Artemisinin Project: Effect of *Artemisia annua* Extract on Safe ESKAPE Pathogen Relatives

WPI

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Abstract

Artemisia annua L. contains a known antimalarial drug called artemisinin and other compounds with antibacterial properties. However, there is not a consensus on which bacteria are susceptible to *A. annua* extracts. This experiment investigated whether an aqueous extract of *A. annua* would be effective against the ESKAPE pathogens. When tested, the extract did not exhibit antibacterial properties compared to aqueous controls, 5% bleach, and chloramphenicol. Although this aqueous extract did not demonstrate antimicrobial properties against the tested bacteria, extracts produced using more nonpolar solvents might be more effective.

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1.0 Introduction

Antibiotic resistant bacteria are a big threat to disease prevention and protection (CDC, 2019). In 2013 the CDC produced their first Antibiotic Resistant (AR) Threats Report, which detailed all of the bacteria thought to be a major threat to American citizens. This report included everything from comparing which drug resistant bacteria pose the biggest threat to how the United States should combat this growing risk (CDC, 2013). In that report, one important note was that, on average, 2.0 million cases and 23,000 deaths were being caused annually by antibiotic resistant bacteria (CDC, 2013). When the CDC conducted the same AR Threats Report in 2019, the average annual cases and deaths in the US caused by AR bacteria had increased substantially. The 2019 report found that, on average, there were now 2.8 million cases and over 35,000 deaths being reported each year (CDC, 2019). From that report, it found that certain strains from each of the six ESKAPE pathogens were on the list of urgent and serious threats for antibiotic resistance (CDC, 2019). This paper looks at *Artemisia annua* tea extracts as an effective treatment against similar strains of bacteria.

1.1 Antibiotics

Antibiotics are drugs or compounds that fight bacterial and viral infections either by killing the microbe, or by stopping them from replicating (CDC, 2020). There are many types of antibiotics but most are effective against bacteria. Some work specifically on Gram-positive bacteria, some work on Gram-negative bacteria, and some work on both. The main mechanisms of action include targeting cell wall synthesis, protein synthesis, RNA synthesis, and metabolic reactions. By disrupting these processes, the antibiotic either kills the bacteria, or stops it from growing (Kapoor, 2017). Antibiotics are used mostly against bacteria, as they are not usually effective against fungus nor against viruses. One class of antibiotics are the ß-lactam antibiotics

that include penicillin and methicillin (Stapleton, 2002). This class works by inhibiting the formation of the peptidoglycans, which are a major component of the bacterial cell wall that provides mechanical stability to the cell (Pandey, 2020). Penicillin, and other ß-lactam antibiotics target the last step in peptidoglycan formation by interrupting enzymes meant to cross-link peptides that hold the cell wall together (Pandey, 2020). The enzymes meant to cause this cross-linking reaction are known as penicillin binding-proteins (PBPs) because instead of carrying out this reaction, they preferentially bind to penicillin. Both Gram-positive and Gram-negative bacteria require the peptidoglycan layer to survive and because of this, ß-lactams can inhibit cell wall growth of both groups of bacteria (Soares, 2012).

1.2 Antibiotic Resistance Mechanisms

Bacteria will eventually evolve resistance to antibiotics. Antibiotic resistance can be caused by misuse of antibiotics, when people take antibiotics unnecessarily or do not follow their prescription and stop taking the antibiotic after they begin to feel better (CDC, 2020). Bacteria can either mutate to become resistant to antibiotics, or they can obtain a plasmid containing antibiotic resistant genes from another bacterial cell.

There are four general categories of antibiotic resistance mechanisms as pictured in Figure 1. Figure 1A shows how bacteria use efflux pumps to remove the antibiotics before they can kill the cell. For instance, some strains of *E. coli* increase the number of efflux pumps they have in their membrane in order to combat antibiotics (Webber, 2003). In another instance as seen in Figure 1B, some antibiotics need to pass through the cell wall and enter the cytoplasm of the cell in order to kill the cell, so some bacteria can modify their cell wall to limit its access into the cell. One example of this is in *Staphylococcus aureus* where some strains use a

glycotransferase enzyme called TarP to modify their cell wall to evade antibiotics and also the immune response. This allows the bacteria to avoid phagocytosis by host cells like macrophages that engulf and kill marked bacterial cells (Gerlach, 2018). Another mechanism involves bacteria destroying the antibiotic with an enzyme that targets it or marks it for degradation as seen in Figure 1C. For example, when *Klebsiella pneumoniae* is exposed to ß-lactam antibiotics and ß-lactamase hydrolyzes them with ß-lactamase, the antibiotics can no longer inhibit cell wall synthesis (Ventola, 2015). The last method whereby bacteria gain resistance is by modifying the affected antibiotic target protein so that the antibiotic is unable to bind as shown in Figure 1D. For example, some strains of *Escherichia coli* become resistant to quinolone antibiotics because of a point mutation in DNA gyrase that prevents quinolone from binding to the gyrase (Hooper, 2015). Instead, the bacteria uses the modified DNA gyrase to carry out replication that quinolone would have blocked (Hooper, 2015). Taken together, these mechanisms make treating antibiotic resistant infections challenging, so sometimes a combination of antibiotics must be used (WHO, 2020).

There are six types of particularly interesting common pathogens that have become resistant to many antibiotics. These pathogens known as “ESKAPE pathogens” cause infections that can be difficult to treat because of their lack of susceptibility to antibiotics, sometimes even resulting in fatal infections. This has created the need for novel antimicrobial compounds, new antibiotics to which the bacteria have not been exposed, thereby making these species more susceptible and therefore treatable. See section 1.7 for a more detailed description of the ESKAPE pathogens.

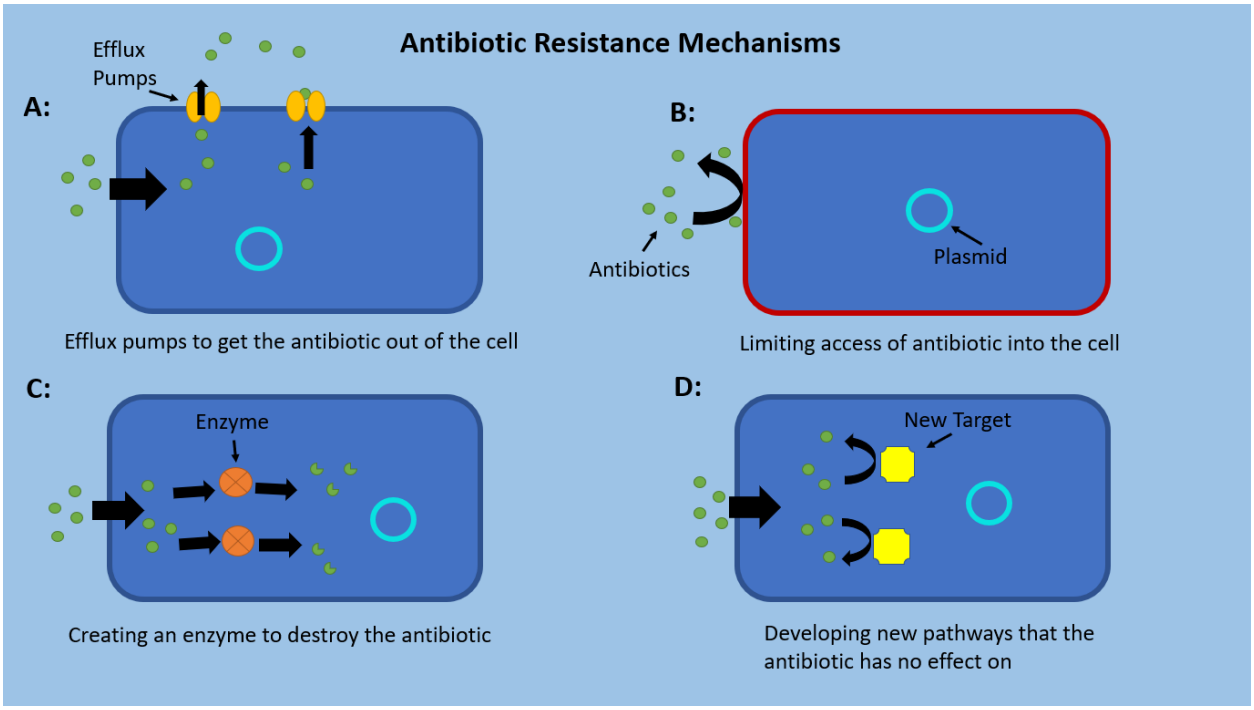

Figure 1: Antibiotic resistance mechanisms. These are the four most common mechanisms in which bacteria develop resistance to antibiotics based on Ventola, 2015.

1.3 *Artemisia annua* L.

Artemisia annua L. (Figure 2) also known as "sweet sagewort", annual wormwood, sweet wormwood, or sweet Annie is an annual herbaceous plant that originated in Southeast Asia, but is now commonly found and cultivated in Asia, India, Central and Eastern Europe, as well as the temperate regions of the Americas, Africa, Australia and in tropical regions. It has been used as a spice, tea, and medicinal plant in Asia for over two thousand years (Septembre-Malaterre, 2020). *A. annua* L. was used as an herbal drug, along with other *Artemisia* species by the Chinese since the 11th century. In 1086CE, the use of *Artemisia* species was clarified by Li Shizhen and Shen Gua. They identified *A. apiaceae* as the species used by ancient Chinese and not *A. annua* which is known today to have a greater concentration of artemisinin but may not have been favored because of extraction methods (Hsu, 2006).

