Comparative study on the antibacterial activities of Neem oil, Mustard oil and Black seed oil against *Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus, Salmonella typhi and Pseudomonas aeruginosa*

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

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Declaration

I hereby declare that the thesis project titled "**Comparative study on the antibacterial activities of Neem oil, Mustard oil and Black seed oil against *Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus, Salmonella typhi and Pseudomonas aeruginosa.*"** submitted by me has been carried out under the supervision of Dr. M. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is based on actual and original work carried out by me. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Abstract

The study was aimed to determine the antimicrobial activity of commercially available Neem oil, Mustard oil and Black seed oil against some disease causing organism such as *Staphylococcus aureus, Klebsiella pneumoniae , Bacillus cereus, Salmonella typhi and Pseudomonas aeruginosa.* This study of antibacterial activity against selected pathogens was done by dilution method, agar disc diffusion method and agar well diffusion method. Pathogens were taken from BRAC University stock culture.

All three essential oils tested showed antibacterial activity against *Staphylococcus aureus, Klebsiella pneumonia* and *Bacillus cereus and Salmonella typhi*. Black seed oil and Neem oil showed highest rate of antibacterial activity even at very low concentration. In case of all four pathogens, inhibition of growth caused by Neem oil was more than 99%. Black seed oil inhibited the growth of *Staphylococcus aureus and Bacillus cereus* by 100% and by 99.97% in case of *Klebsiella pneumonia.* Mustard oil exhibited antibacterial activity against the tested bacteria by dilution method but no zone of inhibition was found by agar disc diffusion or agar well diffusion method. *Pseudomonas aeruginosa* exhibited very low degree of sensitivity to Mustard oil and Black seed oil. However, Neem oil showed antibacterial activity against *Pseudomonas aeruginosa* by dilution method only.

The results showed that all these oils can be a good source of antibacterial agents. The encouraging results also indicate that these oils should be exploited as natural antibiotic for the treatment of several infectious diseases caused by these pathogens, and could be useful in understanding the relations between traditional cures and current medicines.

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List of Abbreviations

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
TSI	Triple Sugar Iron
MR	Methyl Red
VP	Voges- Proskauer
CFU	Colony Forming Unit

Chapter 1

Introduction

Introduction:

1.1 Overview

In this era of emergence of multi drug resistant organisms and a decrease in newer antibiotics, the ancient healing methods by using traditional oil can be considered a very effective way. People's perception towards traditional medicine has also changed and is very encouraging. Medicinal plants have been used for curing diseases for many centuries in different indigenous systems of medicine as well as folk medicines. Moreover, medicinal plants are also used in the preparation of herbal medicines as they are considered to be safe as compared to modern allopathic medicines (Ahmad et al., 2013). World health organization estimates that 80% of the population living in the developing countries relies exclusively on traditional medicine for their primary health care. More than half of the world's population still relies entirely on plants for medicines, and plants supply the active ingredients of most traditional medical products (Kumar & Navaratnam, 2013). Higher and aromatics plants have traditionally been used in folk medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeasts (Hulin et al., 1998). Most of their properties are due to essential oils produced by their secondary metabolism. Essential oils and extracts from several plant species are able to control microorganisms related to skin, dental caries, and food spoilage, including Gram-negative and Gram-positive bacteria. Many countries have maintained research programs to screen traditional medicines for antimicrobial activity, as is the case of India, Palestin , Africa, Honduras , Jordan , Cuba and Italy . Plants from Brazilian biomes have also been used as natural medicines by local populations in the treatment of several tropical diseases, including schistosomiasis, leishmaniasis, malaria and fungal and bacterial infections (Sartoratto et al., 2003).

1.2 Oils as treatment agent:

Essential oils have long been known for their ability to heal, but few people realize that they can even kill off bacteria, viruses, and fungi, fighting off and preventing a host of infections, treating skin conditions and more. Essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties (Burt, 2004). Some oils have been used in cancer treatment. Some other oils have been used in food preservation, aromatherapy and fragrance industries (Sylvestre et al., 2005). 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:

Hospital-acquired infections and antibiotic-resistant bacteria continue to be major health concerns worldwide. The oils can be a great way to fight bacteria without having to experience potentially severe side effects that come with antibiotics. In fact, they're generally much safer than taking a prescription antibiotic and also will help to address the worldwide problem of antibiotic resistance as well (Warnke et al., 2009).

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).

According to Romm, "In the U.S., antibiotic resistance caused more than two million illnesses in 2013, according to a report by the Centers for Disease Control and Prevention, and an estimated 23,000 deaths," and they've also amounted to an extra $20 billion in healthcare costs and it's only poised to get worse: a recent report commissioned by the U.K. government estimates that drug-resistant microbes could cause more than 10 million deaths and cost the global economy $100 trillion by the year 2050 (Rodreguez, 2015).

1.4 General Characteristics of Black seed

The seeds of *N. sativa* and their oil have been widely used for centuries in the treatment of various ailments throughout the world. It is an important drug in the Indian traditional system of medicine and among Muslims, it is considered as one of the greatest forms of healing medicine. It is also recommended for use on regular basis in Tibb-e-Nabwi (Prophetic Medicine) (Ahmad et al., 2013). *N. sativa* is an annual flowering plant which grows to 20-90 cm tall, with finely divided leaves, the leaf segments narrowly linear to threadlike. The flowers are delicate, and

usually coloured white, yellow, pink, pale blue or pale purple, with 5-10 petals. The fruit is a large and inflated capsule composed of 3-7 united follicles, each containing numerous seeds (Goreja, 2003) . Seeds are small dicotyledonous, trigonus, angular, tubercular, black externally and white inside, odor slightly aromatic and taste bitter.

Figure 1: Black Seed

1.4.1 Classification of Black seed (Khare., 2004):

Kingdom - Plantae

Subkingdom - Tracheobionta

Superdivision - Spermatophyta

Phylum - Magnoliophyta

Class -Magnoliopsida

Subclass -Magnoliidae

Order - Ranunculales

Family - Ranunculaceae

Genus - *Nigella*

Species - *N. sativa*

1.4.2 Chemical composition of Black seed oil

Nigella sativa seed contains fixed oil that ranges between 28% to 36% and chiefly composed of unsaturated fatty acids that are arachidonic, eicosadienoic, linoleic and linolenic and saturated fatty acids that includes palmitic, stearic and myristic . The seed oil contains compounds such as cholesterol, campesterol, stigmasterol, β-sitosterol, α-spinasterol, (+)-citronellol, (+)-limonene, p-cymene, citronellyl acetate, carvone, nigellone, arachidic, linolenic,

linoleic, myristic, oleic, palmitic, palmitoleic and stearic acids . Seed oil contains fixed oils like linoleic acid (55.6%), oleic acid (23.4%) and palmitic acid (12.5%) and volatile oils like trans-anethole (38.3%), p-cymene (14.8%), limonene (4.3%), and carvone (4.0%) (Dinagaran et al., 2015).

1.4.3 Medicinal uses of Black seed

Researchers have found many beneficial properties of Black seed. Medicinal uses of the seeds of *N. sativa* are given below (Al- Ali et al., 2008);

1. It is used in the treatment of various diseases like bronchitis, diarrhea, rheumatism, asthma and skin disorders.
2. It acts as a liver tonic, anti- diarrheal, appetite stimulant, emmenagogue.
3. It is used in digestive disorders, to increase milk production in nursing mothers to fight parasitic infections, and to strengthen immune system.
4. Seeds are useful in the treatment of worms and skin eruptions.
5. Oil is used as an antiseptic and local anesthetic externally. Roasted black seeds are given internally to stop the vomiting (Morsi, 2000).

1.5 General Characteristics of Neem

Azadirachta indica is locally known as Neem. It is a tree in the mahogany family of *Meliaceae*. It has one or two species in the genus of *Azadirachta.* It is native to India, Bangladesh, Thailand, Nepal and Pakistan. It is growing well in tropical and sub-tropical regions. The neem oil is isolated from its fruits and seeds (Akerele, 1993) ; (Ghimeray et al., 2009) Neem is most important medicinal plant that has been declared worldwide as the "Tree of the 21st century" by the United Nations (Hossain et al., 2013). The neem tree is an attractive broad leaved evergreen which can grow up to 30m tall with spreading branches covering some 10 m across. Both the bark and leaves also contain biologically active molecules but not high levels of azadirachtin which is found mainly in the seed kernels. Mature trees may produce some 2 kg of seed per year. The tree is now grown in most tropical and sub-tropical areas of the world for shade, for reforestation programmes and in plantations for the production of compound which have toxic, antifeedant and repellent properties against insects (Mordue et al., 2000).

Figure 2: Neem oil

1.5.1 Classification of Neem (Plant Database, USDA):

Kingdom - Plantae

Subkingdom - Tracheobionta

Superdivision - Spermatophyta

Division - Magnoliophyta

Class - Magnoliopsida

Subclass - Rosidae

Order - Sapindales

Family - Meliaceae

Genus - *Azadirachta*

Species - *Azadirachta indica*

1.5.2 Chemical composition of Neem oil:

Neem oil contains 29.27% of lipids, 12.10% of proteins and 43.28% of parietal constituents. The azadirachtin content is at 2.24 g.kg-1. It has 96.82% of the lipids and 92.20% of the proteins. The oil contains nine fatty acids, including four major ones. These are oleic acid (41.91±0.69%), linoleic acid (19.59±0.44%), stearic acid (18.71±0.46%) and palmitic acid (15.59±0.27%). The oil consists of two major triglycerides: SOL by a proportion of 52.93% and

POL by a proportion of 36.61%. The protein composition of neem seeds reveals the presence of seventeen amino acids. The major compounds are glutamic acid (23.65±0.2%), aspartic acid (9.62±0.04%), glycine (8.64±0.09%), leucine (8.09±0.11%), serine (7.19±0.24%) and alanine (7.14±0.06%) (Djibril et al., 2015).

1.5.3 Medicinal uses of Neem

Due to its medicinal importance it is used to prepare formulated medicine for the treatment of a variety of human ailments. Those are given below;

1. Traditionally, people used to clean their teeth with neem twigs.
2. Drink of its juice is considered as a good tonic to increase appetite and cure fever or to kill intestinal worms.
3. Traditionally, neem is also widely used in Indian Ayurvedic medicine system for the treatment of incurable diabetes (Ping, Yong & Ming, 2002).
4. Therapeutically, its crude extracts from bark and leaves have been used in folk medicine to control diseases such as leprosy, intestinal helminthiasis and respiratory system (Prieto et al., 1999).
5. Besides these uses, there are several other reports on the biological and pharmacological actions such as antiviral, antibacterial, antifungal, anti-inflammatory, antipyretic, antiseptic and antiparalitic uses (Britto & Sheeba, 2013)

1.6 General Characteristics of Mustard

Mustard is an annual herb with seedlings that emerge rapidly, but then usually grow slowly. Plants cover the ground in 4 to 5 weeks with favorable moisture and temperature conditions. The tap roots will grow 5 ft into the soil under dry conditions, which allows for efficient use of stored soil moisture. Plant height at maturity varies from 30 to 45 in. depending on type, variety, and environmental conditions. Mustard is a cool season crop that can be grown in a short growing season. Varieties of yellow mustard usually mature in 80 to 85 days whereas brown and oriental types require 90 to 95 days. Soils prone to crusting prior to seedling emergence can cause problems. This crop will not tolerate water logged soils since growth will be stunted. Dry sand

and dry, sandy loam soils should also be avoided. Seed will germinate at a soil temperature as low as 40°F (Oplinger et al., 1991).

Figure 3: Mustard oil

1.6.1 Classification of Mustard (Plant Database, USDA):

Kingdom - Plantae

Subkingdom - Tracheobionta

Superdivision - Spermatophyta

Division - Magnoliophyta

Class - Magnoliopsida

Subclass - Dilleniidae

Order - Capparales

Family - *Brassicaceae*

Genus - *Brassica juncea*

1.6.2 Medicinal uses of Mustard (Grieve, 1931):

1. Mustard is used in the form of poultices for external application near the seat of inward inflammation, chiefly in pneumonia, bronchitis and other diseases of the respiratory organs.
2. It relieves congestion of various organs by drawing the blood to the surface, as in head affections, and is of service in the alleviation of neuralgia and other pains and spasms.
3. Mustard Leaves, used instead of poultices, consist of the mustard seeds, deprived of fixed oil, but retaining the pungency-producing substances and made to adhere to paper.
4. Oil of Mustard is a powerful irritant and rubefacient, and when applied to the skin in its pure state, produces almost instant vesication. It is a very useful application for chilblains, chronic rheumatism, colic, etc.
5. Hot water poured on bruised Black Mustard seeds makes a stimulating footbath and helps to throw off a cold or dispel a headache. It also acts as an excellent fomentation.
6. Internally, Mustard is useful as regular and mild aperients, being at the same time an alternative.
7. The bland oil expressed from the hulls of the seeds, after the flour has been shifted away, promotes the growth of the hair and may be used with benefit externally for rheumatism.

1.6.3 Chemical composition of Mustard oil

Mustard seeds have high-energy content, having 28–32% oil with relatively high protein content (28–36%). The amino acid composition of mustard protein is well balanced; it is rich in essential amino acids. Mustard seeds until now have been used mainly for condiment production, however, this advantageous chemical composition and its relatively low price offer wide possibilities for utilization of this valuable seed, for example, in human foods as additive and to feed animals. Mustard oil has a special fatty acid composition, it contains about 20–28% oleic acid, 10–12% linoleic, 9.0–9.5% linolenic acid, and 30–40% erucic acid, which is indigestible for human and animal organisms. The high erucic acid content of mustard seed could be reduced by breeding, some low erucic acid content genotypes are in cultivation in several countries. Mustard oil is rich in tocopherols, as a consequence of their antioxidant characteristic, they act as a preservative against rancidity (Moser et al., 2009).

1.6.4 Infection caused by clinical pathogens against which antibacterial activity of oils are tested:

Infection occurs when viruses, bacteria, or other microbes enter our body and begin to multiply. Disease occurs when the cells in our body are damaged as a result of infection and signs and symptoms of an illness appear (Janeway et al., 2001).

Table 5: Infection caused by clinical pathogens used in this study

S.No	Test pathogens	Infections
1.	*Staphylococcus aureus*	Cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections. (Tong et al., 2015)
2.	*Pseudomonas aeruginosa*	malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia (Bodey et al., 1983)
3.	*Salmonella typhi*	causes food poisoning and typhoid (*Hema et al.*, 2013)
4.	*Bacillus cereus*	causes skin infetion, food poisoning, food-borne intoxications and also causes ocular infections (*Hema et al.*, 2013)
5.	*Klebsiella pneumoniae*	neumonia, septicaemia, meningitis, rhinoscleroma, ozaena, sinusitis, otitis, enteritis, appendicitis and cholycystitis (Sikarwar, 2011)

1.8 Resistance of bacteria:

Antibiotic resistance has become a major clinical and public health problem within the lifetime of most people living today. Confronted by increasing amounts of antibiotics over the past 60 years, bacteria have responded to the deluge with the propagation of progeny no longer susceptible to them (Levy, 2002). 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 *Staphylococcus 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. The highest percentage of methicillin resistance was determined as 30% in 2009, and the resistance was determined to have decreased in subsequent years (20%, 16%, 13%, 19%, and 21%) (Rağbetli et al.,2016)

1.8.2 Resistance of *Klebsiella pneumoniae*

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).

