Antimicrobial activity of Tea Tree oil *(Melaleuca alternifolia)* against skin infection causing bacteria and comparison of its effectiveness with that of the Eucalyptus oil, Lemongrass oil and conventional antibiotics.

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

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DECLARATION

I hereby solemnly declare that the thesis project titled **"Antimicrobial activity of Tea Tree oil (*Melaleuca alternifolia*) against skin infection causing bacteria and comparison of its effectiveness with that of the Eucalyptus oil, Lemongrass oil and conventional antibiotics."** submitted by the undersigned has been carried out under the supervision of Dr. M. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University.

The presented dissertation is based on original research work carried out by myself and has not been submitted to any other institution for any degree or diploma. Any reference to work done by any other person or institution or any material obtained from other sources have been accordingly cited and referenced.

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ABSTRACT

The last several years it's seen that rising number of bacteria is developing resistance to synthetic antibiotics which hinders combating infective health conditions. It's known to everyone that most antibiotics no longer work; infections are getting harder or impossible to control. It is high time to find alternative to antibiotics from natural sources.

Tea Tree oil (TTO) is known to have antibacterial effects and this study was aimed to determine the abilities to control the growth of some skin infection causing bacteria and also compared the antimicrobial effectiveness of Eucalyptus oil (ECO), Lemongrass oil (LGO) and some common antibiotics those are using for bacterial infection. This study of antibacterial activity against selected pathogens was done by Broth dilution method and Agar well diffusion method. Pathogens (*Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus vulgaris, Aeromonas hydrophila, Escherichia coli, Streptococcus pneumoniae, Bacillus subtilis, Klebsiella pneumonia* and *Streptococcus agalactiae*) were taken for this study.

The inhibition of bacterial growth after 24 hours incubation exhibits greater results than 6 hours incubation in most of the cases. **Tea Tree oil** showed **100%** inhibition against almost all the bacteria selected for this study whereas **Eucalyptus oil** showed minimum **21.87%** against *E.coli* and maximum **100%** inhibition against *P. vulgaris* and *A. hydrophila* and the another essential oil that is **Lemongrass oil** exhibited minimum **72.72%** against *S. agalactiae* and maximum **100%** inhibition against *S. aureus, P. vulgaris* and *A. hydrophila*. TTO showed noticeable zone against all the tested bacteria in well diffusion method. On the other hand, ECO and LGO didn't exhibit satisfactory results in well diffusion method; most of the bacteria exhibited very low degree of sensitivity moreover others were resistant to eucalyptus and lemongrass oil. At last, there was a comparison between tea tree oil and some broad spectrum antibiotics by well diffusion method. Tea tree oil exhibited observable zone against all the bacteria contrariwise, among nine antibiotics only two of them were showed noticeable zone of inhibition to all the bacteria tested others didn't exhibit any zone of inhibition to multiple bacteria. From above results, it is expected that natural elements will gradually take place of antibiotics as conventional antibiotic become resistant day by day against emerging bacteria.

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List of Abbreviations:

TTO	Tea Tree oil
ECO	Eucalyptus oil
LGO	Lemongrass oil
SSTIs	Bacterial skin and soft tissue infections
MHA	Mueller Hinton Agar
BHIB	Brain Heart Infusion Broth
NA	Nutrient Agar
MDR	Multi Drug Resistant
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
HPLC	High Performance Liquid Chromatography
WHO	World Health Organization
EMB	Eosin Methylene Blue Agar
MAC	MacConkey Agar
MSA	Manitol Salt Agar
BAP	Blood Agar Plate
TSI	Triple Sugar Iron
MR	Methyl Red
VP	Voges- Proskauer
CFU	Colony Forming Unit

Chapter 1

Introduction

Introduction

1.1 Overview

The world seems to be running out of antibiotics. While any antimicrobial resistance is concerning, the increasing incidence of antibiotic-resistant Gram-negative bacteria has become a particular problem as strains resistant to multiple antibiotics are becoming common and no new drugs to treat these infections will be available in the near future (Schneider *et al*., 2017). There's a larger problem—the problem of resistance is also due to an abuse of antibiotics. Many people will go to a doctor and demand an antibiotic when they have a cold or a flu, for which these antibacterial compounds are useless. In many countries it is possible to buy antibiotics over the counter. Often, if people are poor, they will not take the full dose—all of that leads to resistance (Moskvitch, 2015).Human skin serves as the first line of defense against microbial infection as a physical barrier. But the skin has an extremely diverse ecology of organisms that may produce infection. Traditionally, pharmacotherapeutic recommendations have been based on bacterial etiology. Unfortunately, most often, the specific bacterial etiology of an SSTI is unknown and clinicians are forced to prescribe empirically. As a result, treatment recommendations based solely on organisms are difficult to apply clinically (Ki and Rotstein, 2008). Tea tree oil has recently emerged as an effective topical antimicrobial agent active against a wide range of organisms. Tea tree oil may have a clinical application in both the hospital and community, especially for clearance of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage or as a hand disinfectant to prevent cross-infection with Gram-positive and Gram-negative epidemic organisms (May *et al*., 2000). The antibacterial properties in eucalyptus essential oil are well established, and its antiseptic nature makes it appropriate for treating wounds like burns, sores, cuts, and abrasions (Yaneff, 2017). Lemongrass may prevent the growth of some bacteria and yeast and it has antioxidant properties. It contains substances that are used to alleviate muscle pain, reduce fever, and to stimulate uterus and menstrual flow. Essential oils are able to penetrate the skin and pass into the blood stream and into different areas of the body for internal therapeutic benefits (Axe, 2015).

1.2 Essential Oils as treatment agent

Essential oils have great medicinal benefits as they contain the essence of herbs and flowers in concentrated form. The aroma molecules are very potent organic plant chemicals that make the surroundings free from disease, bacteria, virus and fungus. Their versatile character of antibacterial, antiviral, anti-inflammatory nature along with immune booster body with hormonal, glandular, emotional, circulatory, calming effect, memory and alertness enhancer, is well documented by many scientists (Ali *et al*., 2015).Essential oils are a rich source of biologically active compounds. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils. Therefore, it is reasonable to expect a variety of plant compounds in these oils with specific as well as general antimicrobial activity and antibiotic potential (Darokar *et al*., 1998).

1.3 Alternative to conventional antibiotics

Nowadays, use of alternative and complementary therapies with mainstream medicine has gained the momentum. Aromatherapy is one of the complementary therapies which use essential oils as the major therapeutic agents to treat several diseases. It came into existence after the scientists deciphered the antiseptic and skin permeability properties of essential oils (Ali *et al*., 2015). Antibiotic resistance is becoming a crisis and according to many experts the growing inability of antibiotics to effectively treat illnesses owing to antimicrobial resistance is one of medicine's most significant challenges of the new century. Not only have antibiotics been oversubscribed, but according to research, the amount of antibiotics consumed by livestock is increasing year on year (Deckard, 2015).

1.4 General Characteristics of Tea Tree Oil

Tea tree oil is derived from the leaves of the tea tree. The tea tree was named by eighteenth century sailors, who made tea that smelled like nutmeg from the leaves of the tree growing on the swampy southeast Australian coast. Do not confuse the tea tree with the unrelated common tea plant that is used to make black and green teas. Tea tree oil is applied to the skin (used topically) for infections such as acne, fungal infections of the nail (onychomycosis), lice, scabies, athlete's foot (tineapedis), and ringworm. It is also used topically as a local antiseptic for cuts and abrasions, for burns, insect bites and stings, boils, vaginal infections, recurrent herpes labialis, toothache, infections of the mouth and nose, sore throat, and for ear infections such as otitis media and otitis externa (WebMD, 2009).

Figure 1.1: Tea Tree oil

1.4.1 Classification of Tea Tree oil (Plant Database, USDA)

Kingdom - Plantae

Sub kingdom - Tracheobionta

Super division- Spermatophyta

Division -Magnoliophyta

Class - Magnoliopsida

Subclass - Rosidae

Order –Myrtales

Family – Myrtaceae

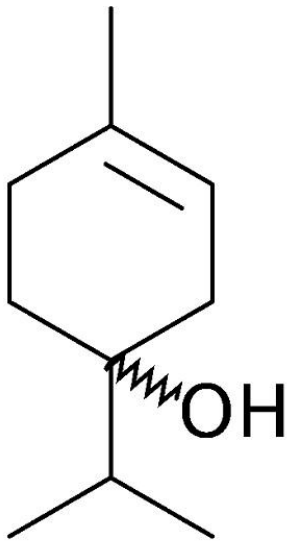
Genus - *Melaleuca L.*

1.4.2 Chemical Composition of Tea Tree oil

TABLE 1. Composition of *M. alternifolia* (tea tree) oil

Component	Composition (%)	
	ISO 4730 range[a]	Typical composition[b]
Terpinen-4-ol	≥30[c]	40.1
γ-Terpinene	10–28	23.0
α-Terpinene	5–13	10.4
1,8-Cineole	≤15[d]	5.1
Terpinolene	1.5–5	3.1
ρ-Cymene	0.5–12	2.9
α-Pinene	1–6	2.6
α-Terpineol	1.5–8	2.4
Aromadendrene	Trace–7	1.5
δ-Cadinene	Trace–8	1.3
Limonene	0.5–4	1.0
Sabinene	Trace–3.5	0.2
Globulol	Trace–3	0.2
Viridiflorol	Trace–1.5	0.1

[a] IOS 4730, International Organization for Standardization standard no. 4730



Terpinen-4-ol

Figure 1.2: Chemical composition of Tea Tree Oil

1.4.3 Medicinal Uses of Tea Tree oil

- **Topical Antiseptic to Treat Wounds and Infections:** It's even been shown in scientific research published in the Journal of Investigative Dermatology in 2006 to be effective at fighting the super-bug MRSA, which is a strain of staph bacteria that is resistant to many common antibiotics (Bright, 2017).
- **Acne Treatment:** Tea tree oil's antimicrobial terpenes content also makes it popular for combating acne. Not only does it kill MRSA or staph infections, but it will also kill *Propionibacterium acnes* that live inside hair follicles and can lead to inflammation and acne (Bright, 2017).
- **Ear Infections:** Melaleuca oil is safe to use topically on almost every part of the body, including the ear canal. For people suffering from ear infections. Dropping a few drips of melaleuca oil into the ear can clear out the infection and it can even reduce pain and speed the healing process (OrganicFacts, 2011).
- **Nail Fungus:** Tea tree oil has the ability to kill parasites and fungal infections, which is why it's so popular for use in fighting toenail fungus, ringworm and athlete's foot.

Apply undiluted tea tree oil twice daily to affected areas like nails or feet to relieve symptoms, and possibly completely heal these unsightly ailments (Bright, 2017)

1.5 General Characteristics of Eucalyptus oil

Eucalyptus is a tree. The dried leaves and oil are used to make medicine. Though eucalyptus is used medicinally for many purposes, there isn't enough scientific evidence so far to rate it as effective for any of them (WebMD, 2009).

Figure 2.1: Eucalyptus oil

1.5.1 Classification of Eucalyptus oil (Plant Database, USDA)

Kingdom - Plantae

Sub kingdom - Tracheobionta

Super division - Spermatophyta

Division -Magnoliophyta

Class - Magnoliopsida

Subclass -Rosidae

Order -Myrtales

Family - Myrtaceae

Genus - *Eucalyptus L'Hér*

Species -*Eucalyptus globulusLabill.*

1.5.2 Chemical composition of Eucalyptus oil

Compounds in Eucalyptis Essential Oil	Mean (%)	Minimum (%)	Maximum (%)
1. 3-Methylbutanal	0.1	0.0	0.7
2. α-Pinene	14.0	1.5	26.7
3. β-Pinene	0.4	0.0	3.4
4. β-Myrcene	0.3	0.0	1.2
5. Limonene	3.6	0.0	13.2
6. p-Menth-2,4(8)-diene	0.3	0.0	1.6
7. 1,8-Cineol	63.8	47.7	75.8
8. p-Cymene	0.5	0.0	2.7
9. Terpinolene	0.1	0.0	0.4
10. Terpinen-4-ol	3.1	0.1	10.1
11. $C_{15}H_{24}$	1.7	0.0	7.4
12. α-Terpinyl acetate	0.6	0.0	4.7
13. Aromadendrene	2.0	0.0	7.1
14. Geranyl acetate	1.4	0.0	7.3
15. Viridiflorene	0.9	0.0	5.5
16. Neryl acetate	0.6	0.0	3.0
17. Elemene, α- or_β-	0.6	0.1	1.4
18. 2-Phenylethyl isovalerate	0.2	0.0	2.5
19. α-Bisabolol	0.6	0.1	3.8
20. Globulol	3.0	0.0	9.8
21. Guaiol	0.3	0.0	4.6
22. Ledol	0.4	0.0	4.9
23. Spathulenol	0.3	0.0	1.7
24. γ-Eudesmol	0.5	0.0	3.7
25. β-Eudesmol	0.8	0.0	5.5

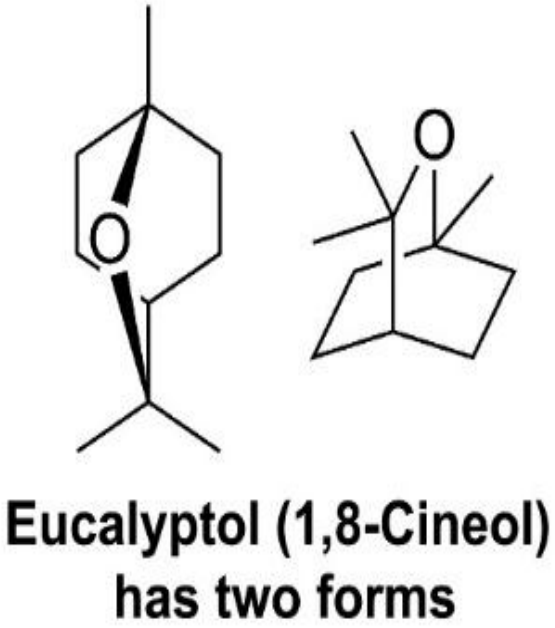


Figure 2.2: Chemical Composition of Eucalyptus oil

1.5.3 Medicinal Uses of Eucalyptus oil

- **Wounds:** Eucalyptus essential oil has antiseptic qualities because of its germicidal quality. Eucalyptus oil is used for healing wounds, ulcers, burns, cuts, abrasions, and sores. It is also an effective salve for insect bites and stings.
- **Respiratory problems:** Eucalyptus essential oil is effective for treating a number of respiratory problems including cold, cough, running nose, sore throat, asthma, nasal congestion, bronchitis, and sinusitis. Eucalyptus oil is antibacterial, antifungal, antimicrobial, antiviral, anti-inflammatory and decongestant in nature, which makes it a good ingredient in many medicines that treat respiratory problems.
- **Dental care:** The antibacterial and antimicrobial potential of eucalyptus has been harnessed for use in some mouthwash and dental preparations. In promoting dental health, eucalyptus appears to be active in fighting bacteria that cause tooth decay and periodontitis (Nordqvist, 2017).

1.6 General Characteristics of Lemongrass oil

Lemongrass is a plant. The leaves and the oil are used to make medicine. Lemongrass is used for treating digestive tract spasms, stomachache, high blood pressure, convulsions, pain, vomiting, cough, achy joints (rheumatism), fever, the common cold, and exhaustion. It is also used to kill germs and as a mild astringent (WebMD, 2009).

Figure 3.1: Lemongrass oil

1.6.1 Classification of Lemongrass oil (Plant Database, USDA)

Kingdom - Plantae

Sub kingdom - Tracheobionta

Super division- Spermatophyta

Division -Magnoliophyta

Class - Liliopsida

Subclass -Commelinidae

Order –Cyperales

Family -Poaceae – Grass family

Genus -*CymbopogonSpreng*.

Species -*Cymbopogonflexuosus*

1.6.2 Chemical Composition of Lemongrass oil

Table 1
Percentage composition (> 1%) of the lemongrass essential oil

Components	Lemongrass oil (%)
Limonene	4.39
Citronellal	1.32
n.i.	2.16
Borneol	2.16
n.i.	1.66
Neral	31.85
Geranial	40.79
Neryl acetate	2.95
Z-caryophyllene	2.71

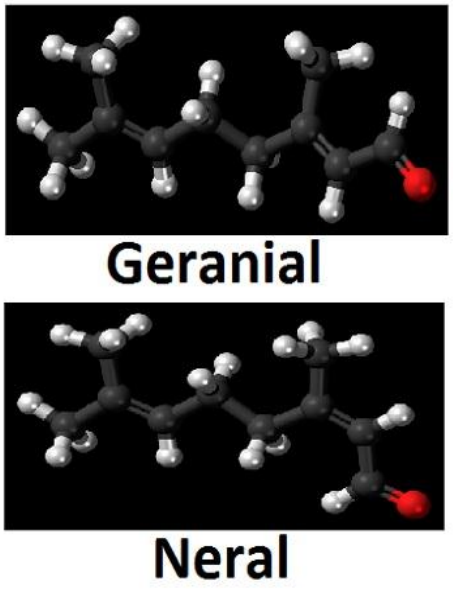


Figure 3.2: Chemical Composition of Lemongrass oil

1.6.3 Medicinal Uses of Lemongrass oil

- **Infections:** Lemongrass can help kill both internal and external bacterial and fungal infections, such as ringworm and athlete's foot. In a 2008 study at Weber State University in Utah, it was found that out of 91 essential oils tested, lemongrass ranked highest in inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Mercola,2017).
- **Fever:** The antipyretic effect of lemongrass oil helps bring down very high fever, especially when it is beginning to reach dangerous levels.
- **Digestive issues:** A diluted lemongrass mixture may assist in facilitating nutrient assimilation and boosts the functioning of the digestive system, which is helpful alleviating bowel problems and digestive disorders. It may also prevent the formation of excessive gas and increases urination, which helps eliminate toxins from the body (Mercola, 2017).

1.7 Skin infection Caused by Clinical Pathogens Used in this Study

Table 1: List of Skin Infection Caused by Clinical Pathogen Used in this Study

Sl no.	Organism	Infections
A.	*Staphylococcus aureus*	Impetigo, Ecthyma (crusted ulcers), Cellulitis and Secondary skin infection of wounds, dermatitis, scabies, diabetic ulcers etc. (Stanway, 2002).
B.	*Streptococcus pyogenes*	Impetigo, Erysipelas and Cellulitis (Stevens & Bryant, 2016)
C.	*Pseudomonas aeruginosa*	Soft tissue infection, Ecthyma Gangrenosum nosocomial infection (Kingsbery *et al.*, 2017)
D.	*Proteus vulgaris*	Intertrigo and Common Secondary Skin Infections (Gonzalez *et al*.,2016)
E.	*Aeromonas hydrophila*	Necrotizing fasciitis and myonecrosis, tissue damage (Markov *et al*., 2007)
F.	*Escherichia coli*	Necrotizing fasciitis, surgical site infections, infections after burn injuries (Petkovšek *et al*.,2009)
G.	*Streptococcus pneumonia*	Superficial skin infections in children (rare case)
H.	*Bacillus subtilis*	Opportunistic Pathogen, Wound infection , Ear infection, Sepsis, Mostly affects infants and Immuno-compromised patients (Kirk, 2009)
I.	*Klebsiella pneumonia*	nosocomial infection, wound infection
J.	*Streptococcus agalactiae*	soft tissue infections in non-pregnant adults

1.8 Resistance of Bacteria to Antibiotics

Antimicrobial resistance occurs naturally over time, usually through genetic changes. However, the misuse and overuse of antimicrobials is accelerating this process. In many places, antibiotics are overused and misused in people and animals, and often given without professional oversight. Without effective antimicrobials for prevention and treatment of infections, medical procedures such as organ transplantation, cancer chemotherapy, diabetes management and major surgery (for example, caesarean sections or hip replacements) become very high risk (WHO, 2016).Antibiotic resistance is a growing and worrying problem associated with increased deaths and suffering for people. Overall, there are only two factors that drive antimicrobial resistance, and both can be controlled. These factors are the volumes of antimicrobials used and the spread of resistant micro-organisms and/or the genes encoding for resistance (Collignon, 2015).

1.8.1 Resistance of *Staphylocossus aureus*

According to the results of susceptibility tests of all isolates of *S. aureus*, they have been identified as sensitive to vancomycin, daptomycin, linezolid, and levofloxacin. While the resistance rates to nitrofurantoin, quinupristin-dalfopristin, and trimethoprim-sulfamethoxazole were determined as 0.3%, 2.4%, and 6%, respectively, resistance rates to penicillin, erythromycin, rifampicin, gentamicin, and clindamycin were determined as 100%, 18%, 14%, 14%, and 11%, respectively (Rağbetli et al., 2016).

1.8.2 Resistance of *Streptococcus pyogenes*

S. pyogenes isolates, 27 (12.7%) were resistant to erythromycin, and one isolate (0.5%) showed intermediate susceptibility (Table I). The resistance rate was higher in isolates from children (18.9%) than in those from adults (10.7%). All erythromycin-resistant strains were also resistant to clarithromycin and vice versa. According to the DIN guidelines, the resistant and susceptible breakpoints for ciprofloxacin are ≥;4 mg/L and ≤;1 mg/L, respectively, although these breakpoints have not been specifically defined for *S. pyogenes*. Applying these DIN breakpoints, the ciprofloxacin resistance rate was 2.8% (Arvand, 2000).

