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Investigating the Growth Kinetics of Gram-Positive and Gram-Negative Bacteria in the Presence of Zinc Oxide Nanoparticles and Curcumin

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by

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This is to certify that we have read the thesis **Investigating the Growth Kinetics of Gram-Positive and Gram-Negative Bacteria in the Presence of Zinc Oxide Nanoparticles and Curcumin** submitted by **Mehmet Baran Karakaplan**, and it has been judged to be successful, in scope and in quality, at the defense exam and accepted by our jury as a MASTER'S THESIS.

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I, **Mehmet Baran KARAKAPLAN**, declare that this thesis titled **Investigating the Growth Kinetics of Gram-Positive and Gram-Negative Bacteria in the Presence of Zinc Oxide Nanoparticles and Curcumin** and the work presented in it are my own. I confirm that:

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Investigating the Growth Kinetics of Gram-Positive and Gram-Negative Bacteria in the Presence of Zinc Oxide Nanoparticles and Curcumin

Abstract

Antimicrobial resistance is one of the leading global health threats for human health according to World Health Organization (WHO). The excess or misuse of antibiotics to combat microorganisms causes the generation of a resistant strain of microorganisms. Because of the low efficiency of antibiotics against resistant strains, researchers have leaned to alternative approaches despite conventional antibiotics. With the rapid development of nanomedicine, diverse approaches were investigated to solve antimicrobial resistance problems using nanoparticles. Metal oxide nanoparticles with their high stability and multi-action mechanisms against microorganisms make them efficient for antimicrobial therapy. One of the most outstanding metal oxide nanoparticles is zinc oxide nanoparticles (ZnO NPs). In many studies, ZnO NPs conclude their antibacterial activity in both gram-negative and gram-positive bacteria strains. Curcumin is an herbal extract natural material which is used in medicine for many years. Although the antibacterial activity of curcumin is demonstrated, its bioavailability and low dispersion characteristic are the limitations for biological applications.

In this study, the antibacterial efficiency of two distinct materials, ZnO NPs, and curcumin, were investigated on gram-negative (*E. coli ATCC 25922*) and gram-

positive (*S. aureus ATCC 29213* and *E. faecalis ATCC 29212*) bacterial species. ZnO NPs were synthesized via the sol-gel method and their characterization was performed using dynamic light scattering (DLS) for size and zeta potential (ζ-potential) analysis and scanning electron microscopy for morphology analysis. The results of characterization demonstrated that ZnO NPs were synthesized successfully with 207.6 nm hydrodynamic size and 16.2 mV ζ-potential. The antibacterial efficiency of ZnO NPs and curcumin were investigated using kinetic optical density (OD) and Alamar Blue Assay on gram-negative (*E. coli ATCC 25922*) and gram-positive (*S. aureus ATCC 29213* and *E. faecalis ATCC 29212*). Also, the growth rate, μ and doubling time t_d were evaluated using growth curves obtained from kinetic OD measurements. Checkboard tests were applied to investigate the combinatory effect of ZnO NPs and curcumin. The results reveal that cell viability of bacterial strains was decreased under the effect of increasing concentrations of curcumin while ZnO NPs are effective for *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* due to Alamar Blue Assay. The growth rate, μ of bacterial strains were attenuated under the effect of ZnO NPs and curcumin individually.

Keywords: Growth rate, doubling time, curcumin, zinc oxide nanoparticles, metal oxide nanoparticles.

Çinko Oksit Nanopartiküller ve Kurkumin Varlığında Gram Pozitif ve Gram Negatif Bakterilerin Büyüme Kinetiğinin İncelenmesi

Öz

Antimikrobiyal direnç, Dünya Sağlık Örgütü'ne (DSÖ) göre önde gelen küresel sağlık sorunlarından birisidir. Antibiyotiklerin bilinçsiz ve fazla kullanımı, dirençli mikroorganizma türlerinin oluşmasına neden olmuştur. Antibiyotiklerin dirençli suşlara karşı etkinliğinin düşük olması nedeniyle araştırmalar geleneksel antibiyotiklerin yerine geçebilecek alternatif yaklaşımlara yönelmiştir. Nanotıbbın hızlı gelişimi ile birlikte, araştırmacılar nanopartikülleri kullanarak çözümler üretmeyi amaçlamışlardır. Metal oksit nanoparçacıklar, yüksek stabilite ve mikroorganizmalara karşı gösterdiği çoklu etki nedeniyle öne çıkmaktadırlar. Bunların en öne çıkanlarından biri çinko oksit nanoparçacıklardır (ZnO NP). Birçok çalışmada, ZnO NP'lari, antibakteriyel aktivitesini hem gram negatif hem de gram pozitif bakteri suşlarında göstermiştir. Kurkumin, tıpta uzun yıllardır kullanılan bitkisel kaynaklı doğal bir malzemedir. Kurkuminin antibakteriyel aktivitesi bilinmesine rağmen, düşük biyoyararlanım ve düşük dispersibilite gibi sorunları mevcuttur.

Bu çalışmada, ZnO NP'ler ve kurkuminin gram negatif *(E. coli ATCC 25922*) ve gram pozitif (*S. aureus ATCC 29213 ve E. faecalis ATCC 29212*) bakteri türleri üzerinde ki antibakteriyel etkinliği araştırıldı. ZnO NP'leri sol-gel yöntemi ile sentezlendi. Boyutu ve zeta potansiyeli (ζ-potansiyel) için dinamik ışık saçılımı (DLS) ve morfoloji analizi için ise taramalı elektron mikroskobu (SEM) kullanıldı. Karakterizasyon sonuçlarına göre, ZnO NP'lerin 207.6 nm hidrodinamik boyut ve 16.2 mV ζ-potansiyeli sahip

olduğu belirlenmiştir. ZnO NP'lerin ve kurkuminin antibakteriyel etkinliği, gram negatif (*E. coli ATCC 25922*) ve gram pozitif (*S. aureus ATCC 29213* ve *E. faecalis ATCC 29212*) üzerinde kinetik optik yoğunluk (Optical Density) ve Alamar Blue Testi kullanılarak incelendi. Ayrıca büyüme hızı (growth rate), μ ve iki katına çıkma süresi (doubling time) t_d, kinetik OD ölçümlerinden elde edilen büyüme eğrileri kullanılarak hesaplandı. Alamar Blue Test sonuçlarına göre, artan kurkumin konsantrasyonlarının etkisi altında bakteri suşlarının hücre canlılığının azaldığı gözlemlenmiştir. Buna karşın ZnO NP, *S. aureus ATCC 29213 ve E. faecalis ATCC 29212* üzerinde etkili olmuştur. Fakat büyüme hızları ve iki katına çıkma süreleri incelendiğin hem kurkuminin hem de ZnO NP antibakteriyel etkisini 3 bakteri suşunda da gösterdiği gözlemlenmiştir.

Anahtar Kelimeler: Büyüme hızı, ikiye katlanma süresi, kurkumin, çinko oksit nanoparçacıkları, metal oksit nanoparçacıkları.

To my dear family…

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Mehmet Baran KARAKAPLAN

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ATCC	American Type Culture Collection
WHO	World Health Organization
ZnO	Zinc Oxide Nanoparticles
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
DLS	Dynamic Light Scattering
OD	Optical Density
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
MIC	Minimum Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
CFU	Colony Forming Unit
PDI	Poly Dispersity Index
MRI	Magnetic Resonance Imaging
(RBITC)	Rhodamine B isothiocyanate

List of Symbols

Zn^{+2}	Zinc Ion
μ	Growth Rate
ζ	Zeta
μg	Microgram
mL	Milliliter
Z’	Z Prime
t_d	Doubling Time
Ag	Silver
Au	Gold

Chapter 1

Introduction

As per World Health Organization (WHO), antimicrobial resistance is one of the leading health issues in the 21st century [1]. The excess use of antibiotics in order to kill the microorganisms or slow down their growth pave the way for the generation of antibiotic-resistant bacteria [2]. Since the available antibiotics are less effective or ineffective [3], antibiotic-based treatments have become insufficient. Additionally, biofilm formations of microbial make them more pathogenic and resistant to treatments due to their easy adaptiveness in a given environment [4].

Chemical approaches and materials which is utilized for medical purposes provide diverse antibiotic alternative materials. These materials are used to treat bacterial infections in order to avoid the generation of resistant strains of bacteria [5]. One of these approaches is nanomaterials which destruct the cell wall of the bacteria [6]. The vaccines which promote the immune system offer preventative approaches against bacterial infections [7]. The other strategy is the peptide nucleic acids which suppress the gene expression of the bacteria [8]. Besides the bio-molecular or material-based solutions for antimicrobial resistance, plasma [9] and photodynamic therapy [10] are used in order to combat resistant strains of bacteria. The biofilm formation and quorum sensing of bacteria engender the survival of the bacteria under extreme conditions [11]. Quorum sensing is one of the ways which provides communication between bacteria [12]. Inhibiting these routes via quorum sensing inhibitors is the strategy that aims to control virulence and so the immune system can easily fight pathogens [13].

The other outstanding approach is antimicrobial peptides [14]. The action mechanism of antimicrobial peptides is attributed to interaction with bacterial cell membrane thus resulting in cell lysis [15]. Also, these structures destroy the bacterial cell via electrostatic interactions which avoids the development of antimicrobial resistance

[16]. Although short half-life of antimicrobial peptides in body fluids are a disadvantage thus limiting the clinical applications, covalent immobilization of positive charged antimicrobial peptides onto suitable hydrogel substrates increases long-term stability [17]. Again the covalent immobilization strategy of antimicrobial peptides paved the way for usage in the clinic. Dutta et al. used the melimine peptides to coat contact lenses and observed high antibacterial activity in humans and rabbits [18].

