Antibiotic properties of Cedarwood Oil

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Table of Contents

Abstract

More than 2.8 million antimicrobial-resistant infections occur each year in the United States. Antimicrobial resistance is an urgent public health issue witnessed globally and we need to do what we can to prevent new resistance from developing and to stop the spread of resistance that already exists. Many plant materials such as cedarwood oil, have shown to have anti-inflammatory and antimicrobial properties. One study in particular found that *Escherichia coli, Bacillus subtilis* and *Bacillus cereus* were sensitive to cedarwood oil and showed bactericidal activity. In the current study we compared the effectiveness of steam distilled and commercially produced oil. The oil from needles and wood of a cedarwood tree were isolated by steam distillation. Antibiotic properties of the compounds were assessed in the Minimum inhibitory concentration (MIC) assay and Kirby Bauer Assay. The homemade steam distilled leaves did not demonstrate antibiotic properties. Commercial cedarwood essential oil demonstrated some antibiotic properties against *B.subtilis* and *S.epidermidis.* Commercial cedarwood essential oil did not demonstrate a significant amount of antibiotic properties against *E.coli, P.aeruginosa, & S.aureus.*

I. Introduction

Antibiotic resistance is when germs, such as bacteria, develop the ability to fight and defeat a drug that was once able to kill it. If the germ is not killed, it continues to grow and can reproduce. In just the United States alone, there have been more than 2 million infections and 35,000 deaths annually. Worldwide, there have been nearly 5 million deaths associated with antibiotic resistance (CDC, 2021). This global crisis has the potential to affect many people at any stage of their life. If antibiotics lose their effectiveness, then the general public health is at risk of not fighting off these infections.

1.Bacteria

Bacteria are microscopic, single celled, living organisms. There are millions of types of bacteria; while the majority are beneficial, others can be harmful. The bacteria found in and on your body are beneficial; these make up your microbiome.(Cleveland Clinic, 2022)
The bacteria in your gut absorb nutrients, break down food and prevent the growth of harmful bacteria. Bacteria that are harmful, can cause diseases. These are called pathogens, and they can reproduce quickly. The majority of bacteria reproduce through binary fission. A bacterium cell duplicates its DNA and divides into two parts, with each new cell receiving one copy of DNA (Britannica, 2019). While bacteria tend to thrive best in warm, moist environments, some bacteria thrive better in hotter and colder temperatures. In addition to temperature, bacteria depend on pH. While most bacteria tend to thrive in neutral or slightly acidic environments, others might need more acidic or salty conditions. Bacteria can be classified by their shape. They can be Cocci (Round), Bacilli (Rod), and (Spiral) Spirochete (Figure 1). They can also be classified by their gram status (Figure 2)(Aryal, 2023)

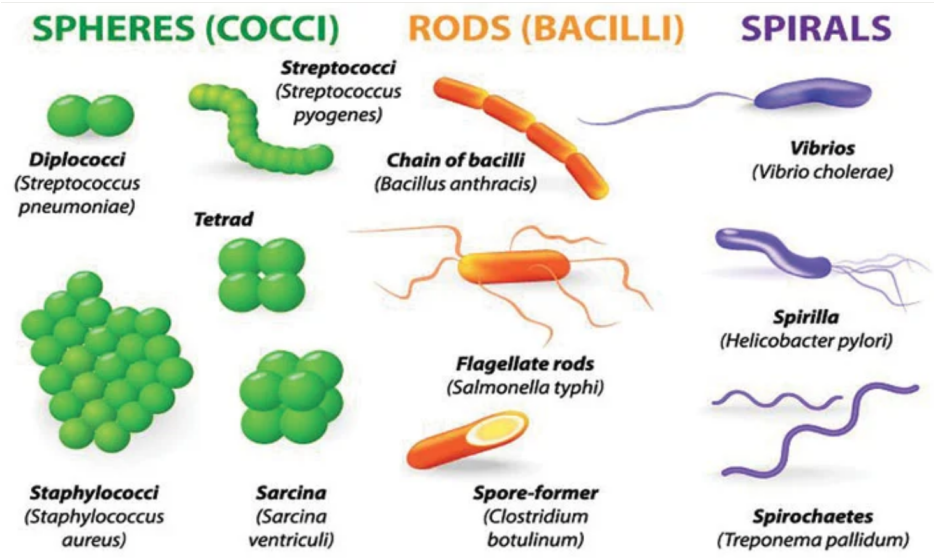


Figure 1: Classification of Bacteria on the Basis of Shape Bacteria can be found in many shapes and sizes. The most common shapes include round, rod and spiral.(Aryal, 2023)

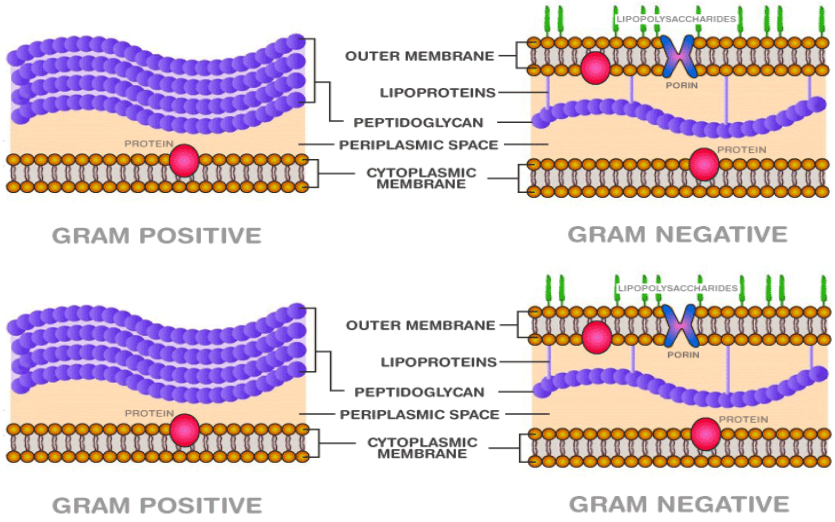


Figure 2: Difference Between Gram Positive and Gram-Negative Bacteria. Gram positive bacteria have a thicker peptidoglycan. Gram negative bacteria have a thinner peptidoglycan and have an outer membrane for protection made up of lipopolysaccharides. **(**Admin, 2022)

1.1 *Bacillus cereus*

Bacillus cereus (B.cereus) is an anaerobic, gram-positive rod-shaped bacterium (Figure 3). This bacterium is found all over the environment. It is found in soil and vegetation. Because it is found in all of these areas, it is likely to contaminate food. If ingested, it can cause intestinal illnesses such as nausea, vomiting and diarrhea. Optimal growth for this bacterium occurs in the range of 82oF (28oC) to 95oF (35oC) and a pH range of 4.9 to 9.3 (Schneider,2017).

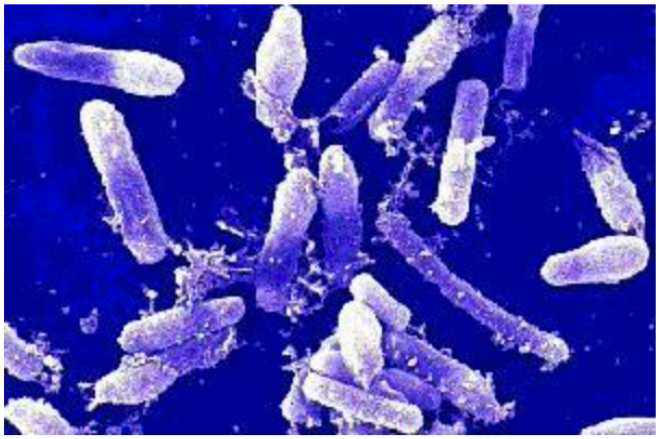


Figure 3. *B.cereus* rods. Visual of what *B.cereus* looks like under a microscope. (Michel, 2023)

1.2 *Bacillus subtilis*

Bacillus subtilis (B. Subtilis) is gram positive bacterium that is commonly found in the human gut and in fermented food (Figure 4). It is a probiotic, therefore has many benefits for our bodies such as aiding metabolic function and fighting off "bad" bacteria that can cause diseases. While *B. Subtilis* was believed to be aerobic, recent studies have found that it can grow anaerobically through nitrate or nitrite respiration (Nakano, 1998).

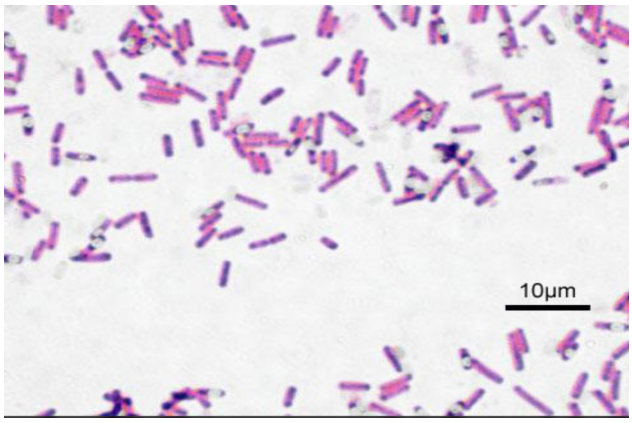


Figure 4. *B.subtilis.* Visual Size and shape of *B.subtilis* under a microscope. (Wikimedia Foundation,2024)

1.3 Staphylococcus epidermidis

Staphylococcus epidermidis (*S.epidermidis*) is coagulase negative, meaning that no clots form and it is an avirulent strain (Figure 5). It is a gram-positive bacterium that has shown to be one of the most common causes of nosocomial infections, which are infections from receiving health care. While *Staphylococcus epidermidis* lives on our skin, this bacterium can invade the human host through cuts and prosthetic devices. When this happens, the microbes travel through the device into the bloodstream. The microbes will then form biofilms, which will protect it from the host's defenses (Lee, 2023).

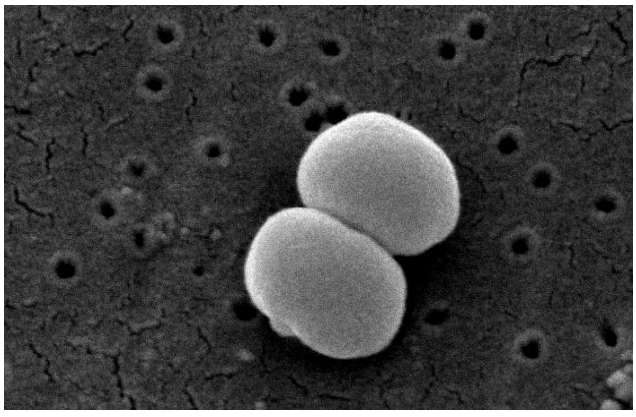


Figure 5. ***Staphylococcus epidermidis.*** Visual of *S.epidermidis* as diplococci. (Wikimedia Foundation.2023)

1.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is found in the environment such as in soil and water (Figure 6). *Pseudomonas aeruginosa* is a universally well-known gram-negative bacterium. While it is considered aerobic, it can grow anaerobically in the presence of nitrate. This bacterium is rod shaped. It is an opportunistic pathogen that is not found frequently in normal hosts; however, it is a major cause of infection with patients who are immunocompromised. They are well known for being antibiotic resistant, which is a significant problem (Krylov,2014). Infections with *P. aeruginosa* are difficult to treat because of their rise in resistance. While *P. aeruginosa* can be treated with phage therapy, there are difficulties that come with it; One being a lack of diverse virulent phages that are active against *P. aeruginosa* (Krylov,2014).

Figure 6. *P.aeruginosa* Visual of *P.aeruginosa* and flagella for movement of the bacteria. (Centers for Disease Control and Prevention, 2019)

1.5 Escherichia coli

Escherichia coli (*E.coli*) is a gram negative, rod shaped, facultative anaerobic bacterium, that can grow both with and without oxygen. (Figure 7) It can be found in contaminated water and food sources. *E.coli* is also found in the intestines of humans and animals. Human

and animal feces pollute the ground, streams, lakes, and rivers, which is how water sources get contaminated. When it comes to water treatment, water systems will use ozone, chlorine and ultraviolet light to kill the *E.coli*. When it comes to contaminated food, *E.coli* can be found in fresh produce, and undercooked ground beef. Produce becomes affected from the runoff from the cattle farms, which contaminate the fields. Ground beef can become contaminated since the *E.coli* in their intestines can get on the meat. While most types of *E.coli* are harmless, some strains can cause diarrhea, vomiting, and stomach cramps. (Mayo Clinic, 2022). While *E.coli* plays a major role in the intestinal microflora, it also contains many pathotypes, which can cause a variety of diseases. There are several different pathotypes that cause intestinal diseases, such as dysentery. Other pathotypes can cause extra-intestinal infections, such as urinary tract infections and meningitis (Kaper et al., 2004).

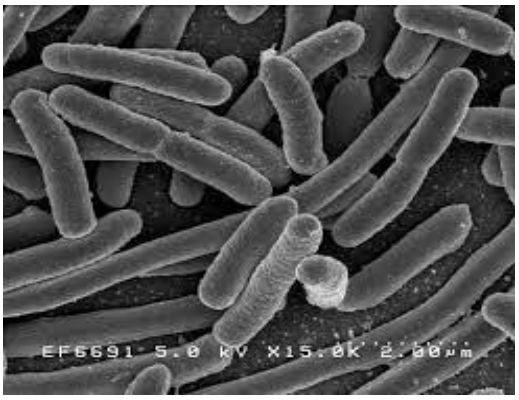


Figure 7. ***E.coli.*** Visual of size and shape *of E.coli* (National Geographic Society, 2023)

1.6 *Staphylococcus aureus*

Staphylococcus aureus (*S.aureus*) is a gram positive, cocci (round) bacterium that can grow aerobically or anaerobically. They appear in clusters that look similar to grapes (Figure 8). *S.aureus* can be found in the environment, but it can also be found on the skin and mucous

membranes such as our nose (Taylor, 2023). On the skin *S.aureus* is harmless, however if it enters the bloodstream, it can cause a variety of infections. Methicillin-resistant *S.aureus* (MRSA) causes staph infections that are challenging to treat due to antibiotic resistance. MRSA often causes skin infections. In some cases, however, it can cause pneumonia and other infections. If untreated, MRSA infections can become severe and cause sepsis (the body's extreme response to an infection) (CDC, 2019).

