

# **Comparative Study on the Antibacterial Activities of Neem Oil, Mustard oil and Black Seed Oil Against Pathogenic *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella Typhi* and *Pseudomonas Aeruginosa***

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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*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The antibacterial activity of these oil against selected pathogens were determined by dilution method, disc diffusion and agar well diffusion method. Pathogens were collected from a tertiary hospital and the pathogenicity was determined by DNase, Coagulase and Blood Agar hemolysis test. All three essential oil tested showed antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumonia* and *Salmonella typhi*. Black seed oil and Neem oil showed the highest rate of antibacterial activity even at very low concentration. In case of all three pathogens, inhibition of growth caused by Neem oil was more than 99%. Black seed oil inhibited the growth of *Staphylococcus aureus* 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 agent. The encouraging results also indicate that these oils should be exploited as natural antibiotic for the treatment of many infectious diseases caused by these pathogens, and will be helpful in understanding the relations between ancient cures and current medicines.

**Keywords:** Natural Antimicrobial agent, Disc diffusion method, well diffusion method, pathogenic bacteria, zone of inhibition

## 1. Introduction

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, Ahmad *et al.*, (2013) showed medicinal plants are also used in the preparation of herbal medicines as they are considered to be safe as compared to modern allopathic medicines. World health organization estimates that 80% of the population living in the developing countries relies exclusively on traditional medicine for their primary health care. Kumar and Navaratnam (2013) investigated 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. Further, Hulin *et al.*, (1998) stated 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. 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. Sartoratto *et al.*, (2004) described many countries have maintained research programs to screen traditional medicines for antimicrobial activity, as is the case of India, Palestine, 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.

*Nigella sativa* of the Ranunculaceae family is a medicinal plant of traditional Indian medicine. Al-Ali *et al.* (2008), found it possesses many pharmacological activities, also considered as one of the peak forms of healing medicine, treatment of various diseases like bronchitis, diarrhea, rheumatism, asthma and skin disorders, used in digestive disorders, to increase milk production in nursing mothers to fight parasitic infection. *Azadirachta indica* is locally known as Neem. Akerele, (1993) has claimed it is a tree in the mahogany family and native to India, Bangladesh, Thailand, Nepal and Pakistan. Again, Ping *et al.*, (2002) has revealed neem is widely used for the treatment of incurable diabetes and Prieto *et al.*, (2002) showed it can be used to control diseases such as leprosy, intestinal helminthiasis and respiratory system. According to Britto *et al.*, (2013) it also have antiviral, antibacterial, antifungal, anti-inflammatory, antipyretic, antiseptic and antiparasitic uses. *Brassica juncea* is locally known as mustard and Grieve (1931) suggested it can be used in inflammation, chiefly in pneumonia, bronchitis and other diseases of the respiratory organs. There are various infection causing pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae* are used in this study to measure the efficiency of these natural oils.

Aims

Aim of this study was to find the antimicrobial activity of Neem oil, Mustard oil and Black seed oil against some pathogenic bacteria such as; *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Pseudomonas aeruginosa*.

## **2. Materials and Methods**

### **Bacterial Strains**

In this study, the used bacterial species were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Salmonella typhi*. All these organisms were collected from a tertiary hospital.

### **Product Tested**

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

### **Conformation of the Pathogenic Bacteria**

Each bacterial strain were subjected to morphological and biochemical confirmation tests to determine the purity of the samples. All the biochemical tests were performed in specific media according to the standard methods described in Microbiology Laboratory Manual by Cappuccino and Natalie (2013). All the bacterial cultures were grown on nutrient agar plates in the incubator at 37°C before the process of any biochemical identification test. Lastly, the pathogenicity was determined by DNase, Coagulase and Blood Agar hemolysis test

### **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 °C 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 °C as well.

### **Long-term Preservation**

For long-term preservation, 500 µl of bacterial culture was grown in Trypticase Soy Broth (Oxoid, England) at 37 °C 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 °C.

### **Methods for Detection of Antibacterial Activity:**

#### **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.

#### **Comparing with the McFarland Solution**

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  $6 \times 10^8$  colonies per ml. A bacterial suspension which matches with McFarland 0.5 is supposed to contain  $1.5 \times 10^8$  colonies per ml as described by McFarland (1907).

### **Detection of Inhibition Rate**

One hundred micro-litre 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 micro-litre 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.

### **Test for Anti-Microbial Activity**

#### **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 discs 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.

#### **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 37° C

#### **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.

#### **Data Analysis**

Data were analyzed using Microsoft excel version 2007.

### **Results**

Clinical strain of the four bacterial species *i.e.* *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Pseudomonas aeruginosa*, obtained from a tertiary hospital were streaked on the respective selective media in order to determine and confirm the cultural properties of the organisms.