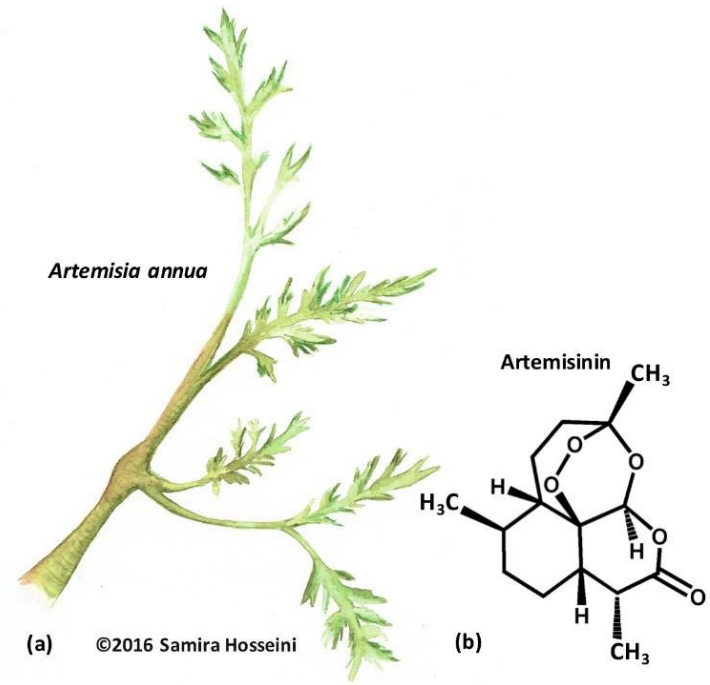

Figure 2: *A. annua* L. and its main constituent artemisinin (Hosseini, 2016)

A. annua contains many chemical compounds with biological activity, including monoterpenes, sesquiterpenes (including artemisinin), phenolics, and coumarins (Septembre-Malaterre, 2020). Studies have also shown biological effects such as antioxidant, antidiabetic, antitumor, antibacterial, antifungal, antiviral, and antiparasitic activity (Krishna, 2008). *A. annua* was traditionally used as an antimalarial and fever-reducing herb mainly in Asia where it was pressed into juice, or made into a tea infusion. The fresh plant was sometimes soaked in water and then wrung out so the phytochemical containing juice could be ingested to treat fevers and joint pain. However, it is thought the Chinese used *A. apiacea* more often than *A. annua* (Hsu, 2006) and the actual antimalarial compound, artemisinin, was not identified until much later.

After the 1950s, malaria caused by *Plasmodium falciparum* was resurging because of emerging strains resistant to chloroquine antibiotics used during that time. In 1967 a Chinese national project under the Project 523 office, was initiated to find a medicine effective against chloroquine resistant *P. falciparum* (Tu, 2011). A team of Chinese scientists tested over 2,000 Chinese herb preparations and found that tea made from qinghao (*Artemisia*) inhibited *P. falciparum* growth (Tu, 2011). After extracting the individual components, the team found a colorless, crystalline molecule that they called qinghaosu, aka artemisinin, in 1972 (Tu, 2011). In the 1980s, the team of scientists was allowed to share this finding with the rest of the world and tell them about *A. annua* and its antimalarial effects. It took until 1993 for a western company to do their own research and manufacturing of artemisinin-derived compounds (Faurant, 2011). Due to the relative safety and availability of *A. annua*, it has recently been tested against various viruses with some success. Among those viruses were herpes simplex virus 1 and 2, Epstein-Barr virus, hepatitis B which showed susceptibility to artemisinin both *in vitro* and *in vivo* (Efferth, 2018). *A. annua* extract was also found to inhibit replication of SARS-CoV-1 and -2 *in vitro* and may provide a potential antiviral drug against COVID-19 and influenza (Cao et al., 2020; Bae et al. 202, Nair et al. 2021).

1.4 Artemisinin

Artemisinin (Figure 2) is a bioactive, organic soluble compound found mainly in *A. annua* with reports of occurrence in some other *Artemisia* species (Klayman, 1985), and can be found and extracted from the plant leaves, stems and flowers (Klayman, 1985). Most of *A. annua'*s medicinal properties are attributed to the compound artemisinin. Artemisinin is a well-known antimalarial drug from the *Artemisia* genus of plants (Krishna, 2008). Artemisinin is

also effective *in vitro* against other parasitic infections, fungi, bacteria, cancer cells, and against viruses (Krishna, 2008; Lin, 2018; Meshnik, 2002). Artemisinin inhibits some viruses like herpes simplex virus type 1, Epstein-Barr virus, hepatitis B and C viruses, and others (Efferth, 2008). Due to its promising antiviral effects, artemisinin is currently being tested as a possible treatment for other viruses including SARS-CoV-2 (Haq, 2020). Several studies specifically found therapeutic advantages to using artemisinin and *A. annua* hot water extracts against COVID-19 *in vivo*, especially when used in conjunction with other antiviral drugs like remdesivir and favipiravir (Cao et al., 2020; Bae et al. 202, Nair et al. 2021).

Artemisinin is a sesquiterpene lactone possessing a trioxane ring system as well as a peroxide functional moiety (Rudrapal, 2016), features also included in artemisinin derivatives. The proposed mechanism of action for artemisinin occurs at the endoperoxide within the ring system of the molecule (Figure 3). This peroxide molecule reduces the respiratory metabolism of bacteria (Lin, 2018). As an antimalarial, the endoperoxide bridge in artemisinin is thought to be targeted for heme-mediated degradation generating carbon centered free radicals and alkylating intermediates (Meshnik, 2002; Tran, 2018). Carbon centered free radicals are thought to target specific proteins on the membranes of disease microbes like malaria (Tran, 2018).

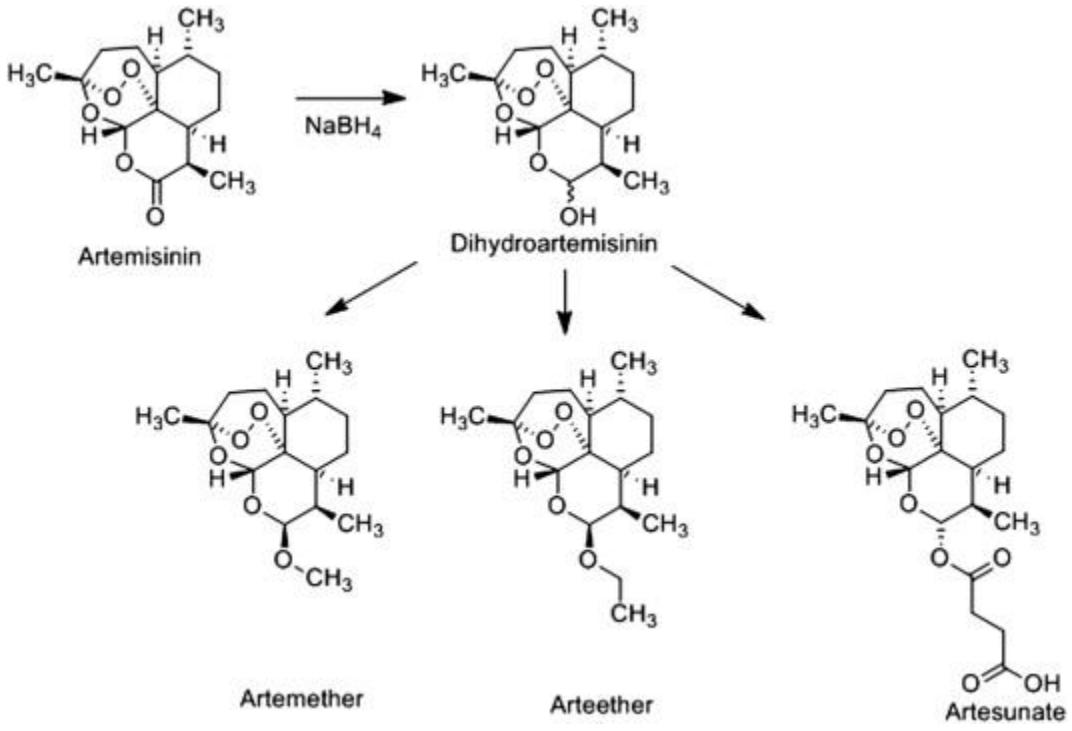

Figure 3: Chemical structure of artemisinin and its derivatives (Li, 2012)

There are a variety of synthetic and semisynthetic derivatives of artemisinin (Figure 3). Of these derivatives, artesunate, dihydroartemisinin (DHA), arteether and artemether are the most important and are used instead of artemisinin because they are more soluble and bioavailable (Dondorp, 2017). Because artemisinin is an organic soluble compound, using the drug in combination with other drugs or targeting areas of the body that have low organic solubility become a major problem. Artesunate, the water-soluble version of artemisinin, and artemether, the fat-soluble version, can help target different areas of the body not reachable with artemisinin (Dondorp, 2017). However, artemisinin consumed using the dried leaves of *A. annua* L. increased artemisinin bioavailability >40-fold compared to pure artemisinin. This seems to occur because the dried *A. annua* leaf delivery inhibits hepatic metabolism like liver P450s using

phytochemicals in the dried leaves (Desrosiers et al, 2020). This means the artemisinin is not metabolized as fast, leading to higher concentrations in the body. Essential oils in the plants also help solubilize artemisinin leading to more artemisinin in the blood (Desrosiers & Weathers, 2016). In addition, phytochemicals in *A. anuua* can facilitate and enhance the transport of artemisinin across the intestine (Desrosiers & Weathers, 2018).

1.4.1 Artemisinin against malaria

Artemisinin is well-established as a treatment against *P. falciparum*, the most deadly form of malaria. The drug is especially useful when introduced in malaria-stricken regions that contain strains of malaria resistant to previously used antimalarials (WHO, 2020). Starting in the 19th century, quinine was used against malaria and then during World War 2 chloroquine started to be used until the 1960s (Tran, 2017). In the 1960s, doctors began noticing chloroquine resistant *P. falciparum*, which led to the discovery of artemisinin (Tran, 2017). Now, malarial strains also resistant to artemisinin are starting to emerge and new drugs need to be developed that can combat this resistance (Peplow, 2020; WHO, 2020). Discovery of new drugs to kill artemisinin resistant malaria may enable scientists to kill other microbes that become resistant to artemisinin.

One method for killing artemisinin resistant malaria is by using an Artemisinin based Combination Therapy (ACT). ACT is characterized by the use of an artemisinin derivative in combination with another antimicrobial agent, e.g. lumefantrine (Dondorp, 2017). Artemisinin and its partner drug help each other in a couple of different ways. First, artemisinin, and its derivatives, have a short half-life in the bloodstream of 2-5 hours (Vires, 1996). However, the artemisinins are very potent against *P. falciparum* in its early stages by reducing the rate of proliferation of the parasites (Dondorp, 2017). After the artemisinins dissipate, and become less effective, the partner drug with a longer half-life (the length of time it takes for half of the

compound to degrade) continues killing the parasite for a longer time and usually via a different mechanism of action (Dondorp, 2017). ACTs are also useful because if that strain of *P. falciparum* is resistant to one of the drugs, the other drug will still be able to work effectively and hopefully prevent spread of the disease back to the mosquito (Dondorp, 2017).