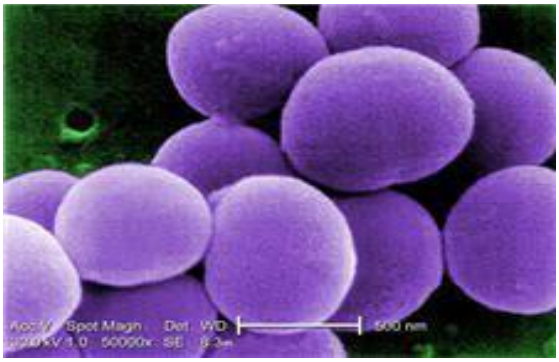


Figure 8. *S.aureus.* Visual of *S.aureus* as grape like features (Centers for Disease Control and Prevention, 2011)

2.Antibiotics

Antibiotics are medications used to treat or prevent bacterial infections. Antibiotics work by stopping the growth (bacteriostatic) or by killing the bacteria (bactericidal). Antibiotics can work by targeting certain parts that the bacteria need to survive, such as a cell wall. Antibiotics can come from natural sources, or they can be synthetic (Cleveland Clinic, 2023). Natural sources include garlic, ginger, and honey. Synthetic antibiotics include quinolones, cotrimoxazole, and sulphonamides (Libretexts, 2022). They can also come in several different forms, such as oral, topical and Injections and intravenous (IV). Oral antibiotics can include tablets, capsules, or liquids. Topical treatment is a cream or ointment applied to the skin. And lastly a healthcare provider can inject medicines through one's muscle or IV in a patient's vein (Cleveland Clinic, 2023).

2.1 Antibiotic History

Before the Antibiotic era, plants were used traditionally to treat infections. Traces of tetracycline (a class of antibiotic) have been found in human skeletal remains from ancient Sudanese Nubia dating 350–550 CE. The intake of tetracycline may have been beneficial since the infectious disease rate in the Sudanese Nubian population was low (Aminov, 2010). In the ancient Egyptian era, molds and extracts from plants were used to treat infections. Until the 19th century, it was unknown that infections were caused by microbes such as bacteria. Microbes that produced antibiotic properties were used to treat infectious diseases in Egypt, China, Serbia, and Greece. In 1550 BC, The Eber's papyrus describes treating infections with moldy bread and medicinal soils (Dutta, 2022).

Fast forward to the antibiotic era. In 1928; The Scottish physician and microbiologist, Alexander Fleming, made a huge impact in the discovery of antibiotics. He was culturing *S.aureus* and noticed mold on his petri dish. Rather than tossing the plate, he studied it. The bacterial colonies seemed to disappear the closer it was to the mold. This suggested that the mold was killing the bacteria. He then was able to identify the mold as penicillium. Penicillin was then mass produced 13 years later and made a huge impact on soldiers in World War II. (Copenhagen, Denmark, 2007)

In the 1940's a new property of antibiotics was found and had a huge impact on livestock. Tetracycline (derived from bacteria found in soil), was added to livestock feed since it had promoted growth of the animals. While Alexander Fleming warned the public about the misuse of these antibiotics, around this time, nobody listened. It was not until the 1960's that *S.aureus* was becoming resistant to penicillin. With that being said other bacteria were also developing antibiotic resistance such as *E.coli*. A committee in the British parliament

investigated to see if the use of antibiotics in livestock feed was promoting growth of resistant pathogenic bacteria. They concluded that there was a link between antibiotic usage in livestock feed and antibiotic resistant bacteria in humans (Copenhagen, 2007).

2.2 Antibiotic mechanisms

Antibiotics target bacteria by targeting their cell wall, inhibiting nucleic acid synthesis, or inhibiting protein synthesis (Figure 9). Antibiotics that target the cell wall are able to directly kill the bacteria; these antibiotics tend to include B-lactams (Strong Medicine, 2013). Antibiotics that inhibit the nucleic acid synthesis indirectly affect protein synthesis. These antibiotics will target the enzyme that transcribes deoxyribonucleic acid (DNA) into messenger ribonucleic acid (mRNA). Lastly, there are antibiotics which inhibit protein synthesis directly. These antibiotics will either target the 50s or 30s ribosomal subunits. Amino acids are unable to form chains or polypeptides, meaning they cannot make protein (Strong Medicine, 2013)

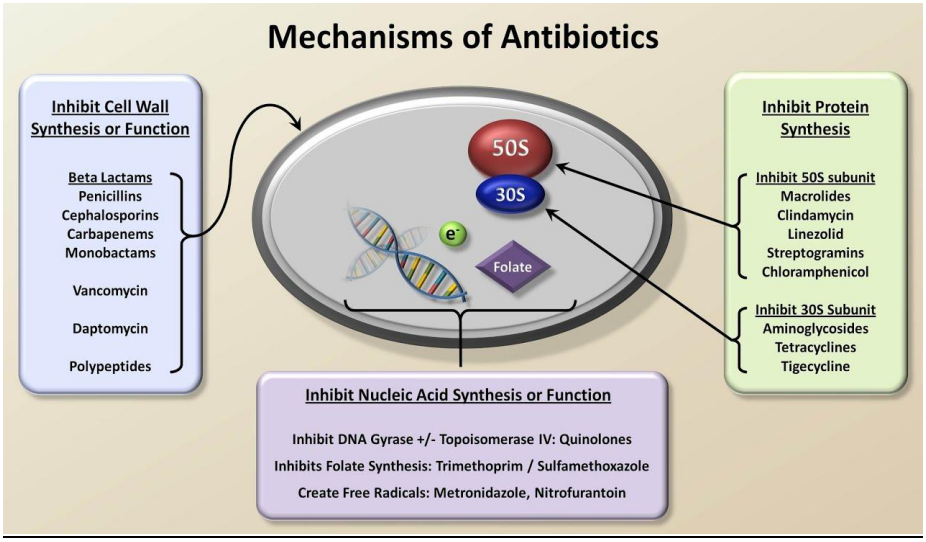


Figure 9 . Mechanisms of Antibiotics A summary of the mechanisms and classification of antibiotics (Strong Medicine,2013)

2.3 Development of Antibiotic resistance

Antimicrobial resistance occurs when microbes such as bacteria, fungi, and viruses evolve to defeat the drugs designed to kill them. The main cause of this global health concern is the misuse of antibiotics. When prescribed antibiotics it is important to take them as directed and to finish them. If antibiotics are not taken properly, then the microbe may develop defenses that will make the drug ineffective. This microbe is then able to live and reproduce (figure 10). With this bacterium reproducing, there are now more bacteria that have these antibiotic resistant traits. These bacteria are then able to transfer their genes via horizontal gene transfer or Vertical Gene Transfer (Figure 11). Vertical gene transfer is when a bacterium passes its traits onto its offspring. Horizontal gene transfer is when a bacterium transfers genes to another organism and still keeps the genes it passes on. In addition to the misuse of antibiotics, the advancement of transportation has led to the rise of antibiotic resistance. With the movement of humans, animals, and goods, bacteria are spreading worldwide and that bacteria may have the mechanisms to defend itself from antibiotics and reproduce. There are also mutations in genes that are able to change the charge of the cell membrane which could inhibit certain reagents.

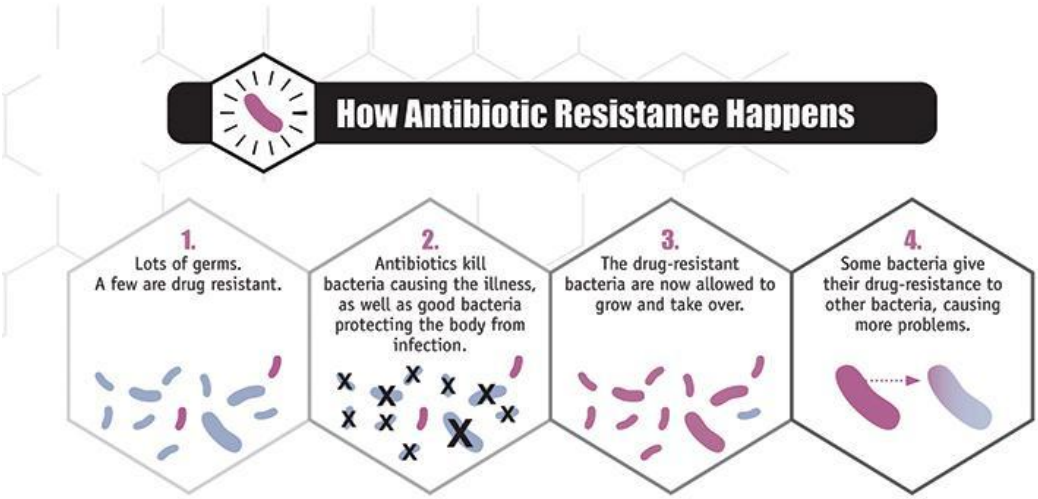


Figure 10. Resistant Bacteria Reproducing. Visual of how antibiotic resistance works and how it spreads from one bacterium to another. (MARR, 2022).

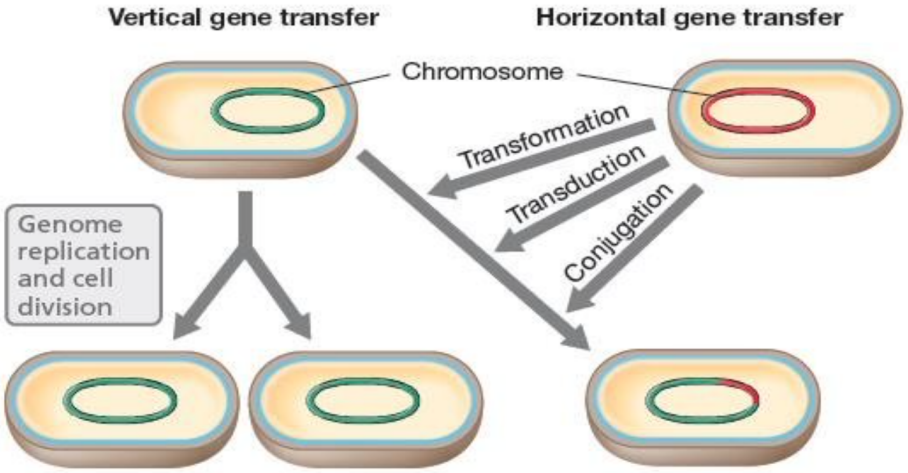


Figure 11 . Difference between Vertical and Horizontal Gene Transfer
Vertical Gene transfer is passing the gene onto its offspring through binary fission. Horizontal gene transfer is one type of bacterium giving its traits to another, while still keeping it. (Eranga, 2015).

2.4 The four types of antibiotic resistance

There are four main mechanisms for antimicrobial resistance: “Limiting uptake of a drug, modifying a drug target, inactivating a drug, and active drug efflux” (NLM, 2018). Gram

negative bacteria are at an advantage with their lipopolysaccharide (LPS) outer membrane and can take advantage of all four mechanisms. Gram positive bacteria on the other hand, lack LPS and do not have the capacity for different types of drug efflux mechanisms (Figure 12).

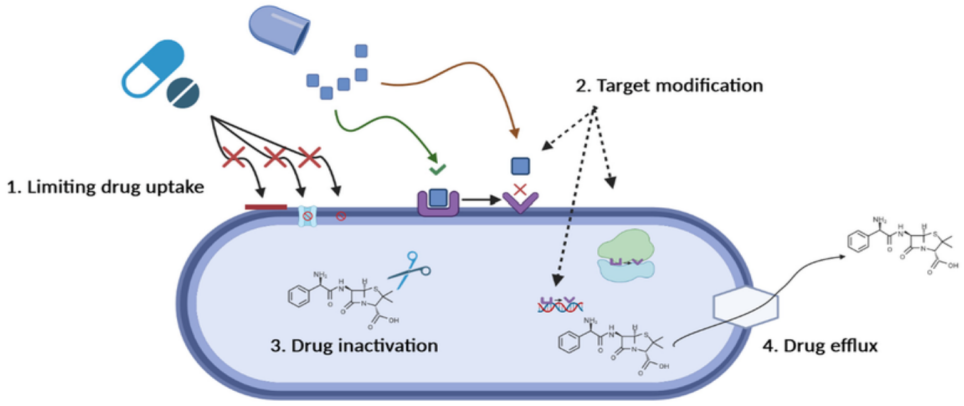


Figure 12. The Four Mechanisms of Antimicrobial Resistance 1. limiting drug uptake (thickened cell wall, porin channels, biofilms); 2. target modification (PBP modification, mutation in targeted structure); 3. drug inactivation (degrading, transferring chemical group); 4. drug efflux through active pumps.(Reygaert, 2018)

In regard to limiting uptake of a drug (Figure 12), the LPS in gram negative bacteria acts as a barrier to different molecules. This feature protects the bacteria from large antimicrobial agents. Some antibiotics can enter the cell through porin channels, which are found on the outer membrane of gram-negative bacteria. The two ways in which changes in porin can limit uptake of a drug is by mutations leading to changes in the selectivity of the channel or reducing the amount of porins (Reygaert, 2018). For pathogenic organisms, the formation of biofilms acts as a layer of protection from a host's immune response and other antimicrobial agents because it is an extra layer for the drug to go through. For the drug to reach its target, it would need to be more concentrated to get through this biofilm. Since the microbes are very close together in this biofilm, there's a chance that the resistant microbes could transfer

their antimicrobial resistant genes throughout the community, therefore making them stronger and more resistant (Reygaert, 2018).

Bacteria have the ability to modify targeted structures which could impact the ability of the drug to bind (Figure 12). These modifications could act as mutations in ribosomal subunits, nucleic acid synthesis, and metabolic pathways (Reygaert, 2018). Ribosomal mutation can affect the ability of the drug to bind to the ribosome, making it resistant. Resistance can also be found in Gram positive bacteria, in which they can change the structure or number of Penicillin binding proteins (PBP), which is used in the resistance of β-lactam drugs (Reygaert, 2018). Changing the number or structure of PBP can affect the amount of drug that can bind. Mutation in any structure that is being targeted is at risk of antibiotic resistance, for the drug cannot bind (Reygaert, 2018).

Inactivation of the drug is the third mechanism for antimicrobial resistance. The two main ways in which a drug is inactivated is by degrading the drug or transferring a chemical group to the drug (Figure 12). Bacteria are able to produce degrading enzymes that can destroy the molecular structure of antibiotics by modification or hydrolysis. Several studies have found that antibiotic degrading enzymes typically include these four types: β-Lactamase, aminoglycoside modifying enzyme, macrolide passivase, and chloramphenicol inactivating enzyme (Yang et al., 2019). An example of transferring a chemical group would be with the drug, tetracycline. This drug can be inactivated by the *tetX* gene. This mechanism uses chemical group transfer and is achieved by transferring the adenyl and acetyl groups to the drug.