Nanoparticles are materials with a high surface to volume ratio and tunable physical and chemical properties. In fact %90 of the total mass of nanoparticles is on the surface [19]. So, a high surface volume ratio could be exploited in different biomedical applications since they are capable of penetrating biological systems and interact with them much more than bulk materials. The application of nanomaterial in medicine is called "nanomedicine". The recent advances in the field of nanomedicine have directed to combat bacteria and their resistant strains [20] in addition to biomedical, pharmaceutical, imaging and drug delivery applications [21–24].

The high surface interaction and penetration ability of nanoparticles could be utilized in various clinical applications. For instance; semiconductor nanomaterials named quantum dots are used for tumor imaging and targeting [25]. In another study, gold (Au) nanoparticles which are able to absorb the X-ray and coated with gadolinium chelates are used in multimodal imaging of X-ray and magnetic resonance (MRI) imaging. The obtained core-shell structure can freely circulate in blood without accumulation in the lungs, spleen and liver [26]. Since the nanoparticle surface can be modified with diverse purposes, fluorescent modifications of nanoparticles serves as optical imaging agents [27]. An example of this goal was achieved by Yang et al. monodisperse silica-coated manganese oxide nanoparticles (NPs) were modified with a fluorescent dye, Rhodamine B isothiocyanate (RBITC), and folate (FA) onto a surface and this structure was used both magnetic resonance and fluorescence imaging.

Also, nanoparticles are good drug delivery agents thanks to their biocompatibility, controllable pore volume, controllable size and high surface to volume ratio [28]. Besides that, their capacity to encapsulate drugs increases the solubility in comparison to conventional drugs [29]. The enhanced permeability and retention effect of tumor tissues means that tumor tissues constrain more nanoparticles, liposomes, proteins

rather than healthy tissues contribute accumulation of nanoparticles in target tumor tissue. The reason for this contribution is regarded with tunable specific size and shape of nanoparticles [30]. Another contribution of nanoparticles to drug delivery approaches is providing controlled and targeted release of the drugs. It is important fact since the controlled release of the drugs can keep the drug concentration in the therapeutic window for long-term treatments [31]. In contrast, burst release of drugs necessary for fast intervention needed disease [32].

Aforementioned, nanoparticles are one most promising materials to combat antimicrobial resistance. One of the strategies to provide antibacterial activity is combining nanoparticles with drug (i.e. antibiotics) molecules and enhancing their release kinetics and antibacterial activity [33]. The most promising nanoparticle in this field is polymeric nanoparticles [33]. They have the ability to decrease the side effect of the drugs [34] and enhance water solubility [35]. Moreover, they are able to overcome biological barriers [36]. Poly-(d,l-lactide-co-glycolide) nanoparticles doped with violacein, an antibiotic and antiviral with poor solubility, were performed on methicillin-resistant *S. aureus* (MRSA). The violacein doped PLGA nanoparticles decreased the minimum inhibitory concentration (MIC) 5 times rather than free violacein. Additionally, the results revealed that the sustained release for violacein could be provided for 5 days [37]. In another study, solid nanoparticles were loaded with Rifampin which was used for tuberculosis therapy. The obtained results established that the MIC of nano-based formulation decreases the MIC 8 times [38]. The antibacterial mechanism of this structure is attributed to the structure of solid lipid nanoparticles can provide easy penetration into the cell wall of the bacteria. Same time, the solid lipid nanoparticles are effective to hinder the P-gp pumps of bacterial strains [39]. It is known that these pumps are one main reason for antimicrobial resistance [40].

Metal oxide nanoparticles are prominent because of their unique physical and chemical properties thus, offering versatility. This versatility of metal oxide nanoparticles makes them efficient antibacterial agents as an alternative to conventional antibiotics [41]. Also, developing resistance against metal oxide nanoparticles is very tough since the mode of action of metal oxide nanoparticles differs from conventional antibiotics [42] and having a multiple action mechanism [43]. Antibacterial activity of metal oxide

nanoparticles is related to their size in addition to the surface area to volume ratio, crystalline structure, and surface chemistry [44], [45]. Besides that, the shape of the nanoparticles influences the antibacterial activity and mode of action [46]. In a study achieved by Pal et al., silver (Ag) nanoparticles demonstrated shape-related antibacterial activity. These shape-related activities generally were attributed to the interaction between nanoparticle and bacterial surfaces. The high aspect ratio of nanoparticles which is placed their long axis parallel to the bacteria membrane increases surface attachment [47]. Hence, the antibacterial activity of nanoparticles, even indirectly, is related to the surface area. The other criteria of antibacterial activity are the size of the nanoparticles [48]. It is known that metal oxide nanoparticles establish better antibacterial activity rather than bulk materials [49,50]. This is because smaller nanoparticles are able to cover the surface of bacteria at a high percentage [51] and the discussion in the literature findings is that nanoparticles smaller than 30 nm are able to penetrate the bacterial membrane and initiate an antibacterial activity [52]. Raza et al. examine the antibacterial activity of Ag nanoparticles at different size and shape on *P.aeruginosa* and *E. coli*, On both bacteria, smaller size Ag nanoparticles demonstrate more antibacterial activity [53].

The Au nanoparticles are another outstanding material that is used for antibacterial therapies by the virtue of their nontoxicity, ability of functionalization and ease of detection [54]. The mechanism of action of Au nanoparticles originated from i) attachment of bacterial membrane and decreasing ATP level ii) inhibition of tRNA [55]. The study by Lima et al. observed % 95 inhibition on *E. coli* and *S. typhi* bacteria colonies treated with 5 nm Au nanoparticles. The factors that direct the antibacterial efficiency were depicted as the roughness and well dispersion of Au nanoparticles [56]. As they do not generate reactive oxygen species (ROS) while exhibiting antibacterial mechanisms, it is safer for mammalian cells [57].

TiO_2 nanoparticles are also among the attractive antibacterial agents, its effectivity is originated from ROS production thus damaging the DNA of organisms [58]. TiO_2 nanoparticles are commonly employed as a preventative strategy to avoid bacterial adherence on medical devices [59]. As a disadvantage TiO_2 nanoparticles can show toxicity, non-toxic polymer coatings were utilized to overcome this challenge [60]. The antibacterial activity of TiO_2 nanoparticles was studied in a variety of studies. In

a study, mesoporous titania was formed as a thin film and loaded with various antibacterial agents. The structure hindered the bacterial attachment and aided to eliminate the adhesion of bacteria due to the hydrophobic structure with and without antibacterial agents [61]. In another study, the antibacterial activity of TiO_2 nanorods was tested against *E. coli ATCC 8739*. After 24 hours of treatment with TiO_2 nanorods, a significant decrease was observed in cell viability [62].

Zinc oxide nanoparticles (ZnO NPs), as an attractive metal oxide nanoparticles with their high stability and biocompatibility [63] were employed in antibacterial solutions very frequently [64–67]. However, ZnO NPs offer multi-action mechanisms against bacterial species, these mechanisms are the production of ROS [68], destruction of cellular integrity [69] and release of zinc ions (Zn^{+2}) [66]. Therefore, the development of bacterial resistance is restricted. So, bacteria are challenged to develop resistance pathways. The action mechanism of ZnO NPs is generally attributed to ROS production, Dutta et al. have investigated the antibacterial property of ZnO NPs resulted in the generated ROS in culture media causing peroxidation of the lipid membrane [18].

While the numerous metal oxide nanoparticles have bactericidal and bacteriostatic activity, various groups of the pathogen can develop antimicrobial resistance against nanoparticles [70]. Nanoparticles are tailored to subdue antimicrobial resistance by combining them with other antimicrobial agents decreases the possibility of developing resistance. The development of resistance against these structures means the generation of multiple and simultaneous gene mutations in the same bacteria [71]. That's why, combinatorial strategies can be beneficial rather than individual ones in order to avoid antimicrobial resistance. The functionalization of nanoparticles with antibiotics is useful for fighting antimicrobial resistance and enhancing the antibacterial activity at low doses [72]. Synergistic effect by the virtue of nanoparticle-antibiotic combinations has been observed in both gram-positive and gram-negative bacteria [73]. The combination of Ag NPs and oregano essential oil inhibited the strains of non-methicillin-resistant *S. aureu*s (non-MRSA) and β-lactamase- and carbapenemase-producing *E. coli* and *A. baumannii* strains [74].

Another approach is the usage of herbal extracts for the synergistic effect against antibacterial resistance [75]. The Ag nanoparticles combined with chitosan and

polyphenol biomolecules enhanced the antibacterial activity synergistically on both gram-negative and gram-positive bacteria [76]. The synergistic effect of ZnO NPs impregnated with ethanolic propolis extracts was investigated and the results revealed that ZnO NPs impregnated with ethanolic propolis extracts demonstrated a synergistic effect on gram-positive bacteria. Rosemary or cinnamon extracts combined with ZnO NPs demonstrate stronger antibacterial activity against *E. coli O157:H7*. Consequently, combinatorial therapies for combating antimicrobial resistance are more efficient in comparison to other strategies due to lower dose usage, synergistic effect and multi-action mechanisms [77]. Besides that, herbal or natural extracts of plants are suitable agents to be combined with metal oxide nanoparticles because of their biocompatibility [78].

