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M.S. Thesis In Genetics and Bioengineering

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ANTIMICROBIAL ACTIVITY SCREENING OF SOME FERROCENE-PAMAM DENDRIMERS

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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M. S. Thesis – Genetics and Bioengineering
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Supervisor: Assoc. Prof. Mustafa Fatih ABASIYANIK

ABSTRACT

The resistance improved in microorganisms against an antimicrobial agent soon after its use demands development of new antimicrobials for the treatment of infectious diseases. As much as discovery of such antimicrobial agents, drug delivery also increase in importance to reduce side effects and effective dosage level of the antimicrobial agents. In this study, the potential antimicrobial activity of ferrocene-amine and three different generations of ferrocene-PAMAM denrimers, as drug delivery instruments, against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Citrobacter freundii, Klebsiella pneumoniae, Bacillus subtilis, Proteus mirabilis, Cryptococcus neoformans, Candida albicans* and *Candida utilis* were investigated. Broth microdilution method was used to determine minimum inhibitory concentration (MIC) values of the test substances against ten pathogenic microorganisms. Antibacterial drugs including kanamycin, tetracycline, streptomycin, erythromycin, nalidixic acid and ampicillin and antifungal drugs including nystatin and amphotericin B were used in the tests as control to understand antimicrobial effectiveness of ferrocene-PAMAM dendrimers relative to effective antibiotics. The results indicated the presence of antimicrobial activity for ferrocene-amine and ferrocene-PAMAM dendrimers against all microorganisms. The MIC values were determined as ranging from 0.0625 µg/mL to 0.125 µg/mL for ferrocene-amine, ranging from 0.25 µg/mL to 0.0625 µg/mL for ferrocene-PAMAM (G2) denrimer and ferrocene-PAMAM (G3) denrimer, ranging from 0.03125 µg/mL to 0.125 µg/mL for ferrocene-PAMAM (G1) denrimer. Minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC) were also determined to reveal that the compounds display either microbiostatic or microbicidal effect on the test microorganisms. Ferrocene-amine (G0) killed *K.pneumoniae, C.freundii, P.aeruginosa, S .aureus, C.neoformans, C.albicans* and *C.utilis;* ferrocene-PAMAM (G1) dendrimer killed *K.pneumoniae, E.coli, B.subtilis, C.freundii, P.aeruginosa, C.neoformans, C.albicans* and *C.utilis;* ferrocene-PAMAM

(G2) dendrimer killed *E.coli, C.freundii, S.aureus, C.neoformans, C.albicans* and *C.utilis* while ferrocene-PAMAM (G3) dendrimer killed *C.freundii, C.neoformans, C.albicans* and *C.utilis.* When the results were assessed, it has been understood that the compounds have a more effective antimicrobial activity against fungal strains than those of against bacterial strains.

Keywords: Antimicrobial Activity, Broth Microdilution Method, MIC, MBC, Ferrocene–PAMAM Dendrimers.

BAZI FERROSİN–PAMAM DENDRİMERLERİNİN ANTİMİKROBİYAL AKTİVİTELERİNİN TARANMASI

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ÖZ

Mikroorganizmalarda bir antimikrobiyal ajana karşı kullanımından kısa bir süre sonra gelişen direnç, infeksiyöz hastalıkların tedavisi için yeni antimikrobiyallerin geliştirilmesini gerekli kılmaktadır. Böyle antimikrobiyallerin keşfedilmesi kadar ilaç taşınımı da istenmeyen yan etkileri ve etkili antimikrobiyal ajan dozu seviyesini azaltmada önem kazanmaktadır. Bu çalışmada ferrosin–aminin ve ferrosin–PAMAM dendrimerlerinin üç farklı jenerasyonunun *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Citrobacter freundii, Klebsiella pneumoniae, Bacillus subtilis, Proteus mirabilis, Cryptococcus neoformans, Candida albicans* ve *Candida utilis'*e karşı potansiyel antimikrobiyal aktiviteleri araştırıldı. Test maddelerinin on patojen mikroorganizmaya karşı minimum inhibe edici konsantrasyonlarını (MİK) belirlemek için broth mikrodilüsyon yöntemi kullanıldı. Kanamisin, tetrasiklin, streptomisin, eritromisin, nalidiksik asit ve ampisilinden oluşan antibakteriyeller ve nistatin ve amfoterisin B' den oluşan antifungaller ferrosin–PAMAM dendrimerlerinin etkili antibiyotiklere kıyasla antimikrobiyal etkinliğini anlamak için testlerde kontrol olarak kullanıldı. Sonuçlar ferrosin–amin ve ferrosin–PAMAM dendrimerleri için tüm mikroorganizmalara karşı antimikrobiyal aktivitenin varlığını gösterdi. MİK değerlerinin ferrosin–amin için 0.0625 µg/ml ile 0.125 µg/ml aralığında, ferrosin–PAMAM (G2) dendrimeri ve ferrosin–PAMAM (G3) dendrimeri için 0.03125 µg/ml ile 0.125 µg/ml aralığında, ferrosin–PAMAM (G1) dendrimeri için 0.03125 µg/ml ile 0.125 µg/ml aralığında olduğu belirlendi. Maddelerin mikroorganizmalar üzerinde mikrobiyostatik ya da mikrobisidal etkilerinin olduğunu ortaya çıkarmak için minimum bakterisidal konsantrasyonları ve minimum fungusidal konsantrasyonları da saptandı. Ferrosin–PAMAM (G3) dendrimeri *C.freundii, C.neoformans, C.albicans* ve *C.utilis*'i öldürürken ferrosin–amin (G0) *K.pneumoniae, C.freundii, P.aeruginosa, S .aureus, C.neoformans, C.albicans* ve *C.utilis*'i öldürdü; ferrosin–PAMAM (G1) dendrimeri *K.pneumoniae, E.coli, B.subtilis, C.freundii, P.aeruginosa, C.neoformans, C.albicans*

ve *C.utilis*'i öldürdü; ferrosin–PAMAM (G2) dendrimeri *E.coli, C.freundii, S.aureus, C.neoformans, C.albicans* ve *C.utilis*'i öldürdü. Sonuçlar değerlendirildiğinde maddelerin fungal türler üzerinde bakteriyel türlere göre daha etkili antimikrobiyal aktiviteye sahip olduğu anlaşıldı.

Anahtar Kelimeler: Antimikrobiyal Aktivite, Broth Mikrodilüsyon Yöntemi, MİK, MBK, MFK, Ferrosin–PAMAM Dendrimerleri.

To my parents and sister who are the true owners of the successes in my life….

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SYMBOL/ABBREVIATION

µg/mL	Microgram per mililiter
API	Active pharmaceutical ingredients
ATCC	American type culture collection
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
HCl	Hydrochloric acid
MAM	Mode of action mechanism
MBC	Minimum bactericidal concentration
MDR	Multi drug resistance
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
PAMAM	Poly (amidoamine)

CHAPTER 1

INTRODUCTION

1.1 A BRIEF HISTORY OF ANTIMICROBIALS

The use of natural or synthetic antimicrobial agents to counteract infections causing diseases spans to ancient times of history which frankincense and myrrh were used by the ancient Egyptians, arsenic was used by the Assyrians and mercury was used by the Arabs [1] There are suggestions about that for paralysing malarial-like symptoms, cinchona bark infusion was used by Peruvian Indians in 17 th century and the active quinine component was isolated in Paris in 1820 [2].

The finding out of the fact that microorganisms are responsible for a variety of diseases that plague humanity from ancient times was realized in 19th century. In 1887, when Pasteur and Joubert were working on an anthrax vaccine, they found the phenomenon of bacteria inhibiting the growth of other bacteria [3]. In 1889**,** 'antibiosis' term was first used by Vuillemin and in the 1940s, 'antibiotic' term that describe the chemical substance involved in bacterial antagonism was introduced by Waksman [3]. In 1899, also, *pyocyanase,* the first antibacterial extract, was introduced by Emmerich and Low. It was used as a local antiseptic in the treatment of diptheria [3]. In 1910, Ehrlich synthesized salvarsan and it was used in the treatment of syphilis. But this drug was a synthetic compound and had limitations regarding safety and efficacy [4].

The remarkable dawn of antimicrobials became about 1930s. In 1928, penicillin was discovered by Alexander Flemming. He recognised that the growth of *Staphylococcus aureus* was inhibited in a zone surrounding a contaminated blue mold from Penicillium genus in culture dishes and reached the conclusion that a microorganism would produce substances that could inhibit the growth of other

microorganisms [4]. In 1935, the sulphonamides were introduced and used against meningitis, streptococcal infections and urinary-tract infections [1].

Although the discovery of penicillin was in 1928 and it was an outstanding agent in terms of safety and efficacy, it did not become clinically relevant until the 1940s [5]. It provided a great advantage for treating wounds and saved the lifes of many soldiers during Second World War. Soon after, an oral form of the antibiotic was introduced and used against Gram-positive bacteria [5]. Streptomycin was obtained from *Streptomyces griseus* in 1944. Chloramphenicol (1947) and neomycin (1949) were developed in the 1940s, as well.

Shortly afterwards the use of penicillin, in the early 1950s, resistant strains of *Staphyloccocus aureus* against penicillin emerged in hospital patients [5]. Alternatively, streptomycin was used to treat tuberculosis [1]. Then, toxicity and ease of resistance acquisition limited its use. Oxytetracycline (1950), erythromycin (1952) and vancomycin (1956) were the other antimicrobials developed during this decade [6].

New penicillin compounds including ampicillin, amoxicillin and flucloxacillin were developed owing to identification and isolation of the penicillin nucleus in 1959. These were effective against both Gram-positive and Gram-negative microorganisms. Yet, then, resistance developed and they have been used for only anti Gram-positive action. Cephalosporins were also developed in this decade and used especially against penicillin-resistant Gram negative bacterial strains [1]. Gentamicin was introduced in 1964 and, as an antibiotic with broad antibacterial spectrum, was succesful to threat acute sepsis. Nalidixic acid, methicillin (1960) and metronidazole (1960) were other antimicrobials developed in this decade [6].

Clavulanic acid which is a compound produced by *Streptomyces* and inhibits β-lactamase, what neutralize penicillines, was discovered in the 1970s. It demonstrates a poor activity against Gram negative and Gram positive bacteria. However, it is co-formulated with broad-spectrum penicillins susceptible to β-lactamase due to being a highly potent inhibitor of β-lactamase [5, 6].

The introduction of antifungal fluconazole in 1982 became a hope in the treatment of candidiasis, especially a problem for immunosuppressed patients, and for life-

threatening fungal infections [7, 8]. It had advantages over other current members of azole group such as being applicable intravenously, being well tolerated by patients and having fewer drug-drug interactions [8]. In 1985, zidovudine became the first licensed antiretroviral agent against AIDS [9].

Linezolid was developed in the late 1990s. It was the first member of the first entirely new antibiotic class, the oxazolidinone antibiotics, in 30 years [7]. It demonstrated a perfect activity against Gram positive microorganisms including resistant strains to vancomycin and even methicillin. In 2001, caspofungin was developed and was the first in a new class of antifungal agents, the echinocandins. It is acting by preventing the synthesis of an essential component of the cell wall of several fungi [10].

In spite of several discovered antimicrobial agents, recently, development of new antimicrobials has increased remarkably. And, the need for new antimicrobials versus increasing resistance among microorganisms is showing an increase.

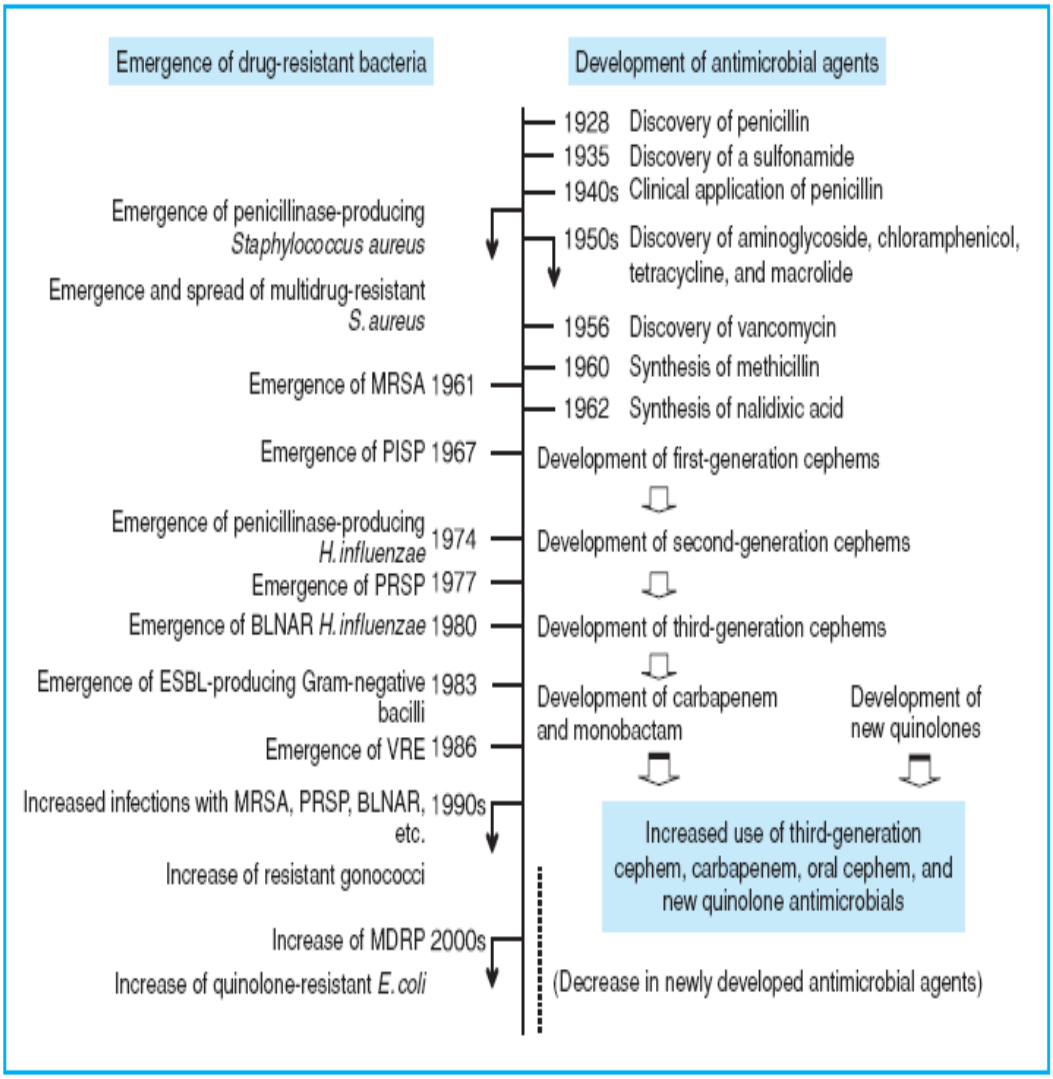

Figure 1.1 Development of antimicrobial agents and emergence of drug resistance in bacteria [4].

1.2 MECHANISMS OF ACTION OF ANTIMICROBIALS

An antimicrobial agent can be either bactericidal or bacteriostatic. Bactericidal refers to killing the bacteria completely while bacteriostatic refers to decreasing bacterial growth dramatically [11].

1.2.1 Action Mechanisms of Antibacterials

There are four main mechanisms of antibacterial agents all of which are selectively toxic [12]. These agents can inhibit; (i) bacterial cell wall synthesis, (ii) bacterial deoxyribonucleic acid (DNA) synthesis, (iii) bacterial protein synthesis and (iv) folate synthesis.

1.2.1.1 Bacterial Cell Wall Structure and Inhibition of Bacterial Cell Wall Synthesis

Most bacteria have a cell wall surrounding their plasma membrane [13]. Many bacterial cell wall contain a substance called peptidoglycan that provides structure and durability to bacterial cell [14]. Peptidoglycan is composed of polysaccharide chains cross-linked by peptides containing D-aminoacids [15].

Bacterial cell walls are divided into two main types according their gram staining under microscope: Gram negative and Gram positive bacteria.

Gram positive bacteria cell walls contain more amount of peptidoglycan that result in deep purple colour after Gram staining while Gram negative bacteria cell walls contain less peptidoglycan causing poorly red colour.

Gram negative bacteria have a lipid layer different from Gram positive bacteria. This lipid layer facilitates an extra advantage for escaping from body's immune system and antibiotic diffusion [11].

The human eukaryotic cells do not contain a cell wall or peptidoglycan. This characteristic makes bacterial cell wall an ideal target for therapy, as an antibacterial acting cell wall do not target human cells which is lack of cell wall [14].

Peptidoglycan is the basic component of the bacterial cell wall. Penicillins and cephalosporins (beta-lactam antibiotics) shut off peptidoglycan synthesis. These antibiotics bind penicillin binding proteins which are all involved in the final stages of the synthesis of peptidoglycan and cause formation of a deficient cell wall structure that results in bacterial cell burst [16, 12].

1.2.1.2 Inhibition of Bacterial DNA Synthesis

DNA replication is required for bacterial cells to multiply and produce new bacterial cells. Some of antibiotics such as quinolones, metronidazole, nitrofurantoin and rifampicin act by inhibiting DNA replication. These antibiotics have to have a highly selective toxicity for only bacterial DNA synthesis, not for human DNA synthesis.

The quinolones interfere with DNA synthesis by inhibiting enzymes which are required for the synthesis [12, 16]. Rifampicin inhibits enzymes taking a role in messenger RNA synthesis. Metronidazole is effective only on anaerobic bacteria and acts by disrupting DNA via some chemical reactions [12].

1.2.1.3 Inhibition of Bacterial Protein Synthesis

Ribosomes, which are organels responsible for protein synthesis of cell, exist in both human and bacterial cells. But, bacterial ribosomes are much more different than those of eukaryotics [11, 12]. Protein synthesis inhibiting antibiotics have more affinity to bacterial ribosomes than to those of humans [12].

Aminoglycosides, tetracyclines and macrolides are main examples of protein synthesis inhibitors [14]. Aminoglycosides display their inhibitor effect by causing misreading of mRNA code therefore resulting in dysfunctional protein. Tetracyclines block transfer RNA molecules and tRNA can not transport essential amino acids for protein synthesis. Macrolides bind to ribosomal subunits and interfere with them to function for protein synthesis [12].

1.2.1.4 Inhibition of Folate Synthesis

Folate is a compulsory component of DNA synthesis process. Bacteria produces their own folate in contrast to human [12]. Some antibiotics such as trimethoprim and sulphonamides interfere with DNA synthesis by inhibiting folate synthesis in bacteria.

1.2.2 Action Mechanisms of Antifungals

Initial drugs in the clinical treatment till 1970s were **two polyene**: amphotericin B and nystatin, **potassium iodide** (1950s), **flucytosine** (1964), which have been used for a long time [17]. But, because of the significant increase in the systemic and life-threatening fungal infections in 1980s caused by wide use of broad spectrum antibiotics, the need for new antifungal agents, especially to treat increased fungal infections correlated with increased numbers of immunosuppressed people, have rised [17, 18]. Since this era, scientists have developed several antifungal agents, having different mode of action mechanisms (MAM) , most are associated with the cell envelope (cell wall and plasma membrane).

1.2.2.1 Fungal Cell Wall Structure and Inhibition of Cell Wall Synthesis

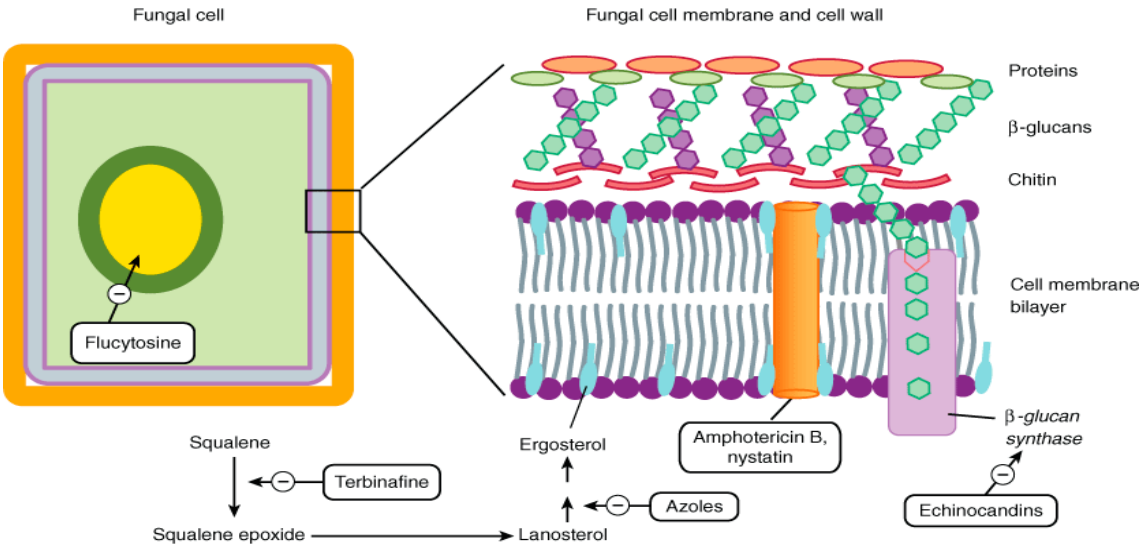

Figure 1.2 Fungal cell wall structure and action mechanisms of antifungals [135].

Fungal cell walls possess a multilayered configuration composed of a chitin layer, a mannoproteins layer and a β-glucan layer (Figure 1.2). β-glucan and mannoprotein layers forms 80 % of the cell wall mass [19, 20].

Mannan, mannoprotein, and β-(1,6)-glucan compose the outer layer, while β-(1,3)-glucan and chitin with some mannoproteins compose the inner layer [21].

Antifungals as inhibitors of cell wall biosynthesis are selective only for fungal cells [22, 17]. Antifungals may target different components of the cell wall such as chitin, mannoprotein and glucan, which are unique to the fungal cells [23]. Consequent defective cell wall give rise to rupture in yeasts and aberrant hyphal growth in molds [24].

1.2.2.1.1 Inhibition of Glucan Synthesis

Glucan is an essential component of the cell wall for its physical features [25]. The most known example of glucan synthesis inhibitors are echinocandins [26]. They act by inhibiting β-1,3-D-glucan synthase which is glucosyltransferase enzyme involved in the generation of beta-glucan of cell wall in fungi [27, 22]. Therefore**,** cells become osmotically sensitive [28, 29].

1.2.2.1.2 Inhibition of Chitin Synthesis

Chitin is one of the microfibrillar components of the cell wall and is made of β-(1,4)-linked N-acetylglucosamine. This structure maintains morphological shape of the cells and strengthens the wall by linking to the glucan. Nikkomycins and polyoxins are the classical inhibitors of chitin synthesis. They function as substrate analogues of UDP-N-acetylglucosamine which is essential for chitin biosynthesis [17]. In addition, some novel antifungals prevents chitin synthesis by inhibiting chitin synthase [30].

1.2.2.1.3 Inhibition of Mannoprotein Synthesis

Mannoproteins play important role in the function of the cell membrane. They contain as much as 50 % carbohydrate. Mannoprotein inhibitors interact with saccharide side of mannoproteins and cause disruption of the cell membrane and leakage of intracellular potassium [17].

1.2.2.2 Inhibition of Sphingolipid Synthesis

Sphingolipid synthesis is a process that occurs in both human and fungus. However, there are some enzymes that are unique for fungal sphingolipid synthesis. Those enymes make fungal sphingolipid synthesis as a target for antifungal agents. Three key enzymes in this pathway are serine palmitoyltransferase, ceramide synthase and inositol phosphoceramide synthase. Inhibition of these key enymes result in interruption of growth and ultimately cell death [17, 31, 32]. Some known examples of antifungals as enzyme inhibitors are (i) sphingofungins, lipoxamycin and viriofungins for serine palmitoyltransferase inhibition; (ii) fumosin B1 and australifungin for ceramide synthase; (iii) khafrefungin, australifungin and rustmicin for inositol phosphoceramide synthase [17, 31, 32, 33, 34, 35, 36, 37, 38, 39].

1.2.2.3 Inhibition of Protein Synthesis

A great degree of similarity presents between human and fungal protein synthesis mechanisms due to eukaryotic nature of fungi. Some antifungals such as sordarins target elongation factors, that are essential for protein synthesis, which are distinct from its mammalian counterparts [17, 40, 41, 42]. Sordarins interact with EF2 (elongation factor 2) and inhibit translation by stabilizing fungal EF2 complex [41, 42, 43].

1.2.2.4 Disruption of Integrity of the Cell Membrane

Ergosterol is the major sterol of fungal cell membrane. It has a cylindrical three-dimensional structure unlike cholesterol in the human cell membrane which has a sigmoid shape. This conformational difference provides polyenes (such as amphotericin B and nystatin) high affinity for ergosterol instead of human cell membrane sterol.

Polyenes interact with fungal membrane sterols and form aqueous pores consisting of an annulus of amphotericin B molecules linked to the membrane sterols [44, 45]. This formation alters membrane permeability and results in leakage of cytoplasmic components and cell death [46, 47].

The ergosterol biosynthesis pathway is the target for azoles and allylamines. It is required for the integrity of the cell membrane whose sterols are lack of methyl groups [48]. Azoles inhibits cytochrome P-450-dependent demethylation of membrane sterols

leading to ergosterol deplition that cause the alteration of the structure and function of the plasma membrane [49]. Furthermore, some azole derivatives demonstrate different action mechanisms through inhibiting membrane-bound enzymes [50, 51]. Allylamines act by inhibiting early steps of ergosterol biosynthesis that cause accumulation of the sterol precursor squalene [46]. The accumulation of squalene increases membrane permeability and cause disruption of cellular organization [52, 53].

1.2.2.5 Some Other Mechanisms

1.2.2.5.1 Inhibition of DNA and RNA Synthesis

5-Fluorocytosine is a fluorinated pyrimidine used as an antifungal. It is converted to 5-fluorouracil (5FU) in the cell once it enters fungal cell. Fluorouracil is incorporated into RNA and distrupts protein synthesis via causing premature chain termination [54]. And 5FU is also an inhibitor of thymidylate synthase which is an essential enzyme for DNA synthesis and nuclear division. Thanks to this, it prevents DNA synthesis, as well [55].

1.2.2.5.2 Microtubule Assembly

The first antimicrobial agent against fungus was griseofulvin of which MAM is interfering with microtubule assembly [56]; benzimidazole was another example of drugs having this kind of MAM [27].

1.2.2.5.3 Others

Coumarine derivatives induce apoptosis by disrupting cytochrome biosynthesis [57]. Indol-3-carbinol displays candidacidal effect by binding fungal DNA [58].

Cruentaren inhibits mitochondrial ATPase activity [59]. Fatty acids such as 6-acetylenic acids blocks beta oxidation pathway [60].

1.3 RESISTANCE

A microorganism is named resistant when it grows in a higher concentration relative to normally effective concentration of an antimicrobial agent [61]. Microbial sensitivity against an antimicrobial is determined by standardized methods that reveal minimum inhibitory concentration (MIC) of agents for microorganism. If defined new breakpoint is above standard MIC values, microorganism is considered as resistant for this antimicrobial agent.

Resistance can be either intrinsic or acquired. Becoming intrinsic of a resistance means presence of the resistance mechanism in all members of a given genus or species while becoming acquired of resistance means presence of the resistance mechanism only in certain isolates of the same genus or species [62].

1.3.1 Resistance in Bacteria

Bacterial genome contains a single mostly circular chromosome and non-chromosomal (accessory) genetic elements. The genetic information required for the life cycle of the bacterium presents in the chromosome while dispensable information genes are carried in the accessory elements (plasmid). An accessory element can carry an antibiotic resistance gene and, as a consequence, can provide an advantage for survival of the bacteria [62]. Hence, bacteria can develop resistance against antibiotics because of mutations in their chromosome or acquisition of a resistance gene via (i) transduction (transfer of a bacterial DNA using a bacteriophage vector and then incorporation of the DNA into host chromosome), (ii) transformation (incorporation of chromosomal DNA, plasmids and other DNAs from dying organisms into the host chromosome), or (iii) conjugation (via plasmids and conjugative transposons) [63, 61]. A mutation can occur in the bacterial chromosome, hence, can give rise to resistance versus antibiotic. This type of resistance can result from an alteration in targets or regulatory pathway components [62].

An alteration occured in the nucleotide sequence of a structural gene which is responsible for the production of a protein, target for certain antibiotic, can result in transformation of the target protein and so can put antibiotic out of action. This kind of resistance acts reversibly and it is revealed when antibiotic presents in the environment

[62,64]. An alteration occured in the motifs for gene expression such as promoters or in regulatory modules can result in alterations that cause resistance emergence. Moreover, some house-keeping genes can generate antibiotic resistance when overexpressed. This kind of resistance occurs independent from the presence of antibiotic [62,64].

