeared against *E. coli, Proteus mirabilis, Pseudomonas aeruginosa, S. aureus, C. albicans* and *Penicillium spp* (Devi, Srinivas, & Rao, 2012).

It has also been reported that *Aloe vera* contains six antiseptic agents: Lupeol, salicylic acid, urea nitrogen, cinnamonic acid, phenols and sulfur, which causes *Aloe vera* to have inhibitory effects against fungi, bacteria and viruses (Surjushe et al., 2008). The anthraquinone aloin present in *Aloe vera* inactivates several enveloped viruses such as herpes simplex, varicella zoster and influenza (Sydiskis et al., 1991).

1.3 Clove

Syzygium aromaticum (Clove) is a dried, aromatic and unopened floral bud of an evergreen tree belonging to the Myrtaceae family. The tree grows 10-20 m in height and is indigenous to India, Indonesia, Zanzibar Mauritius and Ceylon (Srivastava & Malhotra, 1991.; Chaieb et al., 2007). Cloves have a unique aroma and flavour which is derived from compounds known as phytochemicals or secondary metabolites (called so because they are secondary to a plant's basic metabolism). Plants usually have a wide variety of secondary metabolites like tannins, alkaloids and flavonoids which have been found to have antimicrobial properties *in vitro* (Lewis & Ausubel, 2006).

Clove is used as a spice that increases taste and variation of food (Bulduk, 2004). It is also used in Ayurveda, Chinese medicine and Western herbalism (Saeed & Tariq, 2008). Clove possesses a sweet and spicy fragrance that is stimulating and revitalizing. Eugenol and carvacrol are phenol compounds found in cloves that can function as mould inhibitors when added to bakery items (Shrivastava et al., 2014).

Figure 1.3: (Right) Clove tree flowers (Image retrieved from: https://en.wikipedia.org/wiki/Clove#/media/File: The_flowers_of_clove_tree_in_Pemba_island.JPG)

(Left) Dried Cloves (Image retrieved from: https://en.wikipedia.org/wiki/Clove#/media/File:ClovesDried.jpg)

1.3.1 Taxonomic hierarchy of clove

Taxonomic hierarchy of clove (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	Myrtales
Family	Myrtaceae – myrtles, myrtacées
Genus	Syzygium P. Br. ex Gaertn.
Species	Syzygium aromaticum (L.)

1.3.2 Therapeutic uses of clove

According to several literature, cloves have many therapeutic uses. Cloves are often used as a carminative in order to increase hydrochloric acid in stomach and to improve peristalsis (Phyllis & James, 2000). In dentistry, the essential oil of clove is used as anodyne for dental emergencies (Cai & Wu, 1996). They control nausea and vomiting, cough, diarrhoea, dyspepsia, flayulence, stomach distension and gastro-intestinal spasm, relieve pain, cause uterine contractions and stimulate the nerves (Shrivastava et al., 2014). In addition to all these, cloves are also highly antiseptic (Blumenthal, 1998), antimutagenic (Miyazawa & Hisama, 2003), anti-inflammatory (M. Kim et al., 1998), antioxidant (Chaieb et al., 2007), antiulcerogenic (Bae et al., 1998), antithrombotic (Srivastava & Malhotra, 1991) and antiparasitic (Yang et al., 2003).

Essential oil extracted from the dried flower buds of cloves is used for acne, warts, scars and parasites (Saeed & Tariq, 2008). It has also been shown by research that clove oil is an effective mosquito repellent (Trongtokit et al., 2005). Clove oil is also used as a topical application to relieve pain and promote healing. It is also used in fragrance and flavouring industries (Chaieb et al., 2007). The oil from clove, however, is toxic to human cells (Prashar et al., 2006). If ingested or injected in sufficient quantity, it has been shown to cause life threatening complications such as Acute Respiratory Distress Syndrome, Fulminant Hepatic Failure and central Nervous System disorder. The lethal oral dose is 3.752 g/Kg body weight (Kirsch et al., 1990). The main constituents of clove essential oil are phenylpropanoides such as carvacrol, thymol, eugenol and cinnamaldehyde (Chaieb et al., 2007).

1.3.3 Antimicrobial properties of clove

Over the past decade, studies have confirmed that cloves can inhibit the growth of both gram positive and gram negative food borne bacteria, yeast and mould (Shrivastava et al., 2014). Several studies have demonstrated the potent antifungal (Park et al., 2007), antiviral (Chaieb et al., 2007) and antibacterial (Cai & Wu, 1996; Bae et al., 1998; Fu et al., 2007) activities of clove extracts.

By using paper disc diffusion method, antimicrobial activities of aqueous, ethanol and acetone extracts of clove were studied against gram negative bacteria such as *Pseudomonas* spp. and *Proteus vulgaris*. It was observed that acetone and ethanol extracts had more potent activity than the aqueous extract (Shrivastava et al., 2014). In a study conducted by Beg and Ahmad, the *in vitro* antifungal activity of clove oil was examined against four fungi: *Alternaria alternata*, *Fusarium chlamydosporum*, *Helminthosporum oryzae* and *Rhizoctonia bataticola* by agar well diffusion method. The test result indicated that all the fungi were highly sensitive to clove oil at a concentration of 100 µl/well (Beg & Ahmad, 2002).

In another study, among the aqueous ethanolic extracts of four medicinal plants against human pathogenic *Escherichia coli, Salmonella typhi, Salmonella paratyphi, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa,* clove was found to be the most effective against *Salmonella typhi* (Joshi et al., 2011). In a study undertaken by Mehrotra et al., ethanol extracts of clove buds were found to inhibit the growth of methicillin resistant *Staphylococcus aureus*, *Vibrio cholera* and *Pseudomonas aeruginosa*. By screening 100 isolates of 10 different species of gram negative bacteria with standard disc diffusion method, Saeed and Tariq (2008) investigated the potential of aqueous infusion, decoction and essential oil of clove. It was observed that aqueous infusion and decoction were most effective in killing *P. aeruginosa,* and clove essential oil was most effective against *V. cholerae.* While many of the bacterial strains were resistant against aqueous infusion and decoction, all of them were highly sensitive to the essential oil.

All these studies were, therefore, effective in determining alternative forms of antimicrobial agents within a spectrum of natural produces. These findings can play very important roles in the treatment of infectious diseases especially in light of the emergence of drug-resistant microorganisms (Joshi et al., 2011).

1.4 Extraction techniques

Several different methods of organic solvent extraction can be adopted in order to separate active ingredients from plant materials: in order to prepare ethanol extracts, ten grams of dried plant material were pounded with mortar and pestle and soaked in 40 ml ethanol for 24 hours in a

shaker incubator. The content was filtered with Whatman no. 1 filter paper, and the residue treated with 40ml absolute ethanol again as mentioned. The entire process was repeated 3 times, and the pooled up filtrates were evaporated to dryness under vacuum using a distillation unit. The dried extracts were reconstituted in 5 ml ethanol and stored at 4ºC until use (Mehrotra, Srivastava, & Nandi, 2010).

In another study, the dried plant materials were ground into fine powder using an electric grinder, and acetone, ethanol and aqueous extracts prepared by dissolving 5g powder in 20ml of each of the solvents by stirring at room temperature. The mixtures were filtered using sterilized Whatman filter paper no.1 and exposed to UV radiation for 24h before storing at 4ºC until use (Shrivastava et al., 2014). For preparation of *Aloe vera* extracts, sometimes 100 grams of the colourless, parenchymatous tissue was mixed in one litre of 2% dimethyl sulfoxide (DMSO) and kept at 4ºC until use (Goudarzi et al., 2015).

In another method, the plant materials were sterilized properly, dried in the oven at 80ºC for 48 hours and then crushed into powder. In the process of maceration, 10g of powdered material was dissolved in 100 ml of organic solvents (ethanol, methanol, acetone and distilled water) and placed in shaking incubator for 48 hours. The extracts were then filtered with muslin cloth and centrifuged at 8000g for 20 minutes. The supernatant was stored at 4 ºC until use (Irshad et al., 2011).

1.5 Bacterial strains selected for the study

Bactericidal activity of aqueous, ethanol, methanol and acetone extracts of *Aloe vera* and clove were observed against the following bacterial species isolated from clinical samples:

1.5.1 *Pseudomonas* spp.

Pseudomonas aeruginosa is a gram-negative, rod shaped bacterium. It is a ubiquitous pathogen capable of infecting virtually all types of tissues (Lyczak et al., 2000). It is an opportunistic human pathogen and its prominence is facilitated by its intrinsic resistance to antibiotics and disinfectants.

1.5.2 *Klebsiella* spp.

Members of the genus *Klebsiella* are gram-negative, nonmotile, rod shaped bacteria found almost everywhere in nature. These bacteria frequently cause human nosocomial infections. The species *Klebsiella pneumoniae* is responsible for a significant portion of hospital acquired urinary tract infections, pneumonia, septicemia and soft tissue infection. Owing to their ability to spread rapidly, these bacteria tend to cause nosocomial outbreaks. Hospital outbreaks of multidrug resistant *Klebsiella* spp. have been increasing steadily over the past years (Podschun & Ullmann, 1998).

1.5.3 *Escherichia coli*

These gram-negative organisms are commensal gut bacteria, but some pathogenic strains can sometimes produce enterotoxins that can cause foodborne diseases and gastrointestinal infections. These organisms can also cause urinary tract infections (Tortora et al., 2010).

1.6 Commercial antibiotics and antibiotic resistance

Antibiotics are natural or synthetic substances that destroy microorganisms or inhibit their growth. Antibiotics are used extensively to treat diseases in plants, animals and humans. After the discovery of penicillin in 1928, many other antibiotics have been discovered and commercially produced. About 100,000 tons of antibiotics are manufactured annually worldwide, and their widespread use has profoundly affected bacterial life on earth. More and more strains are becoming resistant every day, and many have already become resistant to multiple drugs and chemotherapeutic agents-the phenomenon of multidrug resistance (Nikaido, 2010).

Antibiotics can be categorized according to their principal mechanisms of action:

- β-lactams (and cephalosporins) interfere with bacterial cell wall synthesis, achieved by a competitive inhibition on PBP (penicillin binding proteins) [Example: Ampicillin, Cefuroxime, Ceftriaxone]
- Macrolides and tetracyclines are bacteriostatic and inhibit protein synthesis in bacteria [Example: Erythromycin, Azithromycin]

- Fluoroquinolones are synthetic antibiotics belonging to the family of quinolones and they function by blocking bacterial DNA replication through inhibition of DNA gyrase [Example: Ciprofloxacin, Levofloxacin, Ofloxacin]
- Aminoglycosides also inhibit bacterial protein synthesis [Example: Amikacin, Gentamicin, Kanamycin]

Other antibiotics work by inhibiting metabolic pathways of bacteria and also by disruption of bacterial membrane structures (Tenover, 2006).

1.6.1 Antibiotic resistance

Treating bacterial infections is becoming increasingly complicated because of the ability of bacteria to develop resistance to antimicrobial agents. Bacteria may be intrinsically resistant to one or none of the classes of antimicrobials, but they may acquire resistance by de novo mutation or through the acquisition of resistant genes from other organisms (Tenover, 2006). Multidrug resistance in bacteria occurs by the accumulation of genes on resistance (R) plasmids or transposons, with each gene coding for resistance to a specific agent, and/or by the action of multidrug efflux pumps, each of which can pump out more than one drug type (Nikaido, 2010).

There are some common biochemical mechanisms of resistance. Mutational alteration of the target protein can occur when bacteria become resistant through mutations that make the target proteins less susceptible to the antimicrobial agents. Fluoroquinolone resistance, for example, is due to mutations in the DNA topoisomerase enzymes (Hooper, 2000). Enzymatic inactivation of the drug occurs when antibiotics of natural origin are inactivated by enzymatic phosphorylation, acetylation, adenylation or enzymatic hydrolysis by β-lactamases. Genes coding for these inactivating enzymes can produce resistance as additional genetic components on plasmids. Other methods might include acquisition of genes for less susceptible target proteins from other species, bypassing the targets or preventing drug access to targets (Nikaido, 2010).

Bacteria from both clinical and non-clinical environments are becoming increasingly resistant to conventional antibiotics. Gram-positive bacteria, predominantly methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp. were the primary concerns

10 years ago, but multidrug resistant Gram-negative bacteria pose the greatest risk to public health today (Kumarasamy et al., 2010). Increase in resistance in Gram-negative bacteria is faster than that in Gram-positive bacteria (Cornaglia, 2009) and there are also fewer new and developmental antibiotics active against Gram-negative bacteria (Baiden et al., 2010). Also, current drug development programs appear to be quite inadequate to provide therapeutic cover in the next 10-20 years (Rice, 2009).

Increase in resistance in Gram-negative bacteria is primarily due to mobile genes on plasmids that can readily spread through bacterial populations (Kumarasamy et al., 2010). Additionally, unprecedented human air travel and migration allow bacterial plasmids and clones to be transported rapidly between countries and continents (Walsh, 2006). Most of this spread, however, is undetected and resistant clones are carried in the normal human flora and only become evident when they are the sources of endogenous infections (Kumarasamy et al., 2010). It has been identified in recent surveys that extended-spectrum β-lactamases (ESBLs) are present in 70-90% of Enterobacteriaceae in India, necessitating the widespread use of reserved antibiotics such as carbapenems [antibiotics used for treatment of infections caused by multidrug resistant bacteria] (Hawkey, 2008).

Carbapenem antibiotics are often the last option for treatment of infections caused by Gram-negative bacteria resistant to other β-lactam agents because of their broad spectra of activity and stability to hydrolysis by most β-lactamases including extended-spectrum β-lactamases (Bush et al., 1995). In *Pseudomonas aeruginosa* resistance to carbapenems often result from reduced levels of drug accumulation or amplified levels of expression of pump efflux (Hancock, 1998). The resistance is occasionally due to production of metallo-β-lactamases (MBLs), which can be either chromosomally encoded or plasmid mediated (Lauretti et al., 1999).

1.6.2 β-Lactamases

β-Lactam antibiotics, including penicillins, cephalosporins and carbapenems, act cytostatically on bacteria by inactivating peptidoglycan transpeptidases irreversibly. The transpeptidases catalyse the cross-linking of peptidoglycan polymers in bacterial cell walls, and cell death can be caused by the inhibition of this polymerisation (Green, 2002). The transpeptidases are members

of the family of penicillin binding proteins (PBPs) from which β-lactamases are likely to have evolved (Massova & Mobashery, 1998). B-Lactamases are enzymes of bacterial origin that degrade β-lactam antibiotics into antimicrobially inert compounds, thereby protecting the organisms from the lethal actions of β-lactam antibiotics (Medeiros, 1997).

β-Lactamases are classified into four different molecular groups- A,B,C and D according to amino acid sequences (Ambler, 1980). Class A, C (AmpC) and D b-lactamases use a catalytically active serine residue for inactivation of β-lactam drugs. The enzymes assigned to molecular class B are metallo-enzymes requiring zinc for their catalytic activity, and they operate through a completely different mechanism (Majiduddin et al., 2002).

Hydrolysis of β-lactam antibiotics by β-lactamases is the most common mechanism of resistance for this class of antibiotics in clinically important Gram-negative bacteria. Penicillins, cephalosporins and carbapenems are included in the preferred treatment regimens for many infectious diseases, so the presence and characteristics of the said enzyme plays a critical role in the selection of appropriate therapy. β-Lactamase production is most frequently suspected in Gram-negative bacterial isolates that demonstrate resistance to β-lactam antibiotics (Bush & Jacoby, 2010).

1.6.3 New Delhi Metallo-beta-lactamase 1 (NDM-1)

Recently, a novel metallo-enzyme, the New Delhi metallo-β-lactamase-1 (NDM-1), has been identified in *Enterobacteriaceae,* which was first reported in India, Pakistan and the United Kingdom. The identification of this enzyme has led to international concern, as its spread represents a new and major challenge in the field of infectious diseases (Kumarasamy et al., 2010). Metallo-β-lactamase enzymes mediate resistance to various β-lactam agents, including carbapenems (Bush & Jacoby, 2010). The NDM-1 enzyme received its name “New Delhi” after it was originally detected in a carbapenem-resistant *Klebsiella pneumoniae* isolated from a urinary tract specimen form a Swedish patient of Indian origin who had travelled to New Delhi (Yong et al., 2009). After its initial identification, NDM-1 producing organisms have been reported in hospitalized patients in Europe, Australia and North America in studies that focused mainly on hospitalized patients returning from South Asian countries-India and Pakistan in

particular (Kumarasamy et al., 2010). International travel and widespread use of multiple healthcare systems contribute to the rapid spread of *bla*$_{NDM-1}$ which has potentially serious consequences (Jovcic et al., 2011).

In order to determine the prevalence of NDM-1 in Gram-negative bacteria isolated from patients in Bangladesh, a study was conducted at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) laboratories, where 1,816 consecutive clinical samples were tested for imipenem-resistant Gram-negative bacteria. The imipenem-resistant isolates were then tested for the *bla*$_{NDM-1}$ gene. Among 403 isolates, 14 (3.5%) were positive for *bla*$_{NDM-1}$, and the predominant species were *Klebsiella pneumoniae, Acinetobacter baumannii* and *Escherichia coli.* All *bla*$_{NDM-1}$ positive isolates were resistant to multiple antibiotics (Islam et al., 2012). In another study conducted in Bangladesh, out of thirty five imipenem resistant isolates, eight (22.86%) were positive for *bla*$_{NDM-1.}$ In analysing the antimicrobial resistant patterns of these organisms it was found that 100% of NDM-1 producers were resistant to amoxicillin, cephradine, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, aztreonam, gentamicin and piperacillin, 87.5% to amikacin, 75% to ciprofloxacin and 62.5% to co-trimoxazole and the combination of tazobactam and piperacillin (Farzana et al., 2013).

