INVESTIGATION OF ANTIMICROBIAL PROPERTIES OF 4-HYDROXYBENZOIC ACID AND ITS APPLICATION IN BONE CEMENT

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ABSTRACT

INVESTIGATION OF ANTIMICROBIAL PROPERTIES OF 4-HYDROXYBENZOIC ACID AND ITS APPLICATION IN BONE CEMENT

The bone cement is composed of polymethylmethacrylate powder and liquid methylmethacrylate which is commonly used in clinical applications. Nosocomial pathogens utilize the bone cement surface to induce infections, so that bone cements are loaded with various antibiotics to inhibit the infection. Nevertheless, the bacteria gain resistance against antibiotics since the antibiotics are released at slow rates. Phenolic acids are secondary metabolites of plants to prevent infections; thus, are promising antimicrobial chemicals against pathogens. The main aim of this study is the investigation of the antimicrobial effect of liquid and powder 4-hydroxybenzoic acid-loaded bone cement on *Escherichia coli*, methicillin-resistant and susceptible *Staphylococcus aureus*. Previously, liquid and powder vancomycin-loaded bone cement had been studied against MRSA and *Candida albicans,* caffeic acid-loaded bone cement is also investigated on the *S. aureus.* This study will be the first investigation that the antimicrobial effect of liquid and powder phenolic acid-loaded bone cement against pathogens. 4-HBA was added directly into polymethylmethacrylate powder and mixed with liquid methylmethacrylate. Cement discs were prepared by metal mold and the antimicrobial assay was carried out to determine the bacterial growth inhibition. The structural features of bone cements such as pores and surface were characterized SEM. The antimicrobial agent release of bone cement was also examined by spectrophotometer and HPLC was used for confirmation. Resistance development against 4-HBA was tested on MRSA and *Acinetobacter haemolyticus* by exposing them to the sub-inhibitory concentrations. The possible genes for the resistance were checked by ddPCR. The significant inhibition of bacterial growth by 4-HBA-loaded bone cement is expected and liquid 4-HBA is expected to be released at a higher rate. Therefore, the phenolic acids may be used as antimicrobial agents in bone cement to inhibit pathogenic bacterial growth.

ÖZET

4-HİDROKSİBENZOİK ASİTİN ANTİMİKROBİYAL ÖZELLİKLERİNİN İNCELENMESİ VE KEMİK ÇİMENTOSUNDA UYGULAMASI

Kemik çimentosu, klinik uygulamalarda yaygın olarak kullanılan polimetilmetakrilat tozu ve sıvı metilmetakrilattan oluşur. Nozokomiyal patojenler, enfeksiyonları indüklemek için kemik çimentosu yüzeyini kullanır, bu yüzden kemik çimentolarına enfeksiyonu önlemek için çeşitli antibiyotikler yüklenir. Ancak antibiyotikler yavaş salındığından dolayı bakteriler antibiyotiklere karşı direnç kazanır. Fenolik asitler, enfeksiyonları önlemek için bitkilerin ikincil metabolitleridir; bu nedenle patojenlere karşı umut verici antimikrobiyal kimyasallardır. Bu çalışmanın temel amacı, sıvı ve toz 4-hidroksibenzoik asit yüklü kemik çimentosunun *Escherichia coli*, metisiline dirençli ve duyarlı *Staphylococcus aureus* üzerindeki antimikrobiyal etkisinin araştırılmasıdır. Daha önce sıvı ve toz vankomisin yüklü kemik çimentosu MRSA ve *Candida albicans'a* karşı çalışılmış, kafeik asit yüklü kemik çimentosu *S. aureus* üzerinde incelenmiştir. Bu çalışma, sıvı ve toz fenolik asit yüklü kemik çimentosunun patojenlere karşı antimikrobiyal etkisinin ilk araştırması olacaktır. 4-HBA, polimetilmetakrilat tozuna doğrudan eklenecek ve sıvı metilmetakrilat ile karıştırılacaktır. Çimento diskler metal kalıp ile hazırlanacak ve bakteri üremesinin inhibisyonunu belirlemek için antimikrobiyal tahlil yapılacaktır. Gözenekler ve yüzey gibi kemik çimentolarının yapısal özellikleri SEM ile karakterize edilecektir. Kemik çimentosunun antimikrobiyal madde salınımı spektrofotometre ile incelenecek ve doğrulama için HPLC kullanılacaktır. 4-HBA'nın direnç gelişimi, MRSA ve *Acinetobacter haemolyticus* üzerinde sub-minimal konsantrasyonlara maruz bırakılarak test edilecektir. Direnç için olası genler ddPCR ile kontrol edilecektir. 4-HBA yüklü kemik çimentosu tarafından bakteri üremesinin önemli ölçüde engellenmesi ve sıvı 4-HBA'nın daha yüksek bir oranda salınması beklenmektedir. Bu sayede fenolik asitler, patojenik bakteri üremesini engellemek için kemik çimentosunda antimikrobiyal ajanlar olarak kullanılabilir.

Dedicated to my beloved family who are always besides me and support me no matter what.

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µg	Microgram
µL	Microliter
2D	Two dimension
2-HBA	2- hydroxybenzoic acid
3-HBA	3-hydroxybenzoic acid
4-HBA	4- hydroxybenzoic acid
ABC transporter	ATP-binding cassette transporter
ATP	Adenosine triphosphate
bp	Base pair
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
Cys	Cysteine
ddPCR	Digital Droplet PCR
ExPEC	Extraintestinal pathogenic *E. coli*
g	Gram
GTP	Guanosine triphosphate
Genta	Gentamicin
h	Hour
HBA	hydroxybenzoic acid
HCA	hydroxycinnamic acid
HPLC	High-Performance Liquid Chromatography
L	Liter
MBC	Minimum bactericidal concentration
MDR	Multidrug resistance
mg	Milligram
MIC	Minimum inhibitory concentration
mL	Mililiter
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
nm	Nanometer

OD	Optical density
PBP	Penicillin binding proteins
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PMMA	Polymethylmethacrylate
ppm	mg/mL
rpm	Rotation per minute
SCC	Staphylococcal cassette chromosome
SCVs	Small colony variants
sec	Second
SEM	Scanning Electron Microscopy
Ser	Serine
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U.S.A	United States of America
WHO	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1. Bacterial Infection

Bacteria are crucial microorganisms that maintain the ecological balance as well as the normal flora of living organisms such as humans. There are various reservoirs for bacteria, such as animals for syphilis, air for tuberculosis, soil for tetanus, food for Vibrio, and water for Shigella disease. Only a few of the bacteria cause infection by colonizing the host. Nevertheless, bacterial infections are considered a major threat to public health. The disease is induced by the abundance of the bacteria in the body or the body's immune response against the bacteria. Transmission of bacterial infections by organisms is easy via air, droplets, vectors, and direct contact with contaminated materials or patients (Doron, Gorbach, 2008).

Prevention of bacterial infections is extremely important in reducing morbidity and mortality, especially since antibiotic resistance is on the rise. Prevention of bacterial infections is divided into three main stages: i) removal of the source of infection; ii) elimination of transmission; and iii) protection of the host. The removal of the source of infection includes proper water treatment, sterilization of the air in the hospital, and protection of food from contamination. Elimination of transmission involves protection from zoonotic diseases through vaccination of animals, proper consumption of meat, and proper equipment for handling. Finally, hosts are protected from infection by passive or active immunization. Passive immunization occurs through the transfer of antibodies against bacteria, active immunization develops through the transfer of a small number of bacteria to the organism, and the immune system produces antibodies against bacteria. Treatment of bacterial infection involves using an antimicrobial agent that inhibits the growth of bacteria (bacteriostatic) or kills the bacteria (bactericidal). The use of antimicrobials kills all the bacteria in the organism, including normal flora, which reduces the immunity of an organism (Doron, Gorbach, 2008).

The Centers for Disease Control and Prevention (CDC) has listed bacterial infections as critical, serious, and concerning due to their importance to healthcare. Critical threats include carbapenem-resistant *Acinetobacter, Clostridioides difficile,* carbapenem-resistant *Enterobacterales,* drug-resistant *Neisseria gonorrhoeae,* etc. Serious threats are based on the infection by drug-resistant *Candida,* multidrug-resistant *Pseudomonas aeruginosa,* drug-resistant *Shigella,* methicillin-resistant *Staphylococcus aureus* (MRSA), drug-resistant tuberculosis, etc. The concerning threats are resistant groups A and B of *Streptococcus*. *As listed,* the most serious bacterial infections are caused by drug-resistant bacteria, . Antimicrobial resistance causes more than 2.8 million infections each year in the U.S., resulting in more than 35,000 mortality (CDC, 2021). This chapter explains *Pseudomonas aeruginosa*, MRSA, *Acinetobacter haemolyticus*, and *Escherichia coli* because these bacteria were used in experimental setups.

1.1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod-shaped, strictly aerobic bacterium belonging to the Pseudomonadaceae family. *P. aeruginosa* is generally found in the environment such as soils and waters; it is a particularly important bacterium because it can induce infection in both plants and animals. *P. aeruginosa* is an opportunistic bacteria that cause acute and chronic infections in immunocompromised patients, especially those with cystic fibrosis and traumatic burns. *P. aeruginosa* causes infections ranging from tissue and urinary tract infections to fatal septic shock and pneumonia. Patients who use ventilators and catheters are at high risk for infection, as are patients with surgical wounds since *P. aeruginosa* can form a biofilm (Moradali et al., 2017). *P. aeruginosa* caused approximately 32,600 infections in hospitalized patients in the U.S., and 2,700 of these resulted in deaths (CDC, 2019). In addition, this bacterium's intrinsic antibiotic resistance mechanisms lead to multidrug resistance and complicate the treatment of infections. Clinical isolates from the last 13 years showed that 28% are multidrug-resistant isolates, and 15% have extensive drug resistance (Reynolds, Kollef, 2021).

The genome of *P. aeruginosa* is highly plastic. Only 17.5% of the genome is considered the core genome. The accessory genome expresses various virulence factors to trigger the infection of the host and increase its survival. The most common features

of virulence factors are i) flagella and pili, ii) quorum sensing and biofilm formation, iii) type 2 secretion system, and iv) type 3 secretion system to inject effector proteins into host cells (Gellatly, Hancock, 2013). Pili and flagella are crucial for bacterial motility and chemotaxis as the initial attachment to the host cell surface. They are also important for the aggregation of the bacteria to form microcolonies on the host and protect themselves from immune cells and antibiotics (Gellatly, Hancock, 2013).

Quorum sensing is a common bacterial mechanism that allows adaptation to environmental changes. Biofilm formation is highly dependent on quorum sensing. The extracellular polymeric substance surrounding the biofilm reduces the penetration of toxic compounds and increases mechanical resistance. The Psl polysaccharide plays an important role in the early phase of the biofilm by forming spiral arrangements and thus increasing the adherence to the host. It also protects the biofilm from phagocytosis and oxidative stress caused by infection. On the other hand, Pal is a polysaccharide that protects the biofilm from aminoglycoside antibiotics (Moradali et al., 2017). Alginate is another virulence factor for *P. aeruginosa*. It is an exopolysaccharide that forms a mucoid biofilm and protects it from environmental changes by increasing its structural stability. Alginate is particularly found in the clinical isolates of cystic fibrosis patients (Reynolds, Kollef, 2021).

The type 2 secretion system (T2SS) of *P. aeruginosa* is responsible for the secretion of exotoxin A, elastase, protease, and phospholipase (Al-Wrafy et al., 2017). Exotoxin A is produced by the *toxA* gene, which inhibits the protein synthesis of the host by modifying elongation factor 2. The impairment of protein synthesis leads to cell death and tissue damage and triggers bacterial. Inhibition of protein synthesis also impairs the host-cell immune response (Gellatly, Hancock, 2013). Two major elastases are produced by *P. aeruginosa:* LasA and LasB. LasB degrades the collagen of the host cell and reduces the physical barrier of the host. It also degrades lung surfactant proteins and inhibits monocytes' chemotaxis. LasA is a staphylolysin that reduces peptidoglycan stability in the cell wall of staphylococci. The type IV protease degrades immunoglobulin and leads to a decrease in the immune response. The other protease is an alkaline protease that inhibits host complement proteins. Finally, phospholipase H is secreted by T2SS, which degrades surfactant lipids and phospholipids of the host cell membrane as well as lyses of erythrocytes (Al-Wrafy et al., 2017).

The type 3 secretion system (T3SS) of *P. aeruginosa* involves the injection of toxins that are produced by bacteria into host cells. Two important exotoxins produced by *P. aeruginosa* are ExoU and ExoS which are injected into the host. ExoU is a phospholipase that impairs the integrity of the plasma membrane and induces necrosis; ExoS affects the organization of the host cytoskeleton (Gellatly, Hancock, 2013). The *lecA* gene encodes lectin, which is important for host attachment and cell-cell interactions that induce biofilm formation. Lectin increases the permeability of epithelial cells and thus the increase in uptake of exotoxin A by the host. Finally, siderophores are secreted via the T3SS. Siderophores are the iron chelators that are critical for the establishment of chronic infections. When iron is depleted in the environment, *P. aeruginosa* sequesters the iron to induce infection and biofilm (Moradali et al., 2017). Because of this multitude of virulence factors, the World Health Organization (WHO) considers *P. aeruginosa* to be one of the critical priority pathogens for the research and development of new antibiotics (WHO, 2020).

1.1.2. *Staphylococcus aureus*

Staphylococcus aureus is a common inhabitant of the body, generally found in the upper respiratory tract and on the skin, but it is also one of the most well-known opportunistic pathogens. *S. aureus* is a Gram-positive, rod-shaped, facultatively anaerobic bacterium that belongs to the Staphylococcaceae family. Even though *S. aureus* is found as commensal in the normal microbiota of 30% of the human population, it can cause skin and respiratory infections and food poisoning (Tong et al., 2015). Infections caused by *S. aureus* range from mild skin infections such as pimples to life-threatening diseases such as meningitis, toxic shock syndrome, bacteremia, and sepsis. After *Escherichia coli, S. aureus* is the second most common bacterium causing bloodstream infections. In the 1940s, *S. aureus* quickly became a serious threat to hospital-acquired infections, and penicillin was used as a treatment. However, *S. aureus* developed resistance to penicillin through the *blaZ* gene that expresses β- lactamase that degrades β-lactam antibiotics. Then, semi-synthetic penicillin was developed in the 1960s, and the methicillin-resistant *S. aureus* (MRSA) was identified within a year of its use. Methicillin resistance is acquired by horizontal gene transfer of the staphylococcal cassette

chromosome *mec* (SCC*mec*). MRSA colonizes dynamically in the host and increases the risk of infection and persistence (Bouiller et al., 2020).

Pathogenic *S. aureus* generally initiates infections via various virulence factors such as toxins and proteins on the cell surface to inactivate antibodies produced by the host defense mechanism. Another significant virulence factor of *S. aureus* is the formation of a biofilm that can adhere to catheters and implants (Kimmig et al., 2021). The *S. aureus* cells within the biofilm are subpopulations called "small colony variants" (SCVs) that grow slowly. These subpopulations arise from spontaneous mutations in housekeeping genes or mutations induced by harsh conditions such as antibiotic treatment. The selective pressure of SCVs leads to increased tolerance. Mutations in *thyA, menB,* and *hemH*, *hemA* genes impair the biosynthesis of thymidine, menadione, and hemin, respectively. The mutations in these genes lead to auxotrophism, which results in phenotypic differences within the subpopulation. The decrease in these biosynthetic pathways impairs the electron transport chain and reduces ATP production. The lack of ATP also reduces metabolic activity, such as lower pigmentation and hemolysis (Melter, Radojevič, 2010).

The core genome is highly conserved in *S. aureus* strains (75%), and the accessory genome is acquired either by horizontal gene transfer or natural selection. The accessory genome confers virulence factors and antibiotic resistance to increase its survival. The major virulence factors of MRSA are i) toxin production by the *hld* and *hlg* genes for δ-hemolysin and γ-hemolysin, respectively, the *ata, etb, etd* genes for exfoliative toxin causing scaffold skin syndrome and the *sea, seb, sec* and *sep* genes for enterotoxin causing food poisoning; ii) adhesion by the *ebh* and *clf* genes that express fibrinogen and extracellular matrix binding proteins, respectively; iii) immune evasion by the *cap* gene for capsular polysaccharide, the *chp* gene for chemotaxis inhibition; the *luk* gene for leukotoxin and the *spa* gene for IgG binding protein; iv) tissue invasion and destruction by the *hys* gene for hyaluronidase and the *sspA* and *sspB* genes for Ser and Cys protease (Turner et al., 2019). The plasticity of the genome confers on *S. aureus* diversity and pathogenicity.

1.1.3. *Acinetobacter haemolyticus*

Acinetobacter haemolyticus is a Gram-negative coccobacillus strictly aerobic bacterium that belongs to the Moraxellaceae family. The genus *Acinetobacter* are generally nonpathogenic environmental bacteria found in soil and water. Environmental strains are generally resistant to carbapenems and β-lactams, and those strains act as an environmental reservoir for antibiotic resistance genes. It is also found in the normal flora of the skin and intestine. However, it is considered an opportunistic bacterium that frequently induces tissue and urinary tract infections as well as more dangerous infections such as pneumonia and bacteremia (Wong et al., 2017). *A. haemolyticus* can degrade gelatin and have a hemolytic activity which forms beta-hemolysis in blood agar. *A. haemolyticus* generally induces infections in hospital intensive care units and less frequently in the community.

The pathogenicity of *A. haemolyticus* is generally acquired by horizontal gene transfer of virulence factors from the environmental reservoir of antibiotic resistance genes and other bacteria in the gut lumen. Type 1 secretion system (T1SS) is found in *A. haemolyticus,* including the ABC transporter, membrane fusion protein, and outer membrane protein. *A. haemolyticus* strains contain multiple *ompA* genes that encode porin and induce apoptosis of the host cell. The *bfmRS* genes encode Chaperone-usher pili, which is important in surface adherence and biofilm formation. Type-IV pilus is another type of pili produced by *A. haemolyticus* that is important in adhesion and twitching motility. Another important virulence factor is lipooligosaccharide, which is produced by *lpx* genes. It is important in serum resistance as it inhibits the complement proteins of the host. *A. haemolyticus* also has a T2SS that can secrete phospholipase D for nutrient uptake and lysis of the host cell (Castro et al., 2020). Some strains of *A. haemolyticus* are associated with endocarditis and verotoxin production and therefore induce bloody diarrhea. Verotoxin belongs to the RNA N-glycosidase protein family that inhibits protein synthesis. The verotoxin is presumably acquired via horizontal gene transfer from other bacteria in the gut lumen. The thin layer of biofilm via pilus is formed in *A. haemolyticus,* especially on medical devices such as catheters (Doughari et al., 2011). With the combination of multidrug resistance and the biofilm formation of *A. haemolyticus*, the infections become deathlier and harder to treat. Together with these

properties of *A. haemolyticus*, it has a high potential to become a threatening pathogen, just like *A. baumannii* (Castro et al., 2020).

1.1.4. *Escherichia coli*

Escherichia coli is the most well-studied microorganism, a common inhabitant of the gastrointestinal tract, and one of the most frequent pathogens in humans. *E. coli* is a Gram-negative, rod-shaped facultatively anaerobic bacterium that belongs to the Enterobacteriaceae family. Even though most *E. coli* are commensal strains and are found in the normal intestinal microbiota of warm-blooded organisms, some serotypes are highly dangerous (Vila et al., 2016). Those serotypes are divided into two broad clinical categories: intestinal pathogenic variants (enterotoxigenic *E. coli* and enterohemorrhagic *E. coli*) and extraintestinal pathogenic *E. coli* (ExPEC) (Sannes et al., 2004). Such serotypes include various virulence factors such as adhesins, invasins, toxins and polysaccharide coats that are dangerous. *E. coli* is mostly linked to urinary tract infections. However, it is also linked to meningitis, skin, bone, and muscle infections, and orchitis. Severe *E. coli* infections generally involve the bloodstream and induce systemic inflammatory response syndrome, the major cause of morbidity, mortality, and cost (Vila et al., 2016).

E. coli is one of the microorganisms with the highest degree of genetic plasticity, of which only 20% of the genome is conserved among all strains. This conserved region is called the core genome that encodes for essential cellular functions. The flexible gene pool of the genome is called an accessory genome. The virulence factor-encoding genes are located on the accessory genome of the *E. coli* and confer bacteria adaptability and fitness. Pathogenic strains of *E. coli* generally have different combinations and multiple virulence factors that confer pathogenicity and antibiotic resistance. This flexible gene pool can expand and is called pathogenicity island, which is present in the pathogenic strain of a given microorganism but absent in non-pathogenic strain. This genomic plasticity leads to phenotypic variation in their accessory traits, such as virulence factors of *E. coli,* and those variations induce differences in strains' clinical behaviors. Commensal *E. coli* strains colonize within the intestine of the host and produce vitamin K and suppress the colonization of pathogenic bacteria. The commensal strains only cause extraintestinal disease if the inoculum is large or the host is immunocompromised, and

they never cause extraintestinal infection. ExPEC strains also colonize within the gut of the host, but they have an adherent and invasive ability to penetrate to extraintestinal body sites, survive and induce disease in those sites. Commensal *E. coli* strains acquire virulence factors to become ExPEC probably via horizontal gene transfer and therefore increase survival via i) attachment to the host, ii) more acquisition of nutrients, iii) competition with other microorganisms, iv) escape from host defense, and v) inducing cellular injury and disease (Braz et al., 2020).

The virulence factors are associated with the pathogenicity and antibiotic resistance of the stains. The main virulence factors of the *E. coli* strain that induce bacteremia are: i) adhesins as *pap* for P-fimbriae, *bmaE* for M-fimbriae, *fimH* for type I fimbriae, *sfa* for S fimbriae; ii) toxins as *hly* for hemolysin, cnf1 for cytotoxic necrotizing factor 1, *cdtB* for cytolethal distending toxin; iii) iron acquisition as *ireA* and *iroN* for novel siderophore receptors, *fyuA* as yersiniabactin receptor, *iutA* for aerobactin system; iv) capsule as *kpsM II* element. In addition, other proteins are involved in pathogenicity, such as the *rfc* gene, which is responsible for O4 lipopolysaccharide synthesis; the *iss* gene for increased serum survival; the *traT* gene for protectin as serum-resistance-associated outer membrane protein; *beA* for invasion of brain endothelium A; and *fliC* gene for flagellar antigen. However, the *ompT* gene is the most associated gene with bacteremia as the OmpT is an outer membrane protease that can cleave the host cell surface proteins and inactivate proteins of the host that are produced for defense (Sannes et al., 2016). Different pathotypes of the *E. coli* strains have different combinations of those virulence factors (Braz et al., 2020).

1.2. Implant Infections and Bone Cement

Surgical site infections are infections that develop after surgery. In particular, medical implants such as catheters and prosthetics increase the risk of infection. Biomedical devices cause 25.6% of healthcare infections in the U.S.A (Arciola et al., 2018). Implant-associated infections are generally caused by opportunistic pathogens during surgical procedures and are generally cleared by the host immune system. The complex niche of pathogen, implant, and the host immune response against pathogen and implant causes implant-infection. Biomedical implants are recognized as foreign materials and cause local immunological responses that induce inflammation,

coagulation, complement system, and fibrous capsule. This response leads to immune suppression that provides bacterial colonization and infection. The surface of biomedical implants also acts as a platform for bacterial adhesion and biofilm formation. The attachment and the biofilm give an advantage to bacteria to escape from the host immune defense (Bistolfi et al., 2011). *S. aureus* is one of the most well-known opportunistic bacteria that cause osteomyelitis by adhering to the implant materials and developing antibiotic resistance as a response to antibiotic treatment. *E. coli* is another orthopaedical implant pathogen that develops resistance against the complement system by producing a long chain of lipopolysaccharide which helps the bacteria to persist in the blood (Arciola et al., 2018).

Bone cement is a polymeric material, polymethylmethacrylate (PMMA), used to fix the implants to bones. During the chemical reaction of liquid monomer with the powder polymer, the temperature increases rapidly, which air is entrapped within the cement and creates different pore sizes. The porous structure of the bone cement is advantageous as it creates an environment for the tissue to grow inside and allows the release of the antibiotic from the bone cement (Bistolfi et al., 2011). Antibiotic-loaded bone cement has been one of the main prevention and treatment methods for orthopedic implant infections since the late 1990s. The first application of antibiotic-loaded bone cement was in 1945, in which penicillin, erythromycin, and gentamicin were loaded into the bone cement was used for the hip implant, and prolonged antibiotic concentration was obtained (Belt et al., 2002). Then, in the 1970s, gentamicin was incorporated into bone cement beads to fill the tissue removal in osteomyelitis (Hendricks et al., 2004).

The promising results have increased the use of antibiotic-loaded bone-cement in implant surgeries. Broad-spectrum antibiotics incorporated into bone cement are generally chosen against Gram-negative and Gram-positive bacteria. Other criteria for antibiotics are their high antimicrobial efficiency, low potential to develop resistance by bacteria against, water solubility, and thermal stability (Belt et al., 2002). However, the use of antibiotics is not recommended by several scientists. First, it may reduce the mechanical properties of the bone cement, and secondly, it may increase antibiotic resistance. Also, the roughness of the bone cement increases the bacterial adhesion to the surface. Recent studies focus on developing antibiotic-loaded bone cement for clinical applications (Bistolfi et al., 2011). One of the critical approaches is the combination of different antibiotics to obtain the synergistic effect of antibiotics against antibiotic-

resistant bacteria. Generally, vancomycin and aminoglycosides are combined for *S. aureus* infections on bone cement (Bistolfi et al., 2011). In another study, daptomycin was used in bone cement to treat Gram-positive bacterial infections. The compressive strength was shown to be sufficient, and the release kinetics was controlled in the first 14 days (Hsu et al., 2014). The extensive research by Gálvez-López and his colleagues focused on the elution kinetics of 11 different antibiotics, vancomycin, gentamycin, daptomycin, moxifloxacin, rifampicin, cefotaxime, cefepime, amoxicillin-clavulanate, ampicillin, meropenem, and ertapenem. Antibiotics of vancomycin, gentamycin, moxifloxacin, and rifampicin were found to be constantly eluted for 30 days.

The addition of rifampicin reduced the mechanical properties of the acrylic bone cement, whereas other antibiotics did not affect the compressive strength (Gálvez-López et al., 2014). The effect of gentamicin-loaded bone cement on *S. aureus* showed reduced adherence of bacteria and lower slimy film formation; hence decreased biofilm is observed after 24 h when gentamicin is used (Belt et al., 2001). Recently, the addition of antibiotic solution to the bone cement is has been a hot topic of research, which aims to increase the release rate of the antibiotic from the bone cement to reduce the local infection. Chang et al.'s study showed that the solution-based antibiotics of vancomycin and amphotericin B had a 3.3-fold higher release rate from the bone cement than the powder-loaded bone cement. However, liquid antibiotics have significantly reduced mechanical properties (Chang et al., 2014). Therefore, the choice of antibiotics for the bone cement requires knowledge of antibiotics, their antimicrobial actions, and resistance mechanisms. Also, other antimicrobial agents, such as phenolic acids, are being highly investigated to overcome antibiotic resistance.

1.3. Antibiotics and Mechanism of Action

The antibiotic is derived from the term “antibiosis,” which means “against life” (Etebu, Arikekpar, 2016). Antibiotics are metabolites of different organisms that target bacterial structures or enzymes to kill the bacteria (bactericidal) or inhibit bacterial growth (bacteriostatic). These low molecular weight molecules are produced by fungi, actinomycetes, and other bacteria under stress conditions such as nutrient limitation and morphological differentiation to protect the organism from other bacteria. However,

antibiotics can also be chemically synthesized, such as quinolones, sulphonamides, or oxazolidinones (Laureti et al., 2013).

The main classification of antibiotics involves in the structures as i) β-lactams, ii) Macrolides, iii) Tetracyclines, iv) Quinolones, v) Aminoglycosides, vi) Sulphonamides, vii) Glycopeptides and viii) Oxazolidinones. β-lactams include Penicillins, Cephalosporins, Monobactams, and Carbapenems. Macrolides are generally broad-spectrum including Erythromycin, Azithromycin, and Clarithromycin. Quinolones include cinoxacin, norfloxacin, ofloxacin, ciproxin, etc. Aminoglycosides are another broad-spectrum antibiotic that includes Streptomycin, Gentamicin, Neomycin, Tobramycin, and Amikacin (Etebu, Arikekpar, 2016).

The classification of antibiotics based on their action involves i) inhibition of cell wall synthesis, ii) disruption of the cell membrane, iii) inhibition of synthesis of proteins and nucleic acids. The cell wall is crucial for protecting the cell from osmotic pressure and harsh environmental conditions. The synthesis of bacterial cell walls requires a transpeptidation reaction catalyzed by penicillin-binding proteins (PBP). β-lactam antibiotics such as penicillin, cephalosporin, monobactams, and carbapenems block the cross-linking of peptidoglycan by inhibiting the peptide bond formed by PBP. Glycopeptides such as vancomycin inhibit peptidoglycan synthesis by directly binding to the peptidoglycan. The disruption of the cellular membrane generally involves targeting unique bacterial lipids. For example, daptomycin causes depolarization of the membrane by creating holes, whereas polymyxin binds to the lipid moiety of lipopolysaccharide. The nucleic acid synthesis is inhibited by blocking the replication or inhibiting the transcription. Quinolones impair the helicase, which unwinds the DNA during replication, inhibiting replication. Other antibiotics inhibit nucleic acid synthesis by targeting the topoisomerase II and IV and blocking the RNA synthesis. Antibiotics that inhibit protein synthesis usually act on either 50S or 30S ribosomal subunits. Antibiotics that inhibit the 50S ribosomal subunit block the initiation or elongation of translation. Oxazolidinones block the initiation of protein synthesis, whereas macrolides block the elongation phase of translation. The 30S ribosome inhibitors block the aminoacyl tRNA to access the ribosome. Tetracycline, streptomycin, and spectinomycin are the main antibiotics that inhibit the 30S ribosomal subunit (Etebu, Arikekpar, 2016).

The first discovery of an antibiotic was penicillin from the soil fungus *Penicillium notatum* in the 1920s, and clinical trials were investigated in 1940. The antibiotic is

considered a revolution in medicine from the 1950s to 1960s, called the “Golden Age”. However, the misuse and wrong dosage of antibiotics caused the development of antibiotic-resistant strains in 1955 (Hutchings et al., 2019). The struggle against infections is still ongoing. The misuse and wrong dosage of antibiotic consumption lead to antibiotic-resistant bacteria development. Antibiotics act on only specific bacterial structures or enzymes, and therefore bacteria can easily adapt to the antibiotic-present environment and develop resistance against the antibiotic by gene regulation such as upregulation of efflux pump or downregulation of porin. These antibiotic-resistant bacteria survive in the presence of antibiotics and cause serious infections (Kapoor et al., 2017).

1.4. Antibiotic Resistance of Pathogens

The misuse of antibiotics against infection induces the rise of antibiotic-resistance bacteria. Some bacterial strains have intrinsic antibiotic resistance mechanisms, but bacteria can acquire antibiotic resistance genes via horizontal gene transfer. The mechanisms of antibiotic resistance include i) modification of the antibiotics, ii) blocking the antibiotic from its target via lower uptake of the antibiotic or removal of the antibiotic from the cell, iii) modification or bypass of the target (Blair et al., 2015).

The enzymes involved in modifying the antibiotics are seen in both Gram-negative and Gram-positive bacteria. Most of those modifying enzymes inhibit the antibiotic via inhibition of protein synthesis. There is a variety of modifications of an antibiotic, such as i) acetylation, ii) phosphorylation, which both generally occur in aminoglycosides, chloramphenicol, iii) adenylation of aminoglycosides. The destruction of antibiotics involves the degradation or inhibition of the antibiotics. The most well-known example is the β-lactamase enzyme which cleaves the amide bond in β-lactam, inhibiting its activity. The reduction in permeability is especially important in Gram-negative bacteria (Munita, Arias, 2016). The hydrophilic antibiotics such as β-lactams and tetracyclines use porin channels to get inside the cells. The change in porin includes i) type of porins, ii) expression level of porins, and iii) function of porin. The efflux pump system pumps antibiotics out of the cell, which is important in Gram-negative and positive pathogens. Tetracycline resistance is one of the most studied mechanisms that pump the tetracycline from the bacteria via proton exchange as an ATP source (Reygaert,

2018). Finally, the bypass of the antibiotic from the target involves protection of the target site or modification of the target site. The example for target protection is TetO/M proteins which are GTPase and serve as elongation factors. Both TetO and TetM interact with the ribosome and remove the tetracycline from its target via its GTPase activity. The protection of the target from tetracycline results in resistance. On the other hand, modification of the target site involves i) mutation in target-encoding genes, ii) enzymatic modification of the binding site, such as methylation, and iii) bypassing the target. The most famous example is β-lactam resistance, as it inhibits the cell wall synthesis via inhibiting PBP. Acquisition of the *mecA* gene that encodes PBP2 causes β-lactam resistance as PBP2 has a low affinity for β-lactams (Munita, Arias, 2016).