Drug efflux systems are the fourth mechanism microbes use for resistance and are activated by environmental signals (Figure 12). This system pumps solutes out of a cell. Gram negative

bacteria can regulate their internal environment by removing the toxins, such as antimicrobial agents (Reygaert, 2018).

2.5 Botanical sources of antibiotics

The use of plants in medicine was initiated centuries ago; biological activity showed benefits to the health of humans as well as antimicrobial, anti-inflammatory, and antioxidant properties. Traditionally, plants have been used to prevent or cure infections; Plants are rich in a wide variety of secondary metabolites. Secondary metabolites do not have a direct role or function in the growth of the plant; however, they aid in plant defense and ecological interactions. The plants are then able to defend against herbivores and use other interspecies defenses. Their antibiotic, antifungal and antiviral properties protect the plant from pathogens. Plant secondary metabolites are classified by their chemical structure and can be divided into four major classes: terpenoids , Phenolic compounds, polyketides, and alkaloids (Cowan, 1999).

Terpenoids are the main bioactive compound found in essential oils. They possess anti-inflammatory, antibacterial, and anticancer activities (Masyita et al., 2022). They are divided into alcohols, esters, aldehydes, ether, ketones, epoxides and phenols. Examples include: carvacrol, linalyl acetate, piperitone, and menthol (Masyita et al., 2022).

Phenolic compounds have a common chemical structure. These are composed of an aromatic ring with at least one hydroxyl substituents that are subdivided into classes. The main groups of phenolic compounds are flavonoids, phenolic acids, tannins, stilbenes, and lignans (ScienceDirect Topics, 2019). Bioactive properties of phenolic compounds have been studied over the years. The most significant biological activity they have is inhibitory effects on mutagenesis and carcinogenesis (Chi-Tang Ho, 1992).

3. Origin of Cedar Trees.

Cedrus, commonly known as Cedar are many species of trees that belong to the family Pinaceae (Figure 13). These trees are native to the mountainous regions of the Mediterranean Basin and Western Himalayas. The genus *Cedrus* is categorized into four different species which include: Atlas Cedar, Cyprus Cedar, Deodar Cedar, and Lebanon Cedar. In the United States, some plants are described as cedar; these are called “false cedar” (Figure 14). These “false cedars" are categorized into many families which include: Bignoniaceae, Caryophyllaceae, Cephalotaxaceae, Cupressaceae, Lycopodiopsida, Meliaceae, and Surianaceae. Junipers are evergreen, coniferous plants. Though they can grow as trees, they are mostly shrubs.(Figure 15)

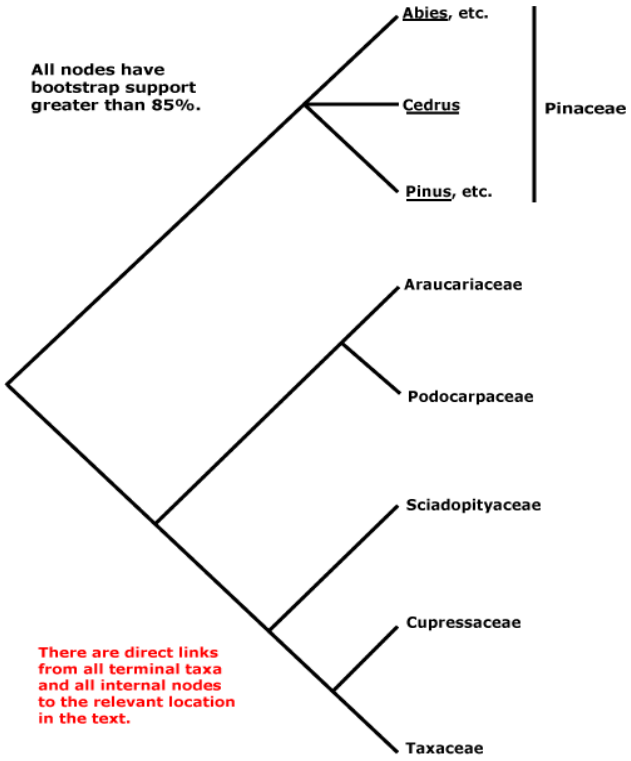


Figure 13 . Phylogenetic Tree of True and False Cedar. The Pinaceae family consists of true cedars and the rest are families of false cedars. For this research we are studying a species of cedar from the Cupressaceae family. Nix, S. (2022, May 23)

Junipers tend to have berry-like cones on the tips of their shoots. In addition, some have spiny needle-like leaves. Juniper trees tend to resemble narrow columns once fully developed (Figure 15). *Virginiana (Easter red cedar)* is *Juniperus*. It is the most common juniper in eastern North America.

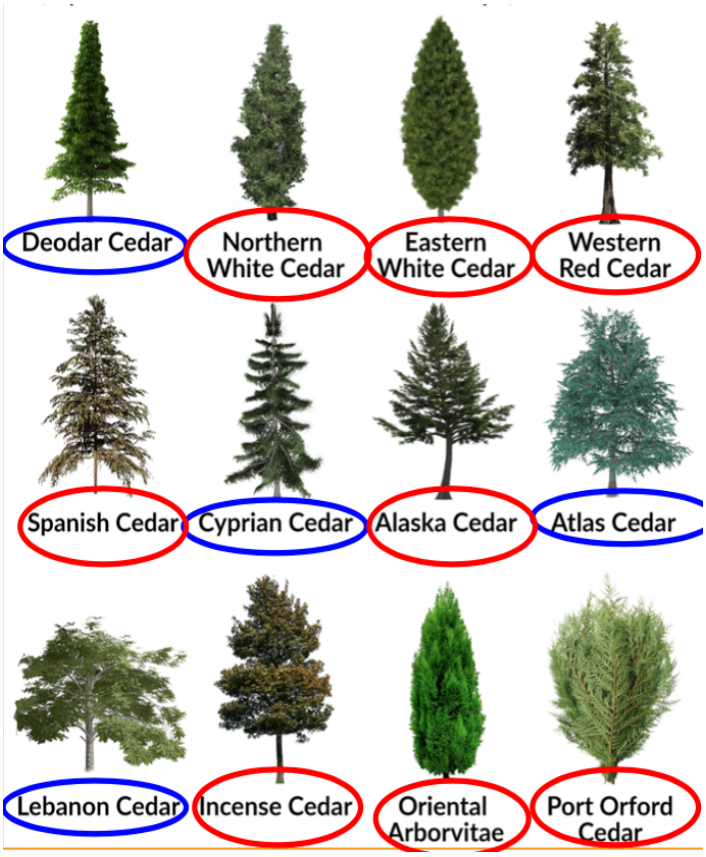


Figure 14 . Types of Cedar Trees. The trees circled in blue are the True cedars. The trees circled in red represent the false cedars.(Coniferous Forest, 2024)

3.1 Traditional Uses For Cedarwood

The cedarwood tree is known for its significance in ancient culture. It was desired for its aromatic qualities and its repellency against insects.

It has been used in perfumery for centuries. Lebanon cedar is referenced in the bible many times, but more specifically, when building King Solomon's temple and sealing David's house. Cedarwood is therefore a symbol for strength and purification. Cedarwood was also used as a base for paints by the ancient Sumerians. Ancient Egyptians used cedarwood oil in their embalming practices and wound healing (Amorós, 1998). Native American tribes and Tibetan cultures had used cedarwood oil in folk medicine for centuries. More specifically, they used it for respiratory ailments and wound healing. Today, cedarwood essential oil has several purposes. It is typically used in cologne, deodorant, shampoo, candles, insect repellent, and also aromatherapy (Team, 2022). Cedarwood oil makes good use as a pesticide. In 1960, the Environmental Protection Agency registered cedarwood oil as a pesticide that repels moths from your home, and a compound found in cedarwood oil called cedrol can repel ticks (WebMD, 2024).

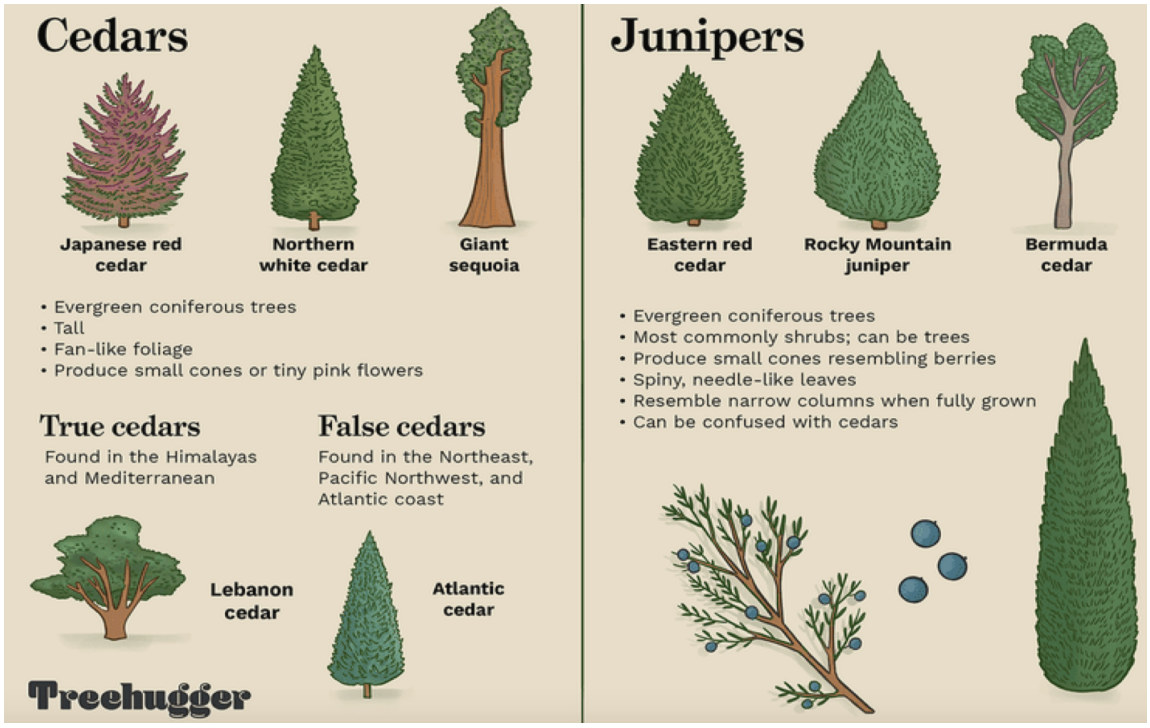


Figure 15 . Difference between cedar and Junipers. Description and visual differences between true cedars and false cedars. (Nix, 2022)

3.2 Cedarwood In Modern Medicine

Physically, cedarwood oil has many benefits. One study found cedarwood oil along with other essential oils treated alopecia, a disease which causes hair loss (Hay,1998). One case study found cedarwood essential oil to improve acne that was resistant to treatment (WebMD,2024). Another study found cedarwood oil can ease pain and inflammation on rodents. In this study, procedures were performed to identify and extract the chemical composition of the essential oil obtained from *Cedrus atlantica* wood. The medicinal properties from the oil were evaluated by determining the pain alleviation and anti-inflammatory properties. In addition to this, the assessment of toxicity was assessed in rodents (Kamaly et al., 2022). Along with physical aspects, cedarwood oil has many benefits for stress and anxiety relief (Zhang et al., 2018). Cedrol, a compound in cedarwood essential oil, was identified through gas chromatography/mass spectrometry. It produced significant anxiolytic effects in mice (Zhang et al., 2018).

The common compounds in the Virginia and Texas oils are cedrol, alpha-cedrene, and thujopsene. The relative percentages vary depending on the origin of the cedar trees used to produce the oil. Western Red cedarwood oil contains methyl thujate and thujic acid. (Figure 16)Cedrol is a sesquiterpene alcohol found in the essential oil of conifer; evidence supports that it reduces stress and anxiety, while also improving sleep. One study found that inhaling cedrol had sedative effects (Kagawal et al., 2003).

Component	CAS No.	Texas Oil (%)	Virginia Oil (%)	Western Red Cedarwood Oil (%)
Thujopsene	470-40-6	60.4	27.6	-
Cedrol	77-53-2	19.0	15.8	-
∀-Cedrene	469-61-4	1.8	27.2	-
∃-Cedrene	546-58-1	1.6	7.7	-
∀-Copaene	3856-25-5	2.8	6.3	-
Widdrol	6892-80-4	1.1	1.0	-
Methyl thujate		-	-	65
Thujic acid		-	-	25
∃-Thujaplicin	499-44-5	-	-	1
∀-Thujaplicin		-	-	1

Source: Lawrence, 1993; Laurel Laboratories, Inc., 2002; Mookherjee & Wilson, 1996

Figure 16 . Chemical Composition of Cedarwood Oils The composition of cedarwood oils varies depending on the species. Texas and virginia oil contain the same compounds, but Western Red Cedarwood Oil contains different compounds.(Lawrence, 1993)

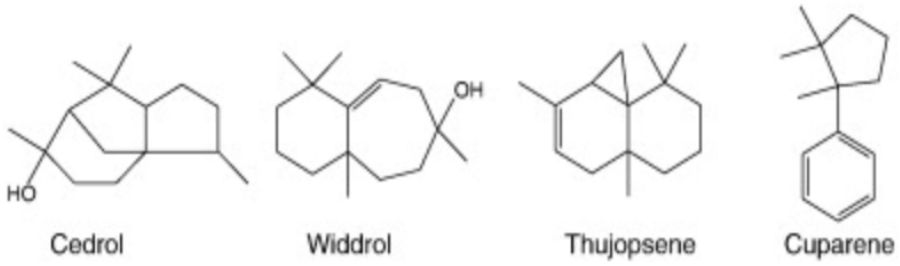


Figure 17. Molecular structures of Compounds Found in Cedarwood Essential oil
These structures are classes of terpenes and terpenoids, which are secondary metabolites. These secondary metabolites play a role in holistic medicine.

3.3 Previous Studies of Cedarwood Oil

Research has found that atlas cedar essential oil has antibiotic activity against some bacteria such as *Eschericia coli.* In this experiment cedarwood (*Juniperus ashei*, Cupressaceae) from

Texas was tested along with Cabreuva oil, Juniper berries, and Myrrh against eleven various strains of Gram-positive and Gram-negative bacteria by using agar diffusion and agar serial dilution methods. The oils showed high inhibitory effects against all tested organisms, except *Pseudomonas*. There was higher activity against Gram-positive strains compared to Gram-negative bacteria. Cabreuva oil from Brazil had similar results, but in comparison with the other oils tested, only when higher concentrations of oil were used. They concluded that because Cabreuva needed more concentration to be as effective, that the other essential oils were more effective against the different strains of bacteria (Wanner et al., 2010).