1.8.3 Resistance of *Bacillus cereus*

Bacillus cereus isolates are generally resistant to β-lactam antibiotics such as ampicillin (98%), oxacillin (92%), penicillin (100%), amoxicillin (100%), and cefepime (100%) but susceptible to other antibiotics (Owusu-Kwarteng, 2017).

1.8.4 Resistance of *Salmonella typhi*

Since 1996, the National Antimicrobial Resistance Monitoring System (NARMS) has identified increasing numbers of Salmonella isolates resistant to amoxicillin/clavulanate, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. The isolates also have decreased susceptibility or resistance to ceftriaxone, an antimicrobial used to treat serious infections in children (CDC, 2009)

1.8.5 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. (Lister, et al., 2009) *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%) (Yayan et al., 2015)

1.9 Literature Review:

Seenivasan Prabuseenivasan, Manickkam Jayakumar, and Savarimuthu Ignacimuthu (2017) conducted a research to evaluate the antibacterial activity of 21 plant essential oils against six bacterial species. The selected essential oils were screened against four gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) and two gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* at four different concentrations (1:1, 1:5, 1:10 and 1:20) using disc diffusion method. Out of 21 essential oils tested, 19 oils showed antibacterial activity against one or more strains. Cinnamon, clove, geranium, lemon, lime, orange and rosemary oils exhibited significant inhibitory effect. Cinnamon oil showed promising inhibitory activity even at low concentration, whereas aniseed, eucalyptus and camphor oils were least active against the tested bacteria. In general, *B. subtilis* was the most susceptible. On the other hand, *K. pneumoniae* exhibited low degree of sensitivity. Majority of the oils showed antibacterial activity against the tested strains.

Abobakr M. Mahmoud, Rehab Mahmoud Abd El-Baky , Abo Bakr F Ahmed and Gamal Fadl Mahmoud Gad (2012) conducted a study aimed to investigate the clinical effectiveness of most of the marketed antimicrobials is found to be threatened by the rapid emergence of multidrug resistant pathogens which increase the need to find alternatives. The objective of their study was to investigate the antibacterial activity of some essential oils against different microorganisms and to study the possible effects between the tested oils and some standard antimicrobials. The antibacterial activity of 11 essential oils was evaluated against *Staphylococcus aureus*, *E. coli*, *Klebseilla pneumoniae* and *Pseudomonas aeruginosa* and 50 clinical strains isolated from different infections each alone and in combination with some standard antimicrobials using well diffusion method. Minimum inhibitory concentrations were determined using linear regression analysis. Results showed that all tested essential oils have good antimicrobial activity. As Coriander oil showed the highest antimicrobial activity against *Staphylococcus aureus* and *E. coli* followed by Origanum and Ivy oil. Cumin oil showed the highest activity against *E. coli* followed by Origanum oil while Chammomile and Onion oil showed the highest activity against *Pseudomonas aeruginosa*. In-vitro interaction between the tested antimicrobials and oils showed variable results against the tested bacteria. The results are of significance in health care system and

microbial diseases treatment. Their study showed that essential oils possessed good antimicrobial activity against the tested strains. Most of essential oils/antimicrobials combinations showed synergistic effects. Essential oils can be used as adjuvant to antibiotic therapy.

Uwimbabazi Francine, Uwimana Jeannette, and Rutanga Jean Pierre (2015) investigated antibacterial activity of Neem plant (*Azadirachta indica*) on *Staphylococcus aureus* and Escherichia coli. Their study was carried out to screen and evaluate antimicrobial activity of leaf and bark extracts of *Azadirachta indica*. Ethanol and aqueous extract of leaves and barks of *A. indica* (Neem) were tested against Escherichia coli and *Staphylococcus aureus* which are known to be resistant to various antibiotics. The efficiency of the extracts was studied and determined by applying different extract concentrations onto the two cultured bacterial strains using the disc diffusion method. Ethanol and aqueous extracts were prepared from both dry and fresh neem's leaves and barks. The susceptibility of tested bacteria to both extracts was determined by measuring the diameter of inhibition zones formed around plates. With the statistical test analysis, the comparison done showed that, Fresh Neem materials was found to be the most showing much effect on both *Escherichia coli* and *Staphylococcus aureus*. Comparing Neem leaves and Neem barks, always their fresh extracts were found more efficient than dry extracts; the same as ethanol extracts were more effective than aqueous extracts in all cases, *S. aureus* was the only bacterium susceptibly affected by these neem extracts used; while *E. coli* didn't respond to any of them. The results showed that the effectiveness of the extracts was dependent of the concentration used thus the increase of extract concentration increased the inhibition zone.

Hussein Ahmed Bakathir and Nageeb Ahmed Abbas (2011) conducted a study to detect the antibacterial effect of *Nigella Sativa* ground seeds with water. In their study they have tried to use the normal human mechanism in digestion by using the ground seeds. A modified paper disc diffusion method was used to test the antibacterial effect of NS seeds. They found clear inhibition of the growth of *Staphylococcus aureus* . It was observed by concentration of 300mg/ml with distilled water (D.W.) as control , this inhibition was confirmed by using the positive control Azithromycin. The inhibition obtained was higher with *Nigella sativa* ground seeds from Hadramout (HNSGS) than with *Nigella sativa* ground seeds from Ethiopia (ENSGS). No inhibition was found in the growth of *E.Coli* and *Enterobacter*. This was emphasized by

using the positive control Ciprofloxacin. The positive inhibition may be attributed to the two important active ingredients of NS, Thymoquinone and melanin.

Shiva Rafati, Mohammad Niakan, and Mohsen Naseri (2014) researched about anti-microbial effect of *Nigella sativa* seed extract against staphylococcal skin infection. The in vivo anti-microbial effect of the Nigella sativa seeds extract at a concentration of 33% on pustules staphylococcal Skin Infections was assessed and compared with standard drug mupirocin on 40 neonates .All neonates were divided and examined into two experimental and control groups randomly. Recovery times were compared between two groups. The mean of recovery time in experimental group was 75/1 with SD= ± 12, and the mean of recovery time in control group was 69/4 with SD = ± 8/7.There was no significant difference in recovery time between two groups (p value = 0/131). In clinical practice, the agent of *Nigella Sativa* recovered as pustular from tissues of all patients. While the extract was as nearly effective as the standard drug, mupirocin, no side effect was observed.

Amanuel Amare, Atsede Hadush, Haftu Aregawi and Nigisti Kide (1999) conducted a research on antibacterial activity of oil extracts of Black Mustard (*Brassica nigra*) seeds against bacteria isolated from fresh juice in selected areas of Axum town. To study antimicrobial activity of oil extracts of *Brassica nigra* seed against fresh juice pathogens. Oil extraction *B. nigra* was prepared to inhibit the fresh juice pathogens. Pathogens that was found in the fresh juice were *B. cereus, S. aureus* and *Salmonella*. The inhibition zone of oil extract *B. nigra* by ethanol was dominated on oil extract *B. nigra* by acetone and positive control Ampicillin. They found *B. nigra* seed is an important for antibacterial activity.

Mélanie Turgis , Jaejoon Han , Stéphane Caillet and Monique Lacroix (2009) investigated antimicrobial activity of mustard essential oil against *Escherichia coli* O157:H7and *Salmonella typhi.* The aim of the study was to investigate how mustard essential oil (EO) affected the cell membrane of *Escherichia coli* O157:H7 and *Salmonella typhi*. Intracellular pH and ATP concentration and the release of cell constituents were measured when mustard EO was in contact with *E. coli* and *S. typhi* at its minimal inhibitory concentration (MIC) and maximal tolerated concentration (MTC). The treatment with mustard EO affected the membrane integrity of bacteria and induced a decrease of the intracellular ATP concentration. Also, the extracellular ATP concentration increased and a reduction of the intracellular pH was observed in both

bacteria. A significantly ($P \leqslant 0.05$) higher release of cell constituent was observed when both bacteria cells were treated with mustard EO. Electronic microscopy observations showed that the cell membranes of both bacteria were apparently damaged by mustard EO. In conclusion, mustard EO affects the concentration of intracellular component, such as ATP in both bacteria and affects the pH suggesting that cytoplasmic membrane is involved in the antimicrobial action of mustard EO. Mustard EO can be used as an effective antimicrobial agent. We have demonstrated that mustard EO affected the cell membrane integrity, resulting in a loss of cell homeostasis.

1.10 Aims and Objectives:

Aims of this study was to find the antimicrobial activity of Neem oil, Mustard oil and Black seed oil on some pathogenic bacteria such as; *Staphylococcus aureus, Klebsiella pneumoniae , Bacillus cereus, Salmonella typhi and Pseudomonas aeruginosa*. In the present situation due to emergence of multi drug resistant organisms alternative options are increasing importance for the treatment of bacterial infections. Another purpose of this study was to find alternative way in the treatment of skin diseases.

On the basis of above context, the objectives of the present study are:

- To determine the antibacterial activity of of Neem oil, Mustard oil and Black seed oil on some pathogenic bacteria.
- To determine the inhibition rate of these oils against *Staphylococcus aureus, Klebsiella pneumoniae , Bacillus cereus, Salmonella typhi and Pseudomonas aeruginosa*.
- To compare the oils with standard antibiotic against these pathogens.

Chapter 2

Materials and Methods

2.1 Working Place

Overall research was performed in the Microbiology Research Laboratory, Departmentof Mathematics and Natural Sciences, BRAC University. All the microbiological works were done inside Biological Safety Cabinet.

2.2 Bacterial Strains

In this study, the used bacterial species were *Staphylococcus aureus, Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Bacillus cereus and Salmonella typhi.* All these organisms were obtained from BRAC University stock culture.

2.3 Product tested

Commercially available Neem oil, Mustard oil and Black seed oil which do not contain any preservatives.

2.4 Conformation of the stock culture:

Each bacterial strain were subjected to morphological and biochemical confirmation tests. Biochemical tests performed were Methyl red test, Voges–Proskauer test, Indole test, Citrate utilization test, Oxidase test, Catalase test and TSI agar 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 37^0C before the process of any biochemical identification test.

2.4.1 Preparation for reviving the bacteria

- Nutrient agar was prepared for each of the five 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. However, in case of *S. typhi* for 48 hours incubation time was followed.

2.4.2 Growth on selective media

All the organisms from their previous culture was streaked on Selective agar plates for confirmation of the pathogen. The plates were incubated at 37 °C for 24 hours. In case of *S. typhi* plates were incubated for 48 hours.

2.4.3 Citrate utilization test

- A small vial was taken into which a slant of the Simmon's citrate agar was prepared and allowed to solidify.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle and carefully streaked onto the slope of the citrate media.
- The vial was incubated at 37^0C for 24 hours.
- Over these 24 hours, if the organism had the ability to utilize citrate, it would change the colour of the media from deep green Prussian blue. A negative result would keep the colour unchanged.

2.4.4 Triple Sugar Iron (TSI)

- TSI media was prepared into a screw capped test tube, and solidified to obtain a slant and butt at the length of the test tube.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle and carefully stabbed at the butt of the TSI (dextrose, lactose & sucrose) followed by slow streaking at the slant.
- The screw caps of the test tube were loosened and the tube was incubated at 37^0C for overnight. After the 24-48 hours of the incubation period, the test tube was examined to observe carbohydrate fermentation, Carbon dioxide (CO_2) and Hydrogen sulfide (H_2S) gas production.

- If the organism is able to ferment all the three sugars, then the butt would turn into yellow colour indicating the production of acid and the subsequent decrease in the pH of the media, whereas a red colour in the slant and butt indicated that the organism being tested is a non-fermenter and the media remains alkaline.
- Presence of bubbles, splitting and cracking of the medium is the indication of CO_2 gas production. A black precipitation in the butt of the tube is the indication of H_2S production.

2.4.5 Catalase test

- 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.
- The glass slides were labeled according to the sample being tested.
- 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.
- An immediate bubble formation indicated a positive result.
- The same procedure was carried out for the clinical and the environmental strains

2.4.6 Oxidase test

- A number of filter papers were taken, and two drops of oxidase reagent (Aminodimethylaniline oxalate) were added onto the filter papers (Whatman, 1MM).
- The filter papers were labeled according to the sample being tested.
- 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).
- 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.

2.4.7 Methyl red (MR) test

- Six ml of dextrose phosphate (MR-VP) broth was prepared in five test tubes.3 ml from each of the test tubes were transferred to another five different empty test tubes.
- All the ten test tubes were labeled according to the sample being tested and the test being conducted.

- 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.
- After the incubation period was over, five drops of the Methyl red reagent was added into the six test tubes (labeled as MR test) to check the pH of the medium.
- Development of a red colour would indicate a positive result, whereas a yellow colour would indicate a negative result.

2.4.8 Voges–Proskaur test

- 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.
- 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.
- Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
- 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 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) at37 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.

2.5 Methods for Detection of antibacterial activity:

2.5.1 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.

2.5.2 Comparing with the McFarland Solution

McFarland solution is an essential material needed before testing the microorganisms for their sensitivity. McFarland standards are used as reference to adjust the turbidity of any given bacterial suspension. This is done to make sure that the number of bacteria is within a given range to standardize the microbial testing. This would also help avoid any error in result, because if the suspension is too heavy or too diluted, an error might occur for any given anti microbial agent.

- The bacterial suspension prepared was compared with the commercially available McFarland solution 2 (for detection of inhibition rate) and McFarland solution 0.5 (for detection of zone of inhibition by agar disc/well diffusion method). A bacterial suspension which matches with McFarland 2 is supposed to contain 6×10^8 colonies per ml. A bacterial suspension which matches with McFarland 2 is supposed to contain 1.5×10^8 colonies per ml (McFarland, 1907).

2.5.3 Procedure of dilution:

- Bacterial suspension matched with McFarland 2 was subjected to 10 fold dilution both in saline and in oil.
- One thousand eight hundred microlitre of oil and saline was taken separately in sets of 7 tubes.
- Two hundred microlitre of bacterial suspension was added to the 1st tube and 200µl solution was transferred in the 2nd tube and this procedure was repeated till 7th tube. Before transferring the solution every tube was subjected to vortex for uniform mixing.

- From the 7th tube 200 µl solution was discarded.

2.5.4 Detection of inhibition rate:

- One hundred microlitre of the both diluted and undiluted samples was spread on the agar plate containing nutrient agar and from each diluted tube containing saline was spread immediately.
- One hundred microlitre from each diluted tube containing oils was spread on agar plate after 24 hour incubation.
- CFU in saline and CFU in oil of each spread plate was counted and compared.
- Rate of inhibition in case of every diluted tube was then calculated and averaged to detect actual inhibition rate.