1.8.3 Resistance of *Pseudomonas aeruginosa*

Treatment of infectious diseases becomes more challenging with each passing year. This is especially true for infections caused by the opportunistic pathogen *Pseudomonas aeruginosa*, with its ability to rapidly develop resistance to multiple classes of antibiotics. Although the import of resistance mechanisms on mobile genetic elements is always a concern, the most difficult challenge we face with *P. aeruginosa* is its ability to rapidly develop resistance during the course of treating an infection. *P. aeruginosa* showed a very high resistance to fosfomycin (81.0%). A similar resistance pattern was seen with ciprofloxacin (70.4%), levofloxacin (66.7%), ceftazidime (33.3%), piperacillin (44.4%), imipenem (55.6%), piperacillin and tazobactam (44.4% , 50.0%), tobramycin (52.0%), gentamicin (44.4%), and meropenem (42.3%) (Lister, *et al*., 2009).

1.8.4 Resistance of *Proteus vulgaris*

P. vulgaris is naturally resistant to ampicillin, narrow-spectrum cephalosporins and cefuroxime, by virtue of production of a similar beta-lactamase. Tigecycline has lesser activity against *P. vulgaris* than against other *Enterobacteriaceae*. *Proteus vulgaris* produces a chromosomally encoded beta-lactamase, referred to as the cefuroxime-hydrolyzing beta-lactamase, which hydrolyzes cephalosporins. The enzyme can be induced by ampicillin, amoxicillin and first generation cephalosporins, weakly induced by carboxypenicillins, ureidopenicillins, cefotaxime and ceftriaxone, and inhibited by clavulanate. Strains of *P. vulgaris* that have a mutation in the regulatory genes of this beta-lactamase produce high levels of the enzyme and are resistant to penicillins, cefuroxime, ceftriaxone and cefotaxime (Armbruster, 2002).

1.8.5 Resistance of *Aeromonas hydrophila*

A total of 319 strains of *Aeromonas hydrophila* were isolated from 536 fish and 278 prawns for a 2-year period. All the strains were tested for resistance to 15 antibiotics and 100% of the strains was resistant to methicillin and rifampicin followed by bacitracin and novobiocin (99%). Only 3% of the strains exhibited resistance against chloramphenicol (Vivekanandha, 2002).The use of antibiotics is one of the most important factors influencing the emergence of resistance in bacterial pathogens. Multi-resistant *A. hydrophila* were isolated from different parts of the world and are reported to be resistant to penicillin and ampicillin (Stratev, 2016).

1.8.6 Resistance of *Escherichia coli*

The ß-lactam antibiotics, especially the cephalosporins and ß-lactam- ß-lactamases inhibitor combinations, are major drug classes used to treat community-onset or hospital-acquired infections caused by *E. coli*, especially due to the ExPECpathotype resistance to aminopenicillins (e.g. ampicillin) and early-generation cephalosporins (e.g. cefazolin) among E. coli is often mediated by the production of narrow-spectrum ß-lactamases such as TEM-1, TEM-2 and to a lesser extent SHV-1 enzyme. Most importantly among E. coli, is the increasing recognition of isolates producing the so-called "newer ß-lactamases" that causes resistance to the expanded-spectrum cephalosporins and/or the carbapenems (Allen *et al*., 1998).

1.8.7 Resistance of *Streptococcus pneumoniae*

The first clinical isolate of *S. pneumoniae* not susceptible to penicillin (MIC, >2 μg/mL) was reported in 1986. About 60% of isolates with intermediate resistance to penicillin also had intermediate resistance to cefotaxime or ceftriaxone, and nearly all isolates were resistant to penicillin were also not susceptible to those two agents (Hsueh and Luh, 2002). Drug resistant *S.pneumonia e*resistant to other antibiotics as well, including erythromycin, Trimethoprim/sulfamethoxazole, Vancomycin, Tetracyclin, Chloramphenicol, Ofloxacin (SNHD, 1996).

1.8.8 Resistance of *Bacillus subtilis*

To investigate the stability of the antibiotic resistance markers present in *B. subtilis* O/C, T, N/R, SIN strains, we considered resistances to Chloramphenicol, Tetracycline, Rifampicin and Streptomycin. Resistances to Tetracycline, Rifampicin and Streptomycin were stably maintained for at least 200 generations in the absence of selective pressure (Mazza *et al*., 1992).

1.8.9 Resistance of *Klebsiella pneumonia*

Based on the pooled data from a number of studies conducted at different part of the globe, antibiotic resistances among KP isolates were found to be 100% for Cephradin, 87.5% for Cefeclor, 84% for Tobramycin, 82.5% for Cefotaxime, and 80.4% for Norfloxacin. Whereas, *K. pneumonia* was found to be more sensitive to Impenem (92.5%), Meropenem (92.5%), Amoxicillin / Clavulanic acid (87.5%), Gatifloxacin (85%), Moxifloxacin (75%) and chloraphenicol (62.8%) (Woldu, 2015).

1.8.10 Resistance of *Streptococcus agalactiae*

Resistance towards clindamycin and erythromycin was as high as 43,75% and 32,20% (Matani*et al*. 2016). Erythromycin and clindamycin resistance was 21.1 and 17.2 %, respectively, in which 69 % had harboured constitutive macrolide, lincosamide and streptogramin B (MLS_B), 17.4 % had inducible MLS_B. The M and L phenotypes were present in 6.8 % each. The methylation of target encoded by *ermB* genes was the commonest mechanism of resistance observed in 55 % of isolates, 38 % of isolates had both

erm*B*and *linB* genes and efflux pump mediated by *mefA* genes was also distributed among the isolates (Bolukaoto, 2015).

1.9 Literature Review

Carson *et al.,* 2006 researched that complementary and alternative medicines such as tea tree (*melaleuca*) oil have become increasingly popular in recent decades. This essential oil has been used for almost 100 years in Australia but is now available worldwide both as neat oil and as an active component in an array of products. This review summarizes recent developments in our understanding of the antimicrobial and anti-inflammatory activities of the oil and its components, as well as clinical efficacy. Specific mechanisms of antimicrobial and anti-inflammatory action are reviewed, and the toxicity of the oil is briefly discussed.

Falci*et al.,* 2015 explored that *Melaleuca* sp. oil and to assess its in vitro inhibitory effect against *Staphylococcus aureus* isolates obtained from lower limb wounds and resistant to several antibiotics. A total of 14 test-tubes containing Mueller-Hinton broth were used to determine the Minimum Inhibitory Concentration (MIC). The experimental study was carried out in triplicate at 37ºC for 18 hours. The Minimum Bactericidal Concentration (MBC), able to killing all the microorganisms, was also determined. Two S. *aureus* isolates were obtained from lower limb wounds of female patients and the identification of the microorganisms (*Staphylococcus aureus*) and the test for susceptibility to the antimicrobial agents were carried out by automation using the apparatus Micro Scan(r). After identification, the isolates were preserved in liquid Trypticase Soy medium, and inoculated for determination of the MIC and MBC. The MIC was 0.2% and the MBC was 0.4%. The *Melaleuca* sp. oil showed antimicrobial properties in vitro against strains isolated from lower limb wounds which were resistant to multiple antibiotics.

Lee*et al.,* 2013 showed in their study that components of TTO-L satisfied the International Organization for Standardization (ISO) 4730 guidelines. TTO-L and its components, terpinen-4-ol, terpinolene, α-terpinene, and α-terpineol, had strong inhibitory activities against *Propionibacterium acnes* and *Staphylococcus aureus*. In the skin irritation assay, TTO-L, terpinen-4-ol, and 1,8-cineole did not cause significant skin irritation at 2% per site. In conclusion, terpinen-4-ol is the major active component responsible for TTO's antibacterial efficacy, while minor components in TTO also contributed to its efficacy. Moreover, we

suggest that a concentration less than 5% is more suitable and safer for treating acne than higher concentrations.

Naik *et al.,* 2010 experimented that effectiveness of essential oil of lemongrass for the treatment of pathogenic organisms. Lemongrass oil was investigated for activity against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, using Agar Diffusion Method and Broth Dilution Method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the Broth Dilution Method. The antibiotic susceptibility test against the test organisms was performed by Disc Diffusion Method. Lemongrass was found effective against all the test organisms except *P.aeruginosa*. Gram positive organisms were found more sensitive to lemon grass oil as compared to gram negative organisms. The test organisms were found inhibited by Lemon grass oil at lower concentrations in Broth Dilution Method as compared to Agar Diffusion Method. The tested organisms, particularly gram-negative organisms had shown high resistance towards different antibiotics whereas they were found to be inhibited by lemongrass oil even at lower concentration. Thus lemongrass oil is effective against drug resistant organisms. It can be suggested that use of lemongrass oil would be helpful in the treatment of infections caused by multidrug resistant organisms.

Bachir *et al*., 2012, to examine the in vitro antimicrobial activities of essential oil of the leaves of *Eucalyptus globulus* (E. *globulus*). The essential oils of this plant were obtained by the hydrodistillation method. The inhibitory effects of this essential oil were tested against *Escherichia coli* and *Staphylococcus aureus* by using agar disc diffusion and dilution broth methods. Results: The results obtained showed that essential oil of the leaves of *E. globulus* has antimicrobial activity against gram negative bacteria (*E. coli*) as well as gram positive bacteria (*S. aureus*). The encouraging results indicate the essential oil of *E. globulus* leaves might be exploited as natural antibiotic for the treatment of several infectious diseases caused by these two germs, and could be useful in understanding the relations between traditional cures and current medicines.

Cox *et al.,* 2000 in Essential oil of *Melaleuca alternifolia* (tea tree) exhibits broad-spectrum antimicrobial activity. Its mode of action against the Gram-negative bacterium *Escherichia coli* AG100, the Gram-positive bacterium *Staphylococcus aureus* NCTC 8325, and the yeast *Candida albicans* has been investigated using a range of methods. In the case of *E. coli* and *Staph. aureus*, tea tree oil also caused potassium ion leakage. Differences in the susceptibility

of the test organisms to tea tree oil were also observed and these are interpreted in terms of variations in the rate of monoterpene penetration through cell wall and cell membrane structures. The ability of tea tree oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control is the most likely source of its lethal action at minimum inhibitory levels.

May *et al.*, 2000 stated that Tea tree oil has recently emerged as an effective topical antimicrobial agent active against a wide range of organisms. Tea tree oil may have a clinical application in both the hospital and community, especially for clearance of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage or as a hand disinfectant to prevent cross-infection with Gram-positive and Gram-negative epidemic organisms. Our study, based on the time-kill approach, determined the kill rate of tea tree oil against several multidrug-resistant organisms, including MRSA, glycopeptide-resistant *enterococci*, aminoglycoside-resistant *klebsiellae*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, and also against sensitive microorganisms. The study was performed with two chemically different tea tree oils. One was a standard oil and the other was Clone 88 extracted from a specially bred tree, which has been selected and bred for increased activity and decreased skin irritation. Our results confirm that the cloned oil had increased antimicrobial activity when compared with the standard oil. Most results indicated that the susceptibility pattern and Gram reaction of the organism did not influence the kill rate. A rapid killing time (less than 60 min) was achieved with both tea tree oils with most isolates, but MRSA was killed more slowly than other organisms.

1.10 Aim and Objectives

The main purposes of this study was to find the antimicrobial activity of Tea Tree oil against skin infectious bacteria as bacterial skin infection is very common and some infections are difficult to control with medication. Following are objectives of the present study.

- To determine the antibacterial activity of Tea Tree oil, Eucalyptus oil and Lemongrass oil against selected organism those are responsible for several skin infection.
- To determine the inhibition percentage of these oils against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Aeromonas hydrophila*, *Escherichia coli*, *Streptococcus pneumonia*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Streptococcus agalactiae.*
- To set a comparison of antimicrobial activity among Tea Tree oil, Eucalyptus oil and Lemongrass oil against these organisms.
- To establish comparative analysis of antimicrobial efficacy between Tea Tree oil and conventional antibiotics against selected organism.

Chapter 2

Materials and Methods

Materials and Methods

2.1 Working place

The laboratory works of this research study were carried out in the laboratory of Microbiology, of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Bacterial Strain

Staphylococcus aureus, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Aeromonas hydrophila*, *Escherichia coli*, *Streptococcus pneumonia*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Streptococcus agalactiae* strains used for this particular research work was collected from BRAC University Microbiology Laboratory. Subculture was prepared from the laboratory culture for using in research purpose. To keep the bacteria viable and to prevent contamination from other microorganisms, subculture was done every week. The freshly cultured colonies were used in the procedure.

2.3 Product tested

Commercially available Tea Tree oil, Eucalyptus oil and Lemongrass oil which do not contain any preservatives as the provider claimed.

2.4 Confirmation of the stock culture:

Each bacterial strain were subjected to morphological and biochemical confirmation tests. Biochemical tests performed were gram staining, indole production test, citrate utilization test, methyl red reaction, vogesproskauer reaction, TSI Fermentation, catalase activity test, oxidase activity test, MIU(motility and urease) test, nitrate reduction test, lipid hydrolysis, starch hydrolysis test . All the biochemical tests were performed in specific media according to the standard methods described in Microbiology Laboratory Manual (Cappuccino, 1996). All the bacterial cultures were grown on nutrient agar plates in the incubator at 37^0 C before the process of any biochemical identification test.

2.4.1 Preparation for reviving the bacteria

- Nutrient agar was prepared for each of the microorganisms.
- The media was prepared and autoclaved at 121 °C for 15 minutes (SAARC).
- After autoclave the media was poured into the plates.
- After incubation, each of the organisms from their previous culture was streaked on nutrient agar plate.

- The plates were incubated at 37 °C for 24 hours.

2.4.2 Growth on selective media

All the organisms from their previous culture were streaked on Selective agar plates for confirmation of the pathogen. The plates were incubated at 37 °C for 24 hours.

2.4.3 Gram Stain

Gram staining was done to detect whether the bacteria is gram positive or gram negative.

2.4.4 Indole Production

1. Using sterile technique, a colony taken from NA was inoculated into an Indole tube, containing peptone broth by means of stab inoculation.
2. The tube was inoculated overnight at 37ºC.
3. After incubation, 10 drops of Kovac's reagent were added and agitated gently.

2.4.5 Citrate Utilization

1. Using sterile technique, a colony taken from NA was inoculated into a vial, containing a slant of Simmon's Citrate agar by means of streak inoculation.
2. The vial was inoculated overnight at 37ºC.
3. Result observed after incubation.

2.4.6 Methyl Red Reaction

1. Inoculate two tubes containing MR-VP Broth with a pure culture of the microorganisms under investigation.
2. Incubate at 35 °C for up to 4 days.
3. Add about 5 drops of the methyl red indicator solution to the first tube.
4. A positive reaction is indicated, if the colour of the medium changes to red within a few minutes.

2.4.7 Voges-Proskaur test

1. All the bacterial samples to be tested were inoculated into 3 ml dextrose phosphate broth (MR-VP broth) which contained dextrose and a phosphate buffer and incubated at 37^0 C for 24 hours.
2. After the incubation period was over, 10 drops of Barritt's reagent A was added to each of the test tubes and the cultures were shaken.

3. Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
4. After 15 minutes, the colours of the cultures were examined and the results were recorded. Appearance of a red colour was taken as a positive result.

2.4.8 TSI Fermentation

1. All the bacteria samples to be tested were inoculated into 3 ml dextrose phosphate broth (MR-VP broth) which contained dextrose and a phosphate buffer and incubated at 37^0 C for 24 hours.
2. After the incubation period was over, 10 drops of Barritt's reagent A was added to each of the test tubes and the cultures were shaken.
3. Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
4. After 15 minutes, the colours of the cultures were examined and the results were recorded. Appearance of a red colour was taken as a positive result.

2.4.9 Catalase Activity

1. A number of autoclaved glass slides were taken, and a drop of the catalase reagent (Hydrogen peroxide) was placed on each of the glass slides.
2. The glass slides were labeled according to the sample being tested.
3. A colony for each of the bacteria to be tested was taken from a nutrient agar plate, and later placed onto the reagent drops on each of the glass slides.
4. An immediate bubble formation indicated a positive result.
5. The same procedure was carried out for the clinical and the environmental strains

2.4.10 Oxidase Activity

1. A number of filter papers were taken, and two drops of oxidase reagent (Aminodimethylaniline oxalate) were added onto the filter papers (Whatman, 1MM).
2. The filter papers were labeled according to the sample being tested.
3. A loopful of each bacterium to be tested (the clinical and environmental strains) were taken from nutrient agar plate and streaked onto the filter paper (Whatman, 1MM).
4. A positive reaction would turn the paper from violet to purple within 1 to 30 seconds. Delayed reactions should be ignored as that might give false positive result.

2.4.11 Motility Indole Urease (MIU)

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

2.4.12 Nitrate Reduction

1. Nitrate broth was inoculated with a heavy growth of test organism.
2. Inoculated broth was incubated at an appropriate temperature for 24 to 48 hours.
3. Added one dropperfull of sulfanilic acid and one dropperfull of a α-naphthylamine to each broth.
    - At this point, a colour change to RED indicates a POSITIVE nitrate reduction test. If apper a red color, then reaction can be stopped at this point.
    - No colour change indicates the absence of nitrite. This can happen either because nitrate was not reduced or because nitrate was reduced to nitrite, then nitrite was further reduced to some other molecule. If reaction doesn't get a red colour, then this test proceed to the next step.
4. A small amount of zinc (a toothpick full) was added to each broth. Zinc catalyzes the reduction of nitrate to nitrite.
    - At this point, a colour change to RED indicates a NEGATIVE nitrate reduction test because this means that nitrate must have been present and must have been reduced to form nitrite.
    - No colour change means that no nitrate was present. Thus no color change at this point is a POSITIVE result (Acharya, 2015).

2.4.13 Lipid Hydrolysis

1. Using the wire loop culture was transferred to the plate.
2. Inverted plates were incubated for 1-3 days at 35°C. All negative results should be incubated for at least 3 days
3. Lipid agar plates were examined. Looked for areas of clearing around the bacterial growth, compared the results to the positive and negative controls.
4. The organism produced a clear zone around the bacterial growth scored it as positive. Otherwise, scored it as negative.

2.4.14 Starch Hydrolysis

1. Soluble starch was dissolved in a small amount of water and is heated slowly with constant stirring. Then all the ingredients were added to it and transferred into a conical flask and sterilized by autoclaving at 121.5 °C for 15 min.
2. The sterilized agar medium was poured into the sterilized Petri plates and allowed to solidify.
3. Each plate was inoculated at the center with the bacterial inoculum.
4. Plates are incubated at 37°C for 24-48 hrs.
5. To test the hydrolysis of starch, each plate was flooded with iodine.
6. The starch in the plate was changed to blue-brown by the iodine reagent. Areas where starch has been digested by bacterial growth exhibit clear halos in the midst of the dark plate, indicating a POSITIVE alpha-amylase, or starch hydrolysis test.
7. Plates containing bacteria without alpha-amylase were uniformly dark, a NEGATIVE result.

2.5 Preparation of stock sample

For short-term preservation, 2 ml of T1N1 agar butt in a vial was inoculated by stabbing bacterial growth of each isolate from nutrient agar plate. Then the vial was kept at 4 ºC for an hour to gelatinize. After an hour, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature and at -20 ºC as well.

Long-term preservation

For long-term preservation, 500 µl of bacterial culture grown in Trypticase Soy Broth (Oxoid, England) at 37 ºC for 6 hours was taken in a sterile cryovial. Then 500 µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20 ºC.

2.6 Methods for Detection of Antimicrobial Activity

2.6.1 Preparation of bacterial suspensions

Using a sterile inoculating loop, one or two colonies of the organism to be tested were taken from the subculture plate. The organism was suspended in 3 ml of physiological saline. The test tube containing the saline was then vortexed to create an overall smooth suspension.

2.6.2 Comparing with the McFarland Solution

The bacterial suspension prepared was compared with the commercially available McFarland solution 1 (for detection of inhibition rate). A bacterial suspension which matches with McFarland 1 is supposed to contain 3×10^8 bacteria per ml. (McFarland, 1907).

2.6.3 Procedure of Dilution

1. At first, prepared two tubes where one of them contain 5ml of Brain Heart Infusion Broth (BHIB) and another tube contained mixture of 4ml of BHIB and 1ml of tested oil.
2. Bacterial suspension matched with McFarland 1 and transferred 10µl per tube prepared earlier.
3. After mixing the broth and suspension well incubated the tube at 37°C for 24 hours.
4. After twenty-four hours nine hundred microliters of saline was taken separately in sets of 7 tubes for Broth and 3 tubes for oil.
5. One hundred microliters of bacterial suspension from broth were added to the 1st tube and 100 µl solution was transferred to the 2nd tube and this procedure was repeated till 7th tube.
6. Similarly, 100 µl of bacterial suspension from broth with oil was added to the 1st tube and 100 µl solution was transferred to the 2nd tube and this procedure was repeated till 3rd tube. Before transferring the solution every tube was subjected to vortex for uniform mixing.

7. As the study planned to detect inhibition rate in two different time interval so the previous step needed to repeat at 6 hours and 24 hours incubation.