Mutations in the chromosomal gene specifying a target enzme can result in overexpression of an enzyme with a reduced affinity for antibiotic [64].

Gene amplification can cause to overexpression of multidrug transporters and drug targets [65]. This type of resistance becomes unstable and is reverted in the absence of drug [66]. Both of intrinsic and acquired resistance mechanisms can affect the same resistance pathways. Types of resistance mechanisms can be listed as (i) the inactivation of antibiotics, (ii) the prevention of antibiotic to reach its target and (iii) the modification of the target.

1.3.1.1 Antibiotic Inactivation

The inactivation of antibiotic prior to reach the target site occurs by means of enzymatic modifications of antibiotic. Some microorganisms produce enzymes that detoxify the drug and inactivate it by cleveage or produce enymes, such as acetyltransferases, phosphotransferases, nucleotidyltransferases, that modify chemical structure of the drug [67]. In both cases antibiotic's capacity to bind to its target can be loosed or reduced [62].

*1.3.1.2 Prevention of Access to the Targ*et

Preventing the drug to reach its target site occurs via either reduced rates of entry or removel of the drug. There are two main mechanisms taking a role in such issue as follows:

A. Porins: The outer membrane of Gram negative bacteria function as a permeability barrier and allows only passive diffusion of some compounds through channels called porins [61, 67]. Mutations can alter membrane permeability by reducing number and/or size of porins [62, 61], and, as a result, can slow down the penetration process of the drug [67].

***B. Membrane Based Efflux Pumps*:** Pomping antibiotics out of the cell via efflux pumps can result in resistance. Efflux pumps are protein channels that export molecules such as antimicrobials out of the cell and function as kidney of the bacterial cell [68]. While exporting, pumps use ATP hydrolisis and/or an ion antiport system as energy sources. Through efflux system, cells limit intracellular antibiotic accumulation and decrease efficacy of even structurally unreleated antibiotics. Efflux pumps are responsible for species- or genus-specific intrinsic antibiotic resistance and also responsible for acquired cross-resistance to several antibiotics via overproduction of the pumps. Efflux system pumps can be either drug–specific or multidrug resistant. Multidrug resistance efflux pumps are usually encoded by the chromosome while drug-specific efflux pumps are encoded by plasmids and hence, drug-specific resistance is transmissible via plasmids. The expression of the genes encoded by plasmids usually becomes sufficient for emergence of resistance without any additional mutations. In contrast, multidrug resistance encoded by chromosome rises mostly owing to increased gene expression as a result of substrate induced transcriptional activation, gene amplification or the occurrence of regulatory mutations [62].

1.3.1.3 Alteration of the Target

The third mechanism of the resistance is alteration of the drug target. This modification can accomplish with alteration of usual target or acquisition of new unsusceptible target. Both lead to lossed or decreased affinity of the drug against its target without interfering with the physiological function of the target [67, 61].

Exchanging residues within the target molecule result in modified target that cause low affinity or no affinity. In some cases, microorganisms can acquire a new unsusceptible target gene or a new target gene with low affinity via genetic mobile elements [67, 61].

1.3.2 Resistance in Fungus

Antifungal resistance can be intrinsic or acquired in fungi, as well. Intrinsic resistance exists naturally without prior presence of the drug while acquired resistance are developed in previously susceptible fungi after they are exposed to the antifungal agent. And, it comes out mostly as a result of altered gene expression [24, 69] because it

has been shown that the appearance of mutations and rearrangements in fungal cell is induced by antifungal pressure [70]. More than one resistance mechanism can function in a single fungal strain. The mechanisms that can function in fungal cells are (i) the alteration of the target, (ii) the prevention of access to the target, (iii) the biofilm formation, and (iv) the development of bypass pathways.

1.3.2.1 Alteration of the Target

Ergosterol is required for membrane fluidity and asymmetry and, consequently, membrane integrity in fungal cells [48]. For the integrity of the cell membrane, inserted sterols must lack of methyl groups. Lanosterol is a precursor of sterol and demethylation of lanosterol, to get functional sterols, is catalyzed by p450-dependent Erg11 protein which is an important enzyme of ergosterol biosynthesis. Mutations in Erg11 genes result in altered protein and prevent binding of drug to enzymatic site of the altered protein or decrease the affinity between drug and protein [71, 72, 73].

Besides alteration of the target, some fungi overwhelmes routine effective concentration of the drug by up-regulating the target enzyme via gene amplification, increased transcription rate, or decreased degradation of the gene product. Hence, the antifungal can no longer effectively inhibit fungal growth [24, 74].

1.3.2.2 Prevention of Access to the Target

As in the case of bacteria, fungi can improve resistance to some antifungals via up-regulation of efflux pumps in the cell membrane, therefore, decrease drug concentration and prevent the drug to reach its target [75, 76].

1.3.2.3 Biofilm Formation

Biofilm is a kind of dense network layer of extracellular matrix formed by differentiated cells on synthetic or natural surfaces. Biofilms can make up a physical barrier against penetration of antifungals and result in resistance to many unrelated antifungals except caspofungin. It has been shown that measurements of drug susceptibilities of microorganisms in biofilms revealed high MIC values for some antifungals [77, 78, 79]. Recent studies about molecular basis for resistance suggest that biofilms contain variable proportions of more tolerant phenotypic variant cells [80, 70].

These cells can break free from drug action without using resistance mechanisms [81]. Moreover, biofilms possess a heterogenous cell population which has cells with different transcriptional activity of genes involved in resistance [82, 83]. And also, drugs can be holded in the matrix of biofilms and so inhibitory effects of drugs can be put out of action [84].

1.3.2.4 Development of Bypass Pathways

In some instances, drug is metabolized by fungal cellular enzymes and toxic metabolites are formed after this process. Accumulation of toxic compounds leads to growth arrest [85]. However, when mutations in the genes that encoding enzymes that are responsible for drug metabolization occur, they prevent the conversion of the drug into their toxic metabolite forms [86]. Therefore, fungal cells that are unable to produce toxic metabolites acquire resistance against drugs.

1.4 INCREASING NEED FOR NEW ANTIMICROBIALS

The need for discovery of new antimicrobials is increasing day by day based on (i) increasing resistance against antimicrobial agents and (ii) decreasing discovery and production of drugs by large companies.

1.4.1 Resistance Problem

Microorganisms have ability for adaptation and development of resistance to antimicrobials [12]. Soon after entry of an antimicrobial into clinical use, resistance emergence for that drug has started to be reported.

Antibiotics are grouped based on their chemical structure. So, members of the same class are closely similar molecules and generally possess the same target and action mechanisms [62]. As a consequence, they are subject to cross-resistance, once resistance was developed against a member of the class or family through target alteration and drug detoxification mechanisms. In this way, microorganisms develop multi drug resistance (MDR), where bacteria are resistant to several different classes of antibiotic agents [62, 12]. And, after a short time, efforts can become insufficient due to

MDR although pharmaceutical companies have responded with new generations or new classes of drugs following identification of resistance to a drug class or a single drug [87].

As mentioned before, resistance can present inherently or can be acquired via horizantal gene transfers by mobile genetic elements (e.g. plasmids, bacteriophages) from different strains. The microorganisms that gained some genetic characteristics, account for their survival, transmit these characteristic determinants to their off springs. It has been shown that the transmission of resistance determinants can occur not only from mother to offsprings but also from Gram positive bacteria to Gram negative bacteria (for example: gene transfer by conjugation from Gram positive cocci to Gram negative bacteria) [88]. This issue makes difficult to counteract widespread presence of resistance and infections of resistant microorganisms.

The incidence of antifungal resistance is moderate when compared to incidence of antibacterial resistance. But, antifungal resistance is also a serious problem because of the limited number of available antifungals. Hence, the need for effective antifungals exists all the time [70].

1.4.2 Decreasing Discovery and Production of Drugs by Large Companies

After the success in commercialisation of penicillin following the Second World War, companies have spent their efforts significantly on antibiotic development for decades. But, new antimicrobial discovery and production is not enough to prevent losses arising from flow of the antibacterial resistance. Treatment for many infections will get harder and harder if the pharmaceutical industry doesn't produce a constant flow of effective new agents [90,91]. However, large pharmaceutical companies either plan to spin off their antimicrobial drug-discovery and development programs or have already spun off into smaller pharmaceutical companies (Figure 1.3) . Most of them entered into collaboration with other antibiotic producer companies instead of creation of new antimicrobials themselves. The view of companies is that profits from antimicrobials are not worth the effort [91]. This view is formed depending on (i) increasing regulatory requirements leading to larger drug trials, (ii) a decrease in the discovery rate of new broad antimicrobials with novel mechanisms of action, (iii) the limited effective life of an antimicrobial because of the emergence of antimicrobial

resistance and (iv) the raising of the bar for the statistical standards needed to show the efficacy of experimental drugs in clinical trials [90,91].

Dissemination of resistance is closely tied to widespread use of antibiotics. It has revealed that antibiotics, notably in the subinhibitory concentrations, can induce modulation of resistance gene expression, promote intracellular or intercellular movements of resistance genes within bacteria, lead to appearance of mutations in fungi and bacteria [62].

Limitation of antimicrobial efficacy through resistance, increased resistance incidence due to widespread use of antimicrobials, rapidity and proliferation of the resistance emergence expose a serious public health concern [12] and especially multi-drug resistant microorganisms infections demand continuous discovery of new antimicrobials [62, 87].

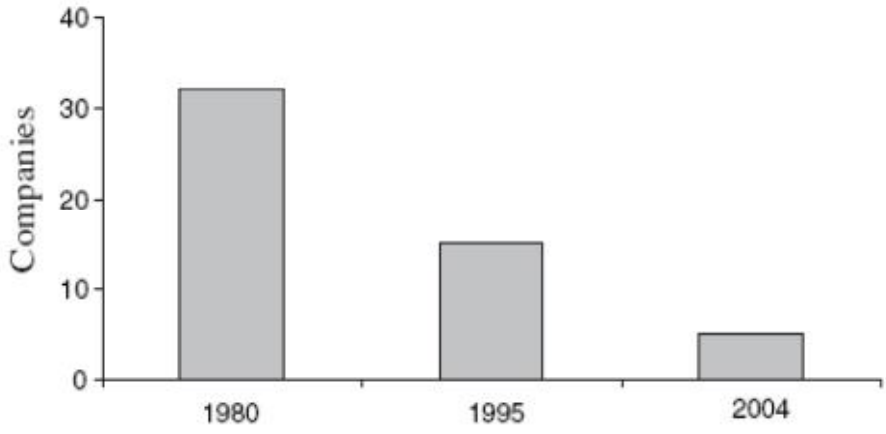

Figure 1.3 Contraction of the pharmaceutical industry in the antimicrobial drug marketing with time [89].

Development of a drug by a company takes approximately ten years. Since developer companies have less time to recoup their research and development studies, shorter lifecycles of drugs due to microbial resistance increases the risk to drug developers. And, since some governments have limited the antibiotic use to fight

resistance issues, these shorter lifecycles restricts sales [90]. All of these factors have shifted big companies to spend their research dollars in more productive ways.

Based on 2006 datas, the number of new antibacterials reaching the drug market has fallen by over 50 % in the last 20 years while resistance among microorganisms are increasing continuously [91]. This number is as critical as, in some recent years no or only one antimicrobial were launched although there are lots of pharmaceutical companies in the drug market. Nowadays, smaller firms have been taking over the drug discovery and development function while big pharma companies have been carrying on their presence in the sector by either acquiring producer companies or inlicensing compounds. Therefore, decreased efforts on antimicrobial drug discovery versus increasing resistance presence are resulting in increasing need for new antimicrobials [90].

1.5 DRUG DELIVERY AND DENDRIMERS

Dendrimers (Dendritic polymers) are described as macromolecules having regularly branched unique 3D structures that provide a high degree of surface functionality [92]. They are entirely defined, 5-10 nanometer sized nanomolecules [92].

1.5.1 Structure of Dendrimers

Dendrimers consist of three different parts: (a) a focal core to which elements are added by repeating serious of chemical reactions, (b) interior layers (generations) or building blocks composed of repeated units and (c) peripheral functional groups (Figure 1.4). The focal core encapsulates various chemicals, demonstrating unparalled futures due to special nanoenvironment surrounded by dendritic branchings. The interior layers consist of repeating units provide a flexible space which will serve to encapsulate small guest molecules. The multivalent functional surface by functional groups interacts with external environment and defines dendrimer's macroscopic properties [93, 94].

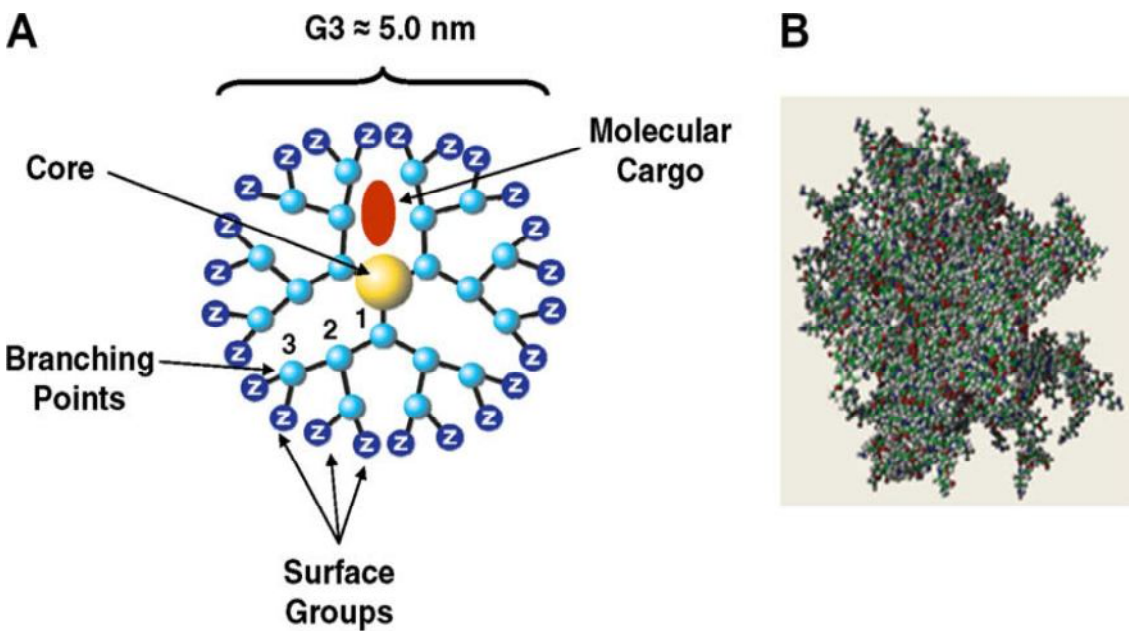

Figure 1.4 (A) Schematic presentation of a dendrimer G3 containing three generations (B) 3D presentation of dendrimer G3 [131].

Generation is the hyperbranching from the centre of the dendrimer to the outer and results in unique structural layers between branching points. The number of branching points from center towards surface is equivalent to generation number. For instance, a dendrimer possessing four branching points from center towards surface is expressed as the 4th generation dendrimer (G4 dendrimer) and the core part of the dendrimer is expressed as generation zero (G0) [92].

The dendrimer shell is the homostructural spatial segment between branching points. The outer shell is the space between the last outer branching point and surface whereas inner shells mean the dendrimers' interior [92].

1.5.2 Properties of Dendrimers

Molecular mass and size of dendrimers can be controlled during synthesis process while the classical polimerization process produces molecules with different size and molecular mass randomly. The size of dendrimers increases regularly and ranges from several to tens of nanometers in diameter. Moreover, the ability to control dendrimer synthesis allows for site-selective functionalization of denrimer [92, 94].

Dendrimers have improved physical and chemical properties. Unlike linear polymers, they form a tightly packed ball in solution, and have predictable three-dimensional structure. Especially at higher generation dendrimers, they possess a densely packed surface consisting of a great number of functionality. Therefore, focal core is encapsulated completely and isolated from the outer environment. Lower generation dendrimers are able to do spherical formation but do not form tightly packed surface [92,94]

The intrinsic viscosity of dendrimer solutions does not increase linearly with mass, when compared with linear polymers of the same molecular weight. They display a maximum viscosity at a specific generation, after this generation, viscosity begins to decrease. This behaviour is due to that dendrimer's shape and surface compaction changes with generation. From lower towards higher generation, shape converts from more open planar-elliptical shape to more compact spherical shape [92, 94,95, 96].

As in the case of in nature which tree-like structures have evolved to maximize the exposed surface area, dendritic structure of the dendrimer allows to have very high molecular surface to volume ratios (up to 1000 m^2/g) [92].

Solubility, reactivity and binding of dendrimers are predominantly influenced by their surface groups which determine their peripheral functionalities. Dendrimers with hydrophilic terminal groups are soluble in polar solvents whereas dendrimers with hydrophobic terminal groups are soluble in nonpolar solvents. A marked difference on solubility and reactivity present between linear polymers and dendrimers. It has been shown that dendrimers have a remarkably higher solubility and chemical reactivity relative to linear polymers [92, 94].

1.5.3 Dendrimers for Different Applications

The first dendrimer was a cascade molecule and introduced into chemistry by Fritz Vogtle and coworkers in 1978 [97]. Since the first discovery, a great number of dendrimers have been developed to create well-defined nanostructures for several applications.

Thanks to multivalent and monodisperse character, dendrimers have created a wide interest in the scope of chemistry and biology. They were used in applications such as drug delivery [98, 99, 100, 101], gene delivery [102, 103], chemotherapy [104, 105], biomimicry [106, 107], photodynamic therapy and some diagnostic applications (as moleculer probes) [108, 109, 110, 111, 112, 113, 114, 115, 116] such as magnetic resonance imaging contrast agents [117, 118, 119, 120 121, 122, 123, 124, 125], X-ray contrast agents [126, 127, 128, 129].

The dendrimer sphere can be expanded to desired size by researchers. The ability to arrange dendrimer's properties according to needs makes these molecules ideal carriers for drugs with small molecule and biomolecules [92].

1.5.4 Drug Delivery

Drug discovery and development can take time up to fifteen years and cost up to $800 millions [130]. Nearly forty percent of newly developed active pharmaceutical ingredients (APIs) are being rejected by authorities because they don't become useful for human due to low bioavailability with low water solubility and/or cell membrane permeability in addition to undesired side effects. Moreover, nearly seventeen percent of launched APIs display effect under optimal levels due to poor bioavailability reasons. It has been tought that site-specific drug delivery and controllable release can allow to optimize drug concentration easily and can reduce undesired localization, therefore, increase the effectiveness of the APIs. In the circumstances, drug delivery, that enhance the bioavailability, carry weight to make problems minimum for APIs [131, 94].

To develop molecular nanostructures with well-defined particle size and shape is an important interest in drug delivery applications. Being of a construct that is in the nanometer range and uniform in size to enhance drugs ability to cross cell membranes and to reduce the risk of undesired clearance from the body through liver or spleen is a desired feature for a drug carrier [131]. In order to apply dendrimers, as a molecule carrying mentioned features, to drug delivery applications, they have to be non-toxic, non-immunogenic and have low biocompatibility. And also, they have to have an appropriate biodistribution, permeability and a tolerance against enzymatic attack in the blood system in addition to have high solubility in aqueous medium. Large dendrimers have a tightly packed surface structure and a free interior space isolated from the

external environment. Cytotoxicity of large dendrimers is predominantly determined by the nature of their surface functionalities. Dendrimers with cationic surface groups destabilize cell membranes and cause cell lysis due to the negatively charged nature of the cell membrane. Furthermore, cytotoxicity is found to be dependent on dose and generation. For example, high generation PAMAM dendrimers have been found to be cytotoxic whereas lower generation PAMAM dendrimers (below 5th generation) have not been found to be cytotoxic [94]. However, dendrimers exhibit lower toxicity than linearpolymers owing to lower adherence of the rigid globular dendrimers to cellular surfaces [132, 94].

1.5.4.1 Mechanisms of Drug Delivery by Dendrimers

Dendrimers offer a very high drug loading capacity and owing to this feature, they are used in drug delivery. Two dendrimer drug delivery mechanisms present: encapsulation of drugs (via physical association) and dendrimer–drug conjugates (via chemical conjugation) [92, 131].

Physical association of drugs or APIs occurs through encapsulation into void spaces (nanoscale container), association with surface groups (nano-scaffolding) or a mixture of both. Small drug molecules are mostly encapsulated into the void space of dendrimers' interior, while larger molecules are mostly adsorbed onto the dendrimer surface [131]. Once deprotection of terminal functionalities occurs, shell structure on the surface opens and guest molecules are allowed to come out [94].

The physical interactions between dendrimer and drug (i) do not alter the chemical structure of drug and so provide a less challenging regulatory path forward; (ii) limit control over release kinetics; (iii) offer a limited drug loading capacity resulting in low drug-to-dendrimer ratio [131].

Formation of dendrimer-drug conjugates via chemical bonding is achieved by three main pathways: (i) conjugation of drugs to the dendrimer surface directly; (ii) conjugation through linker molecules and (iii) becoming an integral part of a dendrimer [131].

Conjugation is achieved via linker molecules in the case of that (i) drug doesn't contain the functional groups required for direct conjugation or (ii) in the presence of a need for a linker molecule to modify solubility profile or release kinetics or reduce congestion of drug molecules on the dendrimer surface which allows a higher degree of conjugation [131].

In some cases, dendrimer is released through certain events at the target site, e.g. tumor site, and drug molecule is conjugated as an integral part of this dendritic carrier [131].

The different ionic nature of the dendrimer drugs due to different functional groups result in different existing times in the lysosomes, and so, intracellular drug release varieties [133, 131].

Dendrimers rise to notice for drug delivery applications because of their well defined three-dimensional structure, the availability of many functional surface groups, their low polydispersity and their ability to mimic [92].

Dendrimers can decrease cytotoxicity, extend systemic circulatory half-life, increase solubility and taking up by cells, facilitate the passive targeting of drugs to target site and lead to the selective accumulation of macromolecules in the target site. Hence, because many drugs have hydrophobic characteristics, dendrimers are successful alternatives to increase bioavailability [92].

Research efforts on this area are focused on antimicrobial, anticancer and anti-inflammatory drugs, recently.

Objectives of the thesis: In this thesis, we would like to display effect of PAMAM dendrimers, as a solubility and cellular permeability enhancer, on antimicrobial activity of ferrocene. And, we would like to reveal antimicrobial activity of ferrocene without additional ligands unlike in the literature only by increasing its solubility in the cell and display certain cidal values of ferrocene for both some bacteria and fungi.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1 Ferrocene-Amine and Ferrocene-PAMAM Dendrimers as Test Substances

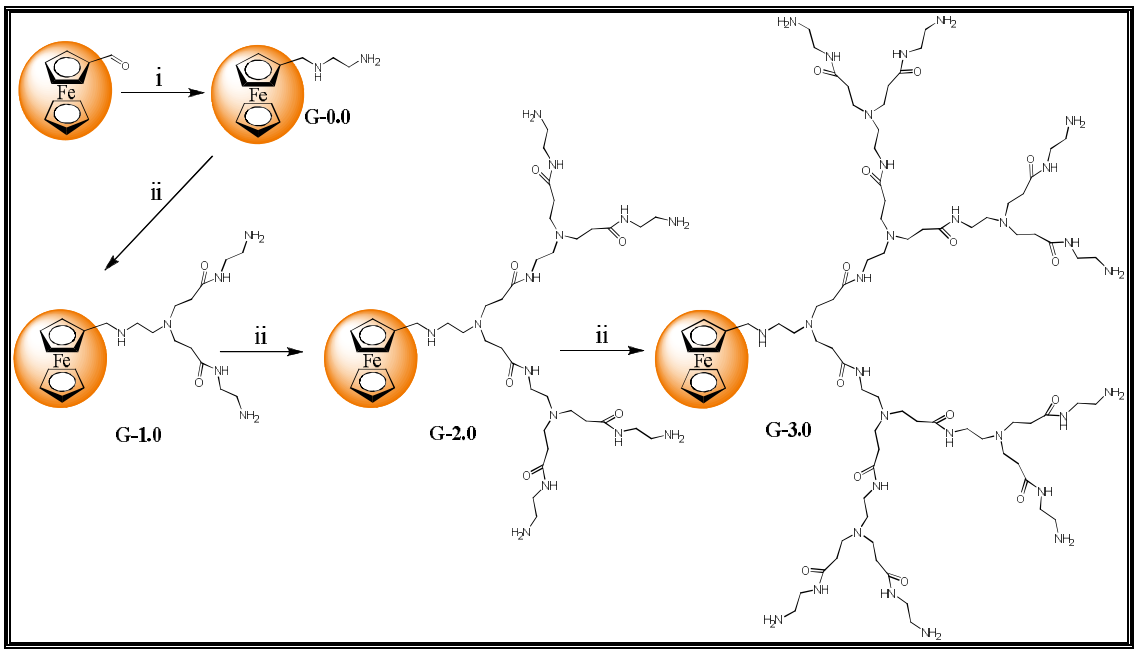

Figure 2.1 Structure of ferrocene-amine (G-0.0) and three different generations of ferrocene-PAMAM dendrimers

Ferrocene-amine, ferrocene-PAMAM (G1), ferrocene-PAMAM (G2), ferrocene-PAMAM (G3) were synthesized in the biotechnology laboratories at Fatih University (Figure 2.1).

2.1.2 Chemicals and Growth Mediums

2.1.2.1 Solvents

Dimethyl sulfoxide (DMSO) was used to solve ferrocene-PAMAM dendrimers and ferrocene-amine.

Hydrochloric acid (2M HCl) was used to solve erythromycin antibiotic according to manufacturer's (Sigma-Aldrich, USA) directions.

2.1.2.2 Antibiotics

All antibiotics as follows were used for comperative assessment of antimicrobial activities of ferrocene-PAMAM dendrimers in broth microdilution tests.

2.1.2.2.1 Antibacterials

For antibacterial activity tests, six antibiotics were used in the experiments. Five antibiotics were purchased from Sigma-Aldrich (USA) while one was from Roche (Switzerland). These antibiotics are as follows: tetracycline hydrochloride (T7660, Sigma-Aldrich), kanamycin sulfate (K1377, Sigma-Aldrich), streptomycin sulfate salt (S2522, Sigma-Aldrich), erythromycin (E5389, Sigma-Aldrich), nalidixic acid sodium (N4382, Sigma-Aldrich) and ampicillin (Roche)

2.1.2.2.2 Antifungals

For antifungal activity tests, two antibiotics were used in the experiments. These antibiotics were nystatin suspension (N1638, Sigma-Aldrich) and amphotericin B solution (A2942, Sigma-Aldrich).

2.1.2.3 Growth Mediums

2.1.2.3.1 LB Broth

LB broth was purchased from Acumedia (Product number: 7279, USA) and used to grow bacterial strains in a liquid media.

Composition of LB broth is as follows:

Enzymatic Digest of Casein…. 10 g/litre

Yeast Extract…….. 5 g/litre

Sodium Chloride..... 10 g/litre

2.1.2.3.2 Nutrient Agar

Nutrient agar was obtained from Merck (Product number: 1.05450.0500, Germany) and used to grow bacterial strains on a solid media.

Composition of nutrient agar is as follows:

Peptone from meat…… 5.0 g/litre

Meat extract…….. 3.0 g/litre

Agar–agar…….... 12.0 g/litre

2.1.2.3.3 Sabouraud 4 % Dextrose Agar

Sabouraud 4 % dextrose agar was purchased from Merck (Product number: 1.05438.0500) and used to grow fungal strains on a solid media.

Composition of sabouraud 4 % dextrose agar is as follows:

Peptone from meat…… 5.0 g/litre

Peptone from caseine…… 5.0 g/litre

D (+) Glucose…….. 40.0 g/litre

Agar-agar …… 15.0 g/litre

2.1.2.3.4 Sabouraud 2 % Dextrose Broth

Sabouraud 2 % dextrose broth is purchased from Merck (Product number: 1.08339.0500) and used to grow fungal strains in a liquid media.

Composition of sabouraud 2 % dextrose broth is as follows:

Peptone from meat…… 5.0 g/litre

Peptone from caseine…… 5.0 g/litre

D (+) Glucose…….. 20.0 g/litre

2.1.2.4 Turbidity Standard

The performance of susceptibility testing requires the use of standard inocula. For this purpose, 0.5 McFarland standard solution which was used as a turbidity standard for preparation of microbial inocula/suspension equal to desired cell number (10^8 CFU/mL) was manually prepared in the laboratory. The chemicals required for its preparation were obtained from Sigma-Aldrich (USA). The standard was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4). Then, it was stored in the dark and at room temperature until its usage.