2.6 Agar disc and well diffusion method

When a filter paper disc or well containing a chemical is placed on the agar the chemical will diffuse from the disc or well into the agar. This diffusion will place the chemical in the agar only around the disc and around the well. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition". Many conditions can affect a disc diffusion susceptibility test. When performing these tests certain things are held constant so only the size of the zone of inhibition is variable. Conditions that must be constant from test to test include the agar used, the amount of organism used, the concentration of chemical used, and incubation conditions (time, temperature, and atmosphere). The amount of organism used is standardized using McFarland 0.5 standard for this method.

2.6.1 Preparation of dried filter paper discs

- Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish after sterilization in autoclave.

2.6.2 Inoculation on the nutrient agar (NA) plate

- A sterile swab was dipped into the bacterial suspension and the test organisms were suspended in 5 ml of nutrient broth.
- The swab was rotated against the side of the tube using firm pressure, to remove excess fluid, but the swab was not dripped wet.
- The dried surface of the Nutrient agar plate 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 .
- The plate was rimmed with the swab to pick up any excess liquid .
- 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.

2.6.3 Placement of the Oil disc and antibiotic disc

- Oil discs containing 20 µl concentration of Neem oil , Black seed oil and Mustard oil was made using filter paper and then placed on the plates using a sterile forcep.
- One sterile antibiotic disc was placed on the surface of an agar plate, using a forcep. The forcep was sterilized by immersing the forceps in alcohol. It was then burnt. The disks were gently pressed with the forcep to ensure complete contact with the agar surface. The disks were placed away from the edge of the plates so that it is easily measured.
- Once all disks are in place, the plates were inverted, and placed them in a 37 °C incubator for 24 hours .

2.6.4 Placement of oil in the well

- Well was made in agar using a borer .
- Twenty microlitre of oil was placed in the well using a pipette .

- Zone of inhibition was then measured after 24 hours incubation at 37º C

2.6.5 Measuring zone sizes:

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

2.7. Data analysis:

Data were analysed using Microsoft excel version 2007.

Chapter 3

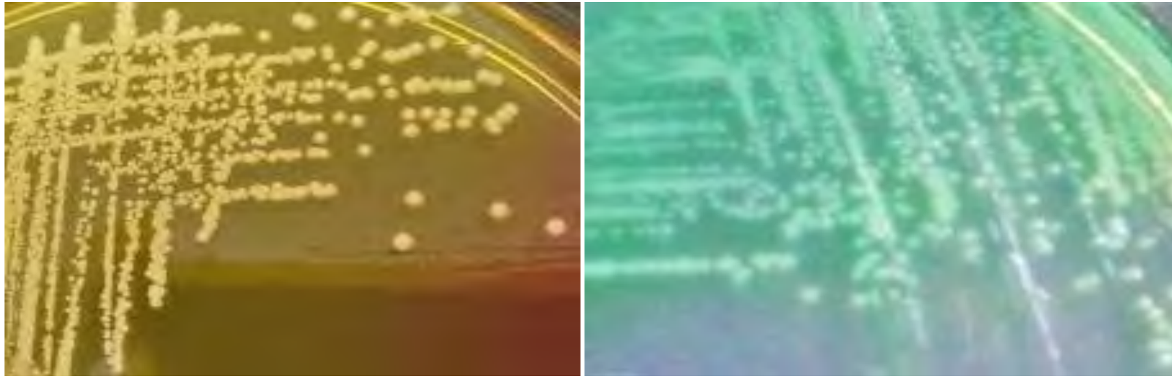
Results

3.1 Confirmation of the clinical strain

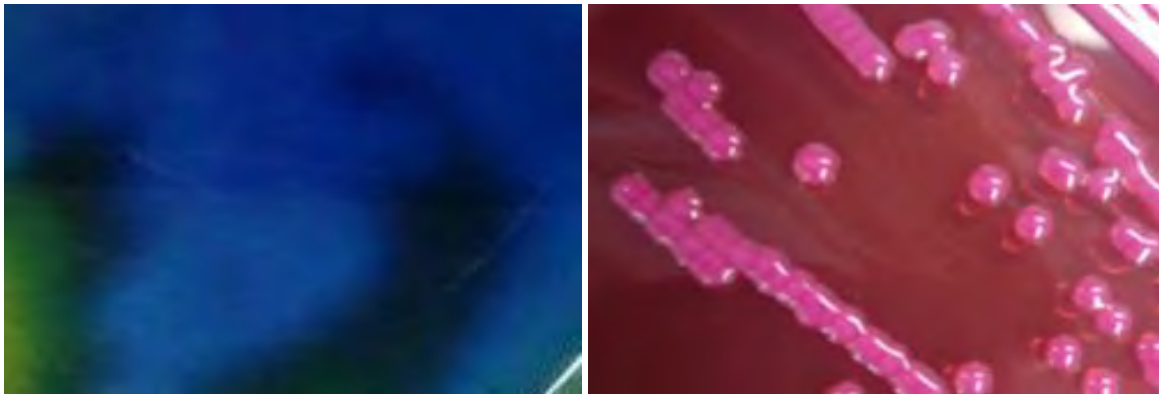
Clinical strain of the seven bacterial species *i.e. Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus, Salmonella typhi and Pseudomonas aeruginosa*, obtained from BRAC University stock culture were streaked in the respective selective media in order to determine and confirm the cultural 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. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms which are not targeted for study.

Table 2 : Cultural characteristics of the pathogens on selective agar

Organisms	**Cultural Characteristics**						
	Media	**Size**	**Margin**	**Elevation**	**Form**	**Pigment**	**Consistency**
Staphylococcus aureus	MSA	Small	Entire	Convex	Circular	Yellow colonies that turn the media Yellow	Smooth
Pseudomonas aeruginosa	Cetrimide	Small	Undulate	Raised	Circular	Green colonies that turn the media greenish	Mucoid
Klebsiella pneumoniae	MacConkey agar	Small	Entire	Pulvinate	Circular	Pink, glossy colonies	Smooth
Bacillus cereus	BC agar	Large	Undulate	Raised	Circular	Blue colonies	Creamy
Salmonella typhi	XLD	Moderate	Entire	Raised	Convex	Red colonies with black center	Smooth



(a) **(b)**



(c) **(d)**

(e)

Figure 4: Growth of selected pathogens on selective agar media (a) *Staphylococcus aureus* on MSA agar *(b) Pseudomonas aeruginosa on* Cetrimide agar (c) *Bacillus cereus* on BC agar *(d)Klebsiella pneumonia* on MacConkey agar (e) *Salmonella typhi* on XLD agar

3.2 Biochemical Test results

The organisms were identified based on following biochemical tests.

Table 3: Biochemical test results of the pathogens

Organisms	Biochemical tests									
	Indole production test	Methyl red reaction test	VogesProskauer reaction test	Citrate utilization test	TSI Fermentation				Catalase activity test	Oxidase activity test
					Slant	Butt	CO_2	H_2S		
Staphylococcus aureus	-	+	+	+	**A**	**A**	-	-	+	-
Pseudomonas aeruginosa	-	-	-	+	**K**	**K**	-	-	+	+
Klebsiella pneumoniae	-	-	+	+	**A**	**A**	+	-	+	-
Bacillus cereus	-	-	-	-	**A**	**A**	-	-	+	+
Salmonella typhi	-	+	-	-	**K**	**A**	-	+	+	-