In Nairobi, Kenya a study found seven carbapenem-resistant NDM-1-positive *Klebsiella pneumoniae* isolates, all of which were recovered from patients hospitalized between 2007 and 2009 at different wards in tertiary care centres. All the isolates were obtained from urine samples and carried *bla*$_{NDM-1}$ carbapenemase gene. These isolates were clonally related and expressed many other resistance determinants, including β-lactamases CTX-M-15, OXA-1, OXA-9 and CMY-6 (Poirel et al., 2011).

1.6.4 OXA-type β-lactamases

The OXA-type (Oxacillin-hydrolysing) enzymes are widespread and have mostly been identified in Enterobacteriaceae and *Pseudomonas aeruginosa.* They usually confer resistance to amino- and ureidopenicillin and possess high-level hydrolytic activity against oxacillin, cloxacillin and methicillin. Their activities are weakly inhibited by clavulanic acid, but sometimes NaCl possesses a strong inhibitory activity (Naas & Nordmann, 1999). Historically, the first

characterised class D β-lactamases were referred to as oxacillinases because they commonly hydrolyse isoxazolylpenicillin oxacillin much faster than classical penicillins, that is, benzylpenicillin (Bush et al., 1995).

The "OXA" designation of the class D beta-lactamases refers to their preferred penicillin substrate. Currently, 121 different variants of class D β-lactamases have been identified on the protein level and 45 of these exhibit carbapenem-hydrolysing activities (Walther-Rasmussen & Høiby, 2006). 24 Ambler class D enzymes, named OXA-1 to OXA-22, AmpS and LCR-1 have been characterised. While some oxacillinases present a significant degree of amino-acid identity (for example, OXA-1 and OXA-4; OXA-10 (PSE-2) derivatives; OXA-2 and OXA-3), most of them are only weakly related (20% to 30% amino-acid identity). Oxacillinases usually display a restricted-spectrum phenotype. However extension of their spectrum towards oxyimino cephalosporins and/or imipenem has been observed mostly as a consequence of point mutations in OXA-2 or OXA-10 derivatives (Naas & Nordmann, 1999).

Classical OXA enzymes confer resistance to carboxypenicillins and ureidopenicillins, but not to ceftazidime. On the other hand, extended-spectrum β-lactamases (ESBLs) that hydrolyse third-generation cephalosporins have been reported with increasing frequency in *Pseudomonas aeruginosa.* These ESBLs often result from amino acid substitutions in OXA enzymes or, less frequently, in TEM or SHV enzymes (Bert e al., 2002). Oxacillinases can be divided into distinct groups (Sanschagrin et al., 1995). OXA group I includes OXA-5, OXA-7, OXA-10 and its derivatives (OXA-11, OXA-14, OXA-16, OXA-17), and OXA-13 and its derivatives (OXA-19, OXA-28). OXA group II includes OXA-2, OXA-3, OXA-15 and OXA-20. OXA group III includes OXA-1, OXA-4, OXA-30 and OXA-31, and OXA group IV is defined by OXA-9. OXA group V consists of the enzyme LCR-1 and OXA-18 does not belong to any of these groups and has very low amino acid identity with the other oxacillinases (Bert et al., 2002).

In a study conducted in Bangladesh, a total of 339 *E. coli* were isolated from patients with urinary tract and wound infections. 40 *E. coli* were identified to have ESBL phenotypes and $bla_{\text{OXA-1}}$ genes were detected in 19 (47.5%) isolates (Lina et al., 2014). In another study, 233 *E. coli* isolates obtained from tap water in Dhaka were analysed for susceptibility to antibiotics and for the presence of genes associated with antibiotic resistance. It was found that out of 22 ESBL producers, 7 were positive for $bla_{\text{OXA-1}}$ and all were positive for $bla_{\text{OXA-47}}$ (Talukdar et al., 2013).

1.7 Objectives

The worldwide prevalence of multi-drug resistant bacterial strains is a major concern today. Looking into alternative sources for potential antimicrobial agents, and a clear understanding of the molecular basis of drug resistance in bacteria are two tools that can help combat such serious issues in infective health environments. There is enormous potential in the study of plants with medicinal relevance like *Aloe vera* and clove. It is also of great significance to try and identify the occurrence of certain genes in bacteria that are associated with multi-drug resistance. Considering these, the specific objectives of this study included the following:

1. Extraction of phytochemicals from *Aloe vera* leaf and clove using organic solvents like ethanol, methanol, acetone and water
2. Analysing the antimicrobial activity of the extracts against some clinically relevant bacteria
3. Comparison of antimicrobial activity of the plant extracts with commercial antibiotics
4. Identification of NDM-1 and OXA-2 genes in multi-drug resistant bacterial species using PCR and gel electrophoresis

Chapter 2:

Materials and Method

Materials and Method

This study was carried out in the laboratory of the Department of Mathematics and Natural Sciences at BRAC University.

2.1 Specimen collection and processing

Aloe vera leaves and dried clove buds were bought from the local markets, washed thoroughly with distilled water and dried. *Aloe vera* leaves, including the inner gel, were then cut into thin slices and dried in an oven at 40-50°C for 1-2 days until the weight became constant. The clove buds were sun dried for a few days. Plant materials were checked periodically for any fungal growth. The dried plant materials were then pounded manually into fine powder using mortar and pestle.

2.2 Preparation of plant extracts

A total of four different types of extracts were prepared each from *Aloe vera* and clove using four organic solvents: ethanol, methanol, acetone and water.

2.2.1 Ethanol extraction

Using an electronic balance, 20 grams of *Aloe vera* leaf powder and 20 grams of clove powder were separately measured and soaked in 100 ml of absolute ethanol in sterile 250 ml conical flasks. After stirring continuously for about a minute the conical flasks' mouths were covered with aluminium foil and made air-tight with parafilm strips. The flasks were then left in shaker incubator (37°C and 150 rpm) for 48 hours.

After 48 hours, the extracts were filtered into sterile 100 ml conical flasks using autoclaved Whatman No.1 filter papers. The filtrates were then heated in water bath at around 78°C until the solvent evaporated to dryness. The remaining solidified extracts were then collected in clean McCartney bottles and re-suspended in ethanol. The bottles were labelled for identification.

2.2.2 Methanol extraction

Measuring with an electronic balance, 20 grams each of *Aloe vera* leaf and clove powder were transferred into 100 ml methanol in sterile 250 ml conical flasks. After stirring continuously for about a minute the conical flasks' mouths were covered with aluminium foil and made air-tight with parafilm strips. The flasks were then left in shaker incubator (37°C and 150 rpm) for 48 hours.

After 48 hours, the extracts were filtered into sterile 100 ml conical flasks using autoclaved Whatman No.1 filter papers. The filtrates were then heated in water bath at around 65°C until the solvent evaporated to dryness. The remaining solidified extracts were then collected in clean McCartney bottles and re-suspended in methanol. The bottles were labelled for identification.

2.2.3 Acetone extraction

Using an electronic balance, 20 grams each of *Aloe vera* leaf and clove powder were separately measured and soaked in 100 ml of acetone in sterile 250 ml conical flasks. After stirring continuously for about a minute the conical flasks' mouths were covered with aluminium foil and made air-tight with parafilm strips. The flasks were then left in shaker incubator (37°C and 150 rpm) for 48 hours.

After 48 hours, the extracts were filtered into sterile 100 ml conical flasks using autoclaved Whatman No.1 filter papers. The filtrates were then heated in water bath at around 56°C until the solvent evaporated to dryness. The remaining solidified extracts were then collected in clean McCartney bottles and re-suspended in acetone. The bottles were labelled for identification.

2.2.4 Aqueous extraction

Using an electronic balance, 20 grams each of *Aloe vera* leaf and clove powder were separately measured and soaked in 100 ml of distilled water in sterile 250 ml conical flasks. After stirring continuously for about a minute the conical flasks' mouths were covered with aluminium foil

and made air-tight with parafilm strips. The flasks were then left in shaker incubator (37°C and 150 rpm) for 48 hours.

After 48 hours, the extracts were filtered using autoclaved Whatman No.1 filter papers. The pooled up filtrates were collected in McCartney bottles and labelled for identification. These extracts were directly used in antimicrobial susceptibility testing.

2.3 Storage and preservation of extracts

The prepared extracts were stored in autoclaved 25 ml McCartney bottles in 4°C refrigerator until use. The bottles were stoppered carefully and labelled accurately prior to storage.

Figure 2.3: Plant extracts stored in McCartney bottles

2.4 Maintenance and preservation of bacterial samples

The bacterial strains used in this study were collected from sputum taken from patients in National Institute of Diseases of the Chest and Hospital (NIDCH), Mohakhali, Dhaka.

The samples (10 *Pseudomonas*, 10 *Klebsiella* and 1 *E. coli*) were streaked on freshly prepared Nutrient Agar plates and incubated for 24 hours. After growth was clearly visible, the plates

were wrapped with parafilm and stored at 4°C until further use. Before each respective experiment, the organisms were freshly subcultured and the 24-hour cultures were used. Purity of the cultures was maintained by regular subculturing.

2.5 Antibacterial activity test of plant extracts

Agar well diffusion method was used for the primary screening of the antimicrobial activity of plant extracts against the target pathogens *in vitro*.

2.5.1 Preparation of Nutrient Agar (NA) plates

In order to prepare 1000 ml of NA, 28 grams of the powder must be added. Keeping this concentration constant, the required volumes of NA were prepared each time. Using an electronic balance the powder was measured prior to adding distilled water. After stirring to break off any lumps, the flask was heated on a Bunsen burner until the solution turned clear and bubbles appeared. After allowing to cool for a few minutes, the mouth of the conical flask containing the media was covered using aluminium foil and autoclaved.

After sterilisation, the media was carefully poured on to sterile petri dishes inside a laminar air flow cabinet. For a medium-sized plate, the volume of NA needed is 20 ml and for large plates the volume is 35 ml.

2.5.2 Preparation of Saline Solution

To make 0.9% saline solution, 0.9 grams of sodium chloride (NaCl) was taken into 100 ml of distilled water. 9 ml of the saline solution were put in each test tube. Several such test tubes were prepared and autoclaved, with the screw cap opened through half turns. When taken out of the autoclave machine, the screw caps were turned fully to close the mouth of the tube to prevent contamination.

2.5.3 Inoculation and agar well diffusion

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

Using sterile cotton swabs, each of the test bacterial strains were lawn cultured on properly labelled NA plates to achieve even growth. The plates were allowed to dry and then a sterile cork borer was used to bore wells in the agar plates. The extracts (100 µl/well) were then loaded in the wells using a micropipette. The plates were then incubated at 37°C for 24 hours.

Antimicrobial activities of the extracts were determined by measuring the diameters of inhibition zones in millimetres produced against the pathogens. The experiments were repeated three times and the mean values were calculated.

2.6 Antibiogram of test organisms using commercial antibiotics

An antibiogram is the result of an antibiotic sensitivity test. Using the following commercial antibiotics an antibiotic sensitivity test for all the test organisms were carried out:

- Imipenem
- Meropenem
- Gentamicin
- Oxacillin
- Cloxacillin
- Ceftazidime
- Ampicillin
- Amikacin
- Kanamycin
- Ciprofloxacin

2.6.1 Preparation of Mueller-Hinton Agar (MHA) plates

In order to prepare 1000 ml of MHA, 38 grams of powder must be added. Keeping this concentration constant, the required volumes of MHA were prepared each time. Using an electronic balance the powder was measured prior to adding distilled water. After stirring to break off any lumps, the flask was heated on a Bunsen burner until the solution turned clear and

bubbles appeared. After allowing to cool for a few minutes, the mouth of the conical flask containing the media was covered using aluminium foil and autoclaved.

After sterilisation, the media was carefully poured on to sterile petri dishes inside a laminar air flow cabinet. For a medium-sized plate, the volume of MHA needed is 20 ml and for large plates the volume is 35 ml.

2.6.2 Inoculation of media and Disk Diffusion

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

Using sterile cotton swabs, each of the test bacterial strains were lawn cultured on properly labelled MHA plates to achieve even growth. After lawn culture, sterile forceps were used to carefully pick up antibiotic disks from the stacks and were placed very carefully on the lawn culture. Care was taken to ensure that the disks are well-spaced in order to prevent overlapping of inhibition zones. Also, once an antibiotic disk was placed, it wasn't picked up or replaced on any other location on the MHA plates.

Antimicrobial activity was determined by measuring the diameter of inhibition zone in millimetre produced against the pathogens. The experiments were repeated three times and the mean values were calculated.

2.6.3 Measuring activity index

The inhibitory effects of ethanol, methanol, acetone and aqueous extracts were determined and compared with that of antibiotics by measuring the activity index (AI) using the following formula:

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

2.7 Identification of multidrug resistant genes

In order to identify multidrug resistant genes, first DNA was isolated from bacteria. Then the DNA was amplified using Polymerase Chain Reaction, and the results were analysed using gel electrophoresis.

2.7.1 DNA Extraction

The following method was used to purify genomic DNA without using any commercial kits:

- 1.5 ml of the overnight culture (grown in LB medium) was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13,500 rpm for 3 minutes to pellet the cells
- The supernatant was discarded without disturbing the pellet
- The cell pellet was then resuspended in 600 µl lysis buffer and vortexed to resuspend completely
- The resuspended cell pellet was then incubated for 1 hour at 37 °C
- After incubation, 750 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and mixed by inverting the tubes until the phases were completely mixed
- Following second round of centrifugation for 5 minutes, three distinct layers were visible: bottom layer of phenol: chloroform: isoamyl alcohol, intermediate layer of proteins, and the top aqueous layer of nucleic acids
- The top layer was carefully transferred to a new tube
- To remove phenol, an equal volume of chloroform was added to the aqueous layer. The tube was again inverted to mix well
- The tubes were centrifuged at 13,500 rpm for 5 minutes
- Approximately 200 µl of the upper aqueous layer containing DNA was transferred to a new tube
- To precipitate the DNA, 3 volumes of cold ethanol was added and mixed gently
- The tubes were incubated at -20°C for 30 minutes
- Next, the tubes were centrifuged at 13,500 rpm for 15 minutes
- The supernatant containing ethanol was discarded and the DNA pellet rinsed with 1 ml 70% ethanol

- Another round of centrifugation was done at 13,500 rpm for 2 minutes
- The supernatant was discarded and the DNA pellet was air-dried
- The DNA was resuspended in 50 µl TE buffer

2.7.2 Polymerase Chain Reaction (PCR)

PCR is a technique used in molecular biology that uses Taq DNA Polymerase enzyme to amplify the quantity of a DNA sample. High temperatures in the denaturation phase denature the double stranded template into single strands. Then in the annealing phase, short oligonucleotides called primers bind to the template strands. The Taq Polymerase then binds to the primers and starts forming new strands by adding complementary nucleotides and thus forming double stranded DNA again.

The following procedures were used to amplify DNA samples:

NDM-1:

- By using the following components 50 µl reaction mixture was made (for 15 samples):

Table 2.7.1: PCR reaction mixture for *bla*$_{\text{NDM-1}}$

Forward Primer	75µl
Reverse Primer	75 µl
Taq Polymerase	3.75 µl
dNTPs	15 µl
Template DNA*	75 µl
10x Reaction buffer	75 µl
Nuclease free water	431.25 µl
Total	750 µl

*Template DNA was added separately to each PCR tube after all the other components

- 50 µl of reaction mixture was transferred to each sterile PCR tube using a micropipette

- The tubes were placed in the PCR machine and the following cycling parameters were used to run the reaction:

Table 2.7.2: Cycling parameters for *bla*$_{NDM-1}$

PCR step	Temperature	Time
Initial denaturation	95°C	10 minutes
Denaturation*	95°C	1 minute
Annealing*	63°C	45 seconds
Extension*	72°C	1 min 30 seconds
Final extension	72°C	10 minutes

* 30 cycles each

- The primers used for *bla*$_{NDM-1}$ (200bp) were:

Table 2.7.3: Primers for *bla*$_{NDM-1}$

Forward primer	5′-ACC GCC TGG ACC GAT GAC CA- 3′
Reverse primer	5′-GCC AAA GTT GGG CGC GGT TG- 3′

- After the run was complete, the PCR products were immediately transferred to -20°C refrigerator and stored there until further use

OXA-2:

- By using the following components 50 µl reaction mixture was made (for 15 samples):

Table 2.7.4: PCR reaction mixture for *bla*$_{OXA-2}$

Forward primer	75µl
Reverse Primer	75 µl
Taq polymerase	3.75 µl
dNTP	15 µl
Template DNA*	75 µl
10x reaction buffer	75 µl
Nuclease free water	431.25 µl
Total	750 µl

*Template DNA was separately added to individual PCR tubes after all other components

- 50 µl of reaction mixture was transferred to each sterile PCR tube using a micropipette
- The tubes were placed in the PCR machine and the following cycling parameters were used to run the reaction:

Table 2.7.5: Cycling parameters for *bla*$_{OXA-2}$

PCR step	Temperature	Time
Initial denaturation	96°C	5 minutes
Denaturation*	96°C	1 minute
Annealing*	65°C	1 minute
Extension*	72°C	2 minutes
Final extension	72°C	10 minutes

*30 cycles each

- The primers used for *bla*$_{OXA-2}$ (702 bp) were:

Table 2.7.6: Primers for *bla*$_{OXA-2}$

Forward primer	5´- TTC AAG CCA AAG GCA CGA TAG -3´
Reverse primer	5´- TCC GAG TTG ACT GCC GGG TTG -3´

- After the run was complete, the PCR products were immediately transferred to -20°C refrigerator and stored there until further use

2.7.3 Agarose Gel Electrophoresis

DNA samples amplified by PCR were analysed by gel electrophoresis- a method that separates PCR products into distinct bands based on size. An electric field allows the DNA samples to run from one end of the gel to other, and a standard ladder DNA is used to identify the bands present by viewing under UV light.