Another method for killing artemisinin resistant malaria may be to use the whole plant as a remedy instead of just extracting artemisinin (Elfawal, 2015). This method may be effective because *A. annua L.* may have a naturally evolved ACT (Elfawal, 2015). One study that used whole leaf extracts from an *A. annua* mutant that lacked artemisinin but contained major flavonoids concluded bioactivity against *P. falciparum* of flavonoids was greatly reduced compared to artemisinin (Czechowski, 2019). Another study came to the same conclusion, but only studied the ring stage of *P. falciparum* suggesting dried-leaf *A. annua* required an interaction with their host to be useful as an antimalarial therapy (Gruessner and Weathers, 2021). In contrast, a different study testing *Artemisia* spp. tea infusions against trophozoites and gametocytes concluded the tea infusions containing little to no artemisinin still had an antiparasitic effect, although not as much as artemisinin containing infusions (Snider and Weathers, 2020). If *A. annua* was able to make a natural ACT against malaria, then it is possible this evolutionary advantage carries over to antibiotic resistant strains.

1.5 Flavonoids

Other possible antibiotic contributors in *A. annua* L. are the flavonoids. Flavonoids are a general category of natural compounds that have varying types of phenolic structures (Panche, 2016). Flavonoids commonly occur as a natural pigmentation for certain plants and fluoresce when excited by UV light (Havsteen, 2002). Three flavonoids: nareingenin, eriodictyol, and

taxifolin inhibited the ß-ketoacyl-acyl carrier protein synthase (KAS III), which is an essential enzyme in bacterial fatty acid synthesis, when tested on Methicillin Resistant *Staphylococcus aureus* (MRSA) (Lee, 2009). Flavonoids also are thought to inhibit reverse transcriptase in viruses and destroy parasitic protozoa while also having a low toxicity towards humans (Havsteen, 2002). The problem with testing flavonoids as antimicrobials is that scientists do not know all of their structures. It is difficult to differentiate among them and flavonoids are challenging to separate from artemisinin in *A. annua* L.

1.6 Gram-negative vs. Gram-positive

Bacteria are classified into two different categories, Gram-negative and Gram-positive. Gram- negative bacteria have a thin peptidoglycan wall surrounded by a lipopolysaccharide outer membrane. Gram-positive bacteria have no outer membrane but do have a peptidoglycan wall significantly thicker than the Gram-negative bacteria (Shilhavy, 2010). The bacteria can be differentiated with a Gram stain. Gram-positive bacteria will retain a purple stain because of their thick cell wall, while the Gram-negative bacteria will not and thus appear pink under a microscope (O'Toole, 2016). Because these bacteria have different wall compositions, they are often treated with different drugs. Antibiotics can have different mechanisms of action, but Gram-positive specific antibiotics usually target and degrade their thick cell wall, while Gram-negative specific antibiotics target other essential processes to kill the bacteria (Shilhavy, 2010).

1.7 ESKAPE Pathogens

A 2002 study estimated 1.7 million hospital acquired infections (nosocomial infections) in that year, with 99,000 deaths worldwide (Klevens, 2002). The infections caused by these bacteria can be life threatening because of the pathogen's ability to resist antibiotic and antimicrobial drugs, which makes them difficult to treat. This resistance can be caused by misuse of antibiotics and antimicrobial drugs. Antibiotic resistant bacteria are becoming harder to treat as their resistance spreads, so it is important to continue to find new antimicrobial compounds to fight against them (Santajit, 2016).

Most nosocomial infections are caused by six types of pathogens. These pathogens are called the ESKAPE pathogens taken from the first letter in their genus name, and because they can “escape” the effects of antibiotics. The ESKAPE pathogens include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp. These are a combination of Gram-positive and Gram-negative infection-causing bacteria (Santajit, 2016), but they all have safe relatives that are less virulent and are easier and safer to study.

1.7.1 *Enterococcus faecium*

E. faecium is a Gram-positive bacteria that can cause urinary tract infections (UTIs), endocarditis, wound infections, and bacteremia, which is an infection in the bloodstream (Mulani, 2019). Enterococcal bacteria are typically found in the GI tract of humans as well as most animals and insects, but can also be early colonizers in the soil of a new environment (Agudelo, 2014). Both *E. faecium* and its safe relative, *E. faecalis* are pathogenic species of bacteria that can gain antibiotic resistance to ampicillin and vancomycin, however *E. faecium* is inherently more antibiotic resistant than *E. faecalis* (Agudelo, 2014). In 2008, *Enterococcus*

species were the cause of approximately 12% of hospital associated infections with *E. faecalis* causing a majority of device-related infections (specifically in central lines, urinary drainage, catheter, and ventilator devices) (Hidron, 2008).

1.7.2 *Staphylococcus aureus*

S. aureus is a Gram-positive bacterium with a coccus shape that forms grape-like clusters. It is commonly found on the skin and in mucus membranes, e.g. in the nose, but can cause skin infections like impetigo. In more severe cases, *S. aureus* can enter the bloodstream through an open wound and cause a wide range of life-threatening infections from bacteremia, to pulmonary infections (Mulani, 2019). Transmission usually occurs from direct contact, and immunocompromised individuals are more susceptible to infection, but healthy people can be infected as well. *S. aureus* infections are usually treated with penicillin antibiotics first, and a combination of antibiotics if the strain is resistant to penicillin (Taylor, 2020).

1.7.3 *Klebsiella pneumoniae*

K. pneumoniae is a Gram-negative, rod-shaped bacterium. It can be found on the skin, and in the mouth and intestines and can cause lung infections (Mulani, 2019). It is encapsulated and protected from harsh environments, e.g. during antibiotic exposure and immune activation, which makes it more virulent than its unencapsulated relatives. For *K. pneumoniae*, the bacterial capsule makes a protective polysaccharide coating around the bacterium that prevents the bactericidal effects of serum and phagocytosis (Struve, 2003). It is the leading cause of hospital-acquired pneumonia, and is especially dangerous for individuals with a compromised respiratory tract typically found in cystic fibrosis patients. It accounts for between 3-8% of all nosocomial bacterial infections (Ashurst, 2020).

1.7.4 *Acinetobacter baumannii*

A. baumannii is a Gram-negative, rod-shaped opportunistic nosocomial bacterium that primarily affects immunocompromised patients. *A. baumannii* can cause respiratory, wound, and urinary tract infections (Mulani, 2019), but mostly causes ventilator associated pneumonia and bacteremia, which can have a mortality rate of 35% (Antunes, 2014). Its pathogenicity is mostly due to its modified surface protein OmpA that binds to host epithelia. *A. baumannii* can form biofilms enabling it to survive antibiotic treatment (Howard, 2012).

1.7.5 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative rod-shaped bacterium, and is motile due to its flagella. It can be found in water and soil as well as on fresh produce. It often forms biofilms to defend against antibiotics and the immune response. This species also uses ß-lactamase as a defense against penicillin. *P. aeruginosa* can cause respiratory infections like pneumonia especially in cystic fibrosis patients, and can cause wound infections and UTIs (Mulani, 2019). It is an opportunistic pathogen and often acquired in the healthcare setting, accounting for 9% of all nosocomial bacterial infections (CDC, 2019).

1.7.6 *Enterobacter* sp.

Enterobacter is a genus of Gram-negative bacteria that are rod-shaped and can survive in the presence or absence of oxygen. *Enterobacter* sp. are found in the GI tract of humans and also in nature (Davin-Regli, 2015). *Enterobacter* sp. are particularly important because they can cause nosocomial opportunistic infections and can gain multi-drug resistance. Species of *Enterobacter* can limit the permeability of their cell membrane and also easily produce enzymes that can cause the degradation/inactivation of many antibiotics (Davin-Regli, 2015). *Enterobacter* sp. can also easily gain many mobile genetic elements that can contribute to this multidrug antibiotic

resistance. While *Klebsiella aerogenes*, previously known as *Enterobacter aerogenes*, can be a pathogenic bacteria that can cause bacteremia, it is less pathogenic than other species related to *Enterobacter* sp. (Davin-Regli, 2015).

1.8 ESKAPE Relatives and Virulence

The measure of pathogenicity of an organism is called virulence. Virulence factors include adherence and invasion ability, and toxin production (Peterson, 1996). Bacteria can have surface components that aid in attaching to or invading the host. Other bacteria protect themselves with a capsule to keep the host from killing it. Once inside a cell, the bacteria can produce exotoxins, or release endotoxins that disrupt the host activity. These factors help bacteria invade their host, avoid host defenses, and cause disease (Peterson, 1996). The ESKAPE pathogens express high virulence and easily cause disease. Because the ESKAPE pathogens are more dangerous to handle and study, their safe relatives are studied instead. These relatives include *Bacillus subtilis, Escherichia coli, Acinetobacter baylyi, Staphylococcus epidermidis, Klebsiella aerogenes,* and *Enterococcus faecalis.* These bacteria are closely related to the ESKAPE pathogens so they react similarly to the ESKAPE pathogens, but they rarely cause disease and are safer for laboratory work (Nichols, 2020). It is also important to note the optimal temperature for these bacteria. The average human body temperature is 37°C, so most pathogens grow best at or around that temperature. However, since the safe relatives are non-pathogenic, they do not necessarily have the same optimal temperatures, as seen in Table 1.

Table 1: Optimum growth temperatures for the ESKAPE pathogens and their nonvirulent relatives (Leung, 2017).