Most nosocomial pathogens have intrinsic antibiotic resistance and acquire resistance genes via horizontal gene transfer. In both ways, multidrug-resistant pathogens have one or more mechanisms against antibiotics that enable them to survive. *P. aeruginosa* is a multidrug-resistance bacterium that has different resistance mechanisms against antibiotics as i) antibiotic-modifying enzymes, ii) reduction in permeability for antibiotics, and iii) efflux pump against antibiotics (Al-Wrafy et al., 2017). The antibiotic modifying enzymes of *P. aeruginosa* include β-lactamase and aminoglycoside-modifying enzymes. The β-lactamase enzyme of *P. aeruginosa* includes AmpC, which induces resistance against cephalosporins and CARB-1, 2, 3, and 4 against carbenicillin. Aminoglycoside-modifying enzymes attack radicals of the aminoglycoside and modify the antibiotic, reducing the antibiotic's binding to the 30S ribosomal subunit. *P. aeruginosa* frequently express aminoglycoside acetyltransferases and aminoglycoside nucleotidyltransferases. Those enzymes generally cause resistance to gentamicin and tobramycin (Castro et al., 2020). The porin formation of the outer membrane of the bacteria enables the uptake of antibiotics within the cell. The OprD porin channel on the outer membrane of the *P. aeruginosa* is specific for carbapenem. The reduction in OprD reduces the permeability against carbapenem, which induces resistance (Moradali et al., 2017). Other porins of *P. aeruginosa* are OprF and OprB, which further confer resistance to aminoglycosides and quinolones (Gellatly, Hancock, 2013). Finally, the efflux pump upregulation by bacteria increases the removal of antibiotics from the cell. *P. aeruginosa* expresses efflux pump from the *mex* gene and contains four multidrug efflux pumps as MexAB-OprM, MexXY/OprM(OprA), MexCD-OprJ, and MexEF-OprN. The efflux pumps of MexAB–OprM, MexEF-OprN, and MexCD-OprJ confer resistance against β-

lactams, MexEF-OprN and MexCD-OprJ involve in fluoroquinolones resistance, and MexXY-OprM is efflux pump against aminoglycoside (Al-Wrafy et al., 2017).

MRSA is a multidrug-resistant bacterium that can develop resistance to multiple types of antibiotics. MRSA can develop resistance against aminoglycosides by expressing aminoglycoside modifying enzymes from *aadD* and *ant* genes. MRSA developed resistance against penicillin by *blaZ* gene that expresses β- lactamase, which degrades the β- lactam antibiotics. The resistance against methicillin, oxacillin, and nafcillin is acquired by horizontal gene transfer *mecA* gene, which codes for Penicillin-binding protein 2 (Bouiller et al., 2020). MRSA also uses chloramphenicol acetyltransferase to modify the enzyme and cause resistance via *cat* gene. On the other hand, *tet* and *otr* genes cause resistance against tetracycline. The *tetM* gene protects the ribosome by encoding elongation factor-like proteins, and *tetK* encodes for an efflux pump that pumps the tetracycline from the cell. The efflux pumps on the outer membrane of the MRSA are the Qac, Nor, and Smr pumps. The NorA/B/C are the chromosomally encoded efflux pumps that confer fluoroquinolone resistance, such as norfloxacin and ciprofloxacin. QacA/B involves developing resistance against trimethoprim, an aminoglycoside, which is encoded by the plasmid of MRSA (Turner et al., 2019).

E. coli is another bacterium that expresses β-lactamase from *blaCTX* that causes resistance against extended-spectrum of β-lactam antibiotics. The enzyme carbapenemase is encoded by the gene blaKPC, which modifies the carbapenem antibiotics and reduces antimicrobial activity. In some cases, co-expression of carbapenemases, NDM, and OXA is observed in *E. coli.* The resistance against aminoglycoside is acquired by the genes of *aac(6'), armA,* and *rmtB*. *E. coli* is also resistant to various fluoroquinolones such as ciprofloxacin and norfloxacin. Quinolones act on the DNA gyrase and topoisomerase IV activity. DNA gyrase is crucial for the negative supercoil of the DNA and is expressed by the *gyrA* gene, whereas topoisomerase IV is encoded by the *parC* gene. So, the mutation in the *gyrA* gene (at Ser 83 and Asp 87) and *parC* gene (Ser 80 and Glu 84) changes the protein target that results in the gain of resistance. The *qnr* genes are also involved in fluoroquinolone resistance as they encode a peptide that protects DNA gyrase and topoisomerase IV. *E. coli* also harbors mechanism for efflux pump and reduced permeability against fluoroquinolones. The OmpF porin on the outer membrane of *E. coli* reduces the permeability, hence decreasing the uptake of fluoroquinolones. The efflux

pump system of *E. coli* includes AcrAB and AcrEF, which cause resistance against quinolones, chloramphenicol, and tetracyclines (Vila et al., 2016).

Acinetobacter is known to be multidrug-resistant. *A. haemolyticus* is one of the most resistant *Acinetobacter* strains after *A. baumannii*. *A. haemolyticus* strains contain conserved aminoglycoside acetyl-transferase gene, which confers aminoglycoside resistance. Another resistance gene is the *blaOXA* gene which is β-lactamase that degrades β-lactams. Most of the *A. haemolyticus* also contain *blaTEM,* another β-lactamase coding gene. It is shown that *A. haemolyticus* efficiently acquires resistance to carbapenem via the *blaNDM*-1 gene (Castro et al., 2020).

Antibiotic resistance has been declared one of the top ten health problems by the WHO. The wrong dosage and misuse of antibiotics are the main causes of antibiotic-resistant bacteria. The antibiotic abundance at the wastewater is another cause of resistance development that causes environmental pathogens resistance. The lack of effective antimicrobials increases the risk of infection during surgery and chemotherapy. The development of new antimicrobials against antibiotic-resistant pathogens is considered an urgent multisectoral requirement by WHO (WHO, 2020).

1.5. Phenolic Acids

Phenolic compounds are secondary metabolites of plants that are not required for biological events such as growth, development, or reproduction; but are important for plants to create responses to their environment. Secondary metabolites of plants are derived from their primary metabolites, such as amino acids, and these secondary metabolites are crucial for protecting the organism from radiation microorganisms and giving odor, color, and flavor to plants (Kumar and Goel, 2019). Phenolics are highly abundant compounds in the human diet as they have high antioxidant properties that reduce oxidative stress caused by tissue damage (Khadem and Marles, 2010). Phenolics are commonly found in all foods such as cereals, oilseeds, fruits such as berries, vegetables such as olives, beverages, and herbs. Besides the antioxidant activities (Kiokias et al., 2020), phenolic compounds have been shown to have anticancer (Anantharaju et al., 2016), antimicrobial (Alves et al., 2013), antifungal (Teodoro et al., 2015), and anti-inflammatory properties (Liu et al., 2020). In addition, phenolics prevent disease by different mechanisms, such as blocking bacterial replication, inducing the

apoptosis of tumors, and activating immune cells such as macrophages and neutrophils (Ferrazzano *et al*., 2011).

Phenolic compounds have one aromatic ring to which one or more hydroxyl groups are attached. The presence of one hydroxyl group attached to the aromatic ring creates phenols, and multiple hydroxyl groups create polyphenols (Kumar and Goel, 2019). Polyphenols are divided into two main groups: flavonoids and non-flavonoids. Flavonoids have a C_6-C_3-C_6 structure resulting from extending the side chain of cinnamic acid and include flavanones, flavones, flavonols, anthocyanidins, chalcones, etc. (Khadem and Marles, 2010). The main role of flavonoids is ecological, as they confer pigment on flowers and fruits and attract pollinators. Non-flavonoid phenolic compounds have one carboxylic acid group attached to the benzene ring and can be classified based on their carbon skeleton as phenols, benzoic acids, cinnamic acids, lignans, coumarins, etc. (Ferrazzano *et al*., 2011).

Phenolic acids are classified into two main groups: hydroxybenzoic acid (HBA) and hydroxycinnamic acid (HCA). Hydroxycinnamic acids have common C_6-C_3 structures that are derived from cinnamic acid. The most known hydroxycinnamic acids are ferulic acid, caffeic acid, *p*-coumaric and sinapic acids. Ferulic acid is one of the most abundant phenolic acids in plants. It is found in plants with up to 50 g of ferulic acid per kg, such as wheat bran, sugar-beet pulp, and corn kernel. Ferulic acid is generally found cross-linked in the polysaccharide of the cell wall, or protein, which enables this compound to be used in gels in the food industry. The antioxidant activity, cholesterol-reducing property, anticancer effect, and antimicrobial activity of the ferulic acid were shown (Ou and Kwok, 2004). Caffeic acid is found in all plants as it is an intermediate molecule in the lignin synthesis pathway. It is commonly abundant in various vegetables such as coffee beans, tea leaves, olives, potatoes, carrots, and propolis. It is shown to have antioxidant, anti-inflammatory, and anticancer activity. The anti-hepatocarcinoma activity is especially important for therapeutical applications as hepatocarcinoma has one of the highest mortality rates (Espíndola *et al.,* 2019). Hydroxybenzoic acids are benzoic acid-derivatives with a common structure of C_6-C_1 and are generally found in soluble form. The *p*-hydroxybenzoic acid, also called 4-hydroxybenzoic acid (4-HBA), vanillic, and syringic acids are the most known hydroxybenzoic acids (Kumar and Goel, 2019). Vanillic acid is found in various plants such as Japanese alder, Mexican cotton, Chinaberry, red sandalwood, ginseng, and some mushroom. Vanillic acid has antioxidant

activity, and it is shown to reduce hepatic fibrosis and inhibit the snake venom, 5'-nucleotidase. 4-HBA is monohydroxy benzoic acid, the solid crystal that is soluble in water. It is another highly abundant phenolic acid in vegetables and fruits such as carrots, coconuts and grapes, mushrooms, and green algae. 4-HBA is used in the production of parabens, which are preservative additives in the cosmetic industry. Isomers of 4-HBA are 3-hydroxybenzoic acid (3-HCA) and 2-hydroxybenzoic acid (2-HCA), also called salicylic acid, the precursor of aspirin. 4-HBA is found to be effective in antifungal, antimutagenic, and antimicrobial activity and has a growth stimulatory effect on green algae (Khadem and Marles, 2010).

The amount of hydroxyl group and its position of phenolic acid determine the effect of phenolic acids on organisms. The higher amount of hydroxyl group leads to higher toxic properties of the phenolic acids. The antioxidant activity of the hydroxycinnamic acid is higher compared to the hydroxybenzoic acid. The CH=CH-COOH group of hydroxycinnamic acid has higher hydrogen donation compared to the carboxyl group of hydroxybenzoic acid (Kumar and Goel, 2019). The lack of hydroxyl group on the ring structure of phenolic acids impairs the antioxidant activity of the phenolic acids. The antimicrobial activity of hydroxycinnamic acids such as ferulic acid, caffeic acid, and hydroxybenzoic acid such as 4-HBA and vanillic acid was investigated on different bacteria and each phenolic acid was found to be effective against different bacteria (Liu, *et al.,* 2020). These varieties of pharmaceutical activities of phenolic acids and their high abundance in nature create an opportunity for a wide range of applications.

1.5.1. Antimicrobial Effects of Phenolic Acids

The antimicrobial effect of phenolic acids is considered as one of the hot topics as the discovery of new antimicrobials is an urgent need against multi-drug resistant pathogens. The hydroxyl group of the phenolic acids is highly reactive against the protein, such as microbial enzymes, and inhibits the activity of the protein. The inhibition of bacterial toxins also makes phenolic acids a good candidate for antimicrobial activity against antibiotic-resistant bacteria (Liu et al., 2008). Phenolic acids create various stress on microorganisms and impair the quorum sensing of the microorganism, hence reduces the expression of the virulence factors. Phenolic acids are also highly abundant in nature, easy to access and safe to use. The antimicrobial effects of phenolic acids on

microorganisms are generally investigated depends on minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and effect on the cellular structures such as membrane (Ferrazzano et al., 2011).

The antimicrobial effect of positional isomers of benzoic acid was investigated on *E. coli.* The MIC and MBC value of benzoic acid and 2-HBA was found as 1 and 2 mg/mL on *E. coli*, respectively. The position of the hydroxyl group changed the MIC and MBC values as 3-HBA and 4-HBA had MIC and MBC values of 2 and 4 mg/mL. The higher effectiveness of the 2-HBA might have resulted from the high partition coefficient, which increases the penetration through the cell membrane. The higher hydroxyl group increased the MIC and MBC value as the 3,4,5-trihydroxybenzoic acid has the value of 4 and 8 mg/mL, respectively (Synowiec et al., 2021). In another study, the MIC value of ferulic acid, which is a hydroxycinnamic acid and gallic acid, which is trihydroxybenzoic acid against 4 pathogenic bacterial strains, *P. aeruginosa, E. coli, S. aureus* and *Listeria monocytogenes* were determined. Gallic acid had the highest antimicrobial activity against *P. aeruginosa* with the MIC value of 500 µg/mL. The MIC values against *E. coli, S. aureus* and *L. monocytogenes* were found as 1500, 1750 and 2000 µg/mL. Ferulic acid has more antimicrobial effect compared to gallic acid as the MIC values of ferulic acid were lower. MIC values of ferulic acid were found as 100 µg/mL for *E. coli* and *P. aeruginosa;* and 1100 and 1250 µg/mL for *S. aureus* and *L. monocytogenes*, respectively. Both phenolic acids causes irreversible changes in the bacterial membrane by decreasing negative membrane charge, damaging the membrane integrity by local pore formation (Borges et al., 2013). The study of chlorogenic acid focused on its antimicrobial potential on Gram-positive bacteria as *Streptococcus pneumoniae, Bacillus subtilis*, *S. aureus* and Gram-negative bacteria as *Shigella dysenteriaea*, *E. coli, Salmonella* Typhimurium. Chlorogenic acid showed the highest antimicrobial activity against *S. pneumoniae* and *S. dysenteriaea* with the MIC value of 20 µg/mL. MIC values were determined as 40 µg/mL against *S. aureus, B. subtilis and Salmonella* Typhimurium. Chlorogenic acid showed the lowest antimicrobial activity against *E. coli* with the MIC value of 80 µg/mL. The permeability of the outer membrane of Gram-negative bacteria was increased with the chlorogenic acid. The increase in the membrane permeability resulted in a higher synergistic effect with hydrophobic antibiotics erythromycin and rifampin. Another effect of membrane permeability is the leakage of the nucleotide from bacteria (Lou et al., 2021). The study of antimicrobial effect of *p*-coumaric acid was investigated on *S.*

pneumoniae, B. subtilis, *S. aureus*, *S. dysenteriaea*, *E. coli* and *S.* Typhimurium, similar to the study of chlorogenic acid. *p*-coumaric acid was found to be the most effective against *S. dysenteriaea w*ith the MIC value of 10 µg/mL. The MIC value was determined as 20 µg/mL for *S. pneumoniae, B. subtilis*, *S. aureus*, and *S.* Typhimurium. Similar to the chlorogenic acid, *p*-coumaric acid had the lowest antimicrobial activity against *E. coli* with a MIC value of 80 µg/mL. *p*-coumaric acid increased the outer membrane permeability of the Gram-negative bacteria *S. dysenteriaea* and increased the susceptibility against antibiotics. *p*-coumaric acid was found to bind to the outer membrane as it has a negative surface charge and chelates the magnesium ion. The chelating Mg^2 leads to impairment in the barrier function and nutrient flow of the membrane. *p*-coumaric was shown to bind to bacterial DNA and impair the adjacent base pairs of DNA and therefore inhibit cellular functions (Lou et al., 2012). Alves and his colleagues focused on the antimicrobial activity of the 12 different phenolic acids from different mushrooms on Gram-negative bacteria as *E. coli, Proteus mirabilis, Morganella morganni, Pasteurella multocida* and *Neisseria gonorrhoeae* and Gram-positive bacteria as Methicillin resistant and susceptible *S. aureus, Staphylococcus epidermidis, Enterococcus faecalis, L. monocytogenes* and *Streptococcus agalactia.* The benzoic acid derivatives from mushrooms include *p*-hydroxybenzoic, 2,4-dihydroxybenzoic acid, protocatechuic, gallic, vanillic and syringic acids and cinnamic acid derivatives include cinnamic acid, *p*-coumaric, *o*-coumaric, caffeic, ferulic and chlorogenic acids. 2,4-dihydroxybenzoic and protocatechuic acid were shown higher antimicrobial activity against most of the Gram-negative and positive bacteria. MRSA had susceptibility against phenolic acids compared to methicillin-susceptible *S. aureus* (MSSA). The MIC values of 2,4-dihydroxybenzoic, vanillic, syringic acids were determined as 0.5 mg/mL and p-coumaric acid with 1 mg/mL against MRSA. These concentrations of phenolic acids had no growth inhibitory effect against MSSA (Alves et al., 2013). Cueva and his colleagues investigated the antimicrobial activity of 13 different phenolic acids on beneficial bacteria *Lactobacillus* spp. and pathogens such as *S. aureus, E. coli*, *P. aeruginosa,* and *Candida albicans.* The most effective phenolic acid against *E. coli* was found to be 3-HBA. The Gram-positive lactobacilli lack an outer membrane and are enclosed with a cellular membrane which is further covered by the thick cell wall. These differences in the cellular structures change the antimicrobial efficiency of phenolic acids. 4-HBA was found to be the most effective against *L. paraplantarum, L. plantarum, L. fermentum, L. fermentum*,

and *L. brevis*. Among the pathogenic bacteria, *S. aureus* was found to be the most sensitive against phenolic acids as all phenolic acids with 1000 µg/mL concentration inhibited the bacterial growth. The most effective phenolic acid against *S. aureus* was found to be 4-HBA. The yeast *C. albicans* was mildly sensitive to phenolic acid. *P. aeruginosa* showed sensitivity towards 3,4-dihydroxybenzoic acid. The most effective phenolic acid against *C. albicans* was 1000 µg/mL benzoic acid, with only 16% inhibition (Cueva et al., 2010).

CHAPTER 2

ANTIMICROBIAL EFFECTS OF 4-HYDROXYBENZOIC ACID ON METHICILLIN-RESISTANT *Staphylococcus aureus* and *Acinetobacter haemolyti*cus

2.1. Introduction

The first discovery of antibiotics was in 1910. After the discovery, within 100 years, antibiotics increased human life for 23 years by saving lives. It was considered one of the remarkable scientific steps in the 20th century. Later in 1928, penicillin was discovered, which was the beginning of the "Golden Age". The antibiotics were used in different fields, such as vet and agriculture. In the 1960s, antibiotics were started to be used as pharmacological agents. However, their misuse and wrong dosage applications led to the accumulation of excessive amount of antibiotics in the soil. This resulted in the selection of the bacteria that were resistant to antibiotics. The usage of different kinds of antibiotics with the wrong dose was the reason for the rise of the human pathogens with multidrug resistance (MDR). From 1960 until today, antibiotic resistance has been increasing, called the "Lean Years". The bacteria with multidrug resistance lead to extensive death worldwide (Hutchings et al., 2019). Therefore, searching for alternative antimicrobial is a hot topic in our era.

The bacteria *A. haemolyticus* is one of the most known Acinetobacter pathogens widely found in nature, such as soil, water, and food. Besides the nature, *A. haemolyticus* are also widely distributed in hospitals, which causes antibiotic-resistant nosocomial infections and associates with different infections via cross-infection among patients (Bai et al., 2020). On the other hand, *S. aureus* is part of the skin flora and mucous membranes besides the environment. As it enters to the bloodstream or any internal tissues, *S. aureus* causes dangerous infections. The isolation of MRSA in 1961 was one the striking event as the infections became harder and harder to treat. More than half of the clinical strains were MRSA within 50 years after discovery (Hiramatsu et al., 2014). The infections with MRSA result in death 64% more than any other infections. According to World Health

Organization, MRSA is one of the high priority (#2) pathogens for the research and development of new antibiotics (WHO, 2020).

The discovery of new antimicrobials is urgent requirement for treatment infections that are caused by multi drug-resistant pathogens. Despite the various studies about new antimicrobial treatments against multi-drug resistant pathogens, the goal has not succeeded. The research and development of novel antibiotics is hard and time-consuming as well as expensive. However, the development of resistance by bacteria is unlimited and much more rapid and robust. The use of natural substances is much easier and more promising than developing new antibiotics. Plant use as a treatment for different diseases is an ongoing traditional medicine from the earliest time. However, with the rise of antibiotic resistance, scientists have even more focused on natural antimicrobial agents to combat the multi-drug resistance. Besides the antimicrobial activity of the plant extracts itself, those natural products can also be used with the combination of commonly used antibiotics with their synergistic effects (Miklasińska-Majdanik et al., 2018). Plants contain numerous compounds such as alkaloids, flavonoids, some essential oils, and phenolic compounds. Phenolic acids are one of the edible secondary metabolites of plants with various properties such as antioxidant (Kiokias et al., 2020), antimicrobial (Alves et al., 2013), antifungal (Teodoro et al., 2015), and anticancer effects (Anantharaju et al., 2016). One of the reasons of antimicrobial effects of the phenolic acids is due to the presence of phenolic hydroxyl groups. Those hydroxyl groups bind to proteins with high affinities, such as microbial enzymes, and inhibit those enzymes that are generally used for infections and antibiotic resistance. The addition of hydroxyl group and lipophilicity to phenolic compounds increases the antimicrobial activity of the given compound (Liu et al., 2008). Another advantage of using compounds from plant extract is their high abundance in nature. Those compounds are eco-friendly and naturally safe. In this study, 4-hydroxybenzoic acid was used as a phenolic compound to test its antimicrobial activity against the *A.haemolyticus* and MRSA.

This experimental part aims to investigate the antibacterial effect of 4-hydroxybenzoic acid on two famous bacteria,*A. haemolyticus* and MRSA. In this chapter of the experimental study, one hypothesis was tested:

1. The phenolic acid, 4-hydrozybenzoic acid, was tested for its antibacterial effect on *A. haemolyticus* and MRSA. If there is antibacterial activity, then the exposure to the proper concentration would inhibit the total bacterial growth.

2.2. Materials and Methods

In this study, methicillin-resistant *Staphylococcus aureus* (MRSA) N315 type II SCC*mecA*, *Acinetobacter heameloyticus* and *Pseudomonas aeruginosa* were used to test the antimicrobial activity of phenolic acid 4-hydroxybenzoic acid with the determination of a minimum inhibitory concentration.

2.2.1. Bacterial Culture Conditions

MRSA and *A. haemolyticus* were grown in tryptic soy broth (TSB) (Sigma-Aldrich 22092) and tryptic soy broth agar (TSA). For long-term storage, MRSA and *A. haemolyticus* stock cultures were maintained in TSB that contains 20% glycerol at –80ºC. For short-term storage, MRSA and *A. haemolyticus* were sub-cultured onto TSA plates weekly and maintained at +4ºC.

2.2.2. Determination of Growth Curve

The single colony of the bacteria was taken from a streak plate and inoculated into 4 mL of TSB for overnight incubation at 37ºC. The overnight grown bacteria were transferred to TSB to obtain the 2% inoculum, which was considered as the initial time (0^{th} hour) in which optical density was measured spectrophotometrically at 600 nm (OD600nm) by the Thermo Multiscan Spectro Reader. Then the 2% inoculum was incubated at 37ºC for 24 hours, in which OD600nm was measured spectrophotometrically every 3 hours of incubation. Besides the optical density measurements, viable cell counts were also determined by the spread plate. During the 24 hour incubation period, 100 µL of the bacteria from the 2% inoculum were spread onto TSA plates every 3 hours. Theplates were incubated overnight at 37ºC to be enumerated and recorded as colony-forming unit per mL (CFU/mL).

2.2.3. The Antimicrobial Effect of 4-Hydroxybenzoic Acid and Gentamicin

In each antimicrobial experiment, bacterial cultures were prepared according to the same protocol. The single colony of bacteria (MRSA, *A. haemolyticus*) from the streak plate was inoculated into 4 mL of TSB and incubated overnight at 37ºC. The OD600nm of the overnight grown bacteria were measured and then transferred to TSB to obtain the 2% inoculum. The OD600nm of the freshly prepared 2% inoculum was also measured with the combination of spread plates. In every experiment, the 2% bacteria inoculums were spread to TSA to determine the initial bacterial load, after which the plates were incubated overnight at 37ºC to be counted and recorded as colony-forming unit per mL (CFU/mL).

2.2.3.1. Preparation of Antimicrobial Solutions

In the antimicrobial experiments, the optical density and the viable cell count methods were used to compare the effectiveness of the antimicrobial agents, which were selected as gentamicin as an antibiotic and 4-hydroxybenzoic acid as phenolic acid. For the experiements gentamicin (BioChemica A1492) and 4-hydroxybenzoic acid (Sigma-Aldrich H20059) were purchased commercially to standardize the experiments. Both gentamicin and 4-HBA are soluble in water. Therefore, different concentrations were prepared by dispersing the powder of gentamicin or 4-HBA into TSB to be further tested on bacteria. The stock solution of gentamicin was prepared as 0.1 mg/mL, and the stock solution of 4-HBA was adjusted as 10 mg/mL. The stock solutions were then stored at -20ºC. Different concentrations of antimicrobial solutions were prepared freshly in TSB from the stocks before each experiment. The initial time was accepted as the inoculum of the 2% bacteria into the antimicrobial containing TSB. The first optical measurement was taken (OD600nm), and after incubation for 24 hours at 37ºC, the OD was measured again.

2.2.3.2 Determination of the Minimum Inhibitory Concentrations of 4-Hydroxybenzoic Acid and Gentamicin

The antimicrobial effects of 4-HBA and gentamicin were determined by optical measurement as well as the viable cell count. MRSA and *A. haemolyticus* strains were grown as indicated in section 2.2.3. The concentrations of 4-HBA and gentamicin solutions were prepared in TSB from the stock solution prior to experiments as described in section 2.2.3.1. The concentration of the gentamicin solution ranged from 5 µg/mL to 200 µg/mL for MRSA and *A. haemolyticus* (as 5, 10, 25, 50, 75, 100 and 200 µg/mL). The concentration of 4-HBA solution ranged from 0.5 mg/mL to 3 mg/mL for MRSA (as 0.5, 1, 1.5, 2, 2.5 and 3 mg/mL) and from 1 mg/mL to 3.5 mg/mL for *A. haemolyticus* (as 1, 1.5, 2, 2.5, 3 and 3.5 mg/mL). The blanks were prepared by TSB with the same concentration of gentamicin or 4-HBA without any inoculation of bacteria. The positive control was the bacterial inoculation into TSB without adding antimicrobial agents. OD600nm was measured for 0, 8, and 24 hours of incubated bacteria to determine the antimicrobial activities of gentamicin and 4-HBA. Concentrations that inhibit the total bacterial growth at the 24^{th} hour were determined as the minimum inhibitory concentration. Tubes that had no turbidity were selected. And then, 100 µL of bacterial cultures were spread onto TSA plates, then incubated overnight at 37^{o}C to be counted and recorded as colony-forming unit per mL (CFU/mL). The experiments were repeated at least twice for each antimicrobial on MRSA and *A. haemolyticus.*

2.2.3.2 Determination of Sub-inhibitory Concentrations of 4-Hydroxybenzoic Acid and Gentamicin

MRSA and *A. haemolyticus* strains were grown as indicated in section 2.2.3. After determining MIC values as described in section 2.2.3.2, the concentrations of the 4-HBA and gentamicin were fine-tuned to find the subinhibitory concentration. The concentrations of 4-HBA and gentamicin solutions were prepared in TSB from the stock solution prior to experiments as described in section 2.2.3.1. The concentrations of the 4-HBA were narrowed down from 1 mg/mL to 1.5 mg/mL (as 1, 1.1, 1.2, 1.3, 1.4 and 1.5 mg/mL). The concentration of gentamicin was adjusted as 1 µg/mL to 5 µg/mL (as 1, 2,

3, 4 and 5 μg/mL). The blanks were prepared by TSB with the same concentration of gentamicin or 4-HBA without any inoculation of bacteria. The positive control was the bacterial inoculation into TSB without adding antimicrobial agents. The concentrations that reduce the optical density by approximately half were selected as subinhibitory concentrations.

2.3. Results and Discussion

2.3.1. Growth Curve of Bacteria

In each experiment with the bacteria, the growth curves of bacteria under normal conditions are extremely important to understand the behavior of each specific bacterium. Since each bacterial strain grows differently at the same temperature and time point, a growth curve must be plotted in order to understand the bacterial growth of a single strain. Bacterial growth was determined by measuring the optical density in every three hours of 24 hours of incubation. At each time point, bacteria were diluted, and 100 μL from the desired concentration were spread onto TSA plates. The growth curve is plotted as the value of OD 600 nm versus time. The growth curves of *Acinetobacter haemolyticus*, *Escherichia coli, Pseudomonas aeruginosa*, MSSA, and MRSA during 24 hours of incubation at 37^{o}C are given below. Since growth curves of MRSA and MSSA were already determined by Keman and Soyer (Keman & Soyer, 2019), in this experiment, the optical density and plate counts were measured only at the 0th, 9^{th,} and 24th hours of incubation.

The growth curve for the *A. haemolyticus* was shown in Figure 2.1.a, in which the exponential phase was continued to the 24 hours of incubation, indicating that the stationary phase occurs after 24 hours. The stationary phase of *E. coli* took place in the first 9 hours of incubation, and then the stationary phase began (Figure 2.1.b). *P. aeruginosa* grew until 15 hours of incubation and then growth was shifted to stationary phase until 21st hour, but then the second exponential phase began (Figure 2.1.c). Considering the information given about the bacterial growth in the literature review, the second exponential phase was not surprising for the *P. aeruginosa*. Thus, in many growth curves, the stationary phase can show little amount of bacterial growth or stationary phase can be followed by exponential phase. This growth behavior indicates that the substrates

within the media were consumed, and the dead cells were used by other bacteria as a carbon source and energy. This pattern of growth curve is generally seen in bacterial cultures with high cell density (Maier, 2009). On the other hand, growth curves of MRSA and MSSA were consistent with the previous findings as the exponential phase occurred in the first 9 hours of incubation in which bacterial growth was shifted to stationary phase (Figure 2.1.d, e).

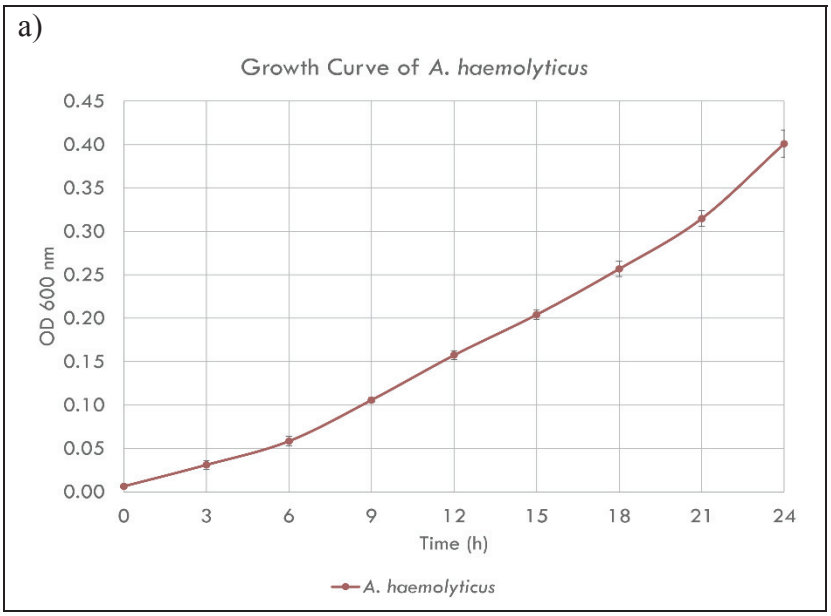

Figure 2.1. Growth curves of (a) *A. haemolyticus*, (b) *E. coli*, (c) *P. aeruginosa*, (d) MSSA and (e) MRSA. Standard deviation was <0.016 for *A. haemolyticus*, <0.012 for *E. coli*, <0.070 for *P. aeruginosa,* <0.028 for MSSA, <0.026 for MRSA.

