The antibacterial activities of Peppermint oil, Mustard oil and Clove oil on *Klebsiella pneumonia, Acinetobacter baumannii, and Pseudomonas taetrolens*

By

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Declaration

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3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

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Abstract

Bacteria are getting highly resistant to antibiotics throughout the time and are getting harder to treat. This project focused on up to which percentage an essential oil can inhibit a multi-drug resistant pathogen, and what are the factors that may vary the outcome. At first, the bacteria sample were collected from tertiary care hospitals. Then biochemical tests were done to confirm their identity. Peppermint oil, mustard oil, and clove oil were selected as essential oils. As pathogenic bacteria, three gram-negative PDR, and MDR bacteria including ***Klebsiella pneumonia, Acinetobacter baumannii, and Pseudomonas taetrol*** were selected which are common pathogens in the Bangladeshi scenario. To determine the antimicrobial activities of the oils, three methods were followed. First, the organisms were exposed to various concentrations (prepared by dilution in physiological saline) of the oil and then spread on different agar media plates. The other two procedures were the disc diffusion and agar diffusion method. Peppermint oil was capable of inhibiting ***Acinetobacter baumannii*****,** ***Klebsiella pneumoniae*****, and** ***Pseudomonas taetrolens*** up to 94.06%, 44.51%, and 61.02% respectively. Mustard oil was capable of inhibiting ***Acinetobacter baumannii*****,** ***Klebsiella pneumoniae*****, and** ***Pseudomonas taetrolens*** up to 51%, 42%, and 38% respectively. Clove oil was found to be most effective and was capable of inhibiting ***Acinetobacter baumannii*****,** ***Klebsiella pneumoniae*****, and** ***Pseudomonas taetrolens*** up to 100%. This project showed the potential of essential oils in treating multi-drug resistant bacteria and the factors that need to be focused on to develop medicine incorporating these oils.

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List of abbreviation

EO	Essential oil
MDR	Multidrug resistant
XDR	Extensively drug-resistant
PDR	Pandrug-resistant
MHA	Mueller Hinton Agar
NA	Nutrient agar
TSI	Triple Sugar Iron
MR	Methyl Red
VP	Voges- Proskauer
MAC	MacConkey Agar
ACB	*Acinetobacter baumannii*
KP	*Klebsiella pneumonia*
PSU	*Pseudomonas taetrolens*
M. oil	Mustard oil
Pm. Oil	Peppermint oil
C. oil	Clove oil
Ab	Antibiotic

Chapter-1

Introduction

1. Introduction

1.1. Background: Essential oil

Essential oils are defined as the extracts of plants that give plants their fragrant aroma. These have been used since ancient days in medicines. Some of the essential oils have excellent antimicrobial properties. This study aims to reveal those oils that can be potentially used in medicines.

Over 17500 species produce essential oils. However, only 300 species of essential oils are commercialized. (Wińska et al., 2019). Essential oils are known to be incorporating a variety of other molecules including oxides, fatty acids, and sulfur derivatives. They are also made of terpenoids containing sesquiterpene and monoterpene, and their oxygenated derivatives are like a complex formation. These terpenoid and phenylpropanoid families may take in about 85% concentration of the oil (Stringaro et al., 2018).

Essential oil being an efficient bioactive compound in research depends on the extraction procedure, period, harvesting procedure, region, and season. (Garzoli et al., 2015) These oils can be produced through steam distillation, fermentation, and extraction. These methods can alter the chemical compound of oils (Wińska et al., 2019).

1.2. Peppermint oil

It belongs to the Lamiaceae family with 30 different species. To get the oil from this plant, dried leaves are distilled with water vapor. The obtained oil is slightly yellow or green. However, it can be transparent in color as well depending on the manufacturer. (Wińska et al., 2019)

1.2.1. Chemical composition

The major components are: (Wińska et al, 2019)

Chemical compound	Concentration
Menthol	30-55%
Mentone	14-32%
Cineol	3.5-24%
Menthyl acetate	2.8-10%
Isomenton	1.5-10%

Menthofuran	1-9%
Limonene	1-5%
Pulegone	4%
Carvone	1%

1.2.2. Antimicrobial properties

Effects on bacteria: peppermint essential oil is used in treating colds, mild spinal gastrointestinal complaints, and to relieve local muscle pain (MH et al., 2015).

Effects on the virus: Recent studies, have showed potential inhibitory effects on HSV-1 and HSV-2. (PS et al., 2003)

Effects on fungus: it has weak antifungal properties on yeast, for example, *C. Albicans*, *C. tropicalis*, *Pichia anomala*, and *S. cerevisiae* (Almeida et al., 2019).

1.2.3. Mode of action

It can be used to treat enterococcus due to the presence of monoterpenes in high concentrations. Especially menthol can affect the hydrophobicity and cell membrane. Monoterpenes can change the protein confrontation. As a result, cellular respiration gets inhibited and the ions cannot be transported at the membrane level. This is responsible for bacterial cell death (Trombetta et al., 2005).

1.3. Mustard oil

It belongs to Brassicaceae family. It is extracted from seeds by distillation. The extracted oil is yellow.

1.3.1. Chemical composition

Fourteen components could be identified in a study. The allyl isothiocyanate was in the highest concentration at about 71.06%. (Peng et al., 2014) The major components are:

Chemical compound	Concentration (%)
Oleic acid	20–28
Linoleic	10–12
linolenic acid	9.0–9.5
erucic acid	30–40
allyl isothiocyanate	71.06

1.3.2. Antimicrobial effects

gram-negative bacteria are more sensitive to this oil, for example, *E. coli, Enterobacter aerogenes, Salmonella enterica serovar Enteritidis, Listeria monocytogenes, Staphylococcus aureus, Lactobacillus fermentum,* and *Bacillus cereus* (Monu et al., 2014).

1.4. Clove oil

It belongs to the Myrtaceae family. It is obtained from *Eugenia caryophyllata* Thunb's undeveloped flower buds by distillation. Its oil is colorless or slightly yellow. Clove oil is a mixture of 23 different compounds, with the three main active ingredients being eugenol, eugenyl acetate, and caryophyllene. Eugenol, for being in the most amount is expected to be the ingredient responsible for antibacterial properties (Wińska et al., 2019).

1.4.1. Chemical composition

The major components are:

Compound	Composition (%)
Eugenol	80.26
Alpha copaene	0.25
Caryophyllene	5.16
Alpha humulene	0.86
Eugenol acetate	8.64
Calamenene	0.15

Beta carrophyllene epoxide	0.52
Cinnamaldehydemethoxy	0.17
Benzothiophene	0.26

1.4.2. Antimicrobial effects

Effects on bacteria: *B. cereus, Typhimuriumrium*, *E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus*, *Staphylococcus epidermidis*, *Proteus mirabilis,* etc can be treated by cove oil (Condò C Anacarso et al., 2020).

Effects on the virus: HSV-1 and HSV-2 viruses can be inhibited by clove essential oil according to research (RR et al., 2009).

1.4.3. Mode of action

According to previous findings, eugenol in clove oil disrupts the cell wall or membrane of a microbe. Then it enters the cell and inhibits the DNA synthesis. As a result, protein cannot be synthesized and the microbe dies. The ATPase synthesis is also decreased by the oil. Also, the beta-galactosidase pathway and bacterial respiratory metabolisms are decreased by clove essential oil. (Wińska et al, 2019)

1.5. Infection caused by study-related bacteria

Bacteria	**Infection**
Klebsiella pneumonia	Pneumonia, Tuberculosis, Aspergillus infection, Malignancy, Acute respiratory distress syndrome (ARDS), Lung abscess, Empyema, and other pleuropulmonary infections (Ashurst et al., 2022)
Acinetobacter baumannii	Skin and soft tissue infection, meningitis, urinary tract infections, bacteremia, and pneumonia(Morris, et al., 2019)

Pseudomonas taetrolens	Urinary tract infection, central nervous system infection, presence in wounds, ears, eyes, and musculoskeletal system, infection (Iglewski et al., 1993)

1.6. Multidrug resistance and emergency

Because of the continuous treatment with antibiotics, the bacteria are getting highly resistant. For this, these bacteria are getting harder to manipulate to treat the infection. When the R plasmid takes up the gene that codes for resistance traits to multiple specific drugs, the organism turns out to be a multidrug-resistant bacteria. It can also be the outcome of the increased genome expression of multidrug efflux pumps. These organisms are capable of surviving in presence of multiple drugs. This is what makes it dangerous to treat (Nikaido et al., 2009). There are some high levels of resistance as well which are PDR and XDR. XDR class includes organisms that are susceptible to one or two classes of antibiotics. PDR class includes organisms that are susceptible to no antibiotic class (Magiorakos et al., 2012).

1.7. Literature review

Delia Muntean et.al. conducted research on peppermint oil to evaluate its effects on hospital-admitted MDR patients. For this, they used the following methods: agar disk diffusion method and microdilution method. Its MIC for *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Acinetobacter baumannii* was about 40 mg/mL which is high. They concluded by identifying peppermint oil as a therapeutic option in treating MDR organisms (Muntean et al., 2019).

Vanessa Lee Rosarior et.al. conducted research on clove oil to evaluate its effects on *Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.* For this they used the following methods: disk diffusion assay, Microwell dilution assay, DPPH, and ABTS radical scavenging assays. They concluded the study by proving it to be a promising antimicrobial agent against both Gram-positive and Gram-negative bacteria (Rosarior et al., 2021).

Amornrat Intorasoot et.al. conducted research on clove oil to evaluate its effects on *Acinetobacter baumannii.* For this, they used the following methods: agar diffusion procedure, minimum inhibitory concentration, and minimum bactericidal concentration (MBC). This research was concluded by indicating the potency of clove oil on *Acinetobacter baumannii* with MBC90 of 1 mg/mL (Intorasoot et al., 2017).

1.8. Objectives

This research aims to figure out,

a. The antimicrobial activity of oil, essential oils including peppermint oil, mustard oil, and clove oil.

b. The growth inhibitory effect of these oils on PDR, XDR, and MDR bacteria that include *Klebsiella pneumonia*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* which are gram negatives in nature

Chapter- 2

Materials and methods

2. Materials and Methods

2.1. Essential oil collection

Commercially available peppermint oil, mustard oil, and clove oil were used from a pharmacy.

2.2. Bacterial strain collection

Klebsiella pneumonia, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* were collected from several tertiary care hospitals named National Institute of Disease of the Chest and Hospital (NIDCH), and Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders Hospital (BIRDEM).

2.3. Identification

For identification, several biochemical tests and growing on specific media were performed under sterile conditions.

2.3.1. Growth in selective media:

For the primary screening of bacterial pure colonies, the sample was grown over MacConkey agar and incubated at 37 ° C for 24 hours. The growth of the bacteria and colony morphology was checked and compared with previous studies to ensure the probable bacterial strain based on lactose fermentation, MacConkey Agar is recommended for use as a selective and differential medium for the isolation of gram-negative bacteria.

2.3.2. Antibiotic susceptibility test

Disc diffusion method was used in determining if the bacterial sample was MDR or above. For this, a well-isolated colony was inoculated and grown on Mueller Hinton agar (MHA). The antibiotic discs were placed on the inoculated media and incubated for 18 to 24 hours at 37 ℃. Here, eleven classes of antibiotic discs were used.

3.3.3. Biochemical tests

2.3.3.1. Gram staining

This test distinguishes between the two types of bacteria: Gram-positive and Gram-negative bacteria by observing stain and morphology. Gram-positive bacteria stain purple, whereas Gram-negative bacteria stain pink. The proper staining procedure maintaining timing was done to avoid error under a microscope.

2.3.3.2. Methyl red test

Bacterial cultures were inoculated in test tubes containing MRVP broth and incubated at 37°C for 24 hours. Before observing the result, 5 drops of methyl red were added to the medium.

2.3.3.3. VP test

Bacterial cultures were inoculated in test tubes containing MRVP broth and incubated at 37°C for 24 hours. To observe the result, a few drops of Barrit's A and Barrit's B were added respectively.

2.3.3.4. Citrate utilization test

Bacterial cultures were streaked in glass vials containing citrate agar and incubated at 37°C for 24 hours.

2.3.3.5. Oxidase test

Bacterial culture was placed on filter paper and a reagent was added to it to see the result instantly.