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

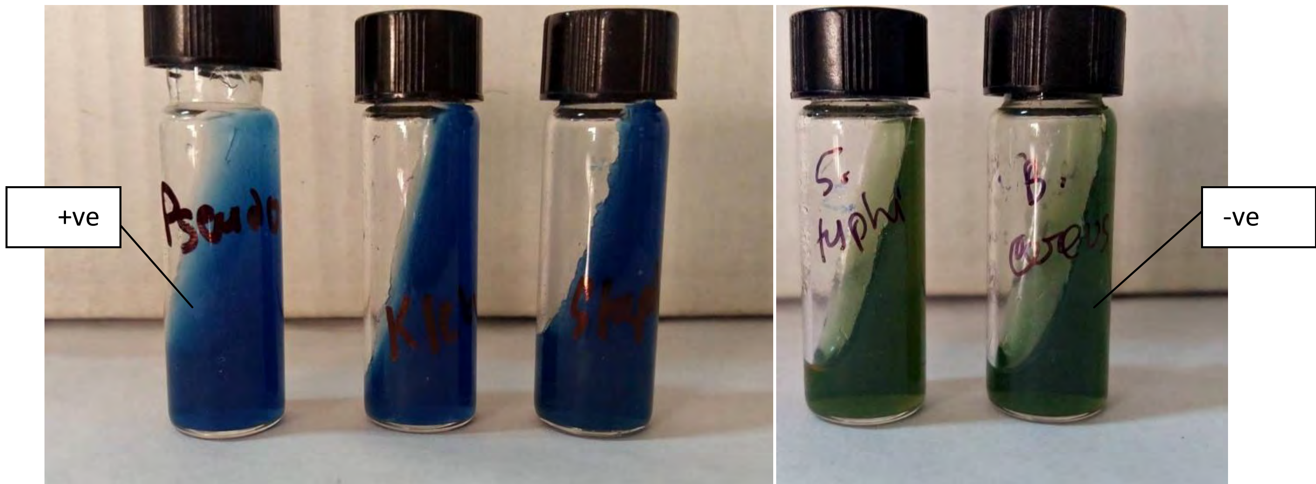


Figure 5: Citrate test result

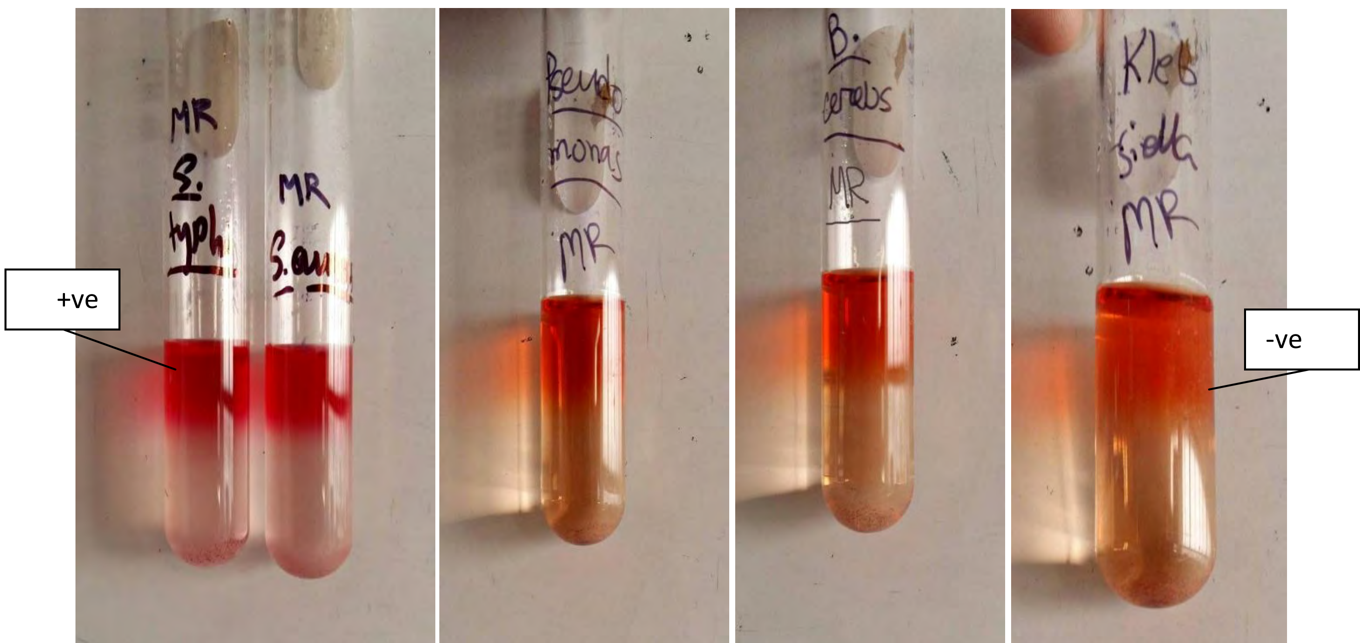


Figure 6: Methyl Red test result

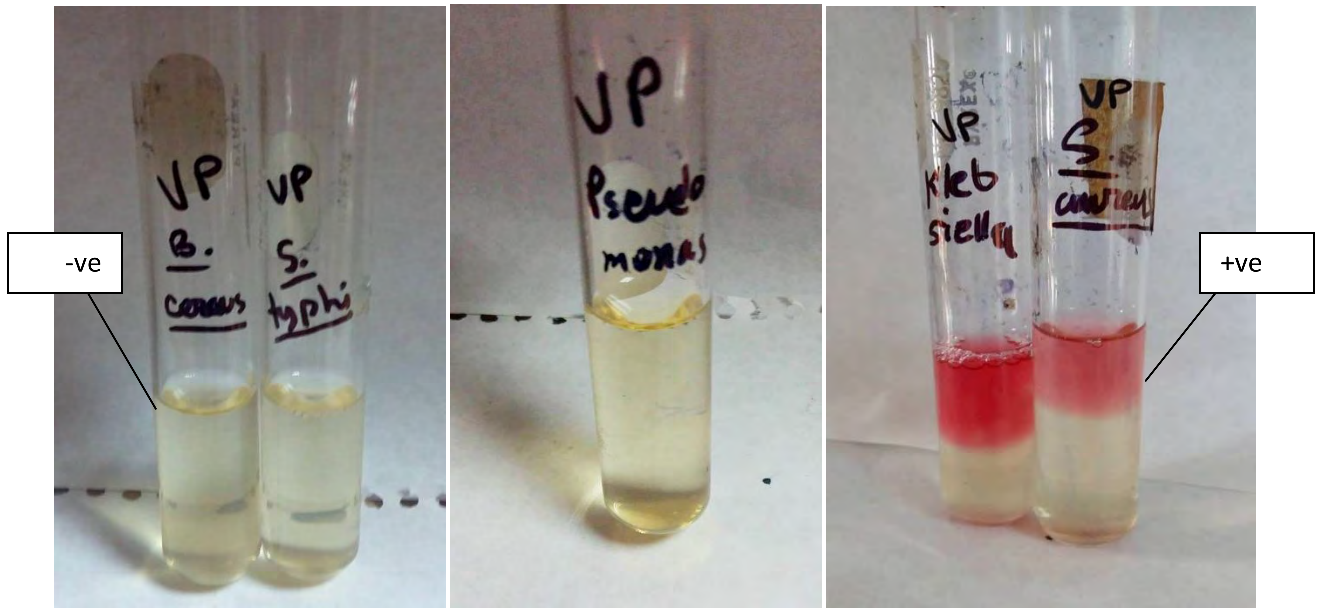


Figure 7: Voges Proskauer test result

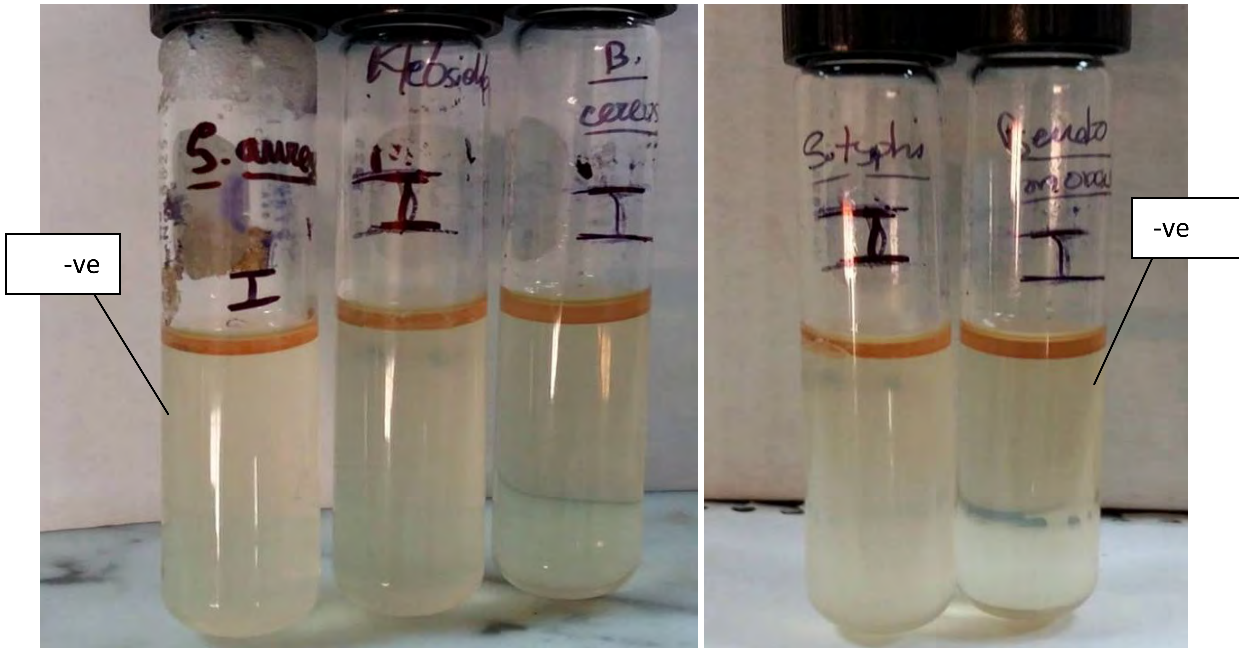


Figure 8: Indole test result

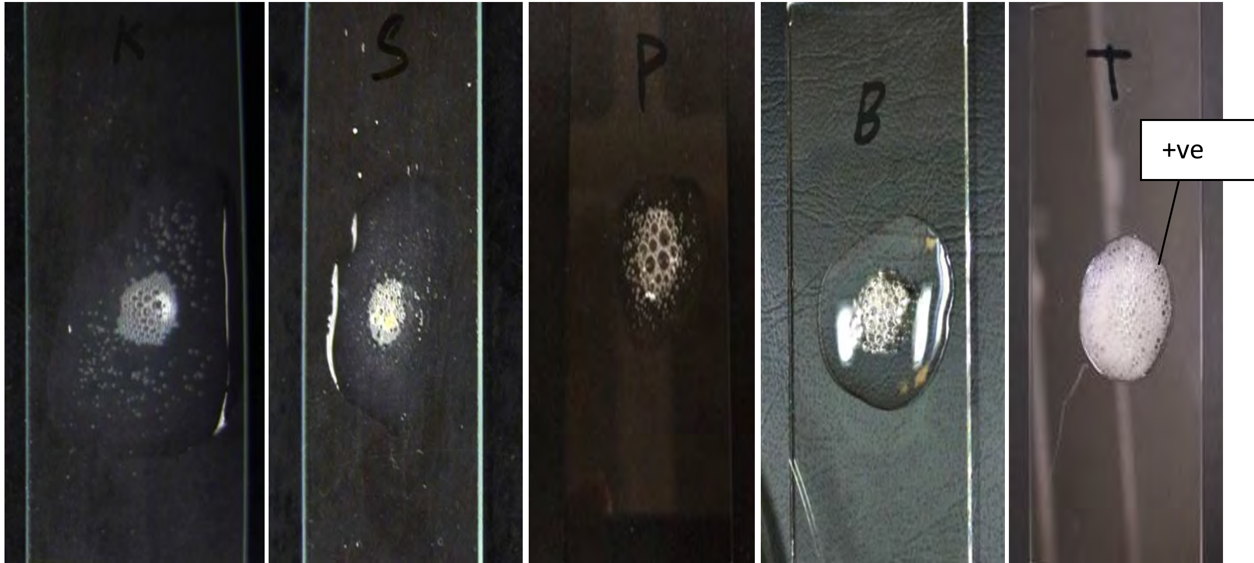


Figure 9: Catalase test result

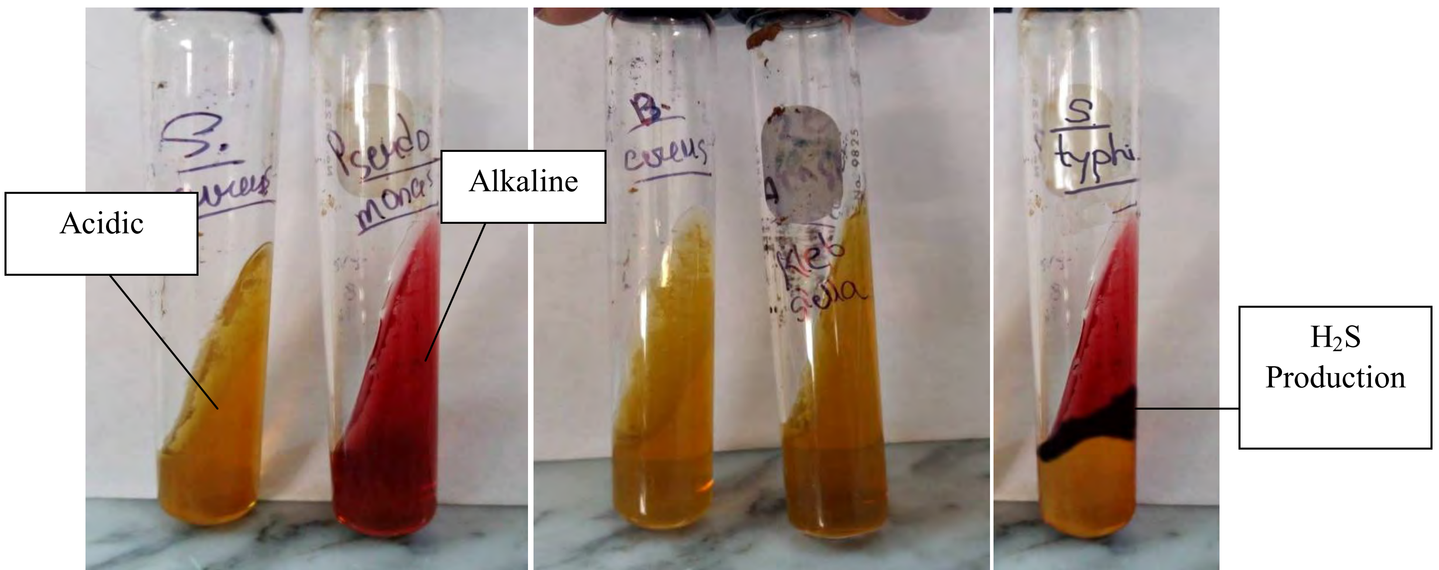


Figure 10: TSI test result

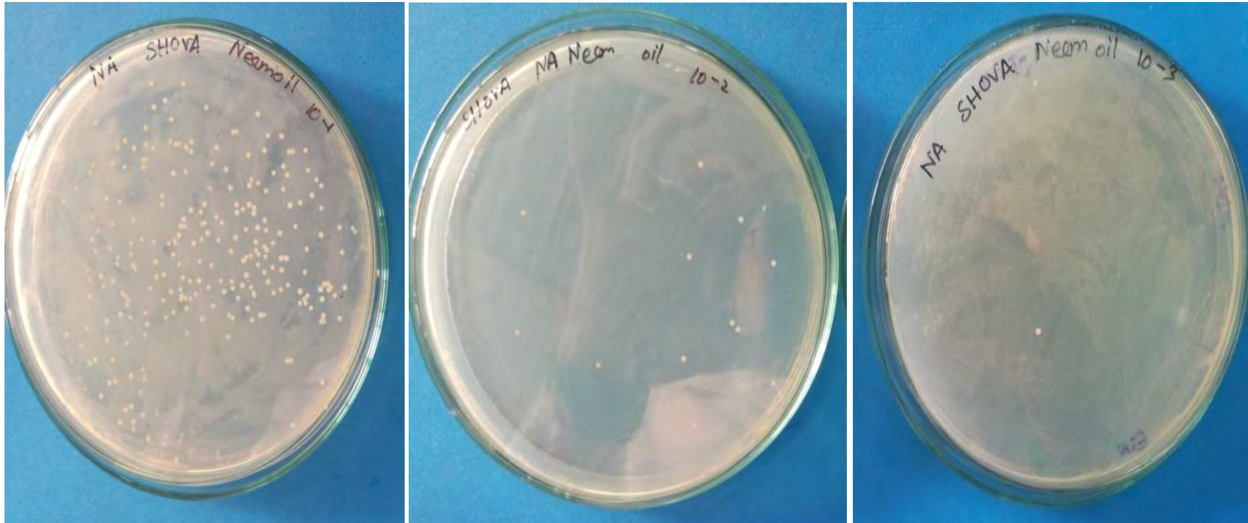
3.3 Comparison of the growth of organisms in saline and oils

Same amount of bacterial suspension were taken and it was serially diluted in saline and in oils. Paraffin oil was not used to dilute Neem, Black seed and Mustard oils because paraffin oil did not allow bacteria to grow. Then 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. Number of colonies of the matted plate and those plates which had more than 300 colonies were determined by back calculation. Number of colonies in countable plates were calculated according to the formula given below;

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

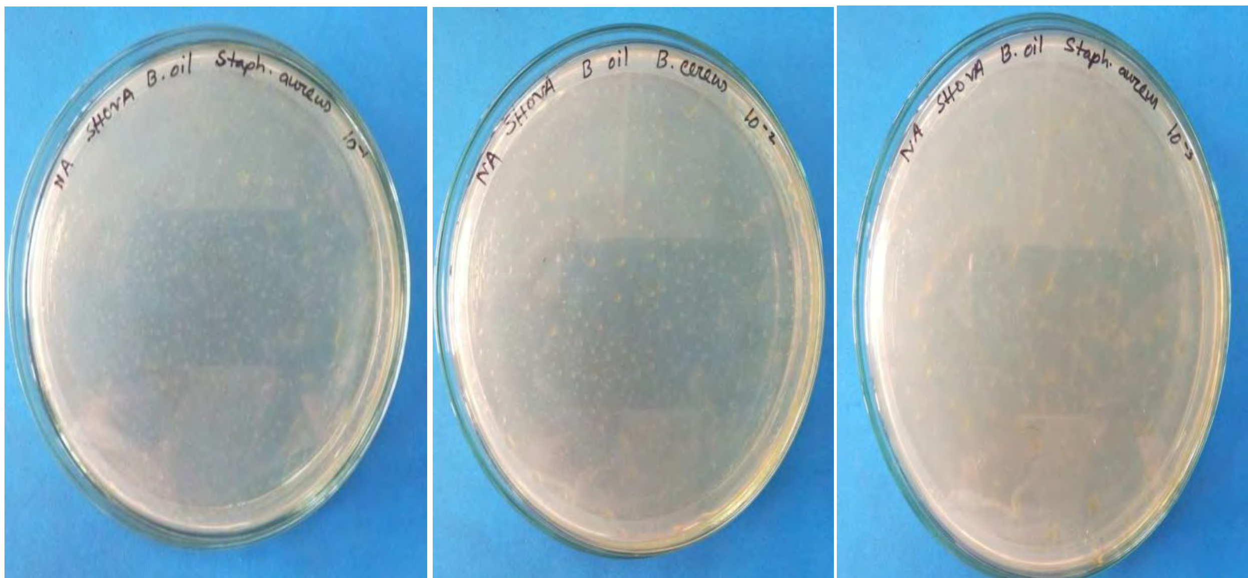
Table 4: Total viable count of *Staphylococcus aureus* in saline and in oils

Organism	**Dilution of the bacterial suspensions with oils and saline**	**Saline CFU/100 µl**	**Neem oil**		**Black seed oil**		**Mustard oil**	
			CFU/100µl	**% of inhibition**	**CFU/100µl**	**% of inhibition**	**CFU/100µl**	**% of inhibition**
Staphylococcus aureus	10^{-1}	6.7×10^{6}	268	99.99	0	100	29.2×10^{4}	95.641
	10^{-2}	6.7×10^{5}	12	99.99	0	100	29.2×10^{3}	95.641
	10^{-3}	6.7×10^{3}	2	99.99	0	100	29.2×10^{2}	95.641
	10^{-4}	6.7×10^{3}	0	100	0	100	292	95.641
	10^{-5}	6.7×10^{2}	0	100	0	100	83	87.612
	10^{-6}	6.7×10^{1}	0	100	0	100	9	86.567
	10^{-7}	6	0	100	0	100	0	100



(a) (b) (c)

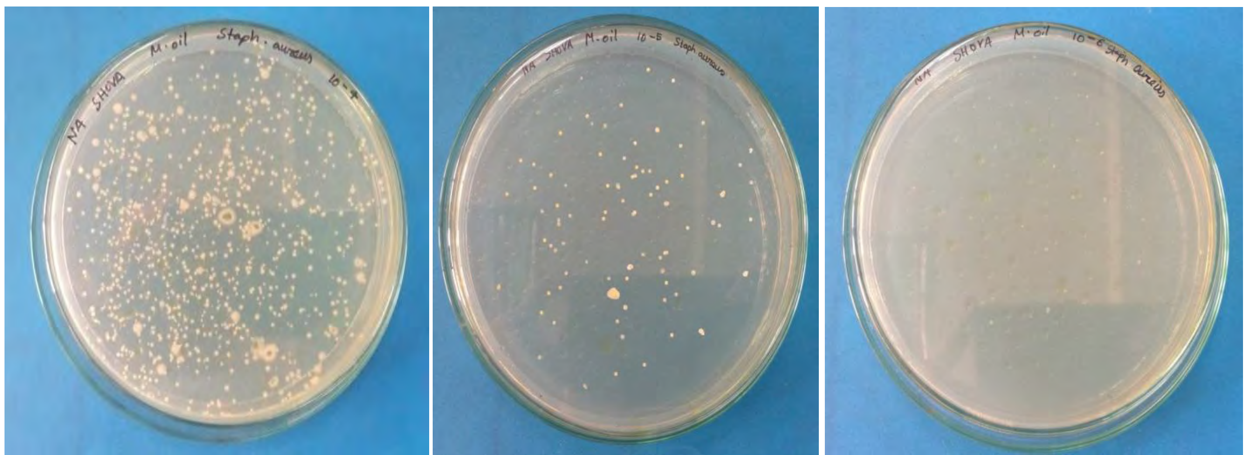
Figure 11: Growth of *Staphylococcus aureus* after incubation with Neem oil at a) 10^{-1} b) 10^{-2} c) 10^{-3} dilution of the suspension.