2.1.3 Equipments and Instruments

In order to determine breakpoints for antimicrobials and ferrocene-PAMAM dendrimers by utilizing optical density values of them, a microplate reader device (BioTek, Power Wave XS, USA) was used.

Sterile, polystyrene, clear 96 well microplates (Greiner bio-one, cell star, Germany) with flat bottom and lid were used for broth microdilution tests.

2.1.4 Microorganisms

2.1.4.1 Bacterial Strains

In order to determine the antimicrobial activity of test compounds, five Gram negative (*E.coli, K.pneumoniae, C.freundii, P.aeruginosa, P.mirabilis*) and two Gram positive (*S.aureus, B.subtilis*) pathogenic bacteria were used in the tests. Their strain numbers are as in the Table 2.1.

Table 2.1 Bacteria used in antimicrobial activity tests and their strain numbers.

	Microorganism	**Strain Number**
1	*Escherichia coli*	JM 103
2	*Staphylococcus aureus*	ATCC 29213
3	*Pseudomonas aeruginosa*	ATCC 27853
4	*Citrobacter freundii*	NRRL B-2643
5	*Klebsiella pneumoniae*	ATCC 4352
6	*Bacillus subtilis*	ATCC 6633
7	*Proteus mirabilis*	CCM 1944

Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Bacillus subtilis strains were supplied by Veterinary Faculty of Istanbul University (Istanbul, Turkey) while *Citrobacter freundii and Escherichia coli* were supplied by Inonu University (Malatya, Turkey).

2.1.4.2. Fungal Strains

In order to determine the antimicrobial activity of test compounds, three pathogenic fungi (yeast) (*C.albicans, C.utilis, C.neoformans)* were used in the tests. Their strain numbers are as in the Table 2.2.

Table 2.2 Fungi used in antimicrobial activity tests and their strain numbers.

	Microorganism	**Strain Number**
1	*Cryptococcus neoformans*	ATCC 90112
2	*Candida albicans*	ATCC 10231
3	*Candida utilis*	KUEN 1029

Cryptococcus neoformans and *Candida utilis* strains were purchased as liyofilized powders from KUKENS (Istanbul University) while *Candida albicans* strain was supplied by Veterinary Faculty of Istanbul University.

2.2. METHODS

2.2.1 Determination of Antimicrobial Activities of Ferrocene-PAMAM Dendrimers and Positive Control Chemicals

2.2.1.1 Preparation of Stock Solutions

Different volumes of the stock solutions were stored at -80 °C in cryogenic vials without the loss of activity for up to 6 months.

2.2.1.1.1 Preparation of Antibiotic Stock Solutions

The antibiotic stock solutions were prepared to a 10 times higher concentration of the antibiotics that would be tested and when they would be used, diluted to appropriate concentration in broth. While preparing stock solutions of antibiotics, manufacturer's guideline regarding antibiotic were followed. Demineralized water as a solvent was used for the preparation of tetracycline hydrochloride (T7660, Sigma-Aldrich), kanamycin sulfate (K1377, Sigma-Aldrich), streptomycin sulfate salt (S2522, Sigma-Aldrich), ampicillin (Roche) and nalidixic acid sodium (N4382, Sigma-Aldrich) stock solutions. 2M hydrochloric acid was used for the preparation of erythromycin (E5389, Sigma-Aldrich) stock solution. Hydrochloric acid was used in a minimal amount by adding dropwise to erythromycin powder untill the powder dissolved and further dilutions of erythromycin were made with water to not to effect antimicrobial activity of the antibiotic.

Antibiotic powders obtained from the manufacturer were not 100 % pure. So the amounts of the antibiotic powders for desired volume were calculated following formula:

$$\text{Weight (mg)} = \frac{\text{volume (mL)} \,.\, \text{desired concentration } (\mu\text{g/mL})}{\text{antibiotic potency } (\mu\text{g/mg})} \qquad (2.1)$$

The needed antibiotic powders were weighed and mixed with the proper solvent via vortex mixer till dissolved. After dissolved, antibiotics sterilized by using sterile 0.20 µm cellulose acetate membrane filters. Then, the prepared stocks in cryogenic vials were labeled with the antibiotic's name and concentration, then, stored at -80 °C till their usage.

2.2.1.1.2 Preparation of Microorganism Stock Solutions

In order to prolong their usage time, stock cultures of bacteria and fungi were prepared. Bacterial culture stocks including *E.coli, K.pneumoniae, C.freundii, P.aeruginosa, P.mirabilis, S.aureus, B.subtilis* were prepared in 50 % glycerol and LB broths while fungal culture stocks including *C.albicans, C.utilis, C.neoformans* were prepared in 50 % glycerol and sabouraud 2 % dextrose broth by inoculation of the fresh culture at a fifty fifty ratio. The prepared stocks in cryogenic vials were labeled with microorganism's name and concentration, then, stored at -80 °C till their usage.

2.2.1.2 Preparation of Growth Mediums

2.2.1.2.1 Preparation of LB Broth (Lennox L Broth)

20 grams of the medium was weighed and dissolved in one liter of demineralized water by heating in a boiling water bath, then, autoclaved at 121 °C for 15 minutes. It was stored at +4˚C till its usage.

2.2.1.2.2 Preparation of Nutrient Agar (Merck)

20 grams of the medium was weighed and dissolved in one liter of demineralized water by heating in a boiling water bath, then, autoclaved at 121 °C for 15 minutes. After it cooled enough to about 45 °C it was poured into petri plates. Petri plates were kept at room temperature for 24 hours to ensure absence of a possible contamination. Then, if contamination were observed on the plates, contaminated ones were discarded and sterile ones were kept at +4 ˚C till further usage.

2.2.1.2.3 Preparation of Sabouraud 4 % Dextrose Agar (Merck)

65 grams of the medium was weighed and dissolved in one liter of demineralized water by heating in a boiling water bath, then, autoclaved at 121 °C for 15 minutes. After it cooled enough to about 45 °C, it was poured into petri plates. Petri plates were kept at room temperature for 24 hours to ensure absence of a possible contamination. Then, if contamination were observed on the plates, contaminated ones were discarded and sterile ones were kept at + 4 °C till further usage.

2.2.1.2.4 Preparation of Sabouraud 2 % Dextrose Broth (Merck)

30 grams of the medium was weighed and dissolved in one liter of demineralized water by heating in a boiling water bath, then, autoclaved at 121 °C for 15 minutes. It stored at +4 °C till its usage.

2.2.1.3 Broth Microdilution Susceptibility Testing

NCCLS standards (M27-A3, M07-A8) were utilized for broth microdilution susceptibility tests.

2.2.1.3.1 Preparation of Microbial Suspensions

Direct colony suspension method was used to prepare all microorganism suspensions.

The required cell concentrations in the microbial suspensions were adjusted by the dilution of the microbial suspensions equal to 0.5 McFarland turbidity standard. In order to obtain equal concentration, firstly optical density values of the initial microbial suspensions were adjusted to optical density value of 0.5 McFarland turbidity standard via making direct colony suspension.

2.2.1.3.1.1 Adjustment of Fungal Suspensions to 0.5 McFarland Standard

A 0.5 McFarland standard is equivalent to $1–5\times10^6$ CFU/mL for yeasts. Fungi to be tested were streaked at least twice onto a sabouraud dextrose agar plate before susceptibility testing is performed to ensure the purity and viability of the test organisms.

All microorganisms were incubated at a proper temperature for their growth during their proper time period as shown in the Table 2.3.

Table 2.3 Incubation times and temperatures for the test fungi.

Microorganism	**Incubation time**	**Incubation temperature**
Cryptococcus neoformans	48 hours	35 °C
Candida albicans	24 hours	35 °C
Candida utilis	24 hours	35 °C

After the purity of the microorganisms was ensured, , 4-5 colonies which are bigger than 1 mm-diameter on agar plate were transfered into sabouraud 2 % dextrose broth and mixed with a vortex mixer in order to prepare direct colony suspension. Colonies were added into this suspension till the turbidity of the inoculums was adjusted to the density of a 0.5 McFarland standard at 530 nm wavelength. The comparison of the turbidity belong to the standard and inoculum suspensions was done by measuring their optical densities in a microplate reader (BioTek, Power Wave XS). After fungal suspensions were adjusted to 0.5 McFarland turbidity standard, the test suspension was prepared by making a 1:100 dilution of prepared suspension which was equivalent to approximately $1–5\times10^6$ CFU/mL, then, followed by a 1:20 dilution with sabouraud 2 % dextrose broth to obtain an 5×10^2 to $2{,}5\times10^3$ CFU/mL fungal suspension as shown in the Figure 2.2. Prepared fungal suspensions with this cell concentration were used in broth microdilution tests immediately.

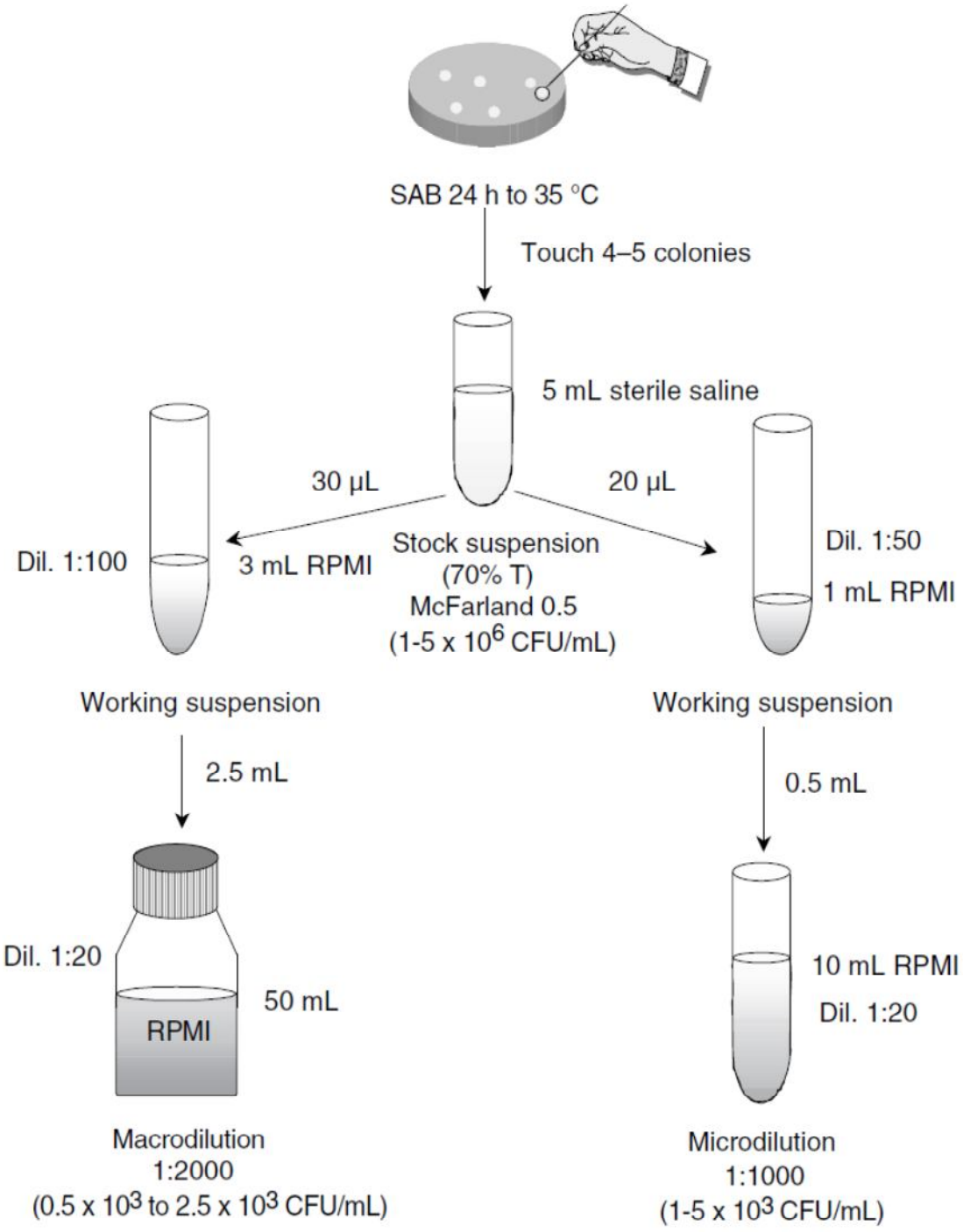

Figure 2.2 Fungal inoculum preparation for broth microdilution method [134].

2.2.1.3.1.2 Adjustment of Bacterial Suspensions to 0.5 McFarland Standard

A 0.5 McFarland turbidity standard correspond to approximately 1×10^8 CFU/mL for bacteria. The density of cell populations in the bacterial suspensions used in broth microdilution tests were approximately 5×10^5. To ensure the purity and viability of the test organisms, bacteria to be tested were streaked at least twice onto a nutrient agar plate before susceptibility testing was performed. All microorganisms were incubated at proper temperature for their growth during a proper time period as shown in the Table 2.4.

Table 2.4 Incubation times and temperatures for the test bacteria.

Microorganism	**Incubation time**	**Incubation temperature**
Escherichia coli	24 hours	37 °C
Staphylococcus aureus	24 hours	37 °C
Pseudomonas aeruginosa	24 hours	37 °C
Citrobacter freundii	24 hours	37 °C
Klebsiella pneumoniae	24 hours	37 °C
Bacillus subtilis	24 hours	30 °C
Proteus mirabilis	24 hours	37 °C

Bacterial suspensions were also prepared as that of fungal suspensions. But, unlike the fungal suspension, after bacterial suspensions adjusted to 0.5 McFarland turbidity standard, they were only 100 fold diluted to obtain a suspension with approximately

concentration of 5×10^5 CFU/mL. Prepared bacterial suspensions with this cell concentration were used in broth microdilution tests immediately.

2.2.1.3.2 Preparation of Antibiotic and Ferrocene-PAMAM Dendrimer Dilutions

Firstly, frozen antibiotic stock solutions were thawed and diluted in broth (LB broth for bacteria, sabouraud 2 % dextrose broth for fungi) to the highest concentration that needed to test the antibiotic by following the formula:

$$M_1 . V_1 = M_2 . V_2 \quad (2.2)$$

Then, the rest of the antibiotic stock solutions were discarded and the highest concentration two fold diluted in a range of determined in the literature for each microorganisms. For two fold dilution, the steps in the Figure 2.3 were used for the preparation of all antibiotic dilutions.

One miligram of ferrocene-amine (G0), ferrocene–PAMAM (G1), ferrocene–PAMAM (G2), and ferrocene–PAMAM (G3) were weighed and dissolved in pure DMSO to obtain final concentrations of 1000 µg/ml (w/v). All samples were two fold serial diluted like in the case of antibiotic solutions.

In the experiments, each dilution was prepared two fold of the desired dilution due to after the addition of microbial suspension in the same volume into the wells of microtiter plates, the final concentration of the antibiotics will be one half of the original concentration in each well.

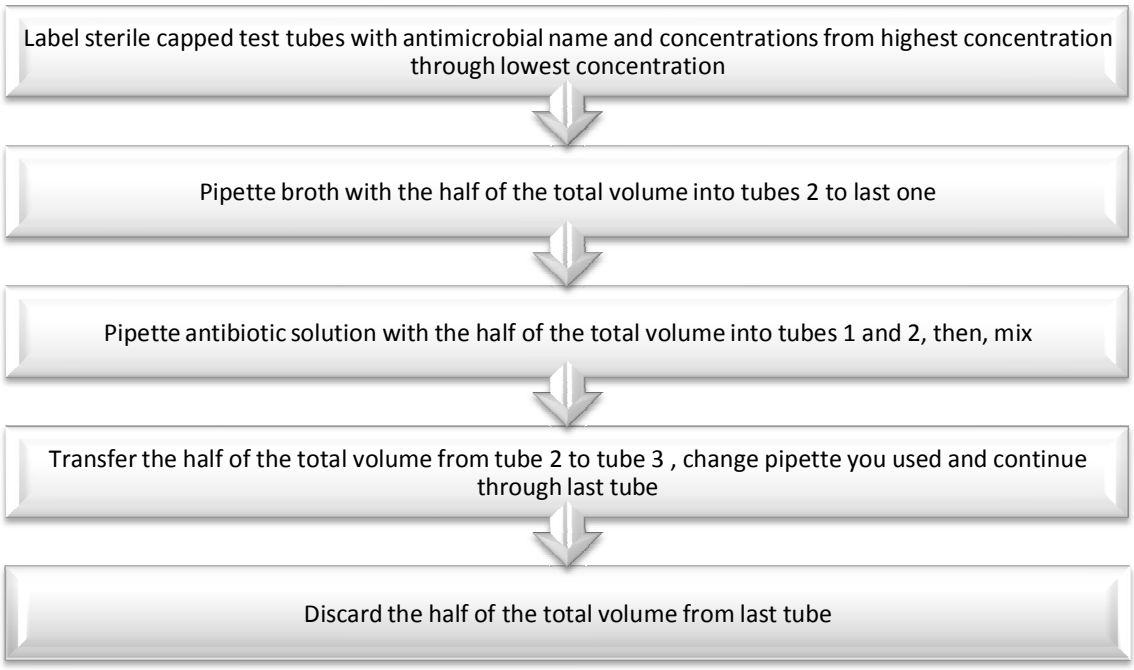

Figure 2.3 The steps of two fold serial dilution preparation of antimicrobials.

2.2.1.3.3 Preparation of 96-well Microtiter Plates for Broth Microdilution Tests

100 µl of each antibiotic and ferrocene-PAMAM dendrimer dilutions were dispensed sequentially into the bottom of each corresponding well in the microtiter plate in triplicate. Microtiter plates were stored at -80 °C till their usage. Before microtiter plate assays were carried out, frozen compounds in the microtiter plates were thawed at room temperature. Then, immediately, 100 µl of standardized bacterial or fungal suspensions were dispensed into all wells except sterility control wells in triplicate. The prepared microtiter plates were incubated at 30 °C for 24 hours for *Bacillus subtilis*, at 37 °C for 24 hours for all other bacteria, at 35 °C for 24 hours for *Candida albicans* and *Candida utilis*, at 35 °C for 48 hours for *Cryptococcus neoformans.*

2.2.1.3.4 Determination of Relative Microbial Growth

In order to determine the microbial growth, optical density (absorbance) values of microbial suspensions were utilized as an indicator of microbial growth. The optical density

of each sample in a microtiter plate was measured at 600 nm. Growth media was used as blank for the absorbance measurements.

2.2.1.3.5. Determination of Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC) and Minimum Fungicidal Concentrations (MFC) of Antibiotics and Ferrocene-PAMAM Dendrimers

MIC is the lowest concentration of a compound that inhibits growth of a particular microorganism in vitro. To determine MIC values of compounds, optical density values for microbial growth in the wells were used. The values that demonstrate a decrease in the absorbance values dramatically were assumed as MIC for such compounds.

MBC is the lowest concentration of a compound that kill more than 99.9 % of a particular bacterium in bacterial suspension in vitro while MFC is the lowest concentration of a compound that kill more than 99.9 % of a particular fungus in fungal suspension in vitro. In order to determine MBC and MFC values of microorganisms, 100 µl of each samples in the wells of micro plates were spreaded onto nutrient agar for bacteria and onto sabouraud 4 % dextrose agar for fungi in the petri dishes. To determine colony forming unit (CFU/mL) values for each microorganism that exposed to antibiotics and ferrocene-PAMAM dendrimers, single colonies grown up on the nutrient and sabouraud dextrose agar plates were enumerated. Ten fold of measured values were assumed as colony forming unit per mililiter for each microorganism in the samples. The concentrations of antibiotics or ferrocene-PAMAM dendrimers that cause no growth of microorganism were recorded as MBC value for bacteria or MFC value for fungi. In the experiments, role of DMSO alone, as a solvent, in the antimicrobial activity was not researched because there is information in the literature about that DMSO have no antimicrobial activity [140,141].

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ANTIMICROBIAL ACTIVITY RESULTS FOR BACTERIA

3.1.1 Antimicrobial Activity Results by Broth Microdilution Tests for *Klebsiella pneumonia*

The results of antimicrobial activity screening of all substances for *Klebsiella pneumonia* are shown in Table 3.1.

Among four ferrocene based substances tested in triplicate in a microtiter plate, all substances demonstrated antimicrobial activity against *Klebsiella pneumonia* and all of them were recorded as showing an negative effect on growth of the test organism, indicating the presence of antimicrobial activity against *K.pneumonia*, when compared to the growth control wells.

Concentration range of antibiotics used in the tests for the test microorganisms was determined according to reference values in the literature. Among the all antibacterial agents used in the tests, streptomycin, nalidixic acid and tetracycline demostrated antimicrobial activity against *K.pneumonia* at the specified concentration range for them while erythromycin, kanamycin and ampicillin did not demostrate antimicrobial activity against the test bacterium at the specified concentration range for them.

Table 3.1 MIC and MBC values of substances at the specified concentration range for *Klebsiella pneumonia.*

Test Substance	Concentration range (µg/mL)	MIC	MBC
Tetracycline	4 - 512	8	-
Kanamycin	0.0625 – 1	>1	-
Streptomycin	0.5 – 64	16	64
Erythromycin	0.125 – 8	>8	-
Nalidixic acid	16- 512	128	-
Ampicillin	0.0625 – 2	>2	-
Ferrocene-amine (G0)	0.00781 – 0.5	0.125	0.25
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.0625	0.5
Ferrocene-PAMAM (G2)	0.00781– 0.5	0.125	-
Ferrocene-PAMAM (G3)	0.00781 – 0.5	0.125	-

MIC values of substances were 16 µg/mL for streptomycin, 128 µg/mL for nalidixic acid and 8 µg/mL for tetracycline, 0.125 µg/mL for ferrocene-amine (G0), 0.0625 µg/mL for ferrocene-PAMAM (G1), 0.125 µg/mL for ferrocene-PAMAM (G2) and for ferrocene-PAMAM (G3) as shown in Figure 3.1, Figure 3.2, Figure 3.4, Figure 3.6. MIC values of kanamycin, erythromycin and ampicillin could not be determined at the concentration range that was tested as shown in Figure 3.3 and Figure 3.5 and Figure 3.7.

MBC values of substances were 64 µg/mL for streptomycin, 0.25 µg/mL for ferrocene-amine (G0) and 0.5 µg/mL for ferrocene-PAMAM (G1) as shown in Table 3.1.

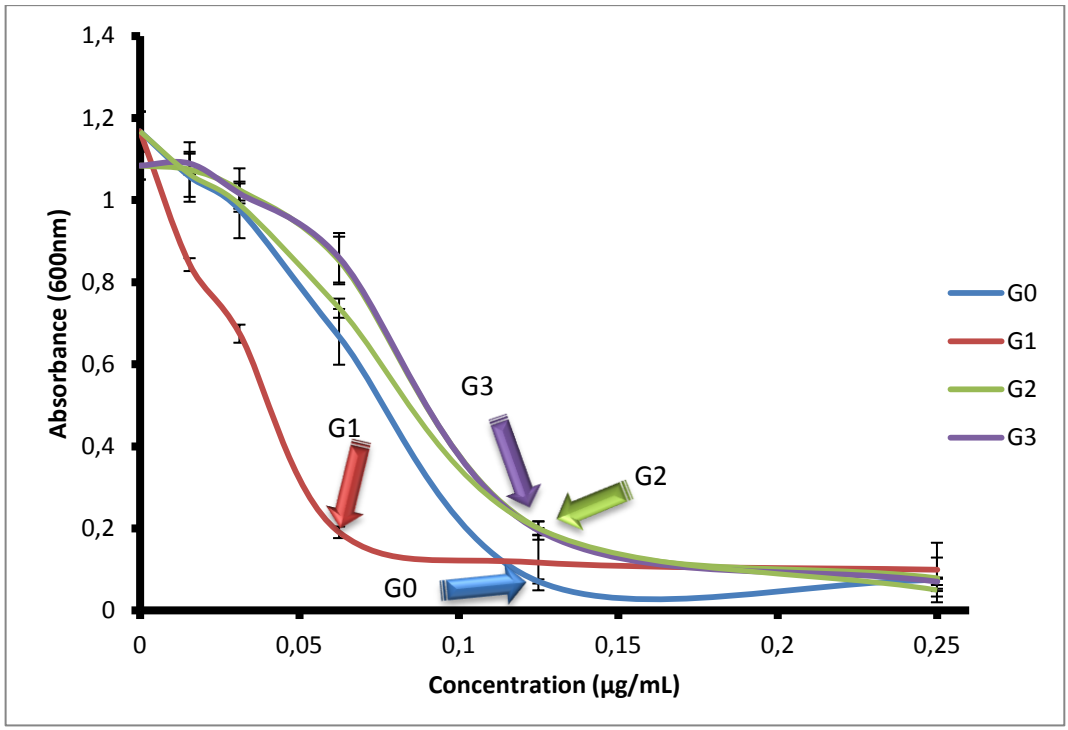

Figure 3.1 Growth of *Klebsiella pneumonia* at different concentrations of ferrocene–amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

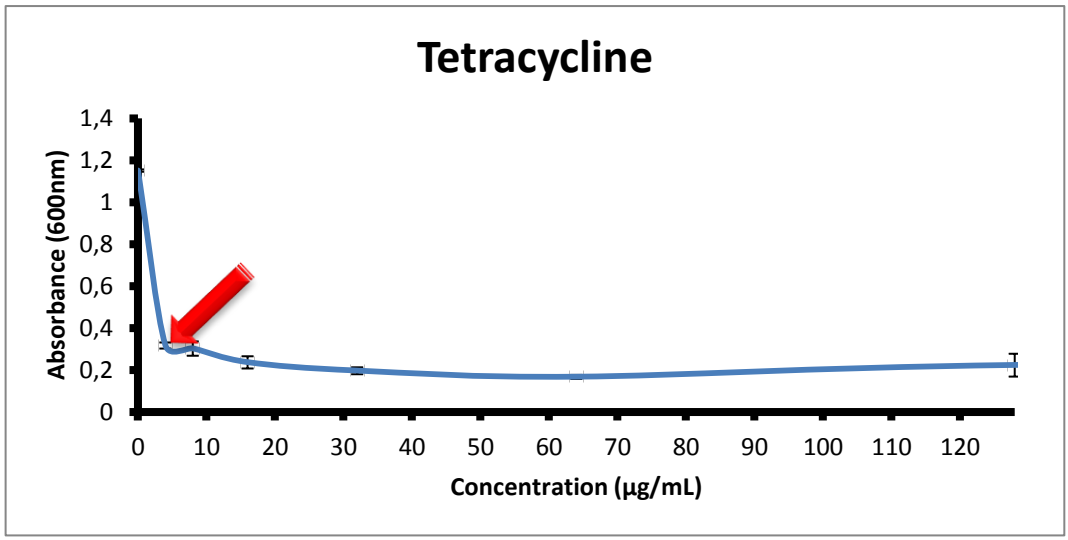

Figure 3.2 Growth of *Klebsiella pneumonia* at different concentrations of tetracycline; MIC value of the antibiotic is indicated with red arrow in the figure.

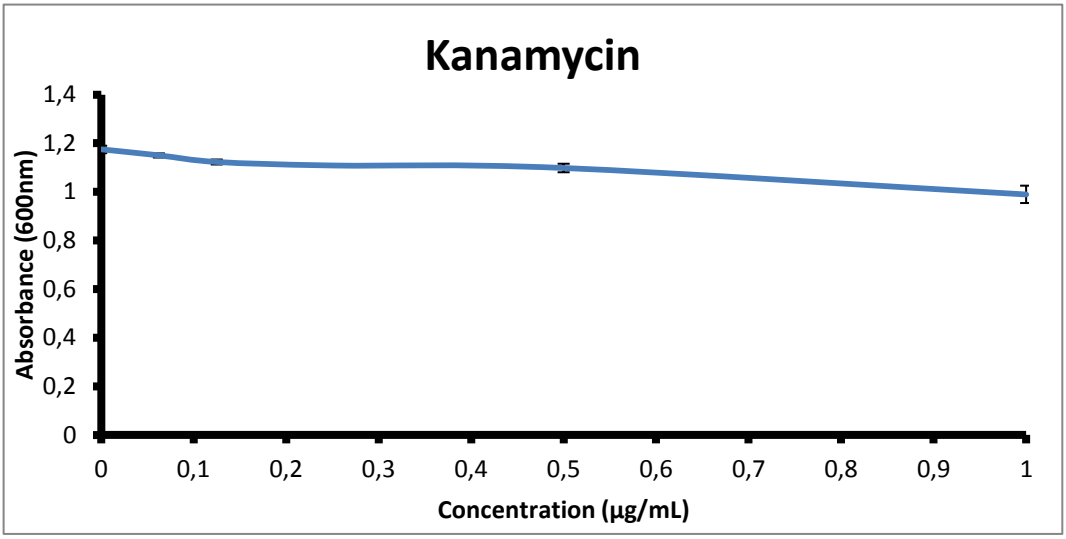

Figure 3.3 Growth of *Klebsiella pneumonia* at different concentrations of kanamycin.