2.3.3.6. Catalase test

A loop full of bacterial culture was smeared on a glass slide containing 3% H_2O_2. The results were observed instantly.

2.3.3.7. TSI test

Bacterial culture was streaked on the surface of the TSI agar slant and stabbed butt down to the bottom. After that, it was incubated at 37°C for 24 hours.

2.3.3.8. Urease test, Motility test, and Indole test by MIU agar

Bacterial culture was stabbed down the MIU agar and incubated at 37°C for 24 hours. Later on, after observing the results of motility and urease, the Kovac's reagent was added to observe the presence of indole.

2.3.3.9. Phenol red glucose

Bacterial culture was inoculated tube containing phenol red glucose with Durham tube and incubated at 37 ºC for 24 hours.

2.3.3.10. Phenol red sucrose

Bacterial culture was inoculated tube containing phenol red sucrose with Durham tube and incubated at 37 ºC for 24 hours.

2.3.3.11. Phenol red fructose

Bacterial culture was inoculated tube containing phenol red fructose with Durham tube and incubated at 37 ºC for 24 hours.

2.3.4. Pathogenicity tests

2.3.4.1. *DNASE test*

Bacterial culture was streaked on the DNase-containing media and incubated at 37 ºC for 24 hours.

2.3.4.2. Hemolysis test

Bacterial vulture was streaked on the blood agar containing media and incubated at 37 ºC for 24 hours.

2.4. Inhibition by essential oils

2.4.1. *Agar well diffusion procedure followed by dose-dependency*

First, bacteria were placed on MHA agar. Then different-sized holes were made on MHA agar for different doses. There the wells were filled up with essential oils in different amounts. They were kept in the refrigerator for two hours. Later on, they were incubated at 37 ºC for 24 hours.

2.4.2. Disc diffusion procedure followed by dose-dependency

First, bacteria were placed on MHA agar. Then discs were submerged and dried with essential oils. After that, the discs were placed on bacteria-containing media and incubated at 37 ºC for 24 hours.

2.4.3. Dilution technique followed by dose-dependency

It was a simple dilution process where bacteria were diluted and essential oils were added based on different concentrations. Four doses were focused on here. Then they were spread on MaConkey agar media and nutrient agar media. To get the result, they were incubated at 37 ºC for 24 hours.

Chapter- 3

Results

3. Results

Three types of oils were used here: peppermint oil, mustard oil, and clove oil. These oils were tested on three gram-negative bacteria: ***Klebsiella pneumonia, Acinetobacter baumannii, and Pseudomonas taetrolens.*** Three different procedures were followed on these bacteria and oils to confirm the antimicrobial activity: disc diffusion procedure, agar well diffusion procedure, and dilution technique. Biochemical tests were done to identify the bacteria along with antibiotic susceptibility test, and pathogenicity tests. The results of all of those tests are interpreted below.

3.1. Confirmation of bacterial strain

The clinical strains were grown on selective MAC agar media. The bacteria fermented the sugar lactose (Lac+) and grew. This is how the gram-negative bacteria are distinguished on MAC agar media. Also, several biochemical tests were done to specify the identify of the bacteria. Moreover, to confirm whether the strains are MDR, antibiotic susceptibility test was carried out. To determine if the bacteria are pathogens, hemolysis test, DNAase test, and coagulase tests were performed.

3.1.1 . Biochemical test to identify the bacteria

Several biochemical tests were performed to identify the organisms as shown in the Table. 1.

Table 1: Biochemical test to identify the bacteria.

Possible Bacterial isolates	Gram staining	MR test	VP test	Citrate test	Oxidase	MIU			TSI				Catalase test	Phenol red glucose		Phenol red sucrose		Phenol red lactose	
						Motility	Indole	Urea	Slant	Butt	Gas	H2S		Glucose	Gas	Sucrose	Gas	Lactose	Gas
Klebsiella pneumonia	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve	(-)ve	(+)ve	Yellow	yellow	(+)ve	(-)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve
Acinetobacter baumannii	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve	(-)ve	(-)ve	red	red	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
Pseudomonas taetrolens	(-)ve	(+)ve	(-)ve	(+)ve	(-)ve	(+)ve	(-)ve	(+)ve	red	black	(-)ve	(+)ve	(-)ve	(+)ve	(+)ve	(-)ve	(-)ve	(-)ve	(+)ve

The results of the table suggest that the organisms we used are *Klebsiella pneumonia*, *Acinetobacter baumannii, and Pseudomonas taetrolens*

3.1.2. Antibiotic susceptibility test results

12 classes of antibiotics were used to check the antibiotic susceptibility test. The classes are: aminoglycosides, carbapenems, cephalosporins, fluroquinons, glycopeptides, penicillins, polypeptides, rifamycins, tetracyclines, monobactams, lincomycins, chloramphenicols.

According to the table, the bacteria ***Klebsiella pneumonia*** is sensitive to tetracycline and doxycycline. This bacteria is only sensitive to one antibiotic class which is tetracyclines out of twelve classes in total. This makes it pan drug-resistant (PDR) bacteria. According to the table, ***Acinetobacter baumannii*** is resistant to all twelve classes of antibiotics. This makes it pan drug-resistant (PDR). According to the table, ***Pseudomonas taetrolens*** is sensitive to meropenem, amoxiclav, and chloramphenicol. This bacteria is sensitive to three antibiotic classes which are carbapenems, penicillins, and chloramphenicol out of twelve classes in total. Also, it is intermediate to one class of antibiotic which is monobactam. This makes it a multidrug-resistant (MDR) bacteria. All the data of antibiotic susceptibility test incorporated table is given below:

Table 2. Antibiotic sensitivity test results

Sl.	Antibiotic	*Klebsiella pneumonia*		*Acinetobacter baumannii*		*Pseudomonas taetrolens*	
		Zone of inhibition	**Interpretation**	**Zone of inhibition**	**Interpretation**	**Zone of inhibition**	**Interpretation**
	Amikacin	0	Resistant	0	Resistant	0	Resistant
	Gentamicin	0	Resistant	0	Resistant	0	Resistant
	Netilmicin	0	Resistant	0	Resistant	6.5	Resistant
	Imipenem	0	Resistant	0	Resistant	11.5	Resistant
	Meropenem	9	Resistant	7	Resistant	29.66	Sensitive
	Ceftazidime	0	Resistant	0	Resistant	0	Resistant
	Cephalexin	0	Resistant	0	Resistant	0	Resistant
	Ciprofloxacin	0	Resistant	0	Resistant	13	Resistant
	Moxifloxacin	0	Resistant	5.75	Resistant	0	Resistant
	Vancomycin	0	Resistant	0	Resistant	0	Resistant
	Amoxiclav/Clavulanic acid	0	Resistant	0	Resistant	20.5	Sensitive
	Penicillin G	0	Resistant	0	Resistant	0	Resistant
	Colistin	12	Resistant	12.5	Resistant	0	Resistant
	Polymixin B	8	Undetermined	12.5	Undermined	0	Resistant
	Rifampicin	0	Resistant	0	Resistant	8	Undetermined
	Tetracyclines	16.5	Sensitive	0	Resistant	6.5	Resistant
	Doxycycline	14	Sensitive	0	Resistant	0	Resistant
	Aztreonam	0	Resistant	0	Resistant	20	Intermediate
	Clindamycin	0	Resistant	0	Resistant	0	Resistant
	Chloramphenicol	0	Resistant	0	Resistant	20.5	Sensitive

3.1.3. Pathogenicity test results

After performing DNase and hemolysis tests, the results were all negative for all three bacteria. Without performing coagulase test, it cannot be confirmed that the bacteria is a pathogen and if it can be harmful. However, after performing two of the tests it can be concluded that three of the bacteria are partially non-pathogenic.

Table 3. DNase test results:

Organism	Result	Interpretation
Klebsiella pneumoniae	(-) ve	Cannot hydrolyze DNA for utilizing it as its carbon and energy source for growth
Acinetobacter baumannii	(-) ve	Cannot hydrolyze DNA for utilizing it as its carbon and energy source for growth
Pseudomonas taetrolens	(-) ve	Cannot hydrolyze DNA for utilizing it as its carbon and energy source for growth

Table 4: Hemolysis test results:

Organism	Result	Interpretation
Klebsiella pneumonia	Gamma	Lack of hemolysis
Acinetobacter baumannii	Gamma	Lack of hemolysis
Pseudomonas taetrolens	Gamma	Lack of hemolysis

3.2. Inhibition by essential oils

3.2.1. Results of Agar well diffusion procedure followed by dose-dependency

In the agar well diffusion procedure, two doses were used to determine the inhibition percentage. The doses are 120 µL/10 ml and 80 µL/10ml. As the doses of oil were close enough, the result was close enough as well. However, the variation in results was visible under different doses of this procedure. As an antibiotic, Kanamycin was used as a reference because it is a broadly used

antibiotic. In agar diffusion method there was no inhibition of the growth of *Acinetobacter baumannii* by peppermint and mustard as showed in the table 5. However, clove oil at a contraction of 80 µL/10 ml was able to significantly inhibit the growth of *Acinetobacter baumannii*, *Klebsiella pneumonia*, and *Pseudomonas taetrolens* showing the zone sizes of 21mm, 13.5 mm, and 14 mm respectively. For dose 120 µL/10 ml it was 25 mm, 16 mm, and 18 mm respectively. The zone sizes varied depending on dosage and bacterial strain. In comparison to antibiotics, clove oil was able to show a significant zone of inhibition when the antibiotic did not work.

Table 5 Inhibition of the growth of *Acinetobacter baumannii* by different concentrations of peppermint, mustard, and clove oil as shown by agar well diffusion method

Essential oil	Dose-80 µL/10 ml	Dose-120 µL/10 ml	Kanamycin
Peppermint oil	0 mm	0 mm	0 mm
Mustard oil	0 mm	0 mm	0 mm
Clove oil	21mm	25mm	0 mm

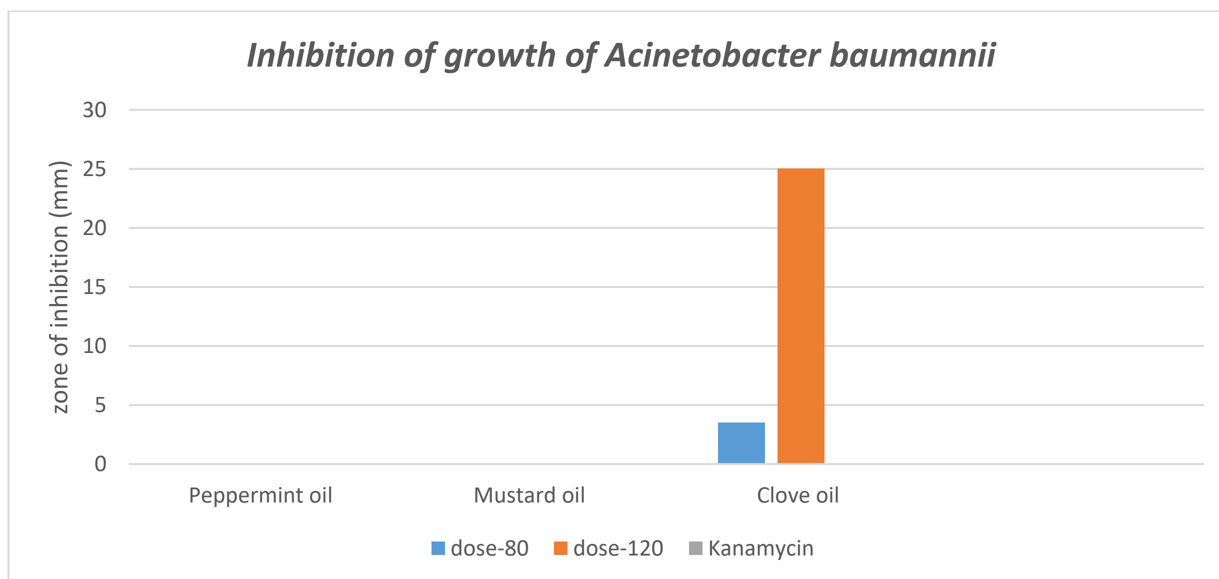

Figure 1 **Inhibition of the growth of *Acinetobacter baumannii* at different concentrations of peppermint oil, mustard oil and clove oil by agar diffusion method**