Curcumin is a hydrophobic and bioactive material that is used in many biological applications [79] and it is a safe herbal extract material with its low toxicity even at high doses [80]. On the other hand, curcumin shows good antibacterial properties against both gram-negative and gram-positive bacteria [81]. Tyagi et al examined the antibacterial efficiency and mode of action of curcumin I on both gram-negative and gram-positive bacteria [82]. This study revealed that curcumin destroys cell membranes of bacteria, thus killing them. Additionally, It is capable of penetrating the bacterial membrane regardless of gram status [64]. As disadvantage curcumin has low bioavailability and absorption, thus being limited its activity biologically. In many studies, ZnO NPs and curcumin were utilized together. Due to the membrane permeability and pharmacological limitations of curcumin, ZnO NPs were utilized in order the increase bioavailability performance [83]. In addition to these contributions, ZnO NPs and curcumin demonstrate a synergistic effect. Besides curcumin, capped ZnO NPs demonstrate higher stability, dispersibility, and low hydrodynamic size rather than pristine ZnO NPs and pristine curcumin [84]. In a nutshell, the combinatorial effect of ZnO NPs and curcumin may establish a synergistic effect in terms of antibacterial activity. Moreover, they can overcome the limitations of each other regarding physical-chemical and biological properties.

To the best of our knowledge, growth rate, μ and doubling time, t_d of *E. coli ATCC 25922* and *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* were not investigated under the effect of curcumin. The synergistic effect of ZnO NPs and curcumin

separately without embedding it in ZnO NP designs were not determined. Examination of growth kinetic and bacterial viability is important to understand the behavior of bacteria under stress which may induce the development of resistance mechanisms. In detail, even the growth rate, μ decrease or doubling time, t_d increase of a bacterial cell, can give the idea of the effect on growth inhibition or cell viability. It is known that the growth kinetics of bacteria are related to antimicrobial resistance and biofilm formation. The biofilms reduce their growth rate in order to resist antibiotics since these antibiotics are designed for rapid-growing planktonic bacteria [85]. The mechanism lying this phenomenon is that slow-growing bacteria target the cell wall synthesis rather than dividing rapidly [86]. Also elongation in the lag phase following with fast high growth rate exponential phase and then higher stationary phases generally related with the stress response of the bacteria [87].

In this Master of Science thesis, we have investigated the antibacterial properties of ZnO NPs and curcumin both combinatorial and separately on gram-negative (*E. coli ATCC 25922*) and gram-positive (*S. aureus ATCC 29213* and *E. faecalis ATCC 29212*) bacterial species. ZnO NPs were synthesized via the sol-gel method. The size and morphology of ZnO NPs were examined using scanning electron microscopy (SEM), the zeta potential (ζ-potential) and size distribution of ZnO NPs using dynamic light scattering (DLS) were determined. The antibacterial activity tests were performed via kinetic optical density (OD) and Alamar Blue Assay. Additionally, growth rate, μ and doubling time, t_d were evaluated through growth curves obtained from OD data. The results revealed that ZnO NPs synthesized successfully with 207.6 nm hydrodynamic size and 16.2 mV ζ-potential. The antibacterial activity determinations revealed that both ZnO NPs and curcumin demonstrate antibacterial activity in terms of growth rate, μ and doubling time, t_d for gram-negative (*E. coli ATCC 25922*) and gram-positive (*S. aureus ATCC 29213* and *E. faecalis ATCC 29212*). The most precise effect of ZnO NPs and curcumin was observed on *S. aureus ATCC 29213*.

Chapter 2

Material and Method

2.1 Synthesis of ZnO Nanoparticles

ZnO NPs were synthesized using the sol-gel method via a study of the Alwan et al. [88] Briefly, 12.6 zinc acetate dehydrate was dissolved completely in 400 mL of distilled water at 50 °C with continuous stirring. Then 600 mL absolute ethanol was added slowly into the stirring solution. Finally, 6 mL hydrogen peroxide (H_2O_2) was added dropwise to this mixture and the resulting solution remained in the stirrer overnight. The resulting solution was washed with distilled water and dispersed in absolute ethanol at 4 °C for further use.

2.2 Characterization Methods of ZnO Nanoparticles

Morphology of the ZnO NPs was examined using SEM. Specimens were prepared by drying a small amount of ZnO NPs dispersed in absolute ethanol and transferring it onto carbon coupons. Then the specimens were monitored using Carl Zeiss 300 VP SEM.

The mean particle size and ζ-potential of the ZnO NPs were investigated using DLS Malvern Nano ZS 90 Zetasizer. 0.25 mg/mL concentration of ZnO NPs in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH=7.2, 25 mM) were dispersed for the ζ-potential and particles size analysis. In both analyses, ZnO NPs were transferred from absolute ethanol into HEPES.

2.3 Antibacterial Activity Determination

The materials used for antibacterial activity determination are ZnO NPs and curcumin. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were used as liquid and solid growth media, respectively. *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* were selected as model bacteria in order to investigate the antibacterial potential of the ZnO NPs and curcumin. Ready to use Resazurin (R&D Systems, USA) was used for Alamar Blue Assay.

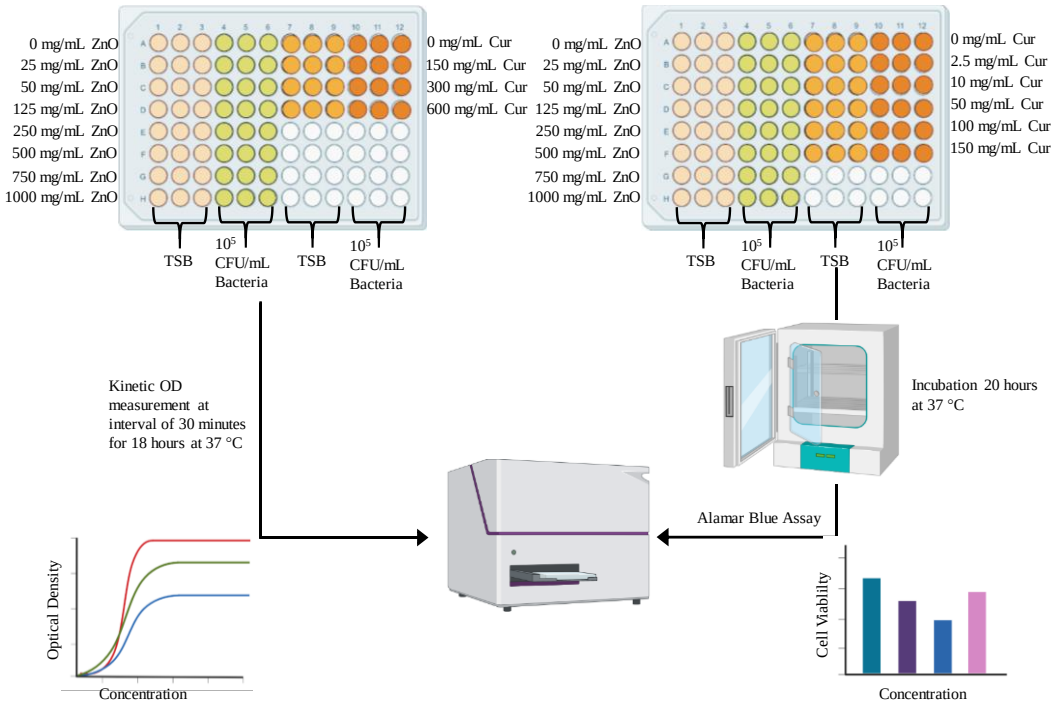

Figure 2.1: Illustration of antibacterial tests

2.3.1 Preparation of Inoculum

The TSB (30 g in 1 L distilled water) was prepared in glass flasks and sterilized via autoclave. In different 15 mL falcons, one colony from strains of each bacterium obtained from TSA (40 g in 1 L) plates were inoculated in 5 mL TSB overnight at 37 °C in the shaker (Unimax1010, Heidolph, Germany) before tests.

2.3.2 Alamar Blue Assay

The bacterium of each species was adjusted to a concentration of 10^5 CFU/mL (from overnight inoculum) in a 96 well-plate containing 125 µL TSB per well. Then, various

concentration of ZnO NPs (25, 50, 125, 250, 500, 750 and 1000 μg/ μL) and curcumin (2.5, 10, 50, 100 and 150 μg/ μL) were used separately in order to treat 10^5 CFU/mL bacterium species. At the same time, 125 μL each concentration of agents and pristine TSB were incubated to be used as blank. Pristine 125 μL 10^5 CFU/mL bacterial solutions were used as a negative control. After 20 hours of incubation at 37 °C, 12,5 μL Ready to use resazurin (R&D Systems, USA) was added to each well. After 2-4 hours incubation with resazurin, fluorescence intensity was measured at values of 542 excitations and 570-670 emission using ClarioStar (BMG-LABTECH, Germany) multi-mode plate reader.