ESKAPE Species	Optimal temp	Safe Species Relative	Optimal temp
Enterococcus faecium	35°C	*Enterococcus faecalis*	35°C
Staphylococcus aureus	37°C	*Staphylococcus epidermidis*	37°C
Klebsiella pneumoniae	37°C	*Escherichia coli*	37°C
Acinetobacter baumannii	37°C	*Acinetobacter baylyi*	37°C
Pseudomonas aeruginosa	37°C	*Bacillus subtilis*	25-35°C
Enterobacter sp.	37°C	*Klebsiella aerogenes*	37°C

1.8.1 Methicillin Resistant *Staphylococcus aureus*

One of the ESKAPE pathogens, *S. aureus* has certain strains notoriously resistant to antibiotics called Methicillin Resistant *Staphylococcus aureus* (MRSA). MRSA is resistant to methicillin, but is also commonly resistant to other similar antibiotics such as vancomycin, oxacillin, and penicillin (Nichols, 2020). Not only is MRSA resistant to a lot of antibiotics, but it can also survive for a long time on surfaces. For example, the bacteria can survive and carry infection on cotton clothes for up to 21 days, longer than 40 days on polypropylene plastic, and up to 3 days on 60% cotton and 40% polyester fabric (Nichols, 2020). Because this species can survive so long on surfaces, MRSA can spread easily at places where there are infected people, e.g. hospitals. MRSA can be killed by other means when it infects surfaces using alcohol-based cleaners or soap and water (Nichols, 2020). However, these stronger cleaners will harm people if they are ingested or applied to an open wound thus finding new antibiotics that MRSA is not resistant to has become an important task.

MRSA is resistant to the entire class of antibiotics similar to methicillin, a penicillin derivative, because it inhibits the function of ß-lactams (Stapleton, 2002). There are two different ways that *S. aureus* can prevent these effects. One possibility is that the bacteria produces ß-lactamase, an enzyme that will break down ß-lactams like penicillin or methicillin. The other way is through the presence of a gene that encodes for the modified protein, PBP2a (Stapleton, 2002). This protein has a higher preferential binding to the other PBPs effectively preventing ß-lactams from inhibiting cell wall construction, allowing for bacterial proliferation (Stapleton, 2002). Thus, an antibiotic that is not a ß-lactam might be effective against MRSA.

1.9 Preliminary Studies on *Artemisia*

As the prevalence of antibiotic resistant bacteria grows, so does the need for novel antimicrobial compounds. Many studies have been conducted on *Artemisia* species, but *A. annua L.* is the only significant source of artemisinin (Furant, 2011). Essential oils are another group of phytochemicals produced by *A. annua*, however they possess lower antimicrobial activity, and are at high concentrations can be toxic (Radulović, 2013). Ingestion of the oils can cause tremors and irregular breathing, so they are less suited to use as a treatment (Radulović, 2013). This suggests that dried leaf extracts or direct ingestion of dried leaves, powdered and encapsulated for ease of delivery, might be possible options as a useful antimicrobial treatment as opposed to essential oils.

A. annua extracts containing the compound artemisinin possess antimicrobial properties, however it is still unclear if the extracts work better against Gram-positive or Gram-negative bacteria. While some studies agree that Gram-positive bacteria are susceptible to artemisinin (Appalasamy, 2014); (Poiată, 2009), there is disagreement about how effective the *Artemisia*

extracts are against Gram-negative bacteria. One study in Romania used *A. annua* taken from aerial parts during the flowering stage. It was powdered before 10g were added to 50ml extracts of ethanol, methanol and hexane. All extracts had moderate to no activity against Gram-negative bacteria (Poiată, 2009). A different study cultured *A. annua* with seeds from Vietnam with MS medium and dried the plants after 8 weeks. The aerial parts were extracted with hexane and the extracts showed activity against the two Gram-negative bacteria they tested, *E. coli* and *Salmonella* sp. (Appalasamy, 2014). The lack of information on *A. annua* extracts effect on Gram-negative vs. Gram-positive and the contradictions between existing studies only supports the need for further study.

1.10 Summary and Hypothesis

This project aims to investigate the susceptibility of ESKAPE relatives to *Artemisia annua L.* extracts and if the extracts will be more effective against Gram-positive bacteria. The need for antimicrobial compounds to combat the rise of antibiotic resistant bacteria is crucial, and testing the extracts against ESKAPE relatives could give more insight into what bacteria are the most susceptible. The **hypothesis** of this study is that artemisinin will have greater antimicrobial activity against Gram-negative bacteria over Gram-positive bacteria. Once the susceptibility of the bacteria to the extracts is discerned, it could possibly be used as a treatment against ESKAPE pathogens in the future. Organisms like *E. coli* and *Saccharomyces cerevisiae* are already being bioengineered to synthesize artemisinin and have been approved to treat infections using ACT (Paddon, 2014). Knowing what types of bacteria are susceptible to artemisinin would be a crucial step in determining possible treatments for those antibiotic resistant bacteria that are still artemisinin susceptible.

2.0 Methods

2.1 Tea Infusion Preparation

The *A. annua* extract used was prepared using the method of hot water extract, which is also called a tea infusion (Poiată, 2009) was produced as follows. First 200ml of water was brought to a boil in a beaker with a hot plate. Then 2g of dried 2018 *A. annua* was added to the boiling water, and thoroughly mixed with a stirring rod. After steeping for 10 minutes, the beaker was taken off the hot plate, and large pieces of plant were sieved out using a 2mm brass sieve. The extract was then strained through multiple filters at 5µm, 1.5µm and the smallest being a 0.22µm filter, aliquoted into 10ml vials, and frozen at -20°C until later use.

2.2 Measuring Total Flavonoid and Artemisinin Concentration

The total flavonoid concentration was found with a Spectrophotometric $AlCl_3$ assay and used quercetin as the standard. A 1:1 solution of 2% $AlCl_3$ to MeOH was made, and different amounts of quercetin were dissolved in 1ml of MeOH ranging from 2µg-20µg, and one blank of just $AlCl_3$ was made. Then 1ml of 1% $AlCl_3$ solution was added to each sample, marbles were placed on top to reduce evaporation, and were incubated for 25 min. The flavonoid content of the extract was calculated using the quercetin standard curve (Appendix B, Figure S1) so the flavonoids were expressed as quercetin equivalents (Weathers, 2014). To find the concentration of artemisinin, six replicates of the 10g dried weight per liter hot water extract were analyzed by gas chromatography-mass spectrometry and averaged.

2.3 Finding Log Phase Growth of Bacteria

After determining the different types of broth that could culture each species, the maximum optical densities were recorded for each type of bacteria and some of them - *K.*

aerogenes, S. epidermidis, and *A. baylyi,* had maximum optical densities (OD_{595}) over 1.0 (Figure S2). The bacteria can continue growing and exceed 1.0, but may be past log phase growth, so 1.0 would be the maximum OD allowed when cultivating bacteria in the future. Also, 0.5 OD would be the minimum OD to take out the bacteria from incubation in order to make sure that each culture was in their log phase growth. 0.5 was specifically taken to be the minimum OD because it was a little less than halfway between the starting and final ODs for each of the bacteria (Figure S2).

2.3.1 Cultivation of ESKAPE bacteria

Initially LB broth was used to culture all of the safe relative ESKAPE strains. One liter of LB broth contains 10g tryptone, 5g yeast extract, 10g sodium chloride (MacWilliams, 2006). To make the solid agar version, the recipe is the same except 15g of agar is substituted for 15mL of water (sigmaaldrich.com). The main difference between Lysogeny Broth (LB) and Lysogeny Agar (LA) is that LA is solid and when bacteria are grown on an LA plate, the bacteria does not grow into the agar because it cannot digest the agar portion of LA (MacWilliams, 2006). Therefore, bacterial growth on the surface of the plate only spreads outwards and a zone of inhibition can be measured empirically by the diameter of that zone.

Another broth used was Todd Hewitt Broth (TH broth). Because *E. faecalis* was known to grow well using TH broth (Medrek and Barnes, 1961), the three safe relatives that did not have sufficient growth after 8 hours were grown using TH broth. TH broth contains 3.1g heart infusion solids, 20g neopeptone, 2g glucose, 2g sodium chloride, 0.4g disodium phosphate, and 2.5g sodium carbonate (Hernandez et al., 2018).

2.4 Testing bacterial susceptibility to an aqueous plant extract

2.4.1 Screening for drug efficacy using the Kirby-Bauer Assay

Agar plates were used to measure zones of inhibition of each bacterial species in response to test drugs. Plates each containing one species of the ESKAPE bacteria were grown until a bacterial lawn was obtained. Bacteria was added to the plate before the disks by pipetting 100µl of a 10^3 dilution of the bacteria while their OD was between 0.5-1 and using beads to gently spread the dilution over the plate. *B. subtilis*, *E. coli*, and *E. faecalis* were grown on TH agar plates. *S. epidermidis, K. aerogenes,* and *A. baylyi* were grown on LB plates. Then the plates were sectioned into thirds by marking the bottom of the plate with a marker, with 1MM Whatman 1cm filter disks centered on each third of the plate. Each disk had an aqueous solution of either tea infusion, pure artemisinin, or water and one of each type of paper disk was put onto a petri dish. If there was a zone of inhibition around one of the disks, that would mean the drug in that disk stopped the bacteria from growing, and the bigger the zone around the disk, the more it killed or inhibited the bacteria. If the pure artemisinin disk had a zone of inhibition, but the plant extract did not, that would likely mean there was not enough artemisinin in the extract to kill or inhibit bacterial growth. If the plant extract inhibited growth, but not the pure artemisinin, then it is likely that other phytochemicals in the plant inhibited that species. The plates were then checked after 24 hours and 48 hours to inspect for zones of inhibition (Appendix A.2).

2.4.2 Comparative growth measurements of the nonvirulent ESKAPE species

To determine the growth kinetics of each species in each drug, we used 96 well CellTreat polystyrene plates (catalog #229196). Each row contained 100µl of a different bacterial species between .5-1 OD, while the top and bottom rows were filled with 200µL water in order to

minimize evaporation. Table 2 depicts the setup of the 96 well plate, with each solution and bacteria mix repeated three times in order to average the results. This was done to compare the effect of the aqueous tea infusion on bacterial growth compared to a solvent control (in this case water), a known bacteriostatic solution (first tetracycline, then chloramphenicol), and a known bactericidal solution (bleach). This way the growth in the tea infusion can be compared to the known agents to see if the tea inhibits growth, kills bacteria, or does nothing.