Table 6. Inhibition of the growth of *Klebsiella pneumonia* by different concentrations of peppermint, mustard, and clove oil as shown by agar well diffusion method

Essential oil	Dose-80 µl/10 ml	Dose-120 µl/10 ml	Kanamycin
Peppermint oil	0 mm	0 mm	0 mm
Mustard oil	0 mm	0 mm	0 mm
Clove oil	13.5mm	16mm	0 mm

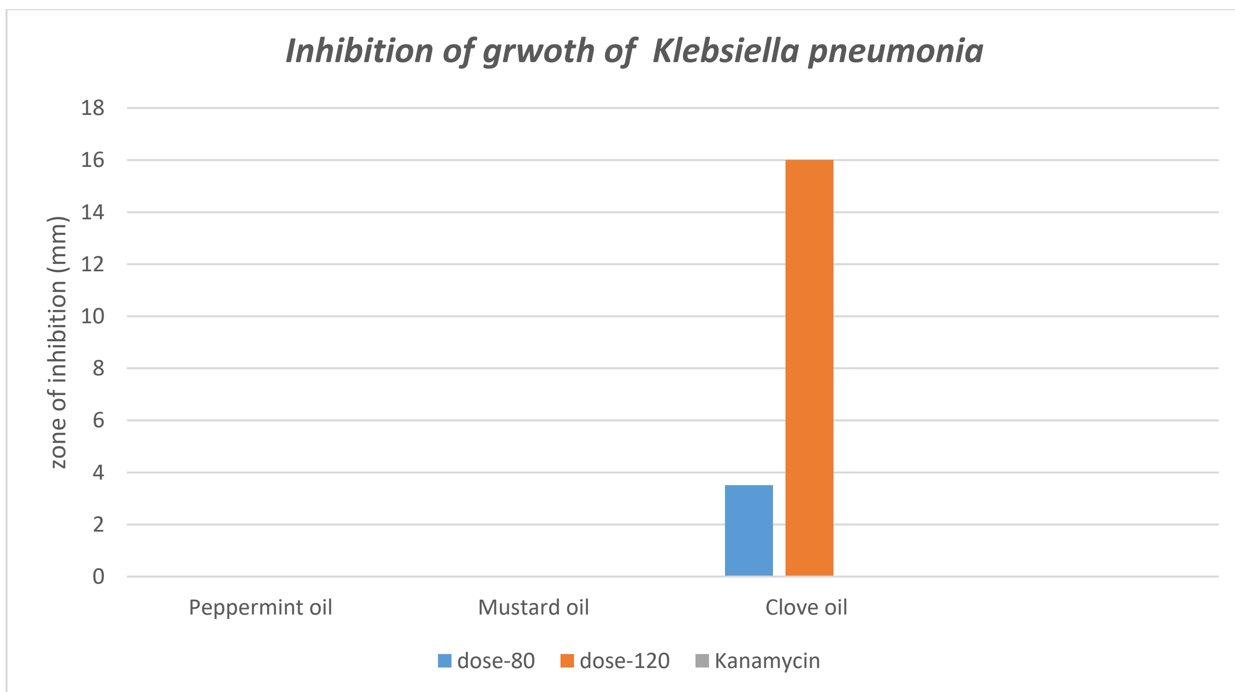

Figure 2: **Inhibition of the growth of *Klebsiella pneumonia* at different concentrations of peppermint oil, mustard oil, and clove oil by agar diffusion method**

Table 7. Inhibition of the growth of *Pseudomonas taetrolens* by different concentrations of peppermint, mustard, and clove oil as shown by agar well diffusion method

Essential oil	Dose-80 µl/10 ml	Dose-120 µl/10 ml	Kanamycin
Peppermint oil	0 mm	0 mm	0 mm
Mustard oil	0 mm	0 mm	0 mm
Clove oil	14mm	18mm	0 mm

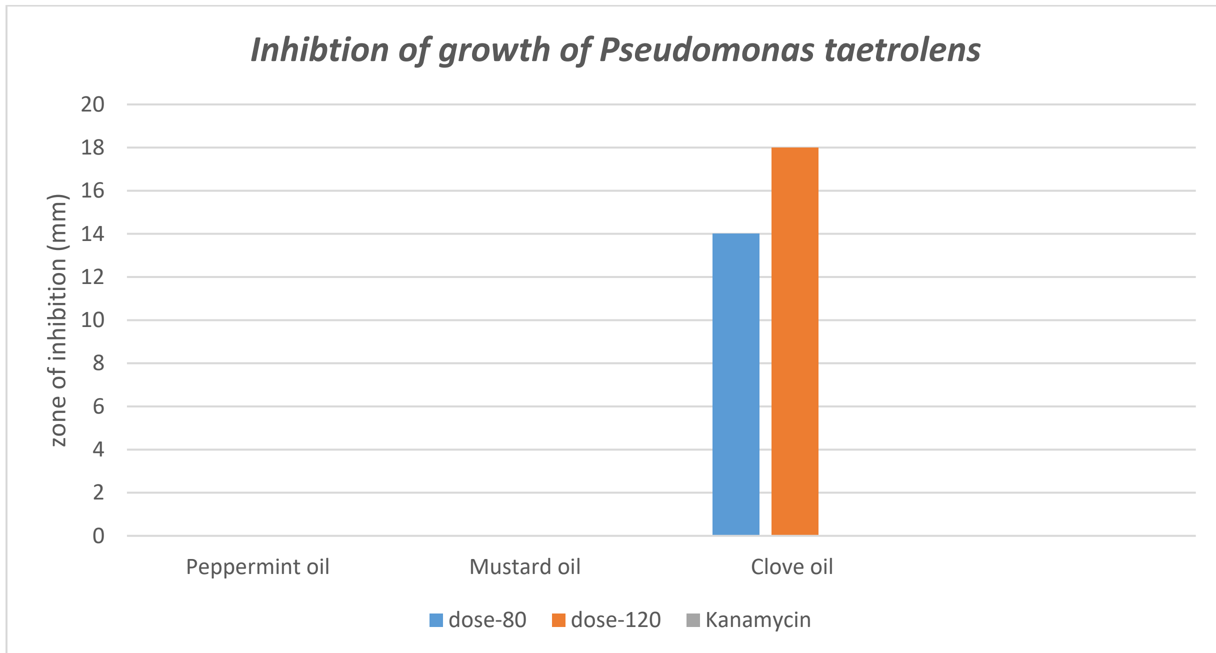

Figure 3: **Inhibition of the growth of *Pseudomonas taetrolens* at different concentrations of peppermint oil, mustard oil and clove oil by agar diffusion method**

3.2.1.4. Inhbition of growth of Klebsiella pneumonia by different oils

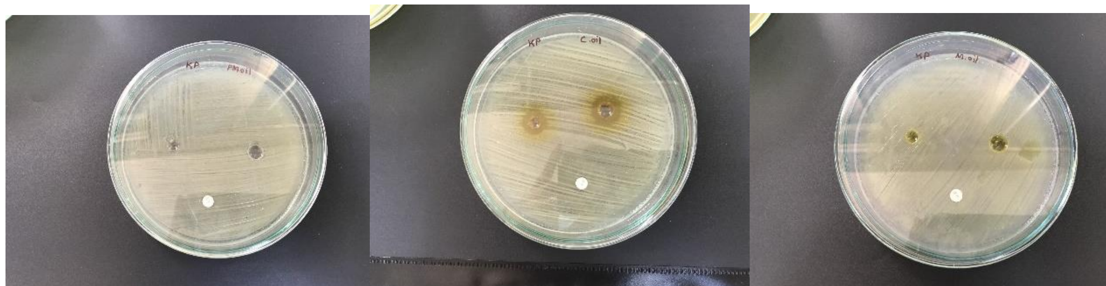

Figure 4: **Inhibition of the growth of *Klebsiella pneumonia* at two different concentrations (80 µl/10 ml and 120 µl/10 ml) of peppermint oil, mustard oil and clove oil by agar diffusion method**

Figure 5: **Inhibition of the growth of A*cinetobacter baumannii* at two different concentrations (80 µl/10 ml and 120 µl/10 ml) of peppermint oil, mustard oil, and clove oil by agar diffusion method with kanamycin disk as reference.**

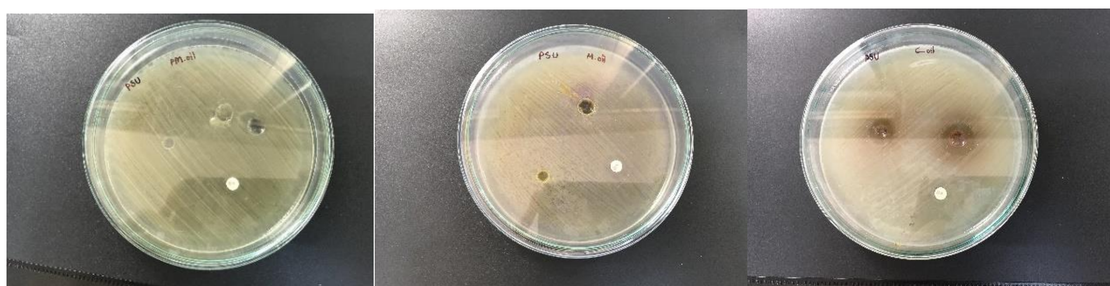

***Figure 6**: Inhibition of the growth of Pseudomonas taetrolens at two different concentrations (80 µl/10 ml and 120 µl/10 ml) of peppermint oil, and mustard oil, and clove oil by agar diffusion method with kanamycin disk as reference.*

3.2.2. Results of the antimicrobial study of essential oils by Disc diffusion procedure

This procedure contained no dose. However, the discs were expected to contain 10 µL of oil each. The results were the similar as the agar well diffusion method. As an antibiotic, Kanamycin was used as it is a broad spectrum one. The result was non-variable in terms of both peppermint and mustard oil. However, clove oil was able to create the zone of inhibition of 22mm, 10.5 mm, and 11 mm for the bacteria *Acinetobacter baumannii*, *Klebsiella pneumonia*, and *Pseudomonas taetrolens* respectively. The zone sizes varied depending on bacterial strain. In comparison to antibiotics clove oil was able to show a significantly higher zone of inhibition when the antibiotic did not work.

Table 8. **Inhibition of growth of bacteria by different oils assessed by disk diffusion method**

Bacteria	**Essential oil**	**Zone of inhibition (mm)**	**Zone of inhibition of Kanamycin (mm)**
Klebsiella pneumonia	Peppermint oil	0	0
	Mustard oil	0	
	Clove oil	22	
Acinetobcter baumannii	Peppermint oil	0	0
	Mustard oil	0	
	Clove oil	10.5	
Pseudomonas taetrolens	Peppermint oil	0	0