**Table 1:** Biochemical test results of the target isolates.

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 <sub>2</sub>	H <sub>2</sub> S		
<i>Staphylococcus aureus</i>	-	+	+	+	A	A	-	-	+	-
<i>Pseudomonas aeruginosa</i>	-	-	-	+	K	K	-	-	+	+
<i>Klebsiella pneumoniae</i>	-	-	+	+	A	A	+	-	+	-
<i>Salmonella typhi</i>	-	+	-	-	K	A	-	+	+	-

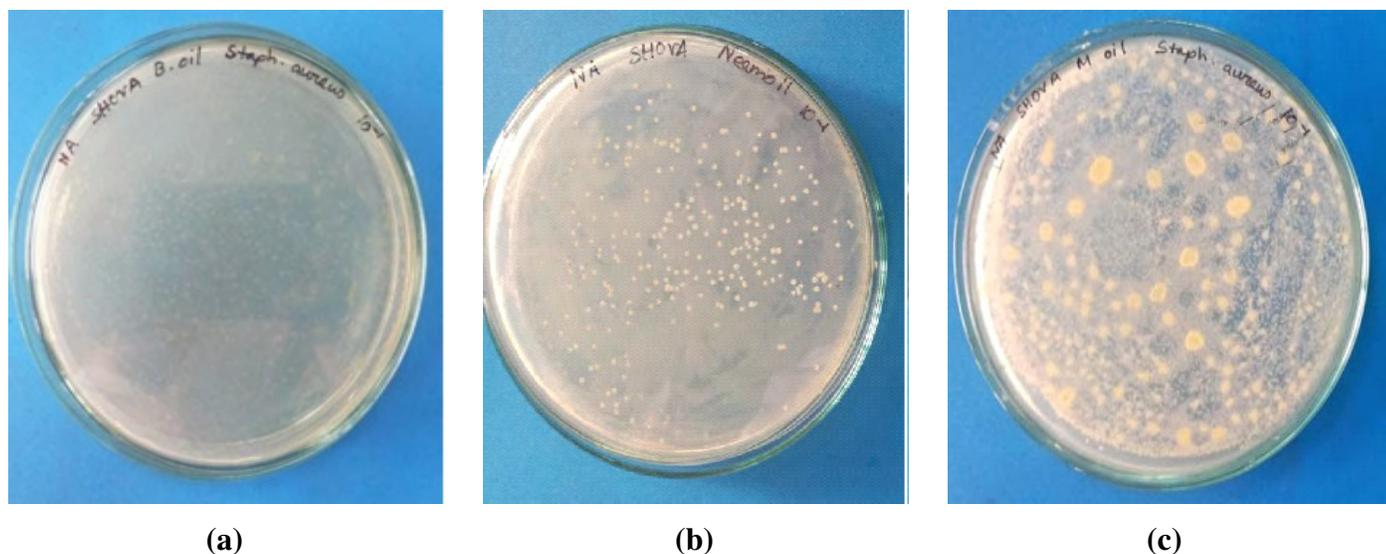
KEY: A= acidic condition, K= alkaline condition, + = positive, - = negative

### Comparison of the Growth of Organisms in Saline and Oils

Bacterial suspensions 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 (100  $\mu$ l) of diluted suspension was taken and spread on Nutrient agar. After incubation oil treated suspensions showed fewer colonies appeared on the agar plate compared to the non-treated suspension in physiological saline that of 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. Numbers of colonies in countable plates were calculated according to the formula given below;

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

**Figure 1:** Growth of *Staphylococcus aureus* after incubation with (a) Black oil at  $10^{-1}$ , (b) Neem seed oil at  $10^{-1}$  and (c) Mustard oil at  $10^{-1}$  dilution of the suspension