- 40ml agarose gel was prepared by dissolving 0.4 grams of agarose powder into 40 ml 1x Tris-EDTA (TE) buffer and heating in microwave oven until the powder dissolved
- Once the gel cooled down a little, 2 µl Ethidium Bromide (EtBr) staining dye was carefully added to it and then the gel was poured onto the gel electrophoresis tray
- A comb was placed near one end to form wells as the gel solidified
- 5µl PCR sample mixed with 2µl loading dye was added in each well, taking care not to spill the contents
- A ladder DNA (100kb) was loaded onto the last well to serve as a standard against which other band sizes would be compared
- The gel was completely submerged in 1x TBE buffer and the machine was connected to an electric supply
- The gel was allowed to run at 80 volts for about 40 minutes
- After the run was complete the gel was visualized under UV light

Chapter 3: Results

3.1 Antibacterial activity test results:

Antibacterial activity of ethanol, methanol, acetone and aqueous extracts of *Aloe vera* leaf and clove were tested against ten *Pseudomonas* samples, ten *Klebsiella* samples and one *E. coli* sample. The results obtained are presented below.

3.1.1 *Aloe vera*

Acetone extract of *Aloe vera* leaf was seen to be most effective most of the time and aqueous extract did not show any activity against *Pseudomonas* samples:

Table 3.1.1: Antimicrobial effects (Inhibition zones) produced by methanol, ethanol, acetone and aqueous extracts of *Aloe vera* leaf against *Pseudomonas* isolates

Pseudomonas isolates	Average zones of inhibition (mm)			
	Crude methanol extract	Crude ethanol extract	Crude acetone extract	Crude aqueous extract
P1	11	12	15	0
P2	12.5	12	14.5	0
P3	14	10	18	0
P4	13	14	18	0
P5	16.5	15	17	0
P6	14.5	15	20	0
P7	18	18.5	23	0
P8	12.5	8	12.5	0
P9	19	14.5	19.5	0
P10	15.5	16	21.5	0

Acetone extract of *Aloe vera* leaf was more effective in average than ethanol or methanol extracts against *Klebsiella* samples. Aqueous extract did not show any antibacterial activity.

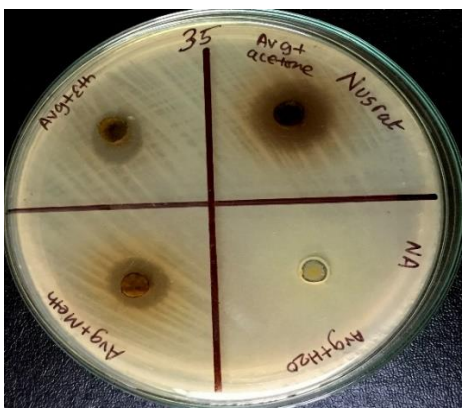
Table 3.1.2: Antimicrobial effects (Inhibition zones) produced by methanol, ethanol, acetone and aqueous extracts of *Aloe vera* leaf against *Klebsiella* isolates

Klebsiella isolates	Average zones of inhibition (mm)			
	Crude methanol extract	Crude ethanol extract	Crude acetone extract	Crude aqueous extract
K1	15	14	16	0
K2	12	15	13.5	0
K3	13.5	14	16.5	0
K4	19.5	11	16.5	0
K5	12.5	17	14.5	0
K6	13.5	11	15	0
K7	16.5	11.5	15	0
K8	15	15.5	18	0
K9	12	14.5	14	0
K10	13.5	13	15	0

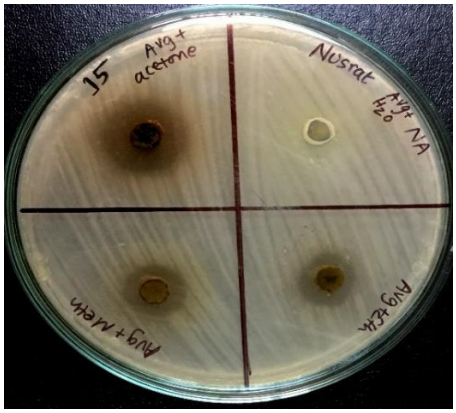
Aqueous extract also did not show any activity against *E. coli* sample.

Table 3.1.3: Antimicrobial effects (Inhibition zones) produced by methanol, ethanol, acetone and aqueous extracts of *Aloe vera* leaf against *E. coli*

Sample	Methanol extract	Ethanol extract	Acetone extract	Aqueous extract
E1	12	13	0	0

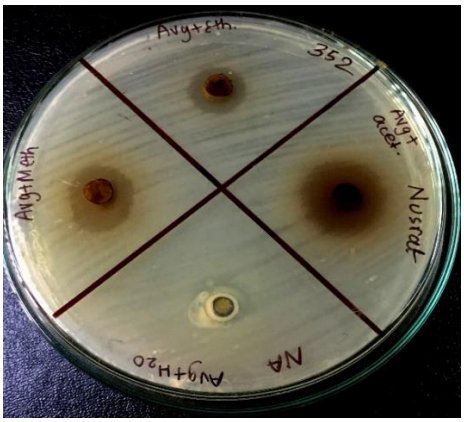


(a)

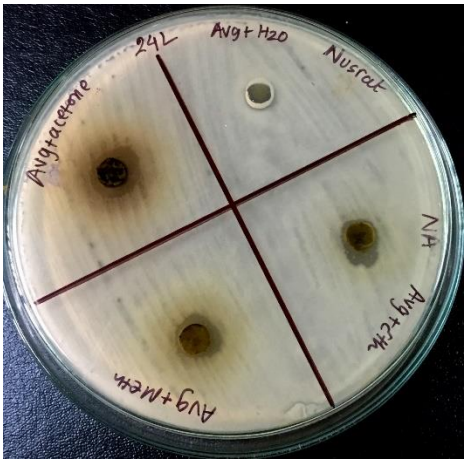


(b)

(c)

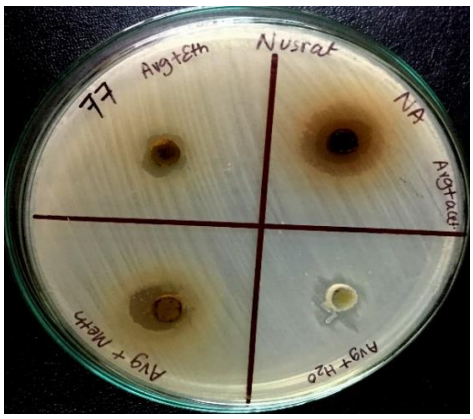


(d)

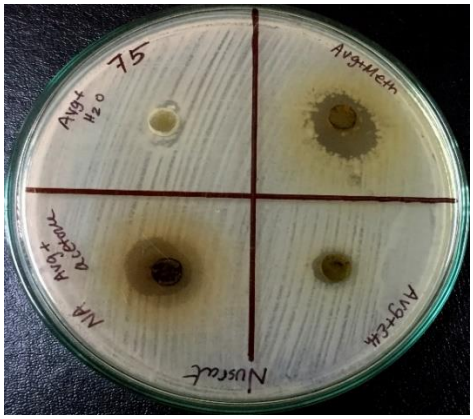


(e)

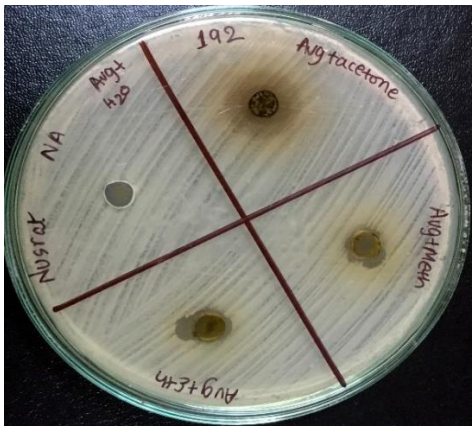
Figure 3.1: Antimicrobial effects of *Aloe vera* leaf ethanol, methanol, acetone and aqueous extracts against *Pseudomonas* isolates (a) P5; (b) P10; (c) P9; (d) P6; (e) P1



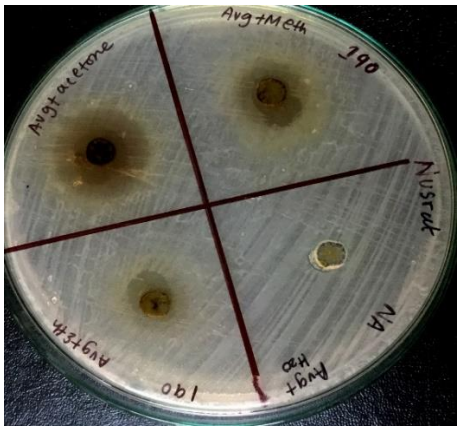
(a)



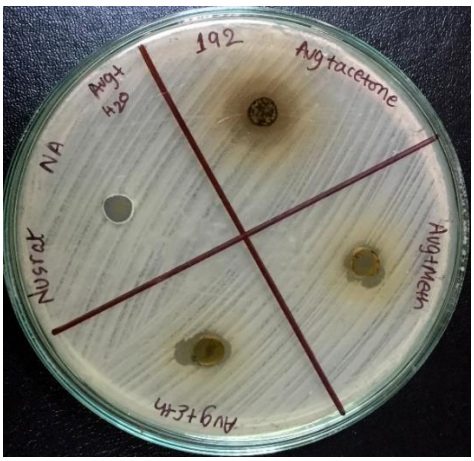
(b)



(c)



(d)



(e)

Figure 3.2: Antimicrobial effects of *Aloe vera* leaf ethanol, methanol, acetone and aqueous extracts against *Klebsiella* isolates (a) K10; (b) K4; (c) K6; (d) K1; (e) K9

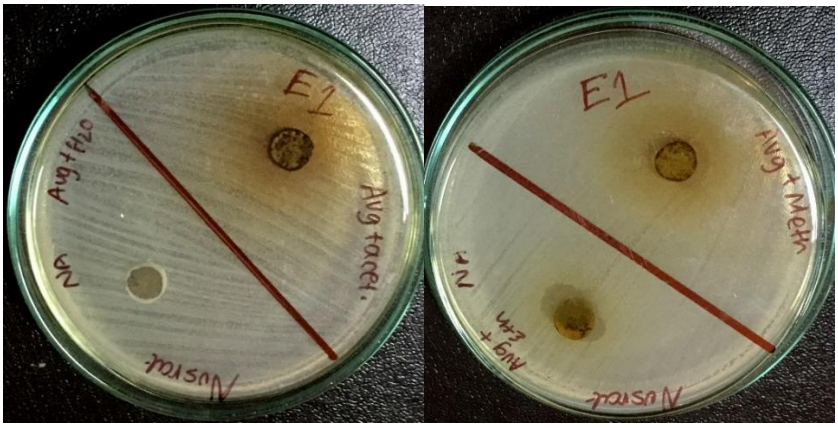


Figure 3.3: Antimicrobial effects of *Aloe vera* leaf ethanol, methanol, acetone and aqueous extracts against *E. coli*

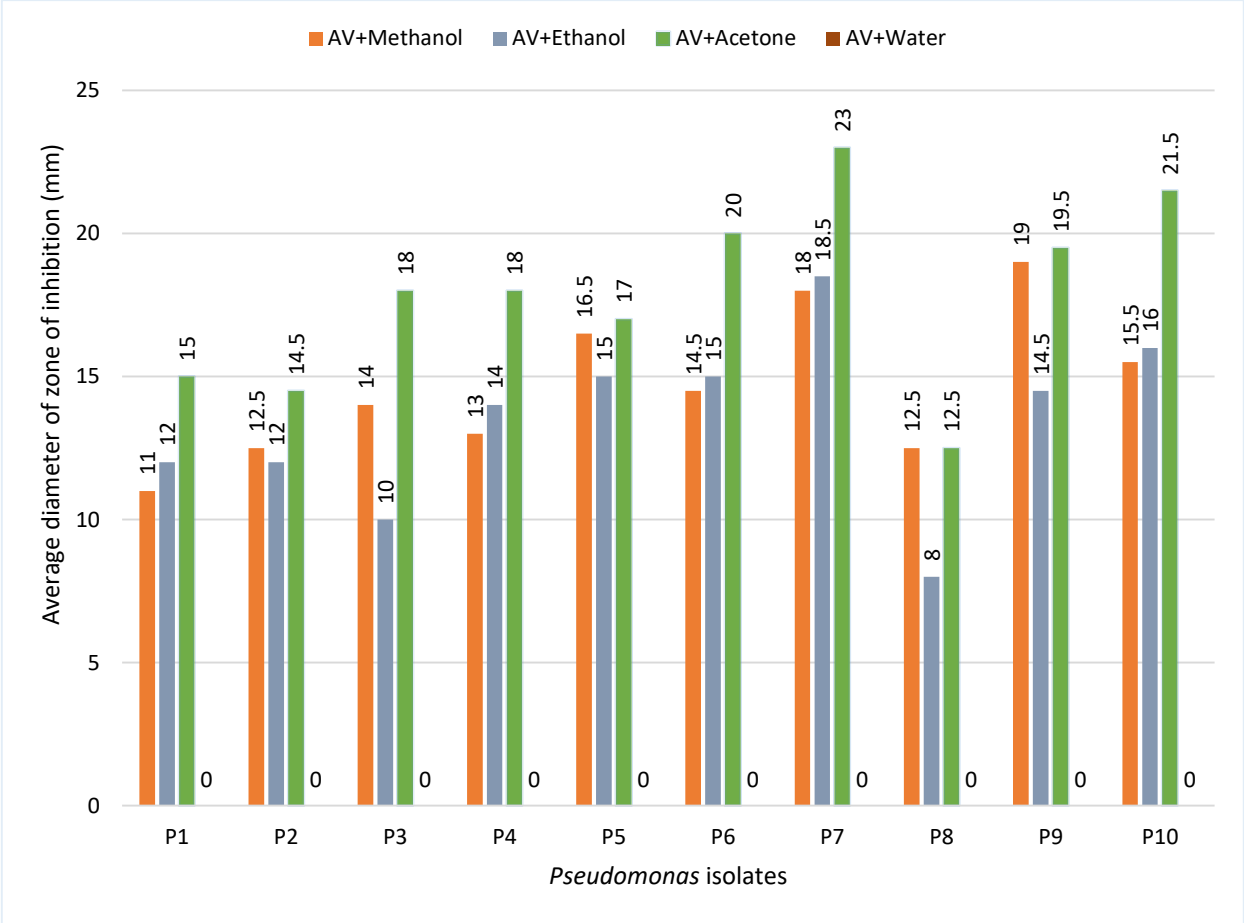


Figure 3.4: Antimicrobial activities of methanol, ethanol, acetone and aqueous extracts of *Aloe vera* leaf against *Pseudomonas* isolates

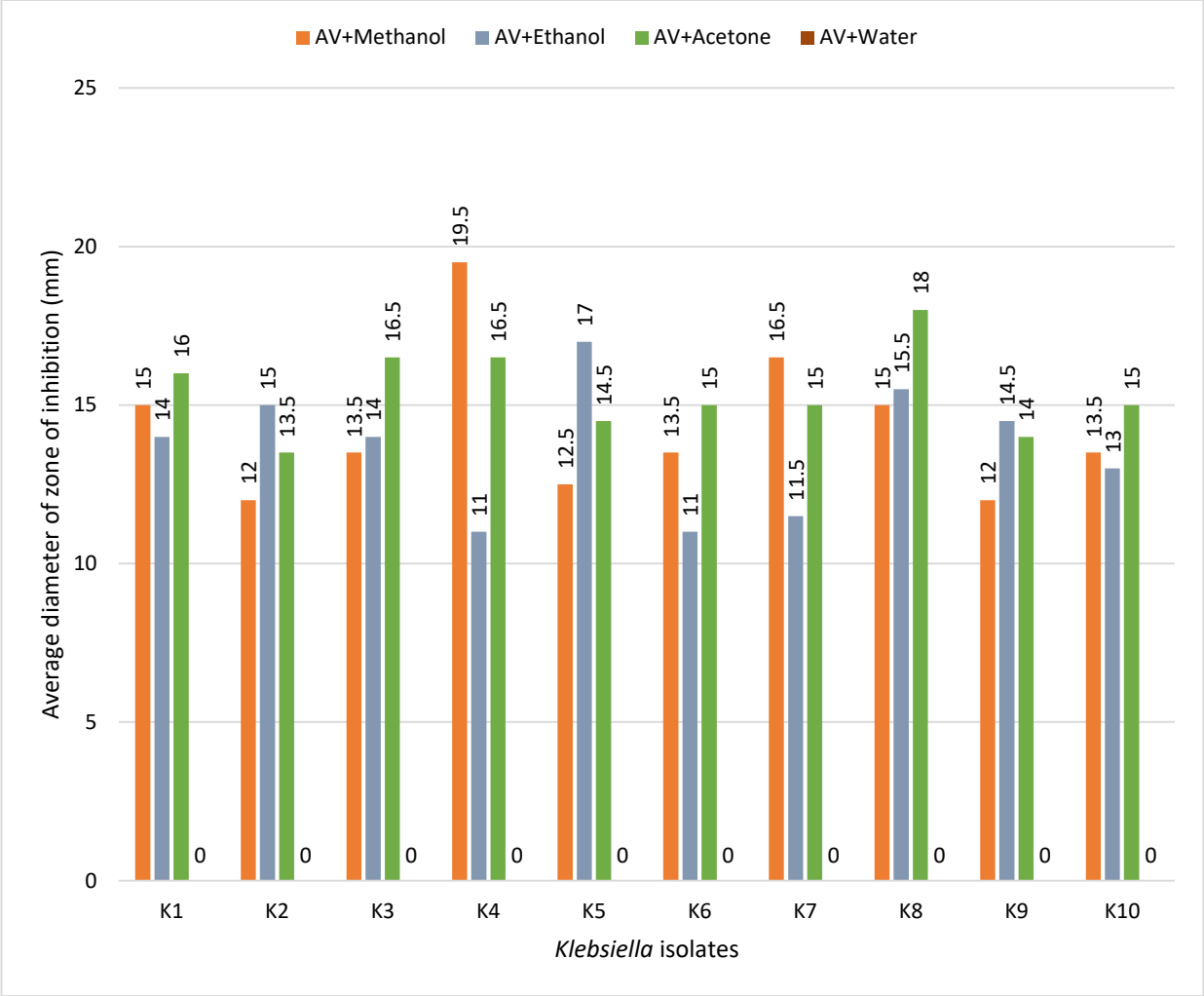


Figure 3.5: Antimicrobial activities of methanol, ethanol, acetone and aqueous extracts of *Aloe vera* leaf against *Klebsiella* isolates

Figures 3.4 and 3.5 above illustrate the antimicrobial activities exhibited by methanol, ethanol, acetone and aqueous extracts of *Aloe vera* leaf against *Pseudomonas* and *Klebsiella* isolates respectively. For *Pseudomonas*, the highest zone of inhibition was produced by acetone extract against sample P7, and for *Klebsiella* the highest inhibition zone appeared for methanol extract against sample K4. Methanol and acetone extracts were more effective than the ethanol extracts. Aqueous extracts showed no activity against the isolates.