(d) (e) (f)

Figure 12: Growth of *Staphylococcus aureus* after incubation with Black seed oil at d) 10^{-1} e) 10^{-2} f) 10^{-3} dilution of the suspension. No growth was seen in any plate.

(g) **(h)** **(i)**

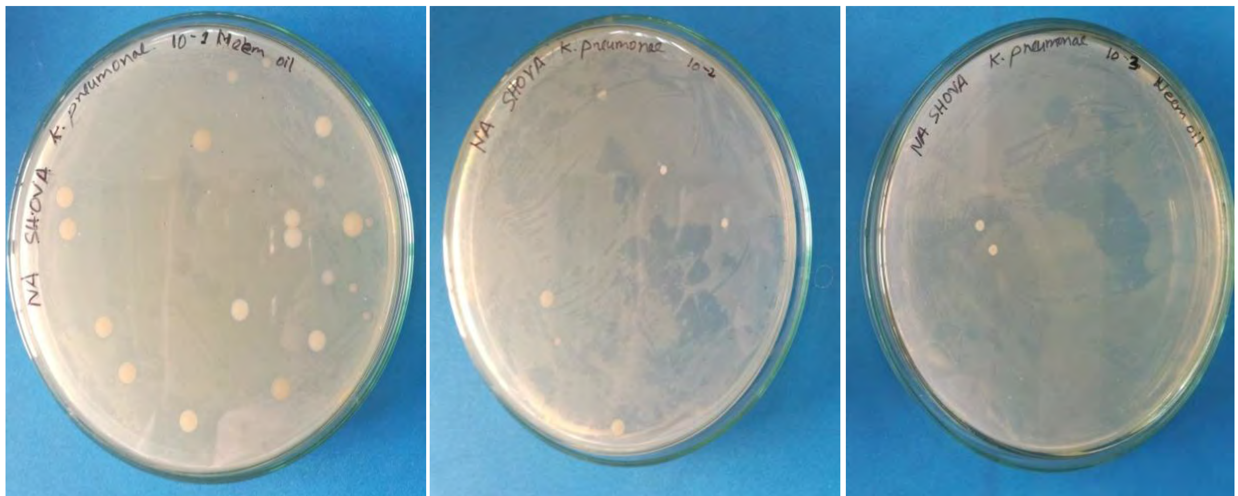


(j) **(k)** **(l)**

Figure 13: Growth of *Staphylococcus aureus* after incubation with Mustard oil at g) 10^{-1} h) 10^{-2} i) 10^{-3} j) 10^{-4} k) 10^{-5} l) 10^{-6} dilution of the suspension.

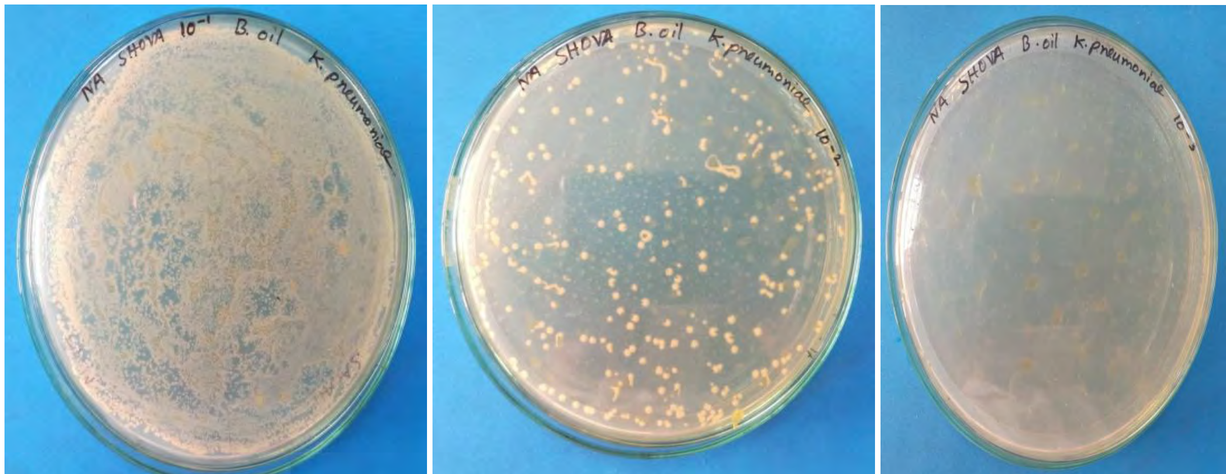
Table 5: Total viable count of *Klebsiella pneumonia* in saline and in oils

Organism	Dilution of the bacterial suspensions with oils and saline	Saline CFU/100 µl	Neem oil		Black seed oil		Mustard oil	
			CFU/100 µl	% of inhibition	CFU/100µl	% of inhibition	CFU/100µl	% of inhibition
Klebsiella pneumoniae	10^{-1}	6.8×10^{6}	23	99.99	1.31×10^{3}	99.98	28.3×10^{2}	99.958
	10^{-2}	6.8×10^{5}	5	99.99	131	99.98	283	99.958
	10^{-3}	6.8×13^{4}	2	99.99	0	100	0	100
	10^{-4}	6.8×12^{3}	0	100	0	100	0	100
	10^{-5}	6.8×10^{2}	0	100	0	100	0	100
	10^{-6}	6.8×10^{1}	0	100	0	100	0	100
	10^{-7}	5	0	100	0	100	0	100



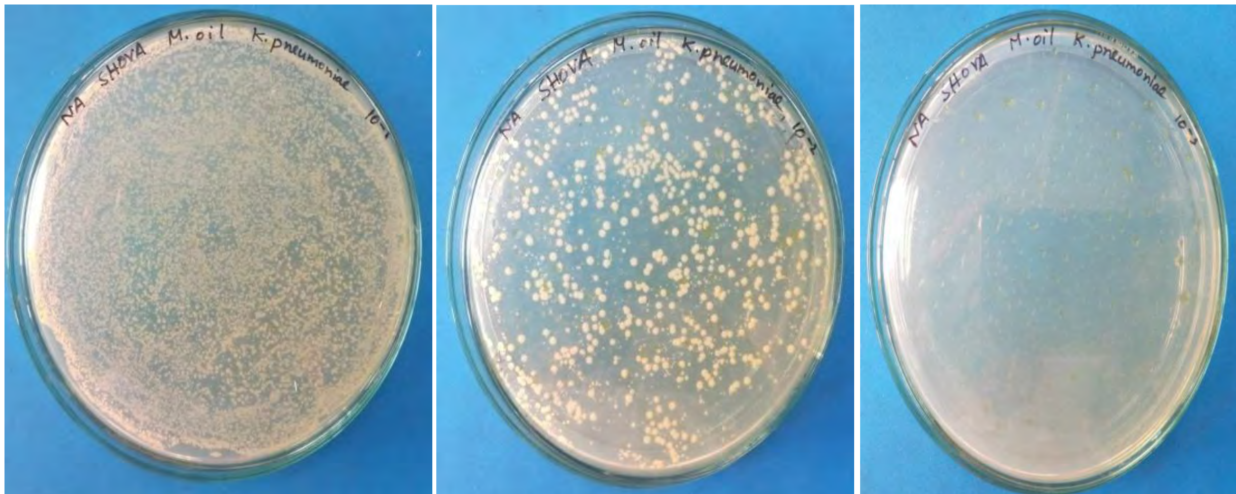
(a) (b) (c)

Figure 14: Growth of *Klebsiella pneumoniae* after incubation with Neem oil at a) 10^{-1} b) 10^{-2} c) 10^{-3} dilution of the suspension.



(d) **(e)** **(f)**

Figure 15: Growth of *Klebsiella pneumoniae* after incubation with Black seed oil at d) 10^{-1} e) 10^{-2} f) 10^{-3} dilution of the suspension.

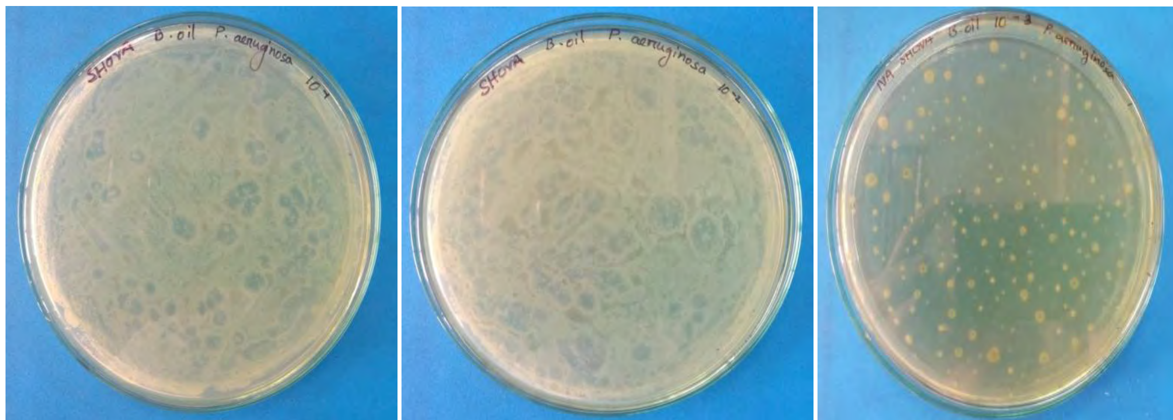


(g) **(h)** **(i)**

Figure 16: Growth of *Klebsiella pneumoniae* after incubation with Mustard oil at g) 10^{-1} h) 10^{-2} i) 10^{-3} dilution of the suspension.

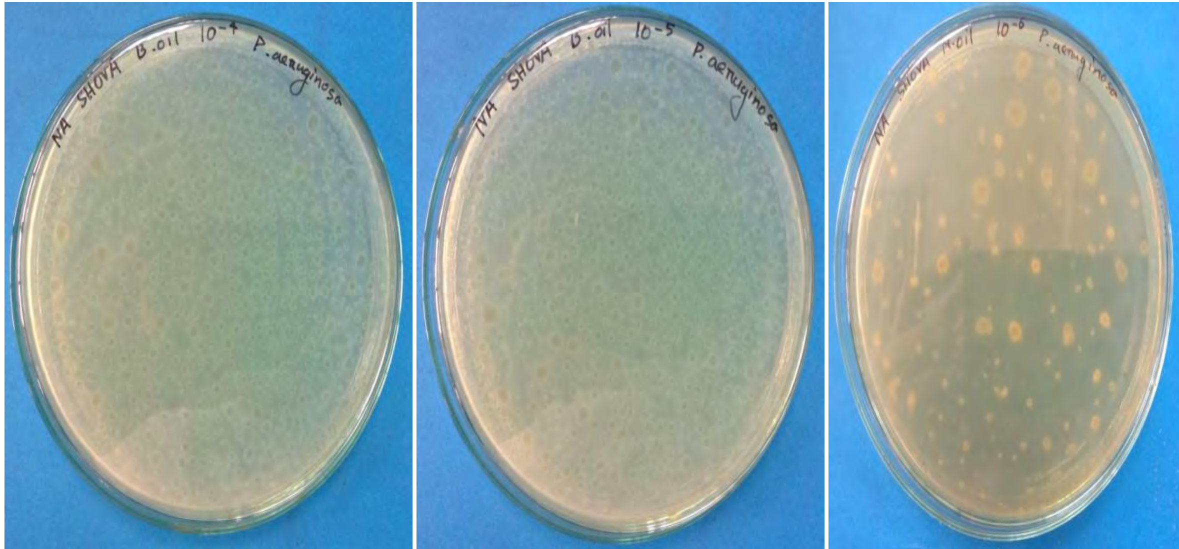
Table 6: Total viable count of *Pseudomonas aeruginosa* in saline and in oils

Organism	Dilution of the bacterial suspensions with oils and saline	Saline CFU/100 µl	Neem oil		Black seed oil		Mustard oil	
			CFU/100 µl	% of inhibition	CFU/100µl	% of inhibition	CFU/100µl	% of inhibition
Pseudomonas aeruginosa	10^{-1}	6.9×10^6	245	99.99	79×10^6	0	61×10^6	0
	10^{-2}	6.9×10^5	66	99.99	79×10^5	0	61×10^5	0
	10^{-3}	6.9×10^4	0	100	79×10^4	0	61×10^4	0
	10^{-4}	6.9×10^3	0	100	79×10^3	0	61×10^3	0
	10^{-5}	6.9×10^2	0	100	79×10^2	0	61×10^2	0
	10^{-6}	6.9×10^1	0	100	79×10^1	0	61×10^1	0
	10^{-7}	7	0	100	79	0	61	0



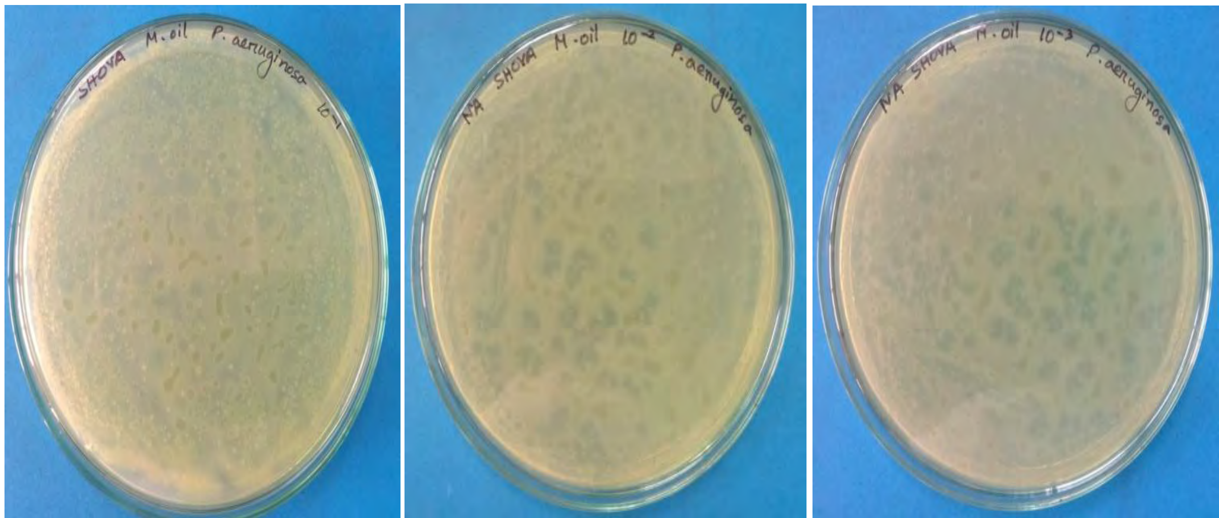
(a) **(b)** **(c)**

Figure 17: Growth of *Pseudomonas aeruginosa* after incubation with Black seed oil at a) 10^1 b) 10^{-2} c) 10^{-3} dilution of the suspension.