A separate study was designed to evaluate commercial cedar essential oil (CEO) (Atlantic cedar), retrieved through hydro distillation from cedarwood, in relationship to its chemical composition and antioxidant, *in vitro* and *in situ* antimicrobial, antibiofilm, and anti-insect activity (Kačániová et al., 2022). Results showed that gram-positive bacteria had inhibition zones with *Staphylococcus aureus* (*S. aureus*) and *Micrococcus luteus* (*M. luteus*). These were found to be more sensitive to cedarwood essential oil compared to the gram-negative bacteria, Salmonella enterica (*S. enterica*). The data also revealed that the main components of the Essential Oil were "δ-cadinene, (*Z*)-β-farnesene, β-himachalene, viridiflorol and himachala-2,4-diene."(Kačániová et al., 2022).

4. Extraction Methods

Steam distillation is a method for extracting essential oils (Figure 18); the process consists of distilling water with volatile compounds (Sruthi et al., 2023). A round bottom flask with water and biomass is heated with a hot plate. The volatile compounds come from the raw material vaporize with steam (Sruthi et al., 2023). The vapor then cools and condenses,

which makes it immiscible with water. This process is used most when generating commercial essential oils (Sruthi et al., 2023).

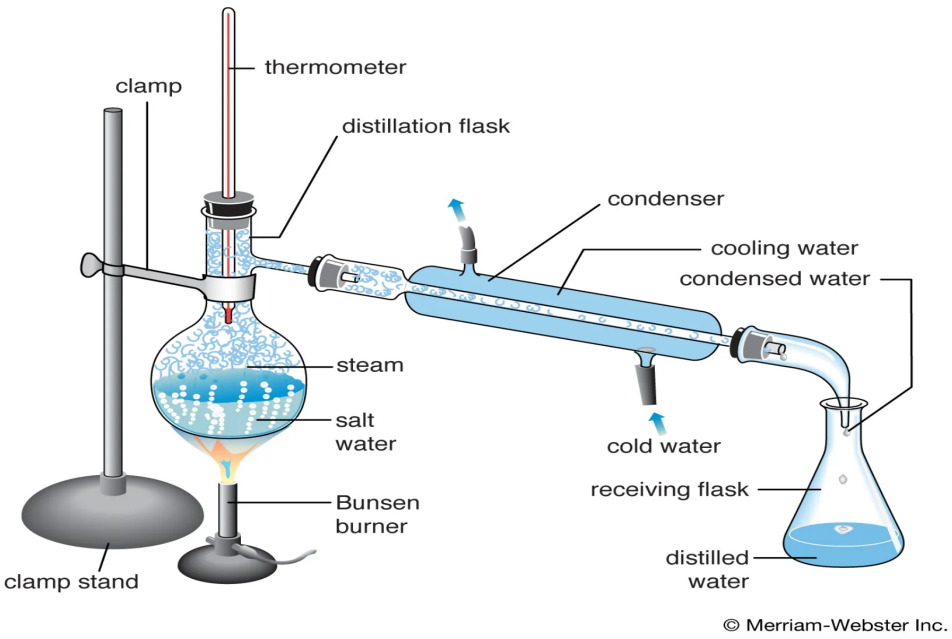


Figure 18. Steam Distillation apparatus . (Britannica,2006)

4.1 Differences between homemade steam distilled leaves and commercial cedarwood oil

Commercial cedarwood essential oil (CCEO) is steam distilled from sawdust and, or wood chips of various species of cedar trees. The variety of cedarwood trees also brings its own essential oils. The essential oils can come from: Atlas Cedarwood *(Cedrus atlantica);* Chinese cedarwood (*Chamaecyparis funebris*)*;* Himalayan cedarwood (*Cedrus deodara);* Port Orford cedarwood (*Chamaecyparis lawsoniana);* Texas cedarwood (*Juniperus ashei);* and Virginian cedarwood (*J. virginiana)* (WebMD, 2021). These essential oils also are known as other common names. The most common cedarwood essential oil in the United States is the Virginian cedarwood, which is also known as the Eastern red cedar. Though Atlas Cedarwood is also a very common essential oil, it is declared an endangered species by

The International Union for Conservation of Nature (WebMD, 2021). With that being said, one is still able to harvest and sell these essential oils, but it is recommended it comes from a sustainable source.

In this research, the homemade steam distilled leaves (HSDL) of the cedar tree are sourced from one species of cedar tree, and more specifically the leaves. When it comes to commercially available cedarwood essential oil, the wood chips can come from the bark and branches. While most cedarwood essential oils specify the species of tree it comes from, some do not.

To better understand the antibiotic properties of cedarwood oil, I wanted to research the effectiveness of CCEO and HSDL, against various gram-positive and gram-negative bacteria. There are many sources of cedarwood oil coming from both true and false cedars. With this research, I wanted to observe the potential antibiotic properties from the leaves of an Easter Red Cedar as well as CCEO.

II. Materials and Methods

Bacteria, media and culture conditions

Reagent plates were made using Mueller Hinton Agar and Nutrient agar. To make the Nutrient Agar, 23 grams of the medium was weighed and added to a flask with 1000 ml of deionized water. The flask was placed in the autoclave and for about one hour. Afterwards the medium was poured into sterile plates. To make the Mueller Hinton Agar, 38 grams of medium was added to a flask that had 1000 ml of Deionized water. The mixture was placed in the autoclave for approximately an hour, and then poured into sterile plates. These plates were stored in the refrigerator until used.

Bacillus Subtilis (*B. subtilis*) (Lot # 15-4921A), Bacillus Cereus (B.cereus), *Staphylococcus aureus (S. aureus), and Pseudomonas aeruginosa (P. aeruginosa)*, were obtained from Carolina Biological Supply Company. These bacteria arrived in a dry powdered form. 2 ml of nutrient broth (NB) was added to the dry powder form of the organism. After mixing, it was transferred to a sterile test tube containing 3 ml of nutrient broth (NB). This totaled 5 ml all together. The test-tube was then placed in a shaker for approximately 18-24 hours for the bacteria to grow. The bacterial cultures were then streaked on nutrient agar (NA) plates to retrieve colonies. The NA plates with the microorganisms were placed in the incubator at 37° for approximately 18-24 hours. The isolated colonies were then inoculated with a sterile loop and placed into test tubes containing 5 ml of NB. These test tubes were placed in the 37°C shaker for 18 hours. This was necessary to isolated colonies for further testing. Cultures were used immediately.

Escherichia coli (E.coli) (Lot # 124500) *and Staphylococcus epidermidis (S.epidermidis) (Lot # 15-5556)* were obtained as a slant tube from Carolina Biological. A sterile inoculating

loop was used to inoculate the microorganisms into a test tube containing 5 ml NB. The test tube was then placed in a shaker for approximately 18-24 hours for the bacteria to grow. The microorganisms were then streaked on a nutrient agar plate to isolate individual colonies. The nutrient agar plate with the microorganism was placed in the incubator at 37°C for approximately 18-24 hours. The isolated colonies were then inoculated into sterile tubes with nutrient broth and were placed into a shaker for about 18-24 hours for the isolated colonies to grow & be used for further testing. Cultures were used immediately.

Steam Distillation

Plant materials were extracted via steam distillation. Apparatus was set up accordingly. 25.02g of cedarwood chips from eastern red cedar was weighed out and added to a round bottom flask with 3 boiling stones. 300ml of distilled water was added to the round bottom flask. Hot Plate was Turned on and water began to boil after an hour. First 10 drops were dispensed, and 50 ml of material was collected . Process was repeated with the HSDL. The Leaves were picked from an Eastern Red Cedar. The leaves were weighed to 25.00g and added to a round bottom. Hot Plate was turned on and water began to boil after an hour. First 10 drops were dispensed, and 50ml of material was collected. Each collection tube was labeled accordingly.

Hexane Extraction

A hexane extraction was used to isolate pure oil from the HSDL. Hexane was completely miscible with the oil and had low toxicity. It also did not produce strong odors, and had a low boiling point, which made it an ideal solvent. 50 ml of steam distilled cedarwood oil from wood chips was added to a separatory funnel along with 30 ml of hexane. Stopper was added on top, and the contents were mixed together and gas was released after every 10 shakes by

opening the stopcock. The separatory funnel was placed back onto the stand and waited to see a clear distinction between the two layers. The aqueous layer was released into an Erlenmeyer flask by opening the stopcock. After all of the aqueous layer was released; Closing the stopcock the organic layer was poured out from the top into a separate Erlenmeyer flask. The contents evaporated until the pure oil was left. Measurements were taken of leftover material. The process was repeated with the HSDL.

Preparation of test disks

The test disks were then prepared with oil from the HSDL, methanol, and the CCEO. A sterile pipette was used to draw up 10u of material and was added to a single sterile filter disk. This step was repeated for each disk.

Kirby Bauer

To test the antibiotic properties of different compounds through diffusion, a Kirby Bauer procedure was followed. *Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa*, *Staphylococcus epidermidis, Staphylococcus aureus, and Escherichia coli* were grown and used in their log growth stage. Three trials of Kirby Bauer were conducted to test the antibiotic properties of each compound. Methanol served as a negative control, and Ciprofloxacin served as a positive control. Sterile technique was used to add 150 ul of each bacterial culture to the center of agar plates. The distribution of the bacteria over the surface of the plate was accomplished using a sterile glass rod. The plates were then covered and set aside to allow the bacterial media to absorb into the agar. After 15 minutes, test disks and positive and negative control disks were added to each plate. 3 plates prepared for each bacterial culture, totaling 18 plates. Afterwards the plates were incubated at 37 Celsius for

24 hours, and the zone of inhibition was measured. Single factor anova was used to analyze the data.

Minimum Inhibitory Concentration (MIC) assay

MIC assay was used to determine the susceptibility of each type of bacteria to the compounds. This test measured the concentration at which bacteria began to grow. The compounds dissolved into the bacteria directly instead of diffusing through a media. Using Falcon 48-well plates,100µl of buffer was added to all wells in columns #1-7. 200µl of buffer was added to the wells of the last column (#8). 100µl of each compound was serially diluted in wells 1-7. Well 8 for each row served as a control, since it only contained the nutrient broth. Subsequently, 100µl of bacteria were added to each well. Bacteria was a 1:10 dilution of 18-hour culture. Each plate was labeled to differentiate between which compounds and bacteria were being used. Three plates were labeled "A", "B", or "C". Plate A was tested with *B.Subtilis* and *S.epidermidis*. Plate B was tested with *S.aureus* and *E.coli*. Plate C was tested with *P.aeruginosa*. The rows and columns of the plates were further differentiated with the compound that was added. The rows were labeled on the side of the plate as 'M' for Methanol, 'C' for Cipro, 'L' for HSDL, and 'O' for the CCEO. The well plates were incubated for 18-24 hours at 37°C. With a sterile inoculating loop, nutrient agar plates were streaked and then incubated for 18-24 hours at 37°C. To measure susceptibility, bacteria either grew from the streak, or there was no bacterial growth.

Polymerase Chain Reaction

A polymerase chain reaction (PCR) was performed on the bacteria to amplify the ribosomal 16s gene directly from a bacteria colony. By amplifying the DNA, the segment was compared with a database to identify the bacteria Five microtubes were labeled with their

corresponding bacteria and one microtube was labeled “C”, for the control. 20 µl of nuclease-free (NF) water was added to each tube. Using a sterile pipette tip, a bacterial colony was collected by lightly touching it with the pipette tip. The bacteria was then added to the tubes with the NF water. Bacteria was not added to the control tube. In separate, labeled microtubes the following were added in this order: 50 µl of Master Mix, 2 µl of 515 F. Primer , 2 µl of 1492 R Prime (Table 1), 1 µl of cells (with the exception of the control tube), 45µl of NF water to the tubes with cells, and 46µl to the control tube.

After the ingredients were added, a microcentrifuge was used to spin the samples down to the bottom of their tubes. The samples were then placed in a thermocycler for 3.5 hours and ran for 30 cycles. The samples go through intervals of denaturing, annealing, and elongation. After this process, the samples were stored at 4 C°

Table 1. Sequences of primers used in PCR

Sequence	Primer
5’ -GTTACCTTGTTACGACTT-3’	Short 515 F Primer
5’- GTGCCAGCMGCCGCGGTAA-3’	Short 1492 R Primer
5’ - GGCTCGAGCGGCCCTTGTTACGACTT-3’	Long 515 F Primer
5’-GCGGATCCTCTAGGCAGCCGCGGTAA-3’	Long 515 F Primer

Gel Electrophoresis

PCR was performed on the bacteria to amplify the ribosomal 16s gene directly from a single colony of all the bacteria. These samples were then analyzed by the gel electrophoresis. This process was used to separate the amplified DNA of the microorganism by their molecular size. The molecules moved through an electrical field; the smaller the molecules moved further in the gel. This method allowed visualization and analysis of the PCR products. This method indicated how large the DNA fragments were to one another. The first step was to

prepare a 1% Tris-acetate-EDTA (TAE) agarose electrophoresis gel. The mixture in the flask was microwaved until there were no particles. The mixture was cooled until the flask could be held without it burning. 1µl of Ethidium Bromide (EtBr) was added to the flask. With parafilm on top, the flask was shaken and then added to the gel electrophoresis tray. A comb was added to create 10 wells. The gel was then cooled and solidified for 45 min. It was then covered with approximately 300 ml of a 1% TAE buffer with 3µl of EtBr added to it. 5µl of each of the PCR samples was added to microtubes with their corresponding labels along with 2µl of 6X dye. A 1 Kb ladder was used to verify the size of the bands and the relative amount of product. 5µl of the ladder was added to a microtube with 2µl of 6X dye. Each of these samples were loaded into the agarose gel wells. Well 2 contained the ladder, well 3 contained *S.aureus*, well 4 had *E.coli,* well 5 had *S.epidermidis*, well 6 contained *B.subtilis*, well 7 had *P.aeruginosa*, and well 8 contained the control. After loading these samples, the test ran for about 30 min-1 hour at about 85-93 volts. Lastly, a UV light was used to look at the DNA fragments.