2.3.3 Kinetic Optical Density (OD) Measurements

The bacterium of each species was adjusted to a concentration of 10^5 CFU/mL (from overnight inoculum) in a 96 well-plate containing 125 μL TSB. Then, various concentration of ZnO NPs (25, 50, 125, 250, 500, 750 and 1000 μg/ μL) and curcumin (150, 300 and 600 μg/μL) were used separately in order to treat 10^5 CFU/mL bacterium species. At the same time, 125 μL from each concentration of agents and pristine TSB were incubated to be used as blank. Pristine 125 μL 10^5 CFU/mL bacterial solutions were used as a negative control. The bacterial sigmoid growth curves were monitored via Synergy HTX (BioTek, USA) multi-mode reader at intervals 30 minutes for 18 and at 37 °C (600 nm)

2.3.4 Evaluation of Growth Rate (μ), Doubling Time (t_d) and Z' Factor

The sigmoid growth curves of three bacteria from the kinetic OD measurements were utilized in order to investigate the growth rate, μ and doubling time, t_d of the bacteria. The specific growth rates, μ give the idea of growth characteristics of same bacterium species under effects different pH, antibacterial agent or temperature, etc. The formula is represented at Equation (2.1) and also, doubling time, t_d (Equation (2.2)) works as well since the doubling time, t_d is the ratio of ln2 to growth rate, μ. [89].

$$\mu = \frac{\log_{OD2} - \log_{OD1}}{t_2 - t_1} \quad (2.1)$$

$$t_{\mathrm{d}} = \frac{\ln 2}{\mu} \quad (2.2)$$

Additionally, the assay performance was examined via calculating the Z' factor of Alamar Blue Assay. Z' factor is introduced by Zhang et al. and defined as a dimensionless number that demonstrates the variability and the dynamic range between positive control wells and negative control wells [90]. Acceptance criteria for the Z' factor are defined as $Z' \geq 0.5$ is excellent, $0 < Z' < 0.5$ is Do-able and $Z' \leq 0$ is unreliable data. (Equation (2.3))

$$Z' = 1 - \frac{3(\sigma_1 + \sigma_2)}{|\mu_2 - \mu_1|} \quad (2.3)$$

σ1=Standard deviation of positive controls, σ2=Standard deviation of negative controls μ1=Mean of positive controls, μ2=Mean of negative controls.

2.4 Investigating the Combinatory Effect of ZnO Nanoparticles and Curcumin

Synergy tests were performed using the checkboard method. The protocol obtained from Orhan et al. [91] was utilized with slight modifications. Briefly, increasing concentrations of ZnO NPs (0, 25, 50, 125, 250, 500, 750 and 1000 μg/μL) and curcumin (0, 2.5, 10, 50, 100 and 150 μg/μL) were dispersed in TSB with 3 times higher concentrations. Then, the bacterium of each species was adjusted to a concentration of $3x10^5$ CFU/mL (from overnight inoculum) and 33 μL of $3x10^5$ CFU/mL bacterial solution were placed into each well of 96 well-plate. After that, 33 μL of each concentration of ZnO NPs (3X higher concentrations) were placed along the rows while 33 μL of each concentration of curcumin (3X higher concentrations) were placed along with the columns. Consequently, bacterial concentration was adjusted to be 10^5 CFU/mL and ZnO NPs and curcumin concentration were adjusted to their 1X concentrations in each well. The 96-well plates were incubated for 20 hours

at 37 °C. At the end of this period, 9.9 µL Ready to use resazurin (R&D Systems, USA) was added each well. After 2-4 hours incubation with resazurin, cell viability was investigated visually (Blue is no viability, pink is viability). The obtained results were used in order to estimate the fractional inhibitory concentrations index (FICI) curcumin and ZnO NPs according to Equation (2.4). FICI values were used to determine whether these combinations were synergistic (FICI≤0.5), antagonistic (FICI>4.0), additive (0.5<FICI≤1), or indifference (1<FICI≤4) [92].

$$FICI = \frac{A}{MIC_A} + \frac{B}{MIC_B} \tag{2.4}$$

In equation (2.3) A and B represents the MIC of each agent in combination, MIC_A and MIC_B are represent the MIC of each agent individually.

Chapter 3

Results and Discussion

3.1 Characteristics of ZnO Nanoparticles

The synthesized ZnO NPs were characterized for their morphology analysis by SEM. Figure 3.1 (a) shows the spherically shaped ZnO NPs and their size with no agglomeration (Figure 3.1 (b)). SEM micrographs indicated that the particle size of ZnO NPs was observed as ~120 nm and smooth spherically shaped which is similar compared to those reported by Alwan et al [88] who is utilized to synthesize our ZnO NPs.

The particle size of the ZnO NPs was performed in order to investigate the particle size distribution of the synthesized sample. The hydrodynamic size of the dispersed nanoparticles from DLS analysis was determined as 207.6 nm ± 55.5 with 0.178 polydispersity index (PDI). ζ-potential value gives the idea of whether a nanoparticle will disperse or agglomerate since the repulsion and attraction properties of a nanoparticle mostly depend on the surface charge. So, this net surface charge is the in a dispersion medium [93]. Obtained results show that ζ-potential of ZnO NPs was 16.2 mV ± 4.23. Although, ζ-potential of the ZnO NPs are below +30 mV, thus considering low colloidal stability because of insufficient electrostatic repulsion force [94], SEM micrographs clearly demonstrate that there is no agglomeration. If the repulsion forces are stronger than the attraction forces, nanoparticles can be dispersible [95]. In addition to electrostatic forces, van der Waals forces and attractive forces (depletion, hydrophobic, etc.) are effective on colloidal stability. The balance between these forces determines colloidal stability [96]. Since ZnO NPs are intrinsically hydrophilic[97], the colloidal stability of ZnO NPs in SEM micrographs can be originated from hydrophilic structure even its ζ-potential is between unstable range (-30 mV-30 mV).

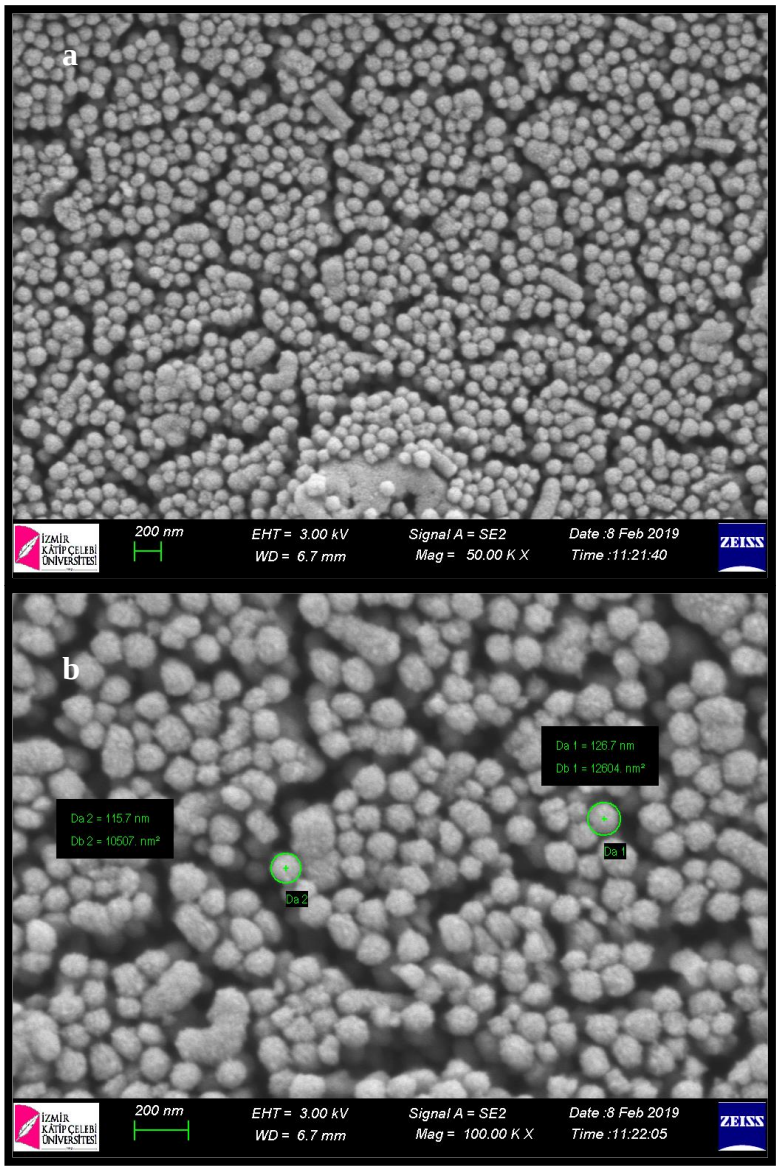

Figure 3.1: The SEM micrographs of ZnO NPs at (a) 100x (b) 50x magnification

3.2 Antibacterial Activity Determination

3.2.1 Alamar Blue Assay

Antibacterial activity of ZnO NPs and Curcumin was assessed against *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* via Alamar Blue Assay. The results of this assessment was shown in Figure 3.2 and Figure 3.3 in terms of cell viability. ZnO NPs does not show any antibacterial activity in *E. coli ATCC 25922*. Moreover, it is observed a promotion. In other study, *Pseudomonas putida KT2440* which is gram-negative bacteria as well *E. coli ATCC 25922* were promoted by

exposing low concentrations (0.5 – 30 µg/mL) of ZnO NPs [65]. In a similar manner, *E. coli ATCC 25922* can survive at low concentrations of ZnO NPs [66], [98]. *S. aureus ATCC 29213* was inhibited by ~%98 at concentrations of 500 µg/µL and 750 µg/µL. This inhibition was lower for *E. faecalis ATCC 29212* (~%55) in comparison to *S. aureus ATCC 29213.*

