Anti-bacterial effect of essential oil extracted from home-grown *Origanum vulgare L* and *Azadirachta indica* tree on pathogenic bacteria

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Biotechnology Program
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It is hereby declared that:

1. The thesis submitted is our own original work while completing a degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

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

4. I/We have acknowledged all main sources of help.

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The thesis/project titled "Anti-bacterial effect of essential oil extracted from home-grown *Origanum vulgare L* and *Azadirachta indica* tree on pathogenic bacteria." submitted by Fall, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the Degree of Bachelor of Science in Biotechnology on 27th October 2022.

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ABSTRACT

Antibiotic resistance has been on the rise for many years now. Multidrug resistance patterns in Gram-negative and Gram-positive bacterial strains are difficult to cure and might even be untreatable with conventional synthetic antibiotics. Furthermore, there is a shortage of novel antibiotics in the market. Therefore, to combat global health crisis, development of new treatment options and alternative antimicrobial therapies, preferably from natural sources, are essential.

Oregano (*Origanum vulgare L)* essential oil (OEO) is known to have antibacterial properties and this study was aimed to find its ability to inhibit the growth of some infectious bacterial strains and also do a comparative analysis with a well-known antibacterial oil; Neem (*Azadirachta indica)* essential oil. This study on antibacterial activity against selected pathogens was carried out using Kill-time and Dosage dependent spread plate method. *Klebsiella pneumonia, shigella, STEC, Salmonella typhi, Aeromonas hydrophila, Staphylococcus aureus* and *Enterococcus faecalis* was selected for this study.

The study showed the average percentage inhibition of each oil is directly proportional with the dosage. OEO showed greater antibacterial activity against the bacterial strains as compared to NEO. For 50µl dosage the average inhibition rate for OEO was 73.3% and for NEO it was only 45.9%. NEO was less effective on bacterial strains; *Klebsiella pneumonia, Shigella, Aeromonas hydrophia, STEC, Staphylococcus aureus* and *Enterococcus faecalis* as compared to OEO. The only bacterial strain NEO was more effective on was *Salmonella typhi*. OEO demonstrated 100% inhibition against *Klebsiella pneumonia, Shigella, Aeromonas hydrophila, STEC, Staphylococcus Aureus* and *Enterococcus faecalis* at varying dosage of oil. Whereas, NEO showed 100% inhibition against *Salmonella typhi* and *Staphylococcus Aureus* at 150µl and 200µl dosage of oil respectively.

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CHAPTER 1

INTRODUCTION

Introduction:

Overview:

Antibiotic resistance has slowly become a world health crisis. Even though antibiotics have transformed medicine and saved countless lives, the crisis of resistant bacteria has been attributed to the misuse and overuse of antibiotics (Ventola, 2015). As well as lack of novel drug development by the pharmaceutical companies, due to diminishing economic incentives and rigorous regulatory requirements. People often visit doctors and demand antibiotics, for minor viral fever or cold, for which these antibacterial compounds are ineffectual. In many countries antibiotics can be bought over the counter from any pharmacy, without a prescription. Furthermore, some people neglect finishing the course of the antibiotic, making the bacterial strain resistant to that specific antibiotic (Moskvitch, 2015). Numerous fields of modern medicine depend on the attainability of potent antibiotics, such as in: Chemotherapy, organ transplantation, intensive care for premature newborns and many other such critical cases require effective antibiotics (Prestinaci *et al*. 2015). The main influencing factor of morbidity and mortality in patients going through these procedures are infections caused due to multidrug-resistant bacterial strains.

Due to the increased emergence of bacterial resistance towards conventional antibiotics, new researches are ongoing to find the most potent natural antibiotics. According to the NHS, every one in ten patients experience digestive problems and one in every fifteen patients are allergic to conventional antibiotics (Dresdon, 2020). Therefore, people are slowly turning to natural antibiotics for treatment. Furthermore, as opposed synthetically produced antibiotics, drug resistance does not develop against naturally occurring antibiotics.

Oregano essential oil (OEO) has become widely recognized for having antimicrobial, antiviral and antifungal properties. Recent studies done on these oil have found cancer suppressing, potent anti-inflammatory, antioxidant and antidiabetic agents. Therefore, these properties have made this oil a potential interest to the cosmetic, food and pharmaceutical industries (López *et al*, 2017). Whereas, neem essential oil (NEO) has been used for millennia as a traditional remedy for a vast range of human ailments. It is recognized across the world as a broad-spectrum antimicrobial agent and pesticide. Research is done on the oil in the fields of food safety, dentistry, bacteriology, virology, mycology as well as parasitology (Wylie & Merrell, 2022).

Essential oils:

Essential oils are vaporous having an oily odor. Essential oils are obtained from different parts of the plant, and they are extracted using various techniques like hydrogen distillation, which is an affordable, easy extraction process and steam distillation. Parts of the plant, including flowers, leaves, stems, bark, and roots, are used to extract essential oils. These oils have a variety of uses. Essential oils are used in aromatherapy and act as an antioxidant, antibacterial, antifungal, analgesic, anxiolytic, and antidepressant. In the cosmetic and industrial sectors, essential oils are used rapidly and are vastly used in the ever-growing perfumery industries. These oils are used in canning and many food products. Essential oils are used as a popular herbal medicine and their aroma is used to improve mood and relieve depression (Irshad et al., 2020).

Essential oils are extracted from various aromatic plants. These plants have become famous because the locals use them to treat diseases. The smell that occurs in plants is due to essential oils. Essential oils are produced from dried, fresh or partially dehydrated plant materials. Moreover, the quality of the essential oil depends on the age of the plant, the parts used for extraction, the stage of the vegetative cycle, the influence of climate, etc. Characterization of essential oils are performed using gas chromatography. Essential oil storage and handling also affect yield and quality, as essential oils accumulate in the sebaceous glands present in plant tissues (Irshad et al., 2020).

Essential oils are used in many products around the world, including ice cream perfumes, baking soda-containing foods, beverages, and cosmetics. Various bioactive components found in essential oils are limonene, geranyl acetate, car-vone etc. which are essential elements of hygiene products. There are an enormous number of essential oils which are enriched with various exceptional characteristics. Some notable essential oils are: peppermint oil, rosemary oil, oregano oil, lemon oil, neem oil, tea tree oil, lavender oil, clove oil etc. However, essential oils are used all over the world and due to its better uses, the global essential oil market is growing rapidly and becoming more and more significant (Irshad et al., 2020).

***Origanum vulgare L.* oil:**

General characteristics of *Origanum vulgare L.* oil:

The members of the genus *Origanum* makes up the most important versatile aromatic and herbaceous medicinal plants, belonging to the family *Lamiaceae*. It was native to Europe, the Mediterranean region, south and western Asia. On the basis of their hydrophilic and hydrophobic characteristics *O.vulgare L.* two main forms of phytochemicals can be found in *O.vulgare L.* (1) Essential oils (EOs) and (2) phenolic compounds. Bioactive compounds such as tannins, terpenoids and sterols can also be found in *O.vulgare L.* *O.vulgare ssp hirtum* most commonly known as Greek Oregano, is one of the most vital subspecies of *O.vulgare L.* due to high quality and high concentration of essential oil. (Saba Soltani, 1970)

The oregano essential oil rich in carvacrol, thymol, p-Cymene, and γ-Terpinene is mostly important for its bactericidal inhibitory activity, antimicrobial antiviral and antifungal activity. Carvacrol and thymol play an important key role as antimicrobial agents in antifungal and antimicrobial activity. Carvacrol (5-isopropyl-2-methylphenol) is a natural monoterpene derivative of cymene and; Thymol (2-isopropyl-5-methylphenol) is a phenolic carvacrol isomeric compound as well as a p-cymene derivative found in essential oil.

The two active chemical compounds can inhibit the growth of both the gram positive and negative bacteria. They can also be applied as an alternative to antimicrobial agents against antibiotic resistant pathogenic bacteria. Recent scientific researchers have confirmed that the oregano essential oils also have antioxidant, anti-cancer, anti-diabetic, anti-inflammatory, hepato-protective activity. A report by Kordali et al stated the effective fungicidal properties of these two chemical compounds against different plant pathogens. (Gonceariuc et al., 2021)

Classification of *Origanum vulgare L.*:

Rank	**Scientific Name and Common Name**
Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* - Vascular plants
Superdivision	*Spermatophyta* - Seed plants
Division	*Magnoliophyta* - Flowering plants
Class	*Magnoliopsida* - Dicotyledons
Subclass	*Asteridae*
Order	*Lamiales*
Family	*Lamiaceae* Martinov Martinov - Mint family
Genus	*Origanum* L. - origanum**P**
Species	*Origanum vulgare* L. - oregano**P**

Table 1 Classification of Origanum vulgare L.

Chemical composition of essential oils of oregano obtained by GC/MS:

Constituent	Yield (%)
α-Thujene	0.29
α-Pinene	0.18
Camphene	0.02
Sabinene	0.18
β-Pinene	0.45
Myrcene	0.27
α -Terpinene	0.82
p-Cymene	1.28
1,8-Cineole	0.96
Limonene	0.55
γ-Terpinene	1.27
Trans-sabinen-hydrate	0.19
Borneol	0.17
Terpinen-4-ol	0.76
α-Terpineol	0.09
Methyl thymyl ether	0.22
Thymol	3.29
Carvacrol	86.06
β-Caryophyllene	0.96
α-Humulene	0.04
Germacrene D	0.06
γ-Cadinene	0.05
β-Bisabolene	0.38
δ-Cadinene	0.10
α-Cadinol	0.03

period ($P < 0.05$).

Figure 1 Chemical composition of essential oils of oregano obtained by GC/MS

Some medicinal uses of oregano essential oil:

1. Tropical ointments are made using thymol, a complex chemical compound present in the oregano essential oil to treat nail fungi, candiasis etc.

2. The powerful antioxidant and anti-inflammatory property of the chemical compounds present in the oregano essential oil help reduce bacterial and fungal infections; hence it can be dissolved in water or used to prepare mouthwash that will help to defend tooth decay, gingivitis and many other general oral infections.

3. Few drops of oregano essential oil at the workplace or home, even combining oregano essential oil with equal parts of carrier oil and massaging it in chest, back, neck can help reduce cough.