2.6.4 Detection of Inhibition Percentage

1. After specific incubation period one hundred microliter of the samples was spread on the agar plate containing nutrient agar from each diluted both Broth and Oil with Broth tube.
2. One hundred microliter from original tube containing oil and broth was spread on agar plate.
3. All the plates were incubated at 37°C for 24 hours.
4. CFU in Oil mixed with broth and CFU in broth of each spread plate was counted and compared.
5. Rate of inhibition in case of every diluted tube was then calculated and averaged to detect actual inhibition Percentage.

2.7. Well Diffusion Method

2.7.1 Preparation of Bacterial Suspension

Using a sterile inoculating loop, one or two colonies of the organism to be tested were taken from the subculture plate. The organism was suspended in 3 ml of physiological saline. The test tube containing the saline was then vortexed to create an overall smooth suspension.

2.7.2 Comparing with the McFarland Solution

The bacterial suspension prepared was compared with the commercially available McFarland solution 0.5 (for detection of zone of inhibition by agar disc/well diffusion method). A bacterial suspension which matches with McFarland 0.5 is supposed to contain 1.5×10^8 bacteria per ml (McFarland, 1907).

2.7.3 Inoculation on Mueller Hinton Agar (MHA) plate

1. A sterile swab was dipped into the bacterial suspension where the test organisms were suspended in 5 ml of saline.
2. The swab was rotated against the side of the tube using firm pressure, to remove excess fluid, but the swab was not dripped wet.

3. The dried surface of the MHA was inoculated by streaking the swab three times over the entire agar surface; the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculum.
4. The plate was rimmed with the swab to pick up any excess liquid.
5. Leaving the lid slightly ajar, the plate was allowed to sit at room temperature at least 3 to 5 minutes for the surface of the agar plate to dry before proceeding to the next step.

2.7.4 Placement of oil in the Well

1. Well was made on agar using a borer.
2. Twenty microliter of oil was placed in the well using a micropipette.
3. Then zone of inhibition was measured after 24 hours incubation at 37º C.

2.7.5 Measuring Zone Sizes

1. Following incubation, the zone sizes were measured precisely using a ruler.
2. All measurements were made while viewing the back of the petri dish.
3. The zone size was recorded on the recording sheet.

2.8 Data Analysis

Data were analyzed using Microsoft excel version 2007.

Chapter 3

Results

Results

3.1 Confirmation of the clinical strain

Clinical strain of the ten bacteria obtained from BRAC University stock culture were streaked on the respective selective media in order to determine and confirm the cultural properties of the organisms. Selective media are formulated to support the growth of one group of organisms, while inhibits the growth of the other organisms.

Table 2: Colony characteristics of the pathogens on selective agar

SL	Organism	Colony Characteristics						
		Media	**Size**	**Margin**	**Elevation**	**Form**	**Pigment**	**Consistency**
A	*Staphylococcus aureus*	Mannitol salt agar	Small	Entire	Convex	Circular	Yellow colonies that turn the media Yellow	Smooth
B	*Streptococcs pyogenes*	Sheep Blood agar	Small	Entire	Flat	Circular	Large deep zone of beta-hemolysis.	Smooth
C	*Pseudomonas aeruginona*	Cetrimide Agar	Small	Undulate	Umbonate	Circular	Green colonies that turn the media greenish	Mucoid
D	*Proteus vulgaris*	MacConkey agar	Medium	Entire	Convex	Circular	Colorless colonies and media turn into yellow.	Smooth
E	*Aeromonas hydrophila*	Sheep Blood agar	Medium-large	Entire	Convex	Circular	Colonies are white or buff in colour	Smooth
F	*Escherichia coli*	EMB agar	Large	Entire	Flat	Circular	Green sheen colonies appear.	Mucoid
G	*Streptococcus pneumoniae*	Sheep Blood agar	Large	Entire	Umbilicate	Circular	Colonies surrounded by zones of alpha-hemolysis.	mucoid
H	*Bacillus subtilis*	TSY agar	Large	Lobate	Flat	Irregular	Sporeforming rods produce colonies	Dry
I	*Klebsiella pneumoniae*	MacConkey agar	Small	Entire	Pulvinate	Circular	Pink, glossy colonies	Smooth
J	*Streptococcus agalactiae*	Sheep Blood agar	Medium	Undulate	Umbonate	Irregular	Small diffused zone of beta-hemolysis.	Creamy

All the sizes of the colonies are given bellow

Large: 4.5 mm (±0.5 mm)

Medium: 3 mm (±0.5 mm)

Small: 2 mm (±0.5 mm)

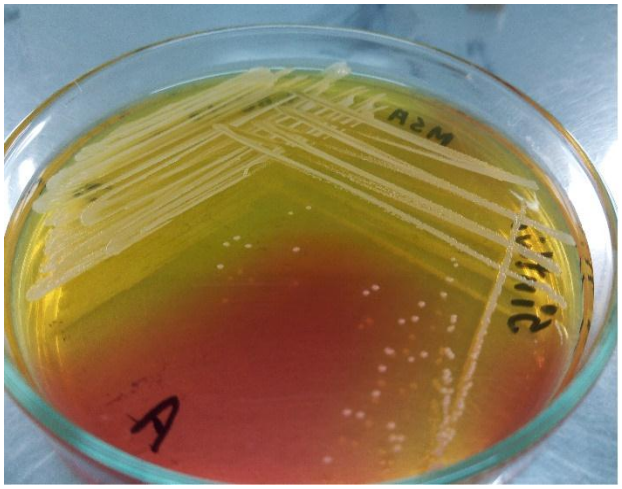


Figure 4.1: *Staphylococcus aureus* on *MSA*

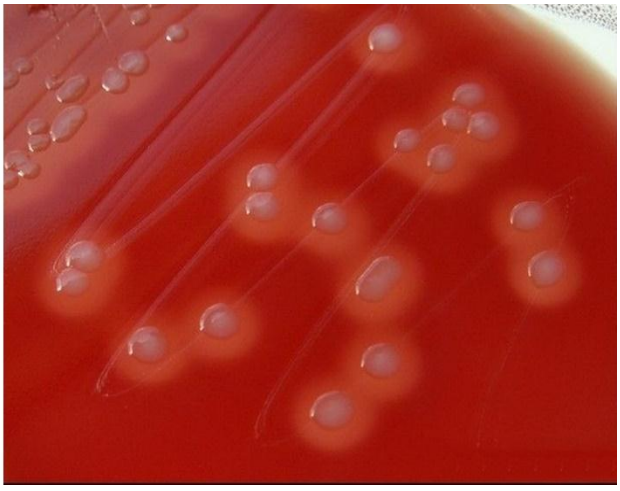


Figure 4.2: *Streptococcus pyogenes* on BAP

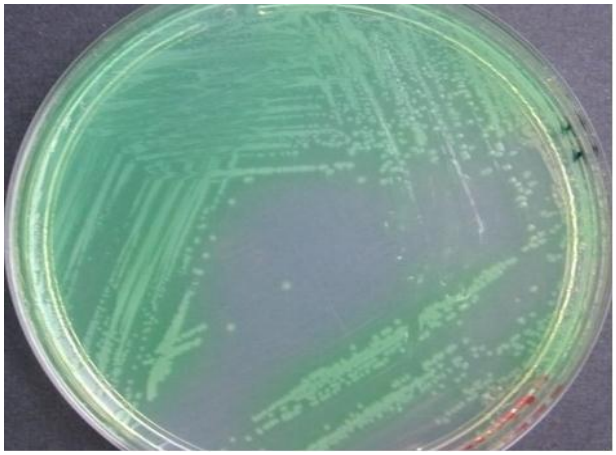


Figure 4.3: *Pseudomonas aeruginosa* on Cetrimide

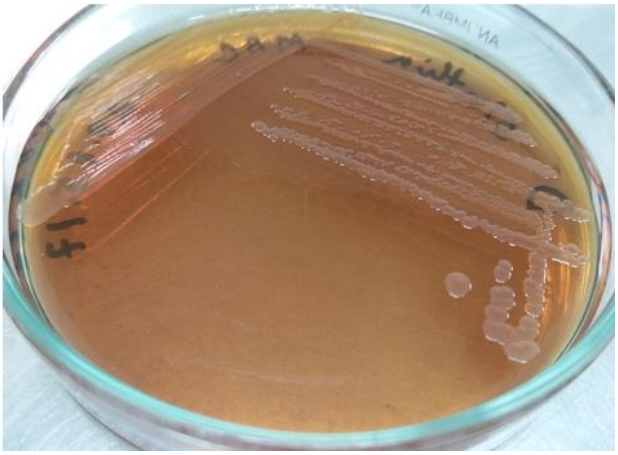


Figure 4.4: *Proteus vulgaris* on MAC Agar

Figure 4.5: *Aeromonas hydrophila* on Blood Agar

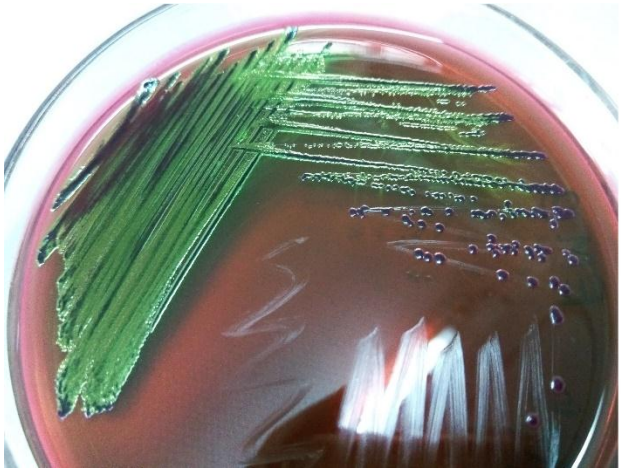
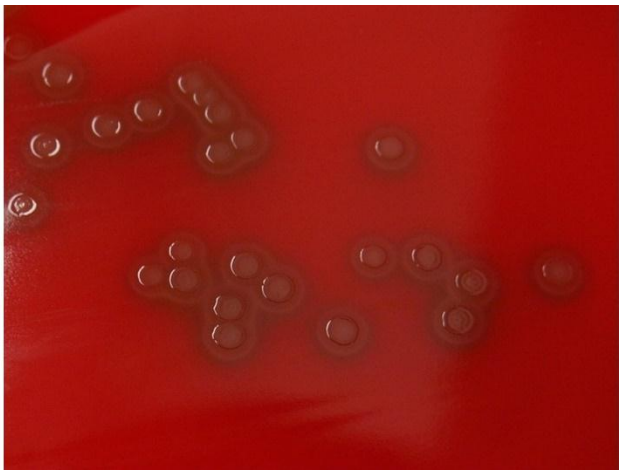


Figure 4.6: *Escherichia coli* on EMB Figure



4.7: *Streptococcus pneumonia* on BAP

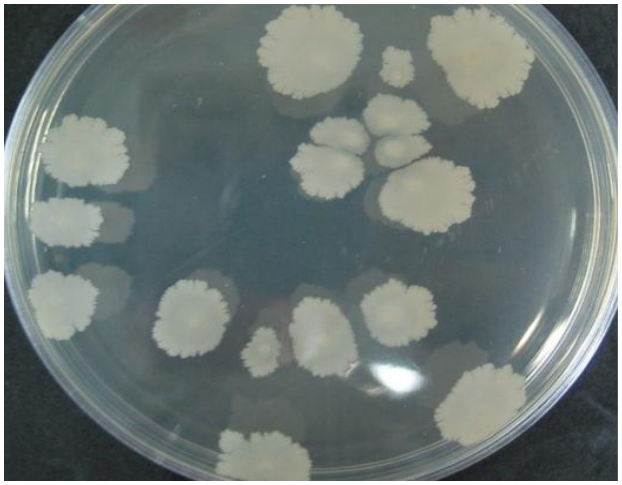


Figure 4.8: *Bacillus subtilis* on TSY agar

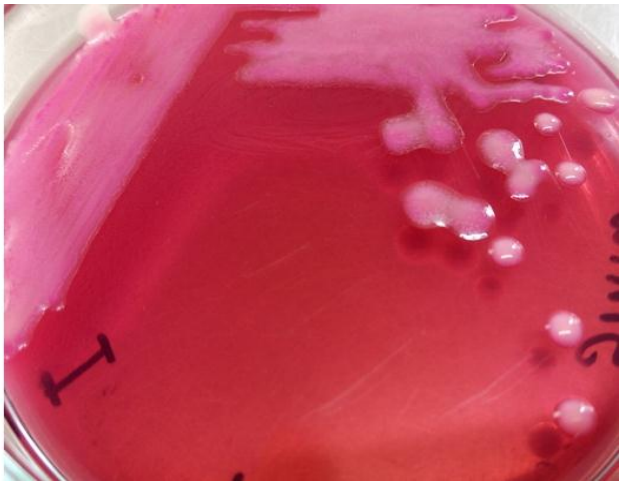


Figure 4.9: *Klebsiellapneumoniae* on MAC

Figure 4.10: *Streptococcus agalactiae* on BAP

Figure 4: Colony characteristics of the pathogens on selective Media

3.2 Biochemical Test results

The organisms were identified based on following biochemical tests.

Table 3: Biochemical test results of the pathogens

Sl no.	Organism	Gram stain	Indole Production	Citrate Utilization	Methyl red reaction	Voges-Proskauer reaction	TSI Fermentation				Catalase Activity	Oxidase Activity	MIU		Nitrate Reduction	Lipid Hydrolysis	Starch Hydrolysis
							Slant	Butt	CO_2 production	H_2S Production			Motility	Urease			
A	*Staphylococcus aureus*	Cocci +	-	-	+	+	A	A	-	-	+	-	-	-	+	+	-
B	*Streptococcus pyogenes*	Cocci +	-	-	+	-	A	A	-	-	-	-	-	-	+	-	-
C	*Pseudomonas aeruginosa*	Rod -	-	+	-	-	K	A	-	-	+	+	+	-	-	+	-
D	*Proteus vulgaris*	Rod -	+	-	+	-	K	A	+	+	+	-	+	+	+	-	-
E	*Aeromonashydrophila*	Rod -	+	-	+	+	A	A	-	-	+	+	+	-	+	+	+
F	*Escherichia Coli*	Rod -	+	-	+	-	A	A	-	-	+	-	+	-	+	-	-
G	*Streptococcus pneumoniae*	Cocci +	-	+	+	-	A	A	-	-	-	-	-	+	+	-	-
H	*Bacillus subtilis*	Cocci +	-	+	-	+	K	A	-	+	+	+	+	-	-	+	+
I	*Klebsiellapneumoniae*	Rod -	-	+	-	-	A	A	+	-	+	+	-	+	+	-	+
J	*Streptococcus agalactiae*	Cocci +	-	-	+	+	A	A	-	-	-	-	-	-	-	-	-

KEY: (A) = acidic condition, (K) = alkaline condition, (+) = positive, (-) = negative.

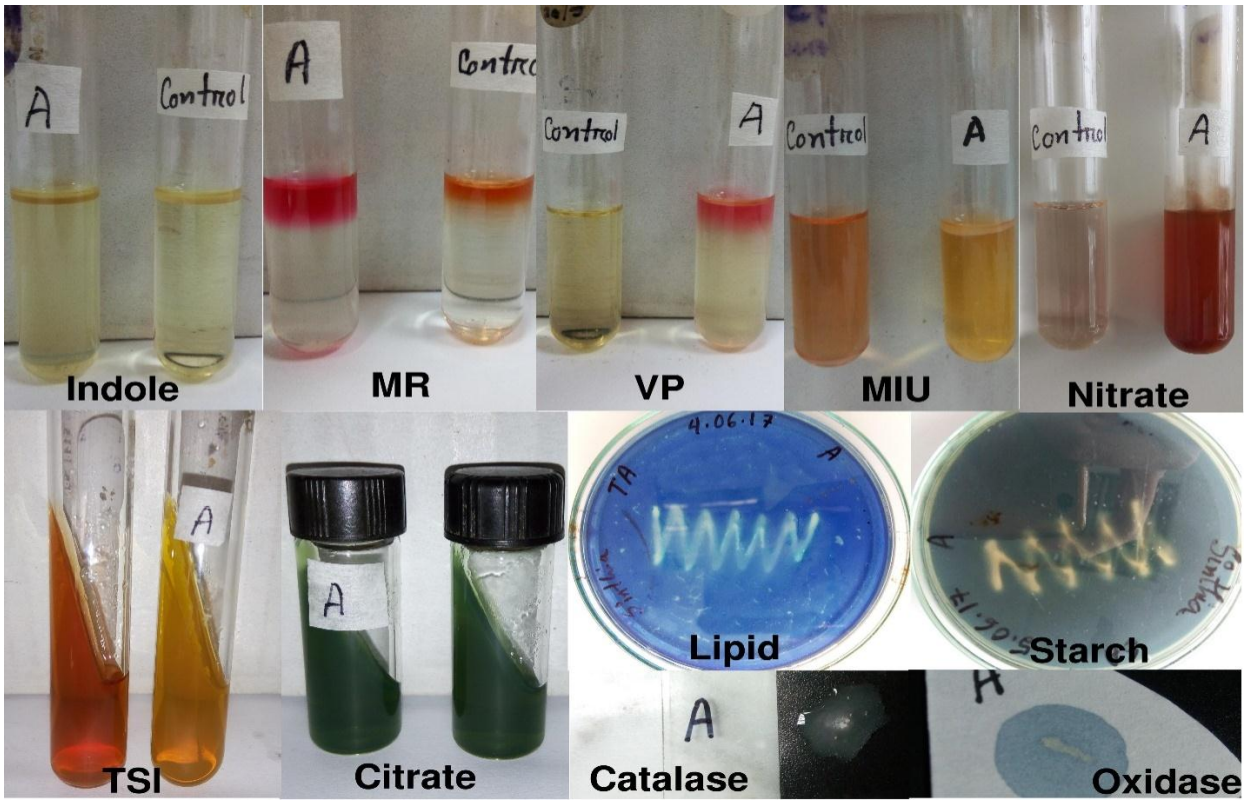


Figure 5.1: Biochemical Test and Identification of *Streptococcus aureus.*

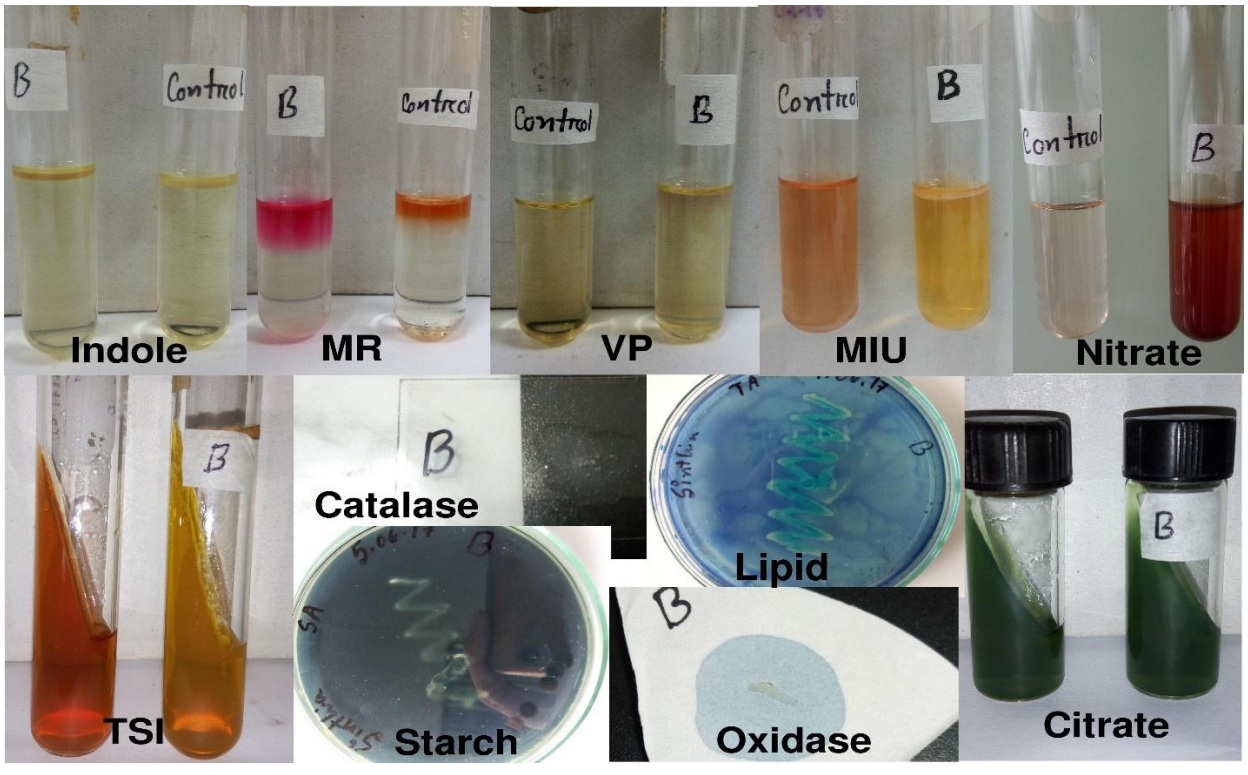


Figure 5.2: Biochemical Test and Identification of *Streptococcus pyogenes.*

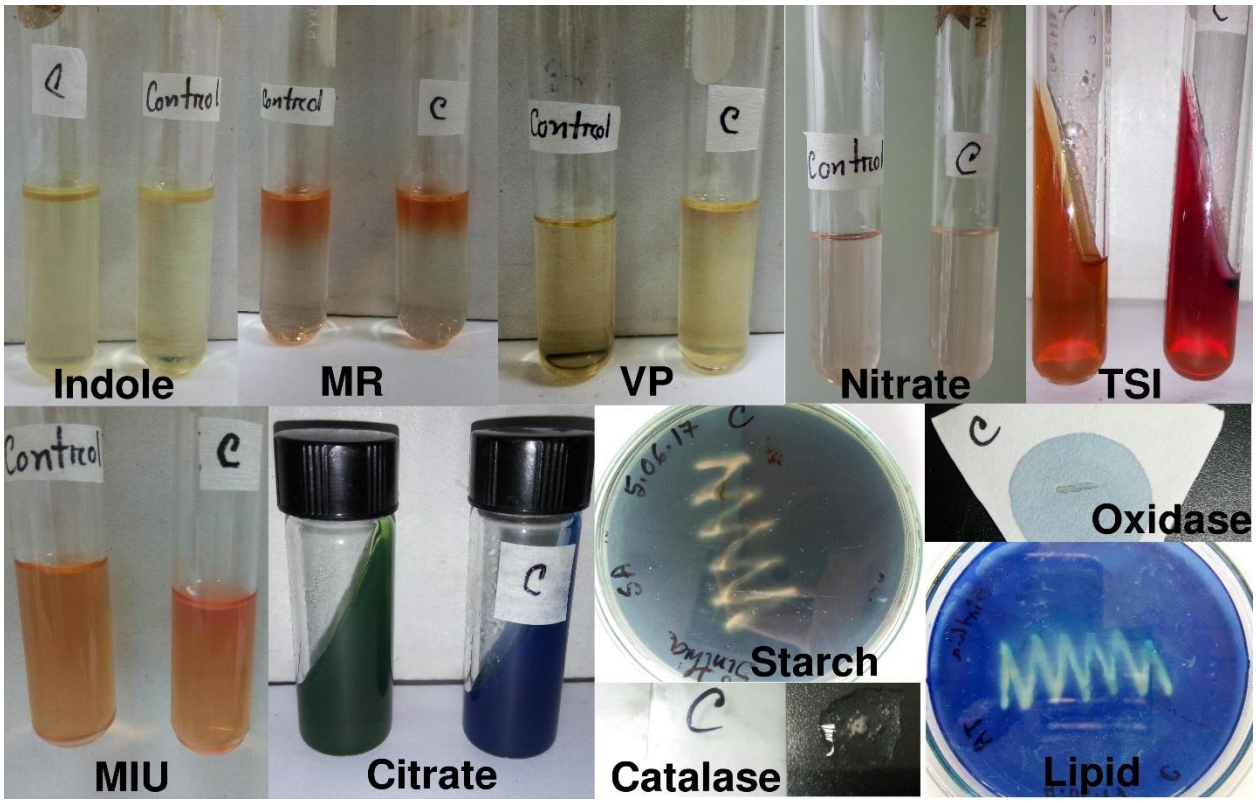


Figure 5.3: Biochemical Test and Identification of *Pseudomonas aeruginosa*..