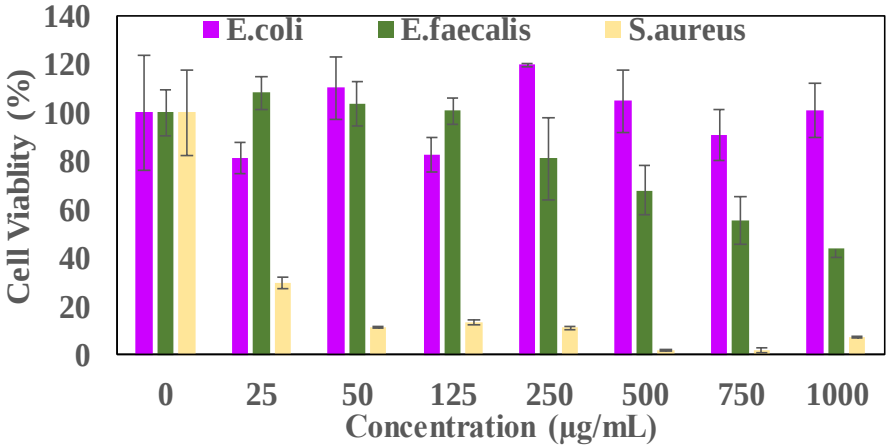

Figure 3.2: Cell viability of *E. coli* (purple) *E. faecalis* (green) and *S. aureus* (yellow) under the effect of ZnO NPs

It is known that curcumin is capable of permeating the cell membrane regardless of gram status, thus leading to cellular lysis [82]. The antibacterial efficacy of curcumin on both gram-negative (*E. coli ATCC 25922*) and gram-positive (*S. aureus ATCC 29213* and *E. faecalis ATCC 29212*) was observed. It decreased the cell viability of *E. coli ATCC 25922 and E. faecalis ATCC 29212* by approximately %95 after treatment with 150 µg/mL curcumin. In contrast, that of *S. aureus ATCC 29213* was %60. Consequently, Alamar Blue assay established *S. aureus ATCC 29213* is the most susceptible to ZnO NPs where *E. coli ATCC 25922* was most resistant and curcumin can demonstrate its antibacterial activity in both gram-negative and gram-positive strains. Even in high concentrations of ZnO NPs, *E. coli ATCC 25922* was not affected. The reason for that can be originated from TSB which is the growth media and gram-negative status of this strain. The other possible mechanism is that ZnO NPs are outsize to penetrate the cell membrane by crossing the outer lipopolysaccharide membrane[99], [100].

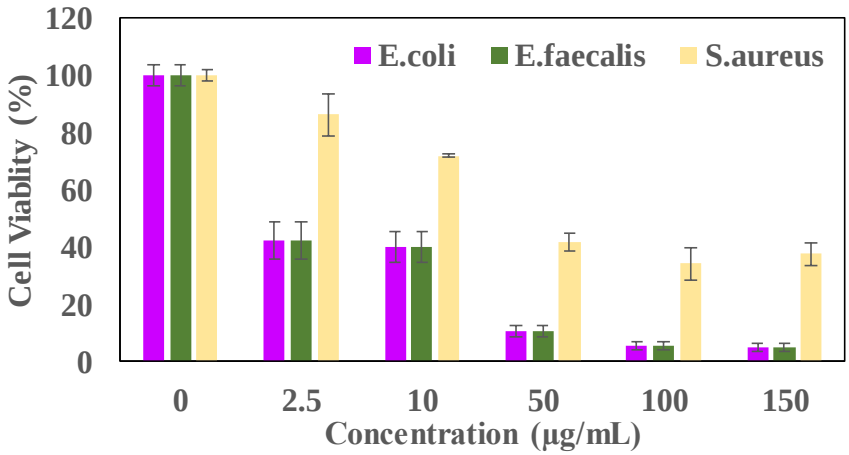

Figure 3.3: Cell viability of *E. coli* (purple) *E. faecalis* (green) and *S. aureus* (yellow) under the effect of curcumin

3.2.2 Kinetic Optical Density Measurements

Kinetic OD measurements showed the effect of ZnO NPs and curcumin on the growth of *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* at different concentrations over a period of 18 hours. A significant decrease was observed on *S. aureus ATCC 29213* treated with 500 µg/mL 750 µg/mL and 1000 µg/mL ZnO NPs. The growth inhibition of *S. aureus ATCC 29213* was %95 for 1000 µg/mL ZnO NPs. However, the growth inhibition of ZnO NPs on *E. faecalis ATCC 29212* and *E. coli ATCC 25922* was not as effective as that of *S. aureus ATCC 29213*. The inhibitions of *E. faecalis ATCC 29212* and *E. coli ATCC 25922* at the end of 18 hours were determined by %30 and %28, respectively for 1000 µg/mL ZnO NPs. The antibacterial efficacy of 1000 µg/mL ZnO NPs applied on *E. faecalis ATCC 29212* was higher in Alamar Blue Assay (Figure 3.2). This discrepancy can be originated from the that OD measures the turbidity of the bacteria. Dead bacteria which is not lysed can be determined as viability.

Although the higher concentrations (150, 300 and 600 µg/µL) of curcumin were used in kinetic OD measurements, the antibacterial efficiency of curcumin of *S. aureus ATCC 29213* decreased in comparison to the Alamar Blue assay. The inhibition percentage at 300 µg/mL and 600 µg/mL was approximately %20. The possible reason for this can be the highest concentrations of curcumin agglomerated in media thus limiting the bioavailability. The situation is valid for *E. faecalis ATCC 29212* and *E. coli ATCC 25922*.

3.2.3 Growth Rate (μ), Doubling Time (t_d) and Z' Factor

The Z' factor evaluations were achieved according to the formula represented in Equation (2.3). Aforesaid, Z' factor value must be Z'>0.5 is excellent, 0<Z'<0.5 is Do-able [101] in order to accept the assay validity. Table 3.1 shows the Z' factor of all tested bacteria with curcumin and ZnO NPs. The obtained results revealed that all Alamar Blue tests are excellent or Doable.

Table 3.1: Z' factor values of Alamar Blue test against bacterial species

	Bacterial Species		
Antibacterial Agent	*E.coli*	*E. faecalis*	*S.aureus*
ZnO NPs	0.49	0.67	0.18
Curcumin	0.87	0.87	0.91

The growth rate, μ and doubling time, t_d enables the describe the growth kinetic of bacteria under the effect of environmental conditions such as antibacterial agents [102]. The determination of antibacterial effect through growth rate, μ was examined in many studies [51,102,103]. The evidence for the antibacterial activity obtained from growth curves is shortening of the log phase [104], elongation in the lag phase [105], or decrease in OD value [106].

The growth curves obtained from treatment of *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* with ZnO NPs at various concentrations were used in order to evaluate growth rate, μ and doubling time t_d. The growth rate, μ of *S. aureus ATCC 29213* decreased with increasing concentrations of ZnO NPs. (Figure 3.4). The growth rate, μ *of S. aureus ATCC 29213* treated with 1000 µg/mL ZnO NPs was lowered by 2.26 fold in comparison to untreated *S. aureus ATCC 29213*. Although we observed a high inhibition (%95) in the investigations treated with 1000 µg/mL ZnO NPs at the end of 18 hours, that of growth of bacteria was not inhibited completely. Doubling time, t_d of *S. aureus ATCC 29213* treated with 1000 µg/mL increase from 1.62 (hour) to 5.23 (hour). These results were confirmed that ZnO NPs can provide good antibacterial activity against *S. aureus ATCC 29213*. In contrast, *E. coli ATCC 25922* and *E. faecalis ATCC 29212* were not affected by the antibacterial activity of

ZnO NPs in terms of growth rate, μ, and doubling time, t_d. There are studies in the literature, represents that ZnO NPs inhibit the *E. coli ATCC 25922* [107]. In the study achieved by Yuan et al. ZnO NPs has 30 nm size. It is clearly known that the size of nanoparticle and antibacterial activity has inverse proportion [108]. Since our ZnO NPs have a bigger size (120 nm), they can be inadequate to generate antibacterial activity. Additionally, bacteria have a negative surface charge [109] and as a possible mechanism, ZnO NPs attach the outer membrane of the bacterial through opposite surface charge interactions [110]. Even though we conclude that ZnO NPs have 16.2 ± 4.23 mV ζ-potential which is opposite to bacterial surface charge in HEPES, we do not know the that of in TSB. The positivity of ZnO NPs cannot be enough to attach to the bacterial surface.

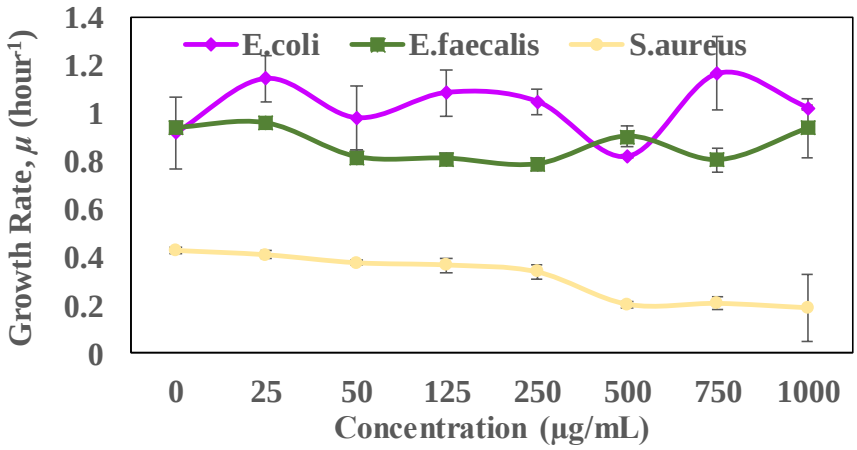

Figure 3.4: Growth rate, μ of *E. coli* (purple) *E. faecalis* (green) and *S. aureus* (yellow) under the effect of ZnO NPs