Azadirachta indica A Juss; Meliaceae oil:

General characteristics of *Azadirachta indica* A Juss; Meliaceae oil:

In general, *Azadirachta* *indica* most commonly known as neem has a diverse medicinal value. It is a fast growing evergreen vascular perennial plant tree that belongs to the Meliaceae family which is native from India. It adapts to both tropical and subtropical climatic conditions. Even though it can grow within soil pH 5.0 to 8.0, it can survive any soil type. A. *indica* is best adapted to deep and permeable sandy soil within a soil pH 6.2 to 7.0. Various parts of this particular tree is of great interest as it has a large number of phytochemicals such as azadirone, azadirachtin, salannin, nimbolide etc each with a wide range of biological and pharmacological characteristics. Constituents of the extracts obtained from its leaves and other parts have been reported to be associated with medical and therapeutic treatment of conditions such as diabetes, cancer, hypertension, heart diseases etc. for their claimed anti-hyperglycemic, antacid, anti-bacterial, anti-fungal, antiviral, antipyretic, anti-diabetic, anti-dermatitis, anti-inflammatory, anti-oxidant, anti-mutagenic, anti-carcinogenic, immune-modulatory, spermicidal etc properties. (Alzohairy, 2016).

Neem extracts contribute to potential effects in both cellular and molecular mechanisms such as detoxification, cell cycle alteration, anti-inflammatory, anti-angio genic, free-radical scavenging, and modulation of a number of signaling pathways. In the leaves of *Azadirachtaindica*, bioactive compounds such as ascorbic acid, amino acids, nimibio, nimbandiol, gedunin, tannin, 6-desacetylnimbinene and n-hexacosanol are present. The first polyphenolic flavonoids purified from fresh leaves of neem were quercetin and -sitosterol which are known to have antibacterial and antifungal activities.

Classification of *Azadirachtaindica*:

Rank	**Scientific Name and Common Name**
Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* - Vascular plants
Superdivision	*Spermatophyta* - Seed plants
Division	*Magnoliophyta* - Flowering plants
Class	*Magnoliopsida* - Dicotyledons
Subclass	*Rosidae*
Order	*Sapindales*
Family	*Meliaceae* Juss. - Mahogany family
Genus	*Azadirachta* A. Juss. - azadirachta**P**
Species	*Azadirachta indica* A. Juss. - neem**P**

Table 2 Classification of Azadirachta indica

<u>Chemical composition of essential oils of *A. indica* obtained by GC/MS:</u>

The chemical composition of the oil samples was investigated by GC/MS. Hydrocarbon constituted 85.36% of the leaves oil. The major compounds were β-Elemene (33.39%), γ- Elemene (9.89%), Germacrene D (9.72%), Caryophyllene (6.8%) and Bicyclogermacrene (5.23%) while the percent of the oxygenated compounds were (5.04%) mainly attributed to sesquiterpene oxide.

<u>Some medicinal uses of *A. indica* essential oil:</u>

Reportedly, ***<u>A.indica</u>*** preparations are efficacious against a wide range of skin diseases, septic sores, infected burns etc. The leaves when applied in the form of decoctions or poultices are recommended for boils, eczema and ulcers. The essential oil can be used for skin diseases such as indolent ulcers, scrofula and ringworm.

In one laboratory study (Khan and Wassilew, 1987) it was shown that neem preparations show toxicity to cultures of a wide range of fungi. For instance, it included members of the following genera, *Trichophyton* known as "athlete's foot" fungus that infects hair, nails and skin; *Epidermophyton* known as "ringworm" that invades both nails and skin of the feet; *Trichosporon* fungus of the intestinal tract; *Candida* a yeast like fungus which is usually a part of the normal mucous flora but can also get out of control, leading to lesions in mouth, skin, hands, lungs etc. In some trials, growth of pathogenic bacteria such as *Staphylococcus aureus* (Schneider, 1986) and *Salmonella typhosa* (Patel and Trivedi, 1962) was suppressed by essential oils extracted from ***<u>A. indica</u>*****.** *Staphylococcus aureus, a Gram* positive bacteria are common causes of food poisoning, abscesses (boils), bristles, furuncles. *Salmonella typhi* a Gram negative bacteria are responsible for typhoid, food poisoning, blood poisoning and intestinal inflammations.

Due to neem's insecticidal properties, they can be applied on wounds that have become infested with maggots, they can also be applied on hair to treat head lice. Dentists have been practicing the use of neem to treat periodontal diseases.

Staphylococcus aureus:

'*Staphylococcus*' came from the Greek word "*staphyle*" which stands for bunch of grapes and berry stands for "kokkos" (Licitra, 2013). *Staphylococcus aureus* is gram positive and spherical. It is observed under microscopic light as clustered like a branch of grapes. It is both anaerobic and facultative anaerobic that gives either yellow or white colored colonies on nutrient agar. However, the yellow colonies produce carotenoids due to production in organisms (Gnanamani et al., 2017). *S. aureus* is salt tolerant that grows in a mannitol salt agar plate that contains 7.5% sodium chloride (Brown et al., 2005).

Staphylococcus aureus is a crucial pathogen causing disease in humans. It is detected in the environment as well as in healthy human individuals' nasal area specifically in mucous membranes. The organism does not usually infect healthy skin. But if they somehow get passed into blood or internal tissues, they can cause several severe infections (Lowy, 1998).

It is one of the most common bacterial infections of human body and a significant cause of multiple human infections, for instance; bacteremia, infective endocarditis, and skin and soft tissue infections including, impetigo, folliculitis, cellulitis, scaly skin syndrome, osteomyelitis, septic arthritis, denture infections, lung infections, gastroenteritis, meningitis, toxic shock syndrome and urinary tract infections and so on. Moreover, *S. aureus* has several strains. According to the strains the site of infection differs, that causes invasive infections and many more toxin-mediated diseases (Tong et al., 2015).

Several factors depend on the treatment of *S. aureus* infections like age of the affected person's infection type, clinical manifestation of the disease, comorbidity, antibacterial susceptibility of infecting organisms etc. Enormous various drugs have been used to treat *S. aureus* infection. Antibiotics like vancomycin, mupirocin, fusidic acid, linezolid, daptomycin, tigecycline, ceftaroline, telavancin, oritavancin actively work for stopping the bacterial growth (Gnanamani et al., 2017). Additionally, diverse medicinal plants have examined the antibacterial properties of herbal plants against multiple drug resistant pathogens in light of the growing awareness of the medicinal value of plants and their herbal component (Kali, 2015). Extracted crude from neem (Gupta, 2016) and oregano has been detected as an active antibacterial agent which helps to inhibit *S. aureus* infections (Cui et al., 2019).

Enterococcus faecalis:

Gram-positive commensal bacteria called *Enterococcus faecalis* is found in the gastrointestinal tract of many different organisms. It is a multi-drug resistant and hospital acquired pathogen (Van Tyne et al., 2013). These bacteria thrive in the intestinal tract's oxygen-depleted, nutrient-rich environment. They are easily found in the environment, at least in part because they shed from their animal hosts (Mundt, 1963). Enterococci are facultative anaerobes, low-GC, and non-sporulating which are the most significant MDR hospital pathogens (Van Tyne et al., 2013). They are the third most frequently isolated pathogen in healthcare (Hidron et al., 2008).

Among 58 species of enterococci so far, the most vital and common one is *E. faecalis* so far (García-Solache & Rice, 2019). This organism is extremely resilient and can withstand a variety of challenging conditions, such as common disinfectants and antiseptics, which contributes to their widespread persistence on inanimate hospital objects (Fiore et al., 2019). Through gut translocation, enterococcal colonization of the gastrointestinal tract is the primary risk factor for severe infections. *E. faecalis* resists killing by the lymph system and are phagocytosed and transported across the wall of intestine (Fernández-Hidalgo & Escolà-Vergé, 2019).

Gelatinase, serine protease, and virulence factors like bacteriocins, cytolysin, gelatinase and serine protease are all secreted by the organism. Moreover, it has the ability to produce toxic oxygen metabolites that can damage cells (Fiore et al., 2019).

Antibiotic named ampicillin is commonly used against *E. Faecalis*. However, it is highly resistant to many antimicrobial agents like cephalosporins, clindamycin, semisynthetic, penicillinase-stable penicillins and aminoglycosides and have the ability to develop resistance genes and mutations (Arias & Murray, 2012). Furthermore, it has been found that the crude from neem showed a significant antimicrobial effect against *E. faecalis* (Bohora et al., 2010). But later it has been stated that the extract was stated that neem extract was inefficient and did not stop the growth of *E. faecalis* till now (Kumar & Sidhu, 2011).

Salmonella typhi:

Salmonella typhi is a gram negative, rod shaped bacteria that only lives in humans. The bacterium exhibits serological positivity for the distinct polysaccharide capsular antigen Vi and the lipopolysaccharide antigens O9 and O12.When compared to Vi-positive strains, antigen Vi-negative strains appear to be less infectious and virulent (Crump et al., 2015).

Typhoid fever is caused by *S. typhi* that is a curse for developing nations for years. After discovering lesions in the abdominal lymph nodes of patients who had died from "gastric fever," Pierre Louis was the first person to use the word "typhoid fever" in 1829. The Greek word "typhus," indicates "smoky," which used to describe the delirium that patients would experience when they had the disease (Barnett, 2016).

Salmonella typhi is typically spread through ingesting food or water contaminated with the excrements of people who carry the organism. The organism must first pass through the stomach's gastric pH barrier before adhering to the small intestine (Parry et al., 2002). Chloramphenicol was the first antibiotic used to treat infections caused by *Salmonella typhi.* Moreover, ofloxacin or ciprofloxacin are now the treatment of choice (Parry et al., 2002).

Klebsiella pneumonia:

Klebsiella pneumoniae is a Gram-negative, rod-shaped, encapsulated, lactose-fermenting that belongs to the family *Enterobacteriaceae* and the genus *Klebsiella*. On MacConkey agar, it appears as a mucoid lactose fermenter (Brabb et al., 2012). *K. pneumoniae* is oxidase-negative, facultative anaerobic, and produces acid and gas from lactose.It is a enteric bacterium that has been found in the intestines of 5% of healthy people (Brabb et al., 2012).

It is typically diagnosed through upper respiratory tract culture. Blood, spleen, liver, peritoneal exudate, and cerebrospinal fluid have all been used to isolate the bacteria. There were few reports of *K. pneumoniae* causing cSSTIs (complex skin and soft tissue infections) (Chang et al., 2008). Moreover, in severe community-acquired pneumonia, *K. pneumoniae* was identified as an independent clinical risk factor for death (Paganin, 2004). However, the specific microbiological factors that cause patients with *K. pneumoniae*-related pneumonia to develop bacteremia remain a mystery.

Additionally, the typical signs and symptoms of bacterial meningitis, such as a fever, confusion, stiff neck, and sensitivity to bright light, are all present in meningitis caused by *K. pneumoniae*.