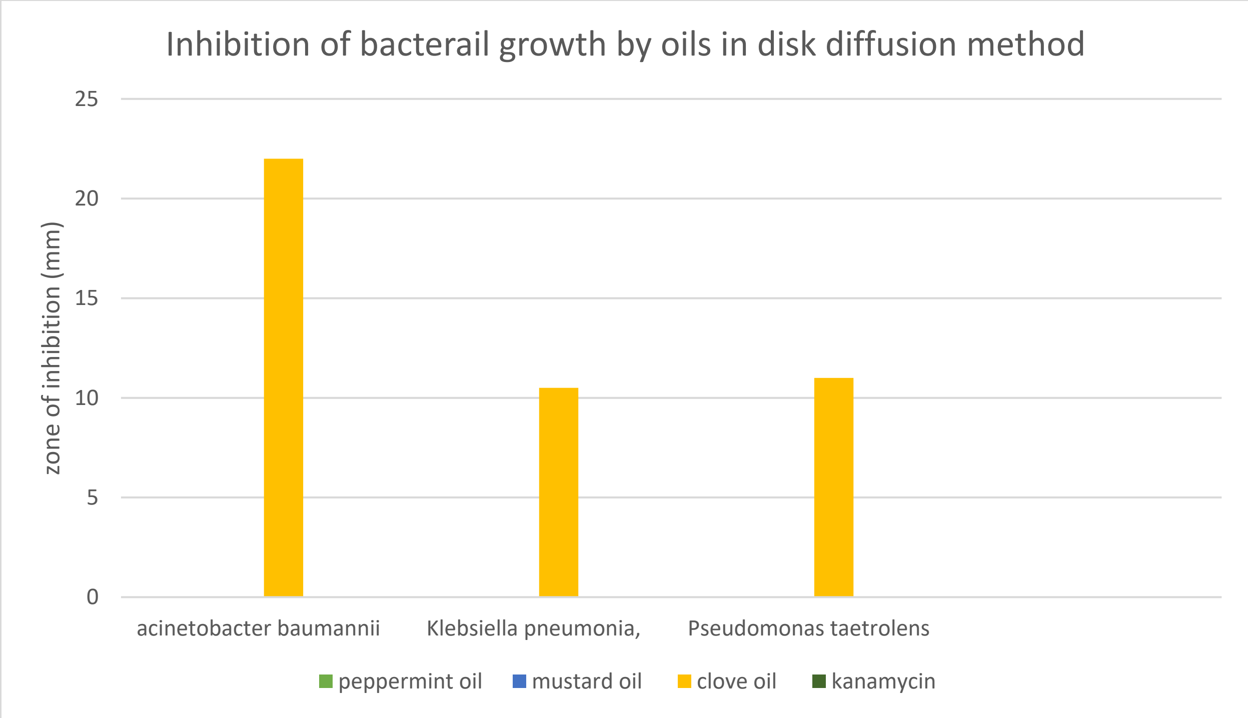

Figure 7: Inhibition of the growth of Acenetobacter baumannii, , Klebsiella pneumonia and Pseudomonas taetrolens at 10 µl/disk of peppermint oil, and mustard oil, and clove oil by agar diffusion method

3.2.2.1. Inhibition of growth of bacteria by different oils assessed by disk diffusion method

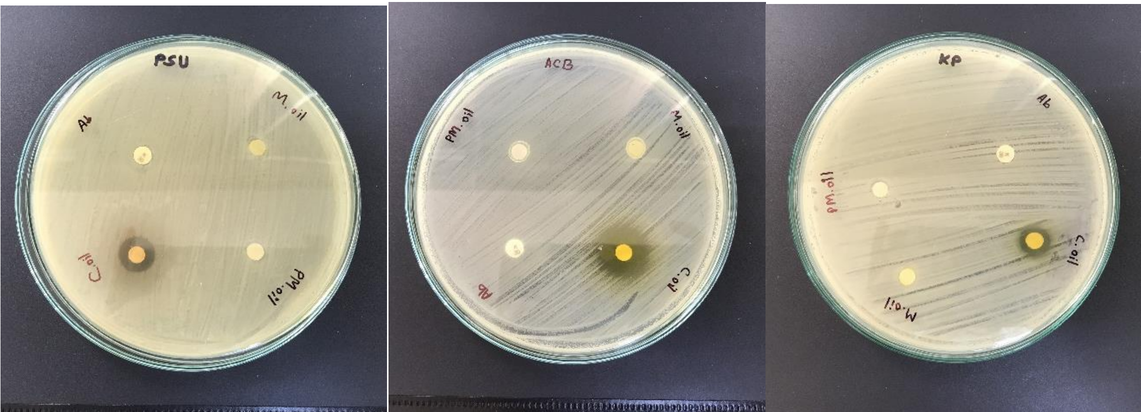

Figure 8: ***Inhibition of the growth of Acenetobacter baumannii, , Klebsiella pneumonia and Pseudomonas taetrolens at 20 µl/disk of peppermint oil, and mustard oil, and clove oil by agar diffusion method***

3.2.3. Results of the antimicrobial study of essential oils by Dilution technique followed by dose dependence

This procedure contained four doses of 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of each oil. Peppermint oil, mustard oil, and clove oil were capable of inhibiting *Acinetobacter baumannii* by 94.06%, 51%, and 100%. The inhibition was above 50% which seemed to be promising. In the case of *Klebsiella pneumonia*, peppermint oil, mustard oil, and clove oil were capable of inhibiting the growth by 44.51%, 42.00%, and 100% respectively. In addition, Peppermint oil, mustard oil, and clove oil were capable of inhibiting the growth by 61.02%, 38%, and 100% *Pseudomonas taetrolens* respectively. However, due to the variability of results at a similar dose, the optimum dose could not be determined. The graphs and charts below show how it varied in a similar dose at different times.

The inhibition rate was determined by the following equation:

Inhibition percentage = (number of colonies of control-number of colonies of oil plated)/ number of colonies of control x 100%

3.2.3.1. Results of antimicrobial study of essential oils by Dilution technique followed by dose dependence on *Acinetobacter baumannii:*

Table 9. Inhibition rate in percentage of *Acinetobacter baumannii* by dilution technique

Essential oil	dose-100 µL/10mL	dose-200 µL/10mL	dose-300 µL/10ml	dose-400 µL/10ml
Peppermint oil	60.38%	55.69%	83.28%	94.06%
Clove oil	100%	100%	100%	100%
Mustard oil	46.00%	66.00%	46.00%	51.00%

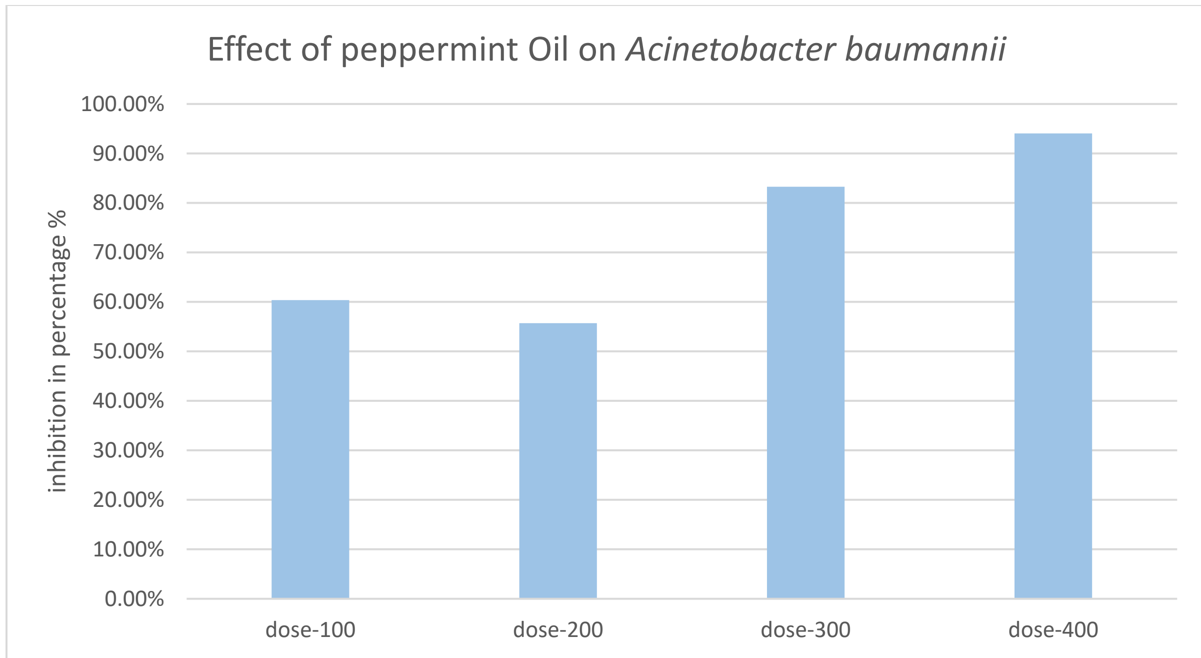

Figure 9: Inhibition of growth of Acinetobacter baumannii at different concentrations of peppermint oil

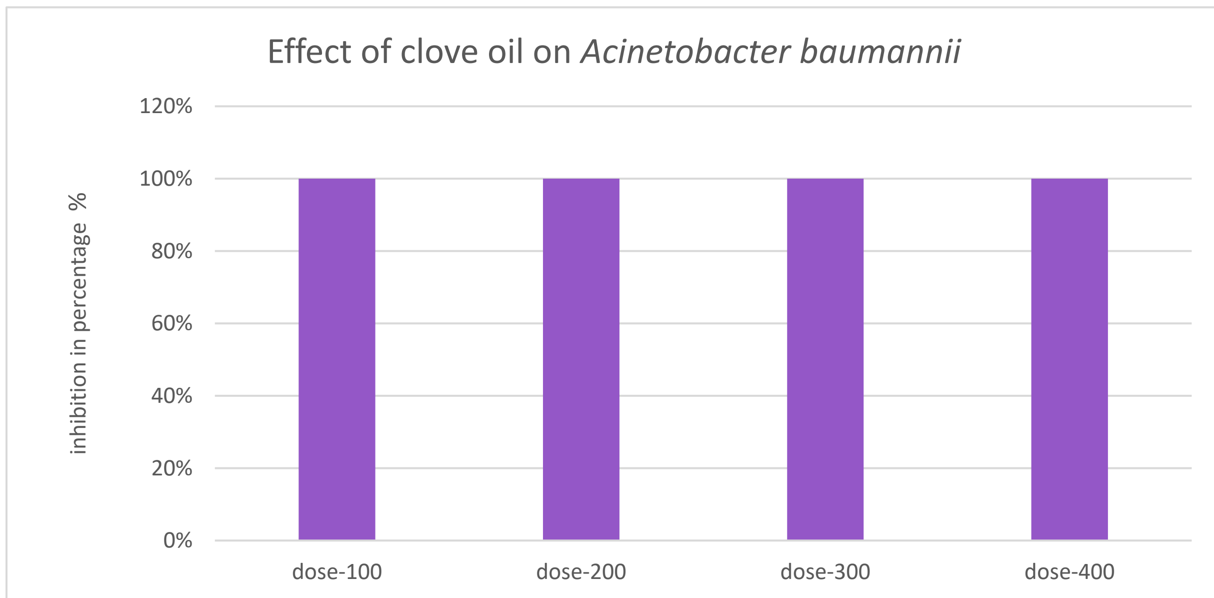

Figure 10: Inhibition of growth of Acinetobacter baumannii at different concentrations of clove oil

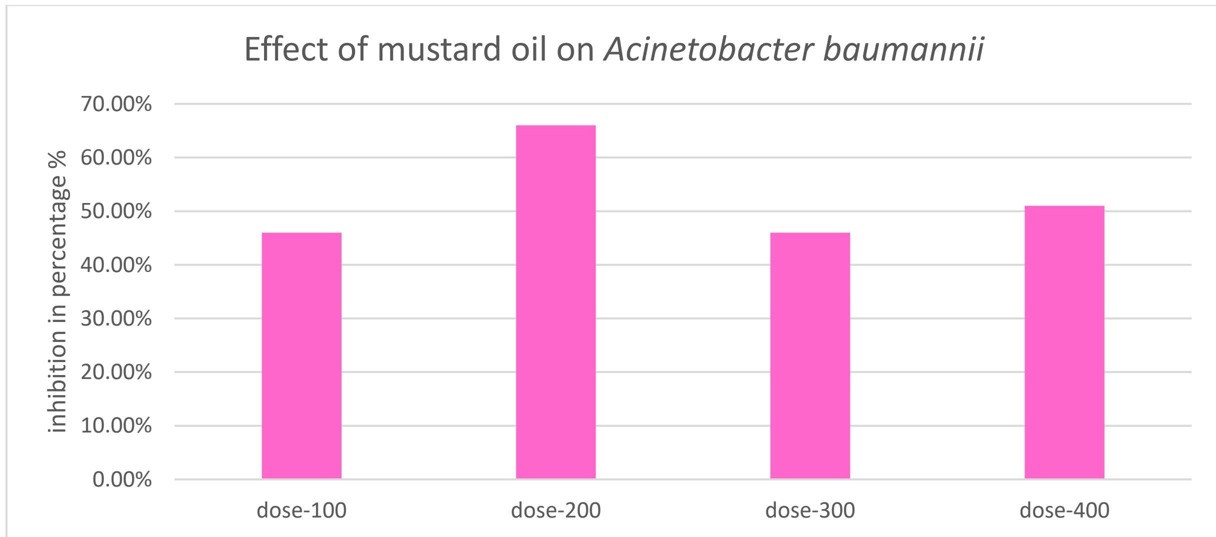

Figure 11: ***Inhibition of growth of Acinetobacter baumannii at different concentrations of mustard oil***