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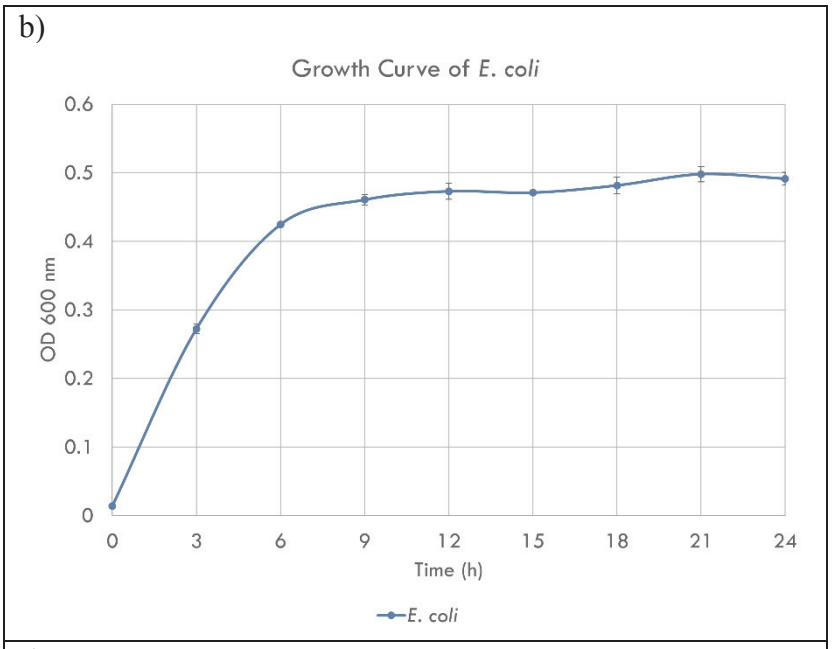

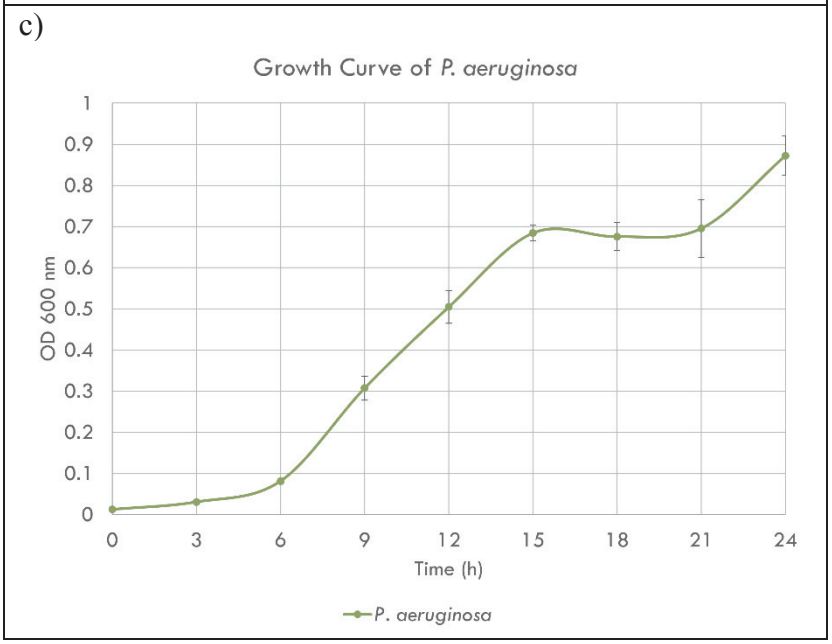

Fig 2.1 (cont.)

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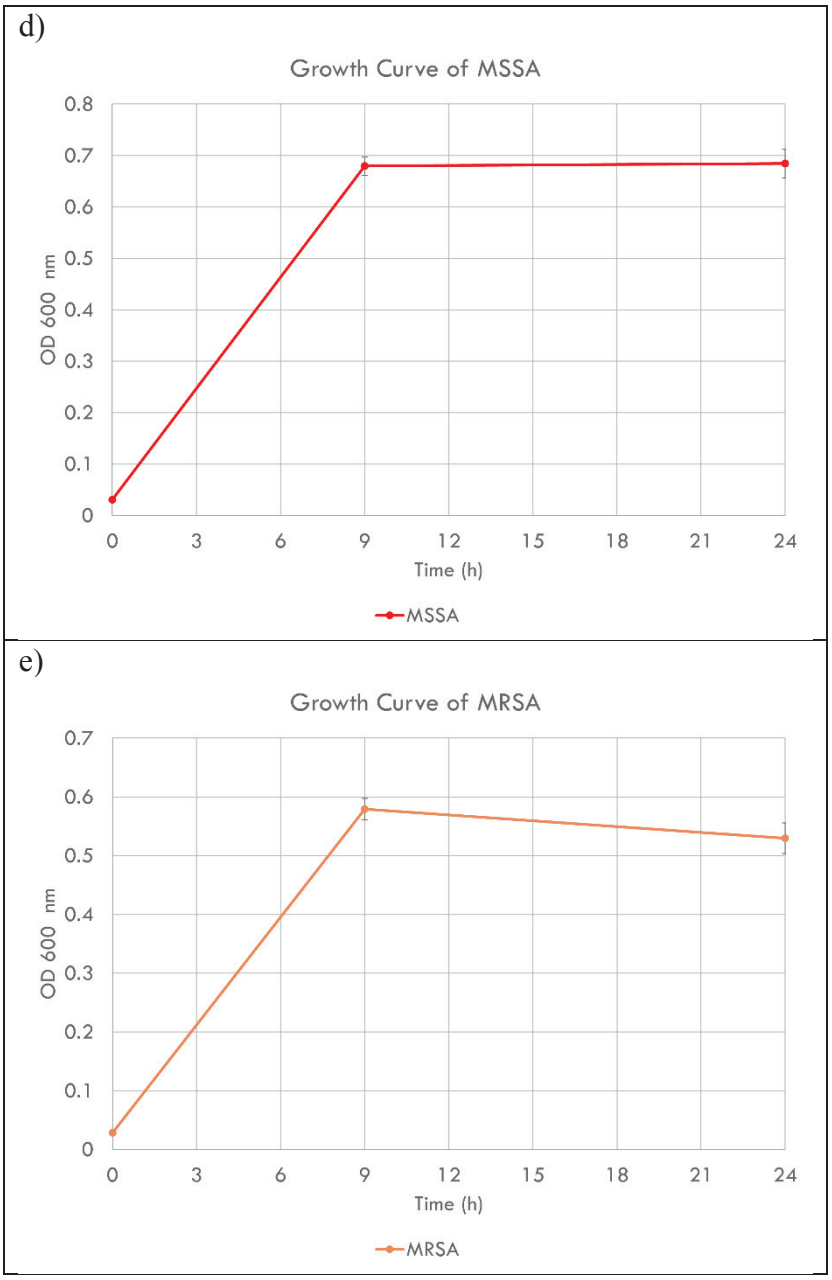

Fig 2.1 (cont.)

Along with the spectrophotometer measurements of optical density every three hours, viable cell counts were also enumerated with spread plate of bacterial culture in every three hours. According to the viable cell counts as shown in Table 2.1., the initial bacterial number for *A. haemolyticus* was approximately 10^7 CFU/mL and reached 10^8 CFU/mL at the end of the 24 hours of incubation. The initial inoculum of the *E. coli* and *P. aeruginosa* was the same as $10^{7,}$ and the bacterial number increased to 10^9 after 24

hours. The bacterial number of MRSA was approximately 10^8 at the initial time, which increased to 10^{10} cfu/ml. On the other hand, the initial inoculum was 10^7 for the MSSA, and the bacterial number increased to 10^{10}. Both MRSA and MSSA bacterial numbers were consistent with the previous findings of the growth curve (Keman & Soyer, 2019).

Table 2.1. Viable count results of bacteria (*A. haemolyticus, E. coli, P. aeruginosa,* MRSA, and MSSA) in every 3 hours during 24 hours of incubation at 37°C.

Spread plate time point (h)	**Viable cell count (CFU/mL)**				
	A. haemolyticus	***E. coli***	***P. aeruginosa***	**MSSA**	**MRSA**
0	9.07×10^6	1.01×10^7	2.7×10^7	7.5×10^7	2.2×10^7
3	1.45×10^7	3.25×10^8	5.1×10^7		
6	6.22×10^7	4.78×10^8	1.6×10^8		
9	1.06×10^8	4.93×10^8	5.23×10^8	1.85×10^{10}	6.33×10^9
12	4.5×10^8	6.1×10^8	9.05×10^8		
15	2.68×10^8	5.6×10^8	8.25×10^8		
18	3.95×10^8	5.6×10^8	9.08×10^8		
21	1.49×10^9	5.25×10^8	1.02×10^9		
24	3.88×10^8	1.3×10^9	1.84×10^9	2.53×10^{10}	1.6×10^{10}

2.3.2. Minimum Inhibitory Concentrations of 4-Hydroxybenzoic Acid and Gentamicin on MRSA and *A. haemolyticus*

The antimicrobial effects of 4-HBA and gentamicin were tested on MRSA and *A. haemolyticus* by optical density measurements during 24 hours of incubation at 37°C. The antibiotic gentamicin was used to compare antimicrobial effects of phenolic acid 4-HBA

and was used to compare the acquisition of resistance. The growth of both bacteria in the presence of 4-HBA and gentamicin was plotted as optical density at 600 nm the versus time point at which the measurements were taken. The growth curves of MRSA and *A. haemolyticus* in the presence of 4-HBA and gentamicin were given in Figures 2.2 and 2.3, respectively.

The effect of gentamicin on *A. haemolyticus* growth was given in Figure 2.2.a. The treatment with gentamicin caused depression of proliferation of *A. haemolyticus* after 8 hours as compared to the control. But it was seen that the gentamicin concentrations higher than 75 µg/mL, totally inhibit the bacterial growth even at the first 8 hours. At the 24^{th} hour of incubation, concentrations higher than the 5 µg/mL have an inhibitory effect on *A. haemolyticus*. On the other hand, 5 µg/mL gentamicin caused depression of proliferation after 24 hours of incubation as seen with the optical density lowered. Minimum inhibitory concentration (MIC) was defined as the lowest concentration which inhibits the total growth of *A. haemolyticus* after 24 hours of incubation. The MIC value of gentamicin was determined as 10 µg/mL; as it was the lowest concentration that inhibited the total bacterial growth.

The growth curve of MRSA under gentamicin exposure was similar to the *A. haemolyticus,* as shown in Figure 2.2.b. Gentamicin reduced the proliferation in 8 hours as compared to the control of MRSA. At the end of the 24 hours, 5 µg/mL gentamicin reduced the optical density but did not inhibit bacterial growth. The concentrations higher than 5 µg/mL completely inhibit the MRSA growth. As the definition suggests, the lowest concentration inhibiting bacterial growth was 10 µg/mL, which was assigned as MIC value. The MIC value was also tested with the viable cell count to ensure no bacterial growth. On the other hand, subinhibitory concentration was determined by the concentration that lowers the optical density approximately into half after 24 hours. The subinhibitory concentration of gentamicin was determined by 5 µg/mL for MRSA*;* this value was used for further experiment of determination of acquisition of resistance in section 2.3.2

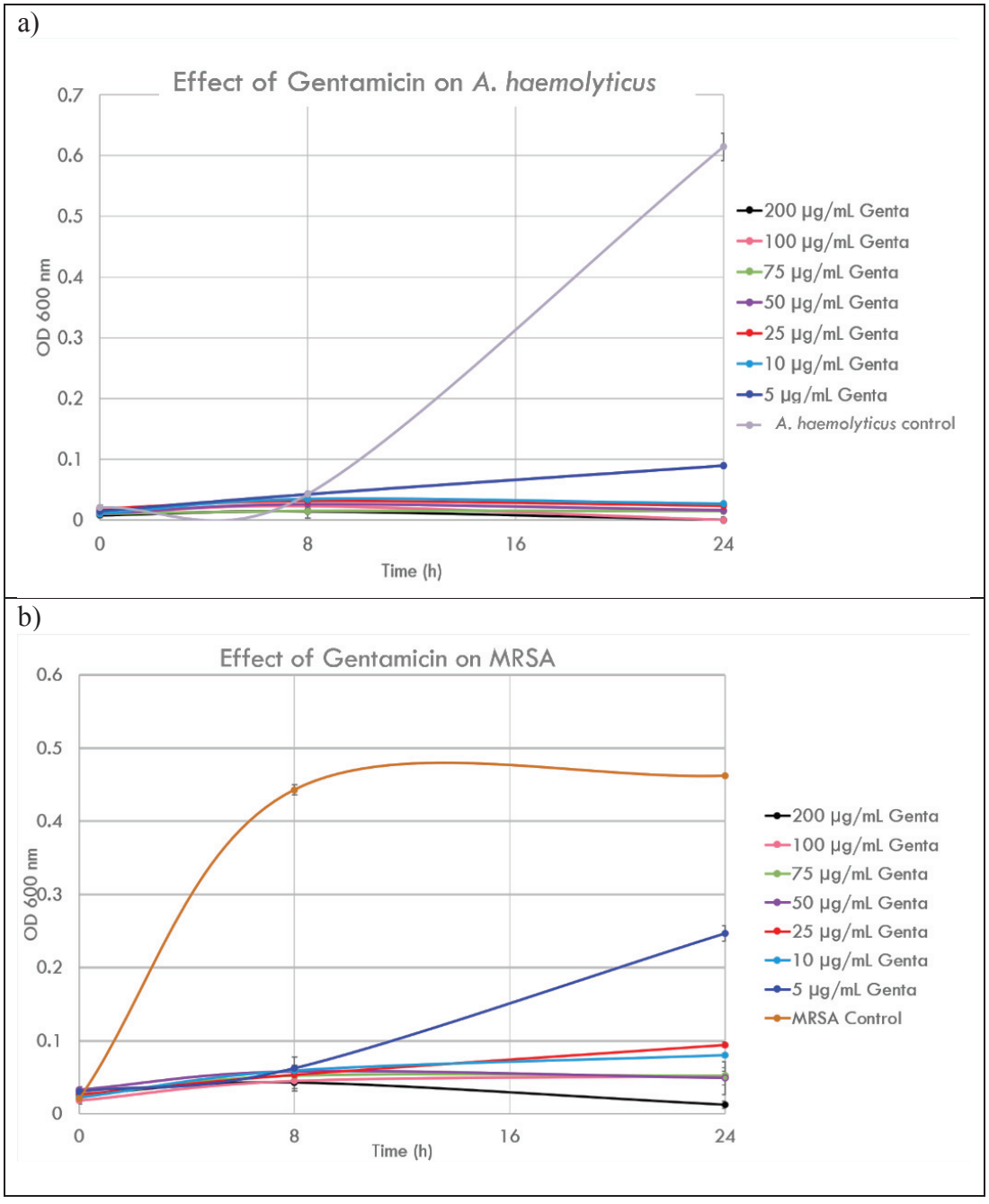

Figure 2.2. Antibacterial effects of gentamicin (indicated as Genta in graph) on *A. haemolyticus* (a) and MRSA (b). Optical densities were measured at 600nm at the incubation periods of 0th, 8th and 24th hour. Standard deviations for different gentamicin concentrations were <0.007 for *A. haemolyticus* and <0.015 MRSA.

The viable cell numbers were tested besides the optical density measurement. The tubes with no turbidity were selected and spread to confirm the MIC values that were already selected based on Figure 2.2. The viable cell counts with the percent inhibitions of different concentrations of gentamicin on *A. haemolyticus* and MRSA were shown in Table 2.2. The 95.69% of growth of *A. haemolyticus* was inhibited by 10 µg/mL of gentamicin. The CFU/mL at the end of the gentamicin treatment was as low as 110. The 5 µg/mL of gentamicin was also not enough to inhibit the total MRSA as the inhibition was as low as 46%. The 10 µg/mL concentration of gentamicin inhibited 82.68% of MRSA, and there was 1 colony of MRSA per mL. The results were consistent with the OD measurements, as predetermined MIC values inhibited the growth of both *A. haemolyticus* and MRSA.

Table 2.2. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation with different gentamicin concentrations.

	A. haemolyticus		**MRSA**	
Gentamicin Concentration	**Percent inhibition**	**Viable cell count (CFU/mL)**	**Percent inhibition**	**Viable cell count (CFU/mL)**
200 µg/mL	100%		97.33%	
100 µg/mL	100%		88.85%	
75 µg/mL	97.68%		88.64%	

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Table 2.2 (continued)

50 µg/mL	97.48%	0	89.4%	
25 µg/mL	96.26%	0	79.55%	0
10 µg/mL*	95.69%	1	82.68%	1.1×10^2
5 µg/mL	85.44%		46.54%	

*: MIC for *A. haemolyticus* and MRSA

The tested concentrations of 4-HBA on *A. haemolyticus* growth were from 1 mg/mL to 3.5 mg/mL. For MRSA, 4-HBA concentrations were selected from 0.5 mg/mL to 3 mg/mL. The effect of 4-HBA on *A. haemolyticus* growth was given in Figure 2.3.a. The treatment with 4-HBA did not affect *A. haemolyticus* in 8 hours as the optical densities of control and different concentrations of 4-HBA were similar. At the 24^{th} hour, 1 mg/mL reduced the bacterial growth approximately in half. But the concentrations of 4-HBA higher than 1 mg/mL inhibited the growth as the optical density was almost zero. The MIC value was determined as higher than 1 mg/mL, and the viable cell count was performed to determine the MIC value depending on the bacterial load within the tubes.

The growth curve of MRSA under 4-HBA treatment was much more different than *A. haemolyticus* as shown in Figure 2.3.b. The concentration of 4-HBA below 1.5 mg/mL retarded the bacterial growth, whereas 1.5 mg/mL of 4-HBA reduced the optical density by more than half. The concentrations from 2 to 3 mg/mL inhibited the MRSA growth. The viable cell count was also performed besides the optical measurements, which were used to confirm the predetermined MIC values based on the bacterial number.

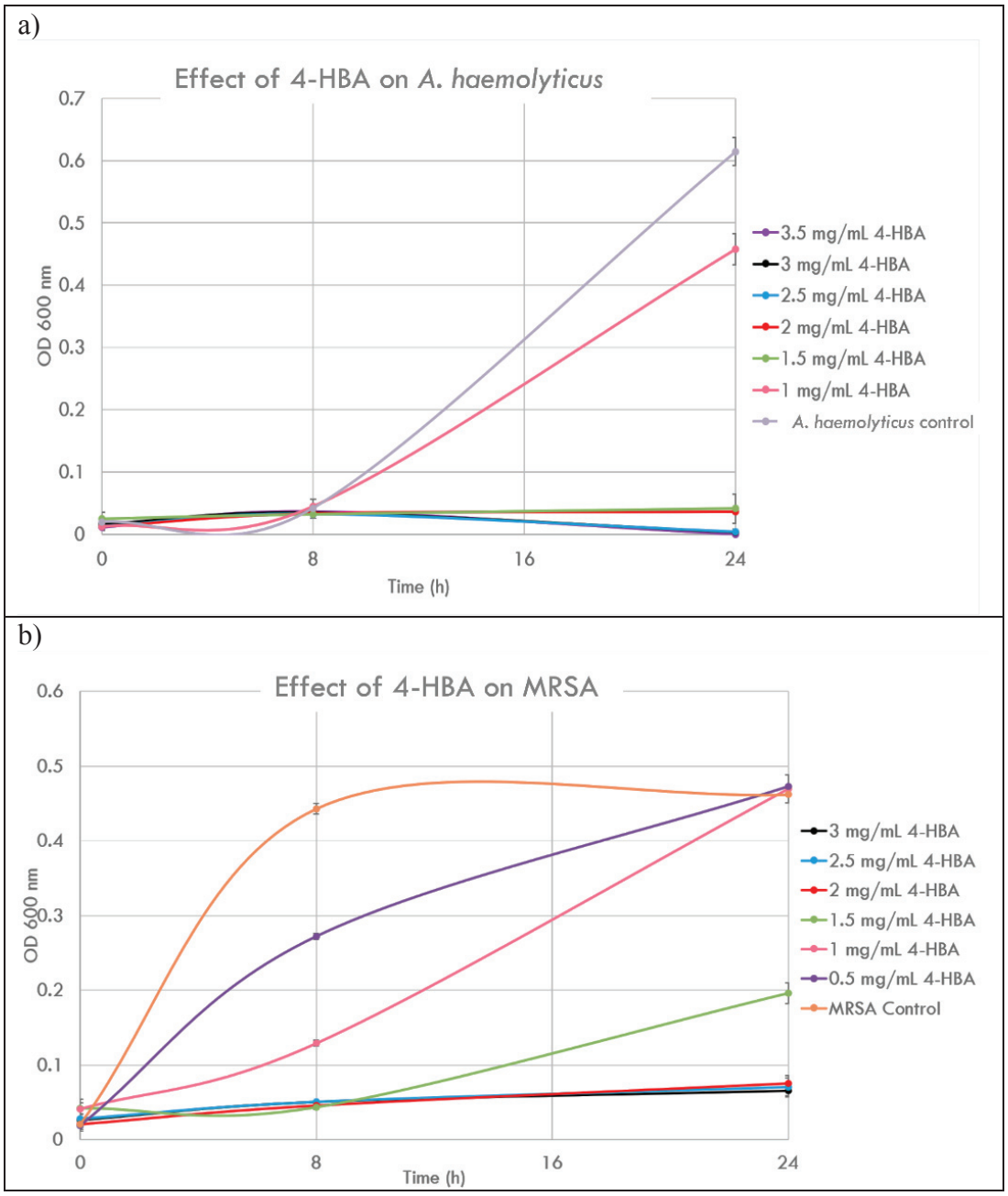

Figure 2.3. Antibacterial effect of 4-HBA on *A. haemolyticus* (a) and MRSA (b). Optical densities were measured at 600nm at the incubation periods of 0^{th}, 8^{th} and 24^{th} hour. Standard deviations for 4-HBA concentrations were <0.025 for *A. haemolyticus* and <0.014 for MRSA.

For the viable cell number, tubes without turbidity were spread plated. The viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation were given in Table 2.3 with the percent inhibitions. Consistent with the optical density measurements, 1 mg/mL of 4-HBA for *A. haemolyticus* was not enough to inhibit the bacterial growth as it inhibited the bacterial growth by only 25%. The concentrations from 1.5 to 2.5 mg/mL were spread plated to determine the MIC values. Even the 1.5 mg/mL 4-HBA containing tube inhibited the bacterial growth 93%, it had too many bacteria to count. But the concentration of 2 mg/mL inhibited 94% of the bacterial growth and there were only 10 colonies per mL. The MIC value was determined as 2 mg/mL for 4-HBA on *A. haemolyticus*. the treatment of MRSA with the 1 mg/mL did not inhibit the bacterial growth at all as the percent inhibition was 0%. The concentration of 1.5 mg/mL was also not enough as 58% of the MRSA growth was inhibited only. The concentrations from 2 to 3 mg/mL were spread plated to determine the MIC value. Both 2 mg/mL and 2.5 mg/mL 4-HBA treated tubes had too many colonies to count. The MIC value of 4-HBA for the MRSA was predetermined as 2 mg/mL from the OD versus time graph, but there was still high number of bacteria. This concludes that the 4-HBA had a bacteriostatic effect on MRSA. Only the concentration of 3 mg/mL had no colony, so the MIC value of 4-HBA was determined as 3 mg/mL for MRSA.

Table 2.3. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation with different 4-HBA concentrations.

	A. haemolyticus		**MRSA**	
Concentrations of 4-HBA	**Percent inhibition**	**Viable cell counts(CFU/mL)**	**Percent inhibition**	**Viable cell counts(CFU/mL**
3.5 mg/mL	99.96%		-	-
3 mg/mL**	99.6%		85.93%	0

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Table 2.3 (cont.)

2.5 mg/mL	99.35%	0	84.70%	5×10^2
2 mg/mL*	94.03%	1×10^1	83.66%	$>10^4$
1.5 mg/mL	93.29%	$>10^3$	57.58%	
1 mg/mL	25.55%		0%	
0.5 mg/mL	-	-	0%	

*: MIC for *A. haemolyticus*
**: MIC for MRSA

From the experimental results of the antimicrobial effects of gentamicin, it can be concluded that both *A. haemolyticus* and MRSA were affected similarly. The concentration of 5 µg/mL was not enough to inhibit the growth of both bacteria. The MIC values of gentamicin were determined as 10 µg/mL for both *A. haemolyticus* and MRSA. The obtained results for MRSA were similar to the study that was carried out by Atashbeyk and his colleagues, in which they found the MIC value of gentamicin as 19.5 µg/mL after 9 hours of incubation of MRSA (Atashbeyk et al., 2014). The incubation period was lower in this study, that might be the reason that the MIC value was higher compared to our results. Logically, more antimicrobials are required to inhibit the total bacterial growth in a limited period such as 9 hours. Other reasons for different MIC values might be due to (i) different initial bacterial load, (ii) different methods to determine MIC, (iii) different strains of MRSA. Also in another study on MRSA obtained from bacterial keratitis showed that the MIC value of gentamicin was found as 0.016 mg/mL and it was found susceptible as 95% (Sueke et al., 2010). But it is already known that MRSA can develop resistance against gentamicin even if it is susceptible at first place (Dacre et al., 1986).

Traub and Sphor found the MIC value of gentamicin as 4 µg/mL for *A. haemolyticus,* which is similar to our finding of 10 µg/mL (Traub & Spohr, 1989). The MIC value was lower than our findings, this might be due to (i) different initial bacterial inoculum, (ii) different methods to determine MIC, (iii) different strains of *A. haemolyticus* as the strains were isolated from clinical patients and the species were identified with API 20E miniature system. Besides the MRSA*, A. haemolyticus* is also another bacterium that can develop resistance against different antibiotics. Japoni and his

colleagues focused on the susceptibility of 88 different Acinetobacter species to 12 different antibiotics including gentamicin. They have found that the 79.5% of the species gained resistance against gentamicin while only 20.5% could not develop any resistance (Japoni et al., 2011).

The treatment with 4-HBA caused different growth curves for *A. haemolyticus* and MRSA as determined MIC were 2 mg/mL and 3 mg/mL, respectively. The concentration of 3 mg/mL inhibited the growth of the MRSA as shown in Figure 2.3.a, but the bacterial load was still high. This can be concluded that at that concentration 4-HBA have a bacteriostatic effect on MRSA. Cho and his colleagues tested the 4-HBA on different bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis*. They found the inhibitory concentration of 50% (IC50) as 926 µg/mL for *S. aureus*, which is almost half of our predetermined MIC for MRSA as 3 mg/mL (Cho et al., 1998). The differences might be due to different strains of *S. aureus,* as MRSA has resistant genes for methicillin and are much more durable under stress. In another study, Alves and his colleagues determined the MIC value of 2,4-dihydroxybenzoic acid as >1 mg/mL for MRSA (Alves et al., 2013). Both 4-hydroxybenzoic acid and 2,4-dihydroxybenzoic acid are derivatives of the benzoic acid. The only difference is 4-hydroxybenzoic acid has the Oxygen atom at the Carbon atom number 4, whereas 2,4-dihydroxybenzoic acid has another Oxygen group at the second Carbon (NIH, 2021b). Keman and Soyer worked on vanillic acid, a derivative of hydroxybenzoic acid. They found the MIC value as 2.5 mg/mL for both MRSA and MSSA (Keman & Soyer, 2019). Vanillic acid is monohydroxy benzoic acid called 4-hydroxy-3-methoxy-benzoic acid, a derivative 4-hydroxybenzoic acid as substituted by a methoxy group at Carbon number 3 (NIH, 2021d). There is no study about the effects of 4-HBA on *A. haemolyticus* yet. But the MIC was found as 2 mg/mL which was similar to the MRSA. Nonetheless, these experimental findings suggest that using phenolic acids as an antimicrobial as an alternative to antibiotics to inhibit bacterial growth and therefore infection.

2.3.3. Sub-Minimum Inhibitory Concentrations of 4-Hydroxybenzoic Acid and Gentamicin against MRSA and *A. haemolyticus*

The MIC value of 4-HBA and gentamicin was already determined as explained in section 2.3.1. The subinhibitory gentamicin concentration was also detected as 5 µg/mL for MRSA. In this experiment, the first step was the determination of the subinhibitory concentration of 4-HBA and gentamicin on *A. haemolyticus* and MRSA. The concentrations of gentamicin (indicated as Genta) were selected from 1 µg/mL to 5 µg/mL for test on the *A. haemolyticus,* as given in Figure 2.4. At the 8th hour, gentamicin did not affect the bacterial growth drastically, but at the 24th hour the concentrations of 1 and 2 µg/mL reduced the optical density by approximately half. The concentrations from 3 µg/mL to 5 µg/mL reduced the optical density significantly.

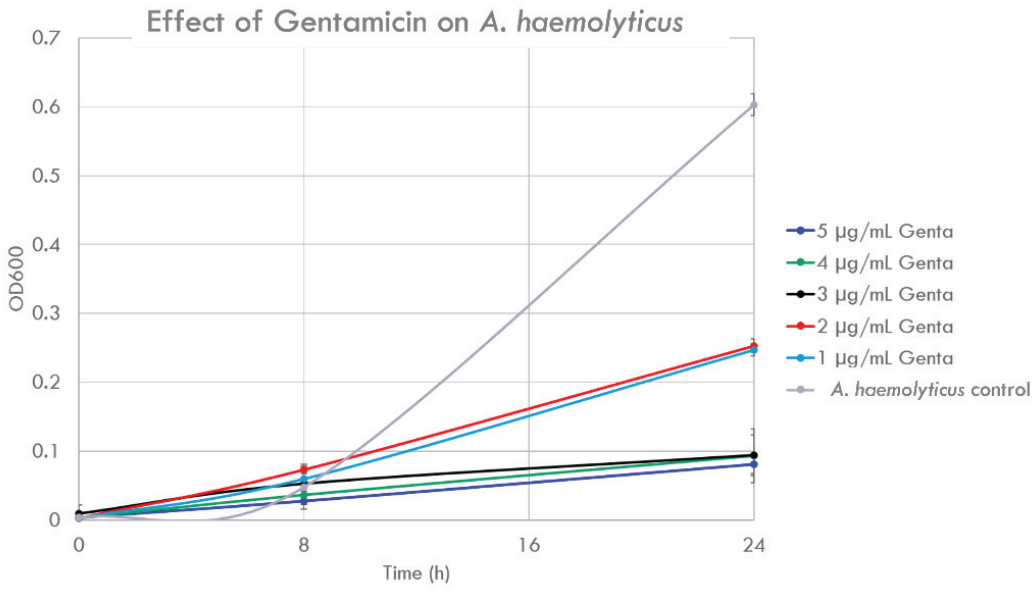

Figure 2.4. Antibacterial effect of gentamicin on *A. haemolyticus.* Optical densities were measured at 600nm at the 0th, 8th and 24th hour incubation periods. Standard deviations for gentamicin concentrations were <0.039 for *A. haemolyticus*.

The percentage of *A. haemolyticus* growth inhibition after 24 hours of incubation with gentamicin was calculated and given in Table 2.4. The concentrations from 3 µg/mL to 5 µg/mL were inhibited the bacterial growth by more than 80%. Both concentrations of 1 and 2 µg/mL gentamicin inhibited the growth by 58 and 59%, respectively. The 2 µg/mL gentamicin was determined as subinhibitory concentration for *A. haemolyticus* as 1 µg/mL gentamicin-containing tube had turbidity.

Table 2.4. Percent inhibitions of *A. haemolyticus* after 24 hours of incubation with different gentamicin concentrations.

Gentamicin Concentration	**Percent inhibition of *A. haemolyticus***
5 µg/mL	86.57%
4 µg/mL	84.52%
3 µg/mL	84.46%
2 µg/mL*	58.09%
1 µg/mL	59.08%

*: subinhibitory concentration for *A. haemolyticus*

The concentrations of 4-HBA were selected from 1 mg/mL to 1.5 mg/mL to test on the *A. haemolyticus* and MRSA; the graphs of growth curves were given in Figure 2.5.a and b, respectively. At the first 8 hours of incubation, all the 4-HBA treated cultures of *A. haemolyticus* showed delayed growth. However, at the 24th hour, all the concentrations higher than 1 mg/mL showed complete inhibition of bacterial growth. The 1 mg/mL 4-HBA was not enough to reduce the bacterial growth in half. As the graph was examined, 1.2 mg/mL showed a less inhibitory effect compared to the 1.1 mg/mL concentration, which might be due to the experimental error.

On the other hand, the same concentrations of 4-HBA resulted in a lower inhibitory effect on MRSA. Unlike the *A. haemolyticus*, MRSA grew in the first 8 hours, but the growth was reduced compared to control. At the 24 hours of incubation, the

concentrations from 1.3 mg/mL to 1.5 mg/mL inhibited the growth significantly. The 1 and 1.1 mg/mL 4-HBA reduced the MRSA growth. However, the 4-HBA with a 1.2 mg/mL concentration reduced the bacterial growth approximately in half.

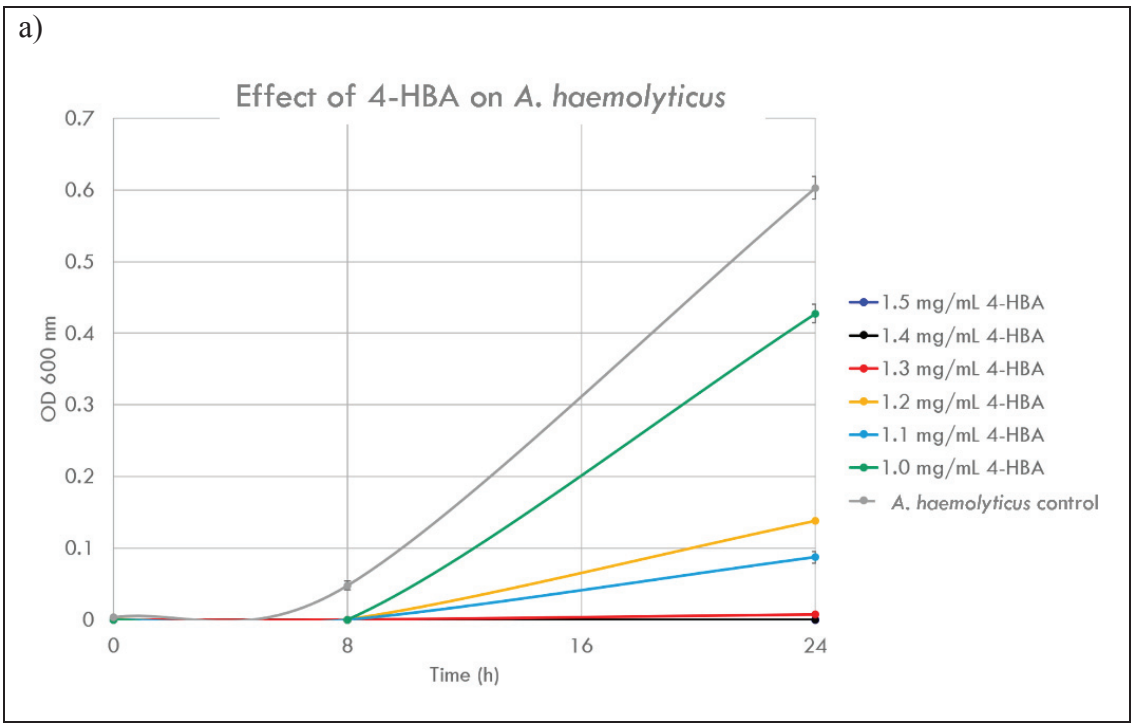

Figure 2.5. Antibacterial effect of 4-HBA on *A. haemolyticus* and MRSA. Optical densities were measured at 600nm at the 0th, 8th and 24th hour incubation periods. Standard deviations for gentamicin concentrations were <0.015 for *A. haemolyticus* and < 0.036 for MRSA.