Fever, chills, fatigue, lightheadedness, and altered mental states are all symptoms of *klebsiella* bloodstream infections (Welch et al., 2020).

However, antibiotics can be used to treat infections caused by *Klebsiella* that are not resistant to drugs. Because there are fewer antibiotics that can treat infections caused by KPC-producing bacteria, it can be difficult to treat them. When *K.pneumoniae* produces an enzyme known as a carbapenemase, antibiotics in the class known as carbapenems will not be able to kill the bacteria and treat (*Klebsiella pneumoniae in healthcare settings,* 2010).

Shigella dysenteriae:

There are four species in the genus *Shigella*, which is in the *Enterobacteriaceae* family. Among those four *Shigella dysenteriae* is one of them (Mumy, 2014). They are non-motile, non-spore-forming, gram-negative rods. One of the most ancient human-specific pathogens with known evolutionary roots is *Shigella*. The direct fecal–oral route is the transmission route for *S. dysenteriae* (Percival & Williams., 2014).

Infected individuals usually have *S. dysenteriae* in their feces or poop. When someone comes into contact with an item that has been contaminated with the bacteria or the infected person's stool, the bacteria is transmitted. Moreover, recent research has demonstrated that *Shigella* is a significant cause of diarrhea, making it clear that it is a major public health concern worldwide (Baker & The H, 2018).

Furthermore, diarrhea, fever, nausea, vomiting, stomach cramp etc. are the most common symptoms. It is also common that it can lead to painful large bowel movements. There may be pus, mucus, or blood in the stool. As a result, *Shigella* cells might lead to dysentery. Young children may experience seizures in rare instances. Although they typically begin between two and four days after ingestion, symptoms may not manifest for up to a week. The symptoms can last for weeks or even longer than that. One of the pathogenic causes of reactive arthritis around the world is thought to be *Shigella* (Gaston & Lillicrap, 2003).

A subset of studies provided scant evidence that ampicillin was more effective than a ciprofloxacin in reducing diarrhea in adults, and that beta-lactams were more effective than fluoroquinolones in reducing diarrhea in children with confirmed *Shigella dysentery*. Moreover, antibiotics like cotrimoxazole, nalidixic acid, pivmecillinam, ceftriaxone, azithromycin etc. have shown effective effect against this bacterial strain at different periods of times. However, due to poor oral absorption, oral gentamicin was found comparatively ineffective (Christopher et al., 2010).

Aeromonas hydrophila:

The gram-negative facultative anaerobic rod *Aeromonas* has morphological similarities to members of the *Enterobacteriaceae* family. At least nine of the fourteen species of *Aeromonas* that have been described are associated with human disease. The pathogen named *A. hydrophila* is the most significant one. The organisms can be found in both fresh and saltwater. Gastroenteritis and wound infections are the two most common diseases caused by this specific organism.

Moreover, *A. hydrophila* has been implicated in approximately 85% of human infections and clinical isolations, according to previous research (Janda & Abbott, 2010). Only habitats with extreme ranges of some specific parameters like extreme saline environments, thermal spring and highly polluted water etc. failed to yield aeromonads as *A. hydrophila* grew over a wide range of temperatures, conductivities, pH, and turbidities (Janda & Abbott, 2010).

Furthermore, *A. hydrophila* have been linked to a wide range of skin and soft tissue infections (SSTIs), from minor skin concerns like pustular lesions to serious or life-threatening infections. The latter symptoms can range from subcutaneous tissue infections to processes affecting the deeper layers of skin and subcutaneous tissues while spreading along fascial planes, which has the potential to cause serious muscle tissue damage (Cui et al., 2007).

However, antibiotics such as tetracycline, sulfonamide, trimethoprim + sulfamethoxazole, or ciprofloxacin are used to treat *A. hydrophila* skin infections. But penicillin usually doesn't work well on it so far (Oakley, 2014).

To determine its efficiency as an antibacterial agent, neem extract was tested against the pathogenic bacteria *Aeromonas hydrophila* in fish. In terms of percentage reduction in bacterial cell population, *A. hydrophila* was the most sensitive to neem (Das et al., 1999).

Shiga toxin-producing E. coli (STEC):

Pathogenic *Escherichia coli* strains are characterized by their ability to Shiga toxin production is called *Shiga toxin-producing E. coli* (*STEC*). It is an important cause of foodborne illness and infections that have been implicated in a wide range of clinical human diseases (Nataro et al., 1998).

This group of *E. coli* is responsible for causing diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans (Nataro et al., 1998). It is passed on to us primarily through absorption of contaminated foods like raw or undercooked meat, raw milk, and contaminated raw vegetables etc. *STECs* can grow at temperatures between 7°C and 50°C, with an optimal temperature of 37°C. However, it can be totally eradicated via cooking food at a temperature of 70°C or higher (WHO, 2022).

Antibiotics named azithromycin, fosfomycin, meropenem etc are widely recommended in the treatment of early STEC as these will be effective in eradicating the production of Shiga toxin as well as kidney failure while removing the pathogens. Moreover, antibiotic resistance activity among STEC strains can have bad effects on these and other similar treatment options, and contribute to the spread of antibiotic resistance genes, especially if encoded (Mir & Kudva, 2019).

The oregano essential oil was found active against all clinical strains of *Escherichia coli*. However, *Origanum heracleoticum L.* essential oil has high antibacterial activity that inhibits the growth of *Escherichia coli* (MediLexicon International, 2022).

LITERATURE REVIEW:

Ionescu, 2018 explored that like old times herbal remedies have been widely used in the treatment of enormous infectious diseases caused by various bacteria, fungi, viruses are common presently as well. Some special compounds of plant origin, such as quinine and artemisinin, have been auspiciously involved in the treatment of deadly infectious diseases. Unfortunately, people's consciousness regarding medical plants has not progressed as promptly as new antibiotics are being introduced day by day. However, the emergence of multi-resistant strains drugs that need new treatment strategies. Also the deficiency of both standardization and reproducibility of herbal plants is considered as a curse for plant based products. Above all, several paths should be reconsidered as a source of significant resources to search for novel active compounds with sustainable antibacterial activity for example: phyto-therapy, the use of adult plants, and gemology, usage of embryonic stem cells etc.

Swamy et al., 2016 showed in their study herbal and aromatic plays a significant role in the international market involving several sectors including pharmaceutical industry, flavoring and perfumery, cosmetics factory and so on. Still now more than 80% of the world's population rely on herbal medicine to treat many different health issues. Moreover, plant-derived oils are rapidly used in the treatment of cardiovascular disease, diabetes, Alzheimer's, cancer and many more. In the human body essential oils can damage the cell membranes of target microorganisms as the membrane permeability becomes higher that results in the leakage of essential intracellular components of that targeted pathogens as well as hamper the cell metabolism and enzyme kinetics. To suppress infectious pathogens synthetic chemicals are demolished to use because of their toxicity and environmental hazards activity. As a result, essential oils are extracted to keep under control of multi-epidemic disease-causing pathogens which can fight against multiple infectious diseases. However, the antibacterial activity is distinguished via various processes as well. Also the efficiency and the effectiveness of essential oils also differs depending on the bacteria (Gram positive or Gram negative).

Singletary, 2010 researched that previously oregano leaves were used as an antiseptic in skin wounds, soothe sore in Greek and Roman times. Moreover, Oregano has also been used as a treatment element for asthma, cramps and diarrhea. Compounds named carvacrol (isopropyl-o-cresol or 5-isopropyl-2-methylphenol) and thymol (isopropyl-m-cresol) are considered to be the major terpene constituents of oregano essential oil including p-cymene (4). - isopropyl toluene) and + -terpinene. Oregano extract and its individual components have demonstrated antibacterial activities in vitro against foodborne pathogens, but their ability to fight infection in humans has not been clearly studied yet. Oregano has various essential and efficient antioxidants, which may in part contribute to the results of preliminary studies in which oregano plays a positive role in the cardiovascular and nervous systems, also reduces inflammatory symptoms, regulates sugar and lipids in the blood.

Truong et al., 2019, found that solvents used in bioactive compound extraction play a crucial role in the yield of extraction, biological activities and contents of chemical compounds present. The experiment to find the best solvent was carried out on Methanol, 99% ethanol, chloroform, dichloromethane, and acetone. The solvents were used to extract bioactive compounds from *Severinia buxifolia* (Rutaceae). Out of them, methanol was proved to be the most effective solvent for extraction. It resulted in the highest extraction yield of 33.2%, followed by distilled water, ethanol, acetone, chloroform and lastly dichloromethane with extraction yields of 27%, 12.2%, 7.2%, and 4.9% respectively. Methanol extract had the highest content of phenolic; 13.36 mg GAE/g DW, flavonoids, alkaloids and terpenoids. Furthermore, methanolic extract had high capacity of antioxidants and in-vitro anti-inflammatory activities. The antioxidant activity was 3 times higher than ascorbic acid extract. The result of this study shows that methanolic extract can be utilized in pharmacognosy as it is capable of obtaining high content of phytochemical constituents.

Aim and Objectives:

The main purposes of this study was to do a comparative analysis between the antimicrobial activity of home-grown Oregano essential oil and Neem essential oil against a range of infectious gram positive and gram negative bacteria. Some infections are very common and some infections are difficult to control with synthetic medications. Following are objectives of the present study.

1. To determine the antibacterial activity of Oregano oil and Neem oil against selected organism those are responsible for infectious diseases.

2. To determine the inhibition percentage of these oils against *Staphylococcus aureus, Enterococcus faecalis, Salmonella Typhi, Klebsiella pneumoniae, Shigella, Aeromonas hydrophila, Shiga-Toxin producing Escherichia coli.*

3. To set a comparison of antimicrobial activity among Oregano essential oil and Neem essential oil against these organisms.

CHAPTER 2

MATERIALS AND METHODS

Materials and Method:

Working Place:

All laboratory work for this study have been done in the laboratory of BRAC University, Mathematics and Natural Sciences Department.

Product tested:

Oil grown from home grown Oregano and Neem plant leaves which contain no synthetic fertilizer or preservatives.

Bacterial Strains:

Staphylococcus Aureus, Enterococcus Faecalis, Aeromonas Hydrophila, Salmonella Typhi, Shiga-Toxin producing Escherichia Coli, Shigella, Klebshiela Pneumoniae were taken from the BRAC University Laboratory. Weekly subculture was carried out to keep the bacteria strains viable and prevent chances of contamination from other microorganism, and kept refrigerated at 4˚Celsius, until needed.

Confirmation of the stock:

Each strain taken from the laboratory stock, were first grown in LA media to increase the number of CFU and then sub cultured using each of their selective media, and the plates were incubated at 37 °C for 24 hours.