3.1.2 Clove

In the present investigation clove extracts gave mixed results against *Pseudomonas* samples. Ethanol, methanol and acetone extracts showed positive results, but aqueous extracts were once again ineffective in killing off any bacteria, except for three samples. Small inhibition zones could be seen even for aqueous clove extracts for sample number 4, 7 and 8.

The results obtained from clove extracts were tabulated as follows:

Table 3.1.4: Antimicrobial effects (Inhibition zones) produced by methanol, ethanol, acetone and aqueous extracts of clove against *Pseudomonas* isolates

Pseudomonas sample number	Average zones of inhibition (mm)			
	Crude methanol extract	Crude ethanol extract	Crude acetone extract	Crude aqueous extract
P1	15	13.5	15	0
P2	17	16	20	0
P3	20	19	21	0
P4	16.5	15.5	14	12
P5	20	20	21.5	0
P6	18.5	19.5	20	0
P7	18.5	17	20	11
P8	17	17	15.5	12
P9	17.5	15.5	12.5	0
P10	15	14.5	20	0

Acetone extracts of clove had more potent bactericidal activity than ethanol and methanol extracts. Aqueous clove extracts showed no antimicrobial activity.

Table 3.1.5: Antimicrobial effects (Inhibition zones) produced by methanol, ethanol, acetone and aqueous extracts of clove against *Klebsiella* isolates

*Klebsiella spp.*sample number	Average zones of inhibition (mm)			
	Crude methanol extract	Crude ethanol extract	Crude acetone extract	Crude aqueous extract
K1	14.5	13.5	15	0
K2	14.5	14.5	14	0
K3	16	13	16.5	0
K4	14.5	15	16.5	0
K5	14	13.5	15	0
K6	14.5	15	16	0
K7	17.5	15	22.5	0
K8	16	16	16	0
K9	13.5	12.5	13.5	0
K10	15	19.5	16	0

Aqueous extract also did not show any activity against *E. coli* sample. However, clove was more effective against *E. coli* than *Aloe vera*.

Table 3.1.6: Antimicrobial effects (Inhibition zones) produced by methanol, ethanol, acetone and aqueous extracts of clove against *E. coli*

Sample	Methanol extract	Ethanol extract	Acetone extract	Aqueous extract
E1	16	15	16	0

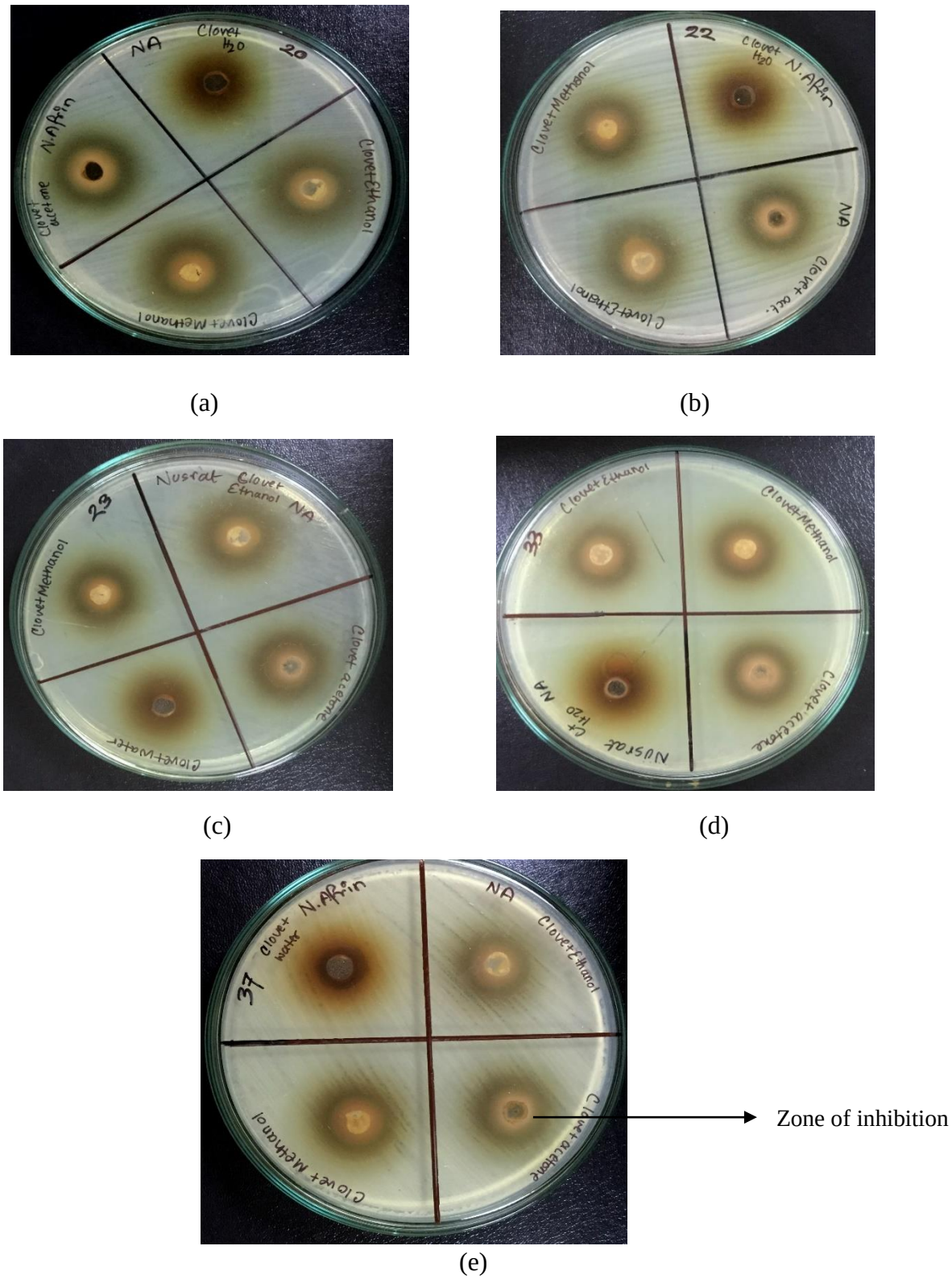
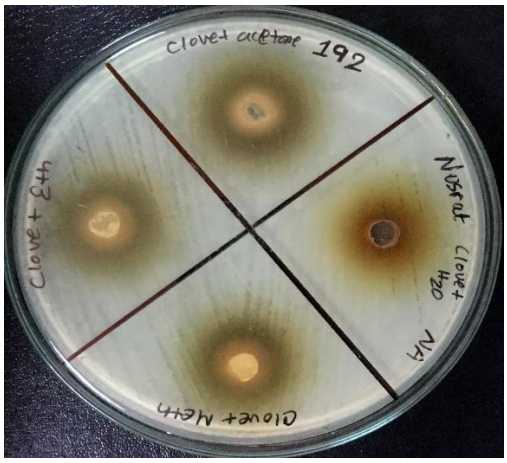
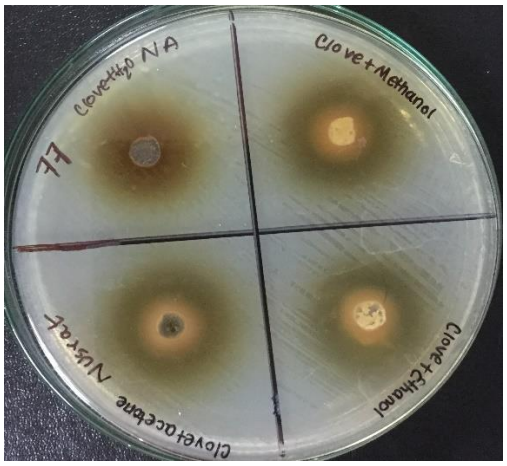


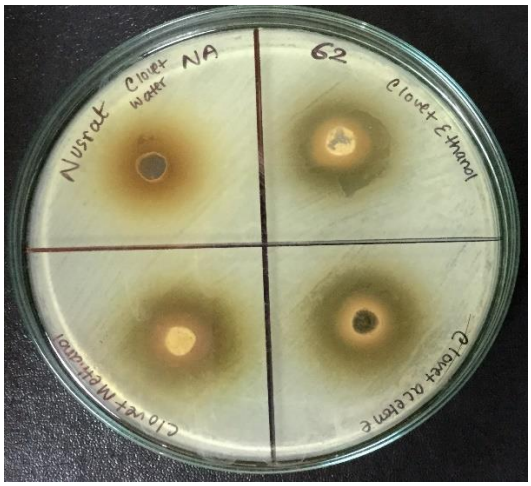
Figure 3.6: Antimicrobial effects of clove ethanol, methanol, acetone and aqueous extracts against *Pseudomonas* isolates (a) P7; (b) P2; (c) P3; (d) P6; (e) P4



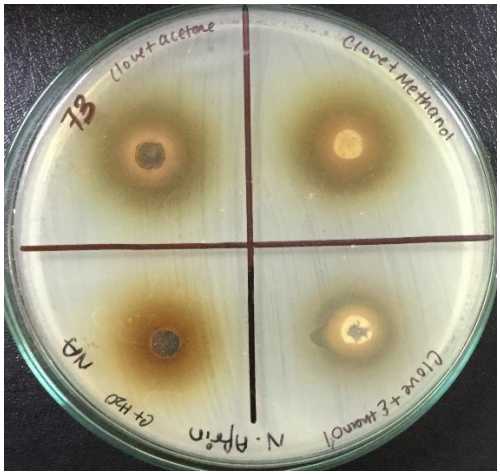
(a)



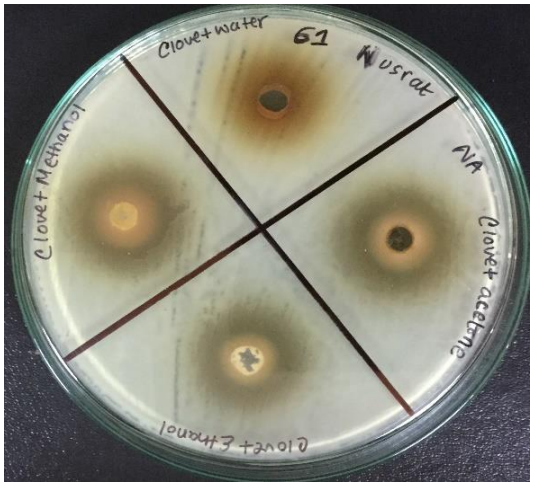
(b)



(c)



(d)



(e)

Figure 3.7: Antimicrobial effects of clove ethanol, methanol, acetone and aqueous extracts against *Klebsiella* isolates (a) K9; (b) K10; (c) K2; (d) K3; (e) K7

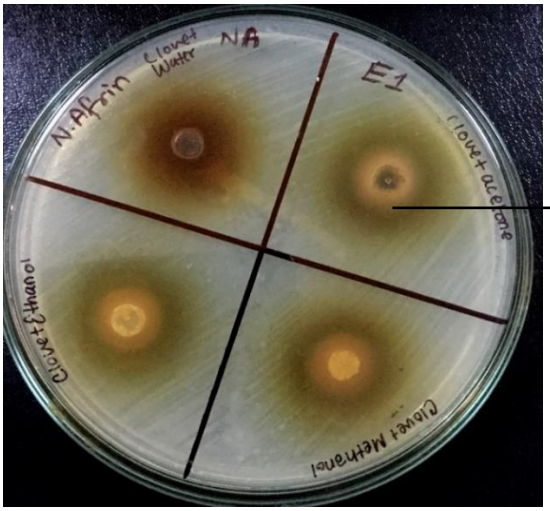


Figure 3.8: Antimicrobial effects of clove ethanol, methanol, acetone and aqueous extracts against *E. coli*

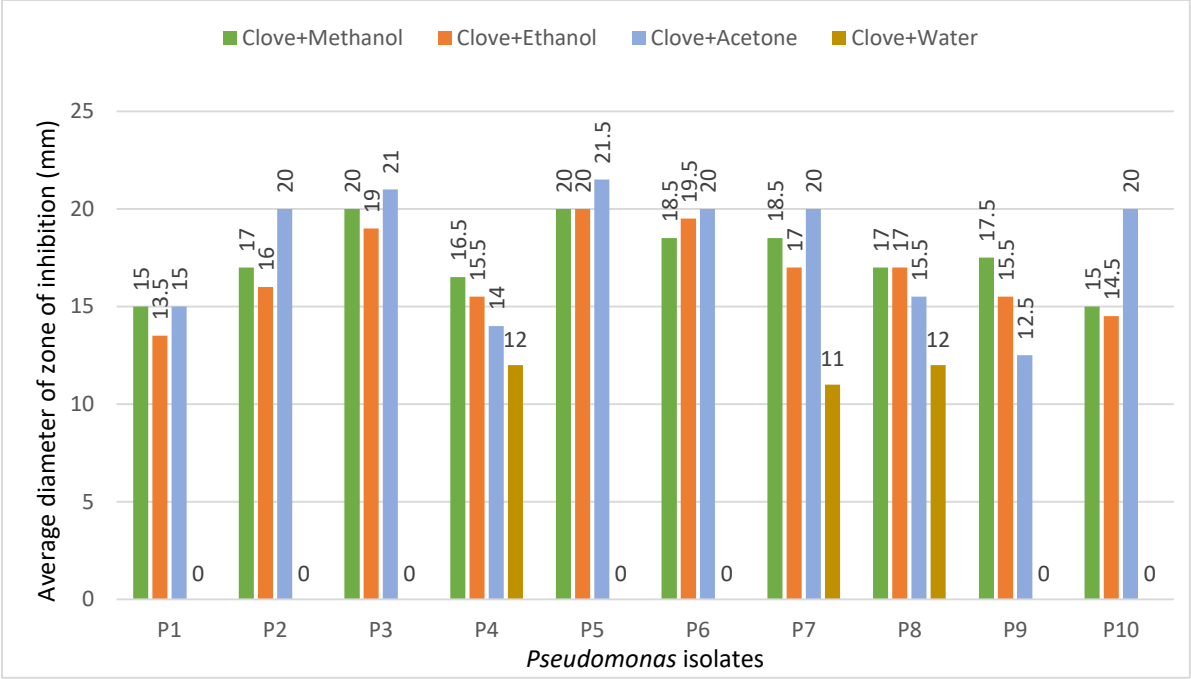


Figure 3.9: Antimicrobial activities of methanol, ethanol, acetone and aqueous extracts of clove against *Pseudomonas* isolates

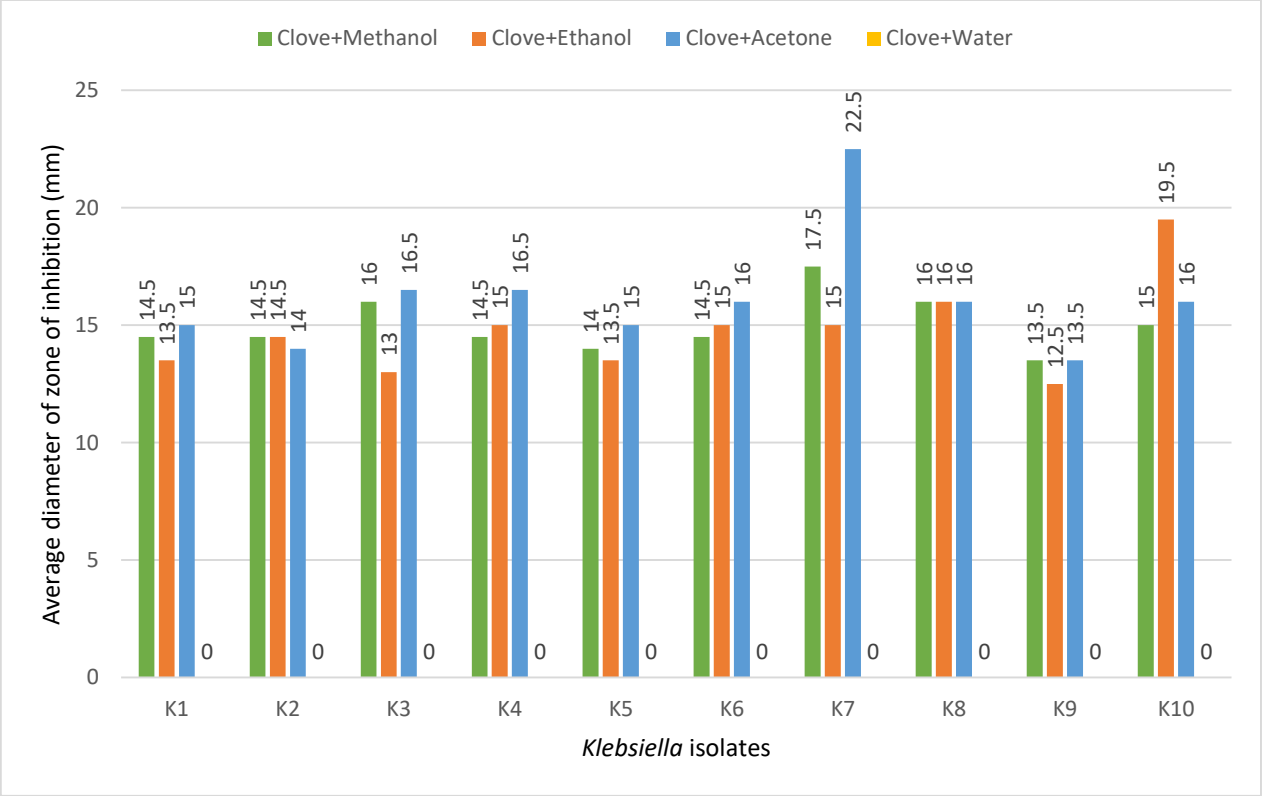


Figure 3.10: Antimicrobial activities of methanol, ethanol, acetone and aqueous extracts of clove against *Klebsiella* isolates