Table 2: Template diagram of the contents within a typical 96 well plate experiment

	1	2	3	4	5	6	7	8	9	10	11	12
controls	100µL Water Blank	100µL T-Tea	100µL S-Chlora	100µL K-Bleach								
B. subtilis	100µL C 100µL W	100µL C 100µL W	100µL C 100µL W	100µL C 100µL T	100µL C 100µL T	100µL C 100µL T	100µL C 100µLS	100µL C 100µLS	100µL C 100µL S	100µL C 100µL K	100µL C 100µL K	100µL C 100µL K
S. epidermidis	100µL C 100µL W	100µL C 100µL W	100µL C 100µL W	100µL C 100µL T	100µL C 100µL T	100µL C 100µL T	100µL C 100µLS	100µL C 100µLS	100µL C 100µL S	100µL C 100µL K	100µL C 100µL K	100µL C 100µL K
A. baylyi	100µL C 100µL W	100µL C 100µL W	100µL C 100µL W	100µL C 100µL T	100µL C 100µL T	100µL C 100µL T	100µL C 100µLS	100µL C 100µLS	100µL C 100µL S	100µL C 100µL K	100µL C 100µL K	100µL C 100µL K
E. coli	100µL C 100µL W	100µL C 100µL W	100µL C 100µL W	100µL C 100µL T	100µL C 100µL T	100µL C 100µL T	100µL C 100µLS	100µL C 100µLS	100µL C 100µL S	100µL C 100µL K	100µL C 100µL K	100µL C 100µL K
E. faecalis	100µL C 100µL W	100µL C 100µL W	100µL C 100µL W	100µL C 100µL T	100µL C 100µL T	100µL C 100µL T	100µL C 100µLS	100µL C 100µLS	100µL C 100µL S	100µL C 100µL K	100µL C 100µL K	100µL C 100µL K
K. aerogenes	100µL C 100µL W	100µL C 100µL W	100µL C 100µL W	100µL C 100µL T	100µL C 100µL T	100µL C 100µL T	100µL C 100µLS	100µL C 100µLS	100µL C 100µL S	100µL C 100µL K	100µL C 100µL K	100µL C 100µL K
Water												

W= water, C= bacterial colony solution, T= tea infusion, S= chloramphenicol (15µg/ml), K= bleach (5% concentrated solution)

Every well has 100µL of its respective bacterial culture (C) and 100µL of the other added solution, besides the controls that had bacterial broth but not colonies. For a typical 96 well plate, the first three columns were given 100µL bacterial culture and 100µL water (W). Columns 4 to 6 contained 100µL of culture and 100µL of tea extract (T). Columns 7 to 9 contained 100µL of culture and 100µL of a known bacteriostatic antibiotic (S) while columns 10 to 12 contained 100µL culture and 100µL of a known bactericidal solution (K). The columns were triplicates in order to average data, and the empty wells were filled with 200µL of water. A plate reader was used at 595nm to determine the absorbances at the start of the experiment, then at 3,6,9,12, and 24 hours after the start.

For the 96 well plate experiments, the bactericidal agent was usually a bleach solution with 0.685% concentration of sodium hypochlorite. The other bactericidal agent used was a combination treatment of streptomycin and penicillin (15µg/ml). This combination is effective against both Gram-positive and Gram-negative bacteria so it should be an effective antibiotic against all ESKAPE safe relatives. Penicillin works by breaking apart the cell wall triggering autolysins to kill the cell, while streptomycin binds to the ribosomes and prevents protein synthesis (Suarez, 2009; Sharma et al., 2007).

The bacteriostatic antibiotics used in the 96 well plate were tetracycline (15µg/ml) and chloramphenicol (15µg/ml). Tetracycline was used because it is a broad-spectrum antibiotic with bacteriostatic effects. Tetracycline binds to ribosomes preventing protein synthesis (Connell, 2003). Chloramphenicol was also used as a bacteriostatic antibiotic, and was found to be more effective than tetracycline when testing against the nonvirulent ESKAPE species. Tetracycline did not inhibit growth of the bacteria in initial experiments, so the more effective chloramphenicol was used in later experiments (Figure S5).

3.0 Results

3.1 Agar plates with paper disks containing tea extract

A. annua tea extracts were tested for anti-ESKAPE activity by creating a series of bacterial lawns for each bacteria. The bacterial lawns were grown using bacteria from the log phase experiments diluted to 10^{-1}, 10^{-2}, and 10^{-3} bacterial colony solution (Appendix A.3).

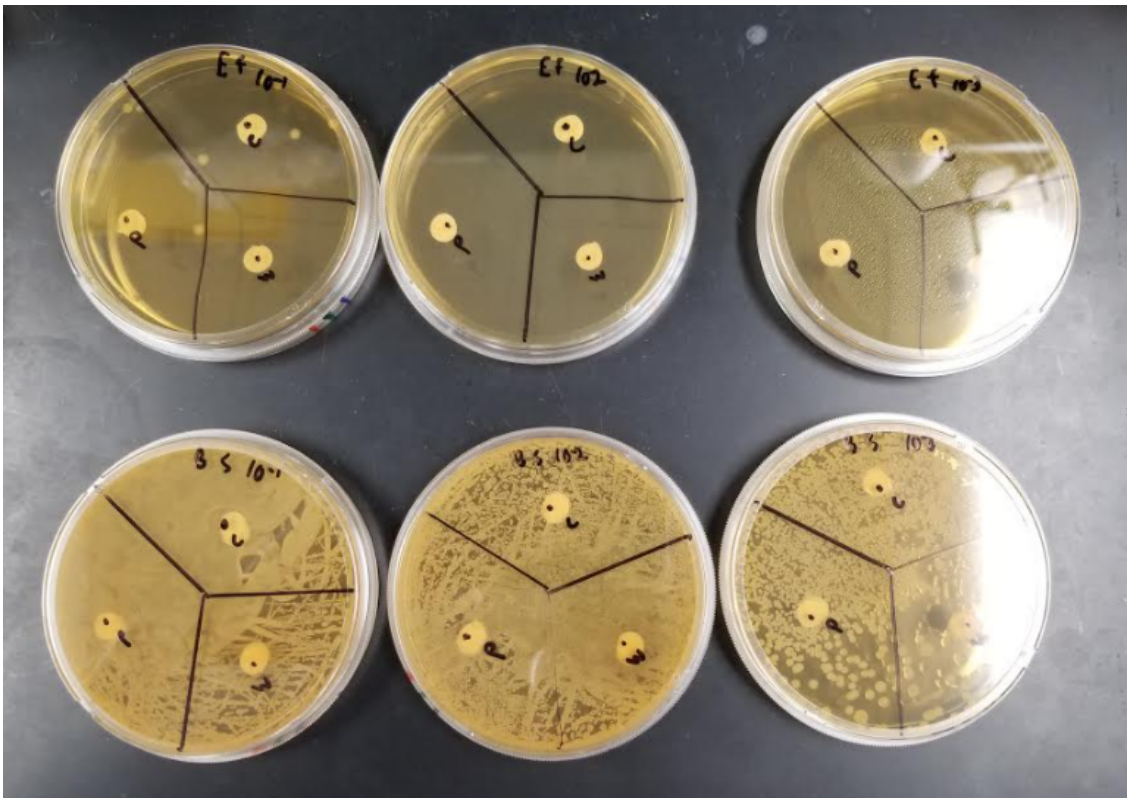

Figure 4: *E. faecalis* (top) and *B. subtilis* (bottom) bacterial lawns after 24 hours of incubation at 35ºC using TH agar plates. Each row left to right shows 10^{-1}, 10^{-2}, and 10^{-3} bacterial dilutions. The three white disks in each plate contain 50 uL of each solution: C, control (water), P, pure artemisinin (0.211mg/ml), W, plant hot water extract (50 uL of a 10 g/L hot water extract).

In Figure 4, the left samples had a concentration of 10^{-1}, the middle samples were 10^{-2}, and the right samples were 10^{-3} of bacterial solution. The top segment of each plate contained a control disk (C), while the left segment contained a disk of pure artemisinin (P), and the right

segment contained a disk with whole plant extract (W). Each disk contained 50µL of their respective solution.

For the bacterial lawns cultured on TH agar, *E. faecalis* did not have enough growth on any of the lawns to detect a zone of inhibition. In contrast, *B. subtilis* had a substantial amount of growth at all concentrations plated and there were no zones of inhibition observed (Figure 4). Both *B. subtilis* and *E. faecalis* bacterial lawns were grown with the same amount of liquid culture, so it is likely that the *E. faecalis* culture did not have a lot of bacteria to begin with.