Staphylococcus Aureus – MSA media

Enterococcus Faecalis – TSA agar media

Aeromonas Hydrophila – MAC agar media

Salmonella Typhi – SSA agar media

Klebshiela Pneumoniae – MAC agar media

STEC – SMAC agar media

Shigella – SSA agar media

Preparation for reviving and maintaining the bacteria:

All the bacterial cultures were grown on LA plates in the incubator at 37ºC

1. LA was prepared for each of the microorganisms.
2. Autoclaved at 121 °C for 15 minutes (SAARC).
3. The media was poured into the petri dishes.
4. After solidification of the media, the bacteria that is needed is taken from a previous culture and was streaked on the LA.
5. The plates were incubated at 37 °C for 24 hours.

Preparation of stock sample:

For short-term preservation

2 ml of soft LA agar in a vial was inoculated after stabbing fresh bacterial colony of each isolate from LA plate. Then the medium was covered with sterile paraffin oil and the vial was refrigerated at 4 ºC until needed.

Long-term preservation:

2 ml of soft LA agar in a sterile cryovial was inoculated after stabbing fresh bacterial colony of each isolate from LA plate. Then 500µl of sterile glycerol was added to the medium and the cryovial was stored at -20 ºC.

OIL PREPARATION:

Collecting and drying leaves:

1. Only the leaves are torn from the plants.
2. They are washed thoroughly.
3. The leaves are cut into tiny pieces (for shorter drying time) using a sterile scissor.
4. The cut leaves are spread out on a large piece of cotton cloth and kept in an air conditioned room for 2 days.
5. After the leaves had dried completely, it was made into powder using a sterile grinder.
6. The powdered leaves are then weighed.

Oil Extraction Process:

Making the Thimble:

1. A piece of sterile cheese cloth is laid flat on a sterile surface.
2. The dried powder is laid on the cloth.
3. The cloth is wrapped around the powder tightly, forming a cylindrical shape.
4. The thimble is then tied at both ends using pieces of the cheese cloth.

Extraction process:

1. The RBF (round-bottom flask) is placed on the top of that hot plate.
2. The Soxhlet extractor is connected to the RBF with clamps, to secure it in place.
3. The thimble is placed at the bottom of the Soxhlet extractor.
4. The Soxhlet extractor is connected with the condenser.

5. The condenser is connected with tubes that allows water input, circulation around the condenser and then the water is let out via the top tube to keep the condenser cool throughout the process.

6. A funnel is placed on top of the condenser, and 250ml methanol is poured into the Soxhlet extractor.

7. When all the methanol soaks the thimble and passes through the siphon tube and falls in the RBF, the hot plate is turned on and the temperature is set at 60˚ Celsius.

The Soxhlet is left to run for 4-5 cycles or until the solvent runs clear through the siphon.

<u>Drying of the oil:</u>

1. The RBF containing the methanol and oil mixture is allowed to cool down

2. Using a funnel and filter paper the solution is filtered into an autoclaved conical flask.

3. The filtered solution is then poured on to weighed autoclaved petri dishes.

4. The uncovered petri dishes are carefully placed into an incubator that is set at 44˚Celsius.

5. The petri dishes are left in the incubator for 24hours.

6. After 24hours, the petri dishes are taken out and weighed again.

7. The weight of the empty petri dishes is subtracted from the weight of the oil containing petri dishes to find the mass of the total oil extracted.

<u>Dissolving the Oil:</u>

The crude oil extracted from the leaves is converted into a liquid consistency by adding 2% DMSO and water. The mixture is vortexed until all the oil is dissolved to make a concentration of 100µg/ml oil.

Kill-Time Test:

1. 4 test tubes are taken and labelled with: **10^0, 10^{-1}, 10^{-2}** and **10^{-3}**.
2. In each tube 9ml BHIB is taken.
3. Bacteria is taken from a fresh culture plate on a loop and added to the tube labeled 10^0.
4. The tube is then vortexed until the colony is completely mixed with the broth and the suspension is compared with 0.5 Mcfarland solution. More bacteria are added if needed.
5. 1ml suspension from 10^0 is taken and pipetted into 10^{-1} tube, and the tube is then vortexed.
6. 1ml suspension from 10^{-1} is taken and pipetted into 10^{-2} tube, and the tube is then vortexed.
7. 1ml suspension from 10^{-2} is taken and pipetted into 10^{-3} tube, and the tube is then vortexed.
8. 2 test tubes are taken, and they are labelled: Tube 1 and 2
9. In tube 1, 9ml BHIB and 1ml bacterial suspension from tube 10^{-3} is taken and vortexed.
10. In tube 2, 4ml BHIB, 5ml oil and 1ml bacterial suspension from tube 10^{-3} is taken and vortexed.
11. The tubes are then placed inside a shaker incubator for 1hr at 37˚ Celsius.
12. After 1 hour the tubes are taken out and 50µl suspension from each tube is spread on MHA media. The plates are then placed in the incubator at 37˚ Celsius for 24 hours.

13. The tubes are again placed inside the shaker incubator for further 30 minutes at 37˚ Celsius.

14. After 30 minutes the tubes are taken out and 50µl suspension from each tube is spread on MHA media. The plates are then placed in the incubator at 37˚ Celsius for 24 hours.

15. The tubes are again placed inside the shaker incubator for the last time for further 30 minutes at 37˚ Celsius.

16. After 30 minutes the tubes are taken out and 50µl suspension from each tube is spread on MHA media. The plates are then placed in the incubator at 37˚ Celsius for 24 hours.

17. After 24 hours the plates are taken out and the colonies are counted.

18. The procedure is repeated 1 more time and average colony count is taken.

Serial Dilution:

1. Four test tubes are taken and labeled with RAW, D1, D2 and D3.

2. In the tube labeled RAW 4ml BHI broth is taken.

3. In each of the tubes labeled D1, D2 and D3 4.5ml BHI broth is taken.

4. Bacteria is taken from a fresh culture plate on a loop and added to the tube labeled RAW.

5. The tube is then vortexed until the colony is completely mixed with the broth and the suspension is compared with 0.5 Mcfarland solution. More bacteria are added if needed.

6. 0.5ml of the suspension from the RAW tube is pipetted into D1 tube.

7. D1 is vortexed to form and uniform suspension of 10^{-1} dilution.

8. 0.5ml of the suspension is pipetted from D1 to D2.

9. D2 is vortexed to form a uniform 10^{-2} dilution.

10. 0.5ml of the suspension is then pipetted from D2 to D3

11. D3 is vortexed to form a uniform 10^{-3} dilution.

Spreading method:

1. 5 Eppendorf tubes are taken for each dilution.

2. 50µl, 100µl, 150µl and 200µl of oil was pipetted into 4 Eppendorf tubes, on the 5th Eppendorf tube no oil was added.

3. 50µl bacterial solution from tube RAW was added to each of the 5 Eppendorf tubes.

4. All the five Eppendorf tubes were closed and vortexed thoroughly for 1 minute, to create a uniform suspension of bacteria and oil.

5. The Eppendorf tubes were then placed inside a 37˚celsius shaker incubator for 2 hours.

6. The same process is followed for dilutions in D1 and D3.

7. After 2hours the Eppendorf tubes are taken out of the incubator and vortexed.

8. 50µl mixture from each Eppendorf tube is pipetted onto respective labelled MHA containing petri dishes.

9. Spreading is carried out using a glass or metal spreader, until all the solution is absorbed by the media.

10. After spreading is complete the plates are then placed into the incubator set at 37˚ Celsius for 24 hours.

11. After the incubation period, colonies of each plate is counted and recorded.

12. The entire process is repeated two more times for each bacterium.

CHAPTER 3

RESULTS

RESULT:

KILL-TIME TEST RESULT:

1ml of 10^{-3} dilution of bacterial suspension was mixed with BHIB and oil with BHIB. The mixtures were incubated for 1 hour, 1.5 hours and 2 hours' time intervals. After each time interval, 50µl from each mixture was plated and incubated for 24 hours at 37˚ Celsius. After incubation the colonies of each plate were counted and the percentage inhibition was calculated.

Pathogen		Incubation Time		
		1 HOUR	1.5 HOURS	2 HOURS
Klebsiella Pneumoniae	BHIB CFU/50µl	TNTC	TNTC	TNTC
	Neem oil CFU/50µl	TNTC	561±189	352±96
	Oregano oil CFU/50µl	640±189	298±69	92±18

Table 3 Average viable colony counts of Klebsiella pneumonia in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation before plating

Pathogen		Incubation Time		
		1 HOUR	1.5 HOURS	2 HOURS
Shigella	BHIB CFU/50µl	760±296	TNTC	TNTC
	Neem oil CFU/50µl	TNTC	120±24.5	65±6
	Oregano oil CFU/50µl	202±96	156±63	67±29

Table 4 Average viable colony counts of Shigella in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation before plating

Pathogen	Incubation Time			
		1 HOUR	1.5 HOURS	2 HOURS
STEC	BHIB CFU/50µl	TNTC	TNTC	TNTC
	Neem oil CFU/50µl	TNTC	780±269	21±6
	Oregano oil CFU/50µl	569±299	322±6	97±3

Table 5 Average viable colony counts of STEC in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation before plating.

Pathogen	Incubation Time			
		1 HOUR	1.5 HOURS	2 HOURS
Salmonella typhi	BHIB CFU/50µl	670±296	768±357	1490±590
	Neem oil CFU/50µl	190±59	25±13	12±6
	Oregano oil CFU/50µl	390±96	331±96	280±188

Table 6 Average viable colony counts of Salmonella typhi in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation before plating.

Pathogen		Incubation Time		
		1 HOUR	1.5 HOURS	2 HOURS
Aeromonas Hydrophila	BHIB CFU/50µl	760±378	TNTC	TNTC
	Neem oil CFU/50µl	TNTC	TNTC	256±152
	Oregano oil CFU/50µl	580±396	156±96	38±16

Table 7 Average viable colony counts of Aeromonas Hydrophila in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation before plating

Pathogen		Incubation Time		
		1 HOUR	1.5 HOURS	2 HOURS
Staphylococcus aureus	BHIB CFU/50µl	490±258	TNTC	TNTC
	Neem oil CFU/50µl	470±199	225±99	120±36
	Oregano oil CFU/50µl	319±185	54±17	34±19

Table 8 Average viable colony counts of Staphylococcus aureus in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation before plating.