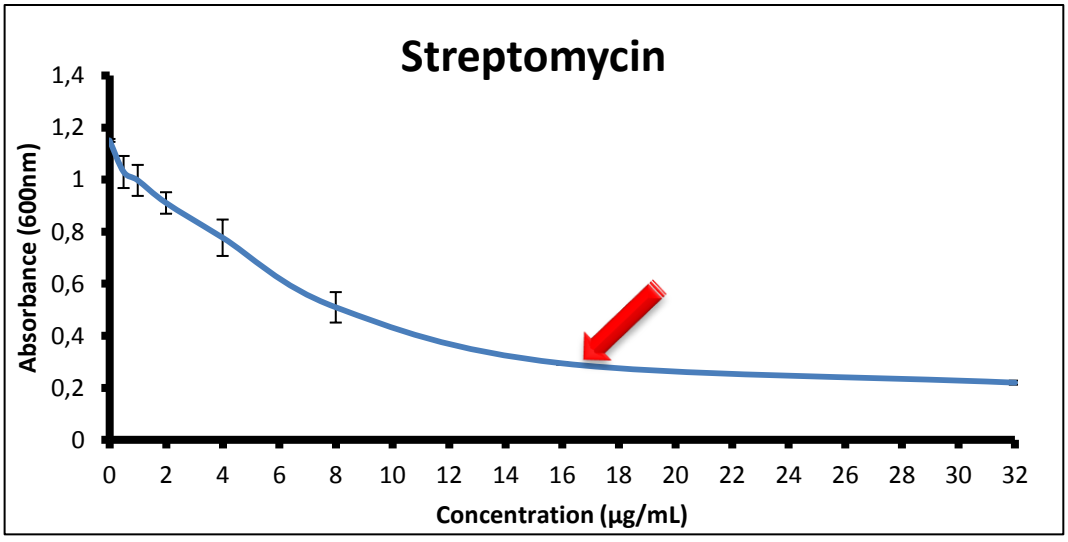

Figure 3.4 Growth of *Klebsiella pneumonia* at different concentrations of streptomycin; MIC value of the antibiotic is indicated with red arrow in the figure.

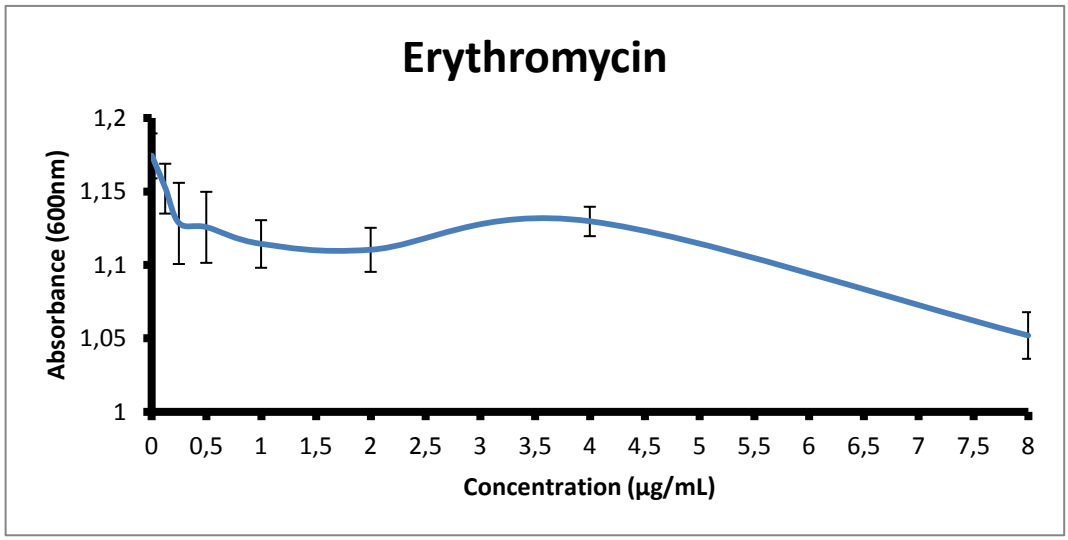

Figure 3.5 Growth of *Klebsiella pneumonia* at different concentrations of erythromycin.

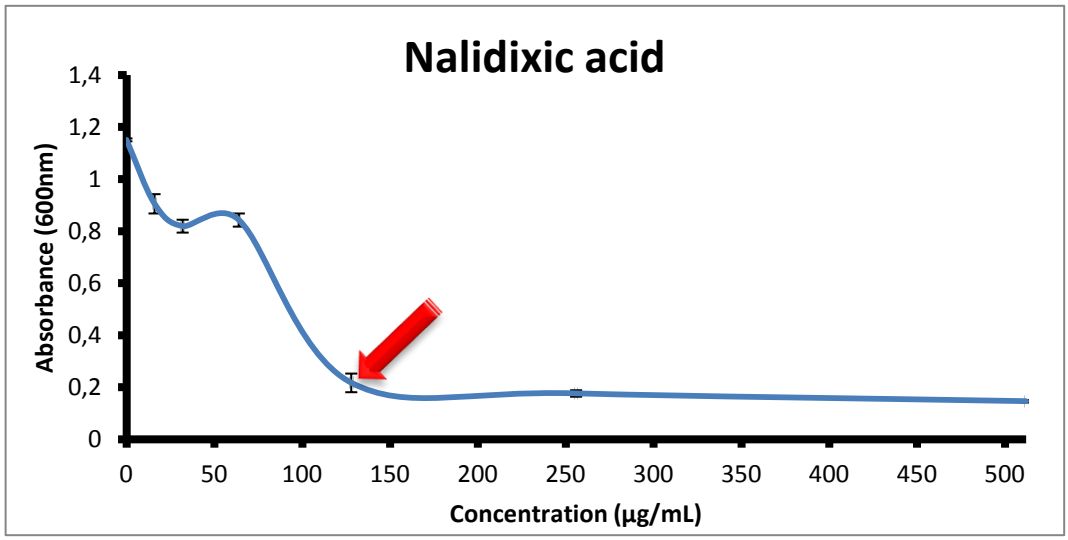

Figure 3.6 Growth of *Klebsiella pneumonia* at different concentrations of nalidixic acid; MIC value of the antibiotic is indicated with red arrow in the figure.

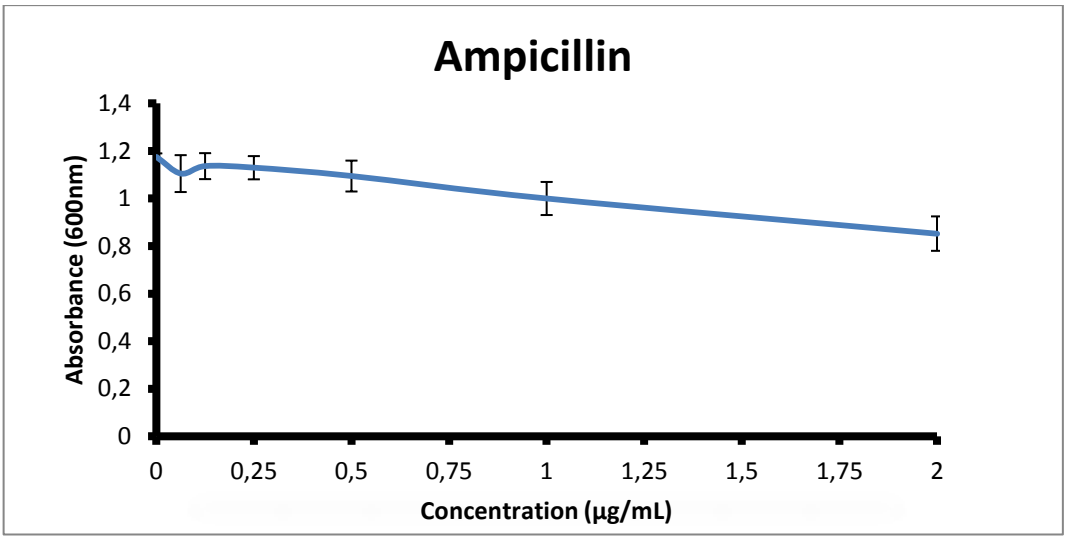

Figure 3.7 Growth of *Klebsiella pneumonia* at different concentrations of ampicillin.

3.1.2 Antimicrobial Activity Results by Broth Microdilution Tests for *Escherichia coli*

The results of antimicrobial activity screening of all substances for *Escherichia coli* are shown in Table 3.2.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Escherichia coli* and all of them were recorded as showing an negative effect on growth of the test organism, indicating the presence of antimicrobial activity against *Escherichia coli*, when compared to the growth control wells.

Concentration range of antibiotics used in the tests for the test microorganisms was determined according to reference values in the literature. Among the all antibacterial agents used, tetracycline, kanamycin, nalidixic acid, ampicillin and erythromycin demostrated antimicrobial activity against *Escherichia coli* at the specified concentration range for them while streptomycin did not demostrate antimicrobial activity against the test bacterium at the specified concentration range for them.

MIC values of substances were 1 µg/mL for tetracycline, 8 µg/mL for kanamycin, 64 µg/mL for erythromycin, 16 µg/mL for nalidixic acid, 16 µg/mL for ampicillin,

0.125 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G2) and for ferrocene-PAMAM (G3), 0.03125 µg/mL for ferrocene-PAMAM (G1) as shown in Figure 3.8, Figure 3.9, Figure 3.10, Figure 3.12, Figure 3.13, Figure 3.14. MIC value of streptomycin could not be determined at the concentration range that was tested as shown in Figure 3.11.

MBC values of substances were 8 µg/mL for kanamycin, 64 µg/mL for erythromycin, 32 µg/mL for ampicillin, 0.5 µg/mL for ferrocene-PAMAM (G1) and for ferrocene-PAMAM (G2) as shown in Table 3.2

Table 3.2 MIC and MBC values of substances at the specified concentration range for *Escherichia coli.*

Test Substance	**Concentration range (µg/mL)**	**MIC**	**MBC**
Tetracycline	0.25 – 8	1	-
Kanamycin	1- 64	8	8
Streptomycin	64 - 512	>512	-
Erythromycin	4 - 512	64	64
Nalidixic acid	0.25 – 16	16	-
Ampicillin	8 - 256	16	32
Ferrocene-amine (G0)	0.00781– 0.5	0.125	-
Ferrocene-PAMAM (G1)	0.00781– 0.5	0. 03125	0.5
Ferrocene-PAMAM (G2)	0.00781 – 0.5	0.125	0.5
Ferrocene-PAMAM (G3)	0.00781 – 0.5	0.125	-

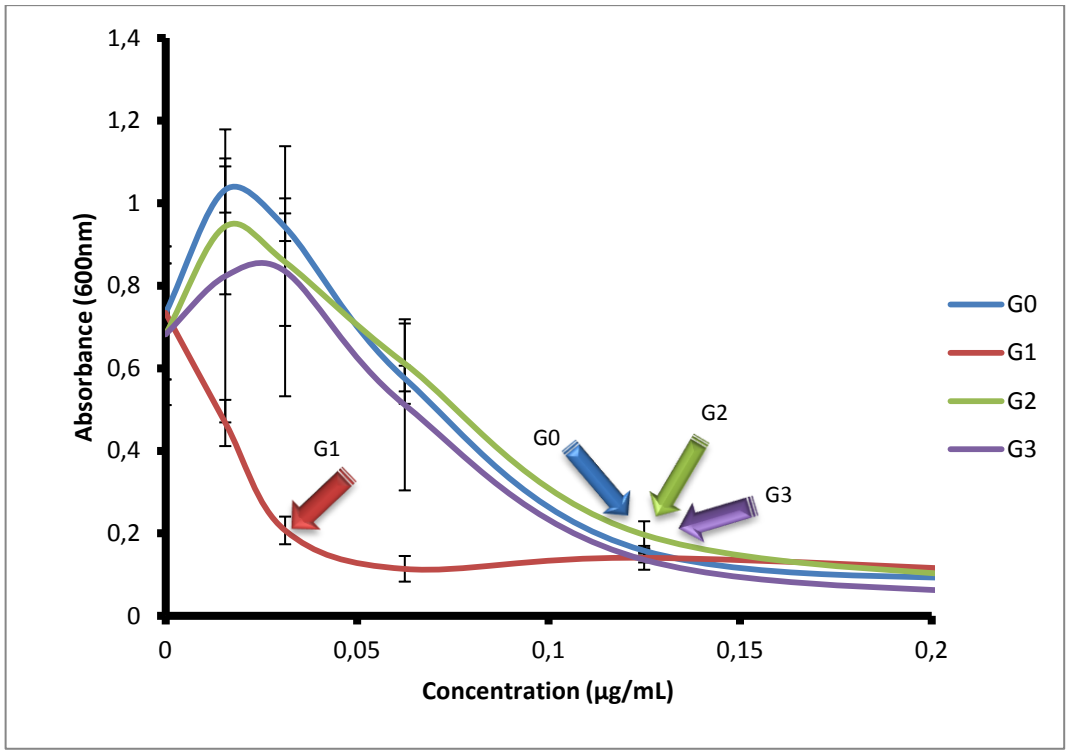

Figure 3.8 Growth of *Escherichia coli* at different concentrations of ferrocene–amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

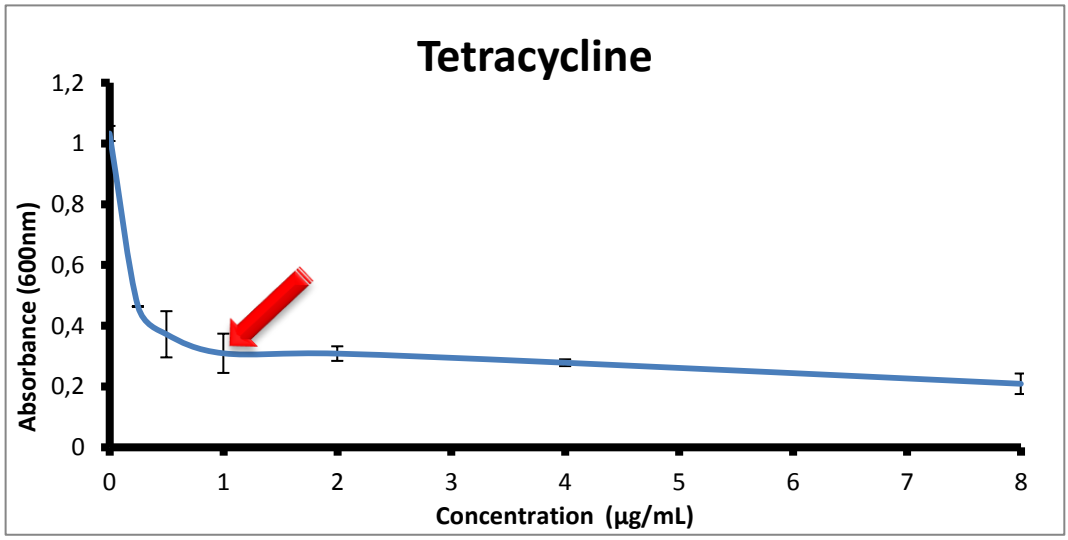

Figure 3.9 Growth of *Escherichia coli* at different concentrations of tetracycline; MIC value of the antibiotic is indicated with red arrow in the figure.

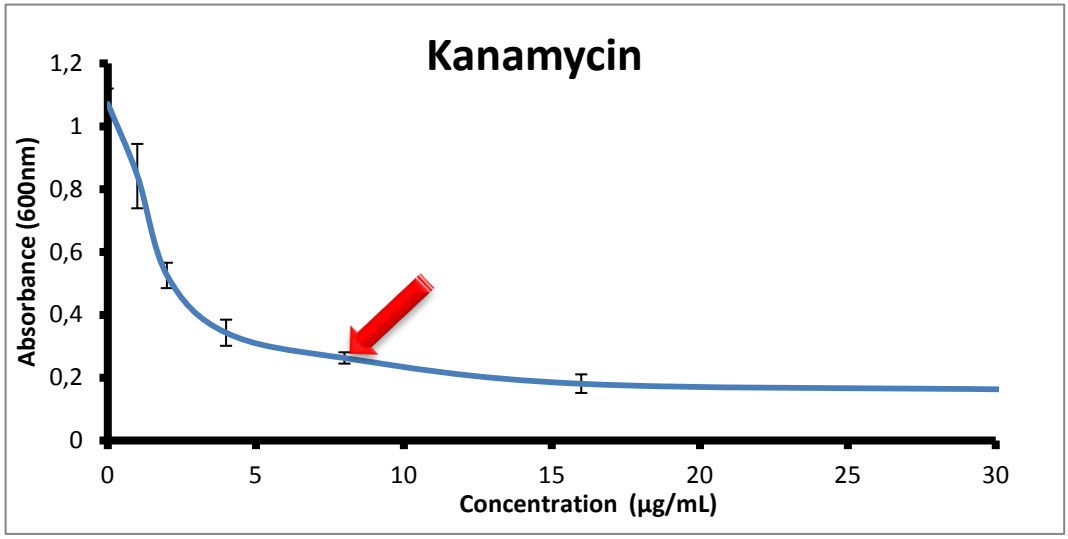

Figure 3.10 Growth of *Escherichia coli* at different concentrations of kanamycin; MIC value of the antibiotic is indicated with red arrow in the figure.

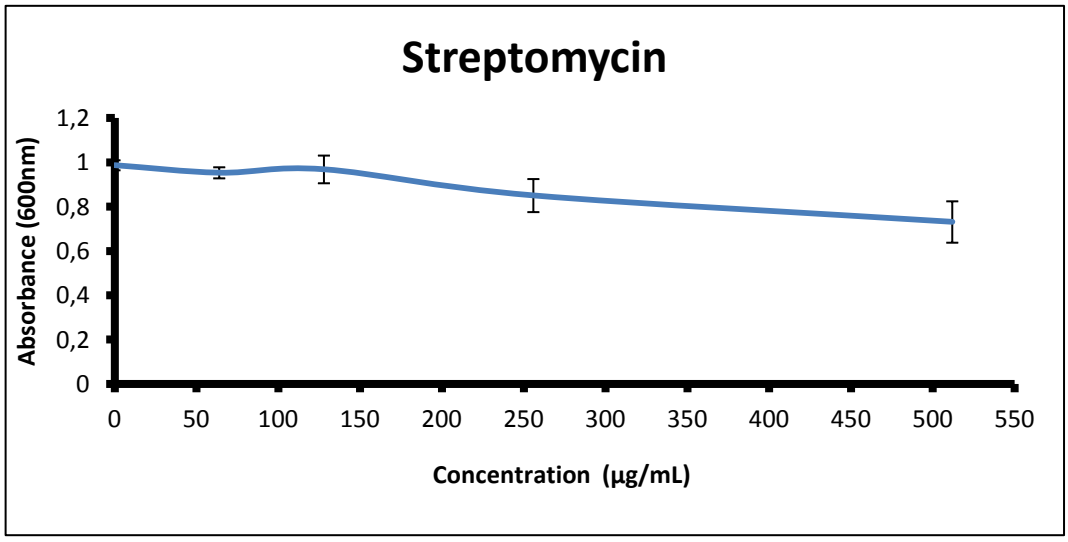

Figure 3.11 Growth of *Escherichia coli* at different concentrations of streptomycin.

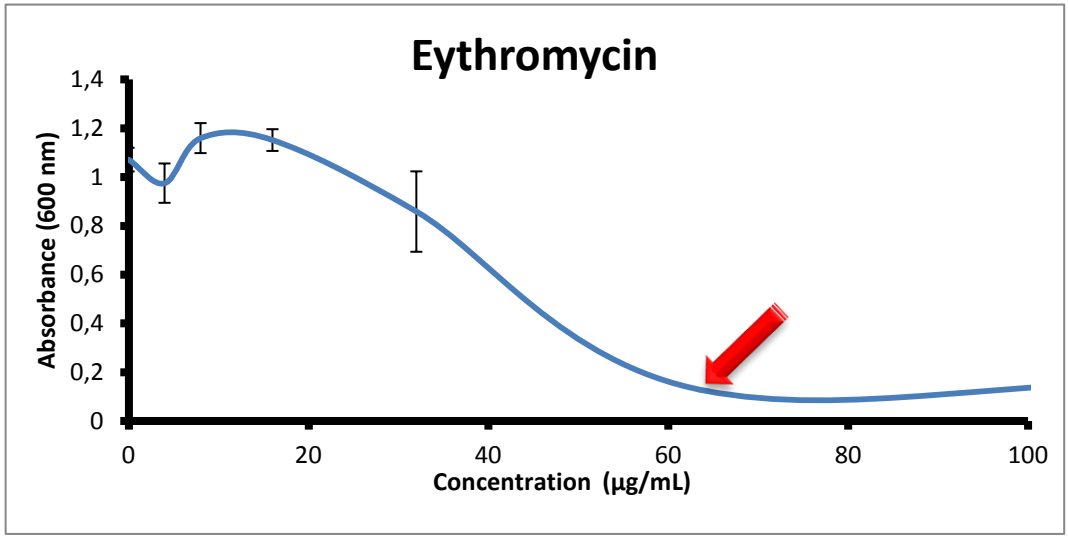

Figure 3.12 Growth of *Escherichia coli* at different concentrations of erthromycin; MIC value of the antibiotic is indicated with red arrow in the figure.

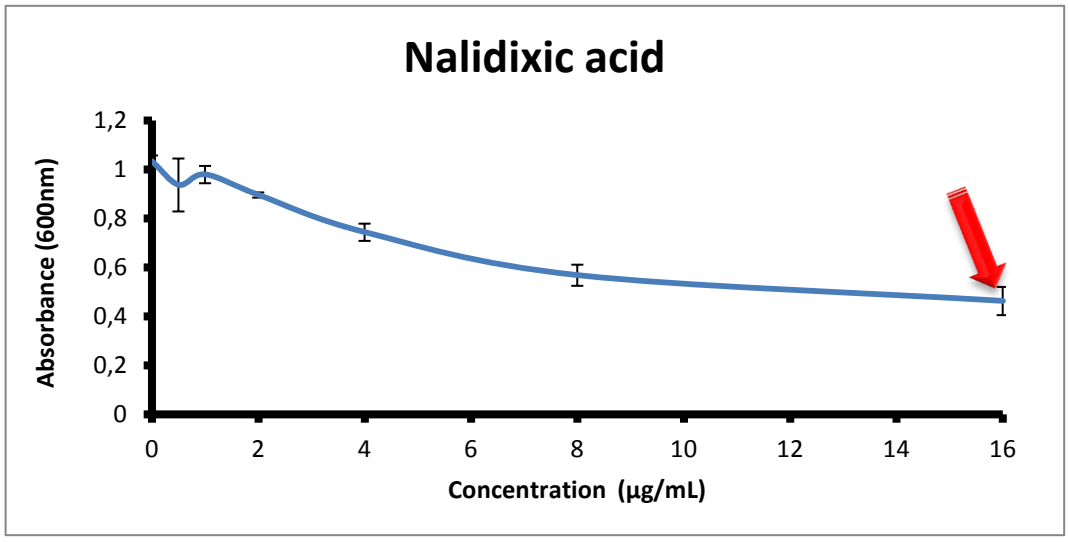

Figure 3.13 Growth of *Escherichia coli* at different concentrations of nalidixic acid; MIC value of the antibiotic is indicated with red arrow in the figure.

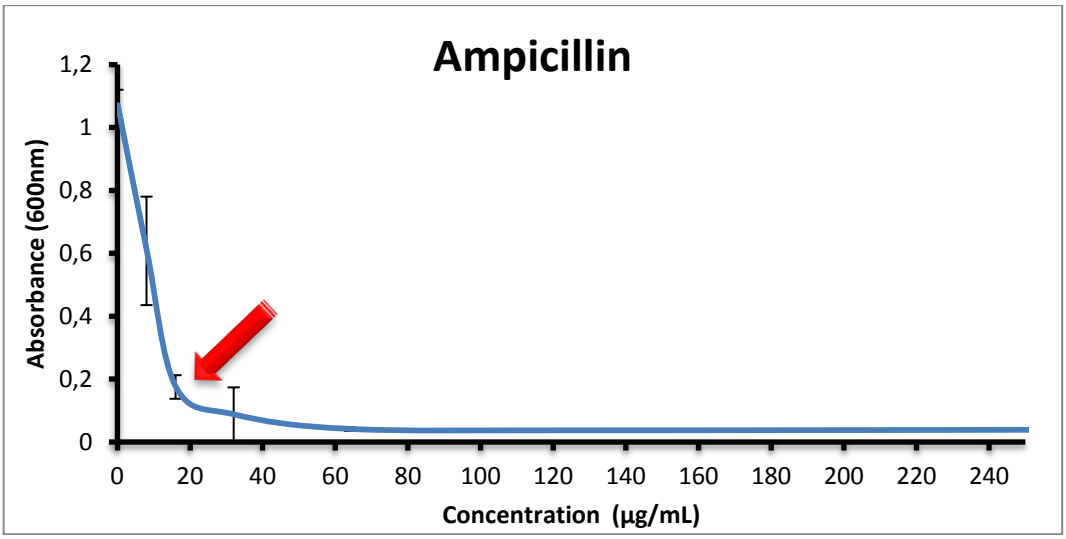

Figure 3.14 Growth of *Escherichia coli* at different concentrations of ampicillin; MIC value of the antibiotic is indicated with red arrow in the figure.

3.1.3 Antimicrobial Activity Results by Broth Microdilution Tests for *Bacillus subtilis*

The results of antimicrobial activity screening of all substances for *Bacillus subtilis* are shown in Table 3.3.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Bacillus subtilis* and all of them were recorded as showing an negative effect on growth of the test organism.

Among the all antibacterial agents used in the tests, tetracycline, kanamycin, streptomycin and nalidixic acid demostrated antimicrobial activity against *Bacillus subtilis* at the specified concentration range for them while erythromycin and ampicillin did not demostrate antimicrobial activity against the test bacterium at the specified concentration range for them.

MIC values of substances were 1µg/mL for tetracycline, 16 µg/mL for kanamycin, 8 µg/mL for streptomycin, 32 µg/mL for nalidixic acid, 0.125 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G2), for ferrocene-PAMAM (G3) and 0.0625 µg/mL for ferrocene-PAMAM (G1) as shown in Figure 3.15, Figure 3.16, Figure 3.17, Figure 3.18, Figure 3.20. MIC values of erythromycin and ampicillin could

not be determined at the concentration range that was tested as shown in Figure 3.19 and Figure 3.21.

MBC values of substances were 16 µg/mL for streptomycin and 0.125 µg/mL for ferrocene-PAMAM (G1) as shown in Table 3.3.