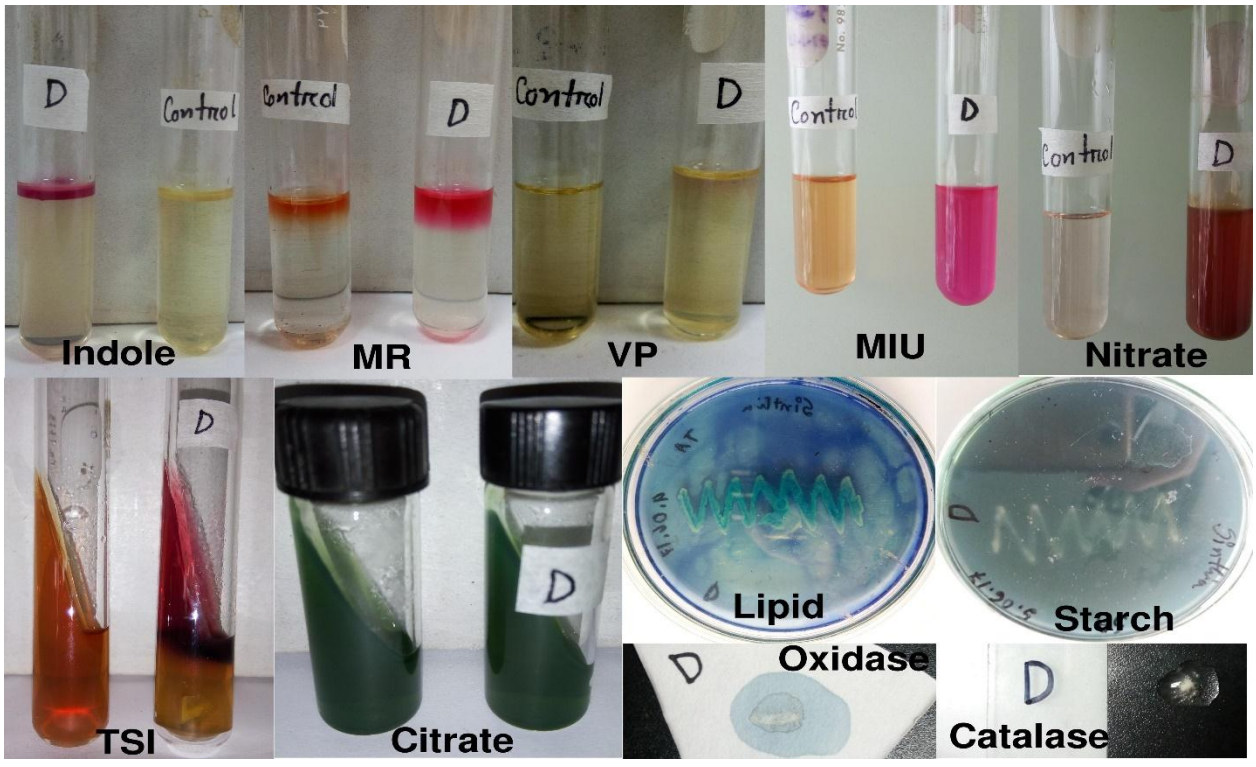


Figure 5.4: Biochemical Test and Identification of *Proteus vulgaris.*

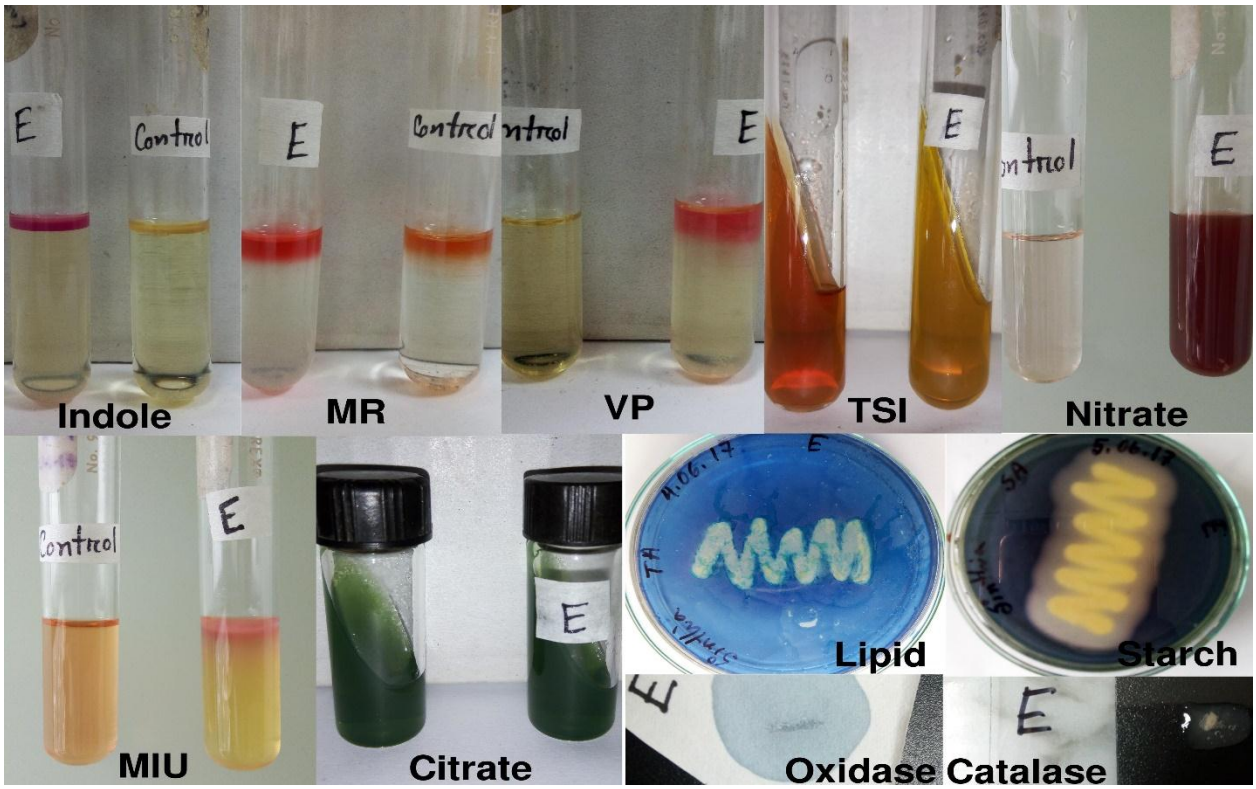


Figure 5.5: Biochemical Test and Identification of *Aeromonas hydrophila.*

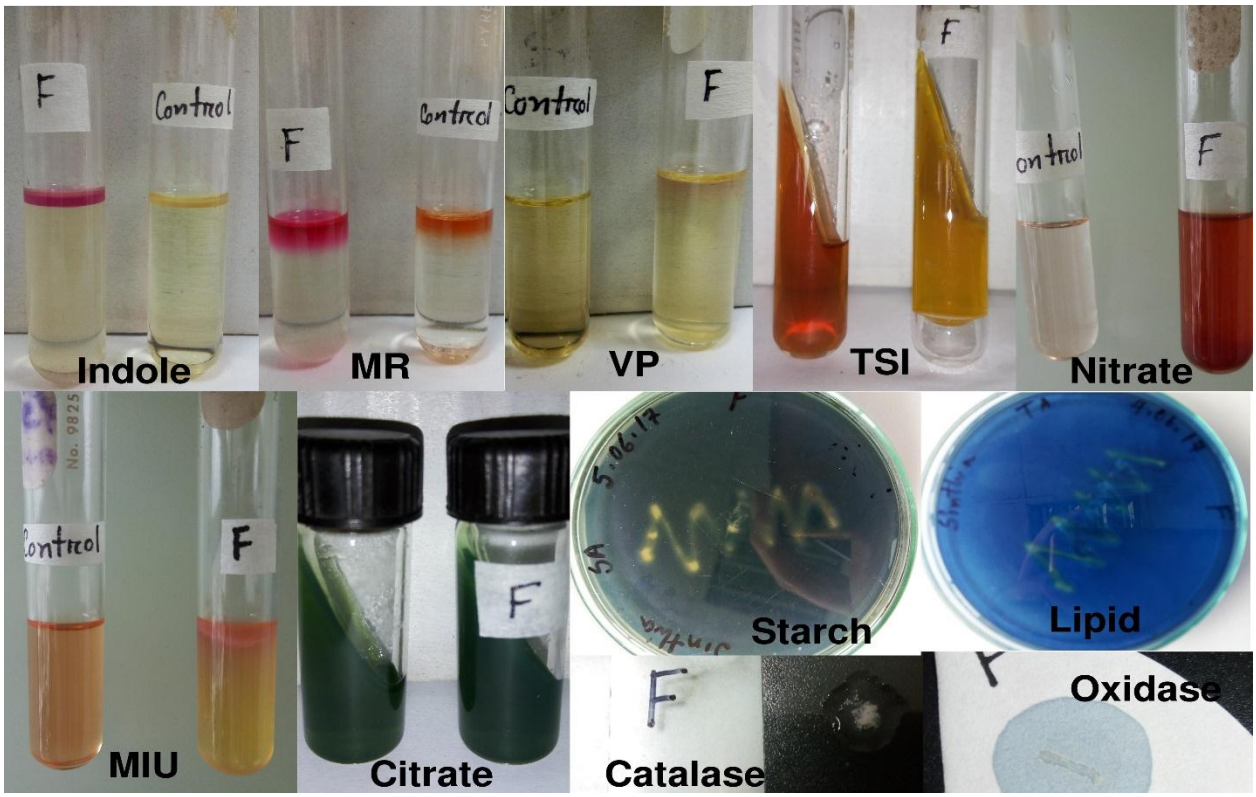


Figure 5.6: Biochemical Test and Identification of *Eschericha coli.*

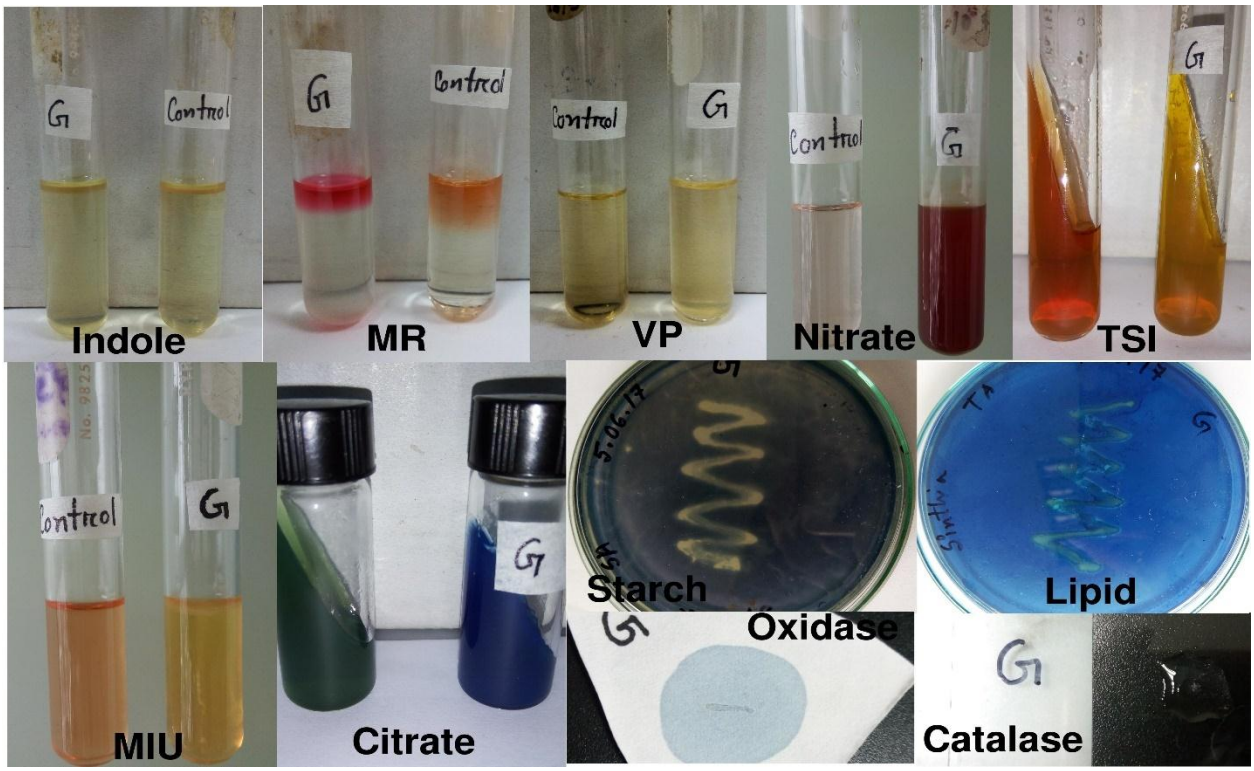


Figure 5.7: Biochemical Test and Identification of *Streptococcus pneumoniae.*

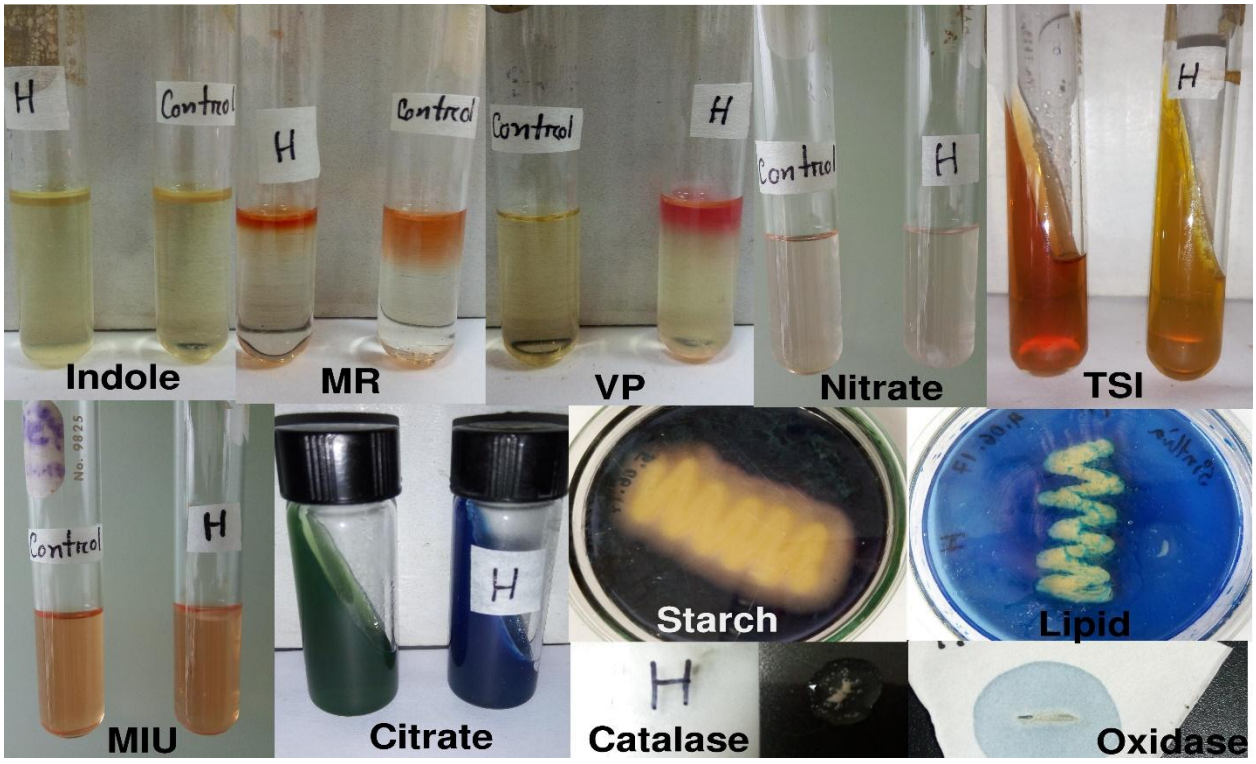


Figure 5.8: Biochemical Test and Identification of *Bacillus subtilis.*

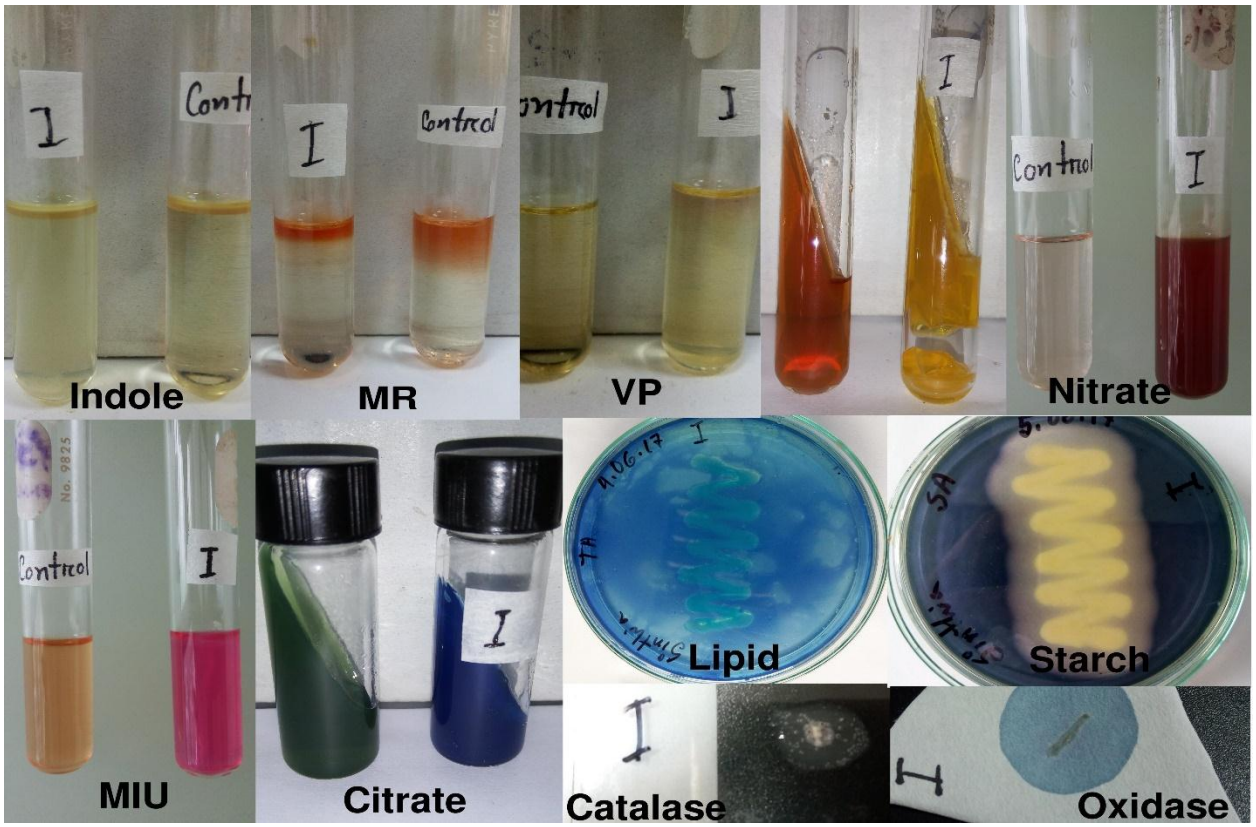


Figure 5.9: Biochemical Test and Identification *of Klebsiella pneumoniae*

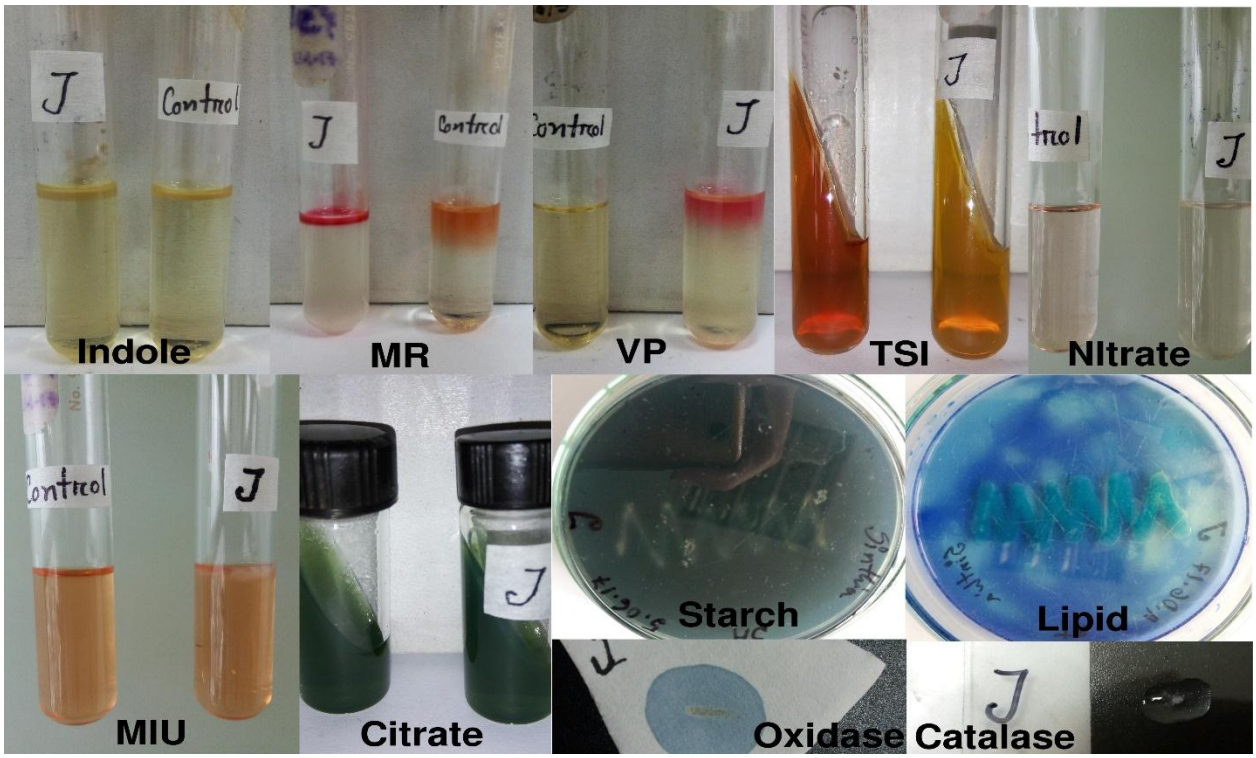


Figure 5.10: Biochemical Test and Identification of *Streptococcus agalactiae.*

Figure 5: Biochemical test results of the pathogens

3.3 Comparison between number of colonies in BHIB and oils with BHIB

Same amount of bacterial suspension were taken incubated BHIB and mixture of oil with BHIB and it was serially diluted in saline. Then the same amount of diluted suspension was taken and spread on Nutrient agar. After incubation, fewer colonies appeared on the agar plate containing oils than saline which indicates that these oils have antimicrobial activity against the selected pathogens. Pathogens were incubated with oils for 6 hours and 24 hours. The number of colonies from each plate was necessary to determine inhibition rate.

Table 4: Total Viable colony count of Organisms in BHIB and BHIB with oils.

Table 4.1: Total viable colony count of *Staphylococcus aureus* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation CFU/100µl	BHIB & Tea Tree oil CFU/100µl		BHIB & Eucalyptus oil CFU/100µl		BHIB & Lemongrass oil CFU/100µl	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Staphylococcus aureus	10^{-1}	TNTC	1	0	6	2	1	0
	10^{-2}	TNTC	0	0	4	0	0	0
	10^{-3}	TNTC	0	0	0	0	0	0
	10^{-4}	276	0	0	0	0	0	0
	10^{-5}	58	___	___	___	___	___	___
	10^{-6}	14	___	___	___	___	___	___

Table 4.2: Total viable colony count of *Streptococcus pyogenes* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation CFU/100µl	BHIB & Tea Tree oil CFU/100µl		BHIB & Eucalyptus oil CFU/100µl		BHIB & Lemongrass oil CFU/100µl	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Streptococcus pyogenes	10^{-1}	TNTC	16	0	0	8	72	3
	10^{-2}	TNTC	2	0	0	0	51	1
	10^{-3}	TNTC	1	0	0	0	16	0
	10^{-4}	216	0	0	0	0	6	0
	10^{-5}	103	___	___	___	___	___	___
	10^{-6}	47	___	___	___	___	___	___

Table 4.3: Total viable colony count of *Pseudomonas aeruginosa* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Pseudomonas Aeruginosa	10^{-1}	TNTC	8	8	78	16	56	203
	10^{-2}	TNTC	3	5	14	5	12	110
	10^{-3}	TNTC	1	1	10	3	1	11
	10^{-4}	288	0	0	6	0	0	3
	10^{-5}	128	___	___	___	___	___	___
	10^{-6}	73	___	___	___	___	___	___

Table 4.4: Total viable colony count of *Proteus vulgaris* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Proteus vulgaris	10^{-1}	TNTC	0	0	2	0	16	0
	10^{-2}	TNTC	0	0	0	0	4	0
	10^{-3}	TNTC	0	0	0	0	1	0
	10^{-4}	292	0	0	0	0	0	0
	10^{-5}	218	___	___	___	___	___	___
	10^{-6}	72	___	___	___	___	___	___

Table 4.5: Total viable colony count of *Aeromonas hydrophila* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Aeromonas hydrophila	10^{-1}	TNTC	0	0	0	0	0	0