Pathogen	Incubation Time			
		1 HOUR	1.5 HOURS	2 HOURS
Enterococcus faecalis	BHIB CFU/50µl	899±366	TNTC	TNTC
	Neem oil CFU/50µl	725±229	512±74	300±65
	Oregano oil CFU/50µl	125±52	51±18	25±9

Table 9 Average viable colony counts of Enterococcus faecalis in BHIB, NEO and OEO after 1hr, 1.5hrs and 2hrs of incubation

Comparison between number of colonies in BHIB and different dosage of oils with BHIB:

Bacterial suspension was incubated for 2 hours with 4 different dosages of 0.1µg/ml concentration of OEO and NEO. 50µl of each mixture was spread plated and incubated for 24 hours at 37˚ Celsius. The colonies were counted to calculate the percentage inhibition of NEO and OEO at dosages.

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µl OEO
Klebsiella Pneumoniae	10^{-0}	TNTC	TNTC	620 ± 30	0	0
	10^{-1}	TNTC	TNTC	4 ± 2	4 ± 1	1 ±1
	10^{-3}	325 ± 20.4	41 ± 4.9	2 ± 2.5	0	0

Table 10 Average viable colony counts of klebsiella pneumoniae in BHIB and BHIB with different doses of oregano essential oil.

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
Klebsiella Pneumoniae	10^{-0}	TNTC	TNTC	TNTC	TNTC	1527 ± 75.6
	10^{-1}	TNTC	TNTC	TNTC	TNTC	720 ± 16.2
	10^{-3}	960 ± 53.1	755 ± 54.8	366 ± 25.3	182 ± 18	137 ± 12.7

Table 11 Average viable colony counts of klebsiella pneumonia in BHIB and BHIB with different doses of neem essential oil

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µlOEO
Shigella	10^{-0}	TNTC	TNTC	TNTC	TNTC	TNTC
	10^{-1}	TNTC	TNTC	TNTC	1210 ± 92.3	890 ± 55.7
	10^{-3}	830 ± 23.7	186 ± 14.3	44 ± 6.5	0	0

Table 12 Average viable colony counts of Shigella in BHIB and BHIB with different doses of oregano essential oil

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
Shigella	10^{-0}	TNTC	TNTC	TNTC	TNTC	TNTC
	10^{-1}	TNTC	TNTC	3572 ± 178	2748 ± 159.1	1550 ± 102
	10^{-3}	1850 ± 130.2	1563 ± 115.5	1208 ±100.4	519 ±16.7	92 ± 6.16

Table 13 Average viable colony counts of Shigella in BHIB and BHIB with different doses of neem essential oil

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µl OEO
Salmonella typhi	10^{-0}	TNTC	TNTC	TNTC	TNTC	1200±329
	10^{-1}	1800±345	1252±265	702±298	470±167	236±98
	10^{-3}	850 ± 24.1	400 ± 46.1	363 ± 13.5	244 ± 13.5	120 ± 9.8

Table 14 Average viable colony counts of Salmonella typhi in BHIB and BHIB with different doses of oregano essential oil

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
Salmonella typhi	10^{-0}	310±45	2±0.65	1±0.06	0	0
	10^{-1}	86±8	8±3.7	1±0.06	0	0
	10^{-3}	40 ± 4.9	2 ± 2.1	1 ± 0.5	0	0

Table 15 Average viable colony counts of Salmonella typhi in BHIB and BHIB with different doses of neem essential oil

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µl OEO
Aeromonas hydrophila	10^{-0}	TNTC	3840±573	154±23	132±19	0
	10^{-1}	TNTC	275±65	164±24	27±12	7±3.5
	10^{-3}	2150 ± 263.8	47 ± 20.8	5 ± 2.1	1 ± 0.5	0

Table 16 Average viable colony counts of Aeromonas hydrophila in BHIB and BHIB with different doses of oregano essential oil

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
Aeromonas hydrophila	10^{-0}	TNTC	TNTC	TNTC	TNTC	TNTC
	10^{-1}	TNTC	6520±798	4200±613	3905±512	3625±552
	10^{-3}	2880 ± 294	1408 ± 139	714 ± 78.8	683 ± 99.6	245 ± 32.3

Table 17 Average viable colony counts of Aeromonas hydrophila in BHIB and BHIB with different doses of neem essential oil

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µl OEO
STEC	10^{-0}	TNTC	747±221	679±68	438±122	290±61
	10^{-1}	TNTC	654±197	478±143	289±177	120±31
	10^{-3}	586 ± 72.3	366 ± 39.6	182 ± 16	137 ± 11	13 ± 3.7

Table 18 Average viable colony counts of STEC in BHIB and BHIB with different doses of oregano essential oil

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
STEC	10^{-0}	TNTC	TNTC	TNTC	TNTC	1502±341
	10^{-1}	TNTC	TNTC	3365±598	2260±324	1570±287
	10^{-3}	1470 ± 207	1002 ± 160	933 ± 173	863 ± 206	740 ± 97.6

Table 19 Average viable colony counts of STEC in BHIB and BHIB with different doses of neem essential oil

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µl OEO
Staphylococcus Aureus	10^{-0}	TNTC	TNTC	TNTC	475 ± 176	35±12.4
	10^{-1}	TNTC	TNTC	1652±334	800±211	529±140
	10^{-3}	1230 ± 209	500 ± 106	188 ± 17.1	0	0

Table 20 Average viable colony counts of Staphylococcus aureus in BHIB and BHIB with different doses of oregano essential oil

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
Staphylococcus Aureus	10^{-0}	TNTC	2912±311	1950±234	1700±445	1620±349
	10^{-1}	TNTC	3670±574	2850±347	1±0.76	0
	10^{-3}	1020 ± 209	317 ± 54.3	4 ± 1.2	3 ± 2.1	0

Table 21 Average viable colony counts of Staphylococcus aureus in BHIB and BHIB with different doses of neem essential oil

Pathogen	Dilution	BHIB	50µl OEO	100µl OEO	150µl OEO	200µl OEO
Enterococcus Faecalis	10^{-0}	TNTC	600±198	19±8	0	0
	10^{-1}	TNTC	8 ± 4	5±2.6	2±1.5	2±1.4
	10^{-3}	2 ± 1.2	0	0	0	0

Table 22 Average viable colony counts of Enterococcus faecalis in BHIB and BHIB with different doses of oregano essential oil

Pathogen	Dilution	BHIB	50µl NEO	100µl NEO	150µl NEO	200µl NEO
Enterococcus Faecalis	10^{-0}	TNTC	TNTC	TNTC	TNTC	1502±354
	10^{-1}	TNTC	TNTC	TNTC	1672±239	900 ± 123
	10^{-3}	1749 ± 295	1020 ± 78.8	312 ± 44.5	298 ± 26.1	231 ± 18.4

Table 23 Average viable colony counts of Enterococcus faecalis in BHIB and BHIB with different doses of neem essential oil

Determination of Inhibition percentage:

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

$$CFU = \frac{\text{NUMBER OF COLONIES} \times \text{RECIPROCAL OF THE DILUTION FACTOR}}{\text{VOLUME OF PLATED SUSPENSION}}$$

$$\text{Inhibition percentage} = 1 - \left(\frac{CFU\ IN\ OIL}{CFU\ IN\ BHIB}\right) \times 100$$

Pathogen	Oil	50µl oil	100µl oil	150µl oil	200µl oil
Klebsiella pneumoniae	NEO	21.9% ± 0.94%	62.2% ± 0.58%	81% ± 0.72%	85.9% ± 0.47%
	OEO	87.4% ± 0.72%	99.3% ± 0.59%	100% ± 0%	100% ± 0%
Shigella	NEO	15% ± 0.65%	35% ± 1.11%	72% ± 0.96%	95% ± 0.04%
	OEO	77.7% ± 1.20%	94.7% ± 0.66%	100% ± 0%	100% ± 0%
Salmonella typhi	NEO	95%±4.54%	97.5%±1.18%	100%±0%	100%±0%
	OEO	53.5% ±3.63%	57.7%±0.26%	71.6%±0.53%	86.0%±0.61%
Aeromonas hydrophila	NEO	50.1%±10.30%	75.2%±0.13%	76.4%±0.95%	91.5%±0.22%
	OEO	97.9%±0.74%	99.8%±0.07%	99.9%±0.02%	100%±0%
STEC	NEO	31%±1.39%	36.1%±3.42%	41.3%±6.04%	48.9%±0.47%
	OEO	37.1%±1.38%	68.6%±1.03%	76.4%±0.85	97.8%±0.40%
Staphylococcus aureus	NEO	68.5%±1.59%	99.6%±0.03%	99.7%±0.17%	100%±0%
	OEO	59.7%±1.23%	84.5%±1.53%	100%±0%	100%±0%
Enterococcus faecalis	NEO	39.3%±14.69%	81.3%±5.71%	82%±04.48%	86%±0.335%
	OEO	100%±0%	100%±0%	100%±0%	100%±0%