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b)

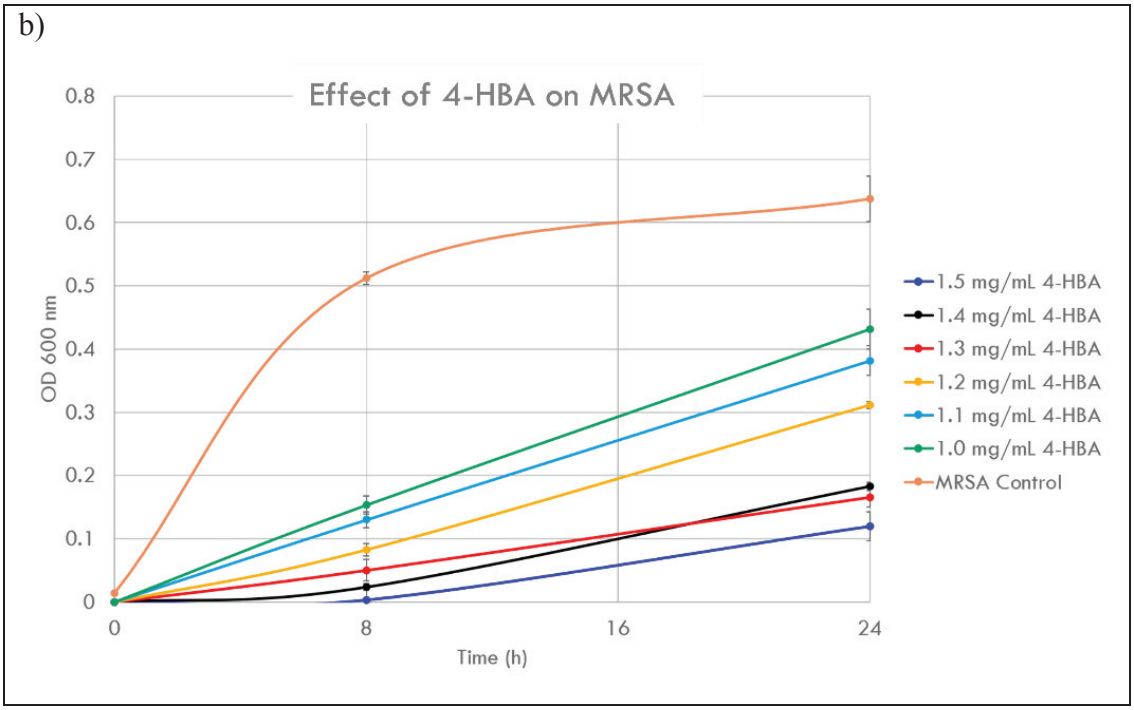

Fig 2.5 (cont.)

The percentage of *A. haemolyticus* and MRSA growth inhibition by 4-HBA treatment was given in Table 2.5. Concentrations from 1.3 to 1.5 mg/mL completely inhibited the bacterial growth of *A. haemolyticus,* whereas the inhibition of MRSA growth was approximately 75%. One can conclude that the *A. haemolyticus* is much more sensitive to 4-HBA compared to MRSA. The 1.2 mg/mL 4-HBA reduced the MRSA growth in half, therefor it was determined as subinhibitory concentration. 4-HBA with 1.2 mg/mL concentration showed less inhibitory effect compared to the 1.1 mg/mL, as 77% and 85%, respectively. This might be due to experimental error, as the subinhibitory concentration for *A. haemolyticus* was selected as 1.1 mg/mL.

Table 2.5. Percent inhibitions of *A. haemolyticus* and MRSA after 24 hours of incubation with different 4-HBA concentrations.

Concentrations of 4-HBA	**Percent inhibition of *A. haemolyticus***	**Percent inhibition of MRSA**
1.5 mg/mL	100%	81.26%
1.4 mg/mL	100%	71.3%
1.3 mg/mL	98.87%	74.09%
1.2 mg/mL**	77.10%	51.18%
1.1 mg/mL*	85.55%	40.15%
1 mg/mL	29.13%	32.38%

*: subinhibitory concentration for *A. haemolyticus*
**: subinhibitory concentration for MRSA

In conclusion, the subinhibitory concentrations of 4-HBA and gentamicin were 1.1 mg/mL and µg/mL for *A. haemolyticus,* respectively. MIC values were predetermined as 2 mg/mL and 10 µg/mL for 4-HBA and gentamicin, respectively. The subinhibitory concentrations of 4-HBA and gentamicin were determined as 1.2 mg/mL and 5 µg/mLfor MRSA, respectively. MIC values were predetermined as 3 mg/mL 10 µg/mL for 4-HBA and gentamicin, respectively.

2.4. Conclusion

This experimental study focused on using 4-hydroxybenzoic acid as an alternative antimicrobial product against antibiotic-resistant bacteria such as *A. haemolyticus* and MRSA. The outcome of this study was the susceptibility of bacteria to phenolic acid 4-hydroxybenzoic acid. The antimicrobial action of phenolic acids is different from antibiotics. Antibiotics commonly target different cellular structures and processes by inhibiting i) the cell wall synthesis, ii) cell membrane function, iii) protein synthesis, iv) nucleic acid synthesis, and v) other metabolic processes that are essential for pathogen

survival (O'Rourke et al., 2020). On the other hand, phenolic acids have different modes of action for inhibiting bacterial growth. Among these, phenolic acids disrupt the quorum sensing of the bacteria (Borges et al., 2014), irreversibly alter the membrane by creating pore and changing surface charge (Borges et al., 2013); bind to the outer membrane and disrupt it, which leads to release cytoplasmic molecules even slightly release nucleotide, which led to cell death. (Lou et al., 2011); bind to bacterial DNA and proteins such as enzymes, inhibiting cellular functions (Alves et al., 2013).

CHAPTER 3

ELUTION KINETICS AND ANTIMICROBIAL ACTIVITY of 4-HYDROXYBENZOIC ACID AND GENTAMICIN LOADED BONE CEMENT ON *Escherichia coli*

3.1. Introduction

Surgical site infections are the infections that occur in the 30 days after surgery. In the case of surgery of implants, infections can occur even up to 1 year after surgery. Those infections generally take place at the site of operation and deep tissue where the operation took place. Most of the time, a high dose of antibiotics is used as a prevention of infection, however, surgical site infections are still one of the significant and common problems as results in prolonged hospitalization and high mortality (National Healthcare Safety, 2021). The rate of infection after surgery may be up to 20%, depend on the surgery and patient profile. In most the cases, surgical site infections are caused by the endogenous flora of the patient itself. The pathogens that cause the surgical site infection are generally *Staphylococcus aureus*, coagulase-negative staphylococci, Enterococcus species and *Escherichia coli* (Owens & Stoessel, 2008). Despite the improvement in technology and prevention in healthcare facilities, the incidence of surgical site infections only reduced to 394 in 2015 from 452 in 2011, just in the United States. 11% of those patients died due to surgical site infection. Upon surgical site infections, 48% of the patients have *Staphylococcus aureus*; 44% of the patients have *Escherichia coli* (Magill et al., 2018).

A higher dose of antibiotics is generally applied to prevent surgical site infections. However, with the reduced immune system of the patients, antibiotics generally cause the development of resistance by bacteria. The study in Greece indicated that among the pathogens that were isolated from surgical site infections, *S. aureus* was highly resistant to ceftriaxone (100%), penicillin (91.36%), amoxicillin (87.5%), and amikacin (80%). *E. coli* was most resistant to cefuroxime (89.50%), cefepime (84.20%) and cefazoline (77.80%) (Călina et al., 2017). The strains isolated from surgical site infections in Egypt showed that 88.2% of the *S. aureus* was methicillin-resistant *S. aureus* (MRSA) which

was completely resistant to vancomycin, penicillin, ampicillin, and oxacillin (Zahran et al., 2017). In Nepal, *S. aureus* and *E. coli* isolates from surgical site infections were multi-drug resistant with the rate of 38% and 40%, respectively (Chaudhary et al., 2017).

One of the most common surgery procedures is orthopedic surgery, in which there were 43 million patients that visited the orthopedic physicians in the United States in 2016-2016 (CDC, 2016). Especially in orthopedic implant surgeries and treatment of joints, bone cements are successfully used. Bone cements are used for anchoring the artificial joints by filling the space between the prosthesis and the bone of the patients. Bone cements are composed of polymer-based materials such as poly-methyl-methacrylate (PMMA) or copolymers. The bone cement powder consists of PMMA beads in which size varies between 5 to 80 μm. After the addition of liquid methyl-methacrylate, polymerization starts, that leads to increase in the temperature of the material. The amount of air bubbles depends on bone cements' polymerization and viscosity rate. The presence of air bubbles results in porous bone cement.

Like any other surgery, antibiotics are highly used in orthopedic surgeries by mixing the antibiotics in the bone cements. The release rate of the antibiotic from bone cement depends on the diffusion rate of the antibiotic, availability, and the size of the pores (van de Belt et al., 2001). Besides the widespread usage of bone cements, there are still concerns as i) bacterial adhesion and biofilm formation, ii) antibiotic resistance, iii) mechanical properties and, iv) release rate of antibiotic from bone cements (Bistolfi et al., 2011). The bone cement provides a surface and attachment point for the microorganisms that have already a tendency to cause biofilm. The bone cements are mostly in contact with the bone and blood, in which any infection of the bone cement will result in entering the microorganisms into bone and bloodstream and can be resulted in death (van de Belt et al., 2001). Similar to the surgical site infections, the addition of antibiotics within the bone cement generally leads to the formation of antibiotic resistant bacteria in the surgical microenvironment.

The aims of this experimental part were 1) the determination of the release rate of phenolic acid and antibiotic from antimicrobial-loaded PMMA bone cement discs, 2) investigation of morphological studies of the antimicrobial-loaded PMMA bone cement, 3) the determination of the antibacterial effect of 4-HBA powder and 4-HBA solution-loaded PMMA bone cement discs on *E. coli* by comparing to well-known antibiotic gentamicin, and finally, 4) the investigation of the synergistic antibacterial effect of 4-

HBA and gentamicin-loaded PMMA bone cement discs on *E. coli.* In this chapter of the experimental study, three hypotheses were tested;

1. As the 4-HBA and gentamicin solubilize in water, the structure of liquid antimicrobial-loaded bone cements would have more porous structure.
2. If liquid antimicrobial-loaded bone cements have more pore, release rate from liquid antimicrobial-loaded bone cement would be higher and it would inhibit bacterial growth more.
3. The surface area of 4-HBA (57.5 Å²) is smaller than gentamicin (200 Å²); therefore, the 4-HBA release from bone cement would be higher (NIH, 2021a, 2021c).

3.2. Materials and Methods

In this study, methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) N315 type II SCC *mecA* and *Escherichia coli* were used to test the antimicrobial activity of phenolic acid 4-hydroxybenzoic acid-loaded bone cement. MRSA, MSSA and *E. coli* were particularly selected as those bacteria were some of the most seen bacteria after surgery as they cause surgical site infections (Owens & Stoessel, 2008). The bacteria *E. coli*, MSSA and MRSA were grown as described in previous chapter 2.2.1. The growth curve of MRSA, MSSA and *E. coli* was also determined in the previous section 2.2.2, as given in Figure 2.1 b, d and e, respectively.

3.2.1. Preparation of Bone Cements

The antimicrobial agents were used in bone cement preparation were i) 1 g gentamicin powder, ii) 2 g gentamicin powder, iii) 10 mL gentamicin solution that contains 2 g gentamicin powder, iv) 2 g 4-HBA powder, v) 10 mL 4-HBA solution that contains 2 g 4-HBA powder, vi) 1 g of gentamicin powder with 1 g of 4-HBA powder and, vii) 1 g gentamicin powder with 2 g 4-HBA powder. The solutions were prepared by addition of 10 mL of sterile ddH_2O to 2g of respective antimicrobial either 4-HBA or gentamicin. The bone cements with the addition of gentamicin were used as a positive

control. The bone cement without any the addition of antimicrobial agent was used as negative control and indicated as “null” during the experimental procedure.

All steps for bone cement preparation were performed under aseptic conditions with sterile material. The solutions, glass materials and pens were autoclaved at 121^{o}C for 15 to 20 minutes. The stainless-steel molds were used to obtain uniform structure and size of each bone cements as 8 mm in diameter and 3 mm in height. The molds were sterilized at 90 ^{o}C for 18 hours. After sterilization, each material was stored in the refrigerator as bone cement preparation must take place in the cold.

Figure 3.1. The stainless-steel molds for uniform structure and sized bone cements.

For standardization of the experimental setups, all bone cements were commercially purchased from Radiopaque Cement Oliga 1. The bone cement kit was coming with 1 g gentamicin, 40 g of PMMA radiopaque powder, 20 mL of liquid methyl methacrylate (MMA). The 40 g of powder of PMMA was mixed with the 20 mL of liquid MMA in the sterile glass container and immediately mixed. The addition of methyl methacrylate starts the exothermic polymerization reaction of bone cement; therefore, the following steps have to be performed quickly. The antimicrobial agent was added and quickly mixed with the bone cement dough. If the used antimicrobial agent was in the solution form, it was vortexed before adding to bone cement. The stainless-steel molds were wet with the sterile ddH_2O to prevent the bone cement to adhere. The bone cement dough was placed between molds and interlocked. At the polymerization step the

temperature of molds increases. After 1 hour, the PMMA bone cements were hardened and got cold. Then the molds were opened and each bone cement disc was taken out of holes of the mold. Bone cement discs were collected in the sterile bottle that was wrapped with aluminum foil to prevent light. Bone cement discs were stored in the refrigerator at -20^{o}C in dark until usage.

3.2.2. Morphological Study of Bone Cement by Scanning Electron Microscopy

The PMMA bone cement powder consists of PMMA beads in whose size varies between 5 to 80 μm. The air bubbles are entrapped in bone cement that results in porous bone cement (van de Belt et al., 2001). The morphology of the porous structure and disc surface of bone cements were characterized by scanning electron microscopy (SEM). Each bone cement was coated with gold for 90 seconds before visualization by Phillips XL-30S FEG SEM. Microscopic images were taken with FEI Quanta 250 FEG. The samples of bone cements were scanned by SEM at accelerating voltage, at 5 kV.

3.2.3. Release Rate of Gentamicin and 4-Hydroxybenzoic Acid by UV-Visible Spectrometry

The release rate of antimicrobials from bone cement discs was performed with UV-visible spectrometry according to the literature. The release rate experiment was modified from the study of Lee and Chang (Lee & Chang, 2015). In this experiment, the release rate of 4-HBA and gentamicin from PMMA bone cement discs was measured. Seven discs of the antimicrobial loaded PMMA bone cement were added to 7 mL of PBS (pH 7.4,) containing tubes. The tubes were wrapped with aluminum foil and incubated at 37^{o}C for 7 days in the dark while shaking at 75 min^{-} for mimicking the human body. In each day, 1 mL of sample was taken and antimicrobial release from bone cements was determined by UV-Vis spectrophotometer at 250 nm for 4-HBA and 320 nm for gentamicin.

3.2.4. Confirmation of Release Rate of 4-Hydroxybenzoic Acid by High Performance Liquid Chromatography

The release rate of the 4-HBA from PMMA bone cement discs was confirmed with the High-Performance Liquid Chromatography (HPLC) technique. As described in the previous section, 7 discs of the antimicrobial loaded PMMA bone cement were added to 7 mL of PBS containing tubes. Therefore, in each 1 mL, the concentration of the antimicrobial released from one individual bone cement was measured. The tubes were wrapped with aluminum foil and incubated at 37^{o}C for 7 days in the dark while shaking at 75 min^{-} for mimicking the human body. In each day, 1 mL of sample was taken. The samples were analyzed with the Agilent 1260 Infinity II HPLC machine. The 95% formic acid (0.1%) and %5 acetonitrile were used as mobile phase in the HPLC. The temperature of HPLC was set to 40^{o}C and samples were analyzed at 254 nm. The 500 ppm (mg/mL) of 4-HBA was used as a standard of the calibration.

3.2.5. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid-Loaded Bone Cements on *Escherichia coli*

Each PMMA bone cements were weighted and 1 g bone cements in total (approximately 7 discs) were added to 5 mL TSB containing tubes. The tubes with the bone cements were pre-incubated for 7 days in the shaking incubator at 37^{o}C for 24 hours shaking at 75 min^{-} to allow the release of the antimicrobial agents from the bone cement discs. The tubes for MRSA and MSSA were pre-incubated for 3 days. The bacteria of MRSA, MMSA and *E. coli* were grown as described in section 2.2.1. The overnight grown bacteria were diluted such that the initial bacterial inoculum was 10^{6} CFU/mL and inoculated into pre-incubated bone cement containing TSB tubes. The initial inoculum for MRSA and MSSA was adjusted as 10^{8} CFU/mL. The tube without any addition of bone cements was used as a negative control. The time of the inoculation was accepted as the initial time (0^{th} hour). The initial optical density was measured at 600 nm and spread plated onto TSA to determine the mode of action of antimicrobial. The initial spread plate from the TSB tubes without any bone cement discs was used to control the initial bacterial number. The TSB with the bone cement discs that do not contain any antimicrobial was

used to determine if the bone cement itself has any antimicrobial effect. After inoculation, tubes were incubated at 37°C for 24 hours. After 24 hours of incubation, the optical density was measured again, and each tube was spread onto TSA to determine the reduction in bacterial growth. Tubes were incubated at 37°C for another 24 hours at 75 min^{-}. After 48 hours of incubation in total, optical density measurement and spread plate were performed again.

3.3. Results and Discussion

During the experiments, 7 different types of PMMA bone cements were used as contained I) 1 g gentamicin powder, ii) 2 g gentamicin powder, iii) 10 mL gentamicin solution that contains 2 g gentamicin powder, iv) 2 g 4-HBA powder, v) 10 mL 4-HBA solution that contains 2 g 4-HBA powder, vi) 1 g of gentamicin powder with 1 g of 4-HBA powder and, vii) 1 g gentamicin powder with 2 g 4-HBA powder.

The solutions were prepared by the addition 2 g of antimicrobial into 10 mL sterile ddH_2O and indicated as "4-HBA liquid" or "Gentamicin liquid" throughout the results. The bone cements with the addition of gentamicin were used as a positive control for 4-HBA. The bone cement without any addition of antimicrobial agent was used as negative control and indicated as "null".

3.3.1. Morphology of Bone Cements by Scanning Electron Microscope

The PMMA bone cement powder includes PMMA beads. The PMMA beads have sizes that vary from 5 to 80 μm. With the addition of the polymerization liquid MMA, polymerization process starts and rises the temperature in which entraps the air bubbles within the bone cements. The presence of the air bubbles provides a porous structure to bone cements. The presence of air bubbles depends on the rate of the polymerization and the viscosity property (van de Belt et al., 2001). The porous structure and the size of the PMMA beads of the bone cement were characterized by scanning electron microscopy. Each bone cement was coated with gold for 90 seconds prior to visualization. The addition of 4-HBA and gentamicin was expected to stabilize the bone cement structure without

affecting the porous structure. However, adding antimicrobial solutions was expected to increase the porous structure.

The bone cement without any addition of antimicrobials was visualized to compare if any addition of antimicrobials changes the surface structure and pore size. The SEM micrograph of null bone cement discs was given in Figure 3.2. The PMMA beads were clearly seen the Figure 3.2.a, as the beads were tightly packed, and the surface was homogenous. Bone cements under 1000X magnification (Figure 3.2.b) revealed that there were numbers of fine grains of globular molecules that cover the surfaces. Those globular structures were PMMA beads which were smaller than 100 μm.

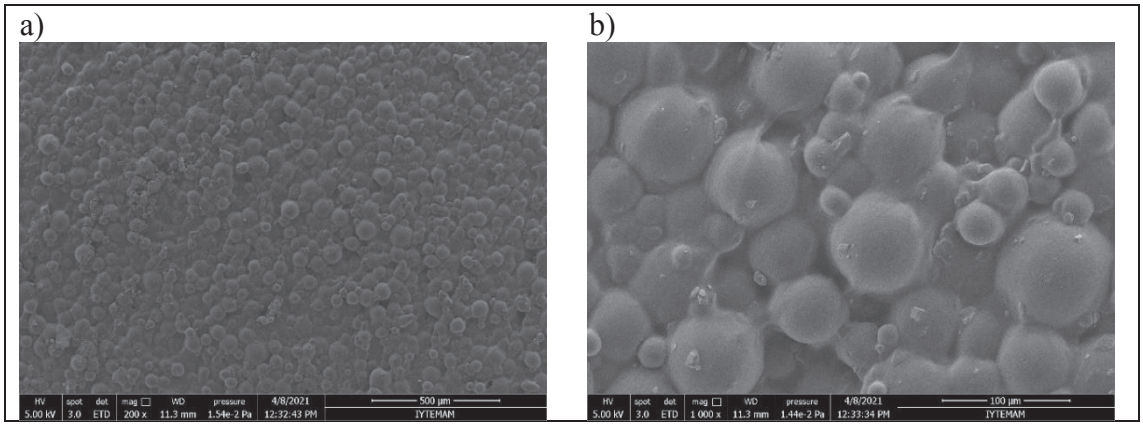

Figure 3.2. SEM micrograph of PMMA bone cement discs without any addition of antimicrobial under 200X (a) and 1000X magnification (b). The null bone cements were used as a negative control.

The SEM images of the PMMA disc surfaces showed that the addition of 1g gentamicin resulted in more heterogenicity of the bone cement as there were more uniform small globules presented in Figure 3.3.b. Compared to null, gentamicin-loaded bone cements had more roughness on the surface. The roughness was resulted from the mixture of gentamicin with the hydrophobic liquid MMA. The null bone cement was more tightly packed compared to gentamicin-loaded bone cement. The bone cement became more heterogenous as the gentamicin was increased to 2 g. The cavity structures were the most presented in the bone cement with gentamicin solution (Figure 3.3.e). The gentamicin solution-loaded bone cements had more pores compared to the powder-loaded

ones. Also compared to the solution, gentamicin powder-loaded bone cements had a smoother surface. The addition of antibiotics increased the porosity of the bone cements. The addition of antibiotic solution even more increased the porosity. Depending on the images, the release of gentamicin from gentamicin solution-loaded bone cement was expected to be at a higher rate, compared to gentamicin powder ones.

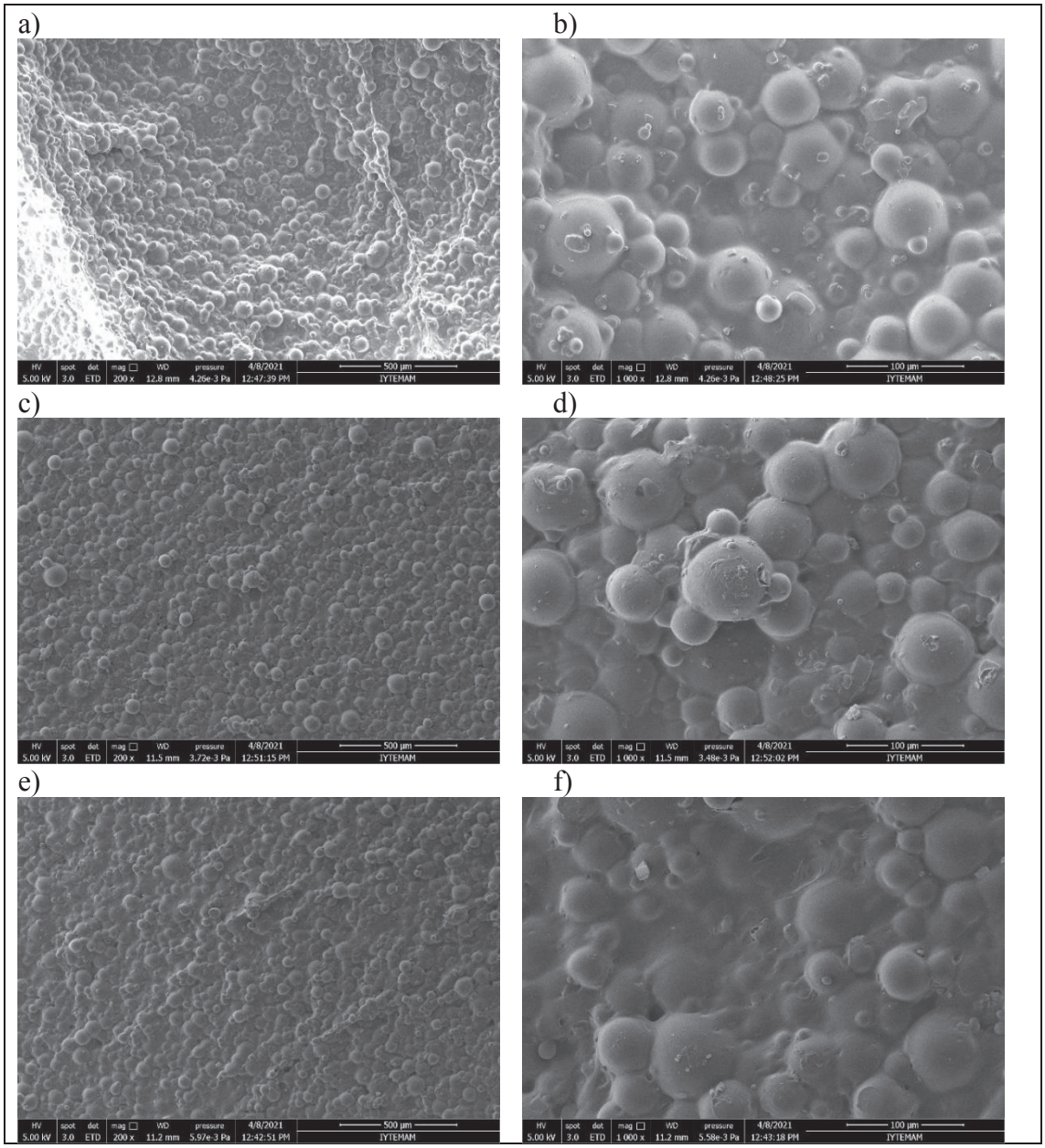

Figure 3.3. SEM micrograph of PMMA bone cement discs with a,b) 1 g gentamicin, c,d) 2 gram gentamicin, e,f) gentamicin solution. The magnification of a, c and e were 200X and b,d and f were 1000X.

The surface area of 4-HBA is 57.5 Å² (NIH, 2021a) which is smaller than gentamicin as the gentamicin is 200 Å² (NIH, 2021c). The addition powder 4-HBA was resulted in a much more compact structure compared to gentamicin addition as 4-HBA filled the gaps but still preserve the porous structure. Therefore, the addition of 4-HBA would increase the mechanical strength of the bone cement. There was still porous structure within the 4-HBA-loaded bone cements as given in Figure 3.4.a. Compared to gentamicin solution-loaded bone cement, loading of 4-HBA solution resulted in much smoother surface morphology (Figure 3.4.c). The 4-HBA loaded-bone cements showed a homogenous structure compared to gentamicin, probably due to the compatibility of the 4-HBA with liquid MMA.

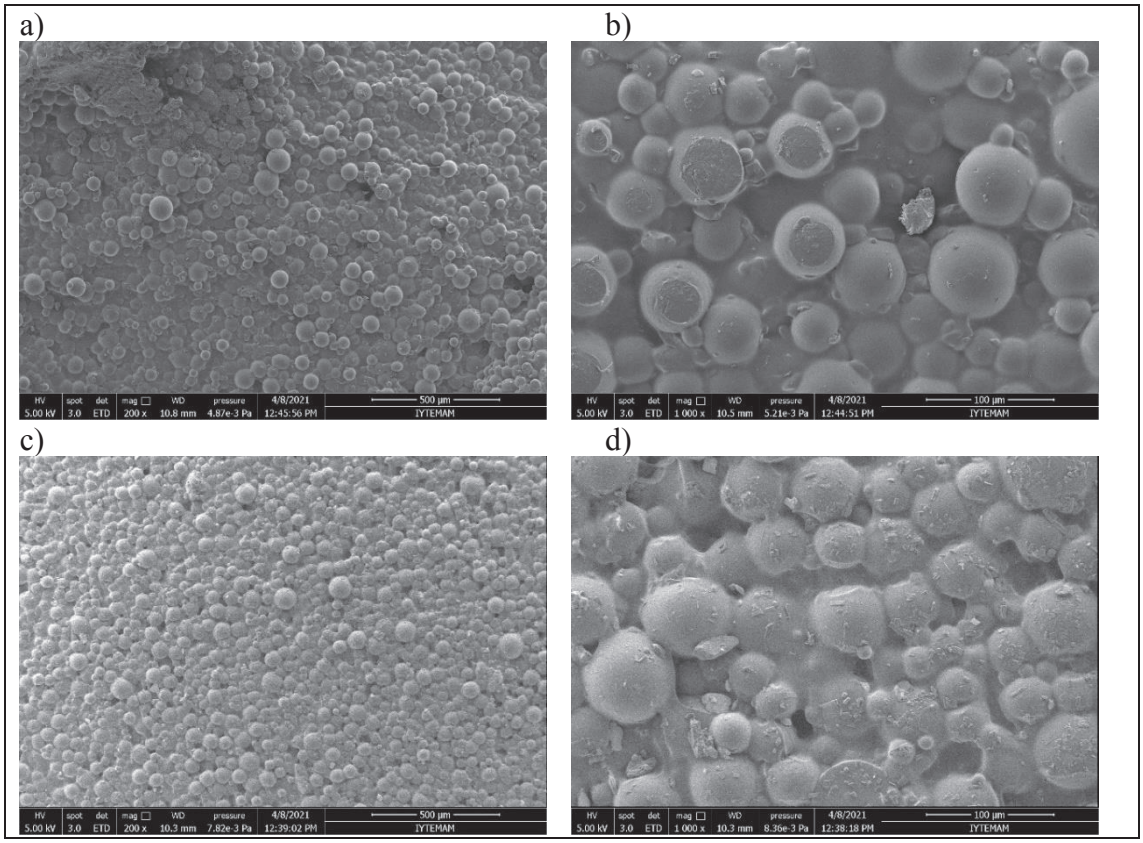

Figure 3.4. SEM micrograph of PMMA bone cement discs with a,b) 2 g 4-HBA, c,d) 4-HBA solution. The magnification of a and c were 200X; b and d were 1000X.

Finally, gentamicin and 4-HBA were combined and loaded into bone cements. Due to difference in their surface area, the bone cement was heterogeneous. However, smaller molecular size of the phenolic acid 4-HBA was important to fill the gaps between the larger molecule of gentamicin and therefore increase the stability. Compared to the 1 g gentamicin-loaded bone cement (Figure 3.3.b), the addition of 1 g of 4-HBA into bone cement (Figure 3.5.b) resulted in drastic changes in the morphology of the bone cement. The globular structures were much denser and distributed uniformly in gentamicin and 4-HBA combination. The surface of the 4-HBA was much smoother as the fine grains of the 4-HBA covered the surface. As the amount of the 4-HBA was increased to 2 g, the porous structures decreased, and the surface of the bone cement discs became rough.

Gandomkarzadeh revealed that the antibiotic increased the porosity and the higher molecular weight of antibiotics resulted in a reduction in compressive strength (Gandomkarzadeh et al., 2020). The reduction in strength was correlated with the drug dose. In that perspective, gentamicin addition was expected to reduce the compressive strength, as it has higher a molecular weight. Also, the addition of more gentamicin would also reduce the mechanical strength of the bone cement. Lee and Chang used the caffeic acid phenyl ester (CAPE) and gentamicin in PMMA bone cements and investigated their morphologies. The surface of bone cements with the gentamicin was found as heterogeneous whereas CAPE-loaded bone cements were homogeneous (Lee & Chang, 2015). Those results were consistent with our findings. Lee and his colleagues also qualitatively analysed the morphology of the poly(dl-lactic-co-glycolic acid) (PLGA) bone cements with the addition of CAPE. The SEM images showed that the addition of CAPE changed the surface morphology as there were small globular molecules were presented (Lee et al., 2013). The addition of 4-HBA in this study also resulted in the addition of small particles on the bone cement disc surface. Porous bone cements are preferred over non-porous as it allows the growth of bone tissues through the cement and also it provides more stable anchorage to bones. In one of the studies by Kim and his colleagues, the PMMA cone cements were morphologically investigated and PMMA beads were observed with the chitosan (Kim et al., 2004). In another study in 2021, lyophilized liquid gentamicin was integrated into the PMMA and compared to the gentamicin powder. Liquid gentamicin addition was denser and showed small pores within the PMMA while powder gentamicin provided a smooth surface to the bone cement discs (Liawrungrueang et al., 2021). The results were consistent with our findings

as the addition of solution-based antimicrobials increased the porous structure. Also, the addition of gentamicin and 4-HBA powder increased the smoothness of the bone cement surfaces.