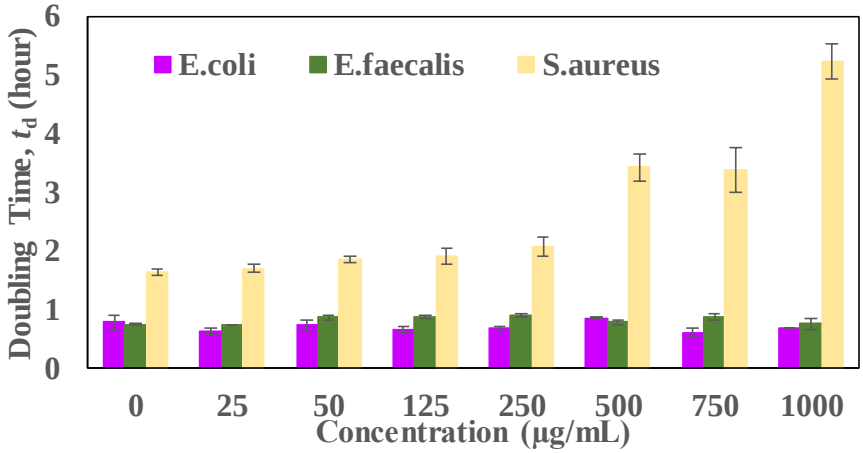

Figure 3.5: Doubling time, t_d of *E. coli* (purple) *E. faecalis* (green) and *S. aureus* (yellow) under the effect of ZnO NPs

The growth curves obtained from treatment of *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *E. faecalis ATCC 29212* with curcumin at various concentrations were used in order to evaluate growth rate, μ and doubling time t_d. According to obtained results, the growth rate, μ of *S. aureus ATCC 29213* did not show any attenuation until the concentration of 300 µg/mL curcumin. At the highest concentration (600 µg/mL) there was a significant decrease such as 1.77 times. This ratio was 1.21 and 1.42 at the concentrations of 300 µg/mL and 600 µg/mL respectively for *E. faecalis ATCC 29212*.

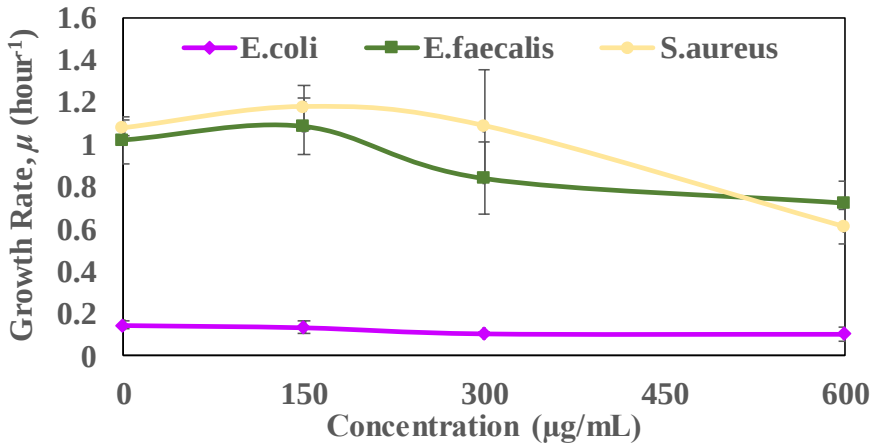

Figure 3.6: Growth rate, μ of *E. coli* (purple) *E. faecalis* (green) and *S. aureus* (yellow) under the effect of curcumin

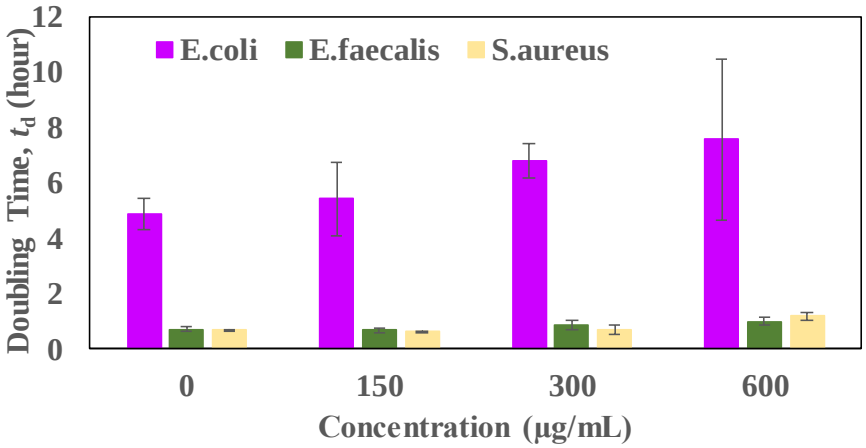

Figure 3.7: Doubling time, t_d of E. coli (purple) E. faecalis (green) and S. aureus (yellow) under the effect of curcumin

Curcumin showed its antibacterial activity on *E. coli ATCC 25922* through decreasing growth rate by 1.40 and 1.43 at the concentrations of 300 µg/mL and 600 µg/mL respectively. Doubling time, t_d is increased for all bacterial species. The increment was generated at 600 µg/mL curcumin by ratio 1.55, 1.78 and 1.42 with respect to the control group of *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *E. faecalis ATCC*

29212, respectively (Table 3.2). Even though a decrease in growth rate is observed, μ or increase in t_d can be accepted as an antibacterial effect [106], a decrease in cell viability cannot occur. For this study, even we observe attenuation in growth rate, μ of *S. aureus ATCC 29213* treated with 600 μg/mL, this attenuation did not affect the growth of *S. aureus ATCC* at the same concentration.

Table 3.2: Growth rate decrease and doubling time increase by ratio according to control groups for highest treatment concentrations

Bacterial Species	ZnO NPs		Curcumin	
	Growth rate	Doubling time	Growth rate	Doubling time
E.coli	0.9	0.87	1.43	1.55
E. faecalis	1	1.01	1.42	1.42
S.aureus	2.26	3.23	1.77	1.78

3.2.4 Checkboard Test

The checkboard test performed on *E. coli ATCC 25922* and *E. faecalis ATCC 29212* reveal that any combinatory effect of ZnO NPs and curcumin cannot inhibit both strains completely. In contrast, *S. aureus ATCC 29213* was affected by the combinatory action of curcumin and ZnO NPs. The MIC of curcumin could not be determined at the concentrations (2.5, 10, 50, 100, 150 μg/mL). The MIC of ZnO NPs alone 1000 μg/mL and the addition of 150 μg/mL curcumin decreased the MIC of ZnO NPs to 125 μg/mL for *S. aureus ATCC 29213*. Thus, the FICI of ZnO NPs on *S. aureus ATCC 29213* was determined as 0.125. If we assume that the MIC of curcumin is 150 μg/mL, FICI value of 150 μg/mL curcumin and 1000 μg/mL ZnO, the FICI value of that combination is 1.125 which means indifference effect. Even the MIC of curcumin is assumed as 150 μg/mL, 2.5 μg/mL curcumin and 750 μg/mL ZnO NPs completely inhibited *S. aureus ATCC 292213* and has 0.76 FICI value which means that additive effect. A study which is performed by Perera et al. is parallel with our results. In the study curcumin-loaded ZnO NPs demonstrate additive effects on gram-positive and gram-negative bacteria. In another study, ZnO NPs conjugated with curcumin showed a synergistic effect on *S. aureus* and the best antibacterial activity concentration of

curcumin was 500 µg/mL. If we assume the MIC of our curcumins on *S. aureus ATCC 292213* as 500 µg/mL, the synergistic effect can be obtained with a combination of 250 µg/mL ZnO NPs and 100 µg/mL curcumin. Also, *E. faecalis ATCC 29212* did not have any complete inhibition. Nevertheless, the cell viability of *E. faecalis ATCC 29212* significantly decreased at combinatorial effects ZnO NPs and curcumin. Due to the results, it is possible to claim that ZnO NPs and curcumin can demonstrate synergistic or additive effects against *E. faecalis ATCC 29212* and *S. aureus ATCC 29213* at specific combinations of concentrations.

While several works that combine ZnO NPs and curcumin for the development of antibacterial approaches were examined [64,83,84], to the best our knowledge there is no study that investigates the antibacterial properties of ZnO and curcumin without any conjugation, capping or loading. Additionally, the growth rate, μ and doubling time, t_d was not utilized in the literature previously to investigate the combinatorial effect of ZnO NPs and curcumin as antibacterial treatments.