Table 24 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl oil

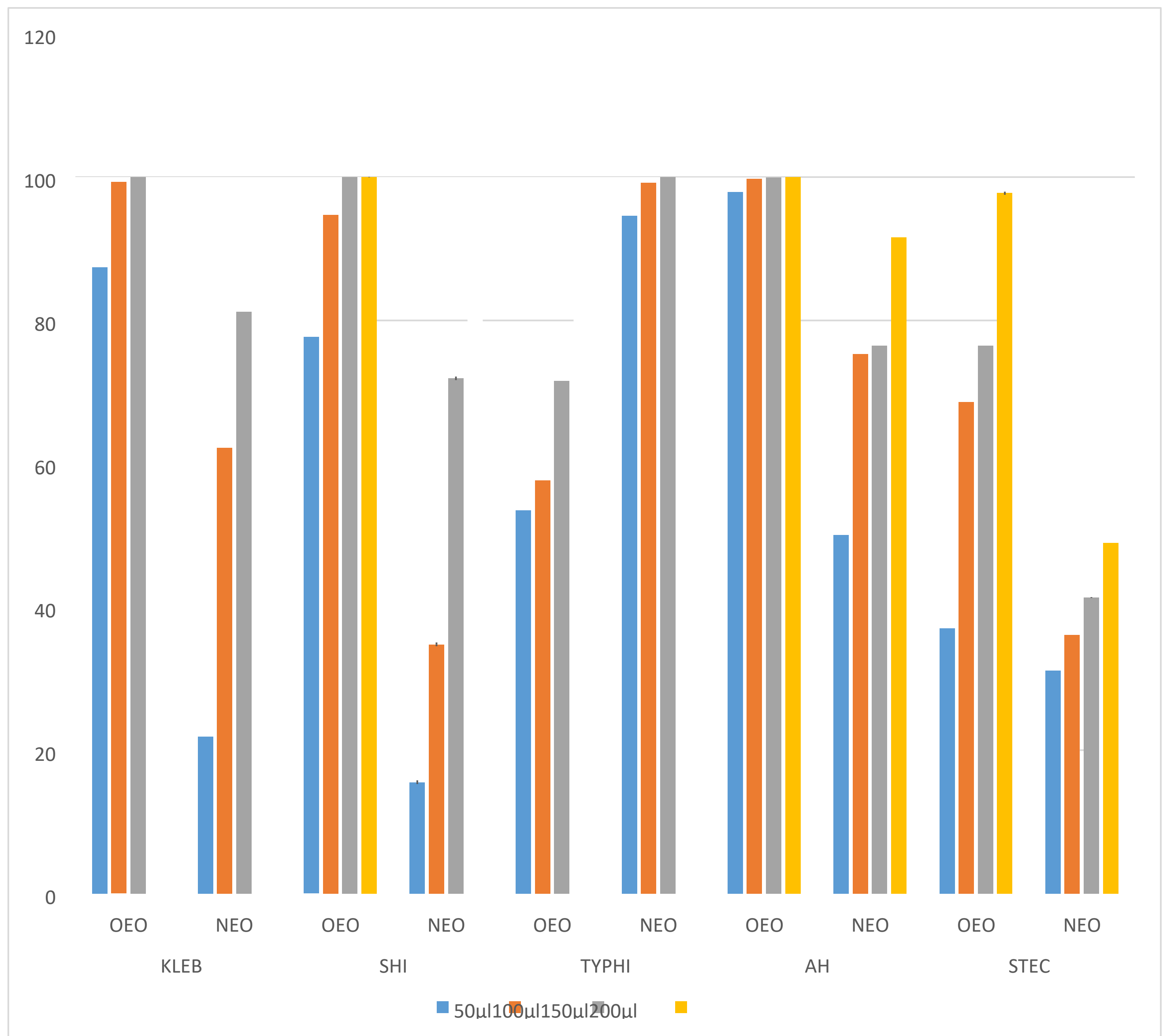

Figure 2 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl dosages OEO and NEO for Gram Negative bacteria

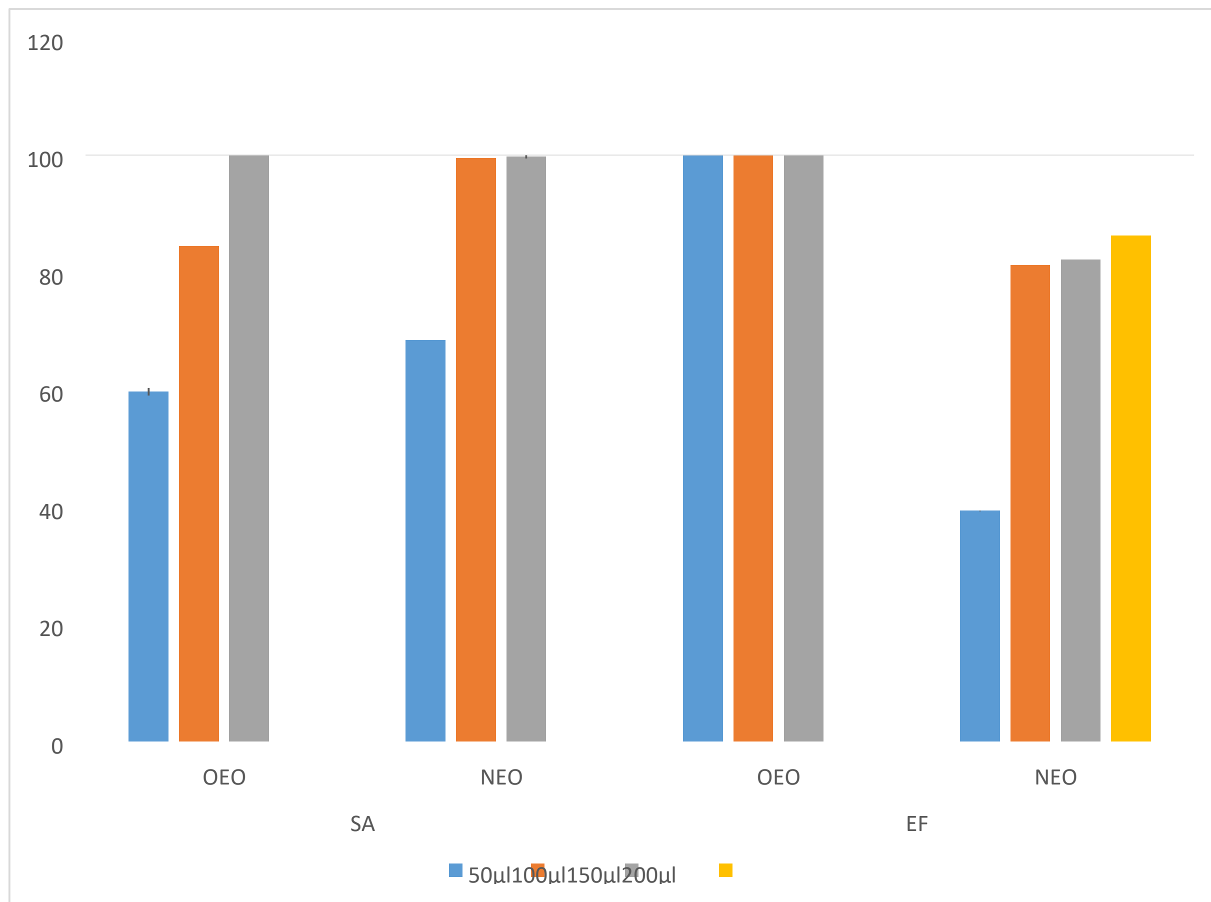

Figure 3 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl dosages for Gram Positive bacteria

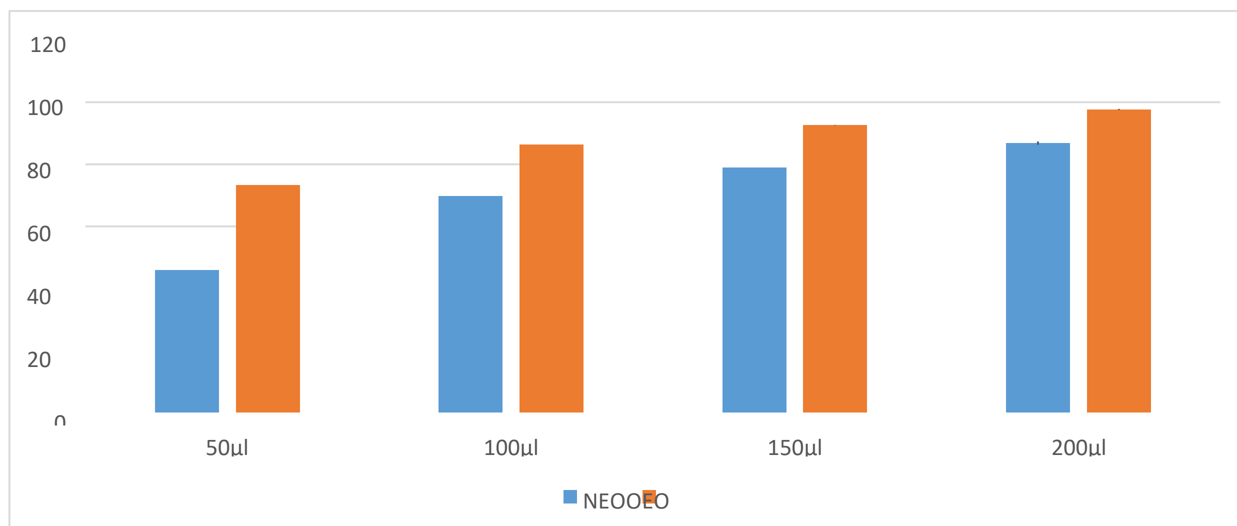

Figure 4 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl dosages

OIL	50µl oil	100µl oil	150µl oil	200µl oil
NEO	45.9%±1.47%	69.8%±1.50%	79%±1.61%	86.8%±0.50%
OEO	73.3%±1.08%	86.4%±1.19%	92.6%±0.05%	97.7%±0.14%

Table 25 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl dosages

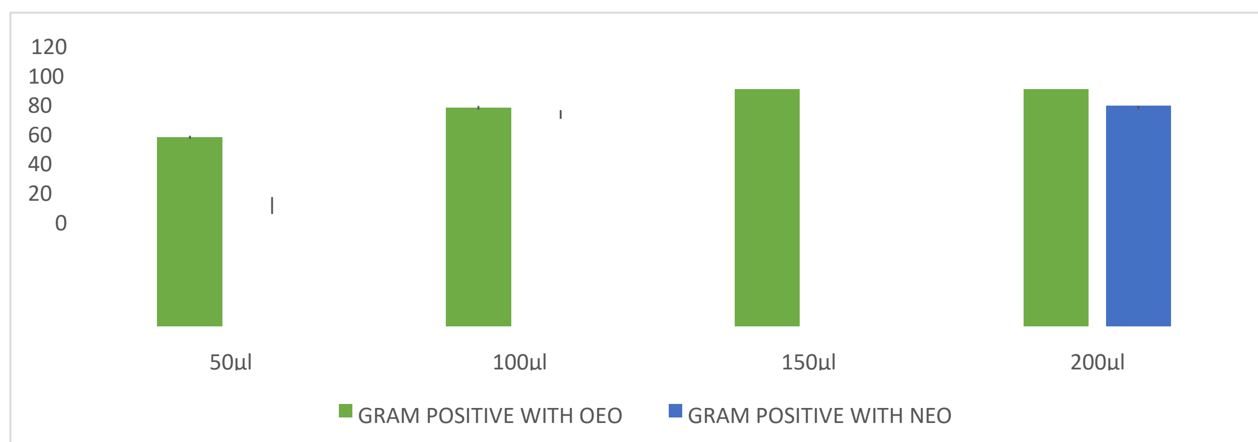

Figure 5 Average percentage inhibition of NEO and OEO 50µl, 100µl, 150µl and 200µl dosages respectively for Gram-Positive bacteria

OIL	50µl oil	100µl oil	150µl oil	200µl oil
NEO	53.9%±6.55%	90.4%±2.87%	91%±2.32%	93.1%±1.68%
OEO	79.9%±0.62%	92.2%±0.76%	100%±0%	100%±0%

Table 26 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl dosages respectively with Gram-Positive bacteria