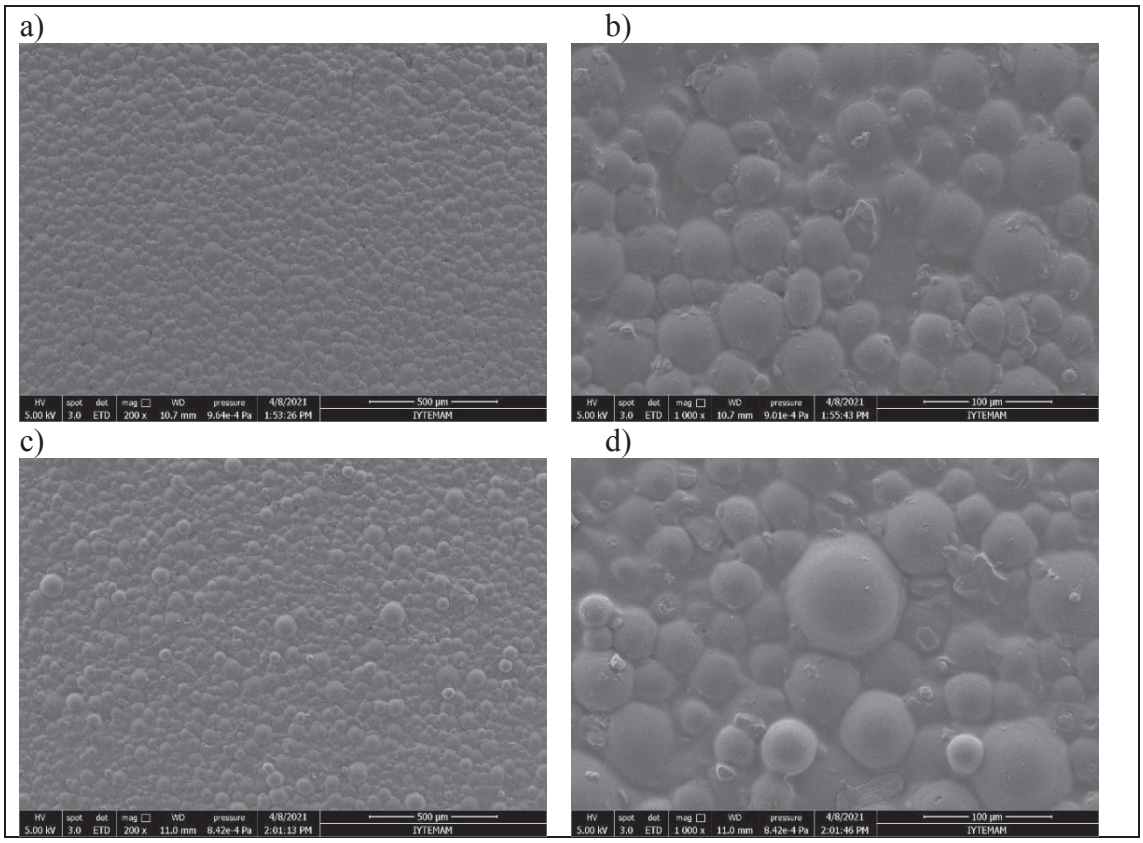

Figure 3.5. SEM micrograph of PMMA bone cement discs with combination of a,b) 1 g 4-HBA with 1 g gentamicin and c,d) 2 g 4-HBA with 1 g gentamicin. The magnification of a and c were 200X; b and d were 1000X.

3.3.2. Release Rate of Gentamicin and 4-Hydroxybenzoic Acid by UV-Visible Spectrometry

The 7 discs of PMMA bone cement were incubated in 7 mL PBS and each day sample was measured with UV-Vis spectrometry. The experimental design of release rate was modified from Lee and Chang (Lee & Chang, 2015). The bone cement without any

addition of antimicrobial was used as a negative control and the graph was given in Figure 3.6.a. The release pattern was not regular as there was only one peak at the 24th hour, there was no significant change in the OD afterward. The release of gentamicin from 1 g and 2 g gentamicin-loaded bone cement was compared in Figure 3.6.b. The porous structures of two bone cements were found similar, consistent with the release pattern. The 2 g gentamicin-loaded bone cement released more gentamicin in to the solution than the 1 g gentamicin loaded bone cement. Both samples reached the peak at the 72nd hours, then oscillation occurred. The powder and solution of gentamicin were compared in Figure 3.6.c. As the porous structure of bone cements was increased in the addition of gentamicin solution, the release of gentamicin from the gentamicin-solution loaded bone cement was higher than the powder-loaded. There was a peak point at the 72nd, which then oscillation was observed. The 4-HBA forms were compared in Figure 3.6.d as powder and solution. The solution-loaded bone cements had higher porous structures and consistent with that there was more release of 4-HBA from solution-loaded bone cements. the release pattern was more regular compared to gentamicin as there were no sharp peaks and oscillations. The combination of gentamicin and 4-HBA includes 1 g gentamicin with 1 g 4-HBA and 1 g gentamicin with 2 g 4-HBA. The release graph of 4-HBA was measured at 250 nm and given in Figure 3.6.e. The release pattern was similar to the previous 4-HBA graph. The major difference was observed in the time period from the initial to 24th hour as bone cement with 2 g 4-HBA had more initial rate. After 24 hours, two of the compounds showed the same pattern. The release graph of gentamicin from combination-loaded bone cement was given in Figure 3.6.f. The release of gentamicin was expected to be the same as both types of bone cements contained 1 g gentamicin. The release of gentamicin from gentamicin containing bone cement was the same as the release from the combination of gentamicin and 4-HBA. The data were consistent.

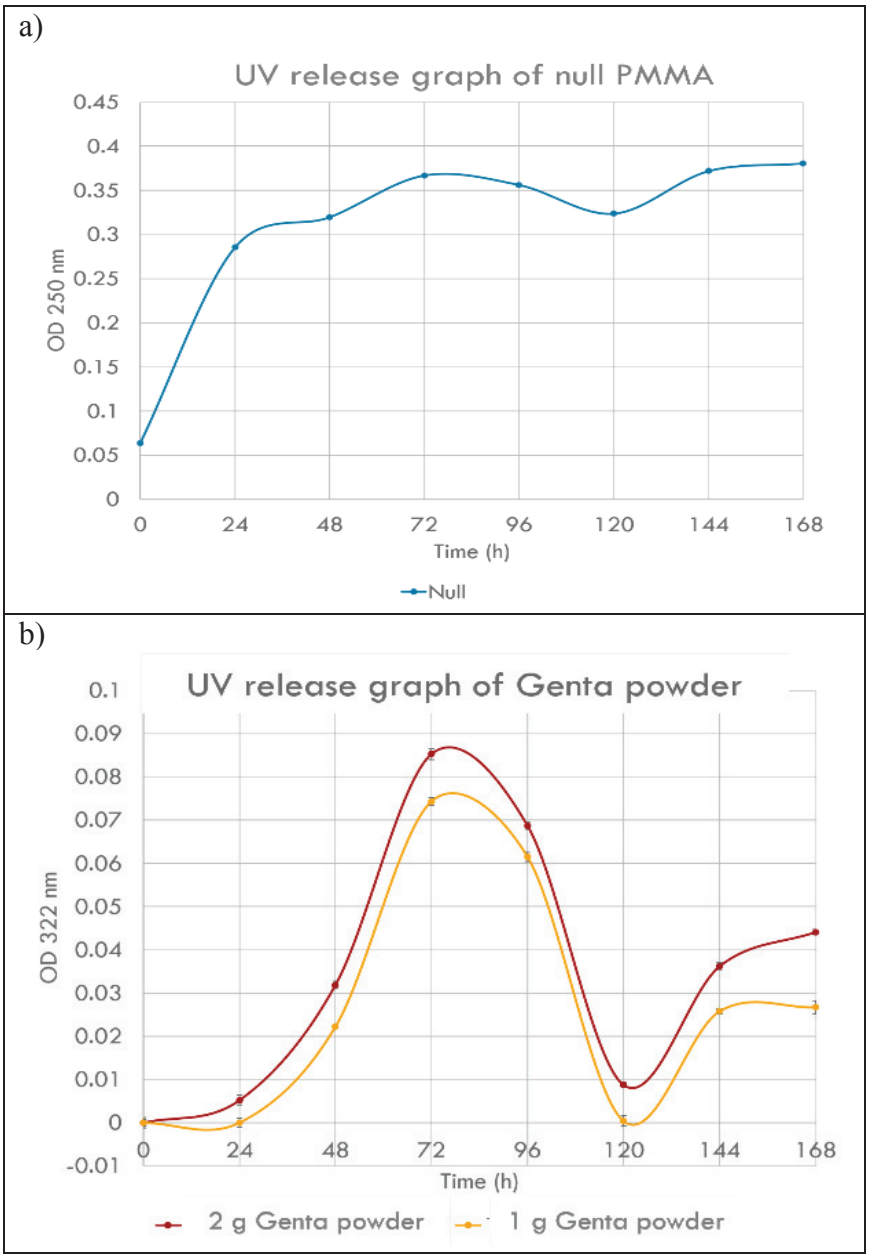

Figure 3.6. The release rate of antimicrobials from PMMA bone cements. a) The null bone cement was used as a negative control for release experiments. The release of gentamicin from bone cements were measured at 322 nm (b, c, f) and 4-HBA was measured at 250 nm (d, e). The standard deviations were <0.0015 in all experiments.

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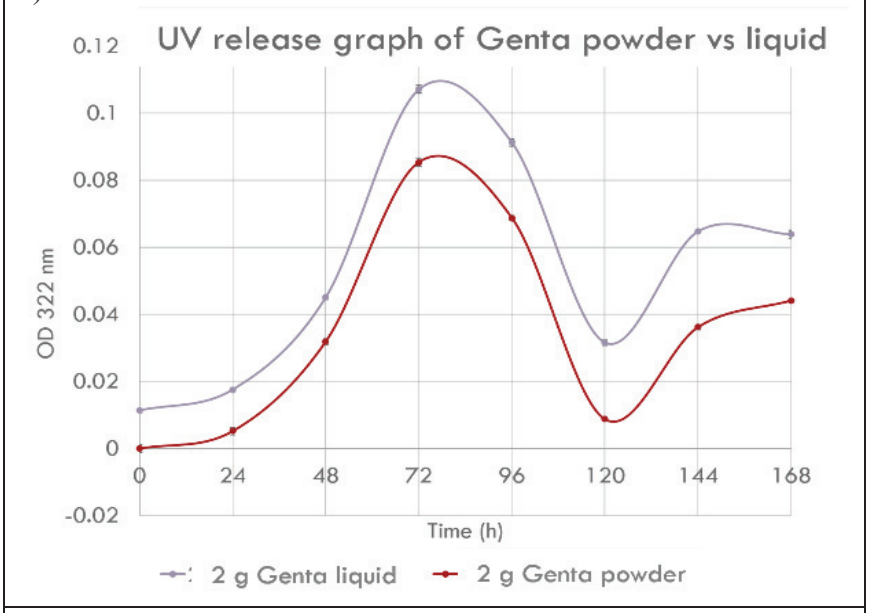
c)



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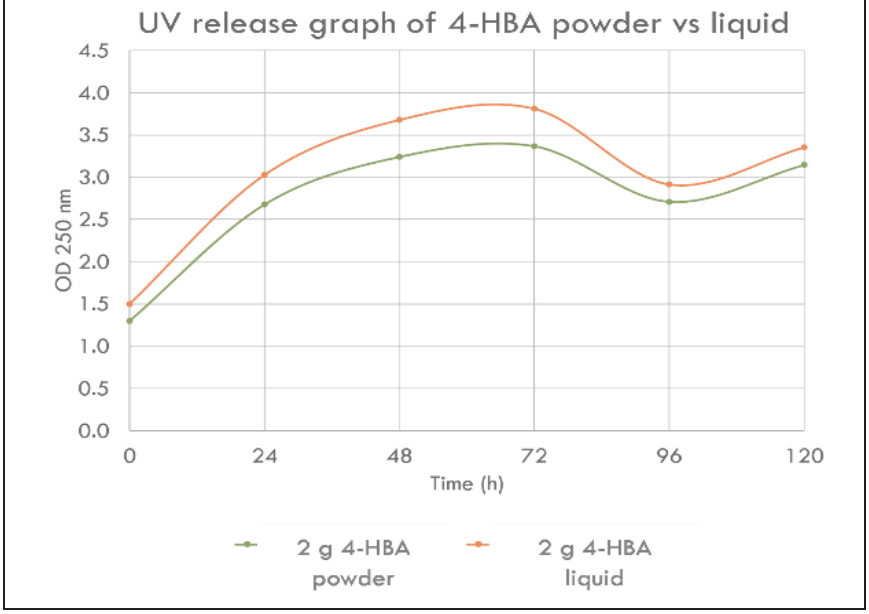

Figure 3.6 (cont.)

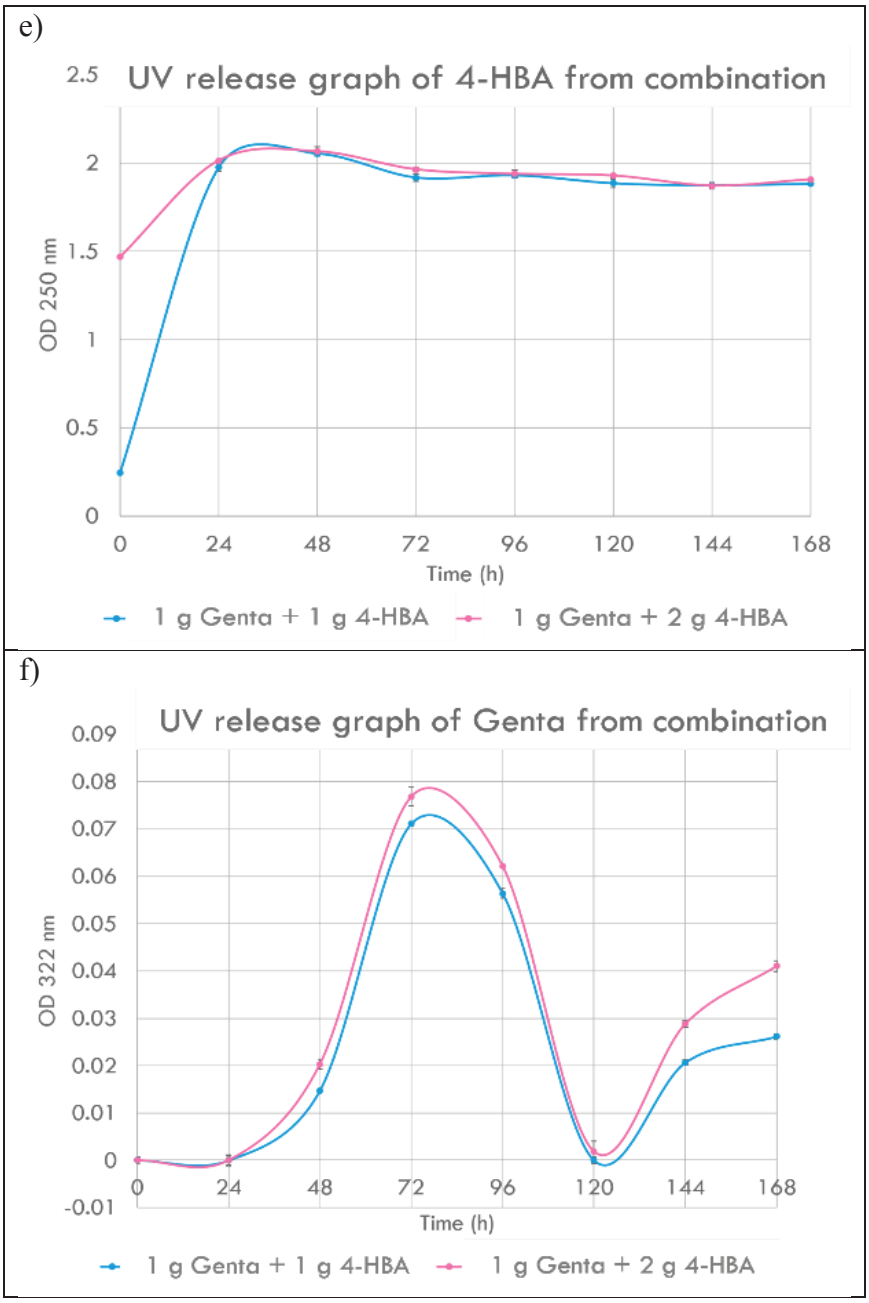

Figure 3.6 (cont.)

The measurement of release rate with UV-Vis spectrophotometer was used by Lee and his colleagues. The release of CAPE from PLGA bone cement was measured after incubation in PBS. The release pattern of CAPE was rapid at the first 8 hours, then controlled release was sustained (Lee et al., 2013). Similar to CAPE, the release of 4-HBA was also controlled. In another study, gentamicin containing PMMA was incubated

in PBS to allow the release. The level of gentamicin was measured by fluorescence polarization immunoassay. Most of the gentamicin was released in the first hours then the release rate was decreased (van de Belt et al., 2000). The results were consistent with our findings as most of the gentamicin was released at 72th hour then the oscillation started. Lopez and his colleagues investigated the release rate of 11 different antibiotics from bone cement by using high-performance liquid chromatography. The release rate was similar to other findings as the antibiotics were released at the first 24 hours and then rapidly decreased (Gálvez-López et al., 2014). The release rate of powder and liquid forms of antibiotics from PMMA bone cement was measured by Chang and his colleagues. Vancomycin and amphotericin B were added to bone cement with powder and liquid form. The use of antibiotics in the liquid form was significantly the increased release rate compared to antibiotic powder. As the liquid form of antibiotics had a higher release rate, the inhibitory zone in the disc diffusion assay was larger (Chang et al., 2014). Same with this paper, the release rate of solution form of both gentamicin and 4-HBA was higher compared to the powder form. It was already expected due to increased porous structure.

According to many studies, the ideal release pattern of antimicrobials from bone cement is the controlled release (Bistolfi et al., 2011; Chang et al., 2014; Lee et al., 2013; van de Belt et al., 2000, 2001). If the antibiotics are released from the bone cement rapidly and then the release pattern decreases, it causes subinhibitory concentration in the microenvironment of surgery, which causes bacterial resistance against antibiotics (van de Belt et al., 2000, 2001). According to our findings, gentamicin was rapidly released from bone cement at 72nd hour. Then the release rate was decreased, and oscillation was observed. As indicated in the previous chapter, MRSA and *A. haemolyticus* gained resistance against gentamicin, also other bacteria were studied and found the ability to develop resistance against gentamicin (Călina et al., 2017). With this perspective, it can be concluded that the release pattern of gentamicin from bone cements may cause resistance at the surgical site. However, 4-HBA provides the desired release pattern as the 4-HBA had a controlled release rate. After surgery, any bacteria that attached to bone cement within the specific period of time will be inhibited by 4-HBA as it was released in a controlled manner. Similar to 4-HBA, caffeic acid had controlled release since it had a homogenous structure and gentamicin had much more rapid release which then stabilized (Lee & Chang, 2015).

3.3.3. Confirmation of Release of 4-HBA with High Performance Liquid Chromatography

The release rate of 4-HBA from PMMA bone cement discs was confirmed by High-Performance Liquid Chromatography (HPLC) technique. The mobile phase was chosen as 95% formic acid (0.1%) and %5 acetonitrile, and samples were analyzed at 254 nm at 40^{o}C. The output graph from the HPLC technique was given in Figure 3.7.as as a response (mAU) versus retention time (minute). The measured sample in this graph was the combination-loaded bone cement with 1 g gentamicin and 2 g 4-HBA which was taken on the 6th day of the experiment. The 500 ppm (mg/mL) of the 4-HBA was used as a calibration standard.

The calibration curve of HPLC chromatogram was obtained by using different standards with the known concentration of 4-HBA, as given in Figure 3.7.b The area under different concentrations of standards versus the concentrations of standards was plotted and the equation was obtained. The equation was then used to calculate the concentration of the sample by using the area under the HPLC graph. To find the respective concentration of the 4-HBA (x), one has to integrate the area under the curve (y) into the equation.

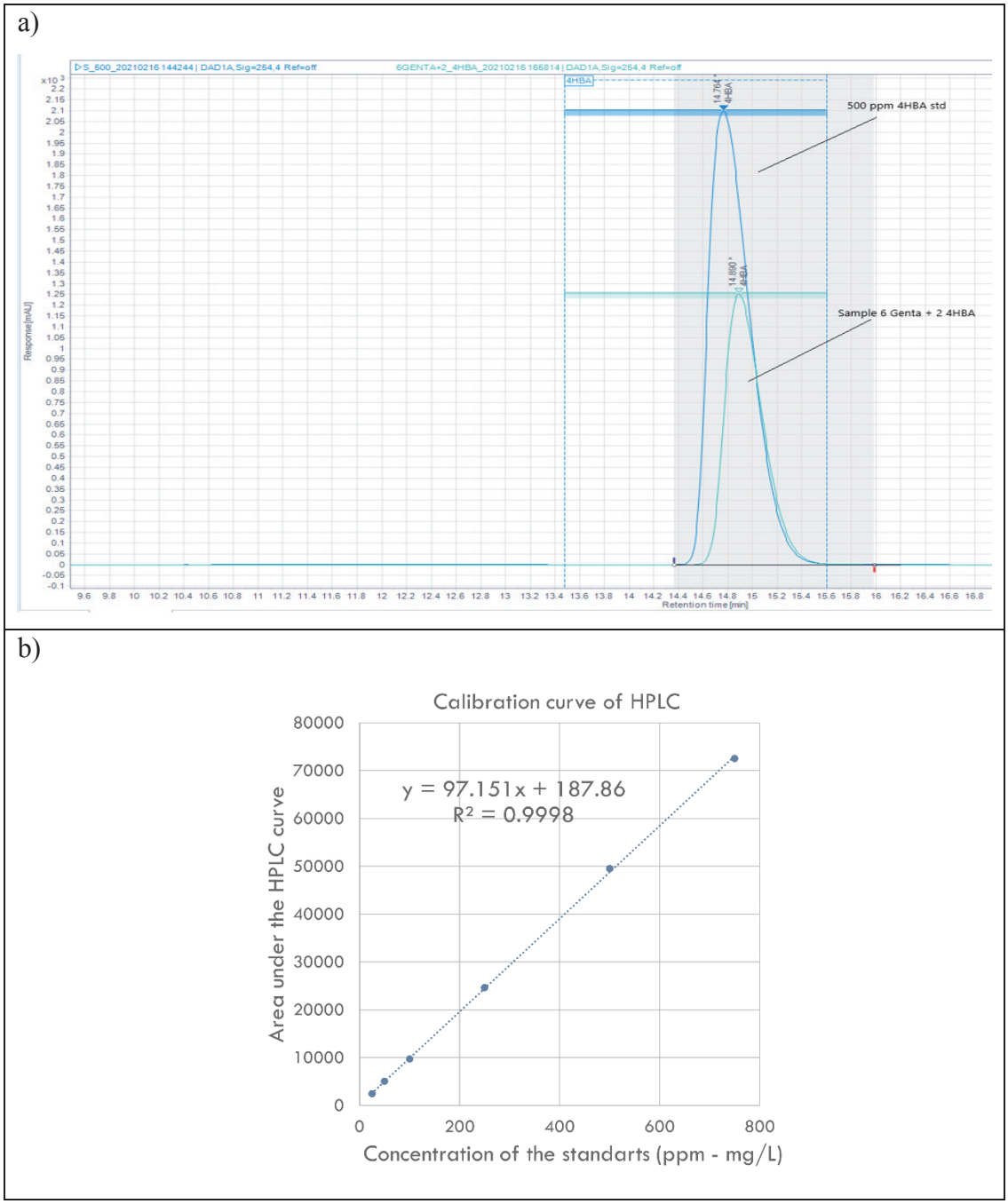

Figure 3.7. The HPLC chromatogram of 4-HBA (a) and the calibration curve (b). The HPLC chromatogram was 6th day sample from the combination-loaded bone cement with 1 g gentamicin and 2 g 4-HBA. The calibration curve of HPLC as area vs standard concentration. The equation obtained from calibration curve was y = 97.151 x + 187.86 (b).

The concentration of 4-HBA was investigated in null bone cements as a negative control. As shown in Figure 3.8.a, there was a background signal in the HPLC chromatogram with the 1.08 mg/L after 168 hours of incubation in PBS. The 1 mg/mL background was normal and acceptable as there were additional compounds within the bone cements which might give a background signal at 254 nm. The release of 4-HBA from powder and solution-loaded bone cements was given in Figure 3.8.b. It was clearly seen that even both bone cements were loaded with 2 g 4-HBA, the release from solution-loaded bone cement was significantly higher ($p=0.044$). After 7 days, 250 mg/mL 4-HBA was released from solution-loaded bone cement, 213 mg/mL 4-HBA was released from the powder-loaded bone cement disc. The release patterns of both powder and solution 4-HBA-loaded bone cement were the same as there were controlled release. These findings were consistent with the results of the UV-Vis spectrophotometer.

For the release of combination loaded-bone cements, the release of 4-HBA from 1 g gentamicin and 2 g 4-HBA was significantly higher ($p=0.001$) compared to 1 g gentamicin and 1 g 4-HBA. At the end of the 7 days, the release rate of 4-HBA was 310 mg/mL for 1g gentamicin and 2 g 4-HBA-loaded bone cement and it was 90 mg/mL for 1 g gentamicin and 1 g 4-HBA-loaded bone cement as shown in Figure 3.8.c. It was already expected as there was more 4-HBA in the combination with 2 g 4-HBA. This finding showed that the results were reliable and consistent. The release rate patterns obtained from HPLC were consistent with those of the UV-Vis spectrophotometer.

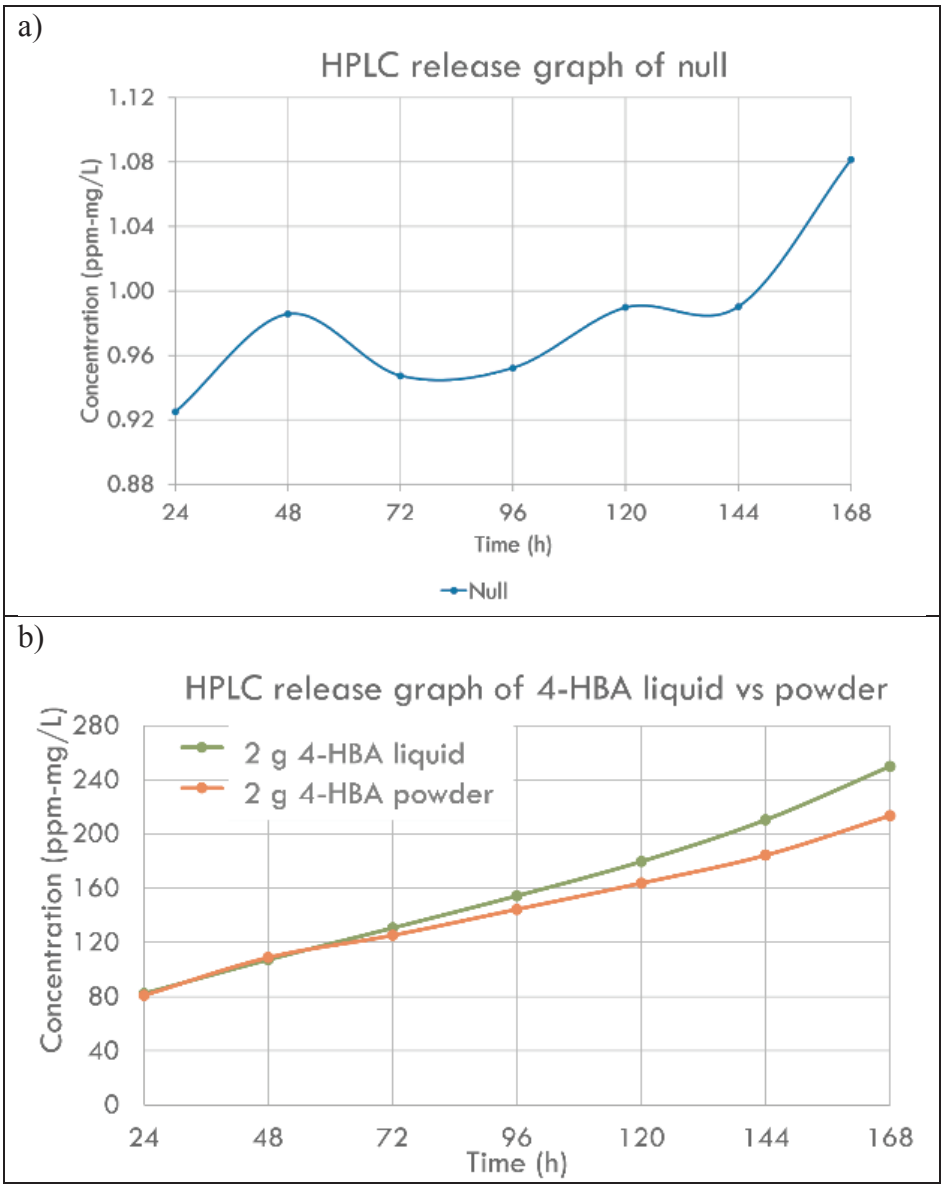

Figure 3.8. HPLC graphs after calibration as mg/mL of 4-HBA versus time. The 4-HBA concentration was quantified in a) null bone cements as negative control; compared between b) 2 g 4-HBA powder and solution-loaded bone cements and c) 1g gentamicin and 1 g 4-HBA-loaded and 1 g gentamicin and 2 g 4-HBA-loaded bone cements. The p values were 0.044 (b) and 0.001 (c).

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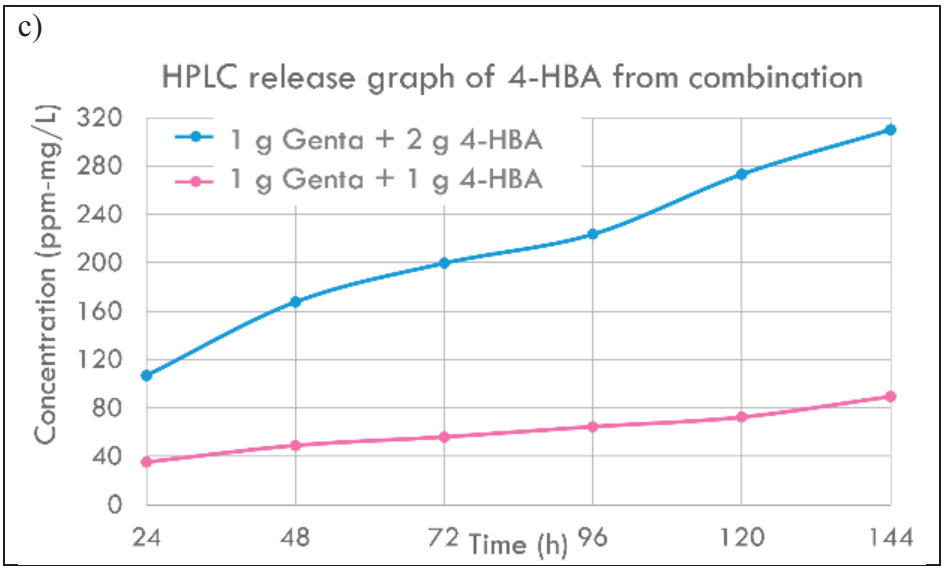

Figure 3.8 (cont)

3.3.4. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid-Loaded Bone Cements on *Escherichia coli*

The investigation of antimicrobial effects of 4-HBA loaded bone cements was performed on *E. coli.* 1 g bone cements were added to 5 mL TSB and preincubated for 7 days at 37°C for 24 hours, shaking at 75 min^{-}. After the 7^{th} day, 10^{6} CFU/mL load of *E. coli* was inoculated to each tube that contained different bone cements. the bone cements that used were i) null bone cement as a negative control, ii) 2 g powder gentamicin-loaded bone cement iii) 2 g liquid gentamicin-loaded bone cement, iv) 2 g powder 4-HBA-loaded and v) 2 g liquid 4-HBA-loaded, vi) combination of 1 g gentamicin with 1 g 4-HBA and vii) combination of 1 g gentamicin with 1 g 4-HBA.