3.2.3.1.1. **Inhibition bacterial growth by essential oils by dilution technique followed by dose dependence on *Acinetobacter baumannii*:**

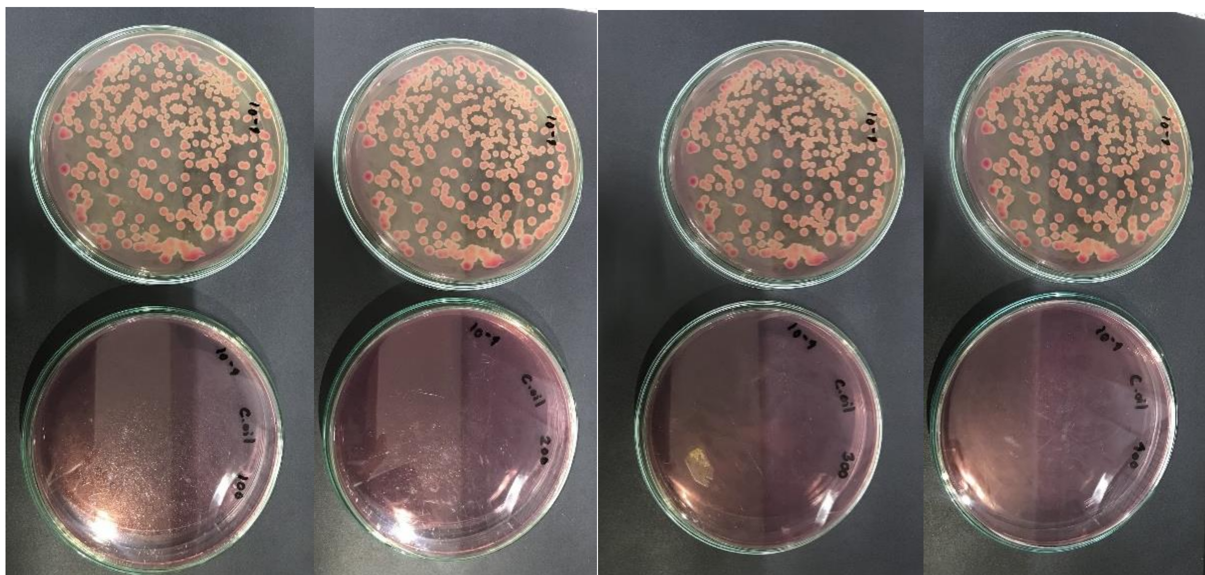

Figure 12: Inhibition of bacterial growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of clove oil on Acinetobacter baumannii

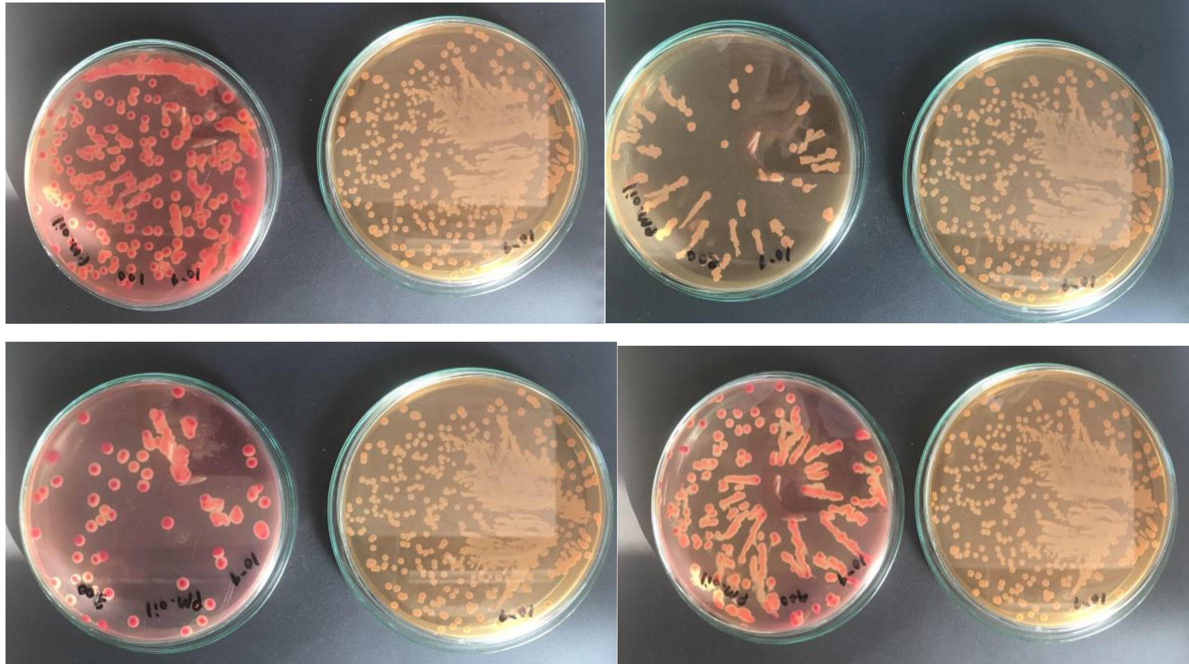

Figure 13: Inhibition of bacterial(Acinetobacter baumannii) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of clove oil

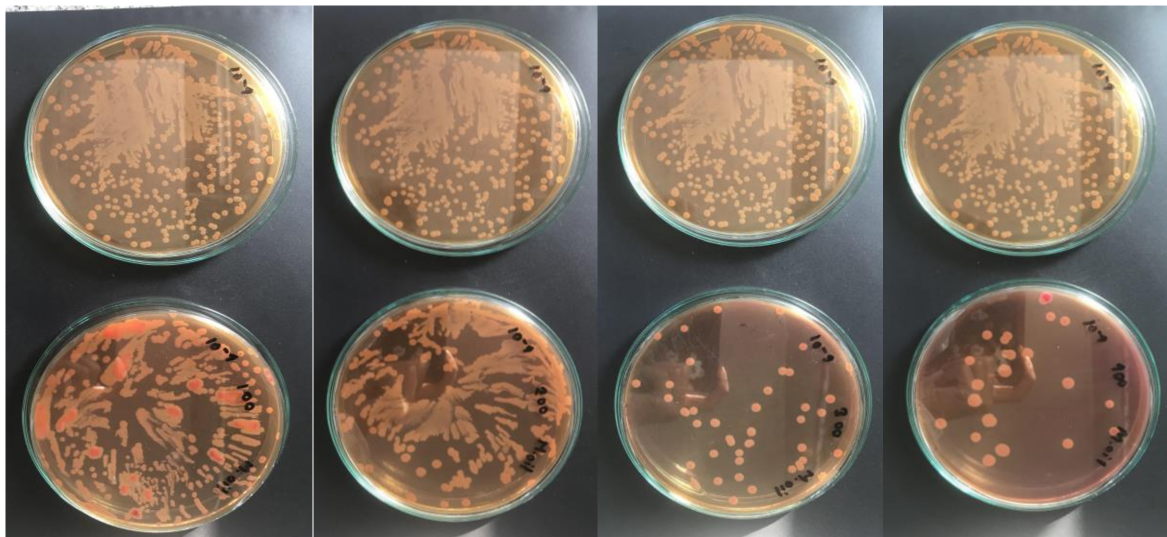

Figure 14: Inhibition of bacterial (Acinetobacter baumannii) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of mustard oil

3.2.3.2. Results of antimicrobial study of essential oils by Dilution technique followed by dose dependence on *Klebsiella pneumonia:*

Table 10. Inhibition rate in percentage of *Klebsiella pneumonia* by dilution technique

Essential oil	dose-100 µL/10 ml	dose-200 µL/10 ml	dose-300 µL/10 ml	dose-400 µL/10 ml
Pepperent oil	20.16%	41.32%	52.90%	44.51%
Clove oil	100%	100%	100%	100%
Mustard oil	35.00%	37.00%	42.00%	42.00%

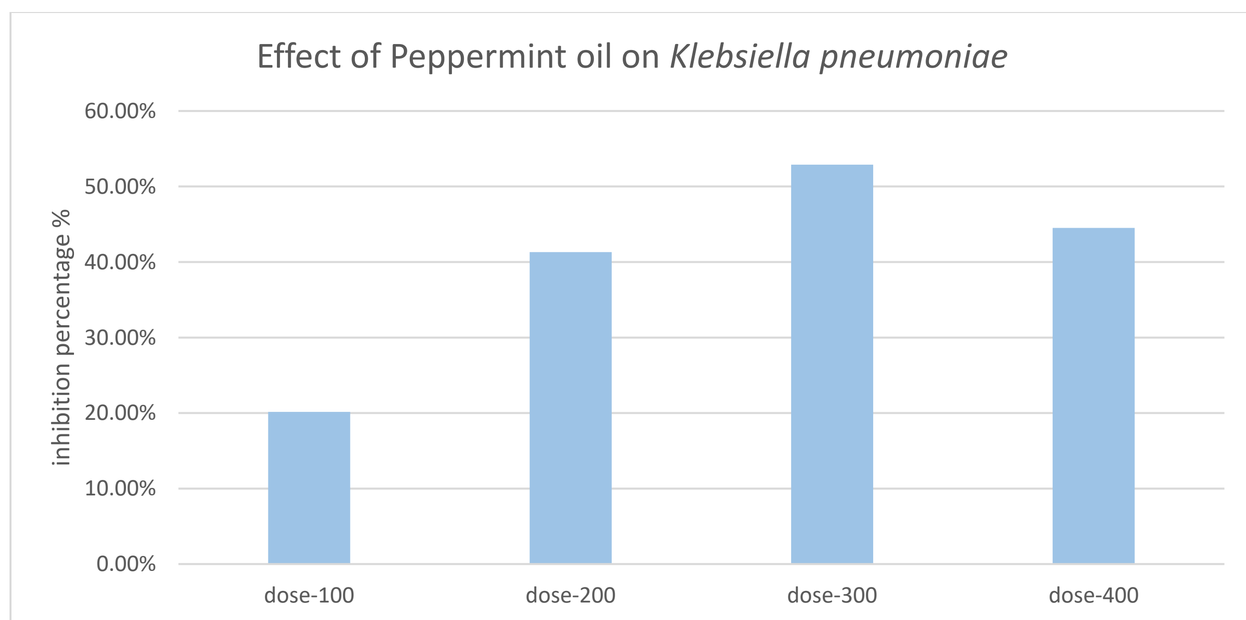

Figure 15:* *Inhibition of bacterial (Klebsiella pneumoniae growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of peppermint oil

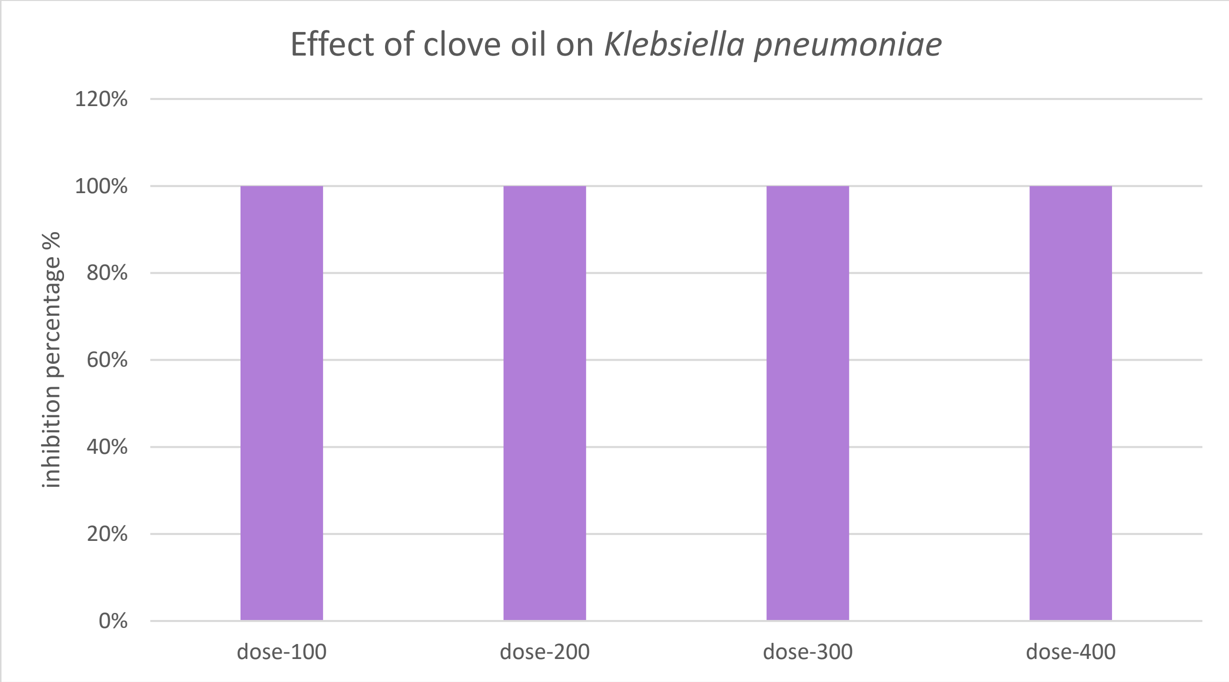

***Figure 16:* Inhibition of bacterial (Klebsiella pneumoniae) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of clove oil**