	10^{-2}	TNTC	0	0	0	0	0	0
	10^{-3}	TNTC	0	0	0	0	0	0
	10^{-4}	248	0	0	0	0	0	0
	10^{-5}	31	___	___	___	___	___	___
	10^{-6}	9	___	___	___	___	___	___

Table 4.6: Total viable colony count of *Escherichia coli* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Escherichia coli	10^{-1}	TNTC	0	0	TNTC	TNTC	0	TNTC
	10^{-2}	TNTC	0	0	TNTC	TNTC	0	446
	10^{-3}	TNTC	0	0	296	355	0	192
	10^{-4}	320	0	0	120	250	0	36
	10^{-5}	232	___	___	___	___	___	___
	10^{-6}	29	___	___	___	___	___	___

Table 4.7: Total viable colony count of *Streptococcus pneumonia* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Streptococcus pneumoniae	10^{-1}	TNTC	270	0	285	6	4	0
	10^{-2}	TNTC	48	0	156	1	0	0
	10^{-3}	TNTC	9	0	46	0	0	0
	10^{-4}	64	0	0	23	0	0	0
	10^{-5}	30	___	___	___	___	___	___
	10^{-6}	9	___	___	___	___	___	___

Table 4.8: Total viable colony count of *Bacillus subtilis* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Bacillus subtilis	10^{-1}	TNTC	0	0	19	20	73	26
	10^{-2}	TNTC	0	0	4	8	11	17
	10^{-3}	TNTC	0	0	2	4	4	5
	10^{-4}	72	0	0	0	0	2	1
	10^{-5}	43	___	___	___	___	___	___
	10^{-6}	23	___	___	___	___	___	___

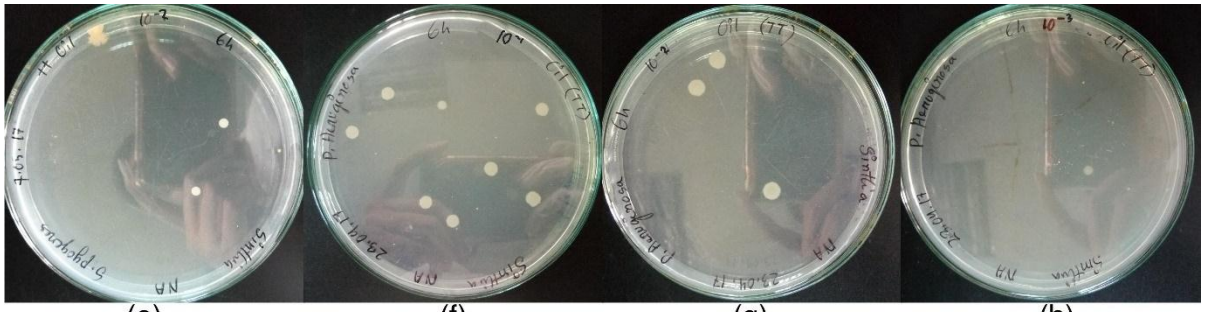
Table 4.9: Total viable colony count of *Klebsiella pneumonia* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Klebsiella pneumoniae	10^{-1}	TNTC	42	0	4	386	336	286
	10^{-2}	TNTC	15	0	0	176	117	121
	10^{-3}	TNTC	9	0	0	112	99	95
	10^{-4}	228	0	0	0	6	38	23
	10^{-5}	94	___	___	___	___	___	___
	10^{-6}	13	___	___	___	___	___	___

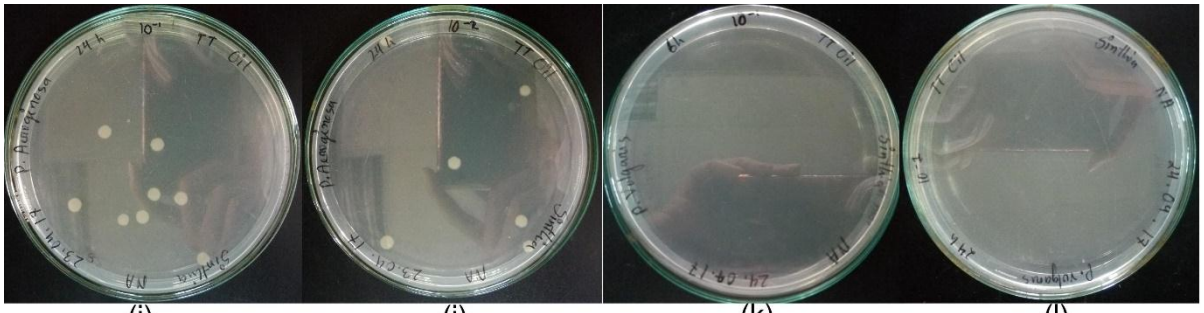
Table 4.10: Total viable colony count of *Streptococcus agalactiae* in BHIB and BHIB with oils

Pathogen	Dilution	BHIB 24 hours incubation	BHIB & Tea Tree oil		BHIB & Eucalyptus oil		BHIB & Lemongrass oil	
			6 Hours	24 Hours	6 Hours	24 Hours	6 Hours	24 Hours
Streptococcus agalactiae	10^{-1}	TNTC	0	17	0	6	365	292
	10^{-2}	TNTC	0	9	0	0	152	129
	10^{-3}	TNTC	0	4	0	0	106	42
	10^{-4}	66	0	0	0	0	52	18
	10^{-5}	38	___	___	___	___	___	___
	10^{-6}	8	___	___	___	___	___	___

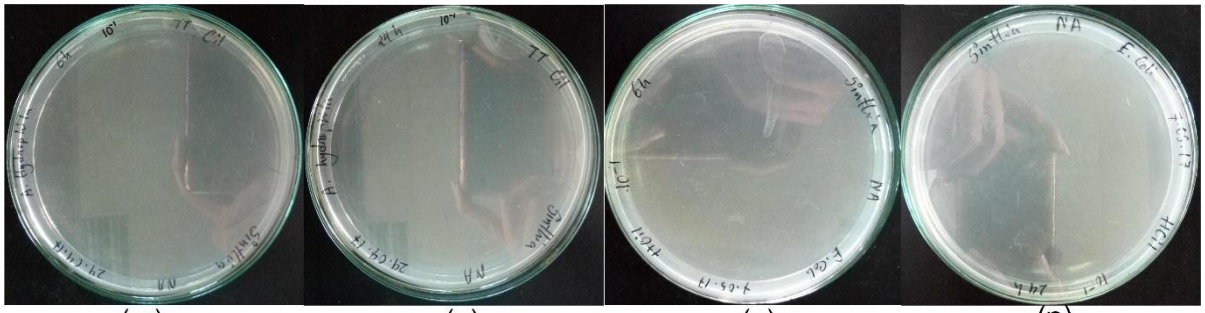
(a) (b) (c) (d)



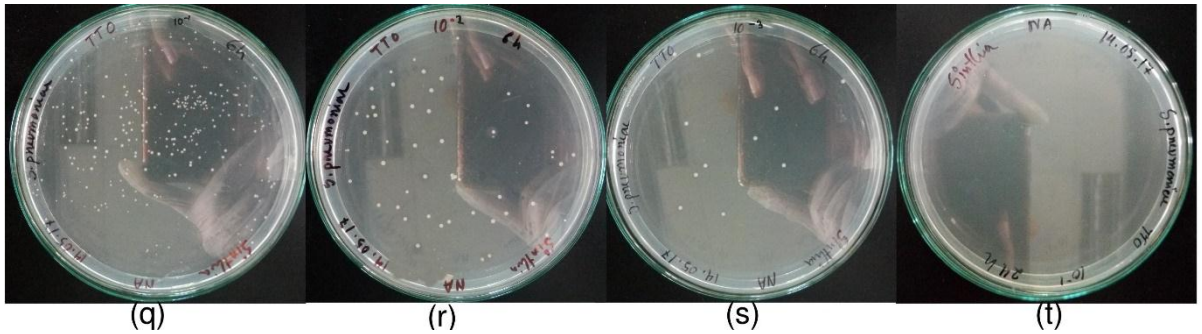
(e) (f) (g) (h)



(i) (j) (k) (l)



(m) (n) (o) (p)



(q) (r) (s) (t)

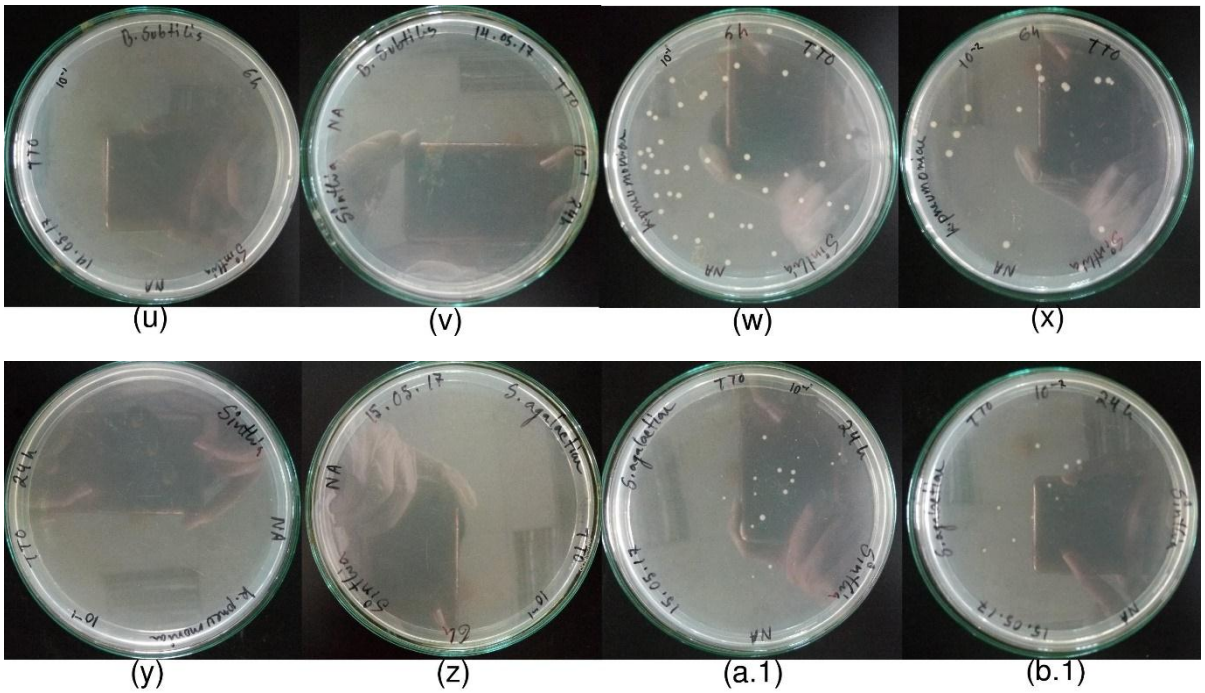
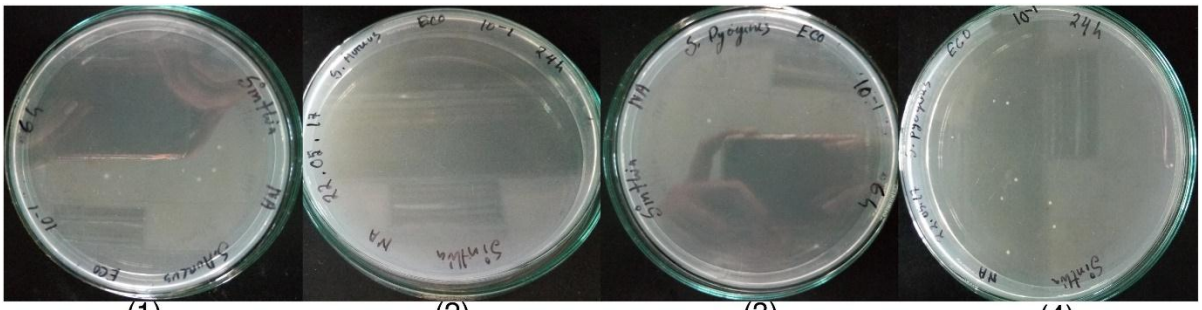


Figure 6.1: Tea Tree oil Inhibition rate

Staphylococcus aureus (a) 6 hours incubation 10^{-1}, (b) 24 hours incubation 10^{-1}
Streptococcus pyogenes (c) 6 hours incubation 10^{-1}, (d) 10^{-2}, (e) 24 hours incubation 10^{-1}
Pseudomonas aeruginosa (f) 6 hours incubation 10^{-1}, (g) 10^{-2}, (h) 10^{-3}, (i) 24 hours incubation 10^{-1}, (j) 10^{-2}
Proteus vulgaris (k) 6 hours incubation 10^{-1}, (l) 24 hours incubation 10^{-1}
Aeromonas hydrophila (m) 6 hours incubation 10^{-1}, (n) 24 hours incubation 10^{-1}
Escherichia coli (o) 6 hours incubation 10^{-1}, (p) 24 hours incubation 10^{-1}
Streptococcus pneumonia (q) 6 hours incubation 10^{-1}, (r) 10^{-2}, (s) 10^{-3}, (t)) 24 hours incubation 10^{-1}
Bacillus subtilis(u) 6 hours incubation 10^{-1}, (v) 24 hours incubation 10^{-1}
Klebsiella pneumoniae (w) 6hours incubation 10^{-1}, (x) 10^{-2},(y) 24 hours incubation 10^{-1}
Streptococcus agalactiae (z) 6 hours incubation 10^{-1}, (a1) 24 hours incubation 10^{-1}, (b1) 10^{-2}