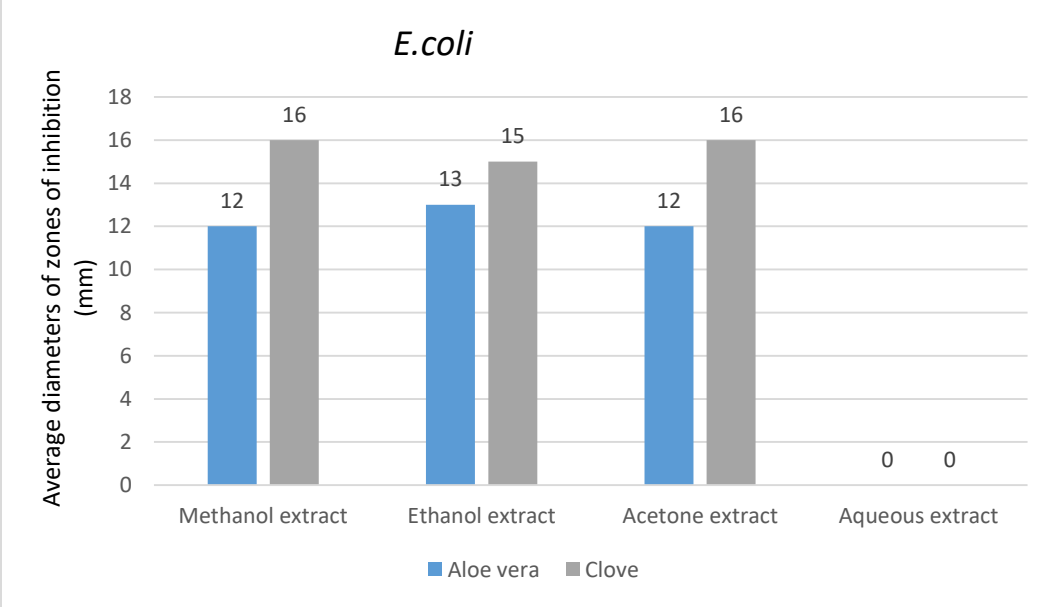


Figure 3.11: Antimicrobial activities of *Aloe vera* leaf and clove extracts against *E. coli*

Figures 3.9 and 3.10 above illustrate the antimicrobial activities exhibited by methanol, ethanol, acetone and aqueous extracts of clove against *Pseudomonas* and *Klebsiella* isolates. For both *Pseudomonas* and *Klebsiella,* the highest zones of inhibition were produced by acetone extracts. Ethanol and methanol extracts showed relatively similar activities.

Figure 3.11 shows antimicrobial activities of *Aloe vera* leaf and clove extracts against *E. coli.* Ethanol, methanol and acetone extracts of clove showed better antimicrobial activity than *Aloe vera* leaf extracts. Aqueous extracts did not possess any antimicrobial activity.

3.2 Commercial antibiotic sensitivity test results

In order to determine the antibiotic sensitivity pattern of the test organisms, ten different antibiotics were used. It was found that most organisms were resistant to multiple drugs. ***Pseudomonas* isolates:**

Table 3.2.1: Average zones of inhibition produced by commercial antibiotics and interpretation of clear zones against *Pseudomonas* isolates

	Average Zones of inhibition (mm) and interpretation of clear zones against *Pseudomonas* isolates									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
IMP	26	13	12	12	25	31.5	12	25	17.5	0
Interpretation	S	R	R	R	S	S	R	S	R	R
MEM	25	0	0	13	25	13	0	14	28	13.5
Interpretation	S	R	R	R	S	R	R	I	S	I
GEN	0	0	0	0	0	0	0	0	14	0
Interpretation	R	R	R	R	R	R	R	R	I	R
OX	0	0	0	0	0	0	0	0	0	0
Interpretation	R	R	R	R	R	R	R	R	R	R
OB	0	0	0	0	0	0	0	0	0	0
Interpretation	R	R	R	R	R	R	R	R	R	R
CAZ	0	0	0	0	0	0	0	0	20	0
Interpretation	R	R	R	R	R	R	R	R	S	R
AMP	0	0	0	0	0	0	0	0	0	0
Interpretation	R	R	R	R	R	R	R	R	R	R
AK	0	0	0	0	0	10	0	0	20	0
Interpretation	R	R	R	R	R	R	R	R	S	R
K	0	0	0	0	0	0	0	0	20	0
Interpretation	R	R	R	R	R	R	R	R	S	R
CIP	32	0	10	0	0	0	10	27	20	0
Interpretation	S	R	R	R	R	R	R	S	I	R

Key: S-Sensitive; R-Resistant; I-Intermediate

Note: IMP: Imipenem, MEM: Meropenem, GEN: Gentamicin, OX: Oxacillin, OB: Cloxacillin, CAZ: Ceftazidime, AMP: Ampicillin, AK: Amikacin, K: Kanamycin, CIP: Ciprofloxacin

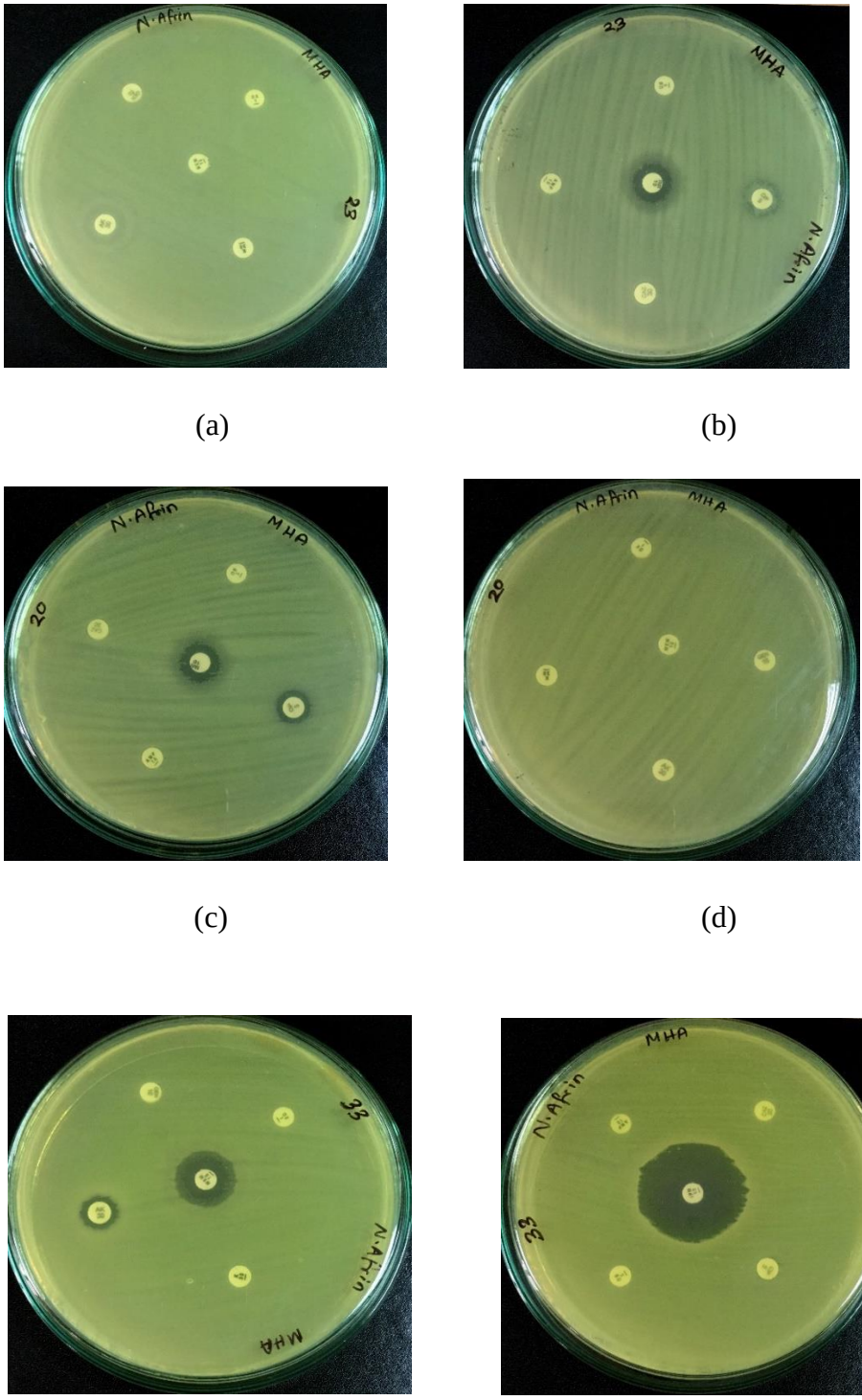


Figure 3.12: Inhibition zones produced by commercial antibiotics against *Pseudomonas* isolates (a), (b) P3; (c), (d) P7; (e), (f) P6

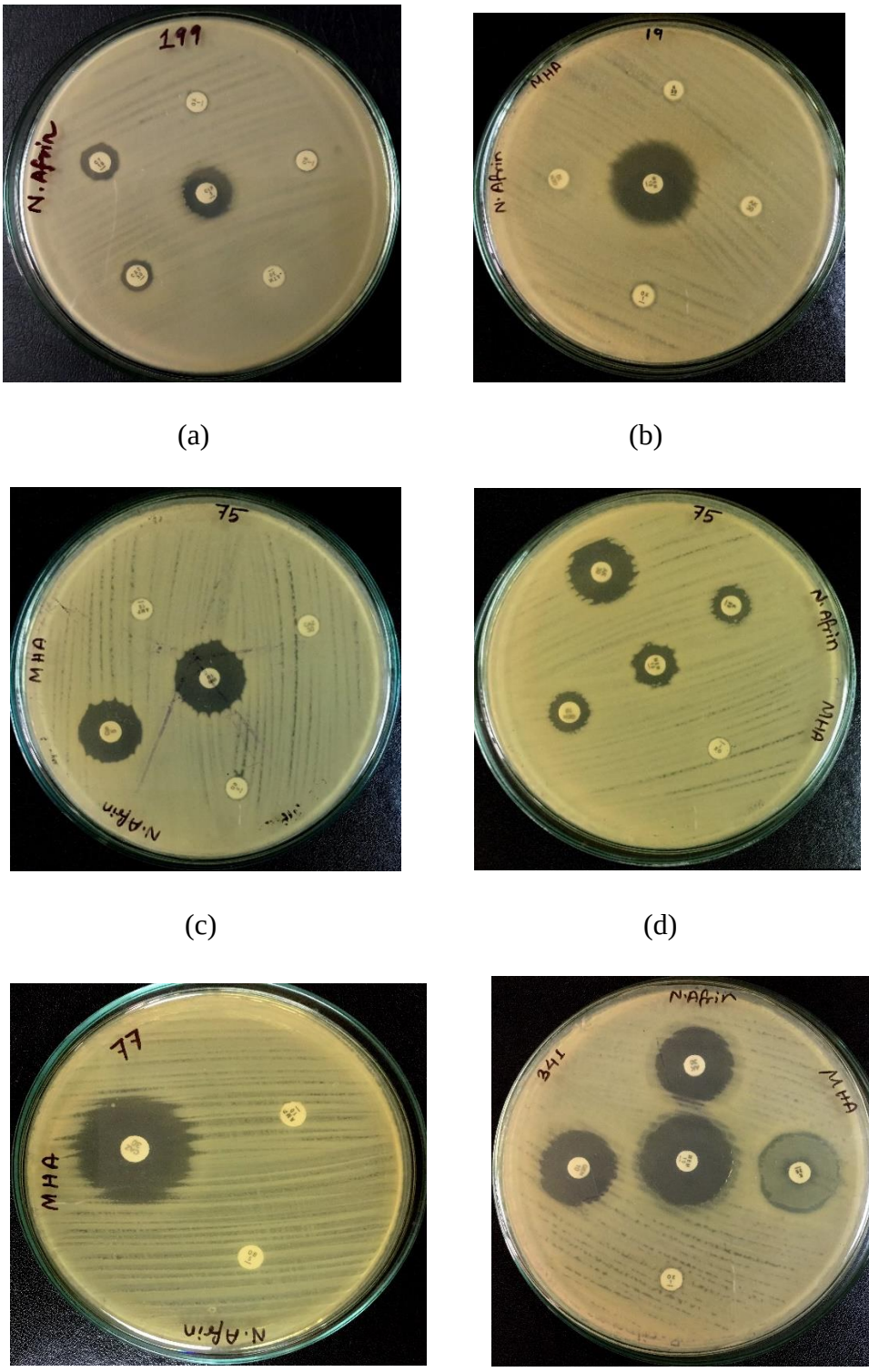
***Klebsiella* isolates:**

Table 3.2.2: Average zones of inhibition produced by commercial antibiotics and interpretation of clear zones against *Klebsiella* isolates

	Average Zones of inhibition (mm) and interpretation of clear zones against *Klebsiella* isolates									
	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10
IMP	17	25	31	20	25	26.5	25.5	31	22	23.5
Interpretation	S	S	S	S	S	S	S	S	S	S
MEM	17	26	28	13	26	24	27.5	30	20	24
Interpretation	S	S	S	S	S	S	S	S	S	S
GEN	21	15	17	11.5	23	0	16	18.5	0	0
Interpretation	S	S	S	R	S	R	S	S	R	R
OX	0	0	0	0	0	0	0	0	0	0
Interpretation	R	R	R	R	R	R	R	R	R	R
OB	0	0	0	0	0	0	0	0	0	0
Interpretation	R	R	R	R	R	R	R	R	R	R
CAZ	0	0	14	0	26	0	0	9.5	0	24.5
Interpretation	R	R	R	R	S	R	R	R	R	S
AMP	0	0	0	0	0	0	0	0	0	0
Interpretation	R	R	R	R	R	R	R	R	R	R
AK	15	19	16	20	24	0	15	21.5	20	22
Interpretation	I	S	I	S	S	R	I	S	S	S
K	0	18	15.5	11.5	23	0	11.5	21	13	19
Interpretation	R	S	I	R	S	R	R	S	R	S
CIP	12	25	27	18.5	30	26.5	16	15	0	33
Interpretation	R	S	S	I	S	S	I	I	R	S

Key: S-Sensitive; R-Resistant; I-Intermediate

Note: IMP: Imipenem, MEM: Meropenem, GEN: Gentamicin, OX: Oxacillin, OB: Cloxacillin, CAZ: Ceftazidime, AMP: Ampicillin, AK: Amikacin, K: Kanamycin, CIP: Ciprofloxacin



(a) (b)

(c) (d)

(e) (f)

Figure 3.13: Inhibition zones produced by commercial antibiotics against *Klebsiella* isolates (a) K8; (b) K6; (c), (d) K4; (e) K10; (f) K5

E. coli

Table 3.2.3: Average zones of inhibition produced by commercial antibiotics against *E. coli* (and interpretation of clear zones)

No.	Average zones of inhibition (mm)									
	IPM	**MEM**	**GEN**	**OX**	**OB**	**CAZ**	**AMP**	**AK**	**K**	**CIP**
E1	24	26	18	0	0	2	16	19	16.5	25.5
Interpretation	S	S	S	R	R	R	I	S	I	S

Key: S-Sensitive; R-Resistant; I-Intermediate

Note: IMP: Imipenem, MEM: Meropenem, GEN: Gentamicin, OX: Oxacillin, OB: Cloxacillin, CAZ: Ceftazidime, AMP: Ampicillin, AK: Amikacin, K: Kanamycin, CIP: Ciprofloxacin

3.3 Activity index of different extracts of *Aloe vera* and clove

Activity index (AI) values estimate the potential of antimicrobial activity of plant extracts by quantitatively comparing them to antibiotic standards (Nimmakayala et al., 2014). AI in this study was calculated against the two carbapenem antibiotics: Imipenem and Meropenem.

***Pseudomonas* spp.**

As shown in figure 3.14, the activity index of methanol, ethanol and acetone extracts of *Aloe vera* against imipenem was fairly high. The better activity index was obtained against imipenem (ranging from 0.42 to 1.92) compared to that of meropenem (ranging from 0.44 to 1.59). For some of the samples, however, the antibiotics had no activity. For such resistant samples no activity index could be calculated.