PCR Clean Up

PCR cleanup was performed to purify the DNA of the PCR sample and remove the unwanted components so that it could be sequenced and compared to the database. The QIAquick PCR Purification Kit was used, and the directions were followed according to the manual.First, ethanol (96-100%) was added to the Buffer PE. 95µl of the PCR sample of the bacteria was added into separate, labeled microtubules. Following this, 475µl of phosphate buffer (PB) was added, this made it a 1:5 ratio. The dye indicator was not added. The QIAquick column was placed into a 2 ml collection tube and centrifuged for approximately 30 seconds to bind the DNA.

Once the samples passed through the filter, the flow-through was discarded. The column was then placed back into the collection tube. 750 microliters of Buffer PE was added to the QIAquick column and centrifuged for approximately 30 seconds to wash the DNA. The flow-through was discarded, and the column was placed back into the tube. The column was centrifuged for one minute to remove the remaining buffer. Afterwards, each column was placed into a new 1.5 ml microcentrifuge tube. 50 microliters of distilled water are added to the center of the QIAquick membrane, allowed to stand for one minute, and then centrifuged to elute the DNA. Finally, these samples were sent in for sequencing to identify the organisms.

Gram Staining

A gram stain procedure was used to identify the bacteria used in the experiment; this ensured that the bacteria used in the experiments were a match to the ones grown from Carolina Biological Supply Company . This identification process was a way to identify the gram status and shape of the bacteria. If the bacteria were purple, it indicated that it was gram-positive. If the bacteria showed up as pink or red, then the bacteria were gram negative. Bacteria can appear as round, rod-shaped, or spiral-shaped.

Using a sterile inoculation loop, a loopful of sterile water is placed on a slide and spread in the center. Following that, a colony of bacteria from a streak plate is added and mixed with the water on the slide. The slide is then dried until and is heat-fixed by passing it through the Bunsen burner flame three times. Afterwards, crystal violet is applied to the slide and stays on for 60 seconds. It is then rinsed off with deionized(DI) water. Iodine is applied to the slide and rinsed off after 60 seconds. Ethyl alcohol is then used to rinse the slide for 5-10 seconds, DI water is used to rinse the alcohol. Following this, Safranin is applied to the slide, and left

on for 45 seconds. DI water is used to rinse the Safranin. These steps were repeated for all of the bacteria. After the slides dried, they were viewed under a microscope under 100x magnification (10x lens). An immersion oil was added to the slide and viewed under 1000x magnification (oil immersion lens). This allowed us to view the shape of the bacteria.

III. Results

3. 1. Antibiotic Tests

3.1.1 Kirby Bauer Assay: *B. Cereus*

One trial of Kirby Bauer was conducted to test the antibiotic properties of CCEO and the HSDL against *B.cereus*. Cipro was used as a positive control and methanol was used as a negative control. The average ZOI for cipro was 16.67mm with a standard deviation of 0.94. No ZOI was observed for HSDL, CCEO, and methanol (figure 19).

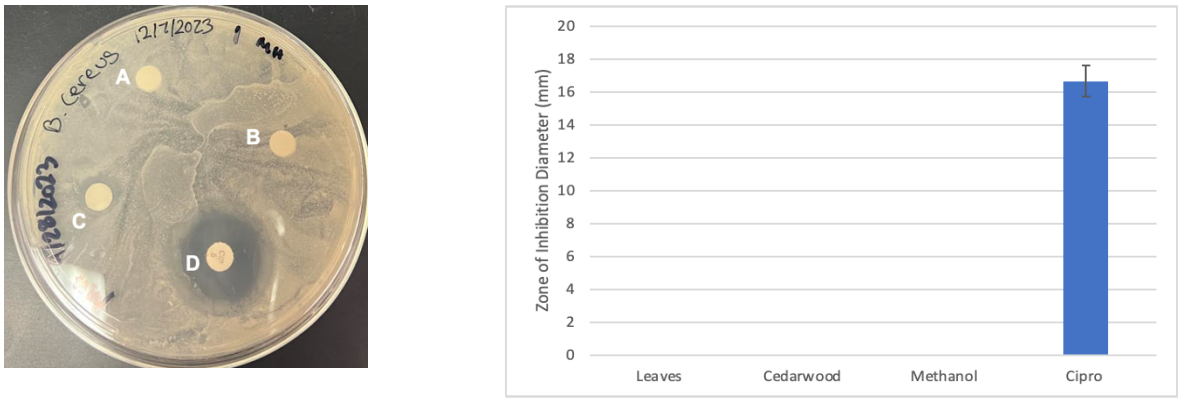
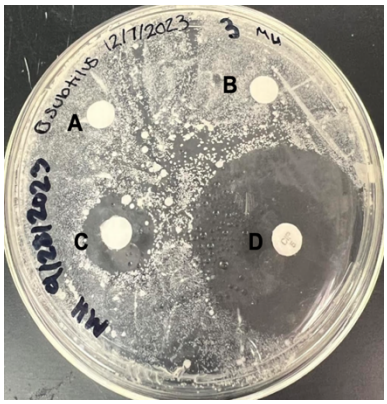


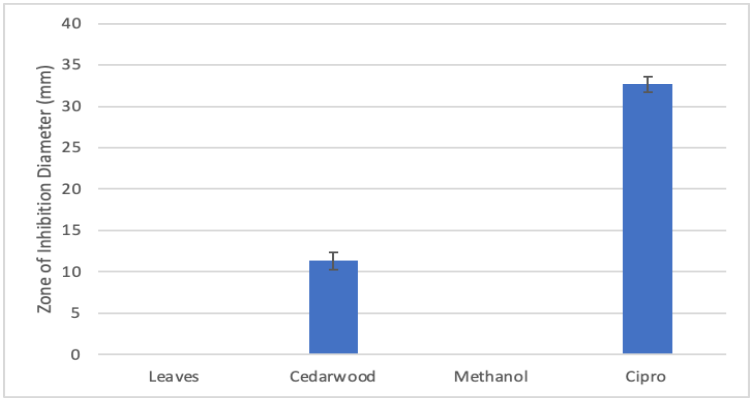
Figure 19.Trial 1 Kirby Bauer *B. cereus* A. Disk A= HSDL; B= methanol; C=commercial essential oil; D= Cipro respectively. The ZOI for disk D=16mm. ZOI for disks A,B, and C= 0mm. **B. Average Zones of inhibition with *B. cereus*** The X-axis represents which compound was used and the y-axis is the Zone of inhibition in mm. Standard deviation was measured. Anova single factor was used to analyze the data. $p < 0.0001$, which is statistically significant. The average ZOI for Cipro in trial 1=16.6667mm +/- .9428.

3.1.2. Kirby Bauer Assay: *B. subtilis*

Three trials of Kirby Bauer were conducted to test the antibiotic properties of CCEO and the HSDL against *B. subtilis*. Cipro was used as a positive control and methanol was used as a negative control. In trial 1, The average ZOI for cipro was 32.67mm with a standard deviation of 0.94. The CCEO had a mean of 11.33mm and a standard deviation of 1.03 No ZOI was observed for the HSDL and methanol (figure 20). In trial 2, the mean for cipro was 21.67 with a standard deviation of 0.58. The mean for the CCEO was 3 mm with a standard deviation of 5.2. No ZOI was observed for the HSDL and methanol (figure 21). In trial 3 the

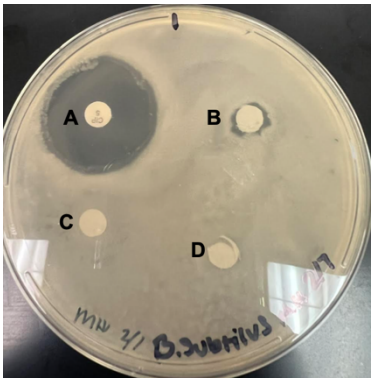
average ZOI for cipro was 26.67mm with standard deviation of 0.47. Again, no ZOI was observed for HSDL, CCEO, and methanol (Figure 22).

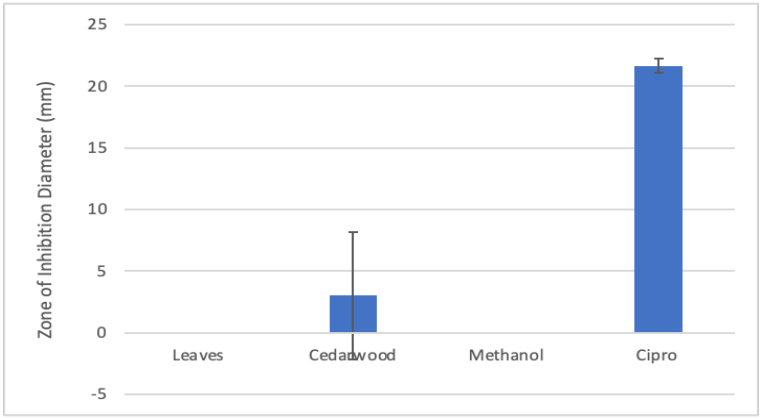




A **B**

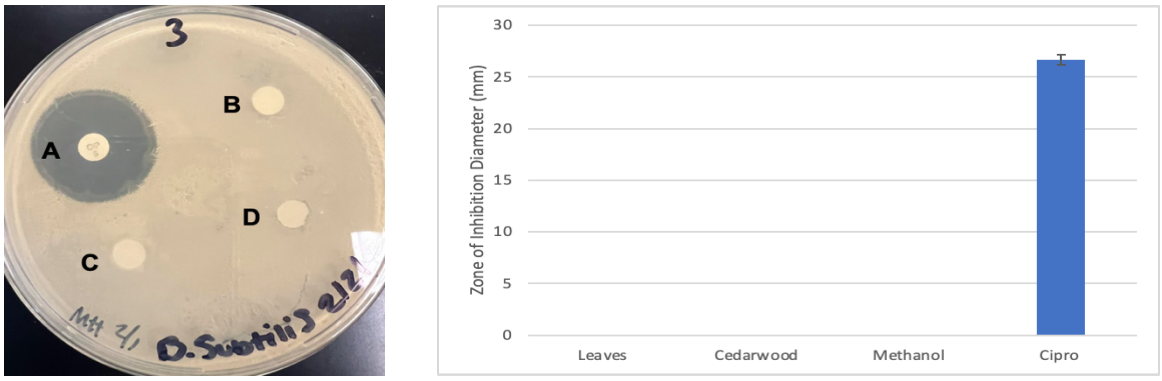
Figure 20.Kirby Bauer: Trial 1 *B. subtilis.* A. Disk A= HSDL; B= methanol; C=CCEO; D= Cipro. The ZOI for disks A and B= 0mm. The ZOI for disk C= 11.5mm. The ZOI for disk D= 34mm. **B.** This graph measures the average zones of inhibition for *B. subtilis* in trial 1..The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 1.36E-10, which is statistically significant. The average ZOI for the CCEO is 11.3333mm +/- 1.0274. The average ZOI for cipro is 32.6667 +/- 0.9428.





A. **B.**

Figure 21.Kirby Bauer Trial 2 *B. subtilis.* A. Disk A= Cipro; B= CCEO; C=methanol; D= HSDL.The ZOI for disks A= 22mm; B= 9mm. C= 0 mm; D= 0mm.**B.**The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 1.884E-05, which is statistically significant. The average ZOI for Cipro= 21.6667 +/- 0.5774 ; The average ZOI for the CCEO is 3 +/- 5.1962 . The average ZOI for the HSDL and methanol are 0.

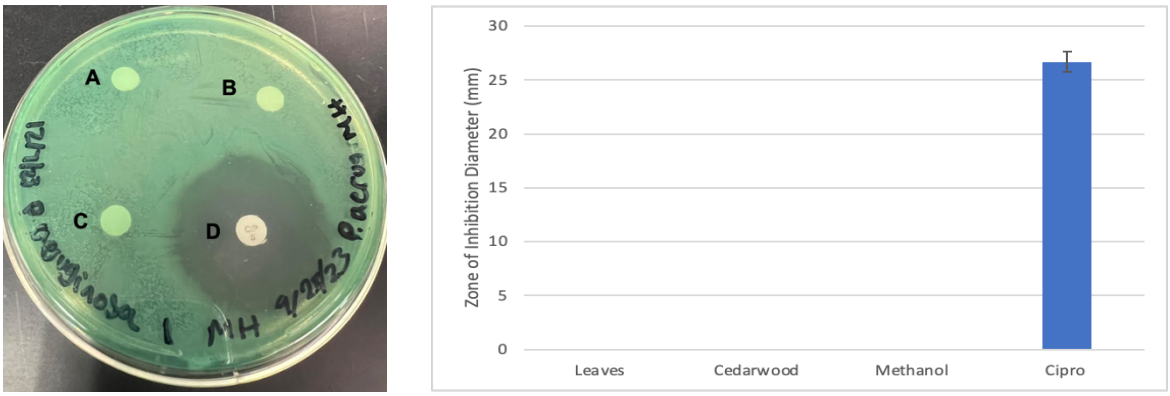


A. **B.**

Figure 22. Kirby Bauer Trial 3 *B. subtilis.* **A.** Disk A= Cipro; B=HSDL; C=Methanol; D= CCEO. The ZOI for disk A = 24mm; B= 0mm; C= 0mm; D= 0mm.**B.**The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 7.4039E-14, which is statistically significant. The average ZOI with cipro is 23.6667 +/- 0.4714 . The average ZOI for the HSDL, CCEO and methanol are 0.

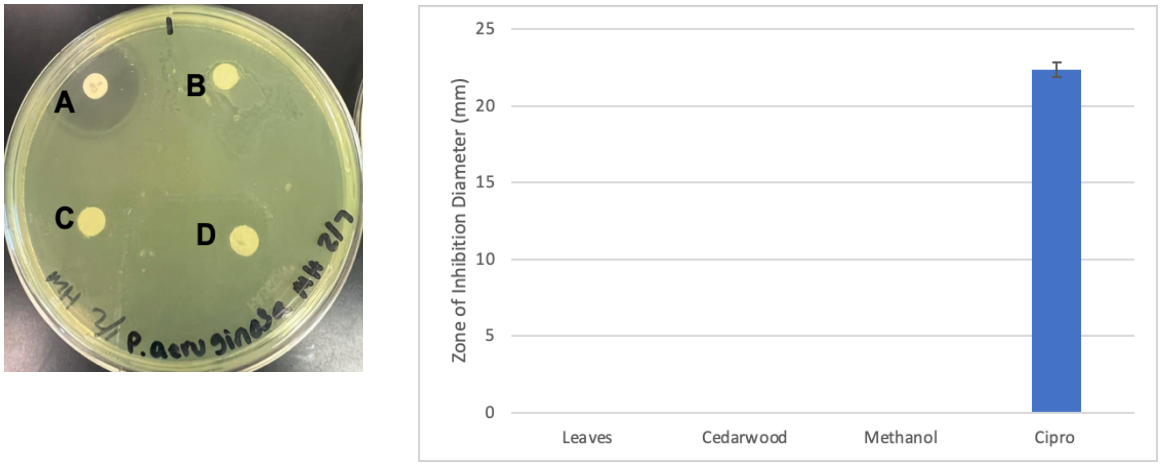
3.1.3. Kirby Bauer Assay: *P.aeruginosa*

Three trials of Kirby Bauer were conducted to test the antibiotic properties of CCEO and the HSDL against *P.aeruginiosa*. Cipro was used as a positive control and methanol was used as a negative control. In trial 1, The average ZOI for cipro was 26.67mm with a standard deviation of 0.94. No ZOI was observed for HSDL, CCEO or methanol (figure 23). In trial 2, the mean for cipro was 22.33mm with a standard deviation of 0.47. No ZOI was observed for HSDL, CCEO or methanol (figure 24). In trial 3 the average ZOI for cipro was 23.67mm with standard deviation of 0.47. No ZOI was observed for HSDL, CCEO or methanol (figure 25).