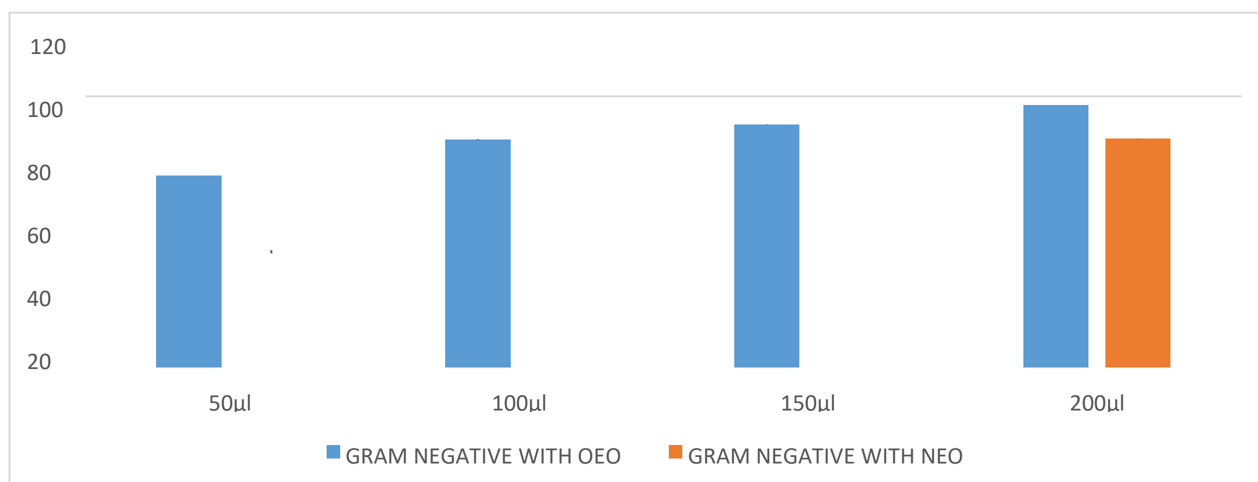

Figure 6 Average percentage inhibition of NEO and OEO 50µl, 100µl, 150µl and 200µl dosages for Gram-Negative bacteria

OIL	50µl oil	100µl oil	150µl oil	200µl oil
NEO	42.7%±0.59%	61.5%±1.11%	74.2%±1.35%	84.3%±0.06%
OEO	70.7%±1.26%	84%±0.17%	89.6%±0.07%	96.7%±0.2%

Table 27 Average percentage inhibition of NEO and OEO at 50µl, 100µl, 150µl and 200µl dosages for Gram-Negative bacteria

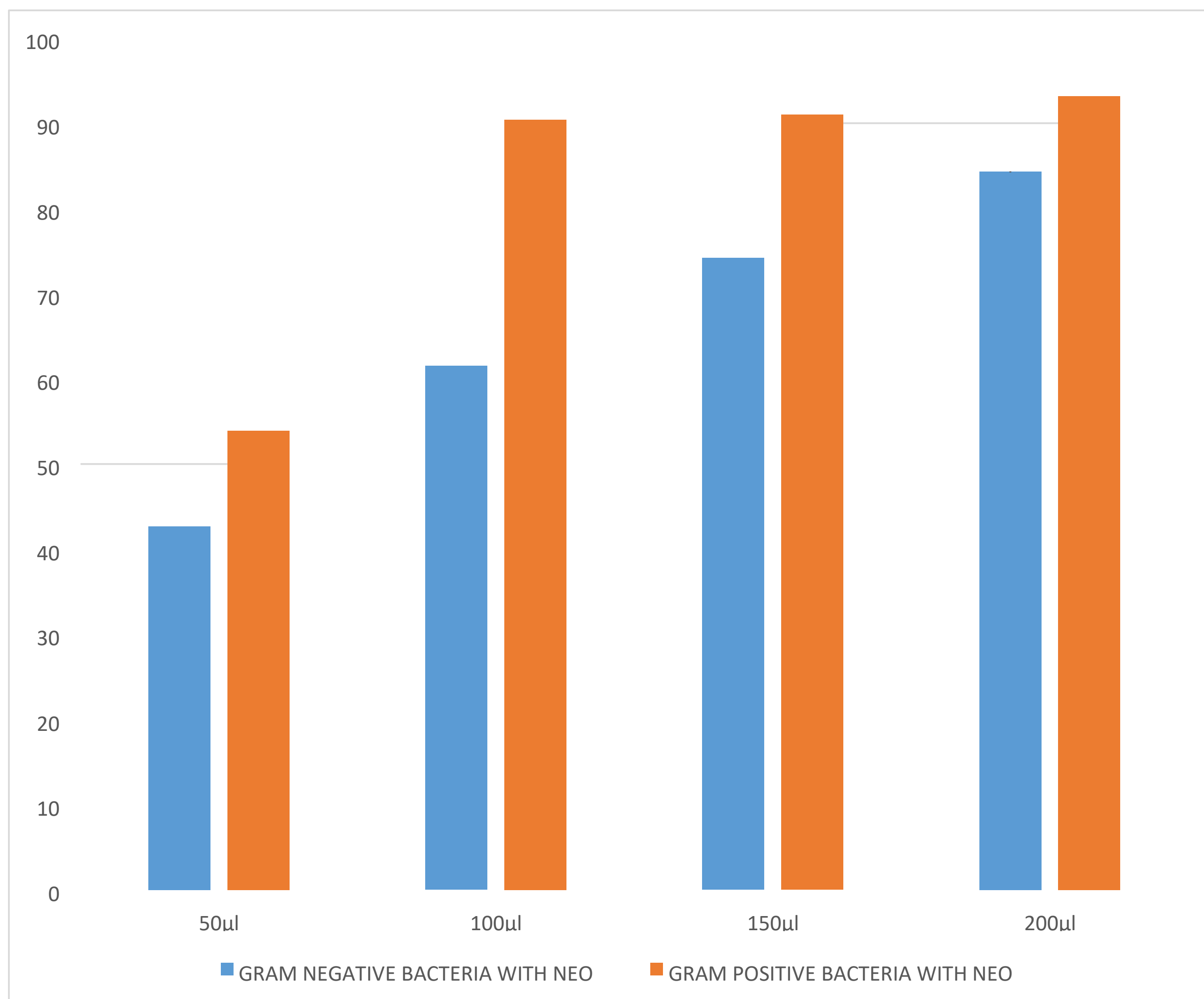

Figure 7 Average percentage inhibition of NEO at 50µl, 100µl, 150µl and 200µl dosages respectively for Gram Positive and Negative bacteria

	50µl oil	100µl oil	150µl oil	200µl oil
Gram Positive	53.9%±6.55%	90.4%±2.87%	91%±2.32%	93.1%±1.68%
Gram Negative	42.7%±0.59%	61.5%±1.11%	74.2%±1.35%	84.3%±0.06%

Table 28 Average percentage inhibition of NEO at 50µl, 100µl, 150µl and 200µl dosages respectively for Gram Positive and Negative bacteria

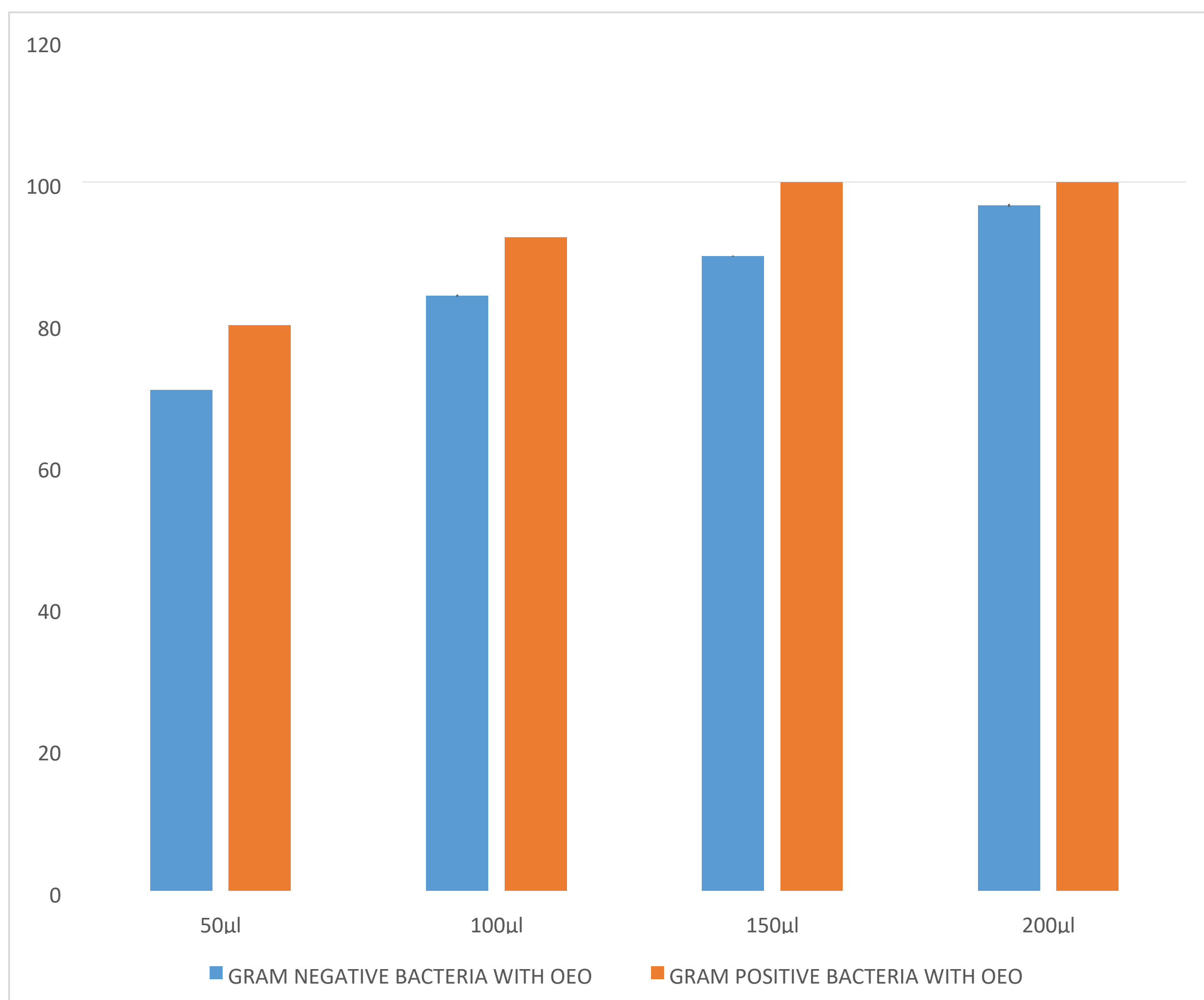

Figure 8 Average percentage inhibition of OEO at 50µl, 100µl, 150µl and 200µl dosages respectively for Gram Positive and Negative bacteria

	50µl oil	100µl oil	150µl oil	200µl oil
Gram Positive	79.9%±0.62%	92.2%±0.76%	100%±0%	100%±0%
Gram Negative	70.7%±1.26%	84%±0.17%	89.6%±0.07%	96.7%±0.2%

Table 29 Average percentage inhibition of OEO at 50µl, 100µl, 150µl and 200µl dosages respectively with Gram Positive and Negative bacteria

CHAPTER 4

DISCUSSION AND CONCLUSION

DISCUSSION:

Essential oil extracted from plants have been in use for a millennium, they are still widely found in food preservatives, natural therapies, pharmaceuticals and as an alternative medicine to synthetic antimicrobial drugs. These plants are readily found in Bangladesh and, especially Neem trees, can be seen all around the south and Southeast Asia. Greek Oregano plant is a lot rarer and not easily available in the country. From this study it can be seen that Oregano essential oil is a potential source of natural antibiotic compounds, which work well against selected bacterial pathogens. This in-vitro study showed that Neem oil and Oregano oil inhibited bacterial growth but their effectiveness is varied.