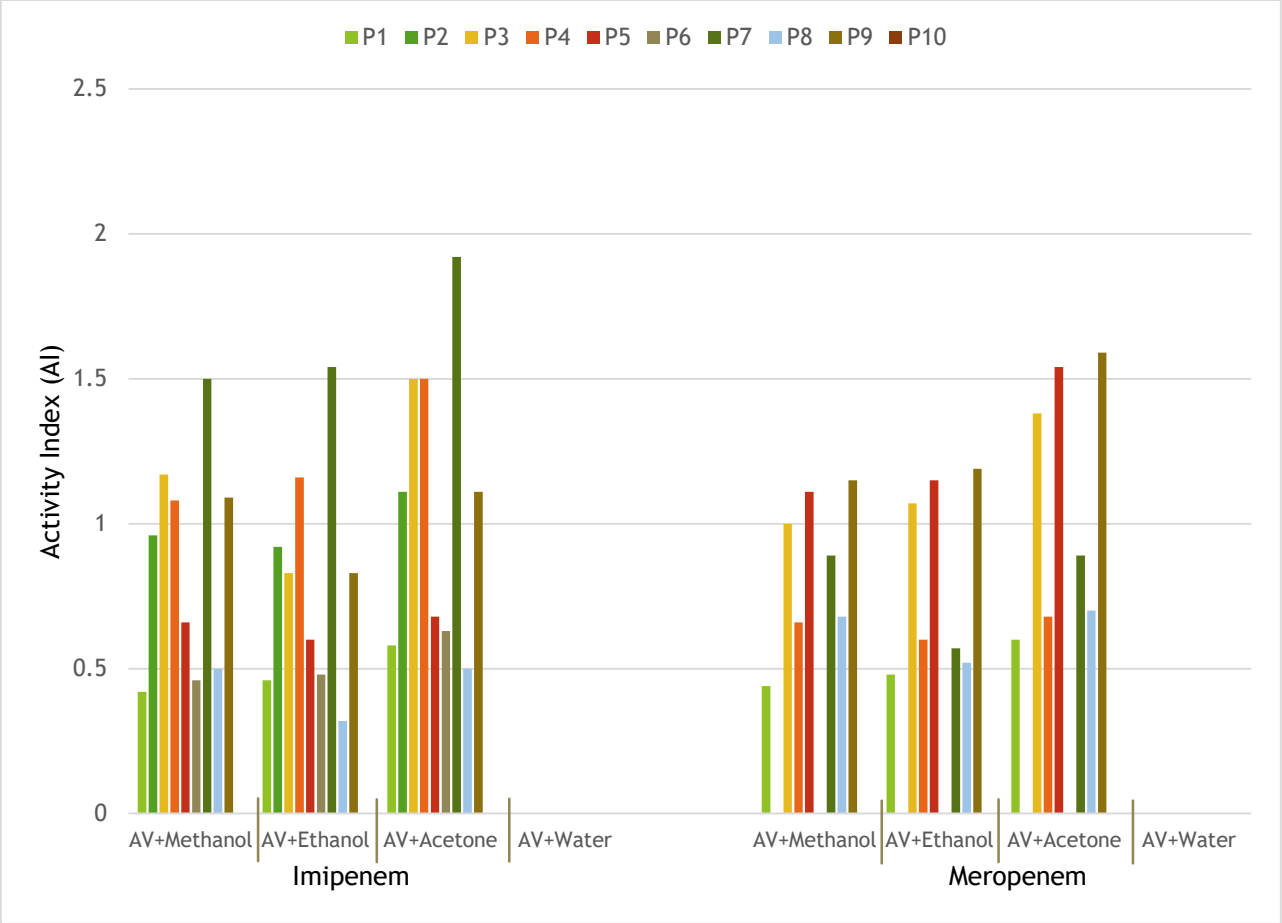


Figure 3.14: Activity Index of *Aloe vera* extracts to imipenem and meropenem against *Pseudomonas* isolates

For clove extracts, the AI compared to imipenem and meropenem was high, particularly against P3. Clove extracts had more potent antibacterial activity than *Aloe vera* extracts, as indicated by the AI values. The AI values for clove against imipenem ranged from 0.48 to 1.75, and against meropenem the range was 0.45 to 1.50, as represented by figure 3.15:

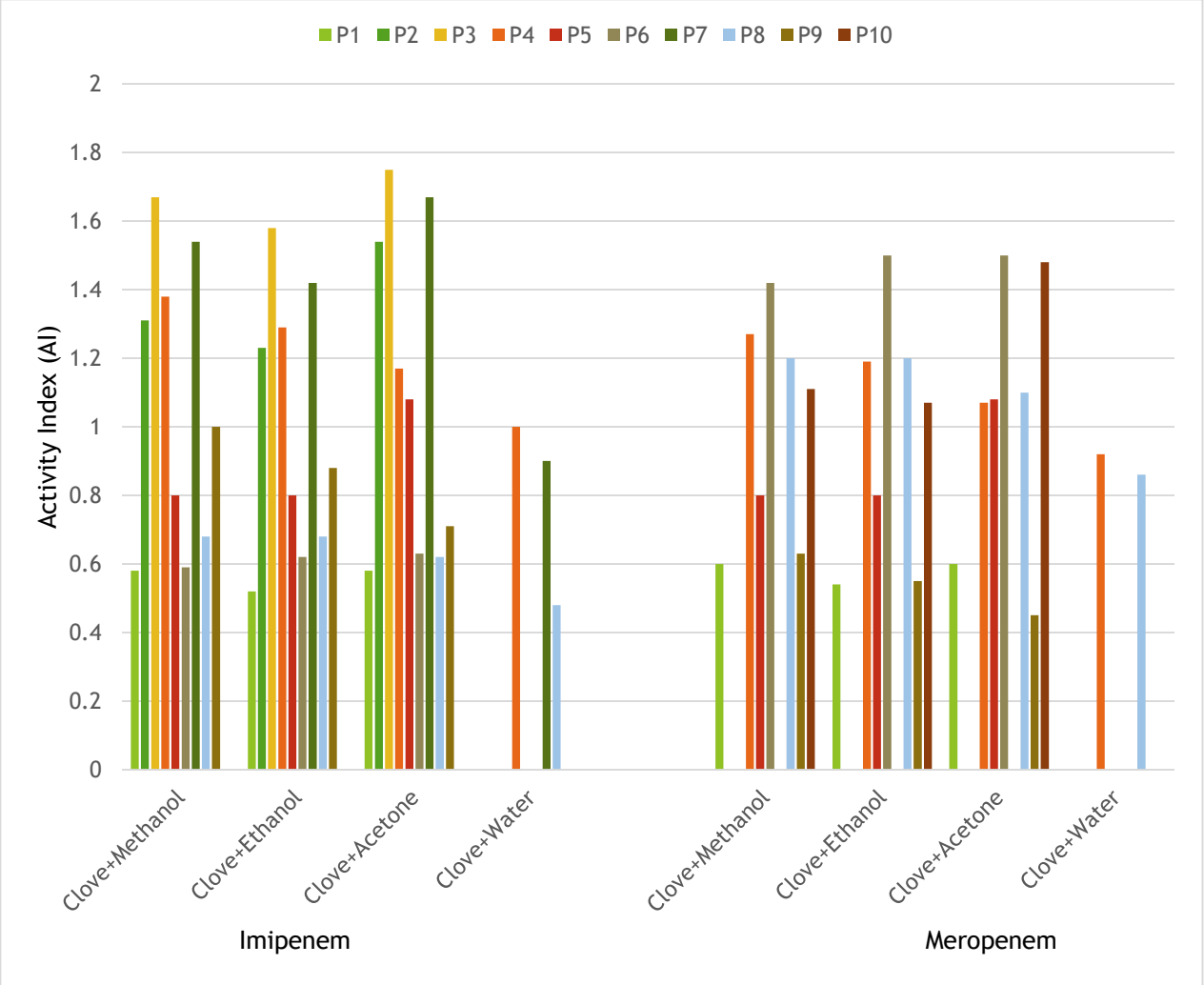


Figure 3.15: Activity Index of clove extracts to imipenem and meropenem against *Pseudomonas* isolates

***Klebsiella* spp.**

The AI values for *Aloe vera* extracts were higher for meropenem (ranging from 0.42 to 1.50) than that of imipenem (ranging from 0.42 to 0.98). The activity index against meropenem was highest for methanol and acetone extracts against K4, as shown in figure 3.16:

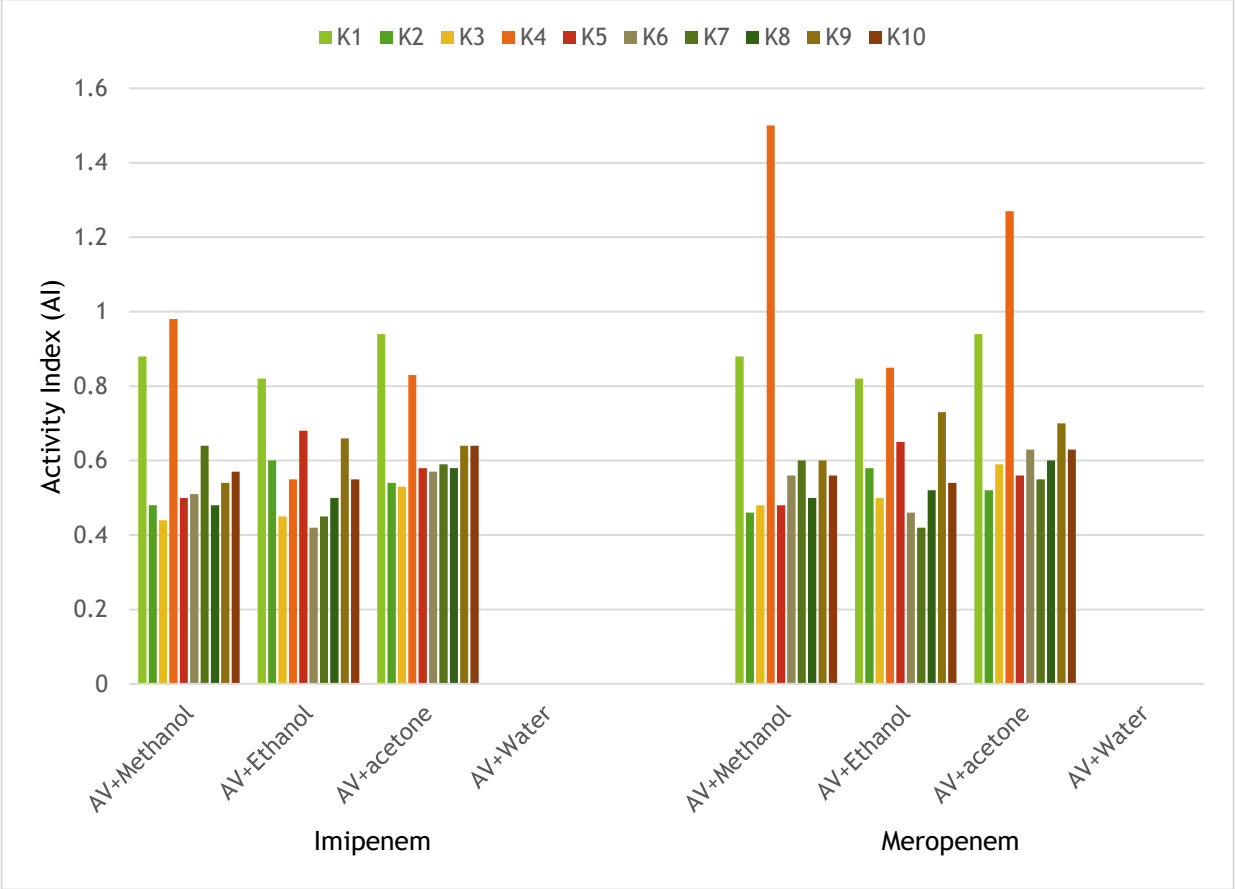


Figure 3.16: Activity Index of *Aloe vera* leaf extracts to imipenem and meropenem against *Klebsiella* isolates

As illustrated by figure 3.17, the AI values of clove extracts were better for meropenem (range: 0.46 to 1.27). The range for imipenem was comparatively lower (0.42 to 0.88). Acetone extracts of clove gave better results than ethanol and methanol extracts. The most significant values were obtained against sample number K4:

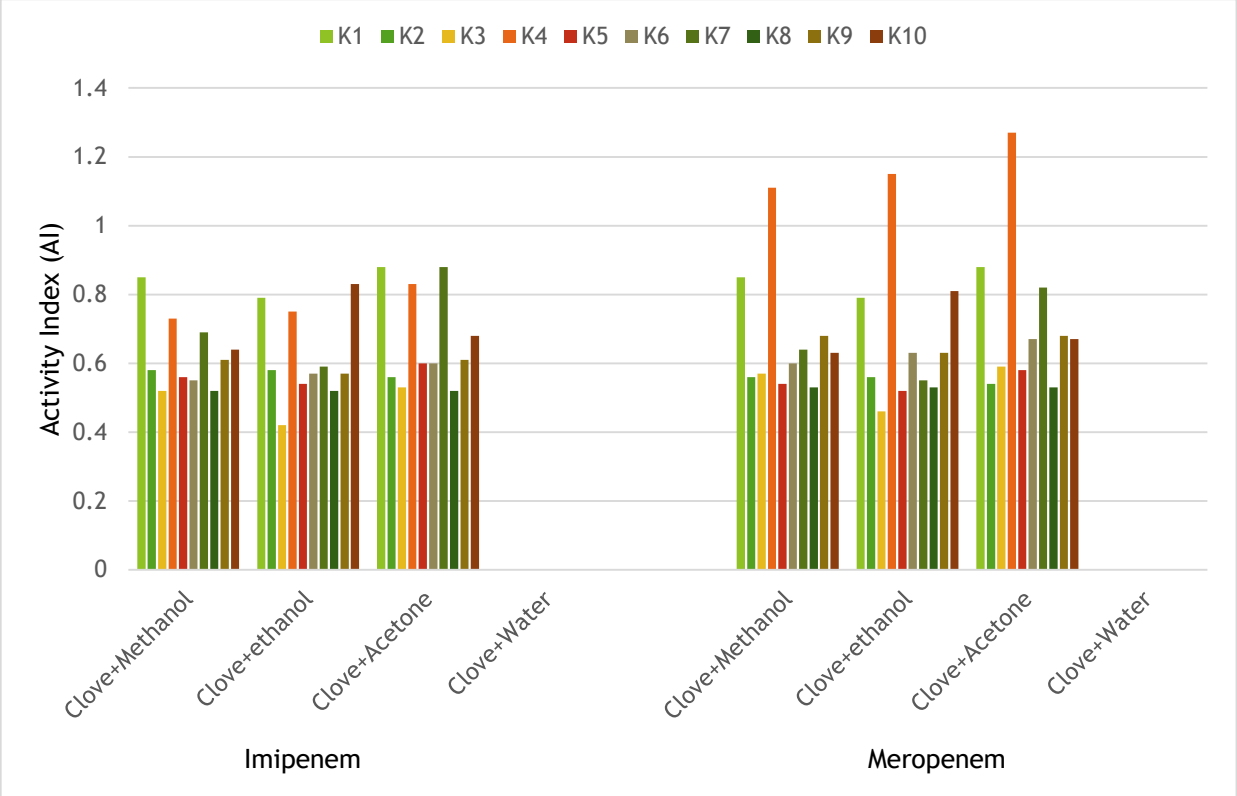


Figure 3.17: Activity Index of clove extracts to imipenem and meropenem against *Klebsiella* isolates

E. coli

AI values for clove extracts against *E.coli* were significantly higher than those for *Aloe vera*. This result was consistent for both imipenem and meropenem. Methanol and acetone extracts were more effective than the ethanol and aqueous ones. For imipenem, the AI values ranged from 0.50 to 0.67, and for meropenem it ranged from 0.50 to 0.62., as shown in figure 3.18 below.

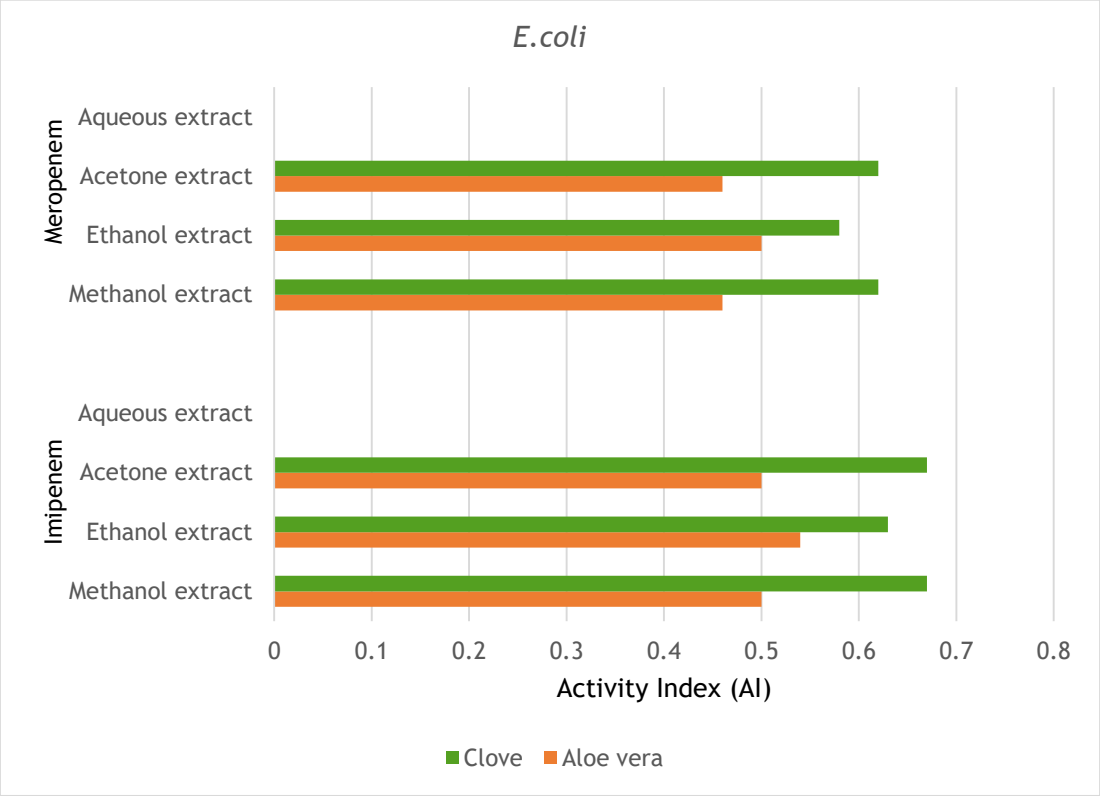


Figure 3.18: Activity Index of *Aloe vera* leaf and clove extracts against *E.coli* for imipenem and meropenem

3.4 Results of molecular analysis of multidrug resistant genes

DNA extraction:

Precipitated DNA appeared as white residue after the extraction process was complete:

Figure 3.19: Result of DNA extraction

Agarose gel electrophoresis

No clear DNA bands could be seen after gel electrophoresis. Only distinct bands of the ladder DNA was observed for both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-2.}}$ This result was obtained for samples P1-P6, K1-K6 and E1.