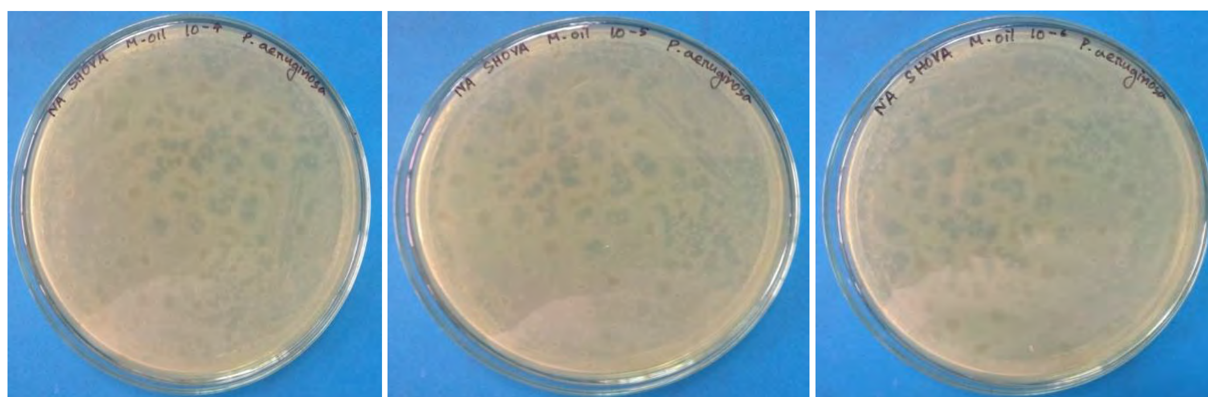
(d) **(e)** **(f)**

Figure 18: Growth of *Pseudomonas aeruginosa* after incubation with Black seed oil at d) 10^{-5} e) 10^{-5} f) 10^{-6} dilution of the suspension.



(g) **(h)** **(i)**

Figure 19: Growth of *Pseudomonas aeruginosa* after incubation with Mustard oil at g) 10^{-1} h) 10^{-2} i) 10^{-3} dilution of the suspension.

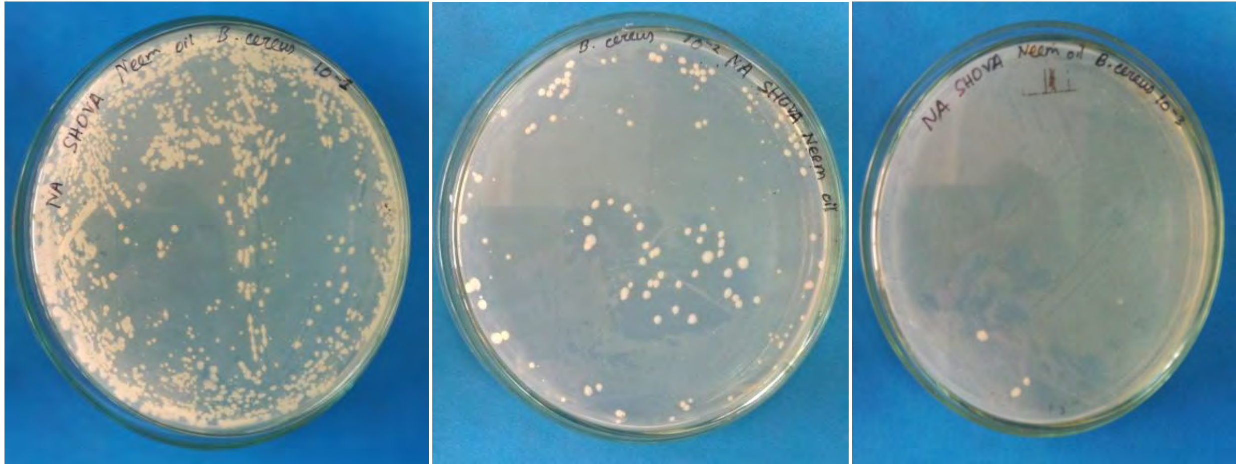


(j) **(k)** **(l)**

Figure 20: Growth of *Pseudomonas aeruginosa* after incubation with Mustard oil at j) 10^{-4} k) 10^{-5} l) 10^{-6} dilution of the suspension.

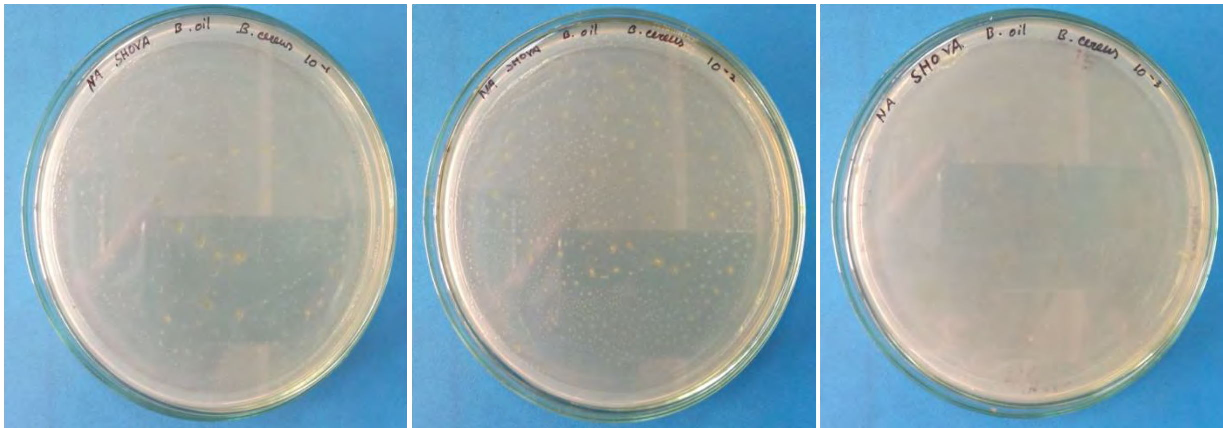
Table 7: Total viable count of *Bacillus cereus* in saline and in oils

Organism	Dilution of the bacterial suspensions with oils and saline	Saline CFU/100µl	Neem oil		Black seed oil		Mustard oil	
			CFU/100µl	% of inhibition	CFU/100µl	% of inhibition	CFU/100µl	% of inhibition
Bacillus cereus	10^{-1}	5.6×10^{6}	1.65×10^{3}	99.97	0	100	1.59×10^{5}	97.16
	10^{-2}	5.6×10^{5}	165	99.97	0	100	1.59×10^{4}	97.16
	10^{-3}	5.6×10^{4}	3	99.99	0	100	1.59×10^{3}	97.16
	10^{-4}	5.6×10^{3}	0	100	0	100	159	97.16
	10^{-5}	5.6×10^{2}	0	100	0	100	16	97.14
	10^{-6}	5.6×10^{1}	0	100	0	100	0	100
	10^{-7}	6	0	100	0	100	0	100



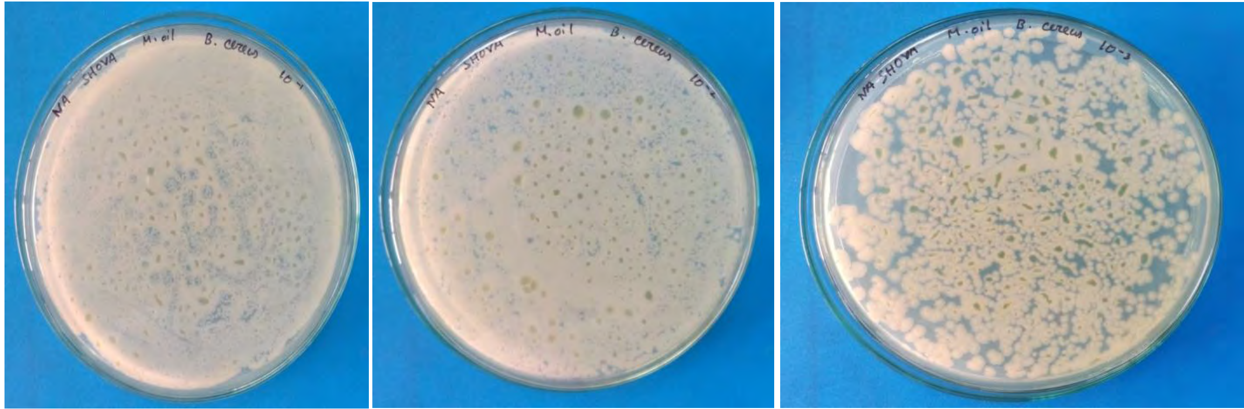
(a) (b) (c)

Figure 21: Growth of *Bacillus cereus* after incubation with Neem oil at a) 10^{-1} b) 10^{-2} c) 10^{-3} dilution of the suspension.



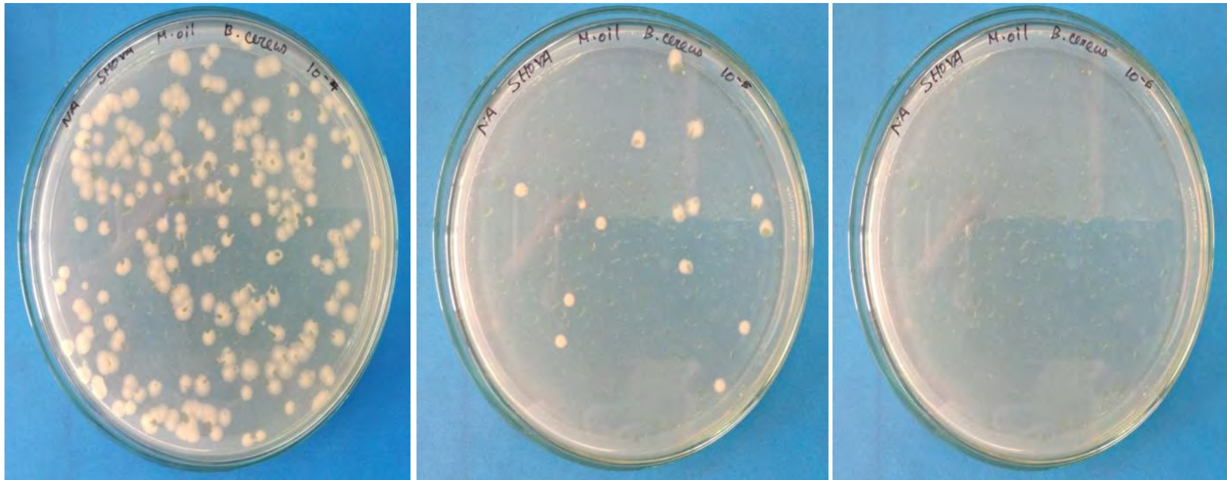
(d) (e) (f)

Figure 22: Growth of *Bacillus cereus* after incubation with Black seed oil at d) 10^{-1} e) 10^{-2} f) 10^{-3} dilution of the suspension.



(g) **(h)** **(i)**

Figure 23: Growth of *Bacillus cereus* after incubation with Mustard oil at d) 10^{-1} e) 10^{-2} f) 10^{-3} dilution of the suspension.



(j) **(k)** **(l)**

Figure 24: Growth of *Bacillus cereus* after incubation with Mustard oil at d) 10^{-4} e) 10^{-5} f) 10^{-6} dilution of the suspension.

Table 8: Total viable count of *Salmonella typhi* in saline and in oils

Organism	Dilution of the bacterial suspensions with oils and saline	Saline CFU/100 µl	Neem oil		Black seed oil		Mustard oil	
			CFU/100 µl	% of inhibition	CFU/100µl	% of inhibition	CFU/100µl	% of inhibition
Salmonella typhi	10^{-1}	6.1×10^{6}	1.86×10^{4}	99.69	1.83×10^{3}	99.97	23.9×10^{2}	99.96
	10^{-2}	6.1×10^{5}	1.86×10^{3}	99.69	183	99.97	239	99.96
	10^{-3}	6.1×10^{4}	186	99. 69	0	100	25	99.95
	10^{-4}	6.1×10^{3}	49	99.19	0	100	4	99.93
	10^{-5}	6.1×10^{2}	2	99.67	0	100	0	100
	10^{-6}	6.1×10^{1}	0	100	0	100	0	100
	10^{-7}	6	0	100	0	100	0	100

3.4 Determination of inhibition Percentage:

Number of colonies found in saline and oils were compared for every dilution to find out the rate of inhibition. Results of each set were then averaged to determine inhibition rate. Formula of calculation of inhibition Percentage is:

$$\frac{\text{CFU in saline} - \text{CFU in oil}}{\text{CFU in saline}} \times 100$$

Table 9: Inhibition of growth of various organisms by different oils

Organisms	Averaged Percentage of inhibition		
	Neem oil	**Black seed oil**	**Mustard oil**
Staphylococcus aureus	**99.99 %**	**100%**	**93.82%**
Pseudomonas aeruginosa	**99.99%**	**0%**	**0%**
Klebsiella pneumoniae	**99.99%**	**99.99%**	**99.98%**
Bacillus cereus	**99.99%**	**100%**	**97.96%**
Salmonella typhi	**99.78%**	**99.99%**	**99.97%**