### Determination of Inhibition Percentage

Number of colonies found in saline and oils suspension was 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 2:** Total viable count of various bacteria 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/100 µl	% of inhibition	CFU/100 µl	% of inhibition
<i>Staphylococcus aureus</i>	10 <sup>-1</sup>	6.7 × 10 <sup>6</sup>	268	99.99	0	100	29.2 × 10 <sup>4</sup>	95.641
	10 <sup>-2</sup>	6.7 × 10 <sup>5</sup>	12	99.99	0	100	29.2 × 10 <sup>3</sup>	95.641
	10 <sup>-3</sup>	6.7 × 10 <sup>4</sup>	2	99.99	0	100	29.2 × 10 <sup>2</sup>	95.641
	10 <sup>-4</sup>	6.7 × 10 <sup>3</sup>	0	100	0	100	292	95.641
	10 <sup>-5</sup>	6.7 × 10 <sup>2</sup>	0	100	0	100	83	87.612
	10 <sup>-6</sup>	6.7 × 10 <sup>1</sup>	0	100	0	100	9	86.567
<i>Klebsiella pneumoniae</i>	10 <sup>-1</sup>	6.7 × 10 <sup>6</sup>	23	99.99	1.31 × 10 <sup>3</sup>	99.98	28.3 × 10 <sup>2</sup>	99.958
	10 <sup>-2</sup>	6.7 × 10 <sup>5</sup>	5	99.99	131	99.98	283	99.958
	10 <sup>-3</sup>	6.7 × 10 <sup>4</sup>	2	99.99	0	100	0	100
	10 <sup>-4</sup>	6.7 × 10 <sup>3</sup>	0	100	0	100	0	100
	10 <sup>-5</sup>	6.7 × 10 <sup>2</sup>	0	100	0	100	0	100
	10 <sup>-6</sup>	6.7 × 10 <sup>1</sup>	0	100	0	100	0	100
<i>Pseudomonas aeruginosa</i>	10 <sup>-1</sup>	6.7 × 10 <sup>6</sup>	245	99.99	79 × 10 <sup>6</sup>	0	61 × 10 <sup>6</sup>	0
	10 <sup>-2</sup>	6.7 × 10 <sup>5</sup>	66	99.99	79 × 10 <sup>5</sup>	0	61 × 10 <sup>5</sup>	0
	10 <sup>-3</sup>	6.7 × 10 <sup>4</sup>	0	100	79 × 10 <sup>4</sup>	0	61 × 10 <sup>4</sup>	0
	10 <sup>-4</sup>	6.7 × 10 <sup>3</sup>	0	100	79 × 10 <sup>3</sup>	0	61 × 10 <sup>3</sup>	0
	10 <sup>-5</sup>	6.7 × 10 <sup>2</sup>	0	100	79 × 10 <sup>2</sup>	0	61 × 10 <sup>2</sup>	0
	10 <sup>-6</sup>	6.7 × 10 <sup>1</sup>	0	100	79 × 10 <sup>1</sup>	0	61 × 10 <sup>1</sup>	0
<i>Salmonella typhi</i>	10 <sup>-1</sup>	6.1 × 10 <sup>6</sup>	1.86 × 10 <sup>4</sup>	99.69	1.83 × 10 <sup>3</sup>	99.97	23.9 × 10 <sup>2</sup>	99.96
	10 <sup>-2</sup>	6.1 × 10 <sup>5</sup>	1.86 × 10 <sup>3</sup>	99.69	183	99.97	239	99.96
	10 <sup>-3</sup>	6.1 × 10 <sup>4</sup>	186	99.69	0	100	25	99.95
	10 <sup>-4</sup>	6.1 × 10 <sup>3</sup>	49	99.19	0	100	4	99.93
	10 <sup>-5</sup>	6.1 × 10 <sup>2</sup>	2	99.67	0	100	0	100
	10 <sup>-6</sup>	6.1 × 10 <sup>1</sup>	0	100	0	100	0	100

Table 2 shows the percentages of inhibition of different bacteria by different oils at different dilution of bacteria in saline and oil. Neem oil at 10<sup>-1</sup> dilution of bacteria inhibited the growth of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi* by 99.99%, 99.99%, 99.99%, 99.97% and 99.69% respectively. In same oil there was no growth from 10<sup>-4</sup> dilution except in case of *Salmonella typhi* and 100% inhibition of growth was observed at 10<sup>-6</sup> dilution. Black seed oil inhibited the growth of *Staphylococcus aureus* at all dilutions. In contrast the same oil did not show inhibition of growth *Pseudomonas aeruginosa* at any dilution. Mustard Oil inhibited the growth of *Klebsiella Pneumoniae* from dilution 10<sup>-3</sup> to 10<sup>-6</sup> but show inhibition 100% in the same dilution. But in contrast the same oil did not show inhibition of growth *Pseudomonas aeruginosa* at any dilution.

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*, *Salmonella typhi* was Chloramphenicol (30 µg) and for *Pseudomonas aeruginosa* was Cefepime (30 µg). 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 3:** Zone of inhibition in response to oils and antibiotics

Organisms	Antibiotic	Zone of inhibition (mm)					
		Neem oil		Black seed oil		Mustard oil	
		Well	Disc	Well	Disc	Well	Disc
<i>Staphylococcus aureus</i>	26	17	9	43	31	0	0
<i>Pseudomonas aeruginosa</i>	29	0	0	0	0	0	0
<i>Klebsiella pneumoniae</i>	24	16	8	48	29	0	0
<i>Salmonella typhi</i>	22	18	8	13	12	0	0

## Discussion

Plant essential oils and extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. Burt (2004) stated it is continuously used as traditional treatment. 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. 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. Jahan *et al.*, (2007) showed in vitro antibacterial activity of neem oil showed 92% susceptibility against *P. aeruginosa*, *S. pyogenes*, *E. coli*, *Proteus* group and *K. aerogenes*. 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.

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 as described by 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. Emeka *et al.*, (2015) investigated 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.

In the present study, the result of anti-bacterial activity of the oils by agar disc diffusion method shown in table 3 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*. According to Bakathir and Abbas (2011) the positive inhibition of Black seed oil may be attributed to the two important active ingredients, Thymoquinone and melanin. Neem oil also showed zone of inhibition. Mishra and Dave (2013) revealed it contains active ingredients like azadirachtin, nimbin, picrin, and sialin. 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, Lister *et al.*, (2009) stated the most difficult challenge with this pathogen is the ability of *P. aeruginosa* to become resistant during treatment of an infection.

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 described by 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* and *Klebsiella pneumoniae* 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.

## Conflict of Interest Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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