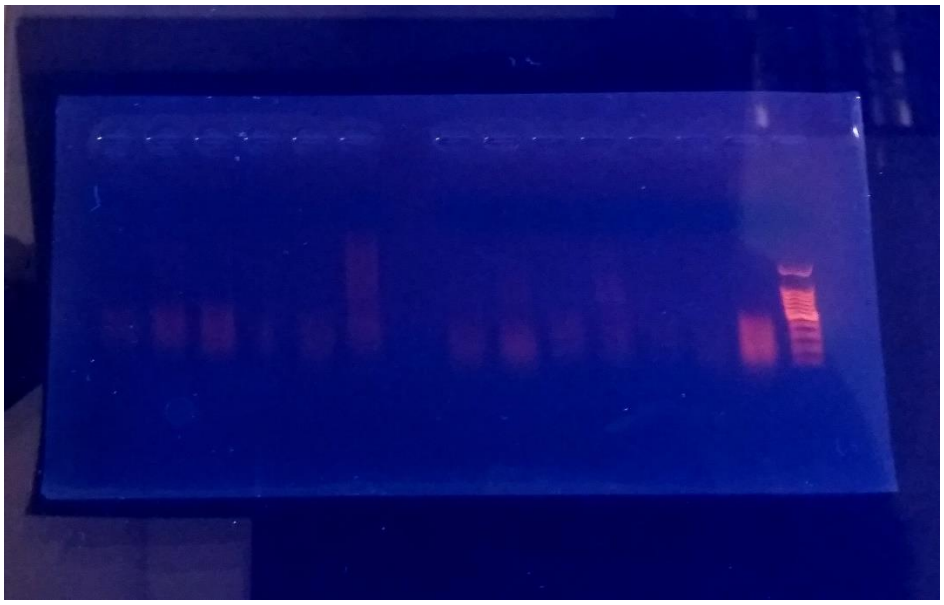


Figure 3.20: Result of agarose gel electrophoresis

Chapter 4: Discussion

Discussion

Methanol, ethanol and acetone extracts of *Aloe vera* leaf were effective against all the tested human pathogens. Firstly, against *Pseudomonas* isolates the most effective *Aloe vera* leaf extract was that of acetone. The largest inhibition zone was obtained for acetone extract against sample number P7 (23mm). Effectiveness of acetone extract was followed by that of methanol extract, with inhibition zones ranging from 11-19.5 mm in diameter. Compared to these, ethanol extract showed a little less effectiveness. Aqueous extract of *Aloe vera* leaf, however, was ineffective in killing any of the *Pseudomonas* isolates. These differences in the antimicrobial effects of various *Aloe vera* leaf extracts may be attributed to varying degrees of solubility of antimicrobial compounds found in *Aloe vera* in different solvents (Goudarzi et al., 2015). However, effect of acetone extract of *Aloe vera* leaf on *Klebsiella* isolates was not as pronounced as it was against *Pseudomonas* isolates. Methanol extract gave the highest zone of inhibition for *Klebsiella,* for sample number K4 (19.5 mm). Ethanol extract of *Aloe vera* leaf showed better activity against *Klebsiella* isolates than against *Pseudomonas* isolates, and once again, aqueous extract remained completely ineffective. Ethanol extract of *Aloe vera* leaf was more effective against *E. coli* than methanol or acetone extracts.

Aloe vera contains acemannan (acylated mannose) which forms a mucilaginous layer around the urinogenital, gastrointestinal and respiratory tracts when orally consumed. The layers trap microbial flora, making them unable to invade the system. *Aloe vera* also has anthraquinones as an active compound, which is a structural analogue of tetracycline. The anthraquinones act like tetracycline and inhibit bacterial protein synthesis by blocking the ribosomal A (where the aminoacylated t-RNA enters) site. The bacteria therefore cannot grow in the media containing *Aloe vera* extracts. Acemannan and anthraquinones both work together *in vivo,* while *in vitro* only anthraquinones are effective. Anthraquinones are soluble in alcohol, acetone, etc. but poorly soluble or insoluble in water. As a result, a significant result was observed against methanol, ethanol and acetone extracts, but not against that of aqueous extract. Some bacteria exhibit less sensitivity in *in viro* conditions, but if *Aloe vera* gel or whole leaf is consumed, then both

acemannan and anthraquinones will work simultaneously, producing more effective results (Pandey & Mishra, 2010).

For clove, it was observed that methanol, ethanol and acetone extracts were effective against all the test pathogens. Acetone extract was most effective against both *Pseudomonas* and *Klebsiella* isolates. This is in accordance with the findings of Shrivastava et al. It was reported that the acetone extract of *Syzygium aromaticum* showed the highest activity against *Pseudomonas* isolates. The result of the study showed that acetone extract of clove had more inhibitory effect than the other extracts, which tends to express that the active ingredients of clove may be better extracted with acetone than other solvents (Shrivastava et al., 2014).

In the present study, it was noticed that for clove, after acetone, methanol extract was most effective, followed closely by ethanol extract. An interesting finding was the effectiveness of aqueous extract of clove against *Pseudomonas* isolates. Clove extracts made using water showed inhibitory activity against three *Pseudomonas* isolates, P4, P7 and P8 with diameters of zone of inhibition being 12mm, 11mm and 12 mm respectively. Apart from this case, no other aqueous plant extract showed any inhibitory activity against any organism. All three (methanol, ethanol and acetone) extracts of clove showed higher degree of activity against *E. coli* than that of *Aloe vera* extracts. In fact, the overall effectiveness of clove extracts against the test organisms was higher than that of *Aloe vera* extracts.

An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Sikkema et al., 1994). The results of the present study does indeed suggest that plant extracts are effective against clinically relevant human pathogens.

The activity of ten different commercial antibiotics were tested against the 21 samples, and it was found that multidrug resistance is a common phenomenon among the organisms. Overall, 100% of the *Pseudomonas* isolates were resistant to oxacillin, cloxacillin and ampicillin; 90% were resistant to gentamicin, ceftazidime, amikacin and kanamycin, and only a very few number of samples were sensitive to imipenem, meropenem and ciprofloxacin. Antibiotic activity was better against *Klebsiella* isolates, as occurrence of antibiotic resistance was less frequent. Although 100% of the *Klebsiella* isolates were resistant to oxacillin, cloxacillin and ampicillin as

well, all of them were sensitive to imipenem and meropenem. Almost all the *Klebsiella* isolates were also resistant to ceftazidime, but sensitive to gentamicin, amikacin and ciprofloxacin. Sensitivity to kanamycin was comparatively less. The *E. coli* sample was sensitive to most of the antibiotics, with resistance only against oxacillin, cloxacillin and ceftazidime.

When comparing the bactericidal activity of antibiotics and plant extracts, the activity index values indicated that the plant extracts are often more effective against the test pathogens. The AI values were only calculated for imipenem and meropenem, as these two antibiotics were effective against most of the test organisms. While many commercial antibiotics were completely incapable of killing most bacteria, the plant extracts (except aqueous) showed positive activity against all the bacterial strains. However, it must be noted that for antibiotics that were effective against the test pathogens, the AI values obtained were very low. This is suggestive of the fact that antibiotics can be more effective than natural plant extracts unless the organisms develop resistance to them. An explanation for this could be that plant extracts and antibiotics follow different mechanisms in order to inhibit bacterial growth.

However, in this investigation, only crude extracts of the plants were used for antimicrobial assay. The concentrations of extracts can be altered, or different extraction methods can be employed to yield different results. Investigation of plant materials for identification of novel antimicrobial agents is necessary in order to combat the increasing incidences of microbial drug resistance.

The molecular analysis of multidrug resistant genes for the test bacteria gave negative results for both $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-2}}$ genes. No distinct bands were observed for either of the genes. Both NDM-1 and OXA-2 are metallo beta-lactamase enzymes conferring bacterial resistance to beta-lactam antibiotics. Bacteria with NDM-1 carbapenemases are highly resistant to many antibiotic classes and can potentially mark the end of treatment with beta-lactams, fluoroquinolones and aminoglycosides-the main antibiotic classes for the treatment of Gram-negative infections (Kumarasamy et al., 2010). There are several reports of NDM-1 producing isolates identified in Bangladesh. The negative results in this study might have one or several explanations.

Production of metallo-β-lactamases (MBLs) can be either chromosomally encoded or plasmid mediated (Lauretti et al., 1999). In this study, however, only chromosomal DNA was analysed

for the presence of NDM-1 gene. It has been suggested that NDM-1 producing isolates often acquire a wide spectrum of uncommon and unrelated resistance genes. The multidrug resistance phenotype, therefore, is not likely to be the result of a single genetic event and is probably selected by the frequent use of various broad-spectrum antibiotics (Islam et al., 2012). *bla*$_{\text{NDM-1}}$ has been reported to be located on broad host-range plasmids. Transmissible plasmids containing *bla*$_{\text{NDM-1}}$ were detected in the majority of samples in a study conducted by Islam et al. In another study, it has been described that the dissemination of *bla*$_{\text{NDM-1}}$ is likely to be linked to a transposon (Tn*125*) and is not plasmid-mediated (Poirel et al., 2012). An analysis of both chromosomal and plasmid DNA samples would have been a better approach for this study. Also, the sample number in the present study was fairly small. A broader spectrum of samples might have provided different results.

In the PCR-based detection of OXA-2 gene in this study, only a single primer set was used. A better process for detection of such genes include PCR-based detection tests followed by analysis of the PCR products by sequencing or restriction with endonucleases chosen to detect restriction site changes generated by point mutations. The main difficulty in applying PCR-RFLP to the characterization of oxacillinases is that they constitute a heterogeneous group of enzymes, including subgroups with large evolutionary distances between them. Therefore, in contrast to other enzymes like TEM or SHV, all oxacillinases cannot be detected using a single primer pair (Bert et al., 2002).

Thus, the absence of positive result in the molecular analysis part of this study can be attributed to the occurrence of multidrug resistant genes in both chromosomal and plasmid DNA. The phenotype of the samples, being extensively drug resistant, might have been coded for by NDM-1 or OXA-2 genes present in the plasmids, but the screening was done using chromosomal DNA only. Also, for OXA, only a single primer set was used. Resistance to oxacillin, cloxacillin and other drugs might have been mediated by a different oxacillinase enzyme coded for by a different gene.

Conclusion:

Organic solvent extracts of *Aloe vera* leaf and clove were effective against *Pseudomonas, Klebsiella* and *E. coli* isolates. Acetone and methanol were the most effective solvents for this

procedure, and aqueous extracts of the two plants possessed little to no antimicrobial activity. Overall, clove extracts were comparatively more potent antimicrobial agents than *Aloe vera* leaf extracts, and a greater susceptibility was noticed among *Klebsiella* isolates than the *Pseudomonas* isolates.

On comparing the results with commercial antibiotics, it was found that most of the test samples were highly resistant to almost all the antibiotics. Plant extracts inhibited bacterial growth more successfully than the commercial antibiotics. NDM-1 and OXA-2 β-lactamase genes, however, were not present in the multidrug resistant bacterial samples analysed during this study.

The results of this investigation lend credence to the folkloric use of certain plants in treating microbial infection and show that *Aloe vera* and clove could be exploited for new potent antimicrobial agents. Extraction of phytochemicals using organic solvents can be a suitable answer to the growing problem of microbial resistance to synthetic antibiotics. It can be hoped that studies like this will contribute to the establishment of compounds that could be used to formulate antimicrobial drugs of natural origin. However, further research is needed for process standardisation and optimisation. Research is also needed to identify, isolate and purify the exact bioactive components that are responsible for such antimicrobial properties of these plants.

As a more pressing problem, the antibiotic resistance scenario must be addressed more frequently. Intensive and indiscriminate use of antibiotics must be avoided at all costs. Regular detection and epidemiological monitoring of both low- and high-risk populations is important for keeping the metallo beta-lactamase genes in check.

References

Agarry, O. O., Olaleye, M. T., & Bello-Michael, C. O. (2005). Comparative antimicrobial activities of *Aloe vera* gel and leaf. *Afr. J. Biotechnol*, *4*(12), 1413–1414. https://doi.org/10.5897/AJB2005.000-3180

Alemdar, S. & Agaoglu, S. (2009). Investigation of *In vitro* Antimicrobial activity of *Aloe vera* Juice. *Journal of Animal and Veterinary Advances*, 8(1), 99-102.

Ambler, R. P. (1980). The structure of beta-lactamases. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *289*(1036), 321–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6109327

Arunkumar, S., & Muthuselvam, M. (2009). Analysis of phytochemical constituents and antimicrobial activities of *Aloe vera* L. against clinical pathogens. *World Journal of Agricultural Sciences*, *5*(5), 572–576. Retrieved from http://www.idosi.org/wjas/wjas5(5)/9.pdf

Bae, E. A., Han, M. J., Kim, N. J., & Kim, D. H. (1998). Anti-Helicobacter pylori activity of herbal medicines. *Biological & Pharmaceutical Bulletin*, *21*(9), 990–2. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9781854

Baiden, F., Owusu-Agyei, S., Webster, J., & Chandramohan, D. (2010). The need for new antibiotics. *The Lancet*, *375*(9715), 637–638. https://doi.org/10.1016/S0140-6736(10)60265-6

Beg, A. Z., & Ahmad, I. (2002). In vitro fungitoxicity of the essential oil of Syzygium aromaticum. *World Journal of Microbiology and Biotechnology*, *18*(4), 317–319. https://doi.org/10.1023/A:1015242901360

Bergamante, V., Ceschel, G.C., Marazzita, S., Ronchi, C. & Fini, A. (2007). *Drug Delivery*, 14, 427-432. Doi: 10.1080/10717540701202960.

Bert, F., Branger, C., & Lambert-Zechovsky, N. (2002). Identification of PSE and OXA beta-lactamase genes in Pseudomonas aeruginosa using PCR-restriction fragment length polymorphism. *The Journal of Antimicrobial Chemotherapy*, *50*(1), 11–8. https://doi.org/10.1093/jac/dkf069

Borrelli, F. & Izzo, A.A. (2000). The Plant Kingdom as a Source of Anti-Ulcer Remedies. *Phytotherapy Research,* 14, 581-591.

Bulduk, S. (2004). *Food Technology.* Ankara, Turkey: Detay Publishing.

Blumenthal, M. (1998). *The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines* (pp. 68). Austin: American Botanical Council.

Bush, K., & Jacoby, G. A. (2010). Updated Functional Classification of beta-lactamases. *Antimicrobial Agents and Chemotherapy*, *54*(3), 969–976. https://doi.org/10.1128/AAC.01009-09

Bush, K., Jacoby, G. A., & Medeiros, A. A. (1995). A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy*, *39*(6), 1211–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7574506

Byeon, S. W., Pelley, R. P., Ullrich, S. E., Waller, T. A., Bucana, C. D., & Strickland, F. M. (1998). Aloe barbadensis extracts reduce the production of interleukin-10 after exposure to ultraviolet radiation. *The Journal of Investigative Dermatology*, *110*(5), 811–7. https://doi.org/10.1046/j.1523-1747.1998.00181.x

Cai, L., & Wu, C. D. (1996). Compounds from *Syzygium aromaticum* Possessing Growth Inhibitory Activity Against Oral Pathogens. *Journal of Natural Products*, *59*(10), 987–990. https://doi.org/10.1021/np960451q

Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nakbi, A. Ben, Rouabhia, M., Mahdouani, K., & Bakhrouf, A. (2007). The chemical composition and biological activity of clove essential oil,Eugenia caryophyllata (Syzigium aromaticum L. Myrtaceae): a short review. *Phytotherapy Research*, *21*(6), 501–506. https://doi.org/10.1002/ptr.2124

Choi, S., Kim, KW., Choi, JS., Han, SK., Park, YI., Lee, SK., Kim, JS. & Chung, MS. (2002).

Angiogenic activity of beta-sitosterol in the ischaemia/reperfusion damaged brain of Mongolian gerbil. *Planta Medica*, 68(4), 330-335.

Cornaglia, G. (2009). Fighting infections due to multidrug-resistant Gram-positive pathogens. *Clinical Microbiology and Infection*, *15*(3), 209–211. https://doi.org/10.1111/j.1469-0691.2009.02737.x

Crew, J.E. (1939). Aloes in the treatment of Burns and Scalds. *Minnesota Medicine,* 22, 538-539.

Dahiya, P. & Purkayastha, S. (2012). Phytochemical Screening and Antimicrobial Activity of Some Medicinal Plants against Multi-drug Resistant Bacteria from Clinical Isolates. *Indian Journal of Pharmaceutical Sciences,* 74(5), 443-450.

Devi, D. L., Srinivas, B., & Rao, B. N. (2012). An evaluation Antimicrobial Activity of Aloe barbadensis Miller (*Aloe vera*) Gel Extract . Abstract : Introduction : Materials and Methods : Discussion :, *21*(21).

Duke, J. *New Crops* (pp. 664-669). Janick, J. & Simon, J.E., (Ed.). New York: Wiley

EL Solh, A.A. & Alhajhusain, A. (2009). Update on the treatment of *Pseudomonas aeruginosa* pneumonia. *Journal of Antimicrobial Chemotherapy,* 64(2), 229-238.

Eshun, K. & He, Q. (2004). *Aloe Vera,* a valuable ingredient for the food, pharmaceutical and cosmetic industries. *Herbs for Health magazine*, 44(2), 91-96.

Essawi, T. & Srour, M. (2000). Screening of some Palestinian medicinal plants for antibacterial activity. *Journal of Ethnopharmacology,* 70(3), 343-349.