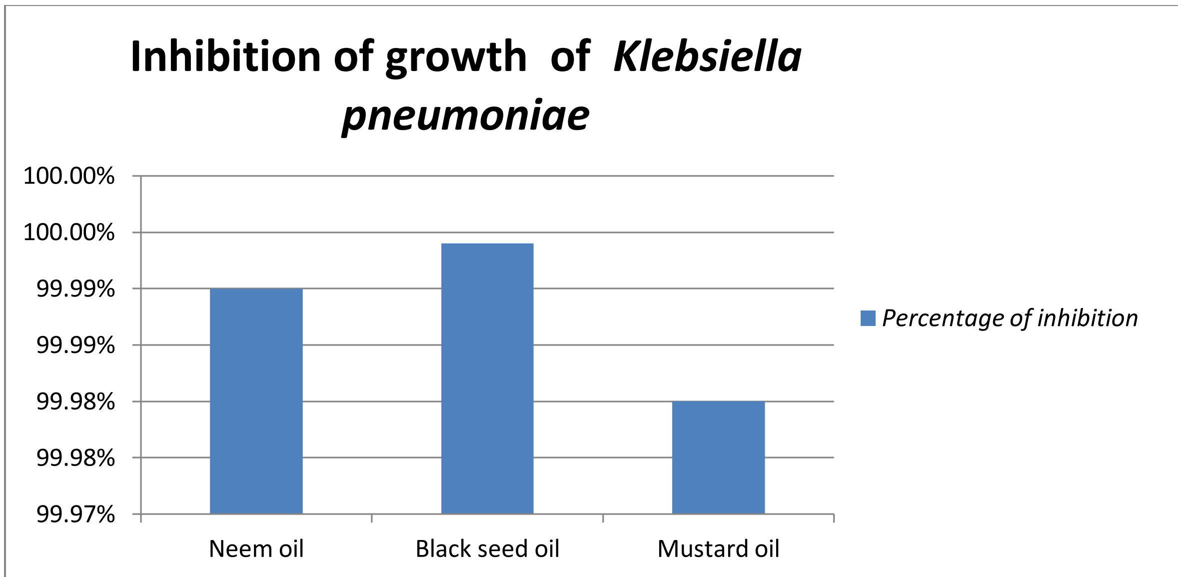


Figure 25: Inhibition of growth of *Klebsiella pneumoniae* caused by the oils

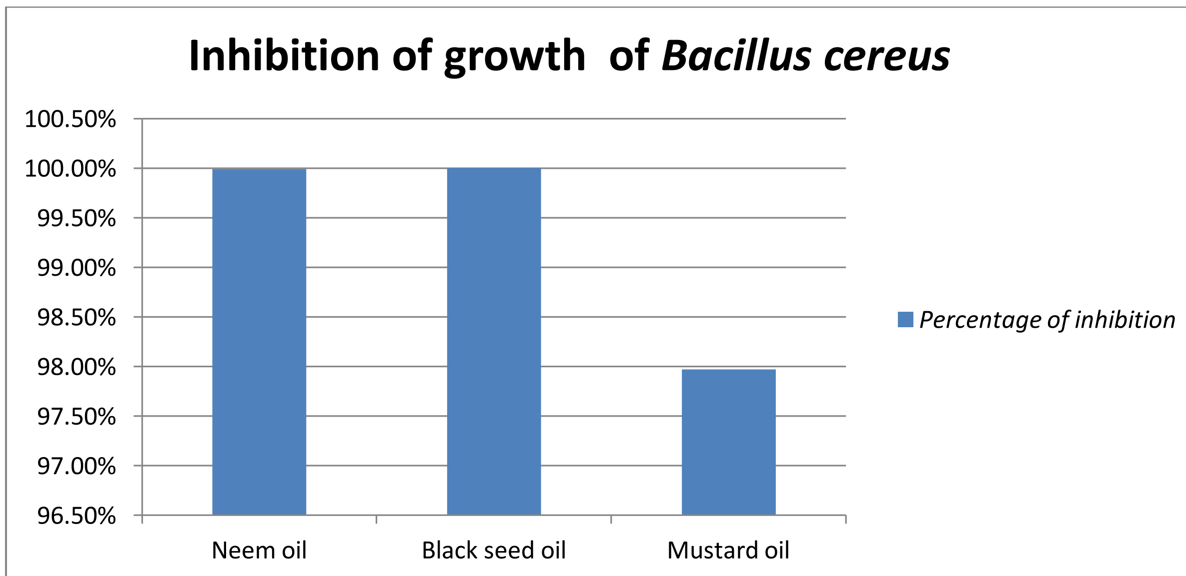


Figure 26: Inhibition of growth of *Bacillus cereus* caused by the oils

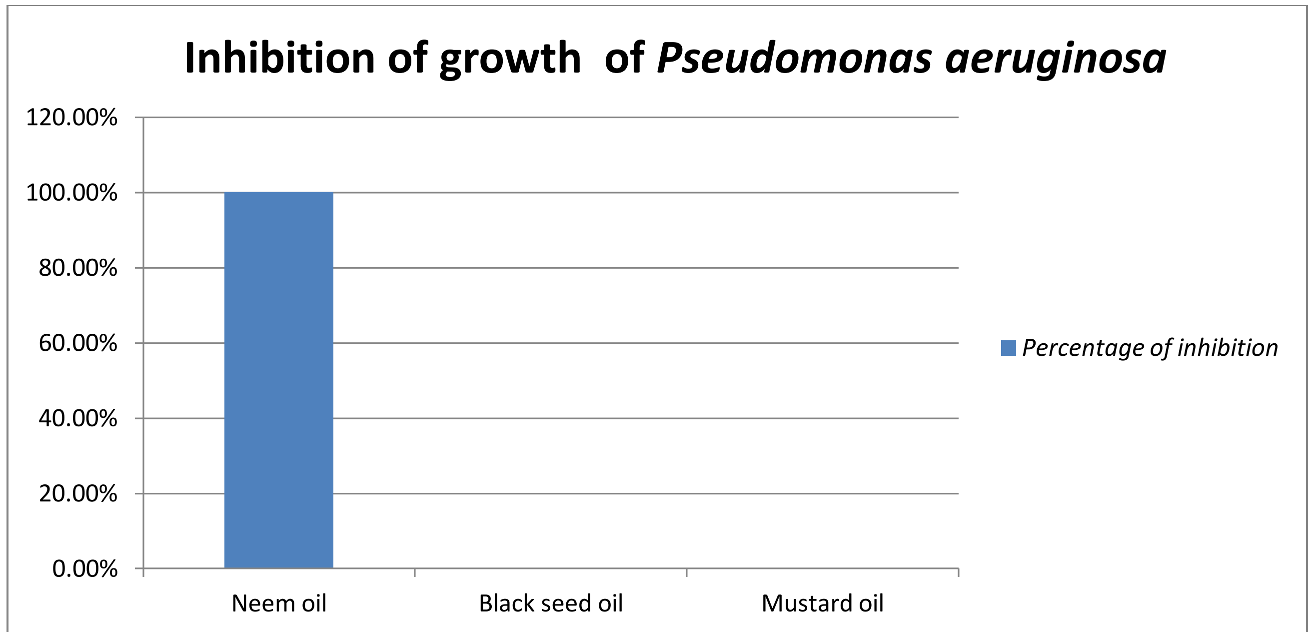


Figure 27: Inhibition of growth of *Pseudomonas aeruginosa* caused by the oils

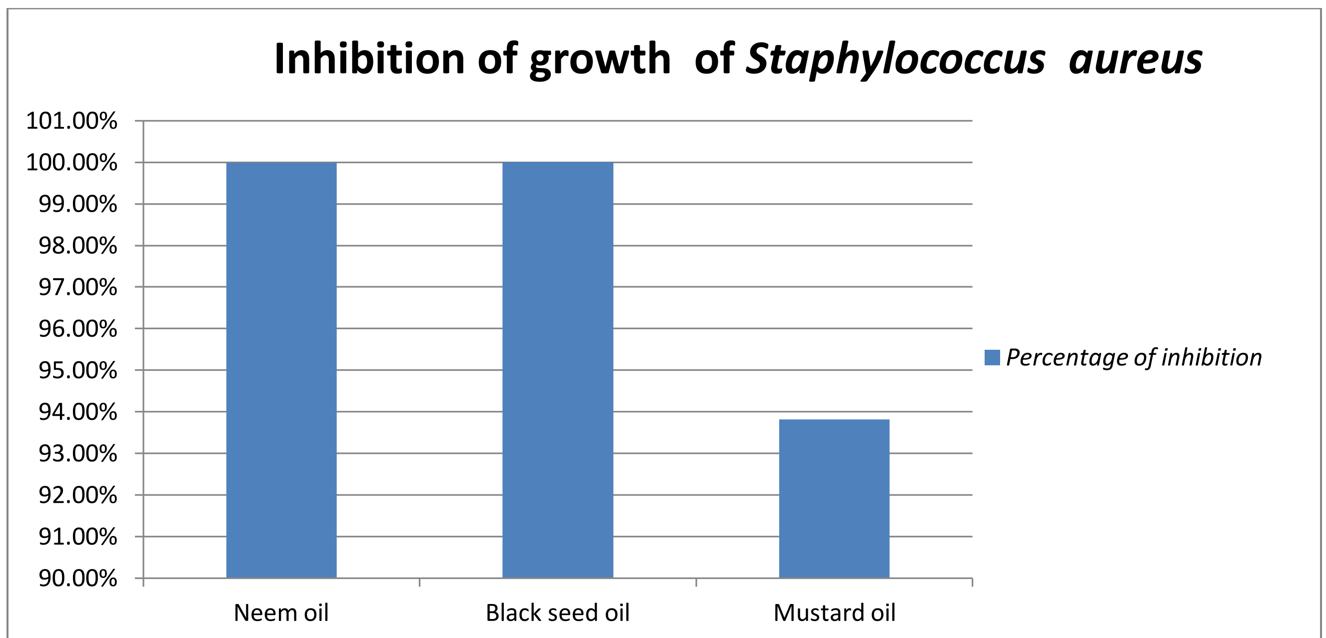


Figure 28: Inhibition of growth of *Staphylococcus aureus* caused by the oils

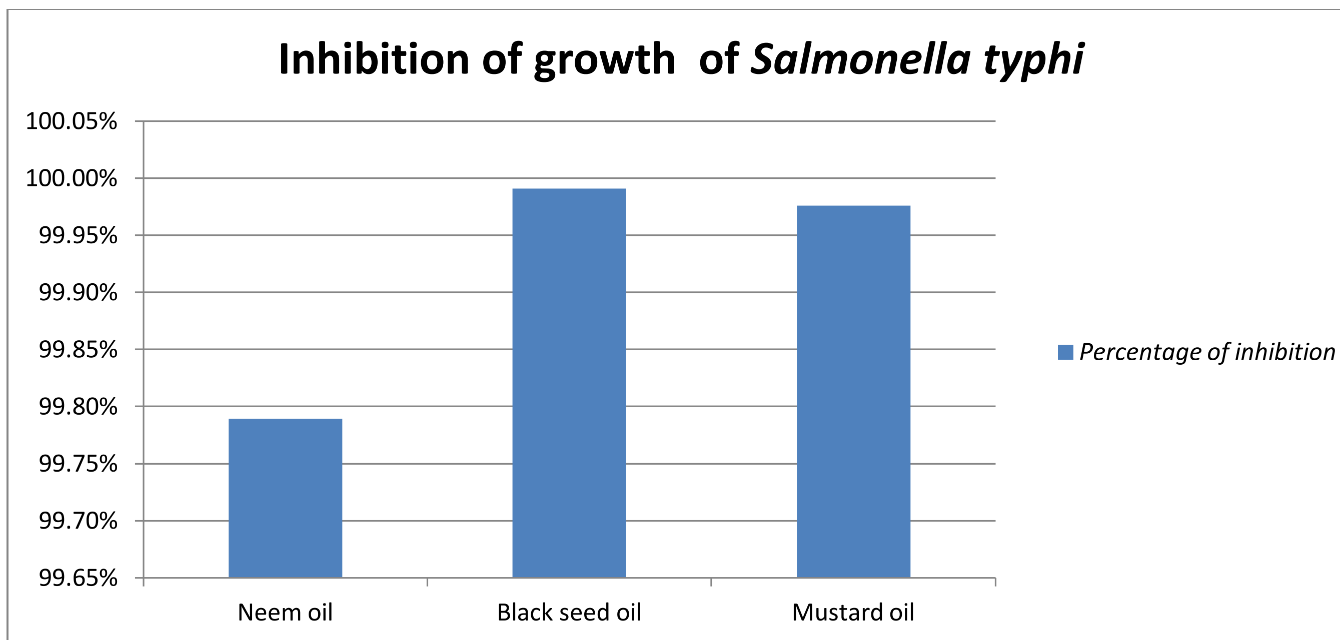


Figure 29: Inhibition of growth of *Salmonella typhi* caused by the oils

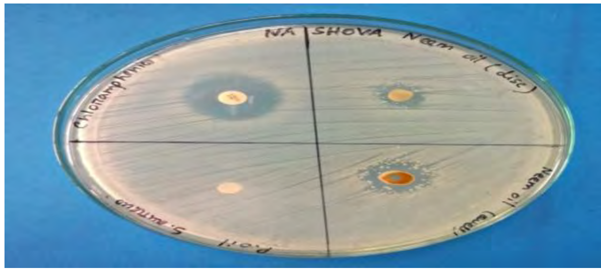
3.5 Selective antimicrobial activity test by means of antibiogram method

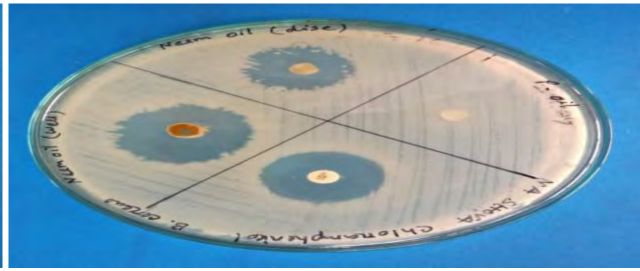
All the five bacterial strains were subjected to the standard disc diffusion test with a control antibiotic and paper disc and well containing oil. Control antibiotic for *Staphylococcus aureus, Klebsiella pneumoniae , Bacillus cereus, Salmonella typhi* was Chloramphenicol and for *Pseudomonas aeruginosa* was Cefepime . The zone diameter of inhibition interprets the resistance and sensitivity of the organisms to the respective antibiotics and oils. Presence of zone of inhibition around oil disc or well containing oil means that these oils have antibacterial activity against the selected pathogens.