Table 3.3 MIC and MBC values of substances at the specified concentration range for *Bacillus subtilis.*

Test Substance	**Concentration range (µg/mL)**	**MIC**	**MBC**
Tetracycline	1 -32	1	-
Kanamycin	16 - 512	16	-
Streptomycin	2- 64	8	16
Erythromycin	0.0625– 0.5	> 0.5	-
Nalidixic acid	1 - 32	32	-
Ampicillin	16-512	> 512	-
Ferrocene-amine (G0)	0.00781– 0.5	0.125	-
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.0625	0.125
Ferrocene-PAMAM (G2)	0.00781– 0.5	0.125	-
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.125	-

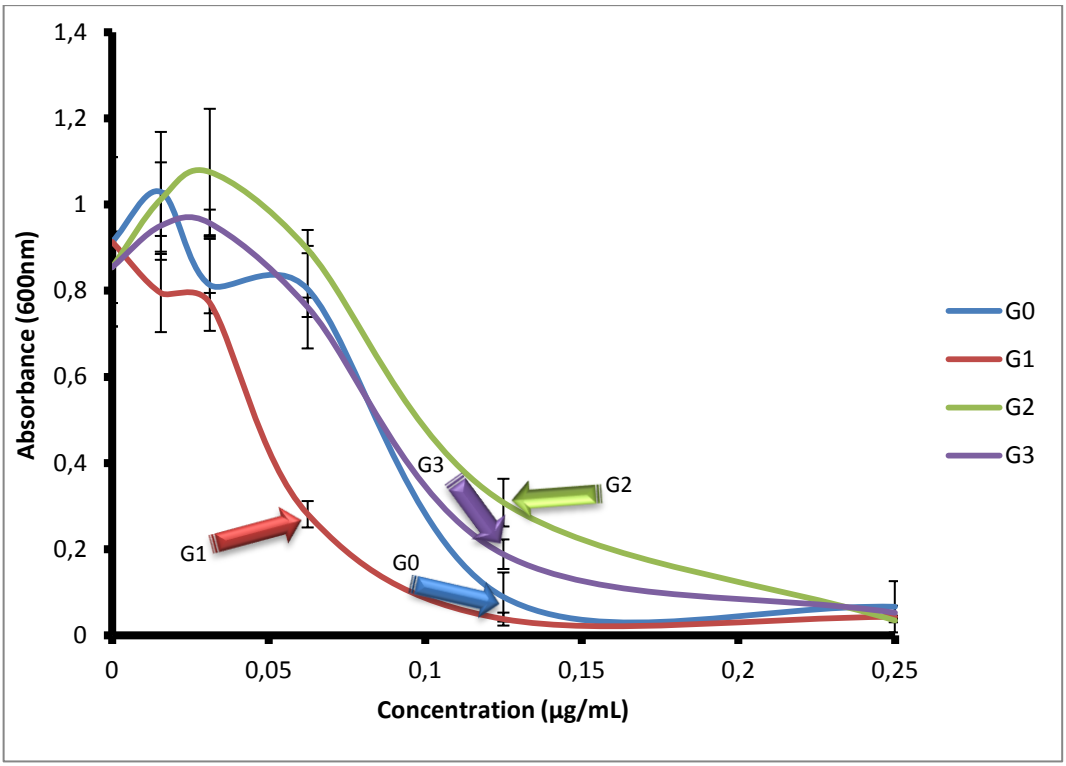

Figure 3.15 Growth of *Bacillus subtilis* at different concentrations of ferrocene–amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

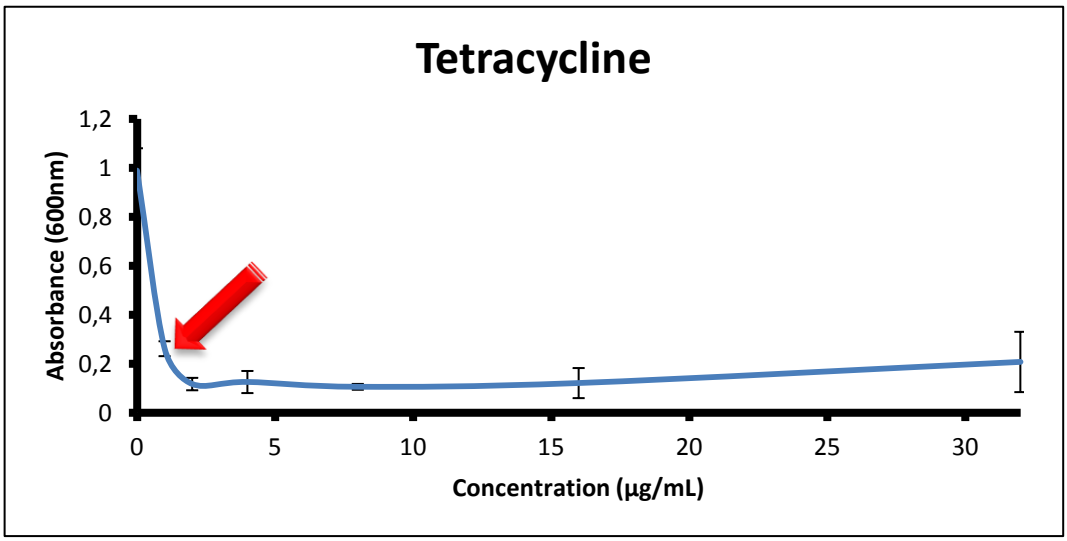

Figure 3.16 Growth of *Bacillus subtilis* at different concentrations of tetracycline; MIC value of the antibiotic is indicated with red arrow in the figure.

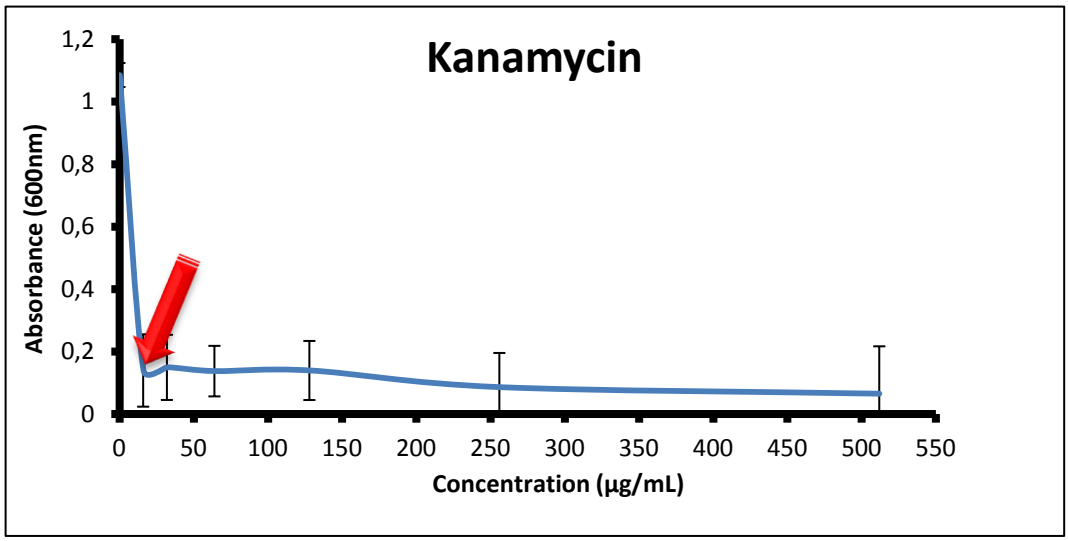

Figure 3.17 Growth of *Bacillus subtilis* at different concentrations of kanamycin; MIC value of the antibiotic is indicated with red arrow in the figure.

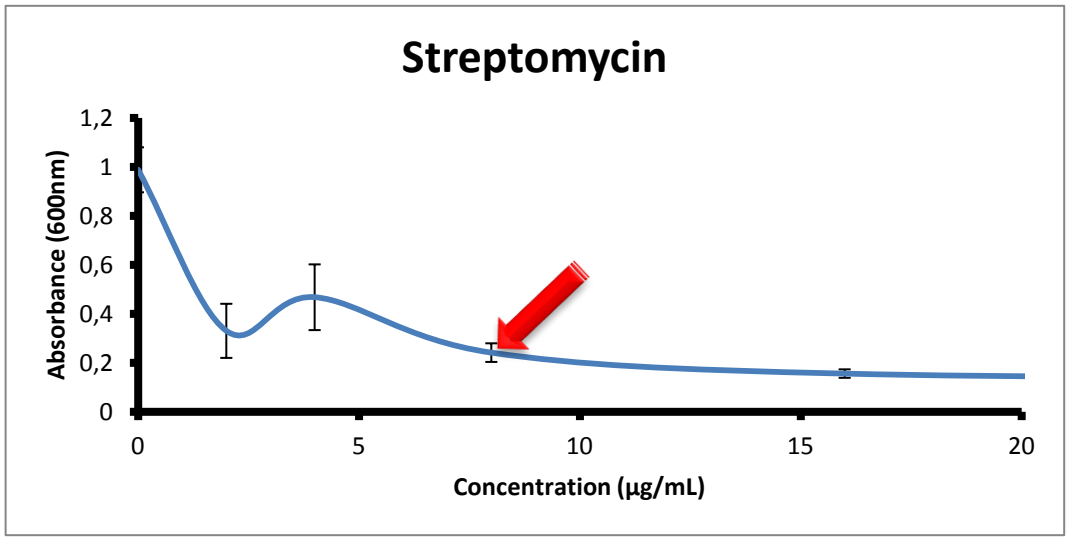

Figure 3.18 Growth of *Bacillus subtilis* at different concentrations of streptomycin; MIC value of the antibiotic is indicated with red arrow in the figure.

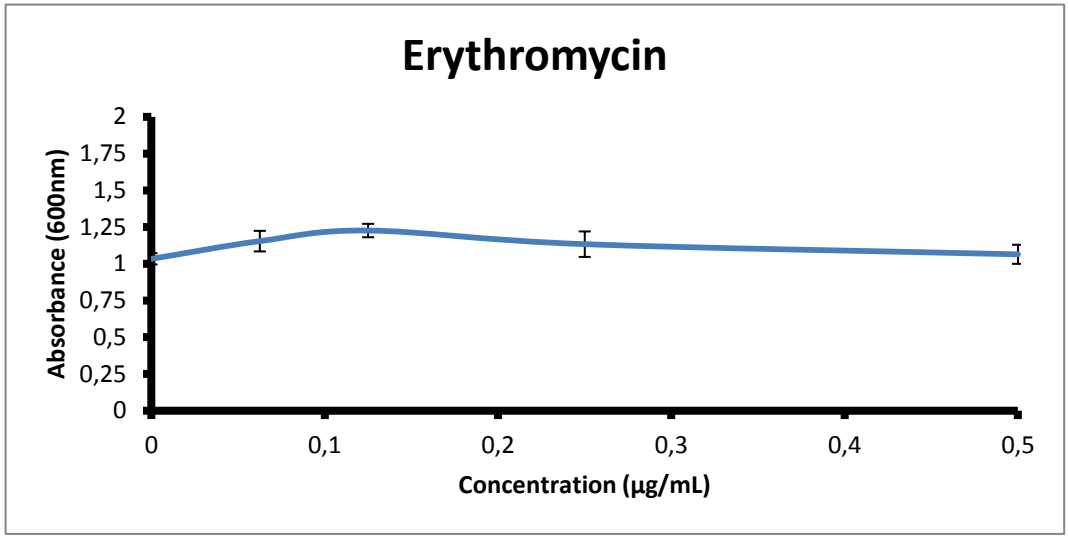

Figure 3.19 Growth of *Bacillus subtilis* at different concentrations of erythromycin.

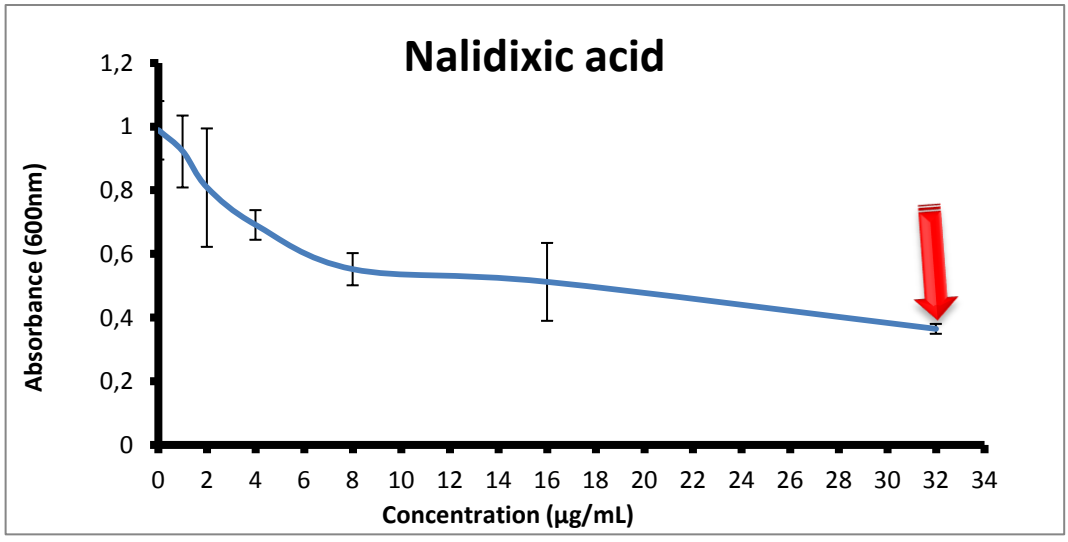

Figure 3.20 Growth of *Bacillus subtilis* at different concentrations of nalidixic acid; MIC value of the antibiotic is indicated with red arrow in the figure.

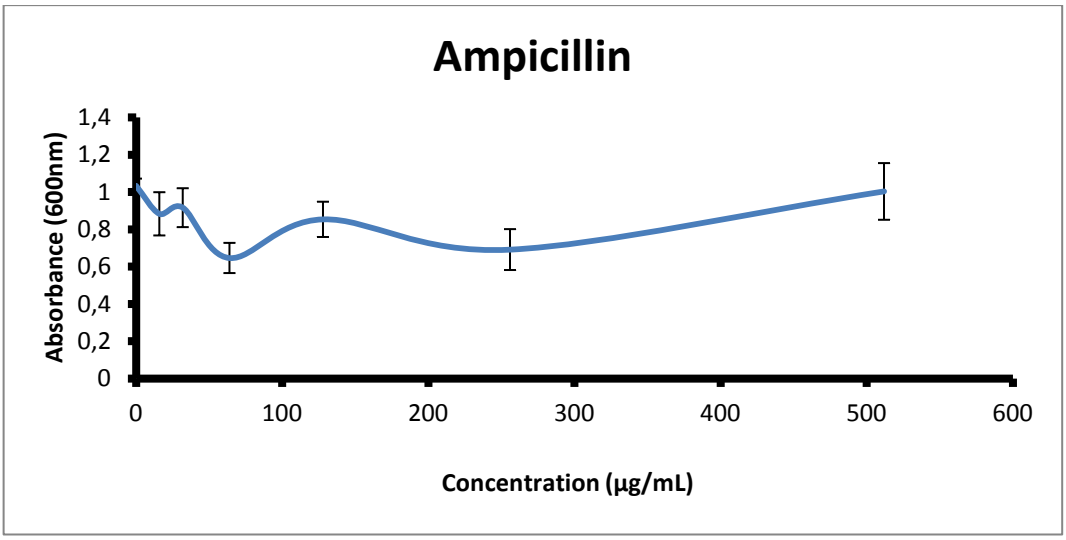

Figure 3.21 Growth of *Bacillus subtilis* at different concentrations of ampicillin.

3.1.4 Antimicrobial Activity Results by Broth Microdilution Tests for *Citrobacter freundii*

The results of antimicrobial activity screening of all substances for *Citrobacter freundii* are shown in Table 3.4.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Citrobacter freundii* and all of them were recorded as showing an negative effect on growth of the test organism.

All antibacterial agents used in the tests (tetracycline, kanamycin, streptomycin, nalidixic acid, erythromycin and ampicillin) demostrated antimicrobial activity against *Citrobacter freundii* at the specified concentration range for them.

MIC values of substances were 4 µg/mL for tetracycline, 8 µg/mL for kanamycin, 32 µg/mL for streptomycin, 512 µg/mL for erythromycin, 64 µg/mL for nalidixic acid, 0.5 µg/mL for ampicillin, 0.125 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G2), for ferrocene-PAMAM (G3) and 0.0625 µg/mL for ferrocene-PAMAM (G1) as shown in Figure 3.22, Figure 3.23, Figure 3.24, Figure 3.25, Figure 3.26, Figure 3.27 and Figure 3.28**.**

MBC values of substances were 512 µg/mL for erythromycin and 0.25 µg/mL for ferrocene-amine (G0), 0.5 µg/mLfor ferrocene-PAMAM (G1), ferrocene-PAMAM (G2) and for ferrocene-PAMAM (G3) as shown in Table 3.4.

Table 3.4 MIC and MBC values of substances at the specified concentration range for *Citrobacter freundii*.

Test Substance	**Concentration range (µg/mL)**	**MIC**	**MBC**
Tetracycline	0.25 – 64	4	-
Kanamycin	2 - 64	8	-
Streptomycin	0.5 – 32	32	-
Erythromycin	8 - 512	512	512
Nalidixic acid	2 - 256	64	-
Ampicillin	0.5– 32	0.5	-
Ferrocene-amine (G0)	0.00781– 0.5	0.125	0.25
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.0625	0.5
Ferrocene-PAMAM (G2)	0.00781 – 0.5	0.125	0.5
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.125	0.5

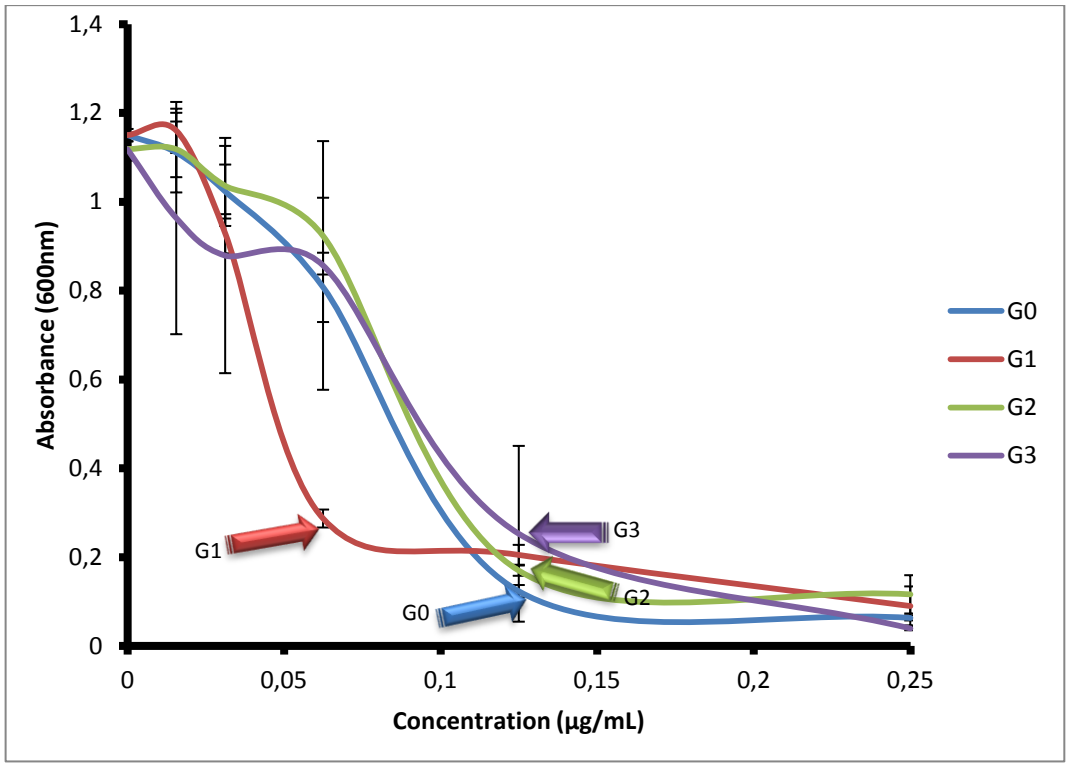

Figure 3.22 Growth of *Citrobacter freundii* at different concentrations of ferrocene-amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

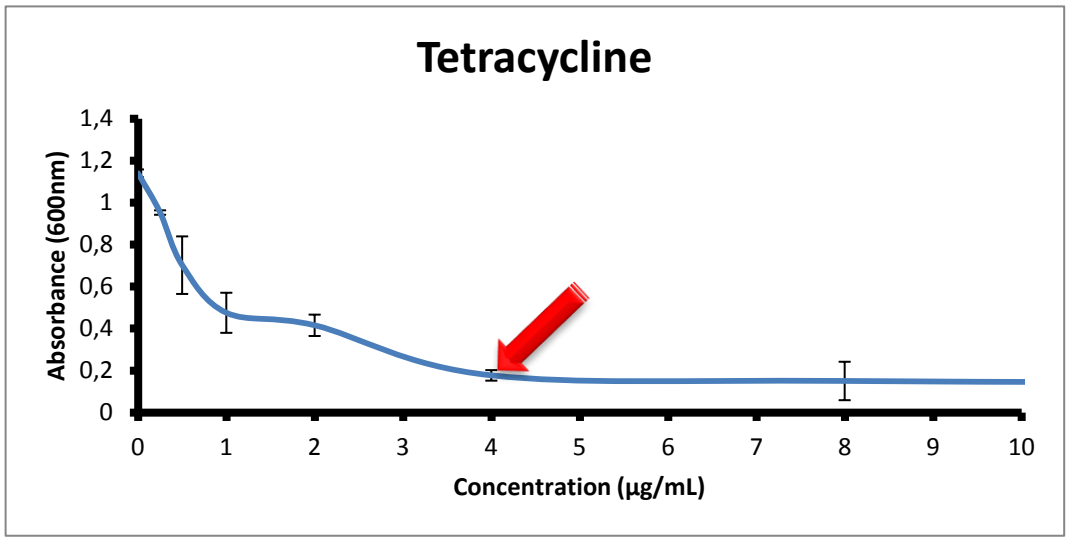

Figure 3.23 Growth of *Citrobacter freundii* at different concentrations of tetracycline; MIC value of the antibiotic is indicated with red arrow in the figure.

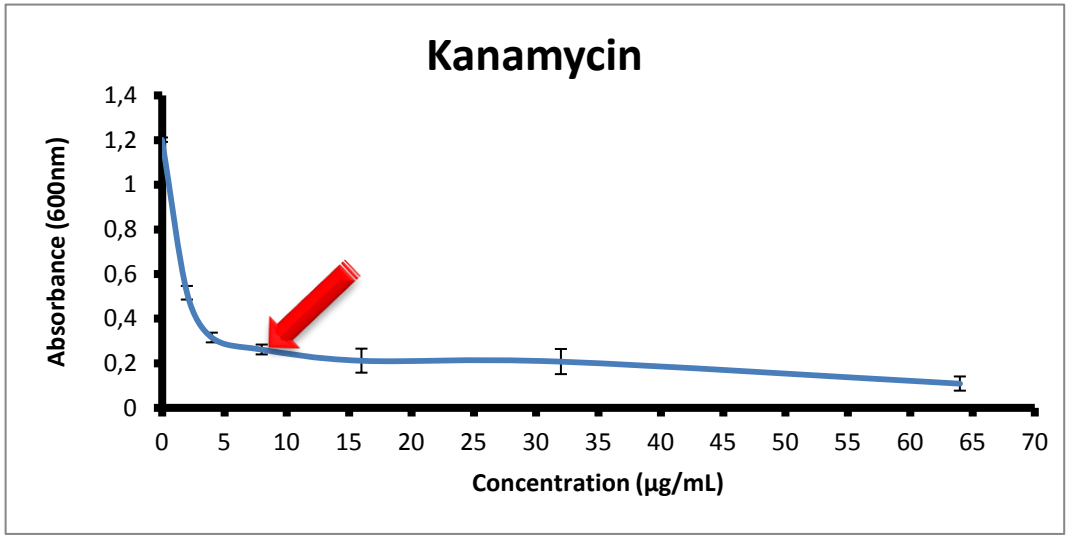

Figure 3.24 Growth of *Citrobacter freundii* at different concentrations of kanamycin; MIC value of the antibiotic is indicated with red arrow in the figure.

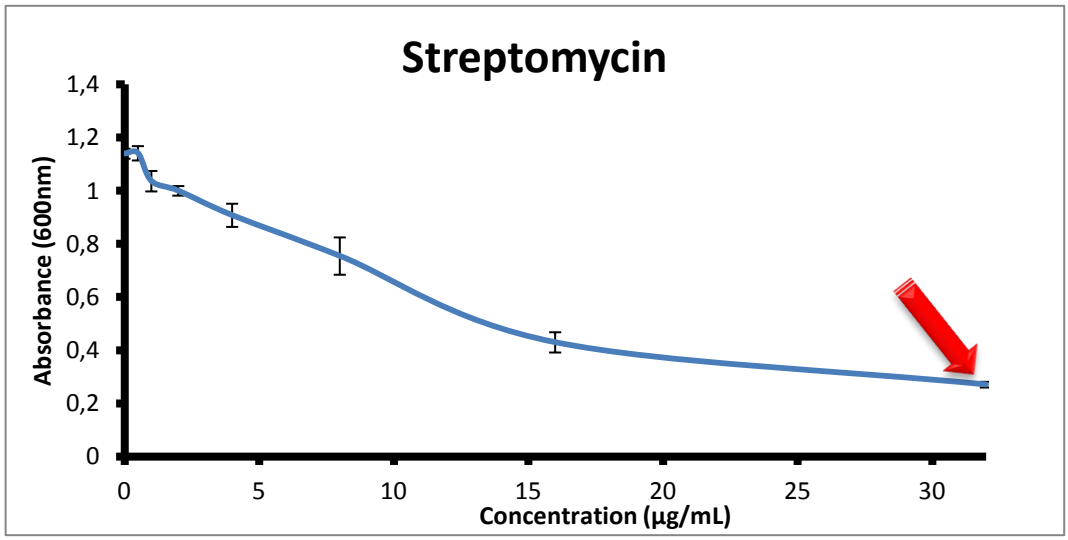

Figure 3.25 Growth of *Citrobacter freundii* at different concentrations of streptomycin; MIC value of the antibiotic is indicated with red arrow in the figure.

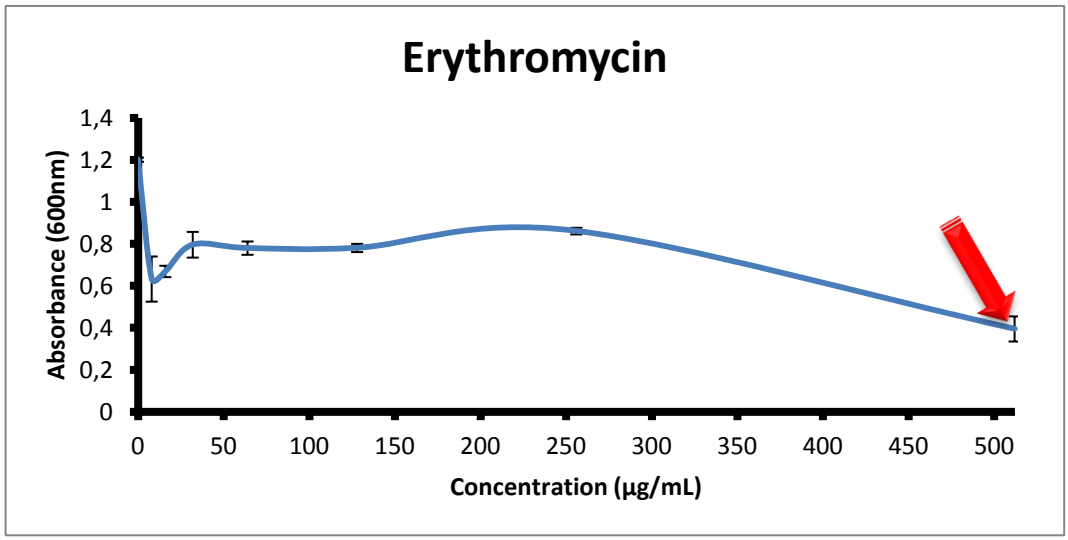

Figure 3.26 Growth of *Citrobacter freundii* at different concentrations of erythromycin; MIC value of the antibiotic is indicated with red arrow in the figure.

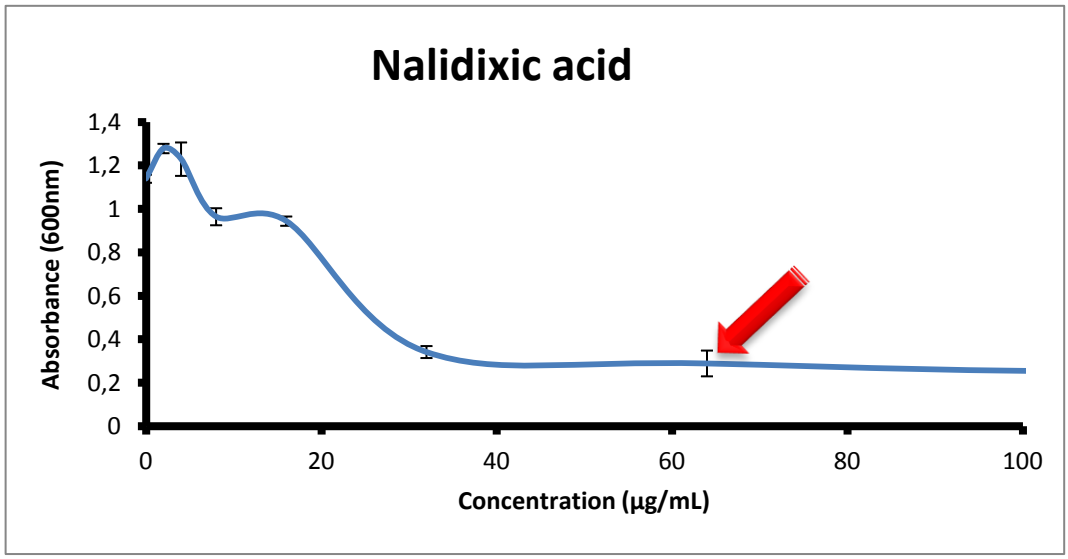

Figure 3.27 Growth of *Citrobacter freundii* at different concentrations of nalidixic acid; MIC value of the antibiotic is indicated with red arrow in the figure.