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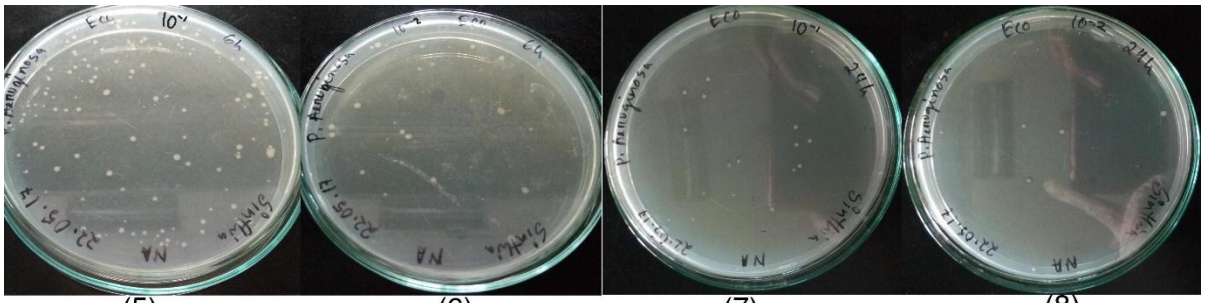
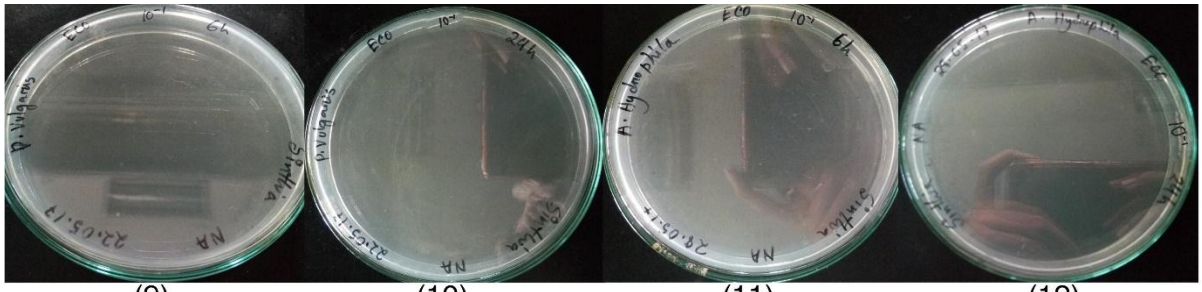
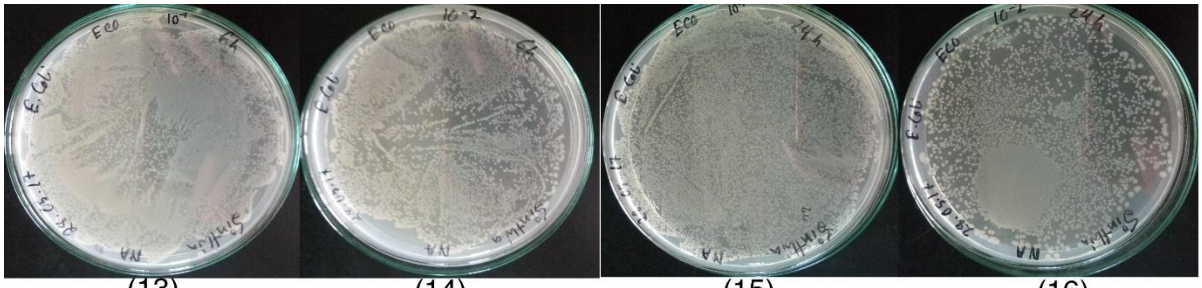


(5) (6) (7) (8)



(9) (10) (11) (12)



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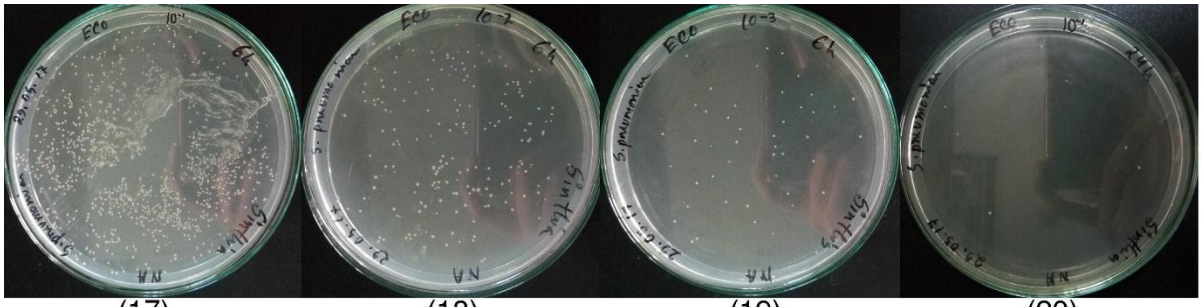

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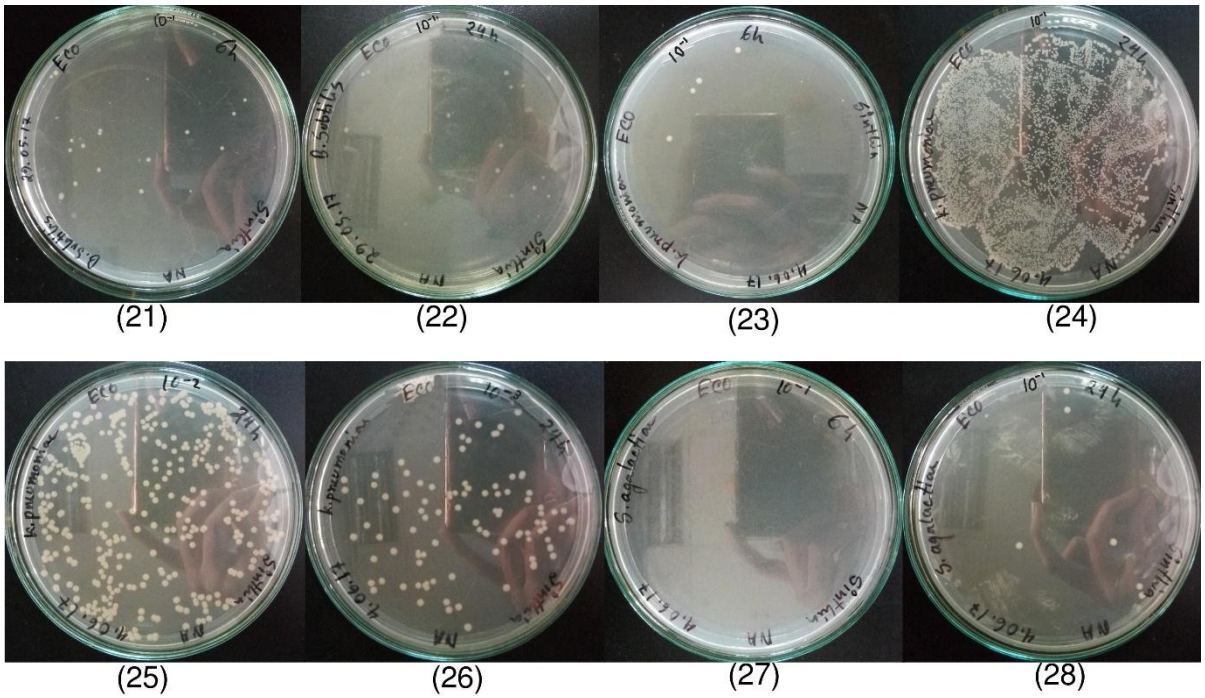
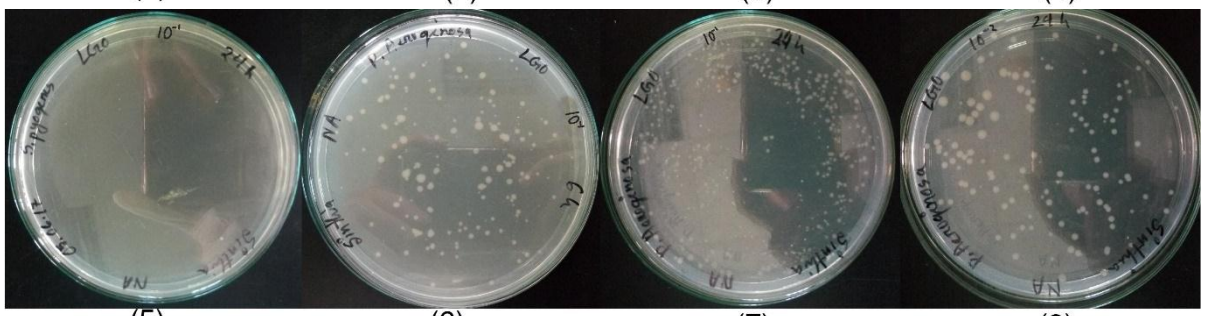


Figure 6.2: Eucalyptus oil Inhibition rate

Staphylococcus aureus (1) 6 hours incubation 10^{-1}, (2) 24 hours incubation 10^{-1}
Streptococcus pyogenes (3) 6 hours incubation 10^{-1}, (4) 24 hours incubation 10^{-1}
Pseudomonas aeruginosa (5) 6 hours incubation 10^{-1}, (6) 10^{-2}, (7) 24 hours incubation 10^{-1}, (8) 10^{-2}

Proteus vulgaris (9) 6 hours incubation 10^{-1}, (2) 24 hours incubation 10^{-1}
Aeromonas hydrophila (11) 6 hours incubation 10^{-1}, (12) 24 hours incubation 10^{-1}
Escherichia coli (13) 6 hours incubation 10^{-1},(14) 10^{-2} (15) 24 hours incubation 10^{-1}, (16) 10^{-2}
Streptococcus pneumonia (17) 6 hours incubation 10^{-1}, (18) 10^{-2}, (19) 10^{-3}, (20) 24 hours incubation 10^{-1}
Bacillus subtilis (21) 6 hours incubation 10^{-1}, (22) 24 hours incubation 10^{-1}
Klebsiella pneumonia (23) 6 hours incubation 10^{-1}, (24) 24 hours incubation 10^{-1}, (25) 10^{-2}, (26) 10^{-3}
Streptococcus agalactiae (27) 6 hours incubation 10^{-1}, (28) 24 hours incubation 10^{-1}.

(1) (2) (3) (4)



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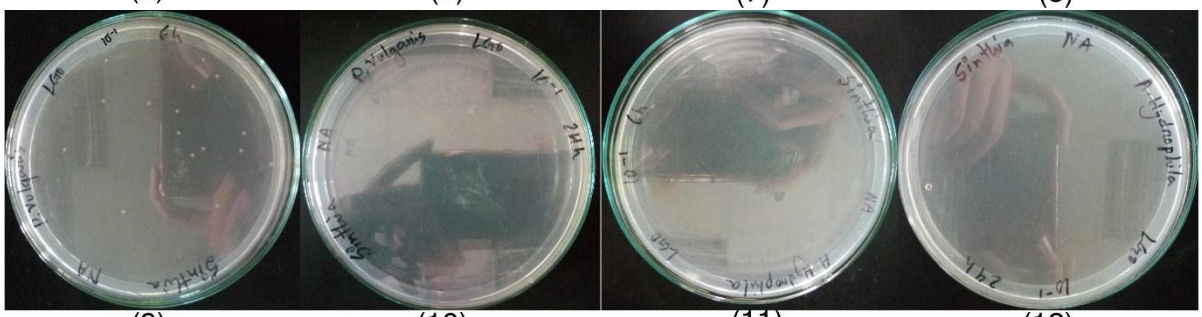

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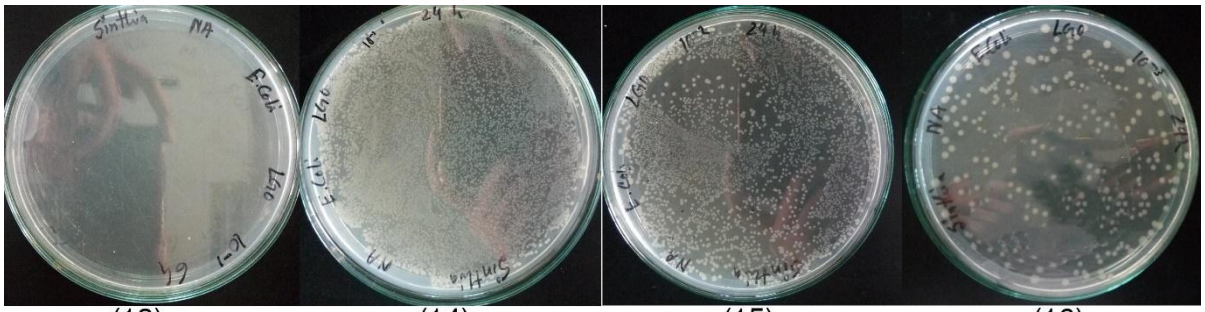
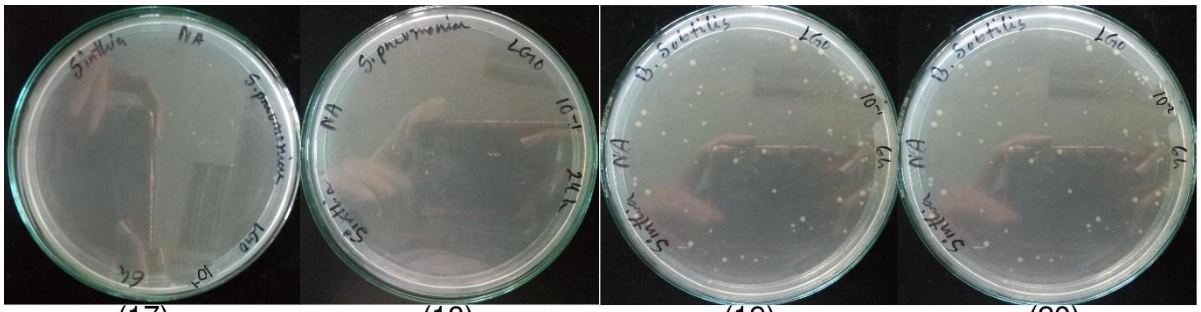


(13) (14) (15) (16)



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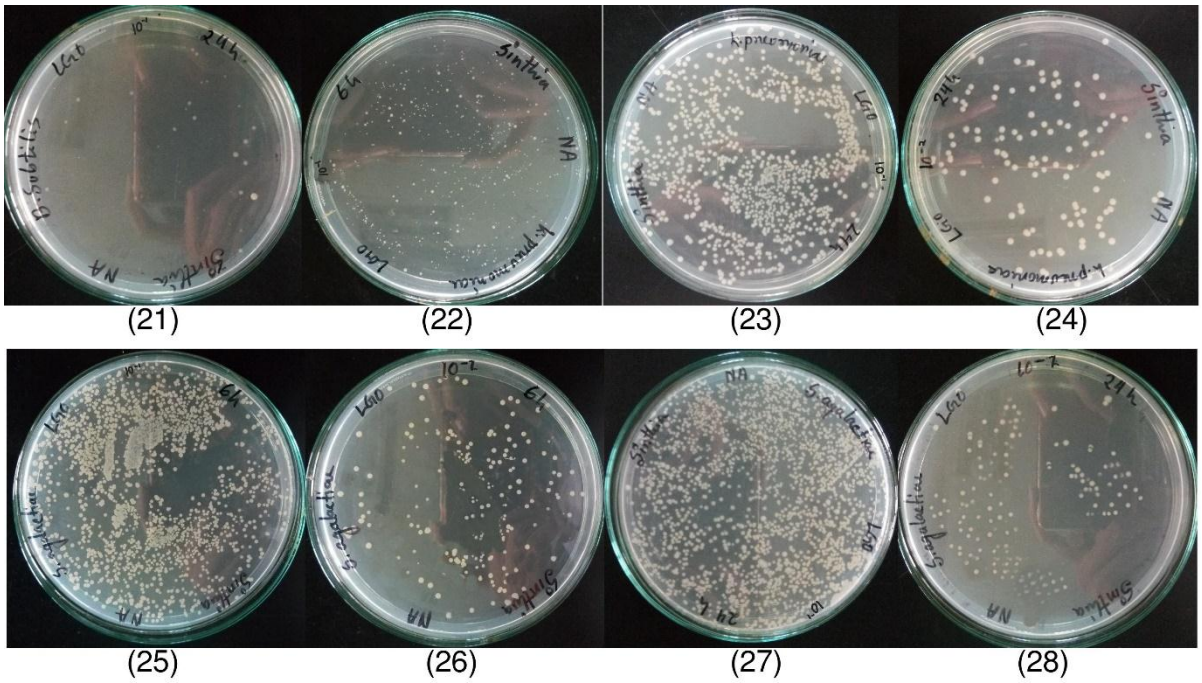


Figure 6.3: Lemongrass oil Inhibition rate

Staphylococcus aureus (1) 6 hours incubation 10^{-1}, (2) 24 hours incubation 10^{-1}
Streptococcus pyogenes (3) 6 hours incubation 10^{-1}, (4) 10^{-2}, (5) 24 hours incubation 10^{-1}
Pseudomonas aeruginosa (6) 6 hours incubation 10^{-1}, (7) 24 hours incubation 10^{-1}, (8) 10^{-2}
Proteus vulgaris (9) 6 hours incubation 10^{-1}, (2) 24 hours incubation 10^{-1}
Aeromonas hydrophila (11) 6 hours incubation 10^{-1}, (12) 24 hours incubation 10^{-1}
Escherichia coli (13) 6 hours incubation 10^{-1}, (14) 24 hours incubation 10^{-1}, (15) 10^{-2}, (16) 10^{-3}
Streptococcus pneumonia (17) 6 hours incubation 10^{-1}, (18) 24 hours incubation 10^{-1}
Bacillus subtilis (19) 6 hours incubation 10^{-1}, (20) 10^{-2}, (21) 24 hours incubation 10^{-1}
Klebsiella pneumonia (22) 6 hours incubation 10^{-1},(23) 24 hours incubation 10^{-1}, (24) 10^{-2},
Streptococcus agalactiae (25) 6 hours incubation 10^{-1}, (26) 10^{-2}, (27) 24 hours incubation 10^{-1}, (28) 10^{-2}.

3.4 Determination of Inhibition percentage

Number of colonies found in BHIB and oils with BHIB were compared to find out the rate of inhibition. Formula of calculation of inhibition Percentage is:

$$\mathbf{CFU} = \frac{\mathbf{Number\ of\ colonies\ \times\ reciprocal\ of\ the\ dilution\ factor}}{\mathbf{Volume\ of\ plated\ suspension}}$$

$$\mathbf{Inhibition\ percentage} = \mathbf{1} - \left(\frac{\mathbf{CFU\ in\ oil}}{\mathbf{CFU\ in\ broth}}\right) \times \mathbf{100}$$

Table 5.1: Inhibition Percentage of Tea Tree oil, Eucalyptus oil and Lemongrass oil.

Pathogens	Tea Tree oil		Eucalyptus oil		Lemongrass oil	
	6 hours Incubation	24 hours Incubation	6 hours incubation	24 hours incubation	6 hours Incubation	24 hours incubation
Staphylococcus aureus	99.99	100	99.99	99.99	99.99	100
Streptococcus pyogenes	99.99	100	100	99.99	99.25	99.99
Pseudomonas aeruginosa	99.99	99.99	99.66	99.89	99.95	99.61
Proteus vulgaris	100	100	99.99	100	99.96	100
Aeromonas hydrophila	100	100	100	100	100	100
Escherichia coli	100	100	62.50	21.87	100	88.75
Streptococcus pneumoniae	99.25	100	64.06	99.99	99.99	100
Bacillus subtilis	100	100	99.72	99.44	97.22	98.61
Klebsiella pneumoniae	99.99	100	99.99	96.11	86.80	92.01
Streptococcus agalactiae	100	99.99	100	99.99	21.21	72.72

Table 5.2: Average Inhibition percentage of Tea Tree oil, Eucalyptus oil and Lemongrass oil against selected pathogens

Experimented oils	Averaged Percentage of inhibition	
	6 hours incubation	24 hours incubation
Tea Tree oil	99.92%	99.99%
Eucalyptus oil	92.59%	91.72%
Lemongrass oil	90.43%	95.16%

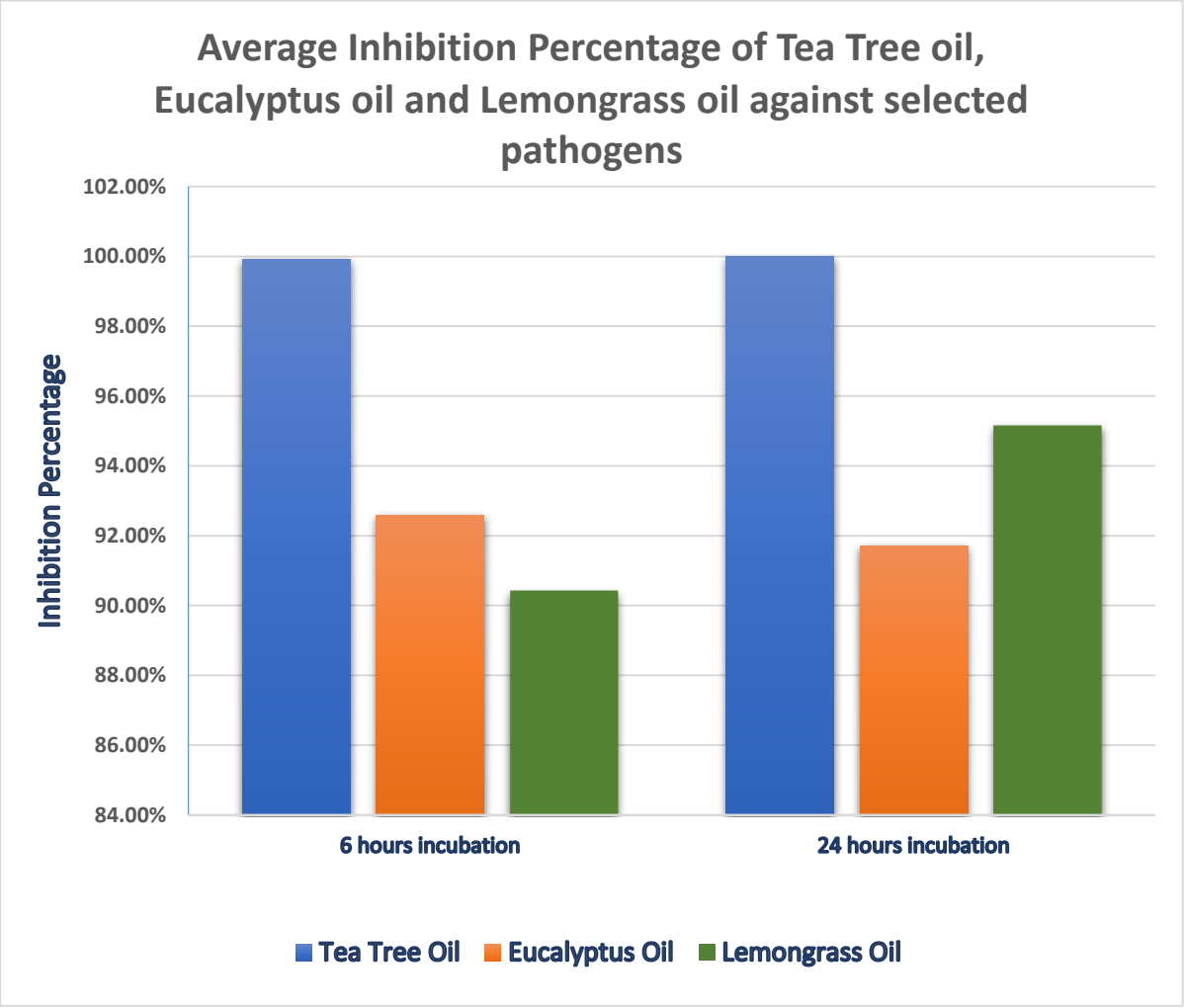


Figure 6.4: Average inhibition percentage of Tea Tree oil, Eucalyptus oil and Lemongrass oil against selected pathogens.