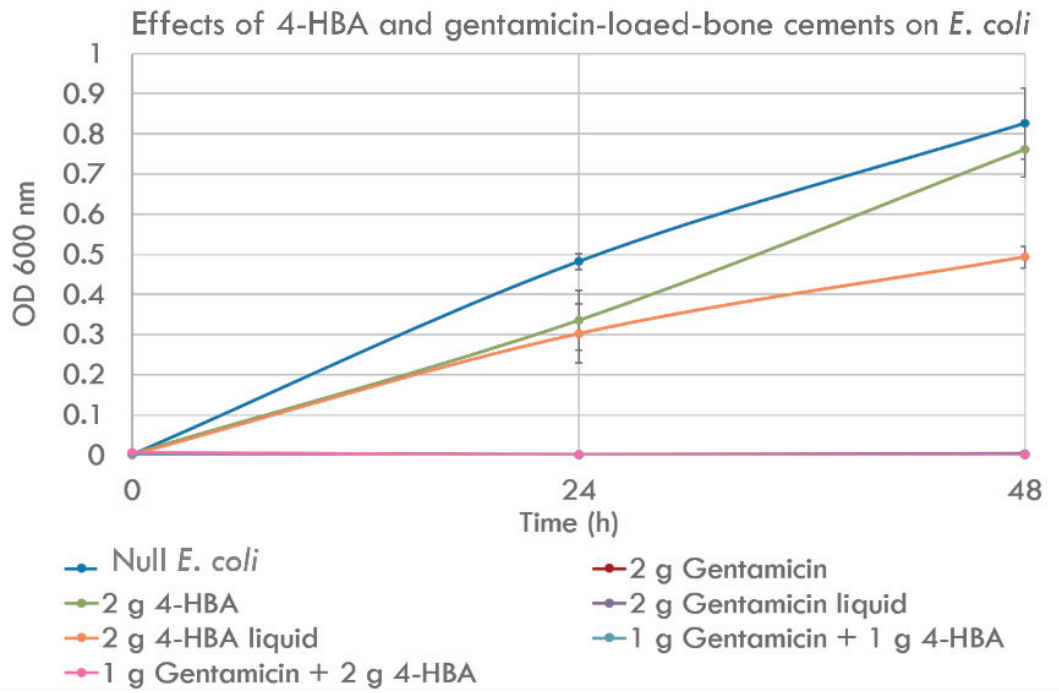

Figure 3.9. Growth curves of *E. coli* in the presence of different bone cements. Optical densities were measured at 0th, 24th and 48th hours for *E. coli*. Standard deviations were lower than 0.0886.

The incubation of *E. coli* with 4-HBA-loaded bone cement resulted in lower optical density at the 24th hour compared to null; however, at the 48th hour the optical density was close control. It can be concluded that even it was effective in first 24 hours, the concentration of 4-HBA was not enough the sustained the inhibition rate after 48 hours of incubation. The optical density was almost reduced to half in the solution form of 4-HBA-loaded bone cement. 4-HBA solution-loaded bone cement was more effective compared to powder one as the optical density was lower at the 48th hour as indicated in Table 3.1. Gentamicin-loaded bone cement discs were also sufficient to inhibit the total growth of *E. coli* at 24th hour and the inhibition was sustained at the 48th hour. The gentamicin was enough to inhibit the bacterial growth as the addition of 4-HBA did not induce any change in inhibition. However, as described in previous sections, 4-HBA is smaller molecular compared to gentamicin and will provide structural strength as well as induce synergistic effects.

Table 3.1. Percent inhibitions of *E. coli* in incubation with bone cements.

Bacteria	**Bone cement**	**Percent inhibition at 24th hour**	**Percent inhibition at 48th hour**
E. coli	2 g Gentamicin	100%	99.9%
	2 g 4-HBA	30.4%	7.8%
	2 g Gentamicin liquid	100%	99.6%
	2 g 4-HBA liquid	37.3%	40.3%
	1 g Gentamicin + 1 g 4-HBA	100%	100%
	1 g Gentamicin + 2 g 4-HBA	100%	100%

The viable cell counts of the *E. coli* at the initial inoculum, after 24 hours and 48 hours of incubation were given in Table 3.2. The bacterial growth in the presence of null bone cement discs was negative control. The initial inoculum of *E. coli* was 10^6 CFU/mL and initial spread was performed after inoculation. Both gentamicin and gentamicin solution-loaded bone cement had 10^3 CFU of *E. coli* per mL. Contrast to MRSA and MSSA, addition of gentamicin-loaded bone cement discs was not resulted in immediate bactericidal effect on *E. coli.* After 24 hours, cell number from control reached to 6.45 x 10^8 CFU/mL, there were 3.66 x 10^8 CFU/mL in the 4-HBA solution-loaded bone cement and 1.68 x 10^8 CFU/mL in powder ones. Normally, solution-based 4-HBA was expected to inhibit the bacterial growth more but within 24 hours both of the forms of 4-HBA inhibited the growth similarly. Similar to the data from MRSA, 24 hours of incubation was not enough to evaluate the effectiveness of the solution-based 4-HBA. After 48 hours, control reached to 10^9 CFU/mL. The incubation of *E. coli* with 4-HBA-loaded bone cement after 48 hours resulted in 6.5 x 10^8 CFU/mL; but the solution form of 4-HBA reduced the bacterial number to 7.5 x 10^7. The results were already expected as the addition of 4-HBA solution to bone cement increased the release from bone cement and inhibited the bacterial growth more. After 24 hours of incubation with bone cements,

gentamicin included bone cements inhibited the total growth of *E. coli.* Similar to the MRSA and MSSA, gentamicin was enough to inhibit the growth as addition of 4-HBA did not induce change in inhibition. However, 4-HBA was also important in the release pattern from bone cement and structural support.

Table 3.2. Viable cell counts of *E. coli* in incubation with bone cements.

Bacteria	**Bone cement**	**Viable cell counts 0th (CFU/mL)**	**Viable cell counts 24th (CFU/mL)**	**Viable cell counts 48th (CFU/mL)**
E. coli	2 g Gentamicin	4.2×10^3	0	0
	2 g 4-HBA	1.29×10^6	1.68×10^8	6.5×10^8
	2 g Gentamicin liquid	5.67×10^3	0	0
	2 g 4-HBA liquid	2.38×10^6	2.3×10^8	7.5×10^7
	1 g Gentamicin + 1 g 4-HBA	1.31×10^5	0	0
	1 g Gentamicin + 2 g 4-HBA	1.8×10^5	0	0

control as null *E.coli* 0th hour: 1.2×10^6 CFU/mL; 24th hour: 6.45×10^8 CFU/mL; 48th hour: 1.47×10^9 CFU/mL

3.4. Conclusion

This part of experimental study was focused on the use of 4-hydroxybenzoic acid as alternative antimicrobial product within the bone cement, the morphological effects of addition of 4-HBA into bone cement, the release rate of 4-HBA from the bone cement. There were two main 4-HBA containing bone cements as 4-HBA powder and 4-HBA solution-loaded bone cement. Gentamicin powder and gentamicin solution were compared to 4-HBA.

The addition of 1 g gentamicin with 1 g 4-HBA and 1 g gentamicin with 2 g 4-HBA were used for combinatory studies. The first conclusion of this chapter was the addition 4-HBA into bone cement resulted in homogeneous loading, preserved porous structure and smooth surface. The addition of gentamicin resulted in heterogenous structure with rough surface. The solution-based bone cements had more porous structure and the surface became rough compared to powder-loaded bone cements. The best morphology was obtained with the combinatorial bone cement with gentamicin and 4-HBA as the porous structure was sustained and the holes, that were created by gentamicin, were filled by smaller molecule 4-HBA, therefore the bone cement has higher structural strength. The second outcome is the release pattern of 4-HBA was ideal as the release was controlled and release rate was sustained in 7 days. However, the release pattern of gentamicin was more rapid, which was followed by slight release pattern. The slow release of gentamicin might cause resistance afterward as bacteria would expose to subinhibitory concentration of gentamicin. As proved by HPLC, solution-loaded bone cements had higher release rate. than powder-loaded bone cements, Even though 4-HBA solution-loaded bone cement had a higher release rate compared to powder-loaded ones, the release pattern was still controlled. from this perspective, a combination of 4-HBA with gentamicin might be effective within the surgical site as gentamicin would kill the bacteria within the surgical site immediately and 4-HBA would be released in controlled manner and prevent any infection afterward. The third outcome is the antimicrobial effect of 4-HBA and gentamicin. As already indicated in the previous section, the minimum inhibitory concentrations of 4-HBA and gentamicin were significantly different as mg/mL as μg/mL, respectively. The 2 grams of 4-HBA was not sufficient to inhibit the total bacterial growth completely, but still inhibited the growth of MRSA, MSSA and *E. coli* at a rate of 55%, 71% and 40%, respectively. Therefore, higher concentrations of 4-HBA solution within the bone cement discs are the most suitable for usage with i) structural strength due to small molecules of 4-HBA, ii) controlled release pattern with higher release rate to release more 4-HBA in extended period of time without inducing any resistance, iii) natural ability to inhibit the bacterial growth and, iii) compatibility with gentamicin within the bone cement.

CHAPTER 4

INVESTIGATION OF RESISTANCE OF METHICILLIN-RESISTANT *Staphylococcus aureus* and *Acinetobacter haemolyti*cus AGAINST 4-HYDROXYBENZOIC ACID AND GENTAMICIN

4.1. Introduction

One of the most famous antibiotic resistant bacteria is methicillin resistant *S. aureus* which is nosocomial pathogen. MRSA gains resistance via horizontal gene transfer of the gene *mecA* which expresses penicillin- binding protein 2 that cause resistance against methicillin (Hiramatsu et al., 2014). MRSA is also frequently resistance to multiple antibiotics such as erythromycin (97%) and clindamycin (97%) in USA which is linked to the *erm* gene. On the other hand, tetracycline resistance in the MRSA isolates is associated with the use in pig industry and linked to the *tet* gene. MRSA can also develop resistance against penicillin by acquiring *blaZ* gene that expresses β-lactamase that degrade penicillin (Bouiller et al., 2020).

Acinetobacter is considered a notorious hospital infectious pathogen since those bacteria develop resistance against multiple antibiotics. The high abundance of *A. haemolyticus* in hospital leads to gene exchange between other bacteria in hospital therefore increases the resistant against different antibiotics day by day (Bai et al., 2020). One of the studies in Portuguese indicated that the *A. haemolyticus* isolates that were taken from clinical patient were resistance to all β-lactams, except cefepime, aztreonam, and ceftazidime. *A. haemolyticus* clinical isolates were also found that OXA-24/40 enzyme was presented in the bacteria. For the resistance, the role of horizontal gene transfer of the blaOXA-40 gene was suspected (Quinteira et al., 2007).

P. aeruginosa is highly abundant in health care facilities, in which leads to infections in the patients that had surgery (Pang et al., 2019). *P. aeruginosa* is also well-known bacteria that have various resistance mechanisms. It has adoptive resistance mechanisms such as the development of biofilm formation of persister cells as it

encounters antibiotics. The intrinsic resistance mechanism of *P. aeruginosa* includes efflux pumps to pump the antibiotics out of the bacteria and enzymes that can degrade antibiotics such as β-lactamase. However, with the aid of horizontal gene transfer, it can also acquire different antibiotic resistance genes (Pang et al., 2019).

In this study, 4-hydroxybenzoic acid was used as phenolic compound to test if the bacteria *A.haemolyticus* and MRSA can develop any resistance against and the ability of resistance was compared to the antibiotic gentamicin. Up to today, there is no experimental study that shows any resistance mechanism of MRSA against any of the tested phenolic acids. There are few studies that investigate the resistance against phenolic acids. *Leptospermum* honey was investigated for its antibacterial activity and development of resistance by transcriptomics on 12 different pathogens, and none of the pathogens did not develop any resistance (Blair et al., 2009). This study was one of the first studies that showed both the antimicrobial effect of natural compounds and the lack of resistance with the comparison of the antibiotic under subinhibitory concentrations. Another study was focused on the food-borne pathogen *Listeria monocytogenes* and the ferulic acid and nisin. Ferulic acid is a hydroxycinnamic acid-derivative compound and found that *L. monocytogenes* could not develop any resistance against it.

On the other hand, nisin was the bacteriocin of *Lactococcus lactis* which kills *L. monocytogenes.* However, the exposure of bacteria to nisin was resulted into increased minimum inhibitory concentration (Takahashi et al., 2015). The recent study of Keman and Ferda showed that *S. aureus* did not develop any resistance against 2-hydroxycinnamic acid and vanillic acid while exposure to vancomycin induced the resistance (Keman & Soyer, 2019). In conclusion, those studies indicated that the exposure of subinhibitory concentrations of antibiotics and bacteriocins induces the development of resistance on different kinds of bacteria. However, the treatment of bacteria with different kinds of phenolic acids does not induce any resistance, except for phenol-degrading bacteria.

The aim of this experimental part was to investigate if the *A. haemolyticus* and MRSA against 4-HBA by comparing to well-known antibiotic gentamicin. In this chapter of the experimental study, two hypotheses were tested;

1) If *A. haemolyticus* and MRSA have the ability to acquire resistance to 4-HBA and gentamicin, continuous exposure of subinhibitory concentration would cause resistance,

2) If *A. haemolyticus* and MRSA cannot develop resistance against 4-HBA, even the presence of phenol degrading enzymes, such as catechol-1,2 dioxygenase and phenol hydroxylase, would not be enough to overcome the antimicrobial activity of phenolic acid.

4.2. Material and Methods

The phenolic acid 4-HBA and antibiotic gentamicin were tested on MRSA and *A. haemolyticus* to detect if the bacteria developed any resistance. The experimental design to induce the resistance of bacteria against 4-HBA and gentamicin was adopted from Blair and colleagues (Blair et al., 2009).

4.2.1. Investigation of Resistance of Methicillin-Resistant *Staphylococcus aureus* and *Acinetobacter haemolyticus* Against 4-Hydroxybenzoic Acid and Gentamicin

Bacteria have the ability to acquire resistance to antibiotics. The usage of antibiotics in the wrong doses and for the wrong purposes leads to the formation of drug-resistant bacteria. Both overuse and exposure to sublethal concentrations of bacteria force the bacteria to develop resistance. In this section, bacteria were exposed to increasing subinhibitory concentrations of phenolic acid and antibiotics to test if they will develop any resistance. MIC values of 4-HBA and gentamicin after each step of transfer to increased concentrations. The acquisition of resistance was determined by final MIC values if it was increased or stable.

In this experiment subinhibitory concentration of 4-HBA against MRSA was selected as 1.2 mg/mL and 1.1 mg/mL was selected for *A. haemolyticus*. The subinhibitory concentration of gentamicin was 2 µg/mL for MRSA and 2 µg/mL for *A. haemolyticus*. The experiment of the acquisition of the resistance against 4-HBA and gentamicin was started with the 2% inoculation from overnight bacterial culture into the TSB that contains subinhibitory and inhibitory concentrations of 4-HBA or gentamicin. For control, 2% inoculum was prepared in the TSB only, without any addition of antimicrobials. The initial optical densities were measured at the 0^{th} hour. Then the 100

µL of the culture was spread onto the TSA plate and incubated overnight at 37°C to be counted in order to have viable cell number that were inoculated into tubes. After 24 hours of incubation at 37°C, optical densities were measured again, followed by spread plate of 100 µL of the bacterial culture which then incubated overnight at 37°C.

After 24 hours of incubation, 2% inoculum from the subinhibitory concentration of antimicrobials was transferred to increased concentration of the antimicrobials as increased subinhibitory concentration, MIC and increased MIC. The 2% inoculum from subinhibitory concentration of antimicrobials was also transferred into 4 mL TSB to be used as control. The increments for the subinhibitory concentration were determined as 0.1 mg/ml for the 4-HBA and 0.5 µg/mL for gentamicin. The increments for the MICs were determined as 0.2 mg/mL for 4-HBA and 5 µg/mL for gentamicin. More precisely, 2% MRSA inoculum from the tube that contains 1.2 mg/mL 4-HBA (subinhibitory concentration) was transferred to 1.3 mg/mL 4-HBA (increased subinhibitory concentration), transferred to 3 mg/mL 4-HBA (determined MIC), transferred 3.2 mg/mL 4-HBA (increased MIC) and transferred to 4 mL TSB for control. For *A. haemolyticus*, the subinhibitory concentration of 4-HBA was determined as 1.1 mg/mL so that the increased subinhibitory concentration was 1.2 mg/mL, predetermined MIC value was 2 mg/mL and the increased MIC for 4-HBA was 2.2 mg/mL. For gentamicin increments, 2% MRSA inoculum from the subinhibitory concentration of gentamicin, which was 5 µg/mL, was transferred to 5.5 µg/mL gentamicin (increased subinhibitory concentration), transferred to 10 µg/mL (determined MIC), transferred 15 mg/mL (increased MIC) and transferred to 4 mL TSB for control. For *A. haemolyticus*, the subinhibitory concentration of gentamicin was determined as 2 µg/mL so that the increased subinhibitory concentration was 2.5 µg/mL, predetermined MIC value was 10 µg/mL and the increased MIC for 4-HBA was 15 µg/mL. As a positive control, 80 µL from previous control was transferred into 4 mL of fresh TSB to make the total bacterial concentration as 2%. The initial optical densities were measured immediately after bacterial transfer into first increased concentration. Subsequently, cultures were incubated for 48 hours at 37°C, which then optical densities was measured again at 600 nm.

To induce the resistance, the subinhibitory concentrations were increased again. 2% inoculum from first increased subinhibitory concentrations was transferred to second increased concentration of the antimicrobials. The 2% inoculum from first increased subinhibitory concentration of antimicrobials was also transferred into 4 mL TSB to be

used as control. As determined before, the increments for the subinhibitory concentration were 0.1 mg/ml for the 4-HBA and 0.5 µg/mL for gentamicin. The increments for the MICs were also determined as 0.2 mg/mL for 4-HBA and 5 µg/mL for gentamicin. Same steps were followed as previously described for transfer to first increased subinhibitory concentrations. The initial optical density was measured initially at 600 nm. Then transferred tubes were incubated for 72 hours at 37°C. After incubation for 72 hours, optical density of bacterial cultures was measured again, and all the test and control tubes were diluted and 100 µL from desired concentration was spread onto TSA plates. Plates were grown overnight at 37°C. The colonies from the plate were counted and noted as CFU/mL.

Then the 72 hours incubated cultures from second increased concentrations of phenolic acid 4-HBA and antibiotic gentamicin were transferred into fresh TSB media as 2% inoculum. The controls were also transferred into fresh TSB. The optical density was measured initially as 0^{th} hour, which tubes were then incubated for 48 hours at 37°C. Finally, after incubation of bacteria in fresh TSB without any addition of phenolic acid and antibiotics, cultures were transferred into tubes with initial subinhibitory concentration and MIC of phenolic acid and antibiotic. The initial optical density was measured at 0^{th} hour and tubes were incubated for 24 hours at 37°C. After the incubation, optical density of bacterial cultures was measured again, and all the tests and controls were diluted and 100 µL from desired concentration was spread onto TSA plates. Plates were grown overnight at 37°C. The colonies from the plate were counted and noted as CFU/mL. These experiments were repeated for at least two times independently for both bacteria as MRSA and *A. haemolyticus*.

4.2.2. Quantitative Analysis of Phenol Hydroxylase and Cathecol-1,2 Dioxygenase Genes by Droplet Digital PCR

The droplet Digital PCR (ddPCR) method was selected to quantitively measure the gene level within the DNA. ddPCR was performed for the confirmation of the resistance experiments in order to determine if bacteria contain the genes that can degrade the phenolic acids, therefore develop resistance against phenolic acids. Two genes were

analysed with ddPCR in this experiment, which express the key enzymes in the phenolic acid degradation as phenol hydroxylase and cathecol-1,2 dioxygenase.

4.2.2.1. Primer Design for ddPCR

Specific primers were designed by the online tool Primer BLAST program by using suitable primers in Refseq database by adjusting the maximum target size to 200. The designed forward and reverse primers were listed in the Table 4.1.

Table 4.1. Primers designed for Droplet Digital PCR.

Targeted gene	**Primers**		**bp long of target gene**
cathecol-1,2 dioxygenase	forward	5'-TCTGGCACGCCAATACCC-3'	259 bp
	reverse	5'-AGGTTGATYTGGGTGGTCA-3'	
phenol hydroxylase	forward	5'-AGCGGCTCRTCGAACAGGAA-3'	227 bp
	reverse	5'-CTCTAMCCCCACATGACSGTG-3'	

4.2.2.2. DNA Isolation from Bacterial Cultures

Bacterial cultures were prepared by the same protocol as describe in section 2.2.3. The single colony of bacteria (MRSA, *A. haemolyticus, P. aeruginosa*) from the streak plate was inoculated into 4 mL of TSB and incubated overnight at 37^{o}C. The DNA was isolated from each bacterium by using QIAamp DNA mini kit (Qiagen). For the isolation of DNA from bacterial cultures, 1 mL of bacterial cultures were centrifuged for 5 minutes at 7500 rpm. Buffer ATL was added to the pellet to the total volume of 180 µL for complete lysis. 20 µL of proteinase K was added and vortexed. Then the mixture was incubated at 56°C for 1-3 hours until the sample was completely lysed. The sample was vortexed occasionally during incubation period to mix the sample. 200 µL of Buffer AL

was added to the sample and mixed with vortex then the sample was incubated at 70°C for 10 minutes. The mixture of the sample with Buffer AL is essential to obtain a homogeneous solution. 200 µL of ethanol (96–100%) was added to the sample and mixed with vortex for 15 seconds. Ethanol must be used in this step since other alcohols may reduce the yield. The mixture was then inserted into the QIAamp Mini spin column which centrifuge the sample at 8000 rpm. The collection tube was obtained and the tube that contained the filtrate was discarded. 500 µL of Buffer AW1 was added to column and centrifuged at 8000 rpm for 1 minute. The collection tube was obtained, and filtrate was discarded. 500 µL of Buffer AW2 was added to column and centrifuged at 14,000 rpm for 3 minutes. The sample was taken from the column and tube that contained the filtrate was discarded. As a final step, 200 µL of distilled water was added to the mixture and incubated at room temperature for 1 minute, then the mixture was centrifuged 8000 rpm for 1 min. This step was repeated one more time.

4.2.2.3. Droplet Digital PCR for Phenol Hydroxylase and Cathecol-1,2 Dioxygenase Genes

Droplet Generation and Droplet digital PCR protocol were performed for absolute quantification of phenol hydroxylase and catechol-1,2 dioxygenase gene expression. 20 µL of PCR mix was prepared that contains 10 µL of 2X ddPCR EvaGreen Supermix (Bio-Rad, cat. no. 1864034), 6 µL of nuclease-free water, 1 µL of both forward and reverse primer and 10 ng of DNA from each sample. Each ddPCR assay mixture was loaded into a disposable DG8 Cartridge (Bio-Rad, cat. no. 1864008) located into a cartridge holder (Bio-Rad, cat. no. 1863051). Then, 70 µL of droplet generation oil for EvaGreen (Bio-Rad, cat. no. 1864005) was loaded into each of the eight oil wells. The cartridge was then covered with a DG8 Gasket (Bio-Rad, cat. no. 1863009) and placed inside the QX200 Droplet Generator (Bio-Rad, cat. no. 1864002). Upon completion of droplet generation, the droplets were carefully transferred to a new ddPCR 96-well PCR plate kit (Bio-Rad, cat. no. 10023379). The plate was heat-sealed with a pierceable aluminum foil (Bio-Rad, cat. no. 1814040) into the PX1 PCR Plate Sealer (Bio-Rad, cat. no. 1814000) and placed in a thermal cycler. Thermal cycling conditions were 95ºC for 5 min, then 50 cycles of 95ºC for 30 s and 57ºC for 1 min and two final steps at 98ºC for 10 seconds and a 4ºC infinite hold. After PCR was completed, the sealed plate was transferred into the plate

holder of the QX200 Droplet Reader (Bio-Rad, cat. no. 1864003). Using QuantaSoft software (Bio-Rad), the analysis was set up and started in order to analyze the droplets with an optical detector. At the end of the plate reading, the resulting data were analyzed with QuantaSoft software v1.7. Specifically, from the 2D amplitude plot, the positive droplets in each well were selected. Finally, the mRNA of phenol hydroxylase and catechol-1,2 dioxygenase amount as copies/μL were obtained.

4.3. Results and Discussion

4.3.1. Investigation of Resistance of Methicillin-Resistant *Staphylococcus aureus* and *Acinetobacter haemolyticus* against 4-HBA and Gentamicin

A. haemolyticus and MRSA were exposed to subinhibitory concentrations of 4-HBA and gentamicin for the confirmation of previous findings before increments of concentrations. The effect of 4-HBA with subinhibitory concentration (subMIC) and MIC on the growth of *A. haemolyticus* and MRSA were given in the Figure 4.1.a and b, respectively. The predetermined subinhibitory concentrations of 4-HBA, 1.1 mg/mL for *A. haemolyticus* and 1.2 mg/mL for MRSA, inhibited the growth around 40% and 56% after 24 hours (Table 4.2). The MIC values of 4-HBA inhibited the bacterial growth more than 90%.

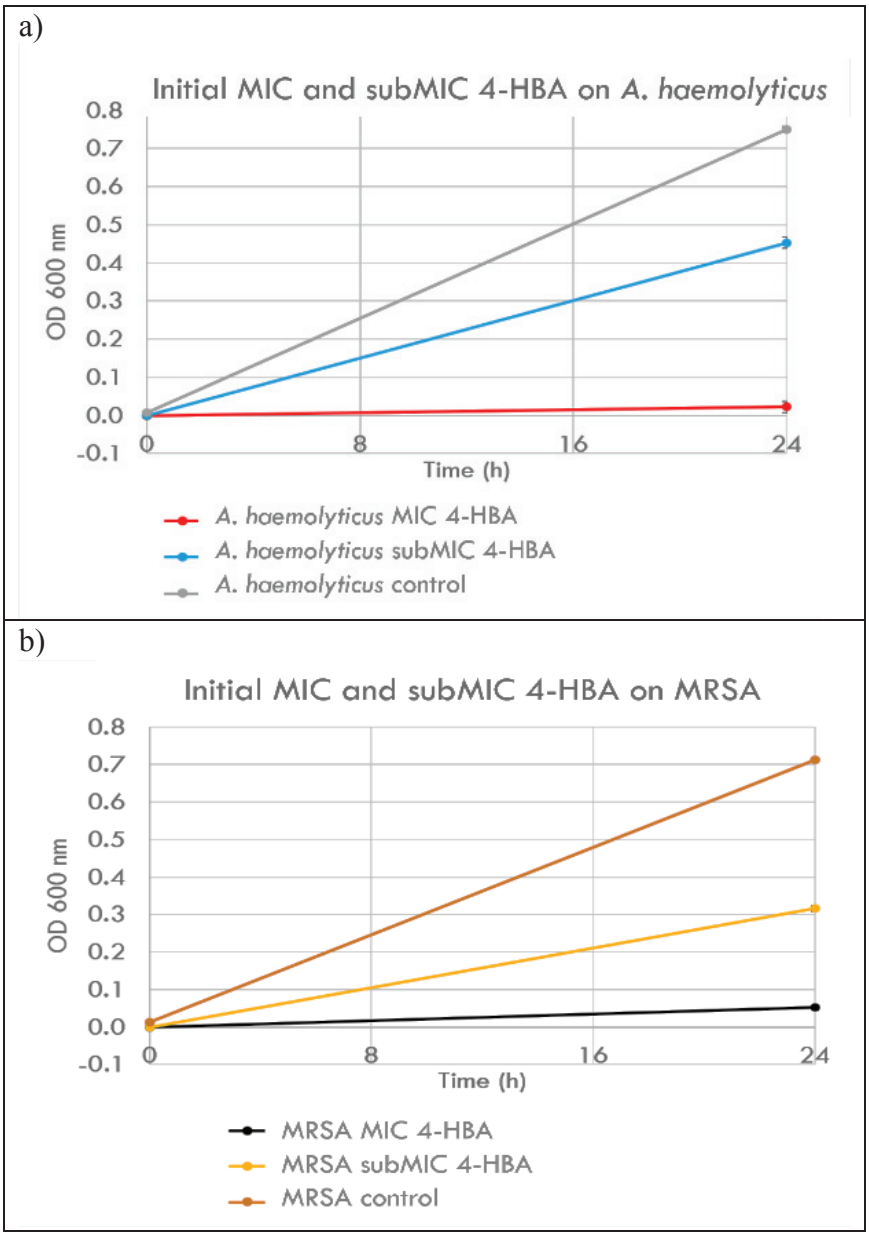

Figure 4.1. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of different concentrations of 4-HBA. Optical densities were measured at 0^{th} and 24^{th} hours. Standard deviations were <0.015 and 0.012 for *A. haemolyticus* and MSSA.

Besides the optical density measurements, cell viability was also tested with spread plate as shown in Table 4.2. the spread plate was performed to determine the initial bacterial load as well as the bacterial number within 4-HBA containing tubes with different concentrations after 24 hours of incubation. The initial load of bacteria was 10^7

CFU/mL for *A. haemolyticus* and MRSA. After 24 hours of incubation, the bacterial numbers reached to 10^8 CFU/mL for *A. haemolyticus* and MRSA. The subinhibitory concentrations of 4-HBA reduced the bacterial number to 10^8 and 10^7 for *A. haemolyticus* and MRSA, respectively. On the other hand, there were no bacteria in MIC containing tubes. The MIC value of 4-HBA reduced the bacterial number 8 log for each bacterium. The findings were similar to the ones obtained in section 2.3.1 and 2.3.2.

Table 4.2. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation with different 4-HBA concentrations.

Bacteria	**Concentrations of 4-HBA**	**Percent inhibition**	**Viable cell counts (CFU/mL)**
A. haemolyticus	MIC 2 mg/mL	97.04%	0
	subMIC 1.1 mg/mL	39.62%	2×10^8
MRSA	MIC 3 mg/mL	92.78%	0
	subMIC 1.2 mg/mL	55.56%	2.19×10^7

initial *A. haemolyticus* 0^{th} h: 1.45×10^7 CFU/mL; MRSA 0^{th} h: 7×10^6 CFU/mL
control *A. haemolyticus* 24^{th} h: 3.9×10^8 CFU/mL; MRSA 24^{th} h: 2.96×10^8 CFU/mL

The predetermined MIC values of gentamicin on *A. haemolyticus* and MRSA were tested again (Figure 4.2). The 10 µg/mL of gentamicin resulted in growth inhibition of both bacteria after 24 hours of incubation. On the other hand, subinhibitory concentrations reduced the optical density by approximately 80%. The percent inhibition of gentamicin was consistent with the previous findings as explained in sections 2.3.1 and 2.3.2.

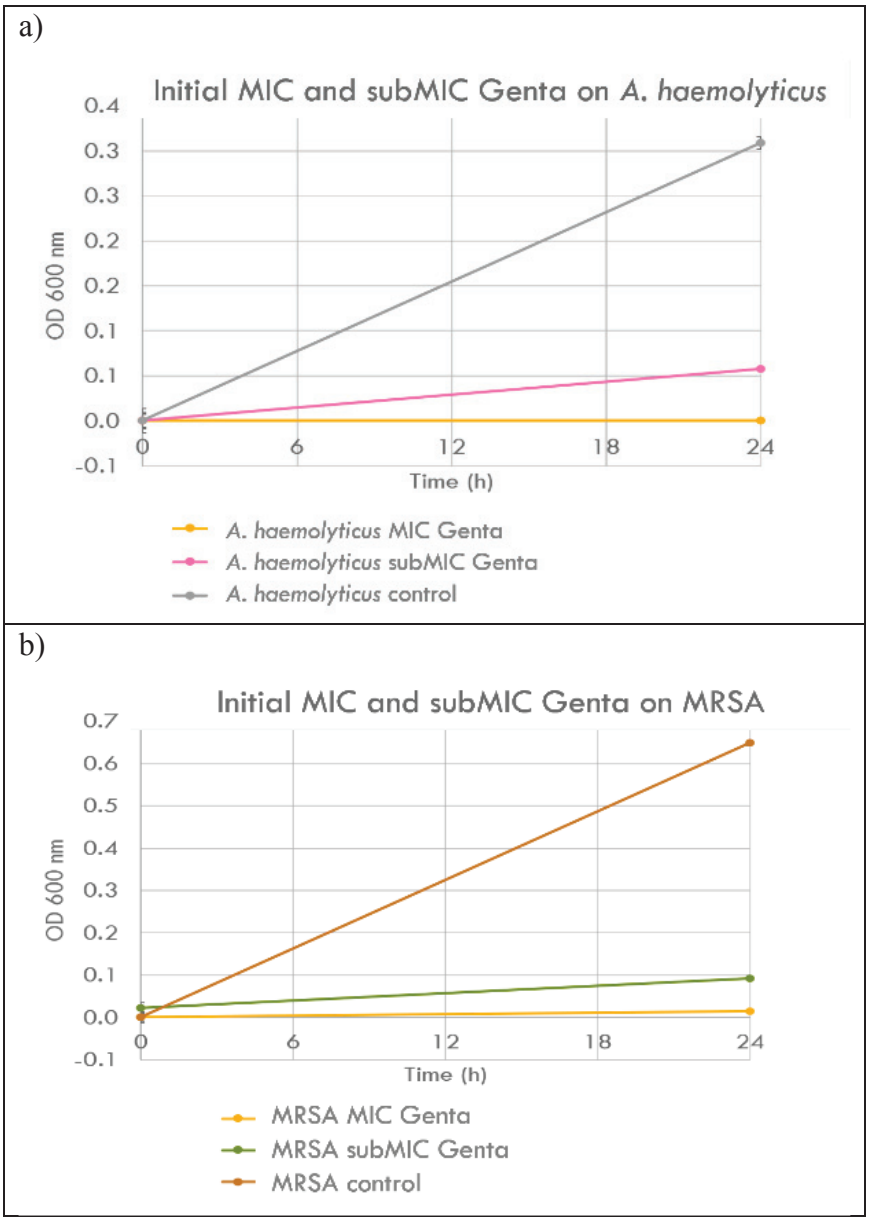

Figure 4.2. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of different concentrations of gentamicin. Optical densities were measured at 0^{th} and 24^{th} hours. Standard deviations were lower than 0.014 and 0.013 for *A. haemolyticus* and MSSA, respectively.