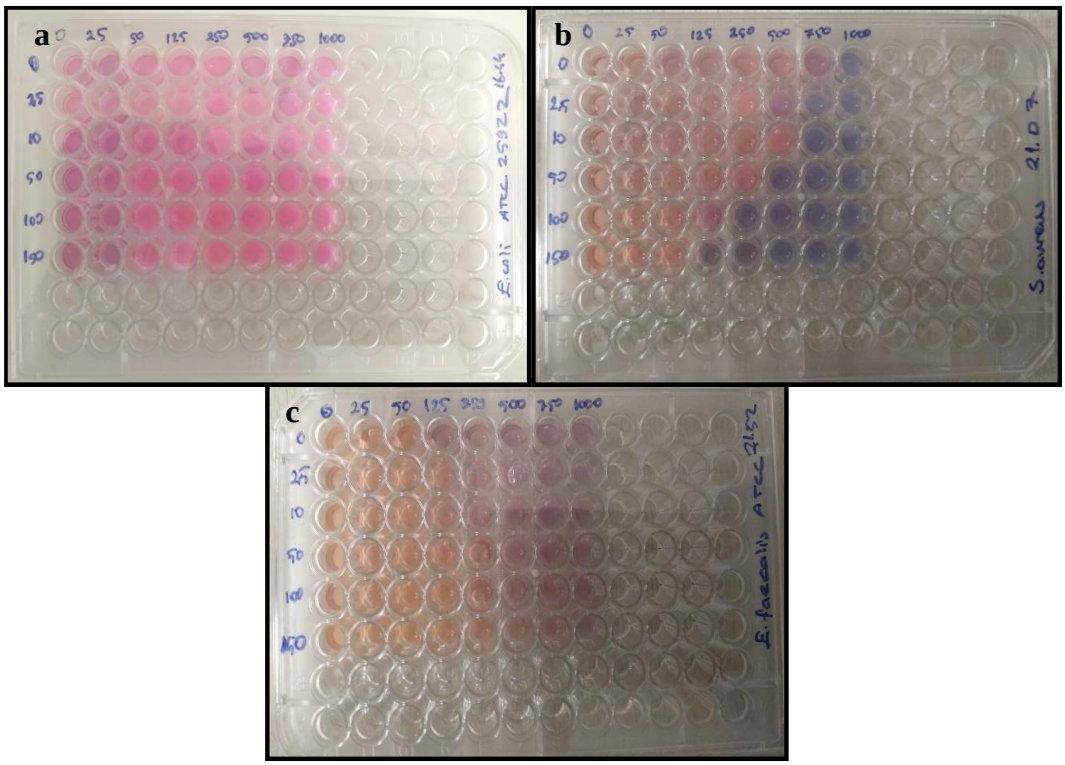

Figure 3.8: Checkboard test on (a) *E. coli ATCC 25922* (b) *S. aureus ATCC 29213* and (c) *E. faecalis ATCC 29212*

Chapter 4

Conclusion

ZnO NPs are successfully synthesized via the sol-gel method. The characterization of ZnO NPs was performed. The ZnO NPs have 16.2 mV ± 4.23 ζ-potential and 207.6 nm ± 55.5 hydrodynamic size with 0.178 polydispersity index (PDI) value. The antibacterial effect of ZnO NPs was not efficient for E. *coli ATCC 25922* in both Alamar Blue and kinetic OD measurements. The growth rate, μ of *S. aureus ATCC 29213* were decreased 2.26 times in comparison to that of the negative control. *E. faecalis ATCC 29212* and *E. coli ATCC 25922* did not demonstrate any significant decrease in terms of growth rate, μ. Curcumin is effective for all bacterial species in terms of reducing the growth rate. The growth rate, μ of *S. aureus ATCC 2921, E. faecalis ATCC 29212* and *E. coli ATCC 25922* was decreased by 1.77, 1.42 and 1.43 fold under the effect of 600 µg/mL curcumin. Even though we observed decreases in growth rare, μ of the bacterial species and this is accepted as an antibacterial effect, it cannot be observed in the growth inhibition. Combinatorial usage of ZnO and curcumin generate a synergistic effect on *S. aureus ATCC 2921* and *E. faecalis ATCC 29212*. This study concluded that ZnO nanoparticles and curcumin can be a promising approach to combat antibacterial resistance.

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Appendix A

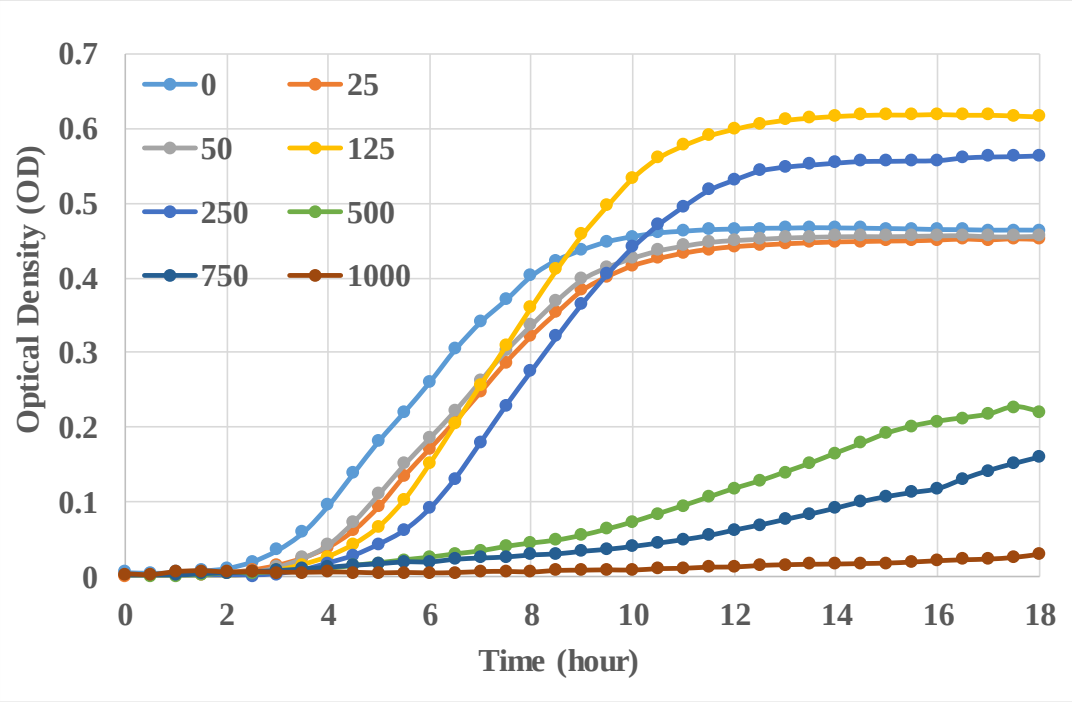

Figure A.1: Growth curves of *S. aureus ATCC 29213* with and without increasing concentrations of ZnO NPs

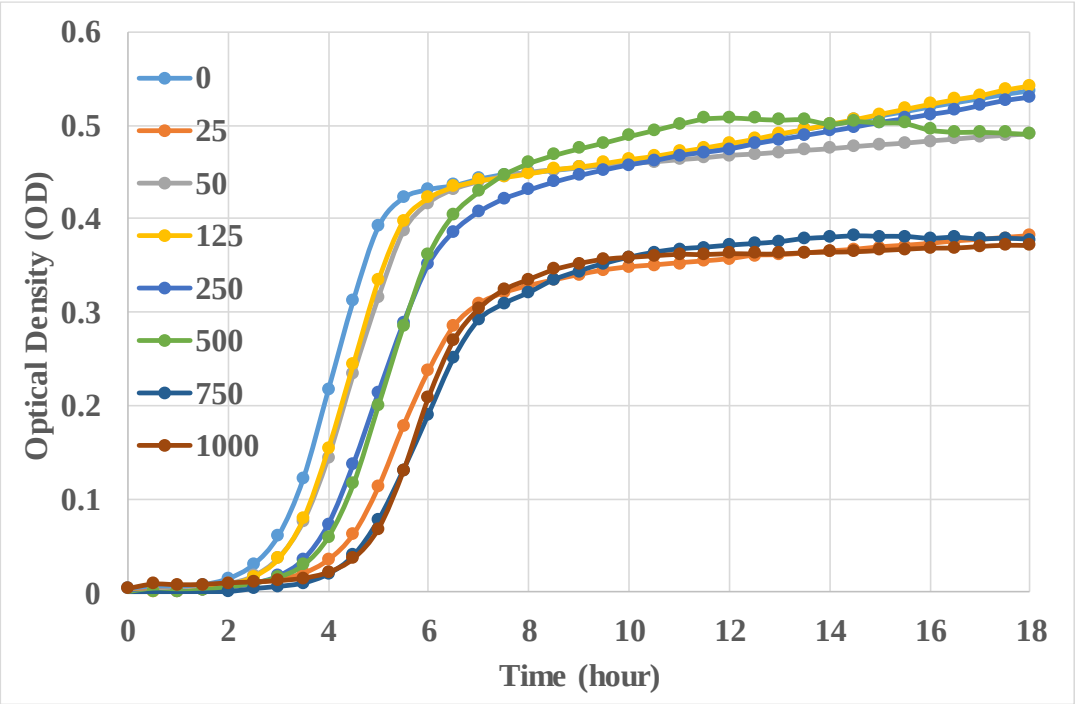

Figure A.2: Growth curves of *E. faecalis ATCC 29212* with and without increasing concentrations of ZnO NPs