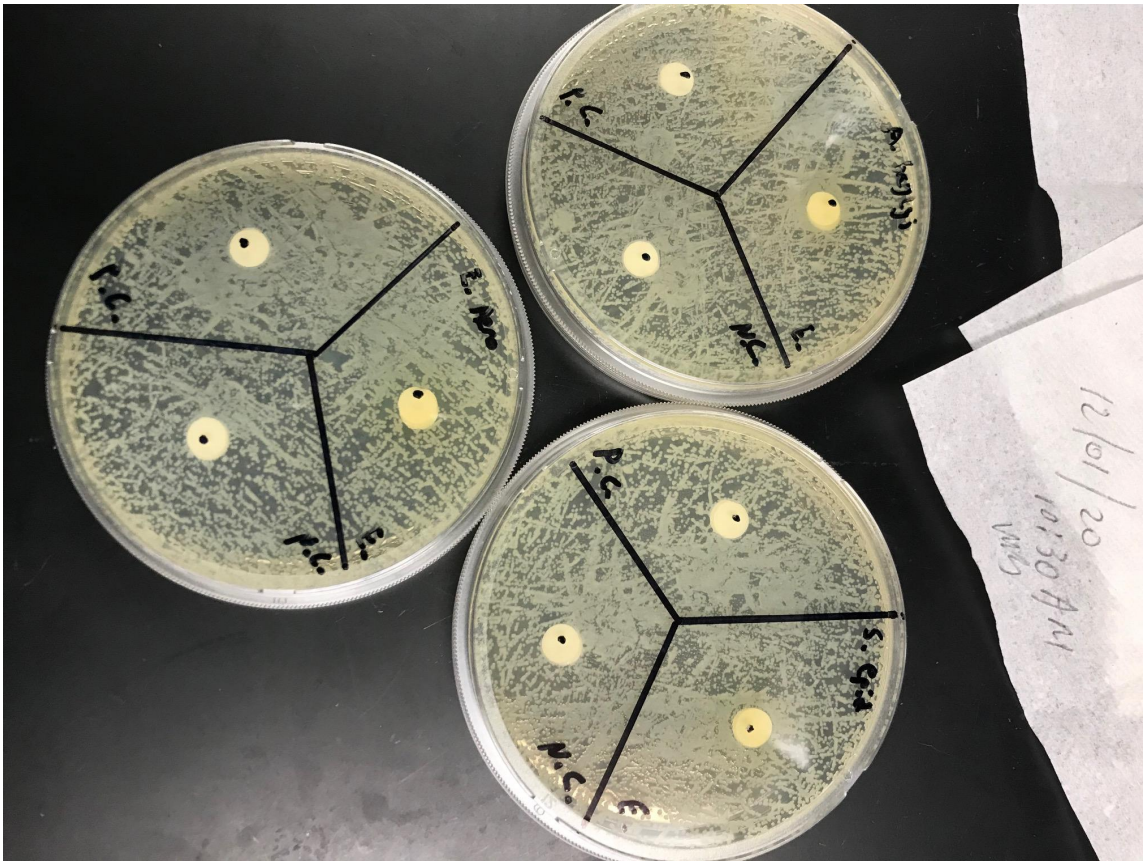

Figure 5: *K. aerogenes* (left), *A. baylyi* (top), and *S. epidermidis* (bottom) bacterial lawns at 10^{-1} bacterial solution incubated for 24 hours at 37°C on LB agar. Three paper disks containing a different chemical were added to each plate indicated by a dot. The top disk for each plate contained 50µL of pure artemisinin (P.C.), the bottom left disk contained 50µL of water (N.C.), and the bottom right disk contained 50µL of tea extract (E.).

The LB agar plates also did not contain any zones of inhibition. For each of the bacteria, the 10^{-1} solution had no visible inhibition around the disks after 24 hours of incubation, which shows that there was no clear distinction between the Gram-positive and Gram-negative bacteria and no apparent inhibition of these bacteria by either artemisinin or the plant extract (Figure 5).

Along with the experiments done in Figure 4 and Figure 5, there was a separate experiment carried out where three plates were grown for each type of bacteria. 18 agar plates were inoculated with one of the ESKAPE pathogen safe relatives (three plates for each pathogen). Directly after inoculation, 200µL of water, tea extract, or pure artemisinin were added onto separate bacterial lawns and spread using glass beads before beginning a 24 hour incubation period at 36ºC. This experiment gathered subjective results looking at the relative densities of colony formation between each bacteria lawn for a species. After a 24 hour incubation, none of the plates seemed to have any significant reduction in colony formation among one another (Figures S3 and S4).

3.2 Growth kinetics of ESKAPE pathogens in liquid culture.

Considering that it is possible there may have been inadequate drug diffusion from the disks into the surrounding agar, we subsequently measured ESKAPE pathogen growth in liquid culture using a 96 well plate (Figure 6). The water control is shown in Figure 6A and the tea infusion in Figure 6B. Growth slowly increases in both water and the tea infusion suggesting no inhibition of the pathogens. In contrast, when the bacteriostatic agent, chloramphenicol (Figure 6C), was added to the bacteria, the optical density of the cultures declined indicating that there was no new growth after inoculation. Growth also declined in the presence of bleach, except for *B. subtilis* that had a surprising and sudden growth spurt at 12 hr (Figure 6D).

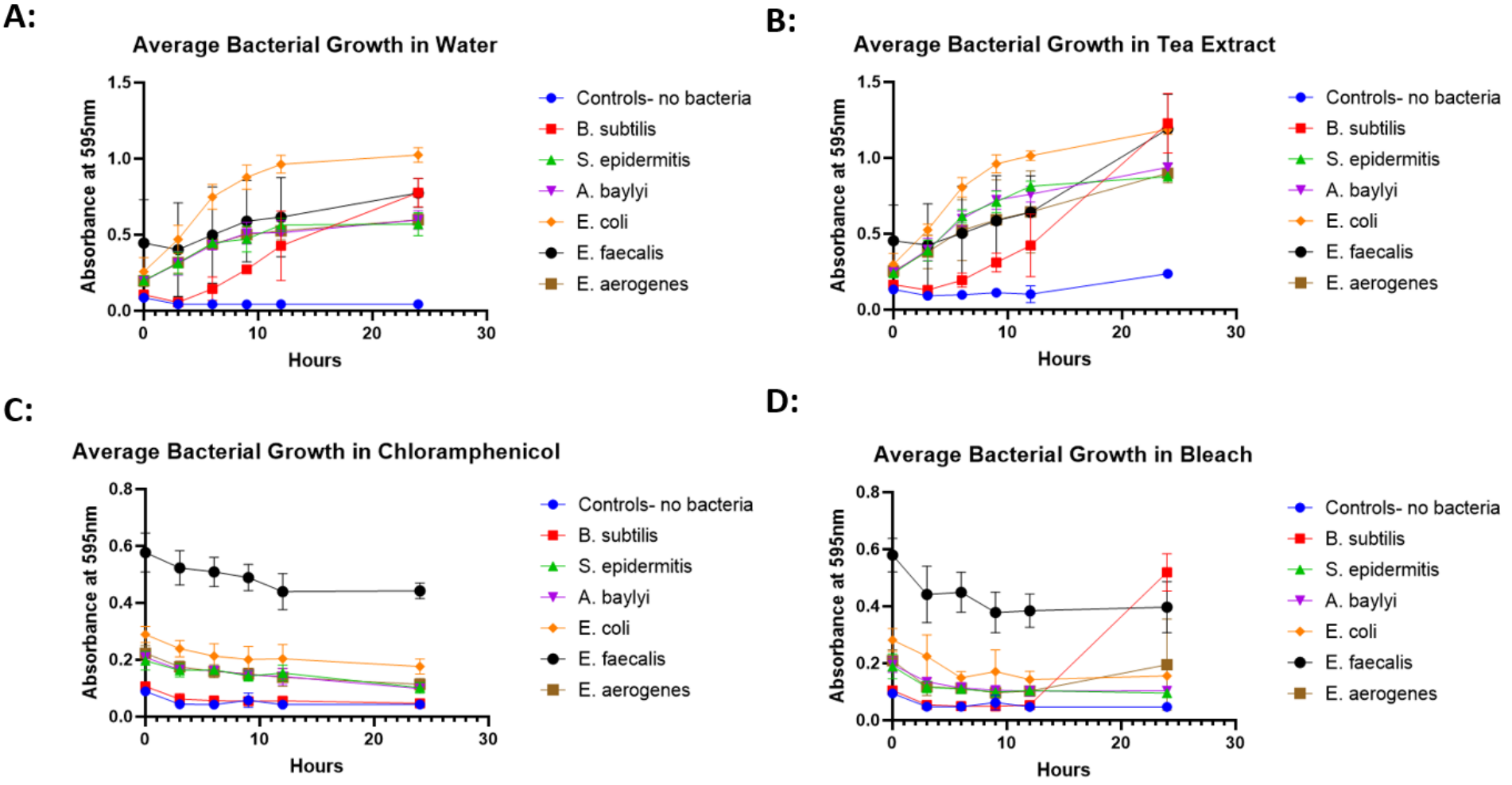

Figure 6: Average of bacterial growth over 24hrs for water, tea infusion, chloramphenicol, and bleach solutions. Error bars represent standard error over three experiments.

4.0 Discussion

From these experiments, the antimicrobial properties of hot water extracts of *A. annua* were evaluated for the six ESKAPE pathogens. These experiments used a hot water extraction method of dried *A. annua* leaves to extract artemisinin and other phytochemicals. In comparison, other researchers used different extraction methods that resulted in what seemed to be a much stronger inhibition of microbes. For example, Ćavar, 2012 used hydrodistillation of the aerial parts of the plant for two hours. That used hot water and steam to free the bioactive compounds to obtain the essential oils, which were then extracted using dichloromethane and dried over anhydrous sodium sulfate (Ćavar, 2012). Subsequently, they observed that their 10mg/mL essential oil extract inhibited many of the bacteria we also tested including *S. aureus*, *B. subtilis*, *E. faecalis*, *E. coli*, and *P. aeruginosa* (Ćavar, 2012). While that study did not measure the relative abundance of artemisinin, they did find that 10mg/mL essential oil solution was most effective against *S. pneumoniae* with a 50mm zone of inhibition (ZOI). They also observed that the essential oil extract created a 20mm ZOI for *S. aureus*, a 20mm ZOI for *B. subtilis*, a 27mm ZOI for *E. faecalis*, a 20mm ZOI for *E. coli*, and a 15mm ZOI for *P. aeruginosa* (Ćavar, 2012) indicating that the extraction for essential oils provided more antimicrobial activity than our aqueous extraction. While pure essential oils can be toxic, *A. annua* essential oil as part of a tea infusion or dried leaf material is non-toxic, and can be safely consumed at naturally occurring levels (Desrosiers et al., 2019; Tisserand & Young, 2013).

Another study used methanol, ethanol, and hexane solvents to extract plant tissues and tested the extracts against bacteria and fungus. They found that alcoholic extracts of *A. annua* were the most effective against the tested microorganisms (Poiată, 2009). Supporting that conclusion, a later study using n-hexane to extract artemisinin from *A. annua* found the extract

was especially active against gram-negative bacteria, and also tested *B. subtilis*, *S. aureus* and *E. coli* (Appalasamy, 2014). That extract contained 6mg/ml of artemisinin and inhibited *B. subtilis* and *S. aureus*.

The average concentration of artemisinin in the hot water extracts for this experiment was 0.133 mg/ml. This was lower than some other studies that used other solvents. One study that used water, methanol, ethanol, and acetone extracts found that water had the lowest concentration of artemisinin at 0.081 mg/ml, while methanol, ethanol, and acetone had 0.171, 0.154, and 0.211 mg/ml of artemisinin, respectively (Kim WS, 2014). Another study that tested methanol, ethanol, and hexane extracts against bacteria and fungus found that alcoholic extracts of *A. annua* were the most effective against the tested microbes (Poiată, 2009). Although that study suggested that artemisinin was not fully extracted from the plant leaves into the hot water, analyses of the extracts used in our study contradicted that conclusion. About 99% of the artemisinin was extracted into the hot water extracts used in this study. There may, however, be other compounds in the plant that do not extract into the hot water and thus use of other solvents are recommended for future work on the efficacy of *A. annua* vs. ESKAPE pathogens.