Fabricant, D. S., & Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives*, *109*(SUPPL. 1), 69–75. https://doi.org/10.1289/ehp.01109s169

Farzana, R., Shamsuzzaman, S. M., & Mamun, K. Z. (2013). Emerging problems in infectious diseases isolation and molecular characterization of New Delhi metallo-beta- lactamase-1 producing superbug in Bangladesh. *J Infect Dev Ctries*, *7*(3), 161–168. https://doi.org/10.3855/jidc.2493

Finch, R., & Hunter, P. A. (2006). Antibiotic resistance - Action to promote new technologies:

Report of an EU Intergovernmental Conference held in Birmingham, UK, 12-13 December 2005. *Journal of Antimicrobial Chemotherapy*, *58*(SUPPL. 1), 12–13. https://doi.org/10.1093/jac/dkl373

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S., & Efferth, T. (2007). Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytotherapy Research*, *21*(10), 989–994. https://doi.org/10.1002/ptr.2179

Gibbons, S. (2004). Anti-staphylococcal plant natural products. *Natural Product Reports*, *21*(2), 263–277. https://doi.org/10.1039/b212695h

Gong, M., Wang, F. & Chen, Y. (2002). Study on application of arbuscular-mycorrhizas in growing seedlings of Aloe Vera (in Chinese). *Journal of Chinese Medical Materials,* 25(1), 1-3.

Goudarzi, M., Fazeli, M., Azad, M., Seyedjavadi, S. S., & Mousavi, R. (2015). *Aloe vera* Gel: Effective therapeutic agent against multidrug-resistant pseudomonas aeruginosa isolates recovered from burn wound infections. *Chemotherapy Research and Practice*, *2015*, no pagination. Retrieved from http://www.hindawi.com/journals/chrp/%5Cnhttp://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed13&NEWS=N&AN=2015249778

Green, D. W. (2002). The bacterial cell wall as a source of antibacterial targets. *Expert Opinion on Therapeutic Targets*, *6*(1), 1–20. https://doi.org/10.1517/14728222.6.1.1

Habeeb, F., Shakir, E., Bradbury, F., Cameron, P., Taravati, M. R., Drummond, A. J., … Ferro, V. A. (2007). Screening methods used to determine the anti-microbial properties of *Aloe vera* inner gel. *Methods*, *42*(4), 315–320. https://doi.org/10.1016/j.ymeth.2007.03.004

Hancock, R. E. (1998). Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *27 Suppl 1*, S93-9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9710677

Hawkey, P. M. (2008). Prevalence and clonality of extended-spectrum β-lactamases in Asia. *Clinical Microbiology and Infection*, *14*, 159–165. https://doi.org/10.1111/j.1469-

0691.2007.01855.x

He, F. (2011). *E. coli* Genomic DNA Extraction. *Bio-protocol* Bio101: e97. http://www.bio-protocol.org/e97

Hooper, D. C. (2000). Mechanisms of Action and Resistance of Older and Newer Fluoroquinolones. *Clinical Infectious Diseases*, *31*(Supplement 2), S24–S28. https://doi.org/10.1086/314056

Integrated Taxonomic Information System. Retrieved from: https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=182653#null

Integrated Taxonomic Information System. Retrieved from: https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=506167#null

Irshad, S., Butt, M., & Younus, H. (2011). In-Vitro antibacterial activity of Aloe Barbadensis Miller (*Aloe vera*). *International Research J. of …*, *1*(2), 59–64. Retrieved from http://www.londoncss.co.uk/scientific-journals.co.uk/web_documents/1010209_aloe_barbadensis.pdf

Ishii, Y., Tanizawa, H., & Takino, Y. (1994). Studies of aloe. V. Mechanism of cathartic effect. (4). *Biological & Pharmaceutical Bulletin*, *17*(5), 651–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7920425

Islam, M. A., Talukdar, P. K., Hoque, A., Huq, M., Nabi, A., Ahmed, D., … Endtz, H. P. (2012). Emergence of multidrug-resistant NDM-1-producing Gram-negative bacteria in Bangladesh. *European Journal of Clinical Microbiology and Infectious Diseases*, *31*(10), 2593–2600. https://doi.org/10.1007/s10096-012-1601-2

Iwu, M.W., Duncan, A.R. & Okunji, C.O. (1999). New antimicrobials of plant origin. In J. Janick (Ed.), *Perspectives on New Crops and New Uses* (pp. 457-462). Alexandria, VA: ASHS Press

Joshi, B., Sah, G. P., Basnet, B. B., Bhatt, R., Sharma, D., Subedi, K., … Malla, R. (2011). Phytochemical extraction and antimicrobial properties of different medicinal plants:

Ocimum sanctum (Tulsi), Eugenia caryophyllata (Clove), Achyranthes bidentata (Datiwan) and Azadirachta indica (Neem). *Journal of Microbiology and Antimicrobials*, *3*(1), 1–7. Retrieved from http://www.academicjournals.org/JMA

Jovcic, B., Lepsanovic, Z., Suljagic, V., Rackov, G., Begovic, J., Topisirovic, L., & Kojic, M. (2011). Emergence of NDM-1 metallo-β-lactamase in Pseudomonas aeruginosa clinical isolates from Serbia. *Antimicrobial Agents and Chemotherapy*, *55*(8), 3929–31. https://doi.org/10.1128/AAC.00226-11

Kirsch, C. M., Yenokida, G. G., Jensen, W. A., Wendland, R., Suh, H., & Bourgault, M. (1990). Non-cardiogenic pulmonary oedema due to the intravenous administration of clove oil. *Thorax*, *45*(3), 235–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2330557

Klein, A.D. & Penney, N.S. (1988). Aloe Vera. *Journal of the American Academy of Dermatology*, 18(1), 714-720.

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., … Woodford, N. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: A molecular, biological, and epidemiological study. *The Lancet Infectious Diseases*, *10*(9), 597–602. https://doi.org/10.1016/S1473-3099(10)70143-2

Lauretti, L., Riccio, M. L., Mazzariol, A., Cornaglia, G., Amicosante, G., Fontana, R., & Rossolini, G. M. (1999). Cloning and characterization of blaVIM, a new integron-borne metallo-beta-lactamase gene from a Pseudomonas aeruginosa clinical isolate. *Antimicrobial Agents and Chemotherapy*, *43*(7), 1584–90. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10390207

Lewis, K., & Ausubel, F. M. (2006). Prospects for plant-derived antibacterials. *Nature Biotechnology*, *24*(12), 1504–1507. https://doi.org/10.1038/nbt1206-1504

Lina, T. T., Khajanchi, B. K., Azmi, I. J., Islam, M. A., Mahmood, B., Akter, M., … Talukder, K. A. (2014). Phenotypic and Molecular Characterization of Extended-Spectrum Beta-Lactamase-Producing Escherichia coli in Bangladesh. *PLoS ONE*, *9*(10), e108735. https://doi.org/10.1371/journal.pone.0108735

Livermore, D. M. (2000). Epidemiology of antibiotic resistance. *Intensive Care Medicine*, *26*

Suppl 1, S14-21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10786953

Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2000). Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. *Microbes and Infection*, *2*(9), 1051–1060. https://doi.org/10.1016/S1286-4579(00)01259-4

M. Kim, H., H. Lee, E., H. Hong, S., J. Song, H., K. Shin, M., H. Kim, S., & Y. Shin, T. (1998). Effect of Syzygium aromaticum extract on immediate hypersensitivity in rats. *Journal of Ethnopharmacology*, *60*(2), 125–131. https://doi.org/10.1016/S0378-8741(97)00143-8

Majiduddin, F. K., Materon, I. C., & Palzkill, T. G. (2002). Molecular analysis of beta-lactamase structure and function. *International Journal of Medical Microbiology*, *292*(2), 127–137. https://doi.org/10.1078/1438-4221-00198

Malar, R.J.J.T., Johnson, M., Beaulah, N.S., Laju, R.S., Anupriya, G. & Ethal, R.J.J.T. (2012). Anti-Bacterial and Antifungal Activity of Aloe vera Gel Extract. *International Journal of Biomedical and Advance Research,* 3(3).

Mann, A., Amupitan, J., & Oyewale, A. (2008). Evaluation of in vitro antimycobacterial activity of Nigerian plants used for treatment of respiratory diseases. *African Journal of …*, *7*(June), 1630–1636. Retrieved from http://www.ajol.info/index.php/ajb/article/view/58747

Marshall, J.M. (1990). Aloe vera gel: What is the evidence? *Pharma Jr,* 24, 360-362.

Massova, I., & Mobashery, S. (1998). Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. *Antimicrobial Agents and Chemotherapy*, *42*(1), 1–17. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9449253

Medeiros, A. A. (1997). Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *24 Suppl 1*, S19-45. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8994778

Mehrotra, S., Srivastava, A. K., & Nandi, S. P. (2010). Comparative antimicrobial activities of Neem, Amla, Aloe, Assam Tea and Clove extracts against Vibrio cholerae, Staphylococcus aureus and Pseudomonas aeruginosa. *Journal of Medicinal Plants Research*, *4*(18), 2473–2478. Retrieved from http://www.academicjournals.org/JMPR

Miyazawa, M. and M. Hisama. (2003). Antimutagenic activity of phenylpropanoides from clove (*Syzygium aromaticum*). *J. Agric. Food Chem.,* 51(22). 6413-6422.

Mohammad, A. (2003). Aloe Vera. *Textbook of Pharmacognosy* (pp. 111-115).

Mukherjee, P. K., & Wahile, A. (2006). Integrated approaches towards drug development from Ayurveda and other Indian system of medicines. *Journal of Ethnopharmacology*, *103*(1), 25–35. https://doi.org/10.1016/j.jep.2005.09.024

Naas, T., & Nordmann, P. (1999). OXA-type beta-lactamases. *Current Pharmaceutical Design*, *5*(11), 865–79. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10539993

Newall, C.A., Anderson, L.A. & Phillipson, J.D. (1996). *Herbal medicines, A Guide for Healthcare Professionals.* London: Pharmaceutical press.

Nikaido, H. (2010). Multidrug Resistance in Bacteria. *Annu Rev Biochem.*, (2), 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923.Multidrug

Nimmakayala, S., Duggirala, S. L., & Puchchakayala, G. (2014). Antimicrobial activity of ethanolic extracts of Justicia neesii. *Bangladesh Journal of Pharmacology*, *9*(4). https://doi.org/10.3329/bjp.v9i4.20571

Pandey, R., & Mishra, A. (2010). Antibacterial activities of crude extract of aloe barbadensis to clinically isolated bacterial pathogens. *Applied Biochemistry and Biotechnology*, *160*(5), 1356–1361. https://doi.org/10.1007/s12010-009-8577-0

Park, M.-J., Gwak, K.-S., Yang, I., Choi, W.-S., Jo, H.-J., Chang, J.-W., … Choi, I.-G. (2007). Antifungal activities of the essential oils in Syzygium aromaticum (L.) Merr. Et Perry and Leptospermum petersonii Bailey and their constituents against various dermatophytes. *Journal of Microbiology (Seoul, Korea)*, *45*(5), 460–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17978807

Phyllis, B. and B. James. 2000. *Prescription for Nutritional Healing, 3rd ed*. (pp 94) Avery Publishing.

Podschun, R., & Ullmann, U. (1998). Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews,*

11(4), 589–603. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9767057

Poirel, L., Bonnin, R. A., Boulanger, A., Schrenzel, J., Kaase, M., & Nordmann, P. (2012). Tn125-related acquisition of blaNDM-like genes in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy*, *56*(2), 1087–9. https://doi.org/10.1128/AAC.05620-11

Poirel, L., Revathi, G., Bernabeu, S., & Nordmann, P. (2011). Detection of NDM-1 Producing Klebsiella pneumoniae in Kenya. *Antimicrobial Agents and Chemotherapy*, *55*(2), 934–936. https://doi.org/10.1128/AAC.01247-10

Prashar, A., Locke, I. C., & Evans, C. S. (2006). Cytotoxicity of clove (Syzygium aromaticum) oil and its major components to human skin cells. *Cell Proliferation*, *39*(4), 241–248. https://doi.org/10.1111/j.1365-2184.2006.00384.x

Rice, L. B. (2009). The clinical consequences of antimicrobial resistance. *Current Opinion in Microbiology*, *12*(5), 476–481. https://doi.org/10.1016/j.mib.2009.08.001

Ro, J. Y., Lee, B. C., Kim, J. Y., Chung, Y. J., Chung, M. H., Lee, S. K., … Park, Y. I. (2000). Inhibitory mechanism of aloe single component (alprogen) on mediator release in guinea pig lung mast cells activated with specific antigen-antibody reactions. *The Journal of Pharmacology and Experimental Therapeutics*, *292*(1), 114–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10604937

Roberts, D. B., & Travis, E. L. (1995). Acemannan-containing wound dressing gel reduces radiation-induced skin reactions in C3H mice. *International Journal of Radiation Oncology, Biology, Physics*, *32*(4), 1047–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7607925

Robicsek, A., Jacoby, G. A., & Hooper, D. C. (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *The Lancet Infectious Diseases*, *6*(10), 629–640. https://doi.org/10.1016/S1473-3099(06)70599-0

Saeed, S., & Tariq, P. (2008). In vitro antibacterial activity of clove against gram negative bacteria. *Pakistan Journal of Botany*, *40*(5), 2157–2160.

Sanschagrin, F., Couture, F., & Levesque, R. C. (1995). Primary structure of OXA-3 and phylogeny of oxacillin-hydrolyzing class D beta-lactamases. *Antimicrobial Agents and*

Chemotherapy, *39*(4), 887–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7785990

Shrivastava, K., Sahu, S., Mishra, S. K., & De, K. (2014). In vitro Antimicrobial Activity and Phytochemical Screening of Syzygium aromaticum. *Asian J. Res. Pharm. Sci*, *4*(1), 12–15. Retrieved from www.asianpharmaonline.org

Sikkema, J., de Bont, J. A., & Poolman, B. (1994). Interactions of cyclic hydrocarbons with biological membranes. *The Journal of Biological Chemistry*, *269*(11), 8022–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8132524

Srivastava, K.C. and N. Malhotra. 1991. Acetyl euginol, a component of oil of cloves (*Syzygium aromaticum* L.) inhibits aggregation and alters arachidonic acid metabolism in human blood platelets. *Prostaglandins Leukot Essent Fatty Acids*, 42(1): 73-81.

Surjushe, A., Vasani, R., & Saple, D. G. (2008). *Aloe vera*: a short review. *Indian Journal of Dermatology*, *53*(4), 163–6. https://doi.org/10.4103/0019-5154.44785

Sydiskis, R. J., Owen, D. G., Lohr, J. L., Rosler, K. H., & Blomster, R. N. (1991). Inactivation of enveloped viruses by anthraquinones extracted from plants. *Antimicrobial Agents and Chemotherapy*, *35*(12), 2463–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1810179

Talukdar, P. K., Rahman, M., Rahman, M., Nabi, A., Islam, Z., Hoque, M. M., … Islam, M. A. (2013). Antimicrobial Resistance, Virulence Factors and Genetic Diversity of Escherichia coli Isolates from Household Water Supply in Dhaka, Bangladesh. *PLoS ONE*, *8*(4), e61090. https://doi.org/10.1371/journal.pone.0061090

Tan, B.K. & Vanitha, J. (2004). Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbs. *Current Medicinal Chemistry*, 11, 1423-1430.

Tenover, F. C. (2006). Mechanisms of Antimicrobial Resistance in Bacteria. *The American Journal of Medicine*, *119*(6), S3–S10. https://doi.org/10.1016/j.amjmed.2006.03.011

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

Trongtokit, Y., Rongsriyam, Y., Komalamisra, N., & Apiwathnasorn, C. (2005). Comparative repellency of 38 essential oils against mosquito bites. *Phytotherapy Research*, *19*(4), 303–309. https://doi.org/10.1002/ptr.1637

Walsh, T. R. (2006). Combinatorial genetic evolution of multiresistance. *Current Opinion in Microbiology*, *9*(5), 476–482. https://doi.org/10.1016/j.mib.2006.08.009

Walther-Rasmussen, J., & Høiby, N. (2006). OXA-type carbapenemases. *Journal of Antimicrobial Chemotherapy*, *57*(3), 373–383. https://doi.org/10.1093/jac/dki482

Wendakoon, C., Calderon, P., & Gagnon, D. (2012). Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity against Human Pathogens. *Journal of Medicinally Active Plants*, *1*(2). https://doi.org/10.7275/R5GH9FV2

West, D. P., & Zhu, Y. F. (2003). Evaluation of *Aloe vera* gel gloves in the treatment of dry skin associated with occupational exposure. *American Journal of Infection Control*, *31*(1), 40–2. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12548256

Woodford, N. (2005). Biological counterstrike: antibiotic resistance mechanisms of Gram-positive cocci. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *11 Suppl 3*, 2–21. https://doi.org/10.1111/j.1469-0691.2005.01140.x

Yang, Y.-C., Lee, S.-H., Lee, W.-J., Choi, D.-H., & Ahn, Y.-J. (2003). Ovicidal and Adulticidal Effects of *Eugenia caryophyllata* Bud and Leaf Oil Compounds on *Pediculus capitis*. *Journal of Agricultural and Food Chemistry*, *51*(17), 4884–4888. https://doi.org/10.1021/jf034225f

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., & Walsh, T. R. (2009). Characterization of a New Metallo- -Lactamase Gene, blaNDM-1, and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in Klebsiella pneumoniae Sequence Type 14 from India. *Antimicrobial Agents and Chemotherapy*, *53*(12), 5046–5054. https://doi.org/10.1128/AAC.00774-09

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

Appendix – II

Reagents

The reagents used in the above procedures were made using the following compositions:

LB medium	1% tryptone 0.5% yeast extract 200 mMNaCl
TE buffer	10 mMTris-Cl (pH 8.0) 1 mM EDTA (pH 8.0)
Lysis buffer	(10 ml) 9.34 ml TE buffer 600 µl of 10% SDS 60 µl of proteinase K (20 mg ml-1)
TBE buffer (1x)	5.4 g Tris-HCl 2.75 g Boric acid 2ml 0.5M EDTA Adjust volume with distilled water pH: 8.0

Appendix – III

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
Table Top Centrifuge	Model: DSC-200A-2 Digisystem Laboratory Instruments Inc. Made in Taiwan
Electronic Balance	RADWAG Wagi ELEktroniczne Model: WTB 200
Refrigerator (4^0C)	Model: 0636 Samsung