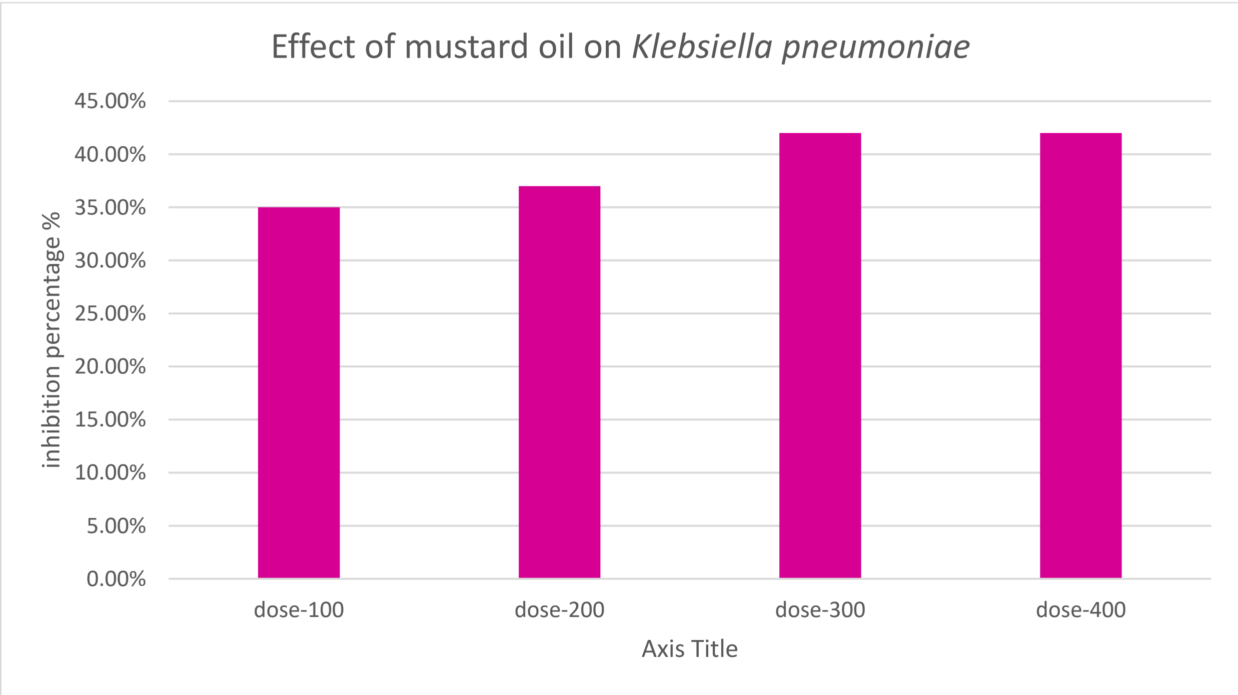

***Figure 17:* Inhibition of bacterial (Klebsiella pneumoniae) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of mustard oil**

3.2.3.2.2. Results of antimicrobial study of essential oils by Dilution technique followed by dose dependence on Klebsiella pneumonia:

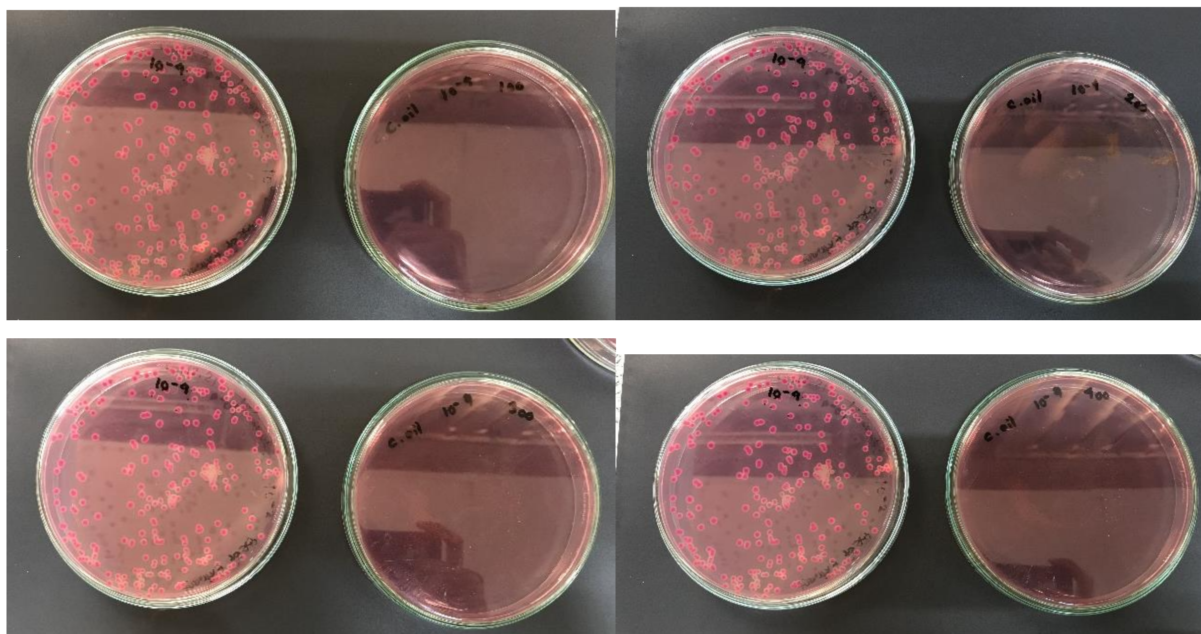

Figure 18: Inhibition of bacterial (Klebsiella pneumonia)growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of clove oil

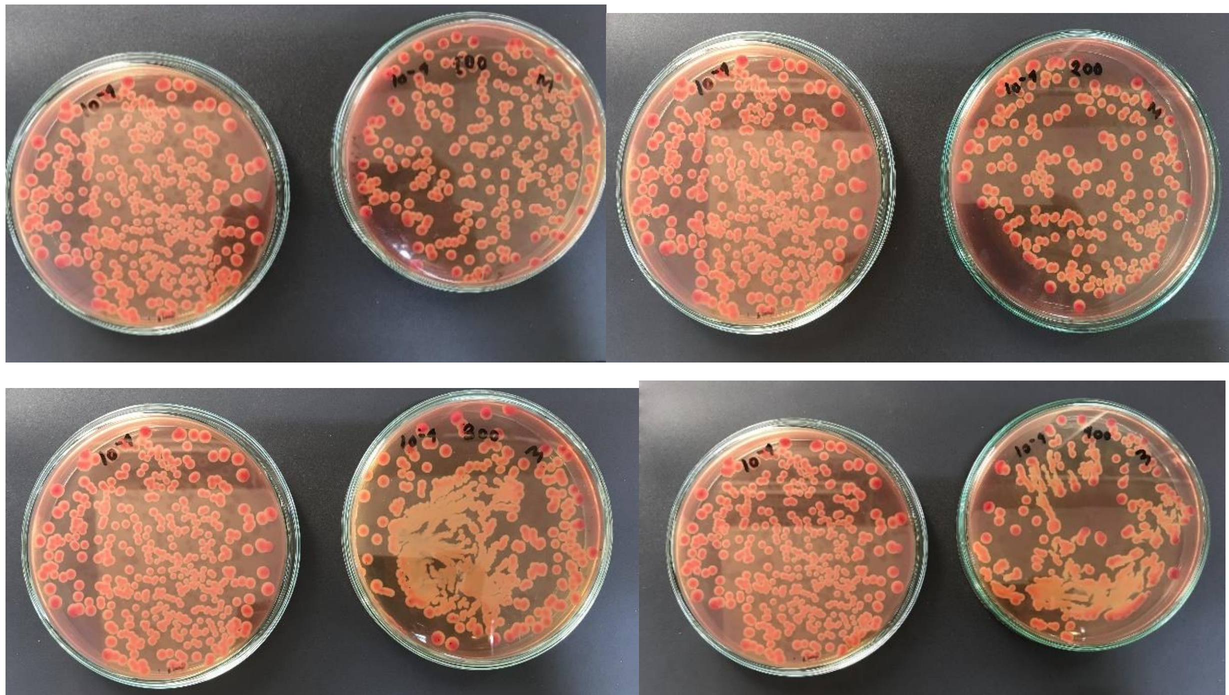

Figure 19: Inhibition of bacterial (Klebsiella pneumonia) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of mustard oil

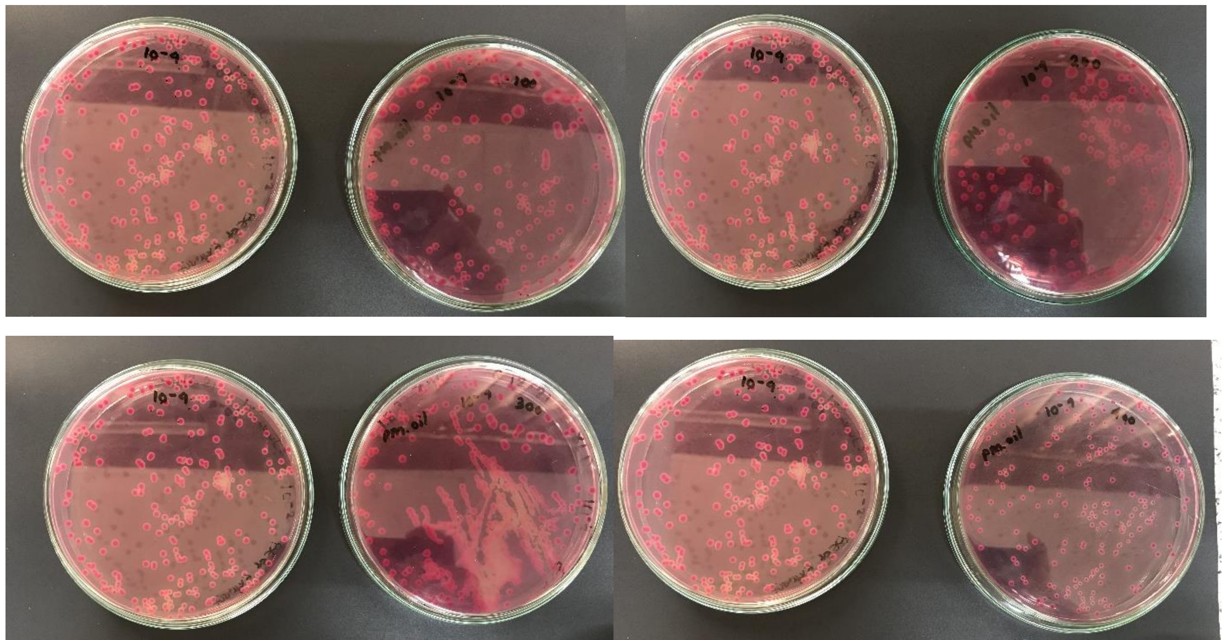

Figure 20: Inhibition of bacterial (Klebsiella pneumonia) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of peppermint oil

3.2.3.3. Results of the antimicrobial study of essential oils by Dilution technique followed by dose dependence on Pseudomonas taetrolens:

Table 11. *Inhibition rate in percentage of Pseudomonas taetrolens by dilution technique*

Essential oil	dose-100 µL/10 ml	dose-200 µL/10 ml	dose-300 µL/10 ml	dose-400 µL/10 ml
Peppermint oil	20.90%	47.31%	40.36%	61.02%
Clove. oil	100%	100%	100%	100%
Mustard. oil	10.30%	13.91%	15.17%	38.00%

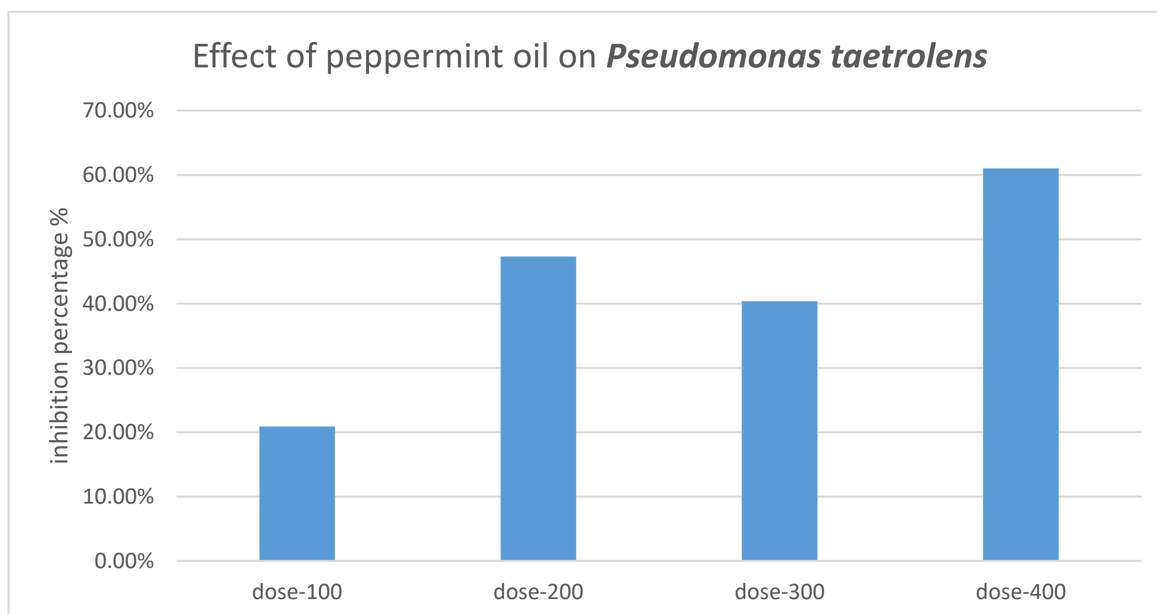

Figure 21: **Inhibition of bacterial growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of peppermint oil on *Pseudomonas taetrolens***

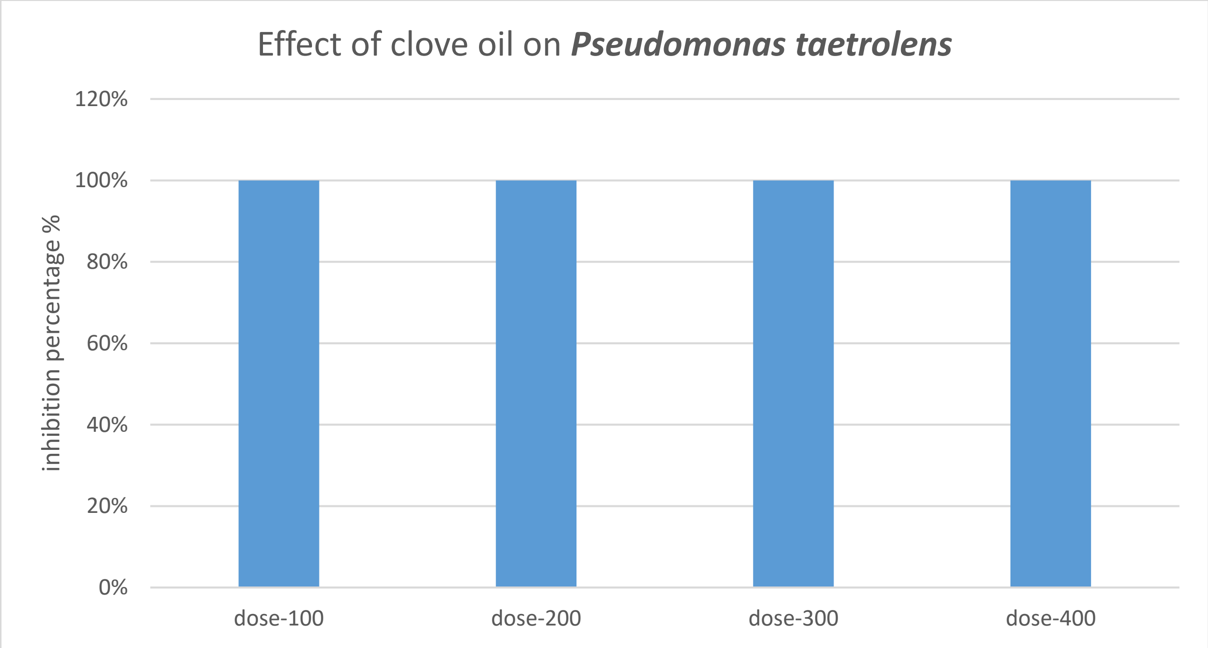

Figure 22: **Inhibition of bacterial growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of clove oil on *Pseudomonas taetrolens***

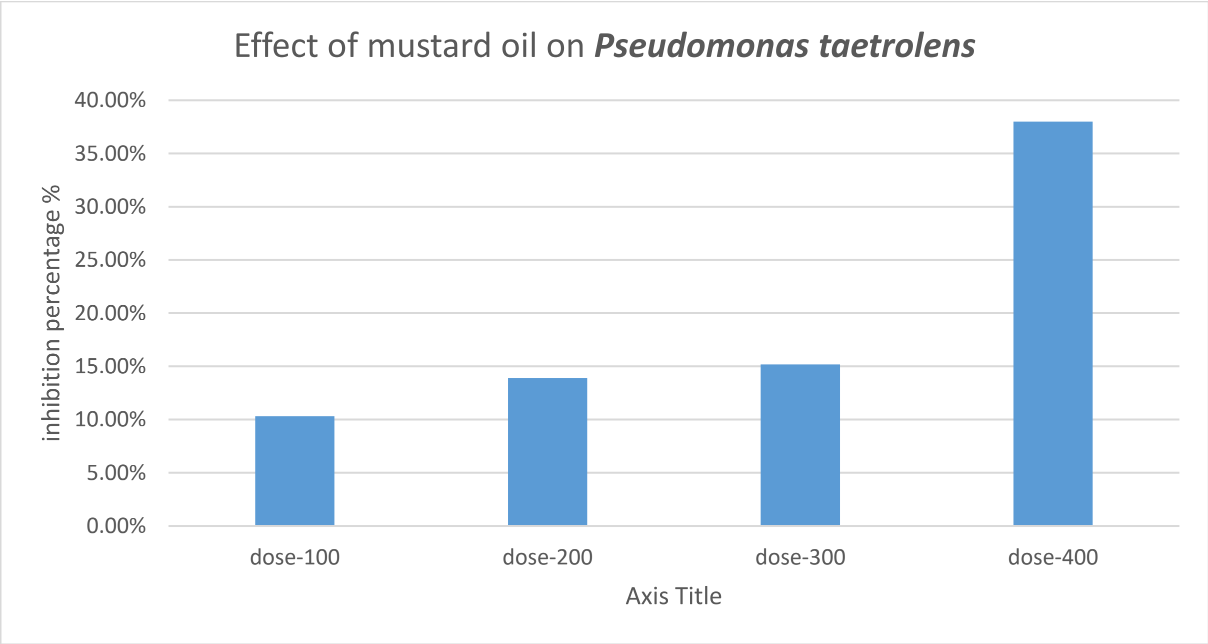

Figure 23: **Inhibition of bacterial growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of mustard oil on *Pseudomonas taetrolens***

3.2.3.3.1. Figures of the results of antimicrobial study of essential oils by dilution technique followed by dose dependence on *Pseudomonas taetrolens*:

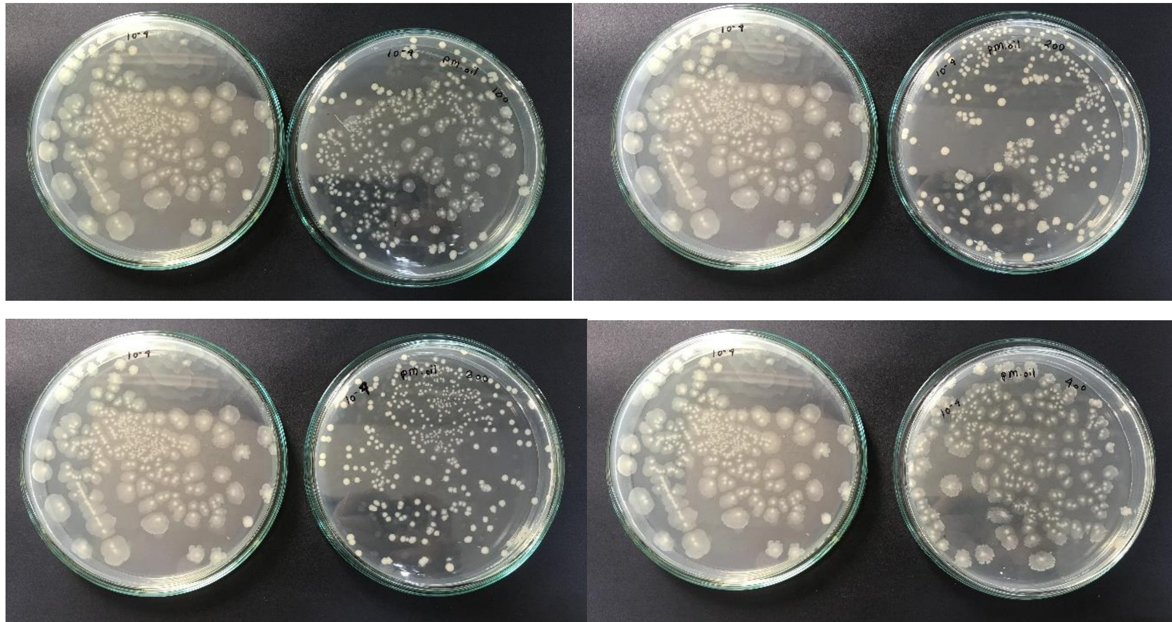

Figure 24: **Inhibition of bacterial (*Pseudomonas taetrolens*) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of peppermint oil**

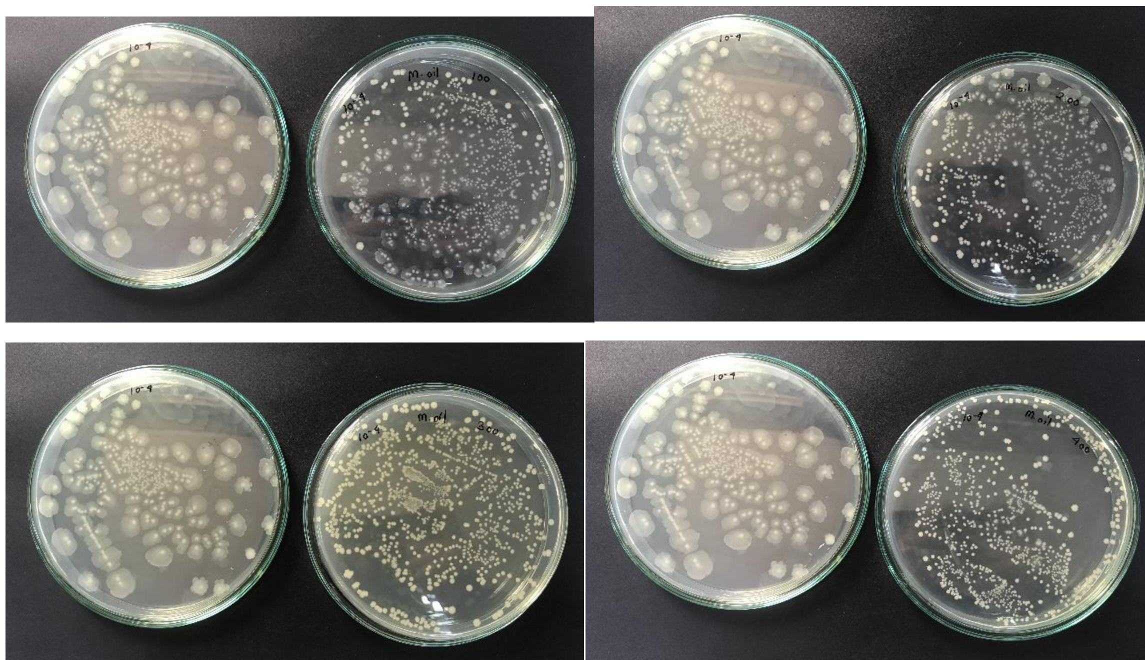

Figure 25: **Inhibition of bacterial (*Pseudomonas taetrolens*) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of mustard oil**