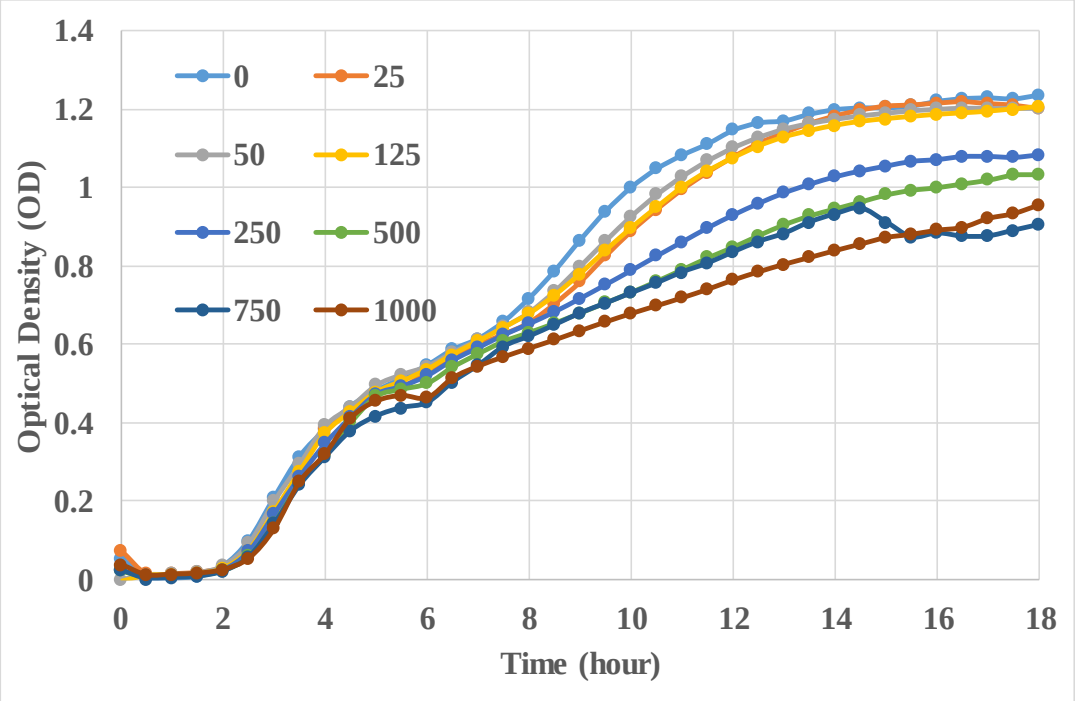

Figure A.3: Growth curves of *E. coli ATCC 25922* with and without increasing concentrations of ZnO NPs

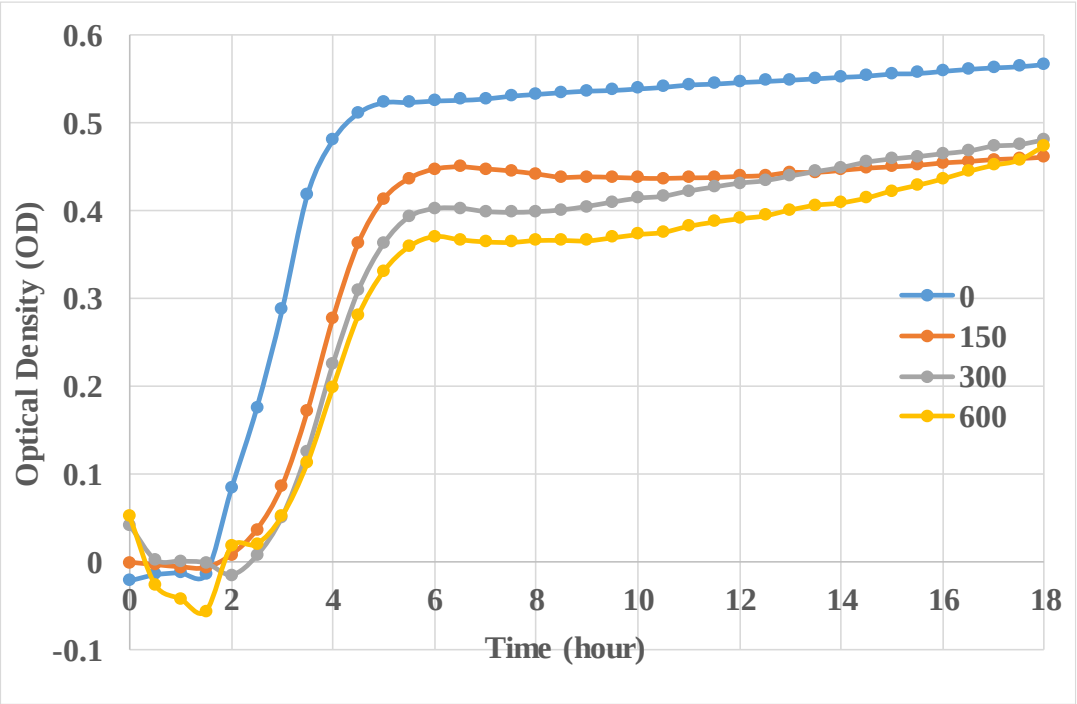

Figure A.4: Growth curves of *S. aureus ATCC 29213* with and without increasing concentrations of curcumin

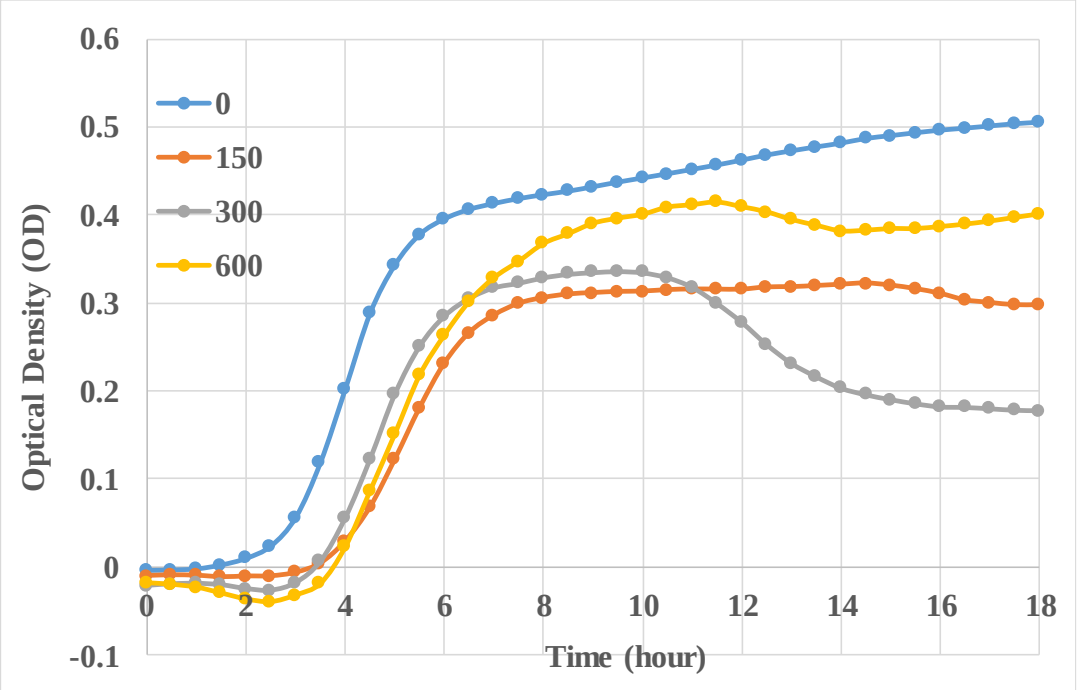

Figure A.5: Growth curves of *E. faecalis ATCC 29212* with and without increasing concentrations of curcumin

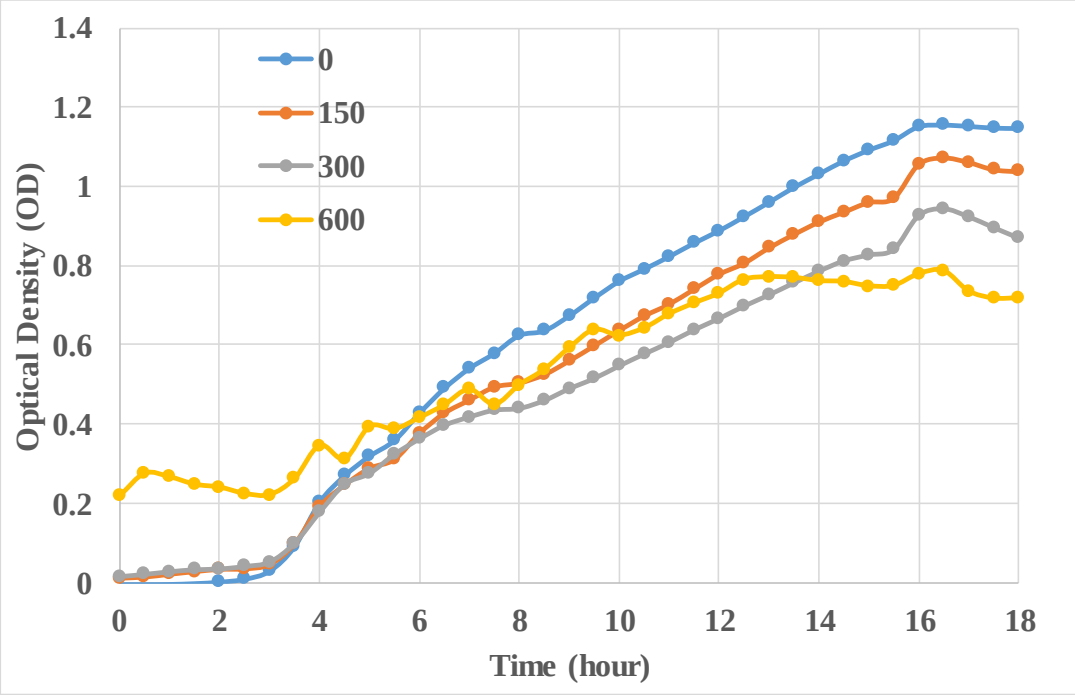

Figure A.6: Growth curves of *E. coli ATCC 25922* with and without increasing concentrations of curcumin

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(International Journal of Nanomedicine)

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(The Journal of The Minerals, Metals & Materials Society)

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Investigating the Growth Kinetics of Gram-Positive and Gram-Negative Bacteria in the Presence of Zinc Oxide Nanoparticles and Curcumin

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