3.5 Comparing Tea Tree oil with Eucalyptus oil and Lemongrass oil by well diffusion method

Comparing antimicrobial activity of Tea Tree oil, Eucalyptus oil and Lemongrass oil was tested and the zone of inhibition was observed and the diameter (mm) was calculated as follows:

Table 6: Zone of inhibition in response to oils and antibiotic control by Well diffusion method

Name of Organism	Control Cefepime (FEP30)	Tea Tree oil (50µl)	Eucalyptus oil (50µl)	Lemongrass oil (50µl)
Staphylococcus aureus	27 mm	16 mm	12 mm	11 mm
Streptococcus pyogenes	34 mm	20 mm	13 mm	15 mm
Pseudomonas aeruginosa	19 mm	22 mm	11 mm	15 mm
Proteus vulgaris	33 mm	19 mm	12 mm	22 mm
Aeromonas hydrophila	34 mm	36 mm	22 mm	17 mm
Escherichia coli	32 mm	24 mm	0	0
Streptococcus pneumoniae	34 mm	19 mm	13 mm	14 mm
Bacillus subtilis	19 mm	22 mm	18 mm	24 mm
Klebsiella pneumoniae	25 mm	19 mm	0	0
Streptococcus agalactiae	10 mm	17 mm	14 mm	16 mm

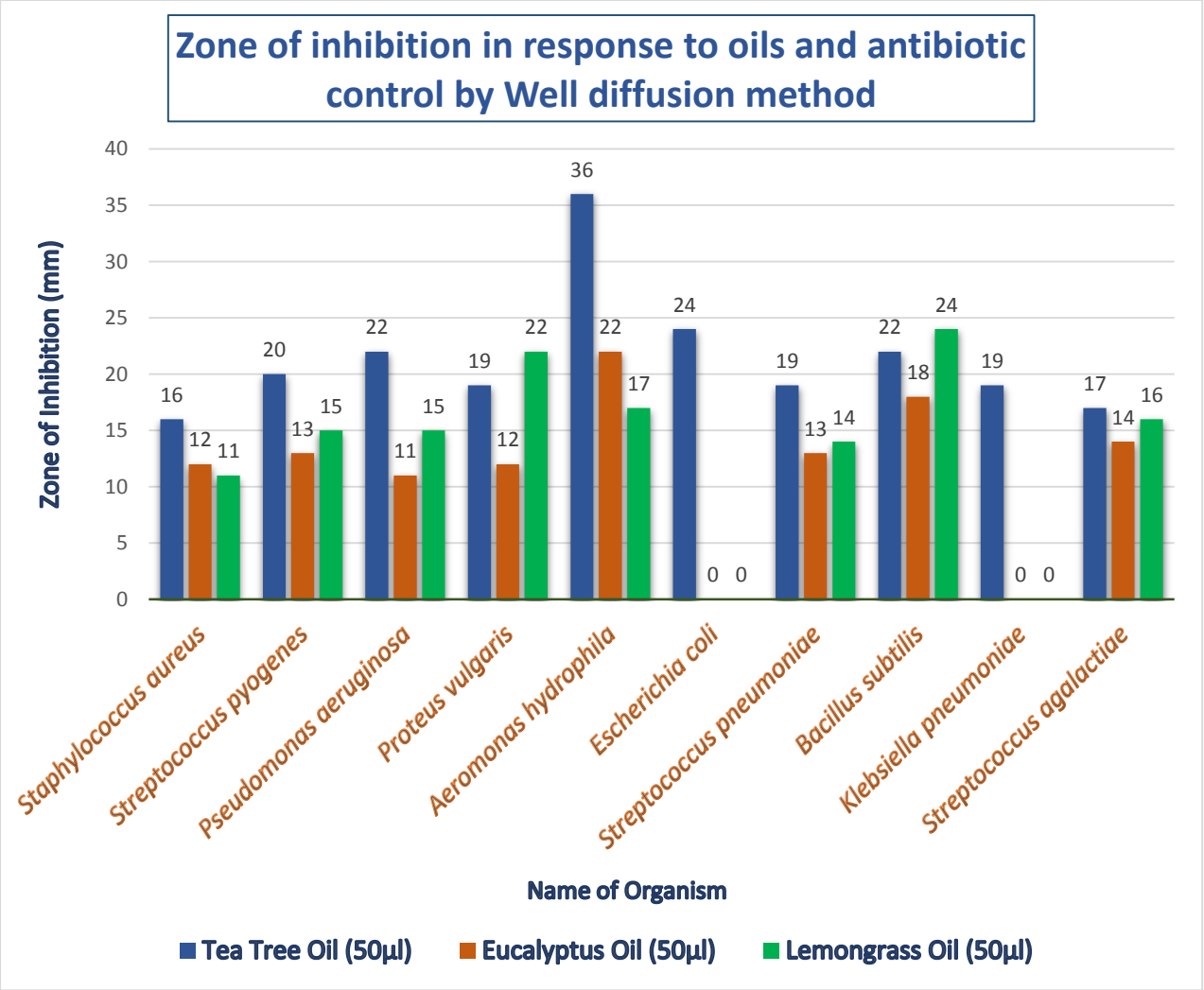


Figure 7.1: Antimicrobial activity of Tea Tree, Eucalyptus and Lemongrass oil against selected Organisms by well diffusion method.

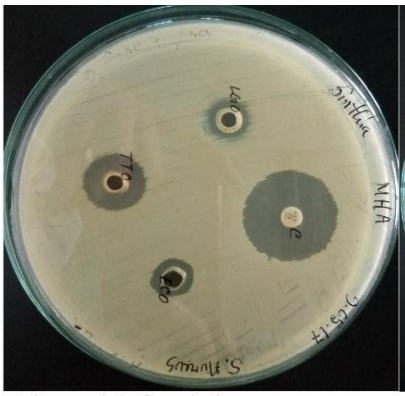


Figure 6.1: Staphylococcus aureus

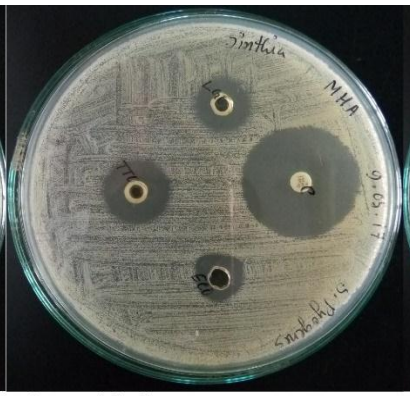


Figure 6.2: Streptococcus pyogenes

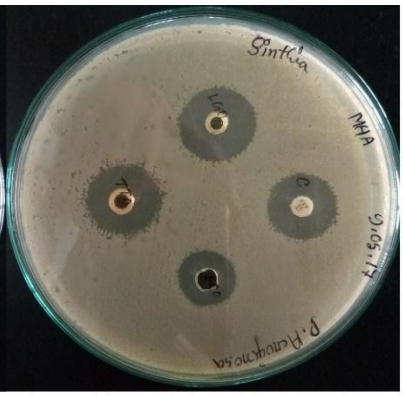


Figure 6.3: Pseudomonas aeruginosa

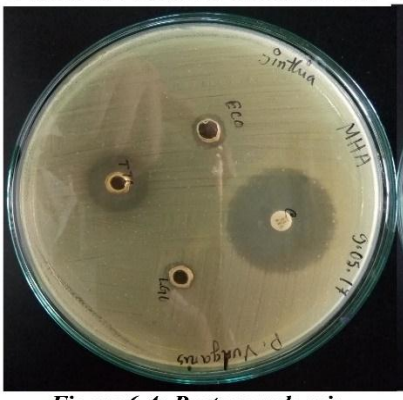


Figure 6.4: Proteus vulgaris

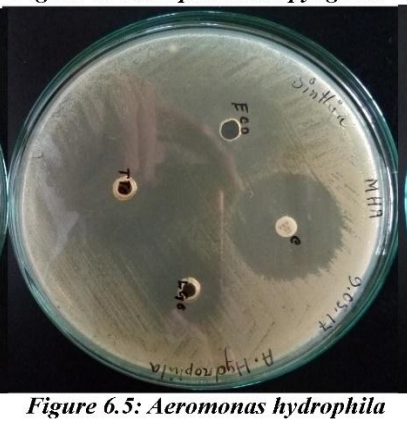


Figure 6.5: Aeromonas hydrophila

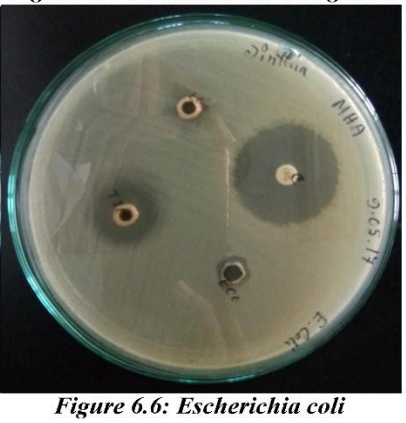


Figure 6.6: Escherichia coli

Figure 6.7:Streptococcus pneumoniae

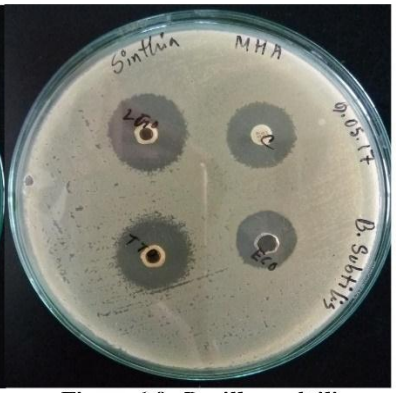


Figure 6.8: Bacillus subtilis

Figure 6.9: Klebsiella pneumoniae

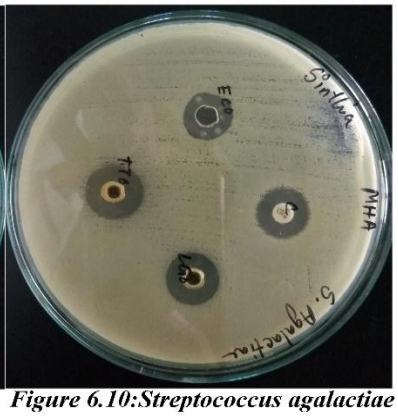


Figure 6.10:Streptococcus agalactiae

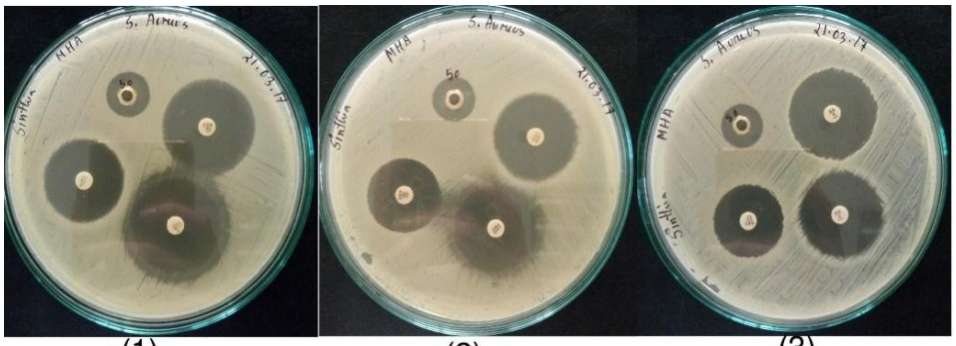
Figure 7.2: Antimicrobial activity of Tea Tree, Eucalyptus and Lemongrass oil against selected Organisms by well diffusion method

3.6 Comparison between Tea Tree oil and conventional Antibiotics

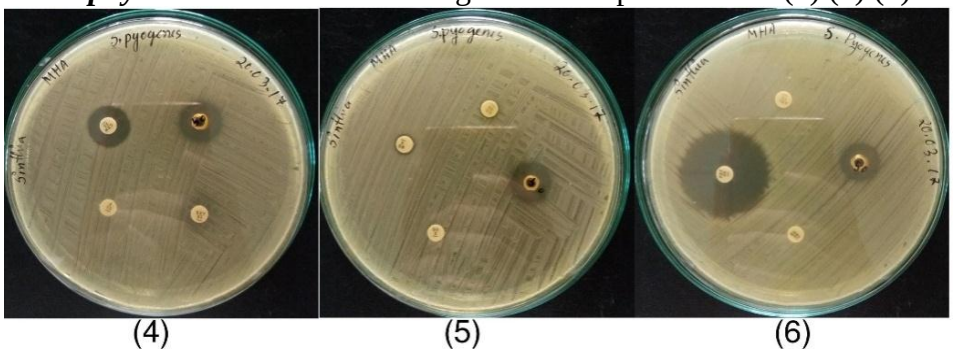
Comparing antimicrobial activity of Tea Tree oil and conventional antibiotics was determined and the zone of inhibition was observed and the diameter (mm) was calculated as follows:

Table 7.1: Zone of inhibition in response to Tea Tree oil (TTO) and conventional antibiotic discs

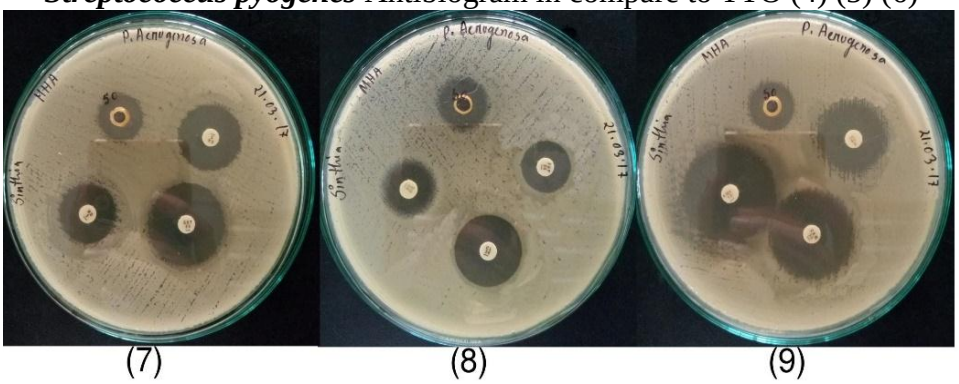
Pathogens / Antibiotics	Zone of Inhibition (mm)									OIL
	Rifampicin	Cefepime	Cephalexin	Erythromycine	Amoxycillin	Sulphamethoazole	Doxycycline	Cefuroxime Sodium	Clindamycin	Tea Tree Oil (50)
Staphylococcus *aureus*	32.6	25.3	33.3	28.6	35.3	24.3	30.6	34.3	29.3	18.6
Streptococcus pyogenes	0	34.2	0	0	0	0	0	14.6	0	17.3
Streptococcus agalactiae	20.3	18.3	24.3	24.3	32.3	29.6	16.6	31.3	24.3	16.3
Pseudomonas aeruginosa	10.3	31.6	0	0	0	0	0	16.3	0	18.6
Proteus vulgaris	17.6	35.3	19.3	14.3	0	21.6	27.6	28.3	0	32.3
Aeromonas hydrophila	8.6	29.3	19.6	0	0	24.3	21.3	23.6	0	24.6
Escherichia Coli	37.6	30.3	37.6	0	38.6	22.3	34.3	35.3	24.6	15.6
Streptococcus pneumoniae	25.6	17.6	28.6	26.3	40.3	27.3	10.3	35.3	23.3	17.6
Bacillus Subtilis	0	24.3	11.3	0	0	9.6	11.6	14.6	0	14.3
Klebsiella pneumoniae	26.6	10.3	35.6	30.3	36.6	38.3	35.3	16.6	28.3	16.6



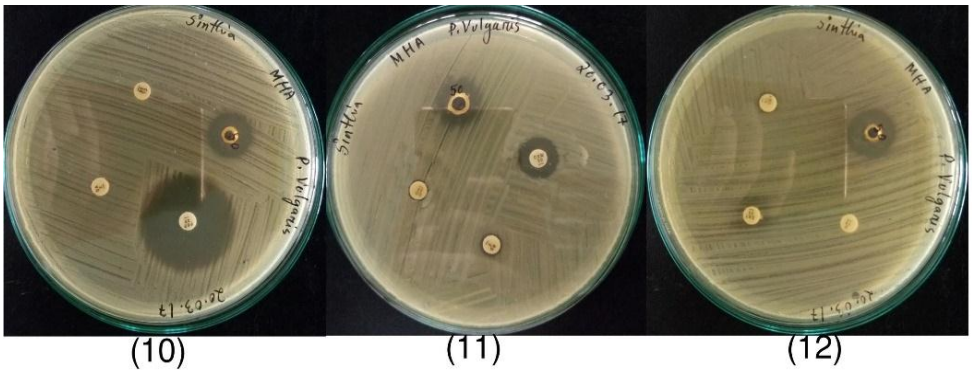
Staphylococcus aureus Antibiogram in compare to TTO (1) (2) (3)



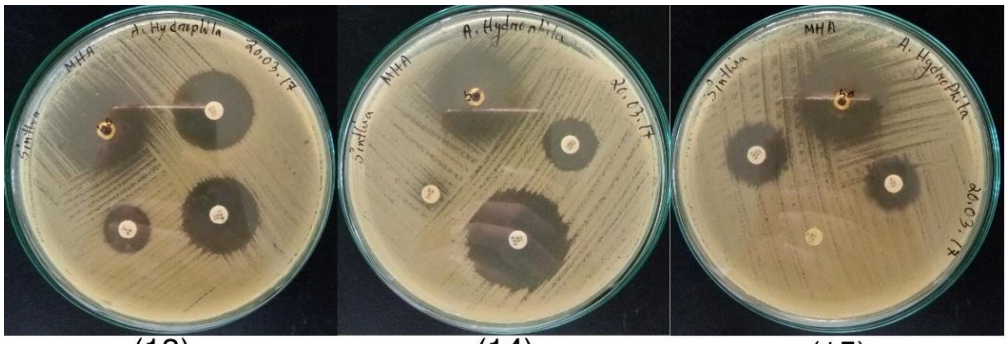
Streptococcus pyogenes Antibiogram in compare to TTO (4) (5) (6)



Pseudomonas aeruginosa Antibiogram in compare to TTO (7) (8) (9)

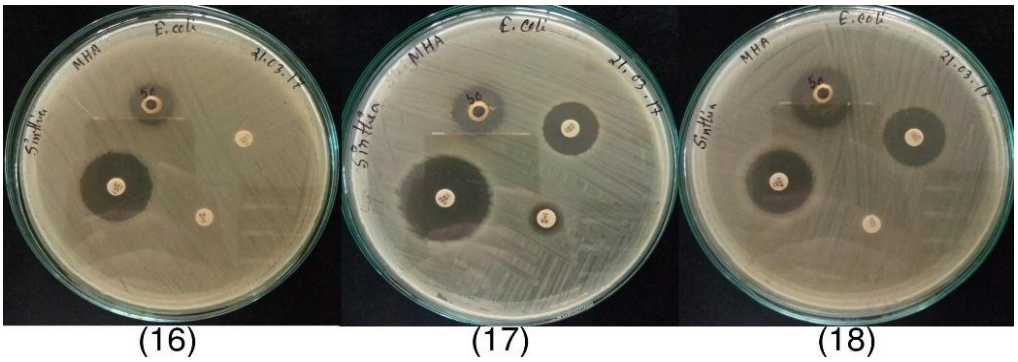


Proteus vulgaris Antibiogram in compare to TTO (10) (11) (12)