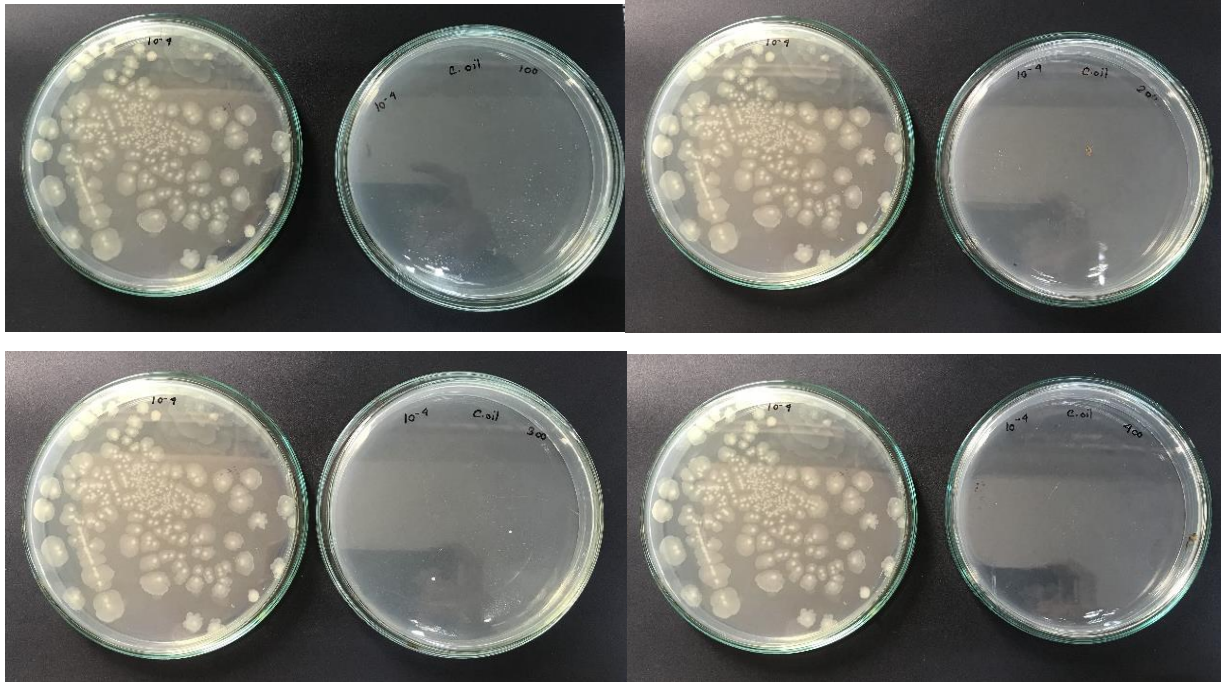

Figure 26: **Inhibition of bacterial (*Pseudomonas taetrolens*) growth at 100 µL/10 ml, 200 µL/10 ml, 300 µL/10 ml, and 400 µL/10 ml of *clove oil***

Chapter-4

Discussion

Discussion

The acquired test results in this study show that bacteria that are categorized MDR or above, can be treated with essential oils. First, peppermint oil was capable of inhibiting *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas taetrolens* up to 94.06%, 44.51%, and 61.02% respectively. Second, mustard oil was capable of inhibiting the growth of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas taetrolens* up to 51%, 42%, and 38% respectively. Third, clove oil was capable of inhibiting *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas taetrolens* up to 100%. After analyzing the data of this study, clove oil is seen to be the most effective one whereas peppermint was moderate and mustard oil was less effective oil.

In the disc diffusion and agar well diffusion process, an antibiotic disc was used side by side. The antibiotic kanamycin is a broadly used antibiotic for infections. When it showed no zone, clove oil was able to show the zone. Even though both peppermint and mustard oil was unable to create a zone on plate with bacterial growth.

In the test results, there are some inconsistencies as well. For example, in dose-dependency, it is ideal to get a higher inhibition rate in higher doses. However, in some of the cases, the higher doses showed a lower inhibition rate. Several reasons can be underlying the inconsistency. It can be the reason oil and water are immiscible. As a result, there can be different compositions in different tubes that might have resulted in inconsistent inhibition rates. Also, there can be an ideal dose for some oil up to which it inhibits organisms. However, these are only the possible reasons. To figure out the exact reason, further research needs to be done.

Peppermint oil was highly effective against *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas.* These were all clinical MDR strain. (Muntal et al., 2019) similar result was seen in this study as well. In this study, peppermint oil was highly effective against *Acinetobacter baumannii*, *Klebsiella pneumonia* in comparison to Pseudomonas strain. Research on clove oil showed high effectiveness of this essential oil on *Klebsiella pneumonia* depending on zone of inhibition in diameter (Ginting et al., 2021). This study also showed inhibition of growth as indicated by zone of inhibition for clove oil on specific bacteria in both agar well diffusion process and disc diffusion process. Another study on clove oil proved that clove oil possessed potent anti-bacterial activity on *Acinetobacter baumannii*. (Intorasoot et al., 2017) This was true for the study

as well. This study found the clove oil was highly effective against *Acinetobacter baumannii*. Delia Muntean et al. investigated the effects of peppermint oil on MDR patients admitted to hospitals. They employed the agar disk diffusion method and the microdilution method for this. It's MICs for *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* were all around 40mg/mL, which is quite high. They came to the conclusion that peppermint oil could be used to treat MDR bacteria. (Muntean et al., 2019) Clove oil was studied by Amornrat Intorasoot et al. to see how it affected *Acinetobacter baumannii*. They employed the agar diffusion approach, the minimal inhibitory concentration, and the minimum bactericidal concentration to accomplish this (MBC). The potency of clove oil on *Acinetobacter baumannii* with MBC90 of 1 mg/mL was demonstrated at the end of the study. (Intorasoot and colleagues, 2017) Vanessa Lee Rosarior et al. investigated the effects of clove oil on *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. They employed the disk diffusion assay, Microwell dilution assay, DPPH and ABTS radical scavenging assays to accomplish this. They found it to be a promising antibacterial agent against Gram-positive and Gram-negative bacteria at the end of the investigation (Rosarior and colleagues, 2021).

The results of this study has both similarities and novelty with the previous scientific studies. The similarities relies on the close results on essential oils and bacterial strain. However, the novelty relies on how different the newly found results are and what might be the reasons of it than the previous ones. The difference in the results of the same oil or bacterial strain can be due to choice of oil. Essential oil being an efficient bioactive compound in research depends on the extraction procedure, period, harvesting procedure, region, and season. (Garzoli et al., 2015) These oils can be produced through steam distillation, fermentation, and extraction. These methods can alter the chemical compound of oils (Wińska et al., 2019). As these oils are commercially produced, little to no information of its compounds are known and so does its quantities. As a result, it was not possible to figure out the exact extraction procedure, period, harvesting procedure, region, and season. These oils might have difference in these factors that alters the efficiency of essential oils on the same strain.

Chapter- 5

Conclusion

Conclusion

The study reveals possible essential oils to treat MDR, XDR, and PDR organisms by incorporating them into medicines. In this study, it has been proven that clove oil can be one of the most effective essential oils to treat harmful bacteria like *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas taetrolens.* These can be deadly from time to time. Antibiotics are not always the solution nowadays as the bacteria are getting more resistant day by day. So, it is important to take a step behind and reconsider the chemical compounds of essential oils to incorporate these into medicines.

This study lacks in finding out the consumable dose of essential oils. There are few studies that could assume the consumable dose. Peppermint oil is well tolerated mild oil to consume, but it might be harmful at higher dosages (Kligler et al., 2007) So, further investigations need to be done to find out if the essential oils used in this research (peppermint, mustard, and clove oil) are safe for human consumption and how to incorporate them into medicines.

Chapter-6

Reference

Reference

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Chapter- 7

Appendix

Appendix

Media composition

1. NA media: 1 liter, Himedia, India

Ingredients	Gms / Litre
Peptone	10.000
Meat extract	10.000
Sodium chloride	5.000
Agar	12.000
pH after sterilization	7.3±0.1

2. MAC media: 1 liter, Oxoid, England

Ingredients	Gms / Litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

3. TSI media: 1 liter, Himedia, India

Ingredients	Gm/L
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30

Casein enzymatic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0

4. MR-VP broth: 1 liter

Ingredients	**Gm/L**
Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0

5. MHA media: 1 liter, HIMEDIA

Ingredients	**gm/L**
Beef extract	2.00
Acid Hydrolysate of Casein	17.50
Starch	1.50
Agar	17.00

6. Simmons Citrate media: 1 liter, Oxoid, England

Ingredients	**Gm/L**
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bactobromthymol blue	0.08

7. Phenol red glucose: 1 liter

Ingredients	Gm/L
Peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Glucose	5.0
Phenol red	0.018
pH	7.4

8. Phenol red lactose: 1 liter

Ingredients	Gm/L
Peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Lactose	5.0
Phenol red	0.018
pH	7.4

9. Phenol red sucrose: 1 liter

Ingredients	Gm/L
Peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Sucrose	5.0
Phenol red	0.018
pH	7.4

10. Blood Agar: 1 liter, HIMEDIA

Ingredients	**Gm/L**
HM peptone B	10.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Blood	5%

11. Dnase agar: 1 liter, HIMEDIA

Ingredients	**Gm/L**
Tryptose	20.0
Deoxyribonucleic acid (DNA)	2.0
Sodium chloride	5.0
Methyl green	0.050
Agar	15.0

12. MIU: 1 liter, HIMEDIA

Ingredients	**Gm/L**
Tryptone	10.0
Dextrose	1.0
Sodium chloride	5.0
Phenol	0.01
Agar	2.0

Reagents

1. **MR reagent:**

Ingredients	**(g/L)**
Polypeptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g
Distilled water	1 L
Final pH	6.9

2. **Voges-Proskauer Reagent A: Barritt's reagent A**

Alpha-Naphthol, 5%	50 gm
Absolute Ethanol	1000 ml

3. V**oges-Proskauer Reagent B: Barritt's reagent B**

Potassium Hydroxide	400 gm
Deionized Water	1000 ml

4. **Oxidase reagent:**

N,N,N1,N1-tetramethyl-p-phenyldiamine-dihydrochloride	100g
Distilled water	10mL

List of antibiotics

Sl.	Antibiotics	Amount per disc	Manufacturer
1.	Amikacin	30	Oxoid
2.	gentamicin	30	Oxoid
3.	netilmicin	30	Oxoid
4.	imipenem	10	Oxoid
5.	meropenem	10	Oxoid
6.	ceftazidime	30	Oxoid
7.	cephalexin	30	Oxoid
8.	ciprofloxacin	5	Oxoid
9.	moxifloxacin	5	Oxoid
10.	vancomycin	5	Oxoid
11.	amoxiclav/Clavulanic acid	30	Oxoid
12.	penicillin G	10	Oxoid
13.	colistin	10	Oxoid
14.	polymixin B	300 units	Oxoid
15.	rifampicin	5	Oxoid
16.	tetracyclines	30	Oxoid
17.	doxycycline	30	Oxoid
18.	aztreonam	30	Oxoid
19.	clindamycin	2	Oxoid
20.	chloramphenicol	30	Oxoid