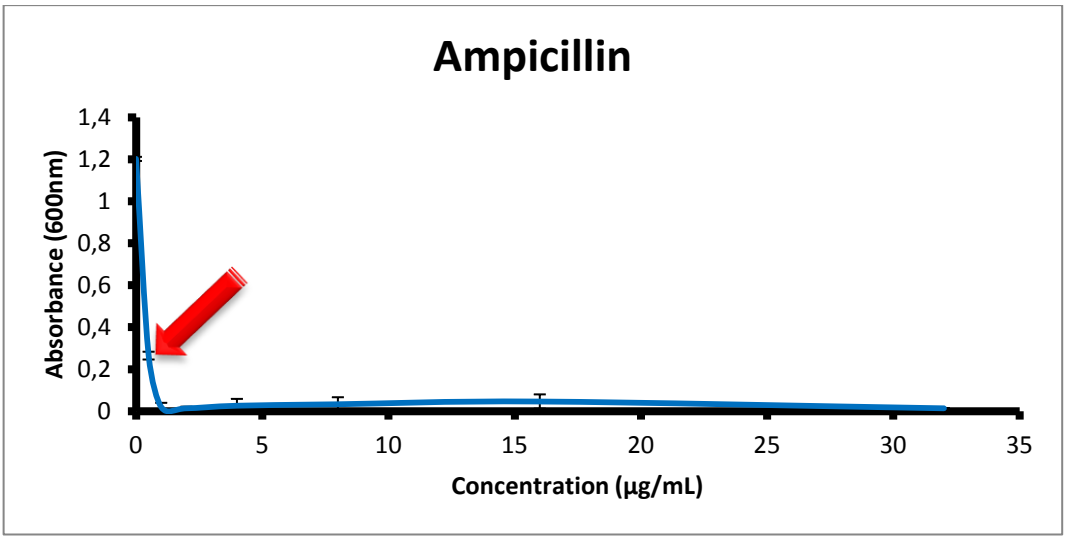

Figure 3.28 Growth of *Citrobacter freundii* at different concentrations of ampicillin; MIC value of the antibiotic is indicated with red arrow in the figure.

3.1.5 Antimicrobial Activity Results by Broth Microdilution Tests for *Proteus mirabilis*

The results of antimicrobial activity screening of all substances for *Proteus mirabilis* are shown in Table 3.5.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Proteus mirabilis* and all of them were recorded as showing an negative effect on growth of the test organism.

All antibacterial agents used in the tests (tetracycline, kanamycin, streptomycin, nalidixic acid, erythromycin and ampicillin) demostrated antimicrobial activity against *Proteus mirabilis* at the specified concentration range for them.

MIC values of substances were 8 µg/mL for tetracycline, 4 µg/mL for kanamycin, 256 µg/mL for streptomycin, 256 µg/mL for erythromycin, 64 µg/mL for nalidixic acid, 2 µg/mL for ampicillin, 0.125 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G1), for ferrocene-PAMAM (G2), for ferrocene-PAMAM (G3) as shown in Figure 3.29, Figure 3.30, Figure 3.31, Figure 3.32, Figure 3.33, Figure 3.34, Figure 3.35.

MBC values of substances were 256 µg/mL for tetracycline, 4 µg/mL for kanamycin, 256 µg/mL for streptomycin and 256 µg/mL for erythromycin while

ferrocene-amine (G0), ferrocene-PAMAM (G1), ferrocene-PAMAM (G2) and ferrocene-PAMAM (G3) did not display bactericidal effect on the test organism at the specified concentration range as shown in Table 3.5.

Table 3.5 MIC and MBC values of substances at the specified concentration range for *Proteus mirabilis.*

Test Substance	**Concentration range (µg/mL)**	**MIC**	**MBC**
Tetracycline	8- 512	8	256
Kanamycin	4 - 512	4	4
Streptomycin	1 - 512	256	256
Erythromycin	16 - 512	256	256
Nalidixic acid	4 -512	64	-
Ampicillin	2 - 32	2	-
Ferrocene-amine (G0)	0,00781– 0,5	0,125	-
Ferrocene-PAMAM (G1)	0,00781– 0,5	0,125	-
Ferrocene-PAMAM (G2)	0,00781– 0,5	0,125	-
Ferrocene-PAMAM (G3)	0,00781– 0,5	0,125	-

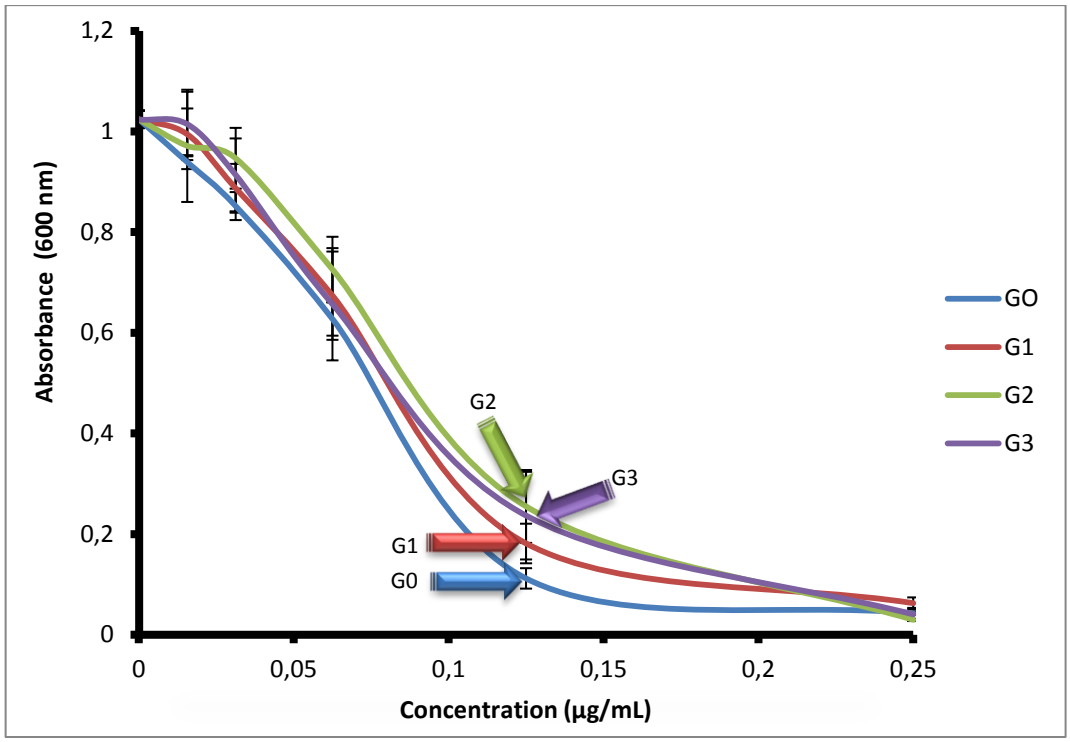

Figure 3.29 Growth of *Proteus mirabilis* at different concentrations of ferrocene-amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

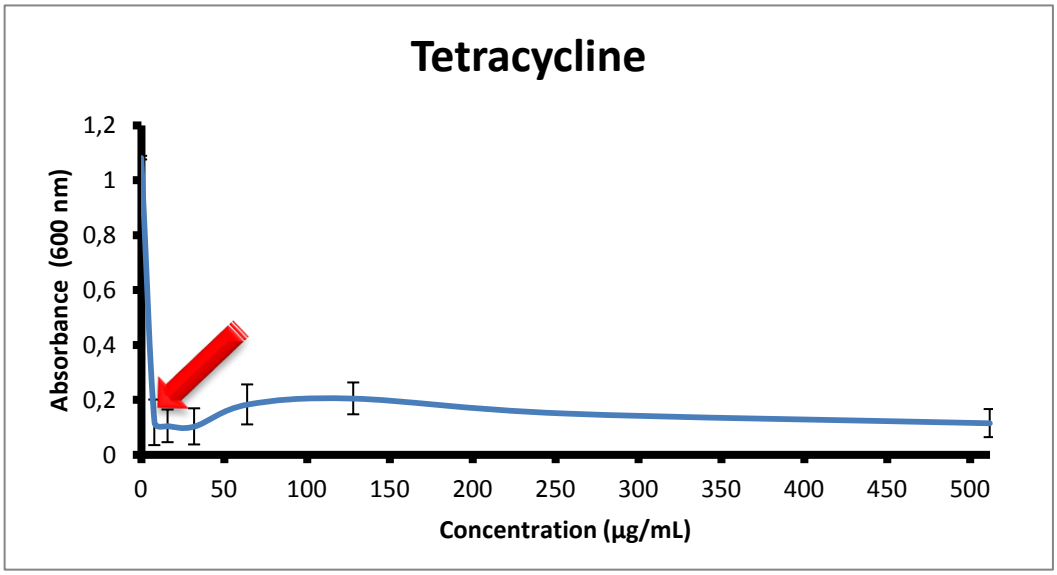

Figure 3.30 Growth of *Proteus mirabilis* at different concentrations of tetracycline; MIC value of the antibiotic is indicated with red arrow in the figure.

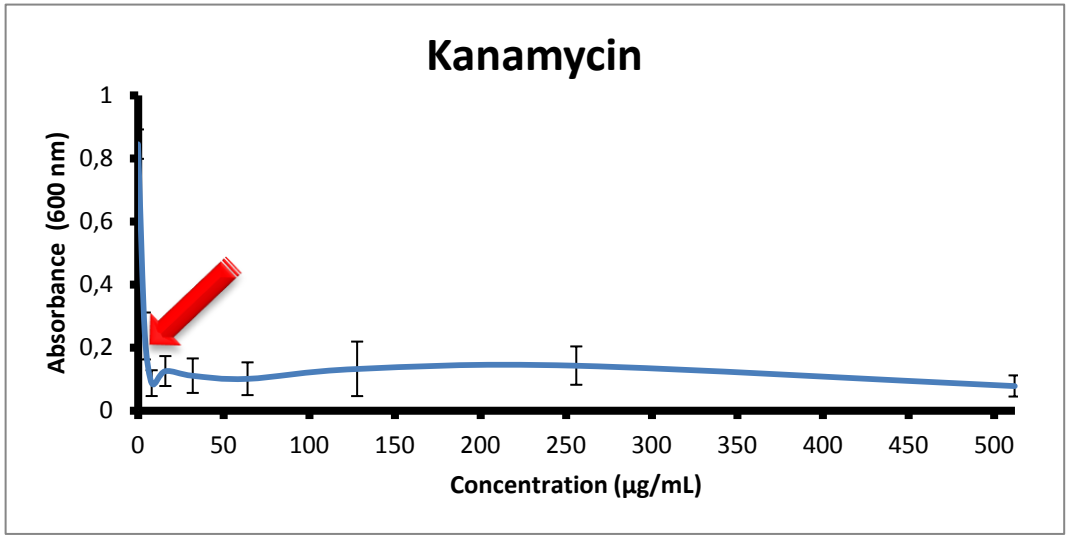

Figure 3.31 Growth of *Proteus mirabilis* at different concentrations of kanamycin; MIC value of the antibiotic is indicated with red arrow in the figure.

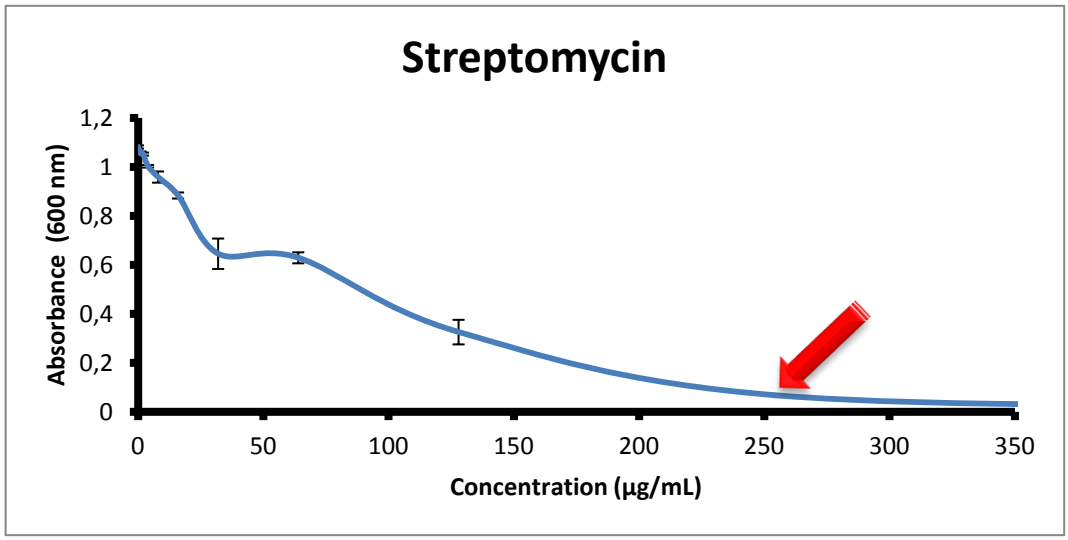

Figure 3.32 Growth of *Proteus mirabilis* at different concentrations of streptomycin; MIC value of the antibiotic is indicated with red arrow in the figure.

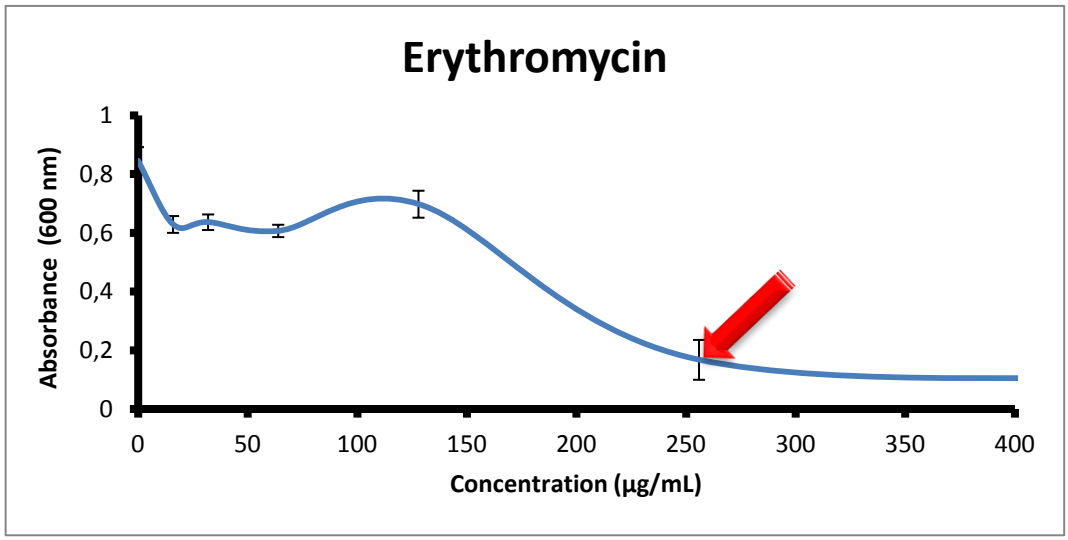

Figure 3.33 Growth of *Proteus mirabilis* at different concentrations of erythromycin; MIC value of the antibiotic is indicated with red arrow in the figure.

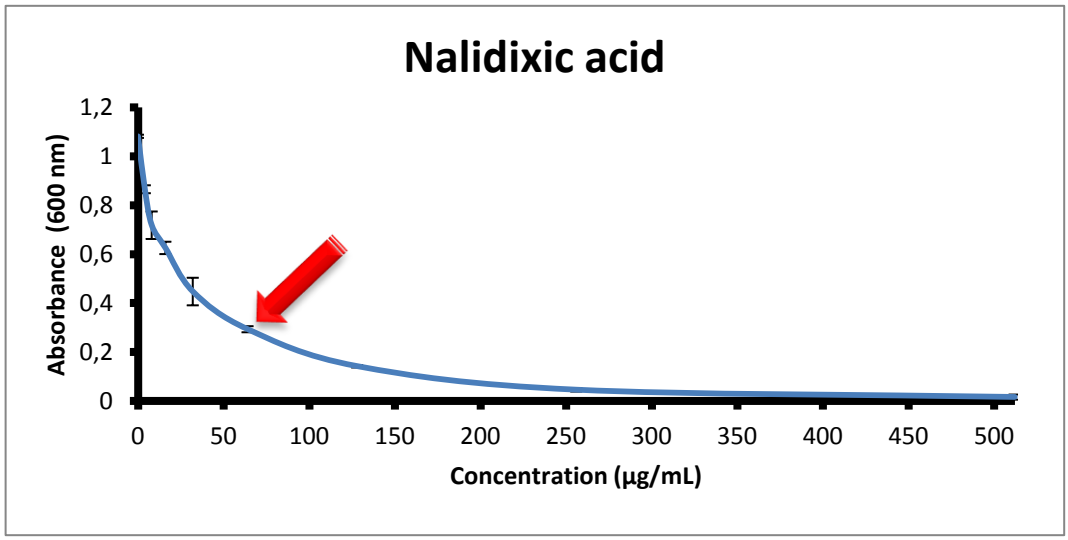

Figure 3.34 Growth of *Proteus mirabilis* at different concentrations of nalidixic acid; MIC value of the antibiotic is indicated with red arrow in the figure.

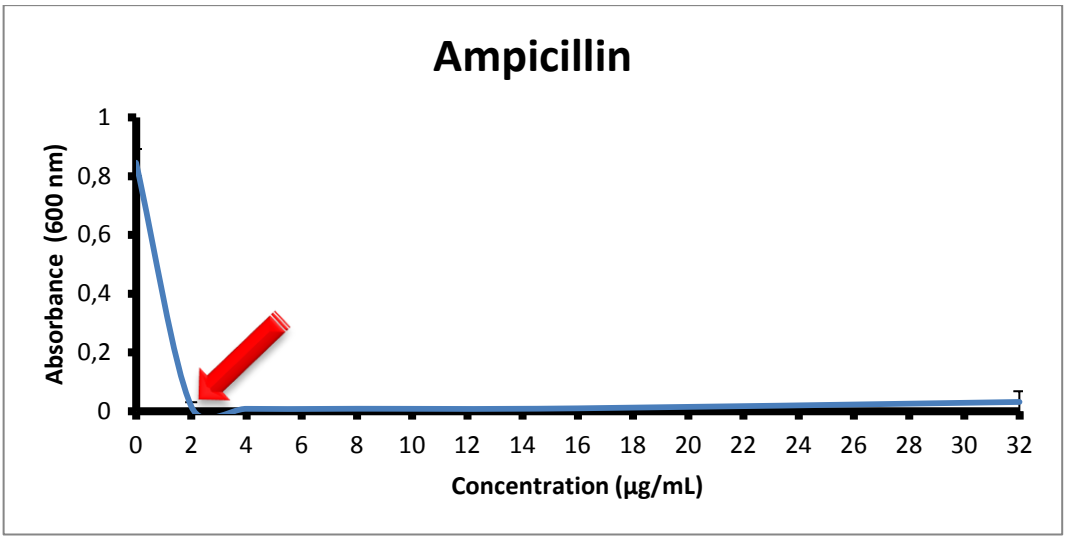

Figure 3.35 Growth of *Proteus mirabilis* at different concentrations of ampicillin; MIC value of the antibiotic is indicated with red arrow in the figure.

3.1.6 Antimicrobial Activity Results by Broth Microdilution Tests for *Pseudomonas aeruginosa*

The results of antimicrobial activity screening of all substances for *Pseudomonas aeruginosa* are shown in Table 3.6.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Pseudomonas aeruginosa* and all of them were recorded as showing an negative effect on growth of the test organism.

Among all antibacterial agents used in the tests, only streptomycin demostrated antimicrobial activity against *Pseudomonas aeruginosa* at the specified concentration range for it while others had no antimicrobial effect on the test organism at the specified concentration range for them.

MIC values of substances were 16 µg/mL for streptomycin, 0.125 µg/mL for ferrocene-amine (G0), ferrocene-PAMAM (G1), ferrocene-PAMAM (G2), ferrocene-PAMAM (G3) as shown in Figure 3.36, Figure 3.39. MIC values of tetracycline, kanamycin, erythromycin, nalidixic acid and ampicillin could not be determined at the concentration range that was tested as shown in Figure 3.37, Figure 3.38, Figure 3.40, Figure 3.41 and Figure 3.42.

MBC values of substances were 64 μg/mL for streptomycin, 0.25 μg/mL for ferrocene-amine (G0) and for ferrocene-PAMAM (G1) while the other substances did not display bactericidal effect on the test organism as shown in Table 3.6.

Table 3.6 MIC and MBC values of substances at the specified concentration range for *Pseudomonas aeruginosa.*

Test Substance	**Concentration range (μg/mL)**	**MIC**	**MBC**
Tetracycline	0.0625 – 4	> 4	-
Kanamycin	0.5 – 8	> 8	-
Streptomycin	16 - 256	16	64
Erythromycin	0.0625 – 1	> 1	-
Nalidixic acid	0.5– 8	> 8	-
Ampicillin	16 - 256	> 256	-
Ferrocene-amine (G0)	0.00781– 0.5	0.125	0.25
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.125	0.25
Ferrocene-PAMAM (G2)	0.00781– 0.5	0.125	-
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.125	-

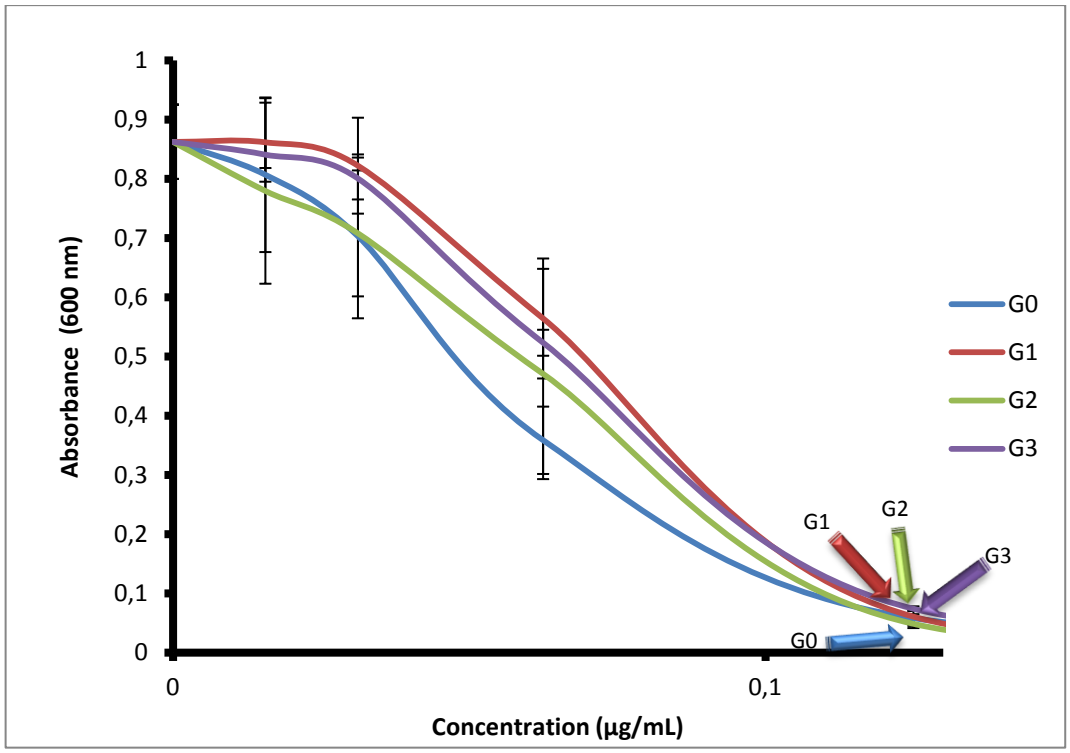

Figure 3.36 Growth of *Pseudomonas aeruginosa* at different concentrations of ferrocene–amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

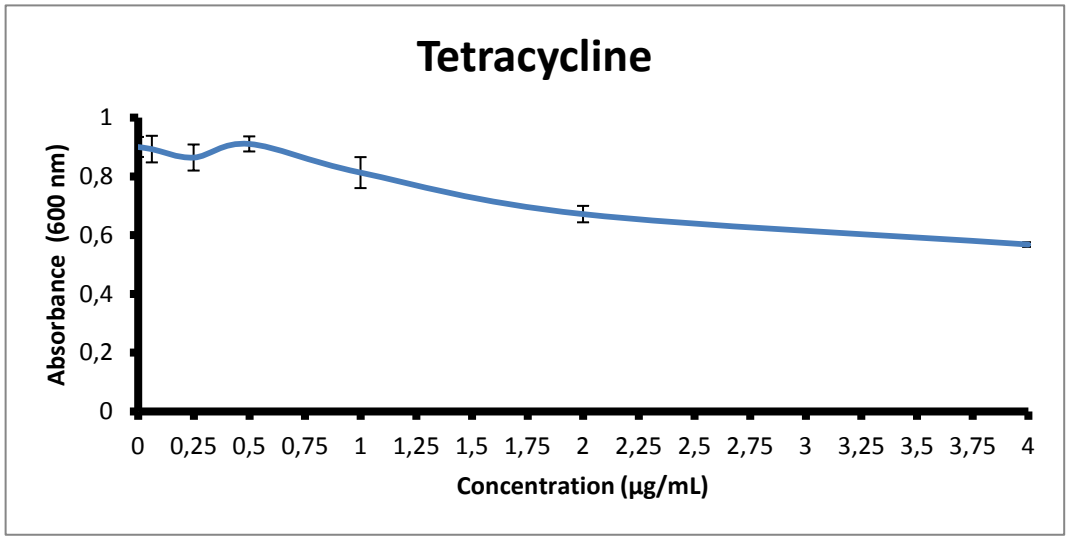

Figure 3.37 Growth of *Pseudomonas aeruginosa* at different concentrations of tetracycline.

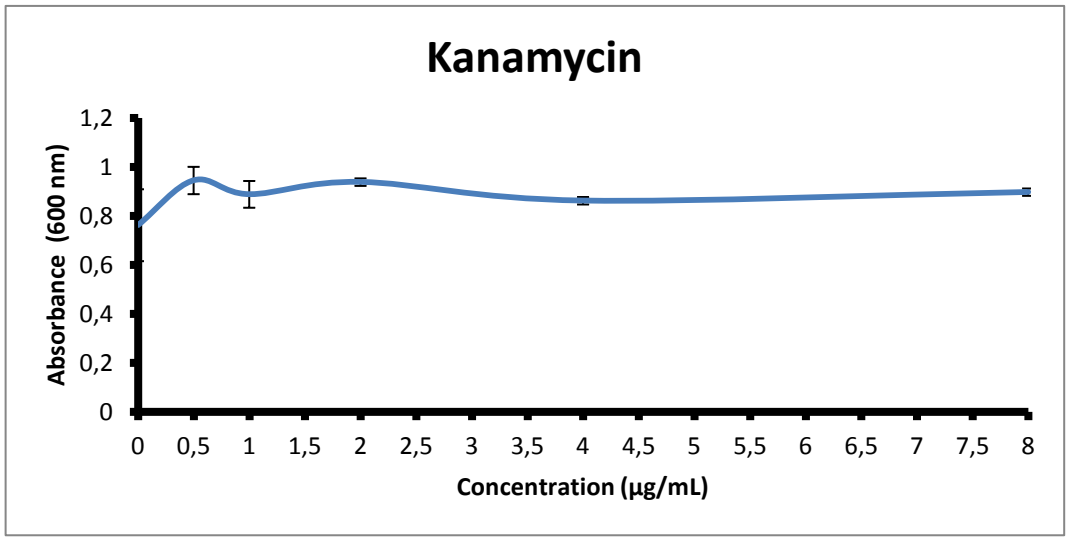

Figure 3.38 Growth of *Pseudomonas aeruginosa* at different concentrations of kanamycin.

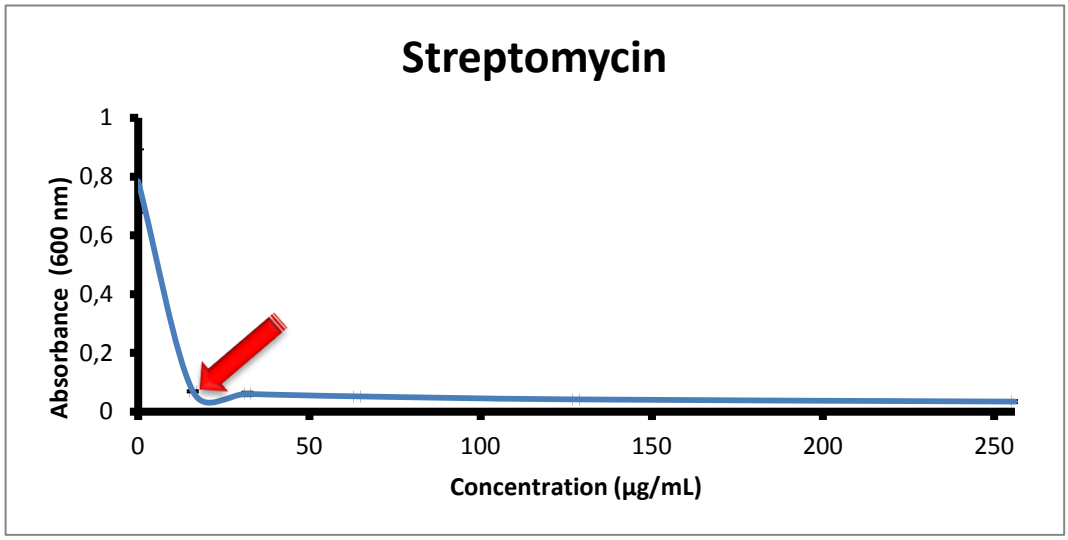

Figure 3.39 Growth of *Pseudomonas aeruginosa* at different concentrations of streptomycin; MIC value of the antibiotic is indicated with red arrow in the figure.