The viable cell count was performed to determine the initial bacterial load and enumerate the colonies of bacteria that were treated with gentamicin (Table 4.3.). The initial load of *A. haemolyticus* was 8 x 10^6 CFU/mL and increased to 9 x 10^9 CFU/mL after 24 hours. The initial bacterial number was 2 x 10^7 for MRSA which increased to 1 x

10^9 after 24 hours of incubation. The subinhibitory concentration of gentamicin reduced the bacterial number to 10^7. There were no MRSA colonies after incubation with MIC and 22 colonies were found in the *A. haemolyticus* tube. The viable cell counts were similar to the ones obtained in sections 2.3.1 and 2.3.2.

Table 4.3. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation with different gentamicin concentrations.

Bacteria	**Concentrations of gentamicin**	**Percent inhibition**	**Viable cell counts (CFU/mL)**
A. haemolyticus	MIC 10 µg/mL	100%	2.2×10^1
	subMIC 2 µg/mL	81.23%	6.55×10^7
MRSA	MIC 10 µg/mL	97.71%	0
	subMIC 5 µg/mL	85.85%	2.7×10^7

initial *A. haemolyticus* 0^{th} hour: 7.8×10^6 CFU/mL; MRSA 0^{th} hour: 1.66×10^7 CFU/mL
control *A. haemolyticus* 24^{th} hour: 8.5×10^9 CFU/mL; MRSA 24^{th} hour: 1.32×10^9 CFU/mL

After increasing the subinhibitory concentration and MIC of 4-HBA, bacterial growth was measured again with optical density following 48 hours of incubation (Figure 4.3). The optical densities of both bacteria were around zero after the first increment in the MIC. The increased MIC values of 4-HBA totally inhibited the bacterial growth of *A. haemolyticus* and MRSA. Also transfer into the initial value of MIC did not induce any resistance since bacteria could not grow either. The increase in subinhibitory concentration of 4-HBA did not change the growth of both bacteria as the percent of inhibition was still around 50%.

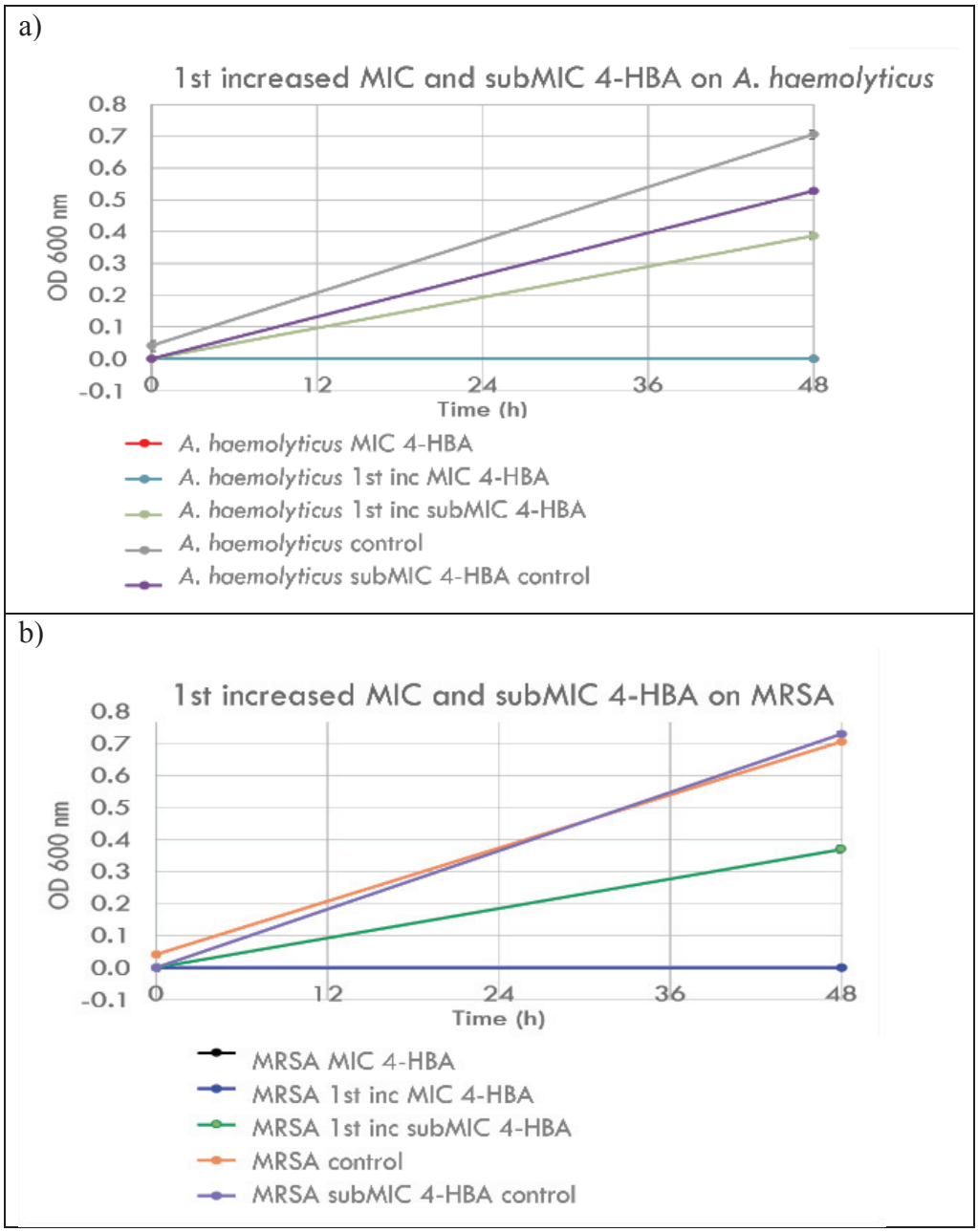

Figure 4.3. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of first increment in concentration of 4-HBA. Optical densities were measured at 0th and 48th hours. Standard deviations were lower than 0.016 and 0.016 for *A. haemolyticus* and MSSA, respectively.

Unlike the increment in 4-HBA, increasing the subinhibitory concentration and MIC of gentamicin changed the bacterial growth after 48 hours (Figure 4.4). The transfer from subinhibitory concentration of gentamicin to initial MIC value caused lower inhibition. The initial treatment of gentamicin with MIC caused total inhibition of bacterial growth for both *A. haemolyticus* and MSSA. After transfer, MIC was not enough to kill bacteria as the bacterial inhibition was around 50%. The transfer from subinhibitory concentration of gentamicin to increased MIC showed total inhibition of MRSA growth. But *A. haemolyticus* could grow 50% after transfer to the increased MIC value.

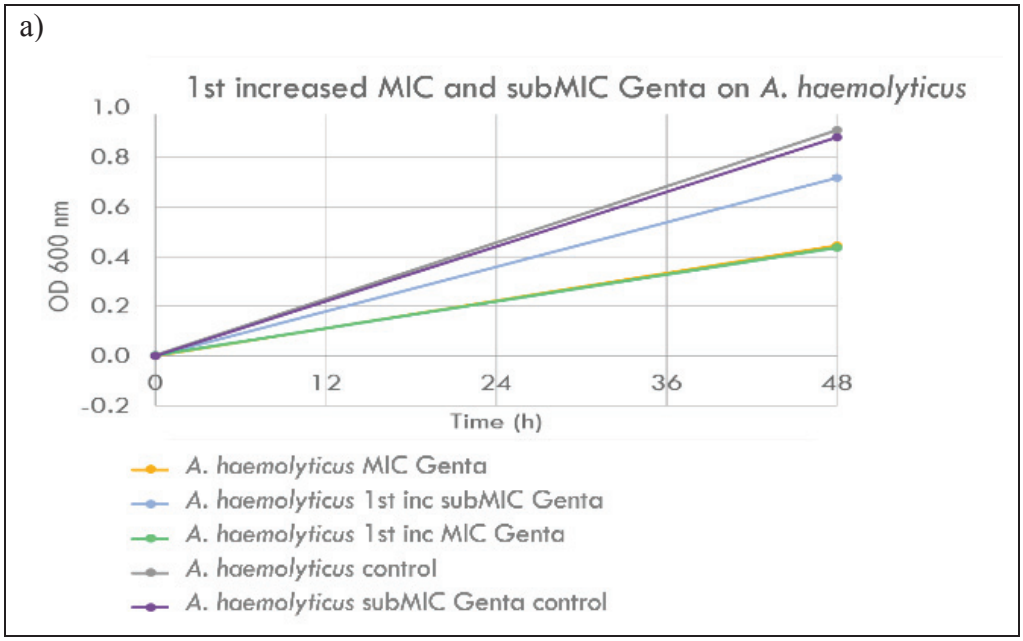

Figure 4.4. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of first increment in concentration of gentamicin. Optical densities were measured at 0^{th} and 48^{th} hours. Standard deviations were lower than 0.011 and 0.013 for *A. haemolyticus* and MSSA, respectively.

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b)

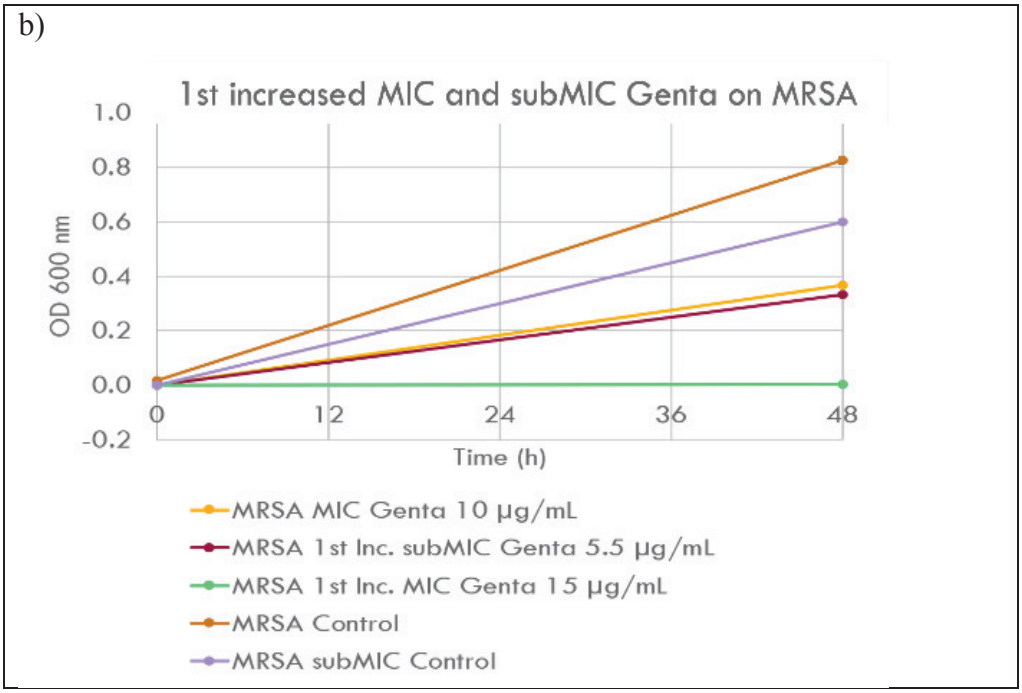

Figure 4.4 (cont.)

According to Table 4.4, increased subinhibitory concentrations of 4-HBA did not change the growth of *A. haemolyticus* and MRSA. The initial MIC value was enough to inhibit the bacterial growth and increased MIC was also effective. The transfer from subinhibitory concentration of gentamicin into increased concentrations caused change in the inhibition. The increased MIC of gentamicin inhibited the MRSA growth. The increased MIC value could not inhibit *A. haemolyticus* growth. The 10 µg/mL of gentamicin was predetermined as MIC value for both bacteria. After exposure to subinhibitory concentration, both bacteria could grow 50%, so the MIC value increased for both bacteria.

Table 4.4. Percent inhibitions of *A. haemolyticus* and MRSA after 48 hours of incubation with first increased 4-HBA and gentamicin concentrations.

Bacteria	Concentrations of 4-HBA	Percent inhibition	Concentrations of Gentamicin	Percent inhibition
A. haemolyticus	MIC 2 mg/mL	100%	MIC 10 µg/mL	51.21%
	Inc MIC 2.2 mg/mL	100%	Inc. MIC 15 µg/mL	51.98%
	Inc subMIC 1.2 mg/mL	45.06%	Inc. subMIC 2.5 µg/mL	21.15%
	Control from 1.1 mg/mL	25.19%	Control from 2 µg/mL	3.12%
MRSA	MIC 3 mg/mL	100%	MIC 10 µg/mL	55.6%
	Inc MIC 3.2 mg/mL	100%	Inc. MIC 15 µg/mL	99.57%
	Inc subMIC 1.3 mg/mL	50.34%	Inc. subMIC 5.5 µg/mL	59.59%
	Control from 1.2 mg/mL	2.22%	Control from 5 µg/mL	27.39%

The control from 1.1 mg/mL and 1.2 mg/mL 4-HBA were transferred into TSB and had similar growth to the control without any antimicrobial. After the first increase in concentration, both bacteria could not develop any resistance against the phenolic acid. But, both bacteria developed resistance against gentamicin as the MIC values increased. The concentration was increased again and the incubation was increased to 72 hours. After the second increase, the growth of both bacteria were inhibited by MIC and second increased MIC (Figure 4.5). Both bacteria could not develop resistance after the second increase in subinhibitory concentration.

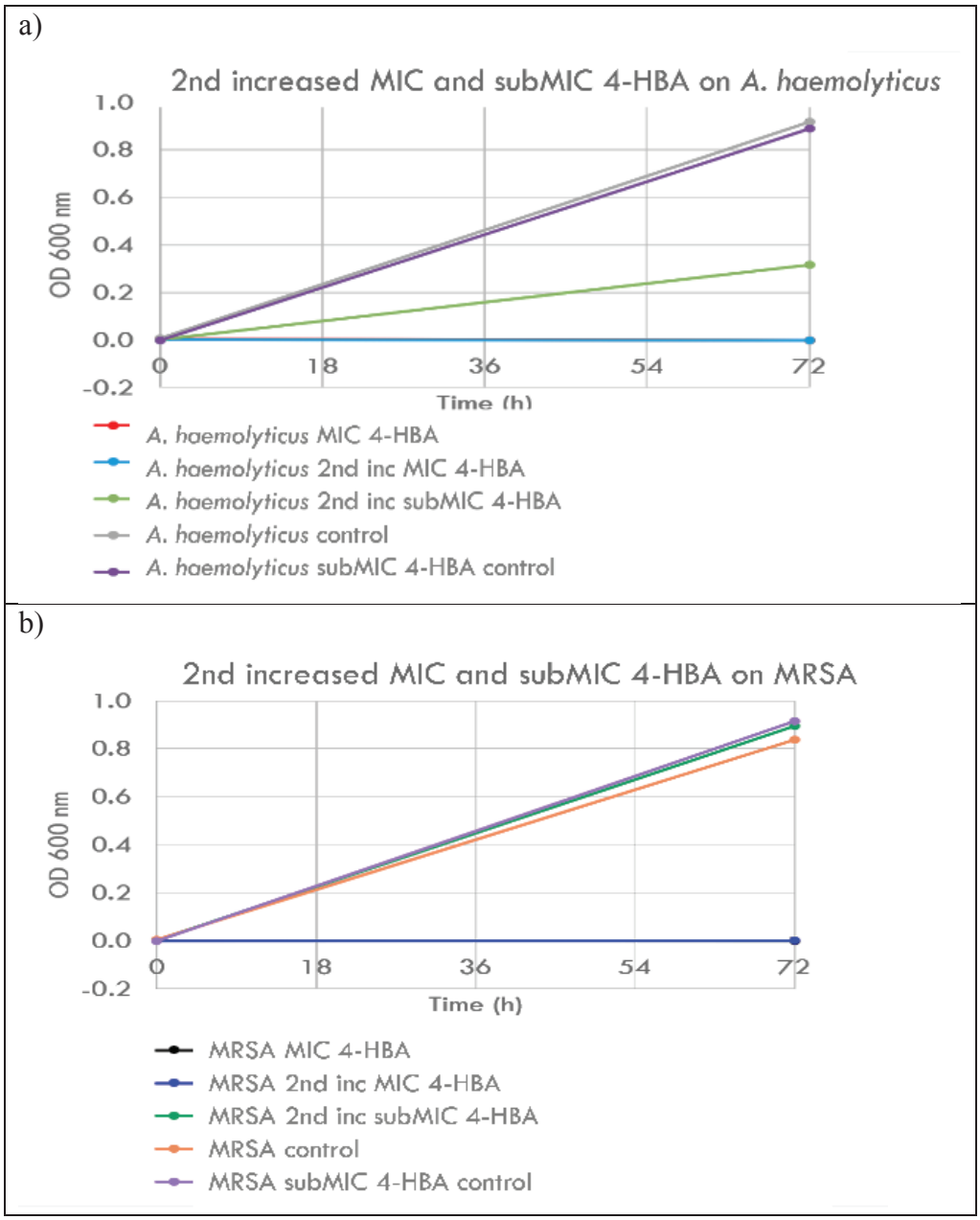

Figure 4.5. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of second increment in concentration of 4-HBA. Optical densities were measured at 0^{th} and 72^{th} hours. Standard deviations were lower than 0.012 and. 0.01 for *A. haemolyticus* and MSSA, respectively.

The control group had the bacterial number as 10^9 CFU/mL. After the second increment in 4-HBA concentration, the cell count was calculated as shown in Table 4.5. After 72 hours of incubation, the predetermined MIC values were still effective for both

bacteria. The final bacterial number of *A. haemolyticus* was 10^3 after exposure to MIC value of 4-HBA, whereas MRSA was reduced to 10^4. As a conclusion, the treatment with 4-HBA at the MIC value resulted in 6 log reduction in *A. haemolyticus* and 5 log reduction in MRSA. Viable cell counts showed that longer incubation with 4-HBA caused more growth inhibition.

Table 4.5. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 72 hours of incubation with different 4-HBA concentrations.

Bacteria	**Concentrations of 4-HBA**	**Percent inhibition**	**Viable cell counts (CFU/mL)**
A. haemolyticus	MIC 2 mg/mL	100%	5.6×10^2
	Inc MIC 2.4 mg/mL	100%	1.6×10^2
	Inc subMIC 1.3 mg/mL	65.47%	7.5×10^6
	Control from 1.2 mg/mL	-9.70%	7.3×10^8
MRSA	MIC 3 mg/mL	100%	1.2×10^4
	Inc MIC 3.4 mg/mL	99.61%	6.6×10^3
	Inc subMIC 1.4 mg/mL	-6.86%	1.8×10^7
	Control from 1.3 mg/mL	-9.44%	1.33×10^8

control *A. haemolyticus* 72^{th} hour: 9.3×10^8 CFU/mL; MRSA 72^{th} hour: 5.3×10^8CFU/mL

The second increment in the gentamicin concentrations resulted in resistance of *A. haemolyticus* and MRSA against the gentamicin as there were no inhibition of both bacteria (Figure 4.6). The bacterial growth of *A. haemolyticus* and MRSA decreased in half after exposure to MIC of gentamicin whereas initially MIC reduced the bacterial growth 100%. The predetermined concentration for MIC was 10 µg/mL. After exposure to subinhibitory concentration, even two-fold MIC values were not enough to inhibit the bacterial growth.

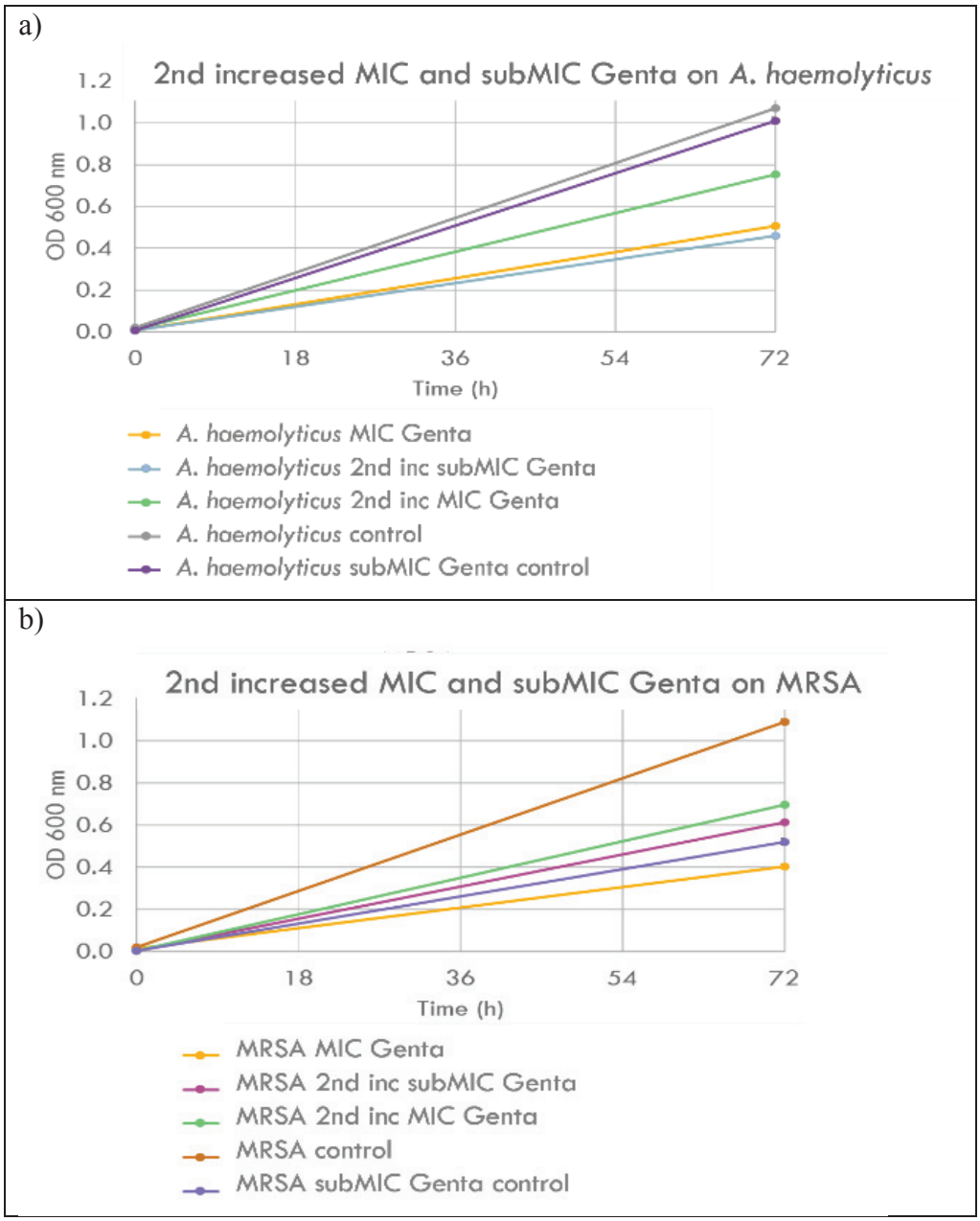

Figure 4.6. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of second increment in concentration of gentamicin. Optical densities were measured at 0^{th} and 72^{th} hours. Standard deviations were <0.015 for *A. haemolyticus* and MSSA.

After the second increment in concentrations, the bacterial number was consistent with the Figure 4.6, as there was obvious development of resistance against gentamicin by both bacteria, as shown in Table 4.6. The *A. haemolyticus* control group had the

bacterial number as 10^8 CFU/mL, and 10^{10} CFU/mL for MRSA. After 72 hours of incubation, the predetermined MIC values were not effective to both bacteria. The exposure to MIC values of gentamicin resulted in no log reduction for *A. haemolyticus* and only 2 log reduction for MRSA. The twofold concentration of MIC as 20 µg/mL was also not effective, it did not induce any reduction for *A. haemolyticus.* As a conclusion, the continuous exposure of gentamicin induced resistance of *A. haemolyticus* and MRSA.

Table 4.6. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 72 hours of incubation with different gentamicin concentrations.

Bacteria	**Concentrations of Gentamicin**	**Percent inhibition**	**Viable cell counts (CFU/mL)**
A. haemolyticus	MIC 10 µg/mL	52.55%	3.8×10^8
	Inc. MIC 20 µg/mL	29.55%	2.48×10^6
	Inc. subMIC 3 µg/mL	56.89%	3.3×10^8
	Control from 2.5 µg/mL	5.56%	3.8×10^8
MRSA	MIC 10 µg/mL	56.89%	3×10^8
	Inc. MIC 20 µg/mL	36.20%	1.17×10^6
	Inc. subMIC 6 µg/mL	43.87%	6.08×10^8
	Control from 5.5 µg/mL	52.25%	1.8×10^{10}

control *A. haemolyticus* 72^{th} h: 4.2×10^8 CFU/mL; MRSA 72^{th} h: 2.1×10^{10} CFU/mL

The continuous exposure to subinhibitory levels of gentamicin resulted in resistance as indicated by increased MIC. The bacteria from gentamicin and 4-HBA were transferred to fresh TSB without any antimicrobials for 48 hours (Figure 4.7 and 4.8). The transfer to fresh TSB was required to determine if the increased MIC of gentamicin was stable or not. The growth curves of both bacteria were similar but the inhibitions were different (Table 4.7). This was expected as the inoculums were taken from tubes

that contained different concentration of antimicrobials and different bacterial loads. Since both bacteria developed resistance against gentamicin, the transfer to fresh TSB resulted in more growth as the initial load was high (Figure 4.8). On the other hand, the MIC of 4-HBA inhibited the bacterial growth, so the transfer to fresh TSB had less bacteria as the initial load was low (Figure 4.7).

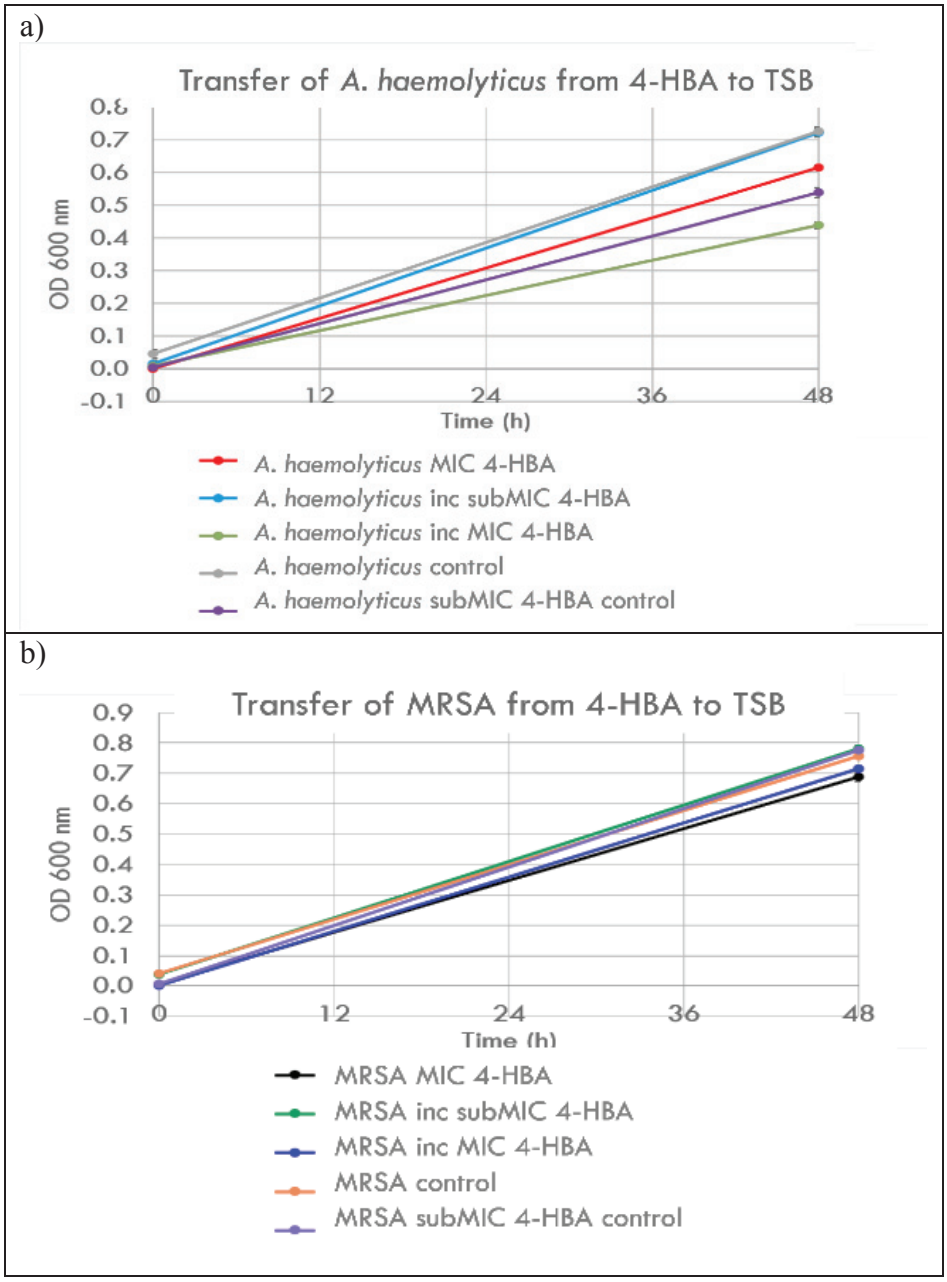

Figure 4.7. Growth of (a) *A. haemolyticus* and (b) MRSA after transfer from 4-HBA to fresh TSB media. Optical densities were measured at 0th and 48th hours.

Standard deviations were lower than 0.015 and 0.017 for *A. haemolyticus* and MSSA, respectively.

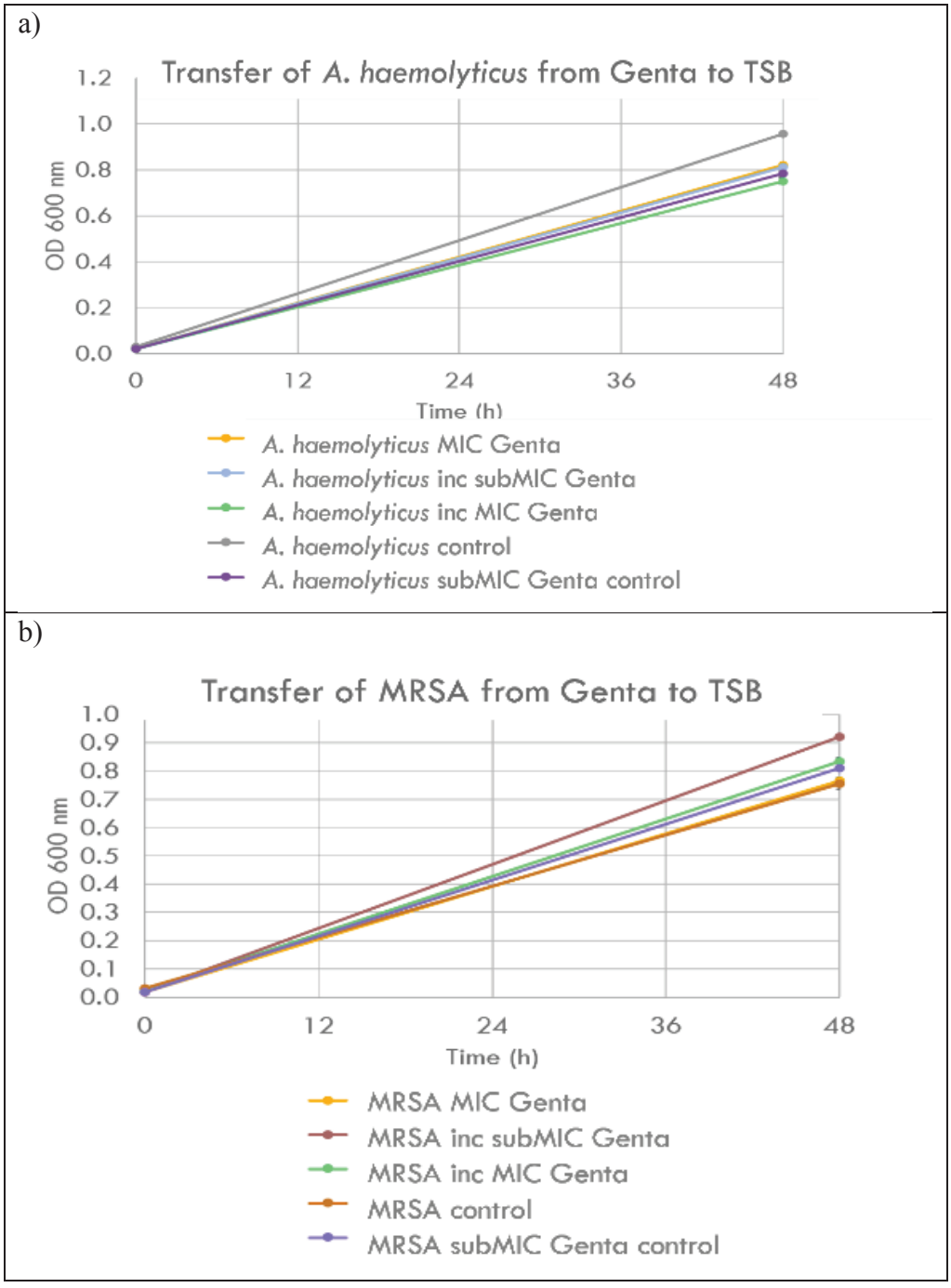

Figure 4.8. Growth of (a) *A. haemolyticus* and (b) MRSA after transfer from gentamicin to fresh TSB media. Optical densities were measured at 0th and 48th hours.