5.0 Conclusion

The hypothesis that hot water extracts would work better against gram-positive bacteria compared to gram-negative bacteria was not supported. The extract was not effective against any of the ESKAPE relatives. The lack of inhibition on the agar plates coupled with bacterial growth in all tested species in the extract solution suggested that a tea infusion (hot water extract) does not kill or inhibit growth of these bacteria. The next steps to further investigate the antimicrobial properties of *A. annua* extracts against ESKAPE pathogens would be to use a different extraction solvent. There have been some antibacterial properties shown when *A. annua* was extracted using either methanol or ethanol (Kim WS, 2014). Another future step could be to test chloramphenicol as a positive control on the solid agar plates in order to validate that the plated bacteria were truly resistant to both *A. annua* extract as well as pure artemisinin.

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Appendix A: Standard Operating Procedure

A.1 *A. annua* tea infusion extract

1. Measure out 2g of dried *Artemisia annua* leaves
2. Boil 200mL of water and infuse the leaves with this boiling water for a total of 10 minutes
3. Use a sieve to discard large pieces of debris from the sample
4. Use a vacuum filtration device with a maximum filtration size of 0.22µm in order to filter out debris.
5. Use sterile technique, store in separate 15mL tubes and freeze extract at -20^{o}C.

A.2 Log phase growth

1. Take the 6 plated ESKAPE relative pathogens
2. Take the LB broth solution and pipette 45mL of broth into 4 different erlenmeyer flasks that have breathable caps.
3. Using the sterile inoculation procedure, inoculate three of the four erlenmeyer flasks with *S. epidermidis*, *A. baylyi*, and *E. aerogenes*.
4. Place them in a shaker incubator set at 37^{o}C with and shake the flasks at 220rpm.
5. Let all four flasks incubate and check optical density every hour four 12 hours.
6. Graph optical density vs. time to find the time of log phase growth for each bacteria
7. Take the Todd Hewitt broth solution and pipette 45mL of broth into 4 different erlenmeyer flasks that have breathable caps.
8. Using the sterile inoculation procedure, inoculate three of the four erlenmeyer flasks with *B. subtilis*, *E. coli*, and *E. faecalis*.
9. Place them in a shaker incubator set at 35^{o}C with and shake at 220rpm. Let all four flasks incubate for 12 hours, checking optical density every hour.
10. Graph optical density vs. time to find the time of log phase growth for each bacteria

A.3 Agar plates to find antibiotic resistance

1. Using the procedure in Appendix A.2, grow separate colonies for all of the ESKAPE safe relatives until they are in the log growth phase. Once they have reached this log growth phase, take them out of the incubator and store them in the 4^{o}C refrigerator until all of the colonies have grown into the log phase.
2. Pipette 1000µL of each colony into 3 separate 1.5mL pipette tubes
3. Fill 5 more 1.5mL tubes with 900µL of water for each strain tested. Pipette 100µL of the first colony into another tube, vortex that tube, then pipette 100µL from the new tube into

another tube. Do this until there are bacterial concentrations of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} for each strain

4. For each strain, pipette 200µL of the 1 times bacterial concentration onto their respective agar plate (TH agar plates for *B. subtilis*, *E. coli*, and *E. faecalis.* LB plates for *S. epidermidis, K. aerogenes,* and *A. baylyi*). Use approximately 6 glass beads for each plate to spread out the liquid across the entire plate.
5. Divide each of these agar plates into three sections using a ruler and a sharpie.
6. There should be one paper disk for each of the plate segments so three paper disks for each of the agar plates.
7. Using aseptic technique, sterilize a set of tweezers and load 50µL of *A. annua* extract onto one third of the total paper disks. In order to load 50µL, there might need to be 2 paper disks layered on top of one another in order to get all 50µL of solution.
8. Load another one third of the paper disks with 50µL of pure artemisinin solution and the final one third with 50µL of water
9. Place one of each disk solution onto each of the agar plates and incubate for 24 hours before recording zones of inhibition.

A.4 Agar plates without paper disks

1. Using the procedure in Appendix A.2, grow separate colonies for all of the ESKAPE safe relatives until they are in the log growth phase. Once they have reached this log growth phase, take them out of the incubator and store them in the 4°C refrigerator until all of the colonies have grown into the log phase.
2. Pipette 1000µL of each colony into 3 separate 1.5mL pipette tubes
3. Fill 5 more 1.5mL tubes with 900µL of water for each strain tested. Pipette 100µL of the first colony into another tube, vortex that tube, then pipette 100µL from the new tube into another tube. Do this until there are bacterial concentrations of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} for each strain.
4. For each strain, pipette 200µL of the 1 times bacterial concentration onto their respective agar plate (TH agar plates for *B. subtilis*, *E. coli*, and *E. faecalis.* LB plates for *S. epidermidis, K. aerogenes,* and *A. baylyi*). Use approximately 6 glass beads for each plate to spread out the liquid across the entire plate.
5. After a few minutes, add another 200µl of 15µg/ml tetracycline solution directly to each plate, and spread out with beads again.
6. Let all of the plates incubate at 37 degrees C for 24 hours and then check for growth.

A.5 98 well plates with tetracycline

1. Grow each bacteria in it's assigned media in a shaker incubator at 37 degrees C, and check every half hour after 3 hours, and take out once the optical density is between 0.5-1.0
2. Once all the bacteria are taken out, make a 50ml, 0.04125% solution of sodium hypochlorite (5% bleach solution), and a 50ml 15µg/ml solution of tetracycline.
3. Load wells according to table 2, using tetracycline instead of chloramphenicol
4. Incubate at 37 degrees C and use a plate reader to measure absorbance at 595nm at 0,3,6,9,12, and 24 hours.
5. Graph growth kinetics in absorbance vs. time

A.6 96 well plates with chloramphenicol

1. Grow each bacteria in it's assigned media in a shaker incubator at 37 degrees C, and check every half hour after 3 hours, and take out once the optical density is between 0.5-1.0
2. Once all the bacteria are taken out, make a 50ml, 0.04125% solution of sodium hypochlorite (5% bleach solution), and a 50ml 15µg/ml solution of chloramphenicol.
3. Load wells according to table 2.
4. Incubate at 37 degrees C and use a plate reader to measure absorbance at 595nm at 0,3,6,9,12, and 24 hours.
5. Graph growth kinetics in absorbance vs. time

Appendix B: Supplementary Figures

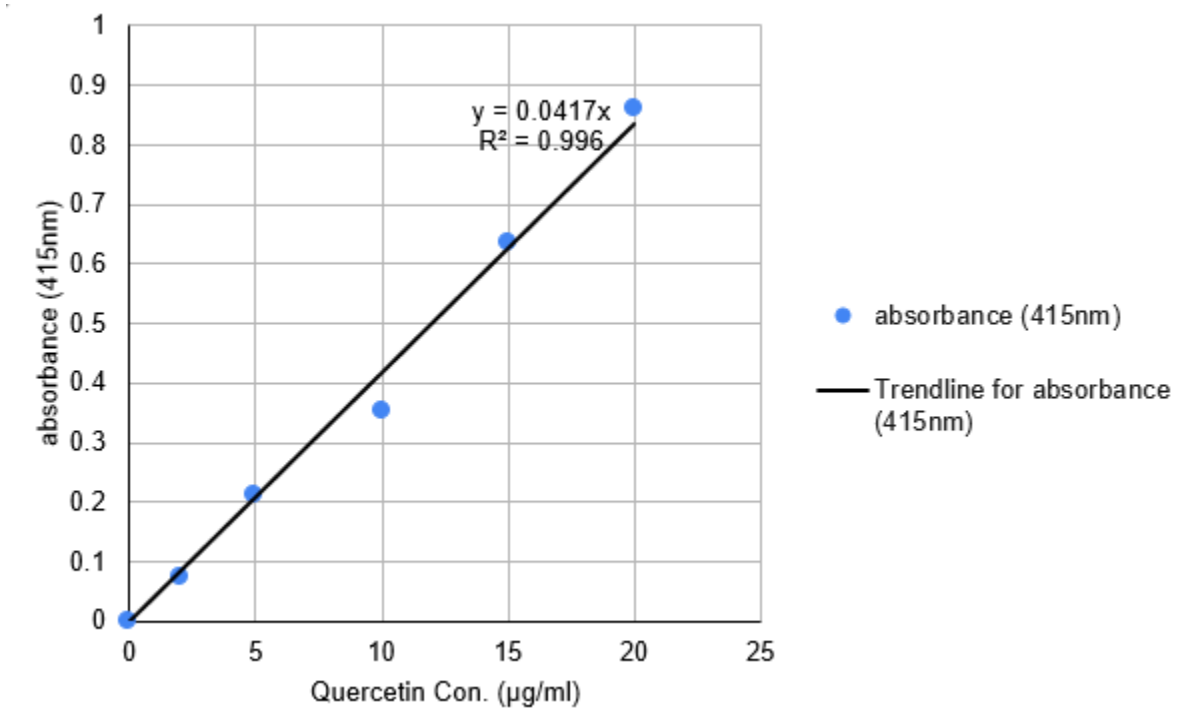

Figure S1: Quercetin Standard curve that was used to calculate the total flavonoid content of the tea extract.