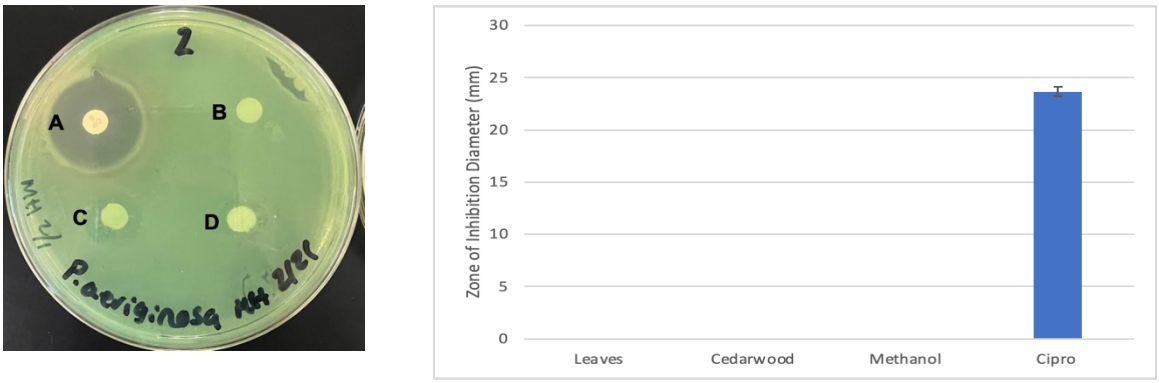
A. **B.**

Figure 23. Kirby Bauer Trial 1: *P.aeruginosa* **A.**Disk A= HSDL; B= methanol; C=CCEO; D= Cipro. **B.**Average Zones of inhibition with *P. aeruginosa.* The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 1.855E-11, which is statistically significant.



A **B.**

Figure 24. Kirby Bauer Trial 2: *aeruginosa.* **A.** Disk A= Cipro; B= CCEO; C=methanol; D= HSDL respectively. **B.** The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 9.6763E-07, which is statistically significant.

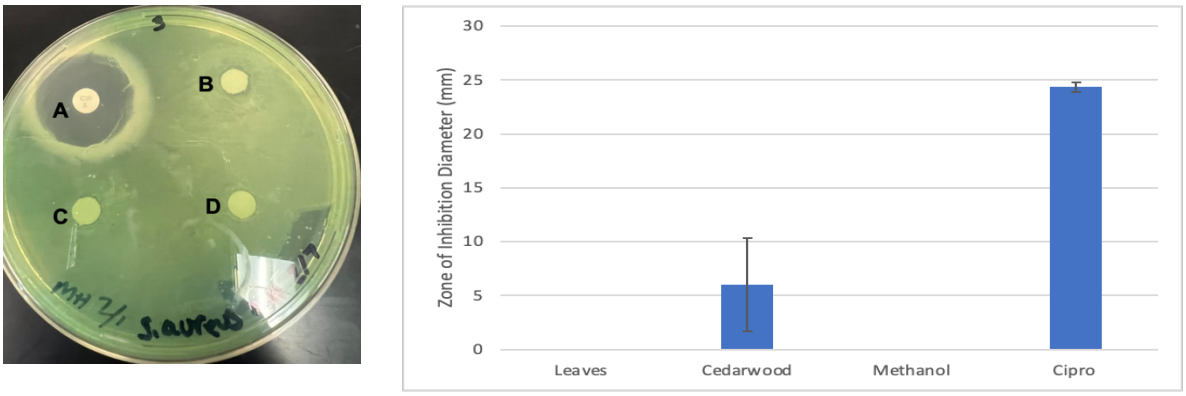


A. **B.**

Figure 25. Kirby Bauer Trial 3: *P. aeruginosa.* A. Disk A= Cipro; B=HSDL; C=Methanol; D= CCEO respectively. **B.** The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 1.9226E-13, which is statistically significant.

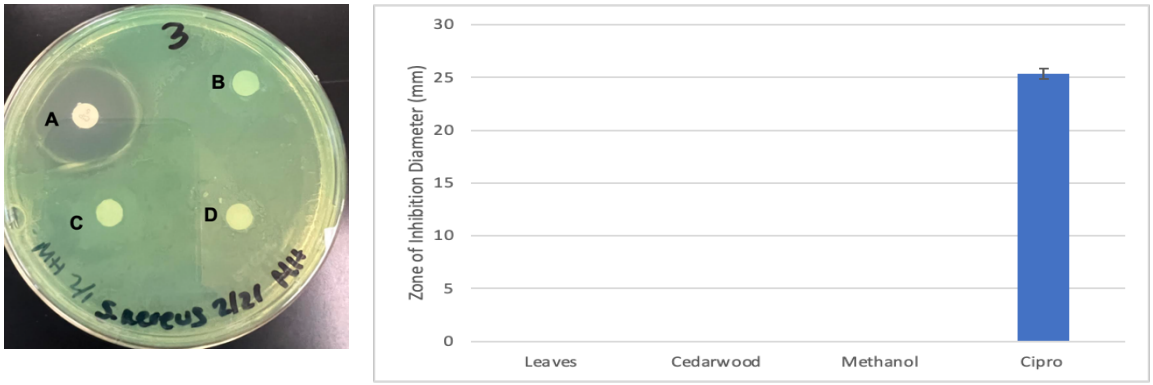
3.1.4. Kirby Bauer Assay: *S. aureus*

Two trials of Kirby Bauer were conducted to test the antibiotic properties of CCEO essential oil and the HSDL against *S. aureus*. Cipro was used as a positive control and methanol was used as a negative control. In trial 1, the average ZOI for cipro was 24.33mm with standard deviation of 0.47. The average ZOI for CCEO was 6mm with a standard deviation of 4.3. No ZOI was observed for HSDL or methanol (Figure 26). In trial 2, the average ZOI for cipro was 25.33mm with standard deviation of 0.47. No ZOI was observed for HSDL, CCEO or methanol (figure 27).



A. **B.**

Figure 26. Kirby Bauer Trial 1: *S.aureus* A. Disk A= Cipro; B= CCEO; C=methanol; D= HSDL.**B.** The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 1.0174E-05, which is statistically significant.



A. **B.**

Figure 27. Kirby Bauer Trial 2: *S. aureus* A. Disk A= Cipro; B= HSDL; C=methanol; D= CCEO. **B.**The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 1.1158E-13, which is statistically significant.

3.1.5. Kirby Bauer Assay: *S. epidermidis*

Two trials of Kirby Bauer were conducted to test the antibiotic properties of CCEO and the HSDL against *S. epidermidis*. Cipro was used as a positive control and methanol was used as a negative control. In trial 1, the average ZOI for Cipro was 21.67mm with a standard deviation of 0.57. The average ZOI for CCEO was 3mm with a standard deviation of 5.2. No ZOI was observed for HSDL or methanol (figure 28). In trial 2, the average ZOI for Cipro

was 29mm with a standard deviation of 0.82. No ZOI was observed for HSDL, CCEO or methanol (figure 29).

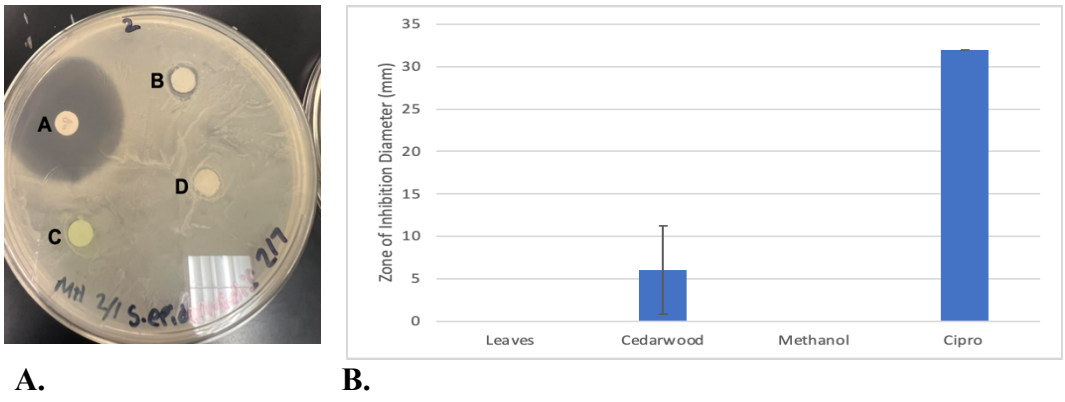


Figure 28 Kirby Bauer Trial 1 *S. epidermidis* A. Disk A= Cipro; B= CCEO; C=methanol; D= HSDL. **B.**The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 9.6763E-07, which is statistically significant.

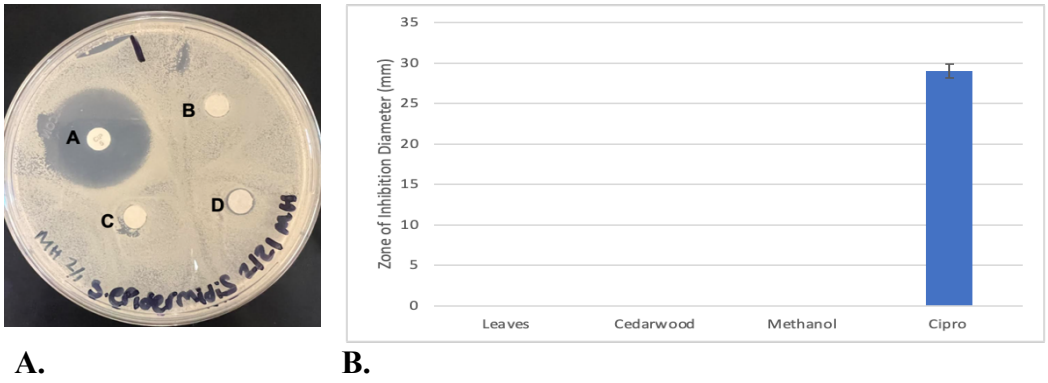
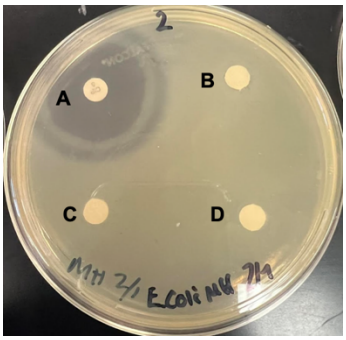


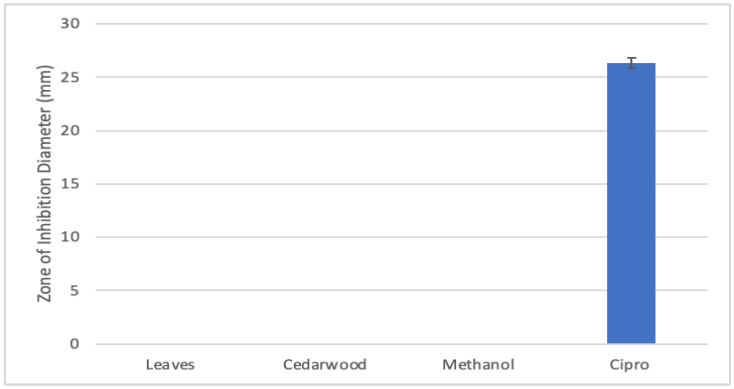
Figure 29. Kirby Bauer Trial 2: *S. epidermidis.* A. Disk A= Cipro; B= HSDL; C=methanol; D= CCEO. **B.** The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 3.0569E-12, which is statistically significant.

3.1.6. Kirby Bauer Assay: *E.coli*

Two trials of Kirby Bauer were conducted to test the antibiotic properties of CCEO and the HSDL against *E, coli*. Cipro was used as a positive control and methanol was used as a negative control. In trial 1, the average ZOI for cipro was 26.33mm with a standard deviation

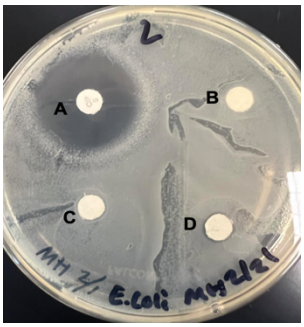
of 0.47. The HSDL, CCEO, and methanol had an average ZOI of 0mm. In trial 2, the average ZOI for cipro was 25.33mm with a standard deviation of 0.94.

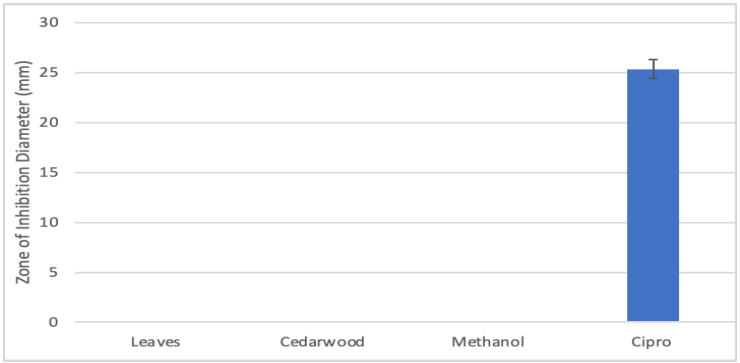




A. **B.**

Figure 30. Kirby Bauer Trial 1: *E.coli* A. Disk A= Cipro; B=CCEO; C=methanol; D= HSDL. **B.** The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 8.1873E-14, which is statistically significant.