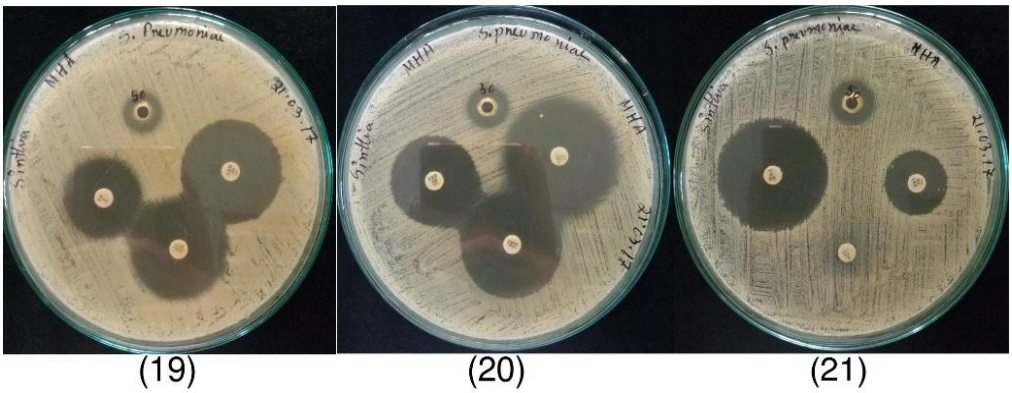
(13) (14) (15)

Aeromonas hydrophila Antibiogram in compare to TTO (13) (14) (15)



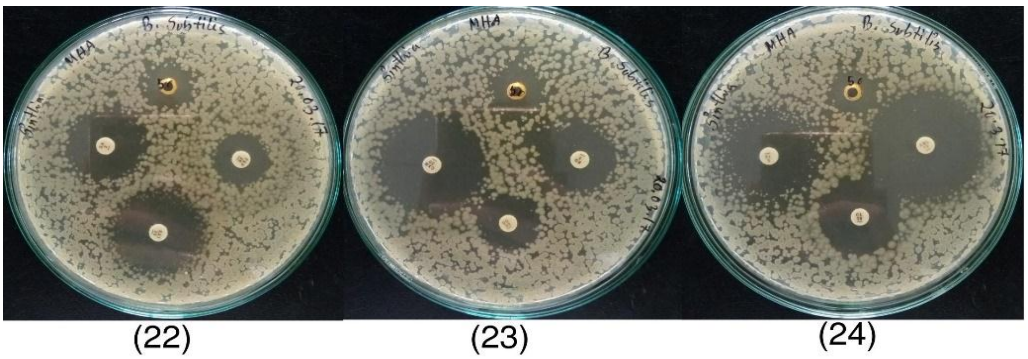
(16) (17) (18)

Escherichia coli Antibiogram in compare to TTO (16) (17) (18)



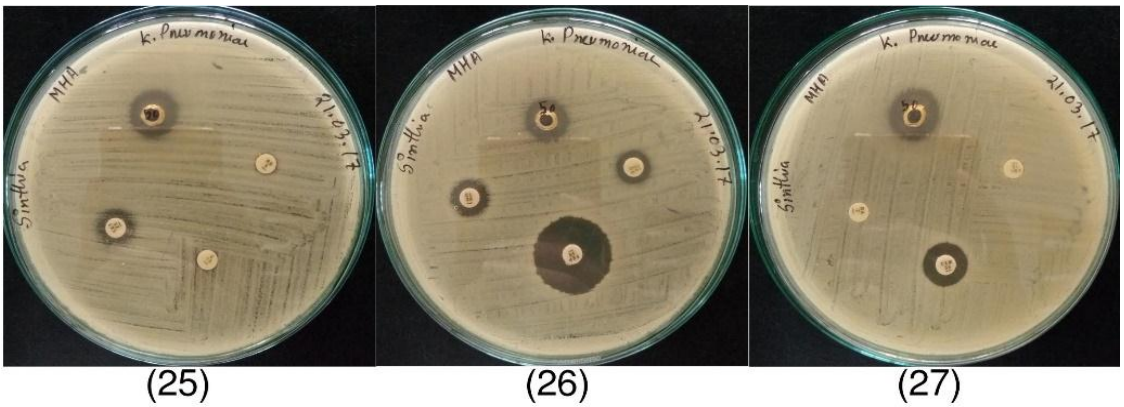
(19) (20) (21)

Streptococcus pneumonia Antibiogram in compare to TTO (19) (20) (21)

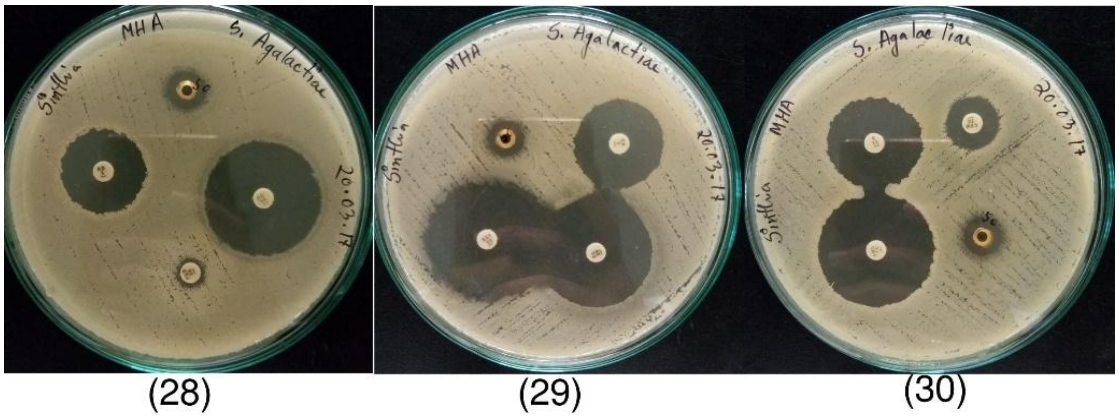


(22) (23) (24)

Bacillus subtilis Antibiogram in compare to TTO (22) (23) (24)



Klebsiella pneumoniae Antibiogram in compare to TTO (25) (26) (27)



Streptococcus agalactiae Antibiogram in compare to TTO (28) (29) (30)

Figure 8.1: Zone of inhibition in response to Tea Tree oil (TTO) and conventional antibiotic discs

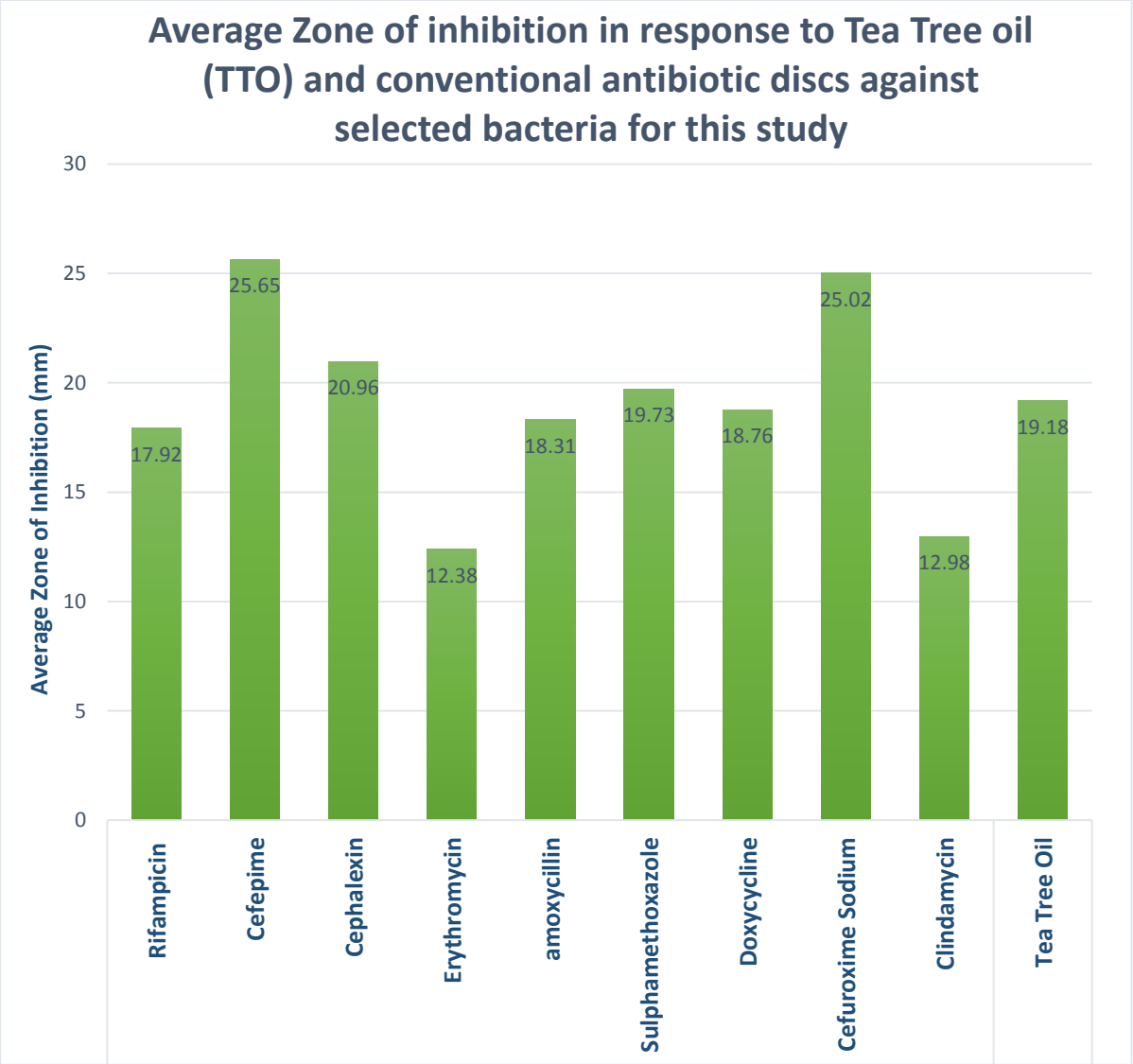


Figure 8.2: Average zone of inhibition in response to Tea Tree oil (TTO) and conventional antibiotic discs against selected bacteria for this study.

Chapter 4

Discussion

Discussion

The appearance of antibiotic resistance organism is increasing vigorously and certainly it's become global concern for researcher. The need of alternatives to antibiotics is heightened to control outburst numbers of antibiotic-resistant strains. New antibiotics need to be discovered or existing antibiotics have to be modified so that these can have broad spectrum activity. However, both the procedures are time consuming as well as expensive. Use of anti-microbial activity of natural products like different extract of plants, essential oils, herbs can be an alternative option to control those microorganisms.

J. May *et al*., 2000 studied with two types of Tea tree oil one is standard tea tree oil another one is chemically cloned tea tree oil. Both of this oil gave more or less same results. The two tree oil was more active against Methicillin sensitive *S. aureus* than (MSSA) Methicillin resistant *S. aureus* (MRSA). This Study based on the time-kill approach, determined the killing rate of tea tree oil against several multidrug-resistant organisms, including MRSA, glycopeptide-resistant enterococci, aminoglycoside-resistant *klebsiellae*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, and also against sensitive microorganisms. A rapid killing time (less than 60 min) was achieved with both tea tree oils with most isolates, but MRSA was killed more slowly than other organisms. In this study, efforts were made to determine the effectiveness of incubation period on antimicrobial activity.

Carson *et al*., 2006 researched that complementary and alternative medicines such as tea tree (melaleuca) oil have become increasingly popular in recent decades. This review summarizes recent developments in our understanding of the antimicrobial and anti-inflammatory activities of the oil and its components, as well as clinical efficacy. Specific mechanisms of antimicrobial and anti-inflammatory action are reviewed, and also the safety and toxicity of the oil is briefly discussed. In this study, effectiveness of the essential oils at various doses weren't studied as there is lack of information on doses that is required to treat diseases.

Fitzpatrick, 2010 compared the antimicrobial effectiveness of fresh garlic (*Allium sativum*), an industrial cleaner and deodorizer Quad 10, and mouthwash Listerine a brand made by McNeil-PPC, Inc., contiguously with tea tree oil. He thought that previous research lacks data as to how tea tree oil compares in its antimicrobial action to other commonly used antibacterial solutions, such as those tested in this experiment. He used a bacterial lawn technique on agar plates for each tested bacterium. Small disks of each of the antimicrobial were placed on plates

of each strain with enough area surrounding each to calculate inhibitory effects. Naik *et al*., 2010 experimented that effectiveness of essential oil of lemongrass for the treatment of pathogenic organisms. Lemongrass oil was investigated for activity against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae*and *Pseudomonas aeruginosa*, using Agar Diffusion Method and Broth Dilution Method. On the other hand in present study, different techniques were used to determine antibacterial activity of TTO, ECO and LGO on selected bacteria responsible for skin infection and a comparative study of the effectiveness of Tea Tree oil with Eucalyptus oil, Lemongrass oil and conventional antibiotics was also performed.

Antibiotic resistant bacteria are difficult to eradicate from skin and *Staphylococci*, *Klebsiellae* by direct contact. From the present study it is clear that Tea Tree oil possess a promising antibacterial activity against ***Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus vulgaris, Aeromonas hydrophila, Escherichia coli, Streptococcus pneumoniae, Bacillus subtilis, Klebsiella pneumoniae***and ***Streptococcus agalactiae***. The results obtained from agar diffusion assay and broth dilution method showed that the inhibition of bacterial growth by **TTO** after incubation for 6 hours ranged from **99.25% to 100%**and after incubation for 24 hours ranged from **99.99% to 100%.** Similarly, the inhibition of bacterial growth by **ECO** after incubation for 6 hours ranged from **62.50% to 100%**and after incubation for 24 hours ranged from **21.87% to 100%.** Likewise, the inhibition of bacterial growth by **LGO** after incubation for 6 hours ranged from **21.21% to 100%**and after incubation for 24 hours ranged from **72.72% to 100%.** The Inhibition percentage of Tea Tree oil, Eucalyptus oil and Lemongrass oil against selected pathogens (Table 5.1) will describe the comparison between these essential oils more evidently.

Mota *et al*., 2014 explored that *Eucalyptus globulus* oil showed higher inhibition than chlorhexidine when applied to *Staphylococcus aureus*, and equal inhibition when applied to the following microorganisms: *Escherichia coli*, *Proteus vulgaris* and *Candida albicans*. Papain (10%) showed lower antimicrobial effect than chlorhexidine in relation *to Candida albicans*. Xylitol showed no inhibition of growth of the tested microorganisms. In this present study, the comparison of antimicrobial activity among Tea Tree oil, Eucalyptus oil and Lemongrass even more clearly described through well diffusion and agar disc diffusion method. It was observed that Tea Tree oil proved the most effective essential oil among these three oil. Tea Tree Oil showed remarkable efficacy against all the organism selected for this

study in well diffusion method. On the other hand, during well diffusion method Eucalyptus and Lemongrass oil didn't exhibit zone of inhibition against ***Escherichia coli*** and ***Klebsiella pneumonia*** where these organisms have great involvement in skin infection. **(**Figure 7.1) presented the overall result of agar diffusion assy.

The use of herbal medicine is becoming popular due to toxicity and side effects of allopathic medicines. This has led to sudden increase in the number of herbal drug manufacturing (Agarwal, 2005). Another important section of this study was to establish comparative analysis of antimicrobial efficacy between Tea Tree oil and conventional antibiotics against selected organism. The purpose of this section was to find out a replacement of broad spectrum antibiotics which has nearly similar ability to control bacterial growth on skin as broad spectrum antibiotics has several side effects and it was done by agar diffusion assay. Nine broad spectrum antibiotics and Tea Tree oil involved in this segment. Undoubtedly Tea Tree oil proved itself as a great substitution of conventional antibiotics as Tea tree oil showed similar efficacy corresponding to seven antibiotics out of nine antibiotics. The claimed statement presented by graphical presentation of average zone of inhibition in response to Tea Tree Oil (TTO) and conventional antibiotic discs against selected bacteria for this study (Figure 8.2).

Lee *et al.,* 2013 found that TTO presented dose-dependent inhibitory effects against the growth of *P. acnes* and *S. aureus*, while the inhibitory effects against *P. acnes* were stronger than those against *S. aureus* .These findings are similar to those shown in Wilkinson and Cavanagh, 2005 and (Carson *et al*., 2006) showed that TTO presented better antibacterial activity toward anaerobic bacteria than aerobic bacteria. They used mass spectrophotometry to separate two major components, terpinen-4-ol and 1,8-cineole, were used to evaluate skin toxicity by a single topical application. On the other hand, in this study, the components were not separated through mass spectrophotometry as they did in their research and also they had applied TTO directly on erythema and edema instead the skin infectious bacteria which was not done in this study. These are the main drawbacks of this study.

Conclusion

The overuse of antibiotics and antimicrobial treatments is creating drug resistance, a public health threat in which bacteria, a fungus, or a virus can become completely resistant to drugs. If this continues for long standard treatments will not work anymore; infections are harder or impossible to control; the risk of the spread of infection to others is increased; and the risk of death is greater—in some cases, twice that of patients who have infections caused by non-resistant bacteria.

Slowly, science is catching up in explaining why tea tree oil is such an effective antimicrobial agent. Over three hundred studies are returned referring to tea tree oil's antimicrobial benefits on skin infections. In this study, it's been proven that Tea Tree oil has noticeable antimicrobial activity against bacteria those are responsible for skin infection in compared to Eucalyptus oil, Lemongrass oil and broad spectrum conventional antibiotics.

In the final analysis, the potential of Tea Tree Oil to be used as natural antimicrobial agent is recommendable as antimicrobial activity against ***Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus vulgaris, Aeromonas hydrophila, Escherichia coli, Streptococcus pneumoniae, Bacillus subtilis, Klebsiella pneumonia*** and ***Streptococcus agalactiae*** were demonstrated. The antibiotics would never be up for use in the first place. Tea tree oil itself as fully effective against all the bacteria used in this study. The development of Tea Tree oil would be a great alternative to conventional antibiotics against bacterial skin infections and many more infectious diseases.

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APPENDIX-I

Media composition

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

1. **Nutrient Agar (Himedia, India)**

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2. **Cetrimide agar (Merck, India)**

Ingredients	Amount (g/L)
Pancreatic digest of gelatin	20.0
Magnesium chloride hexahydrate	1.4
Potassium sulfate anhydrous	10.0
Cetrimide	0.3
Agar-Agar	13.0

3. **Tryptone soy broth, (Oxoid, England)**

Ingredients	Amount (g/L)
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

4. **MR-VP broth**

Ingredients	Amount (g/L)
Peptone	7 g
Dextrose	5 g
Potassium phosphate	5 g

5. MacConkey agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

6. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bactobromthymol blue	0.08

7. Triple sugar iron agar (Himedia, India)

Ingredients	Amount (g/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

8. Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Sucrose	5.0
Lactose	5.0
Di-potassium phosphate	2.0
Eosin Y	0.14
Methylene blue	0.065
Agar	13.50

9. Mannitol Salt agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Manitol	10.0
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

10. Xylose Lysine Deoxycholate agar (Himedia,India)

Ingredients	Amount (g/L)
L- lysine	5.0
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium deoxycholate	2.50
Yeast extract	3.0

11. Mueller Hinton agar

Ingredients	Amount (g/L)
Beef Extract	2.00
Acid Hydrolysate of Casein	17.50
Starch	1.50
Agar	17.00

12. Brain Heart Infusion Broth(BHIB)

Formula	Amount (g/L)
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0

APPENDIX-II

Buffers and reagents

1. Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored a t4^{o}C.

2. Methyl redreagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4^{o}C.

3. Barritt'sreagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4^{o}C.

Solution B

40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminum foil and stored at4^{o}C.

4. Oxidasereagent

100 mg of N,N,N^{1},N^{1}-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^{o}C.

APPENDIX-III

Instruments

The important equipment used through the study are listed below:

Autoclave	**SAARC**
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Oven, Model:MH6548SR	LG, China
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Refrigerator (4^{o}C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Water bath	Korea
Weighing balance	ADAM EQUIPMENT™, United Kingdom

APPENDIX – IV

List of Antibiotics

Name	Concentration	Short Form	Manufacturer
Rifampicin	5 µg/disc	RD5	Oxoid
Cefepime	30 µg/disc	FEP30	Oxoid
Cephalexin	30 µg/disc	CL30	Oxoid
Erythromycin	15 µg/disc	E15	Oxoid
Amoxycillin	10 µg/disc	AML10	Oxoid
Sulphamethoxazole	25 µg/disc	SXT25	Oxoid
Doxycycline	30 µg/disc	DO30	Oxoid
Cefuroxime Sodium	30 µg/disc	CXM30	Oxoid
Clindamycin	2 µg/disc	DA2	Oxoid