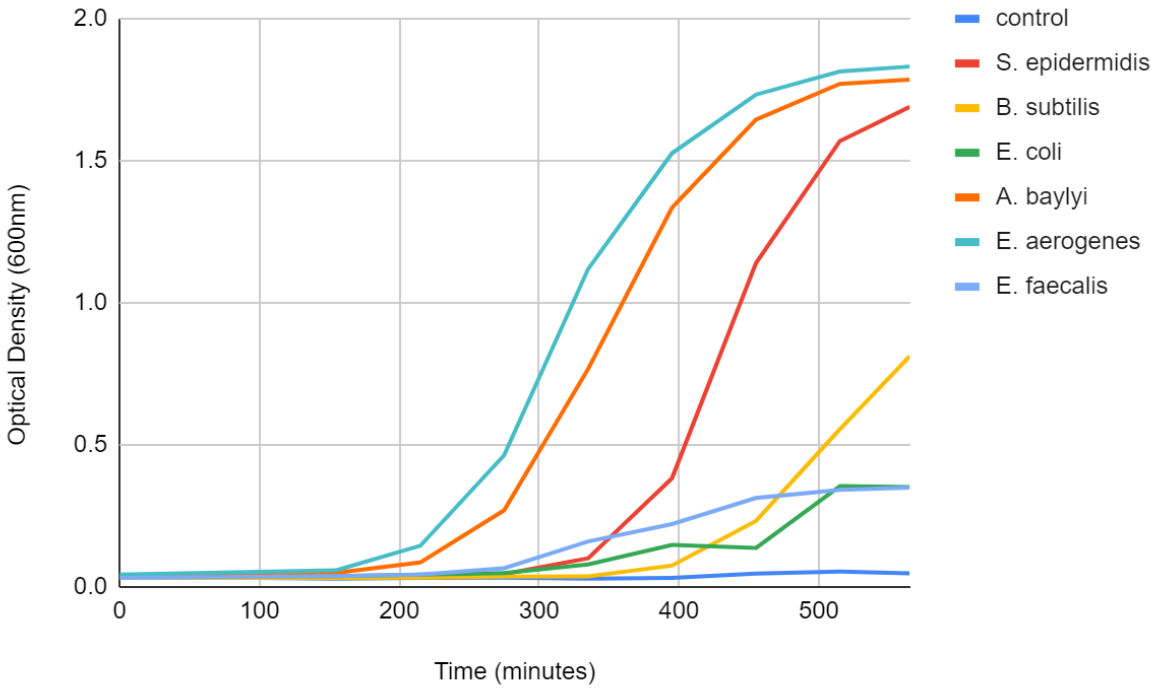

Figure S2: LB broth log phase growth experiment at 37C: log phase growth for *E. aerogenes*, *A. baylyi*, and *S. epidermidis* was found to be around 6 hours, and they begin to plateau around 8 hours after incubation.

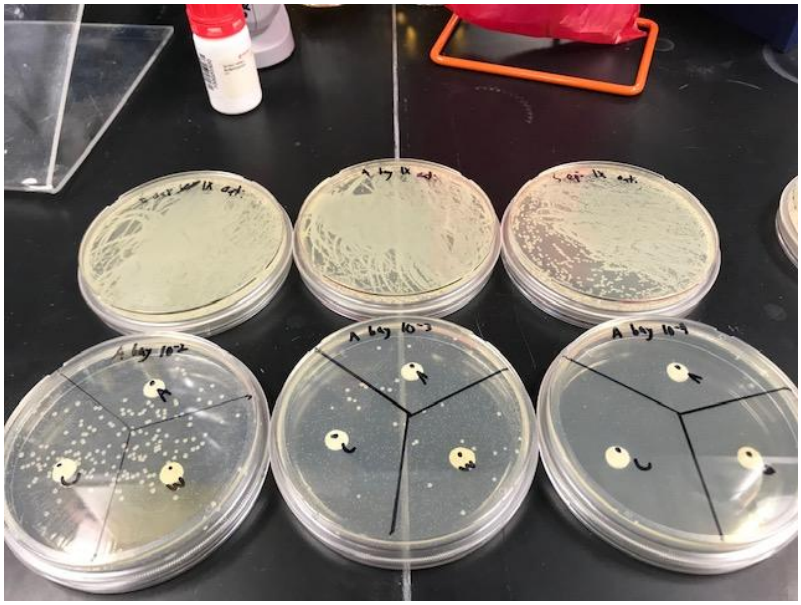

Figure S3: *E. aerogenes* (top left), *A. baylyi* (top middle), and *S. epidermidis* (top right) on LB plates at 10^{-1} bacterial concentration with 200µL of pure artemisinin extract. These plates were incubated at 37°C for 24 hours. Also shown is *A. baylyi* (bottom) at 10^{-2}, 10^{-3}, and 10^{-4} bacterial colony solution concentration.

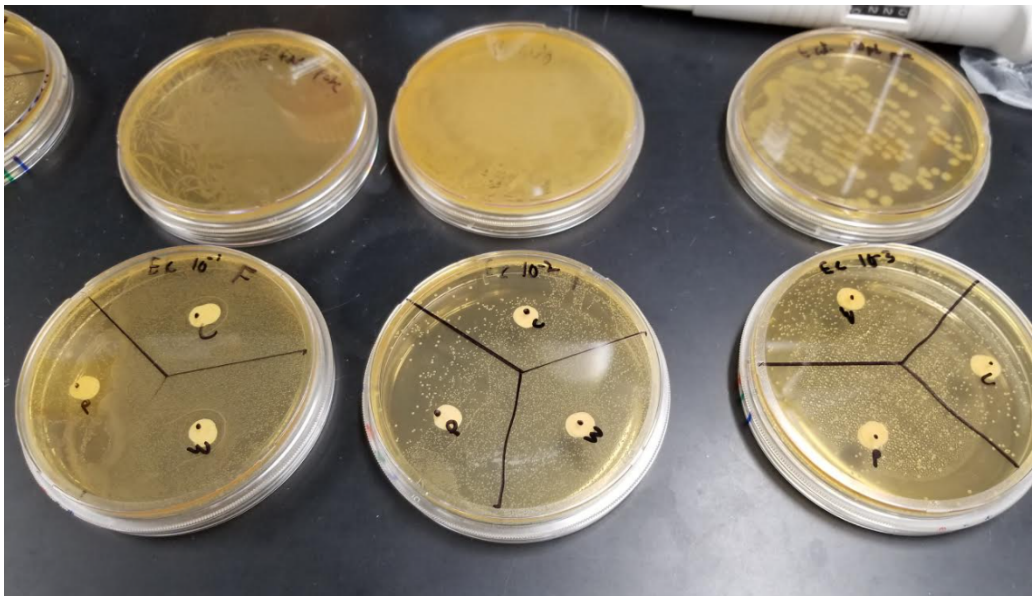

Figure S4: *E. faecalis* (top left), *B. subtilis* (top middle), and *E. coli* (top right) plated on TH agar plates with 200µL of pure artemisinin. Also shown is *E. faecalis* plated at 10^{-1} concentration on left, 10^{-2} in the middle, and 10^{-3} on right. Top part of each plate was control disk (C), left side was pure artemisinin (P), and right side was whole plant extract (W)

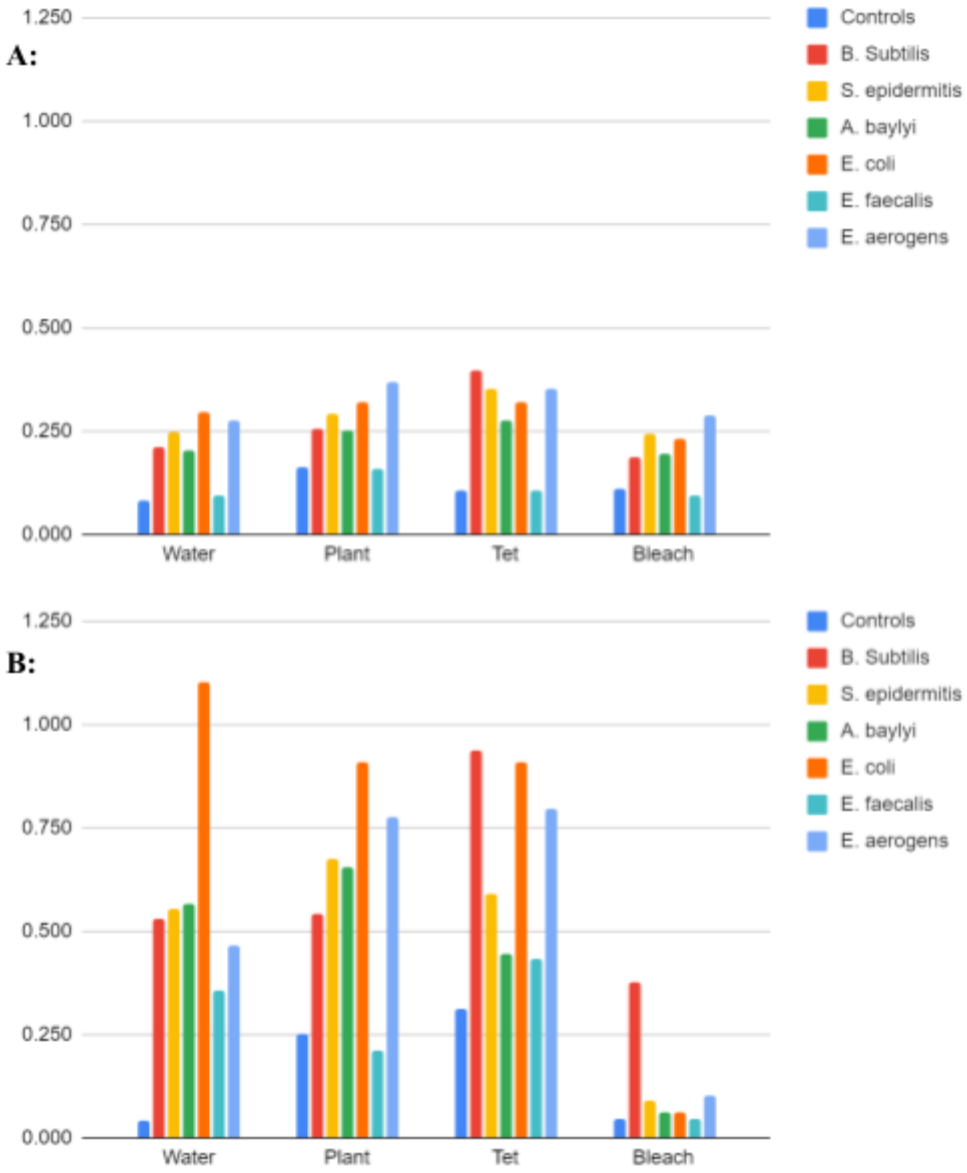

Figure S5: 96 well plate absorbance at 595nm for all bacterial samples at start (panel A) and after 24 hours (panel B). The plate was run using the same layout in Table 2 except tetracycline was used instead of chloramphenicol.