A. **B.**

Figure 31. Kirby Bauer Trial 2: *E. coli* A .Disk A= Cipro; B= HSDL; C=methanol; D= CCEO. **B.** The X-axis represents which compound was used and the y-axis is the ring of inhibition in mm. Anova single factor was used to analyze the data. The p value is 2.8391E-11, which is statistically significant.

3.2 MIC Assay

Another test that was used to look into the antibiotic properties of cedarwood oil was a MIC Assay. The purpose of this test is to determine the susceptibility of the bacteria to each of

the compounds. These results determine the concentration at which the compound is not effective against the bacteria. The well plates were 48 well plates (6x8); The rows were numbered 1-8 from top to bottom (Figure 33). The compound was diluted across the well. The wells in the last column for all of the rows changed between having cells for two rows and and not adding cells to the next two rows. The well plates were incubated for 18-24 and then evaluated for bacterial growth (Figure 32). Each plate was labeled A-C accordingly. Rows are numbered from top to bottom with rows 1-4 of plate A containing *B. subtilis.* Rows 5-8 of plate A contained *S. epidermidis.* Rows 1-4 of plate B contained *S. aureus.* Rows 5- 8 of plate B contained *E.coli.* Rows 1-4 of plate C contained *P. aeruginosa.* Rows 5-8 of plate C did not contain anything, which is why the wells are completely clear.

Each well was streaked onto an agar plate and incubated. The results represent if bacteria grew where the plate was streaked. If there was no bacterial growth, such as number 1 (Figure 34 B.) then the data for the dilution factor table is distinguished as 0 . If there was minimal bacterial growth, like what is seen in number 4 (Figure 34 A), the data was recorded as 1+. If there was regular bacterial growth from the streak, such as number 8 in (Figure 35 C) , then the data was recorded as 2+. For each bacteria tested, the wells with cipro did not have any regular growth of bacteria (figures , meaning that the bacteria was susceptible at all concentrations of the compound. The wells with methanol had regular growth starting at ¼ dilution for *S. epidermidis* (Table 3) , whereas growth was seen at the first dilution (1/2) in wells with *S. aureus, E. coli, B. subtilis* and *P. aeruginosa* (tables 1,2,4 and 5). The wells that contained CCEO (cedarwood) had regular bacterial growth starting at 1/16 for *S. epidermis and P. aeruginosa* (table 3 and 6) and 1/8 for *B. subtilis* (table 2). Alternatively,

regular growth for *E. coli* and *S. aureus* was observed at ¼ (tables 5 and 6). The wells that contained HSDL (leaves) had regular bacterial growth at ¼ for all of the bacteria (tables 2-6).

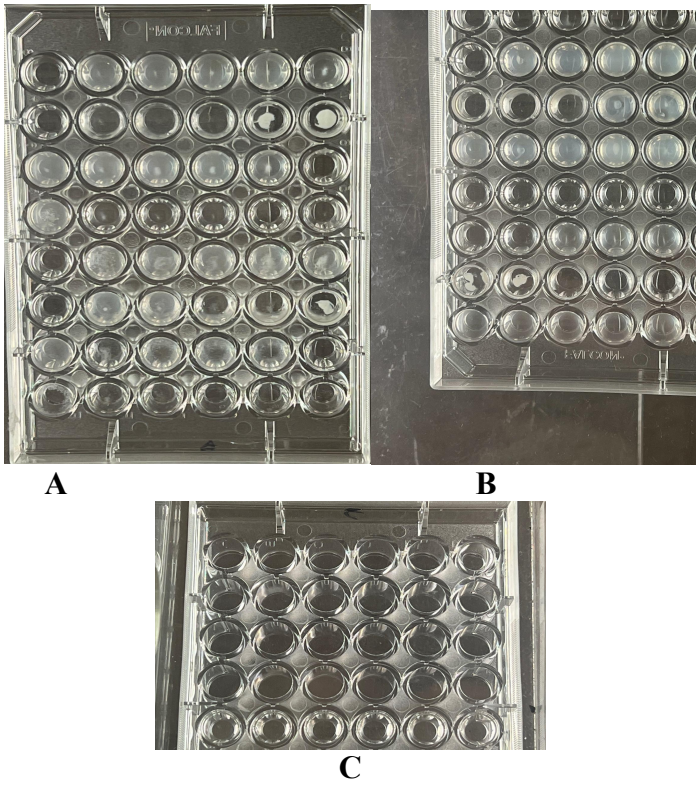


Figure 32. Well plates with bacteria and compounds after an 18–24-hour incubation period. Each plate is labeled A-C accordingly. Rows 1-4 of plate A contains *B. subtilis.* Rows 5-8 of plate A contain *S. epidermidis.* Rows 1-4 of plate B contain *S. aureus.* Rows 5- 8 of plate B contain *E.coli.* Rows 1-4 of plate C contain *P. aeruginosa* Rows 5-8 of plate C do not contain anything.

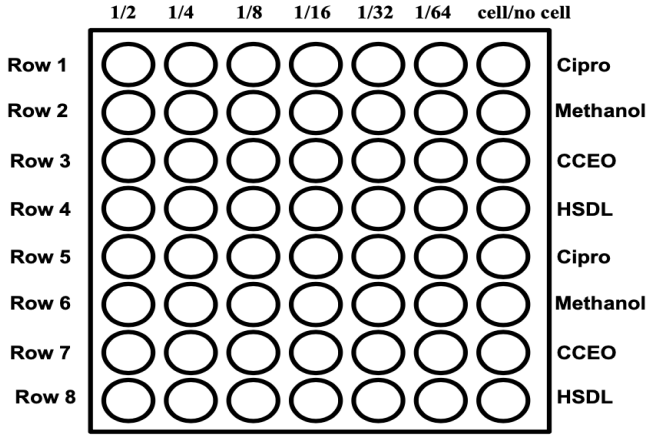
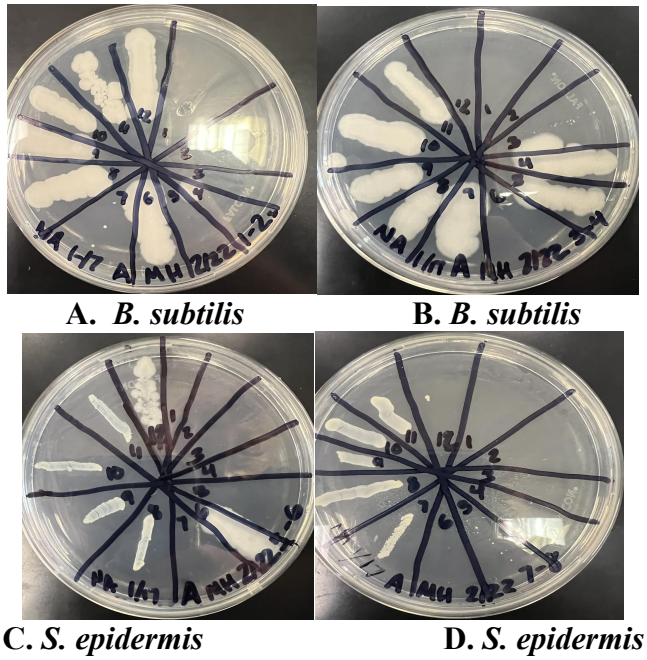


Figure 33. Well Plate for MIC Assay. Rows are labeled going down the plate. The columns are labeled with their corresponding dilution factor. Each row has a corresponding compound that is diluted across the plate.



A. *B. subtilis* B. *B. subtilis*

C. *S. epidermis* D. *S. epidermis*

Figure 34. Streaks for MIC Assay On Nutrient Agar plates. Samples of each well MIC assay plates were streaked onto nutrient agar plates. In figure A. *B. subtilis* with Cipro in streaks 1-5. Methanol is the compound used in streaks 7-11. Figure B *B. subtilis* with Cedarwood oil in streaks 1-5. The HSDL is in streaks 7-11. In Figure C, *S. epidermis* with Cipro in streaks 1-5 is Cipro. Methanol is in streaks 7-11 is methanol. In Figure D *S. epidermis* with cedarwood oil in streaks 1-6. HSDL in streaks 7-11 On each plate, quadrants 6 and 12 were positive controls (with cells and no test compound) or negative control (no cells).

Table 2. Dilution Factor Table for *B. subtilis*

Dilution factor is labeled across the top of the table with the compounds labeled down the left side of the table. Inhibition of bacteria is represented with a "-". Minimal bacteria growth is represented with "1+" i.e., ; Regular growth is represented with "2+"

	1/2	1/4	1/8	1/16	1/32	1/64	Cell/ No Cell
Cipro	-	-	-	1+	-		2+
Methanol	1+	2+	2+	2+	2+		2+
Cedawrwooc	-	-	1+	2+	2+		-
Leaves		2+	2+	2+	2+	2+	-

Table 3. Dilution Factor Table for *S. epidermidis*

Dilution factor is labeled across the top of the table with the compounds labeled down the left side of the table. Inhibition of bacteria is represented with a "-". Minimal bacteria growth is represented with "1+"; Regular growth is represented with "2+"

	1/2	1/4	1/8	1/16	1/32	1/64	Cell/ No Cell
Cipro	-	-	-	-	-		2+
Methanol	-	2+	2+	2+	2+		2+
Cedawrwooc	-	-	-	1+	1+		-
Leaves		2+	2+	2+	2+	2+	-

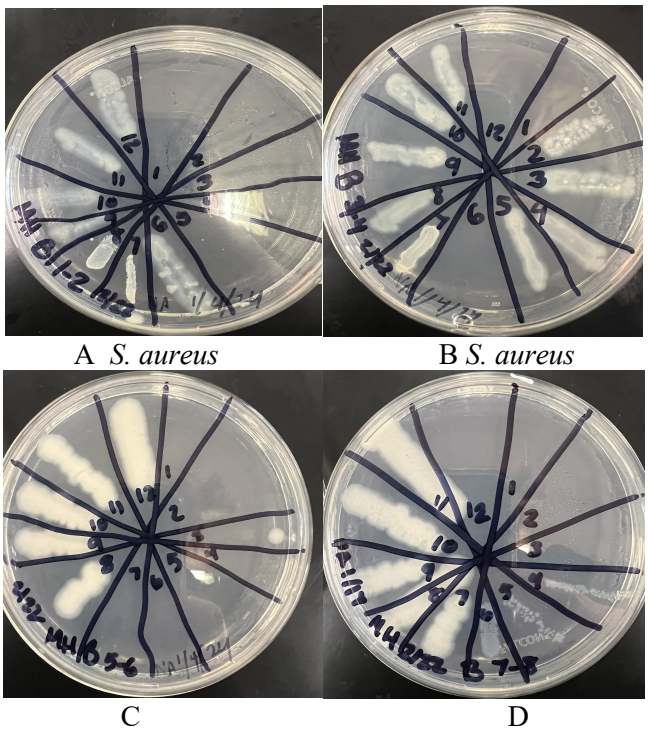


Figure 35. Streaks for MIC Assay on Nutrient Agar plates. Samples of each well MIC assay plates were streaked onto nutrient agar plates. In figure A *S. aureus* with Cipro in streaks 1-5. Methanol is the compound used in streaks 7-11. Figure B *S. aureus* with Cedarwood oil in streaks 1-5. The HSDL is in streaks 7-11. In Figure C, *E. coli* with Cipro in streaks 1-5 is Cipro. Methanol is in streaks 7-11 is methanol. In Figure D *E. coli* with cedarwood oil in streaks 1-6. HSDL in streaks 7-11 On each plate, quadrants 6 and 12 were positive controls (with cells and no test compound) or negative control (no cells).

Table 4. Dilution Factor Table for *S. aureus*

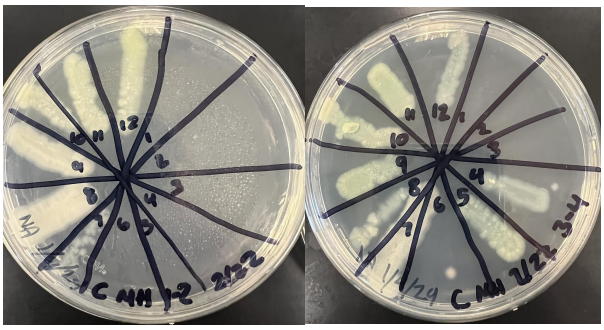
Dilution factor is labeled across the top of the table with the compounds labeled down the left side of the table. Inhibition of bacteria is represented with a "-". Minimal bacteria growth is represented with "1+"; Regular growth is represented with "2+"

	1/2	1/4	1/8	1/16	1/32	1/64	Cell/ No Cell
Cipro	-	-	-	-	-		2+
Methanol	2+	2+	2+	2+	2+		2+
Cedawrwooc	-	2+	2+	2+	2+		-
Leaves		2+	2+	2+	2+	2+	-

Table 5. Dilution Factor Table for *E.coli*

Dilution factor is labeled across the top of the table with the compounds labeled down the left side of the table. Inhibition of bacteria is represented with a "-". Minimal bacteria growth is represented with "1+"; Regular growth is represented with "2+"

	1/2	1/4	1/8	1/16	1/32	1/64	Cell/ No Cell
Cipro	-	-	-	-	-		2+
Methanol	2+	2+	2+	2+	2+		2+
Cedawrwooc	-	2+	2+	2+	2+		-
Leaves		2+	2+	2+	2+	2+	-



A *P. aeruginosa* **B.** *P. aeruginosa*

Figure 36.Streaks for MIC Assay On Nutrient Agar Samples of each well MIC assay plates were streaked onto nutrient agar plates. Samples of each well MIC assay plates were streaked onto nutrient agar plates. In figure A *P. aeruginosa* with Cipro in streaks 1-5. Methanol is the compound used in streaks 7-11. Figure B *P. aeruginosa* with Cedarwood oil in streaks 1-5. The HSDL is in streaks 7-11. On each plate, quadrants 6 and 12 were positive controls (with cells and no test compound) or negative control (no cells).