Table 10: Zone of inhibition in response to oils and antibiotics

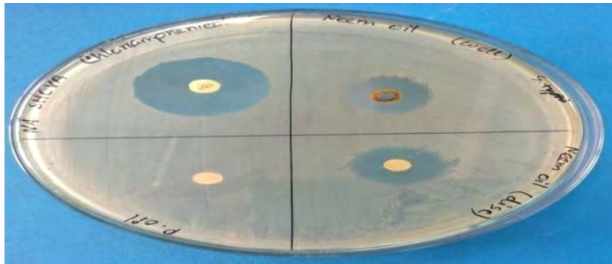
Organisms	Zone of inhibition (mm)							
	Antibiotic	Paraffin oil	Neem oil		Black seed oil		Mustard oil	
			Well	Disc	Well	Disc	Well	Disc
Staphylococcus aureus	**26**	**0**	**17**	**9**	**43**	**31**	**0**	**0**
Pseudomonas aeruginosa	**29**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Klebsiella pneumoniae	**24**	**0**	**16**	**8**	**48**	**29**	**0**	**0**
Bacillus cereus	**23**	**0**	**24**	**19**	**45**	**36**	**0**	**0**
Salmonella typhi	**22**	**0**	**18**	**8**	**13**	**12**	**0**	**0**

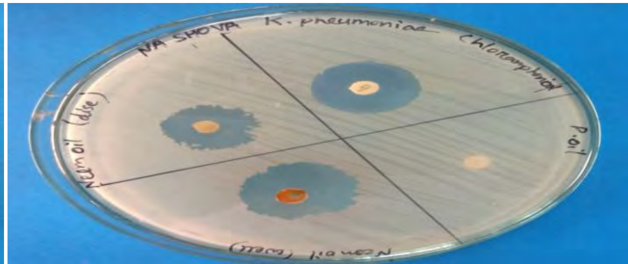
Neem oil:





(a) (b)





(c) (d)

(e)

Figure 30: Zone of inhibition of Neem oil and antibiotic

(a) **Zone of inhibition in response to Neem oil and antibiotic in case of** *Staphylococcus aureus*

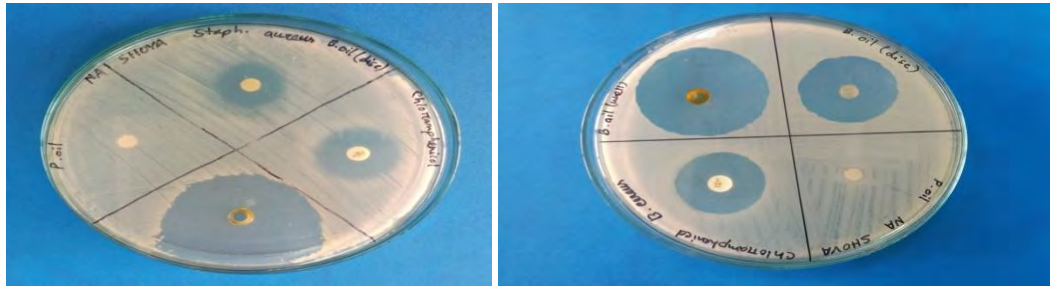
(b) **Zone of inhibition in response to Neem oil and antibiotic in case of** *Bacillus cereus*

(c) **Zone of inhibition in response to Neem oil and antibiotic in case of** *Salmonella typhi*

(d) **Zone of inhibition in response to Neem oil and antibiotic in case of** *Klebsiella pneumoniae*

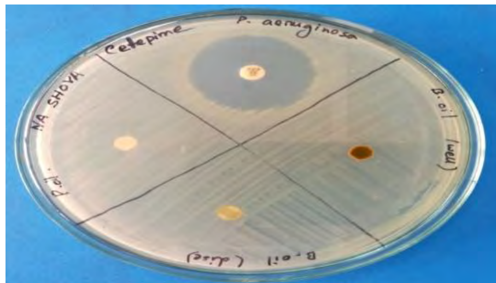
(e) **Zone of inhibition in response to Neem oil and antibiotic in case of** *Pseudomonas aeruginosa*

Black seed oil:



(a) **(b)**

(c) **(d)**

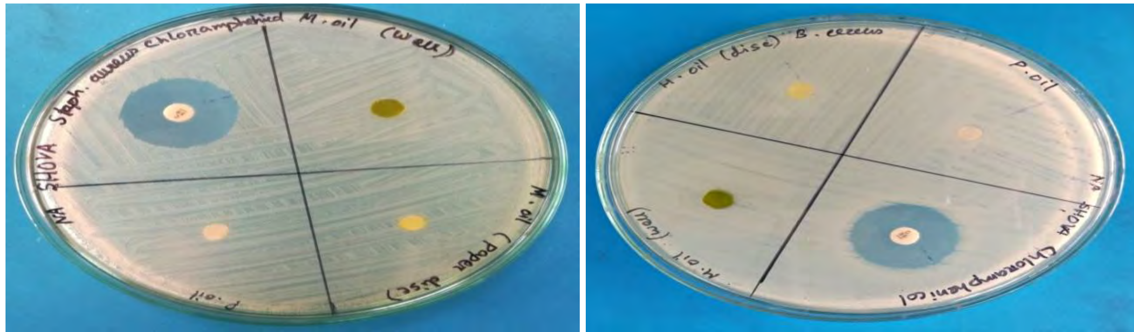


(e)

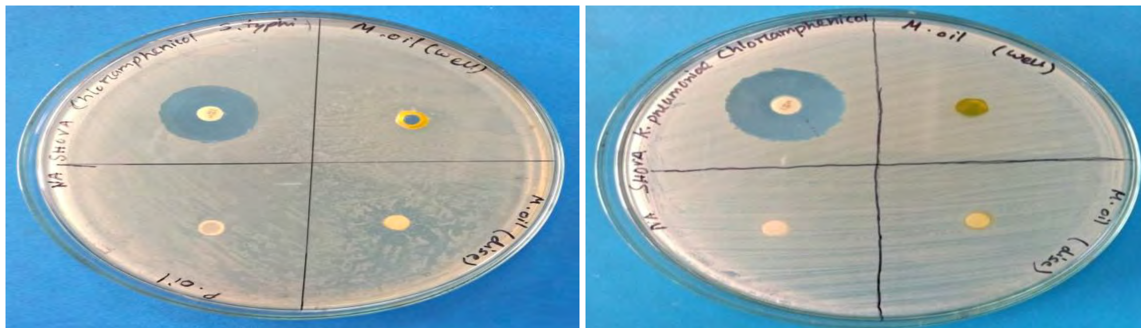
Figure 31: Zone of inhibition of Black seed oil and antibiotic

- **(a) Zone of inhibition in response to Black seed oil and antibiotic in case of** *Staphylococcus aureus*
- **(b) Zone of inhibition in response to Black seed oil and antibiotic in case of** *Bacillus cereus*
- **(c) Zone of inhibition in response to Black seed oil and antibiotic in case of** *Salmonella typhi*
- **(d) Zone of inhibition in response to Black seed oil and antibiotic in case of** *Klebsiella pneumoniae*
- **(e) Zone of inhibition in response to Black seed oil and antibiotic in case of** *Pseudomonas aeruginosa*

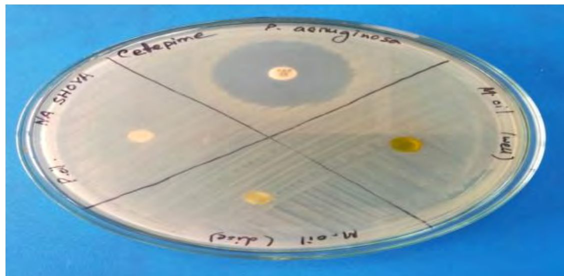
Mustard oil:



(a) **(b)**



(c) **(d)**



(e)

Figure 32: Zone of inhibition of Mustard oil and antibiotic

(a) **Zone of inhibition in response to Mustard oil and antibiotic in case of** *Staphylococcus aureus*

(b) **Zone of inhibition in response to Mustard oil and antibiotic in case of** *Bacillus cereus*

(c) **Zone of inhibition in response to Mustard oil and antibiotic in case of** *Salmonella typhi*

(d) **Zone of inhibition in response to Mustard oil and antibiotic in case of** *Klebsiella pneumoniae*

(e) **Zone of inhibition in response to Mustard oil and antibiotic in case of** *Pseudomonas aeruginosa*

Chapter 4

Discussion and Conclusion

Discussion:

Plant essential oils and extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. It is continuously used as traditional treatment (Burt., 2004). These oils are available in Bangladesh and are potential sources of novel antimicrobial compounds against selected bacterial pathogens. This in vitro study showed that Neem oil, Black seed oil and Mustard oil inhibited bacterial growth but their effectiveness varied.

In this study, Inhibition rate of Neem, Black seed and Mustard oil was measured. Neem oil exhibited more than 99% inhibition rate against the selected bacterial strains. Black seed oil exhibited more than 99% inhibition rate against the selected bacterial strains except *Pseudomonas aeruginosa.* Black seed oil did not have any antibacterial activity against *Pseudomonas aeruginosa.* Mustard oil exhibited more than 93% inhibition rate against the selected bacterial strains except *Pseudomonas aeruginosa*. Among all the oils tested in this work, Neem oil and Black seed were most effective as an antibacterial agent.

In another study carried out by Tuhin Jahan, Zinnat Ara Begum and Sayeeda Sultana in 2017, Neem oil was prepared by steam distillation process and its effect against *S. aureus, S. typhi, E. coli* and *P. aeruginosa* was examined by detection of MIC by using 'broth dilution method' and by detection of bacterial susceptibility by 'Agar disc diffusion method.' The MIC against *S. aureus, S. typhi, E. coli* and *P. aeruginosa* was at 1:32, 1:16, 1:32 and 1:8 dilution. The average diameter of zone of inhibition against *S. aureus* with neem oil was 19 mm whereas it was 30 mm with cefepime. *S. typhi, E. coli* and *P. aeruginosa* exhibited zone of inhibition. Among all the test bacteria *S. aureus* had lowest MIC. In vitro antibacterial activity of neem oil showed 92% susceptibility against *P. aeruginosa, S. pyogenes, E. coli, Proteus* group and *K. aerugenes*. The MICs were varying between ¼ to 1/64 dilution. Inhibitory zones of 13-30 mm were obtained with 65.5% strains while 26.5% strains showed zones of 8-12 mm (Zahan et al., 2007).

An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Knobloch et al., 1986).

Extensive leakage from bacterial cells or the exit of critical molecules and ions may lead to death. This can be the reason of higher inhibition rate of oils.

A research done by Emeka LB, Emeka PM and Khan TM in 2015, evaluated the susceptibility of multidrug resistant *Staphylococcus aureus* to *Nigella sativa* oil. *Staphylococcus aureus* was isolated from 34 diabetic patient's wounds attending the Renaissance hospital, Nsukka, Southeast Nigeria. The isolates were characterized and identified using standard microbiological techniques. Isolates were cultured and a comparative In vitro antibiotic susceptibility test was carried out using the disk diffusion method. Of the 34 samples collected, 19 (56%) showed multidrug resistance to the commonly used antibiotics. *Nigella sativa* oil was then studied for antibacterial activity against these multidrug resistant isolates of *Staphylococcus aureus* in varying concentration by well diffusion method. Black seed oil showed pronounced dose dependent antibacterial activity against the isolates. Out of 19 isolates, 8 (42%) were sensitive to undiluted oil sample; 4 (21%) of these showed sensitivity at 200 mg/ml, 400 mg/ml and 800 mg/ml respectively. Eleven (58%) of the isolates were completely resistant to all the oil concentrations.

The result of anti-bacterial activity of the oils by agar disc diffusion method shown in table 14 revealed that Neem oil and Black seed oil possesses an effective antibacterial activity against both gram positive and gram negative bacteria except *Pseudomonas aeruginosa*. In agar disc diffusion test , Neem oil and Black seed oil showed zone of inhibition but Mustard oil did not show any zone. Size of the zone of inhibition of Antibiotic disc was in between 22-29 mm. In most cases, Neem oil gave smaller zone compared to antibiotic discs. On the contrary, Black seed oil gave larger zone of inhibition than the control antibiotic disc (Chloramphenicol). None of the oils showed zone of inhibition in case of *Pseudomonas aeruginosa.* The positive inhibition of Black seed oil may be attributed to the two important active ingredients, Thymoquinone and melanin (Bakathir and Abbas, 2011). Neem oil also showed zone of inhibition. It contains active ingredients like azadirachtin, nimbin, picrin, and sialin (Mishra and Dave, 2013). Mustard oil showed high inhibition rate by dilution method but did not show any zone of inhibition against any organism. Mustard oil's viscosity can be a reason for this result. It is a possibility that the oil did not diffuse from the disc or well into the agar. As a result, no zone of inhibition was found.

None of the oil exhibited zone of inhibition against *Pseudomonas aeruginosa* . Inhibition rate of Black seed oil and Mustard oil was 0%. Though Neem oil showed more than 99% inhibition at dilution method against *Pseudomonas aeruginosa,* no zone of inhibition was found. These results indicate that *Pseudomonas aeruginosa* is not much sensitive to the oils tested.

Pseudomonas aeruginosa presents a great challenge in the clinical environment because of its antibiotic resistance and prevalence of infection in patients with open wounds and compromised immune systems. Its biofilms are difficult to destroy and its survival persists within and without its host. Another ability of *P. aeruginosa* is to develop antibacterial resistance through mutational changes in the function and production of chromosomally encoded resistance mechanisms. Furthermore, the most difficult challenge with this pathogen is the ability of *P. aeruginosa* to become resistant during treatment of an infection (Lister et al., 2009).

The oils were not subjected to any dilution by using paraffin oil because in a separate test done with only paraffin showed inhibition of growth. So, the results would not have been accurate using paraffin as a diluent. According to a research, Paraffin is great source for storage of bacteria stock culture but it is not suitable for bacterial growth because it has a strong inhibitory effect and weak killing effect (Hartsell, 1953).

The demonstration of activity against other bacteria is an indication that the oil can be a source of bioactive substances that could be of broad spectrum of activity. The fact that the oil was active against *Staphylococcus aureus, Klebsiella pneumoniae* and *Bacillus cereus* is also an indication that it can be a source of very potent antibiotic substances that can be used against drug resistant microorganisms prevalent in hospital environments.

Further studies are required to confirm this antibacterial activity and to separate the active constituents and evaluate their antibacterial activity. It would be great if the exact antimicrobial compounds could be identified from the oils. Accordingly, the compounds need to be identified and purified using high performance liquid chromatography (HPLC) or other high throughput technique.

Conclusion

From this study, it can be concluded that many essential oils possess antibacterial activity. Neem oil and Black seed has the most potential bactericidal properties. The present investigation together with previous studies provides support to the antibacterial properties of these oils. It can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agents. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of this oil as an antibacterial agent in topical or oral applications.

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Gadgets

List of gadgets that were used during the study

Instrument	**Manufacturer**
Weighing Machine	Adam equipment,
UK Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS20R	Daihan Scientific Companies, Korea
Freezer	Siemens,
Vortex Machine	VWR International
Microwave Oven	Model:MH6548SR LG, China

Media composition:

The composition of the media used in the present study is given below.

1. Nutrient Agar

Ingredients	Amount (g/L)
Peptone	5.0
Yeast Extract	2.5
NaCl	5.0
Agar	15.0

2. MSA agar

Ingredients	Amount (g/L)
Sodium Chloride	75.0
Proteose Peptone	10.0
Mannitol	10.0
Beef Extract	1.0
Phenol Red	0.025
Agar	15.0

3. TSI agar

Ingredients	Amount (g/L)
Yeast extract	3.0
Lab-Lemco’ powder	3.0

Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

4. Citrate agar

Ingredients	Amount (g/L)
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000

5. Cetrimide agar

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

6. XLD agar

Ingredients	Amount (g/L)
Lactose	7. 5
Sucrose	7.5
Sodium Thiosulfate	6.8
L-Lysine	5.0
Sodium Chloride	5.0
Xylose	3.75
Yeast Extract	3.0
Sodium Deoxycholate	2.5
Ferric Ammonium Citrate	0.8
Phenol Red	0.08
Agar	15.0

7. **MacConkey agar**

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

8. **MR-VP broth**

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

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 at 4^oC.

2. **Methyl red reagent**

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^oC.

3. **Oxidase reagent**

Hundred of N,N,N1,N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^oC.

4. **Catalase Reagent**

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.