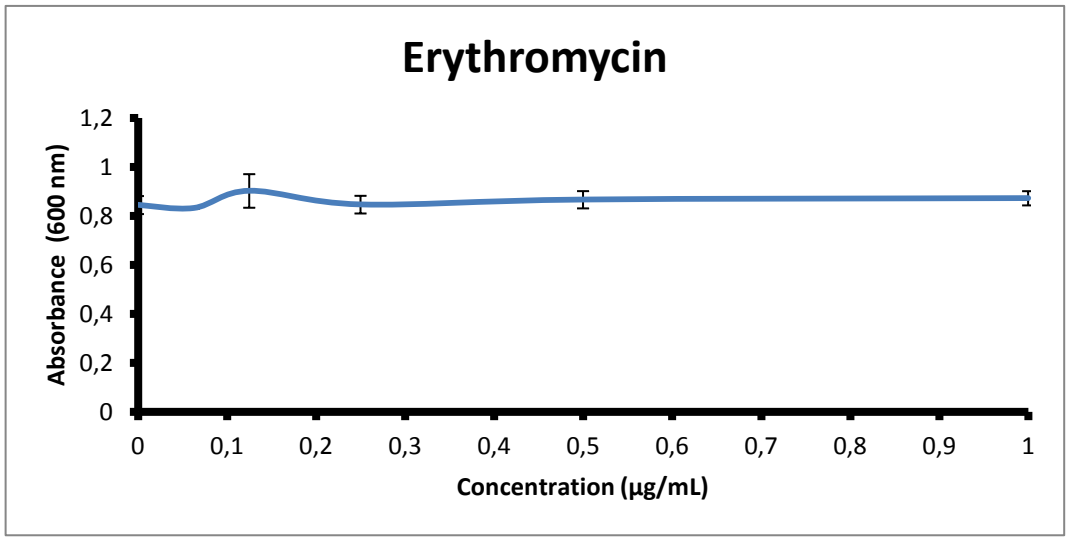

Figure 3.40 Growth of *Pseudomonas aeruginosa* at different concentrations of erythromycin.

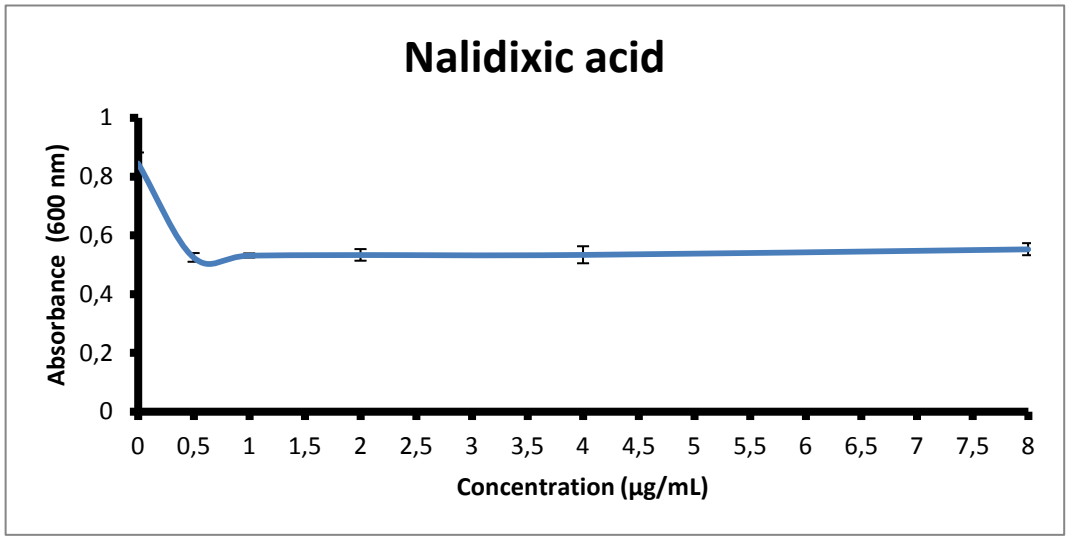

Figure 3.41 Growth of *Pseudomonas aeruginosa* at different concentrations of nalidixic acid.

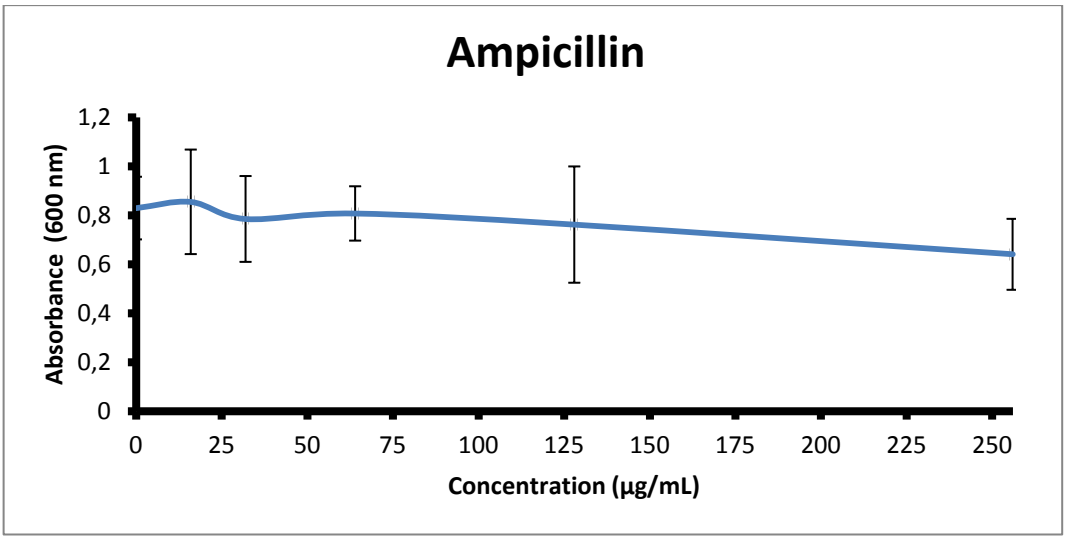

Figure 3.42 Growth of *Pseudomonas aeruginosa* at different concentrations of ampicillin.

3.1.7 Antimicrobial Activity Results by Broth Microdilution Tests for *Staphylococcus aureus*

The results of antimicrobial activity screening of all substances for *Staphylococcus aureus* are shown in Table 3.7.

Among four ferrocene based substances tested, , all substances demonstrated antimicrobial activity against *Staphylococcus aureus* and all of them were recorded as showing an negative effect on growth of the test organism.

Among the all antibacterial agents used in the tests, tetracycline, kanamycin, streptomycin, erythromycin and ampicillin demostrated antimicrobial activity against *Staphylococcus aureus* at the specified concentration range for them.

MIC values of substances were 512 µg/mL for tetracycline, 4 µg/mL for kanamycin, 256 µg/mL for streptomycin, 512 µg/mL for erythromycin, 16 µg/mL for ampicillin, 0.125 µg/mL for ferrocene-amine (G0) and for ferrocene-PAMAM (G1), 0.25 µg/mL for ferrocene-PAMAM (G2) and for ferrocene-PAMAM (G3) as shown in Figure 3.43, Figure 3.44, Figure 3.45, Figure 3.46, Figure 3.47, Figure 3.49. MIC value of nalidixic acid could not be determined at the concentration range that was tested as shown in Figure 3.48.

MBC values of substances were 256 μg/mL for streptomycin, 512 μg/mL for erythromycin, 16 μg/mL for ampicillin, 0.5 μg/mL for ferrocene-amine (G0) and ferrocene-PAMAM (G2) while tetracycline, kanamycin, nalidixic acid, ferrocene-PAMAM (G1) , and ferrocene-PAMAM (G3) did not display bactericidal effect on the test organism at the specified concentration range as shown in Table 3.7.

Table 3.7 MIC and MBC values of substances at the specified concentration range for *Staphylococcus aureus.*

Test Substance	**Concentration range (μg/mL)**	**MIC**	**MBC**
Tetracycline	64 - 512	512	-
Kanamycin	4 - 512	4	-
Streptomycin	16 - 256	256	256
Erythromycin	16 - 512	512	512
Nalidixic acid	32 - 512	> 512	-
Ampicillin	16 - 256	16	16
Ferrocene-amine (G0)	0.00781– 0.5	0.125	0.5
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.125	-
Ferrocene-PAMAM (G2)	0.00781– 0.5	0.25	0.5
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.25	-

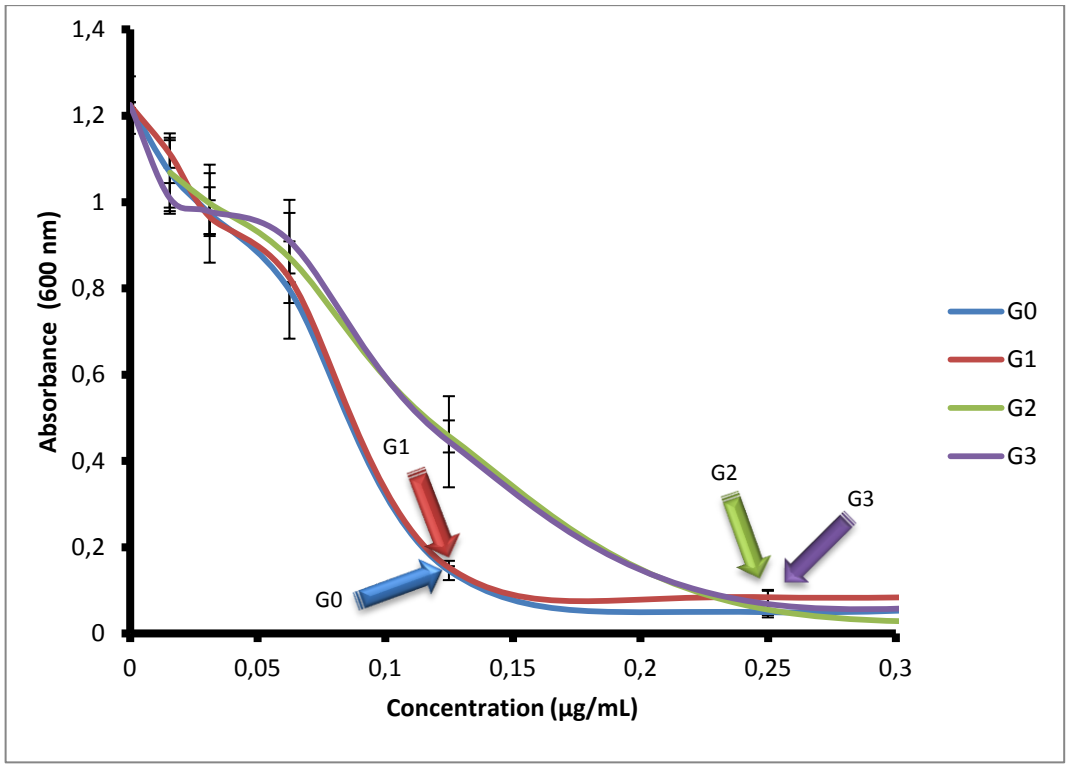

Figure 3.43 Growth of *Staphylococcus aureus* at different concentrations of ferrocene-amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

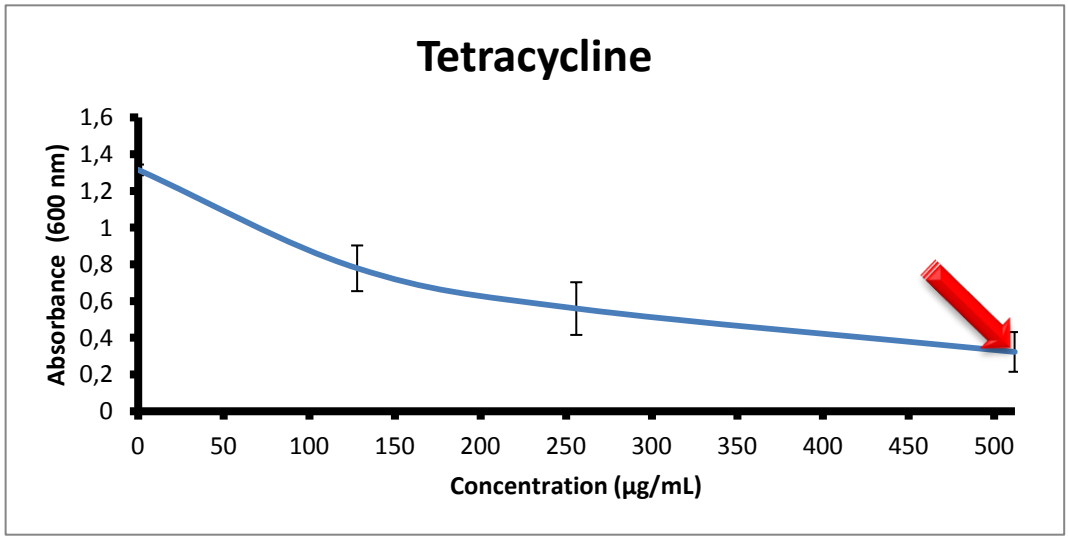

Figure 3.44 Growth of *Staphylococcus aureus* at different concentrations of tetracycline; MIC value of the antibiotic is indicated with red arrow in the figure.

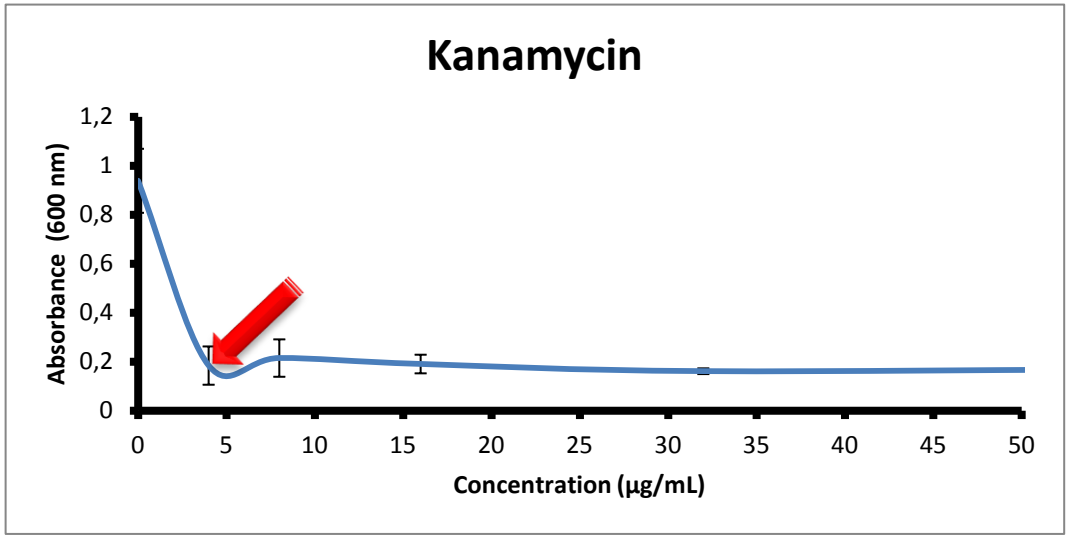

Figure 3.45 Growth of *Staphylococcus aureus* at different concentrations of kanamycin; MIC value of the antibiotic is indicated with red arrow in the figure.

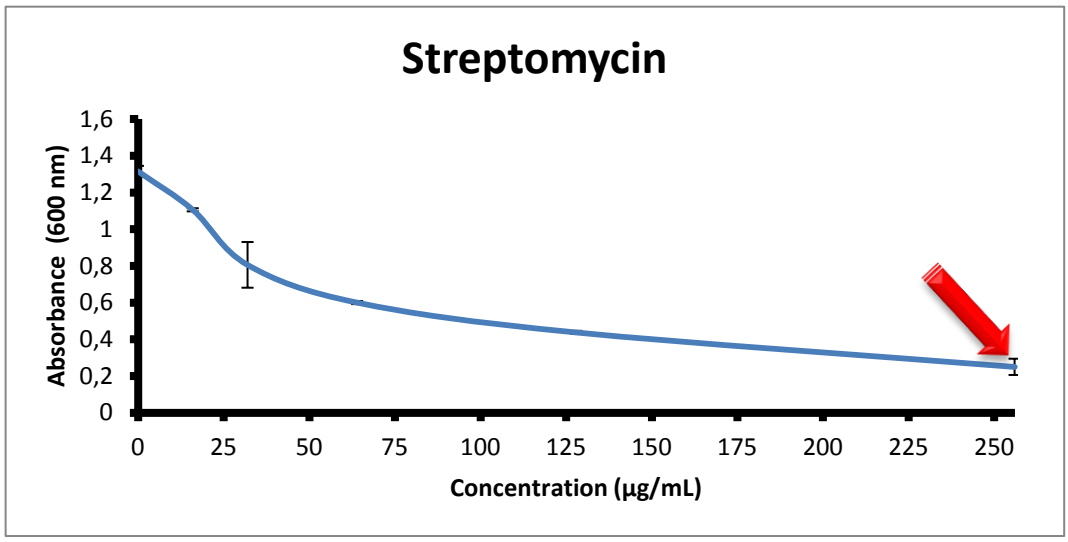

Figure 3.46 Growth of *Staphylococcus aureus* at different concentrations of streptomycin; MIC value of the antibiotic is indicated with red arrow in the figure.

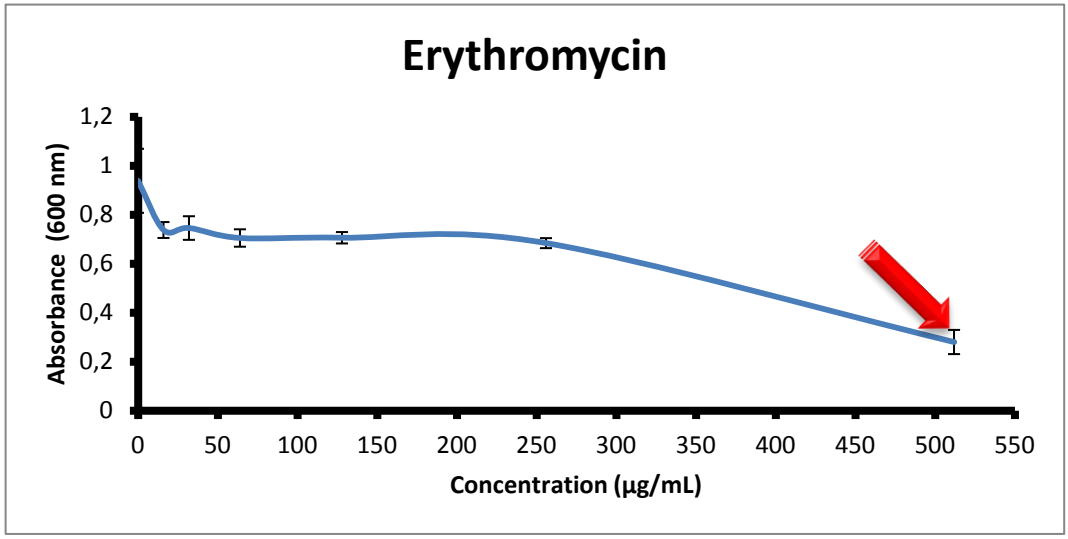

Figure 3.47 Growth of *Staphylococcus aureus* at different concentrations of erythromycin; MIC value of the antibiotic is indicated with red arrow in the figure.

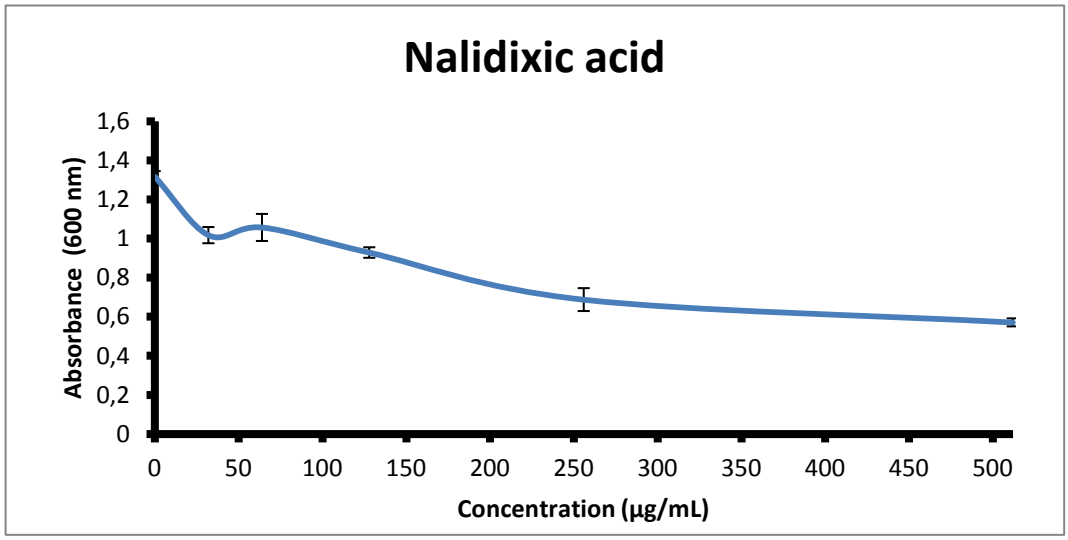

Figure 3.48 Growth of *Staphylococcus aureus* at different concentrations of nalidixic acid.

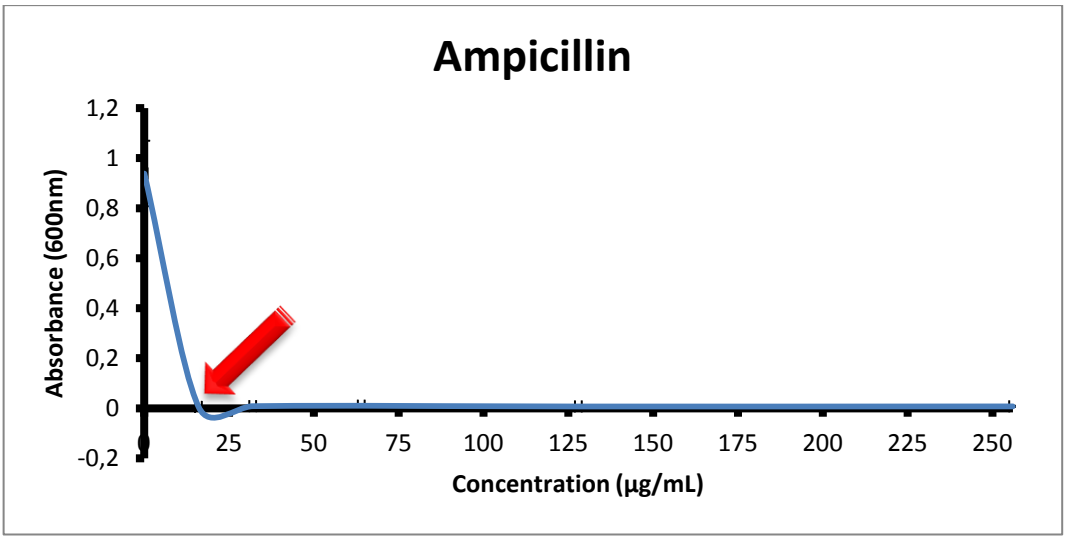

Figure 3.49 Growth of *Staphylococcus aureus* at different concentrations of ampicillin; MIC value of the antibiotic is indicated with red arrow in the figure.

3.2 ANTIMICROBIAL ACTIVITY RESULTS FOR FUNGI

3.2.1 Antimicrobial Activity Results by Broth Microdilution Tests for *Candida albicans*

The results of antimicrobial activity screening of all substances for *Candida albicans* are shown in Table 3.8.

Among four ferrocene based substances tested, , all substances demonstrated antimicrobial activity against *Candida albicans* and all of them were recorded as showing an negative effect on growth of the test organism.

Only one of the antifungal agents used in the tests, amphotericin B, demostrated antimicrobial activity against *Candida albicans* at the specified concentration range for it.

MIC values of substances were 0.5 µg/mL for amphotericin B, 0.125 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G2), for ferrocene-PAMAM (G3) and 0.0625 µg/mL for ferrocene-PAMAM (G1) as shown in Figure 3.50, Figure 3.52. MIC value of nystatin could not be determined at the concentration range that was tested as shown in Figure 3.51.

MBC values of substances were 0.25 μg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G2), for ferrocene-PAMAM (G3) and 0.5 μg/mL for ferrocene-PAMAM (G1) as shown in Table 3.8.

Table 3.8 MIC and MBC values of substances at the specified concentration range for *Candida albicans.*

Test Substance	**Concentration range (μg/mL)**	**MIC**	**MBC**
Nystatin	0.125– 2	>2	-
Amphotericin B	0.0625 – 1	0.5	-
Ferrocene-amine (G0)	0.00781 – 0.5	0.125	0.25
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.0625	0.5
Ferrocene-PAMAM (G2)	0.00781– 0.5	0.125	0.25
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.125	0.25

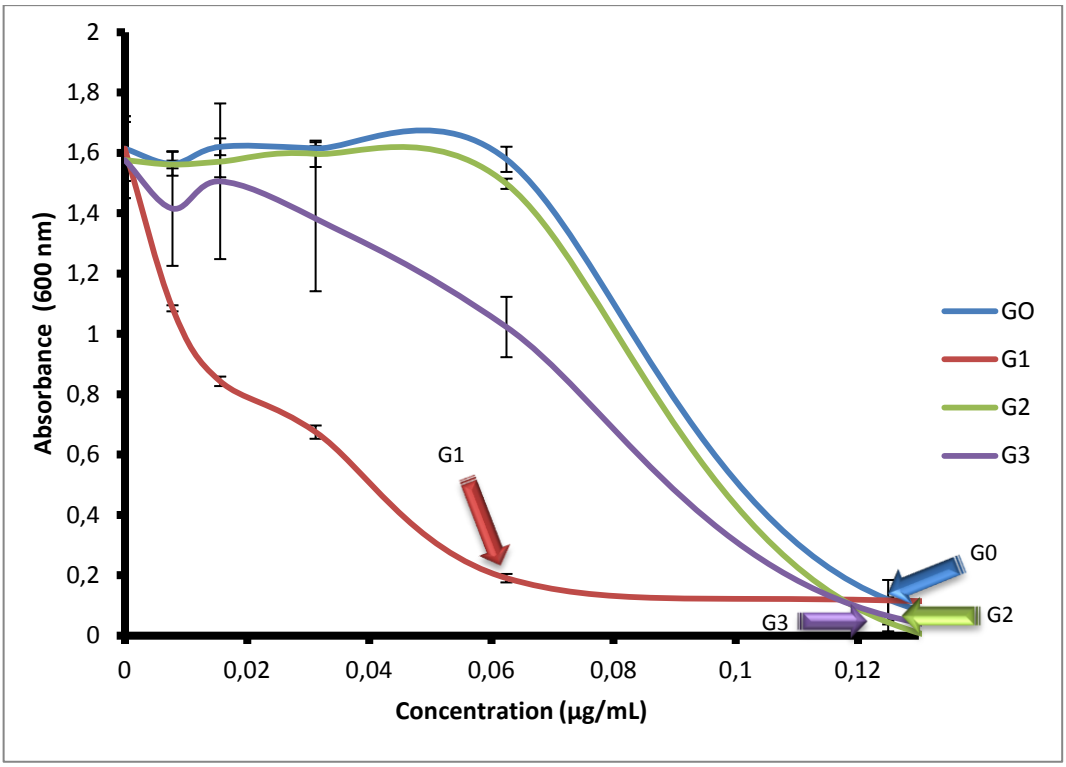

Figure 3.50 Growth of *Candida albicans* at different concentrations of ferrocene-amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

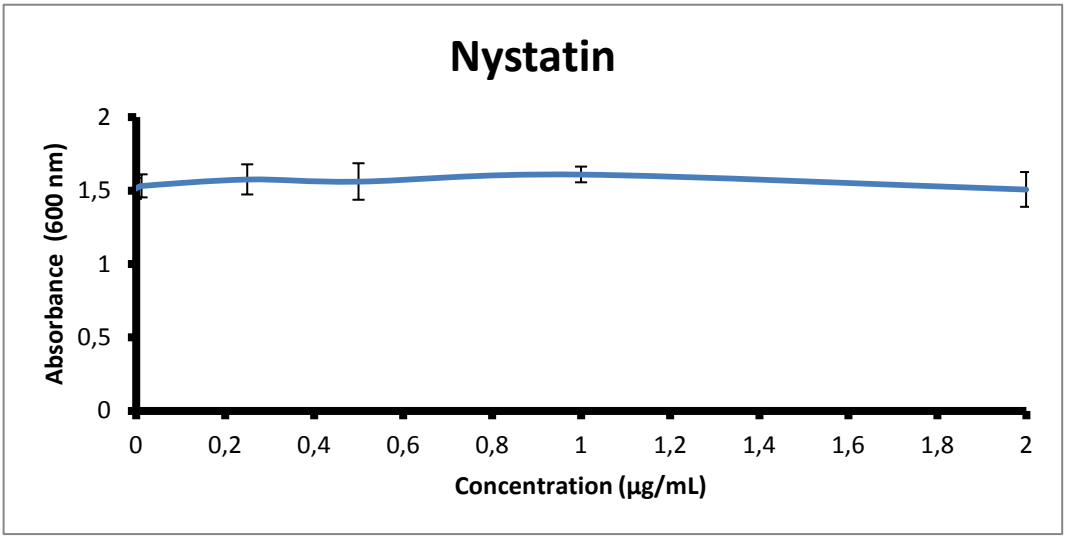

Figure 3.51 Growth of *Candida albicans* at different concentrations of nystatin.