Table 6. Dilution Factor Table for *P. aeruginosa*

Dilution factor is labeled across the top of the table with the compounds labeled down the left side of the table. Inhibition of bacteria is represented with a "-". Minimal bacteria growth is represented with "1+"; Regular growth is represented with "2+"

	1/2	1/4	1/8	1/16	1/32	1/64	Cell/ No Cell
Cipro	-	-	-	-	-		-
Methanol	2+	2+	2+	2+	2+		2+
Cedawrwooc	-	-	-	2+	2+		-
Leaves		2+	2+	2+	2+	2+	2+

4. Gram Stain

The results of our gram stains indicated the bacteria we worked with , based on color and shape. The results for *S. aureus* (Figure 37) show that the bacteria were stained purple and was cocci shaped. This is what would be expected of a gram stain of *S.aureus.* The results for *S. epidermidis* (Figure 38) show that the bacteria were stained purple and are cocci shaped. This is what would be expected of these bacteria. The results for *E.coli* show the bacteria is stained pink and is rod shaped. (Figure 39) This is what would be expected of a gram stain of *E. coli.* The results for *P. aeruginosa* show that the bacteria are stained pink and are rod shaped (Figure 40) This is what would

be expected of a gram stain of *P. aeruginosa.* The bacteria also had diplobacilli features. The results for *B. subtilis* show that the bacteria are stained purple and are rod shaped (figure 41). This is what would be expected of a gram stain of *B. subtilis* .

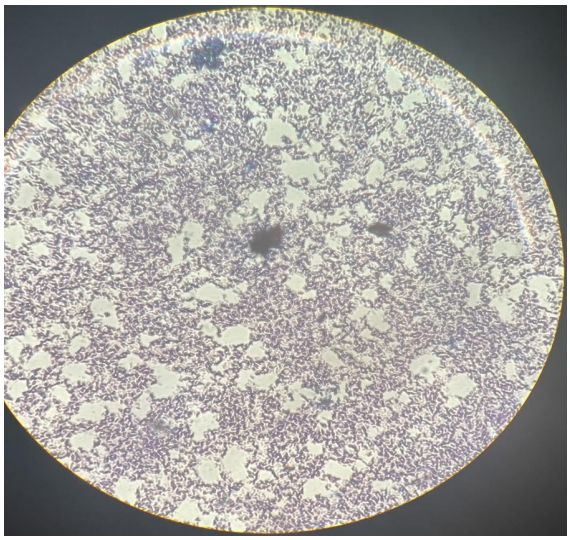


Figure 37. Gram Stain Results of *S. aureus* 1000X magnification .
The bacteria is stained purple and is cocci shaped. This is what would be expected of a gram stain of *S. aureus.* Some of the bacteria is arranged to have streptococci features.

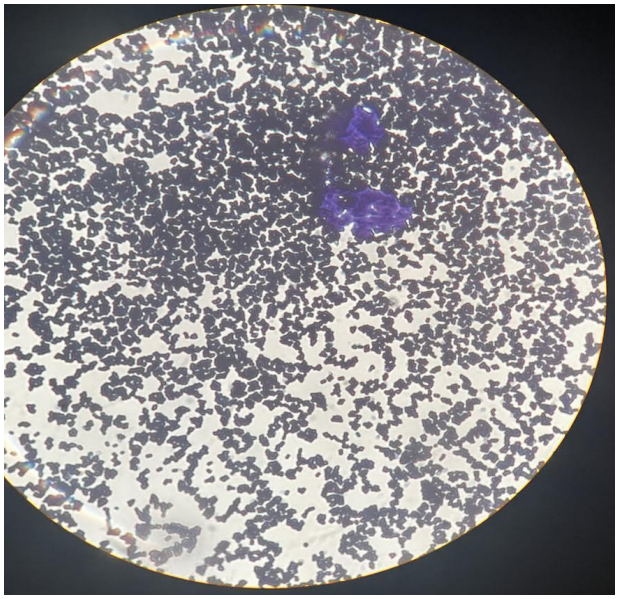


Figure 38. Gram Stain Results of *S. epidermidis* 1000X magnification
The bacteria is stained purple and is cocci shaped. This is what would be expected of a gram stain of *S. epidermidis.*

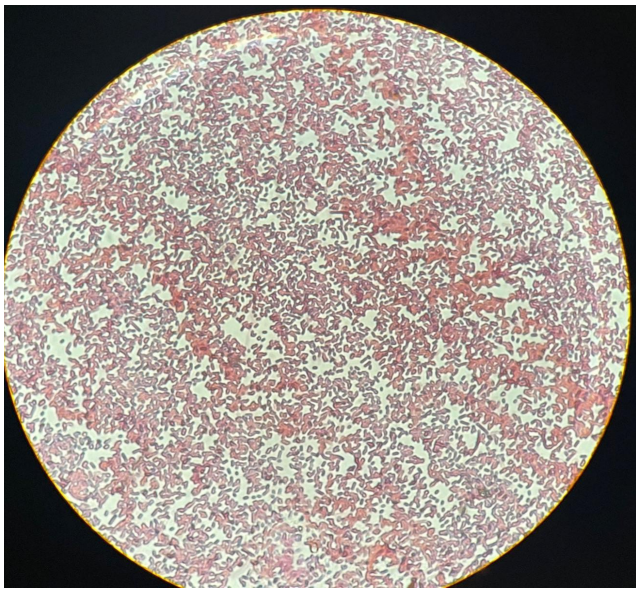


Figure 39. Gram Stain Results of *E. coli.* 1000X magnification
The bacteria is stained pink and is rod shaped. This is what would be expected of a gram stain of *E. coli.* Some of the bacteria is found to be arranged as diplobacilli.

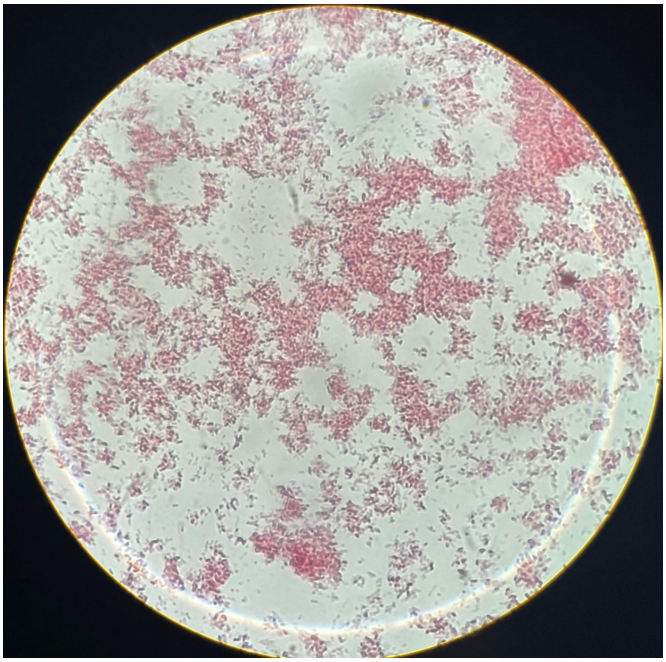


Figure 40. Gram Stain Results of *P. aeruginosa* 1000X magnification
The bacteria is stained pink and is rod shaped. This is what would be expected of a gram stain of *P. aeruginosa.* The bacteria is found to have diplobacilli features.

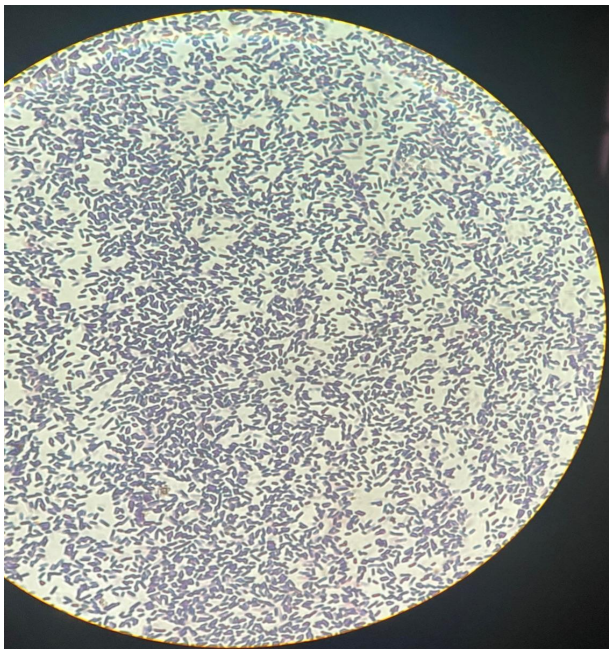
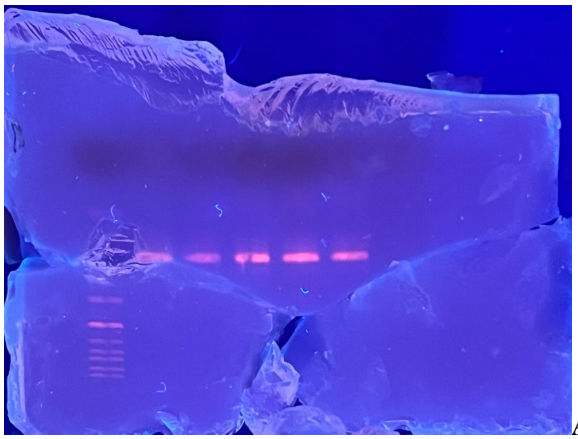


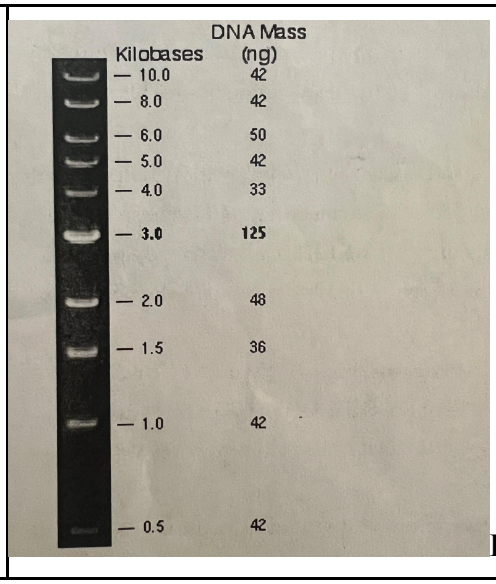
Figure 41. Gram Stain Results of *B .subtilis* 1000X magnification
The bacteria is stained purple and is rod shaped. This is what would be expected of a gram stain of *B. subtilis* .

Gel electrophoresis

The results for this experiment showed that the DNA for all of the microorganisms reached 8.0Kb which is equivalent to 42ng (Figure 42). DNA bands are present which ensures us there is amplified DNA and that the sample can be sent for sequencing. The gel broke apart right before being placed under the uv light, which is why it is pieced together.



A



B

Figure 42. Agarose gel with UV light after gel electrophoresis. A From left to right the wells contained the following: (2) 1 Kb ladder, (3) *S. aureus*, (4) *E. coli,* (5*) S. epidermidis,* (6) *B. subtilis,* (7) *P. aeruginosa,* (8) control. **B. 1Kb DNA Ladder visualized by EtBr staining on .8% TAE agarose gel** This serves as a reference for approximating the mass of the DNA.

IV. Discussion

The CCEO demonstrated some antibiotic properties in the Kirby Bauer test with *B.subtilis*, *S. aureus* and *S.epidermidis*. There was no antibiotic activity of CCEO or the HSDL against the other bacteria. The results for the Kirby Bauer were very different then the results from the research from Wanner. The only bacteria that cedarwood essential oil did not have antibiotic properties against in their experiment was with *Pseudomonas.* With that being said it is possible that the commercial oil used in my experiment is not from an Atlas cedarwood tree. In the MIC assay, the CCEO showed highest antibiotic activity against *P. aeruginosa* and *S. epidermidis* and to a lesser extent *B. subtilis*, with minimal activity against*, S.aureus* and *E. coli.*

Since CCEO is produced from a variety of species of trees and from several parts of the tree, the next step would be to look into different species of cedar trees and test if different parts or species are more effective.

Lastly it would be beneficial to have a better understanding of which compounds were in the HSDL. The oil did not have any antibiotic effects against any of the bacteria. With that being said, it is possible that the compounds that may cause these bioactive properties were not evaporated during the steam distillation process, which may have led to these results. If these compounds were not able to vaporize, then those compounds would not have been collected in the oil. In addition, there are likely different compounds in the leaves then the bark, which could indicate that these leaves did not contain compounds that have these antibiotic properties.

Conclusion

In conclusion the CCEO showed higher rates of antibiotic activity when compared with the

HSDL. Since the CCEO could be any variety of species and come from various parts of the plant, it is likely that the compounds are working together and produce a synergistic effect against these bacteria. The HSDL came from one species of cedarwood tree and it is isolated from one part of the tree. If we had used the wood chips and different species of trees, we may have yielded different results. Since different species of cedarwood trees contain different chemical compositions, it is possible that the leaves from the Easter Red Cedar did not contain some of the bio- active compounds, such as alpha cedrene, beta cedrene, and cedrol.

Limitations

There were limitations in this research that should be addressed, for future research. To have a better understanding of the concentration of molecules that were present in my essential oils, it would have been ideal to use a gas chromatography mass spectrophotometer. It is possible that the compounds in the oil were not able to diffuse in the Kirby Bauer, and understanding the concentration of the molecules in these compounds could help us identify why we got the results we did.

In addition, we would like to continue research on the effectiveness of freshly made antibiotic disks versus already prepared disks. In trial one, the disks were prepared with their compounds the day of the Kirby Bauer. In trials 2 and 3 the disks were prepared days prior to their use. There were larger zones of inhibition of CCEO on *B. subtilis* in trial 1 than the other trials. This suggests that there may be an antibiotic compound that evaporated after the disc had been sitting for a few days.

Since the microplate reader for the MIC assay was not working at the time, we had to come up with a new method to study the susceptibility of the bacteria to the applied

compound. While streaking the plate from each well was effective, it is more prone to human error and therefore, it would have been ideal to use the microplate reader. In addition, it would be important to do more than just one trial of a MIC assay. More MIC assay trials would have been ideal to compare results.

Future Research

Further research could look into true versus false cedars, to study the effectiveness of those that are native to the Himalayas and Mediterranean basin.

Along with conducting more trials, it would be beneficial to test against different bacteria. Most of the bacteria that were tested were gram-positive and we were not able to determine if the gram-status of the bacteria had an effect with the compound. We got better results with *B. subtilis,* and we were able to see a cloudy inhibition with *B. cereus,* indicating bacteriostatic effects. The next step would be to test against more bacillus bacteria.

Implications

This research has increased our knowledge and understanding of cedarwood essential oil, and the impacts it has on bacteria. What makes this research stand out is that the leaves were being analyzed for their antibiotic properties rather than the bark/branches. Discovering the benefits of Cedarwood Essential Oil has a huge impact on everyone who is interested in studying medicinal properties of plants. Researching the antibiotic properties of cedarwood oil is a step in the right direction for combating antibiotic resistance.

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