Standard deviations were lower than 0.015 and 0.017 for *A. haemolyticus* and MSSA, respectively.

The percent of inhibition of bacteria that were transferred into fresh TSB after 48 hours of incubation were given in Table 4.7. The percent of inhibition that gentamicin caused was much lower than the 4-HBA. Especially negative values of MRSA indicated that the continuous exposure of gentamicin caused severe resistance. Those tubes had much more bacterial growth compared to the control group, as the gentamicin caused stress to bacteria to grow more.

Table 4.7. Percent inhibitions of *A. haemolyticus* and MRSA after 48 hours of incubation in the fresh TSB.

Bacteria	**4-HBA Transfer to Fresh TSB**	**Percent inhibition**	**GentamicinTransfer to Fresh TSB**	**Percent inhibition**
A. haemolyticus	From MIC	15.28%	From MIC	14.35%
	From inc. MIC	39.63%	From inc. MIC	21.58%
	From inc. subMIC	0.54%	From inc. subMIC	15.27%
	From control subMIC	25.74%	From control subMIC	18.23%
MRSA	From MIC	9.09%	From MIC	-1.46%
	From inc. MIC	5.57%	From inc. MIC	-10.52%
	From inc. subMIC	-3.21%	From inc. subMIC	-22.31%
	From control subMIC	-9.44%	From control subMIC	-7.66%

Finally, after the bacteria were grown in fresh TSB for 48 hours, those cultures were transferred into predetermined subinhibitory concentration and MIC of antimicrobials. The effect of exposure to initial concentration of 4-HBA to *A.*

haemolyticus and MRSA were given in Figure 4.9. The growth of *A. haemolyticus* and MRSA in the presence of initial concentration of 4-HBA were similar to the initial results as discussed in Figure 4.2. The MIC values were effective again and inhibited the bacterial growth of both bacteria by 100% even after continuous exposure to 4-HBA. The subinhibitory concentrations also reduced the bacterial growth in half. The same growth pattern was also seen in the first exposure to 4-HBA (Figure 4.2).

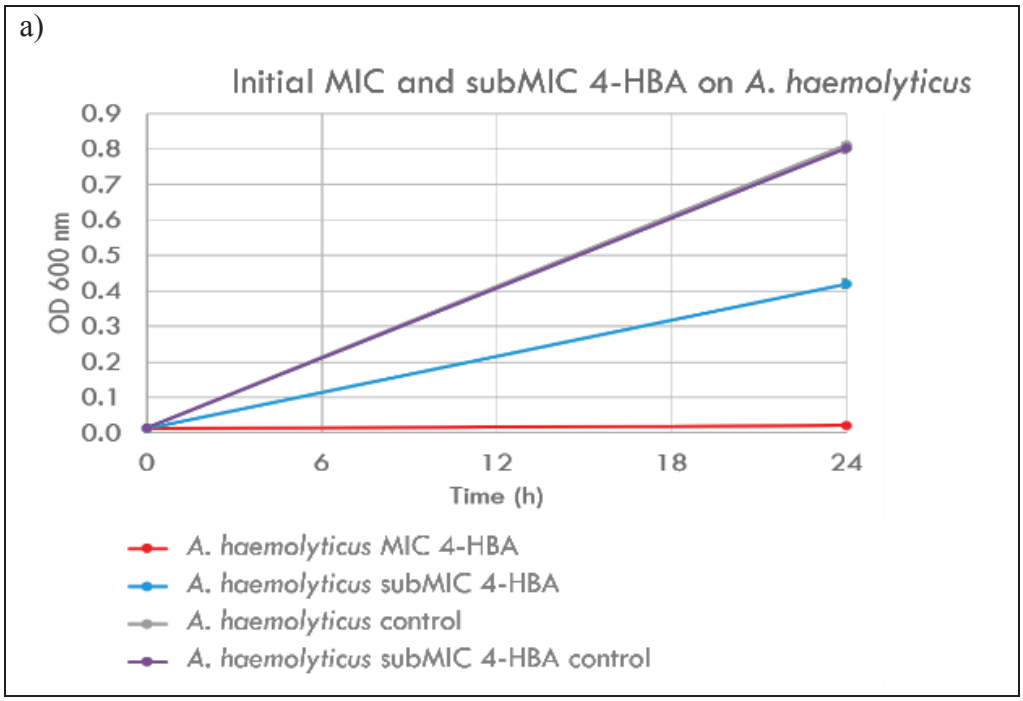

Figure 4.9. Growth of (a) *A. haemolyticus* and (b) MRSA in the presence of initial concentrations of 4-HBA. Optical densities were measured at 0^{th} and 24^{th} hours. Standard deviations were lower than 0.013 and 0.009 for *A. haemolyticus* and MSSA, respectively.

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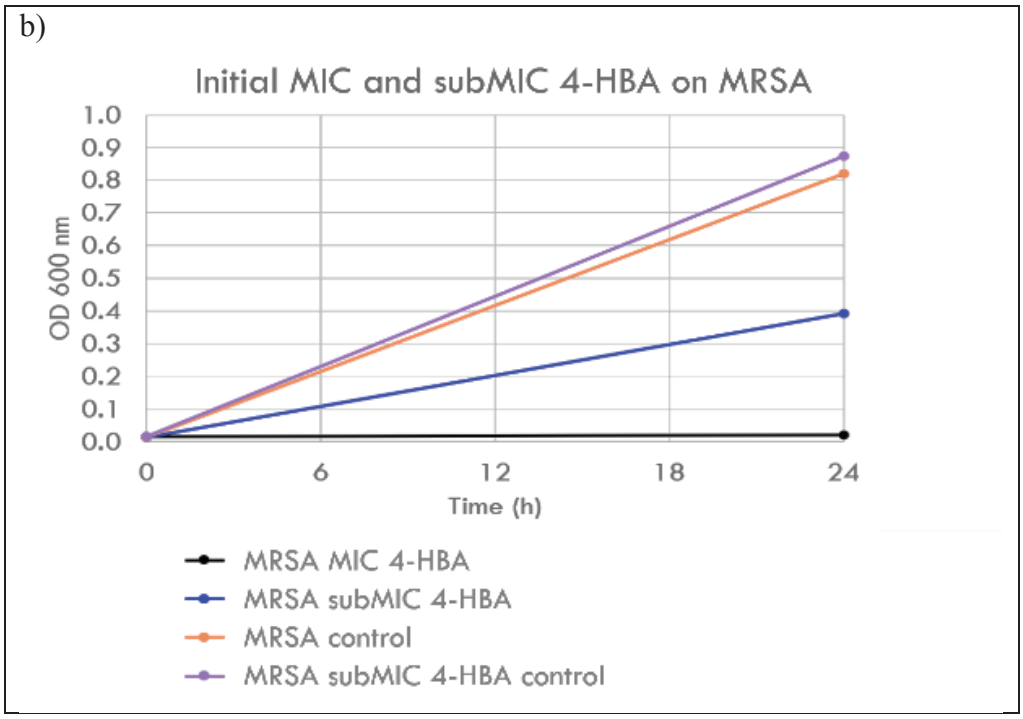

Figure 4.9 (cont.)

The percent of inhibition and viable cell counts of bacteria after 24 hours of incubation in initial 4-HBA concentrations were given in Table 4.8. The inhibition level was 98% for the *A. haemolyticus* as it was treated with the initially predetermined MIC value. The growth inhibition was 98% for MRSA, which it was found as 93% initially. The subinhibitory concentrations of 4-HBA reduced the bacterial growth by 48% and 52% for *A. haemolyticus* and MRSA, respectively. In the initial findings, the bacterial inhibitions were 40% and 56%. The percent of inhibitions were similar to the initially found ones. The control had the bacterial number of 10^9 CFU/mL for both *A. haemolyticus* and MRSA. The MIC value caused 5 log reduction, which was also similar to the initially found results. Those findings indicated that the both *A. haemolyticus* and MRSA did not develop any resistance against 4-HBA after continuous exposure to increasing subinhibitory concentration.

Table 4.8. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation with initial 4-HBA concentrations.

Bacteria	**Concentrations of 4-HBA**	**Percent inhibition**	**Viable cell counts (CFU/mL)**
A. haemolyticus	MIC 2 mg/mL	97.54%	8×10^3
	subMIC 1.1 mg/mL	48.2%	5.3×10^4
	Control from subMIC	0.99%	8.1×10^8
MRSA	MIC 3 mg/mL	97.61%	2.6×10^4
	subMIC 1.2 mg/mL	52.21%	5.3×10^4
	Control from subMIC	-6.77%	7.7×10^8

control *A. haemolyticus* 24th hour: 2.7×10^9 CFU/mL; MRSA 24th hour: 1.6×10^9 CFU/mL

The effects of exposure to an initial concentration of gentamicin to *A. haemolyticus* and MRSA were given in Figure 2.10. None of the concentrations were enough to inhibit the bacterial growth, as most of the growth curves were very similar. The MIC values of gentamicin reduced the optical densities to zero in the initial experiment before any exposure to subinhibitory concentrations (Figure 4.2). After continuous exposure to subinhibitory concentrations, the MIC concentrations were not enough to inhibit the bacterial growth.

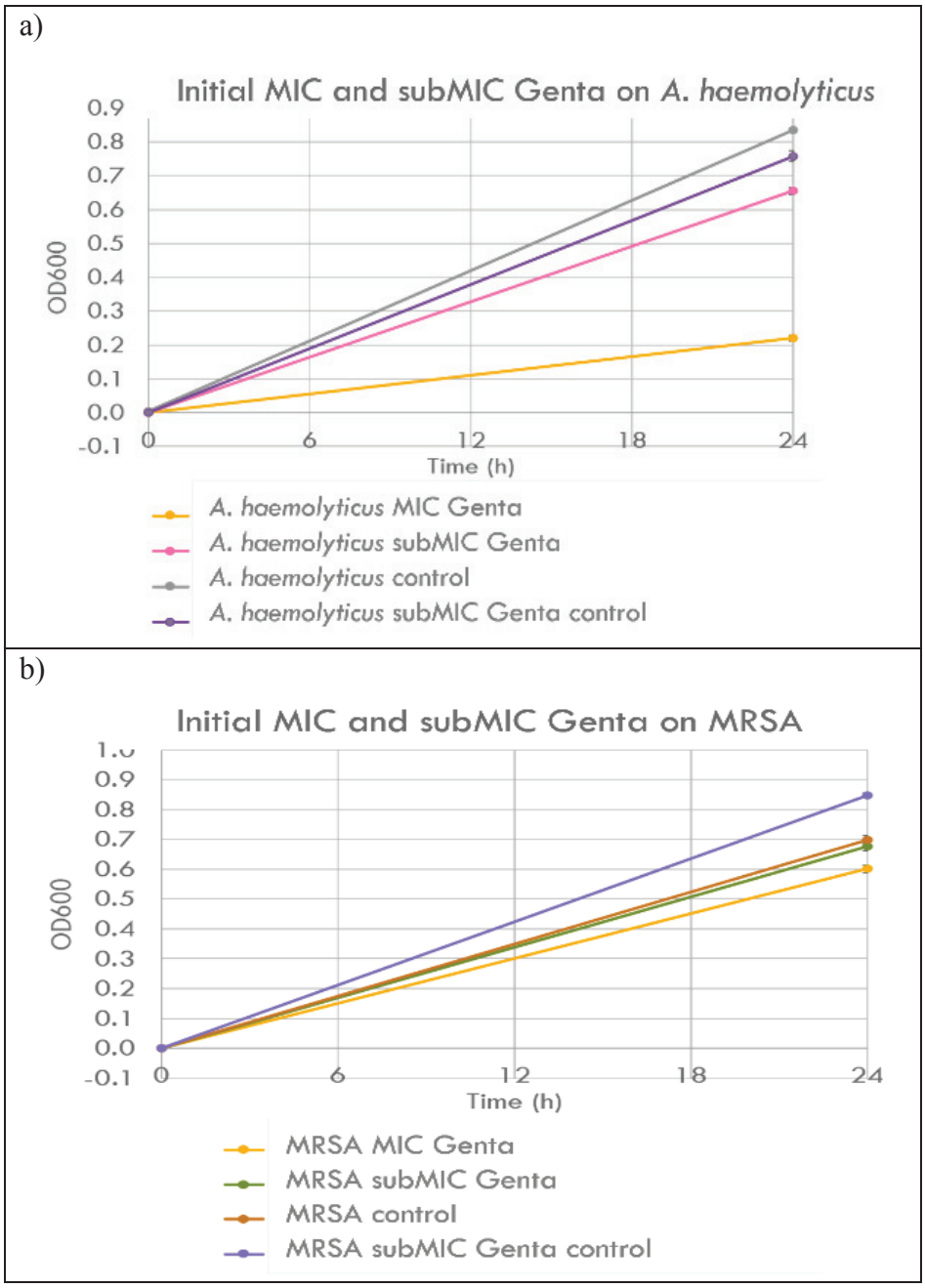

Figure 4.10. Growth of (a) *A. haemolyticus* and (b) MRSAin the presence of initial concentrations of gentamicin. Optical densities were measured at 0th and 24th hours. Standard deviations were lower than 0.015 and 0.014 for *A. haemolyticus* and MSSA, respectively.

The percent of inhibition and viable cell counts of bacteria after exposure to initial gentamicin concentrations were given in Table 4.9. The inhibition level was 74% for the *A. haemolyticus* as it was treated with the initially predetermined MIC value. Previously, it was found as 100%. The growth inhibition was 14% for MRSA, which it was found as 98% initially. The initial findings for viable cell counts were much more different as there were no colonies within the tubes with MIC. But after continuous exposure of gentamicin, there were 1.3 x 10^5 CFU/mL of *A. haemolyticus* and 5.6 x 10^6 for MRSA. For the subinhibitory concentrations, the initial findings were 81% and 86% inhibition of bacterial growth of *A. haemolyticus* and MRSA, respectively. After continuous exposure, the percent of inhibition reduced to 22% and 3%. These findings showed that both *A. haemolyticus* and MRSA developed resistance against gentamicin.

Table 4.9. Percent inhibitions and viable cell counts of *A. haemolyticus* and MRSA after 24 hours of incubation with initial gentamicin concentrations.

Bacteria	**Concentrations of Gentamicin**	**Percent inhibition**	**Viable cell counts (CFU/mL)**
A. haemolyticus	MIC 10 µg/mL	73.64%	1.3 x 10^5
	subMIC 2 µg/mL	21.52%	3.6 x 10^8
	Control from subMIC	9.28%	4.5 x 10^8
MRSA	MIC 10 µg/mL	13.99%	5.6 x 10^6
	subMIC 3 µg/mL	3.20%	2.8 x 10^8
	Control from subMIC	-21.34%	4.5 x 10^8

control *A. haemolyticus* 24^{th} h: 2.4 x 10^9 CFU/mL; MRSA 24^{th} h: 1.3 x 10^9 CFU/mL

The development of resistance against antibiotic is already well known, so the resistance against gentamicin was not surprising. The resistance against antibiotics is the major problem in treatment of different infections such as MRSA infections. Those infections become more and more hard to treat as the antibiotic treatments are generally

unsuccessful (Basak et al., 2016). Both *A. haemolyticus* and MRSA developed resistance against the gentamicin after continuous exposure to subinhibitory concentration. Their resistance profiles and resistance levels were similar. After exposure to subinhibitory concentrations of gentamicin, the percent of inhibition of the MIC value was reduced drastically. The initial treatment of *A. haemolyticus* with 10 μg/mL gentamicin reduced the growth by 100%. However, after continuous exposure, the growth inhibition reduced to 53% after 72 hours of incubation. MRSA had similar pattern as the initial concentration as 10 μg/mL gentamicin inhibited the growth by 98%. This inhibition was reduced to 57% after the exposure to subinhibitory concentration. After transferring of bacteria into fresh TSB without any addition of antimicrobial, the MIC values for gentamicin were changed. After transferring into initial concentration of gentamicin, the growth of MRSA was inhibited by 14% and *A. haemolyticus* was inhibited by 77%. These findings may conclude that the more incubation period with gentamicin leads formation of more resistant bacterial strains.

This study is one of the antimicrobial studies in which the findings show that bacteria could not develop resistance against the tested antimicrobial compounds. Similar results were obtained in the study of Keman and Soyer, in which vanillic acid and o-coumaric acid were tested on MRSA and MSSA (Keman & Soyer, 2019).

4.3.2. Quantitative Analysis of Phenol Hydroxylase and Catechol-1, 2 Dioxygenase Genes by ddPCR

Droplet Generation and Droplet digital PCR protocol were used for absolute quantification of phenol hydroxylase and catechol-1,2 dioxygenase gene expression in *A. haemolyticus, P. aeruginosa* and MRSA. The agarose gel was given in Figure 4.11. The negative control has no band, and the ladder was used. *A. haemolyticus* had no bands for phenol hydroxylase, whereas *P. aeruginosa* and MRSA had bands at the same molecular weight of 230 bp, which corresponds to the gene that codes for phenol hydroxylase. On the other hand, MRSA did not contain any gene that codes for catechol-1,2 dioxygenase, as there were no bands in the agarose gel. *A. haemolyticus* and *P. aeruginosa* had bands in the agarose gel at molecular weight of 260 bp, this indicated that DNA of those two

bacteria have the respective gene that is used for encoding the enzyme catechol-1,2 dioxygenase. The quantification of the number of genes per µL was performed by ddPCR.

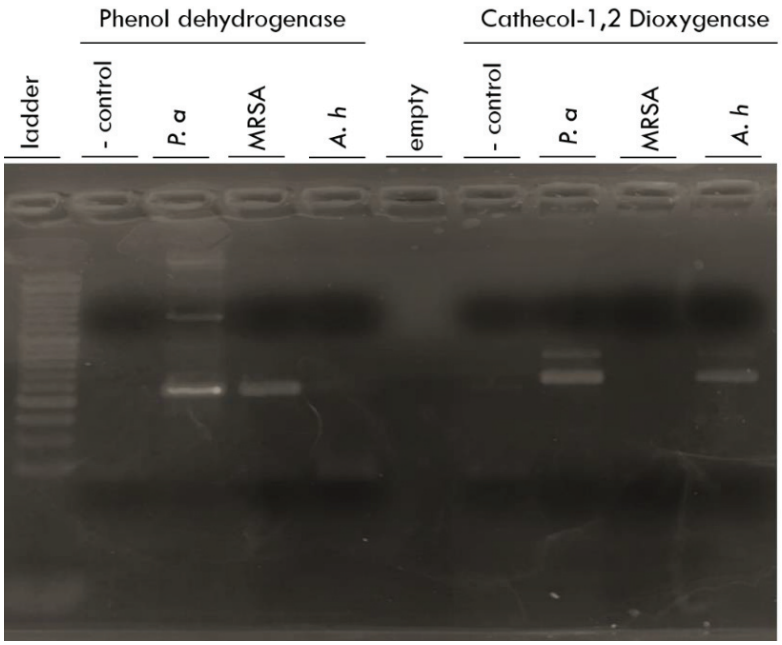

Figure 4.11. Agoraose gel results of Droplet Digital PCR. (- control: negative control, P.a : *Pseudomonas aeruginosa*, A.h: *Acinetobacter haemolyticus*).

For Droplet Generation and Droplet digital PCR, 20 µL samples were used in order to calculate how much of respective genes were found in the same amount of sample. Each droplet was analysed by optical detector. The data were analysed by QuantaSoft software v1.7. The 2D amplitude plot was obtained and positive droplets were selected. The final mRNAs of phenol hydroxylase and catechol-1,2 dioxygenase were determined as copies per µL. The 2D amplitude plot of catechol-1,2 dioxygenase was given in Figure 4.12. The concentration of the genes that codes for catechol-1,2 dioxygenase in negative control (A10) was close to 0. The amount of the gene was higher in *A. haemolyticus* (B10) sample, which is consistent with the findings of the agarose gel as there were a band. The highest concentration of the gene was seen in the well C10, which is the well for *P. aeruginosa.* It was also consistent with the agarose gel as there was thicker band compared to the *A. haemolyticus.* Finally, the amplitude from the MRSA was low, which confirmed that there were almost no genes code for catechol-1,2 dioxygenase.

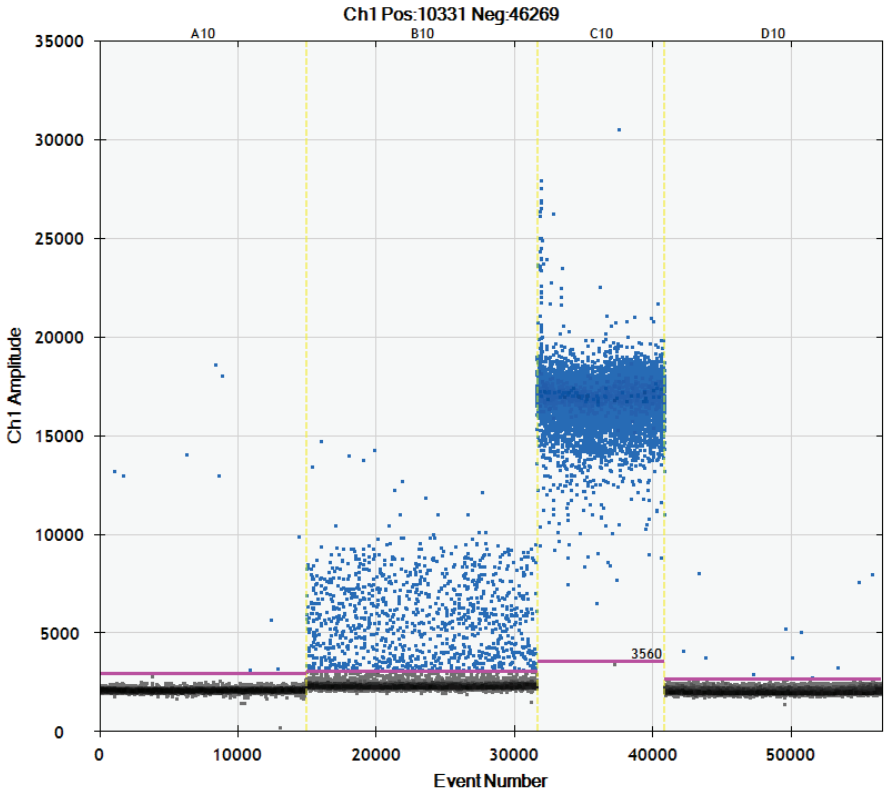

Figure 4.12. 2D amplitude graph of ddPCR of the catechol-1,2 dioxygenase. Data were analysed with QuantoSoft software. The wells were represented as A10, B10, C10 and D10 which corresponds to the negative control, *A. haemolyticus, P. aeruginosa* and MRSA, respectively.

The concentrations of the gene that codes for catechol-1,2 dioxygenase were calculated by QuantoSoft software. The concentration of the gene in negative control (A10) was 0.78. It was close to zero, but due to EvaGreen dye, there were some background signals in the analysis. The concentration of the gene was highest in the *P. aeruginosa* sample as there were 10700 copies of the gene were found in 1 µL (Table 4.10). On the other hand, no genes were found in the MRSA sample as the value was 0.83, which was close to the negative control. There were approximately 81.1 gene copies that codes for catechol-1,2 dioxygenase in 1 µL of the *A. haemolyticus* sample.

Table 4.10. Concentration (copies/µL) of the catechol-1,2 dioxygenase gene.

Samples	**Concentration (copies/µL)**
Negative control	0.78
A. haemolyticus	81.1
P. aeruginosa	10700
MRSA	0.83

The 2D amplitude plot of phenol hydroxylase was given in Figure 4.13. The concentration of the genes that codes for phenol hydroxylase in negative control (A10) was close to 0. The same statement was also true for *A. haemolyticus* (B10) sample, since there were few dots. Both of the data were consistent with the agarose gel as there were no bands correspond to the gene that codes for phenol hydroxylase. The highest concentration of the gene was again in the well C10, belongs to *P. aeruginosa* sample. There were also some dots in the MRSA indicated that there were few copies of the genes. It was also consistent with the agarose gel as there were bands in both samples of *P. aeruginosa* and MRSA.

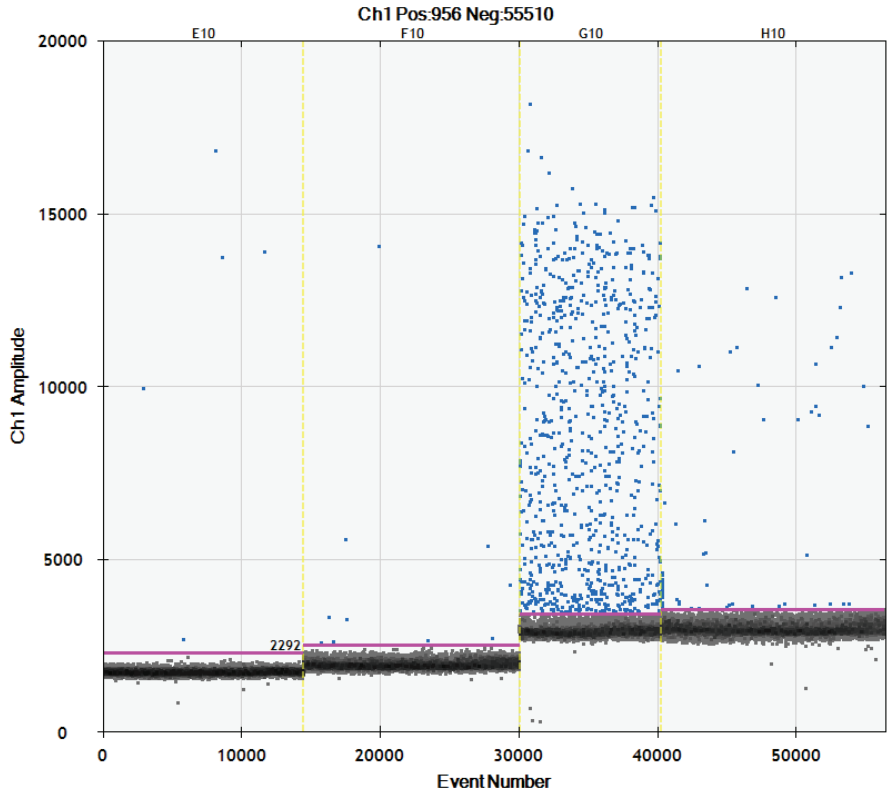

Figure 4.13. 2D amplitude graph of ddPCR of the phenol hydroxylase. Data were analysed with QuantoSoft software. The wells were represented as A10, B10, C10 and D10 which corresponds to the negative control, *A. haemolyticus, P. aeruginosa* and MRSA, respectively.

The concentrations of the gene that codes for phenol hydroxylase was calculated by QuantoSoft software as the same way of calculating the catechol-1,2 dioxygenase. The concentration of the gene in negative control (A10) was 0.41. It was close to zero just in Table 4.11, but there was also same background due to EvaGreen. The concentration of the gene in the the *A. haemolyticus* sample was close to negative control as 0.75. The concentration of the gene was highest in the *P. aeruginosa* sample again as there were 106 copies per µL. There were approximately 4.6 gene copies in 1 µL in the MRSA sample that codes for phenol hydroxylase.

Table 4.11. Concentration (copies/µL) of the phenol hydroxylase gene.

Samples	Concentration (copies/µL)
Negative control	0.41
A. haemolyticus	0.75
P. aeruginosa	106
MRSA	4.6

Even both of the *P. aeruginosa* and MRSA had gene that codes for phenol hydroxylase, MRSA couldn't develop any resistance against phenolic acid 4-HBA. On the other hand, *A. haemolyticus* contains catechol-1,2 dioxygenase, it also couldn't develop any resistance against the phenolic acid. The phenols were degraded by the phenol degradation pathway, in which the phenol was oxygenated with the phenol hydroxylase enzyme to form catechol. Then the ring within the catechol was cleaved by the enzyme catechol-1,2 dioxygenase. The final products were mineralized into carbon dioxide. Since *A. haemolyticus* and MRSA don't contain both phenol hydroxylase and catechol-1,2 dioxygenase at the same time, both bacteria couldn't develop any resistance against used phenolic acid.

4.4. Conclusion

This experimental study was focused on the use of 4-hydroxybenzoic acid as an alternative antimicrobial product against antibiotic resistant bacteria as *A. haemolyticus* and MRSA. The first conclusion of this study was the ability of bacteria to develop resistance against antibiotic, which is gentamicin in this study. The results were consistent with the general information about antibiotic resistant bacteria. The second outcome was the susceptibility of bacteria to phenolic acid 4-hydroxybenzoic acid. The results were exciting and promising as the susceptibility to 4-hydroxybenzoic acid was maintained by both *A. haemolyticus* and MRSA. The development of resistance against

antibiotic and phenolic acid might be due to i) different chemical properties of two compounds, ii) different resistance mechanisms against antibiotic and phenolic acid or ii) different inhibition mechanisms of bacterial growth by phenolic acid.

The antimicrobial action of phenolic acids is different than antibiotics. Antibiotics commonly targets different cellular structures and processes by inhibiting i) the cell wall synthesis, ii) cell membrane function, iii) protein synthesis, iv) nucleic acid synthesis and, v) other metabolic processes that are essential for pathogen survival (O'Rourke et al., 2020). On the other hand, phenolic acids have different mode of action for inhibiting the bacterial growth. Among these, phenolic acids disrupt the quorum sensing of the bacteria (Borges et al., 2014), irreversibly alter the membrane by creating pore and change surface charge (Borges et al., 2013); bind to outer membrane and disrupt it which leads to release cytoplasmic molecules even slightly release nucleotide, which led to cell death. (Lou et al., 2011); bind to bacterial DNA and proteins such as enzymes , so inhibits cellular functions (Alves et al., 2013). Therefore, the development of resistance against phenolic acids by *A. haemolyticus* and MRSA is not expected. Nonetheless, the molecular study of antimicrobial action mechanisms is essential to understand the individual targets of pathogens.

4.5. Future Aspects

For the general view, this study includes the

i) The antibacterial effect of phenolic acid 4-HBA and antibiotic gentamicin on *A. haemolyticus* and MRSA,

ii) The antibacterial effect of the solution/powder form of 4-HBA/gentamicin-loaded PMMA bone cement on *E. coli,*

iii) The synergistic effect of gentamicin and 4-HBA-loaded PMMA bone cement on *E. coli,*

iv) The structural properties of the PMMA bone cement via SEM,

v) The release rates of the solution/powder form of 4-HBA/gentamicin -loaded PMMA bone cements via UV and HPLC,

vi) The investigation of development of resistance against gentamicin and 4-HBA on MRSA, *A. haemolyticus,*

vii) Quantification of resistance genes catechol 1,2-dioxygenase and phenol hydroxylase were via ddPCR on MRSA, *A. haemolyticus, P. aeruginosa*.

The significance of this study is the first investigation of the antimicrobial effect of 4-HBA-loaded bone cement, the development of resistance profile against 4-HBA. This study the first comparative research of liquid and powder form of phenolic acid 4-HBA-loaded bone cement against pathogens. Finally the synergistic effect of phenolic acid with the antibiotics within the bone cement was investigated for the first time.

The results of experimental procedures were promising as bacterial growth of *E. coli* was significantly inhibited by 4-HBA loaded bone cement. The release pattern of liquid antimicrobial loaded bone cement was higher and more controlled compared to the powder antimicrobial loaded cement. *A. haemolyticus* was found to have high copy number of catechol-1,2 dioxygenase. But *A. haemolyticus* and MRSA haven't developed resistance to 4-HBA, while gentamicin has induced resistance. These results showed that bacteria are more susceptible to development of resistance against antibiotic gentamicin. Therefore, phenolic acids may be used as antimicrobial agents in bone cement to inhibit the pathogenic bacterial growth.

The antimicrobial effect of 4-HBA-loaded bone cement on different pathogens such as MSSA is still under investigation in Soyer Laboratory. The cytotoxic effect of gentamicin and 4-HBA on healthy cell lines may be determined with the MTT cell proliferation assay. The antimicrobial action mechanism of phenolic acids is still under investigation as phenolic acids act on different cellular structures and pathways. The antimicrobial effect of 4-HBA can be further investigated by transcriptomics and proteomics to understand the action mechanism of 4-HBA. Finally in vivo experiments of phenolic acid loaded bone cement can be performed on different organisms such as mice.

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