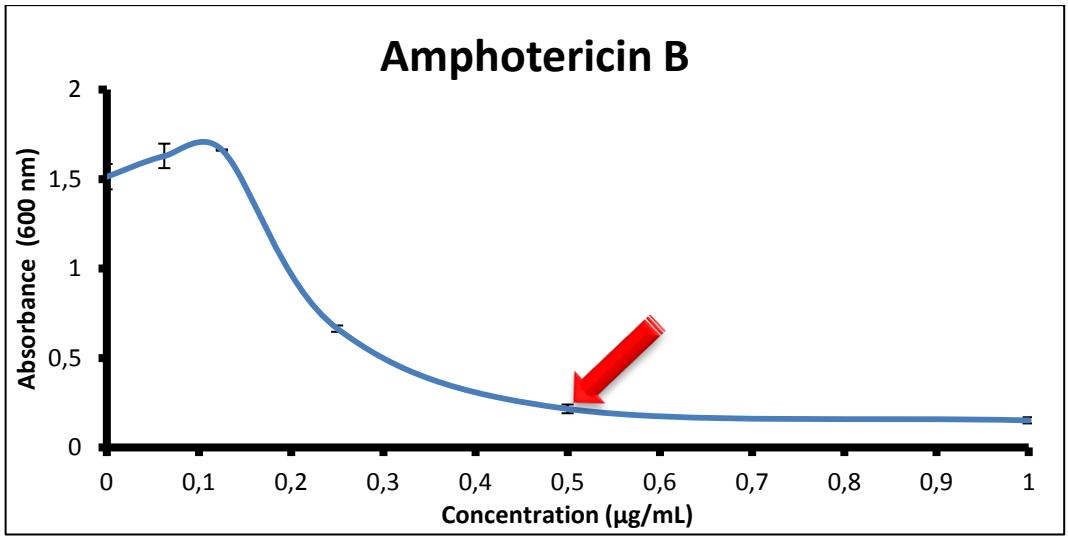

Figure 3.52 Growth of *Candida albicans* at different concentrations of amphotericin B; MIC value of the antibiotic is indicated with red arrow in the figure.

3.2.2 Antimicrobial Activity Results by Broth Microdilution Tests for *Candida utilis*

The results of antimicrobial activity screening of all substances for *Candida utilis* are shown in Table 3.9.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Candida utilis* and all of them were recorded as showing an negative effect on growth of the test organism.

Only one of the antifungal agents used in the tests, amphotericin B, demostrated antimicrobial activity against *Candida utilis* at the specified concentration range for it.

MIC values of substances were 2 µg/mL for amphotericin B, 0.0625 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G1), for ferrocene-PAMAM (G2) and for ferrocene-PAMAM (G3) as shown in Figure 3.53, Figure 3.55. MIC value of nystatin could not be determined at the concentration range that was tested as shown in Figure 3.54.

Table 3.9 MIC and MBC values of substances at the specified concentration range for *Candida utilis.*

Test Substance	**Concentration range (µg/mL)**	**MIC**	**MBC**
Nystatin	1– 16	>16	-
Amphotericin B	2- 32	2	8
Ferrocene-amine (G0)	0.00781– 0.5	0.0625	0.125
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.0625	0.125
Ferrocene-PAMAM (G2)	0.00781 – 0.5	0.0625	0.25
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.0625	0.125

MBC values of substances were 0.125 µg/mL for ferrocene-amine (G0), for ferrocene-PAMAM (G1), for ferrocene-PAMAM (G3) and 0.25 µg/mL for ferrocene-PAMAM (G2) as shown in Table 3.9.

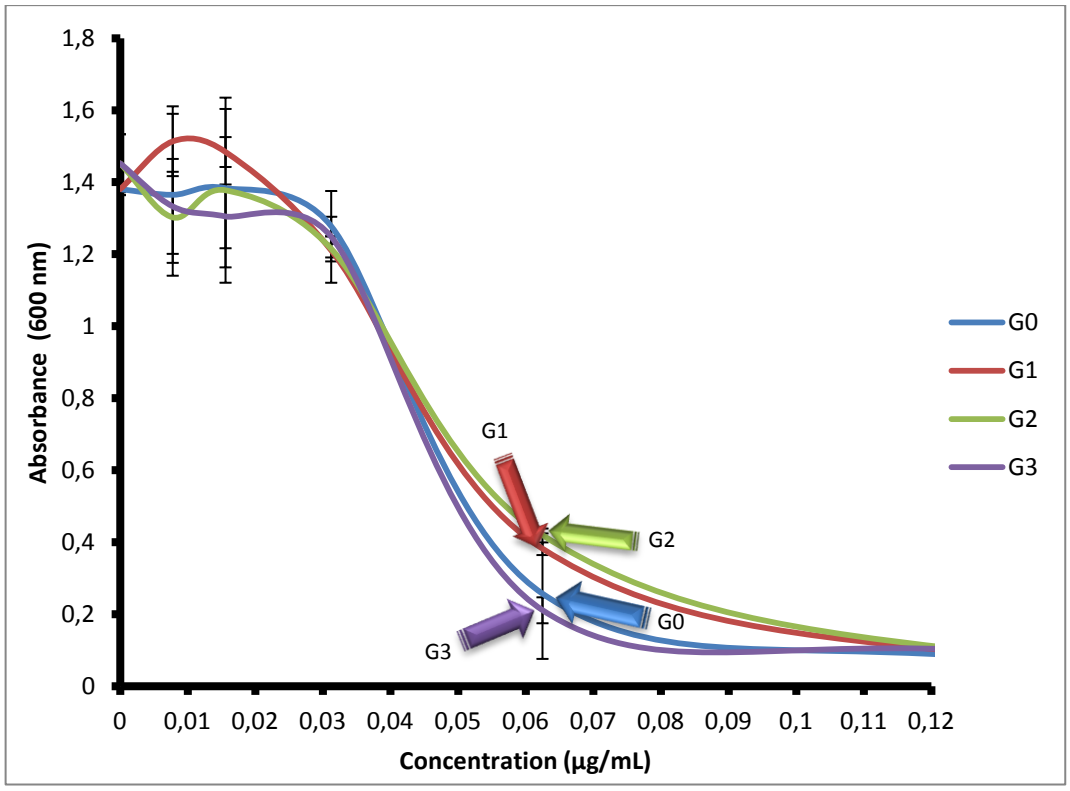

Figure 3.53 Growth of *Candida utilis* at different concentrations of ferrocene–amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

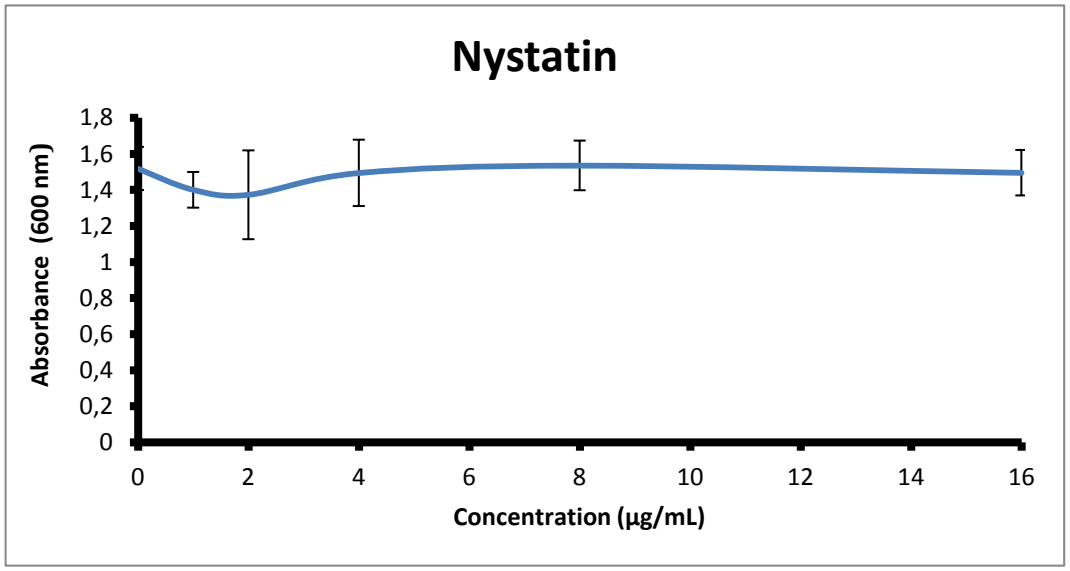

Figure 3.54 Growth of *Candida utilis* at different concentrations of nystatin.

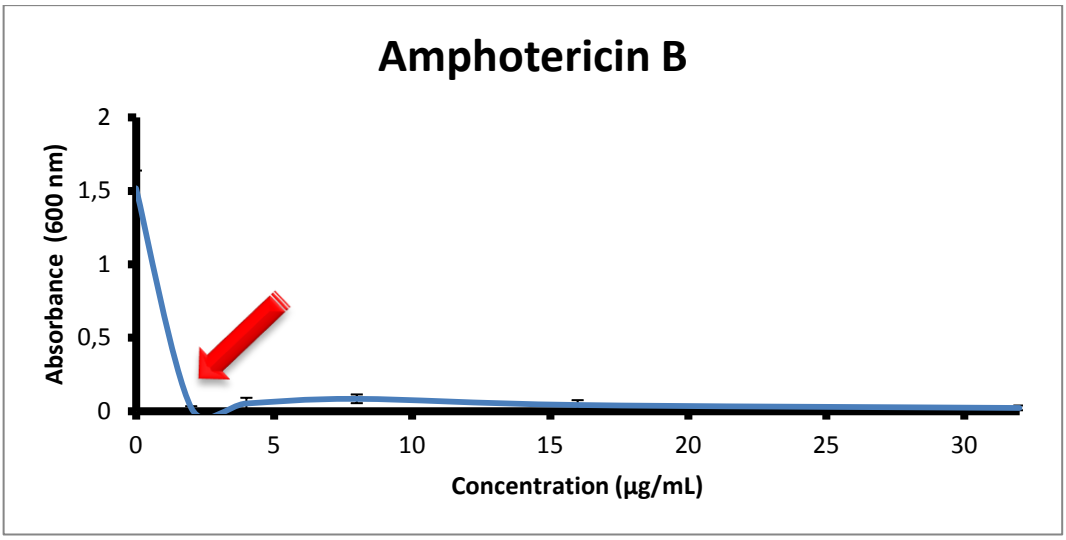

Figure 3.55 Growth of *Candida utilis* at different concentrations of amphotericin B; MIC value of the antibiotic is indicated with red arrow in the figure.

3.2.3 Antimicrobial Activity Results by Broth Microdilution Tests for *Cryptococcus neoformans*

The results of antimicrobial activity screening of all substances for *Cryptococcus neoformans* are shown in Table 3.10.

Among four ferrocene based substances tested, all substances demonstrated antimicrobial activity against *Cryptococcus neoformans* and all of them were recorded as showing an negative effect on growth of the test organism.

Both of the antifungal agents used in the tests demostrated antimicrobial activity against *Cryptococcus neoformans* at the specified concentration range for them.

MIC values of substances were 0.125 µg/mL for nystatin, 4 µg/mL for amphotericin B, 0.0625 µg/mL for ferrocene-amine (G0) and for ferrocene-PAMAM (G3); 0.125 µg/mL for ferrocene-PAMAM (G2), and 0.03125 µg/mL for ferrocene-PAMAM (G1) as shown in Figure 3.56, Figure 3.57, Figure 3.58.

MBC values of substances were 4 µg/mL for amphotericin B, 0.125 µg/mL for ferrocene-amine (G0), ferrocene-PAMAM (G2), ferrocene-PAMAM (G3) and 0.0625 µg/mL for ferrocene-PAMAM (G1) as shown in Table 3.10.

Table 3.10 MIC and MBC values of substances at the specified concentration range for *Cryptococcus neoformans.*

Test Substance	**Concentration range (µg/mL)**	**MIC**	**MBC**
Nystatin	0.125- 2	0.125	-
Amphotericin B	4 - 64	4	4
Ferrocene-amine (G0)	0.00781 – 0.5	0.0625	0.125
Ferrocene-PAMAM (G1)	0.00781– 0.5	0.03125	0.0625
Ferrocene-PAMAM (G2)	0.00781 – 0.5	0.125	0.125
Ferrocene-PAMAM (G3)	0.00781– 0.5	0.0625	0.125

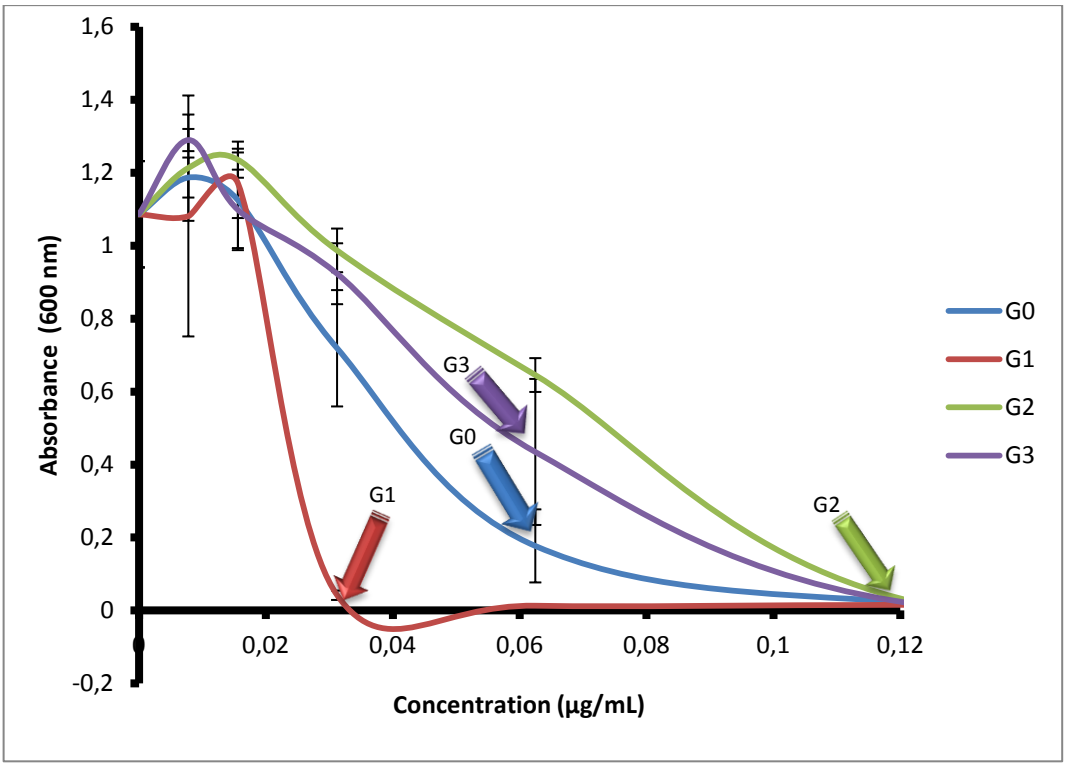

Figure 3.56 Growth of *Cryptococcus neoformans* at different concentrations of ferrocene–amine and ferrocene–PAMAM dendrimers, MIC values of the compounds are indicated with arrows in the figure (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation).

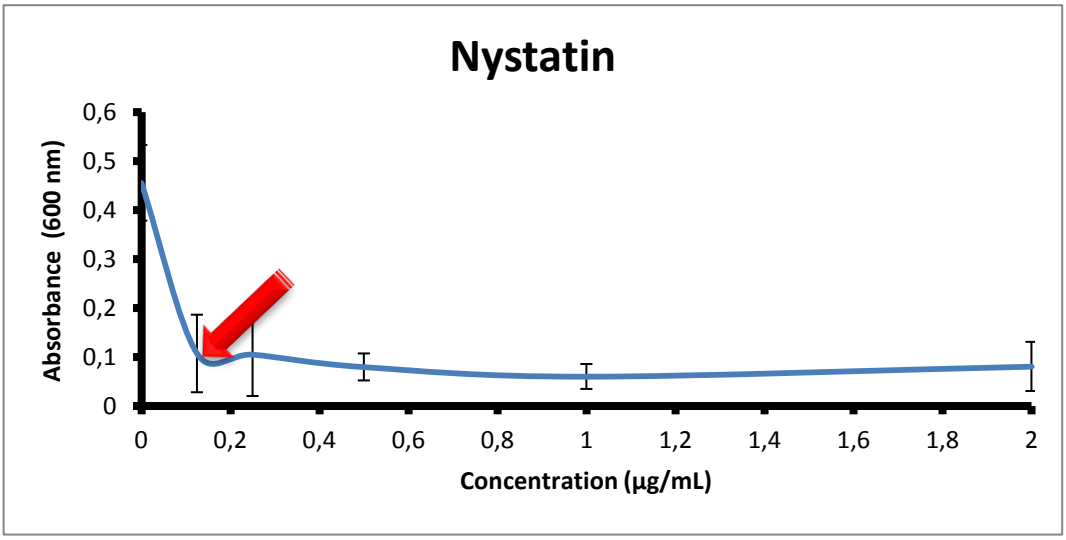

Figure 3.57 Growth of *Cryptococcus neoformans* at different concentrations of nystatin; MIC value of the antibiotic is indicated with red arrow in the figure.

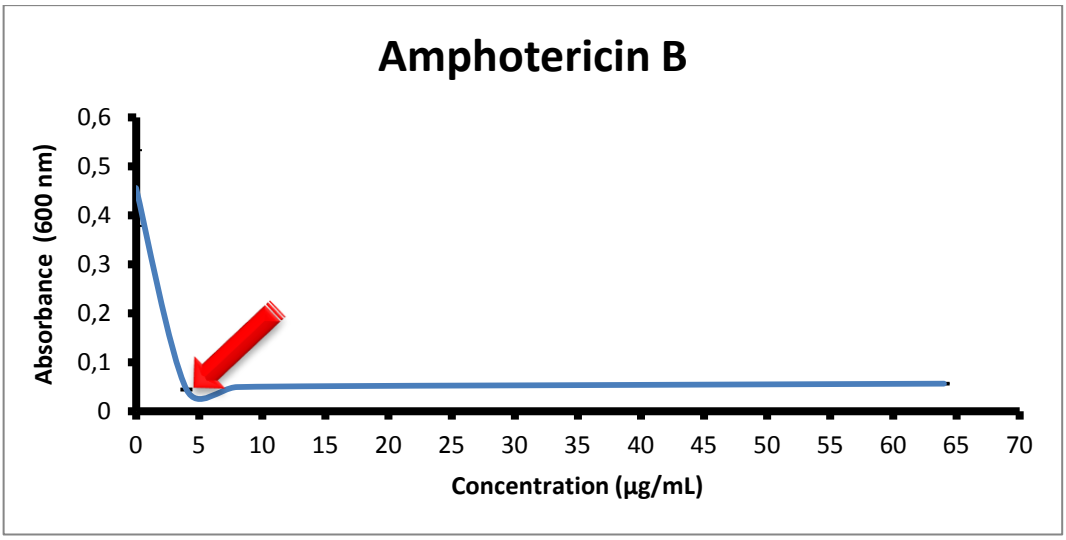

Figure 3.58 Growth of *Cryptococcus neoformans* at different concentrations of amphotericin B; MIC value of the antibiotic is indicated with red arrow in the figure.

3.3 DISCUSSION

Different applications of ferrocene brought out it interest by scientists in the last decade. Ferrocene compounds were used in catalysis, in the construction of chemical sensors and biosensor systems, in the preparation of biologically active compounds especially in medicine [136, 137, 138]. They have been shown effective against different types of cancer and they are expected to use in treatment of some cancer types [136]. Furthermore, some ferrocenyl substituted heterocyclic compounds have been reported to have a high fungicidal activity [139]. So, ferrocene can be incorporated into various organic molecules to increase its molecular activity. In the in vitro and in vivo experiments, some of such substances have been proved to be much more active against microorganisms than parent forms [137, 140].

Application of ferrocene in medicine attracted us to research it as an antimicrobial agent with a drug delivery instrument: PAMAM dendrimer. Since the water solubility of ferrocene is not good, we used PAMAM dendrimers as solubility and cellular permeability enhancers in order to obtain a serious antimicrobial activity [91,93,94,100]. When we compared the MIC, MBC and MFC results for bacteria and fungi, of which results are given in Table 3.11, Table 3.12, Table 3.13 and Table 3.14, with results for various ferrocene derivatives in the literature, it is clear that ferrocene-

PAMAM dendrimers display a much higher activity against both bacteria and fungi. According to literature, various ferrocene derivatives demonstrated their antimicrobial activity against *B.subtilis, E.coli, S.aureus, P.aeruginosa and C.albicans* at 60 µg/mL to 1 mg/mL concentration [136, 137, 138, 141]. In the ferrocene-PAMAM dendrimers experiments, these substances displayed their antimicrobial activity at 0.0625 to 0.125 for *B.subtilis*, at 0.0312 to 0.125 for *E.coli*, at 0.125 to 0.25 for *S.aureus*, at 0.125 for *P.aeruginosa* and at 0.0625 to 0.125 for *C.albicans*. In the literature, there were no data about the bactericidal and fungicidal concentrations of ferrocenyl compounds. In this study, different from previous data, we revealed MBC and MFC values of ferrocene-PAMAM dendrimers.

Table 3.11 MIC values of all substances for bacteria (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation, T.: tetracycline, K.: kanamycin, S.: streptomycin, E.: erythromycin, N.: nalidixic acid, A.: ampicillin).

MIC (µg/mL)	G0	G1	G2	G3	T.	K.	S.	E.	N.	A.
K.pneumonia	0.125	0.0625	0.125	0.125	8	>1	16	>8	128	>2
E.coli	0.125	0.0312	0.125	0.125	1	8	>512	64	16	16
B.subtilis	0.125	0.0625	0.125	0.125	1	16	8	> 0.5	32	> 512
C.freundii	0.125	0.0625	0.125	0.125	4	8	32	512	64	0.5
P.mirabilis	0,125	0,125	0,125	0,125	8	4	256	256	64	2
P.aeruginosa	0.125	0.125	0.125	0.125	> 4	> 8	16	> 1	> 8	> 256
S.aureus	0.125	0.125	0.25	0.25	512	4	256	512	> 512	16

Table 3.12 MIC values of all substances for fungi (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation, Nysta.:Nystatin, Ampho.B.: Amphotericin B).

MIC (µg/mL)	G0	G1	G2	G3	Nysta.	Ampho.B
C.utilis	0.0625	0.0625	0.0625	0.0625	>16	2
C.albicans	0.125	0.0625	0.125	0.125	>2	0.5
C.neoformans	0.0625	0.03125	0.125	0.0625	0.125	4

Table 3.13 MBC values of all substances (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation, T.: tetracycline, K.: kanamycin, S.: streptomycin, E.: erythromycin, N.: nalidixic acid, A.: ampicillin).

MBC (µg/mL)	G0	G1	G2	G3	T.	K.	S.	E.	N.	A.
K.pneumonia	0. 25	0.5	-	-	-	-	64	-	-	-
E.coli	-	0.5	0.5	-	-	8	-	64	-	32
B.subtilis	-	0.0125	-	-	-	-	16	-	-	-
C.freundii	0. 25	0. 5	0. 5	0.5	-	-	-	512	-	-
P.mirabilis	-	-	-	-	256	4	256	256	64	2
P.aeruginosa	0.25	0.25	-	-	-	-	64	-	-	-
S.aureus	0. 5	-	0.5	-	-	-	256	512	-	16

Table 3.14 MFC values of all substances (G0: Ferrocene-amine, G1: Ferrocene-PAMAM dendrimer first generation, G2: Ferrocene-PAMAM dendrimer second generation, G3: Ferrocene-PAMAM dendrimer third generation, Nysta.:Nystatin, Ampho.B.: Amphotericin B).

MFC (µg/mL)	G0	G1	G2	G3	Nysta.	Ampho.B
C.utilis	0.125	0.125	0. 25	0.125	-	8
C.albicans	0.25	0.5	0.25	0. 25	-	-
C.neoformans	0.125	0.0625	0.125	0.125	-	4

PAMAM dendrimers tend to make ferrocene more effective and potent antimicrobial agents, hence ferrocene can inhibit or kill micrrorganisms even at very low concentration ranges. The possible explanation of this is that PAMAM dedrimers, correlated with generation number, increase the solubility of ferrocene in water and uptake of ferrocene by cells. Apart from these, surface features of dendrimers may be another reason of increased antimicrobial especially fungicidal activity.

CHAPTER 4

CONCLUSIONS

Ferrocene is a compound displaying antimicrobial activity on some microorganisms and PAMAM dendrimers are instruments that enhance solubility of drugs in the cells and used for drug delivery applications. In this study, we investigated antimicrobial activity of ferrocene – PAMAM dendrimers against seven pathogenic bacterial strains and three pathogenic fungal strains.

Antimicrobial activity screening analysises were performed by using broth microdilution method that measures the growth of microorganisms via a micro plate reader at 600 nm. According to results, for both fungi and bacteria, ferrocene – amine and three different generations of ferrocene – PAMAM dendrimers were much more effective against microorganisms at least ten fold lower concentrations than that of antibiotics. Among all test substances, not all but generally ferrocene – PAMAM (G1) dendrimer had the highest antimicrobial activity against bacteria except *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis.* Its MIC value against *S.aureus* was equal to ferrocene – amine and higher than others (G2 andG3) while MIC values for *P.aeruginosa and P.mirabilis* were same for the all test substances. Ferrocene – PAMAM (G1) dendrimer had also highest activity against fungi except *C.utilis* for whose MIC values were equal to all others. Antimicrobial activity of ferrocene – PAMAM (G2) dendrimer, ferrocene – PAMAM (G3) dendrimer, and ferrocene – amine were very close to each other.

The test substances had either bacteriostatic or bactericidal effect for bacteria while they had fungicidal effect even at very low concentrations for all fungal organisms used in the study. The success of substances at even low concentrations may be because of the fact that dendrimers are good drug delivery instruments.

The cause of the noteworthy fungicidal effect of PAMAM dendrimers at low concentrations may be the structure of their surface. When a positively charged surface of PAMAM dendrimer interacts with the negatively charged surface of eukaryotic cell membrane, this interaction may result in disruption of cell membrane and, therefore, a toxic effect on the cell.

In the experiments, test substance solutions were prepared according to having equal total moleculer weight per mililiter for all test substances. Due to that, the ferrocene amount in the solutions decreased while generation number of PAMAM dendrimers, so PAMAM amount, in the solutions increased. In conclusion, the antimicrobial activity is higher in the first generation of ferrocene – PAMAM dendrimers possibly because of the higher ratio of ferrocene moiety in G1 PAMAMs while G2 and G3 did not show a high antimicrobial activity as much as G1 because of decreasing ferrocene amounts with increased PAMAM amount. In addition, despite of high ferrocene ratio, ferrocene-amine did not show high activity as G1 because of the absence of PAMAM dendrimers. But, all of them may be used as antimicrobial agents as they were more successful than the control antibiotics notably.

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