Different methodologies were used to determine the effectiveness of NEO and OEO, such as: Spread plate technique to determine effect of dosage on bacteria, Agar well diffusion, Agar disk diffusion and kill-time test. Out of the four methods, Agar well diffusion and Agar disk diffusion gave the least definitive and conclusive results. In most cases, zone of inhibition could not be noticeable around the well/disk of oil. One reason could be that the oils were too viscous to diffuse through the agar media. Therefore, spread plate technique and kill-time test were used to do the comprehensive comparative study on the antibiotic characteristics of NEO and OEO.

In the kill-time test it was clearly seen that, as the incubation time before plating was increased the inhibition rate also increased for both the oils. This test was necessary to perform to decide on the ideal pre-plating incubation time for the spread plate method. It was seen that almost all the bacterial strains started producing countable colonies when they were incubated for 2 hours prior to plating. Therefore, for the spread plate method 2 hours pre-plating incubation time was administered, as countable colonies are essential to calculate percentage inhibition of the oils.

Furthermore, it was also found that on an average OEO had greater inhibition rate for each incubation time compared to NEO. These findings are consistent with the result of the dosage dependent inhibition rate experiment. For better display of the effect of incubation time on bacterial growth, more incubation time intervals should be added, to find out whether the inhibition rate ever becomes 100% or the growth spikes up again in the case of each bacteria with each oil.

A range of bacterial strains (5 Gram-negatives and 2 Gram-positives) were tested against increasing dosage of NEO and OEO with a concentration of 100µg/ml. For each bacterial strain, as the dosage of oil increased the inhibition percentage increased. OEO had the greatest effect on *Enterococcus faecalis*, as the inhibition was 100% with only 50µl of OEO. OEO was second most effective on *Aeromonas hydrophila*. It was extremely sensitive to OEO, and showed an increase from 97.9% to 100% inhibition when the dosage was raised from 50µl to 200µl. *Klebsiella pneumonia, Shigella* and *Staphylococcus aureus* were also completely inhibited at dosage 150µl and above. OEO was least effective on *Salmonella typhi*, which had an inhibition of 53.5% with dosage 50µl and the inhibition increased to 86% with 200µl oil dosage. As OEO was active against *Klebsiella pneumonia* and *Staphylococcus aureus* it indicates that it is a potent source of antibacterial compounds that has the potential to be used against hospital acquired drug resistant microorganisms (Sultana et al.,2019).

Unlike OEO, NEO was the most effective against *Salmonella typhi.* It had a 95% inhibition rate with 50µl dosage of NEO and the inhibition increased to 100% with dosage of 150µl and above. NEO was second most effective on *Staphylococcus aureus,* as even though the inhibition was only 68.5% at 50µl dosage, the rate spiked to 99.6% then to 100% with dosages 100µl and 200µl respectively. *STEC* was the most resistant against NEO, only 48.9% inhibition was seen for 200µl dosage.

The average percentage inhibition of each oil demonstrated a directly proportional relationship with the dosage. Overall, OEO showed greater antibacterial activity against the bacterial strains as compared to NEO. For 50µl dosage the average inhibition percentage for OEO was 73.3% and for NEO it was only 45.9%. Furthermore, for the highest dosage of 200µl, NEO had an average percentage inhibition of 86.8% whereas, OEO has an average inhibition of 97.7%. NEO was less effective on bacterial strains; *Klebsiella pneumonia, Shigella, Aeromonas hydrophia, STEC, Staphylococcus aureus* and *Enterococcus faecalis* as compared to OEO. The only bacterial strain NEO was more effective on was *Salmonella typhi*. OEO demonstrated 100% inhibition against *Klebsiella pneumonia, Shigella, Aeromonas hydrophila, STEC, Staphylococcus Aureus* and *Enterococcus faecalis* at varying dosage of oil. Whereas, NEO showed 100% inhibition against *Salmonella typhi* and *Staphylococcus Aureus* at 150µl and 200µl dosage of oil respectively.

Through data analysis, it was seen that OEO is more effective against gram-positive bacterial strains than NEO. Both the gram-positive bacteria tested in this experiment were more sensitive to OEO than NEO. Only *Staphylococcus aureus* was 100% inhibited by NEO with a dose of 200µl. On average, OEO showed 100% inhibition at 150µl dosage and above, whereas on average NEO demonstrated an inhibition of 93.1% at 200µl dosage.

For gram-negative bacteria the trend remains consistent as before. Bacterial strains overall showed more sensitivity towards OEO as compared to NEO. At 50µl dosage of OEO, on average 70.7% inhibition was found, whereas with the same dosage of NEO, only 42.7% inhibition was seen. The highest inhibition for both the oils were for 200µl dosage.

Furthermore, NEO had greater inhibition percentage against gram-positive bacteria as opposed to gram-negative bacterial strains. Same is also true for OEO. Therefore, it can be deduced that OEO and NEO on average are more effective against gram-positive bacterial strains. Further research should be carried out by taking equal number of gram-positive and negative bacteria and do more comparative analysis.

Knobloch et al., (1986) described that the high inhibition rate could be the result of one of the characteristics of these essential oils. That is their hydrophobicity. This feature allows them to create partition in the lipids of the membrane and mitochondria of bacterial cell. This hinders the cell structure and makes them more permeable, resulting in extensive leakage of critical ions and molecules from bacterial cell, which may lead to cell death.

The oils were diluted and dissolved in 2% DMSO, because DMSO at concentration less than 2% is not antibacterial and does not interfere with the effectiveness of the oils. Methanol and acetone were not used to dilute the oil because a separate test was done on them, and they both showed varying degrees of inhibition of growth. Paraffin should not be used as a diluent because it has a potent inhibitory effect and weak killing effect on bacteria (Hartsell,1953).

Further studies are necessary to confirm these antibacterial traits, and to identify, segregate and purify the different active components of the oils by high performance liquid chromatography (HPLC) or other high throughput technique, to evaluate their individual antimicrobial properties.

CONCLUSION:

The world is quickly moving towards the era of absolute synthetic antibiotic resistance. To combat this potential deadly catastrophe, novel and natural antibiotics has to be churned out on a rapid scale. For that, in depth research on antimicrobial properties and components of all kinds of plant essential oil is crucial. Many research over the years have proved the varying effectiveness of a wide range of essential oils. Neem has been used for centuries mainly in every south Asian household to treat a wide range of ailments and diseases. In this study, it has been proven that Oregano, a non-native plant of Bangladesh that can be easily grown in any home nursery, has stronger antibacterial effect than the Neem plant.

Data analysis of this study demonstrated that Neem essential oil and Oregano essential oil can be recommendable as it showed promising inhibitory activity against *Klebsiella pneumonia, Shigella, Salmonella typhi, Aeromonas hydrophila, Shiga-Toxin producing Escherichia Coli, Staphylococcus aureus* and *Enterococcus faecalis.* Additional, in vivo studies and clinical trials is required to confirm and investigate the potential of these oils as an antimicrobial agent in tropical as well as oral applications.

CHAPTER 5

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APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121˚C for 15 mins.

1. Luria Agar Ingredients	**Amounts (g/L)**
Sodium Chloride	5.0
Yeast	5.0
Tryptone	10.0
Agar	15.0

2. Nutrient Agar (Himedia, India) Ingredients	**Amounts (g/L)**
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

3. Mannitol Salt Agar Ingredients	**Amounts (g/L)**
Peptone	10.0
Mannitol	10.0
Sodium Chloride	75.0
Lab-lemco powder	1.0
Agar	15.0
Phenol Red	0.025
4. MacConkey Agar (Oxoid, England) Ingredients	**Amounts (g/L)**
Peptone	20.0
Lactose	10.0

Sodium Chloride	5.0
Bile salts	5.0
Agar	12.0
Neutral Red	0.075

5. Mueller Hinton Agar Ingredients	**Amounts (g/L)**
Beef extract	2.0
Acid hydrosylate of casein	17.5
Starch	1.5
Agar	17.0

6. Brain Heart Infusion Broth Ingredients	**Amounts (g/L)**
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium Chloride	5.0

7. Salmonella Shigella Agar Ingredients	**Amounts (g/L)**
Beef Extract	5.0
Enzymatic digest of casein	2.5
Enzymatic digest of animal tissue	2.5
Sodium Chloride	8.5
Lactose	10.0
Bile salts	8.50
Sodium Thiosulfate	8.50
Ferric Citrate	1.0
Brilliant Green	0.00033
Neutral Red	0.025
Agar	13.5

8. Trypticase Soy Agar Ingredients	**Amounts (g/L)**
Tryptone	15.0
Enzymatic digest of Soybean	5.0
Sodium Chloride	5.0
Agar	15.0

9. Sorbitol MacConkey Agar Ingredients	**Amounts (g/L)**
Peptone	17.0
Proteose peptone	3.0
D-sorbitol	10.0
Sodium Chloride	5.0
Bile salts mixture	1.0
Neutral salts	0.03
Crystal violet	0.001
Agar	13.5

APPENDIX-II

Instruments

All the equipment used in this research work is listed below:

The important equipment used through the study are listed below: Autoclave	**SAARC**
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (100-1000µl)	Eppendorf, Germany
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Oven, Model:MH6548SR	LG, China
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Refrigerator (4˚C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Water bath	Korea
Weighing balance	ADAM EQUIPMENT™, United Kingdom